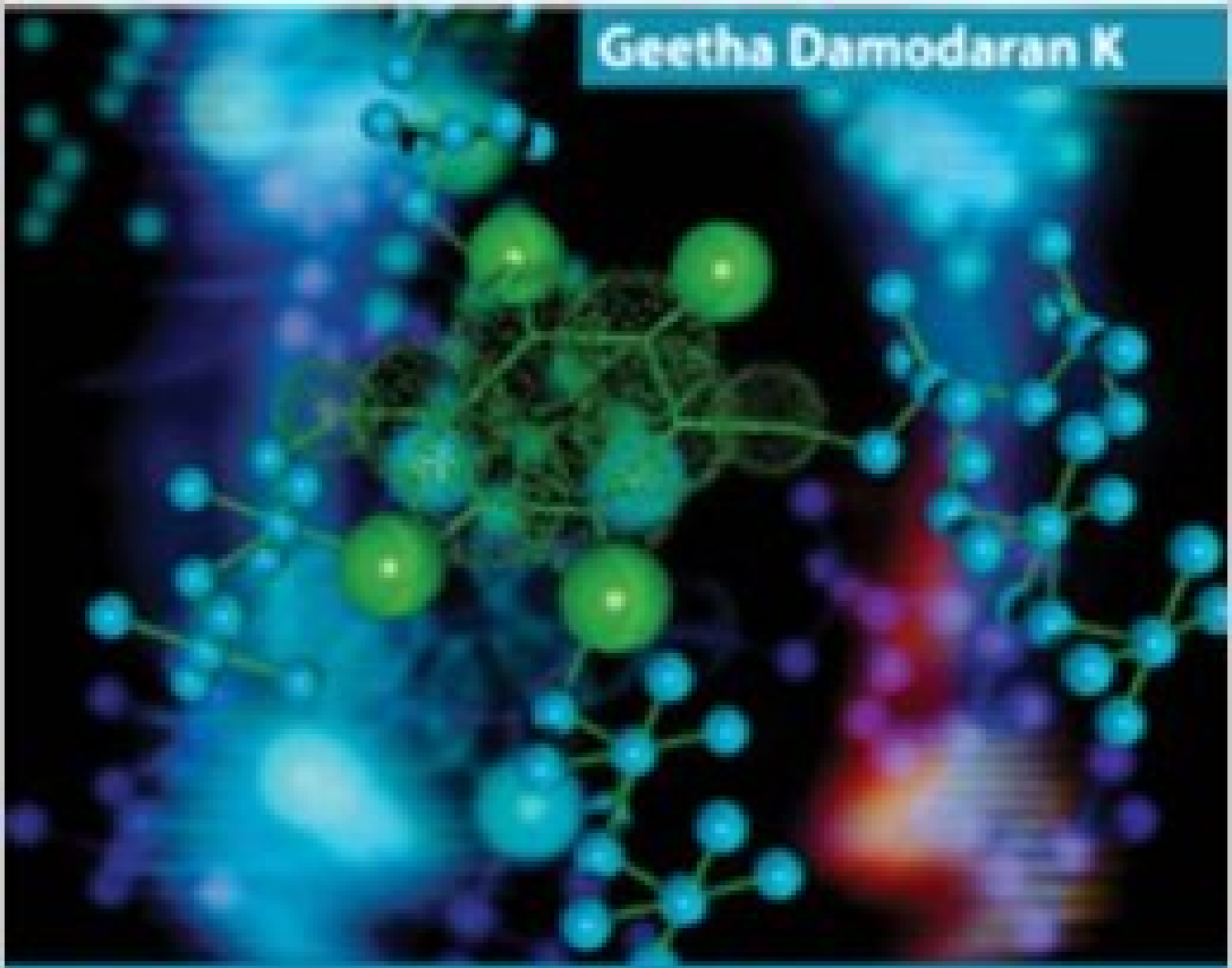
PRACTICAL BIOCHEMISTRY

Geetha Damodaran K



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Practical Biochemistry

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To
my father (late) Sri KV Damodaran

Preface

Nearly two decades of teaching experience have driven me to write this book. I realized that if an illustrated book is available, students will be able to recollect the experiments done earlier, to face the different types of questions during practical examinations. Hence all the items in this book are illustrated.

The contents of this book are structured in the practical examination-oriented manner. The major sections are qualitative experiments, quantitative experiments, charts, spotters and objective structured practical examination questions. All the tests are provided with diagrams and interpretations. This will help the students to understand each concept thoroughly and enable them to use it as an instant doubt clearing book. I hope it will be very useful for day-to-day studies and exam preparations.

Details of reagent preparations given along with the respective chapters are useful for the staff involved in the laboratory preparation of practical sessions. This part will also help to improve the level of understanding of students about the reagents they are using for various experiments in the laboratory.

Questions provided with the chapters are useful for having better clarity and grasp of the topic. Moreover, it will definitely boost the confidence of students to face the examination. Chapters on charts and spotting and OSPE questions are useful for self-training of such type of evaluation methods.

I warmly welcome the views of those using the book and I shall be grateful to the readers for bringing to my notice of mistakes for corrections, in future editions of the book.

Geetha Damodaran K

Acknowledgments

I would like to thank God for enabling me to do this work. I thank my parents, teachers for molding me to reach this level. I extend my gratitude to my colleagues for their support. I should thank my husband Dr PK Balachandran for constantly persuading me to write.

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SECTION ONE

Qualitative Analysis

Reactions of Carbohydrates

1

1A. REACTIONS OF MONOSACCHARIDES

INTRODUCTION

Carbohydrates are aldehyde or ketone derivatives of polyhydric alcohols. They are widely distributed in plants and animals. Plants synthesize glucose by photosynthesis and it is converted mainly to storage form, the starch and structural frame work form, the cellulose.

Animals largely depend on plant source to obtain carbohydrates though they can synthesize carbohydrates from non carbohydrates sources *like glycerol and amino acids* in their body (gluconeogenesis).

The glucose is the major form of carbohydrate absorbed from the gut in humans.

According to the metabolic status it has different fates–

- catabolized to release energy
- polymerized to form the storage fuel—the glycogen
- sometimes converted to other sugars like fructose and galactose.

Different carbohydrates are present in intracellular and extracellular fluids and are excreted in urine when the concentration of them rises in the blood as in certain diseases (glucose in urine in diabetes mellitus, fructose in urine in fructosuria , galactose in urine in galactosemia). Hence, it is essential to understand the tests for their detection.

The classification of **carbohydrates** will be useful for the detection of various types of carbohydrates by different chemical tests.

CLASSIFICATION

1. Monosaccharides: Cannot be hydrolyzed into simpler carbohydrates. They are classified into trioses,tetroses,pentoses, hexoses, heptoses based on the number of carbon atoms present in them. They are again divided into aldoses and ketoses based on the functional group present in them (see Table 1A-1).

Table 1A-1: Classification of Monosaccharides

Monosaccharides	*Aldoses*	*Ketoses*
Trioses	Glycerose	Dihydroxyacetone
Tetroses	Erythrose	Erythrulose
Pentoses	Ribose	Ribulose
Hexoses	Glucose	Fructose

2. Disaccharides: Give rise to two monosaccharide units upon hydrolysis

E.g.: Sucrose (glucose + fructose)
Lactose (glucose + galactose)
Maltose (glucose + glucose)

3. Oligosaccharides: Yields less than ten monosaccharides.

E.g.: Maltotriose (3 glucose units),
Raffinose (glucose + fructose + galactose)

4. Polysaccharides: Contain more than ten monosaccharide units

(i) Homopolysaccharides (consisting of same type of monomeric units)

Polymer of glucose: Starch, glycogen, cellulose

Polymer of fructose: Inulin

(ii) Heteropolysaccharides (consisting of different types of monomeric units)

Proteoglycans, e.g. Heparin (D-glucosamine sulfate + D-sulfated iduronic acid)

Hyaluronic acid (D-β glucuronic acid + N-acetylglucosamine).

REACTIONS OF MONOSACCHARIDES

Monosaccharides possess one or more hydroxyl groups and an aldehyde or keto group. Therefore many reactions of monosaccharides are the known reactions of alcohols,aldehydes or ketones. Many of the reactions shown by monosaccharides are exhibited by higher carbohydrates also. Differences in the structures of sugars often affect the rate of a reaction and sometimes the ability to react.

The reactions described below, are applied in the identification of sugars.

The reactions due to hydroxyl group:

- Dehydration (e.g. Molisch test, Rapid furfural test, Seliwanoff's test)

The reactions due to carbonyl group:

- Reduction (e.g. Benedict's test, Barfoed's test)
- Condensation (e.g. Osazone test)

1. Molisch Test (α-Naphthol Reaction) (Fig. 1A-1)

Procedure: To 5 ml of sugar solution in a test tube add two drops of Molisch reagent. Mix thoroughly. Add 3 ml of concentrated sulphuric acid along the sides of the test tube by slightly inclining the tube, thus forming a layer of acid (acid being heavier goes down beneath the sugar solution) in the lower part.

Observation: A **reddish violet ring** appears at the junction of two liquids.

Inference: Indicates presence of a carbohydrate and hence the presence of monosaccharide.

Principle: Concentrated acid dehydrates the sugar to form furfural (in the case of pentoses) or furfural derivatives (hexoses and heptoses) which then condense with α-naphthol to give a **reddish violet** colored complex

Application of the test: Used as a general test to detect carbohydrates.

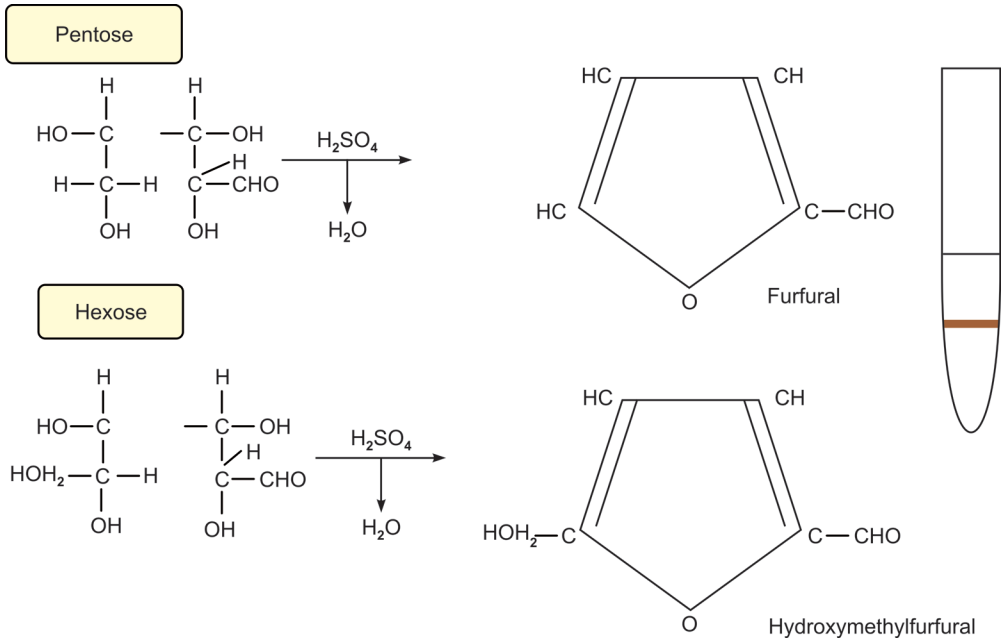
Aberrant Observations

1. Instead of a violet ring in the Molisch test, appearance of **dark brown color** indicates **charring of sugar** due to the **heat generated during the addition of acid** (acid water interaction generates heat). It will become obvious when the concentration of the sugar solution is high. To avoid charring, dilute the sugar sample solution with water as depicted in figure 1A-2 and repeat the Molisch test.

2. Appearance of a **green color** while doing the test, which persist even after completion of the test suggest excess use of Molisch reagent than required or due to the presence impurities in the reagent.

2. Benedict's Test (Fig. 1A-3)

Procedure: To 5 ml of Benedict's reagent in a test tube add exactly 8 drops of the sugar solution. Mix well. Boil the solution vigorously for two minutes or place in a boiling water bath for three minutes. Allow the contents to cool by keeping in a test tube rack. Do not hasten cooling by immersion in cold water.



Furfural and hydroxymethylfurfural condense with phenolic (alpha naphthol in Molisch test) compounds to give rise to colored products.

Fig. 1A-1: Chemistry of Molisch test

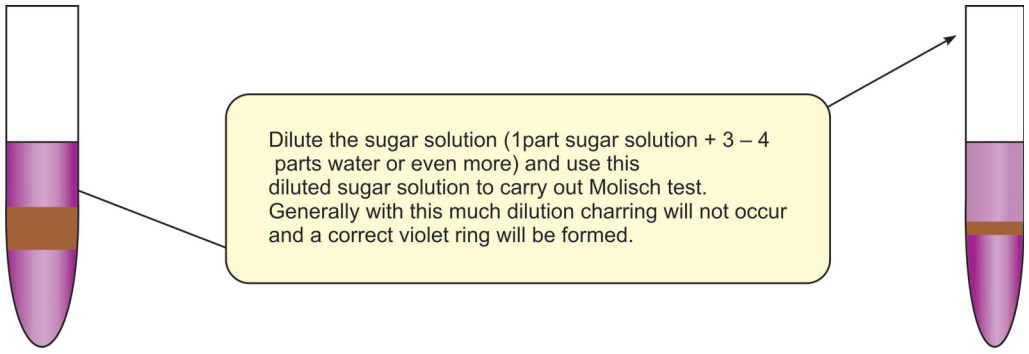


Fig. 1A-2: Method to avoid charring

Observation: The entire body of the solution will be filled with a precipitate, the color of which varies with the concentration of the sugar solution—green, yellow, orange or red.

In the absence of reducing substance, blue color of the Benedict's reagent remains as such. **The test is sensitive up to 0.1-0.15 gm% of sugar solution (that is Benedict's test will not be**

positive with solutions containing less than 0.1-0.15 gm% of sugar).

Inference: Reducing monosaccharides, glucose, fructose, galactose and mannose give a positive reaction with Benedict's reagent.

The color of the precipitate give an idea about the concentration of the sugar solution as shown below.

Blue – absence of reducing sugar
Green – up to 0.5 gm%
Yellow – > 0.5 to 1.0 gm%
Orange – > 1.0 to 2.0 gm%
Brick red – ≥ 2 gm%

Thus, Benedict's test is described as a semi-quantitative test.

Principle: (see Fig. 1A-4) Carbohydrates with a free aldehyde or keto group have the ability to reduce various metallic ions. In this test cupric ions are reduced to cuprous ions by the enediols formed from sugars in the alkaline medium of Benedict's reagent.

Benedict's reagent contains copper sulphate, sodium citrate and sodium carbonate.

Copper sulphate dissociate to give sufficient cupric ions (in the form of cupric hydroxide) for the reduction reactions to occur.

Sodium citrate keeps the cupric hydroxide in solution without getting precipitated.

Sodium carbonate (Na_2CO_3) make the pH of the medium alkaline.

In the alkaline medium sugars form enediols which are powerful reducing agents. They reduce blue cupric hydroxide to insoluble yellow to red cuprous oxide.

Application of the test: To detect reducing sugars. It is widely used in detecting glucose in urine even though not specific for glucose.

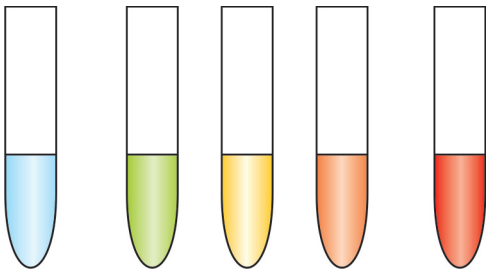


Fig. 1A-3: Benedict's test at different sugar concentrations

3. Barfoed's Test (Fig. 1A-5)

Procedure: To 5 ml of Barfoed's reagent in a test tube add 0.5 ml of sugar solution. Mix well. Keep in a boiling water bath for **2 minutes**. Keep the tube in a test tube rack and examine for precipitate after 10-15 minutes.

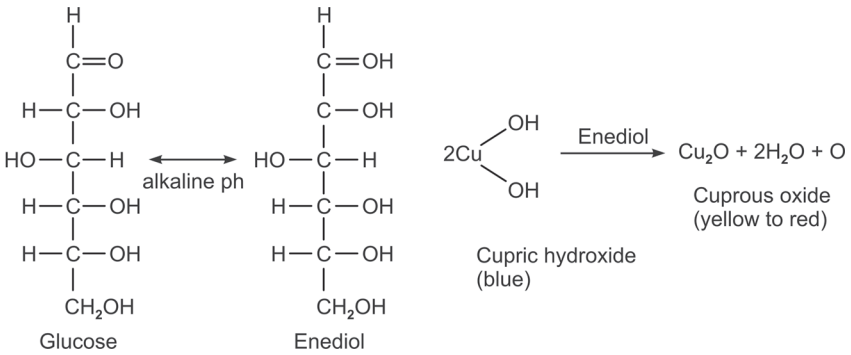


Fig. 1A-4: Chemistry of Benedict's test

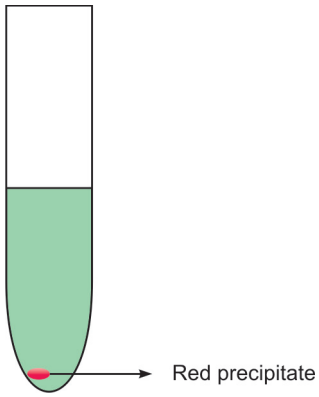


Fig. 1A-5: Barfoed's test

Observation: A **red** precipitate clinging to the bottom most part of the test tube forms, in the presence of a monosaccharide**.**

Inference: The test is answered by monosaccharides only, e.g. glucose, fructose, galactose, mannose.

Principle: It is a reduction test. Reducing property owes to the carbonyl group (aldehyde or keto group). Barfoed's reagent is copper acetate in acetic acid.

Difference between Barfoed's test and Benedict's test: Barfoed's test differs from Benedict's test with respect to the pH of the medium. It is alkaline in the case of Benedict's and acidic in the case of Barfoed's test. In the acid medium monosaccharides enolize much more readily than disaccharides and these enediols reduce cupric ions released by copper acetate of Barfoed's reagent to produce a red precipitate.

Points to Ponder

- It is important to keep the time limit (2 minutes) prescribed for Barfoed's test otherwise disaccharides will also respond to the test positively.
- Disaccharides when present in high concentrations (> 5 gm%) also will give positive response
- Unlike the Benedict's test, Barfoed's test is unsuitable for testing sugars in urine or any fluids containing chloride.
- The red precipitate is formed at the bottom of the tube. To see the precipitate, lift the tube to the eye level, otherwise the precipitate formed adhering to the bottom most part of the tube may escape notice.

Application of the test: Useful to distinguish between monosaccharides and disaccharides.

Chemistry of the test: Reduction reaction as shown under Benedict's test.

4. Rapid Furfural Test

Procedure: To 2 ml of sugar solution add 6 drops of Molisch reagent and 3 ml of concentrated HCl. Boil for **30 seconds** only.

Observation: Positive reaction is indicated by the development of **violet** color (Fig. 1A-6).

Inference: Development of violet color within **30 seconds** of boiling indicates presence of a keto sugar, e.g. fructose.

Principle: A dehydration reaction which owe to the hydroxyl groups of the sugar. Concentrated HCl being weaker than concentrated sulphuric acid, dehydrate ketoses (e.g. fructose) more readily than aldoses to form hydroxymethyl furfural, which then condenses with α-naphthol to form a violet colored complex.

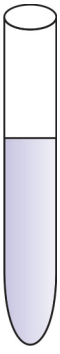


Fig. 1A-6: Positive rapid furfural test

Chemistry of the test: Dehydration reaction as shown under Molisch test.

Aberrant reaction: If red color develops instead of violet color due to charring action of acid, dilute the sugar sample with water and conduct the test with diluted sugar solution (Fig. 1A-7).

Application of the Test

- For the detection of ketoses.
- Useful for differentiating ketoses from aldoses.

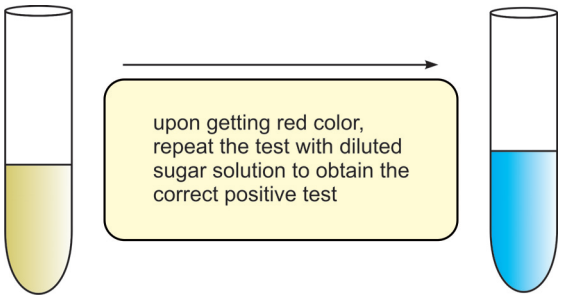


Fig. 1A-7: Method to avoid charring response in rapid furfural test

5. Seliwanoff's Test

Procedure: To 5 ml of Seliwanoff's reagent in a test tube add 5 drops of fructose solution and heat the contents to just boiling.

Observation: Positive reaction gives a **red color** within half a minute (Fig. 1A-8).

Inference: This test is given by ketoses. e.g. fructose.

Fig. 1A-8: Positive Seliwanoff's test

Principle: A dehydration reaction due to the hydroxyl groups of the sugar. Selivanoff's reagent is resorcinol in dilute hydrochloric acid. Ketoses (e.g. fructose) are more readily dehydrated by HCl than the aldoses to form hydroxymethyl furfural which then condenses with resorcinol of Seliwanoff's reagent to form a **red colored** complex.

Points to Ponder

- The test is sensitive up to 0.1 gm% of fructose in the absence of glucose.
- In the presence of glucose, the test becomes less sensitive to fructose.
- Large amounts of glucose gives the same color.
- If the boiling is prolonged a positive reaction may occur with glucose because of Lobry de Bruyn-van Ekenstein transformation of glucose into fructose in the presence of acid.

The precautions to be followed to get a positive test for fructose are given below:

1. Concentration of HCl used must be less than 12%.
2. The reaction must be observed ***within 20 to 30 seconds of performing the test.***
3. Those reactions occurring after 20 -30 seconds, must not be taken into account.
4. Glucose must not be present in amounts more than 2% or else it will interfere with the test.

6. Osazone Test

Procedure: To 5 ml of sugar solution in a test tube add 300 mg (one or two scoopfuls) of phenyl hydrazine mixture. Shake well. Heat in a boiling water bath for **15 minutes**. Then take the tube out of the water bath and allow cooling at room temperature by placing it in the test tube rack.

Avoid showing under the tap water because rapid cooling disturbs crystallization where as slow cooling ensures crystallization (ideally within the water bath itself).

Observation: Crystals are formed readily (within 1-5 minutes) at the room temperature in the case of mannose. For other sugars minimum time required in minutes in the water bath for the formation of insoluble yellow osazone is given in the Table 1A-2.

Look under the microscope to view the crystals (see Fig. 1A-9).

Table 1A-2: Time of Formation of Osazones

Sugar	*Time (minutes)*
Glucose	5
Fructose	2
Galactose	20

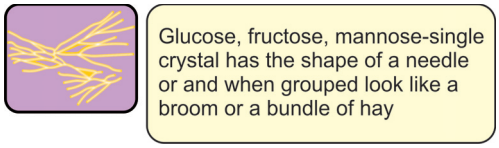


Fig. 1A-9: Osazone crystals

Inference: Glucose, fructose, mannose yield the same shaped phenyl osazone crystals because of the elimination of differences in configuration about the carbon atoms 1 and 2 during osazone formation.

Principle: The reaction involves the carbonyl carbon (either aldehyde or ketone as the case may be) and the adjacent carbon. One molecule of sugar reacts with one molecule of phenyl-hydrazine to form phenylhydrazone which then reacts with two additional phenyl hydrazine molecules to form the osazones as shown in the figure 1A-10.

Points to Ponder

If the solution appears red after heating process, it indicates that the solution has become concentrated in the boiling process and no crystals will separate in the concentrated form. So dilute with water for the separation of crystals.

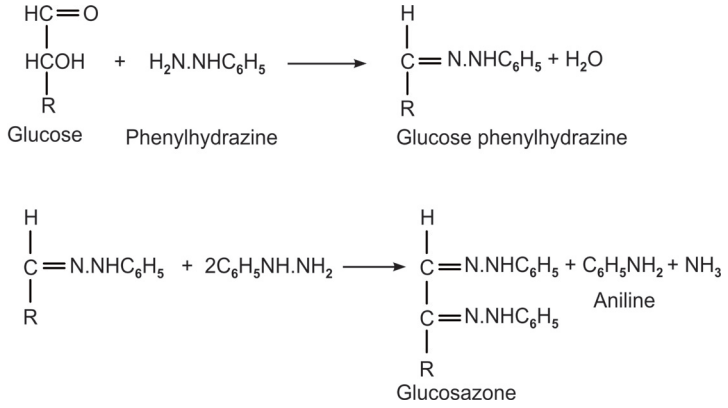


Fig. 1A-10: Chemistry of Osazone test

1B. REACTIONS OF DISACCHARIDES

INTRODUCTION

Disaccharides are glycosides in which both components are monosaccharides. The general formula of common disaccharides is $C_{12}H_{22}O_{11}$. The common disaccharides studied are detailed below.

Maltose (α-D-glucopyranosyl-(1→4) α-D-glucopyranose) (Fig. 1B-1): Maltose yield 2 glucose molecules upon hydrolysis. Maltose is formed from the hydrolysis of starch by the action of the enzyme maltase. It is also produced as an intermediate product of mineral acid hydrolysis of starch. It is dextrorotatory, exhibits mutarotation, reduces metallic ions in alkaline solutions. Like other disaccharides maltose is hydrolyzed by dilute acid leading to the formation of two molecules of glucose. With phenyl hydrazine maltose forms maltosazone.

Examples for other disaccharides that produce only glucose upon hydrolysis:

- *Cellobiose* a β glucoside with 1,4 linkage derived from partial hydrolysis of cellulose.
- *Gentiobiose*, a β glucoside with 1,6 linkage derived from roots of Gentiana lutea.
- *Trehalose*, α glucoside with 1,1 linkage obtained from yeast and mushrooms.
- *Isomaltose*, α glucoside with 1,6 linkage formed as a side product of hydrolysis of starch by amylase enzyme.

Lactose (β-D-galactopyranosyl-(1→4) β-D-glucopyranose) (Fig. 1B-2): Lactose give rise to one molecule of glucose and galactose upon enzymatic (lactase) or acid hydrolysis. Lactose is normally present in milk and in the urine of women during later half of pregnancy and during lactation. It is dextrorotatory, shows mutarotation in solution. It reduces metallic ions, forms lactosazone with phenylhydrazine. It is a galactoside since the carbon number 1 of galactose is involved in the β galactoside bond with the carbon number 4 of glucose.

Sucrose (α-D-glucopyranosyl-β-D fructo-furanoside): (see Fig. 1B-3).

Hydrolysis of sucrose yields one molecule of glucose and one molecule of fructose. Sucrose is dextrorotatory. After hydrolysis by enzymes or weak acids , it becomes levorotatory. This is because of the formation of fructose upon hydrolysis, which is strongly levorotatory than the glucose .Thus the change of optical rotation of sucrose solution from dextro to levo rotation upon hydrolysis is known as **inversion** and the mixture of glucose and fructose obtained is called **invert sugar**.

Sucrose do not reduce metallic ions (do not answer Benedict's and Barfoed's tests) and also do not form osazone with phenylhydrazine.

But *prolonged boiling* with phenylhydrazine in acid medium will form osazone due to the reaction of products of hydrolysis of sucrose with

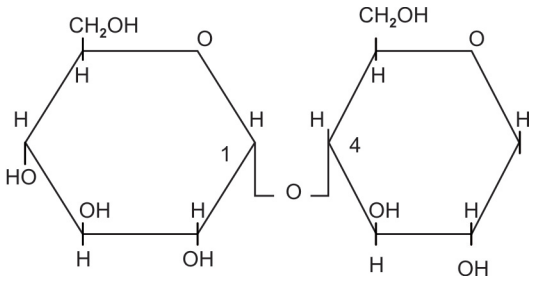


Fig. 1B-1: Maltose (α-D-glucopyranosyl-(1→4) α-D-glucopyranose)

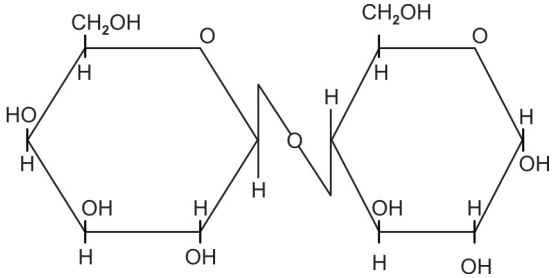


Fig. 1B-2: Lactose (β-D-galactopyranosyl-(1→4) β-D-glucopyranose)

phenylhydrazine and **not due to the reaction of intact sucrose** molecules with phenylhydrazine.

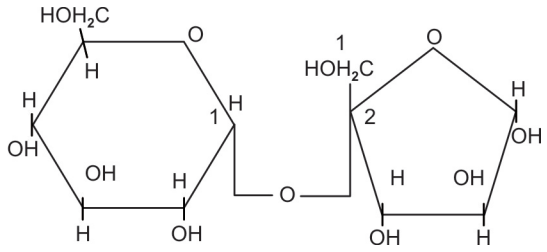


Fig. 1B-3: Sucrose – (α-D-glucopyranosyl – β-D fructofuranoside)

REACTIONS OF DISACCHARIDES

1. Molisch Test

Principle: Response of the disaccharides: All the disaccharides that are experimented routinely give the positive reaction – ***reddish violet ring*** *as this is a general test to detect the presence of carbohydrate.*

Procedure:
Observation:
Inference:
} Same as given under monosaccharides

2. Benedict's Test

Procedure:
Observation:
Inference:
} Same as given under monosaccharides

Response of the Disaccharides

Based on Benedict's test disaccharides are classified into:

1. Reducing disaccharides, e.g. Lactose, Maltose. These disaccharides have a free carbonyl (keto/aldehyde) group which is not involved in glycosidic linkage will reduce cupric ions in the alkaline medium as explained under the monosaccharides, E.g. Lactose, maltose.

2. Nonreducing disaccharides
E.g. Sucrose, Trehalose. These are the disaccharides in which the functional groups of constituent monaosaccharides are in linkage.

3. Barfoed's Test

Procedure:
Observation:
Inference:
} Same as given under monosaccharides

Principle: Response of the disaccharides: Disaccharides will not reduce cupric ions in the weak acid medium within the prescribed keeping time of 2 minutes in the boiling water bath and do not give a positive response to the test.

Application: Useful to differentiate monosaccharides from disaccharides.

Points to Ponder

- If the heating time is prolonged disaccharides will also give a positive response to Barfoed's test.
- If the concentration of disaccharide solution is high, Barfoed's test tends to become positive.

4. Osazone Test

Procedure: Same as given under monosaccharides except for the period for which the reaction tube to be placed in the boiling water bath – it is **45 minutes** for disaccharides.

Lactose gives a characteristic yellow puff shaped lactosazone crystals (see Fig. 1B-4).

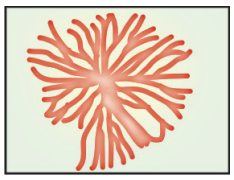


Fig. 1B-4: Lactosazone (Puff shaped)

Maltose: Individual crystals of maltosazone looks like a yellow colored petal and when grouped looks like a sun flower (see Fig. 1B-5).

Fig. 1B-5: Maltosazone (Petal shaped)

Inference

Lactose → Puff shaped lactosazone crystals
Maltose → Petal shaped or sunflower shaped maltosazone crystals
Sucrose → Will not form osazone

Principle: Reducing disaccharides with a reactive carbonyl group condense with phenyl hydrazine to form respective osazone crystals with characteristic shapes as detailed above.

Application: Useful to differentiate disaccharides.

5. Seliwanoff's Test

Procedure: Same as given under monosaccharides.

Observation: Sucrose gives **bright red color** (see Fig. 1A-8) whereas lactose and maltose do not give red color.

Inference: Sucrose upon acid hydrolysis by the HCl in the Seliwanoff's reagent yields a keto sugar, fructose. Fructose being a keto sugar gives positive response to Seliwanoff's test as described under monosaccharides. Whereas lactose (galactose + glucose) and maltose (glucose + glucose) contain no keto sugar and cannot give positive response to this test upon acid hydrolysis by the HCl present in the Seliwanoff's reagent.

Principle: The disaccharide sucrose contains glucose and fructose. Fructose formed from sucrose upon acid hydrolysis by the HCl of Seliwanoff's reagent, is dehydrated by the acid HCl to form hydroxymethyl furfural which then condenses with the **resorcinol** of Seliwanoff's reagent to form a **red colored** complex.

6. Rapid Furfural Test

Procedure: Same as given under monosaccharides.

Observation: Sucrose gives **violet color** (see Fig. 1A-6) whereas **lactose** and **maltose** do not give violet color.

Inference: Sucrose upon acid hydrolysis by the HCl added in the test yields a keto sugar fructose. Fructose being a keto sugar gives positive response to Rapid furfural test as described under monosaccharides. Where as lactose (galactose + glucose) and maltose (glucose + glucose) contain no keto sugar and cannot give positive response to this test .

Principle: The disaccharide sucrose contains glucose and fructose . Fructose formed from sucrose upon acid hydrolysis by the HCl, is dehydrated by the same HCl to form hydroxymethyl furfural which then condenses with the **α-naphthol** of Molisch reagent to form a **violet colored** complex.

7. Specific Sucrose Test (Fig. 1B-6)

Procedure: It is done in two steps.

Step 1: Hydrolysis

To 5 ml of sucrose solution add 1 drop of thymol blue indicator and one or two drops of dilute HCl to make the solution acidic as shown by the development of pink color. Divide it into two equal parts. Boil one part for 1 minute and the other part is kept as control. Neutralize both parts

by adding 2% sodium carbonate drop by drop until a blue color develops.

Step 2: Benedict's Test

Perform Benedict's test with each portions.

Observation: Unboiled sucrose solution will not give a positive response to Benedict's test where as boiled portion gives a positive response.

Inference: Sucrose is hydrolyzed by HCl in the first step to form glucose and fructose and the medium is neutralized by the 2% sodium carbonate.

In the second step, products of acid hydrolysis reduce cupric ions to red cuprous oxide.

Precautions

1. Avoid adding excess acid because it will dehydrate sugar to form furfural derivatives and that will interfere the test.
2. Always remember to add alkali as per the test procedure since neutralization of acidic pH is needed for getting correct reaction in the second step.

Thymol blue indicator contains two components that work at acid **range (pH range 1.2-2.8; color change – red to yellow)** and **at alkaline range (pH range 8.0-9.6; color change – yellow to blue).**

1C. REACTIONS OF POLYSACCHARIDES

INTRODUCTION

The polysaccharides are complex carbohydrates of high molecular weight, which on hydrolysis yields monosaccharides or products related to monosaccharides. The various polysaccharides differ from one another with respect to their constituent monosaccharide composition, molecular weight and other structural features.

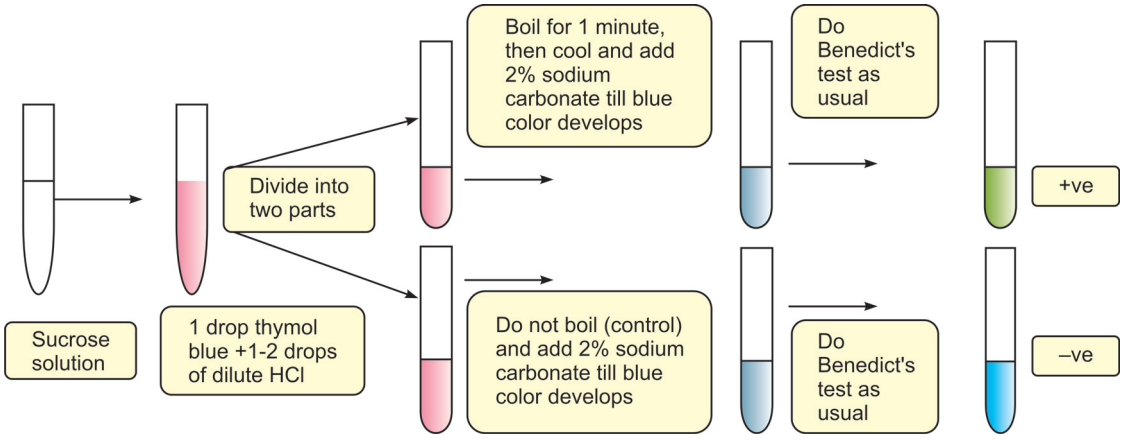


Fig. 1B-6: Specific sucrose test

In all types the linkage between the monosaccharide units is the glycosidic bond.

This may be α or β which join the respective units through $1 \rightarrow 2, 1 \rightarrow 3, 1 \rightarrow 4$ or $1 \rightarrow 4$ linkages in the linear sequence or at branch points in the polymer.

Polysaccharides are **classified** based on the type of monosaccharide units present in them.

1. Homopolysaccharide

Contains only one type of monosaccharide, e.g. Starch, Glycogen.

2. Heteropolysaccharide

Contains more than one type of monosaccharide units, e.g. Glycosaminoglycans (heparin, hyaluronic acid).

We will discuss the reactions of starch in this chapter in order to understand the chemical properties of polysaccharides in general.

REACTIONS OF STARCH

1. Molisch Test

Procedure:

Observation and Inference: Same as given under monosaccharides.

Principle: The test is answered by all furfural yielding substances and hence all the carbohydrates.

2. Iodine Test

Procedure: To 2-3 ml of starch solution add 2 drops of dilute (0.05 N) iodine solution. Observe the changes on heating and on subsequent cooling.

Observation: Deep blue color appears which then disappears on heating and then reappears on cooling (see Fig. 1C-1).

Inference: Starch forms a adsorption complex with iodine to give a blue color. The blue color disappears on heating due to the breaking of the Iodine starch adsorption complex and appears on cooling due to reformation of the adsorption complex.

3. Benedict's Test

Procedure: Same as given with monosaccharides.

Observation: No colored precipitate.

Inference: Starch is a nonreducing carbohydrate.

4. Starch Hydrolysis Test

Procedure: Take 25 ml of starch solution in a beaker. Add 10 drops of concentrated HCl and boil gently. At the end of each minute , transfer a drop (using glass tube) of the solution on to a plate for doing the iodine test and 3 drops to 5 ml of Benedicts solution (Set tubes containing 5 ml of Benedict's reagent in series) . Continue until the iodine test becomes negative. Then place the tubes for the Benedict's test in the boiling water bath for 3 minutes.

Observation: See Table 1C-1.

Inference: Starch upon hydrolysis by HCl gives the following products.

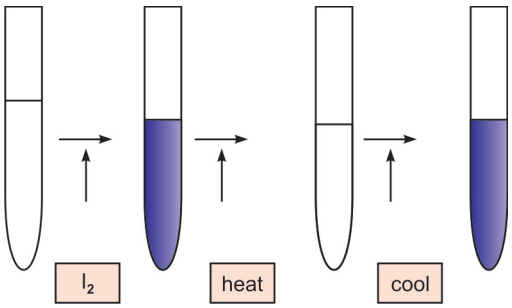


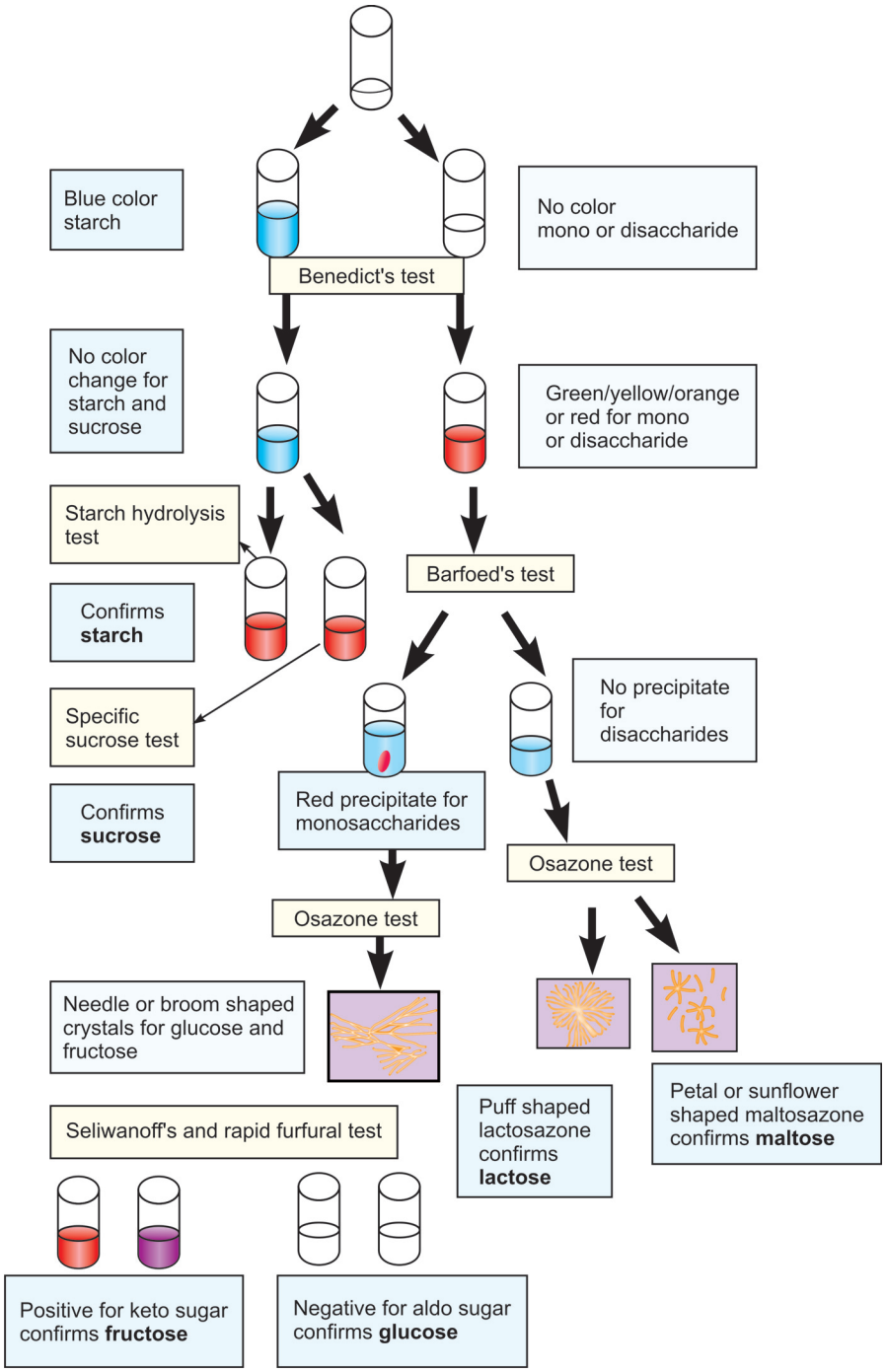
Fig. 1C-1: Iodine test

Table 1C-1: Response of Starch Hydrolysis Test

Time in minutes	*Color with I_2*	*Benedict's test*	*Hydrolysis*	*Product*
1	Blue	Blue	No reduction	Starch
5	Violet	Green	Reduction starts	Amylodextrins
8	Reddish violet	Red	Initiation of reduction	Amylo and erythrodextrins
12	No color	Red	Partial reduction	Achrodextrins
20	No color	Red	Complete reduction	Glucose

Starch → Soluble starch → Amylodextrins → Erythrodextrins → Achrodextrins → Maltose → Glucose. When the hydrolytic stage reaches to the level of formation of maltose and glucose iodine test becomes negative and Benedict's test becomes positive.

1D. IDENTIFICATION OF UNKNOWN CARBOHYDRATES



1E. QUESTIONS

1. Name the following:
   a. General test for detecting carbohydrates
   b. Reduction test for monosaccharides
   c. Sugars giving positive response for Rapid furfural test and Seliwanoff's test
   d. The disaccharide yielding puff shaped osazone crystals
   e. Tests based on reduction property of sugars
   f. The test used to detect sugar in urine
   g. Reducing disaccharides
   h. Nonreducing disaccharides
2. Give the principle of the following tests:
   a. Molisch test
   b. Benedict's test
   c. Barfoed's test
   d. Osazone test
   e. Iodine test for starch
   f. Rapid furfural test
   g. Seliwanoff's test
3. Give the ingredients of following reagents:
   a. Molisch's reagent
   b. Benedict's reagent
   c. Barfoed's reagent
   d. Seliwanoff's reagent
4. Benedict's test is described as a semiquantitive test. Explain.
5. Unlike Benedict's test, Barfoed's test is not suitable for testing glucose in urine. Why?
6. Give the difference between Benedict's and Barfoed's test.
7. Why do glucose, mannose and fructose give similar osazone crystals?
8. Sucrose do not form osazone crystals with osazone test. Why?
9. Make a scheme for the detection of an unknown carbohydrate solution.

1F. REAGENT PREPARATION

1. Molisch's Reagent: Dissolve 5 g of α-naphthol in 100 ml of 95% of alcohol.

2. Benedict's Qualitative Reagent: Heat to dissolve 173 g sodium citrate and 100 g sodium carbonate in about 800 ml of water in a conical flask. Transfer to a graduated cylinder through a folded filter paper placed in a funnel or beaker of 1L capacity. Dissolve 17.3 g copper sulfate in about 100 ml of water. Add the copper sulfate solution slowly with constant stirring to the carbonatecitrate solution and make up to 1L.

3. Barfoed's Reagent: Dissolve 13.3 g neutral copper acetate crystals in 200 ml water. Pass through a filter paper placed in a funnel to remove the particles if present to another graduated beaker. Then add 1.8 ml glacial acetic acid.

4. Seliwanoff's Reagent: Dissolve 0.05 g resorcinol in 100 ml dilute HCl .

5. Phenylhydrazine Mixture: Mix 2 parts phenylhydrazine hydrochloride and 3 parts sodium acetate by weight thoroughly in a mortar (Mixture with longer shelf life may be prepared by using equal weights of phenylhydrazine hydrochloride and anhydrous sodium acetate).

6. 0.1 N iodine Solution: Dissolve 1.27 g iodine and 3 g pure KI (potassium iodide) crystals in 100 ml distilled water. Dilute 1:10 in distilled water before use.

7. Glucose, Fructose, Lactose, Maltose, Sucrose, Starch Solutions: 1% solutions -Weigh 1 gm of respective sugars and dissolve in 100 ml of water.

Reactions of Proteins

2

2A. GENERAL REACTIONS OF PROTEINS

INTRODUCTION

Proteins are the most abundant organic molecules (carbon containing) in the living system. They offer structural and dynamic function .They are polymers of amino acids linked by covalent peptide bonds. Proteins ingested undergo digestion and get absorbed as amino acids into the portal vein and reaches liver and then to other tissues.

They are used mainly for protein synthesis as dictated by the genes of respective tissues (differential expression). Some amino acids undergo specific metabolic reactions to produce specialized compounds.eg; epinephrine and nor epinephrine formed from Tyrosine, Serotonin from Tryptophan.

House keeping proteins like aldolase have longer half life where as ***regulatory proteins*** like HMG CoA reductase have shorter half lives. After their life span proteins are catabolized to release nitrogen which ultimately get converted into urea and excreted in the urine where as the carbon skeletons may be utilized for other purposes like gluconeogenesis.

Proteins are classified into fibrous (offer mainly structural function) eg: fibrinogen, troponin, collagen, myosin and globular proteins (offer mainly dynamic functions), e.g. Hb, enzymes, peptide hormones, plasma proteins. Proteins are present in all types of body fluids.

Proteins have to be studied in different ways. During routine analytical laboratory work, two types of reactions are practiced.

1. **Precipitation reactions**
2. **Color reactions**

1. PRECIPITATION REACTIONS OF PROTEINS

Proteins have to be precipitated for different purposes during routine laboratory work. Two such situations are described below:

- For its own identification and estimation, e.g. Proteins are excreted in urine in various forms of kidney dysfunction. According to the degree of kidney damage different proteins are excreted in urine. In the early stages low molecular weight albumin is excreted. As the disease progresses high molecular weight globulin starts excreting.
- For the analysis of other compounds in the specimen, proteins are first precipitated out.

Proteins form emulsoid colloidal solutions (colloid solutions are formed by particles with a diameter ranging from 1 µm to 200 µm). Emulsoids (here proteins) in general possess two

stability factors – charge and water of hydration either of these prevent aggregation and precipitation of proteins. The electrical charges carried by the proteins may be changed in sign or magnitude by changing the acidity or alkalinity of the solution causing them to precipitate. The inorganic salts like ammonium sulfate act as dehydrating agent, there by removing the shell of hydration of the proteins. The dehydration is also carried out by organic solvents like alcohol and ether.

(i) Precipitation by Salts

Inorganic salts when added to the protein solutions, water of hydration around the protein molecules is removed causing aggregation of protein molecules leading to their precipitation. Proteins are lyophilic colloids as they have much affinity for the dispersion medium.

(a) Half saturation test with saturated ammonium sulfate solution:

Procedure: To 3 ml of protein solution add an equal volume of saturated ammonium sulfate solution. Mix and allow to stand for 5 minutes. Filter (for this take a round filter paper of 5 cm radius and fold it to form a cone to make it fit into a funnel. Then place this funnel over a test-tube and pour the contents of the tube through the filter. Perform Biuret test with the filtrate using an equal volume of **40% sodium hydroxide** and 2 drops of 1% $CuSO_4$.)

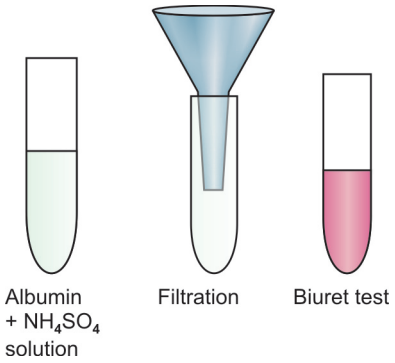


Fig. 2A-1: Half saturation test—Albumin

Observation: Upon doing Biuret test with the filtrate, violet color forms.

Inference

- Albumin is not precipitated by half saturation with ammonium sulfate**.**
- Globulins are precipitated

Principle: The molecular weight of the albumin is much less than the globulin so albumin is not precipitated by half saturation (see Fig. 2A-1) whereas high molecular weight globulins are precipitated.

Points to Ponder: Use 40% sodium hydroxide for doing Biuret test. (In the routine Biuret test 5% sodium hydroxide is used) Here the filtrate contains ammonium sulfate. Ammonium ions form a deep blue cuprammonium ion, $[Cu(NH_3)_4^{++}]$ which will mask the violet color of Biuret test. To avoid this 40% NaOH is used.

(b) Full saturation test with ammonium sulfate crystals:

Procedure: To 5 ml of protein solution, keep on adding ammonium sulfate crystals and at the same time shaking the tube till a few crystals remain at the bottom of the test tube. Filter (for this take a round filter paper of 5 cm radius and fold it to form a cone so as to fit it in a funnel. Then place this funnel over a test-tube and pour the test tube contents through the filter. Perform Biuret test with the filtrate using an equal volume of 40% sodium hydroxide and 2 drops of 1% $CuSO_4$.)

Observation: Upon doing Biuret test with the filtrate no purple or violet color develops.

Inference: The protein (e.g. Albumin) is completely precipitated by full saturation with ammonium sulfate. Upon filtration no protein passes into the filtrate, to be detected by the Biuret test.

Principle: Neutral salt, (e.g. ammonium sulfate) precipitate proteins by salting out which involves the removal of the shell of hydration causing precipitation of proteins. Higher the molecular weight lesser will be salt required for the precipitation. Here globulins have much higher molecular weight than albumin so that albumin require only saturated solution where as globulins require addition of further amount of salt for complete precipitation (see Fig. 2A-2).

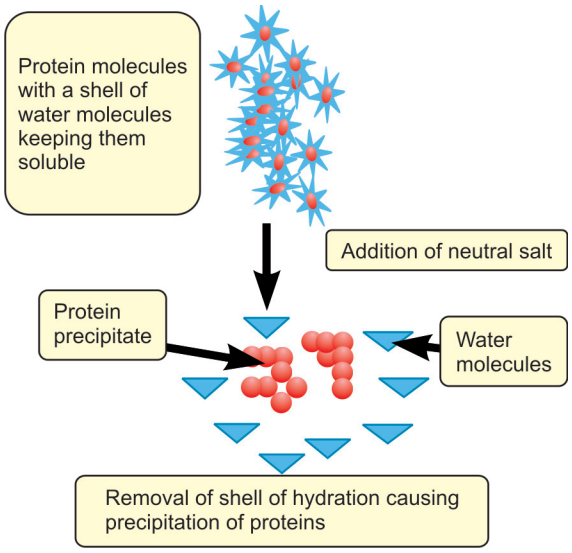


Fig. 2A-2: Salting out

(ii) Precipitation by Heavy Metals

(a) Precipitation by 10% Lead acetate:

Procedure: Take 3 ml protein solution; add 2 drops of 5% NaOH. Mix well and add 2 ml of 10% lead acetate solution.

Observation: White precipitate forms.

Inference: Proteins are precipitated by positively charged lead ions.

Principle: (see Fig. 2A-3.) The isoelectric point of a protein is that pH at which the net charge on the protein is zero. If the pH of the medium is made alkaline the proteins acquire net negative charge and if the pH of the medium is made acidic the proteins acquire net positive charge. In this test upon **adding alkali** proteins gain **negative charge a**nd they form ionic bond with **positively charged metal ions** leading to precipitation of proteins.

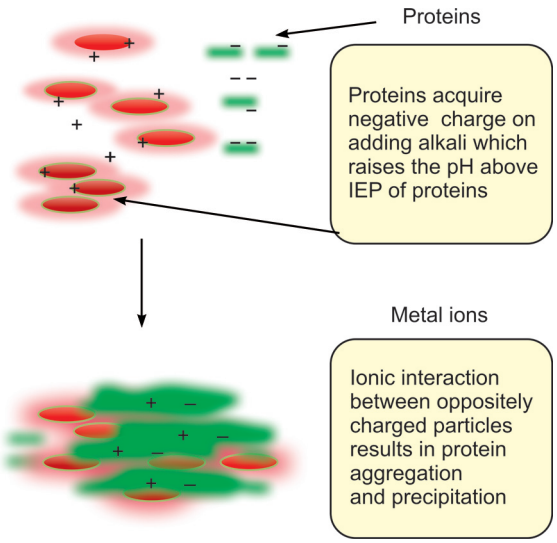


Fig. 2A-3: Mechanism of precipitation of proteins by metal ions

(b) Precipitation by 10% $CuSO_4$ solution:

Procedure: To 3 ml of protein solution add 2 drops of 5% NaOH. Mix well and add 10% $CuSO_4$.

Observation: A light blue precipitate forms

Inference: Proteins are precipitated by positively charged copper ions .

Principle: The same as that of precipitation by lead acetate.

(c) Precipitation by 10% $ZnSO_4$ solution:

Procedure: Take 3 ml of protein solution, add 2 drops of 5% NaOH. Mix well and add 2 ml of 10% $ZnSO_4$ solution.

Observation: An intense white precipitate forms.

Inference: Proteins are precipitated by positively charged zinc ions.

Principle: The same as that of precipitation by lead acetate (Fig. 2A-4).

(iii) Precipitation by Anionic Reagents (Alkaloids)

(a) Precipitation by metaphosphoric acid:

Procedure: Take 3 ml of protein solution in a test tube and add a few drops of metaphosphoric acid.

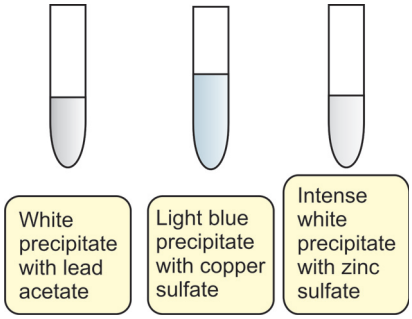


Fig. 2A-4: Precipitation of proteins by different metal ions

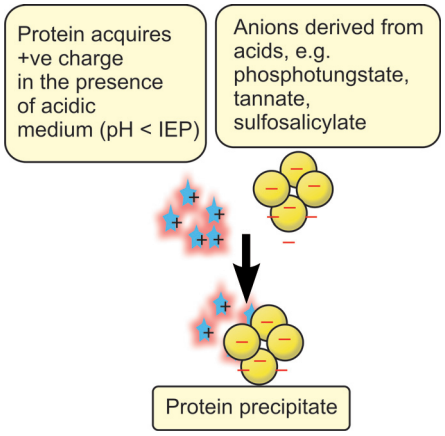


Fig. 2A-5: Mechanism of precipitation of proteins by anionic agents (alkaloids)

Inference: Metaphosphoric acid in solution forms acid anion. Proteins become positively charged. Hence positively charged protein ions and negatively charged acid anions derived from metaphosphoric acid combine to form insoluble complex leading to precipitation.

Principle: (Fig. 2A-5) Alkaloids when dissolved lower the pH of the medium and they themselves form anions. Proteins in this acidic medium acquire positive charge and they complex with negatively charged ions in the medium. These complexes are insoluble and they are precipitated.

(iv) Precipitation by Organic Solvents

(a) Precipitation by ethanol:

Procedure: Add 2 ml of ethanol to 1 ml of protein solution taken in a test tube and mix well.

Observation: Cloudy precipitate forms

Inference: Proteins are precipitated due to removal of water of hydration.

Principle: Organic solvents cause precipitation of proteins by the removal shell of hydration surrounding the proteins (Fig. 2A-6).

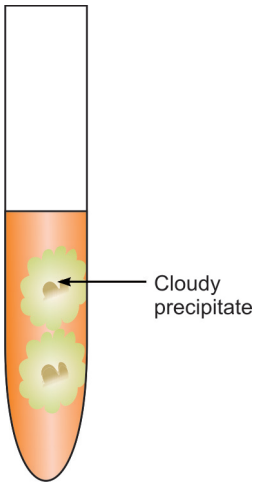


Fig. 2A-6: Precipitation by organic solvents

(v) Precipitation by Heat

Procedure: Take a test tube and fill protein (albumin) solution up to two thirds. Heat the upper one third portion of protein solution column. Note whether any precipitate has

appeared. Irrespective of the presence or absence of the development of the precipitate, add 2% acetic acid drop by drop. Note whether the precipitate formed earlier (if any) became intensified or appeared upon adding acetic acid.

Observation: White coagulum formed on initial heating intensifies on adding acetic acid (see Fig. 2A-7).

Inference: Albumin is denatured by heating and is precipitated by acetic acid.

Principle: Heating caused denaturation. Disruption of secondary, tertiary, quaternary structures maintained by noncovalent forces (hydrogen bonds, ionic interactions, van der waals forces, hydrophobic interactions) causes denaturation.

Aggregation of denatured protein is referred to as coagulum. Denaturation may be reversible in some cases (not always).But coagulation is always irreversible. Addition of acetic acid lowers the pH of the medium towards the isoelectric pH (pI) of the albumin **(IEP of different proteins: Human albumin – 4.7, egg albumin–4.9, human globulin–6.4, casein–4.6).** At pI proteins are least soluble. So the denatured protein get precipitated upon adding acetic acid.

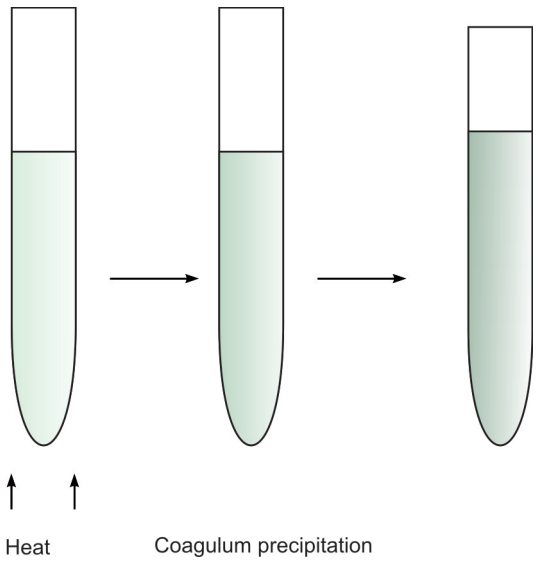


Fig. 2A-7: Precipitation by heating and influence of pI

(vi) Precipitation by Strong Mineral Acids

Procedure: Take 2 ml of concentrated HNO_3 or concentrated HCl in a test tube. Add 2 ml of protein solution along the sides of the test tube slowly.

Observation: White ring forms at the junction of two liquids.

Inference: Albumin as well as globulins are precipitated by strong mineral acids.

Principle: Strong acids causes denaturation and precipitation of proteins.

Points to Ponder: Precipitation by HNO_3 is named as **Heller's test**. It is used as a test for detecting protein in urine or other body fluids.

TEST TO DEMONSTRATE DENATURATION AND COAGULATION

Procedure:

Step 1 (See Fig. 2A-8)

Take 3 test tubes and add 9 ml of a clear salt free albumin solution in them .Mark A,B and C on them.

To the test tube marked A add 1 ml of 0.1 N HCl, to the test tube B add acetate buffer (pH – 4.7) and to the test tube C add 1 ml of 0.1 N NaOH.

- **Heat** tube B in a **boiling water bath** for **15 minutes.**
- Cool

Step 2 (See Fig. 2A-9)

To the tubes A and C add 10 ml of acetate buffer solution (pH 4.7)

- Filter of the precipitates in each tube
- Wash the precipitate obtained in the filter paper with distilled water.

– Precipitate in tubes A and C are denatured egg albumin. Precipitate in the tube B is coagulated protein.

Step 3 (See Fig. 2A-10)

– **Suspend** each of the precipitates in **10 ml of distilled water** and divide each suspension **into 3 parts.**
– To the **first part add dilute HCl** drop by drop, to the **second add dilute NaOH** and **heat the third** part of the suspensions drawn from tubes A and B in a boiling water bath for 15 minutes.
– Cool and check the solubility of the precipitate in dilute acid and alkali.

Observation: Precipitate forms at pI brought about by the addition of acid or alkali. This precipitate dissolves readily in a few drops of dilute acid or alkali. The coagulated protein obtained from tube B remains insoluble upon adding dilute acid or alkali.

Inference: Proteins have got a primary structure as dictated by amino acid sequence bonded by peptide bonds and location of disulfide bonds if any. Functional form of proteins are achieved by higher orders of protein structure (secondary, tertiary and quaternary). These are conferred by noncovalent forces hydrogen bonds, hydrophobic interactions, electrostatic interactions and van der waals interactions.

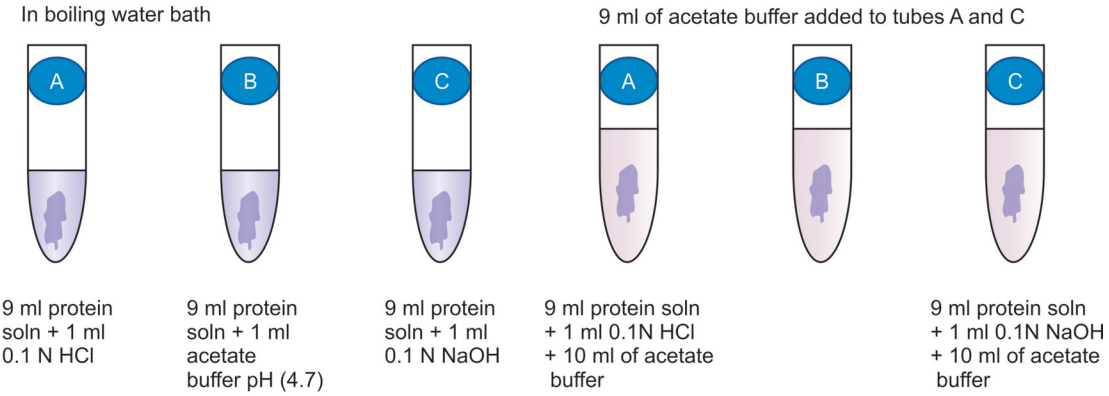
Changes in higher orders of protein structure leading to loss of protein function is caused by denaturation. Denaturing agents can be chemicals (mineral acids, alkalies urea) or physical (heat, uv radiation, ultrasonic waves, shaking, stirring).

Denatured proteins flocculate at or near the pI which is reversible at room temperature. But if it is heated, the floccules form large tenacious masses of coagulated protein .The coagulated proteins are not redissolved by treatment with dilute acids or alkalies.

Denaturation is the **primary change – flocculation** (reversible sometimes) and **coagulation** (irreversible) are visible manifestations of **denaturation.**

2. COLOR REACTIONS OF PROTEINS AND AMINO ACIDS

Proteins react with a variety of reagents to form colored products because of their constituent peptide bonds and amino acids.These reactions are useful for quantitative and qualitative studies



Precipitates formed in tubes A and C are due to denaturation and that formed in B is due to coagulation

Figs 2A-8 and 2A-9: Test to demonstrate coagulation and denaturation—step 1 and step 2

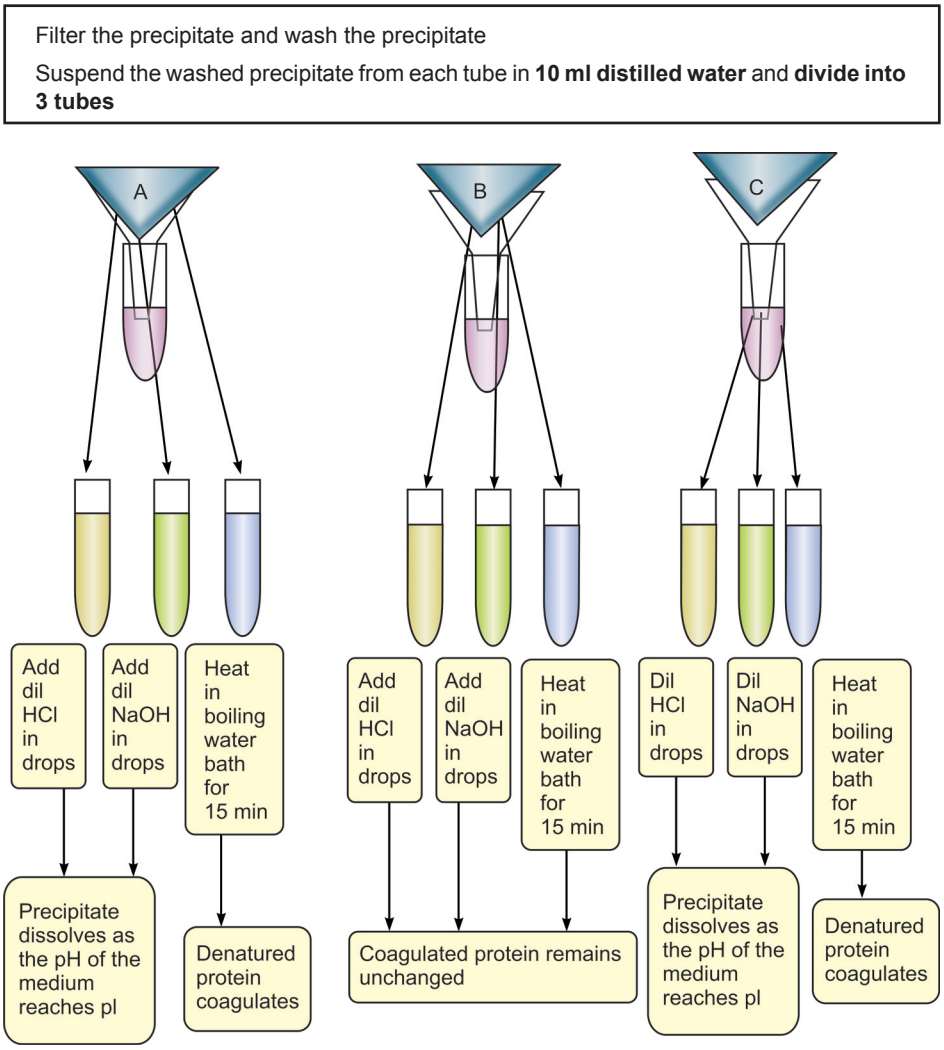


Fig. 2A-10: Test to demonstrate coagulation and denaturation—step 3

of proteins. By quantitative studies the concentration of the proteins are estimated. Qualitative studies help to know the presence of proteins or specific amino acids present in the protein. They are useful mainly in the following situations

1. For the diagnosis of aminoacidurias: Individual amino acids undergo unique catabolic pathways and the deficiency of any enzyme of these pathways lead to accumulation of compounds proximal to the defective step causing disorders called aminoacidurias. For instance phenylketonuria due to phenylalanine hydroxylase deficiency causes elevated blood levels of phenylalanine in the blood and urine. Study of aminoacidurias need identification of

abnormally elevated specific amino acids in the body fluids. Study of color reactions of amino acids are useful in the diagnosis of aminoacidurias.

2. For the nutritional assessment: Out of twenty standard amino acids, only eight are essential and the rest of the twelve amino acids are nonessential in adults. Those proteins containing all the essential amino acids are considered to be good quality proteins eg; egg albumin. Hence for the making nutritional assessment (roughly) of proteins also the study of reactions of amino acids are helpful.

3. To detect the presence of proteins or amino acids in biological fluids or in fluids with unknown composition: This chapter deals with different color reactions of amino acids.The color reactions are due to reaction between constituent radical or groups of the amino acids and the chemical reagents used in the test. Amino acid composition of different proteins is different. Depending on the nature of amino acids contained in a protein, the response and the intensity of the color reactions varies.

(a) Biuret Test (Fig. 2A-11):

Procedure: To 2-3 ml of protein solution add an equal volume of 10% sodium hydroxide solution, mix thoroughly. Then add 0.5% copper sulfate solution drop by drop, mixing between drops until a purplish violet color is obtained.

Observation: Purplish violet color develops.

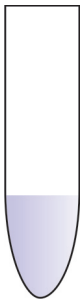


Fig. 2A-11: Biuret test

Inference: The biuret reaction is given by substances which contain two carbamyl groups ($-CONH_2$) joined either directly together or through a single atom of nitrogen or carbon. Positive reaction indicates that the given protein solution contains at least two peptide bonds.

Chemistry of the reaction: The biuret test is given by those substances containing two carbamyl groups ($-CONH_2$) joined either directly or by a single nitrogen or carbon atom. The purplish violet colour is due to the formation of a copper coordination complex (Fig. 2A-12).

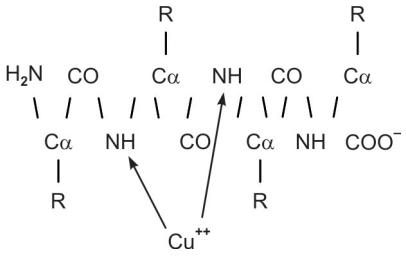


Fig. 2A-12: Copper coordination complex

The molecule should have a minimum of two peptide bonds to give copper coordination complex that impart **violet** color to test mixture**.** *[This reaction is first carried out with the compound biuret formed by the condensation of 2 molecules of urea upon heating. This compound contains two peptide bonds as shown below.]*

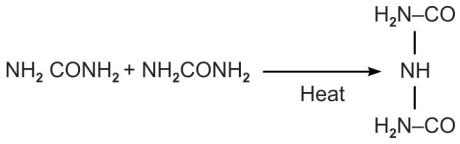


Fig. 2A-13: Biuret

Biuret *(a nonprotein, formed from urea on heating; biuret formed gives violet color with copper sulfate solution in the alkaline medium) (See Fig. 2A-13).*

Proteins give **violet color** with biuret test since there are several pairs of CONH groups in the molecule (see Fig. 2A-14).

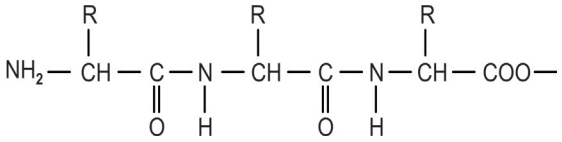


Fig. 2A-14: CONH groups in the peptide

Points to Ponder

- If too much copper sulfate solution is added blue colored copper hydroxide will be formed and that will mask the violet color.
- If magnesium sulfate is present in the test solution it forms magnesium hydroxide and interferes with the test
- If much ammonium sulfate is present excess of alkali have to be used.
- The color depends on the nature of the protein.
  - Proteoses - Purple
  - Peptones - Pink

(b) Ninhydrin Test:

Procedure: To 1 ml of protein solution (pH must be between 5 and 7) in a test tube add 2-3 drops of freshly prepared 0.1% ninhydrin (triketohydrindene hydrate) solution. Heat the solution to boil for 2 minutes and allow to cool.

Observation: Blue color with α-aminoacids and yellow color with iminoacid – proline.

Inference: Blue color is due to the formation of a complex - Ruhemann's purple formed between N-terminal nitrogen and ninhydrin.

Chemistry of the reaction (see Fig. 2A-15): α-amino acids reacts with ninhydrin to form aldehyde, hydrindantin, ammonia and carbon dioxide. Then one molecule of hydrindantin, ninhydrin and ammonia complex to form Ruhemann's purple.

Proteins give a faint blue color. In the case of proteins amino terminal nitrogen participate in the action.

Application: Staining of amino acids in paper chromatography.

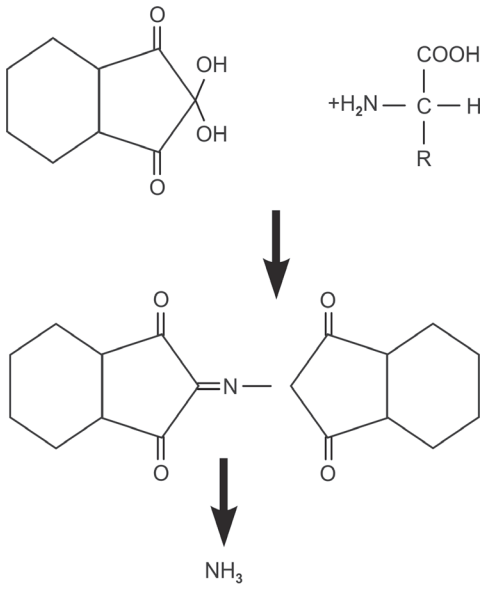


Fig. 2A-15: Chemistry of ninhydrin reaction

SPECIFIC COLOR REACTIONS USED TO IDENTIFY THE SIDE CHAIN (R) GROUPS OF AMINO ACIDS

(c) Xanthoproteic Reaction (Fig. 2A-16):

Procedure: Add 1 ml of concentrated nitric acid to 2-3 ml of test protein solution. Heat to boil. Cool and pour half of the solution into another tube.

One tube is kept as control and the other as test, so as to understand the development of even faint color. To one tube add 40% NaOH or liquor ammonia (ammonium hydroxide) in excess.

Observation: A white precipitate forms on adding nitric acid, which on heating turns **yellow** and then dissolves to impart yellow color to the solution. Upon adding alkali the color deepens to attain **orange** colour.

Interpretation: Addition of nitric acid causes denaturation of proteins to get white precipitate. Yellow color due to nitration of benzene ring of amino acids – **tryptophan and tyrosine**

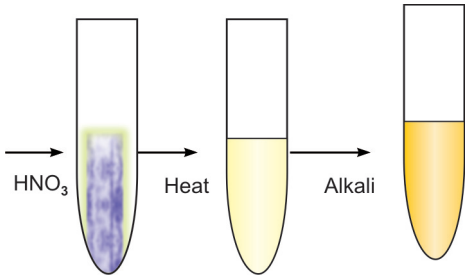


Fig. 2A-16: Xanthoproteic test

(Fig. 2A-17) . Addition of alkali increases the ionization of compounds hence the color deepens to get final orange color.

Points to Ponder

- This test cannot be employed for urine testing as the final color of the test and the natural color of urine are similar.
- The aromatic amino acid **Phenylalanine will not** give a positive response to the test even though it contains benzene ring.

(d) Millon's Test:

Procedure: To 2 ml of protein solution in a test tube add 2 ml of 10% mercuric sulfate ($HgSO_4$) in 10% sulphuric acid. Boil for 30 seconds. A precipitate may form at this stage. Add a few drops of 1% $NaNO_2$ and gently warm.

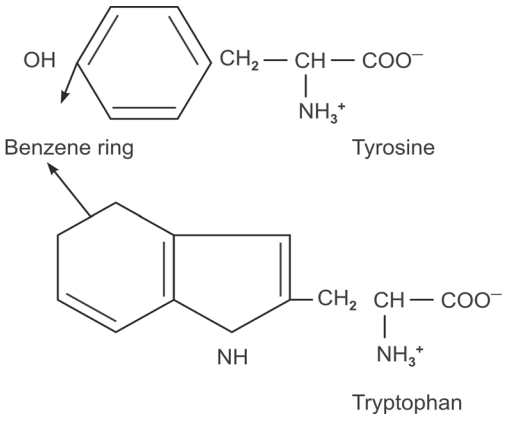


Fig. 2A-17: Tyrosine and Tryptophan

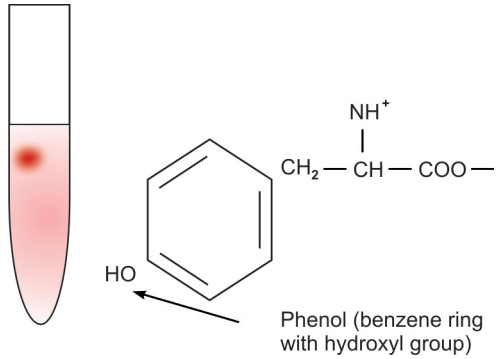


Fig. 2A-18: Millon's test

Observation: Red precipitate forms and solution turns **red**. Amino acid solutions gives red color without a precipitate (see Fig. 2A-18).

Inference: Protein contains the amino acid Tyrosine which contains a phenolic radical.

Principle: The protein precipitated by mercuric sulfate in acidic medium to form mercury – protein complex (metalloprotein complex). Nitrous acid is formed by the reaction between sodium nitrite and sulphuric acid. This nitrous acid causes nitration of phenolic groups of tyrosine .Warming enhances nitration process and intensifies the color.

(e) Aldehyde Test (Glyoxylic Acid , Hopkins – Cole Reaction):

Procedure: Take 2-3 ml of test solution add 2 drops of 1/500 formaldehyde (HCHO) and 1 drop of 10% mercuric sulfate in sulfuric acid. Mix well.

Add 3 ml of concentrated sulfuric acid through the sides of the test tube.

Observation: A **purple** ring develops at the junction of two layers (see Fig. 2A-19).

Inference: The **purple color** is due to the indole ring (see Fig. 2A-20) of the amino acid tryptophan.

Principle: Mercuric sulfate in sulphuric acid act as an oxidizing agent and it oxidizes the indole ring of tryptophan. Then formaldehyde react with the oxidized indole ring to form purple colored complex.

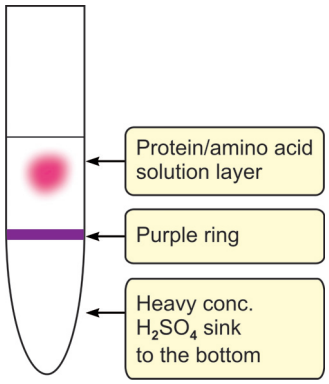


Fig. 2A-19: Aldehyde test

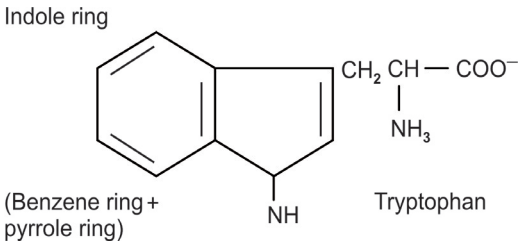


Fig. 2A-20: Tryptophan

(f) Sakaguchi's Test:

Procedure: Add 5 drops of 5% sodium hydroxide to 5 ml of protein solution. Shake well. Add 2-4 drops of Molisch's reagent.

Observation: A **bright red** (see Fig. 2A-21) color develops.

Inference: The protein solution contains aminoacid with guanidino group (Arginine).

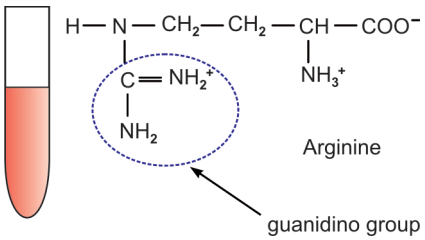


Fig. 2A-21: Sakaguchi's test

Principle: Molisch reagent is α-naphthol in alcohol. Sodium hydroxide provides alkaline pH. At the alkaline pH guanidino group of arginine combines with α-naphthol to form bright red color.

(g) Sulfur Test (Lead Blackening Test):

Procedure: To 3 ml of protein solution add 3 ml of 40% NaOH and boil for 3 minutes. Cool, add 1 ml of lead acetate solution.

Observation: Solution turns **dark brown** (see Fig. 2A-22).

Inference: This test is answered by "S" containing aminoacids – cysteine and cystine but not methionine because of the placement of S in the thio ether linkage (Fig. 2A-23).

Principle: Upon boiling with strong alkali the organic sulfur in the cystine and cysteine is converted into sulfide (here Na_2S). The sodium sulphide react with lead acetate to form black lead sulphide (PbS) and solution turns **brownish black.**

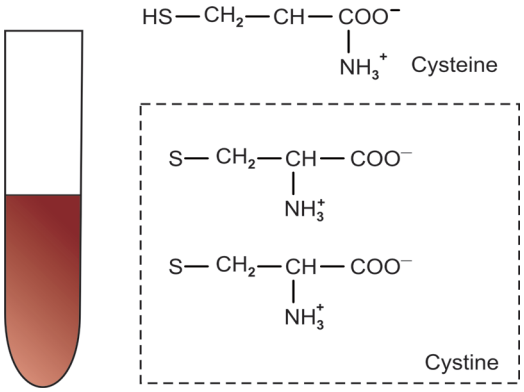


Fig. 2A-22: Sulfur test and cysteine and cystine

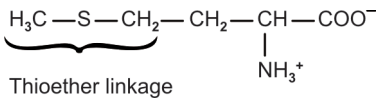


Fig. 2A-23: Methionine

Points to Ponder: Casein and gelatin gives a negative response due to the deficiency of cysteine in them.

(h) Pauly's Test:

Procedure: To 0.5 ml of 0.5% sulfanilic acid add an equal volume of 0.5% freshly prepared sodium nitrite. Allow to stand for 1 minute and add 1 ml of protein solution. Mix well and add 1 ml of 10% Na_2CO_3 to make the solution alkaline.

Observation: Cherry red or orange red color may be observed.

Inference: Cherry red color indicates presence or predominance of histidine and orange red color shows the presence or predominance of tyrosine in the solution (Fig. 2A-24).

Principle: Diazotized sulfanilic acid when complexes with imidazole ring of histidine gives cherry red colored complex and when it complexes with phenolic group of tyrosine yields orange red color (Fig. 2A-25).

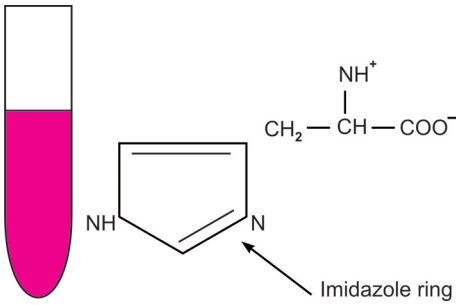


Fig. 2A-24: Cherry red color of histidine

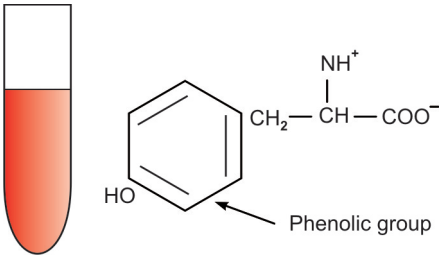


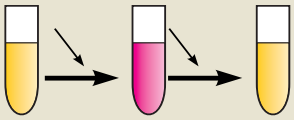
Fig. 2A-25: Orange red color of tyrosine

2B. REACTIONS OF ALBUMIN (TABLE 2B-1)

Albumins are compact roughly spherical in shape and have axial ratios not more than 3 (that is the ratio of their shortest to longest dimensions). Hence albumins come under **globular proteins.** They are soluble with definite molecular weight. Albumins of interest are serum albumin of blood, lactalbumin of milk and ovalbumin of egg . It is also present in pulses. They are soluble in solute free water and coagulable on heating. They are not precipitated by half saturation with salts away from isoelectric pH.

Egg albumin has a molecular weight of 45,000 Kda and it's isoelectric point (pI) is 4.55-4.9. It is commonly employed in the laboratory to carry out experiments to study the properties of albumin in general. Human serum albumin has got a molecular weight of 69,000 Kda and its pI is 4.7.

Table 2B-1: Reactions of Albumin

Reaction	*Observation*	*Inference*
PRECIPITATION REACTIONS		
1. Isoelectric precipitation: Take 10 ml of protein solution in a test tube. Add 2-3 drops of Chlorophenol red (pH range – 5.0-6.6; color range – yellow to red).The purpose of adding the indicator is to get pH around 5.0. Look at the color change. 2% Na_2CO_3 2% acetic acid  With chlorophenol red, the yellow color denotes pH either equal to 5 or less than 5. So even if a yellow color is observed the pH may not be 5, it may be less than 5 also. In order to make sure of the required pH 5 add 2% Na_2CO_3 in drops until a pink color forms and then add 2% acetic acid in drops till the solution turns just yellow. If it is red or pink, we could infer that the prevailing pH is either 6.6 or more than that as indicated by the indicator. Add 2% acetic acid in drops till the yellow color just develops. Boil the above solution	White coagulum	At pI, albumin is denatured. Upon heating denatured protein aggregate to form visible change named coagulation.
2. Heller's test: To 2 ml of concentrated nitric acid in a test tube (*keep the mouth of test tube away from your face and others)* add gently equal amounts of albumin solution	A white ring at the junction of two liquids forms	Stratification of Albumin solution over strong mineral acid causes denaturation and precipitation of albumin at the point of contact that is at the junction between two layers.
3. Half saturation test: To 5 ml of albumin solution add equal volume of saturated ammonium sulfate solution .Shake vigorously for 2 minutes. Keep it for 5 more minutes. Filter and collect the filtrate.Perform Biuret test with the filtrate.To 2ml of the above filtrate taken in a test tube add 2ml 40 % NaOH and 1% $CuSO_4$ drop by drop.	Violet color	Albumin being relatively small in size (MW 69000 Kda) is not completely precipitated by saturated solution of ammonium sulfate and hence go into the filtrate.

(*Contd.*)

(*Contd.*)

4. Full saturation test: To 5 ml of albumin solution add ammonium sulfate crystals and shake well till some crystals remain at the bottom of the tube. Keep it for 5 minutes and filter. Collect the filtrate.Do Biuret test with the filtrate .To 2ml of the above filtrate taken in a test tube add 2ml 40 % NaOH and 1% $CuSO_4$ drop by drop.	No violet color	Albumin is completely precipitated by full saturation with ammonium sulfate crystals and so all the albumins get retained in the filter paper without going into the filtrate. Hence the filtrate devoid of albumin do not produce a positive Biuret reaction.
5. Heat and acetic acid test: Take a test tube and fill protein (albumin) solution up to two thirds .Heat the upper one third portion of protein solution column . Note whether any precipitate has appeared . Irrespective of the presence or absence of the appearance of the precipitate add 2% acetic acid drop by drop. Note whether the precipitate formed earlier (if any) has intensified or appeared upon adding acetic acid.	Precipitate formed on heating become denser on adding acetic acid	Heating caused coagulation of albumin and the addition of acetic acid lowered the pH of the medium towards the isoelectric pH (pI) of the albumin and enhanced the precipitation

COLOR REACTIONS

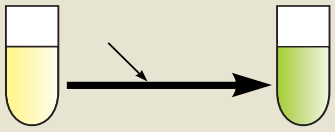
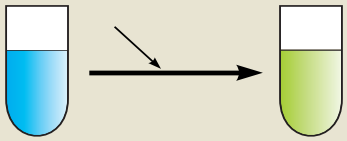
1. Biuret reaction: (see Section 2A)	Violet color	Albumin contains more than 2 peptide bonds
2. Xanthoproteic reaction: (see Section 2A)	Deepening of yellow color	Albumin contains Tryptophan and Tyrosine. Benzene ring of these amino acids are giving the reaction.
3. Millon's test: (see Section 2A)	Red color	Albumin contains the phenolic group containing amino acid, Tyrosine
4. Aldehyde test: (see Section 2A)	Violet or purple ring at the junction of two liquids	Indole ring containing amino acid Tryptophan is present in albumin
5. Sakaguchi's test: (see Section 2A)	Bright red color	Albumin contains the guanidino group
6. Sulfur test: (see Section 2A)	Brownish black solution	Indicates the presence of cysteine in albumin
7. Pauly's test: (see Section 2A)	Orange red	Indicates predominance of Tyrosine in albumin

2C. REACTIONS OF CASEIN (TABLE 2C-1)

Casein is the main protein present in the milk. It is a phosphoprotein and constitute one-third of proteins of human milk , five sixth of proteins of cow's milk and three fourths of proteins of goat's milk. Casein is secreted by mammary gland only. Contains all the essential amino acids.

It is less soluble and is made soluble at acid or alkaline pH and precipitate when the pH is brought to isoelectric point (4.6). It is also precipitated by half saturation with ammonium sulfate and it is not coagulated by heat. Casein act like a suspensoid, the particles of it flocculate when their charges are neutralized.

Table 2C-1: Reactions of Casein

Reaction	*Observation*	*Inference*
PRECIPITATION REACTIONS		
1. Isoelectric precipitation: Take 4 ml of protein solution in a test tube. Add 2-3 drops of Bromocresol green (pH range 4.0 – -5.6; color range – yellow to blue).The purpose of adding the indicator is to get pH around 4.6 Look at the color change. 2% sodium carbonate  2% acetic acid  If it is yellow that means pH is ≤ 4.0. Then add 2% sodium carbonate drop by drop till the solution turns light green. (pH around 4.6) If it is blue that means pH is ≥ 5.6. Then add 2% acetic acid drop by drop till the solution turns light green. (pH around 4.6)	Precipitate seen	When the pH reaches 4.6 (pI of casein) casein get precipitated
2. Half saturation test: To 5 ml of Casein solution add equal volume of saturated ammonium sulfate solution. Shake vigorously for 2 minutes. Keep it for 5 more minutes. Filter and collect the filtrate. Perform Biuret test. To 2 ml of the above filtrate taken in a test tube add 2 ml 40% NaOH and 1% $CuSO_4$ drop by drop.	No violet color with Biuret test.	Casein is completely precipitated by saturated solution of ammonium sulfate. So the filtrate do not contain any casein as it is completely filtered off due to precipitation. Hence the Biuret test done with filtrate becomes negative.

(*Contd.*)

(*Contd.*)

Color Reactions: All the color reactions will be positive **except the Sulfur test**. Sulfur test will be faintly positive because only 0.3 g of cysteine/cystine is present in 100 gms of casein where as in egg albumin about 2.5g cysteine/cystine present in 100 gm of albumin.

Specific Tests for Casein

3. **Neumann's test (detect organic phosphorous):** To 5 ml of casein solution add 0.5 ml of 40 % NaOH. Heat for one minute and cool it keeping in a rack. Add 0.5 ml of concentrated nitric acid. Add 1ml of saturated ammonium molybdate solution.	Canary yellow precipitate	Casein is digested by heating with sodium hydroxide thereby inorganic phosphorous (Pi) is released from it. Ammonium molybdate react with Pi in the acidic medium provided by the concentrated nitric acid to form ammonium phospho molybdate which is canary yellow in color.

2D. REACTIONS OF GELATIN (TABLE 2D-1)

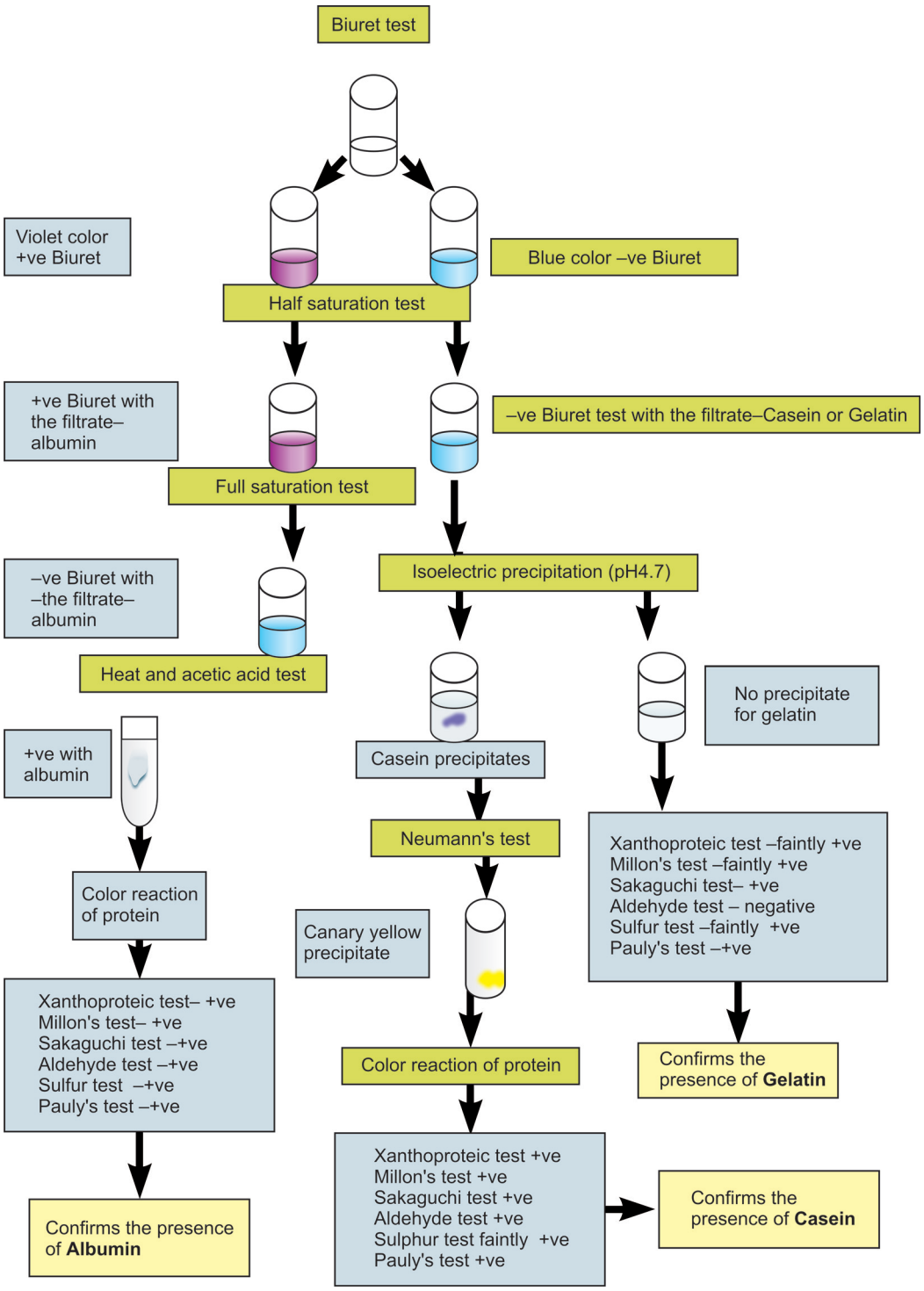
Gelatin is derived from fibrous protein collagen which is abundantly present in the ligaments, bones and teeth. Upon treating with boiling water and subsequent cooling of collagen give rise to gelatin which is free from carbohydrate moieties. It has low biological value since it is lacking the essential amino acid tryptophan and contains very low amounts of phenyl alanine, tyrosine and cysteine. The **isoelectric point for gelatin is about pH 4.7**.But it is quite soluble in water even at it's isoelectric point (IEP).

Table 2D-1: Reactions of Gelatin

Reaction	*Observation*	*Inference*
PRECIPITATION REACTIONS		
1. **Half saturation test:** To 5 ml of gelatin solution add equal volume of saturated ammonium sulfate solution. Shake vigorously for 2 minutes. Keep it for 5 more minutes. Filter and collect the filtrate. Perform Biuret test. To 2 ml of the above filtrate taken in a test tube add 2 ml 40% NaOH and 1% $CuSO_4$ drop by drop.	No violet color with Biuret test	Gelatin is completely precipitated by saturated solution of ammonium sulfate. So the filtrate do not contain any gelatin as it is completely filtered off due to precipitation. Hence the Biuret test done with filtrate becomes negative.

Color Reactions: Except **Aldehyde test, Millon's test and Sulfur test** all the other color reactions will be positive because Tryptophan is absent in gelatin and Tyr and Cys present in very low amounts.

2E. IDENTIFICATION OF UNKNOWN PROTEINS



2F. QUESTIONS

1. Name the following:
   a. General test for detecting proteins
   b. Test to detect peptide linkage
   c. A phosphoprotein
   d. Test to detect the presence of Tryptophan
   e. Test to detect the presence of Arginine
   f. Test to detect the presence of Tyrosine
   g. Test to detect the presence of Cysteine
   h. Test to detect the presence of Cystine
   i. Color produced by α-amino acid and imino acid with ninhydrin
2. Give the principle of the following tests:
   a. Biuret test
   b. Ninhydrin test
   c. Half saturation test
   d. Full saturation test
   e. Isoelectric precipitation of proteins
   f. Sakaguchi's test
   g. Millon's test
   h. Xanthoproteic test
   i. Aldehyde test
3. Give brief answers:
   a. Denaturation vs coagulation
   b. Structural alterations of proteins in denaturation
   c. Mechanism of precipitation of proteins by salts like ammonium sulfate
   d. Mechanism of precipitation of proteins by alkaloids
   e. Mechanism of precipitation of proteins by alcohol
   f . Uses of color reactions in practicing clinical medicine
   g. Applications of precipitation of proteins in the clinical chemistry laboratory.
   h. Rationale of using egg white as a part of treatment of mercury poisoning
   i. What are the amino acids detected by sulfur test?
   j. Why Methionine is not responding to Sulfur test?
   k. What is the reason for using 40% NaOH for doing Biuret test with filtrate obtained after half and full saturation with ammonium sulfate?

2G. REAGENT PREPARATION

1. 1% Albumin Solution: Dissolve 1 g of albumin in 100 ml of water .Ideally prepare on the day or on the day before experimentation and store in the refrigerator.

2. 1% Casein Solution: Dissolve 1 g of casein in 100 ml of 0.1 N NaOH. Ideally prepare on the day or on the day before experimentation and store in the refrigerator.

3. 1% Gelatin Solution: Dissolve 1 g of gelatin in 100 ml of water. Ideally prepare on the day or on the day before experimentation and store in the refrigerator.

4. Ammonium Molybdate Solution: Dissolve 100 g molybdic acid in 144 ml of ammonium hydroxide (specific gravity 0.90) and 271 ml water. Slowly with constant stirring pour the solution into 489 ml of nitric acid (specific gravity 1.42) and 1148 ml water. Keep in a warm place for several days till a portion heated to 40° C deposits no yellow precipitate of ammonium phosphomolybdate. Decant the solution from any sediment and keep in glass stoppered bottles.

5. Lead Acetate Solution (10%): Dissolve 10 gm of lead acetate in 100 ml of distilled water.

6. Sodium Hydroxide 1 N Solution: Sodium hydroxide always contains sodium carbonate as an impurity derived from CO_2 from air. Besides it is hygroscopic (absorb water from atmosphere). So direct preparation of solution out of it will not give the concentration required for the purpose.

Molecular weight of NaOH = 23 + 16 + 1 = 40

Equivalent weight of NaOH = 40/1 = 40

Therefore for making 1N NaOH solution dissolve.

100 g reagent grade sodium hydroxide pellets in 100 ml of distilled water in a flask and cap it and leave overnight at room temperature. The carbonate will settle out as an insoluble precipitate. Filter the solution through a sintered glass filter.

Pipette 75 ml of it and make upto 1 L in a 1000ml volumetric flask and dilute to 1000 ml with distilled water. Mix well.

Prepare 1N oxalic acid by dissolving 63.035 g of pure crystalline oxalic acid in distilled water and make upto a volume of 1L.

To make 1N NaOH – Take 10 ml of 1N oxalic acid in a conical flask and titrate against the NaOH solution prepared in the burette using Phenolphthalein as an indicator (color range – colourless to red, pH range - 8-9.8)

If the titre value (Number milliliters of NaOH required to neutralize 10 ml of acid) is 9.5.

Then the normality of NaOH solution,

= 1 N × 10/9.5 = 1.05 N

To prepare 1N NaOH,

= 1000 ml × 1 N/Normality of the solution

= 1000ml × 1N/1.05 N = 952.4

Take 952.4 ml NaOH solution into a 1L measuring cylinder and dilute to 1000ml by adding distilled water.

7. Sodium Hydroxide 0.1 N Solution: Dilute 100 ml of 1 N NaOH to 1 litre with distilled water.

8. 40% Sodium Hydroxide Solution: 1 N NaOH solution is equivalent to 40%.

9. 10% Copper Sulfate Solution: Weigh 10 g copper sulfate into a few ml of distilled water taken on a 100 ml flask and dissolve it and make up to 100 ml with distilled water.

10. 0.5% Copper Sulfate Solution: Weigh 0.5 g copper sulfate into a few ml of distilled water taken in a 100 ml flask and dissolve it and make up to 100 ml with distilled water.

11. 10% Zinc Sulfate Solution: Weigh 10 g zinc sulfate into a few ml of distilled water taken in a 100 ml flask and dissolve it and make up to 100 ml with distilled water.

12. 10% Mercuric Sulfate in Sulphuric Acid: Dissolve 10 g of sulfuric acid in 100 ml of 10 % sulfuric acid.

13. 0.1% Ninhydrin Solution: Dissolve 0.1 g of ninhydrin in 100 ml of acetone.

14. 1% Sodium Nitrite ($NaNO_2$): Dissolve 1 g sodium nitrite in 100 ml of water.

15. 1/500 Formaldehyde: Dissolve 1 ml of formaldehyde in 500 ml of distilled water.

16. Liquor Ammonia:

17. 0.5% Sulfanilic Acid: Dissolve 0.5 g of sulfanilic acid in 100 ml of 2% HCl.

18. 10% Sodium Carbonate: Dissolve 10 g of sodium carbonate in 100 ml of distilled water .

19. 2% Sodium Carbonate: Dissolve 2g of sodium carbonate in 100 ml of distilled water.

20. Chlorophenol Red : (pH range 5 - 6.6; color range – yellow to red): Add 0.1 g chlorophenol red and 4.8 ml of 0.05 N NaOH to 250 ml of distilled water.

21. Bromocresol Green (pH range 3.8-5.4; color range – yellow to green): Add 0.1 g Bromocresol green and 2.9 ml of 0.05 N NaOH to 250 ml of distilled water.

22. Phenolphthalein (pH range 8.3-10; color range – colorless to red): Dissolve 0.1 g in 100 ml of 95% ethanol.

Reactions of Lipids

3A. GENERAL REACTIONS OF LIPIDS

INTRODUCTION

Lipids are naturally occurring heterogeneous group of substances found in all vegetables and animal matter. They are insoluble in water and soluble in solvents like ether, chloroform, boiling alcohol and benzene. Lipids are esters of fatty acids or substances capable of forming such esters. To understand much about the different (Table 3A-1) types of lipids, classification of lipids is useful. Plasma lipids are present as complexes with protein molecules which make the lipids soluble and are called lipoproteins.

Table 3A-1: Classification of Lipids

1. Simple lipids-esters of fatty acids with various alcohols		*2. Complex lipids – esters of fatty acids containing groups in addition to an alcohol and fatty acids*			*3. Precursor and derived lipids*
a. Fats	*b. Waxes*	*a. Phospholipids*	*b. Glyco Spingolipids*	*c. Other complex lipids*	
Esters of fatty acids with glycerol, e.g. oils are fats in the liquid form	Esters of fatty acids with higher molecular weight monohydric alcohols	Contains fatty acids, an alcohol and a phosphoric acid residue, some contain nitrogen containing bases, e.g. glycerophospholipids (alcohol-glycerol) Sphingophospholipids (alcohol-sphingosine)	Contains fatty acid, sphingosine and carbohydrate, e.g. galactocerамide	e.g. aminolipids, sulfolipids, lipoproteins	Fatty acid, glycerol, steroids, other alcohols, fatty aldehydes, ketone, hydrocarbons, fat soluble vitamins, hormones

REACTIONS OF FATS AND FATTY ACIDS

1. SOLUBILITY TEST

Procedure: Take 4 dry tubes and arrange them in a test tube stand. Add 2 ml each of **water, ether, chloroform and benzene** into 4 different test tubes. Add one drop of gingili or coconut oil into each tube and shake well.

Observation: Droplet of oil will be seen in the tube containing water. It disappears in other tubes

Inference: In water, oil broken into droplets and being less dense than water float on the surface.

In other solvents, oil dissolves.

Principle: Water is polar in nature where as oil is hydrophobic. So oil does not dissolve in water but dissolves in fat solvents like ether, chloroform and benzene.

2. Grease Spot Test

Procedure: Place a drop of gingili oil upon a piece of ordinary writing paper.

Observation: A translucent spot develops.

Inference: Lipids are greasy in nature.

3. Acrolein Test (Fig. 3A-1)

Procedure: Take 2 scoopful of potassium bisulphite ($KHSO_4$) in a clean dry test tube. Add 4 drops of gingili oil on the salt and heat gently at first and then more strongly.

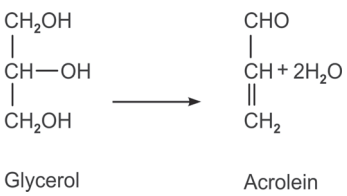


Fig. 3A-1: Formation of acrolein

Observation: An irritating odor develops.

Inference: Glycerol part of the oil is dehydrated to **acrolein** having a irritating odor.

4. Emulsification Test

Procedure: Add one drop of oil into 2 ml of water in a test tube and shake well. Observe and add 4-6 drops of soap solution and shake well.

Observation: Upon shaking with water, oil floats on the surface. On adding soap solution oil get dispersed in water.

Inference: Surface tension of water is lowered by soap solution and this help the droplets of oil to get dispersed and suspended in water (emulsification).

Points to Ponder: Bile salts aid in digestion and absorption of lipid in the gut by emulsification. Bile salts are salts of bile acid and they act as emulsifying agents.

5. Saponification (Table 3A-2)

Procedure:

- Take a clean dry test tube and add 0.5 ml of oil and add 2-3 ml of ethyl alcohol to it and mix well.
- Then add 10 ml of 10% alcoholic NaOH solution. Shake well and keep in a boiling water bath for 15 minutes.
- Take the test tube out of boiling water bath. keep it in rack for another 15 minutes and add 15 ml of water. Shake thoroughly.
- Divide the contents into 4 equal parts and add into 4 tubes marked A, B, C and D.
- To 'A' add 3 ml of concentrated HCl and shake well.
- To 'B' add 4 ml of saturated NaCl solution.
- To 'C' add 3 drops of $CaCl_2$ solution
- "D" will serve as control

Observation and Inference:

Table 3A-2: Observation and Inference of Saponification		
Tube A	White precipitate	Addition of HCl liberates fatty acids which will be seen as white precipitate since it is insoluble in water
Tube B	Pale white layer rises up	Added NaCl reacts with fatty acids to form sodium salts of fatty acids
Tube C	White precipitate	Calcium salt of fatty acid (insoluble in water) are formed on adding $CaCl_2$

Principle: Fats are generally composed of esters of fatty acids and can be hydrolyzed to glycerol and fatty acids by different agents like lipase, superheated steam, long continued action of air and light or boiling with alkali. In this test alkali is used to hydrolyze fat. This process of hydrolyzing triacylglycerol into glycerol and fatty acids by the any one of the above said means is known as **saponification**. Metallic salts of higher fatty acids are called soaps. Ordinary hard soaps are sodium soaps. Potassium soaps are soft soaps. Calcium and magnesium form insoluble soaps.

Application

- The cleansing (detergent) action of soaps is due to their ability to lower surface tension and to cause emulsification of oily material which can then be easily washed away.

Saponification number of a fat is the number of milligrams of KOH required to neutralize free or combined fatty acid in 1 g of fat. It is determined by saponification and titration of excess alkali and is a measure of the mean molecular weight of the fatty acids in a fat.

6. Halogenation Test (Fig. 3A-2)

Procedure:

- Take two test tubes and mark A and B respectively.
- Add 5 ml of chloroform to both tubes.
- Add 6-8 drops of oleic acid in tube A and a scoopful of palmitic acid in tube B and shake well.
- Add a few drops of fresh bromine water and shake well.

Observation and Inference (see Table 3A-3).

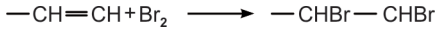


Fig. 3A-2: Halogenation

Table 3A-3: Observation and Inference of Halogenation Test		
Tube A	Orange yellow color of bromine water vanishes	Oleic acid (C 18:1) is an unsaturated fatty acid and take up bromine atoms at the double bonds
Tube B	Orange yellow color	Palmitic acid is a saturated fatty acid (C 16), hence cannot take up bromine atoms due to the absence of double bonds

Principle: The unsaturated fatty acids posses double bonds and they take up halogens like bromine or iodine at their double bonds.

Application: Iodine number is a measure of amount of unsaturation present in a fat. It is expressed as a number of **grams of iodine** absorbed by **100 g of fat**.

CHOLESTEROL

1. Identification by microscopy: Cholesterol has a characteristic shape which can be appreciated by microscopy. Cholesterol crystals have a rhombic shape with a notch at one corner.

Application: Microscopic examination of body fluids suspected to contain cholesterol help to detect it's presence by the rhombic crystals notched at one corner.

COLOR REACTION OF CHOLESTEROL

1. Salkowski's Reaction (H_2SO_4 Test) (Fig. 3A-3)

Procedure: Dissolve a few crystals of cholesterol in 2 ml of chloroform in a dry test tube and add an equal volume of concentrated H_2SO_4 gently along the sides of the tube. The acid being heavier goes down.

Observation: The acid layer develops a yellow color with a green florescence. A play of colors from bluish red to cherry red to purple develops in chloroform layer.

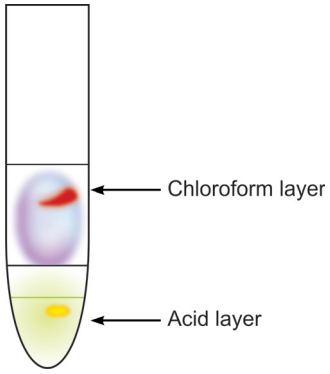


Fig. 3A-3: Salkowski's reaction

Inference: Cholesterol is dehydrated by concentrated sulfuric acid to form 3,5 cholestadine or 2,4 cholestadine. They polymerize and react with sulfuric acid to form their sulfuric acid derivatives giving rise to a play of colors.

2. Liebermann-Burchard Reaction (Acetic Anhydride Sulfuric Acid Test) (Fig. 3A-4)

Procedure: Dissolve a few crystals of cholesterol in 2 ml of chloroform in a dry test tube. Add 10 drops of acetic anhydride and 1-3 drops of concentrated sulfuric acid.

Observation: The solution becomes red, then blue and finally bluish green in colour.

Principle: Acetic anhydride dehydrate cholesterol which then reacts with cholesterol to give the color.

Points to Ponder

Dry glassware must be used for cholesterol experimentation.

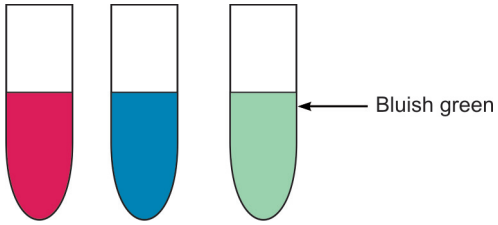


Fig. 3A-4: Liebermann-Burchard reaction

3B. QUESTIONS

1. Name the following:
   a. Derived lipids
   b. Complex lipids
   c. Phospholipids
   d. Emulsifying agent of digestion and absorption of lipids in the intestine

e. Test to detect the presence of glycerol in fat
f. Insoluble soap
g. Soft soap
h. Test to detect the presence of unsaturated fatty acid
i. Two reactions to detect cholesterol

2. Give the principle of the following tests:
   a. Acrolein test
   b. Emulsification test
   c. Liebermann-Burchard reaction
   d. Salkowski's reaction
   e. Halogenation test
3. Give brief answers:
   a. Classify lipids
   b. What are oils?
   c. Role of emulsification of fat in digestion
   d. What is saponification and saponification number?
   e. Iodine number

3C. REAGENT PREPARATION

1. **Chloroform:** As such from the container used for laboratory purpose.
2. **Ether:** As such from the container used for laboratory purpose.
3. **Benzene:** Benzene as such from the container used for laboratory purpose.
4. **Oil:** Gingili or coconut or any oil of convenience.
5. **Potassium bisulfite:** Solid potassium bisulfite as such I used.
6. **Ethyl alcohol:** Such from the container used for laboratory purpose.
7. **Saturated NaCl:** Take water in a beaker or cylinder. Dissolve sodium chloride until some crystals remain undissolved.
8. **Bromine water:** Add a few drops of liquid bromine(caution: very corrosive) to 100 ml water. Prepare fresh (Fading of color indicates inactive reagent).
9. **Palmitic acid:** As such from the container used for laboratory purpose.
10. **Oleic acid:** As such from the container used for laboratory purpose.
11. **Acetic anhydride:** As such from the container used for laboratory purpose.
12. **Concentrated HCl:** As such from the container used for laboratory purpose.

Reactions of Urea

4

4A. GENERAL REACTIONS OF UREA

INTRODUCTION

Urea is the end product of protein catabolism. It is a non protein nitrogen (NPN). Non protein nitrogen of the blood and other body fluids include the nitrogen containing constituents which are not precipitated as protein, e.g. NPN of blood are urea, uric acid, creatinine, creatine, aminoacids, glutathione and other compounds.

The sources of the constituent atoms of urea: Carbon from carbon dioxide, one nitrogen from ammonia and the other nitrogen from aspartate. The full set of enzymes required for the formation of urea occurs only in hepatocytes (liver cells) (Fig. 4A-1). Hence, urea is synthesized exclusively in the liver. It is mainly excreted in urine 80-90% of total nitrogen of human urine is present as urea. *Unlike other NPN, the concentration of urea in urine decreases* when protein intake is restricted. The amount of urea excreted by a normal adult is about 30 g per day. Urea content increases with high protein diet. Blood urea level decreases in liver diseases since it is the **sole organ** concerned with its synthesis.

Physical Properties of Urea Solution

Appearance	-	clear
Color	-	colorless
Odor	-	odorless

Reaction to litmus - No change

CHEMICAL REACTIONS

1. Alkaline Hypobromite Test

Procedure: To 3 ml of urea solution add a few drops of alkaline hypobromite solution (3 ml concentrated NaOH + 2 ml bromine water)

Observation: Brisk effervescence

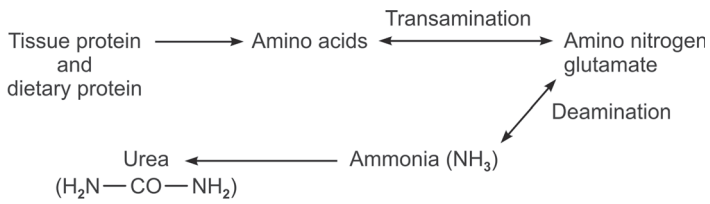


Fig. 4A-1: Formation of urea

Inference: Nitrogen is present in urea and is liberated as nitrogen gas.

Principle: Sodium hypobromite decomposes urea to CO_2 and N_2. Carbon dioxide is absorbed by the excess sodium hydroxide and the nitrogen is evolved and causes the brisk effervescence (see Fig. 4A-2).

Points to Ponder: All ammonium compounds and all compounds containing amino group ($-NH_2$) release N_2 when treated with alkaline hypobromite as in this test (Fig. 4A-3).

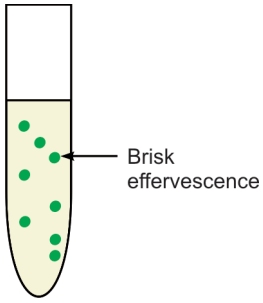


Fig. 4A-2: Alkaline hypobromite test

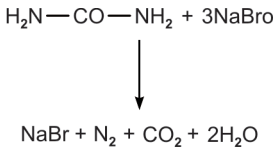


Fig. 4A-3: Alkaline hypobromite reaction

2. Specific Urease Test (Fig. 4A-4)

Procedure: To 2 ml urea solution in a test tube add a drop of phenol red indicator.

- Add 2% Na_2CO_3 solution drop by drop till a pink color develops. (pH range of Phenol red –6.8 – 8.4 and color range is yellow to red).
- Add 2% acetic acid drop by drop till the pink color just disappears indicating the pH nearer to 6.8.
- Add a 1 ml of urease enzyme extract.
  - Hold the tube with palm in order to impart heat into the contents of the tube. (optimum temperature of urease enzyme – 37°C).
  - Set a control tube taking the entire ingredients except urea solution.

Observation: Pink color develops in the tube containing urea and no change in control tube.

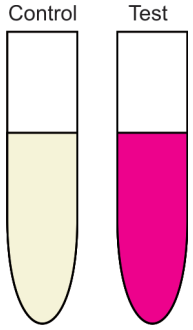


Fig. 4A-4: Specific urease test

Inference: Urease decomposes urea to ammonium carbonate. Ammonium carbonate being basic raises the pH. Phenol red used in this test will show pink to red color at the basic pH.

$$\text{Urea} \xrightarrow[\text{Urease}]{H_2O} \text{Ammonium carbonate}$$

4B. QUESTIONS

1. Name the following:
   a. Number of nitrogen atoms in urea
   b. Sources of nitrogen in urea
   c. Source of carbon in urea
   d. Cells producing urea in the body
   e. Concentration of urea in blood in normal adult
   f. Two tests to detect urea from a specimen
   g. Main route of excretion of urea from the body
   h. Three non protein nitrogen compounds in the blood
2. Give the principle of the following tests:
   a. Alkaline hypobromite test
   b. Specific urease test

3. Give brief answers:
   a. Formation of urea by hepatocytes
   b. Urea cycle disorders
   c. Biochemical principles of treatment of urea cycle disorder
   d. Why blood urea is elevated in kidney diseases

4C. SAMPLE PREPARATION

1. Sodium hydroxide concentrated (1N): Dissolve 40 g reagent grade sodium hydroxide pellets in a few ml of distilled water to make 1 liter. Cool allow to stand for 3 days or so. Decant the solution into a bottle fitted with siphon and a calcium chloride tube to prevent entry of carbon dioxide. Standardize by titration with an acid of known strength using methyl red as an indicator (secondary standardization or potassium biphthalate of known strength (primary standardization).

2. Sodium hypobromite (alkaline hypobromite): Mix 25 ml of liquid bromine with 250 ml of 40% NaOH.

3. 2% acetic acid: Dissolve 2 ml of glacial acetic acid (99.8%) in 100 ml of water.

4. 2% Na_2CO_3:

5. Urease enzyme: Grind 10 g horse gram or jack bean or soya bean with 100 ml 30% alcohol.

6. Phenol red indicator: Dissolve 1 g in 95 ml alcohol and 0.1 N NaOH.

Reactions of Creatinine

5

5A. GENERAL REACTIONS OF CREATININE

INTRODUCTION

Creatinine is a anhydride of creatine. It is a constant constituent of normal human urine. It is a non protein nitrogen (NPN). Non protein nitrogen of the blood and other body fluids include the nitrogenous constituents which are not precipitated as protein, e.g. Some NPN of blood are urea, uric acid, creatinine, creatine, aminoacids, glutathione and other compounds.

Blood level of creatinine is 0.7-1.2 mg%. Rate of excretion of creatinine in urine in an adult is 1-1.8 g. Foods such as meat and fish contain significant amount of creatinine especially after cooking. The level of creatinine on a creatinine free diet is almost constant for a given individual and is independent of the total nitrogen excreted in these conditions. It can be expressed as creatinine coefficient or the daily excretion of creatinine in mg per kg body weight. The endogenous creatinine is formed from creatine phosphate which is concerned with muscle contraction. Creatinine content of urine is decreased in muscular dystrophies and increased in conditions associated with increased tissue catabolism like fever.

Creatinine is the least variable non protein nitrogenous (NPN) constituent of the blood (Fig. 5A-1).

Physical Properties

Appearance - clear
Color - colorless
Odor - odorless

Reaction to litmus – acidic (the creatinine solution provided in mild acid).

Solubility – soluble in 12 parts of cold water; more soluble in warm water and in warm alcohol.

Reactivity: Forms salts with strong mineral acids.

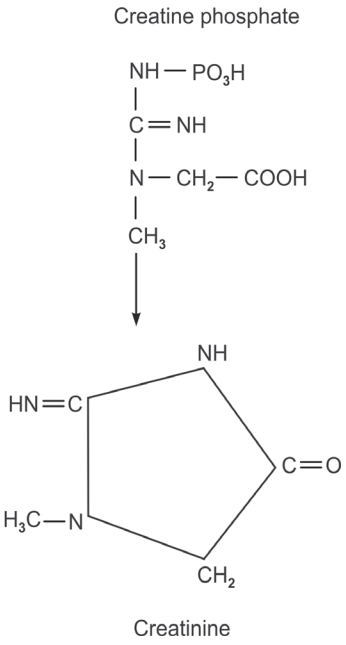


Fig. 5A-1: Formation of creatinine from creatine

CHEMICAL PROPERTIES

1. Nitroprusside Acetic Acid Test

Procedure: Take 5 ml of creatinine solution in a test tube and add a few drops of sodium nitroprusside. Render the solution alkaline by adding NaOH. A ruby red color appears and soon turns yellow. To this yellow solution add an excess of acetic acid and apply heat.

Observation: A green color forms and changes to blue color.

Inference: The color is due to the formation of prussian blue.

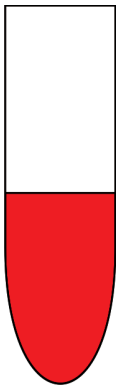


Fig. 5A-2: Jaffe' test

2. Jaffe' Test (Picric Acid Reaction) (Fig. 5A-2)

Procedure: To 5 ml of creatinine solution add 1 ml of 1% picric acid and 10 drops of 10% NaOH. Shake well and keep it for a few minutes.

Observation: An orange red color forms.

Inference: Creatinine forms creatinine picrate in alkaline medium which is red in color.

5B. QUESTIONS

1. Give an outline of formation of creatinine.
2. What is the reference range of creatinine in the blood ?
3. Give the rate of excretion of creatine in urine?
4. On creatinine free diet it's excretion in urine is constant in an individual. Explain.
5. What are the creatinine rich food items?
6. What is creatinine clearance test? What is it's significance in clinical diagnosis?
7. What is the reaction used to estimate the creatinine in body fluids?
8. Give the application of Jaffe' test.

5C. QUESTIONS

1. **Creatinine Solution:** Dissolve 100 mg creatinine in 0.1 N HCl and make upto 100 ml.
2. **1% Picric Acid:** Dissolve 1 g anhydrous picric acid in 100 ml distilled water.

Uric Acid

6

6A. GENERAL REACTIONS OF URIC ACID

INTRODUCTION (FIG. 6A-1)

Uric acid is the end product of purine catabolism. It is a non protein nitrogen (NPN). Non protein nitrogen of the blood and other body fluids include the nitrogenous constituents which are not precipitated as protein, e.g. Some NPN of blood are urea, uric acid, creatinine, creatine, amino acids, glutathione and other compounds.

Blood level of uric acid is 2-7 mg%. Over production of uric acid in the body is due to increased formation and break down of purine nucleotides. Uric acid in the plasma is filtered by the glomeruli. Almost all of the uric acid filtered is reabsorbed at proximal convoluted tubules (PCT) and some secreted at the distal part of the PCT and there is further reabsorption of uric acid in distal convoluted tubules (DCT). Final urine contains about 10% of the filtered uric acid. Above pH 5.75 most of the uric acid molecules are ionized as urate ion and are more soluble than non ionized uric acid seen at pH below 5.75. This kind of solubility exist in urine as well as in other body fluids. Rate of excretion of uric acid in an adult is about 0.5 – 1g in urine per day. But it varies with purine content of the diet. The uric acid content of the urine is relevant in relation to the formation of uric acid calculi. Intake of alkali carbonates and citrates or base forming foods increase the pH of urine and enhance the solubility of uric acid in urine and prevent calculi formation (Fig. 6A-2).

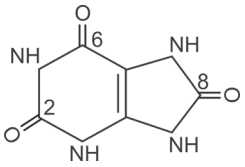


Fig. 6A-1: Uric acid (2,6,8 trioxypurine)

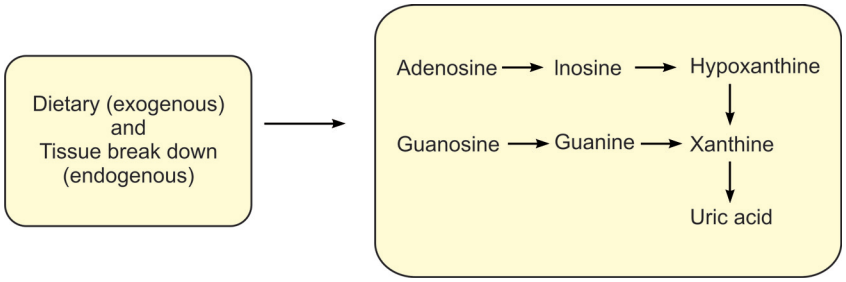


Fig. 6A-2: Formation of uric acid from purine nucleotides

PHYSICAL PROPERTIES

Appearance - transparent
Color - white
Odor - odorless

Reaction to litmus – alkaline (the uric acid solution provided in mild alkali)

Solubility – insoluble in alcohol, ether, soluble in boiling water to some extent (1:1800), insoluble in cold water; soluble in alkali, concentrated sulphuric acid.

CHEMICAL REACTIONS

1. Benedict's Uric Acid Test

Procedure: To 5 ml of test solution add 1 ml of 1% Na_2CO_3 and a few drops of Benedict's uric acid reagent.

Observation: Intense blue color (Fig. 6A-3).

Inference: Uric acid reduces phosphotungstic acid to tungsten blue in alkaline medium.

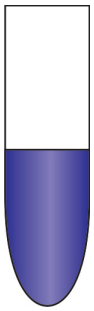


Fig. 6A-3: Benedict's uric acid test

Inference: Uric acid reduces phosphotungstic acid to tungsten blue in alkaline medium.

2. Schiff's Test

Add a drop of 3% ammoniacal silver nitrate at the center of a circular filter paper. Then add a drop of uric acid solution on to it.

Observation: Black color appears (see Fig. 6A-4).

Inference: Uric acid reduces silver nitrate to metallic silver in alkaline medium.

Fig. 6A-4: Schiff's test

3. Murexide Test

Procedure: Add 2 or 3 drops of concentrated nitric acid to a pinch of uric acid in a small evaporating dish and evaporate to dryness by heating on a water bath. A red or yellow residue forms. Then add a drop of a dilute ammonium hydroxide on the edge of the residue and to the opposite edge, add a drop of potassium hydroxide.

Observation: Purplish red color with ammonium hydroxide and purplish violet color with potassium hydoxide (Fig. 6A-5).

Principle: In this reaction uric acid is oxidized to dialuric acid and alloxan which condense to form alloxantin. The alloxantin so formed reacts with ammonium hydroxide to form ammonium purpurate or murexide which is purplish red in color. With potassium hydroxide a purplish violet color is produced due to the formation of potassium salt of ammonium purpurate.

Application: Murexide test is useful to detect uric acid calculi.

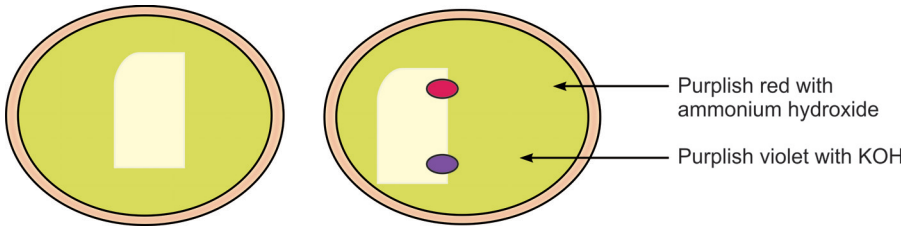


Fig. 6A-5: Murexide test

6B. QUESTIONS

1. Give an outline of formation of uric acid in human body.
2. What is the reference range of uric acid in the blood?
3. Give the rate of excretion of uric acid in urine.
4. Uric acid tend to form stone easily in acid pH of urine. Explain.
5. Give the principle of Benedict's uric acid test.
6. Give the principle of Schiff's test.
7. What is the chemistry of murexide test?
8. Application of Murexide test.

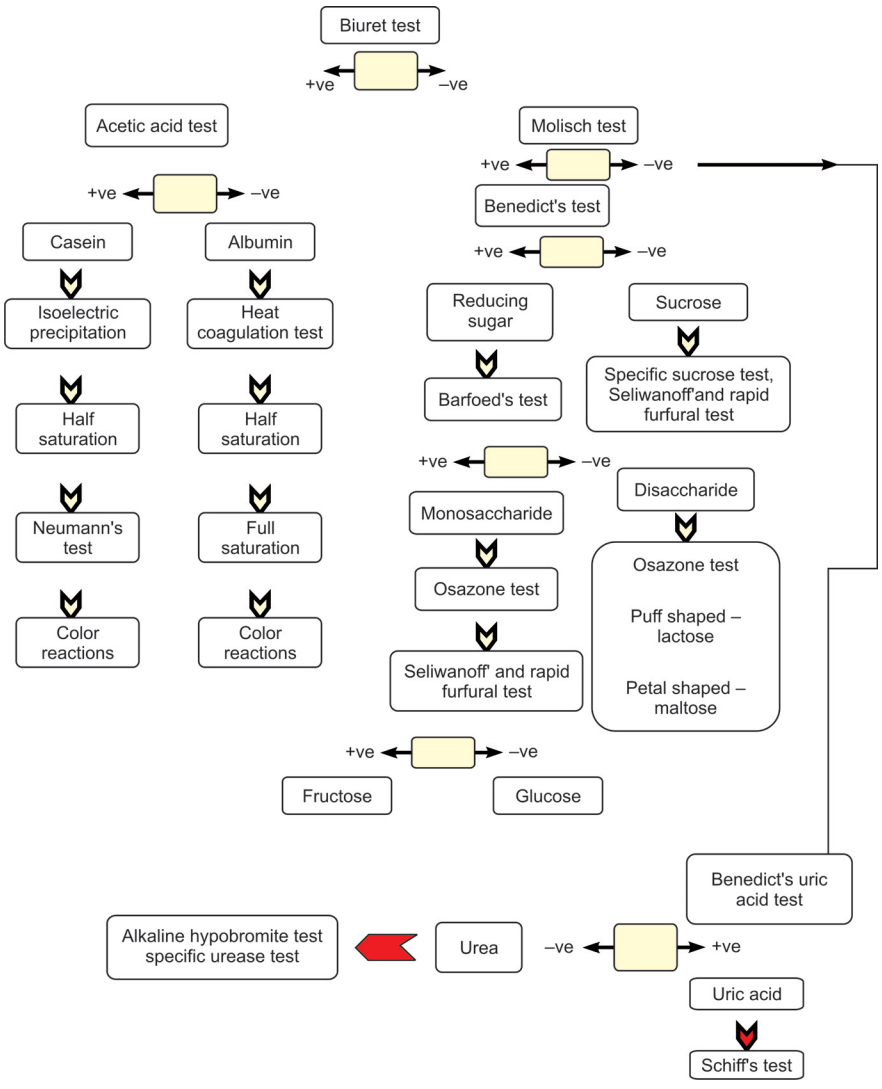
6C. REAGENT PREPARATION

1. **Benedict's Uric Acid Reagent:** Dissolve 100 g of pure sodium tungstate in a few ml water in a one litre pyrex glass flask and add 50 g pure arsenic acid (arsenic pentoxide – As_2O_5) followed by 25 ml 85% orthophosphoric acid and 20 ml of concentrated HCl, boil for 20 minutes, cool and make upto 1 L.
2. **1% Na_2CO_3:** Dissolve 1 g sodium carbonate in 100 ml of water.
3. **3% Ammoniacal Silver Nitrate:** Dissolve 26 g of silver nitrate in about 500 ml water, add enough ammonium hydroxide to redissolve the precipitate which forms upon the addition of ammonium hydroxide initially. Then make up the volume to 1 L with water.

Scheme for Identification of Biologically Important Substance in a Given Solution

7

SCHEME



Urine Analysis

8A. ANALYSIS OF NORMAL CONSTITUENTS OF URINE

INTRODUCTION

Urine is the ultra filtrate of plasma formed when the blood perfuse the two kidneys. Glomerulus filters plasma and the volume of glomerular filtrate amount to 180 L in 24 hours for an adult. Tubules of the kidney modify the glomerular filtrate by reabsorption and secretion of water and solutes to produce final urine volume of 1-2 L per day. Glomerular filtration rate is about 120 ml per minute. Thus, the kidneys retain essential substances and excrete waste products from the body. By this process it also helps in maintaining the acid base balance.

Clinical laboratory analysis of urine can provide information of kidney dysfunction (e.g. Nephrotic syndrome, glomerular nephritis) and about certain systemic diseases (e.g. Phenyl ketonuria, Diabetes mellitus) in an individual.

SPECIMEN COLLECTION

For getting correct analytical results , care must be taken in the collection of urine and transportation of it to the laboratory. Urine should be collected in clean sterile containers with a tightly fitting lid to avoid spillage, evaporation and contamination. Specimen containers should be labeled with name, age, date and time of collection.

The best urine specimen is the first voided urine in the morning since it is the most concentrated urine. Mid stream specimen is to be collected.

PHYSICAL EXAMINATION OF URINE (TABLE 8A-1)

Physical examination of urine is to be carried out prior to routine analysis which includes assessment of **volume, appearance, odor, color, pH and specific gravity.** Careful interpretation of these physical properties gives us a lot of information regarding various types of illnesses.

1. Volume: Normal adults excrete about 750 to 2000 ml of urine. It is influenced by fluid and salt intake, perspiration, respiration and functional status of cardiovascular and renal systems.

Oliguria: A decreased urine output is called oliguria.

Causes of Oliguria

– Prerenal causes
  - low blood pressure, shock, bleeding, fluid deprivation

– Renal causes
  • acute tubular necrosis, poisons causing renal damage, renal vascular disease
– Post renal causes
  • calculi, tumors compressing urinary tract from within or outside, prostate enlargement

Polyuria

An increased output of urine is referred to as polyuria.

Causes of Polyuria

– Conditions leading to excretion of a large amount of solutes along with isoosmotic amount of water
– Excessive salt intake, diabetes mellitus
– Deficiency of antidiuretic hormone
– Excessive fluid intake
– Intake of diuretics

2. Appearance: Normal urine is clear (transparent).

Causes of Cloudiness

Urine may become cloudy due to the presence of amorphous **phosphates** which will disappear or due to **urates** in urine. The cloudiness caused by phosphate appear and that due to urates disappear upon heating.
– Pus cell (white blood cells) clears on filtering
– Bacteria or fungi cleared by centrifugation
– Colloidal suspension of fat (as in chyluria) which cannot be cleared off by usual filtering or centrifugation.

3. Odor: Normally fresh urine has a faint aromatic smell.
– Upon standing, strong ammoniacal odor develop due to formation of ammonia by the decomposition of urea.
– Presence of ketone bodies (acetone and acetoacetic acid)in urine produces a fruity odor

4. Color: Normal color of urine varies from colorless to deep yellow. The color of urine is conferred by urochromes and urobilin. The intensity of the color varies with degree of dilution – dilute urine is pale yellow and concentrated urine is deep yellow.

Change in color of urine is observed in different clinical conditions - a few examples are given below:
• Deep or brownish yellow – bile pigments (jaundice)
• Red color – Intact red cells, free hemoglobin or myoglobin
• Black color – alcaptonuria, melanuria

5. pH: In a healthy person the pH of the urine varies from 4.6 to 8 depending on many factors like dietary intake and metabolic activities. Most often the urine pH is acidic around 6.0 due to the presence of sulfates, phosphates, chlorides and nonvolatile organic acids.

Vegetarian diet produces alkaline urine. On keeping urine, it becomes alkaline due to the formation of ammonia by the decomposition of urea.

Measurement of pH

– ***Litmus paper*** – In acid urine blue litmus turns red and in alkaline urine red litmus turns blue.
– ***pH paper*** which has a wide range of colors from 4.5 to 7.5.
– ***Dip sticks*** uses a combination of indicators methyl red and bromophenol blue which give a range of different colors from orange to green to blue as the pH rises from pH 5.0 to pH 9.0.

Points to Ponder: Extremely acidic or alkaline urine suggest the possibility of poorly collected urine.

6. Specific gravity: Specific gravity of urine serves to assess the concentration ability of the kidneys.

Increased specific gravity more than 1.030 seen in

- Dehydration
- Diabetes mellitus
- Congestive heart failure
- Proteinuria
- Adrenal insufficiency

Decreased specific gravity seen in

- Hypothermia
- Diuretic therapy

Fixed specific gravity: The specific gravity of urine is identical to the glomerular filtrate around 1.010. It is seen in patients with chronic kidney disease (CKD).

Presence of substances with high molecular weight substances like proteins and glucose in the urine impart much higher specific gravity than due to the excessive excretion of crystalloids.

Measurement of specific gravity is done by

- **Urinometer** (see it in the section - spotters)
- **Refractometer** used in higher laboratories

DEMONSTRATION OF INORGANIC CONSTITUENTS OF URINE (TABLE 8A-2)

The main inorganic constituents of urine are Na^+, K^+, Ca^+, Mg^{++}, NH_4^+, Cl^-, phosphates and sulfates.

- Chief inorganic constituent of urine is **chloride**. It is derived from salts of the diet. Rate of excretion of chloride in urine **10 -15 g per day**. It's content in urine is increased in addison's disease in which there is aldosterone deficiency so that reabsorption of sodium and chloride are defective and so get excreted in urine.
- The **sulfates** of urine derived from sulfur containing amino acids. Rate of excretion of inorganic sulfates in urine **0.8-1 g/day**.
- **Calcium** is excreted at the rate of **0.1-0.3 g per day**.
- Phosphates derived from inorganic phosphates in the diet – phosphoproteins, nucleoproteins and phospholipids. It is excreted at the rate of **1 g per day**.

1. Test for Chloride

Procedure: Acidify 2 ml of urine with 2 drops of concentrated HNO_3 and add 2 ml of silver nitrate solution.

Observation: White precipitate.

Inference: A white precipitate of silver chloride (AgCl) forms. Nitric acid prevents precipitation of salts other than chloride like silver urates and silver phosphates.

2. Test for Sulfates

Procedure: Acidify 3 ml of urine with 2-4 drops of concentrated HCl and add 1 ml of barium chloride solution.

Observation: White precipitate.

Inference: A white precipitate of barium sulfate. HCl prevents precipitation of phosphates.

3,4. Test for Calcium and Phosphates (Fig. 8A-1)

Procedure:

- Take 10 -12 ml of urine in a boiling tube. Add 3 ml of strong ammonia solution and boil till white precipitates of calcium and magnesium are formed.
- Filter through a filter paper placed in a funnel placed over a test tube.
- Wash the precipitate thus collected in the filter paper by just pouring a few ml of water through the filter paper.
- Then take the funnel with the filter paper in situ, and place it over another test tube.

- Add 3 ml hot acetic acid through the filter paper placed over the test tube so that precipitate in the filter paper get dissolved in hot acetic acid and will be collected in the test tube underneath.
- Divide it into 2 parts
- ***To detect calcium:*** To one part add 1 ml of potassium oxalate
- ***To detect phosphates:*** To the other part add a drop of concentrated HNO_3 and a few drops of ammonium molybdate solution. Boil.

Observation: white precipitate forms in the test is meant for **calcium**.

Fine lemon yellow (canary yellow) precipitate forms in the tube is meant for detecting **phosphates**.

Inference: Calcium forms a white precipitate of calcium oxalate on addition of potassium oxalate.

On boiling with ammonium hydroxide phosphates of calcium and magnesium are precipitated. These are then filtered and redissolved in hot acetic acid.

Phosphates react with ammonium molybdate to form canary yellow colored ammonium phosphomolybdate in the presence of HNO_3.

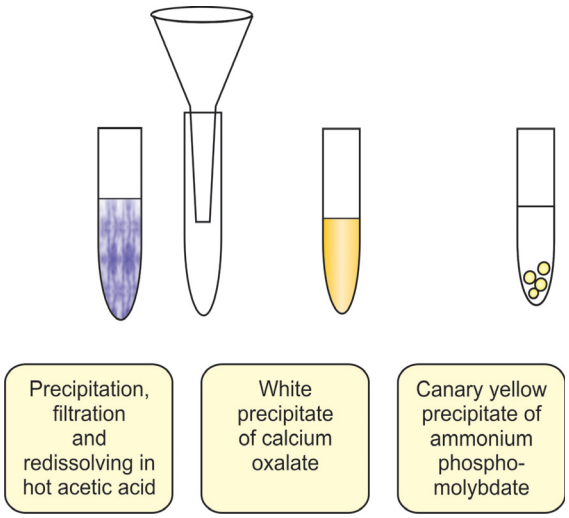


Fig. 8A-1: Test for calcium and phosphate

5. Test for Ammonia

Procedure: To 10 ml of urine add a drop of phenolphthalein and make just alkaline by adding 0.1 N NaOH in drops. Hold a glass rod dipped in phenolphthalein at the mouth of the test tube and heat the contents of the tube.

Observation: The phenolphthalein indicator at the tip of the glass rod turns pink.

Inference: Ammonium salts give off ammonia in alkaline medium and the ammonia vapors emerging from the tube turn the phenolphthalein indicator to show pink color since ammonia is alkaline (Color range of phenolphthalein – colorless to pink; pH range - 8.3-10).

ORGANIC CONSTITUENTS OF URINE

Important organic constituents in urine are urea, uric acid, ethereal sulfates, creatinine, organic sulfates, urinary pigments.

1. Urea: The amino acids released as a result of protein breakdown are transdeaminated to release ammonia. The toxic ammonia is converted to less toxic urea in the liver. Urea in the blood is denoted as blood urea nitrogen (BUN). Urea is filtered at the glomerulus and 40-50% of the filtered urea is reabsorbed by the proximal renal tubules.

Causes of increased urea content in urine

1. High protein diet
2. Conditions leading to increased tissue break down (increased protein catabolism), e.g. fever, diabetes mellitus, adrenal cortical hyperactivity.

Causes of decreased urea content in urine

Liver diseases: In affections of liver synthetic function is disturbed and urea is formed in low amounts.

BUN in blood: 5-17 mg% (1.8-6.1 mmol/L)

Urea content in urine in healthy subjects – **7-16 g/day**.

2. Uric Acid: It is the catabolite of dietary or tissue purine nucleotides. Plasma levels of uric acid is variable and it is higher in males than in females. It is completely filterable and it is reabsorbed at the PCT and secreted at DCT.

Uric acid content in urine is raised in

1. High purine diet
2. Conditions where there is increased tissue turn over without any impairment of kidney function –leukemia and other malignancies
3. Gout
4. Cortisone therapy

Uric acid level in blood

Males: 3.6 – 7.7 mg% (214 – 458 µmol/L)
Females: 2.5 – 6.8 mg% (149 – 405 µmol/L)

Uric acid content in urine: 300 – 800 mg/day on an average diet.

3. Creatinine: The compound creatine phosphate is formed from liver, kidneys and pancreas and is carried by the blood to other tissues, especially utilized in muscles and brain. About 1-2% of creatine in muscle undergo spontaneous conversion to form cyclical anhydride of creatine, the creatinine. Creatinine is filtered by the glomerulus and a small amount of creatinine is reabsorbed in the proximal convoluted tubule and secreted in the distal convoluted tubule in small amounts. Hence the measurement of creatinine excretion can be used to assess the glomerular filtration function of the kineys. The amount of creatinine formed in the body at a point of time depends on age, sex and muscle mass and to a minor extent on the content of creatine in the diet (meat muscle rich in creatine). But daily variations of creatinine levels in blood and excretion in urine are very minimal. Because of this fact, creatinine levels are useful to assess the kidney function.

Creatinine level in blood

Males: 0.6 – 1.0 mg% (57 – 92 µmol/L)
Females: 2.5 – 6.8 mg% (50 – 81 µmol/L)

Creatinine content in urine: On an average diet 1-2 g/day (nearer to higher limit in males and to lower limit in females)

Raised creatinine levels in blood: Seen in renal failure, nephritis.

High creatinine levels in urine seen in:

- Myopathies
- Fever
- Muscle injuries

4. Organic Sulfates: Urinary sulfates are of three types:

(i) Inorganic sulfates which come from metabolism of sulphur containing amino acids.

(ii) Ethereal or organic sulfates: This constitute 10% of total sulfates excreted in urine. The different ethereal sulfates seen in human urine are conjugated phenols, phenol sulfuric acid, p-cresol sulfuric acid, skatoxyl sulfuric acid and indoxyl sulfuric acid (indican). Altogether the excretion rate is **0.04 to 0.1 g per day**. In health the ratio of sum of ethereal and neutral sulfates to inorganic sulfate is about 1: 10.

Formation of ethereal sulfates: Phenols are produced during putrefaction of protein material in the intestine. Phenol then reaches liver where it is conjugated to form phenol potassium sulfate and excreted as such in urine. Indole and skatole get oxidized to indoxyl and skatoxyl respectively and conjugated and then excreted in urine. Action of intestinal bacteria on tryptophan leads to the formation of indoxyl sulphuric acid and it is excreted in the urine as potassium salt (indican) (Fig. 8A-2). Excretion rate of ***indican*** alone give

rough estimate of intestinal putrefaction. Excretion rate of indican in normal individuals **10-20 mg/day**. Excretory rate increases with high meat diet and decreases with high carbohydrate diet.

Pathological increase seen with intestinal obstruction which causes stagnation of intestinal contents and putrefaction there upon and in situations where there is bacterial decomposition of body proteins – gangrene, putrid pus formation.

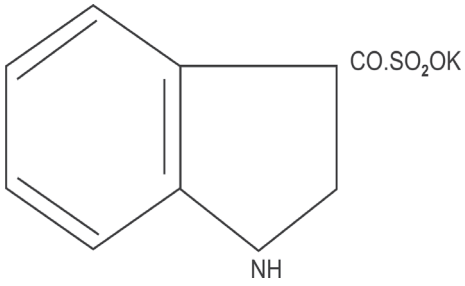


Fig. 8A-2: Indoxyl sulphuric acid

(iii) Neutral sufates: It is produced from endogenous sources and its rate of excretion do not change with diet. Generally sulfur in these compounds is in unoxidized or neutral state. The compounds coming under this category sre cystine, methyl mercaptan, ethyl sulfide, thiocyanates, taurine derivatives. Neutral sulfur content of normal human urine is 5-25% of total sulfur content **(0.08 – 0.16 g/day)**. Its content in urine is raised in cystinuria.

5. Urobilinogen: After the life span of 120 days red blood cells undergo lysis and hemoglobin is released in the reticuloendothelial system (spleen, bone marrow, kupffer cells in liver). Bilirubin released is transported by albumin to the liver and there get conjugated with glucuronic acid to form bilirubin glucuronide (conjugated bilirubin) which then passes to the intestine via common bile duct.

In the intestine, the conjugated bilirubin is reduced by intestinal bacteria to urobilinogen – a term used to include d-urobilinogen, mesobilirubinogen and stercobilinogen. The greater part of urobilinogen thus formed is excreted in the feces as faecal urobilinogen. The rest of the urobilinogen is reabsorbed into the portal circulation and reaches liver. The greater part of this fraction is re excreted by the liver in the bile as urobilinogen. A small part enters the systemic circulation and is excreted in the urine as urine "urobilinogen".

DEMONSTRATION OF ORGANIC CONSTITUENTS OF URINE (TABLE 8A-3)

1. Test for Urea

1. Alkaline hypobromite.
2. Specific urease test (see Chapter 4—Reactions of Urea).

2. Test for Uric Acid

1. Benedict's uric acid test
2. Schiff's test
3. Murexide test (see Chapter 6—Uric Acid).

3. Test for Creatinine

Jaffe' test (see Chapter 5—Reactions of Creatinine)

4. Test for Ethereal Sulfates

Procedure: To 5 ml of urine add 2 ml barium chloride and 2 ml hydrochloric acid. Mix well and filter. Divide the filtrate into two tubes. Boil the contents in one tube. Carefully look for the turbidity developing in the tubes.

Principle: Hot HCl hydrolyzes ethereal sulfate to inorganic sulfate which then gives precipitate with barium chloride.

5. Test for Urobilinogen—Ehrlich's Test

Procedure: To 5 ml urine add 1 ml Ehrlich reagent, mix well and keep for 5 minutes.

Observation: Red color develops.

Principle: Urobilinogen reacts with p-dimethylaminobenzaldehyde of the reagent to form the red colored complex.

Inference: Normal urine gives a faint red color due to the presence of trace amounts of urobilinogen. It is excreted increasingly in urine in hemolytic jaundice where the RBCs are destroyed at a higher rate.

Points to Ponder: Urobilinogen in urine get oxidized to urobilin upon keeping and hence stored urine may not answer the test. Fresh urine is preferred for testing urobilinogen (Fig. 8A-3).

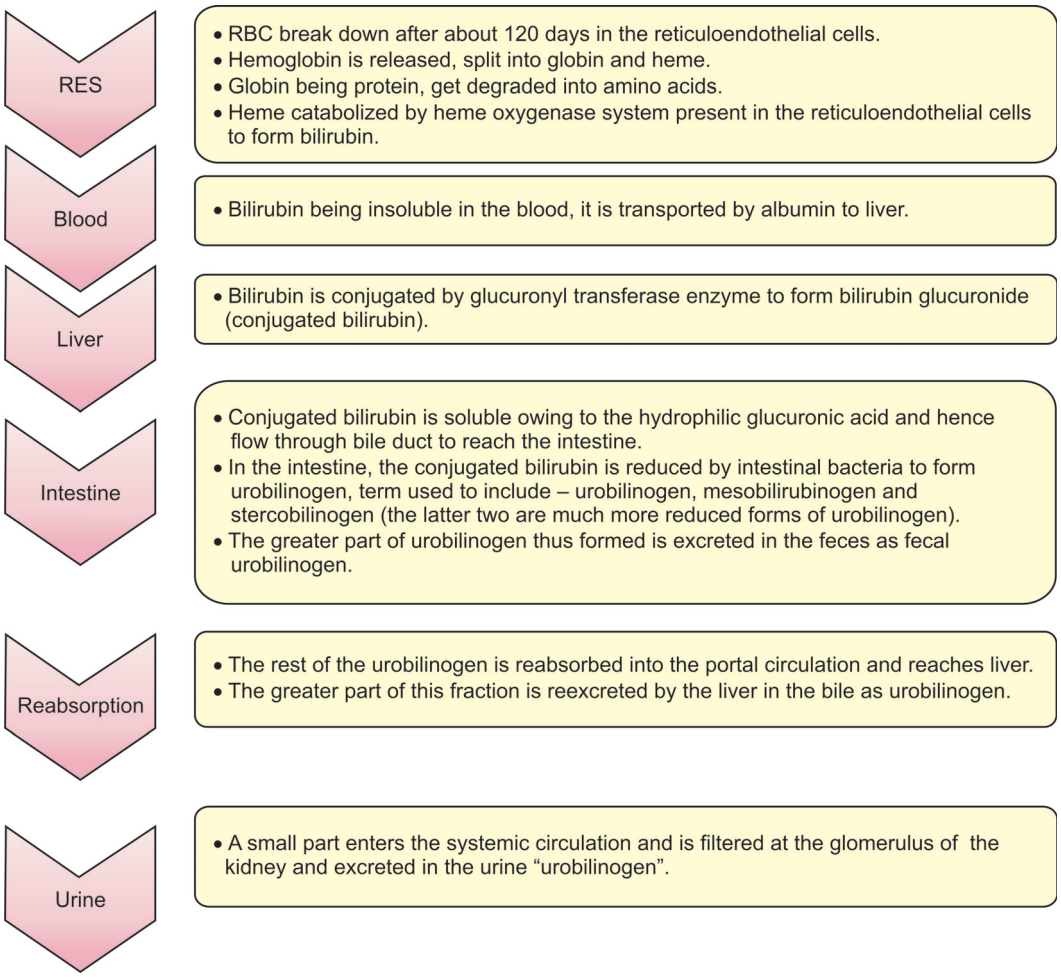


Fig. 8A-3: Urobilinogen formation and excretion in urine

ANALYSIS OF NORMAL CONSTITUENTS OF URINE

Table 8A-1: Physical Properties of Normal Urine

No.	*Experiment*	*Observation*
1	Appearance	Clear
2	Color	Amber yellow
3	Odor	Ammoniacal smell
4	Reaction to litmus	(mostly acidic) Blue litmus turns red
5	Specific gravity	1.015 – 1.025

Table 8A-2: Test for Inorganic Constituents in Urine

No.	*Experiment*	*Observation*	*Inference*
1	Test for chloride	White precipitate	Precipitate due to silver chloride
2	Test for sulfate	White precipitate	Precipitate due to barium sulfate
3	Test for calcium	White precipitate	Precipitate due to calcium oxalate
4	Test for phosphate	Canary yellow precipitate	Precipitate due to ammonium phosphomolybdate
5	Test for ammonia	Phenolphthalein at the tip of the glass rod turned pink	Ammonia vapors emerging from the tube turns the phenolphthalein pink

Table 8A-3: Test for Organic Constituents in Urine

No.	*Experiment*	*Observation*	*Inference*
1	*Test for urea*		
	a) Alkaline hypo-bromite test	a) Brisk effervescence	a) Due to evolution of N_2 gas
	b) Specific urease test	b) Pink color	b) Urea split by urease enzyme to form ammonia making the medium alkaline. In the alkaline medium phenol red used in the test give pink color.
2	*Test for uric acid*		
	a) Benedict's uric acid test	a) Intense blue color	a) Phosphotungstic acid reduced by uric acid to tungsten blue
	b) Schiff's test	b) Black color	b) Silver nitrate reduced by uric acid to metallic silver
3	*Test for creatinine* Jaffe' test	Orange red color	Due to the formation of creatinine picrate
4	*Test for ethereal sulfate*	White precipitate in trace	Precipitate due to barium sulfate
5	*Test for urobilinogen*— Ehrlich's test	Red color	Urobilinogen reacts with p-dimethyl aminobenzaldehyde to give red color

8B. ANALYSIS OF ABNORMAL CONSTITUENTS OF URINE

Clinical laboratory analysis of urine is useful for diagnosis of several clinical conditions e.g. diabetes mellitus, phenyl ketonuria, Maple syrup urine disease, alcaptonuria. Advantage of urine examination is, it involves no pain or any disturbance to the patient. Properly collected, analyzed and interpreted urine laboratory tests are valuable for the practice of Modern Medicine.

1. GLUCOSE

Benedict's Test (see Chapter 1—Reactions of Carbohydrates)

Procedure: To 5 ml of Benedict's reagent taken in a test tube, add 8 drops of urine. Shake well and boil for 1 or 2 minutes or keep it in a water bath for 5 minutes.

Observation: A colloidal precipitate forms and the color of which may be green, yellow, orange or red depending on the in urine. concentration of sugar (see Fig. 1A-3).

Interpretation: In the presence of over 0.2-0.3 percent of glucose, the precipitate form readily. In the absence of glucose the solution may remain clear or will show a turbidity due to precipitated urates.

Color of the precipitate give an idea about the concentration of the sugar solution as shown below.

Blue – absence of reducing sugar
Green – up to 0.5 gm%
Yellow – 0.5 to 1.0 gm%
Orange – >1.0 to 2.0 gm%
Brick Red – ≥ 2 gm%

Normally glucose is absent in urine. Appearance of glucose in urine is referred to as glucosuria. Glucosuria occurs in

- Diabetes mellitus: The high level of glucose in blood crosses the renal threshold for glucose (around 180 mg%) and get excreted in urine.
- Renal glucosuria: Here renal threshold for glucose is lowered. Glucose appears in urine even if it's level remain within normal limits. This may happen in pregnancy and in inherited lowered renal threshold for glucose. It is differentiated from diabetes mellitus by testing blood and urine simultaneously for glucose and if needed glucose tolerance test.

Points to ponder: Benedict's test being a nonspecific test since it involves reduction of cupric ions to cuprous ions by a reducing agent. In the urine several such reducing agents may occur. Such substances are given below.

Carbohydrate substances like fructose, galactose, lactose and pentoses and non-carbohydrate substances like ascorbic acid, homogentisic acid. Presence of glucose can be confirmed by specific test, using glucose oxidase enzyme.

Clinistix: Stiff cellulose strip which turns from red to purple when dipped into urine containing glucose, detect 0.1% glucose or less. It is more sensitive than Benedict's test. Urine containing low amounts of glucose escape detection by the reduction test but detected by clinistix.

Principle of clinistix: Oxidation of glucose by glucose oxidase to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide acted

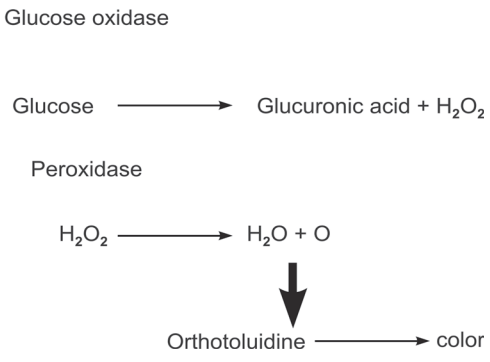


Fig. 8B-1: Principle of clinistix

upon by peroxidase to produce nascent oxygen which in turn acts upon the chromogen (e.g. orthotoluidine) to produce color (Fig. 8B-1).

2. PROTEIN

Heat Coagulation Test (Fig. 8B-2)

Procedure: Fill 3/4th of the test tube with urine Heat the upper 1/3 rd of the urine column by a small flame, so that lower 2/3rd will serve as control. Add a drop of 30 % (v/v) acetic acid to it.

Observation: White turbidity or coagulum.

Interpretation: White turbidity if disappears on addition of acetic acid indicates the presence of phosphates or carbonates. If the white turbidity formed remains or appears or intensifies on adding acetic acid points towards the presence of albumin. Addition of acetic improves the formation of turbidity since the acidification brings the pH of the medium towards 4.7 (IEP of albumin).

Points to ponder: There are chances to miss presence of albumin in the urine if the pH of urine is high and it is not brought down by adding acetic acid. Isoelectric point of human albumin is 4.7.

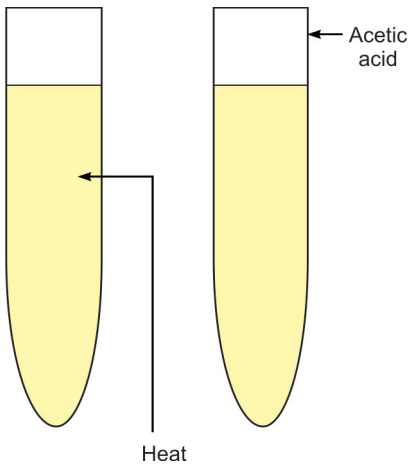


Fig. 8B-2: Heat coagulation test

Normal urine contains less than 250 mg per 24 hours and it escape detection by the usually employed methods. Pathologically different proteins detected in urine-albumin, myoglobin, fibrin and oxyhemoglobin.

- The proteinuria is most commonly seen due to leakage of serum albumin since it is the most abundant and smallest protein in the serum.
- Albumin most often appears in urine due to altered structure of glomerulus in various kidney diseases.
- Albumin may appear in urine by entering below the kidneys (not by glomerular filtration) from blood, exudates or lymph is called **false albuminuria**.
- **Benign proteinuria:** It is transient and not associated with any kidney disaease. Occurs with severe exercise and cold bath.
- **Orthostatic albuminuria:** Albumin appears in urine after prolonged standing

Albustix: Is a stiff cellulose strip impregnated at one end with indicator tetrabromphenol blue buffered at pH around 3 which has a yellow color at pH 3.0. Presence of protein turns it into green - blue. Buffer maintains the pH at 3 and hence pH of urine do not interfere. If protein is absent, the color will be yellow. In the presence of protein the color varies from green to blue. Highly alkaline urine and stale urine (due to the formation of ammonia) may overcome the buffering action of the strip and give a false positive response.

3. KETONE BODIES

Rothera's Test (Fig. 8B-3)

Procedure: Saturate 5 ml of urine with ammonium sulfate crystals and add 2 drops of freshly prepared 2% sodium nitroprusside solution or a little of sodium nitroprusside powder. Shake well. Add 1 ml of liquor ammonia through the sides of the test tube.

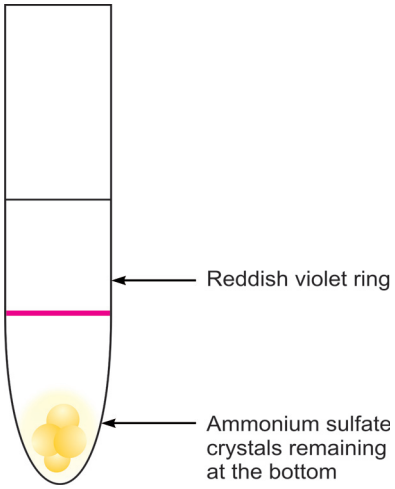


Fig. 8B-3: Rothera's test

Observation: Reddish violet ring at the junction of two liquids.

Principle: Acetone and acetoacetic acid react with sodium nitroprusside (nitroferricyanide) in the presence of alkali to produce a purple color.

Inference: Ketone (acetone) bodies include acetone, acetoacetic acid and β-hydroxybutyric acid. To detect the latter a modified test has to be done. (oxidize the β-hydroxybutyric acid with hydrogen peroxide to form acetoacetic acid. Add a few drops of acetic acid to 2 ml of 1:1 diuted urine with distilled water. Boil for few minutes to discard the acetone and the acetic acid present in the urine. Then add 1 ml of hydrogen peroxide warm gently and carry out Rothera's test. It will give a positive response if β-hydroxybutyric acid is present in the urine.) Normal urine contains approximately 20 mg per 24 hours only. Ketone bodies are produced excessively in the body in starvation and in uncontrolled diabetes mellitus and starvation.

4. BLOOD

Benzidine Test

Procedure: Take 2-3 ml of urine in a test tube. Boil for 5 minutes and cool it. Mix equal volumes of benzidine solution (2-3 ml) and hydrogen peroxide in a test tube and add the boiled cooled specimen of urine into the reagent mixture.

Observation: A transient blue color appears.

Inference and Principle: Peroxidase activity of heme oxidises hydrogen peroxide to release the nascent oxygen which acts upon benzidine to form blue colored compound.

Interpretation: Presence of blood in urine indicates either hematuria (intact RBCs in urine seen in kidney diseases) or hemoglobinuria (Hb in urine).

Points to Ponder

- Benzidine is a carcinogen. So care should be taken while handling the reagent.
- H_2O_2 deteriorates rapidly, so freshly prepared H_2O_2 should be used.
- Boiled cooled urine must be used for the test otherwise peroxidase enzymes of leucocytes present in the urine will interfere with the test.

Strip Test for Detecting Blood or Heme: It is based on the peroxidase activity of heme which splits H_2O_2 to form nascent oxygen which in turn oxidize the chromogen (usual chromogen used is tetramethyl benzidine or orthotolidine) to form the color - on dipping in urine gives a yellow color in the absence of heme (either in RBC or in free Hb) and blue green in the presence of heme. Ascorbic acid and nitrites interfere with it. Formalin if used as a urinary preservative will also give a false negative test.

5. BILE SALT

Hay's Test

Procedure: Take 5 ml of urine in a test tube and sprinkle sulfur powder on the surface of urine.

Observation: Sulfur powder sink to the bottom.

Inference: Bile salts are present in urine otherwise the sulfur powder would have remained on the surface of urine column.

Principle: Bile salts reduce the surface tension. Hence the sulfur powder sinks to the bottom.

Interpretation: Salts of taurocholic acid and glycocholic acid present in the bile regurgitate into blood whenever there is obstruction to bile flow (obstructive jaundice) and will appear in urine. This test is useful to differentiate obstructive jaundice from hemolytic jaundice.

Obstuctive jaundice seen with biliary atresia, obstruction of bile duct due to stones or tumors and obstructive phase of hepatic jaundice.

6. BILE PIGMENT

Modified Fouchet's Test (Fig. 8B-4)

Procedure: To 10 ml urine add 1 ml $MgSO_4$ and boil. While boiling add 10% $BaCl_2$ drop by drop till maximum precipitate is got. Filter and discard the filtrate. Take the filter paper from funnel and dry it by moping it over another paper. After drying add 2 drops of Fouchet's reagent to the precipitate.

Observation: A blue/green color in the presence of bile pigments.

Principle: The precipitate obtained is that of $BaSO_4$ to which bille pigment if any would have adsorbed to it. When Fouchet's reagent to the precipitate (Ferric chloride in Trichloroacetic acid) is added, $FeCl_3$ oxidizes bilirubin to biliverdin and Fe^{3+} (ferric ions) is converted to Fe^{2+} (ferrous ions). This gives the color.

Interpretation: Positive Fouchet's test indicates the presence of conjugated bilirubin in the urine (Only the conjugated bilirubuin can appear in urine in a person with normal kidney). Conjugated bilirubin appears in urine in cases of **obstructive jaundice** and **obstructive phase of hepatocellular jaundice.** So this test is useful to differentiate obstructive jaundice from hemolytic jaundice where it will be negative or weakly positive.

Fig. 8B-4: Modified Fouchet's test

7. UROBILINOGEN

Ehrlich's Test

Procedure: Add 1 ml of Ehrlich's reagent (2% Para dimethylaminobenzaldehyde in 20% HCl) to 10 ml of freshly voided urine. Shake well and keep it in the rack for 5 minutes for the color development.

Observation: Normal urine give only a faint **red** color.

Principle: Urobilinogen forms a colored adduct with Para dimethyl aminobenzaldehyde

Interpretation: Intensity of red color is related to the concentration of urobilinogen in the following manner (see Fig. 8B-5).

No red color: Urobilinogen absent.

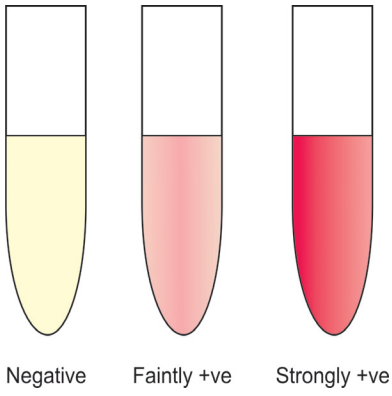


Fig. 8B-5: Ehrlich's test

Faint pink color: Urobilinogen present in normal amounts.

Distinctly red color: Urobilinogen present in increased amounts.

Points to Ponder: Bilirubin if present in the same sample may also react in the same way as that of urobilinogen. In order to avoid this remove bile pigments by adding 2 ml 10% Calcium chloride solution to the urine. Filter and carry out the test with the filtrate.

8C. QUESTIONS

1. Give brief answers:
   a. Significance of odor of urine
   b. Polyuria
   c. Oliguria
   d. Importance of observing the color of urine
   e. Normal pH range of urine
   f. Influence of diet on the pH of urine
   g. Physiological range of specific gravity
   h. Physiological causes of change in volume of urine
   i. Pathological causes oliguria and polyuria
2. Give the excretion rate of the following in urine in a normal person.
   a. Calcium
   b. Phosphates
   c. Sulfates
   d. Chloride
   e. Uric acid
3. Give the principle of the following tests:
   a. Test for chloride
   b. Test for sulfates
   c. Test for calcium
   d. Test for phosphates
4. Give brief answers:
   a. Source of sulfates in urine
   b. Importance of Rothera's test in clinical medicine
   c. Biochemical principle of treatment of urea cycle disorder
   d. Tests useful in the differential diagnosis of jaundice

8D. REAGENT PREPARATION

1. **Concentrated Nitric Acid:** Supply from the bottle (16 N).
2. **3% Silver Nitrate Solution:** Weigh 3 g silver nitrate and add to a small volume of distilled water taken in a 100 ml volumetric flask, shake well and make upto 100 ml.
3. **10% Barium Chloride Solution:** Weigh 10 g Barium chloride and add to a small volume of distilled water taken in a 100 ml volumetric flask, shake well and make upto 100 ml.
4. **2% Potassium Oxalate:** Weigh 2 g Barium chloride and add to a small volume of distilled water taken in a 100 ml volumetric flask, shake well and make upto 100 ml.
5. **Ammonium Molybdate Solution:** Dissolve 100g of molybdic acid in 144 ml of ammonium hydroxide (sp. gravity 0.9) and 271 ml water. Pour the solution thus obtained slowly with constant stirring to 489 ml nitric acid (sp. gravity 1.42) and 1148 ml water. Keep the mixture in warm place for several days. Check the adequacy of keeping in the following manner. Take about 5 ml of this solution in a tube and heat upto 40°C and if no yellow precipitate of ammonium phosphomolybdate is forming, it can be considered as fit for use.
6. **Urease Solution:** Grind 10 gm horse gram or jackfruit seeds (rich sources of urease enzyme)

with 100 ml 30% ethanol using a mortar and pestle.

7. **Fouchet's Reagent:** Dissolve 25 g trichloroacetic acid in about 50 ml of water and add 10 ml of 10% ferric chloride and make upto 100 ml with water.
8. **Ehrlich's Reagent:** Dissolve 2 g p-dimethylaminobenzaldehyde in 100 ml of 20% hydrochloric acid.
9. **Phenolphthalein (pH range–8.3-10, color range – colorless to red):** Dissolve 1 g in 50% alcohol.

Spectroscopy

9

9A. SPECTROSCOPIC EXAMINATION OF HEMOGLOBIN PIGMENTS

SPECTROSCOPY AND SPECTROSCOPE

Spectroscopy involves observation and study of absorbed or emitted light by means of a spectroscope. Spectroscope is an instrument used to study the absorption spectra of various substances. It consists of a prism that refracts the light or gratings for diffraction of light and an arrangement for rendering the rays parallel and a telescope that magnifies a spectrum (Fig. 9A-1).

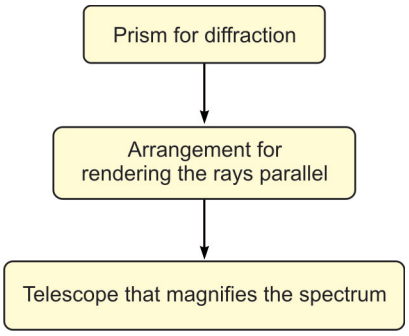


Fig. 9A-1: Essential parts of a spectroscope

Many biological substances have characteristic light absorbing properties because many molecular groupings have characteristic light absorption pattern which can be used for detection and quantitative assay. These substances can be identified by studying their light absorption properties, e.g. Hemoglobin, porphyrins.

Spectroscope consists of a prism situated behind an adjustable eyepiece. When a beam of light is allowed to pass through the prism it disperses light into solar spectrum. When a colored solution is inter placed in the path of the light entering the prism through the slit of the spectroscope, it will alter the appearance of the spectrum. If the solution absorbs light of particular wavelength correspondingly dark bands will be produced in the spectrum. It is possible to identify specific substances by accurately positioning the absorption bands produced in the spectrum by the solution.

Various types of spectroscope are available. For the identification of Hb derivatives or porphyrins in biological fluids, a simple hand held spectroscope is used.

In the solar spectrum several vertical dark lines, called Fraunhofer lines are seen. These dark lines are caused by the absorption of the white light from the hotter regions of the sun by chemical elements present in the cooler parts of the sun. They are designated as A, B, C, D, E ..... like that from the red end of the spectrum.

Chromosphere: This is a layer of sun's atmosphere surrounding the photosphere which is visible during a total eclipse. The chromosphere is several miles thick and has an estimated temperature of 20000 K.

Photosphere: The visible, intensely luminous portion of the sun which has an estimated temperature of 6000 K.

The spectroscope consists of a long tube (slit tube) and a short tube (tube holder). Long tube contains the optical system and the short tube contains the wavelength scale. Adjust the slit in the long tube by adjusting the knurled ring. Slit should be very narrow. Then the instrument is directed to sunlight. When it is viewed through eyepiece the spectrum is visible. By adjusting the slit tube vertical narrow absorption bands can be viewed in the spectrum. The wavelength scale can be adjusted using the tube holder, so that graduations in the scale appear at short focus. Graduations are done in nanometers (nm). Set the scale with reference to D line (i.e. 590 nm marking should correspond to D line in the spectrum). D line is the most important of the fraunhofer lines. D line corresponds to absorption band of sodium with a wavelength of 590 nm. This marking is extended downwards. Deviations in the D line can be adjusted using the adjusting screw.

Principle: A beam of sun light is dispersed into 7 components (VIBGYOR) using a prism incorporated in the spectroscope. When a tube containing relevant Hb solution is kept against the spectroscope, the substance in solution will absorb a certain portion of the visible light and that will be seen as dark bands in the spectrum when viewed through the spectroscope. The bands produced will be characteristic of the molecular nature of the substance (Fig. 9A-2).

Procedure: Set the spectroscope for reading. Take the solution to be examined in a clean dry test tube and hold it vertically in front of the instrument. View the bands produced and note their position.

Precautions and General Rules

1. Spectroscope should be adjusted properly
2. Bright light should be used
3. Before colored pigments are examined it has to be diluted in the right concentration. In higher concentrations the bands are darker and broader. In low concentrations the bands are narrow and sharp.
4. Bands when viewed on spectroscopic examination are read from the red end of the spectrum as α, β, γ and so on.
5. Position of bands is read from the scale. The scale reading against the mid point of the band is read.

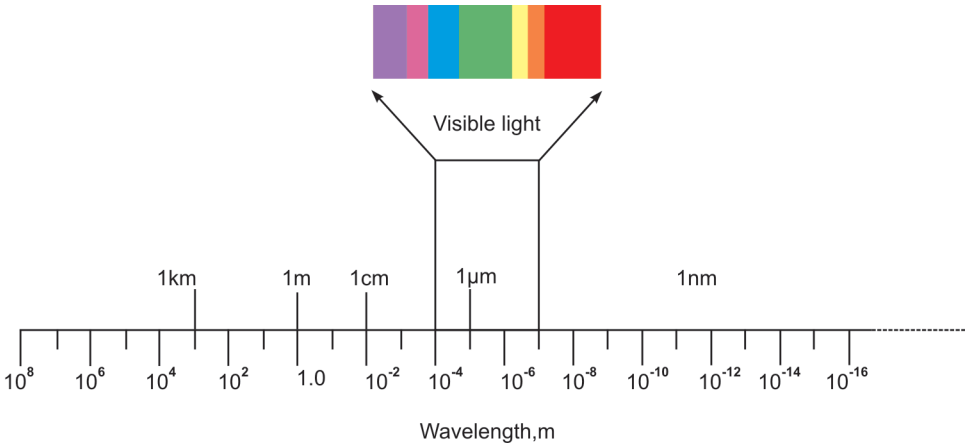


Fig. 9A-2: Electromagnetic spectrum and visible light

Identification of Different Hb Derivatives

1. Oxyhemoglobin: Two atoms of oxygen are taken up by each atom of iron. One iron atom is attached to each monomer of Hb. So a tetramer Hb molecule can take up 4 molecules of oxygen. One gram of Hb can combine with 1.36 ml of oxygen at NTP. Oxy Hb loses oxygen when exposed to a low oxygen pressure or upon treating with ammonium sulphide or sodium dithionite ($Na_2S_2O_4$).

Color: Orange red.

1. ***Spectroscopy:*** 2 bands seen. α band at 577 nm in the yellow region and β band at 541 nm in the green region (γ band at 413 nm in the violet region). Alpha band is narrow and sharp and beta band is broad and hazy. Generally alpha and beta bands are looked for as the gamma band will not be distinguishable.

2. ***Schumm's test:***

Principle: Involves reduction of oxy Hb to Hb by the action of reducing substances.

Procedure: Take 2 ml of the specimen containing oxy Hb in test tube. Add 1 ml of ether to cover the surface in order to avoid contact with air and then add 0.2 ml of ammonium sulfide solution. Examine spectroscopically.

Observation: Alpha band disappears

3. ***Addition of sodium dithionite:***

Principle: Involves reduction of oxy Hb to Hb by the action of reducing substances.

Procedure: Take 2 ml of the specimen containing oxy Hb in a test tube and add a pinch of sodium dithionite. Note the color change and examine spectroscopically.

Observation: Orange red color changes into purple.

Clinical Application: In the diagnosis of ***intravascular hemolysis***.

Hb released from the cells is bound to haptoglobin(a glycoprotein) and haptoglobin Hb complex is rapidly removed by reticulo-endothelial system. When the binding capacity of the haptoglobin is exceeded (40-160 mg of Hb/dl), free Hb accumulates in the plasma. Renal threshold of free Hb is 0.15g/dl. When the concentration of free Hb in the plasma exceeds 0.15 g/dl, free Hb starts appearing in urine. In such cases plasma and urine shows the presence of oxy Hb.

2. Deoxyhemoglobin (Reduced Hb): Formed by deoxygenation of Hb.

Color: Purple.

1. ***Spectroscopy:*** A single broad band .The mid point of this band corresponds to 565nm in the green region

2. ***Upon shaking vigorously*** the tube containing reduced Hb solution, it forms oxy Hb.

It is obvious by the change in color to orange red and on spectroscopic examination gives the bands of oxy Hb.

3. Carboxy Hb(Carboxyl Hb): Formed by the action of carbon monoxide on hemoglobin and oxy hemoglobin.CO has high affinity towards Hb than O_2. So it can displace oxygen from oxy Hb to form carbonyl Hb.

Color: Pink.

1. ***Spectroscopy:*** 2 bands seen. α band at 570 nm in the yellow region and β band at 535 nm in the green region (γ band at 418 nm in the violet region). Alpha band is narrow and sharp and beta band is broad and hazy. Generally alpha and beta bands are looked for as the gamma band will not be distinguishable (see Fig. 9A.3). The bands look very much similar to oxy Hb but can be differentiated from it by the following tests.

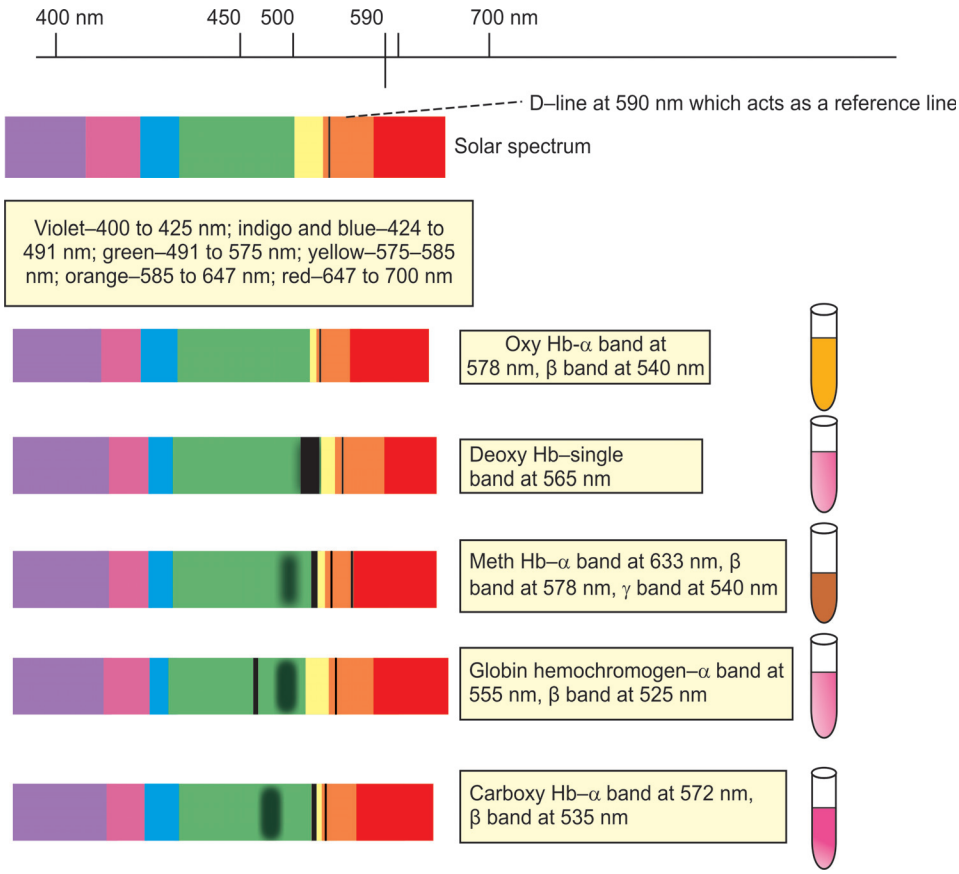


Fig. 9A-3: Identification of Hb derivatives by spectroscopy

2. ***Addition of reducing agents*** like sodium dithionite and ammonium sulfide to carbonyl Hb solution will not produce any effect where as oxy Hb will be converted into deoxy Hb as indicated by the color change (purple color for deoxy Hb, orange red color for oxy Hb). But heat and strong reducing agents can convert carbonyl Hb into hemoglobin.

3. ***Addition of alkalis:*** Mix equal volumes of Hb solution and 25% sodium hydroxide. Oxy Hb turns brown but carboxy Hb remains as such.

Points to Ponder

1. Blood collected from patients suspected of CO poisoning should be taken under oil in order to avoid contact with atmospheric oxygen or by vaccutainers.
2. Carboxy Hb being photolabile, it must be collected in amber colored bottles.

4. Methemoglobin: The iron in meth Hb is in the ferric state and hence it cannot transport and deliver oxygen. Normally only 1% of Hb exist as meth Hb. Meth Hb reductase reduce meth Hb formed in the cells to Hb and there by only low levels of meth Hb is seen in the tissues (maximum of 1% of the total Hb). It's concentration in the body fluids may be raised in congenital meth hemoglobinemia and in dapsone poisoning, known as meth hemoglobinemia. Meth Hb is induced by cerain drugs like phenacetin,

sulfonamides, primaquine. Treatment is by giving reducing agents like methylene blue, vitamin C etc.

If the red cells containing meth Hb undergo hemolysis, meth Hb will be liberated and can be filtered by the glomerulus and reaches urine. In such situations meth Hb may be present in tissues, plasma and urine.

Color: Reddish brown.

1. ***Spectroscopy:*** 3 bands are seen. The characteristic alpha band in the orange region at 630 nm, beta band at 578 nm in the green region and gamma band at 541nm in the green region.

2. ***Addition of reducing agent, the sodium dithionite:*** This reduces meth Hb and oxy Hb to reduced Hb. But carboxy Hb and Sulph Hb are not affected.

3. ***Addition of alkalis:*** Add 2 drops of ammonia solution to 5 ml of meth Hb solution.

This causes conversion of meth Hb to alkaline meth Hb. Alkaline meth Hb do not produce a band at 630 nm.

5. Globin Hemochromogen (Heme + denatured globin): This is an important derivative of Hb. It absorbs light and give visible bands even in high dilutions. So it is useful to test the presence of traces of blood in body fluids in clinical practice and to aid in the detection and confirmation of suspected blood stains in forensic medicine. For this purpose, the sample (1 in 100 dilution) should be treated with 3 drops of 5% NaOH. Then heat the solution. It will give yellow solution due to the formation of alkali hematin (hematin - ferric iron + protoporphyrin; heme - ferrous iron + protoporphyrin). Upon addition of alkali, meth Hb is formed which then decomposes into globin and hematin. Cool and add a pinch of reducing agent like sodium dithionite with gentle shaking in order to obtain pink colored globin hemochromogen. (reducing agent reduces hematin to heme to give heme + denatured globin = globin hemochromogen)

Color: Pink.

1. ***Spectroscopy:*** Two characteristic bands are seen- alpha band at 555 nm in the green region and beta band at 525 nm in the green region itself. Unlike oxy Hb, in the case of globin hemochromogen α band is more intense and the β band is faint.

Clinical application: Fetal Hb is relatively resistant to alkali so it will help to distinguish blood rich in HbF from blood containing predominantly adult hemoglobin (Hb A).

HEMIN CRYSTALS

Hemin – chloride of hematin
Hematin – ferric iron + protoporphyrin

Preparation: Place a drop of blood on a clean glass slide and make a smear on the slide. Place a cover slip over that. Add one drop of Nippe's fluid at one side of the cover slip so that fluid permeates to the undersurface of the cover slip by capillary action. Show the slide over the flame so that the fluid under the cover slip evaporates almost completely. Look under low power and high power of microscope. The hemin crystals will be seen as brown colored rhombic shaped crystals (see Fig. 9A-4).

Mechanism: Nippe's fluid contains KCl, KBr and KI and glacial acetic acid. Upon heating with Nippe's fluid globin is denatured and heme is oxidized to hematin which is then converted into hematin chloride. Hematin chloride is otherwise known as Hemin.

Clinical application: To differentiate between blood stain from other stains.

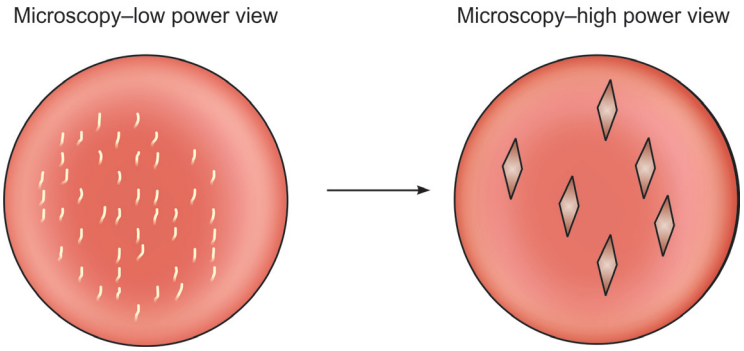


Fig. 9A-4: High power view of hemin crystals

9B. QUESTIONS

1. Name the following:
   a. Hemoglobin derivative containing ferric iron.
   b. Accidental dapsone poisoning causes elevation _____ Hb derivative in blood.
   c. Suffocation due to Carbon monoxide is because of the formation of _____ Hb derivative in blood.
   d. Which is the ionic state of iron in oxy Hb, meth Hb and in globin hemochromogen
   e. What will happen to oxy Hb when it is exposed to reducing substances?
   f. One hemoglobinopathy producing meth hemoglobinemia.
   g. Name two drugs that may cause meth hemoglobinemia.
   h. Dark brown color is imparted to the blood due to the presence _____ Hb derivative in blood.
2. How will you identify the following Hb derivatives
   a. Oxy Hb
   b. Deoxy Hb
   c. Carboxy Hb
   d. Meth Hb
   e. Globin hemochromogen
3. Give short answers:
   a. Relevance of identification of globin hemochromogen in clinical and forensic practice.
   b. What are the precautions to be taken while taking blood samples for carbonyl Hb screening?
   c. What is the mechanism of the formation of meth Hb formation?
   d. What are fraunhofer lines. Which is the fraunhofer line used as a reference line in the identification of Hb derivatives?
   e. How will you prepare hemin crystals from suspected blood spot from the crime site?
   f. Draw the high power view of hemin crystals.
   g. What is the importance of hemin crystals in forensic medicine?

9C. REAGENT PREPARATION

Anticoagulated blood is required for making different Hb derivatives.

1. Oxyhemoglobin: Add 2 ml of blood to 100 ml of distilled water in a conical flask and mix well

till a clear solution is obtained. 3-5 ml of thus obtained oxy Hb solution may be taken in test tubes for spectroscopic examination.

2. Deoxy Hb solution: Prepare oxy Hb solution as detailed above and add in small quantities (a pinch) of sodium dithonite into the conical flask till the orange red color transform into a purple color.

3. Carboxy Hb solution: Prepare by passing CO through oxy Hb solution.

4. Meth Hemoglobin solution: Prepare 1 in 50 dilution of blood and take 100 ml of it in a conical flask. Add a pinch of potassium ferricyanide crystals into the conical flask and stir well so as obtain a black tea colored solution.

5. Globin hemochromogen: Prepare 1 in 100 diution of blood and take 100 ml of it in a conical flask. Add 3-4 ml 5% NaOH solution to it, so as to get a brown solution and heat gently till brown color becomes a yellowish brown due to the formation of alkali hematin. Cool and add a little sodium dithionite with simultaneous gentle shaking to get pinkish globin hemochromogen solution.

Reactions of Milk

10

10A. REACTIONS OF MILK

INTRODUCTION

Milk is secreted by mammary gland during lactation. After expulsion of placenta, levels of oestrogen and progesterone declines abruptly. The sudden drop in oestrogen initiates lactation. Prolactin also is involved in producing milk. Major components of milk are casein, lactalbumin, lactoglobulin, lactose, fat, electrolytes (sodium, potassium, chloride, calcium, magnesium, phosphorus and very little amounts of iron.) and vitamins (vitamin A, D, B_1, B_2, niacin and vitamin C).

REACTIONS OF MILK

Precipitation of Casein from Milk

Take 20 ml of milk in a 100 ml conical flask or beaker. Add an equal volume of water and 2% acetic acid in drops till a maximum precipitate is obtained. Stir well with a glass rod to break up the precipitate. Keep for 5 minutes and filter through a Whatman No: 1 filter paper. Collect the precipitate containing casein and dry between folds of filter paper. Filtrate contains lactose and minerals. Acetic acid is added in the above procedure to lower the pH to the isoelectric pH of casein (4.6).

TESTS WITH THE PRECIPITATE

1. Test for fats: Grease Spot Test: Add a portion of the precipitate to 3 ml of ether and perform grease spot test (see Chapter 3—Reactions of Lipids).

TESTS WITH THE FILTRATE

2. Test for lactalbumin and lactoglobulin-Heat coagulation test:

Procedure: Take 10 ml of filtrate in a test tube. Heat the top 1/3 rd over a flame

Observation: A fine coagulum forms

Principle: Lactalbumin and lactoglobulin present in the filtrate are heat coagulable.

3. Test for lactose: Do Benedict's test and Osazone test with the filtrate.

4. Test for calcium and phosphorus:

Procedure: To 10ml filtrate add 5 drops of concentrated ammmonia and heat to boil. Cool. A gelatinous precipitate forms. Filter and discard the filtrate. Add 5 ml warm 16% acetic acid through the sides of the filter paper to dissolve the precipitate. Divide the solution into 2 portions

in two test tubes. To one add 2 ml of 2% potassium oxalate. To the second tube add 1ml concentrated HNO_3 and 3 ml ammonium molybdate reagent. Warm the solution.

Observation: A white precipitate forms in the first tube and a canary yellow colour in the second tube.

Principle: White precipitate due to calcium oxalate formation and canary yellow color due to ammonium phosphomolybdate.

10B. QUESTIONS

1. Name the hormones and mention their role in the secretion of milk.
2. Which are the proteins and carbohydrates of the milk?
3. Name the minerals present in the milk.
4. Name the trace element which is present scarcely in the milk.
5. Mention 4 vitamins present in the milk.
6. How will you precipitate out casein from milk?
7. How will you demonstrate lactalbumin and lactoglobulin in the milk?
8. How can you show that lactose is present in the milk?
9. Describe the experiment to test the presence of calcium and phosphorous in the milk.

10C. REAGENT PREPARATION

1. Concentrated Ammonia: Dispense from the 15 N liquor ammonia bottle.

2. 2% Potassium Oxalate: Weigh 2 g Potassium oxalate add to a small volume of distilled water taken in a 100 ml volumetric flask, shake well and make up to 100 ml.

SECTION TWO

Quantitative Analysis

Principles of Colorimetry

11

11A. INTRODUCTION TO QUANTITATIVE ANALYSIS

In many diseases blood levels or urinary excretion values of certain constituents may be varied from normal as a manifestation of the diseased condition. Such changes if measured quantitatively can be used in diagnostic and prognostic purposes. By normal value, it is meant the amount of a particular constituent present in the body fluids of clinically healthy individuals. The normal value is affected by a number of factors such as age, sex, type of the individual, genetic make up etc. Hence we usually use the term **normal ranges or reference ranges.** These investigations or biochemical assays which help to reach at a clinical diagnosis constitute what is meant by **clinical chemistry.**

Usually the samples used for routine analysis in a disease are blood, urine, CSF, pleural and peritoneal fluid. Careful attention is needed in all stages of investigation from the collection of samples to the estimation of constituents to avoid false results. Interpretation of results requires information regarding complaints, management and the conditions under which samples are collected and the method of assay.

COLLECTION OF BLOOD SPECIMEN

Capillary or venous blood is taken for almost all the determinations made on blood.

Whole blood is used for determination of pH, analysis of blood gases and determination of concentration of Hb or its derivatives. But majority of the clinical investigations are carried out using ***serum or plasma.***

Serum

For separating the serum, blood is collected in clean dry containers and allowed to clot at room temperature. After sometime, the clot retracts and the serum separates as a supernatant layer. This supernatant layer is removed carefully using capillary pipettes into dry bottles. Serum has the advantage that no anticoagulant is necessary but it takes time for the separation of serum. During this period CO_2 is lost and K^+ and phosphates move from cells to serum. Hence for electrolyte estimations, plasma is preferred (immediate separation can be done).

Plasma

To prepare plasma collect the blood in a container with anticoagulant and then centrifuge. The cells

settle down and the plasma separates. The difference between the plasma and the serum is that the serum is devoid of fibrinogen and other clotting factors where as plasma contains fibrinogen.

Anticoagulants usually used are heparin, potassium oxalate and sodium citrate. Specimen for glucose estimation must contain specific antiglycolytic preservatives such as sodium fluoride (inhibits enolase enzyme there by inhibiting glycolysis).

Hemolyzed sample when used for estimation will give wrong values. Therefore during collection of sample, care should be taken to prevent hemolysis. It is better to carry out the investigations immediately after collecting the sample especially in the case of enzyme assays. If the analysis cannot be carried out immediately, serum has to be preserved in cold. At 4°C it is stable up to a week. But if it is kept frozen the constituents are stable for a longer period.

Sera for bilirubin and calcium estimations need special precautions. Bilirubin is rapidly destroyed by light (photolabile) and hence should be protected from light by keeping in amber colored bottles. Serum for calcium estimations should be kept in glass containers and preserved in refrigerators.

COLLECTION OF URINE SAMPLE

Single specimen of urine is used for qualitative tests. But for quantitative tests 24 hour urine samples are required. 24 hour urine samples are collected in bottles containing suitable preservatives. eg: concentrated HCl, thymol, toluene etc. Refrigeration is the best method of urine preservation.

COLORIMETRY

Colorimetry means measurement based on the intensity of the color. Clinical chemistry estimations are usually carried out by colorimetric procedures. Many substances of biological and medical interests are colored. Colorless constituents can be converted to colored derivatives or colored complexes by subjecting them to undergo specific reactions. If a colored substance present in a solution absorbs light in the visible region of the spectrum, the amount of light absorbed depends on the intensity of the color which in turn influenced by the concentration of the substance in solution.

Quantitative estimation of substance by measurement of the intensity of their colored solution is known as colorimetric analysis or colorimetry. In this procedure the color intensity of the solution is compared with that of a standard solution. For this purpose, colorimeters, the instruments that match the colors of unknown and standard solutions are used.

These are of two types.

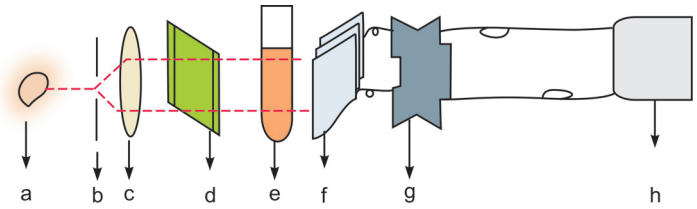
1. Visual colorimetry
2. Photoelectric colorimeters

In visual colorimeters, the colored solution of an unknown concentration is compared with a standard solution of identical color.

PHOTOELECTRIC COLORIMETRY

It is the widely used method for determining the concentration of biochemical compounds. This utilizes the property that when white light passes through a colored solution, some wavelengths are absorbed more than the others. Many compounds are not colored themselves but can be made to absorb light in the visible region by reaction with suitable reagents. These reactions are specific and sensitive enough to measure compounds present in low concentration like millimoles per liter.

- The depth of the color is proportional to the concentration of the compound being measured.



a. Light source
b. Slit
c. Condenser lens
d. Monochromator (gelatin filter)
e. Cuvette holding the colored solution
f. Photocell
g. Amplifier
h. Galvanometer or digital read out

Fig. 11A-1: Diagrammatic representation of photoelectric colorimeter

- Amount of light absorbed is directly proportional to the intensity of the color and hence concentration of the substance. Figure 11A-1 gives diagrammatic representation of photoelectric colorimeter.

Beer-Lambert's law is a combined law of light absorption practiced in carrying out photometric assays.

When a ray of monochromatic light of initial intensity 'I_0' passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light **'I'** is less than **'I_0'.** There is some loss of intensity of light due to scattering by particles in solution and reflection at the interfaces but mainly by absorption by the solution.

The relationship between the I and I_0 depends on:

- the path length of the absorbing medium **'l'**
- the concentration of the absorbing solution **'C'**

These two factors are related in the laws of Lambert and Beer (See Fig. 11A-2).

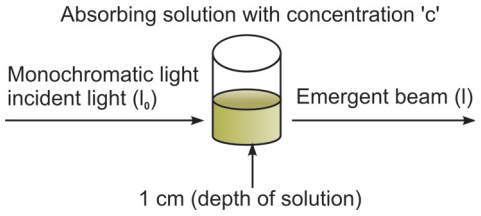


Fig. 11A-2: Incident and emergent light

Lambert's law: when monochromatic light passes through a solution, the intensity of light transmitted decreases exponentially with increasing path length.

$$I = I_0 e^{-K_1 l}$$

Beer's law: when a ray of monochromatic light passes through a solution, the intensity of light transmitted decreases exponentially as the concentration of the medium increases

$$\mathbf{I = I_0 e^{-K_2 c}}$$

These two laws are combined together in Beer-Lambert's law.

$$I = I_0 e^{-K_3 c l}$$

Transmittance

The ratio of intensities is known as the transmittance (T) and this is usually expressed as a percentage.

Percent transmittance = $\mathbf{I/I_0 \times 100 = e^{-K_3 c l}}$

This is not very convenient which is evident from the negative exponential curve obtained by plotting percent transmittance against concentration (Fig. 11A-3A).

Extinction

If logarithms are taken instead of a ratio, then

$$\log_e I_0/I = K_3 c l$$

$$\log_{10} I_0/I = K_3 c l/2.303$$

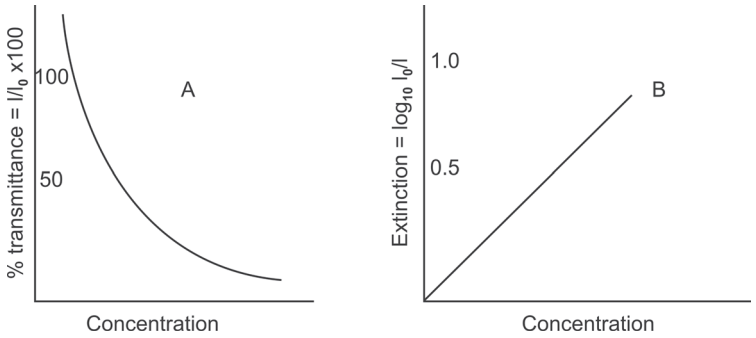


Fig. 11A-3: Percent transmittance vs concentration and absorbance vs concentration

The expression $\log_{10} I_0/I$ is known as extinction (E) or absorbance (A). The extinction is sometimes referred to as the optical density. But this term is not recommended.

Therefore, E = Kcl.

If Beer-Lambert's law is obeyed and 'l' is kept constant then a plot of extinction against concentration gives a straight line passing through the origin (Fig. 11A-3B) which is convenient than the curve for transmittance.

Some colorimeters and spectrophotometers have two scales—a linear one of percent transmittance and a logarithmic one of extinction (Fig. 11A-4).

The extinction scale is linearly related to concentration and is used in the construction of standard curve. With the aid of such a standard curve the concentration of a unknown solution can easily be determined from its extinction.

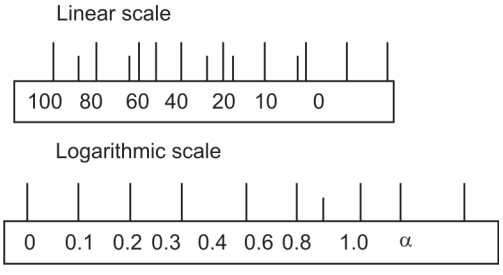


Fig. 11A-4: Linear and logarithmic scale

Molar Extinction Coefficient

$$E = k\,cl$$

If $l = 1$ cm; $c = 1$ mol/L

$E = K$ (the molar extinction coefficient)

The molar extinction coefficient is characteristic for a compound. Molar extinction coefficient 'K' is thus the extinction given by 1 mol/L in a light path of 1 cm and is written as E 1mol/1 cm and has a dimension of 1 liter mol^{-1} cm^{-1}.

Specific Extinction Coefficient

Molecular weights of some compounds such as proteins and nucleic acids in a mixture are not available and this case specific extinction coefficient is used. This is the extinction of 10 g/l of the compound in the light path of 1 cm (E 10 g per liter/1 cm)

Limitations of Beer-Lambert's Law

Sometimes a nonlinear plot is obtained of extinction against concentration and it is due to one of the following conditions not being fulfilled

- light must be of narrow wavelength range and preferably monochromatic

- wavelength of the light used should be at the aborption maximum of the solution which gives greatest sensitivity.
- there must be no ionization, association, dissociation or solvation of the solute with concentration or time
- the solution is too concentrated giving an intense color. The law only holds up to a threshold maximum concentration for a substance.

PHOTOELECTRIC COLORIMETER (SEE FIG. 11A-2)

1. **Light source:** Usually a tungsten lamp to provide light over a range 200-700 nm.
2. **Absorption filters:** These absorb selective wavelengths. Gelatin filters offer a range of alternate wavelengths.
   Examples:
   Blue filter – provides a light ray around 425 nm
   Green filter – provides a light ray around 525 nm
   Red filter – provides a light ray around 690 nm
3. **Sample holder or cuvette:** These are glass test tubes made of scratch proof glass. It should be dirt free and of uniform diameter.
4. **Photocell:** This is a device which convert light energy to electrical impulse. Light passing through the cuvette containing the solution falls on the surface of a sensitive photocell and this photocell convert light energy to electrical impulse. This electrical impulse is proportional to the emergent light.
5. **Galvanometer:** The photocell is connected to a galvanometer whose scale is graduated in % transmittance or as optical density (OD) units.

Measurement in a Photoelectric Colorimeter

In photoelectric colorimeter, absorbance of a substance is found out by measuring the percentage of incident light that is transmitted by the solution.

% Transmittance = Intensity of emergent light/Intensity of incident light × 100

A more satisfying way of expressing % transmittance is by optical density.

$$\mathbf{OD = -\log T}$$

The emergent light rays are passed through a photocell which will convert the light energy into electrical impulses. The current thus generated is measured by galvanometer.

Beer's law states that optical density is directly proportional to the concentration. Thus if a graph is plotted with concentration on X axis and optical density on Y axis a straight line will be obtained and the concentration could be directly read.

When Beer's law is obeyed,

$$\frac{\text{Concentration of unknown}}{\text{Concentration of standard}} = \frac{\text{OD of unknown}}{\text{OD of standard}}$$

∴ Concentration of unknown = OD of unknown/OD of standard × concn of standard

This is the **principle** of all colorimetric reactions. To measure the concentration of a substance in test solution 3 solutions have to be prepared.

1. Test solution which is to be analyzed
2. Standard solution prepared from known quantity of the substance to be estimated
3. Reagent blank containing all the reagents but without the substance to be estimated.

Reagent blank compensates for non-specific color produced by color of reagents or impurities present in the reagent. In practice the instrument is first set at zero reading by using reagent blank.

Precautions: It is important that the solution should not be cloudy or turbid and should not contain any bubbles since all these will affect percent transmittance.

Selection of filter: It depends on the color of the solution to be tested. Filter with complement color is used.

Examples:

Color of the solution	Color of filter
Purple	Green (505-555)
Bluish green	Red (650-700)
Yellow	Blue (430-475)

11B. QUESTIONS

1. What is colorimetry ?
2. Mention two types of colorimetry.
3. What do you mean by photoelectric colorimetry?
4. Give the principle of photoelectric colorimetry.
5. State Beer-Lambert's law.
6. What do you mean by extinction coefficient (absorbance) of a substance?
7. Mention the parts of a photoelectric colorimeter.
8. What is meant by monochromatic light?
9. Why extinction is preferred than transmittance in the photoelectric colorimetric techniques employed for estimating the concentration of various substances?
10. What are the limitations of Beer-Lambert's law?

Determination of Blood Sugar

12

12A. DETERMINATION OF GLUCOSE CONCENTRATION

Blood glucose determination is commonly done in the Clinical chemistry laboratories at the outset of increasing incidence of Diabetes mellitus. It is used to diagnose hyperglycemic conditions like diabetes mellitus and hypoglycemic situations.

SPECIMEN

Whole blood, plasma or serum. The container should contain potassium oxalate-sodium fluoride mixture (3 parts potassium oxalate and one part sodium fluoride-add 3 mg/ml blood). Potassium oxalate prevents clotting by precipitating calcium and sodium fluoride (prevent glycolysis within the cells by inhibiting enolase enzyme of glycolytic pathway) for collecting whole blood and plasma. If serum is to be used, it should be separated immediately after clotting to avoid getting falsely low values of glucose concentration.

METHODS

1. Folin-Wu method
2. Ortho toluidine method
3. Glucose oxidase method

The first two methods are chemical methods and the third one is enzymatic.

1. Folin-Wu Method

Principle: A protein free filtrate is heated with alkaline cupric tartartate solution. Glucose present in the specimen reduces cupric ions to cuprous ions which precipitate as insoluble cuprous oxide. The amount of cuprous oxide formed is measured by the reduction of phosphomolybdate to **molybdenum blue.**

Reagents Required

1. **King's isotonic diluent:** used instead of water to minimize the interference of non glucose reducing substances present inside the red cells.
2. **Sodium tungstate (10 g/dL)**
3. **Schaffer–Hartman alkaline copper tararate**
4. **Phosphomolybdic acid**
5. **Glucose standard solution (100 mg%)**

Procedure

1. **Deproteination of test sample:** Take 0.2 ml of whole blood or serum or plasma into 3.5 ml of King's isotonic diluent using a pipette. Then pipette 0.3 ml of sodium tungstate (10 g/L). Mix well and centrifuge (thus original blood is diluted 20 times.) Transfer

2 ml of supernatant protein free filtrate into Folin – wu tube.
Mark this tube as 'T' for test sample.

2. **Setting up blank (B) and standard (S) tubes**
   Blank (B) tube: Pipette 2.0 ml distilled water into'B' tube
   Standard (S) tube: Pipette 2.0 ml working standard into 'B' tube
3. To all the three tubes (B, S and T) add 2 ml alkaline copper tartarate reagent solution.
   Mix well. Keep the tubes in a boiling water bath for 10 minutes. Then cool the tubes quickly by keeping in water without shaking. Add 2.0 ml of phosphomolybdic acid to each tube. Then the precipitated cuprous oxide dissolves and dilute it to the mark 12.5 ml on the Folin–Wu tube with distilled water. Mix thoroughly by inverting the tubes.

Reading

- Select blue filter for colorimeter or 420 nm wave length in a spectrophotometer.
- Adjust the reading (absorbance or optical density) to zero using the Blank solution
- Take the absorbance reading of standard (100 mg%)
- Take the absorbance reading of test

Calculation

Glucose concentration in mg/dl in the test sample =
absorbance of T/absorbance of S × 100

2. Orthotoluidine Method

Specimen: Plasma or serum (whole blood is not suitable)

Principle: Glucose condenses with ortho toluidine in glacial acetic acid at 100°C to form N- glucosylamine which is blue-green in color. Glucose concentration is proportional to the intensity of the color.

Reagents Required

1. **Orthotouidine reagent**
2. **Glucose standard solution (100 mg%)**

Procedure

- Set 3 dry tubes by marking B, S and T on them
- Add 5.0 ml ortho toluidine reagent in each tube.
- Add 0.2 ml distilled water to the tube labeled 'B'.
- Add 0.2 ml standard to the tube labeled 'S'.
- Add 0.2 ml plasma or serum to the tube labeled 'B'.
- Place all the tubes in a boiling water bath for exactly 12 minutes.
- Then cool the tubes for 5 minutes in cold water

Reading

- Select orange filter/red filter for colorimeter or 630 nm/600 nm wave length in a spectrophotometer.
- Adjust the reading (absorbance or optical density) to be zero using the Blank solution
- Take the absorbance reading of standard (100 mg%)
- Take the absorbance reading of test (the final color is stable for 1 hour)

Calculation

Glucose concentration in mg/dl in the test sample =
absorbance of T/absorbance of S × 100

3. Glucose Oxidase (GOD/POD) Method

Specimen: Plasma

Principle: Glucose is converted to gluconic acid and hydrogen peroxide by the enzyme glucose oxidase. Hydrogen peroxide then split to form water and nascent oxygen. The nascent oxygen then combine with a chromogen (e.g.

4-aminophenazone + phenol) forms a pink color. Glucose oxidase enzyme specifically act on glucose. So this method gives true value of glucose levels.

Reagents

1. Reagent kits based on GOD/POD method is commercially available. Use any one of them and follow the instructions given in the leaflet.
2. Glucose standard (100 mg %)

Procedure

- Label B, S and T on three separate tubes for blank, standard and test respectively.
- Add 2.0 ml distilled water into the 'B' tube
- Add 0.2 ml standard and 1.8 ml distilled water in 'S' tube. Mix well
- Add 0.2 ml plasma and 1.8 ml distilled water in 'T' tube. Mix well
- Set another 3 sets of tubes marked B, S and T. and add 5ml of coloring reagent each into these tubes
- Pipette 0.2 ml each from (the former set) B, S and T tube into the corresponding tubes containing coloring reagent.
- Incubate all the tubes at 37°C for 15 or 25 minutes at room temperature.

Reading

- Choose green filter (in colorimeter) or select 515 nm(in spectrophotometer)
- Zero the colorimeter with blank and take the absorbance readings of T and S

Calculation

Plasma glucose (mg/dL) =
absorbance of T/absorbance of S × 100

Points to Ponder

- Whole blood is not a preferred specimen for the blood glucose determination.
  Glucose being water soluble, it get associated with water. Water content in plasma is 93% and in RBC 73%. Hence whole blood if used, the glucose values obtained vary with packed cell volume of the blood. Other merit of using plasma is that since it contains no cells , the problem of consumption of glucose by the cells do not arise.
- For GOD/POD method, blood must be anti-coagulated with EDTA and separation of plasma must be done within 30 minutes. Sodium flouride, the antiglycolytic chemical is not preferred since it will inhibit the enzyme constituent of the reagent kit. So in order to minimize the effect of glycolysis, the anticoagulated blood must be centrifuged within 30 minutes to separate the plasma.

Interpretation: Blood glucose level is determined by several factors – absorption, storage and utilization of glucose. The level of glucose in the blood varies with time of food intake. But irrespective of the amount and type of food taken its level in the blood is kept within a range in normal people. When food is taken it's level increases and excess glucose converted to glycogen there by blood glucose is kept within normal limits. Between meals and during exercise glycogen is broken down to raise the blood levels towards normal as the blood glucose is utilized by the tissues.

So the normal values are expressed in the fasting (≤ 126 mg%) and 2 hr post prandial states (≤ 200 mg%) .

12B. GLUCOSE TOLERANCE TEST

The oral glucose tolerance test evaluates glucose clearance from the blood circulation after oral glucose loading under standard conditions. The Committee on statistics of the American Diabetes Association (ADA) has standardized the test.

Standard Conditions

1. A minimum carbohydrate intake of 150 g/day for 3 days should be taken before the test. (Otherwise carbohydrate intolerance will be lowered giving false result for the test)
2. The subject should be on 8-16 hour fast before testing.
3. The person should be on routine activities and not bedridden (because inactivity decreases glucose tolerance).
4. The person should be peaceful without any emotional stress.
5. Should avoid exercise
6. Should be free of illness since illness will reduce glucose tolerance. Abnormalities involving thyroxin, growth hormone, cortisol and catecholamines will interfere the test.
7. Drugs like oral contraceptives, hypoglycemic agents (sulfonylureas, insulin),diuretics, salicylates and other agents like tobacco and caffeine will interfere the test. These must be stopped prior to the test.
8. Timing of the test: Best time is between 7 am and noon.
9. Age must be considered during interpretation of the test. Adjustments for age should be done.
10. Glucose load should consists of glucose only and other forms of carbohydrate are not recommended.

During the test: Patient should sit quietly. Should not smoke (smoking elevates blood glucose)

Indications for Oral Glucose Tolerance Test (OGTT)

Not recommended as a routine test. There are specific indications for doing OGTT.

1. Diagnosis of gestational diabetes mellitus
2. Diagnosis of impaired glucose tolerance
3. Diagnosis of renal glucosuria
4. Population studies for collecting epidemiological data.

Plasma glucose levels in OGTT in normal, diabetes mellitus and IGT are given in Table 12B-1.

Procedure

- Collect fasting blood and urine samples
- **Dose:** 75 gm for adults and 1.75 g/kg body wt for children, may be dissolved in 250-300 ml of water and it may be taken within 15 minutes time. Can be flavored with lime or orange juice.
- Collect blood and urine samples at ½ hour intervals after the intake of glucose load for up to 2 hours.
- Test urine for glucose by either Benedict's qualitative test or glucose oxidase based strip test (ideal) and estimate plasma glucose in all samples.

Interpretation

Normal Glucose Tolerance

Urine glucose: Absent in all the samples

Table 12B-1: Plasma glucose levels in OGTT in normal, diabetes mellitus and IGT

	Normal	*Diabetes mellitus*	*Impaired glucose tolerance (IGT)*
Fasting	< 6.1 mmol/L (110 mg%)	>7 mmol/L (126 mg%)	More than 6.1 mmol/L (110 mg%) but less than 7 mmol/L (126 mg%)
1 hour	< 9 mmol/L (160 mg%)	Not set	Not set
2 hour	< 7.8 mmol/L (140 mg%)	>11.1 mmol/L (200 mg%)	>11.1 mmol/L (200 mg%)

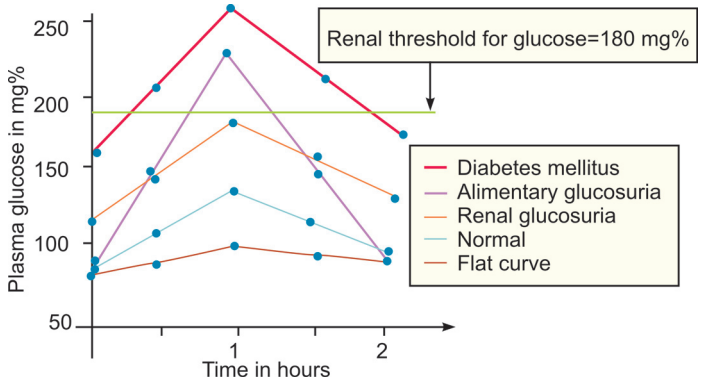


Fig. 12B-1: Oral glucose tolerance test curve

Fasting blood glucose: < 7.0 mmol/l (126 mg%)

2 hour postprandial blood glucose: < 11.1 mmol/l (200 mg%)

Other Conditions causing Abnormal Glucose Tolerance

1. **Increased glucose tolerance (flat GTT curve)** (see Fig. 12B-1): Normally after an glucose load, blood glucose values rise to peak at 60 minutes and then fall to near fasting levels at 2 hours. But in some cases blood glucose show a minimal rise only at 60 minutes. The curve obtained in malabsorption, hypopituitarism, Addison's disease and hypothyroidism

2. **Alimentary glucosuria or lag curve** (see Fig. 12B-1): Exaggerated rise in glucose value after ingestion of glucose load within 1-1½ hours – even cross the renal threshold for glucose and therefore glucose get excreted in urine. But by 2 hours blood glucose level goes down to normal levels or even to hypoglycemic levels. This is due to increased rate of glucose absorption from the gut as a result of conditions leading to rapid emptying of stomach causing increased rate of glucose absorption from the gut, e.g. hyperthyroidism, partial gastrectomy.

3. **Renal glucosuria** (see Fig. 12B-1): It occur in persons with lowered renal threshold for glucose. Normal renal threshold for glucose is 180 mg%. In some individuals it is lowered so that at normal blood glucose levels, glucose get excreted in urine. Most often it is discovered by chance. The condition will not cause any harm to the person. It is frequently encountered in the third trimester of pregnancy.

12C. QUESTIONS

1. What are the methods used to estimate glucose in the blood. Which is the best method among them? Why?
2. Give the normal values of fasting and 2 hr postprandial blood glucose?
3. Name the chemicals to be added to the container in which blood sample is to be collected and sent to the laboratory?
4. What is the principle of Folin wu method of blood sugar estimation?

5. Give the principle of Glucose oxidase method of blood glucose estimation.
6. What is the WHO criteria for the diagnosis of diabetes mellitus?
7. What are the different types of diabetes mellitus?
8. Give the principle of ortho toluidine method of blood glucose estimation.
9. What is Glucose tolerance test. Give 3 indications for doing GTT?
10. What is impaired glucose tolerance test? How will you confirm it?
11. What are the instructions to be given to a patient while going for an OGTT?
12. Give briefly the procedure of OGTT.
13. What do you mean by renal glucosuria?
14. What is meant by alimentary glucosuria?
15. When will you get a flat curve on GTT? Mention the conditions causing it.
16. What should be the blood glucose values in order to say that a person is not diabetic?

12D. REAGENT PREPARATION

1. **King's isotonic diluent:** Preparation of Solution A - 7% copper sulfate solution: Dissolve 7 g of copper sulfate in 100 ml of distilled water. Solution B - Sodium sulfate solution: Dissolve 30 g of hydrated sodium sulfate or 13.23 g of anhydrous sodium sulfate in a few ml of distilled water in a measuring cylinder and make upto 1000 ml with distilled water. King's isotonic diluent is prepared by mixing 45 ml of 7% copper sulfate solution with 480 ml of sodium sulfate solution. This solution is stable indefinitely if stored at 25-30 °C.

2. **Sodium tungstate (10 g/dL):** Dissolve 10 g of sodium tungstate in about 80 ml of distilled water and then make up to 100 ml distilled water.

3. **Schaffer–Hartman alkaline copper tartarate:**

Solution A: Dissolve 13 g crystalline copper sulfate in water and make up to 1000 ml. It is stable for 1 year at 25-30°C.

Solution B: Dissolve 50 g sodium bicarbonate in 600 ml distilled water in a 1000 ml volumetric flask. Add 40 g sodium carbonate (anhydrous) and shake well to dissolve completely. Dissolve 36.8 g of potassium oxalate by adding small amounts in warm distilled water. Add this to sodium bicarbonate-carbonate mixture. Dissolve 24 g of potassium sodium tartarate in a little amount of distilled water and add this to the mixture in the flask. Then make upto 1000 ml with distilled water and mix well. It is stable for 1 year at 25-30°C.

4. **Phosphomolybdic acid:** Dissolve 20 g sodium hydroxide in 400 ml distilled water in 1 L boiling flask with a long stem. Add 35 g molybdic acid and 5 g sodium tungstate and dissolve. Boil vigorously for 45 minutes to remove ammonia present in the molybdic acid. Add a few glass beads to prevent bumping and spilling during boiling. Cool and transfer to 500 ml volumetric flask. Dilute to 350 ml with distilled water. Add 125 ml ortho phosphoric acid and shake well. Make up to 500ml with distilled water. It is stable for 1 year at 25-30°C.

5. **Stock glucose standard (1 g/dL):** Weigh 1 g dry anhydrous glucose (dextrose) and dissolve it in 80 ml of saturated benzoic acid (saturated benzoic acid is prepared by dissolving 2.5 g in 1000 ml of hot distilled water) in a 100 ml volumetric flask. Then make up to 100 ml with saturated benzoic acid solution. In order to dry the dextrose it should

be kept in an oven at 80°C for about 4 hours. Cool it in a closed vessel before weighing.

6. **Working standard (10 mg/dL):** Prepared by diluting 1.0 ml of stock glucose standard to 100 ml with saturated benzoic acid in a 100 ml volumetric flask. Benzoic acid act as a preservative. Hence it is stable for 3-4 days.

7. **Orthotoluidine reagent:** Dissolve 1.5 g of thiourea in 940 ml of glacial acetic acid. Add 60 ml of orthotoluidine. Mix well and store in amber colored bottle. This reagent should be kept for 24 hours before using. It is stable for 6 months at 20-25°C. Handle this reagent carefully since it is highly corrosive.

8. **Saturated benzoic acid solution:** Dissolve 2.5 g benzoic acid in hot distilled water and make up to 1000 ml in a volumetric flask. This solution is stable indefinitely if stored at 25-30°C.

Determination of Urea

13

13A. DETERMINATION OF UREA CONCENTRATION

Urea is the end product of protein catabolism. Deamination of amino acids release ammonia which is detoxified in the liver to form urea (Fig. 13.1). More than 90% of urea produced is excreted in urine and the rest through gastrointestinal tract and skin. It is filtered freely at the glomeruli and neither actively reabsorbed nor secreted by the tubules. However, 40-70% of the filtered urea re-enters plasma by passively diffusing out of the renal tubule into the interstitium. Urine flow rate also influences this back diffusion. Higher the urine flow lesser the back diffusion. Hence urea clearance underestimate glomerular filtration rate (GFR). Besides, urea level is dependent on diet and hepatic synthesis of urea.

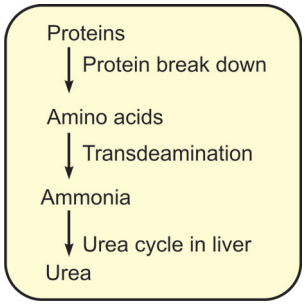


Fig. 13A-1: Formation of urea

If the diet is rich in protein more urea will be formed and excreted.

Specimen: Serum or anticoagulated whole blood or plasma.

Method used: Diacetyl monoxime-Thiosemicarbazide method.

Principle: Urea present in the protein free filtrate (obtained by adding trichloro acetic acid) upon reacting with diacetyl monoxime give a colored condensation product (diazine) in the presence of strong acid medium. The presence of thiosemicarbazide and ferric ions help to intensify and stabilize the reaction. Upon initial hydrolysis diacetyl monoxime release diacetyl and hydroxylamine. Urea reacts with diacetyl. The hydroxylamine is prevented from interfering the reaction by ferric ions. Thiosemicarbazide intensify the color reaction. The initial color of the condensation product diazine is yellow and this color is intensified to red by thiosemicarbazide.

Reagents Required

1. **Trichloroacetic acid (3 g/dl):** used as protein precipitant
2. **Urea standard**
3. **Color reagent**

Procedure

To prepare protein free filtrate: Add **0.2 ml blood, serum or plasma** to **1.8 ml 3% trichloracetic acid** (0.2 ml blood diluted to 2 ml; Dilution = 2/0.2 = 10). Use this protein free filtrate as test solution in the procedure (usually protein free filtrate is provided for the students, in that case this step can be avoided) Take 3 test tubes and label T (test), S (standard) and B (blank) and proceed as given in the table (Table 13A-1).

Table 13A-1: Procedure of urea estimation

	T (ml)	S (ml)	B (ml)
Protein free filtrate	0.2	nil	nil
Std urea solution	nil	0.2	nil
Color reagent	5.2	5.2	5.2

Keep these three tubes in a boiling water bath for 20 minutes. Cool the tubes to room temperature. Take the reading in a photoelectric colorimeter using green filter.

Calculation

Concentration of urea in 0.2 ml blood

$$= \frac{\text{OD of T} - \text{OD of B}}{\text{OD of S} - \text{OD of B}} \times \text{concentration of std urea solution}$$

Concentration of urea in 100 ml blood (mg%)

$$= \frac{\text{OD of T} - \text{OD of B}}{\text{OD of S} - \text{OD of B}} \times \text{concentration of std} \times 100/\text{vol of serum taken}$$

$= T/S \times 30 \times 100/0.2$ mg%

Interpretation

Blood urea concentration in normal individuals is between 15-40 mg%. Near upper limits of normal range are seen with high protein intake.

The concentration of urea in the whole blood is slightly less than that in plasma or serum. Urea being soluble is distributed in intracellular and extracellular water. Since there is less water inside the blood cells, the concentration of urea in the whole blood is lower. Measurement of urea alone is less useful in diagnosing kidney diseases because it's blood level is influenced by dietary proteins and hepatic function.

But its diagnostic value improves with serum creatinine values.

Sometimes urea may be expressed in terms of BUN (blood urea nitrogen) 1 gram mole (molecular weight expressed in grams) of urea ($NH_2 - CO - NH_2$) [i.e. 60 g of urea contains 28 g of nitrogen].

For converting urea expressed in mg% to urea in milli moles/L the conversion factor is 0.357 (to convert 30 mg% into millimoles/L, multiply with 0.357 (0.357 × 30 = 10.1 m mol/l)

Utility in Clinical Medicine

High levels of urea in the blood: It is referred to as uremia : It is seen with disorders of kidney in which the GFR is reduced.

Causes of uremia: See Fig. 13A-2.

Causes of Low Levels of Blood Urea

- Low protein intake
- Conditions leading to hemo dilution
- Severe liver disease causing impaired urea cycle

13B. QUESTIONS

1. What is the method used to estimate urea in the blood? Give its principle.
2. Give the normal values of blood urea.
3. Why do the level of urea in the whole blood lower than that of plasma or serum?

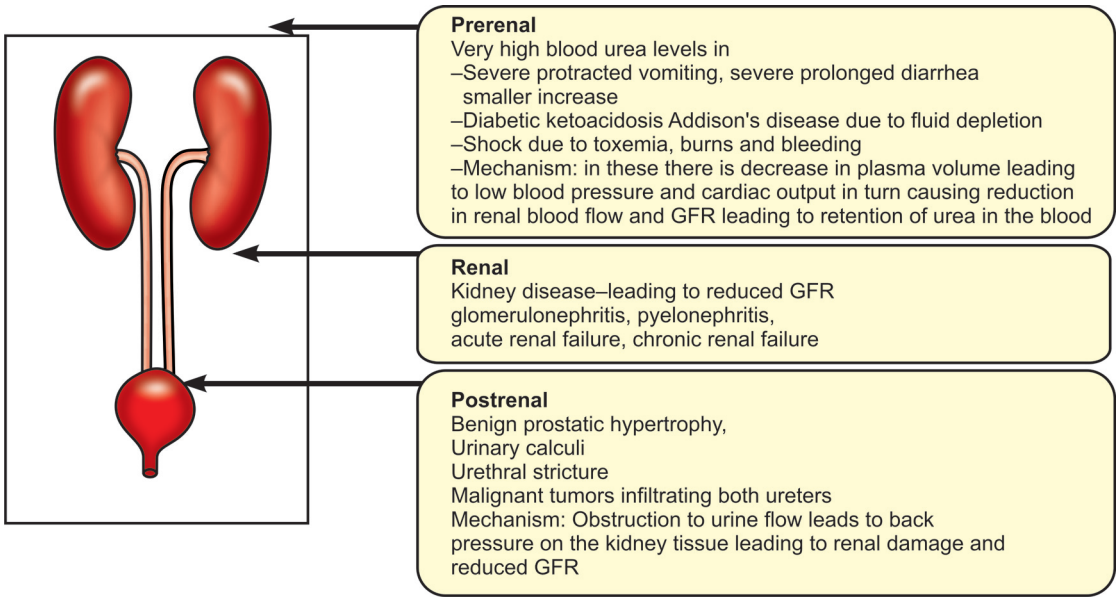


Fig. 13A-1: Causes of uremia

4. What is uremia? What are the different causes of uremia?
5. Is blood urea level a critical diagnostic marker of kidney disease? If not give reason.
6. What are the pre renal causes of uremia? Explain the mechanism of the causation of uremia in these conditions.
7. Mention three renal causes of uremia
8. Mention three post renal causes of uremia. Explain the mechanism.
9. Name the factors affecting urea level in the blood in a normal person.
10. Urea clearance underestimate GFR. Explain.

13C. REAGENT PREPARATION

1. **Trichloroacetic acid (TCA) (10 g/dl):** Weigh 10 g of TCA and dissolve in a few ml of distilled water and make up to 100 ml with distilled water. It is stable up to 1 year.
2. **Orthophosphoric acid (specific gravity 1.750, purity 85-90%):** It is corrosive and should be handled with care.
3. **Ferric chloride (5 g/dl):** Dissolve 5g of anhydrous ferric chloride in 80 ml of distilled water. Add 1 ml of concentrated sulfuric acid make up to 100 ml with distilled water.
4. **Concentrated sulfuric acid (specific gravity 1.840, purity 98%):** It is corrosive and should be handled with care.
5. **Diacetyl monoxime (2.5 g/dl):** Dissolve 2.5 gm diacetyl monoxime in distilled water and make up to 100 ml with distilled water in a volumetric flask. If stored in amber colored bottles at 25-30° C, it is stable up to 6 months
6. **Thiosemicarbazide (0.25 g/dl):** Weigh out 0.25 g of thiosemicarbazide and dissolve in a few ml of distilled water and make up to 100 ml with distilled water in a volumetric flask. If stored at 25-30° C, it is stable up to 6 months.

7. **Benzoic acid solution (2.5 g/L):** Add about 2.5 g of benzoic acid to 1 L of hot distilled water.
8. **Urea standard: Stock standard (1 g/dl):** Weigh accurately 1 g of pure urea (AR) dissolve in a few ml of benzoic acid solution and make up to 100 ml with benzoic acid solution in a standard flask store at 25-30°C.

   Working standard: Dilute 3 ml of stock standard to 100 ml in a standard flask with benzoic acid solution.
9. **Acid reagent :** Take 300 ml of distilled water in a volumetric flask and add with caution 24 ml of concentrated sulfuric acid and 60 ml of orthophosphoric acid and 3 ml of ferric chloride solution (5g/dL). It is stable for 24 hours only.
10. **Color reagent:** Since this reagent is stable for 3 hrs only, make only required volume. Mix 75 ml acid reagent, 50 ml distilled water, 2.5 ml diacetyl monoxime (2.5g/dL) and 0.6 ml of thiosemicarbazide (0.25g/dL).

Determination of Creatinine

14

14A. DETERMINATION OF CREATININE CONCENTRATION

Creatine, methyl guanidoacetic acid is synthesized in the liver and kidney and carried by the blood to muscular tissues and brain and converted to creatine phosphate. Energy needed for muscular contraction is provided by ATP break down to form ADP. The ATP is regenerated from ADP by the action of creatine kinase. This regeneration of ADP by the hydrolysis of creatine phosphate is called Lohmann's reaction (see Fig. 14A-1). During this process creatine phosphate is converted to creatine. Creatine in turn converted by spontaneous dehydration into creatinine. About 2% of the total creatine is converted to creatinine per day so that the rate of creatinine formation is constant in an individual as it is related to muscle mass.

Creatinine is filtered at the glomerulus and reabsorbed by the tubules. So that at low plasma concentrations, as in a normal individual, no creatinine appears in urine. Creatinine is also filtered at the glomerulus. It is reabsorbed at PCT in very small amounts and secreted in the tubules to a minor degree.

Method used: The method used for the determination of serum creatinine is based on the Jaffe' reaction.

Specimen: Serum or plasma. Whole blood should not be used because blood cells contains more substance that interfere with Jaffe' reaction.

Principle: In Jaffe' reaction a yellow-red colored product is formed when creatinine is allowed to react with an alkaline picrate solution. The

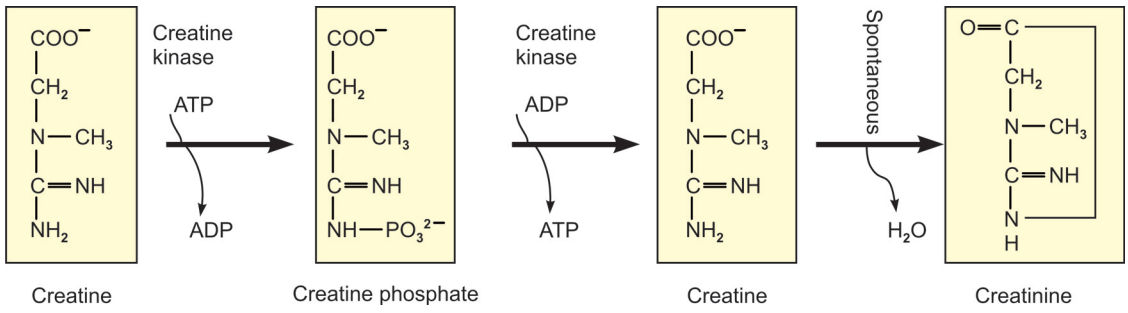


Fig. 14A-1: Formation of creatine phosphate, Lohmann's reaction and creatinine formation in the muscle

absorbance of the complex is measured with green filter in a colorimeter or at 505 nm in a spectrophotometer.

Reagents required: All reagents should be of analytical reagent grade (AR).

1. Sodium hydroxide solution (2.8 g/dL or 0.7 mol/L)
2. Picric acid solution (0.04 mol/L)
3. Acid tungstate reagent
4. Stock creatinine standard (100 mg/dL)
5. Working creatinine standards (1 mg/dL)

Procedure (Fig. 14A-2)

Preparation of protein free supernatant: Add **0.5 ml of plasma or serum** to 4 ml of acid tungstate solution taken in a centrifuge tube. Mix well and centrifuge at 3000 rpm for 10 minutes to get a clear supernatant.

Most often protein free sample will be provided for the students and if so, this step can be omitted

Set tubes T, B and S in the following manner

- Transfer 3 ml of the supernatant into the test tube labeled 'T'
- Add 3.0 ml of distilled water into a test tube labeled 'B'
- Add **0.5 ml of working standard (1 mg/dL)** + 2.5 ml distilled water into test tube labeled 'S'
- Then add 1.0 ml picric acid solution and mix well
- Add 1.0 ml sodium hydroxide (0.7 mol/L) and shake well.
- Keep all the tubes at room temperature for 15 minutes.
- Adjust the reading in the colorimeter to zero with the blank, using green filter in the colorimeter (**520 nm**) and read the absorbance of test and standard against the blank.

Calculation

Concentration of creatinine in 100 ml blood (mg%)
= OD of T/OD of S × con. of std in mg%
× 100/volume of sample
= OD of T/OD of S 0.05 × 100/0.5
= OD of T/OD of S × 10 mg%

INTERPRETATION

Reference range serum – 0.7 to 1.4 mg%.

Determination of creatinine levels in the serum or plasma is unaffected by dietary pattern and state of hydration of the body. More over it is mainly excreted through urine.

The plasma/serum creatinine increases with renal diseases – nephritis, nephrotic syndrome,

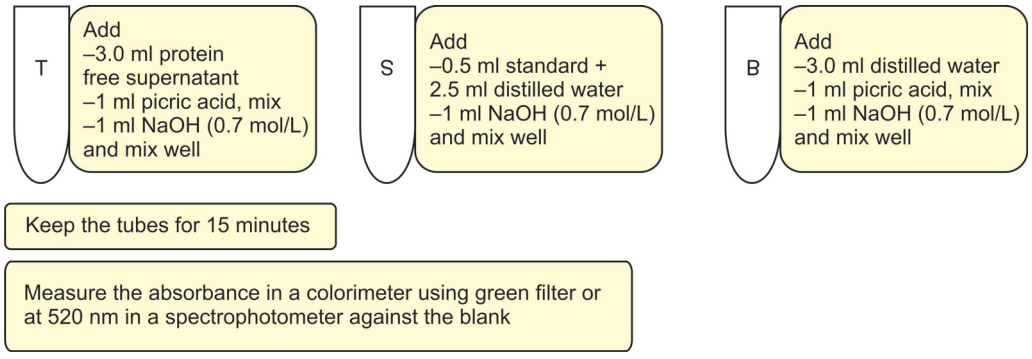


Fig. 14A-2: Procedure of method based on Jaffe reaction—creatinine assay

acute and chronic renal failure and other types of renal insufficiency caused by drugs and toxins.

CREATININE CLEARANCE TEST

It is a test done to assess the renal function. The affection of glomerular filtration membrane is the most important cause of reduced renal function. When glomerulus is involved in disease processes, glomerular filtration will be reduced and consequently excretion of waste products will be lowered. So assessment of glomerular filtration is a good tool to recognize any altered renal function. For this renal clearance of an ideal substance is looked for.

Renal clearance is the volume of plasma from which a substance is cleared completely by the kidneys per unit of time.

Urine is formed by filtration at the glomeruli. Except the cellular portion, proteins and lipids and all other constituents of blood pass to the ultrafiltrate at the level of glomerulus. Subsequent passage through the tubule allows most of the water, glucose, sodium, calcium, phosphorous and chloride to get reabsorbed into the blood stream to maintain normal levels in the blood. Mainly the nitrogenous waste products are excreted in urine.

At the level of kidneys, the possible mechanisms dealt by different types of constituents in the blood are given below.

1. filtration at the glomeruli and reabsorption by the tubules
2. filtration at the glomeruli and excretion by the tubules
3. filtration at the glomeruli only and no change (neither reabsorption nor secretion) at the level of tubules - whatever filtered is passed into the urine.

Creatinine belongs to the 3rd group. It is produced within the body and released into the body fluids. It is filtered at the glomerulus and reabsorbed at PCT in small amounts and secreted in the tubules to urine to a minor degree.

To measure creatinine clearance a **timed urine** and **blood specimens** are required. The volume of urine is measured in terms of ml and creatinine is measured in mg/dl in both urine and serum/plasma specimens.

The creatinine clearance is then calculated by using the formula,

$$\text{Creatinine clearance (ml/min)} = \frac{\text{Creatinine in urine (U) (mg/ml)} \times \text{V (ml/mt)}}{\text{Creatinine in serum (P) (mg/ml)}}$$

Creatinine clearance can also be calculated by using Cockcroft and Gault formula if serum creatinine concentration, age and weight of an individual are known.

Creatinine clearance = (140 – age) × K ÷ 72 × serum creatinine (mg/dl)

K = 0.85 for women and 1.0 for men.

Reference interval for creatinine clearance as per Cockcroft and Gault formula

Men - 94-140 ml/mt/1.73 m^2

Women - 72-110 ml/mt/1.73 m^2

Points to Ponder

Since creatinine is produced as a result of muscle contraction, it's concentration in the body fluids is related to muscle mass. Hence men have higher creatinine levels than women.

URINE CREATININE DETERMINATION

Since the creatinine level in the body fluids is not influenced by the diet, and is excreted at constant rate in an individual, it's measurement in urine is useful to check the reliability of 24 hour urine collections.

14B. QUESTIONS

1. What is the method used to estimate creatinine in the blood? Give its principle.
2. Give the normal value of serum creatinine.
3. What is the reason for lower creatinine concentration in females?
4. What is the source of creatinine in the body?
5. Why creatinine is preferred than blood urea in assessing renal function?
6. What do you mean by creatinine clearance? How will you use it?
7. What is the normal creatinine clearance?
8. Give reference interval of creatinine clearance.
9. What are the different the uses of estimating creatinine in urine?
10. What is Lohmann's reaction?

14C. REAGENT PREPARATION

1. **Sodium hydroxide solution (2.8 g/dL or 0.7 mol/L):** Take 28 g of sodium hydroxide in a measuring cylinder and dissolve in a few ml of distilled water and make up to 1 L with distilled water. Store in a stoppered polyethylene bottle. It is stable up to 1 year at 25-30°C

2. **Picric acid solution (0.04 mol/L):** Dissolve 9.16 g of hydrated picric acid or 8.25 g of anhydrous picric acid in a little of distilled water in a measuring jar and make up to 1 liter. Transfer to an amber colored bottle and it is stable for 1 year at room temperature.

Precaution: Dry picric acid is explosive on percussion. Do not use ground glass stoppers for the containers in which picric acid is kept. Discharging picric acid waste through copper pipes, will cause formation of copper picrate accumulation of which may cause explosion.

3. **Sodium tungstate – 5%:** Dissolve 5 g of sodium tungstate dihydrate (Na_2WO_4) in a few ml of distilled water in a measuring cylinder and make up to 100 ml distilled water.

4. **Stock creatinine standard (100 mg/dL):** Dissolve 100 mg pure anhydrous creatinine in a few ml of hydrochloric acid (0.1 mol/L) in a volumetric flask and make up to 100 ml with the HCl (0.1 mol/L). It is stable for 6 months at 2-8°C.

5. **Working creatinine standard:** Dilute 1 ml of stock standard to 100 ml in a standard flask using HCl (0.1 mol/L). This solution contains 0.01 mg of creatinine/ml (1 mg/100 ml).

Determination of Total Protein and Albumin

15

15A. DETERMINATION OF SERUM TOTAL PROTEIN AND ALBUMIN

Proteins are polymers of L-amino acids. There are numerous proteins in our body concerned with different functions. Here we shall discuss plasma/serum proteins. Plasma and serum are both fluid part of the blood. **Plasma** is the supernatant, obtained upon addition of an anticoagulant to the blood where as **serum** is the supernatant obtained when the plain blood specimen is allowed to clot. Hence serum is devoid of fibrinogen where as plasma contains fibrinogen.

Plasma/serum proteins comprise a complex mixture of different proteins. The important proteins present in the plasma/serum are:

1. Albumin
2. Globulin
3. Conjugated proteins such as lipoproteins
4. Fibrinogen (absent from serum)

Important biological functions of different kinds of proteins are given below:

1. Oncotic pressure - albumin
2. Transport of molecules - albumin (bilirubin), transferrin (iron)
3. Hormone function - TSH
4. Coagulation - fibrinogen
5. Defense - antibodies
6. Nutritional - albumin
7. Catalytic - enzyme proteins

Liver is involved in the synthesis of albumin, fibrinogen, prothrombin , other clotting factors and other several proteins coming under alpha and beta globulins.

Determination of total protein method used: Biuret method.

Specimen: Serum or plasma.

Principle: Proteins form purple coloured complex with cupric ions in alkaline solution.

The biuret test is given by those substances containing two carbamyl groups (CONH) joined either directly or by a single nitrogen or carbon atom. The purplish violet colour is due to the formation of a copper coordination complex (see Fig. 2A-12).

The molecule should have a minimum of two peptide bonds to give copper coordination complex that impart **violet** color to test mixture.

Reagents Required

1. Sodium chloride 0.9% (Normal saline)
2. Biuret reagent
3. Protein standard: 5 g%

Procedure

Set tubes labeled as T, S and B. For further steps see Fig. 15A-1.

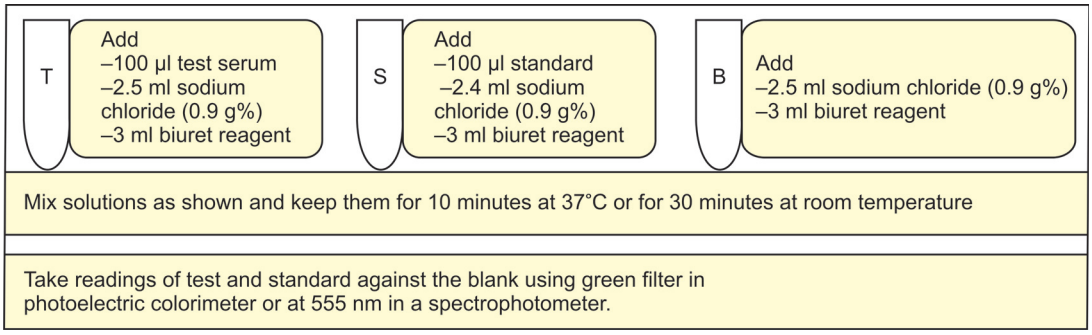


Fig. 15A-1: Procedure of biuret method for total protein estimation

Calculation

Concentration of Total Protein in 100 ml serum (g %)
= T/S × con of std × 100/vol. of serum × 1/1000
= T/S × 5 mg × 100/0.1 × 1/1000
= T/S × 5 g%

Concentration of standard = 5 g% = 5000 mg/100 ml = 500 mg/10 ml = 50 mg/1 ml = 5mg/0.1 ml.

DETERMINATION OF ALBUMIN

Method: Reinhold's method using Biuret reagent.

Specimen: Serum.

Principle: The globulins are precipitated using 28% sodium sulphite solution. This globulin free albumin solution reacts by the same principle given along with total protein determination.

Reagents

1. Precipitating agent
2. Biuret reagent
3. *Standard albumin solution (6 g%):* Commercial lyophilized standard or prepared from powdered human albumin.

Procedure (Fig. 15A-2)

Precipitation of globulins: Pipette 0.2 ml of serum in a centrifuge tube and add 5.8 ml sodium sulphite solution and add 3 ml ether. Shake well and keep for 5 minutes.

Centrifuge so that bottom layer will contain albumin. For the students globulin free albumin solution is usually provided. In that case the above step is not needed. For further steps see Fig. 15A-2.

Calculation

Concentration of Total Protein in 100 ml serum (g%)
= OD of T/OD of S × con. of std × 100/volume of serum taken × 1/1000
= T/S × 6 mg × 100/0.1 × 1/1000
= T/S × 6 × 1000 × 1/1000
= T/S × 6 g%

0.1 ml std contains 6 mg of albumin
6000 mg (6 g) in 100 ml
600 mg in 10 ml
60 mg in 1ml
6 mg in 0.1 ml

INTERPRETATION

Total proteins include albumin, globulin and fibrinogen. Fibrinogen is absent in serum.

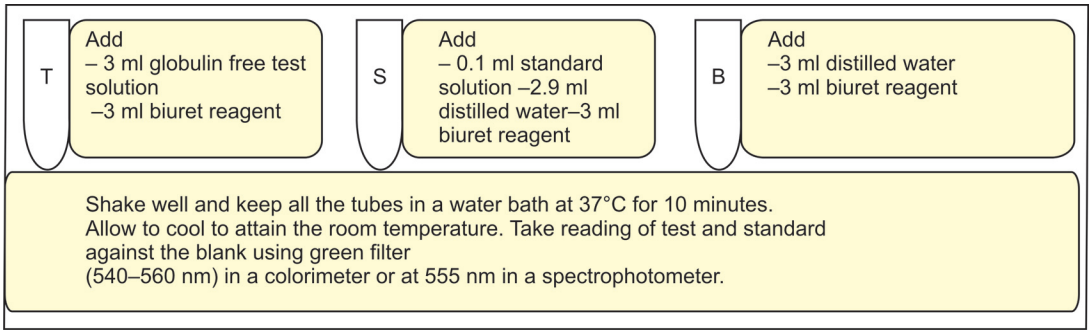


Fig. 15A-2: Procedure of albumin estimation

Reference Range

Total Protein - 6.3 – 7.9 g%
Albumin - 3.7 – 5.3 g%

Globulin (can be calculated from total protein and albumin values)
Globulin = Total protein – Albumin
Albumin Globulin ratio—1.5-2.5 : 1

SERUM TOTAL PROTEINS

Increased

- in dehydration

Decreased

- in over hydration.
- in cases with low albumin accompanied by no increase in globulin

SERUM ALBUMIN

Decreased in

1. Loss of albumin, e.g. Nephritic syndrome, protein loosing enteropathy, burns, severe hemorrhage
2. Malabsorption of protein from the alimentary tract, e.g. malignancies of stomach , intestines and pancreas, enteritis
3. Decreased synthesis in liver diseases, e.g. cirrhosis
4. Increased catabolism of proteins (negative nitrogen balance), e.g. shock due to any cause, febrile illness, untreated diabetes mellitus, hyperthyroidism

GLOBULINS

Increased in

- Advanced liver disease
- Multiple myeloma
- Chronic infections, e.g. rheumatoid arthritis, Tuberculosis,
- Macroglobulinemia

ALBUMIN GLOBULIN RATIO

Albumin Globulin ratio—1.5-2.5 : 1.

Note: A decrease in albumin or a rise in globulin may give low A:G ratio, but total protein remains within normal limits

A:G ratio reversal seen in cases where albumin is low, e.g. chronic liver diseases like cirrhosis or cases in which globulins are produced excessively.

15B. QUESTIONS

1. Name the method used in the estimation of total protein.
2. Name the serum protein fractions.
3. Name the protein fraction absent from serum.
4. Give the reference ranges of serum total protein and albumin.
5. How will you approximately find out the globulin concentration, from serum total protein and albumin values?
6. Name the conditions in which total protein levels are low.
7. Name four conditions in which albumin levels are low.
8. What is A: G ratio? Give its importance.

15C. REAGENT PREPARATION

1. **Sodium chloride 0.9% (Normal saline):** Dissolve 9 g NaCl in a few ml of distilled water in a measuring cylinder or volumetric flask and make upto 1000 ml .This is stable at room temperature for 5-6 months.

2. **Biuret reagent:** Dissolve 4 g NaOH in 400 ml of distilled water. Add 4.5 g of sodium potassium tartarate and mix to dissolve. Then add 1.5 g cupric sulphate pentahydrate ($CuSO_4$ 5 H_2O) followed by 4.5 g potassium iodide. Transfer the solution into a 500 ml volumetric flask or measuring cylinder and make upto 500 ml with distilled water. Keep in a brown bottle at room temperature. It is stable up to 6 months.

3. **Protein standard:**

- Human serum pools are not recommended due to the risks of Hepatitis B and HIV.
- Lyophilized (freeze dried) protein standards are available commercially. But it is costly.
- It can be prepared from less costly dried bovine albumin.

Weigh about 5.3 g (a little excess of wanted quantity) of bovine albumin powder. Dry it overnight in an oven at 60°C. Then from this dried powder, weigh out 5 g and add this into a beaker containing 25-30 ml of normal saline (NaCl 0.9 g%). Stir gently to dissolve it. Then transfer it to a standard flask of 50 ml capacity. Then make up the volume to 50 ml with saline. This gives a protein standard of 10 g% strength. This standard solution is stable for 6 months at 2-8°C.

Working protein standard: Pipette out 5 ml of stock standard into a 10 ml standard flask and make up to 10 ml with normal saline.

Determination of Cholesterol

16

16A. DETERMINATION OF TOTAL CHOLESTEROL

Cholesterol is steroid with a alcoholic group. It is a tetracyclical compound containing cyclopentano perhydro phenanthrene ring (see Fig. 16A-1). It is found in all types of cells.

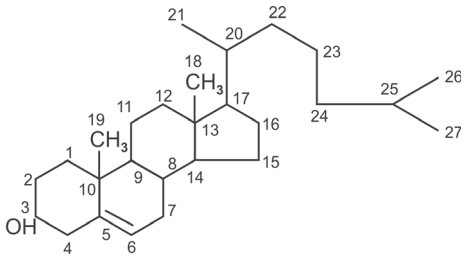


Fig. 16A-1: Structure of cholesterol

Cholesterol in the body is derived from exogenous (diet) and endogenous source. Several physiologically important compounds are derived from it, e.g. vitamin D, bile acids, steroid hormones.

DETERMINATION OF TOTAL CHOLESTEROL

1. Photometric Method

Using reaction with Ferric chloride and Sulphuric acid (Zak's method) (Fig. 16A-2).

Specimen

Serum separated from plain blood collected in a dry bottle.

Principle

Serum is treated with ferric chloride-acetic acid reagent to precipitate the proteins. The protein free filtrate is treated with sulfuric acid and acetic acid. The cholesterol present in the protein free filtrate is oxidized and dehydrated by ferric chloride, acetic acid and sulfuric acid to a **red** colored compound. The intensity of the color is proportional to the cholesterol content in the serum. It is read at 540 nm (green filter).

Reagents Required

1. Ferric chloride acetic acid reagent
2. Concentrated sulfuric acid (analytical grade)
3. Cholesterol standard (working) – 0.04 mg/ml.

Calculation

Concentration of standard in 5 ml std solution
= 5 × 0.04 mg/ml = 0.2 mg/ml

Serum cholesterol in 100 ml serum (mg%)
= Reading of test/Reading of standard × concentration of std × 100/volume of serum taken
= Reading of test/Reading of standard × 0.2 × 100/0.1
= Reading of test/Reading of standard × 200

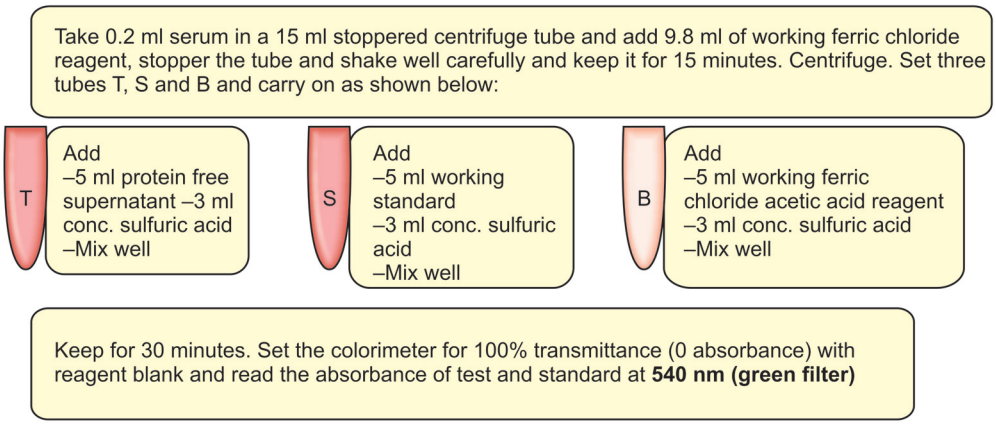


Fig. 16A-2: Procedure for Zak's method of cholesterol estimation

2. Enzymatic Method

Specimen

Serum separated from plain blood collected in a dry bottle.

Principle

Cholesterol esterase is used to free the cholesterol from cholesterol esters. The free cholesterol is oxidized by cholesterol oxidase producing hydrogen peroxide (H_2O_2) which gives a pink color on reacting with phenol and 4 aminoantipyrine.

Cholesterol ester hydrolase

Cholesterol ester + H_2O $\longrightarrow$ Cholesterol + free fatty acid

Cholesterol oxidase

Cholesterol + O_2 $\longrightarrow$ Cholesterol – 4 – en-3-one + H_2O_2

Peroxidase

H_2O_2 + Phenol + 4 aminoantipyrene $\longrightarrow$ Quinoneimine dye + H_2O

Procedure

3-10 ml of serum or plasma added to single reagent containing all enzymes and other ingredients and incubated under controlled conditions as specified in the brochure provided with the commercial reagent kits. The pink color developed is read **at 500 nm.**

Interpretation

Desirable level suggested by National Education Programme (NCEP) of Total cholesterol.

Adults < 200 mg%

Children and adolescents < 170 mg%

Alterations in Total Cholesterol

Hypercholesterolemia: It is very common. Seen commonly in

1. Diabetes mellitus
2. Nephrotic syndrome
3. Obstructive jaundice
4. Hypothyroidism
5. Hypopituitrism – small increase

Hypocholesterolemia: It is uncommon

1. Hyperthyroidism
2. Anemias
3. Hemolytic jaundice
4. Malabsorption syndrome
5. Severe wasting
6. Acute infections
7. Terminal states

16B. QUESTIONS

1. Give the importance of cholesterol in the body.
2. Describe the structure of cholesterol.
3. Name 3 biologically important compounds derived from cholesterol.
4. Name two food items rich in cholesterol.
5. Mention two methods by which serum total cholesterol can be estimated.
6. Give the principle of Zak's method using ferric chloride.
7. Give the principle of enzymatic method of total cholesterol estimation.
8. Give the desirable serum cholesterol level recommended by NCEP in adults, adolescents and children.
9. What are the major causes of hypercholesterolemia?
10. What are the major causes of hypocholesterolemia?

16C. REAGENT PREPARATION

1. **Ferric chloride acetic acid reagent:** Dissolve 0.05 g ferric chloride ($FeCl_3 6H_2O$) in 100 ml analytical grade glacial acetic acid in a graduated cylinder.

2. **Concentrated sulfuric acid (analytical grade)**

3. **Cholesterol standard stock:** Dissolve 100 mg cholesterol in 100 ml glacial acetic acid in a standard flask (100 ml)

4. **Working cholesterol standard:** Dilute the stock standard 1 to 25 with ferric chloride acetic acid reagent (0.04 mg/ml).

Determination of Uric Acid

17

17A. DETERMINATION OF URIC ACID CONCENTRATION

Uric acid is the major end product of catabolism of purine bases—adenine and guanine nucleotides of cellular DNA and RNA (endogenous). It is also formed from dietary nucleic acids (exogenous) (Fig. 17A-1). Uric acid from endogenous source constitutes about 400 mg and from exogenous source it is about 300 g.

Uric acid in the blood is filtered at the glomerulus and fully reabsorbed in the proximal tubule. The uric acid secreted in the distal convoluted tubule which is partly reabsorbed and partly excreted in urine.

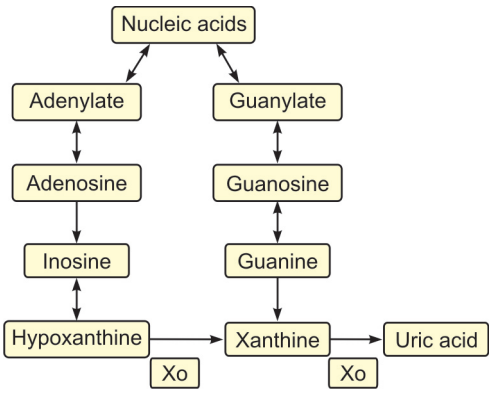


Fig. 17A-1: Formation of uric acid

An understanding of solubility characteristics of uric acid is important to know the uric acid crystallization and stone formation. The first pka (dissociation constant) of uric acid is 5.75 [the second pka is at 9.8 which do not come in the range of any physiological significance]. *Above this pH, uric acid exist as urate ion which is more soluble than the unionized form (uric acid).*

Below the urine pH 5.75, it exist mainly in unionized form which is insoluble and tend to crystallize when the concentration of it in body fluids crosses saturation points.

Method Used

Phosphotungstic acid method.

Specimen

Serum, plasma (collected using the anticoagulant oxalate).

Phosphotungstic acid is preferred as protein precipitant. If tungstate or sulfate anions are used turbidity will be imparted. Usually protein free filtrate would be provided for the students to carry out the estimation experiment.

Principle

Uric acid is oxidized to allantoiin and carbon dioxide by a phosphotungstic acid reagent in alkaline medium and phosphotungstic acid is in

turn reduced to **tungsten blue** in the reaction. The intensity of the color developed is measured at wavelengths of **650–700 nm** in a spectrophotometer or by using red filter in an photoelectric colorimeter. Protein free filtrate is to be used to avoid turbidity and the quenching of the absorbance.

Reagents Required

1. Sodium tungstate 10 g/dL
2. Sulfuric acid (0.33 mol/L)
3. Phosphotungstic acid reagent
4. Standard uric acid solution (stock) (1 mg/ml)
5. Uric acid working standard (5 mg/dL)

Procedure

Preparation of protein free filtrate: Mix 1 ml of serum or plasma with 8.0 ml of distilled water, 0.5 ml of 0.33 molar H_2SO_4 and 0.5 ml of sodium tungstate (10 g%) in a tube and filter (1:10 dilution).

Usually for the students protein free filtrate is supplied in the laboratory so the above step could be skipped.

Set tubes T, S and B for test, standard and blank respectively.

Proceed as shown in the figure 17A-2.

Calculation

Concentration of uric acid in 100 ml blood (mg%)

$$= \frac{\text{OD of T}}{\text{OD of S}} \times \text{con. of std in mg\%} \times \text{dilution factor}$$

$$= \frac{\text{OD of T}}{\text{OD of S}} \times 5 \text{ mg\%} \times 10$$

INTERPRETATION

Reference range serum uric acid is 4.4 – 7.6 mg% (0.26 – 0.45 mmol/L) in males and 2.3 – 6.6 mg% (0.13 – 0.39 mmol/L) in females (conversion factor for converting mg% values to mmol/L , multiply by 0.059). The level of uric acid gradually increases with age in both sexes especially after menopause in women. Men with serum uric acid levels more than 9.0 mg% are more prone for developing gouty arthritis.

Rate of uric acid excretion in individuals with unrestricted purine diet is 250-750 mg per day. This may decrease to 400 mg/day upon a purine free diet. That is the importance of restriction of purine rich foods in cases of hyperuricemia.

Hyperuricemia: It is defined by serum or plasma uric acid levels greater than 7 mg% in men or greater than 6.0 mg% in women.

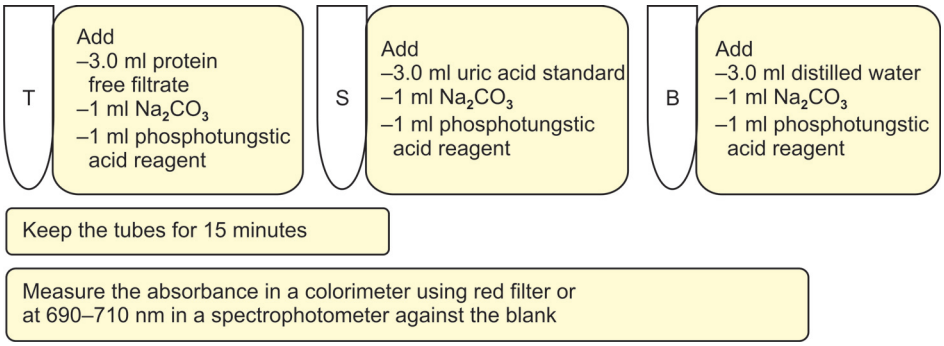


Fig. 17A-2: Procedure of phosphotungstic acid method—Uric acid assay

Causes of Hyperuricemia

Increased formation

Primary causes
- Inherited metabolic disorders, e.g. Lesch-Nyhan syndrome

Secondary causes
- Excess dietary intake
- Increased nucleic acid turn over, e.g. malignancy, psoriasis

Decreased Excretion

- Primary (idiopathic) causes
- Secondary causes
  1. Chronic renal failure
  2. Lactic acidosis
  3. Thiazide diuretics therapy

HYPOURICEMIA

It is defined by serum or plasma uric acid levels less than 2 mg%.

It is rare where as hyperuricemia is common.

Causes

- Severe hepatocellular disease with reduced synthesis of purines
- Defective renal tubular reabsorption of uric acid, e.g. Fanconi's syndrome (congenital)

Acquired renal tubular damage due to toxic agents like radio opaque contrast media, cancer chemotherapy, over treatment with allopurinol.

17B. QUESTIONS

1. Name the method used in the estimation uric acid in the serum. Give it's principle.
2. Give the normal values of serum uric acid in males and females.
3. What are the factors affecting serum uric acid level in a normal person?
4. What is hyperuricemia? What are the different causes of it?
5. Define hypouricemia. Name the conditions in which it is seen.
6. What is the rationale of giving alkalizer in patients with uric acid calculi?
7. What is gout? What do you mean by tophi?
8. Give the reason for getting high uric acid levels in the serum in patients with malignancy.
9. Name some purine rich foods, the intake of which to be restricted in patients with hyperuricemia.
10. Name one drug used to treat hyperuricemia that act at the level of xanthine oxidase. Describe it's mechanism of action.

17C. REAGENT PREPARATION

1. Phosphotungstic Acid Reagent

Weigh 40 g of molybdenum free sodium tungstate AR and dissolve in 250-300 ml distilled water. Slowly add concentrated 88-93% pure ortho phosphoric acid cautiously. Reflux gently for 4 hours. Cool to room temperature. Add 300 ml distilled water. Add 32 g of lithium sulfate monohydrate into this. Mix and make up to 1 L. Store in a refrigerator.

2. Sodium Tungstate (10 g/dL)

Take 10 g of sodium tungstate ($Na_2WO_42H_2O$) AR in a volumetric flask and dissolve in a few ml of distilled water and make upto 100 ml.

3. Sodium Carbonate (14 g/100 ml)

Weigh 70 g of anhydrous sodium carbonate AR and add it to a few ml of water in a beaker and dilute to 500 ml. Transfer it to a polyethylene bottle.

4. Uric Acid Stock Standard (100 mg/dL)

Weigh accurately 100 mg of uric acid AR and 60 mg of lithium carbonate AR (Li_2CO_3) and add them into a volumetric flask. Add a few ml of distilled water and warm gently to dissolve the solids added. Cool and make up to 100 ml with distilled water. It is stable for many months if refrigerated.

5. Working Uric Acid Standard (6 mg/dL)

Pipette 0.5 ml of uric acid stock standard and add it into a 100 ml standard flask and make up to 100 ml using distilled water. It is stable for 2-3 days if refrigerated.

Determination of Bilirubin

18

18A. DETERMINATION OF SERUM BILIRUBIN

Bilirubin is an orange yellow pigment derived from heme. Daily bilirubin production is approximately 250 – 300 mg in humans from all sources. (85% heme released from senescent RBCs in the reticuloendothelial system, 15% from RBC precursors destroyed in the bone marrow and from catabolism of other heme containing proteins such as peroxidases, cytochromes and myoglobin).

Bilirubin is bound to albumin and transported to the liver. Inside the hepatocytes bilirubin is conjugated with glucuronic acid by UDP glucuronyl transferase to produce bilirubin glucuronides which then are excreted in bile into the intestine. In the intestine bilirubin glucuronides are hydrolyzed by β glucuronidase to form unconjugated bilirubin which is then reduced by anaerobic intestinal microorganisms to form colorless urobilinogens which includes urobilinogen, stercobilinogen, mesobilinogen. About 20% of urobilinogens produced are reabsorbed from intestine and enters enterohepatic circulation. 2- 5% of this enters the systemic circulation and appears in urine as urobilinogen (see Fig. 18A-1). Stercobilinogen is excreted in feces.

Biliverdin is formed by mild oxidation of bilirubin. It is formed spontaneously when bilirubin is oxidized by exposure to air in alkaline solution or oxidized with ferric chloride in acetic acid or is treated with H_2O_2. It is dark green in color.

Urobilins are formed by mild oxidation of urobilinogen on exposure to air or to mild oxidizing agents. Urobilins are reddish orange in color. It is formed readily when urine is exposed to light and air and its formation is slow at alkaline pH.

DETERMINATION OF SERUM BILIRUBIN

Method Used

Method of Malloy and Evelyn.

Specimen

Serum

Principle

It is based on the formation of purple azobilirubin when bilirubin reacts with diazo reagent introduced by Van den Bergh hence called as **Van den Bergh reaction**. Van den Bergh reaction consists of two types of reactions - direct and indirect reactions. The water soluble bilirubin glucuronides (conjugated bilirubin) react

immediately with diazo reagent but unconjugated bilirubin reacts very slowly and requires an accelerator like methanol. Methanol releases albumin bound bilirubin and exposes the carboxyl groups to diazo reagent. Total bilirubin value (conjugated + unconjugated bilirubin) given by direct and indirect reaction. The intensity of the color developed is directly proportional to the concentration of the bilirubin in the serum and is read at 540 nm.

Reagents

1. Diazo reagent
2. 1.5% HCl
3. Bilirubin standard: 0.1mg/ml
4. Absolute methanol

Calculation (Fig. 18A-2)

OD of test (direct bilirubin)
= OD of Dt – OD of Db

OD of standard
= OD of S – OD of B

Concentration of standard
= 10 mg/100 ml = 1 mg/10ml
= .1 mg/1ml = 0.01 mg/0.1 ml
= 0.02 mg/0.2 ml

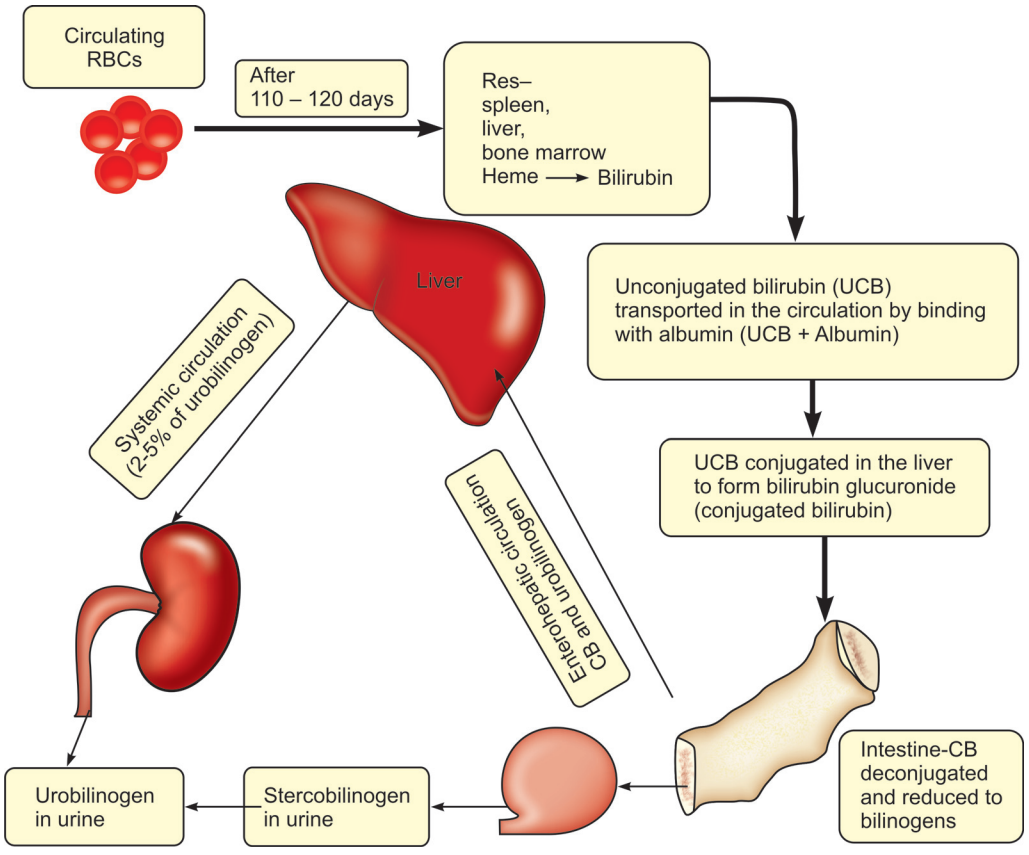


Fig. 18A-1: Formation and fate of bilirubin

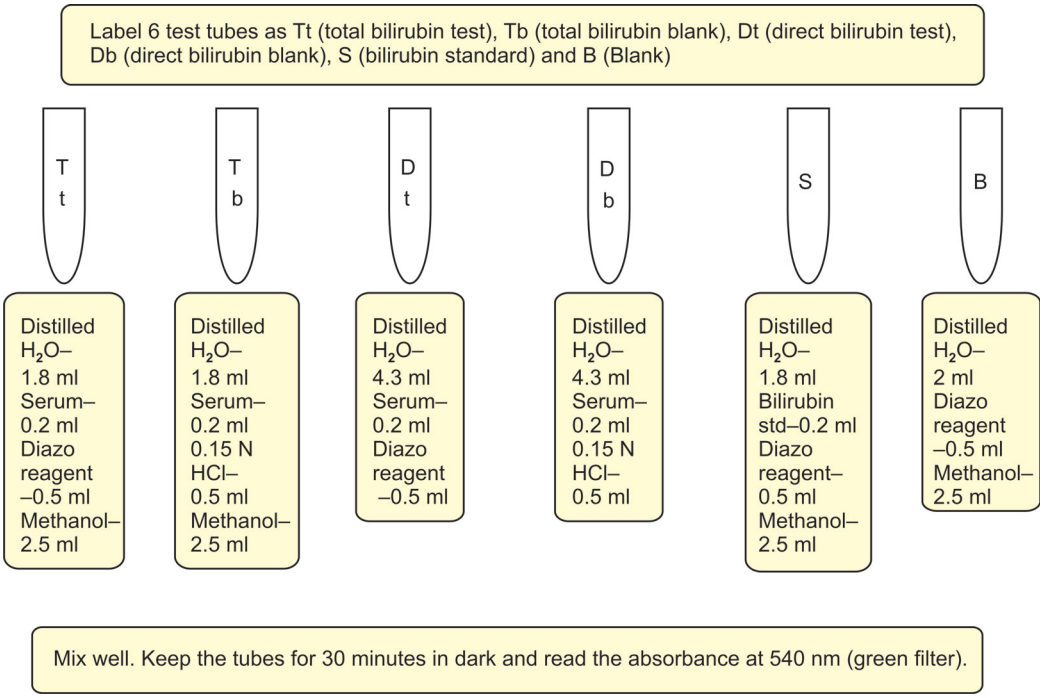


Fig. 18A-2: Procedure of bilirubin estimation

Concentration of direct bilirubin
= OD of test/OD of std × concn of std/ volume of sample × 100 mg %
= OD of test/OD of std × 0.02/ 0.2 × 100 mg %
= OD of test/OD of std × 10 mg %

OD of test (Total bilirubin)
= OD of Tt – OD of Tb

OD of standard
= OD of S – OD of B

Concentration of total bilirubin
= OD of test/OD of std × concn of std/ volume of sample × 100 mg %
= OD of test/OD of std × 0.02/ 0.2 × 100 mg %
= OD of test/OD of std × 10 mg %

INTERPRETATION

Reference Range

Total bilirubin – 0.3-1.2 mg%
Direct bilirubin – 0-0.2 mg%

Defects in bilirubin metabolism results in jaundice. (bilirubin > 2 mg%). It may be due to

i. Excessive production eg: hemolytic jaundice – here erythrocytes undergo hemolysis excessively to produce heme and bilirubin in excess.
ii. Disorders of liver causing defective conjugation or defective secretion into bile eg; different kinds of hepatitis.

iii. Defective secretion due to obstruction of biliary pathways eg: biliary atresia, stones in common bile duct, carcinoma head of pancreas pressing the common bile duct.

18B. QUESTIONS

1. Explain how bilirubin is formed in the body.
2. Describe the excretion of bilirubin from the body.
3. Give the disturbances in which bilirubin levels in body fluids goes high.
4. What is jaundice? Give three major types of jaundice.
5. How will you diagnose jaundice in terms of serum bilirubin?
6. How will you estimate bilirubin? What is the basis of this?
7. What is Van den Bergh reaction?

18C. REAGENT PREPARATION

1. Diazo reagent A: Dissolve 1 g of sulfanilic acid in 15 ml of concentrated hydrochloric acid and make upto 1 liter with water.

2. Diazo reagent B: Dissolve 0.5 g of sodium nitrite in water and make upto 100 ml. Prepare freshly at frequent intervals.

3. Diazo reagent: Prepare freshly before use by adding 0.3 ml solution B to 10 ml diazo reagent solution A.

4. 1.5% HCl (v/v): 1.5 ml concentrated HCl in 100 ml water.

5. Absolute methanol: Dispense from the bottle.

6. Standard solution of bilirubin: Dissolve 10 mg in 100 ml chloroform. For the working standard dilute 1 ml to 100 ml with 95% ethanol to give a working standard of 0.8 mg% concentration. The purity of bilirubin standards varies with suppliers. Hence instead of true bilirubin methyl red standard can be used.

Determination of Transaminases

19

19A. DETERMINATION OF SERUM TRANSAMINASES

Transaminases catalyzes the transfer of amino group from an α-amino acid to an α-oxoacid leading to the formation of a different α-amino acid and a different α-oxoacid. All the primary alpha amino acids except (lysine, threonine, proline) can undergo such trasmination reactions catalyzed by different types of transaminases. Out of **these aspartate aminotransferase (AST) (EC 2.6.1.1**) (Former name Glutamate oxaloacetate transaminase – GOT) and **alanine aminotransferase (ALT) (EC 2.6.1.2)** (Former name Glutamate pyruvate transaminase –GPT) Reactions catalyzed by these enzymes are shown in the Figure 19A-1.

Transaminases are present in most of the tissues in the body. They are the enzymes of cytoplasm. They are present in the plasma of healthy individuals. Enzymes along with other molecules are retained in the cells by metabolically active plasma membrane. Integrity of the plasma membrane depends on the availability of cellular currency - the ATP. When the ATP synthesis is impaired due to deficiency of fuels (oxidizable substrates) or anoxia, cell membrane function (integrity) deteriorates. Molecules leak out of the cells. Cytoplasmic enzymes appear in the blood earlier than membrane or organelle bound enzymes.

Assay of Alanine Aminotransferase

Method Used

Colorimetric method using 2,4 Dinitrophenyl hydrazine.

Principle

Alanine aminotransferase (SGPT) catalyze transamination of L-alanine with α-ketoglutarate to form L-glutamate and pyruvate using pyridoxal phosphate as coenzyme.

To estimate ALT activity serum is treated with alanine and alpha ketoglutarate (substrates). The 2,4 dinitrophenylhydrazine added, will react with the pyruvate formed in the reaction in alkaline medium to form reddish brown hydrazone. The intensity of the color is proportional to the enzyme activity of the serum.

Reagents

1. Phosphate buffer – 0.1M pH 7.4
2. Substrate – L Alanine 0.2 M in 0.1 M phosphate buffer pH 7.4
3. Pyruvate standard (2 mmol/ml)

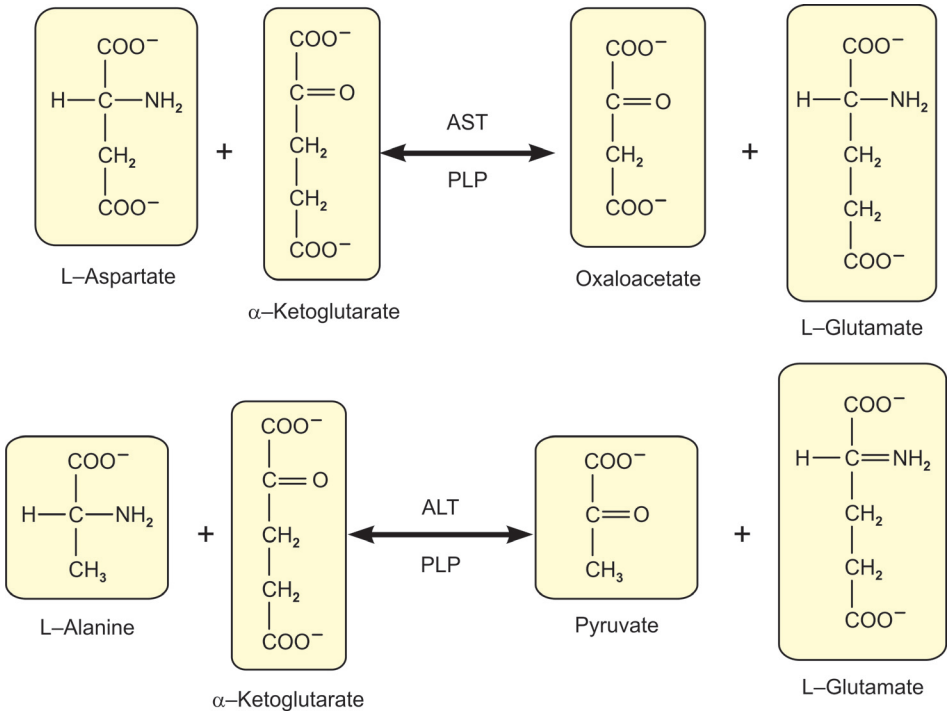


Fig. 19A-1: Reactions catalyzed by AST and ALT

4. 2, 4 DNPH (dinitrophenylhydrazine)–1 mM/L
5. NaOH 0.4 M

Procedure

See Figure 19A-2.

Calculation

ALT activity in the serum

$$= \frac{\text{OD of T} - \text{OD of C}}{\text{OD of S} - \text{OD of B}} \times \text{Concentration of std in mmol} \times 1000/\text{volume of serum} \times 30 \text{ (incubation time)}$$

$$= \frac{\text{OD of T} - \text{OD of C}}{\text{OD of S} - \text{OD of B}} \times 0.4/0.2 \times 1000/30$$

$$= \frac{\text{OD of T} - \text{OD of C}}{\text{OD of S} - \text{OD of B}} \times 66.66 \text{ IU/L}$$

Assay of Aspartate Aminotransferase

Method Used

Colorimetric method.

Principle

Aspartate aminotransferase (SGOT) catalyze transamination of L-aspartate with α-ketoglutarate to form L-glutamate and oxalo acetate (OA)using pyridoxal phosphate as coenzyme. To estimate AST activity, serum is treated with aspartae and alpha ketoglutarate (substrates). 2.4 dinitrophenylhydrazine is added will react with the OA formed in the reaction in alkaline medium to form reddish brown hydrazone. The intensity of the color is proportional to the enzyme activity of the serum.

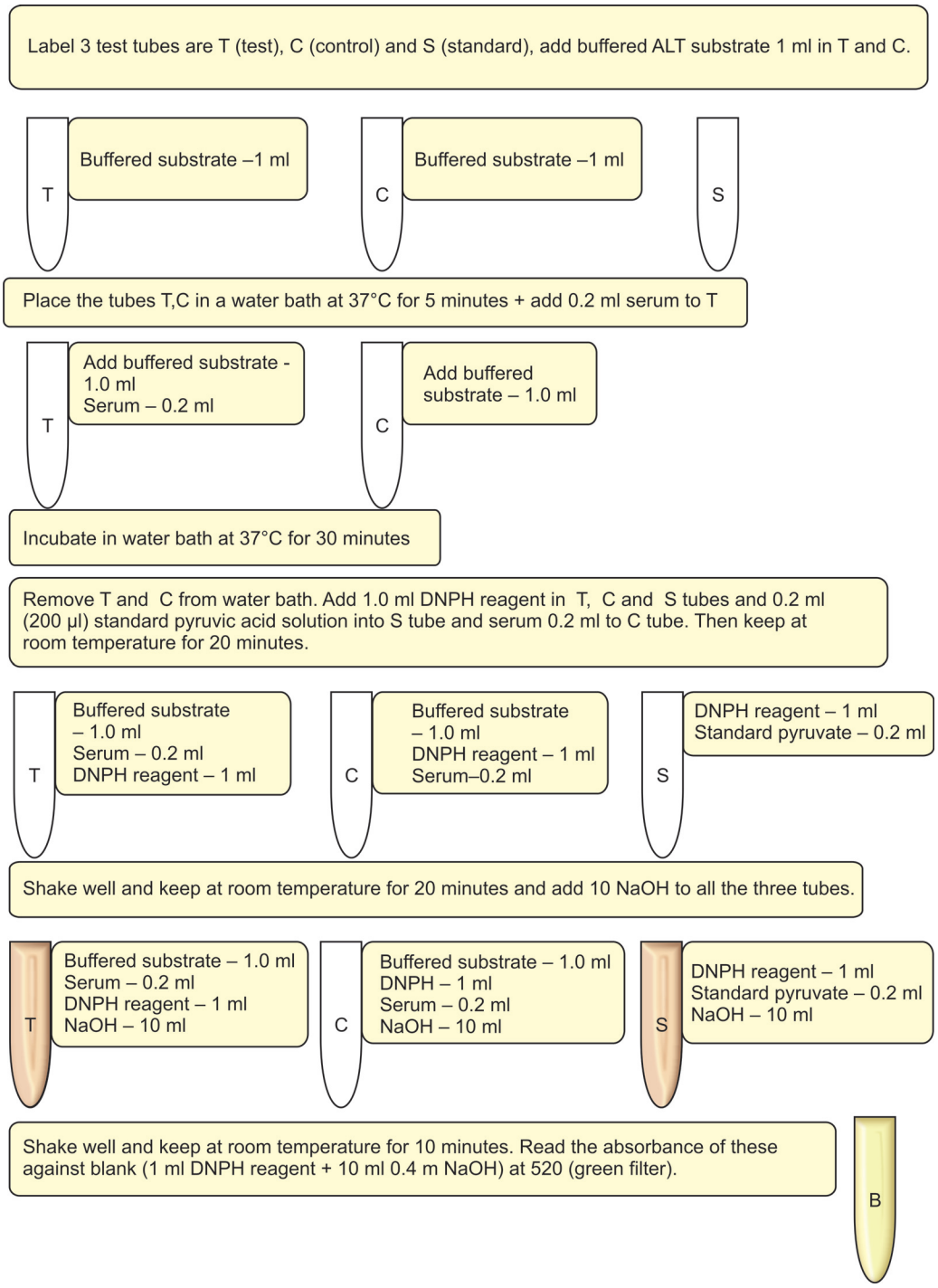


Fig. 19A-2: Procedure of alanine transaminase estimation

Reagents

1. Phosphate buffer substrate – 0.1M pH 7.4
2. Substrate – L Aspartate 0.2 M and 2mM alpha ketoglutarate in 0.1M phosphate buffer pH 7.4
3. Oxaloacetate standard (2 mmol/ml)
4. 2,4 DHPH (dinitrophenylhydrazine)–1 mM/L
5. NaOH 0.4 M

Procedure

See Figure 19A-3.

Points to Ponder

Collect blood in dry containers. Hemolyzed specimen will give falsely high AST and ALT activities (Table 19A-1).

Calculation

AST activity in the serum

$$= \frac{\text{OD of T} - \text{OD of C}}{\text{OD of S} - \text{OD of B}} \times \text{Concentration of std in mmol} \times 1000/\text{volume of serum} \times 60 \text{ (incubation time)}$$

$$= \frac{\text{OD of T} - \text{OD of C}}{\text{OD of S} - \text{OD of B}} \times 0.4/0.2 \times 1000/60$$

$$= \frac{\text{OD of T} - \text{OD of C}}{\text{OD of S} - \text{OD of B}} \times 33.33 \text{ IU/L}$$

INTERPRETATION

Table 19A-1: Reference Interval

Enzyme	*Adult serum level*
AST	10–30 U/L
ALT	10–40 U/L

Highest activity of AST (7800 times the normal serum level) is in the myocardium and next in the liver (7000 times the serum level) and next in skeletal muscle (5000 times). Where as highest activity of ALT (2850 times the normal serum level) is in the liver and next in the kidney (1200 times the serum level) and only 450 times the serum level in the myocardium and 300 times in skeletal muscle. In clinical practice both AST and ALT are assayed for diagnosing liver diseases and AST is used for evaluating ischemic heart disease occasionally.

ALT and AST in Hepatic Disorders

1. Viral hepatitis and other types of liver diseases.

   In hepatocellular diseases except viral hepatitis, transaminases are elevated to produce ALT/AST ratio less than 1. This ratio is known as **De Ritis ratio**. It becomes elevated to unity or greater than 1 in cases of infectious hepatitis and other types of inflammatory diseases of liver. In cirrhosis liver the ratio is elevated slightly depending on the degree of hepatocellular necrosis. In terminal cirrhosis it is less than 1.
2. Primary and secondary carcinoma: 5-10 times the normal activity of ALT and AST is observed.
3. *Toxic hepatitis:* Very high (20 times) activity of both enzymes.

AST in Ischemic Heart Disease

Serum AST activity rise only 6-8 hours after the onset of chest pain and it peaks around 18-24 hours and fall to within the reference range by 4th–5th day in cases where no fresh infarct has been developed.

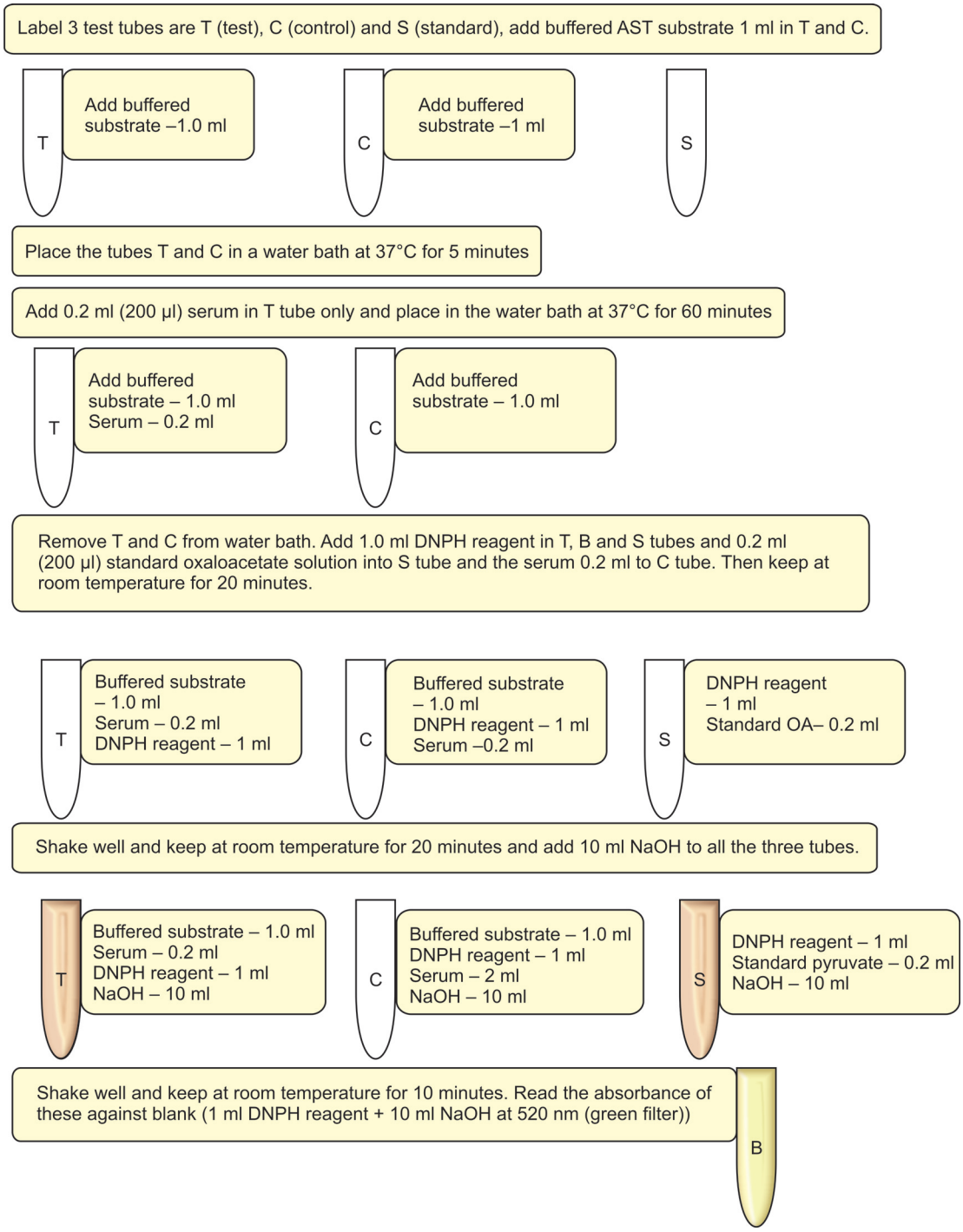


Fig. 19A-3: Procedure of aspartate transaminase estimation

19B. QUESTIONS

1. Describe the catalytical role of transaminase.
2. Name two tissues each in which high activity of AST and ALT are observed.
3. Give the reference range of serum AST and ALT in adults.
4. Give the principle of a method employed in the assay of serum transaminases.
5. What are diagnostic uses of ALT and AST?
6. What is De Ritis ratio? What is the application of using the De Ritis ratio in evaluating different types of hepatic diseases?
7. Name one technique that will help in the separation of different isoenzyme fractions in the serum.
8. What is the pattern of rise of serum AST in myocardial infarction?
9. What is the precaution to be taken during the collection of blood for assay of transaminases?

19C. REAGENT PREPARATION

1. **Phosphate buffer – 0.1M pH 7.4:** Dissolve 14.9 g disodium hydrogen phosphate dihydrate (11.9 g of anhydrous disodium hydrogen phosphate) and 2.2 g of anhydrous potassium dihydrogen phosphate in a few ml of distilled water and in a volumetric flask or cylinder or beaker and make upto 1000 ml with distilled water. Check the pH after adding 900 ml water and if the pH is less than 7.4 add a small amount of disodium hydrogen phosphate. If the pH is more than 7.4 add a pinch of potassium dihydrogen phosphate.

It is stable for 2 months at 2-8°C.

2. **Buffered substrate for ALT (SGPT): L Alanine - 0.2 M in 0.1M phosphate buffer pH 7.4:** Dissolve 1.78 g DL alanine and 30 mg alpha keto glutaric acid in 20 ml of phosphate buffer (pH 7.4) and add 1- 1.25 ml of 10% NaOH to adjust the pH to 7.4 in a beaker. Transfer the contents to a volumetric flask and rinse the container with phosphate buffer and add that also to the volumetric flask and make the volume to 100 ml with phosphate buffer. Add 1 ml chloroform as a preservative. And keep at 2-8°C. It is stable upto 2 weeks.

3. **Pyruvate standard (2 µmol/ml):** Dissolve 220 mg sodium pyruvate in phosphate buffer and make up to 100 ml with phosphate buffer in a volumetric flask. Take 10 ml of this into another volumetric flask and dilute to 100 ml with phosphate buffer to get 2 µmol/ml pyruvate working standard. The working standard must be aliquoted into 5 ml sized containers in the freezer.

4. **2,4 DHPH (dinitrophenylhydrazin) 1 mM/L:** Dissolve 200 mg DNPH in hot 1N HCl in a beaker. Allow to cool and make up to 1 L with 1 N HCl. Store at 2- 8°C in amber colored bottles. Stable up to 6 months.

5. **NaOH 0.4 M/L:** Dissolve 16 g NaOH in a little distilled water in a beaker and make up to 1 L with distilled water. Keep in a stoppered polythene bottle.

6. **HCl – 1 N:** Dilute 90 ml concentrated HCl to 1 L with distilled water in a graduated cylinder.

7. **NaOH 1 M/L:** Dissolve 40 g NaOH in a little amount of distilled water in a beaker and make upto 1 L with distilled water. Keep in a stoppered polythene bottle.

8. **Buffered substrate for AST (SGOT):** Dissolve 2.66 g DL aspartic acid and 30 mg α-ketoglutaric acid in 20.5 ml of 1M NaOH solution in a small beaker. Adjust the pH to 7.4 by adding addition NaOH in drops with stirring. Transfer to a 100 ml volumetric flask, rinsing the beaker and making up to 100 ml with phosphate buffer (pH 7.4, 0.1M). Add 1 ml chloroform as preservative. Stable up to 2 weeks at 2–8°C.

Determination of Alkaline Phosphatase

20

20A. DETERMINATION OF SERUM ALKALINE PHOSPHATASE

Alkaline phosphatase is an enzyme that catalyze hydrolysis of monophosphoric esters to liberate phosphoric acid at alkaline pH (optimum pH 10). Several isoenzymes of ALP are recognized, e.g. those derived from liver, bones, intestine, kidney and placenta. Of this, the isoenzyme derived from the liver constitutes the major fraction in the serum normally. Next comes the one derived from skeleton, then intestine. During pregnancy placental isozyme also will be there in pregnant women.

Assay of serum alkaline phosphatase is useful routinely in the diagnosis of hepatobiliary disease and diseases of skeletal system associated with increased osteoblastic activity.

ASSAY OF ALKALINE PHOSPHATASE

Method Used

Method of King and Armstrong.

Specimen

Serum

Principle

Disodium phenylphosphate is acted upon by alkaline phosphatase in the serum and is hydrolyzed to release phenol and sodium phosphate. The phenol then reacts with 4- aminoantipyrine in the presence of potassium ferricyanide to give purple color and is read photometrically at 525 nm (green filter).

Reagents

1. 0.01 M disodium phenylphosphate
2. 0.1 M bicarbonate buffer pH 10
3. Buffered substrate
4. 0.6% 4 - aminoantipyrine
5. 2.4% Potassium ferricyanide
6. 0.01 mg/ml phenol working standard

Calculation (Fig. 20A-1)

OD of test = OD of Test – OD of Control
OD of standard = OD of S – OD of B
Concentration of standard = 0.01 mg
Phenol liberated by 0.1 ml serum
= OD of test/OD of std × concentration of std
= OD of test/OD of std × 0.01 mg
Phenol liberated by 100 ml serum
= OD of test/OD of std × 0.01 mg
× 100/vol. of serum taken
= OD of test/OD of std × 0.01 × 100/0.1 mg
= OD of test/OD of std × 10 mg

The enzyme activity is expressed in **King-Armstrong unit (KAU)**. One KAU corresponds to the production of 1 mg phenol in 15 minutes in this analytical procedure.

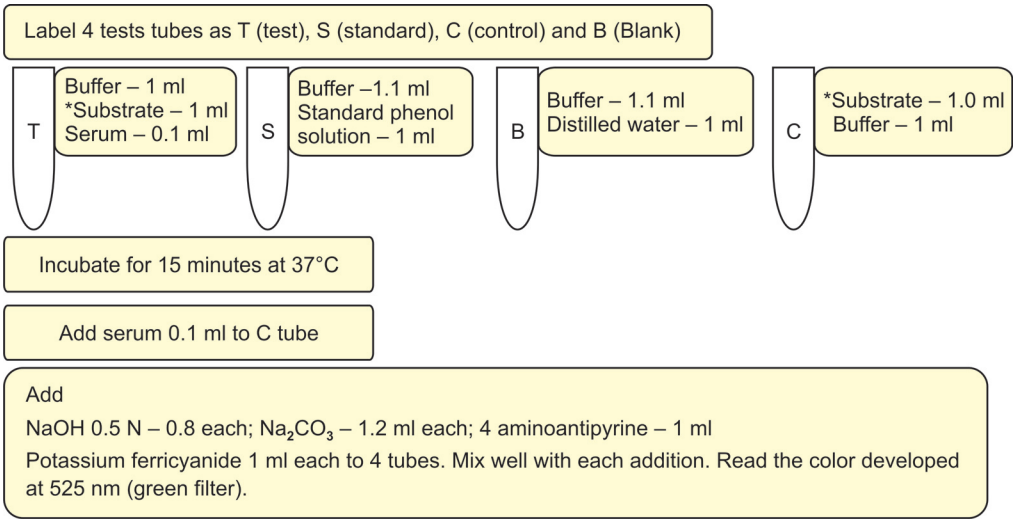


Fig. 20A-1: Procedure of alkaline phosphatase estimation

∴ Serum alkaline phosphatase activity = OD of test/OD of std × 10 KAU/100 ml.

INTERPRETATION

Reference range: 3–13 KAU/100 ml.

High Alkaline Phosphatase Activity

1. Physiologically
   - First few months of life
   - Pubertal growth spurt
2. Pathologically
   a. Bone diseases - where osteoblasts are active ie when bone regeneration is taking place.
      - Rickets
      - Osteomalacia
      - Malabsorption of vitamin D and Calcium
      - Paget's disease (osteitis deformans)
      - Osteogenic sarcoma
      - Secondary deposits in bone

   b. Diseases of liver and biliary tract
   5/nucleotidase help to differentiate between high ALP activity due to bone and hepatic diseases.
   5/nucleotidase activity will be raised in ALP rise of hepatic origin but will be normal in those with bone diseases.

ISOENZYMES OF ALP

Multiple forms of an enzyme are referred to as isoenzymes.

True isoenzymes are multiple forms of an enzyme catalyzing the same reaction but encoded by different genes producing different structure of enzymes. But many posttranslational modifications cause hetergeneity of various enzymes. ALP isoenzymes are due to both genetic and nongenetic modifications. Genetic loci of ALP are in chromosome 1 and 2. The locus of commonly encountered ALP isozymes (liver, bone, kidney and intestine) are located in

chromosome 1 and that of placenta in chromosome 2. The former four have different degree of sialation (the number of sialic acid residues attached to the enzyme protein). These isoforms in general can be measured by different types of analytical techniques – electrophoresis, chromatography, chemical inactivation, immunochemical methods and methods based on differences in the catalytical properties of isoenzymes.

20B. QUESTIONS

1. Describe the catalytical role of alkaline phosphatase.
2. What is the pH optimum of alkaline phosphatase?
3. Where is this enzyme located inside a cell?
4. What are the different enzymes of alkaline phosphatase?
5. Normally which isoenzyme predominate in the serum?
6. What is the role of alkaline phosphatase in the diagnosis of jaundice?
7. Name one technique that will help in the separation of different isoenzyme fractions in the serum.
8. Name one method by which you can determine the activity of ALP in the serum.
9. Explain the KAU unit used to express the ALP activity in the serum.

20C. REAGENT PREPARATION

1. Disodium phenyl phosphate 0.01 M: Dissolve 1.09 g in distilled water and make upto 500 ml in a 750 ml – 1 L beaker. Heat to boil quickly, then cool, add a 1 ml chloroform and keep in a refrigerator.

2. Sodium carbonate – sodium bicarbonate buffer pH 10 (0.1 M): Dissolve 3.18 g anhydrous sodium carbonate (Na_2CO_3) and 1.68 g sodium bicarbonate ($NaHCO_3$) in a few ml of water in a beaker and make upto 500 ml.

3. NaOH 0.5 N: Dissolve 20 g in 1 L of distilled water.

4. Sodium bicarbonate 0.5 N: Dissolve 42 g in 1 L of distilled water.

5. 4 – aminoantipyrine 0.6%: Dissolve 0.6 g in 100 ml of distilled water.

6. Potassium ferricyanide 2.4%: Dissolve 2.4 g in 100 ml of distilled water.

7. Phenol stock standard 0.1%: Dissolve 1 g of pure crystalline phenol in 0.1 N HCl and make upto 1 L with acid. Store at 4°C.

8. Phenol working standard 0.001% (.01 mg/ml): Dilute 1 ml stock phenol to 100 ml with water in a standard flask. Prepare whenever necessary , if any excess store at 4°C.

9. Buffered substrate for use: Mix equal volumes of (1) and (2) [Disodium phenyl phosphate 0.01M and sodium carbonate – sodium bicarbonate buffer pH 10 (0.1 M)].

Determination of Calcium

21

21A. DETERMINATION OF CALCIUM CONCENTRATION

Calcium is found mainly in the skeleton and teeth. It is also present in plasma and other body fluids. In blood 50% of calcium is free, 40% protein bound and 10% is complexed with diffusible ions like bicarbonate, lactate, phosphate and citrate. About 32% of total blood calcium is albumin bound and 8% is bound to globulins. Calcium binds to negatively charged sites on proteins. The charge of proteins is pH dependent. For example, alkalosis leads to more basic pH which in turn causes increase in negative charge on proteins enhancing calcium binding. This lowers the level of free calcium in the blood. The reverse happens with acidosis. Thus calcium is distributed among three plasma pools. Calcium in the plasma is redistributed among these three pools depending on protein concentrations, changes in pH and changes in free and total calcium concentrations in the serum.

Intracellular calcium participate in muscle contraction, hormone secretion, second messenger of hormone action, metabolic activities, enzyme actions, exocytosis and cell division.

A decrease in serum free calcium (either due to actual decrease or due to relative decrease caused by alkalosis) causes increased neuromuscular excitability and tetany where as an increase in free calcium reduces neuromuscular excitability (See Fig. 21A-1).

DETERMINATION OF CALCIUM

1. Photometric Method—O-Cresolphthalein Method

Specimen

Serum separated from plain blood collected in a dry bottle.

Principle

In alkaline pH, o-cresolphthalein complexone (CPC) forms a red chromophore with calcium. The intensity of the color is a measure of concentration of calcium in the serum. The serum sample is diluted with acid to release protein bound and anion bound calcium. Interference with the divalent magnesium ions are reduced by addition of 8-hydroxyquinoline and measurement of absorbance near 580 nm.

Reagents Required

1. **CPC reagent:** Containing o-cresolphthalein complexone (CPC), HCl, dimethyl sulphoxide and 8-hydroxyquinoline.

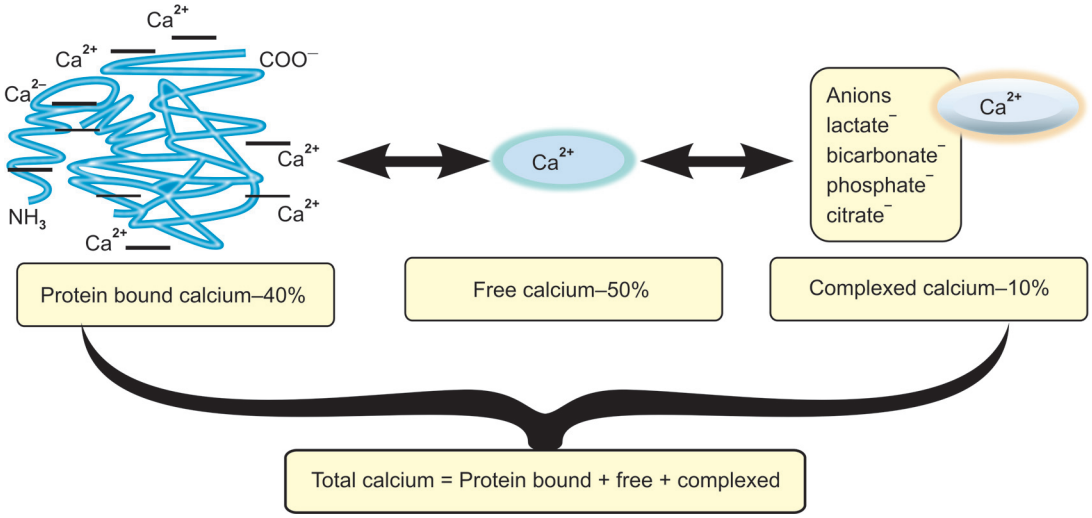


Fig. 21A-1: Three plasma pools of calcium

2. **Dimethylamine reagent:** Containing KCN and dimethylamine.
3. **Calcium–Standard solution:** 0.02 mg/ml = 2 mg%.

Procedure

See Figure 21A-2.

Calculation

OD of Test = OD of Test – OD of blank

OD of Standard = OD of Standard – OD of blank

Concentration of std in 0.1 ml = 0.002 mg/0.1ml

Volume of the sample = 0.1 ml/5 = 0.02

Concentration of calcium in 100 ml serum (mg%)

= OD of test/OD of std × concentration of std/ volume of sample × 100 mg%

= OD of test/OD of std × 0.002/0.02 × 100 mg%

= OD of test/OD of std × 10 mg%

2. Titration Method—Method of Clark and Collip (1925)

Specimen

Serum separated from plain blood collected in a dry bottle.

Principle

The calcium in the sample is precipitated as calcium oxalate by treating with ammonium oxalate. Excess ammonium oxalate is washed off with dilute ammonium hydroxide. The calcium oxalate precipitate is then dissolved in 1 N sulfuric acid. To form oxalic acid. It is then titrated with standard permanganate solution to find out the amount of oxalic acid which is equated with cacium present in the serum. The end point is indicated by a pink color. The titer value is used to calculate the concentration of calcium.

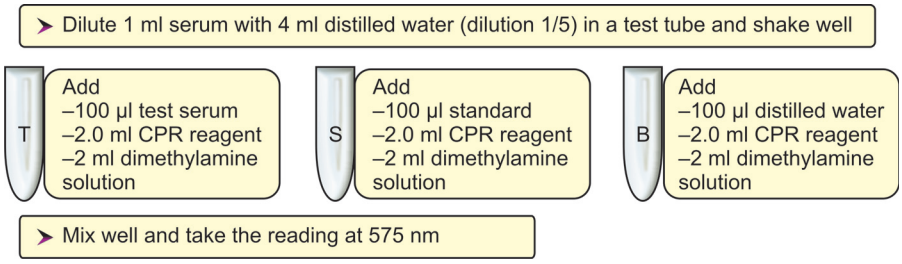


Fig. 21A-2: Procedure of CPC method for total calcium estimation

Reagents

1. 4% ammonium oxalate
2. 2% ammonium hydroxide
3. 1N H_2SO_4
4. $KMnO_4$ - 0.01 N: Prepare freshly before use by diluting stock 0.1 solution.

Procedure

- Take 2 ml serum in centrifuge tube.
- Add 2 ml distilled water and 1 ml of 4% ammonium oxalate to it. Mix well to achieve complete precipitation in half an hour.
- Centrifuge at 2000 rpm for 30 minutes.
- Discard the supernatant fluid without disturbing the precipitate. Invert the tubes over a filter paper to drain off the remaining supernatant for 5 minutes.
- Add 3 ml of 2% ammonia down the sides of the tube and mix the precipitate in it.
- Centrifuge again and pour off the supernatant
- Add 2 ml of 1 N sulphuric acid and dissolve the precipitate in it using the glass rod used previously.
- Warm by placing it in a water bath.
- Titrate it against 0.01 N permanganate take in a micro burette graduated to 0.02 ml, to get pink color that persists for a minute (gives the titre value of test).
- Perform blank titration of 0.01 N permanganate taken in a micro burette against 2 ml of 1 N sulphuric acid taken in a dry test tube to the same end point (gives the titre value of blank).
- The difference between these titrations gives the volume of 0.01 N permanganate required to titrate the calcium oxalate precipitate.

Calculation

1 ml of 0.01 N permanganate is equivalent to 0.2 mg of calcium. Here 2 ml serum is used.

Mg of calcium per 100 ml of serum

= (Titer value of test – Titer value of blank) × 0.2 × 100/2

= (Titer value of test – Titer value of blank) × 10

INTERPRETATION

Reference Range in Adults

Total calcium – 8.6 – 10.3 mg%
Free calcium – 4.6 – 5.3 mg%

Alterations in Serum Calcium Levels

The level of serum calcium is affected by

- Defective absorption from the intestine

- Altered parathyroid hormones
- Changes in serum phosphorous concentration
- Changes in serum protein concentration
- Altered pH

Low Serum Calcium Levels

Alkalosis: Lowered calcium in the blood often manifest as tetany. It is commonly seen with alkalosis.

Hypoparathyroidism: It occurs more commonly after thyroidectomy due to the removal of a considerable part of this glandular tissue along with thyroid gland.

Rickets: Due to defective calcium absorption. Associated finding in rickets is low phosphorous levels.

Steatorrheas: Due to different causes like ideopathic, celiac disease, sprue leads to defective absorption leads to low serum calcium levels.

High Serum Calcium Levels

Hypercalcemia occurs due to excessive release of calcium from skeleton, intestine or kidney into extracellular fluid compartment. Different conditions leading to hypercalcemia are given below:

- Primary hyperparathyroidism
- Malignancies
- Hyperthyroidism
- Hypothyroidism
- Acute adrenal insufficiency
- Renal failure, immobilization
- Increased serum proteins
- Lithium therapy
- Vitamin D and vitamin A overdose

Points to Ponder

Several calculations are used to adjust total calcium determinations to correct for variations in protein concentration. One such calculation is given below.

Corrected total calcium in mg% = Total calcium + 0.8 (4 – Albumin in g%)

But the factors like effects of pH, binding kinetics, fatty acids, other substances bound by albumin, heparin and anions like citrate, bicarbonate, lactate, phosphate, pyruvate, β hydroxybutyrate, sulfate limit the usefulness of total and corrected calcium. So if possible instead of going for mathematical corrections to get the real values, it is better to determine free calcium directly by ion selective electrode (ISE).

21B. QUESTIONS

1. Mention two methods for estimating calcium in the serum. Give the principle of each method.
2. What are the different functions offered by calcium in the body?
3. Name the 3 different pools in the blood in which calcium is distributed. Give main factors affecting the redistribution in different pools.
4. What is the role of serum proteins in maintaining normal free calcium level?
5. Alkalosis causes tetany. Explain.
6. What are the anions that bind with calcium?
7. What is normal serum calcium level?
8. What are the major causes for hypocalcemia?
9. What are the different causes of hypercalcemia?
10. What is the method available to assay cacium directly so that influence of compounding factors can be eliminated?

21C. REAGENT PREPARATION

1. **CPC reagent:** Dissolve 40 mg CPC in 1 ml concentrated HCl in a 1 liter volumetric flask. Add a few drops of distilled water to dissolve it. Add 50 ml dimethyl sulphoxide used as wash. Add 2.5 g 8-hydroxyquinoline and mix well to dissolve it fully and make up the volume to 1 L.

2. **Dimethyl amine reagent:** Dissolve 500 mg KCN and add 40 ml dimethylamine and make the volume 1 L with distilled water.

3. **Standard calcium solution:** 0.02 mg/ml

4. **Ammonia 2% (v/v) solution (ammonium hydroxide):** Dilute 2 ml ammonia SG. 0.88, to 100 ml with water.

5. **Potassium permanganate, 0.01 N (stock):** Dissolve 3.162 g pure potassium permanganate in a litre of distilled water. Allow to stand for a few days and filter through a glass wool. Standardize against 0.1 N oxalic acid solution.

6. **Potassium permanganate, 0.01 N:** Prepare freshly before use by diluting the stock solution.

7. **1 N H_2SO_4:** Add 27.8 ml concentrated sulfuric acid to 950 ml distilled water in a volumetric flask. Cool. Make up to 1 L with distilled water. Standardize against 1 N NaOH using phenolphthalein as an indicator.

Determination of Phosphorus

22

22A. DETERMINATION OF INORGANIC PHOSPHORUS

Phosphorus is found either in the inorganic or organic phosphate in the human body.

In the soft tissues mostly it is seen as organic phosphates and in the bone as inorganic phosphate (a major component of hydroxyapatite). Its concentration in plasma ranges from 2.5 to 4.5 mg/dl of inorganic phosphate. For diagnostic purposes inorganic phosphate concentration is measured.

In the serum, phosphate exists as monovalent (H_2PO_4) and divalent phosphate (HPO_4^{2-}) anions. The pH influences the ratio of H_2PO_4/HPO_4^{2-}.

In the serum, it is existing in different pools (Fig. 22A-1):

- 10% protein bound
- 35% complexed with sodium, calcium and magnesium
- The rest (55%) is in free form.

Organic phosphate esters are present inside the cellular elements of the blood.

Apart from forming part of structural framework of the body, the phosphate has many other biochemical and physiological functions in the body.

- Forming high energy phosphate bond
- Constituent of cyclic adenine and guanine nucleotides and NADP
- Essential element of biomembranes (as part of phospholipids), nucleic acids, phosphoproteins (casein and some other proteins)
- In gene transcription and cell growth
- Regulation of enzyme action (covalent modification of enzymes by phosphorylation and dephosphorylation)

DETERMINATION OF INORGANIC PHOSPHORUS

1. Photometric Method—Method of Fisk and Subba Rao

Specimen: Serum separated from plain blood collected in a dry bottle.

Principle: Serum is treated with trichloracetic acid to get protein free filtrate. Protein free filtrate is then treated with acid molybdate reagent to form phosphomolybdic acid. The hexavalent molybdenum of phosphomolybdic acid is reduced by 1, 2, 4 aminonaphtholsulphonic acid to give a blue compound which is estimated photometrically.

Reagents Required

1. Trichloroacetic acid (10%)
2. Sulfuric acid 10 N
3. 0.25% 1, 2, 4 aminonaphtholsulphonic acid
4. Stock standard phosphate solution
5. Working standard solution

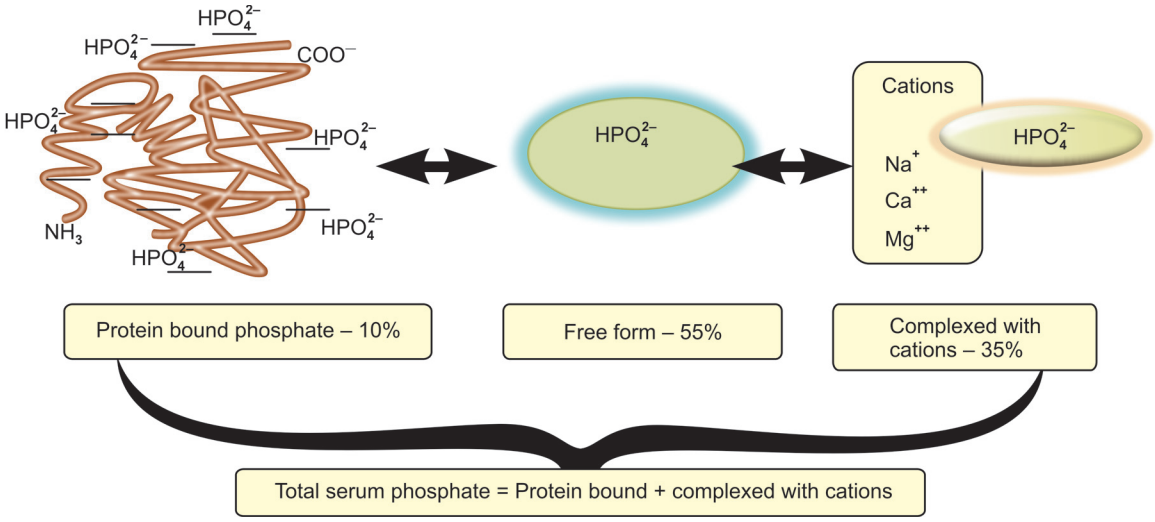


Fig. 22A-1: Different pools of phosphate in the serum

Procedure (Fig. 22A-2)

Preparation of protein free filtrate: take 2 ml serum in a dry test tube and add 8 ml TCA (dilution factor = 10/2 = 5). Mix well and keep for 5 minutes and filter to obtain a protein free filtrate.

Use this protein free filtrate for phosphate estimation.

Calculation

5 ml standard contains 0.04 mg P and 5 ml of protein free filtrate is equivalent to 1 ml serum

Mg of inorganic phosphorus per 100 ml serum

= Reading of test/Reading of standard × 0.04 × 100

= Reading of test/Reading of standard × 4

INTERPRETATION

Reference Range

Adults – 2.5-4.5 mg%
Children – 4-6 mg%

ALTERATIONS IN INORGANIC PHOSPHORUS IN SERUM

Hypophosphatemia

The term hypophosphatemia is used when the serum inorganic phosphate concentration is less than 2.5 mg%. Clinical manifestations depend on duration and extent of the deficiency. Since phosphate is a component of energy currency of the – ATP, cellular functions are impaired in hypophosphatemia.

It leads to muscle weakness, respiratory failure decreased cardiac output. At very low concentrations, i.e. when it goes below 0.5 mg% rhabdomyolysis, lysis of RBC, mental confusion and even coma may occur. Chronic hypophosphatemia causes rickets in children and osteomalacia in adults.

1. **Oral or intravenous hyperalimentation and insulin:** Carbohydrates induce insulin secretion which enhances transport of phosphate from extracellular fluid into the cells leading to a fall in serum phosphate.

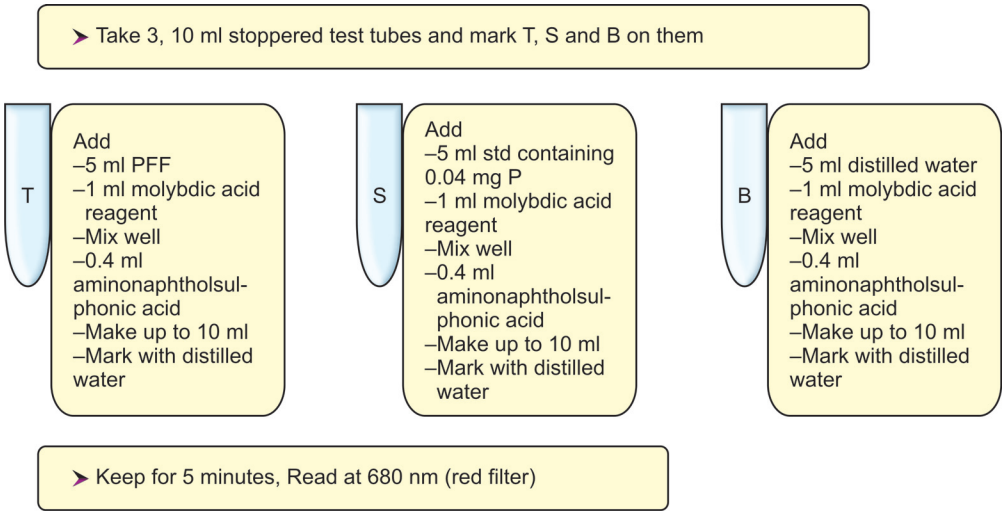


Fig. 22A-2: Procedure for phosphate estimation

2. **Respiratory alkalosis:** Promote an intracellular shift of phosphate from extracellular fluid leading to a fall in it's level in the serum.

3. **Lowered renal threshold for phosphate**
- Primary and secondary hyperparathyroidism
- Fanconi's syndrome
- X-linked hypophosphatemia

4. **Intestinal loss**
- Malabsorption
- Ingestion of antacids containing aluminium and magnesium which bind with phosphate making it unsuitable for absorption.

5. **Acidosis :** For example, ketoacidosis, lactic acidosis.

Acidosis leads to catabolism of organic phosphates to form inorganic phosphates which then pass into plasma and get excreted in urine leading to depletion of phosphates from the interior of the cells.

Hyperphosphatemia

Elevated phosphate causes a decrease in serum calcium concentration which may lead to tetany and seizures. An increase in serum phosphorus is seen in the following conditions.

1. Renal failure: A decrease in glomerular filtration rate hinder excretion of phosphate in urine leading to hyperphosphatemia.
2. Hypoparathyroidism and acromegaly: Enhances tubular reabsorption of phosphates.
3. Aggressive phosphate therapy
4. Release of phosphate: Occurs in rhabdomyolysis, chemotherapy.

Points to Ponder

Serum or plasma should be separated soon after blood collection. Ester phosphates present in the cells hydrolyze to form inorganic phosphates which diffuse out of the cell causing an elevation of it's level in the serum or plasma.

22B. QUESTIONS

1. How will you estimate inorganic phosphate in the serum? Name one method, give its principle.
2. What will happen to phosphate value if plasma or serum separation of blood sample is delayed? Give the reason.

3. Name the 3 different pools in the blood in which serum phosphorus is distributed.
4. Give the reference range of serum phosphate in adults and children.
5. What is hypophosphatemia? Name different conditions leading to hypophosphatemia.
6. What is hyperphosphatemia? Name different conditions leading to hyperphosphatemia.
7. Why do you get hypophosphatemia in IV infusions of glucose?
8. What are the manifestations of hyperphosphatemia?
9. What are the manifestations of hypophosphatemia?
10. Why do you get hypophosphatemia in respiratory alkalosis?
11. Renal failure causes hyperphosphatemia. Explain.

22C. REAGENT PREPARATION

1. Trichloroacetic acid (TCA) 10%: Dissolve 10 g of reagent grade TCA in water and make up to 100 ml in a 100 ml conical flask or cylinder.

2. Sulfuric acid 10 N: Add 450 ml concentrated sulfuric acid slowly into 1300 ml of distilled water.

3. Molybdic acid reagent: (2.5% ammonium molybdate in 3 N sulfuric acid.) Dissolve 25 g ammonium molybdate in 200 ml distilled water and transfer it to a 1 L flask containing 300 ml 10 N sulfuric acid. Dilute to 1 L with water.

4. Sodium bisulphite 15% solution: Freshly prepared solutions may be turbid so keep it for 2 days to make it clear.

5. Sodium sulphite 20% solution: Dissolve 200 g sodium sulphite ($Na_2SO_3 7H_2O$) in 380 ml water. Filter and keep it in a stoppered bottle.

6. 0.25% 1,2,4 aminonaphtholsulphonic acid: Add 0.5 g to 195 ml of 15% sodium bisulphite and 5 ml of 20% sodium sulphite. Stopper and shake well to dissolve it. Store in an amber colored bottle in cold. Solution is stable up to 4 weeks.

7. Standard phosphate solution: Dissolve 0.351 g of pure potassium dihydrogen phosphate in water in a liter flask add 10 ml of 10 N sulfuric acid and make up to the mark with water. Five ml contains 0.4 mg. This is stable.

8. **Working standard:** Dilute the stock standard 1 to 10 so that 1 ml contains 0.008 mg phosphorus.

Determination of Titrable Acidity and Ammonia in Urine

23

23A. DETERMINATION OF AMMONIA AND TITRABLE ACID IN URINE

The pH of the blood is normally maintained within narrow limits (7.35-7.45). Hydrogen ions are produced as a result of metabolic activities. Sulfur containing amino acids, phospholipid, glucose and other nutrients get catabolized to release sulfuric acid, phosphoric acid and other organic acids (fixed acids). These acids ionize to form respective anions and H^+.

Aerobic metabolism produces CO_2 and water. The carbonic anhydrase enzyme catalyze the conversion of CO_2 and water to form carbonic acid (volatile acid) which then dissociate to H^+ and HCO_3^-.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

In the lungs the reverse reaction takes place and the CO_2 is exhaled. The hydrogen ions produced by the dissociation of fixed acids are either consumed by other reactions in the body or dealt by regulatory mechanisms involving buffers, lungs and kidneys.

Different mechanisms are available in the kidney to maintain normal pH of the blood. They are:

a. Excretion of H^+ ion and generation of HCO_3^-
b. Reabsorption of HCO_3^-
c. Excretion of titrable acid
d. Excretion of ammonium ions

Excess H^+ ions produced are secreted into the tubular fluid and there it is buffered by HPO_4^{2-} and accounts for titrable acidity (30 mmol/day).

(The titrable acidity is mainly due to acid phosphate and to a minor extent due to weak organic acids like uric acid and oxalic acid). The remainder is excreted as ammonium ion (NH_4^+ 40 mmol/day). Ammonia required for this purpose is released from renal tubular epithelial cells. In the renal tubular epithelium the glutaminase enzyme acts upon glutamine to release ammonia with the formation of glutamic acid.

Ammonia in urine arises from the hydrolysis of glutamine by glutaminase enzyme in the renal tubular cells and the activity of which is increased during acidosis. NH_3 thus formed diffuse into the renal tubular lumen and there it bind with H^+ ions to form ammonium ions and these NH_4^+ cannot diffuse back. The rate of NH_4^+ ion excretion is controlled by H^+ concentration in the blood. Therefore in acidosis NH_4^+ ions are excessively excreted in urine.

Titrable acidity, alone cannot give an exact measure of acid secretion. But along with urinary ammonia, titrable acidity of urine is useful in assessing the severity of acidosis. A more

accurate measure is obtained by comparing the pH of blood and urine. A pH meter is useful to measure the pH of the blood and urine.

DETERMINATION OF TITRABLE ACIDITY AND AMMONIA

Method Used: Titration Method

Principle

Titrable acidity of urine is expressed in terms of the amount of standard alkali required to bring the urine from its original pH to the phenolphthalein end point (that is around the pH 8.5-9). The urine is titrated with standard sodium hydroxide solution, using phenolphthalein as an indicator. Potassium oxalate is added to precipitate the calcium, and to remove it completely as calcium oxalate. This would help to avoid interference by the precipitation of calcium phosphate upon neutralization of urine. Ammonia is estimated by **formol titration method.** Upon addition of neutral formaldehyde to a solution containing ammonium salts, H^+ ions are liberated which can be titrated with standard alkali of known strength. When formaldehyde is added, it will form an adduct with any nitrogenous material having two or more hydrogen bound to nitrogen (hexamethylene tetramine) and an equivalent quantity of acid. Hence, ammonium salts in urine react with formaldehyde to release free acids (shown below) which are also titrated. The titer value is proportional to the ammonia content in urine.

$$4\,NH_4Cl + 6\,HCHO \leftrightarrow N_4(CH_2)_6 + 6\,H_2O + 4\,HCl$$

Reagents Required

1. Potassium oxalate (pulverized)
2. Phenolphthalein solution:
3. Sodium hydroxide (NaOH) – 0.1 N
4. Neutralized formalin

Procedure

(See Fig. 23A-1).

Estimation of titrable acidity: Pipette 25 ml urine in a 200 ml Erlenmeyer flask and add 5 g of finely powdered potassium oxalate and 1-2 drops of a 1% phenolphthalein solution to the fluid. Shake well. Titrate against N/10 NaOH until a faint permanent pink color remains. Note the end point and titre value. Let the titre value be X ml.

Estimation of ammonia: Add 10 ml of neutral formalin to the above urine obtained after titration. The faint pink color disappears due to the liberation of acid upon addition of formalin.

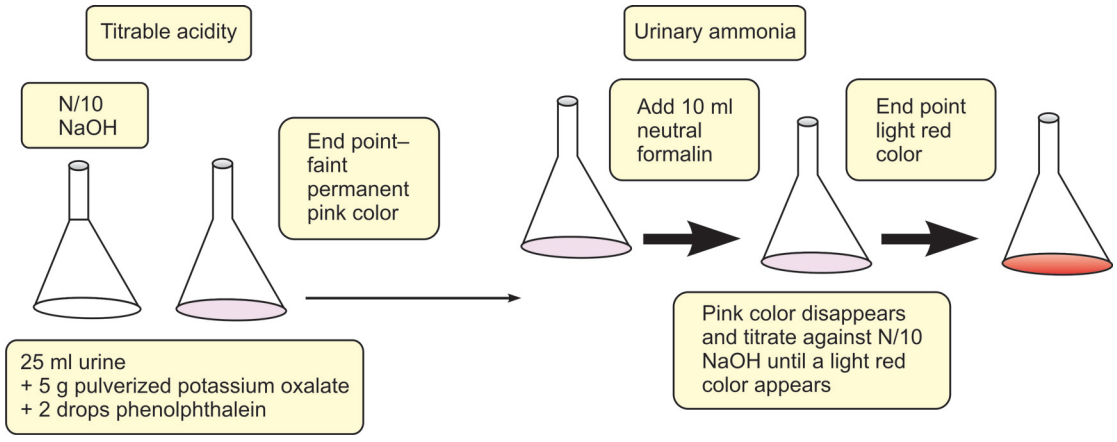


Fig. 23A-1: Titration method for determining titrable acidity and urine ammonia

Titrate the contents to light red against N/10 NaOH. Let the titre value be Y ml.

Calculation

Titrable acidity

- Volume of NaOH in ml used up in the titration to neutralize the titrable acids in 25 ml of urine = X ml
- Volume of NaOH in ml that may be used up in the titration to neutralize the titrable acids in 100 ml of urine = X × 100/25 ml = 4X ml of N/10 NaOH
- Let the 24 hours urine volume be U ml

Titrable acidity of urine = 4X × U/100 ml/day

Ammonia

- Volume of NaOH in ml used up in the titration for 25 ml urine = Y ml 0.1 N Na OH
- Volume of NaOH in ml that may be used up for 100 ml urine = Y × 100/25 = Y × 4 ml 0.1 N NaOH

1 ml 0.1 N NaOH is equivalent to 1.7 mg ammonia

Concentration of ammonia in 100 ml urine = (Y × 4 × 1.7) mg of ammonia

24 hour urine ammonia = (Y × 4 × 1.7) × U/100 mg

INTERPRETATION

Titrable acidity normally ranges from 200-500 ml of N/10 NaOH (20-50 meq /L) per day. Normally, it is influenced by the diet. It is low on vegetable diet (base forming) and high on acid forming diet. e.g. meat, milk, cheese, rice and whole wheat products, etc.

In fasting it may rise to 800. Urine samples collected shortly after meals will be more alkaline due to alkaline tide.

Bacterial decomposition of the urea to form ammonia decrease the acidity of urine. Exposure of urine to atmosphere also cause the same change.

Acidity of urine is increased in metabolic acidosis and also with administration of mineral acids, acid phosphates and ammonium chloride.

It is decreased in metabolic alkalosis.

Urinary ammonia—normal daily excretion rate ranges from 0.5-0.8 g/day that is 2-5% of the total nitrogen excreted daily.

It is excreted as ammonium ion (NH_4^+). It represents a mode of excretion of hydrogen ion in urine. As in the case of titrable acidity, acid forming foods increase and base forming food decrease the rate urinary ammonia excretion.

Metabolic acidosis increase and metabolic alkalosis decrease the urinary ammonia content.

The sum of titrable acidity and ammonia excretion in urine give an idea about the severity of acidosis and the renal capacity to conserve base.

23B. QUESTIONS

1. What is the normal pH of blood? Name the various mechanisms by which it is maintained.
2. What are the modes by which kidneys excrete unwanted H^+ ions from the body?
3. Explain titrable acidity. What is the normal titrable acidity of urine?
4. Name the situations in which titrable acidity of urine is raised.
5. Name the conditions in which you get low titrable acidity.
6. What is the technique used for determining titrable acidity?
7. What is the purpose of adding potassium oxalate to urine prior to titration?
8. Describe mechanism of H^+ ion elimination in urine as ammonium ion.

9. What is the role of glutaminase enzyme in the H^+ ion elimination in urine as ammonium ion?
10. What are the conditions in which urinary ammonia is increased?
11. Describe the principle of urinary ammonia determination by titration method.
12. What is the range of urinary ammonia concentration in a healthy person?
13. What would be the change produced in the urinary ammonia level:
    i. If the urine sample is putrified by bacterial action?
    ii. If the urine sample is taken in a open container and kept in the laboratory for many hours?

23C. REAGENT PREPARATION

1. **Potassium oxalate (pulverized)**
2. **Phenolphthalein solution (pH range 8.3-9) (color range – colorless to red):** Dissolve 100 mg in 100 ml of 50 % ethyl alcohol.
3. **Sodium hydroxide (NaOH) – 0.1 N:** Dissolve 40 g reagent grade sodium hydroxide pellets in a few ml distilled water in a 1 L beaker or cylinder and make up to I L with distilled water. Keep it for two days and decant the solution into a bottle fitted with siphon. Standardize against by titration with an acid of known strength using methyl red as an indicator.
4. **Neutralized formalin:** Neutralize 40% formalin with 1/20 N NaOH using phenol red as an indicator until a light red is obtained.

Determination of Urine Chloride

24

24A. DETERMINATION OF URINE CHLORIDE

Chloride is the major extracellular anion (98-107 mmol/L). Sodium and chloride together forms the major osmotically active constituents in plasma. Hence, it has a prominent role in water distribution, osmotic pressure and cation – anion balance in the ECF (extracellular fluid).

The source of chloride in the body is diet. It is absorbed from the intestinal tract and reaches plasma. They are filtered at the glomerulus and passively absorbed along with Na^+, in the PCT (proximal convoluted tubule). The chloride pump in the thick ascending limb of loop of Henle promotes active reabsorption of Cl^- and passive reabsorption of Na^+. Excess chloride in the body is excreted in the urine.

Sample: 24 hours urine collected without adding any preservatives. It may be stored at 2-4 °C or frozen for delayed analysis.

DETERMINATION OF URINE CHLORIDE

Method Used: Titration Method

Principle

Urine is acidified with nitric acid and chlorides precipitated with a measured excess of silver nitrate solution. The silver chloride formed is filtered off. The filtrate contain the silver nitrate which is not consumed in the silver chloride formation. The concentration of this silver nitrate in the filtrate is found out by titrating the filtrate with standard ammonium thiocyanate solution using ferric ammonium sulphate (ferric alum) used as an indicator. Silver nitrate reacts with thiocyanate to form ferric thiocyanate. When all the silver nitrate is used up, the red color of ferric thiocyanate begin to appear. The appearance of this red color is the end point of this procedure.

$$AgNO_3 + NaCl \leftrightarrow AgCl + NaNO_3$$

$$AgNO_3 + NH_4CNS \leftrightarrow AgCNS + NH_4NO_3$$

$$6\ NH_4CNS + (NH_4)_2\ SO_4\ Fe_2\ (SO_4)_3 \leftrightarrow 2Fe\ (CNS)_3 + 4\ (NH_4\ )_2\ SO_4$$

Reagents

1. Siver nitrate standard solution 0.17 N.
2. Ammonium thiocyanate standard solution 0.17 N.
3. Ferric ammonium sulfate $(NH_4)_2\ SO_4Fe_2\ (SO_4\ )_3\ 24\ H_2O$ (ferric alum) saturated solution
4. Concentrated nitric acid

Procedure

Pipette 5 ml urine, 2 ml concentrated nitric acid (to prevent the precipitation of silver urates and silver phosphates), 10 ml silver nitrate solution

and 10 drops of saturated ferric alum solution into a 25 ml conical flask. Mix gently and titrate against 0.17 N thiocyanate solution till reddish brown color due to the formation of ferric thiocynate is obtained and that should last for at least 30 seconds. Note the titer value.

Repeat the titration to get at least two consecutive concordant values.

Calculation

Titrable acidity

Titre value for 5 ml urine = X ml

Volume $AgNO_3$ taken for titration = 10 ml

Volume of $AgNO_3$ reacted with chloride in urine to form AgCl = 10 – X

1 ml $AgNO_3$ ≈ 1 ml standard thiocyanate ≈ 10 mg NaCl ≈ 6 mg Cl^-

(10 – X) ml $AgNO_3$ ≈ 10 × (10 – X) mg NaCl or 6 × (10 – X) mg Cl^-

5 ml urine contains = 10 × (10 – X) mg NaCl or 6 × (10 – X) mg Cl^-

Therefore 100 ml urine contains

= 10 × (10 – X) × 100/5 mg NaCl
or 6 × (10 – X)100/5 mg Cl^-

= 10 × (10 – X) × 20 mg NaCl
or 6 × (10 – X) 20 mg Cl^-

= 200 × (10 – X) mg NaCl
or 120 × (10 – X) mg Cl^-

INTERPRETATION

Urine chloride normally ranges from 8-15 g per day.

Chloride excretion rate is in proportion to dietary salt in healthy state.

High urine chloride is observed in

- High salt intake
- *Addison's disease:* Impairment of tubular reabsorption of chloride causing raised levels in urine and low chloride levels in serum

Low urine chloride is observed in:

- Low salt intake
- Cushing's syndrome
- Steroid therapy
- Kidney disease with edema causes retention of chloride in blood with less excretion in urine.

24B. QUESTIONS

1. What is the source of chloride in the body?
2. How chloride is absorbed and excreted?
3. Give the normal levels of chloride in the blood and urine.
4. Name one method by which urine chloride can be estimated and give its principle.
5. What is the normal excretory rate of urine chloride?
6. Name two situations in which you get high chloride content in urine.
7. What is the reason for getting high levels of chloride in urine in cases of Addison's disease?
8. Name two conditions leading to low values of chloride in urine.

24C. REAGENT PREPARATION

1. **Siver nitrate standard solution 0.17 N:** Dissolve 29.061 g $AgNO_3$ in a few ml of distilled water and make up to 1 L. 1 ml of this solution is equivalent to 10 mg NaCl or 6 mg Cl.

2. **Ammonium thiocyanate standard solution:** Dissolve 13 g ammonium thiocyanate in 1 liter water. Take 20 ml standard solution (0.17 N), 4 ml concentrated nitric acid and 5 ml ferric alum

solution in a flask and dilute to 100 ml with distilled water and titrate against ammonium thiocyanate solution. Silver nitrate reacts with thiocyanate to form ferric thiocyanate. When all the silver nitrate is used up, the red color of ferric thiocyanate begins to appear. The appearance of this red color is the end point of this procedure. Note the titer value. Dilute ammonium thiocyanate solution with distilled water to make 1 ml of that solution is equivalent to 1 ml 0.17 N $AgNO_3$ solution.

3. **Ferric ammonium sulfate $(NH_4)_2\ SO_4Fe_2\ (SO_4)_3\ 24\ H_2O$:** Saturated solution in distilled water.

SECTION THREE

Charts

Charts

25

25A. ACID-BASE DISORDERS

Q. 25A-1

Sheela aged 19 years brought to the casuality at 11 AM with dizziness, tingling of fingers, sweating, breathing heavily and nausea.

On examination: Hyperventilation, carpo pedal spasm were found.

Laboratory data:

pH	7.55
pCO_2	20 mm Hg
HCO_3^-	24 mq/L
H_2CO_3	0.6 meq/L

a. What kind of acid-base disorder is this girl suffering from? Explain.

b. What are the common causes of this kind of disorder?

c. Give the compensatory mechanisms available in the body to correct this sort of acid-base imbalance.

Ans. 25A-1:

a. Respiratory alkalosis (uncompensated)

Here pH = 7.55 (normal pH range = 7.35 - 7.45) indicating alkalosis.

pCO_2 = 20 mm Hg (Normal p CO_2 = 40 mm Hg) which is less than normal; (Normal serum HCO_3^- = 22-26 mmol/L).

HCO_3^- = 24 meq/L. Showing a modest decrease.

∴ The acid-base disorder is respiratory alkalosis.

The probable reason in this case is hysterical over breathing leading to wash out of CO_2 causing to ↓ pCO_2, elevation of pH and ↑ HCO_3^-/H_2CO_3 ratio (in this case it is 24/0.6 = 40 : 1 but normally it is 20 : 1).

Tingling of fingers and carpopedal spasm is due to a reduction in ionized calcium caused by increased binding of calcium to albumin in alkaline pH of extracellular fluid.

b. Common cause of respiratory alkalosis is functional hyperventilation due to anxiety disorders.

It also occurs in cases where the respiratory center in the medulla is over stimulated as in encephalitis, intracranial surgery, salicylate poisoning, high altitude (mountain climbers) and chronic liver disease leading to metabolic encephalopathy.

c. In respiratory alkalosis, there is loss of CO_2 leading to ↓ pCO_2 and ↑ HCO_3^-/H_2CO_3 ratio. In an attempt to return the pH towards normal, kidneys excrete more HCO_3^- in urine, so that bicarbonate-carbonic acid ratio is brought low. It is evident from the Henderson-Hasselbalch equation shown below.

$pH = pKa + HCO_3^-/H_2CO_3$.

Q. 25A-2

Simon 75 years who has undergone an intracranial surgery and his acid base parameters are given below. Interpret it.

Laboratory data:

pH	7.5
pCO_2	20 mm Hg
HCO_3^-	20 meq/L
H_2CO_3	0.6 meq/L

Ans. 25A-2:

a. Respiratory alkalosis

Here pH = 7.5 indicating alkalosis (normal pH range = 7.35-7.45).

pCO_2 = 20 mm Hg which is less than normal (Normal pCO_2 = 40 mm Hg);

HCO_3^- = 20 meq/L which is lower than normal. Hence, it is respiratory alkalosis.

The probable reason in this case is the intracranial surgery causing respiratory centre stimulation leading to wash out of CO_2 and causing decrease in pCO_2, elevation of pH and rise in HCO_3^-/H_2CO_3 ratio (in this case, it is 24/0.6= 33.3:1 but normally it is 20:1).

Normal values of acid base parameters are as follows:

pH	7.35 – 7.45
pCO_2	40 mm Hg
HCO_3^-	24 meq/L (22-26 meq/L)
H_2CO_3	1.2 meq
HCO_3^-/H_2CO_3	20:1

Q. 25A-3

Ramakrishnan, 60-year-old, a known smoker attended casuality with exacerbation of bronchial asthma. The acid base analysis report is given below. Give your interpretation:

Laboratory data:

pH	7.04
pCO_2	90 mm Hg
HCO_3^-	24 meq/L
H_2CO_3	2.7 meq/L

Ans. 25A-3: Respiratory acidosis

Here pH = 7.04 indicating acidosis (normal pH range =7.35-7.45).

Asthma attacks are characterized by episodes of airway obstruction leading to retention of CO_2, leading to increased pCO_2, decreased pH, decreased bicarbonate-carbonic acid ratio and normal bicarbonate in the initial phase.

Renal compensatory mechanisms tend to retain bicarbonate in the blood which in turn helps to raise the bicarbonate-carbonic acid ratio and the pH of the blood.

Causes of respiratory acidosis: Generally respiratory acidosis is caused by disorders that interfere with the respiratory activity – pneumonia, asthma, pulmonary edema, emphysema, apnea. Morphine and barbiturate poisoning cause depression of respiratory centre leading to respiratory acidosis.

Q. 25A-4

A woman complaining of intractable vomiting suspected of suffering from pyloric stenosis receiving treatment showed following acid base data on day 1 and day 2.

Comment on

Laboratory data on day 1:

pH	7.6
pCO_2	40 mm Hg
HCO_3^-	35 meq/L
H_2CO_3	1.2 meq/L

Laboratory data on day 2:

pH	7.55
pCO_2	45 mm Hg
HCO_3^-	28 meq/L
H_2CO_3	1.35 meq/L

Ans. 25A-4:

Day 1 – Metabolic alkalosis (uncompensated)
Day 2 – Metabolic alkalosis (partially compensated)

Here pH = > 7.6 indicating alkalosis on both days (normal pH range = 7.35-7.45).

Due to loss of acid by vomiting, the bicarbonate-carbonic acid ratio has been raised to produce alkalosis. The respiratory center responds by decreasing the respiratory rate and depth by the influence of alkaline pH on the

medullary respiratory center. The kidney reacts by excreting more bicarbonate in urine. This response by kidneys and respiratory systems causes decrease in HCO_3^-/H_2CO_3 ratio. These changes are evident on day 2 indicating partially compensated metabolic alkalosis.

Metabolic alkalosis is due to either an excess of base or a loss of acid. This causes rise of HCO_3/H_2CO_3 ratio and pH.

The conditions causing loss of acid from the stomach in severe vomiting, aspiration of gastric contents and loss of acid through urine in diuretic drug therapy (carbonic anhydrase inhibitors and potassium sparing drugs) can lead to metabolic alkalosis. The endocrine disorders primary aldosteronism and Cushing's syndrome also cause hypokalemic alkalosis. (retention of sodium and loss of potassium – hypokalemia in turn causes shift of protons into the ICF → alkalosis).

Q. 25A-5

Kumaran, 58-year-old peon in a private firm has been suffering from diabetes mellitus for the past 20 years. He was taking irregular treatment for DM. On one Sunday he was brought to the casuality in a stuperosed state. O/E: stuperosed, fruity smell +, Kussmaul's type of breathing + Urine – Rothera' s test +ve and Blood glucose – 450 mg% . Acid base data of his blood is given below. Comment on the acid base status of the patient.

Laboratory data:

pH	7.2	Na^+	140 mmol/L
pCO_2	40 mm Hg	K^+	4 mmol/L
HCO_3^-	15 meq/L	HCO_3^-	22 mmol/L
H_2CO_3	1.2 meq/L	Cl^-	102 mmol/L

Ans. 25A-5:

Metabolic acidosis: Low pH indicates acidosis of metabolic origin (identifiable by decreased plasma bicarbonate) In the uncontrolled diabetes mellitus ketoacids (acetoacetate, beta hydroxy-butyric acid and acetone) are produced excessively which are acidic in nature. Bicarbonate is consumed for the buffering of the excess acids. This in turn leads to decrease in the ratio of bicarbonate- carbonic acid ratio resulting in drop in pH.

Compensatory response is hyperventilation caused by stimulation of respiratory centre by low pH which in turn helps raising the pH towards normal by lowering pCO_2.

Causes of metabolic acidosis: Normally the sum of anions is equal to the sum of cations. But it is not possible to measure all the anions .Hence there is a difference between measured cations and anions which is described as anion gap.

Anion gap = $[Na^+ + K^+] - [HCO_3^- + Cl^-]$

The apparent gap is due to proteins, sulfates and phosphates in a healthy person .The **normal anion gap is 7–16 mmol/L**

In this case the anion gap is 20. Therefore, it is a case of high anion gap acidosis due to the abnormal accumulation of anions (ketone bodies)

Different types of metabolic acidosis can be differentiated on the basis of anion gap.

1. High anion gap acidosis

Ketoacidosis – starvation, diabetes mellitus
Renal failure → uremia
Lactic acidosis, Salicylate toxicity

2. Normal anion gap acidosis

Gastrointestinal fluid loss – diarrhea
Renal tubular acidosis

25B. JAUNDICE

Q. 25B-1

Resmi, 18 years staying in a college hostel, brought to the outpatient clinic of medical college complaining fever, head ache, nausea and yellowish discoloration of sclera O/E: Febrile, jaundice + Liver palpable

Laboratory data:

Blood		**Reference values**
Total bilirubin (TB)	6 mg%	(0.3–1.2 mg/dl)
Conjugated bilirubin (CB)	2.6 mg%	(0.1–0.4 mg/dl)
ALP	200 IU/L	(40–125 IU/L)
ALT (SGPT)	- 80 IU/L	(10–35 IU/L)
AST (SGOT)	- 70 IU/L	(8–20 IU/L)
Urine		
Bile salts	- Positive	
Bile pigment	- Positive	
Urobilinogen	- Trace	

What kind of illness, the girl is suffering from? Evaluate the clinical condition by the laboratory data provided?

Ans. 25B-1:

The girl is suffering from hepatic jaundice.

The **clinical features** fever, headache and nausea are suggestive of an infection and the finding of liver enlargement with yellowish discoloration of sclera suggests hepatic jaundice.

Laboratory data confirms the hepatic origin of jaundice.

Serum bilirubin levels: Elevated in **TB** levels (> 2.0 mg%) suggest jaundice. Hepatocyte dysfunction affecting the glucoronyl transfease activity has caused elevation of unconjugated bilirubin **(UCB)** (6-2.6 = 3.4 mg%) and the delayed clearance of **CB** due to blockade of biliary micro channels by inflammation leading to a slight hike in its level.

Serum enzymes: Rise in **transaminase** shows injury to hepatocyte and its release from the cytoplasm of hepatocytes due to infection. Slight elevation of **alkaline phosphatase** points towards the release of membrane bound ALP resulting from the pressure effect produced by inflammatory swelling of biliary lining cells caused by infection.

Urinary findings of **positive bile salts** and **bile pigments** again indicate that the patient is in the obstructive phase of hepatic jaundice (i.e. infection causing inflammation of lining cells of biliary canaliculi causing regurgitation of biliary contents into the blood stream. When the blood levels of these compounds crosses the renal threshold for that substance, it get excreted in urine – thus CB and bile salts are excreted in urine)

Urobilinogen is in trace means not strongly +ve indicates that it is not supporting any hemolytic disease. The test for urobilinogen is not negative suggestion that there is no severe obstruction as in biliary stone, strictures to cause complete obstruction of biliary flow into the intestine.

How to differentiate hepatic jaundice from obstructive jaundice due to stones, tumors or other obstructions in the biliary tract?

- **Serum bilirubin values:** In obstructive jaundice the level CB will be much higher than the hepatic jaundice and UCB values remain within normal limits.
- **Enzymes:** Transaminase values generally remain within normal range but ALP values will be very high in obstructive jaundice.
- **Urinary findings:** Bile salts + ve and CB + ve and urobilinogen will be absent. Due to biliary obstruction CB cannot reach the intestine in obstructive jaundice and hence urobilinogen cannot be formed as in the normal situation. Urine will be giving –ve response to Ehrlich's test (the test for urobilinogen) and the patient will complain of passing clay coloured stools (due to the absence of stercobilinogen in feces).

Q. 25B-2

Kurinji, 45 years old woman, a tribal hailing from Waynaud district came with severe tiredness and severe pain all over the body O/E: Pallor+, jaundice +, Hepatosplenomegaly +. Based on

clinical and laboratory data what is your provisional diagnosis? What other tests do you require to confirm the diagnosis?

Laboratory data:

Blood

Hb	7 g%	
Reticulocytes	10%	
Sickling test	+ve	
Total bilirubin (TB)	10 mg%	(0.3 – 1.2 mg/dl)
Conjugated bilirubin(CB)	0.6 mg%	(0.1 – 0.4 mg/dl)
Unconjugated bilirubin	9.4 mg%	(0.2 – 0.7 mg/dl)
ALP	45 IU/L	(40 – 125 IU/L)
ALT (SGPT)	14 IU/L	(10-35 IU/L)
AST (SGOT)	20 IU/L	(8 – 20 IU/L)

Urine

Bile salts	- Negative
Bile pigment	- Positive
Urobilinogen	- Strongly positive

Ans. 25B-2:

The woman is suffering from hemolytic jaundice probably due to sickle cell disease.

Total bilirubin and UCB (10 -0.6 = 9.4 mg%) are high – suggesting increase in bilirubin not due to any obstruction in the biliary passages.

Serum enzyme studies show normal activities indicating that hepatocytes are not involved in the disease process there by excluding hepatic jaundice.

Bile salts negative and this shows that the jaundice is not due to obstructive type.

Urobilinogen strongly + ve - it is due to increased rate of RBC break down producing maximum amount of conjugated bilirubin getting secreted into the intestine and converted to urobilinogen in increasing amounts which then absorbed from the intestine into the blood and excreted in urine excessively. The positive sickling test, tribal origin of the woman and the kind of pain suggestive of sickling crisis and strongly suggests sickle cell disease.

It is to be confirmed by **Hb electrophoresis** to show the presence of Hb S, if possible **globin chain separation** to demonstrate abnormal 'S" globin chain.

Q. 25B-3

Meenakshi, 58 year old woman c/o pain in the upper right side of the abdomen, fever with chills,pruritus and passing dark urine and clay coloured stools. O/E: Jaundice +, scratch marks on the skin +, Fever + No other findings obtained. From the following laboratory data given an explanation about her disease.

Laboratory data:

Blood

Total bilirubin (TB)	- 12 mg%	(0.3–1.2 mg/dl)
Conjugated bilirubin (CB)	- 10 mg%	(0.1–0.4 mg/dl)
ALP	- 200 IU/L	(40–125 IU/L)
ALT (SGPT)	- 30 IU/L	(10–35 IU/L)
AST (SGOT)	- 18 IU/L	(8–20 IU/L)

Urine

Bile salts	- Positive
Bile pigment	- Positive
Urobilinogen	- Negative

Ans. 25B-3:

The lady is suffering from obstructive jaundice (cholestasis).

Total bilirubin and CB is high – suggesting obstruction in the biliary passages leading to cholestasis.

Serum enzyme studies shows high **ALP** indicating obstructive type of jaundice and normal **transaminases** giving an idea that hepatocytes are unaffected by the disease process.

Urine – Test for Bile salts (Hay's test) +ve – supporting the diagnosis of obstructive jaundice. Obstruction of biliary passages causing stasis of

it's contents leading to regurgitation of it's constituents into the blood there by elevating the concentration of CB and bile salts in the blood. Bile salt has a tendency to get deposited in the skin causing intense pruritus and CB will be excreted in the urine giving the test for **CB (Modified Fouchet's test) as positive.**

Test for urobilinogen is negative – Due to obstruction in the biliary passages biliary contents cannot reach the intestine there fore intestinal bacteria cannot act upon CB to form bilinogens (urobilinogen and stercobilinogen) leading to Ehrlich's test for urobilinogen negative and clay colored stools.

Q. 25B-3

Serum bilirubin (mg%)	*ALP (IU/L)*	*Transaminases (IU/L)*	*Urine-bilirubin*	*Urine-bile salt*	*Urine-urobilinogen*	*Comment on the condition*
TB – 8 CB – 3.5 UCB – 4.5	150 IU/L	ALT- 60 AST - 50	Present	Present	Trace	a) ?
TB – 7 CB – 0.3 UCB – 6.7	88 IU/L	ALT- 25 AST - 15	Absent	Absent	Increased	b) ?
TB – 0.8 CB – 0.3 UCB – 0.5	60 IU/L	AST - 10 ALT- 20	Absent	Absent	Trace	c) ?
TB – 14 CB – 13 UCB – 1.0	280 IU/L	AST - 18 ALT- 30	Present	Present	Absent	d) ?

Ans. 25B-4:
(a) Hepatic jaundice (b) Hemolytic jaundice (c) Normal (d) Obstructive jaundice.

25C. DIABETES

Q. 25C-1
A 50-year-old executive attended out patient clinic with complaints of passing large volumes of urine at frequent intervals, unusual thirst and overeating and a feeling of weight loss for the past 3 months. Interpret the results. What other tests you like to do?

Urine
Dipstick test for glucose - Positive

Blood
Fasting plasma glucose - 150 mg% (8.3 mmol/L)
2- hour post load plasma glucose concentration - 250 mg% (13.88 mmol/L)
HbA_{1c} - 9.5%

Ans. 25C-1:
The person is suffering from diabetes mellitus. He is experiencing classical symptomatology of diabetes mellitus – polyuria, polydypsia, polyphagia and weight loss.

The laboratory data support the diagnosis. As per American Diabetes Association –Adult Treatment Panel III (ATP III) guidelines, the diagnosis of DM can be made if any of the following is there:

1. Classic symptoms of diabetes mellitus and random (regardless of the time of the preceding meal) plasma glucose concentration ≥ 200 mg% (11.1 mmol/L)
2. Fasting plasma glucose (FPG) ≥ 126 mg% (7 mmol/L)
3. 2-hour post load plasma glucose concentration ≥ 200 mg% (11.1 mmol/L)

In the above case FPG and 2-hour post load plasma glucose have crossed the normal limits. So the diagnosis of Diabetes mellitus can be made.

The following test will help better evaluation regarding the diabetic state of the individual.

1. **HbA_{1C} –** Give an idea about the glucose level in the previous 3 months and also it can serve as an index of long-term glycemic control.

During consistent elevation of plasma glucose, hemoglobin undergo nonenzymatic glycation at an increased rate. So the level of HbA_{1c} give an idea about the glucose level over the previous 3 months (since the lifespan of RBC is 120 days).

The desired level of HbA_{1C} is 7.0%.

Roughly the plasma glucose levels corresponding to HbA_{1C} concentrations are given below:

HbA_{1C} (g%)	Plasma glucose (mg%)
6	135
7	170
8	205

NB: 1% rise in HbA_{1C} corresponds to 35 mg% rise in plasma glucose

2. **Lipid profile**

Total cholesterol, HDL – Cholesterol, LDL-Cholesterol and triglycerides will help estimating the risk of developing atherosclerotic vascular disease. If the levels are not within desirable limits, lipid lowering drugs should be started.

Desirable levels of lipid parameters

TC	< 200 mg%
HDL-C	> 40 mg% in men; > 50 mg% in women
LDL-C	< 100 mg%
Triglycerides	< 150 mg%

3. **C- Peptide assay –** Though not routinely done for routine management of patients with diabetes, it is important in clinical research.

- It can give an idea about the insulin secretion capacity of an individual.
- It gives an estimate of endogenous insulin secretion in patients on insulin therapy. C-peptide (connecting peptide) is produced during post-translational modification of endogenous proinsulin and it is not present in commercial insulin preparations of insulin.

Q. 25C-2

Two brothers Peter and Joseph aged 44 and 38 came to attend a preventive health check up camp because of strong family history of diabetes mellitus. They were examined and after preliminary investigations they were undergone oral glucose tolerance test. The relevant details of the test report are given below. Give your interpretation and guidelines if any, for further follow-up.

	Peter (44 years)	Joseph (38 years)
Urine dipstick test for glucose	Negative	Negative
Fasting plasma glucose	118 mg% (6.55 mmol/L)	115 mg% (6.38 mmol/L)
2-hour post load plasma glucose concentration during OGTT	138 mg% (7.66 mmol/L)	150 mg% (8.33 mmol/L)

Ans. 25C-2:

Peter is suffering from impaired fasting glucose (IFG).

IFG is a stage of impaired glucose homeostasis. To diagnose IFG, the glucose values should be as shown below.

- Fasting plasma glucose concentrations of 100 – 125 mg% (5.6 - 6.9 mmol/L)
- 2 hour plasma glucose < 140 mg%

Joseph is suffering from impaired glucose tolerance (IGT).

IGT is a stage of impaired glucose regulation manifesting biochemically as glucose tolerance above the normal limits but lower than the cut off values to diagnose diabetes mellitus.

To diagnose IGT the following two criteria must be met

- Fasting blood glucose <126 mg% (7 mmol/L)
- 2-hour post load plasma glucose concentration during OGTT between 140 and 199 mg% (7.77 mmol/L and 11.05 mmol/L)

IGT is a transient stage between normal glucose tolerance and type II diabetes mellitus.

Significance of recognizing IFG and IGT: Both IFG and IGT are associated with increased risk of developing diabetes in future. Risk is more when both coexist in one individual.

Lifestyle modifications (exercise, healthy food habits) are effective in delaying or even preventing the onset of diabetes in persons with IFG and/or IGT.

Indications for OGTT

1. Diagnosis of gestational diabetes mellitus (GDM)
2. Patients having symptoms suggestive of DM but fasting blood sugar values inconclusive between 100-126 mg%
3. Pregnant ladies with past history of big baby more than 4 kg or a past history of miscarriage
4. To rule out benign renal glucosuria
5. For the diagnosis of IGT.

TEST:

Instructions:

- 3 days of unrestricted carbohydrate diet (>150 g) to sensitize the β-cells of pancreas
- Test is done in the morning (between 7 and 9 hours) after 10 -12 hours of overnight fast (intake of water is permitted)
- Smoking and physical activity should be avoided.

Procedure:

- Patient should be seated comfortably
- Withdraw blood in the fasting state for testing plasma glucose level
- Give a glucose load 75 gm for adults and 1.75 g/Kg body weight for children (maximum of 75 g) dissolved in 300 ml of water and drunk within 5 minutes. Then every 30 minutes withdraw blood for 2 hours after the glucose load.

Urine may be collected with every blood sample collection to test the presence of glucose.

To rule out diabetes mellitus

- FPG < 100 mg% and 2 hours plasma glucose after an ordinary meal <140 mg%
  If the values are not conclusive go for an OGTT
- FPG < 100 mg% and 2 hour plasma glucose <140 mg% with an OGTT

Q. 25C-3

Mary, 42-year-old working woman have undergone a health check up to join a health insurance scheme. A part of the investigation report is given below for evaluation. What is your impression? Is there any additional test required?

Urine

Dipstick test for glucose (fasting and post glucose load urine specimens) - Negative

Blood

Fasting plasma glucose - 118 mg%

Ans. 25C-3:

Fasting plasma glucose value suggest impaired fasting glucose (which is considered to be a forerunner of type 2 diabetes mellitus).

Further evaluation need study of tolerance of glucose under standard conditions – i.e. Oral glucose tolerance test (OGTT).

Q. 25C-4

Suhara 35 years old obese (Ht: 160 cm, Wt: 72 kg)pregnant lady visited Obstetrics and Gynaecology OPD in the **second semester** for check up. It was her second pregnancy. She told that her first baby weighed 4.3 kg and her mother died of diabetes mellitus. She had no other complaints and on examination also she appeared normal except for the obesity. What should be done for her?

Ans. 25C-4:

Being obese, having told the history of giving birth to an overweight baby and family history diabetes mellitus, Suhara is at risk of having gestational diabetes mellitus (GDM).

She should be screened for GDM.

Screening for GDM should be routinely done on all pregnant women ≥ 25 years or < 25 years with any one risk factor .

For that administer **50 g** oral glucose at any time of the day without any regard to food intake. Withdraw blood **after 1 hour** of 50 g glucose load.

If plasma glucose is < 140 mg % tolerance to glucose can be considered to be normal and if ≥ 140 mg% (7.7 mmol/L), further evaluation by glucose tolerance test is required.

OGTT in Pregnancy

- Carry out in the morning after 8–14 hour fast
- Withdraw fasting blood sample
- Administer 100 g glucose orally in 300 ml of water
- Withdraw blood at 1 hour interval for 3 hours after glucose load and check the concentration of glucose in the plasma
- A least 2 values should go beyond the limits shown below
  Fasting – 95 mg%
  1 hour – 180 mg%
  2 hour – 155 mg%
  3 hour – 140 mg%
- If the results are within normal limits but clinically suggestive of GDM, repeat the test in the third trimester also.

Q. 25C-5

A Krishnan, 75-year-old senior citizen, a retired bank officer came to Medicine OPD with the following results after a routine check up.

Urine

Dipstick test for glucose - Positive

Blood

Random plasma glucose - 164 mg% (9.1 mmol/L)

Upon going through the laboratory data, he was advised to undergo OGTT. The report is given below:

Plasma glucose		*Urine - Dipstick test for glucose*
Fasting	102 mg% (5.66 mmol/L)	Negative
½ hour	178 mg% (9.88 mmol/L)	Positive
1 hour	168 mg% (9.33 mmol/L)	Positive
1 ½ hour	158 mg% (8.77 mmol/L)	Positive
2 hour	108 mg% (6 mmol/L)	Negative

Go through the laboratory results and give your interpretation and the advice.

Ans. 25C-5:

Mr. Krishnan is suffering from **renal glucosuria.**

The first report showed random plasma glucose 164 mg% which is within normal limits.

But at that plasma glucose value his urine showed positive response to glucose. It can happen when the renal threshold for glucose diminishes with age. Normally the renal threshold for glucose is 180 mg%. When it is lowered the glucose start appearing in urine at lower plasma glucose levels as in this case.

This assumption has to be confirmed by OGTT. OGTT showed passing of glucose in urine even when the plasma glucose level is 158 mg% (at 1½ hour) which is much lower than the normal renal threshold for glucose.

Q. 25C-6

Meenakshi, 72 years who has been on oral antidiabetic drugs for more than 20 years attended the casualty unit of Medical College with complaints of fatigue, sweating , palpitation and tremors. Upon examining the patient quickly, a sample of blood was withdrawn for plasma glucose estimation and the patient was given 2 doses of 100ml of 25% dextrose at 15 minutes interval. The patient showed dramatic improvement. The laboratory report came afterwards which is shown below. Give your diagnosis and explanation of the clinical situation.

Blood

Plasma glucose - 45 mg% (2.5 mmol/L)

Ans. 25C-6:

The patient was suffering from hypoglycemia because of the following reasons:

1. She had been on antidiabetic drugs. Her advanced age and long standing diabetes might have caused impairment of renal function to cause diminished clearance of the antidiabetic drugs. It has led to the over action of glucose lowering effect.
2. Symptoms were neuroglycopenic in nature resulting from glucose deprivation of central nervous system.
3. Plasma glucose value is suggestive of hypoglycemia.

The lower limit of fasting plasma glucose is normally around 70 mg% (3.9 mmol/L). Glucose levels less than 55 mg% with symptoms which are relieved promptly after the glucose intake can be documented as hypoglycemia.

Hypoglycemia triggers sympathoadrenal discharge. This leads to adrenergic symptoms mediated by norepinephrine released by postganglionic neurons and epinephrine released from adrenal medulla-(palpitation, tremor and anxiety). Cholinergic symptoms (sweating, hunger and paresthesias) are mediated by acetylcholine released from sympathetic postganglionic neurons. These symptoms can be declared to be due to hypoglycemia only when they are associated with low plasma glucose concentration and the disappearance of such symptoms after the glucose level is raised. This is referred to as Whipple's triad which constitute the following:

1. Symptoms suggestive of hypoglycemia
2. Low plasma glucose concentration
3. Relief of symptoms after the glucose level is raised

Demonstration of a normal plasma glucose concentration when the patient manifests symptoms **excludes** the possibility of a hypoglycemic insult.

Q. 25C-7

A person lying on the street in a comatosed state was brought to the casualty by some people unrelated to the patient. Papers from his pocket showed that he was a diabetic on oral antidiabetic treatment

O/E : Comatosed, intense smell of alcohol +

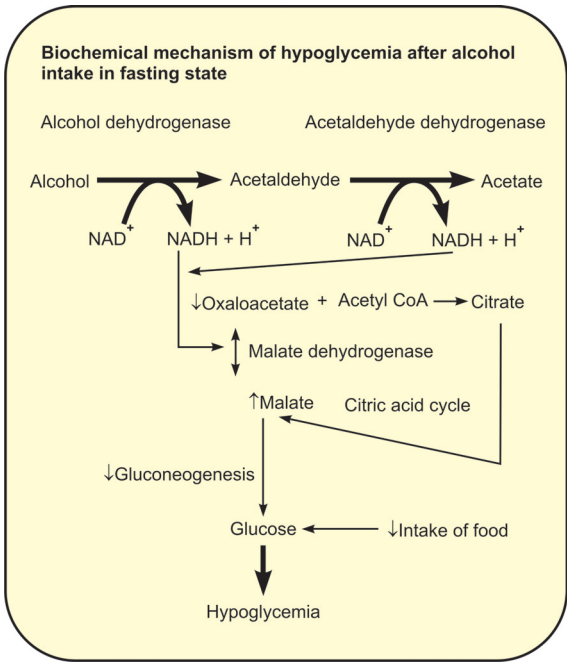
Blood is taken for preliminary investigations and for plasma glucose estimation

Plasma glucose - 45 mg% (2.5 mmol/L)

What is your diagnosis? Is alcohol consumption related to his illness? Explain.

Ans. 25C-7:

Diagnosis: Hypoglycemic coma



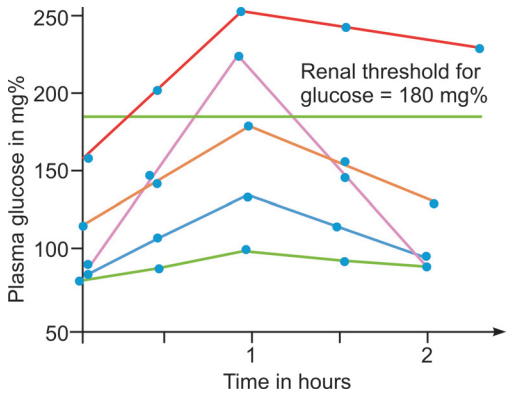
Excessive alcohol consumption by a person who is skipping meals can lead to hypoglycemia. During oxidation of alcohol NAD^+ is reduced to $NADH^+ + H^+$ causing increased NAD/NADH ratio which in turn inhibit the formation of oxaloacetate, there by diminishing the concentration of OA available for gluconeogenesis. Along with diminished endogenous production of glucose and reduced intake cause lowering of blood glucose leading to hypoglycemia.

Q. 25C-8

OGTT performed in different clinical situations are given below as line graphs in separate colors. Identify the clinical situation attributable to each graph line.

Ans. 25C-8:

Diabetes mellitus: FPG level is more than the normal FPG (Normal FPG : < 110 mg%) and 2 hour post load plasma glucose is > 200 mg%.



Lag curve (Alimentary glycosuria): Exaggerated rise in blood glucose following an oral glucose load and rapid fall in its level in the blood to touch the normal level at 2 hours. Transient glycosuria occurs due to the peak level crossing the renal threshold for glucose.

Renal glycosuria: It is seen due to lowered renal threshold for glucose, caused by reduced renal tubular reabsorption of glucose.

Flat curve (increased glucose tolerance): Very little rise in blood glucose levels after glucose load. It is seen in patients with hypoactivity of some endocrine glands, e.g. hypopituitarism, Addison's disease and in malabsorption.

Normal: Glucose level within normal limits.

25D. IN BORN ERRORS OF METABOLISM

Q. 25D-1

3 months old home delivered male baby, brought to the pediatrics OP. O/E : Appearance : doll like face with fat cheeks, thin extremities, protuberant abdomen; system examination – massive hepatomegaly, enlarged kidneys, spleen and heart are not enlarged; Histopathological examination of biopsy of the liver: distended hepatocytes with glycogen and lipid vacuoles.

Laboratory report

Blood

Fasting plasma glucose	- 35 mg%	Reference limit (60–100 mg%)
ALP	- 40 IU/L	(40–125 IU/L)
ALT	- 140 IU/L	(40–125 IU/L)
AST	- 26 IU/L	(8–20 IU/L)
Uric acid	- 10 mg%	(2–5.5 mg%)
TG	- 250 mg%	(30–100 mg%)
Cholesterol	- 300 mg%	(114–203 mg%)
Lactate	- 90 mg%	(normal–4.5–20 mg%)

Urine: Dipstick test for glucose–Negative

Based on clinical and laboratory data what is your diagnosis and explain. Suggest further tests required for this case?

Ans. 25D-1: von Gierke's disease (Fig. 25D-1)

It is a inherited disorder of carbohydrate metabolism in which **glucose 6 phosphatase enzyme is deficient** in liver, kidney and intestine. This leads to **inadequate conversion of glucose 6 phosphate to glucose.** Normally during short fasting, liver glycogen breakdown to release glucose 6 phosphate which is acted upon by glucose 6 phosphatase to release glucose into the blood to maintain blood glucose levels within normal limits. But in von Gierke's disease due to the deficiency of glucose 6 phosphatase , glucose is trapped in the tissues in the form of glucose 6 phosphate and hence glucose cannot be released into the blood to prevent it's level going low. This leads to hypoglycemia after a short fast. Glucose 6 phosphate trapped in the tissues pass through Hexose Monophosphate pathway excessively so that surplus amount of NADPH + H^+ and pentoses are produced which favor the excessive synthesis of purine nucleotides and their break down to cause elevated uric acid. The uric acid passing through the glomerular filtrate compete

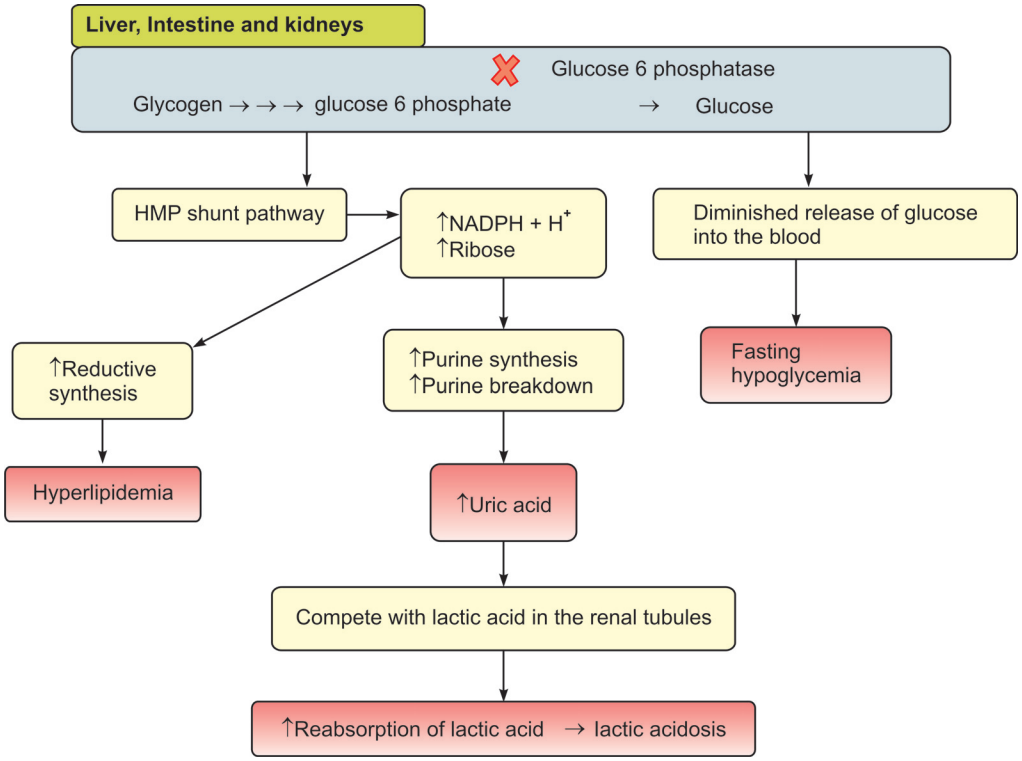


Fig. 25D-1: Biochemical derangements in von Gierke's disease

with lactic acid and minimize it's excretion leading to retention of lactic acid and lactic acidosis. Due to very active Hexose Monophosphate pathway, a part of the reducing equivalents produced in excess amounts are diverted to reductive synthesis (fatty acid and cholesterol synthesis → hyperlipidemia).

Definitive diagnosis:

1. **Liver biopsy:** Enzyme assay in the hepatic tissue; Low glucose 6 phosphatase
   HPE: Distended hepatocytes with glycogen and lipid vacuoles.
2. **Noninvasive method:** Gene based mutation analysis

Q. 25D-2

A male baby aged 6/12 referred from peripheral hospital, presenting with vomiting, jaundice and failure to thrive admitted to pediatric ward of Medical College. Examination revealed cataract in both eyes and hepatomegaly.

Laboratory report

Blood

Plasma galactose - ↑
Plasma glucose - Normal

Urine

Galactose - Positive

Based on clinical and laboratory data what is your diagnosis?

Ans. 25D-2:

Galactosemia: Severe form of galactosemia is due to deficiency of galactose 1 phosphate uridyl transferase leading to accumulation of galactose 1 phosphate in the liver. This will in turn inhibit galctokinase and glycogen phosphorylase. Inhibition of galactokinase will cause accumulation of free galactose and its reduction to dulcitol. Dulcitol due to the osmotic effect causes cataract. The inhibition of glycogen phosphorylase will cause accumulation of glycogen leading to hepatomegaly. The bilirubin uptake and conjugation is affected to cause unconjugated hyperbilirubinemia and jaundice.

Q. 25D-3

A 30 years male attended OPD of orthopedics department with complaints of low back pain, stiffness of knee joint, greyish discoloration of helix of the ear and darkening of urine on exposure to air.

Lab report:

Urine

Ferric chloride test - Positive
Benedict's test - Positive

Urine – developed black color upon keeping in a test tube.

What is your possible diagnosis?

Ans. 25D-3:

Alkaptonuria: It is a autosomal recessive condition due to the deficiency of homogentisate oxidase leading to accumulation of homogentisic acid and it's consequent oxidation by polyphenol oxidase to benzoquinone acetate which undergo polymerization to form black colored alkaptone bodies. These alkaptone bodies get deposited in cartilages and joint surfaces to cause discoloration and arthritis.

Q. 25D-4

1 year old female child with c/o delayed mile stones, hyperavtivity, tremors, convulsions and one or two hypopigmented areas.

O/E Delayed mile stones, MR +, mousy odor +. What is your clinical diagnosis? What all tests are needed to make a diagnosis?

Ans. 25D-4: Phenylketonuria (PKU)

Blood

Guthrie test
Blood phenylalanine

Urine

Ferric chloride test

Guthrie test and ferric chloride test will be positive and blood phenylalanine will be raised

to become > 20 mg% (Normal blood phenylalanine – 1 mg%)

More about PKU: It is due to deficiency of phenylalanine hydroxylase enzyme as a result of genetic mutation. Due to this phenylalanine cannot be converted to tyrosine and the level of Phenyl in the blood increases. Phenylalanine passes through aberrant pathways to produce **phenyl ketones** excessively to produce ketosis and mousy odor to the baby. Early recognition of the disease and low phenylalanine diet can minimize the mental retardation.

Q. 25D-5

Mallika, 32 years who is hypopigmented attended ophthalmology clinic with defective vision and feeling discomfort in the eye especially during day time. What is the possible diagnosis and the biochemical abnormality of this disease?

Ans. 25D-5:

Albinism: Normal skin color is due to the effect of four biochromes –

1. Melanin (major determinant)
2. Reduced hemoglobin (blue)
3. Oxyhemoglobin (red)
4. Carotenoids (yellow – exogenous from diet)

Melanin being the major determinant of the skin color, the variations in the amount and distribution of melanin in the skin forms the basis of three common human skin colors – black, brown and white.

Albinism may be due to either absence of tyrosinase (tyrosinase negative albinism) or deficiency of tyrosinase in melanocytes leading to failure of melanin synthesis in the epidermis, hair bulb and eye. It is inherited as autosomal recessive manner. Prevalence 1/20000.

C/F: Skin is white or pink, hair white and pigmentation is lacking in the eye. Poor eye sight, photophobia, nystagmus are common. In the tropics albinos are prone for photo ageing and squamous cell carcinoma at an earlier age.

Diagnosis: Straight forward due to the presence of hypopigmentation.

Histology shows clear cells in the basal layer that fail to stain with Fontana. In tyrosinase positive albinism dopa reaction is positive(shows deficiency of tyrosinase) and in tyrosinase negative albinism dopa reaction is negative (shows absence of tyrosinase).

Q. 25D-6

A 6 years mentally disabled male child with defective vision was admitted to Ophthalmic ward and further examination.O/E: MR +, Eyes myopia, ectopia lentis (dislocation of lens) +, glaucoma +; Skeletal system: features of severe osteoporosis + (knock knee, genu valgum, vertebral and foot deformities) Vascular system: Cardivascular lesions +.Give your provisional diagnosis based on these features. What further supporting biochemical test you need to establish the diagnosis?

Ans. 25D-6: Homocystinuria

Biochemical Test: Silver nitroprusside test done on urine sample will give a magenta colour if the sample is positive for homocystine.

Further confirmation may be done by measuring enzyme activities in fibroblasts or lymphocytes if such laboratory facilities are available.

More about homocystinuria: Inherited homocystinuria is mostly due to **cystathionine β synthase** deficiency causing elevated plasma levels of methionine and homocysteine and their increased excretion in urine. Homocyseine accumulated forms disulfide bonds between two molecules of homocysteine to form **homocystine** which is the predominant form seen in plasma and urine. Accumulation of homocysteine causes disruption of collagen and elastin synthesis causing widespread manifestations in the body. Reduced myelination in the brain leads to mental retardation.

Biochemical basis of treatment: Diet low in methionine and rich in cysteine is recommended. Some times pyridoxal phosphate in high doses will relieve symptoms.

Q. 25D-7

A three years old baby referred from peripheral hospital to Pediatric casuality of Medical college with complaints of seizures, lethargy, vomiting and a peculiar smell of burnt sugar. Acid base blood analysis revealed acidosis. What is the possible diagnosis? What all biochemical tests will help you in the diagnosis?

Ans. 25D-7: Maple syrup urine disease (MSUD)

Biochemical Test:

1. Dinitrophenylhydrazine test performed on urine specimen will give a yellow white precipitate
2. Ferric chloride test - Gray blue color

More about MSUD:

It is due to defective **branched chain keto acid decarboxylase enzyme deficiency**. It is inherited as an autosomal recessive disorder. Incidence of this disorder is about 1/200,000. Reduced oxidative decarboxylation of branched chain amino acids (leucine, valine and isolecine) due to branched chain keto acid decarboxylase enzyme deficiency result in accumulation of these amino acids and their α keto or α - hydroxy acid derivatives causing metabolic acidosis.

Q. 25D-8

A Filipino tourist aged 40 years, came to attend a private hospital with complaints of abdominal pain and diarrhoea. He said that he had taken milk based food items on the previous day. He recalled that he had similar episodes in the past upon taking diary products in mild form. What is your assumption about the disorder the person suffering from?

Ans. 25D-8: Lactose intolerance due to intestinal lactase deficiency

Biochemical Test:

1. Oral lactose tolerance test: Patient is asked to come in the morning after a 8 – 10 hours fast. Fasting blood sample taken for glucose estimation. Patient is given 50 g oral dose of lactose. Blood samples are withdrawn at 5, 10, 15, 30, 45 and 60 minutes after ingestion. An increase in blood glucose concentration of at least 25 mg% over the fasting glucose concentration indicates, normal intestinal lactase activity (lactase acting on lactose to produce glucose and (galactose which are absorbed into the blood). Patients with intestinal lactase deficiency will not show any increase in glucose concentration after lactose administration.

2. Direct evidence by histochemical studies of intestinal villous biopsy: Reveal no lactase activity in cases of lactose intolerance. This is not routinely done.

More about lactose intolerance: Lactase deficiency is the most common disorder of carbohydrate digestion. It may be congenital or aquired.Infants will manifest as profuse diarrhea on introduction of milk. Acquired variety which will be manifested later in life is common among Ashkenazi Jews, Arabs, Filipinos and Japanese.

Biochemical derangements: Due to lactase deficiency, the lactose ingested remains in the intestinal lumen causing increase in the osmotic pressure of the intestinal contents. Lactose is fermented by intestinal bacteria in the lower intestine to produce gas, lactic acid and fatty acids which also exert osmotic effect causing extrusion of water into the intestinal lumen causing diarrhoea.These lead to abdominal distension, flatulence, abdominal pain, diarrhoea, dehydration and electrolyte disturbances.

Biochemical principle of treatment: Avoidance of diary products.

Q. 25D-9

A Filipino tourist aged 40 years, came to attend a private hospital with complaints of abdominal

pain and diarrhea. He said that he had taken milk based food items on the previous day. He recalled that he had similar episodes in the past upon taking diary products in mild form. What is your assumption about the disorder the person suffering from?

Ans. 25D-9:

Tay-Sachs disease: It is a rare inherited autosomal recessive ganglioside storage disorder. Deficiency of **hexosaminidase** will cause lysosomal deposition of ganglioside in glial and ganglion cells causing cell death.

- Demyelination of white matter and spinal cord leading neurological manifestations.
- Neuron destruction leading to retinal thinning (produces cherry red spot detectable by fundus examination) and blindness.

Tay Sachs disease is diagnosed by **lowered activity of hexosaminidase A** in the blood and identification of **cherry red spot** in the fundus by ophthalmoscopic examination.

Q. 25D-10

A 40-year-old man referred from periphery to Dermatology OP as a case of pellagra complaining of head ache and unsteadiness of gait for further investigations.

O/E: Erythematous eczematous skin lesions +, ataxia +.

Investigations: Urine for aminoacid analysis : neutral aminoaciduria

What is your diagnosis?

Ans. 25D-10:

Hartnup disease

Incidence: 1/24000

Mode of Inheritance: Autosomal recessive

Defect: **Defective neutral amino acid transporter** located in the renal tubular and intestinal epithelial cells.

C/F: Pellagra like skin lesions, variable neurological manifestations and passing excessively neutral **amino acids** (alanine, serine, threonine, valine, leucine, isoleucine, glutamine, asparagine) and aromatic (phenyla alanine, tyrosine, tryptophan and histidine) amino acids in urine.

Diagnosis: Demonstration of neutral and aromatic aminoacids in urine by chromatography.

Q. 25D-11

A boy aged 7 years came for consultation to Pediatric OP with complaints of abnormal movements, tendency for self mutilation and joint pains. Investigations showed high uric acid levels in the blood and low activity of hypoxanthine guanine phosphoribosyl transferase deficiency. What is your diagnosis?

Ans. 25D-11: Lesch-Nyhan syndrome

Mode of Inheritance: X-linked recessive affecting males only

Defect: Defective hypoxanthine guanine phosphoribosyl transferase of salvage pathway so that feed back inhibition of denovo synthetic pathway become reduced leading to excessive production and break down of purine nucleotides leading to hyperuricemia.

C/F: Impaired motor function, athetosis and abnormal mental status – violent behavior and self mutilation.

Q. 25D-12

A man aged 58 years came with history of recurrent attacks of joint pain especially at big toe. On examination his right metatarso phalangeal joints are swollen and tender.

On investigation uric acid level was found to be 12 mg%. What is your diagnosis?

Ans. 25D-12:

Adult gout: It is metabolic disease affecting middle aged – elderly men and women due to increased body pool of urate. It is characterized by episodic joint pains due to deposition of monosodium urate crystals in joints and

connective tissue (*tophi*) and urate stones in the kidneys (nephrolithiasis).Metatarsophalangeal joint of big toe is first affected.

Investigations: Serum uric acid will be elevated.

Aspiration of affected joints or tophaceous deposits → needle shaped strongly negative birefringent crystals on polarized light microscopy.

25E. PORPHYRIAS

Q. 25E-1

Kumar, 40 years was brought by his wife to the outpatient clinic of Medical College with complaints of recurrent attacks of abdominal pain, head ache, palpitation and unpredictable behavior. His wife recalled that his illness got aggravated whenever he takes alcohol. The following test in the urine was done. Give your most probable diagnosis? Explain the biochemical mechanism of the disease process.

Urine

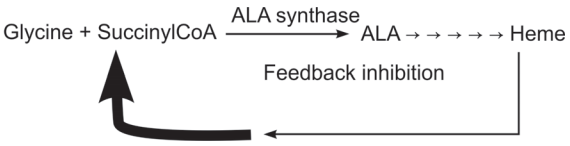
Watson-Schwartz test - Positive
for Porphobilinogen

Ans. 25E-1:

The clinical picture and urine test suggest the possibility of **acute intermittent porphyria** due to the deficiency of the enzyme hydroxy methylbilane synthase (Older names – PBG deaminase and uroporphyrinogen 1 synthase)

Porphyrias are rare disorders due to enzyme deficiencies of heme biosynthetic pathway and most often associated with specific mutations which show a **dominant mode of inheritance** except in the case of **congenital erythropoietic porphyria(CEP)**. CEP is inherited as autosomal recessive manner.

Mechanism of diseases process—Heme biosynthetic pathway is regulated by product feedback inhibition by heme of the **rate limiting enzyme ALA synthase**.



When any of the enzymes of the pathway is deficient, heme will not be formed in sufficient amounts to cause feedback inhibition of the key enzyme. Hence ALA synthase activity continue to produce precursors proximal to the deficient enzyme in excessive amounts. This kind of accumulation of precursors of the pathway cause different types clinical features (different types of porphyrias) based on the site of defect.

In this case urine was + ve for PBG. In the heme biosynthetic pathway, the PBG is the substrate of the enzyme hydroxymethyllbilane synthase. From this we could infer that PBG is accumulated in the blood and excreted in urine due to the deficiency of hydroxymethyllbilane synthase. The enzyme deficiency is detectable in erythrocytes.

Common precipitating factors include alcohol, exogenously administered steroid drugs and endogenously produced steroids, fasting.

Biochemical principle of treatment is to inhibit the key enzyme by giving heme.

Q. 25E-2

Revi, 43 years who used to attend the medical college hospital from childhood onwards with a progressive disease, came to attend medicine OP due to exacerbation of his illness.

O/E : Disfigurement of fingers and nose +
Reddish brown teeth which fluoresce on exposure to ultraviolet light
Skin lesions mostly on exposed parts of the body, Splenomegaly.

Investigations:

Blood

- ↑ Uroporphyrin I and coproporphyrin I
Uroporphyrinogen synthase activity - ↓

Urine
- ↑ Uroporphyrin I and ↑ coproporphyrin I

Comment on the type of illness the person suffering from?

Ans. 25E-2:
Congenital erythropoietic porphyria.

It is due to deficiency of uroporphyrinogen synthase activity. This is inherited as autosomal recessive manner and it is the most severe type of porphyria.

The disease can be detected in utero by analyzing the amniotic fluid porphyrins (type I) and uroporphyrinogen synthase activity.

Q. 25E-3
Mother noticed pale lips, fatigue of her 4 year old baby girl and brought her to a pediatrician. While examining the baby, the doctor noticed toy in the baby's hand which was notorious for high lead content. The mother told that the child was given treatment for intestinal worms and iron supplementation before. But the child remains pale. O/E: Pallor + No other findings.

Investigations:
Urine – delta aminolevulinic acid ↑
Erythrocyte Zinc protoporphyrin - ↑
Blood lead level – 50 µg/dl (normal – upto 25 µg/dl)

What is your diagnosis? Explain.

Ans. 25E-3: Lead poisoning (Plumbism)
Toxic effects of lead are due to its ability to inhibit ALA dehydratase and ferrochelatase and hence the substrates of these enzymes tend to accumulate. They are ALA and protoporphyrin respectively. Protoporphyrin combines with zinc to form zinc protoporphyrin (ZPP).

C/F: Behavioral changes-irritability, hyperactivity, learning disabilities or generalised symptoms like loss of appetite, nausea, muscle weakness, fatigue, pallor, headache.

Biochemical tests:
1. Urine - δ - amino levulinic acid
2. Erythrocyte zinc protoporphyrin
3. Blood lead assay

25F. VITAMINS AND MINERALS

Q. 25F-1
A women aged 47 years suffering from menoorhagia for the past one year came with complaints of tiredness, headache O/E: Pallor + and her hemoglobin = 6 g%. What is the problem with the women? Evaluate.

Ans. 25F-1:
The woman most probably suffering from **iron deficiency anemia** due to chronic blood loss for the past one year.

Chronic blood loss by hemorrhage (excessive menstruation, peptic ulcer, hemorrhoids) is the most common cause of iron deficiency among adults. Hookworm infestation is another major cause.

As iron deficiency develops, iron is released from storage compound ferritin. This will continue until iron stores are depleted leading to anemia.

C/F: Distorted appetite (pica) with cravings for ice, earth and in extreme cases spooning of finger nails (koilonychia) may occur and also Plummer-Vinson syndrome (partial occlusion of opening of esophagus).

Biochemical assessment of iron deficiency:
1. Ferritn assay in blood – Will be lowered (ferritin is the storage form of iron)
2. TIBC (Total iron binding capacity) – Will be raised

(TIBC is a measure of iron binding capacity of transferrin when fully saturated with iron. Normal range – 250 -450 µg/dL)

Q. 25F-2

Pokker, a business man, aged 52 years came with complaints of weariness, joint pains, darkening of skin color, yellowish discoloration of sclera O/E: Hyperpigmentation on the face, neck, extensor aspects of lower part of the forearms, dorsal aspects of hands, lower legs +, Hepatomegaly +, splenomegaly +, gynecomastia, testicular atrophy +

Investigations:		*Normal range*
Fasting Plasma Glucose: 140 mg%		
2 Hour Post Prandial Plasma Glucose: 240 mg%		
TB	3.0 mg%	
Direct bilirubin	1.2 mg%	
ALP	134	(40–25 IU/L)
AST	25	(8–20 IU/L)
ALT	40	(10–35 IU/L)
Serum iron µg/dl	200 µg/dl	(50–150)
TIBC µg/dl	150	(250–370)
Transferrin saturation %	90	(22–46)
Serum ferritin µg/L	- 5000	(20–250)

What is your impression?

Ans. 25F-2:

Hemochromatosis: Clinical features and investigations are supporting hemochromatosis.

It is a inherited disorder (autosomal recessive) of iron metabolism in which an inappropriate increase in iron absorption results in deposition of excessive amounts of iron in parenchymal cells leading to tissue damage and later organ dysfunction and are manifested as cirrhosis of liver, diabetes mellitus (due to pancreatic damage), arthritis, hypogonadism and cardiomyopathy.

Genetic basis: It is caused by mutant gene – most commonly by *HFE mutant gene.*

C/F: Hepatomegaly in the absence of any significant symptoms and with only minimal derangement of liver functions.

Excessive skin pigmentation (bronzing) is due to increased melanin and iron in the dermis. **Diabetes mellitus** occurs in more than 50 % of the patients which is due to direct damage of pancreatic islet cells by iron deposition.

Arthropathy is also common and the reason for its association with hemochromatosis is not clearly known.

Cardiomyopathy: Heart is diffusely enlarged and is prone to develop congestive cardiac failure.

Hypogonadism: Common in both sexes and is manifested as sparse body hair , impotence, gynecomastia and testicular atrophy in males and amenorrhea in females.

Biochemical tests for diagnosis: Ferrokinetic studies shown below are useful

1. Plasma iron - when it is between 180 – 300 mg/dl the disease will be symptomatic
2. TIBC - is decreased in hemochromatosis often approaches zero (Normal range - 250 – 370)
3. Percent Transferrin saturation (Serum iron/ TIBC × 100) - > 50%
4. Serum ferritin µg/L - >1000 indicate hemochromatosis

In this case, all the tests done are conforming to hemochromatosis.

Q. 25F-3

A 28-year-old man admitted with cirrhosis, neurological features suggestive of damage to basal ganglia, behavioral changes, Kayser-Fleischer ring in the cornea and sunflower cataracts for further investigation?

Based on these data what is your provisional diagnosis? What biochemical investigations are needed in this case?

Ans. 25F-3: Wilson's disease

Biochemical investigations required:

1. Serum ceruloplasmin (ceruloplasmin bound copper) - Low
2. Serum total copper - Low

3. Serum ceruloplasmin free copper - High [Free copper = Total copper (µg/dl) – ceruloplasmin (mg%)]
4. Urine copper - High

More about Wilson's disease: It is an autosomal recessive disorder and is caused by mutations in the ***ATP7B*** gene which encode a membrane bound copper – transporting ATP ase. In general its incidence is – 1/35000.

Deficiency of ATP7 B transporter protein impairs biliary copper excretion leading to retention of copper in the liver. Accumulated copper exerts oxidant damage. Defective copper incorporation to apoceruloplasmin will cause its premature breakdown leading to low serum ceruloplasmin and low serum copper. As a result free copper level increases as there is no sufficient copper binding protein ceruloplasmin in the serum to bind with. This will lead to deposition of free copper in various tissues like brain (basal ganglia lesions + psychiatric manifestations), cornea (Keyser-Fleischer ring) and liver (cirrhosis) leading to hepatic failure.

Q. 25F-4

A few case presentations are given below. Give the possible diagnosis

1. A lady hailing from Tamil Nadu engaged in garbage disposal presenting with edema of legs and face and breathlessness. Upon taking dietetic history, she said that she prefers polished rice only.
   Biochemical Test: Erythrocyte transketolase activity – Low
2. A man coming from Rajasthan engaged in construction work came to attend Medicine OPD of Medical College with *diarrhea* and he also complained about the forgetfulness and unstable mood he had noticed recently. About the diet: He was taking mainly sorghum based diet. O/E: *erythematous lesion* on the face and feet +, *Hyperpigmentation around the neck* +, *Ataxia* +.
3. A woman came with complaints of night blindness and dry eyes to Ophthalmic OP.
   O/E: Biot's spots (patches with dried soap bubble appearance) on the conjunctiva +, Skin is dry and rough Investigations: Serum Vitamin A level – Low; Retinol binding protein - Low

Ans. 25F-4:

1. Wet beriberi: Eating polished rice for long period by nutritionally compromised individuals are prone for developing niacin deficiency which is known as beriberi. It is of two types-

Dry beriberi - mainly neurological manifestations

Wet beriberi - predominantly of cardiovascular manifestations

RDA: 0.5 mg/1000 calories (since it's coenzyme form (TPP) is a cofactor of many enzymes of energy yielding catabolism of nutrients like carbohydrates and fat.

2. Pellagra: Deficiency of niacin causes diarrhea, dementia and dermatitis.

Niacin for the body is obtained from the diet and also from tryptophan. Consumption of sorghum aggravates niacin deficiency. Sorghum is rich in leucine. Leucine inhibits **quinolinate phosphoribosyl transferase** which is **rate limiting enzyme of nicotinamide nucleotide** (nucleotide form of niacin) synthetic pathway from tryptophan.

3. Vitamin A deficiency

RDA : Children 400-600 µg/day; Adults 750 µg/day

Q. 25F-5

After going through the following case studies give your diagnosis and explanation.

1. A 6-year-old female presenting with bow legs, pigeon chest bossing of frontal bones. Her plasma Vitamin D level – Low
2. A 2-year-old male child admitted to pediatric OP, with features of bleeding tendency – echymosis, hemorrhage in the

mucus membranes. Investigations showed prolonged prothrombin time and delayed clotting time. The condition improved with vitamin K administration.

Ans. 25F-5:

1. Rickets due to Vitamin D deficiency: The active form of vitamin D, Calcitriol promotes absorption of Calcium and phosphorous from the intestine. Vitamin D deficiency is characterized by inadequate mineralization of bone causing deformation of weight bearing bones.

Vitamin D is formed mainly from **7 – dehydrocholesterol** present in the skin on exposure to sunlight. Dietary sources are egg yolk,liver and fish liver oils

RDA; 400 IU (10mg)/day

2. Vitamin K deficiency: Vitamin K is needed for the post translational carboxylation of glutamic residues of proteins - clotting factors II, VII, IX and X; protein C; protein S; osteocalcin of bone; matrix Gla protein of vascular smooth muscle to make them functionally active. Hence deficiency of Vitamin K causes defective clotting of blood.

RDA of Vitamin K – 50-100 µg/day.

Dietary sources: Green leafy vegetables, margarine and liver (intestinal bacterial flora will also synthesize vitamin K)

Phyloquinone from plants (Vitamin K_1), Menaquinone (Vitamin K_2) from bacterial source and synthetic forms relate to menadione (Vitamin K_3).

Q. 25F-6

Give explanation for the following:

1. Fluoride has got anticariogenic effect
2. Iodine deficiency causes goiter
3. Vitamin C deficiency causes scurvy
4. Phytanic acid oxidase deficiency causes Refsum disease.

Ans. 25F-6:

1. Cariostatic effects of fluoride may be due to the following effects

- Help in apatite crystal formation
- Stimulation of enamel surface process
- Decreased enamel solubility
- Decreased bacterial enzyme activity

2. Dietary iodine deficiency (common in hilly areas like Himachal Pradesh) results in decreased secretion of thyroid hormones which by feed back mechanism via thyrotropin causes hyperplascia glandular tissue in an attempt to maintain adequate hormone secretion.

Some examples for goitrogens: cassava root which contains thiocyanate (inhibit iodine trapping enzyme of thyroid gland).

3. Ascorbic acid (vitamin C) act as a cofactor of the enzyme protocollagen hydroxylase which catalyze hydroxylation of prolyl and lysyl residues of collagen. This causes inadequate intercellular connective tissue substance. This is manifested as swollen tender bruised skin, mucous membranes and joints.

4. Peroxisomal phytanic acid oxidase concerned with α-oxidation of branched fatty acids is deficient in Refsum disease. In the affected individuals phytanic acid get accumulated over time, upon consumption of green leafy vegetables dairy products, meat and fish (originally derived from chlorophyll). Phytanic acid get integrated into the myelin causing disintegration of myelin sheath leading to neuronal damage. It is manifested as demyelinating neuropathy, sensineural deafness, cerebellar ataxia and retinitis pigmentosa causing night blindness.

25G. TUMOR MARKERS

Q. 25G-1

What are tumor markers? Give 5 clinical applications of tumor markers.

Ans. 25G-1:

Tumor markers are substances found in increased amounts in the blood, other body fluids

or in the tissues and may suggest the existence of a particular type of cancer.

Clinical applications: Screening for cancer in asymptomatic individuals, clinical staging of cancer, for estimating disease progression, evaluation of the outcome (success) of treatment and detection of recurrence of cancer after treatment.

Q. 25G-2

For the following type of tumor markers, give one example of malignancy detectable by each

1. Carcinoembryonic antigen
2. Alpha-fetoprotein
3. CA 125
4. Estrogen and progesterone receptors

What are tumor markers? Give 5 clinical applications of tumor markers.

Ans. 25G-2:

1. Carcinoembryonic antigen - Colorectal carcinoma
2. Carcinoembryonic antigen - Hepatocellular carcinoma
3. CA 125 - Endometrial (uterine) carcinoma
4. Estrogen and progesterone receptors – Carcinoma breast

25H. WATER AND ELECTROLYTES

Q. 25H-1

Anto was travelling by bus from Kottayam to Kasargod. He experienced severe vomiting during the journey. Upon reaching Kasargod he felt intense thirst, weakness and dizziness and complained of reduced urine output. He was brought to near by hospital and was examined by the duty medical officer O/E : confused, dry mouth, reduced skin turgor, tachycardia – Hemogram showed hemoconcentration and high MCV.

Laboratory data:

Plasma sodium	125 mmol/L
Blood urea	54 mg%
Plasma creatinine	1 mg%
Plasma osmolality	230 mOsm/kg
Urine sodium	Decreased

What kind of water and electrolyte imbalance this man suffering from? Explain.

Ans. 25H-1: Hyponatremia (plasma sodium = < 135 mmol/l)

Sodium loss occurs in vomiting. General symptoms of hyponatremia are weakness, apathy, lassitude, headache, giddiness and gastrointestinal symptoms like anorexia, nausea and vomiting and cardiovascular signs like hypotension, tachycardia.

With marked sodium loss mental confusion, delirium, delusion, stupor or coma may develop

Laboratory findings:

- Hemoconcentration + Mean corpuscular volume (MCV) – high due to entry of water into the cells (loss of $Na^+ \rightarrow \downarrow$ osmotic pressure of extracellular water) $\rightarrow \downarrow$ Mean corpuscular Hb concentration.
- Serum Na^+ - low (in spite of hemoconcentration) low Na^+ help to differentiate sodium loss from water loss where you will get high hematocrit with high serum Na^+ level
- Blood urea – high (Hyponatremia $\rightarrow \downarrow$ osmotic pressure of extracellular fluid (ECF), water move into the cells causing reduced plasma volume $\rightarrow \downarrow$ renal blood flow and decreased GFR leading to $\uparrow$ blood urea
- Low urinary sodium along with hyponatremia indicates low blood volume causing an increased retention of Na^+ ions by the kidneys
- Plasma osmolality – A low serum/plasma osmolality indicates low solute concentration (osmolality) of ECF. (**normal plasma osmolality → 275 – 290 mOsm/Kg of water**)

A low serum osmolality can occur in water excess or sodium loss or a combination of both. Serum osmolality can be measured directly by freezing point osmometer or can be found out indirectly using the calculation

Calculated serum osmolality (mOsm/kg) = 1.86 Na^+ + Glucoe/18 + BUN/2.8 (normally the calculated serum osmolality is 5-8 mOsm/kg less than osmolality measured by osmometer)

Conditions causing hyponatremia:

1. Loss of gastrointestinal secretions : vomiting, dysentery, diarrhea.
2. Loss through skin: Excessive sweating, exudative skin lesions, burns.
3. Sequestration of sodium within the body, e.g. Small bowel obstruction – large volumes of intestinal fluid retained in the lumen of the intestine, severe burns.
4. Sodium loss through kidneys eg: chronic renal disease, Syndrome of inappropriate secretion of antidiuretic hormone (SIADH) – Continuous secretion of ADH causes retention of water and increased circulating blood volume which in turn causes decrease in the secretion of aldosterone and increase in the secretion of atrial natriuretic factor. This causes kidneys to excrete large amounts of Na^+ in urine leading to hyponatremia.
5. Diabetic ketoacidosis: Loss of glucose through urine accompanied by Na^+ leading to hyponatremia.
6. Drugs: E.g. Carbonic anhydrase (in the kidney) inhibitors

   Carbonic anhydrase

   $CO_2 + H_2O \longrightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$

   When carbonic anhydrase is inhibited H^+ ions are retained instead of Na^+ and K^+
7. Severe hemorrhage lead to hyponatremia.

Q. 25H-2

Devaky, 40 years was brought to casualty because of vomiting , diarrhea, muscle cramps.

On examination she was dehydrated. ECG findings due to delayed depolarization (inversion of T wave, prominent U wave and ST segment depression) are seen.

Plama K^+ - 2.8 mmol/L; Plasma Na^+ - 130 mmol/L

What is the predominant electrolyte disturbance the patient suffering from?

Ans. 25H-2: Hypokalemia (Because plasma Potassium = < 3.5 mmol/l)

Potassium and sodium loss occur in vomiting and diarrhea. Here ECG findings suggest the hypokalemia. Normal Plasma K^+ - 3.5 – 5 mmol/L

Q. 25H-3

Go through the laboratory data of a patient admitted with diabetic ketoacidosis, given below and interpret.

pH	-	7.34
Plasma glucose	-	400 mg%
Plasma Na^+	-	135 meq/L
Plasma K^+	-	5 meq/L
Plasma HCO_3^-	-	20 meq/L
Plasma Cl^-	-	92 meq/L
Urine: Rothera's test	-	+ ve

Ans. 25H-3: High anion gap metabolic acidosis

Metabolic acidosis because of low pH, low plasma bicarbonate (being used up for buffering ketoacids) due to diabetic ketoacidosis (plasma glucose 400 mg%).

High anion gap

Anion gap $= (Na + K) - (HCO_3^- + Cl^-)$

$= (135 + 5) - (20 + 92)$

$= 140 - 112 = 28$ meq/L

It is high due to accumulation of ketoacids (normal anion gap = 12 meq/L).

25I. BIOCHEMICAL PARAMETERS OF RENTAL FUNCTIONS

Q. 25I-1

Bushra, 5-year-old admitted with puffiness of face, weakness and hypertension admitted for further evaluation.

Biochemical investigations

Urine	
Protein	4 g/24 hours
M/E	granular and epithelial casts +
Blood	
Total protein	4.5 g% (normal range at this age 6-8%)
Albumin	2.0 (normal range at this age 3.8-5.4%)
Cholesterol	300 mg% (normal range at this age 120-200%)
Blood urea	50 mg%
Serum creatinine	1.5 mg%

Ans. 25I-1: Nephrotic syndrome. The patient has constellation of clinical and laboratory findings suggestive of nephrotic syndrome.

Edema +; Hypertension +; Hypoalbuminemia +; Proteinuria > 3 g% per day +; hypercholesterolemia +.

Characteristic group of findings that constitute nephrotic syndrome (nephrosis) are heavy proteinuria (> 3.5 g%/day), hypoalbuminemia, hypercholesterolemia, minimal hematuria, oedema and hypertension. This can be caused by different types of renal conditions with evidence of inflammation, without any evidence of inflammation (minimal change nephritis) or with proliferative changes.

Q. 25I-2

Kumar, 40 years who used to have recurrent attacks of sore throat in the past developed rapid onset of headache, malaise, loin pain, hematuria, proteinura, hypertension, diminished urine output and peripheral edema, admitted in the Medicine ward for evaluation. Previous throat swab culture report showed that the sore throat he had in the past, was due to streptococcal infection.

Biochemical investigations

Urine	
- Protein	- 2 g/24 hours
- M/E	RBC +++; Pus cells +++
Blood	
- Total protein	- 5 g%
- Albumin	- 3.5 g%
- Cholesterol	- 150 mg%
- Blood urea	- 70 mg%
- Serum creatinine	- 2.2 mg%
- ASO titre	- ↑ (evidence of β hemolytic streptococci)
- C_3 complement	- ↓ (Consumed in immune complex formation)
- GFR	- (76 – 120 ml/minute at 40 years)

Ans. 25I-2: Glomerulonephritis

Streptococcal infection is notorious for causing immune mediated injury to glomerular filtration membrane. The antigens present on the nephritogenic streptococci (precursor of exotoxin B, prescribing antigen (PA-Ag), and streptokinase) have biochemical affinity for glomerular basement membrane (GBM)and there they bind with antibodies and further immune mediated reactions causing damage to GBM affecting glomerular filtration consequently renal function.

The patient has the **classical features of acute glomerulonephritis** because of **sudden onset, hematuria, pyuria, edema, hypertension** and **oliguria**.

Creatinine clearance can be estimated by Cockcroft-Gault formula (the value should be multiplied by 0.85 for women because of lower muscle mass)

$$\text{Creatinine clearance} = \frac{(140 - \text{age}) \times \text{Body weight in Kg}}{\text{Plasma creatinine (mg\%)} \times 72}$$

Q. 25I-3

Validate the diagnostic potential of the following biochemical parameters in assessing renal function.

1. Plasma urea and urea clearance
2. Serum creainine and creatinine clearance
3. Serum cystatin

Ans. 25I-3:

1. Plasma urea and urea clearance is influenced factors not related to the kidney . Urea is the end product of amino acid catabolism. Its level is influenced by the hydration of the body (dehydration causes spurious elevation); protein rich diet, increased protein catabolism, muscle wasting, bleeding into the GI tract (cause high urea level).

Urea is filtered by glomeruli and the urea upon reaching the renal tubules diffuse out of the tubules passively to re-enter the plasma. It causes under estimation of glomerular filtration rate if it is calculated based on urea concentrations in plasma and urine.

Urea clearance = [U × V]/P

(where U = urea concentration in urine in mg%; V = volume of urine; P = urea concentration in plasma in mg%)

Urea clearance (normal) = 75 ml/mt which is falsely lower than the true creatinine clearance.

∴ Plasma urea and urea clearance will not give a real status of the kidney disease.

2. Serum Creatinine and creatinine clearance: Creatinine formed as a result of muscle contraction hence it's level is related to muscle mass of an individual. (But the meat intake by the individual influence its blood level up to 10%). Due to this fact its level remains constant in an individual. Moreover creatinine is produced endogenously and released into the body fluids at constant rate. It is freely filtered at the glomerulus and a small amount is secreted by the proximal convoluted tubule (PCT) and hence creatinine clearance exceed inulin clearance (by a factor of 1.1). Inulin is freely filterable at the glomerular filtration membrane, neither reabsorbed nor secreted by the tubules. **Inulin clearance is considered to be gold standard marker of GFR.** The draw back of inulin clearance in clinical practice is that it requires exogenous administration.

Because of this, serum creatinine is the most widely used marker for GFR since its validity is closer to the true value. GFR is related directly to the urine creatinine concentration and inversely to serum creatinine since,

Creatinine clearance = $U_{cr}/P_{Cr} \times 24$ urine volume

3. Cystatin C: It is a cysteine protease inhibitor produced almost at a constant rate by all nucleated cells.Its level is not affected by diet at all. It is constitutively expressed by the cells. It is freely filtered at the glomerulus and not reabsorbed or secreted by the tubules. Because of these reasons it is more specific and sensitive than creatinine.

Q. 25I-4

What is the difference between urine specific gravity and urine osmolality?

Ans. 25I-4:

Specific gravity compares the density of a solution (thus the weight and size) to that of an equal volume of water (exact number of solute particles are not taken for calculation).

Osmolality measures exact number of solute particles in solution and is a weight/weight relationship (independent of the size). Osmolality is measured by change in freezing point caused by the number of solutes present in the solution. Therefore, when there are solutes with large molecular weight in urine (proteins, glucose), specific gravity (Normal range 1003 – 1030) will disproportionately increase, but osmolality (Normal range 500 – 1200 mosm/L) will not. Specific gravity of 1040 may be obtained in a child

with proteinuria (e.g. nephrotic syndrome). But this is not attainable by human kidney. Hence renal concentrating ability is better checked by measurements of osmotic concentration of urine – that is by measuring osmolality of urine using freezing point depression osmometer.

25J. BIOCHEMICAL DIAGNOSIS OF MYOCARDIAL INFARCTION

Q. 25J-1

Karim, 60-year-old, a shopkeeper who had severe chest pain in the morning. He was brought to the hospital by the fellow workers from the work place in the evening of same day itself. He was admitted in Medicine ward and a blood sample taken from him sent for enzyme analysis and the report is given below.

Investigation Report

- AST - 55 IU/L (Reference range: 8-20 U/L)
- CK - 450 U/L (Reference range: 20-110 U/L in males)
- LDH - 180 IU/L (Reference range: 100-190 U/L)

What is your inference?

Ans. 25J-3: The clinical presentation and enzyme studies indicate myocardial infarction. Patient was brought to the hospital within one day of the onset of chest pain. Myocardial injury causes release of creatine kinase **(CK) within 3-6 hours** and aspartate transaminase **(AST) within 4-8 hours** of myocardial infarction (MI) from cardiac muscle cells. The high **CK activity** (peak at 24 -36 hours) will persist in circulation **for 3 days** and that of **AST (peak at 24 hours)** for 3-4 days.

Activity of **LDH** will rise slowly from the basal level only **after 10 -12 hours** after the onset of MI and show **peak activity by 48-72 hours** and high activity will continue up to **5-10 days**.

In this case, since the blood sample is drawn within 12 hours of the onset of chest pain only CK and AST activities in the serum are elevated.

To understand the time course of cardiac marker enzyme activity, see Figure 25J-1 and Table 25J-2.

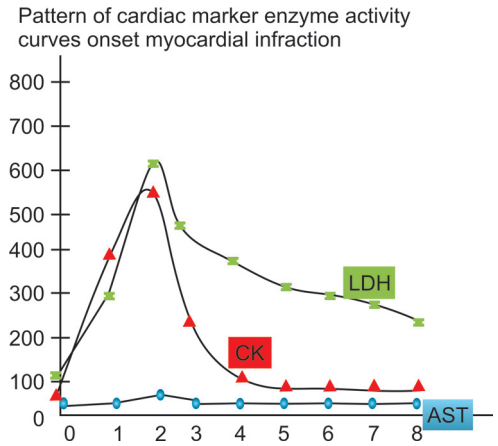


Fig. 25J-1: Characteristic of cardiac marker enzymes

Q. 25J-2

Vinayak, 40 years, a business man upon reaching the airport, seized with sudden onset of chest pain, profuse sweating and nausea. He was rushed to nearest hospital with all modern facilities.

It took approximately 75 minutes to reach the hospital .

What cardiac marker you prefer to do at this period ? Why?

Ans. 25J-2:

Myoglobin: Serum levels of myoglobin rise above the reference interval **by 1 hour** after the onset of myocardial infarction with **peak** between **4 and 12 hours**. This is cleared rapidly by kidney within 12 hours.

Points to Ponder

- If the person had any muscle injury or even an intramuscular injection , the myoglobin will be raised due to its release from skeletal muscle.

Table 25J-1: Time Course of Cardiac Marker Enzyme Activity			
Enzyme	*Time elapsed to exceed the upper reference limit*	*Peak elevation (hours)*	*Return to baseline level (days)*
Creatine kinase (CK)	3-6	24-36	3
Aspartate transaminase	4-8	24	3-4
Lactate dehydrogenase	10-12	48-72	5-10

If myoglobin levels remains within normal range within 2-4 hours of the onset of chest pain, recent myocardial injury can be excluded.

Q. 25J-3
What are isoenzymes? What is the role of isoenzymes in the diagnosis of myocardial infarction?

Ans. 25J-3: Isoenzymes are a group of related enzymes catalyzing the same reaction but having different molecular structures and characterized by different physical, biochemical and immunological properties.

Creatine kinase catalyzes the reaction between creatine and adenosine triphosphate to form phosphocreatine. It consists of 2 subunits - M and B and has 3 isoenzymes. They are

CK-1– (CK-BB predominantly in brain)

CK-2 (CK MB – mainly in myocardium and a small percentage in skeletal muscle

CK-3 (CK-MM) mainly in skeletal muscle.

Hence **CK-2 (CK-MB) assay** would be more specific of myocardial injury. It's level start to rise by 3-6 hours, peak at 24 -36 hours and come down to basal level by 3 days.

Lactate dehydrogenase catalyze the reversible reaction between pyruvate and lactate. There are 5 isoenzymes for it (composed of two (M and H)subunits in different proportions)-

LDH-1 (H_4 - predominantly in heart)

LDH-2 (H_3M – mainly in kidney, brain, RBC)

LDH-3 (H_2M_2 – mainly in spleen, lungs, kidney, RBC)

LDH-4 (HM_3 – mainly in spleen, lungs, kidney, RBC)

LDH-5 (M_4 – predominantly in skeletal muscle, skin)

Normally serum LD- 2 is present in greatest amounts.

There is a marked increase in the proportion of LDH-1 level in serum after myocardial infarction. The increase of LDH-1 over LDH-2 in serum after myocardial infarction is called **flipped pattern**. This finding is useful in diagnosing MI.

Q. 25J-4
Abdulla aged 36 involved in an accident, sustained severe crush injury and was on treatment in a hospital. He developed central chest pain radiating to left arm and neck while in the hospital. ECG findings are inconclusive. What type of cardiac marker would be useful in this case?

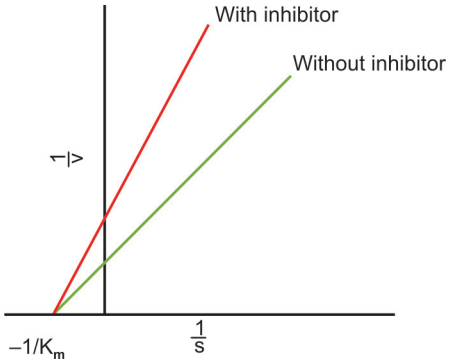
Ans. 25J-4: Cardiac Troponins (cTn T or cTn I)
CK- 2 activity of the heart is 10- 20% of total CK activity and that of skeletal muscle is 2%. After skeletal muscle injury, skeletal muscle fraction of CK-2 will increase considerably. In this situation, CK-2 values will not be reliable to diagnose MI. Cardiac troponins is the best available marker in this case.

Cardiac troponin T (cTn T) or Cardiac troponin I (cTn I) will serve this purpose.

cTn T and cTn I increase above the reference limit at 4-8 hours. cTn T and cTn I can remain elevated for 5 and 10 days respectively.

25K. ENZYMOLOGY

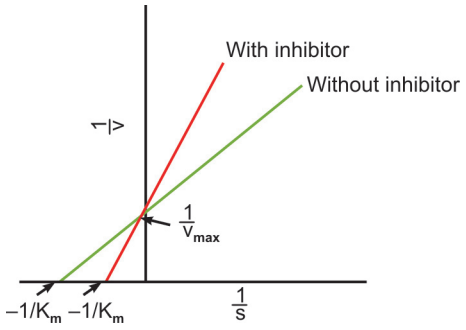
Q. 25K-1
Study the following Lineweaver-Burk plot and comment on it.



Ans. 25K-1: Noncompetitive inhibition
Inhibitor is not a structural analog of the substrate. It binds to the enzyme at a site other than the active site. The binding causes a conformational change in the structure of the enzyme which in turn alters the active site so that it cannot bind with its natural substrate. Noncompetitive inhibition can be reversible or irreversible depending on the type of bond formed. If the inhibitor binds to the enzyme by weak bonds, the inhibition is reversible. But if it is by covalent bonding the inhibition became irreversible. It causes decrease in V_{max} but produces no change to K_m value. Increasing substrate concentration cannot reverse the inhibitory effect.

Clinical significance:
- Mainly poisons act by uncompetitive inhibition, e.g. iodoacetate, cyanide, heavy metal ions (lead, mercury)
- Therapeutic application: British antilewisite used as an antidote for heavy metal poisoning.
- Laboratory use: Inhibition of enolase of glycolysis by fluoride, is utilized while collecting blood for glucose estimation.

Q. 25K-2
Study the following Lineweaver-Burk plot and comment on it.

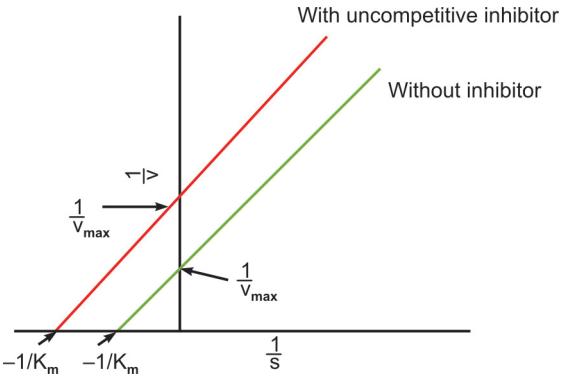


Ans. 25K-2: Competitive inhibition
Inhibitor is a structural analog of the substrate and so the inhibitor competes with substrate for binding to the active site of a specific enzyme. It is possible to reverse the competitive inhibition by increasing the substrate concentration. Here V_{max} of the reaction is not altered but K_m is increased.

Clinical significance:
Therapeutic application: Many widely used drugs act by this principle of competitive inhibition.

Examples:
- Sulphonamides
- Methotrexate

Q. 25K-3
Study the following Lineweaver-Burk plot and comment on it.



Ans. 25K-3: Uncompetitive inhibition

Inhibitor binds to the ES complex to form an enzyme substrate inhibitor complex that does not generate products. Increase in substrate concentration form more ES complex to which inhibitor binds and wasting the enzyme and substrate without relieving the inhibition.

Application: The principle of uncompetitive inhibition is used in the identification and estimation of placental isoenzyme of alkaline phosphatase (Regan enzyme). In this method, uncompetitive inhibition of placental ALP by phenylalanine is utilized.

SECTION FOUR

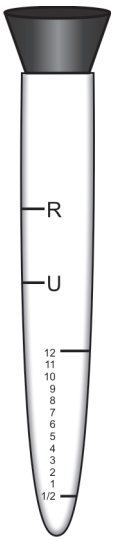
Spotters

Spotters

26

26A. INSTRUMENTS

26A-1. ESBACH'S ALBUMINOMETER



Spotter-1

Q. 26A-1

i. Identify the instrument.
ii. What is it used for?
iii. Briefly note down the procedure adopted.

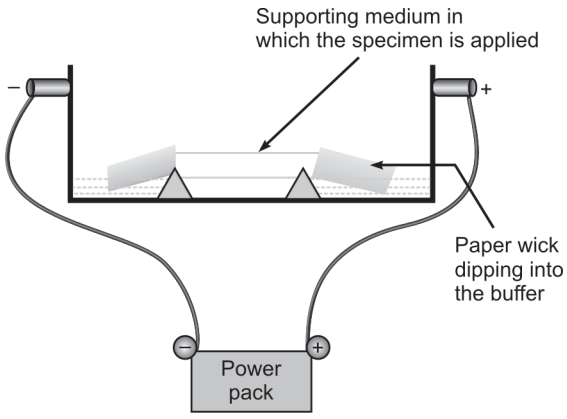
Details of Esbach's Albuminometer

Use: Useful for approximate quantitative estimation of protein in urine.

How to use: It is a glass tube with markings, fitted with a cork. Add urine up to mark 'U' and Esbach's reagent up to 'R'. Allow to stand for 24 hours. Protein picrate would be precipitated and settled at the bottom. The scale impregnated on the tube is calibrated in **grams of protein per liter of urine**. If the urine specimen is concentrated, dilute it with water to a specific gravity of about 1.008-1.010 and follow the same procedure and multiply the reading by dilution factor.

Esbach's reagent: 5 g of picric acid and 10 grams of citric acid dissolved in distilled water.

26A-2. ELECTROPHORETIC TANK



Spotter-2

Q. 26A-2

i. Identify the instrument.
ii. What is it used for?
iii. Write the principle of the technique used.

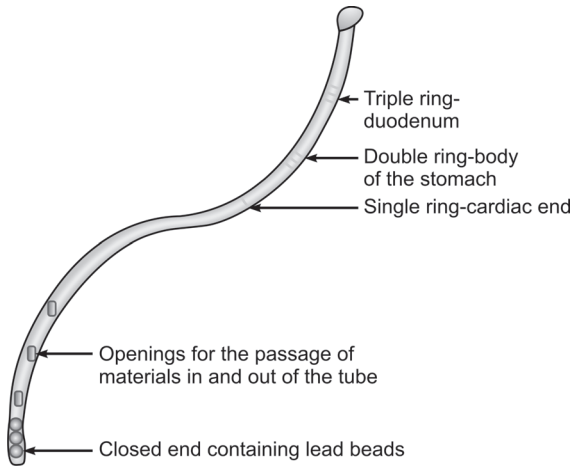
Details of Electrophoretic Tank

Use:

1. Separation of serum proteins, hemoglobins
2. Isoenzyme separation (creatine kinase, lactate dehydrogenase)
3. DNA studies, e.g. separation of DNA fragments as in southern blotting.

Principle: It is based on the movement of small charged particles through an electrolyte subjected to an electric field. If there are differently charged particles they will move in opposite directions. The positively charged will migrate to the cathode and the negatively charged to the anode. The rate of migration of charged particles depend on the amount of charge, mass and shape.

26A-3. RYLE'S STOMACH TUBE



Spotter-3

Q. 26A-3

i. Identify the spotter.
ii. Give two uses of it.
iii. What is the aim of incorporating lead beads at the closed end?

Details of Ryle's Stomach Tube

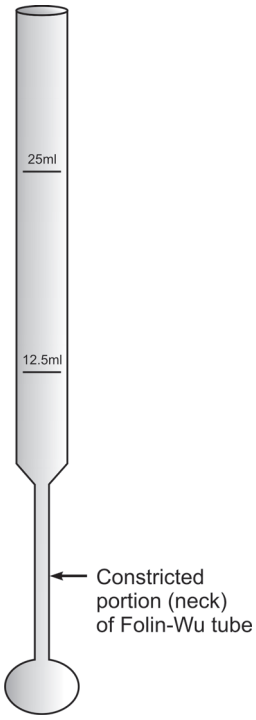
It is a plastic tube of 4 mm external diameter. It has a closed end containing 3 small lead beads and there are holes on the tube, a short distance from the closed end through which the stomach contents can enter the tube, during aspiration and leave the tube, during feeding. The markings on the tube would help to indicate how far the tube had been reached. During introduction of Ryle's tube through mouth when the **single ring** marking reaches the lips one can assume that the end has reached cardiac orifice of the stomach. Approximation of the **double line** with lips indicates that the tip has reached the body of the stomach or almost to the pylorus. Longer tubes contains a **triple rings** and when this ring comes in contact with the lips it means that the end of the tube has entered the duodenum.

Use:

1. Aspiration of gastric contents.
2. Feeding, e.g. In cases of coma postoperative cases

Significance of lead beads at the closed end: In doubtful cases of misplaced tube, the person can be passed though X-rays to assess the position of the tip of the tube as the lead beads being radio-opaque, will cast a shadow.

26A-4. FOLIN-WU SUGAR TUBE



Spotter-4

Q. 26A-4

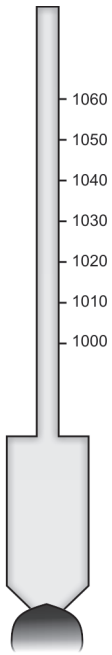
i. Identify the tube.
ii. What is it used for?
iii. What is the importance of the constricted portion of the tube?

Details of Folin-WU Sugar Tube

It is a glass tube designed by Folin and Wu to **estimate glucose** by **alkali copper reduction method.** In this method, proteins are precipitated by tungstic acid. Reduction of alkaline copper by sugar of the specimen to form cuprous copper is the principle of the reaction. Then the cuprous copper is oxidized to cupric oxide and the molybdic acid is reduced to molybdenum blue. It is colorimetrically measured.

Significance of the constricted portion of the tube (neck): To minimize reoxidation of cuprous oxide by atmospheric oxygen, during the period before adding phophomolybdic acid (during incubation in water bath) Folin and Wu designed the special tube with constricted neck.

26A-5. URINOMETER (HYDROMETER)



Spotter-5

Q. 26A-5i

i. Identify spotter.
ii. What is it used for?
iii. Give the temperature corrections to be made on the observed reading.

Spotter-6

Q. 26A-5ii

i. Name the instrument used to measure the specific gravity of urine?
ii. Give the normal range of urine specific gravity and mention two physiological factors affecting it.

iii. Give one pathological condition each for low and high specific gravity of urine.

Spotter-7

Q. 26A-5iii

i. Read the specific gravity of the given sample of urine.
ii. From the value find out the total solid present in the given urine sample.
iii. What is meant by fixed specific gravity? Name the conditions in which it occur.

Details of Urinometer (Hydrometer)

The specific gravity test measures the density of urine relative to the density of water. It varies directly with grams of solutes excreted per liter. It reflects the ability of the kidney to concentrate glomerular filtrate. Specific gravity of urine is determined directly with urinometer. It has a slender neck and a stem with a specific gravity scale usually covers the range from 1.000 to 1.060.

Procedure

- Fill the cylinder about three fourth full with specimen and place on a level surface
- Insert the urinometer into the cylinder
- Read the specific gravity directly from the scale on the stem
- Take the reading coinciding lowest point of the meniscus
- It is calibrated ordinarily at 16° C (look for the calibration temperature of the urinometer provided).
- Observations made at any other temperature must be subjected to correction to obtain the true specific gravity. For making the correction, add 0.001 for every 3°C rise above 16°C and subtract 0.001 for every 3°C dip below 16°C .

Specific Gravity of the Urine of Normal Individuals: 1.015-1.025

The value fluctuate with water intake and rate of perspiration. It is low with intake of large amount of fluids and it is high with increased perspiration and with low intake of fluids.

Specific gravity of urine will vary in different clinical situations.

High Specific Gravity

1. Acute nephritis–due to excretion high amount of proteins in urine.
2. Diabetes mellitus – due to excretion of glucose in urine.

Low Specific Gravity Leading to Specific Gravity Less than 1010 (Fixed Specific Gravity)

1. Diabetes insipidus—Where there is ADH deficiency causing excretion of dilute urine
2. Chronic renal failure – Inability concentrate urine.

The total solids excreted in urine may be roughly calculated by **Long's coefficient (2.6)**.The solid content in 1 L of urine is obtained by multiplying the last two figures of specific gravity at 25°C by 2.6, e.g. if the specific gravity of a urine sample is 1.020 and the 24 hours urine volume is 1200 ml.

The calculation for the total solids in 24 hour urine would be = 20 × 1200/1000 × 2.6 = 62.4 g of solids.

26A-6. pH METER

Spotter-8

Q. 26A-6

i. Identify the instrument.

ii. What is it used for?
iii. Mention the essential parts of the instrument.

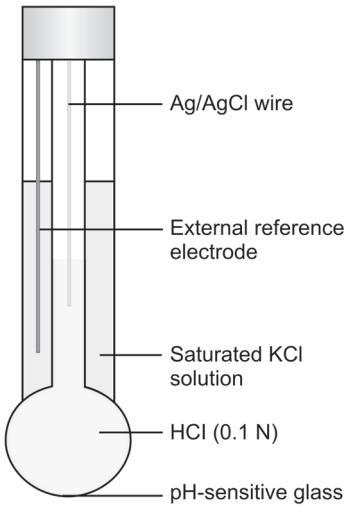
Details of pH Meter

pH determination is essential in the diagnosis and monitoring of acid base balance disorders. Use of pH meter allows accurate determination of pH of body fluids and laboratory reagents.

Principle: It is based on the measurement of electromotive force (emf) generated between two electrodes due to difference in $[H^+]$ concentration. One is reference electrode of known potential and the other is unknown electrode sensitive to hydrogen ion concentration. The electrode potential generated by $[H^+]$ concentration in an unknown solution is measured against a standard hydrogen electrode potential. A special pH sensitive glass is used in this instrument.

Here the measuring electrode is the glass electrode. Inside the glass is a sliver wire covered with AgCl paste dipped in 0.1 N HCl and it servel as the internal reference electrode.

An electric potential is generated when the thin glass membrane separates unknown solution outside and the known solution inside.



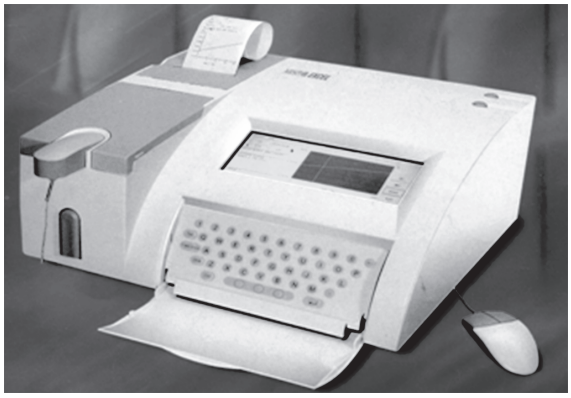
The difference in the potential is amplified and converted into direct pH reading on the display unit (digital read out).

26A-7. SEMIAUTOMATIC BIOCHEMISTRY ANALYZER

Spotter-9

Q. 26A-7

i. Identify the instrument and give its principle.
ii. What is it used for?
iii. Mention the essential parts of the instrument.



Details of Photoelectric Colorimetry

Principle: Beer-Lambert' Law

Lambert's law: when a ray of monochromatic light passes through an absorbing medium; its intensity decreases exponentially as the length of the medium increases

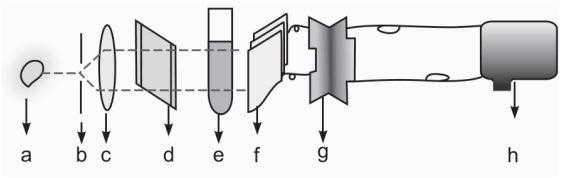
$$I = I_0 e^{-K1l}$$

Beer's law : when a ray of monochromatic light passes through an absorbing medium it's intensity decreases exponentially as the concentration of the medium increases

$$I = I_0 e^{-K2C}$$

These two laws are combined together in Beer-Lambert's law.

$$I = I_0 e^{-K3Cl}$$



Parts of Photoelectric Colorimeter

a. Light source
b. Slit
c. Condenser lens
d. Monochromator (gelatin fitter)
e. Cuvette holding the colored solution
f. Photocell
g. Amplifier
h. Galvanometer or digital read out

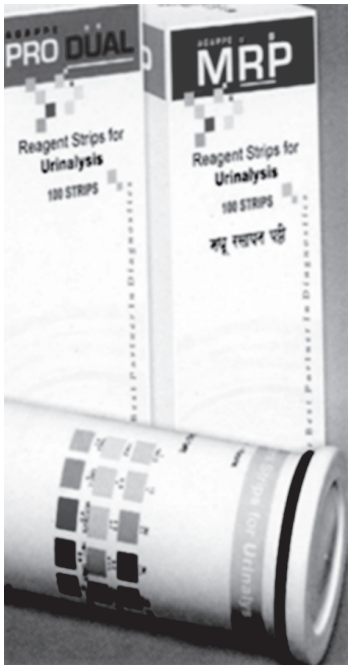
Use: Estimation of biologically important compounds of various body fluids.

26A-8. DRY CHEMISTRY STRIP TEST FOR URINE GLUCOSE

Spotter-10

Q. 26A-8

i. Identify the strip.
ii. What is it used for and give its principle?
iii. Mention its advantage over Benedict's test.



Test to determine glucose in urine in strip form based on a vegetable peroxidase and glucose oxidase.

Principle: The strip is impregnated with o-toluidine, peroxidase, glucose oxidase and a chromogenic substrate.

$$\text{Glucose} \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O + [O]$$

Colorless chromogenic substrate

$$\xrightarrow{[O]} \text{Colored compound}$$

The color developed is proportional to the glucose concentration in urine.

The color developed is compared with the standard chart provided to know the corresponding glucose concentration. It is superior to routine tests based on reducing property of sugars (e.g. Benedict's test) because the glucose oxidase specifically acts on glucose. Hence, the glucose oxidase based test is useful to confirm the presence of glucose.

26B. REAGENTS

26B-1

Spotter-11

Q. 26B-1
Name the ingredients of this reagent and the test done by it. Give the role of each ingredient.

Ans.
- Sodium citrate, sodium carbonate and copper sulfate.
- Benedict's test to detect reducing sugars in urine
- Sodium carbonate – provide alkaline medium; Copper sulphate – yield cupric ions; Sodium citrate – keep the cupric ions in solution.

26B-2

Spotter-12

Q. 26B-2
i. What are the ingredients of this reagent?
ii. What is the role of the test done by this reagent in the identification of biologically important solutions?

Ans.
- Copper acetate in acetic acid. It is used to do Barfoed's test.
- Barfoed's test is used to differentiate monosaccharides from disaccharides.

26B-3

Spotter-13

Q. 26B-3
Name the ingredients of this reagent and give its application in the clinical chemistry.

Ans.
- Sodium arsenophosphotungstate in HCl.

- It is used for doing Benedict's uric acid test to identify uric acid in biological fluids.

26B-4

Spotter-14

Q. 26B-4
Name the ingredients of this reagent and give its application in the clinical chemistry.

Ans.
- Resorcinol in HCl
- Selivanoff's test
- Principle of Selivanoff's test: HCl being a weak acid dehydrate keto sugars more readily than aldose sugars to yield furfural or furfural derivatives which in turn condense with resorcinol to form a red colored complex. Hence useful to differentiate ketoses from aldoses.

26B-5

Spotter-15

Q. 26B-5
i. What are the ingredients of this reagent?
ii. What is its use in the clinical chemistry laboratory?

Ans.
- Sodium potassium tartarate, cupric sulphate, potassium iodide and sodium hydroxide in water.
- Estimation of serum proteins.

26B-6

Spotter-16

Q. 26B-6
Name the ingredients of this reagent and two tests performed using this.

Ans.
- α-naphthol in alcohol.
- Molisch test, a general test for carbohydrates.
- Rapid furfural test to detect ketosugars which is useful to differentiate ketoses and aldoses.

26B-7

Spotter-17

Q. 26B-7
Name the ingredients of this reagent and its use.

Ans.
- Trichloroacetic acid and ferric chloride in water.
- It is used to perform Modified Fouchet's test to detect bilirubin in urine.

26B-8

Spotter-18

Q. 26B-8
Name the ingredients of this reagent and its use.

Ans.
- Para dimethylaminobenzaldehyde in hydrochloric acid.
- It is used to perform Ehrlich's test to detect urobilinogen in urine.
- Ehrlich's test will be strongly positive in hemolytic jaundice.

26C. INDICATORS

26C-1

Spotter-19

Q. 26C-1
Mention the pH range, color change and use of this indicator in the clinical chemistry laboratory.

Ans.
pH range: 3.8-5.4

Color range: Yellow to blue

Use: Isoelectric precipitation of casein at pH 4.6.

26C-2

Spotter-20

Q. 26C-2
Mention the pH range, color change and use of this indicator in the clinical chemistry laboratory.

Ans.
pH range: 4.8-6.8

Color range: Yellow to red

Use: Heat coagulation test for albumin - Isoelectric precipitation of albumin.

26C-3

Spotter-21

Q. 26C-3
Mention the pH range, color change and use of this indicator in the clinical chemistry laboratory.

Ans.
pH range: 1.2-2.8

Color range: Red to yellow

Use: Specific test for sucrose.

26C-4

Spotter-22

Q. 26C-4
Mention the pH range, color change and use of this indicator in the clinical chemistry laboratory.

Ans.
pH range: 6.8-8.4

Color range: Yellow to red

Use: Specific urease test—to provide the optimum pH of 6.8 for the urease enzyme used in the test.

26D. CRYSTALS

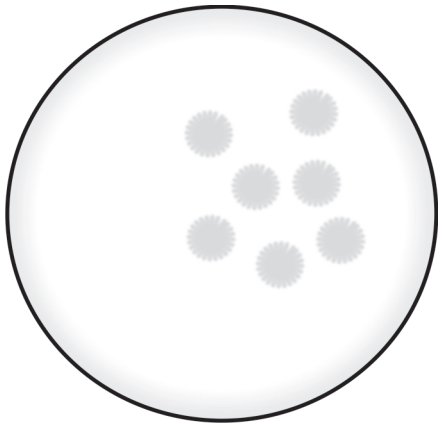
26D-1

Spotter-23

Q. 26D-1
Identify the crystal. Name three biologically important compounds giving this type of crystals. Name the test done to obtain this.

Ans.
- Needle shaped osazone crystal
- Glucose, fructose and mannose
- Osazone test

26D-2

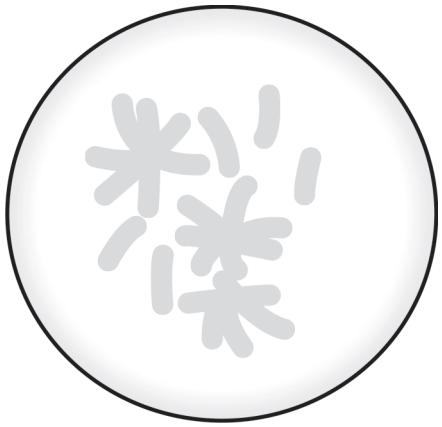


Spotter-24

Q. 26D-2
Identify the crystal and name the compound giving this type of crystal. Mention three conditions in which you may get this compound in urine.

Ans.
- Puff-shaped lactosazone crystals are given by lactose.
  Lactate may be excreted in urine during lactation and in the third trimester of pregnancy and it is also common in the urine of neonates.

26D-3

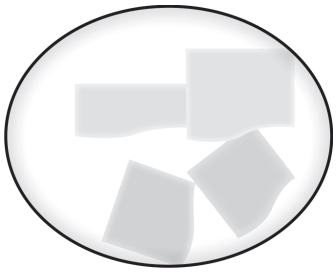


Spotter-25

Q. 26D-3
Identify and describe the shape of the crystal and name the compound giving this type of crystal.

Ans.
- Maltosazone crystal- Individual crystals of Maltosazone look like a yellow colored petal, when grouped, looks like a sun flower.
- Maltose

26D-4

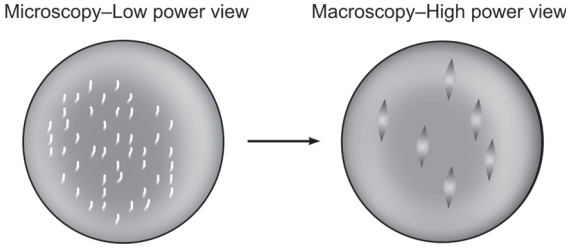


Spotter-26

Q. 26D-4
Identify the crystal and name three biologically important compound formed from it present in the body.

Ans.
- Cholesterol crystals - Rhombic crystals notched at one corner.
- Bile acids, Calcitriol, Sex hormones (e.g. estrogen , progesterone, androgen)

26D-5



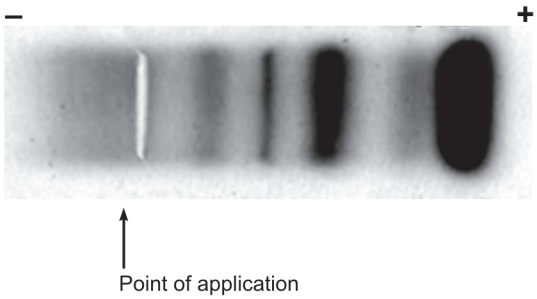
Spotter-27

Q. 26D-5
Identify the crystal; describe the chemical nature of the crystal and its application.

Ans.
- Hemin crystals (Rhombic shaped) Hemin is heme in which iron has been oxidized to the ferric form.
  Hemin – chloride of hematin (where as Hematin is – ferric iron + protoporphyrin)
- **Application:** Mainly in the forensic medicine to differentiate between blood stain from other stains.

26E. SEPARATION TECHNIQUES

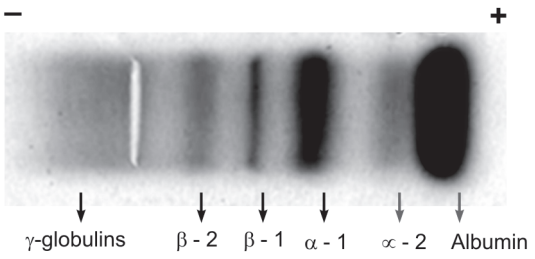
26E-1



Spotter-28

Q. 26E-1
i. Identify the strip and the bands and give the principle of the technique used.
ii. Name 5 biologically important substances separated by this technique.

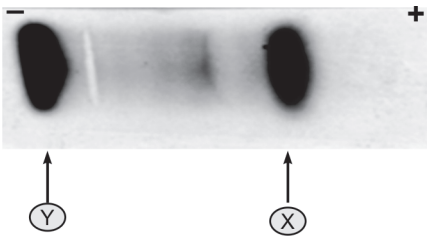
Ans.
- Electrophoretogram of serum proteins.
- Bands read from the anode: Albumin, α-1 globulins, α-2 globulins, β-1 globulins, β-2 globulins and γ-globulins as shown below:



Principle: Charged particles migrate through an electrolyte when subjected to an electric field and the rate of migration is dependent on net charge of the molecule, size and shape of the molecule, strength of the electrical field, type of supporting medium and prevailing temperature

Analytes separated by electrophoresis in the clinical laboratory: Proteins, isoenzymes (Lactate dehydrogenase,Creatine kinase), nucleic acids, oligonucleotides and organic acids are some examples.

26E-2



Spotter-29

Q. 26E-2
Identify the strip and name the bands marked as X and Y and comment on.

Ans.

- **Serum protein electrophoretogram,** showing a **monoclonal band** at the **gamma region.**
- **X** – Albumin; **Y** – Monoclonal band (Paraprotein)

Most common cause for producing monoclonal band is **multiple myeloma.**

More about paraproteinemia: Normally several different clones of plasma cells produce immunoglobulins so that a faint broad band is obtained at the gamma region. Immunoglobulins generally occupy the mid region of gamma band. Increase in immunoglobulins can produce two different types of electrophoretic representations. Infections can stimulate a variety of clones of plasma cells which produce different immunoglobulins leading to an appearance of a diffuse band on serum protein electrophoresis which is referred to as **polyclonal band.**

But if a single clone of malignant plasma cells proliferate as in multiple myeloma to produce a single type of Ig (immunoglobulin) or a part of Ig (kappa or lambda light chain), it can cause an intensely staining band more common in the gamma region and is referred to as **"M" (monoclonal band).** See Figure 26E-1.

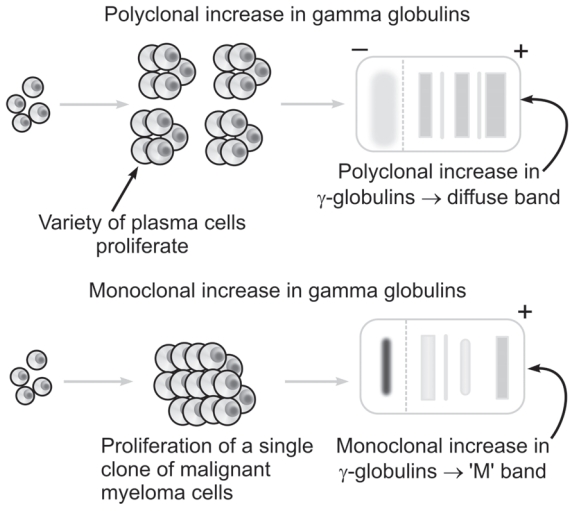
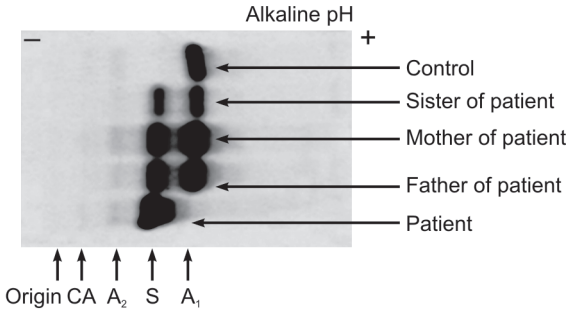


Fig. 26E-1: Two types of increases in immunoglobulins

26E-3



Spotter-30

Q. 26E-3

A male child aged 7 years exhibited symptoms and signs of hemolytic anemia and the sickling test was found to be positive for him. Then the whole family screened by Hb electrophoresis against a normal control. Interpret the electrophoretogram. (CA – carbonic anhydrase; A_2 – Hemoglobin A_2; S – Hemoglobin S; A_1 – Hemoglobin A_1)

Ans.

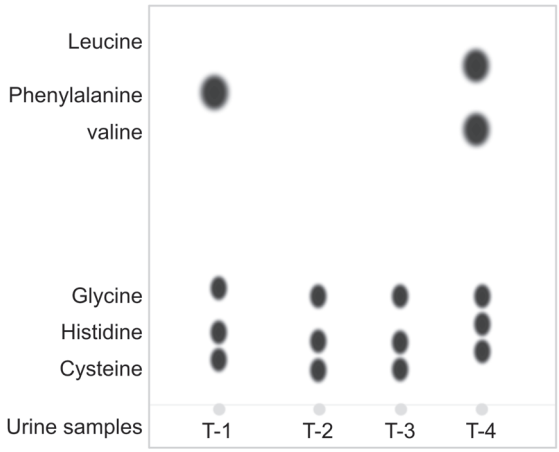
- Patient is suffering from homozygous sickle cell disease, because electrophoretogram indicates the presence of Hb S only and no other bands suggestive of Hb A_1.
- Father, mother and sister are heterozygous for Hb S since both Hb S and Hb A_1 are separated out upon electrophoresis.

26E-4

Spotter-31

Q. 26E-4

Identify and comment on.



Ans.

Urine paper aminoacidogram (Urine amino acid paper chromatogram)

- T-1 – suggestive of Phenylketonuria due to phenylalanine hydroxylase enzyme deficiency because a spot suggestive of Phenyl is seen
- T-2 – Normal (only the amino acids which are excreted normally are seen).
- T-3 – Normal (only the amino acids which are excreted normally are seen).
- T-4 – Maple syrup urine disease due to branched chain amino acid decarboxylase deficiency since spots indicative of branched chain amino acids (leucine and valine) are visualized on the chromatogram.

More about Paper Chromatography

- **Principle:** It involves physical method of separation in which the components (solutes) of a mixture are separated by their differential distribution between stationary and mobile phases.
- After Chromatographic run, distance travelled by the solvent and the solute along the support medium is measured from the point of application. The ratio of distance travelled by the solute to the distance travelled by the solvent is a constant for a particular solute under particular laboratory conditions. The

ratio is called **R_f value (ratio of fronts)** of the amino acid which is specific for the amino acid and hence useful in the identification of amino acids.

$$R_f \text{ value} = \frac{\text{Distance travelled by a amino acid from the point of application}}{\text{Distance travelled by the solvent}}$$

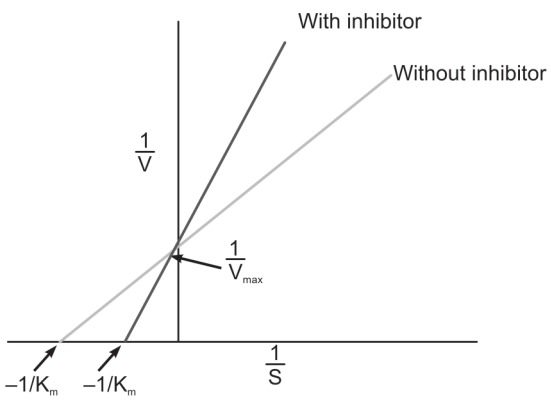
Routine application: Paper chromatography is used routinely in the clinical chemistry laboratory for analyzing urine for aminoaciduria (separation and identification of aminoacids – e.g. Phenyl ketonuria ,maple syrup urine disease, homocystinuria) and glycosuria (separation and identification of sugars).

Types of Chromatography Based on the Mechanisms Applied

Partition chromatography; Ion exchange chromatography; Adsorption chromatography; Affinity chromatography.

26F. GRAPHS

26F-1



Spotter-32

Q. 26F-1
Identify the graph and comment on.

Ans. Lineweaver-Burk plot showing competitive inhibition because here Vmax is not changed but Km is increased.

Application in Clinical Medicine

Drug designing – examples of drugs used, based on the principle of competitive inhibition are sulphonamides and methotrexate.

Treatment of methanol poisoning by ethanol is based on competitive inhibition.

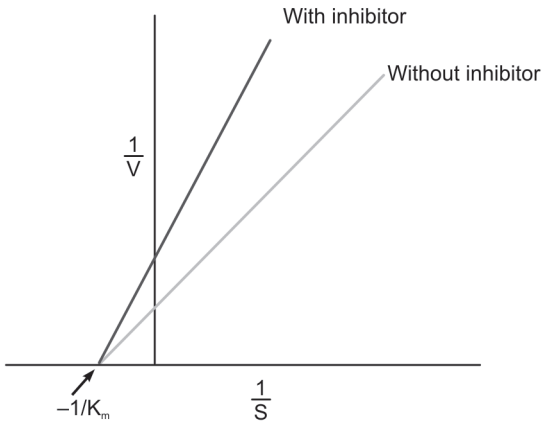
More Details

Ethanol $\xrightarrow{\text{Alcohol dehydrogenase}}$ Acetaldehyde

Methanol ⟶ Formaldehyde (highly toxic causes optic neuritis leading to blindness)

As shown above the same enzyme alcohol dehydrogenase is concerned with metabolism of ethanol and methanol. When methanol is ingested it will be metabolized to toxic formaldehyde. To counteract this ethanol is given to compete with methanol for alcohol dehydrogenase so as to decrease the conversion of methanol to formaldehyde.

26F-2



Spotter-33

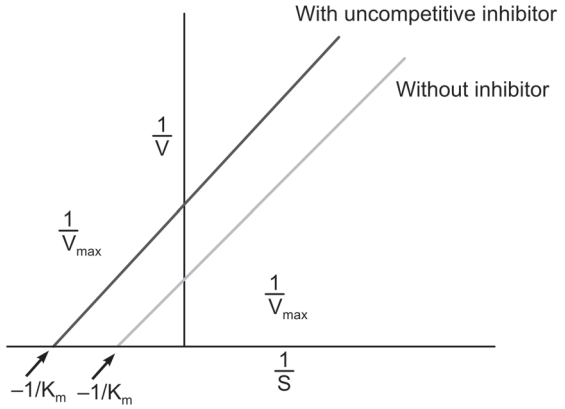
Q. 26F-2
Identify the graph and comment on.

Ans. Lineweaver-Burk plot showing non-competitive inhibition because here V_{max} is decreased but there is no change in K_m.

Clinical Significance

- Mainly poisons act by noncompetitive inhibition, e.g. iodoacetate, cyanide, heavy metal ions (lead, mercury)
- Therapeutic application: British anti lewisite used as an antidote for heavy metal poisoning.
- Laboratory use: Inhibition of enolase of glycolysis by fluoride, is utilized while collecting blood for glucose estimation.

26F-3



Spotter-34

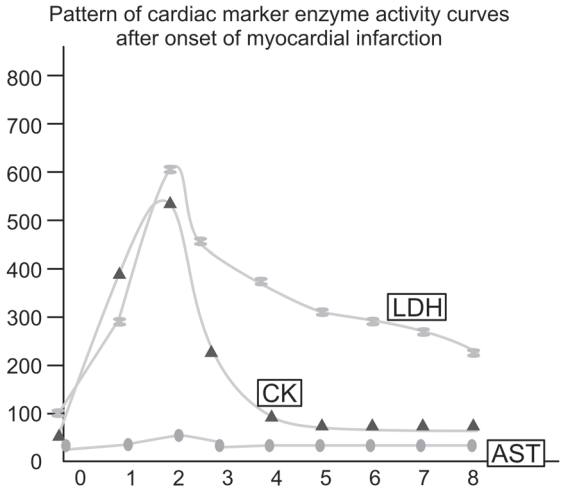
Q. 26F-3
Identify the graph.

Ans. Lineweaver-Burk plot showing uncompetitive inhibition because here both V_{max} and K_m are decreased.

26F-4

Spotter-35

Q. 26F-4
Suppose a patient comes to the hospital 6 hours after the onset of chest pain,



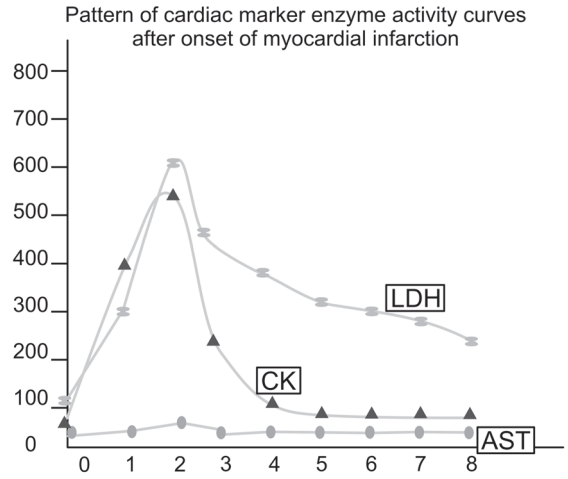
- which enzyme assay would be useful to diagnose myocardial infarction?
- name a nonenzyme cardiac marker that can be used at this time of presentation and mention its merits or demerits against the enzyme marker.

Ans.
- Creatine kinase (The CK-MB, the isoenzyme of creatine kinase is more specific than total CK)
- Nonenzyme cardiac marker - Troponin T .
- Merit of Troponin T over CK or CK MB- Troponin T assay help to eliminate a false diagnosis of myocardial infarction in patients with elevated CK levels after skeletal muscle injuries.

26F-5

Spotter-36

Q. 26F-5
A diabetic patient who had been feeling uneasiness for the past 4 days came for cardiology consultation. The cardiologist suspected myocardial infarction in the patient (Because long standing diabetes will cause



neuropathy due to which the pain of myocardial infarction will not be experienced by the patient) Which enzyme will help on the 5th day and mention the more specific isoenzyme of it? What is meant by flipped pattern?

Ans.

– Lactate dehydrogenase
– LDH -1

In normal serum LD- 2 is present in greatest amounts.

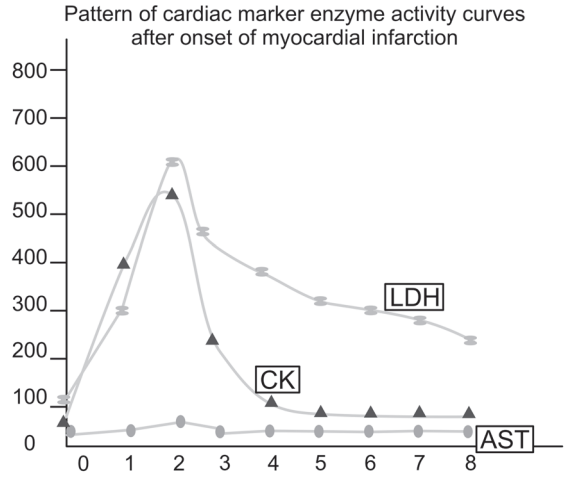
There is a marked increase in the proportion of LDH -1 level in the serum after myocardial infarction. The increase of LDH -1 over LDH-2 in serum after myocardial infarction is called **flipped pattern**. This finding is useful in diagnosing MI.

26F-6

Spotter-37

Q. 26F-6

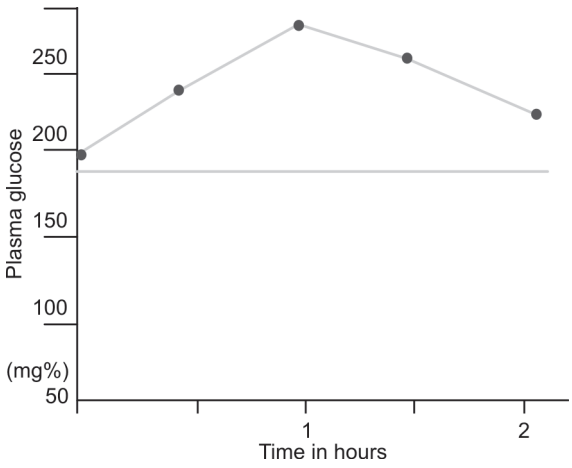
i. After going through the graph, select the marker if any, that would be useful in the diagnosis of myocardial infarction in the first one hour after the onset of chest pain?
ii. If no enzyme marker is available, name any other marker useful in this context.



Ans.

- No enzyme marker is available to serve as a cardiac marker of myocardial infarction in the first hour after the onset of chest pain.
- Myoglobin is useful in the first hour.

26F-7



Spotter-38

Q. 26F-7

Identify the graph and interpret.

Ans.

– Oral glucose tolerance test (OGTT) graph showing the curve indicative of **diabetes mellitus**

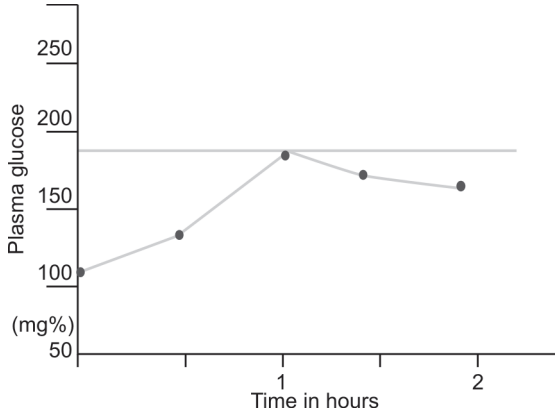
Fasting plasma glucose (FBG) > 126 mg% (7.0 mmol / L) and 2 hour post load glucose level > 200 mg% (11.1 mmol/L) is diagnostic of diabetes mellitus.

In this case FBG is 190 mg% (10.55 mmol/L) and 2 hour post load glucose level 220 mg% (12.22 mmol/L).

Points to Ponder: To convert glucose value in mg% to mmol /L divide the value in mg% by 18

$$= \frac{\text{Glucose in mg\%}}{18}$$

26F-8



Spotter-39

Q. 26F-8

Identify the graph and give your opinion.

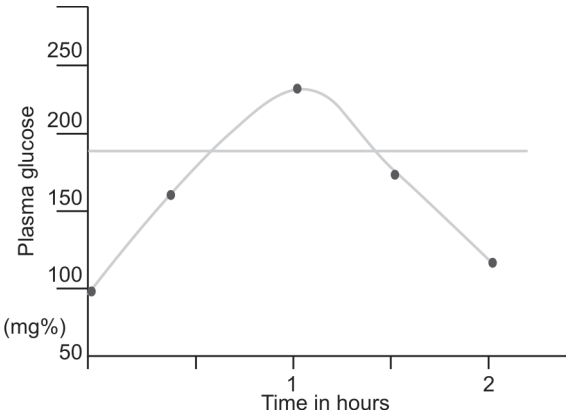
Ans.

– Oral glucose tolerance test (OGTT) graph suggesting **IGT (impaired glucose tolerance)**.

When the fasting plasma glucose (FBG) between 110 and 126 mg% and 2 hour post load glucose level between 140 and 199 mg% is suggestive of impaired glucose tolerance (IGT).

In this case, FBG is near 115 mg% and 2 hour post load glucose level 170 mg%.

26F-9



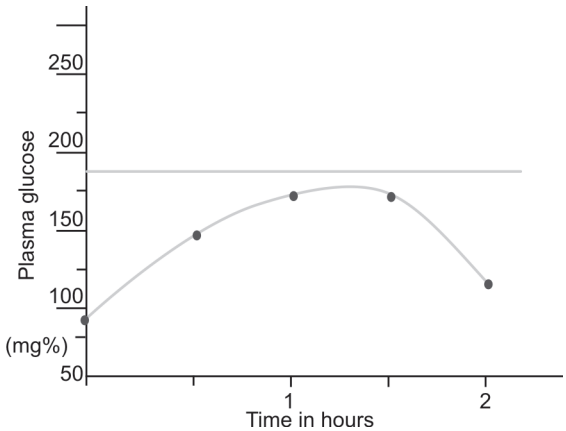
Spotter-40

Q. 26F-9

Identify the graph and give your opinion.

Ans. Oral glucose tolerance test (OGTT) graph showing **lag curve suggesting alimentary glucosuria.** Exaggerated rise in blood glucose following an oral glucose load and rapid fall in it's level in the blood to touch the normal level at 2 hours. Transient glucosuria occurs due to the peak level crossing the renal threshold for glucose.

26F-10



Spotter-41

Q. 26F-10
Identify the graph and comment on it. What are indications for doing this test? What is the glucose load to be given for conducting this test?

Ans.
– Oral glucose tolerance test (OGTT) graph showing **normal tolerance to glucose load**. It is said to be normal when the fasting blood glucose is below 110 mg% (here it is 90 mg%) and 2 hour post load glucose is less than 140 mg% (here it is 100 mg%).

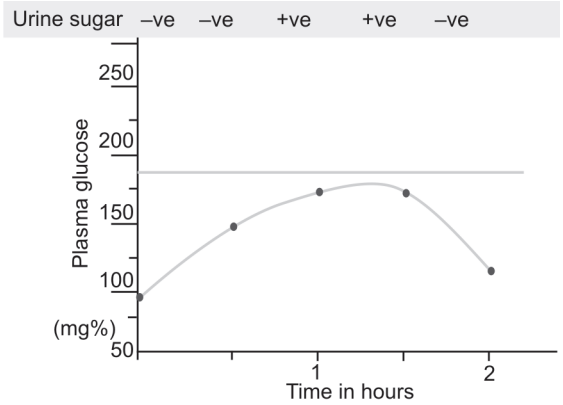
Indications for OGTT

1. Diagnosis of GDM (gestational diabetes mellitus).
2. Patients having symptoms suggestive of DM but fasting blood sugar values inconclusive between 100-126 mg%.
3. Pregnant ladies with past h/o big baby more than 4 kg or a past h/o miscarriage.
4. To rule out benign renal glucosuria.
5. Diagnosis of IGT.

– **Glucose load** 75 g for adults and 1.75 g/kg body wt for children (maximum of 75 g) dissolved in 300 ml of water.

In pregnancy to rule out gestational diabetes mellitus, the glucose load is 100 mg.

26F-11

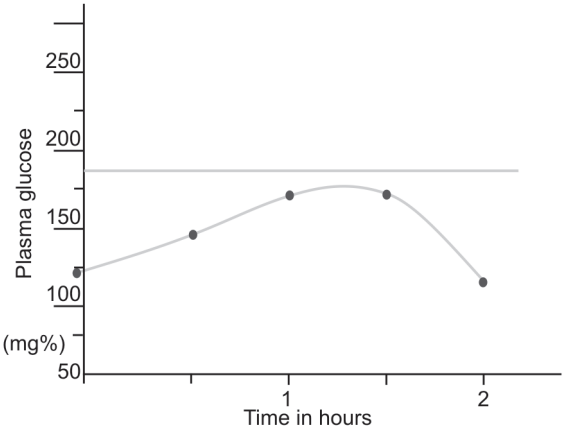


Spotter-42

Q. 26F-11
Identify the graph and interpret the curve.

Ans.
– Oral glucose tolerance test (OGTT) graph suggestive of renal glucosuruia because all the plasma glucose values are within normal limits but the test for glucose is positive in two samples indicating renal glucosuria due to lowered renal threshold for glucose which is around 180 mg% in a normal person.

26F-12



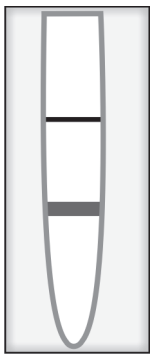
Spotter-43

Q. 26F-12
Identify the graph and comment on it.

Ans.
– Oral glucose tolerance test (OGTT) graph showing **impaired fasting glycemia.**
– Impaired fasting glycemia is said to occur when the plasma glucose level is above normal but below the cut off value practised to designate diabetes mellitus (that is between 110 and 126 mg%).
– In this case fasting glucose is 124 mg% and other values are within normal limits.

26G. TESTS

26G-1



Spotter-44

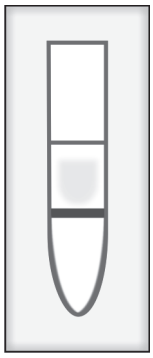
Q. 26G-1
Identify the test, give its principle and its application.

Ans. Molisch test

Principle: Concentrated acid dehydrates the pentoses to form furfural, and furfural derivatives in the case of hexoses and heptoses which in turn condenses with a-naphthol to give a **reddish violet** colored complex

Application of the test: Used as a general test to detect carbohydrate.

26G-2



Spotter-45

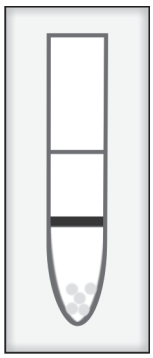
Q. 26G-2
Identify the test, give the principle and its use in the laboratory.

Ans. Aldehyde test

Principle: Mercuric sulphate in sulphuric acid act as a oxidizing agent and it oxidizes the indole ring of tryptophan. Then formaldehyde react with the oxidized indole ring to form purple colored complex.

Application of the test: Used to detect indole ring containing amino acid, Tryptophan.

26G-3



Spotter-46

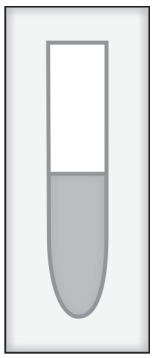
Q. 26G-3
Identify the test and its application in clinical medicine.

Ans. Rothera's test

Principle: Acetone and acetoacetic acid react with sodium nitroprusside (nitroferricyanide) in the presence of alkali to produce a **purple color.**

Application of the test: Used to detect ketone bodies (acetoacetic acid and acetone) in urine, e.g. diabetic ketoacidosis, starvation ketoacidosis.

26G-4



Spotter-47

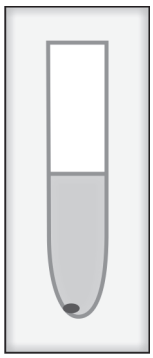
Q. 26G-4
Identify the test and comment on it.

Ans. Benedict's test

Principle: It is a semiquantitative test. The color of the precipitate indicates the concentration of sugar in solution. Here the color of the precipitate is green which approximately corresponds to the concentration of 0.5 g%. Carbohydrates with a free aldehyde or keto group have the ability to reduce various metallic ions. Here the cupric ions are reduced to cuprous ions by the enediols formed from sugars in the alkaline medium of Benedict's reagent.

Application of the test: Used to differentiate between reducing and nonreducing sugars.

26G-5



Spotter-48

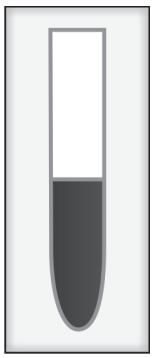
Q. 26G-5
Identify the test, give the principle and its use in the laboratory.

Ans. Barfoed's test

Principle: It is a reduction test. Reducing property owes to the carbonyl group (aldehyde or keto group) of sugars. Barfoed's reagent is copper acetate in acetic acid. In the acid medium monosaccharides enolize much more readily than disaccharides and these enediols reduce cupric ions released by copper acetate of Barfoed's reagent.

Application of the test: Used to differentiate monosaccharides from disaccharides.

26G-6



Spotter-49

Q. 26G-6
Identify the test, give the principle and its use in the laboratory.

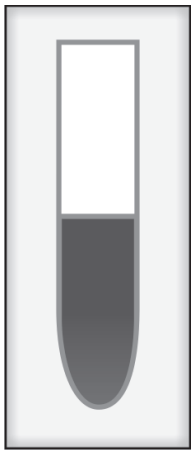
Ans. Rapid furfural test

Principle: A dehydration reaction due to the hydroxyl groups of the sugar.

Concentrated HCl being weaker than concentrated sulfuric acid, dehydrate ketoses (e.g. fructose) more readily than aldoses to form hydroxymethyl furfural which then condenses with α-naphthol to form a violet colored complex.

Application of the test: Used to differentiate ketoses from aldoses.

26G-7



Spotter-50

Q. 26G-7
Identify the test, give the principle and its use in the laboratory.

Ans. Rapid furfural test

Principle: A dehydration reaction due to the hydroxyl groups of the sugar. Concentrated HCl being weaker than concentrated sulphuric acid , dehydrate ketoses (eg: fructose) more readily than aldoses to form hydroxymethyl furfural which then condenses with α-naphthol to form a violet colored complex .

Application of the test: Used to differentiate ketoses from aldoses.

26G-8

Spotter-51

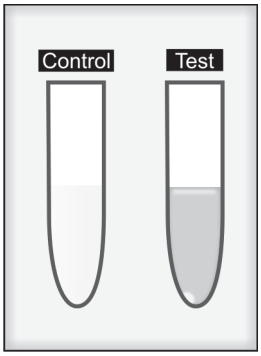
Q. 26G-8
Identify the test, give the principle and its use in the laboratory.

Ans. Biuret test

Principle: The biuret test is given by those substances consisting of molecules containing two carbamyl groups ($-CONH_2$) joined either directly or by a single nitrogen or carbon atom . The purplish violet colour is due to the formation of a copper coordination complex.

Application of the test: Used as a general identification test of peptides and proteins.

26G-9



Spotter-52

Q. 26G-9
Identify the test and interpret.

Ans. Xanthoproteic test. Detect amino acids containing benzene ring, e.g. tryptophan, tyrosine

Principle: Addition of nitric acid causes denaturation of proteins to get white precipitate. Yellow color due to nitration of phenyl group (C_6H_5) of amino acids – **tryptophan and tyrosine**. Addition of alkali increases the ionization of compounds hence the color deepens to get final orange color.

Application of the test: For the detection of benzene containing amino acids in the test samples.

26G-10

Spotter-53

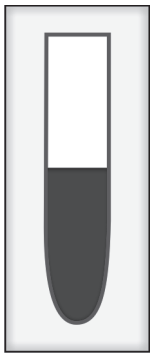
Q. 26G-10
Identify the test, give the principle and its use in the laboratory.

Ans. Millon's test

Principle: The protein precipitated by mercuric sulfate in acidic medium to form mercury – protein complex (metallo protein complex). Nitrous acid is formed by the reaction between sodium nitrite and sulfuric acid. This nitrous acid causes nitration of phenolic groups of tyrosine. Warming enhances nitration process and intensifies the colour to give reddish color

Application of the test: Used to detect the presence of Tyrosine in protein solutions or amino acid mixtures.

26G-11



Spotter-54

Q. 26G-11
Identify the test, give the principle and its use in the laboratory.

Ans. Sulfur test

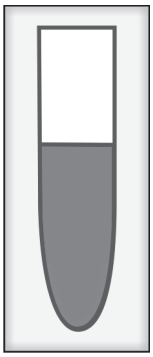
Principle: Upon boiling with strong alkali the organic sulfur in the cystine and cysteine is converted into sulphide (here Na_2S). The sodium sulphide react with lead acetate to form black lead sulphide (PbS) and the solution turns **brownish black.**

Application of the test: Used to detect the presence of sulfur containing amino acids– cysteine and derived amino acid cystine. It cannot detect the sulfur containing amino acid methionine due to the presence of thio ether bond.

26G-12

Spotter-55

Q. 26G-12
Identify the test, give the principle and its use in the laboratory.

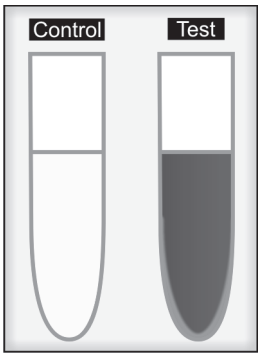


Ans. Sakaguchi's test

Principle: Molisch reagent contains α-naphthol in alcohol. Sodium hydroxide provides alkaline pH. At the alkaline pH guanidino group of arginine combines with α-naphthol to form bright red color.

Application of the test: Used to detect the presence of the semi essential amino acid arginine.

26G-13



Spotter-56

Q. 26G-13

Identify the test, give the principle and its use in the laboratory.

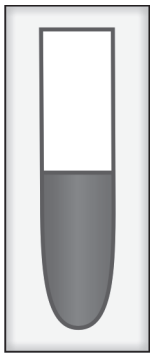
Ans. Specific urease test

Principle: Urease decompose urea to ammonium carbonate. Ammonium carbonate being basic, raises the pH. Phenol red used in this test will show pink to red colour at the basic pH.

$$\text{Urea} \xrightarrow[\text{Urease}]{H_2O} \text{Ammonium carbonate}$$

Application of the test: Used for the identification of urea.

26G-14



Spotter-57

Q. 26G-14

Identify the test, give the principle and its use in the laboratory.

Ans. Benedict's uric acid test

Principle: Uric acid reduces phosphotungstic acid to tungsten blue in alkaline medium.

Application of the test: Used for the identification of uric acid.

26G-15

Spotter-58

Q. 26G-15
Identify the test, give the principle and its use in the laboratory.

Ans. Schiff's test

Principle: Uric acid reduces silver nitrate to metallic silver in alkaline medium.

Application of the test: Used for the identification of uric acid.

26G-16

Spotter-59

Q. 26G-16
Identify the test, give the principle and its use in the laboratory.

Ans. Murexide test

Principle: In this reaction uric acid is oxidized to dialuric acid and alloxan which condense to form alloxantin.The alloxantin so formed reacts with ammonium hydroxide to form ammonium purpurate or murexide which is purplish red in color. With potassium hydroxide a purplish violet color is produced due to the formation of potassium salt of ammonium purpurate.

Application of the test: Used for the identification of uric acid.

26G-17

Spotter-60

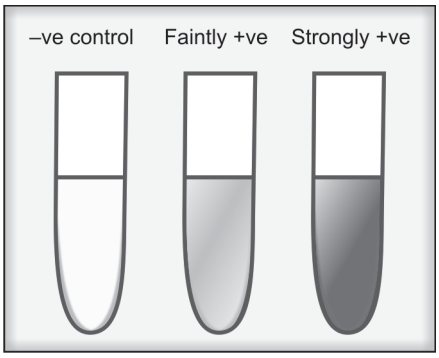
Q. 26G-17
Identify the test, give the principle. What is it used for?

Ans. Modified Fouchet's test

Principle: The precipitate obtained is that of $BaSO_4$ to which bile pigment, if any would have adsorbed on to it. To this when Fouchet's reagent (Ferric chloride in Trichloroacetic acid) is added. $FeCl_3$ oxidises bilirubin to biliverdin and Fe^{3+} is converted to Fe^{2+}. This gives the bluish green color.

Application of the test: Used to detect bilirubin in urine (conjugated bilirubin excreted in urine in obstructive jaundice and hepatic jaundice).

26G-18



Spotter-61

Q. 26G-18
Identify the test, give the principle and its use in the laboratory.

Ans. Ehrlich's test

Interpretation of the test: No red color: Urobilinogen absent.

Faint pink color: Urobilinogen present in normal amounts.

Distinctly red color: Urobilinogen present in increased amounts.

Principle: Urobilinogen forms a colored adduct with para dimethyl aminobenzaldehyde.

Application of the test: Used for the identification of urobilinogen in urine. It is faintly positive in normal urine and strongly positive in hemolytic jaundice.

26G-19

Spotter-62

Q. 26G-19
Identify the test, give the principle and its use in the laboratory.

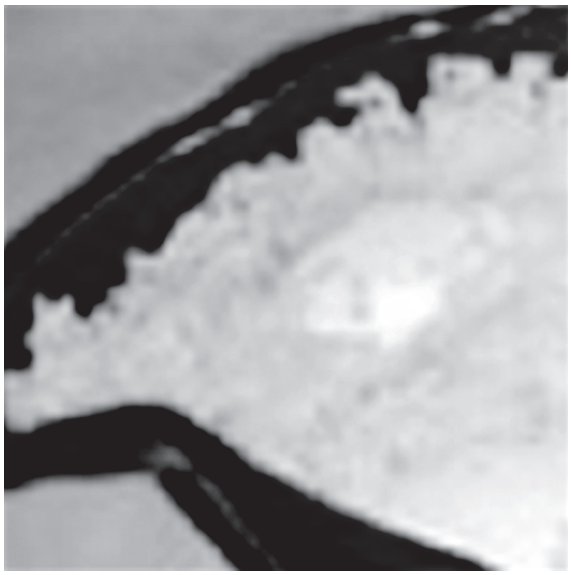
Ans. Jaffe test

Principle: Creatinine forms red colored creatinine picrate with picric acid in alkaline medium.

Application of the test: Used for the identification and estimation of creatinine in biological fluids.

26H. NUTRITION

26H-1



Spotter-63

Q. 26H-1
Name the limiting amino acids and biological value of rice. What is the harm in taking polished rice for long periods especially by persons on poor diet?

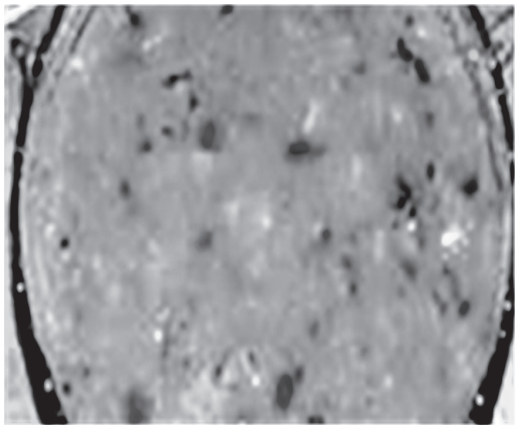
Ans.

- Limiting acids – lysine
- Biological value – 64%
- Thiamine (vitamin B_1) deficiency causes beriberi.

26H-2

Spotter-64

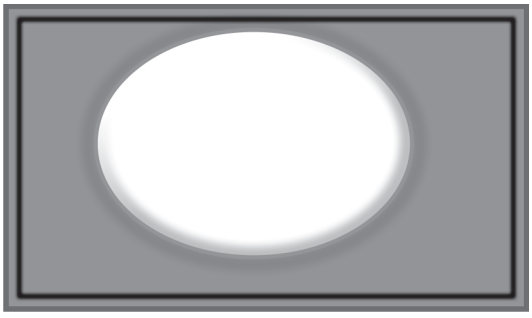
Q. 26H-2
Name the limiting amino acids, biological value of dhal. What do you mean by mutual supplementation of proteins?



Ans.

- Limiting acids – Methionine
- Biological value – 56%
- Mutual supplementation of proteins – Cereals are deficient in the essential amino acid lysine and pulses deficient in methionine. Generally the deficiency of these amino acids does not occur due to the habit of taking mixed diet - combination of cereals and pulses, e.g. iddli, chappathi + dhal.

26H-3



Spotter-65

Q. 26H-3

i. What is meant by biological value of a protein?
ii. Give the biological value of egg.
iii. What is the harm in taking raw eggs?

Ans.

- Biological value = $\frac{\text{Retained nitrogen}}{\text{Absorbed nitrogen}} \times 100$
- Biological value of egg – 90%
- A protein avidin which has a high affinity to biotin is present in egg white. When raw egg is consumed, this egg white avidin will bind with dietary biotin tightly in the intestine there by blocking the absorption of biotin by intestinal mucosa leading to biotin deficiency.

Avidin being heat labile, cooking of egg will denature avidin and there by abolish the affinity toward towards biotin.

26H-4

Spotter-66

Q. 26H-4

i. Name the vitamin present in the lemon that is involved in the collagen synthesis.
ii. What is the role of this vitamin in the collagen synthesis? Name the deficiency manifestation of this vitamin.

Ans.

- Ascorbic acid (Vitamin C)
- Ascorbic acid is a cofactor of prolyl and lysyl hydroxylase involved in the hydroxylation of these amino acids in the protein collagen.

Hydroxylysine and hydroxyproline are essential for the formation of crosslinks in the collagen that confer tensile strength to the collagen fibers.

26H-5

Spotter-67

Q. 26H-5
What is the harm in having sorghum as staple diet?

Ans.
Leucine content of sorghum is very high. Leucine inhibits the key enzyme QPRTase of nicotinamide nucleotide synthetic pathway, there by blocking the conversion of niacin to it's active form – nicotinamide adenine dinucleotide leading to deficiency manifestations - pellagra.

26H-6

Spotter-68

Q. 26H-6
Including fish in the diet is considered to be beneficial to health. Give reasons.

Ans. Fish oils are rich in omega -3 fatty acids (e.g. Timnodonic acid, cervonic acid). They lower the plasma triglyceride concentration, protect against thrombosis and reduce inflammation.

26H-7

Spotter-69

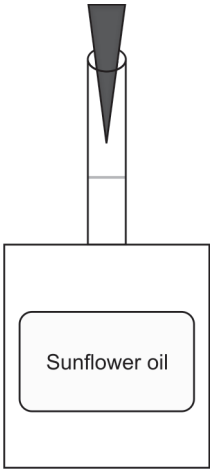
Q. 26H-7
i. What is the provitamin present in carrot?
ii. What is the earliest deficiency manifestation of this vitamin?
iii. Give its RDA in adults and children.

Ans.
- β carotene is the provitamin of vitamin A
- Vitamin A deficiency causes night blindness
- Children – 400-600 µgm/day; Adults – 750-1000 µgm/day.

26H-8

Spotter-70

Q. 26H-8
i. What is the most abundant type of fatty acid in the sunflower oil?
ii. Why this kind of oils are recommended for cooking?



Ans.

- Sunflower oil contains 63% polyunsaturated fatty acid and it is a good source of essential fatty acids-linoleic acid and linolenic acids.
- Consumption of PUFA helps reducing serum cholesterol level - by the up regulation of LDL receptors there by enhancing the hepatic uptake of LDL and reducing the level of atherogenic lipoprotein fraction, LDL producing an anti athergenic effect.

26H-9

Spotter-71

Q. 26H-9

Give the RDA of the following vitamins in adults:

1. Pyridoxine
2. Folic acid
3. Vitamin C
4. Thiamine

Ans.

1. Pyridoxine	2-2.5 mg/day
2. Folic acid	200 µg/day
In pregnancy	400 µg/day
3. Vitamin C	75-100 mg/day
4. Thiamine	1-1.5 mg/day

26H-10

Spotter-72

Q. 26H-10

What is the advantage of including high fiber containing green leafy vegetables in the diet?

Ans. High fiber content in the diet reduces serum total cholesterol and LDL and raises HDL fraction–thereby help reducing atherogenic process in the blood vessels.

26H-11

Spotter-73

Q. 26H-11

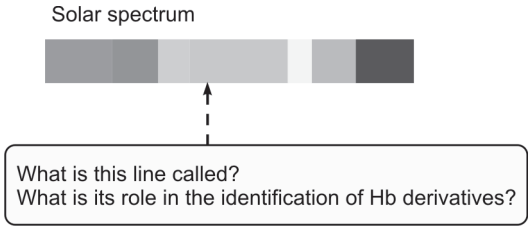
i. Name one branched fatty acid present in the green leafy vegetables.
ii. How is it metabolized?
iii. Name the condition in which you will get defective metabolism of such branched fatty acids.

Ans.

- Phytanic acid
- α-oxidation
- Refsum's disease

26I. SPECTROSCOPY

26I-1



Spotter-74

Q. 26I-1

Ans.

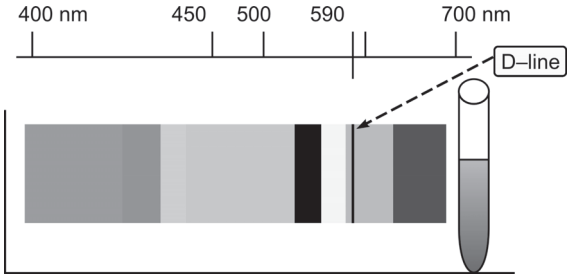
- D line (the most prominent of Fraunhofer lines)
- D line is used as the reference line in the identification of Hb derivatives by spectroscopic examination

26I-2

Spotter-75

Q. 26I-2

The solution of Hb derivative and Hb absorption spectrum is shown. Identify the Hb derivative.

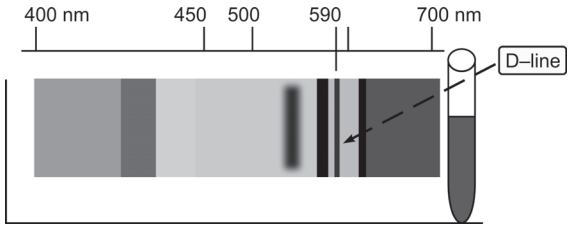


Ans.

- **Deoxy Hb**
- **Color of the solution :** Purple.

Spectroscopy: A single broad band in the green region. The mid point of this band corresponds to 565 nm in the green region.

26I-3



Spotter-76

Q. 26I-3

The solution of Hb derivative and Hb absorption spectrum is shown. Identify the Hb derivative.

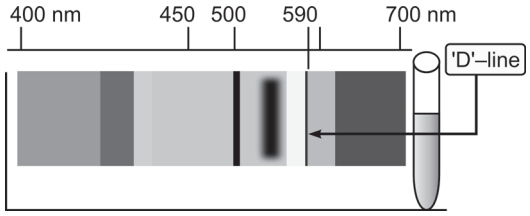
Ans.

- **Meth Hb**
- **Color of the solution:** Reddish brown.

Spectroscopy: 3 bands are seen. The characteristic alpha band in the orange region at 630 nm beta band at 577 nm and gamma band at 541 nm in the in the green region.

26I-4

Spotter-77



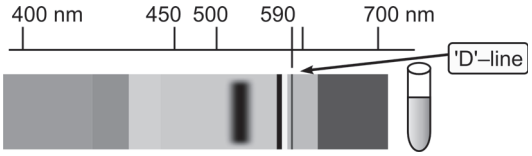
Q. 26I-4
The solution of Hb derivative and Hb absorption spectrum is shown. Identify the Hb derivative and give its application.

Ans.
- Globin hemochromogen; color of the solution: Pink

Spectroscopy: Two characteristic bands are seen- alpha band at 555 nm in the green region and beta band at 525 nm in the green region itself.

Application: Fetal Hb is relatively resistant to alkali so it will help to distinguish blood rich in HbF from blood containing predominantly adult hemoglobin (Hb A).

26I-5



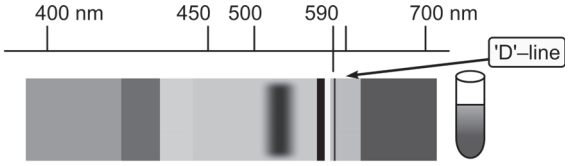
Spotter-78

Q. 26I-5
The solution of Hb derivative and Hb absorption spectrum is shown. Identify the Hb derivative.

Ans.
- **Oxy Hb**
- **Color of the solution :** Orange red

Spectroscopy: 2 bands characteristic of oxy Hb are seen. α band at 577 nm in the yellow region and β band at 541 nm in the green region.

26I-6



Spotter-79

Q. 26I-6
The solution of Hb derivative and Hb absorption spectrum is shown. Identify the Hb derivative and its importance.

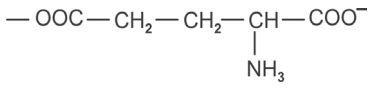
Ans.
- **Carboxy Hb**
- **Color of the solution:** Pink

Spectroscopy: 2 characteristic bands are seen. α band at 570 nm in the yellow region and β band at 535 nm in the green region.

Importance: Identification of carboxy Hb will help in the diagnosis of carbon monoxide poisoning

26J. CONCEPTUAL QUESTIONS

26J-1



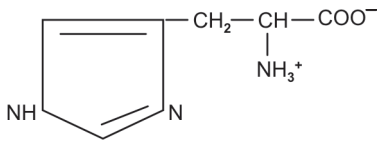
Spotter-80

Q. 26J-1
i. Name this biomolecule.
ii. What is the consequence produced if this molecule in the 6th position of β globin chain of hemoglobin is replaced by valine?

iii. Name one biochemical method to detect such abnormality in an individual.

Ans.
- Glutamic acid
- Formation of HbS in the body leading to sickle cell anemia
- Hb electrophoresis

26J-2



Spotter-81

Q. 26J-2
Hb has large buffering capacity owing to the presence of the molecule shown in the figure. Name the molecule and explain how it confers this property to hemoglobin.

Ans.
- Histidine
- pK_a value of ionisable imidazole side chain of histidine is 6.1 which is closer to the physiological pH range of blood. pK_a value of all the other 19 primary amino acids are far away from the physiological pH of blood, i.e. 7.35-7.45. 38 Histidine residues are present in a molecule of Hb. Hence the buffering property of Hb is mainly dependent on histidine residues.

26J-3

Single letter abbreviations of a polypeptide is given below.

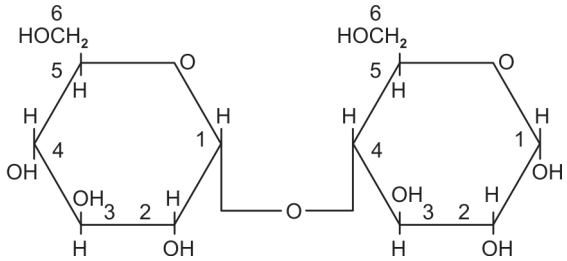
A V L D F W Q

Spotter-82

Q. 26J-3
Name the amino acids in order from the aminoterminal end.

Ans. Alanine, valine, leucine, aspartic acid, phenylalanine, tryptophan, glutamine

26J-4



Spotter-83

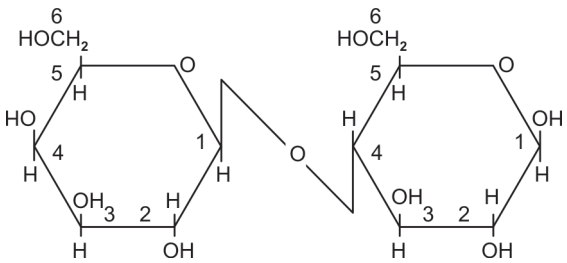
Q. 26J-4
Identify the sugar, name the linkage and mention the name of the intestinal enzyme concerned with its digestion.

Ans.
- Maltose
- O-α-D–Glucopyranosyl-(1→4)α-D–glucopyranose
- Maltase

26J-5

Spotter-84

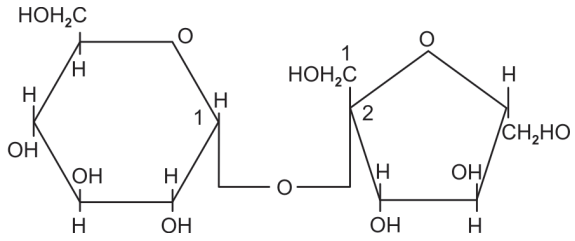
Q. 26J-5
Identify the sugar, name the linkage and mention the name of the intestinal enzyme concerned with its digestion.



Ans.
- Lactose

- *O*-β-D – Galactopyranosyl-(1→4) β-D – glucopyranose
- Lactase

26J-6



Spotter-85

Q. 26J-6

Identify the sugar, name the linkage and mention the name of the intestinal enzyme concerned with its digestion.

Ans.

- Sucrose
- *O*-α-D – Glucopyranosyl-(1→2) β-D – fructofuranoside
- Sucrase

26J-7

Spotter-86

Q. 26J-7

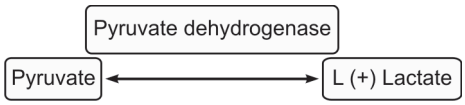
i. What is the disaccharide present in the mushroom?

ii. Why some individuals develop diarrhea, vomiting and abdominal pain upon consuming edible mushrooms?

Ans.

- Trehalose
- Those developing hypersensitivity reactions on taking edible mushrooms may be due to intestinal trehalase deficiency (that digest trehalose) leading to retention of trehalose which is then fermented by intestinal bacteria to release osmotically active substances that draws water into the lumen leading to diarrhea, vomiting, abdominal distension and abdominal pain.

26J-8



Spotter-87

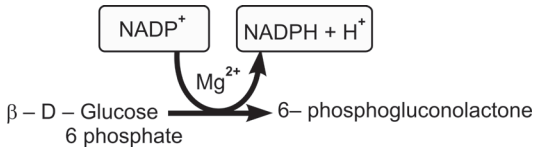
Q. 26J-8

Name the three inhibitors of this reaction and mention the metabolic derangement caused by the deficiency or inhibition of pyruvate dehydrogenase enzyme.

Ans.

- Arsenic ions, mercuric ions and deficiency of thiamine
- Lactic acidosis

26J-9



Spotter-88

Q. 26J-9

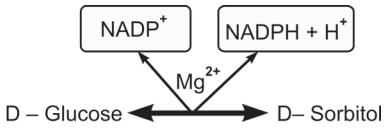
i. Name the enzyme catalyzing this reaction and the pathway to which it belongs to.

ii. What are the implications of this enzyme?

Ans.

- Glucose-6-phosphate dehydrogenase (G6PD) catalyze this reaction. This reaction is a part of Hexose Monophosphate (HMP) shunt pathway
- G6PD is the rate limiting enzyme of HMP shunt pathway. Its deficiency produces hemolytic anemia, when susceptible individuals are given oxidant drugs, e.g. antimalarial primaquin, aspirin, sulphonamides.

26J-10



Spotter-89

Q. 26J-10

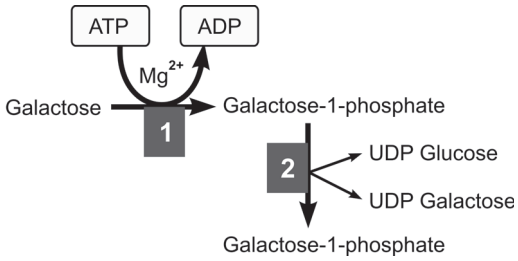
What is the enzyme catalyzing this reaction? Name one condition in which its activity is increased and mention one important consequence of that.

Ans.

- Aldose reductase
- Diabetes mellitus
- Diabetic cataract

26J-11

Spotter-90



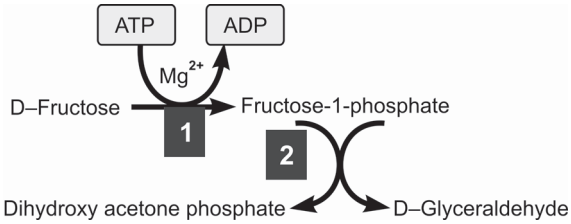
Q. 26J-11

Name the enzymes catalyzing reaction (1) and reaction (2) and the deficiency manifestation of these enzymes.

Ans.

- **Galactokinase** catalyze reaction (1) and **galactose 1 phosphate uridyl transferase** reaction (2)
- Galactosemia

26J-12



Spotter-91

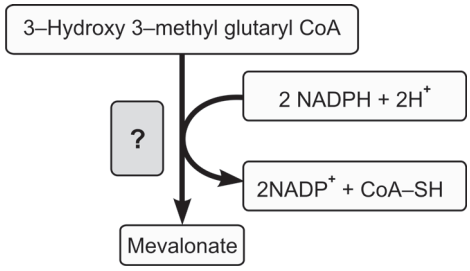
Q. 26J-12

1. Name the enzymes catalyzing reaction 1 and reaction.
2. Name the deficiency manifestation of these enzymes.

Ans.

- **Fructokinase** catalyze reaction (1) and **aldolase B** the reaction (2)
- Fructokinase deficiency causes **essential fructosuria** and aldolase B deficiency causes **hereditary fructose intolerance.**

26J-13



Spotter-92

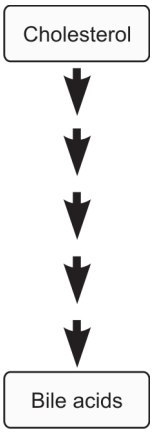
Q. 26J-13

i. Name the enzyme catalyzing the reaction.
ii. Cite the pathway to which this reaction fit in as a rate limiting step.
iii. Name two endogenous substances and one drug that inhibit this enzyme and state the purpose of administration of that drug.

Ans.

- HMG Co A reductase
- Cholesterol biosynthetic pathway
- Endogenous substances – Cholesterol, bile acids;
- Drug: Simvastatin coming under statin group of drugs used for lowering serum cholesterol

26J-14



Spotter-93

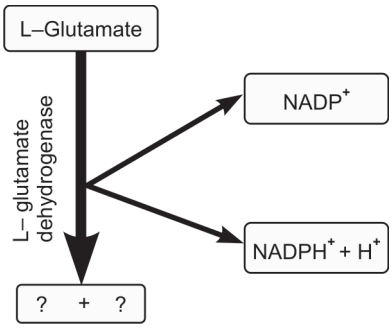
Q. 26J-14

i. Name the enzyme catalyzing the rate limiting step of bile acid synthesis and two factors inhibiting the activity of this enzyme.
ii. Name two primary bile acids and two secondary bile acids.

Ans.

- Enzyme catalyzing the rate limiting step: 7-α hydroxylase
- Inhibiting factors – Vitamin C deficiency and bile acids
- Primary bile acids – Taurocholic acid and glycocholic acid
- Secondary bile acids – Deoxycholic acid and lithocholic acid

26J-15



Spotter-94

Q. 26J-15

Name the products of this reaction and two factors inhibiting it.

Ans.

- Products-α-Ketoglutarate and ammonia
- Two inhibiting factors – ATP and GTP

26J-16

Clinical condition	*Type of bilirubinemia*	*Defect*
Neonatal physiological jaundice	a. ?	b.?
Crigler-Najjar syndrome type I	c. ?	d. ?
Gilbert's syndrome	e ?	f ?
Dubin Johnson syndrome	g ?	h ?

Spotter-95

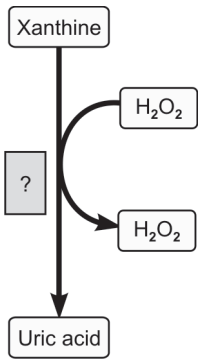
Q. 26J-16
Go through the table and answer accordingly.

Ans.
a. Unconjugated hyperbilirubinemia
b. Accelerated hemolysis around the time of birth and immature hepatic function (uptake, conjugation and secretion of bilirubin)
c. Unconjugated hyperbilirubinemia
d. Severe deficiency of UDP glucuronyl transferase activity in the hepatocytes
e. Unconjugated hyperbilirubinemia
f. Mild deficiency of UDP glucuronyl transferase activity in the hepatocytes
g. Conjugated hyperbilirubinemia
h. Defective ATP dependent organic anion transporter

26J-17

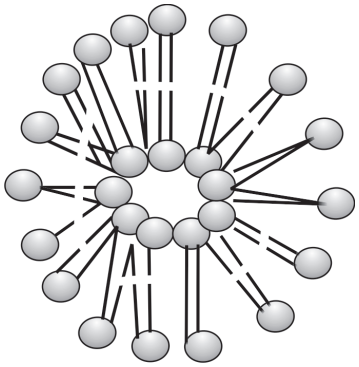
Spotter-96

Q. 26J-17
i. Name the enzyme catalyzing this reaction.
ii. What is the importance of this enzyme in the treatment of gout?



Ans.
- Enzyme catalyzing the reaction – Xanthine oxidase
- The drug allopurinol (a purine analog) inhibit xanthine oxidase and it helps lowering the uric acid level in gout.

26J-18



Spotter-97

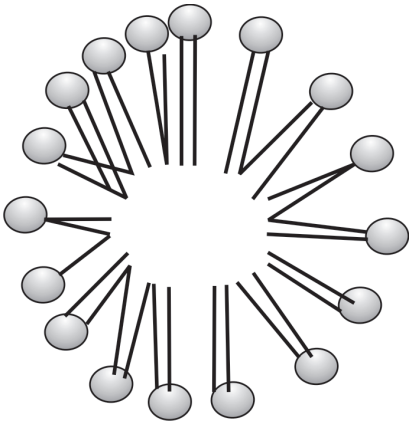
Q. 26J-18
i. Identify the structure.
ii. How it can be formed?
iii. What is its use in the medical field?

Ans.
- Lipsome. It consists of spheres of lipid bilayers that enclose a aqueous medium.
- It can be formed by sonicating amphipathic lipids in an aqueous medium.
- By combining liposomes with tissue specific antibodies and can be used as carriers of drugs in circulation and can be targeted to specific organs. It may be very useful when drugs with side effects have to be used for therapeutic purpose as in cancer treatment.

26J-19

Spotter-98

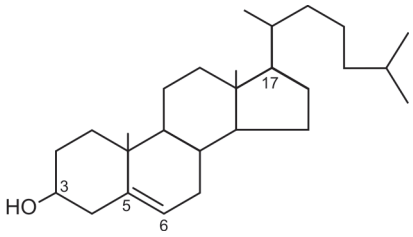
Q. 26J-19
i. Identify the structure.
ii. How can it be formed?
iii. What is its significance?



Ans.

- Micelle. It is a sphere of lipid monolayer
- Major lipids of the diet, triglycerides and phospholipids hydrolyze to form amphipathic lipids and are emulsified to micelles. Micelles are formed when a critical concentration of amphipathic lipids (fatty acids, phospholipids, sphingolipids, bile salts and cholesterol in an aqueous medium) reaches in an aqueous medium.
- Allow absorption of lipids and fat soluble vitamins from the gut.
  (Fat soluble vitamins if present also associate with micelle. These micelles carrying the fat soluble nutrients being soluble transported through the aqueous environment of intestinal lumen and come in contact with the intestinal brush border there by allowing absorption of fat soluble nutrients through intestinal mucosal epithelium).

26J-20



Spotter-99

Q. 26J-20

i. Identify the compound.
ii. Name two important compounds derived from it in the body.
iii. What is the rate limiting enzyme of its synthetic pathway?

Ans.

- Cholesterol (3-hydroxy-5, 6–cholestene)
- Vitamin D and steroid hormones
- HMG Co A reductase

SECTION FIVE

OSPE (Objective Structured Practical Examination) Questions

OSPE (Objective Structured Practical Examination) Questions

27

27A. OSPE - QUALITATIVE EXPERIMENTS

Q. 27A-1
A solution suspected to contain a carbohydrate is provided. Do a test to detect its presence.

Ans.

Guideline:
- Test for detecting the presence of carbohydrate – Molisch's test
- Do the test and write down the procedure, principle, observation and inference.

NB
- Mouth pipetting should be avoided while taking the reagents
- The solution on either side of the ring test must be nearly equal
- The ring should be violet in color

Q. 27A-2
Glucose solution is provided in a numbered beaker/test tube. Show that it is a carbohydrate by doing one test and an aldose by doing two tests.

Ans.

Guideline:
- To show that glucose is a carbohydrate – Do Molisch's test.
- To demonstrate glucose is an aldose – Do Seliwanoff's test, Rapid furfural or Foulger's test (any two).
- Do the test and write down the procedure, observation and inference.

Q. 27A-3
Glucose solution is provided in the numbered beaker. Give the approximate concentration of the solution by doing the appropriate test.

Ans.

Guideline:
- Do Benedict's test
- Write down the approximate concentration of the solution by noting the color of the precipitate.
- Do the test and write down the procedure, principle observation and inference.

Q. 27A-4
Fructose solution is supplied. Demonstrate that fructose is a carbohydrate and a keto group containing sugar.

Ans.

Guideline:
- To show that fructose is a carbohydrate – Do Molisch's test.
- To demonstrate fructose is a ketose – Do Seliwanoff's test and Rapid furfural test.

- Do the tests and write down the procedure, principle observation and inference.

Q. 27A-5
A disaccharide solution is supplied. Demonstrate the presence of a reducing disaccharide in the given solution and identify it.

Ans.

Guideline:
- To demonstrate the presence of disaacharide – Do Benedict's test to show the reducing nature and Barfoed's test to prove it to be a disaccharide.
- To identify the disaccharide – Do Osazone test and by noting the shape of the crystal identify it as lactosazone or maltosazone.
- Do the tests and write down the procedure, principle observation and inference.

Q. 27A-6
Do a test to show that the given solution contains sucrose.

Ans.

Guideline:
- Do Specific sucrose test
- Do the test and write down the procedure, principle observation and inference.

Q. 27A-7
Name the homopolysaccharide present in rice and do a test to demonstrate its presence in the given solution.

Ans.

Guideline:
- Homopolysaccharide present in rice - starch
- Do iodine test which will give a deep blue color for starch and will disappear on heating and reappear on cooling.
- Do the test and write down the procedure, principle observation and inference.

Q. 27A-8
Name two non reducing sugars and write the monomers in them. Demonstrate the presence of a non reducing sugar in the sample provided.

Ans.

Guideline:
- Two non reducing sugars: Sucrose – monomers of sucrose are glucose and fructose; Trehalose – monomer is glucose (two glucose residues)
- To show non reducing nature : Do Benedict's test – it will be negative .
- Do the tests and write down the procedure, principle observation and inference.

Q. 27A-9
Demonstrate that starch is a nonreducing carbohydrate.

Ans.

Guideline:
- Do Molisch test to show it as a carbohydrate and Benedict's test to demonstrate the non reducing nature.
- Do the tests and write down the procedure, principle observation and inference.

Q. 27A-10
A protein solution is given. Demonstrate its precipitation by inorganic salt and organic solvent.

Ans.

Guideline:
- Do precipitation test with ammonium sulfate and ethanol
- Do the tests and write down the procedure, principle observation and inference

Q. 27A-11
A protein solution is given. Demonstrate its precipitation by a heavy metal and an anionic reagent.

Ans.

Guideline:

- Do precipitation test with heavy metals by using 10 % lead acetate or 10 % $CuSO_4$ **or** 10 % $ZnSO_4$ and by anionic reagent by using metaphosphoric acid
- Do the test and write down the procedure, principle observation and inference

Q. 27A-12

Do Heller's test with the protein solution. Write down its application in clinical chemistry laboratory.

Ans.

Guideline:

- Do Heller's test

Procedure: Take 2 ml of concentrated HNO_3 or concentrated HCl in a test tube. Add 2 ml of protein solution along the sides of the test tube slowly.

Observation: White ring forms at the junction of two liquids.

Inference: Albumin as well as globulins are precipitated by strong mineral acids.

Principle: Strong acids causes denaturation and precipitation of proteins

Application: It is used as a test for detecting protein in urine or other body fluids

Q. 27A-13

Demonstrate that the given solution is a protein solution by doing a chemical test and also give its principle.

Ans.

Guideline:

- Do Biuret test.
- Do the test and write down the procedure, principle observation and inference.

Q. 27A-14

Name the amino acid from which catecholamines are synthesized. Demonstrate its presence in the given solution by doing two tests.

Ans.

Guideline:

- Catecholamines are synthesized from the amino acid Tyrosine.
- Two tests: Xanthoproteic test and Millon's test.
- Do the tests and write down the procedure, principle observation and inference.

Q. 27A-15

Protein solution is supplied. Do a test to demonstrate its presence in the solution and give its principle.

Ans.

Guideline:

- Do Biuret test.
- Write down its principle.

Q. 27A-16

Demonstrate the presence of indole ring containing essential amino acid in the solution provided. Name a biologically important compound and a vitamin formed from it in the body.

Ans.

Guideline:

- Do aldehyde test
- Biologically important compound - Serotonin
- Vitamin - Niacin

Q. 27A-17

Name the amino acid participating both in the urea cycle and creatine synthesis and also serving as a substrate of NOS (nitric oxide synthase enzyme) and demonstrate it in the given sample. Name the characteristic side group of this amino acid.

Ans.

Guideline:

- Arginine
- Do Sakaguchi's test
- Guanidino group

Q. 27A-18
Name sulfur containing primary amino acids. Do a test to demonstrate any one of them in the given sample.

Ans.

Guideline:
- Cysteine and methionine
- Do sulfur test which will detect cysteine but not methionine due to the presence of thioether bond in it.

Q. 27A-19
Name the amino acid from which the histamine is formed and show its presence in the sample provided.

Ans.

Guideline:
- Histidine
- Do Pauly's test

Q. 27A-20
Demonstrate two aromatic amino acids in the albumin solution supplied.

Ans.

Guideline:
- Two aromatic amino acids – Tryptophan and Tyrosine.
- Do aldehyde test for tryptophan and Millon's test for tyrosine

Q. 27A-21
Perform isoelectric precipitation of albumin with the given solution.

Ans.

Guideline:
- Do isoelectric precipitation test of albumin

Q. 27A-22
Do heat and acetic acid with the albumin solution and write down the principle of the test.

Ans.

Guideline:
- Do heat and acetic acid test

Q. 27A-23
Demonstrate that the isoelectric point of casein is 4.6.

Ans.

Guideline: Do isoelectric precipitation using the indicator Bromocresol green (pH range 4.0 – -5.6; color range – yellow to blue). The purpose of adding the indicator is to get pH around 4.6. Adjust the color to green by adding weak acid or base to get green color which corresponds to pH 4.6 and at this point precipitation occurs (see qualitative section for further details about the test).

Q. 27A-24
Demonstrate the presence of phosphorus in casein.

Ans.

Guideline:
- Do Neumann's test (see Chapter 2 on Reactions of Proteins)

Q. 27A-25
Prove that the given protein solution is albumin and not casein.

Ans.

Guideline:
- Do any one of the following tests,

Half saturation test – albumin do not precipitated completely where as casein is fully precipitated with half saturation with ammonium salt.

Sulfur test (see Chapter 2 on Reactions of Proteins).

Neumann's test (see Chapter 2 on Reactions of Proteins).

Q. 27A-26
Prove that the given substance is fat by doing any two tests.

Ans.

Guideline:
- Do solubility test, grease spot test or acrolein test (see Chapter 3 on Reactions of Lipids).

Q. 27A-27
Two specimens of oils are supplied. Identify them as saturated or unsaturated type.

Ans.

Guideline:
- Do Halogenation test (see Chapter 3 on Reactions of Lipids)

Q. 27A-28
Identify the crystal and draw the shape of it. Name two chemical tests to identify it.

Ans.

Guideline:
- Cholesterol crystal-rhombic crystals notched at one corner
  1. Salkowski's reaction (H_2SO_4 test).
  2. Libermann Burchard reaction (acetic anhydride sulfuric acid test) (see Chapter 3 on Reactions of Lipids).

Q. 27A-29
Do two tests to prove that the given solution contains urea.

Ans.

Guideline: Do alkaline hypobromite test and specific urease test (see Chapter 4 on Reactions of Urea).

NB: Remember to put a control while doing specific urease test.

Q. 27A-30
Do two tests to prove that the given solution contains urea.

Ans.

Guideline: Do alkaline hypobromite test and specific urease test

Remember to put a control for doing specific urease test (see Chapter 4 on Reactions of Urea).

Q. 27A-31
Show that the given solution contains creatinine and write down the principle of the test.

Ans.

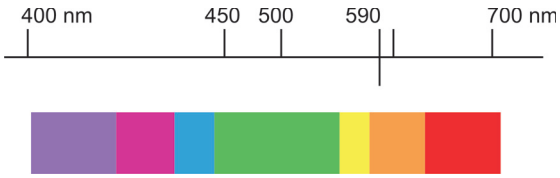
Guideline:
Do Jaffe' test (picric acid reaction)

Q. 27A-32
Demonstrate the presence of uric acid in the given solution.

Ans.

Guideline:
Do Benedict's uric acid test and Schiff's test.

Q. 27A-33
Identify the Hb derivative by spectroscopy and mark the position of the band in the visible spectrum.



Ans.
Guideline: Any one of the following Hb derivative may be given for identification purpose. Mark the characteristic absorption bands on the visible spectrum.

Oxy Hb: Two bands, α band at 578 nm and β band at 540 nm

Deoxy Hb: Single broad band at 565 nm

Meth Hb: α band at 633 nm, β band at 578 nm and γ band at 540 nm

Globin hemochromogen: α band at 555 nm, β band at 525 nm.

Q. 27A-34
Demonstrate that the milk contains reducing disaccharide lactose.

Ans.
Guideline: Do Benedict's test and Osazone test.

Q. 27A-35
Demonstrate the presence of calcium and phosphorus in milk.

Ans.
Guideline: Do the test for calcium and phosphorus as mentioned in Chapter 10 on Reactions of Milk.

Q. 27A-36
Check the pH and specific gravity in the given sample of urine.

Ans.

Guideline: Check the pH of urine by red and blue litmus paper and specific gravity by urinometer.

Q. 27A-37
Demonstrate the presence of chloride in the normal urine supplied. Mention the normal excretion rate and the condition in which its concentration in urine becomes high.

Ans.

Guideline: Do test for chloride in urine
Normal excretion rate: 10-15 g/day
Its content in urine is increased in Addison's disease in which there is aldosterone deficiency which causes reduced reabsorption of sodium and chloride leading to their excessive excretion in urine.

Q. 27A-38
Make a report of 5 physical properties of the sample of urine provided.

Ans.

Guideline: Look for appearance, color, odor , pH and specific gravity and make a report of them.
For details see Chapter 8 on Urine Analysis.

Q. 27A-39
Demonstrate the presence of inorganic sulfate in the normal urine supplied. Mention the normal excretion rate and the source of it.

Ans.

Guideline: Do test for sulfate (see Chapter 4 on Reactions of Urea).
Rate of excretion - 0.8 -1.0g/day
Source - Sulfur containing amino acids

Q. 27A-40
Demonstrate the presence of calcium in the normal urine supplied. Mention the normal excretion rate and the condition in which its concentration in urine becomes high.

Ans.
Guideline: Do test for calcium (see Chapter 4 on Reactions of Urea).
Rate of excretion - 0.1-0.3g/day (100-300 mg/day)
Increased rate of excretion seen in hyperparathyroidism (300-700 mg/day), multiple myeloma (300-500 mg/day).

Q. 27A-41
Demonstrate the presence of phosphorus in the normal urine supplied. Mention the normal excretion rate and the condition in which its concentration in urine becomes high.

Ans.

Guideline: Do test for phosphorus
Rate of excretion - 1g/day; Phosphates derived from inorganic phosphates in the diet – phosphoproteins, nucleoproteins and phospholipids.

Increased rate of excretion seen in hyperparathyroidism

Q. 27A-42

Demonstrate the presence of urea in the normal urine supplied. Mention the normal excretion rate and the condition in which its concentration in urine becomes high and low.

Ans.

Guideline: Do alkaline hypobromite test or specific urease test.

Rate of excretion - 7-16 g/day

Increased rate of excretion seen in high protein diet, conditions leading to increased tissue break down (increased protein catabolism) eg: fever, diabetes mellitus, adrenal cortical hyperactivity.

Decreased rate of excretion seen in severe liver diseases. In affections of liver when urea synthetic function is disturbed, urea is formed in low amounts.

Q. 27A-43

Demonstrate the presence of uric acid in the normal urine supplied. Mention the normal excretion rate and the condition in which its concentration in urine becomes high and low and give the normal serum level.

Ans.

Guideline: Do Benedict's uric acid test or Schiff's test.

Rate of excretion : 300 – 800 mg/day on an average diet

Increased rate of excretion: High purine diet, conditions where there is increased tissue turn over without any impairment of kidney function –leukemia or other malignancies, gout, cortisone therapy

Normal serum level

Males: 3.6 – 7.7 mg% (214 – 458 µmol/L)

Females: 2.5 – 6.8 mg % (149 – 405 µmol/L)

Q. 27A-44

Demonstrate the presence of creatinine in the normal urine supplied. Mention the normal excretion rate and the condition in which its concentration in urine becomes high and give the normal serum level.

Ans.

Guideline: Do Jaffe's test.

Rate of excretion -1 to 2 g/day (nearer to higher limit in males and to lower limit in females).

Increased rate of excretion Myopathies, Fever, Muscle injuries.

Normal serum level: 0.7–1.4 mg%

Q. 27A-45

Mention a catabolite of heme present in normal urine. Do a test to demonstrate it. Name the condition in which it is excessively excreted in urine.

Ans.

Guideline:

- Urobilinogen
- Ehrlich's test (given in the chapter on reaction of urine). Place a control along with the test since urobilinogen is present only in traces normally.
- It is excessively excreted in **hemolytic jaundice** due to increased rate of break down of RBCs leading to release of heme and it's catabolism to cause excretion of large amounts of urobilinogen in urine.

Q. 27A-46

Name a possible urinary marker of intestinal obstruction. What is its normal excretory rate? Demonstrate its presence in normal urine.

Ans.

Guideline:

- Indican (an organic sulfate) Indican is potassium salt of Indoxyl sulfuric acid. It is

formed from tryptophan. It is a organic sulfate (ethereal sulfate). (see Chapter 4 on Reactions of Urea)
- Excretory rate in normal urine - 0.04 to 0.1 g per day
- Do the test for organic sufate (ethereal sulfate) [given in the chapter on reactions of urine]

Q. 27A-47

Demonstrate the presence of ammonia in the urine. Name two sources of ammonia in the body.

Ans.

Guideline:
- Do test for ammonia (given in the Chapter 4 on Reaction of Urea)
- Catabolism of amino acids derived from proteins and catabolism of other nitrogen containing molecules like purines and pyrimidines.

Q. 27A-48

A man aged 60 years came with complaints of polyuria, polydypsia, polyphagia and loss of weight. Urine specimen of the patient is available. Do a simple test to arrive at a provisional diagnosis. Write down the specific tests to confirm the diagnosis.

Ans.

Guideline:
- Provisional diagnosis is diabetes mellitus
- Do Benedict's test
- Estimation of fasting and 2 hour postprandial blood glucose are needed to make a diagnosis of diabetes mellitus. Fasting blood glucose > 7 mmol/L (126 mg%) and postprandial 2 hour blood glucose >11.1 mmol/L (200 mg%) diagnostic of diabetes mellitus.

Q. 27A-49

Raman aged 58 years, a farmer having the history of diabetes mellitus for 10 years taking irregular treatment came with vomiting, tiredness, disorientation and convulsions. What is your probable diagnosis? Do two simple tests with the urine of the patient to establish your provisional diagnosis.

Ans.

Guideline:
- The patient may be suffering from a complication of Diabetes mellitus called diabetic ketoacidosis
- Do Benedict's test to detect sugar in urine and Rothera's test to detect ketone bodies in urine

Q. 27A-50

A boy aged 5 years was brought to the out patient department with complaints of puffiness of face, edema of legs, reduced urine out put. What can be the diagnosis? Urine of that child is supplied. Do the most appropriate test to support your diagnosis? What other blood test would be helpful to confirm the diagnosis.

Ans.

Guideline:
- Nephrotic syndrome is the provisional diagnosis
- Do Heat and acetic acid to detect proteins in urine which will be positive in a case of nephritic syndrome
- Blood tests that can support above diagnosis are serum albumin (which would be lowered); serum cholesterol and lipoproteins (which would be elevated).

Nephrotic syndrome is characterized by heavy proteinuria (Total protein > 3g/24 h or albumin > 1.5 g/24 h), hypoalbuminemia, hypercholesterolemia and massive edema. Edema is the result of decreased oncotic pressure due to loss of protein. The transudation of salt and water into the interstitial spaces causes a decrease in plasma volume that in turn causes the kidneys to retain sodium. Abnormalities in lipid metabolism

occur in the form of ↑total cholesterol, ↑VLDL, ↑HDL. Inspite of ↑HDL cholesterol (HDLc), cholesterol remains high due to reduced activity of lecithin cholesterol acyl transferase activity (LCAT) there by reducing reverse cholesterol transport leading to hypercholesterolemia.

Nephrotic syndrome results from a variety of causes – minimal change glomerulonephritis, membranous glomerulonephritis, drugs, infection, systemic lupus erythematosis, diabetic nephropathy.

Q. 27A-51

A boy aged 10 years admitted with complaints of sudden onset of passing red colored urine, hypertension and pedal oedema. The boy had a history of pyoderma on the skin about 2 months back. The urine collected from the boy is supplied. What is your provisional diagnosis? Do relevant tests with urine supplied and write down the relevant investigations to be done in the blood in order to reach definitive diagnosis.

Ans.

Guideline:

- Provisional diagnosis – Acute glomerulonephritis.
- Do heat and acetic acid to detect proteins and benzidine test to detect blood in urine.
- Investigations to be done in blood are blood urea and serum creatinine – these will be raised in glomerulonephritis.

Acute glomerulonephritis (GN) is characterized by the rapid onset of hematuria, proteinuria, reduced GFR and sodium and water retention leading to edema and hypertension. Many of the cases of acute GN is related to infection of skin or pharynx with group A β hemolytic streptococcal infection. This causes immune mediated injury to glomerular capillaries leading to GN. Immune mediated damage to glomerular barrier causes leakage of proteins into the urine and due to reduced GFR, nitrogenous waste products like urea and creatinine are retained in the blood causing uremia or azotemia.

Q. 27A-52

A girl aged fifteen studying in tenth standard came with complaints of head ache, decreased appetite, yellowish discoloration of sclera and fever. On examination the girl is febrile and jaundiced. Liver is slightly enlarged. Urine specimen of the patient is supplied.

Do the relevant investigations to establish a provisional diagnosis.

What other tests you like to do to confirm the diagnosis?

Ans.

Guideline:

- The patient may be suffering from hepatic jaundice.
  Do Modified Fouchet's test and Hay's tests which will be positive and the Ehrlich's test will be weakly positive or negative.
- Serum bilirubin and serum transaminases and alkaline phosphatase will help in making differential diagnosis of jaundice.

Total Bilirubin will be raised; Conjugated bilirubin will be raised due to obstruction of bile flow through the inflamed biliary canaliculi.

Serum transaminases (ALT and AST) and alkaline phosphatase (ALP) will be raised. But activity of transaminases is much higher than ALP favoring inflammation of liver tissue due to hepatitis (infection of liver).

Q. 27A-53

A 50 year man hailing from Attapadi of Palghat district came with complaints of joint pains and severe cramps in the legs, jaundice and hematuria.

What all relevant investigations you will do with his urine?

Do one test that will give clue for diagnosis.

What other blood tests are needed to diagnose the type of jaundice?

Ans.

Guideline: The history is strongly suggestive of hemolytic jaundice. Leg cramps, hematuria, joint pains along with jaundice is suggestive of sickling crisis related to sickle cell anaemia.

- **Urine:** *Ehrlich's test for urobilinogen* would be strongly positive and suggestive of hemolytic jaundice (increased rate of RBC break down →↑ heme release and catabolism →↑ urobilinogen in urine). Benzidine test for blood will also be positive.
  Hay's test for bile salts and Modified Fouchet's test for bilirubin will be negative since there is no obstruction to biliary flow in this case.
- In order to prove the presence of excess urobilinogen in urine due to hemolytic disease do Ehrlich's test.
- Do estimation of serum total bilirubin (TB), conjugated bilirubin (CB) and unconjugated bilirubin (UCB). The TB and UCB will be highly raised and CB will be marginally raised. Serum hepatic enzymes (ALT, AST and ALP) assay will show normal pattern since liver is not affected.

Q. 27A-54

A 52-year-old fat lady came with abdominal pain, jaundice and itching.

Blood report is given below.

TB – 8 mg%; CB – 6.5 mg%; UCB – 1.5 mg%

Serum AST (SGOT) - 15 IU/L (Reference interval 8 -20 IU/L)

Serum ALT (SGPT) - 30 IU/L (Reference interval 7 -35 IU/L)

Serum ALP - 180 IU/L (Reference interval 25 -75 IU/L)

The urine of the patient is supplied. Do two relevant tests to supplement the blood tests to arrive at a diagnosis.

Ans.

Guideline: The history suggestive of obstructive biliary disease characterized by abdominal pain, jaundice with conjugated hyperbilrubinemia and itching due to bile salt deposition in the skin.

Do Hay's test for bile salts and Modified Fouchet's test for bilirubin which would be positive in this case suggesting obstructive jaundice.

27B. OSPE-QUANTITATIVE EXPERIMENTS

Q. 27B-1

A 10-year-old boy admitted with tiredness, hematuria, oedema of legs, oliguria and hypertension. Urine protein +

What is your provisional diagnosis?

Estimate a relevant parameter in the serum to arrive at a diagnosis.

Ans.

Guideline:

- Provisional diagnosis: Glomerulonephritis.
- Estimate serum creatinine in the blood which will be raised in GN due to ↓ GFR

Q. 27B-2

A 50-year-old man admitted with tiredness, hematuria, oedema of legs, oliguria and hypertension. Urine protein +

What is your provisional diagnosis?

Name two parameters that would be raised in the blood due to reduced GFR of such cases.

Estimate any one of the parameter suggested by the examiner

Ans.

Guideline:

- Provisional diagnosis: Glomerulonephritis.
- Serum creatinine and blood urea

Q. 27B-3

50-year-old Mary admitted with puffiness of face with tiredness, edema of legs.

Serum cholesterol - ↑

Urine protein – Total protein > 3 g/24h; Albumin > 1.5 g/24 h

What is your provisional diagnosis?

Estimate one blood parameter that will fulfill the criteria for the diagnosis of the disease

Ans.

Guideline:

- Provisional diagnosis: Nephrotic syndrome
- Serum albumin

The diagnostic criteria for nephritic syndrome are **proteinuria (Total protein > 3 g/24h; Albumin > 1.5g/24 h), hypoalbuminemia, hypercholesterolemia** and **edema**. In order to show hypoalbuminemia, serum albumin estimation is to be done.

Q. 27B-4

A 45-year-old bank officer attended out patient clinic with complaints of polyuria, polyphagia, polydypsia and loss of weight.

What may be the probable diagnosis

Estimate a relevant parameter for making a diagnosis

Write down the criteria for establishing a diagnosis of diabetes mellitus

Ans.

Guideline:

- Probable diagnosis: Diabetes mellitus
- Blood sugar estimation

Criteria for the diagnosis of Diabetes mellitus

Any one of the following is diagnostic.

1. Classic symptoms of diabetes (polyuria, polyphagia, polydypsia and loss of weight) and random (regardless of the time of the preceding meal) plasma glucose concentration ≥ 200 mg%
2. Fasting plasma glucose ≥ 126 mg%
3. Two hour post load plasma glucose ≥ 200 mg% during OGTT.

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