PREFACE

UGC (Open and Distance Learning Programmes and Online Progra With its grounding in the "guiding pillars of Access, Equity, Equality, Affordability and Accountability," the New Education Policy (NEP 2020) envisions flexible curricular structures and creative combinations for studies across disciplines. Accordingly, the UGC has revised the CBCS with a new Curriculum and Credit Framework for Undergraduate Programmes (CCFUP) to further empower the flexible choice based credit system with a multidisciplinary approach and multiple/ lateral entry-exit options. It is held that this entire exercise shall leverage the potential of higher education in three-fold ways – learner's personal enlightenment; her/his constructive public engagement; productive social contribution. Cumulatively therefore, all academic endeavours taken up under the NEP 2020 framework are aimed at synergising individual attainments towards the enhancement of our national goals.

In this epochal moment of a paradigmatic transformation in the higher education scenario, the role of an Open University is crucial, not just in terms of improving the Gross Enrolment Ratio (GER) but also in upholding the qualitative parameters. It is time to acknowledge that the implementation of the National Higher Education Qualifications Framework (NHEQF), National Credit Framework (NCrF) and its syncing with the National Skills Qualification Framework (NSQF) are best optimised in the arena of Open and Distance Learning that is truly seamless in its horizons. As one of the largest Open Universities in Eastern India that has been accredited with 'A' grade by NAAC in 2021, has ranked second among Open Universities in the NIRF in 2024, and attained the much required UGC 12B status, Netaji Subhas Open University is committed to both quantity and quality in its mission to spread higher education. It was therefore imperative upon us to embrace NEP 2020, bring in dynamic revisions to our Undergraduate syllabi, and formulate these Self Learning Materials anew. Our new offering is synchronised with the CCFUP in integrating domain specific knowledge with multidisciplinary fields, honing of skills that are relevant to each domain, enhancement of abilities, and of course deep-diving into Indian Knowledge Systems.

Self Learning Materials (SLM's) are the mainstay of Student Support Services (SSS) of an Open University. It is with a futuristic thought that we now offer our learners the choice of print or e-slm's. From our mandate of offering quality higher education in the mother tongue, and from the logistic viewpoint of balancing scholastic needs, we strive to bring out learning materials in Bengali and English. All our faculty members are constantly engaged in this academic exercise that combines subject specific academic research with educational pedagogy. We are privileged in that the expertise of academics across institutions on a national level also comes together to augment our own faculty strength in developing these learning materials. We look forward to proactive feedback from all stakeholders whose participatory zeal in the teaching-learning process based on these study materials will enable us to only get better. On the whole it has been a very challenging task, and I congratulate everyone in the preparation of these SLM's.

I wish the venture all success.

Professor Indrajit Lahiri
Vice Chancellor

NETAJI SUBHAS OPEN UNIVERSITY

Four Year Undergraduate Degree Programme

Under National Higher Education Qualifications Framework (NHEQF) & Curriculum and Credit Framework for Undergraduate Programmes
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Course Title: Analytical Clinical Biochemistry
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Unit 1 Basics of carbohydrates

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1.1 Objectives:

After completion of this unit one can understand the followings

1. Proper definition of Carbohydrate.
2. Primary Functions of Carbohydrate.
3. Biological Roles of Carbohydrate.
4. Drawing proper Howarth Projection of Various Carbohydrates.

5. Alcoholic and Lactic Acid Fermentation.

1.2 Introduction:

Carbohydrates are the most abundant biomolecules in nature. Carbohydrates are very useful for human beings. Many of us eat the meat of the animals they are fed, or they are consumed directly as food like bread, rice, and potatoes. Cellulose makes up a considerable portion of both wood and the cotton that we wear. The primary component of paper, whose significance in contemporary society is hard to gauge, is carbohydrates. In nature, green plants use a process known as photosynthesis to create carbohydrates. The green pigment chlorophyll, which is found in plant leaves, catalyses the process that turns carbon dioxide and water into glucose. Sunlight from the sun provides the energy required to accomplish this conversion. We simply cover the fundamental concepts of carbohydrates in this unit.

1.3 Definition:

1.3.1. Classical Definition:

The name carbohydrates have been assigned to all the above type of substances. The general formula for most of them could be written as C_n (H_2 O) $_n$, and thus they may be regarded as hydrates of carbon.

Limitation of this classical definition:

(i) This definition was not found to be correct because many sugars such as rhamnose ($C_6H_{12}O_5$) and fucose ($C_6H_{12}O_5$) subsequently became known. These sugars do not have the hydrogen and oxygen atoms in 2 to 1 ratio as is found in water and hence cannot be called hydrates of carbon.

(ii) Many compounds of carbon, hydrogen and oxygen (e.g. HCHO, CH_3COOH) have the last two elements in the same ratio as in water, but they are not carbohydrates.

1.3.2 Modern Definition:

A more accurate description subsequently emerged according to which carbohydrates are regarded as polyhydroxy aldehydes or polyhydroxy ketones or substances that can be hydrolysed to either of them. It may, however, be noted that the aldehydic or ketonic groups in carbohydrates do not occur as free aldehyde or keto groups but usually exist in the form of hemiacetal or hemiketal groups, respectively.

1.4 Classification in Details:

The names of most of the simple carbohydrates end in –'ose'; those with an aldehyde group are known as aldoses, and those with a keto group, ketoses. Carbohydrates, which are also called saccharides, have been classified into three major groups.

a) Monosaccharides: Monosaccharides are the simplest carbohydrates and include three, four-, five-, and six carbon sugars. They cannot be hydrolysed further to smaller units. They are further classified according to the number of carbon atoms and the nature of the carbonyl group present. Monosaccharides having three-, four-, five-, and six-carbon atoms are known as trioses, tetroses, pentoses and hexoses, respectively. It, therefore, follows that in order to classify a monosaccharide, we must know the type of carbonyl group and the number of carbon atoms. Glucose, for instance, is an aldohexose as it has an aldehyde group and six carbon atoms. Fructose, on the other hand, is a ketohexose, i.e., it contains a keto group and a chain of six carbon atoms. Monosaccharides are colourless, water-soluble compounds sweet in taste and cannot be hydrolysed to smaller molecules. They occur either in the free or combined states and contain 3 to 8 carbon atoms. These are of two types:

(i) **Aldoses** are monosaccharides which contain an aldehyde group (-CHO).

(ii) **Ketoses** are monosaccharides which contain a keto group (>C=O).

b) Oligosaccharides (Greek: Oligos = few): The hydrolysis of these carbohydrates yields two or more monosaccharide molecules. They have further been subdivided into the following sub groups:

(i) Disaccharides: The disaccharides are hydrolysed to give two molecules of monosaccharides, e.g., sucrose which produces glucose and fructose on hydrolysis.

(ii) Trisaccharide: Three molecules of monosaccharides are obtained by the hydrolysis of a trisaccharide, e.g., raffinose which produces glucose, fructose and galactose on react with water.

3. Polysaccharides: Polysaccharides are high-molecular weight carbohydrates having general formula (CHO), where n =100-3000, i.e., they contain several monosaccharide units. In contrast to monosaccharides (and disaccharides), which are water soluble and sweet in taste (and hence commonly referred to as sugars), the polysaccharides are tasteless, water-insoluble substances. Hydrolysis of a polysaccharide yields many molecules of monosaccharides, e.g., starch gives many molecules of glucose.

Alternatively, they are classified as follows:

(a) Sugars and non-sugars: The carbohydrates may be broadly divided into sugars and non-sugars. All the monosaccharides and oligosaccharides, such as glucose, fructose and cane sugar are crystalline, water-soluble solids which are sweet in taste. These are called sugars. Non-sugars on the other hand are polysaccharides which include starch, cellulose, etc., and are amorphous, insoluble in water and tasteless substances.

(b) Reducing and non-reducing sugars (or carbohydrates): It would be worthwhile to point out here that all monosaccharides and nearly all disaccharides (a prominent exception being sucrose) reduce Fehling solution and Tollen's reagent and are, therefore, called as reducing sugars (or carbohydrates) while others which do not reduce these reagents are called non-reducing sugars (or carbohydrates).

1.5 Primary Functions of Carbohydrates:

(i) Energy production: Carbohydrates are the main sources of energy for all organisms. Many cells prefer glucose as a source of energy versus other compounds like fatty acids. Cells convert glucose into carbon dioxide and water and the energy is transferred to ATP in a process known as cellular respiration. Brain cells and red blood cells are exclusively dependent on glucose to produce energy. Energy production from dietary carbohydrates is roughly 4 kcal/g.

(ii) Energy storage: In animals, extra carbohydrates are transformed into glycogen which serves as reserve food material to satisfy the immediate energy demands of the body. In plants, starch serves as the storage form of energy.

(iii) Markers of cellular identity: Glycoproteins and glycolipids are components of cell membranes and receptors.

(iv) Structural components: These include cellulose of plants, exoskeleton of insects, cell wall of microorganisms. Carbohydrates provide structural support in animals as components of skin, connective tissue, tendons.

(v) Building macromolecules: Carbohydrate is the constituent of genetic material like DNA and RNA in the form of deoxyribose and ribose sugars. Excess carbohydrate is converted to fat.

(vi) Participation in metabolism of other biomolecules: The presence of adequate glucose spares the breakdown of proteins to amino acids for synthesizing glucose needed by the body. Moreover, increase in blood glucose stimulates release of the hormone insulin, which directs cells to use glucose. Thus, the use of lipids as an energy source is inhibited.

(vii) Medicinal use: Diet rich in fibre, a type of carbohydrate, promotes good digestive health. Carbohydrate-based or modified therapeutics are used extensively in cardiovascular and haematological treatments. Fondaparinux is a fully synthetic analogue according to the Penta saccharide domain of heparin and widely used for the treatment of deep vein thrombosis in clinic. A number of FDA approved antibiotics, antifungal and antiviral drugs contain carbohydrate moieties as part of their structures. Streptomycin, was the first example of the class of aminoglycoside antibiotics. Azithromycin, dihydrostreptomycin gentamycin are some popular aminocyclitol antibiotics currently in use.

1.6 Biological Importance of Carbohydrates:

Two chemical processes are ultimately referred to the chemistry of life:

(1) The use of radiant solar energy to drive chemical reactions that produce oxygen and reduced organic compounds from CO_2 and H_2O.

(2) The oxidation of the reduced products of (1) with production of CO_2, H_2O and energy.

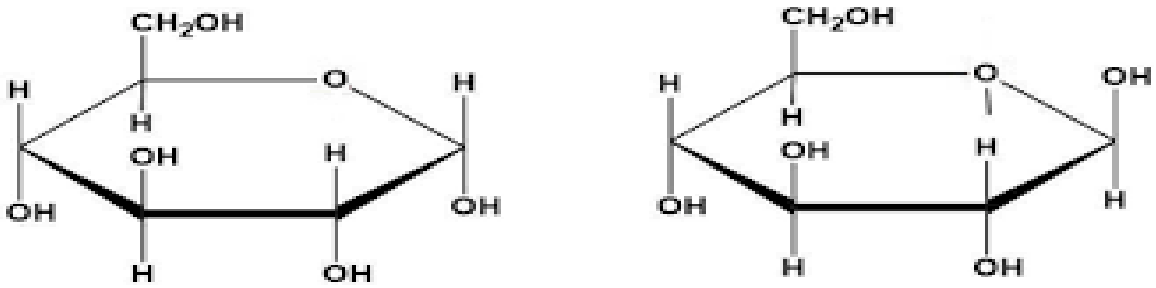
Photosynthesis is initiated by the capture of solar energy, usually referred to as “light harvesting”. In the leaf of a plant, the simple compounds CO2 and H2O are combined to form the sugar, (+)-glucose. A large number of organic pigments, including chlorophylls, carotenoids, phycoerythrin, and phycocyanin (in green plants and algae) are clustered together in pigment-protein complexes called photosystems. These pigments collectively absorb most of the sunlight reaching the earth and transform light into chemical energy.

$$\mathbf{6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 60_2}$$

Thousands of glucose molecules can then be combined to form much larger molecule of cellulose, which constitutes the supporting framework of the plant. (+) Glucose molecules can also be combined to form the large molecules of starch, which serves as food source to the growing plant.

When eaten by animals, the starch is broken down into original (+) glucose units. These can be carried by bloodstream to the liver to be recombined into glycogen (animal starch). Animals, such as human beings, are not capable of photosynthesis and so they derive their energy by running the above-mentioned reaction backwards, degrading the glucose to, ultimately, carbon dioxide and water. The overall reaction of glucose oxidation is the reverse of the overall reaction for its formation.

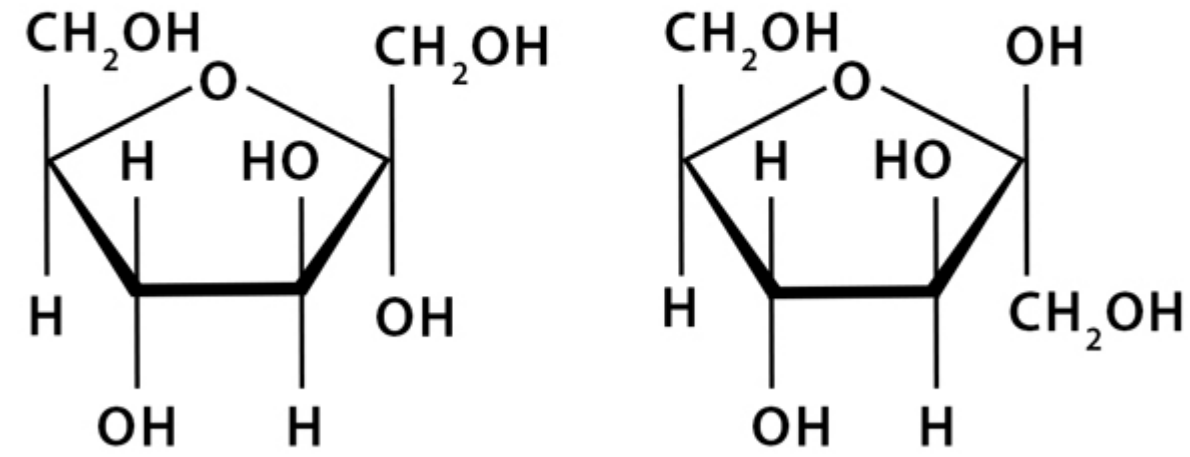
1.7 Howarth structure of Various Carbohydrates:



Haworth projection of Glucose

Haworth Projection

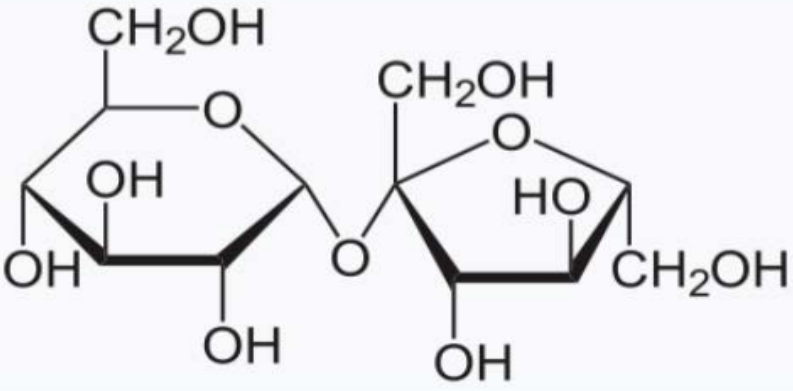
Fructose



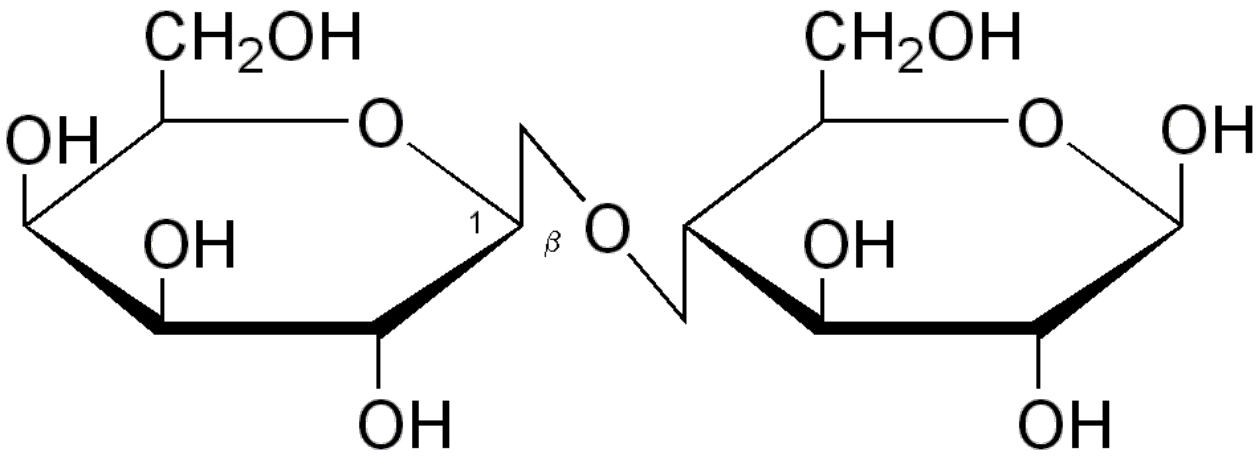
α-D-Fructofuranose β-D-Fructofuranose

ChemistryLearner.com

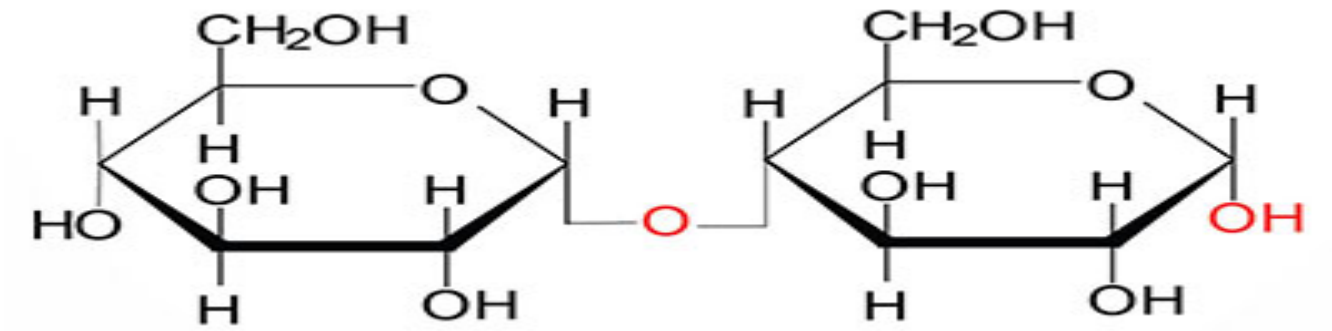
Sucrose



Lactose



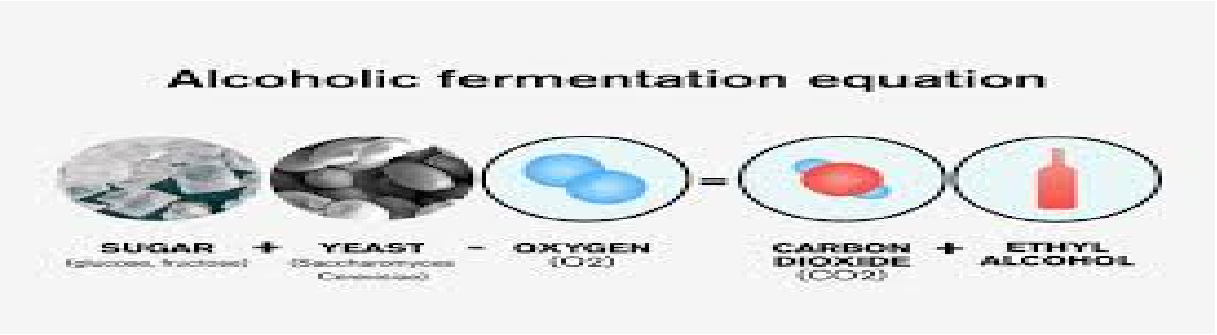
Maltose



1.8 Alcoholic fermentation:

1.8.1 Definition:

Alcoholic fermentation is a complex biochemical process during which yeasts convert sugars to ethanol, carbon dioxide, and other metabolic byproducts that contribute to the chemical composition and sensorial properties of the fermented foodstuffs.



1.8.2 Agent:

It is a well-known fact that the most widely used agent for the process of alcoholic fermentation is S. cerevisiae. This yeast is commonly used as a microbial starter in different fermentation industries.

At the time of alcoholic fermentation of fruits and juices, the S. cerevisiae becomes a dominant species as a result of their strong selective environment, given the low pH and high ethanol and sugar concentrations with anaerobic conditions.

1.8.3 Place:

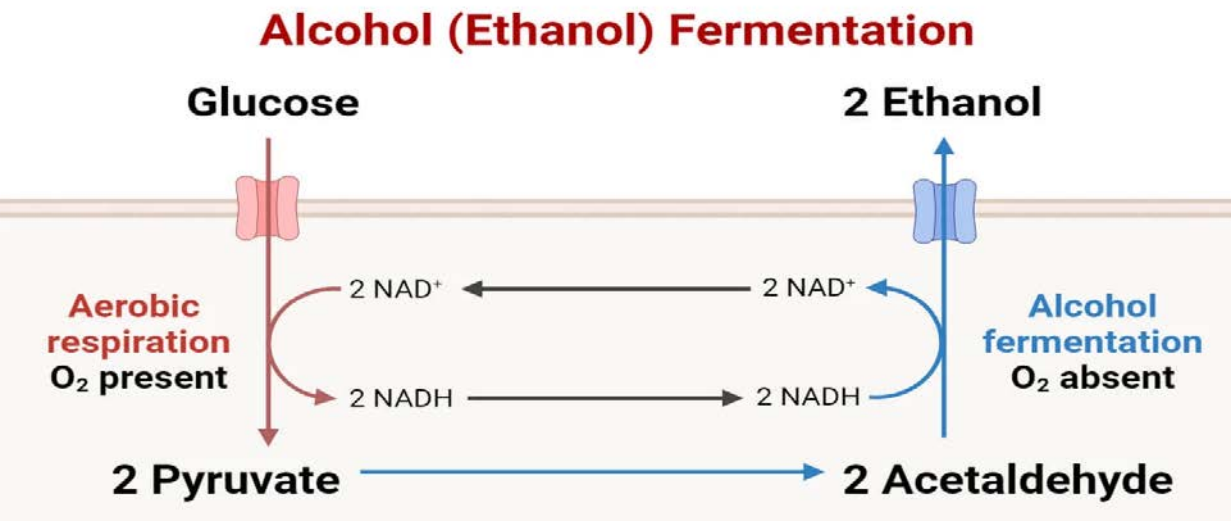
The process of alcoholic fermentation occurs within the cytoplasm

1.8.4 Product:

Initially, pyruvate is decarboxylated into ethanal by pyruvate decarboxylase. The enzyme requires cofactors in the form of magnesium and thiamine pyrophosphate. Hence, alcohol dehydrogenase reduces ethanal to ethanol, thus recycling NADH to NAD +.

In Saccharomyces cerevisiae, there are three isoenzymes of alcohol dehydrogenase, however, isoenzyme I is mainly involved in the conversion of ethanal to ethanol. Zinc is used as a cofactor by Alcohol dehydrogenase.

The final products of alcoholic fermentation are ethanol and carbon dioxide. Both are transported to the exterior of the cell by the process of simple diffusion. Apart from ethanol, some other compounds are generated all through the process of alcoholic fermentation, such as esters, higher alcohols, succinic acid, glycerol, 2,3-butanediol, diacetyl, acetoin.



1.9 Lactic acid fermentation:

1.9.1 Definition:

Lactic acid fermentation is a type of anaerobic respiration (or fermentation) that breaks down sugars to produce energy in the form of ATP. It is called anaerobic because it occurs in the absence of oxygen Lactic acid fermentation was a method used to preserve dairy products, vegetables, and meat for extended periods of time before the advent of refrigeration and modern canning practices and today is also utilized in industrial fermentation.

1.9.2 Applications:

Lactic acid fermentation is the best method used for food preservation. Lactobacillus is the most commercially used bacteria for this process. They are used in the production of pickles, sour beer, fermented fish, yoghurt, etc.

1.9.3 Place:

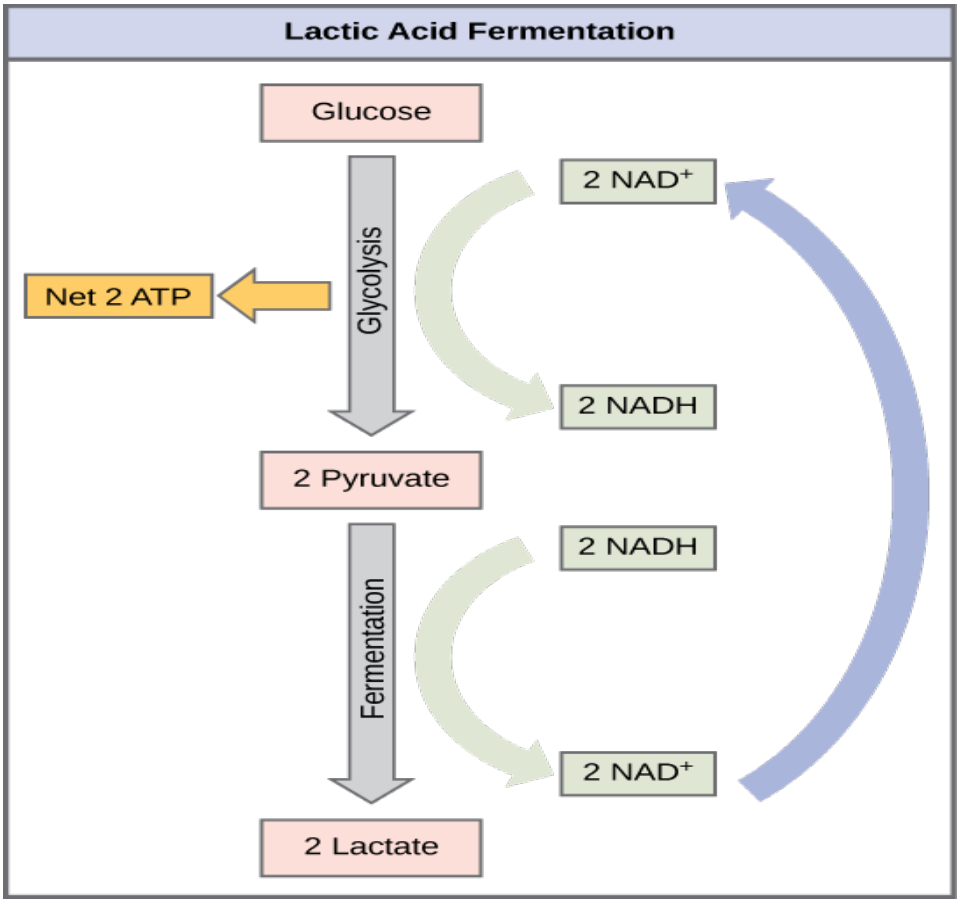
Lactic acid occurs in a few animal cells and bacterial organisms. Generally preferred in animal cells, cardiovascular respiration doesn't include lactic acid fermentation. However, in circumstances where the oxygen supply is insufficient, mainly in muscle groups, during periods of strenuous work, cells must go through respiration. This anaerobic respiration process creates lactic acid as a byproduct that collects in muscle tissue. Increased acidity leads to a burning sensation felt in the muscles during a workout. This prevents individuals from overworking parts of their muscles.

Contrary to popular opinion, the lactic acid doesn't cause delayed-onset of muscle tissue discomfort. After the muscle mass activity is completed, lactic acid is removed from the muscles through the bloodstream. It goes to the liver, where it goes through chemical reactions to produce pyruvic acid, which is utilised to produce energy. Aside from muscle tissue, this process occurs in purple bloodstream cells because they lack mitochondria and thus cannot create energy aerobically. Lactic acid fermentation gives rise to lactic acid bacteria (LABs) inside bacterial organisms.

Although fermentation is crucial in allowing LABs to produce energy in anaerobic problems, it also helps them reduce the level by forming lactic acid. The decreased pH makes their environment unsuitable for most other microorganisms and thus decreases the competition experienced by the LABs.

1.9.4 Steps:

1. The glucose or 6-carbon molecule is broken down into Glyceraldehyde 3-phosphate, and then to 3-Phosphoglyceric acid.
2. During this, NAD^+ is converted into $NADH+H^{+\cdot}$
3. The 3-Phosphoglyceric acid forms Phosphoenol pyruvic acid, which later forms the Pyruvic acid.
4. Net 2 ATP molecules are formed in this process (glycolysis).
5. This Pyruvic acid is reduced to Lactic acid with the help of reducing agent $NADH+H^+$, which deoxidises to NAD+.
6. This process produces two lactate/lactic acid molecules from two pyruvate/pyruvic acid molecules. This reaction happens in the presence of the enzyme lactate dehydrogenase.



1.10 Summary:

The most prevalent biomolecules in nature are carbohydrates. Humans benefit greatly from carbohydrates. They are taken directly as food such as bread, potatoes and rice, or are fed to animals, whose meat is eaten by many of us. Carbohydrates are regarded as polyhydroxy aldehydes or polyhydroxy ketones or substances that can be hydrolysed to either of them. Carbohydrates can be classified into several categories depending on reducing nature, hydrolysis, taste etc. This unit also shows the Howarth formulae of various mono as well as disaccharides. All living things rely on carbohydrates as their primary energy source. Beside this it plays significant role in storage, metabolism etc. Alcoholic fermentation takes place inside the cytoplasm. One kind of anaerobic respiration (or fermentation) that converts carbohydrates into ATP for energy is lactic acid fermentation.

1.11 Model Questions:

1. Give modern definition of carbohydrate.
2. Give two examples of reducing sugars.
3. What do you mean by alcoholic fermentation?
4. Draw Howarth projection of sucrose and glucose.
5. Give two primary functions of carbohydrates.
6. Draw a flow chart for lactic acid fermentation.
7. Write basic difference between aldose and ketose.
8. Explain biological role of carbohydrate.
9. Classify carbohydrate depending on taste with proper examples.
10. Discuss about the place where lactic acid fermentation occur.

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Unit 2 Carbohydrate Metabolism

Index

2.1 Objectives:

To gather deep knowledge on the followings.

1.Difference between catabolism and Anabolism.

2.Total Glycolysis process.

3.TCA cycle pathways and features.

4. Difference between Glycolysis and Krebs Cycle.

5. Biological Importance of Glycolysis and Krebs Cycle.

6. Total ATP production in both processes.

2.2 Introduction:

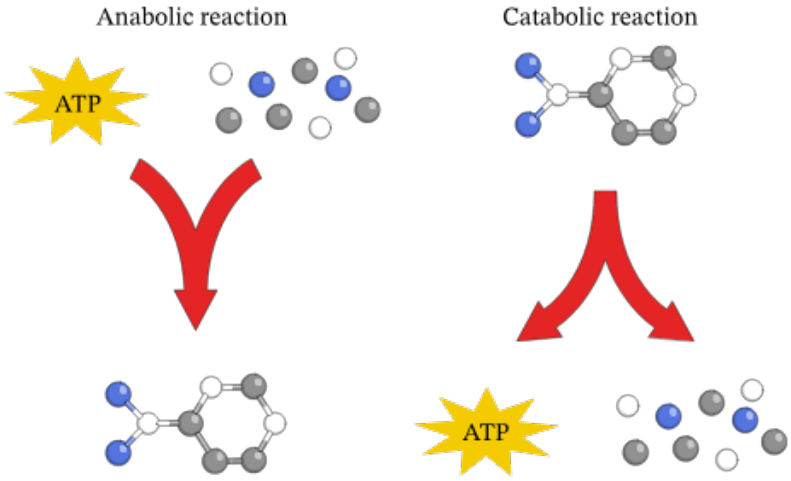
Metabolism is a term that is used to describe all chemical reactions involved in maintaining the living state of the cells and the organism. Metabolic pathways are series of connected enzymatic reactions that produce specific products. Their reactants, intermediates, and products are referred to as metabolites. The three main purposes of metabolism are:

- Conversion of food to energy to run cellular processes.
- Conversion of food/fuel to building blocks for proteins, lipids, nucleic acids, some carbohydrates.
- Elimination of nitrogenous wastes.

2.3 Catabolism and Anabolism:

1. **Catabolism** or degradation is a process in which energy rich complex macro-molecules are broken down to smaller, easier to absorb molecules. Energy is released during this process. Examples of catabolic processes are proteins becoming amino acids, glycogen breaking down into glucose and triglycerides breaking up into fatty acids.

2. **Anabolism** or biosynthesis is a process in which complex biomolecules are synthesized from simple precursors. This process requires energy. Examples include the formation of polypeptides from amino acids, glucose forming glycogen and fatty acids forming triglycerides.



2.4 Glycolysis:

The metabolism of glucose begins with the catabolic pathway called glycolysis (Greek: glykus, sweet + lysis, splitting) where glucose is oxidized and broken down to pyruvate in order to obtain energy as ATP. It is an oxygen independent process. Under aerobic conditions, the pyruvate formed by glycolysis is further oxidized by the citric acid cycle (discussed in the next section) and oxidative phosphorylation to CO_2 and water. Under anaerobic conditions, however, the pyruvate is instead converted to a reduced end product, lactate. Glycolysis is the only pathway that is taking place in all the cells of the body. It occurs in the cytosol of cell cytoplasm of both prokaryotic and eukaryotic cells of biological systems.

Glycolysis is alternately known as the Embden-Meyerhof-Parnas's pathway to memorialize the work of three investigators Gustav Embden, Otto Meyerhof, and Jacob Parnas. In the pathway of glycolysis, a single molecule of glucose (with six carbon at-oms) is degraded in a series of enzyme-catalysed reactions to yield two molecules of pyruvate (CH_3COCOO^-, each with three carbon atoms) and H^+ ions along with production of a small quantity of energy. The free energy released in the process is harvested to synthesize the high-energy molecules ATP from ADP and Pi. Thus, glycolysis is a pathway of chemically coupled phosphorylation reactions.

2.4.1 Sequence of Glycolysis:

Glycolysis is a sequence of 10 enzymatic reactions which is divided into two stages: energy investment and energy recovery. In the preparatory stage of glycolysis, which encompasses its first five reactions (1-5), glucose utilizes two ATPs, in an "energy investment". The second stage of glycolysis, the "energy recovery" stage, comprises its last five reactions (6-10) where energy is released in the form of 4 ATP molecules and 2 NADH molecules. Overall, 2 ATP molecules are produced per molecule of glucose during glycolysis.

The ten steps of glycolysis can be"desc'Ibed In terms of their substrates, products and enzymatic mechanisms. Mainly four basic reactions are involved catalysed by glycolytic enzymes: isomerization (catalysed by isomerase/mutases), phosphorylation (catalysed by

kinases), dehydration (catalysed by a dehydratase or hydrolase acting in reverse), aldol cleavage (catalysed by an aldolase).

Reaction 1 is the phosphorylation of glucose using the first ATP. Enzyme hexokinase (HK) catalyses the transfer of a phosphoryl group from ATP to glucose to form glucose-6-phosphate (G6P), a more reactive form of glucose. The plasma membrane is impermeable to glucose-6-P and, therefore, keeps the substrate within the cell. This is an irreversible reaction, dependent on ATP and Mg2+.

Reaction 2 is the isomerization of an aldose to a ketose. Enzyme phosphoglucose isomerase (PGI) catalyses the conversion of glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P). This change from phosphoglucose to phosphofructose allows the eventually facilitates the cleavage of C-C bond splitting the sugar into two three-carbon molecules.

Reaction 3 is the phosphorylation of fructose-6-phosphate (F6P). Enzyme phosphofructokinase (PFK) uses the second ATP for phosphorylating F6P to yield fruc-tose-1,6-bisphosphate [FBP or F1,6P]. This is an irreversible and regulatory step. The enzyme phosphofructokinase is a rate-limiting enzyme.

Reaction 4 is the aldol cleavage, where the C-C bond of the 6-carbon com-pound is split into two different 3-carbon compounds. Enzyme aldolase catalyses the cleavage of FBP to form trioses glyceraldehyde-3-phosphate (GAP) (an aldose) and dihydroxyacetone phosphate (DHAP) (a ketose).

Reaction 5 is a reversible isomerization reaction. Only one of the products of the aldol cleavage reaction, GAP, continues along the glycolytic pathway. However, DHAP and GAP are ketose-aldose isomers just as are F6P and G6P. Enzyme triose phosphate isomerase (TIM) interconverts dihydroxyacetone phosphate (DHAP) to glyceraldehyde-3-phosphate (GAP). Thus, two molecules of glyceraldehyde 3-phos-phate are obtained from one molecule of glucose.

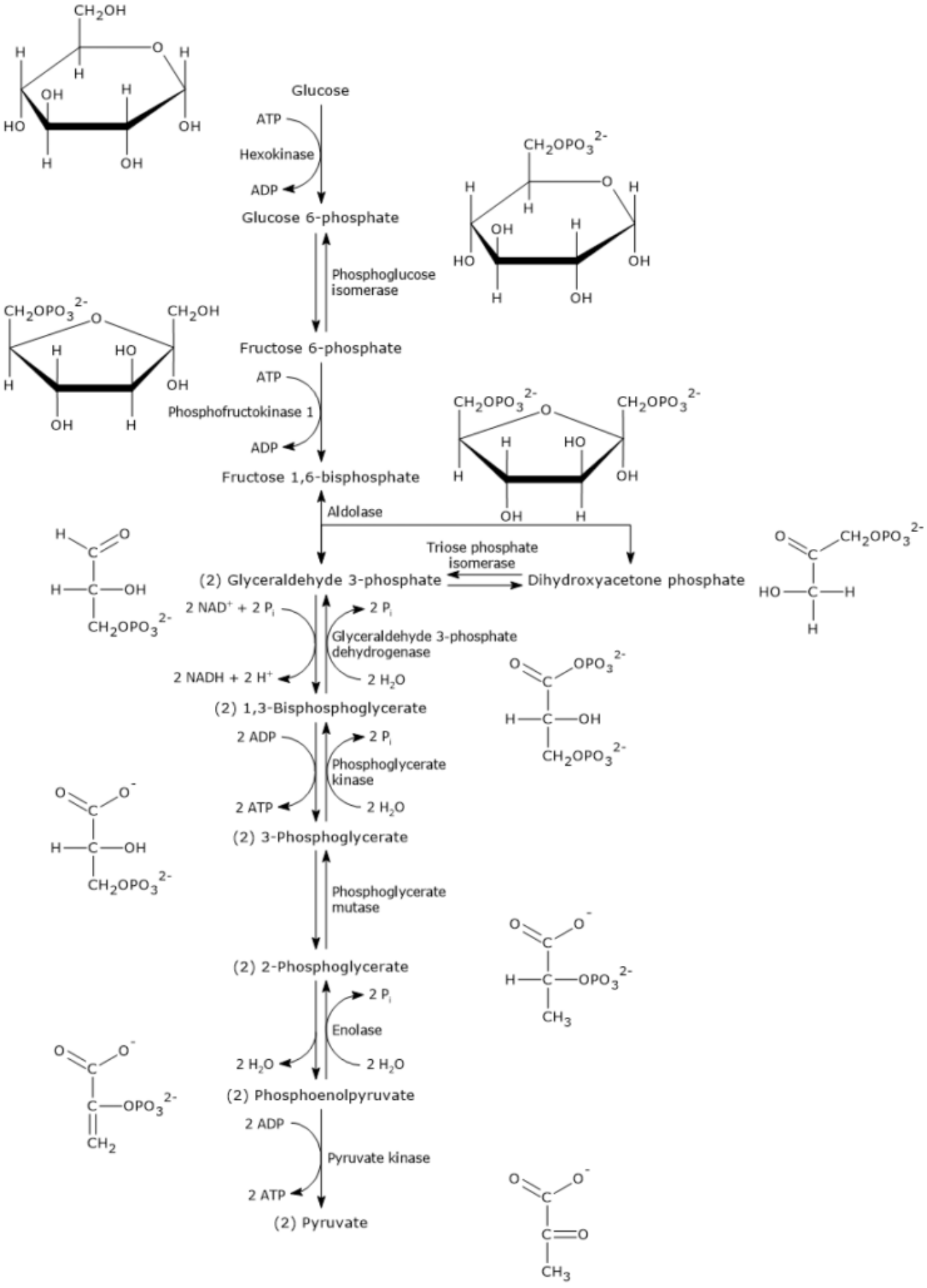
Reaction 6 is the oxidative phosphorylation of GAP by NAD and Pi catalysed by enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is one of the three energy-conserving or forming steps of glycolysis. This exergonic reaction is important as it is involved in the synthesis of the first “High-Energy” intermediate 1,3-bisphosphoglycerate (1,3-BPG) and NAD+ is reduced to coenzyme NADH.

Reaction 7 is transfer of phosphate from 1,3-biphosphoglycerate to ADP. This step generates the first two ATP molecules together with 3-phosphoglycerate (3PG). This step is a good example of substrate level phosphorylation, since ATP is directly synthesized from the substrate without the involvement of electron transport chain. Enzyme phosphoglycerate kinase (PGK) catalyses this reaction which is reversible, being a rare example among the kinase reactions.

Reaction 8 is the reversible isomerization of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) due to the shift of phosphoryl group. This step is catalysed by enzyme phosphoglycerate mutase (PGM).

Reaction 9 is the formation the second "High-Energy" intermediate Phosphoenol pyruvate (PEP) from dehydration of 2-phosphoglycerate (2PG). This reaction is catalysed by the enzyme enolase.

Reaction 10, the final reaction, yields pyruvate and ATP (two molecules). First, PEP is made into a transient intermediate of enol pyruvate; which is spontaneously isomerized into keto pyruvate, the stable form of pyruvate. This reaction is irreversible. Enzyme pyruvate kinase catalyses the transfer of a phosphoryl group from Phosphoenol pyruvate to ADP, thus forming ATP. This is again an example of substrate level phosphorylation.


Glucose
ATP
Hexokinase
ADP
Glucose 6-phosphate
Phosphoglucose isomerase
Fructose 6-phosphate
ATP
Phosphofructokinase 1
ADP
Fructose 1,6-bisphosphate
Aldolase
Triose phosphate isomerase
(2) Glyceraldehyde 3-phosphate
Dihydroxyacetone phosphate
2 NAD+ + 2 Pi
2 Pi
Glyceraldehyde 3-phosphate dehydrogenase
2 NADH + 2 H+
2 H2O
(2) 1,3-Bisphosphoglycerate
2 ADP
2 Pi
Phosphoglycerate kinase
2 ATP
2 H2O
(2) 3-Phosphoglycerate
Phosphoglycerate mutase
(2) 2-Phosphoglycerate
2 Pi
Enolase
2 H2O
2 H2O
(2) Phosphoenolpyruvate
2 ADP
Pyruvate kinase
2 ATP
(2) Pyruvate

2.4.2 Products of Glycolysis:

ATP: The initial investment of 2 ATP per glucose in Stage I (reaction 1 and 3) and the subsequent generation of four ATP by substrate-level phosphorylation (reaction 7 and 10) give a net yield of 2 ATP per glucose.

NADH: Reaction 6 is one of the three energy-conserving or forming steps of glycolysis. NAD+ is reduced to coenzyme NADH by the H- from glyceraldehyde 3-phosphate. Since two moles of glyceraldehyde 3-phosphate are formed from one mole of glucose, two NADH are generated in this step.

Pyruvate: The two pyruvate molecules produced through the partial oxidation of each glucose are still relatively reduced molecules.

2.5 Importance of Glycolysis:

Glycolysis is the first phase of cellular respiration; the process by which cells convert the energy in food into the energy of ATP. Cells that lack mitochondria and /or adequate oxygen supply are completely dependent on glycolysis to produce ATP from glucose. Glycolysis is the only source of energy in erythrocytes, cornea, lens etc. In strenuous exercise, when muscle tissue lacks enough oxygen, anaerobic glycolysis forms the major source of energy for muscles. Cells containing mitochondria use glycolysis as a preparatory pathway for the complete oxidation of glucose to carbon dioxide with the production of larger amounts of ATP (net yield of only 2 ATP per glucose molecule versus 32 ATP per glucose molecule).

The intermediates of glycolysis provide branch point to other pathway, therefore are useful for the synthesis of amino acids and fat. Within the cells of modern-day organisms that carry out glycolysis, what happens after glycolysis depends mostly on the presence or absence of oxygen.

2.6 Fate of Pyruvate:

Anaerobic pathway: If oxygen is absent, then anaerobic respiration follows. Anaerobic respiration means “without oxygen.” Rather than being oxidized, pyruvate will undergo anaerobic homolactic fermentation to lactate or anaerobic alcoholic fermentation to ethanol. Fermentation doesn’t create additional energy for the cell but it does regenerate NAD+, making it possible for the cell to continue carrying out glycolysis, which creates a small amount of ATP.

Aerobic pathway: If oxygen is present, then aerobic respiration follows. Aerobic means "with oxygen." Be totally oxidized to CO_2 through the Link Reaction and the Krebs/TCA cycle. Pyruvate is converted to acetyl CoA which is oxidized via TCA cycle and forms CO_2 and H_2O.

2.7 Steps and Reaction Sequences of Krebs Cycle:

Step 1: The first step is an aldol condensation of acetyl-CoA and oxaloacetate to yield citrate in a reaction catalysed by citrate synthase.

Step 2: Citrate isomerizes to the more easily oxidized secondary alcohol, isocitrate in a two-step reaction catalysed by aconitase. The reaction sequence involves a dehydration, producing enzyme-bound cis-aconitate intermediate. This step is followed by a hydration, which effectively transfers citrate's hydroxyl group to an adjacent carbon atom.

Step 3: Oxidation of isocitrate to oxalosuccinate and then decarboxylation to aα-ketoglutarate in a reaction catalysed by isocitrate dehydrogenase. This is the first step in which oxidation is coupled to NADH production and also the first CO_2-generating step.

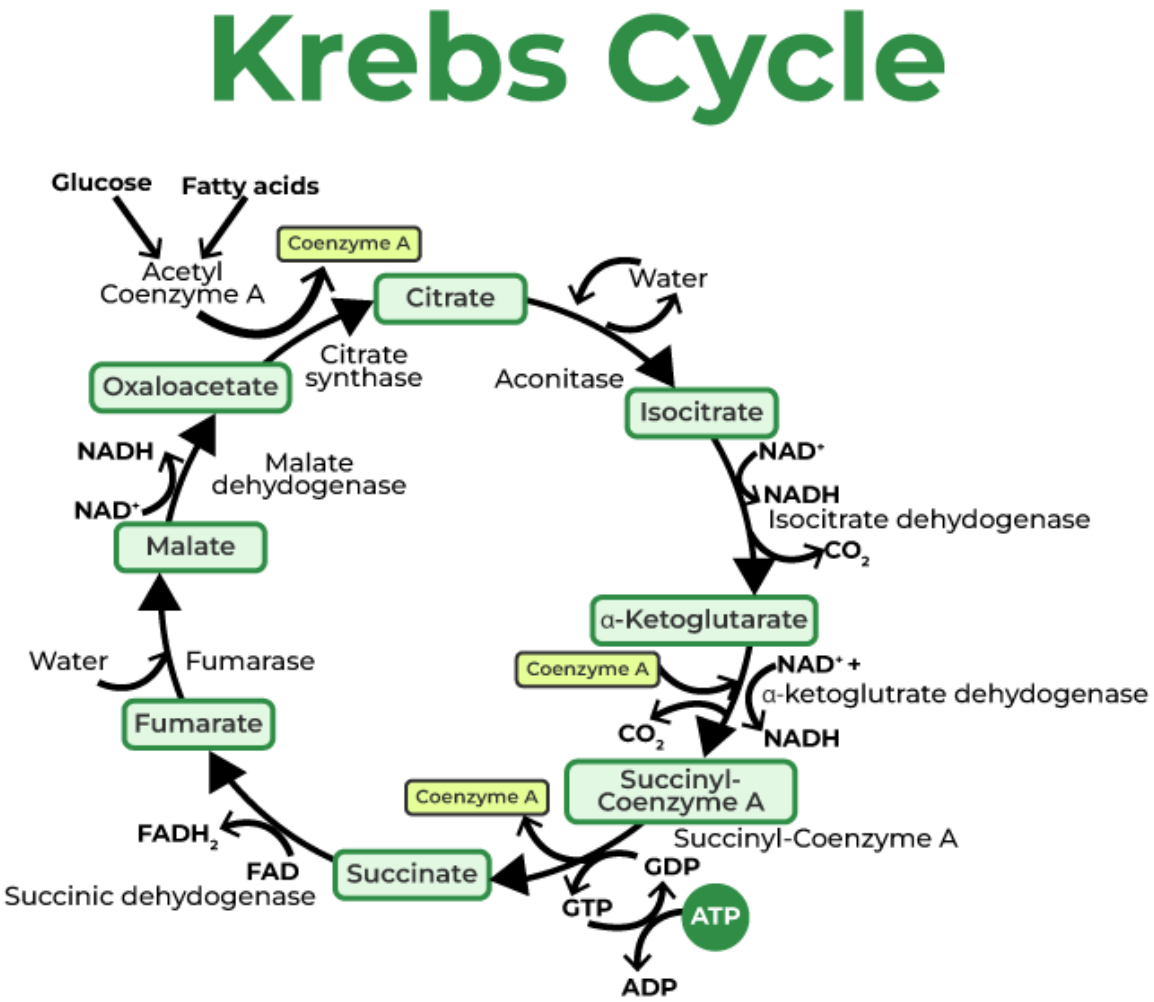
Step 4: a-Ketoglutarate oxidatively decarboxylates to succinyl-coenzyme A by the multi-enzyme complex a-ketoglutarate dehydrogenase. The reaction involves the reduction of a second NAD^+ to NADH and the generation of a second molecule of CO2. At this point in the cycle, two molecules of CO_2 have been produced. But note that these CO_2 molecules enter the citric acid cycle as a component of oxaloacetate rather than of acetyl-CoA.

Step 5: This is a substrate level phosphorylation. The "high-energy" compound succinyl-coenzyme A is converted to succinate by succinyl-CoA synthetase. This re-action is coupled with the phosphorylation of GDP to GTP. The free energy of the thioester bond is thus conserved in this reaction by the formation of "high-energy" GTP in mammals and ATP in plant and bacteria. GTP is rapidly converted to ATP through the action of the enzyme nucleoside diphosphate kinase. By this point in the citric acid cycle, one acetyl equivalent has been completely oxidized to two CO_2 ,two NADH and one GTP (equivalent to one ATP) have also been generated. In the remaining reactions of the cycle, succinate is oxidized back to oxaloacetate for another round of the cycle.

Step 6: This is oxidation of succinate's central single bond to a trans double bond. Succinate dehydrogenase catalyses this dehydrogenation reaction yielding fumarate. Simultaneously redox coenzyme FAD is reduced to FADH2.

Step 7: Fumarase then catalyses the hydration of fumarate's double bond to yield malate.

Step 8: In this final reaction catalysed by malate dehydrogenase, oxaloacetate is regenerated by oxidizing malate's secondary alcohol group to the corresponding ketone. At this stage, concomitant reduction of a third NAD^+ to NADH takes place. The oxaloacetate that is consumed in the first step of the citric acid cycle is regenerated in the last step of the cycle. Thus, an endless number of acetyl groups can be oxidized through the agency of a single oxaloacetate molecule.



2.8 Features of the Cycle:

a) Amphibolic pathway:

Citric acid cycle is a good example of an amphibolic pathway because it functions in both degradative/catabolic and biosynthetic/anabolic processes. The first reaction of the cycle, in

which oxaloacetate (a four-carbon compound) condenses with acetate (a two-carbon compound) to form citrate (a six-carbon compound) is typically ana-bolic. The next reaction, which is intramolecular rearrangement, produces isocitrate. The following two reactions, namely the oxidation of isocitrate to a-ketoglutarate followed by its oxidation to succinyl-CoA, are typically catabolic. Carbon dioxide is lost in each step and succinate (a four-carbon compound) is produced.

b) Anaplerotic role:

Biosynthesis of many vital cellular constituents such as glucose, lipid and amino acid utilize citric acid cycle intermediates. For example, heme is synthesized from succinyl CoA and aspartate from oxaloacetate. Reactions that deplete citric acid cycle intermediates are called cataplerotic reactions (Greek: cata, down+ plerotikos, to fill). Reactions that replenish citric acid cycle intermediates are called Anaplerotic reactions (filling up, Greek: ana, up). Anaplerotic reactions are essential to maintain the concentrations of the 4-carbon units in the cell. The main reaction of this type is catalysed by pyruvate carboxylase, which produces oxaloacetate from pyruvate.

$$\text{Pyruvate} + CO_2 + \text{ATP} + H2O \leftrightharpoons \text{Oxaloacetate} + \text{ADP} + \text{Pi}$$

The other important Anaplerotic reactions are conversion of glutamate to alpha ketoglutarate and aspartate to oxaloacetate through transamination.

2.9 Significance of Krebs Cycle:

► The TCA cycle plays a central role in the breakdown, or catabolism of organic fuel molecules-i.e., glucose and some other sugars, fatty acids, and some amino acids.

► The major significance of the citric acid cycle is to act as the final common pathway for the oxidation of carbohydrates, lipids and proteins since all of them are metabolized to acetyl CoA. It is a major source of ATP production in cells. Much of the free energy liberated during the oxidation of carbohydrate, lipids and amino acids is made available through this cycle.

► TCA cycle is of further significance since it has dual or amphibolic role thus providing precursor compounds for biosynthesis of other biomolecules, nucleo-tides, cytochromes etc.

NADH and FADH2 are vital products of the citric acid cycle. These molecules then transfer their energy to the electron transport chain, a pathway that is part of the third stage of cellular

respiration. The electron transport chain in turn releases energy so that it can be converted to ATP through the process of oxidative phosphorylation.

2.10 Differences Between Glycolysis and Krebs Cycle:

Glycolysis	Krebs cycle
1. The process of glycolysis occurs in the cytoplasm of the cell.	1. Krebs cycle takes place in mitochondria.
2. In glycolysis, one molecule of glucose is oxidized step-by-step to produce two molecules each of pyruvic acid, ATP, $NADH_2$ and water.	2. In Krebs cycle, molecule of acetyl-co-A is completely oxidized and in the process CO_2, H_2O, $NADH_2$, $FADH_2$ and ATP is produced.
3. Glycolysis can take place in both aerobic and anaerobic respiration.	3. Krebs cycle takes place only during aerobic respiration.
4. The first step in cellular respiration is glycolysis where glucose is converted into pyruvate.	4. The second step in cellular respiration is Krebs cycle.
5. Two molecules of pyruvate are obtained in glycolysis.	5. Pyruvate is converted into CO_2 and H_2O during Krebs cycle.
6. Two molecules of ATP are used up in glycolysis.	6. ATP molecules are not used up in Krebs cycle.
7. Four molecules of ATP are produced in glycolysis.	7. Two molecules of ATP are produced in Krebs cycle.
8. CO_2 is not produced during glycolysis.	8. CO_2 is produced in Krebs cycle.

2.11 Total ATP formation calculation in Both Processes:

The re-oxidation of NADH and $FADH_2$ by O_2 through the mediation of the electron-transport chain and oxidative phosphorylation will ultimately drive synthesis of ATP. For every NADH that passes its electrons on, approximately 2.5 ATP molecules are produced from ADP + Pi. For every $FADH_2$, approximately 1.5 ATP molecules are produced.

When one molecule of glucose is converted to two molecules of pyruvate by glycolysis, two molecules of ATP are generated and two molecules of NAD+ are reduced. The NADH molecules yield approximately 5 molecules of ATP on passing their electrons to the electron-transport chain. However, due to NADH transport over the mitochondrial membrane, each NADH produces net 1.5 ATP instead of usual 2.5. So Total ATP generated 2+5= 7, alternatively 2+3 = 5

When the two pyruvate molecules are converted to two acetyl-CoA molecules by the pyruvate dehydrogenase complex, the two molecules of NADH produced in that process also eventually give rise to 5 ATP molecules.

In citric acid cycle, approximately 9 ATP (3 x 2.5 for NADH +1.5 for $FADH_2$)are generated in oxidative phosphorylation when the four pairs of electrons are eventually transferred to O. Besides, there is one substrate level phosphorylation producing one GTP (or ATP). Thus, one turn of the citric acid cycle ultimately generates approximately 10 molecules of ATP. Considering two turns of the citric acid cycle (one for each acetyl-CoA group), ATP generated 20. To summarize, in a eukaryotic cell, complete oxidation of glucose through glycolysis plus citric acid cycle can potentially yield 32 or 30 molecules of ATP.

TOTAL ATP Production from one Glucose molecule

	Glycolysis: (Net yields)	
Stage I.	**ATP**	**2 ATP**
	2 NADH+H⁺ → 2 $FADH_2$ (to ETC)	**3 ATP**
Stage II.	**Conversion of pyruvate to ACoA**	
	2 NADH + H⁺ (to ETC)	**5 ATP**
Stage III.	**TCA cycle**	
	ATP (at one site)	**2 ATP**
	NADH+H⁺ at three steps (to ETC)	**15 ATP**
	$FADH_2$ at one step (to ETC)	**3 ATP**

Total ATP from one molecule of glucose = 30 ATP

2.12 Summary:

All of the chemical processes necessary to keep the cells and the organism alive are collectively referred to as metabolism. The two major classes of this metabolism are anabolism and catabolism. The catabolic process known as glycolysis, which starts the metabolism of glucose, oxidises and breaks it down to pyruvate, which is then converted into ATP. Energy investment and energy recovery are the two phases of glycolysis, a series of ten enzyme processes. The breakdown, or catabolism, of organic fuel molecules, such as glucose and some other sugars, fatty acids, and amino acids, is mostly mediated by the TCA cycle. In a eukaryotic cell, complete oxidation of glucose through glycolysis plus citric acid cycle can potentially yield 32 or 30 molecules of ATP.

2.13 Model Questions:

1. Give difference between Catabolism and anabolism.
2. 'Krebs Cycle is a good example of amphibolic pathway'- Comment.
3. Write four differences between Glycolysis and Krebs Cycle.
4. Write the significance of TCA cycle.
5. Calculate total ATP production in both processes.
6. What products are obtained in Glycolysis?
7. Write the process of Glycolysis through flow chart diagram.
8. Draw Krebs Cycle.
9. Write the purpose of metabolism.
10. What is another name of Krebs Cycle and Why?

2.14 References:

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Unit 3: Basics of Proteins

Index

3.1 Objectives:

After studying this unit one can realize the followings

1. The importance of protein in biological system.
2. Several types and examples of amino acids and proteins.
3. All four levels of protein structure.
4. Functions of various proteins in human physiology.
5. About denaturation and renaturation.

3.2 Introduction:

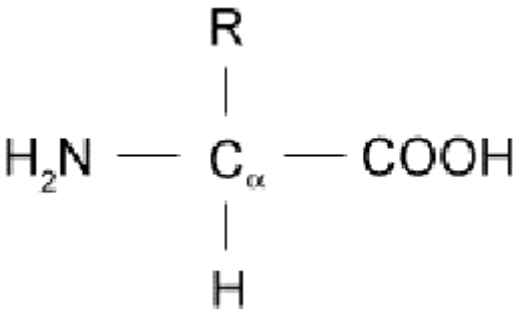
Living organisms produce three types of organic polymers, called biopolymers, which are essential for life processes. These are the polysaccharides (already discussed, Chapter 1), nucleic acids and proteins (Greek word, proteios means first). The choice of the name proteins rests upon the fact that they constitute the essential part of living organisms. Our muscles, skin, nerves, blood, etc., are all made up of various types of proteins. Besides, the enzymes-called the biological catalysts are also proteins. Chemically, the proteins are polyamides of α-amino carboxylic acids. In fact, a single protein molecule is made up of hundreds or even thousands of amino acid units. Over 100 amino acids have been isolated from natural sources. A vast majority of these amino acids have amino group attached to the α-carbon atom with respect to carboxylic group, while the third bond of the a-carbon is attached to a hydrogen atom and the fourth one is joined to a group I with about 100 variations. Of the amino acids isolated from living materials, about twenty odd have been found to be the naturally occurring components of proteins. In the present chapter, we shall discuss the general aspects of the chemistry of amino acids together with that of proteins.

3.3 About Amino Acids and Its Classification:

Amino acids represent one of the most important classes of naturally occurring compounds, because they form the fundamental structural units of proteins. As mentioned above, all naturally occurring amino acids have an amino group located at α-position with respect to the carboxylic group. Proline and hydroxyproline are exceptions as in these molecules the amino group forms a part of the pyrrolidine ring. In addition to the essential structural features, i.e., the presence of amino and carboxylic functions, some amino acids contain benzene or heterocyclic ring systems, phenolic or alcoholic group or even sulphur.

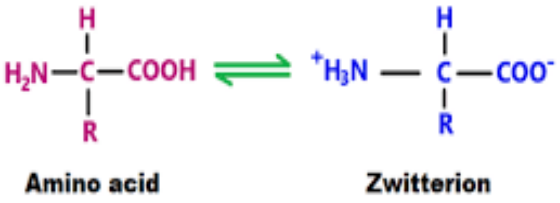
3.3.1 Structure:

An inspection of the structure of a typical α-amino acid shows that there is a chirality centre at the α-carbon atom, and we shall discuss subsequently that all naturally occurring α-amino acids belong to L-series. The planer projection formula of a typical α-amino acid is shown below:



3.3.2 Dipolar Nature:

We know that addition of an amine to a carboxylic acid gives the ammonium salt of the carboxylic acid. This takes place by the transfer of a proton from the carboxylic group to the amino group. However, when both these groups form part of the same molecule, as indeed is the situation in amino acids, the proton transfer occurs internally to give what is called internal salt, ‘dipolar ion’ or ‘zwitterion’.



3.3.3 Evidence in Favour of The Dipolar Ionic Structure:

Because of the dipolar ionic structure, amino acids exhibit the following typical properties:

(1) They are highly soluble in water and practically insoluble in common organic solvents.

(ii) They melt at temperatures above 473 K and that too with decomposition.

(iii) Their aqueous solutions behave like solution of substances having high dipole moments.

(iv) The spectral studies do not show the presence of free NH, and NH, and COO groups are present. COOH groups but indicate that

(v) Because of the dipolar nature they are amphoteric, i.e., they can accept a proton from a stronger acid as well as donate a proton to a stronger base.

3.3.4 Classifications:

3.3.4.1 Based on the structure:

Amino acids with aliphatic side chains: Glycine, alanine, valine, leucine and isoleucine.

Hydroxyl group containing amino acids: Serine, threonine and tyrosine.

Sulphur containing amino acids: Cysteine (with sulfhydryl group) and methionine (with thioether group).

Acidic amino acids and their amides: Aspartic acid and glutamic acid (dicarboxylic monoamino acids), asparagine and glutamine (respective amide derivatives).

Basic amino acids: Lysine, arginine (with guanidino group) and histidine (with imidazole ring).

Aromatic amino acids: Phenylalanine, tyrosine and tryptophan, histidine.

Imino acids: Proline (with imino group).

3.3.4.2 Based on the polarity

There are four main classes of amino acids based on polarity, i.e. the interaction of the R group with water molecules at physiological pH

(1) **Amino acids with non-polar (hydrophobic) side chain:**

These amino acids have a non-polar hydrophobic side chain. They do not provide protons or participate in hydrogen or ionic bonding. E.g.: glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine.

(b) Amino acids with uncharged polar side chain:

These amino acids are more soluble in water. They are hydrophilic in nature. The functional groups can make hydrogen bonds with water. E.g. glycine, serine, threonine, tyrosine, cysteine, asparagine and glutamine. The polarity of serine, threonine is due to the presence of hydroxyl groups; the polarity of asparagine and glutamine is due to the amide group and the sulfhydryl group (thiol group) is responsible for cysteine.

I Amino acids with polar, negatively charged side chain:

These amino acids will have a net negative charge at neutral pH. E.g. aspartic acid and glutamic acid. These amino acids will have one more carboxyl group, which contribute to negative charge at neutral pH.

(d) Amino acids with polar, positively charged side chain:

These amino acids will have a net positive charge at pH 7. They accept protons. E.g.: lysine, arginine and histidine. Lysine contains a second amino group at e-position on the aliphatic side chain. Arginine contains a positively charged guanidino group and histidine contains an imidazole group.

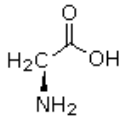
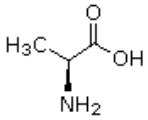
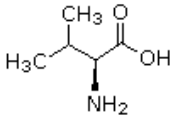
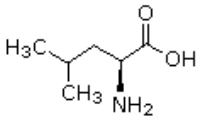
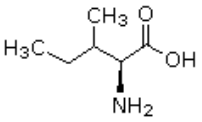
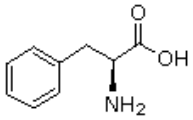
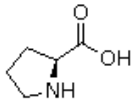
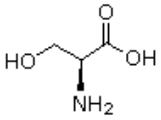
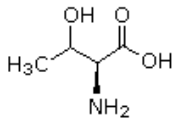
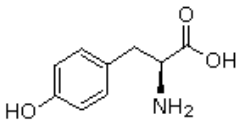
3.3.4.3 Based on the metabolic pathways:

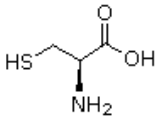
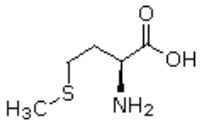
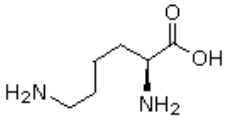
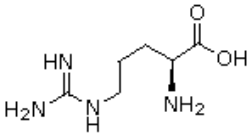
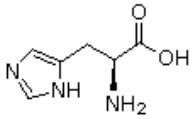
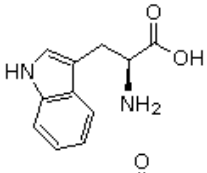
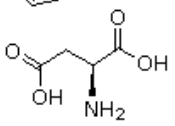
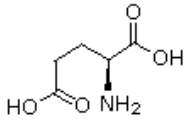
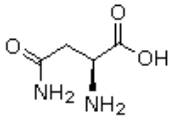
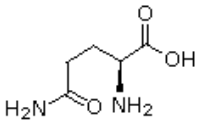
The amino acids may be divided into two groups based on their catabolic path-ways:

(a) Glucogenic amino acids: They are degraded to the intermediates pyruvate, ketoglutarate, succinyl-CoA, fumarate, or oxaloacetate and are therefore glucose precursors.

(b) Ketogenic amino acids: They are broken down to acetyl-CoA, which is the precursor of ketone bodies.

In humans, two amino acids, leucine and lysine, are exclusively ketogenic. Five more are both ketogenic and glucogenic: phenylalanine, isoleucine, threonine, tryptophan and tyrosine. The remaining thirteen are exclusively glucogonia.

Name	Formula	Abbreviations	
Glycine		Gly	G
Alanine		Ala	A
Valine		Val	V
Leucine		Leu	L
Isoleucine		Ile	I
Phenylalanine		Phe	F
Proline		Pro	P
Serine		Ser	S
Threonine		Thr	T
Tyrosine		Tyr	Y

Name	Formula	Abbreviations	
Cysteine		Cys	C
Methionine		Met	M
Lysine		Lys	K
Arginine		Arg	R
Histidine		His	H
Tryptophan		Trp	W
Aspartic Acid		Asp	D
Glutamic Acid		Glu	E
Asparagine		Asn	N
Glutamine		Gln	Q

3.4 Isoelectric Point:

3.4.1 Definition:

The isoelectronic point or isoionic point is the pH at which the amino acid does not migrate in an electric field. This means it is the pH at which the amino acid is neutral, i.e. the zwitterion form is dominant.

3.4.2 Values of Isoelectric Point for Several Amino Acids:

Each amino acid has a different isoelectric point, which is influenced by the number and type of charged groups present in the molecule. Amino acids with acidic side chains, such as aspartic acid and glutamic acid, have low isoelectric points, while those with basic side chains, such as arginine and histidine, have high isoelectric points.

Amino acid	pKa_1	pKa_2	pKa_3	pI
Glycine	2.34	9.60	---	5.97
Alanine	2.34	9.69	---	6.00
Valine	2.32	9.62	---	5.96
Leucine	2.36	9.60	---	5.98
Isoleucine	2.36	9.60	---	6.02
Methionine	2.28	9.21	---	5.74
Proline	1.99	10.60	---	6.30
Phenylalanine	1.83	9.13	---	5.48
Tryptophan	2.83	9.39	---	5.89
Asparagine	2.02	8.80	---	5.41
Glutamine	2.17	9.13	---	5.65
Serine	2.21	9.15	---	5.68
Threonine	2.09	9.10	---	5.60
Tyrosine	2.20	9.11	---	5.66
Cysteine	1.96	8.18	---	5.07
Aspartic acid	1.88	9.60	3.65	2.77
Glutamic acid	2.19	9.67	4.25	3.22
Lysine	2.18	8.95	10.53	9.74
Arginine	2.17	9.04	12.48	10.76
Histidine	1.82	9.17	6.00	7.59

3.4.3 Significance:

At the isoelectric point, the molecule has no net charge, which means it is not attracted or repelled by charged particles in the environment. This may affect its solubility, as charged molecules tend to dissolve more readily in polar solvents. If the pH of the solution is below the isoelectric point of the amino acid, the molecule will have a net positive charge and will be attracted to negatively charged surfaces, making it less soluble. Conversely, if the pH is above the isoelectric point, the molecule will have a net negative charge and be repelled by a negatively charged surface, also decreasing its solubility.

In addition to solubility, the isoelectric point can affect the behaviour of molecules during chromatography and electrophoresis. These techniques rely on the separation of molecules based on their charge and size, and the isoelectric point can be used to predict where molecules will move in these systems. For example, during isoelectric focusing, proteins are separated based on their isoelectric point in a pH gradient. As proteins move through the gradient, they will reach a region where the pH is equal to their isoelectric point, causing them to stop moving because they have no net charge and thus can be separated based on the protein's isoelectric point.

3.5 Detail classification of Proteins:

3.5.1. Based on Chemical Composition:

(a) Simple proteins: These are also known as homoproteins, are made up of only amino acids. Examples are albumin, globulin, glutelin, prolamin, histone, globin, protamine.

(b) Conjugated proteins: They are sometimes also called heteroproteins, contain in their structure a non-protein portion, referred to as a prosthetic group or cofactor. There are several types of conjugated proteins.

► **Glycoproteins:** Covalently bound one or more carbohydrate units to the poly-peptide backbone. Example – mucins (saliva), blood group antigens.

► **Chromoproteins:** Contain coloured prosthetic groups. Typical examples are haemoglobin and myoglobin which bind four and one heme groups respectively, Chlorophylls which bind a porphyrin ring with a magnesium atom at its centre, Rhodopsin which bind retinal (allows the retina for monochromatic vision in low light conditions).

►**Phosphoproteins:** Bind phosphoric acid to serine and threonine residues. Generally, they have a structural function such as tooth dentin, or reserve function, such as casein of milk and vitellin of egg yolk.

►**Nucleoproteins:** These are proteins attached to nucleic acids, e.g. nucleo histones, nucleo protamine's. The DNA carries negative charges, which combines with positively charged proteins.

► **Lipoproteins:** Combination of proteins and lipids. Example — serum lipoproteins, membrane lipoproteins.

► **Metalloprotein:** Combination of proteins and metal ions. Examples are Hemo-globin (Iron), Cytochrome (Iron), Tyrosinase (Copper) and Carbonic anhydrase (Zinc).

I Derived proteins: These are proteins derived from the partial or complete hydrolysis of simple or conjugated proteins by acid, alkali or enzymes. Examples are coagulated proteins and peptides.

3.5.2. Based on Shape:

(a) Fibrous proteins:

They are fibre like in shape. Their structure consists of β-pleated sheets held together by intermolecular hydrogen bonding. These proteins are insoluble in water as they contain, both internally and on their surface, many hydrophobic amino acids. They are resistant to digestion. They have primarily mechanical and structural functions, providing support to the cells as well as the whole organism. They serve as chief structural materials of animal tissues a function to which their insolubility and fibre forming tendency suit them. The presence of hydrophobic amino acids on their surface facilitates their packaging into very complex supramolecular structures. Examples:

► **Collagen:** It strengthens connective tissue, surrounds blood vessels, prevents cracks in teeth and bones.

 α-Keratins: It constitute almost the entire dry weight of nails, claws, beak, hooves, horns, hair, wool and a large part of the outer layer of the skin

 Elastin: It provides elasticity to tissues such as tendons and arteries

► **Myosin:** It helps to grow and maintain muscles

► **Fibroin:** One of main component of silk.

(b) Globular proteins:

Most of the proteins belong to this class. They have a compact and more or less spherical/oval structure, more complex than fibrous proteins. Their structure consists of α-helix held together by intramolecular hydrogen bonding. In this regard, motifs, domains, tertiary and quaternary structures are found, in addition to the secondary structures. They are generally soluble in water but can also be found inserted into biological membranes (transmembrane proteins), thus in a hydrophobic environment. They are digestible. At the intestinal level, most of the globular proteins of animal origin are hydrolysed almost entirely to amino acids.

They serve a variety of functions related to the maintenance and regulation of life processes, functions that require mobility and hence solubility. They act as:

- enzymes
- hormones
- membrane transporters and receptors
- transporters of triglycerides, fatty acids and oxygen in the blood
- immunoglobulins or antibodies
- grain and legume storage proteins

Examples of globular proteins are cytochrome c, albumins, globulins, glutelin, prolamins, histones, globin (myoglobin, haemoglobin), protamine, lectins.

3.5.3. Based on Biological Functions:

► **Enzymes (biochemical catalysts):**

In living organisms, almost all reactions are catalysed by specific proteins called enzymes. Examples — amylase, pepsin, trypsin, lipase and many more.

► **Transport protein:**

Many small molecules, organic and inorganic are transported in the bloodstream and extracellular fluids, across the cell membranes and inside the cells from one compartment to

another, by specific proteins. Examples: Haemoglobin a protein molecule in the red blood cells, which carries oxygen from the lungs to the tissues for respiration.

Transferrin a blood plasma protein that transfers iron from storage sites in the body to locations where cells synthesizing iron proteins reside.

Lipoproteins — transport lipids within the bloodstream.

Membrane carriers — facilitate the movement of charged and polar molecules and ions across the lipid bilayer structure of the cell membranes.

► Storage protein:

They serve as isms. Biological reserves of metal ions and amino acids, used by organ. Examples: Ferritin intracellularly stores excess iron in a non-toxic form. Ovalbumin an amino acid storage protein. Glutelin a major rice seed storage protein (SSP).

► Structural protein:

Proteins have pivotal role in the stabilization of many structures. Examples: a-keratins, collagen and elastin, myosin, fibroin. Proteins

► Contractile protein:

They generate movement. An example is myosin, responsible for the contraction of the muscle fibres. They are also involved in nerve transmission. An example is the receptor for acetylcholine at synapses.

► Genetic protein:

Some proteins are involved in the regulation of gene expression. They control development and differentiation. An example is the nerve growth factor (NGF).

► Hormonal protein:

They are regulatory molecules involved in the control of many cellular functions, from metabolism to reproduction. Examples are insulin, glucagon and thyroid-stimulating hormone (TSH).

►Immuno protein:

The antibodies or immunoglobulins are glycoproteins that protect against harmful agents. They can recognize antigens expressed on the surface of viruses, bacteria and other infectious agents.

► **Storage of energy:**

Proteins, and in particular the amino acids that constitute them, act as energy storage, second in size only to the adipose tissue, that in particular conditions, such as prolonged fasting, may become essential for survival.

3.5.4. Based on solubility:

Simple proteins yield on hydrolysis, only amino acids. These proteins are further classified based on their solubility in different solvents such as water, salt and alcohol as well as their heat coagulability.

Albumins are readily soluble in water, dilute acids and alkalies. They are coagu-lated by heat.

Globulins are insoluble or sparingly soluble in water but their solubility is greatly increased by the addition of neutral salts such as sodium chloride. These proteins are coagulated by heat.

Prolamins are insoluble in water but soluble in 70-80% aqueous alcohol.

Glutelin are insoluble in water and absolute alcohol but soluble in dilute alkalies and acids.

Histones are small and stable basic proteins. They contain fairly large amounts of basic amino acid, histidine. They are soluble in water, but insoluble in ammonium hydroxide. They are not readily coagulated by heat.

Protamine is the simplest of the proteins. They are soluble in water and are not coagulated by heat. They are basic in nature due to the presence of large quantities of arginine.

Albuminoids are characterized by great stability and insolubility in water and salt solutions.

3.6 Biological Importance of Proteins:

Proteins are at the centre of action in biological processes. Proteins are essential for the main physiological processes of life and perform functions in every system of the human body. A protein’s shape determines its function. Nearly all the molecular transformations that define cellular metabolism are mediated by protein catalysts. Proteins also perform regulatory roles, monitoring extracellular and intracellular condition and relaying information to other cellular

components. In addition, proteins are essential structural components of cells. Proteins are vital for the growth and repair and they have enormous diversity of biological functions.

► All enzymes are proteins.

► Some hormones are derived from proteins, for example insulin that regulates glucose absorption into cells, secreted by beta cells of the pancreas, deficient in diabetics.

► They act as structural components such as keratin of hair and nail, collagen of bone etc.

► Proteins are the molecular instruments through which genetic information is expressed.

► They execute their activities in the transport of oxygen and carbon dioxide by haemoglobin and special enzymes in the red cells. Transport proteins present in the cell membrane play a role in nutrient transport.

► They function in the homeostatic control of the volume of the circulating blood and that of the interstitial fluids through the plasma proteins.

► They are involved in blood clotting through thrombin, fibrinogen and other protein factors.

► They act as the defence against infections by means of protein antibodies/immuno globulins produced by white blood cells in response to a specific antigen.

► They perform hereditary transmission by nucleoproteins of the cell nucleus.

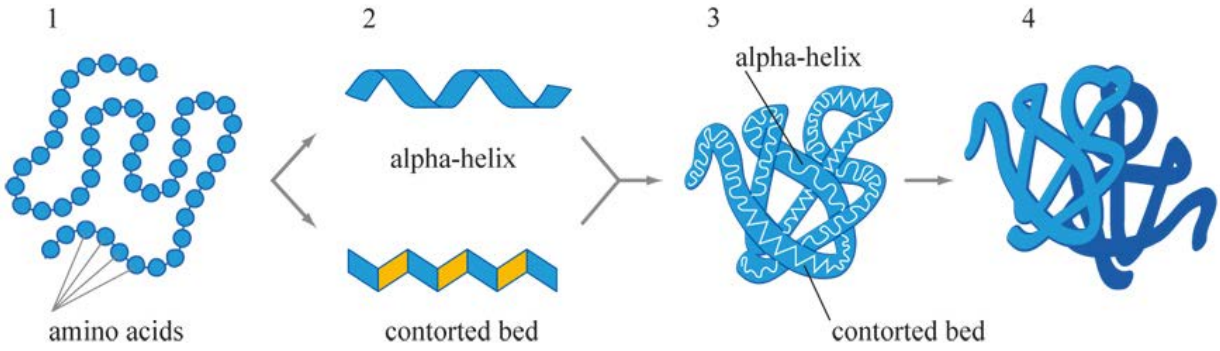
► Storage proteins can be mobilized and utilized for the maintenance and growth of organisms. They are particularly prevalent in plant seeds, egg whites and milk. Actin, myosin act as contractile protein important for muscle contraction.

► Some of the amino acids in proteins can be disassembled and provide energy when carbohydrate and fat intake is inadequate.

3.7 Four Levels of Protein Structure:

Like all polymeric molecules, proteins can be described in terms of levels of organization; in this case, their primary, secondary, tertiary, and quaternary structures. Primary structure means the linear sequence of amino acids in polypeptide chain. Secondary structure is concerned with local spatial arrangement of a polypeptide’s backbone atoms without regard to the conformations of its side chains. Tertiary structure denotes overall arrangement and interrelationship of the various regions or domains of a single polypeptide chain. Quaternary

structure results when the proteins consist of two or more polypeptide chains held together by non-covalent forces. Protein structures are determined by X-Ray Crystallography, Nuclear Magnetic Resonance and Cryo-Electron Microscopy.



3.7.1. Primary structure:

A protein's primary structure is the linear sequence of amino acids within a poly-peptide chain or chains if the protein consists of more than one polypeptide. The primary structure is held together by peptide bonds that are made during the pro-cess of protein biosynthesis. In general, proteins contain at least 40 residues or so; polypeptides smaller than that are simply called peptides. The largest known single polypeptide chain belongs to the 35,213-residue titin a giant (3906 kD) protein that helps to arrange the repeating structures of muscle fibres. The primary structure of a protein drives the folding and intramolecular bonding of the linear amino acid chain leading to the natural three-dimensional shape of the protein molecule. So the higher levels of organization are dependent on the primary structure.

DNA carries the genetic information for making proteins. The four bases A, T, Cand G make up the genetic code. The base sequence ultimately determines the unique sequence of amino acids in each peptide chain (Chapter 5). Even changing just one amino acid in a protein's sequence can affect the protein's overall structure and therefore function. For instance, sickle cell anaemia, an inherited disease that affects red blood cells, is related to a single amino acid change (glutamic acid replaced by valine) in one of the polypeptide chains that make up haemoglobin.

3.7.2. Secondary structure:

The next level of protein structure, secondary structure, refers to local folded structures that form within a polypeptide due to interactions between atoms of the backbone. Polypeptide's backbone conformation can be specified by the torsion angles (rotation angles or dihedral

angles) about the C-N bond and the C-C bond of each of its amino acid residues. There are several steric constraints on the torsion angles of a polypeptide backbone that limit its conformational flexibility. The pioneer work by Indian scientist G. N. Ramachandran can determine sterically allowed values of angles, thereby indicating the allowed conformations of polypeptides. The Proteins most common secondary structures are the α-helix and the B-sheet, described by scientists Linus Pauling and Robert Corey in 1951. The a-helix and the □-sheet allow the polypeptide chain to adopt favourable angles. They are repetitive regular structures because they are composed of sequences of residues with repeating angular values. Both structures are held in shape by hydrogen bonds formed between the carbonyl oxygen of one amino acid and the amino hydrogen of another. In a-helix, the polypeptide chains are stabilized by intramolecular hydrogen bonding whereas □-sheet is stabilized by intermolecular hydrogen bonding. The following figures describes two major fundamental structural motifs of protein structure: α-helix and β-sheet.

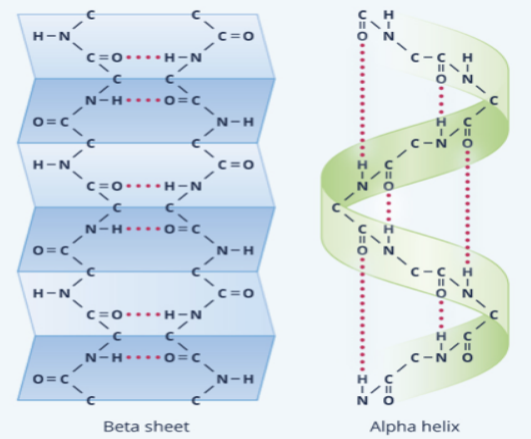


Fig 1. Secondary Structure: Notice how the beta sheet has two amino acid chains running side by side, whilst the alpha helix has coiling. The red dots are hydrogen bonds which hold together both the beta pleated sheets and the alpha helix.

3.7.2.1 About alpha helix:

The first protein structure proposed by Pauling and Corey is known as alpha helix, named after the fibrous protein, alpha keratin. The helix is the only polypeptide helix that has both favourable hydrogen bonding pattern. The alpha helix is a spiral structure (Fig. 2.5a). The

polypeptide bonds form the backbone and the side chains of amino acids extend outward from the outer helical surface. It is stabilized by extensive intramolecular hydrogen bonding.

The backbone hydrogen bonds are arranged in such a way that the peptide C=O bond of the nth residue points along the helix axis toward the peptide N-H group of the (n + 4) th residue (e.g., the carbonyl of amino acid 1 would form a hydrogen bond to the N—H of amino acid 5). This results in a strong hydrogen bond that has the nearly optimum N...O distance of 2.8 Å. This pattern of bonding pulls the polypeptide chain into a helical structure that resembles a curled ribbon. Amino acid side chains project outward and downward from the helix to avoid steric interference with the polypeptide backbone and with each other. The hydrogen bonds are roughly parallel to the axis of the helix.

There are 3.6 amino acid residues in each turn of an a-helix, which means that there is one residue every 100 degrees of rotation (360/3.6). Each residue is translated 1.5 Å along the helix axis, which gives a vertical distance of 5.4 Å between structurally equivalent atoms in a turn. The alpha-helix ideally has torsion angles $ = -57° and Y = -47°. It is right-handed; i.e., it turns in the direction that the fingers of a right-hand curl when its thumb points in the direction that the helix rises.

Certain amino acids disrupt the regularity of the a-helical backbone conformation. Amino acids whose -R groups are too large (tryptophan, tyrosine) or too small (glycine) destabilize a-helices. Proline also destabilizes a-helices because its back-bone nitrogen is bonded to its cyclic side group and cannot participate in hydrogen bonding. Proline and glycine are sometimes called as “helix breaker”. Some amino acid residues in a peptide sequence (e.g., alanine, leucine, glutamic acid and histidine) promote a-helical assembly.

The alpha-helix is abundant in transport proteins like haemoglobin and myoglobin. Storage protein ferritin has a huge hollow spherical structure, with a wall mostly made up of a-helical peptide chains. Alpha-helix is virtually absent in chymotrypsin.

3.7.2.2 About β-Pleated Sheets:

The second major type of polypeptide secondary structure is known as □-Pleated Sheets (□ comes after a). Here, neighbouring segments of polypeptide chains are held together by intermolecular hydrogen bonds, forming a sheet-like structure. The hydrogen bonds are formed between carbonyl and amino groups of adjacent back-bones, while the -R groups on each polypeptide chain alternately extend to opposite sides of the sheet. The polypeptide chains in

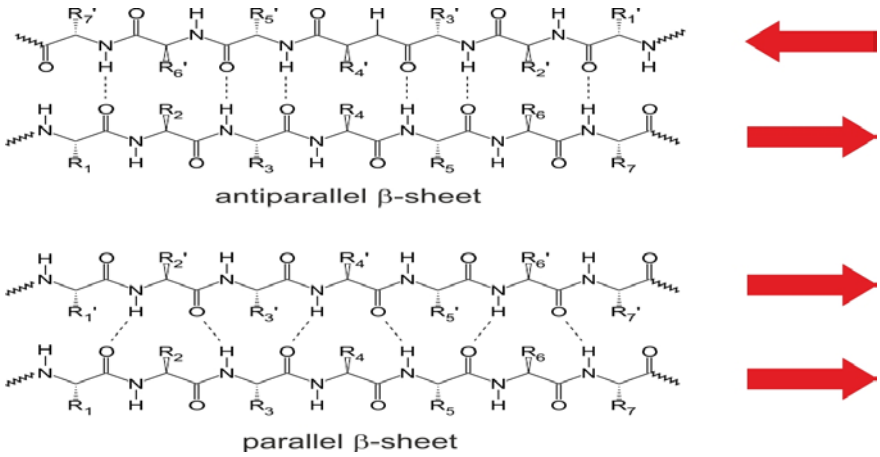
beta-sheet are almost fully extended. The distance between adjacent amino acids is 3.5 Å. The conformations in which these B-structures are optimally hydrogen bonded vary somewhat from that of the fully extended conformation (= 4 = ±180°). They therefore have a rippled or pleated edge-on appearance and for that reason are sometimes called “pleated sheets”

. Thus, each strand of a □-sheet has a two-residue repeat with a repeat distance of 7.0 Å. B-Sheets in proteins contain 2 to as many as 22 polypeptide strands, with an average of 6 strands. Each strand may contain up to 15 residues, the average being residues.

There are two varieties of Sheets:

1. The antiparallel β-sheet: In this neighbouring hydrogen-bonded polypeptide chains run in opposite directions.

2. The parallel β-sheet: In this the hydrogen-bonded chains extend in the same direction.

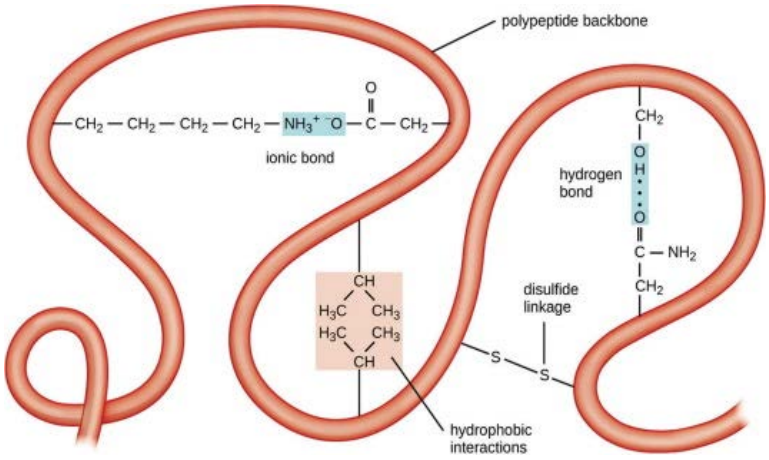


Amino acids such as tryptophan, tyrosine, and phenylalanine, which have large ring structures in their -R groups, are often found in □-pleated sheets, perhaps because the B-pleated sheet structure provides plenty of space for the side chains. Beta-pleated sheet is the major structural motif in proteins like silk fibroin (antiparallel), flavodoxin (parallel) and carbonic anhydrase (both).

3.7.3. Tertiary structure:

The overall three-dimensional structure of a polypeptide is called its tertiary structure.The tertiary structure of a protein describes the folding of its secondary structural elements and

specifies the positions of each atom in the protein, including those of its side chains. The tertiary structure is maintained by hydrogen bonding, eletonic (electrostatic) bonding, dipole-dipole interactions and van der Waals forces basically, the whole range of non-covalent interactions. Ionic bonds are formed by charged groups of acidic and basic amino acids. Positive charges (NH_3) are provided by of lysine, guanidinium group of arginine and imidazolium group of histidine while negative charges (COO) are provided by beta and gamma carboxyl groups of aspartic and glutamic acids. In hydrogen bonds, polar -R groups on the amino acids form bonds with other polar -R groups. Hydrophobic interactions are very important to this structure, in which neutral amino acids with nonpolar, hydrophobic -R groups cluster together on the interior of the protein. On the other hand, hydrophilic amino acids are on the surface of the protein to interact with surrounding water molecules. Finally, there's one special type of covalent bond that can contribute to tertiary structure: the disulfide bond. A disulfide bond (-S-S-) is a covalent linkage between the sulfhydryl groups (-SH). Oxidation of two cysteine amino acids results in a disulfide bond. Cystine is a sulphur-containing amino acid obtained by the oxidation of two cysteine molecules which are then linked via a disulfide bond. The disulfide bond serves as a mechanical linkage that allows proteins to retain their three-dimensional structure. This bond is much stronger than the other types of bonds that contribute to tertiary structure. They act like molecular "safety pins," keeping parts of the polypeptide firmly attached to one another.

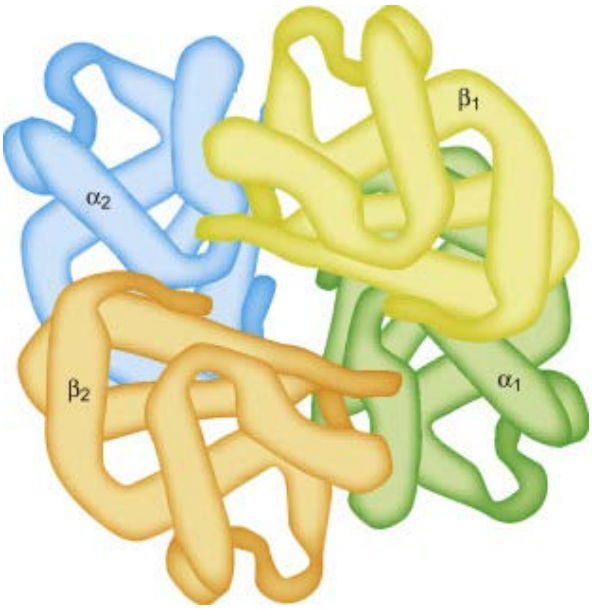


3.7.4. Quaternary structure:

Some proteins, particularly those with molecular masses >100 kD, consist of two or more polypeptide chains. Proteins with more than one subunit are called oligomers, and their identical units are called protomers. These polypeptide subunits associate with a specific

geometry. The spatial arrangement of these subunits is known as a protein's quaternary structure. A number of tertiary structures may fold into a quaternary structure.

The arrangement of the two alpha and two beta polypeptide chains in haemoglobin is an example of the structure of a protein. Other examples of proteins with quaternary structure include aspartate transcarbomylase, lactate dehydrogenase, DNA polymerase.

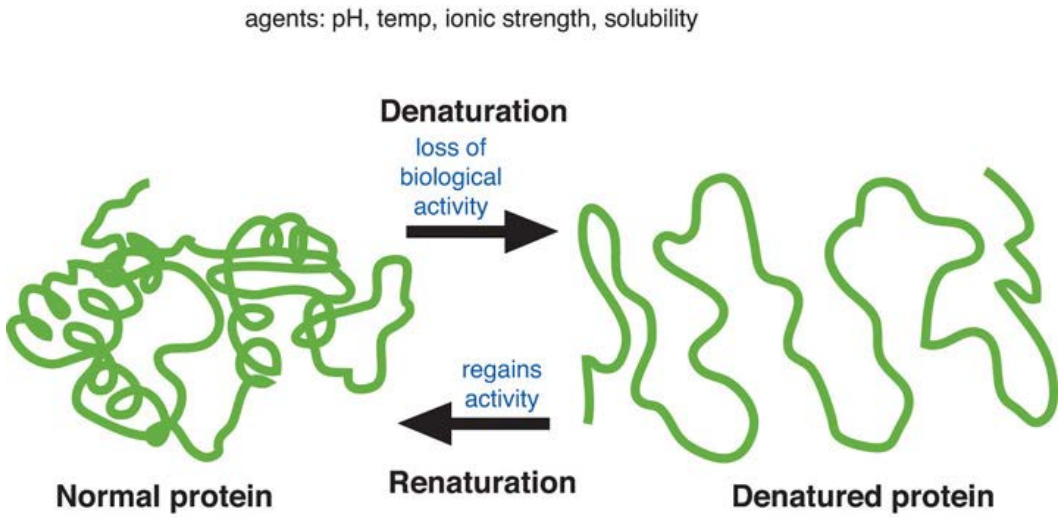


3.8 Denaturation of Proteins:

Sometimes, partial or complete unfolding of the native (natural) conformation of the polypeptide chain is caused by heat, mechanical action, acids or organic solvents Denaturation is the term used for any change in the three-dimensional structure of a protein that renders it incapable of performing its assigned function. In many instances the process of irreversible denaturation leads to coagulation. Coagulation of protein is defined as the change in the structure of protein to a semi-solid viscous precipitate. All the proteins are not heat coagulable, only a few like the albumins, globulins are heat coagulable. Enzymes may also cause protein coagulation e.g. cheese making.

We have encountered denaturation in our daily life while frying an egg. The heat from a pan denatures the albumin protein in the liquid egg white and it becomes insoluble. The clear egg white turns opaque as the albumin denatures and coagulates. Another example is heating milk. You may have noticed that when you heat milk to just below boiling point, a skin forms. This

is due to the milk protein coagulating. The low conformational stabilities of native proteins make them easily susceptible to denaturation.



3.8.1. Characteristics of Denaturation:

Denatured proteins can exhibit a wide range of characteristics, from conformational change and loss of solubility to aggregation due to the exposure of hydrophobic groups.

(1) The phenomenon of denaturation causes disorganization (unfolding) of native protein structure resulting in the loss of secondary, tertiary and quaternary structure of proteins

(ii) But the primary structure with its peptide linkages remains intact.

(iii) Denaturation involves the breaking of many of the weak linkages (e.g., hy-drogen bonds) or chemical bonds (disulphide bonds) within a protein molecule that are responsible for the highly ordered structure of the protein in its natural (native) state. So structure of denatured protein is looser and more random.

(iv) During the process, solubility is decreased while precipitability of the protein is increased.
(v) Denaturation causes loss of biological function of the protein.

(vi) Denatured proteins have more exposed sites for action of proteolytic enzymes. Since cooking leads to denaturation of proteins, cooked foods are more easily digested.

(vii) Denaturation is usually irreversible.

(viii) Denatured proteins can sometimes be re-natured when the physical agent is removed.

3.8.2. Factors Responsible for Denaturation:

Proteins can be denatured by a variety of conditions and substances:

(1) Heating causes a protein's conformationally sensitive properties, such as optical rotation, viscosity, and UV absorption, to change abruptly over a narrow temperature range. Such a sharp transition indicates that the entire polypeptide unfolds or "melts" cooperatively, that is, nearly simultaneously. Most proteins have melting temperatures that are well below 100°C.

(ii) pH variations alter the ionization states of amino acid side chains, thereby-changing protein charge distributions and hydrogen-bonding requirements.

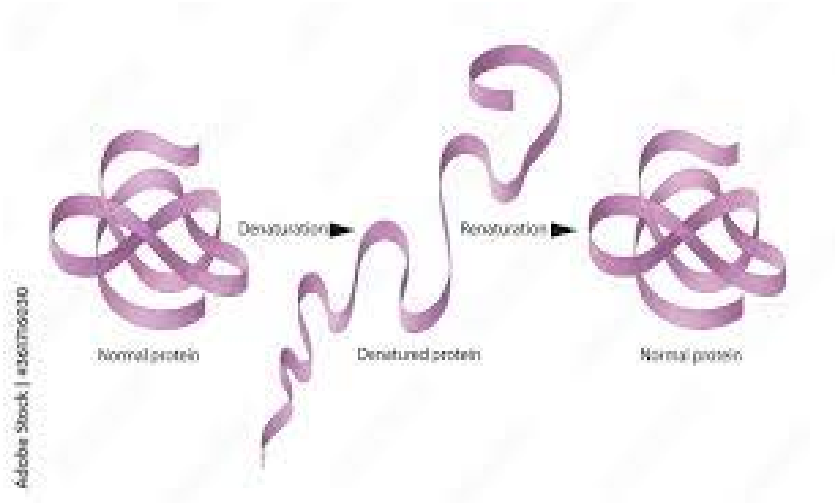
(iii) Detergents associate themselves with the non-polar residues of a protein, thereby interfering with the hydrophobic interactions responsible for the protein's native structure.

(iv) The chaotropic agents guanidinium ion and urea, in concentrations in the range 5 to 10 M, are the most commonly used protein denaturants. Chaotropic agents are ions or small organic molecules that increase the solubility of nonpolar substances in water.

3.9. Renaturation:

Many denatured proteins can be renatured. Given the proper circumstances and enough time, a protein that has unfolded under sufficiently gentle conditions can refold and may again exhibit biological activity.

In 1957, the novel experiment of Christian Anfinsen on ribonuclease A (Rnase A), a 124-residue single-chain protein, established that proteins can be denatured reversibly. In an 8 M urea solution containing 2-mercaptoethanol. In order to renature it, first Rnase A is completely unfolded and its four disulfide bonds are reductively cleaved the urea and reductant are dialyzed away. Then the resulting solution is exposed to O2 at pH 8 which oxidizes the -SH groups to form disulfides again. This yields a protein that is virtually 100% enzymatically active and physically indistinguishable from native Rnase A.



3.10 Summary:

Proteins are chemically polyamides of α-amino carboxylic acids; in fact, a single protein molecule is composed of hundreds or even thousands of amino acid units; all naturally occurring α-amino acids belong to the L-series. The proton transfer that occurs in any amino acid internally gives internal salt, ‘dipolar ion’, or ‘zwitterion’. Fibrous proteins are fibre-like in shape, with a structure consisting of β-pleated sheets held together by intermolecular hydrogen bonding and these proteins are insoluble in water. Both proteins as well as amino acids can be classified into several categories as mentioned in this unit in detail. Globular proteins are more complex, with a compact, roughly spherical/oval structure and they are held together by intramolecular hydrogen bonding; and they have an α-helix. The basic, secondary, tertiary, and quaternary structures of proteins are the levels of organisation that may be used to characterise them. From structural changes and lack of solubility to aggregation brought on by the exposure of hydrophobic groups, denatured proteins can display a broad spectrum of traits. In our everyday lives, we have come across denaturation when cooking an egg. It is possible to renature a lot of denatured proteins. Renaturation is the process by which a protein that has unfolded under mild enough conditions can refold and potentially regain biological function given the right conditions and sufficient time.

3.11 Model Questions:

1. Write the general structure of α-amino acid.

2. Why amino acids are called alpha amino acids?

3. Give differences between fibrous and globular proteins.

4. Write three biological roles of proteins.

5. Give examples for transport protein and storage proteins each.

6. Compare and contrast the structural features of α-helix and β-pleated sheet structures of proteins.

7. Mention the forces that are responsible for the stability of secondary structure of protein.

8. what do you mean by denaturation of protein?

9. Write two evidences behind zwitterionic structure of amino acid.

10. Give examples of Hydroxyl group containing amino acids.

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Unit 4 Isolation and Characterization of Carbohydrates and Proteins

Index

4.1 Objectives:

After reading this unit students can understand the followings

1. How carbohydrates are detected through qualitative ways.

2. Familiar with several qualitative tests.

3. How one can qualitatively identify proteins.

4. Gather idea on some processes like ion exchange chromatography, electrophoresis etc.

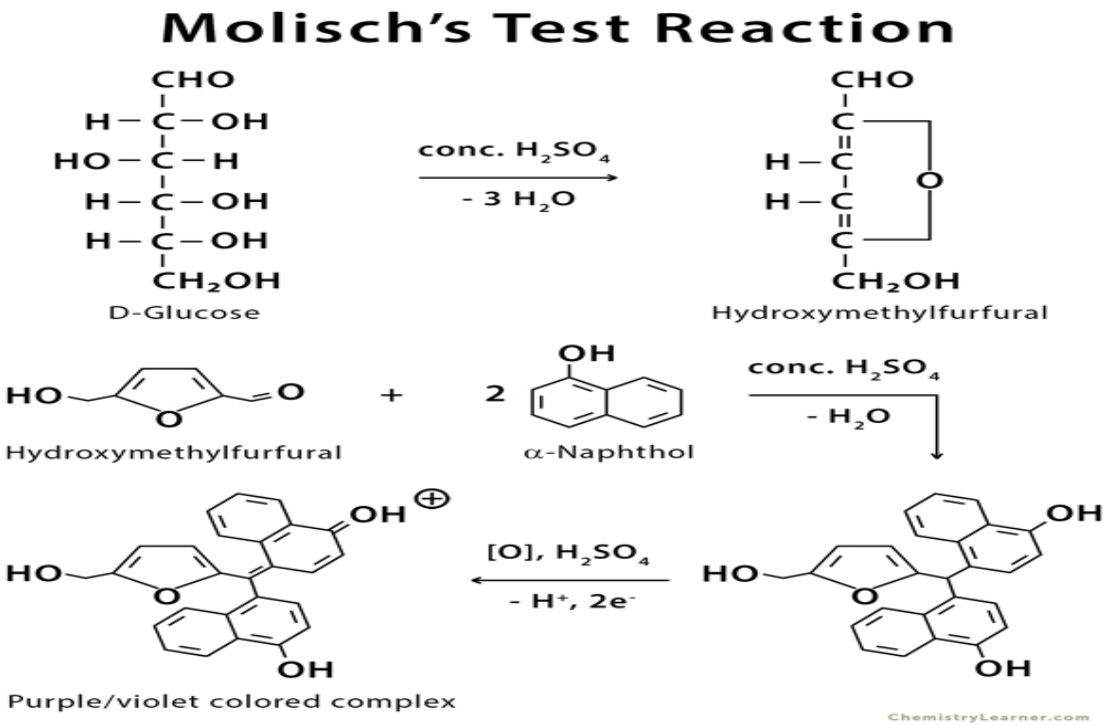
4.2 Introduction:

Carbohydrates and proteins are two important biomolecules controlling biological features of animal kingdom. Up to a particular range both are beneficial for human being. But the excess accumulation of both injurious to health. So, the detection of both is very much needful. In modern science both carbohydrates and proteins can be detected through qualitative as well as quantitative ways. This unit only deals with some famous qualitative ways of detection of carbohydrates and proteins.

4.3 Qualitative Detection of carbohydrates:

4.3.1 Molisch Test: 2 mL of the carbohydrate solution is taken in a clean and dry test tube. To this solution, 2 drops of an ethanolic solution of a-naphthol (Molisch Reagent: it is prepared by dissolving 2 g of α-naphthol in 20 mL of 95% of ethanol) is added and mixed by shaking. The test tube is inclined and 2 mL of concentrated sulfuric acid along the side of the test tube is added so as to form two layers. Appearance of reddish violet or purple coloured ring at the junction of the two layers indicates the presence of carbohydrate in the sample solution.

When a carbohydrate of aldopentose series or aldohexose series or any oligosaccharide is treated with concentrated sulfuric acid, it undergoes dehydration to yield furfural or 5-hydroxymethyl furfural. This furfural derivative undergoes condensation with α-naphthol under acidic condition to give the coloured products. The coloured reaction can be represented roughly in the following form:



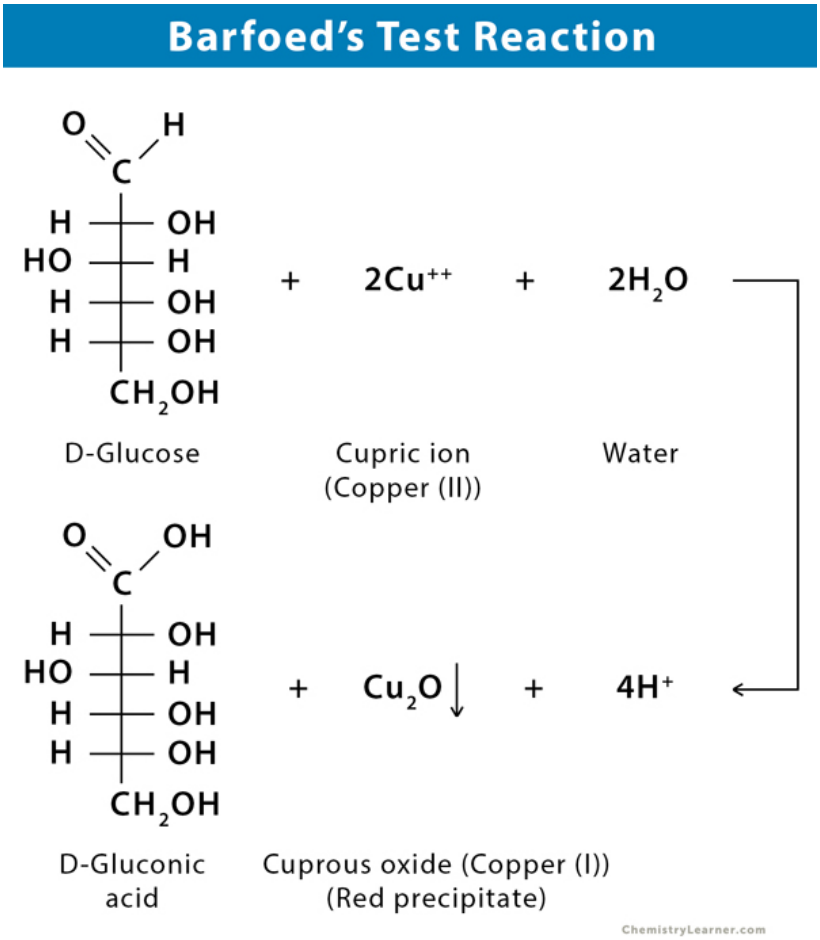
In this connection it may be mentioned that Molisch test is a sensitive but non-specific test and is given by all types of carbohydrates.

4.3.2 Benedict's Test: 5 mL of Benedict's solution (1 litre of Benedict's solution can be prepared on dissolving 100 g of anhydrous sodium carbonate, 173 g of sodium citrate and 17.3 g of copper (II) sulfate pentahydrate in distilled water) is taken in a clean and dry test tube. To this solution, 10 drops of carbohydrate solution is added. The reaction mixture is gently heated in hot water bath or over flame for about two minutes. This reaction mixture is then allowed to cool at room temperature. Change in coloration of solution from blue to green turbidity to yellow turbidity or the formation of a reddish precipitate within three minute indicates the presence of reducing sugars such as glucose or fructose or maltose.

Reducing sugar in the straight chain form contains an aldehyde group or a keto group. Under alkaline condition the reducing sugar tautomerizes to an enediol. Enediol is a strong reducing agent and thus reduces cupric ion in Benedict's solution to cuprous ion and itself is oxidized to sugar acid:

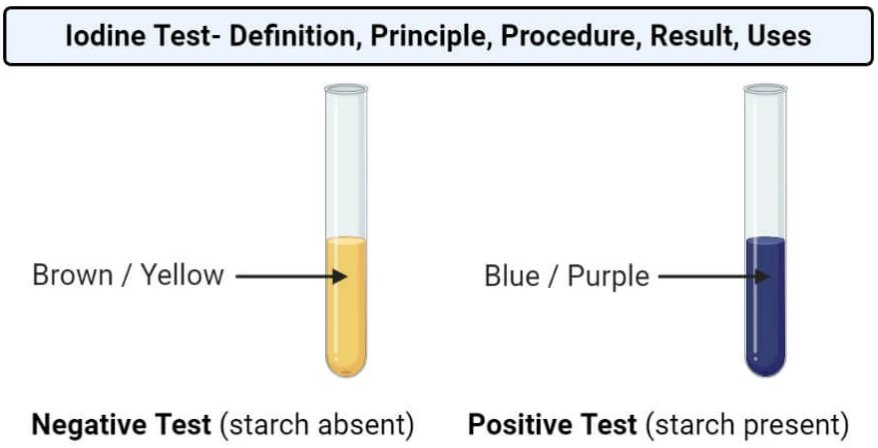
Now cuprous ion combines with hydroxide ion to first from the yellowish cuprous hydroxide which on heating gives the red precipitate of cuprous oxide.

4.3.3Barfoed's Test: 2 mL of Barfoed's Reagent (it is prepared freshly by dissolution of 1.33 g of cupric acetate in 20 mL of distilled water followed by filtration with addition of 4 drops of glacial acetic acid to the filtrate) is taken in a clean and dry test tube. To this solution 2 mL of sample solution is added. The reaction mixture is heated in hot water bath for just 3 minutes (over-heating should be avoided) and then the test tube is cooled under running water. Formation of scanty red precipitate indicates the presence of a monosaccharide in the sample solution.



Monosaccharide can reduce cupric ions even in weakly acidic conditions (pH ~ 4.5) to the red cuprous oxide just as in case of Benedict's test. This test is used to distinguish monosaccharides from disaccharides, even the reducing ones, by controlling the time of heating and pH of the reaction medium. Monosaccharides react very fast (within 3 minutes) whereas disaccharides react very slowly (about 8 minutes). Point is to be noted that there are a number of reducing disaccharides as well as non-reducing disaccharides. Since disaccharides contain acid labile acetal linkage, they respond favourably to Barfoed's test on prolonged heating due to hydrolysis under acidic condition. Evidently, this test can be utilized to distinguish glucose, a monosaccharide from sucrose, a non-reducing disaccharide.

4.3.4 Iodine Test (specific test for polysaccharides containing amylose such as glycogen and starch): To 3 mL of 1% aqueous solution of starch, 2 drops of 0.05 N iodine solution (it is prepared by dissolving 1.27 g of iodine and 3 g of pure potassium iodide crystals in 100 mL distilled water followed by dilution to 200 mL with 2% aqueous solution of potassium iodide) is added. Appearance of deep blue colour indicates presence of polysaccharides containing amylose. The colour disappears on heating but comes back after cooling the solution.



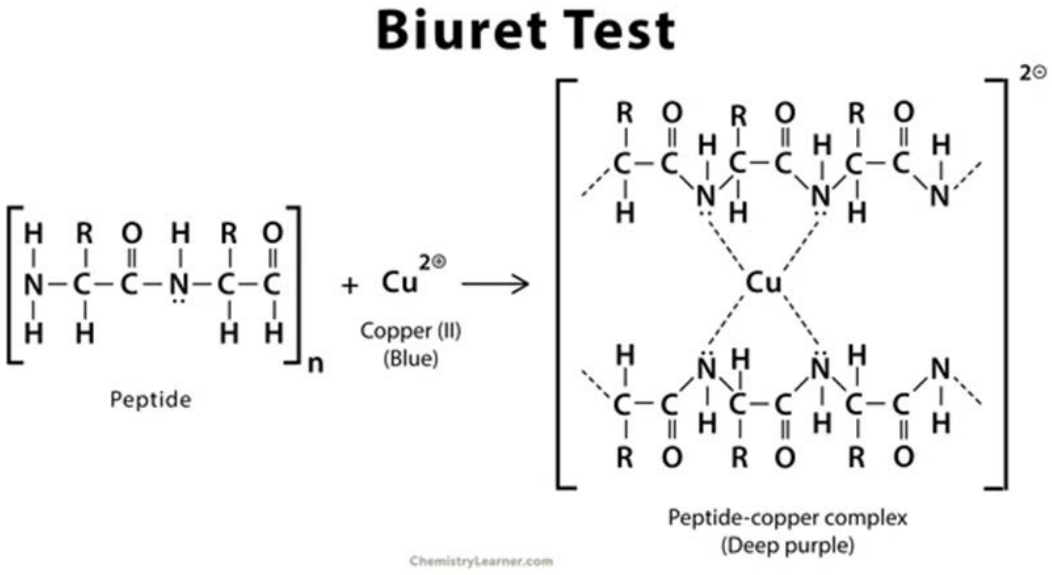
Starch and glycogen are made up of two components, water soluble amylose and water insoluble amylopectin. Of these two components, it is the linear amylase that is responsible for complexing with iodine-iodide mixture to furnish the blue colour. In this complex, amylose forms a helical structure with a central cavity that provides enough space to accommodate iodine atoms. They line up to produce a linear polyiodide chain which is deeply coloured.

4.4 Qualitative Detection of Proteins:

Proteins are polymers of amino acids. These are complex organic compounds containing nitrogen, hydrogen, carbon, oxygen and sulphur. Proteins are abundant in our everyday food, e.g. egg, soya bean, pulses, fish, milk etc. Presence of proteins can be confirmed qualitatively by several tests. Due to the presence of characteristic side chains in them, certain amino acids exhibit typical colour reactions that form the basis for their identification. The following tests are performed for the qualitative analysis of proteins.

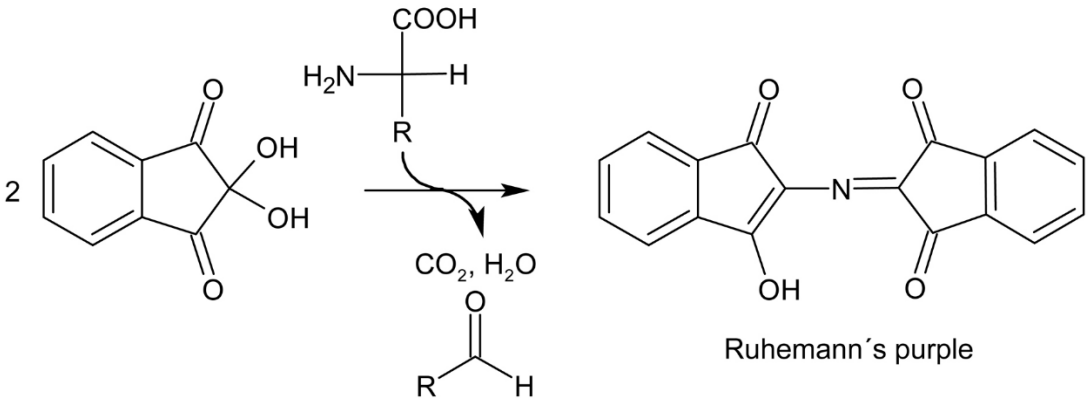
4.4.1 Biuret test: To perform this test to 2 mL of protein solution (10% egg-white or albumin), 2 mL 5% NaOH and 3 drops of 1% CuSO4 solution are added. In another test tube, which serves as the control, the same reagents are mixed with 2 mL of distilled water instead of the protein solution. Appearance of purple-violet or pink colour, as opposed to the blue colour in the control solution, indicates the presence of a protein.

Here Cu (II) ion forms a coordination complex with the proteins because of the presence of peptide bonds. Actually, the same colour is developed with any compound that has at least two CO-NH (peptide) linkage, such as biuret ($NH_2CO\text{-}NHCO\text{-}NH_2$) which is derived by condensing two molecules of urea. As a matter of fact, the test is named after this compound. As mentioned previously, the success of the test depends upon having at least two peptide bonds in the sample molecule so individual amino acids or dipeptides do not respond to this test. The complex formed between a protein and Cu (II) is believed to have the following structure:



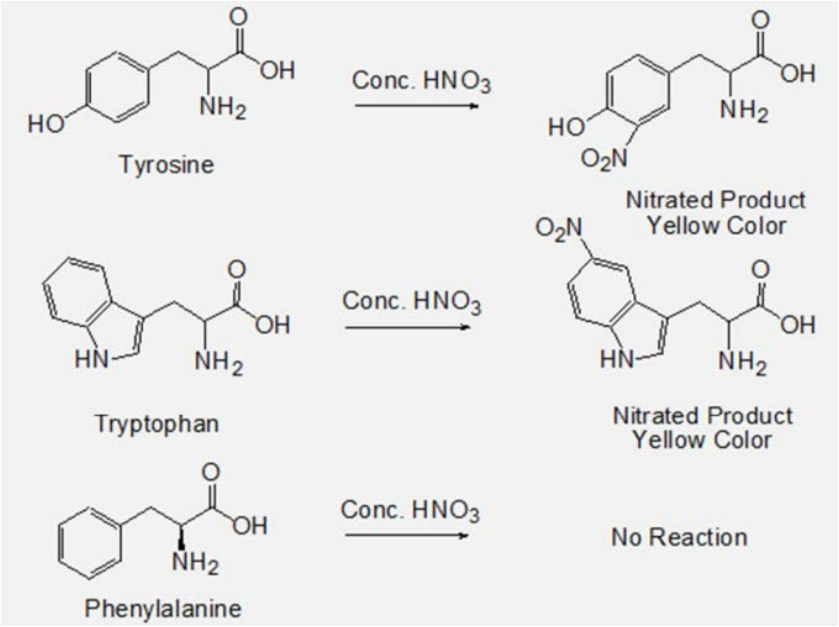
The colour varies depending on the number of peptide linkages; albumin/globulin give violet, proteoses purple and peptones dark pink colour indicating that albumin/globulins have largest number of peptide linkages and peptones the least.

4.4.2 Ninhydrin test: To carry out this test, 2-3 drops of freshly prepared 0.1% ninhydrin solution (in acetone) is added to 1 mL of protein solution taken in a test tube. The solution is boiled for 2 minutes and allowed to cool. Appearance of faint blue colour indicates presence of protein. Free amino acids give deep purple colour, except proline, that affords yellow colour. Here ninhydrin reacts with the free amino group of a-amino acids or proteins and after a series of steps finally affords the dye called Ruhemann's purple that is responsible for the colour:



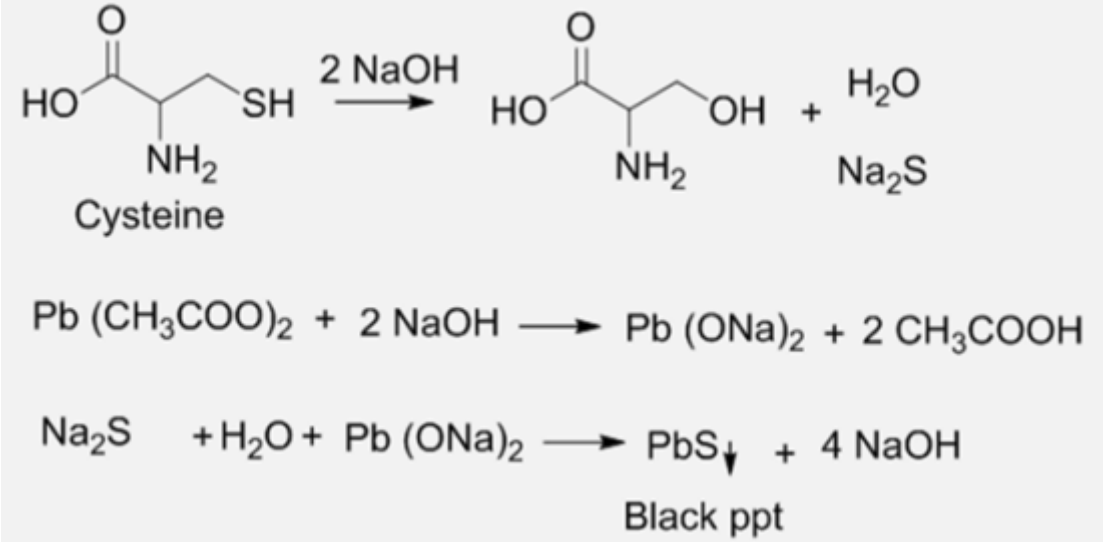
Ruhemann´s purple

4.4.3 Xanthoproteic Test: To carry out this test, 1 mL concentrated HNO3 is added to 2 mL of protein solution. The contents of the test tube are boiled. A yellow colour appears. The solution is then cooled and to it excess 40% NaOH solution is added. The yellow colour changes to orange. This indicates presence of aromatic amino acids such as tyrosine and tryptophan in the protein sample. The xanthoproteic test utilizes a nitration reaction which identifies the presence of activated benzene ring. The appearance of yellow colour is due to the nitration of the aromatic rings present in the two amino acids mentioned. Upon addition of alkali, the nitrated rings' substituents are ionized and colour turns to orange. This is because of extended conjugation which shifts the absorption maxima to a higher wavelength. The reaction is demonstrated using a tyrosine residue as an example:



Phenylalanine does not undergo nitration under this condition. This reaction is responsible for development of yellow stains upon the skin in case of accidental exposure to nitric acid.

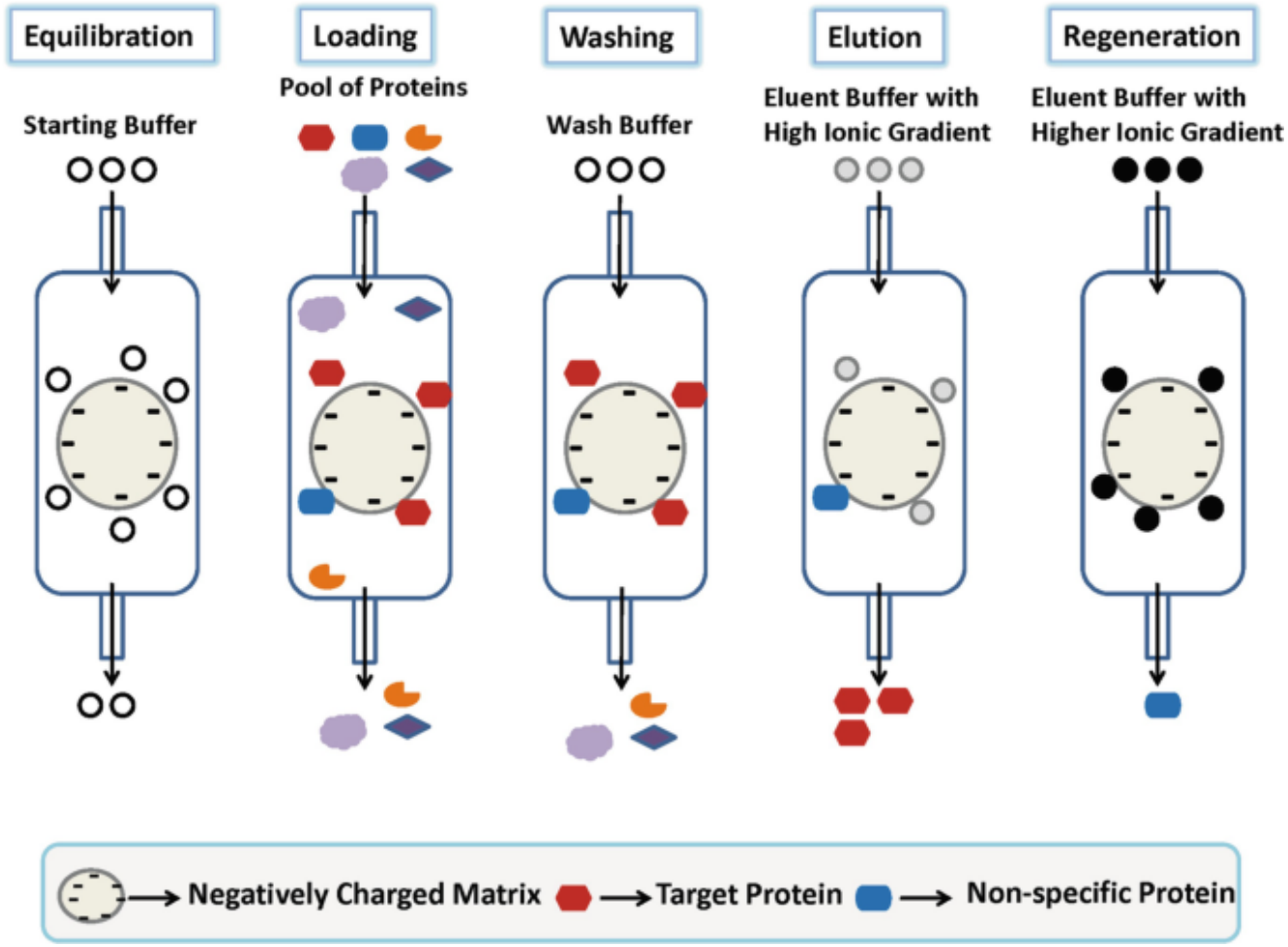
4.4.4 Lead acetate test (for sulphur-containing amino acids): This test is specific for sulphur-containing amino acid residues such as cysteine and cystine. To perform this test, 2 mL of 40% NaOH is added to 2 mL of sample solution and boiled for 3 minutes, cooled, then 2-3 drops of lead acetate solution is added. Appearance of black precipitate indicates presence of sulphur-containing amino acids. Here the organic sulphur in cysteine and cystine is released as inorganic sulphide ions which form the black precipitate of lead sulphide:



Methionine does not give this test as the sulphur group in this amino acid is in thioether linkage, which is difficult to break, and not released by treatment with alkali. Albumin and keratin respond favourably to this test, but casein (containing methionine) does not.

4.4.5 Ion Exchange Chromatography:

Chromatography involves interaction with mobile and stationary phases. Proteins and other polyelectrolytes (polyionic polymers) that bear both positive and negative charges can bind to both cation and anion exchangers. The binding affinity of a particular protein depends on the presence of other ions that compete with the protein for binding to the ion exchanger and also on the pH of the solution, which influences the net charge of the protein.

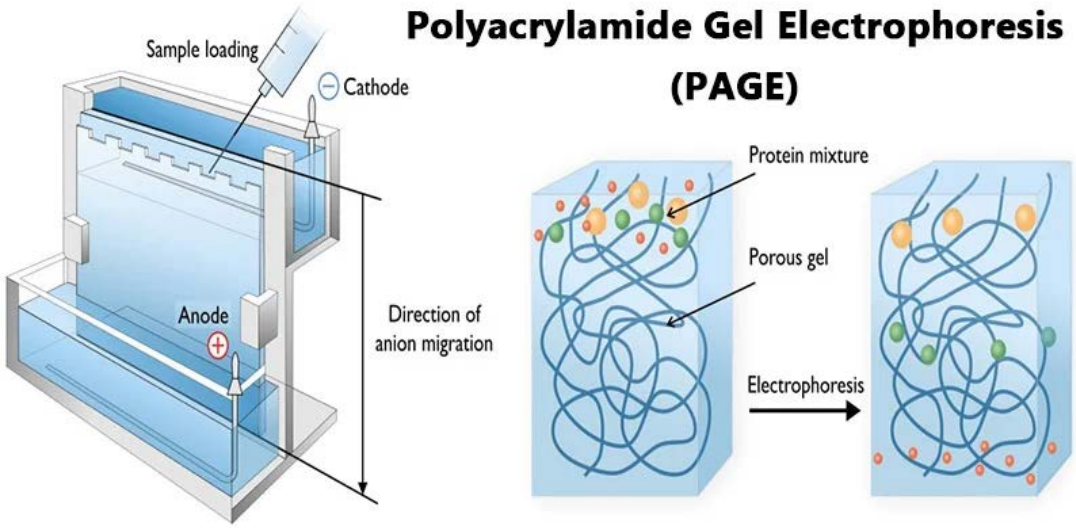


In purifying a given protein, the pH and the salt concentration of the buffer solution in which the protein is dissolved are chosen so that the desired protein is strongly bound to the selected ion exchanger. A small volume of the impure protein solution is applied to the top of a column containing the ion exchanger. The column is then washed with the buffer. Various proteins bind to the ion exchanger with different affinities. As the column is washed with the buffer, a process known as elution, those proteins with relatively low affinities for the ion exchanger move through the column faster than the proteins that bind to the ion exchanger with higher affinities.

This occurs because the progress of a given protein through the column is retarded relative to that of the solvent due to interactions between the protein molecules and the ion exchanger. The greater the binding affinity of a protein for the ion exchanger, the more it will be retarded. Thus, proteins that bind tightly to the ion exchanger can be eluted by changing the elution buffer to one with a higher salt concentration (and/or a different pH), a process called stepwise elution. With the use of a fraction collector, purification of a substance can be effected by selecting only those fractions of the column effluent that contain it.

4.4.6 Electrophoresis:

Electrophoresis, the migration of ions in an electric field is widely used for the analytical separation of biological molecules. Electrophoresis separates molecules ac-cording to charge and size.



Polyacrylamide gel electrophoresis (PAGE) of proteins is typically carried out in polyacrylamide gels with a characteristic pore size, so the molecular separations are based on gel filtration (size and shape) as well as electrophoretic mobility (electric charge). The pH of the gel is high enough (usually about pH 9) so that nearly all proteins have net negative charges and move toward the positive electrode when the current is switched on. Molecules of similar size and charge move as a band through the gel. The separated bands may be visualized in the gel by an appropriate technique, such as soaking the gel in a solution of a stain that binds tightly to proteins.

4.5 Summary:

Two significant macromolecules that regulate the biological characteristics of the animal world are proteins and carbohydrates. Both are advantageous to humans up to a certain point. However, too much of either can be harmful to one’s health. Therefore, it is imperative that both be detected. Both qualitative and quantitative methods can be used in modern research to identify proteins and carbs. Amino acid polymers make up proteins. Nitrogen, hydrogen, carbon, oxygen, and sulphur are all included in these complex organic molecules. Our daily

foods, such as eggs, soy beans, lentils, seafood, and milk, are rich in proteins. A number of assays can qualitatively establish the presence of proteins. Some amino acids have typical colour responses that serve as the foundation for their identification because of the existence of distinctive side chains in them. Only a few well-known qualitative methods of protein and carbohydrate detection are covered in this unit.

4.6 Model questions:

1. Give Molisch test for detection of carbohydrates qualitatively with relevant reaction.

2. What is Benedict's solution and how it is prepared?

3. Write Barfoed's test reaction.

4. Which test is specific for detection of polysaccharide and how it carried out?

5. Write the structure of complex obtained in Biuret test.

6. Write the structure of Ruhemann's purple and how you get it?

7. Which test is carried out to detect sulphur contain amino acid and write relevant reaction.

8. Write the full form of PAGE.

9. Write a short note on Ion-exchange chromatography.

10. Explain xanthoproteic test and give its limitation.

4.7 References:

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Unit 5 Basics of Enzymes

Index

5.1 Objectives:

One can understand the following after completion of this unit.

1. Classification and nomenclature system of enzyme.

2. Application of enzymes in several sectors.

3. Definition of apoenzyme, Holoenzyme, Prosthetic group etc.

4. Several classes of enzyme inhibition.

5. Clear idea on Biocatalyst and ribozymes.

5.2 Introduction

Enzymes are complex protein substances produced by living organisms that catalyse chemical reactions without being destroyed. Living systems are shaped by enormous variety of biochemical reactions, nearly all of which are mediated by a series of remarkable biological

catalysts known as enzymes. Chemically, enzymes are generally globular proteins. Enzymes catalyse all aspects of cell metabolism. This includes the digestion of food, in which large nutrient molecules (such as proteins, carbohydrates and fats) are broken down into smaller molecules, the conservation and transformation of chemical energy and the construction of cellular macromolecules from smaller precursors. Enzymes may act outside the producing organism and find application in industrial and medical fields.

5.3 Properties of Enzymes:

Enzymes allow chemical reactions to occur fast enough to support life. They are very efficient in their action; extremely high rates of chemical reaction are achieved by enzymes. Enzymes are only present in small amounts in the cell since they are not altered during their reactions. They are highly specific for their substrate. Generally, there is one specific enzyme for each specific chemical reaction. Most of the enzyme catalysed reactions are reversible. The reversibility of the reaction depends upon the requirements of the cell. In some cases, there are separate enzymes for forward and reverse reaction. They are affected by temperature and pH. There are cofactors and coenzymes that are necessary for some enzymatic catalysis.

5.4 IUBMB Nomenclature and Classification:

Enzymes are commonly named by appending the suffix ‘ase’ to the name of the enzyme's substrate or to a phrase describing the enzyme's catalytic action. Thus, urease catalyses the hydrolysis of urea and alcohol dehydrogenase catalyses the oxidation of primary and secondary alcohols to their corresponding aldehydes and ketones by removing hydrogen. Moreover, many enzymes, such as catalase were given names that provide no clue to their function.

The International Union of Biochemistry and Molecular Biology (IUBMB) initiated standards of enzyme nomenclature which recommend that enzyme names indicate both the substrate acted upon and the type of reaction catalysed. The systematic name of an enzyme is the name of its substrate(s) followed by a word ending in -ase specifying the type of reaction the enzyme catalyses according to its major group classification. Enzymes are classified and named according to the nature of the chemical reactions they catalyse. There are six major classes of reactions that enzymes catalyse. Moreover, for each class, there are many subclasses and sub-subclasses as well.

EC1.Oxidoreductases: Catalyse redox reactions i.e., transfer of electrons from reductants (electron donors) to oxidants (electron acceptors).

$$A_{red} + B_{ox} \leftrightharpoons Aox + B_{red}$$

Major subclasses:

► Oxidases: Use oxygen as an electron acceptor but do not incorporate it into the substrate. Example: Cytochrome P450

► Dehydrogenases: Use molecules like NAD^+, $NADP^+$, FAD for transferring hydrogen. Example: Alcohol dehydrogenase.

► Peroxidases: Catalyse the reduction of hydrogen peroxide

► Hydroxylases: Add hydroxyl groups to its substrates

 Oxygenases: Incorporate oxygen from molecular oxygen into organic substrates

► Reductases: Catalyse reductions.

EC2.Transferases: Catalyse the transfer or hydrogen among some substrates. exchange of certain groups other than hydrogen among some substate.

$$A\text{-}B + C = A + B\text{-}C$$

Major subclasses:

► Methyltransferase: Transfer one-carbon units between substrates

►Aminotransferase: Transfer $-NH_2$ from amino acids to keto acids

► Transaminase: Transfer nitrogenous groups

► Kinase (phosphotransferases): Energize molecules by transferring phosphoryl group from high energy ATP molecules to specific acceptor molecule

► Phosphorylase: Transfer phosphoryl group from inorganic phosphate (Pi) to an acceptor

EC3.Hydrolases: Accelerate the hydrolytic cleavage of C-O, C-N, C-C and some other bonds of substrates.

$$A\text{-}B + H_2O \rightarrow A\text{-}H + B\text{-}OH$$

Major subclasses:

► Lipase: Hydrolysis of triglycerides to produce fatty acids and glycerol

► Phosphatase: Cleave a phosphoric acid monoester into a phosphate ion and an alcohol

► Protease: Cleave the peptide bonds within proteins

► Urease: Hydrolysis of urea

EC4.Lyases: Promote the removal of a group (CO_2, H_2O, NH_3) from the substrate by cleaving C-C, C-O, C-N, and other bonds leaving double bonds or rings and also catalyse its reverse reaction, adding groups to double bonds.

$$AX\text{-}BY \leftrightharpoons A\text{-}B + X\text{-}Y$$

Major subclasses:

► Decarboxylase: Produce CO2 via elimination reactions

► Aldolase: Produce aldehydes via elimination reactions

► Hydratase: Add water to a double bond

► Synthase: Link two molecules without involvement of ATP

EC5.Isomerases: Facilitate the conversion of geometry or structure within a molecule.

$$A\text{-}B\text{-}C \leftrightharpoons A\text{-}C\text{-}B$$

Major subclasses:

► Racemases: Catalyse racemization

►Epimerases: Catalyse epimerization

► Cis-trans isomerases: Isomerization of geometric isomers

►Mutases: Catalyse intramolecular rearrangements

EC6.Ligases: Catalyse the synthesis of one molecular compound from two molecular substrates using hydrolysis of ATP or other tri-phosphate.

$$A + B + ATP \rightarrow A\text{-}B + ADP\text{-}+ Pi$$

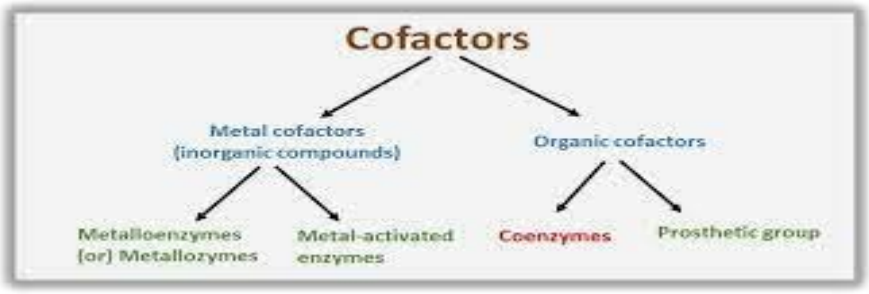
5.5 Cofactors, Apoenzyme, Holoenzyme, Coenzyme:

Cofactors are catalytically essential non-protein molecules or ions associated to the enzyme. Cofactors play an integral role in a number of cellular metabolism reactions playing both structural and functional roles to aid in the catalysis. Some enzymes require several cofactors.

Cofactors are considered as helper molecules for a biochemical reaction and can be either inorganic or organic in nature. These include (a) metal ions and (b) organic molecules.

A catalytically active enzyme-cofactor complex is called a **holoenzyme.** The enzymatically inactive protein resulting from the removal of an organic or inorganic cofactor from the holoenzyme is referred to as an **apoenzyme.**

Holoenzyme (active) = apoenzyme (inactive) + cofactor



Metal ion cofactors: Enzymes that require metal ions for their catalytic activity fall into two classes- metalloenzymes and metal-activated enzymes.

(1) **Metalloenzymes** are a broad group of enzymes that use a metal cation as a cofactor in the enzyme active site. They contain tightly bound metals that do not dissociate during isolation or dialysis of the enzyme under conditions where activity is retained. Metal ions, such as Cu2+, Fe^{3+} or Zn^{2+} are common cofactors. The essential nature of these cofactors explains why organisms require trace amounts of certain elements in their diets.

(ii) **Metal-activated enzymes** require a metal ion cofactor, but exhibit a lower affinity for the metal. So, the metal ion is not tightly held and can be exchanged easily with other ions.

The largest group of metal-activated enzymes contains the phosphotransferases that catalyse the transfer of the terminal phosphoryl group of ATP to an acceptor molecule that can be an alcohol, carboxylic acid, nitrogenous compound or a phosphorylated compound (ref. kinase). Their essential requirement for a bivalent metal ion is satisfied by Mg^{2+} or Mn^{2+}. The role of bivalent metal ions in the activation of phosphotransferases is to form an Mg2+-ATP complex that then acts as the true substrate (instead of ATP) for the reaction. Thus, the binary complex formed by the interaction of the enzyme and its nucleotide substrate is an enzyme-nucleotide-metal complex.

(b) Organic molecules as cofactors: The organic part can also be classified de-pending on how tightly they bind to an enzyme. According to International Union of Pure and Applied Chemistry (IUPAC), loosely-bound cofactors are termed coenzymes and tightly-bound cofactors are termed prosthetic groups. It is important to emphasize that there is no sharp division between loosely and tightly bound cofactors. Indeed, many such as NAD^+ can be tightly bound in some enzymes, while it is loosely bound in others.

(i) Coenzyme: A coenzyme is a substance that works with an enzyme to initiate or aid the function of the enzyme. Coenzymes are organic molecules and quite often bind loosely to the active site of an enzyme and assist in substrate recruitment. Coen-zymes are small, non-protein molecules that provide a transfer site for a functioning enzyme. They participate in enzymatic reactions as a dissociable carrier of chemical groups or electrons. Coenzymes cannot function on their own and require the presence of an enzyme. In order to complete the catalytic cycle, the coenzyme must return to its original state. Loosely bound cofactors can be regenerated in a subsequent reaction catalysed by a different enzyme. In that case, the cofactor can also be considered a substrate or co-substrate.

(ii) Prosthetic groups: Prosthetic group is a non-protein portion of another molecules, act as structural elements and act as charge carriers. Prosthetic group protein that is required for the protein's biological activity. They help proteins bind tightly or even covalently to the active site of enzyme and it is not easily separable by dialysis. An example of a prosthetic group is heme in haemoglobin, myoglobin, and cytochrome. The iron (Fe) found at the centre of the heme prosthetic group allows it to bind and release oxygen in the lung and tissues, respectively.

Vitamins are essential in very small (trace) amounts for the maintenance of normal metabolism. They generally cannot be synthesized at adequate levels by the body and must be obtained from the diet. The absence or shortage of a vitamin may result in a vitamin-deficiency disease. Coenzymes are often vitamins or made from vitamins. For example, Flavin adenine dinucleotide (FAD) is a vitamin derivative that participates in several intracellular oxidation-reduction reactions. It is covalently bonded to succinate dehydrogenase (SDH), a Krebs cycle enzyme. Some examples are listed.

5.6 Specificity of Enzyme:

Enzymes are highly specific in their action in comparison to chemical catalyst. Specificity is a characteristic property of the active site. The specificity of an enzyme is determined by the

functional groups of the substrate, the functional groups of the enzyme, and the physical proximity of these functional groups. It should be not that not all enzymes are highly specific. Particularly, digestive enzymes such as pepsin and chymotrypsin are able to act on varied types of proteins consumed as food. On the other hand, thrombin, which reacts only with the protein fibrinogen, is part o a very delicate blood-clotting mechanism. Thus, it must act only on one substrate in order to maintain the proper functioning of the system.

Enzymes show different types of specificity such as, Reaction specificity, Bond specificity, Substrate specificity and Stereoscopic specificity.

(i) Reaction specificity: The same substrate can undergo different types of reactions; a separate enzyme is needed for catalysing each reaction. This is referred to as reaction specificity. An amino acid can undergo transamination, oxidative deamination. decarboxylation, racemization etc. using different enzymes for each of these reactions.

(ii) Bond Specificity: Enzymes showing bond specificity are specific to substrates having similar bonds and similar structures. Bond specificity is also called relative specificity, a-amylase can hydrolyse the 1,4-glycosidic linkage in starch and glycogen Lipase can hydrolyse ester bonds between glycerol and any fatty acid. Most of the proteolytic (proteinase) enzymes exhibit bond specificity towards the peptide formed by any amino acid in the protein.

(iii) Group Specificity: The enzyme is specific to a group in structurally similar compounds. Group Specificity is also known as moderate specificity and is more than Bond Specificity. The digestive enzymes pepsin, trypsin and chymotrypsin are typical examples of this class. Pepsin can hydrolyse a peptide bond where amino group is contributed by an aromatic amino acid such as phenyl alanine, tyrosine and tryptophan. Trypsin can hydrolyse peptide bond in which amino group is contributed by any basic amino acid such as arginine, lysine and histidine. Chymotrypsin can hydrolyse a peptide bond in which carboxyl group is contributed by aromatic amino acid.

(iv) Substrate specificity: The substrate specificity is also absolute specificity. Some enzymes are absolutely specific which means they are capable of acting only on one substrate. For example, urease acts only on urea to produce ammonia and carbon dioxide. Carbonic anhydrase brings about the union of carbon dioxide with water to form carbonic acid. Thrombin reacts only with the protein fibrinogen, is part of a very delicate blood-clotting mechanism.

(v) Geometric specificity: Enzymes vary considerably in their degree of geometric specificity. The same enzyme can act on different substrates having similar molecular geometry, although with different efficiencies. For example, alcohol dehydrogenase catalyses the oxidation of ethanol (CH_3CH_2OH) to acetaldehyde (CH_3CHO) faster than it oxidizes methanol (CH_3OH) to formaldehyde (HCHO).

(vi) Stereospecificity: Enzymes are highly specific both in binding chiral substrates and in catalysing their reactions. This stereospecificity arises because enzymes, by virtue of their inherent chirality (proteins consist of only L-amino acids), form asymmetric active sites.

5.7 Enzyme Inhibitors:

The activity of enzymes can also be regulated by more specific inhibitors. Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Substances that reduce an enzyme's activity in this way are known as inhibitors. The binding of an inhibitor can stop a substrate from entering the enzyme's active site and/or hinder the enzyme from catalysing a reaction.

Importance of enzyme inhibition:

► Understanding regulation of enzyme activity in living cells.

► Elucidation of the cellular metabolic pathways by accumulation of intermediates.

► Helps in identification of catalytic or functional groups present at the enzyme active site

► Providing information on enzyme's substrate specificity.

► Studying the mechanism of catalytic activity

A large part of the modern pharmaceutical resource consists of enzyme inhibitors. Blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance. Such inhibitors can be used for the treatment of various disorders and diseases including asthma and chronic obstructive pulmonary disease, cardiovascular diseases, gastrointestinal disorders, hepatitis virus infection, herpes virus infections, human immunodeficiency virus (HIV)/ acquired immune deficiency syndrome (AIDS) and rheumatoid arthritis and related inflammatory diseases. Enzyme inhibitors are also used in pesticides. There are two broad categories of enzyme inhibition:

1. Irreversible inhibition and 2. Reversible inhibition.

5.7.1. Irreversible inhibition:

Irreversible enzyme inhibitors, bind to the enzyme so tightly that they permanently block the enzyme's activity. Such inhibitors bind covalently with the enzymes and deactivate them irreversibly. In this type of inhibition, the hindrance is of permanent nature, so these are also termed as catalytic poisons or inactivators. These inhibitors are usually toxic poisonous substances. Iodoacetate or Arsenate is irreversible inhibitor of the enzymes like papain and glyceraldehyde 3-phosphate dehydrogenase. Many specific enzyme inhibitors have been developed as therapeutic agents.

Penicillin, one of the most widely used antibiotics, functions by interfering with the synthesis of cell walls of reproducing bacteria. It does so by inhibiting an enzyme-transpeptidase. Human cells are not affected because they have cell membranes, not cell walls.

The nerve gases, especially Di-isopropyl fluorophosphate (DIFP), irreversibly inhibit biological systems by binding to a serine —OH group situated at the active sites of certain enzymes like serine proteases, acetyl choline esterase.

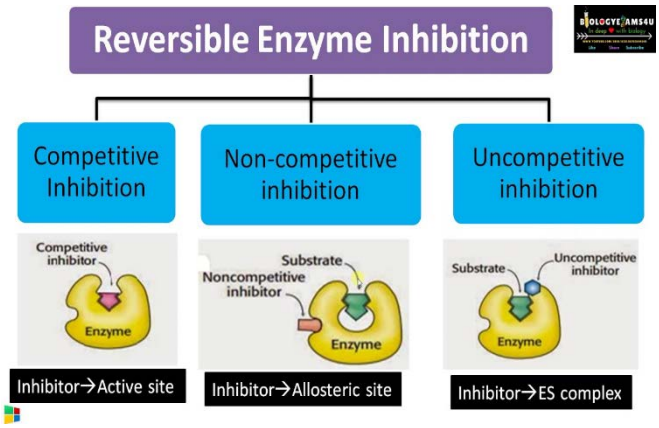
■ **Suicide inhibition:** Suicide inhibition is a special group of irreversible inhibition. A suicide inhibitor is a relatively inert molecule that is transformed by an enzyme into a more potent form that irreversibly inactivates the same enzyme.

Aspirin irreversibly inhibits the activity of the enzyme called cyclooxygenase (COX) which leads to the production of Prostaglandins (PGs), responsible for sensations of pain and processes of fever and inflammation. Aspirin acts by acetylating the hydroxyl of a serine residue in the active site of the COX enzyme. This makes Aspirin different from other NSAIDs (such as Diclofenac and Ibuprofen), which are reversible inhibitors.

The antiviral drug Acyclovir (ACV) has in-vitro inhibitory activity against the herpes simplex 1 and 2 viruses and Varicella-zoster virus. It is selectively converted into ACV-monophosphate by viral thymidine kinase itself. Further phosphorylation by cellular kinases generates the active triphosphate form, ACV-triphosphate. It targets the viral DNA polymerase, thereby inhibiting viral replication. 5-Fluorouracil, which is used in treatment of cancerous tumours, acts as a suicide inhibitor of thymidylate synthase.

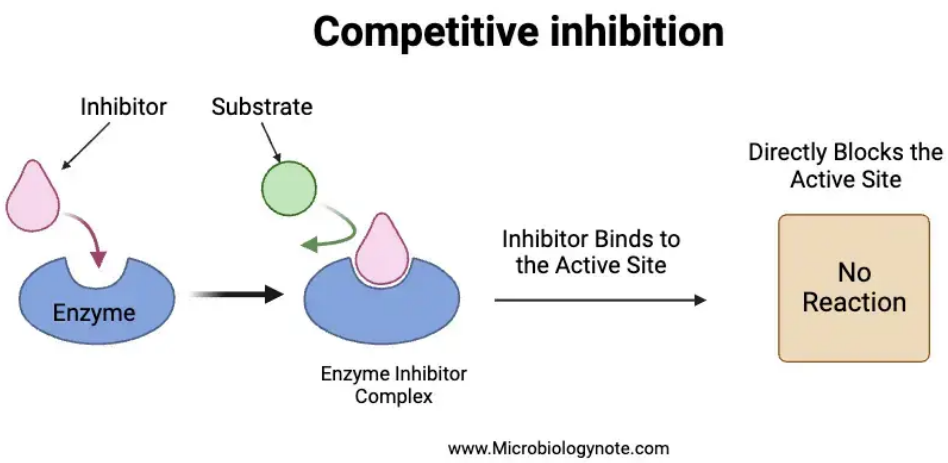
5.7.2. Reversible inhibitors:

Reversible inhibitors bind non-covalently to enzymes, and the enzyme inhibition can be reversed if the inhibitor is removed. Many different types of inhibition can occur depending on what the inhibitors bind to.



5.7.2.1 Competitive inhibition: A substance that competes directly with a normal substrate for an enzyme's substrate-binding site is known as a competitive inhibitor. Such an inhibitor usually structurally resembles the substrate and consequently occupies the active site of the enzyme. The effect of inhibitors can be overcome by increasing the substrate concentration at the binding site.

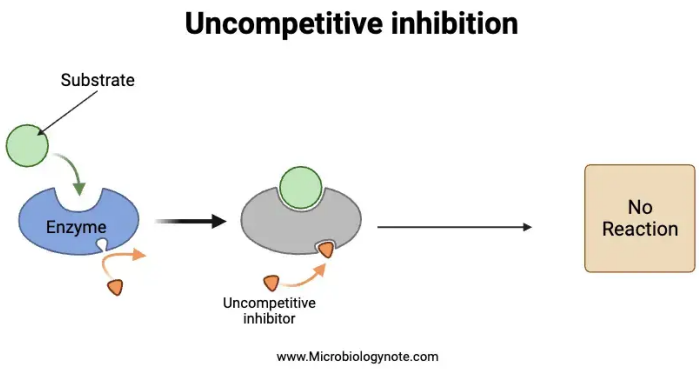
The enzyme succinate dehydrogenase (SDH) is a classic example of competitive inhibition with succinic acid as its substrate. Succinate dehydrogenase, a citric acid cycle enzyme that converts succinate to fumarate, is competitively inhibited by the compounds, namely, malonic acid, glutaric acid and oxalic acid, which structurally resembles succinic acid but cannot be dehydrogenated.



The simplest form of Sulpha drugs, sulphanilamide is an example of competitive inhibitor which acts as competitive antagonist of p-aminobenzoic acid (PABA) in the biosynthesis of folic acid. Sulphonamides replace PABA in the active site of the enzyme dihydropteroate synthetase blocking the metabolic pathway. This results in the in-ability of the microorganism to synthesize nucleotides and other metabolites essential for growth.

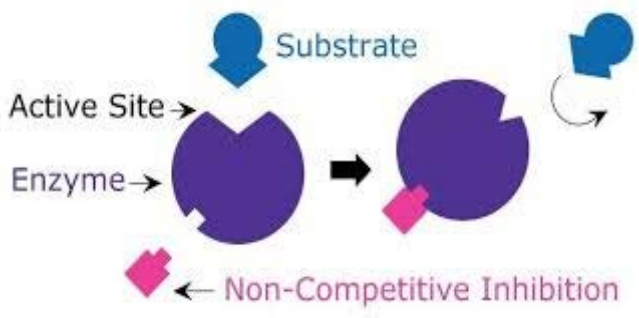
Statins such as Atorvastatin, Simvastatin helps to reduce plasma cholesterol level by targeting HMG CoA reductase. Discoumarol which is widely used as an anti-coagulant targets Vit K-epoxide reductase.

5.7.2.2 Uncompetitive inhibition: Uncompetitive inhibition is unique in that the inhibitor is binds exclusively to the enzyme-substate complex with little or no affinity for the free enzyme. This could imply that the binding site for the inhibitor is accessible only after the enzyme has bound to its substrate. This binding to an allosteric site changes the conformation of the enzyme in such a way that it loses its catalytic activity. The affinity of uncompetitive inhibitor is greatest at saturating concentration of substrate as then concentration of ES complex is high. Uncompetitive inhibitor binds directly to the enzyme-substrate complex but not to the free enzyme. Example: L-phenylalanine is the uncompetitive inhibitor for the enzyme alkaline phosphatase.



Nevirapine and Efavirenz provide interesting examples of clinically relevant uncompetitive inhibitors. The enzyme, Reverse Transcriptase is vital for virus replication. These Anti-HIV drugs are No Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), used in the treatment of AIDS.

5.7.2.3 Non-competitive and mixed inhibition: Non-competitive inhibitors bind to an allosteric site of the enzyme (a site on the enzyme which is not the active one). This results in a conformational change of the protein, distorting the active site. So as long as the non-competitive inhibitor is bound, the enzyme remains catalytically inactive. In fact, the inhibitor does not interfere with the enzyme-substrate binding, but the catalysis is prevented. Here, concentration of the substrate is meaningless. KM remains unchanged while Vmax will be lower.



For example, heavy metal ions (Ag^{+}, $Pb^{2+,}$ Hg^{2+} etc.) can non-competitively inhibit the enzymes by binding with cysteinyl sulfhydryl group of enzymes. Alanine non-competitively inhibits the enzyme pyruvate kinase.

5.8 Role of Enzyme in Green Chemistry:

Green chemistry is the design of chemical products and processes that reduce or eliminate the generation of hazardous substances. The goal of green chemistry is to design safer, more efficient, and less expensive chemical processes. Fundamental to the GC concept is the idea of sustainability - reducing environmental impacts and conserving natural resources for future generations. Therefore, green chemistry practices are intended to provide economic and environmental benefits to society as a whole. It is necessary for any chemical process, whether industrial or laboratory scale, to fulfil the following principles and guidelines of Green Chemistry:

Waste Prevention: Preventing waste is better than treating or cleaning up waste after it is created.

Atom economy: Synthetic methods should try to maximize the incorporation of all materials used in the process into the final product. This means that less waste 11-will be generated as a result.

Less hazardous chemical synthesis: Synthetic methods should avoid using or generating substances toxic to humans and/or the environment.

Designing safer chemicals: Chemical products should be designed to achieve their desired function while being as non-toxic as possible.

Safer solvents and auxiliaries: Auxiliary substances should be avoided wherever possible, and as non-hazardously as possible when they must be used.

Design for energy efficiency: Energy requirements should be minimized and processes should be conducted at ambient temperature and pressure whenever possible.

Use of renewable feedstocks: Whenever it is practical to do so, renewable feed-stocks or raw materials are preferable to non-renewable ones.

Reduce derivatives: Unnecessary generation of derivatives-such as the use of protecting groups-should be minimized or avoided if possible; such steps require additional reagents and may generate additional waste.

Catalysis: Catalytic reagents that can be used in small quantities to repeat a reaction are superior to stoichiometric reagents (ones that are consumed in a reaction).

Design for degradation: Chemical products should be designed so that they do not pollute the environment; when their function is complete, they should break down into non-harmful products.

Real-time analysis for pollution prevention: Analytical methodologies/need to be further developed to permit real-time, in-process monitoring and control before hazardous substances forms a

Inherently safer chemistry for accident prevention: Whenever possible, the sub-stances in a process and the forms of those substances, should be chosen to minimize risks such as explosions, fires and accidental releases.

5.9 Biocatalysis:

Biocatalysis is one of the most important tools for green chemistry. Biocatalysis is environmentally benign, and, because it can catalyse otherwise difficult transformations, it can eliminate multiple steps involved in complex chemical synthesis. Eliminating the steps reduces waste and hazards, improves yields, and cuts costs. Biocatalysis refers to the use of living (biological) systems or their parts to speed up (catalyse) chemical reactions. In biocatalytic processes, natural catalysts, such as enzymes, perform chemical transformations on organic compounds. Both enzymes that have been more or less isolated and enzymes still residing inside living cells are employed for this task.

Nowadays, a number of industrial processes use biocatalysis to produce valuable optically active pharmaceuticals, plant protecting agents and fragrance and cosmetics. The biological nature of enzymes makes them less hazard-fine chemicals, such as pus to health and less toxic to the environment than chemical catalysts. This favours their employment in the food and beverage industries too. The enzyme technology provided a new route for manufacturing bulk and high added-value products utilizing enzymes, in order to meet needs such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. The enzyme-based processes have gain momentum in various sectors such, as in washing and bioremediation, or for analytical and diagnostic purposes. They often result in shorter, less wasteful, environmentally and economically appealing processes when compared to conventional chemical syntheses.

5.10 Biological Importance of Enzymes:

1. Medical Enzymes:

(a) Therapeutic enzymes: Some enzymes are used in the treatment of various diseases.

► Collagenase for treating skin ulcers

► Glutaminase for treating Leukaemia

► Lactamase for treating penicillin allergy

► Streptokinase/ Urokinase for removing blood clots

(b) Diagnostic/Analytical reagents:

Enzymes can serve as eminent analytical reagents due to their highly specific nature and efficiency. They can be used in quantitative determination of substrate concentration. Alcohol dehydrogenase is one of the most useful enzymes for bioanalytical applications. There are many other examples of enzymes that can be used in diagnosis.

►Arginase: For L-arginine levels in plasma and urine

► Cholesterol esterase: For serum cholesterol levels

► Creatine kinase: For cardiac and skeletal malfunction

► Glycerol-3-phosphate dehydrogenase: Determination of serum triglycerides

► Uricase: For uric acid

(c) Biosensors:

Biosensors are electronic monitoring devices that make use of an enzyme's specificity and the technique of enzyme immobilisation. One of the most widely used biosensors is the glucose biosensor. The enzyme which can be used in glucose detectors is glucose oxidase.

2. Industrial Applications:

(a) Pharmaceuticals

► Insulin for controlling blood sugar

►Protease capsule for quicker healing and better stamina.

► Various digestive enzymes

(b) Food and brewing industry:

►Bread-making: Amylase in flour, maltase in yeast

►Cheese production: Rennin

►Alcoholic drinks: Amylase, maltase, glucanase

►Dairy products: Proteases, lactase

► Bakery: Glucose oxidase

(c) Paper and pulp industry:

Enzymes used in pulping increases the yield of fibre, lessen energy requirements and provide specific modifications to the fibre. Cellulases, hemicelluloses and pectinases have been used in paper and pulp industry.

(d) Agriculture industry:

Enzymes have been used as natural pesticide, herbicide, fertilizer and for odour remover and also added to animal feed to improve digestion of livestock. Enzymes are sprayed in animal farms to reduce infection and to keep insects away.

(e) Detergents:

The oily dirt is removed using lipases, the amylases and proteases are also added to the detergents to remove the stains.

(f) Textile:

Enzymes are used to facilitate contaminant removal and increase bond strength in recycled fibres. Peroxidases, laccases, cellulase and xylanase are some important enzymes used in fiber recycling.

3. Biofuel:

Biofuel production contributions to energy security and renewable energy targets worldwide. Enzymes can convert grains into fuel ethanol and also can assist the conversion of biomass for cellulosic ethanol. A new enzymatic trans-esterification process has been developed to address

prevailing concerns about biodiesel production technology. Lipases (triglyceride hydrolases) are mainly used for biodiesel production.

5.11 Ribozymes:

Ribozymes, also known as catalytic RNA, are catalytically active RNA molecules or RNA-protein complexes, in which solely the RNA provides catalytic activity. They are regarded as non-protein enzymes. The well-established natural ribozymes known to date are Ribonuclease P, peptidyl transferase 23S rRNA, hairpin, hammerhead, hepatitis delta virus (HDV), Varkud Satellite (VS). Ribozymes are found in the ribosome where they link amino acids during protein synthesis. Ribonuclease P (RNaseP) is a ribonucleoprotein nuclease which cleaves the tRNA precursors to generate mature 5' end of tRNA.

5.12 Summary:

Living things manufacture complex proteins called enzymes, which catalyse chemical processes without being destroyed. All facets of cell metabolism are catalysed by enzymes. The suffix "ase" is frequently added to the name of an enzyme's substrate or to a phrase that describes the enzyme's catalytic activity in order to give it a name. For every particular chemical reaction, there is often just one particular enzyme. The majority of processes catalysed by enzymes may be reversed. These days, biocatalysis is used in many commercial processes to create useful optically active medicines, plant protection agents, fragrances, and cosmetics. Ribozymes, often referred to as catalytic RNA, are RNA molecules or RNA-protein complexes that are catalytically active but in which only the RNA exhibits catalytic activity. Lastly, this unit highlights of several biological applications of enzymes.

5.13 Model Questions:

1. Highlight some properties of enzymes.

2. What do mean by ‘transferases? Discuss the subclasses of it.

3. Define prosthetic group and holoenzyme.

4. Write the relation between co-factor and apoenzyme.

5. Discuss reaction and substate specificity of enzyme.

6. Highlight importance of enzyme inhibition.

7. What is suicide inhibition?

8. Compare noncompetitive and uncompetitive enzyme inhibition.

9. How green chemistry and enzymes are co-related?

10. Write four applications of enzymes.

11. What is biocatalyst?

5.14 References:

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Unit 6 Mechanism and Kinetics of Enzyme

Index

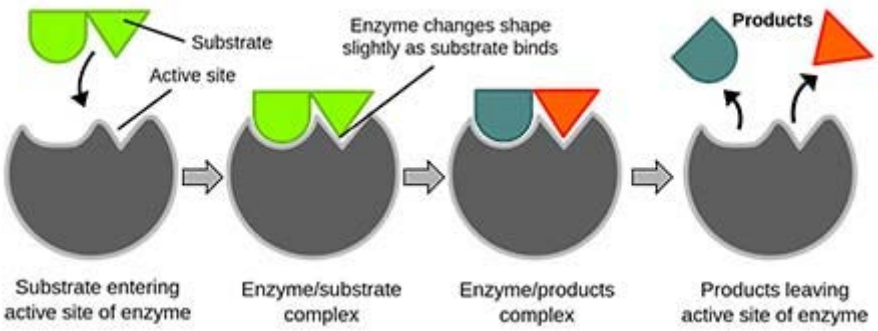
6.1 Objectives:

After completion of this unit student can understand

1. Influence of Enzyme on reaction rate.
2. Different types of catalytic mechanism.
3. Two types of models regarding enzyme mechanism.
4. Kinetics of Enzyme action.
5. Factors influencing enzyme activity.
6. The Michale-Menten equation.

6.2 Introduction:

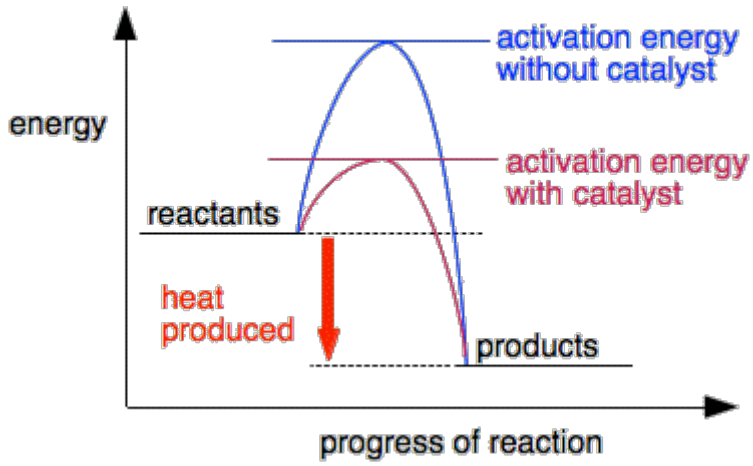
An enzyme attracts substrates to its active site, catalyses the chemical reaction by which products are formed, and then allows the products to separate from the enzyme surface. The combination formed by an enzyme and its substrates is called the enzyme-substrate complex.



Enzymes are only expressed as activities and there are various methods for the estimation of enzyme activities. Specific activity is the number of enzyme units present per milligram of protein. In modern methods, activity of an enzyme is expressed in Katals (kat). One Katal (catalytic activity) is defined as the number of moles of substrate transformed per second per litre of sample. Turnover number is the number of substrate molecules transformed per unit time by a single enzyme molecule. The turnover number is often about 1000 substrate molecules processed per second per enzyme molecule, although turnover numbers between 1 and 10,000 are known.

6.3 Energy Profile of Reactions Catalysed by Enzymes:

Enzymes accelerate a particular reaction because they lower the energy of activation the energy that must be supplied for molecules to react with one another. Enzymes, like other catalysts, reduce the energy of the transition state; i.e. they stabilize the transition state of the catalysed reaction. So, the activation energy for the reaction is lower and the reaction occurs with a faster rate. However, the energy of reactants and products are not changed by enzymes. So, enzymes do not affect the change in free energy (ΔG) of the reaction. The equilibrium constant which depends only on the difference in energy level between reactants and products, is also not changed by enzyme catalysis.



6.4 Types of Catalytic Mechanism:

The types of catalytic mechanisms that enzymes employ have been classified as:

(1) **Acid-base catalysis:** General acid catalysis is a process in which proton transfer from an acid lowers the free energy of a reaction's transition state. A reaction may also be stimulated by general base catalysis if its rate is increased by proton abstraction by a base. The amino acid, histidine is the most effective contributor for protein buffering action. Protonated form of histidine functions as an acid and its conjugated form as a base. Ribonuclease (Rnase) that hydrolyses RNA to its component nucleo-tides by cleaving phosphodiester bonds, is a classic example of acid-base catalysis.

(ii) Covalent catalysis: Some enzymes accelerate reaction rates through the formation of a catalyst-substrate covalent bond. Such enzymes possess nucleophilic (negatively charged) or electrophilic (positively charged) groups which attacks the substrate. This results in transient covalent binding of the substrate to the enzyme. The primary amine catalysed decarboxylation of acetoacetate is an example of covalent catalysis which proceeds through Schiff base formation between amino and carbonyl group. The enzyme functional groups that participate in covalent catalysis include unprotonated amino group of Lys, imidazole moiety of His, thiol group of Cys, carboxyl function of Asp and hydroxyl group of Ser. In addition, co-enzymes often form covalent bonds with the substrates. Several coenzymes, notably thiamine pyrophosphate and pyridoxal phosphate, function in association with their apoenzymes as covalent catalysts.

(iii) Metal Ion Catalysis: Nearly one-third of all known enzymes require metal ions for catalytic activity. This group of enzymes includes the metalloenzymes, which contain tightly

bound metal ion cofactors, most commonly transition metal ions such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, or Co^{2+}. On the other hand, metal-activated enzymes loosely bind metal ions from solution, usually the alkali and alkaline earth metal ions Na^{+}, K^{+}, $Mg^{2+,}$ or $Ca^{2+.}$

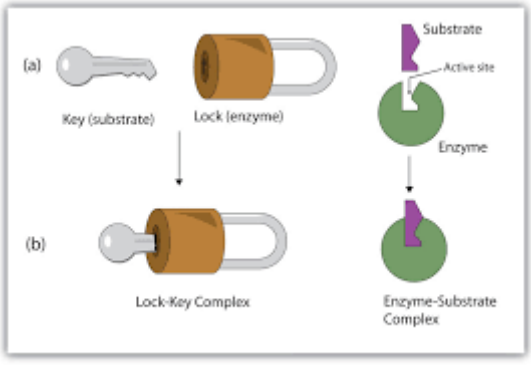
(iv) Proximity and Orientation Effects: Reactants must come together with the proper spatial relationship for a reaction to occur. Enzymes enhance reaction rates by decreasing the entropy of reactants causing them to come closer. So, this is also known as entropy effect.

(v) Preferential Binding of the Transition State Complex: An enzyme may bind the transition state of the reaction it catalyses with greater affinity than its substrates or products. The substrate strain theory proposed that enzymes induce strain on their substrate and the energy level of the substrate is raised towards the transition state geometry.

6.5 Various Models for Enzyme Action:

Two theories have been proposed to explain the binding of substrate with an enzyme at specific active site. This understanding further accounts the fact that an enzyme could perform biochemical reaction so efficiently with greater specificity.

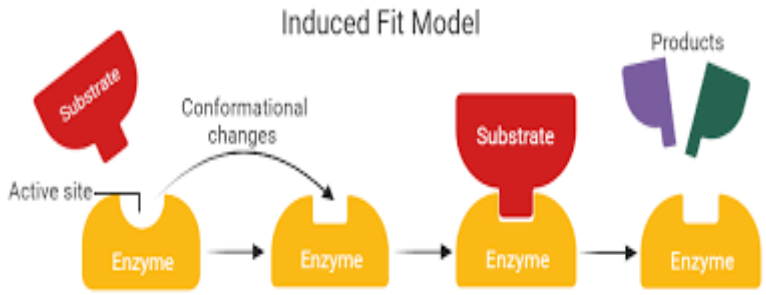
6.5.1 "Lock and Key" Model: To explain the observed specificity of enzymes, in 1894 Emil Fischer proposed that both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another (Fig. 3.6a). This is often referred to as "the lock and key" model. This early model explains enzyme specificity but fails to explain the stabilization of the transition state that enzymes achieve. Also, an unfortunate feature is the rigidity of the active site.



6.5.2 Induced Fit Model: In 1958, Daniel Koshland suggested a modification to the lock and key model; since enzymes are rather flexible structures, the active site is continuously reshaped

as the substrate interacts with the enzyme. As a result, the substrate does not simply bind to a rigid active site. The substrate induces conformational changes in the enzyme. The amino acid side-chains that make up the active site are moulded into the precise positions that enable the enzyme to perform its catalytic function.

The metalloenzyme carboxypeptidase A, €h helps break down proteins by cleaving the peptide bond at carboxy-terminal (C-terminal) end, is a good illustration of the induced-fit theory. When the substrate binds, the active site of carboxypeptidase A undergoes structural rearrangement to bring the groups that participate in catalysis into the correct orientation. In some cases, such as glycosidases, the substrate molecule also changes shape slightly as it enters the active site. This model explains the allosteric regulation and competitive inhibition on enzymes.

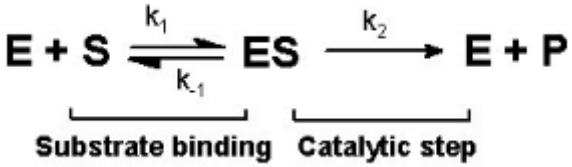


6.6. The Michaelis-Menten Equation:

The study of enzyme kinetics began in 1902 when Adrian Brown reported an investigation of the rate of hydrolysis of sucrose as catalysed by the yeast enzyme invertase:

$$\text{Sucrose} + H_2O \rightarrow \text{Glucose} + \text{Fructose}$$

It was observed that when the sucrose concentration is much higher than that of the enzyme, the reaction rate becomes independent of the sucrose concentration; i.e. the rate is zero order with respect to sucrose. So, the overall reaction is composed of two elementary reactions in which the substrate forms a complex with the enzyme that subsequently decomposes to products and enzyme:



Here E, S, ES, and P symbolize the enzyme, substrate, enzyme-substrate complex, and products, respectively (for enzymes composed of multiple identical subunits, E refers to active sites rather than enzyme molecules).

According to this model, when the substrate concentration becomes high enough to entirely convert the enzyme to the ES form, the second step of the reaction becomes rate limiting and the overall reaction rate becomes insensitive to further increases in substrate concentration.

The general expression for the velocity (rate) of this reaction is

$$V = d[P]/dt = k_2\ [ES]$$

The overall rate of production of ES is the difference between the rates of the elementary reactions leading to its appearance and those resulting in its disappearance:

$$d[ES]/dt = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

Consider two possibilities.

1. Assumption of equilibrium: In 1913, Leonor Michaelis and Maud Menten assumed that $k_{-1} >> k_2$, so that the first step of the reaction achieves equilibrium.

$$Ks = \frac{K_{-1}}{k1} = \frac{[E][S]}{[ES]}$$

Here Ks is the dissociation constant of the first step in the enzymatic reaction. In recognition of the importance of this pioneering work, the non-covalently bound enzyme-substrate complex ES is known as the Michaelis complex.

2. Assumption of steady state: Except the initial stage of the reaction (transient phase) [ES] remains approximately constant until the substrate is nearly exhausted. Hence, the rate of synthesis of ES must equal its rate of consumption; i.e. [ES] maintains a steady state. One can therefore assume that [ES] is constant; i.e.

$$d[ES]/dt = 0$$

This so-called steady-state assumption was first proposed in 1925 by George E. Briggs and John B.S. Haldane.

Kinetic expressions for overall reactions must be formulated in terms of experimentally measurable quantities. The quantities [ES] and [E] are not, in general, directly measurable but the total enzyme concentration Er is usually readily determined.

$$[E]_T = [E] + [ES]$$

The rate equation for our enzymatic reaction is then derived as follows.

$$K_1 \ ([E] - [ES])\ [S] = (k_{-1} + k_2)\ [ES]$$

$$[ES]\ (k_{-1} + k_2 + k_1 \ [S]) = k_1 \ [E]_T\ [S]$$

Dividing both sides by k_1 and solving for [ES],

$$[ES] = [E]_T\,[S] / (K_M + [S])$$

where K_M, which is known as the Michaelis constant, is defined as $K_M = (k_{-1}+k_2)/ k_1$

The initial velocity of the reaction can then be expressed in terms of the experimentally measurable quantities $[E]_T$ and [S]:

$$V_0 = (d[P]/dt)_{t=ts} = k_2 \ [ES] = k_2[E]T[S]/ (K_M + [S])$$

At lower substrate concentration, $[S] << K_M$

$$V_0 = k2[E]_T[S]/ K_M$$

The reaction is now 1st order with respect to substrate.

The maximal velocity of a reaction, V_{max}, occurs at high substrate concentrations when the enzyme is saturated (KM << [S]), that is, when it is entirely in the ES form:

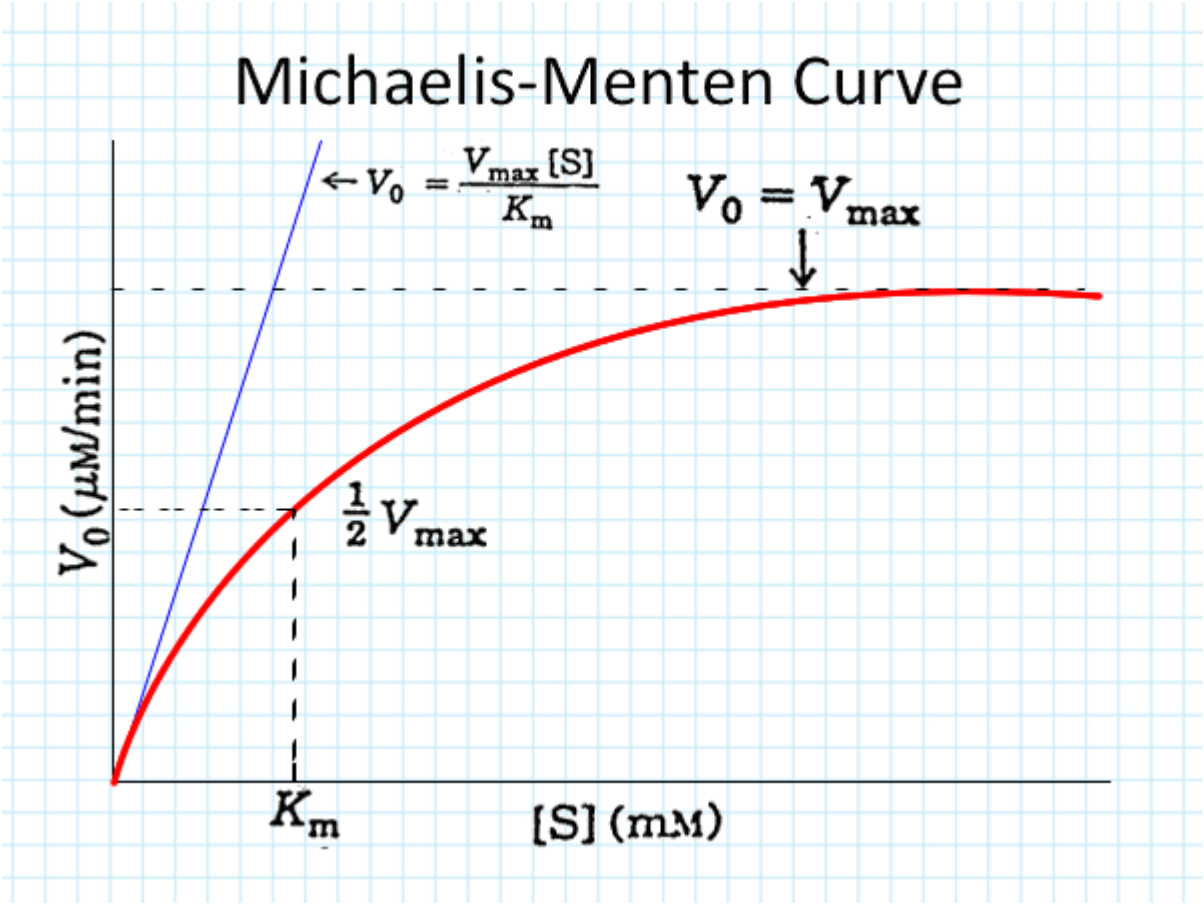
$$V_{max} = k_2[E]_T$$

The reaction is zero order for with respect to substrate for a given amount of enzyme. Combining both equations, we can obtain

$$V_0 = Vmax\ [S] / (K_M + [S]) \text{ ---------- (1)}$$

This expression, the Michaelis-Menten equation (1), is the basic equation of enzyme kinetics.

It describes a rectangular hyperbola as shown in the following figure.



6.6.1 Significance of the Michaelis-Menten Constant:

At the substrate concentration where [S] = K_M, $v_o = V_{max}/2$

K_M or the Michaelis-Menten constant is defined as the substrate concentration (expressed in moles/I) when the rate of the enzyme catalysed reaction is half of the maximum rate under enzyme catalysis.

K_M value is a constant and a characteristic feature of a given enzyme (comparable to a thumb impression or signature). It is a representative for measuring the strength of binding of the substrate to the enzyme molecule i.e., the strength of ES complex. If an enzyme has a small value of K_M, it achieves maximal catalytic efficiency at low substrate concentrations.

The magnitude of K_M varies widely with the identity of the enzyme and the nature of the substrate. It is also a function of temperature and pH. The Michaelis constant can be expressed as

$$K_M = \frac{K_{-1}}{K_1} + \frac{K_2}{K_1} = K_s + \frac{K_2}{K_1}$$

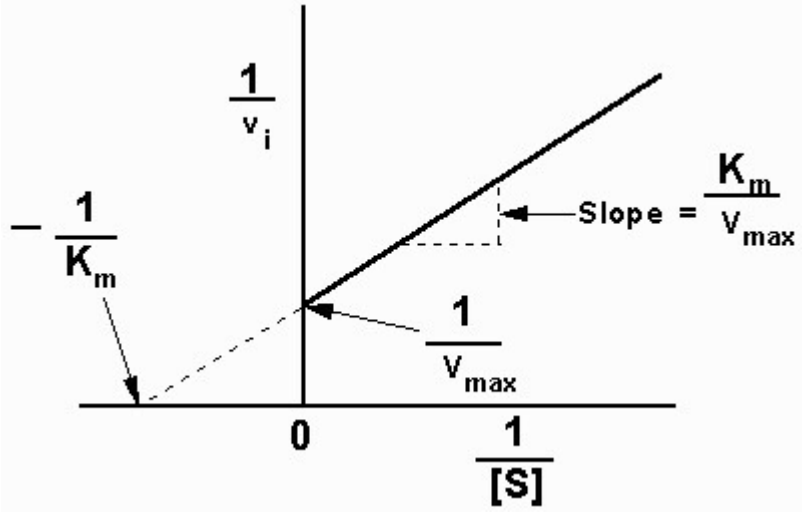
Since Ks is the dissociation constant of the Michaelis complex, as Ks decreases, the enzyme's affinity for substrate increases. KM is therefore also a measure of the affinity of the enzyme for its substrate providing $k2/k_1$ is small compared with Ks. A low K_M value indicates a strong affinity between enzyme and substrate, whereas a high Km value reflects a weak affinity between them.

6.7. Analysis of Kinetic Data:

A useful method for determining the values of Vmax and K_M, which was formulated by Hans Lineweaver and Dean Burk, uses the reciprocal of Eq. (1)

$$\frac{1}{v_0} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

This is a linear equation in $\frac{1}{V_0}$ and 1/[S]. The following plot of these quantities is known as Lineweaver-Burk or double-reciprocal plot. The slope of the line is K_M/Vmax, the $\frac{1}{V_0}$, intercept is 1/Vmax and the extrapolated 1/[S] intercept is $-1/K_M$. The double reciprocal plot is useful in understanding the effect of various inhibitions.



An enzyme's kinetic parameters provide a measure of its catalytic efficiency. The catalytic constant of an enzyme may be defined as

$$K_{cat} = \frac{V_{max}}{[E]_T}$$

This quantity is also known as the **turnover number** of an enzyme because it is the number of reaction processes (turnovers) that each active site catalyses per unit time. For the Michaelis-Menten model, $k_{cat} = k2$.

6.8 Factors Influencing Enzyme Activity:

The various factors which affect enzyme activity are explained below.

i) Enzyme Concentration: Velocity of a reaction is increased proportionately with the concentration of enzyme, provided substrate concentration is sufficient.

ii) Substrate Concentration: As substrate concentration is increased, the velocity is also correspondingly increased in the initial phases. As more substrate is added, all enzyme molecules are saturated. Further increase in substrate cannot make any effect, so the curve flattens afterwards.

iii) Product Concentration: When product concentration is increased, the reaction is slowed, stopped or even reversed. End product inhibition is caused when product combines with the active site of the enzyme.

iv) Sensitiveness to Heat and Temperature: Enzymes are very sensitive to heat this is the reason for preserving food and vegetables in the refrigerator. Velocity of and temperature. They are labile. Correct temperature for the utmost activity optimum temperature. An enzyme reaction increases with increase in temperature up to a maximum and then declines. A bell-shaped curve is usually observed.

Temperature coefficient or Q10 is defined as increase in enzyme velocity when the temperature is increased by 10°C. For a majority of enzymes, Q10 is 2 between 0°C and 40°C. Increase in temperature results in higher activation energy of the molecules and more molecular (enzyme and substrate) collision and interaction for the reaction to proceed faster. The optimum temperature for most of the enzymes is between 35°C-40°C. However, a few enzymes (e.g. Taq DNA polymerase, muscle adenylate kinase) are active even at 100°C. Some plant enzymes like urease have optimum activity around 60°C. This may be due to very stable structure and conformation of these enzymes. In general, when the enzymes are exposed to a temperature above 50°C, denaturation leading to derangement in the native (tertiary) structure of the protein and active site are seen. Majority of the enzymes become inactive at higher temperature (above 70°C).

v) Effect of pH: Increase in the hydrogen ion concentration (pH) considerably influences the enzyme activity and a bell-shaped curve is normally obtained. Hydrogen ions influence the enzyme activity by altering the ionic charges on the amino acids (particularly at the active site), substrate, ES complex etc.

Each enzyme has an optimum pH at which the velocity is maximum. Below and above this pH, the enzyme activity is much lower and at extreme pH, the enzyme becomes totally inactive.

Most of the enzymes of higher organisms show optimum activity around neutral pH (6-8). However, enzymes can function at different pH, like acid phosphatase (4-5) and alkaline phosphatase (10-11). The digestive enzymes for example, salivary amylase acts best at pH 6.8, pepsin acts best at pH 2 etc. Sometime a change in pH causes the reverse reaction, e.g. at pH 7.0 phosphorylase breaks down starch into glucose 1-phosphate while at pH 5 the reverse reaction occurs. Enzymes from fungi and plants are most active in acidic pH (4-6).

(vi) Effect of Inorganic Activators: In presence of certain inorganic ions, some enzymes show higher activity. Thus, chloride ions activate salivary amylase and calcium activates lipases.

6.9 Summary:

Enzyme activities can be estimated using a variety of techniques, and enzymes are only expressed as activities. The quantity of enzyme units per milligram of protein is known as specific activity. According to contemporary techniques, an enzyme's activity is measured in katals (kat). Because they reduce the energy of activation—the energy required for molecules to react with one another—enzymes speed up certain reactions. Enzymes are used in many different kinds of catalytic processes. Two hypotheses have been put forth to explain how a substrate binds to an enzyme at a particular active site. The kinetics of every process catalysed by an enzyme are based on the Michaelis-Menten Equation. Any enzyme-catalysed reaction's kinetic data is examined using a Lineweaver-Burk or Double-Reciprocal plot.

6.10 Model Questions:

1. Define turnover number of any enzyme.

2. Draw energy profile diagram for any reaction catalysed by enzyme and compare it with graph for same reaction in absence of enzyme.

3. Explain acid-base and metal ion catalysis.

4. Describe induce fit model for explaining enzyme action.

5. Deduce Michaelis-Menten equation.

6. Explain the significance of Michaelis-Menten Constant.

7. What is Lineweaver-Burk plot?

8. How pH influences enzymatic activity?

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Unit 7 Lipids

Index

7.1 Objectives:

After studying this unit one can understand

1. Several classes of lipids.

2. Biological significance of lipids.

3. Importance of fatty acids in human body.

4. Structural features of liposomes, phospholipids and triglycerides.

5. Clear idea on lipid bilayer.

7.2 Introduction:

Lipids (Greek: lipos, fat) are highly important to the body as metabolic fuel reserve, besides their role as the fundamental components of cellular and organelle membranes. Lipids constitute about 15-20% of the body weight in humans. Lipids are a heterogeneous group of organic compounds that are grouped together because they do not interact appreciably with water. A diverse group of organic compounds including fats, oils, certain vitamins and hormones, and most non-protein membrane components are lipids.

Lipids are substances of biological origin that are soluble in organic solvents such as chloroform, ether and methanol, but are insoluble or only sparingly soluble in water. Hence, they are easily separated from other biological materials by extraction into organic solvents and may be further fractionated by such techniques as adsorption chromatography and thin layer chromatography. Biological lipids are not large macro-molecular polymers (e.g., proteins, nucleic acids and polysaccharides). They are mostly small molecules. Glycerol and fatty acids are the basic building blocks of fats (lipids).

7.3 Biological Importance of Lipids:

Lipids perform several important functions:

1. They serve as the storage form of energy in the body (triglycerides).

2. Lipids are the structural components of bio membranes and regulate the mem-brane permeability (phospholipids and cholesterol).

3. They help in absorption of fat-soluble vitamins (A, D, E and K).

4. Lipids are important as cellular metabolic regulators (steroid hormones and prostaglandins).

5. Lipids protect internal organs by providing a cushioning effect and also cause (thermal) insulation against changes in external temperature (subcutaneous fat).

6. They give shape and smooth appearance to the body.

7. Improve taste and palatability of food.

8. They act as surfactants, detergents and emulsifying agents (amphipathic/amphiphilic lipids).

9. Lipids are focal to brain activity in structure and in function. They facilitate the signalling of electrical impulses throughout the brain.

10. Lipids serve as chemical messengers between cells, tissues, and organs (steroid hormones).

7.4 Classification of Lipids:

These are broadly classified as simple lipids, complex, derived and miscellaneous lipids, which are further subdivided into different groups.

- **Simple lipids:** They are esters of fatty acids with various alcohols. These are mainly of two types:

(a) Fats and oils: They are esters of fatty acids with glycerol. Triacylglycerols/triglycerides belong to this class. Oils are fats in the liquid state at room temperature.

Fats: solid at room temperature, triacylglycerols of saturated fatty acids.

Oils: liquid at room temperature, triacylglycerols of unsaturated fatty acids

(b) Waxes: They are esters of fatty acids (usually long chain) with alcohols other than glycerol. Higher monohydroxy aliphatic alcohols such as cetyl alcohol are most commonly found in waxes.

- **Complex (or compound) lipids:**

These are esters of fatty acids with alcohols, but they contain additional groups such as phosphate, nitrogenous base, carbohydrate, protein etc. Depending on the extra groups, they are further divided as follows:

(a) Phospholipids: In addition to alcohol and fatty acids, they contain phosphoric acid and frequently a nitrogenous base. It is subdivided into two categories:

(i) Glycerophospholipids/ phosphoglycerides: These phospholipids contain glycerol as the alcohol e.g., lecithin, cephalin.

(ii) Sphingophospholipids: Sphingosine is the alcohol in this group of phospholipids, e.g., sphingomyelin.

(b) Glycosphingolipids/sphingoglycolipids: These lipids contain, in addition to fatty acid and alcohol, carbohydrate (Glycolipids) and nitrogenous base. The special alcohol present is sphingosine; hence they are called as glycosphingolipids. Glycerol and phosphate are absent. Examples - cerebrosides, gangliosides.

(c) Lipoproteins: They are macromolecular complexes of lipids with proteins. Examples: chylomicrons, VLDL, LDL and HDL.

(d) Other complex lipids: Sulfolipids, amino-lipids and lipopolysaccharides are among the other complex lipids.

- **Derived lipids:**

These are the derivatives obtained on the hydrolysis of group 1 and group 2 lipids which still possess the characteristics of lipids. They are classified further into fatty acid, alcohol (glycerol, sphingosine), steroid hormones, lipid soluble vitamins (vitamin E and K.).

- **Miscellaneous lipids:**

These include a large number of compounds possessing the characteristics of lipids which cannot be grouped under any of the above headings.

► Aliphatic hydrocarbons such as iso-octadecane (in liver fat), pentacosane (in bees wax).

► Terpenes.

► Carotenoids.

- **Neutral Lipids:**

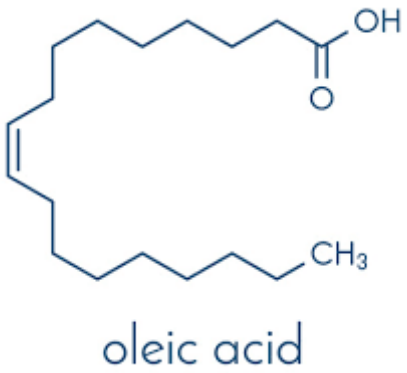
The lipids which are uncharged are referred to as neutral lipids. They are hydrophobic molecules. Examples are mono-, di-, and triacylglycerols, cholesterol and cholesteryl esters.

7.5 Structure and Function of Fatty Acids:

Fatty acids belong to the group of derived lipids. They are the simplest form of lipids. They occur in esterified form as the major components of the various lipids. Free fatty acids are very rare in the human body and are formed only during metabolism.

7.5.1 Structure:

Fatty acids are carboxylic acids with long-chain hydrocarbon side groups. Saturated fatty acids do not contain double bonds, while unsaturated fatty acids contain one or more double bonds. Over half of the fatty acid residues of plant and animal lipids are unsaturated and are often polyunsaturated. Fatty acids with one double bond are monounsaturated fatty acids (MUFA) and those with two or more double bonds are collectively known as polyunsaturated fatty acids (PUFA). The physical properties of fatty acids vary with their degree of unsaturation. Unsaturated fatty acids also exhibit geometrical isomerism at the double bonds. All the naturally occurring fatty acids have the cis configuration.



oleic acid

Saturated fatty acids are highly flexible molecules that can assume a wide range of conformations because there is relatively free rotation about each of their C-C bonds. They are named by adding the suffix 'anoic' after the hydrocarbon. The two carbon acetic acid and four carbon butyric acid are important metabolic intermediates. In higher plants and animals, the predominant fatty acid residues are those of the C16 and C18 species. Examples are Palmitic acid (Hexadecanoic acid): $CH_3(CH_2)_{14}COOH$ (saturated) and Stearic acid (Octadecanoic acid): $CH_3(CH_2)_{16}COOH$ (saturated).

7.5.2 Biological Functions of Fatty Acids:

Fatty acids contribute to the modulation of the structure and function of biological membranes, including elasticity, membrane organization and ion permeability. There-fore they may facilitate neurotransmission and neuronal function. Polyunsaturated fatty acids are critical for brain development. Deregulation of fatty acids is responsible for numerous brain disorders, such as neurodegenerative diseases like Alzheimer disease, mental retardation, stroke and trauma. In the reproductive system, fatty acids are required for proper reproductive health;

women who lack proper amounts may stop menstruating and become infertile. Omega-3 and omega-6 essential fatty acids help regulate blood clotting and control inflammation in the joints, tissues and blood-stream. Omega-3 fatty acids reduce the risk of heart attack or stroke. Their beneficial effects regarding the cardiovascular diseases are mediated by their anti-arrhythmic, lipid lowering, anti-thrombotic and anti-inflammatory properties.

Fatty acids play major role in preparation of toiletries and cosmetics. Soaps are sodium and potassium salts of fatty acids. These are obtained by hydrolysis of vegetable or animal oils and fats with strong alkaline solution (NaOH or KOH). The fatty acids concerned with soap making are: lauric, myristic, palmitic, stearic, linoleic, oleic, linoleic and linolenic acids. Potassium salt of long chain fatty acid is known as soft soap, as it produces more lather. Potassium oleate is used as toilet soap and shaving soap. Zinc stearate is used in talcum powders because it is water repellent. Topical products incorporating essential fatty acids include moisturizing creams and lotions, lipsticks, shampoos and therapeutic skin preparations for dryness and eczema.

7.6 Triglycerides (Triacylglycerols):

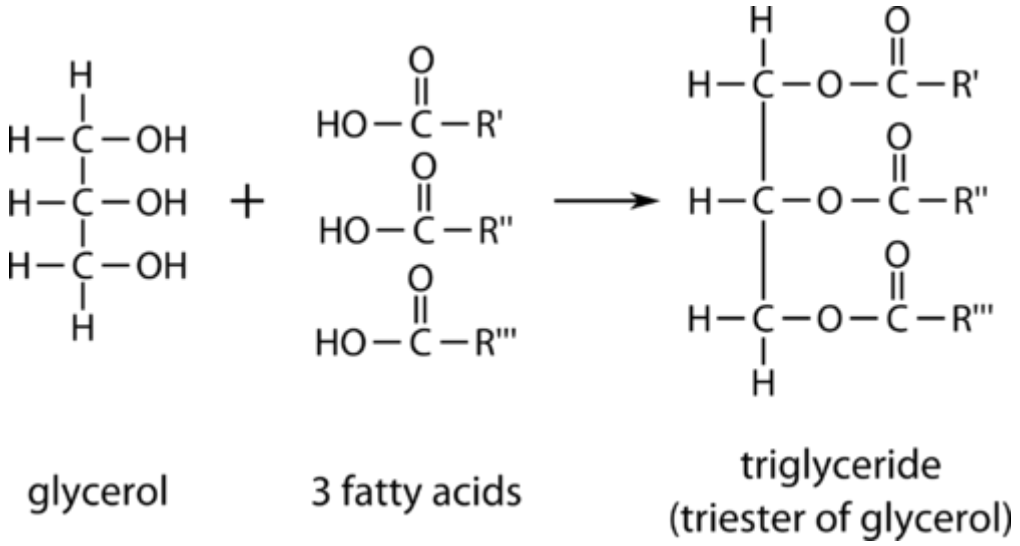
Triacylglycerols belong to the group of simple lipids. The fats and oils that occur in plants and animals consist largely of mixtures of triacylglycerols (also referred to as triglycerides or neutral fats).

7.6.1 Structure:

These are esters of glycerol in which all the three -OH groups of glycerol are esterified with fatty acids. Depending upon the fatty acid residues in glycerol, there are two types of fats namely simple triglycerides and mixed triglycerides.

3 fatty acids + 1 glycerol = triglyceride

If all three fatty acids are the same, the triglyceride is called simple triglycerides. Example: triolein (oleic acid as fatty acid) found in Olive oil. If glycerol is esterified with more than one fatty acid, then the fat is named as mixed triglycerides. Generally, two hydroxyl groups are esterified to similar fatty acid and the third with a different one. Example: 1,3-dipalmitoyl-2-olein, 1-palmitoyl-2, 3-distearin.



7.6.2 Properties of Triglycerides:

They are insoluble in water and hydrophobic in character. Hydrolysis of triglycerides is important for digestion of dietary fat and mobilization of TAG from adipose tissue. The process of hydrolysis is catalysed by hydrolase enzymes, lipases. Triacyl glycerol is stepwise hydrolysed to glycerol and three fatty acids.

(a) Saponification: The process of hydrolysis of triglycerides by alkali is known as saponification which results glycerol and soaps. Saponification number is defined as the number of milligrams of potassium hydroxide required to hydrolyse (saponify) one gram of fat. It is an indication of the molecular weight of the fat and is inversely proportional to it.

$$\text{fat} + KOH \rightarrow \text{glycerol} + \text{salt of fatty acid (soap)}$$

(b) Iodine number: Iodine number of a fat is used to test the purity of fats and oils. It is defined as the number of grams of iodine taken up by 100 grams of fat. It is an index of the degree of unsaturation - higher the iodine number, higher is the degree of unsaturation, e.g. iodine number of butter is 28, and that of sunflower oil is 130.

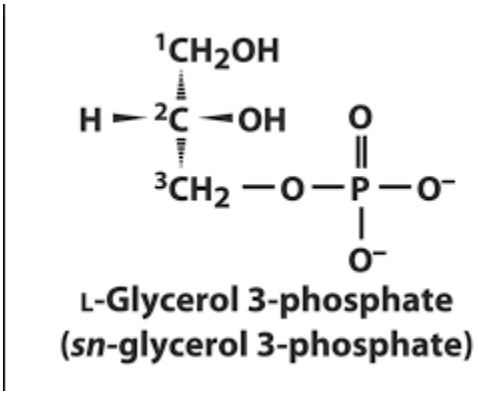
(c) Rancidity: The term rancidity refers to the appearance of an unpleasant smell and taste for fats and oils. Hydrolytic rancidity occurs due to partial hydrolysis of the triacyl glycerol molecules while oxidative rancidity is the result of partial oxidation of unsaturated fatty acids. Many natural fats and oils may contain antioxidants (e.g. vitamin E), which prevent the occurrence of oxidative rancidity. PUFA are more easily oxidized, so have a higher tendency to become rancid. Vegetable oils with a high content of PUFA are usually preserved with addition of antioxidants.

7.6.3 Functions of Triglycerides:

Triacylglycerols primarily function as energy reservoirs in animals and are there-fore they are most abundant class of lipids even though they are not components of biological membranes. Triacylglycerols are highly concentrated form of energy yield-ing 9 kcal/g or 38 kJ/g as compared carbohydrates (approximately 4 kcal/g or 17 kJ/g). They are stored in the adipose tissue. The fat reserve of normal humans (men 20%, women 25% by weight) is sufficient to meet the body's caloric requirements for 2-3 months. Triacylglycerols control the body's internal climate, maintaining a constant temperature. Those who don't have enough fat in their bodies tend to feel cold sooner and are often fatigued. However, excess fat in the body leads to obesity. Elevated triglyceride level, a condition known as hypertriglyceridemia, is associated with coronary artery disease. So, the levels of triglyceride should be monitored by lipid panel profile. Triacylglycerols also help the body to produce and regulate hormones. For example, adipose tissue secretes the hormone leptin, which regulates appetite.

7.7 Phosphoglycerides/ Glycerophospholipids:

The simplest glycerophospholipids, in which X = 1, are phosphatidic acids. In the glycerophospholipids that commonly occur in biological membranes, the additional polar group -X can be choline, ethanolamine, serine or inositol. This polar end is hydrophilic. Usually, glycerophospholipids contain saturated C16 and 18 fatty acids at the C1 position and the C2 position is occupied by an unsaturated C16 to 20 fatty acids. These fatty acid chains give phospholipids hydrophile properties, Glycerophospholipids are therefore amphiphilic/amphipathic molecules with nonpolar aliphatic “tails" and polar phosphoryl-X "heads".



L-Glycerol 3-phosphate
(*sn*-glycerol 3-phosphate)

7.7.1 Functions of different Types of Phospholipids:

The main glycophospholipids and their functions are listed below:

► Phosphatidic acid are present only in small amounts in biological membranes, it is an intermediate in the synthesis of triacylglycerols and phospholipids.

► Lecithin (phosphatidylcholine) is phosphatidic acid with choline as the base, It is the most abundant phospholipid in the cell membrane. It is involved in membrane structure and lipid transport.

► Cephalin (phosphatidylethanolamine) has nitrogenous base ethanolamine. They have a role in blood coagulation.

► Phosphatidylinositol is an important component of cell membranes which helps in cell signalling. It also mediates certain hormones like oxytocin, vasopressin.

► Phosphatidylserine, which contains the amino acid serine, plays a key role in cell cycle signalling.

► Plasmalogen serves as endogenous antioxidants, protecting other PL, lipid and lipoprotein particles from oxidative stress.

► Cardiolipin (Di phosphatidylglycerols) is an important component of inner mitochondrial membrane and essential for mitochondrial function. It possesses antigenic properties.

7.7.2 Biological Importance of Phospholipids:

The biological importance of phospholipids is enormous. Phospholipid bilayers are critical components of cell membranes and regulate permeability of the membranes. In the mitochondria, lecithin, cephalin and cardiolipin are responsible for maintaining the conformation of electron transport chain components and thus cellular respiration. Phospholipids help by preventing the accumulation of fats in the liver (fatty liver). Phospholipids participate in the absorption of fat from the intestine. It plays a major role in the transportation and removal of cholesterol from the cells. They are essential for the synthesis of different lipoproteins and thus participate in the transport of lipids. It generates arachidonic acid which is precursor of eicosanoids. They are also involved in blood coagulation and signal transmission. They act as surfactants in the respiratory system.

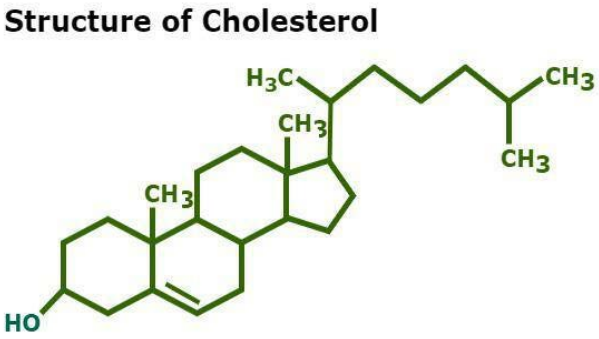
7.8 Cholesterol:

Cholesterol is a derived type of lipid which is biosynthesized by all animal cells. It is often known as animal sterols because it is exclusively found in animals. It is a waxy substance that

is present in blood plasma and in all animal tissues. Cholesterol was first isolated from bile (chole: bile).

7.8.1 Structure:

Chemically, cholesterol is an organic compound belonging to the steroid family. Steroids, which are mostly of eukaryotic origin, are derivatives of cyclopentanoperhydrophenanthrene Cholesterol is further classified as a sterol because of its C3-OH group. Its polar -OH group gives it a weak amphiphilic character, whereas its fused ring system provides it with greater rigidity than other membrane lipids. It is a white, crystalline substance that is odourless and tasteless.



7.8.2 Biological importance of cholesterol:

Cholesterol is a major component of animal plasma membranes, constituting 30 to 40 mol% of plasma membrane lipids. Due to its structural rigidity, it plays a vital role in membrane structure and fluidity. Its most important function is in reducing the permeability of the cell membrane. Cholesterol helps to restrict the passage of molecules by increasing the packing of phospholipids.

It is an essential ingredient in the structure of lipoproteins in which form the lipids in the body are transported. It is abundant in blood plasma lipoproteins, where 70% of it is esterified to long-chain fatty acids to form cholesteryl esters such as cholesteryl stearate (Fig. 4.8). Fatty acids are transported to liver as cholesteryl esters for oxidation.

Cholesterol is a poor conductor of heat and electricity, since it has a constant. It is present in abundance in nervous tissues. It appears that cholesterol functions as an insulating cover for the transmission of electrical impulses in the nervous tissue.

In mammals, cholesterol is the metabolic precursor of all other steroids in the body. All classes of steroid hormones are derivatives of cholesterol: glucocorticoids (e.g. cortisol),

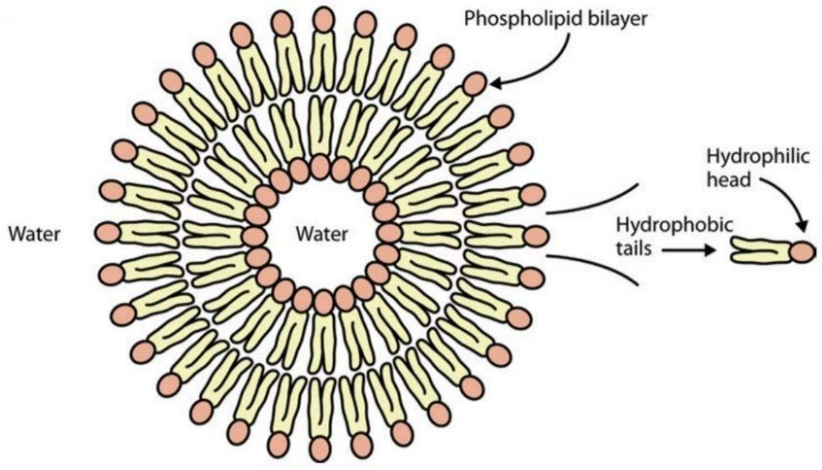
mineralocorticoids (e.g. aldosterone), progestins (e.g. progesterone), andro-gens (e.g. testosterone) and estrogen (e.g. estradiol). Cholesterol is also the precursor from which the body synthesizes vitamin D. Another major use of cholesterol is the synthesis of bile acids. These are synthesized in the liver from cholesterol and are secreted in the bile. They are essential for the absorption of fat from the contents of the intestine.

Cholesterol is much maligned for its adverse effect on human health. High levels of LDL cholesterol led to the development of atherosclerosis: cholesterol-rich deposits (plaques) that form on the inside of blood vessels and predispose to heart attacks. Cholesterol in the bile can crystallize to form gall stones that may block the bile ducts.

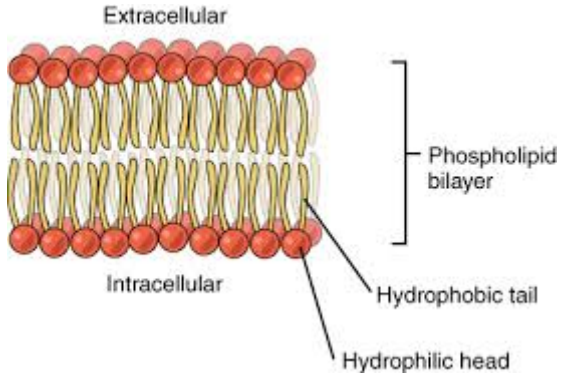
7.9 Lipid bilayer formation:

Molecules such as proteins, nucleic acids and carbohydrates have an affinity for water and are called hydrophilic ("water-loving"). Lipids, however, are hydrophobic ("water-fearing"). Some lipids are amphipathic - part of their structure is hydrophilic and another part, usually a larger section, is hydrophobic. Such lipids exhibit a unique behaviour in water: they aggregate to form micelles and bilayers. The physical proper-ties of lipids in bilayers are of concern because these aggregates form the structural basis for biological membranes.

Let us first consider the packing behaviour of single chain amphipathic lipids. A micelle is stable packing of amphipathic molecules, or molecules that have a head and a nonpolar tail in aqueous solutions. Single-tailed lipids tend to form micelles. A micelle is a spherical structure where the polar groups (heads) orient themselves. aqueous phase while the non-polar tails orient towards the opposite directions leading to the formation of micelles.



Phospholipids tend to form bilayers in water. In the overall molecule there is a hydrophilic area described as its head (the phosphate-containing group), which has a polar character or negative charge and an hydrophobic area containing two tails of the fatty acids, which has no charge. The individual molecules assemble into a bilayer in water in order to minimize the contact of hydrophobic tails to the surrounding water. In biological membrane, the polar heads of the lipid bilayer are directed to the external aqueous phase on either side of the cell while the hydrophobic tails usually occur towards the centre of the membrane. The formation of lipid bilayer is the structural basis of biological membranes

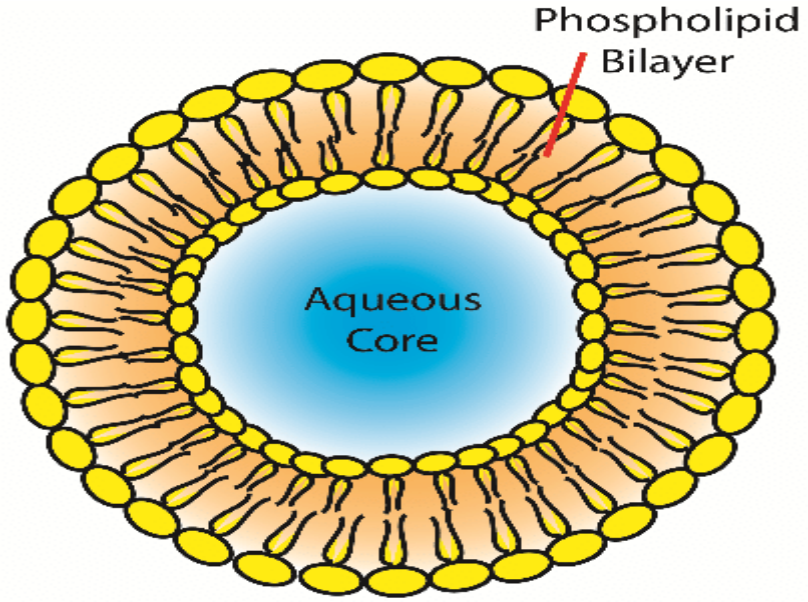


7.10 Liposomes and their Biological Function:

Liposomes can be defined as spherically shaped microscopic vesicles that con-sist of an aqueous core surrounded with phospholipid bilayer membranes, which closely resemble the structure of cell membranes. The general structure of liposomes is shown in Fig. 4.11. The inner core of liposomes consists of hydrophilic parts of the phospholipids, where hydrophilic molecules can be incorporated. On the other hand, lipophilic molecules tend to remain in the lipid portion of the phospholipid bilayer.

7.10.1 Biological functions and underlying applications:

Liposomes composed of synthetic lipids and/or lipids extracted from biological ources (e.g., lecithin from egg yolks) have been extensively used as models to study ell membranes and organelles.



Whether composed of natural or synthetic lipids, liposomes are biocompatible and biodegradable which make them suitable for biomedical research. The unique feature of liposomes is their ability to compartmentalize and solubilize both hydrophilic and hydrophobic materials by nature. These liposomes can encapsulate the therapeutic molecules such as drugs, vaccines, enzymes, proteins, oligonucleotides, genetic mate-rial and therefore they have been widely investigated as drug delivery systems. Various studies have been conducted for the design of successful liposomes-based oral delivery of the insulin hormone. Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. Drug distribution is then controlled primarily by properties of the carrier and no longer by physicochemical characteristics of the drug substance only. Subsequent research on their stability and drug interactions resulted in several commercial liposome products in the market in recent times.

7.11 Lipid Membrane:

The most abundant component of the fluid mosaic model is lipids. The three major classes of membrane lipids are phospholipids, glycolipids and cholesterol.

The plasma membranes of animal cells contain four major phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin), which together account for more than half of the lipid in most membranes. These phospholipids are asymmetrically distributed between the two halves of the mem-brane bilayer. The outer leaflet of the plasma

membrane consists mainly of phosphatidylcholine (Lecithin), sphingomyelin and glycolipids. The predominant phospholipids of the inner leaflet are phosphatidylethanolamine (Cephalin) and phosphatidylserine with phosphatidylinositol in minor quantity. Most of the fatty acids in the phospho-lipid tails are unsaturated which introduce kinks (bents) into the hydrocarbon chains. The long hydrocarbon chains of the fatty acids therefore move freely in the interior of the membrane. This allows the membrane to be soft and flexible (cis bonds are bent).

Cholesterol is a major membrane constituent of animal cells. It is inserted into a bilayer with its polar hydroxyl group close to the phospholipid head groups and makes up about 20% of the molecules of the membrane. This helps to make the membrane more rigid and adds strength. It lowers the membrane permeability and also maintains membrane fluidity. Depending on temperature, cholesterol has distinct effects on membrane fluidity.

7.12 Summary:

Various lipids play their individual roles in human body. During starvation they basically supply the energy for all kinds of physical and biological work. There are many natural occurring fatty acids which also play significant biological roles. Hydrolysis of triglycerides is important for digestion of dietary fat. Phospholipids are essential for production of lipoproteins which has very important transportation role. Cholesterol is one of major member of lipid membrane. On the other hand, the physical properties of lipids in bilayers are of concern because these aggregates form the structural basis of cell membrane.

7.13 Model Questions:

1. What do you mean by lipids?

2. Write four biological roles of lipids.

3. Write basic difference between fat and oil.

4. Give two examples of complex lipids.

5. Write structural features of fatty acids.

6. Define iodine number.

7. Write the biological importance of liposomes

8. Explain bilayer structure of lipids.

9. Write the basic structure of cholesterol.

10. Write a short note on glycerophospholipids.

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Unit 8 Lipoproteins and Hormones

Index

8.1 Objectives:

After careful reading this chapter one can understand the followings:

1. Biological Functions of various peptide hormones.

2. Main sources of hormones in human body.

3. Compare and contrast steroid and peptide hormone.

4. Biological role of lipoproteins

5. Various types of lipoproteins and their functions.

8.2 Introduction:

Cholesterol and triglycerides are insoluble in water. They are solubilized in association with proteins to form lipoproteins - the form in which lipids are transported in the blood stream. Free lipids are undetectable in blood. On the other hand, Endocrine glands are ductless glands of the endocrine system that secrete their products, hormones, directly into the blood. Endocrine glands are known as the ductless glands because of the fact that their secretions are

released directly into the blood, not to any tubes or ducts. The major glands of the endocrine system include the pineal gland, pituitary gland, pancreas, ovaries, testes, thyroid gland, parathyroid gland, hypothalamus and adrenal glands. Hormones are chemical messenger molecules synthesised in ductless endocrine glands of higher animals and are released into the bloodstream in response to external stimuli. The circulatory system carries these hormones to their target cells in which these will produce a response.

8.3 Lipoproteins:

8.3.1 About Lipoproteins:

Lipoproteins are molecular complexes that consist of lipide and proteins (conjugated proteins). The protein components of lipoproteins are known as apolipoprotein or just apoproteins. They function in the blood plasma as transport vehicles for lipids. Lipoproteins deliver the lipid components (cholesterol, triacylglycerol etc.) to various tissues for utilization. Lipoproteins are complex particles that have a central hydrophobic core of non-polar lipids, primarily cholesterol esters and triglycerides. This hydrophobic core is surrounded by an amphiphilic coating consisting of phospholipids, free cholesterol and apolipoproteins.

Their hydrophobic areas are inward, toward the lipid core. Their hydrophilic charged areas that face outward are oriented toward the aqueous environment. This enables the lipoprotein to be carried in the blood. Lipoproteins are synthesized mainly in the intestines and liver. They are trans ported throughout the body of all vertebrates and insects to tissues where they are required. The peripheral tissues take up the various components before the remnants return to the liver.

8.3.2 Classification of Lipoproteins:

The lipid and protein composition are reflected in the density of each lipoprotein (lipid molecules being less dense than proteins). Therefore, density is the operational basis of defining the lipoprotein classes. Measuring density also provides the basis of separating and purifying lipoproteins from plasma for study and diagnosis. The lipoproteins in plasma are classified into five broad categories on the basis of their functional and physical properties.

1. Chylomicrons: They are the least in density and the largest in size among the lipoproteins. They consist of highest (99%) content of lipid and lowest (1%) content of protein. They are triglyceride rich particles synthesized in the intestine. They transport exogenous (externally supplied; in this case, dietary) triacylglycerol to peripheral tissues.

2. Very low-density lipoproteins (VLDL): The liver synthesizes triacylglycerols from excess carbohydrates. VLDLs are produced in liver and intestine. They are re-sponsible for the transport of endogenous (internally produced) triacylglycerol from the liver to the tissues.

3. Intermediate density lipoproteins (IDL): The removal of triglycerides from VLDL by muscle and adipose tissue results in the formation of IDL particles which are enriched in cholesterol.

4. Low density lipoproteins (LDL): They are derived from VLDL and IDL particles and they are even further enriched in cholesterol. LDL carries the majority of the cholesterol that is in the circulation. They transport cholesterol from liver to peripheral tissues. High LDL concentration in blood leads to atherosclerosis and has a direct correlation with cardiovascular diseases. Hence it is also called "bad cholesterol". High density lipoproteins (HDL): HDL is densest because it has the highest protein-to-lipid ratio. They are mostly synthesized in liver. HDL particles transport endogenous cholesterol from peripheral tissues to liver (reverse cholesterol transport) for excretion through bile. Hence it is also called "good cholesterol".

8.3.3 Biological Role of Lipoproteins:

The primary function of lipoproteins is the transportation and delivery of fatty acids, triacylglycerol and cholesterol to and from target cells in many organs. Within the circulation, lipoprotein particles undergo continuous metabolic processing, have variable composition and physical structure. The major lipid components of chylomicrons and VLDL are triacylglycerol,

whereas the predominant lipids in LDL and HDL are cholesterol and phospholipids respectively. The protein part of lipoprotein is known as apoprotein.

Apolipoproteins have four major biological roles. They guide the formation of lipoproteins and help solubilize the hydrophobic lipids (apo A-I, B100, and B48). They provide structural integrity of the lipoprotein (apo B, E, A-I, and A-II). They also serve as enzyme cofactors for specific enzymes involved in the metabolism of lipoproteins (apo A-I, A-V, C-I, C-II, and C-III). They act as ligands for binding or docking to specific receptors (apo A-I, B100, and E). Table 2 gives a summary of the characteristics of the lipoprotein classes and shows the correlation between composition and density.

8.3.4 Clinical Significance of Lipoproteins:

Lipids are insoluble in the blood, so it must be bound to lipoproteins in order to be transported. Lipoproteins in blood plasma have been intensively studied because they are the mode of transport for cholesterol and triglycerides. Low-density lipoproteins (LDLs) transport cholesterol from its site of synthesis in the liver to the body's cells, where the cholesterol is separated from the LDL and is then used by the cells for various purposes. High-density lipoproteins (HDLs) transport excess or unused cholesterol from the body's tissues back to the liver, where the cholesterol is broken down to bile acids and is then excreted. LDL-bound cholesterol is primarily responsible for the atherosclerotic buildup of fatty deposits on the blood vessel walls. This narrows the arteries and increases the risk for heart attack and stroke. On the other hand, HDL particles may actually lower the risk for heart disease and stroke.

8.4 Steroid or Lipid-derived Hormones:

Most lipid hormones are derived from cholesterol, so they are structurally similar to it. The primary class of lipid hormones in humans is the steroid hormones. Chemically, these hormones are usually ketones or alcohols; their chemical names will end in "-ol" for alcohols or "-one" for ketones. Steroid hormones are insoluble in water and soluble in lipids; they are carried by transport proteins in blood. As a result, they remain in circulation longer than peptide hormones. They bind to receptors inside either the cytoplasm or nucleus of the target cell, to form an active receptor-hormone complex. This activated complex will move into the nucleus and bind directly to DNA, modifying the transcription for gene expression.

Common examples of steroid hormones are those released by the gonads, primary reproductive organs. These include female sex hormone-progesterone and estrogen and male sex hormone -

testosterone. These hormones help to develop and maintain both the reproductive system and female/male characteristics.

Other examples of steroid hormones include aldosterone and cortisol, which are re-leased by the adrenal glands. Aldosterone is essential for sodium conservation in the kidney, salivary glands, sweat glands and colon. It plays a central role in the homeo-static regulation of blood pressure, plasma sodium (Na^+), and potassium (K^+) levels. Cortisol has a very important role in helping the body respond to stress. It increases sugars (glucose) in the bloodstream, enhances brain's use of glucose and increases the availability of substances that repair tissues.

Bioactive vitamin D or calcitriol is a steroid hormone that has long been known for its important role in regulating body levels of calcium and phosphorus and in mineralization of bone. Note that it is not a true vitamin because it can be synthesized endogenously through ultraviolet exposure of the skin.

8.5. Peptide hormones:

8.5.1 About peptide hormones:

Peptide hormones are molecules that have as their core structure one or more polypeptide chains ranging from three to hundreds of amino acids in size. The number of different peptide hormones exceeds that of the other major hormone classes, le., steroid and amine hormones. Peptide hormones can be synthesized and secreted by specialized cells of endocrine glands or by cells within tissues or organs whose main function is not the synthesis of hormones, e.g., gut, heart, kidneys, and adipose tissue. After their synthesis, peptide hormones are stored in membrane-bound secretory vesicles, thus enabling their rapid secretion and onset of action when required. Once secreted, most peptide hormones are not bound to carrier proteins in the circulation. Thus, they are subject to rapid degradation by serum proteases, resulting in shorter half-life and duration of action compared to steroid hormones. Peptide hormones are water-soluble and, unlike steroid hormones, do not readily cross hydrophobic cell membranes. Most peptide hormones exert their actions by binding to specific receptors located on the surface of target cells. The binding of peptide hormones to the extracellular domain of plasma membrane receptors induces the initiation of intracellular signal transduction processes, leading to the opening of ion channels and/or activation of enzymes involved in the production of second messengers and phosphorylated proteins causing specific cellular responses.

8.5.2 Features of peptide Hormones:

The structure of peptide hormones is that of a polypeptide chain. The peptide hormones also include molecules that are short chains of amino acids or small proteins. Polypeptide hormones are water-soluble and insoluble in lipids (lipophobic). These hormones cannot pass through plasma membranes of cells; therefore, their receptors are found on the surface of the target cells. The receptor complex activates a series of intracellular molecules called second messengers, which initiate cell activity. This process is called signal transduction, because the external signal (hormone) is transduced via internal intermediaries. Peptide hormones have a short half-life, meaning they break apart quickly. This allows organisms to use peptide hormones to direct processes quickly and efficiently, without the signal lingering for a long time.

8.5.3 Examples of Some Peptide Hormones and their Biological Role:

Examples of peptide hormones include insulin, glucagon, leptin, ADH and oxytocin. ADH (antidiuretic hormone) produced by the hypothalamus in the brain and released into the blood from the posterior pituitary gland (storage). One of the most common examples of peptide hormones are the pancreatic islet hormones insulin, glucagon and somatostatin that control our fuel metabolism. The pancreas is a large glandular organ dedicated to producing digestive enzymes - such as trypsin, chymotrypsin, RNase A, a-amylase, and phospholipase A2 - that are secreted via the pancreatic duct into the small intestine. However, ~1 to 2% of pancreatic tissue consists of scattered clumps of cells known as islets of Langerhans, which comprise an endocrine gland that functions to maintain energy homeostasis.

ADH constantly regulates and balances the amount of water in our blood. It acts to maintain blood pressure, blood volume and tissue water content by controlling the concentration of urine excreted by the kidney.

8.5.3.1 Insulin:

Insulin is one of the most commonly known peptide hormones, produced by beta cells of the pancreatic islets. It regulates the metabolism of carbohydrates, fats and protein by promoting the absorption of glucose from the blood in liver, fat and skeletal muscle cells. Insulin favours glycolysis by activating the three key glycolytic enzymes. It inhibits gluconeogenesis. When you eat, your body breaks food down into sugar and sends it into the blood. Insulin then helps move the sugar from the blood into your cells. When sugar enters your cells, it is either used

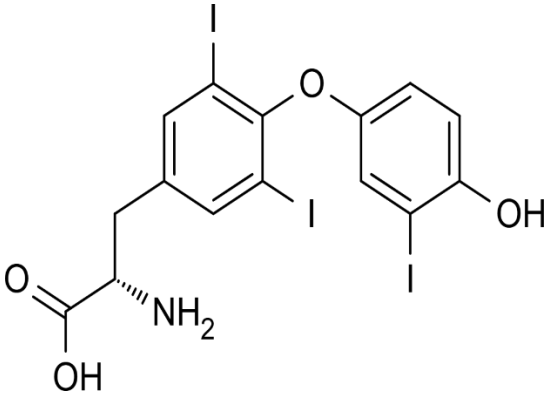
as fuel for energy right away or stored for later use. Insulin is released to stop blood sugar levels rising too high (hyperglycaemia), Decreased or loss of insulin activity results in diabetes mellitus, a condition of high blood sugar level.

8.5.3.2 Glucagon:

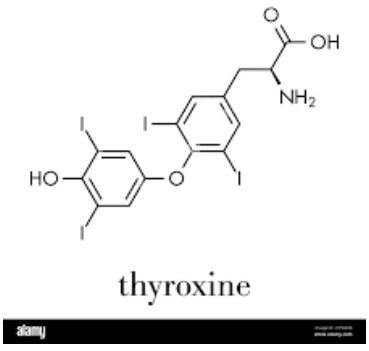
On the other hand, hormone glucagon inhibits glycolysis and favours gluconeogenesis. Glucagon is released to stop blood sugar levels dropping too low (hypoglycaemia). Glucagon is secreted in response to low blood glucose and shows essentially the opposite effects of insulin. It stimulates the liver to release glucose through the breakdown of glycogen (glycogenolysis) and the synthesis of glucose from non-carbohydrate precursors (gluconeogenesis). It also stimulates adipose tissue to release fatty acids through lipolysis.

8.5.3.3 Thyroid-Stimulating Hormone (TSH):

Another very important peptide hormone is the thyroid-stimulating hormone (TSH, also known as thyrotropin) that helps in maintaining the balance of the triiodothyronine (T3) and thyroxine (T4) hormones released from the thyroid gland. Thyroid hormones control a group of biological functions and act on virtually every cell in the body. These act to increase the primary metabolic rate, affect protein synthesis, help regulate long bone growth and neural maturation, and increase the body's sensitivity to catecholamines such as adrenaline. These hormones are essential to proper development and differentiation of all cells of the human body. They also regulate protein, fat, and carbohydrate metabolism, affecting how human cells use these energetic compounds. Stimulation of vitamin metabolism is also their responsibility.



T3



T4

8.5.3.4 Some other Peptide Hormones:

Evidently regulation of synthesis and secretion of these hormones is vital and their balance needs to be carefully maintained for optimum function. Two glands in the brain, hypothalamus and pituitary, communicate to maintain this balance. The hypothalamus, in the base of the brain, produces thyrotropin-releasing hormone (TRH). TRH is another peptide hormone which consists of only three amino acid residues. TRH stimulates the anterior pituitary gland to produce TSH. Somatostatin, another peptide hormone, is also produced by the hypothalamus, and has an opposite effect on the pituitary production of TSH, decreasing or inhibiting its release. TSH is a glycoprotein with two subunits carrying 92 and 118 amino acid residues in the a- and the B-subunits respectively.

TSH binds to the TSH-receptor in the thyroid gland and stimulates the gland to produce the thyroid hormones. The concentration of thyroid hormones T3 and T4 in the blood regulates the hypothalamus release of TRH and pituitary release of TSH; when T3 and T4 concentrations are low, hypothalamus produces TRH which in turn increases TSH production that eventually leads to thyroid releasing more T3 and Ta hormones.

Conversely, when T3 and T4 concentrations are high, they inhibit TRH and TSH production from hypothalamus and pituitary glands. This is an example of a negative feedback loop. In this way the hypothalamic-pituitary-thyroid (HPT) maintains the appropriate levels of thyroid hormones.

If somehow the said balance is disrupted and the thyroid gland does not produce enough thyroid hormones, it results in hypothyroidism. The most common cause behind this condition is too little iodine in the diet. On the other hand, if excessive thyroid hormones are produced by an overactive thyroid gland, hyperthyroidism is observed. These conditions can be detected by measuring the TSH, Ts and Ts concentrations in blood plasma, Hypothyroidism is generally associated with an increased level of TSH while TSH level is decreased for hyperthyroidism. The most common treatment of hypothyroidism is levothyroxine, a man-made version of the thyroid hormone thyroxine (T4). It acts just like the hormone your thyroid gland normally makes

8.6 Summary:

Triglycerides and cholesterol are insoluble in water. The form in which lipids are carried in the bloodstream is called lipoprotein, which is created when they solubilise in conjunction with proteins. Complex particles known as lipoproteins have a hydrophobic core of non-polar lipids,

mainly triglycerides and cholesterol esters. Phospholipids, free cholesterol, and apolipoproteins make up the amphiphilic coating that envelops this hydrophobic core. Based on their morphological and functional characteristics, the lipoproteins in plasma are divided into five major groups. Transporting and delivering fatty acids, triacylglycerol, and cholesterol to and from target cells in various organs is the main job of lipoproteins. The atherosclerotic accumulation of fatty deposits on the blood vessel walls is mostly caused by LDL-bound cholesterol. This causes artery narrowing and raises the risk of stroke and heart attack. However, HDL particles may actually reduce the risk of stroke and heart disease. For the kidney, sweat glands, salivary glands, and colon to save salt, aldosterone is necessary. The hormone cortisol plays a critical function in assisting the body's reaction to stress. The beta cells of the pancreatic islets create insulin, one of the most well-known peptide hormones. By encouraging the absorption of glucose, it controls the metabolism. Side by side there are other several peptide hormones which plays their important biological role as mentioned in this unit.

8.7 Model Questions:

1. What are lipoproteins?

2. How lipoproteins are classified?

3. Write the full form of TSH, VDL, HDL.

4. Discuss the biological role of lipoproteins.

5. Write a note on ‘steroid Hormones’.

6. Give the chemical name and structure for T3 and T4.

7. Write the function of Insulin in human body.

8. What is the biological role of Vitamin-D.

9. Write down the major functions of ‘Apolipoproteins’.

10. What do you mean by ‘good’ and ‘bad’ cholesterol?

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Unit 9 Nucleic Acid: DNA & RNA

Index

9.1 Objectives:

After fruitful completion this unit student can understand

1. Chemistry behind DNA and RNA structure.

2. Difference between DNA and RNA from various angles.

3. Biological Functions of DNA and RNA.

4. Replication and Transcription process.

5. Clear idea on Genetic Code.

9.2 Introduction:

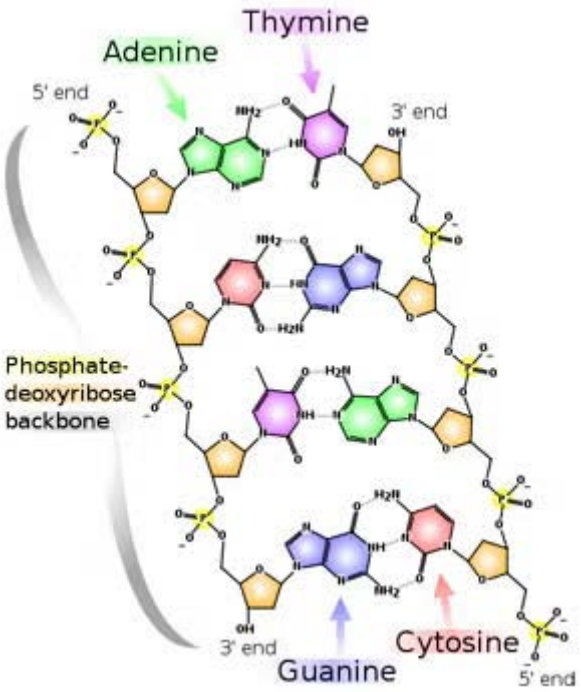
Primarily, nucleic acids are concerned with the storage and expression of genetic information. They are the most important macromolecules for the continuity of life. They direct the process of protein synthesis, thereby determining the inherited characteristics of every living thing. The two main types of nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

9.3 Chemical structure of DNA:

DNA is composed of subunits called deoxynucleotides. A nucleotide is made up of a sugar (2'-deoxy-D-ribose/deoxyribose), a phosphate group, and one of four nitrogenous bases: adenine (A), thymine (T), guanine (G) or cytosine (C).

DNA nucleotides assemble in chains linked by covalent bonds which form between the hydroxyl group (3' position) of one deoxyribose sugar of one nucleotide and the phosphate group (5' position) of the next. The linkage between individual nucleotides is named phosphodiester bond, because the phosphate is esterified to two ribose units. This arrangement makes a chain of alternating deoxyribose sugar and phosphate groups in the DNA polymer - a structure known as the sugar-phosphate backbone.

Polynucleotides have directionality. The terminal residue whose C5' is not linked to another nucleotide is called the 5' end, and the terminal residue whose C3' is not linked to another nucleotide is called the 3' end. By convention, the sequence of nucleotide residues in a nucleic acid is written, left to right, from the 5' end to the 3' end.



9.3.1 Chargaff's Rules:

The first reliable methods for the compositional analysis of DNA were discovered in the late 1940s by Erwin Chargaff. The most important clue to the DNA structure comes from Chargaff's rule.

- A, T, C and G were not found in equal quantities
- DNA base composition varies from one species to another species.
- Individuals of the same species have the same base composition.
- For a particular species, base composition is independent of the tissue from which the DNA is taken as well as the organism's age, its nutritional state, or any other environmental factor
- DNA has equal numbers of adenine and thymine residues (A = T) and equal numbers of guanine and cytosine residues (G = C). This relationship is known as Chargaff's rules.

The double helical structure of DNA derives its strength from Chargaff's rule.

9.3.2 Watson and Crick Model of DNA:

The determination of the structure of DNA by Watson and Crick in 1953 is often said to mark the birth of modern molecular biology. For understanding the structure of DNA, James Watson, Francis Crick and Maurice Wilkins were awarded the Nobel Prize in Medicine in 1962.

The structure of DNA, as represented in Watson and Crick's model, is a double-stranded, antiparallel, right-handed helix. The structure is similar to a spiral stair case. The sugar-phosphate backbones of the DNA strands make up the outside of the helix, while the nitrogenous bases are found on the inside, lying perpendicular to the axis of the helix. The bases form hydrogen-bonded pairs that hold the DNA strands together. The base pairs are stacked with their planes, providing extra stability to the double helical structure.

■Major features of the Watson-Crick Model of DNA:

i. The DNA consists of two poly(deoxyribo)nucleotide chains (strands) that is twisted about a common axis into a double helix.

ii. The two helices wind in such a way so as to produce two interchain spacings or grooves of unequal width: a major or wide groove and a minor or narrow groove.

iii. The two strands DNA are antiparallel, which means that it is composed of two strands that run alongside each other but point in opposite directions; one strand runs in the 5' → 3' direction, while its partner runs 3' → 5'. In a double-stranded DNA molecule, the 5' end (phosphate-bearing end) of one strand aligns with the 3' end (hydroxyl-bearing end) of its partner, and vice versa.

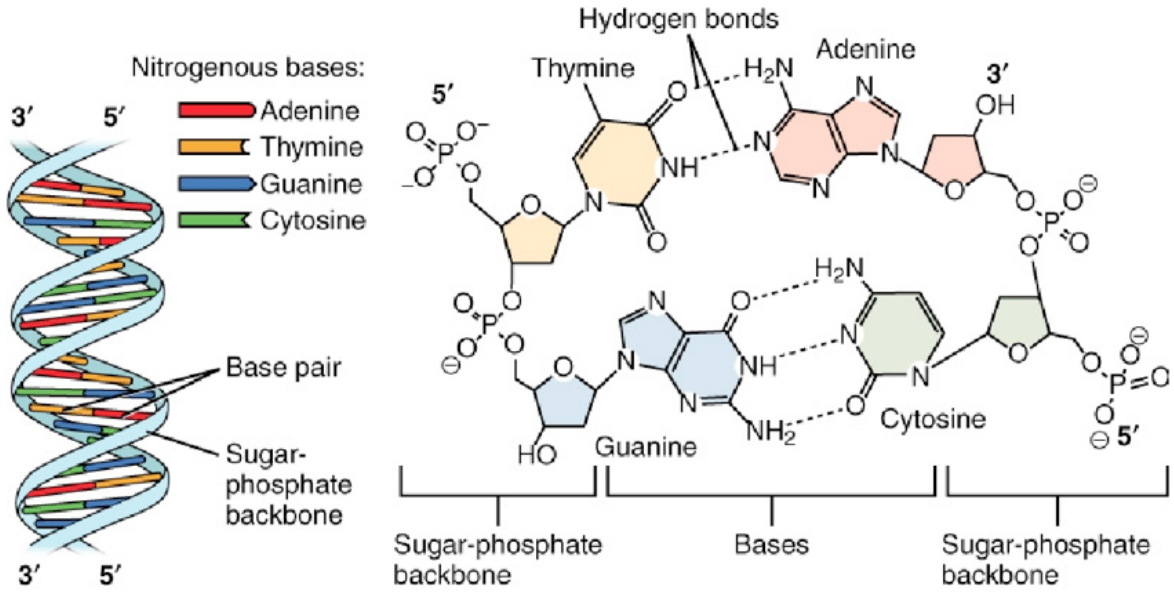
iv. The two strands of DNA twist around each other to form a right-handed helix (the turns run clockwise looking along the helical axis).

v. Each DNA base is hydrogen bonded to a base on the opposite strand to form a planar base pair. G is always hydrogen bonded (forms a base pair) with C and vice versa, whereas A always forms a base pair with T and vice versa. The A-T pair has two hydrogen bonds while G-C pair has three hydrogen bonds. The G-C is stronger by about 50% than A-T. So, the two strands are not identical but complementary to each other; that is, the sequence of one strand implies the sequence of the other. These hydrogen bonding interactions, a phenomenon known as complementary base pairing, result in the specific association of the two chains of the double helix.

vi. Each strand of DNA has a hydrophilic deoxyribose phosphate backbone (3'-5' phosphodiester bonds) on the outside (periphery) of the molecule while the hydrophobic bases are stacked inside (core).

vii. Structural parameters: The bases are nearly perpendicular to the helix axis, and adjacent bases are separated by 3.4 Å. The helical structure repeats every 34 Å, so there are 10 bases (= 34 Å per repeat/3.4 Å per base) per turn of helix. There is a rotation of 36 degrees per base (360 degrees per full turn/10 bases per turn). The width (or diameter) of a double helix is 20 Å (2nm).

viii. In DNA, the base sequence is of paramount importance. The precise sequence of bases along a polynucleotide chain carries the genetic information.

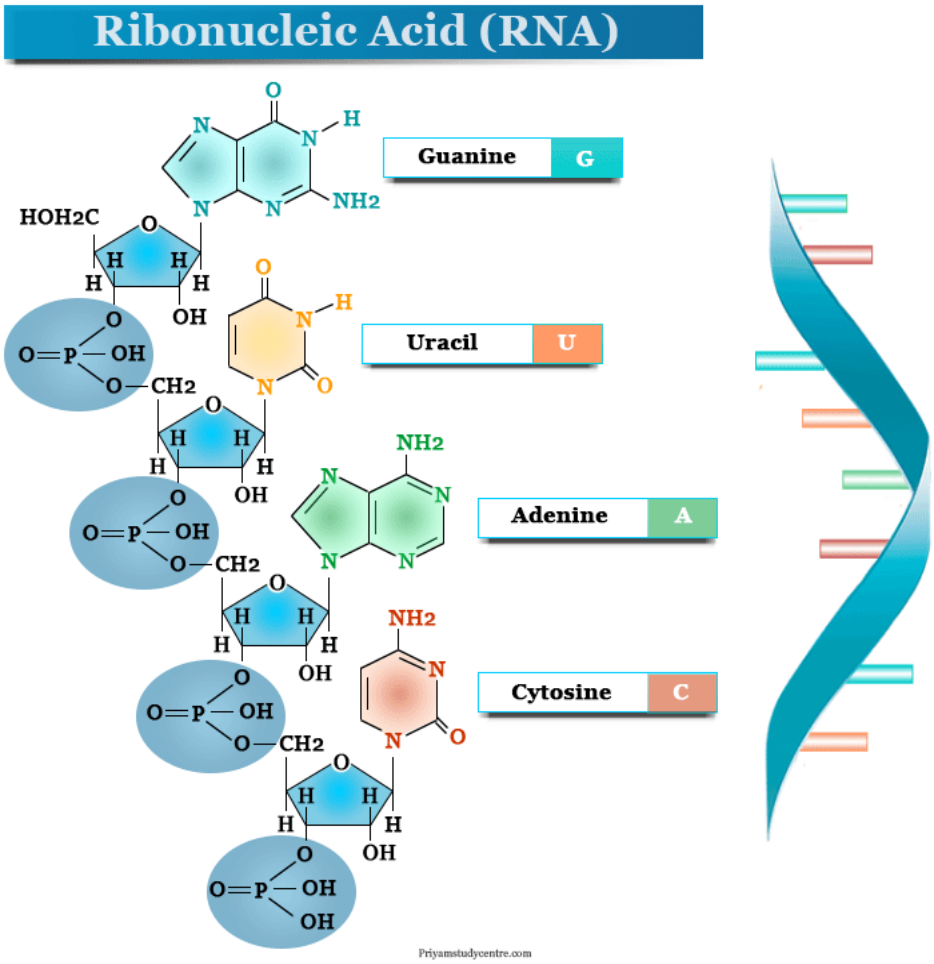


9.4 Chemical Structure of RNA:

The structural features of RNA are very similar to that of DNA, with a few differences. A ribonucleotide in the RNA chain contains ribose (the pentose sugar), one of the four nitrogenous bases and a phosphate group. Each kind of RNA is a polymeric molecule made by stringing together individual ribonucleotides through phosphodiester bonds. The 5'-phosphate group of one nucleotide are joined to the 3'-hydroxyl group of the previous nucleotide. Like DNA, each RNA strand has the same basic structure, composed of nitrogenous bases covalently bound to a sugar-phosphate backbone.

However, the sugar in RNA is ribose instead of deoxyribose (ribose contains one more hydroxyl group on the second carbon), which accounts for the molecule's name. RNA consists of four nitrogenous bases: adenine, cytosine, uracil, and guanine. Uracil is a pyrimidine that is structurally similar to the thymine, another pyrimidine that is found in DNA. Like thymine, uracil forms base-pair with adenine.

However, unlike DNA, RNA is usually a single-stranded molecule. RNA strands are shorter than DNA strands. Even though RNA is single stranded, most types of RNA molecules can fold upon itself. The folds are stabilized by intramolecular base pairing between complementary sequences within the RNA strand creating a three-dimensional structure essential for their function. About 50% of cellular RNA is distributed in the ribosomes; 25% in cytoplasm; 15% in mitochondria and the rest 10% in nucleus.



9.5. Biological Roles of DNA and RNA:

DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) are very similar molecules that serve very different functions.

First and foremost, DNA is an informational macromolecule. DNA is the chemical basis of heredity and may be regarded as the cell's reserve bank of genetic information. It is a suitable genetic material for long-term storage. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). DNA sections called genes indirectly code for proteins, which give structure and function to human bodies.

Diseases and medical conditions can be interpreted and understood using DNA: gene mutations that damage DNA have adverse effects on the health and well-being of an individual. The battle against cancer also relies on manipulating DNA replication.

The primary role of Ribonucleic acid (RNA) is to transmit the genetic code from DNA through three types RNA to ribosome for making protein. So, it functions as an adapter molecule in protein synthesis. It replaces DNA as a carrier of genetic codes in some viruses. Certain RNAs are able to catalyse chemical reactions.

9.6 Differences between DNA and RNA:

	DNA	RNA
Acid Name	**D**eoxyribo**N**ucleic	**R**ibo**N**ucleic
Stability	Very stable (long "life")	Less stable (short "life")
Found in	Nucleus	Nucleus and cytosols
	Mitochondria (most eukaryotes)	
	Plastids (plant cells)	
Function	Static, digital genetic data storage	Dynamic, many varied funvtions
Copier Enzyme	DNA polymerase	RNA polymerase
Structure	Long nucleotide chain	Short nucleotide chain
	Two complementary strands	One strand
	A-, B- or C-form helix	A-form helix only
"Backbone"	Inorganic phosphate	
	Deoxyribose (**D** in DNA)	Ribose (**R** in RNA)
Nucleobases	**T**hymine, **C**ytosine, **A**denine, **G**uanine	**U**racil replaces **T**hymine
Base Pairing	A↔T (Adenine to Thymine)	A↔U (Adenine to Uracil)
	G↔C (Guanine to Cytosine)	
EM Radiation	Somewhat UV sensitive	Relatively UV resistant

9.7 Protein Synthesis from DNA via RNA:

The central dogma of molecular biology is an explanation of the flow of genetic information within a biological system. It is popularly stated as "DNA makes RNA, and RNA makes protein". The central dogma of molecular biology suggests that DNA maintains the information to encode all of our proteins, and that RNA convert this code into polypeptides. Three general classes of RNA molecules are involved in ex-pressing the genes encoded within a cell's DNA.

Messenger RNA (mRNA) or template RNA is a type of ribonucleic acid which is synthesized on the surface of DNA template by the process of transcription and has its base sequence complementary to DNA. It carries the genetic information or 'message' (hence its nomenclature) for the assembly of amino acids from DNA to ribosomes, the site of protein synthesis. They are generally degraded quickly.

Transfer RNA (tRNA) molecules carry amino acids to the ribosomes during protein synthesis.

Ribosomal RNA (rRNA) molecules form the core of a cell's ribosomes. They direct the catalytic steps in the protein biosynthesis. They are very stable.

The biosynthesis of protein from DNA through all three types RNA involves two basic steps (Fig. 5.8).

• Transcription is the first step in decoding a cell's genetic information. In transcription, the DNA sequence of a gene is transcribed (copied) to make a mRNA.

• After the transcription of DNA to mRNA is complete, translation or the reading of these mRNAs to make proteins begins. In translation, the sequence of nucleotides in the mRNA is "translated" into a sequence of amino acids to form a polypeptide (protein chain). Translation is controlled by various enzymes that recognize specific nucleotide sequences.

Cells fall into one of two broad categories: prokaryotic and eukaryotic. The single-celled organisms of the domains Bacteria and Archaea are classified as prokaryotes (pro = before; karyon- = nucleus). Animal cells, plant cells, fungi, and protists are eukaryotes (eu = true). Prokaryotes lack a cell nucleus whereas a eukaryotic cell has a membrane-bound nucleus and the DNA is contained inside the cell's nucleus where it is transcribed into RNA.

The overall scheme of protein synthesis is similar in between bacteria and eukaryotes. The major structural and mechanistic themes recur in all living cells. However, eukaryotic protein synthesis needs more protein components than does prokaryotic protein synthesis, and some aspects are more complex.

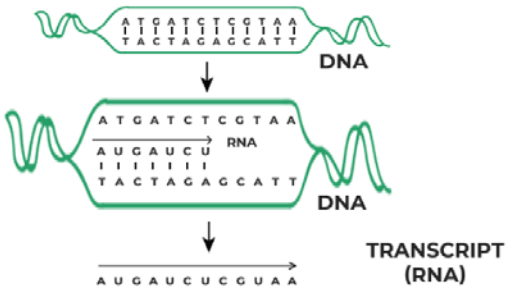
9.8 Transcription:

9.8.1 Overview of transcription:

Transcription involves synthesis of RNA chain representing one strand of a DNA duplex. The genetic information stored in DNA is expressed through RNA. For this purpose, one of the two

strands of DNA serves as a template and produces working copies of RNA molecules. Template strand is also known as antisense or noncoding strand because its sequences complementary to that of the RNA. The other DNA strand which does not participate in transcription is referred to as coding strand or sense strand because the same base sequence (with the exception of T for U) is transcribed onto primary mRNA.

RNA synthesis is catalysed by the enzyme RNA polymerase (RNAP). Transcription starts when RNA polymerase binds to a special region of the DNA, the promoter. Promoter regions in prokaryotic and eukaryotic genomes are predicted to have several common structural features, such as lower stability, higher curvature and lesser bendability as compared with their neighbouring regions. The promoter surrounds the first base pair that is transcribed into RNA, the start points (+1). Sequences prior to the start point are described as upstream of it; those after the start point (within the transcribed sequence) are downstream of it. Before the transcription start site '-' denotes upstream region and '+' denotes downstream region. Conservation of very short consensus sequences is a typical feature of promoters in both prokaryotic and eukaryotic genomes. There are two well conserved consensus sequences on the coding strand which function as recognition sites for RNA polymerase to bind. The most conserved sequence is at -10 base pair upstream which has a consensus sequence of TATAAT (known as TATA box, alternatively called Pribnow box after its discoverer David Pribnow). Upstream sequences around position -35 also have a region of sequence similarity, TTGACA.



9.8.2 Stages of Transcription:

(i) Initiation:

Holoenzyme attaches with the DNA and slides along it. When it reaches to a promotor, o factor identifies it at both -10 and -35 sequences present in the coding strand. o factor can bind tightly with these sequences. RNA polymerase initiates transcription after binding to a promoter site on DNA. In the first step, when RNA polymerase binds to DNA, DNA is still in the double

stranded form known as closed promoter complex. For transcription to occur, the closed promoter complex must isomerize into an open promoter complex, where the DNA double helix is opened up (unwind) into a transcription bubble (Fig. 5.12). The unwinding is caused by the hydrogen bonding between promoter and sigma factor. The first opening of DNA occurs at TATA sequences at -10 position because A/T rich sequences (TATAAT) have only double H-bonds.

(ii) Elongation:

σ factor separates from holoenzyme and the core enzyme then slides along the template DNA strand. As the complementary bases pair up, the core enzyme collect nucleotides one by one according to the base pairing rule and join them to the 3' end of the growing RNA molecule. Thus, during elongation the transcription bubble moves along DNA and the RNA chain is extended in the 5'→ 3' direction.

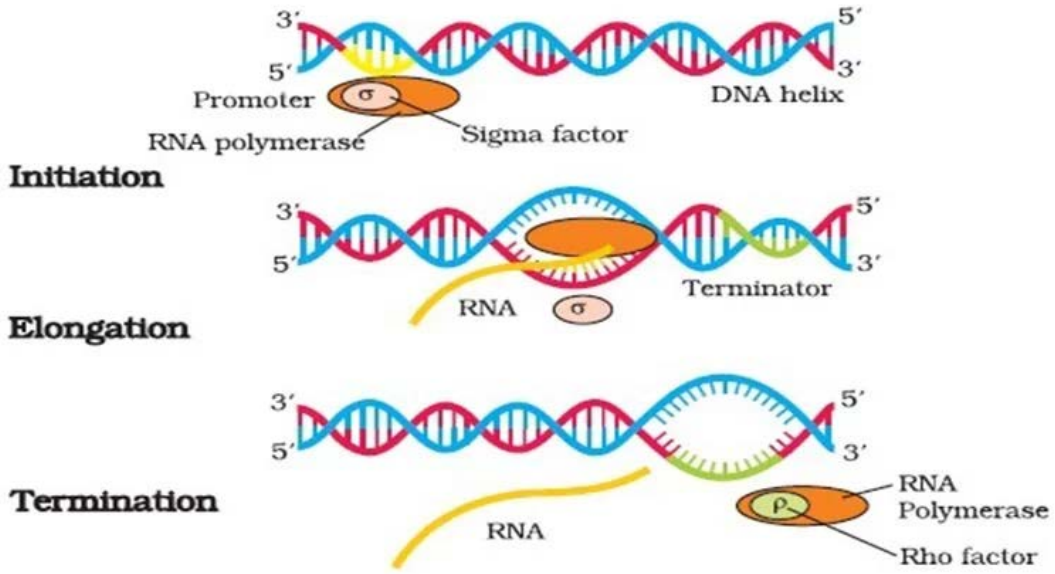
(iii) Termination:

At the termination portion of the gene, the mRNA transcript is complete. DNA duplex reforms, and RNA polymerase and mRNA dissociate from each other. Two types of termination are identified.

(a) Rho (p) dependent termination: A termination protein, named p factor, can recognize specific signals and binds to the growing RNA (and not to RNA polymerase) or weakly to DNA. The attachment is ATP dependent process and in the bound state it acts as ATPase. The p factor is responsible for the dissociation of RNA polymerase from DNA, thus terminates transcription and releases the newly formed RNA.

(b) Rho (p) independent termination: The termination in this case is brought about by the formation of hairpins of newly synthesized RNA. This occurs due to the presence of palindromes.

A palindrome is a word that reads alike forward and backward e.g. madam, level. The presence of palindromes in the base sequence of DNA template in the termination region is known. The RNA transcript of this region, called an intrinsic terminator, therefore folds to form hairpin structures (due to complementary base pairing) that cause termination of transcription.



9.8.3 Difference in Prokaryotic and Eukaryotic Transcription:

In prokaryotes, which lack membrane-bound nuclei, transcription occurs in the cytoplasm of the cell. There is a little or no processing of mRNA transcripts; the mRNA is translated directly as it comes off the DNA template. In prokaryotic organisms, transcription, translation, and mRNA degradation can all occur simultaneously; ribosomes can attach to mRNA while it is still being transcribed (Fig. 5.13). On the other hand, in eukaryotes, transcription occurs in the nucleus of the cell and the mRNA transcript needs to be processed within the nucleus before it can be fully functional. The strand that is made during transcription includes two regions: exons code for a protein whereas introns are non-coding section. Introns need to be removed and

9.9 Genetic Code:

The correspondence between nucleic acid sequences and polypeptide sequences in known as Genetic code. Genetic code is the sequence of nucleotides in deoxy-ribonucleic acid (DNA) and ribonucleic acid (RNA) that determines the amino acid sequence of proteins.

DNA is the repository of genetic information. The DNA is organized into genes: the fundamental units of genetic information. Though the linear sequence of nucleotides in DNA contains the information for protein sequences, proteins are not made directly from DNA. A messenger RNA (mRNA) molecule is synthesized from the DNA and directs the formation of the protein.

9.9.1 The Triplet Hypothesis:

George Gamow proposed that a group of 3 successive nucleotides in a gene might code for one amino acid in a polypeptide.

► RNA is composed of four nucleotides: adenine (A), guanine (G), cytosine (C), and uracil (U).

► Three adjacent nucleotides constitute a unit known as the codon, which codes for an amino acid For example, the sequence AUG is a codon that specifies the amino acid methionine.

► There are 64 possible permutations, or combinations, of three-letter nucleotide sequences that can be made from the four nucleotides (43). These 64 unique sequences of nucleotides are more than enough to cover the 20 amino acids.

► Of these 64 codons, 61 represent amino acids. There are three stop codons/nonsense codons in the genetic code.

Initiator/start codons: The codons which act as "chain initiation" signals or starter codons for synthesis of a polypeptide chain are known as initiator codons. The most common start codon is AUG. However, prokaryotes (Archaea and Bacteria) are permitted to use alternate start codons, mainly GUG and UUG. The start codon always codes for methionine in eukaryotes and Archaea, and N-formyl methionine (fMet) in bacteria, mitochondria and plastids.

Nonsense/stop codons: The codons which act as "chain termination" signals to end the translation of a particular polypeptide chain are known as nonsense codons. It causes the release of the polypeptide chain from the ribosome. Three such codons are UAA, UAG, UGA.

► The AUG codon, in addition to coding for methionine, is found at the beginning of every mRNA and indicates the start of a protein. Thus, every polypeptide typically starts with methionine.

Note: codon AUG can also appear later in the coding sequence of an mRNA, where it simply specifies the amino acid methionine.

 mRNA codons are read from 5' to 3', and they specify the order of amino acids in a protein from N-terminus (methionine) to C-terminus.

9.9.2 Characteristics of The Code:

- **Degenerate:**

Methionine and tryptophan are the only two amino acids that are coded for by just a single codon (AUG and UGG, respectively). The other 18 amino acids are coded for by two to six codons. Because most of the 20 amino acids are coded for by more than one codon, the code is called degenerate; one amino acid has more than one codon. For example, proline is represented by four different codons: CCU, CCC, CCA, and CCG.

- **Non-overlapping:**

The genetic code is read from a fixed point as a continuous base sequence and it is non-overlapping, meaning that each nucleotide is part of only one codon nucleotide cannot be part of two adjacent codons. a single

- **Unambiguous:**

A particular codon always stands for the same amino acid; hence the genetic code is highly specific or unambiguous, e.g. UGG is the codon for tryptophan.

- **Universal:**

Furthermore, the genetic code is nearly universal, with only rare variations re-ported. The same codons are used to code for the same amino acids in all the living organisms. Thus, the genetic code has been conserved during the course of evolution.

- **Codon-anticodon recognition:**

The codon of the mRNA is recognized by the anticodon of tRNA. They pair with each other in antiparallel direction (5' → 3' of mRNA with 3' 5' of tRNA). The usual conventional complementary base pairing (A-U, C-G) occurs between the first two bases of codon and the last two bases of anticodon. The third base of the codon is rather lenient or flexible with regard to the complementary base. The reduced specificity at the last position is known as third base degeneracy.

- **Wobble hypothesis:**

The pairing of codon and anticodon can wobble at the third letter. This is due to the fact that the third base (3'-base) in the codon often fails to recognize the specific complementary base in the anticodon (5'-base). Actually, the first base in anticodon can form non-Watson-Crick

base pairing with the third position of the codon. The allowed pairing of 5' end base of anticodon (of tRNA) with the 3'-end base of codon (mRNA) is given in Table 3.

Wobble hypothesis explains the degeneracy of the genetic code, i.e. existence of multiple codons for a single amino acid. Wobble hypothesis is the phenomenon in which a single tRNA can recognize more than one codon. Although there are 61 codons for amino acids, the number of tRNAs is far less (around 40) which is due to wobbling. 5.6

9.10 Replication:

9.10.1 DNA Replication (Prokaryotic):

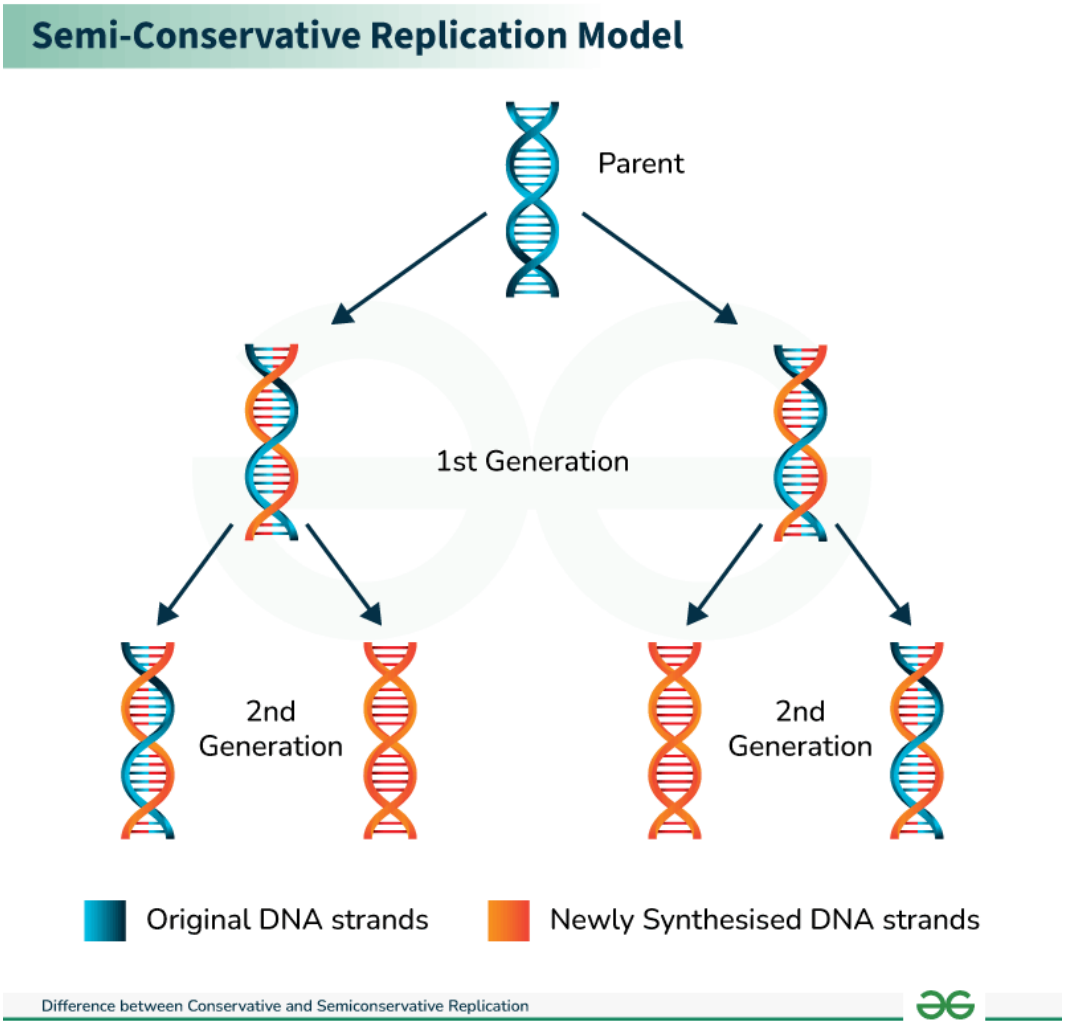
DNA replication is a fundamental genetic process that is essential for cell division during growth or repair of damaged tissues. Replication is a process in which DNA copies itself to produce identical daughter molecules of DNA. Replication is carried out with high fidelity which is essential for the survival of the species. Even mild disturbances in its dynamics could potentially lead to inhibition of DNA replication and termination of cell growth. Also, it conserves the entire genome for the next generation. Here, we will focus on DNA replication as it takes place in the bacterium E. coli, but the mechanisms of replication are similar in humans and other eukaryotes.

9.10.2 Basics of DNA Replication:

Watson and Crick's discovery that DNA has two strands complementary to each other provided a hint as to how DNA is replicated. During cell division, each DNA molecule has to be perfectly copied to ensure identical DNA molecules to move to each of the two daughter cells. The double-stranded structure of DNA suggested that the two strands might separate during replication with each strand serving as a template from which the new complementary strand for each is copied, generating two double-stranded molecules from one.

9.10.3 Semiconservative Model of Replication:

Each strand in the double helix acts as a template for synthesis of a new, complementary strand. Each one of the newly synthesized DNA has one-half of the parental. DNA (one strand from original) and one-half of new DNA (Fig 5.21). This type of replication is known as semiconservative since half of the original DNA is conserved in the daughter DNA. The base pairing rule is always maintained. The first experimental evidence for the semiconservative DNA replication was provided by Meselson and Stahl in 1958.



9.10.4 Process of Replication:

Replication occurs in three major steps:

- Opening of the double helix and separation of the DNA strands
- Priming of the template strand, and
- Assembly of the new DNA segment

During separation, the two strands of the DNA double helix uncoil/unwind at a specific location called the origin of replication on the cell's circular chromosome. The two complementary strands of DNA separate at the site of replication to form a replication bubble (Fig. 5.22). In the replication bubble, the DNA synthesis occurs in both the directions (bi-directional) from the point of origin. The separation of the two strands of parent DNA results in the formation of a Y-shaped structure called replication fork (Fig. 5.23), Two replication forks are formed at the origin of replication and these get extended bi-directionally as replication proceeds.

9.11 Summary:

Nucleic acids are primarily involved in the expression and storage of genetic information. They are the macromolecules that are most crucial to the continuation of life. They control the production of proteins, which determines the hereditary traits of all living organisms. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the two primary forms of nucleic acids. Erwin Chargaff developed the first trustworthy techniques for DNA compositional analysis in the late 1940s. According to Watson and Crick's model, DNA is a double-stranded, right-handed, antiparallel helix. The construction resembles a spiral staircase. The nitrogenous bases are located on the interior of the helix, perpendicular to the helix's axis, whereas the sugar-phosphate backbones of the DNA strands form the helix's exterior. The DNA strands are held together by hydrogen-bonded pairs formed by the nucleotides. The double helical structure is given additional stability by the base pairs' stacking with their planes. With a few exceptions, RNA and DNA share many structural similarities. One of the four nitrogenous bases, a phosphate group, and ribose (the pentose sugar) are all found in a ribonucleotide in the RNA chain. Phosphodiester bonds are used to join individual ribonucleotides to form the polymeric molecule that is RNA. DNA is a macromolecule that contains information. The chemical foundation of heredity, DNA, may be thought of as the cell's genetic information storehouse. The main function of ribonucleic acid (RNA) is to transfer the genetic code from DNA to the ribosome for protein synthesis via three different forms of RNA. The process of transcription entails creating an RNA chain that represents one strand of a DNA duplex. RNA expresses the genetic information contained in DNA. Genetic code is the relationship between polypeptide and nucleic acid sequences. The nucleotide sequence of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) that establishes the amino acid sequence of proteins is known as the genetic code.

9.12 Model Questions:

1. Name only the base pairs present within DNA and RNA.

2. Write some important features of Watson-Crick model.

3. What do you mean by Genetic Code?

4. Give three characteristics of Genetic code.

5. What is Wobble hypothesis?

6. Give differences between DNA & RNA.

7. Describe protein synthesis from DNA via RNA.

8. What are Initiator and Nonsense Codons?

9. What is replication fork?

10. Discuss semiconservative model of replication.

9.13 References:

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Unit 10 Basics of Blood

Index

10.1 Objectives:

After proper studying this unit one can understand the following

1. Function and properties of all components of blood.

2. Factors responsible for blood coagulation.

3. Several types of anaemia.

4. All pathways of blood coagulation.

5. Compare and contrast WBC & RBC.

10.2 Introduction:

Blood is composed of plasma and blood cells. Three types of blood cells - erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets) are suspended in the plasma with other particulate matter. Blood has three main functions: transportation, protection and regulation. The kidneys filter unwanted sub-stances from the blood and produce urine to excrete them. The test results of blood analysis are useful for detecting certain diseases and conditions. They also help to check the function of organs such as the liver and kidneys. Blood test reports guide the therapy of a patient and show how well treatments are working. Blood is a body fluid which is considered as a connective tissue. It consists of 55% plasma and 45% "formed elements" including blood cells and platelets. The formed elements are so named because they are enclosed in a plasma membrane and have a definite structure and shape. Blood circulates throughout our body through three types of vessels: arteries, veins and capillaries.

10.3 Physical Properties of Blood:

- It is slightly denser and more viscous than water.
- Blood contains various chemical constituents such as glucose, proteins, lipids, globulin, fibrinogen, urea, amino acids, uric acid, creatinine, hormones, vitamins, electrolytes, etc.
- Normally, blood pH is maintained in narrow range of 7.38-7.42, making it slightly alkaline. The average pH of plasma is 7.4. If the blood pH falls below 7.38, the condition is referred to as an acidosis, and above 7.42, as an alkalosis. Blood pH values below 7.0 or above 7.8 are life threatening.

10.4 Biological Importance of Blood:

Blood is specialized for performing various functions transportation, regulation of body temperature etc. It makes up 8% of our body. An average adult possesses around 5-6 litres of blood. Evidently blood has three main functions: transportation, protection and regulation. Blood has many different functions:

 Absorbs oxygen from the lungs and transports it to different cells of the body. The waste carbon dioxide moves from the blood to the lungs and exhaled.

► Transports nutrients from the digestive tract and storage sites to the rest of the body. Hormones secreted by the endocrine glands are also transported to their target organs. Brings waste products to the kidneys and liver, which filter and clean the blood. Regulates internal body temperature by absorbing or releasing heat (Homeo-stasis).

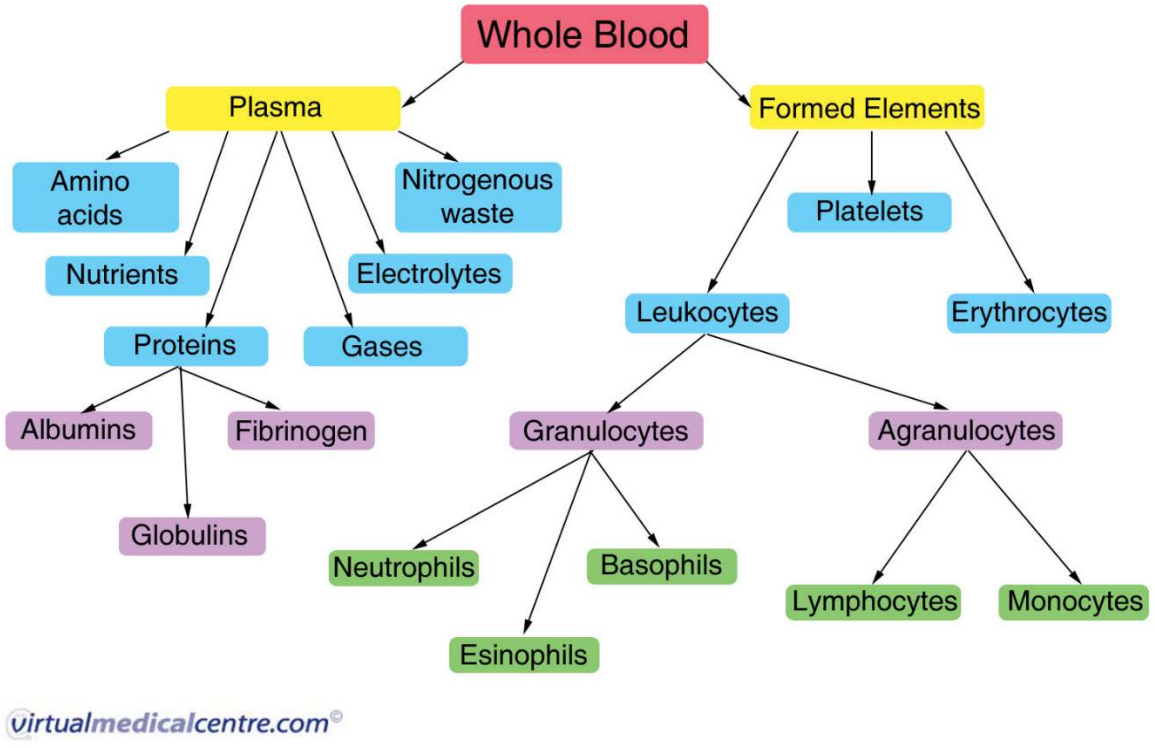
► Forms blood clots to prevent excess blood loss at the site of injury.

► Produces special kinds of proteins called antibodies, which identify and fight pathogens invading the human body.

► Regulates pH by interacting with acids and bases, and water balance by transferring water to and from tissues.

10.5 Composition and Functions of Blood Components:

Blood is both a tissue and a fluid. The formed elements are cells and cell fragments suspended in a liquid matrix called plasma, which makes the blood a fluid. The three classes of formed elements are the erythrocytes (red blood cells), leukocytes (white blood cells), and the thrombocytes (platelets). The red blood cells constitute about 45 percent of the volume of the blood, and the remaining cells (white blood cells and platelets) less than 1 percent. The composition of blood is shown in following chart.



10.5.1 Plasma:

Plasma is a straw-coloured fluid matrix (54.3% by volume) in which blood cells are suspended. It is slightly alkaline and viscous. It is made up of approximately 90% water as well as

electrolytes such as sodium (Na^+) and potassium (K^+) and proteins. It also contains some organic substances in dissolved form like glucose, proteins, fats, urea, hormones, enzymes etc.

10.5.1.1 Proteins:

These are the most abundant substance in plasma by weight and play a part in a variety of roles including clotting, defence and transport. The protein in plasma includes antibodies to assist in the body's defence system against disease and infection. Each individual type of proteins has its own specific properties and functions in additions to their overall collective role:

 Albumins: These are the smallest and most abundant plasma proteins. Albumin plays a significant role in maintaining blood volume and body fluid distribution. Reductions in plasma albumin content result a fall in osmotic pressure. This leads to a gain of fluid in the interstitial space (space within the tissue), which may occur in nutritional, liver and kidney disease. Albumin also helps many substances such as drugs, hormones and fatty acids to dissolve in the plasma by binding to them.

► **Globulins:** They act as transport protein. The globulins include HDL and LDL which play important parts in the regulation of cholesterol and hence have a large impact on cardiovascular disease. Immunoglobulin, also known as antibody, is a critical part of the immune system by specifically recognizing and binding to particular antigens, such as bacteria or viruses, and aiding in their destruction.

► **Fibrinogen:** This is a soluble precursor of a sticky protein called fibrin which plays a key role in coagulation of blood.

10.5.1.2 Amino acids: These are formed from the breakdown of tissue proteins or from the digestion of digested proteins.

10.5.1.3 Nitrogenous waste: Being toxic end products of the breakdown of substances in the body, these are usually cleared from the bloodstream and are excreted by the kidneys at a rate that balances their production.

10.5.1.4 Inorganic salts: These include electrolytes such as chlorides, bicarbonates, phosphates of sodium, potassium and calcium. Sodium ions, which are most abundant among this, account for the blood's osmolarity: a measure of the body's electrolyte-water balance.

10.5.1.5 Dissolved nutrient: Those absorbed by the digestive tract are transported in the blood plasma. These include glucose, amino acids, fats, cholesterol, phospholipids, vitamins and minerals.

10.5.1.6 Gases: Some oxygen and carbon dioxide are transported by plasma. Plasma also contains a substantial amount of dissolved nitrogen.

10.5.2 Formed elements:

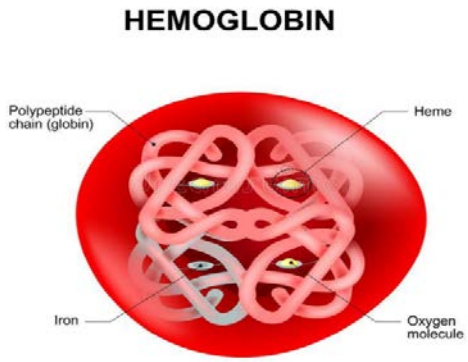
The blood cells or formed elements consist of red blood cells (RBCs), white blood cells, (WBCs), and platelets.

10.5.2.1 Red Blood Cells:

Red blood cells (RBCs), also known as erythrocytes, are by far the most abundant cells in the blood. In humans, red blood cells have a biconcave disk shape, and do not contain nucleus. They can easily change shape without breaking, which helps them fit through the various blood vessels. They contain the plasma oxygen-transport protein haemoglobin - an iron containing pigment heme attached to protein globin (Fig. 6.3b). The prosthetic group heme is iron porphyrin complex called iron protoporphyrin IX. Adult haemoglobin is a tetramer since it has 4 polypeptide chains consisting of two a-subunits (a_1 and a_2 : 141 amino acids) and two □-subunits (B_1 and B2: 146 amino acids). Biosynthesis of heme is initiated in mitochondria with the condensation of glycine and succinyl CoA. The iron in haemoglobin which gives blood its red colour is in +2 oxidation state. Oxygen molecules coordinate to Fe (II) and this helps in the transportation of the oxygen to different tissues and organs of the human body. The binding of oxygen to haemoglobin is a reversible reaction:

$$Hb + 4O_2 \leftrightharpoons Hb\text{-}O_2$$

In lungs, where oxygen concentration is high, oxyhaemoglobin is formed. But in tissues where oxygen concentration is low, oxyhaemoglobin dissociates to release oxy-gen. Once the oxygen is delivered, iron then helps red blood cells to carry 20-25% carbon dioxide from different organs and tissues back to the lungs for exhalation. The percentage of whole blood volume that is made up of red blood cells is called the haematocrit and is a common measure of red blood cell levels.



Vitamin B12 (cobalamin) and Vitamin B9 (Folic acid) are required for complete maturation of RBCs. The lifespan of RBC is about 120 days. Old or damaged red blood cells are broken down in the liver and spleen, and new ones are produced in the bone marrow. Production of red blood cells is controlled by erythropoietin, a hormone produced primarily by the kidneys in response to low oxygen levels (hypoxia) in blood. The plasma membrane of a mature RBC has glycoproteins and glycolipids that determine a person's blood type.

RBC count: 4.1 to 5.1 million cells/mm³ for women, 4.5 to 5.9 million cells/mm³ for men

The normal range for haemoglobin: For men, 13.5 to 17.5 grams per decilitre. For women, 12.0 to 15.5 grams per decilitre. Low count of RBCs results in anaemia.

10.5.2.2 White Blood Cells:

White blood cells (WBCs), also called leukocytes, are much fewer in number than red blood cells. WBCs comprise 1% of the total blood volume. They have nuclei and are colourless because they are devoid of haemoglobin. These cells act as a defence system against any infections in the human body. Stem cells in the bone marrow are responsible for producing white blood cells. The lifespan of WBC is 12-20 days. After that, they are destroyed in the lymphatic system.

These cells are divided into two different groups, named for their appearance un-der a microscope, granulocytes which contain granules in their cytoplasm and agranulocytes which lack granules in their cytoplasm. Granulocytes consist of neutrophils, eosinophils and basophils while agranulocytes includes monocytes and lymphocytes. Each type of white blood cells is given a specific defence task to fight against foreign objects. Neutrophils are the one of the body's main defences against bacteria. They kill bacteria through the process of phagocytosis. Eosinophils have a role in allergic reactions and release enzymes that disable parasites.

Basophils secret histamine during allergic reactions. They prevent blood clotting since they contain heparin which is an anticoagulant. Monocytes enter the tissue, where they become larger and turn into macrophages. There they can phagocytize bacteria throughout the body. These cells also destroy old, damaged and dead cells in the body. Lymphocytes are complex cells that produce antibodies and direct the body's immune system. T lymphocytes help regulate the function of other immune cells and directly attack various infected cells and tumours. B lymphocytes make antibodies, which are proteins that specifically target bacteria, viruses, and other foreign materials. Lymphocytes are different from the other WBCs because they can recognize and have a memory of invading bacteria and viruses.

WBC count: 4,300 and 10,800 cells/mm^3 of blood.

Leukopenia is a low white blood cell count that can be caused by damage to the bone marrow from things like medications, radiation, or chemotherapy. A high white blood cell count may indicate the medical conditions like asthma attack, rheumatoid arthritis, or infections. Leukaemia is a blood cancer caused by a rise in the number of white blood cells in body.

10.5.2.3 Platelets:

These are circular or oval, colourless tiny fragments of bone marrow cells. They lack nuclei. They are also known as thrombocytes. They are fragments of cytoplasm, which are derived from the megakaryocytes of the bone marrow. They are smallest of the three major blood cell types. The main function of platelets is to form blood clots to stop or prevent bleeding. When the platelets are activated, they aggregate to form a platelet plug which covers the wound and prevents blood from leaking out. Platelets are a natural source of growth factors necessary to maintain the linings of blood vessels. The average lifespan of a platelet is normally just 5 to 9 days. The nor-mal range for platelets is 1.5 - 4 lakhs per cubic millimetre. But since platelets are so small, they make up just a tiny portion of the blood volume. A higher-than-normal number of platelets can cause unnecessary clotting, which can lead to strokes and heart attacks. Conversely, lower than normal counts can lead to extensive bleeding. Dengue fever can result in a drop in platelet counts to as low as 20,000 to 40,000.

10.6 Blood Coagulation:

10.6.1 About Blood Coagulation:

Blood is a necessary component of human body and loss of this fluid may be life threatening. Blood coagulation is an important process of forming a clot to stop bleeding.

Haemostasis (commonly known as blood coagulation, heme means blood and stasis means to halt) is the physiological process that stops bleeding at the site of an injury while maintaining normal blood flow elsewhere in the circulation. Haemostasis requires both platelets and the coagulation system. At sites of vessel injury, bleeding is minimized by the formation of a haemostatic plug consisting of platelets and fibrin. This process is strictly regulated so that it is activated within seconds of an injury but must remain localized at the site of injury. There are two main components of haemostasis.

The first stage, primary haemostasis, is characterized by two processes: blood vessel constriction (vasoconstriction) and platelet plug formation. Vasoconstriction is the body's first response to injury in the vascular wall. When injury occurs, vessel walls constrict, causing reduced blood flow to the site of injury. Platelets aggregate to the site of the vessel injury. They stick together acting as a "plug". Platelets also activate the process of blood clotting or coagulation, which is body's major defence mechanism against blood loss.

Secondary haemostasis refers to the formation of fibrin mesh through coagulation cascade which strengthen and stabilize the primary platelet plug. In essence, blood clotting is achieved by conversion of a soluble plasma protein fibrinogen to the insoluble mesh-like cross-linked network of fibrins. This occurs through a sequence of processes mediated by several coagulation factors (clotting factors).

10.6.2 Blood Coagulating Factors:

The coagulation factors are numbered in the order of their discovery. There are 12 principal coagulation factors in all, and each of these has been assigned a Roman numeral I to XIII. Factor VI was subsequently found to be part of another factor.

Factor	Name
I	Fibrinogen
II	Prothrombin
III	Tissue thromboplastin
IV	Ionized Calcium (Ca2+)
V	Labile Factor or proaccelerin
VII	Stable Factor or proconvertin

VIII	Antihemophilc Factor A
IX	Plasma thromboplastin component, Christmas Factor, Antihemophilc Factor B
X	Stuart-power Factor
XI	Plasma thromboplastin antecedent (PTA)
XII	Hageman factor
XIII	Fibrin-stabilizing factor

Majorly of the coagulation factors are precursors of proteolytic enzymes known as zymogens that circulate in an inactive form. Non-enzymatic coagulation factors can be cofactors for enzymatic coagulation factors or can just be a protein substrate (Factor I or fibrinogen). Most of the coagulation factors are produced by liver. These factors activate each other through a series of reactions that make up the coagulation cascade which ultimately leads to fibrin. By convention, the activated form of the coagulation factor is denoted by a small "a" after the factor number. Calcium and platelet phospholipid (phosphatidylserine) membrane are required for the proper functioning of the coagulation cascade. Platelets provide a source of phospholipid and a binding surface upon which the coagulation cascade proceeds. Vitamin K helps to regulate the process of blood coagulation (in German-Koagulation; hence the name K for this vitamin) by assisting in the conversion certain coagulation factors into their activated forms. The liver must be able to use Vitamin K to produce factors II, VII, IX, and X. Vitamin K deficiency is associated with impaired coagulation function and excessive bleeding and haemorrhage (internal bleeding, often severe). This can be caused by poor diet, malabsorption in the intestines, or liver failure.

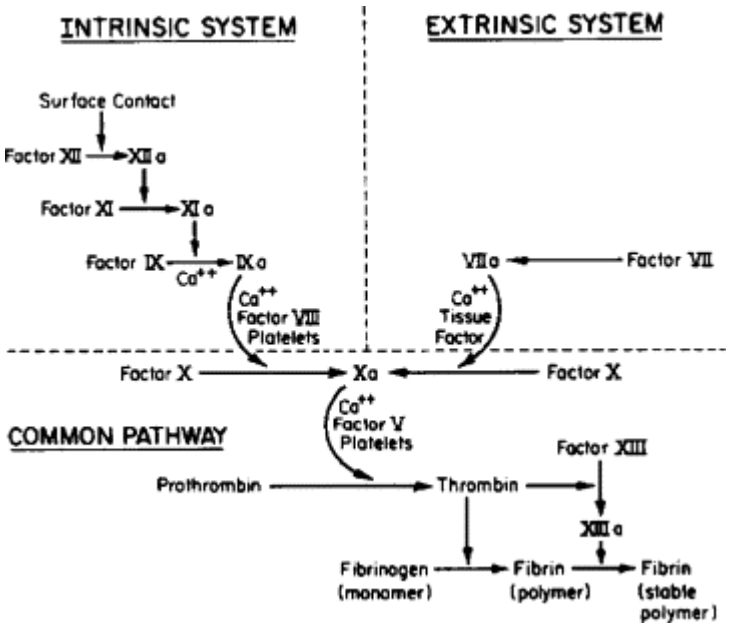
10.6.3. Pathways of Blood Coagulation:

Coagulation can be initiated through the activation of two separate pathways, extrinsic and intrinsic pathways, which meet and finish the pathway of clot production in what is known as the common pathway.

Intrinsic Pathway (contact activation pathway): The intrinsic pathway responds to internal damage of vascular system. This pathway is actually triggered when plasma comes into contact with certain types of artificial surfaces and is activated by plate-lets, exposed endothelium, chemicals, or collagen. It involves factors XII, XI, IX, VIII and Ca^{2+}.

Extrinsic Pathway (tissue factor pathway): The extrinsic pathway is activated through tissue factor released by endothelial cells after external trauma that causes blood to escape from the vascular system. It involves factors III, VII and $Ca^{2+.}$

Both pathways result in the production of factor X. The activation of this factor marks the beginning of the so-called common pathway of coagulation, which ultimately activates fibrinogen into fibrin leading to the formation of a clot. The common pathway factors are X, V, II, I, XIII. Prothrombin is a vitamin K-dependent coagulation factor, which is enzymatically cleaved to thrombin by activated factor X in the presence of calcium ions and other clotting factors. Thrombin in turn, acts as a serine protease and catalyses the conversion of fibrinogen (factor I) -a soluble plasma protein-into long, sticky threads of insoluble fibrin (factor I). The fibrin threads form a mesh that traps platelets, blood cells, and plasma. Within minutes, the fibrin meshwork begins to contract, squeezing out its fluid contents. This process, called clot retraction, is the final step in coagulation. It yields a resilient, insoluble clot that can withstand the friction of blood flow.



10.7 Anaemia:

10.7.1 About:

Anaemia is a blood disorder in which there is a deficiency of actual or available haemoglobin in blood. There is a reduced oxygen capacity leading to oxygen deficiency. RBCs play great role in anaemia. Actually, RBCs carry haemoglobin that attaches to oxygen in the lungs and carries it to the tissues throughout the body. It is diagnosed when a blood test shows a

haemoglobin value of less than 13.5 gm/dl in a male or less than 12.0 gm/dl in a female. Anaemia can be temporary or long term, and can range from mild to severe. A Hb level below 8 mg/dl results severe anaemia. The causes of anaemia may be classified as impaired RBC production, increased RBC destruction (haemolytic anaemia), blood loss and fluid overload (hypervolemia).

Anaemia is associated with the symptoms of apathy (dull and inactive), sluggish metabolic activities, retarded growth, loss of appetite, weakness, shortness of breath, fast or irregular heartbeat, headache, dizziness or light-headedness, cold hands or feet, pale or yellow skin and chest pain.

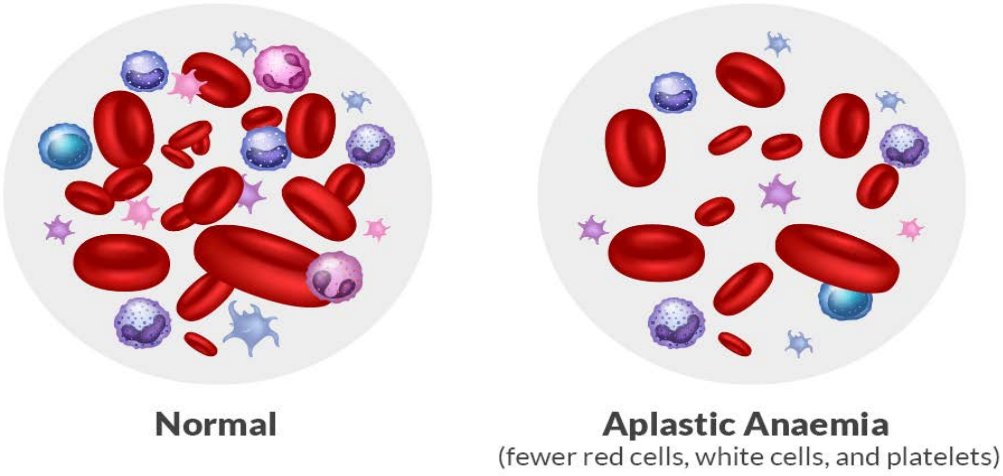
10.7.2 Classification:

a. Deficiency Anaemia: This is the most common type of anaemia caused by the inadequate amount of iron in the body. Without adequate iron, our body can't produce enough haemoglobin for red blood cells. This type of anaemia occurs in many pregnant women. It is also caused by blood loss, such as from heavy menstrual bleeding, ulcer, cancer and regular use of some over-the-counter pain relievers, especially aspirin.

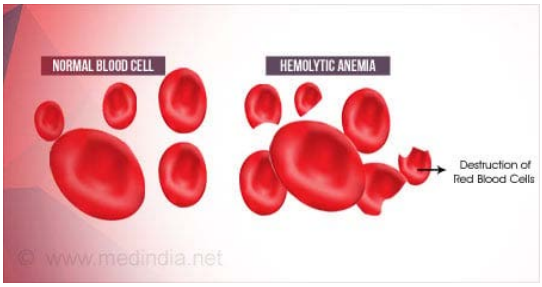
b. Vitamin Deficiency Anaemia: Besides iron, our body needs folic acid (B9) and vitamin B12 to produce enough healthy red blood cells. A diet lacking in these and other key nutrients can cause decreased red blood cell production. Also, some people who consume enough B12 aren't able to absorb the vitamin. This can lead to vitamin deficiency anaemia, also known as pernicious anaemia.

Iron and vitamin deficiency anaemia can be prevented by eating a diet high in iron-rich foods, folate, vitamin B12 and vitamin C. Also, Iron tablets can help restore iron levels in our body.

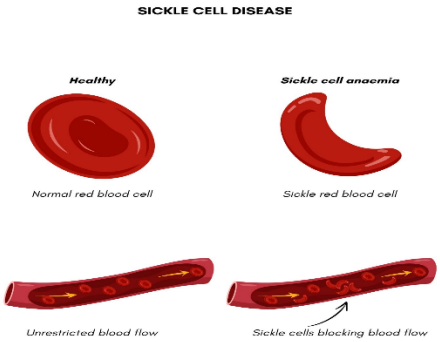
c. Aplastic Anaemia: This rare, life-threatening anaemia occurs when the bone-marrow stops producing enough blood cells (RBCs, WBCs and platelets). This type of anaemia arises due to defect in stem cells in bone marrow. Causes of aplastic anaemia include infections, certain medicines (e.g. Chloramphenicol), autoimmune diseases and exposure to toxic chemicals.



d. Haemolytic Anaemia: This group of anaemia develops when red blood cells are destroyed faster than bone marrow can replace them. Certain blood diseases increase red blood cell destruction. Haemolytic anaemia may due to mechanical causes such as leaky heart valves or aneurysms, infections, autoimmune disorders or congenital abnormalities in the RBCs. Thermal lability of glucose-6-phosphate dehydrogenase results in haemolytic anaemia.



e. Sickle Cell Anaemia: This genetic disease is a haemolytic anaemia. It is caused by a defective form of haemoglobin due to a single amino acid change in the □-chain of haemoglobin. The genetic defect responsible is the replacement of hydrophilic glutamic acid at □-6 with the hydrophobic valine. This forces red blood cells to assume an abnormal crescent (sickle) shape (Fig. 6.5). These irregular blood cells die prematurely, resulting in a chronic shortage of red blood cells. Biochemically, this disease results in severe anaemia and progressive damage to major organs in the body (heart, brain, lungs, joints).



f. Certain Other Diseases: Cancer, HIV/AIDS, rheumatoid arthritis, kidney disease, Crohn's disease and other acute or chronic inflammatory diseases - can interfere with the production of red blood cells. For example, some patients with kidney disease develop anaemia because the kidneys are not making enough of the hormone erythropoietin to signal the bone-marrow to make new or more RBCs. Chemo therapy for treating cancer often impairs the body's ability to make new RBCs, and anaemia often results from this treatment. A variety of diseases, such as leukaemia and myelofibrosis, can cause anaemia by affecting blood production in bone marrow.

10.7.3 Care towards Anaemia Prevention:

To keep a healthy and diverse diet:

- Eat iron-rich foods, including lean red meats, fish and poultry, legumes (e.g. lentils and beans), fortified cereals and dark green leafy vegetables;
- Eat foods rich in vitamin C (such as fruits and vegetables) which help the body absorb iron; and
- Avoid foods that slow down iron absorption when consuming iron-rich foods, such as bran in cereals (wholewheat flour, oats), tea, coffee, cocoa and calcium.

If you take calcium and iron supplements, take them at different times during the day.

People with heavy menstrual bleeding should see their doctor for treatment. Doctors may recommend iron supplements or hormonal contraceptives.

Some infections can cause anaemia. Wash your hands with soap and water and use clean toilets to reduce the risk of infection.

Malaria can also cause anaemia. People living in places where malaria is common should follow prevention advice from local health authorities. Seek prompt treatment if you suspect you have malaria.

10.8 Summary:

Blood is specialised for a number of tasks, including temperature control and transportation. It comprises 8 percent of our body. The typical adult has five to six litres of blood. Blood clearly serves three primary purposes: regulation, protection, and transportation. Blood is a fluid as well as a tissue. The blood is a fluid because of the cells and cell fragments that are created and suspended in a liquid matrix known as plasma. Erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets) are the three kinds of components that are created. Since blood is an essential part of the human body, losing it might be fatal. One crucial step in creating a clot to halt bleeding is blood coagulation. Each of the twelve major coagulation factors has been given a Roman numeral, ranging from I to XIII. Later, it was discovered that factor VI was a component of another factor. Two distinct paths, the intrinsic and extrinsic pathways, can be activated to start coagulation. These pathways meet and complete the clot generation pathway, which is referred to as the common pathway. A blood condition known as anaemia occurs when there is insufficient real or accessible haemoglobin in the blood. The causes of anaemia may be classified as impaired RBC production, increased RBC destruction (haemolytic anaemia), blood loss and fluid overload (hypervolemia).

10.9 Model Questions:

1. What is the pH of blood?

2. Write four differences between WBC & RBC.

3. Write the name of factor V and IX.

4. what is sickle cell anaemia?

5. How blood helps in transportation?

6. How blood gets coagulate?

7. Write the role of albumin and fibrinogen.

8. Write the normal range of haemoglobin present in human body.

9. Write about any two types of anaemia.

10. Give a chart for whole composition of blood.

10.10 References:

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Unit 11 Blood Analysis

Index

11.1 Objectives:

After complete study of this unit one can understand the following:

1. The procedure of collecting blood through vein.

2. The technique and container used for preserving collecting blood.

3. The process of determining sugar, urea, creatinine in blood.

4. The normal range of urea, bilirubin, sugar etc in blood.

5. Anticoagulant used in blood.

11.2 Introduction:

Blood is the body fluid that is used most frequently for analytical purposes. Three-general procedures for obtaining blood are venipuncture, arterial puncture and skin puncture. The technique used to obtain the blood specimen is critical in order to maintain its integrity. Venous blood is commonly used for majority of biochemical investigations. It can be drawn from a superficial vein in the upper limb. Arterial blood is mostly used for the identification of metabolic, respiratory, and mixed acid-base disorders, where CO_2 levels require understanding or monitoring. Radial, brachial, or femoral arteries are the most common site for arterial blood. The venules and arterioles join together in capillary beds forming a mixture of venous and arterial blood. So, blood collected by skin puncture is a mixture of arterial and venous blood along with interstitial and intracellular fluids. Skin puncture is usually carried out in infants or when very little blood is required. Capillary blood is collected from the tip of the thumb or finger or from ear lobe by using a sterile needle.

11.3 Blood Collection:

11.3.1 Techniques for Venipuncture:

Generally, for common biochemical tests, blood is collected through venipuncture. A suitable vein of left arm is selected for puncture. Veins of antecubital fossa, in particular, the median cubital and cephalic veins are preferred. If one arm has an intravenous line, the other arm is used to draw a blood specimen. At first the venipuncture site is cleaned with 70% isopropanol solution or 1% iodine saturated swab-stick. Then a tourniquet is applied several inches above the puncture site only for one minute. Then the needle of the syringe at approximately 15° to the arm is inserted into the vein. The tourniquet is released when blood begins to flow. The sample volume to be collected depends upon the number and type of tests being performed. Generally, 3-5 ml blood is required for many investigations. If the tests are run on automated instruments, then less volume of blood may be sufficient.

Serum [Serum = plasma - clotting factors (fibrinogen)] is the liquid obtained after blood is allowed to clot, whereas plasma is obtained after treating blood with anticoagulation compounds. Serum composition is the same as that of plasma except that serum lacks fibrinogen.

11.3.2 Collection Tubes:

Blood is collected in various collection tubes called vacutainers which are sterile glass tubes with a coloured rubber stopper. In order to streamline the sample collection and testing process, most blood collection tubes contain compounds to preserve the sample in the state required for the specific test ordered. If whole blood or plasma is required, premeasured amounts of anticoagulants are added to the tubes during the manufacturing process. When serum is required, silicone-coated tubes or tubes containing clot-activating factors can be used to hasten clot formation. Other specialized tubes are used in order to inhibit metabolic processes that occur in the blood. The various tube types are indicated by different coloured tops, as indicated in following Table.

Tube Type	**Additive**	**Stopper Colour**
Serum tubes (without additive)	Silicone Coated interior	Red
	Uncoated Interior	Red
Serum Tubes (with additive)	Thrombin (dry additive)	Orange
	Particulate clot activator	Yellow/red
Whole blood/ plasma tubes	K_2EDTA	Lavender
	Citrate, trisodium	Light blue
	Sodium Fluoride/potassium oxalate	Grey
	Heparin, Lithium	Green

11.3.3 Procedure for Plasma Preparation:

Draw blood from patient and pour it in vacutainer with an appropriate anticoagulant. Mix blood with anticoagulant properly and allow the tubes to stand for 10 min. Then, the sample is centrifuged for speed separation and packing of cells. The supernatant is the plasma.

11.3.4 Procedure for Serum Preparation:

Draw blood from patient. Select vacutainer without anticoagulant and allow it to stand for 20-30 min so that clot is formed. Centrifuge the sample at 3000 rpm which affects a greater packing of cells. Various cells along with clot will settle in the form of pellet at the bottom of the tube. The supernatant is the serum.

To carry out precise estimation, dilution of blood sample is often necessary. For this purpose, normal saline is used but not distilled water. If distilled water is used to dilute blood, then all the RBCs will explode. The blood will become haemolyzed. This is because of osmosis. The RBC's membrane is semipermeable and its interior has a lot of substances dissolved. So, if we put a cell into distilled water then by osmosis water will penetrate into the cell, increasing its volume until it bursts. Normal saline is isotonic with blood plasma to avoid this to happen.

11.4 Preservation of Blood Samples:

Alteration in the concentration of a constituent in a stored specimen can result from various processes such as:

- Adsorption on the glass container.
- Evaporation if the constituent is volatile.
- Water shifts due to the addition of anticoagulants.
- Metabolic activities of the erythrocytes and leukocytes (accelerated by hemoly-sis) inducing O_2 consumption and CO2 production, hydrolysis, glycolysis and finally degradation.
- Microbial (fungal/bacterial) growth.

Preservation of sample is necessary to keep samples until time of examination in a state similar to that when it was obtained. Refrigeration of the samples is recommended when laboratory examination will be performed within hours. Changes in concentration of volatile substances such as O, and CO_2 are prevented or at least hindered by collection and storage of sample under anaerobic conditions. The problem of microbial growth appears when the sample is to be stored for longer than one day either at room or refrigerator temperature. This can be solved by four alternative courses of action:

- collection and storage under sterile condition
- freezing of the sample
- extreme alteration of pH
- addition of an antibacterial agent

Often after collection of blood it is preserved by using chemical preservatives for prevention of chemical changes such as glycolysis and for prevention of microbial growth. In 1923, Sander introduced the combination of 10 mg NaF + 1 mg thymol/ml of blood for preservation. The presence of thymol essentially controlled microbial growth so that non-sterile specimens are

stable for all determinations (except non-protein nitrogen) for at least two weeks. Chlorobenzene and bromobenzene have also been coupled with F and have been claimed to be superior to thymol.

11.5 Anticoagulants Used During Blood Collection:

(a) Ethylenediaminetetraacetic acid: This anticoagulant is used at a concentration of 2 mg/dl of blood volume, It removes calcium ions by chelation and block coagulation. It is used mainly for haematological studies.

(b) Heparin: Heparin inhibits conversion of prothrombin to thrombin. Heparin is present naturally in blood and hence acts as ideal anticoagulant. It increases the activity of antithrombin. For every ml of blood sample, 0.2 ml of heparin may be used.

(c) Sodium fluoride: This anticoagulant is considered when glucose estimations are carried out in blood samples. Sodium fluoride inhibits glycolysis by inhibiting activity of enolase enzyme and hence preserves blood glucose levels. It is generally combined with potassium oxalate because of its poor anticoagulant action.

(d) Sodium or potassium oxalate: Sodium, potassium, and even lithium oxalates precipitate calcium ions and inhibit blood coagulation. Potassium oxalate is more water soluble and is used at concentration of 5-10 mg/5 ml of blood.

11.6 Estimation and Interpretation of Data for Blood sugar, Urea, Creatinine, Cholesterol and Bilirubin:

11.6.1. Blood Sugar:

Glucose is a natural sugar in the body that is used for energy. During digestion most foods are converted to a simple sugar called glucose for energy. For glucose to pass for the blood stream into cells we need a hormone called Insulin which is produced by pancreas. In Diabetes there is less Insulin production or a problem with body’s ability to use Insulin (Insulin resistance) Hence there is a high level of glucose in the blood. If not treated it can cause complications like heart disease (blockage), heart attacks, kidney damage, stroke etc.

11.6.1.1 Regulation of Blood Glucose:

Blood sugar regulation is the process by which the levels of blood sugar, primarily glucose, are maintained by the body within a narrow range. The tight regulation is referred to as glucose

homeostasis. The levels of glucose in blood are monitored by many tissues, but the cells in the pancreatic islets are among the most well-understood and important. The two well-known hormones involved are insulin, which lowers blood sugar, and glucagon, which raises it. Insulin (hypoglycaemic hormone) is of prime importance in regulating blood sugar. A rise in blood sugar level stimulates insulin secretion and it lowers the blood sugar in several ways. It increases glucose uptake by tissues, promotes glycolysis for utilisation of glucose, reduces glycogenolysis and gluconeogenesis in the liver. When sugar levels fall, secretion of glucagon (hyperglycaemic hormone) is stimulated. It is antagonistic to insulin and increases blood sugar by enhancing glycogenolysis and gluconeogenesis in the liver and inhibiting glycolysis. Hyperglycaemic hormones like adrenaline, cortisol also regulate blood glucose level in the body. As hyperthyroid-ism leads to hyperglycaemia (Grave's disease), thyroid hormones act against insulin and tend to raise blood sugar by increasing hepatic glycogenolysis and stimulating gluconeogenesis. Growth hormone (GH) is also antagonistic to insulin in its effect on carbohydrate metabolism. It has diabetogenic effects as it raises blood sugar by inhibiting the uptake and utilization of glucose by muscles. It reduces insulin sensitivity.

11.6.1.2 Glucose oxidase-peroxidase method / Trinder's method:

Estimation of glucose is the most common analysis done in clinical laboratories. The blood is collected using an anticoagulant (potassium oxalate) and an inhibitor of glycolysis (sodium fluoride). Plasma is the preferred sample for glucose estimation. Plasma is separated from cells rapidly to avoid glucose loss. Capillary blood from fingertips may also be used for glucose estimation by test strips.

The glucose oxidase (GOD) method (enzymatic method) is the one most widely used. Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid and H_2O_2. The liberated H_2O_2 is broken down to H_2O and nascent O_2 by the peroxidase (POD) enzyme. 4-Aminoantipyrine, an oxygen acceptor, takes up the oxygen and together with phenol forms a pink-coloured quinonimine complex. The intensity of the colour is measured by colorimeter at 520 nm. The intensity of colour formed is proportional to the glucose concentration of the sample.

$$\text{Glucose} + \text{GOD} \rightarrow \text{Gluconic acid} + H2O2$$

$$H_2O_2 + \text{POD} \rightarrow H_2O + [O]$$

$$[O] + \text{4-aminoantipyrine} + \text{Phenol} \rightarrow \text{Chromogen (coloured)}$$

Present day autoanalyzer use this method. Thus, reagents for such estimation are available in the form of kit supplied by various companies.

1. Phosphate buffer: 0.1 M potassium phosphate buffer of pH 7.0.

2. Enzyme reagent: Dissolve 18 mg of 4-aminoantipyrine and 36 mg solid phenol in 100 ml of 0.1M phosphate buffer. Add GOD (1500 U) and POD (100 U). Store in brown bottle at 4°C.

3. Standard glucose solution (100 mg/dl): Dissolve 100 mg glucose in 100 ml distilled water.

The glucose concentration in the sample can be calculated by using the following equation:

Plasma glucose = [OD of test x amount of standard (mg) × 100]/ OD of standard x volume of sample (ml) = x mg/dl

The instrument named glucometer is used for measuring the approximate level of glucose in the blood. Most glucometers are based on electrochemical technology and have two essential parts: an enzymatic reaction and a detector. It requires the users to perform the blood glucose measurement by pricking their fingers and uploading the sample onto the test strip. Establishing accuracy of glucometers is difficult because glucose is unstable in capillary whole blood. However, they are highly useful for self-monitoring of blood glucose (SMBG) by diabetic patients at home.

10.6.1.3 Clinical Significance:

Plasma glucose analysed at any time of the day, without any prior preparations, is called random plasma glucose. Sugar estimated in the early morning, before taking any breakfast is called fasting plasma glucose. Fasting state means, glucose is estimated after an overnight fast (12 hours after the food) (post-absorptive state). The test done about 2 hours after a good meal is called post-prandial plasma glucose (Latin prandium = meal). For the majority of healthy individuals, normal blood sugar levels are as follows: between 72-99 mg/dl when fasting and up to 140 mg/dl at two hours after a meal. However, test results vary with age.

Failure to maintain blood glucose in the normal range leads to conditions classified as either hyperglycaemic (high glucose concentration) or hypoglycaemic (low glucose concentration).

When plasma glucose level is above the normal range, it is known as hyperglycaemia. Hyperglycaemia is associated with diabetes mellitus and hyperactivity of thyroid, pituitary, and

adrenal glands. The glucose concentration in fasting samples of 150-200 mg/dl is very suggestive of diabetes mellitus, and over 200 mg/dl is almost diagnostic. Diabetes mellitus is a metabolic disease due to absolute or relative insulin deficiency. Diabetes mellitus is characterized by abnormally high concentration of plasma glucose and glucosuria. In spite of this high blood glucose, the entry of glucose into the cell is inefficient. Hence, all cells are starved of glucose.

Type 1: It is due to decreased insulin production. Circulating insulin level is very low. These patients are dependent on insulin injections. Insulin is a peptide, so digestive enzymes in stomach and intestine will break insulin before it gets into our bloodstream. Therefore, in order to avoid digestion, it has to be given by injection.

Type 2: The disease is due to the decreased biological response to insulin, otherwise called insulin resistance. So, there is a relative insulin deficiency. About 60% of patients are obese. Diet and exercise are the first line of management of diabetes. Oral hypoglycaemic agents are used in Type 2 diabetes. They are mainly of two types: sulphonyl urea and biguanides. The combination of glimepiride and metformin is widely prescribed for effective blood glucose control. Glimepiride is a sulfonylurea which works by increasing the amount of insulin released by the pancreas in order to lower the blood glucose. Metformin is a biguanide which works by lowering glucose production in the liver, delaying glucose absorption from intestines and increasing the body's sensitivity to insulin.)

11.6.1.4 Complications of Uncontrolled Diabetes:

- **Vascular Diseases:** Increased risk of stroke and heart disease.
- **Damage of small blood vessels:** complications in eyes (retinopathy and blind-ness) and kidneys (renal Pathy).
- **Neuropathy:** Peripheral neuropathy with paraesthesia, a burning or prickling sensation usually felt in the hands, arms, legs, or feet, is very common. Neuropathy may lead to risk of foot ulcers and gangrene. Hence, care of the feet in diabetic patients is important. Infection and wounds take longer time to heal.

When the blood glucose values are below the normal range, it is called hypoglycaemia. Hypoglycaemia occurs most frequently as a result of overdosage with insulin. Also, starvation and severe exercise may produce hypoglycaemia. Alcohol rapidly affects glucose levels in the blood stream which can be a challenge for those dealing with diabetes mellitus. Alcohol

dehydrogenase, the main enzyme in the catabolic reaction of alcohol, produces NADH which triggers lactic acid formation. This increase in NADH leads to a decrease in glucose level. When the plasma glucose is below 50 mg/dl, it is a very serious condition. Hyperglycaemia is harmful in the long run; while hypoglycaemia even for a short while is dangerous, and may even be fatal.

11.6.2. Blood Urea:

Urea is a white crystalline compound also known as carbamide. Urea is the major end product of protein metabolism. Urea production is used by the body to remove potentially toxic products of nitrogen metabolism.

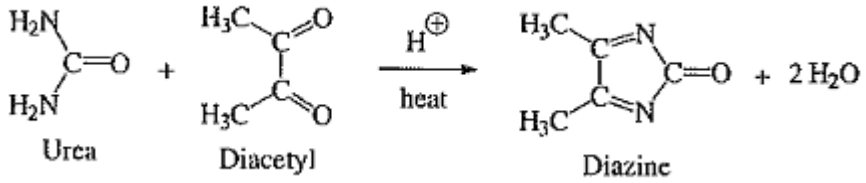
Ammonia is a product of amino acid metabolism - the nitrogen of amino acids is removed as ammonia. It is highly toxic to the body, so it must be removed from body. Ammonia is converted to urea and detoxified. Urea is generated in liver through urea cycle and transported to kidneys for excretion in urine.

$$NH_3 + CO_2 + \text{Aspartate} \rightarrow \text{Urea} + \text{Fumarate}$$

Urea represents about 45-50% of the non-protein nitrogen (NPN) of blood and 80-90% of the total urinary nitrogen excretion. Blood urea estimation is commonly used to assess renal function.

11.6.2.1 Diacetyl monoxime (DAM) method/ Fearon reaction:

This direct method involves the reaction of urea with diacetyl monoxime to give a condensation product. Firstly, diacetyl is released from diacetyl monoxime, and then urea of serum or plasma reacts with diacetyl in hot acidic medium to give a yellow coloured diazine derivative. Diazine is stabilized as pink/red-coloured compound by thiosemicarbazone and ferric ions. The final pink product has absorption maximum at 520 nm in proportion to amount of urea. The major advantage of this direct method is that ammonia does not interfere.



11.6.2.1.2 Reagents used in this method:

1. Acid reagent: Sol. A - Dissolve 0.5 g of ferric chloride (FeCl3) in 2 ml of distilled water. Add 10 ml H3PO4 acid to it. Then mix and make final volume to 25 ml with distilled water. Store at room temperature in a brown bottle.

Sol. B - Prepare 20% aqueous solution of H2SO4. Then mix 0.25 ml of solution A with 500 ml of solution B to prepare acid reagent.

2. Colour reagent:

Sol. A Prepare 2% diacetyl monoxime solution in distilled water.

Sol. B Prepare 0.5% thiosemicarbazone solution in distilled water.

Mix 35 ml of solution A with 35 ml of solution B, and make up to 500 ml with distilled water. Store in a brown bottle at room temperature.

3. Stock urea standard: Prepare 100 mg/dl urea solution in distilled water.

11.6.2.1.3 Calculating Formula:

The urea concentration can be calculated by using the following equation:

Amount of urea in blood = [OD of test x amount of standard (mg) × 100 × dilution factor]/ [OD of standard x volume of sample (ml) × 1000] = x mg/dl

11.6.2.2 Clinical Significance:

The normal range of blood urea is 10-40 mg/dl. The total daily excretion of urea is about 30-40 g. Increased urea production occurs on high protein diets or after gastrointestinal haemorrhage and when there is increased tissue breakdown as observed in starvation, trauma, and inflammation. The capacity of the normal kidney to excrete urea is high, and in the presence of normal renal functions, urea levels rarely rise above normal despite increased production. Azotaemia is a biochemical designation referring to any significant increase of NPN compounds, mainly urea and creatinine, in the plasma. Very high plasma urea concentration accompanied by renal failure is called uraemia. It is evidently fatal if not treated by dialysis or kidney transplantation. Uraemia is associated with several disorders which may be prerenal (diabetic coma), renal (acute glomerulonephritis) and post-renal (enlargement of prostate

gland, tumours are elevated when the glomerular filtration rate is markedly reduced and when the protein intake is higher than 200 g per day. The post renal conditions obstruct the arene outflow through ureters, bladder, or urethra. In diseases such as hydronephrosis, congenital cystic kidneys, renal tuberculosis, hypervitaminosis D increase in urea levels is seen, the extent of which depends on the extent of kidney tissues damaged. Decrease in blood urea level is rare. This usually indicated severe liver disease and is seen, for example, in viral hepatitis with extensive necrosis. Decreased levels are found in liver failure and pregnancy.

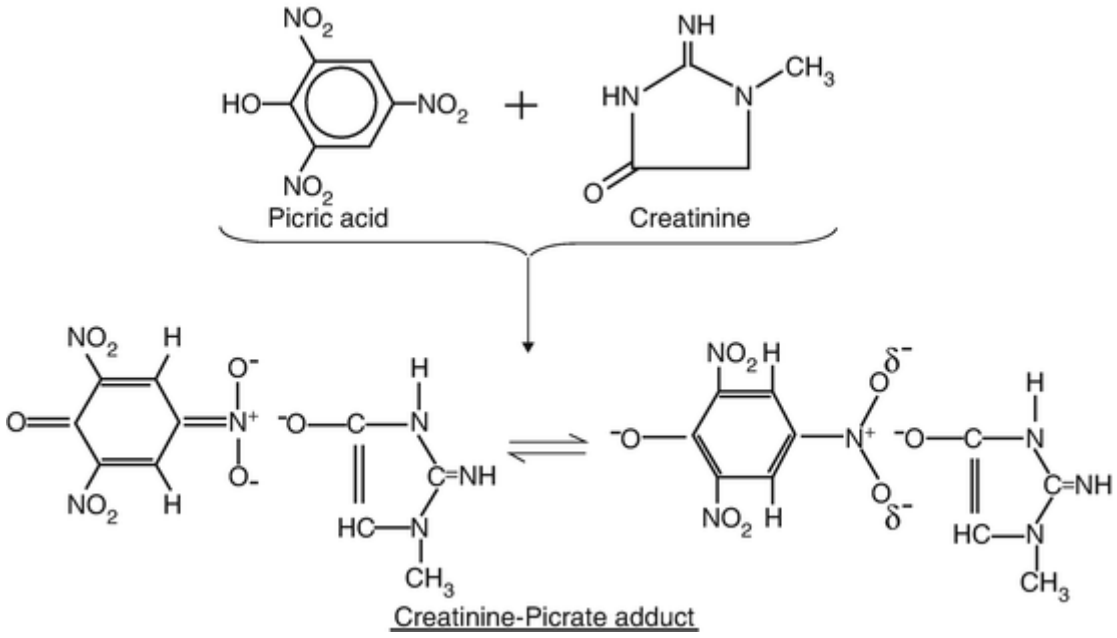
11.6.3 Creatinine:

Creatine is a naturally occurring nitrogenous organic acid that helps to supply energy to muscle cells. Creatine also acts as a buffer. Creatinine is the metabolic waste product resulting from the breakdown of creatine. Chemically creatinine is the anhydride form of creatine. It is formed by spontaneous, non-enzymatic cyclization of creatine and phosphocreatine in skeletal muscle. It is produced at a constant rate depending on muscular mass which is higher in male than female.

Creatinine is eliminated by glomerular filtration through the kidneys and excreted in urine without tubular reabsorption. In renal dysfunction, the ability of the kidneys to filter creatinine is diminished leading to a rise in serum creatinine. Serum creatinine is not influenced by endogenous and exogenous factors, as is the case with urea. Therefore, estimation of serum creatinine is considered to be a more specific and sensitive indicator for the evaluation of kidney function.

11.6.3.1 Jaffe's Alkaline Picrate method:

Creatinine reacts with picric acid in an alkaline solution to form a reddish coloured complex. The reaction is commonly known as the Jaffe reaction and the red coloured product as the Janovski complex, which is basically a 1:1 adduct. The colour produced from the sample is then compared in a colorimeter at wavelength of 530 nm with that produced by a known amount of creatinine under the same condition. Urinary creatinine can also be determined by employing the same principle.



11.6.3.1.2 Reagents:

1. 2/3 N H_2 SO_4

2. **10% sodium tungstate:** Dissolve 10 g sodium tungstate in final volume of 100 ml distilled water.

3. **0.04 M picric acid:** Dissolve 9.3 g pure dried picric acid in water, and make final volume to 1000 ml.

4. 0.75 N NaOH

5. Stock creatinine standard 100 mg/dl: Dissolve 100 mg of pure creatinine in 100 ml of 0.1 N HCL

11.6.3.1.3 Method and Formula:

For serum, add 4 ml of 2/3 N H_2 SO_4 to 0.5 ml of serum, and add 0.5 ml of 10% sodium tungstate solution. Mix well and keep for 10 min. Centrifuge at 3000 rpm for 5 min. Supernatant is used for the estimation of creatinine.

The creatinine concentration can be calculated by using equation:

= [OD of test x amount of standard (µg) × 100] / [OD of standard x volume of sample (ml) × 1000] = x mg/dl

11.6.3.2 Clinical Significance:

Normal value of serum creatinine: For adult male: 0.7-1.4 mg/dl, For adult female: 0.6-1.3 mg/dl. Normal range of creatinine in urine is 90-150 mg/l of urine.

Serum creatinine level more than 1.5 mg/dl indicates impairment of renal function. Serum creatinine level increases in muscular dystrophy and different types of muscular tissue diseases, fever and starvation. There is considerable muscle wasting in diabetes mellitus resulting in high creatinine levels. Creatinine levels are also raised in urinary tract obstruction, due to blockage of creatinine excretion through urine. Decrease in serum creatinine levels is seen in decreased muscle mass with aging and in pregnancy.

11.6.4 Cholesterol:

Cholesterol plays a major role in human heart health. High cholesterol in serum is a leading risk factor for cardiovascular disease such as coronary heart disease and Stroke. Cholesterol can be good or bad. HDL is called "good cholesterol" that is good for the cardiovascular system and LDL is called "bad cholesterol" that is bad for the cardiovascular system. These are the form in which cholesterol travels in the blood. LDLs have little protein and high levels of cholesterol whereas HDL has a lot of protein and very little cholesterol. LDL is the main source of artery clogging plaque. Too much LDL cholesterol in the blood causes atherosclerosis, the disease process that underlies heart attacks, HDL actually works to clear cholesterol from the bloodstream and return it to the liver where it is broken down and passed out of the body.

Excess cholesterol in the bloodstream can form plaque (a thick, hard deposit) in artery walls. The cholesterol or plaque build-up causes arteries to become thicker, harder and less flexible, slowing down and sometimes blocking blood flow to the heart. When blood flow is restricted, angina (chest pain) can result. A heart attack will result when blood flow to the heart is severely impaired and a clot stops blood flow completely. Also, gallstones happen when substances like cholesterol or bilirubin harden in gallbladder, an organ responsible for making bile (a digestive fluid that helps break down fats before they enter intestines).

11.6.4.1 Cholesterol Oxidase-Peroxidase Method:

The determination of total cholesterol level in serum is based on enzymatic assays using the phenol-amino antipyrine fluorometric probes (Trinder and Allain method). In the reaction, the cholesterol esterase hydrolyses cholesterol ester to free cholesterol and fatty acid. The free cholesterol is oxidized to cholest-4-en-3-one and hydrogen per-oxide by cholesterol oxidase.

Peroxidase catalyses the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a red coloured quinonimines derivatives. Colour intensity is directly proportional to the amount of cholesterol having a maximum absorption at a wavelength of 520 nm.

Cholesterol ester + H_2 O + cholesterol esterase = Cholesterol + Fatty acid

Cholesterol + cholesterol oxidase = Cholest-4-en-3-one + H_2O_2

$2H_2O_2$ + 4-Aminoantipyrine + Phenol + peroxidase = Quioneimine dye + $4H_2O$

11.6.4.2 Clinical Significance:

The standard test of cholesterol is done after 9-12 hours fast without food, liquids or pills. Total cholesterol levels less than 200 mg/dl are considered desirable for adults. A reading between 200 and 239 mg/dl is considered borderline high and a reading of 240 mg/dl and above is considered high.

LDL cholesterol levels should be less than 100 mg/dl. Levels of 100 to 129 mg/dl are acceptable for people with no health issues but may be of more concern for those with heart disease or heart disease risk factors. A reading of 130 to 159 mg/dl is borderline high and 160 to 189 mg/dl is high. A reading of 190 mg/dl or higher is considered very high.

HDL levels should be kept higher. A reading of less than 40 mg/dl is considered a major risk factor for heart disease. A reading from 41 mg/dl to 59 mg/dl is considered borderline low. The optimal reading for HDL levels is of 60 mg/dl or higher.

Cholesterol levels are increased in cardiovascular diseases, diabetes and cancers. In pregnancy, the increase in cholesterol levels may reach 20-25% of the normal value. A body mass index (BMI) of 30 or higher puts you at an increased risk of higher cholesterol. The level of cholesterol in blood is related to the development of atherosclerosis. To prevent atherosclerosis, lifestyle changes are required. The primary prevention of coronary heart disease can simply be achieved by following some guidelines such as regular exercise, diets rich in green leafy vegetables, avoiding trans fatty acids (TFA), reduced alcohol consumption, cessation of smoking, maintaining proper weight, control of hypertension, diabetes and dyslipidaemia.

Statin drugs are very effective for lowering LDL cholesterol levels and have few immediate short-term side effects. They are competitive inhibitors of HMG CoA reductase and block the production of cholesterol in the liver itself. These drugs are the first line of treatment for most people with high cholesterol. Examples of statins include: (1) Altocor, (2) Baycol

(cerivastatin), (3) Crestor, (4) Lipitor (atorvastatin), (5) Lescol (Fluvastatin), (6) Mevacor (lovastatin), (7) Pravachol (pravastatin), (8) Zocor (simvastatin).

11.6.5 Bilirubin:

The bile pigment, Bilirubin, is the end-product of heme catabolism. There are two forms of bilirubin: water-soluble (conjugated or direct) and water-insoluble (unconjugated or indirect). Bilirubin has no function in the body and is excreted through bile. Measurement of bilirubin is an important test of excretory function of liver.

The senescent RBCs are broken down liberating the haemoglobin. From haemoglobin, the globin chains are separated and hydrolysed to amino acids as well as the Fe2+ iron is removed and oxidized to Fe3+ to be taken up by transferrin. The porphyrin ring is broken down in liver, spleen and bone marrow to bile pigments. A linear tetrapyrrole, biliverdin is first formed from the porphyrin part of heme in reticuloendothelial cells of liver and is further reduced to bilirubin called unconjugated bilirubin. This unconjugated bilirubin is insoluble in water. The lipophilic bilirubin is therefore transported in plasma bound to albumin. Inside the liver cell, bilirubin is conjugated by the action of glucuronyl transferase to produce bilirubin monoglucuronide and diglucuronide. The bilirubin conjugated with glucuronic acid is water soluble and it exerted through bile. The conjugated bilirubin present in bile passes along the bile ducts into the intestine. Here it is reduced by bacterial action and is also deconjugated, mainly in the colon to a tetrapyrrole, urobilinogen. Urobilinogen is recycled through the body and a part of it is excreted through the urine.

11.6.5.1 Van den Bergh Method:

Bilirubin in serum is coupled with diazotized sulphanilic acid to form a red-purple coloured compound called azobilirubin under acidic conditions (pH = 5). The colour thus produced is measured in a colorimeter at 540 nm and the intensity of the colour is proportional to the bilirubin concentration in serum. The water-soluble bilirubin glucuronide (direct or conjugated bilirubin) reacts easily with reagents such as diazotized sulphanilic acid. But the water insoluble unconjugated bilirubin (indirect bilirubin) requires the presence of 50% alcohol in order to react with the diazotized sulphanilic acid. In this experiment, the direct bilirubin is estimated in the absence of the solubilizing agent and then further bilirubin estimation in the presence of the solubilizing agent will give the total bilirubin level. The indirect or unconjugated bilirubin is then found by difference.

$$NaNO_2 + HCl \rightarrow NaCl + HNO_2$$

11.6.5.1.1 Reagents and Calculation:

1. Diazo reagent:

Sol. A - Dissolve 100 mg sulphanilic acid in 100 ml of 1.5% HCl.

Sol. B 0.5% aqueous solution of sodium nitrite. Diazo reagent is prepared by mixing 10 ml of solution A with 0.3 ml of solution B. Prepare just before use.

2. Diazo Blank: 1.5% v/v aqueous solution of HCl.

3. Bilirubin standard (10 mg %): Dissolve 10 mg of bilirubin powder in 5 ml of 0.1 N NaOH, and add 80 ml of 2% Bovine Serum Albumin (BSA) solution in saline. Then add 5 ml of 0.1 N HCl, and mix and make the volume 100 ml with 2% BSA solution. Store in brown bottle at 4°.

mg of bilirubin in blood/dl = ([OD of test - control) × amount of standard × 100] / [(OD of standard - control) × volume of sample (ml) × 1000] = x mg/dl.

11.6.5.2 Clinical Significance:

Normal serum bilirubin level ranges from 0.2-1 mg/dl. The unconjugated bilirubin is about 0.2-0.5 mg/dl, while conjugated bilirubin is only 0-0.2 mg/dl. Bilirubin analysis is a part of liver function test. High bilirubin indicates Gilbert's syndrome, hepatitis, blockage of bile ducts.

If the bilirubin level in blood exceeds 1 mg/dl, the condition is called hyperbilirubinemia. When the bilirubin level exceeds 2 mg/dl, it diffuses into tissues producing yellowish discoloration of sclera, conjunctiva, skin and mucous membrane resulting in a disease called jaundice. The most common cause for jaundice is viral hepatitis, gall-stones and tumours. Drugs like primaquine, novobiocin, chloramphenicol, and Rogens and pregnanediol may interfere in the conjugation process and may cause jaundice.

Physiological jaundice is an acquired hyperbilirubinemia. It is also called as neo-natal jaundice. In all newborn infants after the 2nd day of life, mild jaundice appears. This transient hyperbilirubinemia is due to an increased rate of destruction of RBCs and also because of the immature hepatic system for the uptake, conjugation and secretion of bilirubin. In such cases, bilirubin does not increase above 5 mg/dl. It disappears by the second week of life.

When blood level is more than 20 mg/dl, the capacity of albumin to bind bilirubin is exceeded. Before the age of 1 year, the blood-brain barrier is not fully matured, and therefore free bilirubin

enters the brain (Kernicterus). It is deposited in brain, leading to mental retardation, fits, toxic encephalitis and spasticity. The binding sites for bilirubin on albumin can be occupied by aspirin, penicillin, etc. Such drugs can, therefore, displace bilirubin from albumin. Hence, care should be taken while administering such drugs to newborn babies to avoid kernicterus.

Barbiturates such as phenobarbital are used in the treatment of neonatal jaundice, as it can induce bilirubin metabolizing enzymes in liver. In some neonates, blood transfusion may be necessary to prevent brain damage. Phototherapy is also used to treat the disease which deals with the exposure of the jaundiced neonates to blue light (440 nm). Phototherapy isomerizes the insoluble bilirubin to more soluble isomers. These can be excreted through urine without conjugation.

11.7 Summary:

The body fluid which is most commonly employed for analytical reasons is blood. Venipuncture, arterial puncture, and skin puncture are the three common methods used to draw blood. To preserve the integrity of the blood sample, the method utilised to collect it is crucial. Most biochemical examinations typically use venous blood. Various collection tubes, known as vacutainers, are sterile glass tubes with coloured rubber stoppers used to collect blood. Most blood collection tubes include substances to keep the sample in the condition needed for the particular test ordered in order to expedite the sample collection and testing procedure. Samples must be preserved in order to be in a condition comparable to that in which they were collected until the time of analysis. When a laboratory test will be conducted within a few hours, it is advised that the samples be refrigerated. This unit explain several methodologies with their clinical significance for estimation of sugar, urea, creatinine, cholesterol and bilirubin in blood for identifying various diseases in human body.

11.8 Model Questions:

1. What do you mean by Type-1 and Type-2 diabetes?

2. Give name of any two anticoagulants used during blood collection.

3. Write the full form of GOD.

4. Give some complications arising due to uncontrolled diabetes.

5. Write down about the clinical significance of Creatinine and Cholesterol.

6. Describe Van den Bergh Method for estimation of blood bilirubin.

7. Write Fearon reaction.

8. What actions are taken for blood preservation?

9. Describe the process of blood collection from human body.

10. Why there is a need for blood preservation?

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Unit 12 Urine Analysis

Index

12.1 Objectives:

After Completion of this unit one can understand

1. How to collect and preserve urine.

2. All about normal and abnormal constituent of urine.

3. Estimation procedure for glucose, ketone bodies and protein in urine.

12. 2 Introduction:

Urine is a liquid waste by-product of the body that the kidneys have secreted in a process called urination and excreted via the urethra. The urinary system is the principal system responsible for water and electrolyte balance and excretion of toxic nitrogenous compounds. Complete urine analysis is important to assess the kidney function. After blood, urine is the most commonly used specimen for diagnostic testing, monitoring of disease status and detection of drugs. Urine is the excretory product of the body produced by the process of filtration, reabsorption and tubular secretion. Urine can be collected and examined easily and presence of certain substances in the urine may indicate the metabolic state of the body.

12.3 Collection and Preservation of Urine Samples:

12.3.1 Urine Collection:

Urine sample is collected in clean vials. Urine specimens may be collected in a variety of ways according to the type of specimen required, the collection site and patient type. The randomly collected sample is usually taken for routine clinical ex-amination, but it is not the preferred choice because of the potential for dilution of the specimen when collection occurs soon after the patient has consumed fluids. First

Morning Specimen is the specimen of choice for urinalysis and microscopic analysis, since the urine is generally more concentrated. Midstream Clean Catch Specimens are strongly recommended for microbiological culture and antibiotic susceptibility testing because of the reduced incidence of cellular and microbial contamination. Timed Collection Specimens may be required for quantitative measurement of certain analytes, including those subjects to diurnal variation. Analytes commonly tested using timed collection include creatinine, urea, potassium, sodium, uric acid, cortisol, calcium, citrate, amino acids, catecholamines, metanephrines, vanillylmandelic acid (VMA), 5-hydroxyindoleacetic acid, protein, oxalate, copper, 17-ketosteroids and 17-hydroxys-teroids. Catheter Collection is an assisted procedure where a foley catheter is inserted into the bladder through the urethra to collect the urine specimen. Specimens may be collected directly from a foley into an evacuated tube or transferred from a syringe into a tube or cup.

Urine collection container cups are available in a variety of shapes and sizes with lids that are either 'snap-on' or 'screw-on'. Leakage is a common problem with low quality products. To protect healthcare workers from exposure to the specimen and protect the specimen from

exposure to contaminants, leak-proof cups should be utilized. Some urine specimen containers have closures with special access ports that allow closed-system transfer of urine directly from the collection device to the tube. Sterile containers are recommended for culture and sensitivity test of urine.

12.3.2 Urine Preservation:

The routine and microscopic examinations of fresh or preserved urine samples are helpful in the diagnosis of several pathological conditions. The specimens that are unpreserved for more than 2 hours or refrigerated for a long time may not be suit-able for chemical urinalysis and conventional (culture based) microbiological testing due to potential bacterial overgrowth and invalidation of bacterial colony counts or errors in chemical urinalysis. A variety of urine preservatives is available that allow urine to be maintained at room temperature while still providing urinalysis test results comparable to those achieved with fresh specimens or those stored under refrigerated conditions. Commonly used preservatives for chemical urinalysis specimens include chlorhexidine, ethyl paraben, thymol and sodium propionate, toluene, formalin. Usually, acidification is done by adding 6(N) HCI, 50% acetic acid or boric acid. Sodium bicarbonate is used to preserve porphyrin and urobilinogen. Light sensitive compounds should be protected by collecting in amber coloured bottle or by covering with aluminium foil or dark coloured paper. Preservation times are typically within the range of 24 to 72 hours. Claims for the duration of stability for specific analytes should be obtained from the manufacture.

Today urinalysis continues to be a powerful tool in obtaining crucial information for diagnostic purposes in medicine. Physical examination of urine includes description of colour, odour, clarity, volume, pH and specific gravity. Chemical examination of urine includes the identification of protein, blood cells, glucose, bilirubin, urobilinogen, ketone bodies, nitrites and leukocyte esterase. Finally, microscopic examination entails the detection of crystals, cells, casts and microorganisms.

12.3.3 Employed Preservatives:

Refrigeration: at 4-6 degree C for 8 hours.

Toluene: 1 ml per 50 ml of urine. It acts by forming a surface layer and it preserves the chemical constituents of urine. It is antimicrobial and good for ketone bodies estimation.

Formalin: 6-8 drops of 40% formalin per 100 ml of urine. It preserves RBCs and pus cells. It is best for microscopic examination (preserve cast and cellular elements). Disadvantage is that it gives false positive test for sugars.

Thymol: 1% of thymol is used. Disadvantage is that it gives false positive test for protein.

Acids: hydrochloric acid, boric acid and sulphuric acid.

12.4 Physical Examination of Urine:

(i) Colour and Odor: The colour of normal urine is pale yellow or amber due to presence of urochrome or urobilin. Slight change in colour occurs in fever, dehydration, jaundice, or vitamin B-complex therapy which adds riboflavin (deep yellow colour). Red to brown colour is observed in haematuria, haemoglobinuria, myoglobinuria and porphyria. Urine turns brown to black in alkaptonuria and melanoma. Normally odour of urine is faintly aromatic. On decomposition, a very unpleasant ammoniacal odour evolves. Food beverages and drugs may impart a specific odour to urine.

(ii) Appearance: Normally freshly voided urine is clear and transparent. But it may become turbid if exposed for a long time due to the bacterial action on urea present in urine to convert it into ammonium carbonate. Phosphate excretion in alka-line urine also makes urine turbid. The presence of white cells, red cells, or epithelial cells makes urine cloudy. Fat globules give urine milky appearance.

(iii) Specific Gravity: Specific Gravity is the relative mass density in urine. It is basically a comparison of density of urine against the density of distilled water at a particular temperature. Specific gravity of urine is a measure of concentrating ability of kidneys and is determined to get information about its tubular function. Specific gravity of normal urine is between 1.002 and 1.026. Specific gravity of urine is measured by urinometer. Solute and temperature affect the specific gravity. Specific gravity increases as solute concentration increases and decreases when temperature increases. It depends upon state of hydration, diet, fluid intake, drugs etc. The specific gravity will be decreased in excessive water intake, in chronic nephritis and in diabetes insipidus. It is increased in diabetes mellitus, in nephrosis and in excessive perspiration.

(iv) Volume: Normal healthy individual excretes about 800-2000 ml of urine/day. Daily urinary excretion depends upon intake of fluid volume, loss of fluid, solute load, climatic

condition, fever, or intake of drugs. Polyuria is defined as the frequent passage of large volumes of urine more than 3 litres a day. Polyuria is usually the result of drinking excessive amounts of fluids (polydipsia), particularly water and fluids that contain caffeine or alcohol. It is also one of the major signs of diabetes mellitus and also indicates kidney disease and hypercalcemia. When the kidneys filter blood to make urine, they reabsorb all of the sugar, returning it to the bloodstream. In diabetes, the level of sugar in the blood is abnormally high. Not all of the sugar can be reabsorbed and some of this excess glucose from the blood ends up in the urine where it draws more water. This results in unusually large volumes of urine. In oliguria, urine output is less than 500 ml/day. Oliguria may be due to fewer intakes of water or due to dehydration or may indicate early renal dysfunction symptoms. In anuria, a complete cessation of urine output (<100 ml/day) is observed. Anuria should be taken care of immediately; otherwise, complete renal failure may take place. Dysuria - Painful or uncomfortable urination, often from urinary tract infections.

(v) pH: Normal range of urinary pH is 4.5-7.5. Normally freshly voided urine is slightly acidic. On standing urine becomes alkaline due to loss of carbon dioxide and production of ammonia from urea. Various factors like heavy meals, heavy exercise, metabolic acidosis, or chronic respiratory acidosis influence urine pH greatly. The causes of the accumulation of acids in urine are dehydration, diabetes mellitus, ketoacidosis, starvation, chronic renal failure, UTI. Calcium oxalate crystals are usually found in acidic urine. On the other hand, urine becomes alkaline in diseased conditions such as severe vomiting, diarrhoea, hyper ventilation and prolonged use of diuretic drugs.

12.5 Normal Chemical Composition of Urine:

12.5.1 Amount of normal constituents:

Urine is an aqueous solution of greater than 95% water, with various organic and inorganic constituents. The normal inorganic constituents are sodium, potassium, calcium, chloride, phosphate, sulphate, bicarbonate etc. The non-protein nitrogen compounds (NPN) are urea (45%); free amino acids (16%), creatine (2.4%), creatinine (1.7%), ammonium nitrogen (1%), uric acid (2.1%). The concentrations of some major constituents are given below:

Urea 9.3 g/L

Chloride 1.87 g/L

Sodium 1.17 g/L

Potassium 0.750 g/L

Creatinine 0.670 g/L

12.5.2 Tests for normal constituents of urine:

Chloride: Addition of few drops of 3% silver nitrate solution to about 3 ml of urine sample followed by 3-5 drops of conc. HNO3 results in the formation of white precipitate of silver chloride.

Phosphates: To 3 ml of urine, add 1 ml of conc. HNO3 and 5 ml of ammonium molybdate and warm gently. A yellow precipitate of phosphomolybdic acid indicates the presence of phosphate.

Sulphates: To 3 ml of urine, add 3-5 drops of 2% barium chloride (BaCl2) solution followed by 5 drops of conc. HCl. A white precipitate of barium sulphate indicates inorganic sulphate.

Ammonia: Boil urine with equal volume of 10% NaOH. Smell of ammonia Tue gas indicates the presence of ammonium ion in the urine sample.

Calcium: Add few drops of glacial acetic acid to about 5 ml of urine followed by addition of 1 ml of 4% ammonium oxalate solution. Calcium is slowly precipitated as oxalate as enveloped shaped crystals that can be observed under microscope.

Urea: To about 3 ml of urine, add two drops of freshly prepared alkaline sodium hypobromite solution. A marked effervescence due to evolution of nitrogen indicates urea.

Creatinine: Detected by Jaffe's Picric Acid Test (discussed in Blood analysis chapter).

Uric Acid: To about 3 ml urine sample saturated with Na_2CO_3, add 2 drops of Folin's phosphotungstic acid reagent. Uric acid reduces phosphotungstatein alkaline solution and forms tungsten blue.

12.6 Abnormal Constituents of Urine:

Pathological constituents are the tissue components and metabolites that normally do not appear in urine) Examples are albumin, sugar, ketone bodies, blood, bile pigments and bile salts and indican. There are several conditions that can cause abnormal components to be excreted in urine or present as abnormal characteristics of urine. They are mostly referred to by the suffix ‘uria’. Some of the more common types of abnormal components urine include:

Protein: Normally, there will not be detectable quantities of albumin in the urine. When urine protein is elevated, the condition is called proteinuria. Albumin is smaller than most other proteins and is typically the first protein that is seen in the urine when kidney dysfunction begins to develop. Physiological conditions that can produce proteinuria include exercise, convulsions, emotional stress and excessive protein diet. Pathological conditions can be classified as pre-renal, renal and post-renal such as kidney diseases, urinary tract infection and tubular diseases.

Blood in urine: Presence of red blood cells in urine is known as Haematuria. It is a condition which occurs in acute glomeruli nephritis, stone in kidney and ureter, renal tuberculosis and carcinoma of kidney. Free haemoglobin in urine is a marker of haemolysis of erythrocytes; this condition is called haemoglobinuria and occurs in malaria, typhoid, haemolytic jaundice, yellow fever, intravascular haemolysis.

Glucose: Excretion of reducing sugars in urine is known as glycosuria. If the level of glucose in blood is above 160-180 mg/dl, glucose is excreted in urine (glucosuria). This value (160-180 mg/dl) is referred to as "renal threshold" for glucose. Glucosuria may be either due to an excessively high glucose concentration in the blood or a reduction in the renal threshold. Diabetes mellitus, renal failure, hyperthyroidism, hypoadrenalism, peptic ulcer, excessive carbohydrate diet are some common causes of this.

Bilirubin and bile salts: Bilirubin is a yellowish pigment found in bile; a fluid produced by the liver. Bilirubin is found in urine in case of jaundice. Bile salts may be excreted in urine without bile pigment in certain stages in liver disease such as hepatitis.

Ketone bodies: When glucose is not available to the body's cells or body can-not use glucose as an energy source, body starts using fat to meet the energy requirements. During starvation and diabetes mellitus, Acetyl-CoA, produced by oxidation of fatty acids in liver, is converted to acetoacetate (AcAc) and 3-8-hydroxybutyrate (3HB). These compounds together with acetone are referred to as ketone bodies.

The ketogenesis process occurs in the liver particularly during periods of low food intake, carbohydrate-restrictive diets, starvation, or in untreated type 1 diabetes mellitus. The hormone glucagon stimulates ketogenesis whereas insulin inhibits. When the rate of synthesis of ketone bodies exceeds the ability of extrahepatic tissues to utilize them, there will be accumulation of ketone bodies in blood, a condition named ketonemia. This leads ketonuria, a medical condition in which ketone bodies are present in the urine. High ketone levels may indicate diabetic

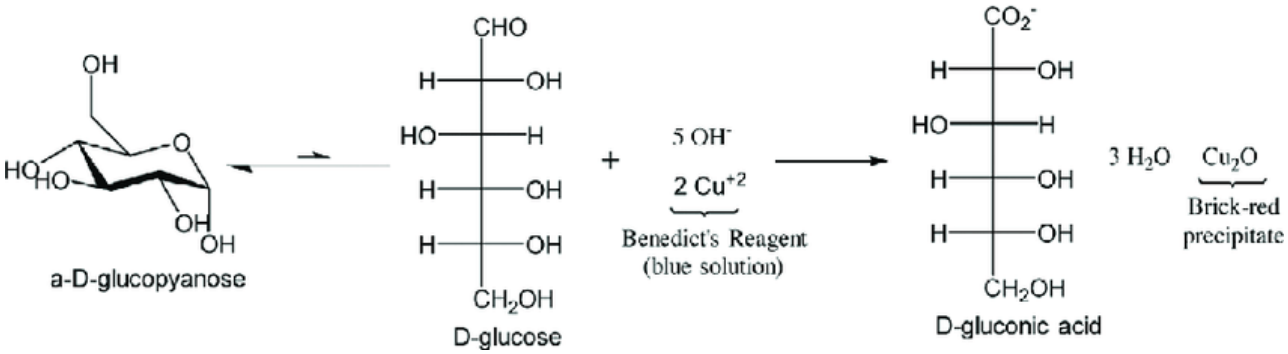
ketoacidosis (DKA), a complication with type 1 diabetes that can lead to a coma or even death. Levels of

12.7 Tests to Estimate Glucose in Urine:

12.7.1 Basic Chemistry:

The reducing property of sugar is based on the presence of free carbonyl carbon of aldehyde or ketone group in them. Most of monosaccharides and disaccharides are reducing sugars, while sucrose is non-reducing sugar. Reducing sugars are capable of reducing Cu2+ (cupric ions) to Cut (cuprous ions) in alkaline medium which produces red precipitate of cuprous oxide or yellow precipitate of cuprous hydroxide. Generally, the following two tests are used to test the presence of sugar in urine sample viz. Benedict's Test and Fehling's Test.

In Benedict's test, Benedict's solution is used as the reagent. Benedict's reagent is a combination of sodium carbonate, sodium citrate and copper (II) sulphate pentahydrate ($CuSO_4.5H2O$). Sodium citrate prevents the spontaneous reduction of $CuSO_4$ while Na_2CO_3 is used to provide alkaline medium. In Fehling's test, Fehling's solution-A and Fehling's solution-B are used as the reagents. Fehling's solution-A is an aqueous solution of copper (II) sulphate, having blue colour, while Fehling's solution-B is clear colourless aqueous solution of sodium potassium tartrate. The free aldehyde or keto group of the reducing carbohydrates reduces cupric ions to cuprous ion with the resultant formation of yellow or red precipitates of cuprous oxide.



12.7.2 Employed Qualitative Reagent:

Solution A: Dissolve 17.3 g of sodium citrate and 10 g of Na2CO3 in about 60 ml of distilled water with the aid of heat.

Solution B: Dissolve 1.73 g of CuSO4 in 10 ml of distilled water.

Solution B is added to solution A gently with constant mixing and final volume is made up to 100 ml with distilled water.

Depending upon the concentration of sugar, green, yellow and brick red precipi-tates of cuprous oxide are formed. Table 2 shows the colour sequence depending upon the concentration of glucose level.

Colour of the Precipitate	Percentage of sugar Present
Blue	Sugar absents
Green	0.5 to 1 %
Yellow	1 to 2 %
Brick Red	2 % or more.

Generally, glucose (sugar) is absent in normal urine. But when the glucose level in blood exceeds the renal threshold of glucose (160 - 180 mg/dl), glucose starts to appear in urine. The presence of glucose in urine is called glucosuria and is usually an indication of diabetes mellitus.

Benedict's test is not recommended or used for diagnosis of glucosuria. This is due to the possibility of a reaction in which the presence of other reducing substances such as lactose, galactose, fructose, xylose etc. creates a false positive. Specific tests are performed to rule out the possibility of presence of these sugars. Normal constituents such as uric acid, creatinine and ascorbic acid (vitamin C) in higher amount as well as thymol, formaldehyde, chloroform also give false-positive result.

12.7.3 Reagent strip method:

These strips are impregnated with glucose oxidase (GOD), peroxidase (POD) and chromogen. In this reaction, glucose is oxidized to gluconic acid and H_2O_2 by the enzyme GOD. H2O2 formed is split into water and nascent oxygen by POD enzyme. This nascent oxygen reacts with the chromogen to form a coloured product. This method, specific for glucose, is more sensitive than benedict’s test. False positive is seen in presence of oxidizing agent like hypochlorite (bleach). False negative is obtained for large amount of ketone, salicylates, severe E. coli infection.

$$GOD + Glucose \rightarrow Gluconic\ acid + H_2O_2$$

$$H_2O_2 + POD = H_2O + [O]$$

$$[O] + o\text{-}toluidine = coloured\ complex$$

12.8 Tests to Estimate Proteins in Urine:

The normal protein excretion in urine is about 30-150 mg/day. The proteins in urine are normally derived from plasma filtrate and the lower lining of urinary tract due to tissue damage. The serum globulins, albumin and proteins secreted by the nephron forms normal urinary proteins. About one third of urinary protein is albumin and remaining includes many small globulins. Proteinuria is an important indicator of kidney disease and the risk of disease progression.

(a) Dipstick test: Dipsticks are plastic strips impregnated with bromophenol blue buffered to pH 3.0 with citrate. The test strips remain yellow in the absence of protein but changes to blue through various intermediate shades of green in presence of increasing protein concentration This is due to divalent anionic form of indicator dye combining with proteins causing further dissociation of yellow monovalent anion into blue divalent anion. The blue-green colour produced is directly proportional to the concentration of proteins in the specimen. The dipstick tests are more sensitive to albumin while, globulins and glycoproteins are less readily detected. Dipstick is useful if urinary proteins are >300-500 mg/day or albumin >10-20 mg/dl.

(b) Heat coagulation test: This is a test for the detection of albumin and/or globulins in urine. When proteins are heated at pH equal to isoelectric point (pl), they get denatured and coagulated. Make the urine slightly acidic with 33% (v/v) aqueous solution of acetic acid and heat the upper (1/3rd) portion until it boils. Appearance of faint turbidity to heavy precipitate indicates positive test. Phosphates also give precipitates, so to rule out false positive result, acetic acid is added. Phosphate get dissolved leaving any coagulated protein still visible.

This is semiquantitative test, so it can be used to measure concentration of proteins to some extent. No turbidity indicates negative result; only turbidity indicates < 30 mg/dl; turbidity with precipitates indicates 100 mg/dl, while turbidity with coagulation indicates proteins >300 mg/dl. If heavy coagulate appears, that indicates >2 g/dl proteins in urine sample.

(c) Sulphosalicylic acid test: Sulphosalicylic acid is an anionic protein precipitant; it reacts with protein cations and causes precipitation. Addition of 5-10 drops of 20% Sulphosalicylic acid to 1 ml of the urine sample causes formation of turbidity or white precipitate if protein is

present. This is a very sensitive test. Uric acid may give false positive test. To rule out this possibility, the sample is heated; if it becomes clear, proteins are absent and turbidity is due to uric acid.

(d) Nitric acid ring test (Heller's test): Layer the urine carefully over few ml of conc. HNO3 in a test tube so as to get a sharp line of demarcation. Proteins give a white colour ring at the junction of fluids. However, urea, uric acid and iodinated organic compounds used for X-rays of the urinary tract can give false positive test.

12.9 Tests to Estimate Ketone Bodies in Urine:

The normal concentration of ketone bodies in blood is about 1-3 mg/dl and their excretion in urine is negligible, i.e. undetectable by routine urine tests. Excretion of ketone bodies in urine is called ketonuria. In untreated diabetes patient and starving patient, the serum ketone body levels may reach 90 mg or above per 100 ml of serum and urinary excretion may reach 5000 mg/24 h urine. In type I diabetes, low insulin levels impair carbohydrate metabolism that leads to accumulation of acetyl CoA and its conversion to ketone bodies. During starvation also increased lipolysis causes overproduction of acetyl CoA which is diverted for overproduction of ketone bodies. Both these conditions result in very high levels of ketone bodies, lowering the blood pH and kidneys excrete very acidic urine.

(a) Rothera's test: Alkaline nitroprusside reacts with keto-group of acetone and acetoacetic acid to form a purple-coloured complex.

Rothera's reagent: Take ammonium sulfate and sodium nitroprusside in 100: 1 ratio. Grind well to mix powder of salts (dry mixture). Saturate 5 ml of urine with solid ammonium sulfate. Add 3 drops of freshly pre-pared sodium nitroprusside followed by 2 ml of liquor ammonia along the sides of the test tube. Development of a purple ring at the junction of the two layers within 30-60 s indicates the presence of acetone or acetoacetic acid in urine. It is not responded by beta hydroxyl butyrate. Strip tests based on the same principle are also available. These strips impregnated with sodium nitroprusside and glycine, react with acetone and acetoacetate to give lavender or purple colour.

(b) Gerhardt’s test for acetoacetic acid: To 5 ml of urine, add dilute ferric chloride solution drop by drop, till a maximum precipitate of ferric phosphate is obtained. This is to eliminate the phosphates which may obscure the colour in the test. Then filter. To the filtrate add excess

ferric chloride. A red colour indicates the presence of acetoacetic acid. This is not a sensitive test. Salicylates will give a false positive test.

(c) Detection of β-Hydroxybutyrate: β-hydroxybutyrate forms acetoacetate on oxidation with H_2O_2 which can be detected with Rothera's test. Add few drops of acetic acid in about 10 ml of urine diluted 1:1 with distilled water. Boil for few minutes to remove acetone and acetoacetic acid. Add about 1 ml of H_2O_2, warm gently and apply Rothera's test. If Rothera's test gives positive result after oxidation with H2O2, then □-hydroxybutyrate is present in urine.

12.10 Summary:

Urine is a bodily waste product that is expelled through the urethra after being secreted by the kidneys during a procedure known as urination. The primary system in charge of maintaining electrolyte and water balance as well as eliminating harmful nitrogenous substances is the urinary system. To evaluate kidney function, a thorough urine analysis is necessary. For urinalysis and microscopic examination, the morning samples is preferred since the urine is often more concentrated. Due to the lower risk of microbial and cellular contamination, midstream clean catch specimens are highly advised for microbiological culture and antibiotic susceptibility testing. There are several urine preservatives that enable urine to be kept at room temperature while still providing urinalysis test results comparable to those achieved with fresh specimens or those stored under refrigerated conditions as mentioned in this unit. This unit explains both normal and abnormal composition of urine. Side by side, this unit also provide reagent, methodology for the estimation of sugar, protein, ketone bodies in urine to detect any abnormalities present within human body.

12.11 Model Questions:

1. Discuss on collection method of urine.

2. Name some preservatives used to preserve urine.

3. What is the pH of urine?

4. write the name of some major constituents of urine with their amount.

5. How chlorine and phosphates are detected in urine?

6. What is heller’s test?

7. What is Rothera reagent?

8. Discuss Dipstick test for estimation of protein in urine.

9. Write the chemistry behind the estimation of glucose in urine.

10. Explain on any two abnormal constituents of urine.

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