National University - Sudan

Faculty of Medicine and Surgery

Department of Biochemistry and Molecular Biology

BIOCHEMSTRY PRACTICAL MANNUAL

General Work Procedure

- Know emergency procedures.
- Never work in the laboratory without the supervision of aII instructor.
- Always perform the experiments or work precisely as directed by the instructor.
- Immediately report any spills, accidents, or injuries to the instructor.
- Never leave experiments while in progress.
- Be careful when handling hot glassware and apparatus in the laboratory
- Hot glassware looks just like cold glassware.
- Never point the open end of a test tube containing a substance at yourself or others.
- Never fill a pipette using mouth suction.
- Always use a pipetting device.
- Make sure no flammable solvents are in the surrounding area when lighting a flame.
- Do not leave lit Bunsen burners unattended.
- Turn off all heating apparatus, gas valves, and water faucets when not in use.
- Do not remove any equipment or chemicals from the laboratory.
- Coats, bags, and other personal items must be stored in designated areas, not on the bench tops or in the aisle ways.
- Notify your instructor of any sensitivities that you may have to particular chemicals if known.
- Keep the floor clear of all objects (e.g., ice, small objects, and spilled liquids).

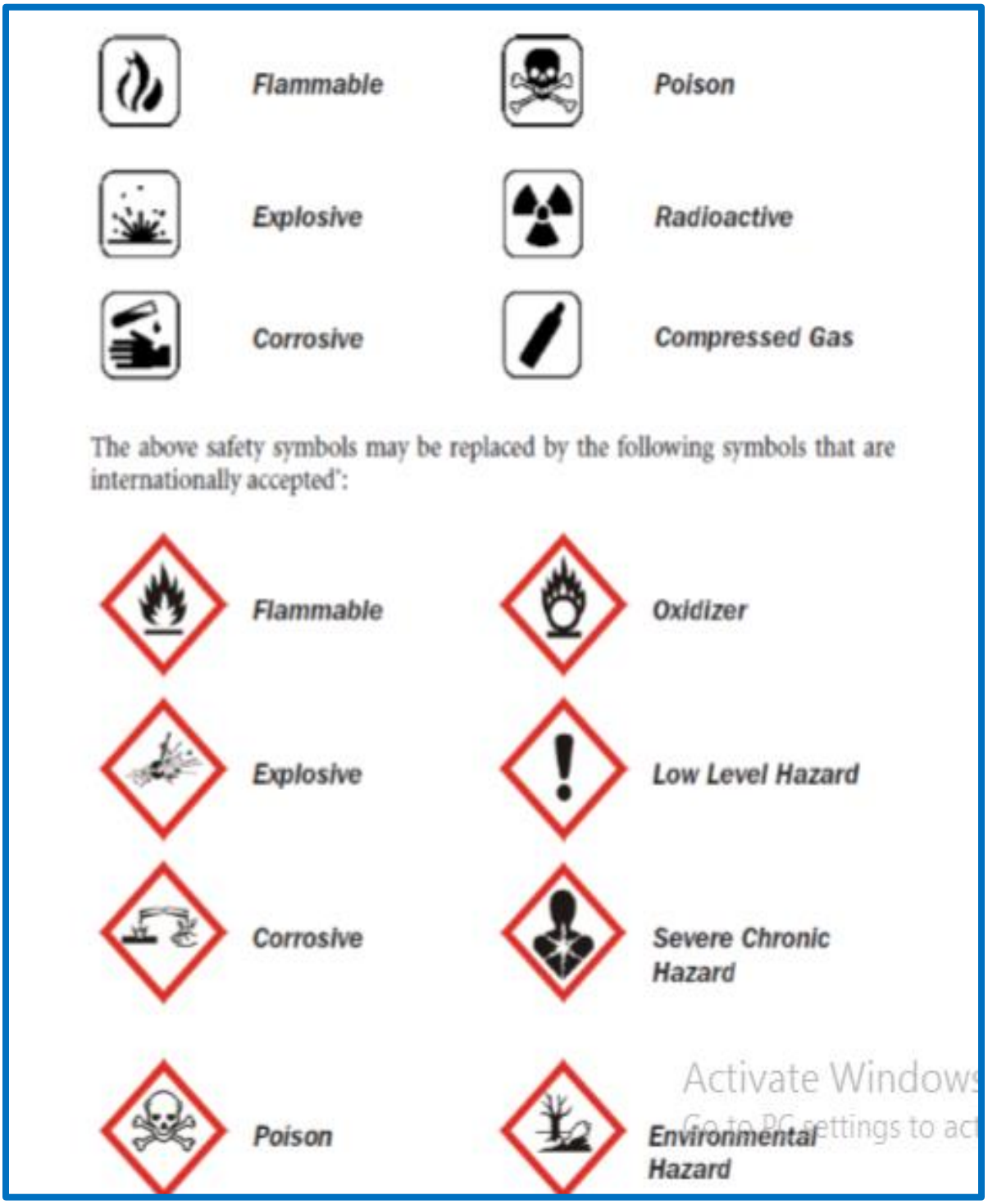
Emergency Procedure

- Know the location of all the exits in the laboratory and building.
- Know how to operate the following:
  - Fire extinguishers
  - Alarm systems with pull stations
  - Fire blankets
  - Eye washes
  - First-aid kits

Chemical Handling:

- Check the label to verify it is the correct substance before using it
- Wear appropriate chemical resistant gloves before handling chemicals.
- Gloves are not universally protective against all chemicals.
- If you transfer chemicals from their original containers, label chemical containers as to the contents, concentration, hazard, date, and your initials.
- Always use a spatula or scoopula to remove a solid reagent from a container.
- Do not directly touch any chemical with your hands.
- Never use a metal spatula when working with peroxides. Metals will decompose explosively with peroxides.
- Hold containers away from the body when transferring a chemical or solution from one container to another.
- Use a hot water bath to heat flammable liquids. Never heat directly with a flame.
- Add concentrated acid to water slowly. Never add water to a concentrated acid.
- Weigh out or remove only the amount of chemical you will need. Do not return the excess to its original container, but properly dispose of it in the appropriate waste container.
- Never touch, taste, or smell any reagents.
- Never place the container directly under your nose and inhale the vapors.
- Never mix or use chemicals not called for in the laboratory exercise.
- Clean up all spills properly and promptly as instructed by the instructor.
- Dispose of chemicals as instructed by the instructor.
- When transporting chemicals (especially 250 mL or more), place the immediate container in a secondary container or bucket (rubber, metal or plastic) designed to be carried and large enough to hold the entire contents of the chemical.
- Never handle bottles that are wet or too heavy for you.
- Use equipment (glassware, Bunsen burner, etc.) in the correct way, as indicated by the instructor.

Appendix A: Common Safety Symbols



Lab Reports:

Lab reports should be filled using the following format or style:

Lab Report Form

Course name ----------

Course number-Lab section

The title of the experiment ----------

Date ----------

Student name ----------

Instructor name ----------

Group no. ----------

All these should be written in the first page.

TILTE: ---------- MARK----------

INTRODUCTION:----------

MATERIALS:----------

METHOD:----------

OBSERVATION AND INTERPRETATION:----------

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Section One

Prepared by:

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BIOCHEMISTRY PRACTICAL

Introduction

Section I:

Biochemistry Practical:

- **Study of biomolecules and their chemical and physical properties.**
- **General and special tests for detection of biomolecules.**
- **Biochemical techniques.**
- **Kinetics of enzyme action and factors that affect the catalytic of the enzyme.**

Practical No. 1

Water and pH

INTRODUCTION:

Water (H_2O) is the medium of biological systems, one must consider the role of this molecule in the dissociation of ions from biological molecules. Water is essentially a neutral molecule but it can be ionized to a small degree. This can be described by a simple equilibrium equation:

$$\mathbf{H_20 \longleftrightarrow H^+ + OH^-} \qquad \textbf{(Eqn. 1)}$$

This equilibrium can be calculated as for any reaction:

$$\mathbf{K_{eq} = [H^+]\,[OH]/\,[H_20]} \qquad \textbf{(Eqn.2)}$$

Since the concentration of H_2O is very high (55.5M) relative to that of the $[H^+]$ and OH, consideration of it is generally removed from the equation by multiplying both sides by 55.5 yielding a new term, $\mathbf{K_w}$:

$$\mathbf{K_w = [H^+]\,(OH)} \qquad \textbf{(Eqn. 3)}$$

This term is referred to as the ion product. In pure water, to which no acids or bases have been added:

$$\mathbf{K_w = 1 \times 10^{-14} M^2} \qquad \textbf{(Eqn. 4)}$$

As $\mathbf{K_w}$ is constant, if one considers the case of pure water to which no acids or bases have been added:

$$\mathbf{[H^+] = [OH) = 1 \times 10^{-7}\ M^2} \qquad \textbf{(Eqn. 5)}$$

This term can be reduced to reflect the hydrogen ion concentration of any solution. This is termed the pH, where:

$$\mathbf{pH = -\log\,(H^+)} \qquad \textbf{(Egn. 6)}$$

<u>pK_a</u>

Acids and bases can be classified as proton donors and proton acceptors, respectively. This means that the conjugate base of a given acid. In biologically relevant compounds various weak acids and bases acid will carry a net charge that is more negative than the corresponding are encountered, e.g. the acidic and basic amino acids, nucleotides, phospholipids etc.

Weak acids and bases in solution do not fully dissociate and, therefore, there is equilibrium between the acid and its conjugate base. This equilibrium can be calculated and is termed the equilibrium constant = K_a. This is also sometimes referred to as the dissociation constant as it pertains to the dissociation of protons from acids and bases.

In the reaction of a weak acid:

$$\mathbf{HA \longleftrightarrow A^-H} \qquad \textbf{(Eqn.7)}$$

The equilibrium constant can be calculated from the following equation:

$$\mathbf{K_a = [H^+]\,[A]/\,[HA]} \qquad \textbf{(Eqn. 8)}$$

As in the case of the ion product:

$$\mathbf{PK_a = -logK_a} \qquad \textbf{(Eqn. 9)}$$

Therefore, in obtaining the -log of both sides of the equation describing the dissociation of a weak acid we arrive at the following equation:

$$\mathbf{-logK_a = -log\,[H^+]\,[A]/\,[HA]} \qquad \textbf{(Eqn. 10)}$$

Since as indicated above-logK, = pK, and taking into account the laws of logarithms:

$$\mathbf{PK_a = -log\,[H^+] - log\,[A]/\,[HA]} \qquad \textbf{(Eqn. 11)}$$

$$\mathbf{pK_a = pH - log[A]/[HA]} \qquad \textbf{(Eqn. 12)}$$

From this equation it can be seen that the smaller the pK_a value the stronger is the acid. This is due to the fact that the stronger an acid the more readily it will give up H^+ and, therefore, the value of (HA) in the above equation will be relatively small.

The Henderson-Hasselbalch Equation:

By rearranging the above equation, we arrive at the Henderson- Hasselbalch equation:

$$\mathbf{pH = pK_a + log\,[A]/[HA]} \qquad \textbf{(Egn. 13)}$$

It should be obvious now that the pH of a solution of any acid (for which the equilibrium constant is known, and there are numerous tables with this information) can be calculated knowing the concentration of the acid, HA, and its conjugate base (A).

At the point of the dissociation where the concentration of the conjugate base (A) = to that of the acid (HA):

$$\mathbf{pH = pK_a + log\,[1]} \qquad \textbf{(Egn. 14)}$$

The log of 1= 0. Thus, at the mid-point of a titration of a weak acid:

$$\mathbf{pK, = pH} \qquad \textbf{(Eqn. 15)}$$

In other words, the term pK_a is that pH at which an equivalent distribution of acid and conjugate base (or base and conjugate acid) exists in solution.

Buffering

It should be noted that around the pK_a, the pH of a solution does not change appreciably even when large amounts of acid or base are added. This phenomenon is known as buffering. In most biochemical studies it is important to perform experiments that will consume H^+ or OH^- equivalents, in a solution of a buffering agent that has a pK_a near the pH optimum for the experiment.

Indicators

Principle these are usually acids of weak strength whose molecules in solution are of different colour than their anion. The colour of indicator solutions depends on the degree of dissociation of the indicator, and on the pH of the solution. Supposing the weak acid indicator is H indicator, it would dissociate.

H^+ Indicator	**=**	**H^+**	**+**	**Indicator**
Colour X		**Hydrogen ion**		**Colour Y**

All acids contain hydrogen ions, so addition of an acid would make the reaction shift from right to left (change of the colour from Y to X) and the addition of alkali (alkali's contain hydroxyl ions) would lead to the production of water (H_20----$H^+ + OH^-$) as the OH radicals will associate with and remove H ions causing a shift to the right of the reaction (change of colour from X to Y). Indicators while some indicators change colour at a precise pH.

Indicators	pK	pH	Colour change
Thymol blue	1.65	2.1-2.8	Red-yellow
Bromophenol blue	3.98	2.8-4.6	Yellow-blue
Methyl red	5.10	4.2-6.3	Red-yellow
Bromocresol purple	6.30	5.2-6.8	Yellow-purple
Phenol red	6.30	6.8-8.4	Yellow-red
Phenolphthalein	9.70	6.8-8.4	Colourless-red

Experiment 1

Objective: To show that H^+ ion concentration depends on the degree of ionization as well as on the concentration of substances which yield H^+ ions:

You are provided with 0.01 N HCL from this solution prepares three (3) serial dilutions as follows:

1. 0.5 ml from 0.01 N HCL diluted to 5 ml with water gives 0.001 N HCI
2. 0.5 ml from 0.001 N HCL diluted to 5 ml gives 0.0001 N HCL
3. 0.5 ml from 0.0001 N HCL diluted to 5 ml gives 0.00001 N HCL
4. To each tube and 3 drops of bromophenol blue and mix
5. Comment on the colour obtained
6. Calculate the pH of the three solutions

Experiment 2:

Buffer solutions demonstrate:

Objective: To show that dilution does not affect the pH of a buffer solution take four test tube and prepare the following mixture of 0.1 N acetic acid and 0.1 M sodium acetate.

Tube No.	1	2	3	4
Volume of 0.1 M sodium acetate mls	5	7	8.5	9
Volume of 0.1 N acetic acid mls	5	3	1.5	1

A second series of 4 tubes are now taken, each containing 8 ml distilled water and 1 ml from the corresponding tube of the above table. Two drops of methyl red are then added to each of the eight tubes is well mixed. The first 4 tubes now show a series of tints ranging from pure red to yellow. Each of the second series will show the same tint as that of the corresponding tubes of the first series, although the solution contents had been diluted 9 times.

Given that the pk of acetic acid is 4.7 calculate the pH of the buffer solution 1-4.

Questions:

1. Calculate the pH of the buffer solution that contains 0.01 mole acetic acid and 0.01 mole acetate ions (pK_a of CH COOH is 4.74).
2. Calculate the pH of the same solution after the addition of 0.01 mole HCL.
3. Calculate the pH of solution (1) after se addition of 0.005 mole hydroxide ions

Experiment 3:

Objective: To show that dilution, though it does not alter the pH of a buffer solution, does affect its buffering capacity.

A mixture is made of 10 ml 0.1 N acetic acids and 0.1 M sodium (5 ml form cache). One milliliter of this mixture is transferred to a second tube then diluted with 9 ml distilled water. 3 drop of methyl red is added to both solutions. The tint should be the same in both tubes. Now add 0.1 NaOH from a burette to each tube and measure the volumes required to change the methyl red colour to the colour at its pk (i.e. from red-yellow) in each tube. With reference to equation pH-pk + log (salt)/ (acid), attempt to explain your results.

Experiment 4

Objective: Determination of pH using indicators into three test tubes pipettes

5 ml of buffer solution pH 2.8, 3.7, 4.6 respectively. To each tube and three drops of bromophenol blue and mix to show the yellow, and the intermediate colours. In the same way setup another three test tubes to show the colours of methyl red in buffers to pH 4.2, 5.2 and 6.3.

Questions:

1. Determine the pH of the unknown A and unknown B using 5 ml of the unknown add one drop of the indicator.
2. Compare the colour of the indicator and the unknown with the colour of each of the buffer solutions to which the indicator was added. The buffer having the same tint as the unknown gives the pH of the unknown.

Experiment 5:

Objective: Determination OF pH of a sample of water:

pH is measured using pH meter, which comprises a detecting unit consisting of a glass electrode, reference electrode, usually a calomel electrode connected by KCl Bridge to the pH sensitive glass electrode and an indicating unit which indicates the pH corresponding to the electromotive force is then detected. Before measurement, pH meter should be calibrated by using at least two buffers.

Equipment required:

1. pH meter.
2. pH electrode filled with KCL solution.
3. Buffer solutions of pH4 and pH 7.
4. Clean beakers.
5. Tissue papers.
6. Distilled water.
7. Thermometer.

pH Meter

Method:

1. Plug in the pH meter to power source and let it warm up for 5 to 10 minutes.
2. Wash the glass electrode with distilled water and clean slowly with a soft tissue.
3. Note the temperature of water and set the same on the pH meter.
4. Place the electrode in pH 7 buffer solution and set the value of 7 on the pH meter turning the Calibrate knob on the meter.
5. Take out the electrode, wash with DW and clean.
6. Dip the electrode in the pH 4 buffer solution. Adjust the value on the pH readout meter by the Slope switch.
7. Repeat with pH 7 and pH4 buffers till a correct and stable reading is displaced.
8. While moving and cleaning the electrode, put the selector switch on standby mode. Turn to pH mode for recording the pH.
9. Now place the electrode in the water sample whose pH is to be determined.
10. You can take a number of simultaneous readings for different samples until the power is on.

Practical No. 2

Carbohydrates

2.1. Introduction:

Carbohydrates are carbon compounds that contain large quantities of hydroxyl groups. The simplest carbohydrates also contain either an aldehyde moiety (these are termed polyhydroxyaldehydes) or a ketone moiety (polyhydroxyketones).

All carbohydrates can be classified as either monosaccharides, disaccharides, oligosaccharides or polysaccharides.

- **Monosaccharides:** Smallest structure; obtained by hydrolysis of larger sugars.
- **Disaccharides:** Polymer of 2 monosaccharides.
- **Oligosaccharides:** Polymer of 3 to 10 monosaccharides.
- **Polysaccharides:** Polymer of more than 10 monosaccharides,

Polysaccharides are much larger, containing hundreds of monosaccharide units. The presence of the hydroxyl groups allows carbohydrates to interact with the aqueous environment and to participate in hydrogen bonding, both within and between chains. Derivatives of the carbohydrates can contain nitrogen, phosphates and sulfur compounds.

Carbohydrates also can combine with lipid to form **glycolipids** or with protein to form **glycoproteins**.

Carbohydrate Nomenclature:

The predominant carbohydrates encountered in the body are structurally related to the aldotriose glyceraldehyde and to the ketotriose dihydroxyacetone. All carbohydrates contain at least one asymmetrical (chiral) carbon and are, therefore, optically active. In addition, carbohydrates can exist in either of two conformations, as determined by the orientation of the hydroxyl group about the asymmetric carbon farthest from the carbonyl. With a few exceptions, those carbohydrates that are of physiological significance exist in the D-conformation. The mirror-image conformations, called enantiomers, are in the L- conformation.

1. Monosaccharides:

The monosaccharides commonly found in humans are classified according to the number of carbons they contain in their backbone structures. The major monosaccharides contain four to six carbon atoms.

Monosaccharides are divided into aldoses and ketoses:

a. Aldoses contain the aldehyde functional groups and are subdivided into D- and L-absolute configurations and include: D Glyceraldehyde (C_3), D-Erythrose (C_4), D-Ribose (C_5), D-Glucose (C_6), D-Mannose (C_6), D-Galactose (C_6).
b. Ketoses contain the ketone functional group and are subdivided into D- and L- absolute configurations and include: Dihydroxyacetone (C_3). D-Ribulose (C_5), D-Fructose (C_6).

The aldehyde and ketone moieties of the carbohydrates with five and six carbons will spontaneously react with alcohol groups present in neighboring carbons to produce intramolecular hemiacetals or hemiketals, respectively. This results in the formation of five - or six-membered rings. Because the five-membered ring structure resembles the organic molecule furan, derivatives with this structure are termed **furanoses.** Those with six-membered rings resemble the organic molecule pyran and are termed **pyranoses**. Such structures can be depicted by either Fischer or Haworth style diagrams. The numbering of the carbons in carbohydrates proceeds from the carbonyl carbon, for aldoses, or the carbon nearest the carbonyl, for ketoses.

The rings can open and re-close, allowing rotation to occur about the carbon bearing the reactive carbonyl yielding two distinct configurations (_ and _) of the hemiacetals and hemiketals. The carbon about which this rotation occurs is the anomeric carbon and the two forms are termed **anomers**.

Carbohydrates can change spontaneously between the _ and _configurations --- a process known as mutarotation. When drawn in the Fischer projection, the configuration places the hydroxyl attached to the anomeric carbon to the right, towards the ring. When drawn in the Haworth projection, the configuration places the hydroxyl downward.

The spatial relationships to the atoms of the furanose and pyranose ring structures and more correctly described by the two conformations identified as the chair form and the boat form. The chair form is the more stable of the two. Constituents of the ring that project above or below the plane of the ring are axial and those that project parallel to the plane are equatorial. In the chair conformation, the orientation of the hydroxyl group about the anomeric carbon of _-D-glucose is axial and equatorial in _-D-glucose.

2. Disaccharides:

a. Disaccharides consist of two monosaccharides joined by a glycosidic linkage
b. Common disaccharides include sucrose, lactose and maltose.

3. Polysaccharides:

1. Polysaccharides generally classified into categories:

1.1 Storage polysaccharides.
1.2 Structural polysaccharides.

1.1. Storage Polysaccharides:

a. In animals, glycogen is the major storage polysaccharide for glucose.
b. In plants, glucose is stored in the form of starch. Starch is found into forms: **amylase** and **amylopectin**.

1.2. Structural Polysaccharides:

a. Cellulose is the most abundant organic compound on the planet.
b. Consists of an unbranched beta 1- 4 linked glucose polymer

c. Chitin is the structural carbohydrate of the exoskeleton of insects and crustacea; also, the cell walls of most fungi and many alga.

2. Polysaccharides may also be classified as:

2.1. **Homoglycans** (polymers with only one type of monomer)

2.2. **Heteroglycans** (polymers with two or more kinds of saccharide monomers).

3. Agarose

Is a high-molecular weight polysaccharide (the neutral, polysaccharide component of agar) composed of alternating [1 → 3] linked D-galactopyranose and [1→ 4] linked 3, 6-anhydro L-galactopyranose resides.

1. Commonly used in electrophoresis procedures.
2. "Beaded" and "crosslinked" forms are commonly used for chromatographic purposes and cell culturing.

4. Selected Heteroglycans:

Heteroglycans are divided into three types:

- Proteoglycans,
- Peptidoglycans
- Glycoprotiens.

SOME FUNDAMENTAL TESTS FOR CARBOHYDRATES

2.1. Molisch's Test for the presence of carbohydrates:

Molisch test is a group test for all **carbohydrates**, either free or bound to **proteins** or **lipids**. It is a sensitive test that requires precision for the detection of carbohydrates.

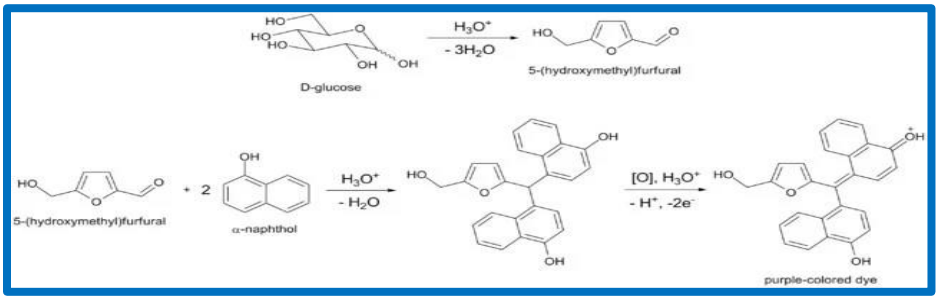
Objectives of Molisch Test

- To detect the presence of carbohydrates in a given sample.
- To distinguish carbohydrates from other biomolecules.

Principle of Molisch Test:

- The reaction is based on the fact that the concentrated acid catalyzes the dehydration of sugars to form furfural (from pentoses) or hydroxymethylfurfural (from hexoses).
- Either of these aldehydes condenses with two molecules of naphthol to form a purple or violet colored complex at the interface of the acid and test layer.
- If the carbohydrate is poly- or disaccharide, a glycoprotein or glycolipid, the acid first hydrolyses it into component monosaccharides, which get dehydrated to form furfural or its derivatives.
- A green ring might be observed if any impurities are present in the reagent as they might interact with the α-naphthol and the acid.
- A rind ring is seen if a concentrated sugar solution is used. This might be due to the charring of the sugar due to the acid.

Reaction:



Reagent:

- Molisch reagent: Dissolve 3.75 g of α-naphthol in 25 ml of Ethanol 99%. This reagent should be prepared fresh.
- Concentrated sulphuric acid
- Test sample

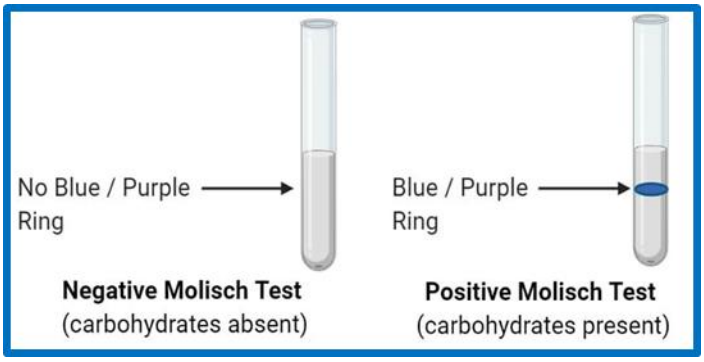
2. Materials required

- Test tubes
- Test tube stand
- Pipette
- Distilled water

Method:

1. Take 2 ml of each distilled water and test sugar solutions in four test tubes separately.
2. Add two drops of Molisch reagent to each tube.Hold the test tube in an inclined position and gently add 1 ml concentrated H_2SO_4 along the wall of the test tube.
3. Do not mix the acid with the solution. A black ring may form if concentrated acid is not added slowly as the heat generated from the reaction can char the carbohydrates.
4. Observe the test tube for the formation of a purple-colored ring at the layer between the solution and the acid.

Observation:



- The formation of the purple colored ring occurs at the interface between the sulphuric acid and the test solution.
- The sulphuric acid remains above the test solution as the acid is denser than the test solution.
- The absence of color indicates a negative result.

Uses of Molisch Test

- Molisch test is used to detect the presence of carbohydrates in different samples.
- It can be used to detect the formation of carbohydrates as a by-product in different reactions and distinguish it from other biomolecules.

Lab results:

- Glucose, maltose, arabinose and starch will all display the purple ring compound at the interface of the acid and solution.

Limitations:

- Not specific to carbohydrates
- Generalized test that cannot distinguish carbohydrates and further testing must be undertaken to identify the carbohydrate.

2.2. Benedict's test for reducing sugars: (Use glucose and sucrose):

Benedict ’s test is a chemical analytical method used for the detection of reducing sugar in a solution. Benedict’s test is a qualitative test often used for the differentiation of **carbohydrates** (saccharides/sugars) into reducing and non-reducing types.
It is widely used to identify monosaccharides (simple sugars) and other reducing sugars. It is used as an alternative to **Fehling’s test.** Identification is based on the development of brick-red color due to the chemical reaction between Benedict’s reagent and reducing sugar. Based on the intensity of the reaction mixture, the concentration of sugar can be determined, but numerical value can’t be estimated. Hence, it is a qualitative and semi-quantitative test.
It is also used for detecting glucose in urine as a presumptive test of diabetes mellitus.

It was discovered by American Chemist/Biochemist Stanley Rossiter Benedict.

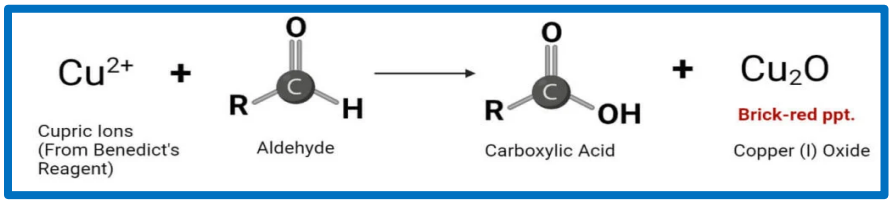
Objectives:

- To detect the presence of reducing sugar in the sample solution
- To diagnose diabetes mellitus by detecting glucose in the urine sample
- To estimate the concentration of reducing sugar in the sample solution
- To differentiate and identify the extracted carbohydrates

Principle:

Sodium carbonate in the Benedict reagent increases the pH of the sample-reagent solution mixture. Under warm alkaline conditions reducing sugars are tautomerism to strong reducing agents, enediols. These enediols reduce the cupric ions (Cu^{2+}) (present as Copper Sulfate ($CuSO_4$)) of Benedict reagent into cuprous ions (Cu^{+}). The cuprous particles are present in form of insoluble Copper (I) oxide or cuprous oxide (Cu_2O) which is of red color. These red-colored copper oxides get precipitated.

Reaction:



The concentration of reducing sugar in the sample differs from the intensity and shade of the color of the reaction mixture. This shade of color can be used to estimate the concentration of reducing sugar in the sample. Color may vary from greenish to yellow to orange-red to brick-red. As the concentration of reducing sugar increases color gradually changes from greenish to yellowish to orange to brick-red.

Requirements of Benedict's Test:

- Sample solution of unknown carbohydrate (or urine sample)
- Test-tubes and test-tube holders
- Pipette
- Bunsen burner
- Benedict's Reagent

Reagent:

1. Measure 17.3 grams of copper sulfate ($CuSO_4$), 173 grams of sodium citrate ($Na_3C_6H_5O_7$), and 100 grams of anhydrous sodium carbonate (Na_2CO_3) (or 270 grams of sodium carbonate decahydrate ($Na_2CO_3.10H_2O$))
2. Put all the measured chemicals in a volumetric flask of 1000 mL.
3. Pour distilled water up to 1000 mL marking.
4. Dissolve all the components properly by shaking gently.

Preparation of Benedict's Reagent:

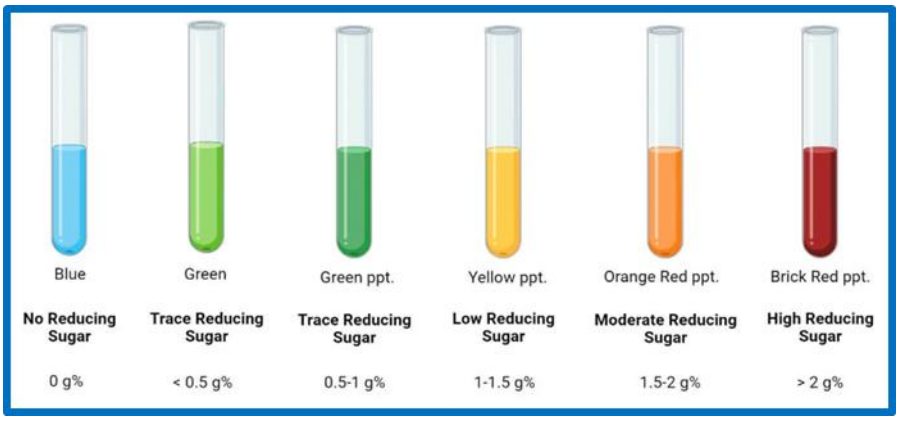
1. Measure 17.3 grams of copper sulfate (CuSO4), 173 grams of sodium citrate (Na3C6H5O7), and 100 grams of anhydrous sodium carbonate (Na2CO3) (or 270 grams of sodium carbonate decahydrate (Na2CO3.10H2O))

2. Put all the measured chemicals in a volumetric flask of 1000 mL.
3. Pour distilled water up to 1000 mL marking.
4. Dissolve all the components properly by shaking gently.

Method:

1. In a clean test tube add 1 mL of sample solution (urine or carbohydrate solution).
2. Add 2 mL of Benedict's reagents over the sample.
3. Place the test tube over a boiling water bath and heat for 3–5 minutes or directly heat over a flame.
4. Observe for color change.

Observation:



Hydrolysis of Disaccharides:

Principle:

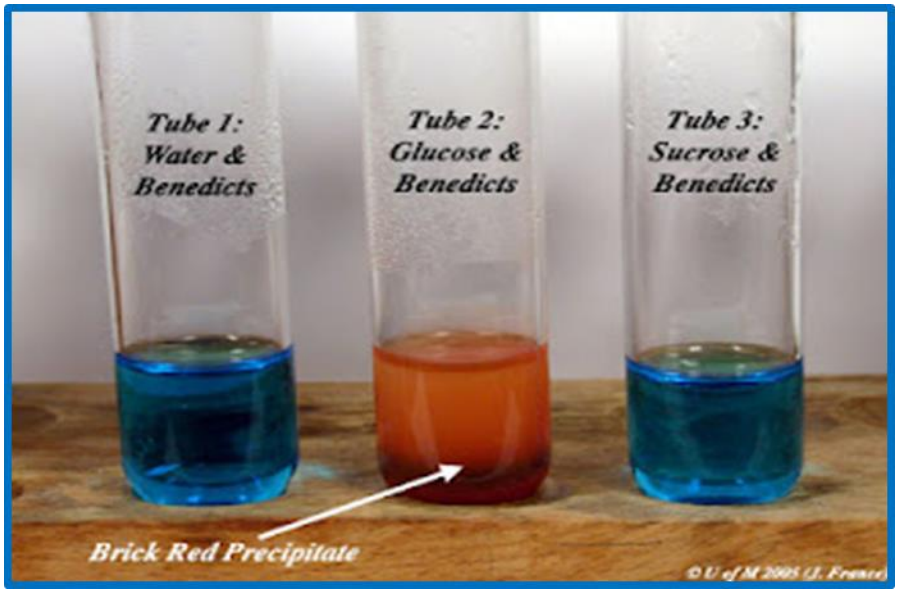
Sucrose is the only non-reducing disaccharide so that it does not reduce Benedict's. Sucrose is therefore hydrolyzed in acid solution to glucose and fructose which are then tested for their reducing ability using Benedict's solution.

Method:

1. Into a test tubes add 2 ml of sucrose solution, glucose solution.
2. Add few drops of dilute hydrochloric acid (make just acid to litmus)
3. Boil for 10 minutes cool

Test for reducing substance by adding Benedict's solution

Observation:



- If the reaction is positive the following results will be obtained:

Shade of Color	Approx. Concentration of Reducing Sugar (in g%)	Indication
Blue	0	No reducing sugar
Green solution	< 0.5	Trace reducing sugar
Green ppt.	0.5 – 1	Trace reducing sugar
Yellow ppt.	1 -1.5	Low reducing sugar
Orange-red ppt.	1.5 – 2	Moderate reducing sugar
Brick-red ppt.	>2	High reducing sugar

Uses of Benedict's Test:

- In biochemistry for analysis and identification of unknown carbohydrate extracts.
- In clinical diagnosis for rapid presumptive diagnosis of diabetes mellitus.
- In quality control for detecting simple sugar and their quantification.

Advantages of Benedict's Test:

- A simple test requiring fewer materials and less time.
- Non-toxic reagents.
- Inexpensive.

Limitations of Benedict's Test:

- False-positive result due to reaction with drugs like penicillin, isoniazid, streptomycin, salicylates, and p-aminosalicylic acid.
- Chemicals in urine like creatinine, ascorbic acid, and urate retard Benedict's reaction.
- The exact concentration of reducing sugar can't be measured; only an estimated semiquantitative value can be indicated.
- Requires further test for identification of the carbohydrate.

2.3. Sugar Fermentation Test:

Carbohydrates are organic molecules that comprise carbon, hydrogen, and oxygen in the ratio (CH2O)n. There are three types of carbohydrates based on their structure and number of carbon atoms present, they are monosaccharides (simple sugar containing 3-7 carbon atoms), disaccharides (comprising of two monosaccharides linked together by the glycosidic bond), and polysaccharides (containing eight or more monosaccharide molecules). The energy is released by the process of catabolism referring to the breakdown of the complex organic molecules. The power of the chemical breakdown of carbohydrates from complex to simple forms is possessed by a large number of bacteria, fungi, and yeasts. However, the utilization of the carbohydrate and its breakdown is different depending upon the enzyme system of the organism. The pattern of fermentation is the characteristics of certain species, genera, or groups of organisms. Due to this reason, the property of fermentative reaction has been extensively used as a method for the biochemical differentiation of microorganisms.

Objectives:

- To demonstrate the ability of microorganisms to ferment the carbohydrate and production of organic acid end products.
- To determine the ability of the microorganism to produce gaseous end products in fermentation.

Principle:

Carbohydrate fermentation is the process by which the microorganism utilizes to produce energy in the form of ATP, the ultimate energy source of the organism. Glucose after entering a cell can be catabolized either aerobically (in the presence of O2), where molecular oxygen serves as the final electron acceptor (oxidative pathway), or anaerobically (in absence of O2) in which inorganic ions can serve as the final electron acceptor (fermentative pathway). The metabolic end products of a carbohydrate fermentation can either be organic acids (lactic, formic, acetic acid) or organic acid and gas (hydrogen or carbon dioxide). Fermentative degradation of the carbohydrates (monosaccharide, disaccharide, and polysaccharide) by microorganisms under the anaerobic condition is carried out in the fermentation tube, which comprises of Durham tube for the detection of the gas production. A fermentation medium is composed of a basal medium containing a specific carbohydrate (glucose, sucrose, or cellulose) along with a pH indicator (phenol red, Andrade's indicator, or bromocresol). When the organism ferments carbohydrates, organic acid products (Lactic acid, formic acid, or acetic acid) are obtained which turns the medium into yellow color with a reduction in the pH (acidic-below pH of 6.8). The change in the pH indicator in the fermentation tube and the gas production in the Durham tube is indicative of the metabolic reaction with the production of acid end product and gas. Color change only occurs and is visible when a sufficient amount of acid is produced, as bacteria may utilize the peptone producing alkaline by-products. The degradation of peptones in the broth may result in the production of alkaline end products, which will change the broth color to pink often at the top of the tube.

Method:

Preparation of Carbohydrate Fermentation Broth:

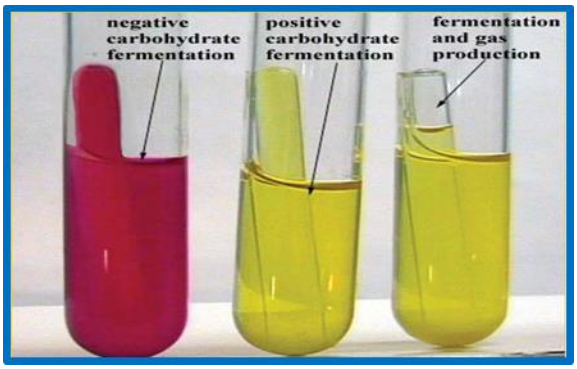
1. Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 ml distilled water and transfer into conical flasks.
2. Add 0.5% to 1% of the desired carbohydrate into all flasks.

3. Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth.
4. Sterilize in an autoclave at 115°C for 15 minutes. (Note: Do not overheat the Phenol red Carbohydrate fermentation broth. The overheating will result in breaking down the molecules and form compounds with a characteristic color and flavor. The process is known as the caramelization of sugar (the browning of sugar).
5. Transfer the sugar into screw-capped tubes or fermentation tubes and label properly.

Inoculation of Bacterial Culture into fermentation medium tube:

1. Inoculate each tube with 1 drop of an 18 hour or 24-hour cultural broth in aseptic condition (keep uninoculated tubes as control tubes).
2. Incubate the tubes at 18-24 hours at 37°C
3. Examine the tube for acid and gas production.

Observation:



Observation	Result	Interpretation
The medium changes to yellow color	Acid production	Organism ferments the given carbohydrate and produces organic acids thereby reducing the pH of the medium into acidic conditions.
The medium changes to yellow color and production of gas formation in the Durham tube	Acid and Gas production	Organism ferments the given Carbohydrate and produces organic acids and gas. Gas production is detected by the presence of small bubbles in the inverted Durham tubes.
No change in color (retains red color)	Absence of fermentation	The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium.

Limitations:

1. Reading after 24 hours may not be reliable if no acid is produced.
2. No color change or a result indicating alkalinity may occur if the organism deaminates the peptone, masking the carbohydrate fermentation evidence.

2.4. Tollens' Test:

Tollens' test is a chemical test used to differentiate reducing sugars from non-reducing sugars. This test is also called the silver mirror test based on the end product of this test.

Objectives:

- To distinguish reducing sugars from non-reducing sugars.
- To detect the presence of aldehyde containing carbohydrates and differentiate them from ketone containing **carbohydrates**.

Principle:

- The Tollen's reagent is the alkaline solution of silver nitrate ($AgNO_3$) mixed with liquid ammonia (NH_3), which results in the formation of a complex.
- The aqueous solution of silver nitrate forms a silver aqua complex where the water acts as a ligand.
- The aqua complexes are then converted into silver oxides (Ag_2O) by the action of hydroxide ions.
- Silver oxide forms a brown precipitate, which is then dissolved by aqueous ammonia resulting in the formation of the $[Ag(NH_3)_2]^+$ complex.
- This complex is the primary component of the Tollen'ss reagent and is a strong oxidizing agent.
- The complex then oxidizes the aldehyde group present in some sugars to form a carboxylic acid.
- At the same time, the silver ions present in the reagent are reduced to metallic silver.
- The reduction of silver ions into metallic silver results in the formation of a silver mirror on the bottom and sides of the test tube.
- However, an α-hydroxy ketone gives a positive Tollen's test as the Tollen'ss reagent oxidizes the α-hydroxy ketone into an aldehyde.

Reaction:

$$2AgNO_3 + 2NaOH \rightarrow Ag_2O \text{ (brown precipitate)} + 2NaNO_3 + H_2O$$
$$Ag_2O \text{ (brown precipitate)} + 4NH_3 + 2NaNO_3 + H_2O \rightarrow 2[Ag(NH_3)_2]NO_3 + 2NaOH$$
$$\text{lucose} + 2[Ag(NH_3)_2]NO_3 + H_2O \rightarrow 2\,Ag \text{ (silver mirror)} + 4\,NH_3 + \text{Gluconic acid} + 2H^+$$

Requirements:

Reagent:

- **Tollen's reagent:** Add 50 ml of 0.1 M $AgNO_3$ to a beaker and to this, add 25 ml of 0.8 M KOH. Now, add sufficient volume of aqueous ammonia in order to dissolve the brown precipitate.
- Test sample.

Materials required

- Test tubes.

- Test tube stand.
- Pipette.

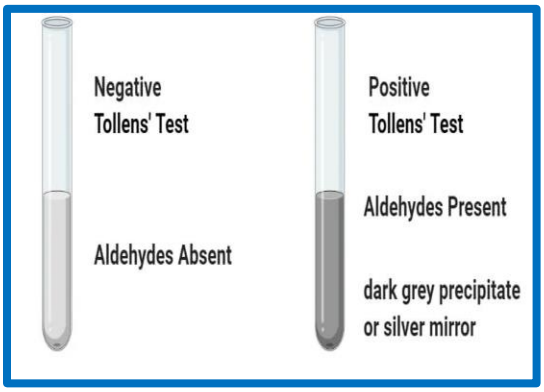
Equipment

- Water bath.

Method:

1. Take two clean, dry test tubes and add 1 ml of the test sample in one test tube and 1 ml of distilled water in another as blank.
2. Add 2 ml of Tollen's reagent to both the test tubes.
3. Keep both the test tubes in a water bath for 1 min.
4. Observe the formation of color and note it down.

Observation:



- The formation of a dark grey precipitate or silver mirror on the bottom and sides of the test tube indicates a positive result, which means that the given sample contains reducing sugars/ aldoses.
- The absence of such precipitate indicates a negative result, which means that the test sample doesn't have reducing sugars/ aldoses/ α-hydroxy ketoses.

Uses of Tollen's test:

- Tollen's test is routinely performed in chemical laboratories for the qualitative organic analysis, which distinguishes aldehydes from ketones.
- This test is also used for the differentiation of reducing sugars from non-reducing sugars.

Limitations of Tollen's test:

- Some carbohydrates that do not have an aldehyde group might give a positive result on Tollen's test because of the isomerization of such sugars under alkaline conditions.

2.5. Seliwanoff's Test:

Seliwanoff's test is used to differentiate between sugars that have a ketone group (ketose) and sugars that have an aldehyde group (aldoses). This test is a timed color reaction specific to ketohexoses.

Objectives:

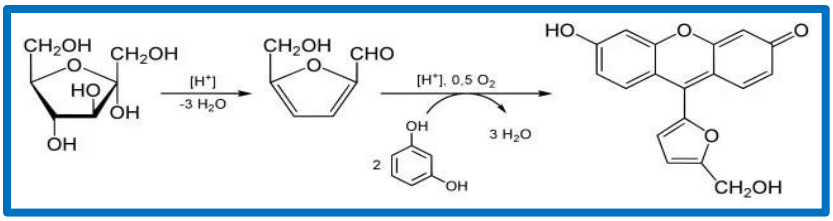
- To detect the presence of ketohexoses in a given sample.
- To distinguish ketoses from aldoses.

Principle:

- The reagent of this test consists of resorcinol and concentrated HCl.
- The acid hydrolysis of polysaccharides and oligosaccharides yields simpler sugars.

- Ketoses are more rapidly dehydrated than aldoses.
- Ketoses undergo dehydration in the presence of concentrated acid to yield 5-hydroxymethyl furfural.
- The dehydrated ketose reacts with two equivalents of resorcinol in a series of condensation reactions to produce a complex (not a precipitate), termed xanthenoid, with deep cherry red color.
- Aldoses may react slightly to produce a faint pink to cherry red color if the test is prolonged.
- The product and reaction time of the oxidation reaction helps to distinguish between **carbohydrates**.
- Other carbohydrates like sucrose and inulin also give a positive result for this test as these are hydrolyzed by acid to give fructose.

Reaction:



Seliwanoff's test with fructose

Requirements:

Reagent:

- **Seliwanoff's reagent:** add 0.05% resorcinol (m-hydroxybenzene) in 3 N HCl. Dissolve 50 mg resorcinol in 33 ml concentrated HCl and make it 100 ml with water.
- Test sample.
- Distilled water.

Materials required

- Test tubes.
- Test tube stand.
- Pipettes.

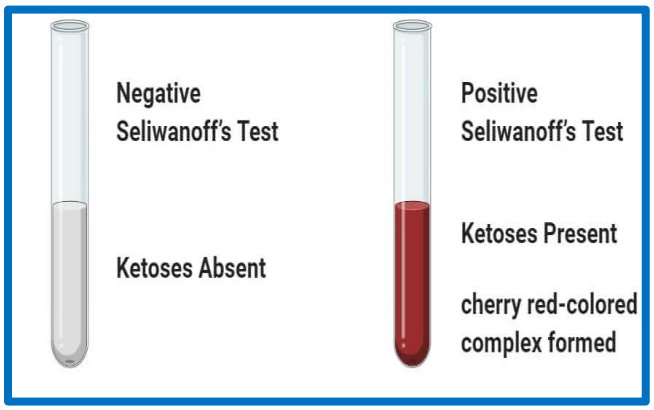
Equipment

- Water bath

Method:

1. Take two clean, dry test tubes and add 1 ml of the test sample in one test tube and 1 ml of distilled water in another as blank.
2. Add 2 ml of Seliwanoffs' reagent to both the test tubes.
3. Keep both the test tubes in a water bath for 1 min.
4. Observe the formation of color and note it down.

Observation:



- The formation of the cherry red-colored complex indicates a positive result which means that the given sample contains ketoses.
- The absence of such color or the appearance of the color after a prolonged period of time indicates a negative result which means that the test sample doesn't have ketoses.

Uses of Seliwanoff's test:

- Seliwanoff's color reaction is used in the method for the colorimetric determination of fructose in fermentation media.
- A modified version of this test can be used for the determination of the concentration of ketoses in a given sample.

Limitations of Seliwanoff's test:

- The high concentration of glucose or other sugar may interfere by producing similar colored compounds with Seliwanoff's reagent.
- Prolonged boiling can transform glucose to fructose by the catalytic action of acid and form cherry red-complex giving a false-positive result.
- This test is a generalized test and doesn't distinguish between specific ketoses, and a separate test is required for the particular ketose sugar identification.

2.6. Mucic Acid Test:

Mucic acid test is a test that is highly specific and is used for the detection of the presence of galactose and lactose. It is also termed galactaric acid that is named after the product of the reaction.

Objectives:

- To detect the presence of galactose and lactose in a given sample.
- To distinguish between the galactose containing saccharides and other sugars.

Principle:

- Monosaccharides upon treating with potent oxidizing agents like nitric acid yield saccharic acids (dicarboxylic acids).
- Nitric acid has the capacity to oxidize both aldehyde and primary alcoholic groups present at C1 and C6 respectively of galactose to yield an insoluble precipitate (rod-shaped crystals) of mucic acid under higher temperature.

- Lactose also yields a mucic acid, due to the hydrolysis of the glycosidic bond between the glucose and galactose subunits of the **carbohydrate**.
- Other monosaccharides like glucose also have a similar structure; however, the resultant precipitate formed in glucose is water-soluble under room temperature.

Reaction:

$$CH_2OH\text{-}(CHOH)_4\text{-}CHO + HNO_3 \rightarrow CH_2OH\text{-}(CHOH)_4\text{-}CHO\text{-}NO_3 + H^+$$

Requirements:

1. Reagent:

- Mucic acid reagent: concentrated nitric acid.
- Test sample (1%).
- Distilled water.

2. Materials required

- Test tubes.
- Test tube stand.
- Pipette.

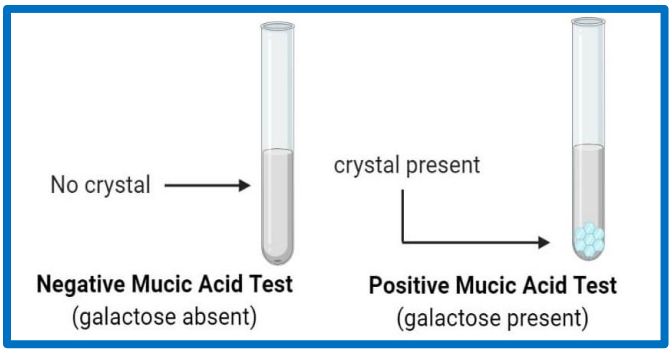
3. Equipment

- Water bath.

Method:

1. Take 6 ml of each distilled water and test sugar solutions in four test tubes separately.
2. Add 1 ml of Mucic acid reagent (concentrated nitric acid) to each tube.
3. Heat the test tubes in the water bath for 1-1/2 hours until the volume of the solution is reduced to 2-3 ml.
4. Let the test tube sit overnight before collecting the results.

Observation:



- The formation of crystal at the bottom of the tube indicates a positive result which means that the sample solution has galactose or its derivatives.
- The absence of such crystals indicates a negative result and represents that the sample doesn't have galactose or its derivative. The solution might still have other carbohydrates.

Uses Mucic acid test:

- The most important use of the mucic acid test is to identify the presence of galactose or its derivatives in the food sample and in synthetics manufacture.
- This test can also be used to detect the presence of lactose or agar-agar.

Limitations:

- This test cannot distinguish between monosaccharides and disaccharide derivations of galactose.
- In some cases, a false positive result might occur due to impurities with carbonyl groups at the terminal ends.

2.7. Fehling's Test:

Fehling's test is a chemical test used to differentiate between reducing and non-reducing sugars. This test can also be used to distinguish ketone functional group **carbohydrates** and water-soluble carbohydrates.

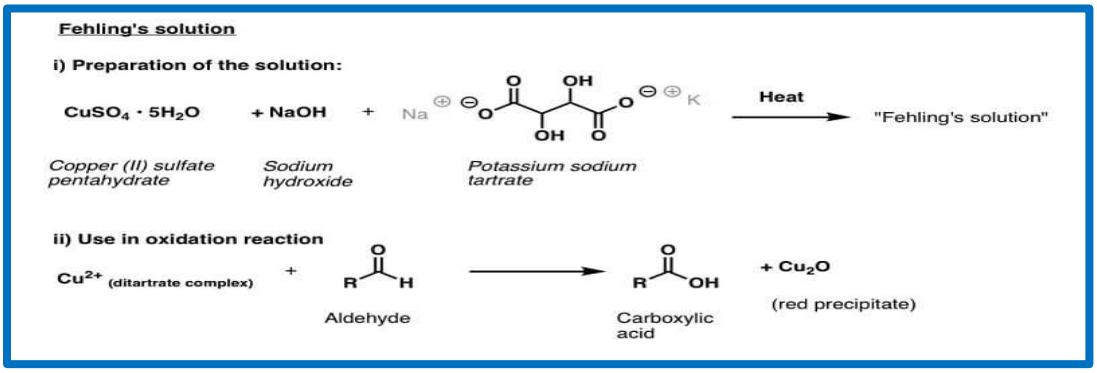
Objectives:

- To detect the presence of carbohydrates in a solution.
- To differentiate between reducing and non-reducing sugars.

Principle:

- The carbohydrates having free or potentially free carbonyl groups (aldehyde or ketone) can act as reducing sugars.
- The Fehling's solution appears deep blue in color and consists of copper sulfate mixed with potassium sodium tartrate and strong alkali, which is usually sodium hydroxide.

Reaction:



Requirements:

1. Reagent:

- Fehling's solution A: Dissolve 7 g of $CuSO_4.7H_2O$ in 100 ml of water.
- Fehlings solution B: Dissolve 24 g of KOH and 34.6 g of potassium sodium tartrate in 100 ml water.
- Fehling's solution: Mix equal volumes of both the solution just before use.
- Sample (5% Glucose, 5% Sucrose, 5% Fructose, 5% Starch, 5% lactose).

2. Materials Required

- Pipettes.
- Test tubes.
- Test tube stand.

3. Equipment

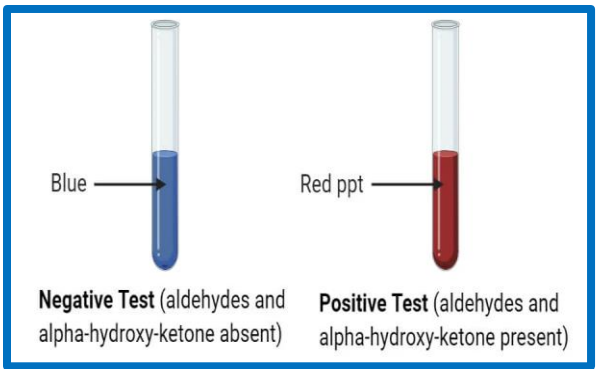
- Water bath.

Method:

1. Take 1 ml of a given sample in a clean, dry test tube. The concentration of the test samples should be 5% (w/v).
2. Take control of 1 ml of distilled water in another tube.

3. Add about 2-3 drops of Fehling's reagent to both the tubes and mix them in a vortex.
4. Keep the test tubes in the water bath for 1-2 minutes.
5. Observe the appearance of color in the test tubes.
6. Note down the appearance of color seen in the test tubes.

Observation:



- The appearance of a reddish-brown precipitate indicates a positive result and the presence of reducing sugars.
- The absence of the reddish precipitate or the appearance of deep blue color indicates a negative result and lack of reducing sugars.

Uses of Fehling's Test:

- Fehling's test is used to distinguish between the presence of aldehydes and ketones in carbohydrates as ketone sugars except alpha-hydroxy-ketone do not react in this test.
- Fehling's test is performed in medical facilities to detect the presence of glucose in urine. This helps to identify whether the patient has diabetes or not.

Limitations of Fehling's Test:

- Aromatic aldehydes cannot be detected by this test.
- This reaction takes place only in an alkaline environment. In an acidic environment, the copper (II) ions would be stabilized and not easily oxidized, thus failing the reaction.

2.8. Bial's Test:

Bial's test is a chemical test performed to detect the presence of pentoses and pentosans (derivatives of pentoses). A derivation of this test termed the Bial's Orchintest is performed to detect the presence of RNA in solutions.

Objectives:

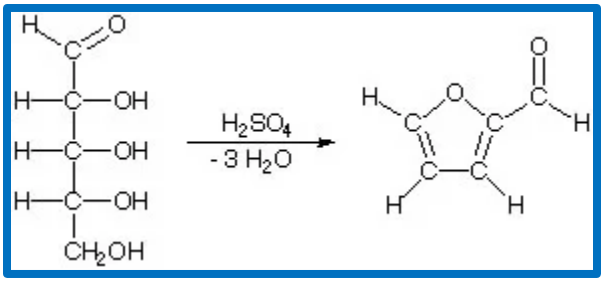
- To detect the presence of **carbohydrates**.
- To distinguish the pentoses and pentosans from other derivatives of carbohydrates like the hexoses.

Principle:

This test is based on the principle that under hydrolysis pentosans are hydrolyzed into pentoses. Further, pentoses are dehydrated to yield furfural, which in turn condense with orcinol to form a blue-green precipitate. In the presence of hexoses, hydroxyfurfural is formed instead of

furfural which upon condensation with orcinol forms a muddy brown colored precipitate. The intensity of the precipitation is directly proportional to the concentration of the pentoses in the sample. The intensity of the color developed depends on the concentration of HCl, ferric chloride, orcinol, and the duration of boiling. The concentration of the sugars is determined by measuring the absorbance of 620 nm wavelength in a spectrophotometer or in a red filter colorimeter.

Reaction:



Requirements:

1. Chemicals/Reagents:

- Bial's Reagent.
- Ribose sugar.
- Other carbohydrates if desired.
- Sample.

2. Materials required

- UV Spectrophotometer.
- Vortex mixer.
- Mantle heater/Water Bath.

3. Glasswares and other equipment:

- Test tubes, Test tube stand, Pipettes, Beaker, Ice Test tube caps, Tissue paper, Wash bottle.

4. Reagents

- **Bial's Reagent:** 300 mg of orcinol is dissolved in 5 ml ethanol. Add 3.5 ml of this mixture to 100ml of 0.1% solution of FeCL3.6H2O. The reagent thus formed is to be stored in a dark bottle and used within a couple of hours.
    - **Ribose stock solution:** 200µg ribose per mL distilled water stock solution of ribose is to be prepared from the stocked solution. Note: Other carbohydrates of the same concentration can be used as samples if desired. If RNA is used, 300 µg/ml of RNA stock solution is added to Tris-EDTA buffer.

Method:

1. Pipette out different volumes (50 µl, 100 µl, and so on) of ribose solution from the supplied stock solution (200µg /ml) into a series of test tubes and make up the volume to 1 mL with distilled water.
2. Take a tube labeled as one as blank containing 1ml of just distilled water and the rest of the tubes labeled 2 to 9 for construction of a standard curve. Tubes 10-15 are for the unknown samples.
3. Add 5 ml of the bial's reagent to each tube and mix well by vortexing.
4. Cool the tubes.

5. Cover the tubes with caps on top and incubate at 90°C for 17 minutes or boiling water bath for 10 minutes.
6. Cool the tubes to room temperature and measure the optical density of the solutions at 620 nm against a blank.
7. Prepare a standard curve of absorbance against ribose concentration.
8. Determine the amount of ribose in the unknown sample by plotting a standard curve of A620 on the Y-axis and concentration of Ribose on the X-axis.

Observation:

two negative tests (left, middle) and a positive test (right)

- The presence of a blue-green complex indicates the presence of pentoses in the sample.
- Using the graph, the concentration of ribose sugar in the sample can be determined. A similar interpretation can be made for RNA detection as well.

Uses of Bial's Test

- This test is used to detect the presence of pentose and pentosans in different samples.
- This test can additionally be used for the quantification of RNA in a sample.

Limitations of Bial's Test

- On prolonged heating, glucoronates might also give a blue-green colored precipitate which might result in false-positive results.
- The color produced might be different with different sugars, and the concentration might not be proportional to the intensity at higher levels.

2.9. Barfoed's Test:

Barfoed's test is a chemical test used to detect the presence of monosaccharides which detects reducing monosaccharides in the presence of disaccharides. This reaction can be used for disaccharides, but the reaction would be very slow.

Objectives:

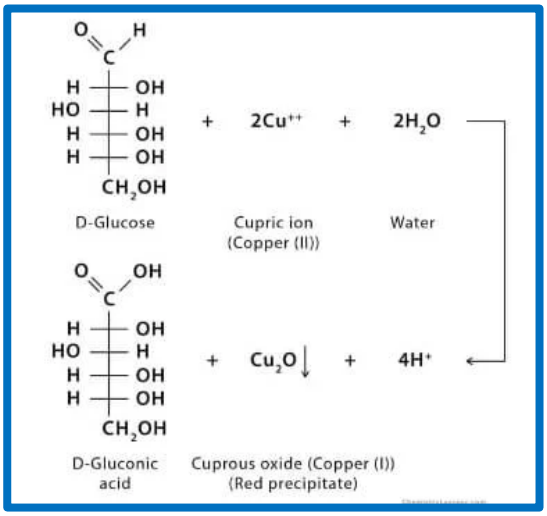
- To detect reducing **carbohydrates.**
- To distinguish reducing monosaccharides from disaccharides.

Principle:

The Barfoed reagent is made up of copper acetate in a dilute solution of acetic acid. Since acidic pH is unfavorable for reduction, monosaccharides, which are strong

reducing agents, react in about 1-2 min. However, the reducing disaccharides take a longer time of about 7-8 minutes, having first to get hydrolyzed in the acidic solution and then react with the reagent. Once the reaction takes place, thin red precipitate forms at the bottom of the sides of the tube. The difference in the time of appearance of precipitate thus helps distinguish reducing monosaccharides from reducing disaccharides.

Reaction:



Requirements:

1. Reagent:

- Barfoed's reagent: 0.33M solution of copper acetate is added to 1% acetic acid. The freshly prepared reagent should be used for the assay.
- Sample.

2. Materials Required:

- Test tubes.
- Test tube stand.
- Pipettes.

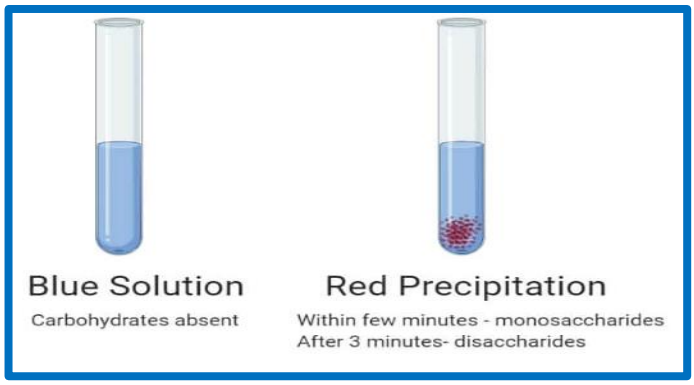
3. Equipment:

- Water bath.
- Vortex.

Method:

1. Take 1 ml of a given sample in a clean, dry test tube. The concentration of disaccharides sample (if used) should not exceed 1% (w/v).
2. Take control of 1 ml of distilled water in another tube.
3. Add about 2-3 drops of Barfoed's reagent to both the tubes and mix them in a vortex.
4. Keep the test tubes in the water bath for 1-2 minutes. The boiling should not be done for more than 2 minutes as the disaccharides might hydrolyze into monosaccharides and give a positive result.
5. Observe the appearance of color in the test tubes.
6. Noted own the time taken for the appearance of color in the tubes.

Observation:



- The presence of red precipitate detects the presence of reducing monosaccharides in the sample.
- If the color appears within the first few minutes, the sample contains reducing monosaccharides.
- However, if the color appears later than the first 3 minutes, the sample is of reducing disaccharides.

Uses of Barfoed's Test:

- This test is used to identify reducing monosaccharides and distinguish the reducing disaccharides from reducing monosaccharides.

Limitation of Barfoed's Test:

- This test cannot be used to detect sugar in urine as urine contains Cl^- ions, which might interfere with the reaction.
- If a higher concentration of disaccharides is present in a sample, it might give a positive result.

2.10. Iodine test:

Iodine test is a chemical test used to distinguish mono- or disaccharides from certain polysaccharides like amylase, dextrin, and glycogen. This test has a variation termed starch-iodine test that is performed to indicate the presence of glucose made by plants in the leaves.

Objectives:

- To detect the presence of polysaccharides, primarily starch.

Principle:

- Iodine test is based on the fact that polyiodide ions form colored adsorption complex with helical chains of glucose residue of amylase (blue-black), dextrin (black), or glycogen (reddish-brown).
- Monosaccharides, disaccharides, and branched polysaccharides like cellulose remain colorless. Amylopectin produces an orange-yellow hue.
- The reagent used in the iodine test is Lugol's iodine, which is an aqueous solution of elemental iodine and potassium iodide.

- Iodine on its own is insoluble in water. Addition of potassium iodine results in a reversible reaction of the iodine ion with iodine to form a triiodide ion, which further reacts with an iodine molecule to form a pentaiodide ion.
- Bench iodine solution appears brown, whereas, the iodide, triiodide, and pentaiodide ion are colorless.
- It is observed that the helix (coil or spring) structure of the glucose chain is the key to this test.
- Further, the resulting color depends on the length of the glucose chains.
- The triiodide and pentaiodide ions formed are linear and slip inside the helix structure.
- It is believed that the transfer of charge between the helix and the polyiodide ions results in changes in the spacing of the energy levels, which can absorb visible light, giving the complex its color.
- The intensity of the color decreases with the increase in temperature and the presence of water-miscible organic compounds like ethanol.
- On heating, the blue color amylase-iodine complex dissociates but is formed again on cooling because the helical structure is disrupted; thereby amylose loses its iodine binding capacity and the blue color.
- The blue color reappears on cooling due to the recovery of iodine binding capacity due to regaining of the helical structure.

Requirements:

1. Reagent:

- Lugol's iodine: 5% elemental iodine is mixed with 10% potassium iodide to form the Lugol's iodine.
- Test sample.

2. Materials Required

- Test tubes.
- Test tube stand.

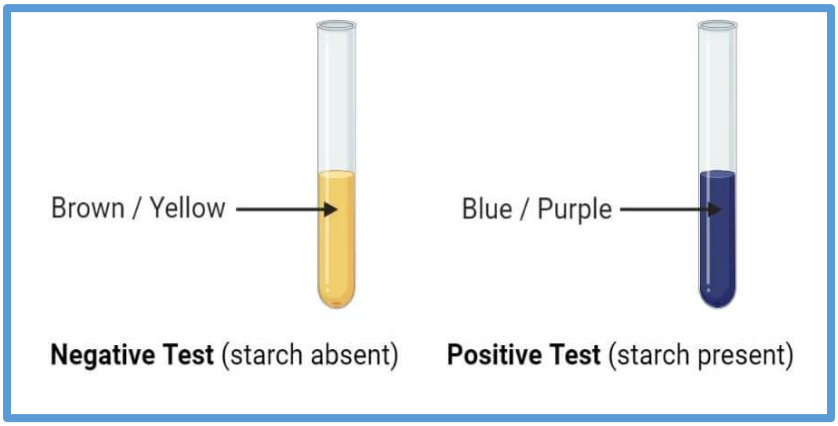
3. Equipment

- Water bath.

Method:

1. Take 1 ml of a given sample in a clean, dry test tube.
2. Take control of 1 ml of distilled water in another tube.
3. Add about 2-3 drops of Lugol's solution to both the tubes and mix them in a vortex.
4. Observe the appearance of color in the test tubes.
5. Heat the test tubes in the water bath until the color disappears.
6. Take the test tubes out for cooling
7. Note down the appearance of color seen in the test tubes.

Observation:



- The appearance of a blue-black or purple color represents a positive test, indicating the presence of starch.
- If there is no change in color, the result is negative and indicates the absence of starch.

Uses of Iodine Test

- This test is used to detect the presence of starch in various samples.
- Similarly, this test is performed to test the process of **photosynthesis** in plants.

Limitations of Iodine Test

- This test cannot be performed under acidic conditions as the starch hydrolyses under such circumstances.
- This test is a qualitative test and doesn't signify the concentration of starch.

Practical No. 3

Lipids

Introduction:

The term lipid is applied to a group of naturally occurring substances characterized by their insolubility in water and their solubility in organic solvents such as chloroform, ethanol, petroleum, ether, etc. Chemically, the lipids are either esters of fatty acids or substances capable of forming such esters. They are very widespread in nature, being found in all vegetable and animal matter. Some members of this group, such as phosphatides and sterols, are found in all living cells where, with the proteins and carbohydrates, they form an essential part of the colloidal complex of the cytoplasm.

Complex lipids are also found in large quantities in brain and nervous tissues, thus indicating the important role played by these substances in living organism. Structurally, these compounds can be classified as follows:

1. Simple Lipids

These are esters of fatty acids with certain alcohols. They are usually further classified according to the nature of the alcohol as follows:

a. **Fats and oils:** These are esters of fatty acids and glycerol (mono-di-and triglycerides).
b. **Waxes**: Esters of fatty acids with long-chain aliphatic alcohols or with cyclic alcohols.

2. Compound or Conjugate Lipids

These are esters of fatty acids, which, on hydrolysis, yield other substances in addition to the fatty acids and an alcohol.

Some important members are:

a. **Phospholipids (Phosphotides):** On hydrolysis, these yield an alcohol, phosphoric acid, fatty acids and nitrogenous base. Examples are phospatidic acid, lecithin, cephalins, etc.
b. **Glycolipids:** These are complex lipids containing carbohydrates in combination with long-chain aliphatic acid or alcohols. Examples are cerebrosides, gangliosides and cytolipids.
c. **Sulpholipids:** These are esters with sulphuric acid.
d. **Lipoproteins:** These lipids are bound to proteins, and are found mainly in mammalian plasma. The lipid moiety consists mainly of cholesterol esters and phospholipids containing principally stearic, palmitic, and oleic acids.

3. Derived Lipids

These are lipids which cannot be further degraded by hydrolysis, as they are found combined in 1 and 2.

a. Fatty acids (saturated and unsaturated)
b. Alcohols (aliphatic alcohols, sterols and alcohols containing ionone ring e.g. vitamin A).
c. Hydrocarbons (compounds having no carboxyl or alcohol groups, and which cannot be saponified).
d. Vitamin D, E, and K with vitamin A, which are the fat soluble vitamins.

LIPIDS FUNCTION IN THE CELL AND ORGANISM:

1. Energy storage: Triglycerides in adipose tissue can readily converted into compounds which yield energy on oxidation.
2. Structural and functional components of the cell membrane. The selective peftileability and integrity of all forms of membranes depend on their content of different lipids (unsaturated fatty acids, phospholipids, sterols and glycolipids are especially important ones).
3. Protection and thermal insulation (subcutaneous adipose tissue).

SOME FUNDAMENTAL TESTS FOR LIPIDS

3.1. Grease-spot Test:

This simple test for lipids has been used for centuries. Lipids that are derived from glycerol and sphingosine, a long-chain base that is the backbone of sphingolipids, will produce translucent "spots" or "stains" on fabrics. If the lipid is not a derivative of glycerol or sphingosine, it will not produce a translucent spot on the fabric. The grease-spot test requires that the lipid be in liquid form.

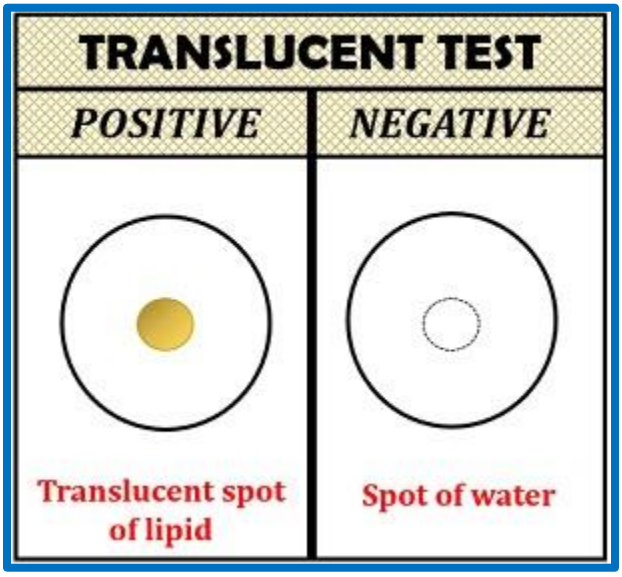
Note: Semi-solid lipids, because of the higher degree of saturation in the fatty acid chains, have melting points higher than room temperature and therefore need to be mildly heated before testing.

Method:

1. Take a filter paper.
2. Add one drop of water at one end and a drop of oil or lipid at the other end.
3. Observe the appearance of a translucent spot on the filter paper.

Observation:

- **Positive result:** Translucent spot will appear on the filter paper.
- **Negative result:** Translucent spot will not appear on the filter paper.



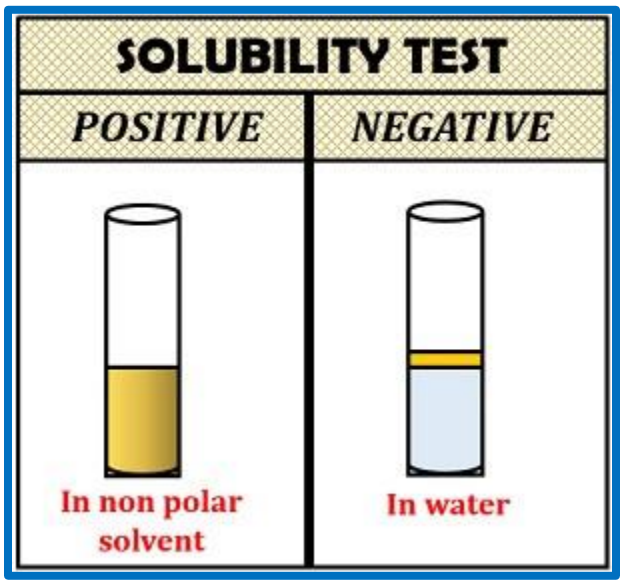
3.2. Solubility of lipids:

Methods:

1. Take the lipid sample in three different test tubes by labelling it as A, B and C.
2. Then, add different solvents like water, ethanol and chloroform in each test tubes A, B and C.
3. Shake the tubes and allow it to stand for 1 minute.
4. Check the solution for whether lipid is soluble or insoluble.

Observation:

- **Positive result**: Lipids are soluble in a non-polar solvent, i.e. chloroform and partially soluble in ethanol which can solubilize upon heating.
- **Negative result**: Lipids are insoluble in a polar solvent, i.e. water



3.3. Emulsification Test

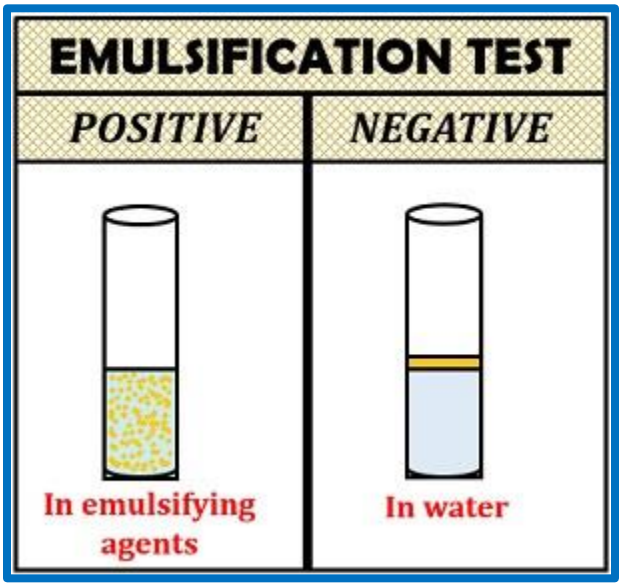
Emulsification test is used to detect the presence of lipids. It is the process that stabilizes the water and oil emulsion by using the emulsifying agents. The lipid or oil in water will appear as the supernatant. The high surface tension of water develops a separate layer by adding emulsifying agents like bile salts, soap etc. Emulsifying agents emulsify the lipid, after which the lipids appear as the tiny droplets suspended in the solution.

Method:

1. Take two test tubes and label it as test tube A and test tube B.
2. Add oil to each of the test tubes.
3. Shake the test tube and allow it to stand for about two minutes.
4. Observe the test tube for the appearance of tiny droplets in the suspension of liquid.

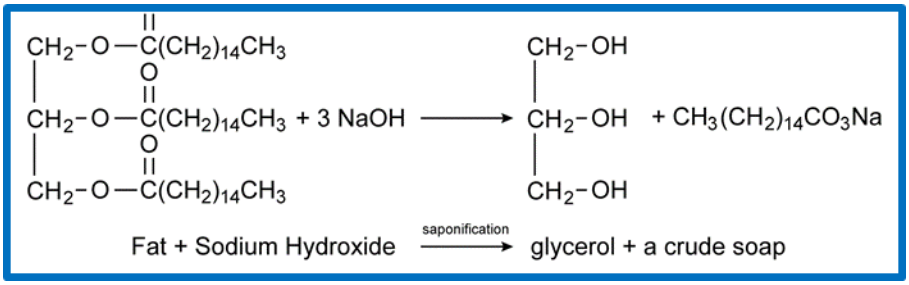
Observation:

- **Positive result:** It gives a permanent or stable emulsion of lipid and water.
- **Negative result:** Oil in water emulsion will form at the top, due to the high surface tension of water.



3.4. Saponification Test:

It is based on the saponification reaction, in which the triglycerides of lipid react with an alkali (NaOH) to produce soap and glycerol in the presence of ethanol. This reaction is also known as alkaline hydrolysis of esters.



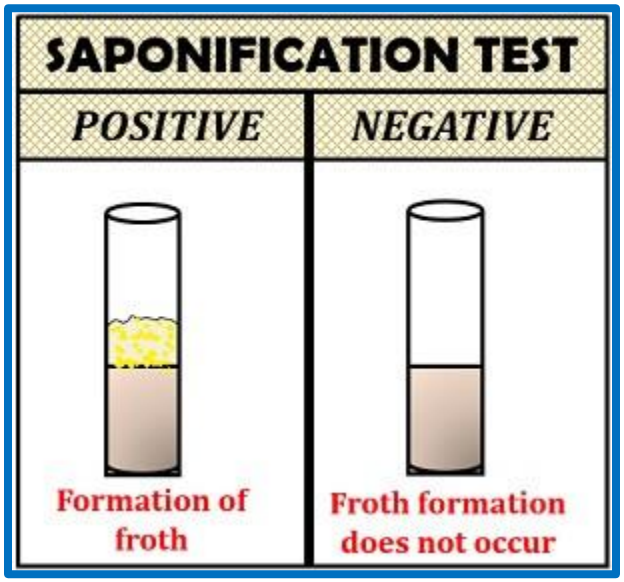
Method:

1. Take a sample of lipid in a test tube.
2. Then, add strong alkali NaOH.
3. Then, boil the solution in a water bath for 5 minutes.
4. At last, add ethanol.

5. Observe the test tube for the appearance of froth.

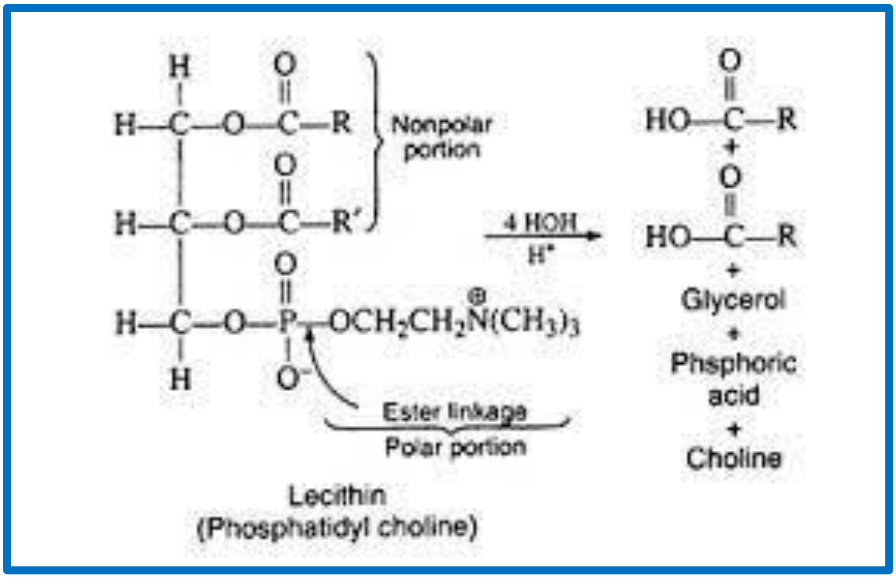
Observation:

- **Positive result:** Froth appears in the test tube.
- **Negative result:** Froth does not appear in the test tube.



3.5. Test for the presence of lipid phosphate (phospholipids):

Hydrolysis of lecithin. Alkali hydrolyses lecithin to glycerol, fatty acids, phosphoric acid and choline. (The fatty acids and phosphoric acid form alkali salts).



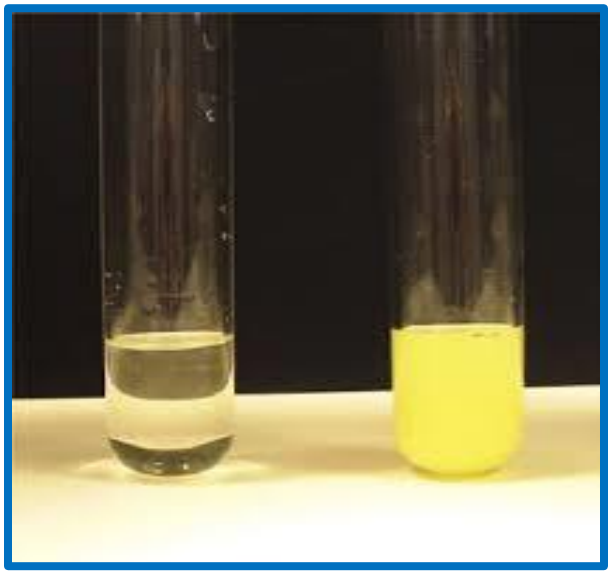
Method:

1. To a 2 ml of compound lipid in a clean test tube, add 5 ml of N NaOH.
2. Heat to boiling.
3. Make acid to litmus with dilute HCL
4. Observe any precipitate. Formation of a precipitate indicates the liberation of free fatty acids. Filter.
5. Test the filtrate for phosphate by boiling with 2 ml ammonium molybdate and 5 drops conc HNO_3.

Observation:

Positive result: A **canary-yellow** precipitate indicates the presence of **phosphate**.

Negative result: The the solution will not change.



3.6. Test for the Presence of cholesterol by the Liebermann-Burchard reaction:

Burchard test was first given by the scientist Liebmann to detect the presence of cholesterol. Cholesterol reacts with the strong concentrated acid, i.e. sulphuric acid and acetic anhydride. Sulphuric acid and acetic anhydride act as a dehydrating and oxidizing agent.

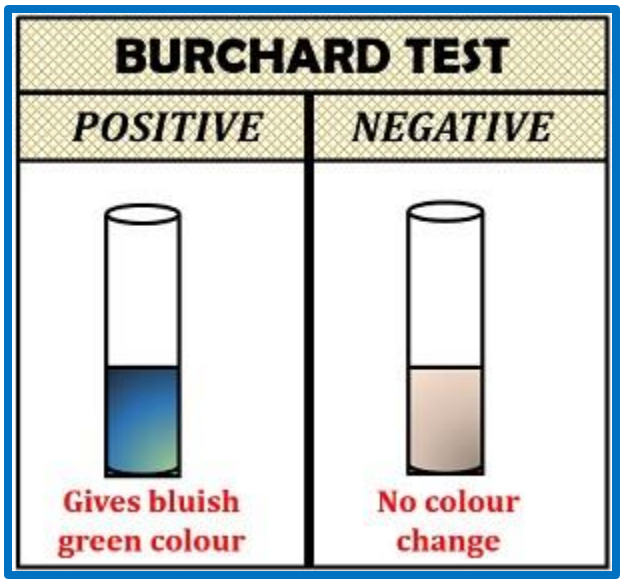
Method:

1. Take crystals of cholesterol in a test tube.
2. Then, add 2 ml of chloroform to dissolve the cholesterol.
3. Add 10 drops of acetic anhydride in a solution and 2-3 drops of concentrated sulphuric acid.

4. Observe the test tube for the appearance of a bluish-green colour.
1. To 1 ml of chloroform solution of lipid, add 5 drops of acetic anhydride.
2. Carefully add 2 ml of conc. H_2SO_4, down the side of the test tube.

Observation:

- **Positive result:** It indicates cholesterol in a sample by giving bluish-green colour to the solution.
- **Negative result:** The colour of the solution will not change.



3.7. Test for the presence of unsaturated fatty acids:

Some fatty acids contain one or more unsaturated linkages and these double bonds have the characteristic property of adding on bromine or iodine.

Unsaturation test is used to detect the unsaturated fatty acids or double bond in a lipid sample. All the neutral fat contains glycerides of fatty acids. Double bonds are found in the structure of unsaturated fatty acids, which becomes saturated by taking up either bromine or iodine. If the lipid contains more unsaturated fatty acids or more double bonds, it will take more iodine.

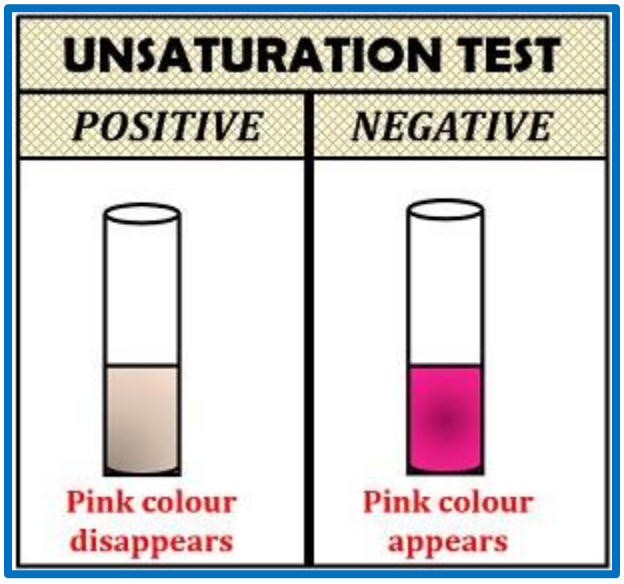
$$H = CH + 2\,Br \rightarrow CHBr - CHBr$$

Method:

1. Take 5 ml of chloroform and 5 ml of Huble's iodine reagent in a beaker, giving pink colour to the solution.
2. Add lipid sample drop by drop and shake vigorously, until pink colour disappears.
3. Count the number of drops added to chloroform and Huble's iodine solution until pink colour disappears. The number of drops determines the taking up of iodine by the unsaturated fatty acid of lipids.

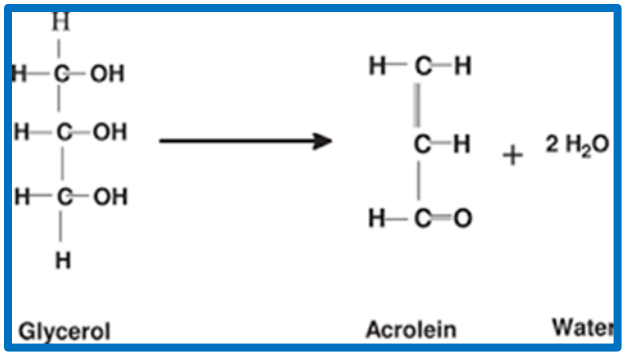
Observation:

- **Positive result:** Pink colour will disappear by the addition of unsaturated fatty acids.
- **Negative result**: Pink colour will not disappear.



3.8. Acrolein test for the presence of glycerol:

Acrolein test is used to detect the presence of glycerol and fat. This test is based on the dehydration reaction, in which the water molecules are removed from the glycerol by adding reagent potassium hydrogen sulphate. The reaction between glycerol and potassium hydrogen sulphate results in acrolein formation, which is characterized physically by the release of the pungent smell.

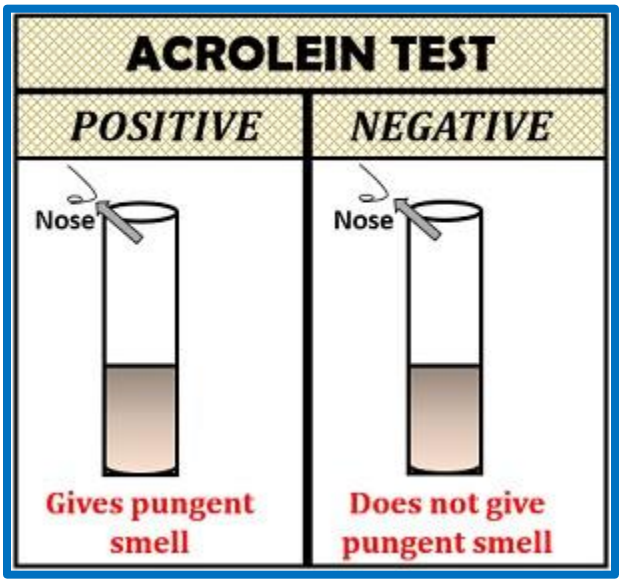


Method:

1. Take 1 ml of the lipid sample in a test tube.
2. Add crystals of potassium hydrogen sulphate.
3. Heat the solution for a few minutes.
4. Smell the test tube for the pungent smell.

Observation:

- **Positive result:** If glycerol present in the sample, it will give a pungent smell.
- **Negative result:** If glycerol is absent in a sample, it will not produce a pungent smell.



3.9. Dichromate Test:

Dichromate test is also used to detect the presence of glycerol. It is based on the principle of an oxidation reaction. In this, glycerol and dichromate ions react to give a brown colour to the solution. Then, the chromic ions oxidize the glycerol and reduce into chromous ions by giving a blue colour to the solution in the presence of nitric acid.

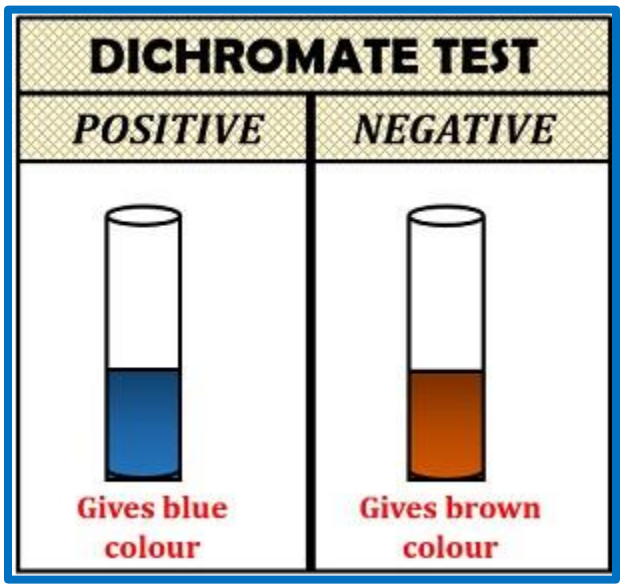
Method:

1. Take 2-3 ml of a sample in a test tube.
2. Then, add a few drops of 5% potassium dichromate solution.
3. After that, add 5 ml of concentrated nitric acid.
4. Observe the test tube for the appearance of a blue colour.

Observation:

- **Positive result:** If the colour of the solution changes from brown to blue, it indicates glycerol.

- **Negative result:** In this, the brown colour will not change into blue.



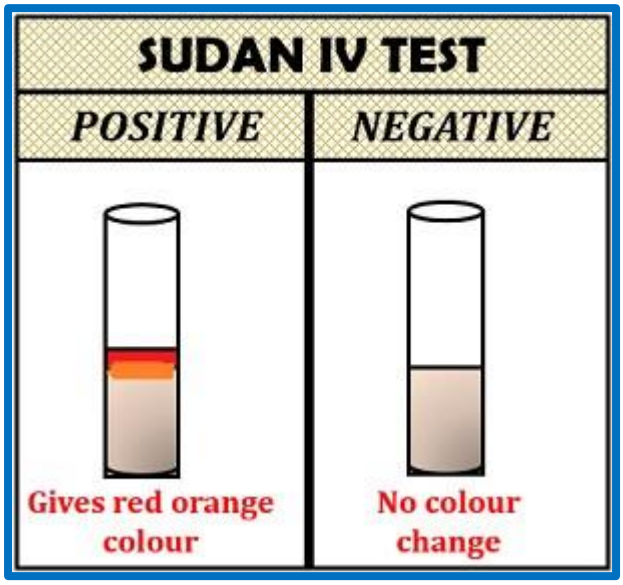
3.10. Sudan IV Test:

Sudan IV test is used to detect the presence of lipid in a solution. This test is based upon the principle of binding and solubility of lipid in non-polar compounds. As Sudan IV is a non-polar stain, the lipid will bind with it and retain the stain's colour by giving a red-orange colour. Sudan IV does not stain or bind to the polar compounds.

Method:

1. Take 1 ml of the lipid sample in a test tube.
2. Then add 1-2 drops of Sudan IV to the solution.
3. Observe the tubes for the appearance of red-orange colour in the solution**.**

Observation:



- **Positive result:** Gives red-orange colour to the solution.
- **Negative result:** The solution of the colour will remain unchanged.

Practical No. 4

Amino Acids and Proteins

Introduction:

Proteins are organic substances of high molecular weight consisting largely (or entirely) of a number of amino acids linked peptide linkage there are twenty different amino acids present in protein each with distinct chemical and structural properties.

Some fundamental tests for Amino acids and Proteins

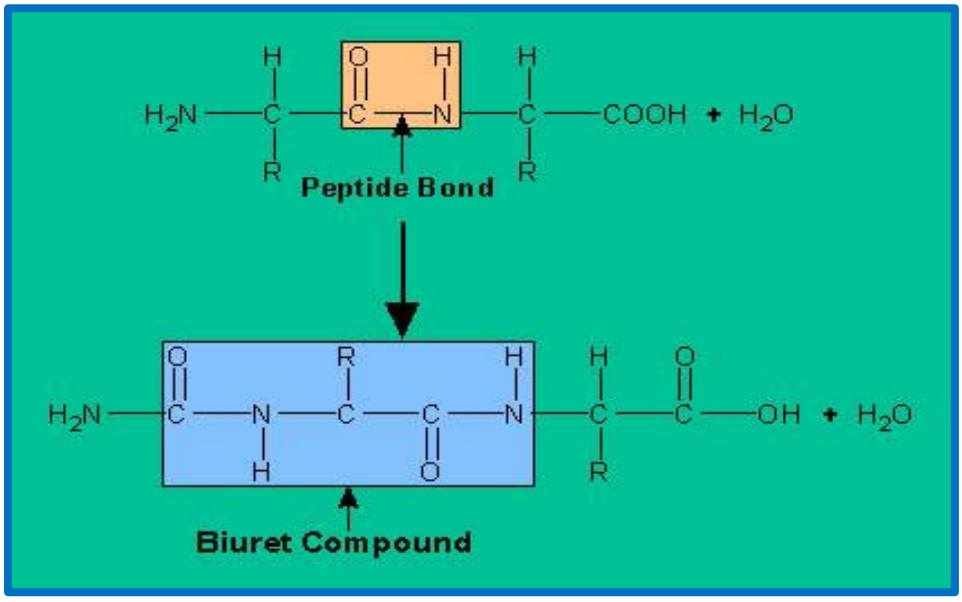
Colour Reactions of Proteins:

These reactions are due to a reaction between someone and more of the constituent radicals or groups of complex protein molecule and the chemical reagent or reagents used in any given test.

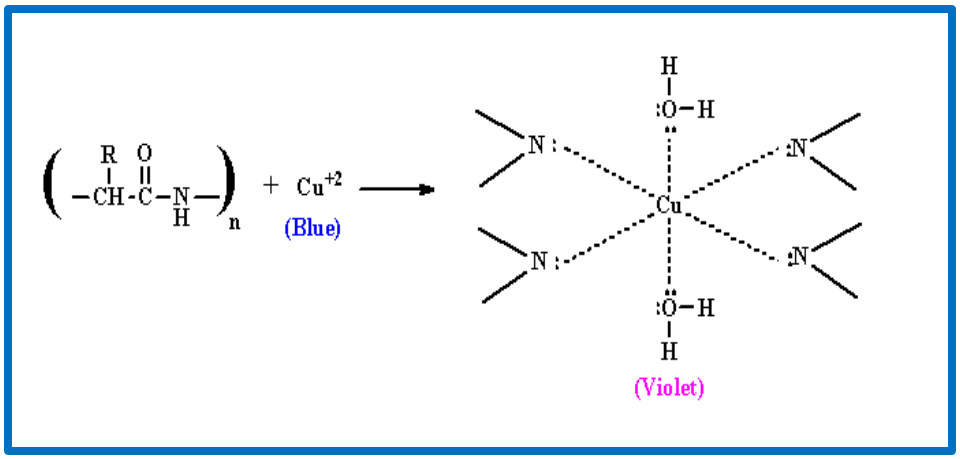
Not all proteins contain the same amino acids and for this reason the various colour tests will yield reactions varying in intensity of colour according to the nature and amount of the groups contained in the particular protein under test.

4.1. Biuret Test: Test for two or more peptide linkages:

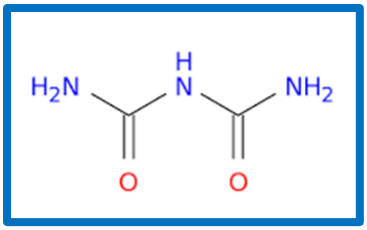
Compounds that contain at least **two peptide bonds** yield **violet colour** with **cupric ions** in alkaline media. All proteins and peptides that have two or more than two peptide bonds give a positive Biuret test. The colour formation is due to the production of a **complex** between Cu and nitrogen atoms of peptide chains.



The complex is believed to have the structure shown below:



The name of the reaction is derived from biuret that has the following structure.



Objectives:

- To detect the protein in the given solution.
- To demonstrate the presence of the peptide bond.

Biuret reagents:

- Copper sulfate (CuSO40
- Sodium hydroxide (NaOH)
- Sodium potassium tartarate (commonly known as Rochelle salt)

Preparation Biuret reagent

- Biuret reagent is prepared by adding NaOH in CuSO4 solution, making it alkaline.

To prepare 1000ml of Biuret reagent:

- Take 1.5 gram of pentavalent copper sulphate (CuSO4) and 6 gram of Sodium potassium tartarate.
- Dissolve them in 500 ml of distilled water.
- Sodium potassium tartarate is a chelating agent and it stabilize the copper ion.
- Take 375 ml of 2 molar Sodium hydroxide.

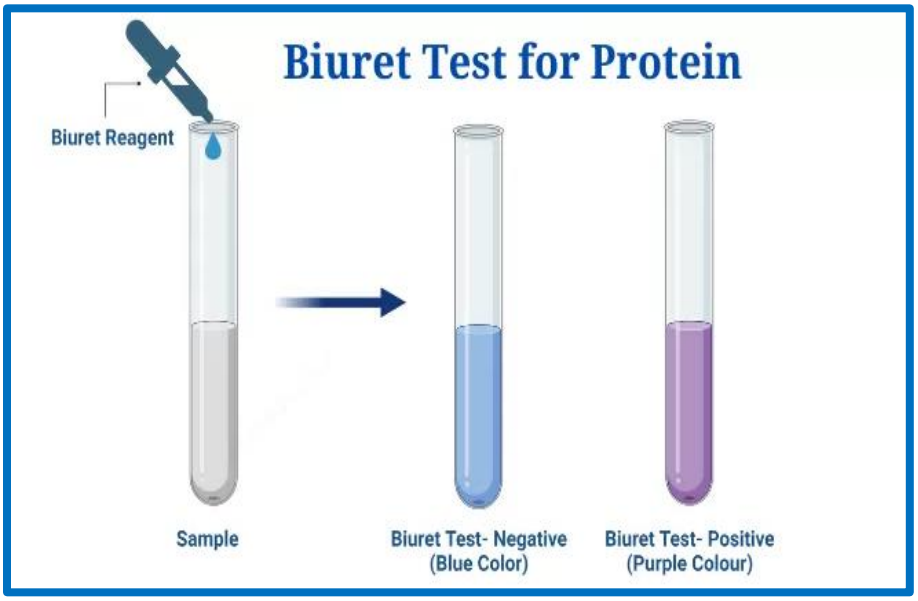
- Mix both the solution in volumetric flask and make it final volume to 1000 ml by adding distilled water.

Method:

Into two test tubes add the following.

1. To one add 2 ml protein solution.
2. To the other add 2 ml amino acid solution.
3. To both tube and few drops of biuret reagent.
4. Most proteins give violet colour some give distinct pink colour.
5. Record your observation and comment.

Observation:



- **Positive result:** Gives **purple or violet** colour to the solution.
- **Negative result:** The solution of the colour will remain blue

4.2. Ninhydrin Test:

Ninhydrin test is a chemical test performed to detect the presence of ammonia, primary/secondary amines, or **amino acids**. This test involves the addition of Ninhydrin reagent to the test sample that results in the formation of deep blue color, often termed as Ruhemann's purple, in the presence of an amino group.

Objectives:

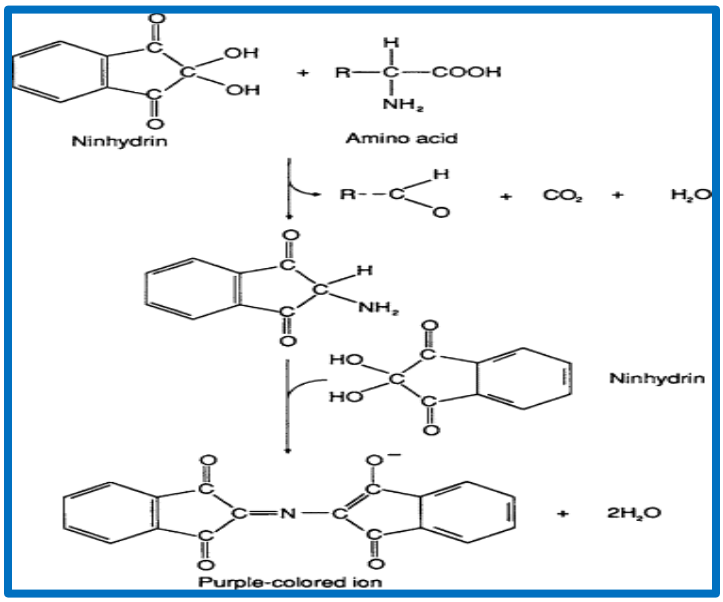
- To detect the presence of amines and amino groups in the test solution.
- To quantify the amino acids present in the sample.
- To distinguish carbohydrates from amino acids.

Principle:

This test is a general test and thus given by all amino acids. This test is due to a reaction between an amino group of free amino acid and ninhydrin. Ninhydrin is a powerful oxidizing agent and its presence, amino acid undergo oxidative deamination liberating ammonia, CO_2, a corresponding aldehyde and reduced form of Ninhydrin (hydrindantin). The NH_3 formed from an amino group reacts with another molecule of Ninhydrin and is reduced product (hydrindatin) to give a blue substance diketohydrin (

Ruhemanns complex). However, in case of imino acid like proline and hydroxyproline, a different product having a bright yellow color is formed. Asparagine, which has a free amide group, reacts to give a brown colored product.

Reaction:



Requirements:

1. Reagents:

- **Ninhydrin reagent:** Dissolve 0.35g of Ninhydrin in 100 ml ethanol (iso-propanol or 1:1 mixture of acetone/butanol may be used instead of ethanol).
- Diluent solvent (for the quantitative test): Mix equal volumes of water and n-propanol.
- Standard solution (1% protein solution)
- Sample solution

2. Materials required:

- Test tubes
- Test tube stand
- Pipette

3. Equipment:

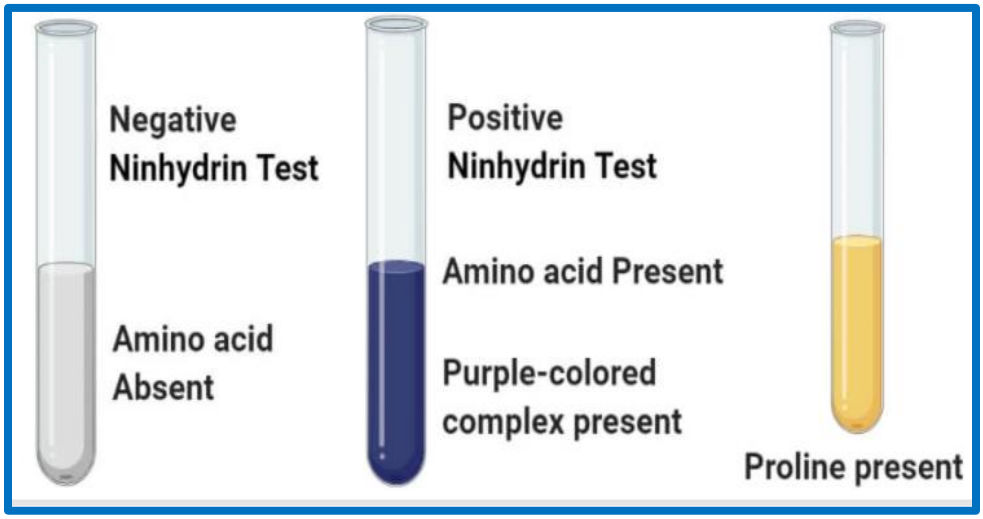
- Water bath
- Spectrophotometer

Method:

1. Prepare 1% amino acid solution in distilled water.
2. Take 1 ml test solution in dry test tube and 1 ml distilled water in another tube as a control.
3. Pour few drops of 2% Ninhydrin in both the test tubes
4. Keep the test tubes in water bath for 5 minutes.
5. Look for the development of blue or violet color.

Note: But in the case of proline and hydroxyproline, yellow color will develop instead of blue color. Similarly, asparagine will give brown color.

Observation:



- The presence of a purple-colored complex in the tube represents a positive result and indicates the presence of amino acid in the sample.
- The absence of the complex in the tube represents a negative result and indicates the lack of amino acids in the sample.
- From the graph, we can determine the concentration of unknown samples.

Uses of Ninhydrin Test

- Ninhydrin test is used to detect the presence of amino acids in unknown samples.
- This test is also used in solid-phase peptide synthesis to monitor the protection for amino acid analysis of proteins.
- As the Ninhydrin test is quite sensitive, it is commonly used to detect fingerprints. It is possible as the terminal amines of lysine residues in peptides and proteins shed off in fingerprints react with Ninhydrin.

Limitations of Ninhydrin Test

- Ninhydrin reacts not only reacts with α-amino groups but also with nitrogen in ammonia and other free amines.
- The Ninhydrin test is not effective to detect high molecular weight proteins as the steric hindrance limits the Ninhydrin from reaching the α-amino groups.

4.3. Pauly's test: to detect aromatic amino acid tyrosine or histidine

Pauly's test is a biochemical test for the detection of tyrosine and histidine where the reagent couples with amines, phenols, and imidazole groups. The test was discovered by and is named after the German Chemist Hermann Pauly. Pauly's test is a specific test for **proteins** containing tyrosine and histidine as the reagent undergoes diazotization with aromatic groups. The reaction is performed under cold conditions as the diazonium compound can only form at cold temperatures. The test is a specific test for proteins, which is performed for further differentiation between amino acids and proteins.

Objectives:

- To detect the presence of tyrosine and histidine-containing proteins.
- To differentiate between histidine and tyrosine from other amino acids.

Principle:

Diazotized sulphanilic acid couples with amines, phenols and imidazole to form highly colored azo compounds. This coupling reaction must be done in cold condition since diazonium compound is formed in cold. Amino acids tyrosine or histidine coupled with diazonium salt in alkaline condition to form red coloured azo dye.

Reaction:

$$\text{Sulphanilic acid} + HNO_2 + HCl \rightarrow \text{Diazonium salt} + 2H_2O$$

$$\text{Diazonium salt} + \text{Tyrosine} \rightarrow \text{Azo dye (red color)}$$

Requirements:

1. Reagent:

- 1% Sulphanilic acid (chilled).
- 10% HCl.
- 5% Sodium nitrite (chilled).
- 10% Sodium carbonate.
- Chilled sample (1% tyrosine, 1% histidine).

2. Materials required:

- Ice bath.
- Vortex.
- Test tubes.
- Test tube stand.
- Pipettes.

3. Equipment:

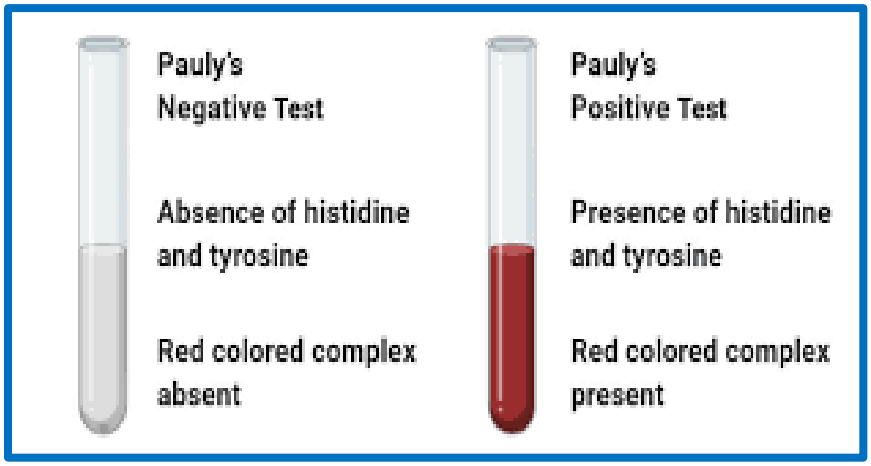
- Water bath.
- Spectrophotometer.

Note: Diazonium compound=Sulfanilic acid + sodium nitrite + sodium carbonate

Method:

1. Take 2ml test solution in dry test tube.
2. Similarly, take 2ml distilled water in another test tube as control.
3. Add 1ml of sulphanilic acid, mix well and keep in ice bath.
4. Now add 1ml sodium nitrite solution to all test tubes.
5. Leave in ice bath for 3 minutes.
6. Make the solution alkaline by adding 5ml of sodium carbonate.
7. Look for the development of red colored complex.

Observation:



- **Positive result:** A positive result is demonstrated by the appearance of a red-colored complex, indicating the presence of histidine and tyrosine in the solution.
- **Negative result:** A negative result is demonstrated by the absence of a red-colored solution, indicating the absence of histidine and tyrosine in the sample.

Uses of Pauly's Test:

- Pauly's test is used to detect the presence of tyrosine and histidine-containing proteins.
- The test also allows the differentiation of histidine and tyrosine from other amino acids.

Limitations of Pauly's Test:

- The formation of diazonium salt occurs at cold temperatures; thus, the test should be performed in the presence of ice.
- The test doesn't allow the differentiation between histidine and tyrosine. Millon's Test can be performed as histidine gives a negative result in Millon's test.

4.4. Hopkin's-Cole test: to detect amino acid tryptophan present in protein:

Hopkin's Cole test is a specific test used for the detection of **indole** ring and thus, tryptophan in proteins. The test is also termed as 'glyoxylic acid test' as the reagent contains glyoxylic acid.

Objectives:

- To detect the presence of indole ring containing amino acid in proteins.
- To detect the presence of tryptophan-containing proteins.

Principle:

The indole group of tryptophan reacts with glyoxylic acid in the presence of conc. H_2SO_4 to give a purple colored complex. Glyoxylic acid is prepared by reducing Oxalic acid with magnesium powder or sodium amalgam. Glacial acetic acid which has been exposed to the sunlight also contains glyoxylic acid and can thus be used for this test.

Reaction:

Tryptophan + Glyoxylic acid → Condensation product + H_2O → Violet-coloured compound + H_2O

Requirements:

Reagent:

1. Hopkin's Cole reagent:

- Glyoxylic acid- It can be prepared by exposing glacial acetic acid to sunlight for a few days.
- Concentrated H_2SO_4.

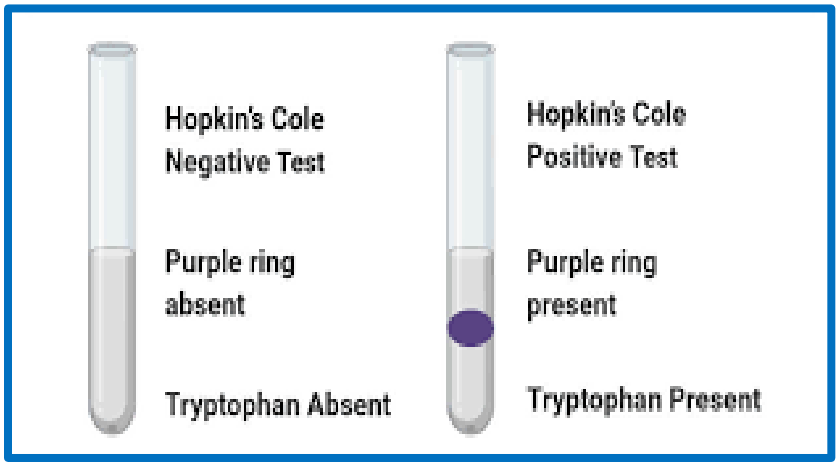
2. Material Required:

- Test tubes.
- Test tube stand.
- Pipettes.

Method:

1. In a test tube, 2 ml of light-exposed glacial acetic acid and 2 ml of the sample liquid are taken.
2. To this, concentrated H_2SO_4 is added along the sides of the test tube held at a slanting position. Two distinct layers of liquid are to be formed without mixing. (*Note: Mouth of the tube must point away from the face, as we should be careful while adding the sulfuric acid.*)
3. The test tube should be observed for the formation of a colored ring at the interface of two layers.

Observation:



- **Positive result:** A positive result is represented by the formation of a purple-colored ring at the junction of two layers. This indicates the presence of tryptophan-containing proteins.
- **Negative result:** A negative result is represented by the absence of a purple-colored ring in the test tube. This indicates the absence of tryptophan-containing proteins.

Uses of Hopkin's Cole Test

- The test is used for the detection of proteins and amino acids in a sample.
- The test is a simple and easy-to-perform test that helps to identify tryptophan from other amino acids.

Limitations

- Compounds like nitrites, chlorates, nitrates, and excess chlorides prevent the formation of the condensation product.

4.5. Ehrlich Test:

A biochemical test used for the detection of amino acid, tryptophan, in a **protein** sample. Ehrlich test is also termed the p-dimethylamino benzaldehyde test, named after the Ehrlich reagent. The test is named after the Nobel Prize winner Paul Ehrlich, who identified this test during the distinction of typhoid from simple diarrhea. It is also called a specific amino acid test as it detects a certain amino acid, tryptophan. This test has been used in laboratories for medical tests, some for drug testing while others for diagnosis of various diseases. Diagnosis of liver diseases, carcinoid syndrome, hemolytic processes, and occlusion of the common bile duct can also be performed with the Ehrich test. One of the common forms of the Ehrlich test is the spot test used for the detection of psychoactive compounds like tryptamines and ergoloids (like, LSD). A positive Ehrlich test is obtained for natural opium as it contains tryptophan. The Ehrlich reagent is also used for the identification of indoles and urobilinogen (found in urine).

Objectives of Ehrlich Test

- To detect the presence of tryptophan in a given sample.
- To distinguish between typhoid and diarrhea.
- To identify the presence of urobilinogen in a urine sample.

Principle:

Ehrlich reagent consists of p-dimethylamino benzaldehyde. The reaction occurring in the test is based on the principle that under acidic conditions, the Ehrlich's reagent undergoes electrophilic substitution. The substitution occurs at the indole or the benzyl pyrrole ring of tryptophan to yield a blue-violet condensation product. The condensation product formed after the reaction is further enhanced by the addition of $NaNO_2$.

Reaction:

Tryptophan + Ehrlich reagent (p-dimethylamino benzaldehyde) → Blue-violet condensation product

Requirements:

Reagents:

- **Ehrlich reagent:** Ehrlich reagent is prepared by the addition of about 0.5 to 2.0 grams of p-dimethylamino benzaldehyde in 50 ml of 95% ethanol. To this, 50 ml 10% H_2SO_4 is added.

- Protein sample.
- Concentration HCl.
- $NaNO_2$.

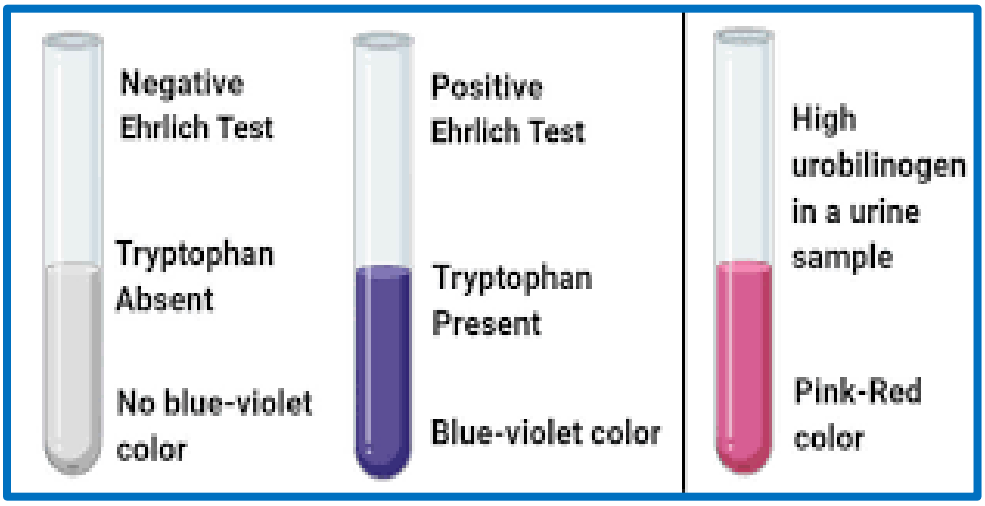
Materials required:

- Test tubes.
- Test tube stand.
- Pipettes.

Method:

1. About 3-4 ml of diluted protein solution or urine sample is added to a test tube.
2. The liquid is then boiled with 1 ml of concentrated HCl.
3. A few drops of the Ehrlich reagent (p-dimethylamino benzaldehyde in H_2SO_4) are added to the test tube.
4. The test tube is then shaken to mix the contents together properly.
5. The test tube is observed for the color change. If a blue-violet color is observed, a few drops of $NaNO_2$ solution are added to the test tube to obtain a blue color.

Observation:



- **Positive result:** A positive result in the Ehrlich test is indicated by the appearance of red to purple or blue-violet color. The color then changes to blue with the addition of $NaNO_2$. This indicates that the sample contains tryptophan.
- **Negative result:** A negative result in the Ehrlich test is indicated by the absence of blue-violet color on the addition of the Ehrlich reagent. This indicates that the sample doesn't contain any tryptophan.

Uses of Ehrlich Test:

- This test is an aldehyde test used for the detection of tryptophan in a protein sample.
- The test is used for the diagnosis of diseases like typhoid and other disorders of the hemolytic process of the obstruction of the common bile duct.
- The test can also be used for the detection of psychoactive compounds and drugs like tryptamines and ergoloids.
- Ehrlich test detects the presence of indoles and urobilinogen. The presence of urobilinogen in high concentration in urine sample helps in the diagnosis of hepatic jaundice and hepatitis.

Limitations of Ehrlich Test:

- The false-negative reaction might occur in the presence of urinary tract infection as nitrites oxidize urobilinogen to urobilin.
- A false negative result during antibiotic therapy as some gut bacteria that produce urobilinogen might get destroyed.
- This test is a specific test for aldehyde or tryptophan, and it doesn't detect other amino acids or proteins.

4.6. Acree-Rosenheim Test:

A biochemical test used for the detection of tryptophan molecules in a **protein** sample. Acree-Rosenheim test is one of the essential tests for the confirmation of formaldehyde in the milk as milk vendors use formaldehyde as a preservative. This test thus helps to understand the condition of the milk sample so as to determine if the surplus milk is not spoiled. A more simplified method of determining the presence of formaldehyde in milk is also available. For the simpler method, the milk can just be heated with a few drops of concentrated HCl to test the detection of formaldehyde. It is also called the aldehyde test as the test is based on the presence of aldehyde molecules in the sample. The test is named after two biochemists; Solomon Farley Acree and Sigmund Otto Rosenheim.

Objectives:

- To detect the presence of tryptophan in a protein sample.
- To detect the presence of formaldehyde in a sample.

Principle:

The reagent for the Acree-Rosenheim test is formaldehyde. The test is used to detect tryptophan in a protein solution. The formaldehyde added to the sample results in a condensation reaction with two tryptophan molecules. The reaction is carried out in acidic conditions by the addition of sulfuric acid (H_2SO_4), which separates two distinct layers in the test tube. The condensation product of the reaction between tryptophan and formaldehyde is violet colored, which indicates the presence of tryptophan molecules.

Reaction:

Tryptophan + Formaldehyde + Tryptophan → Coloured condensation product + H_2O

Requirements:

1. Reagents:

- Protein sample (1% tryptophan or other amino acids).
- Dilute Formaldehyde (CH_2O).
- Sulfuric acid (H_2SO_4).

2. Materials Required

- Test tubes
- Test tube stand
- Pipettes

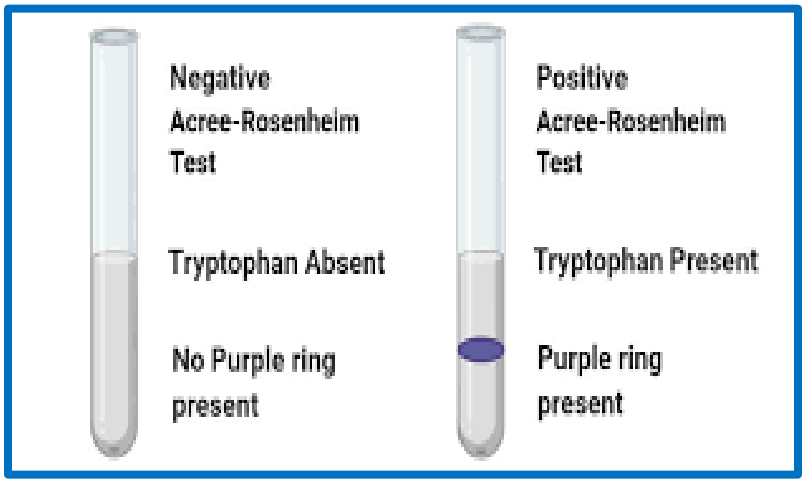
Method:

1. About 2-3ml of protein solution is added in a test tube. If egg albumin is to be used, 5-10% solution is to be prepared.
2. To the test tube, a few drops of dilute formaldehyde are added.
3. Thereafter, few drops of concentrated sulfuric acid are added from the side of the test tubes.
4. The shaking of the test tube should be avoided to prevent the mixing of the two layers.
5. The formation of the violet-colored ring at the junction of two layers is observed.

For milk samples with preservatives:

1. In the case of milk samples, a few drops of concentrated acid (H_2SO_4) are added to the milk sample.
2. The mixture is then heated to obtain a purple-colored solution.

Observation:



- **Positive result:** Positive result of the Acree-Rosenheim test is represented by the appearance of a purple ring at the junction of two layers, indicating the presence of tryptophan.
- **Negative result:** Negative result of the Acree-Rosenheim test is represented by the absence of the purple ring at the junction of two layers.

Uses of Acree-Rosenheim test:

- The test is used to detect the presence of distinct proteins and amino acids in a sample.
- This test is the standard test for the detection of tryptophan in a sample.
- A modified version of this test is also used for the detection of formaldehyde in a milk sample.

Limitations of Acree-Rosenheim test:

- The test can only be used for the detection of tryptophan, so a negative result doesn't rule out the presence of other amino acids.
- Gelatin might give a negative Acree-Rosenheim test as it is deficient in tryptophan.
- The shaking of the test tube might result in the mixing of the two layers, which might result in false-negative results.

4.7. Millon's test:

Millon's test is an analytical test used for the detection of the **amino acid** tyrosine, which is the only **amino acid** containing the phenol group. Millon's test is a specific test for tyrosine, but it is not a specific test for **protein** as it also detects the phenolic group present in other compounds as well.

Objectives:

- To detect the presence of tyrosine-containing proteins in a given sample.
- To detect the presence of phenol-containing compounds.
- To differentiate tyrosine from other amino acids.

Principle of Millon's test:

Compounds containing hydroxybenzene radical react with Millon's reagent to form red complexes. The only amino acid having hydroxybenzene ring is tyrosine. Thus, this test is specific for the amino acid tyrosine and the protein containing this amino acid. Tyrosine when reacted with acidified mercuric sulphate solution gives yellow precipitate of mercury-amino acid complex. On addition of sodioum nitrate solution and heating, the yellow complex of mercury-amino acid complex converts to mercury phenolate which is in red color.

Requirements:

1. Reagent:

- **Millon's reagent:** Millon's reagent consists of mercuric nitrate and mercurous nitrate dissolved in nitric acid and distilled water.

Preparation of Millon's reagent: Dissolve 160 grams of mercuric nitrate and 160 grams of mercurous nitrate in 400 ml concentrate nitric acid solution. The reagent is then made to 1000 ml by the addition of 600ml distilled water. The formula can be adjusted to suit the performance parameters.

- Sample (1% tyrosine)

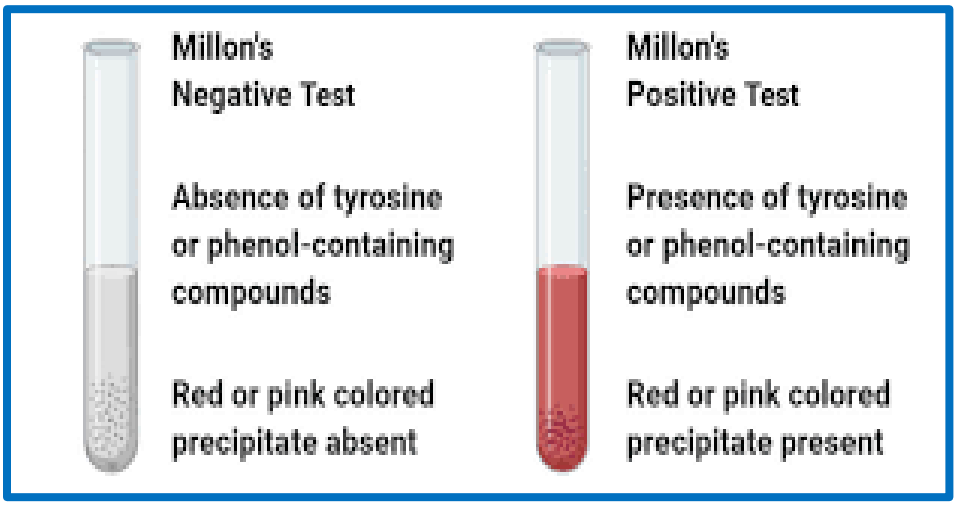
2. Materials Required

- Test tubes.
- Test tube stand.
- Pipettes.
- Water bath.

Method:

1. Take 1ml test solution in dry test tube.
2. Similarly, take 1ml distilled water in another test tube as control.
3. Add 1ml of Millon's reagent and mix well.
4. Boil gently for 1 minute.
5. Cool under tap water.
6. Now add 5 drops of 1 % sodium nitrite.
7. Heat the solution slightly.
8. Look for the development of brick red precipitate.

Observation:



- **Positive result:** A positive result in the Millon's test is demonstrated by the formation of a red or pink colored precipitate. This indicates the presence of tyrosine or tyrosine containing protein.
- **Negative result:** A negative result in the Millon's test is demonstrated by the absence of colored precipitate in the test tube. This indicates the absence of tyrosine or tyrosine-containing protein.

Uses of Millon's Test:

- Millon's test is used for the detection of tyrosine-containing proteins in a given sample.
- The test also helps in the differentiation of tyrosine from other amino acids.
- The test is useful in the detection of casein protein and the protein found in raw meat.

Limitations of Millon's Test:

- Compounds like salicylic acid and phenolic compounds give a positive result to this test; thus, any other phenol compounds that might be present in the test tube should be avoided. Tests like the Biuret test and Ninhydrin test should be performed for confirmation.
- The presence of chlorine in the solution might interfere with the reaction; thus, the test cannot be performed on a sample containing chlorides.
- The formation of a white or yellow precipitate might be observed immediately after the addition of Millon's reagent due to the denaturation of proteins by mercuric ions.

4.8. Lead sulfide test:

A biochemical test for the detection of **amino acids** like cysteine and cystine. The test is a specific test for the detection of amino acids containing sulfur, S-S group in cysteine, and S-H group in cystine. The test is also called a lead acetate test as the reagent for the test is lead acetate. Even though the test is specific for the detection of sulfur-containing amino acids, methionine doesn't give a positive result in this test.

Objectives:

- To detect the presence of sulfur-containing amino acids in a sample.

- To detect **protein**-containing cysteine and cystine in a given sample.
- To distinguish between sulfur-containing and non-sulfur containing amino acids.

Principle of lead sulfide test:

When cysteine is heated with strong alkali like NaOH, some of the sulphur is converted to sodium sulphide (Na2S) which can be detected by precipitation as lead sulphide (PbS) from alkaline solution.

Reactions:

$$\text{Cysteine} + 2\text{NaOH} \rightarrow \text{Serine} + \text{Na2S} + \text{H2O}$$

$$\text{Pb(CH3COO)2} + \text{NaOH} \rightarrow \text{Pb(OH)2} + 2\text{CH3COONa}$$

$$\text{Pb(OH)2} + 2\text{NaOH} \rightarrow \text{Pb(ONa)2} + 2\text{H2O}$$

$$\text{Na2S} + \text{Pb(ONa)2} + \text{H2O} \rightarrow \text{PbS (black precipitate)} + 4\text{NaOH}$$

Requirements:

1. Reagent:

- 2% lead acetate solution in water.
- 40% NaOH.
- Sample.

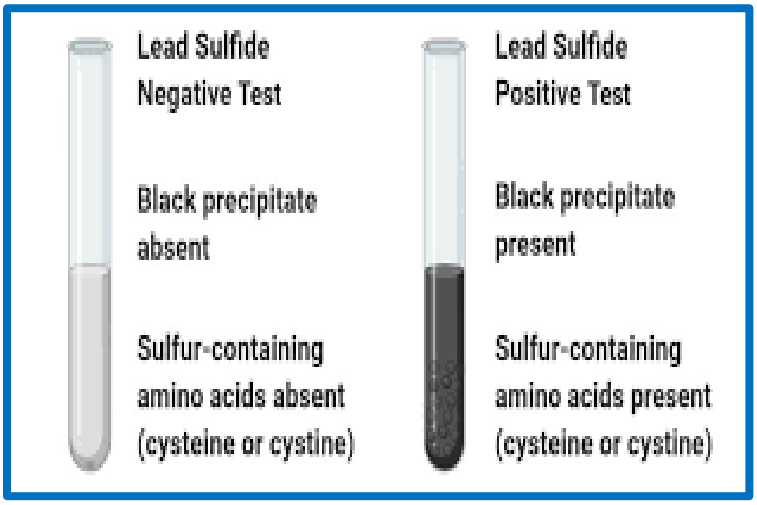
2. Material Required:

- Test tubes.
- Test tube stand.
- Pipettes.

Method:

1. Take 1ml test solution in dry test tube.
2. Similarly, take 1 ml distilled water in another test tube as control.
3. Add 2ml of 40 % NaOH and mix well.
4. Now add 1ml Foli's reagent (lead acetate) t to all test tubes.
5. Heat over the flame of Business burner.
6. Look for the development of black precipitate.

Observation:



- **Positive test:** A positive test in the Lead sulfide test is represented by the formation of black precipitate at the bottom of the test tube. This indicates the presence of cysteine or cystine in the solution.
- **Negative test:** A negative result in the Lead sulfide test is represented by the absence of black residue in the test tube. This indicates the absence of cysteine or cystine.

Uses of Lead Sulfide Test:

- The test is used to detect sulfur-containing amino acids like cysteine and cystine.
- It helps to distinguish between different groups of amino acids.
- The detection of cystine in urine is a pathological symptom of diseases like cystine stones in the kidneys and bladder.

Limitations of Lead Sulfide Test:

- Methionine doesn't give a positive result in this test as the sulfur in the thioester bond in methionine is not released by the treatment with NaOH.
- The addition of excess lead acetate to the solution might result in white-colored precipitation.

4.9. Sullivan and McCarthy's test:

The test is a specific test for methionine which has a high degree of specificity towards methionine as it gives negative results with all other amino acids. The test, however, gives a positive result with glycylmethionine. It is a desirable method for methionine detection as it is a rapid and simple method for the assay of methionine even if fermented extract. Sullivan and

McCarthy's test is a color reaction of proteins which indicates the presence of a certain molecule by the development of a particular color with a particular reagent.

Objectives of Sullivan and McCarthy's Test:

- To detect the presence of the amino acid methionine or methionine-containing proteins.
- To differentiate methionine from other sulfur-containing amino acids like cysteine and cystine.

Principle:

The Sullivan and McCarthy's test is based on the reaction between nitroprusside and alkaline solution of methionine under acidification. The test was developed when it was discovered that tryptophan and histidine both produce a red color with nitroprusside under similar conditions. Thus, acid is added to the sample solution in order to destroy any tryptophan present by the process of acid hydrolysis. Similarly, it has been stated that the addition of glycine eliminates the detection of histidine. The red color is obtained after the addition of sodium nitroprusside to an alkaline solution of methionine followed by the acidification of the reaction.

Requirements:

1. Reagent:

- 10% sodium nitroprusside.
- 5N Sodium hydroxide.
- 6N HCl.
- 2% glycine.
- Sample solution.

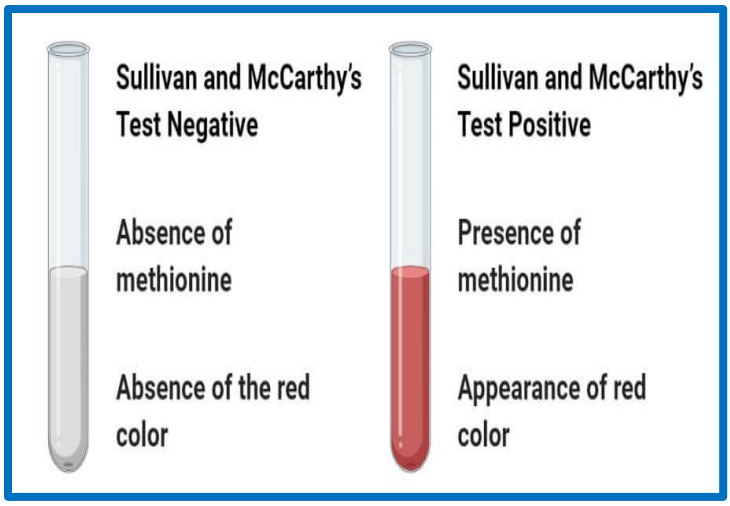
2. Materials required:

- Water bath.
- Ice bath.
- Vortex.
- Test tubes.
- Test tube stand.
- Pipettes.

Methods:

1. A few drops of sodium hydroxide are added to 1 ml of test solution in a test tube.
2. To this, a few drops of 2% glycine and 10% sodium nitroprusside solution is added.
3. The solution is then mixed together by placing it in a vortex for 2 minutes.
4. The test tube is then placed in a water bath at 40°C for 15 minutes.
5. The tube is then cooled in ice-cold water for 5 minutes followed by the addition of 0.5ml of 6N HCl.
6. The contents are then mixed together in a vortex, and the test tube is allowed to stand at room temperature for 15 minutes. The observation is then made.

Observation:



- **Positive result:** The positive result is represented by the appearance of the red color. This indicates the presence of methionine.
- **Negative result:** The negative result is represented by the absence of the red color. This indicates the absence of methionine.

Uses of Sullivan and McCarthy's Test:

- Sullivan and McCarthy's test is used for the detection of methionine either in a free form or in proteins.
- The test can be modified for quantification where the test sample can be matched against the standard methionine solution.

Limitations of Sullivan and McCarthy's Test:

- The test might give a positive result with commercial leucine as it might contain some traces of methionine.
- The test might not give reproducible results with standard solutions of methionine.

4.10. Sakaguchi test:

Is a biochemical test consisting of colorimetric reaction for the detection and quantification of guanidinium groups, used as a qualitative test for arginine that is either free or in protein. The test was discovered by and named after the Japanese Food Scientist Shoyo Sakaguchi in 1925. Sakaguchi test is an example of color reactions or test that is performed for the detection of **amino acids** or **proteins**. The test is a specific test for arginine where the guanidinium group of arginine reacts with 1-naphthol or α-naphthol to produce a colored product. It is a qualitative test, and the quantification of arginine is hindered in this test due to the slow rate of color development and destruction of some guanidinium groups by the reagent.

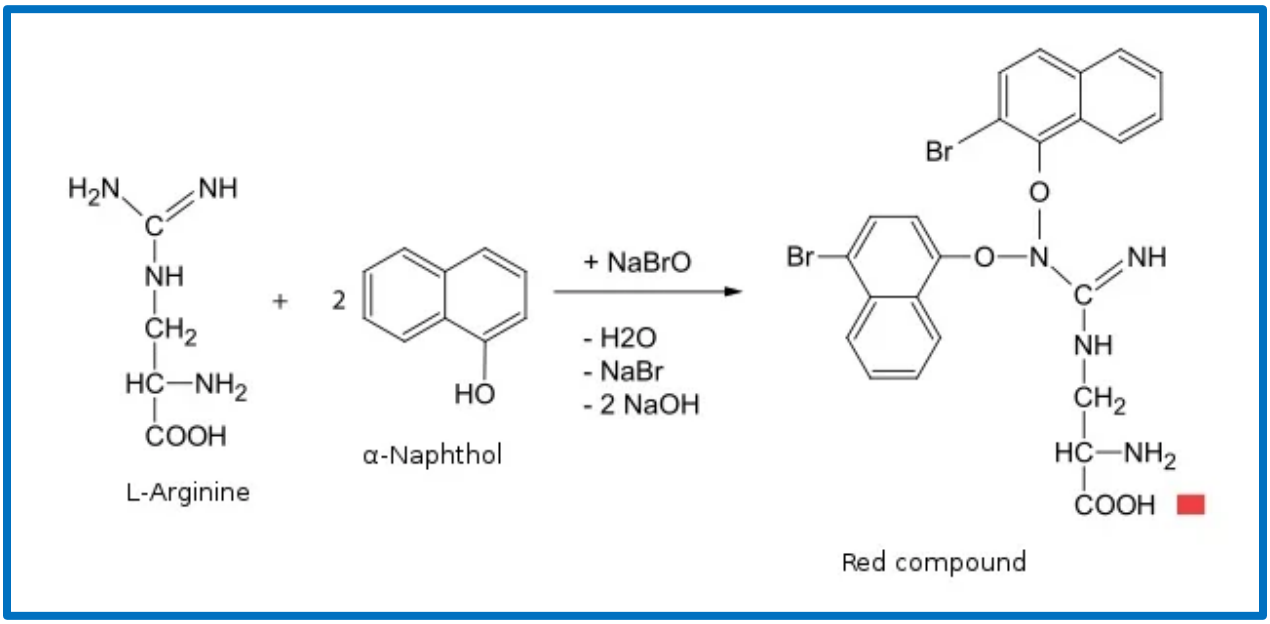
Objective:

- To detect the presence of arginine in either free form or in proteins.

Principle:

Sakaguchi test is based on the principle of reaction between 1-naphthol and the guanidinium groups in arginine, in the presence of an oxidizing agent. The exact mechanism of the reaction is not yet known; however, the reaction results in the formation of a red-colored complex due to the formation of an indole-like structure. The Sakaguchi reagent consists of sodium hypobromite and 1-naphthol. The sodium hypobromite acts as an oxidizing agent that facilitates the hydrogen bonding between two arginine molecules.

Reaction:



Requirements:

1. Reagent:

- **Sakaguchi reagent:** 1% 1-naphthol in alcohol with a few drops of 10% sodium hypobromite solution of bromine water.
- 40% NaOH.
- Sample (0.1% of arginine or 0.1% of creatine).

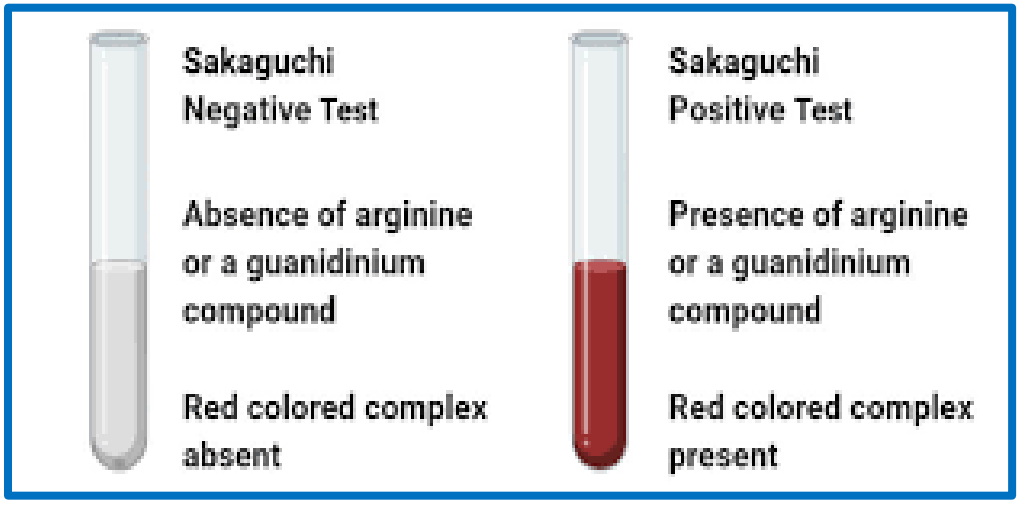
2. Material required:

- Test tubes.
- Test tube stand.
- Pipettes.

Method:

1. About 3 ml of the test solution is added in a test tube, to which 1 ml of 40% NaOH is added and mixed correctly.
2. Then, two drops of 1-naphthol are added to the same test tube and mixed thoroughly.
3. Now, 4-5 drops of the 10% sodium hypobromite or bromine water is added.
4. The test tube is observed for the development of color.

Observation:



- **Positive result:** A positive result on the Sakaguchi's test is demonstrated by the formation of red color. This indicates the presence of an arginine or guanidinium compound.
- **Negative result:** A negative result in the Sakaguchi's test is demonstrated by the absence of red color. This indicates an absence of arginine or a guanidinium compound.

Uses of Sakaguchi Test:

- Sakaguchi's Test is a biochemical test for the detection of arginine in the free or combined form in proteins.
- The test is qualitative but can be made quantitative by the addition of urea that stabilizes the colored product.

Limitations of Sakaguchi Test:

- Quantitative analysis of the colored product is not possible with this test as the rate of Sakaguchi's reaction is slow.
- Similarly, some of the guanidinium groups in the solution might be destroyed by the hypochlorite, resulting in difficulties in testing results.

4.11. Nitroprusside test:

Is a biochemical test used for the detection of the free –SH groups in **amino acids** or the cysteine amino acid in a protein. The test is a specific test for cysteine as it is the only amino acid with a free –SH group in the structure. The test, however, is not specific to proteins and amino acids as it can detect compounds like ketones. The test is a colored reaction of amino acid where the free –SH group in the amino acid reacts with the nitroprusside reagent to form a red-colored complex. Cysteine and methionine are two amino acids with –SH groups but the –SH group in the methionine molecule is involved in the thioester linkage which is difficult to break and thus, cannot be detected by this test. The test can also be used for the differentiation between cysteine and cystine as cysteine gives a positive result to this test, but cystine doesn't.

Objectives:

- To detect the presence of amino acid cysteine in a protein solution.
- To differentiate between the amino acids cysteine and cystine.
- To detect the presence of cysteine in the urine sample and diagnose cystinuria.

Principle:

The test is based on the detection of free sulfur atoms in a solution as a result of the degradation of the –SH group. Nitroprusside is a complex anion consisting of an octahedral ferrous center which is surrounded by five tightly bound cyanide groups. The molecule, when combined with a sulfur atom forms a violet or red-colored complex. The action of a strong alkali releases SH group foud in cysteine molecule. The sulfur then reacts with the nitroprusside ion to form a red-colored compound. Some proteins might not give a positive result immediately, and in that case, the solution should be heated as these proteins might require heat coagulation to release the –SH group.

Reaction:

$$\text{Cysteine} + 2NaOH \rightarrow \text{Serine} + Na_2S + H_2O$$

$$Na_2[Fe(CN)_5NO] + Na_2S \rightarrow Na_4[Fe(CN)_5NOS] \text{ (red coloured complex)}$$

Requirements:

1. Reagent:

- 2% freshly prepared Sodium Nitroprusside.
- Concentrated Sodium hydroxide.
- Sample (1% cysteine).

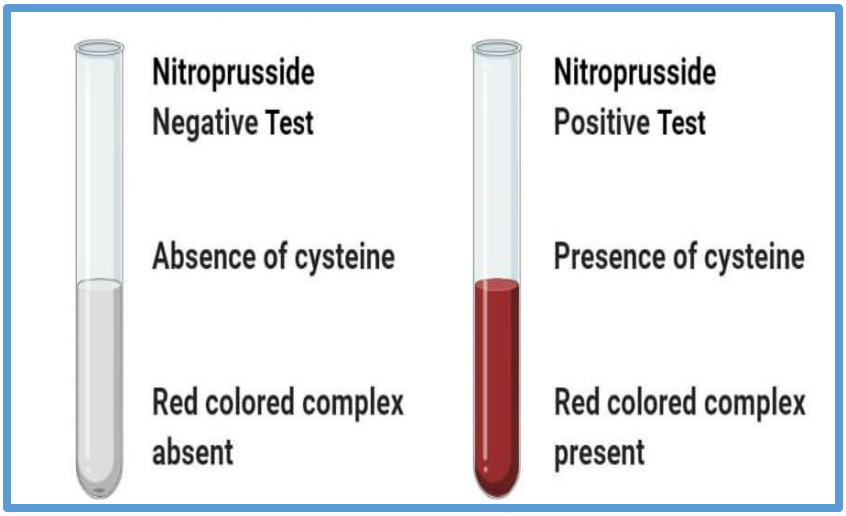
2. Materials Required:

- Test tubes.
- Test tube stand.
- Pipettes.
- Water bath.

Method:

1. 2 ml of the amino acid solution or the sample is added to a test tube.
2. To the test tube, 0.5 ml of the freshly prepared sodium nitroprusside is added and mixed thoroughly.
3. Now, 0.5 ml of the concentrated sodium hydroxide is added. If a positive result is not observed immediately, the test tube can be heated in a water bath for 2 minutes.
4. The test tube is observed for the color change or the formation of a colored complex.

Observation:



- **Positive result:** A positive result in the nitroprusside test is indicated by the appearance of a red-colored complex. The color formation confirms the presence of cysteine.
- **Negative result:** A negative result in the nitroprusside test is indicated by the absence of a red-colored solution. This indicates the absence of cysteine.

Uses of Nitroprusside Test

- Nitroprusside test is an analytical test for the detection of cysteine or cysteine-containing proteins in a sample.
- The test can also be used in the diagnosis of cystinuria as the presence of cysteine in urine is a pathological feature of the disease.
- The nitroprusside test also detects compounds like ketoacids and ketones in **blood** and urine that helps in the determination of the degree of **ketonuria** and ketonemia.

4.12. Isatin test:

Is a biochemical test for the detection of **amino acids** like proline and hydroxyproline. The test is a specific test for specific amino acids that produces a colored addition product (also called adduct) with the Isatin reagent. The detection of imino acid proline for a long time had been unsatisfactory as methods like the ninhydrin test can only detect proline after isolation from other amino acids. Later in 1971, Fouad N. Boctor came up with a reaction between proline and isatin which can be used to qualitatively and quantitatively determine the amount of proline. The distinction between proline and hydroxyproline cannot be made by this test as isatin reacts with both of the compounds to produce a colored product.

Objectives:

- To detect the presence of proline and hydroxyproline-containing proteins in a sample.
- To distinguish proline and hydroxyproline from other amino acids.

Principle:

Isatin reagent used in the test works as a visualizing agent that provides different colors with different amino acids in the chromatography technique. The isatin reaction between proline and hydroxyproline produces a characteristic blue-colored product. The reaction between imino acids like proline and hydroxyproline and isatin (1H-indole-2,3-dione) yields a blue-colored adduct.

The reaction is a simple addition reaction where the combination of isatin and proline produces a colored product, indicating the presence of imino acids.

Requirements:

1. Reagent:

- **Isatin reagent:** A 0.02% solution of isatin is prepared by dissolving isatin crystals in 0.5 M sodium citrate buffer at the pH ranging from 3 to 11.
- Sample.
- Concentrated HCl.

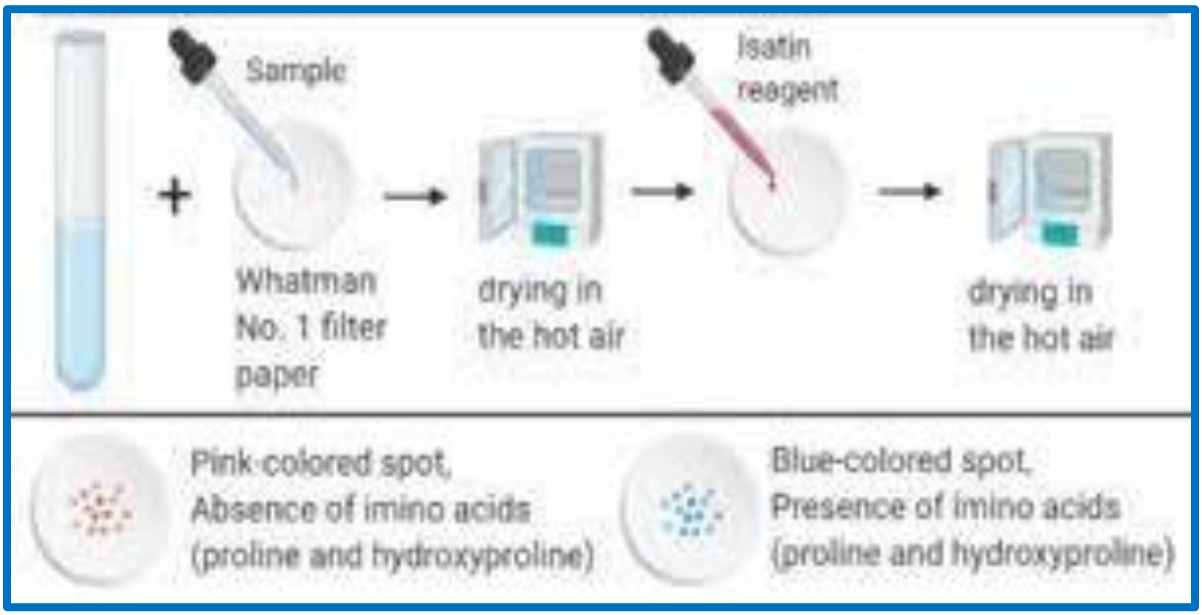
2. Materials required:

- Whatman No. 1 filter paper.
- Hot air oven.
- Pipettes.

Methods:

1. A drop of the sample (imino acid) solution is added to a Whatman No. 1 filter paper and dried by placing it in a hot air oven.
2. Now, a drop of isatin reagent is added to the dried spot, and the process of drying in the hot air oven is repeated.
3. The paper is then observed for the formation of a blue-colored spot.

Observation:



- **Positive result:** A positive result of the Istain test is represented by the formation of a blue-colored spot on the filter paper. This indicates the presence of imino acids.
- **Negative result:** A negative result of the Isatin test is represented by the formation of a pink-colored spot on the filter paper. This indicates the absence of imino acids.

Uses of Isatin Test:

- The test is used for the detection of proline and hydroxyproline amino acids in a sample solution.
- Isatin test is one of the standard tests for the detection of polynuclear hydrocarbons and phenols in polluted air.
- The method is simple, fast, and suitable for the estimation of proline content in larger samples.

Limitations of Isatin Test:

The color observed in the test should be observed carefully, and the test should be repeated if a mixed color is observed.

4.13. Xanthoproteic test:

Xanthoproteic test is a **biochemical test** for the detection of **amino acids** containing phenolic or indolic groups like phenylalanine, tyrosine, and tryptophan (aromatic amino acids). The test is named Xanthoproteic test due to the formation of a yellow precipitate of xanthoproteic acid. The term 'Xantho' refers to 'yellow', so the test is often termed as the Yellow Protein Test. The test gives a positive result for amino acids containing benzene rings or other aromatic groups. The test is a qualitative test that provides information only on the presence or absence of the amino acids.

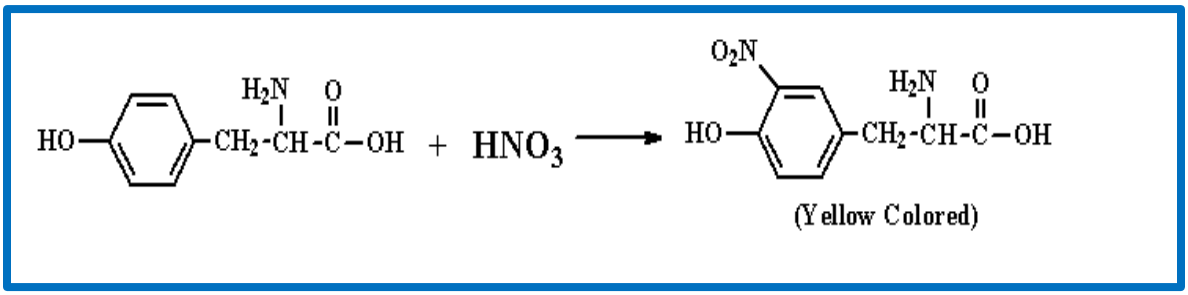
Objectives:

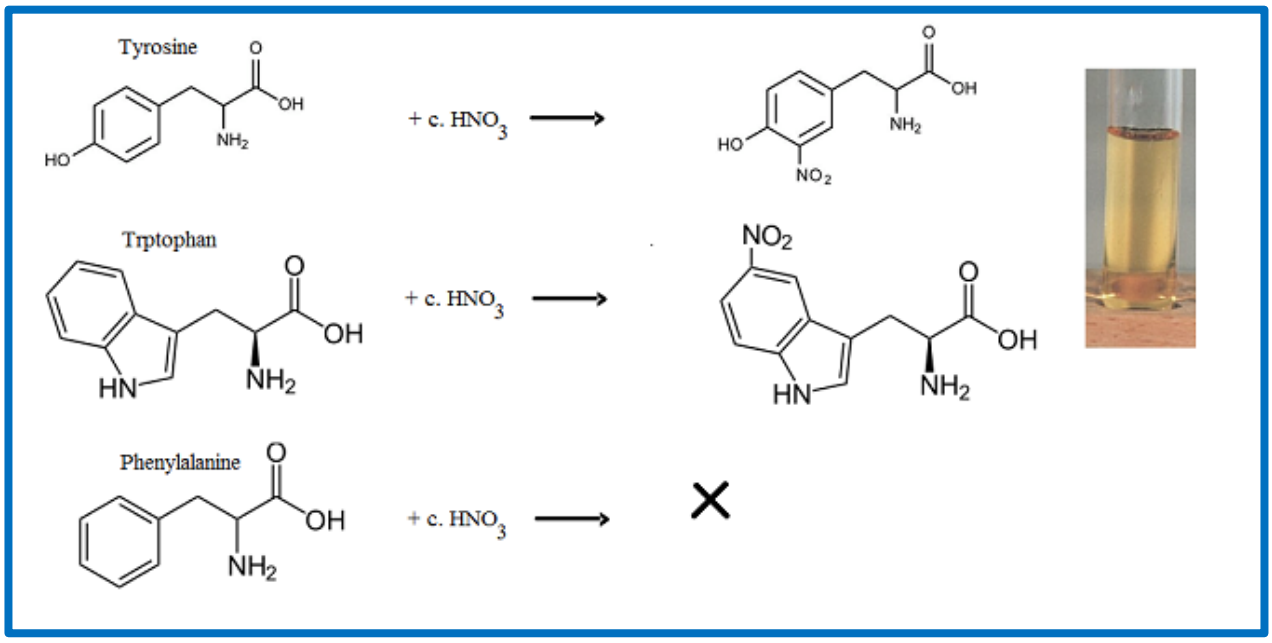
- To detect the presence of aromatic groups-containing amino acids like tyrosine and tryptophan.
- To differentiate tyrosine and tryptophan from other amino acids.

Principle:

Xanthoproteic test is used to detect amino acids containing an aromatic nucleus (tyrosine, tryptophan and phenylalanine) in a protein solution which gives yellow color nitro derivatives on heating with conc. HNO3. The aromatic benzene ring undergoes nitration to give yellow colored product. Phenylalanine gives negative or weakly positive reaction though this amino acid contains aromatic nucleus because it is difficult to nitrate under normal condition. On adding alkali to these nitro derivative salts, the color change from yellow to orange.

Reaction:





Requirements:

1. Reagent:

- Concentrated Nitric acid.
- 40% NaOH.
- Test solution

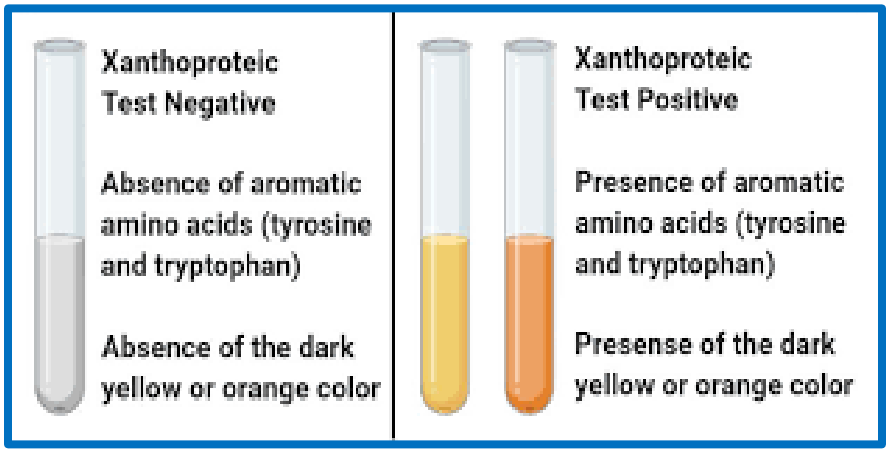
2. Material Required:

- Test tubes.
- Test tube stand.
- Pipettes.

Method:

1. About 1 ml of the sample solution is taken in a test tube. To this, the same amount of concentrated nitric acid is added.
2. The test tube is allowed to cool down to room temperature. If the sample is a protein solution, a white precipitate might develop due to the denaturation of proteins.
3. Then, 1 ml of 40% NaOH solution is added to the test tube and observed for color change.

Observation:



- Positive result: The appearance of a dark yellow or orange-colored solution represents a positive test. This indicates the presence of aromatic groups in the proteins and amino acids.
- Negative result: The absence of a dark yellow or orange-colored solution represents a negative test. This indicates the absence of aromatic groups in proteins and amino acids.

Uses of Xanthoproteic test:

- This is a biochemical test for the detection of proteins and amino acids.
- The test allows the differentiation of aromatic amino acids from non-aromatic amino acids.

Limitations of Xanthoproteic test:

- Even though phenylalanine is an aromatic group-containing amino acid, it doesn't give a positive Xanthoproteic test due to the highly stable phenyl group.

4. B. Physical Properties of proteins:

Denaturation:

It is a change in the chemical, physical and biological properties of a protein from those of a native state. It is involves intramolecular rearrangement with the loss of specific configuration of native protein. Denaturation of globular proteins often involves the folding of the polypeptide chains changing the tertiary structures as well as destruction of secondary structure by cleavage of hydrogen bonding that hold together the polypeptide chains. Furthermore unfolding of the molecules result in the unmasking of certain functional group.

Denaturating agents and the usual changes:

1. Physical agents: heat - mechanical agitation - UV light
   Changes like: decrease in solubility - increase in viscosity of the solution-decrease in the rate of diffusion. Complete denaturation is irreversible.

2. Chemical agents: acids and alkalies - organic solvents – salts of heavy metals - detergents - urea. Some of these are reversible changes.

3. Biological change: Enzymatic and hormonal activity is usually destroyed by complete denaturation.

Precipitation of Proteins:

1. Precipitation by heat:

4.15. Heat coagulation test:

A **biochemical test** performed to determine the presence of proteins like albumin and globulin in protein. Coagulation of proteins as a response to heat is a common phenomenon. The heat coagulation of proteins occurs in one of the two stages; denaturation and agglutination or the separation of the denatured protein in a particular form. The heating of coagulable proteins at their isoelectric pH, a series of changes occur to the proteins; dissociation of subunits or the quaternary structure, uncoiling of the polypeptide chains ultimately leading to the matting together of uncoiled polypeptide chains. Processes like coagulation and flocculation are superficial visible manifestations of changes that occur in proteins during the denaturation process.

Coagulation of proteins is an irreversible process that is maximum at the isoelectric pH of the proteins. Heat coagulation of proteins is an important clinical test for the detection of proteinuria. It is simple and less time-consuming. Both qualitative and quantitative estimation of proteins can be done with the heat coagulation method. The quantitative analysis of coagulation can be performed by measuring the coagulum formed on the test tube.

Objectives:

- To detect the presence of proteins in a given sample.
- To detect the presence of albumin, globulin, and other proteins present in a urine sample.

Principle:

The principle of heat coagulation test is the change in the structure of proteins as a result of heat and change in pH. Heating a protein in acidic medium results in denaturation of protein due to the breaking of certain bonds responsible for the tertiary and quaternary structure of proteins. However, in the case of coagulable proteins, when these proteins are heated at their isoelectric pH, the polypeptide chains become uncoiled and matt with each other to form an insoluble mass. The mass formed doesn't dissolve back to the liquid. The process of coagulation is maximum at the isoelectric point, and the mass of the coagulum might differ with the particle size and the concentration of proteins in the sample. For the heat coagulation test of albumin and globulin, chlorophenol red is used which adjusts the pH of the sample to the isoelectric point of albumin. The reagent for this test also contains acetic acid, which helps in the breaking of peptide bonds present in the protein molecule, facilitating coagulation.

Requirements:

1. Reagent:

- Chlorophenol red indicator.
- 1% acetic acid.
- Sample.

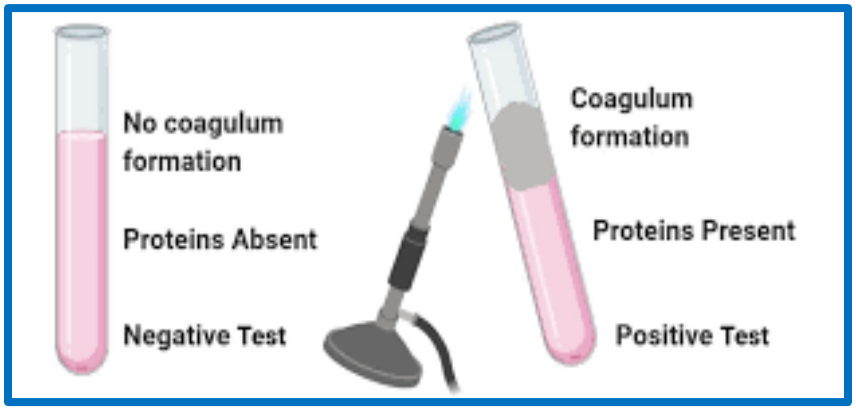
2. Materials required:

- Test tubes.
- Test tube stand.
- Pipettes.

Methods:

1. About two-thirds of the test tube is filled with the given sample.
2. To the test tube, 1-2 drops of chlorophenol red indicator is added drop by drop and mixed properly.
3. When a purple color is observed on the test tube, 1% acetic acid is added drop by drop until the color changes to pale pink.
4. The test tube is then inclined slightly so as to heat the upper portion of the fluid.
5. The tube is observed for the formation of the coagulum.

Observation:



- **Positive result:** A positive result of the heat coagulation test is represented by the formation of a dense coagulum at the upper part of the solution. The lower part of the solution acts as a control.
- **Negative result:** A negative result of the heat coagulation test is represented by the absence of coagulum at the upper layer. This indicates the absence of albumin and other proteins in urine.

Uses of Heat coagulation test of proteins:

- Heat coagulation test is used to detect the presence of albumin and globulin protein in the urine sample. As albumin and globulin in urine are observed under many pathological conditions, their presence can be conclusively established by this test, which aids in the diagnosis of diseases.
- The test is one of the most commonly used methods for the detection of proteins in the urine.

Limitations of Heat coagulation test of proteins:

- In some cases, the test might give a positive result for other coagulable proteins that might be present in the urine.
- It is imperative that the pH of the solution should be around the isoelectric pH of the proteins suspected to be in the sample.

2. Precipitation by complex acid:

4.16. Heller's nitric acid test:

Heller's test is a biochemical test performed to detect proteins in a sample by the denaturation of those **proteins** by the addition of strong acids. Heller's test usually uses concentrated nitric acid for the denaturation of proteins. The test is performed for clinical purposes to detect abnormal proteins in biological fluids, including urine. Heller's test is a type of **precipitation test** where the precipitation is brought about by denaturation.

Objectives:

- To detect the presence of proteins in a given sample.
- To detect proteins in biological fluids like urine and blood.
- To identify albumin and globulin that might be present in urine.

Principle:

The test is based on the principle of precipitation of proteins, which in this case takes place in the presence of mineral acids like nitric acid. Protein precipitation by acids relies on the changes in the pH of the solution. As all proteins have a defined isoelectric point or pI value, changes in the pH of the solution affect the structure of the protein. The addition of acids to a solution reduces its pH value. As the value decreases, protein molecules become positively charged due to the proton capture by amino groups present in the proteins. In an aqueous state, the hydration sphere that surrounds the protein becomes disrupted due to the charges. The disruption brings about an imbalance in the structure of the protein, resulting in precipitation. The addition of acid to a protein sample causes precipitation of proteins at the point where the acid comes in contact with the protein solution. As a result, a white coagulated ring is formed between the two layers of protein solution and the mineral acid (HNO_3).

Requirements:

1. Reagent:

- Nitric acid (HNO_3).
- Sample.

2. Materials required:

- Test tubes.
- Test tube stand.
- Pipettes.

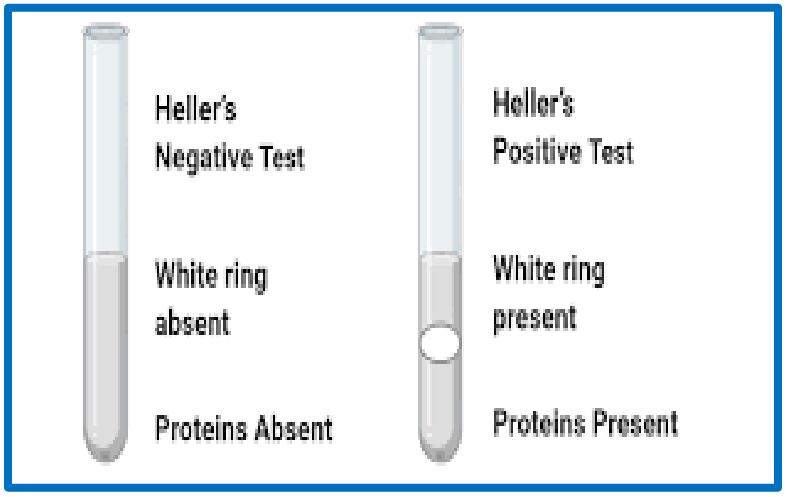
Reaction:

- When native protein solution is treated with Concentrated HNO3, white precipitate ring is obtained due to denaturation of protein.

Method:

1. In a clean and dry test tube, 2 ml of concentrated nitric acid is taken.
2. To this, 2 ml of urine or other sample is added. The sample should be poured from the sidewall of the test tube in an inclined position in order to form a layer of the sample above the nitric acid.
3. The test tube is then observed for the formation of a white ring at the junction of the two layers.

Observation:



- **Positive result:** A positive result is represented by the formation of a white ring (precipitated protein) at the junction of the two distinct layers. This indicates the presence of proteins in the given sample.
- **Negative result:** A negative result is represented by the absence of a white ring. This indicates the absence of protein in the sample.

Uses of Heller's Test:

- Heller's test is a biological test used for the detection of proteins in biological fluids.
- The test is better than other similar tests as the test requires a small amount of urine sample.

Limitations of Heller's Test:

- The concentrated acid used in the test is corrosive and thus, should be handled carefully.
- The test is qualitative and doesn't give the concentration of protein present in the sample.

3. Precipitation by heavy metals in the presence of an alkali (Lead Sulfide Test):

4. Precipitation by Salt:

4.17. Salt Saturation Test:

Salt Saturation Test is a test for the precipitation of proteins and their differentiation from other proteins by using the salting-out technique. Salting out is the process of precipitation of proteins by increasing the concentration of salt in the solution. The salts used in this technique are mostly neutral mineral salts like $MgSO_4$, Na_2SO_4, and $(NH)_2SO_4$. The salt solubilization test also helps in the differentiation of proteins as some proteins precipitate under lower salt concentrations while others require higher concentrations. The reagent used for the test might differ depending on the preference with salts like ammonium sulfate being used due to their increased solubility. The salt saturation test can be performed either as half saturation or full saturation depending on the type of proteins being detected. In the half-saturation, the solution is just half saturated with the salt, but in full saturation, the solution is completely saturated with the salt.

Objectives:

- To detect the presence of proteins in a given sample.
- To differentiate between proteins like albumin and globulin.
- To purify and fractionate large batches of proteins based on their solubility.

Principle:

Proteins are colloidal in nature due to the presence of a large number of electric charges which creates repulsion, preventing the coalescence of the particles. Similarly, a shell of hydration is also formed around each protein molecule where the proteins are surrounded by films of water molecules. The mechanism of the salting-out technique is the preferential solvation of salt molecules by the exclusion of the hydration layer around the protein molecules. When organic salts are added to the solution, the effective water concentration available for the proteins decreases which eventually leads to the precipitation of the proteins. There are two different mechanisms involved in the process.

At first, the higher concentration of mineral salts osmotically removes water from the hydration layer around proteins, which then depletes the immobilized layer of water surrounding each of the protein particles. Secondly, the cations and anions of the mineral salts bind with the respective counterionic groups on the protein particles to reduce the surface charges of the proteins. Both of these mechanisms aid in the aggregation and precipitation of protein particles. The concentration of the salt required for the precipitation of proteins depends on the surface area of the proteins. Smaller molecules like albumin have a larger surface area which requires a higher concentration of salt, whereas larger molecules like casein, gelatin, and globulin have a smaller surface area and require smaller concentrations.

Requirements:

1. Reagent:

- Solid Ammonium sulfate
- Saturation solution of Ammonium sulfate
- 40% sodium hydroxide
- 1% copper sulfate solution
- Sample solution

2. Materials required

- Test tube
- Test tube stand
- Dropper
- Pipettes
- Filter paper

Method:

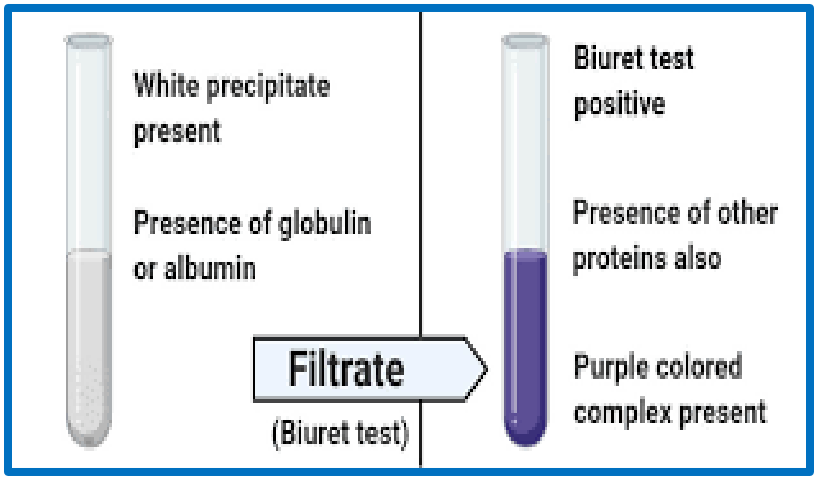
For Half Saturation

1. In a test tube, 3 ml of the test solution is taken. To this, 3 ml of the saturated solution of ammonium sulfate is added.
2. The solution is mixed correctly and allowed to stand for about 5 minutes.
3. The solution is then filtered to remove the residue formed from the rest of the filtrate.
4. The **Biuret test** is performed on the rest of the filtrate.

Full Saturation

1. In a test tube, 3 ml of the test solution is taken. To this, some amount of solid ammonium sulfate is added.
2. The solution is then mixed by shaking until the solid salt dissolves. The solid ammonium sulfate is added continuously until no more of the salt can be dissolved.
3. The solution is then allowed to stand for five minutes, followed by the filtration of the residue.
4. The Biuret test is performed on the filtrate.

Observation:



Half Saturation

- The formation of a white precipitate in the solution under half-saturation indicates a positive result. This result confirms the presence of globulin in the solution.
- The absence of the white precipitate represents a negative result, indicating the absence of globulin in the sample.
- If a purple color is formed during the Biuret test, it shows that other proteins are present in the solution. A negative Biuret test indicates the absence of other proteins.

Full Saturation

- The formation of a white precipitate in the solution under full saturation indicates a positive result. This result confirms the presence of albumin in the solution.
- The absence of the white precipitate represents a negative result, indicating the absence of albumin in the sample.
- If a purple color is formed during the Biuret test, it shows that other proteins are present in the solution. A negative Biuret test indicates the absence of other proteins.

Uses of Salt Saturation Test:

- Salt saturation test can be used for the detection of proteins like albumin and globulin.
- As the precipitation of proteins in the test is due to reduced solubility and not denaturation, precipitated proteins can be used to detect the concentration of the proteins.
- The test can also be used as a method of protein purification for the removal of bound lipid or nucleic acids.
- The mineral salts used in the test help in the stabilization of proteins by preferential solvation. Salts like $(NH)_2SO_4$ inhibit bacterial growth and protease activity on the proteins as well.

Limitations of Salt Saturation Test:

- While using solid ammonium sulfate, lumps might be formed in the solution. In order to avoid that, mortar and pestle should be used.
- The use of low-grade mineral salts might cause contamination with heavy metals that inhibit the precipitation process.
- Online calculators can be used to determine the amounts of solid ammonium sulfate required for saturation.
- The use of ammonium sulfate might cause acidification of the solution, which slows down the process; thus, Tris buffer can be added.

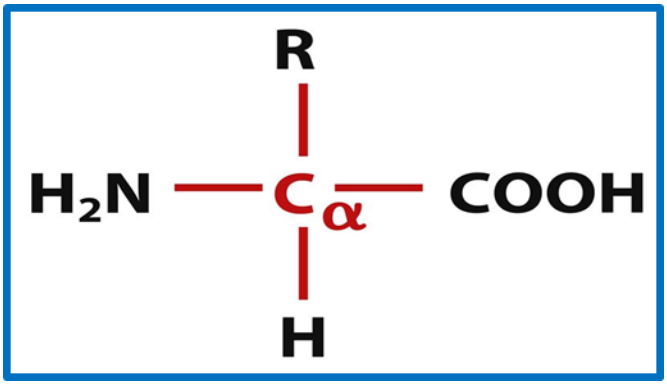
4. C. <u>The Titration of the Amino</u>

Introduction:

A titration curve of an amino acid is a plot of the pH of a weak acid against the degree of neutralization of the acid by standard (strong) base. Consider the ionization of a weak organic acid such as acetic acid by NaOH.

As more of the strong base (titrant) is added to the aqueous solution, more of the weak acid is converted to its conjugate base. During this process, a buffer system forms and the pH of the system will follow the HendersonHasselbalch relationship.

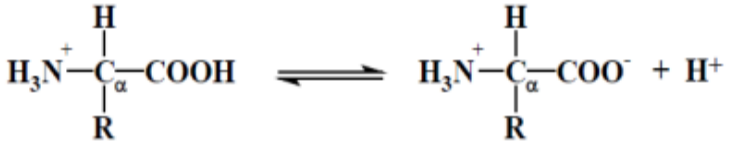
Based on the number of plateaus on a titration curve, one can determine the number of dissociable protons in a molecule. The one plateau observed when acetic acid is titrated indicates that it is a monoprotic acid (i.e., has only one dissociable H^+). Many organic acids are polyprotic (have > one dissociable $H^{+)}$. The protein building blocks, amino acids, are polyprotic and have the general structure:



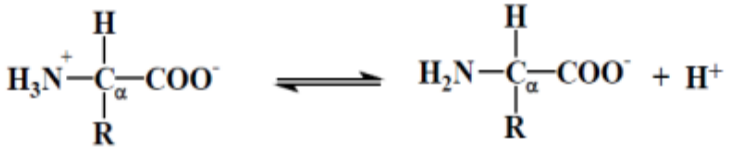
The majority of the standard amino acids are diprotic molecules since they have two dissociable protons: one on the alpha amino group and other on the alpha carboxy group. There is no dissociable proton in the R group. This type of amino acid is called a “simple amino acid”. A simple amino acid is electrically neutral under physiological conditions.

NOTE: Under this definition it is possible to have a simple amino acid which is triprotic. Which of the 20 common or standard amino acids are simple & triprotic? Ionization of a diprotic amino acid will proceed as follows:

Dissociation 1:



Dissociation 2:

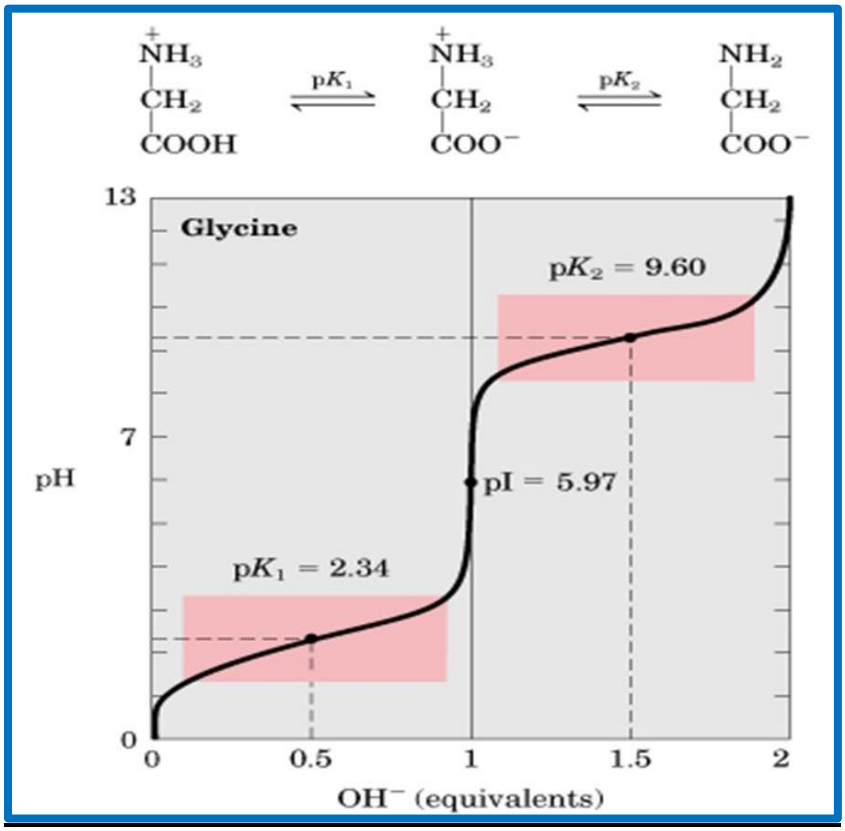


The order of proton dissociation depends on the acidity of the proton: that which is most acidic (lower pKa) will dissociate first. Consequently, the H + on the α-COOH group (pKa 1) will dissociate before that on the α-NH 3 group (pKa 2). The titration curve for this process looks similar to the following:

Objectives:

1. To determine the titration curve for an amino acid.
2. To use this curve to estimate the pKa values of the ionizable groups of the amino acid and the amino acid's pI.

Titration curve:



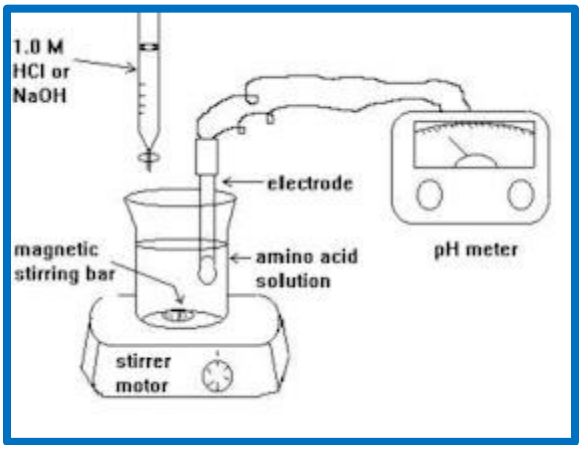
This curve reveals, in addition to the same information observed with a monoprotic acid, an additional characteristic of polyprotic acids and that is the pH at which the net charge on the molecule is zero. This pH defines the isoelectric point (pI) of the molecule, a useful constant in characterizing and purifying molecules. Using a titration curve, the pI can be empirically determined as the inflection point between the pKa of the anionic and cationic forms. Mathematically, the pI can be determined by taking the average of the pKa for the anionic and cationic forms. The ionic form of the molecule having a net charge of zero is called the zwitterion. A few amino acids are classified as triprotic. This is because, in addition to the ionizable protons of the αCOOH and α-NH 3 groups, they also have a dissociable proton in their R group. Although triprotic amino acids can exist as zwitterions, under physiological conditions these amino acids will be charged. If the net charge under physiological conditions is **negative**, the amino acid is classified as an **acidic** amino acid because the R group has a proton that dissociates at a pH significantly below pH 7. The remaining triprotic amino acids are classified as **basic** amino acids due to a) their having a net **positive** charge under physiological conditions and b) an R group dissociable proton with a pKa near or greater than pH 7. Titration curves for triprotic amino acids generate the same information as those for the diprotic amino acids. The pI for a triprotic amino acid can be determined graphically, although this is somewhat more challenging. Graphical determination, as was the case with the diprotic acids, requires one to know the ionic forms of the amino acid and finding the inflection point between the cationic and anionic forms. Mathematically, the pI for an acidic amino acid is the average of pKa 1 and pKa

R (the pKa of the dissociable proton in the R group); for a basic amino acid, it is the average of pKa 2 and pKa R.

4.18. Determine the titration curve for an amino acid

Method:

1. Using a 25-mL graduated cylinder or serological pipet, transfer 25 mL of a 0.2 M amino acid solution to a 150 - 250 mL beaker. Set up the apparatus as shown below:



2. Titrate the amino acid with 1.0 M HCl (titrant)

a. Determine the pH of the amino acid solution before the addition of titrant.

b. Initially add approximately 0.5 mL of the titrant to the amino acid at a time. Record the data IN **YOUR NOTEBOOK** as indicated below.

mL 1.0 M HCl pH

0.0, 0.5, 1.0, etc.

Note: In the beginning, the pH will change very dramatically with each addition of titrant. As you get closer to the pKa of the ionizable group, the pH will change much more slowly. When this phenomenon occurs, add **1 mL** of titrant at a time.

c. After the addition of each volume of HCl, stir the solution briefly.

d. Turn the stirrer off and measure the pH using the pH meter.

e. Continue with the titration until the pH ~1.5. 3.

3. Repeat steps 1 and 2 above, this time using 1.0 N NaOH as the titrant for a fresh 25-mL sample of the same amino acid.

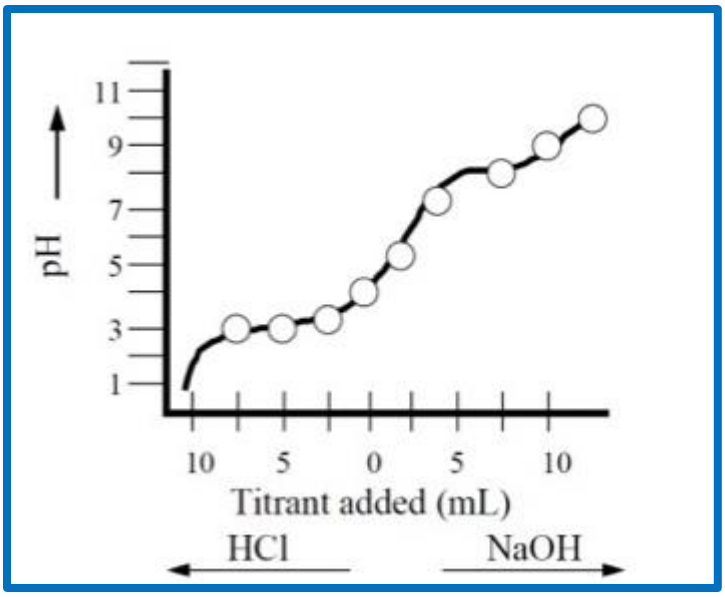
4. Record the data **IN YOUR NOTEBOOK** until you get to pH ~13.

mL 1.0 M NaOH pH

0.0, 0.5, 1.0, etc.

5. Estimate the amino acids pKa values of the ionizable groups and its pI.

6. Using Microsoft Excel (or some similar program), construct your titration curve plotting **pH versus mL of acid and base** added to the amino acid solution as indicated below.



7. On your curve, designate the buffer region(s), pKa(s), and the amino acids pI.
8. From your graph, estimate the pKa values of the ionizing groups and the pI of the amino acid.

Practical No. 5

Chromatography

Introduction:

Chromatography is the name originally applied, by the Polish Botanist Tswett in 1906, to techniques by which the members of a group of similar substances are separated by a continuous redistribution between two phases, one stationary and the other moving. A variety of attractive forces between the stationary phase and the substances to be separated, leads to selective retardation of later relative to the moving phase. Under suitable conditions, the resulting different rates of migration can bring about complete separation of the substances.

A sheet of paper, or a thin layer of powdered insoluble material, can serve as a support for the stationary phase, because they both contain a certain amount of water as part of their structure. If a liquid mobile phase moves along the paper or thin layer, it causes a separation partly by means of the partition effect. However, the supporting medium is not inert and often influences the separation by its property of adsorbing some or all of the components of the mixture to different degrees on its surface. The final separation observed is due to both partition and adsorption effects. Partition may be considered as a driving force moving the compounds forward along the support and adsorption as a retarding force holding the compounds back. The nature and composition of the mobile phase together with different supporting media have a great effect on these two forces and this permits an almost infinite variation in the chromatographic system. Paper is composed of almost pure cellulose, but the thin layer can be prepared from silica gel, celite, alumina, cellulose powder or other materials.

In this layer chromatography, a specially prepared chemical substances is, mixes with water and deposited as a thin layer on a sheet of glass whose dimensions may be up to 20 cm^2. The wet thin layer is spread over the glass plate and is dried in the oven to produce a dry adherent layer.

Thin Layer Chromatography: Basics

- Thin Layer Chromatography (TLC) is a technique used to analyse small samples via separation
    - For example, we could separate a dye out to determine the mixture of dyes in a forensic sample
- There are 2 phases involved in TLC - stationary phase and mobile phase
- **Stationary phase:**
    - This phase is commonly thin metal sheet coated in alumina (Al_2O_3) or silica (SiO_2)
    - The solute molecules **adsorb** onto the surface
    - Depending on the strength of interactions with the stationary phase, the separated components will travel particular distances through the plate
    - The more they interact with the stationary phase, the more they will 'stick' to it
- **Mobile phase:**
    - Flows over the stationary phase
    - It is a polar or nonpolar liquid (solvent) or gas that carries components of the compound being investigated
    - Polar solvents - water or alcohol
    - Non-polar solvents - alkanes
- If the sample components are coloured, they are easily identifiable
- We can examine the plate under UV light using ninhydrin to identify uncoloured components.

Conducting a TLC analysis:

- **Step 1:**

  Prepare a beaker with a small quantity of solvent

- **Step 2:**

  On a TLC plate, draw a horizontal line at the bottom edge (in pencil)

  This is called the **baseline**

- **Step 3:**

  Place a spot of pure reference compound on the left of this line, then a spot of the sample to be analysed to the right of the baseline and allow to air dry

  The reference compounds will allow identification of the mixture of compounds in the sample

- **Step 4:**

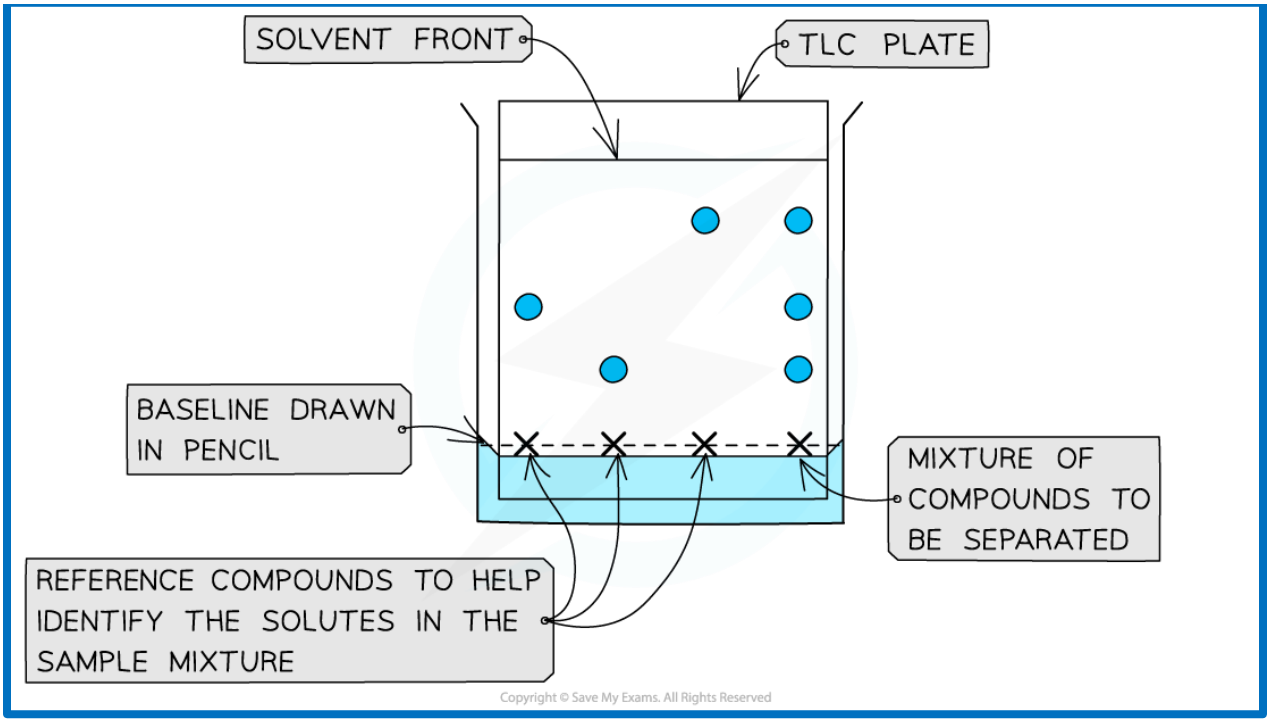
  Place the TLC plate inside the beaker with solvent - making sure that the pencil baseline is lower than the level of the solvent - and place a lid to cover the beaker

The solvent will begin to travel up the plate, dissolving the compounds as it does

- **Step 5:**

  As solvent reaches the top, remove the plate and draw another pencil line where the solvent has reached, indicating the **solvent front**

  The sample's components will have separated and travelled up towards this solvent front



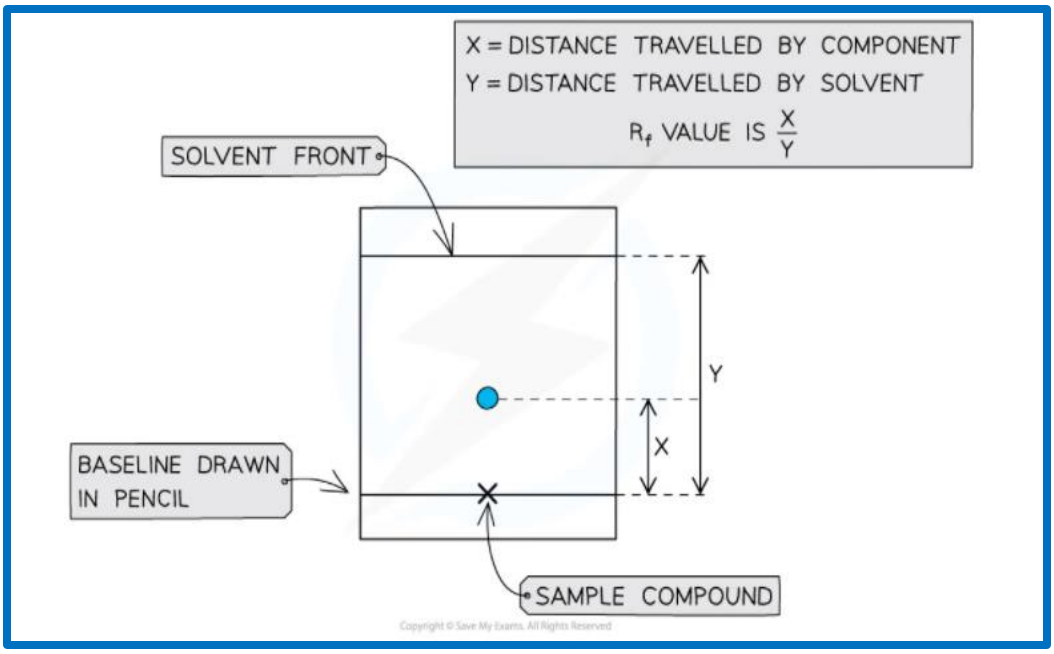
A dot of the sample is placed on the baseline and allowed to separate as the mobile phase flows through the stationary phase; the reference compound/s will also move with the solvent

R_f values:

- A TLC plate can be used to calculate R_f values for compounds.

$$R_f = \frac{\textit{Distance travelled by component}}{\textit{Distance travelled by solvent}}$$

- These values can be used alongside other analytical data to deduce composition of mixtures.



R_f values can be calculated by taking 2 measurements from the TLC plate

Thin layer chromatography (TLC) has a number of advantages when compared to paper chromatography (PC):

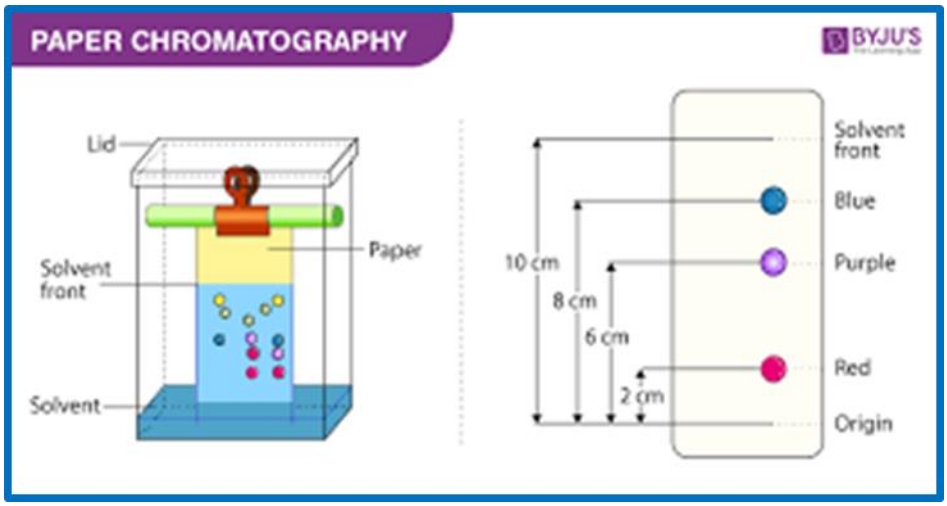
1. Substances, e.g. lipids, which cannot be separated on paper (cellulose), can often be separated on one of the other chemicals used to prepare the plates.
2. Thin layer chromatography is usually faster than paper chromatography.
3. The separated substances are usually more compact and, therefore sharper separations of otherwise overlapping substances are obtained with TLC.

PAPER CHROMATOGRAPHY

Paper Chromatography, in analytical chemistry, technique for separating dissolved chemical substances by taking advantage of their different rates of migration across sheets of paper. It is an inexpensive but powerful analytical tool that requires very small quantities of material.

The method consists of applying the test solution or sample as a spot near one corner of a sheet of filter paper. The paper is initially impregnated with some suitable solvent to create a stationary liquid phase. An edge of the paper close to the test spot is then immersed in another solvent in which the components of the mixture are soluble in varying degrees. The solvent penetrates the paper by capillary action and, in passing over the sample spot, carries along with it the various components of the sample. The components move with the flowing solvent at velocities that are dependent on their solubility in the stationary and flowing solvents. Separation of the components is brought about if there are differences in their relative solubility in the two solvents. Before the flowing solvent reaches the farther edge of the paper, both solvents are evaporated, and the location of the separated components is identified, usually by application of reagents that form coloured compounds with the separated substances. The separated components appear as individual spots on the path of the solvent. If the solvent flowing in one direction is not able to separate all the components satisfactorily, the paper may be turned 90° and the process repeated using another solvent.

Paper chromatography has become standard practice for the separation of complex mixtures of amino acids, peptides, carbohydrates, steroids, purines, and a long list of simple organic compounds. Inorganic ions can also readily be separated on paper. *Compare* thin-layer chromatography.



There are two types of paper chromatography:

1. Ascending technique:

In this method the solvent move vertically up the paper by capillary action. The sample spots are spotted in a position just above the surface of the solvent. The spots move at different rates because at differences in their solubility in the solvent and their adsorption to the stationary phase.

2. Descending technique:

In the technique the end of the paper near which the sample spots are located is help in a trough at the top of the tank and the rest of the paper allowed to hang vertically the solvent in the trough moves down wards under gravity, this technique is faster than ascending one.

Ascending amino acid chromatography procedure

1. Cover the bench with a sheet of clean paper then laid a sheet of chromatography paper over the above paper.
2. Using a pencil rule a line across the chromatography paper at a distance of 1 inch from the bottom edge.
3. On the line above mark 3 crosses 1.5 inches apart from each other.
4. Label these origins Phe, Lys and M (mixture) or any other amino acid provided.
5. On to the cross place one small drop of the appropriate amino acid solution by capillary tube and allow the spot to dry.
6. Place 50 ml of the solvent in the bottom of the tank.
7. Fold the paper (cylindrical shape).
8. Carefully lower the paper into the rank, the paper must sit vertically and should not dip more than 0.25 inch into the solvent.
9. Close the tank and allow running of approximately one hour.
10. Remove the paper, make a solvent mark to indicate the solvent front and allow the paper to air dry.
11. Dip the dry paper in the acetone solution of ninhydrine.
12. Allow to dry and heat in an oven for 2-5 minutes the amino acid will appear as purple spots.
13. Compare the positions of the spots from the unknown with those from known and so deduce the composition of the mixture.
14. Calculate the RF valves of the amino acids (measure the centre ofthe spot or the point of greatest density 11 the spot is elongated).

$$RF = \frac{\text{Distance solute has moved from the origin}}{\text{Distance solvent has moved from the origin}}$$

Terminology:

1. Mobile phase: a solvent or gas used to separate the component e.g. Butanol acetic acid water (4:1:1)
2. Stationary phase: usually a solid or liquid adsorbent, e.g. water, silica gel.
3. Solvent front: distance moved by mobile phase.
4. RF Value (relative/fraction) defined as a ratio of the distance the solute has traveled from the point of origin to the distance traveled by the solvent front.

ELECTROPHORESIS

Introduction:

In 1948, a Swedish Physical Biochemist, Arne Tiselius was awarded the Nobel prize in chemistry for the discovery of proteins in blood serum and for studying the properties of proteins through electrophoresis. Till now, electrophoresis continues to be an important technique to identify and characterize biological macromolecules. Amino acids, peptides, proteins and nucleic acids possess ionisable groups and can be made to exist in solution as cations or anions. When a mixture of these components is subjected to an electric field, they migrate differently and can be separated.

Principle:

Electrophoresis is defined as the migration of charged particles, under the influence of an electric field at a definite pH. In a mixture of proteins, each protein with its electrical charge will move differently in an electric field. Electrophoresis is used for the analysis of large molecules (proteins and nucleic acids) and simpler charged molecules (peptide, simpler ions).

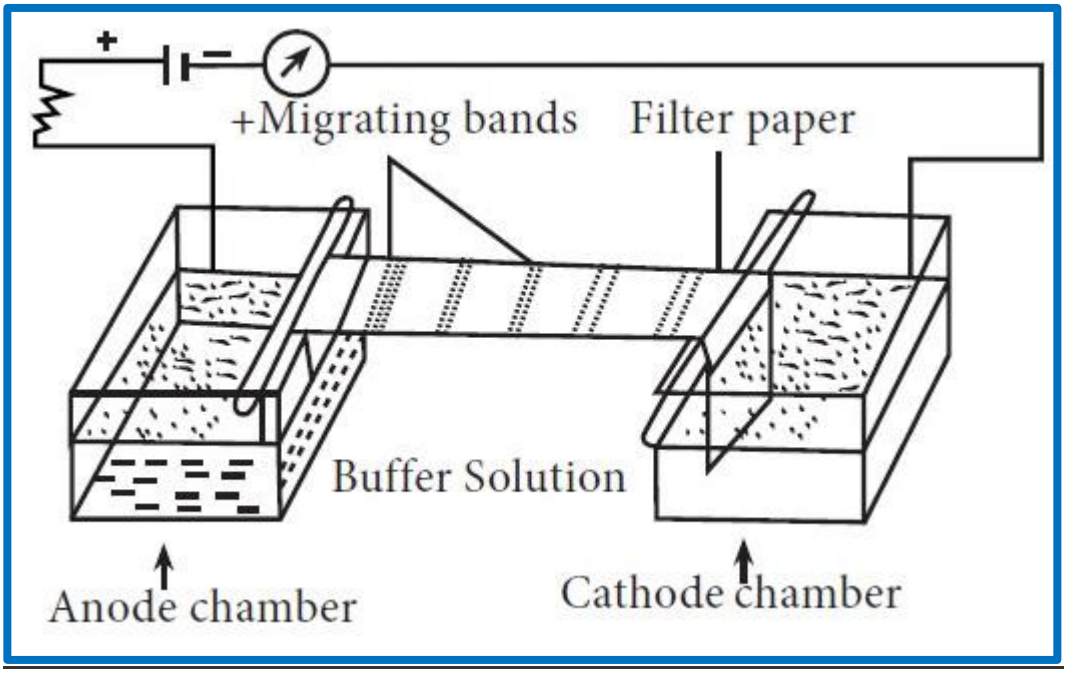
Factors that affect the rate of migration of a protein are:

- Molecular charge (net charge).
- Molecular shape and size.
- Strength of the electrical field.
- Ionic strength, viscosity, and temperature of the medium.

Types of Eelectrophoresis:

1. Paper electrophoresis.
2. Cellulose acetate electrophoresis.
3. Capillary electrophoresis.
4. Gel electrophoresis.
5. Agarose gel electrophoresis, Polyacrylamide Gel Electrophoresis (SDS PAGE, Native PAGE and two- dimensional electrophoresis).

1. Paper electrophoresis:



Paper electrophoresis

2. Gel Electrophoresis:

2, 1, Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel is prepared from acrylamide and bis-acrylamide in a suitable buffer. Polymerization of Acrylamide and bisacrylamide is achieved by a free radical reaction promoted by N,N,N',N'tetramethylethylenediamine (TEMED). This free radical process is initiated by Ammonium per sulfate (APS) used in gel. Acrylamide and bisacrylamide monomers are weak neurotoxin whereas, the polymerised polyacrylamide is non-toxic. While handling acrylamide solutions, care should be exercised and spectacles, gloves and mask should be worn.

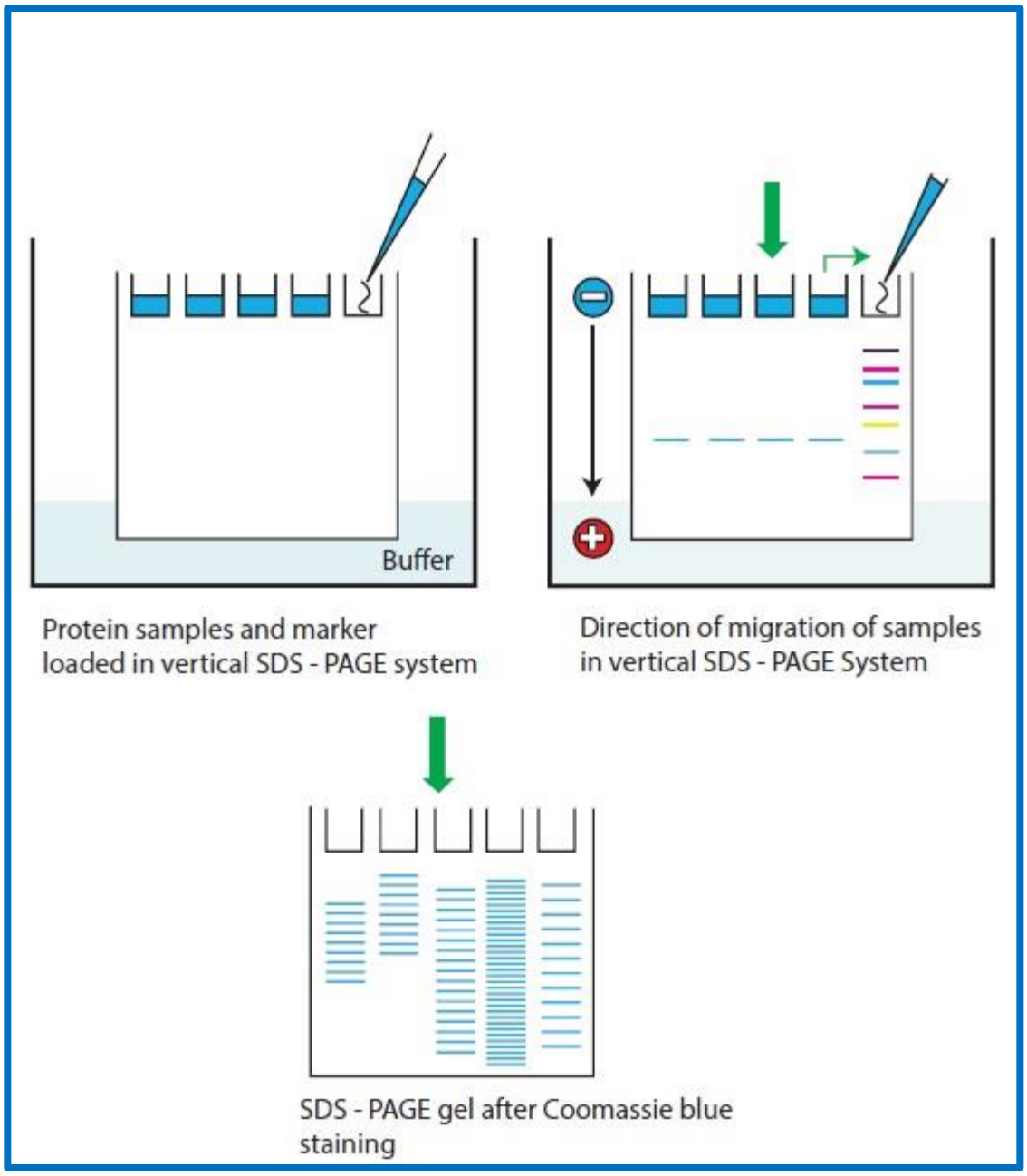
2.2. Sodium dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis:

Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is an electrophoretic technique very commonly used in Biochemistry, Molecular biology and forensic science. This technique was first described by Laemmli in the year 1970 and till now dominates in scientific research.

Electrophoresis apparatus: The electrophoretic apparatus consists of a reservoir tank to fill running buffer, transparent insulating cover, gel plates, spacers and gel comb to form wells. Platinum electrodes provide even current with the help of a regulated power pack. The gel is packed in-between two glass plates with the help of spacers. Clear wells are obtained using

comb. Samples are layered in the little slots cut in the top of the gel slab using gel comb. Buffer is cautiously layered over the samples, and a voltage is applied to the gel using power pack for a period of usually 1-3 h. The proteins migrate in the gel depending upon their electrophoretic mobility, which is dependent on the size.

Electrophoresis



SDS-PAGE

Protein samples to be run on SDS-PAGE are added to sample solubilizing buffer containing beta mercaptoethanol (disrupt disulphide bridges), SDS, glycerol (to make the solution denser and enable proteins to sink in the gel) and bromophenol blue (tracking dye).

SDS -PAGE contain resolving gel, used for separation of proteins and stacking gels for concentrating the proteins prior to entry into resolving gel. Sodium dodecyl sulphate (SDS) is an anionic detergent, which binds to proteins, and provides a constant negative charge per unit mass. Protein-SDS complexes will therefore move towards the anode during electrophoresis and their

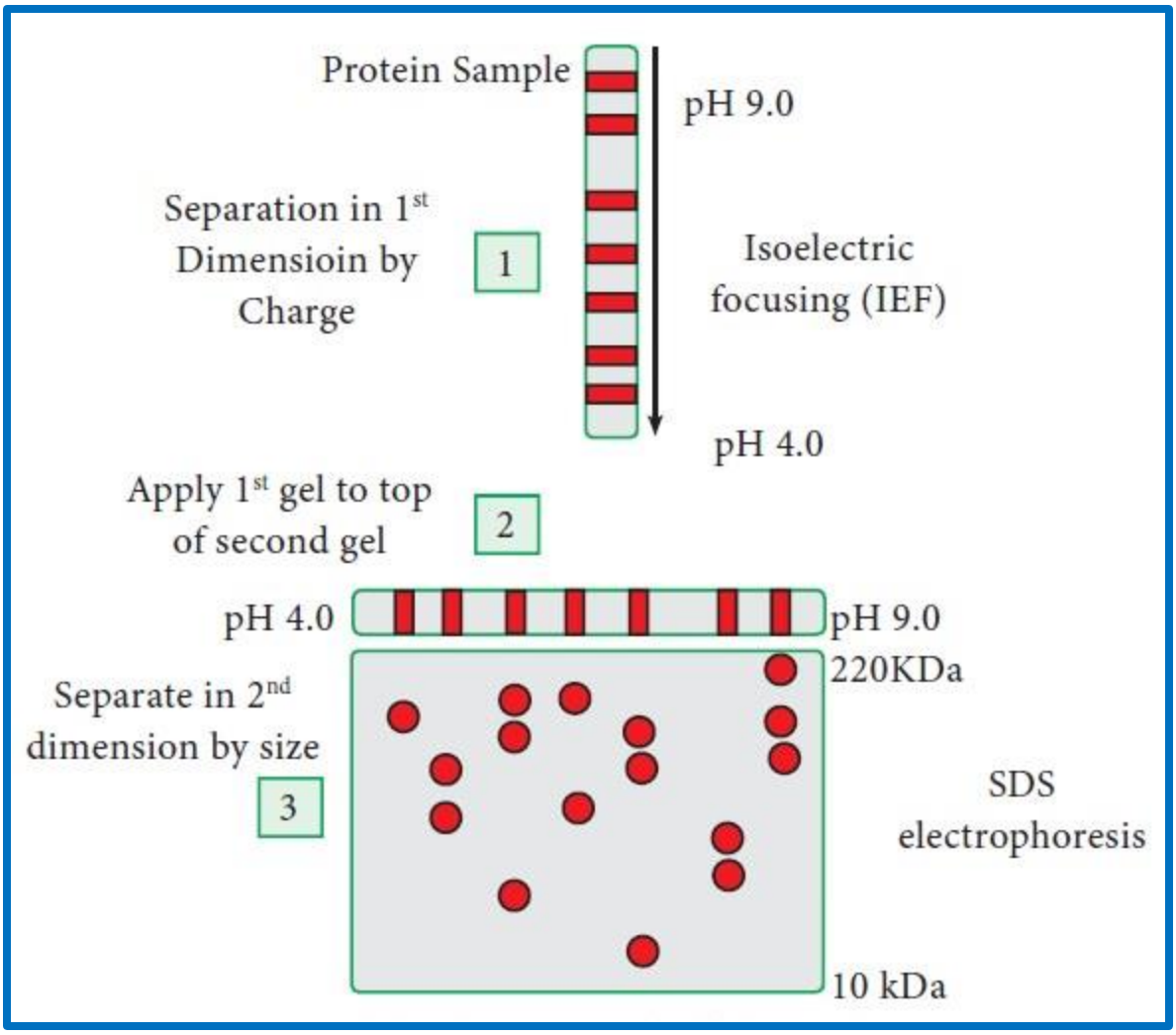
mobilities are inversely proportional to the log of their molecular weights. Since the SDS impart proteins have the same charge per unit length, all proteins travel with the same mobility. However, as the mixture of proteins pass through the resolving gel, the proteins separate, owing to the molecular sieving properties of the gel. The smaller proteins move fast as they can pass through the pores of the gel. But larger proteins move slowly since they are retarded by frictional resistance due to sieving effect of the gels. When the dye reaches the bottom of the gel, the current is turned off. After electrophoresis, the gel is carefully removed from the glass plate, immersed in buffer and stained with appropriate stain solution.

Protein staining: Proteins can be detected using Coomassie Brilliant Blue G250 (CBB) solution. CBB dye stains protein with a detection limit of 40µg. For proteins of less quantity, another sensitive detection known as silver staining (1-5ng detection limit), can be performed.

Applications: SDS–PAGE is used to determine the molecular weight of proteins. To achieve this, a standard mixture of proteins of various molecular weight (molecular weight ladder) was added for direct comparison of migration distance. The molecular weights around 15-200kDa can be analyzed in this manner.

2.3. Two -Dimensional gel electrophoresis:

Two dimensional gel electrophoresis was introduced by O'Farell in the year 1975. It is a combination of two techniques, iso electric focusing and SDS-PAGE. Iso-electric focusing is an electrophoretic technique where proteins are separated based on their iso-electric point (pI). pI is the pH at which the amino acid does not migrate in an electric field (zwitterion form). When a gradient of pH is applied to a gel, and electric field applied to it, one end becomes more positive than the other. Relatively at all pH other than its iso electric point, proteins have a charge (positive or negative) and will be pulled to the opposite side of the gel. In two dimensional electrophoresis, proteins are separated based on isoelectric point and molecular mass. To accomplish this, proteins are first separated by isoelectric focusing where they are separated by their respective isoelectric point. Second dimension of separation is achieved through SDS-PAGE, where proteins are separated according to their molecular weight. Each spot on the resulting 2D gel correspond to single protein species present in the sample.



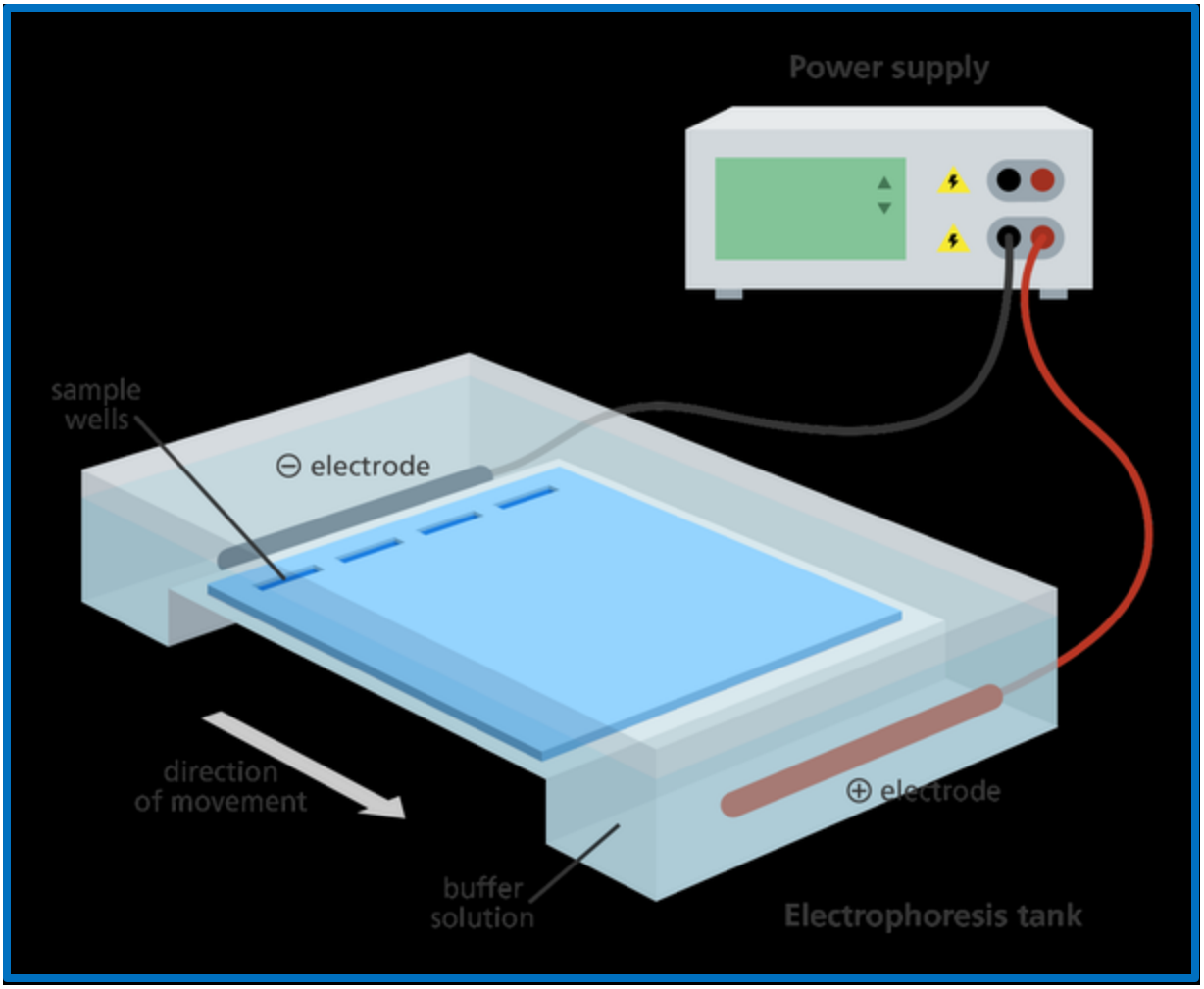
Two Dimensional Gel Electrophoresis

Applications: SDS–PAGE can be used to determine the molecular weight of proteins. To achieve this, a standard mixture of proteins of various molecular weight (molecular weight ladder) was added for direct comparison of migration distance. The molecular weights around 15-200kDa can be analyzed in this manner. Another application of SDS PAGE is to check the purity of a protein sample. Presence of a single band denote the protein sample is pure.

2.4. Agarose gel electrophoresis:

Agarose is one of the several components that can be separated from agar. The major source of agar is certain species of sea weed. Agarose is a linear polymer made up of alternating units of galactose and 3,6- anhydrogalactose. Agarose gels are completely transparent when cooled to room temperature.

In Agarose gel electrophoresis, DNA or RNA molecules can be separated based on their size. This is achieved by the movement of negatively charged nucleic acid molecules through an agarose matrix in a horizontal electrophoresis. Molecules with smaller size move faster and migrate farther as compared to longer ones. The distance between DNA or RNA bands of a given length is determined by the percentage of agarose in the gel.

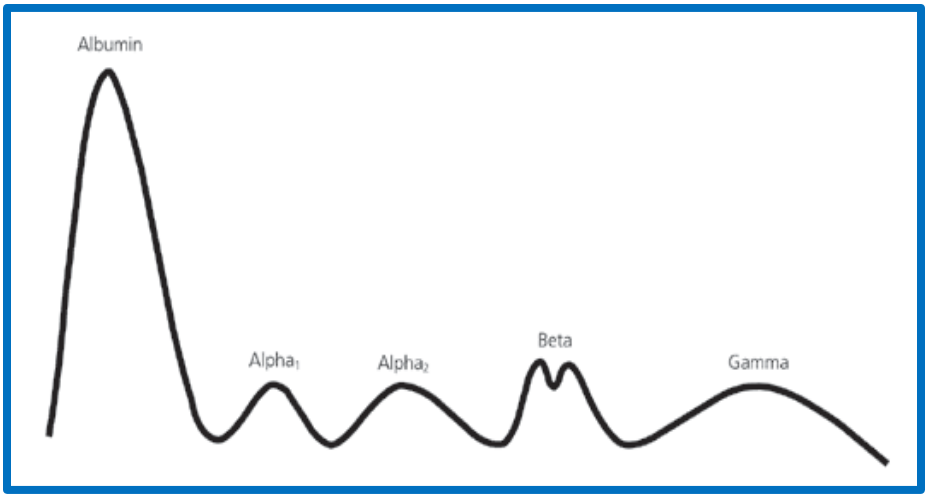


Agarose Gel Electrophoresis

Components of Serum Protein Electrophoresis:

The pattern of serum protein electrophoresis results depends on the fractions of two major types of protein: albumin and globulins. Albumin, the major protein component of serum, is produced by the liver under normal physiologic conditions. Globulins comprise a much smaller fraction of

the total serum protein content. The subsets of these proteins and their relative quantity are the primary focus of the interpretation of serum protein electrophoresis.



Typical normal pattern for serum protein electrophoresis.

Electrophoresis plays an important role in clinical diagnosis. Most body fluids can be separated into well-defined components by electrophoresis. Certain pathological conditions can produce changes that can be detected by the absence, the position or the amounts of these components after electrophoresis. For example, the separation of serum proteins into albumin and globulin fractions, has important diagnostic significance. Variations in protein fractions from their normal values occur in macroglobulinemia and multiple myeloma. The normal albumin/globulin is 1.1/1 to 2.1/1 while in the abnormal conditions above, the A/G ration is less than 1 e.g. 0.25/1 to 0.4/1 in macroglobulinemia, and 0.65 to 0.85/1 in multiple myeloma.

Plasma protein levels display reasonably predictable changes in response to acute inflammation, malignancy, trauma, necrosis, infarction, burns, and chemical injury. This so-called “acute-reaction protein pattern” involves increases in fibrinogen, $alpha_1$-antitrypsin, haptoglobin, ceruloplasmin, CRP, the C3 portion of complement, and $alpha_1$ acid glycoprotein. Often, there are associated decreases in the albumin and transferrin levels.

Experiment:
Cellulose Acetate Electrophoresis strips (Using John Tank).

Objective:
To determine the value of electrophoresis for the separation of macromolecules, namely the protein of human blood serum.

Material:
1. Barbiton buffer pH 8.6 - 1.84 9 barbituric acid, 10.3 9 of its sodium salt per liter of water.
2. Ponceau's 0.2% in 3% TCA.
3. 5% acetic acid
4. Cellulose acetate strips.

Method:
1. Place 100 ml of buffer in the electrophoresis tank.
2. Handle the cellulose acetate strip with forceps and lay on a clean paper.
3. Pencil a line very lightly across the width 3 cm from the edge, this line will be the origins for the application of the sample.
4. Moisten die strip with buffer, by dropping it gently on the surface of the buffer contained in the Perspex tank for 5 minutes.
5. Blot the strip on a sheet of clean filter paper to remove excess buffer.
6. Place the strip across the bridge and turn on the power supply and leave for 5 minutes to activate the cellulose acetate then turn off.
7. Apply the sample on the cathode side apply 200 volts for 30 minutes.
8. Turn off the power supply.
9. Remove the strips rapidly and drop on the dye solution, leave for 2-5 minutes.
10. Remove and wash out excess, dye with 5% acetic and then water until the back ground is clear.

SEPARATION OF SUBSTANCES OF DIFFERING MOLECULAR SIZES

In biochemical work it is often desired to separate large molecules such as proteins from small molecules such as simple sugars, peptides, inorganic ions, etc. one method of effecting this involves an **ULTRA FILTRATION.**

Another method **GEL PERMEATION** or process of differential diffusion across a membrane **DIALYSIS** and **FILTRATION** uses a gel-like material called sephadex. You will carry out separations using both of these procedures and compare the results obtained. The test material will be a simple one – a mixture of a coloured protein (haemoglobin, M. Wt-65.000) with small organic ions (a salt).

Proteins isolated from natural sources are often contaminated with organic ions, buffer material, etc., which must be removed if pure protein is required; so the separation of your simple mixture closely resembles many real problems.

For the dialysis, the ions will be sodium and chloride. For the gel permeation, the ions will be potassium and chromate. The chromate is yellow so its separation from the reddish-brown colour of haemoglobin is easy.

DIALYSIS THROUGH MEMBRANES

In dialysis the solution of large and small ions is placed in a closed bag of acetate film, which is a material permeable only to water, small molecules and small ions. The membrane bag is then placed in a vessel containing distilled water, and the diffusible species pass through the membrane. If the outside fluid (diffusate) is changed frequently, all the diffusible ions and molecules will eventually be remove from the bag, leaving behind the non-diffusible substances. Dialysis is used in ‘Artificial Kidney' to remove waste products from the blood of patients with impaired urinary excretion.

DIALYSIS

You are provided with a solution of haemoglobin (10 mg/ml), and a solution of sodium chloride (1 M). Prepare the mixture for separation by taking 2 ml of the protein solution and 2 ml of the salt solution, Pipette 2 ml of the mixture into a piece of dialysis tube previously wetted in distilled water and knotted at one end. Now knot the other end so that the mixture is enclosed in the dialysis bag. Place the bag in??? Containing 50 ml distilled water and invert, at frequent intervals, to ensure that the bag is completely immersed. Replace the outside liquid with fresh distilled water every 10 minutes. Store about 5 ml from each diffusate for later examination.

Examination of diffusate for chloride and haemoglobin:

1. Chloride: take one ml of the sample and add a few drops of dilute nitric acid followed by a drop of silver nitrate solution. A white precipitate of silver chloride indicates the presence of chloride in the sample.
2. Haemoglobin: although this coloured protein can be detected visually at considerable dilution, a more sensitive test is required for very small amounts. Ask the demonstrator about HEMASTIX.

Principle of hemastix:

Haem portion of the haemoglobin acts as a catalyst for the conversion of a colourless dye on the stix to a blue oxidation product. If hemastix is not available, test for the presence of haemoglobin by pipetting 0.5 ml of the diffusate and adding 3 ml of salicylic acid solution.

Hemastix

GEL PERMEATION

Sephadex is an insoluble macromolecular material which forms a three-dimensional network enclosing solvent (gel). The pores or spaces are of such small dimensions that only species of low molecular weight can penetrate into the solvent within the gel. Large molecules are 'excluded.

If a mixture of small and large molecules is applied to the top of a column sephadex gel, followed by eluting “washing" solvent, only the small molecules with permeate into the pores of the gel. The large molecules will be completely excluded from the eluting solvent. They can be collected as they emerge free from the small molecules which, because they are distributed in a large volume of fluid, namely the fluid both side and inside the gel, travel more slowly. As washing continues, the small molecules too will be eluted, and can be collected.

Sephadex is manufactured with different pore sizes, so that separaticns are not confined to low against high molec'ilar weight species, but can be applied to separate, say molecular weight of 10 from those of 10.

Experiment

You are provided with a sephadex gel of the type which excludes species of molecular weight greater than 5000. Take a small piece of glass-wool and carefully role it into a ball and use it as a plug for the pipette which you shall use as a column. Using another dropper pipette draw some of the already hydrated sephadex into the column. Clip the column to a burette stand. Do not allow the gel to dry. 1 ml of the albumin and 1 ml of M ammonium sulphate. Apply a small amount of (0.25 ml) of the mixture to the top of the column. Elute using the buffer provided. Collect each 5 drops in a separate 10 ml test tube and examine for albumin and sulphate as follows:
For SO_4 add a few drops of barium chloride and observe the precipitate. For albumin and equal volume of 10% NaOH and 3 drops of 2% $CuSO_4$ Observe the colour developed. A pink-purple colour indicating the presence of protein.

Results

1. Did sephadex separate the protein from the salt?
2. Can you suggest other method for separation of these two Inolecules?
3. What is the material of the sephadex?
4. What is the basis of separation achieved by the used of sephadex?
5. What is the significance of the designations sephadex G-25, G-100 etc?
6. Could gel-permeation be used to determine the molecular weight?

COLORIMETRY

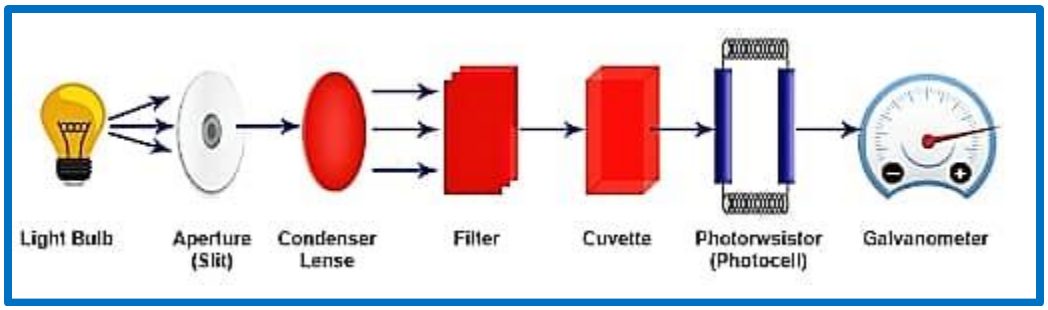
Colorimeter is a light-sensitive device that helps certain **solutions** absorb a particular **wavelength of light** in colorimetry. It is used to measure the absorbance and transmittance of **light** that passes through a liquid. Colorimeter can also be used to determine the concentration of a coloured compound in a solution. The principle of colorimeter is based on the fact that coloured compounds can absorb a certain wavelength of light when monochromatic light is passed through them. The working of a colorimeter is based on the concept of **Beer-Lambert's law**. It was invented by Louis J Duboscq in the year 1870.

Colorimeter

Components of Colorimeter:

The main parts that make up the colorimeter are as follows:

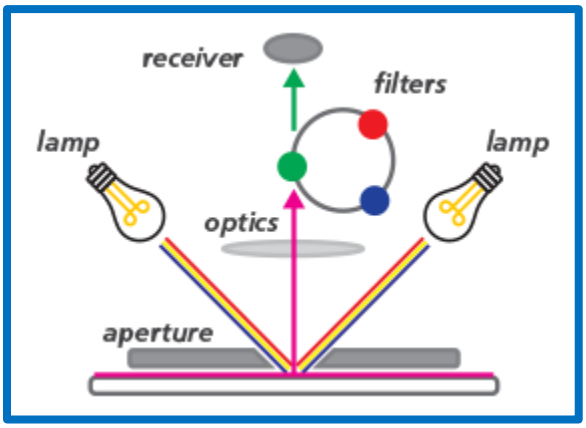
- **Source of Light:** Tungsten filament is commonly used as a light source in colorimeters.
- **Monochromator:** It is used to split the light into different wavelengths and select the particular wavelength under observation.
- **Sample Holder:** This is where the cuvettes or test tube containing the colour sample solution is placed. These are made of glass at visible wavelengths. **Photo Detector System:** This system produces an electric signal when light falls into it which is in turn reflected as a reading in the **galvanometer**.
- **Measuring Device:** The galvanometer is used as the measuring device where it converts the electrical signals into readings that correspond to the intensity of light.



Components of Colorimeter

Working of Colorimeter

1. Colorimeter has to be calibrated first by using the standard solutions of the known concentration of the solute that is to be determined in the test solution. The standard solutions are poured into the cuvettes which are then placed in the sample holder.



Working of Colorimeter

2. A beam of light of a certain wavelength specific to the assay is directed towards the test solution. Before it reaches the test solution, it passes through a series of **lenses** and filters. The lens helps in accurate navigation of the beam of light. The filters split the incoming light into different wavelengths and allow the required wavelength to reach the cuvette containing the test solution.
3. The monochromatic light (light of one wavelength) reaches the test solutions and some of the light gets reflected, some would get absorbed and the remaining would pass through the test solution and falls onto the photodetector. The photodetector sends the pulses to the galvanometer. The galvanometer reads the electrical signals from the detector and displays them in digital form. The reading corresponds to the absorbance or the optical density of the test solution.
4. To determine the absorbance or optical density of the test solution, the following formula is used.

We know that, $A = \in cl$

For standard and test solutions, $\in$ and l are constant.

Therefore:

- $A_T = C_T$ …(i)
- $A_s = C_s$ …(ii)

Cross multiplying (i) and (ii):

- $A_T \times C_s = C_T \times A_s$
- **$C_T = (A_T / A_s) \times C_s$**

Where:

- $C_T \rightarrow$ Concentration of test solution.
- $C_s \rightarrow$ Concentration of standard solution.
- $A_T \rightarrow$ Absorbance or Optical density of test solution.
- $A_s \rightarrow$ Absorbance or Optical density of the standard solution.

- Colorimeter is widely used in the medical industry to estimate biochemical samples such as blood, urine, cerebral spinal fluid, plasma, serum, etc.
- They are used to analyse the colour contrast and brightness in mobile, computer and television screens to provide users with the best viewing experience.
- It also finds its application in the paints and textile industries.
- Colorimeter is used in the food and food processing industry.
- It is used in the printing industry to measure the quality of print paper and printing ink.

Uses of Colorimeter:

- They are also used to test the water quality and screen for the identification of chemical substances such as chlorine, fluorine, cyanide, iron, molybdenum, etc.
- They are used in jewelry to measure diamond quality.
- Colorimeter is used to measure the concentration of haemoglobin in **blood** samples.
- It helps to monitor the nutrient concentration in the **soil** for plant growth.
- Colorimeter is also used in the pharmaceutical industry to identify substandard products and drugs.

Advantages of Colorimeter:

The benefits of using a colorimeter are:

- Colorimeter is a cheap and efficient method of quality analysis.
- Portable colorimeters are available which makes them convenient to use.
- Quantitative analysis of coloured compounds can be easily done by using Colorimeter.

Disadvantages of Colorimeter:

There are some disadvantages of using colorimeter also which are as follows:

- It becomes a tedious process to identify the concentration of colourless compounds.
- Since Colorimeter measures the absorbance of wavelength only in the **visible spectrum of light** (400nm to 700nm), it does not work in the **ultraviolet** and **infrared spectrum**.
- It is not possible to set a specific wavelength; rather a range of spectrum has to be set to measure the absorbance.

Determination of the absorption spectrum of haemoglobin.
The absorbance of a 5 g/dl solution of haemoglobin is measured at different wavelength. By potting absorbance is against wavelength a grave called an absorption spectrum is obtained.

Method:

1. Switch on the instrument allows 5 minute warming up.
2. Fill a cuvette with 1 cm of the top with water and another cuvette with haemoglobin solution (5 g/dL).
3. Piace a colour filter 490 nm in the slot provided in the colorimeter.
4. Place the cuvette containing dist, water at the sample tube position and adjust the galvanometer needle to read zero absorbance.
5. Replace the tube containing water with that containing the haemoglobin solution. Note the reading of the absorbance scale. This being the absorbency of haemoglobin solution at 490 nm.
6. Repeat, with other liter 520/540/... 71 0 every time you change the filter adjust absorbance to zero with distilled water. Tabulate your results as follow:

Wavelength nm	Absorbance
490	
520	
540	
580	
600	
700	

7. Plot a graph with wavelength as abscissa and absorbance as ordinate and from this graph read off the absorbance at which maximum absorption occur.

The determination of concentration of a haemoglobin solution

The determination of the concentration of compound in tissues and in biological fluids is fundamental to an understanding of metabolic process and also to diagnosing illness.

Method:

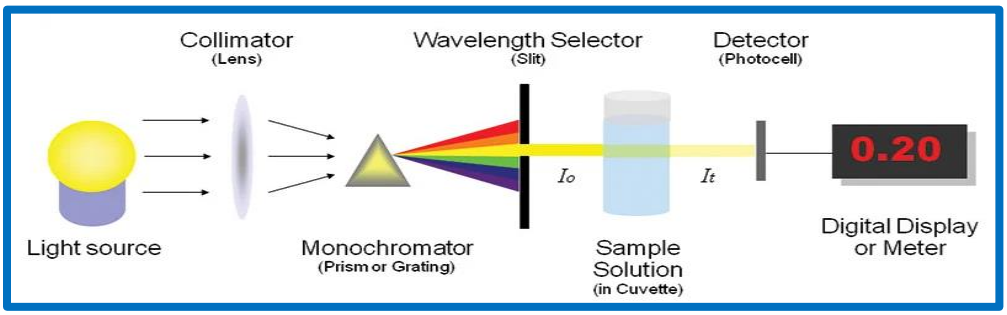
1. From the 10 g haemoglobin solution provided prepare a serial dilution following the table below:

Tube Number	1	2	3	4	5	6
Water (ML)	5	4	3	2	1	-
Haemoglobin(ML 10 g/dl)	-	1	2	3	4	5

2. Using the filter which gives highest absorption (from the previous experiment) determines the absorbance of each tube using tube no. 1 as blank.
3. Add zero using tube no. 1 (distilled water). Now plot a grave of absorbance (ordinate) against haemoglobin concentration. (Abscissa).
4. This is known as standard, curve of calibration then determines the absorbance of unknown from the graph obtained above, read off the concentration of the unknown solution.
5. NB: the unknown concentration can be calculated from the formula.
6. Unknown concentration = Absorbance of unknown X Concentration of standard Absorbance of standard
7. Apply the above formula using absorbance and concentration of tube no. 6 as standard calculates the concentration of the unknown. The result obtained in both condition should be the same.

Spectrophotometry

- A spectrophotometer is an instrument that measures the amount of light absorbed by a sample.
- Spectrophotometer techniques are mostly used to measure the concentration of solutes in solution by measuring the amount of the light that is absorbed by the solution in a cuvette placed in the spectrophotometer.
- Scientist Arnold J. Beckman and his colleagues at the National Technologies Laboratory (NTL) invented the Beckman DU spectrophotometer in 1940.



Components of Spectrometer

Principle of Spectrophotometer:

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device.

1. In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.
2. By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.
3. The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength.

Instrumentation of Spectrophotometer:

The essential components of spectrophotometer instrumentation include:

1. A table and cheap radiant **energy source**

- Materials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.

2. A **monochromator**, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.

- A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

Prisms:

- A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent.
- Two types of Prisms are usually employed in commercial instruments. Namely, 600 cornu quartz prism and 300 Littrow Prism.

Grating:

- Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

3. **Transport vessels** (cuvettes), to hold the sample.

- Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as "CUVETTES".
- Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz.

4. A Photosensitive **detector** and an associated **readout system.**

- Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it.
- Radiation detectors generate electronic signals which are proportional to the transmitter light.
- These signals need to be translated into a form that is easy to interpret.
- This is accomplished by using amplifiers, Ammeters, Potentiometers and Potentiometric recorders.

Applications:

Some of the major applications of spectrophotometers include the following:

- Detection of concentration of substances.
- Detection of impurities.
- Structure elucidation of organic compounds.
- Monitoring dissolved oxygen content in freshwater and marine ecosystems.
- Characterization of **proteins.**
- Detection of functional groups.
- Respiratory gas analysis in hospitals.
- Molecular weight determination of compounds
- The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

Types of Spectrophotometer:

1. **Fluorometers:** It is used to measure the fluorescence discharge once the given sample is exposed to a single wavelength of light.
2. **Atomic absorption spectrophotometer:** A flame fumes the water from the specimen causing it to separate into ions. The dissociation leads to changes in the intensity of light as seen by the detector. Hence, help in finding out the concentration of the sample. Atomic absorption spectrophotometer's high precision analysis is useful in toxicology, environmental testing, and quality control laboratories.

There are present different types of Spectrophotometer. They are classified based on their application/uses.

3. **Visible light spectrophotometer:** use visible light from a tungsten lamp; mainly used for routine laboratory practice, particularly the portable and bench-top spectrophotometer models.

4. **UV vis spectrophotometer:** As compared to visible light spectrophotometer, this type of microscope contains a second lamp. UV/Visible spectrophotometer can measure up to 1100 wavelengths. It has different characteristics such as scanning function, user interface, integral printer, and multiple cell setting.

   - The UV/Visible spectrophotometer is divided into three classes such as;

- **Single beam:** In a single beam spectrophotometer, a reference standard is used to measure light intensity before and after the sample is loaded. These are typically less expensive and have higher sensitivity.

- **Double beam:** In a double beam spectrophotometer the beam is split with one beam passing through the standard and the other through the sample to compare intensities. The double beam spectrophotometers tend to be more stable and easier to use.

- **Split beam:** In this type of spectrophotometer, the light emitted by the same monochromator is split into two beams, one of which reaches the detector directly and the other passes through the sample and reaches the other detector. The advantage of this instrument is that it monitors errors in the light source, but does not eliminate the effects of the reference.

5. **Near-infrared spectrophotometer:** This type of microscope is used to measure the response of a sample when exposed to infrared light. It gives a non-invasive analysis and a quantitative finding with only the least specimen preparation. Those solid samples have a high absorbance, they are mainly monitored by using this Near-infrared spectrophotometer.

6. **Nuclear Magnetic Resonance spectroscopy:** It is used to identify the structure of organic compounds. It gives structural detail of the entire molecule as well as dynamic information of organic reactions.

7. **Mercury spectrophotometer/analyzer:** Used to measure the amount of mercury in water.

Types of Spectrophotometer

Practical No. 6

Enzymes

Introduction

Many reactions in the cell occur very rapidly, at about neutral pH, low concentrations of reacting substance, and at temperature between 37°C-41°C according to the organism. It has been found that most of these reactions catalyzed by large organic molecules called enzymes.

Enzymes are mostly protein catalysts that increase the velocity of a chemical reaction and are not consumed during the reaction they catalyzed. They are highly specific, interacting with one or a few substrate and catalyzing only one type of chemical reaction. Enzyme molecules contain special pockets called the active sites. The active sites contain amino acid side chains that create a three dimensional surface complementary to substrate. The active site binds to the substrate, forming an enzyme-substrate (ES) complex. (ES) is converted to enzyme-product (EP) complex, which subsequently dissociates to enzyme and product.

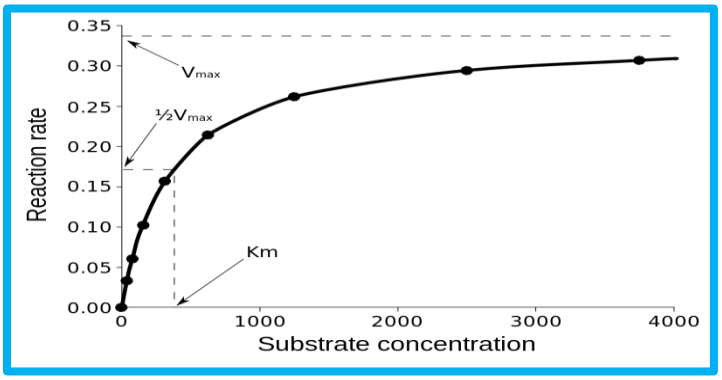
$$E + S \rightleftharpoons ES \rightarrow P + E$$

Some enzymes associate with a non-protein cofactor that is needed for enzymatic activity, these include metal ions (e.g. Zn, Fe) and organic molecules, known as coenzymes (e.g. NAD, FAD, coenzyme A).

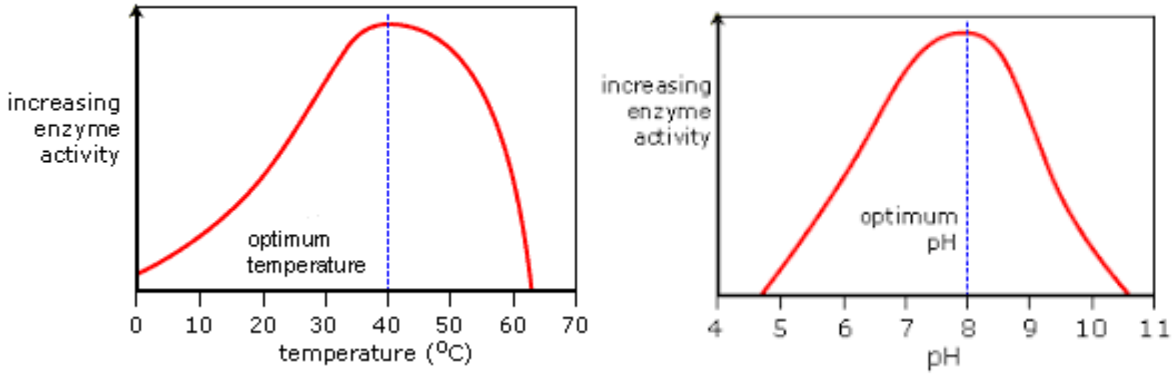
There are multiple factors affecting the rate of enzyme-catalyzed reactions, thus different enzyme shows different responses to changes in substrate concentration, temperature and pH.

1. ***Substrate concentration*:** The rate or velocity or a reaction (V) is the number of substrate molecules converted to product per unit time and is usually expressed as micro mole product formed per minute. The rate of an enzyme-catalyzed increases with the substrate concentration until a maximal velocity (V max) is reached. The leveling off of the reaction rate at high substrate concentrations reflects the saturation with the substrate of all available binding sites on the enzyme.

Reaction Velocity:



2. ***Temperature:*** The rate of enzyme-catalyzed reaction initially increases as the temperature rises owing to increased kinetic energy of the reacting molecules. Eventually, however, the kinetic energy of the enzyme exceeds the energy barrier for breaking the weak hydrogen and hydrophobic bonds that maintain its secondary-tertiary structure. At this temperature denaturation, with an accompanying loss of catalytic activity, predominates. Enzymes, therefore exhibit an optimal temperature at which the reaction is maximal.



3. pH: Hydrogen ion concentration has a well-marked effect on enzymatic activity, most enzymes showing optimum activity at a definite pH (fig. 2). This optimum pH is dependent upon a number of factors:

1. Stability of the enzyme protein at different hydrogen ion concentrations.
2. Effect of pH on the ionization of the enzyme protein, and
3. Effect of pH on the combination of enzyme and substrate.

EXPERIMENT ON FACTORS THAT AFFECTS CATALYTIC ACTIVITY

Enzyme reaction:

6.1. Preparation of salivary amylase:

1. Rinse out the mount with a little warm water.
2. Take 15 ml of warm water (40°C) and circulate in the mouth for at least 1 minute.
3. Collect the washing in a beaker.
4. Repeat (b) and (c).
5. Dilute the combined washing with two volumes of water.
6. Filter and use the filtrate as your enzyme for the following experiments.
7. Divide the filtrate into two portions and boil one portion to be used as control.

EFFECT OF TEMPERATURE ON AMYLASE:

Method:

1. Place a few drops of iodine in the holes of the plate.
2. In two test tubes add 5 ml of starch solution (buffered pH 7).
3. In one tube add 3 ml of amylase solution (your enzyme), and into the other tube add 3 ml of boiled amylase (control).
4. Mix both tubes and place in water batch, at 38°C (room temperature).
5. Using a glass rod remove immediately a drop of a mixture from each tube and place against iodine drop, both tubes should give a blue colour, if no colour is obtained, then the amylase solution is too much concentrated and must be diluted with equal volume of water.
6. At interval of one minute remove few drops of amylase solution from each tube and place against iodine drops.

Observation:

A. The **blue** colour given by the control is given each time doing it once at start, and at the end of the experiment.
B. In case of the other tube, the colour becomes **reddish brown, pink** and finally disappears, (this is the time taken to reach the chromic point)

C. This experiment is the same as the above experiment except that it's done at different temperatures. Now repeat steps as experiment above using different temperature e,g. ice, 60°C.

Questions:

1. What are your conclusions?
2. What is the optimum temperature of the enzyme?

Effect of the pH of the medium on amylase:

You are provided with different pH solution pH_4 (acid) pH_7, (neutral) pH_9, alkaline.

1. Mix 3 ml of unbuffered starch and 1 ml of buffer pH_4, incubate at 38°C for 5 minutes.
2. Add 2 ml of amylase enzyme mix then immediately remove few drops and place against iodine drops as above and repeat at interval of one minute until the colour disappear.
3. Record the time required to reach the chromic point.
4. Now repeat with pH_7, and pH_9.
5. **Plot a graph of $\frac{1}{t}$ vs pH;** where the time taken to each the chromic point.

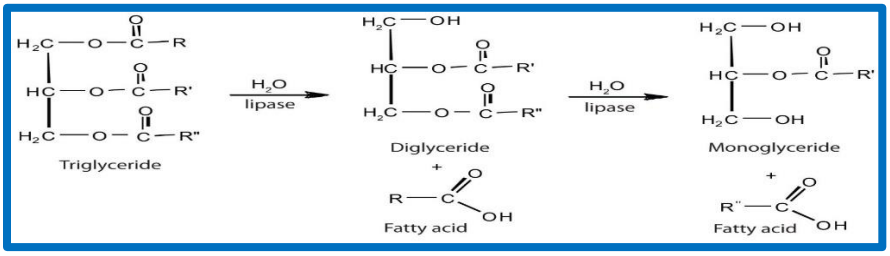
6.2. Kinetics of lipase action:

Introduction:

Dietary lipids are insoluble in water, hence, the enzymatic hydrolysis of lipids, such as triacylglycerols, can occurs only on the surface or lipids droplet-that is- at the interface between the lipid droplet and the surrounding aqueous solution. Therefore, before ingested triacylglycerols can be absorbed through the intestinal wall, they must be converted from the insoluble macroscopic fat particles into finely dispersed microscopic micelles. Bile salts are synthesized from cholesterol in the liver, stored in the gallbladder, and released in the small intestine after ingestion of a fatty meal. Bile salts act as biological detergent, converting dieter fats into mixed micelles of bile salts and triacylglycerols. Micelle formation increases the fraction of lipid molecules accessible to the action of water-soluble lipases in the intestine.

Principle:

- Milk contains fats and the pancreatic preparation contains lipase. On the addition the lipase starts hydrolyzing the milk fats with the release of free fatty acids. On titration of the above mixture with sodium hydroxide, salts of fatty acids are formed.
- The process of hydrolysis proceeds gradually till all the active sites of the enzyme have been saturated with the substrate molecules.



Materials:

1. Whole boiled milk diluted with distilled water 1:1.
2. Crude preparation of pancreatin:

a. Dissolve 1 g of pancreatin in 100 ml of 0.4% NaHCO;.
b. Check the pH of the solution with phenolphthalein, it should be slightly alkaline.

1. 0.1% solution of phenolphthalein.
2. 50 ml of bile.

Method:

1. Into two beakers labeled as "test" and “control" pipette 10 ml of diluted milk each.
2. In the test breaker add 1 ml pancreatin and in the control beaker add I ml of distilled water.
3. Immediately, at zero time, pipette out 2 ml of mixture in the test beaker into a conical flask.
4. Add 2 or 3 drops of phenolphthalein and titrate against 0.01 N NaOH till the colour changes to pink. Repeat with the control.

5. Repeat the titration of both the test and control solutions at regular 10 minutes intervals starting from zero time for 40 minutes.
6. Fill your titration data as in the table shown below and draw the plot of number of NaH against time starting from zero time.

Time	Test (mls of NaOH)	Control (mls of NaOH)	Test-control
0	2.0	2.0	0
10	25	25	0.5
20	3.0	3.0	1.0
30	3.5	3.5	1.5
40	Etc	Etc	Etc

Practical No. 7

Vitamins

Introduction:

Vitamins are essential organic food stuff required for certain metabolic functions, thus must be provided in the diet in trace amounts (<50 mg/day) for normal health.

Some vitamins can be synthesized in the body but in very tiny amount and hence need to be supplied in the diet e.g.

- Nicotinic acid (vitamin B_3) synthesized tryptophan in the body.
- Vitamin D from 7-dehydrochoesterol in skin.
- Vitamin K by intestinal microbial flora.

Classification:

1. Fat soluble vitamin (A, D, K and E).

2. Water soluble vitamin

Characteristics of water and fat-soluble vitamins:

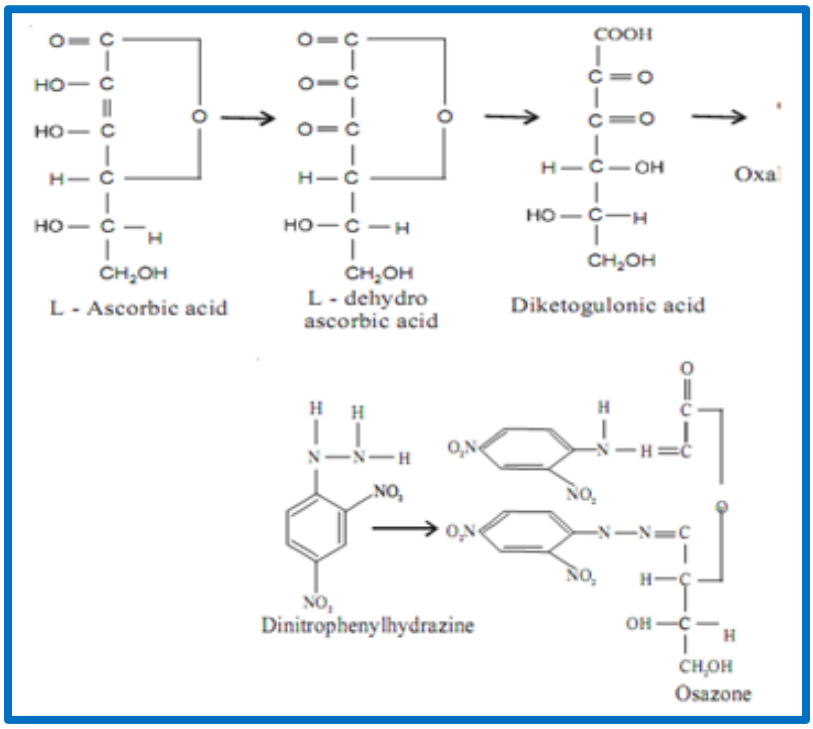
Characteristics	Fat soluble vitamins	Water soluble vitamin
1. Solubility	Insoluble in water	Soluble in water
2. Absorption	Accompanies dietary fat absorbed digestion and absorption	Fat Freely
3. Transport	Needs carrier in blood e.g. vitamin D needs binding protein	No carrier (except vitamin B_{12})
4. Storage	Stored. DR (Daily Recommended Requirement) is low. Deficiency diseases appears after a time.	Not stored Any excess appears in urine. DR is high. Deficiency appear readily appear e.g. scurvy (vitamin C deficiency) starts in 4 months
5. Hyper-vitaminosis	Is common due to storage	Rare
6. Thermal stability	High	Thermolabile

Water Soluble Vitamins:

Ascorbic acid (vitamin C):

Ascorbic acid can be synthesized, in, all mammals except the primates including mammals (human beings) and guinea pig. Lake of ascorbic acid in the diet of primate leads to the disease known as scurvy in which the body showed decreased ability for wound repair. Ascorbic acid is found in fresh fruits and vegetables.

The quantity of ascorbic acid required in all mammals per day by man to prevent the development of scurvy is 10-15 mg. most individual normally intake from 30.80 mg/ day in their diet, daily output is 20-30 mg excess ascorbic acid that is not needed by the body is excreted in the urine. Thus, the estimation of ascorbic acid in the urine gives an indication of its amount in the body.



Osazone formation from ascorbic acid

Experiments:

7.1. Estimation of ascorbic acid in urine:

Principle:

Ascorbic acid is oxidized to dehydroascorbic acid by cupric sulphate, the dehydroascorbic acid in strongly acid solution reacts with 2.4-dinitrophenyI hydazine (2.4 DNPA) form dinitro-hydrazone. The hydrazone in the presence of strong sulphuric acid develops red colour which can be measured at 408 nm by colorimeter.

Method:

1. Label 3 test tubes (Test, Standard, and Blank).
2. Add the following to the above test tubes.

	Test	Standard	Blank
Urine	1 ml	--	--
Standard	--	1 ml	--
Water	--	--	1 ml
2.4 DNPH	0.5 ml	0.5 ml	0.5 ml
H_2SO_4	2	2	2

3. Leave at room temperature for 10 minutes and read standard and test against blank at 490 nm using the colorimeter.

7.2. Estimation of Riboflavin in aqueous solution:

Riboflavin in aqueous solution shows a yellow - green fluorescence under UV light. This property can be used to estimate riboflavin.

Method:

1. Examine the fluorescence of 1 mg riboflavin per 20 ml water under the UV lamp.
2. Place the solution in glass tubes which can show little fluorescence.
3. Use water as a control.

Vitamin A deficiency:

- Vitamin A is a fat-soluble vitamin. Night blindness and exophthalmia develop in man if vitamin A is absent from the diet.
- Rich sources of vitamin A include eggs, liver, fish Oils, milk and vegetables.
- Vitamin A and carotene in chloroform solution from a transient blue colour with antimony trichloride. This reaction is the basis for the quantitative estimation of Vitamin A in the tissues and food stuffs.

7.3. Quantitative estimation of Vitamin A in the tissues and food stuffs:

Method:

1. In two test tubes, add 3 ml of dry chloroform solution of cold liver oil and olive oil.
2. To each tube, add 1 drop of acetic anhydride (to ensure anhydrous conditions. Otherwise a precipitate of antimony oxychloride will be formed)

3. Add 3 ml of a saturated of antimony chloride in chloroform.
4. Record your observation, and complete the vitamin A content of the 2 tubes.

SECTION TWO

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Clinical biochemistry laboratory

Introduction:

Clinical biochemistry laboratory deals with laboratory applications to help in diagnosis of various diseases in correlation with clinical symptoms.

The chemical constituents of various body fluids such as blood (serum or plasma) urine, cerebrospinal fluid (CSF), and other body fluids are analyzed and these constituents have normal level in the body fluids. Any elevation or decrease in these level indicates abnormality in the organ concerned with this constituent and each organ have a specific test, etc.

The clinical biochemistry laboratory tests in relation to the various clinical conditions can give the following:

1. Reveal the causes of the disease.

2. Suggest effective treatment.

3. Assist in monitoring progress of pathological condition, and help in assessing response to treatment.

Practical No. 1

The Composition of Normal Urine

By regulation the extent excretion or/and re-absorption of substances in glomerular filtration the kidney plays an important part in maintenance of the normal composition of body fluids. Disorder and diseases which causes changes in the body fluid usually affect the composition of the normal urine. However, since the urine formed by a normal person varies considerably during the day, for reasonable accurate analysis it is necessary to use 24 hours urine sample, the volume of which is usually1 to 1.5 liter. Since bacterial growth occurs readily in urine it is necessary to add preservative such as chloroform or other preservative to the 24 hours urine sample.

Tests carried out on a sample of urine:

1.1. Determination of pH:

Method:

1. Determine the pH by the use of pH indicator paper. It is usually acidic (5.5-6.5)

1.2. Specific gravity (S. G.):

Method:

1. Use a hydrometer to estimate the S.C of the urine sample
2. Correct the reading for temperature marked on the hydrometer (15°C), Add one unite in the third decimal place for every 3°C above the standard temperature.
3. Normal range for random urine sample is (1.003-1.035) 24-hour urine (1.015-1.030).
4. Calculate the approximate concentration of solid by means of formula:

   Total solid in gram/liter = 2600 (S.G of urine - S.G of water).

Chemical Tests for urine:

1.3. Inorganic constituents of urine:

1.3.1. Chloride:

Method:

1. To 2 ml of urine add 3 drops of concentrated nitric acid.
2. Then add 3 drops of silver nitrate.

Observation:

A w**hite** precipitate is formed (Ag CI), if chloride is present.

1.3.2. Calcium and Phosphate:

Method:

1. To about 10 ml of urine add 5 drops of concentrated ammonia and boil.

Observation:

A **white** precipitate of calcium and phosphate is formed.

2. Filter off and dissolve the filtrate in dilute acetic acid (spread the filter paper in small beaker then add the acid).

3. Divide this into two parts:

1. To one part, add 2 ml of 5% ammonium oxalate solution, a precipitate indicates the presence of calcium (calcium oxalate is formed).
2. To the other part add 5 drops of **c**oncentrated, nitric acid and 2 ml of 5% ammonium molybdate and boil.

Observation:

A **yellow** color is formed indicating the presence of phosphates.

1.3.3. Ammonia:

Method:

1. Into 3 ml of urine add 3 drops of phenolphthalein and few drops of sodium carbonate solution until the mixture Become red (pH 9).
2. Boil and test for ammonia by holding a Drop of HCI on a glass rod at the mouth of tube until white Fumes of ammonium chloride are seen.

1.3.4. Sulphate:

Method:

1. Into 3 ml of urine add 3 drops of HCl.
2. Add 8 Drops of Barium chloride.

Observation:

A **milky** precipitate of Barium sulphate is formed indicating the presence of sulphate.

1.4. Organic Constituent of Urine:

1.4.1. Urea:

This is the major nitrogenous substance in urine and is derived from the catabolism of proteins and amino acid. Normally, about 30 g is excreted per day on a diet containing about 100 proteins.

Experiments of urea:

a. Conversion of Urea into Biuret:

Methods:

1. Heat a little solid urea in a dry test tube very gently over small flame.
2. As the urea melts and ammonia is evolved and biuret is formed.
3. Cool and dissolve the biuret in 3 ml of dilute Na OH
4. Add 4 drops of dilute Cu SO, solution.

Observation:

Note the colour of the copper salt of biuret:

$$2\ NH\text{-}CO\text{-}NH_2 \text{ ---------- } NH_3 + NH_2\text{-}\ CO\text{-}NH_2$$

b. Action of Alkaline Hypobromite:

Methods:

1. To 5 ml of urine add few drops of alkaline sodium hypobromite.
2. Effervescence occurs, N_2 being given off and CO_2 observed by alkali.

c. Action of Urease:

Methods:

1. To 2 ml of urine add 4 drops of phenol red and 1 ml of urease solution.
2. Mix and allow to stand at room temperature.

Observation:

The mixtures becomes **red** as the enzyme converts urea into carbon dioxide and ammonia.

$$H_2N\text{-}CO\text{-}NH_2 + H_20 \xrightarrow{\text{urease}} CO_2 + 2NH_3.$$

1.4.2. Uric acid:

This is a very poorly soluble substance which is the end product of purine metabolism. About 0.5-1.0 g is executed each day.

Phosphotungstic test:

Methods:

1. To 3 ml of urine add 3 drops of Bendict's uric acid reagent (arseno-phosphotungsticacid very toxic) and 4 drops of sodium carbonate.

Observation:

The mixture become **blue** if uric acid present.

(Note this test is not the same as Bendicts test for reducing substances).

1.4.3. Creatinine:

This is the anhydride of creatine of muscle. The 24th excretion is roughly 1.0 - 1.5 but is very constant for an individual and reflects his muscular development.

Gaff's test:

Methods:

1. To 3 ml urine add 4 drops of saturated picric acid.
2. Add 4 drops of sodium hydroxide.

Observation:

Creatinine picricate is formed, giving an **orange** color which change to yellow on acidification with HC

1.5. Normal Ranges for some Common Biochemical Analyses in Blood and urine:

1.5.1. Normal Ranges for some Common Biochemical Analyses in Blood:

Amylase	4.25 units/1	4.25 units/l
Bicarbonate	24-32.mEq/1	24-32 mmol/l
Bilirubin (total)	0.3-1.2 mg%	5.13-20.5 mol/l
Calcium (total)	8.8-10.8 mg%	2.2-2.7 mmol/l
Chloride	100-106 mEq/1	100-106 mmol/l
Cholesterol	145.275 mg%	3.8.7.15 mmol/
Cortisol	6.22 -8%	166.607 nmol/l
Creatinine	0.6-1.4 mg%	53-124 mol
Creatinine phosphokinase (CPK)	7-70 lu/l	7-70 lu/l
	7-44 lu/I	7-44 lu/l
Folic acid	6-15 ng/ml	14-34 mmol/l
Glucose (fasting)	60-105 mg%	3.3 5.8 mmol/l
Glucose 6-phosphate dehydrogenase	5 15 units	5.15 units
Immuglobulins		
S.IgG	720-1500 mg%	7.2-15 units
S.IgA	90-35 mg%	0.9-3.25 units
S.IgM	45-150 mg%	10.7.38_mol/l
Iron	60.210 - 8%	10.7.38_mol/l
Iron binding capacity (total)	240-405-8%	43-72.5 mol/l
Lactic dehydrogenase (LDH)	100-300 lu/l	100-300 lu/l
Lipid (total)	400 -800 mg%	4.8g/l
Luteinising hormones	6.18 mU/ml	6.18 lu/l
5-Nucleotidase	1.5 17 lu/	1.5 17 lu/l

Osmolality	278-290	278-290
	Mosmol/Kg	Mosmol/Kg
pH whole blood	7.36/7.42	36.42 nmol/l
pCO_2	34.45 mmHg	4.5-6.0 kPa
pO_2	90-110 mmHg	12-14.6 kPa
Phosphate	2.5-4.5 mg%	0.8-1.45 mmol/
Phosphatase acid (ACP)	Up to 11 lu/	Up to 0.18 mol.s'R
Phosphatase alkaline (ALP)	50-150 lu/l	0.83-2.5 mol s'R
Potassium	3.5-50 mEq/1	3.5-5.0 mmol/
Protein (total)	62-7.9 g%	62-79 g/l
Albumin	3.4-5,0 g%	34-50 g
Sodium	134-146mEq/l	134-146 mmol/l
Thyroid stimulating hormone TSH	1.6 - 1.2 U/ml	1.6-1.2-10 lu/l
Thyroxin (T_4)	5-11.5 - 8%	64.5-148 nmol/l
Transaminase SGOT	1.44 units/ml	1.33 lu/l
SGOP	05-10 units/ml	1-20 lu/l
Tri-lodothyronine (T_3)	150-250 ng%	2.3-3.9 nmol/l
Urea	17-40 mg%	2.8-6.6 mmol/l
Nitrogen	10-20 mg%	1.6-3.3 mmol/l
Uric acid	1.9-6.4 mg%	0.11-0.38 mmol/l
Vitamin A	0.15-0.6	0.5-2.1 _ mol/l
Vitamin B	200-800 pg/ml	147-590 pmol/l

Enzymes:

- **S.G.O.T. (AST)** 12 IU/l
- **S.G.P.T. (ALT)** 12 IU/l
- **Alk. Phosphatase** 3-13 KAU/100 ml

1.5.2. Normal Ranges for some Common Biochemical Analyses in Urine:

Constituent (Urine)		Present normal Values	Conversion factor	Normal values in SI units
Na*	Total output	200 mEq	1.0	200 mnol
K*	Total output	70 mEq	1.0	70 mmol
Urea	Total output	25g	16.6	415 mmol
Protein	Total output	00.05g	1.0	0.05 mmol
Ca*		10 mEq	0.500	5 mmol
Inorg. P.	Total output	60 mEq	0.500	30 mmol
Uric acid	Total output	400 mEq	0.00595	2.38 mmol
VMA	Total output	<7mg	5.05	<35_ mol
5-HIAA	Total output	3-7 mg	5.24	16-37_mol
Xylose		Over 5g/5h	0.00665	Over 33.3mmol/5h
Creatinine	Total output	1-2g	8.84	9-18 mmol

Practical No. 2

Normal Constituent of Blood

2.1. Detection of Haemoglobin:

Haemoglobin is the pigment of blood which is found in the erythrocytes. It functions in the transport of oxygen and carbon dioxide and in addition is an important buffer.

Method

1. Into a clean tube add 1 ml glacial acetic acid and 1 ml of diluted blood
2. Add a spatula of O-TOLIDINE mixture.

Observation:

A **blue** colour should develop immediately.

3. Carry out control test omitting blood (use water instead of blood).
4. Explain the test.

2.2. Detection protein in serum:

Method:

1. Place 1 ml of diluted serum into a test add small piece of litmus paper.
2. Add 1-2 drops of dilute acetic acid till the litmus become red.
3. Heat.

Observation:

Note the formation of coagulum. Albumin and globulin present in serum are heat coagulable.

1. Place 3 ml dil serum in a test tube
2. Adds 3 ml of saturated ammonium sulphate solution.

Observation:

A precipitate forms, this is globulin.

3. Filter through filter paper fully saturated by adding solid ammonium sulphate.

Observation:

Note that further precipitation now occurs. This is albumin.

- How you explain these results?

2.3. Detection of enzyme in blood:

1. R.B.C Catalase:

This enzymes catalyses the decomposition of H_2O_2, as follows:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2.$$

Method:

1. Use your own blood. Take a drop of blood from the thumb on a glass slid add a drop of hydrogen peroxide from a burette.

Observation:

Note the evolution of oxygen as hydrogen peroxide decomposes.

Other constituents will be determined in the coming practical.

Practical No. 3

Abnormal Constituents of Urine

3.1. Detection of proteins:

In the normal kidney only a small amount of low molecular weight protein is filtered at the glomerulus. Glomerular membrane prevents the passage of high molecular weight proteins such as albumin (mol. Wt 69000) and gamma globulin (mol. Wt 180000). After filtration most of the proteins is reabsorbed in the tubules. About less than 150 mg protein per 24 hours urine is generally excreted, which is not detected by chemical methods.

There are two main mechanisms by which proteinuria (presence of proteins in urine) can occur:

a. glomerular damage
b. Defect in the reabsorption process of the tubules.

In the glomerular damage the capillary walls become more permeable, large molecular weight proteins mainly albumin is passed in the urine. In diminished tubular reabsorption, low molecular weight proteins are not completely reabsorbed and hence they are present in urine.

Proteinuria may be due to pre-renal, renal or post-renal clinical conditions. Dehydration, intestinal obstruction and myocardial infarction are examples of the pre-renal conditions. The renal conditions include nephritic syndrome, hypertension and lipoid nephrosis. In post-renal conditions, proteins may be added in urine as it passes along the urinary tract. Lesions of the renal pelvis, bladder, prostate and urethra can all lead to such condition.

Tests for proteins:

3.1.1. Boiling Test:

Method:

1. Make the urine slightly acid to litmus by adding dilute acetic acid drop by drop (as protein precipitate in the acidic p/H).
2. Place about 10ml of urine in a test tube
3. Heat the upper part of the urine to boiling.
4. If protein is present it coagulates and can be detected by comparing it with the unboiled part lower down the tube.

3.1.2. Sulphosalicylic acid test:

Method:

1. To 5ml of urine add 0.5ml of sulphosalicylic acid solution.
2. If protein is present a white precipitate will form.

BENCE JONES PROTEIN:

This is a very low molecular weight protein (about 44000), hence it is easily filtered through the normal glomerulus. It has unusual solubility properties. It precipitates when heated to 40-60_ C, but becomes soluble again when boiled, and reappear after cooling. This protein is usually associated with multiple myeloma, where there is malignant proliferation of plasma cells, usually, in bone marrow.

3.1.3. Brad show's test:

Method:

1. To about 3ml of concentrated HC1 2ml of clear urine through the test tube wall.

2. A white ring will form at the junction between the two fluids if protein is present.
3. Dilute the 1:10 and repeat the test.
4. If same ring is formed this may indicate the presence of B. J. P. which should be confirmed by further test.

3.1.4. Detection of reducing sugars:

A small amount of glucose (2-20mg) may be present in fasting urine which is not detectable by chemical methods. The presence of chemically detectable amount of glucose in is called glycosuria. The quantity of glucose that appears in urine is dependent upon

1. Blood glucose level.
2. The rate of glomerular filtration.
3. The degree of tubular reabsorption.

The normal renal threshold for glucose is up to180mg/dl /910mMol/1). When the glucose level exceeds the normal renal threshold the tubules cannot re-absorb all of filtered glucose and glucosuria occurs. The main reason for glycosuria is hyperglycemia. Diabetes mellitus is the main reason for hyperglycemia.

In renal glucosuria which is due to defect in the re-absorption ability of the renal tubules or due to subsequent lowered renal threshold, in this case glucose appears in the urine, blood glucose is however normal. There are various other non pathological cases of transitory glycosuria, these conditions include:

1. Pregenancy-due to lowered threshold.
2. Stress and anxiety.
3. Alimentary glycosuria which may occur in patients with gastrectomy, or patients with hyperthyroidism, in both conditions there is rapid delivery of glucose to the small intestine and rapid absorption.
4. There are other reducing sugars which can appear in the urine. Galactose Fructose, lactose, most probably due to inborn errors of metabolism.

3.1.4. Benedict's test:

Method:

1. To 2ml of Benedict reagent add 4 drops of urine
2. Boil the urine for two minutes in a boiling water bath allow cooling spontaneously.

Observation:

1. If the test is negative the colour will remain as it is,
2. If positive the following change in the colour will be seen:

- Green without precipitate (p.pt) = Trace.
- Green with p.pt = (1+).
- Yellow = (2++)
- Orange (3+).
- Red or brick red = (4+) or more.
- The change in colour from green to red is directly proportional to the amount of reducing substance present in the urine i.e. as the amount sugar increase the colour change from green---red.

3.1.5. Detection of ketone bodies:

Whenever there is inadequate carbohydrate in the diet or a defect carbohydrates metabolism the body tends to metabolize increasing amount of fatty acids. Due to this the other intermediary products, such as ketone bodies increase in the blood and hence appear in urine causing ketonuria.

Ketone bodies are acetone, acetoacetic acid, and beta hydroxybutyric acid.

Diabetes mellitus is the most important disorder in which ketonuria occurs. Starvations can also ketomuria.

a. Gerhard's test for acetoacedic acid:

1. To 5 ml of urine 10% add ferric chloride solution drop wise.
2. **Method:**

Observation:

1. A **purplish** colour is given by acetoacetic acid.
2. Acetone does not give positive Gerhard's test.

b. Rothera's test for acetoacetic and acetone:

Method:

1. On a glass slide put some acetone powder.
2. Add 3-5 drops of urine.

Observation:

A **purple** colour indicates the presence of acetoacedic acid and acetone.

3.1.6. Detection of bile constituents in urine:

Human bile secreted by liver contains the following substances, bile pigments, bile salt and cholesterol, in addition to some other substances of minor quantities.

Bile pigments, biliverdin and bilirubin give the bile its characteristic golden or brownish yellow colour. They are derived from heme of haemoglobin and other heme proteins e.g. myoglobin, catalase, cytochrome, they are metabolized to be transported to the liver where it conjugate with glucuronic acid and become water soluble then excreted via bile duct into the intestine where it is hydrolyzed and reduced by bacterial flora to urobilinogen. Some eliminated in the feces while some is reabsorbed in the portal circulation and excreted via the kidney.
In some types if jaundice where the serum bilirubin exceeds the normal level bilirubin appears in the urine, in some types urobinogen is increased.

3.1.7. Test for bile pigments:

a. Iodine test:

Method:

1. Place 3 ml of urine in a test tube
2. Add some tincture iodine on the wall of the tube.

Observation:

A **green** ring at the junction of the two liquids. Indicates the presence of bilirubin.

b. Fouchet's test:

Method:

1. To 5 ml of urine add 3 ml of 10% barium chloride and mix.
2. Filter, spread the filter paper on another filter paper allows to drain
3. Add drops of Fouchet's reagent.

Observation:

A **greenish blue** colour indicates the presence of bilirubin.

c. Nitric acid test:

Method:

1. Place 5 ml of conc. Nitric acid in a test tube
2. Adds 2 ml of urine on the wall of the tube.

Observation:

If bilirubin is present **green blue** and **violet** rings are seen at the junction of the two liquids.

3.1.8. Test for bile pigment derivatives:

a. Ehrlich test for urobilinogen:

Method:

1. To 5 ml of fresh urine add 1 ml of Ehrlich reagent.

Observation:

If urobilinogen is present a **red** colour develops on standing within 2-3 minutes.

b. Test for bile salts:

1. Hav's test:

Method:

1. Place 5 ml of urine in a test tube sprinkles some dry sulfur powder on the surface of the urine.

Observation:

The sulfur will sink if bile salts present but remain floating if they are absent.

2. Petterkofer's test:

Method:

1. Place 4 ml urine in a test tube adds 4 drops of sucrose solution.
2. Carefully pour about 2 ml of conc. Sulfuric acid on side of the tube.

Observation:

A **purple ring** is obtained when bile salts are present.

3.1.9. Paper strip and tablet test for some pathological constituents of urine:

In recent years detection of pathological constituents in urine has been greatly simplified by the commercial availability of strips of filter paper impregnated with special reagents for the detection of glucose, acetone, protein, bilirubin, pH, S.G, etc.

a. Test for Glucose (Clinistick):

Method:

1. Dip the test end of the clinistick strip into the urine and quickly remove it.

Observation:

If glucose is present (even in amounts as less as 0.1%) it will change according to the amount of glucose present.

b. Test for Acetone (Acetest):

Method:

1. Dip the strip as before.

Observation:

Note the colour change after 30 seconds.

c. Test for Protein (Albustic):

Method:

1. Dip the test end of the albustic strip into the urine and remove it immediately.

Observation:

A **green blue** color developing immediately indicates the presence of protein.

d. Test for Bilirubin (Ictotest):

Method:

1. Dip as before and note the colour change.

e. Test for Blood (Occultest);

Method:

1. Place 1 drop of urine in center of the test paper provided
2. Put 1 occultest table in center of moist area and 2 drops of water on table.

Observation:

Diffuse area of **blue** colour on test paper around the table developing within 2 minutes indicates the presence of blood or haemoglobin.

Occulets tables contain o-tolidin, strontium peroxide, calcium acetate, tartaric acid, and sodium bicarbonate.

Note:

- These tests are simple, sensitive, reliable, if not slightly expensive for routine uses.
- Combistix which give protein, sugar, bile, Acetone, urobilinogen, blood, pH, etc., in one single step is available.

3.1.10. Determination of Serum Urea by Diacetyl:

***Monoxime*:**

Widely used screening test for the evaluation of kidney function. The test Determination of serum urea (or blood urea nitrogen BUN) is the most is frequently requested along with serum creatinine, since simultaneous determination of these two compounds appears to aid in the differential diagnosis of pre-renal, renal, and post renal problems.

Principle:

- When urea is heated with substances such as diacetyl, CH, COCOCHZ. Containing two adjacent carbonyl groups colored compounds are formed.
- Diacetyl monoexime CH, COC=NOHCH, has usually been used because of its great stability.
- In the determination it decomposed to give hydroxylamine and diacetyl which then condenses with urea.
- A colored compound will be produced; the intensity of the color is proportional to the urea concentration.
- Normal range 15 - 45 mg/dl in normal adult.

Method:

1. Measures 0.1 ml serum into 1 ml distilled water mix.
2. Measures 0.1 ml standard urea into 1 ml distilled water.
3. Prepare three test tubes as follows:

	Test	**STD**	**Blank**
Distilled water	-	-	0.5ml
Diluted serum	0.5	-	-
Diluted STD	-	0.5	-
Color reagent	4.0	4.0	4.0ml

4. Mix and place in boiling water for 10-20 minutes cool.
5. Read Test and STD against blank at 520 nm.

Calculation:

- Unknown concentration mg/dl serum=A of Test X conc of STD/A of STD
- Concentration of STD = 30 mg/dl.

3.1.11. Estimation of Creatinine in Urine:

Creatinine is removed from plasma by glomerular filtration and its significant extent. Determination of creatinine clearance is a highly sensitive test for measuring the GFR.

Principle:

- Creatinine the anhydride of creatine reacts with alkaline sodium picrate to give an orange - red colored solution.
- The ccolour produced by known volume of urine is proportional to the conc. Of creatinine of Urine.

Method:

1. Dilute the urine 1:100 with distilled water.
2. Prepare three test tubes as follows:

	Test	STD	Blank
Diluted urine	3	-	-ml
Creatinine STD	-	3	-
Distilled water	-	-	3
Picric acid	1	1	1
IN NaOH	1	1	1

3. Mix stand for 15 minutes - read Test: STD against blank at 520 nm.

Calculation:

- Urine creatinine = A. Test X 1 X 100/A. STD
- 1 mg/dl = concentration of STD
- 100 = dilution factor
- Normal range 0.4 - 1.8 g/24 hour urine in normal adult.

Practical No. 4

Estimation of Serum Calcium

Principle:

- Calcium reacts with o-cresolphthalein complexone in an alkaline medium to form a purple colored complex which is proportional to the concentration of calcium present.

Method:

1. Dilute serum and standard as follows:-
2. Serum 0.1 +0.9 ml of distilled water. Dilute STD as serum.
3. Prepares three test tubes as follows:

	Test	STD	Blank
Diluted serum	0.1	-	Ml
Diluted STD	-	0.1	-
Distilled water	-	-	0.1
Colour reagent	2.0	2.0	2.0ml

4. Mix and incubate at room temperature for 10 minutes.
5. Read intensities of Test and standard against blank at 580 nm.

Calculation:

- Calcium concentration = A. Test X 10/ A.STD
- Concentration of STD 10 mg/dl (5 m Eq/l 2.5 mmol/l)

Practical No. 5

Estimation of plasma inorganic phosphate

- Normal range 2.5 - 5 mg/100 ml plasma in normal adult.

Principle:

- After removal of the protein the plasma supernatant is treated with molybdate reagent to form a yellow colour compound.
- The yellow phosphomlybdate is reduced with stannous chloride to give a blue colour, the intensity of which is directly proportional to the amount of phosphate present in the plasma.

Method:

1. Test: to 2.4 ml tricchloro-acetic acid in a centrifuge tube and 0.1 ml of plasma shake well and centrifuge for 2 minutes.
2. Label three test tubes as follows:

Variable	Test	STD	Blank
Supernatant	1.0	-	-ml
STD (.002 mg P/ml	-	1.0	-
Distilled water	2.0	2.0	3.0
Molybdate reagent	0.2	0.2	0.2
Stannous chloride	0.1	0.1	0.1 ml

3. Shake the contents of the test tubes after each addition.
4. Leave for 10 minutes.
5. Read Test and STD against the blank at 710 nm.

Calculation:

- Inorganic phosphate concentration mg/100 ml plasma = A. TestX 5/A. STD

Practical No. 6

Glucose Tolerance Test (G.T.T)

Free glucose either derived from dietary carbohydrate or formed in the liver from other hexoses, or from glycogen, or from glucose phosphate ester, passes in systemic blood stream as blood glucose and transported to all cells of the body for utilization as energy or for other maintained between 70-110mg/dl (4-6mmol), partly by glycogen metabolic purpose. In the fasting state blood glucose level is usually breakdown in liver and partly by gluconeogenesis occurring mostly in liver but also in the kidneys. The hormones mainly concerned with blood glucose homeostasis are insulin, glucose, somatostatin, growth hormone, adrenaline and cortisol. Of these hormones insulin has the most marked effects in man and is only hormone with lowering effects on blood glucose. All other hormones mentioned above tend in general to antagonize the action of the insulin.

The renal threshold of blood glucose is 180mg/dl (10mmole). blood glucose above 10mmol/L is called hyperglycemia in which the blood glucose raises above the renal threshold and causes glycosuria. But not all glycosuria indicates hyperglycemia, blood sugar may be normal and sugar is present in urine due to renal defect (renal glycosuria). Also, can be due to lowered renal threshold as in pregnancy.

G.T.T.

To test the ability of the body to utilize glucose in blood circulation The main value of GTT is that it may help to establish the diagnosis of diabetes mellitus, or impaired glucose tolerance at a time when the metabolic abnormality is mild. It is particularly valuable in the diagnosis of impaired glucose tolerance which can be found in late pregnancy, obesity, severe infections (especially staphylococcal), Cushing's syndrome. Conn's syndrome, Acromegaly, thyrotoxicosis, gross liver damage.

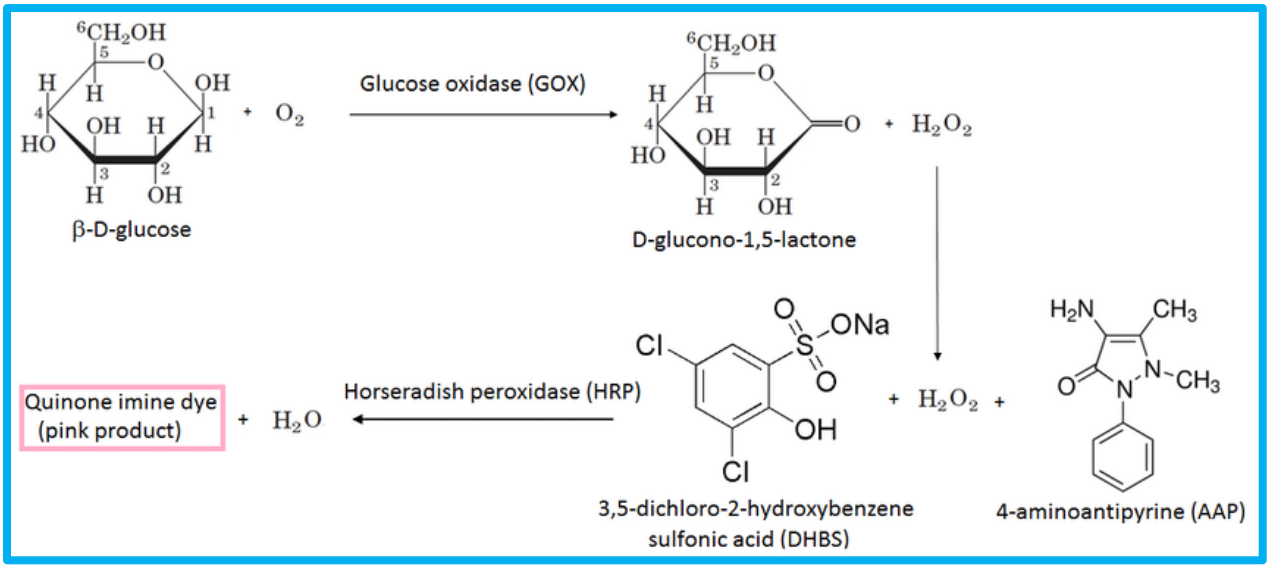
Several precautions must be observed in performing the GTT:

1. The patient should be on his normal diet for at least 3 days before the test.
2. Fasting 12-14 hours (overnight).
3. Ne smoking before or during the test.
4. The patient should be on rest hour before doing the test.
5. Collect fasting blood and urine sample.
6. Give the patient the standard dose of glucose 75g glucose/300ml water (1.75g/kg.).
7. Collect blood and urine sample after 30minutes, 1 and 2 hours.
8. All blood samples are tested for glucose level and urine for glucose and acetone.
9. Draw the GTT curve and comment.

The following types of GTT curves can be obtained:

a. The normal curve: In the normal individuals the fasting blood glucose level is 70- 10mg\dl. The blood glucose rises up to 180mg/dl one hour after the standard dose of glucose then it falls by the second hour to a level below 140mg\dl. There is no glucose or acetone in urine.
b. The diabetic curve: In diabetes mellitus the fasting blood glucose level is above 140mg\dl one hour above 200mg, two hours start to fall but the level is above 180mg\dl.
c. Flat curve: Flat response to GTT describes the response when the blood level fails to rise significantly. The fasting blood glucose level is normal or low and throughout the test the level dose not varies by more than +10 mg\dl. A flat curve is usually found in patients who have malabsorption, as in coeliac disease, in patient who are suffering from myxoedema (which reduce carbohydrate absorption), or who have deficiency in hormonal insulin antagonists as in Addison's disease and hypopituitarism. There is no glycosuria.
d. Lag storage curve: The fasting blood glucose level is normal. There is a steep rise in blood glucose; the maximum level found at 30 minutes is above 180mg/dl. The curve then falls sharply and hypoglycaemic level may be reached before 120 minutes. Transient glucosuria is usually found. This pattern of response is due to rapid absorption of glucose from the intestine, the tissue mainly the liver is unable to attain a sufficiently rapid uptake to match the rapid absorption. In other words, there is a lag in the initiation of normal homeostatic processes, particularly storage of glucose as glycogen. It may be seen after gastric surgery (eg gastrectomy) or in patients with severe liver disease, in patient with thyrotoxicosis and is sometimes seen in apparently healthy individuals.
e. Rena! glucosuria. This term is applied to patients who exhibit glycosuria at some point in the GTT, although the blood glucose level at all time in the normal level, it can be due to lowered renal threshold or defects in renal tubular reabsorption.

Determination of blood glucose using oxidase peroxidase and 4-aminophenzone:



Method:

1. Label five test tube as follows:

Tubes label	Fasting	1 hour	2 hours	STD	Blank	
Fasting plasma	0.2	-	-	-	-	ML
1 hr plasma	-	0.2	-	-	-	
2hrs plasma	-	-	2	-	-	
STD glucose 100mg/dl	-	-	-	0.2	-	ML
Color reagent	2.0	2.0	2.0	2.0	2.0	

2. Incubate at room temperature for 10-15 minutes

3. Read absorbance of test and STD against blank at 520 nm

4. Calculate the concentration of the glucose at each sample then construct the GTT curve.

Calculation:

Un known concentration =A of test X 100

Mg/100 plasma A of STD

A= absorbance

Diagnosis of Diabetes mellitus:

1. Glucosuria: The simplest one, but can give false positive or false negative. What are they?
2. Fasting blood glucose: It diagnoses D.M. but it can be normal in mild D.M.
3. Two-hour postprandial blood glucose: Simplest screening test for D. M. (A modified G.T.T.)
4. Glucose Tolerance test:
    a. Detection of mild D. M.
    b. A certain renal threshold for glucose.
5. Random blood glucose level. Valuable in follow up but a normal result dose not excludes diabetes. Consider the following result which is obtained from specimens that has been taken randomly from different patients. Investigation required blood glucose and urine general.

Patient A:

- Blood glucose 300 mg/dl
- Urine contains glucose and ketone bodies.
- Diagnosis: uncontrolled but uncomplicated diabetes mellitus.

Patient B:

- Blood glucose 400mg/dl.
- Urine contains glucose, ketones and proteins.
- Diagnosis: Uncontrolled, long standing D.M. with renal complication.

Patient C:

- Blood glucose 100mg./dl.
- Urine contains glucose, proteins, free amino acids, and increased phosphate excretion.
- Diagnosis: Fanconi syndrome, which is a group of disorders with proximal renal tubular disorder. It could be idiopathic, or cystinosis, Wilson's disease, Nephrotic syndrome, Multiple myeloma, etc.

Patient D:

- Blood glucose 100mg/dl.
- Urine contains proteins only. (More than 200mg/24 hour).
- Diagnosis: Nephrotic syndrome.

Patient E:

- Blood glucose 50mg/dl.
- Urine contains ketones only.
- Diagnosis: Prolonged starvation.

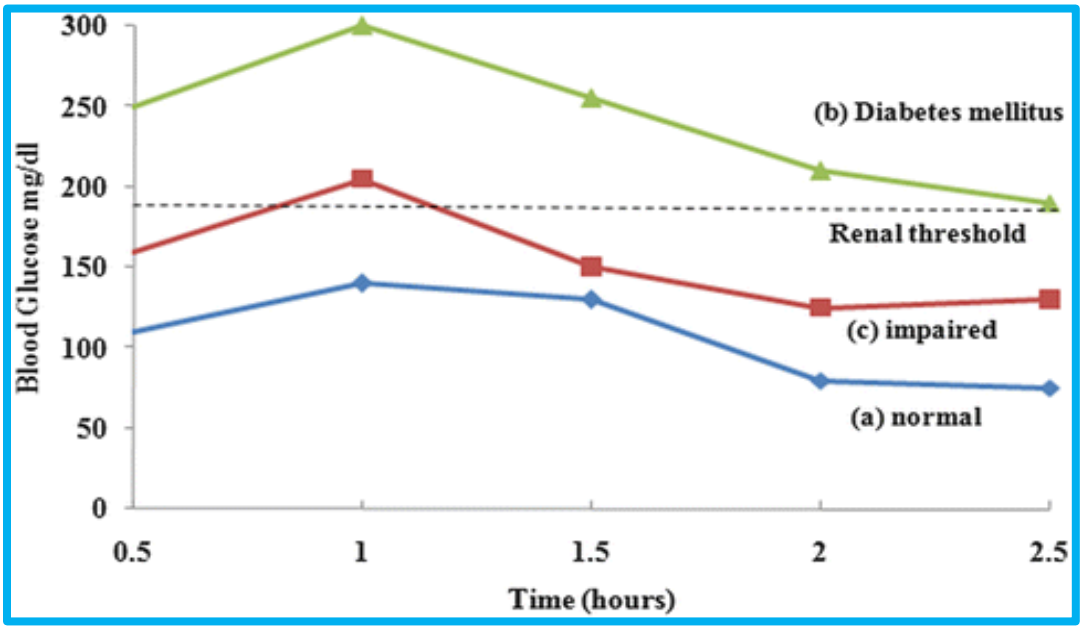
Q Explain in biochemical terms the findings of the above cases, and their relation to the disease process involved.

G.T. T. Results:

Patient	Time in Minutes			
	Blood	Glucose	Mg/dl	
	0	30	60	120
Patient: A	80		160	90
Urine: S	-		-	-
Patient: B	150		300	250
Urine: S	+		+++	+++
Urine: K	-		++	+
Patient: C	80		90	85
Urine: S	-		-	-
Urine: K	-		-	-
Patient: D	80	200	120	60
Urine: S	-	+	-	-
Urine: K	-	-	-	-
Patient: E	80		160	70
Urine: K	-		++	++
Urine: S	-		-	-

S = Sugar **K = Ketones**

G.T.T. Curves:



Practical No. 7

Serum Cholesterol

Case serum report:

Ali (47 years) is director of a commercial bank in Khartoum. He wakes up one night with severe pain across his chest which persisted, he was transferred to hospital, and he sweated profusely and had a slight rise in temperature. On taking the history the doctor in charge found that Ali is diabetic with moderate hypertension. He smoked 50 cigarettes per day. on examination his blood pressure was 180/100 mmHg, he has cardiac arrhythmia (ventricular fibrillation). He immediately received 15 mg porphin IM, followed by treatment for the arrhythmia.

An ECG was made and a blood sample obtained. He was advised to have full bed rest for two weeks in hospital. ECG and blood samples were obtained daily thereafter.

ECG: On 2nd day the following was observed ST Segment elevation. T waves inversion and broad Q waves.

Serum: On admission serum cholesterol was 420 mg/dl. On 2nd day his CPK was 150 IU/L (normal up to 40 IU/L). On 3rd day SGOT was 130 IU/L (normal up to 20 IU/L).

Follow up: After 2 months during which Ali stopped smoking and had a rigid control of his diet, blood pressure and D.M., he was allowed to resume work.

Diagnosis: Myocardial infarction (coronary heart disease).

Now:

1. You are given Ali's serum sample (after 2 months) determine the total cholesterol level.
2. Write a comment about Ali's condition based on your finding.

CPK: creatine phosphokinase

SGOT: serum glutamic oxalo-acetate transaminase

Determination of Serum Total Cholesterol

Principle:

Proteins are precipitated and cholesterol and its esters extracted then added to the extract and the intensity of the colour that develops is directly proportional to concentration the cholesterol. And is measured by a warm ferric chloride acetic acid reagent. Concentrated sulphuric is using colorimeter.

Method:

1. Label two centrifuge tubes as follows Test, STD.

	TEST	STD
Serum	0.10	- ml
Cholesterol STD	-	0.1 ml
FeCl, acetic acid reagent ml	5.0	5.0 ml

2. Mix and leave for 5 minutes then centrifuge for 2 minutes.

3. Label three test tubes as follows:

	Test	STD	Blank
Supernatant	All	All	-
FeCL, acetic acid	-	-	5 ml
Con, H_2SO4	3 ml	3 ml	3 ml

4. Mix leave for 10 minutes

5. Read the absorbance of test and standard against the blank at 520 nm.

Calculation:

Serum cholesterol mg/dl = $\frac{\text{Absorbance of Test X 200}}{\text{Absorbance of STD}}$

Standard cholesterol concentration = 200 mg/dl

Normal range 150 - 250 mg/dl (depend on the method used)

Ferric chloride acetic acid reagent (0.05%)

Discussion

Lipoprotein:

Class	Electrophoresis	Chemical composition	Apoprotein
Chylomicron	Omega	Mostly TAG	A, B
VLDL	Pre-beta	Mostly TAG	B, C, E

LDL	Beta	Mostly cholesterol	B
HDL	alpha	Mostly cholesterol	A, C, E

Sources of Cholesterol:

1. Exogenous. From animal diet - egg yolk, dairy products, meat (600-800 mg/day)
2. Endogenous. All nucleated cells have ability to synthesize cholesterol, the Renovo synthesis occurs in many tissues especially in the liver.

Hyperlipidaemias:

1. Primary inherited)
2. Secondary (acquired)

Both may lead to atherosclerosis, and predispose to cardiovascular disease.

Secondary Hyperlipidaemia: due to abnormal dietary intake.

	Cholesterol	T.A.G
Obesity	+	+
D.M.	+	+
Alcoholism		+
Hyperthyroidism		+
Biliary obstruction		+
Pancreatitis		+

Treatment of hypercholesterolaemia:

1. Dietary restriction
   -Decrease cholesterol and saturated fatty acids
   -Increase polyunsaturated fatty acid
2. Bile salt sequestration: Cholestyramine resin binds bile salt.
3. New drug stops cholesterol: synthesis in the liver (inhibition of H.MG.CoA reductase by Lovastatine, Mevastatine)

Practical No. 8

Plasma Proteins

The following five patients were suspected of having an abnormality in plasma protein levels in blood. Please estimate the level of total proteins, albumin in serum and urine proteins.

a. A middle-aged man was referred from Gadarif hospital for investigation. He had low grade fever for a few weeks, fatigability and excessive weight loss. On examination was done to exclude kalazar.
b. A young boy, 12 years old felt ill for a few weeks. His mother noticed that his face was swollen and brought him to hospital.
c. A male child 3 years old was brought to the paediatrics department because his mother noticed over a number of weeks that he was refusing food, becoming lethargic, with swollen feet and changes in the color of both skin and hair.
d. A young man felt ill for a few days, with low grade fever, occasional vomiting and puffy face.
e. An elderly man complained of backache for a few months. He was found to be anaemic. Bone marrow examination revealed increased plasma cells. X-ray of the skull showed punched out areas.

Normal values:

Total protein	6.3-8.2 gm/dl plasma
Total protein	6.3-8.2 gm/dl plasma
Albumin	4.0-5.7 gm/dl plasma
Globulin	1.5-3.0 gm/dl
Fibrinogen	0.2-0.4 gm/dl plasma
Albumin/globulin	1.3-1.8 average 1.6

Estimation of plasma total proteins:

Principle:

Proteins are made up of amino acids linked together by peptide bonds complex with the cupric ions in the biuret reagent to give a purple colour to protein.

Method:

1. Set up three test tubes as follows:

	Test	STD	Blank
Plasma	0.01	-	-ml
STD T protein	-	0.10	-
Dist, water	-	-	0.10

Biuret reagent	5.0	5.0	5.0 ml

2. Mix well the contents of each tube.

3. Allow standing at room temperature for 10 minutes.

4. Read absorbency of test and STD against blank at 540 nm.

Calculation:

$$\text{Plasma total proteins gm/dl} = \frac{\text{A of Test X Cons. of STD}}{\text{A of STD}}$$

Estimation of plasma albumin:

Principle:

Proteins bind dyes and the latter change colour as a function of constant. Bromocresol green changes colour from yellow to green at pH the protein concentration provided that the pH of the solution remains 3.8.

Method:

1. Set up test tubes as follows.

	Test	STD	Blank
Distilled water	0.4	0.4	0.1 ml
STD albumin	-	0.1 ml	-
Plasma albumin	0.1	-	-

2. Mix well and pipette into a corresponding set of two set tubes labelled as test and STD 0.1 ml of contents (blank as it is).

3. To each tube (test, STD, blank) add 5 ml of bromocresol green reagent and mix well.

4. Read absorbency of test and STD against blank at 600 nm.

Calculation:

$$\text{Plasma albumin concentration gm/dl plasma} = \frac{\text{A of test X cons of STD}}{\text{A of STD}}$$

A=absorbency

Plasma proteins:

Results:

Patient and diagnosis	Plasma proteins (g/dl)		Urine proteins
	Total protein	Albumin	
A. Kalazar	9.0	4.0	Free
B. Nephrotic syndrome	5.0	2.0	Positive
C. Kwashiorkor	5.0	2.0	Negative
D. Nephritis	7.0	4.0	Positive
E. Multiple myeloma	11.0	4.0	Bence Jones Positive

Blood plasma in particular performs many functions that are absolutely critical for the maintenance of health

• Once the blood has clotted (coagulated), the remaining liquid phase (serum) lacks the clotting factors that are normally present in plasma but have been consumed during the process of coagulation.

Plasma: consist of water, electrolytes, metabolites, nutrients, proteins, and hormones.

• Plasma proteins can be separated into three major groups;

a. Fibrinogen
b. Albumin
c. Globulins

By the use of varying concentration of sodium or ammonium sulphate.

• The most common method of analyzing plasma proteins is by electrophoresis.

• Plasma proteins are synthesized in the liver. However, the globulins are synthesized in plasma cells (on membrane – bound polyribosomes)

Almost all plasma proteins are glycoproteins. Albumins are the major exception; it does not contain sugar residues.

Plasma proteins exhibit polymorphism (is a mendlian that exist in the population in at least two phenotypes).

• Albumin is the major protein in human plasma (3.4-4.7 g/dl) and makes up approximately 60% of the total plasma protein.

• Albumin has an ellipsoidal shape i.e.; it does not increase the viscosity of the plasma as much as an elongated molecule such as fibrinogen does. Because of its relatively low molecular mass, and high concentration albumin is responsible for 75-80% of the osmotic-pressure.

• Haptoglobins prevent loss of free haemoglobin into the kidney.

Practical No. 9

Estimation of Serum Uric Acid

Introduction:

Uric acid is main end product of purine metabolism. Nucleic degraded by variety of enzymes initially to nucleotides, these mix with intracellular pool or further broken down to nucleosides and then to free bases. Some of the bases to formed, mainly hypoxanthine, guanine and adenine, are partly converted to uric acid and excreted as urate. Alternatively, these bases are salvaged. Reused for nucleotide synthesis i.e.

Base + PRPP —HGPRT→ PPi + Nucleotide

The common causes of hyperuricaemia are:

a. Increased purine catabolism as in primary gout.
b. Increased cell breakdown as in leukaemia and in the treatment of malignancy by radiotherapy and cytotoxic drugs.
c. Impaired excretion of uric acid, secondary to renal disease.

Notice - Hyperuricaemia can also cause renal disease.

The following patients were referred to the clinical biochemistry laboratory for measurement of their serum uric acid.

a. A middle-aged man referred from the surgical outpatient department he was complaining of pain in his left big toe. The pain had been intermittent over the past two years.
b. A young boy referred from the orthopaedics department suffering from multiple big joints pain.
c. A patient referred from department of internal medicine where he was being treated for leukaemia (cancer of WBC) using radio therapy. This is his third uric acid estimation during the past month.
d. An old man of 70 years, suffering from renal failure referred from renal urine.
e. A young child, 4 years old who had mental deficiency, a tendency to self-mutilation. Aggressive behaviour, athetosis and spastic paraplegia. Uric in the

Please divide yourself into group of 2-3 students and estimate the level of sera provided.

Assemble into large groups and discuss your results.

Estimation of Serum Uric Acid

Principle:

When sodium carbonate and dilute phosphotungstic acid are added to a protein free serum, the uric acid present in serum reacts with the phosphotungstic acid to give a blue colour the intensity is proportional to the concentration uric acid.

1. Use serum or plasma because substances that interfere with colour reaction are found in the red cells.

2. Potassium oxalate (anticoagulant) should not be used, because of the formulation of crystalline precipitate during the analysis (Normal range 2.0 -7.0 mg/dl)

Method:

1. Preparation of protein free serum: into a centrifuge tube add 4 ml of distilled water, 0.5 ml of serum, 0.5 ml of 0.7 N sulphuric acid and 0.25 ml of 10% sodium carbonate mix centrifuge for 5 minutes.

2. The following 3 test tubes.

	Test	STD	Blank
Protein free serum	3	-	-ml
STD uric acid 1 mg/dl	-	3	-
Distelled water	-	-	3
Sodium carbonate	1	1	1
Phosphotungstic acid	1	1	1 ml

3. Mix incubates at room temperature for 10 minutes.

4. Read the absorbance of Test and STD against the blank.

Calculation:

$$\text{Uric acid concentration} = \frac{\text{A of Test X 10 mg/dl serum}}{\text{A of STD}}$$

Uric acid results:

A. Gout: Primary hyperuricaemia

. Middle age man family history of alcoholism, debauchery.

Increase in purine synthesis.

B. Rheumatic arthritis: no increase in uric acid.

C.Secondary hyperuricaemia: Increased turnover of nucleic acid. due to rapidly growing malignant cells and their destruction by radiotherapy

D.Renal failure: hyperuricaemia due to reduced excretion of urates by failing kidneys.

E. Juvenile hyperuricaemia: (Lesh-Nyhan Syndrome). This is a very rare inborn error of metabolism .

That is sex linked and recessive.

Young man with high uric acid due to deficiency of enzyme hypoxanthine and other bases cannot be recycled to form purine nueclotides so uric acid production from them increased.

Practical No. 10

Liver Function Test

Case report:

A. Amna, a middle age housewife had a history of recurrent pain in the right hypochondrium for three years. The pain was usually accompanied by bilious vomiting but subsided in few days, to flare up a month or two later. Three weeks ago she had a severe attack of pain. This time however, she developed jaundice which continued to deepen although the pain has disappeared.

B. Ali, a 19 years old university student was admitted to Khartoum hospital with suspected anaemia and jaundice. He had left his home town in old Wadi Halfa three weeks previously. Two weeks after his arrival in Khartoum he had developed a persistent headache, anorexia, nausea and severe backache. He had bouts of shivering, a temperature of 40°C and he was delirious. Malaria was then suspected and subsequently confirmed by identification of the parasite in the blood smear. He was then prescribed primaquine by the doctor in charge. Three days later he noticed that his urine was dark. The following day abdalla continued to complain of weakness abdominal and back pain. His sclerae were yellow.

C. Abdalla Deng, causal labour felt sick for a few days. He had a low grade fever, aches and pains all over his body. On the 10th day his condition worsened. He had bouts of vomiting and noticed that his urine was dark few days later his friend noticed that he had developed jaundice and brought him hospital.

D. Mansour, a farmer from Gezira felt ill for some time and on examination of his stool Bilharzia was diagnosed. The doctor sent him to laboratory for investigations before giving him treatment for

Bilharzia.

For all patients please do the following:

A. Determine the level of serum bilirubin.

B. The activity of the enzymes serum transaminases, ALT, AST and alkaline phosphatase.

Urine: Examination for bilirubin and urobilinogen.

10.1. Estimation of Serum Bilirubin:

The quantitative Van Den Bergh Reaction (Malloy and Evelyn):

Principle:

Bilirubin forms a red coloured compound (called azobilirubin) with Ehrlich's diazo reagent. The red colour is compared with that given by a known amount of bilirubin in similar way.

Method:

1. Set four test tubes as follows:

	Test	Test blank	STD	STD blank	
water	1.8	1.8	1.8	1.8	ml
serum	0.20	0.20	-	-	ml
STD	-	-	0.20	0.20	ml
Diazo reagent	0.50	-	0.50	-	ml
Blank reagent	-	0.50	-	0.50	ml
Methanol	2.5	2.5	2.5	2.5	ml

2. Mix and keep in a dark for 30 minutes.

3. Read the absorbency of test and standard against its blank at 540 nm.

Calculation:

$$\text{Mg bilirubin/100 serum} = \frac{\text{A. Test X Conc of STD}}{\text{A of STD}}$$

A = Absorbency .

Normal range (0.6 - 1 mg/dl).

10.2. Determination of Serum Aspartate Aminotransferase (S.AST — S.GOT)

In myocardial infarction, hepatic cirrhosis and in infective hepatitis the activity of S.AST rises due to leakage of the enzyme from the damaged cells. The occurrence of abnormally high amounts of this enzyme in the serum is an indication of damage to cardiac or hepatic tissues.

S.GOT = Serum Glutamate Oxaloacetate Transaminse.

Normal range: Adults up to 20 U/1 at 37°C.

Reagent:

- Buffer substrate
- 2, 4 Dinitrophenylhydrazine (2, 4 DNPH).
- 0.4 N Sodium hydroxide (0.4 N NaOH)

Principle:

Aspartate + Ketoglutrate $\xrightarrow{\text{AST}}$ Oxaloacetate + Glutamate

Method:

1. Prepare test tubes as follows.

Variable	Test	STD	Blank
Solution - (A)	0.5ml	0.5ml	0.5 ml
Serum	0.1	-	-
Standard	-	0.1	-
Distilled water	-	-	0.1 ml

2. Mix and incubate at 37°C for one hour.

3. Add 0.5 ml of solution (B).

4. Mix and leave at room temperature for 20 minutes.

5. Add 5 ml of solution (C).

6. Mix well leaves for 5 minutes.

7. Read the absorbency of test and standard against blank at 520 nm.

Calculation:

$$\text{Serum - AST activity U/l (at 37°C)} = \frac{\text{A -of Test X Con of STD.}}{\text{A-of STD}}$$

A = Absorbency

King defined the unite of transaminase activity as that convert one mg of alpha amino acid into one mg of keto acid in one hour at 37°C.

10.3. Colorimeteric Determination of Alkaline phosphatase

Principle:

Colorimeteric determination of alkaline phosphatase activity according to the following reaction.

Phenylphosphate. $\xrightarrow[\text{PH 10}]{\text{Alkaline Phosphatase}}$ Phenol + Phosphate

The phenol liberated is measured in the presence of 4 aminoantipyrine and potassium ferricyanide. The presence of sodium arsenate in the reagent stops the enzyme reaction.

Normal Range: Adults 3- 13 Kind and King U/100 ml

Method:

1. prepare 3 test tubes as follows:

Variable	Test	STD	Blank	conc.
Buffer substerate	2	2	2	2
Serum	0.1	-	-	-
STD phenol 10 mg/ dl	-	0.1	-	-
Blocking reagent	0.5	0.5	0.5	0.5
Potassium ferricyanide	0.5	0.5	0.5	0.5

2. Mix after each addition.

3. Let stand for 10 minute in a dark place.

4. Read test and STD against blank at 520 nm.

Calculation:

$$\text{Unknown activity kind @ King unit/100 ml serum} = \frac{\text{A-Test X 10}}{\text{A-STD}}$$

A = Absorbance

Liver Function Result:

A. Amna:

Serum: Bilirubin	15 mg/dl
Serum Alk, Phosphatase	50 KAU/di
Serum AST.	10 U/I
Urine: Bilirubin.	+++
Urobilinogen.	Absent
Serum: Bilirubin	15 mg/dl

Diagnosis: Obstructive Jaundice.

- High serum bilirubin. Urine contains bilirubin bu: no urobilinogen.
- High Alk. Phosphatase (indicating biliary obstruction).
- Normal or slightly raised serum AST (little cell destruction)

B. Ali:

Serum bilirubin	**5**
Alk. Phosphatase	4
Serum AST	8
Urine: bilirubin	Nil
Serum bilirubin	5
Urobilinogen:	Excess

Diagnoses: Hemolytic Jaundice. Excessive RBCs destruction

- In this case both malaria and G6PD deficiency.
- Moderately raised serum bilirubin.
- Normal serum Alk. Phosphatase and S.AST.
- Urine: contains no bilirubin but excess urobilinogen.

C. Abdalla Deng:

Serum bilirubin	15 mg/dl
Serum AST	200 UI
Serum Alk.phosphatase	12 KAU/di
Urine bilirubin	+++
Urobillinogen	Normal

Diagnosis: Viral Hepatitis:

Destruction of hepatic cells due to viral infection.

Very high serum AST (cell destruction)

Serum Alk, Phosphatase Normal

Practical No. 11

Inborn errors of metabolism

Cases Scenarios:

1. Amna Mohamed was born to healthy parents after a normal delivery in hospital on March 3, 2003. She was breast-fed but on second day she refused her feed and vomited several times. The following day she developed diarrhea and started losing weight. She continued to be fretful, taking her feeds reluctantly and vomiting frequently and on 8th of March she was found to be jaundiced. In the course of next the few days the jaundice deepened. On 16th of March cataracts were noticed in her lenses. Her condition did not improve and her doctor suggested she might have an inherited disorder. He sent her urine for metabolic screening

2. Ali Taha. Age 8 years was brought to hospital at his parents request at the time his sister was under investigation for presumed hereditary metabolic disorder. He was knock-kneed and his feet were abnormally arched. He had fine, brittle sparse hair and dislocated lenses. His mental capacity was very low, he was neither clean nor dirty and he could say a few words, his urine was sent for amino acid metabolic screening.

3. Ibrahim Ahmed. Age 4 years was brought to hospital for investigation he was deaf and mentally subnormal. On investigation he was found to have renal failure. All common causes of renal

disease were excluded. The treating paediatrician suspected an association of his renal condition with an inherited disorder and sent his urine for screening of inborn error of metabolism.

Screening tests for detection of inborn errors of metabolism.

Introduction

The diseases which are known to be caused by inborn errors of metabolism are clinically so diverse that it may appear confusing to consider them collectively. However, the common factor that justifies grouping them together is that the correct diagnosis can always be established by appropriate biochemical tests even when no clinical evidence exists for the underlying metabolic disorder. This program of rapid, easy and inexpensive urine test can be applied to large numbers of samples to detect abnormal metabolites and this way select the patients who require further investigation.

A. Smell: Characteristic odours have been reported in several metabolic disorders

Summary of these disorders is shown below:

Disease	Odour	Compound
Phenyl ketonuria	Mouse-like	Phenylactate
Maple syrup disease	Maple syrup (sweetich)	Branched chain disease ketoacids
Cysinuria	Sulphurons	Hydrogen sulphide
Homocysinuria	Sulphurons	Hydrogen sulphide
Isovaleric acidaemia	Sweaty feet (cheesy)	Isovaleric acid

B. Ferric chloride test:

Reagent:

10% ferric chloride (brown bottle).

Method:

Into 3 ml of urine add few drops of ferric chloride solution,

Result:

Positive result will give blue or green colour. The colour developed for one to two minutes, it then gradually fades. The produced is not specific, a list of diseases and compounds which give a positive result is given below.

Disease	Compound	Ferric chloride test positive
Phenyl ketonuria	Phenyl pyruvic acid	Blue - green
Tyrosinosis	p-hydroxyphenyl pyruvic acid	Transient blue-green
Histidinaemia	Imidazole pyruvic acid	Grey-green
Maple syrup urine	Alpha-keto isovaleric acid	Blue

Disease	Compound	Ferric chloride test positive
Alkaptonuria	Homogentisic acid	Transient blue-green
Others	Conjugate bilirubin	Bluish-green
	Melanin	Grey-black
	Salicylates	purple

C. Reducing substances:

Bendict's test:

Method:

1. Into 2 ml Bendict's reagent adds 4 drops of urine, boil for 2 minutes.

Result:

Change in the colour will be according to the concentration of reducing substance present in the urine.

- Blue colour = negative
- Green without precipitate (p, pt) = Trace, with (p, pt) = +
- Yellow = 2 +
- Orange = 3+
- Red or brick red = 4+ or more.

Other tests for reducing substances include: clinistest taule. Glucose oxidase strips for glucose, etc.

Some examples of reducing substances are:

1. Glucose: D.M. renal glucosuria, Fanconi's syndrome, cystinosis.

2. Galactose: Galactosuria, Fanconi's syndrome, cystinosis.

3. Fructosaemia (aldolase deficiency), and essential fructosuria.

4. Lactose: Lactose deficiency.

5. Xylulose: Pentosuria.

6. Homogentisic acid: Alkaptonuria.

7. Drugs: Ascorbic acid.

D. Cyanide nitroprusside test for sulpher containing amino acids:

Reagent:

- Ammonium Hydroxide (Bench reagent)
- 5% Sodium Cyanide
- 5% Sodium Nitroprusside.

Method:

1. Into a test tube transfer 2.5 ml of urine add 2-3 drops of (a) to alkalinize the urine
2. Add 1 ml of (b) mix stand for 20 minutes.
3. Add one drop of (c).

Results:

- A pink-red (Beet colour) within 5 minutes indicates abnormal urine.
- Compound which possess free sulfhydryl groups of disulfide bond give a positive result.

1. Cystine (Cystinuria).
2. Homocysteine (Homocystinuria).

E. Isatin test for Proline:

Reagents:

- 20 mg isatin in 9 ml acetone and 4 ml of glacial acetic acid.
- 1 N hydrochloric acid.

Method:

1. Dip a filter paper in (a) allow to dry.
2. Place one drop of urine on the filter paper and dry in oven (100°C).
3. Dip in (b) and wash with water.

Results:

- A deep blue colour of the urine spot suggest that praline present in excess (Hyperprolinaemia, Hydroxy prolinaemia).

F. Toluidine blue test for Mucopolysaccharides:

Reagents:

- Toluidine blue: Toluidine blue 100 mg + 5 ml acetic acid completes with distilled water to 500 ml.
- Ethanol.

Method:

1. Place two drops of urine on a piece of filter.
2. Allow to dry
3. Then soak in (a) for one minute.
4. Dip the paper in (b) to decolorize thebackground
5. Allow the paper to dry and observe the colour.

Result:

- Excess mucopolysaccharides give reddish-purple colour.
- The background remains pale blue.
- Positively in a variety of mucopolysaccharidosis syndromes e.g. Hurler syndrome type 1. Hunter ect.

G. Nitrosonaphthol test for tyrosine metabolites:

Reagents:

- N nitric acid (1 vol. nitric acid in 5 vol. water)
- 2.5% sodium nitrite in water.
- 1% ritrosonaphthol in 95% ethanol.

Method:

1. To 1 ml of (a) add one drop of (b) and 10 drops of urine.

Results:

- An orange - red colour within 3-5 minutes indicato positive results.

1. Tyrosine transaminase deficiency.

2. Tyrosinosis.

H. Dintrophenylhydrazine test for keto acid:

Reagents:

- 100 mg 2-4 Dintrophenylhydrazine in 100 ml 2 N Hcl (brown bottle)

Method:

1. Add 10 drops of reagent to 1 ml of clear urine.

Results:

1. Phenyl ketonuria (phenyl pyruvic acid)

2. Maple syrup urine disease (branched chain keto acids).

3. Tyrosinosis (p-hydroxyphenyl pyruvic acid & lactic acid)

4. Histidinaemia (Imidazole pyruvic acid).

I. Ehrlich's aldehyde for porphobilibogen

Reagent:

- 2% p-dimethylamino benzaldehyde in 2N hydrochloric acid

Method:

1. Add 1 ml of reagent to 5 ml of urine. Examine for colour after 10 minutes

Results:

- A pink colour is given by urobilibogen and porphobilinogen.
- Add 5 ml of cholorform to the tube and shake.
- The pink colour of porphobilinogen is not extracted in the chloroform layer.

1. Acute porphyria.

2 Acute intermittent porphyria

3. Acute hereditary porphyria acid.

Further investigation done:

1. Amna:

- On screening a reducing substance was found in urine.
- Glucose was measured using two different methods.

1. Ferricyanide reduction method.
2. Glucose oxidase method

Results:

- Total reducing sugar: 170 mg/dl
- Blood glucose: 55 mg/dl normal: 70-110 mg/dl

Sugar chromatography was performed. What do you think the sugar identified would be?

2. Ali Taha:

- The cyanide nitroprusside test for sulpher containing amino acids was positive.
- On ion exchange chromatography the presence of an abnormal large concentration of sulfur containing amino acids was confirmed.
- A loading test of methionine was carried out.
- The patient excreted a much larger proportion of the administered methionine than did a normal control.
- Homocystine was detected in urine.

3. Ibrahim Omer:

- Satin test for proline was positive.
- The patient condition remained the same.
- The renal failure did not improve.