UNIT 4 MOLECULES OF THE CELL

Structure

4.1 INTRODUCTION

So far you have learnt about the structure of the cell and the various techniques by which the cell structure is studied. You have also learnt about the function of various cellular organelles. A proper understanding of cell is possible only when we correlate the structure with chemical composition and functions of various cellular organelles. This unit will deal with the simple molecules and methods of isolating large molecules whereas the next unit will deal with the structure of large molecules, the macromolecules, present in a cell.

We begin with the description of various molecules present in a cell. Water is the most abundant of the cell molecules. The concept of its ionisation with reference to hydrogen ion concentration is discussed first. The structure of simple organic molecules like sugars, amino acids, nucleotides and lipids have been discussed along with the common methods used for their isolation and determination of concentration of macromolecules.

It is assumed that you are familiar with logarithms, capillary action, and radioactive isotopes, before studying this unit.

Objectives

After studying this unit, you will be able to:

- list different types of molecules and their numbers, present in a cell,
- state the properties of water, define and calculate pH,
- describe briefly the structure of monomeric sugars, amino acids, nucleotides and fatty acids,
- state the methods used for determination of concentration of macromolecules present in a cell,
- calculate the specific activity/concentration of a macromolecule in a biological fluid, and
- suggest methods for separating macromolecules on the basis of their size, charge, solubility and molecular affinity.

4.2 TYPES OF MOLECULES PRESENT IN A CELL

Cells in different tissues of an organism vary in their structure and function. Consequently, cells are of many types, such as muscle cells, blood cells, nerve cells in animals and meristematic cells and vascular cells in plants. Yet all the cells show considerable uniformity in their basic biochemical make-up. In other words, the chemical components of various types of cells are very similar and it is only the arrangement of components that causes a great diversity among cells, and consequently among tissues and organisms. Cells

contain various simple as well as complex molecules. Table 4.1 gives molecular composition of a cell. Let us now study some of these molecules briefly.

Table 4.1
Molecular Composition of a Cell

Type of molecules	Per cent of total mass		Number of types of molecules in a bacterium
	Bacterial cell	Mammalian cell	
Water (H_2O)	70	70	1
Macromolecules (proteins)	15	18	3000
RNA	6	1.1	300
DNA	1	0.25	1
Sugars and precursors	3	2.0	200
Lipids and precursors	2	5	50
Amino acids and precursors	0.4	—	100
Nucleotides and precursors	0.4	—	200
Inorganic ions (like Na^+ Mg^{2+}, Ca^{2+}, PO_4^{3-})	1	1	20
Other small molecules (like vitamins)	0.2	3	500

4.2.1 Water

Water is the most abundant of all the molecules in a living cell and is absolutely necessary for life (Table 4.1). It is difficult to imagine the existence of any form of life in which water can be replaced by any other molecule. Uniqueness of water lies in its (i) structure, (ii) capacity as an excellent solvent, (iii) role as a medium in which all biochemical reactions occur.

Chemical reactions in the body are very sensitive to changes in the pH as most chemical reactions of life occur at neutral pH. Blood and most of the body fluids have pH values about 7.4 except for the stomach contents which have a pH value about 2.0. pH of a solution is measured in terms of its hydrogen ion (H^+) concentration, which indicates whether a solution is acidic or basic.

The unique properties of water are due to its molecular structure. In water two hydrogen atoms are covalently held to an oxygen atom and hence dissociation of hydrogen would be very difficult. Consequently, pure water exhibits very little ionisation. (See Fig. 3.1 and 3.2 of Unit 3, Block I of Ecology Course.)

Ionisation of Water

In pure water, for every 550 million undissociated water molecules one molecule dissociates into one hydrogen ion (H^+) and one hydroxyl ion (OH^-).

$$H_2O \text{ === } H^+ + OH^-$$

The equilibrium constant between the dissociated and undissociated molecules may be represented as follows:

$$\frac{[H^+]\ [OH^-]}{[H_2O]} = K_{eq} \qquad \text{.... Eq. 4.1}$$

K_{eq} = equilibrium dissociation constant.

H,OH and H_2O in brackets indicate molar concentrations.

The molar concentration of water in one liter of water is 1000/18=55.5, so that Equation 4.1 becomes

$$55.5 \times K_{eq} = [H^+]\ [OH^-]$$

or

$$Kw = [H^+]\ [OH^-] \qquad \text{.... Eq. 4.2}$$

One mole of a substance means 6.023×10^{23} molecules of that substance; mole is thus a quantity like a dozen and one mole of hydrogen weighs 21 g and 1 mole of water weighs 18 g.

where Kw is the ionic product of water and is estimated as $=1.0 \times 10^{-14}$ at 25° C.

Since the concentration of both H^+ and OH^- is exactly equal, the concentration of each ion in pure water will be

$[H^+] = [OH^-] = 1 \times 10^{-7}$ moles/litre

The pH scale is based on ionic product of water (Kw).

Taking logarithm of the quantities we get,

$-\log K_w = -\log [H^+] - \log [OH^-]$ .... Eq. 4.3

Since $-\log Kw$ at 25°C is 14, the Equation 4.3 may be simplified as $[H^+] + [OH^-] = 14$.

$-\log K_w = -\log [H^+] - \log [OH^-]$
$-\log 10^{-14} = -\log 10^{-7} - \log 10^{-7}$
$14 = 7+7$
$= 14.$

pH

Acidity or basicity of a solution can be calculated in terms of its hydrogen ion concentration. Concentration of hydrogen ions in a solution is generally represented in terms of pH. pH is defined as the negative logarithm of hydrogen ion concentration. The concept of pH is very useful in understanding the change in hydrogen ion concentration in and around the cellular environment.

Let us see how we can calculate pH from hydrogen ion concentration. For example, a hydrogen ion concentration of 10^{-7} moles/litre means

$pH = -\log [H^+]$
$= -\log [10^{-7}]$
$= 7.0.$

It is important to note that pH can vary only between 0 and 14, and 7.0 is the neutral pH. Table 4.2 gives the pH scale along with the pH of some common substances. You will learn about the importance of pH in all biochemical reactions in further units of this course.

Table 4.2
The pH Scale

	H^+ Concentration (M)	pH	OH^- Concentration (M)	Example
	1.0	0	10^{-14}	1 M HCl
	10^{-1}	1	10^{-13}	Gastric juice
	10^{-2}	2	10^{-12}	Lemon juice
Acids	10^{-3}	3	10^{-11}	Vinegar
	10^{-4}	4	10^{-10}	Tomato juice
	10^{-5}	5	10^{-9}	Black coffee
	10^{-6}	6	10^{-8}	Rain water
Netural	10^{-7}	7	10^{-7}	Pure water
	10^{-8}	8	10^{-6}	Sea water
	10^{-9}	9	10^{-5}	Baking soda
	10^{-10}	10	10^{-4}	Alkaline desert ponds
Bases	10^{-11}	11	10^{-3}	Liquid soap
	10^{-12}	12	10^{-2}	Household ammonia (or Lime water)
	10^{-13}	13	10^{-1}	
	10^{-14}	14	1.0	1 M NaOH

SAQ 1

Calculate the pH of an aqueous solution in which 0.5 cm^3 (ml) of dilute hydrochloric acid has been added to 1 dm^3 of pure water. Assume that this dilute hydrochloric acid has the concentration of 2 moles/dm^3 and is completely ionised in solution.

.....

.....

.....

.....

.....

4.2.2 Basic Organic Molecules

In a living system, a large number of organic molecules are present. Diversity of organic molecules is due to the property of the carbon atom which in association with itself and with other atoms forms an unlimited number of compounds with different properties. Four major classes of organic compounds found in biological systems are: carbohydrates, proteins, nucleic acids and lipids. In this unit you will study about the monomers of these compounds in detail.

Monomers of Carbohydrates: Before you study at length about monomers of carbohydrates, you would like to know what carbohydrates are. Carbohydrates form the most abundant class of organic compounds. They are the compounds containing carbon, hydrogen and oxygen. The hydrogen and oxygen in these compounds are generally present in the ratio of two to one atom as in water.

Monomer: The basic subunit from which, by repetition polymers are made. For example, amino acids are the monomers of proteins, glucose is the monomer of glycogen.

Carbohydrates can be divided into classes based on the size of the molecules. **Monosaccharides**, the basic units of carbohydrates are simple sugars having the general formula $C_nH_{2n}O_n$ where n can vary from three to eight. Depending on the number of carbon atoms, they are called as trioses (C_3), tetroses (C_4), pentoses (C_5), hexoses (C_6) and so on. **Disaccharides** consist of two monosaccharide units joined together. **Oligosaccharides** include the sugars with up to 10 monosaccharide units. **Polysaccharides** are also made of monosaccharides joined together but may contain thousands of units.

$C_nH_{2n}O_n$ means that any monosaccharide molecule contains equal numbers of O and C atoms and twice as many H atoms.

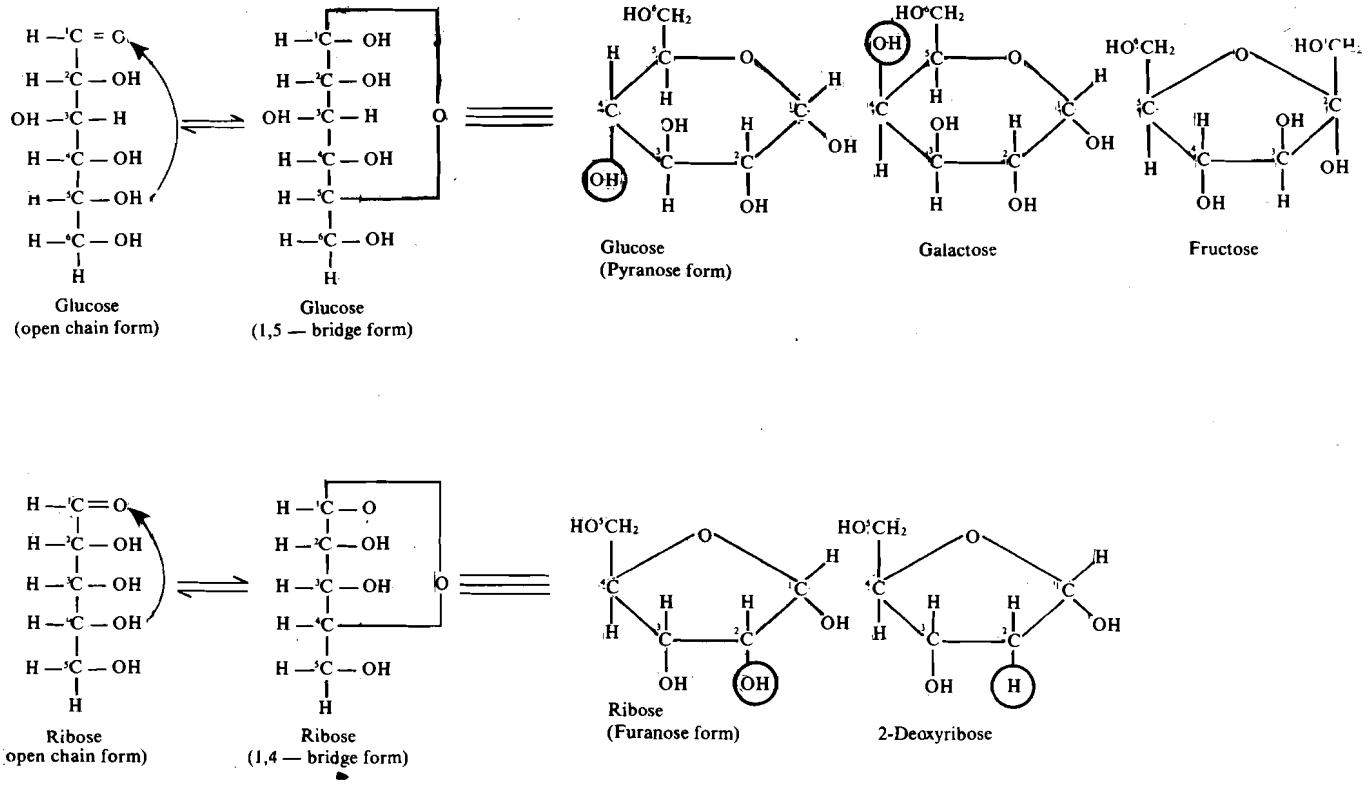


Fig. 4.1 : Structure of some monosaccharides. Monosaccharides in solutions exist in equilibrium as either an open form or a ring form. The ring form is predominant and it explains many chemical properties which are not explained by the open structure. Among hexose sugars glucose and galactose are aldoses and fructose is a ketose sugar. Pentoses are also aldoses. Fructose occurs in a furanose (4 carbon atom ring) form in sucrose but in pyranose form in free solution.

Aldoses are the monosaccharides which have aldehyde group in the 1 position — Glucose and galactose are aldoses. Ketoses are monosaccharides which have a ketose group in the 2 position. Fructose is a ketose.

Pyranose is six membered ring whereas furanose is a five membered ring.

Glucose, fructose, galactose, ribose and deoxyribose are examples of monosaccharides (Fig. 4.1). Sucrose, lactose and maltose are examples of disaccharides. The table sugar or sucrose is composed of glucose and fructose, whereas milk sugar or lactose is composed of glucose and galactose. It is interesting to note that the milk sugar lactose can be digested by the

enzymes produced in the intestine of children but in several groups of adults this enzyme is missing. The deficiency of enzyme causes intolerance of lactose and hence of milk. It is found that the percentage of lactose intolerance is more in adult populations which have not used milk and dairy products in childhood than those who have used them. Starch, cellulose and glycogen (all composed of glucose) are examples of polysaccharides about which you will study more in Unit 5.

Monomers of Proteins: Proteins are complex, diverse and high molecular weight polymers, found in the cell. The monomeric units of proteins are amino acids.

Amino acids join each other in a specific manner to form an unbranched, linear polymer known as polypeptide. Thousands of proteins that are present in a body are made-up of only 20 different naturally occurring amino acids (Figure 4.2). Amino acids are important not only in the synthesis of proteins but also in general metabolism.

In each amino acid, the alpha carbon atom is attached to a carboxyl group, an amino group and a side chain. Presence of both a positively and a negatively charged group in the same molecule of an amino acid is known as a " zwitterion" (German: Zwitter= hybrid, both). The variation in the different amino acids is due to presence of side chain (R) attached to the alpha carbon atom. On the basis of the side chains, i.e., polar, non-polar, uncharged polar, positively or negatively charged polar groups, amino acids are classified into four groups (Fig. 4.2).

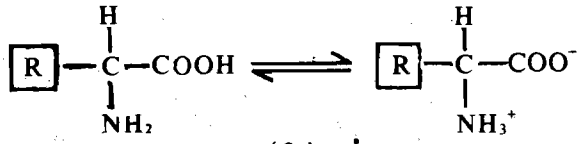
Monomers of Nucleic Acids

The nucleic acids are large complex polymers like proteins. There are two types of nucleic acids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) found within the cell. They are made-up of four types of nucleotides. Each nucleotide is made of a sugar, a phosphate and a nitrogenous base (Table 4.3). There are only 2 kinds of sugars in nucleotides: ribose and 2-deoxyribose (Fig. 4.3). There are five types of nitrogenous bases: cytosine, thymine, uracil, adenine and guanine. The nucleotides are joined together by a specific linkage ($3' \rightarrow 5'$ phosphodiester linkage) to form a linear, unbranched chain of polynucleotides. Table 4.3 gives the components and names of some nucleic acids.

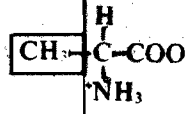
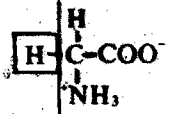
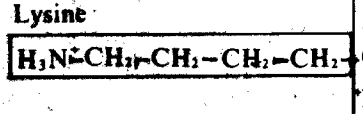
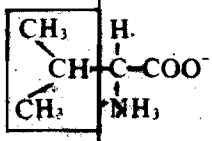
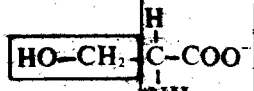
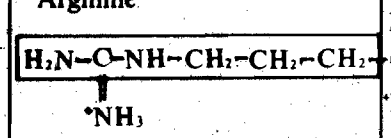
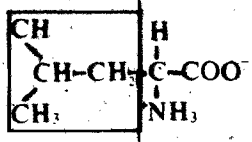
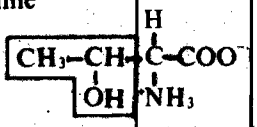
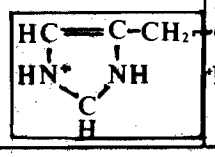
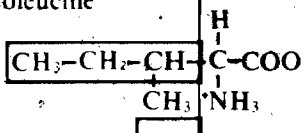
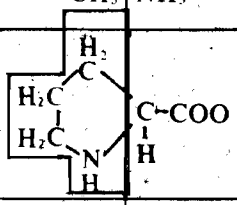
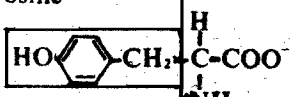
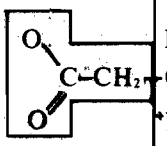
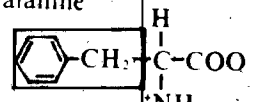
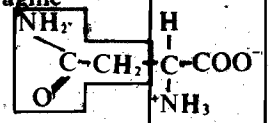
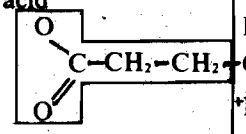
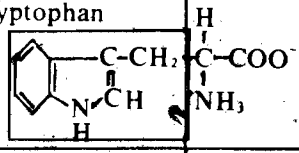
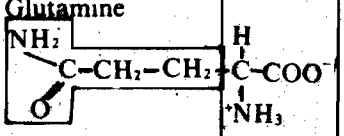
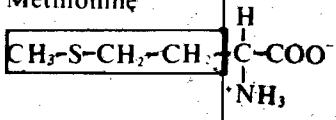
Table 4.3
Nomenclature of nucleosides and nucleotides
Base + Sugar = Nucleoside
Nucleoside + Phosphate = Nucleotide

Base	Nucleoside	Nucleotide
	Purines (Pu)	
Adenine (A)	Adenosine (rA)	Adenylic acid or adenosine monophosphate (AMP)
	Deoxyadenosine (dA)	Deoxyadenylic acid or deoxyadenosine monophosphate (dAMP)
Guanine (G)	Guanosine (rG)	Guanylic acid or guanosine monophosphate (dGMP)
	Deoxyguanosine (dG)	Deoxyguanylic acid or deoxyguanosine monophosphate (dGMP)
	Pyrimidines (Py)	
Cytosine (c)	Cytidine (rC)	Cytidylic acid or cytidine monophosphate (CMP)
	Deoxycytidine (dC)	Deoxycytidylic acid or deoxycytidine monophosphate (dCMP)
Thymine (T)	Thymidine (dT)	Thymidylic acid or thymidine monophosphate (TMP)
Uracil (U)	Uridine (rU)	Uridylic acid or uridine monophosphate (UMP)

- *Note that the names of purine nucleosides end in—osine and the names of pyrimidine nucleosides end n –idine.*
- *Each nucleotide has two names.*
- *'r' stands for ribose and 'd' stands for deoxyribose.*



(a)

Nonpolar R group		Uncharged polar R group		Positively charged polar R group (Basic)	
Alanine		Glycine		Lysine	
Valine		Serine		Arginine	
Leucine		Threonine		Histidine	
Isolcucine		Cysteine			
				Negatively charged polar R group (Acidic)	
Proline		Tyrosine		Aspartic acid	
Phenylalanine		Asparagine		Glutamic acid	
Tryptophan		Glutamine		at pH 6.0—7.0	
Methionine					

(b)

Fig. 4.2 : a) Structure of a typical amino acid. The central or alpha carbon atom of each amino acid is covalently bonded to four groups i.e., hydrogen atom, an amino group, an acid (or carboxyl) group and a side chain called an R group.
b) Structure of twenty amino acids.

Monomers of Lipids

Lipids are heterogenous groups of compounds usually formed by a combination of a fatty acid with glycerol. Fatty acids are the characteristic components of lipids present in both animal and vegetable fats such as ghee, coconut oil and mustard oil. Fatty acids are long linear chain organic compounds containing 4-24 carbon atoms. Nearly all fatty acids in

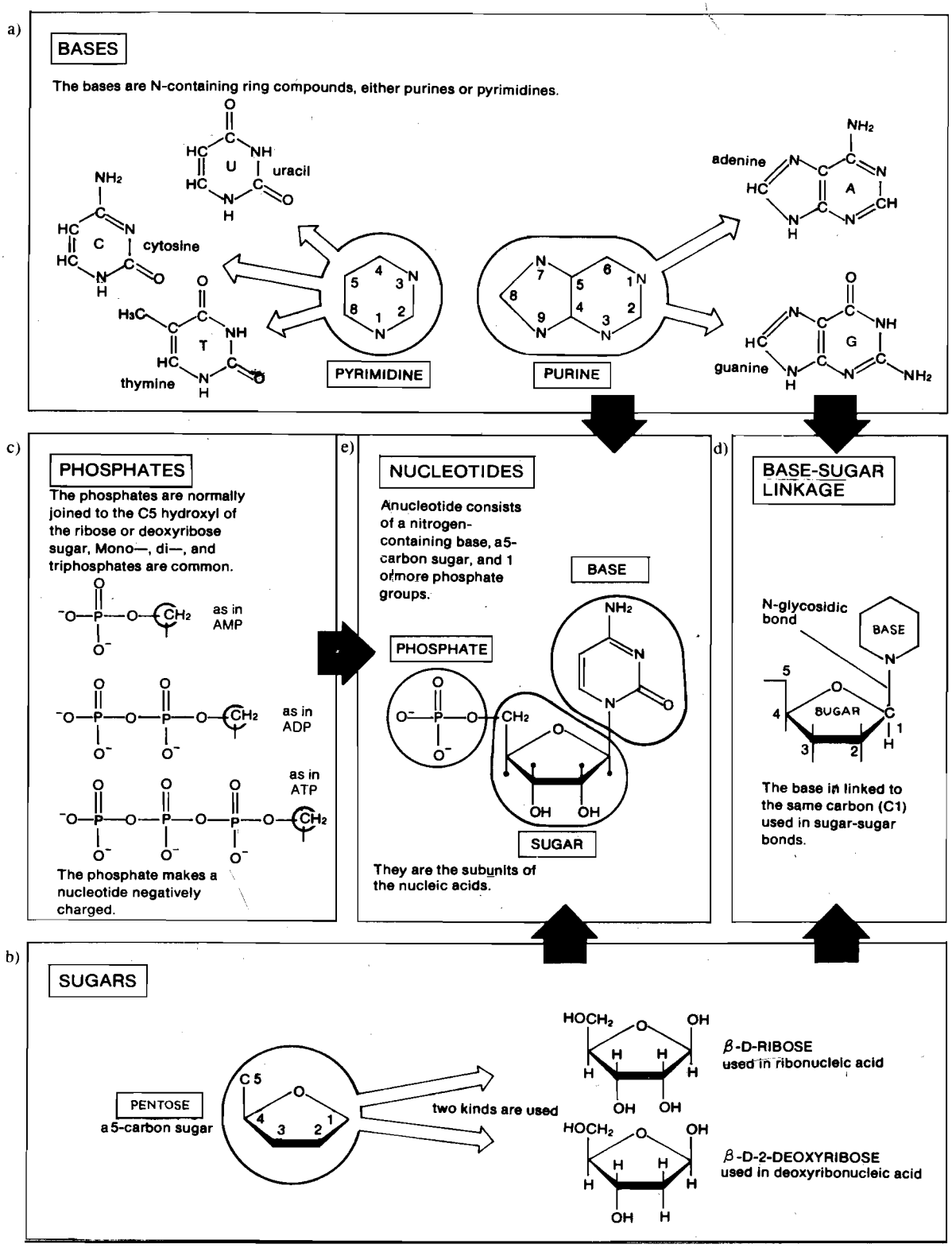


Fig. 4.3 : Components of nucleic acids.

a) Bases. The parent compounds of the two classes of nitrogenous bases are the heterocyclic compounds, pyrimidine and purine. Pyrimidine is a 6-membered ring with nitrogen atoms at 1 and 3 position and purine is a fused ring system of nine atoms with nitrogen atoms at 1,3,7 and 9 positions. Cytosine, thymine and uracil are three pyrimidine bases. Adenine and guanine are two purine bases.

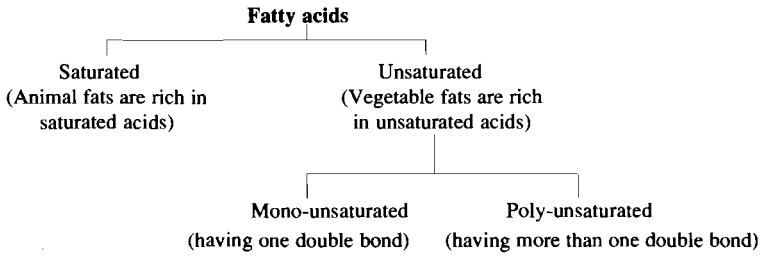
b) Sugars. Two types of pentose sugars are present in nucleic acids, D-ribose in ribonucleic acid (RNA) and 2- deoxy - D-ribose in deoxyribonucleic acid (DNA). Both sugars occur in their furanose forms in nucleotide. Note the position of H and OH at carbon atom 2 in ribose and deoxyribose sugars.

c) Phosphates. Phosphate group is attached to the hydroxyl group of carbon atom 5 of pentose sugar in a single nucleotide. Presence of this phosphate group makes a nucleotide negatively charged.

d) Nucleoside. The pentose is joined to the base by –N- glycosyl bond which is formed between carbon atom 1 of the pentose and nitrogen atom 1 of pyrimidine or nitrogen atom 9 of purine.

e) Nucleotide. Esterification of phosphate group with carbon atom 5 of the pentose in a nucleoside gives rise to a nucleotide, the subunit of nucleic acids.

nature have an even number of carbon atoms, but those with 16 and 18 carbon atoms are most abundant in oil. Fatty acids have a single carboxyl group and a long, non-polar hydrocarbon chain. The presence of non-polar chain makes lipids insoluble in polar solvents such as water. The long hydrocarbon chain may be fully saturated with carbon atoms joined by single covalent bonds or it may be unsaturated with double bonds between carbon atoms. The classification of fatty acids is shown below.



Apart from mono and poly-unsaturated fatty acids, a third class of unsaturated fatty acids is **prostaglandins** (PG). These were first discovered in seminal plasma and are physiologically very important. Cyclisation of central carbon chain of poly-unsaturated fatty acids forms a cyclopentane ring at one end of the molecule (Fig 4.4)

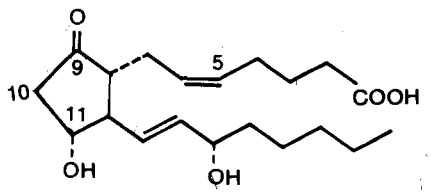


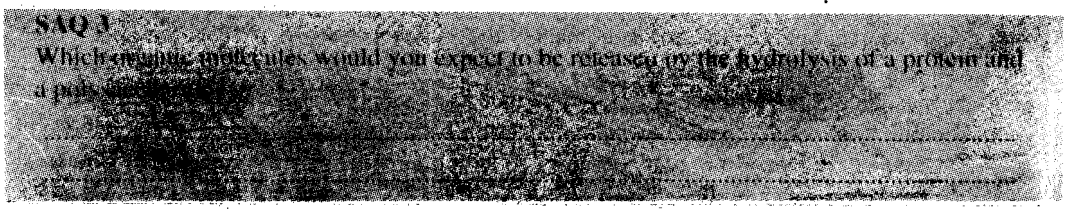
Fig. 4.4 : Prostaglandin E_1 (PGE_1). Variations in the substituent groups attached to the rings give rise to different types in each series of prostaglandins designated as A,B, etc. For example, PGE_1 has a keto group in position 9.

Table 4.4 gives the source, structural formulae and names of some naturally occurring fatty acids.

Table 4.4
Some naturally occurring fatty acids
Saturated fatty acids ($C_nH_{2n+1}COOH$)

Number of carbon atoms in chain	Formula	Common name	Source
12	CH_3- $(CH_2)_{10}$- COOH	Lauric acid	Cinnamon, coconut oil
14	CH_3-$(CH_2)_{12}$ - COOH	Myristic acid	Palm kernel, coconut oil
16	CH_3-$(CH_2)_{14}$ - COOH	Palmitic acid	Common in all animal and plant fats
18	CH_3-$(CH_2)_{16}$ - COOH	Stearic acid	and most abundant in animal fats
	Unsaturated fatty acids		
No. of carbon atoms in chain	Formula	Common name	Source
18	CH_3-$(CH_2)_7$ - CH = CH$(CH_2)_7$ - COOH One double bond	Oleic acid	Found in nearly all fats
18	CH_3-$(CH_2)_4$ - CH = CH-CH_2-CH= CH-$(CH_2)_7$ - COOH Two double bonds	Linoleic acid*	Seed oils like peanut, soyabean
18	CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH Three double bonds	Linolenic acid*	Found frequently with linoleic acid but particularly in linseed oil
20	CH_3-$(CH_2)_4$-CH=CH-CH_2-CH = CH-CH_2-CH = CH-CH_2-CH = CH$(CH_2)_3$ -COOH Four double bonds	Arachidonic acid*	Particularly in peanut oil

- These are called essential fatty acids, because they cannot be produced in the body in adequate amount and hence they must be provided in diet.



4.3 ISOLATION AND DETECTION OF MACROMOLECULES

Properties of any macromolecule can be studied easily if it is in a pure form. For this we need to extract the macromolecules from cells. This is a difficult process because macromolecules occur in a mixture with other cytoplasmic extraneous matter and are susceptible to destruction and damage during extraction procedure. Therefore, to keep the macromolecules intact, the extraction should be done carefully. Purification requires constant monitoring, to ensure that after every purification step the concentration of desired macromolecule increases and that of extraneous matter decreases.

4.3.1 Monitoring the Purification Procedure

There are several methods to ensure recovery of macromolecules during purification.

UV Absorption : Measurement of **UV absorption** with a spectrophotometer is useful in monitoring the purification of nucleic acids or proteins. Nucleic acids have the maximum absorbance at 260 nm (wavelength) and the proteins at 280 nm. A higher absorbance at a particular wavelength indicates a larger quantity of the specific macromolecule.

Catalytic activity is defined as the conversion of 1 micro mole substrate into product in 1 minute under known conditions.

Catalytic Activity: It can be measured to monitor the purification of enzymes. For example, the enzyme arginase, which synthesises urea is monitored in this way. After each purification step, a sample is taken and treated as follows.

$$\text{Arginine} + \text{water} \xrightarrow{\text{Arginase}} \text{urea} + \text{ornithine}$$

$$\text{Urea} + \text{colour reagent (Thiosemicarbozide + Diacetyl mono oxim)} + \text{acid reagent } (FeCl_3.H_3PO_4 + H_2SO_4) \longrightarrow \text{Red coloured complex}$$

The colour intensity is estimated at 520 nm in a colorimeter. The amount of urea formed indicates the amount of arginase present.

Ligand Binding : It is a useful way to monitor macromolecules that have specific binding sites for other molecules. Haemoglobin and starch purification can be measured in this manner.

Molecule + ligand ——Molecule-ligand complex — Quantity of complex is measured

For example,

Haemoglobin + O_2 ——Oxyhaemoglobin (Red Colour) ——Red colour intensity measured by colorimeter at 578 nm

Starch + iodine ——Starch amylose-iodine complex (Blue colour) —— Blue colour intensity measured by colorimeter at 600 nm

Radioactive isotopes: Isotopes with an unstable nucleus that emits ionising radiation, important as tracers in biology.

Isotope Tracers: They can also be used to assay the purification of macromolecules. This technique is more suited for monitoring a newly synthesised macromolecule.

First an appropriate precursor of the molecule with a radioactive isotope is incorporated in the macromolecule while it is being synthesised; for example, ^{3}H-thymidine for DNA and ^{135}I for thyroxine. The macromolecule, thus, becomes radioactive and can be traced due to the incorporated isotope. Finally, the level of radioactivity may be determined in the extract with the help of instruments such as Gieger-Muller counter or Scintillation counter.

4.3.2 Specific Activity

Specific activity measures the purity of the macromolecule in a mixture. Specific activity is defined as the **activity per unit mass**. Let us take an example to illustrate the concept. If a cell extract is estimated to contain 10 'units' of enzyme arginase and 50 mg of protein then the specific activity of arginase is 10 units/50 mg or 0.2 units/mg protein. After a purification step, the extract contains 5 'units' of the enzyme and 10 mg of protein, and therefore, the specific activity of enzyme is 5 units/ 10 mg or 0.5 units/mg protein.

In this example we find that during the process of purification 5 units of enzyme activity and

40 mg of protein are lost but specific activity has increased from 0.2 units/mg protein to 0.5 units/mg protein. This clearly indicates that a higher proportion of contaminant (non-arginase) proteins are removed.

4.4 METHODS OF SEPARATION

Macromolecules can be separated from a mixed molecular population based on the differences in their size, solubility, electric charge and specific binding affinity for other molecules using a variety of techniques.

4.4.1 Separation Based on Molecular Size

Several methods are used to separate macromolecules depending on their size. Proteins can be separated from small molecules and ions by **dialysis** through a semipermeable membrane. Depending upon the pore size (different membranes have different pore sizes), molecules of certain size can pass through a membrane. When the osmotic pressure responsible for passage of molecules through a semipermeable membrane is not sufficient, mechanical pressure is applied to force the molecules through. This process is known as **ultrafiltration**.

Centrifugation

It is a technique used for separation of particles like cellular organelles and macromolecules by rotation in a centrifuge. Separation of macromolecules is achieved by centrifugation at a very high speed, i.e. ultracentrifugation at 80,000 revolution per minute. A mixture containing particles and macromolecules is applied on the top of a salt solution in a

Supernatant: (floating above). The liquid above solid matter in the bottom of a tube, beaker etc.

Density gradient: A solution of different density gradation usually used in centrifugation to separate different kinds of cells, particles, or macromolecules.

Svedberg Unit: The unit of sedimentation equal to 10^{-13} seconds. The number of S units of a molecule or particle in a given centrifugal field is related to the weight, shape and density of the molecule or particle.

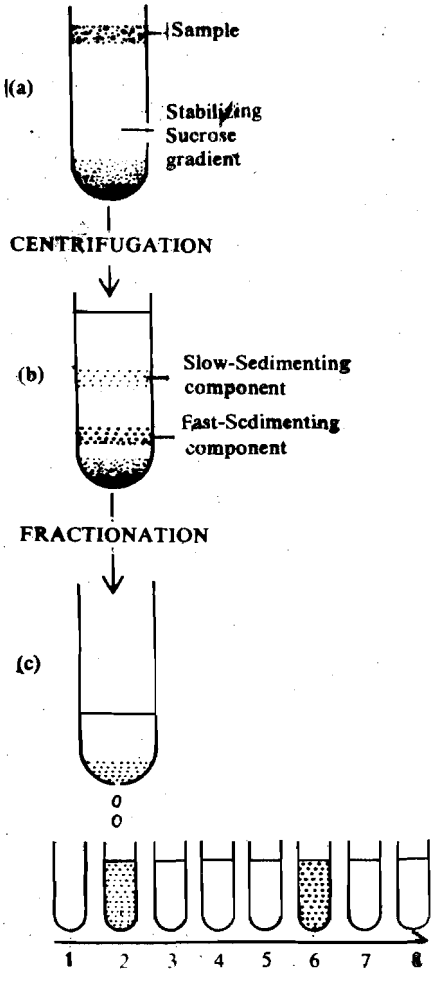


Fig. 4.5 : The fractionation of proteins by ultracentrifugation on sucrose density gradient.
a) The sample has been layered on top of the centrifuge tube containing the continuous gradient of sucrose, which increases in concentration towards the bottom.
b) The sample has been centrifuged and separated into two components which sediment at different rate.
c) A hole has been made at the bottom of the plastic centrifuge tube to collect different fractions drop by drop. Figure shows that the fraction 1 (Tube 3) is already collected and fraction 2 (Tube 7) is being collected. In other tubes only buffer solution without any protein fraction has been collected.

centrifuge tube and is subjected to centrifugation. The density gradient in the solution is created by adding the concentrated and highly soluble materials, for example, sucrose for protein and ceasium chloride for nucleic acids. This gradient helps in the formation and stabilisation of distinct bands of different molecular fractions which sediment at different rates. These bands can be individually collected (Fig. 4.5). The sedimentation rate depends on the size, shape and molecular weight of the components and is called as sedimentation coefficient. It is represented by Svedberg unit or S value.

Column Chromatography

This chromatographic method is used for separating molecules on the basis of size differences (i.e. molecular weight). This technique is also popularly known as **molecular exclusion technique or gel filtration.** In this technique, a sample containing a mixture of molecules is loaded over a sieve like column packed with porous beads of specific size. The flow of molecule is retarded due to weak interactions such as van der Waals forces and hydrogen bonding with the medium. The Sephadex is an inert stable and reusable highly hydrated polysaccharide in the form of beads. Through this column, molecules move according to their molecular weight. Larger molecules are excluded first as they do not enter the beads of the gel and are localised in the solution in between the beads. Small molecules are trapped in the beads, hence they are eluted much later (Fig. 4.6).

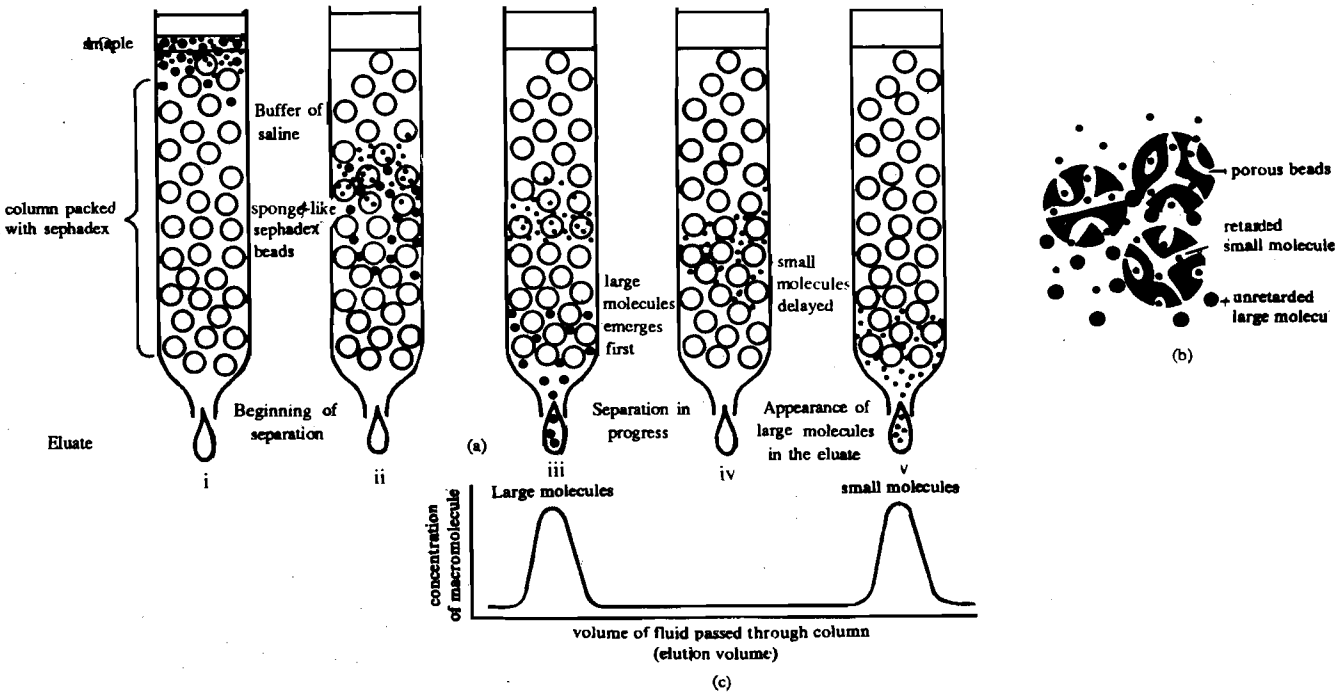


Fig. 4.6 : a) Column chromatography by molecular exclusion technique using sephadex column. (i) Glass column packed with gel particles and overlaid with sample containing molecules of various sizes (small an large dots). (ii-iii) Molecules with molecular weights in excess of the gel exclusion limit percolate between the gel particles, whereas smaller molecules may enter the gel phase. (iv-v) As a result, molecules reach th bottom of the column in order of decreasing size.
b) Schematic drawing of column chromatography.
c) The elution pattern' as it is read on a UV spectrophotometer indicating the presence of proteins in various fractions.

SAQ 4

In the following table molecular weights of some proteins are given. Observe the data and number the sequence (like 1,2,3 etc.) in which the following proteins would be eluted from Sephadex gel column. Sephadex G-200 is the generally used gel for this range of proteins. (**Hint:** Heavy molecular weight compounds are eluted first.)

Number	Protein	Mol weight
	Cytochrome C	13,370
	Aldolase	149,100
	Haemoglobin	64,500
	Myosin	524,800
	Catalase	221,600

SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis is a technique to separate the molecules on the basis of their total charge. Such molecules when placed between two electrodes in polyacrylamide gel medium migrate to the oppositely charged electrodes. For example, in SDS polyacrylamide gel electrophoresis, a protein molecule is treated with high concentration of SDS (Sodium Dodecyl Sulfate) which dissociates the polymer into negatively charged monomers by breaking the non-covalent bonds. The amount of SDS bound and hence the negative charge on each monomer is in proportion to the size of the monomer. Consequently, in an electric field a larger protein molecule moves faster than a small protein molecule towards the anode or the positively charged electrode (Fig. 4.7).

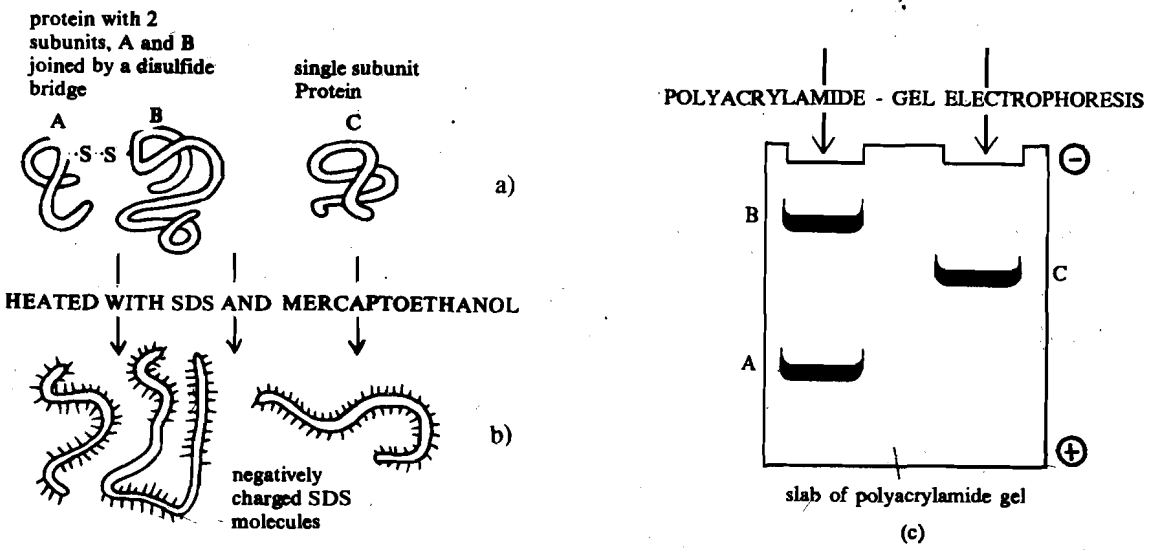


Fig. 4.7 : SDS-Polyacrylamide gel electrophoresis.
a) A polymeric protein consisting of four monomers is treated with SDS.
b) Each monomer is bound with SDS.
c) When allowed to move in an electrical field, the large monomers move faster towards the anode (positively charged electrode) as they have more SDS bound to them.

4.4.2 Separation Based on Solubility

Macromolecules can be separated and purified on the basis of their solubility which depends on pH, ionic strength, dielectric properties of the solvent and temperature.

As you have learnt in previous section, chromatography is a technique of molecular separation in which molecules migrate between a mobile (soluble) and a stationary (matrix) phase. The separation of molecules in a mixture depends upon their relative solubilities in the two phases and their binding strength with either of the phases.

Paper Chromatography is the commonly used technique to separate small molecules like sugars, amino acids, etc. A mixture of molecules is applied as a spot to a sheet of adsorbent paper, such as a filter paper. A solution containing a mixture of two solvents, such as water and alcohol is allowed to flow through the paper by capillary action. Different molecules move at different rates depending on their solubility in the solvent. The separated molecules can be seen on paper (Fig. 4.8).

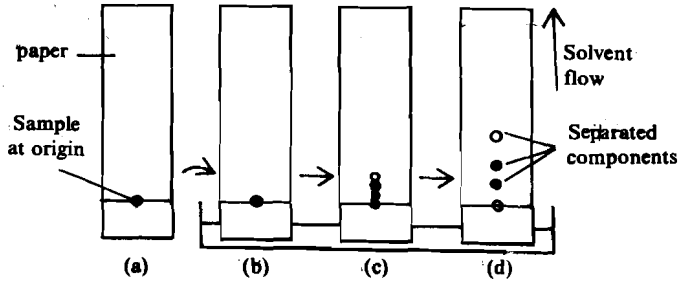


Fig. 4.8 : Paper chromatography. The sample is applied to a paper strip and allowed to dry (a). When the paper is dipped in the solvent (b), the solvent rises up due to capillary action (c). Different components move according to their relative solubility in the solvent (d).

Isoelectric Precipitation: For each protein molecule there is a pH at which it has no net electric charge and hence does not move in an electric field (Fig. 4.9). This is known as its isoelectric pH. At this pH, the protein molecule is least soluble and forms a sharp band, when a mixture of proteins in a fixed gradient is subjected to electric field. Different proteins have different isoelectric pH values.

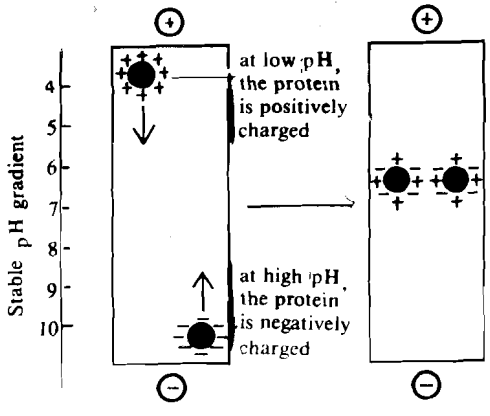


Fig. 4.9 : Principle of isoelectric precipitation and isoelectric focusing. At low pH, the protein is positively charged because excess H^+ ions uncharge the carboxyl group (COO^-) to form COOH and, therefore, the amino groups (NH_3^+) give the protein a net positive charge. Similarly at high pH, the protein acquires a negative charge due to uncharged NH_2 group and negatively charged carboxyl groups (COO^-). At its isoelectric point, the negative and positive charges are equal thus the protein has no net charge.

Salt Fractionation technique is based on the fact that in a solution different proteins precipitate at different concentrations of certain salts such as ammonium sulphate. Therefore, the concentration of ammonium sulphate in a solution containing protein is gradually increased to precipitate different proteins.

4.4.3 Separation Based on Charge

Electrophoresis is the process of separation of molecules whose net charge depends upon the pH of the surrounding medium. Biological molecules having a net charge, move in an electric field. A negatively charged molecule moves towards anode and the positively charged one towards cathode. This is known as electrophoresis. A mixture of macromolecules is applied to a supporting medium such as paper or polyacrylamide gel, and an electric current is passed through the medium. Molecules separate out after an appropriate time and are detected with the help of sepecific staining reagents (Fig. 4.10).

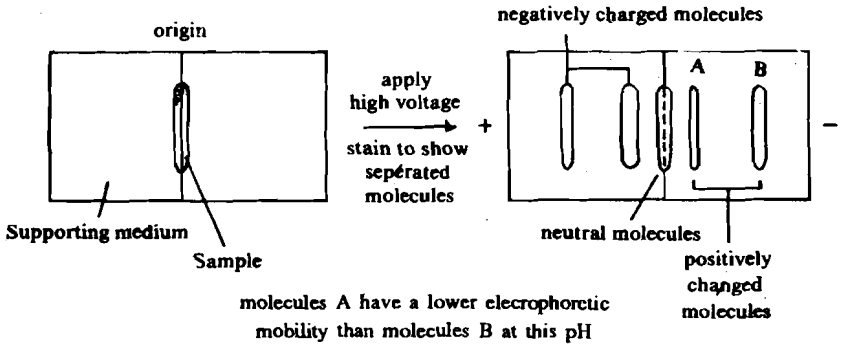


Fig. 4.10 : A sample of protein molecules is applied in the centre of the supporting medium like paper, cellulose acetate, polyacrylamide gel etc. On application of electric current, positively charged molecules move towards the cathode while negatively charged molecules move towards anode. Neutral molecules do not move at all.

Isoelectric focusing is another type of electrophoresis carried out in a pH gradient created in a column by mixing different buffers. A protein mixture is applied to a polyacrylamide gel having a pH gradient and subjected to an electric field. Each protein moves in the pH gradient till it arrives at its own isoelectric pH and stays on there forming a sharp band. The bands can be further separated, and the band containing a protein is dissolved in the appropriate buffer solution (Fig. 4.9).

4.4.4 Separation Based on Molecular Affinity

Based on molecular affinity, macromolecules can be separated by ion-exchange chromatography and affinity chromatography.

Ion-exchange Chromatography

Ion-exchange chromatography is based on the difference between the macromolecules with respect to their **size** and **electric charges at a given pH**. A chromatography column consists of a long column of synthetic resin comprising electrically charged molecules. Diethyl amino ethyl cellulose (DEAE-Cellulose) is positively charged (anion-exchanger) and carboxymethyl cellulose (CM-Cellulose or CMC) is a negatively charged (cation-exchanger) resin, at pH 7.0. When a protein mixture is passed through a DEAE column, the negatively charged molecules bind strongly and positively charged and uncharged molecules are eluted out (Fig. 4.11). Similarly by using CMC, positively charged protein molecules can be purified.

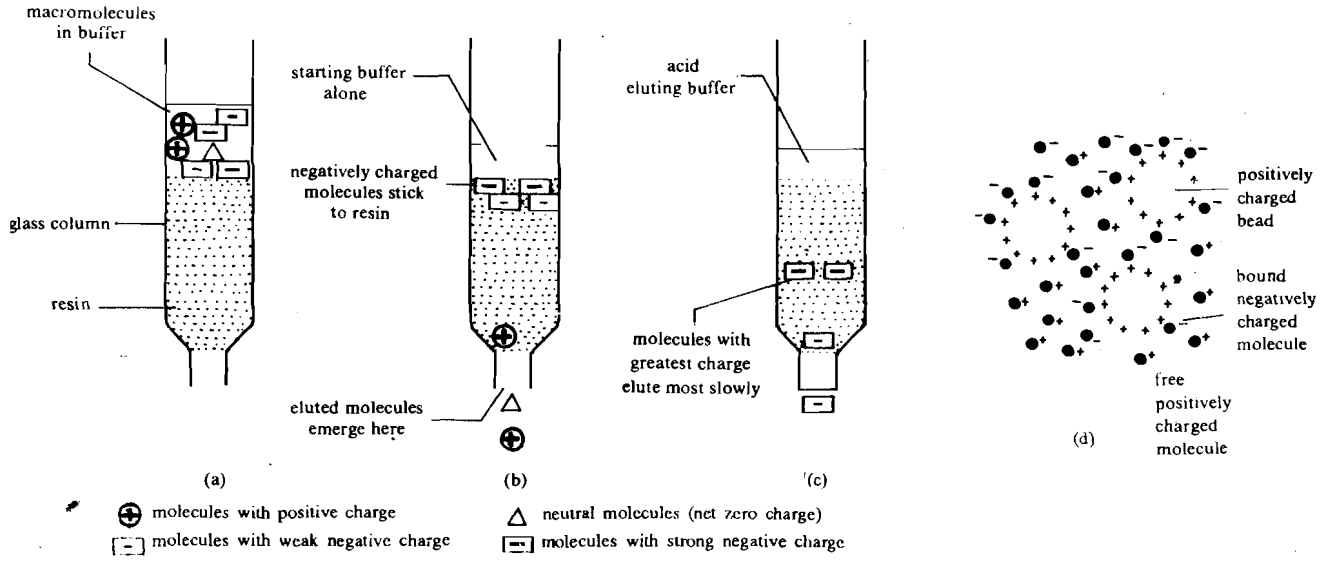


Fig. 4.11 : a) A sample is layered on a positively charged resin (resins are organic compounds prepared or synthesised by polymerisation of specific compounds such as cellulose).
b) Negatively charged molecules stick to the DEAE— cellulose resin and positively charged and neutral molecules are eluted out.
c) To get the negatively charged protein out of the column, acidic buffer (H^+) is added which binds to negatively charged molecules and helps in their elution from the column.
d) Principle of ion-exchange chromatography.

Affinity Chromatography

Affinity Chromatography is another powerful means of purifying proteins. This technique utilises the high affinity of many proteins for specific chemical groups. It is like ion

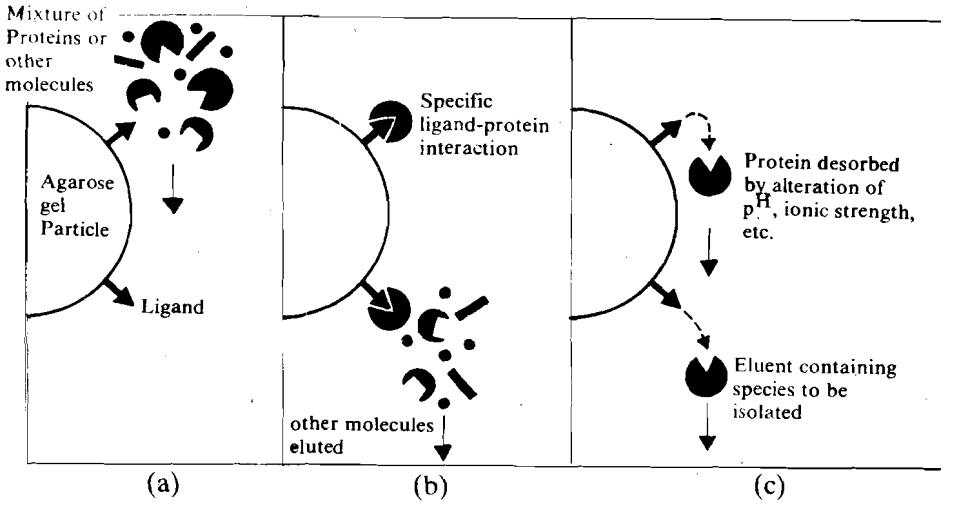


Fig. 4.12: Principle of affinity chromatography.

exchange chromatography but based on covalent bonding by appropriate chemical reaction between insoluble material (Polysaccharide—agarose beads) and specific ligand (such as an antibody molecule or a substrate of the enzyme). When a mixture of molecules is passed through such type of affinity column, those with high affinity for the ligand are retained whereas others move out. The molecules attached to the ligand are dissociated and eluted out by change in pH of the eluting solution (Fig. 4.12). Various biological molecules such as enzyme, receptor molecules are purified from their complex mixture by using this method.

SAQ 5

Protein A has a molecular weight 80,000 while protein B has 50,000. Protein A has a net charge –3 at pH 7 and 0 at pH 6. Protein B has net charge –3 both at pH 7 and pH 6. Which of the following methods may be used to separate these proteins? Give reasons for the preferred method.

a) Centrifugation
b) Ion-exchange chromatography
c) Paper chromatography
d) Affinity chromatography
e) Sephadex gel filtration

.....

.....

.....

.....

.....

4.5 SUMMARY

In this unit you have studied the

- Chemical composition of a cell in order to better understand the structure and function of the cell and its components.
- Ionisation property of water, the simplest and most abundant of cell molecules, and the method to calculate the ionic product of water (Kw) and hydrogen ion concentration (pH).
- Structure of the basic molecules found in a cell, i.e. monosaccharides, amino acids, nucleotides and simple lipids.
- Quantity of macromolecules in a sample can be estimated by various methods like UV absorption, catalytic activity, ligand binding and use of isotopic tracers.
- Macromolecules can be separated on the basis of their molecular size, solubility etc. by different techniques such as centrifugation, chromatography, electrophoresis and fractionation.

4.6 TERMINAL QUESTIONS

1) Ionisation of a base produces OH^- and not H^+ ions. Why then does addition of a base such as KOH change the pH of a solution?

2) Calculate the pH of an aqueous solution in which 1.5 cm^3 of calcium hydroxide $Ca(OH)_2$ has been added to 2 dm^3 of pure water. Calcium hydroxide has the concentration 3 moles/dm^3 and is completely ionised in solution.

3) What is the difference between a saturated and an unsaturated fatty acid?

4) A cell extract contains 40 mg of protein and 200 units of an enzyme. In one experiment, after gel filtration, a particular fraction contains 3 mg of proteins and 120 units of enzyme. In the second experiment after ion-exchange chromatography, one fraction contains 20 mg of protein and 180 units of enzyme, which of the two methods is to be preferred for purification ?

4.7 ANSWERS

Self-assessment Questions

1) i) Since HCl is completely ionised, the concentration of H^+ ions (in HCl) will be the same as the concentration of HCl, that is, 2 moles/dm^3.

   ii) Since 0.5 cm^3 of the HCl is added to 1 dm^3 (1000 cm^3) of water, the concentration of H^+ ions will become $[H^+] = 0.5/1000 \times 2$ mol/dm^3 = 10^{-3} mol/dm^3
   So pH = $-\log (10^{-3})$
   = 3

   iii) We have ignored the negligible contribution of 10^{-7} moles/dm^3 from ionisation of water.

2) b)

3) By hydrolysis of protein and polysaccharide, amino acids and monosaccharides are released respectively.

4) The sequence in which proteins would be eluted from a Sephadex gel column is:

Number	Protein	Mol weight
1	Myosin	5,24,800
2	Catalase	221,600
3	Aldolase	1,49,100
4	Haemoglobin	64,500
5	Cytochrome C	13,370

5) Method a), b) and e) can be used to separate these proteins. Centrifugation (a) can work because proteins have different molecular masses. Ion-exchange chromatography (b) can work at pH 6 when charges are different and not at pH 7 when the charges are same. Sephadex gel filtration (e) can work because proteins have different molecular weights. Sephadex gel filtration is preferred in such a case because other two methods are more time consuming, costly and less efficient.

   Paper chromatography (c) cannot work because in this problem nothing regarding solubility is given. Affinity chromatography (d) cannot work because affinity of protein for any ligand is not mentioned.

Terminal Questions

1) The OH^- ions produced by ionisation of a base react with free H^+ ions present in a solution. The decrease in the concentration of H^+ ions is seen as an increase in pH.

   $KOH \longrightarrow K^+ + OH^-$

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

2) $Ca(OH)_2 \rightleftharpoons Ca^{2+} + 2OH^-$
   This equation implies that 1 mol of $Ca(OH)_2$ ionises to give 2 mol of OH^-. So $[OH^-]$ from 1 dm^3 of 3 mol of Ca $(OH)_2$ is 6 mol.
   Since 1.5 cm^3 of $Ca(OH_2)$ has been added to 2 dm^3 of pure water, the concentration of OH will become
   $[OH^-] = 1.5/2000 \times 6 = 4.5 \times 10^{-3}$ mol
   p OH = $-\log (4.5 \times 10^{-3})$
   $= 3 - \log 4.5$
   $= 3 - 0.6532$
   $= 2.34$
   pH $= 14 - pOH$
   $= 14 - 2.34$
   $= 11.66$

3) In saturated fatty acids carbon atoms are joined by single covalent bonds. In unsaturated fatty acids at least one double bond is present between the two carbon atoms.

4) Since specific activity measures the purity of an enzyme, we must calculate the specific activity. Specific activity in cell extract (before purification)
   = 200 units/40 mg
   = 5 units mg^{-1}

Specific activity after gel filtration
= 120 units/3 mg
= 40 units mg^{-1}
Specific activity after ion-exchange chromatography
= 180 units/20mg
= 9 units mg^{-1}

We see that specific activity has increased 8 times in case of gel filtration and about 2 times after ion-exchange chromatography. Hence gel filtration is the preferred method although the absolute enzyme activity recovered after ion-exchange chromatography is more than that recovered after gel filtration.