MICROBIAL BIOCHEMISTRY AND ANALYTICAL TECHNIQUES

M.Sc. MICROBIOLOGY

SEMESTER-I, PAPER-II

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MICROBIAL BIOCHEMISTRY AND ANALYTICAL TECHNIQUES

First Edition : 2025

No. of Copies :

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Published by:

Prof. V. VENKATESWARLU
Director, I/c
Centre for Distance Education,
Acharya Nagarjuna University

Printed at:

FOREWORD

Since its establishment in 1976, Acharya Nagarjuna University has been forging ahead in the path of progress and dynamism, offering a variety of courses and research contributions. I am extremely happy that by gaining 'A+' grade from the NAAC in the year 2024, Acharya Nagarjuna University is offering educational opportunities at the UG, PG levels apart from research degrees to students from over 221 affiliated colleges spread over the two districts of Guntur and Prakasam.

The University has also started the Centre for Distance Education in 2003-04 with the aim of taking higher education to the door step of all the sectors of the society. The centre will be a great help to those who cannot join in colleges, those who cannot afford the exorbitant fees as regular students, and even to housewives desirous of pursuing higher studies. Acharya Nagarjuna University has started offering B.Sc., B.A., B.B.A., and B.Com courses at the Degree level and M.A., M.Com., M.Sc., M.B.A., and L.L.M., courses at the PG level from the academic year 2003-2004 onwards.

To facilitate easier understanding by students studying through the distance mode, these self-instruction materials have been prepared by eminent and experienced teachers. The lessons have been drafted with great care and expertise in the stipulated time by these teachers. Constructive ideas and scholarly suggestions are welcome from students and teachers involved respectively. Such ideas will be incorporated for the greater efficacy of this distance mode of education. For clarification of doubts and feedback, weekly classes and contact classes will be arranged at the UG and PG levels respectively.

It is my aim that students getting higher education through the Centre for Distance Education should improve their qualification, have better employment opportunities and in turn be part of country's progress. It is my fond desire that in the years to come, the Centre for Distance Education will go from strength to strength in the form of new courses and by catering to larger number of people. My congratulations to all the Directors, Academic Coordinators, Editors and Lesson-writers of the Centre who have helped in these endeavors.

Prof. K. Gangadhara Rao
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Acharya Nagarjuna University.

SEMESTER.I

M.SC. MICROBIOLOGY

1O2MB24 - MICROBIAL BIOCHEMISTRY AND ANALYTICAL TECHNIQUES

UNIT-I

Lesson 1 Carbohydrates – Structure and physicochemical properties of mono and oligosaccharides. Structures and biological importance of structural and storage polysaccharides.

Lesson 2 Lipids– Physicochemical properties of fatty acids, Triacylglycerols, Glycolipids, Phospholipids, Lipid aggregations (micelles, monolayers, bilayers, liposomes).

Lesson3: Nucleic Acids: Structure of DNA and RNA. Renaturation and denaturation of DNA, cot values of DNA.

UNIT-II

Lesson 4 Aminoacids: classification, amino acid properties, essential amino acids, Biological significance.

Lesson 5 Proteins: peptide bond and types of peptides, peptides of non-protein origin, three dimensional structure of proteins (Primary, Secondary, Tertiary, Quaternary).

Lesson 6 Chaperones, denaturation and renaturation of proteins.

UNIT-III

Lesson 7 Enzymes – Nature and outline classification of enzymes, Binding energy, activation energy, rates of reactions, MM equation, factors influencing the enzyme action,

Lesson 8 Mechanism of enzyme action, enzyme inhibitors, allosteric enzymes, isoenzymes,ribozymes, abzymes.

Lesson 9 Protein purification and characterization methods, methods of lipid separation and analysis.

Lesson10 Spectroscopy – Principles and applications of UV-Vis, NMR, ESR and Mass spectroscopy.

Lesson11 Centrifugation: Instrumentation for centrifugation, principles and applications of differential and density gradient centrifugation.

UNIT-V

Lesson 12 Chromatography – Principles and applications of adsorption, ion exchange, gel filtration, affinity and ion exchange chromatography.

Lesson 13 Electrophoresis – Principles and applications of Polyacrylamide, Agar, Pulsefied, and Immuno electrophoresis.

SUGGESTED BOOKS:

1. Nelson and Cox 2000. Lehninger Principles of Biochemistry.
2. Moat, A. Gand J.N. Foster.1999. Microbial Physiology.
3. Wilson, K and J.Walker 1995. Practical Biochemistry. Principles and Techniques.4 th ed.
4. Upadhyay, A., Upadhyay, K and Nirmalendru Nath. 2003. Biophysical Chemistry –Principles and Techniques.
5. David Freifeilder and W. Freeman 1982. Physical Biochemistry – Applications to Biochemistry and Molecular Biology. 2 nd ed.
6. Caldwell, D.R. 1995. Microbial Physiology and Metabolism.

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CENTRE FOR DISTANCE EDUCATION

ACHARYA NAGARJUNA UNIVERSITY

Model Question Paper

M.Sc. Degree Examination

MICROBIOLOGY- I SEMESTER

MICROBIAL BIOCHEMISTRY AND ANALYTICAL TECHNIQUES

Time: 3 hours **Maximum Marks: 70**

Answer ALL Questions **(5x14 = 70 marks)**

UNIT-I

1. a) Give an account on structure and physicochemical properties of carbohydrates.

OR

b) Explain the morphological types, physical and chemical nature of DNA.

UNIT-II

2. a) Write an account on classification and properties of amino acids.

OR

b) Describe the primary, secondary, tertiary and quaternary structures of proteins.

UNIT-III

3. a) Give an account on outline classification and nature of enzymes.

OR

b) Explain the protein purification and characterization methods.

UNIT-IV

4. a) Write an account on principle, instrumentation and applications of UV-Vis spectroscopy.

OR

b) Describe the principle and applications of differential and density gradient centrifugation.

UNIT-V

5. a) Define chromatography. Explain the principle and applications of ion exchange chromatography.

OR

b) What is electrophoresis. Describe the principle and applications of Polyacrylamide gel electrophoresis.

CONTENTS

LESSON 1
CARBOHYDRATES

Objectives: To Understand basic concept of carbohydrates

1.1 INTRODUCTION

Carbohydrates are the most abundant group of biological molecules. They represent a source of energy for living organisms, readily usable form like glucose or as reserve storage form e.g. starch, glycogen. Some carbohydrates have a structural role (Cellulose, Chitin, hyaluronic acid) while others have an important biological role like signal recognition (glycans of glycoproteins and glycolipids).

Carbohydrates constitute about 60 to 90% of the dry matter of plants. Chemically, they contain the elements Carbon, Hydrogen and Oxygen. A large number of the carbohydrates corresponds to the formula $C_n(H_2O)_n$, but there are some exceptions. Carbohydrates are broadly defined as polyhydroxy aldehydes or ketones and their derivatives or as substances that yield one of these compounds on hydrolysis.

1.2 CLASSIFICATION

Carbohydrates are broadly divided into three major groups as follows:

1. Monosaccharides, 2. Oligosaccharides and 3. Polysaccharides

1.2.1 Monosaccharides

These are often called "Simple Sugars" which cannot be hydrolyzed into smaller units. Monosaccharides are compounds which possess a free aldehyde (C-CHO) or ketone (=CO) group and two or more hydroxyl (-OH) groups. Their general formula is $C_n(H_2O)_n$. Monosaccharides are colorless, crystalline solids that are freely soluble in water but insoluble in nonpolar solvents. The monosaccharides may be subdivided into trioses, tetroses, pentoses, hexoses, heptoses etc., depending upon the number of carbonaroms they possess and as aldoses or ketoses, depending upon whether they contain aldehyde or ketone group.

Some of the important monosaccharides

Name	**Formula**	**Aldoses**	**Ketoses**
Trioses	$C_3H_6O_3$	Glyceraldehydes	Dihydroxyacetone
Tetroses	$C_4H_8O_4$	Erythrose	Erythrulose
Pentoses	$C_5H_{10}O_5$	Ribose	Ribulose
Hexoses	$C_6H_{12}O_6$	Glucose	Fructose
Heptoses	$C_7H_{14}O_7$	Glucoheptose	Sedoheptulose

Both the characters, i.e., the number of carbon atoms and the nature of functional groups may also be present in one sugar. For example, glycerose is an aldotriose; ribulose, a ketopentose and glucose, an aldohexose. The most abundant monsaccharide in nature is the 6-carbon sugar, D-glucose.

1.2.2 Oligosaccharides

These are compound sugars that yield two to ten molecules of the same or different monosaccharides on hydrolysis. Accordingly, an oligosaccharide yielding two molecules of monosaccharide on hydrolysis is designated as a disaccharide, and they are yielding three molecules of monosaccharides as a trisaccharide and so on. The general formula of disaccharides is $C_n(H_2O)_{n-1}$ and so on. A few examples are:

Disaccharides - Sucrose, Maltose, Lactose, Cellobiose, Trehalose
Trisaccharide - Rhamninose, Gentianose, Raffinose
Tetrasaccharides - Stachyose
Pentasaccharides - Verbascose

1.2.2 Polysaccharides

These carbohydrates yield more than ten molecules of monosaccharides on hydrolysis. These may be further classified, depending on whether the monosaccharides molecules produced as a result of the hydrolysis of polysaccharides are of the same type (homopolysaccharides) or of different types (heteropolysaccharides). Their general formula is $(C_6H_{10}O_5)_x$.

Some of the common examples are:

Homopolysaccharides - Starch, glycogen, inulin cellulose, chitin, pectin
Heteropolysaccharides - Gums, Hemicelluloases

1.3 Structural aspects of monosaccharides

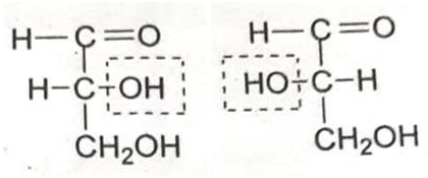
1.3.1 Asymmetric Carbon Atom

A carbon atom to which four different atoms or groups of atoms are attached is said to be asymmetric or chiral. All monosaccharides except dihydroxyacetone has asymmetric carbon atom.

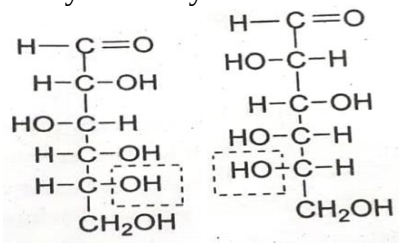
1.3.2 Isomerism

The presence of asymmetric carbon atoms allow the formation of isomers. The compounds which have the same structural formula but differ only in spatial configuration are called stereoisomers. The number of asymmetric carbon atoms (n) determines the possible isomers of a given compound which is equal to 2^n. Glucose contains 4 asymmetric carbons and thus has 16 isomers.

The triose sugar, glyceraldehydes is the simplest monosaccharide, which is generally used as the reference carbohydrate to represent the structure of all other carbohydrates Fig.1.1. It has only one asymmetric carbon atom and it can, therefore, exist in two isomeric forms.



D-Glyceraldehyde L-Glyceraldehyde



D-Glucose L-Glucose

Fig 1.1 D and L Isomers

The D and L isomers are mirror images of each other. The designation of an isomer as D- or L-form is determined by its spatial configuration to the parent compound. When the OH group around the carbon atom adjacent to the terminal primary alcohol carbon (carbon atom 5 in glucose) is on the right, the sugar is a member of D series. When it is on the left, it is a member of the L series.

Optical Isomerism

The presence of asymmetric carbon atoms in the compound also confers optical activity to it. When a beam of polarized light is passed through an optically active solution, it may be rotated either to the right or to the left, depending upon the type of optical isomer present. A compound rotating the plane of polarized light to the right is called as dextrorotatory and is designated by a plus (+) sign. When the rotation of the beam is to the left, it is said to be levorotatory and is designated by a minus (-) sign.

When equal amounts of dextrorotatory and levorotatory isomers and present, the resulting mixture has no optical activity, since the activities of each isomer cancel the other. Such a mixture is said to be racemic or a DL mixture. The separation of optically active isomer from a racemic mixture is called resolution.

Structures of D-aldoses

The structures of some important aldoses starting from D-glyceraldehyde is given below:

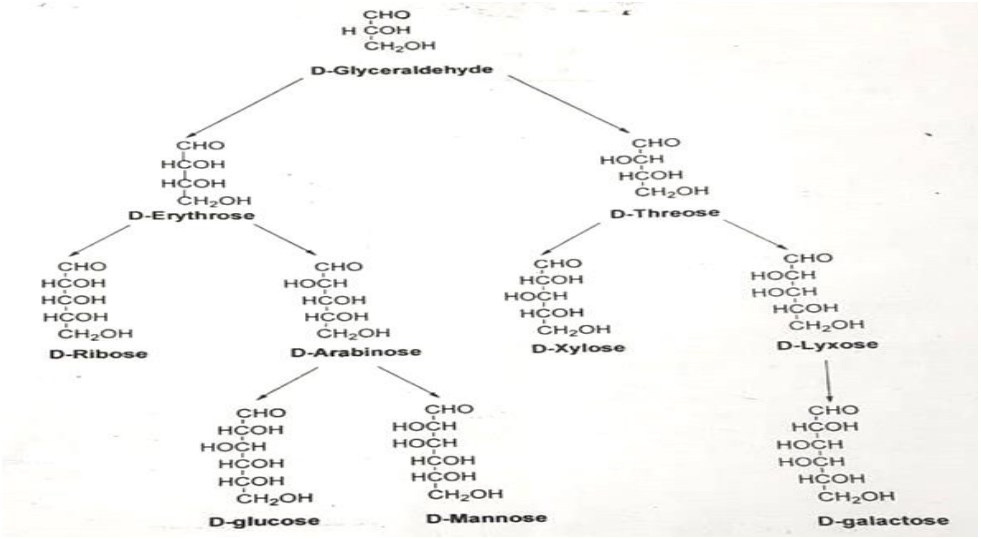


Fig 1.2 Structures of D-aldoses

Structures of D-Ketoses

Starting from Dihydroxyacetone (triose), there are five keto-sugars which are physiologically important. Their structures are given below:

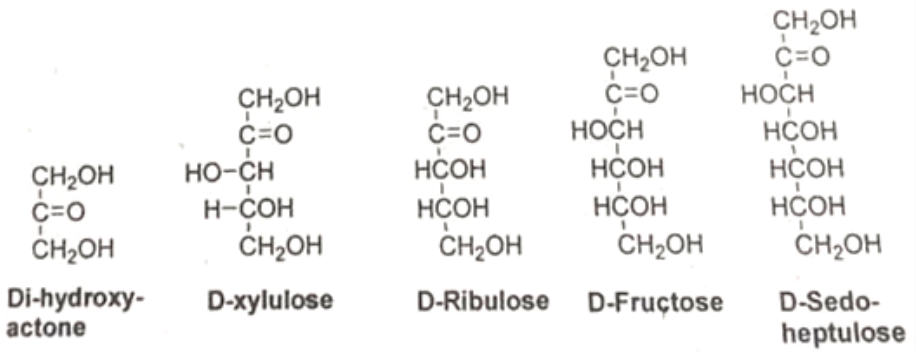


Fig 1.3 Structures of D-Ketoses

Epimers

If two monosaccharides differ from each other in their configuration around a single specific carbon (other than anomeric) atom, they are referred to as epimers to each other. For example, glucose and galactose are epimers with respect to carbon 4 (C_4 – epimers). They differ in the arrangement of – OH group at C_4. Glucose and mannose are epimers with regard to carbon 2 (C_2 – epimers) (Fig – 1.4).

The interconversion of epimes is known as epimerization and a group of enzymes – namely – epimerases catalyse this reaction.

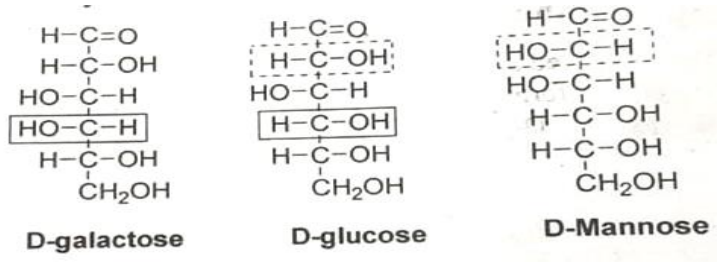


Fig. 1.4 Structures of glucose epimers

α- and β-Anomers

The hydroxyl group of monosaccharides can react with its own aldehyde or keto functional group to form hemiacetal and hemiketal. For example, in case of glucose, the aldehyde group of glucose at C_1 reacts with alcohol group at C_5 to form two types of cyclic hemiacetals, namely α and β forms. The α and β cyclic forms of D-glucose are known as anomers. They differ from each other in the configuration only around C_1 known as anomeric carbon (hemiacetal carbon). The anomeric form with the –OH on C-1 to the right (or pointing downward in Haworth projection formula) is known as α-D-glucose, the other with the –OH to the left is referred β-D-glucose (Fig – 1.5)

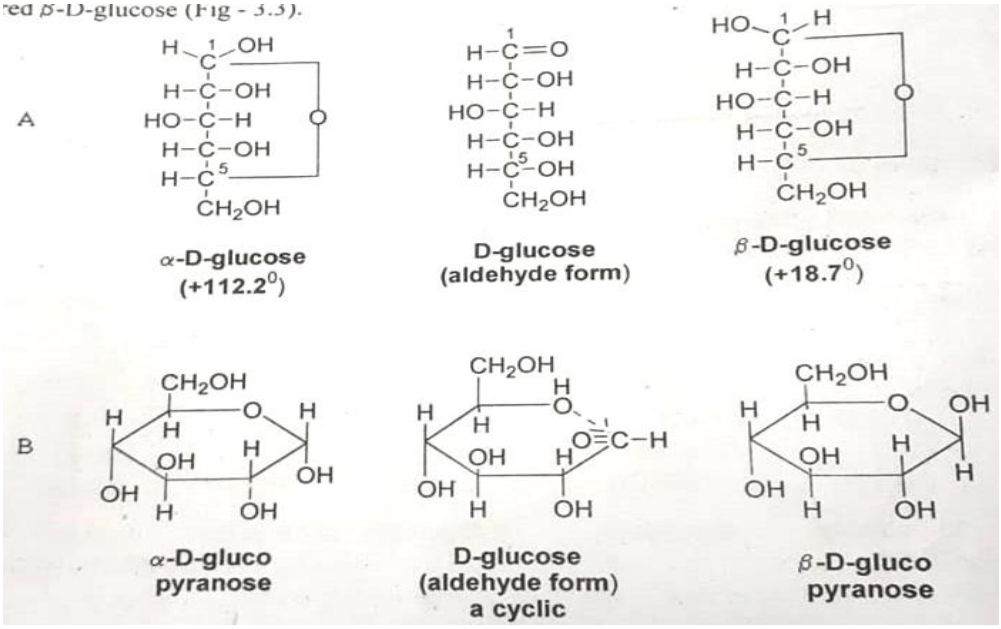


Fig – 1.5 Structures of α- and β-Anomers

1.3.3 Mutarotation

The α and β anomers of glucose have different optical rotations. The specific optical rotation of a freshly prepared glucose (α anomer) solution in water is $+112.2^0$ which gradually changes and attains an equilibrium with a constant value of $+52.7^0$. The optical rotation of β-D-glucose is $+18.7^0$.

Mutarotation is defined as the intra conversion of α and β-glucose in solution with change of optical activity.

1.3.4 Pyranose and Furanose Ring Structure

Haworth, in 1929, proposed a scheme in which all sugars forming six-membered rings are called pyranoses and those forming five-membered rings furanoses Fig – 1.6. The cyclic forms of glucose are known as α-D-glucopyranose and α-D-glucofuranose.

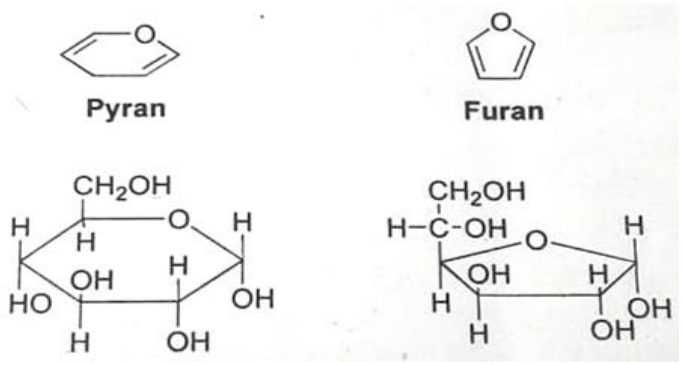


Fig – 1.6: Structure of Pyran, Furan, α-D-glucopyranose and α-D-glucofuranose

1.4 GLYCOSIDES

Glycosides are compounds formed by the condensation reaction between a sugar and the hydroxyl group of another carbohydrate. The bond connecting the anomeric carbon to the acetal oxygen is known as the glycosidic bond. Glycosides are found in many drugs, spices. Some are important in medicine because of their action on the heart (cardiac glycosides). Other glycosides include antibiotics such as streptomycin.

1.5 OLIGOSACCHARIDES

Oligosaccharides are complex sugars, in which a limited number of monosaccharides (2-10) are linked together by glycosidic bonds. Depending on the number of molecules of monosaccharides formed during hydrolysis, these molecules are referred as disaccharides, trisaccharides etc.,

1.5.1 Disaccharides

These are formed by the union of two monosaccharides. They are held together by glycosidic bond between the first carbon of one monosaccharide and with the second or the fourth carbon of another monosaccharide. The disaccharides are of two types.

1. Reducing disaccharides with free aldehyde or keto group eg. Maltose & Lactose.
2. Non-reducing disaccharides with no free aldehyde or keto group e.g. sucrose, trehalose.

Maltose (Malt Sugar): Maltose is made up of two α-D-glucose units, united by the α-1, 4-glycosidic linkage. The hemiacetal hydroxyl group of the second glucose is free and exhibits the reducing property. Maltose is produced when starch is hydrolyzed by amylases or by acid hydrolysis.

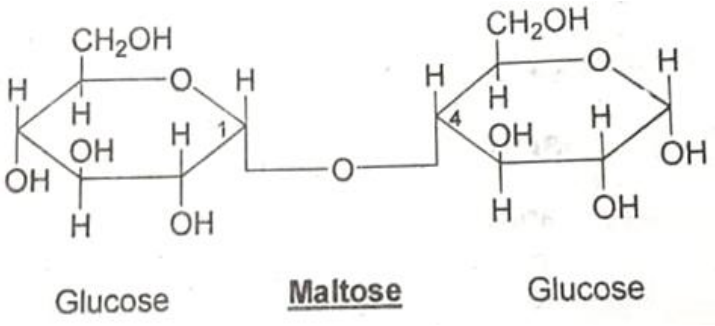


Fig 1.7 (α-D-glucosyl (1 → 4) α-D-glucose)

Cellobiose is another disaccharide, which is identical in structure to maltose, except that the former has β (1 → 4) glycosidic linkage. Cellobiose is formed during the hydrolysis of cellulose, apolysaccharide

Lactose (Milk Sugar) : It is found in the milk of mammals. It is formed by the union of a molecule of D-galactose and one molecule of D-glucose by β-1, 4-galactosidic bond. The hemiacetal group of glucose remains free abd exhibits the reducing property. Lactose is hydrolyzed by the intestinal enzyme lactase to glucose and galactose.

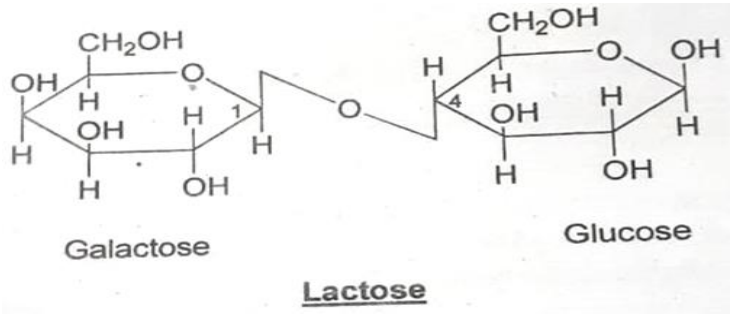


Fig 1.8 (β-D-galactosyl (1 → 4) β-D-glucose)

Sucrose (Cane Sugar): This is the most abundantly distributed non-reducing disaccharide. It is found in several plants, and is particularly abundant in sugar-beet and sugar cane. Sucrose is made up of α-D-glucose and β-D-fructose. The two monosaccharides are held together by a glycosidic bond (α_1→ β_2), between C_1 of α-glucose and C_2 of β-fructose. The reducing groups of both glucose and fructose are involved in glycosidic bond, hence sucrose is a non-reducing sugar.

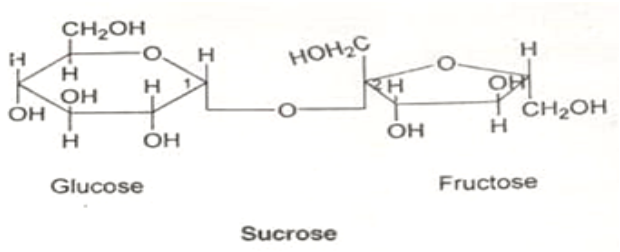


Fig 1.9(α-D-glucosyl (1 → 2) β-D-fructose)

1.5.2 Higher Oligosaccharides

The mose common trisaccharide found in plants in raffinose. It is present in cotton-seed meal and sugar beet in considerable amounts. On hydrolysis, reffinose yields glucose, fructose and galactose in equimolar amounts.

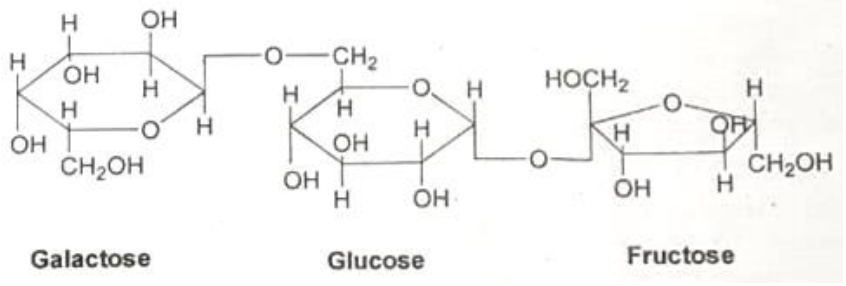


Fig1.10Structure of Raffinose

One of the abundant tetrasaccharides in plants is stachyose. Stachylose was first isolated from the rhizomes of Stachys tuberifera, and was found to coexist with raffinose and

other related oligosaccharides in various organs of a large variety of plant species. Stachyose is considered as a major storage and transport sugar in woody plants, cucurbits and legumes.

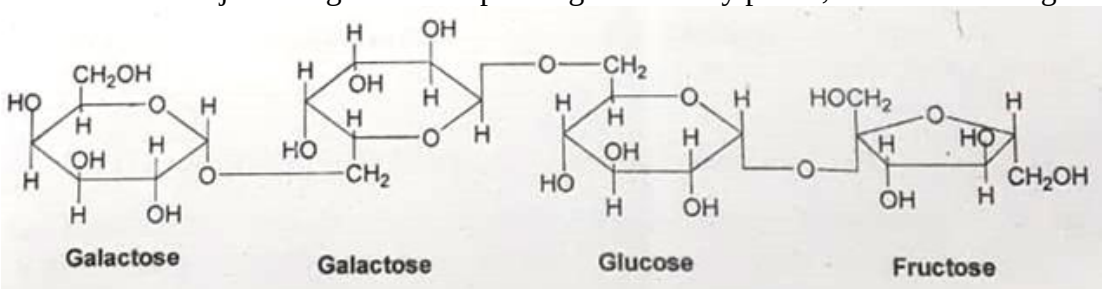


Fig 1.11Structure of Stachylose

1.6 POLYSACCHARIDES

Polysaccharides are formed by the condensation of a large number of molecules of monosaccharides or their derivatives, held together by glycosidic bonds. Thus polysaccharides are high molecular weight compounds. Some are linear, while others are branched polymers. They are widely distributed in plants and function both as structural components of the cell wall and as storage products in seeds, fruits and leaves.

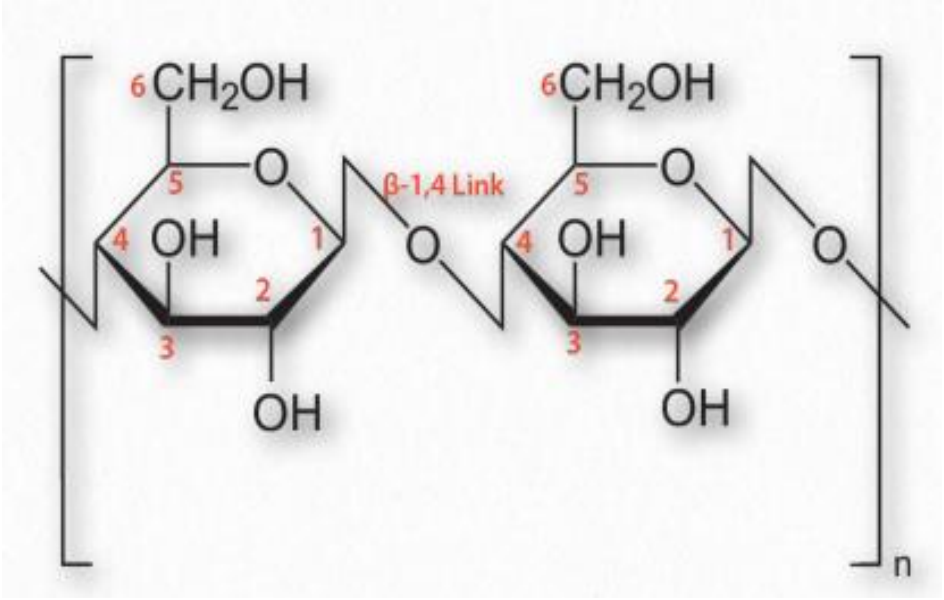


Figure1.12 Chitin with β-1,4 links between N-acetylglucosamine sugars

Polysaccharides are of two types – Homopolysaccharides, Hetero polysaccharides.

1.6.1 Homopolysaccharides

Homopolysaccharides on hydrolysis yield only one type of monosaccharide. Homopolysaccharides are named based on the nature of the monosaccharide unit, for example glucosans are polymers of glucose whereas fructionasans are polymers of fructose.

Starch: Functions both as a long-term and a short-term storage polysaccharide in plants. It is stored on a long-term basis in many seeds, tuber and rhizomes, only being utilized when these structures germinate. It also forms a short-term storage polymer in chloroplasts during periods of active photosynthesis and exported from the leaves as sucrose.

Starch is always formed and stored as starch grains in plastids; in non-photosynthetic tissues, it is stored in amyloplasts. Starch has two components, amylase and amylopectin. Amylose is a polysaccharide with linear chains and is made up of D-glucose units joined by α-1, 4-glycosidic linkages. Its molecular weight varies from 1,50,000 to 6,00,000. The chains are of different lengths and can combine through hydrogen bonds between the hydroxyls, to form compact structures.

Amylopectin is a branched polysaccharide withα (1-6) branch chains. Its molecular weight can reach several million. Amylopectin is formed of main chains like those of amylase (α-1, 4-glycosidic linkages) but with side chains joining them by α-1, 6-glycosidic linkages. The branches have the same structure as the main chains and are made up of 20 to 25 glucose units.

Glycogen: It is the storage form of carbohydrate found in liver and muscles of animals and human beings. The structure of glycogen is the same as that of amylopectin. The branches in glycogen, however are more and therefore contains more of α-1, 6-glycosidic linkages. The average length of chains varies from 10 to 15 glucose units. Its molecular weight can attain several tens of millions.

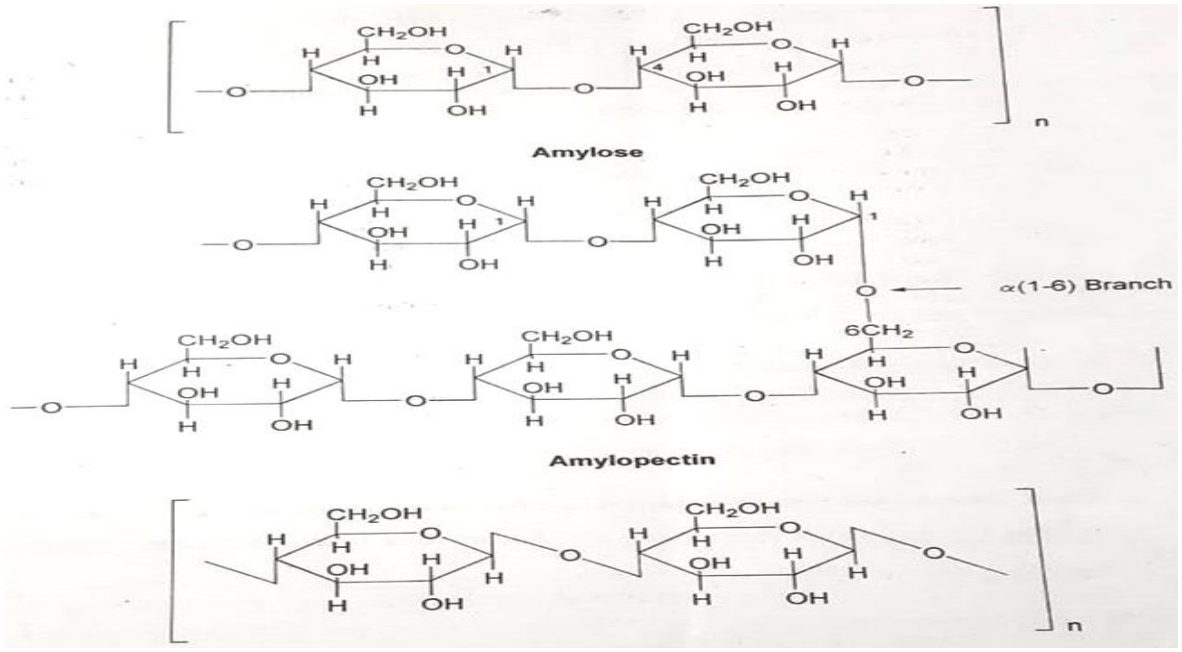


Figure 1.13 Structure of Homopolysaccharides

Cellulose: Cellulose is found only in plants, and it is the most abundant biopolymer in plant kingdom. It is the main constituent of the cell walls of plants. Cellulose molecules are unbranched chains of D-glucopyranose residues linked by β-1, 4-glycosidic bonds (and not α-1, 4 isononyls). The chains are closely attached to one another by hydrogen or van der walls type bonds thus forming compact and insoluble fibrous structures which allow the use of cellulose in industry (paper, fibers, fabrics etc.).

Structure of Cellulose

Chitin: It is a polymer found in cell walls of fungi, Chitin molecules are long, unbranched chains of N-acetyl-D-glucosamine residues linked by β, 1 → 4 glycosidic bonds. As a structural polysaccharide, chitin is found in the exoskeleton of some invertebrates e.g. crustraceans, insects.

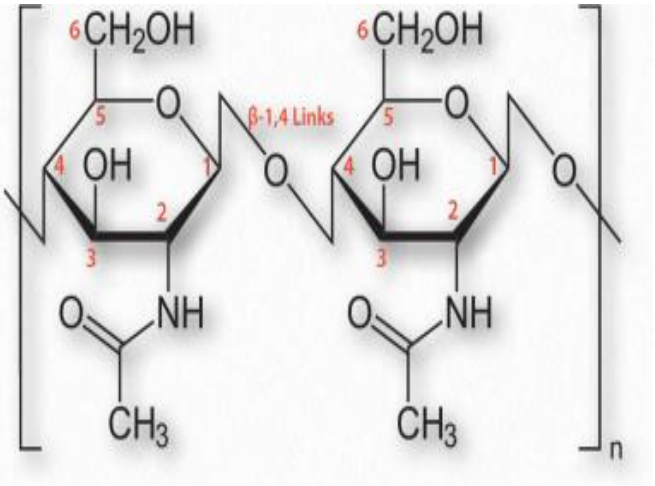


Figure 1.11 Chitin with β-1,4 links between N-acetylglucosamine sugars

Dexatrans: These polysaccharides are made up of α-glucose units linked in 1, 6, Shorter chains are grafted (by 1, 4 linkages) on this main chain. Dextrans are found in various bacteria.

Fructosans: are polymers of β-D-fructofuranose and they are of two types, inulin type I which the fructofuranose residues are linked together by β, 2 → 1 glucosidic linkages, and the levan type in which fructuofuranose units are joined by β 2 → 6 glycosidic linkages.

The inulin-type fructosans are found in the leaves, stems and roots of a number of monocotyledonous plants, of which the gramineae constitute the most important family. They function as temporary storage polysaccharides.

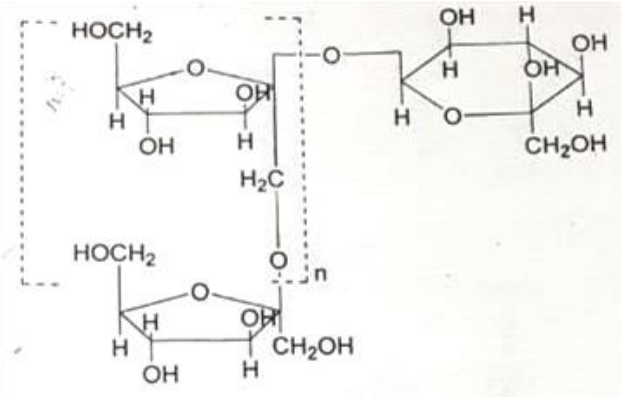


Fig1.14 Structure of Inulin

Peptins are polymers of D-galacturonic acid (α-1, 4-linkages) where some of the free carboxyl groups are, either partly or completely, esterified with methyl alcohol and others are combined with calcium or magnesium ions. Pectins occur as intercellular substances in the tissues of young plants and are particularly abundant in ripe fruits.

1.6.2. Heteropolysaccharide:

A polysaccharide that contains different types of monosaccharides is known as aeteropolysaccharide. Some of the important heteropolysaccharides are:

a) Gums: These are highly branched complex carbohydrates composed of various sugars such as arabinose, galactose, rhamnose, and mannose; however, they can also be mixed with proteins or resins, characterized by having colloidal properties. Gums are mainly extracted from plants, seeds, trees, and shrubs (e.g., Arabic, karaya, and cashew gums; however, they are also produced by bacteria. Gums are gelling, thickening, emulsifying, and stabilizing agents, with applications in the food, textile, pharmaceutical, cosmetic product, coating, encapsulant, and film industries.

b) Hemicelluloses are polysaccharides in plant cell walls that have beta-(1-->4)-linked backbones with an equatorial configuration. Hemicelluloses include xyloglucans, xylans, mannans and glucomannans, and beta-(1-->3,1-->4)-glucans. These types of hemicelluloses are present in the cell walls of all terrestrial plants, except for beta-(1-->3,1-->4)-glucans, which are restricted to Poales and a few other groups. The detailed structure of the hemicelluloses and their abundance vary widely between different species and cell types. The most important biological role of hemicelluloses is their contribution to strengthening the cell wall by interaction with cellulose and, in some walls, with lignin. These features are discussed in relation to widely accepted models of the primary wall

1.6.3 Algal Polysaccharides

Polysaccharides are the most abundant macromolecules in the structure of algae, since they are found as mucopolysaccharides, i.e., structural and energy storage molecules. Although between 4% and 76% of the dry weight of algae corresponds to polysaccharides, the content varies depending on the species of algae; for example, green algae have lignin, cellulose, and hemicellulose, brown algae have only cellulose, and red algae are made of dietary fiber. Some polysaccharides found in algae are described below.

- Agar is a thermoreversible material composed of a linear chain of galactopyranose units linked by (1–4) bonds. This gelatinous substance has gelling, thickening, texturizing, and stabilizing properties, and it is mainly used in the food industry (e.g., beverages, confectionery, dairy products, and dressings) and in bacteriological and biotechnological processes (e.g., culture media).
- Carrageenans and galactans are extracted polysaccharides from marine red algae with very similar characteristics. Galactans are composed of a chain of galactoses linked by (1–6) bonds with (1–3) branches, while carrageenans have their main chain of galactoses linked by (1–3) bonds and branches with (1–4) links. Both polysaccharides are used as gelling and thickening agents in the food industry due to their rheological properties. In addition, they are important in the medical, pharmaceutical, and cosmetic areas due to their antiviral, antitumor, and anticoagulant activity.
- Alginate is a heteropolysaccharide that is extracted mainly from brown algae, consisting of guluronic and mannuronic acids. It is characterized by its resistance and flexibility, which give it high viscosity and stability, as well as gelling properties. In addition, it is valued for its antibacterial activity, biodegradability, nontoxicity, and biocompatibility. Its industrial application is directed particularly toward the generation of particles, matrix materials, encapsulants, or biocontrol agents.

1.7 FUNCTIONS OF CARBOHYDRATES

Carbohydrates are important biopolymers and participate in a wide range of functions. They serve as a source of energy for living organisms, readily usable like glucose or functions as reserve food ex. Starch in plants, glycogen in animals.

- Glucose is a constituent of polysaccharides like starch, glycogen, cellulose and
- Disaccharides like maltose, lactose and sucrose.
- D-xylose and L-arabinose are important components of the complex polymers of cell walls.
- Erythrose is a precursor of shikimic acid and some aromatic compounds.
- D-ribose and D-deoxyribose are components of RNA and DNA respectively.
- The disaccharide, sucrose is a principal product of CO_2 fixation during the photosynthetic reactions.
- Sucrose has enormous economical value as a leading agricultural commodity and as a nutrient to most living organisms. It is used as a chemical material and as food and a sweetener.
- Sucrose provides substances for energy and synthesis of certain constituents and other storage compounds such as starch and fructans.
- Glycosides have physiologically important functions, being present in many drugs, spices and as constituents of animal tissues.
- The cardiac glycosides – digoxin and digitoxin are important in curing heart ailaments. Other glycosides include antibiotics such as streptomycin used in the treatment of tuberculosis.
- The trisaccharide raffinose occurs widely in higher plants, particularly present in leaves, stems and storage organs like seeds, roots and rhizomes.
- The fructans function as storage carbohydrates in many plants and accumulate in roots, tubers, or bulbs of plants belonging to liliaceae and compositae. Inulin occurs in tubers of chicory, dahlia bulbs, onion and garlic.
- Fructans are of great economic and nutritional value being present in large quantities in stems and leaf sheaths of pasture and fodder grasses.
- Starch is the principal reserve carbohydrate of green plants and is found in high concentration in cereals, potatoes, legumes and other vegetables.
- Cellulose is the chief constituent of the woody fibrous portion of plant material and is the most abundant of carbohydrate in nature.
- Cellulose is the main constituent of paper and cloth. It is also the basic material in the manufacture of several synthetic fibres like rayon.
- Cellulose is converted into cellulose nitrate, which is used in propellant explosives. Cellulose acetate is used in fabrics, cellulose plastics and shatter proof glasses, and carboxy methyl cellulose is added to ice creams, cosmetics and medicines to emulsify and to give smooth taste.
- Bacterial dextrans are chemically cross-linked to form gets like sephadex which are widely used in separation of various biomolecules.
- Agar is a polysaccharide present in seaweeds. It dissolves in hot water and sets to a gel on cooling, thus it is used extensively in biological laboratories as a culture medium for bacteria and fungi. Agar-agar is also used in the preparation of medicines and in cosmetics and leather industry.
- Pectins which are present in apples, lemons and other fruits function as important jelling agents.

1.8 SUMMARY

Carbohydrates may be defined as polyhydroxy alcohols with aldehydes or ketones and their derivatives. They are widely distributed both in plant and animal tissue and form source of energy for living organisms.Carbohydrates are classified into three major groups – monosaccharides, oligosaccharides and polysaccharides. The monosaccharides are subdivided into different types based on the presence of functional groups (aldoses or ketoses) and the number of carbon atoms (trioses, tetroses, pentoses, hexoses and heptoses).Glyceraldehydes is the simplest carbohydrate and it serves as a reference molecule to write the configuration (D and L forms) of all other monosaccharides. If two monosaccharides differ in their structure around a single carbon atom, they are known as epimers. Glucose and mannose are C_2 – epimers.The most abundant monosaccharide in nature is the 6-carbon sugar, D-glucose. It exists as α and β anomers with different optical rotations. Mutarotation is defined as the interconversion of α and β anomeric forms with change in the optical rotation.Disaccharides are the most common oligosaccharides, which are of two types – reducing disaccharides e.g. lactose and maltose and the non-reducing sucrose.Polysaccharides are the polymers of monosaccharides or their derivatives, linked together by glycosidic bonds. Homopolysaccharides are made up of same type of monosaccharide for e.g. starch, cellulose, inulin). Heteropolysaccharides are composed of a mixture of few monosaccharides or their derivatives (e.g., gums, Hemicellulose).Glycoproteins are carbohydrate – Protein conjugates. They perform a wide variety of functions, including their role as enzymes, hormones, structural proteins and receptors.

1.9 Self Assessment

1. Give an account of the structural configuration of monosaccharides, with special reference to glucose.
2. What are oligosaccharides? Give the structure and function of important oligosaccharides in plant.
3. Define and classify carbohydrates with suitable examples.
4. Enumerate the functions of carbohydrates.
5. Describe the structure of any two homopolysaccharides.

1.10 Suggested Readings

1. Díaz-Montes, E. Polysaccharides: Sources, Characteristics, Properties, and Their Application in Biodegradable Films. Polysaccharides 2022, 3, 480–501. https://doi.org/10.3390/ polysaccharides3030029

Prof A. Amrutha valli

LESSON 2

LIPIDS

2.0: Objectives: Understand basic concept of lipids

2.1: Introduction

2.2: Classification of lipids- Simple and compound lipids

- **2.2.1. Simple lipids**
- **2.2.2. Compound Lipids-Lecithin, Phospholipids, Glycolipids, Sulfolipids, Sterols, Terpenoids, Triglycerides**
- **2.2.3. Properties of Fatty acids**

2.3. Lipid Aggregations – Micelles, Mono layer, Bilayer, Liposomes

2.4Summary

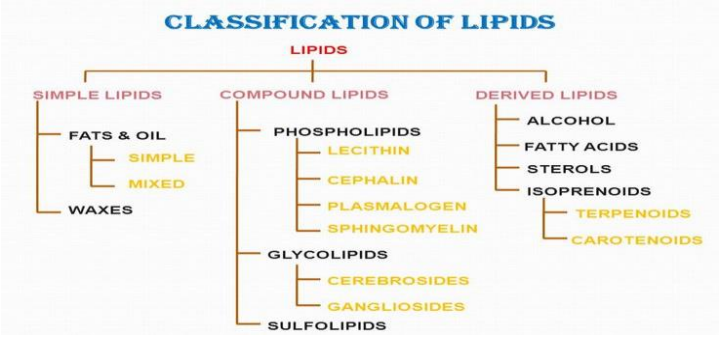
2.5Self Assessment

2.6Suggested Readings

2.1 Introduction:

The word lipid is derived from a Greek word "lipos" which means FAT. These are heterogenous group of compounds. Unlike proteins, nucleic acids, and polysaccharides, lipids are not polymers rather they are small molecules. They are the chief storage form of energy, besides their role in cellular structure and other biochemical functions. Lipids may be regarded as organic substances relatively insoluble in water, soluble in organic solvents, actually or potentially related to fatty acids and utilized by the living cells. Fat is stored in adipose tissue, where it also serves as a thermal insulator in the subcutaneous tissues. Fatty acid derivatives serve as vitamins (A, D, E&K) or hormones. It act as an energy/food reservoir (Triacylglycerol). Several proteins are covalently modified by fatty acids. Structural components of biological membranes (lipoprotein, phospholipids &sphingomyelins). Lipids act as important cellular metabolic regulators (PG's & steroid hormones) Lipids are compounds in inner mitochondrial membrane and participate in Electron transport chain.

2.2. Classification of lipids: Lipids can be classified according to their hydrolysis products and according to similarities in their molecular structures. Three major subclasses are recognized: as, Simple lipids. Complex lipids& Derived lipids



2.2.1 Simple Lipids:

Mainly of two types:
(a) **Fats and oils** which yield fatty acids and glycerol upon hydrolysis.
(b) **Waxes**, which yield fatty acids and long-chain alcohols upon hydrolysis.

Fats and oils: Both types of compounds are called triacylglycerols because they are esters composed of three fatty acids joined to glycerol, trihydroxy alcohol.
The difference is on the basis of their physical states at room temperature. It is customary to call a lipid a fat if it is solid at 25°C, and oil if it is a liquid at the same temperature.These differences in melting points reflect differences in the degree of unsaturation of the constituent fatty acids.
They are present in free state in small quantities, but involved in ester (or sometimes amide) linkages in large quantities. As a general rule, they are monocarboxylic, straight unbranched chain acids containing an even number of carbon atoms (between 4 and 36). They may be saturated or unsaturated and sometimes hydroxylated or branched.

Saturated Fatty Acids (SFA):
Saturated fatty acids are the simplest form of fats that are unbranched linear chains of CH2 groups linked together by carbon-carbon single bonds with a terminal carboxylic acid.
The term 'saturated' is used to indicate that the maximum number of hydrogen atoms are bonded to each carbon atom in a molecule of fat.The general formula for these acids is CnH2n+1COOH.
Fatty acids obtained from an animal source are mostly even-numbered linear chains of saturated fatty acids. They usually have a higher melting point and remain in the solid-state at room temperatures. They are majorly found in animal fat like butter, meat, and whole milk. But some saturated fatty acids are also found in vegetable sources like vegetable oil, coconut oil, and peanut oil.

Unsaturated Fatty Acids (UFA): Unsaturated fatty acids are more complex fatty acids with bent hydrocarbon chains linked together by one or more carbon-carbon double bonds with a terminal carboxylic acids group.The term 'unsaturated' indicates that the carbons atoms do not have the maximum possible hydrogen atoms bound to carbon atoms. Due to the presence of double bonds, the cis and trans conformation of these molecules are existed. The unsaturated fatty acids found in the human body always exist as in cis conformation

Unsaturated fatty acids have a lower melting point when compared to saturated fatty acids, andthus exist in the liquid state at room temperatures. Most vegetable oils and fish oils are some of the important sources of unsaturated fatty acids

Unsaturated fatty acids are numbered from the terminal carboxyl (carbon 1) to the CH_3 group (carbon n). The double bond is indicated by the sign Δ, accompanied by the number corresponding to the first carbon atom participating in the double bond. The sign is being increasingly used and followed by the number of double bonds; the position of the latter being indicated within brackets.
In a biochemical nomenclature carbon 1 is the terminal methyl. The place of the last double bond is indicated by ω followed by the number of atoms of the carbon existing up to this double bond.
In practically all biological unsaturated fatty acids, the double bond, has a cis isomerism.

A. Monounsaturated Fatty Acids (contain one double bond)

Oleic acid (C_{18}), the structure is CH_3-$(CH_2)_7$-CH = CH-$(CH_2)_7$-COOHand double bond is observed between carbon atoms C_9 and C_{10}, which is abbreviated as: (C_{18}, Δ^9 or 18 :1(9) or 18 ω 9).

B. Polyunsaturated Fatty Acids (PUFA) (contain more than one double bond):

In the most common of such fatty acids, the non-conjugated double bonds are separated by a methylene group. Plants can however contain fatty acids with conjugated double bonds, for example, eleostearic acid.

Linoleic acid (C_{18}, $\Delta^{9,12}$ or 18:2 (9,12) or 18 ω 6)

CH_3-$(CH_2)_4$-CH = CH-CH_2-CH = CH-$(CH_2)_7$-COOH

Linolenic acid (C_{18}, $\Delta^{9,12,15}$ or 18 : 3 (9,12,15) or 18 ω 3)

CH_3—CH_2—CH = CH—CH_2—CH = CH—CH_2—CH = CH—$(CH_2)_7$—COOH

Arachidonic acid (C_{20}, $\Delta^{5,8,11,14}$).

Docosahexaenoic acid (C_{22} $\Delta^{4,7,10,13,16,19}$).

Eleostearic acid (C_{18}, $\Delta^{9,11,3}$).

In mammals, PUFA can have up to 22 carbon atoms and 6 double bonds, but in plants, these acids do not exceed 18 carbon atoms and 4 double bonds.

Wax is an ester of long-chain alcohol (usually mono-hydroxy) and a fatty acid. The acids and alcohols normally found in waxes have chains of the order of 12-34 carbon atoms in length. They may contain monoesters, diesters and the lactones. The fatty ester class also has subclasses that include important biochemical intermediates such as fatty acyl thioester-CoA derivatives, fatty acyl thioester-acyl carrier protein (ACP) derivatives, fatty acylcarnitine (esters of carnitine), and fatty adenylates, which are mixed anhydrides.

2.2.2. Compound Lipids

Complex lipids are esters of fatty acids with alcohols and contain additional groups. These additional components could be phosphate, nitrogenous bases, carbohydrates, proteins, among others.

Complex lipids are subdivided as:

a. *Lecithins*:Lecithins are mixtures of glycerophospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidic acid.

b. *Phospholipids:* These lipids are characterized by the presence of phosphoric acid, often accompanied by a nitrogenous base, in addition to the usual alcohol and fatty acids.

Lecithin

Lecithin was first isolated in 1845 by the French chemist and pharmacist Théodore Gobley. In 1850, he named the phosphatidylcholine *lécithine*.Gobley originally isolated lecithin from egg yolk and established the complete chemical formula of phosphatidylcholine in 1874 and demonstrated the presence of lecithin in a variety of biological materials, including venous blood, human lungs, bile, roe, and brains of humans, sheep and chicken.

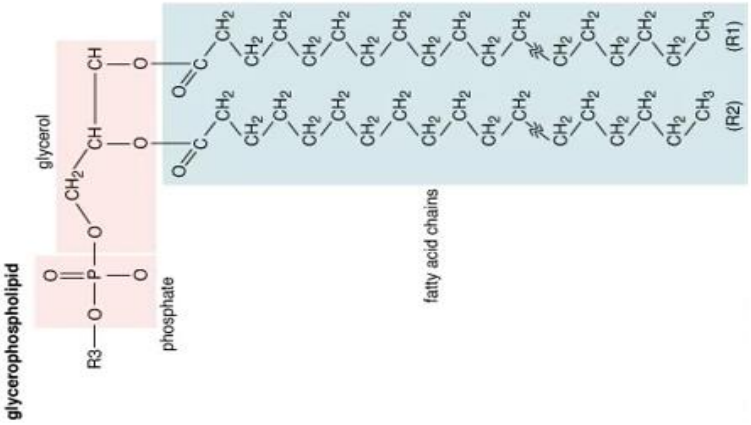
In the pharmaceutical industry, it acts as a wetting agent, stabilizing agent and a choline enrichment carrier, helps in emulsification and encapsulation, and is a good dispersing agent. It

can be used in manufacture of intravenous fat infusions and for therapeutic use.In the paint industry, it forms protective coatings for surfaces with painting and printing ink, helps as a rust inhibitor, is a colour intensifying agent, catalyst, conditioning aid modifier, and dispersing aid; it is a good stabilizing and suspending agent, emulsifier, and wetting agent, helps in maintaining uniform mixture of several pigments, helps in grinding of metal oxide pigments, helps as a spreading and mixing aid, prevents hard settling of pigments, eliminates foam in water-based paints, and helps in fast dispersion of latex-based paints.
Lecithin also may be used as a release agent for plastics, an anti-sludge additive in motor lubricants, an anti-gumming agent in gasoline, and an emulsifier, spreading agent, and antioxidant in textile, rubber, and other industries.

Phospholipids: Phosphate containing several lipids are listed as,

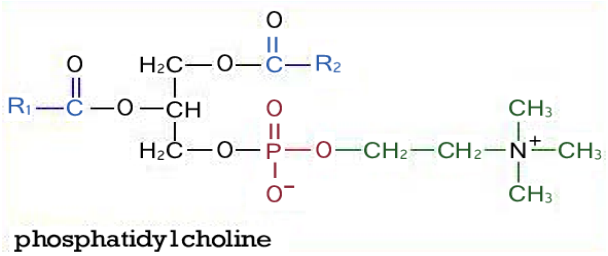
a) Glycerophospholipids

Glycerophospholipids or phosphoglycerides are glycerol-based phospholipids. They are the main component of biological membranes in eukaryotic cells. Two major classes are known from bacteria and eukaryotes and a separate family for archaea.



b) Phosphatidylcholine

Phosphatidylcholines (PC) are a class of phospholipids that play essential role in biological membranes and lipid metabolism. Structurally, they consist of a glycerol backbone linked to two fatty acid chains and a phosphate group with a choline molecule attached. Phosphatidylcholines are major constituents of cell membranes and contribute to membrane integrity, fluidity, and permeability. They act as surfactants in the lungs, facilitating the expansion and contraction of alveoli during respiration. Additionally, phosphatidylcholines are involved in intracellular signaling pathways, lipid transport, and cell-cell interactions. They also serve as precursors for the synthesis of other important molecules, such as platelet-activating factor (PAF) and sphingomyelin.



Phosphatidylcholines are abundant in dietary sources such as egg yolks, soybeans, and peanuts, and they are also synthesized endogenously by the body through the Kennedy pathway. Dysregulation of phosphatidylcholine metabolism has been implicated in various diseases, including liver disorders, cardiovascular diseases, and cancer. As a result, phosphatidylcholines have garnered significant attention as potential targets for therapeutic interventions and biomarkers for disease diagnosis and prognosis.

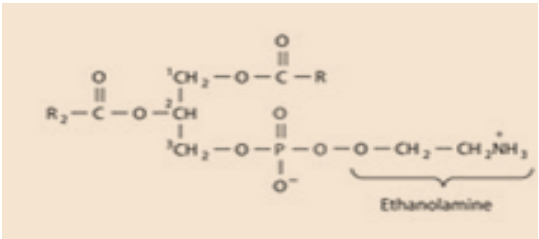
a) ***Phosphatidylethanolamine***

Phosphatidylethanolamine (PE) is a type of phospholipid found in biological membranes and plays crucial roles in cellular structure, function, and signaling. Structurally, phosphatidylethanolamines consist of a glycerol backbone linked to two fatty acid chains and a phosphate group with an ethanolamine molecule attached.

Phosphatidylethanolamines are integral components of cell membranes and contribute to membrane fluidity, integrity, and permeability. They also play a key role in membrane curvature and membrane fusion events, such as exocytosis and endocytosis. Additionally, phosphatidylethanolamines are involved in various cellular processes, including lipid metabolism, vesicular trafficking, and protein trafficking.

Phosphatidylethanolamines are synthesized via the Kennedy pathway, a series of enzymatic reactions that occur in the endoplasmic reticulum and mitochondria-associated membranes. They can also be generated through the decarboxylation of phosphatidylserine by phosphatidylserine decarboxylase enzymes.

In addition to their structural and functional roles, phosphatidylethanolamines have been implicated in several physiological processes and diseases. They are involved in the regulation of membrane proteins and enzymes, as well as in cell signaling pathways, including apoptosis and autophagy. Dysregulation of phosphatidylethanolamine metabolism has been associated with various human diseases, including neurodegenerative disorders, metabolic syndrome, and cancer. Overall, phosphatidylethanolamines are essential constituents of cell membranes and play diverse roles in cellular physiology, making them important targets for further research into their functions and potential therapeutic applications.



c) Phosphatidylinositol

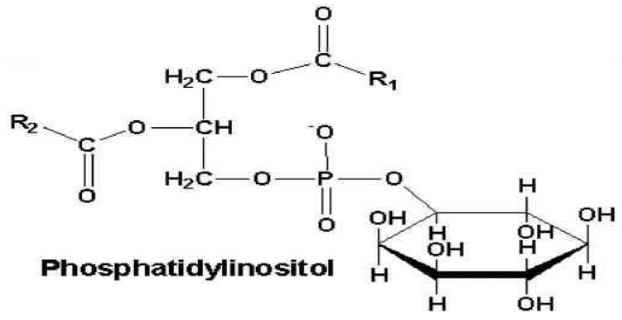
Phosphatidylinositol (PI) is a phospholipid found in cell membranes, where it serves as a precursor for important signaling molecules involved in various cellular processes. Structurally, phosphatidylinositol consists of a glycerol backbone linked to two fatty acid chains and a phosphate group, with an inositol molecule attached to the phosphate group.

One of the key functions of phosphatidylinositol is its role as a precursor for phosphoinositide, which are phosphorylated derivatives of phosphatidylinositol. Phosphoinositide play critical role in cellular signaling, including the regulation of membrane trafficking, cytoskeletal dynamics, and cell proliferation and survival. They act as second messengers in response to extracellular signals, such as hormones and growth factors, and help relay these signals to intracellular effectors.

Phosphatidylinositol is synthesized through the action of phosphatidylinositol synthase enzymes, which catalyze the transfer of inositol from phosphatidylinositol monophosphate to diacylglycerol. Once synthesized, phosphatidylinositol can be further modified by specific kinases and phosphatases to generate different phosphoinositide species, each with distinct cellular functions.

In addition to its role in phosphoinositide signaling, phosphatidylinositol is also involved in other cellular processes. It contributes to the structural integrity and fluidity of cell membranes and serves as a substrate for the synthesis of other important lipid molecules, such as phosphatidylinositol phosphate and phosphatidylinositol bisphosphate.

Dysregulation of phosphatidylinositol metabolism has been implicated in various human diseases, including cancer, neurodegenerative disorders, and metabolic disorders. Abnormalities in phosphoinositide signaling pathways can disrupt cellular homeostasis and contribute to disease pathogenesis.



d) Phosphatidylserine

Phosphatidylserine (PS) is a type of phospholipid that is present in cell membranes, particularly in the inner leaflet. Structurally, phosphatidylserine consists of a glycerol backbone linked to two fatty acid chains and a phosphate group, with serine attached to the phosphate group.

Phosphatidylserine plays several important roles in cellular functions. Firstly, it contributes to the structural integrity and fluidity of cell membranes, and helps to maintain membrane stability and facilitates the movement of proteins and other molecules across the membrane. Additionally,

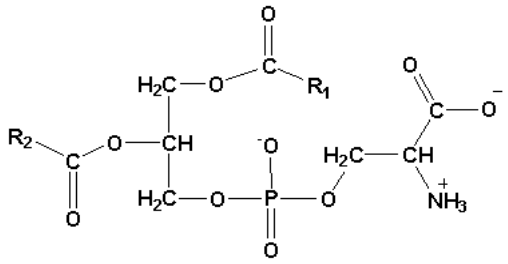
phosphatidylserine is involved in cell signaling and communication. It acts as a signaling molecule itself and is also a precursor for the synthesis of other signaling molecules.

One of the notable functions of phosphatidylserine is its involvement in cell signaling pathways related to apoptosis, or programmed cell death. During apoptosis, phosphatidylserine is translocated from the inner to the outer leaflet of the cell membrane, where it serves as an "eat-me" signal for phagocytic cells, triggering the clearance of apoptotic cells without inducing an inflammatory response.

Phosphatidylserine is also known to interact with various proteins and enzymes involved in cellular signaling pathways, such as protein kinase C (PKC) and Raf-1 kinase, modulating their activity and function. These interactions contribute to the regulation of processes such as cell proliferation, differentiation, and survival.

In addition to its roles in cell signaling and apoptosis, phosphatidylserine has been implicated in various physiological processes, including blood clotting, neurotransmission, and synaptic function in the nervous system. It also found in high concentrations in the brain, where it is thought to play a role in cognitive function and memory.

Further, phosphatidylserine has gained attention for its potential health benefits, particularly in the form of dietary supplements. Some studies suggest that phosphatidylserine supplementation may support cognitive function, reduce the risk of age-related cognitive decline, and improve mood and stress response.



Phosphatidylserine

a) *Phosphatidic acid*

Phosphatidic acid (PA) is a phospholipid that serves as a key intermediate in lipid metabolism and cell signaling pathways. Structurally, phosphatidic acid consists of a glycerol backbone linked to two fatty acid chains and a phosphate group, with one of the hydroxyl groups of glycerol esterified with phosphoric acid.

Phosphatidic acid plays diverse roles in cellular function. One of its primary functions is as a precursor for the synthesis of other phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, through the Kennedy pathway. This pathway involves the stepwise addition of head groups to the phosphatidic acid backbone, resulting in the formation of various phospholipid species essential for membrane structure and function.

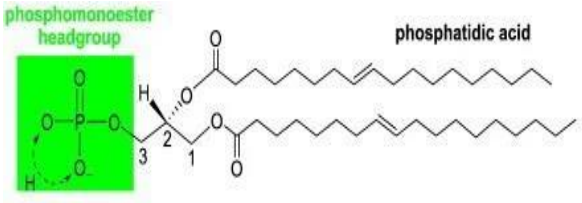
In addition to its role in lipid biosynthesis, phosphatidic acid serves as a signaling molecule involved in the regulation of numerous cellular processes. It acts as a second messenger in intracellular signaling pathways, where it interacts with various proteins and enzymes to modulate their activity and function.

One of the well-characterized functions of phosphatidic acid is its involvement in the regulation of protein kinases, particularly members of the AGC kinase family, such as protein kinase D

(PKD) and mammalian target of rapamycin complex 1 (mTORC1). Phosphatidic acid binds to these kinases and promotes their activation, leading to downstream signaling events that regulate cell growth, proliferation, and survival.

Phosphatidic acid also plays a role in membrane trafficking and vesicle formation. It has been implicated in the regulation of membrane curvature and fusion events during vesicular transport processes, such as endocytosis, exocytosis, and intracellular vesicle trafficking.

Furthermore, phosphatidic acid has been linked to cellular stress responses, including responses to nutrient deprivation, oxidative stress, and heat shock. It modulates the activity of stress-responsive proteins and transcription factors, contributing to the adaptive cellular response to environmental stimuli.

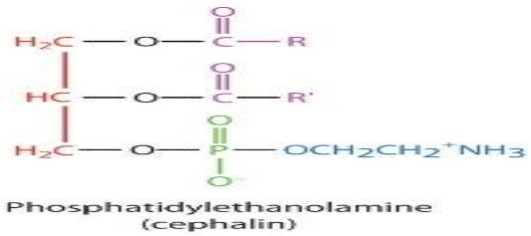


b) *Cephalin*

Cephalin is a term historically used to describe a group of phospholipids found primarily in the brain and nervous tissue. It encompasses various phospholipids, including phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. These phospholipids play crucial roles in the structure and function of cell membranes, particularly in neuronal cells.

Structurally, cephalin molecules consist of a glycerol backbone linked to two fatty acid chains and a phosphate group. One of the hydroxyl groups of glycerol is esterified with phosphoric acid, while the other is esterified with either ethanolamine (in the case of phosphatidylethanolamine), serine (in the case of phosphatidylserine), or choline (in the case of phosphatidylcholine). This composition contributes to the amphipathic nature of cephalin molecules, allowing them to form lipid bilayers that constitute cell membranes.

Cephalin phospholipids are involved in various cellular processes, including cell signaling, membrane trafficking, and neurotransmitter release. They also serve as precursors for the synthesis of other bioactive molecules and play important roles in maintaining membrane integrity and fluidity.



c) *Plasmalogens*

Plasmalogens are a subclass of glycerophospholipids, which are a type of phospholipid found in cell membranes. They are characterized by the presence of a vinyl ether bond at the sn-1 position of the glycerol backbone, instead of the typical ester bond found in other phospholipids. This unique structure gives plasmalogens distinct biochemical properties and functional roles in cellular processes.

Structurally, plasmalogens consist of a glycerol backbone linked to two fatty acid chains and a phosphate group. The vinyl ether bond at the sn-1 position is typically attached to a long-chain fatty alcohol, such as ethanolamine or choline. This structure confers unique physical properties to plasmalogens, including increased membrane fluidity and resistance to oxidative stress.

Plasmalogens are abundant in various tissues, particularly in the brain, heart, and skeletal muscles. They are highly enriched in the lipid rafts of cell membranes, where they play important roles in membrane organization, signaling, and protein trafficking. Additionally, plasmalogens are involved in cellular processes such as membrane fusion, vesicle trafficking, and neurotransmitter release.

Beyond their structural roles, plasmalogens also serve as a reservoir for bioactive lipid mediators and precursors for the synthesis of signaling molecules such as platelet-activating factor (PAF) and eicosanoids. These lipid mediators play critical roles in inflammation, immune response, and cardiovascular health.

Research has shown that alterations in plasmalogen levels are associated with various diseases, including neurodegenerative disorders, cardiovascular diseases, and metabolic syndromes. Decreased plasmalogen levels have been implicated in the pathogenesis of conditions such as Alzheimer's disease, Parkinson's disease, and cardiovascular diseases.

d) *Sphingomyelin*

phingomyelin is a type of sphingolipid, a class of lipids found in cell membranes. Structurally, sphingomyelin consists of a sphingosine backbone, a fatty acid chain, a phosphate group, and a choline or ethanolamine head group. It is synthesized through the condensation of serine and palmitoyl-CoA, followed by a series of enzymatic modifications.

Sphingomyelin is primarily found in the plasma membrane of cells, where it contributes to the structural integrity and fluidity of the membrane. It is particularly abundant in the myelin sheath that surrounds nerve fibers, where it plays a crucial role in insulating and protecting nerve cells.

In addition to its structural role, sphingomyelin is involved in various cellular processes, including cell signaling, membrane trafficking, and lipid metabolism.

One of the unique features of sphingomyelin is its ability to act as a substrate for sphingomyelinases, enzymes that hydrolyze sphingomyelin to produce ceramide and phosphocholine or phosphoethanolamine. This enzymatic reaction generates ceramide, a bioactive lipid that serves as a second messenger in signaling pathways regulating cell proliferation, differentiation, and apoptosis.

Sphingomyelin metabolism is tightly regulated, and dysregulation of sphingomyelin metabolism has been implicated in the pathogenesis of various diseases. For example, deficiencies in enzymes involved in sphingomyelin metabolism are associated with lysosomal storage disorders such as Niemann-Pick disease. Additionally, alterations in sphingomyelin levels have been observed in neurodegenerative diseases, cardiovascular diseases, and cancer.

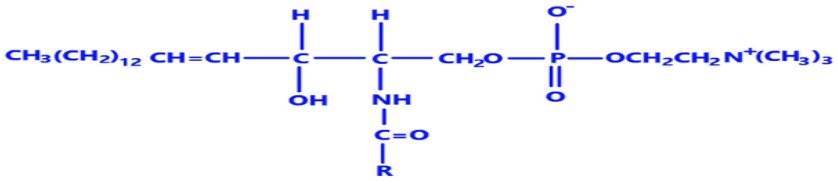


Fig: Sphingomyelin's structure

Glycolipids

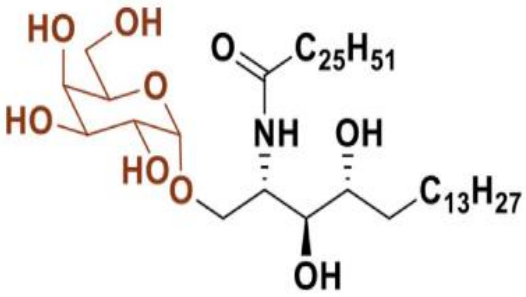
Glycolipids are a class of lipids that contain a carbohydrate moiety attached to a lipid molecule. They are important components of cell membranes and play crucial roles in cell-cell recognition, signaling, and immunity. Two major subclasses of glycolipids are cerebrosides and gangliosides.

a. *Cerebrosides:*

Cerebrosides are glycosphingolipids composed of a single sugar molecule (monosaccharide) attached to a ceramide backbone. The ceramide portion consists of a sphingosine or dihydrosphingosine backbone linked to a fatty acid chain. The sugar moiety can vary, with common examples including glucose and galactose. Cerebrosides are primarily found in the plasma membrane of cells, especially in the nervous system, where they play essential roles in cell-cell recognition, adhesion, and signaling. They are particularly abundant in the myelin sheath that surrounds nerve fibers, contributing to the insulation and integrity of nerve cells.

b. *Gangliosides:*

Gangliosides are more complex glycosphingolipids that contain a sialic acid (N-acetylneuraminic acid) residue in addition to the sugar and ceramide components. They are highly enriched in the outer leaflet of the plasma membrane, particularly in nerve cells and neuronal tissues. Gangliosides are crucial for the proper functioning of the nervous system, where they participate in cell signaling, neuronal development, and synaptic transmission. Additionally, gangliosides play roles in cell adhesion, immune response modulation, and regulation of cell growth and proliferation. Due to their diverse functions, alterations in ganglioside metabolism have been implicated in various neurological disorders, including neurodegenerative diseases and developmental disorders.



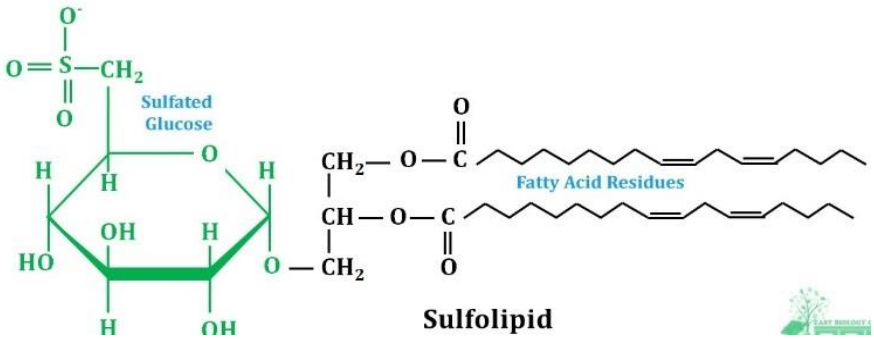
Sulfolipids

Sulfolipids are a class of lipids that contain a sulfur atom in their structure, distinguishing them from other types of lipids. They are primarily found in certain types of bacteria, algae, and plant tissues, where they play an important rolein membrane structure and function.

The structure of sulfolipids typically consists of a hydrophobic lipid tail, often derived from fatty acids, and a hydrophilic head group containing a sulfur atom bonded to a sugar molecule. The sulfur atom is usually in the form of a sulfonic acid or sulfate group. The sugar moiety can vary, with common examples including glucose, galactose, or other monosaccharides.

Sulfolipids are known to contribute to the stability and integrity of cellular membranes, similar to other membrane lipids such as phospholipids. They may also participate in cell-cell recognition, adhesion, and signaling processes. In photosynthetic organisms like algae and plants, sulfolipids are often associated with chloroplast membranes and are involved in photosynthesis-related functions.

One of the most well-studied sulfolipids is sulfoquinovosyl diacylglycerol (SQDG), which is found in the thylakoid membranes of chloroplasts in photosynthetic organisms. SQDG is important for the structure and function of the photosynthetic apparatus, and it has been implicated in the regulation of photosynthetic processes, including light harvesting and electron transport.

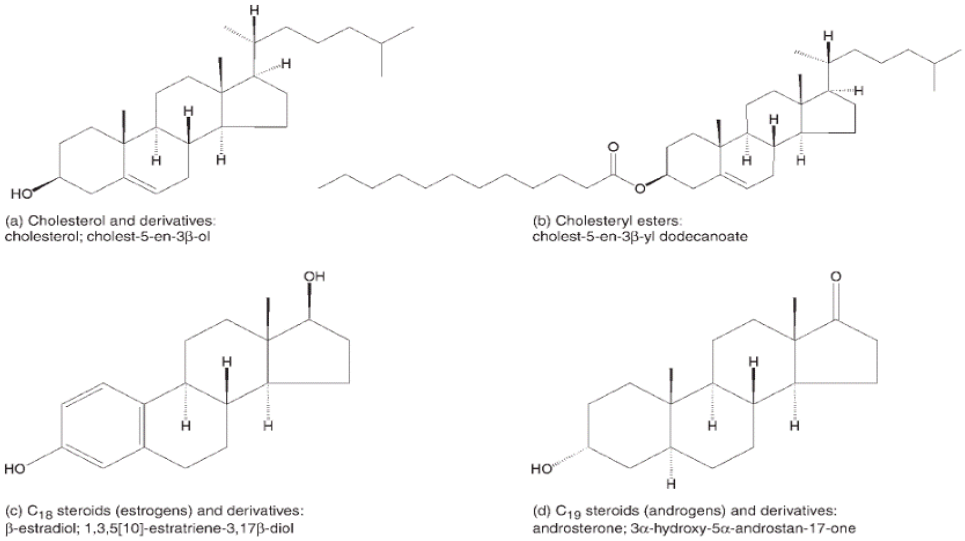


Sterols

This is a very important group of lipids found in practically all eucaryotes. More than hundred sterols are known of which cholesterol is the principal sterol of vertebrates, ergosterol which is a natural precursor of vitamin D,stigmasterol, sitosterol, important sterols in plants.

By esterification of the alcoholic group by fatty acids, sterols give sterides. In general, in normal physiological conditions, the quantity of steridesin a given tissue is very small compared to free sterols (blood is an exception to this rule). Sterides exist only as traces in biological membranes. Their accumulation in the latter is pathological (atheroma). Plants contain an appreciable part of their sterols conjugated with a saccharide like glucose (sterylglucosides). The saccharide is linked by its reducing group to the alcohol group in position 3 of the sterol.

There are many examples of unique sterols from plant, fungal, and marine sources that are designated as distinct subclasses in this schema (Table 8). The steroids, which also contain the same fused four ring core structure, have different biological roles as hormones and signaling molecules. They are subdivided on the basis of the number of carbons in the core skeleton. The C18 steroids include the estrogen family, whereas the C19 steroids comprise the androgens such as testosterone and androsterone. The C21 subclass, containing a two carbon side chain at the C17 position, includes the progestogens as well as the glucocorticoids and mineralo corticoids. The secosteroids, comprising various forms of vitamin D, are characterized by cleavage of the B ring of the core structure, hence the “seco” prefix (32). Additional classes within the sterols category are the bile acids (33), which in mammals are primarily derivatives of cholan 24-oic acid synthesized from cholesterol in the liver and their conjugates (sulfuric acid, taurine, glycine, glucuronic acid, and others). Sterol lipid structures are shown in Fig. 6



(a) Cholesterol and derivatives: cholesterol; cholest-5-en-3β-ol

(b) Cholesteryl esters: cholest-5-en-3β-yl dodecanoate

(c) C_{18} steroids (estrogens) and derivatives: β-estradiol; 1,3,5[10]-estratriene-3,17β-diol

(d) C_{19} steroids (androgens) and derivatives: androsterone; 3α-hydroxy-5α-androstan-17-one

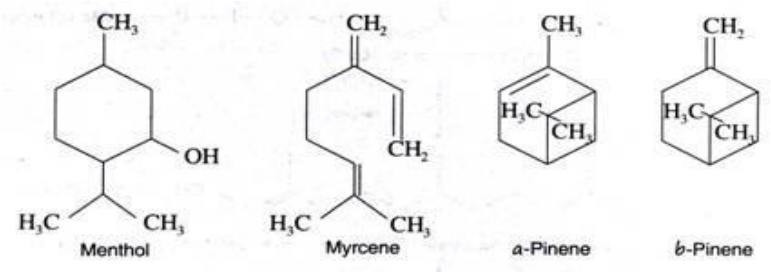
Isoprenoids

Isoprenoid, any of a class of organic compounds composed of two or more units of hydrocarbons, with each unit consisting of five carbon atoms arranged in a specific pattern. Isoprenoids play widely varying roles in the physiological processes of plants and animals. They also have a number of commercial uses.

Isoprenoids in living organisms range in function from pigments and fragrances to vitamins and precursors of sex hormones. One of the most familiar natural substances, rubber, is a polyisoprene. Other commercially valuable isoprenoids are those used as flavourings, solvents, and raw materials for chemicals.

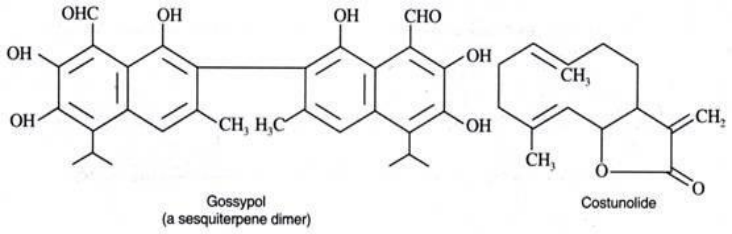
The five-carbon unit that constitutes the basic building block of isoprenoids is a hydrocarbon called isoprene. Isoprene (2-methyl-1,3-butadiene) is a branched-chain unsaturated hydrocarbon, unsaturated meaning it contains one or more double bonds between carbon atoms. Isoprene has in fact two carbon-carbon double bonds. Isoprenoids contain from two to many thousands of isoprene units. The carbon backbone of an isoprenoid can have one or more functional chemical groups, such as hydroxyl and carbonyl, attached to it. These contribute to the diversity of isoprenoids.

The name *terpene* specifically refers to naturally occurring compounds that are derivatives of a single isoprene unit. The smallest terpene molecules, those containing 10 carbon atomsare called monoterpenes. The larger molecules, increased by one isoprene unit at a time, are called sesquiterpenes, diterpenes, triterpenes, and tetraterpenes, respectively. The monoterpenes are mostly volatile, which accounts for their fragrances. Terpenes of higher molecular weight are less volatile, although sesquiterpenes contribute to the flavours of some foods.
Menthol is chief monoterpene component of peppermint oil. Essential oils are obtained from plants by steam distillation and find their use in flavouring foods and in perfumery. Structures of some common monoterpenes are given as follows



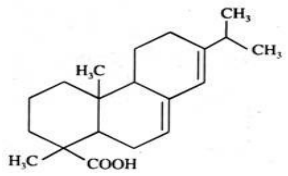
Structure of Monoterpenes

Monoterpene esters pyrethroids occurring in leaves and flowers of Chrysanthemum possess strong insecticidal properties and are used on commercial scale in making insecticides.
Sesquiterpenes (C_{15}) are largest group of isoprenoids which have great structural variations. Many sesquiterpenes co-exist with monoterpenes in essential oils in higher plants. Some sesquiterpene lactones such as costunolide found in the glandular hairs of sage brush and sunflower are feeding deterrents to herbivores. An aromatic sesquiterpene dimer gossypol found in cotton is known to provide resistance to insect, fungal and bacterial pathogens.



Structure of sesquiterpenes

Diterpenes (C_{20}) are plant resins produced by conifers such as pines and certain leguminous trees such as Hymenaeacourbrail, contain appreciable amount of diterpene abietic acid. These diterpenes function as chemical deterrents to predators and also help in healing the wounds caused by insect bites.



Abietic acid (diterpene)

Triterpenes (C_{30}) and their derivatives such as steroids represent another vast group of isoprenoids or terpenoid compounds. The steroids usually have a tetracyclic or pentacyclic molecular structure and many of them are modified to contain fewer than 30 – C atoms.

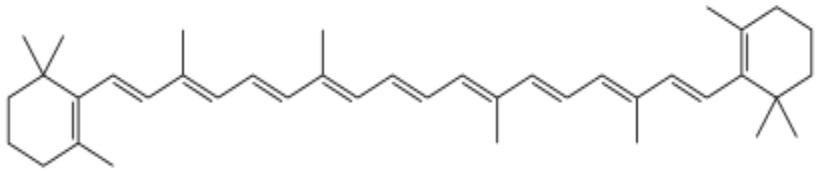
Some steroids such as plant sterols (e.g., sitosterol) have primary function in plant cells being part of their cell-membranes while others are defensive secondary products. Examples of the latter category are various Phyto ecdysones, limonoids, cardenolides, sapogenins, sterol alkaloids and steroid hormones.

Polyterpenes $[C_5]_n$ are high molecular weight components found in plants as natural products. Of these, rubber is best known. Other examples are gutta and chicle. Their function in plants is to provide defence against herbivores and to help in wound healing.

Carotenoids

Carotenoids are yellow, orange, and red organic pigments that are produced by plants and algae, as well as several bacteria, archaea, and fungi. Carotenoids give the characteristic color to pumpkins, carrots, parsnips, corn, tomatoes, canaries, flamingos, salmon, lobster, shrimp, and daffodils. Over 1,100 identified carotenoids can be further categorized into two classes – xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons and contain no oxygen).Carotenoids serve two key roles in plants and algae by absorbing light energy for photosynthesis, and provide photoprotection via non-photochemical quenching.Carotenoids that contain unsubstituted beta-ionone rings (including β-carotene, α-carotene, β-cryptoxanthin, and γ-carotene) have vitamin A activity by converting in to retinol.

Carotenoids are tetraterpenoids, meaning they are composed of eight isoprene units. They consist of a series of conjugated double bonds, which give them their characteristic color and enable them to absorb light energy across a broad range of wavelengths. Carotenoids can be classified into two main groups based on their chemical structure: carotenes, which are purely hydrocarbons, and xanthophylls, which contain oxygen atoms in addition to carbon and hydrogen.



Structure of beta carotene

Carotenoids play crucial roles in capturing light energy and transferring it to chlorophyll molecules for photosynthesis. They absorb excess light energy and protect chlorophyll from damage caused by reactive oxygen species produced during photosynthesis. Additionally, carotenoids participate in photoprotection by dissipating excess energy as heat and scavenging harmful free radicals.

Carotenoids are essential nutrients in the human diet, as they cannot be synthesized by the body and must be obtained from food sources. Certain carotenoids, such as beta-carotene, alpha-carotene, lutein, zeaxanthin, and lycopene, have been associated with various health benefits, including reduced risk of chronic diseases such as cardiovascular disease, age-related macular degeneration, and certain types of cancer. Carotenoids also contribute to skin health and may enhance immune function.

Carotenoids are abundant in a wide range of fruits and vegetables, particularly those with red, orange, yellow, and dark green colors. Some common food sources of carotenoids include carrots, sweet potatoes, spinach, kale, tomatoes, peppers, mangoes, papayas, and citrus fruits.

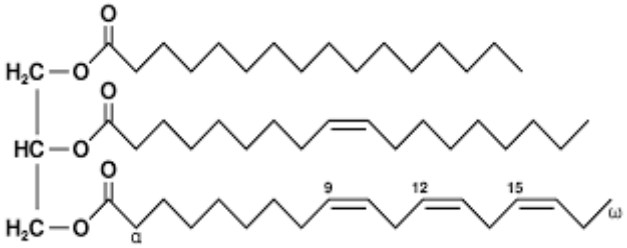
Triglycerides

Triglycerides are esters in which three molecules of one or more different fatty acids are linked to the alcohol glycerol; they are named after the fatty acid components; for example, tristearin contains three molecules of stearic acid, while oleo distearin contains one molecule of oleic acid and two molecules of stearic acid.

Three alcohol groups can react with three long chain carboxylic acids, commonly referred to as fatty acids. Monoglycerides or monoacylglycerols are generated when only one ester bond is created. A diglyceride or diacyl glycerol is formed when two carboxylic acid molecules react to generate two ester bonds. A triglyceride or triacylglycerol is formed when three carboxylic acids react to generate three ester bonds.

Triglyceride Structure

Fatty acids can be metabolised for energy by tissues or stored as energy in the form of triglycerides. The stored triglycerides are digested in response to energy demands, and the unsaturated fatty acids are released into the circulatory system and delivered to the tissues.



2.2.3 Properties of Fatty Acids:

Physical Properties

- Fatty acids are soluble in organic solvents such as benzene, chloroform, and alcohol
- Solubility is inversely proportional to carbon chain length, increasing as the carbon chain length decreases and decreasing as the carbon chain length increases
- Fatty acids are insoluble in water
- They are bad conductors of heat
- Long chain saturated fatty acids are solid at room temperature, while long chain unsaturated fatty acids are liquid at room temperature
- Unsaturated fatty acids show cis-trans isomerism due to presence of double bonds
- Boiling points of saturated fatty acids keeps rising with increasing chain length

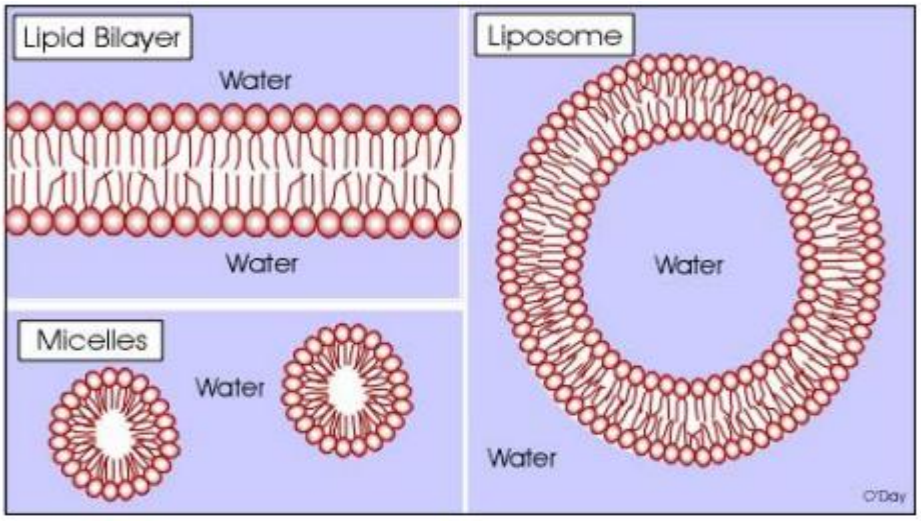
Chemical Properties

- **Hydrolysis**: Hydrolysis by alkalis such as NaOH or KOH generates sodium or potassium salts of fatty acids
- **Hydrogenation**: Oils containing unsaturated fatty acids undergo hydrogenation when exposed to high temperature, pressure, and finely divided nickel. This process converts oils into solid fats.

- **Hydrogenolysis**: Hydrogenolysis involves the splitting of fat by hydrogen. In this process, hydrogen is passed through fat under pressure in the presence of copper-chromium catalyst.
- **Halogenation**: Halogenation involves treating unsaturated fatty acids with halogens such as chlorine and iodine. During this process, the unsaturated fatty acid takes up iodine or other halogens at their double bond site, which is an indication of its unsaturated state.
- **Rancidity**: When fats that are stored for long periods of time come in contact with light, moisture, heat, and air, they undergo chemical changes that result in them developing an unpleasant rancid odor.
- **Emulsification**: Emulsifying agents such as bile juice secreted by the liver, water, proteins, soaps, and gums, break down large-sized fat molecules into smaller molecules in a process known as emulsification.

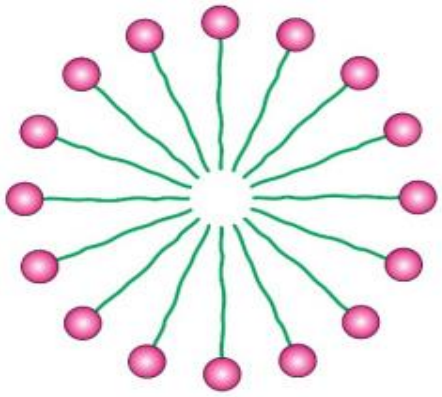
2.3 Lipid Aggregations – Micelles, Mono layer, Bilayer, Liposomes

Lipids are amphipathic molecules i.e. they have both hydrophilic and hydrophobic parts. Such molecules are also called amphiphiles. They rarely exist as monomers. Hence, they spontaneously form various types of aggregate structures in aqueous environments. Some of these structures are monolayers, micelles, reverse micelles, liposomes, and bilayers. These lipid aggregates are biologically significant as they form the structural basis for biological membranes. Biological membranes define the cellular boundaries and allow cells to exist as separate entities distinct from their environments. Membranes also enclose most of the subcellular organelles. Biomembranes not only form the cellular compartments but also mediate transport of essential nutrients and metabolites into or out of these compartments. The various types of lipid aggregates are shown in the figure that follows.



Micelles

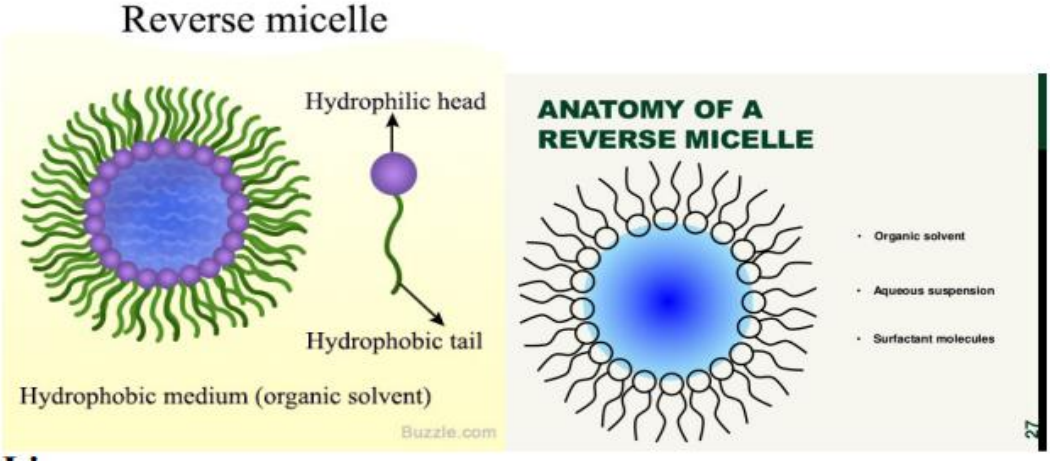
A micelle is an aggregate of surfactant molecules (amphiphilic molecules) dispersed in a liquid colloid. In aqueous environments, lipids form spherical structures in which the hydrophobic tails are buried in the centre of the structure and hydrophilic head groups are sticking out at the surface. This arrangement eliminates unfavorable contacts between water and the hydrophobic tails but permits the solvation of the polar head groups.



Micelle formation is a cooperative process; an assembly of just a few amphiphilic molecules cannot protect its tails from water. So, dilute aqueous solutions of amphiphiles do not form micelles until their concentration exceeds a certain critical micelle concentration (CMC). The value of the CMC depends on the identity of the amhiphile and the solution conditions. Micelles may be categorized into various types, based on their shapes: ¬ Spherical micelles- Ellipsoids¬ Cylinders ¬ Reverse micelles ¬ planar structures –Unilamellar vesicles Single-tailed lipids tend to form micelles. At the air-water interface, the hydrophobic tails of a lipid monolayer avoid association with water by extending into the air.

Reverse micelles

Reverse micelles form when amphiphilic molecules aggregate in nonpolar solvents instead of water. In these aggregates, the hydrophilic heads are buried in the core of the micelle and the hydrophobic tails are on the outside interacting with the solvent. Hence, they are also known as 'inside out' vesicles.

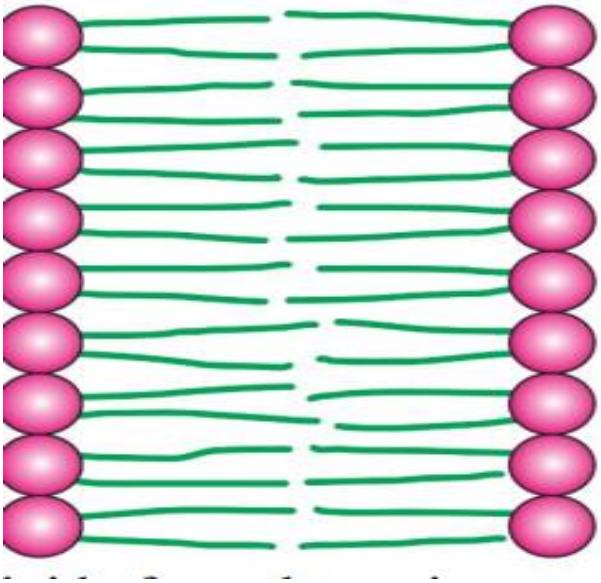


Liposomes

Liposomes are spherical vesicles made of lipid bilayers; they may be used as vehicles for delivery of drugs and other therapeutic agents into the human body.

Lipid bilayers

Double tailed lipids e.g. phospholipids and sphingolipids usually form bilayers. A bilayer has a hydrophobic core and the hydrophilic heads stick out at the surfaces. The bilayer may be divided into outer and inner leaflets.
The schematic figure of a lipid bilayer is shown next.



2.4 Summary

Lipids are regarded as organic substances relatively insoluble in water, soluble in organic solvents. Lipids are chief storage form of energy play a major role in cellular structure and other biochemical functions.

Lipids are classified into Simple lipids, Complex lipids & Derived lipids depending on their hydrolysis products .Simple lipids include, 1) **Fats and oils**, which yield fatty acids and glycerol upon hydrolysis and 2) **Waxes**, which yield fatty acids and long-chain alcohols upon hydrolysis .Complex lipids are esters of fatty acids with alcohols and associated with additional groups like phosphate, nitrogenous bases, carbohydrates, proteins .Complex lipids are subdivided into 1) Lecithins, mixtures of glycerol phospholipids including phosphatidyl choline, phosphatidyl ethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidic acid. 2) Phospholipids, lipids with phosphoric acid, often accompanied by a nitrogenous base, in addition to the usual alcohol and fatty acids. Glycolipids contain a carbohydrate moiety attached to a lipid molecule. They are important components of cell membranes and play a crucial roles in cell-cell recognition, signaling, and immunity. Two major subclasses of glycolipids are cerebrosides and gangliosides. Fatty acid synthesis starts with acetyl-CoA production in the mitochondria. It can occur either through glucose metabolism through glycolysis in the cytoplasm or β-oxidation of fatty acids in the mitochondria. Four steps in the synthesis includes, 1. Synthesis of Acetyl-CoA, 2. Formation of Malonyl-CoA, 3. Fatty Acid Chain Elongation and 4. Termination

2.5 SELF ASSESSMENT:

1) What are lipids and write about the classification of lipids?
2) What are Triglycerols and properties of fatty acids?
3) How do the lipids play an important role in biological system?

2.6 Suggested Readings:

1) Leininger's Principles of Biochemistry (2000) by Nelson, David Land Cox, MM, Macmillan/worth, NY.
2) Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte W Pratt, John Wiley & Sons, NY.
3) Biochemistry IIIrd (1994) by Lubert Stryer, WH Freeman and Co., San Francisco.
Outlines of Biochemistry (1987) by Eric E Conn, PK Stumpf, G Bruening and Ray H Doi, John Wiley & Sons, NY.

Prof A. Amrutha Valli

Lesson 3

NUCLEIC ACIDS

Objectives: understand the detailed structure of DNA and RNA and their types

3.1 Introduction

Nucleic acids include DNA and RNA. Deoxyribonucleic acid (DNA) is the heredity material found in humans and all living organisms. It is a double-stranded molecule and has a unique twisted helical structure.RNA is a single stranded mixed polymer of four types of ribotides linked together by 3′, 5 – phosphodiester bonds. They are adenylate (AMP), guanylate (GMP), cytidylate (CMP) and uridylate (UMP). The difference in the composition of bases in DNA and RNA is that in DNA guanine, cytosine, adenine, thymine are present, but in RNA in the place of thymine, Uracil is present and the remaining all are same.

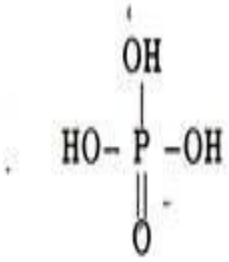
3.2. Chemical composition of DNA

Chemical analysis of highly purified DNA has shown that it is made of four kinds of monomeric building blocks, each of which contains three types of molecules:
(i) Phosphoric acid,
(ii) Pentose sugar, and
(iii) Organic bases.

(i) Phosphoric Acid:

Phosphoric acid has three reactive hydroxyl groups (—OH) of which two are involved in forming sugar phosphate backbone of both DNA or RNA. A phosphate group binds to the 5′ carbon of one and 3′ carbon of the other neighbouring pentose sugar molecule to make the phosphate diester. The phosphate makes a nucleotide negatively charged.

Therefore, a DNA becomes a polyanionic structure:



(ii) Pentose Sugar:

DNA contains β D 2′-deoxyribose sugar. It is a five-carbon sugar; hence it is a pentose sugar. Since one oxygen atom at the 2′ carbon is missing it get its name 2′-deoxy. The 2′- deoxy-containing backbone is more resistant to hydrolysis than the normal form.
In deoxyribose sugar, the hydroxyl group on the carbon that carries the aldehyde group can rapidly change from one position to another. The two positions are called α and β.

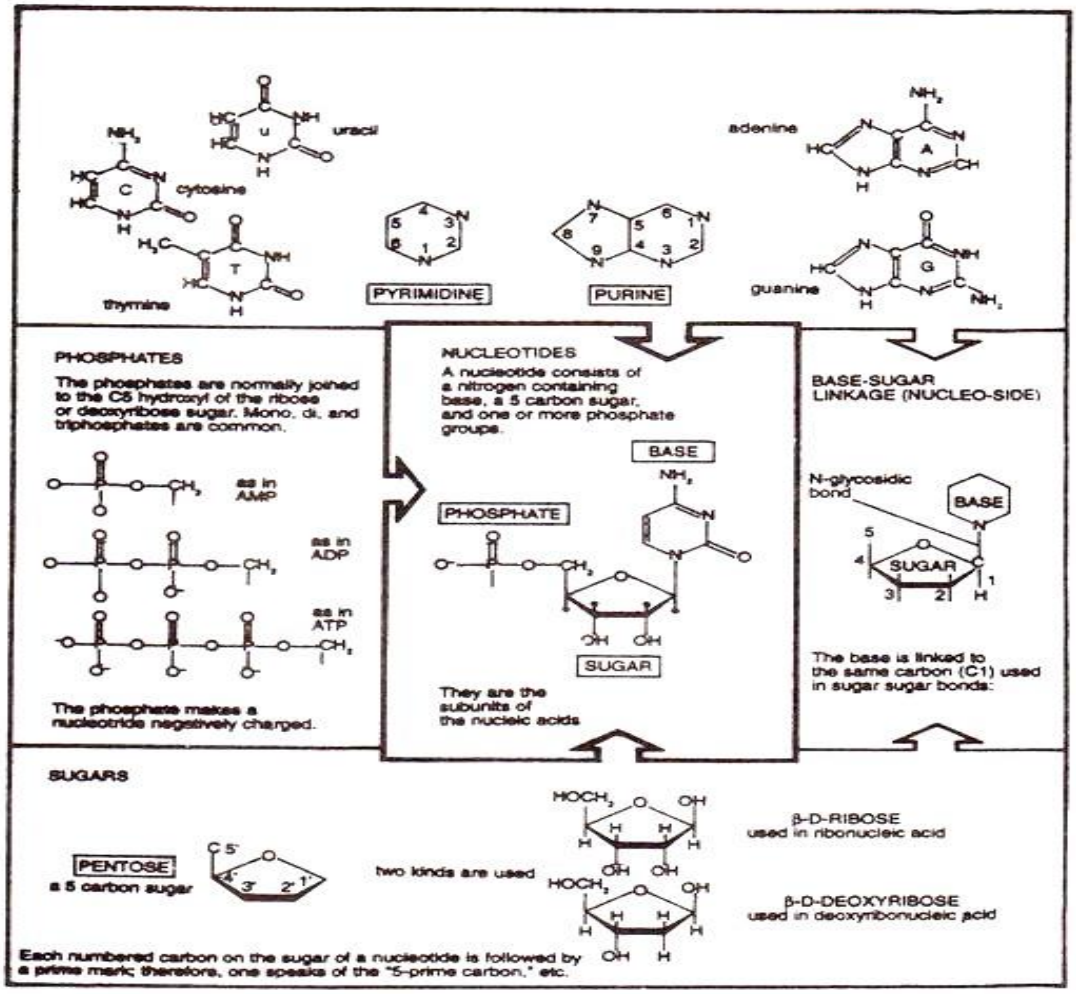
Deoxyribose sugar is always present in the ring form in the structure of DNA. The ring form is derived from heterocyclic furan (C_4H_{40}) structure. The carbon atoms of the deoxyribose are numbered from the end closet to the aldehyde and the numbers are given as 1′, 2′, 3′, 4′ and 5′ in order to differentiate them from the corresponding position in DNA bases.
It is also explained that each numbered carbon on the sugar is followed by a prime mark, therefore one speaks of 5 prime or 3 prime carbon etc.

(iii) Organic Bases:

Different types of heterocyclic nitrogen containing ring compounds are found in the structure of DNA. They are called simply bases because they can combine with H^+ in acidic solution. They are also referred as nitrogenous base due to presence of nitrogen.
The organic bases of DNA can be classified into two major groupson the basis of their structures.:
(a) Pyrimidines&(b) Purines

(a) Pyrimidines: Pyrimidine bases are made of a six-membered pyrimidine ring which is similar to the benzene ring except that it contains nitrogen in place of carbon at the positions of 1 and 3. Three pyrimidine derivatives are uracil, thymine and cytosine.

Their names are commonly abbreviated by three capital letters such as U, T and C, respectively. While cytosine and thymine are commonly found in DNA, cytosine and uracil axe found in RNA. In RNA, thymine is replaced by uracil. Thymine is characteristically present in DNA because thymine ensures stability of the genetic message. Otherwise, retention of the uracil would result in mis-pairing and mutagenesis on subsequent replication.



The chemical configuration on the bases, phosphates, nucleotides, base sugar linkage and sugar (Courtesy J D Watson et al)

All pyrimidine bases have a common keto- oxygen at position 2. In cytosine, an amino group (-NH_2) is present at position 4. An additional keto-oxygen is present at position 4 in uracil. But thymine contains a keto-oxygen at position 4 and a methyl group (CH_3) at position 5 (Fig). All pyrimidine bases have a hydrogen atom at the position 1 which is involved in their linkage with carbon 1 of pentose sugar.

(b) Purines:

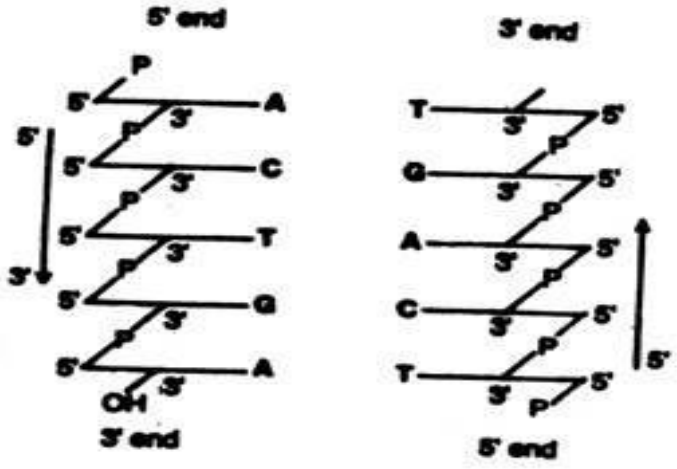
Purine is a derivative of pyrimidine. It consists of a pyrimidine ring and a five-membered imidazole ring (having nitrogen at 7 and 9 position) which are fused together at 5 and 4 position. There are two purine compounds—adenine (A) and guanine (G)—found in the structure of DNA. Adenine has an amino group (-NH_2) at 6 position while guanine has a keto group and an amino group at, 6 and 2 positions of carbon, respectively (Fig. 10.3). The pentose sugar is joined to the base by a β-N glucosidic bond between carbon atom 1 of the pentose and nitrogen atom 9 of purine bases.

3.3 Chemical compostion of RNA:

- RNA is a biopolymer of nucleotides bonded with each other via a phosphodiester bond.
- The nucleotide that makes up the RNA are also referred to as Ribose nucleotide due to the presence of ribose sugar in their structure. Overall, RNA is composed of a ribose sugar, phosphate, and nitrogenous base.
- Ribose sugar is a cyclical structure made up of five carbons and one oxygen atom. This sugar contains two OH-groups at 2' Carbon and 3' Carbon.
- This ribose sugar is attached to a nitrogenous base via hydrogen bonding.
- There are four nitrogenous bases namely: Adenine (A), Guanine (G), Uracil (U), and Cytosine (C).
- These nitrogenous bases pair complementarily with each other: G with C and A with U.

3.4. Double-Helical Structure of Normal DNA:

In 1953 J. D. Watson and F. H. C. Crick proposed a precise three-dimensional model of DNA structure based on the X-ray crystallography data of Franking and Wilkins and the base equivalences rule formulated by Chargaff. The Watson-Crick model of DNA structure postulated that two right-handed polydeoxyribonucleotide chains or strands are coiled in helical fashion around the same axis, thus forming a double helix.



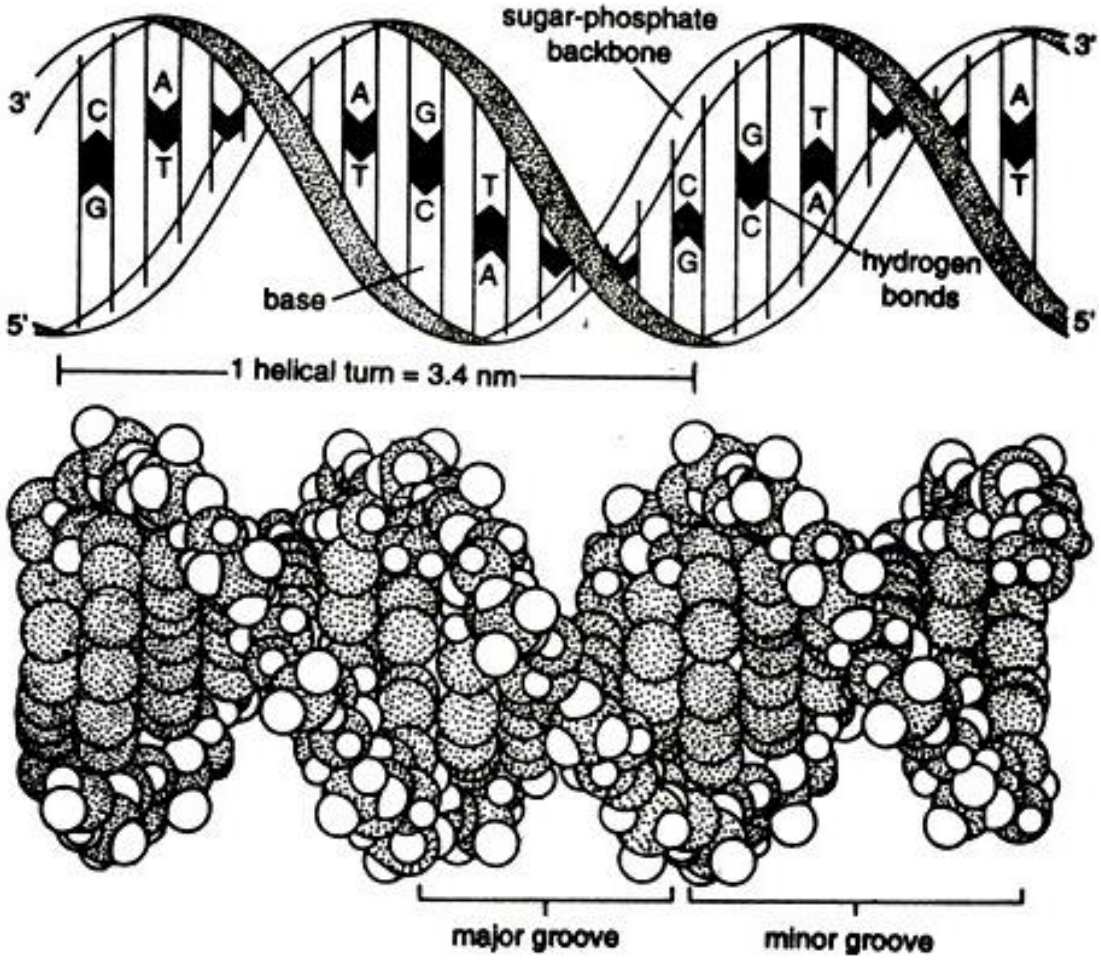
Segment of DNA duplex showing the antiparallel orientation of the complementary chains

The orientation of the two strands is antiparallel i.e., if one strand is oriented in the p-3′ → 5′-p direction, the other strand is oriented in the p-5′ → 3′-p direction. The coiling of the two strands is such that they cannot be separated except by unwinding the coils; such coiling is termed plectonemic.

In honour of this outstanding work Watson, Crick and Wilkins were awarded the Noble Prize in 1962 and one strand of double-stranded DNA is sometimes called Watson (W) and its compliment is called Crick (C). The two strands are held together by mobile hydrogen bond

between the pair of bases facing each other and stacking interaction between flat aromatic surfaces of the bases.

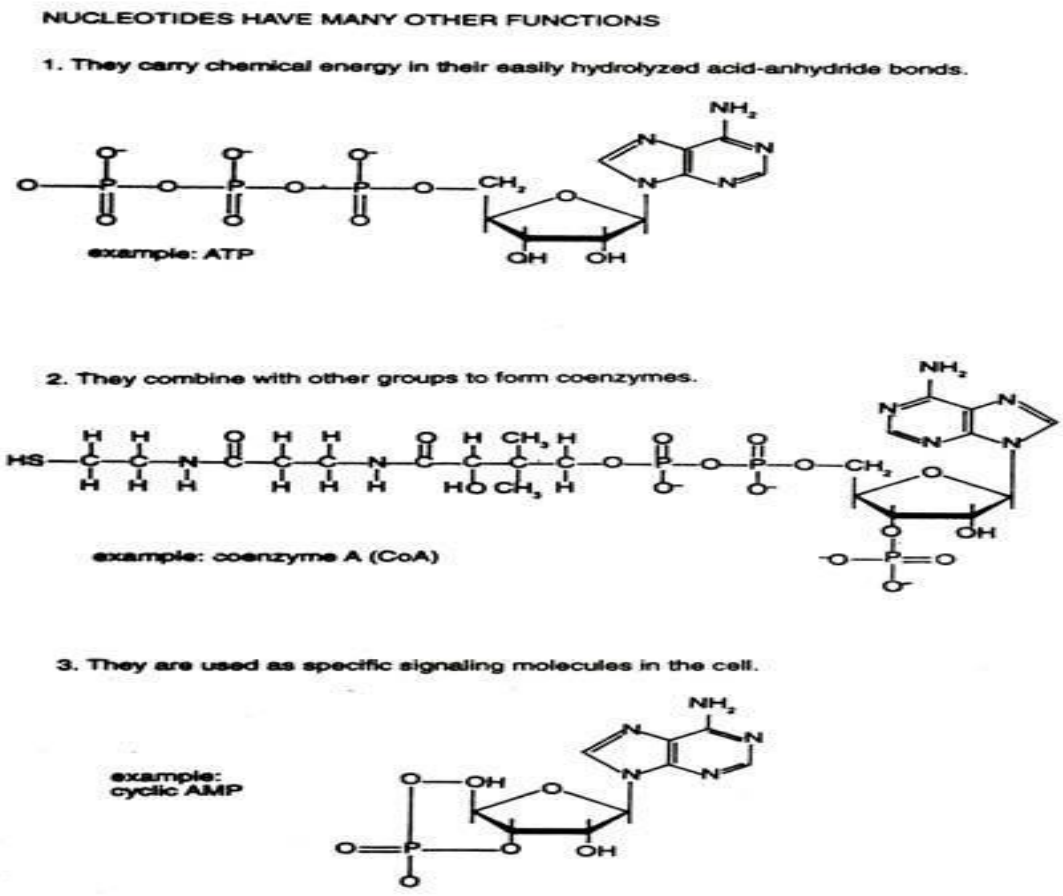
The sugar-phosphate backbone of the two poly-de-oxribo-nucleotide chains is connected as in a ladder, on the outside, the rungs being purine and pyrimidine bases stacked on the inside of the double helix. The two antiparallel polynucleotide chains are not identical in either base sequence or composition. Instead, the two chains are complementary to each other



Two antiparallel strands that are completary in their sequence are paired in a right -handed double helix with about 10nucleotide pairs per turn.

The bases on the antiparallel strands are held together in precise register: A is paired with T by two hydrogen bonds; G is paired with C by three hydrogen bonds. Neither A-G nor C-T pairs are found in DNA. Therefore, the base pair complementarity is a consequence of the size, shape and chemical composition of the base.

Beside this they are strongly hydrogen bonded base pairs than A- G and C-T pairs thus giving maximum fit and stability.



The double helix is stabilized not only by hydrogen bonding of complementary base pairs but also by stacking interactions between the flat aromatic surfaces of the bases. The latter interaction also stabilizes the helical structure against the electrostatic repulsive forces between the negatively charged phosphate groups.

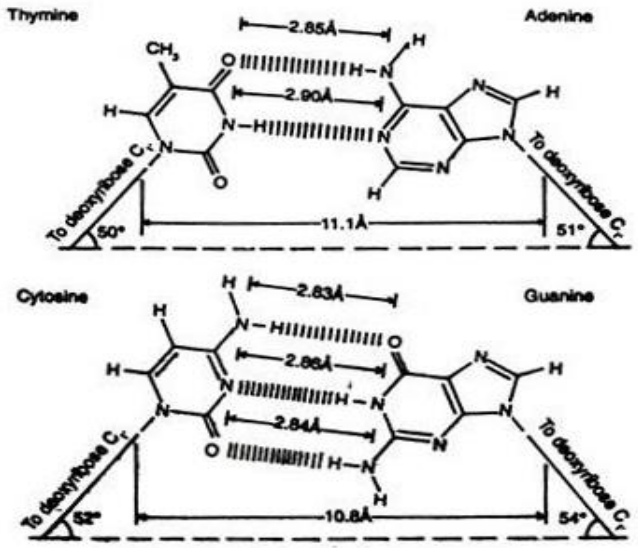
This stabilising energy may be equal to or greater than that of the hydrogen bonds connecting the bases between chains. Some authors suggest that hydrophobic and Van der Waals' interactions between adjacent base pairs also contribute significantly to the overall stability of the double helix.

Two distinctions among the bases of de-oxy-ribonucleotides are crucial to the secondary structure of DNA. One rest on the presence of keto (C = 0) and amino ($-NH_2$) groups that provide opportunities for hydrogen bonding. In case of AT pairing, amino group of C6 of adenine is linked by hydrogen bond with keto group of C4 of thymine). The inter atomic distance is 2.86 A.

In case of GC pairing, keto group of C6 and amino group of C2 of guanine are linked by hydrogen bonds with amino group of C_6 and keto group of C2 of cytosine, respectively. For these two hydrogen bonds, inter atomic distances are 2.83 A and 2.84A, respectively.

The second hydrogen bond can be formed between first nitrogen of A and third nitrogen of T in case of AT whereas, in case of GC, third bond is formed between nitrogen of the first position of both bases. The inter-atomic distances are 2.90 A in case of second bond of AT and 2.86 A in case of third bond of GC.

The bases are also set at particular angle with sugar. Both A and T are set at 51° and 50° angles with sugar molecule, respectively. But G and C make 54° and 52° angle with sugar, respectively.



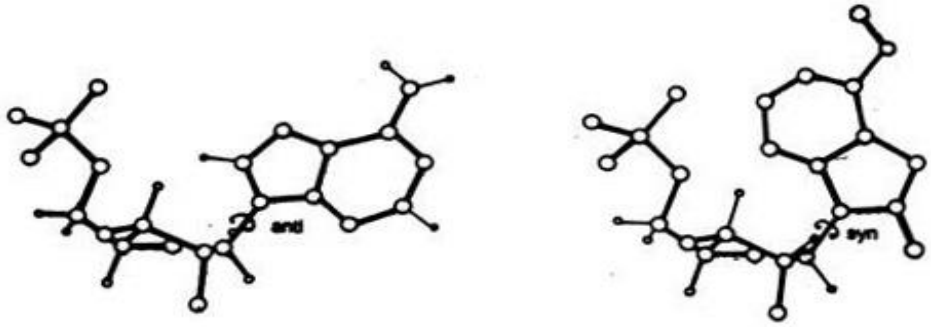
Hydrogen-bonded base pairs.
Interatomic distance and angles are nearly the same fot AT and GC pairs

The second important distinction among the bases is that they come in two sizes: the pyrimidine's T and C are smaller than the purines A and G. However, the base pairs (ATorGC) are nearly identical in size. The AT and GC base pairs have not only the same size but very similar dimension.

Thus the two types of base pairs occupy the same amount of space (11.1 A in case of AT and 10.8 A in case of GC) allowing a fairly uniform dimension throughout the DNA double helix. These are also confirmed by X-ray crystallographic studies.

This study also reveals that the stacked bases are regularly spaced 3.4 A apart along the helix axis. The helix makes a complete turn or a pitch every 34 A, thus there are about 10 base pairs per turn and each base pair is rotated 36° relative to its neighbour.

An important element of symmetry in the helix is the rotation about the N-glycosidic bond. The conformations designated anti occur more frequently in normal DNA than those conformations that are 180° opposite as designated in the Fig. The average diameter of DNA helix is 20 A. On the outside of the molecule, the space between inter-wind strands forms a relatively narrow minor and a wide major groove.

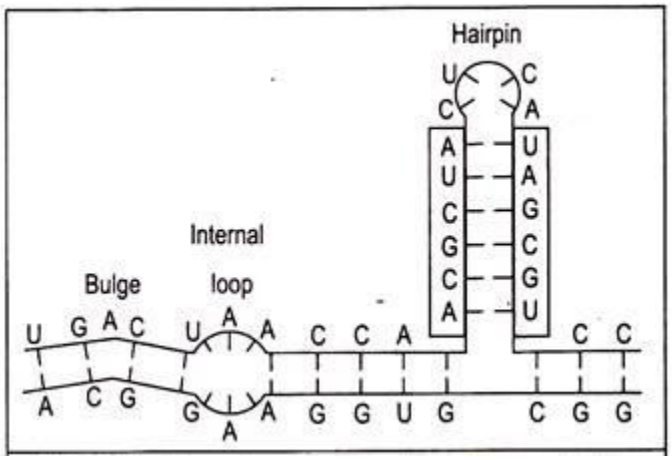


Rotation about the N-C glucosidic bond. The anti-conformation is favoured over the syn

The right-handed double helical structure of DNA proposed by Watson and Crick is the biologically important one. It is also considered as the standard normal DNA structure which is designated as B-form DNA to differentiate it from other forms of DNA discovered later on.

3.5 Structure of RNA;

- The primary structure of RNA is the same as that of DNA. It is also a polynucleotide chain with 5′-3′ sugar phosphate links. But the sugar is ribose and generally it exists as a single-stranded molecule. For that reason, it does not have the one-to-one ratio between the complementary bases. The amount of purines is not equal to that of pyrimidines.
- One of the four major bases in RNA is uracil (U) instead of thymine. Biophysical properties of RNA show much less secondary structure. However, when a sequence of bases is followed by a complementary sequence in the same chain, the polynucleotide may fold back on itself to generate an antiparallel duplex structure, known as a hairpin.
- It has a stem, the base-paired double helical region, and a loop with unpaired bases at one end (Fig. 3.55). In these regions G can also pair with U, but it is not as strong as G-C pair.



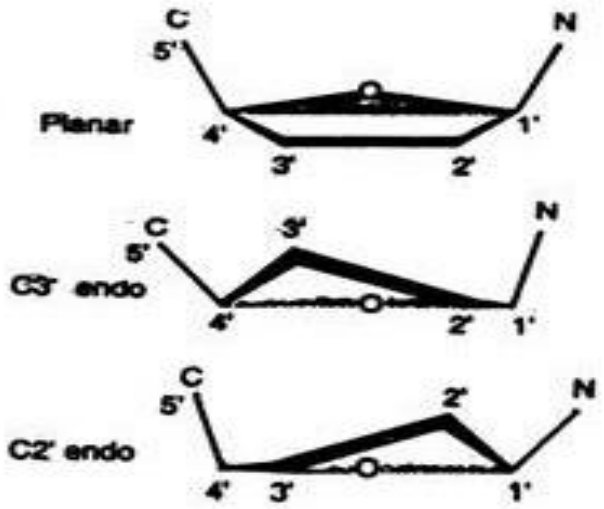
Secondary structure of RNA

- Cells contain three types of RNA-messenger RNA (mRNA), ribosomal RNA(rRNA) and transfer RNA (tRNA). Messenger RNA serves as the template for protein synthesis. It is a very heterogeneous class of molecules and very unstable. It constitutes 2 – 5 per cent of the total RNA of the normal cell. It was first detected by Hershey (1956). The name and concept of messenger RNA was first given by F. Jacob and J. Monod (1961).
- When molten DNA is slowly cooled with some specific m-RNA, DNA- RNA hybrid molecules are formed, suggesting that m-RNA is formed from the template strand of the DNA duplex.
- It is very heterogeneous in size because genes or groups of genes vary in length. In prokaryotes, the translation of mRNA molecule very often begins before the completion of its transcription, because mRNAs are both transcribed as well as translated in the 5′ to 3′ direction and both the processes are not separated by nuclear membrane.

3.6. Different Forms of DNA

(i) A-DNA or Right-Handed DNA: The A-structure is induced in DNA in 70% to 75% ethanol and is found in fibres of DNA in a dehydrated state. The primary difference between A and B helices lies in the sugar ring conformation (pucker). The sugars are C'_3-endo in the A-form but C'_2 endo in the B-form

This altered sugar pucker shortens the distance between adjacent phosphate on one strand by about 1°A. Thus A-DNA has between 11 and 12 base pairs per helix pitch rather than the 10.5 typical of B-form.



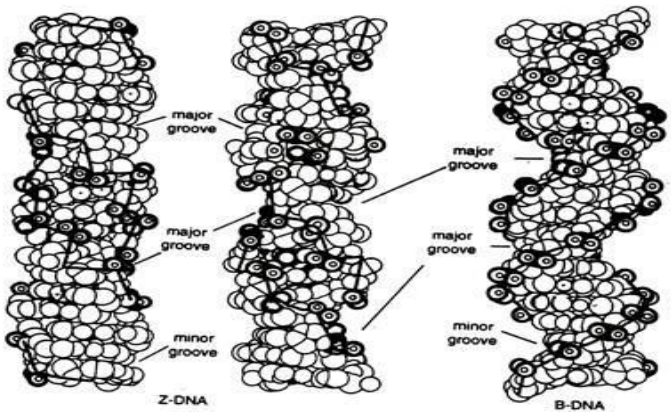
Views of the C2…endo and C2 forms are shown in perpendicular to the paper

The second major difference in A-DNA is that the base pairs are displaced from the central helix axis, towards the major grooves. The base pair in B-DNA are essentially centered over the helix axis. In the A-structure, they are shifted by side nearly 5 A from the centre, resulting in a ribbon-shaped helix with a cylindrical open core and a very deep but narrow major groove.

The shape of the functional groups of the bases within these grooves undoubtedly render A and B forms clearly distinguishable by protein interacting with the DNA. Base stacking—both intra and inter strand—is the key element in stabilising the A helix.
The helix geometry of A-DNA at a glance contains:
1. The length of one pitch is 28.15 A^0.
2. There are 11-12 base pairs per pitch.
3. The axial rise per base pair is 2.56 A^0.
4. There is a tilting of base pairs from the axis of the helix of 20.2.

(ii) Z – DNA or Left-Handed DNA:

Z-DNA is a new family of DNA structure which is left-handed, i.e., the strands of DNA are twisted towards left-side instead of the usual right-handed twisting. B-form is twisted clockwise whereas Z-form is twisted anti-clockwise. Therefore, two possible helical forms of DNA are mirror images of each other.



Side views of Z-DNA and B-DNA

Alexander Rich and his colleagues of USA have discovered the Z-form of DNA. Factors that promote the Z-structure include methylation, bromination, specific DNA binding protein and sufficient torsional stress as in negatively supercoiled DNA. The Z-DNA was so-named because of the zigzag course of the backbones.

Z-DNA differs from the A and B forms not only in its left-handedness, but also in the orientation of the glycosyl bonds. The syn and anti-orientations alternate, in contrast with the all-anti conformation in A- and B- DNA.

The pyrimidine nucleotides are in the standard anti conformation with a C'_2-endo sugar pucker, while the purine residues are syn and contain a C'_3-endo conformation. The Z-form is more likely to occur in alternating purine-pyrimidine sequences because steric repulsion makes it less-favourable for a pyrimidine to adopt the syn conformation.

The crystal structures have a strong dinucleotide repeat unit due to the large alternation in helix twist between the C and G in a CpG step which is about 15° while that between the G and C in the subsequent GpC is close to 45°.

Both B-DNA and A-DNA have two types of grooves—one major and one minor. But Z- DNA has the single groove which is quite deep, extending to the axis of the helix. The diameter of Z-DNA is about 18 A^0 and the length of pith is 45 A^o.
The basic helix geometry of Z-DNA is:
1. The length of one pitch is 45 A^o.
2. Number of base pairs/pitch is 12.
3. The axial rise per base is 3.7 A^o.
4. Tilting of base pairs from the axis of helix is 7°.
5. Diameter of the helix is 18 A^o.
Its presence has also been established in the chromosome of the fruit-fly Drosophila melanogaster and also in the chromosomes of some other organisms.

There is growing evidence that certain DNA regions rich in guanine and cytosine within long molecules do adopt a Z-like conformation. This peculiar form of DNA is presumed to have a role in the regulation of gene activity.

It could be a recognition signal for some important function of DNA. To understand the function of Z-DNA in Drosophila melanogaster chromosomes, research on synthetic DNA was conducted.

It has been demonstrated that purine-pyrimidine sequences can undergo transitions between right- handed B-conformation depending on the salt concentration or on chemical modification of the bases. The reversibility suggests that Z- DNA has some regulatory role.

For example, some nucleotide sequences can be established in the Z-form by being methylated. Methylation and de-methylation are very important in controlling the activity of genes.

There could be many possibilities by which the Z- DNA controls the gene regulations such as when certain control sites are established in the Z-form by methylation—regulatory protein binds to the site and keeps gene turned off; de-methylation might switch the site to the B-conformation, causing the regulatory molecule to let go.

Identification of Z-DNA:

Rich et al could raised antibodies in a rabbit that were highly specific for Z-DNA and did not react at all with the B-form.
By comparing ultraviolet micrographs that reveal the bands and inter-bands of polytene chromosomes, it was established that the fluorescent segments are the inter-bands and not the bands. Therefore, there must be Z-DNA in the inter-bands.

(iii) C-Form DNA:

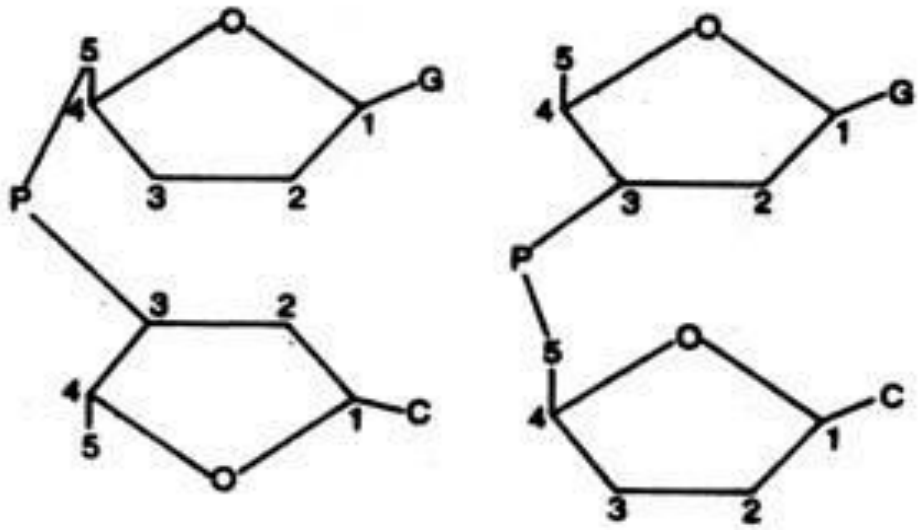
The size of helix of C-form DNA is greater than A type of DNA but is smaller than B-DNA. It is about 31 A^o. There are 9.33 base pairs per turn of the helix. There is an axial rise of base pairs of 3.03 A^o with a tilting of about 7.8° C-form is found at 66% relative humidity: in presence of Li^+ (Lithium) ions.

(iv) D-Form and E-Form DNA:

There are some other forms of DNA called D-form and E-form found rarely as extreme variants. In case of D-form there are 8 base pairs per turn of helix. An axial rise of base pairs is 3.03 A^0 with tilting of about 16.7°. In case of E-form, there are 7.5 base pairs per turn of helix.

Resemblances and Differences Between B-DNA and Z-DNA:

1. Both B-DNA and Z-DNA are of double helical structure.
2. In both DNAs two strands are antiparallel.
3. G Ξ C pairing is present in both B-DNA and Z-DNA.
4. B-DNA is right-handed whereas Z-DNA is left-handed.
5. The sugar-phosphate backbone in B-DNA is regular while in Z-DNA it follows zigzag course.
6. One complete helix is 34 A in B-DNA while it is 45 A^0 in Z-DNA.
7. In Z-DNA, adjacent sugar residues exhibit → opposite orientations while in B-DNA they are in same orientation. This results in dinucleotide units in Z-DNA as against mononucleotide units in B-DNA.



Orientation of sugar residue in Z-DNA and B-DNA

8. In B-DNA there are ten base pairs at each turn while there are twelve base pairs at each turn of Z-DNA.
9. The diameter of B-DNA is 20 A^0 whereas it is 18 A^0 in Z-DNA.
10. Bases are more closer to the axis in Z-DNA than B-DNA.
11. The glycosidic torsion angle is anti in B- DNA and syn in B-DNA.
12. Sugar pucker is C_2 endo for B-DNA and C'_3 endo for Z-DNA in deoxyguanosine.
13. The angle of twist per repeating unit, i.e., dinucleotide is 60° as against 36° in B- DNA.
14. Base pair tilt is 6° in B-DNA whereas it is 7° in Z-DNA.

The differences mentioned above are summarised in Table

Characteristics	B-DNA	Z-DNA
Coiling	right-handed	left-handed
Base pair per turn	10(10 monocleotide units)	12(6 dinucleotide units)
Helical diameter	20 Å	18 Å
Helix pitch	34 Å	45 Å
Tilt of base pair	6^o	7^o
Sugar phosphate backbone	regular	zig-zag
Axial rise per base pair	3.4 Å	3.8 Å
Rotation per residue	36^o	60^o
Glycosidic torsion angle		
deoxyguanosine	*anti*	*syn*
deoxycytidine	*anti*	*syn*
Sugar pucker		
deoxyguanosine	C_2' *endo*	C_3' *endo*
deoxycytidine	C_3' *endo*	C_2' *endo*
Distance of P from axis		
dGpC	9.0 Å	8.0 Å
dCpG	9.0 Å	6.9 Å
Orientation of sugar	Alternate	Opposite

Comparison of B-DNA and Z-DNA

Functions of DNA

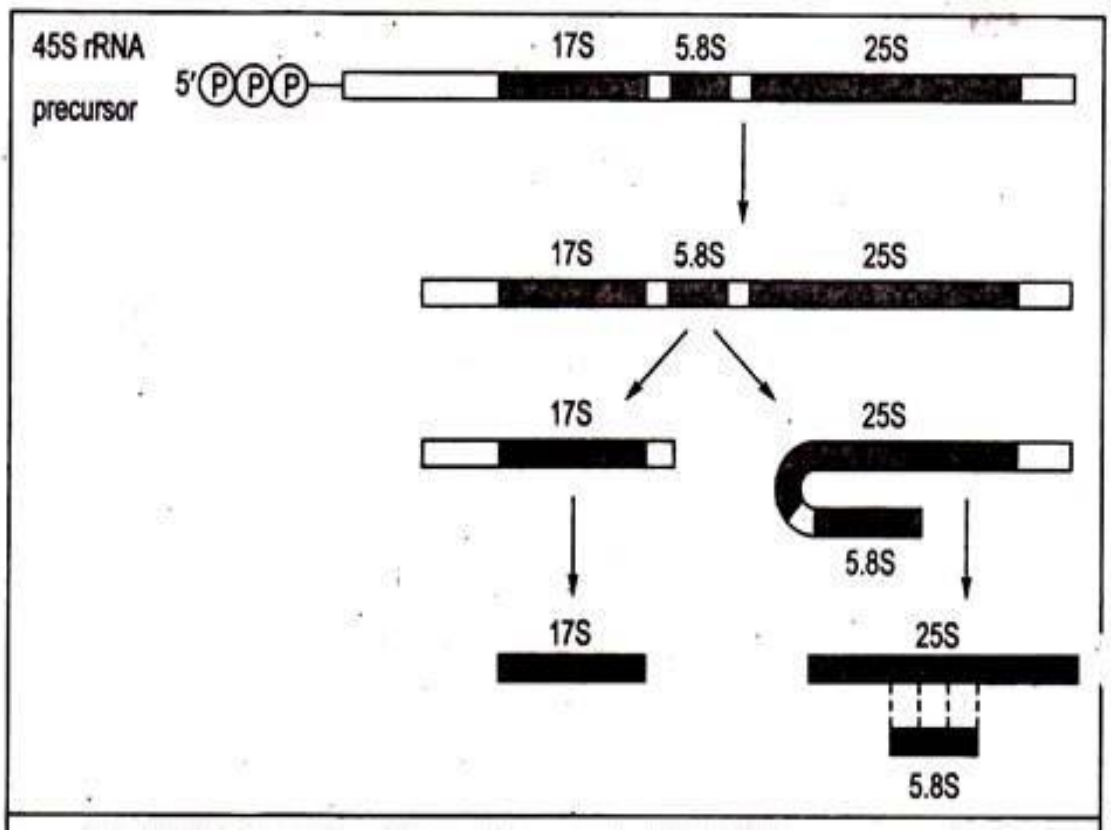
1. DNA stores complete genetic information that requires to specify an organism.
2. It is the source of information that is needed in order to synthesize cellular proteins, and other macromolecules required by an organism.
3. It is responsible for identifying and determining the individuality of the given organism.
4. It can also be taken as a targeted element during the diagnosis of a particular disease.
5. It can replicate to give rise to two daughter cells and transfer one copy to the daughter cells during cell division. Thus, maintaining the genetic materials from generation to generation

3.7 Different types of RNA

(i) Ribosomal RNA

- The bulk of cellular RNA is ribosomal RNA. It is the principal component of ribosomes, but its definite role in protein synthesis is not clear. Plants, algae, and photosynthetic protists each contain three classes of ribosomes: cytoplasmic, plastid, and mitochondrial. In E. coli ribosomes three types of rRNA are found. They are called 5S, 16S and 23S due to their sedimentation behaviour.
- Each ribosome contains one molecule of each of these rRNA species. Eukaryotic ribosomes, however, contain one molecule each of the 5S, 7S, 18S, and 28S rRNA species. Ribosomal RNA is the most abundant of the 3 types of RNA in the cell.
- Ribosomal RNA is single stranded, but it also shows high degree of secondary modifications due to the formation of double strands between complementary regions and hair pin loops. Each loop contains duplex stems. The loops and the stems provide the specific vectorial binding sites for various ribosomal proteins and other enzymes for protein synthesis.

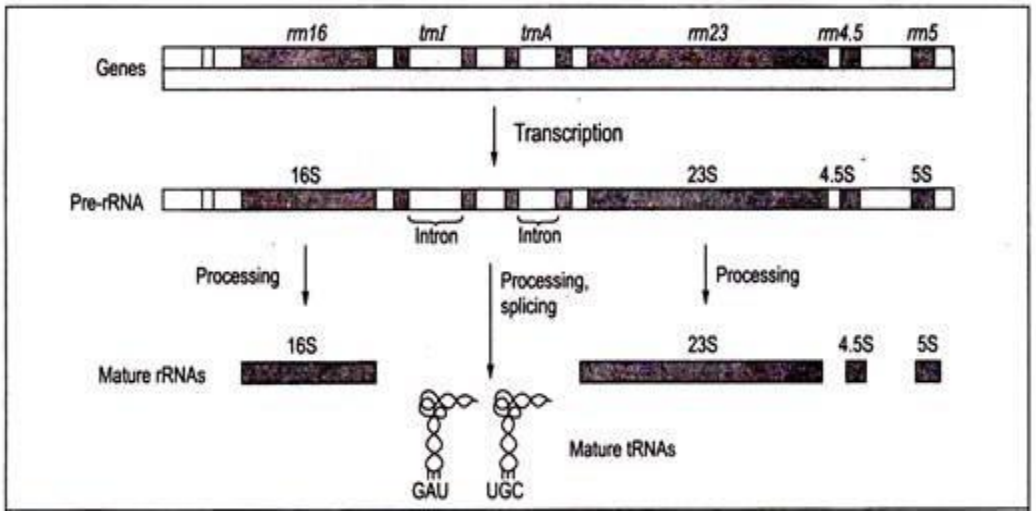
- The 3′ end of 16S rRNA isolated from E. coli has a Shine and Dalgarno sequence which serves as mRNA binding site in the 30S ribosome. This sequence helps to recognize the starting end of the mRNA. The 5S rRNA has a sequence which is complementary to TΨC sequence of all tRNAs. So, 5S RNA is essential for the binding of tRNA to the ribosomes.
- The processing of pre-rRNAs includes nucleolytic cleavage and methylation. Some eukaryotic rRNA transcripts undergo splicing by the intron itself (self-splicing, ribozyme).
- The transcription units in the nuclear rRNA gene cluster that encode the 17S, 5.8S, and 25S rRNA molecules are transcribed by RNA polymerase I into a single long precusor molecule, which then undergoes a series of cleavages and methylation steps to yield 17S, 5.8S, and 25S rRNA molecules.
- Transcription of the 5S rRNA from the corresponding nuclear genes is independent and is catalyzed by RNA polymerase III. It requires no processing.
- The 17S, 5.8S, and 25S rRNAs are produced from a common 45S rRNA precursor molecule by processing reactions catalyzed by several RNases. During processing, the 25S and 5.8S rRNAs become hydrogen bonded and remain paired after the processing is complete as described in the following figure.



Processing of the nuclear-encoded 45S rRNA precursor molecule giving rise to three mature rRNAs

- Four plastid rRNA molecules are encoded as a polycistronic transcription unit that also includes the two tRNA genes encoding $tRNA^{ala}$ and $tRNA^{lle}$ in the spacer region that separate the 16S and 23S rRNA, each of which contains a long intron. Through a complex processing pathway the precursor RNA is cleaved into 16S, 23S, 4.5S, and 5S rRNAs and the tRNA precursors.

- The cleaved tRNA precursors are then processed into functional $tRNA^{lle}$ and $tRNA^{ala}$ molecules. The tRNAs are transcribed by RNA polymerase III from clusters of nuclear genes, but unlike 5S rRNA they need processing as shown in the figure.



Processing of chloroplast pre-rRNA from plants. The chloroplast rRNA operon encodes two tRNAs in the spacer regions separating 16S and 23S rRNA

(ii). Transfer RNA (tRNA)

- It is also called soluble or sRNA. There are over 100 types of tRNAs. Transfer RNA constitutes about 15% of the total RNA. tRNA is the smallest RNA with 70-85 nucleotides and sedimentation coefficient of 4S. The nitrogen bases of several of its nucleotides get modified e.g., pseudouridine (ψ), dihydrouridine (DHU), inosine (I).
- This causes coiling of the otherwise single-stranded tRNA into L-shaped form (three dimensional, Klug, 1974) or clover-like form (two dimensional, Holley, 1965). About half of the nucleotides are base paired to produce paired stems. Five regions are unpaired or single stranded— AA-binding site, T ψ C loop, DHU loop, extra arm and anticodon loop.

(a) Anticodon:
It is made up of three nitrogen bases for recognising and attaching to the codon of mRNA.

(b) AA-Binding Site: It lies at the 3′ end opposite the anticodon and has CCA— OH group. This CCA group is added after the transcription (5′ end bears G). Amino acid or AA-binding site and anticodon are the two recognition sites of tRNA.

(c) T ψ C Loop:
It contains pseudo uridine. The loop is the site for attaching to ribosomes,

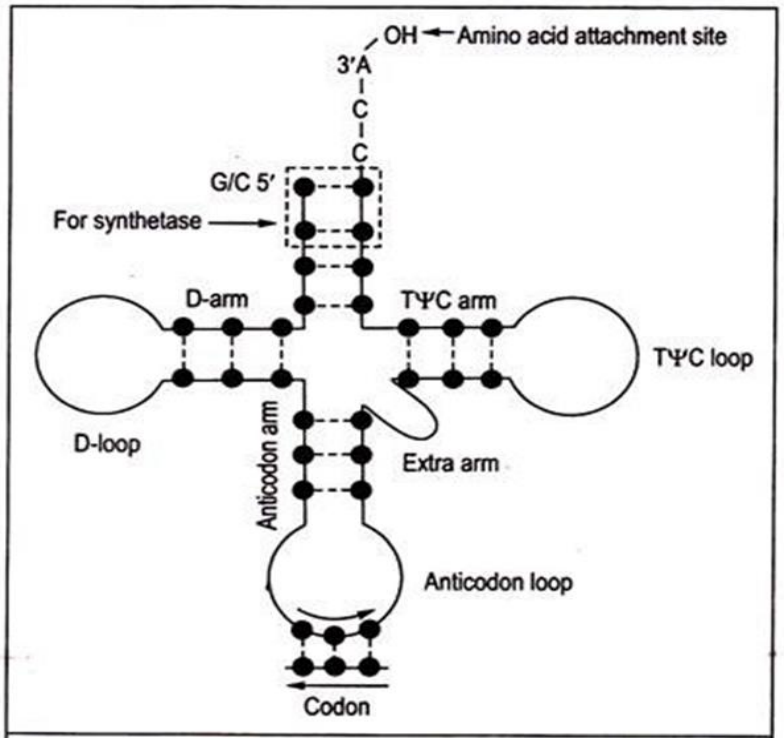
(d) DHU Loop:
The loop contains dihydrouridine. It is binding site for aminoacyl synthetase enzyme,

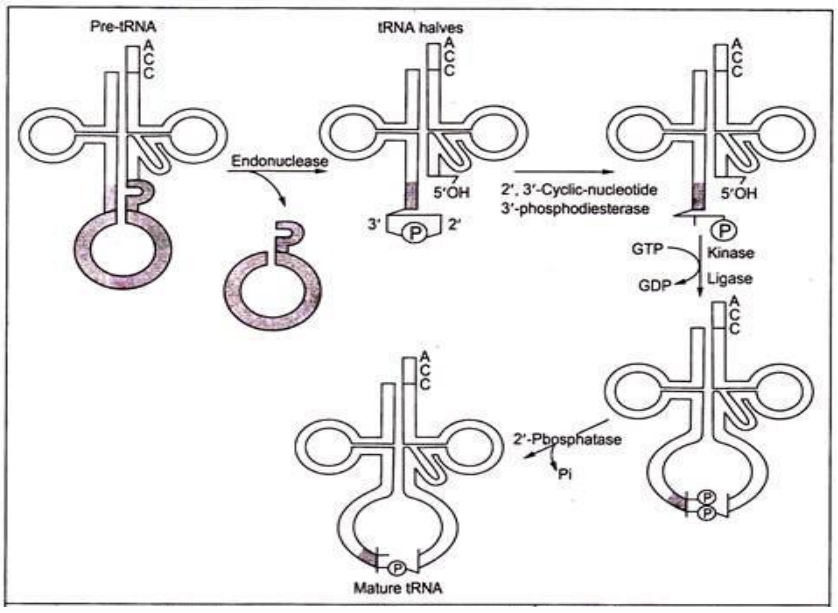
(e) Extra Arm:
It is a variable site arm or loop which lies between T ψ C loop and anticodon. The exact role of extra arm is not known.

Functions:

(i) tRNA is adapter molecule which is meant for transferring amino acids to ribosomes for synthesis of polypeptides. There are different tRNAs for different amino acids. Some amino acids can be picked up by 2-6 tRNAs. tRNAs carry specific amino acids at particular points during polypeptide synthesis as per cidons of mRNA.
Codons are recognised by anticodons of tRNAs. Specific amino acids are recognised by particular activating or aminoacyl synthetase enzymes,
(ii) They hold peptidyl chains over the mRNAs.

Clover leaf structure of tRNA

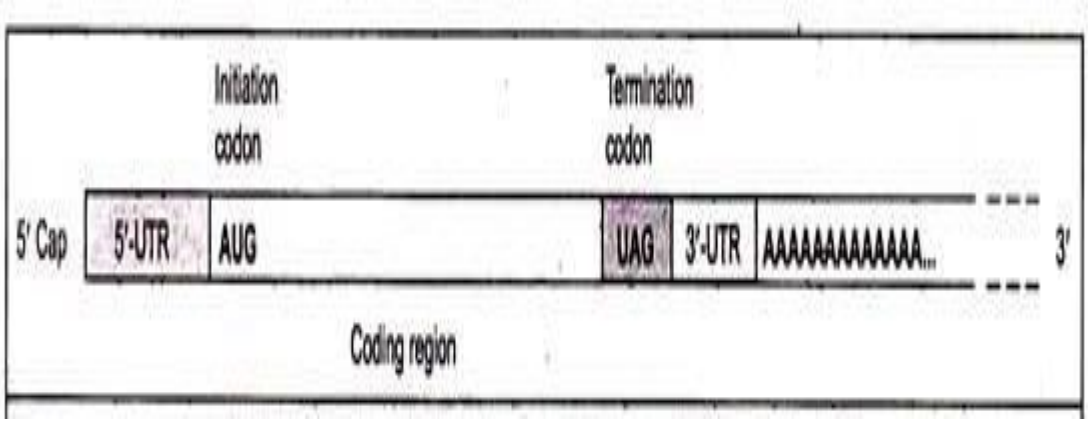




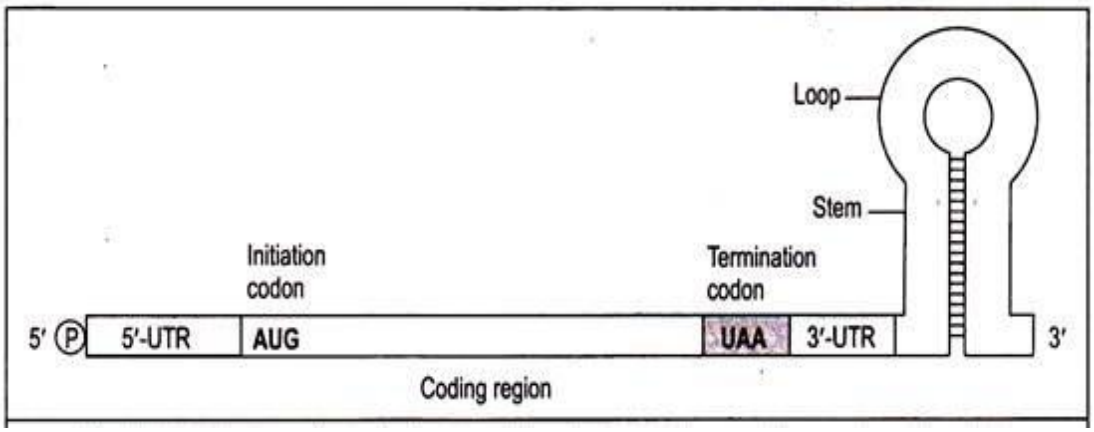
The splicing pathway of pre-tRNA

(iii). Messenger RNA (mRNA):

- It is a long RNA which constitutes 2-5% of the total RNA content. It brings instructions from the DNA for the formation of particular type of polypeptide. The instructions are present in the base sequence of its nucleotides. Ii is called genetic code. Three adjacent nitrogen bases specify a particular amino acid.
- Formation of polypeptide occurs over the ribosome. mRNA gets attached to ribosome. tRNAs are induced to bring amino acids in a particular sequence according to the sequence of codons present over mRNA. mRNA has methylated region at the 5′ terminus.
- It functions as a cap for attachment with ribosome. Cap is followed by an initiation codon (AUG) either immediately or after a small noncoding region. Then there is coding region followed by termination codon (UAA, UAG or UGA). There is then a small noncoding region and poly A area at the 3’ terminus. An mRNA may specify only a single polypeptide or a number of them.



Structure of a typical mature, nulear-encoded, eukaryotic mRNA



Structure of a typical mature chloplast mRNA resembling prokaryotic mRNA

- The former is called monocistronic while the latter is known as polycistronic. Polycistronic mRNA is more common in prokaryotes. Eukaryotic mRNA is usually monocistronic.
- The life time of mRNA is also variable. In some lower forms it is from a few minutes to a few hours. On the other hand the mRNAs of higher forms seem to have a long life. It is several days in case of young red blood corpuscles which continue to form haemoglobin even when nucleus has degenerated.

Functions:

(i) mRNA carries coded information for translation into polypeptide formation.
(ii) Through reverse transcription it can form compact genes which are used in genetic engineering. The phenomenon also occurs in nature and has added certain genes in the genomes,
(iii) It has a cap region for attachment to ribosome.

3.8 Renaturation and Denaturation of DNA:

In simple term, denaturation is a process where nucleic acid or proteins lose the quaternary structure, tertiary structure and secondary structure by application of some external stress or compound.DNA denaturation, also called DNA melting is the process where double stranded DNA unwinds and separates into single strands through the breaking of hydrophobic interactions between the bases and hydrogen bonds.

So, denaturation can be described as the process of formation of single stranded DNA from double stranded helical DNA. DNA denaturation may be induced by high temperature or applying some chemicals such as urea and formamide. Biologically-induced denaturation is observed during DNA replication and transcription. Thermal denaturation of DNA is often termed as melting. In temperature mediated denaturation, with the increase in thermal energy, the molecular motion also increases. It results in the breaking of hydrogen bonds and other forces that stabilize the double helix. Then the strands separate driven apart by the electrostatic repulsion of the negatively charged deoxyribose-phosphate backbone of each strand. Near the denaturation temperature, a small increase in temperature causes a rapid loss of the multiple weak interactions holding the strands together. DNA from different cell types have different

melting points. The melting point or midpoint temperature (Tm) is the temperature at which half the DNA is unwound.If several samples of DNA are melted, it is found that the TM is highest for those DNAs that contain the highest proportion of G-C. The Tm of DNA from many species varies linearly with G-C content.This relationship between Tm and G-C content arises due to the fact that guanine and cytosine form three hydrogen bonds in base pairing, whereas adenine and thymine form only two.These differences depend on several factors. Molecules that contain a greater proportion of G-C pairs require higher temperature to denature. It is because the three hydrogen bonds in G-C pairs make these base pairs more stable as compared to A-T pairs with only two hydrogen bonds. A DNA that consists entirely of AT base pairs melts at about 70° and another DNA that has only GC base pairs melts at over 100°.As such, percentage of G-C base pairs in a DNA sample can be estimated from the Tm.

The ion concentration also influences the Tm as negatively charged phosphate groups in the two strands are protected by positively charged ions. When the ion concentration is low, this protection is decreased, thus increasing the repulsive forces between the strands and reducing the Tm. There are several equations for determining the Tm, out of which the simplest being the method of Wallace. This is suitable for short strands of base pairs (14-20) and is the equation used by many researchers. This very simple method assigns 2°C to each A-T pair and 4°C to each G-C pair. The Tm then is the sum of these values for all individual pairs in a DNA double strand. This takes into account that the G-C bond is stronger than the A-T bond. For longer strands one of the other formulas must be used. Formula: Tm = 2 °C(A + T) + 4 °C(G + C) = °C Tm In another case, extreme pH value denature DNA at low temperature. At low pH value, the bases become protonated and thus positively charged and repel each other. At high pH, bases lose protons and become negatively charged again repelling each other.

Renaturation Denaturation of homogenous DNA is reversible. When heated solutions of DNA are slowly cooled, annealing occurs with return of the coil to helical configuration. This process is called Renaturation. In short, renaturation is the process of formation of double stranded DNA from single stranded denatured complementary DNA strands. It involves reannealing or formation of hydrogen bonds between complementary base pairs.The single stranded DNA molecules produced from denaturation form random coils without an organized structure. When there is lowering of temperature, increase in the ion concentration or neutralization of pH, the two complementary strands may reassociate into a perfect double helix. This process dependent on time, the DNA concentration and the ionic concentration. If the two DNA strands are not related in sequence, they will remain as random coils and will not renature. However, they will not inhibit complementary DNA partner strands from finding each other and renaturing.

3.10 SUMMARY

Deoxyribonucleic acid (DNA) is double-stranded molecule and is a heredity material found in humans and all living organisms. It is composed of Phosphoric acid, Pentose sugar, and Organic bases. DNA contains a pentose sugar, β D 2′-deoxyribose sugar in backbone of DNA and hence it is more resistant to hydrolysis than the normal form. The ring form is derived from heterocyclic furan (C_4H_{40}) structure. Different types of heterocyclic nitrogen containing ring compounds are found in the structure are (a) Pyrimidines (Three pyrimidine derivatives are uracil, thymine and cytosine, however in DNA only thymine and cytosine were found) (b)

Purines, adenine (A) and guanine (G)—found in the structure of DNA. In 1953 J. D. Watson and F. H. C. Crick proposed a precise three-dimensional model of DNA structure based on the X-ray crystallography data.

3.11 SELF ASSESSMENT:

1. Write the composition and structure of double stranded DNA
2. Write a note on variations in the structure of DNA

3.12 SUGGESTED READINGS:

1. Leininger's Principles of Biochemistry (2000) by Nelson, David L and Cox, M M, Macmillan/worth, NY.
2. Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte W Pratt, John Wiley & Sons, NY.
3. Biochemistry III ed (1994) by Lubert Stryer, WH Freeman and Co., San Francisco.
4. Outlines of Biochemistry (1987) by Eric E Conn, P K Stumpf, G Bruening and Ray H Doi, John Wiley & Sons, NY

Prof A. Amrutha Valli

Lesson 4

AMINO ACIDS

Objectives :

Tounderstand types of amino acids and analyze general properties of amino acids.

4.1 INTRODUCTION

Amino acids are divided into three goups.

1. **Protein or Standard Amino Acids:** They are 20 in number and all of them are constituents of proteins.
2. **Non-Standard Amino Acids:** These are found in proteins. All f them are derived from standard amino acids. These amino acids are created by modification of standard residues already incorporated into polypeptide. Some of the examples are given below.
3. **Non-Protein Amino Acids:** Over 300 amino acids have been found in cells. They are not constituents of proteins and exhibit variety of functions. Ornithine and citrulline which are not found in proteins are intermediates in the bio-synthesis of arginine and urea cycle.

Only one of the non-protein amino acids is regularly present in plants, the ubiquitous, g amino butyric acid. The other non protein amino acids are of more restricted occurrence.

Amino acids are building blocks of proteins. Twenty amino acids in different combination and sequences produce wide variety of proteins. Different organisms (bacteria to complex plants and animals) can make widely diverse products such as enzymes, hormones antibiotics, transport proteins, nutrient proteins, antibodies, toxins and other substances having distinct biological functions.

Over 300 amino acids occur in nature are also derived from amino acids, only 20 amino acid take part in the protein synthesis.

Nearly, 12000 known alkaloids are synthesized from amino acids. In most cases alkaloids are formed from L. amino acids such as Tryptophan, Tyrosine, Phenylalanine, Lysine and Arginine, either alone or in combination with a steroidal or terpinoid type of moiety.

Some of the important alkaloids such as ajamalcineajamaline, strychnine, quinine, vincamine, vincristine, vinblastin, yohimbine, berberine are very important because they are used as drugs,

antibiotics, insecticides, herbicides, attractants for pollination as chemical defence in plants, toxins to insects and as feeding deterrents.

Thus synthesis of amino acids directly or indirectly control various aspects of plant growth and development. All amino acids have common names in some cases derived from source from which they were first isolated. Glycine (Greek glykos, "Sweet"); Asparegine was first found in asparagus, tyrosine was first isoland from chees (its name is derived from the (Greek tyros, "Cheese").

4.2STRUCTURE AND PROPERTIES

All twenty amino acids found in proteins are a-amino acids. They have a carboxyl groups (-COOH) and an amino (-NH_2) group bounded to the same carbon atom (-carbon). In all amino acids except glycine, the a-carbon atom is bound to four different groups, a carboxyl group (-COOH) an amino (-NH_2) group, an R group and a hydrogen atom. For glycine, the R group is another hydrogen atom. Amino acids differs from each other in the side chains (R-groups).

Every object has a mirror image. Many pairs of objects that are mirror images can be super imposed on each other. In the other case the mirror image objects cannot be super imposed on one another but are related to each other as the right hand is to the left. Such non super imposable mirror images are said to be chiral (from the greek "hand"); many important bio-molecules are chiral (Fig.6.1). Glycine is not chiral (achiral) because of this symmetry. In all other commonly occurring amino acids the a-carbon has four different groups bonded to it therefore they are chiral giving rise to two non super-impossible mirror image forms, these two forms represent a class of sterioisomers called enantiomers.

The two forms of glyceraldehydes are taken as the basis of classification of amino acids into L and D form (in Latin Laevus and dexter meaning "Left and Right" respectively). The two isomers of each amino acids are designated L and D amino acids on the basis of similarity to the glyceraldehydes standard. In the L-form of glyceraldehydes the hydroxide group is on the left side of the molecule and in the D-form it is on the right side. (First projection). In an amino acid, the position of amino group on the left or right side of the a-carbon determines the L or D designation. The amino acids present in all the proteins is of L-form, although D-amino acids occur in nature, in some antibiotics and in the bacterial cell walls.

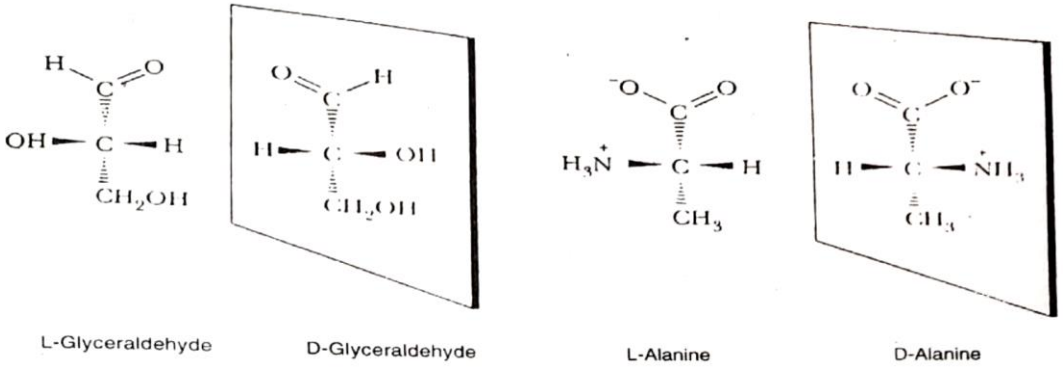


Fig. 4.1: Chiral Compounds

The R group determines the identity of the particular amino acid. R-groups vary in structure, size and electric charge, and influence the solubility of the amino acids in water.

The side chain of the simplest amino acid glycine is a hydrogen atom and in this case also two hydrogen atoms are bonded to the $\propto-$carbon atom. In all other amino acids the side chain is larger and more complex. Side chain carbon atoms are designated with letters of Greek alphabet, counting form the a-carbon. These carbon atoms are in turn numbered – β, γ, δ, ε in carbons a terminal carbon atoms is referred to as w carbon because this is the last alphabet. The standard amino acids have been assigned three letter abbreviations and one letter symbols which are used as shorthand to indicate the composition and sequence of amino acids polymerized in proteins.

The polarity of R groups varies widely from totally nonpolar or hydrophobic (water insoluble) to highly polar or hydrophilic (water soluble). Within each class there are graduation of polarity, size and shapes of R groups.

Amino acids with uncharged polar side chains are relatively hydrophilic and are usually on the outside of the proteins, while the side chains on non-polar amino acids tend to cluster together on the inside. Amino acids with basic or acidic side chains are polar they are nearly always found on the outside of protein molecules.

Aromatic amino acids phynyalanine, tyrosine, tryptophan, show absorption maxima in the year ultraviolet region. The absorption bands arise from the interaction of radiation with electrons in the aromatic rings. The near ultraviolet absorption properties of proteins are determined solely by the content of these three aromatic amino acids. In solution, UV absorption can be quantified with the help of a conventional in UV-Vis-Spectrophotometer and used as a measure of the concentration of proteins.

All amino acids show asymmetry.

Amino Acids can act as acids and bases.

When an amino acid is dissolved in water it exists in solution as dipolar ion or Zwitterion (in German for "hybrid ion"). A Zwitter ion can act as either an acid (proton donor) or a base (proton acceptor).

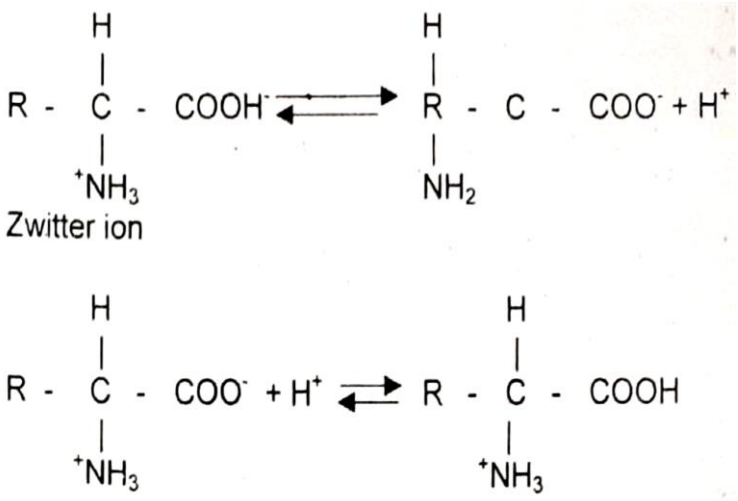


Fig: 4.2 Zwitter Ion

Non ionic form does not occur in significant amounts in aqueous solutions. Thus, substances having this type of dual nature are amphoteric and are often called as ampholytes (from amphoteric electrolytes).

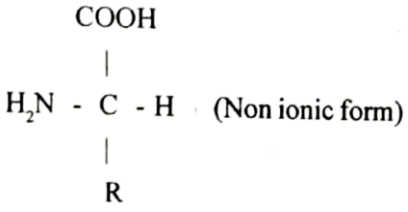


Fig: 4.3 Non ionic form

Alanine, a simple mono amino, mono carboxyl a-amino and is a diprotic and when fully protonated it has two groups the – COOH group and ther – NH_3^+ group, that yield protons.

Titration of Amino Acid

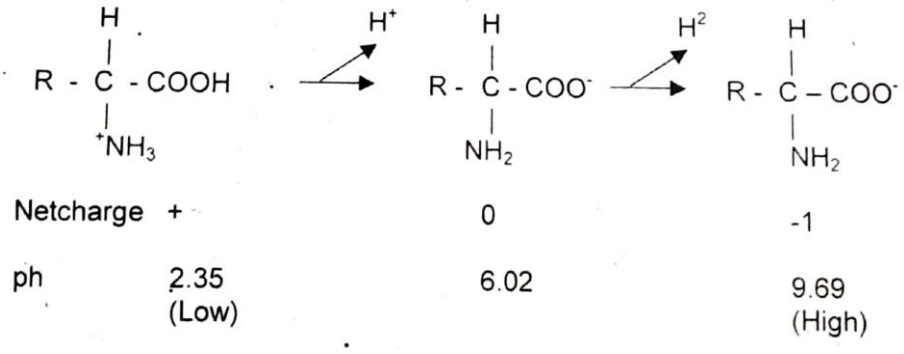


Fig: 4.4

When amino acids placed in solution at pH 7, the amino group and carboxyl groups are both ionized. This state of ionization varies with pH. In acid solution eg. pH 1 there are plenty of protons about and the carboxyl group is not ionized. In alkaline solution eg. pH 11 less protons about wo the amino group is not ionized but the carboxyl group is ionized.

Ionization of amino acids is an important property. This alteration in ionization of amino acids gives amino acids at least 2 characteristic pK values, excluding any contribution from the side group. Alanine shows two pK values pK = 2.35 due to the ionization of carboxyl group and pK2 = -9.69 due to deionization of the amino group.

Change in net charge of an amino group can be seen clearly a titration curve for an amino acid i.e., **amino acids have characteristic titration curves**. Acid base titration involved the gradual addition or removal of protons.

At low pH values, molecules of amino acids bear net charge of +1 due to ionization of amino group. As the pH increases the carboxyl group begins to ionize. For alanine with pK.1 = 2.35, at pH 2.35 then 50% of the molecules as CO_2H and 50%, as COO_2. Further, increase in pH upto pH 6.02 and all the CO_2H has been ionized and the net charge is zero. This is the Iso-

Electric Point (IEP). Further, increase in pH and amino group begins to deionise. For alanine with pK_2 at 9.69, at pH 9.69 50% of them are as NH_2 and 50% as NH_3^+.

(pH and pKa (pK_1 and pK_2) are simply convenient notation for proton conservation and the equilibrium constant for ionization, respectively).

pKa is a measure of the tendency of a group of to give up a proton with the tendency deceasing ten fold as the pKa increased by one unit.

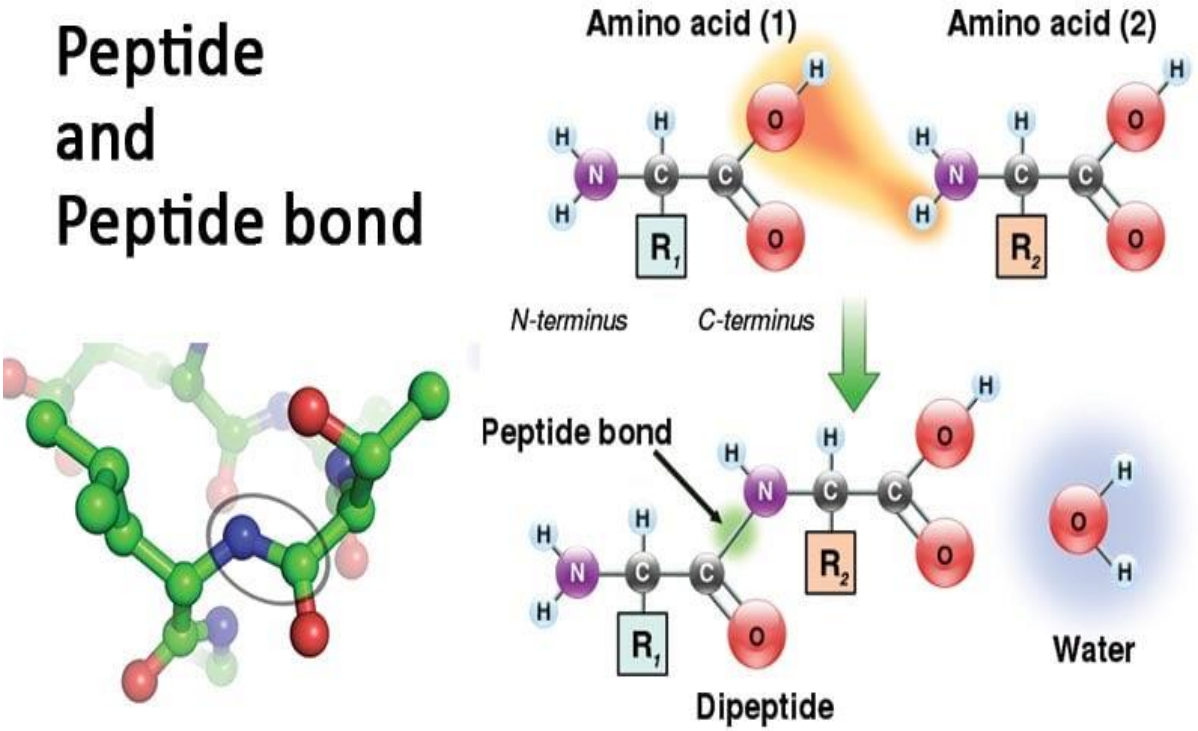
If the pH of a solution is known then it is possible to workout the state of ionization of the amino acid. The state of ionization of an amino acid provides vital biological properties to many proteins and enzymes and for this reason cells and enzymes cannot tolerate wide changes in pH. If the state of key amino acid is altered then this causes a loss of biological activity. Ability to take up and release protons gives amino acids some buffering capacity to resist severe changes in pH.

Amino acids can be separated by paper chromatography, thin layer chromatography and also by ion exchange chromatography and can be detected by a reagent known as Ninhydrin.

4.3 Peptide Bonds

- A peptide is a short-chain made up of **amino acid** which, together with other peptides, forms a protein.
- The number of amino acids in a peptide can range from two amino acids to fifty amino acids.
- Based on the number of amino acids present in the peptide, peptides are of many types; peptides with ten or fewer amino acids are termed oligopeptides, and the peptides with more than ten amino acids are termed polypeptides.
- Polypeptides with around 100 amino acids are then considered proteins.
- A peptide bond is a special type of amide bond formed between two molecules where an α-carboxyl group of one molecule reacts with the α-amino group of another molecule releasing a water molecule.
- The peptide bond is also referred to as the isopeptide bond where the amide bond forms between the carboxyl group of one amino acid and the amino group of another amino acid at other positions than the alpha.
- The process of formation of the peptide bond is an example of a condensation reaction resulting in dehydration (removal of water).
- Peptide bonds are covalent bonds that exist between any two amino acids resulting in a peptide chain.
- A partial double bond exists between carbon and nitrogen of the amide bond which stabilizes the peptide bond.
- The nitrogen involved in the bond donates its lone pair to the carbonyl group resulting in a resonance effect.
- The resonance is highly stabilizing as the electrons can be delocalized over multiple atoms resulting in a resonance structure.
- Thus, the resonance structure stabilizes the bond but also limits the rotation around the amide bond due to the partial double bond.

- Peptide bonds have a planar configuration that undergoes very little movement around the C-N bond but the other single bonds on either side of the C-N bond exhibit a high degree of rotational motion.



4.4 CLASSIFICATION

Based on R-groups, amino acids are classified according to several criteria namely solubility in water which influenced the polarity of their side chains. Polar or nonpolar nature of side chains; presence of an acidic or basic group in the side chain; charged or uncharged; hydrophilic or hydrophobic or presence of functional groups in the side chains and the nature of these groups (Fig.4.5).

Hydrophilic (water soluble), amino acids have ionized or polar side chains. At neutral pH arginine and lysine are positively charged; because they have extra amino group, in addition to that present in all the amino acid (basic amino acids); aspartic acid and glutamic acid are negatively charged (because they have extra carboxyl groups in their side chains in addition to the already present in the all amino acids); acidic amino acids. The four amino acids are the prime contributors to the overall charge of a protein. The fifth amino acid is histidine which has an inudozole side chain, at pH 5.8, histidine residues are protonated andthis carry positive charge whereas at pH 7.8 it is present as uncharged (basic amino acids).

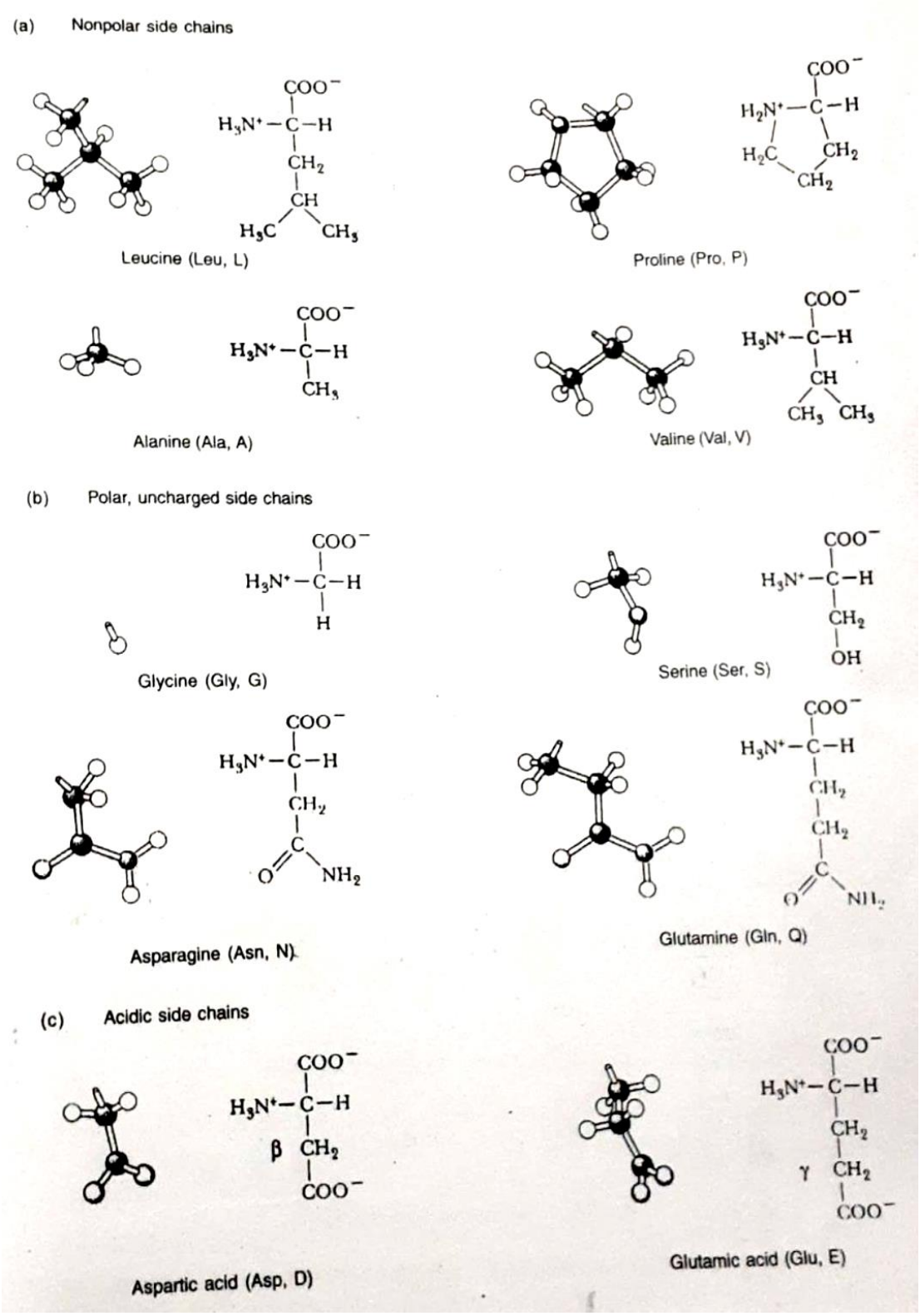


Fig: 4.5 Non polar side chains and polar uncharged side chains

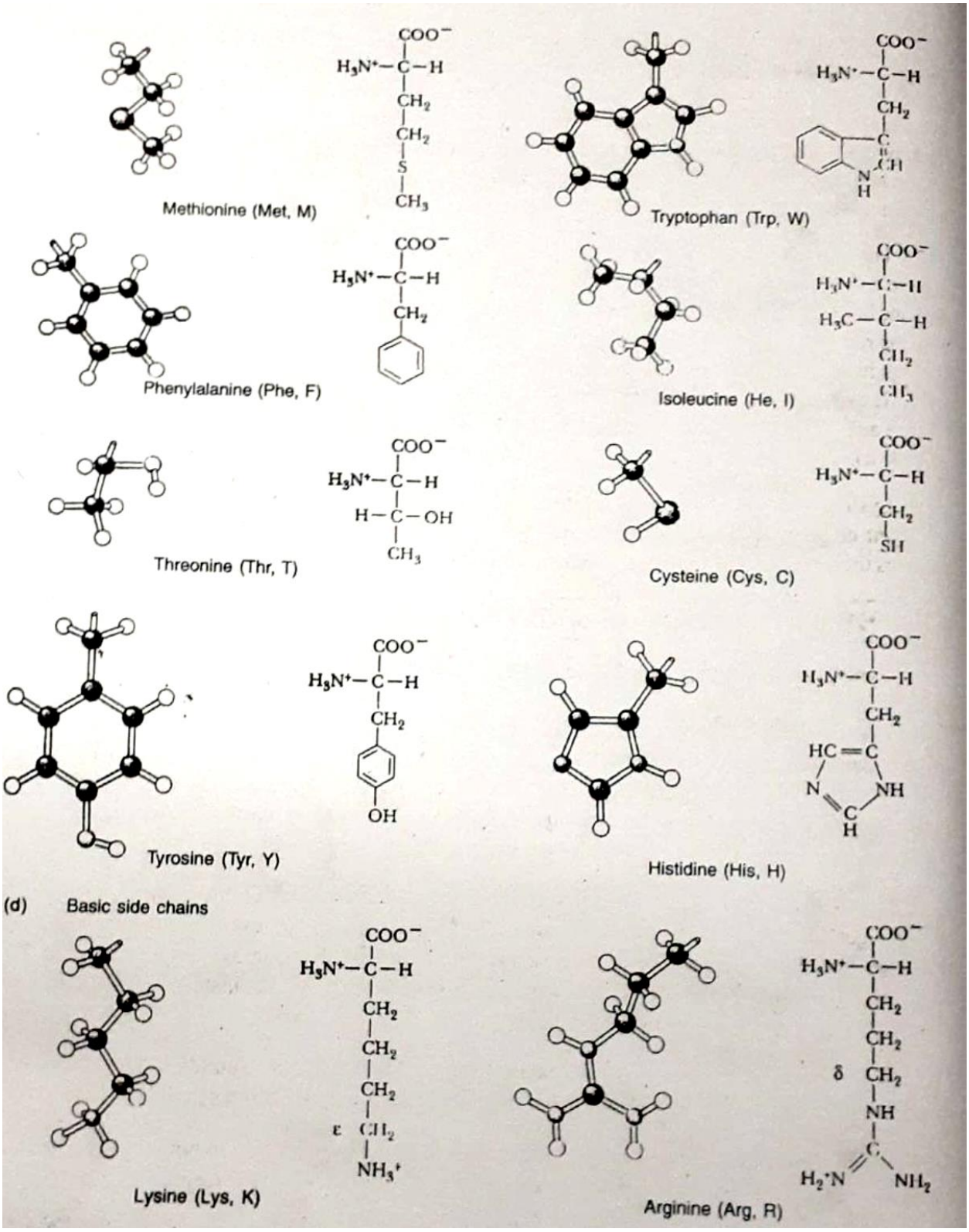


Fig. – 4.6 : Classification of Amino Acids

Asparagine and glutamine are uncharged but have polar amide groups with extensive hydrogen bonding capacities. Similarly, serine and threonine are uncharged but have polar hydroxyl groups, which also participate in hydrogen bonds with other polar molecules. The amino acid glutamine and asparaginesare considered to be derivatives of glutamic acid and aspartic acid respectively because the charged and polar amino acids are hydrophilic. They are usually found at the surface of water-soluble proteins where they contribute not only to the solubility of the protein in water but also form binding sites for charged molecules.

Hydrophobic (water hating) amino acids have aliphatic side chains (aliphatic refers to absence of benzene ring or related structure), which are insoluble or only slightly soluble in water. The side chains of alanine, valine, leucine, isoleucine, and methionine consists entirely of hydrocarbon, except for sulphur atom in metheonine and all of them are non polar. Phenylalanine, tyrosine and tryptophan have large bulk, aromatic side groups. (contain a cyclic group similar to a benzene ring rather than aliphatic) because of their non polar release, the hydropholic amino acids pack into the interior of the proteins away from aqueous environment.

The side chains of cysteine contain a reactive sulfhydryl (-SH) group, which can oxidize to form a disulfide (S-S) bond to a second cystine. Glycine and proline exhibit special roles in proteins because of unique properties of their side chains. When the sulfhydryl group in cystine remains reduced, this amino acid is very hydrophobic. Glycine which has a single hydrogen atom as its R group and because its small size allows it to fit into tight spaces. Proline has a aliphatic cyclic ring that is produced by the formation of a covalent bond between its 'R' group and the amino group of a-carbon atom. Proline is a very rigid and its presence creates a fixed knick in a polypeptide chain.

Lysine, arginine, glutamic acid, aspartic acid and alanine, valine leucine are the most abundant charged and hydrophobic amino acids. Some amino acids are more abundant in proteins than others. Cystine, tryptophan and methionine are rare amino acids.

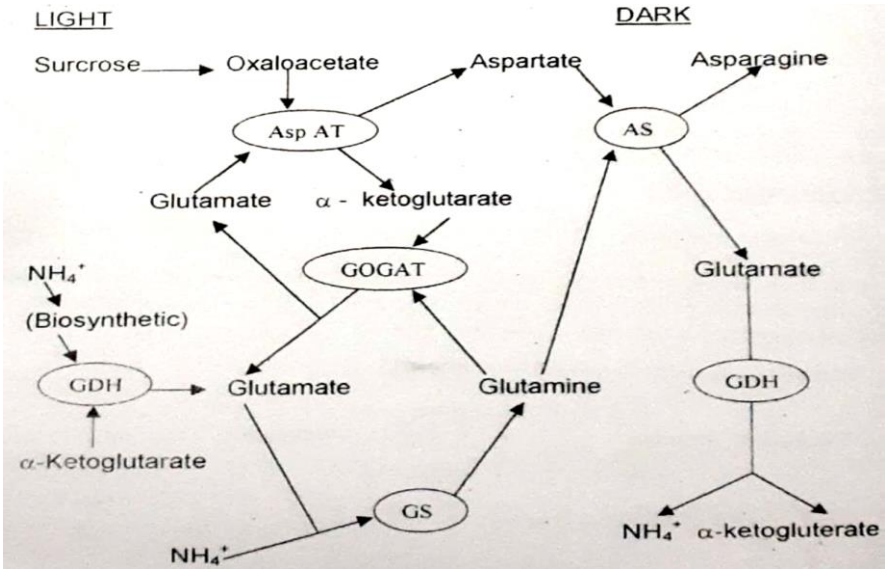


Fig. – 4.7 : Light and Carbon Regulation of Amino Acids

The assimilation of inorganic nitrogen into amino acids is controlled both by light and metabolic factors. For example, the genes for nitrate reductace and nitrite reductace are induced by light and by sucrose. Similarly, light regulates the expression of genes involved in assimilation of ammonium into glutamine and glutamate particularly chloroplast GS_2 and Fd-GOGAT. In contrast, light represses the expression of genes for AS. These regulation may be mediated by the plant photoreceptor, phytochrome. Light induced synthesis of carbohydrates appears to effect the expression of genes involved in nitrogen assimilation. Sucrose or glucose also appears to effect the expression of genes for GS_2 or Fd-GOGAT. In contrast, sucrose represses the expression of AS genes when supplied to dark adopted maize explants or whole Arabidopsis plants. Sucrose induction of GS expression is repressed by amino acids, whereas amino acids relieve sucrose repression of AS gene expression.

4.5 Biological Significance of Amino Acids:

Amino acids are the fundamental building blocks of life, crucial for various biological processes, including protein synthesis, enzyme formation, and the production of hormones and neurotransmitters. They are essential for tissue growth, repair, and metabolism, playing a vital role in sustaining life.

Here's a more detailed look at their significance:

1. Protein Synthesis: Amino acids are the monomers that combine to form proteins, which are essential for a wide range of functions, including structural support, enzyme catalysis, and defense mechanisms. The specific sequence of amino acids in a protein determines its unique three-dimensional structure and function.
2. Enzyme Formation: Many enzymes, which are biological catalysts, are also made of amino acids. They accelerate chemical reactions in the body, making them essential for various metabolic processes.
3. Hormone and Neurotransmitter Production: Some amino acids are precursors for the synthesis of hormones and neurotransmitters, which are crucial for regulating various physiological processes, including growth, metabolism, and mood.
4. Tissue Growth and Repair: Amino acids are essential for building and repairing body tissues. They are used to create new cells and replace damaged ones, contributing to overall health and well-being.
5. Energy Source: Amino acids can also be metabolized to provide energy during periods of fasting or when energy demands are high. They can be broken down to produce glucose or other metabolic intermediates.
6. Other Roles:

- **Nitrogen Handling:**

  Amino acids are involved in the metabolism of nitrogen, which is an essential element for building various biomolecules, according to the National Institutes of Health (NIH).

- **Cellular Structure and Function:**

  Amino acids contribute to the structure and function of cells, playing a role in various cellular processes.

- **Neurotransmission:**

  Some amino acids, like glutamate and GABA, act as neurotransmitters, facilitating communication between nerve cells.

4.6 SUMMARY

Amino acids can be conveniently divided into protein, nonprotein and modified forms. Protein amino acids are twenty in number and all of them are constituents of proteins. Non protein amino acids are over 300, they are not constituents of proteins and exhibit variety of functions. Modified form of amino acids is protein amino acids, which are modified after they have been incorporated into proteins. Twenty protein amino acids are building blocks of proteins. All the amino acids found in proteins are a amino acids i.e., they have a carboxyl group and an amino group bonded to the same carbon atom. In all amino acids except glycine, the a-carbon is bonded to 4 different groups, a carboxyl group, an amino group, R group and a hydrogen atom. In glycine, R-group is another hydrogen atom. Amino acids differ from each other in their R-groups (side chains) i.e., R-group is characteristic for each amino acid.

R group determines their identity. R-groups vary in structure size and electric charge, which interfere in the solubility of the amino acids in water. Amino acids are classified on the basis of several criteria such as acid base properties, hydrophobic or hydrophilic, 'R' group, polar and non-polar nature and based on functional groups etc.

All amino acids are chiral except glycine which is achiral molecule. Amino acids are of L and D forms but protein have only L forms. They exist as dipolar ions (Zwitterions). They can act as acids and as bases. Amino acids differ in their acid base properties, they have characteristic titration curves. Aromatic amino acids absorb light in the near ultra-violet region.

Amino acids can be separated by paper chromatography, thin layer chromatography and also by ion exchange chromatography and can be detected by a reagent known as Ninhydrin.

Two amino acids are joined together to form a peptide bond, many amino acids are joined together to form polypeptides (proteins).

Biosynthesis of amino acids requires carbon skeletons, which are derived from glycolysis, photosynthetic carbon reduction, oxidative pentose phosphate pathway and citric acid cycle. Further from these carbon skeletons (mostly organic acid products) all the 20 amino acids are synthesized. Ammonia produced from nitrate reduction, symbiotic N_2 fixation or other sources is toxic to plants and therefore this is assimilated into other nitrogenous compounds. Glutamine synthetase and glutamate synthase catalyse the initial steps in ammonia assimilation.

From the initial steps of ammonia assimilation, glutamate, glutamine are produced. From these, all other amino acids are formed through transamination reactions. Glutamate, glutamine and asparagines are used to transfer nitrogen from source organs to sink tissues. Light and carbon metabolism regulate the assimilation of nitrogen into amino acids.

4.7 SELF ASSESSMENT

I. Answer the following questions in about 30 lines

1. Write the biosynthesis of amino acids with special reference to GS and GOGAT.
2. Write an essay on classification of amino acids.

II. Answer the following questions in about 15 lines.

1. What are the general properties of amino acids.
2. Describe regulation of amino acid biosynthesis.
3. Give a short note Glutamine Synthetase.

4.8 Suggested Readings

1. Leininger's Principles of Biochemistry (2000) by Nelson, David Land Cox, MM, Macmillan/worth, NY.
2. Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte W Pratt, John Wiley &Sons, NY.
3. Biochemistry IIIed (1994) by Lubert Stryer, WH Freeman and Co., San Francisco.

Outlines of Biochemistry (1987) by Eric E Conn, P K Stumpf, G Bruening and Ray H Doi, wiley &S ons, NY.

Prof V. Umamaheswara Rao

Lesson-5
PROTEINS

Objectives: After going through this unit, you will be able to:

- classify proteins
- describe the structure of proteins
- discuss the isolation and purification of proteins and.
- analyze the method of column chromatography.

5.1 INTRODUCTION

Proteins may be defined as macromolecules, which are polymers of amino acids, joined together with peptide linkage. Proteins are chief constituents of all living matter. All proteins contain Carbon, Hydrogen, Oxygen, Nitrogen and Sulphur and some contain Phosphorous also. Proteins are the fundamental constituents of all the living cells and are involved in cell functions. Enzymes are made up of proteins. They are involved in many biochemical functions of the living cells.

5.2 CLASSIFICATION OF PROTEINS

Proteins may be classified into three main groups:- Simple, Conjugated and Derived proteins.

5.2.1 Simple Proteins

These are proteins, which on hydrolysis yield only amino acids. These include the following groups.

a) **Albumins:** These are soluble in water heat labile and precipitated at high salt concentrations.
   Ex: Serum albumin, egg albumin and Lact albumin (Milk)
b) **Globulins:** These are insoluble in water, soluble in dilute salt solutions of strong acids and bases e.g. Serum globulin, Tissue globulin and Vegetable globulin like Tuberin (Potato) and legumin (Peas).

c) **Glutelins:** These are insoluble in water but soluble in dilute acids and alkalis. They are coagulated by heat.
Eg: Glutenin (Wheat) and Oryzenin (Rice).
d) **Prolamines:** These are insoluble in water but soluble in 60 to 80 percent alcohol.
Eg: Gliadin (Wheat), Zein (Maize).
e) **Histones:** These are basic proteins, which are soluble in water and dilute acids.
Eg: Histones (Thymus).
f) **Protamines:** These are also basic proteins and are soluble in water, dilute acids and alkalis. They are not coagulated by heat.
Eg: SturunSalmine (Sperm of Fish)
g) **Scleroproteins:** These are insoluble in water and dilute acids and alkalies.
Eg: Elastin and Keratin

5.2.2 Conjugated Proteins

These are proteins, which contain some non-protein group called "Prosthetic group which can be separated from the protein part by carrying out hydrolysis very carefully. Conjugated proteins include the following groups.

a) **Nucleo Proteins:** In this group of proteins the prosthetic group is a nucleic acid. They are soluble in water. Eg. Ribosomes and Nucleohistones
b) **Lipoproteins:** These are combination of proteins with lipids. Eg: Lipoproteins of blood, Lipoproteins of egg-yolk and cell membranes.
c) **Glycoproteins:** These are combination of proteins with carbohydrates. They are also known as Muco-proteins. Eg: Mucin (Saliva) and egg white.
d) **Chromoproteins:** They contain prophyrin (with a metal ion) as their prosthetic group. Eg: Hemoglobin, Hemocynin, Myoglobin and Cytochrome.
e) **Phosphoproteins:** They contain phosphorous radical as a prosthetic group. Eg: Casein (Milk) and Ovovitelin (Egg yolk)
f) **Metalloproteins:** They contain metal ions as their prosthetic group. The metal ions found in metalloproteins are Iron, Magnesium, Copper, Zinc and Manganese. Eg: Hemogloblin, Chlorophyll and Ferrition.

5.2.3 Derived Proteins

When proteins are hydrolysed by acids, alkalines or enzymes, the degradation products obtained from them are called "Derived Proteins". They are further classified as (A) Primary derivatives and (B) Secondary derivatives.

A. Primary Derivatives

a) **Proteins:** They are derived in the early stage of protein hydrolysis by dilute acids alkalies or enzymes. Eg: Fibrin from Fibrinogen.
b) **Metaproteins:** They are derived in the later stage of hydrolysis by slightly stronger acids and alkalis. Eg: Acid and Alkali Metaloproteins.
c) **Coagulated Proteins:** These are denatured proteins formed by the action of heat, X-rays and Ultraviolet rays. Eg: Cooked proteins and coagulated albumins.

B. Secondary Derivatives

They are precipitated by saturation with ammonium sulphate, but are not coagulated by heat. Eg: Albumose from albumin, globulose from globlin and peptides like Glycyl-alkanine and leucyl-glutamic acid.

5.3 STRUCTURE OF PROTEINS

Proteins are built from several smaller units called polypeptides. These polypeptides are made up of amino acids. The number of amino acids in a protein may vary considerably. A peptide is composed of two or more amino acids joined together through peptide bond. The term peptide bond is applied to the amide link between amino acid residues. Although more than 100 amino acids are identified only 20 of them participate in the synthesis of proteins. The structure of proteins is considered by their structural configuration and several levels of organization. Based on these different configurations the structure of proteins can be derived as primary, secondary, tertiary and quaternary structures of proteins.

5.3.1 Primary Structure

The peptide bond is formed by the amino acids linked by carboxyl group of one amino acid with the $\propto -$ amino group of another amino acid as shown below:

$H_2N-CH_2-CO-NH-CH_2-COOH$

Glycyl – Glycine

The primary structure ultimately becomes as shown below:

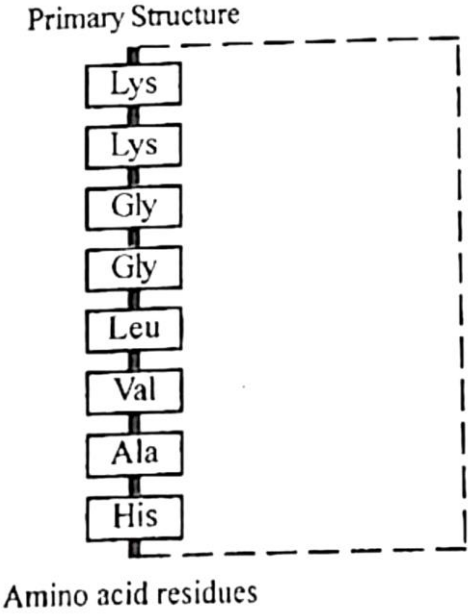


Fig. 5.1 : Primary Structure of Protein

5.3.2 Secondary Structure

Most long peptide chains are folded or coiled in a number of ways. This brings about a second level of organization called the secondary structure. Globular proteins indicate a coiled structure in which peptide bonds occur in a regular manner. In many proteins, the most common form of coiling is the right-handed alpha helix. Only the right-handed $\propto -$ helix exists in nature since it is far more stable form of L-amino acids than the left handed helix. The secondary structure is shown in figure 5.2.

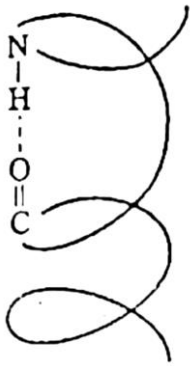


Fig. 5.2 : Secondary Structure of Protein

$\propto$−helix: In the structure of $\propto$−helix it has been found that R groups on the $\propto$−carbon atoms exist outward from the center of helix. In this helical form there are 3.6 amino acid residues per turn of the helix. The $\propto$−helix is stabilized by intramolecular hydrogen bonds between an amide nitrogen (-NH group) and the Carbonyl oxygen (-C=O group) of the fourth amino acid residue away in the peptide chain. Another form of secondary structure is the β-pleated sheet.

β-Pleated Sheet: This results from hydrogen bonding between two peptide chains. The chains may be parallel or anti-parallel. In a β-pleated sheet when the adjacent polypeptide chains run in opposite directions (N to C terminus) the structure is termed an anti-parallel β-pleated sheet. But when the chains run in the same direction, it is termed parallel. The β-conformation is found in fibrous protein. Most heat treatment and stretching of $\propto$−Keratin converts it into β-Keratin by breaking the stabilizing hydrogen bonds. An extended parallel chain β-pleated structure is formed. The anti-parallel structure is found in silk fibroin, which has a repeating sequence of 6 residues.

(Gly-Der-Gly-Ala-Gly-Ala).

Regions of β-pleated structure are present in many proteins and both parallel and anti-parallel forms occur.

5.3.3 Tertiary Structure

In many globular proteins the secondary structural motifs of $\propto$−helix or β-pleated sheet from recognizable super-secondary motifs (Fig). Figure illustrates several super secondary motifs β - $\propto-\beta_1$ – into stands of β-sheet connected by an $\propto$−helix; the β-harpin composed of anti-parallel β-sheet connected by short region of loop and the ‘Greekay’ motif named for its resemblance to a decorative motif on ancient Geek vases. Repetitions of these super secondary motifs can then form structures such as the regularly repeating β - $\propto-\beta$ units, of an entire protein.

The term “Tertiary structure” reflects the spatial relationships between secondary structural elements. The polypeptide chain may undergo coiling and folding to produce the Tertiary Structure. The way of the structure fold has an important bearing on the properties of the protein. The folding brings together active amino acids, which are otherwise scattered along the chain, and may form a distinctive cavity or cleft in which the substrate is bound. In proteins, consisting of a simple polypeptide chain Ex: Myoglobin the tertiary structure determines the overall shape of the molecule. Hence, proteins are called ‘globular proteins’ if

they are very compact and fibrous, proteins form long thin threads. Tertiary structure is shown in (Fig. 5.3).

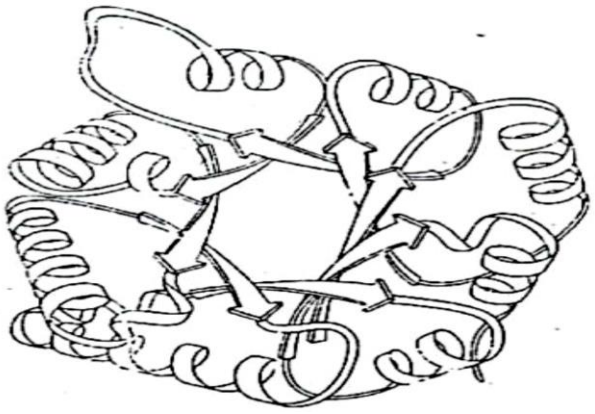


Fig. – 5.3: Tertiary Structure of Protein

The tertiary structure is maintained by a number of bonds which are of different types like a) Disulphide bonds b) Hydrogen bonds c) Electrostatic and d) Hydrophobic bonds.

a) Disulphide Bonds: These bonds give certain rigidity to the protein molecule. They confer additional stability. A disulphide bond is formed between two cysteine residues present in the same or different polypeptides. These disulphide bonds confer additional stability to specific conformations of proteins such as enzymes. (eg. Ribonuclease) and structural proteins (eg. Keratin).

b) Hydrogen Bodns: These are weak but since they are numerous they give considerable stability to the protein molecule. These bonds can be formed by sharing hydrogen between amide Nitrogen and Carbonyl oxygen of the peptide backbone. They are also formed between groups present in the side chain. The side chains of aspartic and glutamic acids, serine and threonine can all form hydrogen bonds.

c) Ionic Bonds (Electrostatic Bonds): Salt (Electrostatic) bonds link oppositely charged R groups of residues and the charged α groups of carboxyl and amino terminal residues. For eg. The R group of lysine (net charge = 1) and aspartate or glutamate (net charge – l) interacts electrostastically to stabilize proteins.

d) Hydrophobic Bonds: These are formed because of the tendency of the non-polar side chains of neutral amino acids to closely associate with one another non-polar side chains of amino acids associate in the interior of globular proteins. These associations are individually extremely weak and are not stoichiometric. Their large number dictates however that hydrophobic interactions contribute significantly to maintaining protein structure. Although hydrophobic interactions do not form true bonds they are of great importance in the formation of tertiary structure.

5.3.4 Quaternary Structure

Quaternary structure is shown by proteins containing more than one polypeptide chain. Two or more polypeptide chains may associate to give rise to the quaternary structure. If the protein consists of identical units it is said to have a homogenous quaternary structure eg.Haemoglobin. Proteins, which consist of more than one polypeptide chain are said to possess quaternary structure. Haemoglobin contains four independent polypeptide chains, two identical α chains and two identical β chains. The chains in haemoglobin are not held together by covalent bonds but by the interactions between the exposed groups of the folded chains. Each of these chains is characterized by its own secondary and tertiary structure. In the adult, the molecule consists of two α and β chains fetal haemoglobin contains two α and

two γ chains. When hemoglobin takes up oxygen there is a change in the structure due to the β chains moving closer together without structural alteration in the chains themselves. Lactic dehydrogenate is another interesting example showing quaternary structure. This protein consists of four sub-units having a molecular weight near 35,000. The subunits are somewhat asymmetrical themselves but form a rather cube like aggregate by occupying positions at the corners of a square Quaternary structure of protein as shown in Fig. 5.4.

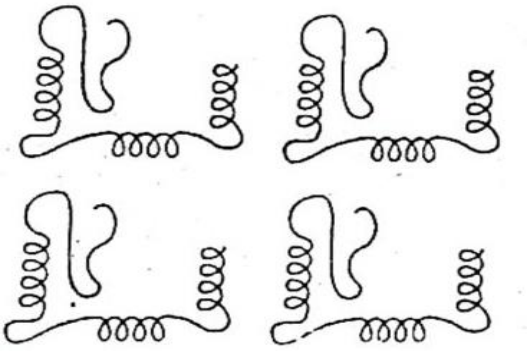


Fig. – 5.4 : Quaternary Structure of Protein

5.4 SUMMARY

Proteins are polymers of amino acids. Proteins are divided into three main groups simple, conjugated and derived proteins and examples of these groups are given. Structure of proteins. There are four structures namely Primary, Secondary, Tertiary and Quaternary. Diagrams of different structures are explained. To study a protein it must be separated or isolated from all other proteins. Methods of Isolation and purification of proteins with examples like column chromatography methods are explained with suitable diagrams.

5.5 SELF ASSESSMENT

I. Answer the following questions in about 30 lines

1. Give a detailed account of classification of proteins with examples.
2. Write an essay on Isolation and purification of proteins.
3. Discuss various forms of structure of proteins with suitable diagrams.
4. Explain briefly the column chromatography methods for fractionation of proteins.

II. Answer the following questions in about 15 lines.

1. Write short notes on Secondary Structure of Proteins with a suitable diagram.
2. Explain briefly the procedure of Column Chromotography methods for fractionation of Proteins.

5.6 Suggested Readings

1) Leininger's Principles of Biochemistry (2000) by Nelson, David Land Cox, MM, Macmillan/worth, NY.
2) Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte WPratt, John Wiley & Sons, NY.
3) Biochemistry IIIed (1994) by Lubert Stryer, WH Freeman and Co., San Francisco. Outlines of Biochemistry (1987) by Eric EConn, PK Stumpf ,G Brueningand Ray H Doi, John Wiley & Sons, NY.

Prof V. Umamaheswara Rao

Lesson-6

CHAPERONES AND DENATURATION AND RENATURATION OF PROTEINS

Objectives:

understand basic concept of chaperons and denaturation and renaturation of proteins

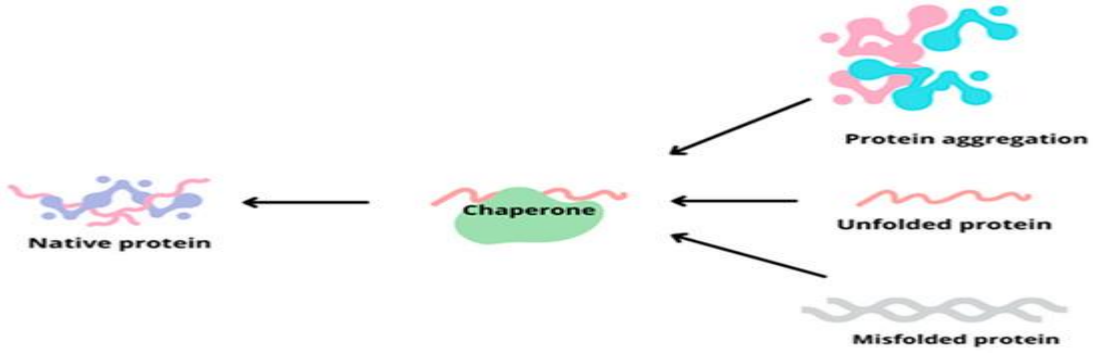
6.1 Introduction

Chaperones are proteins that assist other proteins in correctly folding, unfolding, assembling, and disassembling. They are essential for maintaining protein homeostasis and ensuring proper cellular function. Chaperones help prevent misfolding, aggregation, and degradation of proteins, ultimately contributing to cellular health and preventing diseases associated with protein misfolding.

Denaturation refers to the disruption of a protein's three-dimensional structure, making it inactive, while renaturation is the process where the protein regains its original structure. Denaturation can be caused by various factors like heat, chemicals, or extreme pH, disrupting the bonds that maintain the protein's shape. In some cases, denaturation is reversible, and the protein can refold into its original, functional state through renaturation.

6.2 Chaperons

Molecular chaperones are proteins and protein complexes that bind to misfolded or unfolded **polypeptide** chains and affect the subsequent folding processes of these chains. All proteins are created at the ribosome as straight chains of amino acids, but must be folded into a precise, three-dimensional shape (conformation) in order to perform their specific functions. The misfolded or unfolded polypeptide chains to which chaperones bind are said to be "non-native," meaning that they are not folded into their functional conformation. Chaperones are found in all types of cells and cellular compartments, and have a wide range of binding specificities and functional roles.



6.2.1 Discovery of Chaperons

Chaperones were originally identified in the mid-1980s from studies of protein folding and assembly in plant chloroplasts. A new protein was identified that was required for correct folding of a large enzyme complex in chloroplasts, yet the mysterious protein was not associated with the final assembled complex. It was quickly determined that this "chaperone" protein directing correct assembly was identical to one of the many proteins expressed at high levels when cells are grown at high temperatures (hence the common alternative name, "heat-shock protein," or Hsp).

It was later discovered that chaperones recognize the non-native, partially misfolded states of proteins that accumulate during high temperature stress. Most chaperones are also abundantly expressed under normal cell growth conditions, where they recognize non-native conformations occurring during both protein synthesis (prior to correct polypeptide chain folding), and later misfolding events.

6.2.2 Chaperon systems

Hsp70 chaperones (so called because their size is approximately 70,000 daltons, or atomic mass units) are a very large family of proteins whose amino acid sequences are very similar, indicating how important their structure is to their function. A single cell or cellular compartment may contain multiple Hsp70 chaperones, each with a specific function. In addition, the Hsp70 chaperones often work in concert with one or more smaller co-chaperone proteins, which serve to modulate the activity of the chaperone.

Some of the well-studied Hsp70 chaperones include DnaK from the bacterium *Escherichia coli*, the Ssa and Ssb proteins from yeast, and BiP (for "binding protein") from the mammalian **endoplasmic reticulum** . Hsp70 chaperones are often located where unfolded polypeptide chains typically appear. For example, Ssb chaperones associate with ribosomes, so that they are close to newly synthesized, unstructured polypeptide chains. It is thought that the binding of Hsp70 chaperones to these unfolded chains prevents inappropriate partial folding until the entire polypeptide chain is available for correct folding.

Hsp60 chaperones (also called "chaperonins") are barrel-shaped structures composed of fourteen to sixteen subunits of proteins that are approximately 60,000 daltons in size. Each subunit has a patch of non-polar amino acid groups lining the inner surface of the barrel; this patch recognizes the exposed non-polar amino acids of misfolded proteins. The binding and **hydrolysis** of ATP triggers conformational changes within the barrel, which (1) unfolds the misfolded conformation and releases the unfolded chain into the center of the barrel, (2) closes the top of the barrel with the binding of a co-chaperone "cap," and thereby (3) provides

a protected environment in which correct folding can occur. Upon dissociation of the co-chaperone, the fully or partially folded protein is released into the general cellular environment.

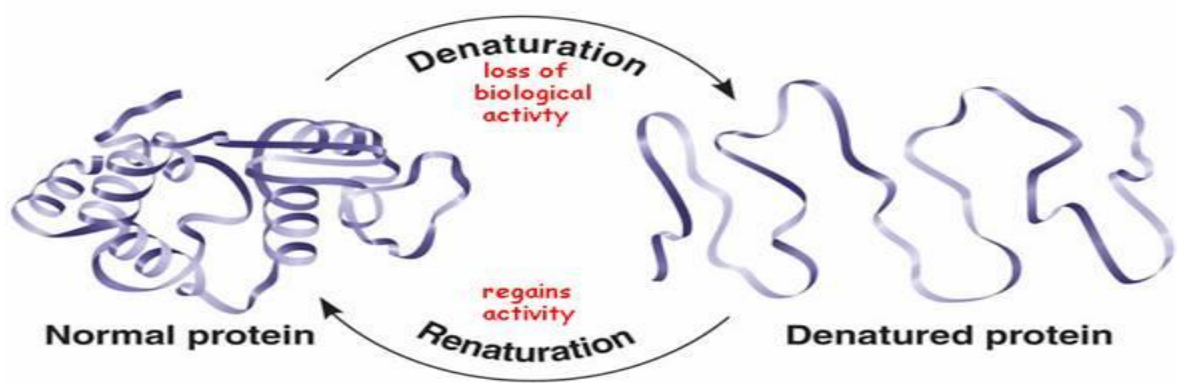
The most extensively studied Hsp60 chaperones include GroEL from *E. coli* and TRiC/CCT from eukaryotic cells. GroEL appears to function as a general chaperone and interacts with 10 to 15 percent of all *E. coli* polypeptide chains, with a definite bias toward proteins that are small enough to fit within its central cavity. TRiC/CCT recognizes a much smaller set of proteins, and appears to play an additional role in the assembly of multiprotein complexes.

6.2.3 Chaperons and Human Diseases

It is clear that molecular chaperones assist with the folding of newly synthesized proteins and correct protein misfolding. Recent studies now suggest that defects in molecular chaperone/substrate interactions may also play a substantial role in human disease. For example, mutations linked to Alzheimer's disease have been shown to disrupt the expression of chaperones in the endoplasmic reticulum. In addition, several genes linked to eye degeneration diseases have recently been identified as putative molecular chaperones.

6.3 Denaturation of Proteins

Protein structures have evolved to function in particular cellular environments. Conditions different from those in the cell can result in protein structural changes, large and small. A loss of three-dimensional structure sufficient to cause loss of function is called denaturation. The denatured state does not necessarily equate with complete unfolding of the protein and randomization of conformation. Most proteins can be denatured by heat, which affects the weak interactions in a protein (primarily hydrogen bonds) in a complex manner. If the temperature is increased slowly, a protein’s conformation generally remains intact until an abrupt loss of structure (and function) occurs over a narrow temperature range. The abruptness of the change suggests that unfolding is a cooperative process: loss of structure in one part of the protein destabilizes other parts. The effects of heat on proteins are not readily predictable. Proteins can be denatured not only by heat but by extremes of pH, by certain miscible organic solvents such as alcohol or acetone, by certain solutes such as urea and guanidine hydrochloride, or by detergents. Each of these denaturing agents represents a relatively mild treatment in the sense that no covalent bonds in the polypeptide chain are broken. Organic solvents, urea, and detergents act primarily by disrupting the hydrophobic interactions that make up the stable core of globular proteins; extremes of pH alter the net charge on the protein, causing electrostatic repulsion and the disruption of some hydrogen bonding. The denatured states obtained with these various treatments need not be equivalent.



6.4 Renaturation of proteins

Protein renaturation is the process of a denatured protein regaining its original, functional three-dimensional structure. It's essentially the reverse of protein denaturation, where the protein's structure is disrupted, often by heat, chemicals, or other factors. Success in renaturation depends on various factors including the protein's sequence, the conditions under which it was denatured, and the chosen renaturation method.
Methods for Protein Renaturation:

- **Dilution:**
  Rapid dilution of a denaturing agent (like urea or guanidine hydrochloride) can allow the protein to refold.
- **Cooling:**
  Gradually lowering the temperature can help proteins refold into their native structure.
- **Dialysis:**
  This technique involves separating denatured proteins from the denaturing agent by passing the protein solution through a semipermeable membrane.
- **Chromatography:**
  Techniques like size exclusion, ion exchange, or affinity chromatography can be used to purify and renature proteins.
- **Chaperones:**
  Proteins called molecular chaperones can assist in the folding process, guiding unfolded proteins to their native structure and preventing aggregation.

6.5 Summary

Chaperones are proteins that assist other proteins in folding, refolding after denaturation, and preventing aggregation.
Denaturation is the process where a protein loses its native structure due to factors like heat or chemicals renaturation is the attempt to recover the native structure after denaturation.

6.6 Self-Assessment

1) How chaperons play an important role in biological systems?
2) what are chaperones and how do they affect in protein renaturation?
3) detailed note on denaturation and renaturation of proteins?

6.7 Suggested Readings

1) Leininger's Principles of Biochemistry (2000) by Nelson, David Land Cox, MM, Macmillan/worth, NY.
2) Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voetand Charlotte WPratt, John Wiley & Sons, NY.
3) Biochemistry IIIed (1994) by Lubert Stryer, WHFreeman and Co., San Francisco.
4) Outlines of Biochemistry (1987) by Eric EConn, PKStumpf, GBrueningand Ray H Doi, JohnWiley&Sons,NY.

Prof V. Umamaheswara Rao

Lesson-7
ENZYMES

Objectives:

After studying this unit you should be able to:describe the role of enzymes in lowering the activation energy and in coupled reactions;list the type of enzymes and cofactors explain the effects of temperature, pH and enzyme concentration on the rate of enzyme action; anddescribe the essential features of allosteric enzymes, and iso enzymes, and assay of enzyme activity.

7.1 INTRODUCTION

You have studied about the biomolecules in Unit 9 which include proteins, carbohydrates and lipids. Membrane proteins act as transporting molecules. The other important function of cellular proteins is to act as enzymes and catalyse biochemical reactions at a rate appropriate to the need of the cell.

In this unit you will study about the nature of enzymes and their functional aspects. Enzymes control the overall metabolic functions of the cell by speeding up the reaction rate. **They do not affect the equilibrium constant and remain unaffected at the end of the reaction.** The reactants bind to a specific site on the surface of enzyme molecule called active site. Enzymes show specificity for their substrate as well as for the reactions. Various factors such as temperature, pH, concentrations of enzymes and substrate affect the rate of enzyme-catalysed reactions. Some enzymes require additional factors for their normal activity. Allosteric enzymes have more than one active site which may be located on the same subunit or on different subunits. The detection and estimation of enzymes can be done by using various techniques which can measure their ability to convert the substrate into products.

Various enzyme-catalysed reactions characterise the metabolism of the cell. Regulation of these reactions is achieved by altering the enzyme activity. In the next unit you will read about the enzyme kinetics and regulation. Before you read this unit, you should refresh your memory about the structure of proteins.

7.2 CHARACTERISTICS OF ENZYMES

Enzymes act as catalysts and influence the rate of reaction in all the living organisms. In fact, essentially all chemical reactions in the living beings are catalysed by enzymes. All the enzymes known till now are proteins except ribozymes with a unique **three dimensional structure** that provides an **active site** for binding the other molecules, i.e. substrates to their surface. Each enzyme normally catalyses a few reactions but most often only one type of reaction. They are required in minute quantity/concentration to convert the substrates into products. The enzyme remains unaffected at the end of the reaction. **The enzyme molecule is much larger than the molecule of its substrate.** The molecular weights of enzymes range from thousands to millions, whereas the molecular weights of substrates are usually in few hundreds. Some enzymes are purely proteins, whereas others require non-protein assistant compounds for their catalytic activity.

Importance of Enzymes

Enzymes play an important role in **metabolism** as you know that all biochemical reactions are enzyme catalysed in the living organisms. They play an important role in **diagnosis**. Level of enzymes in blood are of diagnostic importance e.g. it is a good indicator in disease such as myocardial infarction in which creatine kinase enzyme level is very high. Enzymes can be used **therapeutically** such as digestive enzymes.

7.2.1 Activation Energy

All the chemical reactions in biological system have an energy barrier which prevents reactions from proceeding in an uncontrolled and spontaneous manner. **The input of energy required to break this energy barrier or to start a reaction is called the activation energy.** For example, a mixture of hydrogen and oxygen will not react with each other until they receive enough energy from a heat source to achieve the activation energy. You may have noticed that a tin of petrol or kerosene oil kept exposed to air at room temperature,would not catch fire unless ignited by a spark. This tiny spark is adequate to supply the activation energy for few molecules to react. The amount of energy released as result of the conversion of the first few molecules of reactant to product is sufficient to activate other molecules. Similarly, chemical reactions taking place in living organisms also require activation energy. In biological systems, **enzymes** are important components which **enhance the chemical reactions by lowering the activation energy** and help the reactions to occur at a rate appropriate to the needs of the cell. **The enzymes speed up the reactions by lowering activation energy**. Enzymes combine with different types of substrates in such a way that they reduce the amount of energy required for a particular reaction (see Fig. 7.1). In the absence of enzymes in your gut, it would take many years instead of few hours for your last meal to be digested. You will read about the mechanism of enzyme action in the following sections.

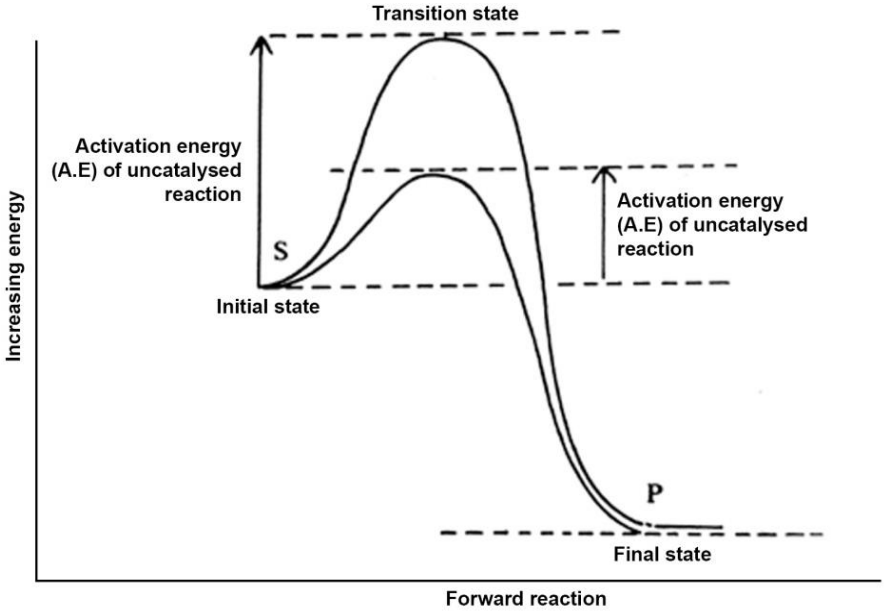


Fig. 7.1: Lowering of activation energy by an enzyme. The activation energy (A.E.) is the energy barrier which the reactants overcome as they react to form the product. The intermediate transition state of enzyme substrate complex lies at the peak of the activation energy barrier.

7.2.2 Coupled Reaction

The energy available to the cells is obtained by burning of food stuff. The breakdown of these food stuffs is carried out by series of reactions, i.e. catabolic reactions in a complex and controlled way. This energy is utilised by the cell for synthetic processes requiring energy, i.e. anabolic reactions which are endergonic in nature. Thus the catabolic reactions which are exergonic in nature and give energy, are coupled to anabolic chemical reactions. This process of coupling maintains a balance in the living systems. The coupling of these reactions is an important characteristic of living organism and is carried out with the help of enzyme-directed reaction pathways. This coupling process can be understood by examining a simple analogy where an energy releasing catabolic chemical reaction is represented by stones falling from a hill top (see Fig. 7.2). The kinetic energy of falling stones would normally be wasted in the form of heat generated when they hit the ground (see Fig. 7.2 a). A part of this kinetic energy can, however, be trapped and used to drive a paddle wheel that lifts a bucket of water, as shown in Fig. 7.2 b. So we can say that the energy producing reaction of a stone falling is directly coupled to the energy requiring reaction of lifting the bucket of water. A part of the energy of falling stones is now used in lifting a bucket of water and the stones hit the ground with less velocity. So less energy is wasted as heat. The potential energy which is stored in the form of a bucket of water can be used for other purposes such as to drive hydraulic machines (Fig. 7.2 c).

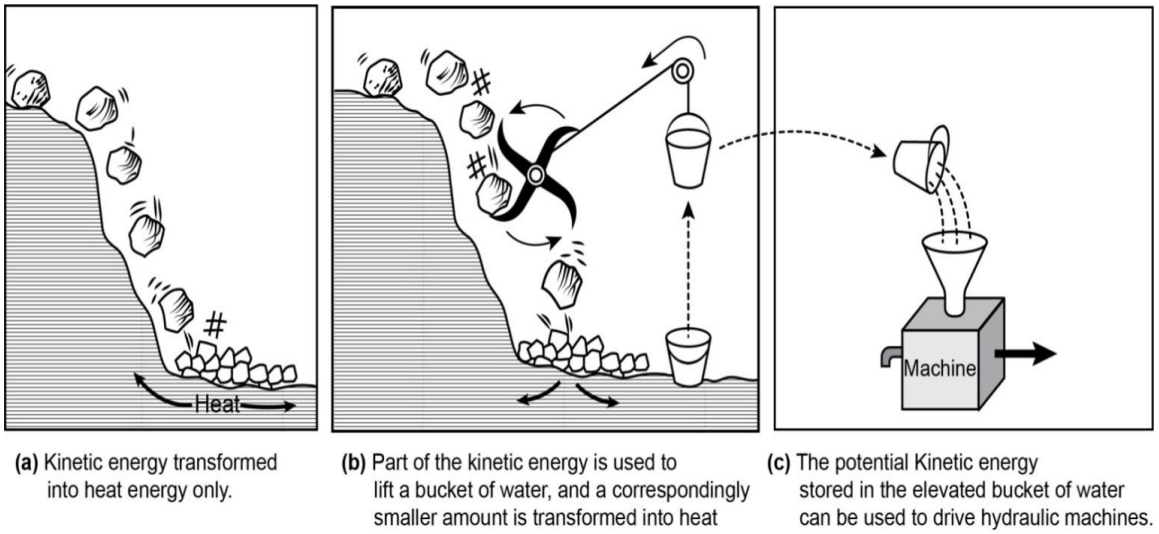


Fig. 7.2: Schematic diagram of a mechanical model indicating the coupling of reactions. Stones falling from the hill top can be compared with the oxidation of food stuffs (a). Part of the energy of falling stones is conserved in the form of lifted bucket of water which can be compared to the synthesis of ATP(b). The conserved energy can be used for energy requiring reaction such as utilisation of ATP during endergonic reactions (c).

In the living organisms, enzymes play the role of paddle wheels and generate and trap energy in the form of high energy phosphate bonds of adenosine triphosphate (ATP). The ATP thus formed is utilised as a source of energy for many energy-dependent processes and is described as an energy currency of the cell. ATP is hydrolysed in neutral aqueous solutions accompanied by release of energy. The first cleavage of ATP to produce ADP and inorganic phosphate, releases 7.3 K. cals of energy per molecule (see Fig. 7.3).

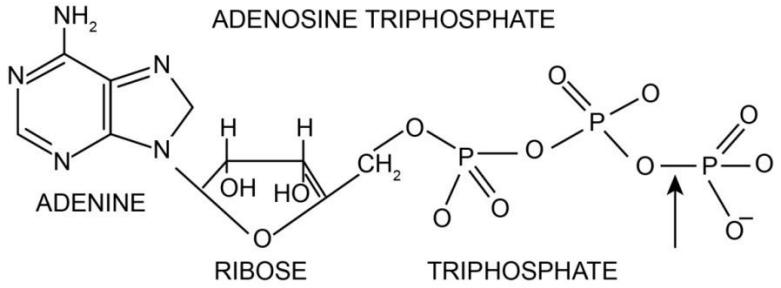


Fig. 7.3: Structural formula of ATP. The first cleavage indicated by an arrow releases 7.3 K. cals of energy per molecule.

7.3 TYPES OF ENZYMES

The Enzyme Commission of the **International Union of Biochemists (IUB)** has classified enzymes into **six categories**on the basis of their functional specificity. Each category is further sub-divided into particular groups consisting of number of enzymes (Table 9.1). Most of the enzymes end as –'ase'. The first part of the name usually indicates the substrate on which the enzyme acts as for example, amylase enzyme acts on the substrate amylose or starch. A few enzymes are still called by their old names, e.g., pepsin, trypsin, chymotrypsin etc.

Table 7.1: Classification of Enzymes (IUB system).

Sl. No.	Enzyme Category	Type of reaction	Examples
1.	Oxidoreducases	Oxidation reduction reactions	***Oxidases:*** transfer hydrogen to oxygen, e.g. cytochrome oxidase $Cyt\ H_2 + 1/2O_2 \rightleftharpoons Cyt + H_2O$ reduced cytochrome oxidized cytochrome ***Dehydrogenases*****:** transfer hydrogen to a molecule other than oxygen. e.g. lactatedehydrogenase $C_3H_6O_3 + NAD \rightleftharpoons C_3H_4O_3 + NADH_3$ lactate pyruvate This reaction occurs in liver following heavy exercise.
2.	Transferases	Transfer a functionally important group from one molecule to another	***Transaminases*****:** transfer amino groups, making new amino from existing ones e.g. asparatate transaminase aspartate + α-ketoglutarate (amino acid) ⇓ (carboxylic acid) glutamate oxaloacetate (amino acid) (carboxylic acid) This occurs in all cells. ***Kinases*****:** transfer phosphate, usually from ATP to another substance e.g. hexokinase ATP + glucose → ADP + glucose-6-phosphate This reaction activates glucose prior to its breakdown in respiration. **Phosphorylases** which add inorganic phosphate without using ATP belong to this category.
3.	Hydrolases	Split molecules in two by the action of water	*All digestive enzymes* fall into this category: pepsin, trypsin etc. e.g. amylase

			$starch_n + H_2O \rightarrow starch_{n-1}$ + maltose These reactions occur in the gut and transform large insoluble food molecules into smaller soluble ones so as to be absorbed. Similar reactions also occur in the lysosomes of cells. *Phosphatases* which remove phosphate groups from organic molecules by hydrolysis also fall in this category.
4.	Lyases	Add or remove groups without involving water	***Carboxylases*:** add CO_2 e.g. ribulosediphosphate carboxylase ribulosediphosphate + CO_2 $\rightarrow$ phosphoglyceric acid This occurs in the stroma of chloroplasts and is the principal mechanism by which atmospheric CO_2 is turned into organic material. ***Decarboxylases*:** remove CO_2 e.g. pyruvate decarboxylase pyruvic acid $\rightarrow$ acetaldehyde + CO_2released (It is an Intermediate step in the conversion of sugars to ethanol during alcoholic fermentation).
5.	Isomerases	Convert one isomer of a compound into a different isomer by redistributing the atoms	***Mutases:*** e.g. phosphoglucomutase glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate This reaction occurs at early steps in the respiration of sugars.
6.	Ligases	Link together two molecules at the expense of ATP	***Synthetases:*** e.g. aminoacylsynthetases Join amino acids to tRNA during protein synthesis.

7.4 Cofactors

As you have read above, all the enzymes are proteinaceous in nature except ribozymes. Most of the enzymes consist of only polypeptide chains. Some enzymes, however, require an additional chemical component for carrying out their catalytic function. These constituents are non-protein and are called **cofactors**. A complete, catalytically active enzyme together with its cofactor is called a **holoenzyme**. The protein part of the holoenzyme is called **apoenzyme,** which is inactive. The cofactors are of three kinds; prosthetic groups, coenzymes and metal ions.

Prosthetic groups are organic compounds and are permanently bound to apoenzymes by covalent bonds. For example, in the enzymes peroxidase and catalase, which catalyse the break down of hydrogen peroxide to water and oxygen, heme is the prosthetic group and is a permanent part of the enzyme's active site.

Coenzymes are also organic compounds, but their binding with apoenzyme is transient by non covalentbond, and occurs only during the catalysis. The same coenzyme molecule may act as cofactor in different enzyme-catalysed reactions. Apart from helping enzymes in their catalytic activity these coenzymes also function as transient carrier, i.e. temporary acceptors for specific atoms or functional groups. **Vitamins are essential components of many coenzymes.** For example, the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) contain vitamin niacin and are electron carriers. You will study about Vitamins in detail in Unit 12 of this course. Coenzyme A (CoA) contains vitamin pantothenic acid and carries (transfers) functional groups such as, an acetyl group.

Metal ions are required by certain enzymes for their catalytic activity. Metal ions such as Mg^{2+}, Mn^{2+} or Zn^{2+} bind with specific side chains at active site and at the same time with the substrate. The binding of metal ions with the substrate helps to break it down into products. For example, zinc is a cofactor for proteolytic enzyme carboxypeptidase. It binds with side chains of amino acid residues in the active site of the enzyme and with a -carboxyl group of substrate amino acid. It is here that the peptide bonds of substrate are broken by the enzyme. Table 9.2 summarises various type of cofactors.

Table 7.2: Some cofactors and their related enzymes.

Cofactor	**Enzyme**	**Reaction**
Prosthetic groups		
Heme	Catalase	$2H_2O_2 \dashrightarrow 2H_2O+O_2$
Heme	Peroxidase	$2H_2O_2 \dashrightarrow 2H_2O+O_2$
Coeznymes		
Flavin mononucleotide (FMN)	Some dehydrogenases	Removal of hydrogen atoms
Thiamine pyrophosphate (TPP)	Some decarboxylases	Removal of CO_2
Metal ions		
Zn^{2+}	Carboxypeptidase	Hydrolysis of proteins
Cu^{2+}	Ascorbic acid oxidase	Ascorbate $\rightleftharpoons$ dehydroascorbate
Mg^{2+}	Hexokinase	Glucose + ATP $\dashrightarrow$ glucose phosphate

7.5 Factors Affecting the Rate of Enzyme Action

Enzymes are capable of enhancing the reaction rate enormously, sometimes a million times compared to uncatalysed reactions. The rates of enzymes catalysed reactions are expressed as the **"turnover number"**. The turnover number is the number of substrate molecules

transformed into product in unit time by a single enzyme molecules transformed into product in unit time by a single catalytic site, and depends upon the enzyme concentration. So if the enzyme concentration is known, the turnover number can be calculated.

$$\text{Turnover number} = \frac{\text{moles of product formed per unit time}}{\text{moles of enzymes}}$$

Turnover numbers of several enzymes are listed in Table 9.4. The highest turnover number is of carbonic anhydrase which converts 3.6×9^6 CO_2 molecules to H_2CO_3 per minute. International Union of Biochemistry has now recommended a new unit for reaction rates called **Katal (kat)**. One Kat is the amount of enzyme that converts one mole of substrate into product per second.

Table 9.4: Turnover Number of Some Enzymes.

Enzyme	Turnover Number
Carbonic anhydrase	3.6×10^6
Acetylcholinesterase	1.5×10^6
Urease	1.0×10^6
Amylase	1.0×10^5
Lactic dehydrogenase	6.0×10^4
Chymotrypsin	6.0×10^3
Lysozyme	3.0×10^1

Rates of enzyme catalysed reactions depend on several factors like temperature, pH, concentration of enzyme and substrate etc.

7.5.1 Effect of Temperature

Within certain limits temperature influences the enzyme catalysed reactions in the same way as it affects ordinary uncatalysed chemical reactions. As the temperature increases, the rate of a chemical reaction increases owing to an increase in the number of activated molecules. But when the temperature rises above a certain limit, it destroys the tertiary structure of the enzyme causing the loss of its activity. Similarly low temperature, such as freezing temperatures, generally inactivate the enzyme. It therefore, follows the rule that for every enzyme under a given set of conditions, there is a temperature at which the activity of the enzyme is at its maximum. This is known as optimum temperature (see Fig. 7.4). Most enzymes are denatured above 50°C. Optimum temperature for mammals and birds is about 35° - 40°C whereas in plants and in other animalss it is between 20° - 35°C. Some procaryotes and certain fishes found in Antartica region are exceptional in that they are adapted to life in hot spring and at freezing cold temperatures. They have optimum temperature at over 80°C and below freezing point respectively.

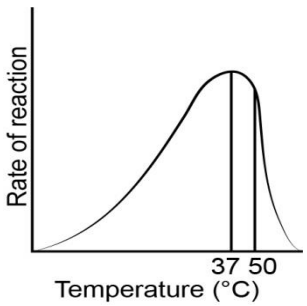


Fig. 7.4: Effect of temperature on the rate of enzyme catalysed reactions. Optimum temperature varies for different enzymes and different species.

7.5.2 Effect of pH

Characteristically, each enzyme has a pH value at which the reaction rate is optimum usually within the pH range 7.0 ± 1.5. This is known as optimum pH at which a certain enzyme causes a reaction to progress most rapidly. On each side of this pH value the reaction is decreased and at certain pH values on both sides an enzyme may be inactivated or even denatured. Therefore, while studying enzymes, buffers are used to keep the enzyme at an optimum pH.
While majority of enzymes function optimally around neutral pH values, some enzymes like pepsin in stomach has an unusually low optimum pH (Fig. 7.5).

Fig. 7.5: Effect of pH on the rate of enzyme catalysed reactions. Proteolytic enzyme pepsin has low optimum pH.

7.5.3 Effect of Enzyme Concentration

As you know, enzymes increase the reaction rate through catalysis. Under constant conditions of temperature, pH and substrate concentration the velocity of the reaction is directly proportional to the amount of enzymes present, i.e., the reaction rate is increased with the increase in enzyme concentration. However, the equilibrium constant for the reaction is not affected by the presence of enzymes.

7.6 Enzyme Kinetics

Enzyme kinetics deals with rates of enzymatic reactions and the various parameters that govern these rates, such as concentration of substrates, concentration of enzyme, pH, temperature and the presence of various substances that may be inhibitors or activators. Study of enzyme kinetics is important because it throws some light on the mechanism of action of an enzyme. Such a study also provides information on the behaviour of an enzyme in the cellular environment and gives us a clue regarding the regulatory mechanisms available to the organism for fine control of enzyme activity.

Measurement of Reaction Rates of Enzymes

The activity of an enzyme can be measured by monitoring the time-dependence of the chemical change that occurs during an enzymatic reaction. The enzyme is incubated with the substrate under optimum conditions. The reaction is observed with the disappearance of the substrate and the formation of a new product. These changes are monitored by withdrawing small aliquots and analysing them for the formation of the product or disappearance of the substrate. In some casessuch measurements can be made directly on the incubation mixture without necessity of withdrawing aliquots. For example, in several oxidation-reduction reaction, where NAD or NADP is the electron acceptor, the progress of the reaction can be continuously monitored by following the reduction of NAD (or NDAP) to the reduced product NADH (or NADPH) which absorbs light at 340 nm. Same principle is applied to monitor many other enzyme catalysed reactions, where either the substrate or the product has a unique and easily measurable absorption spectrum.
Let us now learn how concentration of substrate can alter rate of biochemical reaction.

7.6.1 Concentration of Substrate

In order to outline the effect of substrate concentration on reaction rate, we shall discuss the reaction between a single substrate and an enzyme for the sake of simplicity. If we consider an enzyme a reactant, then this is equivalent to a chemical reaction between two chemical

substances i.e., enzyme and its substrate. However, there is an important difference between enzymic and nonenzymic systems with respect to the dependence of rate on the reactant concentrations. For example, if we carry out a nonenzymic reaction between two reactants A and B, keeping the concentration of A constant and changing the concentration of the B, then the initial rate of product formation will be directly proportional to the concentration of the reactant B. If on the other hand the conditions of an enzymic reaction are so arranged that the enzyme concentration is kept constant and the concentration of substrate is changed, then the initial reaction rate varies hyperbolically with increasing substrate concentrations. The rate reaches a maximum velocity at high substrate concentration which remains unaffected by further increase in substrate concentrations. This is illustrated in Figure 7.6.

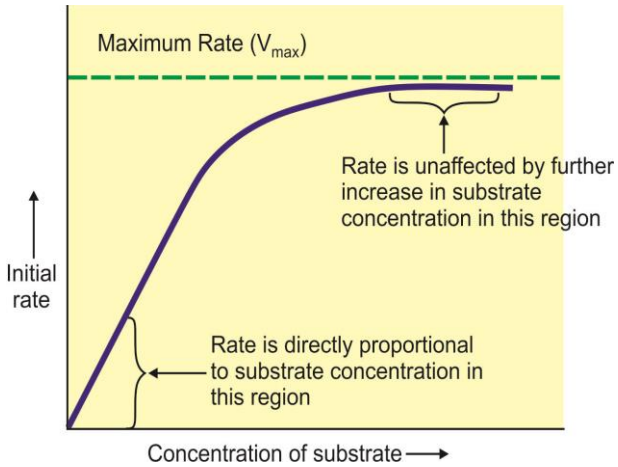


Fig. 7.6: Variation of reaction rate with substrate concentration keeping enzyme concentration constant.

The fact that in an enzymic system, as opposed to a nonenzymic system, the reaction velocity reaches a saturating value at high substrate concentrations can be explained as follows. In a nonenzymatic reaction, the reaction rate is dependent on the number of effective collisions between the reactants. The number of such effective collisions would increase in direct proportion to the concentration of one of the reactants, if the concentration of the other reactant is kept constant. On the other hand, in an enzymic, if the concentration of the other reactant is kept constant.The effective collision between the enzyme and the substrate leads to the formation of an enzyme-substrate complex, in which the substrate is firmly bound to the enzyme at its active site. This complex then breaks down to give the product (P) and release the original enzyme. Since the number of active sites is limited by the enzyme concentration (held constant), the reaction rate will increase with substrate concentration only till all these sites are filled. Under these conditions the system will give maximum possible rate for reaction. Thereafter, there will be no further increase in the rate of reaction.

The shape of the curve as shown in Fig. 7.6, depicting the dependence of reaction rate on the substrate concentration, is a hyperbola, Michaelis and Menton were the first to recognise this relation for enzyme catalysed reactions and put the same in a mathematical form in 1913. This equation for enzyme kinetics is known as the **Michaelis-Menton equation:**

$$\text{Rate of reaction } v = \frac{V_{max} \times [S]}{[S] + K_m}$$

Where [S] is the molar concentration of the substrate and V_{max} is the maximum possible rate for a given enzyme concentration, K_m, also called Michaelis constant, is numerically equal to the concentration of the substrate at that stage when the observed rate of the enzyme

catalysed reaction is one half of the maximum rate i.e., $v = \frac{1}{2}V_{max}$. This will be clear to you from Figure 7.7. From the above equation, it is clear the rate will tend to acquire a maximum value when [S) is very high.

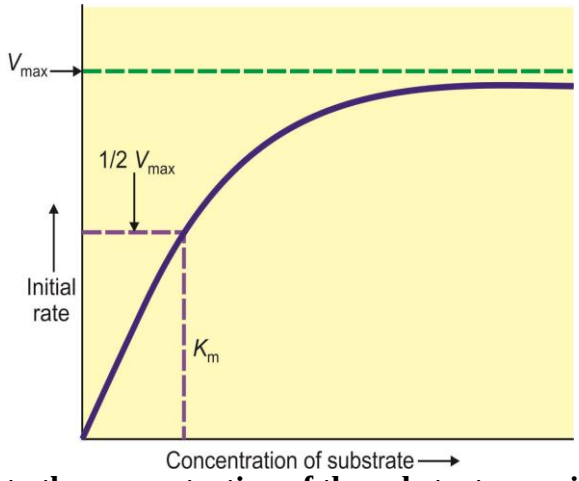


Fig. 7.7: K_m is equal to the concentration of the substrate required to give an initial reaction rate corresponding to half of V_{max}.

We shall now briefly explain what V_{max} and K_m convey in enzyme kinetics.

Significance of V_{max}

At V_{max}, all the enzyme molecules have formed enzyme-substrate complex (ES) and are continuously catalysing the conversion of substrate into the product. Thus at V_{max} value the enzyme is fully saturated. V_{max} values can be used to compare the activity of various enzymes, if they happen to catalyse the same reaction.

Significance of K_m

As we have mentioned already, K_m is equal to the concentration of the substrate at $v = \frac{1}{2}V_{max}$.

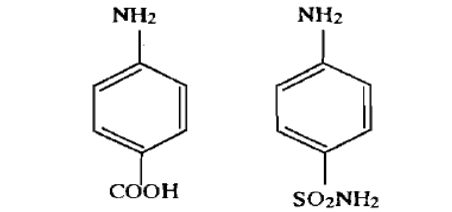
Since at V_{max} all the enzyme molecules have formed enzyme-substrate complex, it, therefore, follows that the concentration of substrate (K_m required to convert half of enzyme molecules to ES complex, is a measure of the affinity of the enzyme for substrate. A small value of K_m signifies the high affinity of the enzyme for the substrate, since a low concentration of substrate would be needed to saturate the enzyme. Similarly, a large value forK_m would indicate a relatively high concentration of substrate for saturating the enzyme, thus signifying a low affinity of the enzyme for its substrate.

You have thus learnt that increase in substrate concentration has a corresponding effect on the rate up to a certain point, beyond which the rate remains unaffected to any further increase in concentration of substrate. We shall now discuss the role of enzyme concentration in the kinetics of enzymic reactions.

7.6.2 Enzyme Inhibition

Enzyme activity is inhibited by several factors, such as unfavourable pH conditions, rise (or sometimes fall) in temperature or presence of protein precipitants, such as alcohol, acetone and trichloroacetic acid. Inhibitory action, caused by these agents is of a general nature and nonspecific. Of greater interest in terms of structure and function of enzymes, are inhibitors known as competitive and noncompetitive inhibitors.

Competitive inhibitors have a structure resembling that of the substrate. The structural similarity between the substrate and the inhibitory compound enables the inhibitor to compete with the substrate for binding to the active site of the enzyme. This prevents the access of the substrate to the active site and the formation of EScomplex. Since the substrates of enzymes are generally metabolites, participating in metabolic transformations in the cell, competitive inhibitors are known as antimetabolites. **One of the best known examples of a competitive inhibitor is sulphanilamide. This is a competitive inhibitor of P-amino benzoate**, a compound utilised in the synthesis of folic acid coenzymes essential for the transfer of one carbon fragments. It is, therefore, easy to understand why sulphanilamide is successfully used as a drug, inhibiting the growth of bacteria. In fact many other drugs also work by acting as competitive inhibitors in enzymatic reactions necessary for the growth and survival of a large number of bacteria.



Another well known competitive inhibitor is malonic acid, which inhibits the oxidation of succinic acid to fumaric acid by the enzyme succinic dehydrogenase. The competitive action of malonic acid against succinic acid can be appreciated by a comparison of their structures, which are closely similar.

It is possible that the active site of the enzyme mistakenly accepts malonic acid instead of succinic acid as its substrate, causing competitive inhibition. You will find a diagrammatic representation of competitive inhibition in given below figure.

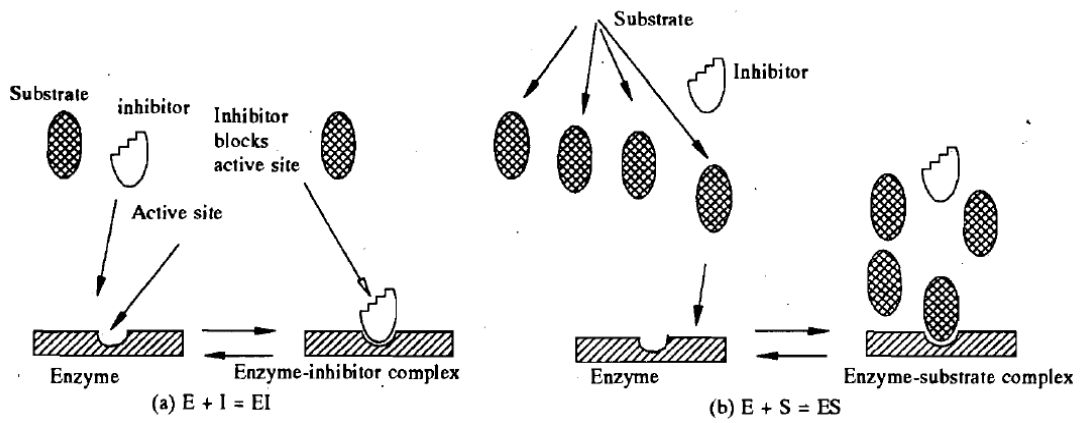


Fig. 7.8: a) Formation of an enzyme-inhibitor complex; b) By increasing substrate concentration, ES concentration gets increased and EI concentration decreases which reverses the effect of the inhibitor.

A characteristic feature of competitive inhibition is that it can be reversed by increasing the substrate concentration. We can detect competitive inhibition by a study of the rate of enzymatic reaction in the presence, and in the absence of a competitive inhibitor. You can observe from Figure 9.15 that V_{max}remains unchanged in the presence of a competitive inhibitor, whereas $K_m$is increased, since a higher substrate concentration would be needed to out-compete with the inhibitor and to achieve the saturation of half of the enzyme molecules.

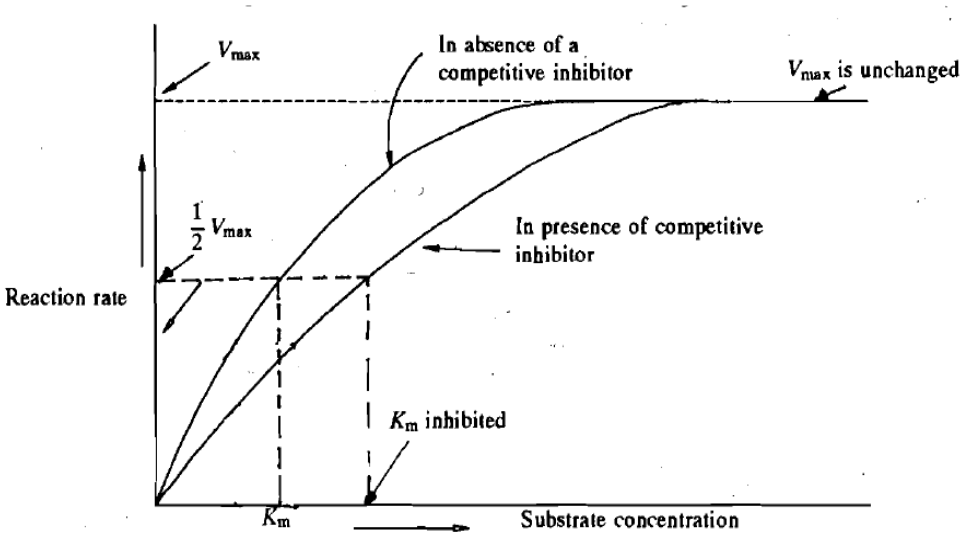


Fig. 7.9: When a competitive inhibitor is present, V_{max}remains unchanged but K_m is increased.

Noncompetitive inhibitors inhibit enzyme action by combining with a group essential for the activity of the enzyme or by removing a metal ion involved in the activity of the enzyme. These compounds act by converting the enzyme into an inactive or less active form. Such inhibitors cannot be displaced by excess substrate and hence are called non competitive inhibitors. Since their action does not involve displacement of the substrate, they have no effect on the $K_m$of the substrate. However, since the inhibitor makes the enzyme less active, $V_{max}$is affected.

7.7 Regulation Of Enzyme Activity

Have you thought of ways by which enzyme activity can be regulated? There are various mechanisms of enzyme regulation as listed below:

1. Enzyme Quantity
2. Allosteric Regulation
3. Feedback Regulation or Inhibition
4. Co-valent Modification
5. Proteolytic degradation

1. Enzyme Quantity

The quantity of enzymes or turnover number is determined by the overall rate of synthesis and rate of degradation of the enzyme. Any change in its quantity can be affected by a change in rate constant for the overall synthesis and degradation processes or both. The concentration of proteins or enzymes remained essentially constant in a state of ‘dynamic equilibrium’. It gets influenced by a wide range of physiologic, hormonal or dietary factors. The turnover number of the enzymes can vary from minutes to hours to days for different enzymes.

Genetic Control: Enzyme synthesis at the gene level

Genes involved in the synthesis of enzymes can be induced or repressed. Induction of enzymes can be done at the gene expression, RNA translation or at the level of post-translational modifications. Hormones or growth factors signal cascade may lead to an increase in the expression or translation of enzyme not present before the signal. Inducers induce the synthesis of enzymes while repressors decrease the production of enzyme. Inducers are generally substrates or structurally similar molecules that initiate the synthesis of enzymes. Inducible enzymes in humans include tryptophan pyrrolase, threonine dehydratase, HMG-CoA reductase and cytochrome P-450. On the other hand, a metabolite or repressor when produced in excess inhibits the synthesis of enzymes involved in its formation. These regulatory molecules block the transcription of mRNA by binding to a part of DNA termed as operator. Repressors are allosteric proteins to which specific molecules can bind to alter their shape and ability to bind DNA. Inducer molecules bind to the repressor molecule and prevent its binding to the operator region of DNA. It allows the transcription of the coding sequences for the enzymes. For example, ***lac*** operon in *E.coli*, lactose acts an inducer to transcribe the synthesis of three enzymes (β-galactosidase, permease and transacetylase) involved in its degradation if it is present in the surrounding environment.

2. Allosteric Regulation

Allosteric means 'other structure or different site'. Allosteric enzymes are the enzymes whose catalytic activity can be modulated by the effector molecules. These molecules do not participate in catalysis directly. They bind at a site other than the active site on the enzyme and can cause activation or inhibition of the enzyme. The binding is reversible, non-covalent and brings about the conformational change in the active site. These conformational changes occur at the tertiary and quaternary levels of protein organization. Allosteric enzymes are generally bigger and composed of multiple subunits having distinct active site and allosteric site. Several studies on X-ray crystallography and site directed mutagenesis have confirmed the existence of two separate sites on variety of enzymes.

Allosteric enzymes exhibit a characteristic sigmoidal saturation curve rather than hyperbolic curve [Michaelis-Menten curve] when v_o is plotted versus [S] on account of cooperativity of structural changes between enzyme subunits (Fig. 7.8). Allosteric enzymes behave in a cooperative system manner with both the substrate as well as modulator. A small change in substrate, inhibitor or activator concentrations brings about a huge change in the rate of reaction. The effectors that increase the catalytic activity are called positive effectors and those that reduce or inhibit the catalytic activity are called negative effectors.

For example, Phosphofructokinase (PFK) is regulated by

a) Negative effectors: High levels of ATP and citrate

b) Positive effectors: High levels of ADP and AMP

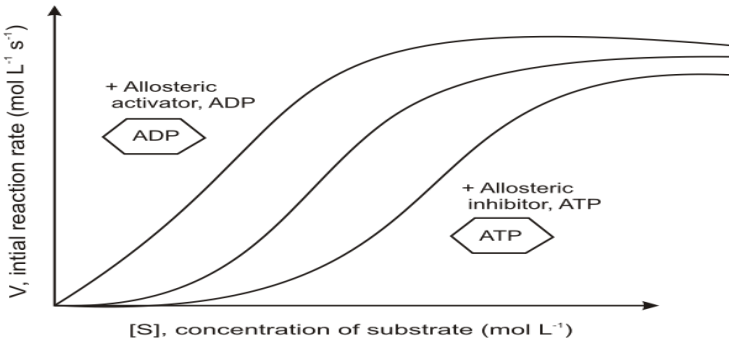


Fig. 7.8: Sigmoidal kinetics of allosteric enzymes.

3. Feedback Regulation or Inhibition

Another interesting aspect of enzyme regulation is the feedback regulation; inhibition of an enzyme in the biosynthetic pathway by the end product of the pathway. This type of inhibition is also termed as feedback inhibition and it develops when metabolic demand for the end product of the pathway gets declined. The end product binds to the regulatory site of the enzyme at the start of metabolic pathway and suppresses its activity. Feedback inhibitors do not bear any structural similarity to the substrates of the enzymes. For example, consider the following reaction:

Enz 1 Enz 2 Enz 3

A----------→ B---------→ C ---------→ D

High concentrations of end product D will act as feedback inhibitor of enzyme Enz 1. Small molecules such as amino acids or nucleotides act as feedback inhibitors in several biosynthetic pathways. In the bacterial enzyme system, L-threonine is converted to L-isoleucine in a sequence of five steps metabolic pathway. Isoleucine binds to the first enzyme in the pathway threonine dehydratase in a non-covalent manner and inhibits its own production.

4. Covalent Modification

Covalent modification is also a means of regulating enzyme activity. The reversible covalent modification process of enzyme regulation involves many target proteins and membrane channels. Modified groups are attached to the enzyme by covalent bond. The covalent modification activates some enzymes as well as inactivates others. Most modifications are reversible. Phosphorylation and dephosphorylation are the most common but not the only means of covalent modification. Enzyme regulation by phosphorylation-dephosphorylation plays a key role in cell signaling. It allows the cell to respond to a signal at its surface and transmits its effect to intracellular enzymes. Phosphorylation cascade is highly selective. Seryl, Threonyl or Tyrosyl residues on the regulatory enzymes are phosphorylated by specific protein kinases. The very nature of protein folding determines whether protein kinase has access to the substrate undergoing phosphorylation. The removal of phosphoryl groups is catalyzed by protein phosphatases. Mammalian cell possesses contain many phosphorylated proteins, several protein kinases and phosphatases that catalyses their interconversion for regulatory control. Phosphorylation influences the functional properties of affected enzyme by altering its three-dimensional structure. Phosphorylation of one enzyme can lead to phosphorylation of a different enzyme which in turn acts on another enzyme, and so on.

Other covalent modifications such as glycosylation, hydroxylation, fatty acid acylation, palmitoylation and prenylation are unique changes that shape structure and localization of enzyme for its lifetime. Hydrophobic acylations can cause the target protein to be associated with a membrane rather than the cytosol.

5. Proteolytic enzymes

Certain enzymes are secreted as precursors in an inactive form and are known as proenzymes or zymogens. Proenzymes help the proteins to be transported or stored in inactive forms that can be converted in active forms at the particular site. Precursor of pepsin is pepsinogen, trypsin is synthesized as trypsinogen and procarboxypeptidase is zymogen of carboxypeptidase. Several other examples include blood clotting enzymes, procollagen and proinsulin. Zymogens underlie the mechanism whereby the levels of enzymes can be readily increased at the post translational level by proteolytic cleavage, an irreversible modification. Generally, cellular and bacterial proteolytic enzymes are synthesized as inactive precursor (zymogen) to prevent undesired protein degradation. Conversion of zymogens to active

form either requires accessory molecules or the autocatalytic processes in response to drop in pH.
Some examples of enzymes and their zymogens are as given below:

Enzyme	**Precursor**	**Function**
Trypsin	Trypsinogen	pancreaticsecretion
Chymotrypsin	Chymotrypsinogen	pancreaticsecretion
Carboxypeptidase	procarboxypeptidase	pancreaticsecretion
Elastase	proelastase	pancreaticsecretion
PhosphalipaseA_2	prophospholipaseA_2	pancreaticsecretion
Pepsin	Pepsinogen	Secreted in gastric juice (Most active in pH range 1-5)

7.8 SUMMARY

In this unit you have studied that:

- Enzymes are proteins that catalyse the biochemical reactions by lowering the activation energy. Enzymes increase the rate of the reactions but do not alter the equilibrium point.
- Enzymes facilitate the coupling of energy releasing exergonic reactions to energy requiring endergonic reactions.
- Enzymes are highly specific with respect to the nature of reaction as well as to their substrate. Enzymes substrate specificity habeen explained by lock and key and induced fit hypothesis.
- Reactions by the enzymes are facilitated on the basis of proximity and orientation of side chains of the substrate and enzyme, acid-base catalysis by the side chain of charged amino acids of active site and by covalent interactions between enzymes and substrates.
- Changes in the concentration of enzymes, substrates, pH and temperature influence the rate of *enzyme* catalysed reaction.
- Some enzymes require additional factors called cofactors for their catalytic activity. Prosthetic groups are organic cofactors and are permanently bound to the enzymes. Coenzymes are also organic compounds but their association is transient. Some enzymes require metal ions for their activity.
- Allosteric enzymes are oligomeric and have more than one active site where the allosteric effector molecules influence their enzyme activity. Influence of one substrate binding on subsequent substrate bindings is known as cooperatively.
- Enzyme activity can be estimated by different procdures such as spectophotometric, fluorometric and isotopic labeling methods.
- The substrate concentration required for half maximal velocity (V_{max}) is called the Michaelis Constant K_m. This is useful parameter for determining catalytic potential of an enzyme under physiological conditions and also gives an idea of the affinity of the enzyme for its substrate.
- Enzyme activity is proportional to the concentration of the enzyme and is dependent on pH and temperature of the medium.

- Enzymes are inhibited by reagents which compete with the substrate for the active site. These substances are known as competitive inhibitors. The other type of substances which inhibit the enzyme by inactivating it are called noncompetitive inhibitors.
- Enzymes are regulated by the end product of a biosynthetic pathway. The final and product inhibits the first enzyme of that pathway by binding to an allosteric site which is distinct from the active site. The inhibition results from a conformational change in the oligomeric structure of the first enzyme.
- In another type of regulation, the amino acid side chains of enzymes are reversibly modified, mainly by phosphorylation or dephosphorylation. This produces an active or an inactive enzyme.
- Sometimes enzymes catalysing the same reaction exist in different electrophoretic forms in different tissues of the same species. These different forms are known as isoenzymes and result due to genetic factors. Lactate dehydrogenase is an interesting case of isoenzymes.
- Enzymes have a diagnostic value in medical sciences. Various enzyme assays are employed to confirm, locate and also indicate the severity of a disease in a human being. Enzymes are used as laboratory reagents and are also employed in treating various diseased conditions.

7.9 SELF ASSESSMENT:

1) What is activation energy?
2) Discuss briefly the variations in the degree of enzyme specificity.
3) How temperature and pH affect the rate of enzyme action?.
4) Explain briefly the functioning of allosteric enzymes.
5) Why is the rate of an enzyme-catalyzed reaction proportional to the amount of *ES* complex?
6) Explain the maximal velocity V_{max} in the v_o vs S graph?
7) Derive double-reciprocal equation from Michaelis-Menten equation and give its importance.
8) How a value for K_m can be obtained from the v_o vs S graph when $v_o = 1/2\ V_{max}$?
9) Describe allosteric regulation of the enzyme activity?
10) What are proteolytic enzymes? Give examples.

7.10 SUGGESTED READINGS:

1. Leininger's Principles of Biochemistry (2000) by Nelson, David L and Cox, M M, Macmillan/worth, NY.
2. Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte W Pratt, John Wiley & Sons, NY.
3. Biochemistry III ed (1994) by Lubert Stryer, WH Freeman and Co., San Francisco.
   Outlines of Biochemistry (1987) by Eric E Conn, P K Stumpf, G Bruening and Ray H Doi, John Wiley & Sons, NY.

Dr Y.R.K V Tirupati Rao

LESSON-8

MECHANISM OF ENZYME ACTION, ENZYME INHIBITORS, DIFFERENT FORMS OF ENZYMES

Objectives;

after learning this unit, you can able to understand the mechanism of enzyme action; enzyme inhibitors, allosteric enzymes, abzymes, ribozymes

8.1 MECHANISM OF ENZYME ACTION

In spite of the excellent information that has accumulated over the last several years on the physical, chemical and structural aspects of enzymes, much remains to be understood about their mechanism of action. Significant information on the mechanisms by which ribonuclease, chymotrypsin, lysozyme and some other enzyme catalyse reaction is, however, available.

The general mechanism of enzyme action can be viewed as having three stages. The enzyme first binds to the substrate followed by the formation of enzyme substrate complex (ES) which finally alters the substrate and forms the product. The events in enzyme catalysed reaction are represented by the equation.

Binding catalysis Release

$$E + S \rightleftharpoons ES \rightleftharpoons P \rightarrow E + P$$

where E is enzyme S is substrate
P is product ES is enzyme substrate complex

As you know an enzyme speeds up a reaction by lowering its activation energy. Enzymes have specific regions on their surface to which the substrate binds and catalysis takes place. This region is called as the active site of the enzyme. The enzyme recognizes and binds the substrate to its active site and forms an activated enzyme substrate complex. The enzyme acts upon the specific bonds within the substrate which results in the formation of the product. Thus the enzymes lower the energy barrier and allow the organisms to carry out reactions at a faster speed.

As enzyme lowers the energy barrier, i.e. activation energy of both the forward and reverse reactions to the same extent. It does not alter a reaction's equilibrium state but merely increases the speed with which the reaction approaches an equilibrium. Furthermore, since a catalyst such as, an enzyme is not permanently changed by participation in a reaction, it comes out exactly as it started, and is ready to catalyse the reaction again. One of the factors responsible for enzyme action is their specificity about which you will read in the following subsection.

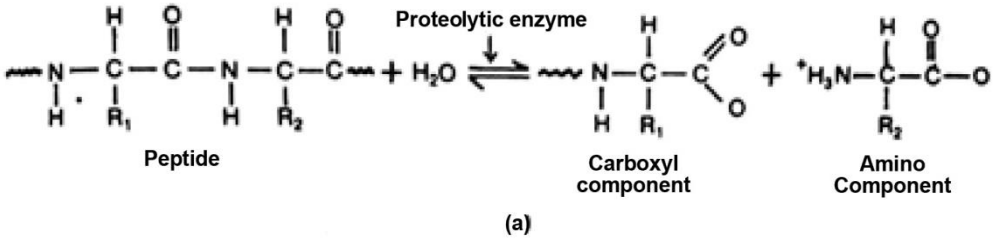
8.1.1 Enzyme Specificity

One of the most significant features of enzymes is their specificity with respect to the nature of the reaction they catalyse and also in the actual reactant, i.e. substrate molecule. Enzymes have varying degrees of specificity for substrates. They may recognize and calatyse**a single substrate, a group of substrates or a particular type of bond. You can understand it better by Table 8.1.**

Table 8.1: Types of Enzyme specificity.

Type of Specificity	Reaction Type	Example
Absolute	Catalyze one type of reaction for a single substrate	Urease catalyzes only the hydrolysis of urea
Group	Catalyze one type of reaction for similar substrate	Hexokinase adds a phosphate group to hexoses
Linkage	Catalyze one type of reaction for a specific type of bond	Chymotrypsin catalyzes the hydrolysis of peptide bonds

For example, proteolytic enzymes catalyse the reactions involving protein digestion, whereas urease and succinic dehydrogenase act exclusively on urea and succinate respectively. Enzyme specificity varies even within a particular group of enzymes. Let us take the example of proteolytic enzymes. The reaction catalysed by these enzymes is the hydrolysis of peptide bond (Fig.8.1a). Subtilisin cleaves any peptide bond joining the amino acids of the substrate, whereas enzyme thrombin catalyses the hydrolysis of peptide bonds only between arginine and glycine (Fig. 8.1b). Still some other proteolytic enzymes have different degree of specificity, for example trypsin breaks peptide bonds only on the carboxyl side, i.e. terminal of amino acids lysine and arginine (Fig. 8.1c). The specificity differences are due to the biological functions of various proteolytic enzymes. Since bacteria use any protein as a source of carbon and nitrogen, subtilisin enzyme found in them can act upon any type of proteins. Enzymes trypsin and chymotrypsin have limited specificity as they break down the proteins into 5 to 20 amino acids long fragments in the mammalian digestive system.



(a)

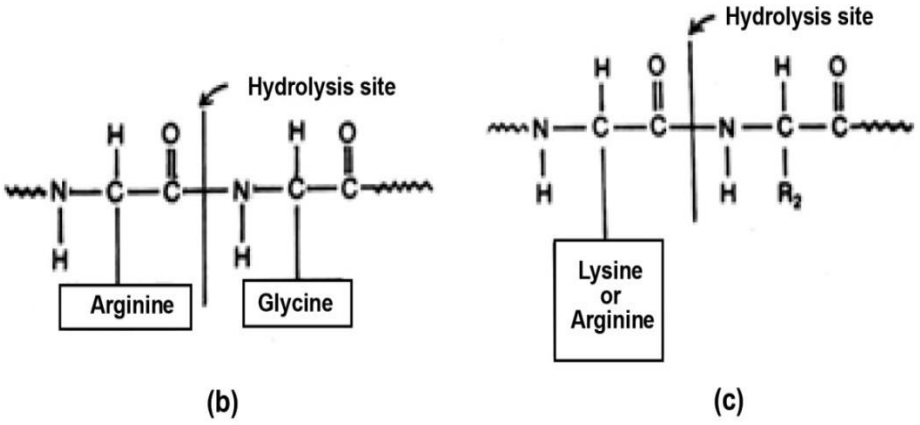


Fig. 8.1: a) Proteolytic enzymes carry out hydrolysis of peptide bonds; b) Specificity of enzyme thrombin; c) Specificity of enzyme trypsin.

Thrombin participates in blood clotting and shows absolute specificity as it splits only a single peptide bond in fibrinogen releasing the fibrin monomer which helps in the formation of fibrin clot. The least specific group of enzymes is hydrolases.

Some enzymes exhibit stereospecificity acting exclusively on different stereoisomers of the same compound e.g., the kidney D-amino acid-oxidase D-isomers of amino acids. However, a small group of enzymes such as racemases catalyse the interconverstion of D and L forms. Some enzymes exhibit geometrical specificity acting only on cis or trans isomers. Cis and trans isomers are geometrical isomers and have different arrangement of their constituent atoms and groups with respect to the double bonds joining the two carbon atoms. For example, maleic acid and fumaric acid have the same molecular formula but differ in the arrangement of the group the C = C bond. As shown in Figure9.5 Maleic acid, with both the carboxylic group on the same side, is the cis form of fumaric acid, the trans isomer in which these groups are on the opposite side.

In view of the specificity exhibited by the enzymes in binding with the substrates, Emil Fischer in 1984 put forward the **lock and key hypothesis.** According to this hypothesis the substrate molecules fit into the active sites of the enzymes like a key fits into the lock. This results in the formation of transient ES-complex which breaks down into enzymes and product (Fig. 8.3 a). The enzyme substrate complex can be isolated from the enzymes that work slowly thus giving the direct evidence for the formation of these ES-complexes.

Although the lock and key model accounts for much of the enzyme substrate specificity, certain observations about enzyme behaviour cannot be explained by this model. For example, sometimes compounds other than the actual substrate bind to the enzyme and fail to form the reaction products. In the 1960's Daniel Koshland proposed the **induced-fit hypothesis** according to which the active site of the enzyme does not initially exist in a shape complementary to the substrate but is induced by the substrate to assume the complementary shape to bind the substrate molecule. Active site of an enzyme is thus regarded as flexible in nature (see Fig. 8.3b). However, a flexible active site is not necessary for all enzyme catalysed reactions as some are adequately explained by the lock and key model.

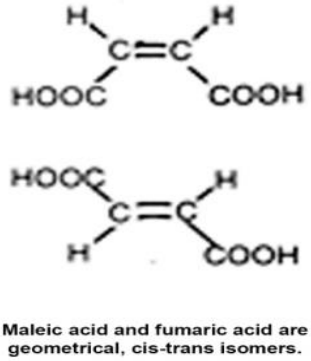


Fig. 8.2: Maleic acid and fumaric acid are geometrical, cis-trans isomers.

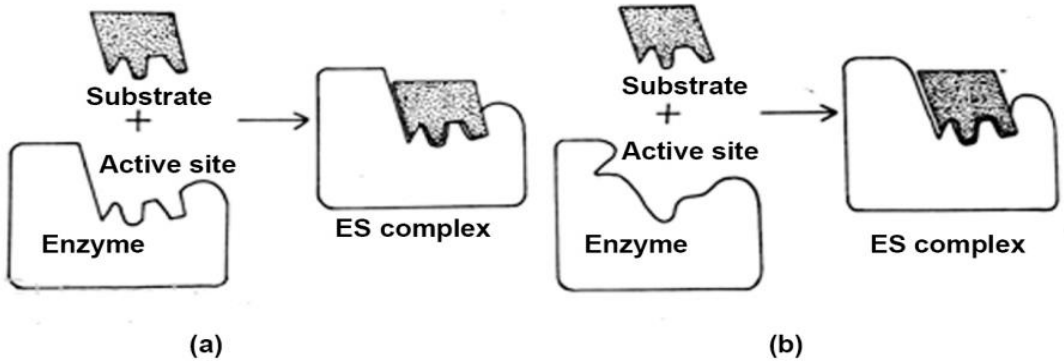


Fig. 8.3: a) Lock and key model: the active site of the enzyme is complementary in shape to that of the substrate. Substrate fits in the active site like a key fits in the lock. b) Induced fit model: the enzyme changes its shape upon binding the substrate. The active site acquires complementary shape to that of substrate only after the substrate is bound.

8.1.2 Molecular Basis of Enzyme Action

The molecular basis of enzyme action depends on its three dimensional structure. The making and breaking of chemical bonds by an enzyme in a reaction are preceded by the formation of an enzyme substrate complex. As you have read earlier, the substrate is bound to a specific region of enzyme called active site. The properties and positions of side chains of the enzymes exposed at the active site determine which substrates will bind to it. The specific amino acid side chains at the active site are not necessarily close to each other in the linear protein chain. Folding of the protein chain, however, brings these groups together. For instance, negatively charged side chain groups, like those of asparatate and glutamate, can be forced together, due to the folding of the enzyme protein molecules in spite of their tendency to repulse each other. This leads to an increase in affinity for the positively charged groups of the substrate. Similarly, some amino acid side chains forced together as a result of folding of peptide chains, may interact through hydrogen bonding, for example in trypsin, a normally non-reactive serine molecule (CH_2OH) becomes highly reactive by acquiring two negative charges (Fig. 9.7).

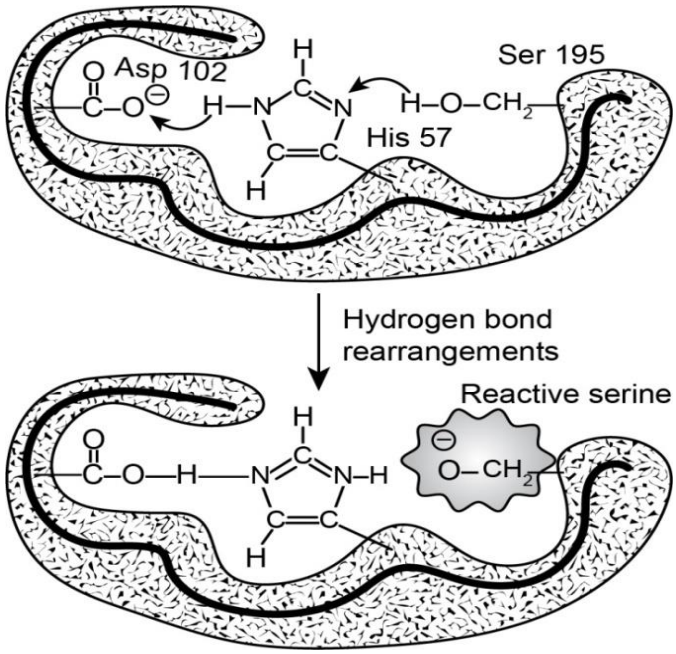


Fig. 8.4: Rearrangements of hydrogen bond makes serine molecule highly reactive in the active site of the enzyme.

When an enzyme is denatured, the native three dimensional structure is disrupted. Amino acid side chains of the active site unfold and separate from the site. This prevents their participation in a chemical reaction thus inactivating the enzyme. Enzyme inactivation is also caused even when only a functional side group is displaced or substituted in the enzyme.
Although enzymes differ widely in structure, specificity and mode of catalysis, various mechanisms operate at the active site of enzymes, each contributing to the lowering of activation energy. These mechanisms can be generalised as follows:

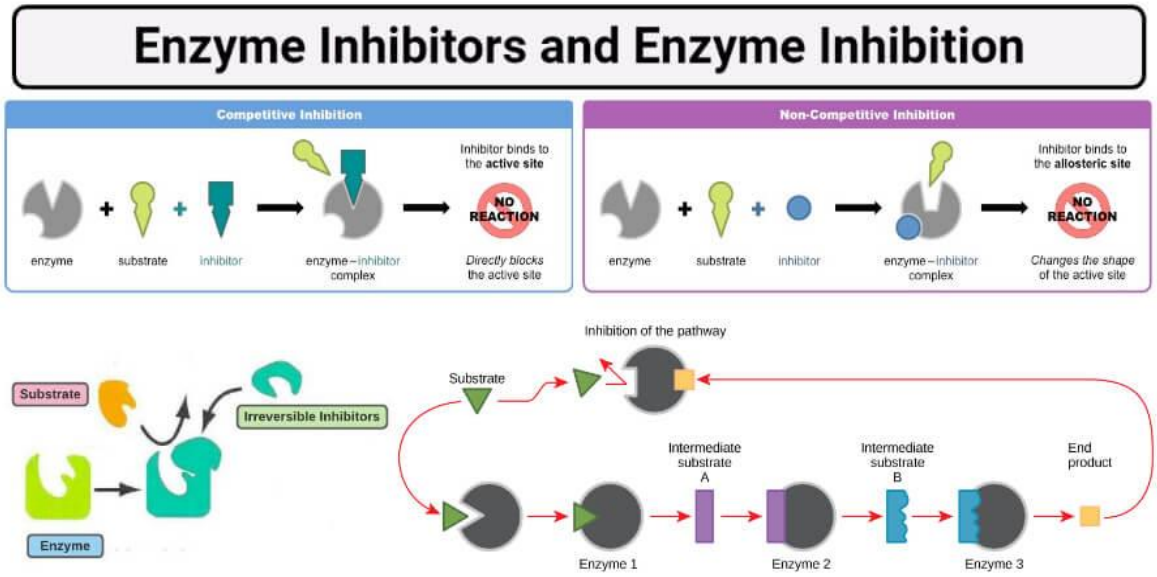
- The binding of reacting molecules with each other brings them in close proximity, thus increasing the chance of a reaction.
- The substrates are bound to the enzyme in such a manner that the formation of several types of temporary non-covalent bonds between them force a redistribution of charges within substrates. This redistribution imposes a strain on specific covalent bonds in the substrates resulting in the breaking of the bond.
- Hydrophobic amino acids eliminate water molecules from the active site to create a water free zone so that non polar groups react more easily.
- Acidic and basic amino acids in the active site of the enzyme transfer the protons to and from the reactants for catalysis of reaction. This is known as acid-base catalysis.
- Enzymes react with the substrate to form transient, high energy unstable covalently linked enzyme substrate-complex which undergoes further reactions to form products.

Although these factors are believed to contribute to the acceleration of reaction rate by various enzymes, the exact mechanism involved is yet to be known.

8.2 ENZYME INHIBITORS

Inhibitors are compounds that convert the enzymes into inactive substances and thus adversely affect the rate of enzymatically-catalyzed reaction is called an enzyme inhibitor, and the process involved is termed enzyme inhibition. Some enzyme inhibitors are normal

body metabolites that inhibit a particular enzyme while other inhibitors may be foreign substances, such as drugs or toxins. The inhibition may be a part of the normal cellular control of a metabolic pathway, a diseased condition, or either a therapeutic measure.

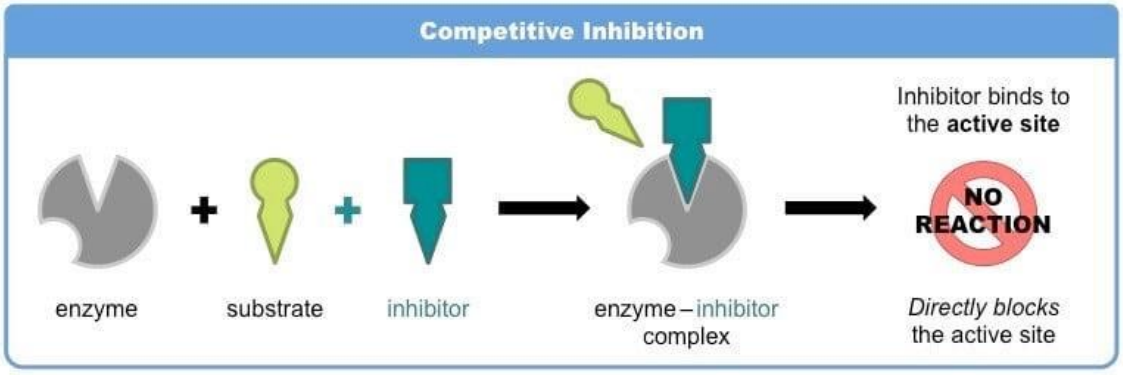


1. Reversible Inhibition

Reversible inhibition is the inhibition of an enzyme caused by reversible inhibitors that dissociate very rapidly from its target enzyme because it becomes very loosely bound with the enzyme. Reversible inhibition is prevented by removing the inhibitor from the enzyme. Reversible inhibitors bind to enzymes with non-covalent interactions like hydrogen bonding and hydrophobic interactions. These weak interactions together result in strong binding. These inhibitors do not undergo chemical reactions but are easily removed by dilution or dialysis. Reversible inhibition are of three types; competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition depending on three different factors:

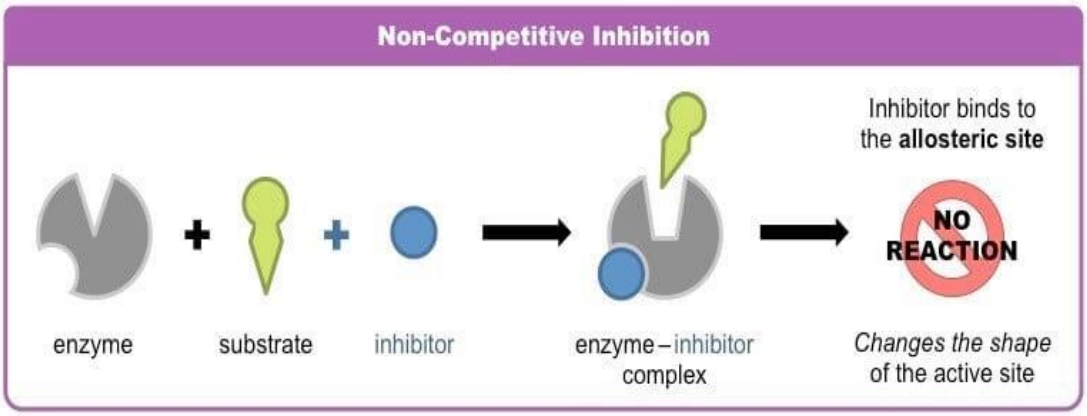
1. whether the inhibition can be overcome by increasing the concentration of the substrate,
2. whether the inhibitor binds at the active site or allosteric site of the enzyme molecule
3. whether the inhibitor binds either with the free enzyme only or with the enzyme-substrate complex only or with either of the two.

a) Competitive inhibition



- Competitive inhibition is the inhibition of enzymatic activity by the competitive binding of inhibitors to the active site.
- The inhibitor is called a competitive inhibitor as it competes with the substrate to bind with the active site.
- The competitive inhibitor has a close structural resemblance with the substrate. The inhibitor may combine with the enzyme (E), forming an enzyme-inhibitor (EI) complex instead of an enzyme-substrate (ES) complex.
- The degree of inhibition depends upon the concentrations of the substrate and the inhibitor.
- Thus, inhibition can be increased by increasing the concentration of inhibitors and can be reduced by decreasing the concentration of the substrate.
- The action of a competitive inhibitor can be reversed at high substrate concentrations because a sufficiently high substrate concentration will successfully compete out the inhibitor molecule in binding to the active site.
- A competitive inhibitor diminishes the rate of the reaction by reducing the proportion of the enzyme molecules bound to a substrate.
- Example: Many microorganisms, like bacteria, synthesize the vitamin folic acid from para-aminobenzoic acid (PABA), and some sulfa drugs that are structural analogs of PABA act as an enzyme inhibitor and occupy the active site of some bacterial enzymes catalyzing this reaction.

b) Noncompetitive inhibition



- Noncompetitive inhibition is the inhibition of enzymatic activity by the binding of inhibitors to the enzyme at a place other than the active site.
- The tern noncompetitive suggests that there is no competition between the substrate and the inhibitor for the binding to the active site and also has no structural resemblance to the substrate.
- The inhibitors involved in this process are termed noncompetitive inhibitors.
- Since inhibitor and substrate may combine at different sites, the formation of both enzyme-inhibitor complex and enzyme-substrate complexes takes place.
- In noncompetitive inhibition, the inhibitor and substrate can simultaneously bind to the same enzyme molecule since their binding sites are different and also, do not overlap.
- During competitive inhibition, the enzyme is inactivated when an inhibitor is bound, whether or not substrate is also present.
- The binding of the inhibitor brings about conformational changes in the active site of the enzyme, which prevents the binding of the substrate molecule.
- Besides, the inhibitor effectively lowers the concentration of active enzymes and hence lowers the rate of reaction.

- Noncompetitive inhibition, unlike competitive inhibition, cannot be overcome by increasing substrate concentration.
- Examples: Various heavy metal ions (Ag+, Hg2+, Pb2+) inhibit the activity of a variety of enzymes like urease.

c) Uncompetitive inhibition

- Uncompetitive inhibition is the inhibition of enzymatic activity by the binding of the inhibitor at an allosteric site like in the case of noncompetitive inhibition but the binding takes place with the enzyme-substrate (ES) complex, and not the free enzyme molecule.
- The mechanism of inhibition involved in uncompetitive inhibition is by the removal of an activated enzyme-substrate complex which causes a decrease in the maximum velocity of the chemical reaction.
- For example, tetramethylene sulfoxide and 3-butylthiolene oxide are uncompetitive inhibitors of liver alcohol dehydrogenase.

8.3 ALLOSTERIC ENZYMES

Some enzymes may have two functionally different binding sites. One of the site, the active site, binds the substrate and catalyses the reaction. The other type of site, known as allosteric or **regulatory site** binds another molecule which is called **effector or modulator.** Such enzymes are known as allosteric enzymes. These enzymes are oligomeric in nature, i.e. they have more than one subunit. The active site and allosteric site may be located on the same subunit or on different subunits of the enzyme and the conformational changes caused by binding are transmitted between subunits. Effector molecules are of two types: positive effectors, i.e. **activators** that enhance enzyme activity and negative effectors or **inhibitors** which inhibit enzyme activity. Binding of effectors causes conformational changes in the allosteric enzymes which influence their catalytic activity (see Fig. 8.10). We can also say that allosteric enzyme action is regulated by effector molecules. These effector molecules can bind at the same allosteric site or at different allosteric sites.

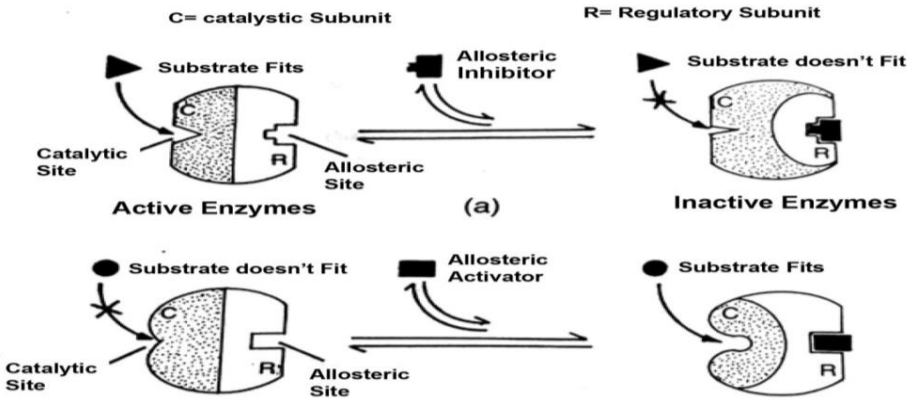


Fig. 8.10: Schematic diagram showing the functioning of allosteric enzymes.

Binding of a substrate to one active site may influence subsequent bindings to other active sites of an enzyme with more than one active sites. This behaviour of enzymes is known as **cooperativity.** When binding of the first substrate molecule promotes binding of subsequent

substrate molecules, it is called **positive cooperativity. Negative cooperativity** is when, after the binding of the first substrate molecule subsequent substrate binding occurs less readily.

8.4 ISOENZYMES

Several enzymes exist in their multiple forms termed **isoenzymes** or **isozymes.** These isoenzymes are not isomers. All isoenzyme forms of a given enzyme catalyse the same reaction but are chemically distinct molecules. They may differ in amino acid composition, amino acid sequence, charge, molecular weight, etc. They usually differ in one or more kinetic properties such as Km, Vmax for their substrate.
Isoenzymes are found in all vertebrates, insects, plants and unicellular organisms. Different tissues may contain different isoenzymes which may differ in their affinity for the substrates. One of the first and best known example of universally occurring isoenzymes is lactate dehydrogenase that exists in five different forms: M_4, M_3H, M_2H_2, MH_3 and H_4. Each of these isozymes is a tetramer made up of four polypeptide subunits. These subunits are of two types and occur in different combinations in the isozymes. These two types of subunits are named as M and H types depending upon their location; M in muscles and H in heart (Fig. 8.11).

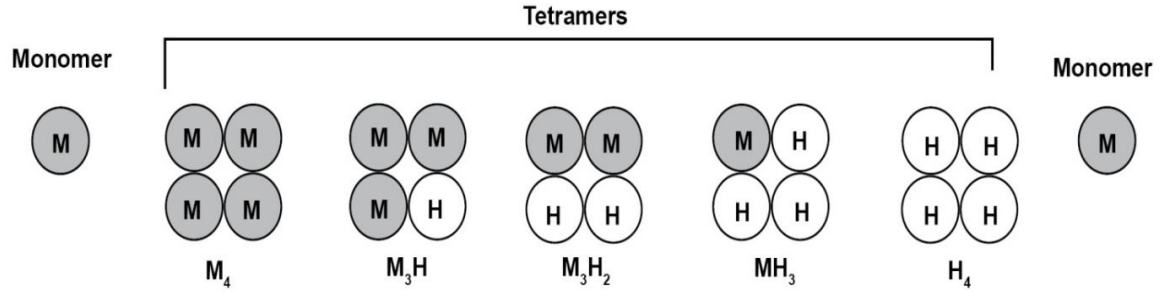


Fig. 8.11: Lactate dehydrogenase enzyme is a tetramer, has five isozymic forms. Each tetramer has M and/or H types of monomers in different numbers.

All the forms of lactate dehydrogenase isoenzymes do not necessarily occur in equal proportion in all the cells as M_4 dominates in muscles and H_4 is predominantly present in the heart. Other isoenzyme forms include oxidases, dehydrogenases. Several regulatory enzymes also exist in isoenzyme forms. The molecular difference of isoenzyme molecules is due to the difference in their locations such as malate dehydrogenase, which occurs in different forms in cystosol and in mitochondria. Analysis of isozymes is frequently used in medical diagnosis.

8.5 RIBOZYMES

A ribozyme is a **ribo**nucleic acid (RNA) en**zyme** that catalyzes a chemical reaction. The ribozyme catalyses specific reactions in a similar way to that of protein enzymes.

Also called catalytic RNA, ribozymes are found in the ribosome where they join amino acids together to form protein chains. Ribozymes also play a role in other vital reactions such as RNA splicing, transfer RNA biosynthesis, and viral replication.

The first ribozyme was discovered in the early 1980s and led to researchers demonstrating that RNA functions both as a genetic material and as a biological catalyst. This contributed to the worldwide hypothesis that RNA may have played a crucial role in the evolution of self-replicating systems. This is referred to as the RNA World Hypothesis and today, many

scientists believe that ribozymes are remnants of an ancient world that existed before the evolution of proteins. It is thought that RNAs used to catalyse functions such as cleavage, replication and RNA molecule ligation before proteins evolved and took over these catalytic functions, which they could perform in a more efficient and versatile way.

Ribozymes have been extensively investigated by researchers to try and determine their exact structure and function. Scientists have developed synthetic ribozymes in the laboratory that are able to catalyze their own synthesis under specific conditions. One example is the RNA polymerase ribozyme. Using mutagenesis and selection, scientists have managed to develop and improve variants of the Round-18 polymerase ribozyme from 2001. The best variant so far is called B6.61, which can add up to 20 nucleotides to a primer template over a period of 24 hours. After 24 hours, the hydrolysis of its phosphodiester bonds causes the ribozyme to decompose.

Ribozymes may also play an important role in therapeutic areas, acting as molecules that can tailor specific RNA sequences, serving as biosensors and providing a useful tool in areas such as gene research and functional genomics. For example, strands of circular ribozymes celled viriods have been discovered and these can have a devastating effect on plants. The viriods replicate by making copies of themselves based on their own genome and their catalytic properties enable them to undergo self-cleavage and send fragments off to colonize and harm areas of a plant by proliferating and using the genetic material that the plant itself needs. Researchers have now identified a site in these viriods that enables them to self-cleave. The site is less than 30 nucleotides in length and has three stems that form a central loop which is referred to as a “hammerhead.” This structure cleaves very specific RNA sequences to release viable RNA daughter strands. Now, hammerheads of just 19 nucleotides in length have been synthesized that act as highly specific catalysts. Similar ribozymes are also being made that could be used to break up RNA viruses and RNA that is required for the transcription and translation of DNA that contains mutations.

Such detailed studies of RNAs have led to rules being established regarding how they achieve target recognition and based on those rules, scientists have managed to adjust ribozymes so that they target and cleave new RNA molecule targets that would not usually undergo cleavage by ribozymes. This raises the exciting possibility that artificial ribozymes could be used as a therapeutic agents to target RNA molecules that cause diseases such as HIV. In models of such diseases, ribozymes have been successful at achieving this and a ribozyme that has been shown to target and break up the RNA that makes up the HIV virus has already been approved for testing in patients with HIV. In the future, ribozymes may also be used as therapeutic agents in the correction of genetic disorders. They could be used to eliminate abnormal proteins before they even exist by attacking and breaking up the molecules of RNA that are needed for their translation and transcription.

8.6 ABZYMES :

Abzymes are catalytic antibodies that have structural complementarity for the transition state of an enzyme-catalyzed reaction. They strongly bind to the transition state, enhancing the reaction rate. 2) Abzymes can be produced artificially or obtained from human and animal serum. They are capable of hydrolyzing proteins, DNA, RNA, and polysaccharides. 3) Examples of abzymes include antibodies that catalyze the hydrolysis of hydroxy esters and esters by binding to cyclic or tetrahedral intermediates that mimic the transition state. Abzymes can also accelerate the biosynthesis of heme by binding to a transition state analog.

8.7 SUMMARY

1. Enzymes are biological catalysts that accelerate biochemical reactions by binding to substrates at their active site, lowering activation energy.
2. Enzyme inhibitors can block or slow enzyme activity,
3. allosteric enzymes have regulatory sites that, when bound by effector molecules, can activate or inhibit the enzyme.
4. Ribozymes are RNA molecules with catalytic activity,
5. abzymes are engineered antibodies that can also catalyze reactions.

8.8 Self Assesment

1) what are enzyme inhibitors?
2) write briefly about Mechnism of Enzyme Action
3) what is meant by an Ribozyme and Abzymes?
4) what is meant by allosteric Enzymes?

8.9 Suggested Readings

1) Leininger's Principles of Biochemistry (2000) by Nelson, David L and Cox, M M, Macmillan/worth, NY.
2) Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte W Pratt, John Wiley & Sons, NY.
3) Biochemistry III ed (1994) by Lubert Stryer, WH Freeman and Co., San Francisco.
Outlines of Biochemistry (1987) by Eric E Conn, P K Stumpf, G Bruening and Ray H Doi, John Wiley & Sons, NY.

Dr Y.R.K V Tirupati Rao

LESSON-9

PROTEIN PURIFICATION AND LIPID SEPARATION

OBJECTIVES:

after completion of this unit, you would come to know briefly about protein purification and lipid separation methods**.**

9.1 INTRODUCTION

Protein purification and characterization is a crucial process in biochemistry and molecular biology, enabling researchers to study the structure, function, and interactions of individual proteins. The initial step, purification, isolates the target protein from a complex mixture, while characterization focuses on analyzing its properties

9.2 PROTEIN PURIFICATION AND 9.3 PROTEIN CHARACTERIZATION METHODS

A protein or other biological macromolecule must be purified before rigorously being studied structurally and functionally. Purifying a single protein from a mixture of as many as 10,000 other cellular or tissue proteins, each containing the same 20 naturally occurring amino acids linked in different orders, is difficult. Proteins differ in size (how many amino acids), charge (how many positively and negatively charged amino acids), sequence, and presence of specific binding sites on the proteins. Any technique that could be used to purify protein must be based on these inherent differences. Once the protein is purified, it must be analyzed by a spectral or electrophoretic technique.

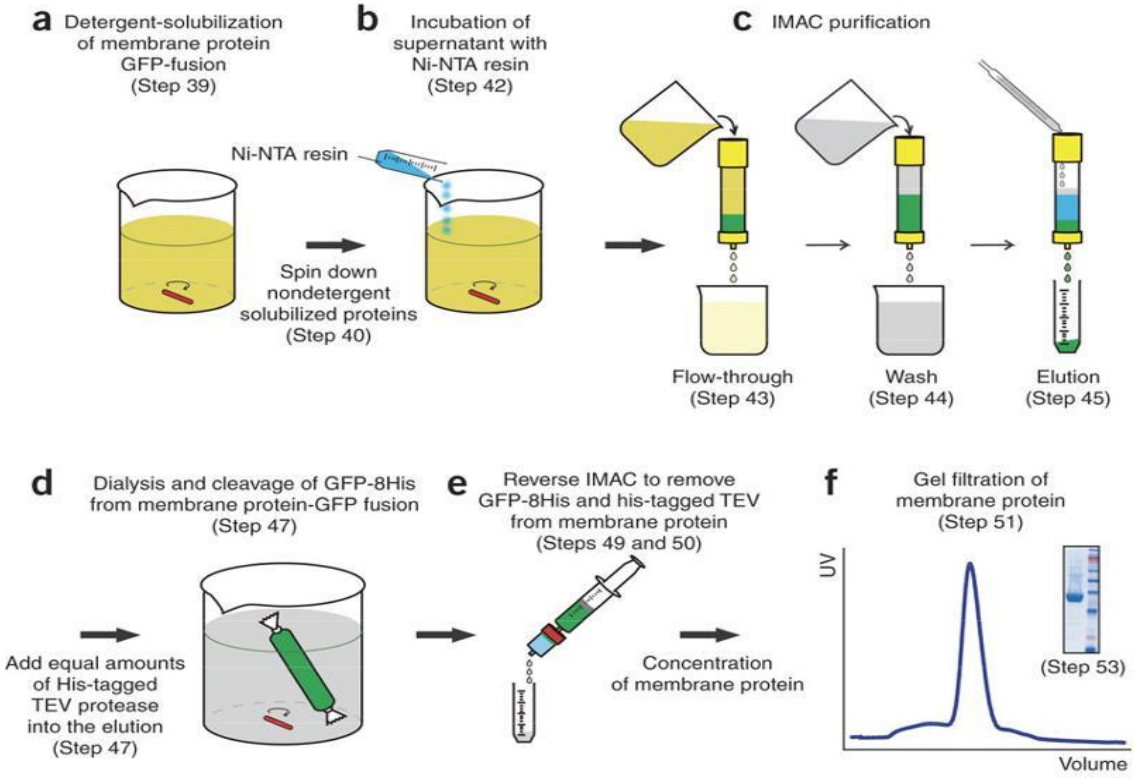
Protein purification is a series of processes intended to isolate and purify a single protein or complex from cells, tissues, or whole organisms. Protein purification is vital for characterizing the function, structure, and interactions of the protein of interest. Separation steps usually exploit differences in protein size, physical-chemical properties, binding affinity, and biological activity.

Protein purification is either *preparative* or *analytical*. **Preparative purifications** aim to produce a relatively large quantity of purified proteins for subsequent use. Examples include the preparation of commercial products such as enzymes (e.g., lactase), nutritional proteins (e.g., soy protein isolate), and certain biopharmaceuticals (e.g., insulin). Many steps and

quality control are required to remove other host proteins and biomolecules that could threaten the patient's health. **Analytical purification** produces a relatively small amount of a protein for various research or analytical purposes, including identification, structural characterization, and studies of the protein's structure, post-translational modifications, and function.

The choice of a starting material is key to the design of a purification process. In plants or animals, a particular protein usually isn't distributed homogeneously throughout the organism; different organs or tissues have higher or lower protein concentrations. Using tissues or organs with the highest concentration decreases the volumes needed to produce a given amount of purified protein. A protein of low abundance or high commercial value is often made using recombinant DNA technology.

It is through protein purification methods that we have been able to study and understand proteins in detail. These methods, or derivatives of the methods, are used in the clinical labs to identify abnormal samples. Protein purification methods use fraction techniques which are in a large part basedon:



solubility;
size;
charge;
binding specificity.

These properties of a protein are derived from the AA properties composing the protein. For example the molecular weight (MW) of a protein is just the summation of the masses of the individual AAs composing the protein. MW is usually expressed in daltons (Da) or kilodaltons (kDa). A Da is the same as an atomic mass unit which is approximately the mass

of a nucleon and is equivalent to 1 g/mol.To begin any sort of purification it is important that an assay be available to identify where the protein of interest is after the fractionation. Assays come in many different forms and depends in a large part on the type of protein you are trying to purify (i.e. is it an enzyme?). Commonly used assay technologies are:

i) spectroscopic (using Bradford reagent or a chromagenic substrate)
ii) immunological (using a antibody that can recognize the protein of interest)

These will be discussed in more detail later.

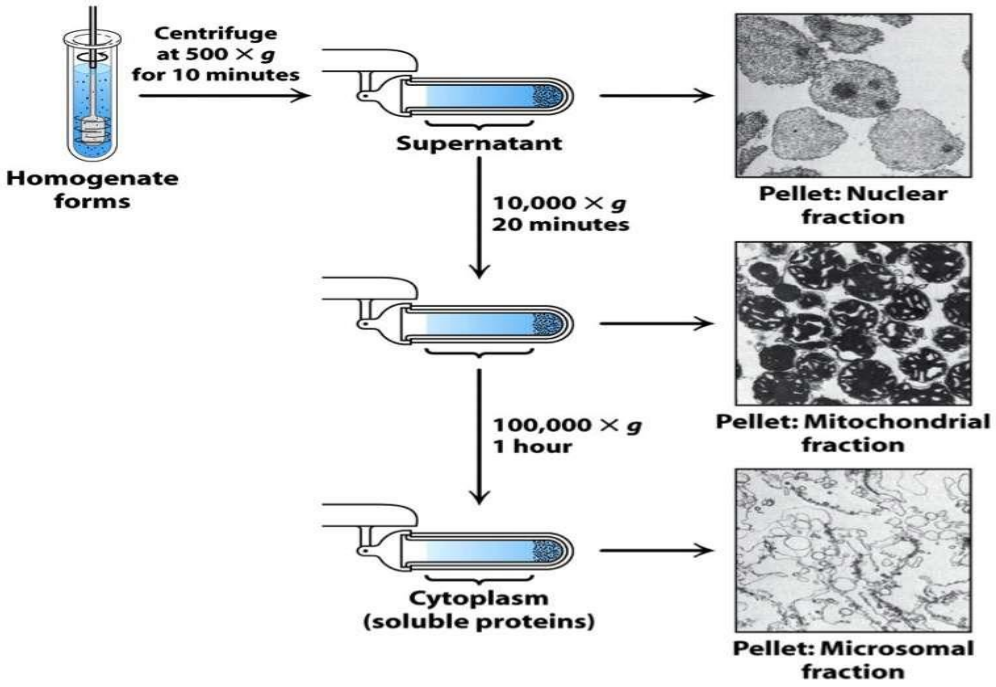
Crude Extracts

To being any sort of purification procedure you need to obtain the material from which you plan the isolate the material. Historically the abundance and ease of isolation dictated which proteins were first studied (e.g. hemoglobin). Also many proteins are common to a large number of species (e.g. metabolic enzymes) so they could be isolated in large abundance from other sources, such yeast or bovine.

Once you have gathered the material containing the protein you want to study it is necessary to generate a crude extract -- for proteins from muscle that would mean grinding it up, for an intercellular protein that would mean breaking the cells open, etc. This is always done in the presence of a buffer and inhibitors. Why? As a scientist you want to control the environment -- keeping the protein you are interested in at a non-denaturing pH, you want to keep it from being cleaved by enzymes that will be released in this process so general inhibitors will be important, etc.

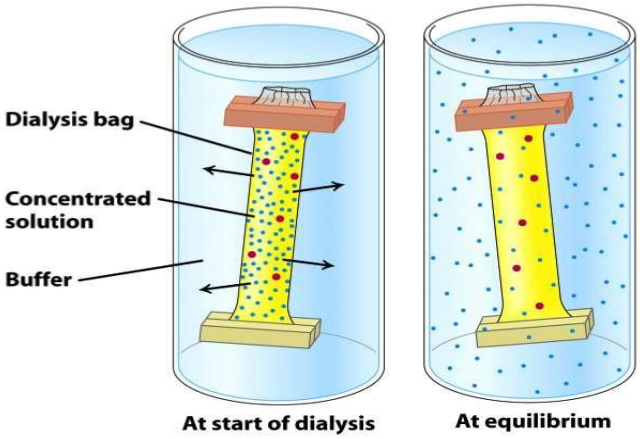
Centrifugation

Generally the first step after forming a crude extract is a simple filtration or centrifugation to remove the large material. Centrifugation is a process that involves the use of the centrifugal force for the sedimentation of mixtures with a centrifuge. This process is used to separate two immiscible liquids with more-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis. Centrifugation alters the effective gravitational force on to tube/bottle so as to more rapidly and completely cause the precipitate ("pellet") to gather on the bottom of the tube. The remaining solution is properly called the "supernatant". The supernatant liquid is quickly decanted from the tube/bottle without disturbing the precipitate.



Differential centrifugation, as shown in the figure, is multiple rounds of centrifugation at increased speeds and time allows for different cellular fractions to be separated.

Dialysis



Dialysis is a procedure for exchanging the solvent around a protein. In general the protein solution is placed inside a semi-permeable membrane (dialysis bag) which is suspended in a larger volume of buffered solution (see image to the right). The key to this procedure working is that the membrane has to be permeable to water and ions, but not to your protein of interest. Thus buffers & salts exchange until an equilibrium is established between the inside & outside of the membrane.

Naturally in medicine the types of dialysis you are likely to see are hemodialysis and peritoneal dialysis which remove wastes and excess water from the blood in different ways. Hemodialysis removes wastes and water by circulating blood outside the body through an external filter containing a semipermeable membrane. The blood flows in one direction and the dialysate flows in the opposite. The counter-current flow of the blood and dialysate

maximizes the concentration gradient of solutes between the blood and dialysate, which helps to remove more urea and creatinine from the blood. The concentrations of solutes (for example potassium, phosphorus, and urea) are undesirably high in the blood, but low or absent in the dialysis solution, and constant replacement of the dialysate ensures that the concentration of undesired solutes is kept low on this side of the membrane. The dialysis solution has levels of minerals like potassium and calcium that are similar to their natural concentration in healthy blood.

In peritoneal dialysis, wastes and water are removed from the blood inside the body using the peritoneal membrane of the peritoneum as a natural semipermeable membrane. Wastes and excess water move from the blood, across the peritoneal membrane, and into a special dialysis solution, called dialysate, placed in the abdominal cavity. Diffusion and osmosis drive waste products and excess fluid through the peritoneum into the dialysate until the dialysate approaches equilibrium with the body's fluids. Then the dialysate is drained, discarded, and replaced with fresh dialysate often 4-5 times pr day.

Column Chromatography

Column chromatography is one of the most powerful fractionation methods. It can separate components of mixtures based upon:

size (gel filtration/size exclusion)

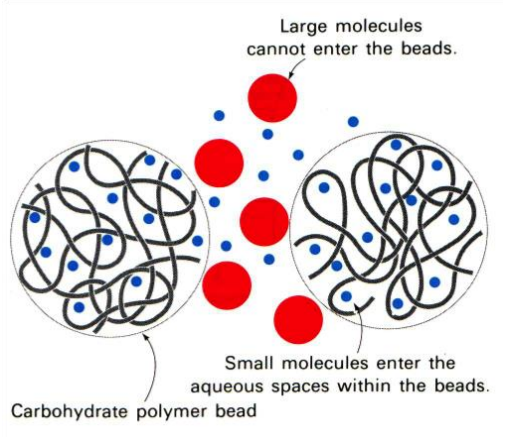
charge (ion exchange)

affinity (a specific binding affinity)

Commonalities between all three types of chromatography methods is that they all use a resin (solid phase) with special chemical properties held in a glass cylinder (called a "column"). A buffered solution (mobile phase) percolates through the column and is collected in tubes ("fractions") upon exiting the column. A protein mixture is applied in the mobile phase & percolates through through the column as an expanding band. Different proteins migrate differently depending on their properties and those of the resin.

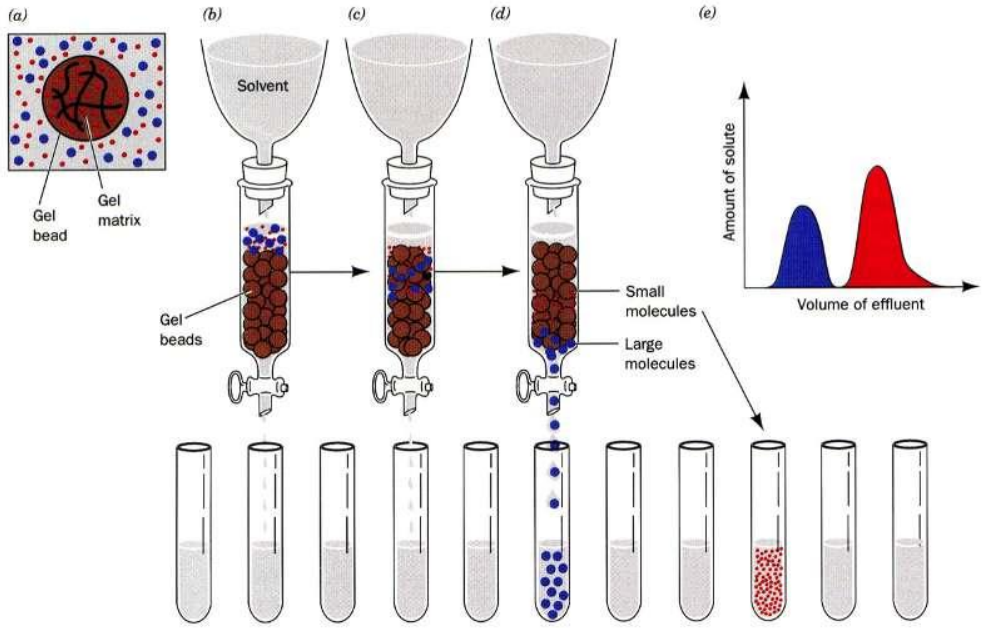
Below we will examine the different forms and examine their particular properties.

Gel Filtration/Size Exclusion



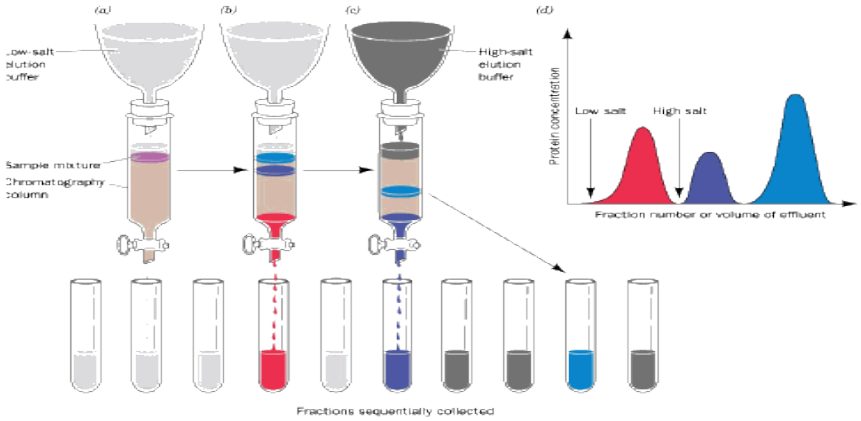
In gel filtration, or as it is sometimes referred to as size exclusion, chromatography the resin are porous (see figure to the left). Some molecules (blue here) can enter the resin and as the

lines try to indicate it is not a straight path through; thus it takes longer for small molecules to traverse the column than large molecules which travel around the outside of the resin. This is highlighted in the figure to the right where big molecules (blue) come off first and smaller molecules (red) later.



Ion Exchange

Ion exchange chromatography is broken in to two types - anion & cation exchangers. There are many different types of moieties that are used from weakly to very strongly charged thus allowing a huge range of molecules the ability to interact.



Ion-exchange chromatography with stepwise elution

Unlike gel filtration chromatography, here proteins directly interact with the resin. So generally the column is equilibrated in a buffer solution to establish a constant pH in the column, then the protein mixture is loaded where all or some of the proteins interact with the resin depending upon their own charge. Buffer is continued to be applied until all proteins not interacting with the resin have been washed off. At that point usually a gradient of increasing salt concentration (disrupts ionic and hydrogen binding) in the buffer is applied to column

allowing the most weakly interacting proteins to release first followed by the more strongly and finally the most strongly interacting. This can also be accomplished by changing the pH of the buffer being applied to the column.

Anion Exchanger

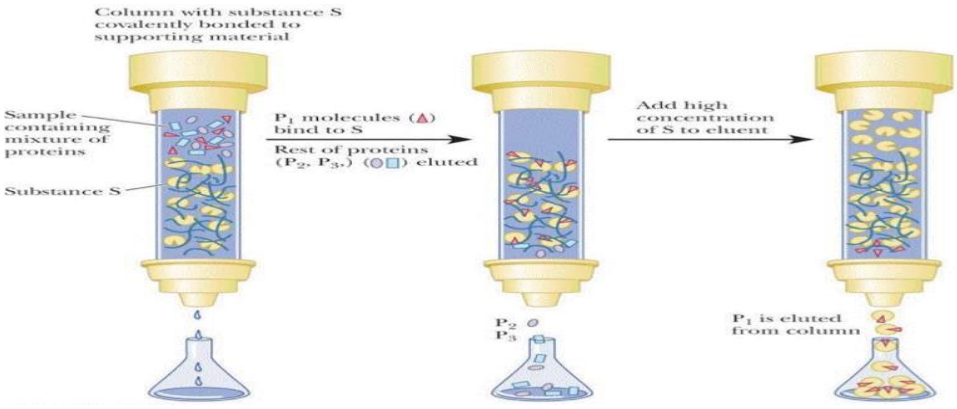
Anion exchanger means that it removes anions from protein mixture so that means the resin must be decorated with positively charged moieties. Before elution begins all positively and uncharged proteins will fall through the column. When you start eluting, first you will knock off the weakly negative proteins (e.g. -1 charge), followed by those with a stronger negative charge (-2), and finally the most negatively charged proteins (-3).

Cation Exchange

It is exactly oppose with a cation exchanger -- here cations are removed from the protein solution so the resin must be negatively charged. Again before elution begins all negatively and uncharged proteins will fall through the column. When you start eluting, first you will knock off the weakly positive proteins (e.g. +1 charge), followed by those with a stronger positive charge (+2), and finally the most positively charged proteins (+3).

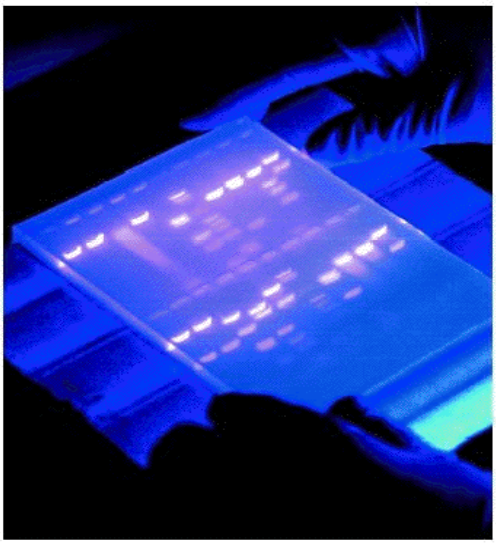
Affinity

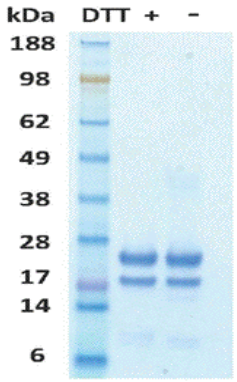
Affinity chromatography requires that you know something specific about your protein -- that it has a specific tag engineered into the sequence, that it binds NAD+, you know the ligand it binds or that you have a specific monoclonal antibody that interacts with your protein. For this type of chromatography your resin is decorated with the antibody, NAD+ or a divalent metal (most popular engineered tag is the 6xHis tag -- 6 His residues at either the N- or C-termini of the protein). Again the column resin is pre-equilibrated in the the appropriate buffer before the protein sample is loaded. It is expected that only the protein of interest will interact. Once all non-interacting proteins have been removed from the column then your protein can be eluted by changing the pH, adding salt, adding a metal chelator, or a high concentration of the ligand.



Electrophoresis

Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a uniform electric field. Thus it separates components of a mixture based on their size amd/or charge.





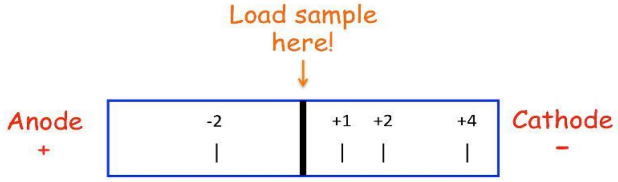
How do you remember which electrode is the cathode and which is the anode?

Simple ... anions travel to the anode and cations travel to the cathode.

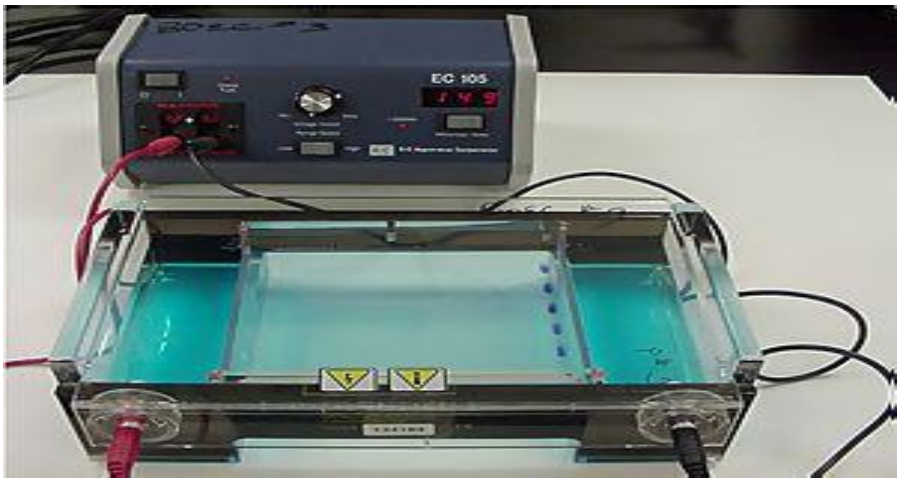
Visualization of proteins on paper or in a gel is an important step in any electrophoresis. Often with DNA the gels are soaked in ethidium bromide which intercalates into DNA and fluoresces under UV light (left image). Proteins may be visualized using silver stain or Coomassie Brilliant Blue dye (right image). In some cases the gels are transferred to a solid support (nitrocellulose) and then probed with specific antibodies (Western Blot).

Paper electrophoresis

Generally used to separate AAs or peptides of differing charge. As shown in the figure, AAs and peptides will separate based on their charge with the most highly charged species moving the furthest.



PAGE (PolyAcrylamide Gel Electrophoresis) -- Native Gel

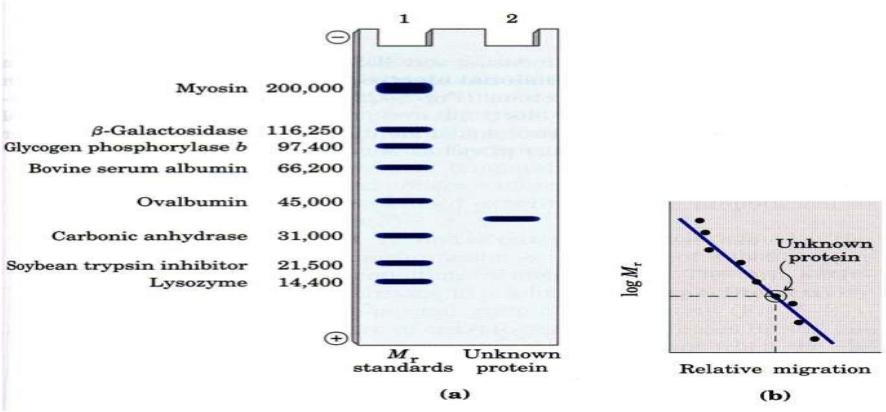


It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. It is a process which enables the sorting of molecules. Using an electric field, molecules (such as DNA) can be made to move through a gel made of agar or polyacrylamide. The electric field consists of a negative charge at one end which pushes the molecules through the gel, and a positive charge at the other end that pulls the molecules through the gel. The molecules being sorted are dispensed into a well in the gel material. The gel is placed in an electrophoresis chamber, which is then connected to a power source (see figure to the left). When the electric current is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel.

The term "gel" in this instance refers to the matrix used to contain, then separate the target molecules. In most cases, the gel is a crosslinked polymer whose composition and porosity is chosen based on the specific weight and composition of the target to be analyzed. When separating proteins or small nucleic acids (DNA, RNA, or oligonucleotides) the gel is usually composed of different concentrations of acrylamide and a cross-linker, producing different sized mesh networks of polyacrylamide. When separating larger nucleic acids (greater than a few hundred bases), the preferred matrix is purified agarose. In both cases, the gel forms a solid, yet porous matrix. Agarose is composed of long unbranched chains of uncharged carbohydrate without cross links resulting in a gel with large pores allowing for the separation of macromolecules and macromolecular complexes.

"Electrophoresis" refers to the electromotive force (EMF) that is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, determined largely by their mass but also their charge and shape which varies widely for proteins. Electrophoretic mobility of small molecules is greater than the mobility of large molecules with the same charge density thus allowing separation. To separate proteins or DNA generally the pH of the buffer and protein mixture is high (~9) so that the proteins carry a net-negative charge. However, because size, charge and shape all play a role in how a molecule will behave in a native gel most scientists use a SDS-PAGE gel which is predictable.

SDS PAGE



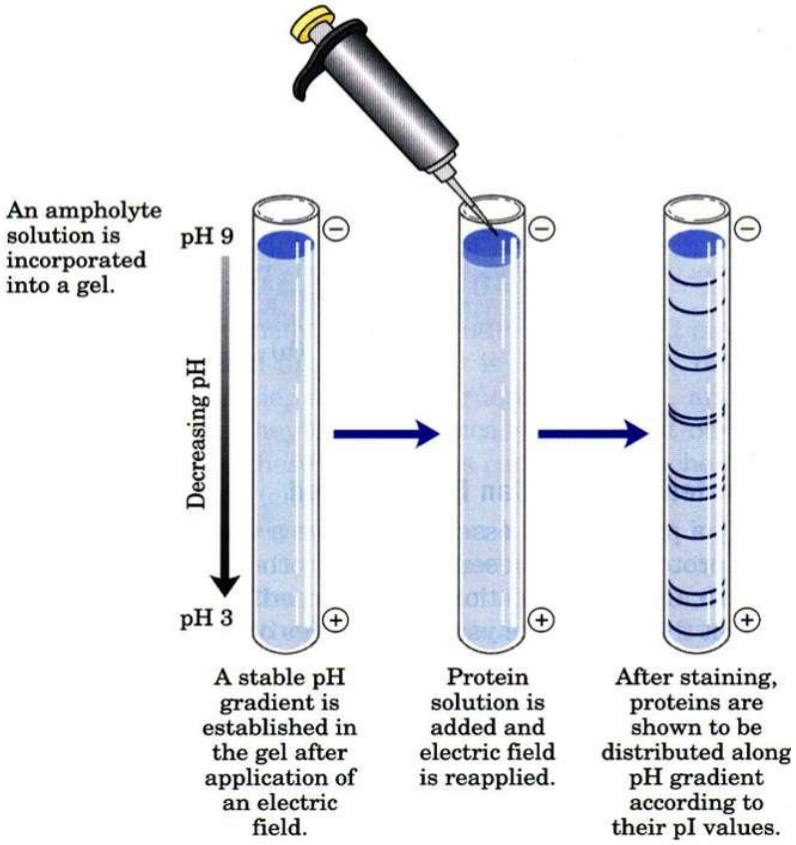
SDS PAGE separate molecules by size because the presence of SDS (sodium dodecyl sulfate) denatures the protein removing 2˚, 3˚ and 4˚ structures (they assume a linear chain) and the

SDS coats the molecules giving them a uniform charge/mass ratio. Most often protein samples are also treated with ß-mercaptoethanol (ß-ME) to break any existing disulfide bonds and to give them a linear chain (1˚ structure).

The presence of standards of known size always a calibration curve to be created that can be used to identify the approximate MW of an unknown protein (band).

IEF (IsoElectric Focusing) electrophoresis

Isoelectric focusing (IEF) is a technique for separating different molecules by differences in their isoelectric point (pI). It is a type of electrophoresis, usually performed on proteins in a gel, that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings. When an IEF gel is poured a pH gradient is

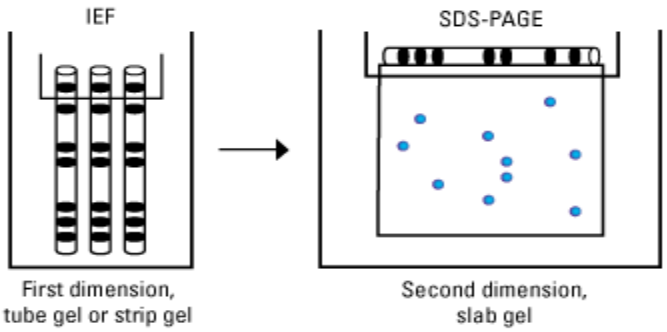


established

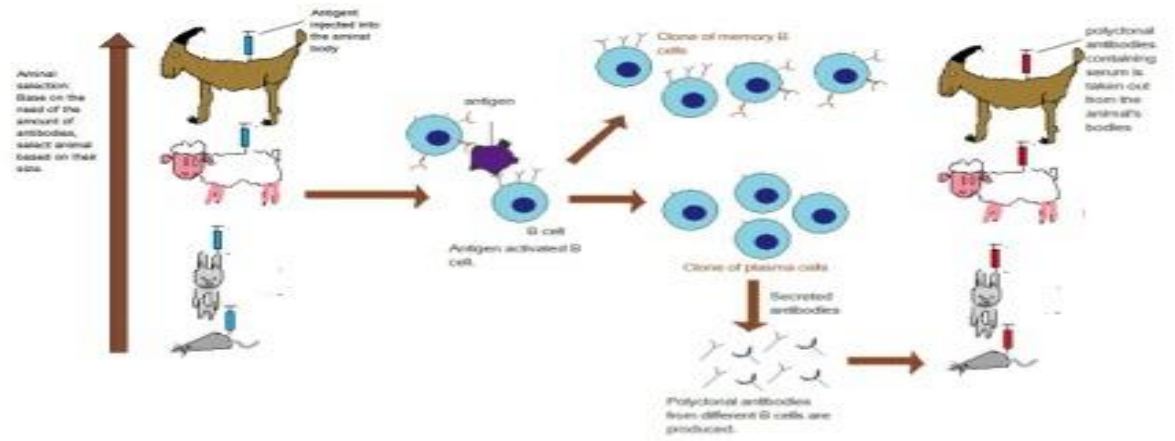
A protein that is in a pH region above its isoelectric point (pI) will be negatively charged and will migrate towards the anode (positive). As it migrates through a gradient of decreasing pH, however, the protein's overall charge will increase until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and so migration ceases (as there is no electrical attraction towards either electrode). As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands.

2D Gel Electrophoresis

Two-dimensional gel electrophoresis (2D electrophoresis) is a form of gel electrophoresis commonly used to analyze proteins in which mixtures of proteins are separated by two properties in two dimensions on gels. As shown in the figure, 2D electrophoresis begins with an IEF gel (in a tube) which separates proteins based on their pI. This is then laid on top of a SDS-PAGE gel (90 degrees from the first separation). Because it is unlikely that two molecules will be similar in two distinct properties, molecules are more effectively separated in 2D electrophoresis than in 1D electrophoresis.



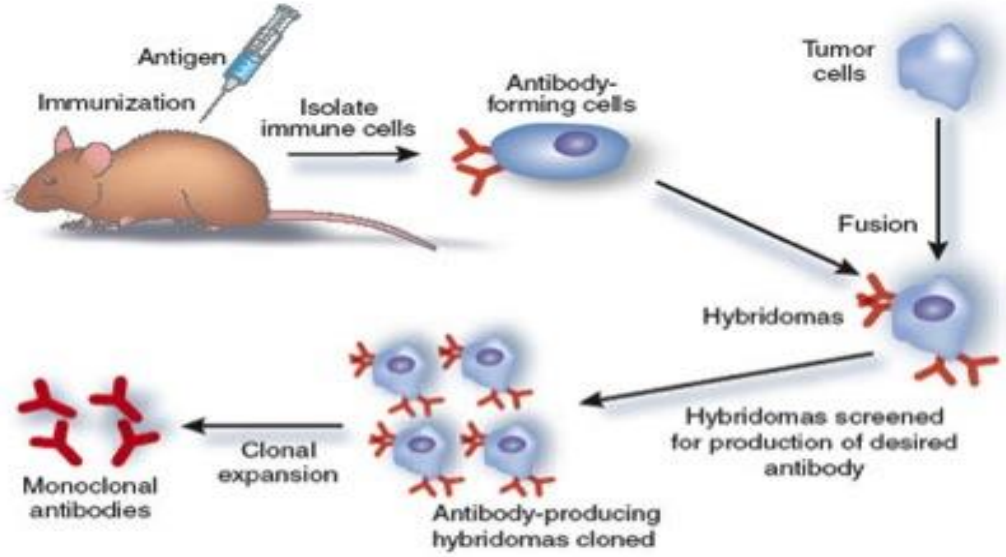
Antibodies



Antibodies are proteins made by B cells (part of the body's immune system) with each B cell producing unique antibodies that recognize a specific epitope on the antigen. An antigen is any substance that provokes an immune response – something foreign or toxic to the body. An epitope is a distinct molecular surface of an antigen capable of being bound by an antibody; for proteins these are divided into two categories, conformational epitopes and linear or sequential epitopes, based on their interaction with the antigen. The normal function of an antibody is to bind foreign substances (antigens) and flag them for destruction. This ability of antibodies to recognize and bind an epitope on an antigen makes them an important tool in research and the clinical laboratory. In fact, more than 30 antibodies are currently used therapeutically.

To generate an antibody the antigen (can be a whole protein or fragments of) are injected into an animal (often a mouse, rabbit, goat or donkey) several times over the course of several months. The key is the antigen must be different enough from the animal's own proteins to allow an immune response to be generated.

A polyclonal antibody actually refers to all the antibodies (IgG) that were in the serum of the host at the time the blood was collected. Since we have stimulated the immune system to produce antibodies, lots of B cells will be producing antibodies to many different epitopes on the antigen. Hence why it is called polyclonal.



A monoclonal antibody is produced by one cell thus they all recognize the same epitope on the antigen. To produce a monoclonal antibody, tumor cells that can replicate endlessly are fused with B cells from an animal which has been stimulated with an antigen. The result of this cell fusion is a "hybridoma," which will continually produce antibodies.

Western Blotting

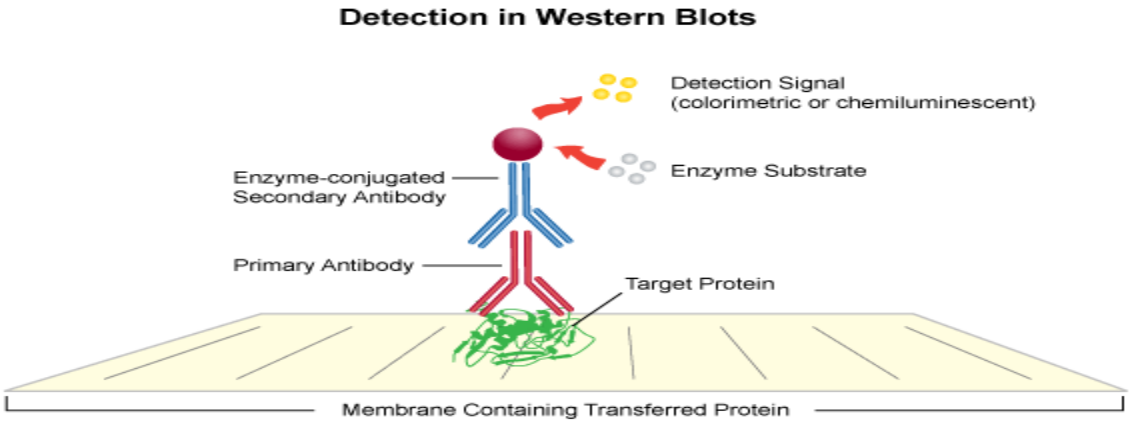
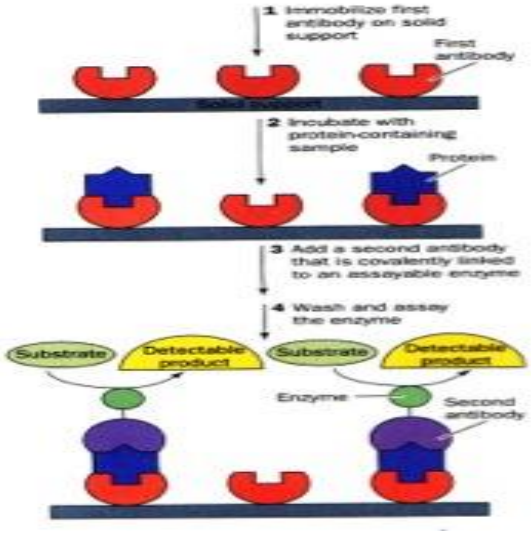


Diagram 2: *Illustration of detection in Western Blots.*

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) is a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture in a semi-quantitative manner.

The first step is to separate the macromolecules using gel electrophoresis (native or SDS-PAGE). After electrophoresis, the separated molecules are transferred to a membrane (usually nitrocellulose). As the membrane will bind any protein (including the antibody you will use to detect your protein of interest), after transferring the sample the membrane must be blocked with a common (cheap!) protein to prevent any nonspecific binding of antibodies to the membrane. Detailed procedures for detection of a protein on a Western blot vary widely. Most laboratories use a indirect detection method, in which a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody which recognizes the primary antibody. Labels include biotin, fluorescent probes, and enzyme conjugates that convert a substrate to a colored product thus staining the membrane.

ELISA (Enzyme Linked ImmunoSorbent Assay)



There are MANY forms of ELISAs. Most frequently used is the "sandwich" form in which an antibody is bound to a well in a microtiter dish. The sample is added, incubated, and then protein which were not captured by the antibody washed away. A labeled secondary antibody which recognizes a different part of the bound antigen can be used to quantify the amount of antigen in the sample.

Mass Spectrometry

Mass Spectrometry is one of the most important analytical techniques available, and part of the power of the technique lies in its ability to detect minute quantities of material - 100 picograms or less. A mass spectrometer determines the mass of a molecule by measuring the mass-to-charge ratio (m/z) of its ion. Ions are generated by inducing either the loss or gain of a charge from a neutral species. Once formed, ions are electrostatically directed into a mass analyzer, where they are separated according to m/z and finally detected. In general the results of a MS or MS/MS experiment are compared to standards in a database to determine the components of the original sample.

9.4 Methods of lipid Separation and analysis

Purification of Lipids

The source of the lipid material to be extracted can be an animal tissue, plant tissue or even microbial.Nevertheless, isolation of lipid follows some common approaches and common

concerns.It is advisable to keep the sample at low temperature (less than -25°C is a good choice) and in an oxygen free/inert atmosphere. The lipids containing unsaturated bonds, as we know, are prone to oxidation.

For the same reason, butylated hydroxy toluene (BHT) is added in the range of 1-10 mg/L of the extracting solvent. Extraction with the organic solvents is invariably the first step in lipid isolation. Keeping the temperature low also minimizes damage to the lipids by enzymatic or non-enzymatic routes.This is especially important for plant tissues which are rich in both polyphenols and polyphenol oxidases.It must be remembered that even in the frozen form, enzymatic degradation cannot be totally stopped.This is best appreciated by recollecting that even frozen food have shelf life.Some of the approaches which have been tried for inactivating endogeneous enzymes are: exposing the tissues to boiling water or steam or even microwaving the sample.

The solvent chosen for extraction depends upon the nature of the lipid to be extracted. Generally, mixture of solvents, rather than a single solvent is tried. Extracting with CHCl3: CH3OH: H2O (in the proportion of 1:2:0.4) is reported to extract practically all kinds of lipids.

The amount of water present in the sample should be factored in while preparing the solvent mixture.These solvents mixed as above form a single phase mixture.The lipid material is homogenized in the above solvent mixture. The rest of the solids separated from the extracted lipids remain in the solvent mixture.Adding either of the three liquids leads to separation of the extracted samples into two phases. Upper aqueous phase has any non-lipid material which got extracted.The lower CHCl3 containing phase has all the extracted lipids. This phase is washed once with water to further remove any adhering non lipid material.Phospholipids etc which are more polar in nature can partition into the water phase. This can be avoided by using salt solutions (at all above steps) in place of water.The CHCl3 phase is dried by treatment with anhydrous Na2SO4. The solvent (CHCl3) thereafter can be removed by evaporation by any of the usual devices.

Selective extraction of neutral lipids from the dried mass can be carried out by extraction with cold anhydrous acetone. This extraction procedure does not extract phospholipids. Dry acetone of low water content is available from good vendors like Aldrich. This low water grade acetone can be further dried by placing over molecular sieves. As has been mentioned before, some free fatty acids are always present in a fat/oil. These fatty acids can be isolated by treating the tissue or liquid extract by dilute alkali. Both aqueous and methanolic solutions have been used.Most of the vegetable oils are obtained by mechanical processing and/or extraction from seeds or by pressing of fruits like olive or olive palm.

Among the solvents (for solvent extraction), hexane is invariably used. Hexane is considered a volatile organic compound (VOC) which hurts the ozone layer. However, despite numerous efforts industry has failed to find a good substitute for hexane.From time to time, some greenner options like aqueous oil extraction and aqueous enzymatic oil extraction have been tried. Adequate improvement in these methods by assistance from microwaves and ultrasonication have also been reported.Economic considerations and reluctance of the industry to invest in development of these greener options have contributed to hexane being continued to be used in the oil industry.

Lipids from various sources

The milk lipids contain ~97% triglycerides and small amount of phospholipids and sterols which originate from membranes of the milk fat globule. Over 400 different fatty acids are detected in the milk fat with estimated number of triglycerides in thousands. Oxo- and hydroxy fatty acids present in milk fat are responsible for forming flavoring ketones, aldehydes and lactones. Short chain fatty acids released if fat globules are damaged (during processing and in transfer through pipelines) cause the unpleasant smell in raw milk. Obtaining milk fat involves separation of fat globules, churning, aggregation of fat globules, separating the butter milk and stabilization of water-in-oil emulsions. Milk fat is liked because of its high and pleasant organoleptic sensations like flavour and texture.

Fish oil is mostly a by product of fish mill industry. It is produced by passing steam over raw fish. The oil is separated from water. Fish oil is known for high degree of double bonds, >50 different fatty acids (C14-C24) and above all for the presence of ω-3 and ω-6 fatty acids. Fish oils contain unsaponifiable lipids like sterols, hydrocarbons, glyceryl ethers, pigments, vitamins and oxidized oil. Industrially fish body oil and fish liver oil is distinguished. Cods and pollack have large livers and are sources of liver oils. In other cases, it is the fish flesh which is the source of the oil. When required as an ingredient or supplement (like ω-3 fatty acids), it refined by processes similar to vegetable oils: neutralization of free fatty acids, bleaching, winterization and deodorization.

Adsorption and Normal phase liquid Partition Chromatography

Silica gel as a chromatographic media has been widely used for analysis of lipids. TLC, low pressure liquid chromatography and HPLC formats have exploited the differences in the number and nature of the polar functional groups in lipids. Silica as a medium has silanol groups which can be free or H-bonded. Silica has layers of water of hydration. To ensure variability in results, it is necessary to remove all but very tightly bound water layer. This dictates choice of the solvent for running the column. Reversible damage to silica columns can occur if polar impurities accumulate. In such cases, the column should be washed with a negative gradient of polar solvents. Solvents containing water at pH <2 or > 7.5 can also dissolve the silica from the surface and would irreversibly damage the column. In the normal phase chromatography, silica can be bonded with some organic moieties like diol, nitro, nitride and alkyl or aryl cyano. Such bonded phases are known to decrease “tailing” of the peaks. It should be noted that even such columns do contain variable amounts of silanol groups which remain and contribute to the separation process. In all HPLC methods, the choice of the detector is based upon not only the compounds to be separated but also by the chice of eluting solvents. UV detectors are useful if the lipid contains conjugated double bonds or aromatic rings or oxidised lipids as a result of
hydroperoxidation. Derivatization techniques like benzoylation of glycolipids or converting fatty acids to aromatic esters have been used to make UV detection possible. RI detectors and evaporative light scattering detectors are of more general use.

Gas Liquid Partition Chromatography

For volatile or low boiling point lipid samples, GC is a convenient and frequently used technique. Again, silica is the material. Fused silica narrow bore column (0.1-0.3 mm internal diameter, 25-100 m in length) with inner walls coated with the liquid phase is used. The distribution coefficient of the lipid molecules between the liquid phase and the carrier gas dictates the separation.The molecules partitioning in the gas phase travels with the gas down

the column. Depending upon the differences in partition coefficient, different materials emerge out of the column at different retention times. The parameters which influence retention times are: nature and flow rate of the carrier gas, column dimensions, column temperature and thickness of the liquid phase. Of these column length is less important as resolution is proportional to √l. Many non volatile molecules can be derivatized and converted into more volatile substances. Againsilylation chemistry is widely used for this purpose and various silylation reagents are commercially available.

Reverse Phase Liquid Partition Chromatography

Here the stationary phase is non polar and the mobile phase is relatively polar. This makes this mode of HPLC very valuable for separation of various lipid molecules of similar kind/class. Some of the detectors used in GC are flame ionization detector, electron capture detector (especially useful for halogen containing lipids), mass spectrometers. Actually GC – MS has emerged as powerful even in lipid analysis. Chain length of the fatty acid component and the number of double bonds present in the chain decides the distribution coefficient of a particular molecule between the two phases. Binding of lipids to the column involves London forces, polar dipole-induced dipole, dipole-dipole and proton acceptor-proton donor interactions. The stationary phase for lipids most frequently used are chemically bonded long chain hydrocarbons to spherical silica particles of 3-10 μm. The most common is the so called C18 or octadecylsilyl (OOS) column. For most lipid separation on such a column, acetonitrile or methanol is mixed with another modifier solvent. The latter is decided on the basis of nature of the lipid being separated. The modifier organic solvent (mixed with either CH3CN or CH3OH) solvates the chain bonded to the silica. This stretches the chains. Solvents like THF (with a low dielectric constant) results in the formation of a kink in the chain. So, the solvent composition decides how much the chains are extended or are in kinks or gauche form.

Ion Exchangers

Some ionic liquids like some phospholipids have been separated on ion exchangers. Again, silica bonded with amine group (anion exchanger) or sulphonic acids (cation exchanger) has given good results. Organic solvents used in the mobile phase change the nature of the chromatography to mixed mode chromatography as adsorption and partition processes come into play.

Silver Ion Chromatography

This is complexation chromatography in which silver ions are part of the stationary phase. This is also called argentation chromatography. The technique is particularly useful in lipid analysis. Silver ions interact with π-electrons of double bonds to form a polar complex. A lipid with larger number of double bonds obviously forms a stronger polar complex and is retained on the column more firmly. These are essentially charge transfer complexes in which the C-atoms with double bonds are donors and silver ions are acceptor of electrons. The free olefinic bond and complexes are in dynamic equlibrium during the chromatographic process. In TLC, silica gel is impregnated with silver nitrate molecules. The same approach can also work in HPLC. However, a more robust approach is to have silica bonded with a moiety like phenylsulfonate residue. The resulting ion exchanger binds silver ions. As a result of steric factors, a double bond in the cis-configuration forms a more stable charge transfer complex with the silver ion. The relative position of double bonds if present also matter.

The most stable are chelates formed with double bonds separated by two methylene groups. Conjugated double bonds result in a less stable complex of the lipid molecule with silver ion. lefinic bonds form stronger complexes then acetylinic bonds. The complex stability is more at lower temperatures. The technique has been extensively used for separation of simple fatty acid mixtures. It is also useful for separation of triglycerides. There is a whole area called "lipidomics" which deals with lipids: isolation, analysis, production and quality control. What we have discussed is just a broad overview to illustrate the kind of concerns which are there in the actual isolation, separation and analysis of lipids. Today GC-MS, capillary electrophoresis-MS and many hyphenated techniques are available which allows one to analyse lipids in detail. Industry, however, goes by established standards and hence the classical parameters are still important and worth knowing.

9.5 SUMMARY

Protein purification isolates a specific protein from a complex mixture, while lipid separation focuses on isolating specific lipids. Both processes utilize various techniques, often based on differences in size, charge, solubility, or binding affinity. Protein Purification is to isolate a target protein from other cellular components. The methods involved in the protein purification are Centrifugation, Precipitation, chromatography, ultra filtration and dialysis. The importance of protein purification is for studying protein function, structure, and interactions.

- Lipid Separation involves the isolation of Specific lipids from a mixture. The methods of lipid separation includes centrifugation, Extraction, Chromatography. The importance of lipid separation is for studying lipid metabolism, structure, and function.

9.6 SELF ASSESSMENT

1) Write about protein purification and Characterization Methods?
2) Write about lipid separation and analysis techniques?

9.7 SUGGESTED READINGS:

1. Leininger's Principles of Biochemistry (2000) by Nelson, David L and Cox, M M, Macmillan/worth, NY.
2. Fundamentals of Biochemistry (1999) by Donald Voet, Judith G Voet and Charlotte W Pratt, John Wiley & Sons, NY.
3. Biochemistry III ed (1994) by Lubert Stryer, WH Freeman and Co., San Francisco.
4. Outlines of Biochemistry (1987) by Eric E Conn, P K Stumpf, G Bruening and Ray H Doi, John Wiley & Sons, NY

Dr Y.R.K V Tirupati Rao

LESSON-10

SPECTROSCOPY

Objective

This lesson mainly deals with properties of electromagnetic spectrum, various spectroscopic techniques (UV-vis, Infrared and Mass spectrophotometry), their principle and applications.

10.1 INTRODUCTION

Spectroscopy deals with interaction of electromagnetic radiations with matter. Spectroscopic techniques now occupy a key position in biochemical studies and some of them are employed routinely and quite extensively. These techniques are extremely useful in characterization of chemical compounds in a sample on the basis of their spectral properties. In the beginning, the spectroscopy was largely confined to use light (visible radiation) for quantitative estimation and in some cases even for identification of biomolecules. With new developments and refinements in instrumentation, the scope of spectroscopic techniques broadened tremendously and now, in addition to visible light, it encompasses examining the behaviour of chemical substances upon their irradiation with regions of electromagnetic radiations such as X-rays, UV-rays, infrared light, microwaves and even radio frequencies. The spectrophotometric methods offer arapid and convenient means of qualitative identification and quantitative estimations of biomolecules even in relatively impure samples.

10.2 Electromagnetic radiation properties

The electromagnetic spectrum is composed of a continuum of waves with different properties (Fig. 10.1). Several regions of the electromagnetic spectrum are of importance in biochemical studies including X-rays (upto 7 nm), the ultraviolet light (UV, 180-340 nm), the visible (VIS, 340-800 nm), the infrared (IR, 1000-100,000 nm), and radio waves (NMR, 106-1010nm).

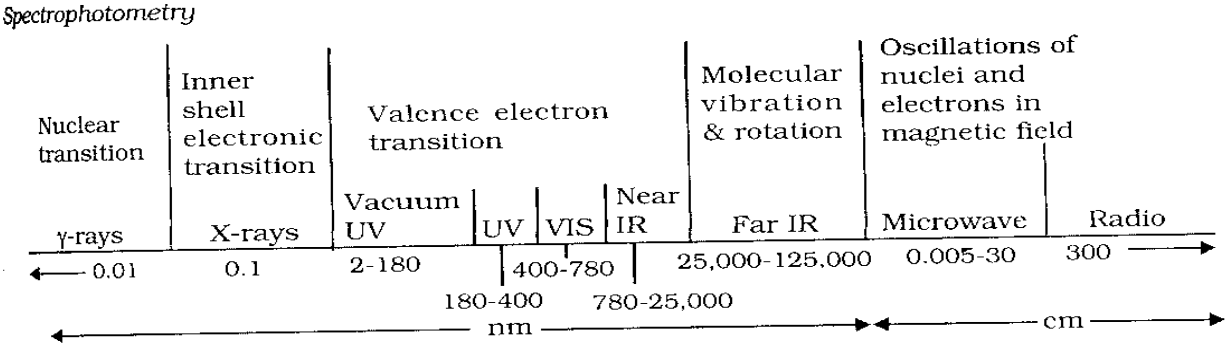


Fig. 10.1 The electromagnetic spectrum – the main regions and their wavelengths. Physical events involved in their production are also indicated. The electromagnetic radiation (Fig. 10.2) is composed of an electrical field component E, and a magnetic field component H, which scillate in planes at right angles to each other and both at right angles to the direction of propagation. The light propagated either in wave form or corpuscular (or photons or quantum) form the wave length (λ) of light, defined by equation 10.1,

Wavelength may be measured in centimeters (cm), micrometers (μm), nanometers (nm), or angstrom units (Ao), where 1 nm = 10-3 □m = 10-6 mm = 10-7 cm = 10-9 m, and 1Ao = 10-8 cm. Sometimes a term, frequency, V, is used rather than wave length to describe a particular radiation. The number of waves passing through afixed point on the time axis per second is known as the frequency, V, of the radiation, usually expressed in hertz (HZ).

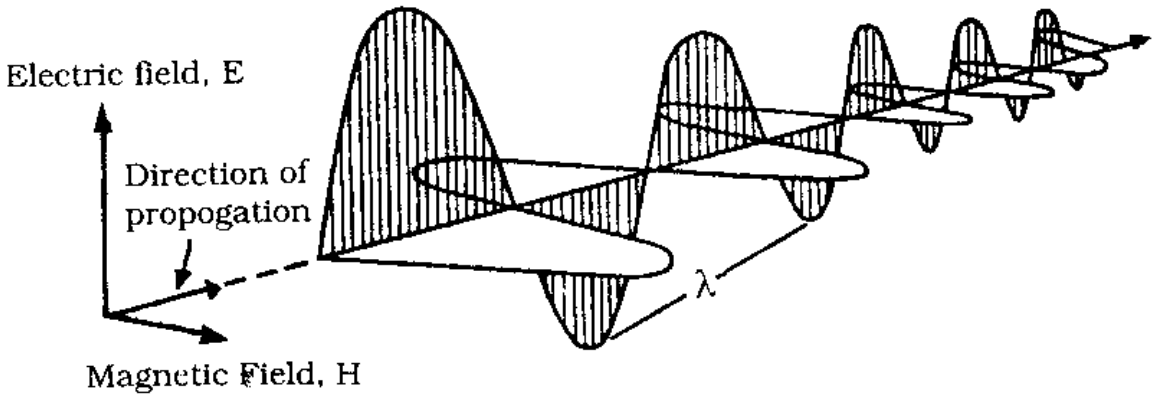


Fig. 10.2 The electromagnetic wave. The magnetic vector (unshaded) and electric vector (shaded)are perpendicular to each other and to the direction of propagation. Wavelength (λ) is the distance between two crests or troughs.

C
□= ----- ... Equation 10.1
V

Where
λ= Wave length
V = Frequency
C = Velocity of the light, 3 x 108 m/sec.
Frequency shares an inverse relationship with the wavelength so that
V = C/ λ… Equation 10.2

Radiation in the infrared region is characterized by another term known as '**Wave number'** and denoted by the symbol □ V. Wave number of light, defined by Equation 11.3 is the number of complete cycles occurring per centimeter.

□V = 1/□… Equation 10.3
The amount of energy, E, associated with energetic particles (or photons) is given by Equation 10.4.

E = hv = h c/□… Equation 10.4

where h is plank's constant, c is the velocity of the light in vacuum. Wave length and frequency share an inverse relationship; this means that as the wave length increases, the energy of the radiation decreases, while the energy increases with the increase in frequency. When a photon of specified energy interacts with a molecule (matter), one of two processes may occur. The photon may be scattered, or it may transfer its energy to the molecule, producing an excited state of the molecule. The former process, called **Rayleigh scattering**, occurs when a photon collides with a molecule and is diffracted or scattered with unchanged frequency. Lights cattering is the physical basis of several

experimental methods used to characterize macromolecules. The other process mentioned above, the transfer of energy from a photon to a molecule, is **absorption**. For a photon to be absorbed, its energy must match the energy difference between two energy levels of the molecule. Molecules possess a set of quantized energy levels, as shown in Fig. 10.3. In Fig. 10.3, different electronic states are shown, a ground state, G, the first excited state, S1, and second excited state, S2. These states differ in the distribution of valence electrons. When electrons catapult from a ground state (G) orbital to an orbital of higher energy in S1, an **electronic transition** occurs. The energy associated with UV and visible light is sufficient to promote the molecules from one electronic state to another, that is, to move electrons from one orbital to another. The atoms in molecules may vibrate and rotate about a bond axis, which gives rise to vibrational and rotational sub-levels. This situation is shown diagrammatically in Fig. 10.3. The electronic transition for a molecule from G to S1, has high probability of occurring if the energy of the photon corresponds to the energy necessary to promote an electron from energy level E1 to energy level E2:

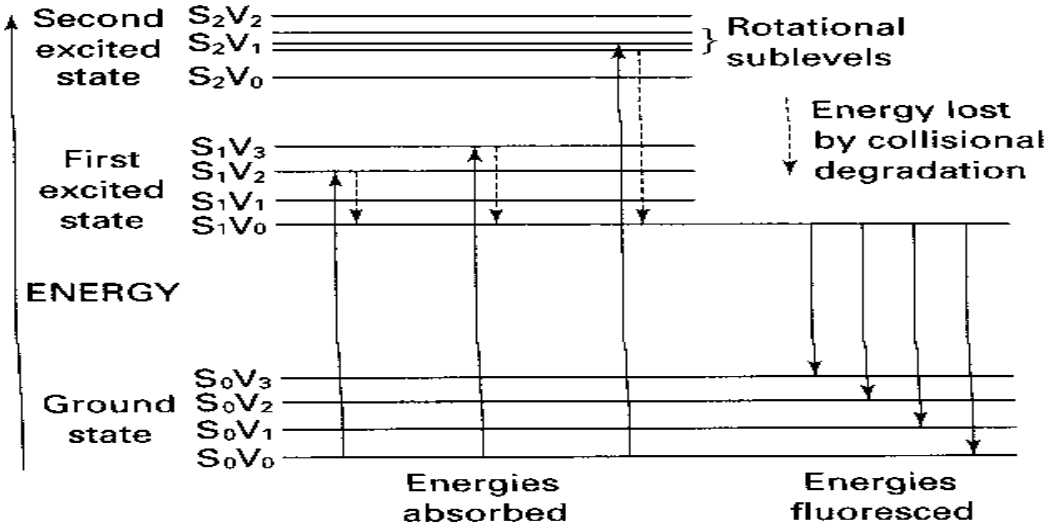


Fig. 10.3. Energy levels and transitions of electrons: in a fluorescent organic molecule.
Note for clarity, rotational sublevels have been indicated only for vibrational sublevel S2V1.

Where Δ E = E2 – E1 = hv,
hc
because of V = c/□□Δ E = ----- … Equation 10.5
Where Δ E is the change in energy state of the electron or the energy of electromagnetic radiation absorbed or emitted by an atom or molecule.
E1 = energy of electron in original state (G)
E2 = energy of electron in final state (S)

10.3 The Laws of Absorption

The absorption of light by any absorbing material is governed by two laws. The first of these laws is known as Bouger-Lambert Law. It states that the amount of light absorbed is proportional to the thickness of the absorbing material and is independent of the intensity of the incident light. It is an exponential function and may be expressed asI_ = C-kb
Io
I = the Intensity of the transmitted light,
Io = the intensity of the incident light,
b = path-length (the absorbing thickness)
and K = the linear absorption coefficient of the absorbing material. The power term in the above relationship can be removed by converting to the logarithmic form. Thus,
I Io

In ---- = - Kb, or In ----- = Kb
Io I
changing to common logarithms we get,
Io
2.303 log10 ---- = Kb … Equation 10.6
I
The second law of absorption is known as the **Beer's law**. This law states that the amount of light absorbed by a substance is proportional to the number of absorbing molecules i.e., the concentration of absorbing solution. This can be mathematically expressed in the form of an equation similar to the one above.

Io
2.303 Log10 ---- = K'C … Equation 10.7
I
Where K' = absorptivity constant, and
C = the concentration of the absorbing material
We can combine the Equations 10.6 and 10.7 for the **Bouger–Lambert Law** and the **Beer's Law**. Here, K and K' merge to become a single constant **a**. The combined equation is
Io
Log10 ---- = abc … Equation 10.8
I
Equation 10.8 has been alternately referred to as the Beer-Lambert Law, **the Bouger-Beer Law**, or more simply, Beer's-Law. This combined law states that "the amount of light absorbed (absorbance or extinction) is proportional to the concentration of the absorbing substance and to the thickness of the absorbing material (path length). The ratio, I/Io, of the intensities of transmitted and incident light gives the transmittance, T (the amount of light which escapes absorption and is transmitted). The reverse, Io/I is known as the absorbance (A) or the optical density (O.D). Absorbance shares a linear relationship with sample concentration. On the other hand, the relationship between transmittance and sample concentration is a non-linear one. It is therefore easier to use absorbance as an index of sample concentration. The two terms are mathematically commutable and so one can be calculated from the other. For this purpose, we rewrite the equation
so that
A (absorbance) = log Io – log I
but Io is always set at 100% and log 100 = 2, so
A = 2-log I … Equation 10.9

10.4 ULTRAVIOLET – VISIBLE ABSORPTION SPECTROPHOTOMETRY

Electron transitions in atoms or molecules (Fig. 11.3) give rise to the electronic spectra generally observed as absorption, emission or fluorescence phenomena in the ultraviolet and visible regions of the electromagnetic spectrum. UV and visible regions of the electromagnetic spectrum and associated techniques are probably the most widely used for routine analytical work and research in biological problems. The instruments that are used to study the absorption or emission of electromagnetic radiation as function of wavelength are called **Spectrometers** or **Spectrophotometers** (colorimeters, if the instrument applies wavelengths only in the visible range).The spectrophotometer is used to measure absorbance experimentally. This instrument

produces light of a preselected wavelength, directs it through the sample, and measures the intensity of light transmitted by the sample. More or less similar optical principles are employed in these instruments. There are, however, some important differences in the specific components used in the various regions of the spectrum. The essential components of a spectrophotometer include: (i) light source, (ii) a monochromator (including various filters, slits and mirrors), (iii) a sample chamber (cuvettes) and(iv) detector and an associated read out system (meter or recorder). All spectrophotometers represent variations of the block diagram shown in Figure 10.4.

Source: Monochromator Sample---Holder---Detector---Amplifier--- Recorder

Fig. 10.4. Block diagram of a Spectrophotometer

10.4.1 Light Source

(a) Sources of ultraviolet light: For absorption measurements in the ultraviolet region, a high pressure hydrogen or deuterium lamp is used. These lamps produce radiation in the range of 200 to340 nm. Xenon lamp may also be used for ultraviolet radiation, but the radiation produced is not as stable as the hydrogen lamp.
Sources of visible light: The light source for the visible region is the tungsten halogen lamp. It emits the radiation with a wavelength range of 350-800 nm. Carbon arc, which provides more intense visible radiation is used in a small number of commercially available instruments.

10.4.2 Wave Length Selectors

All the sources discussed above produce continuous radiation over wide range of wavelengths. However, the laws of absorption in the strict sense apply only to monochromator radiation. Thus, absorption of narrow band width will show greater adherence to Beer's law. Narrow band widths are made possible by using wavelength selector(s). Wavelength selectors are of two types namely filters and monochromators.

(a) Filters: Filters operate by absorbing light in all other regions except certain limited range of wavelengths, which they reflect. This limited range is known as the band width of the filter. Gelatin filters are made of a layer of gelatin, coloured with organic dyes and sealed between glass plates. Most modern filter instruments use tinted-glass filters. Filters resolve polychromatic light into a relatively wide band-width of about 40 nm and are used only in colorimeters. The disadvantage of glass filters is their low transmittance of 5-20%.

(b) Monochromators (Fig. 10.5): The name itself indicates a monochromator resolves polychromatic light into its individual wavelengths and isolates these wavelengths into very narrow bands. The essential components of a monochromator are: (a) an entrance slit which allows polychromatic light from the source, (b) Lens or mirror which collimates the polychromatic light onto the dispersion device, (c) a wavelength resolving device like a prism or
a grating which breaks the radiation into component wavelengths, (d) a focussing lens or mirror,
and (e) an exit slit which allows the monochromatic beam to escape. A monochromator employing a prism for dispersion. The effective band width of the light emerging from the monochromator depends mostly upon the dispersing element (prism or a diffraction grating) and
the slit widths of both the entrance and the exit slits. Narrow slit widths isolate narrow bands.
However, the slit width also limits the radiant power which reaches the detector. Prisms or diffraction gratings are the effective resolving units.

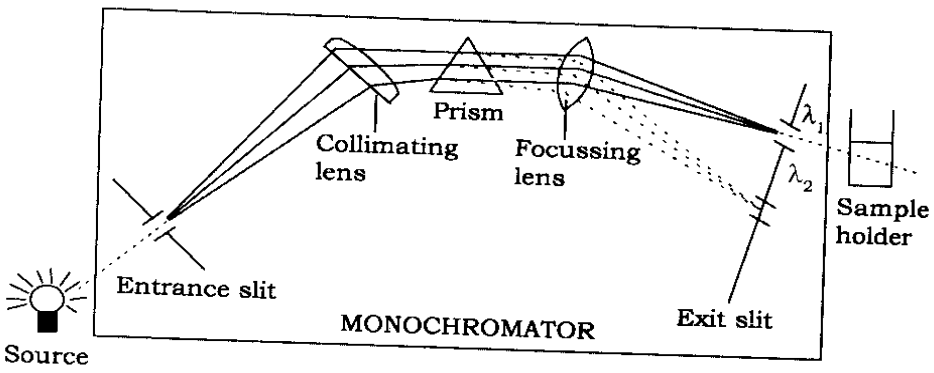


Fig. 10.5 Prism monochromator

(c) Prism: A prism disperses polychromatic light from the source into its component wavelengths by its ability to refract different wavelengths to a different extent. Because of prism disperses the short wavelengths more and long wave lengths less, the wavelengths at the red end of the spectrum are not fully resolved. This is a major disadvantage of a prism. Two types of prisms, namely 60o cornu quartz prism and 30o Littron prism are usually employed in commercial instruments. Simple glass prisms are used for visible range. For ultraviolet region silica, fused silica or quartz prisms are used. Flourite is used in vacuum ultraviolet range.

(d) Gratings: Gratings (Fig. 11.6) are often used in the monochromators of spectrophotometers operating in UV, and visible regions. The grating possesses a highly aluminized surface etched with a large number of parallel grooves which are equally spaced. These grooves are also known as lines. The principle behind dispersion of radiation by grating is that it resolves light into its component wavelength by virtue of constructive reinforcement and destructive interference of radiation reflected. Very often, the monochromator consists of both, a prism anda grating. The prism placed before the grating is known as the fore prism. It preselects aportion of the spectrum which is then allowed to be diffracted by the grating. The major advantage of diffraction grating monochromators is that their resolving power is far superior to that of prisms. In addition, they yield a linear resolution of spectrum which is not possible when prisms are used.

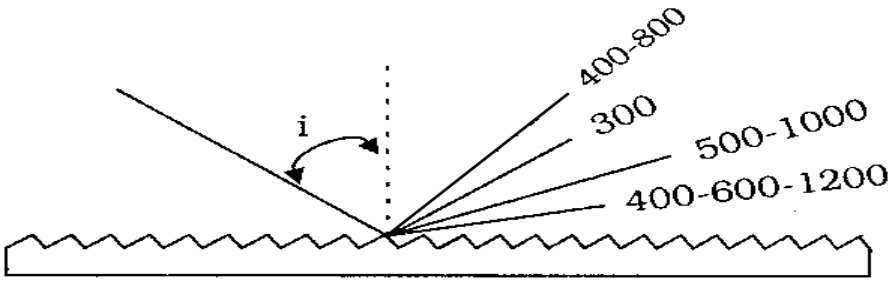


Fig. 10.6 Diffraction grating and the dispersion of polychromatic radiation

10.4.3 Sample chambers

Samples to be analyzed in the UV or visible region are usually solutions or gases and are put in cells known as **Cuvettes**. Most of the spectrophotometric studies are made in solution. Cuvettes meant for visible region are made up of either ordinary glass or some times quartz. Since glass absorbs in the UV region, quartz or fused silica cells are used in this region. Standard path length of these cuvettes is usually 1 cm. The surface of the cuvettes must be kept clean; finger print smudges and traces of previous samples, by causing interference in the optical path, might cause serious errors in quantitative measurements. The most important factor in choosing the solvent is that the solvent should not absorb (optically transparent) in the same region as the solute. Oftenly used solvents in the UV and visible regions are water, methyl-, ethyl-, isopropyl-alcohols, chloroform, hexane etc.

10.4.4 Detection Devices

The intensity of the light that passes through the sample under study depends on the amount of light absorbed by the sample. In the UV and visible regions there are three basic kinds of light sensitive detectors involved in the measurement of intensity of the light that passes through the sample. They are photocells, phototubes and photomultiplier tubes (PMT).

(a) Photovoltaic or barrier layer cells: It employs semi-conductor materials. Although a number of materials are used in photocells (cadmium sulphide, silicon, selenium) selenium based photocells are most common. A typical photocell consists of a thin coating of selenium over athin transparent silver film on a steel base. This arrangement ensures that electrons pass easily from selenium to silver but not in the reverse direction. Due to the inability of electrons comove away from the silver film, the silver acts as the collecting electrode for electrons liberated from selenium by

the incident radiation. The steel plate functions as the other elecrode. The current flowing between the two electrodes is then measured by a microammeter. Fig. 10.7showed selenium based photovoltaic cell. Photocells have a long life and are inexpensive and reliable. They are widely used in colorimeters but their use in spectrophotometers is becoming limited.

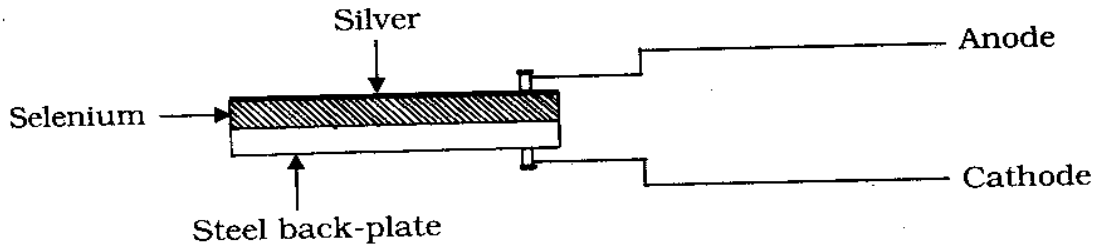


Fig. 10.7 Selenium based photo voltaic cell

(b) Phototubes or photo emissive tubes: The essential components of phototube include (a) an evacuated glass envelope (with a quartz window), (b) a semicylindrical cathode, and (c) a centrally located metal wire anode. The quartz window allows the passage of radiation which strikes the photo emissive surface of the cathode. The energy of the photon is transferred to the loosely bound electrons of the cathode surface. The electrons become excited and finally leave the surface and travel towards the anode causing current to flow in the circuit. If the electron collection is 100% efficient, the phototube current should be proportional to the light intensity. Phototube and its circuit is shown in Fig. 10.8. Phototube currents are small and require amplification.

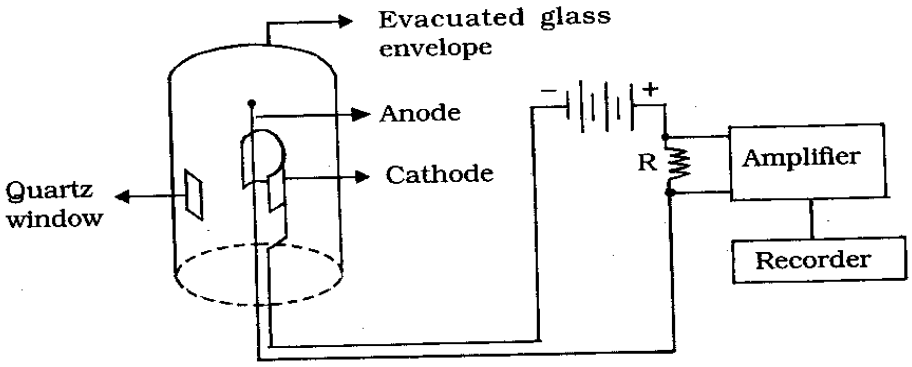


Fig. 10.8 Diagram of a photo emissive tube; R stands for resistance

(c) Photomultiplier tubes (PMT) (Fig. 10.9): These detectors are designed to amplify the initial photoelectric effect and are suitable for use at very low light intensities. A PMT consists of (a) an evacuated glass tube which contains the anode and cathode, and (b) Dynodes. High voltage (1000 volts) difference exists between anode and cathode. As the radiation strikes the photocathode, electrons are liberated and the applied potential difference accelerates the electrons towards the first dynode. Each successive dynode is at a higher electrical potential and thus acts as an amplification stage for the original photon. The applied voltage causes sufficient electron acceleration to knock out other electrons from each dynode surface. The liberated electrons are dragged on to the next dynode. By the time electrons arrive at the collecting anode, the initial photo electric current is amplified by a factor of approximately 106.In practice, PMT's are used only for low light intensities. At higher light intensities photomultipliers exhibit great instability. Photomultipliers are widely used in all modernspectrophotomers.

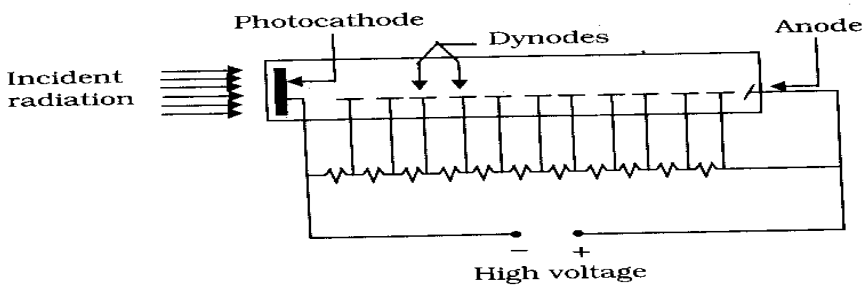


Fig . 10.9 A photomultiplier tube

A new technology has been introduced during the past few years that greatly increases the speed of spectrophotometric measurements. New detectors called photodiode arrays are being used in modern spectrometers; Photodiodes are composed of silicon crystals that are sensitive to light in the wavelength range of 170-1100 nm. Upon photon absorption by the diode, a current is generated in the photodiode that is proportional to the number of photons. Linear arrays of photodiodes are self-scanning and have response times on the order of 100 milliseconds; hence, an entire UV-VIS spectrum can be obtained with a brief exposure of the sample to polychromatic light.

10.4.5 Amplification and read out

Light detectors can generate electronic signals which are proportional to the transmitted light. These signals need to be translated into a form that is easy to interpret. This is performing by using amplifiers, ammeters, potentiometers and potentiometric recorders. Modern, research-grade spectrometers are completely under the control of a computer. Hence, by operating the button, one can obtain the UV-VIS spectrum of a sample displayed on a computer screen in less than 1 second. In addition, these modern instruments with computers used to carry out several functions, such as subtraction of solvent spectrum, spectral overlay, storage, differentiate spectra, derivative spectra and calculation of concentrations and rate constants.

10.5 Double beam operation

Voltage fluctuations inducing fluctuations in the source intensity can cause large scale errors in spectrophotometer operation. To overcome this situation, double beam spectrophotometers have been designed (Fig. 10.10). In the double beam device, the source beam is split in to two parts prior to the sample container and after the monochromator, one of the split beam passing through the blank, or reference cell, at the same time, the other passes through the sample. This approach obviates any problems of variation in light intensity as both reference and sample would be affected equally. The resultant measured absorbance is the difference between the two transmitted beams of light recorded by the matched detectors. Double beam devices are more sophisticated mechanically and electronically as compared to the single beam devices, and are also more expensive.

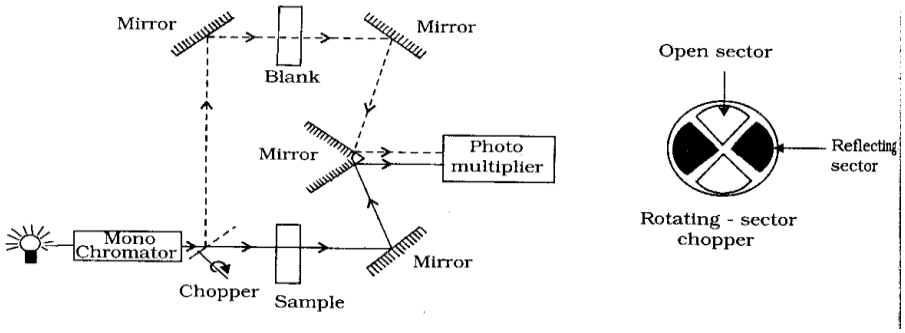


Fig. 10.10 Optical arrangements of a Double beam instrument.
A rotating sector chapter is also shown**.**

10.6 Dual wavelength Spectrophotometer

Dual wavelength spectrophotometry refers to the photometric measurement of substance by passing radiation of two different wavelengths through the sample before reaching the detector. Light from two different sources is allowed to be resolved into two different wavelengths are made to pass through the sample by a complex arrangement of a large number of mirrors (Fig. 10.11). In this device, only a single detector is used. It is always photomultiplier tube.

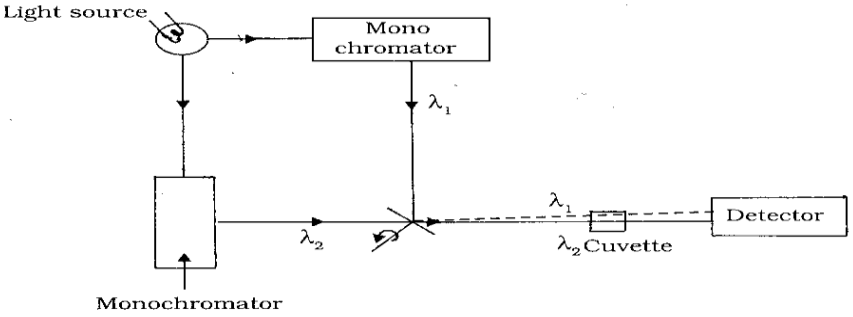


Fig. 10.11 Optical arrangements in a dual wavelength spectrophotometer

10.7 Applications of UV-vis Spectrophotometer

Although many different types of operations carried out on UV-vis spectrophotometer all applications fall in one of two categories:
(a) Measurement of absorbance at a fixed wave length.
(b) Measurement of absorbance as a function of wavelength. Measurements at a fixed wavelength are most often used to obtain quantitative information. Absorbance measurements as a function of wavelength provide qualitative information.

10.8 Summary

Electromagnetic radiation is composed of both an electric vector and magnetic vector (which gives rise to the name), which oscillate in planes at right angles to each other and mutually at right angles to the direction of propagation. The interaction of electromagnetic radiation with matter (solutions or crystals of biomolecules) resulting processes like absorption and fluorescence (or emission). Both processes have led to the development of fundamental techniques like visible, UV and infrared spectrometry. UV-visible spectrophotometry is an important technique for molecular structure elucidation and quantification of compounds (biomolecules)..

10.9 Self Assessment

1. What are the differences between the visible and UV spectrophotometry?
2. Explain the differences between a spectrophotometer that uses a phototube for a detector and one that uses a photodiode array detector.
3. Discuss and describe the principle and instrumentation for UV absorption spectrophotometry?
4. Why can you not use a glass cuvette for absorbance measurements in the UV spectra ranges?

10.10 Suggested Readings

1. Boyer, R. (2003). Modern experimental biochemistry, 3rd edition, Pearson and Education Publishers, New Delhi, pp.1-467.
2. Upadhyay *et al*. (2003). Biophysical Chemistry – Principles and Techniques, 2nd edition, Himalaya Publishing House, pp.1-602.
3. Wilson, K. and John Walker (2000). Practical Biochemistry – Principles and Techniques, 5th Edition, Cambridge University Press, pp.1-770.
4. Sawhney, S.K. and Randhir Singh (2001). Introductory Practical Biochemistry. First edition, Narosa Publishing House, New Delhi, pp.1-435.

Dr J. Madhavi

Lesson-11

ESR AND MASS SPECTROSCOPY

Objective :

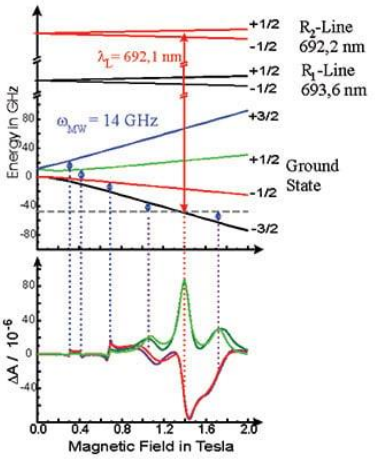
This lesson mainly deals with properties of electromagnetic spectrum, various spectroscopic techniques (ESR and Mass spectrophotometry), their principle and applications.

11.1 INTRODUCTION

Spectroscopy deals with interaction of electromagnetic radiations with matter. Spectroscopic techniques now occupy a key position in biochemical studies and some of them are employed routinely and quite extensively. These techniques are extremely useful in characaterization of chemical compounds in a sample on the basis of their spectral properties. Inthe beginning, the spectroscopy was largely confined to use light (visible radiation) for quantitative estimation and in some cases even for identification of biomolecules. With new developments and refinements in instrumentation, the scope of spectroscopic techniques broadened tremendously and now, in addition to visible light, it encompasses examining the behaviour of chemical substances upon their irradiation with regions of electromagnetic radiations such as X-rays, UV-rays, infrared light, microwaves and even radio frequencies. The spectrophotometric methods offer a rapid and convenient means of qualitative identification and quantitative estimations of biomolecules even in relatively impure samples.

11.2 ESR SPECTROSCOPY

- Electron Spin Resonance (ESR) also known as Electron Magnetic Resonance (EMR) or Electron Paramagnetic Resonance (EPR) is a branch of absorption spectroscopy in which radiations having frequency in the microwave region (0.04 – 25 cm) is absorbed by paramagnetic substances to induce transitions between magnetic energy levels of electrons with unpaired spins.
- ESR is based on the fact that atoms, ions, molecules or molecular fragments which have an odd number of electrons exhibit characteristic magnetic properties. An electron has a spin and due to spin there is magnetic moment.
- Since its discovery in 1944 by E.K. Zavoisky, EPR spectroscopy has been exploited as a very sensitive and informative technique for the investigation of different kinds of paramagnetic species in solid or liquid states.



11.2.1 Principle of Electron Spin Resonance:

The phenomenon of electron spin resonance (ESR) is based on the fact that an electron is a charged particle. It spins around its axis and this causes it to act like a tiny bar magnet. When a molecule or compound with an unpaired electron is placed in a strong magnetic field The spin of the unpaired electron can align in two different ways creating two spin states ms = ± ½.Thealignment can either be along the direction (parellel) to the magnetic field which corresponds to the lower energy state ms = – ½ Opposite (antiparallel) to the direction of the applied magnetic field ms = + ½The two alignments have different energies and this difference in energy lifts the degeneracy of the electron spin states. The energy difference is given by:

$\Delta E = E+ - E- = hv = gm\beta B$

Where,

h = Planck's constant (6.626 x 10-34 J s-1)

v = the frequency of radiation

ß = Bohr magneton (9.274 x 10-24 J T-1) B = strength of the magnetic field in Tesla

g = the g-factor which is a unit less measurement of the intrinsic magnetic moment of the electron, and its value for a free electron is 2.0023.

An unpaired electron can move between the two energy levels by either absorbing or emitting a photon of energy {\displaystyle h\nu } hv such that the resonance condition, hv = Δ E, is obeyed. This leads to the fundamental equation of EPR spectroscopy.

11.2.2Working of Electron Spin Resonance:

- Although the equation permits a large combination of frequency and magnetic field values, the great majority of EPR measurements are made with microwaves in the 9000–10000 MHz (9–10 GHz) region.
- EPR spectra can be generated mostly by keeping the photon frequency fixed while varying the magnetic field incident on a sample.
- A collection of paramagnetic centers, such as free radicals, is exposed to microwaves at a fixed frequency.
- By increasing an external magnetic field, the gap between the and energy states is widened until it matches the energy of the microwaves.
- At this point the unpaired electrons can move between their two spin states. Since there typically are more electrons in the lower state, due to the Maxwell–Boltzmann distribution, there is a net absorption of energy.

- It is this absorption that is monitored and converted into a spectrum.

11.2.3 Instrumentation of Electron Spin Resonance:

KLYSTRONS

- Klystron tube acts as the source of radiation.
- It is stabilized against temperature fluctuation by immersion in an oil bath or by forced air cooling.
- The frequency of the monochromatic radiation is determined by the voltage applied to klystron.
- It is kept a fixed frequency by an automatic control circuit and provides a power output of about 300 milli watts.

WAVE GUIDE OR WAVEMETER

- The wave meter is put in between the oscillator and attenuator.
- To know the frequency of microwaves produced by klystron oscillator.
- The wave meter is usually calibrated in frequency unit (megahertz) instead of wavelength.
- Wave guide is a hollow, rectangular brass tube. It is used to convey the wave radiation to the sample and crystal.

ATTENUATORS

- The power propagated down the wave guide may be continuously decreased by inserting a piece of resistive material into the wave guide. This piece is called variable attenuator.
- It is used in varying the power of the sample from the full power of klystron to one attenuated by a force 100 or more.

ISOLATORS

- It’s device which minimizes vibrations in the frequency of microwaves produced by klystron oscillator.
- Isolators are used to prevent the reflection of microwave power back into the radiation source.
- It is a strip of ferrite material which allows micro waves in one direction only.
- It also stabilizes the frequency of the klystron.

SAMPLE CAVITIES

- The heart of the ESR spectrometer is the resonant cavity containing the sample.
- Rectangular TE120 cavity and cylindrical TE011 cavity have widely been used.
- In most of the ESR spectrometers, dual sample cavities are generally used This is done for simultaneous observation of a sample and a reference material.
- Since magnetic field interacts with the sample to cause spin resonance the sample is placed where the intensity of magnetic field is greatest.

COUPLERS AND MATCHING SCREWS

- The various components of the micro wave assembly to be coupled together by making use of irises or slots of various sizes.

CRYSTAL DETECTORS

- Silicon crystal detectors, which converts the radiation in D.C has widely been used as a detector of microwave radiation.

MAGNET SYSTEM

- The resonant cavity is placed between the poles pieces of an electromagnet.
- The field should be stable and uniform over the sample volume.
- The stability of field is achieved by energizing the magnet with a highly regulated power supply.
- The ESR spectrum is recorded by slowly varying the magnetic field through the resonance condense by sweeping the current supplied to the magnet by the power supply.

MODULATION COIL

- The modulation of the signal at a frequency consistent with good signal noise ratio in the crystal detector is accomplished by a small alternating variation of the magnetic field.
- The variation is produced by supplying an A.C. signal to modulation coil oriented with respect the sample in the same direction as the magnetic field.
- If the modulation is of low frequency (400 cycles/sec or less), the coils can be mounted outside the cavity and even on the magnet pole pieces.
- For higher modulation frequencies, modulation coils must be mounted inside the resonant cavity or cavities constructed of a non-metallic material e.g., Quartz with a tin silvered plating.

DISPLAY DEVICES

- In order to observe the signal a system is connected different devices can be used.

11.2.4 Applications of ESR Spectroscopy:

Studying free radicals:

ESR can detect and characterize free radicals in both chemical and biological systems. This is crucial in understanding radical reactions, oxidative stress, and the effects of free radicals on biomolecules.

Analyzing transition metal complexes:

ESR is used to study the electronic structure and magnetic properties of transition metal complexes, which are often involved in catalytic and biological processes.

Examining molecules in the triplet state:

ESR can investigate molecules with two unpaired electrons, which are important in photochemistry and other processes.

Studying spin labels:

ESR spin-labeling provides information about the local environment and dynamics of biomolecules, such as proteins and membranes.

Identifying irradiated food:

ESR can detect free radicals generated by irradiation, helping to identify if food has been treated with ionizing radiation.

Characterizing nanomaterials:

ESR is used to study the generation of electrons and reactive oxygen species during nanomaterial-mediated photocatalysis.

Monitory enzyme activity:

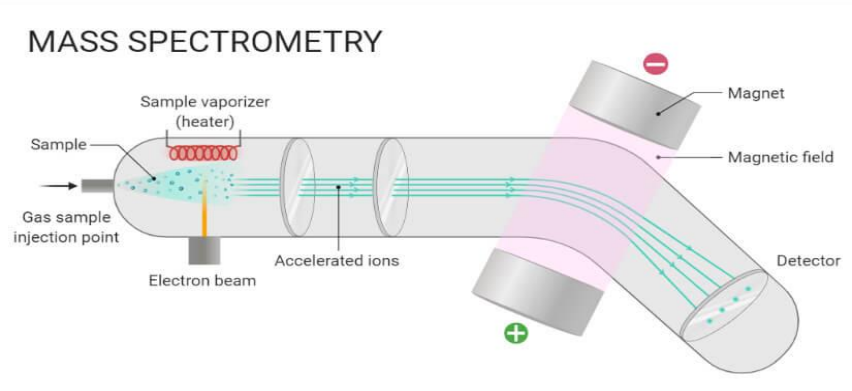
ESR can be used to monitor enzyme activity by examining the oxidation states of metal cofactors.

Studying drug delivery systems:

ESR can characterize the microenvironment within drug delivery systems, providing information about viscosity, polarity, and other properties.

11.3 Mass Spectroscopy

Mass Spectroscopy is an extremely useful technique as it can provide fairly detailed information about the structure of the molecules with very small amounts of the substance (10-6 to 10-9 g).



11.3.1 Principle of Mass spectrometer

This technique essentially involves ionization of the parent molecule to give parent molecular ion and fragmentation of these ions to give fragment ions. This is achieved by bomboarding the sample with a beam of electrons. All the ions produced generally have positive charges (rarely negative charges). These ions, which possess a certain amount of kinetic energy, if subjected to a magnetic field will be deflected to different degrees depending upon their mass/charge (m/z) values. This is the basis of separation by mass spectrometry. If the charge is kept constant, the ions are deflected by angles which are inversely proportional to the square roots of the masses of the ions. All mass spectrometers) are essentially composed of three parts:

(i) ionization chamber or source

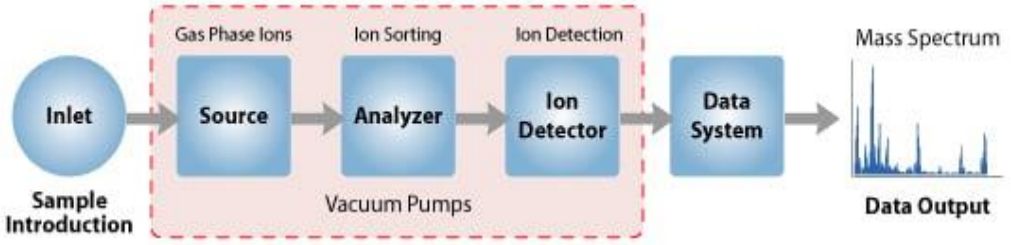
(ii) a mass analyser

(iii) a detector

11.3.2 Working of Mass Spectroscopy

- In a typical procedure, a sample, which may be solid, liquid, or gas, is ionized, for example by bombarding it with electrons.
- This may cause some of the sample's molecules to break into charged fragments. These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field:
- Ions of the same mass-to-charge ratio will undergo the same amount of deflection.
- The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio.
- The atoms or molecules in the sample can be identified by correlating known masses (e.g. an entire molecule) to the identified masses or through a characteristic fragmentation pattern.

11.3.3 Instrumentation and steps of mass spectroscopy



A. Sample Inlet

- A sample stored in the large reservoir from which molecules reach the ionization chamber at low pressure in a steady stream by a pinhole called "Molecular leak".

B. Ionization

- Atoms are ionized by knocking one or more electrons off to give positive ions by bombardment with a stream of electrons. Most of the positive ions formed will carry a charge of +1.
- Ionization can be achieved by :
- Electron Ionization (EI-MS)
- Chemical Ionization (CI-MS)
- Desorption Technique (FAB)

C. Acceleration

- Ions are accelerated so that they all have the same kinetic energy.
- Positive ions pass through 3 slits with voltage in decreasing order.
- Middle slit carries intermediate and finals at zero volts.

D. Deflection

- Ions are deflected by a magnetic field due to differences in their masses.
- The lighter the mass, the more they are deflected.
- It also depends upon the no. of +ve charge an ion is carrying; the more +ve charge, the more it will be deflected.

E. Detection

- The beam of ions passing through the mass analyzer is detected by a detector on the basis of the m/e ratio.
- When an ion hits the metal box, the charge is neutralized by an electron jumping from the metal onto the ion.
- Types of analyzers:
- Magnetic sector mass analyzers
- Double focussing analyzers
- Quadrupole mass analysers
- Time of Flight analyzers (TOF)
- Ion trap analyzer
- Ion cyclotron analyser

11.3.4 Applications of Mass Spectroscopy

- Environmental monitoring and analysis (soil, water, and air pollutants, water quality, etc.)
- Geochemistry – age determination, soil, and rock composition, oil and gas surveying
- Chemical and Petrochemical industry – Quality control
- Identify structures of biomolecules, such as carbohydrates, nucleic acids
- Sequence biopolymers such as proteins and oligosaccharides
- Determination of the molecular mass of peptides, proteins, and oligonucleotides.
- Monitoring gases in patients' breath during surgery.
- Identification of drug abuse and metabolites of drugs of abuse in blood, urine, and saliva.
- Analyses of aerosol particles.
- Determination of pesticides residues in food.

11.4 Summary

ESR spectroscopy, also known as electron paramagnetic resonance (EPR), is a technique that analyzes the absorption of microwave radiation by paramagnetic substances, which are those containing unpaired electrons. This absorption occurs when an external magnetic field is applied, causing the energy levels of the unpaired electrons to split. By measuring the absorbed microwave radiation, ESR provides information about the electronic structure, chemical environment, and dynamics of unpaired electrons, particularly in free radicals, transition metal ions, and other paramagnetic species. The mass spectrum is essentially dependent upon the thermodynamic stability of the ions produced and collected during a massspectrometric experiments. Mass spectrometry is widely used to measurement of protein molecular weight and may also be used to sequence polypeptides of 25 residues or fewer.

11.5 Self Assessment

1. What are the differences between the ESR and Mass spectrophotometer?
2. Discuss the principle of mass spectrophotometer and its applications?
3. Discuss the principle of ESR spectroscopy and its applications?

11.6 Suggested Readings

1. Boyer, R. (2003). Modern experimental biochemistry, 3rd edition, Pearson and Education Publishers, New Delhi, pp.1-467.
2. Upadhyay *et al*. (2003). Biophysical Chemistry – Principles and Techniques, 2nd edition, Himalaya Publishing House, pp.1-602.
3. Wilson, K. and John Walker (2000). Practical Biochemistry – Principles and Techniques, 5th Edition, Cambridge University Press, pp.1-770.
4. Sawhney, S.K. and Randhir Singh (2001). Introductory Practical Biochemistry. First edition, Narosa Publishing House, New Delhi, pp.1-435.

Dr J.Madhavi

LESSON-12
CENTRIFUGATION

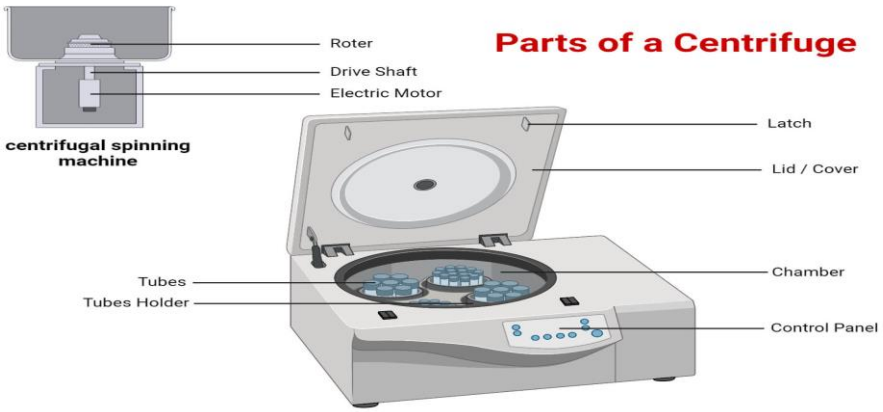
12.0 Objective:

To study about various centrifugation techniques that are employed in molecular biology.

12.1 INTRODUCTION

Centrifugation is a mechanical process which involves the use of the centrifugal force to separate particles from a solution according to their size, shape, density, medium viscosity and rotor speed.[] The denser components of the mixture migrate away from the axis of the centrifuge, while the less dense components of the mixture migrate towards the axis. Chemists and biologists may increase the effective gravitational force of the test tube so that the precipitate (pellet) will travel quickly and fully to the bottom of the tube. The remaining liquid that lies above the precipitate is called a supernatant or supernate.

12.2 INSTRUMENTATION FOR CENTRIFUGATION

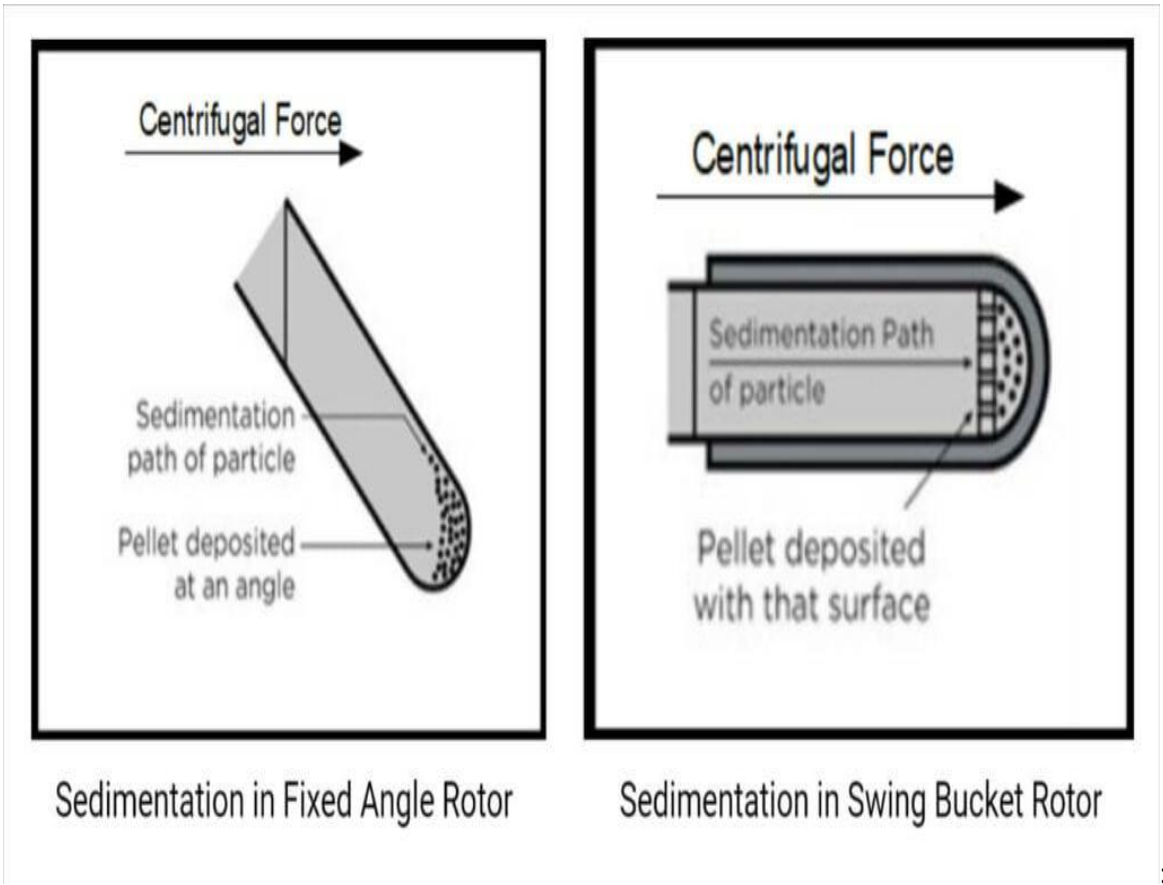


1. **Motor:** The motor is the powerful central component of the centrifuge that creates the spin.

2. **Rotor assembly:** A drive shaft and a rotor comprise the rotor assembly. The drive shaft provides support for the rotor components. The rotor head is attached to the motor, which bears the containers to house the tubes containing the sample to be centrifuged. It converts electrical energy to mechanical energy. Two rotors with different diameters can have the

same rotational speed. Varying radii and angular momentum results in a difference in the acceleration of such rotors. Thus, relative centrifugal force (rcf) is regarded as the accepted standard unit for the rotation speed. There are mainly three types of rotors:

a) Fixed angle rotors: These rotors hold the tubes at an angle of 14 to 40° to the vertical such that particles travel a short distance while moving radially outwards and are used in differential centrifugation. The sedimentation takes place at the walls of the tubes at an angle since the sedimentation direction is the same as the direction of centrifugal force. The pellets (cluster of sediments) later settle at the corner of the base and the wall surface after colliding with the wall surface.



:

b) Swinging bucket/ Horizontal rotors: These rotors, along with the centrifuge tubes, swing out to a horizontal position during the time of acceleration such that particles travel a longer distance, thereby facilitating easier separation of supernatant from the pellet. These types of motors are employed in density gradient centrifugation.

c) Vertical rotors: These hold the tubes vertically, i.e., parallel to the motor axis, and the particles move shorter distances with shorter periods for separation. It is used for isopycnic and density gradient separation; however, it is not considered useful for pelleting because the pellets are spread out along the entire outer wall of the tube by centrifugal force.

3. **Containers:** Several types of containers, such as test tubes, blood bags, cuvettes, centrifuge tubes, etc., are held in the rotors such that the sample rotates along as the rotor rotates.

4. **Control Panel:** It serves the purpose of controlling different parameters such as temperature, rotational speed (rcf or rpm), etc.

5. **Latch:** When a tube breaks, or there are other issues with the centrifuge while running, the latch keeps the lid closed.

6. **Lid:** The centrifuge will only spin if the lid is closed and locked to prevent mishaps.

12.3 PRINCIPLE OF CENTRIFUGATION:

The centrifuge utilizes the sedimentation principle due to gravitational force. The centrifugation technique uses a centrifugal field to separate particles suspended in a liquid medium. These are put in the centrifuge's rotor either in bottles or tubes. Sedimentation is a process whereby gravity causes suspended particles to separate from fluids. The suspended substance may consist of powder or clay-like particles.

Simple filtration filters particles larger than 5 micrometers from those less than 5 micrometers, which start Brownian motion and do not sediment under gravity. These particles can be separated with the help of the central force.

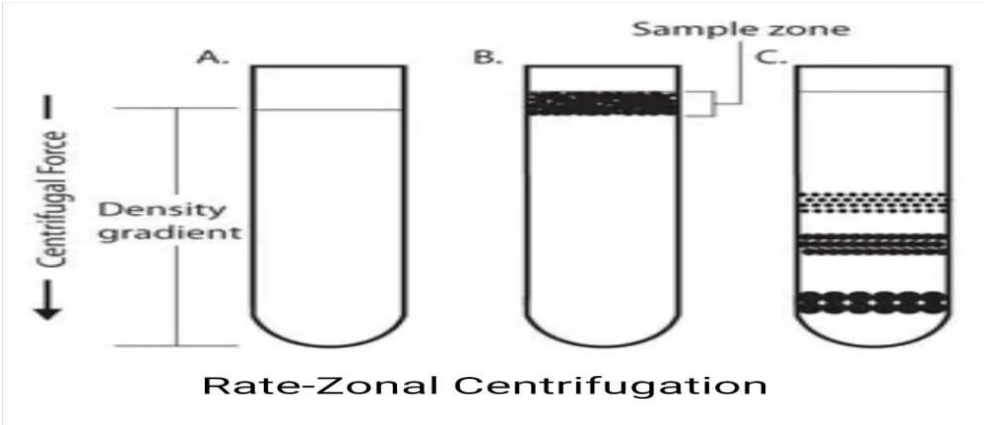
12.4 DENSITY GRADIENT CENTRIFUGATION

It separates particles based on their buoyant density or sedimentation rate. A sample mixture is placed on the top of a preformed liquid density gradients such as CsCl for DNA banding and isolation of plasmids, nucleoproteins, and viruses; NaBr and NaI for fractionation of lipoprotein; Per coll, Ficoll, Metrizamide, Dextran for separation of whole cells and sucrose solution for the separation of DNase, RNase and Protease.

The two subtypes of density gradient centrifugation are rate-zonal and isopycnic centrifugation.

Rate-zonal centrifugation

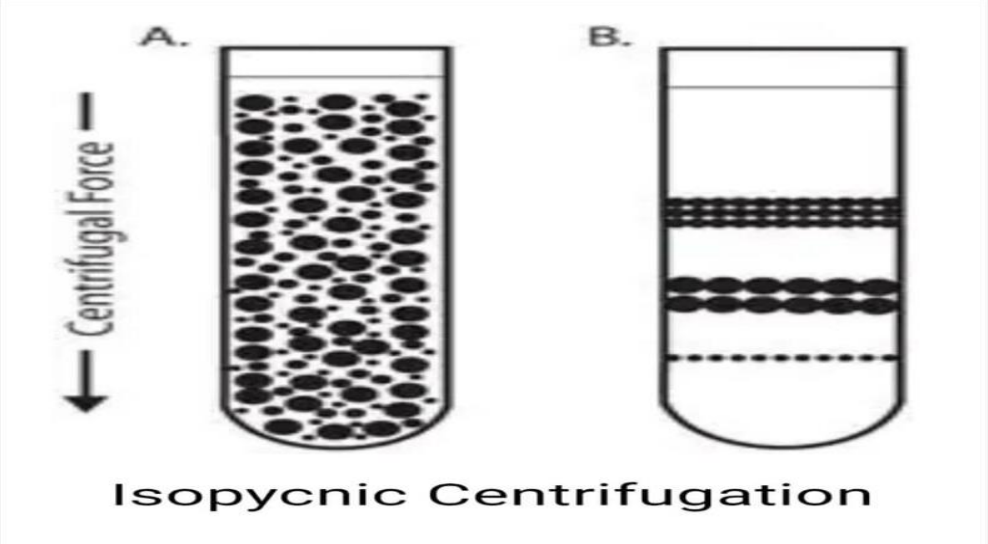
On top of a density gradient, the sample is overlaid as a small zone. Depending on their mass, particles travel under centrifugal force at various speeds. Size and mass are the main determinants of how quickly particles settle. As the band of particles descends through the density medium, zones with particles of comparable size develop as the faster sedimenting particles pass the slower ones.



Isopycnic centrifugation

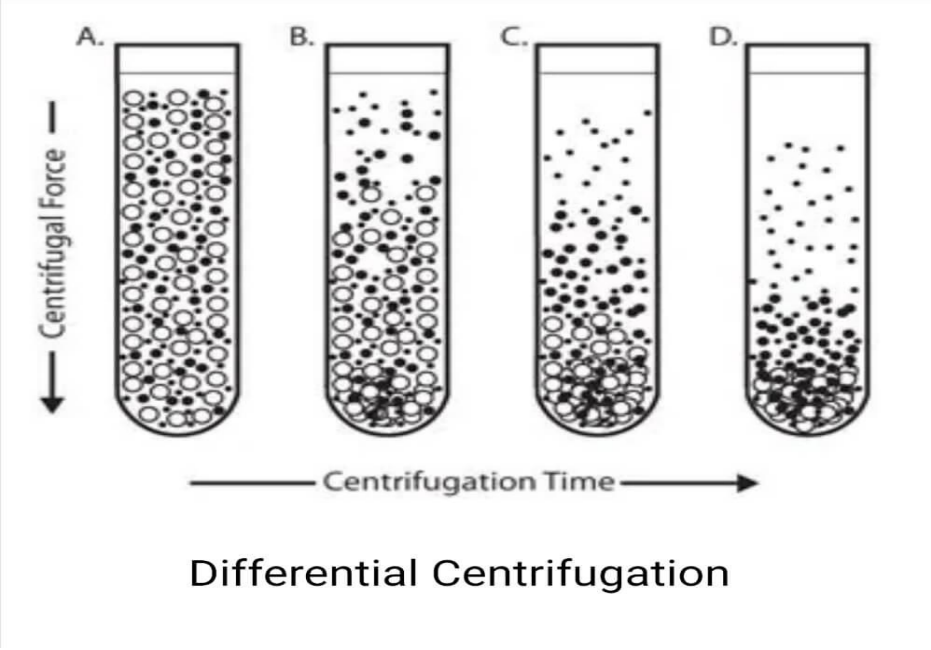
Particles are separated exclusively based on their density in an isopycnic separation, also known as buoyant or equilibrium separation. It is necessary for the gradient medium to have a higher density than the particles that need to be separated.

Particles migrate under the influence of centrifugal force from a uniformly mixed sample and density gradient until their densities are equal to those of the surrounding medium. After centrifugation, particles of a certain density settle until their density equals that of the gradient media (i.e., the equilibrium position).



12.5 DIFFERENTIAL CENTRIFUGATION

It separates particles based on shape, size, and density. A suspension of particles with varying densities or sizes will sediment at varying speeds, with the larger and denser particles sedimenting more quickly. Following a series of rising centrifugal force cycles on a suspension of cells, a series of pellets containing cells with a decreasing sedimentation rate will result.



Applications of centrifuge:

Centrifuges are employed in chemistry, biology, biochemical, and clinical laboratories, such as testing the sedimentation rates of various blood cells.

- These are utilized in dairy industries to separate cream (fat) from milk, and this process is known as churning.
- Giant centrifuging machines are used in water treatment, where it spins the mud and sludge out of the water to produce cleaner water. Likewise, solid matter is separated from freshly drilled-out petroleum in oil rigs.
- Centrifugation is used to produce biological products and bulk drugs and perform biopharmaceutical analysis of drugs.
- It is applied in removing water from lettuce after washing it in a salad spinner and separating chalk powder from water.

12.6 Summary

Centrifugation is a process that separates components of a mixture based on their size, shape, density, and other factors by utilizing centrifugal force. This force, generated by rapidly spinning a sample, causes denser components to move away from the center and settle down, while lighter components remain in suspension. There are different types of centrifugation techniques which includes differential centrifugation and density gradient centrifugation.

12.7 Self Assessment

1) what is centrifugation and write about Principles of centrifugation?
2) What are different types of centrifugation?
3) Write briefly about isopycnic centrifugation?
4) Discuss about Rate Zonal centrifugation?

12.8 Suggested Readings

1) Biophysical Chemistry by Upadhaya and Upadhya Nath, 3rd Edition, Himalaya Publications.
2) Practical Biochemistry by Keith Wilson and Walker 5th Edition, Cambridge University Press.
3) A Biologist Guide to Principles & Techniques of Biochemistry, Keith Wilson and Goulding

Dr J.Madhavi

Lesson-13

CHROMATOGRAPHY

Objective:

To study about various chromatography techniques that are employed to separate the components of sample mixture.

13.1. INTRODUCTION

Michael Tswett, a Russian biochemist, who separated chlorophyll from a mixture of plantpigments in 1906, first developed chromatography. Because of the nature of chlorophyllpigments in the sample, each band had a distinctive color. Thus the name of the process wascoined from the Greek words i.e. Chromo means *Color* and graphy means *to write*.In any chromatographic method two phases are common. They are, one is stationaryphase while the other is mobile phase. The mobile phase either moves over the surface orpercolates through the interstices of the stationary phase. The sample mixture, introduced intothe mobile phase undergoes repeated interactions or partitions between the stationary and mobilephases while being carried through the system by the mobile phase. Different components ofsample mixture interact with the two phases differentially on the basis of small differences intheir physico-chemical properties. Since these different rates of interactions govern the migrationof the sample components through the system, each one of the components migrate at a differentrate. The compound, which interacts more with the mobile phase and least with the stationaryphase, migrates fast. The component showing least interaction with the mobile phase whileinteracting strongly with the stationary phase migrates slowly. This differential movement of thecomponents is responsible for their ultimate separation from each other.

13.2. TECHNIQUES OF CHROMATOGRAPHY

There are two basic techniques of chromatography
(a) Plane chromatography
(b) Column chromatography,
In plane chromatography the stationary phase is coated onto a plane surface. There aretwo types of plane chromatography (1) Paper chromatography (2) Thin layer chromatography. Inpaper

chromatography the stationary phase is supported by cellulose fibers of the paper sheet. Inthin layer chromatography the stationary phase is coated onto a glass or plastic surface. But incase of column chromatography as opposed to plane chromatography the stationary phase ispacked into a glass or plastic column. Each of these techniques has their specific advantages,applications and mode of operation.

13.3. PAPER CHROMATOGRAPHY

A.J.P.Martin and Richard Synge first developed paper chromatography in 1941. Thistechnique has played an important role in biochemical analysis due to its ability to separate smallmolecules such as amino acids and oligopeptides.

Nature of Paper

The paper commonly used consists of highly purified cellulose. Cellulose, a homopolysaccharide of glucose, contains several thousands of anhydro-glucose units linked through
oxygen atoms. Treating the paper with 0.1 N HCl and drying it before chromatography is carried out may remove any organic and inorganic impurities present on the paper. This gives betterresults.

Apparatus And Paper Development

The apparatus required for paper chromatography are
(a) Support for the paper
(b) Solvent trough
(c) An Airtight chamber in which the chromatogram is developed,

Different Techniques Used In Paper Chromatography

The sample is applied to the paper as a small spot. The sample is applied to the paperbefore dipping the paper into the eluting solvent. Any device, which can transfer a small volumeof sample, can be used for spotting. Generally used devices are platinum loop, capillary tube or amicropipette. Of these three devices platinum wire is most preferable because it can be reusedwith several substances after heating on a flame. A micropipette can also be reused after its tiphas been disposed and a new tip applied. For some methods the sample may be applied as anarrow streak at right angles to the flow of solvent.
There are two main techniques, which may be employed for the development of paperchromatography.
(1) Ascending chromatography
(2) Descending chromatography,
In both cases the solvent is placed in the base of a sealed tank or glass jar to allow thechamber to become saturated with the solvent vapour. After equilibration of the chamber isachieved the development of chromatography may be started.

Ascending Technique

If the development is to be performed by the ascending technique, the paper is allowed tohang in or suspended in a manner that the base of the paper is in contact with the solvent at thebase of the chamber. The sample spots should be in a position just above the surface of thesolvent, so that as

the solvent moves vertically up the paper by capillary action, separation of thesample is achieved.

Descending Technique

In the descending technique, the end of the paper near which the samples are located isheld in a trough at the top of the tank and the rest of the paper allowed to hang vertically but notin contact with the solvent in the base of the tank. Adding the solvent to the trough startsdevelopment. Separation of the sample is achieved as the solvent moves downward undergravity.Ascending technique has two advantages (a) The set up required for it is very simple (b)The resolution of sample by ascending technique is somewhat better as compared to thedescending technique. This is because in ascending technique, two forces are acting on thesolute; these are capillary force, which makes it move up and the gravitational force, whichopposes this movement. Under the influence of these two forces, the sample components areresolved better than in the descending technique. The only disadvantage in ascending techniqueis it is very slow. The descending technique on the other hand is much faster than ascendingtechnique.

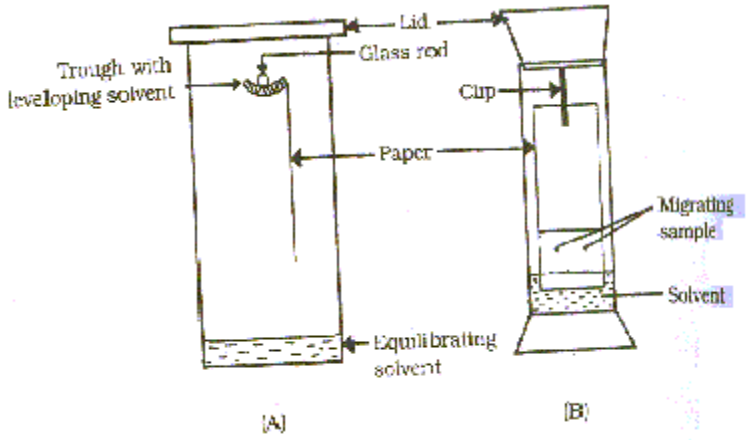


Figure 13.1. Methods of paper chromatography (A) Descending (B) Ascending
(Courtesy from Upadhaya and Upadhaya Nath)

Radial Technique

In this method the sample is spotted at the center of a circularly cut disc of paper, which is placed horizontally. The center of paper is connected with a wick to the solvent, which is placed at the base of the jar. The solvent rises up the wick and then on to the paper through capillary action. The sample components now move outward radially forming concentric circles of increasing diameters. The resolution of components by this technique is sharper. But this is less used compared to ascending and descending techniques.

Two Dimensional Chromatography

The paper, with the sample applied as a spot close to the corner is developed in the normal fashion by either ascending or descending techniques. The development is continued until the faster moving component or solvent front approaches the end of the paper. The paper is removed and the solvent is allowed to evaporate. This paper is then turned 900 and developed a second time with another solvent having totally different eluting properties. Thus components that could not be separated serving one solvent above can be easily separated by this procedure.

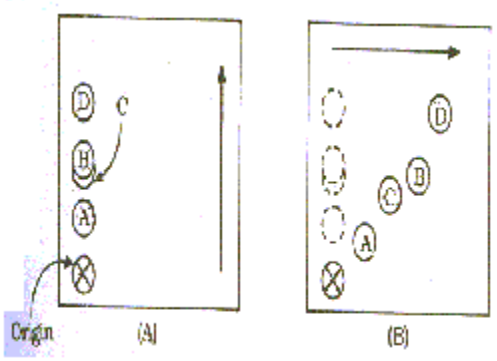


Figure 13.2: Two Dimensional chromatography (A) First development in the direction indicatedby the arrow does not resolve B & C completely (B) Second development in adirection at right angles to the first using a different solvent system resolves allcomponents completely. (Courtesy from Upadhaya and Upadhaya Nath)

Choice of Solvent System

Usually in paper chromatography, the stationary phase is water since it is very well adsorbed by cellulose. The mobile phase, which is less polar, flows over the polar stationary phase. The mobile phases used in paper chromatography are usually a mixture of various solvents such as alcohols, acids, esters, ketones, phenols, amines and hydrocarbons etc. The solvents are selected in such a way that the resolution of sample components is satisfactory. Aqueous + organic solvents used as a solvent system

Ex water :Butanol : Acetic acid (4:5:1ratio)

water : t-amyloalcohol :Pyridin (6:7:7) ratio

Detection

After the solvent has migrated to an appropriate distance the chromatography is stopped and the paper is removed from the solvent and dried. The separated materials on paper if not colored may be detected by various methods. Radioactively labeled compounds located by radiation detection methods. If the compounds are fluorescent they can be seen under UV light. Colourless compounds may be visualized by spraying chromatograms with color producing reagents.

Ex. Alpha amino acids form purple color with ninhydrin reagent but protein forms yellow color with the same reagent.

The identification of a given compound may be made on the basis of the distance traveled by the solute relative to the distance moved by the solvent. This rate, which reflects the distribution coefficient of the given solute, is known as the retardation factor (Rf).

$$Rf = \frac{\text{The distance moved by the solute}}{\text{The distance moved by the solvent}}$$

In case of Carbohydrates the term Rf is replaced by Rg for convenience.

$$Rg = \frac{\text{The distance moved by carbohydrates}}{\text{The distance moved by glucose}}$$

Since each compound has specific Rf (Rg) value, this value can be exploited to detect theunknown compound by matching its retardation factor value to those of the known compounds.

Applications

(1) The technique of paper chromatography has been widely used in identification and separation of biomolecules.
(2) It is also used in the control of purity of pharmaceuticals.
(3) It has been widely used in the detection of contamination in foods and drinks.
(4) It is used in the study of ripening and fermentation.
(5) It is also used in the detection of drugs and dopes in animals and humans.

13.4. Thin Layer Chromatography (TLC)

This technique is similar to paper chromatography technique. In "TLC" a thin layer acts as stationary phase and solvent acts as mobile phase. A thin layer of finely divided substance is deposited on to a flat glass plate. The sample to be separated is spotted at one end. The plate isdipped into the solvent in a glass jar and the development carried out by ascending technique.After the development the layer can be dried and the components detected by various methods. Thin layer chromatography may be either carried out by the adsorption principle i.e., thinlayer is prepared by an adsorbent such as "Kieselguhr" or alumina or by the partition principle ifa substance such as silica gel prepares the layer.

(a) Preparation of Thin Layer

The glass plate, which the thin layer is prepared, should be even and is thoroughly washed and dried before application of layer. The material of which the thin layer is to be made. Ex. Silica gel (or) Kieselguhr is severally mixed with water in such a proportion that a thick suspension is formed known as "slurry". This slurry is applied to a plate surface as a uniform thin layer by means of a "plate spreader", starting at one end of the plate and moving to the other in an unbroken uniform motion. Thickness of thin layer influences resolution power of "TLC".
Thus for analytical separations, the thickness of layer is usually 0.25 mm; and for preparative separations the thickness of the layer might be about 5mm. Although thin layer technique can be used for many different types of chromatographic separations the most widely used technique is usually "adsorption type". While preparing stationary phase for adsorption chromatography calcium sulphate is mixed with the slurry. The binding helps in better adhesion of the stationary phase to the glass or foil plate. The plates are dried after application of the slurry. If adsorption chromatography is to be performed heating at 110□C for several hours activates the thin layer.

(b) APPARATUS AND PLATE ARRANGEMENT

TLC requires glass plate, TLC chamber, solvent, and substance for thin layer preparation,spreader and lid for TLC chamber.
Different solvent and adsorbent combinations are available for TLC.

Adsorbent Solvent Compound
separated

Silica gel – G Petroleum ether / diethyl ether **:**
Acetone 90 :10 : 1
Triglycerides
Silica gel – G 96% Ethanol / Water 70 : 30 Amino acids
Kieselguhr – G Ethylacetate / propane - 1 - ol
65 :35
Mono and Di
saccharides.

(C) DETECTION

After preparing the thin layer the sample to be separated is applied at one end and after drying, the plate is dipped in the solvent in a glass jar. After the plate development, the thin layer can be dried and the separated components can be detected by various methods. If the components are radio-labeled it is detected by autoradiography technique. If the components are colorless they can be visualized by spraying the chromatograms with color producing reagents. The techniques specific for detection of TLC are:

(a) Spraying the plate with 25 - 50% H_2SO_4 in ethanol and heating. This results in charring of most of the compounds, which show up as "brown spots".

(b) Iodine vapour is also used extensively as colour producing reagent in TLC. As in paper chromatography in TLC also compounds are identified on the basis of rates between distances traveled by solute relative to the distance traversed by the solvent.

$$Rf = \frac{\text{The distance traveled by the solute}}{\text{The distance moved by the solvent}}$$

Each compound has a specific Rf value. This can be used to detect the unknowncompounds by comparing its Rf value to Rf values of known compounds.

Applications

(1) One of the greatest advantages of TLC is the speed at which the separation is achieved.Generally 10-30 minutes are sufficient. However with certain compounds 90 minutes may be required. Compared to paper chromatography TLC is more convenient, faster and highly reproducible.

(2) It has been widely used to determine the complexity of mixture.

(3) TLC is also used to study the courses of reactions.

(4) TLC has been used to identify drugs, contaminants and adulterants.

(5) It has also been widely used to resolve plant extracts and many other biochemical preparations.

13.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

“HPLC” is the technique performed in column (it is an example of columnchromatography) and the resolution power of “HPLC” is very high compared with otherconventional chromatographic techniques. Other conventional chromatographic techniquesrequire long time. To reduce the time of experiments, flow rate has to be increased. Althoughthis reduces the time of the experiment, it also reduces the efficiency of chromatographictechnique. In this the sample components undergo lesser number of equilibrations betweenmobile phase and stationary phase. To overcome this

problem column chromatography techniqueperformed in long columns. To increase the flow rate the pressure has to be increased on mobilephase. But commonly used supports in column chromatography cannot tolerate high pressures;there by causing flow rate abnormalities. All these problems were resolved with the developmentof high performance liquid chromatography. This method uses high pressures up to 8000 PSI.(Pressure for square inch). Therefore the flow rate is high and experimental time is shortedconsiderably and the resolution power is high. The technique may be used with small amounts ofsample, (Pico or even femto gram) level. The technique is primarily stable for analyticalpurposes but can be used as a preparative technique also. It is particularly popular for the separation of polar compounds such as drug metabolites. The greatest advantage of "HPLC" isthat it may employ the principles of adsorption, partition, ion exchange, exclusion and affinitychromatography. This makes it an extremely versatile technique and explains its emergence asthe most popular chromatographic technique.

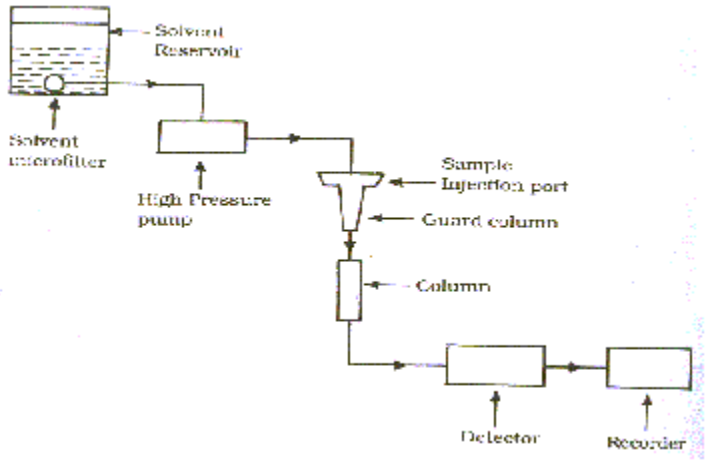


Figure 13.3: Schematic diagram of HPLC system.
(Courtesy from Upadhaya and Upadhaya Nath)

Instrumentations

Six major components needed to perform HPLC are:
(A) A solvent reservoir to store the mobile phase.
(B) High pressure pump to push the mobile phase through the column.
(C) A device to inject the sample into the mobile phase.
(D) A column in which the separation will take place.
(E) A detector used in detecting the concentration of the sample components as they come out of the column.
(F) A potentiometric recorder to produce a chromatogram.

Solvent Reservoir AndThe Solvents

Solvent reservoir in "HPLC" apparatus should contain enough volume of mobile phase for repetitive analysis. It must have a provision for degassing the solvents and it must be inert to the solvent. In "HPLC" apparatus solvent degassing is performed by heating (or) by application of vacuum (or) by treating it with ultrasonic sounds. Generally glass and steel containers of 0.5 – 2.0 liter capacity are suitable as solvent reservoirs.
The type of separation determines the choice of mobile phase. Isocratic separations perform with a single solvent (or) a fixed proportion mixture of two solvents. For gradient

elution, the solvent composition changes continuously, during chromatography.
All solvents to be used in HPLC must be extra pure. Since even the smallest impurity interferes with the detection systems or they block the column. To provide extra pure reagents for HPLC, solvent reservoirs provided with a micro filter.

Pumping Systems

To provide high pressures in "HPLC" different types of pumping systems available. A good pumping system should give pulse less stable flow, constant volume delivery. It should provide solvent flow rates of 0.5 ml per minute for most HPLC models.
Commonly used pumping systems for HPLC are
1. Holding coil
2. Pneumatic amplifier
3. Moving fluid type
4. Reciprocating piston
5. Syringe pump,

Sample Injection

In HPLC sample is introduced on to the column as in conventional types ofchromatographic techniques. Two methods are available for sample applications in HPLC.(a) Stop plane injections: In this method sample is introduced with the help of micro syringe.Preferably the sample is injected when the pressure has dropped to almost one atmosphere,after switching the pump off. This technique is known as stop flow injection. Alternatively,the sample can be injected while the system is under high pressure.(b) In the second method the sample applied directly on to the HPLC column with the help of asmall volume metal loop. The sample is thus carried spontaneously with the eluant to thecolumn.

Column

The columns for HPLC are made up of stainless steel, glass, aluminum, copper or PTFE(Poly tetra fluoro ethylene). But stainless steel columns are suitable; because they can withstandhigh pressures up to 8000psi.straight columns of between 20-50 cm in length are generally used.Short columns are required for liquid adsorbent and liquid-liquid chromatography. Whereasother models of HPLC requires long columns. The internal diameter of the column is usually1-4mm. The columns usually posses an internal mirror finish which allows, efficient packing. Thepacking material is supported by a porous stainless steel (or) teflan plug / disc at the end of the
column.
In "HPLC" three forms of columns are available. They are
(i) Micro porous supporters
(ii) Pellicular supporters
(iii) Bonded phases,

Guard Column

Resolution power of HPLC is so high that sample preparation before chromatography isnot necessary. Thus serum or other biological materials can be applied to the column without anypre treatment. This however, clogs the column after a free application as the column duringseparation retains many undesirable components of the biological sample. To overcome

thisproblem a short column (2-10cm) precedes the main column. This short column is known as“guard column” and its function is to retain these biological components, which would otherwise
clog the main column. The packing of the guard column can be replaced at regular intervals.

Column Packing Procedure

For HPLC, the packing must be uniform without any cracks (or) channels for obtainingoptimum separations. Usually a method known as “high pressure slurry technique” is used forpacking the column. In this method packing material is prepared in a suitable solvent. Thecolumn is sealed with a porous plug at the bottom. The slurry is now pumped into the column athigh pressure. The column so packed, is then equilibrated for a long time by passing thedeveloping solvent through it. The technique can be extended to hard gels. For soft gel, howeverthis technique cannot be applied because pressure results in the fracture of gel particles. Thesegels therefore have to be filled into the column under gravity in a way similar to conventionalchromatography.

Detectors

In HPLC a small quantity of sample is applied to the column. Therefore the sensitivity ofthe detector must be high and stable. U.V., visible photometers can be used for HPLC. Thesedetectors are inexpensive and sensitive, and insensitive to normal flow and temperaturefluctuations, and well suited for gradient elution. However they are sample selective. Thesedetectors are suitable to detect macromolecules, which absorb at 250nm or 280nm. By receiving
the signals from the detectors record chromatogram.

Applications

- Due to high speed of resolution and reproducible it has been widely used for separation
- of macromolecular mixtures.
- It is mainly used for separation of carbohydrates, proteins, lipids, fatty acids and nucleic
- acids.
- This technique has been widely used for purification of proteins from cell extracts.
- HPLC separates the microorganisms from culture supernatants.
- By using this technique we can separate different RNA’s from mixtures of RNA’s.
- It has been widely used to separate synthetic corticosteroids such as Hydrocortisone, and
- progesterone.
- HPLC is used in separation of drugs in horse plasma.
- It has been widely used in separation of sugars in food materials.
- It is also useful for separation of di and tri carboxylic acids such as succinic acid,
- Fumaric acid, Malic acid, citric acid, α – Ketoglutaric acid and oxalic acid.
- It is also used in separation of D and L isomers of amino acids.

8.6. GAS LIQUID CHROMATOGRAPHY (GLC)

The basis for the separation of the compounds in gas liquid chromatography is thedifference in the partition coefficients of volatilized compounds in the liquid stationary phaseand Gaseous mobile phase.

Gas liquid chromatography is a form of column chromatography where the stationaryphase is a non-volatile liquid. Here, the stationary phase is liquid phase. This liquid phase isdispersed over a surface of an inert solid support. The solid support which is coated on to theinside surface of a long column is inert to the sample compounds and does not react with them inany way. Liquid phase interacts with sample components. A gas stream called carrier gas flowscontinuously through this column at a flow rate, which is controlled. When a small quantity ofvolatile sample is introduced in to the gas, the gas promptly carries it on to the column. In thecolumn the sample components become distributed between liquid and the gas phases. Thesecomponents therefore travel more slowly than the carrier gas because they are being retarded byvirtue of their interaction with the liquid phase. The retarding effect is different for differentcomponents. The sample distributing more in the liquid phase is retarded more and thecomponent, which preferred the gas phase, is retarded less. These separated componentseventually elute out of the column and reach the detectors which reads the concentration of agiven components present in the carrier gas and converts it to an equivalent electrical signal. Themagnitude of the detector signal as measured by a continuous recorder when plotted against thetime taken by the particular component to elute out of the column produces a pattern called achromatogram. This chromatogram is made up of a number of peaks each of which is due to acomponent of the sample.The characteristic position of peak is measured in terms of the volume of gas that has traversed through the column between the time that the sample was applied and the time at which theparticular component emerged from the column. This gas volume has been given the termretention volume Vr. The area of the peak is usually proportional to the concentration of thecomponent.

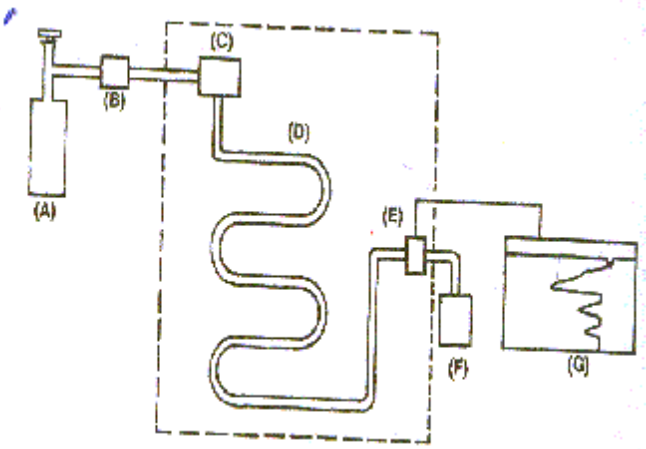


Figure 13.4: Schematic diagram of a gas chromatograph, (A) Carrier gas tank (B) Pressure regulator (C) Sample injection chamber (D) Column detector (E) Detector (F) Fraction collector and (G) Recorder.
(Courtesy from Upadhaya and Upadhaya Nath)

Carrier Gas

In GLC, the carrier gas constitutes the mobile phase and provides transportation for the sample components through the apparatus. The gas must be chemically inert and pure. Gas used at high density gives a better separation but takes a long time. The choice of gas usually depends on the requirements of the detector and also on the availability of the gas. Most commonly used gases

are nitrogen and argon. Purity of carrier gas is critical because even a small impurity can give rise to noise in the detector. The usual contaminant is water vapor. A soap film flow meter is used almost exclusively to measure the flow rate. The gas is usually passed through the column at a flow rate of 40-80 cm3/minute.

Columns

Two distinct types of columns are commonly used, packed columns and open tubular columns. The open tubular columns are also known as capillary columns. Packed columns are stainless steel, copper or glass tubing 1.6, 3.2, 6.4 or 9.5 mm bore, and any where between 1-15 meter in length.

Open tubular or capillary column have an open unrestricted path for the carrier gas with in the column. These columns are about 15-30 meter in length with an inside diameter of about 0.25 mm. These open tubular columns are of two types. One is wall coated open tubular column and the second support coated open tubular columns (Slot).

Solid Support

An ideal support should be chemically inert. It should ideally have a high specific surface. In addition it must be thermally stable and mechanically strong. The most commonly used supports are derived from diatomaceous earth and Teflon. Deactivation of all diatomaceoussupportsis necessary for most applications. Acid washing is effective in removing mineral impurities. This treatment is needed for if the sample is non polar.

Liquid Phase

A good separation will occur only when the sample dissolves well in the liquid stationary phase. So since the gas phase is inert, the separation occurs only in the liquid phase. Thus to select the liquid phase it is necessary to match the polarity of the stationary phase and the sample components.

The requirements for a good liquid phase are

1) It must be non-volatile at the temperature it is to be used.
2) It should be thermally stable.
3) It should provide appropriate partition-coefficient values for the components of interest.
4) It should be completely inert towards the solutes.

Coating the Support

To prepare the column, correct amount of liquid phase dissolved in a low boiling solvent is added to the solid support. The mixture is then heated very slowly with continuous stirring in order to evaporate the solvent. The last traces of solvent are removed under vacuum. Columns are filled by pouring the packing into straightened columns. The column is gently shaken and tapped all the while to ensure an even packing. The pressure applied is 5 psi. Both ends are plugged with glass wool and the column is bent or coiled to an appropriate shape that will fit in the oven.

Sample Preparation And Introduction

If the samples are non polar or have a very low polarity they may not need any pretreatment. Sample should ideally be introduced in the vapour form. Size of the sample varies depends upon the column. Solid samples are much more difficult to introduce. The best method is to seal them

into a thin vial which can then be introduced in to the injection port and then crushed from outside.

DETECTORS

These are located at the exit of the separation column. The detectors detect the presence of the individual components as they leave the column. The most commonly used detectors are

1) Flame Ionization Detector

This is most widely used detector. It measures all organic compounds and it can detect as low as one nano gram of any given compound. Hydrogen used as a carrier or introduced into the detector through elsewhere is burnt to give a nearly colorless flame. The jet of which forms one electrode. The other electrode is mounted just near the tip of the flame and consists of a platinum wire. The flame changes color, the moment a separated component comes out of the column and into the flame. The sample components become ionized in the flame and give rise to a current between the electrodes.

2) Electron Capture Detector

This detector has radioactive source (63Ni), which ionizes the carrier gas coming out of the column. The electrons produced give rise to a current across the electrodes to which a suitable voltage is applied. When a sample component, which has the ability to capture electrons, comes out of the column, it captures the ionized electron there by causing a drop in the current. This change in the current is measured and recorded. It is mostly used to measure polyhalogenated compounds, particularly pesticides such as DDT, dieldein and aldein. It is very sensitive and can detect as little as one pico gram of these compounds.

3) Thermionic Emission Detector

This detector employed fuel poor hydrogen plasma. This low temperature source suppresses the normal flame ionization response of the compounds not containing nitrogen or phosphorus. A non-volatile rubidium silicate bead is centered about 1.25 cm above the plasma jet. This bead is electrically heated to about 600-8000C. It is used to measure organo phosphorous pesticides.

Retention Time And Qualitative Analysis

Retention time aids in qualitative analysis in gas chromatography under standard conditions of temperature, gas flow, gas compressibility etc. The time taken for a compound to emerge from a column is constant and is known as the retention time. The separation of two components of a sample is a function of the ratio of their retention times. This ratio is known as separation factor. The separation factor is a function of the stationary phase and can be varied in order to improve resolutions by varying the stationary phase.

Applications Of Gas Liquid Chromatography

Apart from the separation of components of tobacco smoke, atmospheric pollutants solvents plant extracts, essential oils, volatile vegetable oils and organic acids etc.

- Gas chromatography is being increasingly used as an analytical tool to study many parameters.
- It is widely used in the field of solution chemistry including study of polymers, Lewis acid base properties, liquid crystals, and adsorption.
- This is also an excellent tool to study thermodynamic properties of solutions.
- It is one of the most widely used to study reaction rates, energies and mechanisms.

 They are used to behave as isotope exchange vessels.
 GLC used to analyze such molecular properties as vapour pressure heat of vaporization molecular weight.

13.7. SUMMARY

Chromatography is the technique used to separate the components of sample mixture. The differential movement of components in sample between stationary and mobile phases is responsible for their ultimate separation from each other. There are two basic techniques of chromatography, plane and column chromatography. There are two types of plane chromatography "Paper and TLC". In paper chromatography the stationary phase is supported by cellulose fibers of the paper sheet and solvent acts as mobile phase. The sample is applied to paper as a small spot. There are two techniques which may be employed for the development of paper chromatography. Those are ascending and descending chromatography. Separation of sample is achieved in ascending chromatography as the solvent moves vertically up the paper by capillary action. In the descending technique, the separation of the sample is achieved as the solvent moves downward under gravity. There are two other techniques in paper chromatography are radial and two dimensional chromatography. This technique has played an important role in biochemical analysis due to its ability to separate small molecules such as aminoacids and oligopeptides. The "TLC" is similar to paper chromatography. In TLC a thin layer acts as stationary phase &and solvent acts as mobile phase. In "TLC" the stationary phase is coated onto a glass or plastic surface. The separation of components and detection is assimilar to ascending paper chromatography. The advantage of "TLC" is the speed at which the separation is achieved. HPLC is an example of column chromatography, and the resolution power of HPLC is very high compared with other conventional chromatography, techniques. This method has high pressures. Therefore the flowrate is high, and experimental time is short and resolution power is high. The greatest advantage of HPLC is that it may employ the principle of adsorption, partition, ion exchange exclusion and affinity chromatography. This makes it an extremely versatile and most popular technique. In "GLC" the stationary phase is non volatile liquid and carrier gas constitutes the mobile phase. Liquid phase is dispersed over a surface of an inert solid support and is present in long column. When a small quantity of volatile sample is introduced into the gas, the gas carriers it into the column. In column the sample components become distributed between liquid and gas phases. The separated components elute out of the column and reach the detector which reads the concentration of a given component present in the carrier. Gas converts it to an electrical signal. The chromatogram is made of no of peaks each of which is due to components of sample. The area of peak is proportional to the concentration of component. The GLC is the most popular technique and havingwide number of applications.

13.8. Model Questions

Essay Type Questions

1. Write about the principle, instrumentation, procedure and applications of high performance liquid chromatography.
2. Give an account on the principle, instrumentation, procedure and applications of gas liquid chromatography.
3. Define partition coefficient and explain the procedure for the separation of lipids by TLC.

Short Answer Questions

Explain the term chromatography and add a note on paper chromatography.

1) Thin layer chromatography.
2) Applications of HPLC
3) Radial paper chromatography.

13.9. Reference Books

1) Biophysical Chemistry by Upadhaya and Upadhya Nath, 3rd Edition, Himalaya Publications.
2) Analytical Chromatography by G.R.Chatwal, 1st Edition, Himalaya Publications.
3) Practical Biochemistry by Keith Wilson and Walker 5th Edition, Cambridge University Press.
4) A Biologist Guide to Principles & Techniques of Biochemistry, Keith Wilson and Goulding.

Dr K. Nagaraju

LESSON-14

ELECTROPHORESIS

OBJECTIVES

By the end of the lesson you will be able to understand the definition, principle and purpose of Electrophoresis and factors affecting electrophoresis and different types of Electrophoresis

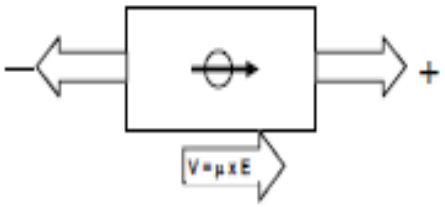
14.1. INTRODUCTION

Many important biological molecules such as amino acids, peptides, proteins, nucleotides and Nucleic acids possess ionisable groups. These can be made exist in solution as electrically charged species, either as cations (+) or anions (-). Even typically non-polar substances such as carbohydates can be given weak charges by derivatisation, for example, as borates or phosphates. Molecules which have a similar charge will have different charge/mass ratios when they have inherent differences in molecular weight. These differences form a sufficient basis for a differential migration when the ions in solution are subjected to an electric field. This is the principle of electrophoresis.
The migration of charged particles in a medium under the influence of an applied electric field. The usual purposes of carrying out electrophoresis are:
(a) to determine the number, amount and mobility of components or to separate them in a given sample
(b) to obtain information about the electrical layers surrounding the particles.
(c) determination of molecular weight of proteins and DNA sequence.

Electorphoresis is a Greek word means, ‘born by electricity’. In 1807 a Russian Physicist, Alexander Reuss observed the electrophoretic movement of some colloidal particles when current was passed through glass tube filled with water and clay. Michael Faraday was also confirmed this discovery.

14.2 PRINCIPLES AND FACTORS AFFECTING ELECTROPHORESIS

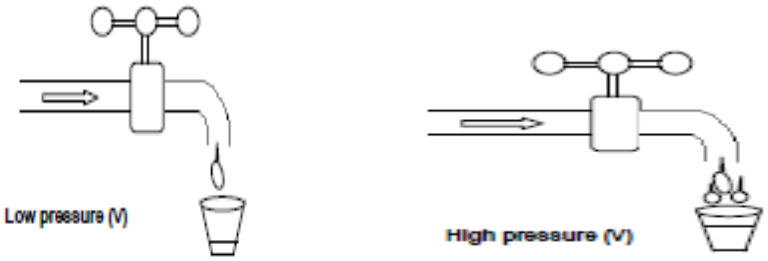
Principle of electrophoresis



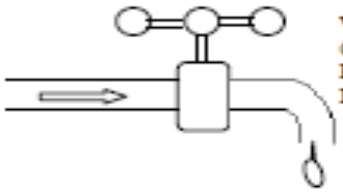
V=μx E where,

V= Velocity of molecule (cm/second)

μ= Electrophoretic mobility (cm2/volt/second)
E = Electrical field strength (Volt/cm)
= a molecule migrating in the electrical field
= migration of cations
Voltage is the fundamental driving force in electrophoresis. A volt is equal to the difference in potential needed to cause a current of one ampere to flow through a resistance of one Ohm. Mobility of molecule increases with increase in voltage. According to Ohm's law:
V = IR or I = V/R
V = volt, I = current and R = resistance



Water tap is analogous to an electrophoresis system.



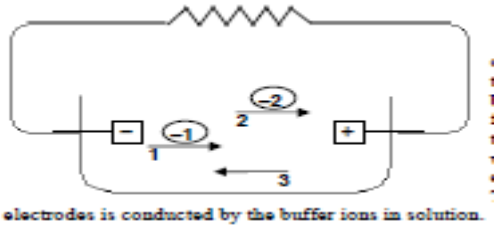
The equipment required for electrophoresis consists of two items
(a) a power pack and
(b) an electrophoretic unit
In an electrophoretic unit, the power pack supplies a direct current between the electrodes. The sample in an aqueous solvent acquires either positive or negative charge. Cations move to the

cathode (-) and anions move to the anode (+). The acquisition of such charges depends on the nature of the particle/molecule and the solvent. The sample must be dissolved or suspended in buffer for electrophoresis and any supporting medium must be saturated with buffer to conduct the current. A buffer is also important to maintain a constant state of ionization, because changes in pH would alter the charge on molecules being separated. For example, the net charge density of a protein depends upon the number of ionizable amino and carboxyl groups and velocity depends on the sign and quantity of net charge density.

The net charge density depends upon chemical groups and their number present in the molecule is modified by the nature of the solvent. The current is maintained throughout the circuit by electrolysis taking place at the electrodes, both of which dip into buffer reservoirs. During electrolysis, OH□ions and H+ ions are produced at the cathode and O2 and H+ ions are produced at the anode.

2e- + 2H2O 2OH + H2
H2O 2H+ + ½ O2 + 2e

The hydroxyl ions produced at the cathode cause an increase in dissociation of the weak acid component (HA) of the buffer mixture. This results in the formation of more A to conduct the current to the anode. At the anode, A ions combine with H+ ions to reform HA and the electrons are fed into the electric circuit. Therefore, most of the current between the electrodes is conducted by the buffer ions in solution.



A charged particle (1) migrates toward the oppositely charged electrode (+). Arrow 2 indicates faster movement of a particle bearing a net charge density of –2. Arrow 3 indicates the direction of frictional force.

Factors affecting electrophoresis

There are certain factors like the charge, size and shape of the sample, composition, concentration or ionic strength and pH, adsorption, electro-osmosis, and molecular sieving of the supporting medium and voltage, current, resistance relationships in electric field. (a) The sample: Electrophoretic mobility of the sample will be dictated by charge/ mass ratio of the sample.

Charge = positive or negative
Mass = size and shape of the molecule

1. *Charge*: Increase in the charge increases the electrophoretic mobility. It depends upon pH of the medium.

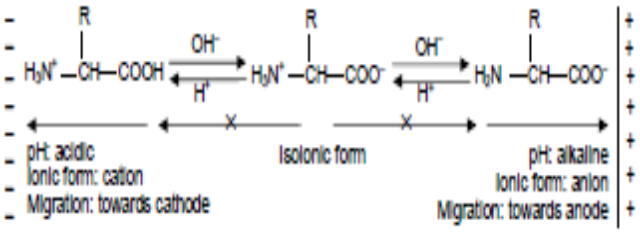
2. *Size*: Bigger the molecule, greater is the frictional and electrostatic forces exerted upon it by the medium of suspension. Larger particles have a smaller electrophoretic mobility than the smaller particles.
1. *Shape*: Round particles move faster than fibrous or other shapes.

(b) The buffer: The pH of the supporting medium will be determined and stabilizes by the buffer.

1. Composition: The commonly used buffers are formate, acetate, citrate, barbitone, phosphate, Tris, EDTA and pyridine. The rates of migration will be altered if the buffer binds with the compounds being separated. So buffer should not bind with the compounds being separated. In some cases binding can be advantageous, for example, borate buffers are used to separate carbohydrates. Some diffusion of the sample is inevitable, because buffer acts as a solvent for the sample, for example, amino acids and sugars. This diffusion can be minimized by avoiding over loading, using a high voltage for a short time and by rapid removal of the supporting medium after the separation has been completed.

2. Concentration (ionic strength): Increased ionic strength of the buffer means a large share of the current being carried by the buffer ions and a meager proportion carried by the sample ions. This situation leads to slower migration of the sample components. The overall current will also increase, there will be heat production. A decrease in ionic strength, would mean larger share of the current being carried by the sample ions leading to a faster separation. Since the overall current will be low, less heat will be produced. In low ionic strength buffers, diffusion tends to be high with concomitant loss of resolution. The chosen ionic strength of the buffer is usually between 0.05-0.10 M.

3. pH: pH shows more effect of ionization of organic compounds whereas only little effect on fully ionized compounds like inorganic salts. The ionization of organic acids increases as pH increases and the ionization of organic bases decreases as pH decreases. Therefore, migration depends on pH. Both the above effects the amino acids that have both acidic and basic properties.



The direction and migration of ampholytes are pH dependents and buffers from pH 1-11can be used to produce the required separation. Generally, the buffer in both reservoirs is normally the same buffer, this is called continuous buffer system. In some forms of gel electrophoresis like SDS PAGE, where the buffer acts as part of the supporting medium and a different buffer may be used in the gel to that in the reservoirs. This is called discontinuous buffer system.

(**c) The supporting medium**: The supporting medium may cause adsorption, electro-osmosis and molecular sieving.

1. Adsorption: This is nothing but attachment of the component on to the surface of supporting medium. It causes tailing of the sample so that it moves in the shape of a comet rather than a compact band. Thus the rate and resolution of the electrophoretic separation can be reduced by adsorption.

2. Electro-osmosis: This results from a relative charge being produced between H2O molecules in the buffer and the surface of the supporting medium. The charge may be caused by surface adsorption of ions from the buffer and the presence of stationary carboxyl groups on paper/sulphonates on agar. This generates a motive force for anions to the anode and cations to cathode carrying along the neutral substances by solvent flow.3. Molecular sieving is the intertwined molecular chains, which are distributed throughout the gel, acts as a sieve like structure. The movement of large molecules is stopped/hindered by decreasing the pore size in case of agar, starch and polyacrylamide gels, where as in case of Sephadex gel, small pores shut out/exclude (leave) larger molecules, therefore they move outside the pores, where as small molecules are tightly held with in the pores.

(d) The electric field

1. Voltage: The distance of the electrodes is d meters and potential difference between them is V volts, therefore Potential gradient = V/D volts m-1. Ions bear a charge q coulombs. The force on this ion is Vq/d newtons. This force causes migration and the rate of migration is proportional to Vq/D. Increase in potential gradient will increase the rate of migration.
2. Current: When a potential difference is applied between the electrodes, a current is generated. Current is measured in coulombs sec-1 or amperes. Current is directly proportional to the voltage. The current in the solution between the electrodes is conducted by the buffer ions. Increase involtage increases the total charge/sec conveyed towards the electrode. The distance migrated by the ions is proportional to both current and time.
3. Resistance: Ohm's law expresses the relationship between current I (measured in Amperes A), voltage V (measured in volts V) and resistance R (measured in Ohms Ω) in which: V/I = R. The current and rate of migration are inversely proportional to the resistance. Resistance will increase with the length of the supporting medium but will decrease with its cross-sectional area and with increasing buffer ion concentration. During electrophoresis the power (comes out from the supporting medium) dissipated in the supporting medium (W, measured in watts) is: W = I2 RIncrease in temperature will cause fall in resistance. Part of this is due to an increase in the mobility of the ions. This heating will produce evaporation of the solvent from the supporting medium causing a decrease in resistance. Increase in buffer ion concentration will result in slowermigration of the sample. Stabilized power packs are used which can automatically maintain either a constantvoltage/constant current. When a constant voltage is applied, the current will increase due to adecrease in resistance of the medium with the rise in temperature.

Consequently, more heat will be produced, more evaporation of solvent and a decrease in resistance. When a constant current is applied, these problems will be solved, but may lead to a drop in voltage due to decreased resistance, reduced rate of migration. If a number of supporting media are run in parallel from one power supply, the total resistance will decrease: I/R = 1/r1 + 1/r2 + 1/r3 + …… 1/rn The voltages used can be low (100-500) or high (500-10,000 v). High voltages are used for the separation of low molecular weight compounds. In low voltage constant voltage or current may be applied, as the heat generated is small and is easily dissipated (paper electrophoresis). Incase of cellulose acetate and other gels, a constant current is used to reduce heat production. Direct current must be used. Enclosing the apparatus under an airtight cover can minimize evaporation.

14.3. TYPES OF ELECTROPHORESIS

14.3.1. Gel Electrophoresis

For the separation of high molecular weight substances like proteins and nucleic acids gels are used as supporting medium. Suitable gels may be prepared by using powdered solids like starch, agar and polyacrylamide. Molecular sieving property of the gel helps to separate proteins, which have similar charge but different sizes and shapes.

Starch gels

Starch gels are of two types, weak high porosity and strong low porosity gels. Weak high porosity gels are prepared by incorporating less than 2 % (W/V) starch in an appropriate buffer and strong low porosity gels are prepared by adding 8 to 15 % starch. In both the cases, the branched chains of the amylo pectin component of starch to intertwine and form a gel.

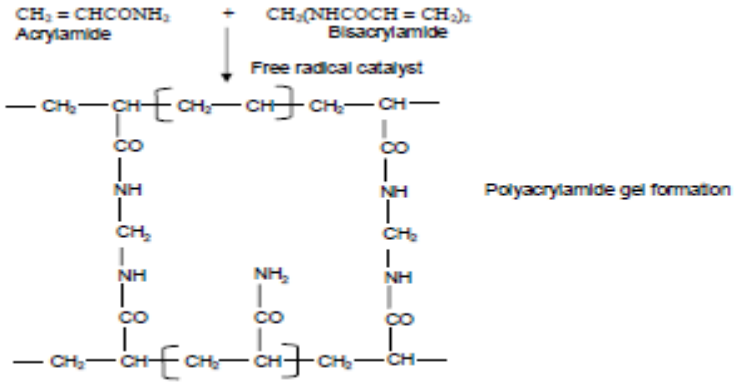
Agar / agarose

Agar is a cheap, non-toxic, complex powdery mixture containing two galactose-based polymers, agarose and agaropectin. 1% (W/V) gel in buffer has a high water content, good fib restructure, large pore size, low fractional resistance and sets at 380 C. Electro-osmosis is severe, because of sulfate content. It should be removed prior to purification. Purified agarose gel is now used for the separation of DNA restriction fragments and DNA because of lack of molecular sieving and electro-osmosis.

Polyacrylamide

Highly toxic synthetic chemicals are used for the preparation of polyacrylamide gels. The chemicals used are: Acrylamide and bisacrylamide, APS (Ammonium per sulfate), TEMED (N,N,N’-tetramethylenediamine). Acrylamide monomer is copolymerized with a crosslinking agent, bisacrylamide. Freshly prepared (0.1-0.3% W/V) APS is used as catalyst. TEMED is an

indicator. It speeds up the rate of gel polymerization. Degasing of the solution is required since molecular oxygen inhibits chemical polymerization. The relative proportions of acrylamide monomer to cross-linking agent determine porosity of a gel.



Gels may be defined in terms of the total percentage of acrylamide present. Low percentage gels have larger pore size, less resistance to the passage of larger molecules. Most protein separations are carried out using gels from 5 to 15 % acrylamide. These gels are useful for macromolecule separation because of their minimal adsorption capacity, lack of electro-osmosis and various types of histochemical analysis.

Equipment

Gels can be run as horizontal slabs or vertical slabs. Tube gels are also used. Power packs used are similar to that of low voltage electrophoresis. Slab gels can carry more samples than tube gels.

Preparation

The gels are cast between two clean glass plates, which are clamped together but held apart by plastic spacers. For agarose and starch 12 x 25 cm with 3 to 6 mm thick gels are prepared. For polyacrylamide, 12 x 14 cm with 1 to 3 mm thick gels are prepared (Fig.14.1).

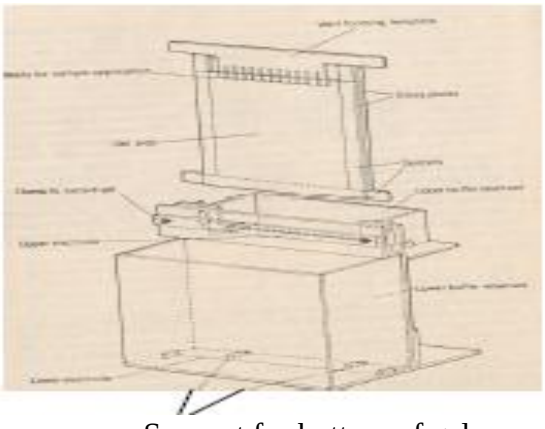


Fig. 14.1. Vertical gel electrophoresis unit

Sample application

Sample solutions are injected with a micro syringe into the wells of a gel. Wells are prepared by inserting a comb into the gel before it sets. For making sure that the sample should sink in the well, sample must contain sucrose or glycerol (10-15%). For monitoring migration, bromophenol blue dye is added. Urea or SDS may be added to facilitate solubilization of proteins and disulfide reducing agents like mercaptoethanol or Dithiothreitol may be added. Only microgram (µg) quantities of proteins and DNA are used.

Running the sample

The horizontal gel should be submerged in the buffer so that buffer allows current to pass directly through it. In vertical systems, gel will be sandwiched between glass plates, is placed in voltage and time required to obtain optimal separations will depend on the nature of the sample and the type of gels used. Marker dyes like bromophenol blue for proteins and ethidium bromide for nucleic acids enable the progress of the run to be monitored. Sodium dodecyl sulfate (SDS) Polyacrylamide gel electrophoresis SDS is an anionic detergent. It binds strongly to proteins and causes denaturation. It gives negative charge to the proteins. Protein-SDS complexes will move toward the anode. Their mobilities are inversely proportional to their molecular weight are also run, the molecular weights of the sample proteins can be determined.

Apparatus and methods

Standard SDS polyacrylamide gels are run vertically. The protein samples are generally dissolved in Tris buffer pH 6 to 8, SDS, 2-mercaptoethnol, glycerol/sucrose (to increase density) and bromophenol blue. Resolution of the protein band is increased by using both stacking and separation/resolving gels. Differences in pH and composition between these two gels cause the samples to be concentrated into narrow bands before separation occurs. Separation is primarily based on differences in size of the proteins.

14.3.2. Gradient gels

Gradient gels contain a *concentration gradient* of acrylamide increasing from around 5 to25 %, with a corresponding decrease in pore size, causing improved resolution of protein bands. The gradients are formed by running high and low concentrations of acrylamide solutions between the gel plates via a gradient mixer. If the pore size is too small, narrow bands will be produced and thus increased resolution. Separation is primarily based on differences in the size of the proteins. This means that samples can be run in which there are wide ranges of molecular weights. When molecular weights are similar, better separation is achieved than in a uniform gel. Though gradient gels can be used without SDS and stacking gels, these should be utilized if optimal separations are required.

Two dimensional gels

Two-dimensional gels enable the protein components of a complex mixture to be separated with still greater resolution. The samples are first partially separated by isoelectric focussing on the basis of differences in isoelectric points, using a cylindrical column of gel. This gel is then applied along the top of the stacking gel for separation to be completed on the basis of

differences in molecular size. Either uniform or gradient SDS gels may be used, depending on requirements. It can resolve a mixture containing 5000 particles into individual species.

Procedure

The mixture is first subjected to isoelectric focussing on a 1 mm diameter gel in a capillary tube. At the conclusion of the isoelectric focussing, the gel is extruded from the column and placed on to the top of the slab gel. The sample is now subjected to SDS acrylamide gel electrophoresis, which separates the proteins according to their molecular weight. Isoelectric point and molecular weight of a protein are in no way connected to each other and thus the technique exploits two properties of protein for separation and thus has a great resolution power. Detection, recovery and estimation Gels from a column are removed by forcing water from a hypodermic syringe around the walls of the column, allowing the gels to be extruded under gentle pressure. Slab gels are removed by introducing a thin metal plate between two gel plates and remove the plates. Before staining, the gels are immersed in a fixative (7 % acetic acid) to guard against diffusion of separated components. The sample is stained with Coomassie Brilliant Blue. The starch gel becomes opaque after staining and so very difficult for direct densitometry. Direct UV absorbency may also be performed. However, polyacrylamide absorbs in the UV range. Inorderto get accurate quantitative results, the compound has to be removed from the supporting matrix. Compounds from the starch gel can be removed by slicing the appropriate portion of the gel, macerating it and then treating it with amylase which solubilizes the starch and leaves the compounds in solution. If the sample has been radioactive labelled, prior to electrophoresis, autoradiography may be performed to detect the portion of the separated components in the gel. Fluorescent staining: - Fluorescent staining includes flouresamine is applied to proteins before electrophoresis, if SDS-containing gels are to be used. In other cases stain can be applied before or after electrophoresis. This method does not give quantitative results because fluorescence decreases with time. Proteins can have varied content of lysin and so the dye would give high results. Densitometry: - After staining, the quantitative estimation of individual components can be carried out by densitometry. Depending on the intensity of the colour the results are made.

14.4. ISOELECTRIC FOCUSSING

Isoelectric focussing was discovered by H. Svensson in Sweden and has high-resolution power. A simple comparison would help to establish the methods supremacy over other methods, when paper electrophoresis resolves plasma proteins into 6 bands, isoelectric focussing resolves it into atleast 40 bands. Protein molecules have a net positive charge in an acidic solution because most aminogroups carry a positive net charge and most carboxylic groups are protonated and electrically uncharged. With a gradual increase in pH, the number of carboxylic groups carrying a negative charge increases, while the number of positively charged groups decreases. At a certain pH value, the isoionic point, the net charge of the protein molecule is zero. The isoionic point of a moleculeis determined by the number of types of proteolytic groups and their dissociation constants. Although there is considerable variation in the isoionic points of proteins, they are generally in the pH range of 3-11. In conventional electrophoresis, the pH between anode and cathode is constant andthe positively charged ions migrate towards the cathode and the negative ions migrate to the anode. In isoelectric focusing on the other hand, a stable pH gradient is arranged, the pH increases gradually from anode to cathode. Protein introduced into

this systemat a point where the pH is lower than the isoionic point will possess a net positive charge and will migrate in the direction of cathode. Due to the presence of the pH gradient, the protein will migrate to an environment of successively higher pH values which in turn, will influence the ionization and net charge of the molecule.

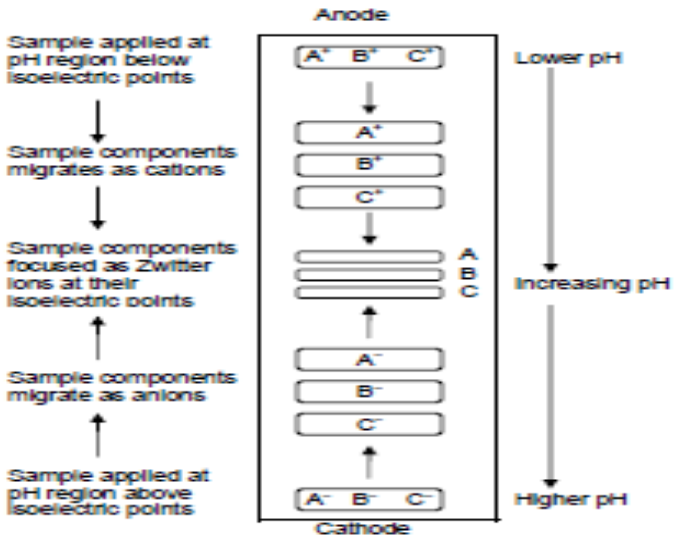


Fig.14.2. The Principle of Isoelectric Focussing.

Finally, the protein will encounter a pH where its net charge is zero and will stop migrating. This is the isoelectric point of the protein. The consequence of this is that, every protein will migrate to and focus at its respective isoelectric point in a stable pH gradient, irrespective of its origin in the apparatus at the time the current was applied. Thus, the point of application and the volume of the protein solution are not critical. Diffusion, which is an obstacle with every other method of electrophoresis, is not a problem with electrofocussing, because focussing effect works against diffusion. Thus, once a final stable focussing is reached, the residual will be retained even if the experiment is continued for a long time (Fig.14.2).

Establishing the pH gradient carrier ampholytes

The pH gradients may be obtained by electrofocussing special buffer substances known as carrier ampholytes. The carrier ampholytes must have the properties:

1. Since carrier ampholytes must dictate the pH course, they should have a certain buffering capacity at their isoelectric point.
2. They should have conductance at their isoelectric point.
3. They should have low molecular weight so that macromolecules can be separated from them easily after electrofocussing.
4. They should be soluble in water. This hydrophilic character will also prevent their binding to hydrophobic regions of proteins.
5. Ideally they should have a low light absorption at 280 nm. This would permit the detection of proteins after electrofocussing by measuring at 280 nm. Carrier ampholytes are isomers and homologs of aliphatic poly amino, poly carboxyl acids. The general formula for a carrier ampholyte is:

R –N –(CH2)n –N – (CH2)n – COOH
where R can be
- (CH2)n – COOH, H or (CH2)n – N – R
It is usually less than 5. Carrier ampholytes are available commercially in mixtures covering a wide pH band or various narrow bands. The pH range of the carrier ampholytes should be chosen such that the pI (isoelectric point) values of the proteins under study lie well within the corresponding pH range. When making the first run with the protein sample it is often advisable to work with the pH range 3-10. Generally an amount of carrier ampholyte which gives a final average concentration of 1% (W/V) in the column is used. For electrofocussing in gels, an average concentration counteracts electroendosmosis. When pH range is outside 6-8, ampholyte concentration of up to 10% have been used in order to obtain a more even distribution of conductivity between electrodes.

Stabilization against convection
As for all other electrophoretic techniques described so far, electrofocussing also needs provision for stabilization of separating protein zones against convecting flow in the solution.
Three ways are in use:
1. Density gradient
2. Gel
3. Zone convectingelectrofocussing (not popular)

1. Density gradient
Density gradients suitable for electrofocussing can be made with many uncharged solutes which are dissolvable in water to a concentration that will increase the density sufficiently. The compounds should not react with proteins and should have a low content of heavy metals. They should be of high purity. Sucrose is the most ideal compound for the formation of density.
gradients as it has a protective action on proteins. It has been used with 50% (W/V) as the densest solution. Maximum solute concentration and thus the maximum density are placed at the bottom of the column. There is a linear decrease in the concentration of the solute as a function of the column height giving rise to approximately linear density gradients. However, non-linear density gradients have also been used. Sucrose cannot, however, be used at all pH ranges since it is destabilized at pH range 10. Glycerol is generally used at such pH ranges other compounds which can be used in formation of density gradients are mannitol, sorbitol, ethylene glycol, dextran and ficoll. The gradient should be unchanged so that they won't effect the pH. They should not react with sample, should not contain heavy metals, and should be pure.

2. Gels
In electrofocussing, the gel serves only as an anticonvectent and not as a molecular sieve. Obviously, the gel concentration should be low to provide larger diameter pores. For large proteins, molecular weight exceeding 200 KD, lower concentration of acrylamide is the preferred for electrofocussing. Agarose and starch gels are not preferred as in these gels, pH gradient drifts considerably during prolonged experiments.

3. Zone convection electrofocussing
The apparatus is made up of two rectangular boxes, the upper one being the cover. The upper surface of the lower box is with ridges, with a height of about 10 mm, separated by depressions and is facing the upper box which has corresponding ridges (Fig. 14.3).

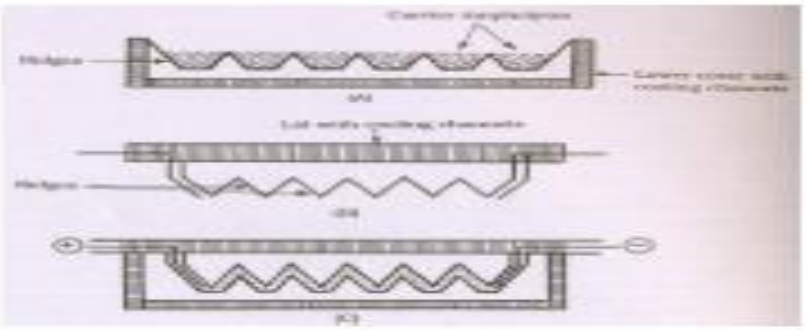


Fig. 14.3. Schematic diagram of Zone convection electrofocussing apparatus. A) Bottom part filled with carrier ampholytes; B) The lid with corresponding ridges; C) The apparatus assembled for electrofocussing.

The ridges and depressions of the two halves fit together leaving a space of few milli meters in between. Thus from one end to the other there will be a narrow wave like channel between the two parts which can be described as series of inter connected broad U-tubes. The carrier ampholyte solution is filled in this space with electrodes situated at the two ends. Both the lid and the bottom part are hollow with channels built into them through which the liquid streams to maintain a constant temperature. When the current is on, a density gradient is formed in each depression by the solute. When proteins become immobile at their isoelectric pH, the density increases locally and the proteins settle down in the depression of the bottom part. When the experiment is over, the cover is lifted and the liquid collects in the depression. Each depression will contain a fraction separated by a ridge. The fractions can now be collected without any possibility of contamination by neighboring fraction.

Procedure: Separation can be carried out in a vertical column or on a horizontal gel plate, but in both cases purpose made equipment is required (Fig.14.4).

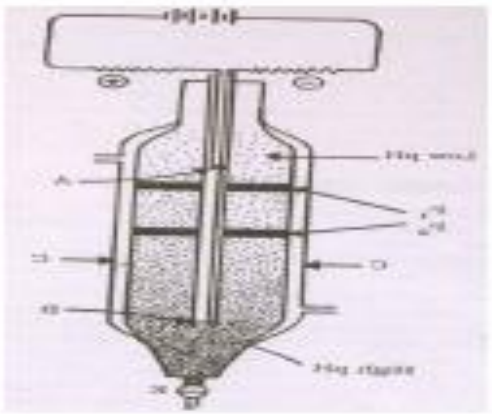


Fig. 14.4. Schematic design of an Isoelectrofocussing apparatus.

A) Anode platinum ring B) Cathode platinum
B) ring C) Water jacket to maintain the

temperature. D) Power pack , E) Valve to empty the column , P1, P2) Protein bands.
The column system in modern days being superceded by the plate system. For preparative purposes, the column mode is still preferred. Water-cooled vertical glass columns are commercially available. These are filled with a mixture of carrier ampholytes suspended in adensity gradient solution (sucrose, glycerol). The anode end of the column is connected to areservoir containing an acidic solution (H3 PO4) and the cathode end is connected to a reservoir filled with an alkaline solution (NaOH). The valves of the reservoir are opened to allow the acidicand the alkaline solutions to diffuse through the column. This results in the formation of a pHgradient between the anode and cathode. The valves are closed and the power is switched on. Thecarrier ampholytes now migrate until they become immobile upon reaching the regions of their corresponding isoelectric pH. These compounds remain fixed in these regions and because of their buffering capacity, the pH gradient is stabilized. Now the sample is applied at the upper end of the column. The charged components of the sample migrate in the electric field till their net charge becomes zero, that is, till they reach their isoelectric pH. The component proteins of the sample remain focussed at the regions of their isoelectric pH. The whole process might take 1-3days. Once the experiment is over, the power is switched off and the sample components are allowed to run off through a valve at the base of the column into a faction collector. The fractions can be analyzed further. Instead of density gradient, polyacrylamide gel impregnated with carrier ampholytes, can be used in the vertical columns. The time required for electrofocussing in the gel is considerablyless than that required in the density gradients (2,3Hours). The carrier ampholytes are mixed with the unpolymerized gel solution and the mixture is allowed to polymerize. The rest of the process remains the same as for the density gradients. Gels are however, used more with the plate mode rather than the column mode. Upto 24 samples can be simultaneously analyzed using the plate system.

Separation of protein from carrier ampholytes
Dialysis against a buffer would effectively remove at least 99% of the ampholytes, but it is a slow process. Gel filtration would give effective separation in a very short time. Sephadex G- 50 is the general choice for the process, others to be ammonium sulfate precipitation of proteins, ion exchange chromatography and partition chromatography by counter current.

14.5. PULSED FIELD GEL ELCTROPHORESIS

Agarose gel electrophoresis can not separate linear double stranded DNA molecules that have a radius of gyration which is larger than the pore size of the gel. These large liner duplex DNA molecules migrate through agarose gels at the same rate irrespective of their size. Agarose stops to perform any molecular sieving effect on these large DNA duplexes. This behavior of large DNA molecules is because of a phenomenon known as Reptation, which means the molecules migrate ‘end-on’ through the matrix. The problem of separating large sized DNA can be removed to some extent by increasing the pore size of the gel. These large pore gels are capable of sieving larger size DNA molecules. Thus, extremely low concentration of agarose (0.1% to 0.2%) have been used to resolve extremely large DNA molecules. This low percentage of gels present two problems of their own:

1. They are very fragile and have to be handled extremely careful.
2. They have to be run very slowly failing which the resolution might be poor.

They are absolutely incapable in separating linear DNA molecules whose size is in excess of 750 kb. The major importance is Histocompatability locus of mammals and therefore, the need to study DNA is well known. This locus occupies several thousands of DNA. Naturally, Norder to study this locus, it is desirable if one can separate it from other genetic material. Inorder to solve this problem, Schwartz and Cantor (1984) developed pulsed-field gel electrophoresis. It uses pulsed, a Hernating orthogonal electric fields.

When such a field is applied to a gel, large DNA molecules become trapped into their separation tubes every time the direction of the electric field is changed. These molecules remain immobilized till they reorient themselves along the direction of the new electric field. The different DNA molecules adopt a behavior consonant with their respective sizes. Large DNA molecules take a larger time to reorient themselves and are consequently retarded more in the new electric field as compared to the smaller DNA molecules. The molecules of DNA whose reorientation times are less than the period of electric pulse can be fractionated in a sizedependentmanner. The resolution of pulsed-field gel electrophoresis depends on

1. The absolute periods of electric pulses.
2. The angles at which the two electric fields are applied to the gel.
3. The relative field strengths of two electric fields and the degree of uniformity of the two electric fields
4. The ratio of the periods of the electric pulses employed to generate the two electric fields. By this method DNA molecules upto 2000 kb in size can be separated.

Instrumentation

The original apparatus used alternately pulsed electric fields are perpendicular orientations and linear electrodes. This type of apparatus does not give proper resolution. The electric field generated is never uniform and so the speed and direction of DNA, which depend son the position at which they are loaded into gel, so no proper resolution at the edges of the gel.

1. Recent innovation is by Carle *et al.*, :- The apparatus designed by them does not use orthogonal or perpendicular arrangement of electric fields. Instead, the apparatus utilizes periodic inversion of a single electric field. So, this method is also called as Field-Inversion Gel Electrophoresis (FIGE). This arrangement produces an electric field, which is uniform in both the directions. The pulse in the forward direction is slightly longer than the one in the reverse direction. This arrangement ensures the migration of the DNA along an absolutely straight track. The ratio between forward and reverse pulses is always maintained as a constant, the absolute lengths of the individual pulses might be varied to improve the resolution. This innovation makes it relatively easy to resolve DNA fragments of upto 2000 kb with fairly good resolution.

2. The innovation by Gardiner *et al*., :- In the apparatus designed by them, a vertical gel apparatus with platinum wire electrode placed on opposite sides of the gel. The electric field is continuouslyswitched between the two electrodes and the DNA moves alternately toward one and then towardanother electrode in a movement, which can be described as zigzag. However, the net result ofsuch a zigzag movement is a straight line and the DNA moves from the place of application to thebottom of gel. All the lanes in the gel experience equivalent electric field. This condition preventsany horizontal distortion of

the resolved DNA bands. The usual arrangement of the electrodes is at an angle of 90o. The length of the electricpulses varies depending upon the size of the DNA to be separated. 10-second pulses used forDNA between 50-500 kb in length, 60 second pulses used for DNA larger than 1000 kb.Extremely useful for DNA molecule upto 9000-kb length can be separated from each othersatisfactorily.As pulsed field gel electrophoresis is a relatively new technique, no single type of apparatus has gained broad popularty till date. Different laboratories use apparatus of different designs depending on their needs. There are several companies, which are marketing suchapparatus.

14.6. SUMMARY

Electrophoresis is the migration of charged particals in a medium under the influence of an applied electric field. The equipment required for electrophoresis consists of two items; power pack and an electrophoresis unit. Power pack supplies direct current between the electrodes. The positive charged samples move to the cathode (-) and negative charged samples move to the anode (+). The buffer that is used to dissolve the sample and in electrophoretic apparatus must be the same. Buffer plays an important role in maintaining constant ionization, as changes in pH would alter the charge on molecules being separated. Voltage is the fundamental driving force in electrophoresis. Volt is equal to the difference in potential needed to cause a current of one ampere to flow through a resistance of one Ohm. Mobility of the molecule increases with increase in voltage. There are certain factors like charge, size and shape of the sample, composition and ionic strength of the buffer, pH, adsorption, electro-osmosis and molecular sieving of the supporting medium, voltage, current and resistance of the electric field, influence electrophoretic mobility. For separation of high molecular weight substances like proteins, nucleic acids gels are used as supporting medium like starch gel, agar or agarose and polyacrylamide gels. Each type of gel has separate compositions, preparations and apparatus are required. Weak high porosity gels are prepared with less than 2% starch and strong low porosity gels are prepared with 8 to15% starch in starch gels. 1% agar or agarose is appropriate for the separation of DNA restriction fragments and DNA. Toxic synthetic compounds like acrylamide, bisacrylamide, APS and TEMED are used for polyacrylamide gels. Most protein separations are carried out using gels from 5 to 15% acrylamide. Only µg quantities of protein and DNA are used. Sample should be added with glycerol (10-15%), which makes the sample to sink in the well, bromophenol blue for monitoring migration, urea or SDS may be added for solubilization of proteins and disulfide reducing agents like mercaptoethanol or dithiothreitol may be added. Marker dyes like bromophenol blue for proteins and ethidium bromide for nucleic acids are used to know the progress of the run to be monitored. For studying denaturation proteins, SDS-polyacrylamide gels are used. SDS causes denaturation of proteins and binds to them and gives negative charge to the proteins. Resolution of proteins is increased by using stacking and resolving gels. Gradient gels contain a concentration gradient of acrylamide increasing from around 5 to25%, with a corresponding decrease in pore size, causing improved resolution of proteinbands. Separation depends on differences in size of the sample. Samples can be run where there are wide ranges of molecular weights. Two dimentional gels are used to separate proteins with greater resolution. Either uniform orgradient SDS gels may be used and it can resolve a mixture of 5000 particals into individual species. Staining the gel with Coomassie Brilliant Blue or UV detection of DNA, fluorescent stainingor by densitometry are used for the detection of the sample .A resolution electrophoresis is isoelectric focussing. Proteins carry a net positive charge.

At acertain pH, the ionic point, the net charge of the protein molecule is zero. The ionic point of a molecule is determined by the number of types of proteolytic groups and their dissociation constant. Diffusion, is a problem in every electrophoretic methods, is not a problem because focusing effect works against diffusion. Electrofocussing needs density gradient, gel and zone convectingelectrofocussing to separateprotein zones against convecting flow in the solution. Gel filtration is an effective separtion. Sephadex G-50 is the general choice for the process. pulsed field gel electrophoresis is used to separate large sized DNA, by increasing the poresize of the gel. 0.1 to 0.2% agarose gels are used.The resolution depends on the absolute periods of electric pulses, angles at which the two electric fields are applied to the gel and the relative field strength of two electric fields and the degree of uniformity of the two electric fields, the ratio of the periods of the electric pulses. Pulsed field gel electrophoresis is a new technique. There are several companies designed depending on the needs.

14.7. Model questions

1. Write the principle behind electrophoresis and what are the factors that influence electrophoresis.
2. Discuss briefly the Isoelectric focusing.
3. Write an essay on different types of electrophoresis
4. What is pulsed field gel electrophoresis.

14.8. Reference Books

1. Biophysical chemistry principles and techniques 4th ed., Keith Wilson and John Walker, Cambridge University Press.
2. Biologists' guide to principles and techniques of practical biochemistry 3rd Ed. by Keith Wilson and Kenneth H. Goulding, Cambridge University Press.
3. Biophysical chemistry :Priciples an

Dr K. Nagaraju