M.Sc. Final Year

Chemistry, MC-07

BIOINORGANIC, BIOORGANIC AND BIOPHYSICAL CHEMISTRY

मध्यप्रदेश भोज (मुक्त) विश्वविद्यालय – भोपाल

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SYLLABI-BOOK MAPPING TABLE

Bioinorganic, Bioorganic and Biophysical Chemistry

Syllabi	Mapping in Book
UNIT - I (a) Biological Cell and its Constituents: Biological cell, Structure and Functions of Proteins and Enzymes, DNA and RNA in Living Systems, Helix Coil Transition. (b) Cell Membrane and Transport of Ions: Structure and Functions of Cell Membrane, Ion Transport through Cell Membrane, Irreversible Thermodynamic Treatment of Membrane Transport, Nerve Conduction. (c) Metal Ions in Biological Systems: Essential and Trace Metals. (d) Na+/ K+ Pump: Role of Metals Ions in Biological Processes. (e) Transport and Storage of Dioxygen: Heme Proteins and Oxygen Uptake, Structure and Function of Haemoglobin, Myoglobin, Hemocyanins and Hemerythrin, Model Synthetic Complexes of Iron, Cobalt and Copper.	**Unit-1:** Biological Cell and its Constituents **(Pages 3-79)**
UNIT - II (a) Bioenergetics: Standard Free Energy Change in Biochemical Reactions, Exergonic and Endergonic Process, Hydrolysis of ATP, synthesis of ATP from ADP. (b) Diffraction Methods: Light Scattering, Low Angle X-Ray Scattering, X- Ray Diffraction and Photo Correlation Spectroscopy, ORD. (c) Bioenergetics and ATP Cycle: DNA Polymerization, Glucose Storage, Metal Complexes in Transmission of Energy, Chlorophylls, Photosystem I and Photosystem II in Cleavage of Eater, Model systems. (d) Electron Transfer in Biology: Structure and function of Metalloproteins in Electron Transport Processes, Cytochromes and Iron-Sulphur Proteins, Synthetic Models. (e) Nitrogenases: Biological Nitrogen Fixation, Molybdenum Nitrogenase, Spectroscopic and Other Evidence, Other Nitrogenases Model Systems.	**Unit-2:** Bioenergetics **(Pages 81-136)**
UNIT - III (a) Statistical Mechanics in Biopolymers: Chain Configuration of Macromolecules, Statistical Distribution End-to-End Dimensions, Calculation of Average Dimensions for Various Chain Structures, Polypeptide and Protein Structures, introduction to Protein Folding Problem. (b) Biopolymer Interactions: Forces Involved in Biopolymer Interactions, Electrostatic Charge and Molecular Expansion Hydrophobic Forces, Dispersion Force Interactions, Multiple Equilibria and Various Types of Binding Processes in Biological Systems, Hydrogen Ion Titration Curves.	**Unit-3:** Biopolymers **(Pages 137-197)**

(c) Thermodynamics of Biopolymer Solutions: Thermodynamics of Biopolymer Solutions, Osmotic Pressure, Membrane Equilibrium, Muscular Contraction and energy generation in Mechanochemical System.

(d) Biopolymers and their Molecular Weights: Evaluation of Size, Shape, Molecular Weight and Extent of Hydration of Biopolymers by Various Experimental Techniques, Sedimentation Equilibrium, Hydrodynamic Methods, Diffusion, Sedimentation Velocity, Viscosity, Electrophoresis and rotational motions.

UNIT - IV

(a) Introduction: Basic consideration, Proximity Effects and Molecular Adaptation.

(b) Enzymes: Introduction and Historical Perspective, Chemical and Biological Catalysis, Remarkable Properties of Enzymes like Catalytic Power, Specificity and Regulation, Nomenclature and Classification, Extraction and Purification. Fischer's Lock and Key and Koshland's Induced-Fit Hypothesis, Concept and Identification of active site by the use of Inhibitors, Affinity Labeling and Enzyme Modification by Site-Directed Mutagenesis. Enzyme Kinetics, Michaelis-Menten and Lineweaver-Burk plots, Reversible and Irreversible Inhibition.

(c) Mechanisrn of Enzyme Action: Transition State Theory, Orientation and Steric Effect, Acid-Base catalysis, Covalent Catalysis, Strain or Distortion, Examples of Some Typical Enzyme Mechanisms for Chymotrypsin, Ribounclease, Lysozyme And Corboxypeptidase A

(d) Kinds of Reactions Catalyzed by Enzymes: Nucleophilic Displacement on a Phosphorus Atom, Multiple Displacement Reactions and the Coupling of ATP Cleavage to Endergonic Processes, Transfer of Sulphate, Addition and Elimination Reactions, Enolic Intermediates in Isomerization Reactions, β- Cleavage and Condensation, Some Isomerization and Rearrangement Reactions, Enzyme Catalyzed Carboxylation and Decarboxylation.

Unit-4: Enzymes and Molecular Adaptation
(Pages 199-262)

UNIT - V

(a) Co-Enzyme Chemistry: Cofactors as Derived from Vitamins, Co-enzymes, Prosthetic Groups, Apoenzymes, Structure and Biological Functions of Co-enzyme A, Thiamine Pyrophosphate, Pyridoxal Phosphate, NAD+, NADP+, FEN, FAD, Lipoic Acid, Vitamin Bl2, Mechanisms of Reactions Catalyzed by the Above Cofactors.

(b) Enzyme Models: Host-guest chemistry, Chiral Recognition and Catalysis, Molecular Recognition, Molecular Asymmetry and Prochirality, Biomimetic Chemistry, Crown Ethers, Cryptates, Cyclodextrins, Based Enzyme Models, Calixarenes, ionophores, Micelles, Synthetic Enzymes or Synzymes.

(c) Biotechnological Applications of Enzymes: Large-Scale Production and Purification of Enzymes, Techniques and Methods of Immobilization of enzymes, Effect of Immobilization on Enzyme Activity, Applications of Immobilized Enzymes, Uses of Enzymes in Food and Drink Industry-Brewing and Cheese-Making, Syrups from Corn Starch, Enzymes as Targets for Drug Design, Clinical Uses of Enzymes, Enzyme Therapy, Enzymes and Recombinant DNA Technology.

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INTRODUCTION

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Bioinorganic chemistry is a field that analyse the role of metals in biology. Bioinorganic chemistry includes the study of both natural phenomena, such as the behaviour of metalloproteins as well as artificially introduced metals, including those that are non-essential, in medicine and toxicology. Bioinorganic chemistry is important in elucidating the implications of electron-transfer proteins, substrate bindings and activation, atom and group transfer chemistry as well as metal properties in biological chemistry.

Bioorganic chemistry is the branch of chemistry which integrates organic chemistry and biochemistry. Bioorganic chemistry is a scientific discipline that combines organic chemistry and biochemistry. It is that branch of life science that deals with the study of biological processes which are related to chemical methods. Protein and enzyme function are examples of these processes. Bioorganic chemistry involves the study of biological processes using chemical methods. Organic chemistry methods are used to synthesize biological molecules and to examine their structure, to investigate biochemical reactions.

Biophysical chemistry is a physical science that uses the concepts of physics and physical chemistry for the study of biological systems. The field of biophysical chemistry seeks a mechanistic understanding of important biological phenomena. Study of physical chemistry in biological processes requires molecular understanding of structure and dynamics. Biophysical chemistry is aimed at the collection and analysis of quantitative data to provide predictive physical models describing biological phenomena occurring at the molecular level.

This book is divided into five units that attempt to give the students the basic idea of bioinorganic, bioorganic and biophysical chemistry, biological cell and membrane, diffraction methods and metal ions in biological system, statistical mechanics in biopolymers and their interaction, thermodynamics of biopolymer solutions, molecular adaptation, applications and mechanism of enzymes and co-enzymes. The book follows the Self-Instructional Mode or SIM format wherein each unit begins with an 'Introduction' to the topic followed by an outline of the 'Objectives'. The detailed content is then presented in a simple and structured manner interspersed with Answers to 'Check Your Progress' questions. A list of 'Key Terms', a 'Summary' and a set of 'Self-Assessment Questions and Exercises' is also provided at the end of each unit for effective recapitulation.

UNIT 1 BIOLOGICAL CELL AND ITS CONSTITUENTS

NOTES

Structure

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1.0 INTRODUCTION

The cell is the basic structural and functional unit of life. Every cell contains of a cytoplasm enclosed within a membrane, which contains many biomolecules, such as proteins and nucleic acids. Proteins are large, complex molecules that play many critical roles in the body. Proteins also receive signals from outside the cell and mobilize intracellular response. Enzymes are protein catalysts that speed biochemical reactions by facilitating the molecular rearrangements that support cell function.

The cell membrane, also called the plasma membrane, is found in all cells and separates the interior of the cell from the outside environment. The cell membrane consists of a lipid bilayer, including cholesterols that sit between phospholipids to maintain their fluidity at various temperatures. The mechanisms that transport ions across membranes are facilitated diffusion and active transport. Facilitated diffusion of solutes occurs through protein-based channels. A nerve conduction study is a medical diagnostic test commonly used to evaluate the function, especially the ability of electrical conduction, of the motor and sensory nerves of the human body.

Metal ions are fundamental elements for the maintenance of the lifespan of plants, animals and humans. Their substantial role in biological systems was recognized a long time ago. Trace metals within the human body include iron, lithium, zinc, copper, chromium, nickel, cobalt, vanadium, molybdenum, manganese and others. Trace metals are metals needed by living organisms to function properly

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and are depleted through the expenditure of energy by various metabolic processes of living organisms.

The sodium–potassium pump is found in many cell (plasma) membranes. Powered by Adenosine TriphosPhate or ATP, the pump moves sodium and potassium ions in opposite directions, each against its concentration gradient. In a single cycle of the pump, three sodium ions are extruded from and two potassium ions are imported into the cell.

A hemeprotein or heme protein, is a protein that contains a heme prosthetic group. They are a very large class of metalloproteins. Hemoglobin or haemoglobin, is the iron-containing oxygen-transport metalloprotein in the red blood cells of almost all vertebrates as well as the tissues of some invertebrates. Hemoglobin in blood carries oxygen from the lungs or gills to the rest of the body. Myoglobin is an iron- and oxygen-binding protein found in the cardiac and skeletal muscle tissue of vertebrates in general and in almost all mammals. Hemocyanins are proteins that transport oxygen throughout the bodies of some invertebrate animals. These metalloproteins contain two copper atoms that reversibly bind a single oxygen molecule. Hemerythrin is an oligomeric protein responsible for oxygen transport in the marine invertebrate phyla of sipunculids, priapulids, brachiopods, and in a single annelid worm genus, Magelona.

In this unit, you will study about the biological cell, DNA and RNA in living system, cell membrane, transport of ions, nerve conduction, sodium–potassium pump, hemeprotein, hemoglobin, myoglobin, hemocyanins, hemerythrin, and model synthetic complexes of iron cobalt copper.

1.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the biological cell
- Elaborate on the DNA and RNA in living system
- Interpret the cell membrane
- Know about the transport of ions
- Analyse the nerve conduction
- Explain about the sodium–potassium pump
- Discuss about the hemeprotein, hemoglobin and myoglobin
- Comprehend the hemocyanins and hemerythrin
- Describe the model synthetic complexes of iron, cobalt, copper

1.2 BIOLOGICAL CELL

Living beings are composed of cell or many cells. A cell is a mass of cytoplasm enclosed by plasma membrane and contain certain organelles besides nucleus. It is a unit of biological entity covered by semipermeable membrane and capable of medium free of other living systems.

Cell is an open system as it allows the entry and exit of matter and energy. It takes up matter for sustenance, growth and division. Organisms are built according to information encoded in a collection of genes. This vast information is packaged into a set of chromosomes that occupies the space of cell nucleus. Genes are more than storage lockers for information, they constitute the blueprint for reconstructing cellular structure. The molecular structure of genes allows for changes in genetic information that lead to variation among individuals which forms the basis of evolution. Exchange of matter and energy between a cell and its surrounding environment is dynamic. By being able to regulate the entry and exit of materials into and out of it and by chemical changes, cell attains homeostasis in which internal levels of materials remain constant. Such an open, steady state system is in contrast to the closed, equilibrium system of non-living world which don't allow the movement of material into and out of them. All of the energy required by life on the earth surface arrives in the form of electromagnetic radiation. The energy of light is trapped by light absorbing pigments present in membranes of photosynthetic cells.

Cells expand an enormous amount of energy by breaking down and rebuilding the macromolecules and organelles of which they made of. The first culture of human cell was begun by George Gey of Johns Hopkins University in 1951. The cells are obtained from a malignant tumour and named HeLa cells. Every cell is enclosed on all sides by a distinct covering called plasma membrane. It keeps the cell contents in place and prevents their mixing up with extracellular materials. This enables the cell to maintain its highly organized structure and carry on reactions in a regulated manner.

In 1809, Lamarck stated that nobody can have life if its constituent parts are not cellular tissue. A French biologist **H.J Dutrochet** stated in 1824 that all organic tissue are globular cells of small size which appear to be united by simple adhesive forces. Thus all tissues, animal and plant organs are only cellular tissue variously modified. In 1831, a Scotch botanist **Robert Brown** discovered the nucleus in orchid root cell. It was found that cells were surrounded by some sort of limiting structure, cell membrane. A French zoologist **Dujardin** discovered in 1835 a semifluid living material in certain protozoans. He called this material sarcode. In 1840, a similar material was noted in plant cells by biologist Purkinje. He proposed the term protoplasm. Theodore Schwann stated that all animals were formed of cells. Schwann found that animal cells had nuclei and were enclosed by thin cell membrane instead of thick cell wall. He defined the cell as a membrane bound nucleus containing structure. He proposed cell hypothesis stating that all plant and animal bodies are composed of cells. The cells of protists fungi, plants and animals have in their cytoplasm many membrane bound compartments called organelles.

The first three organelles are surrounded by double membrane known as envelope. The part of cytoplasm excluding compartments are eukaryotic cells. The cell of bacteria and blue green algae don't contain membrane bound compartment called single compartment cell called prokaryotic cell. The lack of compartments show primitive nature of these organisms.

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Statements of Cell Theory

It states that:

- All living things are composed of minute units, the cells.
- A cell is a mass of protoplasm containing a nucleus and bounded by cell membrane and by cell wall.
- All cells are basically alike in structure and metabolic activities.
- The function of an organism is the result of the activities and interaction of its constituent cells.

Shortcomings of the Cell Theory

- Bacteria and blue green algae don't have organized nucleus. Their genetic material is not enclosed by nuclear envelope and lies in the cytoplasm
- Protozoans are not cellular. They are acellular.
- Certain fungi, have hyphae composed of a multinucleate mass of cytoplasm without division into cells.
- Some tissue have a good deal of nonliving material, the matrix, between the cells.

Cell Properties

Organization

Organism is the sum of its component cells, and its activities are the sum of the activities of its cell. New function emerge with each increasing level of organization. Just as molecules have different properties from its constituent atoms, tissue have different function from its cells, organs have different activities from its component tissue, and organism have different properties from its organ system.

Cellular Totipotency

Every cell contains a complete set of genes in its nucleus. If the nucleus of fertilized egg of frog is removed and replaced with the nucleus from skin cell, normal development starts and perfect frog is produced. This experiment shows that the nuclei of all cells are totipotent, i.e., they have a complete genetic information.

Autonomous Existence

Each cell take up food material as micromolecules and synthesizes living matter. Each cell itself disposes of its own worn out components with the help of lysosomes, each cell has its own life span, and each cell respires and draws oxygen from the environment and oxidizes food molecules to liberate energy which stores as ATP molecules.

Differentiation, Dedifferentiation and Redifferentiation

The cells at certain sites in the body of multicellular organism remain undifferentiated and retain the power of mitotic divisions to provide for growth. Such cells are known as undifferentiated cells. At certain times, the specialized cells revert to actively dividing state for producing new cells. This process is known as dedifferentiation. A stem cutting of a plant, say rose when fixed in the soil, sprouts

new roots by dedifferentiation and grows into a new plant. The new cells produced by the dedifferentiation once again differentiate into the cells of various tissue which make up into new parts in place of lost ones. This change in cells is called redifferentiation.

Various Chemical Reactions

Cells function like miniature chemical plants. The simplest bacterial cell is capable of different chemical transformations, which occur at any rate in the inanimate world. All chemical changes that take place in cells require enzymes that increase the rate at which reaction occurs. The sum total of all the reaction in cell represent cell metabolism.

Response to Stimuli

Cells within a multicellular plant or animal respond to stimuli. Most cells are covered with receptors that interact with substance in highly specific ways. Cell possess receptors to hormones, extracellular materials and to substance on surface of other cell. A cell receptors provide pathways through which external agents can evoke specific response in target cells. Cell respond to stimuli by changing their metabolic activities.

Cells Evolve

It is presumed that cells evolved from some type of precellular life form, which in turn evolve from non-living organic material. If we observe the features of bacterial cell living in intestinal tract, cell that is part of lining of intestinal tract, we can see the difference between the two cells. Both are evolved from common ancestral cell that lived more than 3 billion years ago. These structures are shared by two distantly related cells, such as their similar plasma membrane and ribosomes are present in ancestral cells.

Self Regulation

Cell regulatory mechanism becomes evident when they break down, for example, the failure of cell to correct mistake when its duplicate its DNA result in debilitating mutation, transform the cell into a cancer cell.

Highly Complex and Organized

The more complex a structure, greater the number of parts that must be in proper place, less tolerance of errors in nature and interaction of parts, the more regulation that must be exerted to maintain the system.

Every cell is enclosed by a distinct covering called plasma membrane which keeps the cell contents properly and prevent their mixing up with extracellular materials which enables the cell to maintain its highly organized structure and to carry on reaction in regulated manner. The exchange of materials allowed by cell membrane is in a selective and regulated manner which keeps the cell contents distinct from surrounding materials. The cells of bacteria and blue green algae do not contain membrane bound compartments in the cytoplasm. Such single compartment cells are called prokaryotic. The lack of compartments shows the primitive nature of organism. The main advantage of compartments is that to enable

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the cell to keep separate the various kinds of chemical reaction occurring in it all the time. Cell is organized mass of protoplasm which is capable of performing all life activities and is separated from environment by protective and selectively permeable envelope. The envelope is made up of cell wall in (plants) and plasma membrane in (all types of cell). Plasma membrane is part of protoplasm while cell wall is product of unit mass of protoplasm. The unit mass of protoplasm contained in cell is called protoplast. Protoplast is differentiated into four parts plasma membrane, cytoplasm, nucleus and vacuoles.

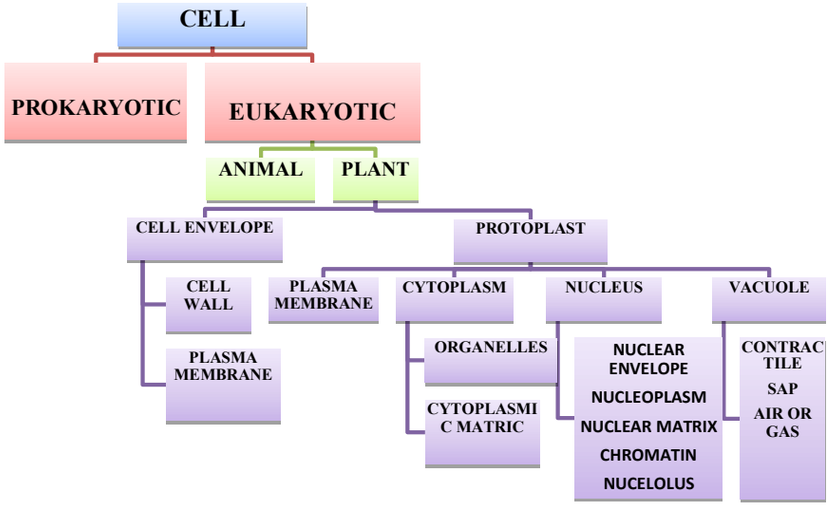

Fig. 1.1 *Cell Classification*

Two main type of cells present are prokaryotic and eukaryotic (Refer Figure 1.1).

Cytoplasm is differentiated into cytoplasmic matrix, cell organelles and cell inclusions. Cytoplasmic matrix (hyaloplasma) is the fluid part of cytoplasm which exists in both sol and gel state respectively called plasmasol and plasma gel. Water constitutes 90% of the matrix. Matrix is the crystallo-colloidal complex where some chemicals are present as true solution and others as colloidal solution, for example, minerals, sugars, amino acids, vitamins, proteins, enzymes, etc.

The autonomic vital movement that occurs in the cytoplasmic matrix also called as protoplasmic streaming or cyclosis. Reason for cyclosis is either sol-gel changes or movement by microfilaments through their molecular motors. The matrix is the seat of synthesis of no of biochemicals like fats, proteins, carbohydrates, coenzymes. Cell organelles exchange materials through cytoplasmic matrix. It helps in distribution of materials inside the cell.

Characteristics that Distinguish Prokaryotic and Eukaryotic Cell

The eukaryotic cell certainly evolved from prokaryotic ancestors. Because of their common ancestry, both type of cell share an identical genetic language, similar set of metabolic pathways and many common structural features. Both type of cells are bounded by plasma membrane that serve as selectively permeable barrier between the living and non-living world. Both type of cell is surrounded by a rigid, non-living cell wall that protects the delicate life form. The genetic material of a prokaryotic cell is present in a nucleoid. In contrast, eukaryotic cell possess a

nucleus, region bounded by a complex membranous structure called nuclear envelope. The chromosomal DNA of eukaryotes unlike prokaryotes is tightly associated with proteins to form complex nucleoprotein material called chromatin. Eukaryotic cell contain array of membrane bound organelles. It includes mitochondria, where chemical energy is made available to fuel cellular activities, an endoplasmic reticulum, where many of cell proteins and lipids are manufactured. Golgi complex, where materials are sorted, modified and transported to specific cellular destination. Plant cell contain additional membranous organelles, i.e., chloroplast which is site of photosynthesis and large vacuole that occupy most of the cell volume. Both the eukaryotic and prokaryotic cells possess ribosomes which are non-membranous particles on which proteins of cell are manufactured. However one major difference between both type of cells are eukaryotic cell divide by complex process of mitosis in which duplicated chromosomes condense into compact structures that are segregated by microtubule containing apparatus called mitotic spindle which allows each daughter cell to receive an equivalent array of genetic material. In prokaryotes, there is no compaction of the chromosome and no spindle. The DNA is duplicated, two copies are separated by the growth of intervening cell membrane. The movement of prokaryotic cell may be accomplished by a thin protein filament called flagellum which protrudes from the cell and rotates.

A Comparison of Prokaryotic and Eukaryotic Cell

Features Held in Common by the both Types of Cells

- Plasma membrane of similar construction.
- Genetic information encoded in DNA using identical genetic code.
- Same mechanism of photosynthesis (between cyanobacteria and green plants).
- Same mechanism for synthesizing and inserting membrane proteins.
- Shared metabolic pathways (for example, glycolysis and TCA cycle).
- Same mechanism for transcription and translation of genetic information including ribosomes.
- Same apparatus for conservation of chemical energy as ATP (located in plasma membrane of prokaryotes and mitochondrial membrane of eukaryotes).
- Protein digesting structure (Proteasomes) of similar construction.

Features of Eukaryotic Cells not found in Prokaryotes

- Complex membranous cytoplasmic organelles (endoplasmic reticulum, Golgi complex, lysosomes, peroxisomes).
- Complex cytoskeletal system (microfilaments, intermediate filaments, microtubules) and associated motor proteins.
- Ability to ingest fluid and particulate matter by enclosure within plasma membrane vesicles.
- Cellulose containing cell wall.

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- Cell division using a microtubule containing spindle that separates chromosomes.
- Presence of two copies of genes per cell.
- Presence of three different RNA synthesizing enzymes (RNA polymerases).
- Sexual reproduction requiring meiosis and fertilization.

Structure of Prokaryotic Cell

A prokaryotic cell is a single envelope system. There is no membrane enveloping the genetic material. It is present in bacteria and blue green algae. Some organisms like mycoplasma, rickettsias and spirochetes are similar to bacteria and are prokaryotes (Refer Figure 1.2). The prokaryotes comes under kingdom Monera and Super kingdom *Prokaryota*. Prokaryotes are divided into two groups: the *Archaea* (Archaebacteria) and the *Bacteria* (Eubacteria). Members of the Archaea are closely related to eukaryotes than they are to the other group of prokaryotes. Archaea include group of organism whose evolutionary ties to one another are revealed by similarities in the nucleotide sequence of their nucleic acid. These species live in extremely inhospitable environment known as extremophiles. It includes methanogens, halophiles, acidophiles and thermophiles, included in the latter group are hyperthermophiles which live in hydrothermal vents of ocean floor. The latest among this group is strain 121, because it is able to grow and divide in superheated water at temperature of 121 degree Celsius. All other prokaryotes comes under domain bacteria. This includes smallest known cell called mycoplasma, only prokaryotes lacking cell wall. The most complex prokaryotes are cyanobacteria, which contain errays of membranes serve as site of photosynthesis.

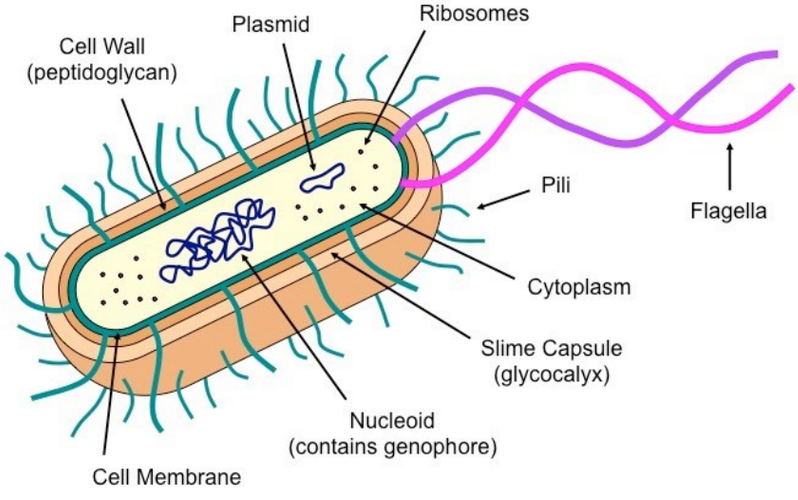

Fig 1.2 *Prokaryotic Cell Structure under Electron Microscope*

Organization of Prokaryotic Cell

Prokaryotic cell mainly contains 3 components:

- Outer covering or cell membrane
- Cytoplasm
- Nucleiod

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Outer Covering: It comprises three layers: inner plasma membrane, middle cell wall and outer slimy capsule.

- **Cell Membrane**: It is thin and flexible, composed of lipids and proteins. It controls the movement of molecules into and from the cell. It carries respiratory enzymes for energy reactions. In bacteria, small infolds of plasma membrane, i.e., mesosomes bear respiratory enzymes and is analogous to mitochondria of eukaryotic cell. In photosynthetic bacteria and in blue green algae, pigment and enzyme molecules that absorb and convert light into chemical energy are associated with plasma membrane and its infolds called photosynthetic lamellae. Photosynthetic lamellae are analogous to chloroplasts of eukaryotic cells. Plasma membrane of prokaryotic cell serve many function which are located in separate membrane bound organelles in eukaryotic cells. Plasma membrane is thought to play role in replication and division of nuclear material. The plasma membrane serve many function which are located in membrane bound organelles in eukaryotic cell. The infolds remains continuous with cell membrane. The prokaryotic cell is non compartmentalized
- **Cell Wall**: The outer covering is surrounded by non-living, rigid cell wall. In gram positive bacteria, cell wall consists of thick layers. It consists of peptidoglycan and teichoic acid. Peptidoglycan consists of complex of oligosaccharide chains and short peptides called murein. The oligosaccharide is made of sugar derivatives acetylglucosamine and acetylmuramic acid joined together by α 1, 4 linkages. Teichoic acid consists of glycerol or ribitol subunits linked by phosphate groups. In gram negative bacteria, the cell wall consists of peptidoglycan, periplasmic space and outer membrane, the peptidoglycan layer lacks teichoic acid. The periplasmic space consists of proteins secreted by cell. The outer membrane consists of lipopolysaccharides. Blue green algae have cell wall structure similar to gram negative bacteria but still they are Gram positive in Gram negative bacteria, i.e., *E.coli*. The peptidoglycan layer is thin about 25Å lacks teichoic acid and lies next to the plasma membrane. The periplasmic space lies outside the peptidoglycan layer and contain proteins secreted by cell. The outer membrane consists of a lipid bilayer and lipopolysaccharides. It is permeable and contain porin proteins which line channels large enough to let chemicals pass.
- **Capsule:** It protects the cell against desiccation and virus attacks. It is present outside the cell wall in form of gelatinous coat.

Cytoplasm: It contains water, proteins, lipids, inorganic ions. It contains enzymes for various biosynthetic reactions and protein forming machinery containing ribosomes, all types of RNA. Ribosomes are the only organelle found in prokaryotic cell. They lie free in cytoplasm and form polyribosome at time of protein synthesis. Cytoplasm do not show streaming movements. It do not show cyclosis, phagocytosis, pinocytosis and exocytosis. They contain gas vacuoles in cytoplasm. They lack well developed organelles except ribosomes (which is 70 S type)

Nucleoid (Genophore): It consists of single chromosome that is coiled, double stranded, circular not associated with histones. Transcription and translation occur in the cytoplasm. There is no nucleolus. It consists of single chromosome, which is coiled forming body. The chromosome is short, simple and attached to membrane.

NOTES

There is a single copy of chromosome. The DeoxyriboNucleic Acid or DNA is double stranded and helical but circular and associated with proteins, replication of DNA is continuous throughout the cell cycle. Transcription and translation occur in the cytoplasm. Mitotic apparatus is not formed during cell division, there is no nucleolus

Plasmids: They are small circular genetic material in addition to nucleiod. They generally code for proteins required by organism in resistance against antibiotics and toxic materials. They are not essential for the growth of the cell. Some encode proteins needed by organism to resist antibiotics.

Pili: Short, rod like non motile structure made up of protein pilin. They are used for attachment to surfaces. Many bacteria in addition to flagella is short, rod like non motile processes called pili or fimbriae on the surface, formed of protein pilin. Pili is used for attachment to surfaces, some bacteria bear tubular sex pili.

Flagella: Many bacteria bear whip like locomotory organelle called flagella. It is locomotory organelle present in prokaryotes composed of spiral chains of subunits of protein named flagellin which is unrelated to actin or tubulin. A bacterial flagellum is about 150Å thick and 10-15µm long. It has two main parts- filament and basal body.

Filament: It has the form of a fixed, spiral resembling a corkscrew. It is composed of intertwined spiral chains of subunits of protein named flagellin, it is unrelated to actin or tubulin.

Basal Body: It comprised of two regions: shaft and hook. The shaft bears a pair of rings in gram positive bacteria and two pairs of rings in gram negative bacteria. The inner ring is embedded in the plasma membrane and fixed to the shaft. The outer ring is attached to the cell wall but is free to slip on the shaft surface called S ring. The bacterial flagellum doesn't have any bounding membrane and grows at the tip. This is in contrast to the eukaryotic flagellum which is bounded by a cell membrane and grows at the base adjacent to the basal body.

Structure of Eukaryotic Cell

Organisation

A eukaryotic cell is two envelope system. First membrane surrounds the cell while second envelope the nucleus and other organelles. It occurs in protists, fungi, plants and animals. The most complex cells are not found inside plants and animals but in single celled protists. Complex unicellular organism represent one evolutionary pathway. An alternate pathway leads to evolution of multicellular organism in which different activities are carried out by different specialized cells, which is formed by the process of differentiation. The pathway of differentiation followed by each embryonic cell depends upon the signals it receives from surrounding environment. Most eukaryotic cell possess single nucleus that contains only two copies of most genes (Refer Figure 1.3).

Size

Human cells range from 20 to 30µm. Nerve cells are the longest in size. Among the smallest are 2µm long Plasmodium vivax. The size of the cell is directly related

to its function. The surface area of cell tells the capacity to exchange material with the environment. Cells and their organelles are defined in micrometers. There are many reasons for cell being small in size. The greater a cells cytoplasmic volume, longer it takes to synthesize the messages required by the cell. As cell increases in size, surface area / volume decreases. The ability of a cell to exchange substance with its environment is proportional to its surface area. If cell grows beyond a certain size, its surface would not be sufficient to take up the substance.

Shape

It may be spherical, polygonal, disc like, oval, spindle like, irregular. The shape is related to its function. Muscle and nerve cell are adapted to their function. Form of cell depends on its function, pressure, age and viscosity.

Number

Majority of organisms have numerous cells so are called multicellular organism while organism made up of single cell is unicellular. Though all multicellular organism begin life as single cell, i.e., zygote.

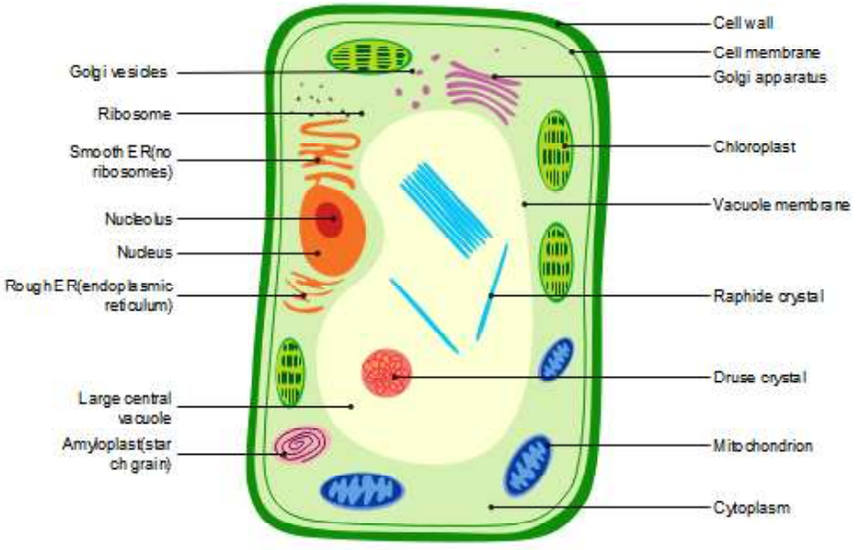

Fig 1.3 *Ultrastructure of a Eukaryotic Cell with Central Vacuole Cell Wall (Plant Cell)*

Physical Structure

It is composed of three layers:

- Cell membrane
- Cytoplasm
- Nucleus

Cell Membrane

All eukaryotic cells are surrounded by elastic living covering called cell membrane. It is composed of lipid protein complex and lack respiratory enzymes. Certain protists, fungi and plant cells have rigid non-living covering called cell wall which protects and supports the cell.

Cytoplasm

The part of cell between nucleus and plasma membrane is called cytoplasm. It consists of semifluid, translucent ground substance cytosol. Cytoplasm shows streaming movements and contain two structures, i.e., organelles and inclusions. Each organelle has its own function which is regulated by the control centre of the cell called nucleus

Inclusions are non-living or deutoplasmic structures that includes stored organic materials (starch grains, aleurone layers, fat droplets and glycogen granules, pigment granules and inorganic crystals.

Nucleus: The genetic material is enclosed by distinct envelope. It is double stranded, helical and linear complexed with histones and non-histones. Replication of DNA occurs during S phase of cell cycle. Transcription occurs in nucleus and translation in cytoplasm. One or more rounded bodies called nucleoli is present in the nucleus. Nucleus controls all the activities of the cell and cell devoid of it dies.

Plasmids: There are no plasmids present in eukaryotic cells. Cilia and flagella have 9+2 plan of microtubules, composed of proteins tubulin. The cilia and flagella move the fluid around them and act as locomotory organelle.

Difference between Prokaryotic and Eukaryotic Cell

Prokaryotic Cells	Eukaryotic Cells
• DNA is naked and circular, not associated with histones	• DNA is linear and associated with histone proteins to form nucleosomes
• Ribosomes are 70 S type	• Ribosomes are 80 S and 70 S in organelles
• Single envelope organization is present	• Two envelope organization is present
• An organized nucleus is absent, instead nucleoid is present	• An organized nucleus is present
• A spindle apparatus is not formed	• A spindle apparatus is generally present
• There is no change in the amount of DNA throughout the life cycle	• There is regular change in amount of DNA in the life cycle
• Transcription and Translation occur in cytoplasm	• Transcription occurs inside nucleus and translation in cytoplasm
• Protein present in flagella is flagellin	• Flagella has tubulin protein
• Pilli and plasmids are found in organisms which help in conjugation	• Both pili and plasmids are absent in eukaryotes
• Flagella if present , are single stranded	• Flagella if present are 11 stranded
• Sap vacuoles are absent, Gas vacuoles may be present	• Sap vacuoles are present. Gas vacuoles are absent
• Examples: Bacteria, Blue green algae, mycoplasma	• Examples: Algae (Except Blue green algae), Fungi, plants and animals

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Structure and Functions of Protein and Enzyme

Proteins are built as chains of **amino acids**, which then fold into unique **three-dimensional shapes**. Bonding within protein molecules helps stabilize their structure, and the final folded forms of proteins are well-adapted for their functions.

The significance of the unique sequence, or order, of amino acids, known as the protein's **primary structure**, is that it dictates the **3-D conformation** the folded protein will have. This conformation, in turn, will determine the function of the protein. We shall examine protein structure at four distinct levels (Refer Figure 1.4) how sequence of the amino acids in a protein (primary structure) gives identity and characteristics to a protein, how local interactions between one part of the polypeptide backbone and another affect protein shape (secondary structure) how the polypeptide chain of a protein can fold to allow amino acids to interact with each other that are not close in primary structure (tertiary structure); and how different polypeptide chains interact with each other within a multi-subunit protein (quaternary structure) found in biological cell.

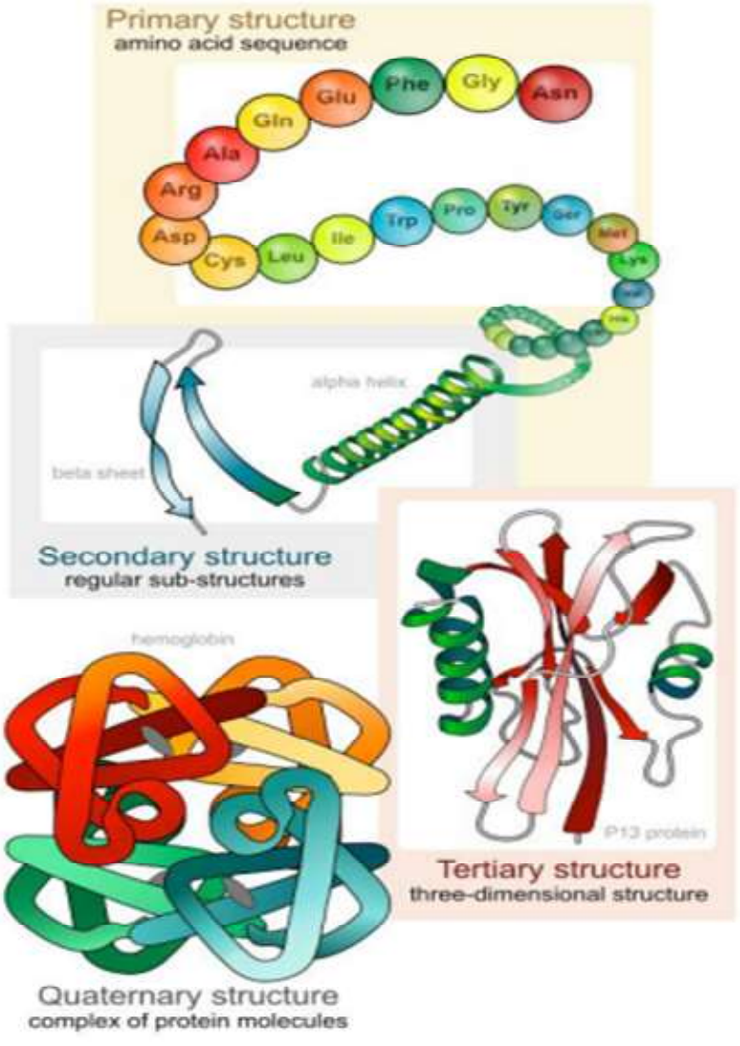

Fig. 1.4 *Four Levels of Protein Structure*

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Proteins are responsible for nearly every task of cellular life, including cell shape and inner organization, product manufacture and waste clean-up, and routine maintenance. Proteins also receive signals from outside the cell and mobilize intracellular response.

Main Functions of Protein in biological Cell

- Protein is necessary in building and repairing body tissues
- Hormone Production
- Hormones are chemicals produced by glands in one part of the body that help coordinate activities and communicate with other areas
- Enzymes
- Immune Function
- Energy

The collection of proteins within a cell determines its health and function. Proteins are responsible for nearly every task of **cellular life**, including cell shape and inner organization, product manufacture and waste clean-up, and routine maintenance. Proteins also receive signals from outside the cell and mobilize intracellular response. They are the workhorse macromolecules of the cell and are as diverse as the functions they serve.

Proteins can be big or small, mostly **hydrophilic** or mostly **hydrophobic**, exist alone or as part of a **multi-unit structure,** and change shape frequently or remain virtually immobile. All of these differences arise from the unique amino acid sequences that make up proteins. Fully folded proteins also have distinct surface characteristics that determine which other molecules they interact with. When proteins bind with other molecules, their conformation can change in **subtle or dramatic ways**.

Protein functions are as diverse as protein structures. For example, structural proteins maintain cell shape, akin to a skeleton, and they compose structural elements in connective tissues like cartilage and bone in vertebrates. **Enzymes** are another type of protein, and these molecules catalyse the biochemical reactions that occur in cells. Other work of proteins like as monitors, changing their shape and activity in response to **metabolic signals** or messages from outside the cell. Cells also secrete various proteins that become part of the extracellular matrix or are involved in **intercellular communication**.

Proteins are sometimes altered after translation and folding are complete. In such cases, so-called **transferase enzymes** add small modifier groups, such as phosphates or carboxyl groups, to the protein. These modifications often shift protein conformation and act as molecular switches that turn the activity of a protein on or off. Many post-translational modifications are reversible, although different enzymes catalyse the reverse reactions. For example, enzymes called **kinases** add **phosphate groups** to proteins, but enzymes called **phosphatases** are required to remove these phosphate groups.

Cells be dependent on thousands of different enzymes to catalyse metabolic reactions. Enzymes are proteins, and they make a biochemical reaction more likely

to proceed by lowering the activation energy of the reaction, thereby making these reactions proceed thousands or even millions of times faster than they would without a catalyst. Enzymes are highly specific to their substrates. They bind these substrates at complementary areas on their surfaces, providing a snug fit that many scientists compare to a lock and key. Enzymes work by binding one or more substrates, bringing them together so that a reaction can take place, and releasing them once the reaction is complete. In particular, when substrate binding occurs, enzymes undergo a conformational shift that orients or strains the substrates so that they are more reactive.

The name of an enzyme usually refers to the type of biochemical reaction it catalyses. For example, proteases break down proteins, and dehydrogenases oxidize a substrate by removing hydrogen atoms. As a general rule, the '-Ase' suffix identifies a protein as an enzyme, whereas the first part of an enzyme's name refers to the reaction that it catalyses.

1.2.1 DNA and RNA in Living System

RiboNucleic Acid or RNA and DeoxyriboNucleic Acid or DNA are polymers constituted of monomers called mononucleotide units. These mononucleotide units are united together by intermolecular dehydration reactions that make phosphate ester bonds. Specialized enzymes catalyse such reactions.

Structure and Properties of DNA

In the early 1950s, four scientists, James Watson and Francis Crick at Cambridge University and Maurice Wilkins and Rosalind Franklin at King's College, determined the true structure of DNA from data and X-ray pictures of a molecule taken by Franklin. In 1953, Watson and Crick published a paper in the scientific journal *Nature* describing their research. They showed that DNA molecule is not only double-stranded, but also has two strands attached around each other forming a coil, or helix. A double helix represents the exact structure of DNA.

Except viruses, in most living organisms, genetic information is stored in DNA. DNA resides in the nucleus of living cells. Four different nucleotide bases occur in DNA, namely Adenine (A), Cytosine (C), Guanine (G) and Thymine (T).

The nucleotide bases of the DNA molecule form complementary pairs with the nucleotides hydrogen bond to another nucleotide base in a strand of DNA, opposite to the original. This bonding is specific, with adenine always pairing with thymine (and vice versa) and guanine always pairing with cytosine (and vice versa).

A unique feature of DNA molecule is that it is able to make exact copies of itself, or is self-replicate. When an organism requires more DNA, such as during reproduction or cell growth, a break in the hydrogen bonds between the nucleotide bases takes place, leading to the separation of two single strands of DNA. New complementary bases are brought in by the cell and paired up with each of the two separate strands, thus forming two new, identical, double-stranded DNA molecules.

DNA is present in cells in the long structures in the nucleus, also known as chromosomes. These chromosomes duplicate before the cells divide and this

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process is known as DNA replication. Chromosomes are condensed thread-like structures of DNA. DNA is present in the eukaryotes inside the cell nucleus.

A small amount of DNA is also available in cell organelles like mitochondria or chloroplasts. On the other hand, in prokaryotes like bacteria and archaea, DNA is present in the cytoplasm. Chromatin proteins, such as histones, compact and organize DNA into chromosomes. A DNA molecule consists of two long polymers, which face in the opposite direction and are known as 'anti-parallel'. This means that in a double helix the direction of the nucleotides in one strand is opposite to the direction of the other strand.

Every sugar moiety is connected to one of the four types of molecules known as bases. Each stand has polarity, that is, a top and a bottom that imparts 3' (three prime) and 5' (five prime) ends. Information is encoded within the sequence of these bases along the backbone. The 5' end has a terminal *phosphate group* while whereas the 3' end has a terminal *hydroxyl group*. Hydrogen bonds that bind the bases attached to the two strands help in stabilizing the DNA double helix. Figure 1.5 shows the DNA molecular topography.

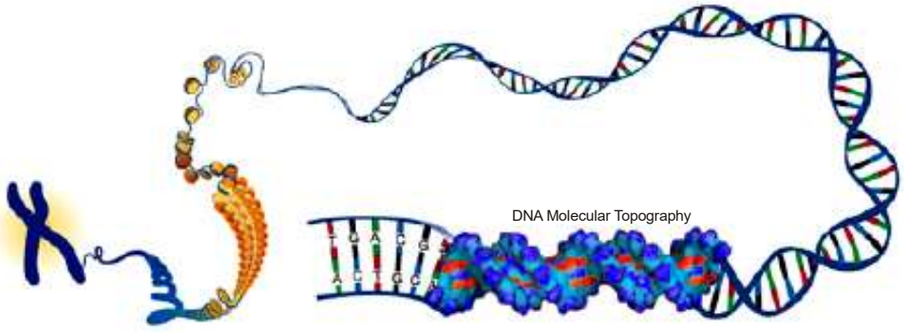

Fig. 1.5 *Molecular Topography of DNA*

Chemically, DNA is a long polymer that is comprised of nucleotide units. The DNA chain is 22–26 Å (Ångströms) wide and each nucleotide unit is 3.3 Å long. DNA polymers are large molecules that may have millions of nucleotides.

DNA exists as paired molecules in living beings. The two polymers, or strands, interweave like vines. This gives it the characteristic double helical shape. A nitrogenous base bonded to a sugar is known as a nucleoside and a base bonded to a sugar and one or more phosphate groups is known as a nucleotide. If multiple nucleotides are bonded together, like in DNA, this polymer is called a polynucleotide.

Nitrogenous base + sugar	**= Nucleoside**
Nitrogenous base + sugar + phosphate group	**= Nucleotide**
Multiple nucleotides linked together	**= Polynucleotide**

Figure 1.6 shows the chemical structure of DNA and the hydrogen bonds are shown as dotted lines.

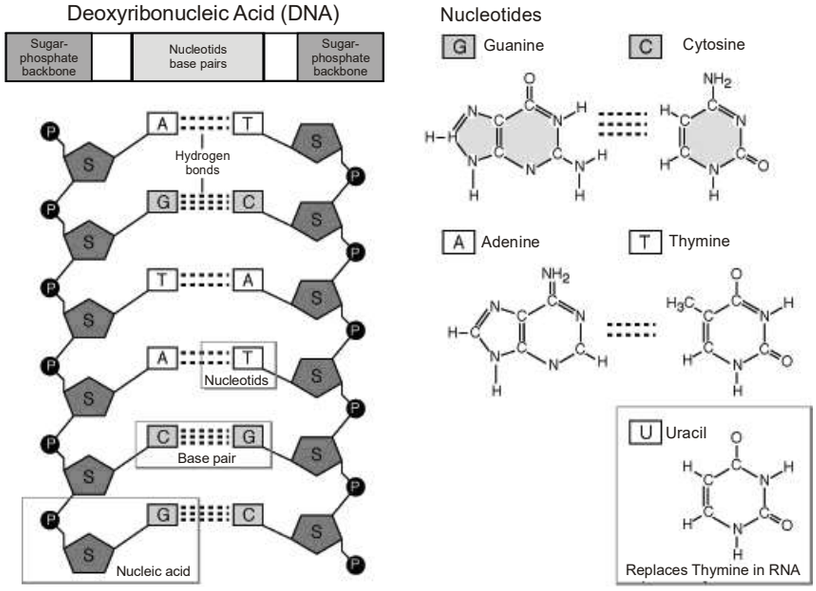

Fig. 1.6 *Chemical Structure of DNA*

The backbone of the DNA strand consists of alternating phosphate and sugar residues. The sugar in DNA is known as 2-deoxyribose, a pentose (five-carbon) sugar. Pentose can be differentiated from ribose as shown in Figure 1.7. In RNA ribose sugar is present in place of deoxyribose. The sugars join together forming phosphodiester bonds between third and fifth carbon atoms of adjacent sugar rings, as shown as Figure 1.8.

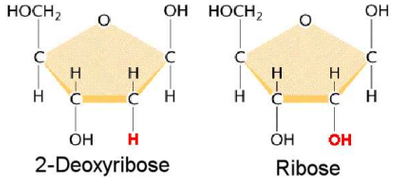

Fig. 1.7 *Difference between 2-Deoxyribose Sugar and Ribose Sugar*

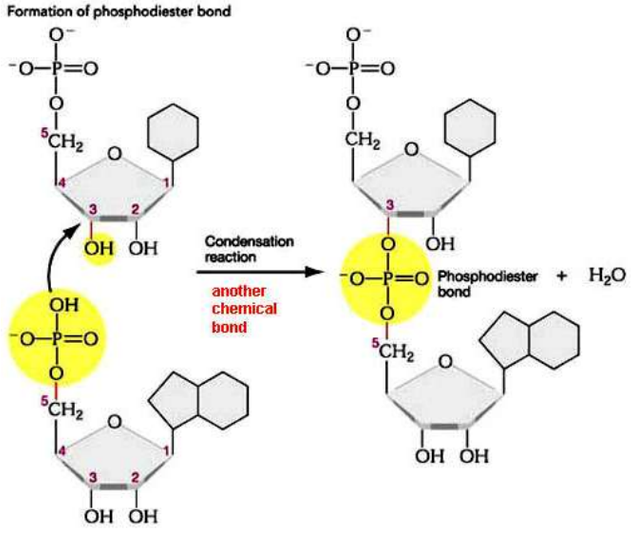

Fig. 1.8 *Formation of Phosphodiester Bond and Polynucleotide*

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Bases in DNA

The bases in DNA are classified into two types:

1. **Purines:** Examples of purines include adenine and guanine (fused five- and six-membered heterocyclic compounds).
2. **Pyrimidines**: Examples of pyrimidines include cytosine and thymine (six-membered rings).

Uracil (U), a fifth pyrimidine base, usually replaces thymine in RNA and differs from thymine because it lacks a methyl group on its ring.

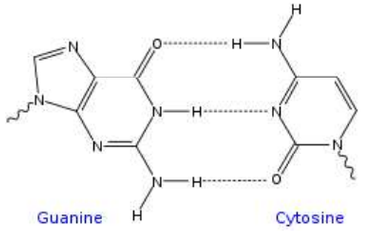
Grooves

A twisted DNA molecule shows two types of grooves that are different based on size. The major groove is 22 Å wide while the minor groove is 12 Å wide. Because it is narrow, the edges of the bases are less accessible in the minor groove in comparison to major groove that leads to lesser interaction. Proteins such as transcription factors that can bind to specific sequences in double-stranded DNA therefore make contacts to the sides of the bases exposed in the major groove normally.

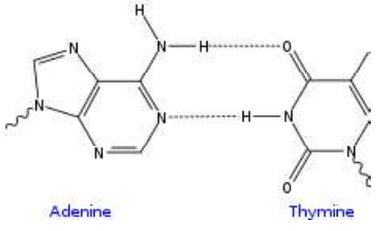
Base pairing

Complementary base pairing is when each type of base on one strand establishes a link with exactly one type of base on the other strand. Purines form hydrogen bonds with pyrimidines. The arrangement of two nucleotides binding together across the double helix is known as a **base pair**.

The double helical DNA strands can be pulled apart like a zipper either by mechanical force or by applying high temperature. The concept of 'Complementarity' causes duplication of all information in the double-stranded sequence of a DNA helix, which is essential in DNA replication. This reversible, specific interaction between complementary base pairs is crucial for all DNA functions in living organisms (Figure 1.9). In this figure, non-covalent hydrogen bonds between the pairs are shown as dashed lines.



(a) GC base pair with three hydrogen bonds



(b) AT base pair with two hydrogen bonds

Fig. 1.9 *Base Pairing*

The two types of base pairs are able to form various numbers of hydrogen bonds. The AT base pair forms two hydrogen bonds while the GC base pair forms three hydrogen bonds. DNA that has high GC content is more stable than DNA that has low GC content. The DNA double helix that separates easily tends to have a high AT content. The strength of the interaction can be calculated by finding the temperature that is needed to break hydrogen bonds. This is known as the melting temperature or T_m value. When all the base pairs in a DNA double helix melt, the strands separate and are there in solution as two completely independent molecules.

Sense and anti-sense

A DNA sequence is referred to as 'sense' if its sequence is the same as that of a messenger RNA copy that is translated into protein. Its opposite is known as the 'anti-sense' when the sequence is on the opposite strand. Some DNA sequences in prokaryotes and eukaryotes, and more in plasmids and viruses, distort the difference between sense and anti-sense strands by having overlapping genes.

Supercoiling

The process of DNA supercoiling involves twisting DNA into a rope-like structure. If the DNA is twisted in the direction of the helix, it is known as *positive supercoiling*. The bases are held more tightly together but if they are twisted in the opposite direction, it is known as *negative supercoiling*. This way the bases come apart more easily. Most DNA have slightly negative supercoiling introduced by enzymes called topoisomerases.

Alternate DNA structures

The many conformations in which DNA exists include A-DNA, B-DNA and Z-DNA (Refer Figure 1.10). The conformation adopted by DNA is dependent on the hydration level, DNA sequence, amount and direction of supercoiling, chemical modifications of bases, type and concentration of metal ions and the presence of polyamines in solution.

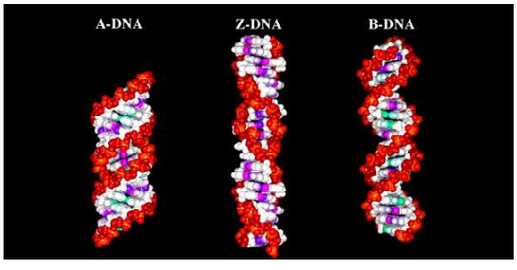


Fig. 1.10 *Structures of A, Z and B DNA (from Left to Right)*

As compared with B-DNA, A-DNA forms a wider, right-handed spiral with a shallow, wide minor groove and a narrower, deeper major groove. The A form occurs under non-physiological conditions in partially dehydrated samples of DNA. DNA segments, where the bases have been chemically modified by methylation, may undergo a larger change in conformation and adopt the Z form. Here, the strands turn about the helical axis in a left-handed spiral, the opposite of the more common B form.

Base modifications

It is supposed that base modifications are involved in packaging. Regions that have low or no gene expression usually have high methylation levels of cytosine bases (Refer Figure 1.11). Despite the significance of 5-methylcytosine, it can deaminate to leave a thymine base. Methylated cytosines are therefore particularly prone to mutations. Other base modifications include adenine methylation in bacteria and the presence of 5-hydroxymethylcytosine in the brain. Deamination converts 5-methylcytosine into thymine.

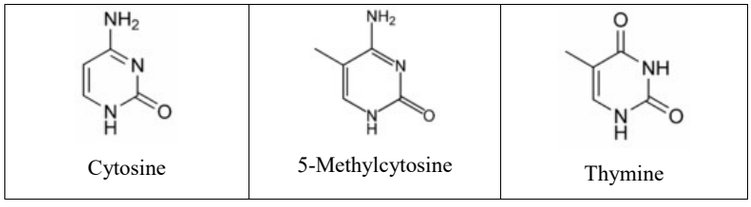


Fig. 1.11 *Structure of Cytosine, 5-Methylcytosin*

Structure and Properties of RNA

RNA has derived its name from the sugar group present in its backbone, that is, ribose. Some of the features of DNA and RNA are common. However, there are also features that differentiate DNA from RNA. Both have a sugar-phosphate backbone with nucleotide bases attached to it. Adenine, cytosine and guanine are common in both. However, RNA lacks thymine. It contains uracil in place of thymine. Further, DNA is a double-stranded molecule, whereas RNA is a single-stranded molecule.

RNA is the key genetic material used in microorganisms, such as viruses. It also plays an important role in the production of proteins in other living organisms. Due to its ablity to move around the cells of living organisms, it functions as a genetic messenger, transmitting the information contained in the cell's DNA to other parts of the cell. This transmission helps in protein synthesis.

Ribonucleic acid (RNA) is a biologically important molecule that consists of a long chain of nucleotide units. Each nucleotide has a nitrogenous base, a ribose sugar and a phosphate. RNA is transcribed from DNA by enzymes known as RNA polymerases and is usually processed further by other enzymes. RNA is considered very important in the synthesis of proteins. Messenger RNA (mRNA), a type of RNA, carries information from DNA to ribosomes, made of proteins and ribosomal RNAs that bind together to form a molecular machine that can read mRNAs and translate the information they carry into proteins. Other RNAs with diverse roles exist – in particular, these regulate which genes are expressed and are also the genomes of most viruses.

Each nucleotide in RNA comprises ribose sugar, with carbons numbered from 1' to 5'. A base is attached to the 1' position, generally Adenine (A), Cytosine (C), Guanine (G) or Uracil (U). Adenine and guanine are purines while cytosine and uracil are pyrimidines.

A phosphate group is attached to the 3' position of one ribose and the 5' position of the next ribose. Phosphate groups have a negative charge at

physiological pH, making RNA a charged molecule. The bases may form hydrogen bonds between cytosine and guanine, between adenine and uracil and between guanine and uracil (Refer Figure 1.12).

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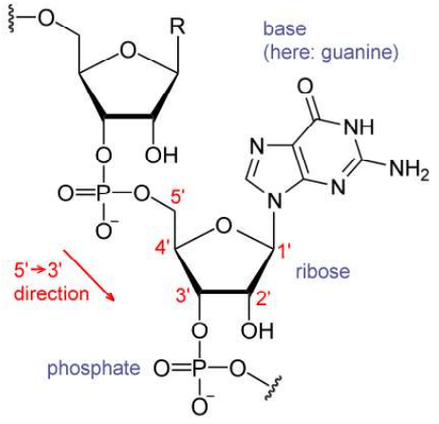

Fig. 1.12 *RNA Nucleotide with Ribose Sugar, Phosphate and Base*

The functional form of single-stranded RNA molecules frequently asks for a certain tertiary structure. The basis for this structure is given by secondary structural elements that are hydrogen bonds within the molecule. This causes many recognizable secondary structure 'Domains', such as hairpin loops, bulges and internal loops. Since RNA is charged, metal ions such as Mg^{2+} are required to stabilize many secondary and tertiary structures.

Comparison with DNA

RNA and DNA are both nucleic acids, but differ in the following ways:

DNA	RNA
1. Long polymer with a deoxyribose and phosphate backbone 2. Four different bases: adenine, guanine, cytosine and thymine	1. Polymer with a ribose and phosphate backbone 2. Four different bases: adenine, guanine, cytosine, and uracil
3. It is a nucleic acid containing the genetic instructions used in the development and functioning of living organisms 4. Stands for deoxyribonucleic acid 5. A double- stranded molecule with a long chain of nucleotides 6. Deoxyribose sugar in DNA is less reactive because of C-H bonds. 7. Stable in alkaline conditions 8. Has smaller grooves where the damaging enzyme can attach which makes it harder for the enzyme to attack DNA. 9. The helix geometry of DNA is of B-Form. 10. Can be damaged by exposure to Ultra-violet rays	3. The main job of RNA is to transfer the genetic code required creating proteins from the nucleus to the ribosome. This process prevents the DNA from leaving the nucleus, so that it stays safe. Without RNA, proteins could never be made. 4. Stands for ribonucleic acid. 5. A single-stranded molecule in most of its biological roles and has a shorter chain of nucleotides 6. Ribose sugar is more reactive because of C-OH (hydroxyl) bonds. 7. Not stable in alkaline conditions. 8. has larger grooves which makes it easier to be attacked by enzymes. 9. The helix geometry of RNA is of A-Form. 10. RNA is more resistant to damage by Ultra-violet rays.

Most biologically active RNAs, such as mRNA, tRNA, rRNA, snRNAs and other non-coding RNAs, have self-complementary sequences that enable parts of the RNA to fold and pair with itself. This causes double helices to form. Structural analysis of these RNAs shows that they are highly structured and their structures, unlike DNA, do not consist of long double helices but rather collections of short helices packed together into structures similar to that of proteins. Thus, RNAs can attain chemical catalysis like enzymes do.

Types of RNA

There are three types of RNA, which are as follows:

1. **Ribosomal RNA (rRNA) (Insoluble RNA):** This form of RNA has the highest molecular weight. It is the most abundant RNA of all types of RNAs. There are two different types of RNA in the prokaryotic and in eukaryotic cells. In prokaryotes the ribosome possesses two different subunits, large and small. Large subunits (50 s) contains two types of rRNA;. 23 s and 5 s named upon the sedimentation behaviour. The smaller subunit (30 s) contains 16 s types.

   The ribosome of eukaryotes also possesses three different types of rRNAs named on the sedimentation behaviour. Larger subunit contains 5 s and 28 s types. The smaller subunit contains 18 s types.

   The mammalian rRNA possesses 5 s, 5.8 s and 28 s types in larger subunit and in smaller subunit it contains only one 18 s types. Figure 1.13 shows the structure of rRNA.

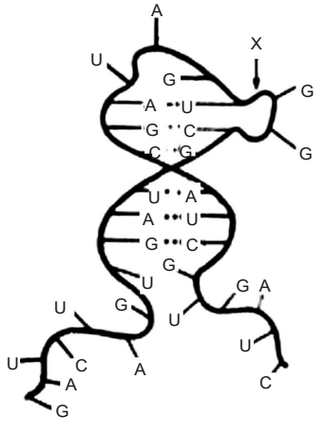

Fig. 1.13 *Structure of rRNA*

2. **Messenger RNA (mRNA) (Template RNA):** Messenger RNA is the most heterogeneous in size and stability. Its amount is 5 per cent of total RNA of the cell. It is synthesized on the surface of the DNA template. This carries the genetic information to assemble the amino acids from DNA to ribosomes, hence it is called messenger RNA. In prokaryotes the mRNA is unstable rather than in eukaryotes. The mRNA is complementary to the DNA template strand. In eukaryotes there are specific 5' cap, which is the recognition site for ribosome subunit, whereas in prokaryotes there is no

specific 5' recognition site. As a result, there can be many ribosomal binding sites in prokaryotes, each one giving rise to a different type of protein.

In mammals the 5' cap is methylated that means a cap of 7-methyle guanosine triphosphate is linked to the 5' end. The 3' end of mRNA possess 20 -250 nucleotides of adenylate residue, which is called poly A tail. This tail probably works as a stabilizer in the cell. Figure 1.14 shows the structure of mRNA.

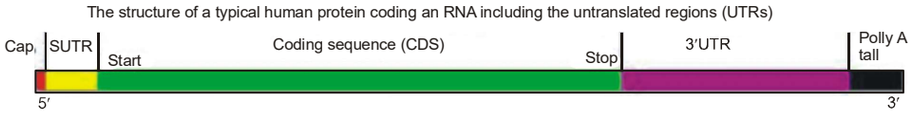

Fig. 1.14 *Structure of mRNA*

3. **Transfer RNA (tRNA) (Soluble RNA):** It is the smallest polymeric form of RNA. It is nearly 15 per cent of total RNA of the cellular RNA. The most important function of tRNA is to act as a specific carrier of activated amino acids to specific site on the protein synthesis. Now, nearly 50 sequences are known. Robert W. Holley presented the clover leaf model for the tRNA. Figure 1.15 shows the structure of tRNA.

   The common structural features of tRNA are as follows:

   - All tRNAs have a common design, consisting of three folds. This gives them a shape of a clover leaf.
   - The molecular weight of all tRNA molecules range from 24,000 to 31,000.
   - tRNAs contain 7-15 unusual bases. Many of them are methylate of unmethylated derivatives of A, U, G, C.
   - The 5' end is phosphorylated and terminal residue is usually guanylate.
   - The base sequence of 3' end of all tRNAs is CCA.
   - Nearly 60 per cent of the nucleotides in tRNAs bases are paired to form double helices.
   - There are 5 groups of bases which are not base paired. These forms loops which are as follows:
     - o 3' CCA terminal region
     - o The Ribothymine-Pseudouracil-cytosine (RPC) loop
     - o The extra arm or little loop
     - o The DiyHdrouracil loop (DHU)
     - o Anticodon loop
   - The unique feature of all the tRNAs is that distance between CCA to anticodon is constant.

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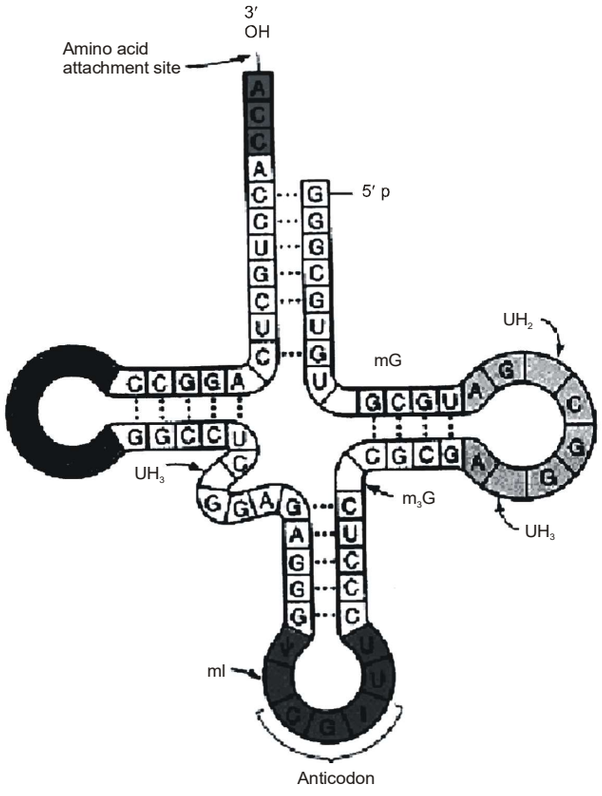

Fig. 1.15 *Structure of tRNA*

DNA is Genetic Material

There is direct evidence that DNA is genetic material. According to Frederick Griffith (1928), there are two types of strains of *dipococcus pneumonia*:

(i) Smoth (virulent strain)

(ii) Rough (avirulent strain)

Smooth strain has a specific polysaccharide capsule and the rough strain lacks this specific capsule. Both these S and R strains are of many types designated as SI, SII, SIII. and RI, RII, RIII , and so on. In his experiment, Griffith injected laboratory mice with live RII strain and the mice suffered no illness. When he injected the SIII strain the mice became ill and died. But when he injected heat-killed SIII, the mice did not fall ill. When he injected a mixture of RII and heat-killed SIII, the mice became ill and died, which he found strange. By conducting a postmortem on the dead mice, he found that the heart of the mice contained both RII and SIII strains.

On the basis of the fact that S form can mutate to R form but R form cannot mutate to S form, he concluded that bacteria must have a transformation of the living RII strain and restoration of the capsule in the presence of heat-killed SIII strain.

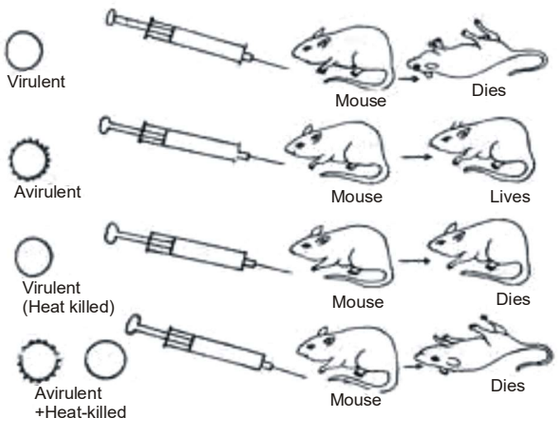

Fig. 1.16 *Bacterial Transfomation*

However, he could not understand the cause of bacterial transfomation. In 1944, Oswald Avery, Colin Macleod and Maclyn McCary demonstrated that it was DNA.

Semi-conservative nature of DNA: The Watson and Crick's model of DNA structure and replication suggested that the replication is semi conservative. According to this method, the two original polynucleotide strands of the duplex will unwind and each will serve as a template for the new strand. Both duplexes that result from replication should be hybrid in nature, each containing an old strand derived from the original strand and a new strand which has been formed during the replication process.

Since each of the two double helices conserves only one of the parent polynucleotide strand, the process is said to be **semiconservative.**

Genetic Code: The DNA molecule is composed of three kinds of moieties phosphoic acids, deoxyribose sugar and nitrogen base. The poly sugar phosphate backbone is always the same but the nitrogenous bases differ from one segment to another. Therefore, the information might depend on their sequence. The sequences of nitrogen bases of a given segment of DNA molecule, have been found to be identical to the linear sequence of amino acids sequence in protein molecule.

First letter	Second letter: U	Second letter: C	Second letter: A	Second letter: G	Third letter
U	UUU, UUC: Phe; UUA, UUG: Leu	UCU, UCC, UCA, UCG: Ser	UAU, UAG: Tyr; UAA, UAG: Stop Stop	UGU, UGC: Cys; UGA: Stop; UGG: Trp	U C A G
C	CUU, CUC, CUA, CUG: Leu	CCU, CCC, CCA, CCG: Pro	CAU, CAC: His; CAA, CAG: Gin	CGU, CGC, CGA, CGG: Arg	U C A G
A	AUU, AUC, AUA: He; AUG: Met	ACU, ACC, ACA, ACG: Thr	AAU, AAC: Asn; AAA, AAG: Lys	AGU, AGC: Ser; AGA, AGG: Arg	U C A G
G	CUU, CUC, CUA, CUG: Val	GCU, GCC, GCA, GCG: Ala	GAU, GAC: Asp; GAA, GAG: Glu	GGU, GGC, GGA, GGG: Gly	U C A G

Fig. 1.17 *Genetic Code*

The properties of the genetic code are as follows:

1. The code is a triplet codon: The nucleotides of mRNA are arranged as a linear sequence of codons, each consisting of three successive nirogenous bases.
2. The codon is non-overlapping: In translating mRNA molecules, the codons do not overlap but read sequentially.
3. The code is commaless: It means that after one amino acid is coded, the second amino acid will be coded automatically by the next three letters.
4. The code is non- ambiguous: Non-ambiguous code means that a particular codon will always code for the same amino acids.
5. The code has polarity: The code is always read in a fixed direction, i.e, 5' 3'direction.
6. The code is degenerate: More than one codon may specify the same amino acid, which is called degeneracy of code.
7. Some codes act as start codons: In most organisms, the AUG codon act as the start codon. In rare cases, GUG also serves as a start codon.
8. Some codes act as stop codons: Three codon UAG, UAA ad UGA are the chain stop or termination codon.
9. The code is universal: Same genetic code is found valid for all organisms ranging from bacteria to man.

A Helix-To-Coil Transition Model

Consider a macromolecule consisting of N units (think residues) comprising ν helical sequences. There is a total of NH helical units and NC = N-N_H coil units. Define the partition function for the melting of one helical unit as $\mathbf{s = exp\ (\Delta H_m / RT)}$ where $\mathbf{\Delta H_m}$ is the enthalpy needed, **R** is the molar gas constant (related to the Boltzman constant $\mathbf{k_B}$ through **Avogadro's number** $\mathbf{N_{Av}}$ as R = $\mathbf{k_B N_{Av}}$) and **T** is the temperature in absolute units. Assume that it takes no enthalpy to form a coil so that the partition function for a coil unit is equal to 1. Define the partition function for the removal of one helical sequence as σ. The partition function for the helix-to-coil melting process is:

$$\mathbf{Z = \Sigma \prod s^{NH} \sigma^{v}}$$

The product $\prod$ is taken over all helical units $\mathbf{N_H}$ and all helical sequences **v** and the summation $\mathbf{\Sigma}$ is taken over all possible configurations (i.e., over all possible unit arrangements to form the macromolecule with **N** units).

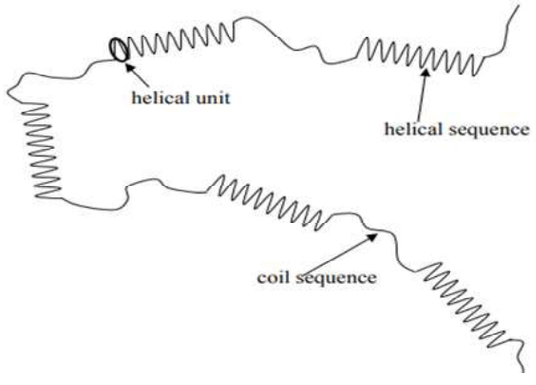

Fig. 1.18 *Schematic Representation of the Helical and Coil Sequences*

1.3 CELL MEMBRANE

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Plasma membrane is the outer membrane covering of cell protoplasts discovered by Schwann (1838). It was called cell membrane by Nageli and Cramer (1855). The name plasmalemma of plasma membrane was given to it by Plowe (1931). Biomembranes are not visible under the light microscope because their thickness is below the resolving power of the microscope. Under electron microscope biomembranes appear to be trilaminar or tripartite. Freeze etching technique has shown that a membrane possesses particles of different sizes. Membrane occur inside the cytoplasm. Internal membranes are rare in prokaryotes. They occur in eukaryotic cells as covering of several cell organelles like nucleus, mitochondria, plastids, lysosomes, Golgi bodies, peroxisomes, etc. Vacuoles are separated from cytoplasm by a membrane called tonoplast. All membranes whether external or internal are called cell membranes or biomembranes. Average thickness of membrane is 75 Å. Biomembranes are selectively permeable for solutes but semipermeable for water.

The asymmetric functioning of such system is of little value to cells. It was unclear for many years as to how a phospholipid bilayer which is only 5 nm thick, could be strong enough to withstand stress on the plasma membrane of most cells. The size of most animal cells are small enough for adhesive forces between water molecules, to maintain spherical cell shape inside a lipid bilayer surface. Plasma membrane is able to lie passively as unreinforced thin lipid barriers at the surface of cell. Monocytes surrounded by thin membranes are deformed as they migrate between vascular endothelial cells in response to injury. Cell membrane face continuous challenge to their integrity from within. An area of membrane about equal to area of entire cell surface turns over every 30 min in many cells due to endocytosis and exocytosis. Endocytosis and exocytosis depend on the cytoskeleton for movement of vesicles to and from plasma membrane and cytoskeletal infrastructure influences the motion of membrane. The first proposal that cellular membranes might contain a lipid bilayer was made in 1925 by two Dutch scientists E. Gorter and F. Grendel. They extracted the lipid from human blood cells and measured the amount of surface area the lipid would cover. Mammalian red blood cell lack both nuclei and cytoplasmic organelles, plasma membrane is the only lipid containing structure and all of the lipids extracted from the cells can be assumed to have resided in the cells plasma membranes. Experiments conducted in 1960 led to the concept of membrane structure known as fluid mosaic model proposed in 1972 by S. Jonathan Singer and Garth Nicolson of University of California, San Diego.

Composition of Biological Membrane

The lipid bilayer is fundamental structure of most biomembranes. Non lamellar lipid structure also form important components of biomembranes. The phospholipids that are present in biomembranes are highly amphipathic, phosphate containing groups esterified that have long chain fatty acyl groups. The phosphate containing groups are electrically charged and are hydrophilic whereas the hydrocarbon side chain of fatty acyl groups are hydrophobic. In the phospholipid bilayer, these hydrophobic side chains extend within each leaflet toward each other to form

hydrophobic interior of biomembranes. In contrast, hydrophilic phosphate containing group seek position in bilayer between hydrophobic interior and either of two external aqueous phases. Animal biomembrane also contain other lipids, such as cholesterol in addition to phospholipids. Cholesterol is less amphipathic than are phospholipids because hydrophilic portion of cholesterol is due to its uncharged and small hydroxyl group rather than to electrically charged and larger phosphate containing group. The protein content of biomembranes varies from 20% to more than 70% depending on the membrane. The ratio of lipid to protein in membrane varies, depending on type of cell membrane (plasma/ endoplasmic/ Golgi), type of organism (bacteria/plant/animal) and type of cell (cartilage/ muscle /liver). Inner mitochondrial membrane has a very high ration of protein / lipid in comparison to red blood cell plasma membrane, which is high in comparison to membranes of the myelin sheath that form wrapping around nerve cell. Inner mitochondrial membrane contains protein carriers of the electron transport chain and relative to other membranes, lipid is diminished.

In contrast, myelin sheath act as electrical insulation for nerve cell it encloses, a function that is carried by thick lipid layer of high resistance. Biomembranes transport is believed to be catalyzed by integral membrane proteins, though their activities are influenced by peripheral proteins. The tertiary and quaternary structure of integral membrane proteins has been studied. For example, aquaporin-1 (CHIP28) monomer, a member of protein family forms water channel in many epithelial and non-epithelial tissue is observed using cryo electron crystallography to form tetramers of four water channel pathways through the plasma membrane. Biomembranes structure is asymmetric as well as heterogeneous (Refer Figure 1.19).

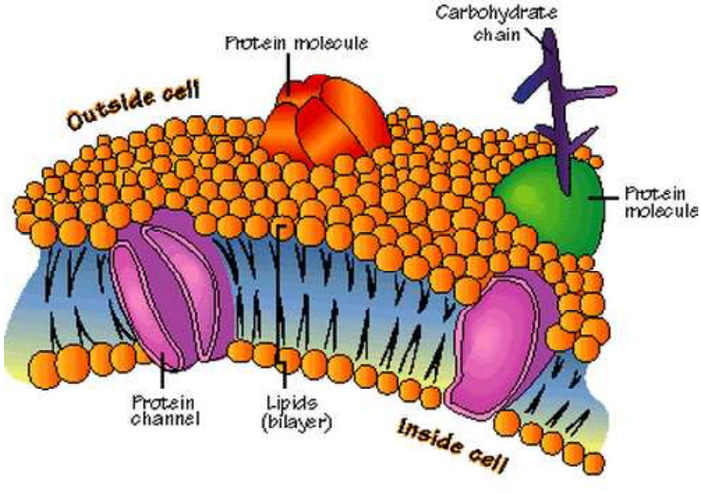

Fig. 1.19 *Biomembranes Structure*

Proteins: Proteins constitute 20 to 70 % of the membrane by mass. They occur in different ways and forms in different sites of plasma membrane. Two distinct types are common. Extrinsic proteins and intrinsic proteins. Extrinsic proteins are attached to the periphery of the lipid bilayer and can be separated from the membrane. Peripheral proteins are mostly made up of amino acids having hydrophilic side chains which act with the polar heads of lipid molecules or with

surrounding water. Intrinsic proteins are partly or wholly embedded in the lipid bilayer. The constituent amino acids of these proteins form hydrophobic bonds with fatty acids of lipid molecules. These proteins are soluble in organic solvents. Due to differential distribution of peripheral proteins, two surfaces of a membrane is always asymmetric. Most integral membrane proteins are inserted into membranes in the endoplasmic reticulum by a process that involves amino acid residue signaling sequences. Integral proteins are transmembrane proteins that is they pass entirely through lipid bilayer and have domains that protrude from both extracellular and cytoplasmic side of membrane (Refer Figure 1.20).

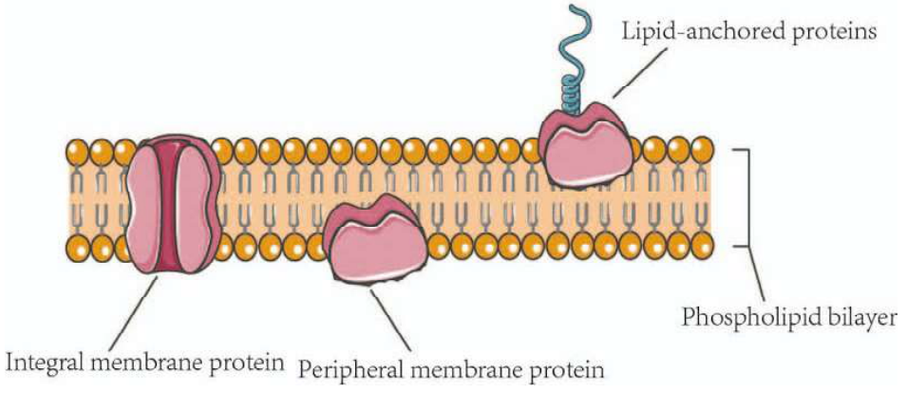

Fig. 1.20 *Protein Membrane*

Some membrane transport proteins undergo large conformational changes during processing and functioning. Peripheral proteins that are located entirely outside the lipid bilayer on either of cytoplasmic or extracellular side, and are associated with surface of membrane by non-covalent bonds. Lipid anchored proteins are located outside the lipid bilayer on either the extracellular surface but are covalently linked to lipid molecule situated within the bilayer. Besides structural proteins, the plasma membrane possess several enzymes which regulate cellular metabolism. More than 30 different types of enzymes are known to occur in association with plasma membrane. The carrier proteins embedded in the membrane help in transport of solutes.

Lipids: Lipids have asymmetric concentration across biomembranes, for example, out of four most abundant category of phospholipid in plasma membrane, the anionic one (phosphatidylserine) and a zwitterionic one (phosphotidylethanolamine) are more concentrated in the inner than the outer leaflet of bilayer. Free energy is required to move these phospholipids against their concentration gradients. The asymmetric distribution of lipids across the plasma membrane is scrambled by normal and artificial process. Cellular activation by stimuli is associated with increase in the cytosolic free Ca^{2+} concentration. Membrane lipids contain wide variety of lipids all of which are amphipathic, .i.e., they contain hydrophilic and hydrophobic regions.

There are three types of membrane lipids:

- Phosphoglycerides
- Sphingolipids
- Cholesterol

Phosphoglycerides: Phospholipids constitute a major portion of lipids (55-57%) in plasma membranes. They belong to mainly two categories. Neutral phospholipids and acidic phospholipids. Examples of neutral phospholipids are lecithin, cephalin, sphingomyelin, etc. Most membrane lipids contain phosphate group which make them phospholipid, most membrane phospholipids are built on glycerol backbone called phosphoglycerides.

Unlike triglycerides, which have three fatty acid and are not amphipathic, membrane glycerides are diglycerides-only two of the hydroxyl group of glycerol are esterified to fatty acid, third is esterified to hydrophilic phosphate group. These phospholipid molecules constitute the structural framework of plasma membrane.

Sphingolipids: A less abundant class of membrane lipids are sphingolipids, they are derivatives of sphingosine, an amino alcohol that contain a long hydrocarbon chain. Sphingolipid consists of sphingosine linked to a fatty acid by its amino group. This molecule is ceramide. The various sphingosine based lipids have additional group esterified to terminal alcohol of sphingosine. If substitution is phosphorylcholine, molecule is sphingomyelin, which is only phospholipid of membrane (Refer Figure 1.21).

Cholesterol: It is an amphipathic molecule like phospholipids having both hydrophilic and a hydrophobic portion. Cell membrane would be too fluid and too permeable to some molecules without cholesterol. Cholesterol is part of steroid ring that is closely attracted to part of fatty acid chain on the nearest phospholipid.

Cholesterol is sterol lipid derived from squalene, forming a major component of animal cell membrane. It is absent in higher plant membranes and bacteria. In animal cells, it affect membrane fluidity.

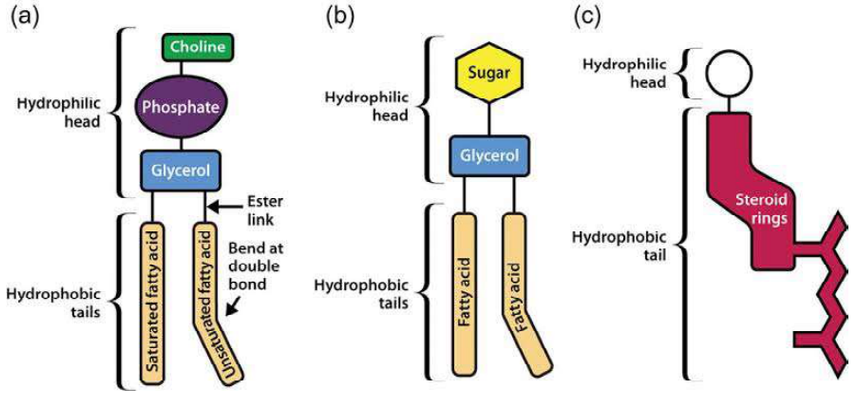

Fig. 1.21 *Structure of Membrane Lipid*

Phospholipid Bilayer

Membrane formation is due to a self-assembly process which is a consequence of amphipathic nature of phospholipid molecules. The polar hydrophilic heads of phospholipids come in contact with water while their hydrophobic tails cannot do so. There are two methods of arrangement pf phospholipids in contact with water-micelle (globule) and bilayer (bimolecular sheet) in which hydrophobic tails are inside and the hydrophobic heads are towards outside. The micelle or globular form would be mechanically unsound as the bulky double tails would not be able

to get adjusted in the center of globule. The driving force for the formation of lipid bilayer is hydrophobic interaction amongst the hydrocarbon tails of lipid molecules. Vander walls forces help in close packing of tails. Polar heads of phospholipids are held to water molecules by electrostatic attractions and hydrogen bonds. Lipid bilayers are also called cooperative structures because their molecules are held together by many reinforcing non-covalent interactions which are predominantly hydrophobic. Because of these interactions lipid bilayers have a tendency to become extensive, close on themselves and the ability of self-sealing (Refer Figure 1.22).

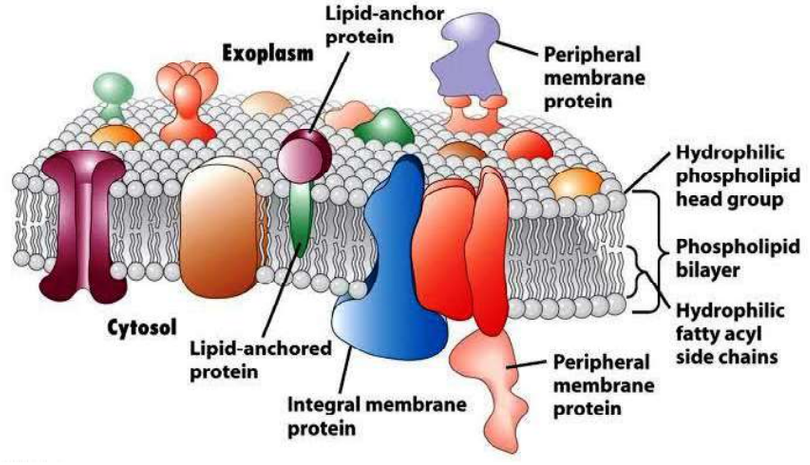

Fig. 1.22 *Lipid Bilayer showing Inner and Outer Leaflets*

Carbohydrates: More than 90% of the membrane carbohydrate is covalently linked to proteins to form glycoproteins Their peripheral association is highly asymmetric. The remaining carbohydrates are covalently linked to lipids to form glycolipids. Unlike proteins and lipids, carbohydrates are not integral component of membranes. Their peripheral association is highly asymmetric. Oligosaccharides are covalently bounded to membrane lipids and integral proteins on their non-cytosolic sides. Oligosaccharides are assembled and transferred to membrane proteins and lipids and modified in the lumen of endoplasmic reticulum and Golgi apparatus. No mechanism is known for assembly and attachment of molecules to segments of proteins and lipids at cytosolic surface of membranes. The carbohydrate rich zone on the surface of cell is known as glycocalyx. Specific glycosylation of some integral membrane proteins appear to be essential for their transport activities.

Proteins penetrate through membranes rather than remaining external to lipid bilayer was derived from results of a technique called freeze fracture replication. Here tissue is frozen solid and struck with blade, which fractures block into two pieces. Once the membrane are split, metals are deposited on their exposed surface to form replica which is viewed in the electron microscope (Refer Figure 5.5).

Functions of Biological Membrane

- Membranes are continuous and enclose compartments. The plasma membrane encloses the contents of cell, whereas the nuclear and cytoplasmic membranes enclose diverse intracellular spaces. The various membrane bound compartments possess different contents. It allows specialized

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activities to proceed without external interference and enable cell activity to be regulated independently.

- Membrane prevent the unrestricted exchange of molecules from one side to another. Membranes provide the means of communication between compartments.
- They form outer boundaries around the cells to maintain environment inside the cell different from those of outside environment.
- Membrane prevent the unrestricted exchange of molecules from one side to the other. At the same time, membrane provide the communication between the compartments they separate
- The plasma membrane contain machinery for physically transporting substance from one side of membrane to another from region where solute is at low concentration to a region where solute is at higher concentration. The plasma membrane is able to transport ions thereby establishing ionic gradients across itself.
- The plasma membrane of organism mediates the interaction between a cell and its neighbours. The plasma membrane allow cells to recognize and signal one another, to adhere when appropriate and to exchange material and information
- Membranes are involved in the process by which one type of energy is converted to another type (energy transduction). Membranes are involved in transfer of chemical energy from carbohydrates and fats to ATP.
- Plasma membrane plays a role in response of cell to external stimuli, a process known as signal transduction. Membranes possess receptors that combine with specific molecules. The interaction of a plasma membrane receptor with external ligand cause the membrane to generate signal that stimulate or inhibit internal activities.
- Boundaries round distinct sub-cellular compartments (Nucleus, Mitochondria, Lysosomes, Golgi bodies, etc.), compartmentalize and segregate intracellular events, and separate cells from one another,
- Membranes mediate regulation of cellular functions by: acting as selective barriers, allowing inside environment of cells or organelles to differ from outside.
- Plasma membrane is selectively permeable outer boundary of cell.
- Plasma membrane contains: Specific systems; Pumps, Channels, Transporters used for exchange of nutrients and other materials with the environment.
- Normal cellular function starts with normal cell membrane, i.e., damage to membrane can affect water balance and ion influx and therefore grossly alter most processes within the cell.
- In nerve cells, the cell membrane takes part in transmission of impulses.
- They provide sheaths for cilia and flagella.
- Secretory, excretory and waste products are thrown out by plasma membrane through exocytosis.

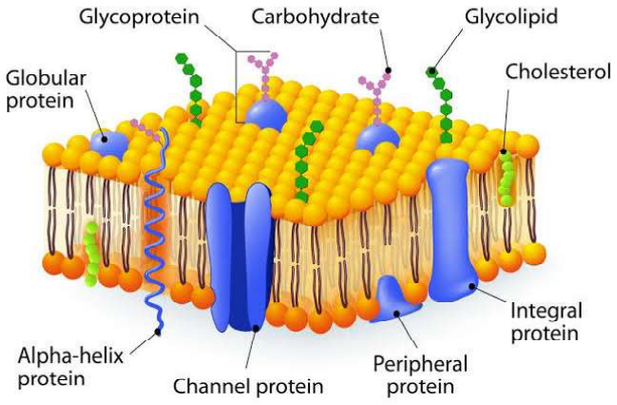

Fig. 1.23 *Biological Membrane with Different Protein Membranes*

1.3.1 Transport of Ions Across Membrane

Fluidity of Plasma Membrane

The physical state of the lipid of a membrane is described by fluidity. Simple artificial bilayer composed of phosphatidylcholine and phosphotidylethanolamine, whose fatty acids are unsaturated. If temperature is kept warm, lipid exist in fluid state. At this temperature, lipid bilayer is described as two dimensional liquid crystal. If the temperature is lowered, point is reached where the bilayer changes. The lipid is converted from a liquid crystalline phase to a frozen gel in which movement of phospholipid fatty acid chains is restricted. The temperature at which this change occurs is called transition temperature.

Saturated fatty acids have a shape of straight, flexible rod. Cis unsaturated fatty acids have crooks in the chain at sites of double bond. Phospholipids with saturated chains pack together more tightly than those containing unsaturated chains. The greater the degree of unsaturation of fatty acids of bilayer, lower the temperature before bilayer gels.

Another factor that influences bilayer fluidity is fatty acid chain length. The shorter the fatty acyl chains of phospholipid, lower is its melting temperature. The physical state of membrane is affected by cholesterol. Because of their orientation within bilayer, cholesterol disrupt the close packing of fatty acyl chains and interfere with mobility.

Cholesterol tends to increase durability while decreasing the permeability of a membrane. Many basic cellular processes including cell movement, cell growth, and cell division, formation of intercellular junction, endocytosis, and secretion depend on the movement of membrane components.

Cell membrane do not exist in rigid state nor any disordered state. They are partly fluid and partly rigid or stabilized. Membrane fluidity is mainly controlled by fatty acid moieties of phospholipids. Fluidity increases with the decrease in fatty acid chain and increase in the number of double bonds. Usually one fatty acid is saturated. Its straight chain helps in packing. The second fatty acid is unsaturated with one or more double bonds. Bends appear in the region of double bonds. Such bends interfere in close packing of phospholipids and increase fluidity. Bacteria

are known to regulate fluidity by changing the number of double bonds and length of saturated acyl chains. Ratio of saturated to unsaturated fatty acid is 1.6 at 42 degree Celsius. Cholesterol and other lipids provide stability to membranes by preventing displacement of phospholipid due to their large size, formation of membrane complexes and interaction with adjacent phospholipids.

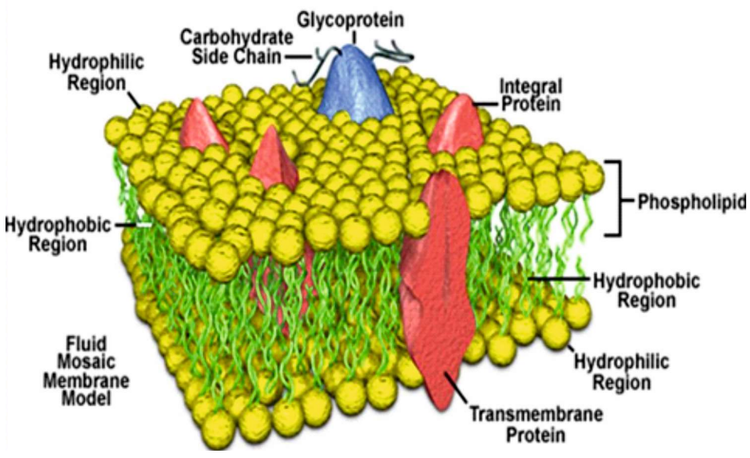

Fig. 1.24 *Fluid Mosaic Model*

Fluid Mosaic Model of Plasma Membrane:

- It is the most acceptable model of a biomembranes proposed by Singer and Nicolson in 1972.
- According to this model, the membrane does not have a uniform disposition of lipids and proteins but is mosaic of two.
- The membrane is not solid but is quasi-fluid. The quasi-fluid nature of the biomembranes is shown by their properties of quick repair, dynamic nature, ability to fuse, expand and contract.
- Fluid mosaic model postulates that the lipid molecules are present in a viscous bilayer as in lamellar model.
- Protein molecules occur at places both inside and on the outer side of lipid bilayer.
- The integral proteins are called intrinsic or integral proteins while the external ones are known as extrinsic or peripheral proteins. Integral proteins account for 70% of the total membrane proteins. They cannot be extracted from the membrane without disrupting the latter.
- The plasma membrane that surrounds these cells has two layers (a *bilayer*) of *phospholipids* (fats with phosphorous attached), which at body temperature are like vegetable oil (fluid).
- Both layers of the plasma membrane have the hydrophilic heads pointing toward the outside; the hydrophobic tails form the inside of the bilayer
- Each phospholipid molecule has a head that is attracted to water (*hydrophilic: hydro* = water; *philic* = loving) and a tail that repels water (*hydrophobic: hydro* = water; *phobic* = fearing.

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- The extrinsic proteins are attached covalently to phospholipid head or non-covalently to transmembrane proteins. They can be separated with mild treatment.
- Proteins provide the structural and functional specificity to the membranes. Membrane proteins provide structural and functional specificity to the membranes.
- Many membrane proteins function as enzymes. Some of them behave as permeases for allowing facilitated diffusion. A few proteins act as carriers because they actively transport different substances across the membrane.
- Some lipids and extrinsic proteins present on the outer side possess small carbohydrate molecules to form glycolipids and glycoproteins. They constitute glycocalyx.
- Conjugated oligosaccharides function as recognition centers, site of attachment, antigens, etc. Oligosaccharides provide negative charge to the outer surface.
- Because cells reside in a watery solution (extracellular fluid), and they contain a watery solution inside of them (cytoplasm), the plasma membrane forms a circle around each cell so that the water-loving heads are in contact with the fluid, and the water-fearing tails are protected on the inside**.**
- The lipid bilayer can exist in fluid state. For flip flop to occur, hydrophilic head group of lipid must pass through internal hydrophobic sheet of the membrane. Thus cells contain enzymes that move certain phospholipids from one leaflet to other. These enzymes play role in establishing lipid asymmetry and reverse the slow rate of passive transmembrane movement. Because lipids provide the matrix in which integral proteins of membrane are embedded, the physical state of lipid is important determinant of mobility of integral proteins.
- Plasma membrane proteins are not totally free to drift around randomly but instead they are subjected to various influences that affect their mobility. Some membranes are crowded with proteins, so random movements of one molecule can be impeded by its neighbors. The plasma membrane possess fibrillary network or membrane skeleton consisting of peripheral proteins situated on the cytoplasmic surface of membrane. Studying the factors that affect membrane protein mobility is to genetically modify cells to produce altered membrane proteins.
- Integral proteins whose cytoplasmic portion have been genetically deleted move much greater distance than their counterparts indicating that barriers reside on cytoplasmic side of the membrane. It suggest that membrane underlying skeleton form network of fences creating compartments that restrict the distance an integral protein can travel. Proteins move across the boundaries from one compartment to another through break in fences.

Evidences for Fluid Mosaic Model

- Fluid mosaic model can explain the presence of different types of permeability and retentively of various cell membranes.

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- It accounts for dynamic nature of biomembranes with their quick repair.
- The change in permeability in different parts of the same membrane can be explained
- The model explains the passage of both electrolytes and non-electrolytes through the biomembranes
- It provides for quick growth, expansion and contraction of the membrane
- The model provides for the occurrence of protein particles both on the surface and interior of cell membranes.

Asymmetry of Biomembranes

- The two surfaces of the biomembranes are not similar, .i.e. , the membranes are asymmetric
- Lipids present in the outer and inner side of the bilayer are commonly different, for example, lecithin on the outer side and cephalin on the inner side of erythrocyte membrane
- The amount and types of extrinsic proteins are different on the two sides. They are more abundant on the inner surface than on the outer surface
- Oligosaccharides are attached to external surface of lipids and proteins of biomembranes. They are absent on the inner side.

Proteins are huge molecules, so their movement within lipid bilayer might be restricted. Phospholipids are small molecules that make up the very fabric of lipid bilayer. When individual phospholipid molecules of plasma membrane are tagged and followed under microscope using ultra high speed cameras, they are confined for brief period and hop from one area to another.

Analysis indicates that phospholipid diffuses freely within one compartment before jumps into neighbouring compartment and then over another fence into an adjacent compartment. Treatment of the membrane with agents that disrupts the underlying membrane skeleton removes some of the fences that restrict phospholipid diffusion. As the contents of a cell are surrounded by its plasma membrane, all communication between the cell and the extracellular medium must be mediated by this structure. In this way, plasma membrane has a dual function. On one hand, it must retain the dissolved material of the cell so that they do not leak out into the environment while on other hand, it must allow the necessary exchange of materials into and out of the cell. The lipid bilayer of membrane is suited to prevent the loss of charged and polar solutes from a cell. Several different processes are known by which substances move across membrane: simple diffusion through lipid bilayer, simple diffusion through an aqueous protein lined channel, diffusion that is facilitated by a protein transporter and active transport which requires an energy driven protein pump capable of moving substance against concentration gradient.

Transport Across Membrane

There are three types of membrane transport,.i.e.,

- Diffusion
- Active Transport
- Bulk Transport

Diffusion: It is a spontaneous process in which a substance moves from a region of high concentration to a region of low concentration, eliminating the concentration difference between the two regions. It is of two types- passive and facilitated.

Passive Diffusion: The membrane plays a passive role in the transport of substances across it. The unassisted movement is from higher concentration side to the side where concentration is lower. Since the membranes have hydrophobic interior, simple or passive diffusion occurs only for small, non-polar molecules. For example, oxygen, carbon dioxide, small uncharged polar molecules like ethanol and glycerol

Facilitated Diffusion: The transport across the membrane is along the concentration gradient but is facilitated by the presence of membrane proteins without involving expenditure of energy. Membrane proteins taking part in facilitated diffusion are of two types, carrier proteins and channel proteins. Both of them are integral membrane proteins with several transmembrane segments.

Water molecules move much more rapidly through cell membrane than do dissolved ions or small polar organic solutes which are non-penetrating. Because of this difference in the penetrability of water vs solutes, membranes are semipermeable. Water moves readily through a semipermeable membrane from a region of lower solute concentration to region of higher solute concentration. The process is known as osmosis, and is demonstrated by placing cell in solution containing non-penetrating solute at a concentration different that present within cell itself. When two compartments of different solute concentration are separated by a semipermeable membrane, the compartment of higher solute concentration is said to be hypertonic relative to the compartment of lower solute concentration, known as hypotonic. When cell is placed into hypotonic solution, cell gains water by osmosis and swells. Conversely, cell placed into hypertonic solution loses water by osmosis and shrinks. It suggests that a cells volume is controlled by the difference between the solute concentration inside the cell and that in the extracellular medium. Once the internal solute concentration equals the external solute concentration, the internal and external fluids are isotonic and no net movement of water into or out of cell occurs.

Channel Protein Mediated Facilitated Diffusion

Ion Channels: Cell membrane contain ion channels that is openings in membrane that are permeable to specific ions. Bert Sakmann and Erwin Neher at the Max Planck Institute in Germany in 1970s and early 1980s developed technique to monitor the ionic current passing through single ion channel. Most ion channels are highly selective in allowing only one particular type of ion to pass through the pore. Most of the ion channels that are identified can exist in either an open or closed conformation such channels are said to be gated. The opening and closing of the gates are subject to complex physiologic regulation and induced by variety of factors depending on particular channel**.**

Three major categories of channels are:

- Voltage Gated Channels
- Ligand Gated Channels
- Mechano Gated Channels

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Voltage Gated Channels: The channels open and close in response to changes in membrane potential are voltage gated channels.

Ligand Gated Channels: Its conformational state depends on the binding of a specific molecule which is not the solute that passes through channel.

Some ligand gated channels are opened or closed following the binding of a molecule to the outer surface of the channel, others are opened or closed following the binding of ligand to the inner surface of channel. For example, neurotransmitter such as acetylcholine, act on outer surface of cation channels, while cyclic nucleotides such as cyclic AMP act on the inner surface of calcium ion channels.

Mechano Gated Channels: Its conformational stated depends on mechanical forces that are applied to the membrane. Members of one family of cation channels, for example, are opened by the movements of stereocilia on the hair cells of the inner ear in response to sound.

Porins: They are present in the outer membrane of mitochondria, chloroplasts and some bacteria. The transmembrane segment of the protein has a closed cylindrical â sheet called â barrel. It is water filled pore that is bigger than the ion channels. It allows passage of small sized hydrophilic molecules and solutes.

Aquaporins: They are membrane channels for the passage of water molecules. Each aquaporin has a central channel lined by 24 transmembrane segments of four proteins. The diameter of the channel is about 0.3 nm, wide enough to allow passage of water molecules. The rate of water movement is several billion molecules per second.

Active Transport: Like facilitated diffusion, active transport depends on integral membrane proteins that selectively bind a particular solute and move it across the membrane in a process driven by changes in protein conformation. Unlike facilitated diffusion, movement of solute against gradient requires the coupled input of energy. The endergonic movement of ions or solutes across the membrane against a concentration gradient is coupled to an exergonic process such as hydrolysis of ATP.

- **Direct or Primary Active Transport**: The active transport is directly coupled to exergonic reaction, most commonly hydrolysis of ATP. Four types of ATPases are P type, V type, F type and ABC type.
- **Indirect or Secondary Active Transport:** It is linked to direct active transport which produces high concentration of sodium ions in animal cells or hydrogen ions in bacterial cells ions on the outside. The ions have tendency to enter the cells as per their electrochemical gradient. They do so by forming symport and antiport systems. In symport, inward passage of hydrogen ions or sodium ions is coupled to passage of organic solutes. An antiport system involves exchange of potassium ions for hydrogen ions and calcium ions for sodium ions.

Bulk Transport: It involves the enclosure of the materials under transport in the vesicles of membrane called carrier vesicles. The vesicles are formed in response to chemical stimuli received in specific areas having receptors. It is of two types- Pinocytosis and Phagocytosis.

Pinocytosis: It is also known as cell drinking. Invagination of the vesicles requires attachment of three types of proteins on the cytosolic side- adapter protein, clathrin and dyanmin. The pinched off vesicle is initially coated and pinosome either burst in cytoplasm, fuse with lysosome or release its content into the vacuole. They are either fluid phase pinocytosis or adsorptive pinocytosis.

Phagocytosis: It is the bulk transport of solid matter like food, foreign particles, and pathogen across the membrane by means of carrier vesicles. Also known as cell eating. The carrier vesicles are known as phagosome. Fusion of lysosome with phagosome produces a food vacuole. The digested substances diffuse out. The vacuole with undigested material is called residual vacuole. The indigestible matter is thrown out of cell through exocytosis or cell vomiting or ephagy.

Other Ion Transport System: Biologists are eagerly awaiting a high resolution structure of Na^+/K^+ ATPase. A great deal can be learnt about P type pump by studying related protein, the Ca^- ATPase whose three dimensional structure has been determined. The calcium pump is present in the membrane of endoplasmic reticulum where it transport Ca^{2+} ions out of cytosol into the lumen of organelle. The transport of Ca^{2+} ions by Ca^- ATPase is accompanied by large conformational changes. The sodium potassium pump is found only in animal cells. Unlike P type pumps, V type pumps utilize the energy of ATP without forming a phosphorylated protein intermediate. It actively transport hydrogen ions across the walls of cytoplasmic organelles and vacuoles. Another diverse group of proteins that transport ions is ATP binding cassette transporter because all the members of this family share homologous ATP binding domain.

1.3.2 Irreversible Thermodynamic Treatment of Membrane Transport

To fully comprehend the membrane transport processes a knowledge is required of the structure of the membrane and of the internal interactions between ions matrix and solvent during such processes. Irreversible Thermodynamics, provides a more rigorous and comprehensive analysis of these measured quantities than any other theoretical method available. This concept is however confined to situations in which the membrane system is near equilibrium and where the resultant phenomenological equations are linear functions of the applied forces. Since ion exchangers are intrinsically heterogeneous the validity of the Onsager Reciprocal Relations must be questioned Stein have given this problem a general treatment which is applicable to systems in mechanical equilibrium. Their conditions for the validity of the Onsager Reciprocal Relations require that flow and forces be measured normal to the membrane surface and that forces are constant over that surface. All local forces must bear a constant relationship to their potential difference across the membrane and this implies that path lengths through the membrane must be equal. These conditions are valid for a homogeneous membrane and it is postulated that the experimental membranes in this study are sufficiently homogeneous on a macroscopic scale to allow the Onsager Reciprocal Relations. Irreversible Thermodynamics may be used to interpret and predict to a high degree of accuracy of the transport and diffusion properties of membranes.

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Irreversible Thermodynamics have shown that for the AMP C60 membrane in a univalent cationic form the analogous electrolyte solution is a good model on which to base predictive calculations. It is hoped to show in this work whether or not the properties of the calcium form may be analyzed to a similar degree of accuracy using calcium chloride solution models. To / 6 To ensure that as far as possible, the sodium and calcium forms of the membranes were under parallel conditions the anion concentration of equilibrium solution was the same in both cases .

Since ion-exchangers are inherently heterogeneous the validity of the O.R.R. must be justified. Snell and Stein have applied a general treatment to systems in mechanical equilibrium. From this work the conditions for the validity of the O.R.R. require that the forces and flows be measured normal to the membrane surface and that these forces must be uniform over that surface. All local forces must bear a constant relationship to the potential difference across the membrane. This means that the diffusional path lengths across the membrane must be equal. This condition implies that the local in homogeneities of the membrane must-be distributed uniformly throughout the matrix and be orders of magnitude smaller than the geometric thickness of the membrane. In effect the membrane must be uniformly heterogeneous on a "microscopic" scale or macroscopically homogeneous. The membrane system consists of four components which are the counter ion, the co-ion, water, and the matrix-fixed sulphonate.

Thermodynamically the flow of substances from one compartment to another can occur in the direction of a concentration or electrochemical gradient or against it. If the exchange of substances occurs in the direction of the gradient, that is, in the direction of decreasing potential, there is no requirement for an input of energy from outside the system; if, however, the transport is against the gradient, it will require the input of energy, metabolic energy in this case. For example, a classic chemical mechanism for separation that does not require the addition of external energy is dialysis. In this system a semipermeable membrane separates two solutions of different concentration of the same solute. If the membrane allows the passage of water but not the solute the water will move into the compartment with the greatest solute concentration in order to establish an equilibrium in which the energy of the system is at a minimum. This takes place because the water moves from a high solvent concentration to a low one (in terms of the solute, the opposite occurs) and because the water is moving along a gradient there is no need for an external input of energy.

A physiological process can only take place if it complies with basic thermodynamic principles. Membrane transport obeys physical laws that define its capabilities and therefore its biological utility.

A general principle of thermodynamics that governs the transfer of substances through membranes and other surfaces is that the exchange of free energy, ΔG, for the transport of a mole of a substance of concentration C1 in a compartment to another compartment where it is present at C2 is:

$$\Delta G = RT \log \frac{C_2}{C_1}$$

When C2 is less than C1, ΔG is negative, and the process is thermodynamically favorable. As the energy is transferred from one compartment to another, except where other factors intervene, an equilibrium will be reached where C2=C1, and where ΔG = 0. However, there are three circumstances under which this equilibrium will not be reached, circumstances which are vital for the in vivo functioning of biological membranes

- The macromolecules on one side of the membrane can bond preferentially to a certain component of the membrane or chemically modify it. In this way, although the concentration of the solute may actually be different on both sides of the membrane, the availability of the solute is reduced in one of the compartments to such an extent that, for practical purposes, no gradient exists to drive transport.
- A membrane electrical potential can exist which can influence ion distribution. For example, for the transport of ions from the exterior to the interior, it is possible that:

$$\Delta G = RT \log \frac{C_{inside}}{C_{outside}} + ZF\Delta P$$

Where F is Faraday's constant and ΔP the membrane potential in volts. If ΔP is negative and Z is positive, the contribution of the term ZFΔP to ΔG will be negative, that is, it will favor the transport of cations from the interior of the cell. So, if the potential difference is maintained, the equilibrium state ΔG = 0 will not correspond to an equimolar concentration of ions on both sides of the membrane.

- If a process with a negative ΔG is coupled to the transport process then the global ΔG will be modified. This situation is common in active transport and is described thus:

$$\Delta G = RT \log \frac{C_{\text{inside}}}{C_{\text{outside}}} + \Delta G^b$$

Where ΔG^b corresponds to a favorable thermodynamic reaction, such as the hydrolysis of ATP, or the co-transport of a compound that is moved in the direction of its gradient.

The fundamental equation which served as starting point for numerous further developments is that of Nernst and Planck. It is based on the assumption that the steady velocity of transport in a viscous medium vector $\boldsymbol{V}$ is directly proportional to the driving force of vector $\boldsymbol{X}$, the proportionality factor being the constant mobility. Thus,

$$\vec{v} = \omega \vec{X} \tag{1}$$

For diffusional flows

$$X_i = -\operatorname{grad} \tilde{\mu}_i \tag{2}$$

Where $\boldsymbol{X}_i$ is the force acting on the ith component and $\tilde{\mu}_i$ its electrochemical potential. The flux of the ith component ($\boldsymbol{J}_i$) is the product of velocity and concentration, or,

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$$\vec{J_i} = c_i \vec{v_i} = c_i \omega_i \vec{X_i} \tag{3}$$

In the case of unidirectional diffusion of a non-electrolyte, Equation (3) yields readily the conventional equation of Fick. Assuming that the system is dilute in the *i*th component, and that ideality may be attributed to the chemical potential is:

$$\mu_i = \mu_i^0 + RT \ln c_i$$

$$X_i = -\frac{d\mu_i}{dx} = -\frac{RT}{c_i}\frac{dc_i}{dx} \tag{4}$$

Inserting Equation (4) into (3) we obtain

$$J_i = RT\omega_i \left(-\frac{dc_i}{dx}\right) = D_i \left(-\frac{dc_i}{dx}\right) \tag{5}$$

Where

$$D_i = RT\omega_i \tag{6}$$

Is the coefficient of diffusion according to the well-known equation of Einstein. For the flow of an electrolyte, the electrochemical potential $\tilde{\mu}_i$, may be written as

$$\tilde{\mu}_i = \mu_i^0 + RT \ln c_i + z_i F\psi \tag{7}$$

Where Ψ the local electrical potential and zi is is the valency of the *i*th component. Inserting Equation (7) into (2) we obtain

$$X_i = -\frac{d\mu_i}{dx} = -\left(\frac{RT}{c_i}\frac{dc_i}{dx} + z_i F \frac{d\psi}{dx}\right) \tag{8}$$

Which upon insertion into (3) gives the renowned Planck expression

$$J_i = RT\omega_i \left[\left(-\frac{dc_i}{dx}\right) + \frac{c_i z_i F}{RT}\left(-\frac{d\psi}{dx}\right)\right] \tag{9}$$

Equation (9) has been tested extensively for diffusion in liquid and solid media.

A limitation of Equation (3), and the derived expressions (8) and (9), is that they deal with independent flows, i.e., with cases in which the transport of each component is not influenced by the concomittant flows passing through the system. It is known, however, since the ***pioneering work*** of Reuss in 1809, that we should in general assume a coupling between flows, which may profoundly change the transport pattern. In his ingenious experiments on the electro kinetic behaviour of porous media, Reuss demonstrated that the flow of electricity is accompanied by a volume flow, while a flow of water, induced by a mechanical pressure head, results in a flow of electrical current. A series of other coupling phenomena were discovered during the XIXth century, such as thermoelectricity, thermo-osmosis

and thermodiffusion. It is therefore evident that a comprehensive theory of membrane transport should treat explicitly the coupling between flows and should provide a measure for the transport interaction.

In the study of biological systems, there is a special interest in the interpretation of coupling between diffusional transport and metabolic processes taking place within the membrane or in its vicinity. Such coupling, known in physiology as active transport, which plays an important role in the regulatory function of cells and tissue, will be considered later.

A suitable theoretical introduction to the analysis of coupling phenomena is provided by the thermodynamics of irreversible processes. Although kinetic and statistical mechanical treatments are better suited for the visualization of the processes under consideration, the statistical mechanics of irreversible processes is still an inadequate tool for the description of condensed systems. Despite its inherent limitation as a formal and 'empty' conceptual system, non-equilibrium thermodynamics has the advantages of simplicity and consistency in making its statements a useful guide in membrane study. It is indeed the apparent simplicity of the equation which misleads the inexperienced who may believe that the thermodynamic statements are trivial and 'Self-Evident' a convenient starting point for the development of the phenomenology of non-equilibrium thermodynamics is the treatment of the total change in entropy, written in the form

$$\mathrm{d}S = \mathrm{d}_e S + \mathrm{d}_i S \tag{10}$$

Where $\mathbf{d}_e\boldsymbol{S}$ is the entropy exchanged with the surrounding and $\mathbf{d}_i\boldsymbol{S}$ is the entropy created within the system by all irreversible processes. In the terms of Equation (10) the second law may be written as

$$\mathbf{d}_i\boldsymbol{S} \geq \mathbf{0} \tag{11}$$

Or, the ***irreversible entropy*** change is positive definite. $\mathbf{d}_i\boldsymbol{S}$ equals zero for equilibria and is positive for all irreversible processes. The thermodynamic description of the rate processes is based on the increase in inner entropy per unit time or on the entropy formation $\mathbf{d}_i\boldsymbol{S}/\mathbf{d}\boldsymbol{t}$. For isothermal processes, it is convenient to use the dissipation function introduced by Lord Rayleigh, $\boldsymbol{\phi} = \mathbf{T}(\mathbf{d}_i\boldsymbol{S}/\mathbf{d}\boldsymbol{t})$, which measures the degradation of free energy per unit time due to irreversibility.

1.3.3 Nerve Conduction

All the living organisms which have lungs for breathing, the physiological respiration involves respiratory cycles of inhaled and exhaled breaths. Inhalation or breathing in is usually an active movement. The contraction of the diaphragm muscle cause a pressure variation, which is equal to the pressures caused by elastic, resistive and inertial components of the respiratory system. In contrast, exhalation or breathing out is usually a passive process. Breathing in, brings air into the lungs where the process of gas exchange takes place between the air in the alveoli and the blood in the pulmonary capillaries.

The fact that the Central Nervous System (CNS) controls the respiratory system was first described by Whytt, a neurologist, who observed an unconscious reflex breathing action. The Central Nervous System (CNS) includes the brain

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and spinal cord. The brain and spinal cord are protected by bony structures, membranes, and fluid. The brain is held in the cranial cavity of the skull and it consists of the cerebrum, cerebellum, and the brain stem. The nerves involved are cranial nerves and spinal nerves.

The ventilatory system is typically designed to bring oxygen into the body, to fuel energy-generation and to remove carbon dioxide, which is a waste product of cellular metabolism. The system is made up of the cortex of the brain, which controls voluntary breathing, the brainstem which is involved with automatic breathing, the spinal cord and motor neurons which transmit nerve impulses, the respiratory muscles which are the effectors of the system and a complex system of feedback receptors and nerves that precisely regulate ventilation process.

Voluntary Breathing Controllers: The signals for voluntary breathing originate in the cerebral cortex. There are centers within the parietal cortex that send the signals for inspiration and expiration to occur. These cortical areas project to the motor neurons in the spinal cord via the cortico-spinal tracts.

Automatic Breathing Controllers: Automatic breathing is controlled by a complex system that includes respiratory centers in the pons and medulla, nerve tracts in the lower brainstem, and the feedback mechanisms that are both chemical and mechanical in nature.

Spinal Cord: The spinal cord and the motor nerves conduct the nerve impulses from the cortex and brainstem to the anterior horn cells of the motor neurons that supply the respiratory muscles. The nerve fiber tracts in the spinal cord that are responsible for voluntary (cortico-spinal tract) and automatic (reticulo-spinal tract) breathing are distinct within the spinal cord.

Lower Motor Neurons: In the Peripheral Nervous System (PNS), the lower motor neuron has its cell body in the spinal cord (anterior horn cell) but exits the spinal cord to become the spinal nerve roots and the nerves that supply the respiratory muscles.

Respiratory Muscles: The respiratory muscles are the mechanical effectors of the breathing system. The respiratory muscles are often divided into 3 major groups: the inspiratory muscles, the expiratory muscles, and the accessory muscles of respiration. The muscles are of the upper airway that maintain patency during the respiratory cycle and are often also considered muscles of respiration. The diaphragm is the major muscle of inspiration as it contributes approximately 70% to inspiratory tidal volume in the normal condition. The innervation of the diaphragm is via the phrenic nerve, which originates from cervical nerve roots.

However, there are several nerves responsible for the muscular functions involved in respiration but the following three types are considered as the important respiratory nerves:

Phrenic Nerves: The nerves stimulate the activity of the diaphragm. They are composed of two nerves, the right and left phrenic nerve, which pass through the right and left side of the heart, respectively. They are autonomic nerves.

Vagus Nerve: This nerve innervates the diaphragm as well as movements in the larynx and pharynx. It also provides parasympathetic stimulation for the heart and the digestive system. It is a major autonomic nerve.

Posterior Thoracic Nerves: These nerves stimulate the intercostal muscles located around the pleura. They are considered to be part of a larger group of intercostal nerves that stimulate regions across the thorax and abdomen. They are somatic nerves.

All these three types of nerves regulate and control the signal of the ascending respiratory pathway from the spinal cord to stimulate the muscles that perform the movements essential for the respiration process.

If any of the above mentioned three respiratory nerves are damaged then they can cause severe health problems, such as paralysis of diaphragm if the phrenic nerves are damaged, irritation to the phrenic or vagus nerves can result in hiccups.

Check Your Progress

1. Give the statements of cell theory.
2. What is cytoplasmic matrix?
3. What do you understand by cell membrane?
4. Define the term genophore.
5. Define protein.
6. Give the main functions of protein in biological cell.
7. Which is the constitutional unit of DNA and RNA?
8. What is the classification of base in DNA?
9. Define the complamentry base pair in DNA.
10. Give the types of membrane lipid.
11. What are the categories of membrane transfort?
12. Define the vagus nerve.

1.4 ROLE OF METAL IONS IN BIOLOGICAL SYSTEM

Metal ions are fundamental elements for the maintenance of the lifespan of plants, animals and humans. Their substantial role in biological systems was recognized a long time ago. They are essential for the maintenance of life and their absence can cause growth disorders, severe malfunction, carcinogenesis or death. They are protagonists as *macro or microelements* in several structural and functional roles, participating in many biochemical reactions, and arise in several forms. They participate in intra and inter cellular communications, in maintaining electrical charges and osmotic pressure, in photosynthesis and electron transfer processes, in the maintenance of pairing, stacking and the stability of nucleotide bases, and also in the regulation of DNA transcription. They contribute to the proper functioning of nerve cells, muscle cells, the brain and the heart, the transport of oxygen and in many other biological processes up to the point that we cannot even imagine a life without metals.

Metals (Cobalt (Co), Nickel (Ni) and Chromium (Cr)) are used in prosthesis manufacturing because of their high mechanic stability and good biological compatibility, but their corrosion products have been shown to elicit biological responses. *Monocytes and macrophages* are the first barrier of the innate immune system, which interact with abrasion and corrosion products, leading to the release of proinflammatory mediators and Reactive Oxygen Species (ROS). The inflammation-relevant changes in monocytes and macrophages after exposure to several concentrations of metal salts ($CoCl_2$, $NiCl_2$, $CrCl_3 \times 6H_2O$) were studied by analysing viability, gene expression, protein release and ROS production. Metal particles, as well as their corrosion products, have been shown to elicit a biological response in the aseptic loosening of endoprosthetic implants. Owing to different metal alloy components, the response may vary depending on the nature of the released corrosion product.

Titanium (Ti), being one of the most abundant elements in the earth's crust with many examples of bioactive properties, is widely used in the cosmetic industry. Titanium Dioxide (TiO_2) Nano Particles (NPs) are often incorporated in sunscreens as physical sun blockers absorbing Ultra Violet (UV) radiation.

Exact needs vary among species, but commonly required plant trace elements include copper, boron, zinc, manganese, and molybdenum. Animals also require manganese, iodine, and cobalt.

Elements are present in different forms in the nature, and these elements are very essential for the body to perform different functions. Trace elements are very important for cell functions at biological, chemical and molecular levels. These elements mediate vital biochemical reactions by acting as cofactors for many enzymes, as well as act as centres for stabilizing structures of enzymes and proteins. Some of the trace elements control important biological processes by binding to molecules on the receptor site of cell membrane or by alternating the structure of membrane to prevent entry of specific molecules into the cell. The functions of trace elements have a dual role. In normal levels, they are important for stabilization of the cellular structures, but in deficiency states may stimulate alternate pathways and cause diseases. These **trace elements** have clinical significance and these can be estimated using different analytical method. The four main electrolytes namely sodium, magnesium, potassium, and calcium constitute about 1.89%, while the rest 0.02% or 8.6 g of an average human adults is made up of 11 typical trace elements. However, this tiny fraction exerts a tremendous influence on all body functions. Most of them mediate vital biochemical reactions by acting as a cofactor or catalyst for many enzymes. They also act as centres of building stabilizing structures, such as enzymes and proteins. The accumulation of metals or deficiency of these elements may stimulate an alternate pathway which might produce diseases. Interaction among the trace elements may also act as a scaffold upon which the etiopathogenesis of many nutritional disorders lie. Although these elements account for only 0.02% of the total body weight, they play significant roles, for example as active centres of enzymes or as trace bioactive substances. Trace elements refers to elements that occurs in natural and perturbed environments in small amounts and that, when present in sufficient bioavailable concentrations are toxic to living organism.

Elements, such as iron, zinc, and selenium are essential components of enzymes where they attract or subtract molecules and facilitate their conversion to specific end products. Few elements donate or accept electrons in redox reactions, which results in generation and utilization of metabolic energy and have an impact on the structural stability and to import certain biological molecules. Iron is involved in the binding, transporting, and release of oxygen in higher animals. Some of the trace elements control important biological processes by facilitating the binding of molecules to their receptor sites on cell membrane, by alternating the structures or ionic nature of membrane to prevent or allow specific molecules to enter or leave a cell and in inducing gene expression resulting in the formation of protein involved in life processes.

Essential Elements for Human Body

Four organic basic elements: H, C, N, O

Quantity elements - Na, Mg, K, Ca, P, S, Cl.

Essential trace elements - Mn, Fe, Co, Ni, Cu, Zn, Mo, Se, I.

Function suggested from active handling in humans, but no specific identified biochemical functions - Li, V, Cr, B, F, Si, As.

Biological Classification of Trace Elements

Classification proposed by Frieden (1981) which divided the elements into micro, trace, and ultra-trace elements based on the amount found in tissues.

Essential Trace Elements: Boron, cobalt, copper, iodine, iron, manganese, molybdenum, and zinc.

Probable Essential Trace Elements: Chromium, fluorine, nickel, selenium, and vanadium.

Physically Promotive Trace Elements: Bromine, lithium, silicon, tin, and titanium.

Categorical Classification of Trace Elements

It is observed that there are at least 29 different types of elements including metal and noN-metals in an adult human body. These 29 elements can be broadly classified into five major groups they are as follows:

Group I: These elements are the basic components of macromolecules, such as carbohydrates, proteins, and lipids. The elements belonging to these groups are carbon, hydrogen, oxygen, and nitrogen.

Group II: These are nutritionally important minerals. They are also called as principal elements or macro elements. Their daily requirement for an adult human is above 100 mg/day. The deficiency of such elements usually proves fatal unless intervened properly. The elements belonging to this group are sodium, potassium, chloride, calcium, phosphorous, magnesium, and sulphur.

Group III: There are the essential trace elements. An element is called as trace elements when their requirement per day is below 100 mg and deficiency leads to disorders and may prove fatal. The elements belonging to this group are copper, iron, zinc, chromium, cobalt, iodine, molybdenum, and selenium. Of these, iodine is a non-metal, while others are metals.

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Group IV: They are additional trace elements. Their role is not clear and they may be essential. The elements belonging to this group are cadmium, nickel, silica, tin, vanadium, and aluminium.

Group V: This group of metals is not essential their presence may produce toxicity. They have no known function in the human body. The elements belonging to this group are gold, mercury, cyanide, and lead.

The trace elements included **Group III** also called as minor elements. Their requirement is below 100 mg/day and their absence may not hinder normal development, but their activity may be substituted by another metal. Analytical methods are used to measure metal concentration in human tissues and body fluids.

The term 'Biomolecule' refers to any organic molecule that is formed by a living organism. This includes large polymeric molecules like proteins, polysaccharides and nucleic acids and small molecules like primary metabolites, secondary metabolites and natural products.

Any living system is primarily composed of six elements, which are as follows:

- Carbon
- Hydrogen
- Oxygen
- Nitrogen
- Phosphorus
- Sulphur

There are some other functional elements, such as Calcium (Ca), Sodium (Na), Magnesium (Mg), Copper(Cu), Zinc(Zn), etc. as shown in Table 1.1. Among these, carbon (C) is the most important element.

Table 1.1 *Chemical Compositions of Important Biomolecules*

Element	Per cent by mass
Oxygen	65
Carbon	18
Hydrogen	10
Nitrogen	3
Calcium	1.5
Phosphorus	1.2
Potassium	0.2
Sulfur	0.2
Chlorine	0.2
Sodium	0.1
Magnesium	0.05
Iron	0.004
Cobalt, Copper, Zinc, Iodine	< 0.05 each
Selenium, Fluorine	< 0.01 each

Carbon (C) is the most important owing to its property to form an infinite number of compounds. This helps carbon to form stable covalent bonds and C–C chains of unlimited length. Carbon has a unique property called catenation, that is, the property of forming bonds with the same element. It is tetravalent in nature and can form single, double and triple covalent bonds to combine with hydrogen, oxygen, sulphur, nitrogen and chlorine to form various types of compounds.

Every living system is built up in a hierarchical manner. Multicellular organisms are made up of organ systems, which are constituted by tissues. These tissues are a collection of cells, which contain organelles. It is after this that the world of biomolecules appears (Table 1.2). Macromolecules (proteins, lipids, nucleic acids and polysaccharides) are formed from monomeric units or building blocks (amino acids, fatty acids, glycerol, nucleotides and monosaccharides).

Table 1.2 *Chemical Compositions of Macromolecules*

Macromolecule	Monomers	Major Functions
Proteins	Amino acids	Fundamental basis of structure and function of cell (static and dynamic function)
Polysaccharide	Monosaccharides	Form of storage of energy to meet short-term demands
Lipid	Fatty acids, glycerol	Form of storage of energy to meet long-term demands; structural components of membranes
Nucleic acids	Nucleotides	Carrier of genetic information

1.5 SODIUM-POTASSIUM PUMP

Na^+/K^+ ATPase Pump

This electrogenic P-type membrane pump carries out the coupled transport of three Na^+ ions outside and two K^+ ions inside the cell. It is a transmembrane heterodimeric Protein and comprises of **α** and **β** subunits, forming intracellular and extracellular domains and five pair of transmembrane helices. The α and β subunits have four isoforms each, expressed in tissue and cell specific manner, and they vary in their sensitivity towards Sodium and the steroid glycoside ouabain (inhibitor of Na^+ /K^+ ATPase in animals). The pump carries out a coupled ion transport accomplished by the hydrolysis of ATP and a subsequent phosphorylation event. It requires Mg^{2+}-ATP as its major substrate and has affinity towards specific ligands. The α subunit is 110 kDa in size and comprises of about 1000 Amino Acid residues. It has four isoforms (α_1, α_2, α_3 and α_4) which differ in their functional properties. The α_1 subunit is a housekeeping isoform expressed in most of the tissues. The saline environment triggers the quantitative expression of specific isoforms depending upon their Sodium binding affinity. The α subunit consists of five pairs of transmembrane helices forming the cation transport path and three cytoplasmic domains, which have the binding sites for Mg^{2+}-ATP and Na^+. These cytoplasmic domains are specified as nucleotide binding sites, phosphorylation site and a actuator site. These transmembrane helices move or rotate along to transport ions across the membrane. The α subunit is an active component of the pump carrying out

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phosphorylation and ion transport. The β subunit is 55 kDa in size composed of 370 Amino Acid residues, of which 30 Amino Acids form a cytosolic loop and 300 Amino Acids are folded to form the extracellular domain. The β subunit has three consecutive glycosylation sites on its extracellular folds. This subunit does not contain any active site for the substrate. It, however, interacts with the α subunit to restore the native structure of the enzyme. There are three S-S bonds present in the extracellular folds of the subunit necessary to provide the K^+ occlusion state of the enzyme.

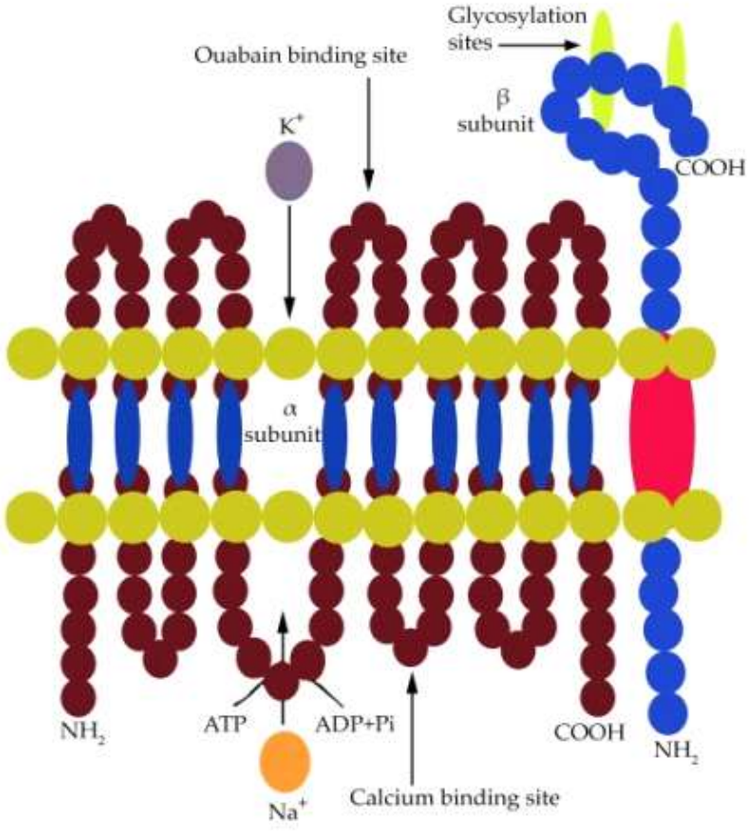

Fig. 1.25 *Structure of the Na^+/K^+ ATPase*

The pump operates through a cycle of phosphorylation-dephosphorylation events accompanied with Na^+ and K^+ binding and hydrolysis of ATP. Sodium binding at the cytosolic loop of the α subunit initiates the Sodium-dependent phosphorylation of the pump, accomplished by hydrolysis of previously bound ATP. This results in the formation of an acyl-phosphate complex between the Pi of ATP released and a specific Asp residue or As portable residue, an α-Amino-Acid residue. This phosphorylated state of the pump, commonly stated as $[E_1P]$, now excludes three Na^+ ions out of the cell. Two Potassium molecules then bind to the extracellular fold of the enzyme and the enzyme attains $[E_2P]$ state. This is followed by occlusion of K^+ into the cell and subsequent dephosphorylation of the pump. Ouabain is a specific glycoside which can bind to the phosphorylated α subunit of the enzyme in the $[E_2P]$ state after it has excluded three Na^+ ions (Refer Figure 1.25).

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Inhibition of the ATPase by Calcium

Calcium is a regulator of the Sodium pump. It inhibits the pump at the physiological concentration range of 0.08-5 μM. In excitable cells, depolarisation causes an increase in $[Ca^{2+}]_{cyt}$, which crosses the threshold level and inhibits specific isoforms of the pump in a concentration and affinity-dependent manner. Calcium at higher concentrations can, however, inhibit the pump in general, irrespective of the isomer. The mechanism of inhibition due to calcium can be explained as follows:

- The α subunit of the enzyme is associated with calcium inhibition, with α_2 isomer having greater affinity for calcium at physiological levels of 0.08-5 μM or even at higher concentration of 10 mM.
- Ca^{2+} in the concentration range of 10^{-5} M competes with magnesium for ATP and lowers the concentration of Mg^{2+}-ATP, which is a rate limiting substrate for the enzyme.
- Calmodulin and Calnaktin are the two Calcium-Binding Proteins which mediate the process of Calcium inhibition of this enzyme.
- Calmodulin and Calnaktin lower the threshold level of Calcium-Mediated inhibition from 100 μM to 2-5 μM of Ca^{2+}.
- These Proteins are unable to inhibit the pump in the absence of Ca^{2+}. Calcium-dependent inhibition of the pump is initiated by Calmodulin and Calnaktin and they interact with at least one or more Proteins in the membrane or cytosol to bring about inhibition of the pump.
- The process of Ca^{2+} inhibition associated with the α subunit of the enzyme is non-competitive and it does not compete with the binding site for Na^{+}.

1.6 TRANSPORT AND STORAGE OF DIOXYGEN

Dioxygen (O_2) plays an important role in the energy metabolism of living organisms. Free oxygen is produced in the biosphere through photolysis (light-driven oxidation and splitting) of water during photosynthesis in cyanobacteria, green algae, and plants. During oxidative phosphorylation in cellular respiration, the chemical energy of oxygen is released as it is reduced to water, thus closing the biological water-oxygen redox cycle.

Haemoglobin is able to transport oxygen within the body due to its unique structure. Its structure consists of four globin subunits: two α and two β subunits. Each subunit contains a heme prosthetic group with an iron bound.

Definition

Haemoglobin is a conjugated chromoprotein which contains haeme as prosthetic group and globin as the apoprotein part. Conjugated proteins are proteins which have a protein part known as **apoprotein** and a **non-protein** part known as prosthetic group. Chromoproteins are proteins which are coloured.

General information about Hb

In adult human beings, the normal concentration of haemoglobin present is 13–18 gm/dl. There is about 750 g of Hb in the total circulating blood of a 70-kg man. One sub-unit of haemoglobin binds to one molecule of oxygen and one Hb has four subunits. 1 g of Hb can carry 1.34 ml of oxygen. Space occupied by Hb in an erythrocyte is about 35 per cent and contributes to about 97 per cent of dry weight. About 27–32 pg of Hb is present in one molecule of erythrocyte. Approximately 6.25 g of Hb is formed and destroyed in the body each day.

History of discovery of Hb

Different aspects of Haemoglobin (Hb) were discovered at different times. In 1940, Hunefeld discovered the Hb in blood. In 1951, Otto Funko was able to isolate Hb crystals by successively diluting Red Blood Cell or RBCs with a solvent such as pure water, alcohol or ether, followed by slow evaporation of the solvent from the resulting protein solution. Hb's reversible oxygenation was found out by Felix Hoppe–Seyler. Its molecular structure was discovered by Max Perutz by x-ray crystallography in 1959. He received Nobel Prize in chemistry in 1962 along with John Kendrew. Functions of Hb were found out by physiologist Claude Bernard.

Hemoprotein

A hemeprotein (or haemprotein), or heme protein, is a protein that contains a heme prosthetic group. They are a very large class of metalloproteins. The heme group discusses the functionality, which can include oxygen carrying, oxygen reduction, electron transfer, and other processes. Heme is bound to the protein either covalently or non-covalently or both. The heme consists of iron cation bound at the centre of the conjugate base of the porphyrin, as well as other ligands attached to the 'Axial Sites' of the iron. The porphyrin ring is a planar dianionic, tetradentate ligand. The iron is typically Fe^{2+} or Fe^{3+}. One or two ligands are attached at the axial sites. The ***porphyrin ring*** has 4 nitrogen atoms that bind to the iron, leaving two other co-ordination positions of the iron available for bonding to the histidine of the protein and a divalent atom.

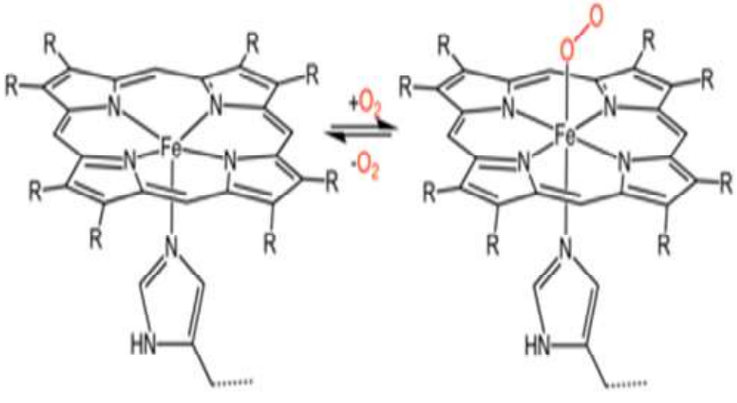

Fig. 1.26 *Binding of Oxygen to a Heme Prosthetic Group*

Hemeproteins probably evolved to incorporate the iron atom contained within the protoporphyrin IX ring of heme into proteins. As it makes hemeproteins responsive to molecules that can bind divalent iron, this strategy has been maintained throughout evolution as it plays crucial physiological functions. Oxygen (O_2), Nitric Oxide (NO), Carbon Monoxide (CO) and Hydrogen Sulphide (H_2S) bind to the iron atom in heme proteins. Once bound to the prosthetic heme groups (Refer Figure 1.26), these molecules can modulate the activity/function of those hemeproteins, affording signal transduction. Therefore, when produced in biologic systems (cells), these gaseous molecules are referred to as gasotransmitters.

Roles of Hemeproteins

- Hemeproteins have diverse biological functions including oxygen transport, which is completed via hemeproteins including hemoglobin, myoglobin, neuroglobin, cytoglobin, and leghemoglobin.
- Some hemeproteins - cytochrome P450s, cytochrome c oxidase, ligninases, catalase and peroxidases - are enzymes. They often activate O_2 for oxidation or hydroxylation.
- Hemeproteins also enable electron transfer as they form part of the electron transport chain. Cyctochrome a, cytochrome b, and cytochrome c have such electron transfer functions.

The sensory system also relies on some hemeproteins including FixL, an oxygen sensor, CooA, a carbon monoxide sensor, and soluble guanylyl cyclase.

1.6.1 Structure and Functions of Myoglobin, Hemocyanin and Hemerythrin

Structure and Functions of Myoglobin

Myoglobin (MB) is an iron- and oxygen-binding protein found in the cardiac and skeletal muscle tissue of vertebrates in general and in almost all mammals. Myoglobin is distantly related to ***haemoglobin***. Compared to haemoglobin, myoglobin has a higher affinity for oxygen and does not have cooperative binding with oxygen like haemoglobin does. In humans, myoglobin is only found in the bloodstream after muscle injury. High concentrations of myoglobin in muscle cells allow organisms to hold their breath for a longer period of time. Diving mammals, such as whales and seals have muscles with particularly high abundance of myoglobin. Myoglobin is found in Type I muscle, Type II A, and Type II B, but most texts consider myoglobin not to be found in smooth muscle.

Myoglobin was the first protein to have its three-dimensional structure revealed by X-ray crystallography. This achievement was reported in 1958 by John Kendrew and associates. For this discovery, Kendrew shared the 1962 Nobel Prize in chemistry with Max Perutz. Despite being one of the most studied proteins in biology, its physiological function is not yet conclusively established: mice genetically engineered to lack myoglobin can be viable and fertile, but show many cellular and physiological adaptations to overcome the loss. Through observing these changes in myoglobin-depleted mice, it is hypothesised that myoglobin function relates to increased oxygen transport to muscle, and to oxygen storage; as well, it serves as a scavenger of reactive oxygen species.

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Myoglobin can take the forms ***OxyMyoglobin (MbO_2), carboxy Myoglobin (MbCO),*** and ***metMyoglobin (met-Mb),*** analogously to haemoglobin taking the forms ***Oxyhemoglobin (HbO_2), CarboxyHaemoglobin (HbCO), and metheMoglobin (met-Hb).***

Like haemoglobin, myoglobin is a cytoplasmic protein that binds oxygen on a haeme group. It harbors only one globulin group, whereas haemoglobin has four. Although its haeme group is identical to those in Hb, Mb has a higher affinity for oxygen than does haemoglobin. This difference is related to its different role: whereas haemoglobin transports oxygen, myoglobin's function is to store oxygen.

Myoglobin belongs to the globin superfamily of proteins, and as with other globins, consists of eight alpha helices connected by loops. Myoglobin contains **153 amino acids**.

Myoglobin contains a porphyrin ring with an iron at its centre. A proximal histidine group (His-93) is attached directly to iron, and a distal histidine group (His-64) hovers near the opposite face. The distal imidazole is not bonded to the iron but is available to interact with the substrate O_2. This interaction encourages the binding of O_2, but not Carbon Monoxide (CO), which still binds about 240× more strongly than O_2.

The binding of O_2 causes substantial structural change at the Fe centre, which shrinks in radius and moves into the centre of N4 pocket. O_2-binding induces 'Spin-Pairing': the five-coordinate ferrous deoxy form is high spin and the six coordinate oxy form is low spin and diamagnetic. Figure 1.27 show the oxygenated myoglobin molecule. This structure shows the structural change when oxygen is bound to the iron atom of the haeme prosthetic group. The oxygen atoms are coloured in green, the iron atom is coloured in red, and the haeme group is coloured in blue.

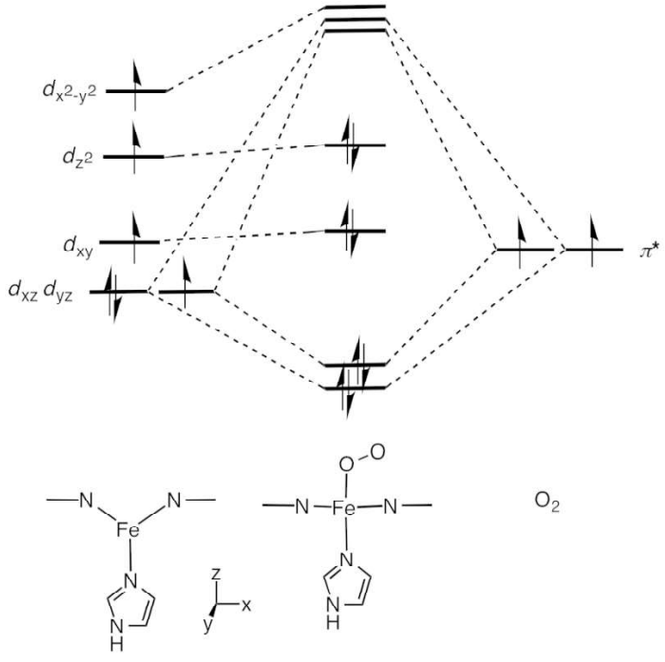

Fig. 1.27 *Molecular Orbital Description of Fe-O_2 Interaction In Myoglobin*

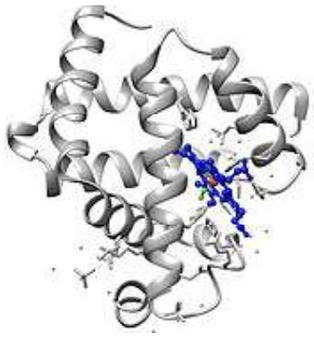

Fig. 1.28 *Oxygenated Myoglobin Molecule*

Structure and Functions of Hemocyanin

Hemocyanins (Hc) are proteins that transport oxygen throughout the bodies of some invertebrate animals. These metalloproteins contain two copper atoms that reversibly bind a single oxygen molecule (O_2). They are second only to haemoglobin in frequency of use as an oxygen transport molecule. Unlike the haemoglobin in red blood cells found in vertebrates, hemocyanins are not confined in blood cells but are instead suspended directly in the hemolymph. Oxygenation causes a colour change between the colourless Cu(I) deoxygenated form and the blue Cu(II) oxygenated form.

Hemocyanins are found only in the Mollusca and Arthropoda: the earliest discoveries of hemocyanins were in the snail *Helix pomatia* (a mollusc) and in the horseshoe crab (an arthropod). They were subsequently found to be common among cephalopods and crustaceans and are utilized by some land arthropods, such as the tarantula Eurypelma californicum, the emperor scorpion, and the centipede *Scutigera coleoptrata*. Also, larval storage proteins in many insects appear to be derived from hemocyanins.

The arthropod hemocyanin superfamily is composed of phenoloxidases, hexamerins, pseudohemocyanins or cryptocyanins, (dipteran) hexamerin receptors. Phenoloxidase are copper containing tyrosinases. These proteins are involved in the process of sclerotization of arthropod cuticle, in wound healing, and humoral immune defense. Phenoloxidase is synthesized by zymogens and are activated by cleaving an N-terminal peptide. Hexamerins are storage proteins commonly found in insects. These proteins are synthesized by the larval fat body and are associated with molting cycles or nutritional conditions. Pseudohemocyanin and cryptocyanins genetic sequences are closely related to hemocyanins in crustaceans. These proteins have a similar structure and function, but lack the copper binding sites.

The evolutionary changes within the phylogeny of the hemocyanin superfamily are closely related to the emergence of these different proteins in various species. The understanding of proteins within this superfamily would not be well understood without the extensive studies of hemocyanin in arthropods.

Although the respiratory function of *hemocyanin* is similar to that of haemoglobin, there are a significant number of differences in its molecular structure and mechanism. Whereas haemoglobin carries its iron atoms in ***porphyrin rings***

(haeme groups), the copper atoms of hemocyanin are bound as prosthetic groups coordinated by histidine residues. The active site of hemocyanin is composed of a pair of copper(I) cations which are directly coordinated to the protein through the driving force of imidazolic rings of six histidine residues. It has been noted that species using hemocyanin for oxygen transportation include crustaceans living in cold environments with low oxygen pressure. Under these circumstances haemoglobin oxygen transportation is less efficient than hemocyanin oxygen transportation. Nevertheless, there are also terrestrial arthropods using hemocyanin, notably spiders and scorpions, that live in warm climates. The molecule is conformationally stable and fully functioning at temperatures up to 90 degrees C.

Most hemocyanins bind with oxygen non-cooperatively and are roughly one-fourth as efficient as haemoglobin at transporting oxygen per amount of blood. Haemoglobin binds oxygen cooperatively due to steric conformation changes in the protein complex, which increases haemoglobin's affinity for oxygen when partially oxygenated. Each hexamer complex was arranged together to form a larger complex of dozens of hexamers. In one study, cooperative binding was found to be dependent on hexamers being arranged together in the larger complex, suggesting cooperative binding between hexamers. Hemocyanin oxygen-binding profile is also affected by dissolved salt ion levels and pH.

Hemocyanin is made of many individual subunit proteins, each of which contains two copper atoms and can bind one oxygen molecule (O_2). Each subunit weighs about 75 kilo Daltons (kDa). Subunits may be arranged in dimers or hexamers depending on species; the dimer or hexamer complex is likewise arranged in chains or clusters with weights exceeding 1500 kDa. The subunits are usually homogeneous, or heterogeneous with two variant subunit types. Because of the large size of hemocyanin, it is usually found free-floating in the blood, unlike haemoglobin.

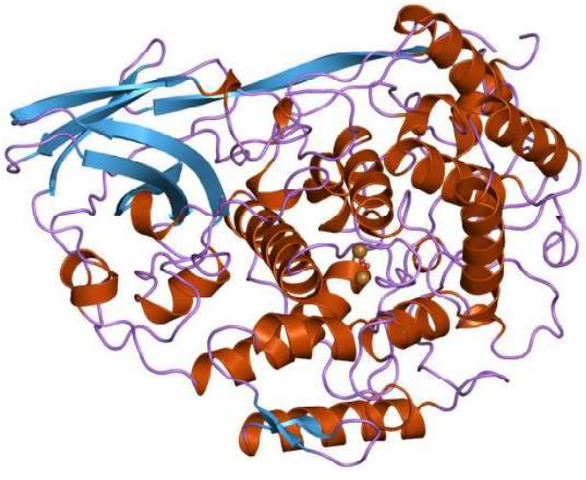

Fig. 1.29 *Crystallographic Analysis of Oxygenated and Deoxygenated States of Hemocyanin*

Structure and Functions of Hemerythrin

Hemerythrin is an oligomeric protein responsible for oxygen (O_2) transport in the marine invertebrate phyla of sipunculids, priapulids, brachiopods, and in a single annelid worm genus, Magelona. Myohemerythrin is a monomeric O_2-binding protein found in the muscles of marine invertebrates. Hemerythrin and myohemerythrin are essentially colourless when deoxygenated, but turn a violet-pink in the oxygenated state.

Hemerythrin does not, as the name might suggest, contain a heme. The names of the blood oxygen transporters hemoglobin, hemocyanin, hemerythrin, do not related to the haeme group (only found in globins), instead these names are derived from the Greek word for blood. Recent evidence has revealed hemerythrin to be a multi-functional protein – contributing to innate immunity and anterior tissue regeneration in worms.

The mechanism of dioxygen binding is unusual. Most O_2 carriers operate via formation of dioxygen complexes, but hemerythrin holds the O_2 as a hydroperoxide (HO_2, or -OOH"). The site that binds O_2 consists of a pair of iron centres. The iron atoms are bound to the protein through the carboxylate side chains of a glutamate and aspartates as well as through five histidine residues. Hemerythrin and myohemerythrin are often described according to oxidation and ligation states of the iron centre shown in following table:

Fe^{2+}—OH—Fe^{2+}	deoxy (reduced)
Fe^{2+}—OH—Fe^{3+}	semi-met
Fe^{3+}—O—Fe^{3+}—OOH^-	oxy (oxidized)
Fe^{3+}—OH—Fe^{3+}— (any other ligand)	met (oxidized)

The uptake of O_2 by hemerythrin is accompanied by two-electron oxidation of the diferrous centre to produce a hydroperoxide (OOH") complex (Refer Figure 1.30). The binding of O_2 is roughly described in this diagram:

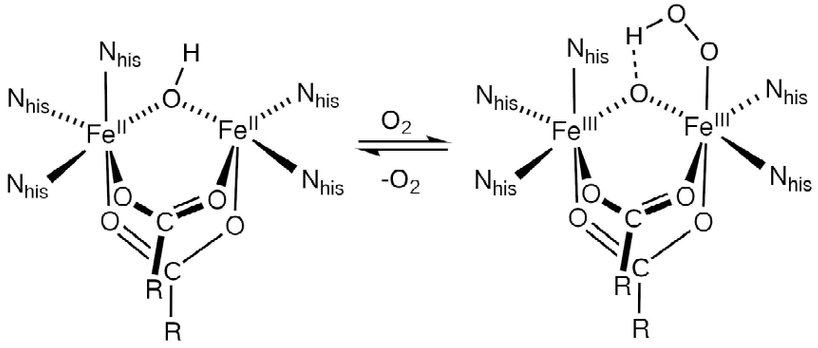

Fig. 1.30 *Active Site of Hemerythrin before and after Oxygenation*

Deoxyhemerythrin contains two high-spin ferrous ions bridged by hydroxyl group (A). One iron is hexacoordinate and another is pentacoordinate. A hydroxyl group serves as a bridging ligand but also functions as a proton donor to the O_2

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substrate. This proton-transfer result in the formation of a single oxygen atom (μ-oxo) bridge in oxy- and methemerythrin. O_2 binds to the pentacoordinate Fe^{2+} centre at the vacant coordination site (B). Then electrons are transferred from the ferrous ions to generate the binuclear ferric (Fe^{3+},Fe^{3+}) centre with bound peroxide (C).

Hemerythrin typically exists as a homooctamer or heterooctamer composed of α- and β-type subunits of 13–14 kDa each, although some species have dimeric, trimeric and tetrameric hemerythrins (Refer Figure 1.31). Each subunit has a four-α-helix fold binding a binuclear iron centre.

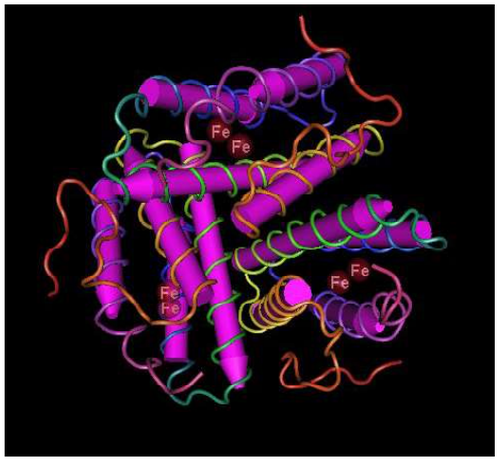

Fig. 1.31 *Trimeric Hemerythrin Protein Complex*

1.6.2 Model Synthetic Complexes of Iron Cobalt Copper

The many synthetic complexes which take up molecular oxygen reversibly can be divided into two groups one is those which fix molecular oxygen in the bridge, dimeric, way, **M: O_2 = 2:1**, and another is in the monomeric way, where **M: O_2 = 1:1**, as in the natural systems. Many dimeric adducts with a bridge of the μ-peroxy type have been known for a long time but monomeric adducts have only been obtained and examined recently. All the oxygen adducts can be divided into three groups according to the geometry of the molecule towards the metal atoms (Refer Figure 1.32) it must be emphasized that most of the models which closely resemble natural systems have been prepared from cobalt.

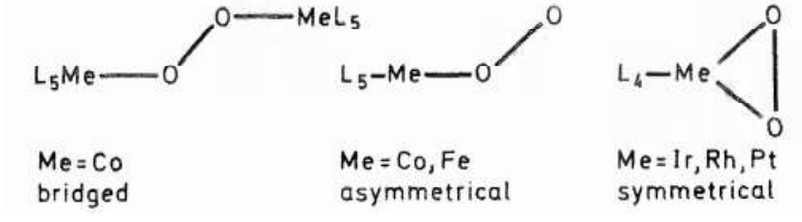

Fig. 1.32 *Synthetic Model of Iron and Cobalt*

Our studies on models of oxygen-carrying systems led to the discovery of new synthetic systems which take up the molecular oxygen reversibly aqueous and in dimethylformamide solutions. These are the cobalt (II) complexes, containing amino acid molecules, and imidazole as ligands. Systems of the type Co—imidazole—amino acid, which are capable of taking up oxygen reversibly, have been obtained with the amino acids listed in Table 1.3. Intensely dark brown coloured oxygen adducts arise on dissolving the polymeric Co^{II} 'Inner' complex of imidazole $[Co(imid)_2]_n$ in an aqueous solution of an amino acid or dipeptide through which oxygen or air is passed, at a temperature close to 0°C. A similar reaction is observed with $[Co(Himid)_2(H_2O)_2CO_3]$ the amino acid has to be in excess to shift the equilibrium in favour of the adduct (Refer Figure 1.33). The 'Active' Co^{II} complex capable of intense oxygen absorption can be obtained in a similar way in an atmosphere of N_2 or Ar, or even O_2 but at higher temperatures (50—60°). The inert gas and temperature cause the displacement of oxygen from the oxygen adducts. On the formation of the oxygen adduct, or 'Active' complex, one imidazole molecule is replaced oxygen or a solvent molecule (Refer Figure 1.41). The unusual ability for rapid oxygen uptake, higher even than the histidine complexes, must be emphasized that system is also unusual because of the perfect reversibility of the process, which allows the oxygen uptake cycle to be repeated many times, without noticeable irreversible oxidation to CO The resistance of solutions of the oxygen adducts towards molecular oxidation to give an inactive monomeric Co^{II} complex depends on the nature of the amino acids. Generally, adducts formed by complexes with dipeptides are less stable. Only in the presence of alpha-amino-carboxylic acid are complexes formed capable of taking up oxygen reversibly.

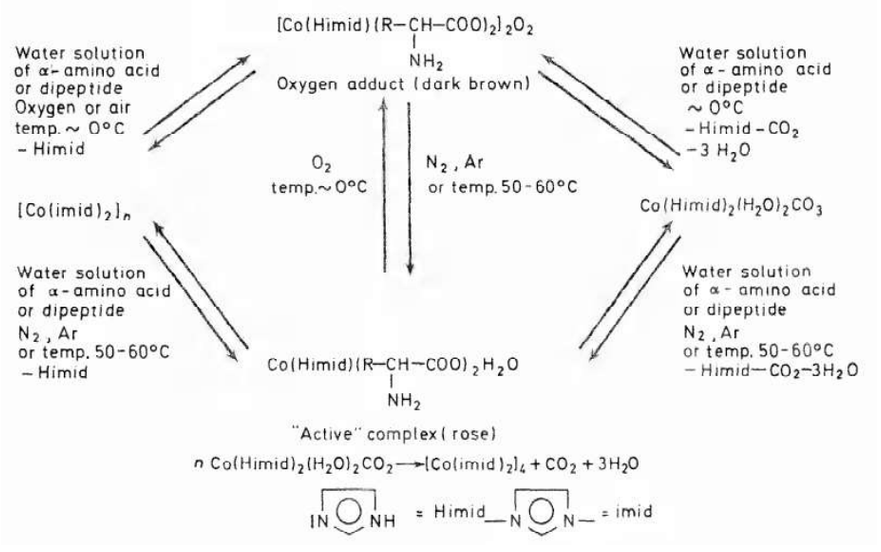

Fig. 1.33 *Scheme of Reactions for Forming 'Active' and Oxygenated Complexes*

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Table 1.3 Neutral Aliphatic Amino Acid and their Substituents

Neutral aliphatic amino acids	Amino acids with substituents
Glycine	Serine
Alanine	Homoserine
Sarcosine	Proline
α-Aminobutyric acid	Hydroxyproline
Valine	Methionine
Norvaline	Citrulline
Isoleucine	Lysine
	Glutamine
	Ornithine
	Arginine
	Asparagine

A negative result is obtained with amino acids and with γ amino-dicarboxylic acids. The presence of an aromatic ring in the β-position, or of a heterocyclic ring, destroys the ability to take up oxygen. This is due most probably to the inductive effect of the ring on the electron density of the α-amino-carboxylic acid group. The basicity of the imidazole group is essential. Replacement of imidazole by weaker bases (pyrazole, benimidazole, purine bases, pyrazine) results in complexes which have lost the ability to take up oxygen. The considerable reactivity of the oxygen adducts must be emphasized.

The photo physical properties of two mononuclear pentaco-ordinated copper(II) complexes formulated as [Cu(L)(Cl)(H_2O)] and [Cu(L)(Br)(H_2O)], HL = (1-[(3-methyl-pyridine-2-ylimino)-methyl]-naphthalen-2-ol) were synthesized and characterized by elemental, physicochemical, and spectroscopic methods. The density function theory calculations are used to investigate the electronic structures and the electronic properties of ligand and complex. The interactions of copper (II) complexes towards calf thymus DNA were examined with the help of absorption, viscosity, and fluorescence spectroscopic techniques at pH 7.40. All spectroscopy's result indicates that complexes show good binding activity to calf thymus DNA through groove binding. The optical absorption and fluorescence emission properties of microwires were characterized by ***fluorescence microscope***. From a spectroscopic viewpoint, all compounds strongly emit green light in the solid state. The microscopy investigation suggested that microwires exhibited optical waveguide behaviour which are applicable as fluorescent nanomaterials and can be used as building blocks for miniaturized photonic devices. Antibacterial study reveals that complexes are better antimicrobial agents than free Schiff base due to bacterial cell penetration by chelation. Moreover, the antioxidant study of the ligand and complexes is evaluated by using 1,1-DiPhenyl-2-PicrylHydrazyl (DPPH) free-radical assays, which demonstrate that the complexes are of higher antioxidant activity than free ligand.

Copper (II) complexes play an important role in the active sites of a large number of metalloproteins in biological systems and potential application for numerous catalytic processes in living organisms that involve electron transfer reactions or activation of some antitumor substances. These processes are also involved in bioinorganic and medicinal chemistry. In fact copper (II) chelates have been found to interact with biological systems and to exhibit antineoplastic activity

[4–6] and antibacterial, antifungal, and anticancer activity. Some copper (II) **N, S, O/N, N**-donor chelators are good anticancer agents due to strong binding ability with DNA base pair. Pyridines are common but vital heterocyclic compounds in organic synthesis, especially as agrochemicals and synthetic intermediates. For example, pyridine derivatives, such as (aromatic) alkoxyl pyridine compounds, amidopyridine, and its derivatives, substituted fused pyridine compounds, have already been widely applied in the fields of agrochemical products. Moreover, pyridine derivatives play a unique role in anthelminthic, acaricide, bacteriocide, and phytocide. For these biological effects we choose the pyridine derivative ligand as a starting material.

1.6.3 Structure and Functions of Haemoglobin

Haemoglobin has two parts. They are:

1. Haeme
2. Globin

Structure of Haeme

Haeme is iron-containing porphyrin nucleus. The porphyrins are complex in nature, having a 'tetra-pyrrole' (four pyrrole rings) structure which are linked by –CH = bridges called 'methynyl' or 'methylidine' bridges. The structure of haeme is shown in Figure 1.34. The valency of iron (Fe) in Hb is six. Iron ion is situated at the centre linking to four nitrogens of the pyrrole rings. The fifth linkage is with the nitrogen of the imidazole ring of Histidine (His) of polypepetide chains, i.e., 'haeme-linked' group. In case of α-chain, histidine is located in amino acid number 87 and in case of β-chain, it is located in His 92. The sixth valence is linked to H_2O in deoxygenated Hb. In case of oxygenated Hb, the H_2O is displaced by O_2.

$$Hb.H_2O + O_2 = Hb.O_2 + H_2O$$

Structure of Globin

The protein part of the Hb is globin. Globin has four peptide chains as shown in Figure 1.34. Each α-chain consists of 141 amino acids and each β-chain consists of 146 amino acids. The protein portion of each of this chain is called globin. Each subunit or globin has a molecular weight of about 17,000 dalton which makes the total molecular weight of the tetramer of about 68,000 daltons.

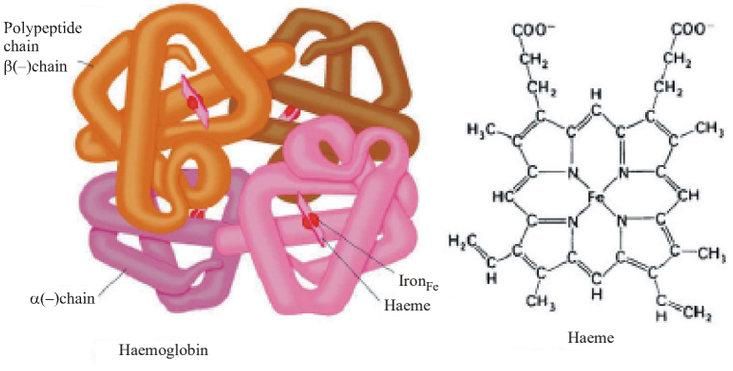

Fig. 1.34 *Structure of Haemoglobin*

Both types of chains are similar in structure. The secondary structure of these four chains is α-helix. Each α-chain and β-chain folds into 8 α-helical segments which further folds to form globular tertiary structure. These four polypeptide chains are arranged in the form of tetrahedron. The four polypeptide chains are then bound to each other by salt bridges, hydrogen bonds and hydrophobic interaction. The folded tertiary structures of helices form pocket. These pockets are known as 'Haeme Pockets.' In each of these four 'haeme pockets,' one haeme is located. Therefore, each Hb molecule contains four 'haeme' units. The gene located for α-chain is situated on chromosome number 16 and the β, γ and δ-chains are located on chromosome number 11.

Types of Haemoglobin

Haemoglobins can be classified mainly into two types. Some Hbs are normal types and some are abnormal variants.

Normal Haemoglobin

There are a variety of normal human haemoglobins which are made up of four sub-units of polypeptides. The polypeptide sub-units are made of different combinations of five different polypeptide chains. The five sub-units are usually known as α, β, γ, δ and ε. Most normal human Hb contains two α-chains with two other β, γ, δ and ε sub-units. Depending on this, they can be classified as follows:

In the Embryo

1. **Gower 1($\zeta_2\varepsilon_2$) and Gower 2 ($\varepsilon_2\gamma_2$):** This is present in foetus in the first three months of pregnancy. There are two α-chains and two ε-chains, i.e., $\alpha_2\varepsilon_2$ in Gower 2.
2. **Hb Portland:** In this case, ε-chains and γ-chains are produced in access and bind with each other to form $\varepsilon_2\gamma_2$. Such foetuses do survive but for some time and commonly die after birth. Such a condition is known as hydrops foetalis.

In Adults

1. **Hb-A1 ($\alpha_2\beta_2$):** It constitutes of about 90–95 per cent of normal haemoglobin in adults. Normal adult Hb consists of two α-chains and two β-chains and they are expressed as $\alpha_2\beta_2$ (Refer Figure 1.35).
2. **Hb-A2 ($\alpha_2\delta_2$):** δ-chain synthesis begins late in the third trimester and in adults, normal range is 1.5–3.5 per cent (Refer Figure 1.35).
3. **Hb-A3:** It is present mainly in the old red cells and it is an altered form of Hb A. It constitutes to about 3–10 per cent of the total adult type.
4. **Hb-A_{1c}(Glycosylated Hb):** It is a glycosylated adult Hb present in increased percentage in the patients of diabetes mellitus. In a normal person, it is about 3–5 per cent but it increases to about 6–15 per cent of total Hb in case of people suffering from diabetes mellitus.

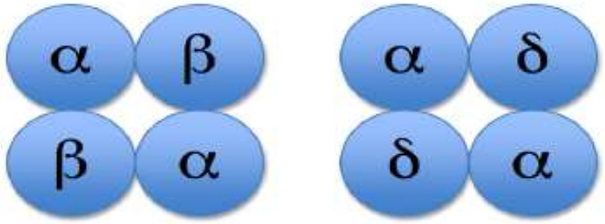

Fig. 1.35 *Structure of Hb in Adults*

Pathologic Mutant Variant

Hemoglobin variants are a part of the normal embryonic and foetal development, but may also be pathologic mutant forms of haemoglobin in a population, caused by variations in genetics. Some well-known haemoglobin variants such as sickle-cell anemia are responsible for diseases, and are considered haemoglobinopathies. Other variants cause no detectable pathology, and are thus considered non-pathological variants.

- **Haemoglobin H (β_4):** It is a variant form of Hb, formed by a tetramer of β-chain, which causes a disease named 'Thalassaemia Major'. Beta thalassaemias are due to mutations in the HBB gene on chromosome 11, also inherited in an autosomal-recessive fashion. The severity of the disease depends on the nature of the mutation. Mutations are characterized as (β^o or β-thalassaemia major) if they prevent any formation of β-chains (which is the most severe form of β-thalassaemia) and they are characterized as (β^+ or β-thalassaemia intermedia) if they allow some β-chain formation to occur. In either case there is a relative excess of α-chains, but these do not form tetramers, rather, they bind to the red blood cell membranes, producing membrane damage and at high concentrations they form toxic aggregates.

  The a-thalassaemias involve the genes HBA1and HBA2, inherited in a Mendelian recessive fashion. It is also connected to the deletion of the 16p chromosome. Alpha thalassaemias result in decreased a-globin production, therefore fewer a-globin chains are produced, resulting in an excess of b-chains in adults and excess g-chains in newborns. The excess b-chains form unstable tetramers (called haemoglobin or Hb of 4 b- chains) which have abnormal oxygen dissociation curves as a- and b-chains are present in haemoglobin about 3 per cent of adult haemoglobin is made of a and b-chains. Just as with b-thalassaemia, mutations can occur which affect the ability of this gene to produce a-chains. This is known as d-thalassaemia.
- **Haemoglobin barts (γ_4):** It refers to the defect in α-chain gene. It causes less expression of α-chain which causes increased amount of γ-chain in respect to α-chain. This γ-chain forms tetramer. Haemoglobin S ($\alpha_2\beta_2^s$) is a case in which the Hb has hereditary defect in β-chain which results in defective Hb causing sickling of RBC. This condition is mostly known as sickle cell disease. This is caused in the homozygous condition (Figure 1.36).

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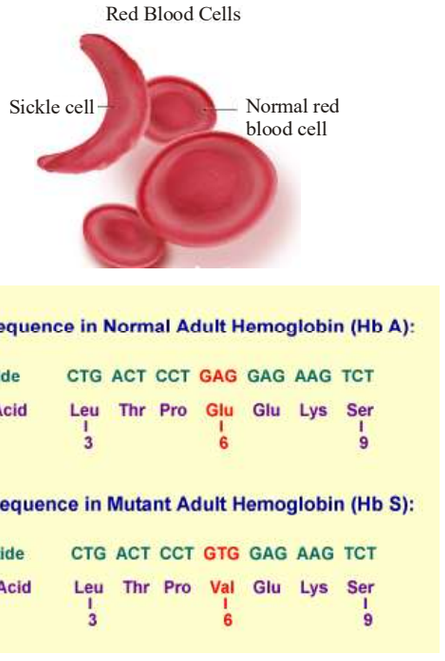

Fig. 1.36 *Sickle Cell*

- **Haemoglobin AS:** It refers to such a heterozygous condition in which one gene is normal adult Hb and another is sickle cell gene. An individual who has such a variety of cells suffers from sickle cell trait.
- **Haemoglobin C ($\alpha_2\beta_2^C$):** Some change in β-chain causes mild chronic haemolytic anaemia. The sixth position of β-chain which contains glutamic acid is replaced by lysine (Refer Figure 1.36).
- **Haemoglobin E ($\alpha_2\beta_2^E$):** If the twenty-sixth position of β-chain containing glutamic acid is replaced by lysine it causes mild chronic hemolytic anaemia.
- **Haemoglobin SC:** It is a heterozygous gene containing one form of sickle gene and another Hb C gene.
- **Haemoglobin D ($\alpha_2\beta_2^D$):** In this type of variant, the glutamic acid in the twenty-sixth position of β-chain is replaced glutamine.
- **Chronic Heinz Body Anaemia (CHBA)**: They are the unstable Hb variants which are formed due to amino acid substitution in the globin chain. The example of α-chain unstable variants are HbTorino, Hb Boyte Heights and Hb Point Philip. The examples of β-chain unstable variants are Hb Belfast, Hb St Luais and Hb Zurich. The γ-chain unstable variants include HbF Poole. All these type of variants cause haemolytic anaemia.
- **Variant with Increased Oxygen Affinity:** In this case, Hb binds very tightly with oxygen, so that oxygen cannot be removed easily. Examples of such variants are: α-chain variant (Hb Chesapeake) and β-chain variant (Hb Olympia).
- **Variant with Decreased Oxygen Affinity:** Hb has less tendency to bind with oxygen. For example, Hb Kansas, Hb Rothschild, Hb Hope.

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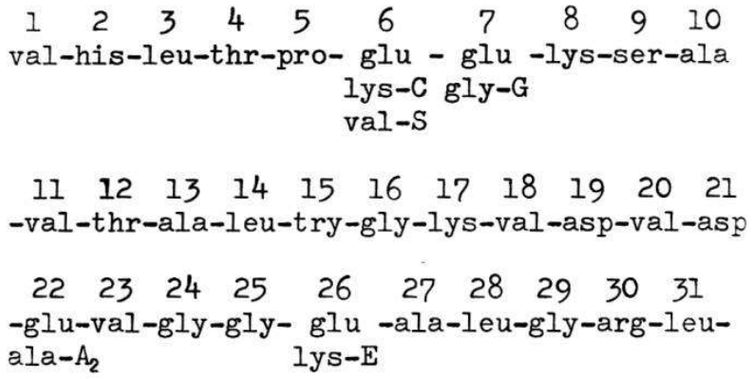

Fig. 1.37 *Mutant-Vaciants of Haemoglobin*

Derivatives of Haemoglobin

The nearest formal oxidation state of iron in Hb-O_2 is the +3 state, with oxygen in the–1 state (as superoxide $.O_2^-$). The diamagnetism in this configuration arises from the single unpaired electron on superoxide aligning anti-ferromagnetically from the single unpaired electron on iron, to give no net spin to the entire configuration, in accordance with diamagnetic oxy-haemoglobin from experiment.

1. **Carboxy haemoglobin:** It is formed when carbon monoxide comes in contact with haemoglobin. The tendency of CO binding to Hb is 200 times more than O_2. This is harmful and life threatening.
2. **Meth haemoglobin:** In this case, iron changes to ferric state and is incapable of binding with oxygen. Normally, the Meth haemoglobin present in blood is about 1 per cent. The oxidation of Hb is stopped by the orientation of haeme pocket. The meth haemoglobin formed is reverted back to ferrous state by enzyme system present in the cell.
3. **Sulph haemoglobin:** It is formed when sulphur combines with the haeme of Hb. It is green in colour and is incapable of carrying oxygen.

Synthesis of Haemoglobin

Hb is synthesized in a complex series of steps. Formation of haeme part occurs in the mitochondria and cytosol of immature RBCs, while the globin part is synthesized by ribosome in cytoplasm. Production of Hb occurs during erythropoiesis of immature RBCs in red bone marrow. During erythropoiesis, even after loss of nucleus, the production of Hb continues till reticulocyte stage.

Synthesis of Haeme

Haemoglobin (Hb) is synthesized in a complex series of steps. The haeme part is synthesized in a series of steps in the mitochondria and the cytosol of immature red blood cells, while the globin protein parts are synthesized by ribosomes in the cytosol.

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Following are the stages involved in synthesis of haeme (Refer Figure 1.38).

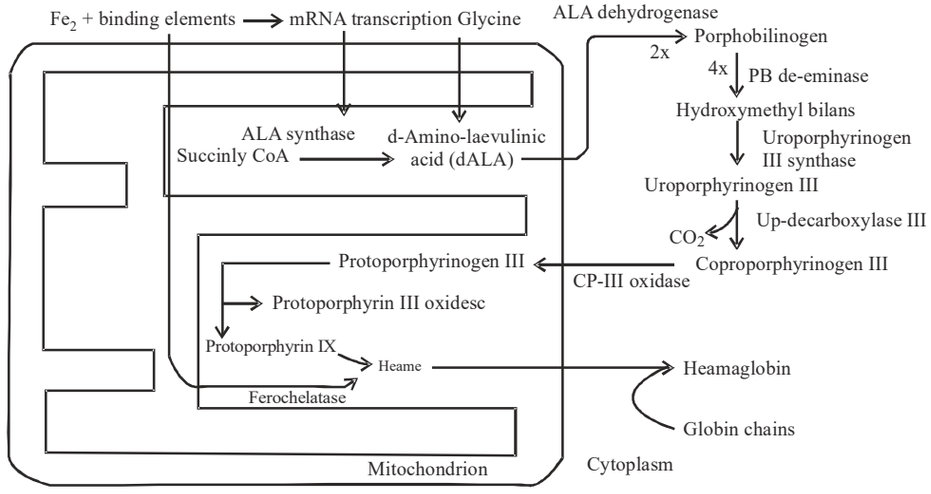

Fig. 1.38 *Synthesis of Haeme*

Biosynthesis of porphyrin: Stages involved in the formation of porphyrin are:

Stage I

- **Synthesis of d-amino laevulinic acid (δ-ALA):** It occurs in mitochondria. First, the succinyl CoA and glycine condense together to form α-amino-β-ketoadipic acid. After that on release of carbon-di-oxide, it forms δ-amino laevulinic acid. This reaction occurs in the presence of δ-ALA synthase. The coenzymes involved in this reaction are pyridoxal phosphate, pathothenic acid (forming CoA–SH) and Mg^{++}.

Stage II

- Synthesis of co-prophyrinogen III and I (cytosolic)
  - o Formation of porphobilinogen: d-ALA then moves into the cytoplasm from mitochondria. Two molecules of d-ALA undergo condensation reaction to form porphobilinogen in presence of enzyme d-ALA dehydrogenase. Co-factor involved is copper.
- Formation of uroporphyrinogen I and III
  - o Uroporphyrinogen I (minor series): In the presence of enzyme, porphobilinogen de-eminase (uroporphylinogen I synthase), four molecules of porphobilinogen condenses, losing four molecules of ammonia and form uroporphyrinogen I or hydroxymethyl bilane.
  - o Uroporphyrinogen III formation (major series): The cyclization of the uroporphyrinogen I occurs in the presence of uroporphyrinogen III synthase with deaminase. The product formed is called uroporphyrinogen III.
  - o Formation of co-prophyrinogen III: In presence of uroporphyrinogen III decarboxylase (removing carbon-di-oxide), the uroporphyrinogen III is decarboxylated to form coporphyrinogen III.

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Stage III

- Formation of protoporphyrin IX

  After the formation of co-proporphyrinogen III, it enters mitochondria. In mitochondria, Coproporphyrinogen III is converted to protoporphyrinogen in the presence of coproporphyrinogen III oxidase. Protoporphyrinogen is then converted to protoporphyrin IX in presence of enzyme protoporphyrin III oxidase.

Stage IV

Fe^{2+} is inserted in central position of protoporphyrin IX by haeme synthetase to form haeme. Haeme is then coupled with protein globin to form haemoglobin.

Catabolism of Haemoglobin

RBC is mainly destroyed in RE cells situated in bone marrow, spleen and liver as shown in Figure 1.39. When RBCs complete their life cycle or become defective, they are broken down and haemoglobin is released. Haemoglobin is then broken down releasing:

- Protein portion globin which is catabolized into free amino acid.
- Iron of haeme enters 'Iron Pool' which is recycled or stored as 'Ferritin.'
- Porphyrin ring is catabolized to form bile pigment, biliverdin and bilirubin in RE cells.

The bilirubin is formed either from 'Haeme' of destroyed erythrocyte or from other sources (such as haeme from haemoglobin synthesis, degradation of haemoglobin within immature erythrocyte, destruction of immature erythrocyte in the bone and breakdown of other haeme pigments such as cytochrome, myoglobin and catalase).

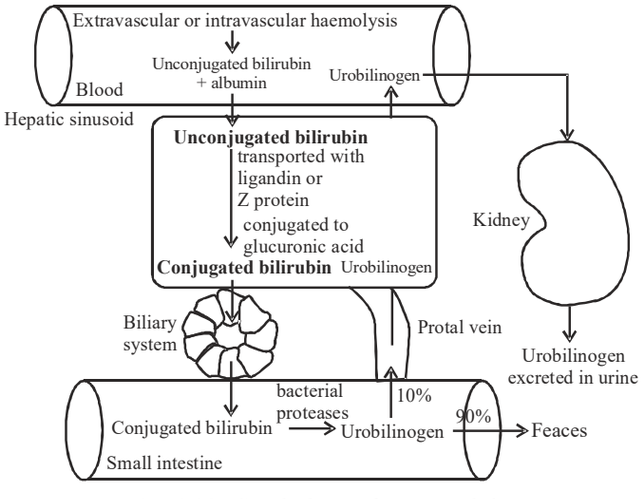

Fig. 1.39 *Catabolism of Haemoglobin*

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Formation of Bile Pigment from Haeme

The pigments of bile are mainly the 'biliverdin' and 'bilirubin' for which bile colour is yellow-green. Among the two, bilirubin constitutes the major portion of bile pigment.

In microsome of RE cells (reticulo endothelial cells), oxidative hydrolysis of the iron-porphyrin ring occurs due to the enzymehaeme–α –methenyloxygenase. It is not yet proved whether globin separates first or after ring opening. The opening of ring helps in removal of Fe++. The ring is being fused at α-methane bridge between pyrrole rings I and II. The first product that is formed in this step is biliverdin and carbon monoxide. This biliverdin is then converted to bilirubin in presence of bilirubin reductase NADH or NADPH as source of hydrogen donor. This has been shown in Figure 1.40.

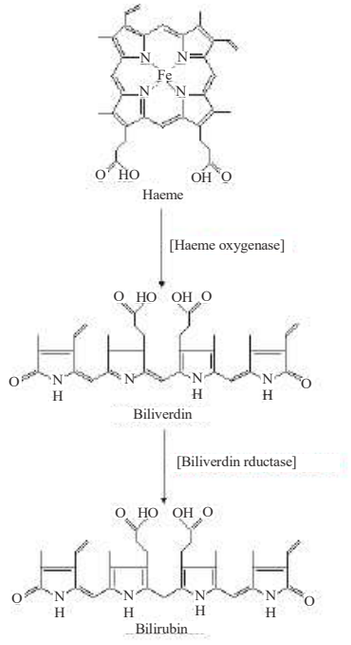

Fig. 1.40 *Formation of Bile Pigment*

Bilirubin is a non-polar compound and is soluble in lipid. When bilirubin binds with albumin by non-covalent bond, it's solubility increases in plasma. Such a form is known as un-conjugated or indirect bilirubin. Albumin acts as a carrier of bilirubin carrying bilirubin from blood to liver.

In liver, bilirubin dissociates from the carrier albumin molecules and enters haepatocytes (liver cell). In smooth endoplasmic reticulum of haepatocyte, bilirubin then conjugates with one or two molecules of glucuronic acid (the acid form of glucose) to form bilirubin monoglucuronide and bilirubin diglucuronide.

The enzyme catalyzing this reaction is glucuronyl transferase and its transfer glucuronic acid from UDP–GA to the propionic acid carboxyl group of bilirubin through 'ester linkage.' Such a process is known as 'Conjugation' and the bilirubin mono and di-glucuronide formed is known as conjugated bilirubin. Conjugated bilirubin is soluble in water and is smaller in size than unconjugated bilirubin because it is attached to albumin. Therefore, the conjugated bilirubin can easily pass through

glomerular filter. Unconjugated bilirubin cannot pass through the filter. For this reason, conjugated bilirubin appears in urine.

The conjugated bilirubin formed in liver is transported to intestine through gallbladder. On reaching caecum and the colon of large intestine, the glucuronides is released from bilirubin and bilirubin is set free by the enzyme β-glucuronidase produced by the bacteria present in that area.

Bilirubin is ultimately reduced in a series of reaction by enzyme system present in intestinal tract which is majorly contributed by anaerobic bacteria in the caecum.

The ultimate product formed is L-stercobilinogen which on exposure to air undergoes auto-oxidation. After auto-oxidation, L-stercobilinogen is converted to L-stercobilin which gives characteristic colour to faeces.

Some of the meso-bilirubinogen is converted to D-urobilinogen which is less stable than stercobilin. After oxidation of D-urobilinogen , it forms urobilin IX which has blue-green or violet coloured pigments.

Entero-Hepatic Circulation of Bile Pigments

The intermediary products which were produced during successive reduction of bilirubin are absorbed from intestine and are returned to liver for re-excretion. Such circulation of bile secretion is called as 'entero-hepatic circulation' (viz. entero means intestine and haepatic means liver) of bile pigments.

Some of these derivatives which are able to escape the entero-hepatic circulation are excreted in urine but majority the is excreted in faeces.

These bile pigments are significant for determining different types of Jaundice. Bilirubin is determined by Vanden–Berg reaction.

Functions of Haemoglobin

Heamoglobin can transport oxygen, carbon-di-oxide and nitric oxide. Heamoglobin carries oxygen from lungs to tissues in oxy-heamoglobin form. After releasing the oxygen in tissue, it takes up carbon-di-oxide and itself form caroboxy-haemoglobin. Haemoglobin then returns to lungs and release the CO_2 and it is able to transport another gas, i.e., nitric oxide. It helps in regulating blood pressure by relaxing the walls of blood vessels which in turn increases the blood flow in vessels. Thus, amount of nitric oxide released affects the contraction and relaxation of blood vessels ultimately affecting the blood pressure. Haeme part of haemoglobin contributes to the formation of bile pigments (see Figure 1.41).

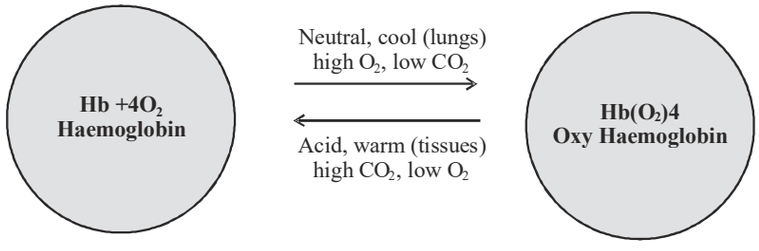

Fig. 1.41 *Formation of Haemoglobin*

The Bohr Effect

The ability of haemoglobin to release oxygen is affected by pH, CO_2 and by the differences in the oxygen-rich environment of the lungs and the oxygen-poor

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environment of the tissues. The pH in the tissues is considerably lower (more acidic) than in the lungs. Protons are generated from the reaction between carbon-di-oxide and water to form bicarbonate:

$$CO_2 + H_2O \longrightarrow HCO_3^- + H^+$$

This increased acidity serves a two-fold purpose. First, protons lower the affinity of haemoglobin for oxygen, allowing easier release into the tissues. As all four oxygen protons are released, haemoglobin binds the two protons. This helps to maintain equilibrium towards the right side of the equation. This is known as the Bohr effect, and is vital in the removal of carbon-di-oxide as waste because CO_2 is insoluble in the bloodstream. The bicarbonate ion is much more soluble and can thereby be transported back to the lungs after being bound to haemoglobin. If haemoglobin could not absorb the excess protons, the equilibrium would shift to the left and carbon-di-oxide will not be removed. In the lungs, this effect works in the reverse direction. In the presence of the high oxygen concentration in the lungs, the proton affinity decreases. As protons are shed, the reaction is driven to the left, and CO_2 is formed as an insoluble gas to be expelled from the lungs. The proton poor haemoglobin now has a greater affinity for oxygen, and the cycle continues (see Figure 1.42).

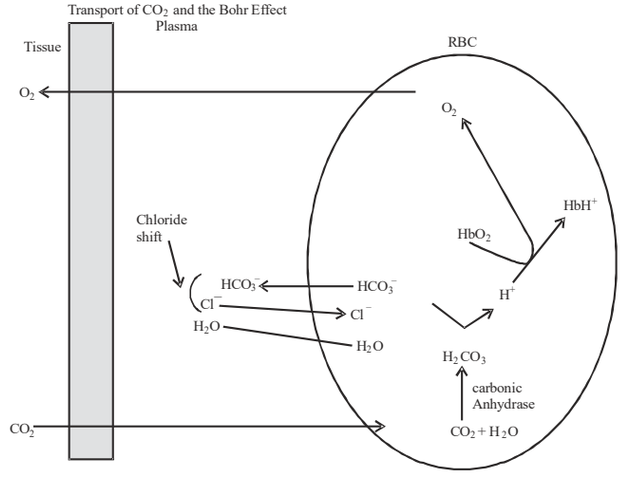

Fig. 1.42 *The Bohr Effect*

Check Your Progress

13. Which trace elements are found in plants in biological system?
14. What is minor elements and why is called so?
15. Define biomolecule.
16. What do you understand by the Na+/K+ pump?
17. How the haemoglobin is able to transport the oxygen within the body of arganism.
18. What is haeme pockets?
19. What do you understand by myoglobin?
20. What is hemerythrin?

1.7 ANSWERS TO 'CHECK YOUR PROGRESS'

NOTES

1. It states that:
   - All living things are composed of minute units, the cells
   - A cell is a mass of protoplasm containing a nucleus and bounded by cell membrane and by cell wall
   - All cells are basically alike in structure and metabolic activities
   - The function of an organism is the result of the activities and interaction of its constituent cells.
2. Cytoplasmic matrix (hyaloplasma) is the fluid part of cytoplasm which exists in both sol and gel respectively called plasmasol and plasma gel. Water constitutes 90% of the matrix. Matrix is the crystallo-colloidal complex where some chemicals are present as true solution and others as colloidal solution, for example, minerals, sugars, amino acids, vitamins, proteins, enzymes, etc.
3. Cell membrane is thin and flexible, composed of lipids and proteins. It controls the movement of molecules into and from the cell. It carries respiratory enzymes for energy reactions.
4. Genophore consists of single chromosome that is coiled, double stranded, circular not associated with histones. Transcription and translation occur in the cytoplasm. There is no nucleolus. It consists of single chromosome which is coiled forming body.
5. Proteins are built as chains of amino acids, which then fold into unique three-dimensional shapes. Bonding within protein molecules helps stabilize their structure, and the final folded forms of proteins are well-adapted for their functions.
6. Main Functions of Protein in biological Cell
   - Protein is necessary in building and repairing body tissues.
   - Hormone Production.
   - Hormones are chemicals produced by glands in one part of the body that help coordinate activities and communicate with other areas.
   - Enzymes.
   - Immune Function.
   - Energy.
7. RiboNucleic Acid or RNA and DeoxyriboNucleic Acid or DNA are polymers constituted of monomers called mononucleotide units. These mononucleotide units are united together by intermolecular dehydration reactions that make phosphate ester bonds.
8. The bases in DNA are classified into two types:
   - Purines: Examples of purines include adenine and guanine (fused five- and six-membered heterocyclic compounds).

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- Pyrimidines: Examples of pyrimidines include cytosine and thymine (six-membered rings).

9. Complementary base pairing is when each type of base on one strand establishes a link with exactly one type of base on the other strand. Purines form hydrogen bonds with pyrimidines. The arrangement of two nucleotides binding together across the double helix is known as a base pair.
10. There are three types of membrane lipids:
    - Phosphoglycerides
    - Sphingolipids
    - Cholesterol
11. There are three types of membrane transport, .i.e.,
    - Diffusion
    - Active Transport
    - Bulk Transport
12. Vagus nerve is the nerve innervates the diaphragm as well as movements in the larynx and pharynx. It also provides parasympathetic stimulation for the heart and the digestive system. It is a major autonomic nerve.
13. Commonly required plant trace elements include copper, boron, zinc, manganese, and molybdenum. Animals also require manganese, iodine, and cobalt.
14. The trace elements included Group III also called as minor elements. Their requirement is below 100 mg/day and their absence may not hinder normal development, but their activity may be substituted by another metal. Analytical methods are used to measure metal concentration in human tissues and body fluids.
15. The term 'Biomolecule' refers to any organic molecule that is formed by a living organism. This includes large polymeric molecules like proteins, polysaccharides and nucleic acids and small molecules like primary metabolites, secondary metabolites and natural products.
16. Na^+/K^+ electrogenic P-type membrane pump carries out the coupled transport of three Na^+ ions outside and two K^+ ions inside the cell. It is a transmembrane heterodimeric Protein and comprises of α and β subunits, forming intracellular and extracellular domains and five pair of transmembrane helices.
17. Haemoglobin is able to transport oxygen within the body due to its unique structure. Its structure consists of four globin subunits: two α and two β subunits. Each subunit contains a heme prosthetic group with an iron bound.
18. The four polypeptide chains are then bound to each other by salt bridges, hydrogen bonds and hydrophobic interaction. The folded tertiary structures of helices form pocket. These pockets are known as 'Haeme Pockets.'
19. Myoglobin (MB) is an iron- and oxygen-binding protein found in the cardiac and skeletal muscle tissue of vertebrates in general and in almost all mammals. Myoglobin is distantly related to haemoglobin.

20. Hemerythrin is an oligomeric protein responsible for Oxygen (O_2) transport in the marine invertebrate phyla of sipunculids, priapulids, brachiopods, and in a single annelid worm genus, Magelona. Myohemerythrin is a monomeric O_2-binding protein found in the muscles of marine invertebrates. Hemerythrin and myohemerythrin are essentially colourless when deoxygenated, but turn a violet-pink in the oxygenated state.

1.8 SUMMARY

- Living beings are composed of cell or many cells. A cell is a mass of cytoplasm enclosed by plasma membrane and contain certain organelles besides nucleus. It is a unit of biological entity covered by semipermeable membrane and capable of medium free of other living systems.
- Cell is an open system as it allows the entry and exit of matter and energy. It takes up matter for sustenance, growth and division. Organisms are built according to information encoded in a collection of genes. This vast information is packaged into a set of chromosomes that occupies the space of cell nucleus.
- The first three organelles are surrounded by double membrane known as envelope. The part of cytoplasm excluding compartments are eukaryotic cells. The cell of bacteria and blue green algae don't contain membrane bound compartment called single compartment cell called prokaryotic cell. The lack of compartments show primitive nature of these organisms.
- The cell walls are small circular genetic material in addition to nucleiod. They generally code for proteins required by organism in resistance against antibiotics and toxic materials. They are not essential for the growth of the cell. Some encode proteins needed by organism to resist antibiotics.
- Cells be dependent on thousands of different enzymes to catalyse metabolic reactions. Enzymes are proteins, and they make a biochemical reaction more likely to proceed by lowering the activation energy of the reaction, thereby making these reactions proceed thousands or even millions of times faster than they would without a catalyst. Enzymes are highly specific to their substrates.
- The name of an enzyme usually refers to the type of biochemical reaction it catalyses. For example, proteases break down proteins, and dehydrogenases oxidize a substrate by removing hydrogen atoms. As a general rule, the '-Ase' suffix identifies a protein as an enzyme, whereas the first part of an enzyme's name refers to the reaction that it catalyses.
- The double helical DNA strands can be pulled apart like a zipper either by mechanical force or by applying high temperature. The concept of 'Complementarity' causes duplication of all information in the double-stranded sequence of a DNA helix, which is essential in DNA replication.
- The nucleotide bases of the DNA molecule form complementary pairs with the nucleotides hydrogen bond to another nucleotide base in a strand of DNA, opposite to the original. This bonding is specific, with adenine always

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pairing with thymine (and vice versa) and guanine always pairing with cytosine (and vice versa).

- A small amount of DNA is also available in cell organelles like mitochondria or chloroplasts. On the other hand, in prokaryotes like bacteria and archaea, DNA is present in the cytoplasm. Chromatin proteins, such as histones, compact and organize DNA into chromosomes.
- A DNA sequence is referred to as 'sense' if its sequence is the same as that of a messenger RNA copy that is translated into protein. Its opposite is known as the 'Anti-Sense' when the sequence is on the opposite strand.
- Plasma membrane is the outer membrane covering of cell protoplasts discovered by Schwann (1838). It was called cell membrane by Nageli and Cramer (1855).
- The asymmetric functioning of such system is of little value to cells. It was unclear for many years as to how phospholipid bilayer only 5 nm thick could be strong enough to withstand stress on the plasma membrane of most cells.
- The lipid bilayer is fundamental structure of most biomembranes. Non lamellar lipid structure also form important components of biomembranes.
- Lipids have asymmetric concentration across biomembranes, for example, out of four most abundant category of phospholipid in plasma membrane, the anionic one (phosphatidylserine) and a zwitterionic one (phosphotidylethanolamine) are more concentrated in the inner than the outer leaflet of bilayer.
- The spinal cord and the motor nerves conduct the nerve impulses from the cortex and brainstem to the anterior horn cells of the motor neurons that supply the respiratory muscles. The nerve fiber tracts in the spinal cord that are responsible for voluntary (cortico-spinal tract) and automatic (reticulo-spinal tract) breathing are distinct within the spinal cord.
- The signals for voluntary breathing originate in the cerebral cortex. There are centres within the parietal cortex that send the signals for inspiration and expiration to occur. These cortical areas project to the motor neurons in the spinal cord via the cortico-spinal tracts.
- Metal ions are fundamental elements for the maintenance of the lifespan of plants, animals and humans. Their substantial role in biological systems was recognized a long time ago. They are essential for the maintenance of life and their absence can cause growth disorders, severe malfunction, carcinogenesis or death.
- Titanium (Ti), being one of the most abundant elements in the earth's crust with many examples of bioactive properties, is widely used in the cosmetic industry. Titanium Dioxide (TiO_2) Nano Particles (NPs) are often incorporated in sunscreens as physical sun blockers absorbing Ultra Violet (UV) radiation.
- Carbon (C) is the most important owing to its property to form an infinite number of compounds. This helps carbon to form stable covalent bonds and C–C chains of unlimited length. Carbon has a unique property called

catenation, that is, the property of forming bonds with the same element. It is tetravalent in nature and can form single, double and triple covalent bonds to combine with hydrogen, oxygen, sulphur, nitrogen and chlorine to form various types of compounds.

- Every living system is built up in a hierarchical manner. Multicellular organisms are made up of organ systems, which are constituted by tissues.
- Calcium is a regulator of the Sodium pump. It inhibits the pump at the physiological concentration range of 0.08-5 μM. In excitable cells, depolarisation causes an increase in $[Ca^{2+}]_{cyt}$, which crosses the threshold level and inhibits specific isoforms of the pump in a concentration and affinity-dependent manner.
- The process of Ca^{2+} inhibition associated with the á subunit of the enzyme is non-competitive and it does not compete with the binding site for Na^{+}.
- Haemoglobin is able to transport oxygen within the body due to its unique structure. Its structure consists of four globin subunits: two α and two β subunits. Each subunit contains a heme prosthetic group with an iron bound.
- Myoglobin (MB) is an *iron- and oxygen-binding protein* found in the cardiac and skeletal muscle tissue of vertebrates in general and in almost all mammals. Myoglobin is distantly related to haemoglobin. Compared to haemoglobin, myoglobin has a higher affinity for oxygen and does not have cooperative binding with oxygen like haemoglobin does.
- Copper (II) complexes play an important role in the active sites of a large number of metalloproteins in biological systems and potential application for numerous catalytic processes in living organisms that involve electron transfer reactions or activation of some antitumor substances.

1.9 KEY TERMS

- **Prokaryotic cell:** A prokaryotic cell is a single envelope system. There is no membrane enveloping the genetic material. It is present in bacteria and blue green algae.
- **Cell membrane:** It is thin and flexible, composed of lipids and proteins. It controls the movement of molecules into and from the cell. It carries respiratory enzymes for energy reactions.
- **Basal body:** It comprised of two regions: shaft and hook. The shaft bears a pair of rings in gram positive bacteria and two pairs of rings in gram negative bacteria. The inner ring is embedded in the plasma membrane and fixed to the shaft.
- **Proteins:** Proteins are built as chains of amino acids, which then fold into unique three-dimensional shapes. Bonding within protein molecules helps stabilize their structure, and the final folded forms of proteins are well-adapted for their functions.
- **Phosphoglycerides:** Phospholipids constitute a major portion of lipids (55-57%) in plasma membranes. They belong to mainly two categories. Neutral phospholipids and acidic phospholipids.

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- **Passive diffusion:** The membrane plays a passive role in the transport of substances across it. The unassisted movement is from higher concentration side to the side where concentration is lower.
- **Phrenic nerves:** The nerves stimulate the activity of the diaphragm. They are composed of two nerves, the right and left phrenic nerve, which pass through the right and left side of the heart, respectively. They are autonomic nerves.

1.10 SELF-ASSESSMENT QUESTIONS AND EXCERCISES

Short–Answer Questions

1. Give the properties of cell in biology.
2. Differentiate between prokaryotic and eukaryotic cell.
3. How do prokaryotic and eukaryotic cells resemble?
4. Give the properties and structures of DNA.
5. What is the molecular structure of Uracil and Adenine?
6. Differentiate between DNA and RNA.
7. Give the types of RNA.
8. Define the cell membrane.
9. Give the evidence for fluid mosaic model.
10. What is nerve conduction?
11. Define the trace metal in biological system.
12. Give the categorical classification of trace elements.
13. Define the Na^+/K^+ pump.
14. Give the structure of haeme.
15. Define the term of myoglobin.
16. Give the synthetic model of iron, cobalt and copper.

Long –Answer Questions

1. Elaborate on the cell structure and history.
2. Give the salient features of eukaryotic cells and prokaryotic cell.
3. Discuss structure and properties of DNA and RNA in living organism.
4. Explain in detail about the cell membrane with the help of their components and their properties.
5. Elaborate on the irreversible thermodynamic treatment of membrane transport.
6. Discuss in detail about the physiology of nerve conduction.
7. Analyse the essential and trace metals of biological system.

8. Illustrate the Na^+/K^+ pump.
9. Describe the transport and storage of dioxygen with the help of examples.
10. Give an elaborated the structure and types of haemoglobin.

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1.11 FURTHER READING

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UNIT 2 BIOENERGETICS

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Structure

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  2.2.2 Hydrolysis of ATP
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2.0 INTRODUCTION

Bioenergetics is the branch of biochemistry that focuses on how cells transform energy, often by producing, storing or consuming Adenosine TriPhosphate (ATP). Bioenergetics processes, such as cellular respiration or photosynthesis, are essential to most aspects of cellular metabolism, therefore to life itself. Exergonic reactions are also called spontaneous reactions, because they can occur without the addition of energy. In other hand endergonic reactions are the reactions where energy enters the system, the free energy here is positive (greater than 0).

ATP hydrolysis is the catabolic reaction process by which chemical energy that has been stored in the high-energy phosphoanhydride bonds in Adenosine TriPhosphate (ATP) is released after splitting these bonds. ATP is the higher energy form (the recharged battery) while ADP is the lower energy form (the used battery).

Diffraction-based techniques such as single-crystal X-ray crystallography, electron microscopy, and neutron diffraction are well established and they have paved the road to the stunning successes of modern-day structural biology. Light scattering methods provide data on biological macromolecules (i.e., proteins, nucleic

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acids) that are complementary with those of size exclusion chromatography, gel electrophoresis, analytical ultracentrifugation and mass spectrometry methods. Small-Angle X-Ray Scattering (SAXS) and Small-Angle Neutron Scattering (SANS) are the two complementary techniques known jointly as Small-Angle Scattering (SAS). X-ray diffraction, a phenomenon in which the atoms of a crystal, by virtue of their uniform spacing, cause an interference pattern of the waves present in an incident beam of X rays. Optical rotatory dispersion is the variation in the optical rotation of a substance with a change in the wavelength of light. It used to find the absolute configuration of metal complexes.

ATP cyclic transformation from ATP to ADP and again back to ATP is called as ATP cycle. A DNA or DeoxyriboNucleic Acid polymerase is a member of a family of enzymes that catalyse the synthesis of DNA molecules from nucleoside triphosphates, the molecular precursors of DNA. Photosystem I (PS I) and photosystem II (PS II) are two multi-subunit membrane-protein complexes involved in oxygenic photosynthesis. The main difference between photosystem I and II is that PS I absorbs longer wavelengths of light (>680 nm) whereas PS II absorbs shorter wavelengths of light (<680 nm).

Electron transfer reactions involve the movement of an electron from one molecular species (the donor) to another (the acceptor) and turn out to be an essential quantum mechanical component in various biological processes.

Nitrogenases are enzymes that are produced by certain bacteria, such as cyanobacteria. These enzymes are responsible for the reduction of nitrogen to ammonia. Nitrogenases are the only family of enzymes known to catalyze this reaction, which is a key step in the process of nitrogen fixation.

In this unit, you will study about the bioenergetics, exergonic and endergonic process, ATP hydrolysis, diffraction methods, light scattering, Small-Angle X-Ray Scattering (SAXS), X-ray diffraction, optical rotatory dispersion, ATP cycle, DNA polymerization, chlorophyll, Photosystem I (PS I) and photosystem II (PS II), electron transfer in biology system, structure and function of metalloprotein, nitrogenases.

2.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the bioenergetics process in biological system
- Elaborate on the exergonic and endergonic process
- Interpret the ATP hydrolysis
- Know about the diffraction and light scattering methods
- Analyse the (SAXS), X-ray diffraction and ORD
- Explain about the ATP cycle
- Discuss about the DNA polymerization
- Comprehend the chlorophyll
- Describe the Photosystem I (PS I) and photosystem II (PS II)
- Analyse the electron transfer in biology system

- Discuss about the structure and function of metalloprotein
- Know about the nitrogenase and biological nitrogen fixation

2.2 INTRODUCTION TO BIOENERGETICS

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Bioenergetics is a field in biochemistry and cell biology that concerns energy flow through living systems. This is an active area of biological research that includes the study of the transformation of energy in living organisms and the study of thousands of different cellular processes such as cellular respiration and the many other metabolic and enzymatic processes that lead to production and utilization of energy in forms such as Adenosine TriphosPhate (ATP) molecules. That is, the goal of bioenergetics is to describe how living organisms acquire and transform energy in order to perform biological work. The study of metabolic pathways is thus essential to bioenergetics.

Bioenergetics is the part of biochemistry concerned with the energy involved in making and breaking of chemical bonds in the molecules found in biological organisms. It can also be defined as the study of energy relationships and energy transformations and transductions in living organisms. The ability to harness energy from a variety of metabolic pathways is a property of all living organisms. Growth, development, anabolism and catabolism are some of the central processes in the study of biological organisms, because the role of energy is fundamental to such biological processes. Life is dependent on energy transformations; living organisms survive because of exchange of energy between living tissues/ cells and the outside environment. Some organisms, such as autotrophs, can acquire energy from sunlight without needing to consume nutrients and break them down. Other organisms, like heterotrophs, must intake nutrients from food to be able to sustain energy by breaking down chemical bonds in nutrients during metabolic processes such as glycolysis and the citric acid cycle. Importantly, as a direct consequence of the First Law of Thermodynamics, autotrophs and heterotrophs participate in a universal metabolic network—by eating autotrophs, heterotrophs harness energy that was initially transformed by the plants during photosynthesis.

In a living organism, chemical bonds are broken and made as part of the exchange and transformation of energy. Energy is available for work (such as mechanical work) or for other processes (such as chemical synthesis and anabolic processes in growth), when weak bonds are broken and stronger bonds are made. The production of stronger bonds allows release of usable energy.

Adenosine TriphosPhate (ATP) is the main 'energy currency' for organisms; the goal of metabolic and catabolic processes are to synthesize ATP from available starting materials , and to break- down ATP into Adenosine DiphosPhate (ADP) and inorganic phosphate by utilizing it in biological processes. In a cell, the ratio of ATP to ADP concentrations is known as the 'energy charge' of the cell. A cell can use this energy charge to relay information about cellular needs; if there is more ATP than ADP available, the cell can use ATP to do work, but if there is more ADP than ATP available, the cell must synthesize ATP via oxidative phosphorylation.

Living organisms produce ATP from energy sources via oxidative phosphorylation. The terminal phosphate bonds of ATP are relatively weak

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compared with the stronger bonds formed when ATP is hydrolyzed (broken down by water) to adenosine diphosphate and inorganic phosphate. Here it is the thermodynamically favorable free energy of hydrolysis that results in energy release; the phosphoanhydride bond between the terminal phosphate group and the rest of the ATP molecule does not itself contain this energy. An organism's stockpile of ATP is used as a battery to store energy in cells. Utilization of chemical energy from such molecular bond rearrangement powers biological processes in every biological organism.

Living organisms obtain energy from organic and inorganic materials, i.e., ATP can be synthesized from a variety of biochemical precursors. For example, lithotrophs can oxidize minerals such as nitrates or forms of sulfur, such as elemental sulfur, sulfhites, and hydrogen sulfhide to produce ATP. In photosynthesis, autotrophs produce ATP using light energy, whereas heterotrophs must consume organic compounds, mostly including carbohydrates, fats, and proteins. The amount of energy actually obtained by the organism is lower than the amount present in the food; there are losses in digestion, metabolism, and thermogenesis.

Environmental materials that an organism intakes are generally combined with oxygen to release energy, although some can also be oxidized anaerobically by various organisms. The bonds holding the molecules of nutrients together and in particular the bonds holding molecules of free oxygen together are relatively weak compared with the chemical bonds holding carbon dioxide and water together. The utilization of these materials is a form of slow combustion because the nutrients are reacted with oxygen (the materials are oxidized slowly enough that the organisms do not actually produce fire). The oxidation releases energy because stronger bonds (bonds within water and carbon dioxide) have been formed. This net energy may evolve as heat, which may be used by the organism for other purposes, such as breaking other bonds to do chemistry required for survival.

(i) Free energy (ΔG) is the useful energy also known as the chemical potential.

(ii) The first law of thermodynamics states that 'the total energy of a system plus its surroundings remains constant'. This is also the laws of conservation of energy. Energy may be transferred from one part to another or may be transformed into an-other form of energy.

(iii) The second law of thermodynamics states that 'the total entropy of a system must increase if a process is to occur spontaneously'.

Entropy

Entropy represents the extent of disorder of the system and becomes maximum when it approaches true equilibrium. Under constant temperature and pressure, the relationship between the free energy change (ΔG) and the change in entropy (ΔS) is given by the following equation which combines the two laws of thermodynamics.

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

where ΔH = the change in enthalpy (heat) and T = the absolute temperature. Under biochemical reactions AH is approximately equal to AE.

Enthalpy

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In a process carried out at constant volume (for example, in a sealed tube), the heat content of a system is equal to internal Energy (E), as no PV (Pressure Volume) work is done. But, in a constant pressure process, the system also expends energy in doing PV work.

Therefore, the total heat content of a system at constant pressure is equivalent to the internal Energy (E) plus the PV (Pressure Volume Product) energy. This is called as enthalpy and is represented by the symbol H. Thus, enthalpy may be defined by the equation,

$H = E + PV$

In simpler words, enthalpy is the total heat content of a system. It reflects the number and kinds of chemical bonds in the reactants and products. Like internal energy, enthalpy is also a function of state and therefore, it is not possible to quantify the absolute enthalpy. However, a change in Enthalpy (ΔH) accompanying a process can be measured accurately. Thus,

$\Delta H = HP - Hr$

(where p = products; r = reactants)

The unit of ΔH is joules/mole (or calories/mole)

The reactions which are accompanied by release of heat energy are called as exothermic reactions. In such cases, there is negative change in enthalpy (-ΔH) from reactants to products. For example, in combustion of glucose to $CO_2^+ H_2O$, large amount of heat is released. Therefore, this is an exothermic reaction with -ΔH. melting of ice into liquid water and its subsequent vaporization into water vapours absorb considerable heat from the surroundings, therefore this is an endothermic reaction with + ΔH.

2.2.1 Exergonic and Endergonic Process

Exergonic Process

An exergonic process is a process in which there is a positive flow of energy from the system to the surroundings. This is in contrast with an endergonic process. Constant pressure, constant temperature reactions are exergonic if and only if the Gibbs free energy change is negative ($\Delta G < 0$).These reactions occur *spontaneously*.

An exergonic reaction is a reaction that releases free energy. Because this type of reaction releases energy rather than consuming it, it can occur spontaneously, without being forced by outside factors. In chemistry terms, exergonic reactions are reactions where the change in free energy is negative. Free energy measures the total amount of energy available in a system; negative changes mean that energy has been released, while positive changes mean that energy has been stored. Reactions where chemical bonds are broken, releasing the energy in those bonds, are often exergonic reactions.

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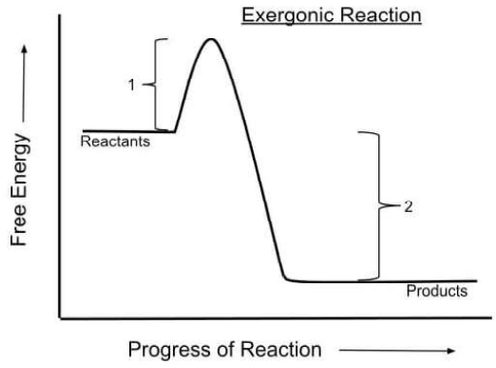

For living things, the chemical bonds in molecules such as sugars, proteins, and fats can be used as energy storage. This can be seen in metabolism, where sugars, proteins, and fats are created by consuming energy from photosynthesis or cellular respiration. It can be seen again when the organism needs energy and these molecules are broken down. Fat contains more energy than sugar by weight because fat molecules contain more chemical bonds than sugar molecules. The more bonds the molecule contains, the more energy can be released by breaking those bonds. Although the breakdown of sugars, fats, and other exergonic reactions are spontaneous – living things use enzymes to speed these reactions up tremendously. The enzymes work by bringing the substrate molecule (such as, a fat or sugar to be metabolized) into an ideal arrangement for the reaction to begin. This lowers the activation energy of the exergonic reaction, making it much more likely to occur.

Exergonic reactions are used by living things to move energy out of 'Storage' in one molecule, such as a sugar or fat, and into an active form, such as ATP. This is done by breaking the chemical bonds in the sugar or fat, and passing its energy in the form of electrons or another currency to a new molecule. The highly efficient process of cellular respiration uses electron transport chains and other highly specialized chemical equipment to create a shocking 38 molecules of ATP from a single glucose molecule.

Examples of Exergonic Process

Glycolysis

Glycolysis is the first process used by *prokaryotes and eukaryotes* alike to turn energy stored in sugar into ATP. For eukaryotes, glycolysis is only the first step in a process that leads to *cellular respiration*; for prokaryotes, glycolysis may be the only means they have of obtaining ATP from glucose. By breaking the chemical bonds that held the two molecules together as one, the enzymes of glycolysis harvest enough energy to produce two molecules of ATP.

Sugars are a good form of energy storage for cells because they are fairly stable; unlike ATP, they do not spontaneously decay and release their energy every time they run into an enzyme that needs energy. Controlling the rate of glucose to ATP conversion allows the cell to control the rate at which it spends the energy it has stored. This can be a lifesaving adaptation in times when food is scarce.

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Cellular Respiration

In eukaryotic cells that practice cellular respiration, the pyruvate molecules left over from glycolysis undergo even more bond-breaking to release even more energy. The bond-breaking equipment of cellular respiration is so advanced that at the end of this process, all that's left is carbon dioxide. Glucose has been broken down into single-carbon units. The energy released by this reaction is harvested to produce a net gain of 30 more molecules of ATP, in addition to the two gained from glycolysis.

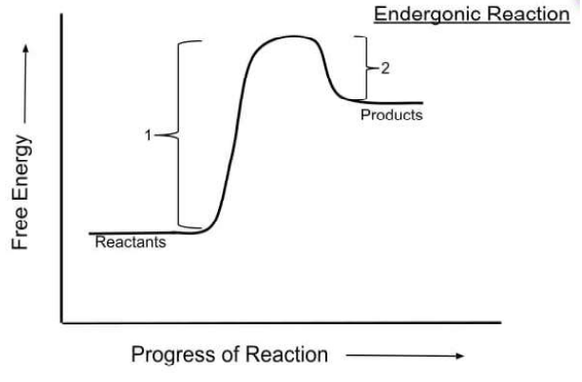
Fatty Acid Catabolism

Fatty acid catabolism refers to the breakdown of fatty acids. For organisms that can afford long-term energy storage, fatty acids are a great way to do it. Fat molecules can contain much more energy than sugar molecules, because they contain many more chemical bonds. While glucose molecules contain 6 carbon atoms, 6 oxygen atoms, and 12 hydrogen atoms bound together – fatty acids contain anywhere from 2 to 26 carbon molecules, and up to 2 hydrogen atoms per carbon. In fatty acid catabolism, these long energy-storing chains are broken down into smaller chunks that can be broken down into carbon dioxide, just like with glucose in cellular respiration.

Endergonic Process

An endergonic reaction is one that requires free energy to proceed. An example of an endergonic reaction of biological interest is photosynthesis. Photosynthetic organisms conduct this reaction by using solar photons to drive the reduction of carbon dioxide to glucose and the oxidation of water to oxygen. The free energy change during a chemical reaction or a physical transformation which takes place at constant pressure is described by the familiar ΔG symbol where G is the Gibbs free energy. By definition, ΔG is positive for an endergonic process (and negative for an exergonic process). When the reaction takes place at constant volume, the free energy change is described by the symbol ΔF, where F is the Helmholtz free energy.

An endergonic reaction is a reaction in which energy is absorbed. In chemistry terms, this means that the net change in free energy is positive – there is more energy in the system at the end of the reaction than at the beginning of it. Because endergonic reactions involve a gain in energy, that energy has to be supplied from an outside source in order for the reaction to occur.



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In **biology**, organisms use endergonic reactions to store energy from outside sources. Photosynthesis, which uses the energy of sunlight to create sugars, is an endergonic reaction. So is fatty acid anabolism, in which the energy from food is stored in fat molecules. In general, reactions that involve creating new chemical bonds are endergonic. The chemical bonds 'Store' the reaction energy until they are broken, at which point some of the energy that was put into the initial reaction is released. This is the principle on which the metabolism of glucose, fatty acids, and other biological fuels is based. Energy from sunlight or another source that was used to create the chemical bonds in sugars, proteins, or fats is liberated when those bonds are broken through processes like glycolysis and cellular respiration.

In general, metabolic reactions that involve creating chemical bonds are called 'Anabolic Reactions'. Metabolic reactions that involve breaking bonds to release energy are called 'Catabolic Reaction'. It is this movement of energy through chemical bonds which allows life to exist. The endergonic reactions of *photosynthesis and chemosynthesis* allow creatures at the bottom of the energy pyramid to survive – and to feed organisms like ourselves, who get their energy by breaking down sugars and fats to liberate that stored energy.

Function of Endergonic Reactions

Endergonic reactions have two important purposes in biology. One is to release energy stored in food molecules, allowing organisms to survive without harvesting all their energy directly from sunlight. The other purpose is to create the building blocks of life: DNA, RNA, proteins, and all the other building blocks of cells must be created through reactions that form new bonds between chemical building blocks. These bond-building reactions are generally endergonic. Organisms need energy to grow because it actually takes energy to produce new materials. For plants, this may mean the sugars, lipids, and nucleic acids that their leaves are made of; for humans, it means the lipids of our cell walls, the protein in our muscles, and of course the DNA in our cells. In most cases, the energy required to build new cells comes from ATP. ATP is a storage molecule for the energy of glucose; which ultimately comes, of course, from the sun through photosynthesizing plants.

Examples of Endergonic Reactions

DNA/RNA Synthesis

DNA and RNA synthesis are fascinating because they do not use ATP the same way more endergonic reactions do. You may recall that DNA has four bases – A, T, C, and G. Rather than being expended and then regenerated during DNA synthesis, ATP is one of the building materials. The process starts out with trisophosphates of each of the base pairs: ATP, TTP, CTP, and GTP. When DNA polymerase moves one of these nucleotide triphosphates into position to attach to the growing DNA strand, one of the nucleotide's phosphate groups breaks off – and is replaced by the formation of a new bond between the nucleotide and the DNA strand.

Protein Synthesis

Protein synthesis is a more typical example of how living things move energy, and add it to reactions to allow new chemical bonds to form. During protein synthesis, a variety of enzymes and ribozymes work together to complete the steps necessary to add an amino acid to a growing protein. In all, about five ATP must be consumed to add a single amino acid to a growing protein.

This process is immensely costly for bacteria; for E. coli cells, about 95% of all the ATP they make is used for protein synthesis. This investment pays off substantially in the long run, since proteins, such as enzymes can drastically lower the activation energy required for thousands of subsequent chemical reactions. The proteins that are made with the energy from ATP allow our metabolisms, muscles, and even our brains and sensory organs to function.

Fatty Acid Synthesis

Fatty acid synthesis uses both ATP and another energy-carrying molecule – NADPH – to supply energy to create fatty acids. Making a fatty acid takes a great deal of energy; it can take 7 ATPs and 14 NADPH to add two carbon molecules to a fatty acid chain, and some fatty acids can have up to 26 carbons. But fatty acids, just like proteins, are necessary for an organism to function and grow; they make up most of the cell and intracellular membranes, as well as serving other purposes.

2.2.2 Hydrolysis of ATP

ATP hydrolysis is the *catabolic reaction process* by which chemical energy that has been stored in the *high-energy phosphoanhydride* bonds in Adenosine TriPhosphate (ATP) is released after splitting these bonds, for example in muscles, by producing work in the form of mechanical energy. The product is Adenosine DiPhosphate (ADP) and an Inorganic Phosphate (Pi). ADP can be further hydrolyzed to give energy, Adenosine MonoPhosphate (AMP), and another Inorganic Phosphate (Pi). ATP hydrolysis is the final link between the energy derived from food or sunlight and useful work, such as muscle contraction, the establishment of electrochemical gradients across membranes, and biosynthetic processes necessary to maintain life. However, when the P-O bonds are broken, input of energy is required. It is the formation of new bonds and lower-energy inorganic phosphate with a release of a larger amount of energy that lowers the total energy of the system and makes it more stable.

Hydrolysis of the phosphate groups in ATP is especially exergonic, because the resulting inorganic phosphate molecular ion is greatly stabilized by multiple resonance structures, making the products (ADP and Pi) lower in energy than the reactant (ATP). The high negative charge density associated with the three adjacent phosphate units of ATP also destabilizes the molecule, making it higher in energy. Hydrolysis relieves some of these electrostatic repulsions, liberating useful energy in the process by causing conformational changes in enzyme structure.

In humans, approximately 60 percent of the energy released from the hydrolysis of ATP produces metabolic heat rather than fuel the actual reactions taking place. Due to the acid-base properties of ATP, ADP, and inorganic

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phosphate, the hydrolysis of ATP has the effect of lowering the pH of the reaction medium. Under certain conditions, high levels of ATP hydrolysis can contribute to lactic acidosis.

Hydrolysis of the terminal *phosphoanhydridic bond* is a highly exergonic process. The amount of released energy depends on the conditions in a particular cell. Specifically, the energy released is dependent on concentrations of ATP, ADP and Pi. As the concentrations of these molecules deviate from values at equilibrium, the value of *Gibbs free energy change* (ΔG) will be increasingly different. In standard conditions (ATP, ADP and Pi concentrations are equal to 1M, water concentration is equal to 55 M) the value of ΔG is between -28 to -34 kJ/mol.

The range of the ΔG value exists because this reaction is dependent on the concentration of Mg^{2+} cations, which stabilize the ATP molecule. The cellular environment also contributes to differences in the ΔG value since ATP hydrolysis is dependent not only on the studied cell, but also on the surrounding tissue and even the compartment within the cell. Variability in the ΔG values is therefore to be expected.

The relationship between the standard *Gibbs free energy change* $\Delta_r G^o$ and chemical equilibrium is revealing. This relationship is defined by the equation $\Delta_r G^o = -RT \ln(K)$, where K is the equilibrium constant, which is equal to the reaction quotient Q in equilibrium. The standard value of ΔG for this reaction is, as mentioned, between -28 and -34 kJ/mol; however, experimentally determined concentrations of the involved molecules reveal that the reaction is not at equilibrium.

In one particular study, to determine ΔG in vivo in humans, the concentration of ATP, ADP, and Pi was measured using nuclear magnetic resonance. In human muscle cells at rest, the concentration of ATP was found to be around 4 mM and the concentration of ADP was around 9 μM. Inputing these values into the above equations yields ΔG = -64 kJ/mol. After ischemia, when the muscle is recovering from exercise, the concentration of ATP is as low as 1 mM and the concentration of ADP is around 7 μM. Therefore, the absolute ΔG would be as high as -69 kJ/mol.

Synthesis of ATP From ADP

ATP synthase is a protein that catalyzes the formation of the energy storage molecule ATP using ADP and Inorganic Phosphate (Pi). It is classified under ligases as it changes ADP by the formation of P-O bond (phosphodiester bond). The overall reaction catalyzed by ATP synthase is:

$$\mathbf{ADP + P_i + 2H^+_{out} \rightleftharpoons ATP + H_2O + 2H^+_{in}}$$

The formation of ATP from ADP and Pi is energetically unfavourable and would normally proceed in the reverse direction. In order to drive this reaction forward, ATP synthase couples ATP synthesis during cellular respiration to an electrochemical gradient created by the difference in proton (H^+) concentration across the inner mitochondrial membrane in eukaryotes or the plasma membrane in bacteria. During photosynthesis in plants, ATP is synthesized by ATP synthase using a proton gradient created in the thylakoid lumen through the thylakoid membrane and into the chloroplast stroma.

Eukaryotic ATP synthases are F-ATPases, running 'In Reverse' for an ATPase. This article deals mainly with this type. An F-ATPase consists of two main subunits, F_O and F_1, which has a rotational motor mechanism allowing for ATP production. ATP synthase is a molecular machine.

2.3 DIFFRACTION METHOD

Diffraction methods are used for the identification and also for the *quantification* of constituents of *crystalline structures*. Diffraction patterns are obtained with an object with crystalline structure is irradiated by X-ray photons, or by other particles (electrons, neutrons, etc.).

Diffraction-based techniques, such as single-crystal X-ray crystallography, *electron microscopy* and *neutron diffraction* are well established and have covered the road to the stunning successes of modern-day structural biology. The major advances achieved in the last 20 years in all aspects of structural research, including sample preparation, crystallization, the construction of synchrotron and spallation sources, phasing approaches and high-speed computing and visualisation, now provide specialists and non-specialists alike with a steady flow of molecular images of unprecedented detail.

X-Ray Diffraction (XRD) is a tool for characterizing the arrangement of atoms in crystals and the distances between crystal faces. The method determined the size of atoms, the lengths and types of chemical bonds, and the atomic-scale differences among various materials, especially minerals and alloys. X-ray powder diffraction is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analysed material is finely ground, homogenized, and average bulk composition is determined. Protein X-ray crystallography is a technique used to obtain the three-dimensional structure of a particular protein by X-ray diffraction of its crystallized form. This three dimensional structure is crucial to determining a protein's functionality. Whereas powder diffraction is a scientific technique using X-ray, neutron, or electron diffraction on powder or microcrystalline samples for structural characterization of materials. An instrument dedicated to performing such powder measurements is called a ***powder diffractometer***.

The basic principle of diffraction methods is based on the *Bragg equation*, which defines the relation between the wavelength of the radiation, the angle of the incident radiation and the crystal lattice geometry of the object irradiated.

2.3.1 Light Scattering

Non-invasive light scattering methods provide data on biological macromolecules (i.e., proteins, nucleic acids, as well as assemblies and larger entities composed of them) that are complementary with those of size exclusion chromatography, gel electrophoresis, analytical ultracentrifugation and mass spectrometry methods. Static light scattering measurements are useful to determine the mass of macromolecules and to monitor aggregation phenomena. Dynamic light scattering measurements are suitable for the quality control and to assess sample homogeneity, to determine particle size, examine the effect of physical and

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chemical treatments, probe the binding of ligands, and study interactions between macromolecules.

Scattering is a term used in physics to describe a wide range of physical processes where moving particles or radiation of some form, such as light or sound, are forced to deviate from a *straight trajectory* by localized non-uniformities (including particles and radiation) in the medium through which they pass. In predictable use, this also includes deviation of reflected radiation from the angle predicted by the law of reflection. Reflections of radiation that undergo scattering are often called *diffuse reflections* and *unscattered reflections* are called specular (mirror-like) reflections. Originally, the term was confined to *light scattering* As more 'Ray' like phenomena were discovered, the idea of scattering was extended to them, so that William Herschel could refer to the scattering of 'Heat Rays'(not then recognized as electromagnetic in nature). John Tyndall, a pioneer in light scattering research, noted the connection between light scattering and acoustic scattering. Near the end of the 19th century, the scattering of cathode rays (electron beams) and X-rays was observed and discussed. With the discovery of subatomic particles and the development of quantum theory in the 20th century, the sense of the term became broader as it was recognized that the same mathematical frameworks used in light scattering could be applied to many other phenomena.

Quantitative modelling of tissue scattering and absorption properties is fundamental to determine light transport and energy deposition in tissue, key to both diagnostic and therapeutic applications of light. The rich spectroscopic behaviour of biological tissue arising from its complex structure, as one main strength of optical interaction with biological tissue and cells compared to other biomedical imaging modalities, has also provided a powerful non-invasive probe to structural alterations in tissue due to disease or physiology. It is hence critical to establish connection between the characteristics of light scattering to the complex structure of tissue and cells.

A fractal continuous random medium model has been proposed for light scattering by biological medium based on the observation that many biological tissues have fractal-like organization and on a macroscopic scale the constituents of tissue have no clear boundaries and merge into a *quasi-continuum structure*. The model has been shown to describe well light scattering by both tissue and cell suspensions. The fractal dimension and the correlation length reflecting both the morphological structure of tissue and the nuclear structure can be detected by *spectroscopic light scattering measurement*. The unified model is currently the only analytical model for light scattering by cells that describes correctly both the Mie resonance structure near forward scattering direction and the fractal scattering at larger angles. Preliminary experimental results show the fractal model can be successfully applied to discriminate accurately normal and malignant prostate tissues

2.3.2 Low Angle X-Ray Scattering

Small-Angle X-Ray Scattering (SAXS) is a small-angle scattering technique by which nanoscale density differences in a sample can be quantified. This means that it can determine nanoparticle size distributions, resolve the size and shape of

(monodisperse) macromolecules, determine pore sizes, characteristic distances of partially ordered materials, and much more. This is achieved by analysing the elastic scattering behaviour of X-rays when travelling through the material, recording their scattering at small angles (typically 0.1 – 10°, hence the 'Small-Angle'in its name). It belongs to the family of Small-Angle Scattering (SAS) techniques along with small-angle neutron scattering, and is typically done using hard X-rays with a wavelength of 0.07 – 0.2 nm. Depending on the angular range in which a clear scattering signal can be recorded, SAXS is capable of delivering structural information of dimensions between 1 and 100 nm, and of repeat distances in partially ordered systems of up to 150 nm. USAXS (Ultra-Small Angle X-Ray Scattering) can resolve even larger dimensions, as the smaller the recorded angle, the larger the object dimensions that are probed.

SAXS and USAXS belong to a family of X-ray scattering techniques that are used in the characterization of materials. In the case of biological macromolecules, such as proteins, the advantage of SAXS over crystallography is that a crystalline sample is not needed. Moreover, the properties of SAXS allow investigation of conformational diversity in these molecules. Nuclear magnetic resonance spectroscopy methods encounter problems with macromolecules of higher molecular mass (> 30–40 kDa). However, owing to the random orientation of dissolved or partially ordered molecules, the spatial averaging leads to a loss of information in SAXS compared to crystallography.

Applications of Small-Angle X-Ray Scattering

SAXS is used for the determination of the microscale or nanoscale structure of particle systems in terms of such parameters as averaged particle sizes, shapes, distribution, and surface-to-volume ratio. The materials can be solid or liquid and they can contain solid, liquid or gaseous domains (so-called particles) of the same or another material in any combination. Not only particles, but also the structure of ordered systems like lamellae, and fractal-like materials can be studied. The method is accurate, non-destructive and usually requires only a minimum of sample preparation. Applications are very broad and include colloids of all types including interpolyelectrolyte complexes, micelles, microgels, liposomes, polymersomes, metals, cement, oil, polymers, plastics, proteins, foods and pharmaceuticals and can be found in research as well as in quality control.

X-Ray Diffraction

X-ray diffraction, a phenomenon in which the atoms of a crystal, by virtue of their uniform spacing, cause an interference pattern of the waves present in an incident beam of X-rays. The atomic planes of the crystal act on the X-rays in exactly the same manner as does a uniformly ruled grating on a beam of light. X-Ray Diffraction Analysis (XRD) is a technique used in materials science to determine the crystallographic structure of a material. XRD works by irradiating a material with incident X-rays and then measuring the intensities and scattering angles of the X-rays that leave the material. Protein X-ray crystallography is a technique used to obtain the three-dimensional structure of a particular protein by x-ray diffraction of its crystallized form. This three dimensional structure is crucial to determining a protein's functionality. Fiber diffraction is a method used to determine the structural information of a molecule by using scattering data from X-rays. Rosalind

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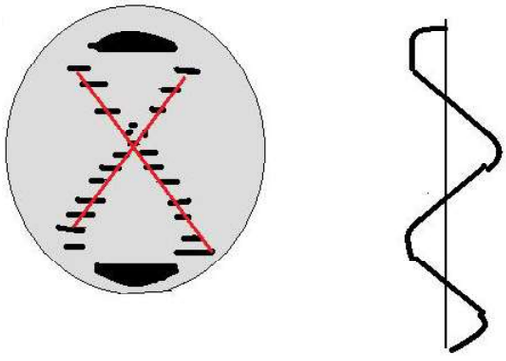
Franklin used this technique in discovering structural information of DNA. The experiment places a fiber in the trajectory of an X-ray beam at right angle. Obtaining a clear diffraction pattern of an object required that the crystal be pure and the X-ray strong enough. However, as Franklin realized, DNA existed in two forms in equilibrium which resulted in a very *unclear diffraction pattern*. These forms were the **A** form, which is the dehydrated form of DNA, and the **B** form, which generated a long and fibrous structure due to humid conditions. Franklin, applying her chemistry background, then proceeded to isolate these two forms using clever laboratory techniques such as "manipulation of the critical hydration of her specimens". The **A** and **B** forms were then separated and subjected to X-ray crystallography obtaining the pictures which would be the basis of Watson and Crick's helical DNA structure.

The diffraction pattern obtained by Franklin and Wilkins showed a X pattern which hinted of a 2 stranded helical form



2.3.3 Photo Correlation Spectroscopy

Dynamic Light Scattering DLS, also called Photon Correlation Spectroscopy (PCS), is a light-scattering technique used to study the average particle size of NPs based on the laser diffraction method. This technique uses the scattering of light from colloidal particles suspended in a liquid medium.

Dynamic Light Scattering (DLS) is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution. In the scope of DLS, temporal fluctuations are usually analysed by means of the intensity or photon auto-correlation function (also known *as photon correlation spectroscopy* or *quasi-elastic light scattering*). In the time domain analysis, the Auto Correlation Function (ACF) usually decays starting from zero delay time, and faster dynamics due to smaller particles lead to faster decorrelation of scattered intensity trace. It has been shown that the intensity ACF is the Fourier transformation of the power spectrum, and therefore the DLS measurements can be equally well performed in the spectral domain. DLS can also be used to probe the behaviour of complex fluids, such as concentrated polymer solutions.

Applications of Photon Correlation Spectroscopy (PCS)

DLS is used to characterize size of various particles including proteins, polymers, micelles, vesicles, carbohydrates, nanoparticles, biological cells and gels. If the

system is not disperse in size, the mean effective diameter of the particles can be determined. This measurement depends on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium. Since DLS essentially measures fluctuations in scattered light intensity due to diffusing particles, the diffusion coefficient of the particles can be determined. DLS software of commercial instruments typically displays the particle population at different diameters. If the system is monodisperse, there should only be one population, whereas a polydisperse system would show multiple particle populations. If there is more than one size population present in a sample then either the CONTIN analysis should be applied for photon correlation spectroscopy instruments, or the power spectrum method should be applied for Doppler shift instruments.

Stability studies can be done conveniently using DLS. Periodical DLS measurements of a sample can show whether the particles aggregate over time by seeing whether the hydrodynamic radius of the particle increases. If particles aggregate, there will be a larger population of particles with a larger radius. In some DLS machines, stability depending on temperature can be analysed by controlling the temperature in situ.

2.3.4 Optical Rotatory Dispersion (ORD) and Circular Dichroism (CD)

The three-dimensional structure of macromolecules in solution can be studied by noting their properties of absorption of polarised light. The plane polarised light is a type of light consisting of waves oscillating in a single plane. This is obtained by passing a beam of light through a Nicol prism or a polarizing screen.

When a plane polarised light is passed through a substance, the polarised light will rotate to a certain angle depending on its structure. It has also been found that this depends on the wavelength of light. Hence, the rate of change of rotation is measured with wavelength of light which is known as Optical Rotatory Dispersion (ORD).

Certain optically active substances have been found to absorb polarised light differently, i.e., differential absorption of Right (R) and Left (L) circularly polarised light. Thus another spectroscopy Circular Dichroism Spectroscopy (CDS) has been developed to investigate the interaction of polarised light and the samples.

Both CD and ORD are almost same but, due to the relative simplicity of CD spectra, CD analysis has gained its superiority. The resolution of CD bands is also superior.

Circularly polarised lights is obtained by superimposing two plane polarised light of the same wavelength which come through the monochromator and Nicol prism. This superimposed light can be resolved into Right (R) and Left (L) waves. Certain substances absorb differentially R and L waves and show refraction with elliptically polarised beam.

ORD and CD are useful in the study of the secondary structure of macromolecules, particularly protein and amino acids in solution. CD spectra is also useful for the study of binding of substrate and inhibitor to the enzyme.

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The helical structure of DNA and protein can be studied with the CD spectra. CD spectrum, is very sensitive to any structural changes of the macromolecule. So, any interaction of protein with nucleic acids can be studied by observing changes in the CD spectra. The transitions between double-stranded and single- stranded nucleic acids can be studied with CD spectrophotometer.

Infra-Red (IR) Spectrophotometry: Infra-red (10^3-10^4 nm) shows vibrational spectra, so molecules under infra-red show different vibrational levels. These vibrational levels change with the bonding characteristics of the compound. For example, vibrations of C – H,-CH_2 and CH_3 will differ.

Similarly, various functional groups like methyl, carbonyl, amide groups, etc. will show different IR spectra. Hence the IR spectra is useful in biochemistry, particularly for the study of macromolecules and membranes, for the identification of drugs, for the study of secondary structure of proteins, such as the number of helical structures present in protein etc.

Check Your Progress

1. What do you understand by bioenergetics?
2. What is enthalpy?
3. Define the term exergonic process.
4. What is an endergonic reaction?
5. Differentiate between anabolic and catabolic reactions.
6. What are diffraction methods?
7. What is the role of XRD in biochemistry?
8. What is Photo Correlation Spectroscopy?

2.4 ATP CYCLE

The compounds we use as chemical fuels to run automobiles are highly reduced organic compounds, such as natural gas (CH_4) and petroleum derivatives. Energy is released when these molecules are burned in the presence of oxygen, converting the carbon to more oxidized state, as in carbon dioxide and carbon monoxide gases. The degree of reduction of a compound is also a measure of its ability to perform chemical work within the cell. The more hydrogen atoms that can be stripped from a "fuel" molecule, the more ATP that ultimately can be produced.

Carbohydrates are rich in chemical energy because they contain strings of $(H-\overset{|}{C}-OH)$ units. Fats contain even greater energy per unit weight because they contain strings of more reduced $(H-\overset{|}{C}-H)$ units.

As the sole building block of both starch and glycogen, glucose is a key molecule in the energy metabolism of plants and animals. The free energy released by the complete oxidation of glucose is very large:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$
$$\Delta G^{0'} = -686 \text{ kcal/mol}$$

By comparison, the free energy required to convert ADP to ATP is relatively small:

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$$ADP + Pi \rightarrow ATP + H_2O$$
$$\Delta G^{0'} = 7.3 \text{ kcal/mol}$$

It is evident from these numbers that the complete oxidation of a molecule of glucose to CO_2 and H_2O can release enough energy to produce a large number of ATPs. As we will see in this chapter later on, upto 36 molecules of ATP are formed per molecule of glucose oxidized under conditions that exist in most cells. For this many ATP to be produced, the sugar molecule is disassembled in many small steps. Those steps in which the free-energy difference between the reactants and products is relatively large can be coupled to reactions that lead to the formation of ATP.

There are basically two stages in the catabolism of glucose, and they are virtually identical in all aerobic organisms. The first stage, **glycolysis**, occurs in the soluble phase of the cytoplasm (the cytosol) and leads to the formation of pyruvate. The second stage is the **tricarboxylic acid** (**TCA**) **cycle** or **Krebs cycle**, which occurs within the mitochondria of eukaryotic cells and the cytosol of prokaryotic cells and leads to the final oxidation of the carbon atoms to carbon dioxide. Most of the chemical energy of glucose is stored in the form of high-energy electrons, which are removed as substrate molecules are oxidized during both glycolysis and the TCA cycle. It is the energy of these electrons that is ultimately used to synthesize ATP.

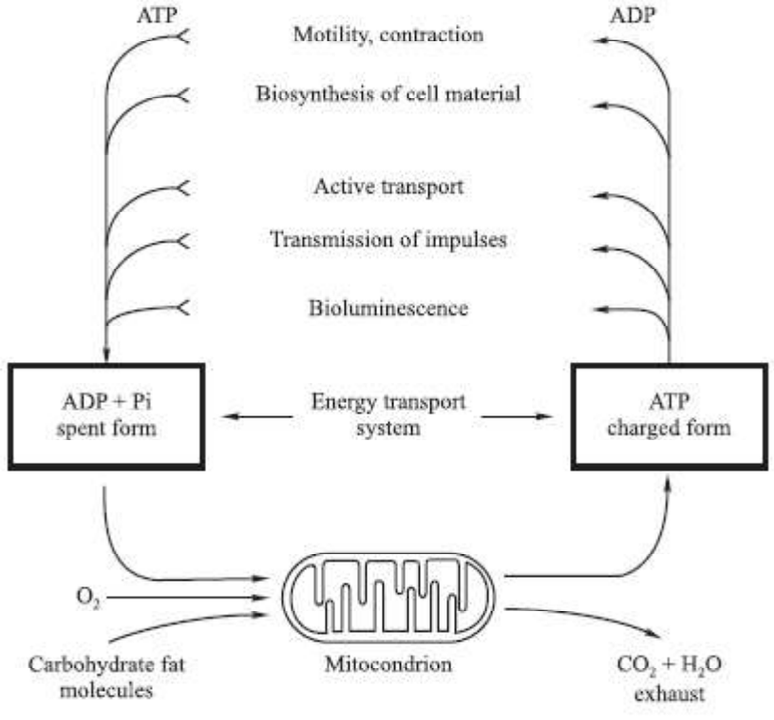

Fig. 2.1 *Diagrammatic Representation of the Energy Liberation in Mitochondrion and its Utilization in Various Cellular Functions*

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Adenosine Triphosphate or ATP

The ATP consists of a purine base **adenine**, a pentose sugar **ribose** and three molecules of the **phosphoric acids**. The adenine and ribose sugar collectively constitute the nucleoside **adenosine** which by having one, two or three phosphate groups forms the adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) respectively. In ATP the last phosphate group is linked with ADP by a special bond known as "**energy rich bond**" because when the last phosphate group of the ATP is broken the large amount of energy is released as shown by the following reaction:

$$\underset{\text{ATP}}{\text{A}-\text{P}\sim\text{P}\sim\text{P}} = \underset{\text{ADP}}{\text{A}-\text{P}\sim\text{P}} + \underset{\text{Phosphate group}}{\text{Pi}} + \underset{\text{Energy}}{7300 \text{ calories}}$$

In the above reaction, we have seen that by the breaking of the energy rich bond about 7300 calories of energy are released, while the common chemical bond releases only 300 calories of energy. The chemical reactions which synthesize the energy rich bond or ~P bond require great amount

of energy which is supplied by the oxidation of the food-stuffs in the mitochondria. The utility

of energy rich phosphate bond (~P) of the ATP is that great amount of energy which is kept stored in

the ready state in a very limited space of the cell. The stored chemical energy is disposed of

very quickly at the time of the need in various cellular functions such as respiratory cycle, protein and nucleic acid synthesis, nervous transmission, cell division, transportation and bioluminescence, etc. (Fig. 2.2).

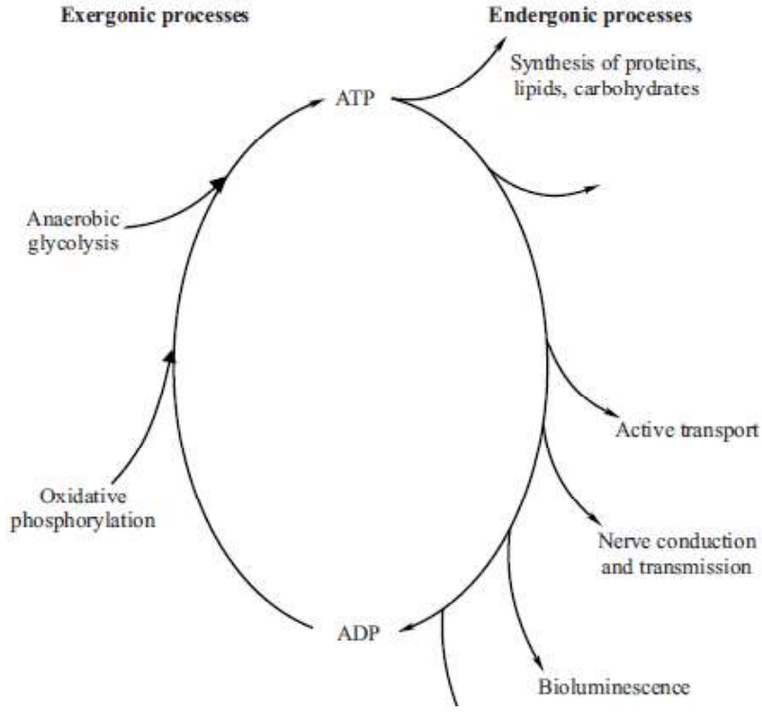

Fig. 2.2 *Diagrammatic Representation of uses and Synthesis of ATP.*

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Because the terminal phosphate linkage in ATP is easily cleaved with release of free energy, ATP acts as an efficient phosphate donor in a large number of different phosphorylation reactions. In this way, ATP acts as a carrier molecule like the acetyl CoA and as coenzyme like the CoA or NAD.

Later on, besides ATP, certain other energy rich chemical compounds have been found to be active in the cellular metabolism. These are **cytosine triphosphate** (**CTP**), **uridine triphosphate** (**UTP**) and **guanosine triphosphate** (**GTP**). These compounds, however, derive the energy from the ATP by nucleoside diphosphokinases. The energy for the production of ATP or other energy rich molecules is produced during the breakdown of food molecules including carbohydrates, fats and proteins (**catabolic** and **exergonic activities**).

Energy transducers. The cell organelles, such as chloroplasts and mitochondria, which convert one form of energy to another are known as **energy transducers**. The plants and animals have not only evolved highly effective energy transducers but have also developed very efficient control systems to regulate the energy transformations and to enable the cells to adjust to variations in environmental conditions.

2.4.1 DNA Polymerization

DNA polymerase is responsible for polymerization of deoxyribonucleotides the building blocks of DNA, during the replication of DNA. During this process, DNA polymerase 'Reads' the existing DNA strands to create two new strands that match the existing ones. DNA polymerase adds nucleotides to the 3' end of a DNA strand, one nucleotide at a time.

Every time when a cell divides, DNA polymerase is required to duplicate the DNA, so that a copy of the original DNA molecule can be passed to each daughter cell. In this way, genetic information is passed down from generation to generation. As stated in earlier unit (replication of DNA), during initiation of DNA replication, an enzyme called Helicase Unwinds the highly coiled double helix DNA molecule by breaking down the Hydrogen bonds between the nucleotide base pairs. Unwinding leads to the opening of the double-stranded helical DNA to two single strands of DNA that can be used as templates for replication.

DNA polymerases have highly conserved structure, as their different catalytic subunits vary among species and are independent of their domain structures. Its shape resembles a right hand with thumb, finger and the palm domains. The major role of finger domain is to bind the nucleoside triphosphates (dATP, dGTP, dCTP, dTTP) with the DNA template bases. The thumb domain helps in translocation and positioning of the DNA whereas the palm domain helps in catalysing the transfer of phosphoryl groups in the phosphoryl transfer reaction. DNA is bound to the palm domain when the enzyme is active. Prokaryotic and Eukaryotic DNA polymerase differs in structure and function of various domains during replication process and is described as follows:

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Prokaryotic DNA Polymerase

Prokaryotic DNA polymerase exists in two forms as core polymerase and Poloenzyme. Core polymerase synthesizes DNA from the DNA template but it cannot initiate the synthesis alone but holoenzyme can accurately initiate DNA synthesis. Following are the types of Prokaryotic DNA polymerases:

DNA Polymerase I (Pol I)*:* DNA polymerase I enzyme (Pol I) is a part of prokaryotic family 'A' polymerases, encoded by the polA gene. This enzyme is involved in excision repair with both 3'–5' and 5'–3' exonuclease activity and processing of Okazaki fragments generated during lagging strand synthesis. Pol I is the most abundant polymerase, accounting for more than 95% of polymerase activity in *E. coli*. Pol I adds about 15-20 nucleotides per second, which shows its poor processivity.

DNA Polymerase II (Pol II): DNA polymerase II belongs to family 'B' polymerase and is a polB gene product also known as DNAA. Polymerase II has 3'–5' exonuclease activity and participates in DNA repair. Pol II is also thought to be a backup to Pol III as it can interact with holoenzyme Proteins and assume a high level of processivity. The main role of Pol II is to direct polymerase activity at the replication fork and helped stalled Pol III bypass terminal mismatches.

DNA Polymerase III (Pol III): DNA polymerase III holoenzyme is the primary enzyme involved in DNA replication in *E. coli* and belongs to family C polymerases. It consists of three parts:

- Pol III Core
- Beta Sliding Clamp
- Clamp-Loading Complex

The core consists of three subunits: α, the polymerase activity hub, ε, exonucleolytic proof-reader and θ, which may act as a stabilizer for. The holoenzyme contains two cores, one for the lagging and leading strand. The beta sliding clamp is present in duplicate, one for each core, to create a clamp that encloses DNA. The third assembly is a seven-subunit (τ2γδδ2 χψ) clamp loader complex (Refer Figure 2.3).

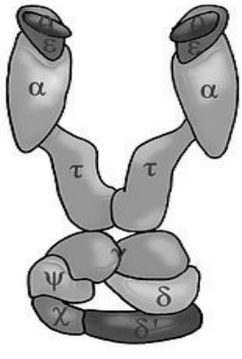

Fig. 2.3 *Structure of DNA Polymerase III in Prokaryotes with Different Subunits*

7DEOH6XEXQLWV RI WKH (FROL '1$ 3RO\PHUDVH ,,, +RORHQ]\PH DQG 7KHL

6XEXQLW	*HQH	3URSHUWLHV DQG)XQFWLRQ
&RUH 6XEXQL'1$ (D	co cSRO\PHUDVH DFWLYLW\ UHTXLUHG IRU '1$ V\QWKHVLV	
H	'1$ 4	coc H[RQXFOHDVH DFWLYLW\ UHTXLUHG IRU SURRIUHDGLQJ
I	8QDVVLJ0D\ KHOS WR DVVHPEOH RWKHU VXEXQLWV	
$FFHVVRU\ 6;'1$;ᴰLW\ W	'1$ GHSHQGHQW $73DVH UHTXLUHG IRU LQLWLDWLRQ 3URPRWHV GLPHUL]DWLRQ	
J	'1$;ᴰ	$VVRFLDWHV ZLWK IRXU SHSWLGHV WR IRUP D '1$ GHSHQGHQW $73DVH UHTXLUHG IRU LQLWLDWLRQ DQG DOVR IDFLOLWDWHV ELQGLQJ RI VXEXQLW

127(6

(XNDU\RWLF '1$ 3RO\PHUDVH

3RO\PHUDVHV 1 DQG %HWD /DPEGD 6LJPD DQG 0X

7KH (XNDU\RWLF SRO\PHUDVH SRO 3RO 1 3RO 3RO D
GHR[\QXFOHRWLG\O WUDQVIHUDVH EHORQJV WR IDPLO\ ; SRO\PHU
KDYH KLJKO\ FRQVHUYHG UHJLRQV WKDW LQFOXGH WZR KHO[K
SOD\ DQ LPSRUWDQW UROH LQ WKH '1$ SRO\PHUDVH LQWHUDFWL
ZLWK GRZQVWUHDP '1$ DQG DQRWKHU PRWLI LQWHUDFWV ZLWK
HQFRGHG E\ 32/% JHQH LV UHTXLUHG IRU VKRUW SDWFK EDVH
DQG 3RO HQFRGHG E\ WKH 32// DQG 32/0 JHQHV UHSHFWLYH
LQ QRQ KRPRORJRXV HQG MRLQLQJ D PHFKDQLVP IRU UHMRLQL
EUHDNV GXH WR K\GURJHQ SHUR[LGH DQG LRQL]LQJ UDGLDWLR
H[SUHVVHG RQO\ LQ O\PSKRLG WLVVXH DQG DGGV Q QXFOHRWLG
IRUPHG GXULQJ 9 ' - UHFRPELQDWLRQ WR SURPRWH LPPXQRORJL

3RO\PHUDVHV . / DQG 0 $OSKD 'HOWD DQG (SVLORQ

)DPLO\ % 3RO\PHUDVHV FRQWDLQ 3RO\PHUDVHV $OSKD 'HOWD
SRO\PHUDVHV SOD\ D PDMRU UROH LQ QXFOHDU '1$ UHSOLFDWLR
DQG '1$ SULPDVH FRPSOH[FRQVLVWV RI IRXU VXEXQLWV WKH &D
WKH 5HJXODWRU\ VXEXQLW 32/$ WKH 6PDOO 3ULPDVH 6XEXQLW
3ULPDVH 6XEXQLWV 35,0 2QFH 51$ SULPDVH KDV V\QWKHVL]HG
GXULQJ '1$ UHSOLFDWLRQ 3RO . LQLWLDWHV DGGLWLRQ RI SRO\Q
LQ ODJJLQJ VWUDQG ZKHUHDV OHDGLQJ VWUDQG LV WDNHQ E\ 3

*HQH 32/' HQFRGHV SRO / DQG FUHDWHV WKH FDWDO\WLF V
32/' DQG 32/' ZKLFK LQWHUDFWV ZLWK 3UROLIHUDWLQJ &H
$QWLJHQ 3&1$ $ '1$ FODPS WKDW DOORZV 3RO / WR SRVVHV
:KHUHDV SRO 0 LV HQFRGHG E\ WKH 32/(WKH FDWDO\WLF

6HOI /HDUQLQJ
0DWHULDO

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all photosynthetic organisms, including green plants, prokaryotic blue-green algae (cyanobacteria), and eukaryotic algae. It absorbs energy from light; this energy is then used to convert carbon dioxide to carbohydrates.

Chlorophyll occurs in several distinct forms: chlorophylls *a* and *b* are the major types found in higher plants and green algae; chlorophylls *c* and *d* are found, often with *a*, in different algae; chlorophyll *e* is a rare type found in some golden algae; and bacterio-chlorophyll occurs in certain bacteria. In green plants chlorophyll occurs in membranous disclike units (thylakoids) in organelles called chloroplasts. The chlorophyll molecule consists of a central magnesium atom surrounded by a nitrogen-containing structure called a porphyrin ring; attached to the ring is a long carbon–hydrogen side chain, known as a phytol chain. Variations are due to minor modifications of certain side groups. Chlorophyll is remarkably similar in structure to haemoglobin, the oxygen-carrying pigment found in the red blood cells of mammals and other vertebrates.

Chlorophyll is a fundamental component of PSI. Chlorophyll is a porphyrin complex of magnesium and is responsible for the green colours of leaves. It plays an important role in receiving light energy and transferring it to redox reaction systems. Chlorophyll is excited from the singlet ground state to the singlet excited state by light, the energy of the excited state is transferred to an acceptor within 10 ps, and the resultant energy reduces an iron-sulphur complex and is finally used for reduction of carbon dioxide in subsequent dark reactions. Since charge separation by photochemical excitation is the most important first stage, studies on photo induced electron transfer are have been actively performed using various kinds of porphyrin compounds as models of chlorophylls. PSI, which obtains oxidizing energy by electron transfer, converts ADP to ATP.

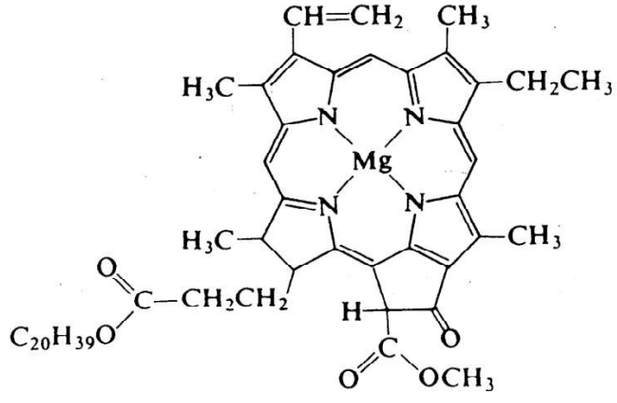

Fig. 2.5 *Structure of Chorophyll*

Metal Complexes in Transmission of Energy

Many biological reactions are known to *involve metal ions*. There are also metals recognized as essential elements, although their roles in living organisms are not clear. *Bioinorganic chemistry*, the study of the functions of metals in biological systems using the knowledge and methods of inorganic chemistry.

The Following List Shows Typical Bioactive Substances Containing Metals:

1. Electron Carriers. Fe: cytochrome, iron-sulphur protein. Cu : blue copper protein.
2. Metal Storage Compound Fe: ferritin, transferrin. Zn : metallothionein.
3. Oxygen Transportation Agent Fe: hemoglobin, myoglobin. Cu: hemocyanin.
4. Photosynthesis. Mg: chlorophyll.
5. Hydrolase. Zn: carboxypeptidase. Mg: aminopeptidase.
6. Oxidoreductase. Fe: oxygenase, hydrogenase. Fe, Mo: nitrogenase.
7. Isomerase. Fe: aconitase. Co: vitamin B12 coenzyme.

The Basis of Chemical Reactions of Metalloenzymes Are:

1. Coordinative activation (coordination form, electronic donating, steric effect),
2. Redox (metal oxidation state),
3. Information communication, and, in many cases, reaction environments are regulated by biopolymers such as proteins, and selective reactions are performed.

Examples of Actions of Metals Other Than By Metalloenzymes Include

1. Mg: MgATP energy transfer
2. Na/K ion pumping,
3. Ca: transfer of hormone functions, muscle contraction, nerve transfer, blood coagulation, are some of the important roles of metals.

The formation of ***glucose and dioxygen*** by the reaction of *carbon dioxide* and *water* is a skillful reaction using photo energy and in which chlorophyll (Refer Figure 2.4), which is a *magnesium porphyrin* and a *manganese cluster complex*, plays the central role. A chloroplast contains Photo System I (PSI) and Photo System II (PSII), which use light energy to reduce carbon dioxide and to oxidize water.

2.4.3 Glucose Storage

Glucose is the main source of fuel for our cells. When the body doesn't need to use the glucose for energy, it stores it in the liver and muscles. This stored form of glucose is made up of many connected glucose molecules and is called glycogen. Glycogen is stored in the liver. When the body needs more energy, certain proteins called enzymes break down glycogen into glucose. They send the glucose out into the body. After your body has used the energy it needs, the leftover glucose is stored in little bundles called *glycogen* in the liver and muscles. Your body can store enough to fuel you for about a day. After you have not eaten for a few hours, your blood glucose level drops. Your pancreas stops churning out insulin. After exercise, the restoration of muscle glycogen occurs in a biphasic manner. During the first phase, glycogen synthesis is rapid (12–30 mmol/g wet weight/h), does not require insulin, and lasts 30–40 minutes if glycogen depletion is substantial.

Certain organic phosphates, formed in the tissues out of anabolic chemical reaction, play a key role in the energy transactions of living organisms.

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The importance of organic phosphates in metabolism was first indicated by the studies of Harden and Young, who found that fermentation of glucose by cell-free yeast juice in-creased rapidly when inorganic phosphorus is added to the medium, which was later converted to organically bound phosphate.

The work of Embden, Meyerhof, Cori and their associates show that organic phosphates concerned with the metabolism of glucose by yeast are also intermediates in the metabolism of **glycogen and glucose** in muscle and other tissues.

The presence of ATP, ADP, CP and other high-energy phosphate compounds in the tissue and their participation in different metabolic processes indicate the importance of organic phosphates in metabolism. The sugar phosphates, although not high-energy compounds, are obligatory intermediates. Their formation involves phosphorylation by the high-energy compound ATP in presence of suitable enzymes.

The term phosphorylation includes all chemical reactions in the body which require combination with phosphoric acid. The reverse changes are called dephosphorylation, where phosphoric acid is dissociated from the compound.

These two processes are probably the reversible reactions of the same enzyme system.

1. Physiological Importance of Phosphorylation

Phosphoric acid enters into the composition of cell protoplasm. Hence, phosphorylation is an essential chemical process for all cells. In addition to this it takes an essential part during absorption and metabolism of different foodstuffs.

2. Oxidative Phosphorylation

This reaction can take place only in presence of intact oxidation process and the energy-yielding (oxidation) mechanism is coupled with energy-harnessing (phosphorylation) mechanism. Uncoupling agents like 2, 4-dinitrophenol inhibit phosphorylation but not oxidation. They elevate the oxygen consumption rate. The second stage in phosphorylation process is transfer of phosphate group from ATP to other intermediates. Oxidative phosphorylation takes place in the cristae of the mitochondria.

Control of Phosphorylation

i. Enzymes

The enzymes that take part in this process are phosphorylase, phosphatase, etc.

ii. Hormones

- Adrenal cortex is believed to be directly responsible for phosphorylation. Glucocorticoids inhibit phosphorylation adrenalectomy accelerate it.
- Anterior pituitary, by its adrenocorticotrophic hormone, may exert a superior control on phosphorylation, through adrenal cortex. The marasmic condition that develops in diseases of adrenal cortex may be partly explained by the consequent disturbance of phosphorylation, which affects absorption, metabolism and nutrition of the body. Growth hormone affects phosphorylation in a similar way as glucocorticoids.

iii. Inorganic Salts

Sodium may have some effects on phosphorylation.

It is believed that adrenal cortex controls phosphorylation by helping the enzymes responsible for the process. But it is also known that many of the defects, seen in diseases of the adrenal cortex, are rectified by giving enough NaCl. For instance, the defective fat absorption in adrenalectomised animals improves by giving NaCl.

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It is interesting to note that it is the same adrenal cortex which also controls Na metabolism. From these observations it may be suggested that adrenal cortex controls phosphorylation not by a direct action on the enzymes but by an indirect influence on Na metabolism.

It is also known that phospholipids are antagonistic to cholesterol. These two compounds are always found to remain together. It is also interesting to note that adrenal cortex which controls phosphorylation and therefore phospholipid formation controls metabolism of sterols.

2.4.4 Photosystem I (PS I) and Photosystem II (PS II)

The process of photosynthesis is largely comprised of biochemical steps categorized in two categories, i.e., the light dependent step (Light Reaction) and light independent step (Dark Reaction). The **Light Reaction** is accomplished by **Oxidation–Reduction Reactions** mediated by electron transfer between Pigment-Protein Complexes. Thus, Oxygen evolution and excitation /energy transfer process is a function of intensity and wavelength of light energy received by chloroplasts. Various physiological investigations have confirmed the presence of PS-I and PS-II photo centres associated with light reaction process. Experiments by Emerson have led to conclusions of the complementary effects of Red and Far Red wavelength in enhancing the rate of Oxygen evolution in light reaction (Quantum yield). The rate of photosynthesis within the range of optimum wavelength does not exhibit any variation associated with values of wavelength. This observation implies that the photon molecules within a fixed wavelength range possess equal efficiency. Emerson's experiments showed interesting results in terms of wavelength dependent regulation of photoreaction. Far Red wavelength (beyond 680 nm) applied in isolation exhibited a drop in the Quantum yield which could be complemented by Red wavelength (680 nm). This phenomenon (Red drop and Emerson enhancement effect) led to the conclusion of parallel existence of two Pigment Systems (PS-I and PS-II) associated with the light reaction.

The Chlorophyll Molecules (Chl A and Chl B) in association with accessory pigments form reaction centre and light harvesting Complex in the two photosystems. The wavelength dependent activation of photosynthetic pigments can be better explained by the action spectrum represented by Quantum yield. This spectrum basically represents rate of photosynthesis (Quantum yield) obtained as a function of wavelength in the visible spectrum. Quantum yield drops at the wavelength near to 500 nm and further exhibits optimum response are red wavelength. However, the decrease in Quantum yield at far red wavelength is due to absence of PS-I functioning. It was further observed that the decrease in photosynthesis rate at 500 nm was attributed to the negligible absorbance of light by chlorophylls.

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However, the absorption of light energy at a range of 500 nm - 600 nm is accomplished by accessory pigments of carotenoids and xanthophylls molecules. Thus, energy transfer from accessory pigments (Light harvesting complex) to reaction center alone does not appear sufficient to drive optimum photosynthesis.

Overview of Pigment and Protein Distribution in the Thylakoid

The photosynthethic pigments present in the chloroplast are usually chlorophylls A, B and carotenoids and xanthophylls. The pigments constitute the photosystems necessary for light harvesting and photochemical reactions. The reaction center, Antennae–Protein Complex and electron carrier Proteins are essentially localized in the thylakoid membrane. The Proteins present in the thylakoid membrane are usually integral membrane Proteins which also possess external hydrophilic domains protruding to the lumen or stroma of the chloroplast. The inner membrane associated domain of the Proteins contains hydrophobic Amino Acids. The pigments necessary for light reaction remain associated in non-covalent interactions with the Proteins in the thylakoids. The maturity of chloroplast is associated with the formation of photosynthetic apparatus within the organelles. The two photosystems and their antennae pigments are spatially separated from each other within the thylakoid membrane. The PS-II associated with light harvesting and photlysis of water is mostly aligned in the grana lamellae. The antennae pigments associated with PS-II also remains in its vicinity. The plastoquinone Proteins remain associated with the plastoglobulin vesicles scattered in the stroma matrix of the chloroplasts. The PS-I centre and its associated antennae pigments remain localized in the stroma lamellae and the edges of grana lamellae. The electron transferring Protein of **Cytochrome b_6/f Complex** is equally scattered both in the **Stroma** and **Grana** region of the **Chloroplast**. It usually connects the two photosystems in its vicinity. The spatial separation of the two photosystems is thus associated with diffusion of electron carrier Proteins between the grana and stroma region of the chloroplast. The protons generated by water oxidation are capable of diffusing into the stroma region of the chloroplast.

This separation of **PS-I and PS-II** has been attributed to increased efficiency of the photosystems. Moreover, the spatial separation implies independent nature of electron transport possible in the two Photo Systems. In the absence of PS-I functioning, the PS-II can function by transferring electrons to the intermediate acceptor of plastoquinone. It has been observed that plant chloroplasts may possess higher amount of PS-II molecules in comparison with PS-I (1.5:1). This ration may, however, change with alteration in light intensity received by leaves. Purple photosynthetic non-oxygen evolving bacteria like *Rhodbacter* or *Rhodopseudomonas* has been reported to possess only a single photosystem.

Molecular Structure of Pigment-Protein Complex in Thylakoid

The molecular structure of photosynthetic pigment and Protein Complex associated in the thylakoid provides important insights to the distribution of electron transfer carriers. Various investigations have revealed the detailed structure of the two photosystems necessary for light reaction in the thylakoid.

PS-I is mostly composed of chlorophyll a associated with Chl B and β carotene molecules attached to Proteins. The PS-I complex is mostly concentrated

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at the stroma lamellae of the thylakid. The reaction center of PS-I is mostly formed of Chl A molecule capable of absorbing wavelength of 700 nm. The associated Chl B and β carotene molecules remaining in the vicinity of the reaction center transfer energy to the core region. PS-I also contain 2-4 moleucles of heme containing Fe-S Proteins which function as the primary electron acceptor. The excited electrons form the reaction center of PS-I are immediately transferred to the Fe-S Proteins. The two major Proteins known as PsaA and PsaB are involved in the electron transfer process in the reaction center of PS-I. The intermediate forms of the Fe-S Proteins are designated as F_X, F_A and F_B. Thus, electrons are usually transferred from a series of Fe-S Proteins with intermediate forms and tnen received by Ferridoxin. The reduced plastcyanin is capable of donating electrons to the P 700 reaction center. The accessory Proteins associated with PS-I are designated as PsaF, PsaD and PsaE which help in association of soluble electron transfer complexes with the PS-I center.

The **PS-II** is mainly composed of Chl A and β carotene. Chlorophyll B is present in comparatively lesser amounts in PS-II. The PS-II component is held in a complex structure of multi-subunit Protein aggregate which bears two reaction centers and one antennae complex. The core region of the reaction centre contains two Proteins known as D_1 and D_2. The two Proteins D1 and D remain in association to each other. The core region of the reaction center in PS-II is composed of specialized Chl A molecules capable of absorbing light at the wavelength of 680 nm. The primary electron acceptor of PS-II is termed as phaeophytin which is a derivative of Chl A molecule without Mg^{2+}. The recation centre molecules along with phaeophytin, plastioquinone and carotenoids remain associated with the membrane Proteins D_1 and D_2. These membrane Proteins have been observed to exhibit sequence similarity to the L and M peptides of Purple Bacteria. Some accessory Proteins like $cytb_{559}$ is associated with the protective function of PS-II. The Quinine Protein (Q) is capable of accepting excited electron from the reaction centre of PS-II. The reaction centre of PS-II is important for its photolytic activity of water. The Proteins associated with PS-II are manganese bound Proteins which form a moiety of four manganese ions bridged by Cl^- in between them. The mangano-Proteins are thus important components of the PS-II structure present in the inner side of thylakoid membrane. These Proteins are generally associated with water oxidation in the presence of light. The manganese ions perform the function of intermediate redox molecules which form a part of the water oxidation clock. The Light Harvesting Complexes (LHC) associated with PS-I and PS-II are usually comprised of Chl A, Chl B, Xanthophylls and Cartenoid molecules. They remain in vicinity of the two photosystems and usually function to transfer energy to the respective reaction centres. The process of PS-I and PS-II mediated electron flow occurs from H_2O to NADP. The electron transfer carriers are usually plastocyanin, $cytb_6$, cytf and Fe-S Protein. The F_1-ATPase is an important component of the thylakoid membrane which involves in ATP hydrolysis and couples proton transfer across the membrane of the thylakoid.

Antennae Pigments are clusters of molecules which belong to the component of LHC I and LHC II associated with intensification of light energy received by chloroplasts. These molecules are also known as Chl A/B Antennae Proteins. Electron microscopic analysis has revealed the molecular structure of

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the LHC molecules. The Protein of LHC contains three **α helical** region which binds to 15 Chl A, Chl B and carotemoid molecules. The Proteins of both LHC I and LHC II have similarities in their Protein sequences and show evolutionary relatedness. The LHC polypeptides remain associated with each other and the pigment molecules remain in vicinity with each other. This aggregation of molecules in the LHC helps in improving the efficiency of electron transfer. The LHC is comprised of both inner and outer regions in the thylakoid membrane. The outer region is associated with the functioning of light energy harvest and the inner region remains in vicinity of the reaction centres of the two photosystems. The process of energy transfer occur form the outer part of LHC, through the inner integral part of LHC associated with the reaction centre. There lays considerable differences among the Protein and pigment composition of LHC I and LHC II. In PS-I the core region of LHC is composed of 100-120 Chl A and 15-20 β carotene molecules. This association remains in the vicinity of P 700 centre. The PS-II associated LHC II contains two major Pigment-Protein Complexes know as CP4 and CP47. Interestingly, the core region of LHC has similarities in their Protein composition while the outer region exhibits variations. The composition of LHC outer region is largely regulated by the intensity of light received by chloroplasts. The algal and higher plant chloroplasts contain two classes of LHC I and LHC II. The detailed analysis of LHC II complex has revealed the presence of four complexes LHCA, LHCB, LHCC and LHCD. The core component of this complex has been reported to be LHCIIb which exists in form of a Trimeric Membrane Protein. Each of the monomers of this LHCIIb is composed of polypeptides associated with lutein. The activity of LHC molecules remaining in association with reaction centre can reversibly dissociate from PS-II when not required. This process is accomplished by Threonine-PhosphoryLation Reactions catalyzed by Protein Kinase Activity. In the antennae complex of PS-II the peripheral Proteins used for energy transduction are commonly CP29, CP26, CP24. These components transfer excited electrons to main core region of antennae and then to PS-II reaction centre.

Concept of Quantasome and Quantum Yield

The photosynthetic unit is comprised of smallest group of pigment molecules which can efficiently induce a photochemical reaction. The effective mechanism of electron ejection from the photosystem is mediated by these pockets of energy or photosynthetic units. Investigations by Arnold and Emerson in the year 1932 revealed that one molecule of O_2 are evolved by the combined action of every 2500 chlorophyll molecules. The energy units of light wavelength trapped within photon molecules are termed a **Quantum.** Thus physiological investigations have reported that about eight quanta of light energy is required for the reduction of one CO_2 molecule during Photosynthesis.

Thus, the concept of **Quantasome** explains the association of 300 molecules of Chlorophyll in each of the photosystems which function as Photosynthetic Units. Each Quantasome is capable of absorbing one Quantum of light energy. Gaffron and Wohl (1936) proposed that the Quantum received by the photosynthetic pockets is transduced to the core region of the reaction centre in each of the photosystems. Kok (1956) stated that the intermediate electron carriers (Cytochrome, Plastocyanin and Plastoquinone) exist in a ratio of one per 300

molecules of Chlorophyll. The number of Oxygen atoms produced by one Quantum of light energy received in the photosystem is termed as **Quantum yield**. Alternately, the number of Quanta required to liberate one molecule of Oxygen is termed as **Quantum requirement**.

Observations have revealed that the absorption spectrum of chlorophyll does not completely coincide with the action spectrum of photosynthesis. This is attributed to the fact that accessory pigments with different absorption maxima (500 nm - 600 nm) are also involved in the process of photosynthesis. In early 1940 experiments conducted by Emerson and his group revealed wavelength dependent variation in the rate of photosynthesis. The Quantum yield measured in isolated cholorolasts of *Chlorella* was examined separately with lights of wavelength 680 nm and > 700 nm. It was observed that wavelength higher than 680 nm resulted in a drop in the Quantum yield. This effect was termed as **Red Drop Effect.** The Far Red region of the visible spectra was found to be responsible for this Red Drop Effect. Subsequently, later investigations in 1957 revealed that supplementation of far red light with wavelengths near to 680 nm could recover the Quantum yield or photosynthetic efficiency. This phenomenon of combined effect of Red and Far Red wavelengths in producing optimum magnitude of Quantum yield was termed as **Emerson Enhancement Effect**. Thus this observation implies the simultaneous functioning of two photosystems in the chloroplasts.

Mechanism of Light Absorption and Energy Transfer During Photosynthesis

The light energy received by chloroplast appears in the form of Quantum which induces energy driven excitation of electron from the reaction centre. The effect of Quantum absorption depends upon the frequency of light energy. The visible spectra of light fall in between the UV (Ultra Violet) and infrared zone which ranges from 400 nm to 700 nm. The absorption of Quantum results in excitation of the outer orbital electrons which initiates the photochemical reaction. The chlorophyll molecules which initially remain in their ground state (S_0) pass through intermediate oxidized stages (S_1) and (S_2). The excited forms of Chl molecule remain in the singlet or triplet stage for a transient time period. The return of Chl molecules into their ground state is accomplished by either emission of fluorescence or phosphorescence. In the later case, the return of excited molecule from triplet to ground state is accompanied by emission of low energy light for longer durations (Phosphorescence). The intensity of fluorescence is higher than that of phosphorescence and is emitted for shorter durations. Fluorescence involves return of electron from its singlet stage to ground state. Among all the types of photosynthetic pigments Chlorophyll A is directly involved in the process of photosynthesis. The other pigments essentially transfer their energy to Chl A molecules present in the reaction centre. The process of energy transfer from the antennae pigments to the reaction centre occurs by the phenomenon of Fluorescence Resonance Energy Transfer (FRET). In this mechanism the fluorescence emission of one molecule appears to be the excitation wavelength of the adjacent molecule. Thus, the excited electron is transmitted across the thylakoid membrane. The efficiency of energy transfer from Chl B to Chl A has been reported to be nearly 100%. However, the transfer of energy from

Phycoerythrin to Chl A possess 80-90% efficiency and lesser for Carotenoids to Chlorophyll A (20-50%).

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2.5 ELECTRON TRANSFER IN BIOLOGY

Electron transfer reactions involve the movement of an electron from one molecular species (the donor) to another (the acceptor) and turn out to be an essential quantum mechanical component in various biological processes.

Electron transfer, or the act of moving an electron from one place to another, is amongst the simplest of chemical processes, yet certainly one of the most critical. The process of efficiently and controllably moving electrons around is one of the primary regulation mechanisms in biology. In this phenomenon the attraction between oppositely charged ions is called an *ionic bond*, and it is one of the main types of chemical bonds in chemistry. Ionic bonds are caused by electrons transferring from one atom to another. Without stringent control of electrons in living organisms, life could simply not exist. For example, photosynthesis and nitrogen fixation (to name but two of the most well-known biochemical activities) are driven by electron transfer processes. It is unsurprising, therefore, that much effort has been placed on understanding the fundamental principles that control and define the simple act of *adding* and *removing* electrons from chemical species.

Transition metals, such as copper and iron play leading roles in electron transport as one-electron redox-active centres within proteins that are used to effectively move electrons around. Well-known examples are the blue copper proteins (Cu^{I}«Cu^{II}), cytochromes (Fe^{II}«Fe^{III} porphyrins), and iron-sulphur proteins (Fe^{II}«Fe^{III} with sulphur ligands). Significant efforts have been placed on developing our understanding of how biological systems control which electron transfer processes are feasible (i.e., reduction potentials) and how fast they will occur (i.e., rate constants). The factors that affect the properties of these important biological electron transfer sites are generally considered as either intrinsic (i.e., an inherent behaviour and property of the site itself) or extrinsic (i.e., modulation of the basic properties by external factors) to the active site. Our recent efforts have served to provide insights into the intrinsic properties of mononuclear iron centres using both experimental and theoretical methods to evaluate their inherent electronic structure and to correlate it to their observed redox properties.

Using small near-tetrahedral $[Fe(SR)_4]^{2-,1-}$ models, we have probed the electronic structure of both the reduced (Fe^{II}) and oxidized (Fe^{III}) complexes to obtain insights into their intrinsic redox properties and implications on the properties of *Rubredoxins*, a class of small electron transport proteins that contain a similar active site. An important question was whether electron transfer in these sites was well-described as a one-electron process. Generally, it is assumed that the removal and/or addition of a single electron is a rather simple process and that it does not significantly alter the electronic structure of a transition metal site. Another way of stating this is that we generally assume that electron transfer is a 'One-Electron' process - our approach to understanding electron transfer processes is rooted in this one-electron approximation. From studies on a related system, it seemed that this assumption might not hold.

In particular, the long-standing puzzle of whether enzymatic glucose sensing involves an enzyme direct electron transfer process was studied. The results indicate

the mechanism is indeed a glucose oxidase direct *electron transfer process* with competitive glucose oxidation and oxygen reduction to detect glucose.

Light Reaction

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The physiological experiments performed by Emerson and Rabinowitch (1960) confirmed the physiological functioning of chloroplasts in response to light wavelength. The process of photochemical reaction is accomplished by electron transport between the two photosystems PS-I and PS-II. The two photosystems contain core reaction centre associated with the functioning of Chl A. The wavelength associated with Chl A in the two photosystems vary as 680 nm and 700 nm in PS-II and PS-I, respectively. The differences thus lie in the efficiency to absorb longer and shorter wavelength of Red Light. Light reaction occurring in the Thylakoids involves Oxidation of Water to Oxygen and transfer of electron to $NADP^+$. The protons liberated from oxidation of water result in the formation of $NADPH_2$. The Chl molecules in P 700 centre are capable of optimum photon absorption in its reduced state. The connecting molecules which transfer electrons between PS-I and PS-II are mainly Copper containing Protein plastocyanin and a Quinone group of carrier termed as Plastoquinone. The cytochrome Proteins involved in this process are mainly cytochrome B6 and cytochrome F.

The Water Oxidation Mechanism of Light Reaction

The most important component of PS-II mediated part of light reaction is the oxidation of Water and liberation of Oxygen (Refer Figure 4.4). PS-II centre receives photons of wavelength 680 nm and upon excitation transfers electron to a chlorophyll derivative known as Pheophytin. Additionally the chlorophyll molecules should essentially return to reduced ground date in order to refunction by absorbing new photon molecules. Thus, the electron is obtained by oxidation of water molecules, which in turn reduces the Chl A molecules. Two water molecules are oxidised by Chl A to liberate four hydrogen ions and four electrons as shown in the given reaction.

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

The water oxidation mechanism accomplished in the PS-I reaction centre is facilitated by intermediate oxidation stages of Mn^+ designated as S_0, S_1, S_2, S_3 and S_4. The oxidation-reduction cycle occurring by the help of these four stages of Mn^+ was reported by Kok *et al* (1970) and is commonly mentioned as the **Water Oxidation Clock** (Refer Figure 2.8)**.** Every step of transition between S_0 and S_4 is a photon-mediated redox reaction. The last step of this water oxidation reaction series involves transformation of S_4 to S_0 accompanied by liberation of Oxygen. The process of water oxidation results in the liberation of four protons from two water molecules. The reaction centre possesses the mangano-protein complex which together with Ca^{2+} and Cl^- forms the catalytic **Oxygen Evolving Complex (OEC)**. The protons liberated are initially stored in the lumen of thylakoid. The OEC is localized towards the inner membrane of the thylakoid and attached to the D_1 and D_2 Proteins present in PS-II which facilitates the entry of protons into the lumen. These protons are later transferred to the stromal region by the activity of F_1 – ATPase. The proton motive force generated due to electrochemical gradient

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thus allows proton movement across the thylakoid membrane. Detailed discussion of the process of proton mediated ATPase activity will be discussed in the following section of the unit. A single PS-II reaction centre containing OEC must be excited four times in order to release a single O_2 from oxidation of two molecules of water. The four electrons obtained from water oxidation are transferred to the P680 site. In this context it is important to understand that an intermediate complex of electron carrier exists between the OEC and P680 centre and is termed as Z and Tyr_Z. Tyr_z transfers the electron to water and again obtains it by oxidizing the cluster of Mn ions. Thus the process of electron transfer from water to Mn ion is completed after four electrons are obtained from water. These electrons are transferred by Mn^+ to Tyr_Z complex.

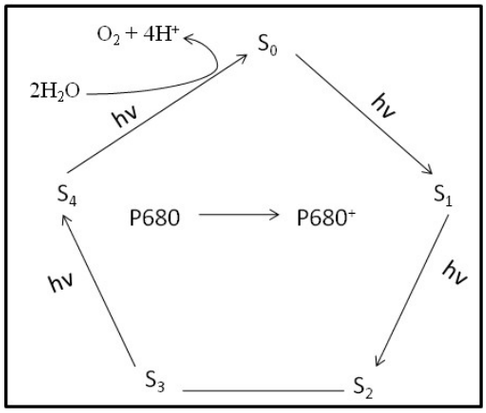

Fig. 2.6 *Water Oxidation Clock*

Pheophytin and Quinone Mediated Electron Transfer Occurs from PS-II

The pheophytin is a specialized molecule of chlorophyll where the central magnesium ion is replaced by two Hydrogen atoms. This molecule by the virtue of its structural differences possesses different spectral properties than chlorophyll. Physiological evidences have confirmed that pheophytin is the immediate electron acceptor of PS-II centre. Furthermore the two quinone molecules present in the electron transfer complex obtain electrons from the pheophytin molecules. The quinine molecules designated as plastoquinone (PQ_A and PQ_B) remains in the vicinity of PS-II reaction centre. The process of oxidation and reduction of plastoquinone occurs in the presence of protons and electrons. The PQ form of plastoquinone is reduced to Plastohydroquinone (PQH_2) by obtaining two electrons from phaeophytin and also two protons from the stromal side of thylakoid membrane. The dynamic nature of PQH_2 results in its dissociation from the reaction centre complex and moves towards the inner part of the membrane. In this region, PQH_2 is reoxidized by transferring the electrons to $Cytb_6f$ complex. The non-polar nature of the quinone Protein results in its diffusible nature across the membranes. The protons of PQH_2 released combine with other protons obtained from water oxidation. This process decreases the pH of the thylakoid lumen. PQH_2 mediated transfer of proton results in the formation of ATP catalysed by the activity of ATPase. Usually two molecules of ATP are generated for each pair of electron passing through PQH_2.

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Electron Transfer through Cytochrome B_6-F and Plastocyanin Connect to PS-I Centre

The process of electron transfer from PQH_2 to PS-I centre is mediated by two intermediate electron carriers called cytb_6f and plastocyanin. The cytochromeb_6f complex is a multi- subunit Protein with prosthetic group attached to it. This complex Protein possess heme group attached to it to the peptide part. Furthermore the cyt Protein complex also possesses the Fe-S centre commonly termed as **Rieske centre**. The mechanism of electron flow from the PQH_2 to cyt complex has been explained by Q cycle. Investigations have revealed that the two electrons released by plastohydroquinone are involved in different pathways. The linear electron transfer chain accounts for the electron which is received by FeS_R (Rieske centre) and forwarded to the cytf complex. The other electron obtained from PQH_2 travels through a cyclic process and is associated with proton pumping activity across the membrane. The electron received by cytf complex is further transferred to Plasto-Cyanin (PC) which is a Blue coloured Copper Containing Protein. The cytb_6f complex is larger in size than quinone and plastocyanin. It is also localized across the stroma and grana region of thylakoid. This result in its immobile nature compare to the other electron carriers (PQ and PC) present in the thylakoid membrane. plastocyanin is a small water soluble copper containing Protein of around 11 kDa in size. It transfers electron between the cytochrome b_6f complex and PS-I centre. The biosynthesis of this Protein is partially regulated by the copper availability of the plant tissue. Plastocyanin is localized in the lumen of the chloroplast. In certain organisms like Cyanobacteria and Algae c-type cytochrome may be present as a substitute of Plastocyanin.

The PS-I centre is a complex aggregation of Proteins which include the reaction centre surrounded by the light harvesting complex or antennae pigment. The core region of antennae pigment in PS-I is comprised of 100 Chl A molecules. The core antennae pigments and PS-II reaction centre are associated with PsaA and PsaB Proteins which are of molecular weight in the range of 65-70 kDa in size. The electron carriers associated with the PS-I centre are strong reductants in nature. Physiological evidences reveal the presence of quinone and phylloquinone associated with Chl A molecule which serves as electron carriers. The Fe-S centre of the reaction centre form an efficient electron acceptor/carrier with three forms FeS_x, FeS_A and FeS_B, respectively. The electron receiving centres of PS-I are designated as A and B which transfer electrons to the water soluble Fe-S Protein called Ferridoxin. Furthermore, Ferridoxin-NADP reductase reduces $NADP^+$ to NADPH. This process terminates the process of electron transfer from PS-II to PS-I. The reduced form of ferridoxin associated with the photochemical reaction also serves in other metabolic pathways like nitrate metabolism and regulation of Carbon Gixation Pathways.

Electron Transport Pathway in Chloroplast

In the earlier sections various electron carriers associated with the two photosystems were discussed in details. The process of electron transfer from PS-II to PS-I operates

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through various intermediate carrier Proteins and is commonly termed as the **Non-Cyclic Electron Flow**. This process does not involve the return of electron into the PS-II reaction centre. However, another pathway of PS-I mediated electron transfer occurs simultaneously to the non-cyclic transfer and hence is termed as **Cyclic Electron Transfer (CET) Pathway**. This pathway involves plastocyanin mediated return of electron to the PS-I centre of chloroplast. Interestingly the formation of $NADPH^{+}$ occurs at the end of the non-cyclic pathway. However, both the electron transport pathways are associated with ATP formation catalyzed by the enzyme ATPase. This process of ATP formation coupled to photochemical reaction is termed as **Photophosphorylation.** The process of photophosphorylation is associated with the cyclic and non-cyclic electron transport pathways and hence termed and **Cyclic and Non-Cyclic Photophosphorylation**. The brief process of electron transport pathways shall be discussed in the following sections of the unit.

Non-Cyclic Electron Transport Pathway

The Magano-Protein associated with the Oxygen Evolving Complex (OEC) has been observed to exhibit enzymatic activity which catalyzes the splitting of water (**Hill reaction**). Mn^{+}, Ca^{2+} and Cl^{-} are associated with the OEC complex. Various compounds like hydroxylamine, ammonia, Carbonyl Cyanide 3-Chloro Phenylhydrazone (CCCP) or heavy metals have been reported to inhibit the process of electron transfer from the PS-II region. The Mn-Protein of OEC mediates the electron transfer from water to the PS-II region. The electron from PS-II region is transferred to pheophytin molecule. The process of electron transport further advances to Quinone and Plastoquinone. The PQH_2-PQ interconversion event associated with the electron transport pathway is coupled to ATP formation. This process is termed as the **Non-Cyclic Photophosphorylation**. A commonly implied inhibitor of electron transport from Quinone to other carriers is **Di-Chlorophenyl-DiMethyl-Urea (DCMU)**. PQH_2 transfers the electrons to the Rieske Center (Fe-S). This Fe-S center is further oxidized by cytochrome (cyt F) followed by electron transfer to the Plasto-Cyanin (PC) complex. The plastocyanin Protein acts as the electron donor for the PS-I center. The PS-I centre transports electrons to the ferridoxin and finally to $NADP^{+}$. This terminates the process of non-cyclic electron transport pathway of chloroplasts.

Cyclic Electron Transport Pathway

The cyclic pathway of electron transport is associated with the PS-I center which transmits the electron across Ferridoxin, Plastoquinone-Fd Complex and Plastocyanin Protein. The cyclic process of electron transport does not involve oxidation of water molecules and it essentially operates independent of the non-cyclic process. Photon-mediated excitation of the P 700 molecules results in transfer of electrons to ferridoxin. The redox reaction associated with electron transfer is further mediated by the activity of Fd-PQ oxido-reductase enzyme and cytochrome b_6f complex. The electron returns to the P 700 center via the plastocyanin molecule. **Paraquat** is a well known inhibitor which blocks the electron transfer from PS-I to $NADP^{+}$. The process of ATP formation in this pathway is energised by proton mediated electrochemical gradient across the

Thylakoid Lumen. Thus ATP formation in this pathway is termed as the **Cyclic-Photophosphorylation** (Refer Figure 2.7)**.**

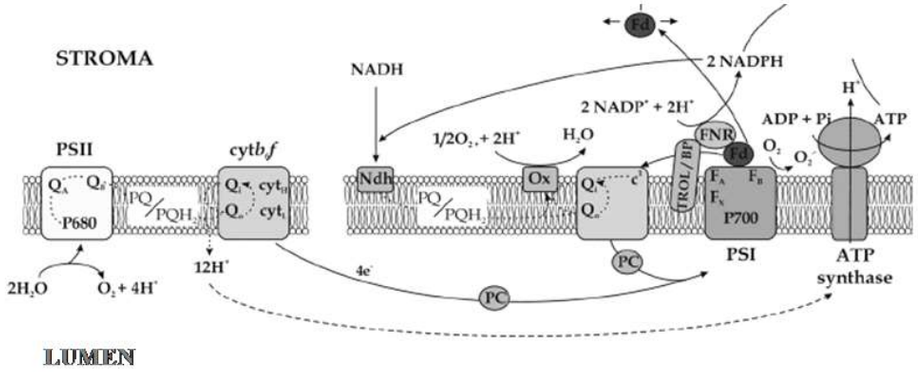

Fig. 2.7 Mechanism of Light Reaction

Proton Transport Across Thylakoid Membrane: Chemiosmotic Theory

The process of proton energised phosphorylation in the thylakoids was initially reported by Daniel Arnon amd co-workers in 1950. The process of light dependent ATP formation in the chloroplasts is thus termed as Photophosphorylation. Physiological investigations in the next decade reported the formulation of **Chemiosmotic Theory** by Peter Mitchell (1960s). Similar kind of mechanism is also associated with aerobic respiration and electron transport chain occurring in the mitochondria. The higher proton concentration in the thylakoid lumen occurs due to proton liberation from water oxidation and plastohydroquinone oxidation. This results in the formation of a proton motive force manisfested as a transmembrane electrochemical gradient across the thylakoid membrane. According to the chemiosmotic theory the difference in ion concentration across the membrane is utilized in the form of free energy. ATPase dependent proton transfer thus couples to ATP formation. According to the Second Law of Thermodynamics, 'Any asymmetric or unequal distribution of energy results in driving a forward reaction'. The thylakoid membranes exhibit a difference in the chemical potential obtained due to concentration gradient of protons. The asymmetric nature of photosynthetic membrane in the chloroplast results in such electrochemical gradient. Higher accumulation of protons in the thylakoid lumen results in its acidic pH, while the stroma region remains alkaline due to fewer protons present there. Earlier investigations before 1960 were performed with isolated chloroplasts in buffer solutions of pH 4.0 and pH 8.0. These buffers equilibrated the pH across the membranes of chloroplast. However, difference in the pH across the thylakoid membrane was associated with ATP generation independent of light perception or electron transport.

According to Mitchell the **Proton Motive Force (*p*)** generated in the thylakoid membrane is the sum total of **Proton Chemical Potential** and **Electric Potential** across the membrane. During the operation of light reaction in the chloroplast the protons accumulating in the lumen result in formation of electrochemical gradient mostly due to the difference in the pH. Physiologists have referred to the process of ATP synthesis where four protons are transported across the membrane coupled to synthesis of one ATP molecule. ATP synthase is mostly localized in the stroma lamellae and grana. The ATPase synthase is a

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400 kDa Protein with two major components called CF_0-CF_1. The CF_0 particle is a hydrophobic membrane spanning domain which provides the channel for passage of protons. The CF_1 particle is composed of various polypeptides which comprise of catalytic domains associated with proton efflux and ATP synthesis. CF_0 region of the enzyme rotates to provide a motor like action associated with proton efflux and ATP synthesis. The polypeptide nature and catalytic sites are mostly similar for ATP synthase associated with chloroplast and mitochondria. However, certain structural differences may exist. The basic mechanism of proton mediated ATP production is similar in both the cases.

2.5.1 Structure and Function of Metalloprotein

Metalloproteins account for nearly half of all proteins in biology. Protein metal-binding sites are responsible for catalyzing some of the most difficult and yet important functions, including photosynthesis, respiration, water oxidation, molecular oxygen reduction, and nitrogen fixation.

Metalloprotein is a generic term for a protein that contains a metal ion cofactor. A large proportion of all proteins are part of this category. For instance, at least 1000 human proteins (out of ~20,000) contain zinc-binding protein domains although there may be up to 3000 *human zinc metalloproteins.*

It is estimated that approximately half of all proteins contain a metal. In another estimate, about one quarter to one third of all proteins are proposed to require metals to carry out their functions. Thus, metalloproteins have many different functions in cells, such as storage and transport of proteins, enzymes and signal transduction proteins, or infectious diseases. The abundance of metal binding proteins may be inherent to the amino acids that proteins use, as even artificial proteins without evolutionary history will readily bind metals. Most metals in the human body are bound to proteins. For instance, the relatively high concentration of iron in the human body is mostly due to the iron in haemoglobin.

Metal Concentrations in Humans Organs (ppm = ug/g ash)

	Liver	Kidney	Lung	Heart	Brain	Muscle
Mn (manganese)	138	79	29	27	22	<4-40
Fe (iron)	16,769	7,168	24,967	5530	4100	3,500
Co (cobalt)	<2-13	<2	<2-8	---	<2	150 (?)
Ni (nickel)	<5	<5-12	<5	<5	<5	<15
Cu (copper)	882	379	220	350	401	85-305
Zn (zinc)	5,543	5,018	1,470	2,772	915	4,688

In metalloproteins, metal ions are usually coordinated by nitrogen, oxygen or sulphur centres belonging to amino acid residues of the protein. These donor groups are often provided by side-chains on the amino acid residues. Especially important are the imidazole substituent in histidine residues, thiolate substituents in cysteine residues, and carboxylate groups provided by aspartate. Given the diversity of the metalloproteome, virtually all amino acid residues have been shown to bind metal centres. The peptide backbone also provides donor groups; these include

deprotonated amides and the amide carbonyl oxygen centres. Lead(II) binding in natural and artificial proteins has been reviewed.

In addition to donor groups that are provided by amino acid residues, many organic cofactors function as ligands. Perhaps most famous are the tetradentate N_4 macrocyclic ligands incorporated into the haeme protein. Inorganic ligands, such as sulphide and oxide are also common.

Metalloproteins are proteins bound by at least one metal ion. Metal ions are usually coordinated by four sites consisting of the protein's nitrogen, sulphur and oxygen atoms. In metalloenzymes, one of the coordination sites is labile.

Many proteins that bind metal ions (metalloproteins) for structural or functional purposes possess metal-binding sites containing aspartate or glutamate side chains or both. Free glutamate and glutamine play a central role in amino acid metabolism.

Metalloprotein in Electron Transport Process

Metalloprotein is a generic term for a protein that contains a metal ion cofactor. A large proportion of all proteins are part of this category. Cytochrome oxidase is the most common example of metalloprotein. Cytochrome c is functionally involved in the electron transport chain of mitochondria. That electron transport is part of the pathway for synthesis of ATP. The role of cytochrome c is to carry electrons from one complex of integral membrane proteins of the inner mitochondrial membrane to another.

Cytochrome

Cytochromes are a major class of haeme-containing Electron Transfer or ET proteins found ubiquitously in biology. They were first described in 1884 as respiratory pigments (called *myohematin or histohematin*) to explain coloured substances in cells. These coloured substances were later rediscovered in 1920 and named *cytochromes*, or cellular pigments. The intense red colour combined with relatively high thermodynamic stability makes cytochromes easy to observe and to purify. As of today, more than **70 000 cytochromes** have been discovered. In addition, due to their small size, high solubility, and well-folded helical structure and the presence of the *haeme chromophore*, cytochromes are one of the most extensively studied classes of proteins spanning several decades.

Cytochromes are present mostly in the inner mitochondrial membrane of eukaryotic organisms and are also found in a wide variety of both Gram-positive and Gram-negative bacteria. Cytochromes play crucial roles in a number of biological ET processes associated with many different energy metabolisms. Additionally, cytochromes are involved in apoptosis in mammalian cells.

2.5.2 Iron–Sulphur Protein

Iron–sulphur proteins (or iron–sulphur proteins in British spelling) are proteins characterized by the presence of iron–sulphur clusters containing sulphide-linked di-, tri-, and tetra iron centres in variable oxidation states. Iron–sulphur clusters are found in a variety of metalloproteins, such as the ferredoxins, as well as NADH

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dehydrogenase, hydrogenases, coenzyme Q – cytochrome c reductase, succinate – coenzyme Q reductase and nitrogenase. Iron–sulphur clusters are best known for their role in the oxidation-reduction reactions of electron transport in mitochondria and chloroplasts. Both Complex I and Complex II of oxidative phosphorylation have multiple Fe–S clusters. They have many other functions including catalysis as illustrated by *aconitase,* generation of radicals as illustrated by SAM-dependent enzymes, and as sulphur donors in the biosynthesis of lipoic acid and biotin. Additionally, some Fe–S proteins regulate gene expression. Fe–S proteins are vulnerable to attack by biogenic nitric oxide, forming dinitrosyl iron complexes. In most Fe–S proteins, the terminal ligands on Fe are thiolate, but exceptions exist.

The prevalence of these proteins on the metabolic pathways of most organisms leads some scientists to theorize that iron–sulphur compounds had a significant role in the origin of life in the iron–sulphur world theory.

Structural Motifs

In almost all Fe–S proteins, the Fe centres are tetrahedral and the terminal ligands are thiolato sulphur centres from cysteinyl residues. The sulphide groups are either two- or three-coordinated. Three distinct kinds of Fe–S clusters with these features are most common.

2Fe–2S Clusters

The simplest polymetallic system, the $[Fe_2S_2]$ cluster, is constituted by two iron ions bridged by two sulphide ions and coordinated by four *cysteinyl ligands* (in Fe_2S_2 ferredoxins) or by two cysteines and two histidines. The oxidized proteins contain two Fe^{3+} ions, whereas the reduced proteins contain one Fe^{3+} and one Fe^{2+} ion. These species exist in two oxidation states, $(Fe^{III})_2$ and $Fe^{III}Fe^{II}$. CDGSH iron sulphur domain is also associated with **2Fe-2S clusters** (Refer Figure 2.8).

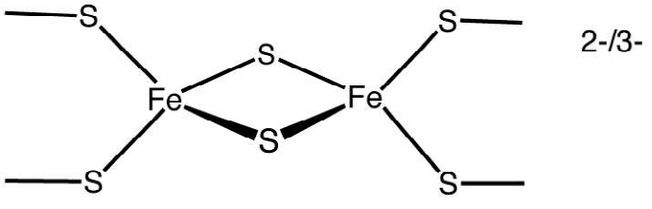

Fig. 2.8 *Structure of 2Fe-2S Clusters*

4Fe–4S Clusters

A common motif features a four iron ions and four sulphide ions placed at the vertices of a cubane-type cluster. The Fe centres are typically further coordinated by cysteinyl ligands. The $[Fe_4S_4]$ ***electron-transfer proteins*** ($[Fe_4S_4]$ ferredoxins) may be further subdivided into low-potential (bacterial-type) and high-potential (HiPIP) ferredoxins.

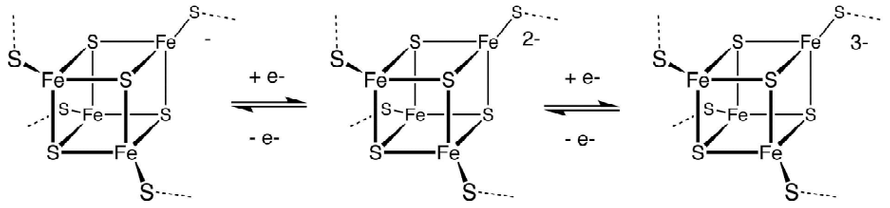

Fig. 2.9 *Structure of 4Fe–4S Clusters*

3Fe–4S Clusters

Proteins are also known to contain $[Fe_3S_4]$ centres, which feature one iron less than the more common $[Fe_4S_4]$ cores. Three sulphide ions bridge two iron ions each, while the fourth sulphide bridges three iron ions. Their formal oxidation states may vary from $[Fe_3S_4]^+$ (all-Fe^{3+} form) to $[Fe_3S_4]^{2"}$ (all-Fe^{2+} form). In a number of iron–sulphur proteins, the $[Fe_4S_4]$ cluster can be reversibly converted by oxidation and loss of one iron ion to a $[Fe_3S_4]$ cluster. E.g., the inactive form of aconitase possesses an $[Fe_3S_4]$ and is activated by addition of Fe^{2+} and reductant.

Other Fe–S Clusters

More complex polymetallic systems are common. Examples include both the 8Fe and the 7Fe clusters in nitrogenase. Carbon monoxide dehydrogenase and the [FeFe]-hydrogenase also feature unusual Fe–S clusters. A special 6 cysteine-coordinated $[Fe_4S_3]$ cluster was found in oxygen-tolerant membrane-bound [NiFe] hydrogenases.

2.6 NITROGENASE

There are numerous types of enzymes, complexes, and other material that operate in living organisms and can be important to their survival.

Nitrogenase, is one enzyme that is produced by certain types of bacteria and is vital to their existence and growth. Nitrogenase is a unique enzyme with a crucial function that is distinct to bacteria that utilize it, has unique structure and symmetry, and is sensitive to other compounds that inhibits its functioning. It is 'an enzymatic complex which enables fixation of atmospheric nitrogen'. The unique structure of nitrogenase is almost completely known because of the extensive research that has been done on this enzyme. Nitrogenase can also bind to compounds other than nitrogen gas, which can inhibit and decrease its production of ammonia to the rest of the organism's body. Without proper functioning, the bacteria that utilize nitrogenase would not be able to survive, and other organisms that depend on these bacteria would also die.

Nitrogenase is unique in its ability to fix nitrogen, so that it is more reactive and able to be applied in other reactions that help organisms grow and thrive. However, nitrogen gas, N_2, is an inert gas that is stabilized by its triple bond , and is difficult for living organisms to use as a source of nitrogen because the molecule's stability. Nitrogenase is used to separate nitrogen gas, N_2, and transforms it into ammonia, NH_3 in the reaction.

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In the form of ammonia organisms have a useable source of nitrogen that is more reactive and can be used to create proteins and nucleic acids that are also necessary for the organism. According to the Peters and Szilagyi, 'Three types of nitrogenase are known, called **Molybdenum (Mo) nitrogenase, Vanadium (V) nitrogenase and iron-only (Fe) nitrogenase' and the molybdenum nitrogenase**,crystal structure, is the one that has been studied the most of the three. The nitrogenase enzyme breaks up a diatomic nitrogen gas molecule using a large number of ATP and 8 electrons to create two ammonia molecules and hydrogen gas for each molecule of nitrogen gas . As a result, the bacteria that utilize this enzyme must expend much of their energy, in the form of ATP, so that they will constantly obtain a steady source of nitrogen. Without nitrogenase's function of fixing nitrogen gas into ammonia, then organisms would not be able to thrive since they would not receive a source of nitrogen for other important reactions.

Many details have been discovered over time about the functioning of nitrogenase, but there has yet to a complete agreement on the structure of nitrogenase. The **iron-molybdenum** cofactor center of the nitrogenase enzyme consists of iron, sulphur, molybdenum, a homocitrate molecule, a histadine amino acid and a cysteine amino acid. Nitrogenase contains various molecules that have different conformations throughout the enzyme giving nitrogenase a unique symmetry.

Nitrogenase is not easily used because of its sensitivity to other compounds, which prevents it from being easily used by other organisms. Nitrogenase contains several iron atoms that could become disrupted if it were to become oxidized by any oxygen gas. In addition, 'nitrogenase has the ability to bond acetylene and carbon monoxide' making the three molecules (acetylene, carbon monoxide, and nitrogen gas) competitive substrates that bind to nitrogenase. The restrictive functionality of nitrogenase makes it only possible for anaerobic organisms to utilize nitrogenase. Rhizobium, normally found in plant roots, is an example of an anaerobic family of bacteria that utilizes nitrogenase. They exist in a symbiotic relationship with leguminous plants to produce nitrogen in exchange for other nutrients and protection from oxygen and other compounds that could inhibit nitrogenase's operation. These limitations of nitrogenase prevent it from being commonly used among organisms, but it is an important element to the bacteria that make use of this enzyme.

Nitrogenase is a unique enzyme that is critical to the bacteria that utilizes this enzyme. Its function is necessary for anaerobic bacteria since it is their main source of nitrogen. However, the high cost of ATP and electrons is another restrictive reason why more organisms do not produce their own source of nitrogen. The specific connections within nitrogenase cause it to lack any symmetric elements in its structure. More research must be done before the complete understanding of nitrogenase and its structure, such as with the possibility and identity to a central atom within nitrogenase. Nitrogenase is a sensitive enzyme that can easily be inhibited by oxygen and other compounds and affects its ability to fix nitrogen. It is the major reason why nitrogenase is strictly found in anaerobic bacteria that is able to utilize nitrogenase for its source of nitrogen. Nitrogenase is simply one enzyme among the multitudes of other enzymes and complexes that is vital strictly to the organisms that utilize it.

2.6.1 Physiology of Nitrogen Fixation

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Symbiotic N_2-Fixing Bacteria

Symbiotic bacteria is the one which forms association with either plant , animal or fungus in which bacteria as well as the host, both are benefited, example - Rhizobium bacteria forms association with the root nodules of higher plants , plant provide shelter to the bacteria and bacteria helps in nitrogen fixation.

The heterotrophic bacteria that fix Di-Nitrogen (N_2) gas from the atmosphere in plant root nodules (symbiotic bacteria) have a mutually beneficial relationship with their host plants. Legumes (pod-bearing plants, such as peas, beans, alfalfa and clovers, etc.) had a beneficial effect upon both companion and whatever crop was planted next in the same soil. It is evident that the fixation of atmospheric nitrogen in the legume is due to the formation of root nodules.

Symbiotic bacteria initially start by infecting root hairs, causing an invagination (enclosing-like sheaths) inward through several cells. Surrounding plant cells proliferate quickly, perhaps because of auxin, a phytohormone produced by the infecting bacteria.

As the bacteria enter the nodule cells, they form enclosing membranes and produce meta-hemoglobin, an oxygen-carrying pigment (the nodule may be pink in cross-section). The hemoglobin like material may be an oxygen sink or trap to keep the bacteria in an anaerobic environment, which is necessary for N_2 fixation.

The Di-Nitrogen (N_2) fixation is performed by the enzymes nitrogenase. This enzyme lowers the activation energy (the energy requires to perform the reaction). The fixation proceeds in reduction stages from di-nitrogen (N = N) through uncertain intermediates HN=NH and H_2N-NH_2 to produce 2 NH_3.

Finally, the ammonium is transformed into some organic compounds such as amino acids. All of this will take place when the nitrogen is bonded to the enzyme(s).

The lifetime of a bacterium may be only a few hours and the bodies of a portion of the bacterial population are continuously dying, decomposing, and releasing NH_4^+ and NO_3^- ions for the utilization by the host plant. Most of the nitrogen fixed is excreted by the bacteria and made available to the host plant and to the other plants growing nearby. The well-known symbiotic bacteria belong to the genus Rhizobium.

Symbiotic heterotrophic bacteria specific to the crop to be grown are frequently applied or inoculated, in a dried powdered from to the crop seed to ensure that nitrogen fixing organisms are present. The same bacterial species will not inoculate all legumes. Sesbania rostrata (dhaincha) was found to form nodules both in roots and stems and it is most important host plant for the symbiotic N_2-fixation.

Recently some plants have been found to have symbiotic relationship with different N_2-fixing bacteria, including blue green bacteria (cyanobacteria), are Digitaria (grass species), water fern for example, azolla (with blue green bacteria), Gunnera macrophylla (with blue green bacteria).

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It has been also reported that bacteria of the genus Klebsiella have been found to be associated in N_2-fixation with various grasses (non-legumes) but none has yet proven to be symbiotic. In addition, may other non-leguminous plants have symbiotic N_2-fixing nodulation (for example, Alnus species, Casuaraina equisetifolia, etc.)

Since the number of host plants is limited, cross inoculation groups have been established. A cross-inoculation group refers to a collection of leguminous species that are capable of developing nodules when exposed to bacteria obtained from the nodules of any member of that particular plant group.

Non-Symbiotic N_2-Fixing Bacteria:

The non-symbiotic nitrogen fixing bacteria do not require a host plant. In 1891, Winogradsky observed that when soil was exposed to the atmosphere, the nitrogen content of the soil was recorded to be increased.

The anaerobic bacterium Clostridium pasteurianum was found responsible for such an increase of the nitrogen content in soil. In 1901, Beijerinck proved that there were also free-living aerobic bacteria, Azotobacter chroococcum that could fix atmospheric nitrogen.

Another bacterial group, Granulobacter (purple colour) obtains nitrogen directly from the atmosphere. The amounts of atmospheric nitrogen fixed by these bacteria are largely variable because of divergent nature of soils.

In aerobic soils of tropical climatic regions, the acid tolerant N_2-fixer Azotobacter beijerinckia is most abundant Azospirillum spp. also fix N_2-non-symbiotically and help to many crops for their growth and yield.

2.6.2 Spectroscopy of Nitrogenases

Nitrogenases are responsible for biological nitrogen fixation, a crucial step in the biogeochemical nitrogen cycle. These enzymes utilize a two-component protein system and a series of iron–sulphur clusters to perform this reaction, culminating at the FeMco active site (M = Mo, V, Fe), which is capable of binding and reducing N_2 to $2NH_3$. Synthetic model chemistry and theory have also played significant roles in developing our present understanding of these systems and are discussed in the context of their contributions to interpreting the nature of nitrogenases.

As a variety of spectroscopic techniques have played key roles in helping to define the geometric and electronic properties of the cofactors, Many of these methods are also readily complemented by theory, and we briefly introduce the application of computational chemistry in N_2ase research with the aim of demonstrating how it has been employed to complement spectroscopic studies. Model chemistry has also been crucial in informing spectroscopic methods, and hence we also review the contributions of molecular models as vital spectroscopic benchmarks. We then elaborate on the present understanding of each of the metal cofactors of N_2ases, focusing on the Mo-dependent form of the enzyme as it has been the most intensely studied. This is followed by a discussion of the alternative N_2ases.

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Electron Paramagnetic Resonance (EPR) Spectroscopy

As a technique, Electron Paramagnetic Resonance (EPR) spectroscopy can be applied to study paramagnetic centres possessing one or more unpaired electrons. To investigate paramagnetic centres with half-integer, *Kramers spin states* (S = 1/2, 3/2, etc.), the more routine (and most commonly available) perpendicular-mode EPR spectrometer may be used. Detection of integer, non-Kramers spin states (S = 1, 2, etc.) requires less common and often significantly more challenging techniques and different instrumentation.

InfraRed (IR) Spectroscopy

InfraRed (IR) spectroscopy is an extremely widespread technique which utilizes electromagnetic radiation in the range of ~20–14/ 000 cm–1 to probe the normal vibrational, translational, and rotational modes of molecular motion. In *bioinorganic systems*, the mid-IR region of 400–4000 cm–1 is utilized to probe vibrational modes involving both metals and their corresponding ligands. Generally, a change in dipole moment of the system is required to absorb infrared radiation, and the energy of this absorbed radiation corresponds to a unique vibrational mode. Given N atoms, the number of possible vibrational modes of a non-linear molecule is calculated as 3N-6. Protein systems naturally have many atoms, which in turn results in an outstandingly high number of possible vibrational modes. As a result, proteins typically have very broad and intense spectra in energetic regions involving the excitation of amide vibrational modes, particularly below 1700–1800cm^{-1}. Because modes directly involving soft metal–ligand bonds (i.e., Fe–S, Mo–S, etc.) are usually low in energy and intensity, they are invariably obscured by modes involving the surrounding protein. However, IR is still particularly useful in detecting the binding and activation of substrates which produce modes outside of this amide window (>1700 cm–1 in D_2O). More specifically, IR has been applied quite successfully to investigate the binding of small molecules such as CO and CN– in the FeMoco cluster through observation of how the energy of bound C≡O and Ca≡N– stretching modes are modulated.

Raman Spectroscopy

Raman spectroscopy provides another method of investigating the vibrational modes of a system. This technique involves the excitation of electrons into a virtual energy state by absorption of an incoming photon, followed by relaxation back to various vibrational sublevels of the ground state accompanied by the emission of a photon in an inelastic relaxation process. The resulting energy difference between the incident photon and observed emitted photons corresponds to the energy of these vibrational modes. The ability to observe such vibrational modes is dependent on whether or not the interaction of the external electric field (from an incident photon source) with the electron cloud of the sample is capable of inducing an instantaneous dipole moment. Hence, the Raman effect results in a different set of selection rules than IR, requiring that a change in polarizability occurs with the vibration of the molecule. For an example of the Raman vs IR active modes in CO_2, as well as examples of Raman spectroscopy applied in bioinorganic chemistry. Resonance Raman (RR) spectroscopy is a particularly powerful derivative of Raman spectroscopy in bioinorganic chemistry. Standard Raman spectroscopy uses a

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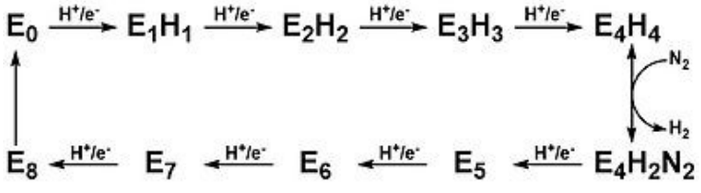
non-selective incident photon energy to excite the system into a virtual excited state.

Magnetic Circular Dichroism (MCD)

Magnetic Circular Dichroism (MCD) spectroscopy also measures the difference between left and right circularly polarized light (referred to as LCP and RCP). However, MCD additionally has an applied magnetic field in parallel to the path of light, resulting in a *Faraday effect* which induces optical activity in all substances rather than just those with chirality. While UV–vis/nIR spectroscopy requires an electronic transition to have a non-zero *electronic dipole moment* in at least one direction (x, y, or z) to gain intensity, MCD spectroscopy requires that a transition is non-zero in two orthogonal directions. The resulting selection rules for MCD are $\Delta m_l = +1$ for LCP and $\Delta m_l = +1$ for RCP light, while $\Delta ms = 0$. An important consequence of these selection rules is that some form of degeneracy in angular momentum, whether orbital or spin in origin, is necessary to gain MCD intensity. In low symmetry systems, orbital angular momentum is quenched. In these cases, only systems with spin degeneracy are able to make a significant contribution to the spectrum. This has made MCD particularly powerful in selectively providing insight into the electronic and geometric structural properties in transition metal complexes and metalloenzymes.

Lowe-Thorneley Kinetic Model

The *reduction of nitrogen* to two molecules of ammonia is carried out at the FeMo-co of Component I after the sequential addition of proton and electron equivalents from Component II. Steady state, freeze quench, and stopped-flow kinetics measurements carried out in the 70's and 80's by Lowe, Thorneley, and others provided a kinetic basis for this process. The Lowe-Thorneley (LT) kinetic model was developed from these experiments and documents the eight correlated proton and electron transfers required throughout the reaction. Each intermediate stage is depicted as E_n where n = 0-8, corresponding to the number of equivalents transferred. The transfer of four equivalents are required before the productive addition of N_2, although reaction of E_3 with N_2 is also possible. Particularly, nitrogen reduction has been shown to require 8 equivalents of protons and electrons as opposed to the 6 equivalents predicted by the balanced chemical reaction.



Intermediates E_0 Through E_4

Spectroscopic characterization of these intermediates has allowed for greater understanding of nitrogen reduction by nitrogenase, however, the mechanism remains an active area of research and debate. Briefly listed below are spectroscopic experiments for the intermediates before the addition of nitrogen:

- **E_0 –** This is the resting state of the enzyme before catalysis begins. EPR characterization shows that this species has a spin of 3/2.

- **E_1** – The one electron reduced intermediate has been trapped during turnover under N_2. *Mçssbauer spectroscopy* of the trapped intermediate indicates that the FeMo-co is integer spin greater than 1.
- **E2 –** This intermediate is proposed to contain the metal cluster in its resting oxidation state with the two added electrons stored in a bridging hydride and the additional proton bonded to a sulfur atom. Isolation of this intermediate in mutated enzymes shows that the FeMo-co is high spin and has a spin of 3/2.[19]
- **E_3** – This intermediate is proposed to be the singly reduced FeMo-co with one bridging hydride and one proton.
- **E_4** – Termed the Janus intermediate after the Roman god of transitions, this intermediate is positioned after exactly half of the electron proton transfers and can either decay back to E_0 or proceed with nitrogen binding and finish the catalytic cycle. This intermediate is proposed to contain the FeMo-co in its resting oxidation state with two bridging hydrides and two sulphur bonded protons. This intermediate was first observed using freeze quench techniques with a mutated protein in which residue 70, a valine amino acid, is replaced with isoleucine. This modification prevents substrate access to the FeMo-co. EPR characterization of this isolated intermediate shows a new species with a spin of ½. ENDOR experiments have provided insight into the structure of this intermediate, revealing the presence of two bridging hydrides.

The above intermediates suggest that the metal cluster is cycled between its original oxidation state and a singly reduced state with additional electrons being stored in hydrides. It has alternatively been proposed that each step involves the formation of a hydride and that the metal cluster actually cycles between the original oxidation state and a singly oxidized state.

2.6.3 Nitrogen Fixation Mechanisms

1. Nitrogenase Types, Structure and Function

The biological conversion of atmospheric nitrogen to ammonia taken place with the help of an enzyme called Nitrogenase. This enzyme is anaerobic in nature and when it comes in contact with oxygen or air, it becomes inert. The proteinaceous enzyme is made up of 2 subunits mainly called larger MoFe protein (2,20,000 dalton mol. wt) and another smaller Fe protein (55000 dalton mol. wt.). Postgate, a British microbiologist named them as Kp_1 and Kp_2. As stated it contains iron and molbydenum atoms. They need Mg^{2+} ions for activation and can convert ATP to ADP during functioning. It is inhibited by ADP and also reduces several other substrates with triple bonded molecules (similar to N≡N). The enzyme can reduce hydrogen ions to gaseous hydrogen, even when N_2 is present, and also have an ability to reduce acetylene to ethylene.

The nitrogenase purified from three species of bacteria showed its following nature as given below. This indicates that nature of enzyme varies in different nitrogen fixing organisms as far as the size of proteins (in both MoFe and Fe components) is concerned.

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(*i*) The Cp Type: According to Postgate, the properties of the nitrogenase are the Cp type (*Clostridium pasteurianum*): It has MoFe (Cp_1) and Fe (Cp_2) proteins which have 2,20000 dalton and 55,000 dalton molecular weight, respectively. The half-life of enzyme is quite short.

(*ii*) The Kp Type (*Klebsiella pneumoniae*): It has MoFe (Kp_1) and Fe (Kp_2) which have 2,18000 daltons and 66,700 dalton molecular weight respectively.

(*iii*) The Ac Type (*Azotobacter chroococcum*): It has MoFe (Ac_1) and Fe (Ac_2) which have 2,27000 and 64,000 dalton molecular weight respectively.

It is observed that the half-life of enzyme of all the three types of Fe units are much shorter than that of FeMo units.

2. Alternative Nitrogenase

Professor P. Bishop and his colleagues in USA obtained evidence that *Azotobacter vinelandii* has a different kind of nitrogenase. The genetic evidences revealed that their normal genes for nitrogenase (*nif* YKDH) deleted. This nitrogenase was later isolated from *A. chroococcum*. It consists of two proteins, one large and heteromeric, one smaller very like the regular Fe-protein; both are sensitive to O_2. The enzyme evolves one molecule of H_2 and reduces acetylene. The larger protein subunit part of enzyme contains *vanadium* in place of Mo ion of the conventional system in this 'new' nitrogenase. V-nitrogenase repressed by Mo suggests that it provides *Azotobacter* with a physiological 'back-up' nitrogen-fixing system for use in case of lack of Mo.

3. Substrates for Nitrogenase

For enzyme activity a suitable substrate is required so as to bind all the active sites of enzyme to get a product. The overall reaction in the enzymic reduction of atmospheric nitrogen to ammonia could be postulated as follow:

$$\underset{\text{Nitrogen}}{N_2\ (N{\equiv}N)} \rightarrow \underset{\text{Di-imide}}{HN{=}NH} \rightarrow \underset{\text{Hydrazine}}{H_2N{-}NH_2} \rightarrow \underset{\text{Ammonia}}{H_3N + NH_3}$$

It is interesting to note that cell free extract of *Azotobacter* and *Clostridium* converted nitrogen in the same way as the free-living bacterial cells. This finally led to the initial isolation and purification of the enzyme from *C. pasteurianum* and *A.chroococcum*. Further, the enzyme was responsible for the adsorption and reduction of N_2 gas.

Although there are several substrates of nitrogenase but except H_2 most of the substrates are nonphysiological substrates because the inhibitors are actually reduced by nitrogenase. Besides this, acetylene (HC=CH) is an important and one of the reliable substrates for measuring the enzyme activity by 'acetylene-reduction' test. It is also important to note that most of the substrates have triple bond in their molecules similar to nitrogen (N≡N).

Following are the substrates reactive to nitrogenase:

Substrate	**Products(s)**
N_2	NH_3
N_2O	H_2O, N_2
N_3	NH_3, N_2

C_2H_2	C_2H_4
HCN	CH_4, NH_3, CH_3, NH_2
CH_3CN	C_2H_6, NH_3

In the reduction of acetylene, the product formed is ethylene i.e. $HC{=}CH \rightarrow H_2C{-}CH_2$ which requires two electrons, whereas reduction of nitrogen to ammonia requires six electrons. Nitrogen is readily reduced in comparison to rest of the substrates.

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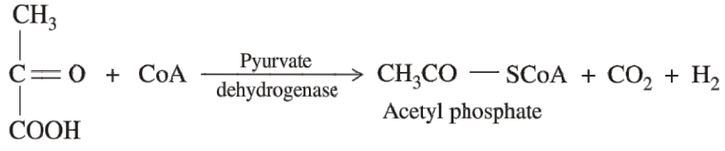
If there is N_2 fixation, simultaneously H_2 is also produced by some of the nitrogen fixing-microorganisms. N_2 fixation is correlated with each other. Nitrogenase reduced the H^+ ion and formed H-D in the presence of deuterium. The H-D reaction suggests the involvement of a bound di-imide intermediate in N_2 fixation.

In view of H_2 evolution during nitrogen fixation, the following reaction has been suggested:

$$N_2 + 8\ H^+ + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

The energy for nitrogenase reaction comes from the cellular metabolic cycles in the form of ATP. This is met out by photophosphorylation, oxidative phosphorylation or phosphoroclastic dissimilation. In later process, keto-acid is dissimilated to acetyl phosphate,CO_2 and H_2.



Pyruvate functions both as electron donor and as energy source. In the phosphoroclastic reactions, pyruvate forms acetyl phosphate which in the presence of ADP gives rise to ATP. The reductants are the naturally occurring electron carrier proteins called *ferredoxin* and *flavodoxin*. Dithionite ($Na_2S_2O_4$) and certain dyes such as methyl viologen and benzyl viologen can also serve as artificial extracellular sources of electron donors. This enzyme system catalyzes the transfer of electrons from pyruvate or hydrogen to ferredoxin or flavodoxin.

(*a*) Ferredoxins or dinitrogenase: Ferredoxins are electron carrier, discovered by Mortenson and Caruahan in the year 1962 from *C. pasteurianum*. It is naturally occurring e^- carrier iron-sulphur (Fe-S) protein (reversible). It has now been isolated from number of cyanobacteria, photosynthetic bacteria and even from higher plants. The ferredoxins are involved in various physiological processes such as photosynthesis in plants and pyruvate metabolism in anaerobic bacteria. The ferredoxin involved in N_2 fixation contains one cluster of four iron and four sulphur atoms in the molecule. Many similar iron-sulphur clusters are the part of iron atoms of nitrogenase. The whole cluster behaves as oxido-reductive unit. The electron paramagnetic resonance indicates that the ferredoxins of aerobic nitrogen fixing bacteria such as *Azotobacter* behave slightly differently from those of anaerobes such as *Clostridium pasteurianum*. In *C. pasteurianum*, ferredoxin is the actual protein which reacts with nitrogenase and provides the reducing power for the conversion of N_2 to NH_3.

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(*b*) Flavodoxins or dinitrogen reductase: The bacteria grow under limited iron supply i.e. nutritional stress condition and produce flavodoxin. It was also isolated in the beginning from *C.pasteurianum*. It is interesting that it was found to replace ferredoxin as an electron carrier in a large number of reactions. An electron carrier named 'azotoflavin' has been isolated from *Azotobacter vinelandii* possessing biological activity similar to ferredoxins. In *K.pneumoniae* and *A.chroococcum*, flavodoxins are the primary reductant to nitrogenase. *A. chroococcum* flavodoxin is blue when half-reduced and colourless when fully reduced. The flavodoxin reduced form is quinone and semiquinone form. This form is unusually stable to oxidation by air, which may be why this protein rather than a ferredoxin is particularly suitable to *Azotobacter's* aerobic way of life. Flavodoxins do not contain iron-atoms; their oxido-reducible centre is yellow, fluorescent molecule called a 'flavin'.

Role of Pyruvate and Ferredoxin-Nitrogenase Reaction. The Fig.14.6 shows the active site of the enzyme for substrate reduction. This enzyme is believed to be composed of a Mo-Fe dinuclear site bridged by sulphur having the proper size and electron characteristics to provide Mo-Fe distance of 3.8 Å. This distance is specific so as to accomodate various nitrogenase substrates including nitrogen and to exclude others. The first reaction in nitrogen reduction is the formation of a linear complex of nitrogen with the Fe atom of nitrogenase. This is followed by transfer of electrons from Mo which is the end point of the electron activating system resulting in the formation of di-imide which is stabilized by hydrogen bonding from the protein as well as the metal nitrogen bonds. Successive addition of electrons produces hydrazine followed by cleavage of N—N bond to fill two molecules of NH_3. The increase in the N—N bond length during reduction is accompanied by compensating changes in the MNN angles so that Mo—Fe distance remains constant.

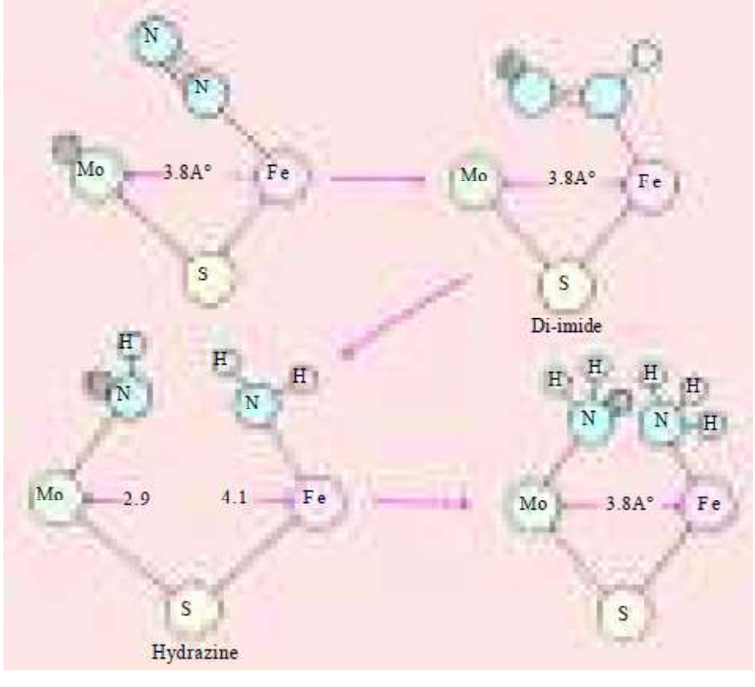

Fig. 2.10 *Proposed Intermediates and Dinuclear Active Sites for N_2 Reduction by Nitrogenase*

Check Your Progress

9. What is cycling of ATP?
10. What is storage of glycogen?
11. What do you understand by electron transfer reactions?
12. What is Metalloprotein and its function?
13. Define the term nitrogenase.

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2.7 ANSWERS TO 'CHECK YOUR PROGRESS'

1. Bioenergetics is a field in biochemistry and cell biology that concerns energy flow through living systems. This is an active area of biological research that includes the study of the transformation of energy in living organisms and the study of thousands of different cellular processes such as cellular respiration and the many other metabolic and enzymatic processes that lead to production and utilization of energy in forms such as adenosine triphosphate (ATP) molecules.
2. The total heat content of a system at constant pressure is equivalent to the internal energy (E) plus the PV (pressure volume product) energy. This is called as enthalpy and is represented by the symbol H. Thus, enthalpy may be defined by the equation, $H = E + PV$.
3. An exergonic process is one which there is a positive flow of energy from the system to the surroundings. This is in contrast with an endergonic process. Constant pressure, constant temperature reactions are exergonic if and only if the Gibbs free energy change is negative ($\Delta G < 0$).These reactions occur spontaneously.
4. An endergonic reaction is a reaction in which energy is absorbed. In chemistry terms, this means that the net change in free energy is positive – there is more energy in the system at the end of the reaction than at the beginning of it. Because endergonic reactions involve a gain in energy, that energy has to be supplied from an outside source in order for the reaction to occur.
5. In general, metabolic reactions that involve creating chemical bonds are called 'Anabolic Reactions'. Metabolic reactions that involve breaking bonds to release energy are called 'Catabolic Reaction'. It is this movement of energy through chemical bonds which allows life to exist.
6. Diffraction methods are used for the identification and also for the quantification of constituents of crystalline structures. Diffraction patterns are obtained with an object with crystalline structure is irradiated by X-ray photons, or by other particles (electrons, neutrons, etc.).
7. Protein X-ray crystallography is a technique used to obtain the three-dimensional structure of a particular protein by x-ray diffraction of its crystallized form. This three dimensional structure is crucial to determining a protein's functionality.

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8. Dynamic Light Scattering DLS, also called Photon Correlation Spectroscopy (PCS), is a light-scattering technique used to study the average particle size of NPs based on the laser diffraction method. This technique uses the scattering of light from colloidal particles suspended in a liquid medium.
9. The process of phosphorylating ADP to form ATP and removing a phosphate from ATP to form ADP in order to store and release energy respectively is known as the ATP cycle. ATP stands for Adenosine TriPhosphate.
10. Glycogen is stored in the liver. When the body needs more energy, certain proteins called enzymes break down glycogen into glucose. They send the glucose out into the body.
11. Electron transfer reactions involve the movement of an electron from one molecular species (the donor) to another (the acceptor) and turn out to be an essential quantum mechanical component in various biological processes.
12. Metalloproteins account for nearly half of all proteins in biology. Protein metal-binding sites are responsible for catalyzing some of the most difficult and yet important functions, including photosynthesis, respiration, water oxidation, molecular oxygen reduction, and nitrogen fixation.
13. Nitrogenase, is an enzyme that is produced by certain types of bacteria and is vital to their existence and growth. Nitrogenase is a unique enzyme with a crucial function that is distinct to bacteria that utilize it, has unique structure and symmetry, and is sensitive to other compounds that inhibits its functioning. It is 'an enzymatic complex which enables fixation of atmospheric nitrogen'.

2.8 SUMMARY

- Bioenergetics is the part of biochemistry concerned with the energy involved in making and breaking of chemical bonds in the molecules found in biological organisms. It can also be defined as the study of energy relationships and energy transformations and transductions in living organisms.
- In a living organism, chemical bonds are broken and made as part of the exchange and transformation of energy. Energy is available for work (such as mechanical work) or for other processes (such as chemical synthesis and anabolic processes in growth), when weak bonds are broken and stronger bonds are made. The production of stronger bonds allows release of usable energy.
- Living organisms produce ATP from energy sources via oxidative phosphorylation. The terminal phosphate bonds of ATP are relatively weak compared with the stronger bonds formed when ATP is hydrolyzed (broken down by water) to adenosine diphosphate and inorganic phosphate.
- Environmental materials that an organism intakes are generally combined with oxygen to release energy, although some can also be oxidized anaerobically by various organisms.
- The first law of thermodynamics states that 'the total energy of a system plus its surroundings remains constant'. This is also the laws of conservation

of energy. Energy may be transferred from one part to another or may be transformed into an-other form of energy.

- In simpler words, enthalpy is the total heat content of a system. It reflects the number and kinds of chemical bonds in the reactants and products. Like internal energy, enthalpy is also a function of state and therefore, it is not possible to quantify the absolute enthalpy.
- An exergonic reaction is a reaction that releases free energy. Because this type of reaction releases energy rather than consuming it, it can occur spontaneously, without being forced by outside factors. In chemistry terms, exergonic reactions are reactions where the change in free energy is negative.
- Protein synthesis is a more typical example of how living things move energy, and add it to reactions to allow new chemical bonds to form. During protein synthesis, a variety of enzymes and ribozymes work together to complete the steps necessary to add an amino acid to a growing protein. In all, about five ATP must be consumed to add a single amino acid to a growing protein.
- Diffraction methods are used for the identification and also for the quantification of constituents of crystalline structures. Diffraction patterns are obtained with an object with crystalline structure is irradiated by X-ray photons, or by other particles (electrons, neutrons, etc.).
- X-Ray Diffraction (XRD) is a tool for characterizing the arrangement of atoms in crystals and the distances between crystal faces. The method determined the size of atoms, the lengths and types of chemical bonds, and the atomic-scale differences among various materials, especially minerals and alloys.
- Non-invasive light scattering methods provide data on biological macromolecules (i.e., proteins, nucleic acids, as well as assemblies and larger entities composed of them) that are complementary with those of size exclusion chromatography, gel electrophoresis, analytical ultracentrifugation and mass spectrometry methods.
- Small-Angle X-Ray Scattering (SAXS) is a small-angle scattering technique by which nanoscale density differences in a sample can be quantified. This means that it can determine nanoparticle size distributions, resolve the size and shape of (monodisperse) macromolecules, determine pore sizes, characteristic distances of partially ordered materials, and much more.
- SAXS is used for the determination of the microscale or nanoscale structure of particle systems in terms of such parameters as averaged particle sizes, shapes, distribution, and surface-to-volume ratio.
- Many biological reactions are known to involve metal ions. There are also metals recognized as essential elements, although their roles in living organisms are not clear. Bioinorganic chemistry, the study of the functions of metals in biological systems using the knowledge and methods of inorganic chemistry.
- Glucose is the main source of fuel for our cells. When the body doesn't need to use the glucose for energy, it stores it in the liver and muscles. This stored

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form of glucose is made up of many connected glucose molecules and is called glycogen.

- Transition metals, such as copper and iron play leading roles in electron transport as one-electron redox-active centres within proteins that are used to effectively move electrons around. Well-known examples are the blue copper proteins (CuI«CuII), cytochromes (FeII«FeIII porphyrins), and iron-sulphur proteins (FeII«FeIII with sulphur ligands).
- Metalloproteins account for nearly half of all proteins in biology. Protein metal-binding sites are responsible for catalyzing some of the most difficult and yet important functions, including photosynthesis, respiration, water oxidation, molecular oxygen reduction, and nitrogen fixation.
- Cytochromes are present mostly in the inner mitochondrial membrane of eukaryotic organisms and are also found in a wide variety of both Gram-positive and Gram-negative bacteria. Cytochromes play crucial roles in a number of biological ET processes associated with many different energy metabolisms.
- As a variety of spectroscopic techniques have played key roles in helping to define the geometric and electronic properties of the cofactors, Many of these methods are also readily complemented by theory, and we briefly introduce the application of computational chemistry in N2ase research with the aim of demonstrating how it has been employed to complement spectroscopic studies.
- As a technique, Electron Paramagnetic Resonance (EPR) spectroscopy can be applied to study paramagnetic centres possessing one or more unpaired electrons. To investigate paramagnetic centres with half-integer, Kramers spin states (S = 1/2, 3/2, etc.), the more routine (and most commonly available) perpendicular-mode EPR spectrometer may be used.
- Raman spectroscopy provides another method of investigating the vibrational modes of a system. This technique involves the excitation of electrons into a virtual energy state by absorption of an incoming photon, followed by relaxation back to various vibrational sublevels of the ground state accompanied by the emission of a photon in an inelastic relaxation process

2.9 KEY TERMS

- **Bioenergetics:** Bioenergetics is a field in biochemistry and cell biology that concerns energy flow through living systems.
- **Entropy:** Entropy represents the extent of disorder of the system and becomes maximum when it approaches true equilibrium. Under constant temperature and pressure, the relationship between the free energy change (ΔG) and the change in entropy (ΔS) is given by the following equation which combines the two laws of thermodynamics.
- **Exergonic process:** An exergonic process is one which there is a positive flow of energy from the system to the surroundings. This is in contrast with an endergonic process. Constant pressure, constant temperature reactions

are exergonic if and only if the Gibbs free energy change is negative ("G < 0).These reactions occur spontaneously.

- **Metalloproteins:** Metalloproteins account for nearly half of all proteins in biology. Protein metal-binding sites are responsible for catalyzing some of the most difficult and yet important functions, including photosynthesis, respiration, water oxidation, molecular oxygen reduction, and nitrogen fixation.
- **Iron–sulphur proteins:** Iron–sulphur proteins (or iron–sulphur proteins in British spelling) are proteins characterized by the presence of iron–sulphur clusters containing sulphide-linked di-, tri-, and tetra iron centres in variable oxidation states.
- **Nitrogenase:** Nitrogenase, is one enzyme that is produced by certain types of bacteria and is vital to their existence and growth. Nitrogenase is a unique enzyme with a crucial function that is distinct to bacteria that utilize it, has unique structure and symmetry, and is sensitive to other compounds that inhibits its functioning.

2.10 SELF-ASSESSMENT QUESTIONS AND EXCERCISES

Short–Answer Questions

1. Define the term bioenergetics
2. What is entropy?
3. Differentiate between exergonic and endergonic process.
4. Define the hydrolysis of ATP.
5. Give the statement of light scattering.
6. Determine the law angel X-ray scattering.
7. Define ORD.
8. What is DNA polymerization?
9. What do you understand by PS I and II?
10. Define the electron transfer in biology.
11. What is iron-sulphur protein?
12. What do you understand by nitrogen fixation?

Long –Answer Questions

1. Discuss about bioenergetics and its importance with appropriate examples.
2. Analyse the exergonic and endergonic process with the help of relevant examples.
3. Elaborate on the hydrolysis of ATP and give the synthesis of ATP from ADP.
4. Analyse the different types of diffraction method with the help of examples.
5. Illustrate the ATP cycle and DNA polymerization.

6. Describe the photosystem I and II with the help of appropriate examples.
7. Give the structure and function of metalloprotein.
8. Explain in detail about the nitrogenase and biological nitrogen fixation.

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2.11 FURTHER READING

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UNIT 3 BIOPOLYMERS

NOTES

Structure

3.0 Introduction
3.1 Objectives
3.2 Statistical Mechanics of Biopolymers
  3.2.1 Chain Configuration of Macromolecules
  3.2.2 Statistical Distribution End to End Dimensions
  3.2.3 Calculation of Average Dimensions of Various Chain Structures
  3.2.4 Peptides
  3.2.5 Protein Folding
3.3 Biopolymer Interactions
  3.3.1 Multiple Equilibria and Binding Process in Biological System
  3.3.2 Hydrogen Ion Titration Curve
3.4 Thermodynamics of Polymer Solutions
  3.4.1 Energy Generation of Mechanical System
  3.4.2 Osmotic Pressure
  3.4.3 Membrane Equilibria and Muscular Contraction
3.5 Biopolymers and their Molecular Weight
  3.5.1 Electrophoresis
3.6 Answers to 'Check Your Progress'
3.7 Summary
3.8 Key Terms
3.9 Self-Assessment Questions and Exercises
3.10 Further Reading

3.0 INTRODUCTION

Biopolymers are polymers produced from natural sources either chemically synthesized from a biological material or entirely biosynthesised by living organisms. The use of biopolymers from different sources has been investigated for many years for pharmaceutical and biomedical applications. The most common macromolecules in biochemistry are biopolymers (nucleic acids, proteins, and carbohydrates) and large non-polymeric molecules such as lipids, nanogels and macrocycles. Synthetic fibers and experimental materials such as carbon nanotubes are also examples of macromolecules.

Protein folding is the physical process by which a protein chain is translated to its native three-dimensional structure, typically a 'Folded' conformation by which the protein becomes biologically functional. Via an expeditious and reproducible process, a polypeptide folds into its characteristic three-dimensional structure from a random coil.

Biopolymer interactions are the basis of all physical processes in living organisms. More precisely, almost all biochemical reactions in living cells are based on molecular interactions between proteins, nucleic acids, sugars, lipids, and low molecular-weight ligands. Several systems we encounter consist of multiple equilibria, systems where two or more equilibria processes are occurring simultaneously. Some common examples include acid rain, fluoridation, and dissolution of carbon dioxide in sea water. Polymer-binding agents are critical for

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the good performance of the electrodes of batteries during cycling as they hold the electroactive material together forming a cohesive assembly by means of mechanical and chemical stability as well as adhesion to the current collector.

Hydrogen-ion titration experiments yield the relative net charge of a protein as a function of pH. To determine absolute net charge, it is necessary to know one charge/pH point exactly. An experimental determination of the pH point at which the protein has zero net charge (the isoelectric point, PI) can be made by IsoElectric Focusing (IEF).

Thermodynamic of biopolymer solution is based upon the quasilattice theory of solutions, and leads to an equation for the free energy of mixing polymeric solute with monomeric solvent which contains a combinatorial entropy of mixing term and a regular solution theory enthalpy of mixing term. The force-developing mechanism is thought to be the transfer of energy from ATP splitting in the globular head of one molecule directly to the hinge region of an adjoining molecule, resulting in a phase transition from crystalline to amorphous within the hinge segment of the second molecule.

An equilibrium state in a system with two solutions of the same solvent and different solute compositions, separated by a membrane permeable only to the solvent, is called an osmotic membrane equilibrium. Muscle contraction is the tightening, shortening, or lengthening of muscles when you do some activity. It can happen when you hold or pick up something, or when you stretch or exercise with weights. Muscle contraction is often followed by muscle relaxation, when contracted muscles return to their normal state.

Molecular weight (M), and Molecular Weight Distribution (MWD), of polymers with emphasis on M and MWD of biopolymers, e.g., carbohydrate polymers, proteins, deoxyribonucleic acid, DNA, and ribonucleic acid, RNA, are reviewed. The M and MWD of biopolymers are compared with those of synthetic polymers.

In this unit, you will study about the biopolymers and statistical mechanics of biopolymers, chain configuration of macromolecules, statistical distribution end-to-end dimension, calculation of average dimension for various chain structure, protein folding problem, biopolymers interactions and force involved in biopolymer interactions, multiple equilibria and binding process, hydrogen ion titration curve, thermodynamic of biopolymer solution, energy generation in mechanochemical system , membrane equilibria and muscular contraction, determination of molecular weight by various types of methods.

3.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the statistical mechanics of biopolymers
- Explain the chain configuration of macromolecules
- Determine the statistical distribution end-to-end dimension
- Calculate average dimension for various chain structure

- Know about the protein folding problem
- Comprehend the biopolymers interactions and force involved in biopolymer interactions
- Elaborate on the multiple equilibria and binding process of biopolymers
- Illustrate the hydrogen ion titration curve
- Describe the thermodynamic of biopolymer solution
- Determine the energy generation of mechanical system
- Know about the membrane equilibria and muscular contraction
- Understand the of molecular weight by various types of methods.

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3.2 STATISTICAL MECHANICS OF BIOPOLYMERS

Biopolymers are natural polymers produced by the cells of living organisms. Biopolymers consist of *monomeric units* that are covalently bonded to form larger molecules. There are three main classes of biopolymers, classified according to the monomers used and the structure of the biopolymer formed: polynucleotides, polypeptides, and polysaccharides. Polynucleotides, such as DeoxyriboNucleic Acid (DNA) and RiboNucleic Acid (RNA), are long polymers composed of 13 or more *nucleotide monomers*. Polypeptides and proteins, are polymers of amino acids and some major examples include collagen, actin, and fibrin. Polysaccharides are linear or branched polymeric carbohydrates and examples include starch, cellulose and alginate. Other examples of biopolymers include natural rubbers (polymers of isoprene), suberin and lignin (complex polyphenolic polymers), cutin and cutan (complex polymers of long-chain fatty acids) and melanin. Biopolymers have applications in many fields including the food industry, manufacturing, packaging, and biomedical engineering.

Types of Biopolymers

Polypeptides: The convention for a polypeptide is to list its constituent amino acid residues as they occur from the amino terminus to the carboxylic acid terminus. The amino acid residues are always joined by peptide bonds. Protein, though used colloquially to refer to any polypeptide, refers to larger or fully functional forms and can consist of several polypeptide chains as well as single chains. Proteins can also be modified to include non-peptide components, such as *saccharide chains* and *lipids*.

Nucleic Acids: The convention for a nucleic acid sequence is to list the nucleotides as they occur from the 5' end to the 3' end of the polymer chain, where 5' and 3' refer to the numbering of carbons around the ribose ring which participate in forming the phosphate diester linkages of the chain. Such a sequence is called the primary structure of the biopolymer.

Sugar: Sugar polymers can be linear or branched and are typically joined with *glycosidic bonds*. The exact placement of the linkage can vary, and the orientation of the linking functional groups is also important, resulting in α- and *β-glycosidic*

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bonds with numbering definitive of the linking carbons' location in the ring. In addition, many saccharide units can undergo various chemical modifications, such as amination, and can even form parts of other molecules, such as glycoproteins.

Statistical Mechanics

Figure 3.1 particle floating in fluid. The particle will undergo *Brownian motion*, or *random movements* as it is randomly pushed around by the molecules around it. The motion of the particle depends on the temperature: if the temperature is increased, the motion of the particle will also be increased. Like Brownian motion, polymers are subject to random molecular forces that cause them to 'Wiggle'. These wiggles are called *thermal fluctuations*. As temperature is increased, the polymers wiggle more. Figure 3.1 show that the schematic depicting Brownian motion of a particle in fluid and thermal fluctuations of a polymer. Both are induced by random forces induced by the surrounding molecules.

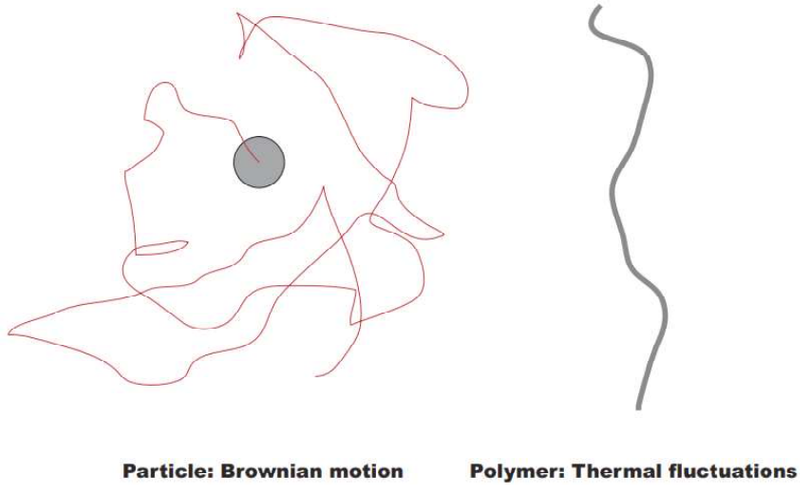

Fig. 3.1 *Schematic Depicting Brownian Motion of a Particle in Fluid and Thermal Fluctuations of a Polymer*

The conformation of a polymer is determined by its *free energy*. Specifically, polymers want to minimize their free energy. As we have seen already, the free energy is defined as Equation 1 are following:

$$\Psi = W - TS \quad (1)$$

W is referred to as the strain energy, or simply as the energy. T is temperature, and S is the entropy. We can see from Equation 1 that a polymer can lower its free energy by lowering its energy W, or increasing its entropy S. Let's look at each of these quantities individually.

Entropy

Within statistical mechanics, entropy has a precise mathematical meaning. Specifically,

$$S = k \ln \Omega \quad (2)$$

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Here, **k** is the *Boltzmann constant* and is equal to 1.38×10^{-23} J/K. **Ω** is defined as the number of microstates for a given macrostate corresponding to some macroscopic quantity. i.e., consider a model polymer consisting of *n* rigid links of size b. The links are connected to each other by freely rotating hinges. The links can be oriented only vertically or horizontally. In this model, we define two lengths. The first length is the contour length L, which is the length of the polymer if it was completely stretched out. In this case, since there are *n* links of size b, then L = *n*b. The second length is the end-to-end length *R*. This is simply the length from one end of the polymer to the other. In this case, *R* must always be less than or equal to *L*. Remember that **Ω** is defined as the number of microstates for a given macroscopic quantity. For example, the different microstates are the different polymer configurations, and the macroscopic quantity of interest is the **end-to-end length R**. Thus, finding **Ω** boils down to counting the number of ways in which our polymer can have a particular end-to-end length. Suppose the polymer is completely stretched out. The polymer will have an end to-end length equal to the contour length, or $\boldsymbol{R = L}$. In this case, there is only one polymer conformation in which this can occur, and so $\boldsymbol{\Omega(R = L) = 1}$. Now suppose that the polymer is not completely stretched out, or $\boldsymbol{R < L}$. In this case, there are multiple polymer conformations that have the same end-to-end length. Thus, $\boldsymbol{\Omega(R < L) > 1}$. The entropy is proportional to ln Ω, so the higher the value of **Ω**, the higher the entropy. Therefore, the unstretched polymer has higher entropy than the stretched out polymer. To decrease its free energy, the polymer wants to increase its entropy, and so it wants to be kinked. Thus, we say that entropically, the polymer wants to be kinked (i.e., it does not want to be stretched out).Figure 3.2 shown that the model polymer with rigid links of size *b*. The links are connected to each other by freely rotating hinges. Other hand Figure 3.3 if $\boldsymbol{R = L}$, there is only one configuration in which this is possible. In contrast, if $\boldsymbol{R < L}$, there are multiple configurations in which this is possible.

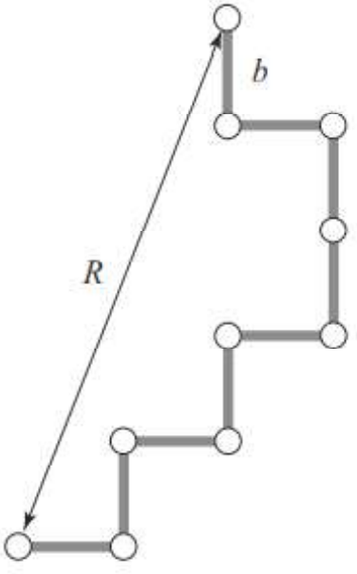

Fig. 3.2 *Model Polymer with Rigid Links of Size* b

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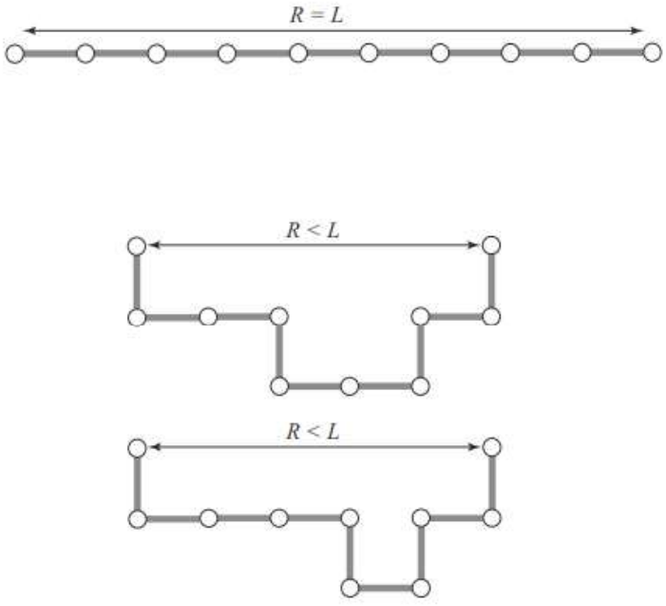

Fig. 3.3 *If R = L, there is Only One Configuration in which this is Possible*

3.2.1 Chain Configuration of Macromolecules

Macromolecule: A macromolecule is a very large molecule, such as a protein. They are composed of thousands of covalently bonded atoms. Many macromolecules are polymers of smaller molecules called *monomers*. The most common macromolecules in biochemistry are biopolymers (nucleic acids, proteins, and carbohydrates) and large non-polymeric molecules, such as lipids, nanogels and macrocycles. Synthetic fibers and experimental materials, such as carbon nanotubes are also examples of macromolecules.

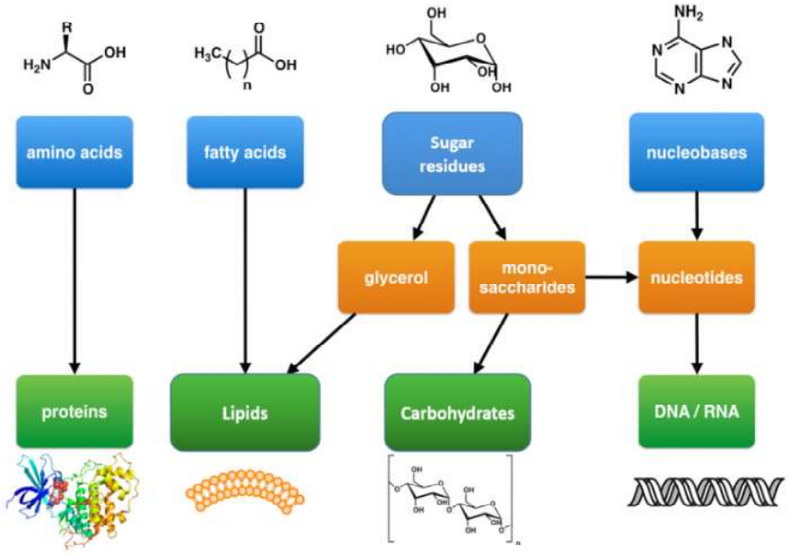

Fig 3.4 *The Molecular Building Blocks of Life are made from Organic Compounds.*

Protein Structure and Function

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Amino Acids and Primary Protein Structure: The major building block of proteins are called *alpha amino acids*. As their name implies they contain a carboxylic acid functional group and an *amine functional group*. The alpha designation is used to indicate that these two functional groups are separated from one another by one carbon group. In addition to the amine and the carboxylic acid, the alpha carbon is also attached to a hydrogen and one additional group that can vary in size and length. In the Figure 3.5 are below, this group is designated as an R-group. Within living organisms there are 20 amino acids used as protein building blocks. They differ from one another only at the R-group position. The basic structure of an amino acid is shown below Figure 3.5:

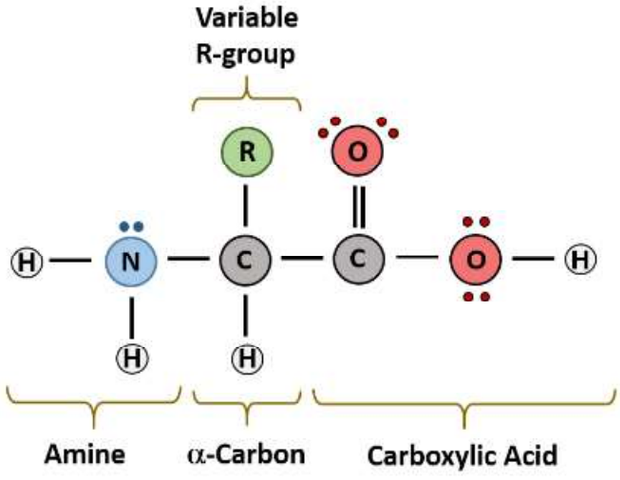

Fig. 3.5 *General Structure of an Alpha Amino Acid*

Within cellular systems, proteins are linked together by a complex system of RNA and proteins called the ribosome. Thus, as the amino acids are linked together to form a specific protein, they are placed within a very specific order that is dictated by the genetic information contained within the RNA. This specific ordering of amino acids is known as the *protein's primary sequence*. The primary sequence of a protein is linked together using dehydration synthesis that combine the carboxylic acid of the upstream amino acid with the amine functional group of the downstream amino acid to form an amide linkage. Within protein structures, this amide linkage is known as the peptide bond. Subsequent amino acids will be added onto the carboxylic acid terminal of the growing protein. Thus, proteins are always synthesized in a directional manner starting with the amine and ending with the carboxylic acid tail. New amino acids are always added onto the carboxylic acid tail, never onto the amine of the first amino acid in the chain. In addition, because the R-groups can be quite bulky, they usually alternate on either side of the growing protein chain in the trans conformation. The cis conformation is only preferred with one specific amino acid known as ***proline.***

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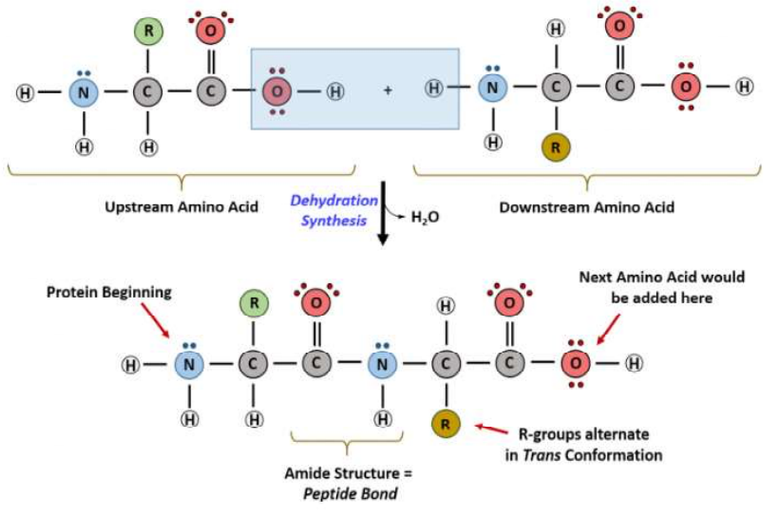


Fig. 3.6 *Formation of the Peptide Bond*

Proteins are very large molecules containing many amino acid residues linked together in very specific order. Proteins range in size from 50 amino acids in length to the largest known protein containing 33,423 amino acids. Macromolecules with fewer than 50 amino acids are known as *peptides*. Figure 3.7 also show the peptides and proteins are macromolecules built from long chains of amino acids joined together through amide linkages.

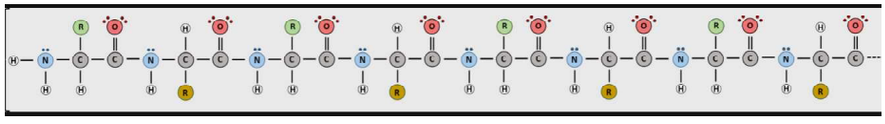
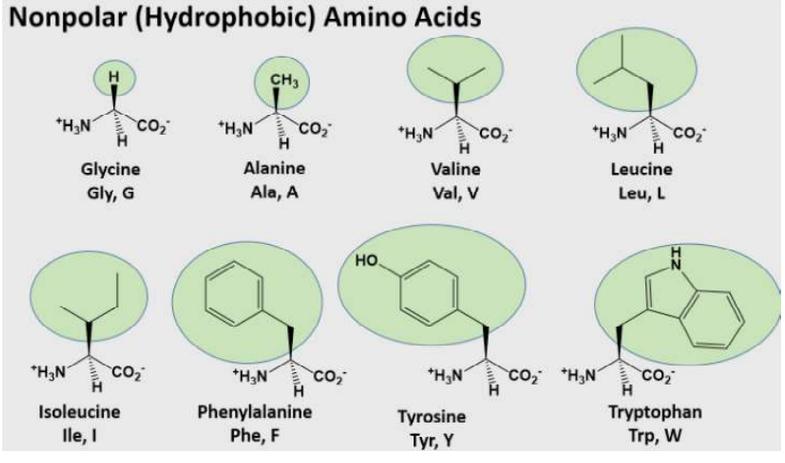


Fig 3.7 *Peptides and Proteins are Macromolecules Built from Long Chains*

The identity and function of a peptide or a protein is determined by the primary sequence of amino acids within its structure. There are a total of 20 alpha amino acids that are commonly incorporated into protein structures (Refer Figure 3.8).



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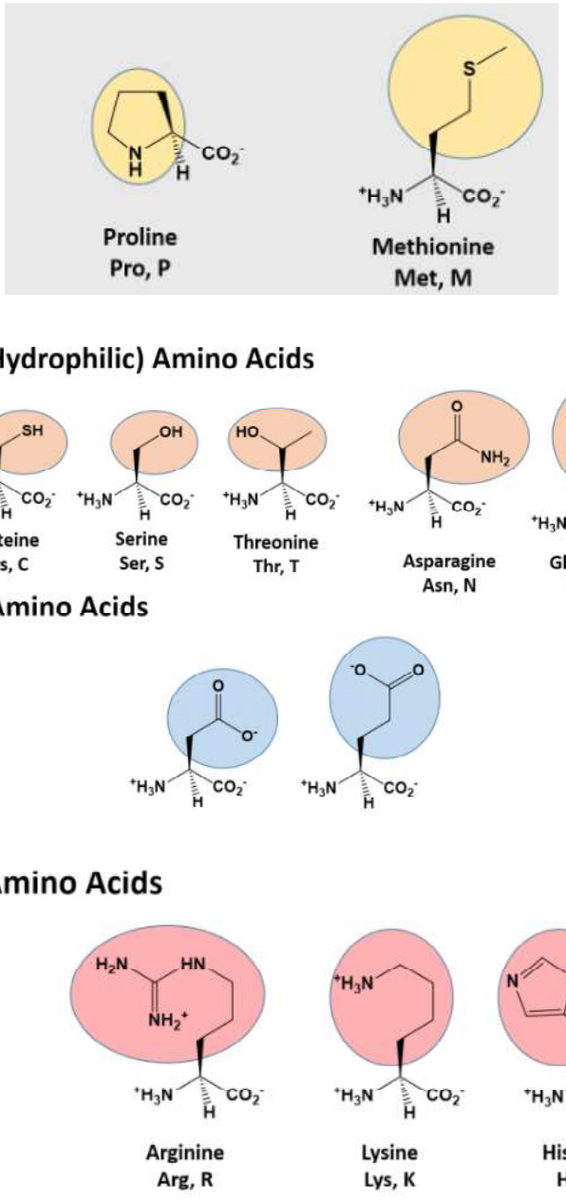

Fig. 3.8 *Structure of the 20 Alpha Amino Acids used in Protein Synthesis*

Within each protein small regions may adopt specific folding patterns. These specific motifs or patterns are called *secondary structure*. Common secondary structural features include alpha helix and beta-pleated sheet (Refer Figure 3.9). Within these structures, intramolecular interactions, especially hydrogen bonding between the backbone amine and carbonyl functional groups are critical to maintain 3-dimensional shape. Every helical turn in an alpha helix has 3.6 amino acid residues. The R groups (the variant groups) of the polypeptide protrude out from the α-helix chain. In the β-pleated sheet, the 'Pleats' are formed by hydrogen bonding between atoms on the backbone of the ***polypeptide chain***. The R groups are attached to the carbons and extend above and below the folds of the pleat. The pleated segments align parallel or antiparallel to each other, and hydrogen bonds form between the partially positive nitrogen atom in the amino group and the partially

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negative oxygen atom in the carbonyl group of the peptide backbone. The α-helix and β-pleated sheet structures are found in most proteins and they play an important structural role. The alpha helix and beta-pleated sheet are common structural motifs found in most proteins. They are held together by hydrogen bonding between the amine and the carbonyl oxygen within the amino acid backbone.

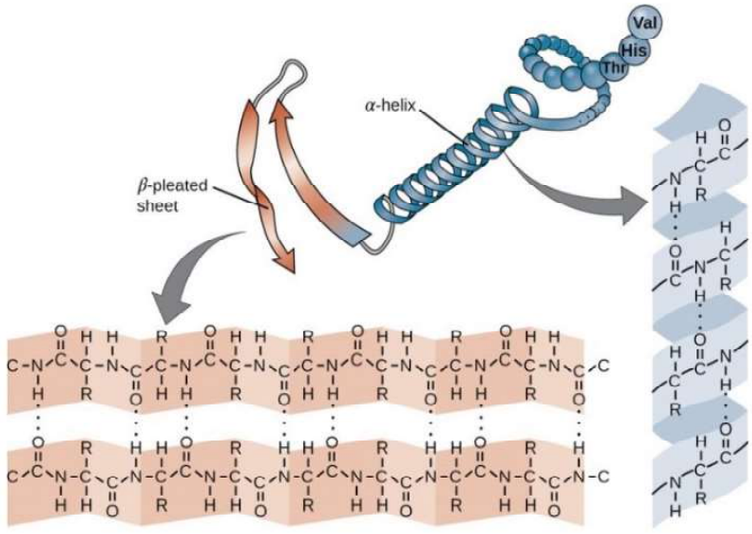

Fig. 3.9 *Secondary Structural Features in Protein Structure*

Hydrolysis is the breakdown of the primary protein sequence by the addition of water to reform the individual amino acids monomer units.

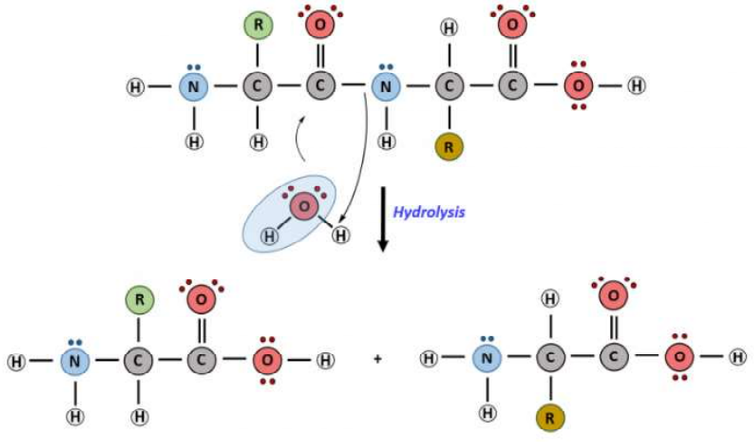

Fig. 3.10 *Hydrolysis of Proteins*

3.2.2 Statistical Distribution End to End Dimensions

We develop expressions for the probability distribution of end-end distances of a ***polymer chain***. We start from a simple random walk in one dimension, and generalize the result to three dimensions. In one dimension, the number of ways of arriving a distance x from the origin after N steps of unit size (n_+ in the positive direction and n_- in the negative) is given by a combinatorial expression.

$$W(N,x) = \frac{N!}{(n_+!)(n_-!)} \quad \textbf{(3)}$$

Where $\mathbf{x = n_+ - n_-}$, $\boldsymbol{N = n_+ + n_-}$ and so $\boldsymbol{n_+ = (N + x)/2}$, and $\mathbf{n- = (N - x)/2.}$ The probability of this occurrence is just the number of ways of realizing the occurrence divided by the total number of possible trajectories.

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$$p(N,x) = \frac{1}{2^N}\frac{N!}{(n_+!)(n_-!)} \quad \textbf{(4)}$$

We consider the natural logarithm of the above probability. Using ***Stirling's approximation***, $N! \cong \sqrt{2\pi N}(N/e)^N$ and assuming that $\boldsymbol{x << N}$ we arrive at the probability distribution function, which is the probability $\boldsymbol{p(N,x)dx}$ that the trajectory of the random walk is terminated within an interval dx from $\boldsymbol{x}$.

$$\begin{aligned} P(N,x) &= \frac{1}{\sqrt{2\pi N}}\exp\left(\frac{-x^2}{2N}\right) \\ \langle x^2\rangle &= \int_{\infty}^{\infty} x^2\, P(N,x)dx = N \\ P(N,x) &= \frac{1}{\sqrt{2\pi\langle x^2\rangle}}\exp\left(\frac{-x^2}{2\langle x^2\rangle}\right) \end{aligned} \quad \textbf{(5)}$$

In three dimensions, the probability $\boldsymbol{P(N,R)}$ is just the product of $\boldsymbol{P(N,Rx)P(N,Ry)P(N,Rz)dxdy\,dz}$ and since $\boldsymbol{< R^2 >= Nb^2}$,

$$P(N,R) = \left(\frac{3}{2\pi Nb^2}\right)^{3/2}\exp\left(\frac{-3R^2}{2Nb^2}\right) \quad \textbf{(6)}$$

So the probability of finding the chain end (terminus of an ideal random walk) in a spherical shell between $\boldsymbol{R}$ and $\boldsymbol{R + dR}$ is $\boldsymbol{P\,(N, R)\ 4\pi R2\,dR}$.

3.2.3 Calculation of Average Dimensions of Various Chain Structures

For any chemical chain reaction, the chain length is defined as the average number of times that the closed cycle of chain propagation steps is repeated. It is equal to the rate of the overall reaction divided by the rate of the initiation step in which the chain carriers are formed.

In *polymer chemistry* the kinetic chain length of a polymer, $\boldsymbol{\nu}$, is the average number of units called *monomers* added to a growing chain during chain-growth polymerization. During this process, a polymer chain is formed when monomers are bonded together to form long chains known as ***polymers***. Kinetic chain length is defined as the average number of monomers that react with an active centre, such as a radical from initiation to termination. This definition is a special case of the concept of ***chain length in chemical kinetics***. For any chemical chain reaction, the chain length is defined as the average number of times that the closed cycle of chain propagation steps is repeated. It is equal to the rate of the overall reaction divided by the rate of the initiation step in which the chain carriers are

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formed. For example, the decomposition of ozone in water is a chain reaction which has been described in terms of its chain length.

In chain-growth *polymerization* the propagation step is the addition of a monomer to the growing chain. The word kinetic is added to chain length in order to distinguish the number of reaction steps in the kinetic chain from the number of monomers in the final macromolecule, a quantity named the degree of polymerization. In fact the kinetic chain length is one factor which influences the average degree of polymerization, but there are other factors as described below. The kinetic chain length and therefore the degree of polymerization can influence certain physical properties of the polymer, including chain mobility, glass-transition temperature, and modulus of elasticity.

Calculating Chain Length

For most chain-growth polymerizations, the propagation steps are much faster than the initiation steps, so that each growing chain is formed in a short time compared to the overall ***polymerization reaction***. During the formation of a single chain, the reactant concentrations and therefore the propagation rate remain effectively constant. Under these conditions, the ratio of the number of propagation steps to the number of initiation steps is just the ratio of reaction rates:

$$\nu = \frac{R_p}{R_i} = \frac{R_p}{R_t}$$

Where $\mathbf{R_p}$ is the rate of propagation, $\mathbf{R_i}$ is the rate of initiation of polymerization, and $\mathbf{R_t}$ is the rate of termination of the polymer chain. The second form of the equation is valid at ***steady-state polymerization***, as the chains are being initiated at the same rate they are being terminated ($\mathbf{R_i = R_t}$).

An exception is the class of living polymerizations, in which propagation is much slower than initiation, and chain termination does not occur until a quenching agent is added. In such reactions the reactant monomer is slowly consumed and the propagation rate varies and is not used to obtain the kinetic chain length. Instead the length at a given time is usually written as:

$$\nu = \frac{[M]_0 - [M]}{[I]_0}$$

Where $\mathbf{[M]_0 - [M]}$ represents the number of monomer units consumed, and $\mathbf{[I]_0}$ the number of radicals that initiate polymerization. When the reaction goes to completion, $\mathbf{[M] = 0}$, and then the kinetic chain length is equal to the number average degree of polymerization of the polymer.

In both cases kinetic chain length is an average quantity, as not all polymer chains in a given reaction are identical in length. The value of $\boldsymbol{\nu}$ depends on the nature and concentration of both the monomer and initiator involved.

Kinetic Chain Length and Degree of Polymerization

In chain-growth polymerization, the degree of polymerization depends not only on the kinetic chain length but also on the type of termination step and the possibility of chain transfer.

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Termination by Disproportionation: Termination by disproportionation occurs when an atom is transferred from one polymer free radical to another. The atom is usually hydrogen, and this results in two polymer chains. With this type of termination and no chain transfer, the number average degree of polymerization **(DP_n)** is then equal to the average kinetic chain length:

$$DP_n = \nu$$

Termination by Combination: Combination simply means that two radicals are joined together, destroying the radical character of each and forming one polymeric chain. With no chain transfer, the average degree of polymerization is then twice the average kinetic chain length:

$$DP_n = 2\nu$$

Chain Transfer: Some chain-growth polymerizations include chain transfer steps, in which another atom (often hydrogen) is transferred from a molecule in the system to the polymer radical. The original polymer chain is terminated and a new one is initiated. The kinetic chain is not terminated if the new radical can add monomer. However the degree of polymerization is reduced without affecting the rate of polymerization (which depends on kinetic chain length), since two (or more) macromolecules are formed instead of one. For the case of termination by disproportionation, the degree of polymerization becomes:

$$DP_n = \frac{R_p}{R_t + R_{tr}} < \nu = \frac{R_p}{R_t}$$

Where R_{tr} is the rate of transfer. The greater R_{tr} is, the shorter the final macromolecule.

3.2.4 Peptides

A **peptide** is a chain of amino acids linked by **peptide bond** or **amide bond**. The role of peptide bond in protein structure was understood by **Hofmeister** (1902). Peptide bond is a special type of amide bond formed between carboxyl (–COOH) group of one amino acid and amino ($–NH_2$) group of next amino acid with the removal of one molecule of water.

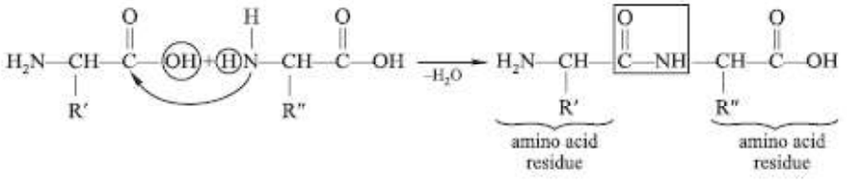

Fig. 3.11 *Formation of a Peptide Bond or Amide Linkage to Join Two Molecules of amino acids.*

A peptide chain may have upto millions of amino acids. Each amino acid in the chain is called a **residue**. Depending on the number of amino acid residues, the peptides are called:

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1. **Dipeptide.** A chain of two amino acids.
2. **Tripeptide.** A chain of three amino acids.
3. **Oligopeptide.** A chain of amino acid upto 10 amino acids.
4. **Polypeptide.** A peptide containing more than 10 amino acids.
5. **Macropeptide.** A polypeptide chain of more than 100 amino acids (Box 7.5).

1. Primary Structure of Peptides

The sequence of amino acids in a polypeptide chain is referred to as its **primary structure**. The two ends of peptide chains are marked, the **N-terminal** on the left and **C-terminal** at the right end of the polypeptide. The N-terminal has amino ($–NH_2$) group of left terminal amino acid and is basic, C-terminal has carboxyl (–COOH) group of the last amino acid and is acidic. These two terminal groups (one basic and one acidic are ionisable).

The primary structure of a polypeptide is very important. The smallest change of even single amino acid in the primary structure often produces remarkable physiological effect. For example, substitution of a single amino acid in the sequence of 100 or more amino acids may change the biological activity having serious consequences.

2. Biological Importance of Peptides

1. Peptides are intermediates in the formation of proteins.
2. Certain peptides serve as growth factors, *e.g.*, folic acid. **Streptogenins** is another peptide which serves as a growth factor for microorganisms.
3. Certain peptides act as hormones, *e.g.*, oxytocin, vasopressin, prolactin, etc.
4. Certain peptides such as **glutathione** participate in controlling oxidation reduction potential.
5. Many peptides such as **penicilin G** have antibiotic activities.
6. Some peptides are constituents of alkaloids. For example, **ergotamine** is a peptide alkaloid from rye ergot (fungi).
7. Some peptides introduce psychiatric disturbances in human beings.

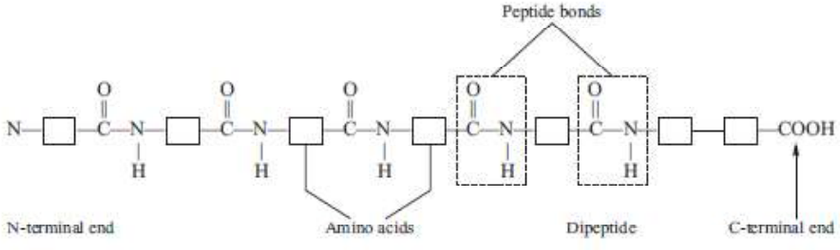

Fig. 3.12 *A Peptide Chain Showing Peptide Bonds between Amino Acids*

Proteins

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Proteins are the most abundant organic molecules of the living system. They occupy a central position in the architecture and functioning of living matter. On this basis, proteins can be broadly grouped as **static** or **structural proteins** (*e.g.*, collagen, elastin, a-Keratin) and **dynamic** or **functional proteins** (*e.g.*, enzymes, hormones, blood clotting factors, immunoglobulins, membrane receptors, storage proteins, motor proteins, etc.).

I. Types of Proteins

A. Classification based on shape of proteins. According to the shape or conformation, two major types of proteins have been recognized:

(*a*) Fibrous proteins. Fibrous proteins are water-insoluble, thread-like proteins having greater length than their diameter. They contain secondary protein structure and occur in those cellular or extracellular structures, where strength, elasticity and rigidity are required, *e.g.*, collagen, elastin, keratin, fibrin (blood-clot proteins) and myosin (muscle contractile proteins).

(*b*) Globular proteins. Globular proteins are water-soluble roughly spheroidal or ovoidal in shape. They readily go into colloidal suspension. They have tertiary protein structure and are usually functional proteins, *e.g.*, enzymes, hormones and immunoglobulins (antibodies). **Actin** of microfilaments and **tubulins** of microtubules are also globular proteins.

B. Classification based on solubility characteristics. According to this criterion proteins can be classified into two main types:

(*a*) Simple proteins. These proteins contain only amino acids in their molecules and they are of following types:

(*i*) Albumins. These are water soluble proteins found in all body cells and also in blood stream, *e.g.*, **lactalbumin**, found in milk and **serum albumin** found in blood.

(*ii*) Globulins. These are insoluble in water but are soluble in dilute salt solutions of strong acids and bases, *e.g.*, **lactoglobulin** found in milk and **ovoglobulin**.

(*iii*) Glutelins. These plant proteins are soluble in dilute acids and alkalis, *e.g.*, **glutenin** of wheat.

(*iv*) Prolamines. These plant proteins are soluble in 70 to 80 per cent alcohol, *e.g.*, **gliadin** of wheat and **zein** of corn.

(*v*) Scleroproteins. They are insoluble in all neutral solvents and in dilute alkalis and acids, *e.g.*, keratin and collagen.

(*vi*) Histones. These are water soluble proteins which are rich in basic amino acids such as arginine and lysine. In eukaryotes histones are associated with DNA of chromosomes to form nucleoproteins.

(*vii*) Protamines. These are water soluble, basic, light weight, **arginine** rich polypeptides. They are bound to DNA in spermatozoa of some fishes, *e.g.*, **salmine** of salmon and **sturine** of sturgeons.

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(*b*) Conjugated proteins. These proteins consist of simple proteins in combination with some non-protein components, called **prosthetic groups**. The prosthetic groups are permanently associated with the molecule, usually through covalent and/or non-covalent linkages with the side chains of certain amino acids. Conjugated proteins are of following types:

(*i*) Chromoproteins. Chromoproteins are a heterogeneous group of conjugated proteins which are in combination with a prosthetic group that is a pigment, *e.g.*, respiratory pigments such as **haemoglobin**, **myoglobin** and **haemocyanin**; **catalase**, **cytochromes**, **haemerythrins**; visual purple or **rhodopsin** of rods of retina of eye and yellow enzymes or **flavoproteins**.

(*ii*) Glycoproteins. Glycoproteins are proteins that contain various amounts (1 to 85 per cent) of carbohydrates. Of the known 100 monosaccharides, only nine are found to occur as regular constituents of glycoproteins (*e.g.*, glucose, galactose, mannose, fucose, acetylglucosamine, acetylgalactosamine, acetylneuraminic acid, arabinose and xylose). Glycoproteins are of two main types: **1. Intracellular glycoproteins** which are present in cell membranes and have an important role in membrane interaction and recognition. They also serve as antigenic determinants and receptor sites. **2. Secretory glycoproteins** are **plasma glycoproteins** secreted by the liver; **thyroglobulin**, secreted by the thyroid gland; **immunoglobulins** secreted by the plasma cells; **ovoalbumins** secreted by the cells of oviduct of hen; **ribonucleases** and **deoxyribonucleases**. **Mucus** and **synovial fluid** are also glycoproteins with lubricative properties.

(*iii*) Lipoproteins. Lipid containing proteins are called **lipoproteins**. Their lipid contents are 40 to 90 per cent of their molecular weight and this tends to affect the density of the molecule. There are four types of lipoproteins: **1. High density lipoproteins (HDL)** or a-lipoproteins; **2. Low density lipoproteins (LDL)** or b-lipoprotiens; **3. Very low density lipoproteins (VLDL)** or pre-b-lipoproteins; and **4. Chylomicrons.** Lipoproteins include some of the blood plasma proteins, various types of membrane proteins, lipovitellin of egg yolk and proteins of brain and nerve tissue.

(*iv*) Nucleoproteins. Nucleoproteins are proteins in combination with nucleic acids (DNA and RNA). However, these proteins are not true conjugated proteins since the nucleic acid involved cannot be regarded as prosthetic groups. Nucleoproteins are of two types: **1. Histones** which are quite similar in all plants and animals. Their highly basic nature accounts for the close associations histones form with the nucleic acids. Histones are involved in the tight packing of DNA molecules during the condensation of chromatin into chromosomes for the mitosis. **2. Nonhistones** have great heterogeneous amino acid composition and are acidic in nature. They have selective combination with certain stretches of nuclear DNA and, thus, are involved in the regulation of gene expression.

(*v*) **Metalloproteins.** Metalloproteins are proteins conjugated to metal ions which are not part of the prosthetic group, *e.g.*, **carbonic anhydrase** enzyme contains zinc ions and amino acids in its molecule; **caeruloplasmin**, an oxidase enzyme containing copper; and **siderophilin** contains iron.

(*vi*) **Phosphoproteins.** Phosphoproteins are proteins in combination with a phosphate group, e.g., casein of the milk and ovovitellin of eggs.

II. Formation of Proteins

Because a molecule of the amino acid contains both basic or amino ($–NH_2$) and acidic or carboxyl (–COOH) group, it can behave as an acid and base at a time. The molecules of such organic compounds which contain both acidic and basic properties are known as **amphoteric molecules**. Due to amphoteric molecules, the amino acids unite with one another to form complex and large protein molecules. When two molecules of amino acids are combined then the basic group ($–NH_2$) of one amino acid molecule combines with the carboxylic (–COOH) group of other amino acid and the loss of a water molecule takes place. This sort of condensation of two amino acid molecules by –NH–CO linkage or bond is known as **peptide linkage** or **peptide bond**. A combination of two amino acids by the peptide bond is known as **dipeptide**. When three amino acids are united by two peptide bonds, they form **tripeptide**. Likewise, by condensation of few or many amino acids by the peptide bonds the **oligopeptides** and **polypeptides** are formed respectively. The various molecules of polypeptides unite to form the **peptones**, **proteases** and **proteins**. Thus, protein macromolecules are the polymers of many amino acid monomers. The size (molecular weight), shape, and function of proteins are determined by the number, type and distribution of the amino acids present in the molecule. Proteins occur in a wide spectrum of molecular sizes from small molecules such as the hormone ACTH (or adrenocorticotrophic hormone) which consists of only 39 amino acids and has a molecular weight of 4500, to extremely large proteins such as haemocyanin (an invertebrate blood pigment) which consists of 8200 amino acids and has a molecular weight greater than 900,000.

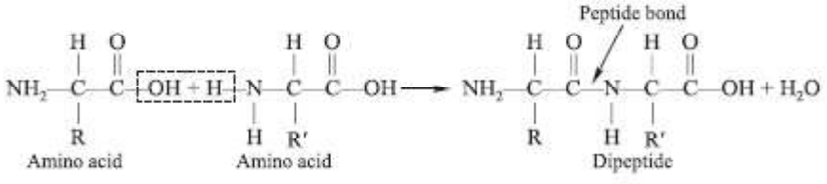

Fig. 3.13 *A Chemical Reaction Showing Formation of a Dipeptide*

III. General Properties of Proteins

Proteins exhibit following general properties:

1. Physical properties

(*i*) **Colloid.** Proteins exist in colloidal state. Their molecules are of large size. Therefore, these are unable to diffuse through plasma membrane.

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(*ii*) Solubility. Because proteins are colloids of large-sized molecules, these form turbid solution in water. Proteins are insoluble in alcohol. They are precipitated by acids in certain concentration.

2. Chemical properties

(*i*) Amphoteric nature. Proteins are **amphoteric**. These behave as acid to alkaline solutions and alkaline to acidic solutions and form salts with them.

(*ii*) Coagulation. On heating proteins are coagulated but temperature for coagulation differs for different proteins.

(*iii*) Optical properties. It is already mentioned that amino acids are optically active except glycine. These are mostly **laevorotatory**. Some of the proteins, haemoglobin and nucleoproteins are dextrorotory.

(*iv*) Hydrolysis. When proteins are boiled with dilute mineral acids in a reflux condenser, the protein molecules gradually break up into simple ones until these are reduced to amino acids. The process of hydrolysis of proteins involves the following steps:

Proteins $\rightarrow$ Proteoses $\rightarrow$ Peptones $\rightarrow$ Amino acids

(*v*) Denaturation. Denaturation of protein is the disruption of secondary, tertiary and quaternary structure of the functional protein resulting in the changes of its physical, chemical and biological characteristics. Various physical and chemical agents such as heat, UV light, ultrasonic waves, high pressure, acids, alkalies, urea, guanidine, ethanol and detergents can cause denaturation of proteins.

The denatured protein becomes insoluble and precipitate out. Their digestability by proteolytic enzymes increases, but their enzymatic or antibody functions are altered.

(*vi*) Colour reactions

(*a*) Biuret reaction. All proteins give **biuret test** with caustic soda and a drop of dilute copper sulphate giving pinkish violet colour.

(*b*) Millon's reagent. Mercury dissolved in strong nitric acid gives a white precipitate, which turns red on heating. The reaction occurs on account of amino acid tyrosine.

(*c*) Rosenheim's reaction. Add a drop of formaldehyde solution to one volume of protein and shake thoroughly. Dilut with one volume of water. When concentrated sulphuric acid, is added slowly a beautiful purple colour appears at the junction. If a drop of ferric chloride solution is added to it the colour is accentuated.

(*d*) Xanthoproteic reaction. Strong nitric acid (HNO_3) gives a white precipitate which turns yellow. The yellow colour changes to orange on addition of alkali. This is due to the presence of phenyl radical.

(*e*) Diazo reaction. Proteins give a red colour on addition of diazobenzene sulphonic acid in a mild alkaline medium. The colour is due to the presence of histidine or tyrosine.

(*f*) Glyoxylic acid reaction or Hopkin's cole reaction. Strong sulphuric acid added to a mixture of protein glyoxylic gives a purple ring at the juction of two. The ring is formed due to the reaction of tryptophan.

3.2.5 Protein Folding

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The structure of proteins is studied under following :

(a) Primary structure

(b) Secondary structure

(c) Tertiary structure

(d) Quaternary structure

Primary structure

The linear sequence of amino acids in the polypeptide chain is known as the primary structure. The determination of the protein structure helps in the analysis of amino acids. The properties of proteins are dependent on the sequence of the amino-acids in the polypeptide chain. Figure 3.14 displays the primary structure of a protein.

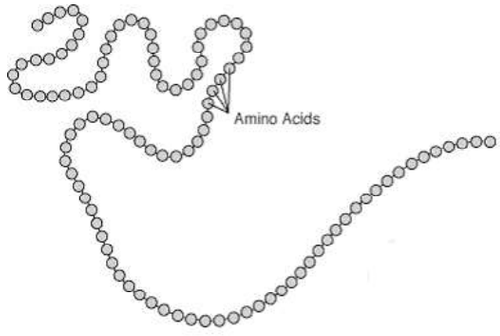

Fig. 3.14 *Primary Structure of a Protein*

Secondary structure

When the peptide chain folds itself in a three-dimensional structure, it is known as Secondary Structure. The secondary structure is dependent on the structural characteristics of the peptide bond. Apart form the peptide bonding, hydrogen bonding that occurs between the nitrogen of one peptide bond and oxygen of another peptide bond, also enhances the stability of the secondary structure of proteins. Proteins fold themselves in two broad classes of structure:

(a) Globular proteins

(b) Fibrous proteins

The globular proteins are compactly folded and coiled whereas the fibrous proteins are filamentous and elongated .Figure 3.15 displays the secondary structure of a protein

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Fig. 3.15 *Secondary Structure of a Protein*

Tertiary structure

The tertiary structure of protein shows a specific three-dimensional structure. The folding regular units of the secondary structure as well as of areas of the peptide chain devoid of secondary structure result in the formation of the tertiary structure. The folded portions are held together by the hydrogen bond formed between the R-group of the oppositely charged ends. The bonds that stabilize the tertiary structure of proteins are:

(a) Van der Waal bonds (if side groups are non-polar)

(b) Hydrogen bonds (if side groups are amino or hydroxyl groups)

(c) Ionic bonds (if side groups are acids or bases)

(d) Covalent bonds (if side groups are cysteine residues)

Figure 3.16 displays the tertiary structure of a protein.

Fig. 3.16 *Tertiary Structure of a Protein*

Quaternary structure

When the proteins contain two or more polypeptide chains, the structure that is formed on the native combination of these polypeptide chains is known as quaternary structure. The primary, secondary and tertiary structures are found in proteins that contain only single polypeptide chain. Figure 3.17 displays the quaternary structure of a protein.

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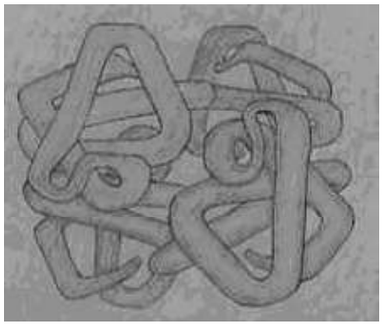

Fig. 3.17 Quarternary Structure of a Protein

Studies of the conformation, function, and evolution of proteins have also revealed the central importance of a unit of organization distinct from the four just described.

This is the **protein domain**, a substructure produced by any part of a polypeptide chain that can fold independently into a compact, stable structure. A domain usually contains between 40 and 350 amino acids, and it is the modular unit from which many larger proteins are constructed.

The smallest protein molecules contain only a single domain, whereas larger proteins can contain as many as several dozen domains, usually connected to each other by short, relatively unstructured lengths of polypeptide chain.

The central core of a domain can be constructed from a helices, from b sheets, or from various combinations of these two fundamental folding elements. Each different combination is known as a **protein fold**. So far, about 1000 different protein folds have been identified among the ten thousand proteins whose detailed conformations are known.Many protein molecules are either attached to the outside of a cell's plasma membrane or secreted as part of the extracellular matrix. All such proteins are directly exposed to extracellular conditions. To help maintain their structures, the polypeptide chains in such proteins are often stabilized by covalent cross-linkages. These linkages can either tie two amino acids in the same protein together, or connect different polypeptide chains in a multi-subunit protein. The most common cross-linkages in proteins are covalent sulfur–sulfur bonds. These disulfide bonds (also called S–S bonds) form as proteins are being prepared for export from cells. Their formation is catalyzed in the endoplasmic reticulum by an enzyme that links together two pairs of –SH groups of cysteine side chains that are adjacent in the folded protein. Disulfide bonds do not change the conformation of a protein but instead act as atomic staples to reinforce its most favored conformation. For example, lysozyme—an enzyme in tears that dissolves bacterial cell walls—retains its antibacterial activity for a long time because it is stabilized by such cross-linkages.

Disulphide bonds generally fail to form in the cell cytosol, where a high concentration of reducing agents converts S–S bonds back to cysteine –SH groups. Apparently, proteins do not require this type of reinforcement in the relatively mild environment inside the cell.

Amino Acid Sequence and Protein Folding

There are an enormous number of ways to make proteins with the same three-dimensional structure. Random mutations can cause amino acid sequences to change

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without a major change in the conformation of a protein. Due to this reason, a current goal of structural biologists is to determine all the different protein folds that proteins have in nature, and to devise computer-based methods to test the amino acid sequence of a domain to identify which one of these previously determined conformations the domain is likely to adopt.

A computational technique called **threading** can be used to fit an amino acid sequence to a particular protein fold. For each possible fold known, the computer searches for the best fit of the particular amino acid sequence to that structure. In many cases, one particular three-dimensional structure will stand out as a good fit for the amino acid sequence, suggesting an approximate conformation for the protein domain. In other cases, none of the known folds will seem possible. By applying x-ray and NMR studies to the latter class of proteins, structural biologists hope to able to expand the number of known folds rapidly, aiming for a database that contains the complete library of protein folds that exist in nature. With such a library, plus expected improvements in the computational methods used for threading, it may eventually become possible to obtain an approximate three-dimensional structure for a protein as soon as its amino acid sequence is known.

Check Your Progress

1. Define the biopolymers.
2. What are the classes of biopolymers?
3. Define the macromolecule.
4. How do you calculate average chain length?
5. What do you mean by tertiary structure of protein?
6. Define the protein fold.

3.3 BIOPOLYMER INTERACTIONS

Surface forces and interactions are a key issue in colloid and surface science, including biopolymer systems. Covalent and ionic bonds determine the structure and composition of materials, but the weaker non-covalent interactions define their functions. Biopolymers are polymers produced by living organisms; in other words, they are polymeric biomolecules. It contains monomeric units that are covalently bonded to form larger structures and there are a number of biophysical techniques for determining sequence information.

Forces Involved In Biopolymer Interactions

The non covalent forces involved in bioadhesion involving flat surfaces, small particles, cells and bio polymers are: Lifshitz Van der Waals (LW), polar (electron acceptor electron donor) or Lewis Acid Base (AB) and Electrostatic forces. LW and AB forces are determined by: (a) direct contact angle measurement with at least three liquids on flat surfaces, or (b) wicking, by capillary rise measurements of at least three liquids along glass plates coated with a thin layer of small particles. This approach yields the LW and AB components of the surface tension, as well as the electron acceptor and the electron donor parameters of its AB component.

Together, these yield the total apolar + polar interfacial energies involved in bioadhesion. EL interaction energies must be obtained separately, by electrokinetic methods. In bioadhesion, the importance of these forces usually is: **AB > > LW > EL**. The electron donor properties of cells and biopolymers are dominant; their electron acceptor parameters are negligible or zero. The degree of hydrophilicity of most materials can be expressed fairly precisely, as the interfacial free energy of attraction between the material and water; it consists principally of LW interactions. The interfacial free energy of interaction between biomaterials and synthetic surfaces, immersed in water is, for the greatest part, due to AB forces.

The surface properties of biopolymers and cells usually are such that a maximum of adhesion should occur onto synthetic polymer surfaces with a surface tension of approximately 30 mJ rn^{2}.

Van der Waals Interaction

The DLVO forces are the Van der Waals forces and the electrostatic double-layer forces. The DLVO theory predicts that the interaction between surfaces with charges of the same sign in a liquid medium is dominated by the repulsion originating from the so-called double-layer of co-ions and counter ions at large separations, whereas the Van der Waals attraction becomes more significant at shorter distances. For surfaces of opposite sign, the DLVO theory predicts attraction at long distances but repulsion at close to molecular contact. Increasing the electrolyte concentration of the medium will cause shielding of the electrostatic repulsion, and the balance between the DLVO forces can be altered towards Van der Waals attraction between the interacting bodies.

Electrostatic Interactions

It is also known as salt bridges, these are formed between the charged interacting residues. In proteins, salt bridges are formed between carboxyl group of aspartic acid or glutamic acid with guanidinium group of arginine or amino group of lysine. Salt bridges in protein are about 2.8Å. Energy contributed by formation of salt bridge in protein structure is approximately 12-17 KJ/mol in water (on the surface) while its formation in the buried hydrophobic environment contributes about 30KJ/mol. In water their strength is less as water can also form ionic interactions with the residues. Thus, ionic interactions forming on the surface of the protein do not contribute much to the protein structure, while its formation in the buried leads to thermally stable protein.

Hydrogen Bonds

Hydrogen bond is week electrostatic interaction with a directional property. Hydrogen bond involves at least one hydrogen atom. In hydrogen bond, there is one hydrogen donor group and a hydrogen acceptor group. In proteins, hydrogen bonds are generally formed between atoms: N-H—O. Hydrogen bond distance is about 3Å (range from 2.7- 3.5 Å). Energy contributed by hydrogen bond in protein structure is approximately 2 to 6 KJ/mol in water and 12- 30 KJ/mol in buried hydrophobic environment.

Hydrophobic Interactions

Hydrophobic interactions also known as Van der Waals interactions, can be seen when non-polar atoms gather together to minimize their contact with water. Oils

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and lipids cluster themselves to form micelle and reduce the surface area of hydrophobic groups and minimize their contact with water. Soluble, globular proteins tend to hide hydrophobic amino acids in the core of the protein, preventing their contact with water molecule. Attractive and repulsive forces in Van der Waals interaction can be described by 'LennardJones' potential.

Total Energy of the Protein: Total energy of protein includes energy contributed by bonded and non-bonded interactions. Energy contributed by bonded interactions includes bond-stretch, bond bend and dihydral angle. While, energy contributed by non-bonded includes electrostatic, Van-der waals and hydrogen bonding.

$$E_{Total} = E_{bonded} + E_{Non\text{-}Bonded}$$

$$E_{Bonded} = E_{Bond\text{-}Stretch} + E_{Bond\text{-}Bend} + E_{Dihydral\ Angle}$$

$$E_{Non\text{-}Bonded} = E_{Electrostatic} + E_{Van\text{-}der_Waals} + EH_{\text{-}Bond}$$

Energy: (Bond-Stretch) when two atoms are connected by a covalent bond, it maintain an equilibrium distance between them. If there is displacement of atoms from the equilibrium position leads to bond stretch energy, which contributes to the total potential energy.

Energy: (Dihydral Angle) Dihydral angle is defined by angle formed by two planes or it can be defined as the angle formed at middle bond of the three bonds connecting four atoms in the space. Deviation from equilibrium bond angle due to rotation about middle bond also contributes towards potential energy of the protein.

Non-Bonded Interactions: The energy potential of non-bonded interactions is due to electrostatic, H-bonds and Van der Waals forces. These interactions are crucial for the stability of the tertiary structure. Energy due to electrostatic forces can be calculated using Coulomb's law. The energy contribution by Van der Waals forces can be calculated using the Lennard-Jones potential. The energy contribution by Van der Waals interactions / hydrophobic interactions is well explained by London dispersion forces. When two non-polar atoms come together, nucleus of one atom attracts the electron cloud of the other atom. Due to this, a dipole moment is induced in both atoms and these dipoles attract each other. But, as these two atoms come very close, electrons of both atoms and nucleus of both atoms repel each other and in best possible case equilibrium is achieved, where the atoms stay at certain distance. At this distance, best attractive force and least repulsive force are maintained. Hydrogen bond is another strong energy potential, which plays important role in protein structure. Total energy of the protein helps in prediction of stability and lowest energy state of the molecule during energy minimization, refinement of NMR and crystal structures. Moreover, it is also helpful in determining binding energy upon interaction of protein-inhibitor, protein-ligand and protein-protein interactions. This kind of energy parameter is used for structure prediction of proteins and simulations of the protein structure.

Dispersion Forces Interaction

Also known as London forces, *dispersion interactions* occur between any adjacent pair of atoms or molecules when they are present in sufficiently close proximity. These interactions account for the attractive forces between non-ionic

and non-polar organic molecules, such as paraffin and many pharmaceutical drugs. The origin of these forces remains unclear, but it is believed that at any given instance, molecules are present in a variety of distinct positions due to thermal oscillations. These positions give rise to a temporary molecular dissymmetry, resulting in a dipole-type characteristic. This instantaneous dipole then induces a polar character to the neighbouring molecules and starts interacting with them.

3.3.1 Multiple Equilibria and Binding Process in Biological System

Multiple equilibria occur when several different local regions of the same phase space are dynamical attractors. The same system dynamics can lead to more than one stable state depending on the location in phase space of the system, and thus on initial conditions.

Multiple Equilibria in Proteins covers the *multiple interactions* between small ions and molecules and a protein molecule.

Chemical reactions are initiated by accidental collision of molecules that have the potential (e.g., sufficient energy) to react with one another to be converted into products:

$$\mathbf{A + B \rightarrow P + Q}$$

In living matter, it cannot be left to chance whether a reaction happens or not. At the precise time, the respective compounds must be selected and converted into products with high precision, while at unfavourable times spontaneous reactions must be prevented. An important prerequisite for this selectivity of reactions is the highly specific recognition of the required compound. Therefore, any physiological reaction occurring in the organism is preceded by a specific recognition or binding step between the respective molecule and a distinct receptor. The exploration of binding processes is substantial for understanding biological processes. The receptors can be enzymes as well as non-enzymatic Proteins, such as serum albumin, membrane transport systems, receptors for hormones or neurotransmitters, or nucleic acids. Generally, receptors are macromolecular in nature and thus considerably larger than the efficacious molecules, the ligands. For the binding process, however, both the macromolecule and its ligand must be treated as equivalent partners (unlike for enzyme kinetics, where the enzyme as catalyst does not take part in the reaction). As a precondition for binding studies, specific binding must be established and unspecific association excluded. There exist various reasons for unspecific binding such as hydrophobic or electrostatic interactions (charged ligands can act as counter ions for the surplus charges of proteins). A rough indicator for specific binding is the magnitude of the dissociation constant, which is mostly below 10^{-3} M (although there are exceptions such as the binding of H_2O_2 to catalase or glucose to glucose isomerase). Specific binding is characterized by a defined number of binding sites n, which is in stoichiometric relationship to the macromolecule. Ligands at high concentrations saturate the binding sites. Structurally similar compounds can displace the ligand from its binding site, while unrelated compounds have no effect. In contrast, unspecific binding has no defined number of binding sites, and the binding process is not saturable.

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Modes of Ligand Binding

Uniform values should be obtained if the rate constants are exclusively determined by diffusion. In reality, however, the values of the rate constants of diffusion-controlled reactions of macromolecules vary within a range of more than five orders of magnitude. The reason for this variation is that, for successful binding of the ligand, random collision with the macromolecule is not sufficient. Both molecules must be in a favourable position to each other. This causes a considerable retardation of the binding process. On the other hand, attracting forces could facilitate the interaction and direct the ligands toward their proper orientation. Under such conditions, rate constants can even surpass the values of mere diffusion control. Quantitative recording of such influences is difficult as they depend on the specific structures of both the macromolecule and the ligand. Theories have been developed to establish general rules for ligand binding. Ligands approach a macromolecule at a rate but only those meeting the correct site in the right orientation will react. If the binding site is considered as a circular area, forming an angle α with the centre of the macromolecule (Refer Figure 3.18a), the association rate constant of will be reduced by the sine of that angle:

$$k_a = 4\pi r_{A+B} D2 \ \ sin \ 5\alpha \qquad (7)$$

The necessity of appropriate orientation between ligand and binding site should be considered by the introduction of a suitable factor, depending on the nature of the reactive groups involved. It is also suggested that the ligand may associate unspecifically to the surface of the macromolecule, where it dissociates in a two-dimensional diffusion to find the binding site (Refer Figure 3.18b). The initial unspecific binding, however, cannot distinguish between the specific ligand and other metabolites, which may also bind and impede the two-dimensional diffusion. The gating model (Refer Figure 3.18c) assumes the binding site to be opened and closed like a gate by changing the conformation of the protein.

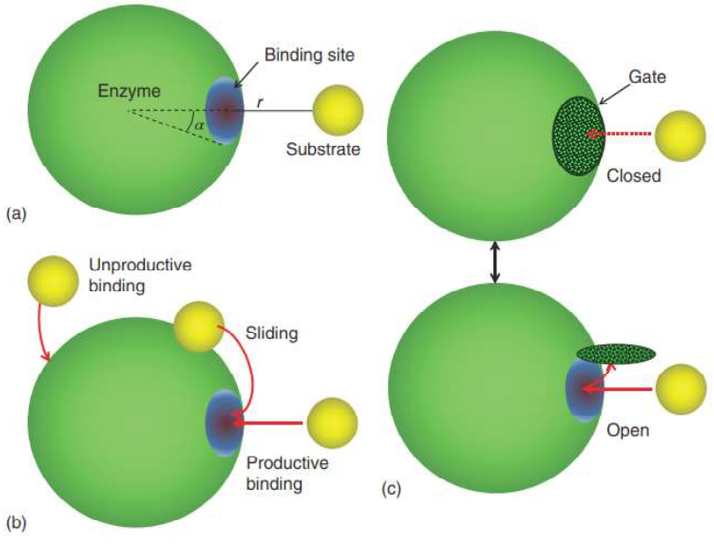

Fig 3.18 *Modes of ligand binding*

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Whereas **(a)** Interaction of a substrate molecule with its binding site on the enzyme. **(b)** Different types of interaction between ligand and macromolecule. **(c)** Gating.

The protein, thus modulating the accessibility for the ligand. A basic limit for the association rate constant for the enzyme substrate is the quotient from the **catalytic constant k_{cat}** and the **Michaelis constant K_m:**

$$\frac{k_{\text{cat}}}{K_{\text{m}}} = \frac{k_{\text{cat}} k_1}{k_{-1} + k_2} \quad \textbf{(8)}$$

For a diffusion-controlled reaction, the value is frequently around 10^8 M"1 s^{-1}. The reaction rate for most enzyme reactions is determined more by the non-covalent steps during substrate binding and product dissociation rather than by the cleavage of bounds.

Interaction between Macromolecules and Ligands

Binding Constants

Binding of a ligand **A** to a macromolecule **E**

$$\text{E} + \text{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{EA} \quad \textbf{(9)}$$

It is described with the law of mass action, applying the association constant K_a

$$K_{\text{a}} = \frac{k_1}{k_{-1}} = \frac{[\text{EA}]}{[\text{A}][\text{E}]} \quad \textbf{(10)}$$

Or its reciprocal value, the dissociation constant K_d

$$K_{\text{d}} = \frac{k_{-1}}{k_1} = \frac{[\text{A}][\text{E}]}{[\text{EA}]} \quad \textbf{(11)}$$

Both notations are used: the association constant more frequently for the treatment of equilibria and the dissociation constant for enzyme kinetics. Here, the dissociation constant is employed throughout.The association constant has the dimension of a reciprocal concentration (M^{-1}); the higher the numerical value, the higher the affinity. Conversely, dissociation constants possess the dimension of a concentration (M), and lower values indicate stronger binding.

Equations (10) and (11) are not quite correct. The concentration terms should be transformed into activity terms by multiplying with an activity coefficient f, for example, $a = f[\text{A}]$. Since f approaches 1 in dilute solutions, this factor can be disregarded for enzyme reactions.

If one reaction component is present in such a large excess that its concentration change during the reaction can be neglected, its concentration can be combined with the rate constant. This applies especially for water, if it takes part in the reaction, especially in hydrolytic processes:

$$A + H_2O \overset{\text{enzyme}}{\rightleftharpoons} P + Q. \quad \textbf{(12)}$$

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Water as a solvent with a concentration of 55.56 mol l^{-1} exceeds by far the nano- to millimolar amounts of all other components in an enzyme assay and any change in its concentration will hardly be detectable. A binding constant for water cannot be determined, and the reaction is treated as if water is not involved:

$$K'_d = \frac{[A][H_2O]}{[P][Q]} = K_d[H_2O]; \quad K_d = \frac{[A]}{[P][Q]}. \quad \textbf{(13)}$$

Hydrogen ions, frequently involved in enzyme reactions, are treated in a similar manner. An apparent dissociation constant is defined:

$$\boldsymbol{K_{app} = K_d[H^+].}$$

Contrary to genuine equilibrium constants, this constant is dependent on the pH value in the solution.

Binding to a Single Site

The binding constants for a distinct system can be determined applying the mass action law (Equations 10 and 11). For this, the concentrations of the free macromolecule **[E],** the free substrate **[A],** and the enzyme–substrate complex **[EA]** must be known, but this is usually not the case. Only the total amounts of macromolecule $\mathbf{[E]_0}$ and ligand $\mathbf{[A]_0}$ added to the reaction can be considered as known. They separate into free and bound components according to the mass conservation principle:

$$\mathbf{[E]_0 = [E] + [EA],} \quad \textbf{(14)}$$

$$\mathbf{[A]_0 = [A] + [EA].} \quad \textbf{(15)}$$

The portion of the ligand bound to the macromolecule $\mathbf{[A]_{bound}}$ can be obtained by binding experiments. If only one ligand molecule binds to the macromolecule as formulated in Equation (9), $\mathbf{[A]_{bound}}$ is equal to [EA]. Substituting Equation (14) into Equation (11) eliminates the free macromolecule concentration [E]:

$$[A]_{bound} = \frac{[E]_0[A]}{K_d + [A]}. \quad \textbf{(16)}$$

This equation describes the binding of a ligand to a macromolecule with one binding site, obviously the only possible mechanism for this case. Many enzymes and macromolecules, however, can bind more than one ligand molecule, and for such cases different binding mechanisms must be considered. They are presented in the following sections, where treatment and evaluation of the respective binding equations is also discussed.

Binding to Identical Independent Sites

General Binding Equation Proteins and enzymes in living organisms are composed mostly of more than one subunit. Often all subunits are identical, and in such cases it can be taken that every subunit carries the same identical binding site for the ligand, so that the number *n* of binding sites is identical to the number of subunits.

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This is a plausible assumption, but it must be kept in mind that in the treatment of binding processes identity means equality of binding constants. If the affinities of binding sites located on non-identical subunits are the same by chance, or if a single subunit possesses more than one binding site with similar binding constants (e.g., due to gene duplication), this will not be differentiated by binding analysis and requires additional experiments.

For the binding of a ligand to a macromolecule with a single binding site only one binding mechanism exists, whereas several modes of binding are possible when the macromolecule carries more than one binding site, as shown in Figure 3.16. If the sites are identical, binding can either proceed independently (Refer Figure 3.19b), or interactions between the sites can influence the binding course (Refer Figure 3.19 c).The same two possibilities exist if the sites are not identical (Refer Figure 3.19 d,e), but in both cases complex binding behaviour is observed. Actually, an independent binding process is already described by Equation (16), since it should make no principal difference whether the binding occurs at a macromolecule with one single binding site or whether *n* independent sites are gathered on the same macromolecule. Figure 3.19 show the modes of binding of a ligand to a macromolecule. Binding to one single site (a) follows a normal binding course. If the macromolecule possesses more binding sites, four different binding modes are possible. If the sites are identical and independent, normal binding occurs (b), whereas characteristic deviations appear if the binding sites interact with one another (c). Deviations also occur for the case of non-identical binding sites even if they are independent (d) and the more if they interact with one another (e).

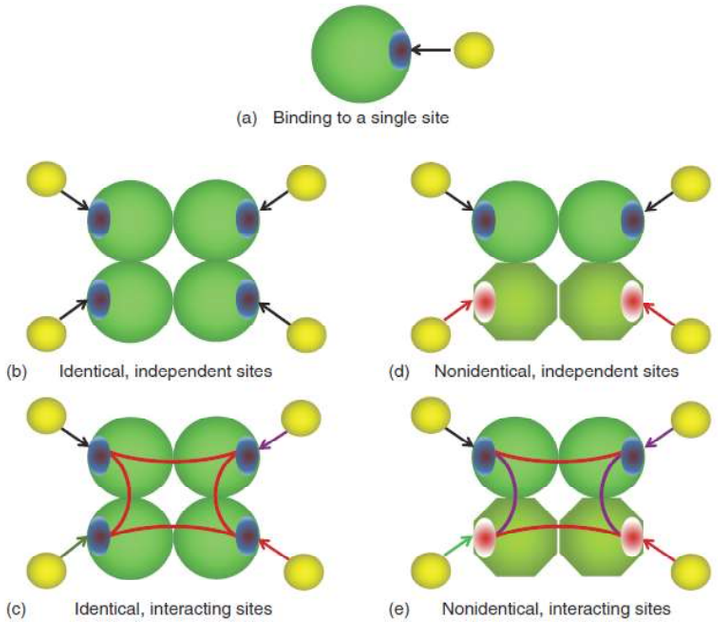

Fig. 3.19 *Modes of Binding of a Ligand to a Macromolecule.*

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For this case, the macromolecule concentration $[E]_0$ in Equation (16) is replaced by $[F]_0$, the total amount of identical binding sites, which is related to the total macromolecule concentration by $[F]_0 = n[E]_0$:

$$[A]_{bound} = \frac{[F]_0[A]}{K_d + [A]} = \frac{n[E]_0[A]}{K_d + [A]}. \quad (17)$$

This equation differs from Equation (16) in two respects .The numerator is extended by the number of binding sites, and **[A]**bound can no longer be equated with **[EA]** but comprises all partially saturated forms of the macromolecule:

$$[A]_{bound} = [EA] + 2[EA_2] + 3[EA_3] + \cdots + n[EA_n] \quad (18)$$

The macromolecule is saturated stepwise:

$$\begin{aligned}
E + A &\rightleftharpoons EA & K_1' &= \frac{[E][A]}{[EA]} \\
EA + A &\rightleftharpoons EA_2 & K_2' &= \frac{[EA][A]}{[EA_2]} \\
EA_2 + A &\rightleftharpoons EA_3 & K_3' &= \frac{[EA_2][A]}{[EA_3]} \\
&\vdots \quad\quad \vdots & & \\
EA_{n-1} + A &\rightleftharpoons EA_n & K_n' &= \frac{[EA_{n-1}][A]}{[EA_n]}
\end{aligned}$$

Each step has its own dissociation constant. If, for independent binding, all individual dissociation constants are taken as equal, Equation (17) is obtained according to the upper derivation. It must be mentioned, however, that this is a simplified derivation, neglecting the fact that the ligand has various modes of orientation between the several binding sites of the macromolecule.

3.3.2 Hydrogen Ion Titration Curve

Titrations are often recorded on graphs called titration curves, which generally contain the volume of the titrant as the independent variable and the pH of the solution as the dependent variable (because it changes depending on the composition of the two solutions).

The equivalence point on the graph is where all of the starting solution (usually an acid) has been neutralized by the titrant (usually a base). It can be calculated precisely by finding the second derivative of the titration curve and computing the points of inflection (where the graph changes concavity. Titration curves do not represent just another way of physically characterizing a protein molecule. More than most other physicochemical methods that are in common use, titration studies tend to emphasize individual differences among proteins, the foundation for any study of hydrogen ion dissociation in proteins is the electrometric titration curve. To obtain such a curve, one begins with a protein solution of known concentration, at an arbitrary reference pH, adds to varying amounts of a strong acid or a strong base, and then measures the new pH attained. In a separate experiment, or by means of calculations based on similar experiments, it is determined that how much acid or base is needed to take a solution, which does not contain protein,

but otherwise has the same initial pH, ionic strength, and volume. The difference between the two amounts is the amount of acid or base that is bound to the protein in going from the reference pH to the final pH, a plot of this quantity versus the final pH is the desired titration curve. In plotting this curve, OH- ions bound are counted as H+ ions dissociated a procedure, which is always permissible in aqueous solutions.

Denition of the Protonation Degree, α, and Dissociation Coefficients, *K*, for the Pyridylamine Oxides

In a pyridylamine oxide molecule, two-step protonation can occur due to the presence of two protonation sites, an amine oxide group and a pyridyl group. Consequently, we defined the protonation degree α as the number of protons bound to a pyridylamine oxide molecule **(0 ≤ α ≤)**. The dissociation constant *K* for monomers is proposed as follows;

$$
\begin{aligned}
&0 < \alpha < 1 \\
&\mathrm{PyAO\,H^+} \rightleftharpoons \mathrm{PyAO} + \mathrm{H^+} \\
&K_1 = [\mathrm{PyAO}][\mathrm{H^+}]/[\mathrm{PyAO\,H^+}] \\
&\mathrm{p}K_1 = \mathrm{pH} + \log([\mathrm{PyAO\,H^+}]/[\mathrm{PyAO}]) \\
&\qquad = \mathrm{pH} + \log(\alpha/(1-\alpha))
\end{aligned}
\tag{19}
$$

$$
\begin{aligned}
&1 \le \alpha < 2 \\
&\mathrm{PyAO\,H_2^{2+}} \rightleftharpoons \mathrm{PyAO\,H^+} + \mathrm{H^+} \\
&K_2 = [\mathrm{PyAO\,H^+}][\mathrm{H^+}]/[\mathrm{PyAO\,H_2^{2+}}] \\
&\mathrm{p}K_2 = \mathrm{pH} + \log([\mathrm{PyAO\,H_2^{2+}}]/[\mathrm{PyAO\,H^+}]) \\
&\qquad = \mathrm{pH} + \log((\alpha-1)/(2-\alpha))
\end{aligned}
\tag{20}
$$

Here, K_1 and K_2 denote the dissociation constants of monomers for the first and second protonation sites, respectively. Similarly, the apparent dissociation coefficient of micelles, Ka, is described by Equations (21) and (22).

$$
\begin{aligned}
&0 < \alpha < 1 \\
&\mathrm{p}K_{a1} = \mathrm{pH} + \log(\alpha/(1-\alpha))
\end{aligned}
\tag{21}
$$

$$
\begin{aligned}
&1 \le \alpha < 2 \\
&\mathrm{p}K_{a2} = \mathrm{pH} + \log((\alpha-1)/(2-\alpha))
\end{aligned}
\tag{22}
$$

For micelles, the K_a values decrease with an increase in α because of the electrostatic repulsion between protonated molecules on the micelle surface, as we later show in the pH titration curves. The intrinsic dissociation coefficients of the micelles K_{M1} and K_{M2} can be obtained as the **p*K*a** values extrapolated to α = 0 and to α =1 for the first and second protonation sites on the micelles, respectively.

Hydrogen Ion Titration

Hydrogen ion titration was performed to evaluate the protonation degree, α, of the monomers and micelles at a given pH α represents the number of protons bound to a pyridylamine oxide molecule. We estimated the dissociation constant for the

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monomer based on Equations (19) and (20) from the relation between α and pH. Three isomers of pyridylamine oxides with an alkyl chain length of 8, C8-x (x=2-4), were used for the monomer titration. The titration curves of pK against α are shown in Figure 3.20. All of the curves of C8-x in the range of $\boldsymbol{\alpha < 1}$ were almost the same, whereas that of C8-2 was different, in the range of $\boldsymbol{\alpha \leq 1}$. The $\boldsymbol{pK_1}$ values estimated from the titration curves were also the same ca. 4.7 for the first protonation of the monomers, as listed in Table 3.1. On the other hand, the pK2 value of C8-2 ca. 1.2 was clearly different from that of C8-3 and C8-4 ca. 2.9 for the second protonation.

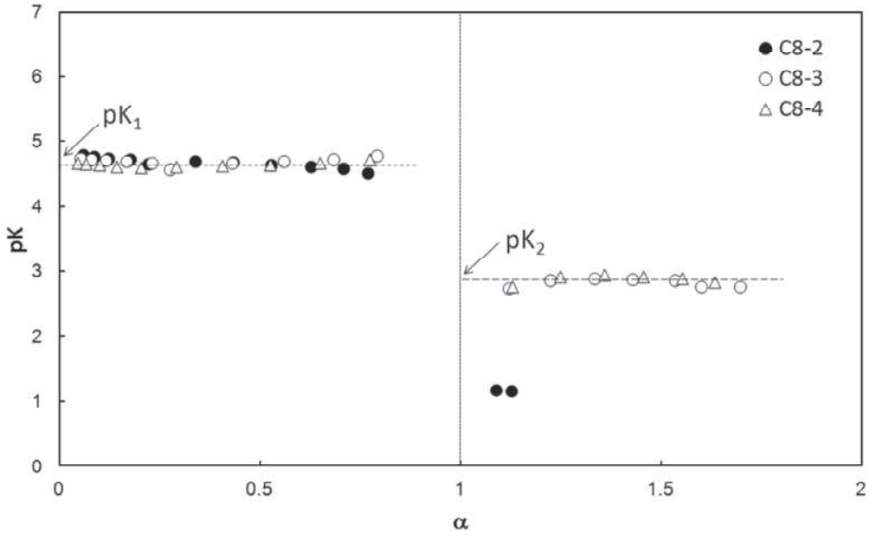

Fig. 3.20 *Hydrogen Ion Titration Curves for Monomers (C8-x)*

Table 3.1 *Dissociation Coefficients for Pyridylamine Oxides at 25°C C_S = 0.1 mol dm^{-3}, CD = 0.02 mol dm^{-3}*

Monomer (C8-x)	pK_1 (±0.1)	pK_2 (±0.1)
8-2	4.7	1.2
8-3	4.7	2.9
8-4	4.6	2.9
Micelle (C12-x and C14-x)	pK_{M1} (±0.1)	pK_{M2} (±0.1)
12-2	5.5	–
12-3	5.7	2.0
12-4	5.6	2.3
14-2	5.9	–
14-3	5.9	1.8

The $\boldsymbol{pK_1}$ values of C8-x were similar to those of simple amine oxide surfactants, such as decyldimethylamine oxides ($\boldsymbol{pK_1}$ = 4.9). Therefore, the first protonation was expected to occur in the amine oxide group. The pH dependence of UV spectra of the pyridyl group also indicated that the first protonation

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occurred in the amine oxide group, as explained later. Here, we concluded that the first protonation occurred in the amine oxide group and the second protonation occurred in the pyridyl group in the pyridylamine oxide molecule. The much smaller value of $\mathbf{p}K_2$ for C8-2 than for C8-3 and C8-4 indicates that the second protonation was less likely to occur on the C8-2 molecule than on C8-3 and C8-4, because of the electrostatic repulsion between charges within the molecule. Although all three titration curves of the C12-x micelles agreed with each other in the range of $\alpha < 0.5$, they provided obvious differences in the range of $\alpha < 0.5$, as shown in Figure 3.18. The protonation behaviour of C14-x micelles other than C14-4 was similar to that of the C12-x micelles （Refer Figure 3.18) The solubility of C14-4 was poor at pH values higher than 4 due to both the symmetrical chemical structure and the hydrophobicity of the long alkyl chain of C14. The C12-2 molecule was bound to at most one proton, same as in the monomer titration of C8-2. That is, binding of two protons to a 2-pyridyl substituent was quite difficult because of the electrostatic repulsion between charges on the molecule. Based on the small $\mathbf{p}K_{M2}$ value (ca. 1), the C14-2 molecule does not easily bind the two protons (Refer Figure 3.21). Comparing the titration curves of monomers with those of micelles, the first protonation was more favourable in micelles than in monomers because of the hydrogen bonding between deprotonated and protonated species. As the protonation continued, however, the protonation degree of the micelles decreased compared with that of monomers at the same pH value.

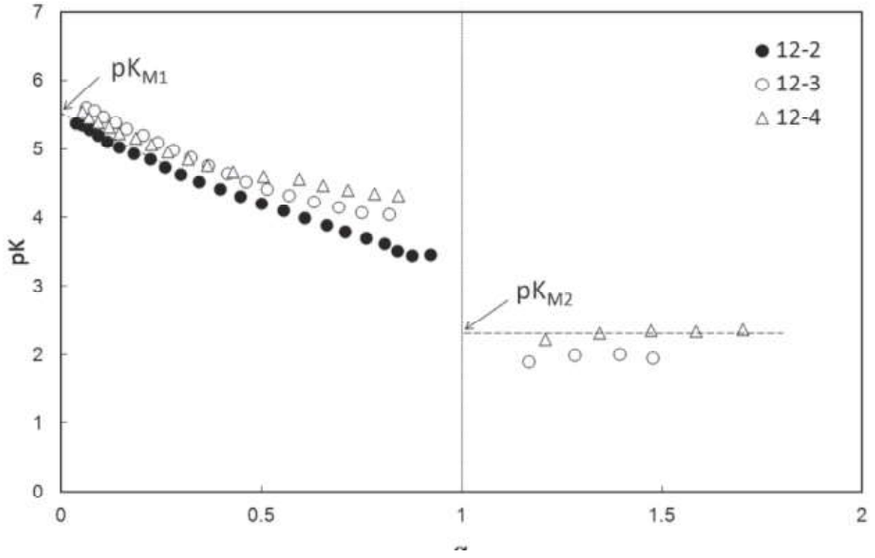

Fig. 3.21 *Hydrogen Ion Titration Curves for Micelles (C12- x)*

The second protonation in micelles was less favourable than that in monomers because of the electrostatic repulsion between charges on the micellar surface.

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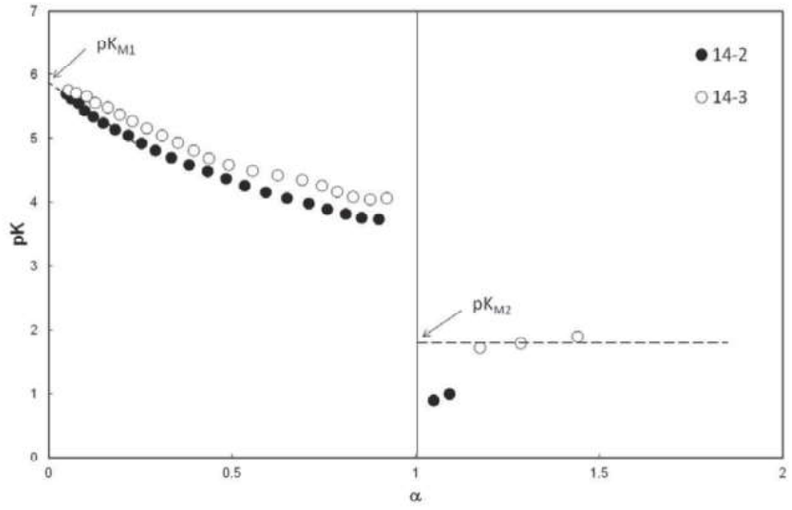

Fig. 3.22 *Hydrogen Ion Titration Curves for Micelles (C14- x).*

The intrinsic dissociation coefficients of micelles, $\boldsymbol{K}_{\mathbf{M1}}$, were evaluated from the extrapolation to $\alpha = 0$ of plots of $\mathbf{p}\boldsymbol{K}$ against α. As listed in Table 3.1, we found little effect of isomers on the values of $\mathbf{p}\boldsymbol{K}_{\mathbf{M1}}$. The longer alkyl chain resulted in a substantial increase in the protonation behaviour, except for in 4-pyridylamine oxides.

Check Your Progress

7. What do you understand by electrostatic interaction?
8. What is the reason of the energy patential of the non-bonded interaction.
9. Why the hydrogen ion titration is performed?

3.4 THERMODYNAMICS OF POLYMER SOLUTIONS

The thermodynamics of biopolymers solutions is based upon the ***quasilattice theory*** of solutions, and leads to an equation for the free energy of mixing polymeric solute with monomeric solvent which contains a combinatorial entropy of mixing term and a regular solution theory enthalpy of mixing term. The widely used ***Flory-Huggins theory*** of polymer solution thermodynamics. It is based upon the ***quasilattice theory*** of solutions, and leads to an equation for the free energy of mixing polymeric solute with monomeric solvent which contains a combinatorial entropy of mixing term and a regular solution theory enthalpy of mixing term. In its original form the theory contains only one adjustable parameter, characteristic of the intermolecular forces between molecules in solution. *Hildebrand's solubility parameter* concept may be used to estimate the value of this parameter.

Flory–Huggins solution theory is a lattice model of the thermodynamics of polymer solutions which takes account of the great dissimilarity in molecular sizes in adapting the usual expression for the entropy of mixing. The result is an equation for the Gibbs free energy change ΔG_M for mixing a polymer with a solvent. Although it makes simplifying assumptions, it generates useful results for interpreting experiments.

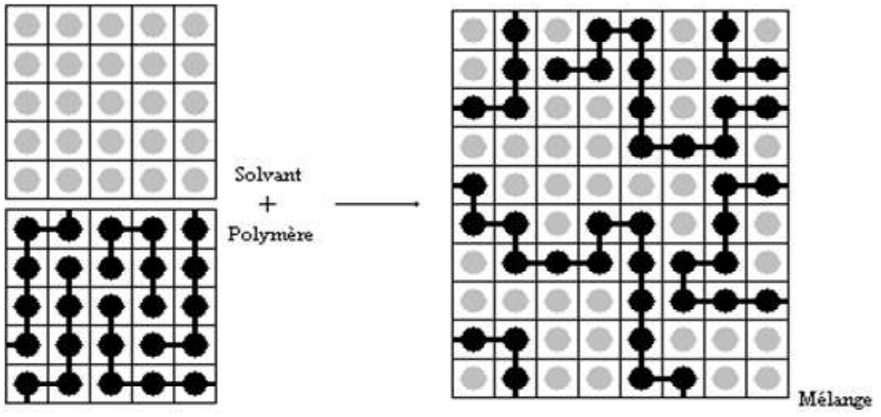

Fig. 3.23 *Mixture of Polymers and Solvent on a Lattice*

Theory

The thermodynamic equation for the Gibbs energy change accompanying mixing at constant temperature and (external) pressure is

$$\Delta G_M = \Delta H_M - T\Delta S_M$$

A change, denoted by Δ, is the value of a variable for a solution or mixture minus the values for the pure components considered separately. The objective is to find explicit formulas for ΔH_M and ΔS_M the enthalpy and entropy increments associated with the mixing process.

The result obtained by Flory and Huggins is:

$$\Delta G_M = RT[\, n_1 \ln\phi_1 + n_2 \ln\phi_2 + n_1\phi_2\chi_{12}]$$

The right-hand side is a function of the number of moles n_1 and volume fraction ϕ_1 of solvent (component 1), the number of moles n_2 and volume fraction ϕ_2 of polymer (component 2), with the introduction of a parameter χ to take account of the energy of interdispersing polymer and solvent molecules. R is the gas constant and T is the absolute temperature. The volume fraction is analogous to the mole fraction, but is weighted to take account of the relative sizes of the molecules. For a small solute, the mole fractions would appear instead, and this modification is the innovation due to Flory and Huggins. In the most general case the mixing parameter, χ, is a free energy parameter, thus including an entropic component.

3.4.1 Energy Generation of Mechanical System

We first calculate the entropy of mixing, the increase in the uncertainty about the locations of the molecules when they are interspersed. In the pure condensed phase's solvent and polymer everywhere we look we find a molecule. Of course,

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any notion of finding a molecule in a given location is a thought experiment since we cannot actually examine spatial locations the size of molecules. The expression for the entropy of mixing of small molecules in terms of mole fractions is no longer reasonable when the solute is a macromolecular chain. We take account of this dissymmetry in molecular sizes by assuming that individual polymer segments and individual solvent molecules occupy sites on a lattice. Each site is occupied by exactly one molecule of the solvent or by one monomer of the polymer chain, so the total number of sites is

$$N = N_1 + x\,N_2$$

N_1 is the number of solvent molecules and N_2 is the number of polymer molecules, each of which has x segments.

For a random motion on a lattice we can calculate the entropy change (the increase in spatial uncertainty) as a result of mixing solute and solvent.

$$\Delta S_M = k[N1 \ln (N_1/N) + N_2 \ln (x(N_2/N)]$$

Where **k** is Boltzmann's constant. Define the lattice volume fractions ϕ_1 and ϕ_2

$$\phi_1 = (N_1/N)$$

$$\phi_2 = (x\,(N_2/N)$$

These are also the probabilities that a given lattice site, chosen at random, is occupied by a solvent molecule or a polymer segment, respectively. Thus

$$\Delta S_m = -k[\,N_1 \ln \phi_1 + N_2 \ln \phi_2\,]$$

For a small solute whose molecules occupy just one lattice site, x equals one, the volume fractions reduce to molecular or mole fractions, and we recover the usual entropy of mixing.

In addition to the entropic effect, we can expect an enthalpy change. There are three molecular interactions to consider: solvent-solvent w_{11} monomer-monomer w_{22} (not the covalent bonding, but between different chain sections), and monomer-solvent w_{12}. Each of the last occurs at the expense of the average of the other two, so the energy increment per monomer-solvent contact is

$$\Delta w = w_{12} - \tfrac{1}{2}\,(w_{22} + w_{11})$$

The total number of such contacts is

$$xN_2 z\phi_1 = N_1 \phi_2 z$$

Where z is the coordination number, the number of nearest neighbours for a lattice site, each one occupied either by one chain segment or a solvent molecule. That is, xN_2 is the total number of polymer segments (monomers) in the solution, so $xN_2 z$ is the number of nearest-neighbor sites to all the polymer segments. Multiplying by the probability ϕ_1 that any such site is occupied by a solvent molecule, we obtain the total number of polymer-solvent molecular interactions. An approximation following mean field theory is made by following this

procedure, thereby reducing the complex problem of many interactions to a simpler problem of one interaction.

The enthalpy change is equal to the energy change per polymer monomer-solvent interaction multiplied by the number of such interactions

$$\Delta H_m = N_1 \phi_2 z \Delta w$$

The polymer-solvent interaction parameter chi is defined as

$$\chi_{12} = z\Delta w / kT$$

It depends on the nature of both the solvent and the solute, and is the only material-specific parameter in the model. The enthalpy change becomes

$$\Delta H_m = kT N_1 \phi_2 \chi_{12}$$

Assembling terms, the total free energy change is

$$\Delta G_m = RT[\, n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_1 \phi_2 \chi_{12} \,]$$

Where we have converted the expression from molecules N_1 and N_2 to moles n_1 and n_2 by transferring the **Avogadro constant** N_A to the gas constant $R = k\ N_A$. The value of the interaction parameter can be estimated from the Hildebrand solubility parameters δ_a and δ_b

$$\chi_{12} = V_{seg}(\delta_a - \delta_b)^2 / RT$$

Where V_{seg} is the actual volume of a polymer segment.

In the most general case the interaction Δw and the ensuing mixing parameter, χ, is a free energy parameter, thus including an entropic component. This means that aside to the regular mixing entropy there is another entropic contribution from the interaction between solvent and monomer. This contribution is sometimes very important in order to make quantitative predictions of thermodynamic properties. More advanced solution theories exist, such as the Flory–Krigbaum theory.

Polymer Blends

Synthetic polymers rarely consist of chains of uniform length in solvent. The Flory-Huggins free energy density can be generalized to an N-component mixture of polymers with lengths r_i by

$$f(\{\phi_i, r_i\}) = \sum_{i=1}^{N} \frac{\phi_i}{r_i} \ln \phi_i + \frac{1}{2} \sum_{i,j=1}^{N} \phi_i \phi_j \chi_{ij}$$

For a binary polymer blend, where one species consists of N_A monomers and the other N_B monomers this simplifies to

$$f(\phi) = \frac{1}{N_A} \ln \phi + \frac{1-\phi}{N_B} \ln(1-\phi) + \chi \phi (1-\phi)$$

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As in the case for dilute polymer solutions, the first two terms on the right-hand side represent the entropy of mixing. For large polymers of $N_A >> 1$ and $N_B >> 1$ these terms are negligibly small. This implies that for a stable mixture to exist χ so for polymers A and B to blend their segments must attract one another.

3.4.2 Osmotic Pressure

Polymers can separate out from the solvent, and do so in a characteristic way. The Flory-Huggins free energy per unit volume, for a polymer with N monomers, can be written in a simple dimensionless form

$$f = \frac{\phi}{N}\ln\phi + (1-\phi)\ln(1-\phi) + \chi\phi(1-\phi)$$

For ϕ the volume fraction of monomers, and $N >> 1$. The osmotic pressure (in reduced units) is:

$$\Pi = \phi/N - \ln(1-\phi) - \phi - \chi\phi^2.$$

The polymer solution is stable with respect to small fluctuations when the second derivative of this free energy is positive. This second derivative is

$$f'' = \frac{1}{N\phi} + \frac{1}{1-\phi} - 2\chi$$

And solution first becomes unstable then this and the third derivative:

$$f''' = -1/(N\phi^2) + 1/(1-\phi)^2$$

Are both equal to zero. A little algebra then shows that the polymer solution first becomes unstable at a critical point at

$$\chi_{CP} \simeq 1/2 + N^{-1/2} + \cdots \qquad \phi_{CP} \simeq N^{-1/2} - N^{-1} + \cdots$$

This means that for all values of $0 < \chi \lesssim 1/2$ the monomer-solvent effective interaction is weakly repulsive, but this is too weak to cause liquid/liquid separation. However, when $\chi > 1/2$, there is separation into two coexisting phases, one richer in polymer but poorer in solvent, than the other.

The unusual feature of the liquid/liquid phase separation is that it is highly asymmetric: the volume fraction of monomers at the critical point is approximately $N^{-1/2}$ which is very small for large polymers. The amount of polymer in the solvent-rich/polymer-poor coexisting phase is extremely small for long polymers. The solvent-rich phase is close to pure solvent. This is peculiar to polymers, a mixture of small molecules can be approximated using the Flory-Huggins expression with $N=1$, and then $\phi_{cp}=1/2$} and both coexisting phases are far from pure.

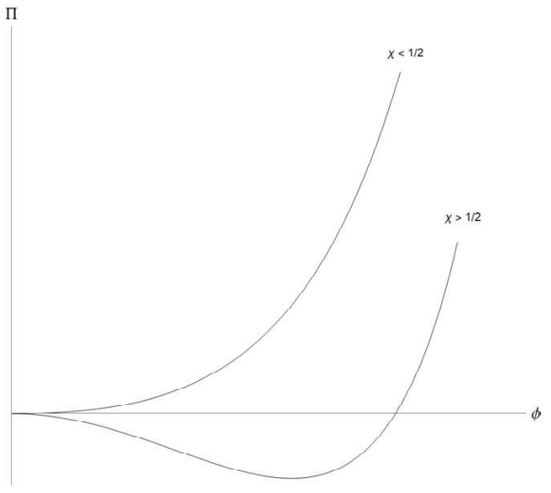

Fig. 3.24 *Osmotic Pressure for a Polymer Solution in two Regimes of Interaction Parameter*

3.4.3 Membrane Equilibria and Muscular Contraction

A semipermeable membrane used to separate two liquid phases can, in principle, be permeable to certain species and impermeable to others. A membrane, however, may not be perfect in this respect over a long time period. We will assume that during the period of observation, those species to which the membrane is supposed to be permeable quickly achieve transfer equilibrium, and only negligible amounts of the other species are transferred across the membrane.

The conditions needed for equilibrium in a two-phase system in which a membrane permeable only to solvent separates a solution from pure solvent. We can generalize the results for any system with two liquid phases separated by a semipermeable membrane: in an equilibrium state, both phases must have the same temperature, and any species to which the membrane is permeable must have the same chemical potential in both phases. The two phases, however, need not and usually do not have the same pressure.

Osmotic Membrane Equilibrium

An equilibrium state in a system with two solutions of the same solvent and different solute compositions, separated by a membrane permeable only to the solvent, is called an osmotic membrane equilibrium.

Consider a system with transfer equilibrium of the solvent across a membrane separating phases **α** and **β**. The phases have equal solvent chemical potentials but different pressures:

$$\mu^{\beta}_{A}(p^{\beta})=\mu^{\alpha}_{A}(p^{\alpha}) \qquad (23)$$

The dependence of μ_A on pressure in a phase of fixed temperature and composition is given by $(\delta_{\mu A}/\delta p)_{T,\{ni\}}=V_A$ where V_A is the partial molar volume of **A** in the phase. If we apply this relation to the solution of phase **β,** treat the partial molar volume V_A as independent of pressure, and integrate at constant temperature and composition from the pressure of phase **α** to that of phase **β**, we obtain

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$$\mu_A^{\beta}(p^{\beta}) = \mu_A^{\beta}(p^{\alpha}) + V_A^{\beta}(p^{\beta} - p^{\alpha})$$

By equating the two expressions for $\mu^{\beta}_{A}(p^{\beta})$ and rearranging, we obtain the following expression for the pressure difference needed to achieve transfer equilibrium:

$$p^{\beta} - p^{\alpha} = \frac{\mu_A^{\alpha}(p^{\alpha}) - \mu_A^{\beta}(p^{\alpha})}{V_A^{\beta}} \quad \textbf{(24)}$$

The pressure difference can be related to the ***osmotic pressures*** of the two phases. The solvent chemical potential in a solution phase can be written $\mu A(p)=\mu^{*}_{A}(p)-V_A\Pi(p)$. Using this to substitute for $\mu^{\alpha}_{A}(p^{\alpha})$ and $\mu^{\beta}_{A}(p^{\alpha})$ in Equation 24, we obtain

$$p^{\beta} - p^{\alpha} = \Pi^{\beta}(p^{\alpha}) - \left(\frac{V_A^{\alpha}}{V_A^{\beta}}\right)\Pi^{\alpha}(p^{\alpha})$$

Equilibrium Dialysis

Equilibrium dialysis is a useful technique for studying the binding of a small uncharged solute species (a ligand) to a macromolecule. The macromolecule solution is placed on one side of a membrane through which it cannot pass, with a solution without the macromolecule on the other side, and the ligand is allowed to come to transfer equilibrium across the membrane. If the same solute standard state is used for the ligand in both solutions, at equilibrium the unbound ligand must have the same activity in both solutions. Measurements of the total ligand molality in the macromolecule solution and the ligand molality in the other solution, combined with estimated values of the unbound ligand activity coefficients, allow the amount of ligand bound per macromolecule to be calculated.

Donnan Membrane Equilibrium

If one of the solutions in a two-phase membrane equilibrium contains certain charged solute species that are unable to pass through the membrane, whereas other ions can pass through, the situation is more complicated than the osmotic membrane equilibrium. Usually if the membrane is impermeable to one kind of ion, an ion species to which it is permeable achieves transfer equilibrium across the membrane only when the phases have different pressures and different electric potentials. The equilibrium state in this case is a ***Donnan membrane equilibrium***, and the resulting electric potential difference across the membrane is called the ***Donnan potential***. This phenomenon is related to the membrane potentials that are important in the functioning of nerve and muscle cells (although the cells of a living organism are not, of course, in equilibrium states). A Donnan potential can be measured electrically, with some uncertainty due to unknown liquid junction potentials, by connecting silver-silver chloride electrodes to both phases through salt bridges.

Muscular Contraction

The interaction between water and the protein of the contractile machinery as well as the tendency of these proteins to form geometrically ordered structures provide

a link between ***water and muscular contraction.*** Protein osmotic pressure is strictly related to the chemical potential of the contractile proteins, to the stiffness of muscle structures and to the viscosity of the sliding of the thin over the thick filaments. Muscle power output and the steady rate of contraction are linked by modulating a single parameter, a viscosity coefficient. Muscle operation is characterized by working strokes of much shorter length and much quicker than in the classical model. As a consequence the force delivered and the stiffness attained by attached cross-bridges is much larger than usually believed.

Energy Generation in Mechanochemical System

Mechanochemistry employs mechanophores (force-sensitive molecules) to produce chemical changes in a material in response to an applied mechanical force. Mechanophores can be linked into the polymer backbone or as crosslinks in a polymer network.

A mechanism for contraction in skeletal muscle is proposed in which the tension-generating site is located within the core of the thick (myosin) filament, specifically within the trypsin-sensitive hinge region of the myosin rod. The force-developing mechanism is thought to be the transfer of energy from ATP splitting in the globular head of one molecule directly to the hinge region of an adjoining molecule, resulting in a phase transition from crystalline to amorphous within the hinge segment of the second molecule. Binding of MgATP at the actinmyosin interface is considered to be a release mechanism. The model leads to out-of-phase oscillating movement of the cross bridges.

Check Your Progress

10. What do you understand by quasilattice theory?
11. State the Flory-Huggins solution theory.
12. Define the osmotic membrane equilibrium.
13. What provide a link between water and muscular contraction?

3.5 BIOPOLYMERS AND THEIR MOLECULAR WEIGHT

To get the number average molecular weight you divide the total weight of the sample by the total number of the molecules.

Since polymerization reactions of both synthetic and natural, can lead to high molecular weight compounds, the reaction chain is however broken by some termination process that usually occurs in a random manner with respect to the size, to which the polymer has already grown. It follows, therefore, that polymers possess a range of molecular weights and that any data for the size or weight of the molecules of polymer must represent some sort of average value. The molecular weights of polymers lend themselves to two types of averages, i.e., (i) number average molecular weights and (ii) weight average molecular weights.

Number Average Molecular Weight: It is defined as the weight of the sample divided by the total number of moles n in the sample, i.e.,

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$$M_n = \frac{\text{Weight}}{n}$$

If a method of molecular weight determination leads to the number of particles, or molecules; that are present, in a given weight of a sample, it will give the number average molecular weight. Let us consider a polymer as made up of fractions consisting of n_1 moles of molecular weight M_1, n_2 moles of molecular weight M_2 and so on, then M_n the number average molecular weight is given by the expression:

$$M_n = \frac{n_1 M_1 + n_2 M_2 + ...}{n_1 + n_2 ....}$$

$$M_n = \frac{\sum_i n_1 M_i}{\sum_i n_i}$$

On the other hand if in an experiment each particle makes a contribution to the measured result according to its molecular weight, we get weight average molecular weight of the polymer, Mw which is defined as,

$$M_w = \frac{\sum_i n_1 M_i^2}{\sum_i n_i M_i}$$

Molecular weight of a polymer can be determined by relative or absolute methods. Many properties of polymers which depend upon molecular weight, such as –solubility, elasticity, absorption on solids, and their tear strength, can be corrected with an average molecular weight. Once correlated, the property can be used as a measure of molecular weight. The weight determination can be done by chemical or physical methods of functional group analysis, by measurement of the colligative properties, light scattering or ultra-centrifugation or by measurement of dilute solution viscosity. In all these methods, except the last, molecular weights can be calculated without reference to calibration by another method and therefore, are absolute methods. Dilute solution viscosity is not a direct measure of molecular weight. All molecular weight methods, except a few end group analysis methods, require solubility of the polymer and all involve extrapolation to infinite dilution of operation in a solvent in which ideal solution behaviour is attained. Some important methods used in practice for determining the molecular weight and size of macromolecules in dilute solutions are discussed below.

Some of the important methods employed for the determination of molecular weights of polymers are:

Osmometry

Osmometry can be used for determining molecular masses from 104 to 106. This is one of the most accurate methods, though due to its lengthy and tedious experimental procedure it cannot be frequently used for fast molecular weight determination. The method, involves the measurement of the osmotic pressure of

solutions at several concentrations. The instrument used for the determination of molecular-weights by this method is called osmometer. In an osmometer, the solvent is separated from the solution by a semipermeable membrane. It then passes through the latter into the solution, until the level of the solution in the capillary stops rising. The osmotic pressure, at this stage, is equal to the weight of the hydrostatic column of the solution.

McMillan and Mayer showed that the osmotic pressure of non-electrolytes could be represented by a power series in the concentrations exactly like the virial equation for a non-ideal gas,

$$\Pi = RT\left(\frac{C}{M_n} + BC^2 + DC^3 + ...\right)$$

Where C is the concentration in mass per unit volume. In a fairly dilute solution of a polymer

$$\frac{\Pi}{C} = \frac{RT}{M_n} + RTBC$$

In low concentrations region a plot of $\frac{\Pi}{C}$ vs should give a straight line. The capillary forces also raise the liquid in the capillary to some extent, to account for these forces a capillary having radius equal to that of osmometer capillary, is immersed in the liquid. This capillary is known as control capillary. The difference in the level of osmometer capillary and control capillary is the measure of osmotic pressure. The two more commonly used methods are:

1. **Static Method:** This method involves the measurement of the equilibrium difference of levels in the osmometer. The osmometer for this method are very simple in design, but equilibrium in such an osmometer is reached after a long time. The concentration of the solution near the membrane may increase as a result of absorption of polymer, at equilibrium. The shortcoming can be eliminated by proper production and storage of membranes. For cross linking polymers this method is usually not recommended.
2. **Dynamic Method:** This method consists of measuring the rate of penetration of solvent through the membrane, depending upon the pressure applied. The main advantage of this method is that the measurements take very less time as compared to the static method.

   Choice of Membrane: A choice of suitable membrane plays an important role in osmometric measurements. The membrane should be permeable to the solvent and impermeable to the polymeric solute. It should satisfy the following two conditions:

   - The membrane should not swell to any great extent in the solvent.
   - It should have sufficient fine pores to allow the solvent molecules to pass through freely.

These days' membranes are prepared from cellophane and specially treated films of denitrated cellulose nitrate. The untreated cellophane membranes have small pore radius and subsequently take long time to reach equilibrium. To increase the pore

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size, the cellophane is treated with ammonia solution or other reagents. For this reason only cellophane is generally used for measuring molecular weights of very low-molecular weight polymers. Denitrated cellulose nitrate membrancs give better and much more satisfactory results.

The polymer is first throughly freed from impurities by reprecipitation before determining the molecular-weight. A stock solution, usually 1 per cent is prepared from a purified polymer. From the stock solution, working solutions of various concentrations, varying from 0.075 per cent to 0.5 per cent are prepared by dilution. One fifth of the one per cent solution is left undiluted for exact determination of its concentration. In order to measure the osmotic pressure, a polymer solution and pure solvent are placed on the opposite sides of a semipermeable membrane. The membrane is so selected as to permit only solvent and not the polymer particles. To establish equilibrium, the pressure on the solution side must be greater. The osmotic pressure, can be measured at several concentrations either by waiting for equilibrium or by measuring and compensating for pressures automatically.

To calculate molecular weight of a polymer the osmotic pressure values obtained for the different solutions are divided by the corresponding concentrations and the resulting reduced osmotic pressures are plotted in p/Cn versus C. The straight line obtained in this way is extrapolated to zero concentration as shown in Figure 3.25. The resulting values A (intercept with the ordinate axis) is substituted in the equation given below and molecular weight is calculated.

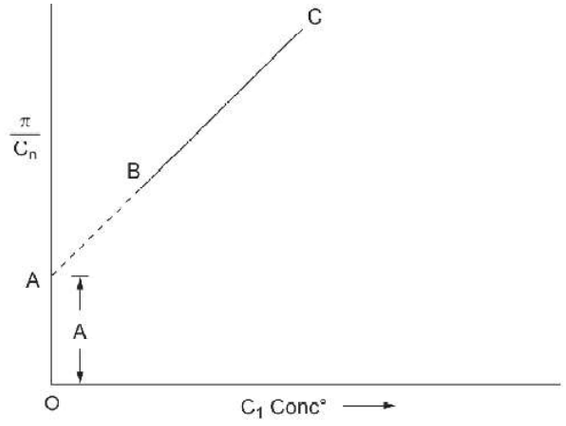

Fig. 3.25 *Variation of reduced osmotic pressure with concentration*

$$\left(\frac{\pi}{C_n}\right)_{C_n \to o} = \frac{RT}{M}$$

or
$$M = \frac{RT}{\left(\frac{\pi}{C_n}\right) C_{n \to 0}}$$

where, π = osmotic pressure (g/cm^2)$h\rho$

h = difference in liquid levels at equilibrium, cm

ρ = density of solvent, g/cm^3

C = concentration, g/cm^3

T = absolute temperature, K

M = molecular-weight, g/mole

R = gas constant, 8.48×10^4 g-cm/(mole) (K)

Viscometry

The first person to draw attention to the usefulness of solution viscosity as a measure of polymer molecular weight was Staudinger, who established the following relationship:

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$$\eta_{sp} = KMC$$

Where, η_{sp} = specific viscosity of polymer solution

K = constant

M = molecular weight of a dissolved polymer

C = concentration of polymer in solution

However, the above relation suffers from two serious drawbacks, first, the coefficient K does not give a constant value, but it depends on the molecular weight of a polymer and secondly, the equation represents the concentration dependence of specific viscosity incorrectly. According to the above equation, the reduced viscosity (ratio of the specific viscosity to the concentration) η_{sp}/C, is independent of concentration.

$$\eta_{sp}/C, = KM$$

But experimental data show that the η_{sp}/C_{vs}. C dependence is represented graphically by a straight line with a definite slope. Hence, it would be more accurate to relate molecular weight of a polymer to intrinsic (viscosity value that is independent of the solution concentration) and not to specific viscosity. Thus, Staudinger's equation can be modified by the following empirical relation,

$$[\eta] = KM^a$$

Where, $[\eta]$= intrinsic viscosity of a solution, K and a are the constants characterizing the given polymer-solvent system.

Intrinsic viscosity is determined from the relative viscosity of the dissolved polymer solutions. Then the specific and reduced viscosities are calculated and a plot of η_{sp}/C_{vs}. C is made. The extrapolation of this line to zero concentration, gives the value of $[\eta]$.K and a are calculated by plotting log $[\eta]$ *vs*. log M, which represents a straight line having slope, a and intercept log K.

Light Scattering

Light scattering accounts for many phenomena including the colours of the sky the rainbow, and of most white materials. A media may be transparent or turbid. The turbidity of a medium is due to scattering of light. The scattering of light occurs whenever a beam of light encounters matter. Similarly, when a polymer is dissolved in a solvent, the light scattered by the polymer far exceeds that scattered by the solvent, and is an absolute measure of molecular weight.

Debye gave a solution for the factor of internal interference $P(\theta)$ for the scattering particles of different shape. The Debye relationship is:

$$\frac{H'C}{K} - \frac{1}{MP(\theta)} + 2A_2C$$

Where H' is a lumped constant including geometric factors and also the change in refractive index with polymer concentration for the particular system being

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investigated. θ is the angle at which the intensity of light is measured and **C** is the concentration. $\mathbf{A_2}$, second virial coefficient and **P(θ)** (function of molecular shape) are derived from data. The light intensity factor **K** is derived from the galvanometer reading $\mathbf{I_g}$. The geometry and the scattering by solvent $\mathbf{I_{gs}}$ is compensated by

$$K = \frac{(I_g - I_{gs})\sin\theta}{1 + \cos^2\theta}$$

Since at **θ = 0, P (θ)** = 1, it is customary to extrapolate to $\boldsymbol{\nu = 0}$ as well as **C = 0.** A plot of $\frac{H'C}{K}$ **vs C** constant **θ** and $\frac{1}{MP(\theta)}$ vs. $\sin^2(\theta/2)$ give intercept 1/M. In Zimm plot, both can be done simultaneously where a network is obtained. This method is more precise and makes it possible to calculate the values of molecular masses of polymers without assumption of the shape of macromolecules in a solution.

The presence of dust particles or gelled polymer particles cause a major practical problem in light scattering. A small concentration of these, obscures the scattering by ordinary polymer particles and result in $\bar{M}_w = \infty$. The removal of dust can be done by ultra centrifugation.

Sedimentation Equilibrium

Sedimentation equilibrium in a suspension of different particles, such as molecules, exists when the rate of transport of each material in any one direction due to sedimentation equals the rate of transport in the opposite direction due to diffusion. Sedimentation is due to an external force, such as gravity or centrifugal force in a centrifuge. It was discovered for colloids by Jean Baptiste Perrin for which he received the Nobel Prize in Physics in 1926.

Colloid

In a colloid, the colloidal particles are said to be in sedimentation equilibrium if the rate of sedimentation is equal to the rate of movement from Brownian motion. For dilute colloids, this is described using the Laplace-Perrin distribution law:

$$\Phi(z) = \Phi_0 \exp\left(-\frac{m^* g}{k_B T} z\right) = \Phi_0 e^{-z/l_g}$$

where

$\Phi(z)$ is the colloidal particle volume fraction as a function of vertical distance z above reference point $z = 0$,

Φ_0 is the colloidal particle volume fraction at reference point $z = 0$,

m^* is the buoyant mass of the colloidal particles,

g is the standard acceleration due to gravity,

k_B is the Boltzmann constant,

T is the absolute temperature,

and l_g is the sedimentation length.

The buoyant mass is calculated using $m^* = \Delta\rho V_P = \frac{4}{3}\pi\Delta\rho R^3$

Where **Δρ** is the difference in mass density between the colloidal particles and the suspension medium, and $\boldsymbol{V_P}$ is the colloidal particle volume found using the volume of a sphere ***R*** is the radius of the colloidal particle).

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Sedimentation Length

The ***Laplace-Perrin distribution law*** can be rearranged to give the sedimentation length l_g**.** The sedimentation length describes the probability of finding a colloidal particle at a height z above the point of reference z**=0.** At the length l_g above the reference point, the concentration of colloidal particles decreases by a factor of e.

$$l_g = \frac{k_B T}{m^* g}$$

If the sedimentation length is much greater than the diameter d of the colloidal particles ($l_g >> d$) the particles can diffuse a distance greater than this diameter, and the substance remains a suspension. However, if the sedimentation length is less than the diameter ($l_g << d$) the particles can only diffuse by a much shorter length. They will sediment under the influence of gravity and settle to the bottom of the container. The substance can no longer be considered a colloidal suspension. It may become a colloidal suspension again if an action to undertaken to suspend the colloidal particles again, such as stirring the colloid.

Determination of Molecular Weight by Hydrodynamic Method

The most popular and widely applied hydrodynamic methods used in electrochemistry are those that involve rotating electrodes such as the Rotating Disk Electrode (RDE) and the Rotating Ring–Disk Electrode (RRDE). RDEs and RRDEs are found in most electrochemical laboratories.

The ***hydrodynamic parameters*** of the major protein fraction, viz. arachin from groundnut, alpha-globulin from sesame seed, brassin (M) from mustard seed and helianthinin from sunflower seed, have been determined in a single solvent system (0.05 M Tris-HCl buffer, pH 7.5 containing 0.5 M sodium chloride): sedimentation coefficient (s0(20,w)) and diffusion coefficient (D0(20,w)) by analytical ultracentrifugation, intrinsic viscosity by Ostwald viscometry and partial specific Volume (V) by densimetry. The molecular weights (M) of the four proteins, calculated using the sedimentation-viscosity and sedimentation-diffusion coefficient methods, were found close to each other.

Determination of Molecular Weight by Diffusion Method

Determination of the aggregation and solvation numbers of organometallic complexes in solution is an important task to increase insight in reaction mechanisms. Thus knowing which aggregates are formed during a reaction is of high interest to develop better selectivity and higher yields. Diffusion-Ordered Spectroscopy (DOSY), which separates NMR signals according to the diffusion coefficient, finds increasing use to identify species in solution. However, there still is no simple relationship between diffusion coefficient and Molecular Weight (MW). Some methods have been developed to estimate the MW but still with a significant error of ±30%. Here we describe a novel development of MW-determination by using an External Calibration Curve (ECC) approach with normalized diffusion coefficients. Taking the shape of the molecules into account enables accurate MW-predictions with a maximum error of smaller than ±9%. Moreover we show that the addition of multiple internal references is dispensable. One internal reference (that also can be the solvent) is sufficient. If the solvent signal is not accessible, 16

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other internal standards (aliphatics and aromatics) are available to avoid signal overlapping problems and provide flexible choice of analytes. This method is independent of NMR-device properties and diversities in temperature or viscosity and offers an easy and robust method to determine accurate MWs in solution.

Chemists have always had a vital interest in the size of molecules. Especially the aggregation and solvation numbers of organometallic intermediates play a dominant role in reaction mechanisms and product forming. Therefore the knowledge of reactive aggregates is a prerequisite to understand how molecules react and why they form which products. Apart from colligative property measurements, such as cryoscopy, Diffusion-Ordered Spectroscopy (DOSY) has gained increasing importance in this area. The physical observable that can be derived from the diffusion NMR experiment is the diffusion coefficient D that is sensitive to size and shape of the molecular species. A number of empirical methods for relating diffusion coefficients to the Molecular Weight (MW) have been proposed. Often the Stokes–Einstein equation and its modifications are useful and enable molecular size estimation of large particles that are much larger than the solvent. Besides that, especially the empirically derived power law is probably the most powerful class of relations which correlates the MW and the diffusion coefficient according to

$$\mathbf{D = KMW^{\alpha}}$$

This power law gives good results but is restricted to a specific class of compounds. Especially the polymer community applied it to estimate the MW distribution of polymer solutions like, e.g., globular proteins, oligosaccharides, polyethyleneoxides and denatured peptides in variable solvents. Even small molecules correlate to the power law, as demonstrated in the work of Crutchfield and Harris.15 Unfortunately this work only allows a MW estimation with a relatively high error of ±30%. For small organometallic molecules Li and Williard *et al.* have used an analogue approach of the power law by introducing at least three internal references to one NMR sample in order to get an Internal Calibration Curve (ICC). For small molecules this method gives much better results. They were able to characterize for example THF-solvated LDA to be dimeric17 and a 1 : 1 mixture of LiHMDS with HMPA. (Hexa Methyl Phosphor Amide) to adopt a disolvated dimeric structure in TOL-d_8 solution.

Determination of Molecular Weight by Sedimentation Velocity Method

Sedimentation velocity is an Analytical Ultra Centrifugation (AUC) method that measures the rate at which molecules move in response to centrifugal force generated in a centrifuge. This sedimentation rate provides information about both the molecular mass and the shape of molecules.

A new method for the direct molecular mass determination from sedimentation velocity experiments is presented. It is based on a non-linear least squares fitting procedure of the concentration profiles and simultaneous estimation of the sedimentation and diffusion coefficients using approximate solutions of the ***Lamm equation***. A computer program, LAMM, was written by using five different model functions derived by Fujita (1962, 1975) to describe the sedimentation of macromolecules during centrifugation. To compare the usefulness of these equations

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for the analysis of hydrodynamic results, the approach was tested on data sets of Claverie simulations as well as experimental curves of some proteins. A modification for one of the model functions is suggested, leading to more reliable sedimentation and diffusion coefficients estimated by the fitting procedure. The method seems useful for the rapid molecular mass determination of proteins larger than 10 kDa. One of the equations of the Archibald type is also suitable for compounds of low molecular mass, probably less than 10 kDa, because this model function requires neither the plateau region nor a meniscus free of solute.

Sedimentation velocity experiments have traditionally been used for samples with relatively high sedimentation coefficients and low diffusion. Such samples give sharp boundaries from which it is relatively easy to extract the sedimentation coefficient, and which permit the separation of multicomponent samples into distinct boundaries. However, many proteins of interest for therapeutic purposes, such as cytokines and growth factors, have molecular masses of only 10–40 kDa. Even at 60000 rpm, such small molecules give very broad boundaries which are difficult to analyse by existing techniques.

Sedimentation Coefficient

The ***sedimentation coefficient*** (s) of a particle characterizes its sedimentation during centrifugation. It is defined as the ratio of a particle's sedimentation velocity to the applied acceleration causing the sedimentation.

$$s = \frac{v_t}{a}$$

The sedimentation speed v_t (in m/s) is also the terminal velocity. It is constant because the force applied to a particle by gravity or by a centrifuge (typically in multiples of tens of thousands of gravities in an ultracentrifuge) is balanced by the viscous resistance (or 'Drag') of the fluid (normally water) through which the particle is moving. The applied acceleration a (in m/s^2) can be either the gravitational acceleration g, or more commonly the centrifugal acceleration $\omega^2 r$. In the latter case, ω is the angular velocity of the rotor and r is the distance of a particle to the rotor axis (radius).

Rotational Motion

One of the dynamical problems studied theoretically and experimentally very extensively is the rotational motion of molecules in liquids. The molecular rotation in gases is solely determined by the ***kinetic energy*** and the moment(s) of inertia of the molecule. Under the action of frequent random collisions with other molecules, the rotation of a particular molecule in a liquid is continuously perturbed, and this is reflected in an exponential time dependence of the correlation function of the orientation of the molecular axes. Generally, at short times of the order of 0.1 ps and less, the motion is determined by the molecular moment of inertia and the temperature, whereas at longer times, the motion is determined by angular momentum exchange due to the frequent collisions with other molecules. This can be described by a friction exerted on the rotating molecule by its neighbours. It was shown by Debye that under certain simplifying assumptions this so-called rotational diffusion can be described by a relaxation time τ_{OR} which is connected to the macroscopic viscosity η of the liquid:

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$$\tau_{OR} = \eta V / kT$$

In this equation **V** is a characteristic volume, the *hydrodynamic volume* of the molecule. If the shape and size of the rotating molecule are known, this relation can be used to probe the local viscosity in liquid systems (e.g., in micelles and membranes). Such a local viscosity can be different from the macroscopic viscosity and is accessible only through measurements done on label molecules.

Since molecular labels are convenient indicators of the local microdynamics of the liquid in their neighbourhood, they can also be used to test theoretical models of liquid-state dynamics. The experimental methods currently used are NMR relaxation, Raman linewidth measurements, dynamic light-scattering spectroscopy, fluorescence anisotropy, and dielectric relaxation. The *theory of rotating molecules* in a liquid medium interacting in an uncorrelated random fashion with the surroundings has been described by models amenable to analytical calculations in the case of simple liquids. The quantities that enter the calculation are molecular moments of inertia, molecular masses, and intermolecular forces. In the case of more complex liquids, the assumption of a diffusive motion in a continuum is made, and the parameters of the model are hydrodynamic quantities that can be compared with the corresponding macroscopic data (e.g., the macroscopic shear viscosity).

The *translational motion* of very large molecules in liquids, such as diluted polymers or supramolecular aggregates like micelles and micro emulsions, has been studied by light-scattering methods to obtain information about the molecular weight and size of the diffusing entity, its polydispersity, the interactions with other species (e.g., ions) or, at higher concentrations, interactions between the diffusing molecules themselves, and, finally, the internal flexibility and the rate of configurational changes.

3.5.1 Electrophoresis

SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules. (Refer Figure 3.26).

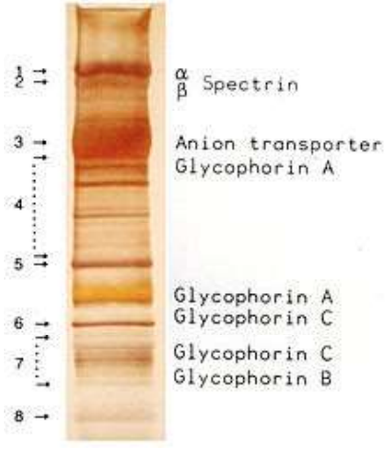

Fig. 3.26 *Proteins of the Erythrocyte Membrane Separated by SDS-PAGE According to Their Molecular Masses*

SDS-PAGE is a discontinuous electrophoretic system developed by Ulrich K. Laemmli which is commonly used as a method to separate proteins with molecular masses between 5 and 250 KDa. The publication describing it is the most frequently cited paper by a single author, and the second most cited overall.

Procedure

The SDS-PAGE method is composed of gel preparation, sample preparation, electrophoresis, protein staining or western blotting and analysis of the generated banding pattern.

Gel Production

When using different buffers in the gel (discontinuous gel electrophoresis), the gels are made up to one day prior to electrophoresis, so that the diffusion does not lead to a mixing of the buffers. The gel is produced by radical polymerisation in a mold consisting of two sealed glass plates with spacers between the glass plates. In a typical mini-gel setting, the spacers have a thickness of 0.75 mm or 1.5 mm, which determines the loading capacity of the gel. For pouring the gel solution, the plates are usually clamped in a stand which temporarily seals the otherwise open underside of the glass plates with the two spacers. For the gel solution, acrylamide is mixed as gel-former (usually 4% V/V in the stacking gel and 10-12 % in the separating gel), methylenebisacrylamide as a cross-linker, stacking or separating gel buffer, water and SDS. By adding the catalyst TEMED and the radical initiator Ammonium Persulfhate (APS) the polymerisation is started. The solution is then poured between the glass plates without creating bubbles. Depending on the amount of catalyst and radical starter and depending on the temperature, the polymerisation lasts between a quarter of an hour and several hours. The lower gel (separating gel) is poured first and covered with a few drops of a barely water-soluble alcohol (usually buffer-saturated butanol or isopropanol), which eliminates bubbles from the meniscus and protects the gel solution of the radical scavenger oxygen. After the polymerisation of the separating gel, the alcohol is discarded and the residual alcohol is removed with filter paper. After addition of APS and TEMED to the stacking gel solution, it is poured on top of the solid separation gel. Afterwards, a suitable sample comb is inserted between the glass plates without creating bubbles. The sample comb is carefully pulled out after polymerisation, leaving pockets for the sample application. For later use of proteins for protein sequencing, the gels are often prepared the day before electrophoresis to reduce reactions of unpolymerised acrylamide with cysteines in proteins.

By using a gradient mixer, gradient gels with a gradient of acrylamide (usually from 4 to 12%) can be cast, which have a larger separation range of the molecular masses. Commercial gel systems (so-called pre-cast gels) usually use the buffer substance Bis-tris methane with a pH value between 6.4 and 7.2 both in the stacking gel and in the separating gel. These gels are delivered cast and ready-to-use. Since they use only one buffer (continuous gel electrophoresis) and have a nearly neutral pH, they can be stored for several weeks. The more neutral pH slows the hydrolysis and thus the decomposition of the polyacrylamide. Furthermore, there are fewer acrylamide-modified cysteines in the proteins. Due to the constant pH in collecting and separating gel there is no stacking effect.

Proteins in BisTris gels cannot be stained with ruthenium complexes. This gel system has a comparatively large separation range, which can be varied by using MES or MOPS in the running buffer.

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Sample Preparation

During sample preparation, the sample buffer, and thus SDS, is added in excess to the proteins, and the sample is then heated to 95°C for five minutes, or alternatively 70°C for ten minutes. Heating disrupts the secondary and tertiary structures of the protein by disrupting hydrogen bonds and stretching the molecules. Optionally, disulfide bridges can be cleaved by reduction. For this purpose, reducing thiols, such as α-mercaptoethanol (α-ME, 5% by volume), Dithiothreitol (DTT, 10 millimolar) or Dithioerythritol (DTE, 10 millimolar) are added to the sample buffer. After cooling to room temperature, each sample is pipetted into its own well in the gel, which was previously immersed in electrophoresis buffer in the electrophoresis apparatus.

Electrophoresis

Electrophoresis chamber after a few minutes of electrophoresis. In the first pocket a size marker was applied with bromophenol blue, in the other pockets, the samples were added bromocresol green

For separation, the denatured samples are loaded onto a gel of polyacrylamide, which is placed in an electrophoresis buffer with suitable electrolytes. Thereafter, a voltage (usually around 100 V, 10-20 V per cm gel length) is applied, which causes a migration of negatively charged molecules through the gel in the direction of the positively charged anode. The gel acts like a sieve. Small proteins migrate relatively easily through the mesh of the gel, while larger proteins are more likely to be retained and thereby migrate more slowly through the gel, thereby allowing proteins to be separated by molecular size. The electrophoresis lasts between half an hour to several hours depending on the voltage and length of gel used.

The fastest-migrating proteins (with a molecular weight of less than 5 KDa) form the buffer front together with the anionic components of the electrophoresis buffer, which also migrate through the gel. The area of the buffer front is made visible by adding the comparatively small, anionic dye bromophenol blue to the sample buffer. Due to the relatively small molecule size of bromophenol blue, it migrates faster than proteins. By optical control of the migrating colored band, the electrophoresis can be stopped before the dye and also the samples have completely migrated through the gel and leave it.

The most commonly used method is the discontinuous SDS-PAGE. In this method, the proteins migrate first into a collecting gel with neutral pH, in which they are concentrated and then they migrate into a separating gel with basic pH, in which the actual separation takes place. Stacking and separating gels differ by different pore size (4-6 % T and 10-20 % T), ionic strength and pH values (pH 6.8 or pH 8.8). The electrolyte most frequently used is an SDS-containing Tris-glycine-chloride buffer system. At neutral pH, glycine predominantly forms the zwitterionic form, at high pH the glycines lose positive charges and become

predominantly anionic. In the collection gel, the smaller, negatively charged chloride ions migrate in front of the proteins (as leading ions) and the slightly larger, negatively and partially positively charged glycinate ions migrate behind the proteins (as initial trailing ions), whereas in the comparatively basic separating gel both ions migrate in front of the proteins. The pH gradient between the stacking and separation gel buffers leads to a stacking effect at the border of the stacking gel to the separation gel, since the glycinate partially loses its slowing positive charges as the pH increases and then, as the former trailing ion, overtakes the proteins and becomes a leading ion, which causes the bands of the different proteins (visible after a staining) to become narrower and sharper - the stacking effect. For the separation of smaller proteins and peptides, the TRIS-Tricine buffer system of Schägger and von Jagow is used due to the higher spread of the proteins in the range of 0.5 to 50 KDa.

Gel Staining

Coomassie-stained 10% Tris/Tricine Gel. In the left lane, a molecular weight size marker was used to estimate the size (from top to bottom: 66, 45, 35, 24, 18 and 9 kDa). In the remaining lanes purified yeast proteins were separated.

At the end of the electrophoretic separation, all proteins are sorted by size and can then be analyzed by other methods, for example protein staining such as Coomassie staining (most common and easy to use), silver staining (highest sensitivity), stains all staining, Amido black 10B staining, Fast green FCF staining, fluorescent stains such as epicocconone stain and SYPRO orange stain, and immunological detection such as the Western Blot. The fluorescent dyes have a comparatively higher linearity between protein quantity and color intensity of about three orders of magnitude above the detection limit, i. e., the amount of protein can be estimated by color intensity. When using the fluorescent protein dye trichloroethanol, a subsequent protein staining is omitted if it was added to the gel solution and the gel was irradiated with UV light after electrophoresis.

Analysis

Protein staining in the gel creates a documentable banding pattern of the various proteins. Glycoproteins have differential levels of glycosylations and adsorb SDS more unevenly at the glycosylations, resulting in broader and blurred bands. Membrane proteins, because of their transmembrane domain, are often composed of the more hydrophobic amino acids, have lower solubility in aqueous solutions, tend to bind lipids, and tend to precipitate in aqueous solutions due to hydrophobic effects when sufficient amounts of detergent are not present. This precipitation manifests itself for membrane proteins in a SDS-PAGE in 'tailing' above the band of the transmembrane protein. In this case, more SDS can be used (by using more or more concentrated sample buffer) and the amount of protein in the sample application can be reduced. An overloading of the gel with a soluble protein creates a semi-circular band of this protein (e. g. in the marker lane of the image at 66 KDa), allowing other proteins with similar molecular weights to be covered. A low contrast (as in the marker lane of the image) between bands within a lane indicates either the presence of many proteins (low purity) or, if using purified proteins and a low contrast occurs only below one band, it indicates a proteolytic degradation of the protein, which first causes degradation bands, and after further

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degradation produces a homogeneous color ('Smear') below a band. The documentation of the banding pattern is usually done by photographing or scanning. For a subsequent recovery of the molecules in individual bands, a gel extraction can be performed.

Archiving

After protein staining and documentation of the banding pattern, the polyacrylamide gel can be dried for archival storage. Proteins can be extracted from it at a later date. The gel is either placed in a drying frame (with or without the use of heat) or in a vacuum dryer. The drying frame consists of two parts, one of which serves as a base for a wet cellophane film to which the gel and a one percent glycerol solution are added. Then a second wet cellophane film is applied bubble-free, the second frame part is put on top and the frame is sealed with clips. The removal of the air bubbles avoids a fragmentation of the gel during drying. The water evaporates through the cellophane film. In contrast to the drying frame, a vacuum dryer generates a vacuum and heats the gel to about 50°C.

Molecular Mass Determination

The proteins of the size marker (black X) show an approximately straight line in the representation of log M over RF. The molecular weight of the unknown protein (red X) can be determined on the y-axis.

For a more accurate determination of the molecular weight, the relative migration distances of the individual protein bands are measured in the separating gel. The measurements are usually performed in triplicate for increased accuracy. The relative mobility (called Rf value or Rm value) is the quotient of the distance of the band of the protein and the distance of the buffer front. The distances of the bands and the buffer front are each measured from the beginning of the separation gel. The distance of the buffer front roughly corresponds to the distance of the bromophenol blue contained in the sample buffer. The relative distances of the proteins of the size marker are plotted semi-logarithmically against their known molecular weights. By comparison with the linear part of the generated graph or by a regression analysis, the molecular weight of an unknown protein can be determined by its relative mobility. Bands of proteins with glycosylations can be blurred. Proteins with many basic amino acids can lead to an overestimation of the molecular weight or even not migrate into the gel at all, because they move slower in the electrophoresis due to the positive charges or even to the opposite direction. Accordingly, many acidic amino acids can lead to accelerated migration of a protein and an underestimation of its molecular mass.

Applications

The SDS-PAGE in combination with a protein stain is widely used in biochemistry for the quick and exact separation and subsequent analysis of proteins. It has comparatively low instrument and reagent costs and is an easy-to-use method. Because of its low scalability, it is mostly used for analytical purposes and less for preparative purposes, especially when larger amounts of a protein are to be isolated.

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Additionally, SDS-PAGE is used in combination with the western blot for the determination of the presence of a specific protein in a mixture of proteins - or for the analysis of post-translational modifications. Post-translational modifications of proteins can lead to a different relative mobility (i.e. a band shift) or to a change in the binding of a detection antibody used in the western blot (i.e., a band disappears or appears).

In mass spectrometry of proteins, SDS-PAGE is a widely used method for sample preparation prior to spectrometry, mostly using in-gel digestion. In regards to determining the molecular mass of a protein, the SDS-PAGE is a bit more exact than an analytical ultracentrifugation, but less exact than a mass spectrometry or, ignoring post-translational modifications, a calculation of the protein molecular mass from the DNA sequence.

In medical diagnostics, SDS-PAGE is used as part of the HIV test and to evaluate proteinuria. In the HIV test, HIV proteins are separated by SDS-PAGE and subsequently detected by Western Blot with HIV-specific antibodies of the patient, if they are present in his blood serum. SDS-PAGE for proteinuria evaluates the levels of various serum proteins in the urine, for example, Albumin, Alpha-2-Macroglobulin and IgG.

Variants

SDS-PAGE is the most widely used method for gel electrophoretic separation of proteins. Two-dimensional gel electrophoresis sequentially combines isoelectric focusing or BAC-PAGE with a SDS-PAGE. Native PAGE is used if native protein folding is to be maintained. For separation of membrane proteins, BAC-PAGE or CTAB-PAGE may be used as an alternative to SDS-PAGE. For electrophoretic separation of larger protein complexes, agarose gel electrophoresis can be used, for example the SDD-AGE. Some enzymes can be detected via their enzyme activity by zymography.

Alternatives

While being one of the more precise and low-cost protein separation and analysis methods, the SDS-PAGE denatures proteins. Where non-denaturing conditions are necessary, proteins are separated by a native PAGE or different chromatographic methods with subsequent photometric quantification, for example affinity chromatography (or even tandem affinity purification), size exclusion chromatography, ion exchange chromatography. Proteins can also be separated by size in a tangential flow filtration or a ultrafiltration. Single proteins can be isolated from a mixture by affinity chromatography or by a pull-down assay. Some historically early and cost effective but crude separation methods usually based upon a series of extractions and precipitations using kosmotropic molecules, for example the ammonium sulfate precipitation and the polyethyleneglycol precipitation.

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Check Your Progress

14. How do you find molecular weight from the number of molecules?
15. Give the uses of osmometry method.
16. What is sedimentation equilibrium?
17. Define the sedimentation coefficient.

3.6 ANSWERS TO 'CHECK YOUR PROGRESS'

1. Biopolymers are natural polymers produced by the cells of living organisms. Biopolymers consist of monomeric units that are covalently bonded to form larger molecules. There are three main classes of biopolymers, classified according to the monomers used and the structure of the biopolymer formed: polynucleotides, polypeptides, and polysaccharides.
2. There are three main classes of biopolymers, classified according to the monomers used and the structure of the biopolymer formed: polynucleotides, polypeptides, and polysaccharides.
3. A macromolecule is a very large molecule, such as a protein. They are composed of thousands of covalently bonded atoms. Many macromolecules are polymers of smaller molecules called monomers. The most common macromolecules in biochemistry are biopolymers (nucleic acids, proteins, and carbohydrates) and large non-polymeric molecules, such as lipids, nanogels and macrocycles.
4. For any chemical chain reaction, the chain length is defined as the average number of times that the closed cycle of chain propagation steps is repeated. It is equal to the rate of the overall reaction divided by the rate of the initiation step in which the chain carriers are formed.
5. The tertiary structure of protein shows a specific three-dimensional structure. The folding regular units of the secondary structure as well as of areas of the peptide chain devoid of secondary structure result in the formation of the tertiary structure.
6. The central core of a domain can be constructed from a helices, from b sheets, or from various combinations of these two fundamental folding elements. Each different combination is known as a protein fold.
7. Electrostatic intrections are also known as salf bridgs, these are formed between the charged interacting residues.
8. The energy potential of non-bonded interactions is due to electrostatic, H-bonds and Van der Waals forces. These interactions are crucial for the stability of the tertiary structure. Energy due to electrostatic forces can be calculated using Coulomb's law.
9. Hydrogen ion titration was performed to evaluate the protonation degree, α, of the monomers and micelles at a given pH. As described above, α represents the number of protons bound to a pyridylamine oxide molecule.

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10. The thermodynamics of biopolymers solutions is based upon the quasilattice theory of solutions, and leads to an equation for the free energy of mixing polymeric solute with monomeric solvent which contains a combinatorial entropy of mixing term and a regular solution theory enthalpy of mixing term.
11. Flory–Huggins solution theory is a lattice model of the thermodynamics of polymer solutions which takes account of the great dissimilarity in molecular sizes in adapting the usual expression for the entropy of mixing. The result is an equation for the Gibbs free energy change??GM for mixing a polymer with a solvent.
12. An equilibrium state in a system with two solutions of the same solvent and different solute compositions, separated by a membrane permeable only to the solvent, is called an osmotic membrane equilibrium.
13. The interaction between water and the protein of the contractile machinery as well as the tendency of these proteins to form geometrically ordered structures provide a link between water and muscular contraction. Muscle power output and the steady rate of contraction are linked by modulating a single parameter, a viscosity coefficient.
14. To get the number average molecular weight you divide the total weight of the sample by the total number of the molecules.
15. Osmometry can be used for determining molecular masses from 104 to 106. This is one of the most accurate methods, though due to its lengthy and tedious experimental procedure it cannot be frequently used for fast molecular weight determination.
16. Sedimentation equilibrium in a suspension of different particles, such as molecules, exists when the rate of transport of each material in any one direction due to sedimentation equals the rate of transport in the opposite direction due to diffusion.
17. The sedimentation coefficient (s) of a particle characterizes its sedimentation during centrifugation. It is defined as the ratio of a particle's sedimentation velocity to the applied acceleration causing the sedimentation.

3.7 SUMMARY

- There are three main classes of biopolymers, classified according to the monomers used and the structure of the biopolymer formed: polynucleotides, polypeptides, and polysaccharides.
- Biopolymers have applications in many fields including the food industry, manufacturing, packaging, and biomedical engineering.
- The convention for a polypeptide is to list its constituent amino acid residues as they occur from the amino terminus to the carboxylic acid terminus. The amino acid residues are always joined by peptide bonds.
- In addition, many saccharide units can undergo various chemical modifications, such as amination, and can even form parts of other molecules, such as glycoproteins.

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- The major building block of proteins are called alpha amino acids. As their name implies they contain a carboxylic acid functional group and an amine functional group.
- Proteins are very large molecules containing many amino acid residues linked together in very specific order. Proteins range in size from 50 amino acids in length to the largest known protein containing 33,423 amino acids.
- In polymer chemistry the kinetic chain length of a polymer, ν, is the average number of units called monomers added to a growing chain during chain-growth polymerization.
- For most chain-growth polymerizations, the propagation steps are much faster than the initiation steps, so that each growing chain is formed in a short time compared to the overall polymerization reaction.
- Some chain-growth polymerizations include chain transfer steps, in which another atom (often hydrogen) is transferred from a molecule in the system to the polymer radical. The original polymer chain is terminated and a new one is initiated.
- The linear sequence of amino acids in the polypeptide chain is known as the primary structure. The determination of the protein structure helps in the analysis of amino acids.
- The smallest protein molecules contain only a single domain, whereas larger proteins can contain as many as several dozen domains, usually connected to each other by short, relatively unstructured lengths of polypeptide chain.
- Disulphide bonds generally fail to form in the cell cytosol, where a high concentration of reducing agents converts S–S bonds back to cysteine –SH groups. Apparently, proteins do not require this type of reinforcement in the relatively mild environment inside the cell.
- A computational technique called threading can be used to fit an amino acid sequence to a particular protein fold.
- Amylopectin recrystallization was observed as an increased amount of fast-relaxing protons, while network strengthening and changes in water levels were noted as a reduced mobility and amount, respectively, of slowly relaxing protons.
- Hydrogen bond is week electrostatic interaction with a directional property. Hydrogen bond involves at least one hydrogen atom. In hydrogen bond, there is one hydrogen donor group and a hydrogen acceptor group.
- When two atoms are connected by a covalent bond, it maintain an equilibrium distance between them. If there is displacement of atoms from the equilibrium position leads to bond stretch energy, which contributes to the total potential energy.
- Also known as London forces, dispersion interactions occur between any adjacent pair of atoms or molecules when they are present in sufficiently close proximity. These interactions account for the attractive forces between non-ionic and nonpolar organic molecules, such as paraffin and many pharmaceutical drugs.

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- The widely used Flory-Huggins theory of polymer solution thermodynamics. It is based upon the quasilattice theory of solutions, and leads to an equation for the free energy of mixing polymeric solute with monomeric solvent which contains a combinatorial entropy of mixing term and a regular solution theory enthalpy of mixing term.
- We first calculate the entropy of mixing, the increase in the uncertainty about the locations of the molecules when they are interspersed. In the pure condensed phase's solvent and polymer everywhere we look we find a molecule.
- Equilibrium dialysis is a useful technique for studying the binding of a small uncharged solute species (a ligand) to a macromolecule. The macromolecule solution is placed on one side of a membrane through which it cannot pass, with a solution without the macromolecule on the other side, and the ligand is allowed to come to transfer equilibrium across the membrane.
- The conditions needed for equilibrium in a two-phase system in which a membrane permeable only to solvent separates a solution from pure solvent.
- An equilibrium state in a system with two solutions of the same solvent and different solute compositions, separated by a membrane permeable only to the solvent, is called an osmotic membrane equilibrium.
- Protein osmotic pressure is strictly related to the chemical potential of the contractile proteins, to the stiffness of muscle structures and to the viscosity of the sliding of the thin over the thick filaments. Muscle power output and the steady rate of contraction are linked by modulating a single parameter, a viscosity coefficient.
- Muscle operation is characterized by working strokes of much shorter length and much quicker than in the classical model. As a consequence the force delivered and the stiffness attained by attached cross-bridges is much larger than usually believed.
- Molecular weight of a polymer can be determined by relative or absolute methods. Many properties of polymers which depend upon molecular weight, such as –solubility, elasticity, absorption on solids, and their tear strength, can be corrected with an average molecular weight.
- In an osmometer, the solvent is separated from the solution by a semipermeable membrane.
- Light scattering accounts for many phenomena including the colours of the sky the rainbow, and of most white materials. A media may be transparent or turbid. The turbidity of a medium is due to scattering of light.
- Sedimentation is due to an external force, such as gravity or centrifugal force in a centrifuge.
- The most popular and widely applied hydrodynamic methods used in electrochemistry are those that involve rotating electrodes such as the Rotating Disk Electrode (RDE) and the Rotating Ring–Disk Electrode (RRDE). RDEs and RRDEs are found in most electrochemical laboratories.

- Since molecular labels are convenient indicators of the local microdynamics of the liquid in their neighbourhood, they can also be used to test theoretical models of liquid-state dynamics.

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3.8 KEY TERMS

- **Biopolymers:** Biopolymers are natural polymers produced by the cells of living organisms. Biopolymers consist of monomeric units that are covalently bonded to form larger molecules.
- **Sugar polymers:** Sugar polymers can be linear or branched and are typically joined with glycosidic bonds.
- **Macromolecule:** A macromolecule is a very large molecule, such as a protein. They are composed of thousands of covalently bonded atoms.
- **Amylopectin recrystallization:** Amylopectin recrystallization strengthened the starch network with concomitant inclusion of water and increased crumb firmness, especially at the beginning of storage.
- **Hydrophobic interactions**: Hydrophobic interactions also known as Van der Waals interactions, can be seen when non-polar atoms gather together to minimize their contact with water.
- **Choice of membrane:** A choice of suitable membrane plays an important role in osmometric measurements. The membrane should be permeable to the solvent and impermeable to the polymeric solute.
- **Colloid:** In a colloid, the colloidal particles are said to be in sedimentation equilibrium if the rate of sedimentation is equal to the rate of movement from Brownian motion.

3.9 SELF-ASSESSMENT QUESTIONS AND EXCERCISES

Short–Answer Questions

1. What do you understand by biopolymers?
2. Define sugar polymers.
3. What do you understand by chain configuration of biopolymers?
4. Give the primary structure of protein.
5. What is protein folding?
6. What do you understand by biopolymer interaction?
7. Define the multiple equilibria.
8. What is binding process in biological system?
9. Define the osmotic pressure.
10. How will you define the muscular contraction?

11. Calculate the molecular weight of biopolymer.
12. What do you understand by electrophoresis process?

Long –Answer Questions

1. Discuss about the biopolymers and their types.
2. Illustrate the chain configuration of macromolecules with the help of examples.
3. Explain in detail about the statistical distribution end-to-end dimension.
4. Describe the protein folding problem.
5. Discuss in detail about the biopolymer interaction with the help of examples.
6. Illustrate the hydrogen titration curve.
7. Analyse the thermodynamics of biopolymer solution.
8. Explain in detail about the energy generation in mechanochemical system and muscular contraction.
9. Calculate the molecular weight with the help of various types of methods.

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3.10 FURTHER READING

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UNIT 4 ENZYMES AND MOLECULAR ADAPTATION

NOTES

Structure

4.0 Introduction
4.1 Objectives
4.2 Molecular Adaptation and Proximity Effect
4.3 Enzymes
  4.3.1 Classification and Nomenclature of Enzymes
  4.3.2 Extraction and Purification of Enzymes
  4.3.3 Theories for Enzyme Action
  4.3.4 Enzyme Kinetics
  4.3.5 Lineweaver–Burk Methods
  4.3.6 Affinity Labelling of Enzyme
  4.3.7 Reversible and Irreversible Inhibitors
4.4 Mechanism of Enzyme Action
  4.4.1 Transitions State Theory of Enzyme
  4.4.2 Steric and Orientation Effect of Enzymes
  4.4.3 Acid-Base and Covalent Catalysis of Enzyme
  4.4.4 Example of Some Typical Enzyme Mechanism
4.5 Kinds of Reactions Catalysed by Enzyme
4.6 Answers to 'Check Your Progress'
4.7 Summary
4.8 Key Terms
4.9 Self-Assessment Questions and Exercises
4.10 Further Reading

4.0 INTRODUCTION

Molecular adaptation studies provide empirical evidence to understand why organismal features exist, to map the evolutionary trajectories that shape populations, and to help predict future adaptations. Proximity effects describe the orientation and movement of the substrate molecules when binding to enzyme active sites, and are most readily observed by comparing equivalent inter- and intramolecular reactions.

Enzymes are diverse types are proteinaceous molecules which function as reaction catalyst of various biochemical reactions of metabolism. In certain cases, enzymes possess higher catalysis power in comparison with inorganic or organic chemical catalysts. The enzymes possess high specificity for the substrates which they metabolize to form products. Enzymes function in mild temperature and pH in aqueous environments. The enzymes essentially function in suitable micro-environments of particular cofactor, pH, and temperature. The specialized feature of the enzyme catalysed biochemical reaction lies in the fact that the enzyme provides the confines of its active site. The chemical transformation of the substrate bound to the active site occurs by biochemical process.

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The idea is very simple; the specific action of an enzyme on a substrate can be explained using a Lock and Key analogy. In this analogy, the lock is the enzyme and the key is the substrate.The main idea is very simple and easy to understand. An enzyme inhibitor is a molecule that disrupts the normal reaction pathway between an enzyme and a substrate.

The Michaelis–Menten equation is the rate equation for a one-substrate enzyme-catalysed reaction. In biochemistry, the Lineweaver–Burk plot (or double reciprocal plot) is a graphical representation of the Lineweaver–Burk equation of enzyme kinetics.

An enzyme attracts substrates to its active site, catalyses the chemical reaction by which products are formed, and then allows the products to dissociate (separate from the enzyme surface). The combination formed by an enzyme and its substrates is called the enzyme–substrate complex. Enzyme catalysis is the increase in the rate of a process by a biological molecule, an 'Enzyme'. Most enzymes are proteins, and most such processes are chemical reactions. Within the enzyme, generally catalysis occurs at a localized site, called the active site.

In this unit, you will study about the molecular adaptation, proximity effects, enzymes, chemical and biological catalysis of enzyme, Fischer lock and key model, properties of enzyme, nomenclature and classification of enzyme, extraction and purification of an enzyme, Michaelis Menten and Lineweaver Burk plot, reversible and irreversible inhibition, mechanism of enzyme action, transition state theory, orientation and steric effect, acid-base and covalent catalysis, example of some typical enzyme mechanism for isoenzymes, carboxypeptidase A, chymotrypsin, ribonuclease, kind of reaction catalysis by enzymes, enzyme catalysed carboxylation and decarboxylation.

4.1 OBJECTIVES

After going through this unit you will be able to:

- Know about the molecular adaptation and proximity effects
- Analyse the extraction and purification of an enzyme
- Discuss about the Michaelis Menten and Lineweaver Burk plot
- Elaborate on the reversible and irreversible inhibition
- Explain the mechanism of enzyme action
- Understand the transition state theory
- Know about the orientation and steric effect
- Interpret the acid-base and covalent catalysis
- Determine the example of some typical enzyme mechanism for isoenzymes, carboxypeptidase A, chymotrypsin, ribonuclease
- Analyse the kind of reaction catalysis by enzymes
- Comprehend the enzyme catalysed carboxylation and decarboxylation

4.2 MOLECULAR ADAPTATION AND PROXIMITY EFFECT

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Molecular adaptation studies provide empirical evidence to understand why organismal features exist, to map the evolutionary trajectories that shape populations, and to help predict future adaptations.

In biology, *adaptation* has three related meanings. Firstly, it is the dynamic evolutionary process that fits organisms to their environment, enhancing their evolutionary fitness. Secondly, it is a state reached by the population during that process. Thirdly, it is a phenotypic trait or adaptive trait, with a functional role in each individual organism, i.e., maintained and has evolved through natural selection.

Advances in *bioinformatics* and *high-throughput genetic analysis* increasingly allow us to predict the genetic basis of adaptive traits. These predictions can be tested and confirmed, but the molecular-level changes, i.e., the molecular adaptation that link genetic differences to organism fitness remain generally unknown. In recent years, a series of studies have started to unpick the mechanisms of *adaptation at the molecular level*. In particular, this work has examined how changes in protein function, activity, and regulation cause improved organismal fitness. Key to addressing molecular adaptations is identifying systems and designing experiments that integrate changes in the genome, protein chemistry (molecular phenotype), and fitness. Knowledge of the molecular changes underpinning adaptations allow new insight into the constraints on, and repeatability of adaptations, and of the basis of non-additive interactions between adaptive mutations.

The study of molecular adaptation has long been fraught with difficulties, not the least of, which is identifying out of hundreds of amino acid replacements those few directly responsible for major adaptations. Six studies are used to illustrate how phylogenies, site-directed mutagenesis, and a knowledge of protein structure combine to provide much deeper insights into the adaptive process than has hitherto been possible. Ancient genes can be reconstructed, and the phenotypes can be compared to modern proteins. Out of hundreds of amino acid replacements accumulated over billions of years those few responsible for discriminating between alternative substrates are identified. An amino acid replacement of modest effect at the molecular level causes a dramatic expansion in an ecological niche. These and other topics are creating the emerging field of 'Paleomolecular Biochemistry'.

The origin and diversification of land plants was one of the most important biological radiations. Land plants are crucial components of all modern terrestrial ecosystems. The first land plants had to adapt to a wide array of new environmental challenges including desiccation, varying temperatures, and increased Ultra Violet or UV radiation. There have been numerous studies of the *morphological adaptations* to life on land. However the molecular adaptations to life on land have only recently gained attention. These studies have greatly benefited from the recent advances in our understanding of the phylogenetic

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relationships between and among the *charophycean algae* and the *basal land plant groups*.

Proximity Effect

Proximity effects describe the orientation and movement of the substrate molecules when binding to enzyme active sites, and are most readily observed by comparing equivalent inter- and intramolecular reactions. This effect of proximity and orientation is analogous to an effective increase in concentration of the reagents and endows the reaction an intramolecular character with a massive rate increase (Refer Figure 4.1). Intramolecular reactions between groups that are tied together in a single molecule are faster than the corresponding intermolecular reactions between two independent molecules. Difference in rates are 3-4 orders of magnitude (intramolecular> intermolecular). This is mostly owing to the differences between the entropy changes that accompany inter- and intramolecular reactions. The entropy of the reactants is reduced in intramolecular reaction, which principally occurs during its preparation process and makes addition or transfer reactions less unfavourable, since the integration of two reactants into a single product could decrease the reduction in the overall entropy. The entropy decrease involved in the formation of the transition state has been moved to an earlier step, the binding of the substrates to form the *enzyme-substrate complex*. However, in the intermolecular reaction the formation of product involves a much larger loss of translational and rotational entropy.

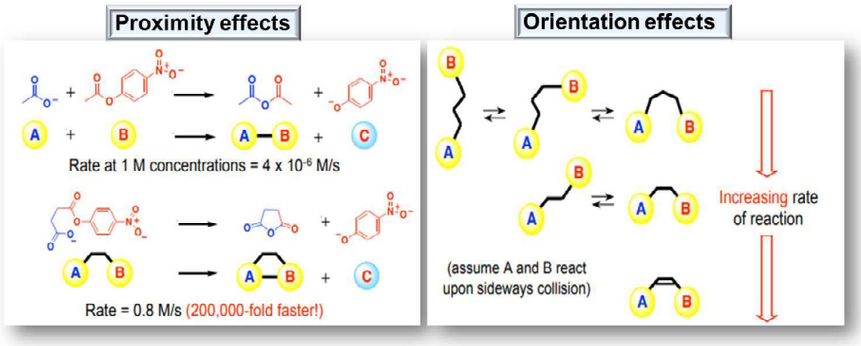

Fig. 4.1 *Proximity and Orientation Effect*

Requirements for Catalysis

It has been stated that a close proximity between a substrate and an enzyme active site does not mean that a catalysis reaction will occur. The enzyme must guide or steer the substrate into the active site in a specific orientation to make the reaction actually occur, which is called orientation. With time elapsing, orientation has evolved and become more efficient and more significant though difficult to quantify. Other requirements for such a reaction to occur are changes in solvation and electronic overlap, as well as overcoming of **Van der Waals forces**. Orientation effects and induction of strain are needed to meet these requirements.

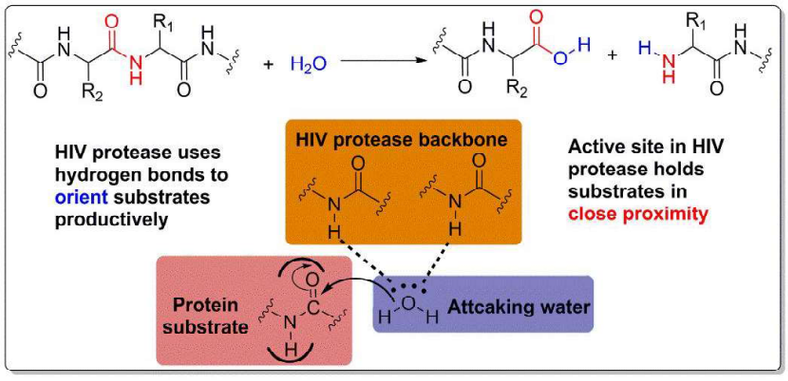

Fig.4.2 *Proximity and Orientation Effects on HIV Protease*

4.3 ENZYMES

Enzymes are diverse types are proteinaceous molecules which function as reaction catalyst of various biochemical reactions of metabolism. In certain cases, enzymes possess higher catalysis power in comparison with inorganic or organic chemical catalysts. The enzymes possess high specificity for the substrates which they metabolize to form products. Enzymes function in mild temperature and pH in aqueous environments. The enzymes essentially function in suitable micro-environments of particular cofactor, pH, and temperature. They sequentially catalyze the steps of biochemical reaction in a metabolic pathway. They convert various nutrient molecules into their derivative forms or function in transformation of chemical energy within the cell. The function of enzyme is also associated with the biosynthesis of macromolecules within the cell. These molecules act as storage compounds or function as structural components of the cell. The study of function of enzymes is necessary in the investigation of various diseases related to metabolism. The absence or hyperactivity of certain enzymes result in physiological disorder manifested as a diseases.

Moreover, various drugs interact with enzymes and accomplish their action in the cell. Enzymes possess immense practical applications in the food industry, food processing and agricultural aspects. Most of the enzymes are chemically Proteins in nature with exceptions for ribozymes which are self-catalytic RNA molecules. The discovery of enzyme unknowingly dates back to the ancient period when ancient Greeks popularized the process of alcoholic fermentation, cheese making and bread leavening. The fact that yeast mediates fermentation of sugary compounds was thought to be resulting from the activity of chemical compound.

However, in 1857 Louis Pasteur reported the importance of yeast as a living organism which undergoes fermentation as a part of its metabolic process. In 1878 Friedrich W. Kuhne coined the word 'Enzyme' which has been derived from the Greek word '*Enzumas*'meaning (the leaving of bready by yeasts). In 1887, Edward Buchner discovered the activity of Zymase necessary for the

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fermentation of Grape Juice. The extract produced in the experiment was a cell free enzyme extract. The initial idea that enzymes are Proteins in nature came from the investigations of James B. Summer in 1926. James isolated the Enzyme Urease in crystalline form from the Jack Bean Seeds (*Canavalia ensiformis)*.

Later on, various other reports on the isolation of pepsin, trypsin, chymotrypsin and chymotrypsinogen led to the conclusion that enzymes are proteinaceous in nature. The catalytic activity of the enzyme depends upon the nature of their native conformation. The primary, secondary, tertiary and quaternary structure of the Protein is important in maintaining the catalytic activity of the enzyme. The quaternary structure formed for an enzyme results in the formation of an active site on its surface. Active sites are usually regions formed by stabilization of various chemical interactions among the Amino Acids. This region binds to particular substrates specific for the enzyme. The active-site mediated binding of substrate is supported by structural complimentary between the substrate and the active site. Enzymes may require the association of various chemical substances known as cofactors. Cofactors are usually inorganic non-protein substances like metal ions (Ca^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+} or Mn^{2+}) which stabilize the enzyme-substrate interaction and help in the catalytic activity of the enzyme (Refer Figure 4.3).

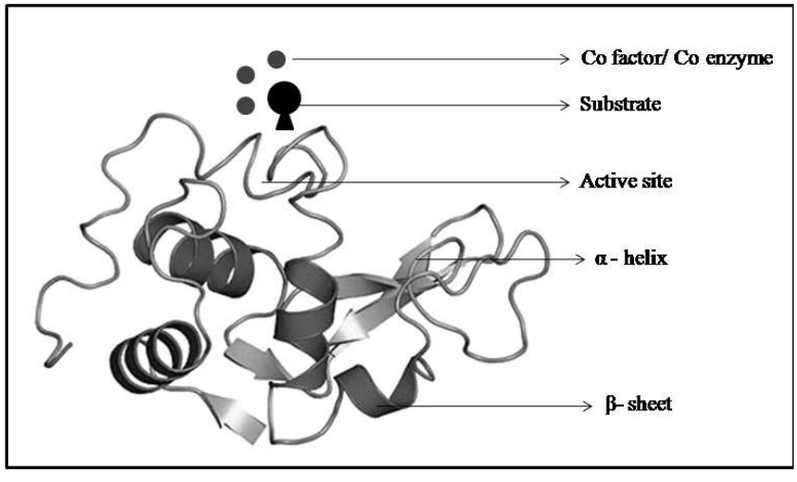

Fig 4.3 *Structure of an Enzyme: Folded in Tertiary Structure*

Coenzymes are, however, metaloorganic compounds which do not bind to the enzyme, but facilitate in its activity (Refer Figure 4.4). These biomolecules are mostly derived from various vitamins and organonutrients which function as carriers of various functional groups. Certain enzymes may require the association of both co-enzymes and cofactors necessary for metabolizing a substrate. The chemical activators which are tightly bound to the enzymes by means of covalent bonds are known as prosthetic groups. The functional catalytic form of the enzyme in association with its cofactors or coenzymes is referred to as the **holoenzyme**. The proteinaceous part of the enzyme excluding the cofactor or coenzyme is known as the **apoenzyme**. The association of the cofactors are required to trigger the catalytic activity of the apoprotein which then constitutes the holoenzyme. The activities of certain enzymes result from their activity modulation by Phosphorylation, Glycosylation or Nitrosylation.

The spatio-temporal regulation of enzymes is important to control any biochemical pathway. Plant enzymes exhibit specific compartmentalizations in respect to their distribution in the cells. The enzymes are non-uniformly distributed in the protoplasm and other organellar components. The site or location of the enzyme in the cell depicts the nature of its action. Most RNA synthesizing enzymes are localized in the nuclelus.

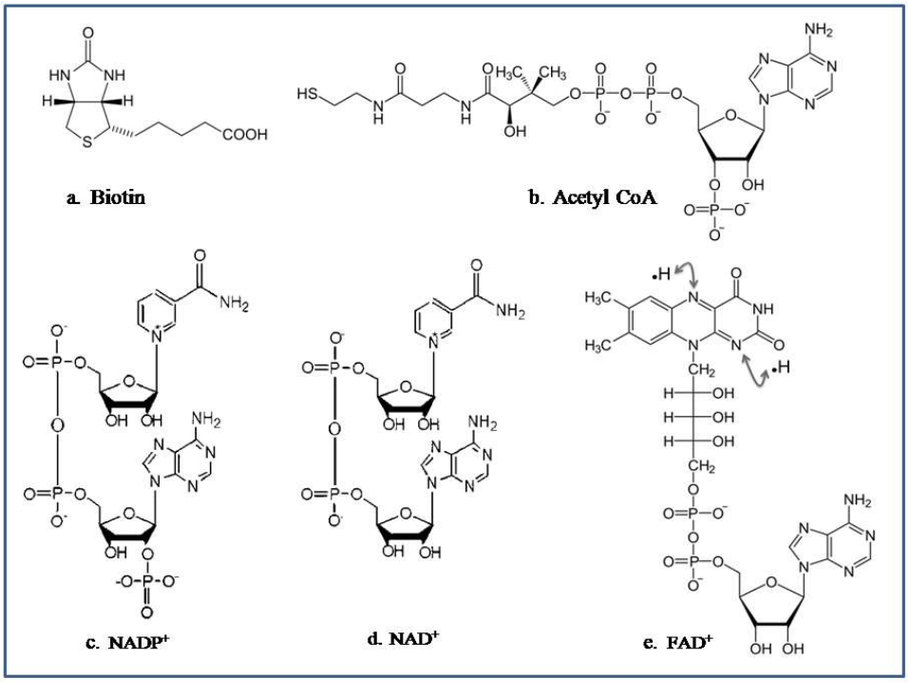

Fig 4.4 *Structure of Various Coenzymes*

The enzymes necessary for translation are mostly present in the cytoplasm. The respiratory enzymes remain both in the cytosol and mitochondria (intermembrane space and matrix). The photosynthetic enzymes are present in the chloroplast. Certain biochemical pathways like photorespiration involve three organelles (chloroplast, mitochondria and peroxysomes) to accomplish their enzymatic reactions. Various enzymes are present in the plasma membrane and function as sites of biochemical reactions. The enzymes have been classified into various classes on the basis of the nature of biochemical reaction catalyzed by them. The class and subclass of the enzyme is being denoted by the Enzyme Commission Number (E.C. Number). This classification is a four level classification which categorizes the enzymes into a class and respective subclasses.

The specialized feature of the enzyme catalyzed biochemical reaction lies in the fact that the enzyme provides the confines of its active site. The chemical transformation of the substrate bound to the active site occurs by biochemical process. The concept of formation of enzyme-substrate Complex was reported by Charles-Adolphe Wurtz in 1880. The biochemical reactions are driven by the availability of free energy. The difference between the ground state and transition state is known as the activation energy. Enzymes present as biocatalysts lower the activation energy of a reaction. This results in easier transformation of a substrate

to its product. The activity of the enzyme increases the rate of the reaction without changing its equilibrium. The intermediate steps of the reaction are sequentially converted to the final state of product. Certain cases where the product is formed by serial reactions are regulated by the step having highest activation energy. The present unit shall elaborate the structure, chemical nature and classification of enzymes in plants. The mechanism of enzyme kinetics is important to understand the nature of substrate transformation and metabolism catalyzed by enzymes.

Enzymes are protein catalysts that speed up the rate of biochemical reactions but do not change the structure of the final product. Like a catalyst, without being used up, the enzymes control the speed and specificity of the reaction, but unlike catalysts, only living cells generate enzymes.

The rate of *biochemical reaction* often influences enzymes like catalysts, so that they can take place at a relatively low temperature. The enzymes are thus known to lower the energy of activation. In certain cases, the biological response is initiated by enzymes.

Historical Background of Enzymes

The historical background of enzymes can be traced as follows:

- Analytical studies of enzymes were initiated in the 19th century, with emphasis on fermentation and digestion.
- In 1810, Joseph Gay-Lussac's discovered ethanol and CO2 formation from sugar fermentation by yeast.
- In 1835, Jacob Berzelius coined the term 'catalysis' (derived from a Greek term which means 'to resolve').
- In 1878, the name 'enzyme' was coined by Fredrich William Kuhne.
- In 1883, enzymes were isolated from cells extracted from yeast.
- In 1894, Emil Fischer's discovered the lock-and-key hypothesis.
- In 1926, James Summer isolated an enzyme known as urease and demonstrated that it is a protein.
- In the mid-1930s, John Norththrop and Moses Kunity showed that there is direct correlation between enzymatic activity and number of protein present in an enzyme. Therefore, it was proved that enzymes are proteins.
- In 1963, the first amino acid sequence of bovine pancreatic ribonuclease A was done after isolation and purification.
- In 1965, the first x-ray structure of hen egg white lysozyme was deduced. Since then, approximately 2000 enzymes have been purified and characterized.

Properties of Enzymes

Properties of enzymes can be classified into:

1. Physical properties
2. Chemical Properties
3. General properties

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Physical Properties of Enzymes

- Physically, enzymes act as colloids or as high-molecular-weight compounds.
- At a temperature below the boiling point of the water, enzymes are killed or inactivated.
- Most enzymes in the liquid medium are inactivated at 60 degrees Celsius.
- Extracting dried enzymes can withstand temperatures of 100 degrees Celsius to 120 degrees Celsius or even higher. Enzymes are, therefore, thermos-labile.
- The optimum activity of each enzyme is always at a particular temperature, which typically varies from 25 degrees Celsius to 45 degrees Celsius. At 37 degrees Celsius, enzyme action is strongest and as temperatures rise above 60 degrees Celsius, enzymes become inactive.

Chemical Properties of Enzymes

Catalytic Properties: Biological catalysts are enzymes. The greater amounts of compounds are catalysed by a small number of enzymes. This means that enzymes are highly capable of turning giant amounts of the substrate into a substance. Enzymes improve the reaction rate and remain unaffected by the reaction they catalyse.

Enzyme Specificity: Enzymes are extremely variable in nature, which means that a specific enzyme can catalyse a specific reaction. For example, only sucrose hydrolysis can be catalysed by Enzyme sucrose.

General Properties of Enzymes

- Enzymes initiate the biochemical reaction rate and accelerate it.
- The activity of enzymes depends on the medium acidity of the (pH specific). At a particular pH, each catalyst is most active. pH 2 for pepsin, pH 8.5 for trypsin, for example. At near neutral pH, most intracellular enzymes act.
- The reaction in either direction can be accelerated by enzymes.
- Both enzymes have active sites involved in biochemical reactions.
- Enzymes, often soluble in water, dilute glycerol, NaCl, and dilute alcohol, are very unstable compounds.
- At the optimum temperature, enzymes work aggressively.
- In nature, all enzymes are proteins, but all proteins may not be enzymes.
- Enzymes lower the molecule's activation energy so that the biochemical reaction can take place at the normal temperature of the body, which is 37 degrees Celsius.

Chemical Nature of Enzymes

Proteins are all enzymes, but all proteins are not enzymes. However, there are several conjugated enzymes bound to the protein portion of the enzyme with a non-protein moiety, which is called the *Apoenzyme*. The portion of the non-protein is known as the cofactor. If the co-factor, like potassium calcium, magnesium, manganese, is of an inorganic type, it is known as the prosthetic group. In general, the prosthetic group is closely bound to the protein portion of the enzyme and it is

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difficult to separate it with a simple technique such as diffusion. The enzyme is called a holoenzyme with the prosthetic group and the Apoenzyme.

If organic moulds such as NADP, NAD, FAD, etc., are co-factor attached to an enzyme protein, it is called a coenzyme. In general, a coenzyme is loosely bound to the *Apoenzyme* and can be isolated easily from the prosthetic group. Often, coenzymes are heat tolerant.

Mechanism of Enzyme Action

The behaviour of the enzyme is greatly influenced by the reaction conditions; unique temperature and pH conditions are needed for most enzymes. The lock and key model will better describe the mechanism of enzyme action and its selectivity.

By providing a surface for the substrate, an enzyme brings down the activation energy of the reaction. The mechanism of enzyme action has been a matter of research ever since their identification. The enzyme provides the substrate with a surface for the response to take place. A complex (intermediate) forms the substrate, which then supplies the substance and the enzyme. There is a *complex configuration* of the substrate that is connected to the enzyme and can only fit into a specific enzyme similar to that of a lock that has a particular key.

4.3.1 Classification and Nomenclature of Enzymes

Nomenclature and classification of enzymes is done on several bases, which are listed as follows:

- **On the Basis of the Substrate Acted upon by Enzyme:** Ducalux named the enzymes by adding suffix –ase to the substrate name (i.e. the substance on which the enzyme acts) in 1883. For example, if the substrate is carbohydrate, the enzymes are named as carbohydrases, if the substrate is lipid, the enzymes is called lipase and nuclease if the substrate is nucleic acid.
- **On the Basis of Type of Reaction Catalysed:** All the enzymes are very highly specific to the reaction they catalyse. On the basis of the type of reaction, the enzymes are named by adding suffix –ase to the name of the reaction. For example, the enzymes catalyzing hydrolysis are named hydrolases, the enzymes catalyzing isomerism are named isomerases, the enzymes catalyzing oxidation are named oxidases, and the enzymes catalyzing dehydrogenation are named hydrogenases and so on.

  The preceding two systems are very simple to understand and easy to learn but both of these do not give complete information; for example, the first one describes only the substrate and the second one only the type of reaction. Therefore, the third system evolved to contain both types of information.
- **On the Basis of Substrate Acted upon and Type of Reaction Catalysed:** According to this method, the name of enzyme contains two parts:
  (i) The name of the substrate
  (ii) The name of the reaction catalysed with the suffix –ase

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For example, the enzyme called succinic dehydrogenase catalyses the dehydrogenation of succinic acid substrate.

- **On the Basis of the Substance that is Synthesized:** According to this method, enzymes are named on the basis of the substance that is synthesized (i.e. product) by adding suffix –ase. For example, rhodonase catalyses the reaction hydrocynic acid and sodium thiosulphate and gives the product rhodonate.
- **On the Basis of Chemical Composition of the Enzyme:** Enzymes have been classified into three different categories on the basis of their chemical composition as follows:

  (i) Enzymes consisting of protein only, for example, pepsin, trypsin, urease, papain and so on

  (ii) Enzymes consisting of a protein and a cation, for example, carbonic anhydrase (which contains Zn++ ion as cation)

  (iii) Enzymes consisting of a protein and a non-protein part

On basis of the nature of prosthetic group: In 1950, Tauber classified the preceding category of enzymes on the basis of the nature of prosthetic group as follows:

a) Iron porphyrin enzymes (e.g. catalase, cytochrome C, peroxidase I and II)

b) Flavoprotein enzymes (e.g. glycine oxidase, pyruvate oxidase, histamine)

c) Diphosphothiamin enzymes (e.g. b carboxylase, pyruvate mutase)

d) Enzymes requiring a coenzyme (e.g. phosporylase)

- **On the Basis of Structure:** The enzymes can be divided into two categories, which are listed as follows:

  (i) Simple protein enzyme: This category of enzymes contains simple proteins only, for example, urease, amylase, and papain and so on.

  (ii) Complex protein enzyme: This category of enzymes contains conjugated proteins. These enzymes contain a protein part called apoenzyme and a nonprotein part called prosthetic group, which is associated with the protein part. These two parts form a holoenzyme.

- **Zymogens:** It is an inactive enzyme precursor requiring a biochemical change (such as a hydrolysis reaction that reveals the active site, or changes the configuration to reveal the active site) for it to become an active enzyme. The biochemical change takes place in a lysosome where a specific part of the precursor enzyme is cleaved in order to activate it. The amino acid chain which is released on activation is known as the activation peptide.

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Zymogens are secreted by the pancreas to prevent the enzymes from digesting proteins in the cells where they are synthesized. Digestive enzymes are secreted by fungi into the environment in the form of zymogens. The external environment has a different pH than the internal environment of the fungal cell. This changes the structure of the zymogen into an active enzyme. Examples of zymogens include angiotensinogen, trypsinogen, chymotrypsinogen and and pepsinogen.

- **On the Basis of Overall Chemical Reaction:** All the preceding classification systems did not provide complete information about enzymes and their related reactions. Therefore, in 1961, International Union of Biochemistry (IUB) used some specific criteria for describing the enzymes (i.e. for classifying and naming).

The main features of IUB classification system are as follows:

This system divides enzymes into six major classes according to the type of reaction catalysed by them.

The name of enzyme comprises two parts, the first part is the name of the substrate and the second part is the name of the type of reaction catalysed which ends with the suffix –ase.

Each enzyme has been allotted a systemic code number, which is called enzyme commission number (EC number).

This system assigns code numbers to enzymes. Every enzyme code consists of the prefix EC followed by four numbers separated by points, with the following meaning (Refer Figure 4.5):

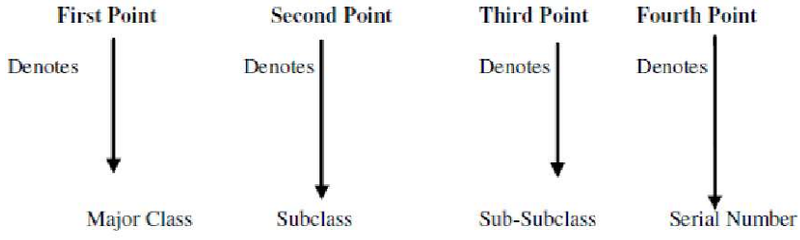

Fig. 4.5 *EC Number of an Enzyme*

i) The first number shows to which of the six main divisions (classes) the enzyme belongs.

(ii) The second number indicates the subclass.

(iii) The third number gives the sub-subclass.

(iv) The fourth number is the serial number of the enzyme in its sub-subclass.

Hence, the EC numbers represent a progressively finer classification of the enzyme. For example, E.C. number 2.7.1.1 represents the class 2, subclass 7, sub-subclass 1 and serial number 1.

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The older system of classification of enzymes was based upon the name of substrate and the nature of reaction catalyzed by the enzyme. The broad group of classification for enzymes were primarily:

- Hydrolyzing Enzymes
- Desmolysing Enzymes

The Hydrolysing Enzymes function to split the molecules by addition of water molecules. The major Hydrolyzing Enzymes associated with the breakdown of Sugars include Invertase, Maltase, Lactase, Cellulose or Inulase. These enzymes were collectively termed as the **Carbohydrates**. The other enzymes of esterases, i.e., Lipase and Phosphatase are involved in hydrolyzing the Ester group. The major proteolytic enzymes associated with hydrolytic reactions include Pepsin, Peptidase, Protease, etc. The Amidases were termed for the enzymes associated with hydrolysis of Amide group, for example, Ureases and Asparaginase. The other class of hydrolyzing enzymes include Phosphorylases.

Apart from the Hydrolysing enzymes, the other category of enzyme was termed as the Desmolysing type. The various types of Desmolysing enzymes include Aldolase, Dehydrogenase, Transphosphorylases, Hydrases, Transaminase, Carboxylase, Peroxidase and Catalases. The category of Desmolysing enzyme was broadly based upon the reactions which involved the breakage of Carbon chain or intra or inter molecular transfers of atoms. However, the older system of classification of enzymes was obscure in terms of clear indication of the type of enzyme. According to the older system of enzymes the names used often did not indicate the type of substrate or reaction they catalyze.

The new system of classification of enzyme involves systematic nomenclature of the enzymes and their categorization according to the reaction catalyzed by them. The Commission on Enzymes of the International Union of Biochemistry (1961) has recommended this new system of classification. According to this system of classification the enzymes are referred by four digit code numbers.

The features of the new system of classification are as follows:

- The enzymes have been categorized into six major classes.
- The major classes have been divided into various subclasses.
- The enzymes are represented by a specific code number consisting of four digits. The first digit represents the major class while the second and third digit represents the subclasses. The last digit denotes the specific name of substrate used and the nature of the reaction.

The major classes of the enzymes are as follows:

1. **Oxido-Reductases:** Enzymes which catalyze oxidation-reduction process.
2. **Transferases:** Enzymes which catalyze group transfer.
3. **Hydrolases:** Enzymes which catalyze isomerisation reactions.
4. **Ligases:** Enzymes catalyzing reactions in which two molecules are linked and coupled. to breakdown of pyrophosphate bond of ATP or similar triphosphates.

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The subclasses of the enzymes are as follows:

1.1 Subclass: Oxidoreeductases acting on the CH.OH group of the donor.

2.1 Subclass: Transferases transferring one carbon group.

3.1 Subclass: Hydrolase acts on the ester links.

4.1 Subclass: Lyase acting on the C-C bond.

5.1 Subclass: Isomerase acts as a epimerase and racemase.

6.1 Subclass: Ligase which forms C-O bonds.

1.1.1 Sub-subclass- Oxidoreductase acting on the CH.CH group of the donor with coenzyme NAD or NADP as acceptors.

2.1.1 Sub-subclass- Transferase which catalyze the transfer of one-carbon groups and a methyl transferase.

3.1.1 Sub-subclass- Hydrolase acting on carboxylic ester links.

4.1.1 Sub-subclass- Lyase (C-C lyase) carboxylase.

5.1.1 Sub-subclass- Isomerase functioning as a racemase and epimerase on Amino Acids and derivatives.

6.1.1 Sub-subclass- Ligases which form C-O bond.

Thus, enzymes like alcohol dehrdogenase or carboxyesterases are represented as 1.1.1.1 and 3.1.1.1, respectively. Pyruvate decarboxylases are represented as 4.1.1.1.

Structure of Enzyme

Enzymes are proteinaceous molecules which assort in the quaternary conformation to function as catalytic units. The functionality of the enzyme is thus maintained by its active site which binds to the substrate and forms a substrate enzyme Complex. Enzymatic Protein molecules thus consist of more than one polypeptide chain and are composed of 10 different types of Amino Acids. The sequence of Amino Acids is specific for each of the enzymes. Mutation in the triplet codon may result in alteration of the Amino Acid composition.

This results in misfolding of Proteins and enzymes which lead to termination in their catalytic activity. Various genetic diseases occur due to non-functionality, hyperactivity or absence of certain metabolic enzymes in the cell. The quaternary structure of the Protein is essential to be maintained by means of stable Sulphide bonds prevalent among the constituent subunits. Protein denaturants like mercaptoethanol, urea and DiThioeriThritol (DTT) may cause disruption of the bonds forming the quaternary structure of Proteins. This may result in loss of catalytic activity of the enzyme. The activity of the enzyme is exhibited at a particular pH and range of temperature. The ambient range of temperature necessary for any enzyme activity usually ranges from 25°C to 37°C.

However, in certain cases enzymes may function at temperatures beyond this range. All enzymes function as biocatalysts in nature. They differ from chemical catalysts in terms of their proteinaceous nature and being products inside the cells. Enzymes act on a specific substrate or a group of substrate to metabolize it into the product. Enzymes which are produced and function inside the cell are termed as endoenzymes. A majority of the enzymes responsible for growth and metabolism

or various cellular processes are usually endoenzymes. The exo-enzymes are secreted by the heterotrophic organisms like Bacteria and Fungi which produce and secrete them at the extracellular region. Cellulase, Pectinase, and Ligninase are enzymes which breakdown Complex organic substances into simpler forms.

Mostly the exoenzymes are digestive or hydrolytic enzymes functioning in breakdown of insoluble compounds to soluble forms. The germinating seeds secrete hydrolytic enzymes which convert insoluble forms of metabolite into soluble sugars in the endosperm. The enzymes which are present in the organism irrespective of its metabolic state are termed as the constitutive enzyme. The inducible enzymes are the ones synthesized in the cell in response to external substrate. The substrate or other chemical agent capable of inducing the synthesis of the enzyme is known as an inducer. The process of inducible expression of enzyme by the action of external substrate is common in bacterial members. In higher plants nitrate reductase is an example of inducible enzyme which functions in presence of external source of nitrate. The activity of Gibberellic Acid stimulates the finction of α-Amylase in the endosperm which breaks down Starch into Sugar forms.

The parts of the enzyme necessary for its functioning are as follows:

- **Protein Part (Apoenzyme):** The Protein part of the enzyme functions in its quaternary or tertiary form of folded Protein. Certain enzymes are composed of subunits of **Proteins** exhibiting similar molecular weight. The Protein part of the enzyme is termed as the apoenzyme which associates with cofactor or prosthetic group to exhibit its catalytic activity.
- **Active Site:** The active site of the enzyme is the most important region necessary to maintain its catalytic activity. This region is specific to the enzyme which may bear more than one active sites together in the same enzyme. The active site is comprised of hydrophilic Amino Acids which orient in specific alignment in the Folded Protein. The active site binds to the specific substrate and catalyzes the reaction. The interaction between the substrate and enzyme is a Complex process. Hydrogen bonds, hydrophobic interactions and ionic bonds facilitate the process of substrate-enzyme Complex formation. The energy derived from the enzyme-substrate binding is termed as the binding energy. In another sense this binding energy is a form of free energy by which the enzyme lowers the activation energy of the reaction.

  Thus, the catalytic power of an enzyme largely depends upon the amount of free energy released in the form of weak bonds and ionic interactions between the substrate and enzyme. The weak interactions are optimised in the process of transition of the substrate into the product. According to Emil Fischer the structure of the enzyme at the active site is complementary to the specific substrate of the enzyme.
- **Prosthetic Group (Non-Protein Part of the Enzyme**): The non-protein part of the enzyme associated with the active site of the enzyme is necessary for the catalytic activity of the enzyme. The prosthetic groups usually consist of an organic compound or inorganic metal ion (Cu, Zn, Mo, etc). The organic compounds usually contain flavin nucleotides or heme containing

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iron groups. The prosthetic group is tightly bound to the enzyme by means of covalent linkage.

- **Cofactor:** The cofactors are not structural part of the enzyme but remain associated with it. The presence of cofactor is essential to accomplish the metabolic reactions catalyzed by the enzyme. The cofactors are not tightly associated with the enzyme are categorized into following two types based upon its structure:
  (i) **Specific Coenzymes:** The various specific coenzymes necessary for the catalytic activity of enzymes include Hydrogen carriers (Oxidation-Reduction Reactions) like Coenzyme I, Coenzyme II, Lipoic Acid, Glutathione or Flavin Adenine Dinucleotide. The other types of coenzymes include Coenzyme A as an Acyl group carrier, Pyridoxal Phosphate as Amino Group carrier and Biotin as a form of CO_2 carrier. Thiamine Pyrophos Phate (TPP) or Coenzymes F are some of the other coenzymes necessary for enzyme activity.
  (ii) **General Activators:** Certain metal ions and inorganic molecules may act as general inducer or activator of enzymes.

4.3.2 Extraction and Purification of Enzymes

Extraction of Enzymes

Many enzyme extracts must, of necessity, be made from green leaves, fruits, and other vegetable tissues that contain large amounts of phenols and polyphenols. These may hinder or, unless precautions are taken, completely prevent a *successful extraction*.

Enzymes are protein molecules that are present in all living things. They speed up and target chemical reactions, in many cases increasing the rate of reaction millions of times. For example, they aid digestion, metabolise and eliminate waste in humans and animals, and play a crucial role in muscle contraction.

Enzymes have been used unknowingly in food production, for example dough making, for centuries. They can be obtained by extraction from plants or animals or by fermentation from *micro-organisms*. They are usually purified but may contain varying traces of the other naturally occurring constituents of these three sources. They are normally added to perform a technological function in the manufacture, processing, preparation and treatment of foods. Examples include enzymes used to break down the structure of fruit so that manufacturers can extract more juice, or to convert starch into sugar in alcohol production.

Historically enzymes are considered to be non-toxic and not of safety concern for consumers since they are naturally present in ingredients used to make food. However, food enzymes produced industrially by extraction from plant and animal tissues, or by fermentation of microorganisms, are assessed for safety.

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The extraction process consists of three parts:

- Grinding, to reduce the size of the plant and increase the contact surface available for the enzymes.
- The enzymatic reaction takes place in a tank stirred and thermostated in the presence of water.
- Centrifugal separation is the last step of the extraction process: it allows to obtain an aqueous extract, a vegetable oil and a cake.
- These three fractions are then worked separately to ensure the *microbiological quality* and *stability* to increase the quality, purity or concentration of certain elements (oligosaccharides, peptides, amino acids, minerals).

Purification of Enzymes

Although initial characterizations of an enzyme in a mixture or sample matrix is practical, such as *activity measurement* and *preliminary quantification*, more fundamental knowledge relies on more advanced studies of the enzyme, which can only performed with *pure enzyme samples*. Pure enzymes also mean easier assays with less interferences. Some analysis methods, such as crystallography, are sensitive to sample purity and give desired results only with the highest samples purity. In large scale production for industrial applications, enzyme purification is directly related to product quality, in addition to regulatory requirements. Therefore, enzyme purification must be thoroughly considered and cautiously operated for both research and production purposes. Many factors could change the efficiency, the yield, and stability of activity during purification, and the effects of these factors vary largely from one enzyme to another.

Almost all samples need to be prepared before the *actual purification*. For the enzymes from cell sources, they need to be fractionated into components before purification. The first step usually involves *homogenization* of cells, which disrupt the cell wall to release the enzyme into the homogenate, along with other components. Depending on the cell type, the homogenization could be easy as in the case of *mammalian tissue* without rigid cell wall, or it may need harsher conditions, such as abrasion, freezing, and high pressure due to the rigid cell wall of the plant tissue. Sometimes, additional hydrolytic enzymes or detergents are added for better extraction. The mixture is then fractionated by centrifugation, yielding a dense pellet of heavy material at the bottom of the centrifuge tube and a lighter supernatant above (Refer Figure 4.6). The supernatant is again centrifuged at a greater force to yield yet another pellet and supernatant. The procedure, called differential centrifugation, yields several fractions of decreasing density, each still containing hundreds of different proteins, which are subsequently assayed for the activity being purified. Usually, one fraction will be enriched for such activity, and it then serves as the source of material to which more discriminating purification techniques are applied. The choice of temperature, pH, buffering salt, buffer strength, ionic strength, osmolarity, additives (EDTA, SDS, non-ionic detergents, etc.), and homogenization technique are important of the success of purification.

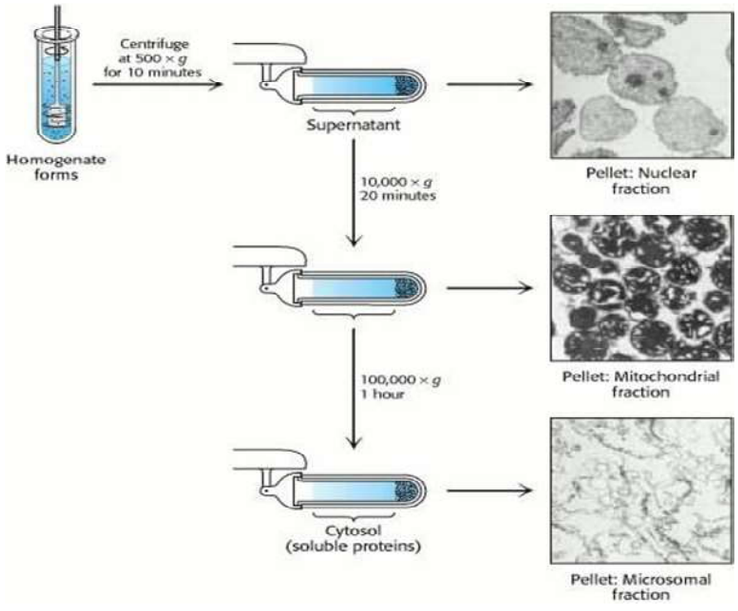

Fig. 4.6 *Differential Centrifugation*

Purification and separation of enzymes are generally based on solubility, size, polarity, and binding affinity. The production scale, timeline, and properties of the enzymes should all be considered when choosing the proper separation method.

Solubility Based Separation

The principle of the type of separation is that enzyme solubility changes drastically when the pH, ionic strength, or dielectric constant changes. For example, most proteins are less soluble at high salt concentrations, an effect called salting out. The salt concentration at which a protein precipitates differs from one protein to another. Hence, salting out can be used to fractionate proteins. Salting out is also useful for concentrating dilute solutions of proteins, including active fractions obtained from other purification steps. Addition of water-miscible organic solvents, such as ethanol or acetone will change the dielectric constant of the solvent and therefore precipitate the desired enzyme. Neutral water-soluble polymers can also be used for the same purpose instead of organic solvents. However, the risks of losing enzyme activity during precipitation and further separation of the added salt or polymer need to be considered.

Size or Mass Based Method

Because enzymes are relatively large molecules, separation based on the size or mass of molecules **favours purification of enzymes**, especially the ones with high molecular weight. Dialysis is a commonly used method, where semipermeable membranes are used to remove salts, small organic molecules, and peptides. Enzyme molecules (red dots) are retained in the dialysis bag and separated from other smaller molecules (blue dots) (Refer Figure 4.7).The process usually needs a large volume of dialysate, the fluid outside the dialysis dag, and a period of hours or days to reach the equilibrium. Counter current dialysis cartages can also be used, in which the solution to be dialyzed flow in one direction, and the dialysate in

the opposite direction outside of the membrane. Similarly, ultrafiltration membranes, which are made from cellulose acetate or other porous materials, can be used to purify and concentrate an enzyme larger than certain molecular weight. The molecular weight is called the molecular weight cut-off and is available in a large range from different membranes. The ultrafiltration process is usually carried out in a cartridge loaded with the enzyme to be purified. Centrifugal force or vacuum is applied to accelerate the process. Both dialysis and ultrafiltration are quick but somewhat vague on distinguishing the molecular weight, whereas size exclusion chromatography gives fine fractionation from the raw mixture, allowing separation of the desired enzyme from not only small molecules but also other enzymes and proteins. Size *exclusion chromatography*, also known as *gel-filtration chromatography*, relies on polymer beads with defined pore sizes that let particles smaller than a certain size into the bead, thus retarding their egress from a column. In general, the smaller the molecule, the slower it comes out of the column. Size exclusion resins are relatively 'Stiff' and can be used in high pressure columns at higher flow rates, which shortens the separation time. Other factors including the pore size, protein shape, column volumes, and ionic strength of the eluent could also change the result of purification.

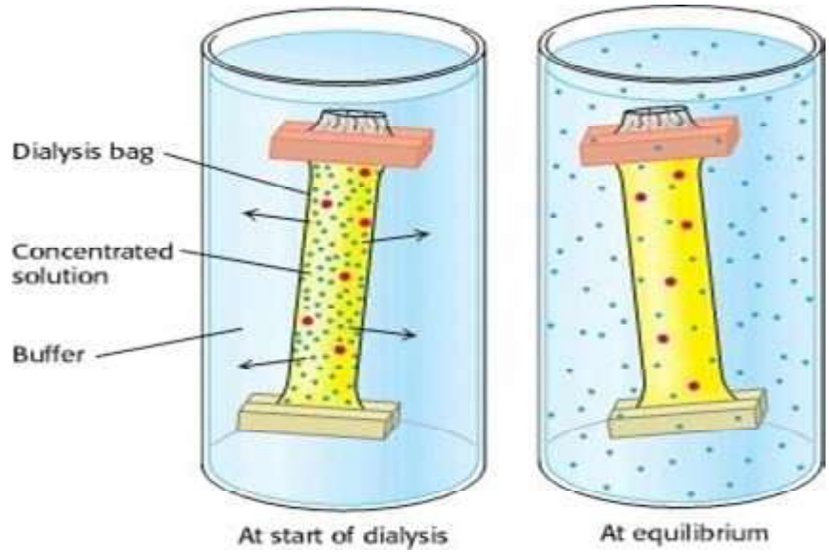

Fig. 4.7 *The scheme of Dialysis.*

Polarity Based Separation

Like other proteins, enzymes can be separated on the basis of polarity, more specifically, their net charge, charge density, and hydrophobic interactions. In ion-exchange chromatography, a column of beads containing negatively or positively charged functional groups are used to separate enzymes. The cationic enzymes can be separated on anionic columns, and anionic enzymes on cationic column.

Electrophoresis is a procedure that uses an electrical field to cause permeation of ions through a solid or semi-solid matrix or surface resulting in separations on constituents on the basis of charge density. The most commonly used methods with a SDS-PAGE matrix are quite well standardized and do not differ much between labs. The distance a protein migrates in SDS-PAGE is inversely proportional to the

log of its molecular radius, which is roughly proportional to molecular weight. Similarly, a matrix with gradient pH can be used in isoelectric focusing separation. A protein moves under the influence of an electrical field and stops upon reaching the pH which is the pI for the protein (net charge = 0). The matrix used can be liquid or a gel poured into either a cylindrical shape, or a flat plate.

Hydrophobic Interaction Chromatography (HIC) employs hydrophobic interactions to distinguish different enzymes, which are adsorbed on matrices such as octyl- or phenyl-Sepharose. A gradient of decreasing ionic strength, or possibly increasing non-polar solvent concentration can be used to elute the proteins, giving fractions that usually contain relatively high-pure enzymes. High-Pressure Liquid Chromatography (HPLC) uses the same principle of separation of HIC, which is filled with more finely divided and tuned materials and thus allows more choices of eluents and results in better separation. Note that HPLC could be based on polarity, affinity, or both.

Affinity or Ligand Based Purification

Affinity chromatography is another powerful and generally applicable means of purifying enzymes. This technique takes advantage of the high affinity of many enzymes for specific chemical groups. In general, affinity chromatography can be effectively used to isolate a protein that recognizes a certain group by (1) covalently attaching this group or a derivative of it to a column, (2) adding a mixture of proteins to this column, which is then washed with buffer to remove unbound proteins, and (3) eluting the desired protein by adding a high concentration of a soluble form of the affinity group or altering the conditions to decrease binding affinity. Affinity chromatography is most effective when the interaction of the enzyme and the molecule that is used as the bait is highly specific. A special example of ligand-affinity chromatography is the Ni-NTA (nickel – nitrolotriacetic acid-agaraose) affinity chromatography. This ligand binds tightly to a 6 amino acid peptide consisting only of Histidines (His6). The cDNA sequence for His6 can be appended to the cDNA coding for a given recombinant protein, thus yielding a recombinant protein which contains a His-TAG. This allows the affinity-purification of such a protein using Ni-NTA without having to design a special ligand-affinity column. Other forms of affinity chromatography include dye-ligand chromatography, immune adsorption chromatography, and covalent chromatography.

After purification, the enzymes need to be concentrated, and sometimes lyophilized to give the pure, stable form distributed as the product or added into the final formulation. The following analysis and quality certification is necessary to confirm the enzyme is the desired one, with reasonable concentration, stability, and activity. The enrichment and certification requires experienced researchers to maintain the enzyme quality and choose the correct characterization method.

4.3.3 Theories for Enzyme Action

The activity of enzyme involves substrate-enzyme interaction and utilization of free energy which helps in the formation of enzyme-substrate Complex. The two main theories proposed for explaining the mode of action of enzyme are Fischer's Lock and Key Hypothesis and Koshland's Induced Fit Theory.

Lock and Key Hypothesis

The structural complementarity in the mode of interaction among the substrate and enzyme has been explained by the Lock and Key Hypothesis. This theory has been widely accepted which explains that the active site possess structural features facilitating the binding of substrate. The specificity of the enzyme-substrate is similar to the Lock-Key specificity. The enzyme catalyzes the formation of product from the transition of the substrate. The enzyme is released free to accept substrate for new reaction (Refer Figure 4.8).

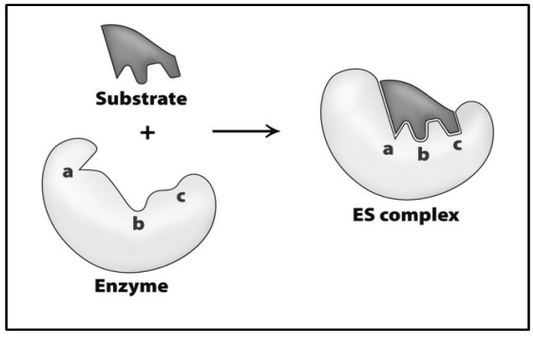

Fig. 4.8 *Lock and Key Hypothesis of Enzyme-Substrate Binding*

Induced Fit Theory

According to this theory the presence of substrate in the vicinity of the enzyme acts as an inducer of the enzyme. The presence of the substrate results in the formation of conformational change in the active site of the enzyme. This results in the formation of chemical interaction between the enzyme and substrate. The substrate is held in the active site by help of Hydrogen bonds. Furthermore, the electrophilic and nucleophilic interactions prevalent in the catalytic groups of the enzyme weaken the bonds formed between the substrate and the active site. Moreover, for non-substrate molecules the active site does not undergo suitable interactions with the molecule present. Thus, the enzymatic reaction does not take place (Refer Figure 4.9).

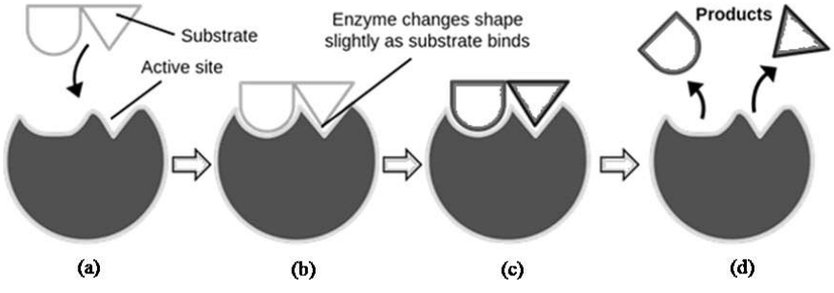

Fig. 4.9 *Induced Fit Theory of Enzyme Action (a-d) Substrate-Induced Changes in the Shape of Enzyme*

4.3.4 Enzyme Kinetics

The enzyme utilizes the binding energy to form the transitory enzyme-substrate Complex. This binding energy is formed due to Hydrogen bond and various ionic

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interactions present between the enzyme and substrate. The binding energy is referred to as the free energy utilized by the enzyme to lower the activation energy of the reaction. Emil Fischer in 1894 proposed that the substrate fits into the active site of the enzyme due to its structural complementarities. This theory was found to possess limitations. Complete structural complementarities between the enzyme and substrate at the active site would, in general, impede the reaction and product formation. According to the modern theories of enzyme action proposed by Michael Polanyi (1921), Haldane (1930), and Linus Pauling (1946) the enzymatic reaction involves formation of a transition state stage established between the Enzyme and the Substrate. Thus, the substrate should essentially gather an increased free energy necessary for undergoing the transformation. Initially the enzyme-substrate Complex forms by weak interactions which are followed by stringer forces operative in the **transition state**.

Thus, the electrophilic interactions occurring at the transition state of the enzyme- substrate Complex is very essential to complete the reaction. The free energy released in this context essentially lowers the activation energy of the reaction. In this case, the summation of two components of energy, i.e., positive free energy and the negative binding energy lower the activation energy of the biochemical reaction. The weak interaction between the enzyme and substrate is the main driving force for the catalytic activity of the enzyme. The larger size of the enzyme helps in providing multiple sites of weak interactions between the active site and the substrate. It has been reported that enzymatic interactions can lower the activation energy by as low as 5.7 kJ/mol. The amount of energy available for formation of weak interaction varies forms 3-30 kJ/mol.

To understand the process of enzyme catalysis in the light of thermodynamics it is essential to know the reasons of higher activation energy in absence of the enzyme. In uncatalyzed reactions usually there in low entropy caused due to less freedom of the molecules. The solvation shell of the biomolecules is formed by Hydrogen bonded water molecules present in the aqueous environment. This results in increased activation energy of the reaction. Higher energy is required to cause structural distortion of the substrate or the alignment of the catalytic groups. This situation is overcome by the free energy and binding energy provided by enzyme-substrate interaction.

According to Arrhenius, low energy rich molecules require higher energy to complete chemical reactions. The stability of the molecules requires to be overcome to undergo transition and transformation into products. This energy required for a chemical reaction is called the **activation energy**. Higher temperature facilitates a chemical reaction occurring due to higher kinetic energy of the molecules. This is caused as a result of thermal agitation. In presence of the enzyme-substrate Complex weak interactions result in breaking the solvation shield of the molecule. Furthermore, the binding energy present helps to compensate any thermodynamic distortion caused by electron redistribution and substrate transformation. Thus, the binding energy is important in imparting enzyme-substrate specificity of a reaction (Refer Figure 4.10).

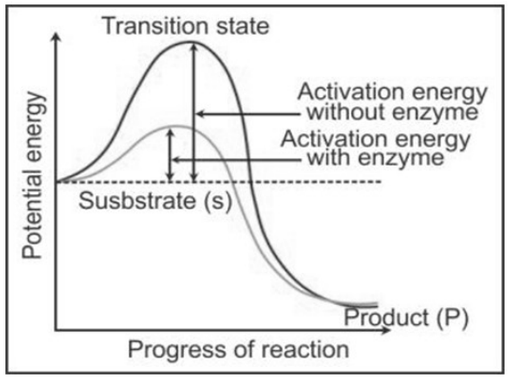

Fig. 4.10 *Lowering of Activation Energy by Enzyme Activity*

The Michaelis-Menten Constant (Mechanism of Enzyme Catalysis)

The mechanism of enzyme-induced catalysis of reaction can be explained by the concept of Michaelis-Menten Equation. The concept explains the regulation of the rate of enzymatic reaction which depends upon the concentration of substrate and available molecules of enzyme in a reaction medium. In situations of increasing substrate concentration and constant enzyme molecules, the reaction rate in relation to the increasing substrate concentration follows a hyperbolic curve. This results due to saturation in the rate of reaction after optimum substrate concentrations. Lower concentration of substrate exhibits a direct proportionality to the reaction rate. This results due to binding of the substrate to the enzyme molecules. However, enzyme molecules become limiting at a later stage which results in the appearance of steady state. At this stage the reaction rate does not increase with increasing the substrate concentrations. The maximum velocity attained by optimum concentration of substrate is denoted as the $V_{max.}$ The substrate concentration necessary for attaining half velocity ($1/2/V_{max}$) of the reaction is known as K_m value for the enzyme. This **K_m value** is known as the **Michaelis-Menten Constant**. This value of K_m is essential to understand the affinity of an enzyme to its substrate. Lower K_m value indicates higher affinity of the enzyme to the substrate.

This results in attaining $1/2V_{max}$ at a lower substrate concentration. During steady state conditions, the kinetic value of enzyme catalyzed reactions show relations between the following parameters:

$$V = \frac{V_{max[S]}}{K_m + [S]}$$

V= Velocity or Rate of Reaction, V_{max} = Maximum Velocity Attained by the Enzyme, [S]= Substrate Concentration, K_m = Michaelis-Menten Constant.

The K_m values of enzymatic reaction may vary according to the pH, temperature and ionic strength of the medium. The amount of coenzyme present in the medium also influences the K_m value. The K_m values are usually calculated in terms of molarity. The K_m of enzymes range from (1 mM -1 μM). The concept of Michaelis-Menten Constant is helpful in understanding the nature of enzyme-substrate reaction. The velocity of the reaction indicates the further requirement of enzyme or substrate molecule to speed up the reaction. Certain enzymes which

can bind to two similar kinds of substrates (Hexose Sugars) will exhibit higher reaction rate for the substrate with lower K_m value. The measurement of K_m value provides indication about the endogenous concentration of the substrate participating in the biological reaction. Enzymes which catalyze reactions with higher availability or high concentration of substrate usually exhibit higher K_m value.

Mechanisms of Enzyme Inhibition

Various metabolic inhibitors are associated with the inhibition of enzyme action partially or completely. The process may appear to be reversible or irreversible process which depends upoon the nature of the inhibitor. The efficiency of the inhibitor depends upon the concentration of the enzyme and substrate.

- **Competitive Inhibitor:** The inhibitors which possess structural similarity to the substrate may compete for the same active site of the enzyme thus preventing the binding of substrate to the enzyme. Prior occupancy of the active site with the application of competitive inhibitor prevents the catalysis of the substrate.

  However, this type of inhibiton is a reversible type, where removal of the inhibitor may restore the activity of the enzyme, for example malonic acid is a competitive inhibitor of the enzyme succininc dehydrogenase which catalyzes the conversion of succininc acid to fumaric acid.
- **Non-Competitive Inhibitor:** The chemical inhibitors which bind to the enzyme at regions different from active sites and distort the functionality of the enzyme. These types of inhibitors usually cause irreversible changes in the structure of the enzyme, for example heavy metals, acid, etc.
- **Allosteric Inhibition (Feedback Inhibition):** Metabolic pathways exhibit the phenomenon of allosteric inhibition. This process involves binding of an end product of the pathway to one of the initial step enzyme thus causing inhibition in its activity. The product which exerts feedback inhibition is completely different in structure in comparison with the substrate of the enzyme. The enzyme undergoing the process of inhibition is termed as **allosteric enzyme**. The allosteric site is different from the active site of the enzyme.

The binding of the product to the allosteric site results in the conformational change in the enzyme which renders the active site to be unsuitable to accept the substrate. The process of allosteric inhibition is reversible with the decrease in the concentration of the end product formed in the pathway. Threonine dehydratase in *E. coli* exhibits the property of allosteric inhibition by the end product of the pathway, i.e., L-Isoleucine.

4.3.5 Lineweaver–Burk Methods

The determination of K_m in the mechanism of enzyme action is a complex process; therefore, simpler methods were devised by Lineweaver and Burk. Two such methods are discussed here:

First method: This method involves plotting the kinetic data as the reciprocals of V and (S). This double reciprocal plot was developed in 1934 by the Hans

Lineweaver and Dean Burk. The following equation has been derived from the reciprocal of Michaelis- Menten equation:

$$1/V = K_m + (S)/V_m \times (S)$$

$$1/V = K_m / V_m \times 1/(S) + 1/V_m$$

After plotting the graph of 1/V versus 1/(S), a straight line is obtained (Figure 4.11). The slope of this curve corresponds to K_m / V_m.

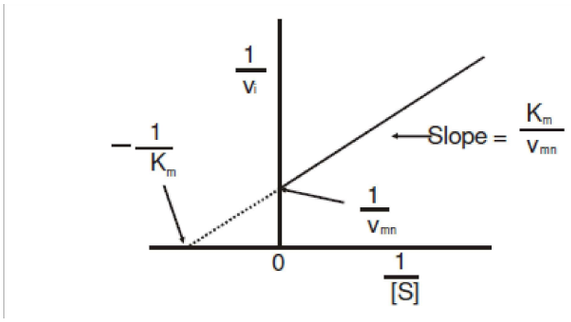

Fig. 4.11 *The Graph of 1/V versus 1/(S)*

Second method: In this method, the Lineweaver-Burk equation is multiplied by (S) on both sides to get the following equation:

$$(S)/V = K_m/V_m + (S)/V_m$$

The plot of (S)/V versus (S) gives a straight line (Figure 4.12). The intercept of the line on (S)/V axis is K_m/V_m and the slope is $1/V_m$.

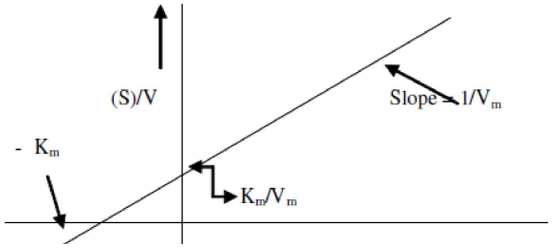

Fig. 4.12 *Plot of (S)/V 3versus (S)*

4.3.6 Affinity Labelling of Enzyme

Affinity labels are a class of enzyme inhibitors that covalently bind to their target causing its inactivation. The hallmark of an affinity label is the use a targeting moiety to specifically and reversibly deliver a weakly reactive group to the enzyme that irreversibly binds to an amino acid residue. The targeting portion of the label often resembles the enzyme's natural substrate so that a similar mode of noncovalent binding is used prior to the covalent linkage. Their usefulness in medicine can be limited by the specificity of the first non-covalent binding step whereas indiscriminate action can be utilized for purposes, such as affinity labelling - a technique for the validation of substrate-specific binding of compounds.

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These labels are not limited to enzymes but may also be designed to react with *antibodies* or *ribozymes* although this usage is less common. Although proteins, such as hemoglobin do not have an active site, binding pockets can be exploited for their affinity and thus be labelled.

Classification

Classical Affinity Labels: This category encompasses the simplest approach of coupling an electrophile with low intrinsic reactivity to a non-covalent binding moiety which frequently mimics the natural substrate. Key to this designation is that the reactivity of the electrophile is not altered by the enzyme and that the non-covalent binding moiety serves to increase the presence and lifetime of the electrophile in the active site (effective molarity). The weakly reactive group may react with functional groups outside of the active site or on other proteins but the selectivity is conferred by the non-covalent binding moiety. Kinetic signatures of this type of inhibitor can be found in saturation because of the covalent reaction (kinact) becomes the rate limiting step at high concentrations of inhibitor. A handful of drugs such as afatinib have gained FDA approval through this approach. The inverse approach of using a weakly nucleophilic inhibitor to attack a protein-bound electrophile has also been studied. This approach has received much less attention due to the lack of protein electrophiles and only those with suitable cofactors can be targeted.

Quiescent Affinity Labels: Quiescent affinity labels represent a promising approach for inhibiting enzymes using 'Masked' reactive functionalities that are only uncovered within the active site. This approach differs from mechanism-based in activators in that the catalysis must be 'Off-Pathway'. One of the best examples to explain this form of catalysis is in the inactivation Dimethylargine DimethylAminoHydrolase (DDAH) by 4-halopyridines. At physiological pH, the 4-halo group has near negligible reactivity with thiolates but upon protonation of the nitrogen, the reactivity increases ~4500-fold. This protonation occurs off-pathway by an aspartate residue that is not normally involved in catalysis.

Photoaffinity Labels: Photoaffinity labels are characterized by non-enzymatic reactivity produced by exposure to light and a non-covalent targeting moiety to enhance the effective molarity of this reactive group in the active site. While this technique appears sound in theory, low degree of labelling is frequently observed primarily due to quenching of the reactive species by solvent or other species in solution. However, this quenching can be advantageous as it is such a fast process that once the reactive species is formed, it will not diffuse to any appreciable extent and will only react with molecules to which it is immediately adjacent. Photoaffinity labels do not show great promise for inhibition or in the use of drugs but are appropriately suited to identify ligand binding sites. Reactive groups such as nitrenes or 2-aryl-5-carboxytetrazoles are often employed to generate highly reactive, nonselective carbenes or moderately selective nitrile-imine intermediates, respectively.

Uses of Affinity Labeling

When characterizing an enzyme, it is essential to identify the amino acid residues responsible for catalysis. While it is clear that X-ray crystallography will provide

more detailed 3-D information about the active site, only a static picture is returned and difficulties can be encountered with co-crystallization of the substrate or mimics due to enzymatic turnover.

The classic example of the use of affinity labels for this purpose is in mapping the topography of the active site of chymotrypsin. Through the use of three different affinity labels that placed reactive groups (halomethyl ketones or phosphofluorides) on different regions of the natural substrate core, the relative positions and identity of three different amino acids could be determined.

Site-Directed Mutagenesis

Site-directed mutagenesis is an invaluable tool to modify genes and study the structural and functional properties of a protein, based on the structure, function, catalytic mechanism, and catalytic residues of enzymes. Site-directed mutagenesis includes single and combinational mutations. It is usually analysed by bioinformatics methods. *Single site-directed mutagenesis* and *multiple mutations* have been used to expedite and simplify methods for mutagenesis. The properties of enzymes can be improved markedly by the combination of site-directed mutagenesis with other methods. Site-directed mutagenesis is the basis for structure and function studies. A 3D structure of the favourite enzyme or, failing that, a robust model is highly recommended. Through site-directed mutagenesis, we have challenged the plasticity of our favourite enzyme as well as its robustness in electrocatalysis or in chemo-enzymatic catalysis involving electron transfer from a transition metal catalyst or from photosensitizers.

4.3.7 Reversible and Irreversible Inhibitors

There are the following two types of organic or inorganic molecules, which act as modifiers of enzyme activity:

1. **Activators**: These molecules are positive modifiers, that is, they increase the activity of enzymes.
2. **Inhibitors**: These molecules decrease the activity of enzymes. Some compounds convert the active enzymes into inactive and thus very adversely affect the rate of reaction, are called enzyme inhibitors and this process is known as enzyme inhibition.

Enzyme inhibitors are molecules or compounds that bind to enzymes and results in a decrease in their activity. An inhibitor can bind to an enzyme and stop a substrate from entering the enzyme's active site and/or prevent the enzyme from catalysing a chemical reaction. There are two categories of inhibitors:

(i) Irreversible Inhibitors

(ii) Reversible Inhibitors

Inhibitors can also be present naturally and can be involved in metabolism regulation. For example, negative feedback caused by inhibitors can help maintain homeostatis in a cell. Other cellular enzyme inhibitors include proteins that specifically bind to and inhibit an enzyme target. This is useful in eliminating harmful enzymes such as proteases and nucleases. Examples of enzymes include poisons and many different types of drugs.

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(I) Irreversible Inhibitors

Irreversible inhibitors covalently bind to an enzyme, because chemical changes to the active sites of enzymes and cannot be reversed. The main role of irreversible inhibitors includes modifying key amino acid residues needed for enzymatic activity. They often contain reactive functional groups such as aldehydes, alkenes or phenyl sulphonates.

These electrophilic groups are able to react with amino acid side chains to form covalent adducts. The amino acid components are residues containing nucleophilic side chains, such as hydroxyl or sulfhydryl groups such as amino acids serine, cysteine, threonine, or tyrosine.

In the first step the inhibitor forms a reversible non-covalent complex with the enzyme or enzyme-substrate complex (EI or ESI). Then, this complex reacts to produce the covalently modified irreversible complex EI*. The rate at which EI* is formed is called the inactivation rate or kinact. Binding of irreversible inhibitors can be prevented by competition with either substrate or a second inhibitor, since the formation of EI may compete with ES (Figure 4.13).

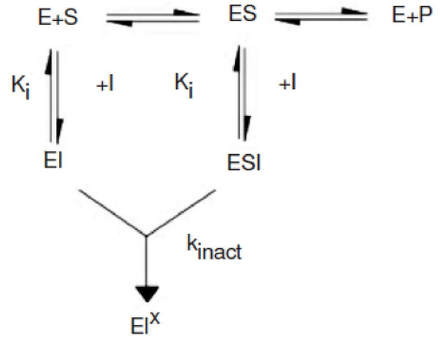


Fig. 4.13 *Irreversible Enzyme Inhibition*

In addition, some reversible inhibitors can form irreversible products by binding very tightly to their target enzyme. These tightly-binding inhibitors show kinetics similar to covalent irreversible inhibitors. As shown in the figure, these inhibitors rapidly bind to the enzyme in a low-affinity EI complex and then undergo a slower rearrangement to a very tightly bound EI* complex. This kinetic behaviour is called slow-binding. Slow binding often involves a conformational change as the enzyme 'Clams Down' around the inhibitor molecule. Some examples of these slow-binding inhibitors include important drugs such as methotrexate and allopurinol.

(II) Reversible Inhibitors

Reversible inhibitors bind non-covalently to enzymes, and many different types of inhibition can occur depending on what the inhibitors bind to. The non-covalent interactions between the inhibitors and enzymes include hydrogen bonds, hydrophobic interactions and ionic bonds. Many of these weak bonds combine to produce strong and specific binding. In contrast to substrates and irreversible inhibitors, reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis.

Types of Inhibitors

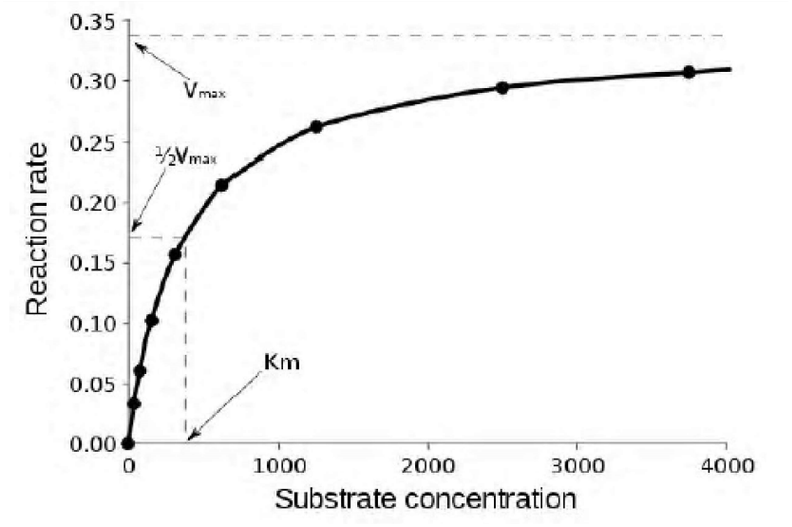
There are three kinds of inhibitors:

(a) Competitive

(b) Non competitive

(c) Uncompetitive/mixed inhibitors

Most reversible inhibitors follow the classic Michaelis-Menten scheme, where an Enzyme (E) binds to its Substrate(S) to form an Enzyme-Substrate Complex (ESC). Km is the Michaelis constant that corresponds to the concentration of the substrate when the velocity is half the maximum. V_{max} is the maximum velocity of the enzyme. Competitive inhibitors can only bind to E and not to ES. They increase K_m by interfering with the binding of the substrate, but they do not affect V_{max} because the inhibitor does not change the catalysis in ES because it cannot bind to ES.



Uncompetitive inhibitors are able to bind to both E and ES, but their affinities for these two forms of the enzyme are different. Therefore, these inhibitors increase K_m and decrease V_{max} because they interfere with substrate binding and hamper catalysis in the ES complex.

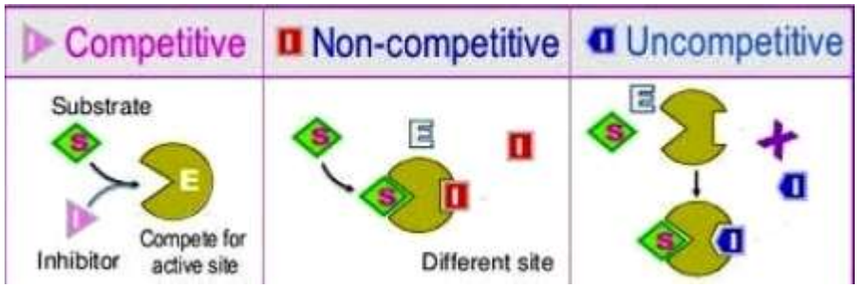


Fig. 4.14 *Types of Enzyme Inhibition*

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Non-competitive inhibitors have identical affinities for E and ES. They do not change K_m, but decreases V_{max}.

Check Your Progress

1. What do you understand by molecular adaption?
2. Define the proximity effect.
3. What is Zymogens?
4. What are the classes of enzymes on the basis of structure?
5. Where are enzymes extracted from?
6. State the induced fit theory.
7. What the concept Michaelis-Menten equation explains?
8. Define the reversible inhibitors.

4.4 MECHANISM OF ENZYME ACTION

The function of enzyme is to increase the rate of reaction in a biological system to sustain life. Consider the following reaction:

$$E + S \longleftrightarrow ES \longleftrightarrow EP \longleftrightarrow E + P$$

Where E, S and P represent enzyme, substrate and product, respectively. ES and EP represent the complex of enzyme with substrate and product, respectively.

Figure 4.15 depicts the change in activation energy in a reaction taking place without enzyme.

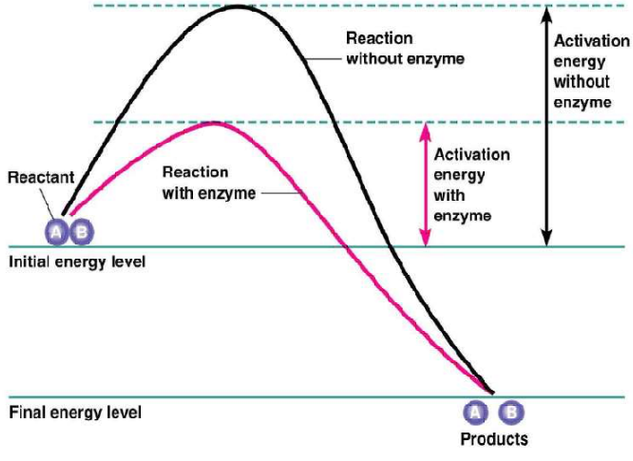

Fig. 4.15 *Process of a Reaction with/without an Enzyme*

Symbols

S = Free energy of substrate

P = Free energy of the product

TS = Transition state

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G*= Activation energy for the two reactions

G = Overall standard free energy change in moving from S-P

According to the reaction coordinate in Figure 4.18, the free energy of P is lower than that of S. Therefore, free energy change is negative for this reaction that favours P. But there exists an energy barrier between the S and P, which is required for the reaction to proceed in either direction. This energy barrier is called the transition state. For the reaction to proceed, the reactant must reach this energy barrier. The difference between the ground state and transition state is known as activation energy.

Therefore, activation energy and rate of reaction are inversely proportional to each other (i.e., if the activation energy is higher, the rate of reaction is slower and vice versa). To overcome this problem, a catalyst is used to enhance the rate of a chemical reaction or, in natural reactions, a catalyst is used by the system itself in the form of enzymes (Refer Figure 4.16). Therefore, a catalyst enhances the rate of a reaction by lowering the activation energy.

Symbols

E = enzymes

ES = enzyme and substrate complex

EP = enzyme and product complex

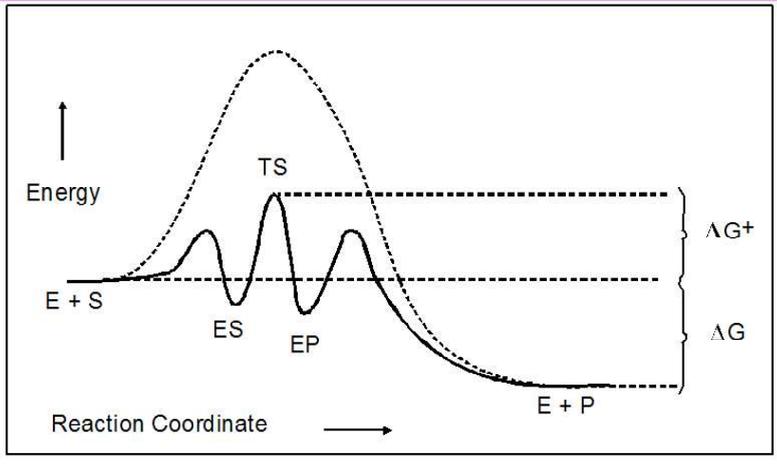

Fig. 4.16 *Process of a Reaction with an Enzyme*

The enzymes do not get used up in the reaction, and the equilibrium point remains unaffected. They only enhance the rate of a reaction. When there are more steps in the reaction, the overall rate is determined by the step with great activation energy, which is known as the rate-limiting step. In Figure 4.19, the ES and EP are intermediates and occupy the valleys in the curve.

4.4.1 Transitions State Theory of Enzyme

The transitions state is the intermediary state of the reaction, when the molecule is neither a substrate nor product. The transition state has the highest free energy, making it a rare and un-stable intermediate. An enzyme helps catalyse a reaction by decreasing the free energy of the transition state. *Transition state theory* is

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most useful in the field of biochemistry, where it is often used to model reactions catalysed by enzymes in the body. For instance, by knowing the possible transition states that can form in a given reaction, as well as knowing the various activation energies for each transition state, it becomes possible to predict the course of a biochemical reaction, and to determine its reaction rate and rate constant.

Enzymes catalyse chemical reactions at rates that are astounding relative to uncatalyzed chemistry at the same reaction conditions. Each catalytic event requires a minimum of three or often more steps, all of which occur within the few milliseconds that characterize typical enzymatic reactions. According to *transition state theory*, the smallest fraction of the catalytic cycle is spent in the most important step, that of the transition state. The original proposals of absolute reaction rate theory for chemical reactions defined the transition state as a distinct species in the reaction coordinate that determined the absolute reaction rate. Soon thereafter, Linus Pauling proposed that the powerful catalytic action of enzymes could be explained by specific tight binding to the transition state species. Because reaction rate is proportional to the fraction of the reactant in the transition state complex, the enzyme was proposed to increase the concentration of the reactive species.

As substrate progresses from the Michaelis complex to product, chemistry occurs by enzyme-induced changes in electron distribution in the substrate. Enzymes alter the electronic structure by protonation, proton abstraction, electron transfer, geometric distortion, hydrophobic partitioning, and interaction with Lewis acids and bases. Analogs that resemble the transition state structures should therefore provide the most powerful non-covalent *inhibitors* known.

All chemical transformations pass through an unstable structure called the transition state, which is poised between the chemical structures of the substrates and products. The transition states for chemical reactions are proposed to have lifetimes near $10^{"13}$ seconds, on the order of the time of a single bond vibration. No physical or spectroscopic method is available to directly observe the structure of the transition state for enzymatic reactions, yet transition state structure is central to understanding enzyme catalysis since enzymes work by lowering the activation energy of a chemical transformation.

It is now accepted that enzymes function to stabilize transition states lying between reactants and products, and that they would therefore be expected to bind strongly any inhibitor that closely resembles such a transition state. Substrates and products often participate in several enzyme catalysed reactions, whereas the transition state tends to be characteristic of one particular enzyme, so that such an inhibitor tends to be specific for that particular enzyme. The identification of numerous transition state inhibitors supports the transition state stabilization hypothesis for enzymatic catalysis. Currently there is a large number of enzymes known to interact with transition state analogs, most of which have been designed with the intention of inhibiting the target enzyme. Examples include HIV-1 protease, racemases, β-lactamases, metalloproteinases, cyclooxygenases and many others.

4.4.2 Steric and Orientation Effect of Enzymes

Steric effects may arise in a number of ways. Primary steric effects result from repulsions between valence electrons or non-bonded atoms. Such repulsions can only result from the energy increase of a group of atoms. The overall steric effect of a chemical reaction may be either favourable or unfavourable. For example, if steric effects in the reactant are larger than in the product (or transition state), then the reaction is favoured (steric augmentation); if the reverse is the case, the reaction is disfavoured (steric diminution).

Enzymes are protein molecules that facilitate vital biological reactions. They can do this because their molecular surfaces contain active sites to which the molecules participating in the reaction (known as substrates) can become temporarily bound.

The active sites are crevices in the enzyme surface, often of a complicated shape. The substrate has the precise shape required to fit the opening, and potential competitors that lack this shape are excluded (Refer Figure 4.21). The need for the substrate to bind to the enzyme surface will often weaken other bonds within the substrate itself, encouraging the changes that the enzyme facilitates. Figure 4.17 show that the model for enzyme Action only one of the three molecules has the shape required to fit the cavity on the enzyme surface; the other two are excluded.

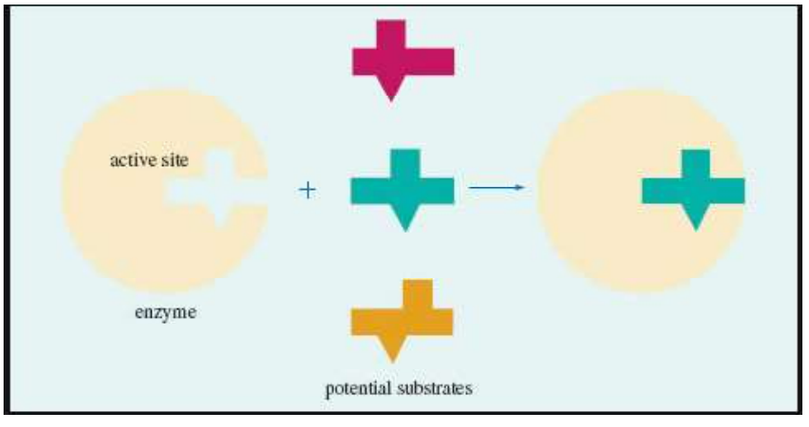

Fig. 4.17 *A Model for Enzyme Action*

Orientation Effect of Enzymes

In addition to proximity effect, a related but distinct strategy used by enzymes to accelerate chemical reactions is to orient substrates into a maximally reactive conformation; this is known as the ***orientation effect***. Simply confining two substrates close to one another does not guarantee a faster reaction rate because in order for two substrates to react they usually must achieve a specific relative orientation. This is similar to you finding your friend in a dining hall. Not only do you need to be in the same area at the same time, but you also need to be facing each other. In the example in Figure 4.22, it is not sufficient to simply tether A and B together; instead, a requirement of their reaction is to orient A and B properly. Many enzymes catalyse reactions not only by holding substrates close together, but

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also by forcing the substrates into an optimal orientation to lower the activation energy needed to reach the *transition state*. Figure 4.18 show the substrates that are restrained into a reactive conformation react more rapidly. Shown are three hypothetical substrates (top, middle, and bottom rows) in which the reactive regions, A and B, are connected by covalent bonds. The reactive conformation in which A and B are close together is drawn in the right-most column for each substrate. The substrate on the top row is the least constrained of the three and can adopt a large number of conformations; consequently, it must lose a large amount of entropy in order to adopt the reactive conformation, making $\Delta S^{\ddagger}$ less favourable. In contrast, the substrate on the bottom row is already constrained into a reactive conformation; consequently, no loss of entropy is needed to adopt the reactive conformation, making $\Delta S^{\ddagger}$ more favourable.

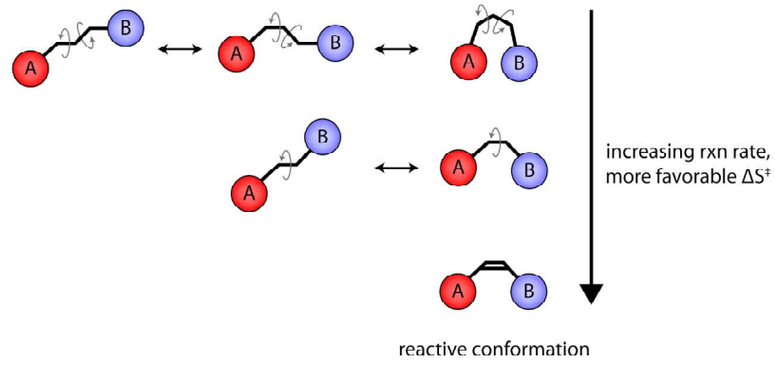

Fig. 4.18 *Substrates that are Restrained into a Reactive Conformation React More Rapidly*

Proximity and orientation effects lower the entropic barrier to forming the transition state ($\Delta S^{\ddagger}$) because they pre-organize the substrates so that they lose less entropy during the formation of the transition state than the free substrates would. In other words, when enzymes bind to substrates they often reduce the entropy of the substrates by constraining them into reactive conformations, thus the favourability of binding between the enzyme and the substrates effectively 'Pre-Pays' for the loss of entropy that is required to form the transition state.

We can quantify the *proximity and orientation effects* using the effective concentration of the reactants in the reactions. The effective concentration is defined as the ratio of the rate constant for the intramolecular reaction (with units of s^{-1}) divided by the rate constant for the intermolecular reaction (with units of $s^{-1}M^{-1}$). Effective concentration has units of Molarity (M) and is a measure of the concentration of a reactant you would have to have in an intermolecular reaction to achieve the same rate as that in the intramolecular reaction with a substrate concentration of 1 M. In the example shown in Figure 4.23 the rate constant for the intermolecular reaction (top) is $k_{inter} = 4 \times 10^{-6}\ s^{-1}M^{-1}$; in contrast, the rate constant of the intramolecular reaction (bottom) is $k_{intra} = 0.8\ s^{-1}$. The effective concentration of A and B in the bottom case is k_{intra}/k_{inter}. In other words, A and B when tethered in close proximity and in the proper orientation by an enzyme react at the same rate as that of the untethered reaction when A or B is an impossibly high 200,000 M. Note that effective concentration takes into account both

proximity and orientation effects; therefore, if the reactants were tethered close to each other but held firmly in the incorrect orientation for the reaction, the effective concentration would not be high. Enzymes use orientation and proximity effects, often with dramatic results, by confining reactants in the proper orientation within the relatively small active site of the enzyme. Figure 4.19 shown the effective concentration measures the rate enhancement of an intramolecular reaction relative to a corresponding intermolecular reaction. (A) Shown are two hypothetical reactions between reactants A and B. The top row shows an intermolecular reaction while the bottom row shows an otherwise equivalent intramolecular reaction. The rate constants of both reactions, k_{inter} and k_{intra}, are indicated. (B) Effective concentration (C_{eff}) equals the ratio of the intramolecular rate constant over the intermolecular rate constant. In this example C_{eff} equals 200,000 M, meaning that the reactants in the intermolecular reaction would have to be present at 200,000 M in order to match the rate of the intramolecular reaction when its reactants are present at 1 M.

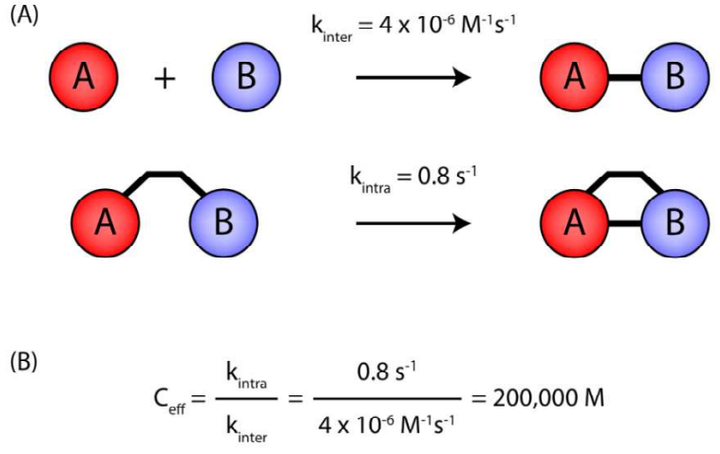

Fig. 4.19 *Effective Concentration Measures the Rate Enhancement of an Intramolecular Reaction Relative to a Corresponding Intermolecular Reaction*

4.4.3 Acid-Base and Covalent Catalysis of Enzyme

In general, an enzyme has an optimum pH. Although most enzymes remain high activity in the pH range between 6 and 8, some specific enzymes work well only in extremely acidic (i.e. pH <5.0) or alkaline (i.e. pH >9.0) conditions.

In **acid-base catalysis**, the chemical reaction is accelerated by the addition of an acid or a base, and the acid or base itself is not consumed in the reaction. Proton transfer is the commonest reaction that enzymes perform. Proton donors and acceptors, i.e., acids and base may donate and accept protons in order to stabilize developing charges in the *transition state*. This typically has the effect of activating nucleophile and electrophile groups, or stabilizing leaving groups. Many acid-base catalysis reactions involve histidine because it has a pK_a close to 7, allowing it to act as both an acid and a base.

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Characteristics of Acid-base Catalysis

The great majority of enzyme reactions, the chemical reactions on which life depends–take place in water, under 'Physiological' conditions, near pH 7. Yet this is where chemical reactions are normally at their slowest. Reactive compounds are readily hydrolyzed by adding strong acid or base in vitro. But this is not a practical proposition for an enzyme: proteins are denatured in the presence of strong acid or base. Nevertheless, the way we think about catalysis by enzymes is based on our understanding of acid–base catalysis in vitro.

The acid-base catalysis is illustrated by the pH–rate profiles shown in Figure 4.20 the three plots are representative of the behaviour of most types of substrate undergoing most sorts of reaction in water. The lower curve (I) represents the reaction in vitro of a typical, unreactive compound: it shows only acid- and base-catalysed reactions, and reaction is very slow at the minimum, near pH 7. For the most reactive compounds, an additional feature (curve II) is a pH-independent region, where the uncatalyzed reaction with water becomes faster than the acid- and base-catalyzed reactions near neutrality. Finally, curve III is the pH–rate profile for a typical enzyme-catalysed reaction: it is much faster than the others (signified by the break in the ordinate), and it is also qualitatively quite different, since now the rate reaches a maximum near pH 7. Enzymes are 'Designed' to operate near pH 7, and typically show pH optima in this region, with rates falling off at higher and lower pH values.

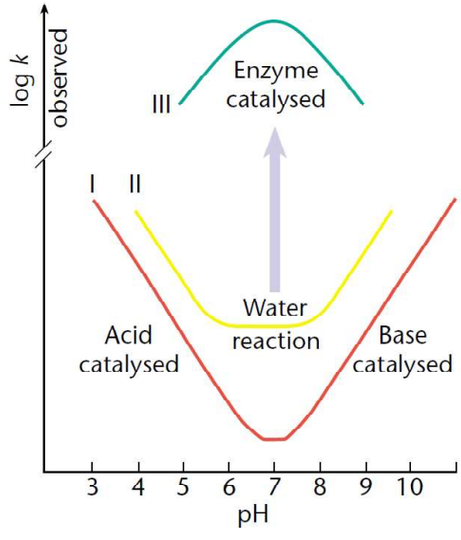

Fig 4.20 *Specific Acid–Base Catalysis and Enzyme Catalysis Compared*

The acid- and base-catalyzed reactions at high and low pH are not directly relevant to catalysis by enzymes. They typically involve activation of the substrate by the addition or removal of a proton in a rapid pre-equilibrium, followed by the rate-determining reaction of the conjugate acid or base.

Mechanism of Acid-base Catalysis

If the substrate is reactive (electrophilic) enough, activation by protonation is not necessary and the attack of water on the neutral molecule is rate determining near

pH 7 (curve II of Figure 4.24). Formally this generates both a positive and a negative charge, and as the reaction proceeds both will be 'Delocalized' into the surrounding solvent via the network of hydrogen bonds. The nucleophile in a hydrolysis reaction is of course a water molecule. As the new C–O bond forms, positive charge develops on the nucleophilic oxygen, and the attached OH protons become more and more acidic, until they can be transferred to solvating water, acting formally as a general base (gb). The negative charge developing on the carbonyl oxygen may similarly be transferred via a hydrogen bond to another water molecule, acting this time as a general acid (ga). This mechanism is always available, though it does not always lead to reaction at an observable rate.

The direct reaction does have the entropic advantage of not involving a third molecule, and a carboxylate anion can–given the right conditions–displace better leaving groups than ethoxide. As long as the intermediate is more reactive than the starting material the result is catalysis of hydrolysis. This mechanism competes equally with general base catalysis for esters with a leaving group of pK_a of ~7, and becomes dominant for derivatives with better leaving groups.

If the leaving group is poor it can be made viable by protonation: complete protonation to form the conjugate acid if the group is sufficiently basic, but involving partial proton transfer in the case of a weakly basic group like OR or OH. This mechanism is involved, though not easily observed, in the breakdown of the tetrahedral addition intermediates involved in the acyl transfer reactions of esters and amides.

Brønsted Acid and Base Groups in Enzymes

The 'Catalytic Machinery' of an enzyme consists of a small number of functional groups brought together in a well-defined three-dimensional arrangement by the tertiary structure of the protein to form the active site. The functional groups concerned are a subset of those available on the side-chains of the naturally occurring amino acids. Under physiological conditions near pH 7, only weak acids and bases, with pK_a within a unit or two of 7, can exist to a significant extent in the acidic or basic form. Thus the strongest acids available on amino acid side chains are the two carboxylic acids; but these will be present at pH 7 almost exclusively as the aspartate and glutamate anions–unless their pK_as are perturbed by their local environment. Similarly, the strongest bases are likely to be protonated unless similarly perturbed. Although such perturbations are not uncommon, the imidazole group of histidine, because it has a pK_a near 7, is at once the strongest acid and the strongest base normally available to enzymes under physiological conditions near pH 7.

A functional group involved in catalysis of a particular step of a particular reaction plays a very specific role, and so can be expected to be active in one particular ionic form: thus reactivity will depend on pH. When two (or more) such groups are involved it is common for one to be active as a general acid, in the protonated form, and the other as a general base or nucleophile, and thus in its basic form. The pH–rate dependence of the catalysed reaction then reflects the fraction of the system with both groups in the active ionic form. At sufficiently high and low pH, a single species is present, as the free base or fully protonated form. But the fraction of the intermediate zwitterionic form increases to a maximum at a

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pH halfway between the two pK_as. If this is the reactive form, the pH–rate profile will also show a maximum at this pH. This is the simplest explanation for a pH optimum for an *enzyme-catalysed reaction*.

Covalent Catalysis

Covalent Catalysis is one of the four strategies that an enzyme will use to catalyse a specific reaction, which involves the formation of a transient covalent bond between a substrate and a residues in the enzyme active site or with a cofactor. In covalent catalysis, an additional covalent intermediate is added to the reaction, and helps to reduce the energy of transition states in the later stages of the reaction. The most common covalent bonds are formed as a result of the attack by an enzyme nucleophilic group on an electrophilic moiety of the substrate that is bound at the active site.

Mechanism

In an enzymatic reaction, covalent catalysis occurs when the substrate become temporarily covalently attached to the enzyme during the catalytic reaction. In this reaction, the enzyme contains a reactive group, usually a nucleophilic residue or an electrophilic residue, which reacts with the substrate through a nucleophilic or electrophilic attack. The nucleophilic groups can be either RCOO-, RNH, ROH that are present on the side-chains of amino acid residues, or the nitrogen atoms of the imidazole ring of histidine residues. The electrophilic moieties of substrates may be acyl, phosphoryl, or glycosyl groups, so the covalent intermediates would be acyl-, phosphoryl-, and glycosyl-enzyme complexes. Enzyme molecules are poor in electrophilic groups, but electrophilic catalysis does occur with those enzymes that contain metals or prosthetic groups that act as electron sinks during catalysis. The charge loss in the reaction during transitional state will then accelerate the hydrolysis.

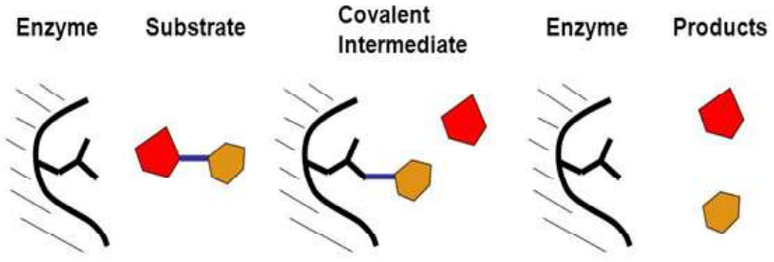


Fig. 4.21 *Mechanism of Covalent Catalysis*

Rather than lowering the activation energy for a reaction pathway, covalent catalysis provides an alternative pathway for the reaction (via to the covalent intermediate) and so is distinct from true catalysis. A true proposal of a covalent catalysis would require, for example, a partial covalent bond to the transition state by an enzyme group, and such effects do not contribute significantly to catalysis.

Covalent Intermediates Formed by Substrates and Cofactors

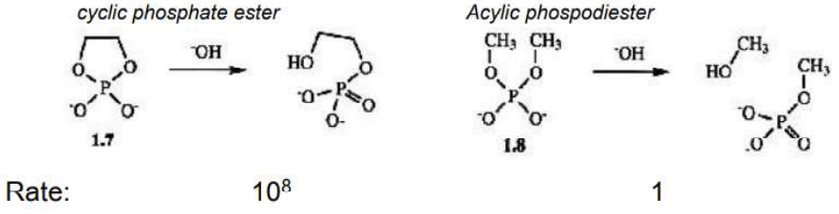
Some enzymes utilize non-amino acid cofactors, such as Pyridoxal Phosphate (PLP) or Thiamine PyrophosPhate (TPP) to form covalent intermediates with reactant molecules. Such *covalent intermediates* function to reduce the energy of

later transition states, similar to how covalent intermediates formed with active site amino acid residues allow stabilization, but the capabilities of cofactors allow enzymes to carryout reactions that amino acid side residues alone could not. Enzymes utilizing such cofactors include the PLP-dependent enzyme aspartate transaminase and the TPP-dependent enzyme pyruvate dehydrogenase.

Strain and Distortion of Enzyme

The activation strain or distortion/interaction model is a tool to analyse activation barriers that determine reaction rates. The energy required to distort the molecules is called the activation strain or distortion energy.

When a substrate bind to the enzyme, it may induce a conformational change in the active site to fit to a transition state. Frequently, in the transition state, the substrate and the enzyme have slightly different structure (strain or distortion) and increase the reactivity of the substrate.



4.4.4 Example of Some Typical Enzyme Mechanism

Isoenzymes

The enzymes that occur in a number of different forms and differ from each other chemically, immunologically and electrophoretically are called 'Isoenzymes' or 'Isozymes'. Isozymes are enzymes that differ in amino acid sequence but catalyse the same chemical reaction. These enzymes usually display different kinetic parameters (e.g. different KM values), or different regulatory properties. The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage are isoforms (closely related variants) of enzymes.

Isozymes are present in the serum and tissues of mammals, amphibians, birds, insects, plants and unicellular organisms.

Enzymes which exist in multiple forms in various tissues and organs but catalyze the same biochemical reaction are referred to as isoenzymes or isozymes. These enzymes possess differences in their molecular structure and kinetic properties. The isoenzymes possess variable molecular weight which is attributed to the differences in their subunit arrangement and Amino Acid composition. Different genes of a single gene family usually encode for isoenzymes in tissues. Isoenzymes comprised of dissimilar subunits are called Heteropolymers. Isoenzymes may vary in their structural feature due to their encoding by alleles of the same gene. The minute differences in the structure of the isoenzymes are also attributed to the post-translational modifications like Glycosylation, N-Nitrosylation or Methylation. The activity of isoenzyme exhibit temporal and spatial regulation in plants and animal tissues. This results in different metabolic

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requirement of the tissues varying with its age and stage of development. The first enzyme reported to possess isoenzyme forms was lactate dehydrogenase. In plants peroxidase has been reported to possess as high as 18 Isoenzyme forms in Maize. This enzyme is a major antioxidative enzyme with detoxification and ion homoeostasis function. The various isoenzymes of peroxidase protects different plant tissues associated with development, senescence or environmental stress. Aspartate Kinase in plants is associated with biosynthesis of Amino Acids like Lysine and Alanine from Aspartate. This enzyme exists in two isozyme forms.

Examples

Isozymes of numerous dehydro-genases, and several oxidases, transaminases, phosphatases, transphosphorylases, proteolytic enzymes, aldolases.

Lysozyme

Lysozyme, also known as *muramidase* or N-*acetylmuramide glycanhydrolase,* is an antimicrobial enzyme produced by animals that forms part of the innate immune system. Lysozyme is a glycoside hydrolase that catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell wall. This hydrolysis in turn compromises the integrity of bacterial cell walls causing lysis of the bacteria.

Lysozyme is abundant in secretions including tears, saliva, human milk, and mucus. It is also present in cytoplasmic granules of the macrophages and the PolyMorphonuclear Neutrophils (PMNs). Large amounts of lysozyme can be found in egg white. C-type lysozymes are closely related to alpha-lactalbumin in sequence and structure, making them part of the same glycoside hydrolase family 22. In humans, the C-type lysozyme enzyme is encoded by the LYZ gene.

Hen egg white lysozyme is thermally stable, with a melting point reaching up to 72 °C at pH 5.0. However, lysozyme in human milk loses activity very quickly at that temperature. Hen egg white lysozyme maintains its activity in a large range of pH (6-9). Its isoelectric point is 11.35. The isoelectric point of human milk lysozyme is 10.5-11.

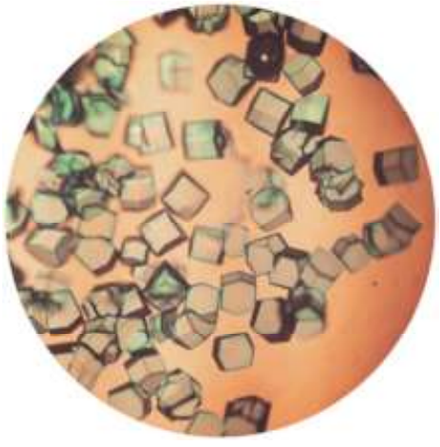

Fig. 4.22 *Lysozyme Crystals Stained With Methylene Blue*

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Function and Mechanism of Lysozyme

The enzyme functions by hydrolyzing glycosidic bonds in peptidoglycans. The enzyme can also break glycosidic bonds in chitin, although not as effectively as true chitinases. Lysozyme's active site binds the peptidoglycan molecule in the prominent cleft between its two domains. It attacks peptidoglycans (found in the cell walls of bacteria, especially Gram-positive bacteria), its natural substrate, between N-AcetylMuramic acid (NAM) and the fourth carbon atom of N-AcetylGlucosamine (NAG).

Shorter saccharides like tetrasaccharide have also shown to be viable substrates but via an intermediate with a longer chain. Chitin has also been shown to be a viable lysozyme substrate. Artificial substrates have also been developed and used in lysozyme.

Phillips Mechanism

The Phillips Mechanism proposed that the enzyme's catalytic power came from both steric strain on the bound substrate and electrostatic stabilization of an oxo-carbenium intermediate. From X-ray crystallographic data, Phillips proposed the active site of the enzyme, where a hexasaccharide binds. The lysozyme distorts the fourth sugar (in the D or -1 subsite) in the hexasaccharide into a half-chair conformation. In this stressed state, the glycosidic bond is more easily broken. An ionic intermediate containing an oxo-carbenium is created as a result of the glycosidic bond breaking. Thus distortion causing the substrate molecule to adopt a strained conformation similar to that of the transition state will lower the energy barrier of the reaction.

The electrostatic stabilization argument was based on comparison to bulk water, the reorientation of water dipoles can cancel out the stabilizing energy of charge interaction. In Warshel's model, the enzyme acts as a super-solvent, which fixes the orientation of ion pairs and provides super-solvation (very good stabilization of ion pairs), and especially lower the energy when two ions are close to each other.

The Rate-Determining Step (RDS) in this mechanism is related to formation of the *oxo-carbenium intermediate.* There were some contradictory results to indicate the exact RDS. By tracing the formation of product (p-nitrophenol), it was discovered that the RDS can change over different temperatures, which was a reason for those contradictory results. At a higher temperature the RDS is formation of glycosyl enzyme intermediate and at a lower temperature the breakdown of that intermediate.

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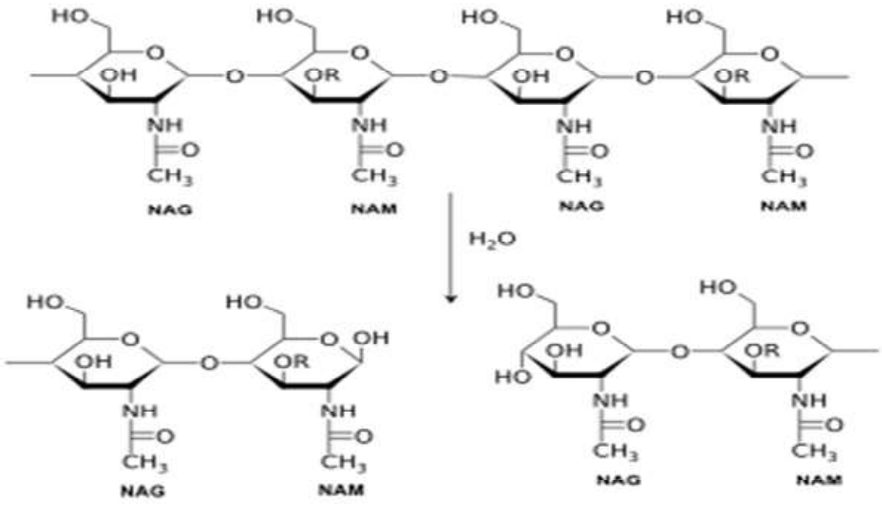


Fig. 4.23 *Overview of the Reaction Catalysed by Lysozyme*

Inhibition

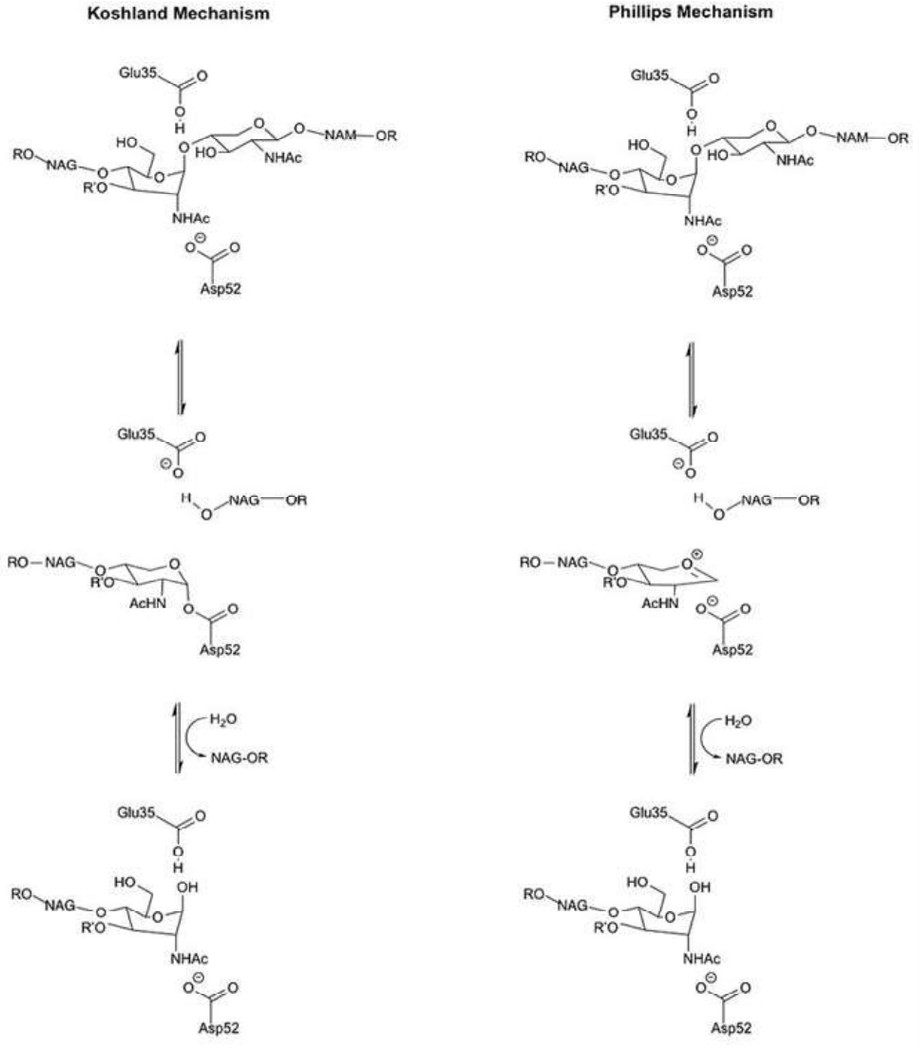
Imidazole derivatives can form a charge-transfer complex with some residues (in or outside active centre) to achieve a competitive inhibition of lysozyme. In Gram-negative bacteria, the lipopolysaccharide acts as a non-competitive inhibitior by highly favoured binding with lysozyme.

Non-Enzymatic Action

Despite that the muramidase activity of lysozyme has been supposed to play the key role for its antibacterial properties, evidence of its non-enzymatic action was also reported. For example, blocking the catalytic activity of lysozyme by mutation of critical amino acid in the active site (52-Asp -> 52-Ser) does not eliminate its antimicrobial activity. The lectin-like ability of lysozyme to recognize bacterial carbohydrate antigen without lytic activity was reported for tetrasaccharide related to lipopolysaccharide of Klebsiella pneumoniae. Also, lysozyme interacts with antibodies and T-cell receptors.

Enzyme Conformation Changes

Lysozyme exhibits two conformations: an open active state and a closed inactive state. The catalytic relevance was examined with Single Walled Carbon Nanotubes (SWCN) Field Effect Transistors (FETs), where a singular lysozyme was bound to the SWCN FET. Electronically monitoring the lysozyme showed two conformations, an open active site and a closed inactive site. In its active state lysozyme is able to processively hydrolyze its substrate, breaking on average 100 bonds at a rate of 15 per second. In order to bind a new substrate and move from the closed inactive state to the open active state requires two conformation step changes, while inactivation requires one step.



Two mechanisms that have beeen proposed for Hen Egg-White Lysozyme. R is an oligosaccharide chain and R' is a petidyl sidechain.

Ribonuclease

Ribonuclease (RNase) is the name of a group of enzymes that change RiboNucleic Acid (RNA) by digesting (cutting) phosphorus-oxygen bonds. The RNases are the subject of wide investigation in the laboratory, though scientists are still learning the many ways they work in living cells. The best-studied RNase is from the pancreas of cattle. Its main portion, called *ribonuclease* A, was the first enzyme whose entire sequence of amino acids was determined. It was also the first protein to be totally synthesized from amino acid.

Pancreatic ribonuclease was first described in 1920 by the American biochemist Walter Jones, who showed that it could digest yeast RNA. It was partially purified in 1938 by the American microbiologist René Jules Dubos and isolated in crystalline form two years later by M. Kunitz. RNase's sequence and three-dimensional structure were determined in 1962 by the American biochemists Christian Anfinsen , Stanford Moore (1913-1982), and William H. Stein, who received the 1972 Nobel prize in chemistry for the accomplishment. Anfinsen

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wanted to learn how the peptide (protein) chain was instructed to fold into its three-dimensional shape. By discovering the amino acid sequence in parts of the molecule, he showed that the sequence itself was all the information needed for folding.

RNases (or ribonucleases) are a class of hydrolytic enzymes that catalyses both the in vivo and in vitro degradation of RiboNucleic Acid (RNA) molecules into smaller components. The nuclease operates at the level of transcription and translation and breaks down the RNA by cleaving the phosphorus-oxygen bonds. RNase enzymes are categorized into two groups:

Exoribonucleases: The exoribonuclease is an exonuclease ribonuclease that degrades RNA by removing terminal nucleotides from either the 52 end or the 32 end of the RNA molecule. It has six families of nucleases, including members such as RNase R, RNase T, and RNase D.

Endoribonucleases: The endonuclease ribonuclease cleaves RNA molecules internally. It can cleave either single-stranded or double-stranded RNA, depending on the enzyme. It has several forms that structurally consist of either single proteins and or a complex of proteins with RNA.

Examples of single proteins are RNase III, RNase A, RNase T1, RNase T2, and RNase H. There are also complexes of the ribonuclease protein and RNA, including RNase P and the RNA-induced silencing complex.

Among all of these RNases, RNase A (or ribonuclease A) is the most commonly studied. It was first isolated from the pancreata of cattle and is also the first enzyme in which a complete amino acid sequence was determined.

The bovine pancreatic ribonuclease (or Ribonuclease A) is also known as a digestive enzyme. It specifically 'Digests' or hydrolyzes RNA polymers by endonuclease cleavage of the phosphodiester bonds. It leads to the formation of covalent links between adjacent ribonucleotide residues in RNA molecules.

Chymotrypsin

Chymotrypsin is a digestive enzyme component of pancreatic juice acting in the duodenum, where it performs proteolysis, the breakdown of proteins and polypeptides. Chymotrypsin preferentially cleaves peptide amide bonds where the side chain of the amino acid N-terminal to the scissile amide bond (the P_1 position) is a large hydrophobic amino acid (tyrosine, tryptophan, and phenylalanine). These amino acids contain an aromatic ring in their side chain that fits into a hydrophobic pocket (the S_1 position) of the enzyme. It is activated in the presence of *trypsin.* The hydrophobic and shape complementarity between the peptide substrate P1 side chain and the enzyme S_1 binding cavity accounts for the substrate specificity of this enzyme. Chymotrypsin also hydrolyses other amide bonds in peptides at slower rates, particularly those containing leucine and methionine at the P_1 position.

Structurally, it is the archetypal structure for its superfamily, the PA clan of proteases.

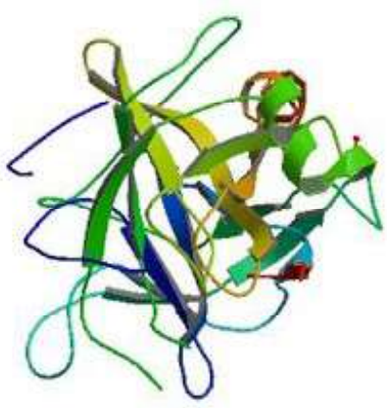


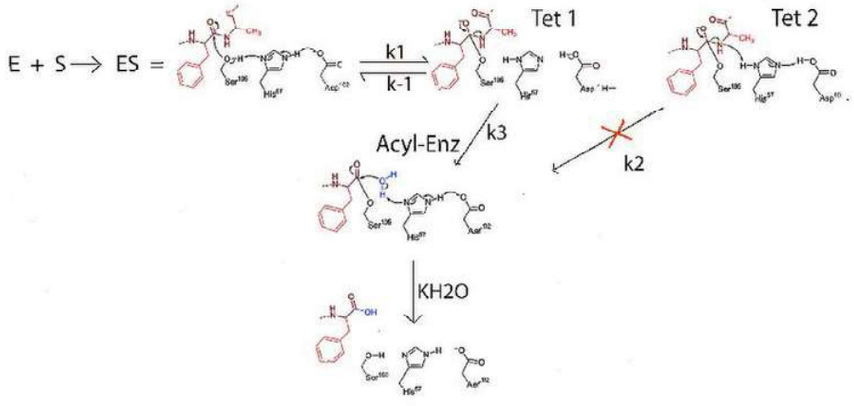
Fig. 4.24 *Crystallographic Structure of Bos Taurus Chymotrypsinogen*

Mechanism

Chymotrypsin is a proteolytic enzyme (serine protease) acting in the digestive systems of many organisms. It facilitates the cleavage of peptide bonds by a hydrolysis reaction, which despite being thermodynamically favourable, occurs extremely slowly in the absence of a catalyst. The main substrates of chymotrypsin are peptide bonds in which the amino acid N-terminal to the bond is a tryptophan, tyrosine, phenylalanine, or leucine. Like many proteases, chymotrypsin also hydrolyses amide bonds in vitro, a virtue that enabled the use of substrate analogs such as N-acetyl-L-phenylalanine p-nitrophenyl amide for enzyme assays. Chymotrypsin cleaves peptide bonds by attacking the unreactive carbonyl group with a powerful nucleophile, the serine 195 residue located in the active site of the enzyme, which briefly becomes covalently bonded to the substrate, forming an enzyme-substrate intermediate. Along with histidine 57 and aspartic acid 102, this serine residue constitutes the catalytic triad of the active site.

Chymotrypsin cleaves peptide bonds by attacking the unreactive carbonyl group with a powerful nucleophile, the serine 195 residue located in the active site of the enzyme, which briefly becomes covalently bonded to the substrate, forming an enzyme-substrate intermediate. Along with histidine 57 and *aspartic acid* 102, this serine residue constitutes the catalytic triad of the active site.

Chymotrypsin catalysis of the hydrolysis of a protein substrate (in red) is performed in two steps. First, the nucleophilicity of Ser-195 is enhanced by general-base catalysis in which the proton of the serine hydroxyl group is transferred to the imidazole moiety of His-57 during its attack on the electron-deficient carbonyl carbon of the protein-substrate main chain (k1 step). This occurs via the concerted action of the three-amino-acid residues in the catalytic triad. The buildup of negative charge on the resultant tetrahedral intermediate is stabilized in the enzyme's active site's oxyanion hole, by formation of two hydrogen bonds to adjacent main-chain amide-hydrogens.



Carboxypeptidase A

A carboxypeptidase is a protease enzyme that hydrolyzes (cleaves) a peptide bond at the carboxy-terminal (C-terminal) end of a protein or peptide. This is in contrast to an aminopeptidases, which cleave peptide bonds at the N-terminus of proteins. Humans, animals, bacteria and plants contain several types of carboxypeptidases that have diverse functions ranging from catabolism to protein maturation.

Carboxypeptidase A usually refers to the pancreatic exopeptidase that hydrolyzes peptide bonds of C-terminal residues with aromatic or aliphatic side-chains. Most scientists in the field now refer to this enzyme as CPA1, and to a related pancreatic carboxypeptidase as CPA2.

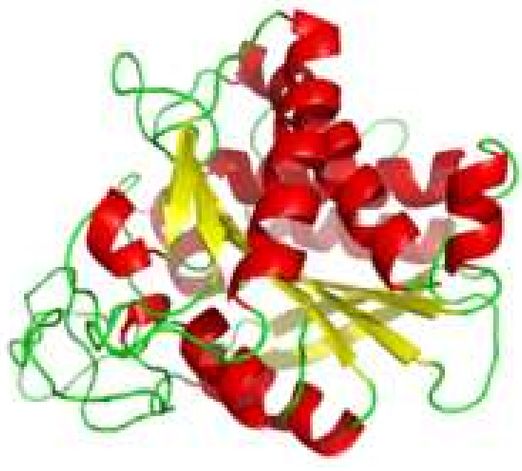


Fig. 4.25 *Carboxypeptidase A*

Types

In addition, there are 4 other mammalian enzymes named CPA-3 through CPA-6, and none of these are expressed in the pancreas. Instead, these other CPA-like enzymes have diverse functions.

- CPA3 (also known as mast-cell CPA) is involved in the digestion of proteins by mast cells.

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- CPA4 (previously known as CPA-3, but renumbered when mast-cell CPA was designated CPA-3) may be involved in tumor progression, but this enzyme has not been well studied.
- CPA6 is expressed in many tissues during mouse development, and in adult shows a more limited distribution in brain and several other tissues. CPA6 is present in the extracellular matrix where it is enzymatically active. A human mutation of CPA-6 has been linked to Duane's syndrome (abnormal eye movement). Recently, mutations in CPA6 were found to be linked to epilepsy.

Mechanism

Classified as a *metalloexopeptidase, carboxypeptidase A* consists of a single polypeptide chain bound to a zinc ion. This characteristic metal ion is located within the active site of the enzyme, along with five amino acid residues that are involved in substrate binding: Arg-71, Arg-127, Asn-144, Arg-145, Tyr-248, and Glu-270. X-ray *crystallographic studies* have revealed five subsites on the protein. These allosteric sites are involved in creating the ligand-enzyme specificity seen in most bioactive enzymes. One of these subsites induces a conformational change at Tyr-248 upon binding of a substrate molecule at the primary active site. The phenolic hydroxyl of tyrosine forms a hydrogen bond with the terminal carboxylate of the ligand. In addition, a second hydrogen bond is formed between the tyrosine and a peptide linkage of longer peptide substrates. These changes make the bond between the enzyme and ligand, whether it is substrate or inhibitor, much stronger. This property of carboxypeptidase A led to the first clause of Daniel E. Koshland, Jr.'s 'Induced Fit' hypothesis.

The S_1 sub-site is where catalysis occurs in CPA, and the zinc ion is coordinated by Glu-72, His-69, and His-196 enzyme residues. A plane exists that bisects the active-site groove where residues Glu-270 and Arg-127 are on opposite sides of the zinc-water coupled complex. The zinc is electron rich due to glutamine ligands coordinating the zinc because before substrate binds, Glu-72 coordinates bidentate but shifts to monodentate after substrate binds. As a result, the zinc metal is not able to deprotonate the coordinated water molecule to make a hydroxyl nucleophile.

Glu-270 and Arg-127 play an important role in catalysis shown in Figure 4.28. Arg-127 acts to stabilize the carbonyl of the substrate that is bound to amino group of phenylalanine. Simultaneously, the water molecule coordinated to zinc is deprotonated by Glu-270 and interacts with the carbonyl stabilized by Arg-127. This creates an intermediate, shown in Figure 2, where the negatively charged oxygen is coordinated to zinc, and through unfavorable electrostatic interactions between Glu-270 and the ionized product facilitates the release of the product at the end of catalysis.

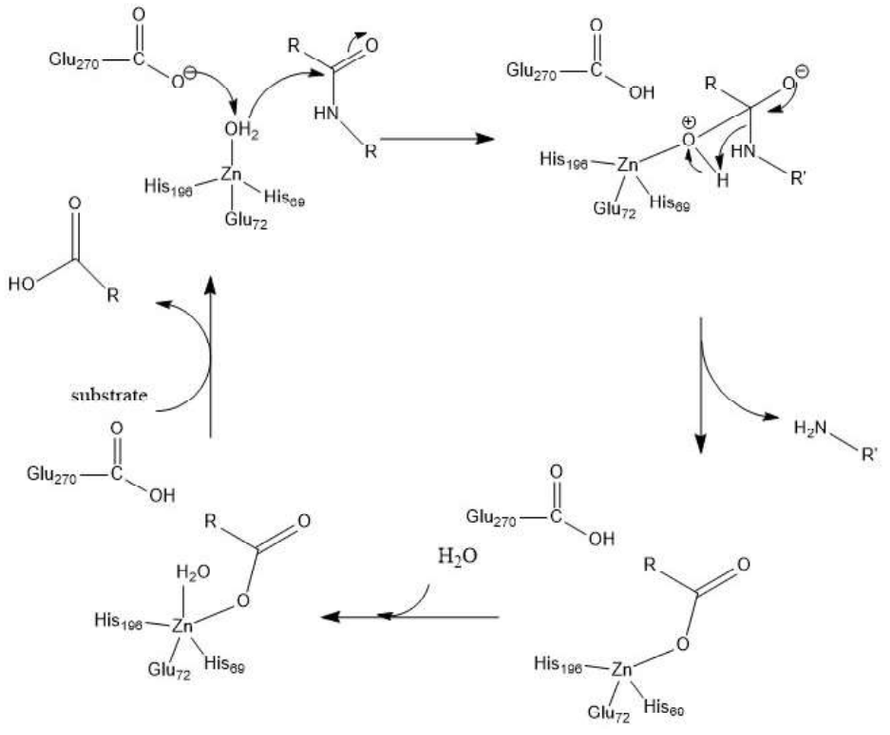


Fig 4.26 *CPA-Catalyzed Proteolysis Promoted by Coordinated Water Molecule*

Applications

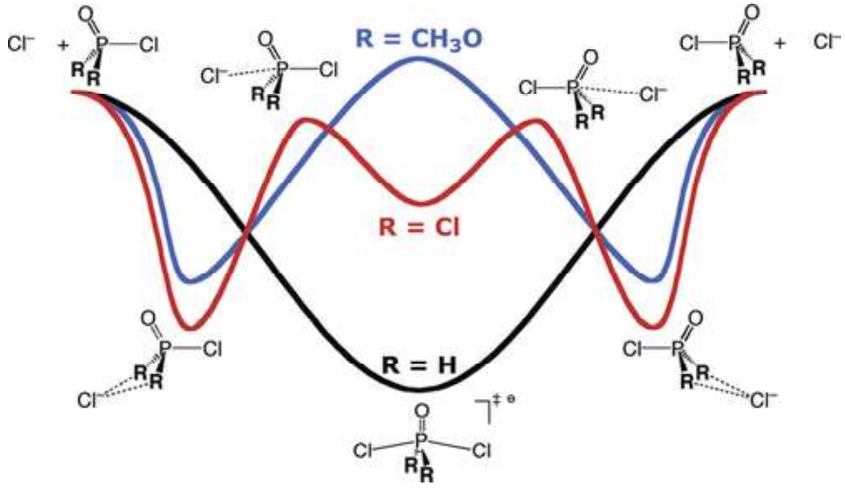
This vast scope of functionality for a single protein makes it the ideal model for research regarding other zinc proteases of unknown structure. Recent biomedical research on collagenase, enkephlinase, and angiotensin-converting enzyme used carboxypeptidase A for inhibitor synthesis and kinetic testing. For example, a drug that treats high blood pressure, Captopril, was designed based on a carboxypeptidase A inhibitor. Carboxypeptidase A and the target enzyme of Captopril, angiotensin-converting enzyme, have very similar structures, as they both contain a zinc ion within the active site. This allowed for a potent carboxypeptidase A inhibitor to be used to inhibit the enzyme and, thus, lower blood pressure through the renin-angiotensin-aldosterone system.

4.5 KINDS OF REACTIONS CATALYSED BY ENZYME

Nucleophilic Displacement at Phosphorus Atom

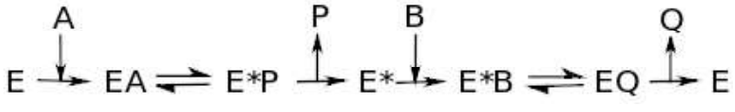
Penta co-ordinate phosphorus species play a key role in organic and biological processes. Yet, their nature is still not fully understood, in particular, whether they are stable, intermediate Transition Complexes (TC) or Labile Transition States (LTS). Through systematic, theoretical analyses of elementary S_N2, S_N2Si, and S_N2 reactions, we show how increasing the co-ordination number of the central atom as well as the substituents' steric demand shifts the S_N2 mechanism stepwise from a single-well potential (with a stable central TC) that is common for

substitution at third-period atoms, via a triple-well potential (featuring a pre- and post-TS before and after the central TC), back to the double-well potential (in which pre- and postbarrier merge into one central TS) that is well-known for substitution reactions at carbon. Our results highlight the steric nature of the S_N2 barrier, but they also show how electronic effects modulate the barrier height. Reaction nucleophilic displacement at phosphorus atom are following:



Multiple Displacement Reaction

The simplest of enzymes will involve one substrate binding to the enzyme and producing a product plus the enzyme. However, the majority of enzymes are more complex and catalyse reactions involving multiple substrates. Binding of two substrates can occur through two mechanisms: sequential mechanism and non-sequential mechanism. In sequential mechanisms both substrates bind the enzyme and the reaction proceeds to form products which are then released from the enzyme. This mechanism can be further subdivided into random and ordered reactions. For random reactions the order in which the substrates bind does not matter. In ordered reactions one substrate must bind the enzyme before the second substrate is able to bind. Non-Sequential mechanism does not require both substrates to bind before releasing the first product. This page will focus on the non-sequential mechanism, which is also known as the 'Ping-Pong' mechanism. It is called this because the enzyme bounces back and forth from an intermediate state to its standard state.The enzyme acts like a ping-pong ball, bouncing from one state to another.



This reaction shows that as substrate A binds to the enzyme, enzyme-substrate complex EA forms. At this point, the intermediate state, E* forms. P is released from E*, then B binds to E*. B is converted to Q, which is released as the second product. E* becomes E, and the process can be repeated. Often times, E* contains a fragment of the original substrate A. This fragment can alter the function of the enzyme, gets attached to substrate B, or both.

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One example of a ping-pong enzyme is low molecular weight protein tyrosine phosphatase. It reacts with the small substrate p-initrophenylphosphate (A) which binds to the enzyme covalently with the expulsion of the product P, the p-nitrophenol leaving group. Water (B) then comes in and covalently attacks the enzyme, forming an adduct with the covalently bound phosphate releasing it as inorganic phosphate. In this particular example, however, you cannot vary the water concentration and it would be impossible to generate the parallel ***Lineweaver-Burk plots*** characteristic of ***ping-pong kinetics***.

Mechanism

Ping-pong mechanism, also called a double-displacement reaction, is characterized by the change of the enzyme into an intermediate form when the first substrate to product reaction occurs. It is important to note the term intermediate indicating that this form is only temporary. At the end of the reaction the enzyme MUST be found in its original form. An enzyme is defined by the fact that it is involved in the reaction and is not consumed. Another key characteristic of the ping-pong mechanism is that one product is formed and released before the second substrate binds. The figure below explains the Ping Pong mechanism through an enzymatic reaction.

Transfer of Sulphate

Protein sulphation occurs exclusively at tyrosine residues. It has been suggested that up to 1% of the tyrosine protein content becomes sulphated, which the most abundant posttranslational modification for tyrosine, with phosphorylation is occurring only on 0.5% of tyrosine protein content. Sulphation occurs mostly on excreted proteins or trans-membrane proteins. Sulphation is catalyzed by Tyrosyl Protein SulphoTransferase (TPST), with PAPS as a cosubstrate (Refer Figure 4.27). Like kinases, sulphotransferases have a biological inverse known as sulphatases. Figure 4.27 shown that the catalytic mechanism of protein sulphation. Tyrosine attacks electrophilic sulphur generating 32 ,52 -ADP as a leaving group.

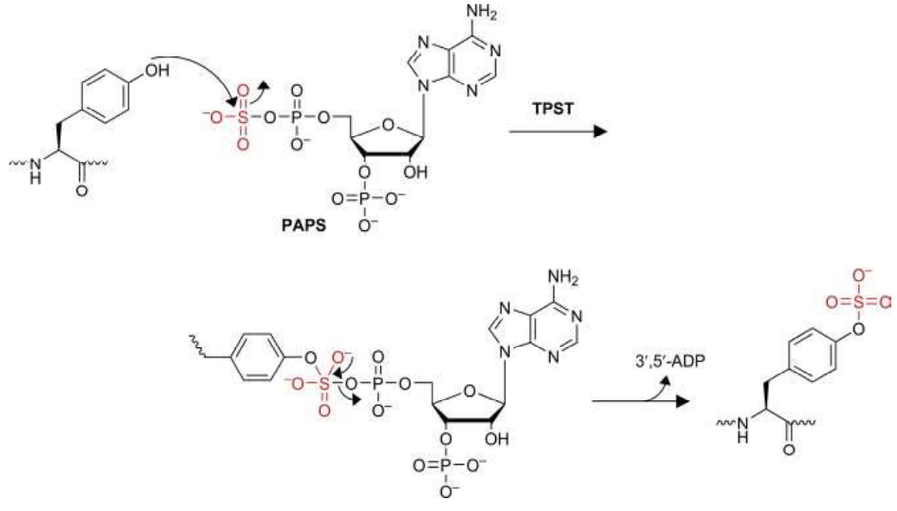

Fig. 4.27 *Catalytic Mechanism of Protein Sulphation*

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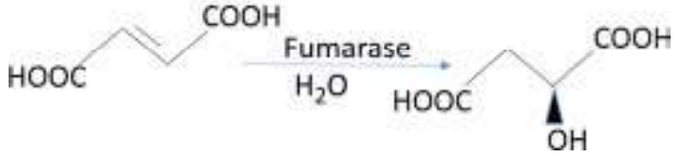
Tyrosine attacks electrophilic sulphur generating 32 ,52 -ADP as a leaving group

Sulphation in most aspects is very similar to phosphorylation, except that sulphation is not involved in intracellular signal transduction, but in other forms of signaling. The mechanism of sulphation is similar to that of phosphorylation as a general base from the enzyme active site that deprotonates the hydroxyl groups of tyrosine residues. The nucleophilic oxygen then attacks the β-position, in contrast to the γ-position in phosphorylation, and releases adenosine 32 , 52 -diphosphate.

Addition and Elimination Reaction Catalysed by Enzymes

Lyases are the enzymes responsible for catalysing *addition and elimination* reactions. *Lyase-catalysed reactions* break the bond between a carbon atom and another atom, such as oxygen, sulphur, or another carbon atom. They are found in cellular processes, such as the citric acid cycle, and in organic synthesis, such as in the production of cyanohydrins. Another example is the use of a fumarase for the production of (S)-malic acid from fumaric acid. A third example is *biocatalytic production* of a cyanohydrin from a ketone that is catalysed by oxynitrilase.

Synthesis of S-malic acid



Beta Cleavage of Enzyme Catalysis

The ***enzymatic cleavage*** of C-C bonds in ***beta-diketones*** is, comparatively, a little studied biochemical process, but one that has important relevance to human metabolism, bioremediation and preparative biocatalysis. In recent studies, four types of enzymes have come to light that cleave C-C bonds in the beta-diketone functionality using different chemical mechanisms. OPH [oxidized poly(vinyl alcohol)], which cleaves nonane-4,6-dione to butyrate and pentan-2-one is a serine-triad hydrolase. Dke1 (diketone-cleaving enzyme from Acinetobacter johnsonii) is a dioxygenase, cleaving acetylacetone to methylglyoxal and acetate. Fumarylacetoacetate hydrolase cleaves fumarylacetoacetate to fumarate and acetoacetate using a water molecule, activated by a catalytic His/Asp dyad, aided by a calcium ion that both chelates the enol acid form of the substrate and indirectly positions the water for nucleophilic attack at a carbonyl group. 6-oxocamphor hydrolase cleaves nonenolizable cyclic beta-diketones and is a homologue of the crotonase superfamily, employing a catalytic His/Asp dyad to activate a water molecule for nucleophilic attack at a carbonyl group on one prochiral face of the diketone substrate, effecting desymmetrizations of symmetrical substrates.

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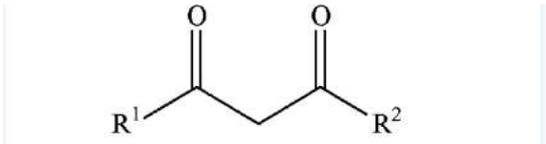


R^1, R^2 = Alkyl; 'neutral' β-diketone

R^1 = alkyl; R^2 = CO_2^-, $CH_2CO_2^-$; β-diketoacid

Condensation Reactions

Generally these reactions are controlled by enzymes such as carbohydrases, proteases, lipases, nucleases, more specific examples of which are fairly well known. In fact the main information which is expected to be known is the type of enzyme and the bonds which it opens. Enzyme-catalysed condensation reactions which initiate rapid peptic cleavage of substrates.

Condensation is a chemical process by which 2 molecules are joined together to make a larger, more complex, molecule, with the loss of water. It is the basis for the synthesis of all the important biological macromolecules (carbohydrates, proteins, lipids, nucleic acids) from their simpler sub-units. It is important not to get condensation and hydrolysis muddled up, as they are in fact opposite processes! Condensation is so called because the product is drawn together from two other substances, in effect getting smaller by losing water. In all cases of condensation, molecules with projecting -H atoms are linked to other molecules with projecting -OH groups, producing H_2O, (H.OH) also known as water, which then moves away from the original molecules.

A-H + B-OH —> A-B + H_2O

Enolic Intermediates in Isomerization Reaction

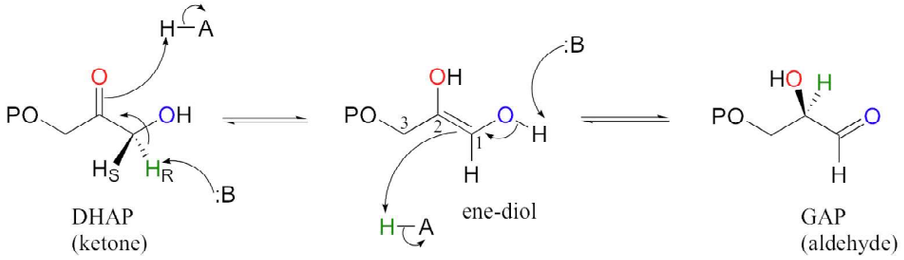
Enols, enolates, and enamines are reactive ***intermediates*** in many ***isomerization reactions***. Isomerizations can involve either the interconversion of constitutional isomers, in which bond connectivity is altered, or of stereoisomers, where the *stereochemical configuration* is changed. Enzymes which interconvert constitutional isomers are called *isomerases*, while those which catalyze the interconversion of enantiomers and epimers are called racemases and epimerases, respectively.

Carbonyl Isomerization

Two important chemical steps in the glycolytic pathway, catalysed by the enzymes phosphoglucose isomerase and triose phosphate isomerase, involve successive *keto-enol tautomerization steps*. In both reactions, the location of a carbonyl group on a sugar molecule is shifted back and forth by a single carbon, as ketones are converted to aldehydes and back again - this is a conversion between two constitutional isomers.

The *triosephosphate isomerase reaction*, in the ketone to aldehyde direction. The ketone species, Di Hydroxy Acetone Phosphate (DHAP) is first converted to its enol tautomer with the assistance of an enzymatic acid/base pair

(actually, this particular intermediate is known as an 'Ene-Diol' rather than an enol, because there are hydroxyl groups on both sides of the carbon-carbon double bond). The initial proton donor is positioned in the active site near the carbonyl carbon, and significantly lowers the pK_a of the alpha-proton.



The second step, leading to Glycer Aldehyde Phosphate (GAP), is simply another *tautomerization,* this time in the reverse direction. However, because there happens to be a hydroxyl group on C_1, the carbonyl can form here as well as at C_2. Most important that DHAP is achiral while GAP is chiral, and that a new chiral centre is introduced at C_2. The catalytic base abstracts the pro-R proton from behind the plane of the page, then gives the same proton back to C_2, again from behind the plane of the page.

In the *phosphoglucose isomerase reaction*, glucose-6-phosphate (an aldehyde sugar) and fructose-6-phosphate (a ketone sugar) are interconverted in a very similar fashion.

glucose-6-phosphate fructose-6-phosphate

The enzyme *ribose-5-phosphate isomerase,* which is active in both the Calvin cycle and the pentose phosphate pathway, catalyses an analogous aldehyde-to-ketone isomerization between two five-carbon sugars.

Stereoisomerization at Chiral Carbons

The *enolate form* of a carbonyl is a common intermediate in a type of stereoisomerization reaction known as an epimerization. The term 'Epimer' refers to a pair of *diastereomers* that differ at a single stereocenter. The five-carbon sugar phosphates ribulose-5-phosphate and xylulose-5-phosphate, for example, are epimers: they are identical except for their stereochemistry at C_3. A reaction in the **Calvin cycle**, which plants use to incorporate, or 'Fix', the carbon atom from carbon dioxide into sugars, involves the interconversion of these two sugar phosphate epimers - in other words, inversion of configuration at C_3.

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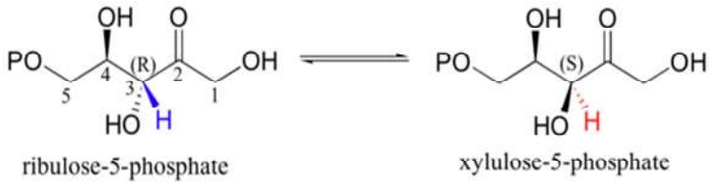
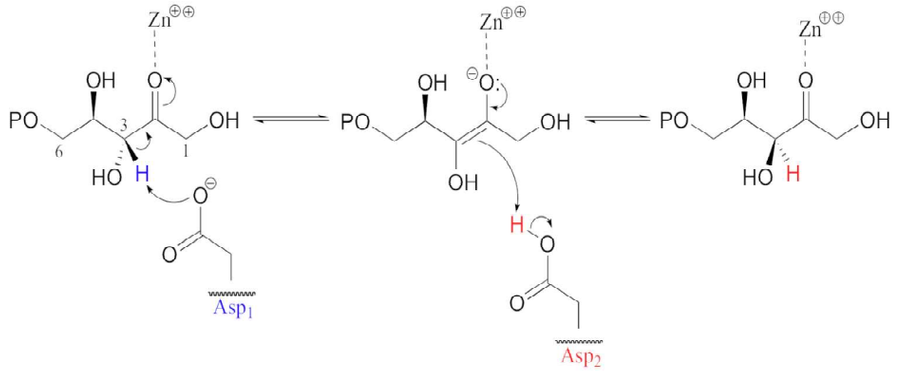


The reaction is simply a deprotonation at C_3 to form an enolate, followed by reprotonation to return to the ketone.



The key to *epimerization* is that deprotonation and reprotonation occur at opposite sides of the planar, sp^2-hybridized intermediate. In the epimerase reaction shown here, a co-ordinated pair of aspartate residues is located at opposite sides of the enzyme's active site. Asp_1, which is ionized at the start of the catalytic cycle, abstracts the C_3 proton from the front side of the plane of the page, converting C_3 from a chiral tetrahedral centre to an achiral, planar group. When reprotonation occurs, it is Asp_2, on the back side of the plane of the page, which donates the proton, resulting in inversion of stereochemistry at C_3.

Come to the thing about this epimerization reaction: the key intermediate is an enolate anion, stabilized by coordination to a zinc cation (a Lewis acid). Contrast this with the phosphoglucase isomerase reaction.Where stabilization of the significant intermediate was achieved by proton donation to form a short-lived enol species.

There are many more examples of reactions in which the stereochemistry of an α-carbon in a carbonyl compound is inverted. The short polypeptides that form part of the peptidoglycan cell wall structure in bacteria contain some D-amino acids, which have the opposite stereochemistry at the a-carbon compared to the L-amino acids that are found almost exclusively in proteins. Enzymes called amino acid racemases catalyse the conversion between the L and D forms of these amino acids. While many amino acid racemases depend on the coenzyme pyridoxal phosphate to stabilize the key carbanion intermediate, others proceed through an *enolate intermediate*. Glutamate racemase, which converts L-glutamate to D-glutamate, is an example.

Alkene Isomerization in the Degradation of Unsaturated Fatty Acids

The oxidation pathway for unsaturated fatty acids (in other words, fatty acids whose hydrocarbon chains contain one or more double bonds) involves the

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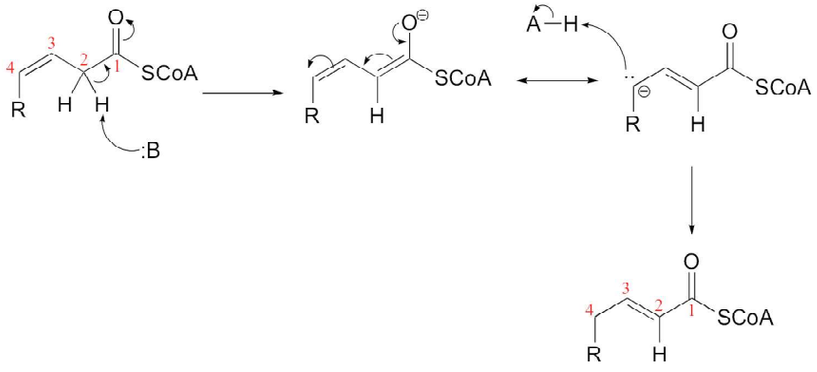
'Shuffling' of the position of carbon-carbon double bonds in the chain. This is accomplished by enoyl CoA isomerase, through an *enolate intermediate*. Here, the cis double bond between C_3 and C_4 is isomerized to a trans double bond between C_2 and C_3.



Enzyme Catalysing Rearrangement

Enzyme catalysing ***optical or geometrical rearrangement*** of atomic groupings without altering molecular weight or number of atom is isomerase. It converts into one form of isomer to the other isomer by changing the intramolecular arrangement by forming or breaking the bond. For, example glucose 6 phosphate is changed to fructose 6 phosphate by enzyme phosphoglucose isomerase (an isomerase) during the process of glycolysis.

glucose-6-phosphate fructose-6-phosphate

Enzyme Catalysed Carboxylation and Decarboxylation

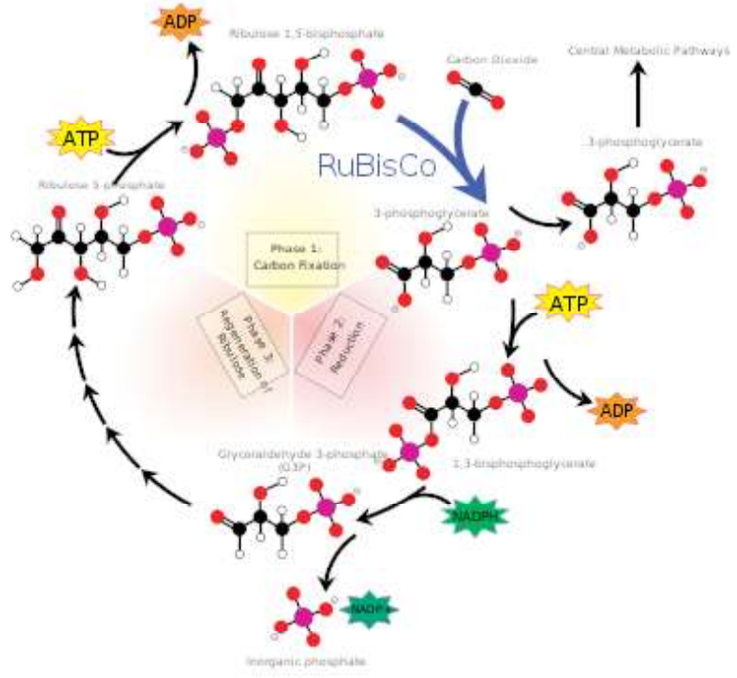
Carboxylation

Carbon-based life originates from carboxylation that couples atmospheric carbon dioxide to a sugar. The process is usually catalysed by the enzyme RuBisCO. Ribulose-1,5-bisphosphate carboxylase/oxygenase, the enzyme that catalyzes this carboxylation, is possibly the single most abundant protein on Earth.

Many carboxylases, including Acetyl-CoA carboxylase, Methylcrotonyl-CoA carboxylase, Propionyl-CoA carboxylase, and Pyruvate carboxylase require biotin as a cofactor. These enzymes are involved in various biogenic pathways. In the EC scheme, such carboxylases are classed under EC 6.3.4, 'Other Carbon—Nitrogen Ligases'.

Another example is the posttranslational modification of glutamate residues, to **γ-carboxyglutamate**, in proteins. It occurs primarily in proteins involved in the blood clotting cascade, specifically factors II, VII, IX, and X, protein C, and protein S, and also in some bone proteins. This modification is required for these

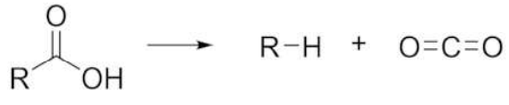
proteins to function. Carboxylation occurs in the liver and is performed by γ-glutamyl carboxylase (GGCX). GGCX requires vitamin K as a cofactor and performs the reaction in a processive manner. γ-carboxyglutamate binds calcium, which is essential for its activity. For example, in prothrombin, calcium binding allows the protein to associate with the plasma membrane in platelets, bringing it into close proximity with the proteins that cleave prothrombin to active thrombin after injury. In following figure show the Calvin cycle showing the carboxylation of ribulose-1,5-bisphosphate.



There are the following six classes as per the IUB system.

Decarboxylation

Decarboxylation is a chemical reaction that removes a carboxyl group and releases Carbon Dioxide (CO_2). Usually, decarboxylation refers to a reaction of carboxylic acids, removing a carbon atom from a carbon chain. The reverse process, which is the first chemical step in photosynthesis, is called carboxylation, the addition of CO_2 to a compound. Enzymes that catalyse decarboxylations are called *decarboxylases* or, the more formal term, carboxy-lyases.



Reaction of Decarboxylation

Decarboxylations are pervasive in biology. They are often classified according to the cofactors that catalyse the transformations. *Biotin-coupled processes* effect the decarboxylation of malonyl-CoA to acetyl-CoA. Thiamine (T:) is the active component for decarboxylation of alpha-ketoacids, including pyruvate:

$$\mathbf{T: + RC(O)CO_2H \rightarrow T{=}C(OH)R + CO_2}$$

$$\mathbf{T{=}C(OH)R + R'CHO \rightarrow T: + RC(O)CH(OH)R'}$$

Pyridoxal phosphate promotes decarboxylation of amino acids. ***Flavin-dependent decarboxylases*** are involved in transformations of cysteine. Iron-based hydroxylases operate by reductive activation of O_2 using the decarboxylation of ***alpha-ketoglutarate*** as an electron donor. The decarboxylation can be depicted as such:

$$RC(O)CO_2Fe^{II} + O_2 \rightarrow RCO_2Fe^{IV}=O + CO_2$$

$$RCO_2Fe^{IV}=O + R'\text{-}H \rightarrow RCO_2Fe^{II} + R'OH$$

(i) Oxidoreductases: These enzymes catalyse oxidation-reduction reactions between two substrates S and S'. An example of these reactions is as follows:

$$S_{reduced} + S'_{oxidized} \longrightarrow S_{oxidized} + S'_{reduced}$$

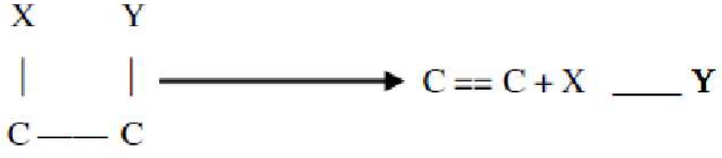
(ii) Transferases: These enzymes catalyse the transfer of a group (G) other than hydrogen between a pair of substrates S and S'. An example of this type of reactions is as follows:

$$S\text{-}G + S' \longrightarrow S + S\text{-}G$$

(iii) Hydrolases: These enzymes catalyse hydrolysis by addition of water across the bond they split. An example of these reactions is as follows:

$$\text{L-arginine} + H_2O \longrightarrow \text{L-ornithine} + \text{Urea}$$

(iv) Lyases: These enzymes catalyse the removal of groups from the substrates by mechanisms other than hydrolysis forming double bonds. An example of such reactions is as follows:



(v) Isomerases: These enzymes catalyse interconversion of optical, geometric or positional isomers by intermolecular rearrangement of atoms or groups. An example of these reactions is as follows:

$$\text{D-glucose-6-posphate} \longrightarrow \text{D-fructose-6-posphate}$$

(vi) Ligases: These enzymes catalyse the linking of two compounds utilizing the energy available from simultaneous breaking of a pyrophosphate bond in Adenosine TriPhosphate (ATP) or a similar compound. An example of these reactions is as follows:

$$\text{ATP} + \text{acetate} + \text{CoA} \longrightarrow \text{AMP} + \text{pyrophosphate acetyle-CoA}$$

Where CoA is coenzyme A.

NOTES

Check Your Progress

9. State the transitions state theory of enzyme.
10. Define the orientation effect of enzyme.
11. Are enzymes acids or bases?
12. What is strain and distortion theory?
13. Define the isoenzyme.
14. What do you understand by chymotrypsin?
15. Is condensation catalyzed by enzymes?

4.6 ANSWERS TO 'CHECK YOUR PROGRESS'

1. Molecular adaptation studies provide empirical evidence to understand why organismal features exist, to map the evolutionary trajectories that shape populations, and to help predict future adaptations.
2. Proximity effects describe the orientation and movement of the substrate molecules when binding to enzyme active sites, and are most readily observed by comparing equivalent inter- and intramolecular reactions.
3. Zymogens is an inactive enzyme precursor requiring a biochemical change (such as a hydrolysis reaction that reveals the active site, or changes the configuration to reveal the active site) for it to become an active enzyme.
4. On the basis of structure enzymes can be classified into Simple protein enzymes and complex protein enzymes.
5. Many enzyme extracts must, of necessity, be made from green leaves, fruits, and other vegetable tissues that contain large amounts of phenols and polyphenols.
6. According to this theory the presence of substrate in the vicinity of the enzyme acts as an inducer of the enzyme. The presence of the substrate results in the formation of conformational change in the active site of the enzyme. This results in the formation of chemical interaction between the enzyme and substrate. The substrate is held in the active site by help of Hydrogen bonds.
7. The concept of Michaeli-Mentur explains the regulation of the rate of enzymatic reaction which depends upon the concentration of substrate and available molecules of enzyme in a reaction medium.
8. Reversible inhibitors bind non-covalently to enzymes, and many different types of inhibition can occur depending on what the inhibitors bind to. The non-covalent interactions between the inhibitors and enzymes include hydrogen bonds, hydrophobic interactions and ionic bonds.
9. The transitions state is the intermediary state of the reaction, when the molecule is neither a substrate nor product. The transition state has the highest free energy, making it a rare and un-stable intermediate. An enzyme helps catalyse a reaction by decreasing the free energy of the transition state.

10. In addition to proximity effect, a related but distinct strategy used by enzymes to accelerate chemical reactions is to orient substrates into a maximally reactive conformation; this is known as the orientation effect.
11. In general, an enzyme has an optimum pH. Although most enzymes remain high activity in the pH range between 6 and 8, some specific enzymes work well only in extremely acidic (i.e. pH <5.0) or alkaline (i.e. pH >9.0) conditions.
12. The activation strain or distortion/interaction model is a tool to analyse activation barriers that determine reaction rates. The energy required to distort the molecules is called the activation strain or distortion energy.
13. The enzymes that occur in a number of different forms and differ from each other chemically, immunologically and electrophoretically are called 'Isoenzymes' or 'Isozymes'. Isozymes are enzymes that differ in amino acid sequence but catalyse the same chemical reaction.
14. Chymotrypsin is a digestive enzyme component of pancreatic juice acting in the duodenum, where it performs proteolysis, the breakdown of proteins and polypeptides.
15. Enzyme-catalysed condensation reactions which initiate rapid peptic cleavage of substrates.

4.7 SUMMARY

- In biology, adaptation has three related meanings. Firstly, it is the dynamic evolutionary process that fits organisms to their environment, enhancing their evolutionary fitness. Secondly, it is a state reached by the population during that process.
- Advances in bioinformatics and high-throughput genetic analysis increasingly allow us to predict the genetic basis of adaptive traits. These predictions can be tested and confirmed, but the molecular-level changes, i.e., the molecular adaptation that link genetic differences to organism fitness remain generally unknown.
- Key to addressing molecular adaptations is identifying systems and designing experiments that integrate changes in the genome, protein chemistry (molecular phenotype), and fitness.
- The study of molecular adaptation has long been fraught with difficulties, not the least of, which is identifying out of hundreds of amino acid replacements those few directly responsible for major adaptations.
- An amino acid replacement of modest effect at the molecular level causes a dramatic expansion in an ecological niche. These and other topics are creating the emerging field of 'Paleomolecular Biochemistry'.
- Intramolecular reactions between groups that are tied together in a single molecule are faster than the corresponding intermolecular reactions between two independent molecules.

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- The entropy of the reactants is reduced in intramolecular reaction, which principally occurs during its preparation process and makes addition or transfer reactions less unfavourable, since the integration of two reactants into a single product could decrease the reduction in the overall entropy.
- The entropy decrease involved in the formation of the transition state has been moved to an earlier step, the binding of the substrates to form the enzyme-substrate complex. However, in the intermolecular reaction the formation of product involves a much larger loss of translational and rotational entropy.
- The rate of biochemical reaction often influences enzymes like catalysts, so that they can take place at a relatively low temperature. The enzymes are thus known to lower the energy of activation. In certain cases, the biological response is initiated by enzymes.
- The optimum activity of each enzyme is always at a particular temperature, which typically varies from 25 degrees Celsius to 45 degrees Celsius. At 37 degrees Celsius, enzyme action is strongest and as temperatures rise above 60 degrees Celsius, enzymes become inactive.
- Biological catalysts are enzymes. The greater amounts of compounds are catalysed by a small number of enzymes. This means that enzymes are highly capable of turning giant amounts of the substrate into a substance. Enzymes improve the reaction rate and remain unaffected by the reaction they catalyse.
- Zymogens is an inactive enzyme precursor requiring a biochemical change (such as a hydrolysis reaction that reveals the active site, or changes the configuration to reveal the active site) for it to become an active enzyme. The biochemical change takes place in a lysosome where a specific part of the precursor enzyme is cleaved in order to activate it.
- Enzymes are protein molecules that are present in all living things. They speed up and target chemical reactions, in many cases increasing the rate of reaction millions of times. For example, they aid digestion, metabolise and eliminate waste in humans and animals, and play a crucial role in muscle contraction.
- Historically enzymes are considered to be non-toxic and not of safety concern for consumers since they are naturally present in ingredients used to make food. However, food enzymes produced industrially by extraction from plant and animal tissues, or by fermentation of microorganisms, are assessed for safety.
- A variety of enzyme purification services are available at creative enzymes. We provide purification and quality analysis in small trial scales or large industrial scales from natural resources or production mixtures, for clinical, therapeutic, research, and chemical industries.
- Purification and separation of enzymes are generally based on solubility, size, polarity, and binding affinity. The production scale, timeline, and properties of the enzymes should all be considered when choosing the proper separation method.

- The activity of enzyme involves substrate-enzyme interaction and utilization of free energy which helps in the formation of enzyme-substrate Complex. The two main theories proposed for explaining the mode of action of enzyme are Fischer's Lock and Key Hypothesis and Koshland's Induced Fit Theory.
- The enzyme utilizes the binding energy to form the transitory enzyme-substrate Complex. This binding energy is formed due to Hydrogen bond and various ionic interactions present between the enzyme and substrate. The binding energy is referred to as the free energy utilized by the enzyme to lower the activation energy of the reaction.
- According to Arrhenius, low energy rich molecules require higher energy to complete chemical reactions. The stability of the molecules requires to be overcome to undergo transition and transformation into products. This energy required for a chemical reaction is called the activation energy.
- The mechanism of enzyme-induced catalysis of reaction can be explained by the concept of Michaelis-Menten Equation.
- Irreversible inhibitors covalently bind to an enzyme, because chemical changes to the active sites of enzymes and cannot be reversed. The main role of irreversible inhibitors includes modifying key amino acid residues needed for enzymatic activity. They often contain reactive functional groups, such as aldehydes, alkenes or phenyl sulphonates.
- Most reversible inhibitors follow the classic Michaelis-Menten scheme, where an Enzyme (E) binds to its Substrate(S) to form an Enzyme-Substrate Complex (ESC). Km is the Michaelis constant that corresponds to the concentration of the substrate when the velocity is half the maximum. V_{max} is the maximum velocity of the enzyme.
- Non-competitive inhibitor is the types of inhibitor which binds to the site (allosteric site) other than the active site of the enzyme. Therefore, there is no competition for the binding site between the substrate and inhibitor. Once the inhibitor binds to the allosteric site, it changes the shape of active site.
- All chemical transformations pass through an unstable structure called the transition state, which is poised between the chemical structures of the substrates and products. The transition states for chemical reactions are proposed to have lifetimes near $10^{"13}$ seconds, on the order of the time of a single bond vibration.
- In acid-base catalysis, the chemical reaction is accelerated by the addition of an acid or a base, and the acid or base itself is not consumed in the reaction. Proton transfer is the commonest reaction that enzymes perform.
- Enzymes which exist in multiple forms in various tissues and organs but catalyze the same biochemical reaction are referred to as isoenzymes or isozymes.
- A carboxypeptidase is a protease enzyme that hydrolyzes (cleaves) a peptide bond at the carboxy-terminal (C-terminal) end of a protein or peptide. This is in contrast to an aminopeptidases, which cleave peptide bonds at the N-terminus of proteins.

NOTES

- Enzyme catalysing optical or geometrical rearrangement of atomic groupings without altering molecular weight or number of atom is isomerase. It converts into one form of isomer to the other isomer by changing the intramolecular arrangement by forming or breaking the bond.

4.8 KEY TERMS

- **Molecular adaptation:** Molecular adaptation studies provide empirical evidence to understand why organismal features exist, to map the evolutionary trajectories that shape populations, and to help predict future adaptations.
- **Proximity effects:** Proximity effects describe the orientation and movement of the substrate molecules when binding to enzyme active sites, and are most readily observed by comparing equivalent inter- and intramolecular reactions.
- **Enzymes:** Enzymes are protein catalysts that speed up the rate of biochemical reactions but do not change the structure of the final product. Like a catalyst, without being used up, the enzymes control the speed and specificity of the reaction, but unlike catalysts, only living cells generate enzymes.
- **Michaelis-Menten concept:** This concept explains the regulation of the rate of enzymatic reaction which depends upon the concentration of substrate and available molecules of enzyme in a reaction medium.
- **Enzyme inhibitors:** Enzyme inhibitors are molecules or compounds that bind to enzymes and results in a decrease in their activity. An inhibitor can bind to an enzyme and stop a substrate from entering the enzyme's active site and/or prevent the enzyme from catalysing a chemical reaction.
- **Reversible enzyme inhibition:** It involves a reversible enzyme inhibitor, which gets loosely bound to an enzyme and dissociates very rapidly.
- **Isozymes:** Enzymes which exist in multiple forms in various tissues and organs but catalyze the same biochemical reaction are referred to as isoenzymes or isozymes.
- **Carboxypeptidase A:** Carboxypeptidase A usually refers to the pancreatic exopeptidase that hydrolyzes peptide bonds of C-terminal residues with aromatic or aliphatic side-chains. Most scientists in the field now refer to this enzyme as CPA1, and to a related pancreatic carboxypeptidase as CPA2.

4.9 SELF-ASSESSMENT QUESTIONS AND EXCERCISES

Short–Answer Questions

1. Define the molecular adaption.
2. What is proximity effect?

3. Define enzyme.
4. Give the physical and chemical properties of an enzyme.
5. What are the important qualities of enzymes?
6. What is zymogens?
7. What do you understand by extraction process of enzyme?
8. Give the size or mass based method for purification of enzyme.
9. State the Lock and Key hypothesis.
10. Define the Michaelis-Menten constant.
11. Define the allosteric inhibition.
12. What do you understand by irreversible inhibitors?
13. What is affinity labelling of enzyme?
14. Define the site-directed mutagenesis.
15. Define the enzyme inhibitor.
16. Give the types of enzyme inhibitor.
17. Differentiate between competitive and non-competitive of enzyme inhibitor.
18. State the Lineweaver–Burk methods.
19. State the transition state theory of enzyme.
20. Define the acid-base catalysis of an enzyme.
21. What do you understand by Ribonuclease (RNase)?
22. What is enolic intermediate in isomerisation reaction?
23. Give the reaction of enzyme catalysed carboxylation.

Long –Answer Questions

1. Elaborate on the molecular adaption and proximity effect.
2. Describe the Classification and nomenclature of an enzyme.
3. Discuss in detail about the structure of an enzyme.
4. Analyse the extraction and purification of enzyme.
5. Illustrate the different types of theories for enzyme action.
6. Explain in detail about the Michaelis-Menten constant.
7. Elaborate on the mechanism of enzyme inhibition and type of inhibitors.
8. Discuss in detail about the affinity labelling of enzyme and its classification.
9. Describe the reversible and irreversible inhibitors.
10. Briefly explain about the Lineweaver–Burk first and second methods.
11. Give the detail information about the mechanism of an enzyme.
12. Analyse the enzyme mechanism for isoenzyme, ribonuclease, chymotrypsin and carboxypeptidase A.
13. Explain in detail about the kinds of reactions catalysed by enzyme with mechanism of reactions.

NOTES

4.10 FURTHER READING

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UNIT 5 CO-ENZYME CHEMISTRY, ENZYME MODELS AND BIOTECHNOLOGICAL APPLICATIONS OF ENZYMES

Structure

5.0 Introduction
5.1 Objectives
5.2 Co-Enzyme Chemistry
  5.2.1 Structure and Biological Functions of Co-Enzyme A
  5.2.2 Thiamine Pyrophosphate and Pyridoxal Phosphate
  5.2.3 NAD+ and NADP+
  5.2.4 Fen and Fad
  5.2.5 Lipoic Acid and Vitamin B12
  5.2.6 Mechanism of Reactions Catalyzed by Above Cofactors
5.3 Enzyme Models
  5.3.1 Host-Guest Chemistry
  5.3.2 Chiral Recognition and Catalysis
  5.3.3 Crown Ethers
  5.3.4 Micelles and Synthetic Enzymes or Synzymes
5.4 Biotechnological Applications of Enzymes
  5.4.1 Techniques and Methods of Immobilization of Enzymes and their Effects on Enzyme Activity
  5.4.2 Enzymes as Targets for Drug Design
  5.4.3 Clinical Uses of Enzymes
5.5 Answer to 'Check Your Progresses'
5.6 Summary
5.7 Key Terms
5.8 Self-Assessment Questions and Exercises
5.9 Further Reading

5.0 INTRODUCTION

A substance that enhances the action of an enzyme. (An enzyme is a protein that functions as a catalyst to mediate and speed a chemical reaction) are known as coenzymes. In technical terms, coenzymes are organic non-protein molecules that bind with the protein molecule (apoenzyme) to form the active enzyme (holoenzyme). Co-enzyme A helps with energy production within the body. Co-enzyme A, a helper molecule, is a non-protein chemical substance needed for the activation of some enzymes, the proteins that catalyze or activate important chemical reactions within the body.

Pyridoxal PhosPhate (PLP) or Pyridoxal 5'-Phosphate, P5P, the active form of vitamin B_6, is a coenzyme in a variety of enzymatic reactions.

Nicotinamide adenine dinucleotide consists of two nucleosides joined by pyrophosphate. NADP+ is a coenzyme that functions as a universal electron carrier, accepting electrons and hydrogen atoms to form NADPH, or nicotinamide adenine

dinucleotide phosphate. Flap EndoNucleases (FENs, also known as 5' nucleases in older references) are a class of nucleolytic enzymes that act as both 5'-3' exonucleases and structure-specific endonucleases on specialised DeoxyriboNucleic Acid or DNA structures that occur during the biological processes of DNA replication, DNA repair, and DNA recombination. In biochemistry, Flavin Adenine Dinucleotide (FAD) is a redox-active coenzyme associated with various proteins, which is involved with several enzymatic reactions in metabolism. Lipoic Acid (LA), also known as á-lipoic acid, Alpha-Lipoic Acid (ALA) and thioctic acid, is an organosulfur compound derived from caprylic acid (octanoic acid). Vitamin B_{12}, also known as cobalamin, is a water-soluble vitamin involved in metabolism.

Enzyme models are generally organic synthetic molecules that contain one or more features present in enzymatic systems. In host–guest chemistry describes complexes that are composed of two or more molecules or ions that are held together in unique structural relationships by forces other than those of full covalent bonds. Chiral recognition is a chemical interaction, frequently occurring in living systems, by which a given chiral molecule (receptor/host) recognizes a particular stereoisomer (substrate/guest). Chiral catalysis is one of the main avenues by which stereoselective synthesis can be achieved in organic chemistry.

Crown ethers are cyclic chemical compounds that consist of a ring containing several ether groups. Enzymes in reverse micelles systems are found owning increased stability. Similarly, enzymes can also be dispersed in a water phase using a micelle system. Synzymes are substances with catalytic capabilities. The name synzyme is derived from synthetic enzyme.

In biotechnology, enzymes are used to make cultures and are used in the development of medicines. Enzymes are also used in the process of food preservation. They can be used in diagnosing diseases or they provide services for the washing and other environmental processes. The term immobilized enzymes refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously."

In this unit, you will study about the co-enzyme chemistry, structure and biological functions of coenzyme A, thiamine pyrophosphate and pyridoxal phosphate, NAD+, NADP+, FEN and FAD, lipoic acid or vitamin B12, mechanism of reactions catalyzed by co-enzyme cofactors, enzyme models, host-guest chemistry, chiral recognition and catalysis, crown ethers, micelles and synthetic enzymes or synzymes, biotechnological applications of enzymes, techniques and methods of immobilization of enzymes and their effects on enzyme activity, enzymes as targets for drug design, clinical uses of enzymes.

5.1 OBJECTIVES

After going through this unit you will be able to:

- Understand the co-enzyme chemistry
- Know about the structure and biological functions of co-enzyme A

- Explain the thiamine pyrophosphate and pyridoxal phosphate
- Discuss about the NAD+ and NADP+
- Describe the FEN and FAD
- Analyse the mechanism of reactions catalyzed by co-enzyme cofactors
- Interpret the enzyme models and host-guest chemistry
- Comprehend the chiral recognition and catalysis
- Elaborate on the crown ethers
- Understand the micelles and synthetic enzymes or synzymes
- Explain the biotechnological applications of enzymes
- Describe the techniques and methods of immobilization of enzymes and their effects on enzyme activity
- Interpret the enzymes as targets for drug design
- Know about the clinical uses of enzymes

5.2 CO-ENZYME CHEMISTRY

Co-enzymes

Co-enzymes are non-protein organic molecules that are mostly derivatives of vitamins soluble in water by phosphorylation; they bind apoenzyme to proteins to produce an active haloenzyme.

Most of the B-vitamins are coenzymes and are essential in facilitating the transfer of atoms or groups of atom between molecules in the formation of carbohydrates, fats and proteins.

Examples are NAD, ATP.

Cofactors as Derived from Vitamins

Vitamins are organic molecules needed for variety of biological functions within the body. The most important function of vitamins is to serve as cofactors (co-enzymes) for enzymatic reactions.

Many organic cofactors are vitamins or molecules derived from vitamins. Most inorganic cofactors are minerals. Cofactors can be oxidized or reduced for the enzymes to catalyze the reactions.

Protein + cofactor (metal ion) = enzyme

Prosthetic Groups

They are non-peptide components of enzymes which are involved in catalysis. They are bound to enzymes (covalently or non-covalently) and their change from one form to another takes place in a single catalytic cycle.

Apoenzymes

In Figure 5.1 shown the co-enzymes participate in catalysis when they bind to the active site of the enzyme (called ***apoenzyme***) and subsequently form the active enzyme

(called ***holoenzyme***). Although co-enzymes activate enzymes they are not considered as substrates of the reaction. The main function of the co-enzyme is to act as intermediate carriers of transferred electrons or functional groups in a reaction.

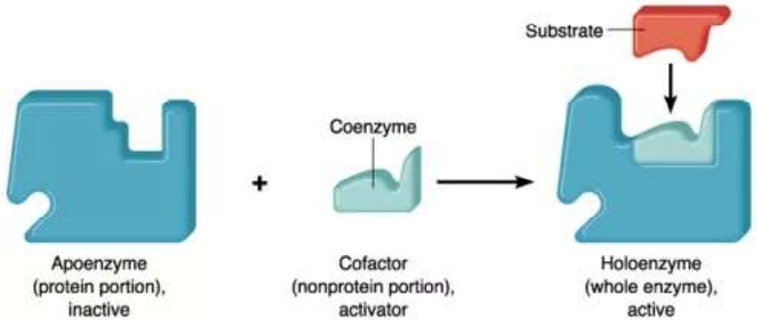


Fig. 5.1 *Formation of Holoengyme*

5.2.1 Structure and Biological Functions of Co-enzyme A

Co-enzyme A or CoA (Refer Figure 5.2) is a small, organic, non-protein molecule that carries chemical groups between enzymes. It may act as an acyl group carrier to form acetyl- CoA and other related compounds. It's another way of transporting carbon atoms within the cell.

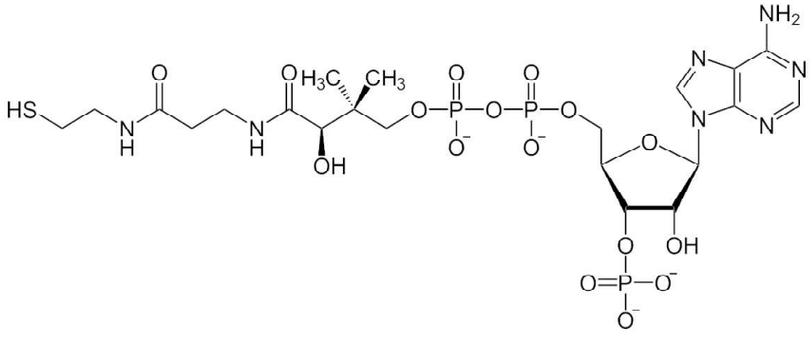


Fig. 5.2 *Structure of Co-Enzyme A*

Pantothenic acid shown in Figure 5.3 is used in the synthesis of CoA. This co-enzyme is formed when the vitamin combines with the derivative of ADP and the amino acid cysteine.

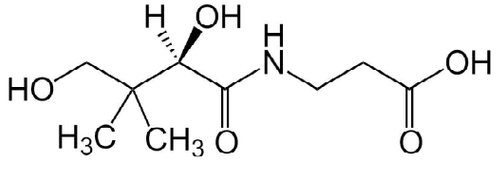


Fig. 5.3 *Structure of Pantothenic acid*

CoA is important in the biosynthesis of many important compounds such as fatty acids, cholesterol.

It is important in energy metabolism for pyruvate to enter the Kerbs cycle or TCA cycle as acetyl-CoA.

5.2.2 Thiamine Pyrophosphate and Pyridoxal Phosphate

Thiamine-Sulphur containing water soluble vitamin of B complex, exists in tissues in the active form of Thiamine Pyrophosphate (TPP). It is an essential co-enzyme involved in the energy extraction and cellular process in catabolism of sugar and amino acid.

Approximately 90% of total thiamine in blood is in erythrocytes.

It is the active form of Vitamin B1. A thiazole ring is found naturally in thiamine and is a heterocyclic compound consisting of a N atom and S atom as a part of the aromatic five-membered ring. It stabilizes charge and electron transfer.

Fig. 5.4 *Structure Thiamine Pyrophosphate*

Pyridoxal Phosphate

It is the active form of vitamin B6. Vitamin B6 is the precursor to coenzyme Pyridoxal Phosphate (PLP) which is essential in transformation of amino acids including transamination, deamination and decarboxylation.

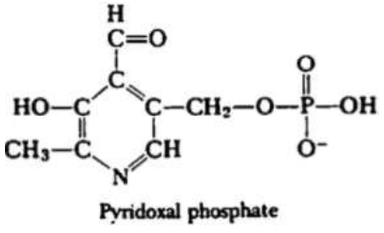


Fig. 5.5 *Structure of pyridoxal phosphate*

It functions as a cofactor in enzymes involved in transamination reactions required for the synthesis and catabolism of the amino acids. It is required as a cofactor in glycogenolysis.

5.2.3 NAD+ and NADP+

NAD+ (Nicotinamide adenine dinucleotide) are coenzymes found in all living cells and are oxidizing agents which are capable of carrying and transferring electrons.

Niacin is required for the synthesis of the active forms of NAD+.

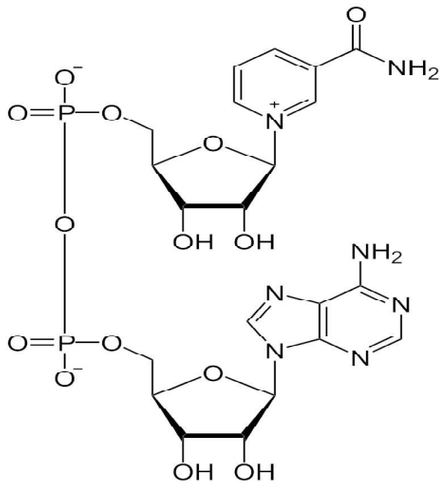


Fig. 5.6 *Structure of NAD+*

There are several riles played by NAD+ in oxidation-reduction reactions, such as transportation of hydrogen from one part to another, conversion of glycolysis into glyceraldehyde, oxidative decarboxylation of pyruvate to lactate, oxidation of acetyl-CoA in TCA cycle.

NADP+

Nicotinamide adenine dinucleotide phosphate (NADP) is the carrier of electrons (protons) in the cells. Therefore, NADP+ serves as the reducing power of the cells in the form of NADP/NADPH2 or an NAD\NADH2.

Reduced substrate + NADP= Oxidised substrate + NADPH2

Oxidised substrate + NADPH2 =Reduced substrate + NADP

NADP + functions as co-enzymes of a large number of oxidoreductase enzyme. They act as electron acceptors during enzymatic removal of hydrogen atoms from specific substrate molecules. Finally, reduced NADP or NAD, i.e., NADPH2 or NADH2 release energy, Adenosine TriPhosphate or ATP is generated. In catabolic reactions like respiration, NAD+/NADH act as electron donor, whereas NADP+/NADPH play roles in anabolic reactions. Structurally, NAD consists of a nicotinamide moiety (the protein which undergoes reversible reduction), two adenine nucleotide. In NADP, a phosphate group is esterified to the second adenine nucleotide at 2-hydroxyl group.

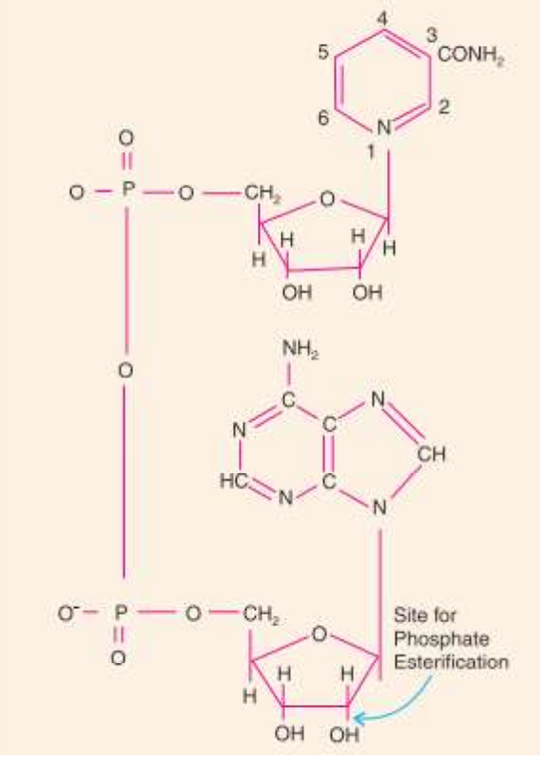


Fig 5.7 *Structure of NADP+*

5.2.4 FEN and FAD

FEN stands for Flap Endonuclease. It is an enzyme involved in the base excision repair pathway and counteracts replication stress, thus blocking the mechanism by which many drugs kill tumor cells.

Structure-specific nuclease with 5'-flap endonuclease and 5'-3' exonuclease activities involved in DNA replication and repair. During DNA replication, it cleaves the 5'-overhanging flap structure that is generated by displacement synthesis when DNA polymerase encounters the 5'-end of a downstream ***Okazaki fragment***. It enters the flap from the 5'-end and then tracks to cleave the flap base, leaving a nick for ligation. Also involved in the long patch base excision repair (LP-BER) pathway, by cleaving within the Apurinic/Apyrimidinic (AP) site-terminated flap. Acts as a genome stabilization factor that prevents flaps from equilibrating into structures that lead to duplications and deletions. Also possesses 5'-3' exonuclease activity on nicked or gapped double-stranded DNA, and exhibits RNase H activity. Also involved in replication and repair of rDNA and in repairing mitochondrial DNA.

FAD

Riboflavin is the precursor for coenzymes, Flavin MonoNucleotide (FMN) and Flavin Adenine Dinucleotide (FAD) and is used to oxidize substrates. FAD (Refer Figure 5.8) contains riboflavin and adenine.

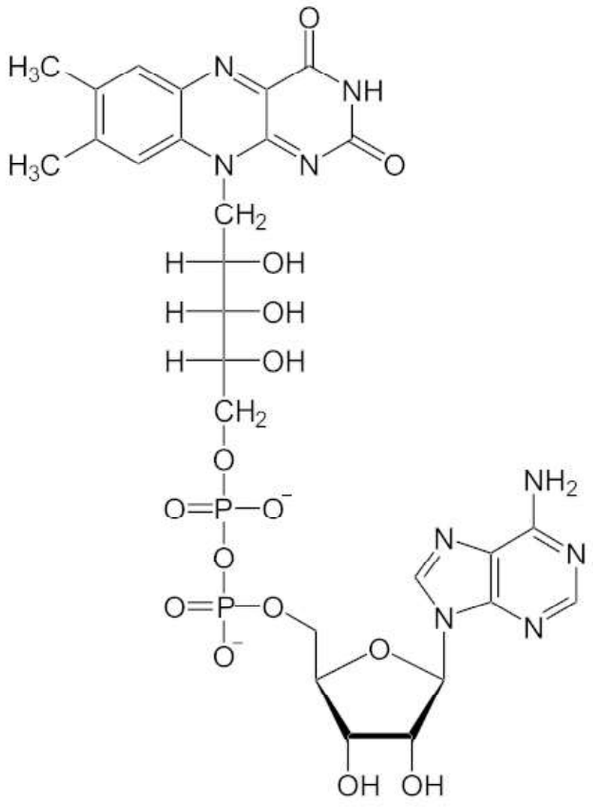


Fig. 5.8 *Structure of Flavin Adenine Dinucleotide*

The enzymes that require FMN and FAD as cofactors are termed as flavoproteins. During enzymatic reactions involving flavoproteins, the reduced forms of FMN and FAD, i.e., FMNH2 and FADH2 are formed respectively.

Syntheses of these cofactors occurs in 2 steps:

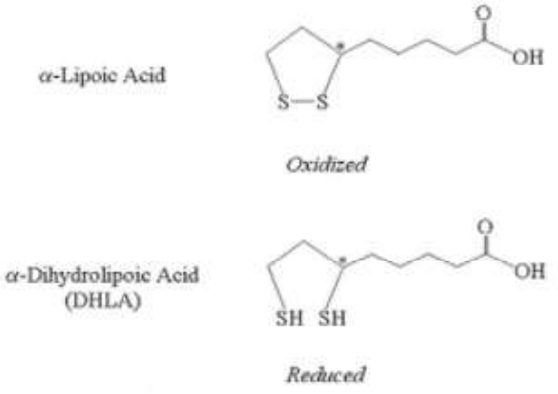
1. FMN is synthesized from riboflavin via the ATP-dependent enzyme RiboFlavin Kinase (RFK). RFK introduces a phosphate group onto the terminal hydroxyl of riboflavin.
2. FMN is then converted into FAD via attachment of AMP (derived from ATP) through the action of FAD pyrophosphorylase.

5.2.5 Lipoic Acid and Vitamin B12

Lipoic acid is an essential cofactor for mitochondrial metabolism and is synthesized using intermediates from mitochondrial fatty-acid synthesis type II, S-adenosylmethionine and iron–sulfur clusters. This cofactor is required for catalysis by multiple mitochondrial 2-ketoacid dehydrogenase complexes, including pyruvate dehydrogenase, -ketoglutarate dehydrogenase, and branched-chain ketoacid dehydrogenase. It plays a critical role in stabilizing and regulating these multi enzyme complexes.

Lipoic Acid (LA) cofactor is covalently bound to protein via an amide bond (lipoamide) to the alpha-amino group of a lysine in a conserved lipoylation domain.

The oxidized form is ready for reaction with different substrates and forms an intermediate until the substrate is released and the reduced form with two sulfhydryls (dihydrolipoamide) is generated. DLD catalyzes the reactivation of the cofactor by a redox reaction with nicotinamide adenine dinucleotide (NAD +).



Vitamin B12

It is composed of a complex tetra pyrrole ring structure and a cobalt ion in the center. Tetrapyrroles are compounds containing four pyrrole rings.

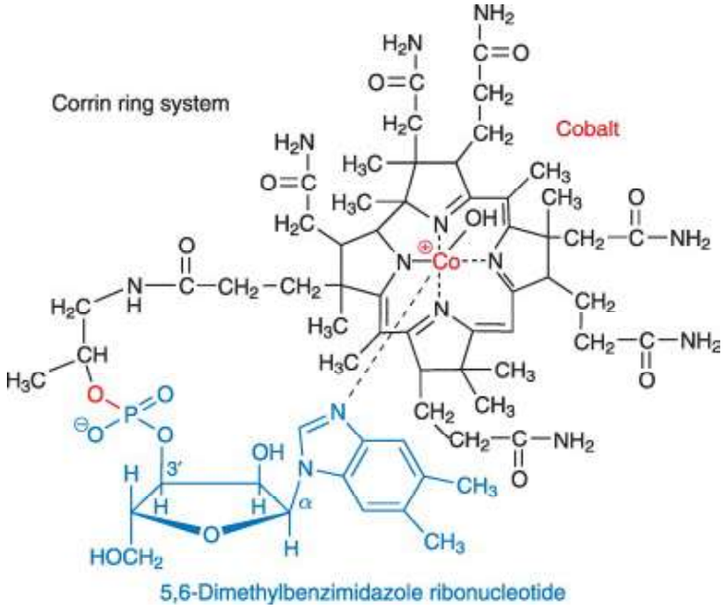


Fig. 5.9 *Structure of Vitamin B12*

Humans are unable to synthesize B12 (Refer Figure 5.9) and is obtained from their diet. It is synthesized exclusively by microorganisms and is found in liver of animals.

There are 2 significant reactions in the body that require vitamn B12 as a cofactor:

1. In TCA (TriCarboxylic Acid) cycle, one of the enzyme methylmalonyl-CoA mutase requires vitamin B12 as a cofactor in the conversion of methylmalonyl-CoA into succinyl-CoA.
2. Conversion of homocysteine to methionine which is catalyzed by methionine synthase.

5.2.6 Mechanism of Reactions Catalyzed by Above Cofactors

NAD+ and NADP+

Nicotinamide co-enzymes are nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+). The both contain nicotinamide (Refer Figure 5.10).

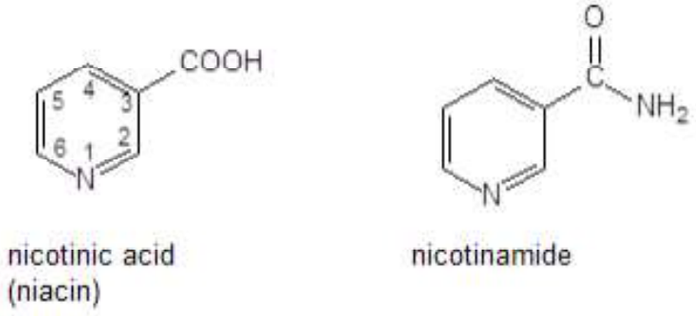


Fig 5.10 *Structure of Nicotinamide and Nicotinic Acid*

The deficiency of *nicotinic acid* (*niacin*) causes the pellagra disease. Nicotinic acid or nicotinamide is essential as a precursor of NAD+ and NADP+ (pyridine nucleotide co-enzymes) (Refer Figure 5.11). Nicotinamide coenzymes play a role in numerous oxidation reduction reactions in the form of electron transfers from and to the metabolite. Pyridine ring NAD+ is reduced by the addition of hydride ion onto C-4 when NAD+ is transformed into NADH (and when NADP+ is transformed into NADPH).

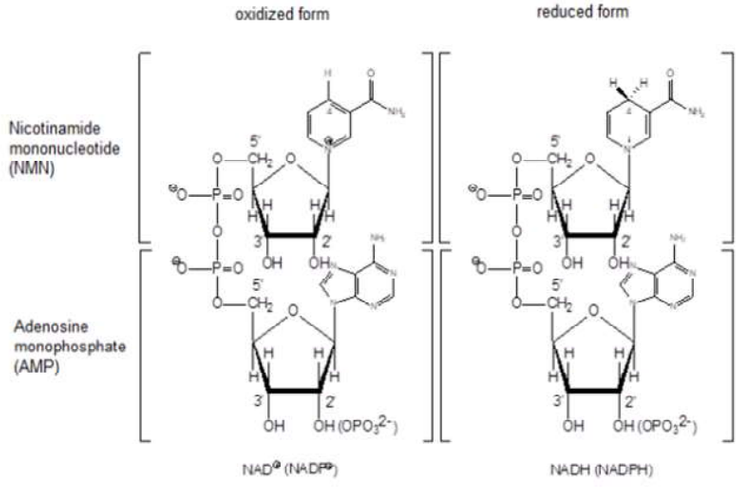


Fig. 5.11 *Oxidised and Reduced Form of NAD+ and NADPH*

NAD+ and NADP+ almost always behave as dehydrogenase substrates. Dehydrogenase catalyzes the oxidation of the substrate by transferring two electrons and proton in the form of hydride ion (H^-) onto C-4 of nicotinamide group NAD+ and NADP+. In this way, the reduced forms are formed (NADH and NADPH), where new C-H bond is created on C-4.

NADH and NADPH (stable in solutions containing oxygen) possess reductive power. The stability of reduced pyridine nucleotides allows them to carry their reduction potential from one enzyme to another; the characteristics not owned by flavin coenzymes. The majority of reactions in which NADH and NADPH are formed are catabolic reactions. The oxidation of NADH in mitochondria is coupled with ATP synthesis. The most significant part of NADPH is used as a reduction agent in biosynthetic reactions.

NADH and NADPH show a maximum in ultraviolet region at 340 nm caused by dihydropyridine ring, while NAD+ and NADP+ do not absorb the light at this wavelength. The appearance and disappearance of the absorbance at 340 nm are useful for the measurement of the rate of the oxidation.

In the mechanism of the oxidation of lactate to pyruvate catalyzed by lactate dehydrogenase (Refer Figure 5.12), the coenzyme accepts the hydride ion on C-4 in nicotinamide group. This phenomenon leads to the bond rearrangement in the ring when electrons are moving to the positively charged nitrogen atom. The enzyme represents acid-base catalyst and the suitable place for binding coenzyme, and the substrate as well. Two hydrogens are moving from the lactate to produce pyruvate. One of these hydrogens are moving to NAD+ as a hydride ion bearing two electrons, and the another is transferring to His-195 as a proton. The second hydrogen is then releasing as H+ to regenerate the base catalyst (His-195).

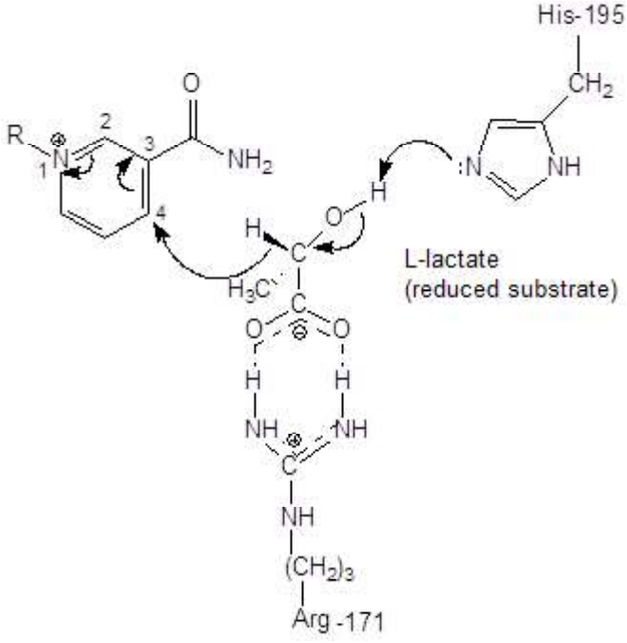


Fig. 5.12 *Mechanism of the Oxidation of Lactate*

FAD and FMN

Coenzymes Flavin Adenine Dinucleotide (FAD) and Flavin MonoNucleotide (FMN) are derived from riboflavin or vitamin B2. Riboflavin is synthesized by bacteria, protozoa, fungi, plants and some animals. Mammals are getting riboflavin from food. Riboflavin consists from ribitol connected to N-10 atom of heterocyclic ring system called isoalloxazine (Refer Figure 5.13(a)). Similarly to NAD+ and NADP+, FAD contains AMP and pyrophosphate bond (Refer Figure 5.13(b)).

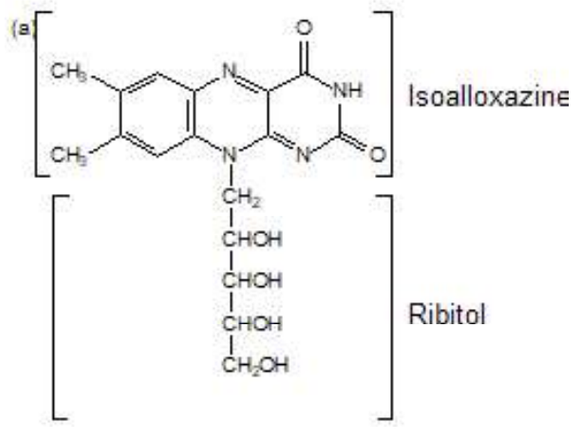


Fig. 5.13 (a) *Structure of Riboflavin*

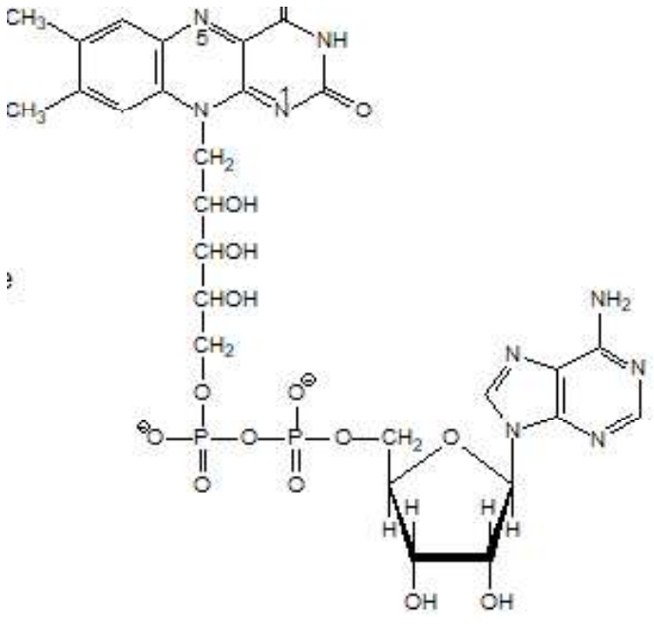


Fig. 5.13 (b) *Structure FAD Contains AMP and Pyrophosphate Bond*

FAD or FMN are necessary as prosthetic groups for many oxidoreductases. Reduced flavin coenzymes can be easily oxidized in the presence of oxygen. FAD and FMN are reduced to FADH2 and FMNH2 by taking proton and two electrons in the form of hydride ion (Refer Figure 5.14). Oxidized enzymes are light yellow as a result of the system of conjugated double bonds of the isoalloxazine cyclic system. Different from NADH and NADPH, which exclusively participate in two-electron systems, FMNH2 and FADH2 are donated electrons (one (FADH• or FMNH• are formed) or two). The intermediates are relatively stable free radicals called semiquinones. Oxidation of FADH2 and FAMNH2 is often coupled with the reduction of metalloproteins containing Fe3+ (in [Fe-S] cluster). Since iron-sulfur cluster can accept only one electron, reduced flavin must be oxidized into two one-electron steps via semi Quinone intermediate.

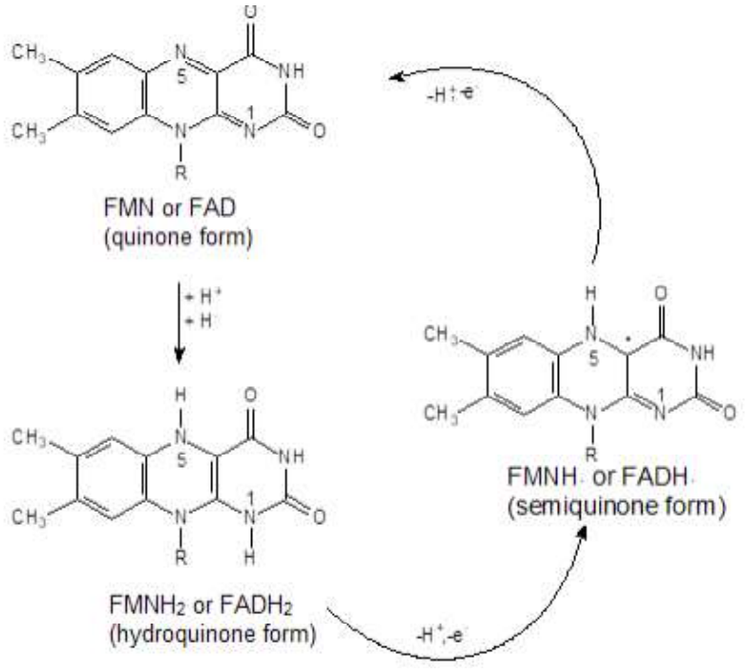


Fig. 5.14 *Reduced form of FADH2 and FMNH2*

Co-enzyme A

Numerous metabolic processes depend on co-enzyme A (CoA, or HS-CoA), including the oxidation of fuel molecule and biosynthesis of some carbohydrates and fats. The coenzyme is involved in the reactions of acyl group transfers. It has three main components: 2-mercaptoethylamine unit with free -SH group, pantothenate vitamin (vitamin B3), and ADP part (Refer Figure 5.15 (a)). Acetyl CoA is energetically rich compound because of the high energy of thioester bond.

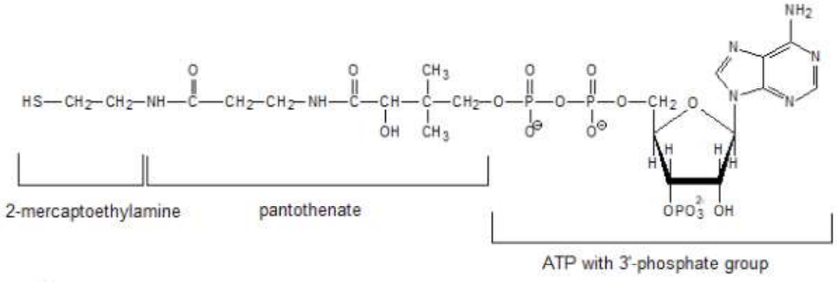


Fig. 5.15(a) *Structure of ATP with 3'- Phosphate Group*

Phosphopantetheine, phosphate ester which contains 2-mercaptoethylamine and pantothenate parts of coenzyme A, is a prosthetic group of a small protein (77 amino acid residues), known as an Acyl Carrier Protein (ACP). The prosthetic group is esterified to ACP via oxygen in the side chain of a serine residue (Refer Figure 5.15 (b)). The intermediates acetylate SH of the prosthetic group ACP in the 'Fatty Acids' biosynthesis.

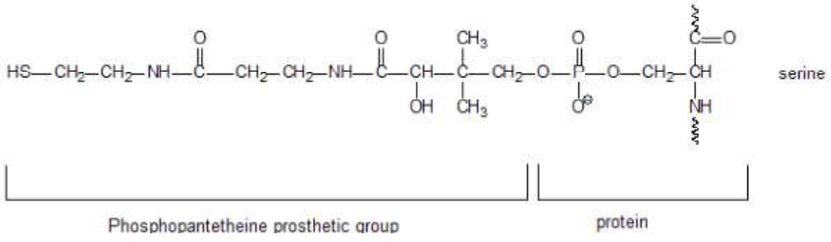


Fig. 5.15 (b) *The Prosthetic Group is Esterified*

Thiamine Pyrophosphate

Thiamine (or vitamin B1) contains pyrimidine ring and positively charged thiazoline ring (Refer Figure 5.16 (a)). In mammals, thiamine is the essential vitamin, wide-spread in the rice peel and other wheat. Its deficiency causes beriberi. Co-enzyme is the Thiamine PyroPhosphate (TPP) (Refer Figure 5.16 (b)). TPP is synthesized from thiamine by the enzymatic transfer of pyrophosphoryl group from ATP.

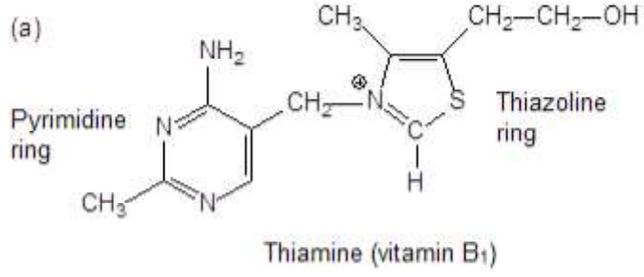


Fig. 5.16 (a) *Vitamin B1*

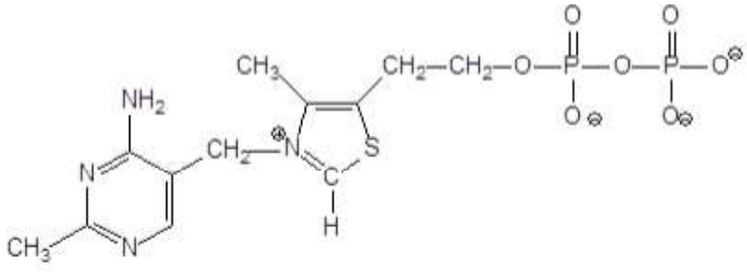


Fig. 5.16 (b) *Co-enzyme is the Thiamine PyroPhosphate (TPP)*

Numerous decarboxylases (carboxylases) require TPP as a co-enzyme (e.g., pyruvate decarboxylase of the yeast) (Refer Figure 5.16 (c)).

TPP is also the co-enzyme involved in oxidative decarboxylation of á-keto acids, except for pyruvates. The first steps in these reactions are occurring according to the mechanism shown in Figure 5.16 (c). Besides, TPP is a prosthetic group for the enzymes known as transketolases, which catalyze the transfer between the sugar molecules of two carbon groups containing keto group.

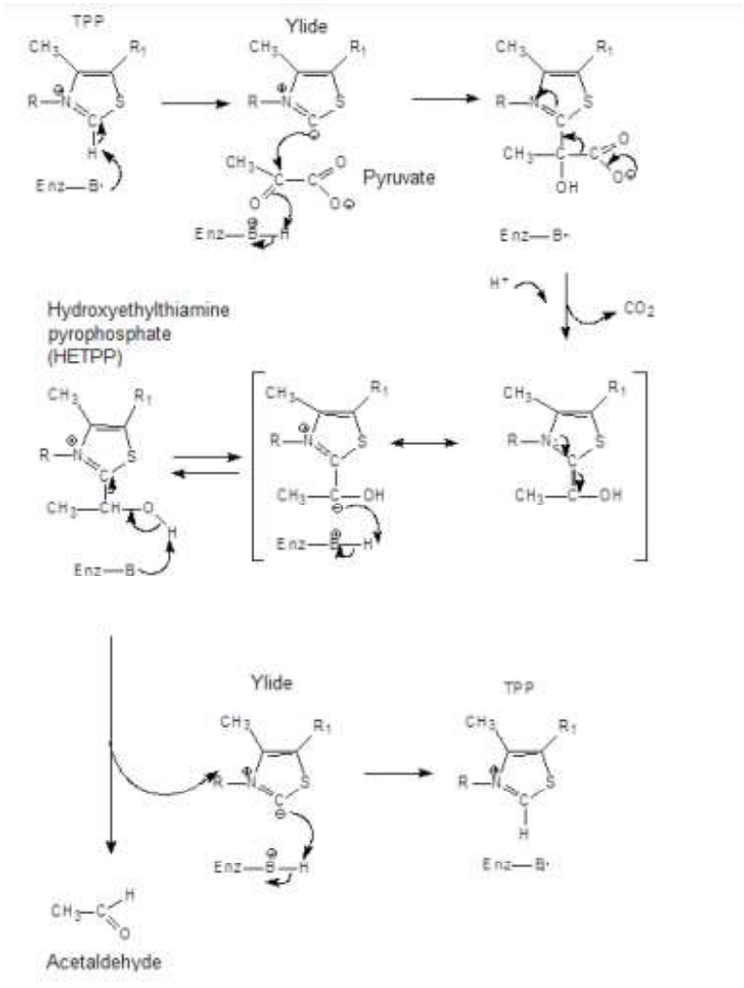


Fig. 5.16 (c) *Co-Enzyme Involved in Oxidative Decarboxylation of á-Keto Acids*

Thiazoline ring of the co-enzyme contains the reactive center. C-2 of TPP has unusual activity; it is acidic despite high pKa in aqueous solution. Experiments show that pKa value for the ionization of HydroxyEthylamine PyrophoSphate (HETPP) (i.e., forming of dipolar carbanion) changes from 15 in water to 6 on the active center of pyruvate decarboxylase. This increased acidity is ascribed to low polarity of active center, which is also responsible for the increased reactivity of TPP itself. The positive charge of thiazoline ring of TPP attracts electrons, weakening the bond between C-2 and hydrogen. The proton is mostly removing by base part of the enzyme. Ionization gives resonantly stable dipolar carbanion known as ylide. Negatively charged C-2 attacks electron-deficit carbonyl carbon of pyruvate substrate, and the first product (CO_2) is releasing. Two carbons of pyruvate are now attached to thiazoline ring as a part of resonance-stabilized carbanion. In the next step, the protonation of carbanion gives hydroxyethylamine pyrophosphate (HETPP). HETPP is separating, releasing acetaldehyde (the second product). TPP is forming again when ylide is protonating from the side of the enzyme (Refer Figure 5.16 (c)).

Pyridoxal Phosphate

The family of B6 vitamins soluble in water consists of three closely connected molecules differing only in the state of the oxidation or amination of the carbon bound to the position 4 of the pyridine ring (Refer Figure 5.17 (a)). Induced deficiency of vitamin B6 in mice causes dermatitis and various disorders connected to the metabolism of proteins; deficiencies of vitamin B6 in humans are rare. Once B6 enters the cell, enzymatic transfer of g-phosphoryl group from ATP forms co-enzyme Pyridoxal 5'-Phosphate (PLP) (Refer Figure 5.17 (b)).

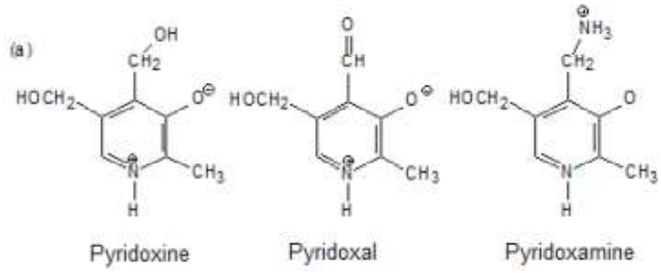


Fig. 5.17 (a) *Carbon Bound to the Position 4 of the Pyridine Ring*

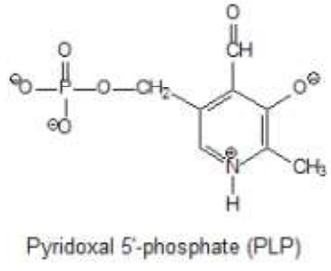


Fig. 5.17 (b) *ATP forms Co-Enzyme Pyridoxal 5'-Phosphate (PLP)*

Pyridoxal phosphate is a prosthetic group for many enzymes which catalyze different reactions, including isomerizations, decarboxylations, and eliminations in side chain or substitutions. In enzymes dependent on PLP, the carbonyl group of the prosthetic group is binding as Schiff base (imine) for alpha-amino group of a lysine residue in the active center (Refer Figure 5.17 (c)).

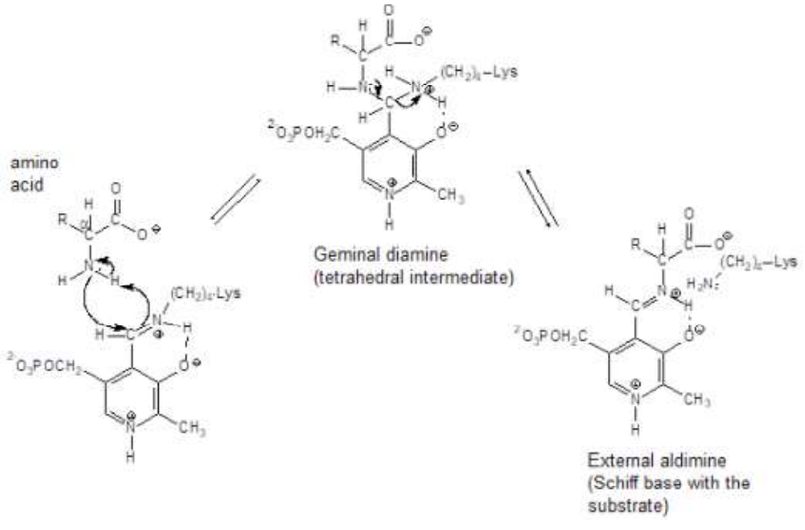


Fig. 5.17 (c) *Lysine Residue in the Active Center*

Transamination is most frequently dependent reaction on PLP, and the mechanism of this type of the reaction is presented in Figure 5.18.

In the first step of the mechanism, amino acid replaces lysine from internal aldimine which binds PLP for the enzyme forming the external aldimine. In the second step, α-hydrogen of the amino acid is taken with the base catalyst by the same lysine residue. Electronic rearrangement leads to the quinoid intermediate. In the third step, the protonation of the intermediate with the lysine residue gives ketoimine. In the fourth step, hydrolysis of the ketoimine gives á-keto acid, which dissociates, and PMP remains bound to the enzyme. If another á-keto acid enters, each step goes in reverse. The amino acid is transferred to á-keto acid, giving new amino acid and regenerates the original PLP form of the enzyme.

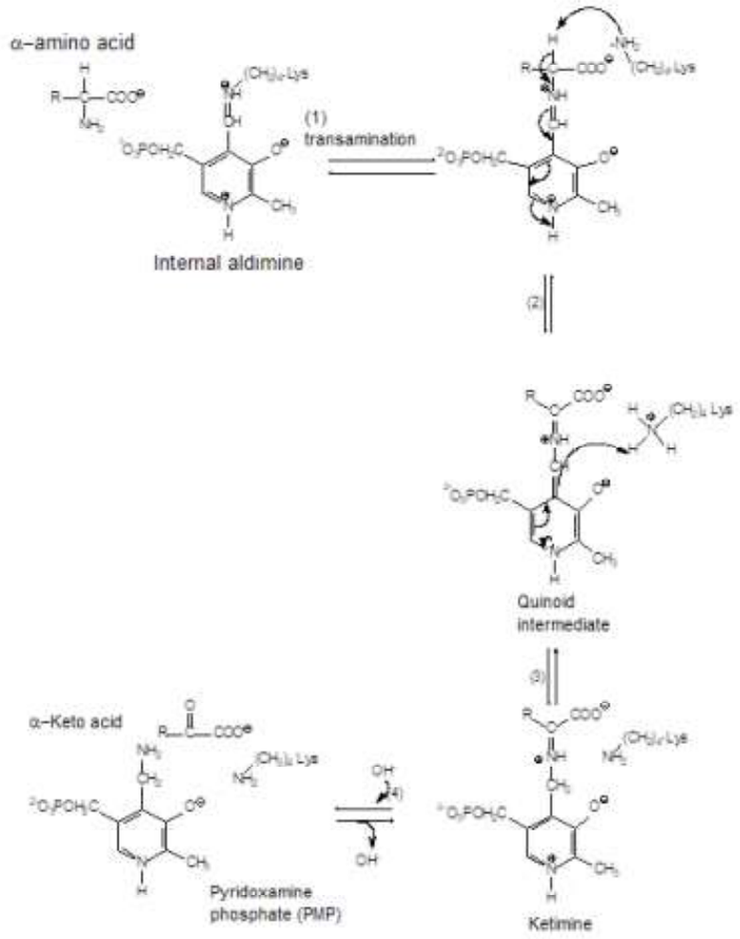


Fig. 5.18 *Mechanism, Amino Acid Replaces Lysine from Internal Aldimine*

Lipoic Acid

Coenzyme lipoamide is a protein binding form of lipoic acid. Although lipoic acid is often described as vitamin B, it seems that the animals are capable of synthesizing it. It is necessary for particular bacteria and protozoa to grow (Refer Figure 5.19).

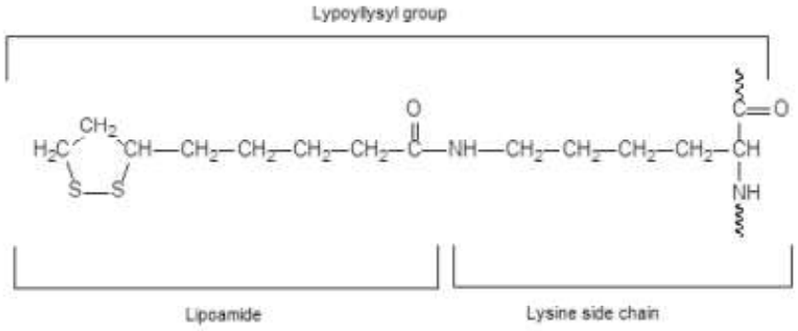


Fig. 5.19 *Structure of Lipoamide and Lysine Side Chain*

It is believed for lipoamide to function as a pendulum which carries acyl groups between active centers in multi-enzymatic complexes. For example, in complex of pyruvate dehydrogenase, disulfide ring of lipoamide prosthetic group reacts with HETPP, binding its acetyl group on sulfur atom attached to C-8 of lipoamide and creating thioester. Then, the acyl group is moved to the sulfhur atom of co-enzyme A giving reduced (dihydrolipoamide) form of prosthetic group.

The last step catalyzed by pyruvate dehydrogenase complex is the oxidation of dihydrolipoamide. In this reaction, NADH is formed by the action of the flavoprotein component of the complex. The actions of multiple co-enzymes of pyruvate dehydrogenase complex show how coenzymes supplying reactive groups and thus increasing catalytic diversity of proteins are used for storing energy and carbon building blocks.

Check Your Progress

1. Define the co-enzyme.
2. Differentiate between apoenzyme and holoenzyme.
3. What do you understand by TPP?
4. Define the NAD+.
5. What is NADP+?
6. What is FEN?

5.3 ENZYME MODELS

Enzyme models are generally organic synthetic molecules that contain one or more features present in enzymatic systems. They are smaller and structurally simpler than enzymes. Consequently, an enzyme model attempts to mimic some key parameter of enzyme function on a much simpler level. To separate out a particular factor responsible for the *catalytic efficiency* of the enzyme within the biological system would be a fabulous task requiring a knowledge of each of the components that would contribute to the overall catalysis. Instead, with appropriate models, it is possible to estimate the relative importance of each catalytic parameter in absence of those not under consideration. One noticeable advantage of the use of 'Artificial

Structures' for modeling enzymatic reactions is that the compounds can be manipulated precisely for the study of a specific property. The state of the art may be further refined by combining those features that contribute most and designing models that actually approach the efficiency of the enzyme. i.e., with the tools of *synthetic chemistry*, it becomes possible to construct a 'Miniature Enzyme' which lacks a macromolecular peptide backbone, but contains reactive chemical groups correctly oriented in the geometry dictated by an enzyme active site. It is often referred to as the biomimetic chemical approach to biological systems. Therefore, ***biomimetic chemistry*** represents the field which attempts to imitate the acceleration and selective characteristic of enzyme-catalyzed reactions. It is hoped that such an approach will eventually bridge or at least reduce the gap between the known complex structures of *organic biomolecules* and their exact functions in life. In order to do this, many factors related to the mechanism of action of a particular enzyme must be known. These include (a) the structure of the active site and the enzyme-substrate complex; (b) the specificity of the enzyme and its ability to bind to the substrate; and (c) the kinetics for the various steps and a knowledge of possible intermediates in the reaction coordinate.

5.3.1 Host-Guest Chemistry

Host-guest chemistry is a branch of supramolecular chemistry that deals with the formation of aggregate structures and their properties. The complex is held by one or a combination of forces which includes hydrogen bonding, ion-dipole, ion-ion, π-π, Van der Waals and hydrophobic interactions. The host generally possesses binding sites, which converge in the complex. Guests can be ionic or neutral, and can include metal ions, ammonium ions, carboxylic acids, amino acid derivatives, and many others.

There are many different types of 'Host-Guest' interaction with various network topologies, such as metal-organic frameworks or zeolites with small molecules, or synthetic cyclic compounds containing a cavity.

The most common examples of 'Host-Guest' chemistry are enzyme-substrate interactions where the substrate fits into the enzyme's active site with complete specificity. In 1894, Emil Fischer proposed the now ubiquitous 'Lock and Key' principle for the active site and the substrate,16 where the 'Lock' represents the host and the 'Key' the guest.

5.3.2 Chiral Recognition and Catalysis

Most of the molecules that mediate the processes of life like enzymes, hormones, antibodies and receptors are chiral. That means, they present exactly the same chemical formula and atomic connectivity but their atomic arrangement in space exists in two mirror-image forms (enantiomers) that cannot be superimposed.

The importance of molecular chirality arises from the fact that the two mirror images of a chiral molecule can have vastly different physiological impacts when ingested by living organisms. This is particularly important for medicinal chemistry. For example, while one enantiomer of ethanbutol is used to treat tuberculosis, the other causes blindness. Even if the enantiomer of a given drug is not dangerous but simply not effective, a patient has to take a double dose of medicine if this is a

racemate (a mixture of equal amount of both enantiomers). As a consequence, there is a strong need to produce many chiral compounds as pharmaceuticals and agrochemicals in enantiomerically pure form. Catalytic processes used for their synthesis are primarily carried out in homogeneous phase. Such catalysts often contain heavy metal centres, which must be removed from the product prior to use, a problem which is avoided by the use of heterogeneous catalysts.

However, a typical drawback of heterogeneous catalysts is that they must be modified to become enantioselective. One common approach is the use of solid surfaces which become enantioselective by the adsorption of organic chiral modifiers. The precise mechanisms by which chiral selectivity is transferred to the substrates are still not known. However, many observations seem to indicate that a major role is played by the formation of ordered supramolecular structures having void spaces which act as enantioselective adsorption sites.

Molecular Recognition

Molecular recognition is a biological phenomenon which realizes special recognition at molecular level in living and chemical systems. The origin of the process lies in the intrinsic capacity of biomolecules to distinguish between other compounds and interact with them on the base of their molecular complementarities.

The process is characterized by the onset of specific non-covalent bonding between the interacting molecules.

The term molecular recognition refers to the specific interaction between two or more molecules through noncovalent bonding such as hydrogen bonding, metal coordination, hydrophobic forces, and Van der Waals forces. In addition to these direct interactions, solvents can play a dominant indirect role in driving molecular recognition in solution. The host and guest involved in molecular recognition exhibit molecular complementarity. Molecular recognition plays an important role in biological systems and is observed in between receptor-ligand, antigen-antibody, DNA-protein, sugar-lectin, RNA-ribosome, etc. An important example of molecular recognition is the antibiotic vancomycin that selectively binds with the peptides with terminal D-alanyl-D-alanine in bacterial cells through five hydrogen bonds. The vancomycin is lethal to the bacteria since once it has bound to these particular peptides they are unable to be used to construct the bacteria's cell wall.

Molecular Asymmetry and Prochirality

An asymmetric molecule is a molecule that has no planes or center of symmetry. The asymmetry of molecules may depend on the presence of the asymmetric atom of carbon; in its absence, by the asymmetry of the entire molecule—for example, in the spirans and in some derivatives of diphenyl. Asymmetric synthesis is a useful method to produce stereoisomeric compounds for pharmaceutical applications because different enantiomers of molecules are known to have different biological activities.

When a tetrahedral carbon can be converted to a chiral centre by changing only one of the attached groups, it is referred to as a 'Prochiral' carbon. The two hydrogens on the prochiral carbon can be described as 'Prochiral hydrogens'. Prochirality is an important concept in biological chemistry, because enzymes can

distinguish between the two 'Identical' groups bound to a prochiral carbon centre due to the fact that they occupy different regions in three-dimensional space.

Molecules which display chirality are called chiral e.g., (S)-(+)-lactic acid is chiral. Chirality as explained and depicted above is associated with this type of molecules is known as centre of chirality and chiral centre at C2 carbon. Some other type of chirality also presents in molecules which do not have chiral centres, but they show chirality due to deymmetrisation of axis and plane.

Biomimetic Chemistry

Biomimetic chemistry deals with chemical systems that can simulate biological phenomena, particularly catalysis and specificity. Recently, increasing attention has been given to making artificial enzymes by purely chemical means. In the future, the field should extend to provide further understanding of living systems and new methods of chemical synthesis.

5.3.3 Crown Ethers

Crown ethers are cyclic compounds that have several ether linkages. A crown ether specifically binds certain metal ions or organic molecules, depending on the size of its cavity.

These crown ethers have cavities ideal for complex formation with metal ions. They can even carry metal ions into solution in organic solvents. 18-crown-6 is the right size for potassium ions, and a solution of $KMnO_4$ and 18-crown-6 in benzene, so-called 'Purple Benzene'(Refer Figure 5.20), is a useful oxidizing agent. The high-yielding oligomerization is a template reaction with a potassium ion holding the two reagents together.

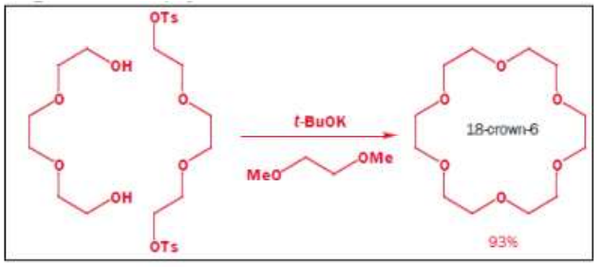


Fig. 5.20 *Structure of Purple Benzene'*

Cryptates

Cryptands are macro-bi- or -poly-cycles able to encapsulate an ion by providing it higher protection because of their cagelike structures.

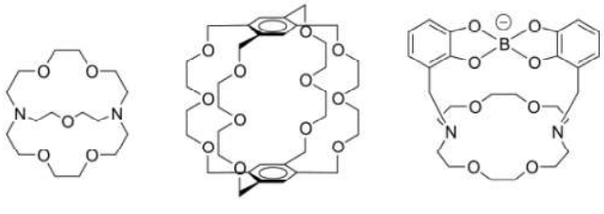


Fig. 5.21 *Cryptates*

Cyclodextrins

Cyclodextrins are a family of cyclic oligosaccharides, consisting of a macrocyclic ring of glucose subunits joined by α-1,4 glycosidic bonds. They are prepared by enzymatic treatment of starch. Commonly, Cyclodextrin Glycosyltransferase (CGTase) is employed along with á-amylase. First starch is liquified either by heat treatment or using á-amylase, then CGTase is added for the enzymatic conversion. CGTases produce mixtures of cyclodextrins.

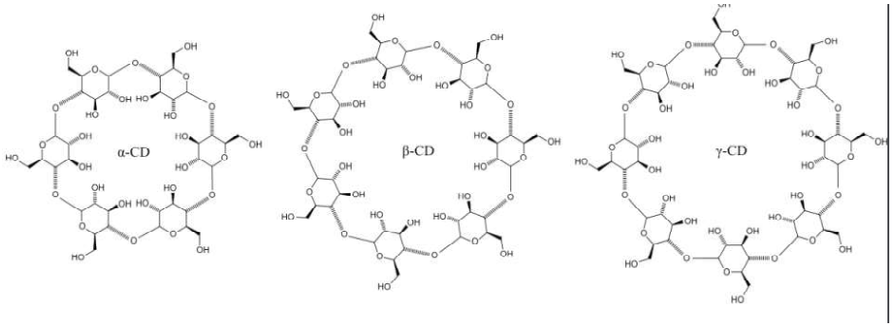
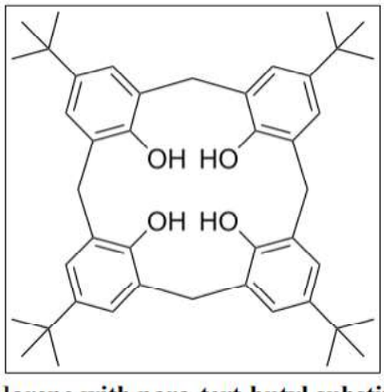


Fig. 5.22 *Cyclodextrins*

Calixarenes

Calixarene (Refer Figure 5.23) is a macrocycle or cyclic oligomer based on a hydroxyalkylation product of a phenol and an aldehyde.

There are several products that use aromatic compounds in the forms of para-alkoxysubstituted benzyl alcohol derivatives, such as p-alkoxybenzyl alcohol, that when reacted with the aid of $AlCl_3$ as a catalyst will result in non-hydroxylated calixarenes.



Calix[4]arene with para-tert-butyl substituents

Fig. 5.23 *Calixarene*

Ionophores

Ionophores are small hydrophobic molecules that dissolve in lipid bilayers and increase their permeability to specific inorganic ions and which facilitate ion passage in or out of cell membranes. They can do this by binding to particular ions and acting as a mobile carrier, escorting through the hydrophobic environment of the

cell. Ionophores are used to increase the permeability of biological membranes and facilitate transfer of molecules into and out of the cells. They create a shield over the ion, enabling it to transverse hydrophobic biological membranes. Ionophores unique molecular properties allow them to be used in a variety of functions.

There are two classes of ionophores; Mobile ion carriers, which are small molecules that physically shield the ion from the surrounding environment and facilitate its movement across the plasma membrane and channel-forming ionophores, which create a pore in the biological membrane to allow the ion pass through.

5.3.4 Micelles and Synthetic Enzymes or Synzymes

A micelle is an aggregate of surfactant molecule dispersed in a liquid colloid. Micelles are organized molecular assemblies of surfactant. Surfactants sometimes called surface active agents or detergents, are extremely versatile chemicals with applications in chemistry, biology and pharmaceuticals science. Surfactants contain both a polar 'Head' group and non-polar long- chain hydrocarbon "tail". The amphiphillic character of surfactants allows for self-association or micellization, whereby the hydrophobic portion forms the micelle core and the polar head groups form the micelle-water interface. A typical micelle in aqueous solution forms an aggregate with the hydrophilic 'Head' regions in contact with surrounding solvent, sequestering the hydrophobic single-tail regions in the micelle centre. This phase is caused by the packing behavior of single-tailed lipids in a bilayer. The difficulty filling all the volume of the interior of a bilayer, while accommodating the area per head group forced on the molecule by the hydration of the lipid head group, leads to the formation of the micelle. Micelles composed of ionic surfactants have an electrostatic attraction to the ions that surround them in solution, the latter known as counterions. Although the closest counterions partially mask a charged micelle (by up to 90%), the effects of micelle charge affect the structure of the surrounding solvent at appreciable distances from the micelle. Ionic micelles influence many properties of the mixture, including its electrical conductivity. Micelles are approximately spherical in shape. The length of the non-polar tail, the nature and size of the polar or ionic head, the acidity of the solution, the temperature and the presence of added salts are the most important factors determining the kind of obtained aggregate. If those parameters are changed, it is possible to change shape and size of the micelles.

Micelles help the body absorb lipid and fat soluble vitamins. They help the small intestine to absorb essential lipids and vitamins from the liver and gall bladder. They also carry complex lipids such as lecithin and lipid soluble vitamins (A, D, E and K) to the small intestine. Micelles are *lipid molecules* that arrange themselves in a spherical form in aqueous solutions. The formation of a micelle is a response to the amphipathic nature of fatty acids, meaning that they contain both hydrophilic regions (polar head shown Figure 5.24 groups) as well as hydrophobic regions (the long hydrophobic chain). Micelles contain polar head groups that usually form the outside as the surface of micelles. They face to the water because they are polar. The hydrophobic tails are inside and away from the water since they are

non-polar. Fatty acids from micelles usually have a single hydrocarbon chain as opposed to two hydrocarbon tails. This allows them to conform into a spherical shape for lesser steric hindrance within a fatty acid. Fatty acids from ***Glycolipids*** and phospholipids, on the other hand, have two hydrophobic chains that are too bulky to fit into the spherical shape as micelles do. Thus, they preferred to form glycolipids and phospholipids as 'Lipid Bilayers'.

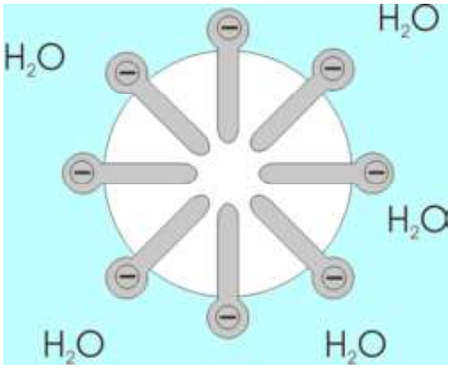


Fig. 5.24 *An Example of Micelles in Polar Solvent*

Micelles form spontaneously in water, as stated above this spontaneous arrangement is due to the amphipathic nature of the molecule. The driving force for this arrangement is the hydrophobic interactions the molecules experience. When the hydrophobic tails are not sequestered from water this results in in the water forming an organized cage around the hydrophobic tail and this entropy is unfavorable. However, when the lipids form micelles the hydrophobic tails interact with each other, and this interaction releases water from the hydrophobic tail and this increases the disorder of the system, and this increase in entropy is favorable.

Enzyme Stabilization by Micelle and Reverse Micelle

Enzymes show great potential in the manufacture of food products, pharmaceuticals and agricultural chemicals. Despite all the advantages, commercial exploitation of enzymatic synthesis remains rather limited. One of the main reasons is the need for a water-rich environment to preserve enzymatic ***bio catalytic activity***. This necessity for an aqueous phase constitutes a significant problem for a number of biochemical reactions of commercial interest. In order to overcome the limitations and to make an enzymatic process attractive, reaction media more suitable than the aqueous phase are necessary to be developed. One of them is the reverse micelle system. Enzymes in reverse micelles systems are found owning increased stability. Similarly, enzymes can also be dispersed in a water phase using a micelle system. Some enzymes showed higher stability and activity in the non-aqueous micro-environment.

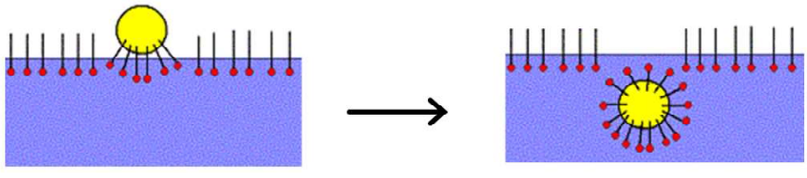


Fig. 5.25 *A Scheme Showing the Formation of Micelles*

Synthetic Enzymes or Synzymes

Synthetic enzymes or Synzymes are substances with catalytic activity. The name synzyme is derived from synthetic enzyme. These are also called artificial enzymes. Current synzymes consist mainly of organic molecules tailored in such a way that they catalyze certain kind of reactions. These molecules bind to a transition state of a substrate in an active site. Like enzymes synthetic enzymes also generally obey Michaelis-Menten Kinetics. Synthetic enzymes are also defined as the synthetic organic molecule prepared to recreate or mimic the active site of an enzyme. Synzymes are designed according to the host molecules such as cyclodextrins, crown ethers or calixarenes etc.

There are 4 Different Type of synzymes

1. Protein derived synzymes
2. Cyclodextrins synzymes
3. Organic synzymes
4. Superoxide Dismutase synzymes

Check Your Progress

7. What do you understand by enzyme model?
8. Define the host-guest chemistry.
9. Give the importance of molecular chirality.
10. Define the biommetic Chemistry.
11. What is crown ether?
12. What do you understand the ionophore?

5.4 BIOTECHNOLOGICAL APPLICATIONS OF ENZYMES

Large-Scale Production and Purification of Enzymes

Biologically active enzymes may be extracted from any living organism: Of the hundred enzymes being used industrially,

- Over a half are produced from fungi
- Rest are from bacteria , animal and plant sources .

There are various ways of enzyme production such as:

1. **Solid Substrate Cultivation:** Also known as Tray cultivation or thin layer culture or Koji fermentation. It is adopted for the extraction of enzymes from fungi, such as Penicillum, aspergillus, etc.
2. **Deep Bed Cultivation:** Microbes are cultivated in Rectangular culture vessels, with the size of 18x200''.
3. **Solid State Cultivation:** Microbes are cultured in a fermenter in order to establish a continuous culture.

The large scale production of enzymes involves culturing micro-organisms in chambers called FERMENTERS or BIOREACTORS.

Micro-organisms are suitable for use in the large scale production of enzymes in fermenters because:

- They have rapid growth rates and are able to produce larger numbers of enzyme molecules per body mass than many other organisms
- Micro-organisms can be genetically engineered to improve the strain and enhance yields
- Micro-organisms are found in a wide variety of different habitats such that their enzymes are able to function across a range of temperatures and pH
- Micro-organisms have simple growth requirements and these can be precisely controlled within the fermenter
- Micro-organisms can utilize waste products such as agricultural waste as substrates

5.4.1 Techniques and Methods of Immobilization of Enzymes and their Effects on Enzyme Activity

Immobilization prevents diffusion of the enzymes in the reaction mixture and permits their recovery from the product stream by simple solid-liquid separation methods. As a consequence, reaction products are free of enzyme and reuse of the enzyme is possible. Another advantage of immobilized enzymes is that they can be used in continuously operated reactors.

Methods of Immobilization

In principle, immobilization of an enzyme can be achieved by fixing it on the surface of a waterinsoluble material, by trapping it inside a matrix that is permeable to the enzyme's substrate and products, and by cross-linking it with suitable agents to give insoluble particles. Bound enzymes may be prepared by covalent coupling to active matrices or by heteropolar and/or Van der Waals binding to adsorbents or ion exchangers. Covalent coupling to activated carrier materials is achieved by methods known in peptide and protein chemistry. The formation of covalent bonds has the advantage of an attachment which is not reversed by pH, ionic strength, or substrate. However, covalent binding offers the possibility that the active site of enzyme may be blocked through the chemical reaction used in the immobilization reaction and the enzyme rendered inactive.

There are a large number of methods of covalent attachment. The groups of enzymes that take part in the formation of the chemical bond are- amino, imino, amide, hydroxyl, carboxy, thiol, methylthiol, guanidyl, imidazole groups, and the phenol ring. Methods have been developed for covalently attaching enzymes to inorganic carriers such as alumina, glass, silica, stainless steel, etc. Adsorption of enzymes at solid surfaces offers the advantage of extreme simplicity. It is carried out according to the principles of chromatography. The conditions of adsorption involve no reactive species and thus do not result in modification of the enzyme. The binding of enzymes, however, is reversible and for this reason adsorbed enzymes

present the problem of desorption in the presence of substrate or increased ionic strength. Commonly used adsorbents include many organic and inorganic materials such as alumina, carbon, cellulose, clays, glass (including controlled-pore glass), hydroxyapatite, metal oxides, and various siliceous materials. Ion exchange resins bind enzyme by electrostatic interactions. The first successful commercial application of immobilized aminoacylase (for resolution of DLamino acids) involved fixing of the enzyme by adsorption to DEAE-Sephadex as carrier.

Inclusion of enzymes in polymer gels, microcapsules, or filamentous structures has the advantage of relatively mild reaction conditions. This method is free from the risk of blocking active site groups on the enzyme molecule by chemical bonds; the enzyme is retained in its native state. The major drawbacks of this immobilization technique are two- retardation of the enzyme reaction due to diffusional control of the transport of substrate and products (particularly with high molecular weight substrates and/or products); and continuous loss of enzyme due to the distribution of pore sizes. Materials used for entrapment include silicone rubber, silica gel, starch, and, preferably, polyacrylamides.

A variation of the inclusion method is encapsulation within semipermeable membranes. Materials, such as collodion polystyrene, cellulose derivatives, and, most commonly, nylon have been used to form thin, spherical, semipermeable membranes shaped into microcapsules which include the enzyme to be immobilized. The size of the capsules can range from ìm to many ìm.

Applications of Immobilized Enzymes

After immobilization of an enzyme, its properties can be changed significantly. Such alterations may be attributed to (1) the physical and chemical nature of the carrier used, (2) the chemical and/or conformational changes in the enzyme structure, and (3) the 'Heterogeneous Nature' of catalysis caused by immobilization.

Effects of altered reactivity include kinetic constants (resulting from a change in activation energy), optimum pH, **Michaelis constant**, and substrate specificity. A matrix charge can affect the hydrogen ion concentration in the locus of the attached enzyme and thus change its apparent pH optimum in one direction or the other, depending on the use of either cationic or anionic carriers, and the apparent Michaelis constant if the substrates are also charged; it is increased if the matrix and the substrate charges are alike and decreased if they are opposite. Changes in enzyme specificity can result from conformational changes in the enzyme molecule caused by the attachment itself.

One of the more important results of enzyme immobilization is the retention of activity for considerable periods of time under suitable conditions of storage. The stability of immobilized enzymes to storage, heat, and pH basically depends on the nature of the carrier surface to which the enzyme is bound.

Among 50 immobilized enzymes, as compared with their soluble counterparts, Melrose (1971) found 30 more stable and 8 less stable than the soluble forms; 12 showed no difference from the free systems. The reasons for the observed increase in stability are not clear. It may be attributed, for example, to prevention of conformational inactivation or to shielding of active groups on the enzyme from reactive groups in solution.

Use of Enzymes in Food and Drink Industry-Brewing and Cheese-Making

Enzymes break down specific components within fruit & vegetables, such as pectin, starch, proteins and cellulose which results in increased yields, shortening of processing time and improving sensory characteristics.

Some examples: Pectinases and Cellulases are used to break down cell walls in fruit and vegetables, resulting in improved extraction and increase in yield. They can also be used to decrease the viscosity of purees or nectars, and to provide *cloud stability' and texture in juices.

Food enzymes aid brewers in cutting down production time and cost while still delivering the quality product that consumers have come to expect. Such enzymes are specifically selected to perform highly specific tasks and improve the overall effectiveness of the process.

Key benefits include:

- Malt separation
- Better extraction yield
- Efficient fermentation
- Beer filtration and stabilization
- Utilization of local raw materials

Syrups from Corn Starch

During starch processing, enzymes help to separate raw milled cereals into polysaccharides gluten and fibres.

Enzymes are also used to further break down the long chains of sugars (polysaccharides) into multiple sugars (e.g., glucose, fructose, sucrose, maltose, maltotriose, raftinose).

Benefits for the Starch Processors

- Efficient and better starch conversion into valuable products: glucose, maltose, high fructose and other Syrups
- Increased capacity utilization during conversion, due to rapid viscosity reduction and low fouling frequency of process equipment such as evaporators
- Improved starch purity due to greater extraction yield from raw materials, and efficient removal of fibres and proteins.
- Energy savings due to less use of process water, lower evaporator costs and decreased production time.

5.4.2 Enzymes as Targets for Drug Design

Enzymes act as a target for drugs for the desired therapeutic effect, which are thereby called **biological targets**. Enzymes offer unique opportunities for drug design that are not available to cell surface receptors, nuclear hormone receptors, ion channels, transporters. Drugs that function as enzyme inhibitors constitute a

significant portion of the orally bioavailable therapeutic agents that are in clinical use today. Likewise, much of drug discovery and development efforts at present are focused on identifying and optimizing drug candidates that act through inhibition of specific enzyme targets. Drugs acting on enzymes can either inhibit them or activate them. Inhibition of enzymes is a strategy which is more commonly used rather than activation of enzymes. 47% of all current drugs inhibit their enzyme targets. Enzyme activity can not only be inhibited; it can also be accelerated through biochemical modification of the enzyme. In this manuscript, the authors provide a system-wise summary of drugs acting on enzymes, microsomal enzymes targeted by drug and newly approved drugs acting on enzymes.

5.4.3 Clinical uses of Enzymes

- Enzymes can be used for Aiding Digestion. Example: Amylases, Proteases and Lipase.
- They can also be used as Deworming agents. Example: Papain.
- Enzymes act as anti-clotting agents like Fibrinolytic and Thrombolytic. Examples: Urokinase and Streptokinase.
- Enzymes can be used as surface disinfectants. Example: Trypsin.
- They can also be used in the diagnosis purpose. Example: Glucose oxidase along with peroxidase to detect the level of glucose (colorimetric estimation).

Enzyme Therapy

Enzyme therapy is a plan of dietary supplements of plant and animal enzymes used to facilitate the digestive process and improve the body's ability to maintain balanced metabolism. Enzyme therapy has improved the lives of many patients, by reducing the burden of their disease. Several studies have sought to determine to what extent optimal clinical outcomes are a function of the prescribed enzyme dose and its resultant costs.

Enzymes used in therapy are:

1. Digestive Enzymes
   a. Chymotrypsin
   b. Bromilain
   c. Rennin
   d. Papain
   e. Cellulase
2. DNase I
3. Alginate Lyase
4. Adenosine Deaminase
5. Dihydrofolate reductase
6. Lipase
7. Streptokinase

Enzymes and Recombinant DNA Technology

Recombinant DNA technology produce recombinant DNA (DNA) using a set of enzymes called recombinant enzymes.

These include -

1. Nucleases- They are of 2 types; Exonuclease which removes the terminal nucleotide of DNA molecule and Endonuclease which breaks the internal phosphodiester bond.
2. Ligases- These are enzymes which join the nucleic acid molecules together. They can join both DNA and RNA.
3. Polymerases
4. DNA modifying enzymes

Goals of recombinant DNA technology:

- To isolate and characterize a gene
- To make desired alterations in one or more isolated gene
- To return altered genes to living cells
- Artificially synthesize new gene
- Alternating the genome of an organism
- Improving human genome

Check Your Progress

13. State the principle of immobilization.
14. What is biological target?
15. What do you understand by enzyme therapy?

5.5 ANSWER TO 'CHECK YOUR PROGRESSES'

1. Co-enzymes are non-protein organic molecules that are mostly derivatives of vitamins soluble in water by phosphorylation; they bind apoenzyme to proteins to produce an active haloenzyme.
2. Co-enzymes participate in catalysis when they bind to the active site of the enzyme (called apoenzyme) and subsequently form the active enzyme (called holoenzyme).
3. Thiamine-Sulphur containing water soluble vitamin of B complex, exists in tissues in the active form of Thiamine Pyrophosphate (TPP). It is an essential co-enzyme involved in the energy extraction and cellular process in catabolism of sugar and amino acid.
4. NAD+ (Nicotinamide adenine dinucleotide) are coenzymes found in all living cells and are oxidizing agents which are capable of carrying and transferring electrons.

5. Nicotinamide adenine dinucleotide phosphate (NADP) is the carrier of electrons (protons) in the cells. Therefore, NADP+ serves as the reducing power of the cells in the form of NADP/NADPH2 or an NAD\NADH2.
6. FEN stands for Flap Endonuclease. It is an enzyme involved in the base excision repair pathway and counteracts replication stress, thus blocking the mechanism by which many drugs kill tumor cells.
7. Enzyme models are generally organic synthetic molecules that contain one or more features present in enzymatic systems. They are smaller and structurally simpler than enzymes.
8. Host-guest chemistry is a branch of supramolecular chemistry that deals with the formation of aggregate structures and their properties. The complex is held by one or a combination of forces which includes hydrogen bonding, ion-dipole, ion-ion, ð-ð, Van der Waals and hydrophobic interactions.
9. The importance of molecular chirality arises from the fact that the two mirror images of a chiral molecule can have vastly different physiological impacts when ingested by living organisms. This is particularly important for medicinal chemistry.
10. Biomimetic chemistry deals with chemical systems that can simulate biological phenomena, particularly catalysis and specificity. Recently, increasing attention has been given to making artificial enzymes by purely chemical means. In the future, the field should extend to provide further understanding of living systems and new methods of chemical synthesis.
11. Crown ethers are cyclic compounds that have several ether linkages. A crown ether specifically binds certain metal ions or organic molecules, depending on the size of its cavity.
12. Ionophores are small hydrophobic molecules that dissolve in lipid bilayers and increase their permeability to specific inorganic ions and which facilitate ion passage in or out of cell membranes.
13. In principle, immobilization of an enzyme can be achieved by fixing it on the surface of a waterinsoluble material, by trapping it inside a matrix that is permeable to the enzyme's substrate and products, and by cross-linking it with suitable agents to give insoluble particles.
14. Enzymes act as a target for drugs for the desired therapeutic effect, which are thereby called biological targets.
15. Enzyme therapy is a plan of dietary supplements of plant and animal enzymes used to facilitate the digestive process and improve the body's ability to maintain balanced metabolism. Enzyme therapy has improved the lives of many patients, by reducing the burden of their disease.

5.6 SUMMARY

- Most of the B-vitamins are coenzymes and are essential in facilitating the transfer of atoms or groups of atom between molecules in the formation of carbohydrates, fats and proteins.

- Prosthetic group are non-peptide components of enzymes which are involved in catalysis. They are bound to enzymes (covalently or non-covalently) and their change from one form to another takes place in a single catalytic cycle.
- The main function of the coenzyme is to act as intermediate carriers of transferred electrons or functional groups in a reaction.
- Co-enzyme A or CoA is a small, organic, non-protein molecule that carries chemical groups between enzymes. It may act as an acyl group carrier to form acetyl- CoA and other related compounds.
- Pantothenic acid is used in the synthesis of CoA. This coenzyme is formed when the vitamin combines with the derivative of ADP and the amino acid cysteine.
- CoA is important in the biosynthesis of many important compounds such as fatty acids, cholesterol.
- Pyridoxal phosphate (PLP) is the active form of vitamin B6. Vitamin B6 is the precursor to coenzyme Pyridoxal Phosphate (PLP) which is essential in transformation of amino acids including transamination, deamination and decarboxylation.
- Pyridoxal phosphate is the functions as a cofactor in enzymes involved in transamination reactions required for the synthesis and catabolism of the amino acids. It is required as a cofactor in glycogenolysis.
- NADP + functions as co-enzymes of a large number of oxidoreductase enzyme. They act as electron acceptors during enzymatic removal of hydrogen atoms from specific substrate molecules.
- In catabolic reactions like respiration, NAD+/NADH act as electron donor, whereas NADP+/NADPH play roles in anabolic reactions. Structurally, NAD consists of a nicotinamide moiety (the protein which undergoes reversible reduction), two adenine nucleotide.
- The enzymes that require FMN and FAD as cofactors are termed as flavoproteins.
- FMN is then converted into FAD via attachment of AMP (derived from ATP) through the action of FAD pyrophosphorylase.
- Lipoic acid is an essential cofactor for mitochondrial metabolism and is synthesized using intermediates from mitochondrial fatty-acid synthesis type II, S-adenosylmethionine and iron–sulfur clusters.
- Vitamin B12 is composed of a complex tetra pyrrole ring structure and a cobalt ion in the center. Tetrapyrroles are compounds containing four pyrrole rings.
- In TCA (TriCarboxylic Acid) cycle, one of the enzyme methylmalonyl-CoA mutase requires vitamin B12 as a cofactor in the conversion of methylmalonyl-CoA into succinyl-CoA.
- NADH and NADPH (stable in solutions containing oxygen) possess reductive power. The stability of reduced pyridine nucleotides allows them to carry their reduction potential from one enzyme to another; the characteristics not owned by flavin coenzymes.

- NADH and NADPH show a maximum in ultraviolet region at 340 nm caused by dihydropyridine ring, while NAD+ and NADP+ do not absorb the light at this wavelength. The appearance and disappearance of the absorbance at 340 nm are useful for the measurement of the rate of the oxidation.
- Numerous metabolic processes depend on co-enzyme A (CoA, or HS-CoA), including the oxidation of fuel molecule and biosynthesis of some carbohydrates and fats. The coenzyme is involved in the reactions of acyl group transfers.
- Phosphopantetheine, phosphate ester which contains 2-mercaptoethylamine and pantothenate parts of coenzyme A, is a prosthetic group of a small protein (77 amino acid residues), known as an Acyl Carrier Protein (ACP).
- Pyridoxal phosphate is a prosthetic group for many enzymes which catalyze different reactions, including isomerizations, decarboxylations, and eliminations in side chain or substitutions.
- The actions of multiple co-enzymes of pyruvate dehydrogenase complex show how coenzymes supplying reactive groups and thus increasing catalytic diversity of proteins are used for storing energy and carbon building blocks.
- An enzyme model attempts to mimic some key parameter of enzyme function on a much simpler level. To separate out a particular factor responsible for the catalytic efficiency of the enzyme within the biological system would be a fabulous task requiring a knowledge of each of the components that would contribute to the overall catalysis.
- The most common examples of 'Host-Guest' chemistry are enzyme-substrate interactions where the substrate fits into the enzyme's active site with complete specificity
- An asymmetric molecule is a molecule that has no planes or center of symmetry. The asymmetry of molecules may depend on the presence of the asymmetric atom of carbon; in its absence, by the asymmetry of the entire molecule.
- Molecular recognition is a biological phenomenon which realizes special recognition at molecular level in living and chemical systems. The origin of the process lies in the intrinsic capacity of biomolecules to distinguish between other compounds and interact with them on the base of their molecular complementarities.
- Molecular recognition plays an important role in biological systems and is observed in between receptor-ligand, antigen-antibody, DNA-protein, sugar-lectin, RNA-ribosome, etc.
- Cyclodextrins are a family of cyclic oligosaccharides, consisting of a macrocyclic ring of glucose subunits joined by á-1,4 glycosidic bonds. They are prepared by enzymatic treatment of starch.
- Ionophores are small hydrophobic molecules that dissolve in lipid bilayers and increase their permeability to specific inorganic ions and which facilitate ion passage in or out of cell membranes.

- Immobilization prevents diffusion of the enzymes in the reaction mixture and permits their recovery from the product stream by simple solid-liquid separation methods. As a consequence, reaction products are free of enzyme and reuse of the enzyme is possible.
- Enzymes break down specific components within fruit & vegetables such as pectin, starch, proteins and cellulose which results in increased yields, shortening of processing time and improving sensory characteristics.

5.7 KEY TERMS

- **Co-enzymes:** Co-enzymes are non-protein organic molecules that are mostly derivatives of vitamins soluble in water by phosphorylation; they bind apoenzyme to proteins to produce an active haloenzyme.
- **NADP+:** Nicotinamide adenine dinucleotide phosphate (NADP) is the carrier of electrons (protons) in the cells.
- **Thiazole ring:** A thiazole ring is found naturally in thiamine and is a heterocyclic compound consisting of a N atom and S atom as a part of the aromatic five-membered ring. It stabilizes charge and electron transfer.
- **FAD:** Flavin MonoNucleotide (FMN) and Flavin Adenine Dinucleotide (FAD) and is used to oxidize substrates. FAD contains riboflavin and adenine.
- **Enzyme models:** Enzyme models are generally organic synthetic molecules that contain one or more features present in enzymatic systems. They are smaller and structurally simpler than enzymes.
- **Artificial enzymes:** Synthetic enzymes or Synzymes are substances with catalytic activity. The name synzyme is derived from synthetic enzyme. These are also called artificial enzymes.

5.8 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short–Answer Questions

1. What is co-enzyme chemistry?
2. Define the apoenzyme.
3. Give the structure of pyridoxal phosphate.
4. What do you understand by lipoic acid?
5. What do you understand by enzyme models?
6. What is host-guest chemistry?
7. Define the prochairality.
8. How will you define the crown ether?
9. Define the synzymes.

10. Determine the purification of an enzyme.
11. Give the uses of enzyme in food and drink industry.
12. What is enzyme therapy?

Long –Answer Questions

1. Discuss in detail about the co-enzyme chemistry with appropriate examples.
2. Illustrate the structure and biological functions of co-enzyme A and thiamine pyrophosphate.
3. Elaborate on the mechanism of reactions catalyzed by co-enzyme factors.
4. Describe the enzyme models with the help of examples.
5. Interpret the chiral and molecular recognition giving applications.
6. Elaborate on the biotechnological applications of enzyme in various fields.

5.9 FURTHER READING

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