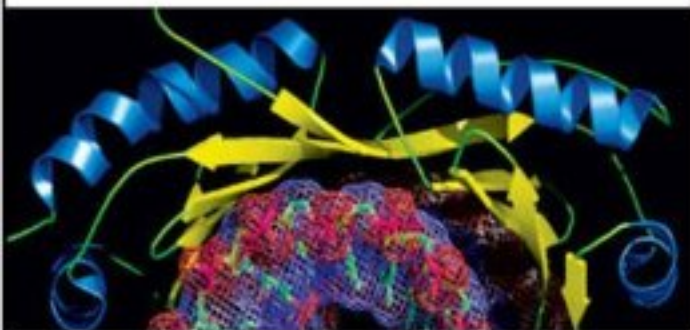


SECOND EDITION

Practical Textbook of

BIOCHEMISTRY

for Medical Students



DM Vasudevan
Subir Kumar Das

NEW DELHI
GATEWAY

**Practical Textbook of
BIOCHEMISTRY
for Medical Students**

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Second Edition

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Dedicated to
With Humility and Reverence,
at the
Lotus feet of the Holy Mother,
Sri Mata Amritanandamayi Devi

Preface to the Second Edition

We are very glad to see that the medical community has well accepted this *Practical Textbook of Biochemistry*, so that the second edition is being published within a short time.

This book is in resonance with the *Textbook of Biochemistry for Medical Students*, by Vasudevan et al, which is now in the 7th edition. That textbook is now accepted not only inside India, but also various other countries in the world. The Spanish edition of the Textbook is already in market and a Russian edition is in preparation. Students are advised to clear the doubts by going through that main textbook.

This practical book is prepared after consulting the syllabi of MBBS course of various universities. The contents are divided into qualitative and quantitative experiments, which the students are supposed to do by themselves in the practical classes. Further, a few more experiments are given, which may not be possible for the student to do by himself/herself. Some of these will be demonstrated in the practical classes. In the end, a few case reports are also included, which will be useful for the students to prepare the practical examinations.

Some of the pictures of the *Textbook of Biochemistry for Medical Students* by Vasudevan et al have been reproduced in this practical book. The remarkable success of the book was due to the active support of the publishers. This is to record our appreciation for the cooperation extended by Sri Jitendar P Vij (Group Chairman) and Mr Ankit Vij (Managing Director), and their associates.

We hope that this practical book is friendly to the students and be useful to the teachers. Suggestions from the teachers are most welcome to improve the contents of this book. Students and teachers are encouraged to contact the authors through Email.

DM Vasudevan
Subir Kumar Das

Preface to the First Edition

The medical community of India has warmly received the “Textbook of Biochemistry for Medical Students” by Vasudevan and Sreekumari. It is now running the 4th edition. There were regular and consistent requests from the student community to have a practical textbook. In order to satisfy this continued demand, this *Practical Textbook of Biochemistry for MBBS Students* is being published.

This book is prepared after consulting the syllabi of MBBS course of various universities. The contents are divided into qualitative and quantitative experiments, which the students are supposed to do by themselves in the practical classes. Further, a few more experiments are given, which may not be possible for the MBBS student to do by himself/herself. Some of these will be demonstrated in the practical classes. In the end, a few case reports are also included, which will be useful for the student to prepare the practical examinations.

We hope that this practical book is friendly to the students and be useful to the teachers. Suggestions from the teachers are most welcome to update the contents in due course.

**DM Vasudevan
Subir Kumar Das**

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PART
A

Qualitative Experiments

1

Colour Reactions of Proteins

Proteins are made up of amino acid residues joined by peptide bonds. Due to their polypeptide structure and different amino acid residues, protein reacts with a variety of reagents to form coloured products. These tests, known as colour reactions of proteins, are of importance in qualitative detection and quantitative estimation of proteins, and of their constituent amino acids in body fluids and other biological materials.

Proteins and amino acids used in different experiments:

1. Egg albumin is an egg protein, which is soluble in water.
2. Casein is the major protein in milk. It is a phosphoprotein with phosphate groups attached to the hydroxyl groups or serine and threonine residues. It is deficient in cysteine.
3. Gelatin is formed from collagen, the connective tissue protein, by boiling with water. It is a rich source of amino acid glycine. It is deficient in tyrosine, tryptophan and cysteine.
4. Metaproteins, proteoses and peptones are partially hydrolysed products of proteins like albumins and globulins. Albumin has relatively low molecular weight. Gelatin, metaproteins, proteoses and peptones are derived proteins.

EXPERIMENT 1. COLOUR REACTIONS OF PROTEINS BASED ON PEPTIDE BONDS AND TYPE OF AMINO ACID RESIDUES

Solution: 10% Egg-white or albumin

Biuret Reaction

Experiment	Observation	Inference
To 2 ml of sample solution, add 2 ml 5% NaOH and 3 drops of 1% CuSO ₄ . Repeat the test with distilled water (control)	Purple-violet or pink colour in test Blue colour in control	Peptide linkages present

- i. The reaction is so named since biuret (NH₂-CO-NH-CO-NH₂) formed by the condensation of two molecules of urea when heated. CO-NH is the peptide linkage in biuret. At least two peptide bonds in the molecule are required for a positive test. Individual amino acids and dipeptides will not answer this test.
- ii. CuSO₄ is converted to Cu(OH)₂ which chelates with peptide linkage in proteins to give the colour.
- iii. Strictly avoid excess addition of CuSO₄. Magnesium and ammonium ions will interfere in this reaction.

- iv. The colour varies depending on the number of peptide linkages; albumin/ globulin give violet, proteoses purple and peptones dark pink colour indicating that albumin/ globulins have largest number of peptide linkages and peptones the least.
- v. This reaction can be used for quantitative estimation of proteins.

Ninhydrin Reaction

Experiment	Observation	Inference
To 1 ml of sample solution, add 0.1% ninhydrin solution, boil and cool	Ruhemann purple colour	Amino acid present

- i. All α -amino acids give purple colour. The imino acids, proline and hydroxyproline give yellow colour. The coloured complex is known as Ruhemann's purple. Glutamine and asparagine produce brown colour.
- ii. α -amino acid + ninhydrin \rightarrow aldehyde + hydrindantin + NH_3 + CO_2 ;
hydrindantin + NH_3 + ninhydrin \rightarrow Ruhemann's purple + $3\text{H}_2\text{O}$.
- iii. Proteins will give a faint blue colour.
- iv. This reaction is often used to detect amino acids in chromatography.
- v. Proteins do not give a true colour reaction; but N-terminal amino group of a protein can react with ninhydrin to produce a faint blue colour.

Xanthoproteic Reaction (for Aromatic Amino Acids)

Experiment	Observation	Inference
To 2 ml of sample solution, add 1 ml conc HNO_3 and boil	Yellow precipitate	Aromatic amino acids, i.e. tyrosine, tryptophan or phenylalanine present
Cool test tube and add 40% NaOH excess	Colour of precipitate and the solution change to orange	

Yellow colour is due to the formation of nitro derivatives of benzene ring containing amino acids (tyrosine and tryptophan), the colour turns orange due to ionization when alkali is added. All proteins usually respond to this test. This reaction is also the basis of yellow stain in skin by nitric acid. Nitration of phenylalanine under these conditions normally does not take place.

Modified Millon's Reaction (Cole's Test)

Experiment	Observation	Inference
To 2 ml of sample solution, add 2 ml 10% HgSO_4 in 10% H_2SO_4 , boil, add 5 drops of 1% sodium nitrite, heat gently	Red PPT of mercury phenolate	Tyrosine present

- i. The colour is due to the formation of nitrated mercury phenolate ion of tyrosine (hydroxyphenyl group) present in proteins.
- ii. Heat coagulable proteins give red PPT, whereas smaller molecules of proteins like peptones give red coloured solution without PPT.
- iii. Gelatin and tapioca both are poor in tyrosine, does not give the test.
- iv. Chloride interferes with this reaction; so it is not suitable for urine test.

Aldehyde Test for Indole Nucleus (Hopkins-Cole's Test)

Experiment	Observation	Inference
To 2 ml of sample solution, add 1 ml of 1:500 (0.2%) formalin, 1 drop 10% HgSO ₄ in 10% H ₂ SO ₄ . Add 2 ml conc. H ₂ SO ₄ slowly and carefully along the side of the test tube. Do not mix	Violet ring at the junction of two liquid layers due to indole ring	Tryptophan present

- Mercuric sulphate cause mild oxidation of indole group of tryptophan, which condenses with an aldehyde to give the coloured complex
- p-Dimethylaminobenzaldehyde and strong hydrochloric acid (Ehrlich's reagent) give dark blue colour.
- Gelatin, poor in tryptophan, does not give the test.

Sakaguchi Test for Guanidine Group (Reaction of Arginine)

Experiment	Observation	Inference
To 2 ml of sample solution add 2 drops of 1% α -naphthol in alcohol, 4 drops of 40% NaOH, and 8–10 drops of bromine water	Bright red colour due to guanidium group	Arginine present

- Instead of NaOH and bromine water, 8 to 10 drops of alkaline hypobromide (NaOBr) can be used as a single reagent.
- Guanidino groups in arginyl residues of proteins react with the α -naphthol and NaOBr to give the coloured complex.
- This test is given by albumin, globulin and gelatin as it contains arginine.

Test for Sulphur-containing Amino Acids

Experiment	Observation	Inference
To 2 ml of sample solution add 2 ml 40% NaOH, boil for 3 minutes, cool, and add 2–3 drops of lead acetate	Black or brown PPT	Cysteine or cystine present

- Avoid excess of lead acetate solutions, which will form white PPT.
- Organic sulphur in cysteine and cystine are released as inorganic S²⁻ ions which form lead sulphide as follows:

$$\text{R-SH} + 2\text{NaOH} \rightarrow \text{ROH} + \text{Na}_2\text{S} + \text{H}_2\text{O}$$

$$\text{Na}_2\text{S} + (\text{CH}_3\text{COO})_2\text{Pb} \rightarrow \text{PbS} + 2\text{CH}_3\text{COONa}$$
- Methionine does not give this test as the sulphur group in this amino acid is in thioether linkage, which is difficult to break, and not released by treatment with NaOH. Albumin and keratin will answer this test, but casein (containing methionine) will not.

Pauly's Test for Imidazole Group and Phenolic Hydroxyl Group

Experiment	Observation	Inference
To 0.5 ml of 0.5% sulphanilic acid add 0.5 ml 1% NaNO ₂ , mix, wait for 1 min, add 1 ml of sample solution	Cherry red colour	Histidine present
Then add 1 ml of 10% Na ₂ CO ₃	Orange red colour	Tyrosine present

Diazobenzene sulphonic acid reacts with the imidazole ring of histidine or the phenolic hydroxyl group of tyrosine to give the coloured products in the alkaline medium.

Test for Phosphoprotein (Neumann's Test) (Test with Casein Solution)

Experiment	Observation	Inference
To 5 ml of sample (casein) solution add 2 drops of chlorophenol red indicator	Dark pink colour	pH > 5.4
<i>Step I</i> Add 1% acetic acid drop by drop Decant the supernatant leaving only the precipitated casein in test tube	Yellow colour with maximum PPT	pH = 4.6, which is the isoelectric pH of casein
<i>Step II</i> Add 12 drops of conc H ₂ SO ₄ , 4 drops of conc HNO ₃ . Heat the test tube continuously and slowly shaking it with caution	Contents of test tube char and turn brown or black	
<i>Step III</i> After no brown fumes are seen in the test tube, add 3 more drops of conc. HNO ₃ and heat	Colour of digest changes to orange	
<i>Step IV</i> Repeat step II, 2 or 3 times until the liquid and fumes turn colourless After cooling, add 5 ml ammonium molybdate	Colour of digest changes from orange to yellow to colourless Very fine canary yellow PPT	Organic phosphorous is converted to inorganic form (PO ₄ ³⁻) Phosphorous is present in casein, it is a phosphoprotein

Air-cool the test tube keeping on test tube rack, not in cold water.

Phosphorous bound with casein is released as inorganic phosphate by digesting with conc H₂SO₄ and conc HNO₃. This inorganic phosphate reacts with ammonium molybdate to produce canary yellow precipitate.

2

Tests Based on Precipitation Reactions of Proteins

Proteins are large molecules with variable sizes, shapes and charges. They can be classified as simple, conjugated and derived proteins. Most simple proteins, especially globular proteins, when dissolved in water, form colloidal solution. A colloid is a system in which the particles have diameters in the range of 1 μm to about 200 μm . The stability of a solution of a lyophobic colloid depends on the electrical charges on the surface of particles, which prevent their coagulation and precipitation. In case of lyophilic colloids, over and above the surface charges, the degree of hydration (shell of water molecules around the particles) also contributes to the stability. Polar groups of the proteins ($-\text{NH}_2$, COO^- , OH^- groups) tend to attract water molecules towards them to produce a shell of hydration. Albumin has a greater degree of hydration than globulins. Purification of enzymes and other proteins usually start with precipitating them from solution. Any factor, which neutralises the charge or removes water of hydration will therefore cause precipitation of proteins. These characteristics of protein molecules depend upon their molecular weights, three dimensional structures and properties of the constituent amino acids.

PRECIPITATION BY SALTS

Supplied sample: 10% egg-white solution

Generally proteins can be precipitated by the addition of salts. When an inorganic salt like ammonium sulphate is added to a solution of protein, it decreases concentration of water molecules available for stabilizing the protein solution and the protein is consequently precipitated. The process is known as “salting out”. Albumin tenaciously holds a large number of molecules of water and, therefore, needs a much higher concentration of salt than globulin to get precipitated. This property of proteins depends upon the type of amino acids that constitute them as well as their sizes and structures, and can be used for separating proteins from each other, such as albumin from globulins. As globulin has higher molecular weight, lower concentration of salt is enough for its precipitation. Thus globulins are precipitated at half saturation of ammonium sulphate or 22% sodium sulphate; but albumin will need full saturation of ammonium sulphate or 28% of sodium sulphate.

Solubility of a protein depends on ionic concentration of the medium. Therefore, the presence of very small quantities of salts will increase the solubility of a protein by diminishing protein-protein interaction. This is called “salting-in.”

a. Half Saturation Test with Ammonium Sulphate

Experiment	Observation	Inference
Step I To 3 ml of sample solution add equal volume of saturated ammonium sulphate solution, mix, let it stand for 5 minutes, then filter	White PPT	Globulins are precipitated by half saturation with ammonium sulphate
Step II To 1 ml filtrate from Step I, add 1 ml 40% NaOH, and 1 drop 1% CuSO ₄	Violet colour	Albumins are not precipitated by half saturation with ammonium sulphate
Step III Take PPT in a test tube, add 2 ml 40% NaOH and 1 drop 1% CuSO ₄	Violet colour	Globulins are precipitated

Filtrate contain high concentration of ammonium ions which interfere in biuret test by forming deep blue cupreammonium ions $[\text{Cu}(\text{NH}_3)_4^{++}]$ which obscure the violet colour produced by proteins. This can be overcome by the use of 40% NaOH and the test is called the modified biuret test.

b. Full Saturation Test with Ammonium Sulphate

Experiment	Observation	Inference
Step IV To the filtrate from step I, add ammonium sulphate crystals in excess (some crystals should be left undissolved after thorough mixing), and filter	White PPT	
Step V To 1 ml filtrate from step IV, add 1 ml 40% NaOH, and 1 drop 1% CuSO ₄	No violet colour	Albumins are precipitated by full saturation with ammonium sulphate
Step VI Take PPT in a test tube, add 2 ml 40% NaOH and 1 drop 1% CuSO ₄	Violet colour	Albumins are precipitated

- i. Albumin and gelatin are precipitated by full saturation with ammonium sulphate; but peptones are not precipitated even by full saturation with ammonium sulphate, because they have smaller molecules.
- ii. Globulins are precipitated by 22% sodium sulphate and albumin by 28% sodium sulphate.

ISOELECTRIC PRECIPITATION

1% Casein Solution

The pH, at which the molecules of a protein bear no net charge, is called its isoelectric pH. The isoelectric pH varies with different proteins. Proteins have minimum solubility at their isoelectric point. Many proteins are precipitated from their solution on adjusting the pH close to their isoelectric point by addition of an acid or alkali. The best example is casein, which forms a flocculent precipitate at its isoelectric pH 4.6; and redissolves, in highly acidic or alkaline solutions. When milk is curdled, the casein forms a white curd, because lactic acid produced by the fermentation process lower the pH to the isoelectric point of casein. Casein is precipitated from milk and the supernatant is called whey.

Experiment	Observation	Inference
I. To 3 ml of casein solution, add 2 drops of bromocresol green indicator	Blue colour	pH > 5.4
II. Add 1% acetic acid drop by drop until the solution turns green in colour	Curdy white PPT	At pH 4.6, casein is precipitated. The indicator colour changes to green at this pH
III. Add excess of 1% acetic acid	Precipitate disappears or reduces	As the pH is far away from isoelectric pH, precipitates of casein dissolve

COAGULATION OF PROTEINS

10% Egg-White or 1% Albumin Solution

Proteins have specific structural organizations. The primary structure refers to the order of amino acids in the polypeptide chain of the protein. The three dimensional conformation of the structure of a protein depends on its primary structure. The subunits of a protein, each possessing its own primary, secondary and tertiary structures, are united together to constitute the quaternary structure of a protein. The weak bonds, involved in the secondary, tertiary, and quaternary structures, are hydrogen bonds, hydrophobic bonds, vander Waals force, ionic bond and disulphide bonds. The disruption of secondary, tertiary and quaternary structures of a protein molecule is called denaturation. The aggregate of denatured proteins is called a coagulum, and the process is called coagulation. Denaturation is sometimes reversible, but coagulation is not. Some proteins when heated, though denatured, are still soluble. They may be precipitated by bringing to isoelectric pH.

Experiment	Observation	Inference
Take 10 ml of sample solution in a test tube. Heat the upper layer of the solution and add 1% acetic acid drop by drop	Cloudy white PPT	Albumin and globulin are coagulated by heat at its isoelectric pH

Albumin and globulin are easily coagulated by heat near or at their isoelectric point. On addition of acetic acid, there is a decrease in pH. When pH approaches the isoelectric pH of albumin/globulin, coagulation occurs spontaneously since the solution is pre-heated. This is called Heat and acetic acid test.

PRECIPITATION BY ORGANIC SOLVENTS

10% Egg-White Solution

Proteins in solution form hydrogen bonds with water. Organic solvents like acetone, ether or ethanol when added to a protein solution in water, reduce the concentration of water molecules available for keeping the proteins in solution and thus decrease the number of hydrogen bonds. The dielectric constant of the medium is also reduced causing aggregation, precipitation and denaturation of proteins. This denaturation does not occur to some proteins at low temperature.

Experiment	Observation	Inference
To 1 ml of sample solution add 2 ml ethanol and mix	Mild cloudy precipitate	Albumin/globulins are precipitated by organic solvents

PRECIPITATION BY HEAVY METALS

10% Egg-White Solution

When the pH of a protein solution is higher than the isoelectric pH of the protein (generally in an alkaline medium), protein molecules become negatively charged anions. Positively charged heavy metal cations may then bind with the negatively charged protein anion, causing their precipitation. Salts of iron, copper, zinc, lead, cadmium and mercury are toxic, because they tend to precipitate normal proteins of the gastro intestinal wall. Raw egg is sometimes used as an antidote for mercury poisoning.

Experiment	Observation	Inference
I. To 2 ml of sample solution add 10% mercuric chloride solution drop by drop	White PPT	Albumin/globulins are precipitated by heavy metals like Hg, Pb and Fe
II. To 2 ml of sample solution add 10% lead acetate solution drop by drop	White PPT	
III. To 2 ml of sample solution add 10% ferric chloride solution drop by drop	White PPT	

- i. If the sample solution is significantly alkaline, its pH should be adjusted to 7–7.5 to avoid formation of metal hydroxides, which interfere with the test.
- ii. Avoid adding excess of heavy metal ions as this may redissolve the PPT due to absorption by the protein molecules, which will give them a positive charge.

PRECIPITATION BY ALKALOIDAL REAGENTS

10% Egg-White Solution

Tungstic acid, phosphotungstic acid, trichloroacetic acid, picric acid, sulphosalicylic acid and tannic acid are powerful protein precipitating agents. These acids lower the pH of the medium, when proteins carry net positive charges. These protein cations are electrostatically complexed with negatively charged ions to form protein-tungstate, protein-picrate, etc. and thick flocculant precipitate is formed. Tanning in leather processing is based on the protein precipitating effect of tannic acid.

Experiment	Observation	Inference
I. To 2 ml of sample solution add 20% sulphosalicylic acid drop by drop	White flocculant precipitate	Albumin/ globulins are precipitated by alkaloidal reagents
II. To 2 ml of sample solution add Esbach's reagent (picric acid + citric acid) drop by drop	Yellow precipitate	
III. To 2 ml of sample solution add 5% tannic acid drop by drop	Brown precipitate	

The test described in (I) is frequently used to identify proteins in body fluids, particularly in urine and CSF.

PRECIPITATION BY STRONG MINERAL ACIDS

10% Egg White Solution

Experiment	Observation	Inference
I. To 2 ml of sample solution add 2 ml conc HNO_3 slowly along the side of the test tube	White ring at the junction of two liquids	Albumin/globulins are precipitated by strong mineral acid
II. To 2 ml of sample solution add 2 ml conc HCl slowly along the side of the test tube	White ring at the junction of two liquids	

Test (I) is called Heller's test and is usually used to identify proteins in body fluids, particularly in urine.

3

Analysis of Individual Proteins

EGG-WHITE PROTEINS

Ten percent egg-white solution

Physical Characters

Experiment	Observation	Inference
i. Appearance ii. Colour iii. Odour iv. Reaction to litmus	Opalescent Whitish Egg-like Red litmus turns blue	Alkaline

Chemical Tests

Experiment	Observation	Inference
1. (a) To 5 ml of sample solution add 2 drops of chlorophenol red indicator Add 1% acetic acid drop by drop	Dark pink or violet colour Solution is colourless or very light pink colour with stringy PPT	pH > 5.4 pH = 5.4
(b) Boil the above solution at pH 5.4	White coagulum	Albumins and globulins are coagulated by heat at pH 5.4

This test can be repeated with bromo cresol green (BCG), which gives blue colour at pH > 5.4, and green colour at pH = 5.4.

Heller's Test

Experiment	Observation	Inference
To 2 ml conc HNO_3 add 2 ml of sample solution slowly along the side of the test tube	White ring at the junction of two liquids	Albumin/globulins are precipitated by strong mineral acid (denatured)

Half-saturation and Full-saturation Tests with Ammonium Sulphate Salt

Experiment	Observation	Inference
(a) To 10 ml of sample solution add equal volume of saturated ammonium sulphate solution, shake vigorously for 2 min, let it stand for 5 min, filter and use filtrate for next test	White precipitate	Globulin is precipitated by half saturation with ammonium sulphate
(b) To 2 ml filtrate (from above) add 2 ml 40% NaOH, and 1% CuSO ₄ drop by drop.	Purple or violet colour	Proteins other than globulins present
(c) To 5 ml of filtrate from test (a), add ammonium sulphate crystals, shake vigorously (some crystals should be left undissolved after thorough mixing), keep for 5 min and filter	White precipitate	Albumin is precipitated by full-saturation with ammonium sulphate
(d) To 2 ml filtrate from step (c), add 2 ml 40% NaOH, and 1% CuSO ₄ drop by drop	No purple or violet colour	All proteins are completely precipitated by full-saturation with ammonium sulphate

Colour reactions with egg white

Students should perform colour reactions of amino acids as described in Chapter 1.

PROTEOSES AND PEPTONES

Two percent peptone solution

Physical Characters

Experiment	Observation	Inference
i. Appearance	Clear	Acidic
ii. Colour	Yellowish	
iii. Odour	Meat-like	
iv. Reaction to litmus	Blue litmus turns red	

Chemical Tests

Experiment	Observation	Inference
1. To 3 ml of sample solution adjust pH to 5.4 using chlorophenol red or bromocresol green indicator, boil	No coagulation	Proteose and peptones are not coagulated by heat
2. To 3 ml of sample solution add 5% tannic acid drop by drop.	Brown PPT	Proteose and peptones are precipitated by tannic acid
3. To 3 ml of sample solution add Esbach's reagent drop by drop	Slight yellow PPT	Esbach's reagent precipitates only proteoses
4. To 3 ml of sample solution add 10% lead acetate solution drop by drop	White PPT	Only peptones are precipitated by lead acetate
5. (a) To 10 ml of sample solution add equal volume of saturated ammonium sulphate solution, shake vigorously for 2 min, let it stand for 5 min, filter and use filtrate for next test	A faint turbidity	Primarily proteose is precipitated by half saturation with ammonium sulphate
(b) To 2 ml filtrate (from above) add 2 ml 40% NaOH, and 1% CuSO ₄ drop by drop	Purple colour	Secondary proteoses and peptones are present in filtrate
(c) To 5 ml of filtrate from test (a), add ammonium sulphate crystals, shake vigorously (some crystals should be left undissolved after thorough mixing), keep for 5 min and filter	Small amount of white PPT	Secondary proteoses are precipitated
(d) To 2 ml filtrate from step (c), add 2 ml 40% NaOH, and 1% CuSO ₄ drop by drop	Rosy pink colour	Peptones are not precipitated

Lower molecular weight proteins (proteoses and peptones) are not coagulated by heat. As the molecules of the proteins become smaller, they require greater concentration of salt for precipitations till finally the stage of peptone is reached.

Colour reactions: All colour reactions are positive.

CASEIN

One percent casein solution in 0.1N NaOH

Physical Characters

Experiment	Observation	Inference
i. Appearance ii. Colour iii. Odour iv. Reaction to litmus	Opalescent Yellowish Non-specific, milk-like Red litmus turns blue	Alkaline

Chemical Tests

Experiment	Observation	Inference
1. (a) To 5 ml of sample solution add 2 drops of chlorophenol red or bromocresol green indicator (b) Add 1% acetic acid drop by drop (c) Add excess of 1% acetic acid (d) Add 2% Na ₂ CO ₃ solution drop by drop	Pink or blue colour with respective indicators. Yellow or green coloured ppt. for respective indicator. Precipitate dissolves. Precipitate reappears	pH > 5.4 pH = 4.6. This being the isoelectric pH of casein, it is precipitated. pH < 4.6 pH = 4.6.
2. (a) To 5 ml of sample solution add equal volume of saturated ammonium sulphate solution, shake vigorously for 2 min, let it stand for 5 min, filter and use filtrate for next test (b) To 2 ml filtrate (from above) add 2 ml 40% NaOH, and 1% CuSO ₄ drop by drop.	White ppt. No violet or purple colour	Casein is precipitated. Casein is completely precipitated.
3. Neumann's test Do as described in Chapter 1, item no.9		

All the colour reactions except for sulphur containing amino acid are positive for casein.

GELATIN

One percent gelatin solution

Physical Characters

Experiment	Observation	Inference
i. Appearance ii. Colour iii. Odour iv. Reaction to litmus	Clear Colourless Odourless No change	Neutral

Chemical Tests

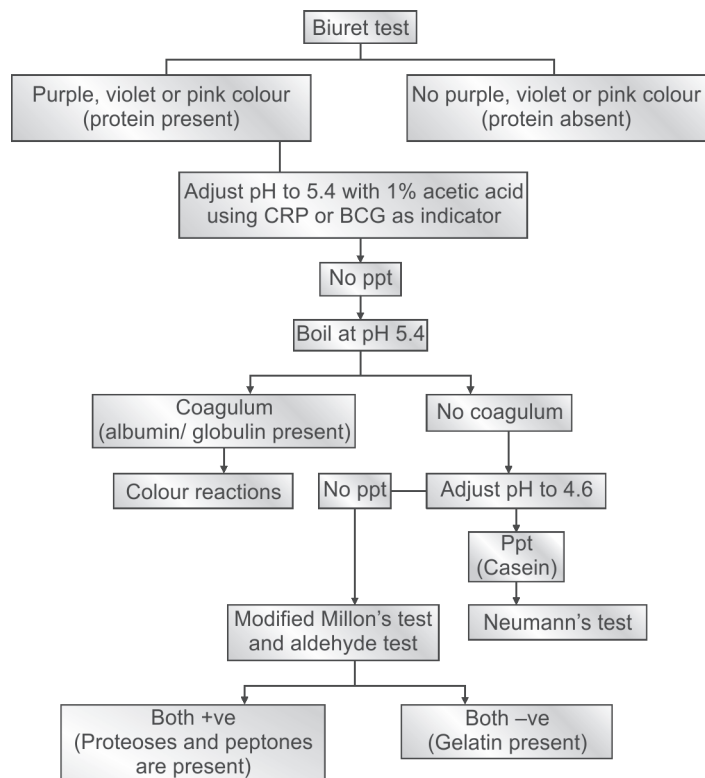
Experiment	Observation	Inference
1. Modified Millon's test (Chapter 1, item no.4)	No red colour	Tyrosine absent
2. To 5 ml of sample solution add equal volume of saturated ammonium sulphate solution, shake vigorously for 2 min, let it stand for 5 min, filter and use filtrate for next test (b) To 2 ml filtrate (from above) add 2 ml 40% NaOH, and 1% CuSO ₄ drop by drop.	White PPT	Gelatin is precipitated by half saturation
3. Aldehyde test (Chapter 1, item no.5)	No violet or purple colour	Gelatin is completely precipitated by half saturation
4. Esbach's test To 2 ml of sample solution add Esbach's reagent (picric acid + citric acid) drop by drop	No purple ring Yellow PPT	Tryptophan present Gelatin is precipitated
5. Sakaguchi test (Chapter 1, item no.6)	Bright red colour	Arginine present

A faint pink colour may develop in Millon's test due to presence of tyrosine as an impurity.

Gelatin obtained by heating collagen. Gelatin is a derived protein, poor in tyrosine, tryptophan and sulphur-containing amino acids. Hence, the colour reactions for these amino acids will never give positive results with gelatin.

4

Identification of an Unknown Protein in a Solution



5

Reactions of Monosaccharides

CARBOHYDRATES

Carbohydrates are polyhydroxy aldehyde or ketone, or compounds that yield these derivatives on hydrolysis. They are classified into monosaccharides (single unit), disaccharides (two units), oligosaccharides (3 to 10 units) or polysaccharides (more than ten units). Monosaccharides can be further classified into trioses, tetroses, pentoses, hexoses, and so on, depending on the number of carbon atoms. They are also grouped into two classes, aldoses (having aldehyde group) or ketoses (having ketone group).

MONOSACCHARIDES

Sample: 1% glucose or 1% fructose solution given separately.

Physical Characters

Experiment	Observation	Inference
i. Appearance.	Clear	Neutral
ii. Colour	Colourless	
iii. Odour	Odourless	
iv. Reaction to litmus	No change	

Chemical Tests

1. Molish test

Experiment	Observation	Inference
To 2 ml of sample solution, add 1 drop of α -naphthol in alcohol and 2 ml conc. H_2SO_4 slowly and carefully along the side of the test tube	A purple ring develops	The sample contains carbohydrates

- A strong dehydrating agent like conc. H_2SO_4 converts sugars to hydroxymethyl furfural. The furfural condenses with phenolic compounds like α -naphthol to give the coloured ring.
- Molish test is given by at least five carbons.
- α -naphthol in alcohol should be freshly prepared.
- Water-acid interaction produces heat and can cause charring of carbohydrates, resulting in formation of

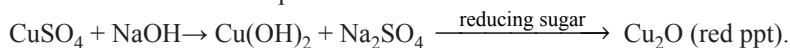
a black ring. Therefore, acid should be layered very slowly and carefully to minimize this interaction. Impurities in the reagent tend to give a green ring, which is negative test.

v. Excess α -naphthol solution also may give green ring.

2a. Fehling's test

Experiment	Observation	Inference
Mix 1 ml of Fehling's A solution to 1 ml of Fehling's B solution, boil, and add 1 ml of sample solution (boil again if necessary)	Green-yellow to orange-red to brown ppt	Glucose and fructose reduces Cu^{2+} to Cu^{1+} in alkaline medium on heating

i. The tartarate from Fehling's (B) solution chelates cupric ion, releasing it slowly for reduction thus preventing the formation of black cupric oxide.



ii. Since uric acid and creatinine also gives a positive test; Fehling's test is not commonly used nowadays.

2b. Benedict's test

Experiment	Observation	Inference
To 5ml of Benedict's reagent add 8 drops of sample solution, boil for 2 min	Green-yellow to brown or orange-red ppt	Glucose and fructose reduces Cu^{2+} to Cu^{1+} in alkaline medium on heating. In turn glucose is oxidized

i. Reducing sugars under alkaline conditions tautomerize and form enediols. The enediols are unstable and decompose to yield a variety of products. 1, 2-enediols will give formaldehyde and a pentose. The chain reaction continues to produce short chain aldehydes, which are powerful reducing agents. They can reduce cupric ion to cuprous form, which is the basis for the Benedict's (and Fehling's) reaction. In order to keep the hydroxide in solution, a metal chelator like citrate (or tartrate) is included in the solution.

ii. Benedict's reagent contains CuSO_4 (to provide cupric ions), Na_2CO_3 (to make the pH alkaline), and Na-citrate (chelates Cu^{2+} and releases it slowly for reduction), thus preventing the formation of black CuO . Sodium citrate acts as a stabilizing agent. Copper is reduced to produce green, yellow, orange or red precipitate.

iii. It is frequently used for detecting sugar in the urine of diabetic patients. Many reducing substances in urine like ascorbic acid can also give positive test.

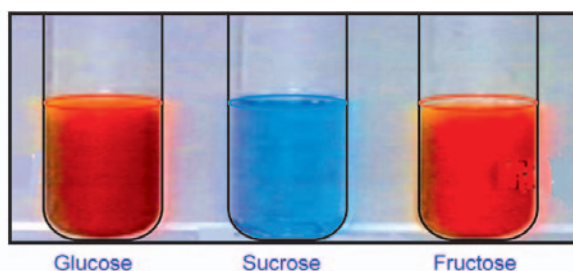


Fig. 5.1: Benedict's test

3. Barfoed's test

Experiment	Observation	Inference
To 5 ml of Barfoed's reagent add 2 ml of sample solution, Keep in boiling water bath for exactly 2 min	Fine red ppt. clinging to the walls of the test tube; some settles down on cooling	Monosaccharides reduces Cu^{2+} to Cu^{1+} in acidic medium on boiling water bath for 2 min

The Barfoed's test depends on the concentration of sugar solution and the time of boiling also. A 5% disaccharides will also give Barfoed's test positive.

4. Rapid furfural test

Experiment	Observation	Inference
To 2 ml of sample solution add 6 drops of α -naphthol in alcohol and 3 ml conc. HCl, boil for 30 sec exactly	Violet colour within 30 sec of boiling, in case of fructose	Ketose (fructose) only responds

- i. Conc. HCl converts hexoses to hydroxymethyl furfural. This conversion is faster for ketoses. The furfural condenses with α -naphthol to give the colour. Prolonged boiling will give a positive test for aldose also.
- ii. The colour develops within 30 sec of boiling. Sometimes, the colour develops on keeping the tubes in the test tube rack for a few minutes.
- iii. This test can differentiate between glucose and fructose.

5. Seliwanoff's test

Experiment	Observation	Inference
To 2 ml of sample solution add 2 ml Seliwanoff's reagent. Boil for 30 sec and cool.	No red colour in case of glucose. Red colour in case of fructose.	Ketose only respond.

- i. Prolonged boiling may also give a positive test for aldose.
- ii. The colour develops within 30 sec of boiling.

6. Osazone test

Experiment	Observation	Inference
To 5 ml of sample solution add 5 drops of glacial acetic acid, a knife-point of phenylhydrazine hydrochloride powder and two knife-point of sodium acetate, mix vigorously, place test tubes in boiling water bath for 30 min, cool and take the crystals on a slide and observe under microscope	Long yellow or greenish yellow needle-shaped crystals	Glucose and fructose form identical osazones insoluble at high temperature.

- i. All reducing sugars will form osazone with excess of phenylhydrazine when kept at boiling temperature. Hydrazones are highly water soluble, but osazones are insoluble.
- ii. Each sugar has characteristic crystal forms of osazone. Glucose, fructose and mannose give similar osazones as their 1st and 2nd carbon atoms are involved in the reaction during osazone formation.

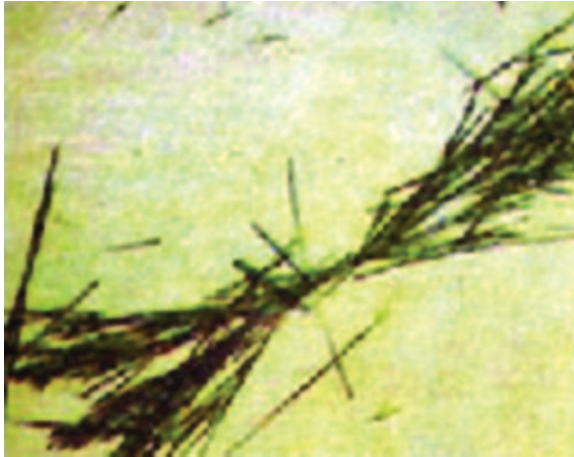


Fig. 5.2: Glucososazone: Needle-shaped crystals arranged like a broom

- iii. Acetic acid and sodium acetate are useful as buffer to maintain pH 5, appropriate for the formation of osazones.
- iv. Osazones of monosaccharides are insoluble in hot solution, and will form crystals. Osazones of disaccharides are soluble in hot water. So they will form crystals only when the test tube is cooled under tap water (Fig. 5.2).

6

Analysis of Disaccharides

Maltose = Glucose + Glucose (α -1,4 glycosidic linkage)

Lactose = Galactose + Glucose (β -1,4 glycosidic linkage)

Sucrose = Glucose + Fructose (α -1,2 glycosidic linkage)

All these three disaccharides can be hydrolysed into their respective monosaccharide units. Maltose is the product of enzyme hydrolysis of starch. Lactose is found in milk.

1% Lactose, 1% maltose, and 1% sucrose solutions are separately provided.

Physical Characters

Experiment	Observation	Inference
i. Appearance ii. Colour iii. Odour iv. Reaction to litmus	Clear Colourless Odourless No change	Neutral

Chemical Tests

Experiment	Observation	Inference
1. Molish test (+ve for all carbohydrate). To 2 ml of sample solution, add 1 drop of α -naphthol in alcohol and 2 ml conc. H_2SO_4 slowly and carefully along the side of the test tube	Violet ring at the junction of two liquids	All three disaccharides are dehydrated to give furfural, which condense with α -naphthol to give the coloured ring
2 a. Fehling's test	a. Red ppt in case of lactose or maltose b. No characteristic ppt for sucrose	a. Lactose and maltose are reducing sugars b. Sucrose is a non-reducing sugar
2 b. Benedict's test	a. Red ppt in case of lactose or maltose b. No characteristic ppt for sucrose	a. Lactose and maltose are reducing sugars b. Sucrose is a non-reducing sugar
3. Barfoed's test	No red ppt	Disaccharides do not respond to this test

- i. Sucrose does not contain any free reducing group.
- ii. Higher concentration of sugar tends to give a Barfoed's positive test.
- iii. Prolonged boiling for more than 2 min in Barfoed's test may give positive test.

Experiment	Observation	Inference
4. Osazone test	a. Brownish yellow powder puff-shaped crystals on cooling b. Greenish yellow sunflower-shaped crystals on cooling	a. Lactose b. Maltose

- i. In the sucrose molecule, the active groups on 1st and 2nd carbon atoms of constituent glucose and fructose molecule are not free; hence sucrose cannot form osazone.
- ii. Osazones of monosaccharides are insoluble in hot solution, unlike those of disaccharides; therefore it should be cooled slowly for forming good crystals. It may take 30 to 45 min during cooling for osazone to separate out (Figs 6.1 and 6.2).
- iii. Osazone test is the only test to distinguish lactose from maltose.

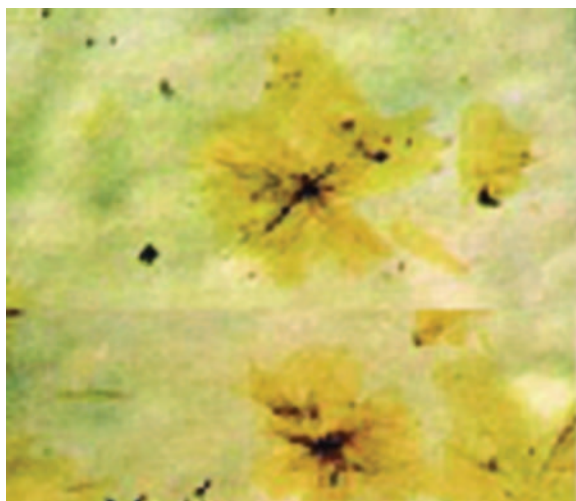


Fig. 6.1: Maltosazone: Sunflower-shaped or petal-shaped crystals

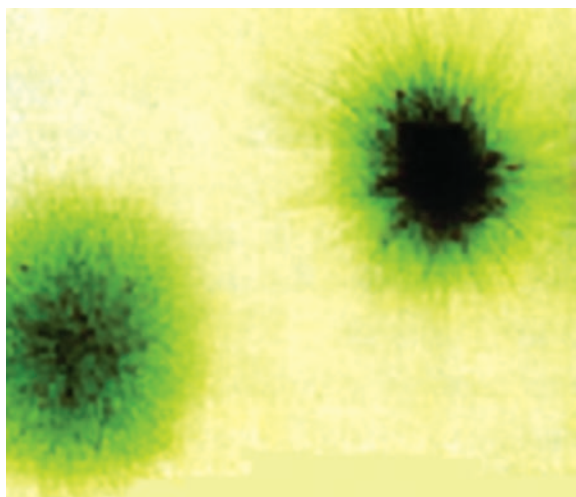


Fig. 6.2: Lactosazone: Hedgehog or "pincushion with pins" or flower of "touch-me-not plant"

7

Analysis of Polysaccharides

Glycogen and starch are made up of amylose (1,4 glycosidic linkages) and amylopectin (1,6 glycosidic linkages). Starch has more of 1,4 glycosidic linkages compared to glycogen which has more branched chains with 1,6 glycosidic linkages. Glycogen is a storage polysaccharide in mammalian liver and muscles. Starch, on hydrolysis by acid (conc. HCl) gives the following products.

Starch → soluble starch → amyloextrins → erythroextrins → achroextrins → maltose → glucose.

On enzyme (amylase) hydrolysis, maltose is the major end product.

One percent solution of starch.

Physical Character

Experiment	Observation	Inference
i. Appearance ii. Colour iii. Odour iv. Reaction to litmus	Opalescent White Odourless No change	Neutral

Chemical Tests

Experiment	Observation	Inference
1. a. To 3 ml of sample solution, add 2 drops of 0.05 N iodine solution b. Heat the solution c. Cool the solution	Deep blue colour Blue colour disappears Colour reappears	Starch present
2. To 3 ml of sample solution, add 1 ml 40% NaOH, mix and add few drops of 0.1 N iodine solution To the above solution add 1 ml conc. HCl	No colour Colour reappears	Iodine is converted to sodium iodide and sodium iodate. Hence, free iodine is not available for starch Acid neutralises the alkali to release free iodine, which can combine with starch

- i. Iodine-starch absorption complex is blue in colour. The complex breaks on heating, and forms again on cooling. Sometimes colour may not reappear on cooling as small amounts of iodine added may vaporise away during heating.

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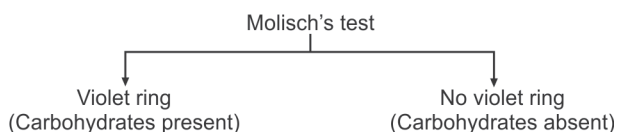
Experiment	Observation	Inference
3. Benedict's test	No coloured PPT	Starch is a non-reducing carbohydrate
4. Rapid hydrolysis of starch and Benedict's test a. To 5 ml of sample solution add 6 drops of conc. HCl, boil for 1 min, cool, add 6 drops of 40% NaOH b. To 5 ml of Benedict's reagent, add 1 ml of above neutralised solution, boil	Red PPT	Starch is hydrolysed by acid to give glucose units

8

Detection of Unknown Carbohydrate

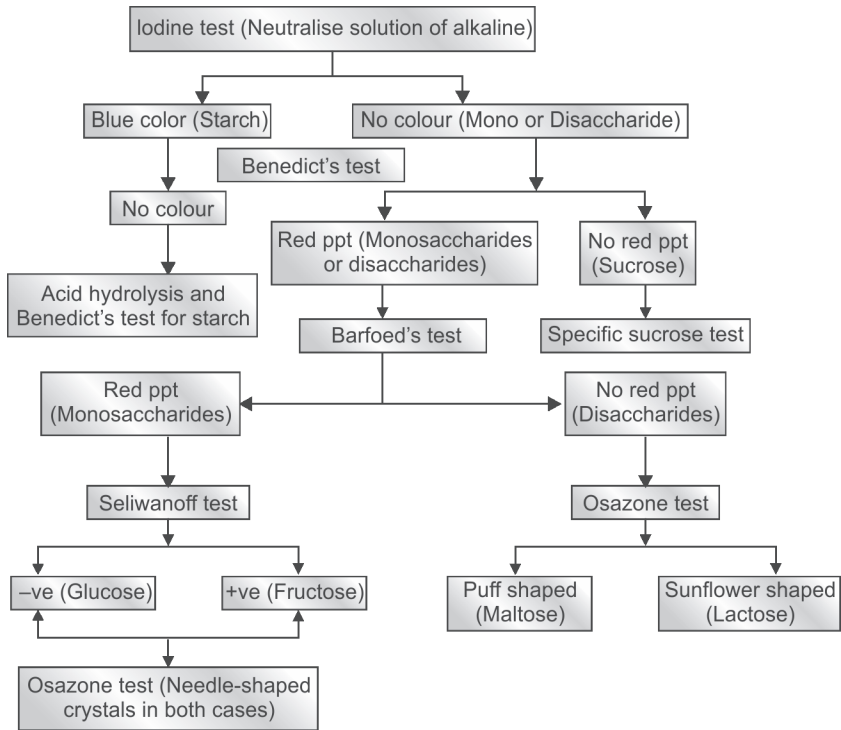
I. Physical Characteristics: (a) Appearance, (b) Colour, (c) Odour, (d) Reaction to litmus

II. Chemical Tests



It is advisable to do 3 more tests as follows, to be sure of absence of carbohydrates in unknown solution.

Experiment	Observation	Inference
Iodine test	No Colour	Glycogen, starch, dextrin absent
Benedict test	No coloured ppt	Glucose, fructose, lactose, maltose absent
Seliwanoff's test	No red colour	Sucrose absent



9

Analysis of Urea

Urea is the end product of catabolism of proteins. It is excreted mainly in the urine. It is neutral in solution. One percent urea solution

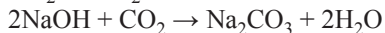
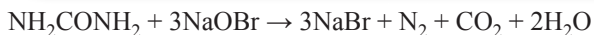
Physical Character

Experiment	Observation	Inference
i. Appearance ii. Colour iii. Odour iv. Reaction to litmus	Clear Colourless Odourless No change	Neutral

Chemical Tests

1. Hypobromite test

Experiment	Observation	Inference
To 3 ml of sample solution add few drops of alkaline hypobromite solution	Effervescence of N ₂	Urea present



The test can also be done by using 1 ml 40% NaOH and a few drops of bromine water instead of NaOBr solution. Reagent must have sufficient bromine.

2. Specific urease test

Experiment	Observation	Inference
To 3 ml of sample solution add 3 drops of phenolphthalein indicator and a knife point of urease powder (or soyabean meal), mix and keep for 15 min	Solution turns pink due to alkalinity	Urea is hydrolysed to NH ₃ and CO ₂ , which form (NH ₄) ₂ CO ₃ to make the solution alkaline. Urease is the enzyme, which acts only on urea

Uric acid is the end product of catabolism of nucleic acids in mammals. It is excreted mainly in the urine. A supplied uric acid is alkaline because it has to be dissolved in an alkali for preparing the solution.

One percent uric acid solution

I. Physical Character

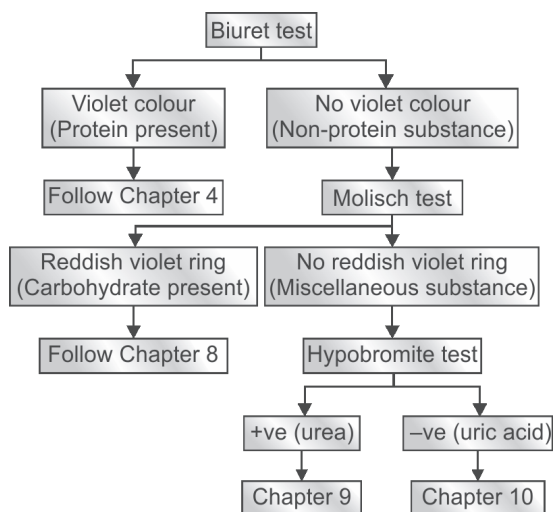
Experiment	Observation	Inference
i. Appearance	Clear	Alkaline
ii. Colour	Colourless	
iii. Odour	Odourless	
iv. Reaction to litmus	Red litmus turns blue.	

Chemical Tests

Experiment	Observation	Inference
1. To 3 ml of sample solution add conc. HCl drop by drop	Cloudy white precipitate	Uric acid is insoluble in acidic medium
2. Schiff's test Moisten a strip of filter paper with 3% ammoniacal AgNO_3 solution. Add to it a drop of uric acid solution	Brown or black stain of colloidal metallic silver on the filter paper	Uric acid reduces silver nitrate to metallic silver in alkaline medium
3. Phosphotungstic acid test To 2 ml of sample solution add a few drops of phosphotungstic acid reagent, and a few drops of 20% Na_2CO_3	Blue colour	Uric acid reduces phosphotungstic acid to blue phosphotungstous acid
4. Benedict's uric acid test To 5 ml of sample solution add 1 to 2 gm of anhydrous Na_2CO_3 powder and 5 to 6 drops of Benedict's uric acid reagent	Intense blue colour	Uric acid reduces phosphomolybdic acid to blue phosphomolybdous acid

Benedict's uric acid solution and Benedict's reagent (for reducing sugar) are different (See APPENDIX).

Identification of Unknown Solution



12

Vitamins A and C

Vitamins are classified into water-soluble and fat-soluble vitamins. Vitamin A is a fat-soluble and vitamin C is a water-soluble vitamin. Vitamin A is a constituent of rhodopsin and other light-receptor pigments of the eye and is also required for growth and maintenance of epithelial, nervous and bone tissues. Vitamin C is a good anti-oxidant and helps in collagen biosynthesis and tyrosine catabolism.

Test for Vitamin A

Cod liver oil in chloroform.

Experiment	Observation	Inference
To 1 ml of sample solution in a dry test tube, add saturated solution of antimony trichloride in chloroform	Blue colour	Vitamin A present

Note: This reaction is known as Carr-Price test. Blue colour soon changes to purple and then to brown.

Test for Vitamin C

Lemon juice or ascorbic acid solution.

Experiment	Observation	Inference
To 2 ml of 0.1% 2, 6-dichlorophenol indophenol dye in metaphosphoric acid, add sample solution drop by drop	Colour of the dye changes from blue to pink to colourless	Vitamin C present

Note: 0.04 mg of dye is reduced by 0.02 mg vitamin C.

Milk is considered as a complete food with most of the nutrients needed for human body. It is the secretion of lactating mammary gland. The secretion during early stages of lactation is known as colostrums. The composition of milk is:

Milk	Water (%)	Solid (%)	Protein (g%)	Fat (g%)	Carbohydrate (g%)
Human	87.5	12.5	1.1	3.8	7.5
Cow	87.2	12.8	3.3	3.8	4.4
Buffalo	83.6	16.4	4.3	6.0	5.3
Goat	87	12.5	3.7	3.5	4.7

The pH of milk is 6.5 to 6.7. Its specific gravity averages 1.03, but changes depending on its dilution with water. The chief protein of milk is casein, which constitutes about 80% of the total milk proteins; the remaining are lactalbumin and lactoglobulin. The proteins of human milk contain more arginine, tryptophan and sulphur containing amino acids. These proteins carry the immune bodies also, which are found in the blood serum. Thus mother's milk transmits immunity to offspring.

Milk fat exists as an emulsion due to presence of proteins in milk. About 90% of the fats are glycerides of higher fatty acids; like palmitic, stearic, oleic and myristic acids. The remaining 10% consist of lower fatty acids; like caproic, caprylic and butyric acids. Milk also contains some cholesterol, phospholipids and free fatty acids. The major carbohydrate is lactose.

Milk is rich in vitamin A and B₂. It is the most important source of calcium and phosphorous; but deficient in iron and copper. The mineral composition in milk is as follows (mg%):

Milk	Ca (%)	Mg (%)	P (%)	Na (%)	K (%)	Cl ⁻ (%)
Human	34	2.2	16	15	55	43
Cow	150	13	100	58	138	100
Buffalo	150	10	100	58	130	60

Physical Character

Experiment	Observation	Inference
i. Appearance	Opaque	Acidic
ii. Colour	White	
iii. Odour	Milky	
iv. Reaction to litmus	Blue litmus turns red	

Bile is formed in the liver, stored in the gallbladder and intermittently discharged into the duodenum. It is golden yellow, viscous, alkaline (pH 7.0 to 8.5) fluid. The daily volume amounts to 700–1200 ml. Important organic constituents are bile salts (Na^+ or K^+ salts of bile acids like glycocholic and taurocholic acids), cholesterol, lecithin, bilirubin and proteins. Inorganic constituents include HCO_3^- , Cl^- , Na^+ and K^+ . The composition of liver bile is: bile salts 1.9%, bilirubin 0.2%, cholesterol 0.06% and lecithin 0.1%.

Physical Character

Experiment	Observation	Inference
i. Appearance	Clear	Alkaline
ii. Colour	Greenish yellow	
iii. Odour	Fishy smell	
iv. Reaction to litmus	Red litmus turns blue.	

Chemical Tests

Experiment	Observation	Inference
1. Tests for bile salts		
a. Hay's sulphur test Take 5 ml of sample solution in one test tube, and 5 ml distilled water in another test tube as control. Sprinkle gently a pinch of sulphur powder to both test tube (avoid shaking)	In bile solution sulphur powder sink	Bile salts reduce the surface tension of the solution to allow the sulphur powder to sink
b. Pettenkoffer's test To 5 ml of sample solution add a small crystal of sucrose, shake to dissolve completely, add 5 ml conc. H_2SO_4 carefully along the side of the test tube	Violet ring at the junction of two liquids	Bile salts present

Sucrose is converted by H_2SO_4 to furfural which then condenses with bile salts to form a violet complex.

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Experiment	Observation	Inference
<p>2. Test for bile pigments</p> <p>a. Gmelin's test To 3 ml of conc. HNO_3 (in a narrow test tube) add 3 ml bile (preferably undiluted) carefully to form a separate layer</p> <p>b. Fouchet's test To 10 ml bile, add 2 drops of saturated MgSO_4 solution, 10 ml 10% BaCl_2, boil, cool and filter Add a drop of Fouchet's reagent to the ppt on the filter paper</p> <p>3. Test for proteins To 5 ml of undiluted bile add glacial acetic acid drop by drop</p>	<p>Play of colours</p> <p>Yellow ppt</p> <p>Yellow ppt changes to blue or green</p> <p>Precipitate</p>	<p>Bile pigments are oxidized to various coloured products, e.g., biliverdin (green), bilicyanin (blue), bilifuscin (red) and choletelin (yellow)</p> <p>Bile pigments absorbed on BaSO_4 ppt are oxidized to coloured products by trichloroacetic acid and FeCl_3 of Fouchet's reagent</p> <p>Proteins present</p>

Analysis of Normal Constituents of Urine

Normal urine is pale yellow in colour. When the output of urine is low, it appears deep yellow. Freshly void urine is clear and transparent. On standing, it may become turbid due to precipitation of phosphate. Normal urine contains both inorganic and organic constituents. The inorganic constituents include Na^+ , K^+ , Ca^{2+} , Mg^{2+} , NH_4^+ , Cl^- , H_2PO_4^- , HPO_4^{2-} , SO_4^{2-} , and traces of HCO_3^- . Normal organic nitrogenous constituents are urea, uric acid, and creatinine. The total non-protein nitrogen varies 10–15 g/d, depending mainly on the protein intake. In addition, detoxified products like indican and ethereal sulphates are found in the urine.

Three to 5 g of sodium and 1 to 3 g of potassium is excreted per day. The reabsorption of sodium by the renal tubules is under adrenal cortical control. The chief inorganic constituent of urine is chloride.

The daily output of calcium in urine is about 0.1 to 0.3 g per day and that of magnesium 0.1 to 0.2 g per day. The phosphates in the urine are mainly derived from the phosphorous of phosphoproteins, nucleoproteins and phospholipids. It may also come from inorganic phosphates in the diet. Phosphates exist in two forms: (i) alkaline phosphates—phosphates of sodium, potassium and ammonium; (ii) earthy phosphates—phosphates of calcium and magnesium. Excess calcium or phosphates may be excreted in some decalcifying diseases of the bone, such as osteomalacia and hyperparathyroidism.

The sulphates in the urine are derived from sulphur-containing amino acids. Inorganic sulphates of the diet are not absorbed in appreciable amounts. Inorganic sulphur in urine is present in oxidized (75 to 95%) and unoxidised (5 to 25%) form. Whereas ethereal sulphate in urine include phenyl sulphate, cresyl sulphate, indoxyl sulphate, skatoxyl sulphate, and indican (potassium indoxyl sulphate). The ethereal sulphate constitutes normally 10% of the oxidized form of sulphates in urine. The total output of ethereal sulphates is ordinarily in between 0.04 to 0.1 g per day.

The most important pigments in urine are urochrome and urobilinogen. Urochrome is a compound of urobilin and urobilinogen. The colour of urine is due to urochrome.

Urea is the chief nitrogenous constituent of urine. It constitutes about 90% of the total nitrogen in urine. It is the end product of protein metabolism. Uric acid in urine represents the end product of purine metabolism in the body. Hippuric acid is formed as a result of detoxification of benzoic acid by conjugation with glycine in liver and kidneys. Benzoic acid may be derived from foods, preservatives or from drugs or may be formed in the intestines from bacterial action on phenylalanine and tyrosine.

FRESH NORMAL URINE

Physical Character

Experiment	Observation	Inference
i. Appearance	Clear	Acidic
ii. volume	Measure and enter	
iii. Colour	Amber yellow	
iii. Odour	Aromatic smell	
iv. Reaction to litmus	Blue litmus turns red	
v. Specific gravity		

DETERMINATION OF SPECIFIC GRAVITY OF URINE

This is done with urinometer. Urinometer (a hydrometer) consists of a thin stem graduated at 60°F (15°C) from 1000 to 1060 corresponding to specific gravities of 1.00 to 1.06 values. Take sufficient urine in the urine glass supplied with the urinometer. Allow the urinometer to float in urine without touching the sides. Observe the reading at the meniscus and note the temperature of the urine.

Suppose the meniscus of the urine coincides with the reading, 1.01 and temperature of urine is 36°C. Since the urine is at a higher temperature compared to calibration temperature, a temperature correction has to be applied as follows: for every 3°C rise above the temperature of calibration, a correction factor of one is added to the last digit of the observed reading. The difference between 36 and 15 is 21°C. This is when divided by 3 gives 7. Thus the corrected specific gravity can be: $1.010 + 0.007 = 1.017$.

Normal volume of urine is 1000 – 2000 ml per day. The volume is influenced by the intake of fluid and the presence of solutes in it. Excessive perspiration and strenuous exercise decrease the volume of urine. The specific gravity of normal urine usually amounts to 1.012 to 1.024. But it can be as low as 1.001 when water intake is high, and as high as 1.040 in case of water deprivation. The specific gravity is directly proportional to the concentration of solutes excreted. Normal urine has pH 4.8 to 7.5 (average pH 6.0). High protein diet makes the urine more acidic whereas it becomes slightly alkaline on a diet rich in vegetables and fruits.

Chemical Tests

Inorganic Constituents

Experiment	Observation	Inference
1. To 3 ml of urine, add 1 ml conc. HNO ₃ and 1 ml silver nitrate solution	Curdy white ppt of AgCl	Chloride present

HNO₃ prevents the precipitation of urates by AgNO₃.

Experiment	Observation	Inference
2. To 5 ml of urine, add 1 ml conc. HCl and 2 ml BaCl ₂	White ppt of BaSO ₄	Inorganic sulphate present

HCl prevents the precipitation of other inorganic salts like phosphates.

Experiment	Observation	Inference
1. a. To 10 ml of urine, add 10 ml of Baryta mixture, filter b. To the filtrate add 5 ml conc. HCl, boil for 5 min, and cool	White ppt White turbidity or faint red colour	Phosphates and inorganic sulphates are directly precipitated by reacting with barium salts Ethereal sulphates (organic) are hydrolysed by conc. HCl to inorganic sulphates which are precipitated as BaSO ₄
2. Tests for Ca ²⁺ and PO ₄ ³⁻ To 10 ml urine add 3 ml strong ammonia solution, boil, filter, dissolve the ppt by pouring 1:5 glacial acetic acid on filter paper	Gelatinous ppt of Ca ₃ (PO ₄) ₂ which is redissolved in the acid	Ca ₃ (PO ₄) ₂ is precipitated in alkaline medium and redissolved in acid medium
a. To one part of the above solution (or undiluted urine) add equal volume of 4% ammonium or potassium oxalate.	White ppt or turbidity	Ca ²⁺ is precipitated as calcium oxalate
b. To the second part add 1 ml conc. HNO ₃ and 5 ml ammonium molybdate solution, and boil	Canary yellow ppt	PO ₄ ³⁻ is precipitated as ammonium phosphomolybdate
3. Test for ammonia To 10 ml urine add 4 drops of phenolphthalein and 5% NaOH drop by drop till a faint pink colour indicates slight alkalinity, boil. Dip a filter paper in phenolphthalein solution and hold it to the vapours from the tube	Colourless phenolphthalein on the filter paper turns pink. The colour disappears after sometime as NH ₃ evaporates	Ammonia present in urine is liberated by heat

- i. Ethereal sulphates do not give a ppt on addition of barium chloride, but only after preliminary hydrolysis.
- ii. Do not add too much NaOH because urea of urine also breaks down to liberate ammonia in alkaline medium.

Tests for Organic Constituents

(For details, see respective section)

Experiment	Observation	Inference
1. Alkaline hypobromite test (See Chapter 9, item no.1).	Effervescence of N ₂	Urea present
2. Specific urease test (See Chapter 9, item no.2).	Urine turns pink	Urea present Uric acid present
3. Schiff's test (See Chapter 10, item no.2)	Black stain on paper	Uric acid present
4. Benedict's uric acid reagent test (See Chapter 10, item no.4)	Intense blue colour	
5. Jaffe's test for creatinine Mix 5 ml saturated picric acid solution with 5 ml 5% NaOH, and divide into two parts		
a. To one part add 5 ml urine	Orange red colour	Creatinine present in urine
b. To other part add 5 ml water	No red colour (control)	
6. Test for hippuric acid To 5 ml urine add a few drops of 5% NaOH and 10% FeCl ₃ solution drop by drop	Light brown ppt of ferric hippurate	Hippuric acid present
7. Test for urobilinogen To 5 ml urine add 1 ml Ehrlich reagent, mix, and keep for 5 min.	Red ppt	Urobilinogen reacts with p-methylaminobenzaldehyde of the reagent to give the coloured complex

- i. Do not add excess NaOH in hippuric acid test, as this will form ferric hydroxide, a buff coloured ppt.
- ii. On standing, urobilinogen of urine is oxidized by air to urobilin, which does not give the test.

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Abnormal Constituents of Urine (Report on Urine)

Many pathological constituents occur in traces in normal urine and escape detection due to low sensitivity of the methods. Their concentrations are increased markedly in the urine in different pathological condition. On standing, urine undergoes bacterial fermentation. It can be preserved under refrigeration or by adding chemicals such as toluene or chloroform. Usually, the analysis is carried out with properly preserved 24 hours' urine specimen. When this is not possible, the early morning specimen can be used.

Physical Characteristics in Pathological Conditions

1. Appearance: Urine is turbid or opalescent if it contains proteins, pus, bacteria, epithelial cells and lipids.
2. Odour: Normal urine has aromatic odour, which turns ammoniacal on prolonged storage. Urine smells sweet for ketone bodies, and maple syrup in maple syrup urine disease.
3. Volume: Urinary output is increased (polyuria) in diabetes and after administration of drugs like digitalis and salicylates. Diminished urinary excretion (oliguria) occurs in nephritis, fever, diarrhea and vomiting. Total suppression of urine formation (anuria) may occur in shock, acute nephritis, incompatible blood transfusion, mercury poisoning and renal stone.
4. Colour: Urine becomes smoky brown when blood is present, yellow when bilirubin is present and black when melanin is present. Urine turns black on standing in alkaptonuria. Urine becomes milky in appearance if pus, bacteria, epithelial cells or lipids are present.
5. pH: Urine is significantly acidic in fever, diabetes, ketoacidosis-alkaline tide.
6. Specific gravity: Normal range 1.015 to 1.025. It is increased in acute nephritis and fever, and decreased in diabetes insipidus.
7. Total solids: Normal range 26–80 g/L. It increases when abnormal constituents are present.

Chemical Constituents

The abnormal constituents which are routinely looked for in urine are albumin, glucose, ketone bodies, bile salts, bilirubin (bile pigments) and blood.

Test for Proteins

The glomeruli of kidneys are not permeable to substances with mol wt 70 kD. The plasma proteins of MW more than 70 kD, hence are absent in normal urine. When glomeruli are damaged or diseased, they become more permeable and plasma proteins appear in urine. The smaller molecules of albumin pass through damaged glomeruli more readily than the heavier globulin and so, when proteins appear in urine, the albumin fraction predominates.

Experiment	Observation	Inference
a. To 3 ml of urine, add 20% sulphosalicylic acid drop by drop	White ppt	Proteins may present
b. Heller's test To 3 ml conc. HNO_3 add 3 ml urine carefully over the acid	White ring at the junction of two liquids	Proteins may present
c. Heat coagulation test (Heat and Acetic acid test) To 10 ml urine in a test tube, heat only upper portion of urine to boil, then add 1% acetic acid along the side of test tube	Turbidity in the upper part due to coagulum, which does not dissolve in acetic acid	Proteins confirmed

- If ppt dissolves on adding 1% acetic acid, it is due to the presence of phosphates in urine. Sometimes urine may be alkaline; in that case heating alone may not precipitate. Acetic acid is to be added to make it acidic.
- The presence of detectable amounts of proteins is characteristic of kidney disease—nephritic syndrome, diabetic nephropathy, hypertensive nephropathy, renal failure as well congestive heart failure.

Test for Reducing Sugar

Experiment	Observation	Inference
To 5 ml of Benedict's reagent add 8 drops of sample solution, boil for 2 min	Green-yellow to brown or orange-red ppt	Reducing sugar is present

Positive Benedict's test usually indicates the presence of glucose. It may also be seen in lactosuria (during pregnancy and lactation), galactosemia and in pentosuria. The identity of different sugars may be established by other relevant tests.

Test for Ketone Bodies

Ketone bodies do not appear in urine because acetoacetic acid, which is produced normally in liver, is completely oxidised in tissues. But if fats are metabolised excessively in liver, as in diabetes and starvation, there will be over production of acetoacetic acid. The tissues are unable to oxidise the excessive amount of acetoacetic acid with the limited supply of oxygen. A part of excess acetoacetic acid is decarboxylated to acetone and remaining circulates in blood as acetoacetic acid and β -hydroxyl butyric acids, which are reversible in equilibrium.

Experiment	Observation	Inference
a. Rothera's test Saturate 5 ml urine with ammonium sulphate crystals, then add 2 drops of fresh 5% sodium nitroprusside and 1 ml strong liquor ammonia slowly along the side of the tube	Purple ring	Acetone or acetoacetic acid or both may present
b. Gerhardt's test To 3 ml urine add few drops of 10% FeCl_3 solution	Port wine colour	Acetoacetic acid may present

- If Gerhardt's test is -ve and Rothera's test is +ve, acetone is present.
- Alkaline urine interfere with Gerhardt's test by forming $\text{Fe}(\text{OH})_3$. Hence neutralise urine with 1% acetic acid.
- Salicylic acid also interferes with Gerhardt's test. To differentiate, repeat test in a well-boiled sample. Boiling converts acetoacetic acid into acetone, which is volatilised, without destroying salicylic acid. A

positive test before boiling and a negative test after boiling indicate the presence of acetoacetic acid in urine.

iv When fat catabolism is excessive as in diabetes or starvation, ketone bodies appear in urine.

Tests for Bile Salts and Pigments

Experiment	Observation	Inference
a. Hay's sulphur test (See Chapter 14, item 1a)	Sulphur sinks	Bile salt present
b. Pettenkoffer's test (See Chapter 14, item 1b)	Purple ring at the junction of two liquids	Bile salt present
c. Gmelin's test (See Chapter 14, item 2a)	Play of colours	Bile pigments present
d. Fouchet's test (See Chapter 14, item 2b)	Colour changes to green or red	Bile pigments present

Test for Blood

Experiment	Observation	Inference
To a pinch of benzidine powder in a test tube, add 2 ml glacial acetic acid and 2ml H ₂ O ₂ , mix and divide it into two parts:		
a. To the 1st part add equal volume of urine	Blue or green colour	Blood is present
b. To the 2nd part add equal volume of water	No blue or green colour	This is the control test

- i. The colour soon changes to brown.
- ii. Peroxidase, released from RBC due to their destruction, acts on H₂O₂ to liberate oxygen that oxidises benzidine to coloured compound.
- iii. This is a very sensitive test but is not specific for blood.
- iv. Benzidine also becomes +ve, if pus cells are present, because of the presence of peroxidase. However, test is -ve if urine is heated before doing the test.
- v. 2 ml 12% benzidine in glacial acetic acid can also be used.
- vi. Blood appears in urine in case of hematuria or due to trauma during surgical manipulation, stone in urinary tract, injury (urethral rupture), infections like tuberculosis, and cancer of urinary system.

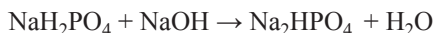
PART
B

Quantitative Experiments

Determination of Titrable Acidity and Ammonia in Urine

PRINCIPLE

Maintenance of intracellular pH in the tissues and in the body fluids is vital for normal cellular function. Kidneys help in maintaining the body acid-base balance by increasing or decreasing the secretion of H^+ in the urine. The H^+ ions secreted by kidneys are buffered in the tubular fluid by HPO_4^{2-} filtered from glomerule and by NH_3 synthesised and secreted by renal tubular cells. The titrable acidity of urine is mainly due to acid phosphates ($H_2PO_4^-$ or NaH_2PO_4) and to a less extent, weak organic acids. It can be determined by titrating urine with a standard alkali using phenolphthalein as the indicator.



Calcium should be removed as Ca-oxalate before titration as it otherwise interferes by being precipitated as calcium phosphate.

Ammonia is synthesised in renal cells by the hydrolysis of glutamine and by transdeamination as well as oxidative deamination. Ammonia is estimated by the formol titration method. In this method, neutralised formaldehyde is added to a solution containing ammonium salts; H^+ ions are liberated and are titrated with a standard alkali. Hexamethylenetetramine $N_4(CH_2)_6$, is other product in this reaction.



REAGENTS

- 0.1N NaOH;
- Potassium oxalate powder;
- Phenolphthalein (0.1% solution in ethanol);
- Formaline, 20% v/v neutralised solution.

PROCEDURE

Estimation of Titrable Acidity

Pipette 25 ml of urine into a 250 ml conical flask and add 2-spatula full potassium oxalate powder to precipitate calcium. Add 2 drops of phenolphthalein mix and titrate with 0.1N NaOH from a burette. Note the titre value (A ml) when a permanent pale pink colour appears. Preserve the contents for 'ammonia' estimation.

Formol Titration of 'Ammonia'

Add 5 ml of neutralised formalin to the above flask. Pink colour disappears due to the liberation of H^+ from NH_4 salts. Titrate the mixture in conical flask with 0.1N NaOH until the pale pink colour reappears. Record the second titre value (B ml).

Repeat Both

Observation

1. Volume of 0.1 N NaOH required to neutralise titrable acidity (A ml).
2. Volume of 0.1 N NaOH required for formol titration (B ml).

Calculations

I. Titrable acidity:

Volume of 0.1 N NaOH required to neutralise the titrable acidity in 25 ml urine = A ml

∴ Volume of 0.1 N NaOH required for 100 ml urine = $A \times 4$ ml

∴ Titrable acidity of 100 ml urine = 4A ml of 0.1 N NaOH.

Assuming 24 h urine output 1500 ml, titrable acidity of urine = $4A \times 15$ ml/day.

II. Total ammonia:

Volume of 0.1 N NaOH required for 100 ml urine = $B \times 4$ ml

Since 1 ml 0.1 N NaOH \cong 1.7 mg of NH_3

∴ Ammonia content of 100 ml urine = $4B \times 1.7$ mg

∴ Ammonia content of urine = $4B \times 1.7 \times 15$ mg/day.

Clinical Significance

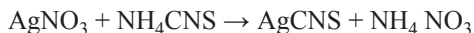
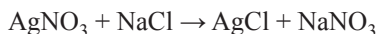
Titrable acidity of urine amounts to 200 to 300 ml/day; urinary ammonia amounts 0.5 to 0.85 g/day. Their values rise on starvation, diabetic ketosis and acidosis. Titrable acidity and ammonia are decreased in alkalosis.

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Estimation of Chlorides in Urine

PRINCIPLE

When a known volume of urine is acidified with HNO_3 , chloride is precipitated as AgCl by adding a measured excess of standard AgNO_3 solution. The amount of AgNO_3 left unused after the precipitation is determined by titrating with standard ammonium or potassium thiocyanate solution, using ferric ammonium sulphate to indicate the end point. Silver with thiocyanate form silver thiocyanate. Excess thiocyanate gives a salmon red colour due to formation of ferric thiocyanate by reacting with ferric ammonium sulphate (ferric alum).



Reagents

- Standard 0.17 N AgNO_3 solution: Dissolve 29.061 g AgNO_3 in distilled water and volume make up to 1 litre. 1 ml of this solution is equivalent to 10 mg NaCl or 6 mg Cl^- .
- Standard 0.17 N ammonium thiocyanate (NH_4CNS) solution: 13 g NH_4CNS in distilled water and volume make up to 1 litre. Now take 20 ml of standard AgNO_3 solution, 4 ml conc. HNO_3 and 5 ml ferric alum solution in a flask, dilute to 100 ml with distilled water and titrate with NH_4CNS solution. Dilute the NH_4CNS solution with distilled water to make 1 ml of that solution is exactly equivalent to 1 ml of 0.17 N AgNO_3 solution.
Standard 0.17 N potassium thiocyanate (KCNS) solution can also be used: Use 16.6 g KCNS instead of 13 g NH_4CNS .
- Saturated solution of ferric alum (Ferric ammonium sulphate): $(\text{NH}_4)_2\text{SO}_4 \cdot \text{Fe}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$.
- Conc. HNO_3

PROCEDURE

In a 25 ml conical flask, take 5 ml of urine, 2 ml conc. HNO_3 (to prevent urate precipitate), 10 ml AgNO_3 (swirl the solution to mix) and 10 drops ferric alum. Titrate this mixture with thiocyanate.

End point: Persistent salmon pink colour for 30 seconds.

Note

- If the first drop of thiocyanate solution gives the colour, indicates the concentration of chloride in urine is high. In that case, repeat the whole procedure using 20 ml AgNO_3 .
- Do not use tap water for washing as it may contain chloride as impurities.
- The end point colour must persist for 30 seconds.

Calculation

Titre value for 5 ml urine = A ml.

Volume of AgNO_3 which reacted with chloride in urine = (10-A) ml

Since, 1 ml $\text{AgNO}_3 \equiv 10 \text{ mg NaCl}$ or 6 mg Cl^-

Thus, (10-A) ml $\text{AgNO}_3 \equiv 10 \times (10-A) \text{ mg NaCl}$ or $6 \times (10-A) \text{ mg Cl}^-$

5 ml urine $\equiv 10 \times (10-A) \text{ mg NaCl}$ or $6 \times (10-A) \text{ mg Cl}^-$

\therefore 100 ml urine $\equiv 10 \times (10-A) \times 20 \text{ mg NaCl}$ or $6 \times (10-A) \times 20 \text{ mg Cl}^-$

Clinical Significance

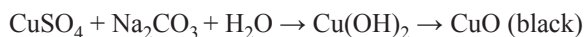
On an average diet, 8–15 g (or 170–250 mEq) of chloride is excreted per day. Normal serum level of chloride is 96–106 mEq/l. Vomiting and diarrhea result in low serum chloride levels and a consequent fall in urinary chlorides. When the serum level is much below 103 mEq of chloride/ litre, the urinary excretion of chloride is low. In Cushing's syndrome and during steroid therapy, decreased urinary excretion of chloride is observed. Retention of chlorides in body fluids lowers urinary chloride in chronic nephritis with oedema, and in pneumonia and inflammation causing large exudates. In Addison's disease, impaired tubular reabsorption of chloride causes significant urinary excretion of chloride even at low serum chloride level like 85 mEq/l.

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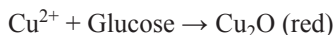
Estimation of Glucose in Urine

PRINCIPLE

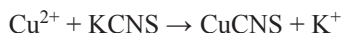
Benedict's quantitative reagent, which is CuSO_4 in alkaline medium, is reduced by glucose in urine to cuprous oxide, and combines with KCNS in the solution to form white ppt of cuprous thiocyanate preventing precipitation of Cu_2O as red ppt. On complete reduction, the blue colour completely disappears to give a green colour, which quickly changes to colourless with an additional drop of urine. The reaction is as follows:



(Prevented by Na-citrate)



(Prevented by potassium ferricyanide)



Note: Na_2CO_3 neutralises any urinary acidity and also liberates CO_2 during titration, which is to an extent useful in preventing reoxidation of cuprous ions.

Reagents

- Benedict's qualitative reagent.
- Benedict's quantitative reagent.
- Na_2CO_3 powder.

Procedure

Part I: Perform Benedict's test with urine as described previously using quantitative reagent. According to the observed changes, dilute the urine sample with distilled water.

Observation	Approx amt of glucose	Dilution advice		Dilution factor (D)
		Undiluted urine	Distilled water	
Green or yellow ppt	0.5–1 g%	50 ml	50 ml	2
Orange or red ppt	1–2 g%	20 ml	80 ml	5
Red ppt with colourless supernatant	> 2 g%	10 ml	90 ml	10

Part II: Pipette exactly 20 ml of Benedict's quantitative reagent in a conical flask. Add about 5 gm Na_2CO_3 powder and a few pieces of glass beads to prevent bumping of the solution on boiling. Boil the solution on a low oxidising flame. Add the diluted urine initially at the rate 0.5 to 1 ml at a time, wait for a few seconds to complete the reaction; and continue with titration.

End point: Complete discharge of blue colour; very faint green or white ppt.

Note: Boiling should be uninterrupted and gentle to avoid bumping.

Calculation

1 ml of Benedict's quantitative reagent is reduced by 2 mg of glucose.

So, 20 ml Benedict's reagent is reduced by 40 mg glucose.

∴ A ml (burette reading) diluted urine \cong 20 ml Benedict's reagent \cong 40 mg glucose.

Or, A/D ml of undiluted urine \cong 40 mg glucose.

∴ 100 ml of undiluted urine \cong $40 \times D/A \times 100$ mg glucose \cong $4 \times D/A$ g glucose.

Clinical significance: Glucose in urine is generally associated with diabetes mellitus.

PRINCIPLE

Colorimetry is frequently used in biochemical estimation. The instrument commonly used is a Colorimeter. It measures the amount of light absorbed by coloured solutions formed from the substance to be estimated. Such a substance must either itself is coloured or form coloured products.

The parts of a Colorimeter include a source of light and a device for selecting lights of narrow wavelength ranges. The common Colorimeters have a set of replaceable filters. In a sophisticated instrument, a diffraction grating or prism replaces the filters so that the transmitted light has a very narrow bandwidth or a single wavelength (monochromatic).

The transmitted light passes through the coloured solution placed in a cuvette or tube. The coloured solution absorbs some light; the residual light falls on a photosensitive detector converts the light into electrical signals of amplitudes directly proportional to the intensity of the impinging light. These signals are measured by a galvanometer and read as optical density or percentage transmittances.

The colour of the solution depends upon the transmitted light. In Colorimetry, filters are so chosen that absorption is maximum at the transmitted wavelength band. Based on complementary colours, filter is chosen. The choice of filter depends also on photosensitivity, concentration, intensity and other experimental factors. When a monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the length of the absorbing medium increases—this is Lambert's law.

$$I = I_0 e^{-kl}$$

Whereas Beer's law states that when a monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the concentration of the absorbing medium increases. $I = I_0 e^{-k_2c}$

These two laws are combined together in the Lambert-Beer's law. $I = I_0 e^{-k_3cl}$.

- Where I_0 is the intensity of incident light,
- I_e is the intensity of emergent light,
- c is the concentration of absorbing solution,
- l is the length of the absorbing medium.

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

$$\% T = \frac{I}{I_0} \times 100 = e^{-k_3cl}$$

If logarithm of ratio are considered then the equation, $\log_e \frac{I_0}{I} = k_3cl$.

$$\log_{10} \frac{I_0}{I} = \frac{k_3cl}{2.303} = Kcl$$

The expression $\log_{10} \frac{I_0}{I}$ is known as the Extinction (E) or absorbance (A). This is some times referred as optical density; $E = Kcl$.

In the experiment, length (l) of the path of light is kept constant for control and test. So the only variable is the intensity of the emergent light, from which we can calculate the concentration of the substance. Plot of extinction (E) against concentration gives a straight line passing through the origin. But plot of percent transmittance against concentration gives a negative exponential curve.

Plot of extinction against concentration is known as standard curve.

The molar extinction coefficient is the extinction given by 1 mol/l of a sample in a light path of 1 cm and is usually written as $E_{1cm}^{1mol/l}$. It has dimension of litre mol⁻¹cm⁻¹.

COLORIMETER

It refers to the measurement of intensity of colour in a solution. The concentration of colourless biochemical compounds and metabolites can be estimated if they are converted into coloured compounds.

Photocolourimeter measure the intensity of transmitted light through a colour solution. The components include:

- Light source: It is usually a tungsten lamp emitting light in the visible range only.
- Filters: Coloured glass filter absorb most of the light and permit light of the complementary colour only with sufficiently narrow wavelength. Selection of filter depends on the colour of the resultant solution. Complement colour of filter is used.

Colour of solution	Colour of filter	Wavelength range (nm)
Bluish green	Red	650–700
Green blue	Orange	600–650
Blue	Yellow	575–600
Violet	Yellow green	555–575
Purple	Green	505–555
Red	Blue green	495–505
Orange	Green blue	475–495
Yellow	Blue	430–475
Yellowish blue	Violet	350–430

- Cuvettes: These are glass tubes of usually 1 cm diameter and uniform thickness in which absorbance is measured.
- Photosensitive detectors: Either a photocell or a phototube may be used to convert the transmitted light into electrical energy.

- e. Measuring device: The photo detector response can be measured by anyone of the following devices, such as galvanometer, ammeter, recorder or digital readout.

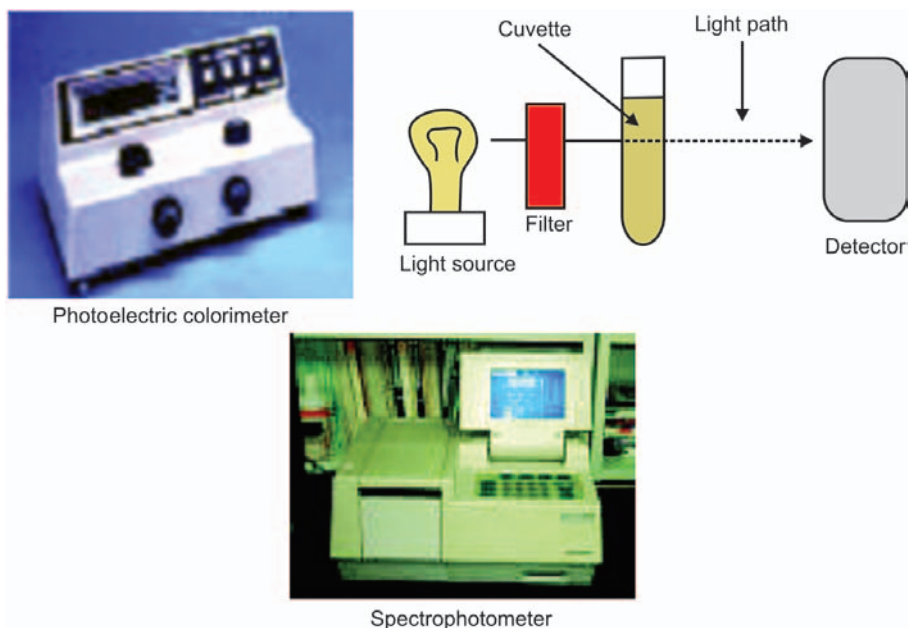


Fig. 20.1: Different colorimeters

USE OF PHOTO ELECTRIC COLORIMETER

Three solutions, 'Blank', 'Test' and 'Standard', are prepared in three different test tubes marked B, T and S respectively.

- 'Test' solution is made by treating a specified volume of the sample with reagents as mentioned in the procedure.
- 'Standard' solution is prepared simultaneously with same reagents, and the same volume of solution with known concentration.
- 'Blank' is prepared with the same reagents in the same way, but with the same amount of solvent without the substance to be estimated.

Calculation: Concentration of the substance in the sample

$$\begin{aligned}
 &= \frac{\text{OD of test solution}}{\text{OD of standard}} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \\
 &= \frac{\text{OD}_T - \text{OD}_B}{\text{OD}_S - \text{OD}_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100
 \end{aligned}$$

Where, OD_T , OD_S and OD_B represents optical density of test, standard and blank solutions respectively.

Verification of Lambert-Beer's Law

Creatinine is not a colour compound and does not absorb any light. But when creatine reacts with alkaline picrate to form red creatinine picrate, which has an absorption maximum at 530 nm.

Materials

- i. Saturated picric acid solution.
- ii. Sodium hydroxide (1 M).
- iii. Creatinine standard (100 mg/l).

Method

Prepare a range of creatinine solution by suitable dilution of the standard creatinine (20, 40, 60, 80, and 100 mg/l) with water.

Take 1 ml each solution in a test tube. Add 1 ml of NaOH and 1 ml picric acid solution, mix thoroughly and allow it to stand for 10 min. Add 7 ml H₂O. Read the absorbance at 530 nm against a reagent blank.

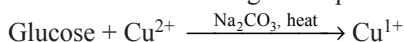
Plot the absorbance or extinction against concentration of creatinine. Determine molar extinction.

Estimation of Blood Sugar

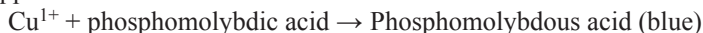
FOLIN-WU METHOD

Principle

Glucose in the protein-free filtrate at higher temperature and alkaline medium reduces Cu^{2+} to Cu^{1+} . The cuprous oxide formed is in turn treated with phosphomolybdic acid, which is reduced proportionally by the cuprous ions to phosphomolybdous acid (molybdenum blue), a blue solution. The intensity of this blue solution is a measure of the amount of glucose present.



Tartarate in the reagent helps to chelate Cu^{2+} and release it slowly for reduction to Cu^{1+} thus preventing its ppt as CuO .



Reagents

- 10% sodium tungstate
- $\frac{2}{3}\text{N}$ H_2SO_4
- Alkaline copper reagent
- Standard glucose [$X = 0.1$ mg/ml].

Sample: Blood collected in a fluoride and oxalate containing tube.

Part I

Preparation of protein free filtrate from blood.

In a test tube, take 7 ml distilled water, 1 ml blood sample, 1 ml 10% sodium tungstate solution and 1 ml $\frac{2}{3}\text{N}$ H_2SO_4 solution (dropwise and with shaking). Thus, the dilution of blood sample is 1 in 10. Let it stand for 10 minutes, filter and collect the filtrate in a dry beaker.

Note

- Oxalate precipitates Ca^{2+} of blood to prevent coagulation; fluoride inhibits glycolytic enzymes of RBC to prevent breakdown of glucose before estimation.
- The Folin-Wu filtrate still contains some polypeptides, which escape precipitation by tungstate. These polypeptide bind Cu^{2+} at their peptide bonds to form coloured complexes and consequently produce some errors in the estimated blood glucose value.

Part II

Label three Folin-Wu tubes as S, T and B for standard, test and blank solutions.

Reagents	S	T	B
Alkaline copper reagent	2 ml Standard 2 ml	2 ml test solutions 2 ml	2 ml distilled H ₂ O 2 ml
Keep tubes in boiling water bath for 8 min and immediately cool			
Phosphomolybdic acid	2 ml	2 ml	2 ml
Add distilled H ₂ O up to 25 ml mark, mix the contents by inverting tube placing your palm tightly over the mouth			

Read the OD at 420–490 nm or blue filter.

Note

- Keeping the tubes in boiling water more than 8 minutes tends to increase the reading due to excess reduction of Cu²⁺.

Calculation

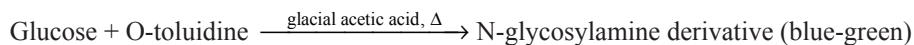
Concentration of glucose in mg/100 ml blood.

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.2}{0.2} 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 100 \text{ mg\%}
 \end{aligned}$$

ESTIMATION OF GLUCOSE BY O-TOLUIDINE METHOD

Principle

Glucose reacts with o-toluidine in glacial acetic acid on heating to yield a blue-green N-glycosylamine derivative. The intensity of this colour is proportional to the concentration of glucose present. The proteins are precipitated in this method with the help of alkaloidal reagent, trichloroacetic acid.



Reagents

- 1% o-toluidine reagent in ethanol,
- 10% trichloroacetic acid,
- Standard glucose solution (0.1 mg/ml).

Part I

Preparation of protein free filtrate (PFF) from blood.

In a dry test tube take 3 ml distilled water, 0.5 ml blood and 1.5 ml 10% TCA. (Dilution of blood = 1 in 10). Mix, keep for 10 minutes, and filter in a dry test tube to obtain a clear solution of PFF.

Part II

Use PFF for blood glucose estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
PFF	1 ml	–	–
Standard glucose solution	–	–	1 ml
3% TCA	–	1 ml	–
O-toluidine reagent	5 ml	5 ml	5 ml

Mix and keep the tubes in boiling water bath for 10 min, cool and read the OD using a colorimeter with red filter of wavelength 630–690 nm.

Note: The solution levels in three test tubes should be below the surface of water in the boiling water bath. Since orthotoluidine is mild carcinogenic, this method is rarely used nowadays.

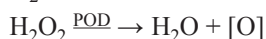
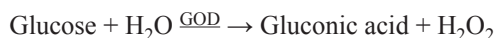
Calculation

Concentration of glucose in mg/100 ml blood.

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} + \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.1}{0.1} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 100 \text{ mg\%}
 \end{aligned}$$

GLUCOSE–OXIDASE METHOD

Glucose oxidase (GOD) acts on glucose to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide is producing nascent oxygen by peroxidase (POD). Nascent oxygen further reacts with a chromogen to produce coloured product, which is estimated colourimetrically.

**Reagents**

- Phosphate buffer, pH 7.0.
- Enzyme reagent: Containing GOD, POD, 4-aminoantipyrine and phenol in phosphate buffer.
- Glucose standard: 100 mg%

Procedure

Use serum for blood glucose estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
Serum	20 μ l	–	–
Standard glucose solution	–	–	20 μ l
Enzyme reagent	2 ml	2 ml	2 ml

Mix and incubate at 37°C for 15 minutes. Take OD at 530 nm and calculate result as above.

Note: Glucose oxidase (GOD) specifically acts on β -D-Glucose.

Glucose oxidase method is the preferred method because:

- i. It is a single step method.
- ii. Only 10 μ l of blood sample can be used for estimation.
- iii. It can be used in semi and fully automated analyser.

Clinical Significance

The fasting blood sugar level estimated by the Folin-Wu method is 80–120 mg% in normal subjects. This is about 10–20 mg higher than true blood glucose level as other reducing substances also produce colour in this method. Hence, this method is rarely used nowadays. The glucose level in normal subjects should be 60–100 mg% by o-toulidine method, whereas 75–110 mg% by GOD-POD method.

Possible reasons for hyperglycemia: (a) Uncontrolled diabetes mellitus, (b) Pancreatitis or pancreatic carcinoma, (c) Sepsis, (d) Asphyxia, (f) Hyperpituitarism, (g) Hyperthyroidism, (h) Emotions like fear, anger, etc.

Possible reasons for hypoglycemia: (a) Insulin overdose in diabetics, (b) Hyperinsulinism, (c) Hypopituitarism, (d) Hypothyroidism, (e) Addison's disease, (f) Starvation, (g) Glycogen storage diseases, and (h) Liver diseases.

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Estimation of Blood Urea (By Diacetyl Monoxime Method)

PRINCIPLE

Urea reacts with diacetyl monoxime ($\text{CH}_3\text{COCNOHCH}_3$) or diacetyl ($\text{CH}_3\text{COCOCH}_3$) under strongly acidic condition in presence of ferric ions and thiosemicarbazide to give a pink coloured complex. Proteins in blood do not interfere as they are precipitated with trichloroacetic acid. The intensity of the pink colour is a measure of the amount of urea present in blood.

Reagents

- 10% trichloroacetic acid.
- Diacetyl monoxime/thiosemicarbazide reagent: Dissolve 1.56 g diacetyl monoxime and 41 mg thiosemicarbazide in 250 ml distilled water, store in brown bottle.
- Phosphoric acid—sulfuric acid—ferric chloride reagent: Dissolve 324 mg of anhydrous FeCl_3 in 10 ml of 56% phosphoric acid. Add 1 ml of this FeCl_3 reagent to 1L of 20% H_2SO_4 .
- Diacetyl monoxime reagent: Mix equal volume of b and c. This is to be freshly prepared.
- Preservative diluent for standard: Dissolve 40 mg phenyl mercuric acetate in about 250 ml water with heating. Transfer the solution into a measuring cylinder. Add 0.3 ml concentrated sulphuric acid and make up to 1 liter with water.
- Standard urea solution: 3 mg urea in 100 ml preservative diluent (0.03 mg/ml).

Sample: Blood in oxalate bulb.

Part I

Preparation of PFF (Protein free filtrate) from blood.

In a dry test tube take 3.4 ml distilled water, 0.1 ml blood and 1.5 ml 10% TCA. (Dilution of blood = 1 in 50). Mix, keep for 10 minutes, and filter in a dry test tube to obtain a clear solution of PFF.

Part II

Use PFF for blood urea estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
PFF	1 ml	–	–
Standard urea solution	–	–	1 ml
Distilled water	–	1 ml	–
Diacetyl monoxime reagent	5 ml	5 ml	5 ml

Mix and keep the tubes in boiling water bath for 15 minutes, cool and read the OD using a colorimeter with a green filter of wavelength 520 nm.

Note: The solution levels in three test tubes should be below the surface of water in the boiling water bath.

Calculation

Concentration of urea in mg/100 ml blood.

$$\begin{aligned} &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\ &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.03}{0.02} \times 100 \text{ mg\%} \\ &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 150 \text{ mg\%} \end{aligned}$$

Other Methods

- Nessler's method: Ammonia reacts with potassium mercuric iodide (Nessler's reagent) to form yellow colour, which is measured at 480 nm.
- Berthelot reaction: Ammonia reacts with sodium hypochlorite and sodium nitroprusside as a catalyst, in alkaline medium to produce blue coloured complex, which is measured at 620 nm.
- Kinetic assay using glutamate dehydrogenase: Ammonia reacts with α -ketoglutarate in presence of glutamate dehydrogenase and NADH. This results in the formation of L-glutamate and NAD^+ . Rate of decrease in concentration of NADH is monitored at 340 nm.

CLINICAL SIGNIFICANCE

The blood urea concentration in normal individual is 15–40 mg%. It increases to the higher side in people whose protein intake is high. Urea is generally excreted in urine by glomerular filtration. When the rate of glomerular filtration is decreased an elevation in blood urea concentration is observed. Severe diarrhoea, vomiting, and excessive fluid loss, decreases the rate of glomerular filtration, consequently increasing blood urea levels. Similarly lower urinary tract obstruction and pathology resulting in decreased glomerular filtration also leads to elevated levels of blood urea.

In renal pathology like chronic acute glomerulonephritis, nephrosis, malignant hypertension, chronic polynephritis, and damage to the kidney tissues due to mercury poisoning or calcium deposition due to hyperthyroidism and hypervitaminosis (Vitamin D) blood urea levels are higher than normal values. Post renal conditions like enlargement of prostate gland, stones in the urinary tract or tumor of the bladder also cause increased blood urea levels.

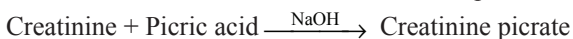
Estimation of Creatinine

BLOOD CREATININE

Principle

Creatinine is produced in muscles from creatine by non-enzymatic irreversible dehydration. Creatine, synthesised in the liver and kidney, passes into the circulation and is taken up almost entirely by skeletal muscles for conversion to creatine phosphate, which serves as the storage form of energy in skeletal muscles. About 2% of total creatine is converted daily into creatinine. The amount of creatinine produced is related to the total muscle mass and remains approximately same in the plasma and urine in day-to-day basis unless muscle mass changes.

Creatinine reacts with picric acid in the presence of an alkali to form orange-red colour of creatinine picrate. Proteins in blood do not interfere as they are precipitated with tungstic acid. The intensity of the orange-red colour is a measure of the amount of creatinine present in blood.



This reaction is known as Jaffe's reaction.

Reagents

(a) 0.04 M picric acid, (b) 0.75 N sodium hydroxide, (c) 10% sodium tungstate, (d) $\frac{2}{3}$ NH_2SO_4 , (e) Standard creatinine solution (0.01 mg/ml) in 0.1 NHCl .

Procedure

Blood collected in oxalate tube.

Part I

Preparation of protein free filtrate (PFF) from blood.

In a dry test tube take 3 ml distilled water, 1 ml blood, 1 ml 10% Na-tungstate and 1 ml $\frac{2}{3}$ NH_2SO_4 (Dilution of blood \equiv 1 in 6). Mix, keep for 10 minutes, and filter in a dry test tube to obtain a clear solution of PFF.

Part II

Use PFF for blood glucose estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
PFF	3 ml	–	–
Standard creatinine solution	–	–	3 ml
Distilled water	–	3 ml	–
Picric acid	1 ml	1 ml	1 ml
NaOH	1 ml	1 ml	1 ml

Mix and keep for 15 minutes. Read the OD using a colorimeter with a green filter of wavelength 520 nm.

Calculation

Concentration of creatinine in mg/100 ml blood.

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.03}{0.5} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 6 \text{ mg\%}
 \end{aligned}$$

Clinical Significance

The normal blood creatinine values are in the range 0.7–1.4 mg%. The value may increase during kidney diseases like acute or chronic renal insufficiency, urinary tract obstruction and impairment of renal function induced by some drugs. Changes in creatinine levels in blood are also known to occur in uremia, nephritis and early stages of muscle wasting diseases.

JAFFE'S REACTION CAN ALSO BE USED TO MEASURE CREATININE IN SERUM/ PLASMA OR URINE

A. Serum separated from blood collected in plain bulb without anticoagulant.

Plasma separated from blood collected in oxalate bulb.

Preparation of PFF as follows:

In a dry test tube take 2 ml distilled water, 2 ml serum (or plasma), 2 ml 5% Na-tungstate and 2 ml $\frac{2}{3}$ NH_2SO_4 (Dilution of blood = 1 in 4). Mix, keep for 10 minutes, and filter in a dry test tube to obtain a clear solution of PFF. Proceed further with PFF as described earlier and calculate as:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.03}{0.75} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 4 \text{ mg\%}
 \end{aligned}$$

B. Urine

24h urine sample is preferred for urine creatinine estimation. Urine is collected in a clean bottle using chloroform as preservative.

Procedure

Dilute 5 ml urine to 50 ml (dilution is 1 in 10).

Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
Diluted urine	5 ml	–	–
Standard creatinine solution	–	–	5 ml
Distilled water	–	5 ml	–
Picric acid	2 ml	2 ml	2 ml
NaOH	2 ml	2 ml	2 ml

Mix and keep for 15 minutes. Read the OD using a colorimeter with a green filter of wavelength 520 nm. Concentration of creatinine in mg/100 ml of diluted urine:

$$= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.5}{5} \times 100 \text{ mg\%}$$

Concentration of creatinine in mg/100 ml of undiluted urine.

$$= \frac{OD_T - OD_B}{OD_S - OD_B} \times 100 \text{ mg\%}$$

Concentration of creatinine in g per 1500 ml (24 h) of undiluted urine:

$$= \frac{OD_T - OD_B}{OD_S - OD_B} \times 100 \times \frac{15}{1000} \text{ g\%}$$

$$= \frac{OD_T - OD_B}{OD_S - OD_B} \times 1.5 \text{ g\%}$$

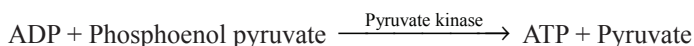
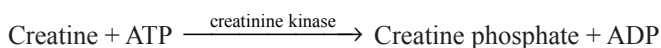
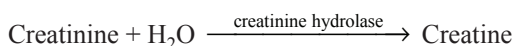
Note

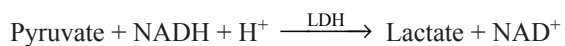
Jaffe's reaction is less specific. Only 80% of the colour develop is due to creatinine in serum. There are a number of other substances present in serum like glucose, urea, uric acid, protein, etc. which react with alkaline picrate and contribute to the colour development, giving rise to higher results. True creatinine value can be determined by getting rid of these non-specific chromogens by the use of Lloyd's reagent (hydrated aluminum silicate). It adsorbs creatinine in acid solutions. After centrifugation, the pellet is resuspended in alkaline medium to elute creatinine, which is then estimated.

Creatinine coefficient is defined as the mg of creatinine excreted in urine/ kg body weight (bw) in 24 hours. Normal level for males 20–26 mg/ kg bw/day, and for females 14–20 mg/kg bw/day. Creatinine coefficient is more precise and is used to assess the muscle mass of an individual.

Enzymatic Method

Serum creatinine can be measured enzymatically based on the following principle:





Clinical Significance

The normal daily excretion of creatinine ranges from 1–2 g. This is not influenced by diet. As creatinine, anhydride of creatine, is related to amount of muscle tissue and to phosphocreatine in the body, its excretion in urine normally remains constant in normal individual. Creatinine clearance test is widely used as a measure of the glomerular filtration rate and it is decreased in advanced renal failure.

Estimation of Uric Acid

Principle

Uric acid is the end product of catabolism of purine bases present in the nucleoproteins. Therefore, formation of uric acid is principally endogenous mainly of tissue nucleoprotein breakdown but some amount is also formed from purine containing compounds present in food. Thus serum uric acid levels are only marginally affected by diet.

Chemically uric acid is 2,6,8 trihydroxypurine. It acts like a dibasic acid (with two pK values 5.75 and 9.8) and can form mono and disodium salts depending on pH. Only pH of 5.75 is possible inside the body such as in renal tubules. At this pH, or above it exists as monosodium urate salt. Thus in plasma, it is mainly as monosodium urate.

The proteins in blood are precipitated by tungstic acid. The uric acid present in PFF reduces phosphotungstic acid in alkaline medium to blue coloured phosphotungstous acid. The intensity of the colour is a measure of the amount of uric acid present in the blood.

Reagents

(a) 10% sodium tungstate, (b) $\frac{2}{3}$ N H_2SO_4 , (c) Phosphotungstic acid, (d) 14% sodium carbonate, (e) Standard uric acid solution (0.1 mg/ml): Transfer 100 mg uric acid to 100 ml of water. Add solid sodium carbonate a little at a time with stirring to dissolve uric acid.

Sample: Blood collected in oxalate tube.

Part I

Preparation of PFF from blood.

In a dry test tube take 8.5 ml distilled water, 0.5 ml blood, 0.5 ml 10% Na-tungstate and 0.5 ml $\frac{2}{3}$ NH_2SO_4 (Dilution of blood = 1 in 20). Mix, keep for 10 minutes, filter in a dry test tube to obtain a clear solution of PFF.

Part II

Use PFF for blood glucose estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
PFF	3 ml	–	–
Standard uric acid solution	–	–	3 ml
Distilled water	–	3 ml	–
14% sodium carbonate	1 ml	1 ml	1 ml
Phosphotungstic acid reagent	1 ml	1 ml	1 ml

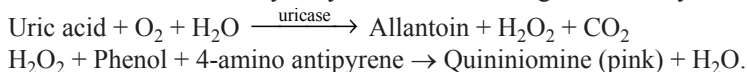
Mix and keep in dark for 15 minutes. Read the OD using a colourimeter with a red filter of wavelength 640 nm.

Calculation

Concentrate of uric acid in mg/100 ml blood.

$$\begin{aligned} &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\ &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.3}{0.15} \times 100 \text{ mg\%} \\ &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 200 \text{ mg\%} \end{aligned}$$

Uric acid can be estimated by enzymatic method using uricase enzyme.



Clinical Significance

Uric acid is an end product of purine (nucleic acid) catabolism. The normal range of uric acid in whole blood is 1.4–4.6 mg%. It is slightly higher in serum in the range of 2–5 mg%. Values in men are slightly higher than women. Increased uric acid levels have been seen in old age, after severe exercise and with high purine diet (meat food). In pathological conditions like gout, leukaemia, and polycythaemia the value tends to increase. Uric acid levels have also been known to increase in impaired renal function.

Haemoglobin in Blood

ESTIMATION

Principle

Haemoglobin in blood is first converted to methaemoglobin by potassium ferricyanide. The methaemoglobin formed is then converted to cyanomethaemoglobin by potassium cyanide of Drabkin's reagent. The intensity of the brown colour of cyanomethaemoglobin is a measure of the amount of haemoglobin present in blood.

Reagents

Drabkin's (Ferricyanide- cyanide) reagent:

Dissolve 200 mg of potassium ferricyanide, 140 mg KCN and 140 mg KH_2PO_4 in 900 ml distilled water. Adjust pH in 7.2 to 7.4 with 0.1 N phosphoric acid or 0.1 N KOH. Store in dark bottle, in cold.

Sample: Blood collected in oxalate tube.

Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
Blood sample	0.02 ml	–	–
Standard Haemoglobin solution	–	–	0.02 ml
Distilled water	–	0.02 ml	–
Drabkin's reagent	5 ml	5 ml	5 ml

Mix and keep for 5 minutes. Read the OD using a colorimeter with a green filter of wavelength 520 nm.

Note: Drabkin's reagent contains highly toxic cyanide.

Calculation

Concentration of haemoglobin in blood

$$\begin{aligned}
 &= \frac{\text{OD}_T - \text{OD}_B}{\text{OD}_S - \text{OD}_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{\text{OD}_T - \text{OD}_B}{\text{OD}_S - \text{OD}_B} \times \frac{0.012}{0.02} \times 100 \text{ mg\%}
 \end{aligned}$$

$$= \frac{OD_T - OD_B}{OD_S - OD_B} \times 60 \text{ mg\%}$$

$$= \frac{OD_T - OD_B}{OD_S - OD_B} \times 0.06 \text{ g\%}$$

Normal values:

- Men: 13–18 g%
- Women: 11–16 g%
- Infant: 14–20 g%
- Children: 11–13 g%

Clinical Significance

The concentration of haemoglobin varies with age, sex, climatic conditions, altitude and physical activity. Decreased values are observed in various anaemias, pregnancy, after severe haemorrhage. Increase is observed in polycythemia and in shock or dehydration, in some cardiac or pulmonary pathology and also after cobalt toxicity.

SPECTROSCOPIC EXAMINATION OF HAEMOGLOBIN AND ITS DERIVATIVES

Haemoglobin is an intracorporeal pigment and does not normally occur in appreciable amounts in plasma (< 10 mg/dl). However, it is not easy in practice to obtain serum or plasma completely free from haemolysis, faint bands of oxyhaemoglobin can often be seen on careful spectroscopic examination of these fluids.

Haemoglobin released from the cells, is bound to a group of glycoproteins known as haptoglobins and the complex formed is rapidly removed by the reticuloendothelial system. When the binding capacity of haptoglobins is exceeded (40–160 mg haemoglobin), free haemoglobin is found in the plasma. Haemoglobin derivatives are compounds of clinical and chemical importance which result when it reacts with substances other than oxygen such as CO, cyanides, acids, alkalis, etc. Being coloured compounds, characteristic absorption properties at different wavelengths are useful in the identification and quantification of different haemoglobin derivatives.

Simple visual inspection of blood specimen can give valuable information, e.g. the blood is cherry red when the pigment presents carboxyhaemoglobin in CO poisoning. The colour is chocolate brown in methaemoglobinemia.

Spectroscope is a simple device that resolves white light into its seven component colours. It consists of a narrow slit through which light enters. A set of prisms resolve the light that can be viewed through an eyepiece. When daylight is viewed through the spectroscope, a few dark lines are seen. The two prominent lines are at 589 and 518 nm. They arise due to absorption of light of particular wavelength by sodium and magnesium respectively, present in solar system.

When a solution of haemoglobin is viewed through a spectroscope, similar dark lines or bands are seen at definite wavelength. They arise due to absorption of light by haemoglobin. The absorption maxima of these lines differ from one haemoglobin derivative to another, which is successfully used in the differential identification of these compounds.

Oxyhaemoglobin

Prepare 1:200 dilution of blood (1 drop in 5 ml of water) and examine with spectroscope. Two bands are seen in the green portion of the spectrum (539 and 577 nm). They are known as alpha and beta band respectively.

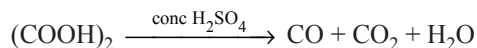
Haemoglobin

This is sometimes referred as reduced haemoglobin. To 1: 100 diluted blood, add a pinch of reducing agent sodium thiosulphite ($\text{Na}_2\text{S}_2\text{O}_4$) and mix gently. The contents turn purple (oxyhaemoglobin is deoxygenated). A single band in the green region with absorption maxima of 565 nm is seen through the spectroscope.

If shaken vigorously, reoxygenation takes place, provided there is not too much reducing agent also, two original bands in the green region will appear through the spectroscope.

Carboxyhaemoglobin

Bubble through the diluted blood solution either coal gas or a mixture of CO and CO_2 obtained by treating oxalic acid with Conc. H_2SO_4



Add a drop of caprylic alcohol (2-octanol) to prevent frothing during the process of bubbling. The solution turns carmine red due to the formation of carboxyhaemoglobin.

Two bands are seen in the green region with absorption maxima 572 and 534 nm through the spectroscope. The small difference in the absorption maxima of bands of oxy and carboxyhaemoglobin can be distinguished with a spectroscope of high resolution.

Test to differentiate carboxyhaemoglobin from oxyhaemoglobin:

- Colour of carboxyhaemoglobin is characteristically cherry red and is much brighter.
- CO has 200 times more affinity for haemoglobin. Add a pinch of solid $\text{Na}_2\text{S}_2\text{O}_4$ to carboxyhaemoglobin solution and mix. Neither is there any change in colour nor is change in position of band. Whereas oxyhaemoglobin gives a single band in green region.

Methaemoglobin

Put 4 drops of blood in 5 ml water. Add a pinch of oxidising agent, potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] and mix gently. The solution turns brown. Haem (Fe^{++}) is oxidised to haematin (Fe^{+++}). A band is seen in the red region with its centre at 638 nm through the spectroscope. Two bands in green and a faint band in the blue region can be seen with a high resolution spectroscope.

Methaemoglobin can be converted to haemoglobin by reduction with $\text{Na}_2\text{S}_2\text{O}_4$.

Haemochromgen

To 1:100 diluted blood, add 2–3 drops of 5% NaOH. Heat very gently until the solution turns yellow. Add a pinch of $\text{Na}_2\text{S}_2\text{O}_4$ and mix. The solution turns pink. Two bands are seen in the green region at 558 and 526 nm through the spectroscope.

Preparation of Haemin Crystals

Upon heating with acid, haemoglobin is denatured and haem is oxidised to haematin. Haematin is finally converted to haematin chloride (haemin).

Spread a drop of blood on slide in the form of a thin film. Dry it over a low flame. Add 2 drops of Nippe's fluid. Place a cover glass in position. Heat gently over low flame until gas bubbles form and the solution boils. Run one or two drops of Nippe's fluid reagent underneath the cover glass. Cool and examine under the microscope. Brown crystals are seen.

Note: It is essential that solution viewed with spectroscope is not too concentrated. At high concentration, two adjacent narrow bands will merge and appear as a single broadband.

26

Estimation of Serum Proteins and Albumin/Globulin Ratio

PRINCIPLE

Proteins, which contain peptide linkages form a complex with copper in alkaline medium giving a violet colour and this reaction, is called the biuret reaction. The intensity of this colour is proportional to the number of peptide linkages present and thus is a measure of the concentration of proteins.

Albumins are estimated in serum using the biuret reaction after precipitation and separation of serum globulins by sodium sulphate. Globulins are precipitated by sodium sulphate. The supernatant albumin is estimated by biuret reaction.

Reagents

- 28% sodium sulphate
- 0.9% saline
- Standard protein solution (bovine serum albumin, 5 mg/ml)
- Dilute biuret reagent.

Sample: Serum separated from blood collected in plain tube with anticoagulants.

Part I

Two dry test tubes labeled P (total proteins) and G (globulin).

	P	G
Saline	3.8 ml	–
28% sodium sulphate	–	3.8 ml
Serum	0.2 ml	0.2 ml

(Dilution 1 in 20). Mix G by inverting tube once only, filter immediately to separate globulins. Do not filter tube P.

Part II

Label 4 test tubes as T (test), A (albumin), B (blank) and S (standard).

	T	A	B	S
Saline	–	–	1 ml	–
Standard protein solution	–	–	–	1 ml
Solution from P	1 ml	–	–	–
Filtrate of test tube G	–	1 ml	–	–
Diluted biuret reagent	5 ml	5 ml	5 ml	5 ml

Mix and keep for 10 minutes read the optical density using a colorimeter with green filter or at 540 nm wavelength.

Calculation

I. Concentration of proteins in serum:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{5}{0.05} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 10000 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 10 \text{ g\%}
 \end{aligned}$$

II. Concentration of albumin in serum:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{5}{0.05} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 10000 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 10 \text{ g\%}
 \end{aligned}$$

III. Globulin in g% = Total protein (in g%) – Albumin (in g%).

IV. A/G ratio = g% albumin/g% globulin.

Normal values: Total protein: 6–8 g/dl, Albumin: 3.5–5 g/dl, Globulin: 2.5–3.5 g/dl, A/G :: 1.2:1–1.5:1.

Note: The sensitivity of biuret method is less and unsuitable for estimation of proteins in mg or μg quantities.

Other Methods

1. Lowry-Folin Ciocalteu (Phosphomolybdic acid and phosphotungstic acid): Tissue protein, measure tyrosine and/or tryptophan residue at 280 nm. Protein content in μg level can be measured. Variation in tyrosine and tryptophan also causes variation in estimation.
2. Micro-Kjeldahl method: This is based on the nitrogen content of protein, which is usually 16% in most cases. Protein nitrogen is acid digested and converted to ammonia that is then estimated by Nesslerization. Nitrogen content is multiplied by 6.25 to calculate protein concentration.
3. Ninhydrin reaction: Proteins do not give true colour, N-terminal amino group of protein react with ninhydrin to produce a blue colour.
4. Albumin can be measured using bromocresol green (Dye binding).

Albumin (Latin, albus= white): It is consisting of 585 amino acids with mol wt 69 kD; having 17 disulfide bonds. It is synthesised in hepatocytes. Therefore, decreases in liver disease. It maintain colloid osmotic pressure, transport hydrophobic substances: bilirubin, NEFA, etc. transport amino acids from liver to extrahepatic cells. It shows buffering capacity (16 Histidine residues maintain).

Clinical Significance

The value of total protein is increased in dehydration, haemoconcentration, multiple-myeloma, rheumatoid arthritis, tuberculosis, and kala-azar, whereas they are decreased in proteinuria, low protein intake, malabsorption, nephrosis, haemorrhage, shock, untreated diabetes mellitus, hyperthyroidism, and in severe liver diseases.

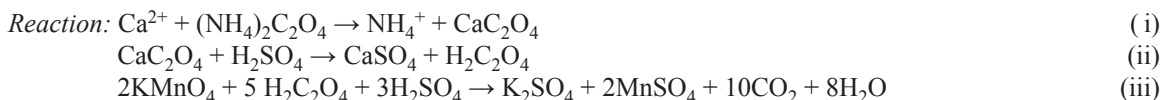
In pregnancy serum albumin level decreases, while the level of globulin increases. Albumin level is decreased (hypoalbuminemia) in malabsorption and malnutrition, low protein intake, haemorrhage, shock, untreated diabetes, albuminuria, hyperthyroidism, and in severe liver diseases and in nephrosis. Globulins increase (hypergammaglobulinemia) in advance liver diseases, multiple myeloma, and chronic infections.

Serum Calcium Estimation

ESTIMATION BY PERMANGANATE TITRATION METHOD

Principle

Calcium in serum is precipitated as calcium oxalate by the addition of ammonium oxalate. The precipitate is washed with dilute ammonium hydroxide to remove any excess of ammonium oxalate. The precipitate is then dissolved in 1N H₂SO₄. The oxalic acid liberated after addition of 1N H₂SO₄ is titrated with standard permanganate solution. The end point of titration is indicated by the formation of a pink colour that should be stable for 30 sec. The titre value is used to calculate the concentration of serum calcium.



Reagents

- 4% ammonium oxalate
- 2% ammonium hydroxide
- 1N H₂SO₄
- Standard 0.01 N KMnO₄ solution.

Sample: Serum separated from blood collected in plain tube without anticoagulant.

Procedure

In two centrifuge tubes take 2 ml distilled water and then add 2 ml serum and 1ml ammonium oxalate. Mix by rotating the tubes between your palm, keep for 30 minutes. Centrifuge at 2000 rpm for 30 minutes. Invert the tubes slowly in one continuous motion and discard the supernatant. Drain the last traces of liquid near the mouth of the tube on a filter paper.

Add 3 ml ammonium hydroxide along the sides of the tube. Mix, centrifuge and decant as indicated earlier. Repeat the procedure.

Add 2 ml 1N H₂SO₄ from the side of the tube rotating it in the process so that H₂SO₄ comes in contact with any precipitate on the surface of the tube. Place the tubes in water bath at 70–80°C for 5 minutes to dissolve the precipitate. Titrate the contents of the tube with standard 0.01N permanganate solution. End point is faint pink colour and stable for 30 seconds.

Repeat the whole process with the second centrifuge tube.

Perform a blank titration with 2 ml 1N H₂SO₄ taken in a dry test tube, kept in a water bath at 70–80°C for 5 minutes. This will indicate the total impurities of oxalic acid in the 1N H₂SO₄.

Calculation

From equation (iii) it is evident that:

$\frac{1}{5}$ mol of KMnO₄ \equiv $\frac{1}{2}$ mol of oxalic acid \equiv $\frac{1}{2}$ mol of CaC₂O₄ (from eqn ii) (iv)

Now, 2 KMnO₄ = K₂O, 2MnO, 5O. i.e., $2\text{Mn}^{+7} + 10\text{e} \rightarrow 2\text{Mn}^{+2}$

\therefore Equivalent weight of permanganate = $[2\text{KMnO}_4/10]$ i.e., $1/5$ molecular weight,

From equation (iv) we have,

1000 ml N KMnO₄ solution can oxidise $\frac{1}{2}$ molecular weight of oxalic acid obtained from $\frac{1}{2}$ molecular weight of Ca-oxalate, which contain 20 g of calcium.

\therefore 1000 ml N KMnO₄ \equiv 20 mg calcium.

\therefore 1 ml 0.01N KMnO₄ \equiv 0.2 mg calcium.

If, amount of KMnO₄ solution required for test sample: T ml (T₁+T₂/2) and amount of KMnO₄ solution required for blank: B ml.

Thus, (T-B) ml of KMnO₄ \equiv (T-B) \times 0.2 mg calcium.

As, 2 ml of serum was taken in each tests,

So, 100 ml serum contains \equiv (T-B) \times 0.2 \times 50 mg calcium.

ESTIMATION BY CRESOLPHTHALEIN COMPLEXONE METHOD

Principle

Cresolphthalein complexone (CPC) is an indicator, which is colourless in neutral or acidic pH but forms a coloured complex with calcium in alkaline medium. The intensity of this colour is a measure of the amount of calcium in serum. Magnesium does not interfere in this reaction as its effect is suppressed by the addition of 8-hydroxyquinoline.

Reagents

- CPC reagent: Dissolve 40 mg CPC in 1 ml Conc. HCl. A few drops of water may be added to help dissolve CPC. Transfer this to a litre volumetric flask with 50 ml dimethyl sulphoxide used as wash. Add 2.5 g 8-hydroxy quinoline, mix to dissolve and make the volume to 1litre.
- Dimethyl amine reagent: Dissolve 500 mg KCN, add 40 ml dimethylamine and make the volume 1L with distilled water.
- Standard calcium solution: 0.02 mg/ml.

Sample: As described in previous experiment.

Procedure

Dilute 1 ml serum with 4 ml distilled water (Dilution 1 in 5), mix thoroughly.

Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
Diluted serum (1:5)	0.1 ml	–	–
Standard calcium solution	–	–	0.1 ml
Distilled water	–	0.1 ml	–
CPC reagent	2 ml	2 ml	2 ml
Dimethylamine solution	2 ml	2 ml	2 ml

Mix by inverting the tubes after placing a paraffin paper piece over the mouth of the test tube and read the OD using a colorimeter with yellowish green filter of wavelength 575 nm.

Calculation

Concentration of calcium in serum:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.002}{0.02} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 10 \text{ mg\%}
 \end{aligned}$$

Note: Do not use turbid or lipemic serum.

Reagents are toxic

Normal level: 8.5–11 mg%.

Clinical Significance

The nondiffusible calcium constitutes approximately 40–50%. The rest is diffusible portion consists of calcium salt of citrate and phosphate. Increase in blood pH decreases the level of ionised calcium without causing a change in total calcium level. In hyperparathyroidism and hypervitaminosis D serum calcium level is increased. In hypoparathyroidism serum calcium level is decreased. Calcitonin, an antagonist to parathyroid hormone decreases serum calcium level. In rickets and osteomalacia serum calcium level is slightly reduced. Serum calcium level can be low in steatorrhea, nephritis and pancreatitis.

Determination of Serum Inorganic Phosphate

PRINCIPLE

In this method, proteins in serum are precipitated with trichloroacetic acid. The protein-free filtrate containing inorganic phosphate is treated with molybdic acid reagent when phosphomolybdate is formed. This is reduced to molybdenum blue by the reducing agent 1-amino-2-naphthol-4-sulfonic acid (ANSA). The intensity of this blue solution is a measure of inorganic phosphate present.

Reagents

(a) 10% trichloroacetic acid, (b) Molybdic acid reagent, 2.5% ammonium molybdate in 3N H_2SO_4 , (c) 1-amino-2-naphthol-4-sulfonic acid (ANSA): Dissolve 500 mg of ANSA to 195 ml 15% sodium bisulphate and 5 ml 20% sodium sulphite. Shake until dissolve. (d) Standard phosphate solution: Dissolve 0.0351 g potassium dihydrogen phosphate in water in a litre flask. Add 800 ml 10% trichloroacetic acid and make up the mark with water. (5 ml = 0.04 mg phosphorous).

Sample: Serum (from blood without anticoagulants).

Part I

Preparation of PFF (Protein free filtrate) from serum

In a dry test tube take 8 ml trichloroacetic acid and 2 ml serum (Dilution of blood = 1 in 5). Mix, keep for 5 minutes, and filter in a dry test tube to obtain a clear solution of PFF.

Part II

Use PFF for blood glucose estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
PFF	5 ml	–	–
Standard phosphorous solution	–	–	5 ml
Distilled water	–	5 ml	–
Molybdic acid reagent	1 ml	1 ml	1 ml
ANSA	1 ml	1 ml	1 ml
Mix and allow to stand for 10 minutes			
Distilled water	3 ml	3 ml	3 ml

Mix and read the OD using a colorimeter with a red filter of wavelength 660–680 nm.

Calculation

Concentration of phosphorous in mg/100 ml serum:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.04}{1} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 4 \text{ mg\%}
 \end{aligned}$$

Normal values:

- Adult: 2.5–4.5 mg%
- Children: 4–6 mg%

Clinical Significance

Decrease in serum inorganic phosphorous is seen in rickets, osteomalacia, hyperparathyroidism and in conditions associated with decrease in the reabsorption of phosphate from the glomerular filtrate (Fanconi syndrome). A transient decrease in blood phosphorous level is seen during increased carbohydrate utilisation. Hyperphosphataemia occurs in hypoparathyroidism, hypervitaminosis D and in renal failure.

29

Estimation of Total Cholesterol in Serum

ZAK'S METHOD USING FERRIC CHLORIDE/ ACETIC ACID REAGENT

Principle

Proteins in serum are precipitated by ferric chloride. The cholesterol present in protein free filtrate is oxidised and dehydrated with ferric chloride, acetic acid and sulphuric acid to a red coloured compound. A measure of the intensity of the colour indicates the concentration of cholesterol in the serum.

Reagents

(a) 0.05 g ferric chloride hexahydrate in acetic acid, (b) Conc. H_2SO_4 . (d) Standard cholesterol solution: (5 ml \equiv 0.2 mg cholesterol), prepared fresh in ferric chloride—acetic acid reagent from stock cholesterol solution (100 mg/100 ml acetic acid).

Sample: Serum (from blood without anticoagulants).

Part I

Preparation of PFF (Protein free filtrate) from serum.

In a dry test tube take 9.9 ml ferric chloride—acetic acid reagent and 0.1 ml serum (Dilution of blood = 1 in 100). Mix by inversion using paraffin film, keep for 5 minutes, centrifuge, use clear supernatant as PFF.

Part II

Use PFF for blood glucose estimation. Label three test tubes as T (test), B (blank) and S (standard).

	T	B	S
PFF	5 ml	–	–
Standard cholesterol solution	–	–	5 ml
Ferric chloride-acetic acid reagent	–	5 ml	–
Conc. H_2SO_4	3 ml	3 ml	3 ml

Mix by swirling, keep for 30 minutes, and read the OD using a colorimeter with a green filter of wavelength 520 nm.

Note: a. The reagents are highly corrosive, handle with care.

b. Pipetting should be done very carefully.

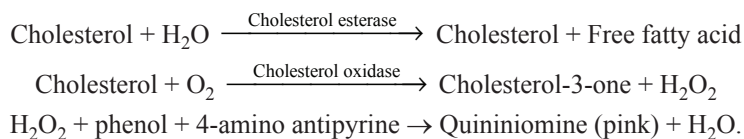
Calculation

Concentration of cholesterol in mg/100 ml serum:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.2}{0.05} \times 100 \text{ mg\%} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 400 \text{ mg\%}
 \end{aligned}$$

ENZYMATIC METHOD

Free cholesterol including that liberated by cholesterol esterase, undergoes oxidation producing H_2O_2 which gives a pink colour on reacting with phenol and 4-aminoantipyrine.



Normal values:

- Adult: Up to 220 mg%
- Children: Up to 150 mg%

Clinical Significance

Hypercholesterolaemia is found in nephrosis, diabetes mellitus, obstructive jaundice, myxoedema, xanthomatosis and hypopituitarism. High fat and high carbohydrate diet increase cholesterol levels. Animal meat, especially red meat and egg are rich in cholesterol levels. Polyunsaturated fatty acids up regulate LDL receptors and enhance the catabolism of cholesterol rich LDL and thus decrease cholesterol level.

Estimation of Serum Bilirubin

PRINCIPLE

Bilirubin in serum reacts with diazotized sulphanilic acid to give purple coloured derivative of azobilirubin. The colour is a measure of the amount of bilirubin in serum. Proteins in serum do not interfere in this estimation and minimal changes if any are eliminated using a control without the diazo reagent. Total bilirubin is measured using methanol as solvent and direct bilirubin is measured with water as solvent because the non-esterified indirect bilirubin, insoluble in water, reacts with the diazo reagent very slowly thus avoiding their interference during the analysis of direct bilirubin.

Reagents

- Diazo reagent A: Dissolve 1 g sulphanilic acid in 15 ml conc. HCl; and make volume 1L with distilled water.
- Diazo reagent B: 0.5% sodium nitrite.
- Diazo colour reagent: Mix 5 ml diazo A in 0.15 ml diazo B (prepare fresh).
- 0.15 N HCl, (e) Methanol, (f) Standard bilirubin in chloroform (1 ml \equiv 0.1 mg).

Sample: Serum (from blood without anticoagulants).

Procedure

Label six test tubes as TT (total test), TC (total control), DT (direct test), DC (direct control), S (standard) and B (blank).

	TT	TC	DT	DC	S	B
Distilled water	1.8 ml	1.8 ml	4.3 ml	4.3 ml	1.8 ml	2 ml
Serum	0.2 ml	0.2 ml	0.2 ml	0.2 ml	–	–
Diazo colour reagent	0.5 ml	–	0.5 ml	–	0.5 ml	0.5 ml
0.15 N HCl	–	0.5 ml	–	0.5 ml	–	–
Methanol	2.5 ml	2.5 ml	–	–	2.5 ml	2.5 ml
Bilirubin standard	–	–	–	–	0.2 ml	–

Mix, keep the tubes for 30 minutes in dark and read the OD using a colorimeter with green filter of wavelength 540 nm.

Note: If bilirubin standard is not available, methyl red solution (0.29 mg%) can be used as an arbitrary standard. The colour of 1 ml of this solution is equivalent to 0.0035 mg bilirubin. The optical density of this standard solution without any treatment is used in calculation and the standard concentration corresponds to 0.017 mg.

Calculation

1. Concentration of direct bilirubin in mg/100 ml serum:

$$\begin{aligned} &= \frac{OD_{DT} - OD_{DC}}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\ &= \frac{OD_{DT} - OD_{DC}}{OD_S - OD_B} \times \frac{0.2}{0.2} \times 100 \text{ mg\%} \\ &= \frac{OD_{DT} - OD_{DC}}{OD_S - OD_B} \times 100 \text{ mg\%} \end{aligned}$$

2. Conc of total bilirubin in mg/100 ml serum:

$$\begin{aligned} &= \frac{OD_{TT} - OD_{TC}}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ mg\%} \\ &= \frac{OD_{TT} - OD_{TC}}{OD_S - OD_B} \times \frac{0.2}{0.2} \times 100 \text{ mg\%} \\ &= \frac{OD_{TT} - OD_{TC}}{OD_S - OD_B} \times 100 \text{ mg\%} \end{aligned}$$

Normal value:

- Total bilirubin: < 1 mg%
- Direct bilirubin: Up to 0.2 mg%.

Clinical Significance

In haemolytic (prehepatic) jaundice, there is a moderate increase in serum total and indirect bilirubin concentration. It may occur due to haemoglobinopathies or Rh incompatibility in children, malaria or due to toxic drug reactions. Obstructive jaundice arising due to carcinoma on the head of the pancreas, gallstones or other causes. In obstructive jaundice indirect bilirubin level markedly increased. In hepatic jaundice, the rise in levels in direct bilirubin. The most common cause is hepatitis virus infection. It may be due to defective conjugation as in chronic hepatitis, Gilbert's disease and Crigler-Najjar's syndrome.

Estimation of Amylase in Serum (Iodometric Method)

PRINCIPLE

A known quantity of starch is incubated with serum for a certain time period when the amylase in serum hydrolyses starch to dextrin and maltose. The difference is the amount of starch before and after incubation with serum amylase is a measure of amylase activity. This difference is measured using iodine solution as the colour reagent.

Unit of amylase activity: One unit of amylase activity is the amount of amylase in 1 ml serum that will digest 1 mg starch at 37°C in 60 minutes.

Sample: Serum (from blood without anticoagulants).

Reagents

- 0.02M phosphate buffer, pH 6.9
- 0.5% starch substrate
- 0.01N iodine solution
- 0.9% saline solution.

Procedure

- In a test tube dilute 1 ml serum to 5 ml with 0.9% saline.
- In three beakers, take 20 ml distilled water and 1 ml 0.1N iodine solution, mix and keep ready.
- In a test tube, take 5 ml 0.02M phosphate buffer (pH 6.9), 4 ml 0.5% starch solution. Keep in water bath at 37°C for 5 min. Add 1 ml diluted serum.
- Mix immediately and remove 0.5 ml of the reaction mixture and put into one of the iodine cylinders. This is Zero Time (B) value.
- Incubate the reaction mixture at 37°C for 60 minutes. Then take 0.5 ml reaction mixture and put it into second iodine solution containing beaker (C).
- Make up the volume of both beakers to 25 ml and read the optical density using a colourimeter with red filter or at 640 nm.

Standard

Take 9 ml phosphate buffer and 1 ml 0.5% starch solution and mix. Take 0.5 ml and add to iodine solution containing beaker (S), make final volume 25 ml and read the optical density.

Calculation

Concentrate of amylase activity/ml serum:

$$\begin{aligned}
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times \frac{0.25}{0.01} \text{ U/ml} \\
 &= \frac{OD_T - OD_B}{OD_S - OD_B} \times 25 \text{ U/ml}
 \end{aligned}$$

Note: Saccharometric method, based on the principle of estimation of reducing sugars formed, by Benedict's quantitative reagent, can also be used for amylase estimation. The reducing sugars are formed due to the action of amylase on starch, which is a non-reducing polysaccharide.

Normal value: 6–35 U/ml

Clinical Significance

Amylase also called diastase is found in urine of normal individuals to the extent of 5–20 U/24h sample. Variations in urinary amylase reflect alterations in serum amylase as long as the kidneys are functioning normally. In renal diseases, serum amylase may increase and urinary amylase may be low. Considerable increase in both urine and serum are seen in acute pancreatitis and in neoplasm of the pancreas. The increase in serum and urinary amylase values may also rapidly decrease without changes in clinical pathology depending upon whether there is duct obstruction or destruction of the secreting tissues in patients with chronic disease of the pancreas. Low values of both serum and urinary amylase may be present in liver diseases.

Estimation of Serum Alkaline Phosphatase

METHOD OF KING AND KING USING PHENYL PHOSPHATE

Principle

Serum alkaline phosphatase acts on disodium phenyl phosphate at pH around 10 to form phenol and phosphate radical. The phenol formed reacts with 4-aminoantipyrine in the presence of alkaline oxidising agent potassium ferricyanide to give purple colour. The amount of phenol formed as indicated by the intensity of the colour developed is a measure of enzyme activity.

Unit of alkaline phosphatase activity: King and Armstrong defined one unit (KAU) of alkaline phosphatase as that liberating 1 mg of phenol from p-phenyl phosphate per 15 minutes per 100 ml serum.

Reagents

- 0.01 M disodium phenyl phosphate (warm to dissolve)
- 0.1 M bicarbonate buffer, pH 9.9
- 0.5 N Na_2CO_3
- 0.5 N NaOH
- 0.6% 4-aminoantipyrine
- 2.4% potassium ferricyanide (0.01 mg/ml).

Sample: Serum (from blood without anticoagulants).

Procedure

Label four test tubes as T (test), S (standard), C (control) and B (blank).

	T	S	C	B
Disodium phenyl phosphate	1 ml	1.1 ml	1 ml	1.1 ml
Distilled water	–	–	–	1 ml
Bocarbonate buffer	1 ml	–	1 ml	–
Standard phenol solution	–	1 ml	–	–
Serum	0.1 ml	–	–	–

Contd...

Contd...

Incubate "test" only for 15 minutes at 37°C

Seum			0.1 ml	
0.5N NaOH	0.8 ml	0.8 ml	0.8 ml	0.8 ml
0.5N Na ₂ CO ₃	1.2 ml	1.2 ml	1.2 ml	1.2 ml
4-aminoantipyrine	1 ml	1 ml	1 ml	1 ml
Potassium ferricyanide	1 ml	1 ml	1 ml	1 ml

Mix and read the optical density using a colorimeter with green filter or 525 nm wavelength.

Calculation

ALP activity as KAU/100 ml serum

$$\begin{aligned}
 &= \frac{OD_T - OD_C}{OD_S - OD_B} \times \frac{\text{Conc of standard}}{\text{Volume of sample}} \times 100 \text{ KAU/ml} \\
 &= \frac{OD_T - OD_C}{OD_S - OD_B} \times \frac{0.01}{0.1} \times 100 \text{ KAU/ml} \\
 &= \frac{OD_T - OD_C}{OD_S - OD_B} \times 10 \text{ KAU/ml}
 \end{aligned}$$

To convert King-Armstrong unit to IU/L:

- Multiply by 1000 and divide by 94 (MW of phenol) to convert to micromole.
- Multiply by 10 to change to litre and divide by 15 to make it per min.

So, ALP activity:

$$= \frac{OD_T - OD_C}{OD_S - OD_B} \times 10 \times \frac{1000}{94} \times \frac{10}{15} \text{ IU/L}$$

METHOD OF BESSEY ET AL USING P-NITROPHENYL PHOSPHATE

Principle

Serum alkaline phosphatase acts on p-nitrophenyl phosphate at around pH 10 to form p-nitrophenol and phosphoric acid. P-nitrophenol gives a yellow colour at alkaline pH and intensity of the colour is proportional to the amount of p-nitrophenol formed and is a measure of the enzyme activity.

Unit of enzyme activity: Bessey and Lowry defined one unit (BLU) of alkaline phosphatase as that liberating one millimole of p-nitrophenol per hour per litre of serum.

Reagents

- 0.01 M p-nitrophenyl phosphate (warm to dissolve)
- 0.1M bicarbonate buffer, pH 10.5
- 0.2N NaOH
- Standard p-nitrophenol (0.001 millimoles/ ml).

Sample: Serum (from blood without anticoagulants).

Procedure

Label three test tubes as T (test), C (control) and S (standard).

	T	C	S
p-nitrophenyl phosphate in bicarbonate buffer, pH 10.5	1 ml	1 ml	–
Standard p-nitrophenol	–	–	1 ml
Incubate at 37°C for 5 minutes			
Serum	0.1 ml	–	–
Distilled water	–	0.1 ml	0.1 ml
Incubate at 37°C for 30 minutes			
0.02N NaOH	10 ml	10 ml	10 ml

Mix and read the optical density using a colorimeter with blue filter or 420 nm wavelength.

Calculation: ALP activity as BLU/ L serum

$$\begin{aligned}
 &= \frac{U - C}{S} \times \frac{\text{Conc of standard in millimoles}}{\text{Volume of sample}} \times 1000 \times \frac{\text{Time of incubation}}{\text{Actual incubation time}} \\
 &= \frac{U - C}{S} \times \frac{0.001}{0.1} \times 1000 \times \frac{60}{30} \\
 &= \frac{U - C}{S} \times 20 \text{ BLU/L}
 \end{aligned}$$

Note

- In general, all nitro derivative of benzene give colour. The benzene ring containing amino acids gives yellow colour, which turns to orange in alkali. The nitrophenol formed by alkaline phosphatase action is also yellow and turns darker in alkaline medium.
- Other phosphatases present in serum do not interfere in this estimation, as the pH of the reaction is quite alkaline.

Normal level: 3–13KAU/100 ml serum (23–92 IU/L); slightly higher in growing children.

Clinical Significance

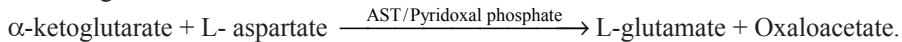
Alkaline phosphatase is found in a number of organs including intestine, bones, liver, and kidneys. The serum enzyme levels markedly increased in bone diseases like rickets, osteomalacia, hyperparathyroidism and Paget disease. Moderate increase is observed in obstructive jaundice, and mild increase in infective hepatitis.

Differences between Alkaline and Acid Phosphatase

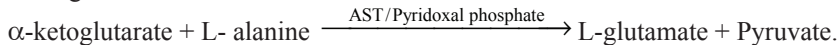
Properties	ALP	ACP
Optimum pH	10	5–6
Sources	Liver, bone, kidney, placenta, intestine	Mainly prostate in males. Minor non-prostatic fractions are from RBC, WBC, platelets, liver, spleen, bone, pancreas, etc.
Separation	Isoenzymes are separated by electrophoresis	Prostatic fraction can be inhibited by tartrate unlike non-prostatic fraction
Stability	Stable	Extremely labile enzyme
Normal level	3–13 KAU	1–3.5 KAU
Significance	Rises in bone and liver diseases	Rises in cancer of prostate and is used for its diagnosis and prognosis

Estimation of Transaminase Activity

Serum aspartate transaminase (AST), also preferably called glutamate oxaloacetate transaminase (SGOT) catalyse the following reaction:



Serum alanine transaminase (ALT), also preferably called glutamate pyruvate transaminase (SGPT) catalyse the following reaction:



These reactions have been used to estimate the concentration of the transaminases in serum.

PRINCIPLE

The transaminases present in serum act on aspartate (AST) or alanine (ALT) when they are incubated together at 37°C in a buffer of around neutral pH, to form the respective keto acids. The keto acids are made to react with 2,4-dinitrophenyl hydrazine (DNPH) in alkaline medium to form reddish- brown complex of hydrazones. The intensity of the colour is proportional to the amount of keto acids present, which, in turn, is proportional to the amount of serum transaminases present. Thus, a measure of the optical density of the coloured solution indicates the concentration of the serum transaminase levels.

Unit of transaminase activity: It is expressed as International units (IU) and is defined as number of micromoles of respective ketoacid formed per minute per litre of serum.

Reagents

- 0.1 M phosphate buffer, pH 7.4.
- SGOT substrate: 200 mM aspartate and 2 mM α -ketoglutarate in 0.1 M phosphate buffer, pH 7.4 with 10% NaOH used to adjust pH.
- SGPT substrate: 200 mM alanine and 2 mM α -ketoglutarate in 0.1 M phosphate buffer, pH 7.4 with 10% NaOH used to adjust pH.
- 0.4 N NaOH.
- 1 mM DNPH reagent.
- Standard pyruvic acid solution (2 micromoles/ml).
- Standard oxaloacetic acid solution (2 micromoles/ml).

Sample: Serum (from blood without anticoagulants).

Procedure

Label three test tubes as TO (test), CO (control) and SO (standard).

	TO	CO	SO
Buffered AST substrate	1 ml	1 ml	–
Incubate at 37°C for 5 minutes			
Serum	0.2 ml	–	–
Incubate at 37°C for 60 minutes			
DNPH reagent	1 ml	1 ml	1 ml
Standard oxaloacetic acid solution	–	–	0.2 ml
Serum	–	0.2 ml	–
Mix and keep at room temperature for 20 minutes			
NaOH	10 ml	10 ml	10 ml
Mix and keep at room temperature for 10 minutes			

Mix and read the optical density using a colorimeter with green filter or 520 nm wavelength. Use 1 ml DNPH reagent and 10 ml 0.4N NaOH as blank.

Label three test tubes as TP (test), CP (control) and SP (standard).

	TP	CP	SP
Buffered ALT substrate	1 ml	1 ml	–
Incubate at 37°C for 5 minutes			
Serum	0.2 ml	–	–
Incubate at 37°C for 30 minutes			
DNPH reagent	1 ml	1 ml	1 ml
Standard pyruvic acid solution	–	–	0.2 ml
Serum	–	0.2 ml	–
Mix and keep at room temperature for 20 minutes			
NaOH	10 ml	10 ml	10 ml
Mix and keep at room temperature for 10 minutes			

Mix and read the optical density using a colorimeter with green filter or 520 nm wavelength. Use 1 ml DNPH reagent and 10 ml 0.4N NaOH as blank.

Calculation

Serum AST activity:

$$\begin{aligned}
 &= \frac{TO - CO}{SO - B} \times \frac{\text{Conc of standard in millimoles}}{\text{Volume of sample}} \times 1000 \times \frac{1}{\text{incubation time}} \\
 &= \frac{TO - CO}{SO - B} \times \frac{0.4}{0.2} \times 1000 \times \frac{1}{60} \text{ IU/L} \\
 &= \frac{TO - CO}{SO - B} \times 33.3 \text{ IU/L}
 \end{aligned}$$

Serum ALT activity:

$$\begin{aligned}
 &= \frac{TP - CP}{SP - B} \times \frac{\text{Conc of standard in millimoles}}{\text{Volume of sample}} \times 1000 \times \frac{1}{\text{incubation time}} \\
 &= \frac{TO - CO}{SP - B} \times \frac{0.4}{0.2} \times 1000 \times \frac{1}{30} \text{ IU/L} \\
 &= \frac{TP - CP}{SP - B} \times 66.6 \text{ IU/L}
 \end{aligned}$$

Note: Though separate standard solution of pyruvate and oxaloacetate have been indicated; only one of the two could be used as standard.

Haemolysed serum samples should be avoided.

Normal level:

- AST: Up to 35 IU/L
- ALT: Up to 40 IU/L.

Clinical Significance

AST is increased to the maximum within 24h of myocardial infarction. Both AST and ALT increase in liver diseases. However, ALT is more specific marker for hepatic disorders.

PART
C

Demonstrations

Determination of pH

PRINCIPLE

Acids may be defined as compounds that yield positively charged hydrogen ions in solution and bases as compounds which yield negatively charged hydroxyl ions in solution. The net concentration of these ions determines the pH of the solution. pH is negative logarithm of hydrogen ion concentration and thus the measurement of hydrogen ion concentration will allow calculation of pH.

pH meter is based on the principle of measurement of electromotive force (EMF) generated between the two electrodes due to the difference in H^+ ion concentration. It is known that if a metal plate is placed in a solution of its own salt, it loses ions into the solution and itself becomes negatively charged as compared to solution. This generates an electrical potential on the metal plate or the electrode. If two different metal electrodes are connected in this way, the difference in their electrode potential can be measured as EMF. Hence, if one of the electrodes is standard electrode (with known potential), the electrode potential of the other can be easily measured.

This can be easily achieved by using standard hydrogen electrode. The standard hydrogen electrode consists of a platinum plate coated with platinum black (to absorb hydrogen) and dipped in 1M HCl solution. Hydrogen gas needs to be constantly passed into it at 1ATM pressure to maintain H^+ concentration. The electrode potential of this is taken as zero. When similar electrode is dipped into a solution of unknown H^+ concentration and connected by means of a bridge of KCl solution, the electrode potential of the unknown can be measured. This potential E is related to pH by the equation $pH = E/0.0591$.

In a day-to-day practice, the use of hydrogen electrode is quite difficult and cumbersome.

Direct measurement of pH can be done using a glass electrode, which consists of a bulb of special glass filled with 0.1N HCl in contact with a suitable metallic electrode (Ag/AgCl). When this bulb is immersed in an unknown solution a potential difference develops between the unknown solution and the HCl in the electrode in contact with platinum wire that passes out of the glass bulb magnitude of which depends on the hydrogen ion concentration of the unknown solution. Thus, the potential difference:

$$E = \frac{RT}{nF} \log \frac{C_2}{C_1}$$

$\frac{RT}{nF}$ is a constant for specific conditions of pH measurement.

Where, R = Gas constant = $8.316 \text{ joules/}^\circ\text{A}$

T = Absolute temperature

n = Valency of ion

F = Faraday.

C_1 and C_2 are the hydrogen ion concentration of the two solutions of which one is known.

The instrument used to measure pH is called pH meter that is specifically calibrated to give the pH (C_2) directly based on E values when R , T , n and F are constant.

Materials Required

Reagent: Standard buffer solution of pH 4.7 and 10.

Apparatus: pH meter with glass electrode.

Procedure

The instrument is turned on and allowed to stabilise for 10–15 minutes. During this period, the electrode bulb should be kept in distilled water. After the instrument is stabilised wash the electrode bulb with distilled water, wipe dry with filter paper and place it in a standard buffer solution. The choice of buffer is dependent on the pH of the unknown solution. After calibrating the pH meter, wash the electrode with distilled water, wipe with filter paper and place it in unknown solution. After the pH measurement knob should be maintained in stand by position, wash the electrode with distilled water and place it in a beaker containing distilled water before turning off the instrument.

Estimation of Serum Electrolytes by Flame Photometry

PRINCIPLE

A dilute solution of serum or standard is sprayed as a fine mist of droplets into a non-luminous oxidising blue flame, which becomes coloured by the characteristic emission of the respective elements present in the solution. Light of a wavelength corresponding to the element being analysed is selected by a light filter or prism system and allowed to fall on a photocell that in turn is connected to a galvanometer to measure the concentration of the element. The intensity of light is proportional to the concentration of respective elements in the solution analysed and can be measured. The instrument used to measure the electrolytes in serum or plasma is called flame photometer (Fig. 35.1).

MATERIALS

Reagents

- Stock standard sodium solution 200 mEq/l: Dissolve 11.69 g pure dry NaCl/ litre of glass distilled water.
- Stock standard potassium solution 10 mEq/l: Dissolve 0.746 g pure dry KCl/ litre of glass distilled water.



Fig. 35.1: Flame photometer

c. Mixed working standards of sodium and potassium prepared as indicated in the table below:

Working Standard	Stock Na ⁺ solution (ml)	Stock K ⁺ solution (ml)	Na ⁺ meq/l	K ⁺ meq/l
I	5.5	2.0	1.1	0.02
II	6.5	4.0	1.3	0.04
III	7.5	6.0	1.5	0.06

Apparatus: Flame Photometer

Procedure

Preparation of sample: Dilute 0.1 ml serum with 9.9 ml glass distilled water and mix. The dilution is 1 in 100.

Estimation of sodium: Insert the Na filter in the instrument. Switch on the instrument. Turn on the gas supply and ignite. Then turn on the air supply. Keep glass distilled water in the cup under 'sample inlet'. Adjust the air pressure; gradually increasing until the distinct individual blue cones of the flame is obtained. Take the working standards and the diluted serum in separate cups labeled accordingly. Keep the blank (glass distilled water) in the sample inlet again and operate the zero control to set the instrument to 'zero'. Now remove blank, keep Standard III and adjust sensitivity knob until a deflection of 80–90 is obtained. Check zero with blank, and read the test and the other standards with the instrument and note the readings.

Estimation of potassium: Replace the sodium filter by the potassium filter. Adopting the same procedure, take the readings for potassium. From the meter readings for standard solutions of respective ions the concentration in the unknown can be calculated. It is also possible to calibrate the instrument for the particular range of concentration of sample using standard solutions and appropriately diluting the unknown sample to get the reading directly.

Normal level:

- Serum sodium 135–150 mEq/l
- Serum potassium: 3.5–5.5 mEq/l.

Note: As the erythrocyte content of potassium is relatively very high, serum should not be haemolysed.

Serum Na⁺ levels

Elevated in dehydration, CNS disease, hyperadrenocorticism with hyperaldosteronism, corticosteroid excess.

Decreased in adrenal insufficiency, renal insufficiency, renal tubular acidosis, trauma or burns, acute or chronic diarrhoea, intestinal obstruction/ fistula, oedema associated with cardiac/ renal disease, hyperglycemia, hyperlipidemia, hyperglobulinemia.

Serum K⁺ levels

Elevated in: Adrenal insufficiency, renal insufficiency, and use of drug like phenformin.

Decreased in: Inadequate intake or absorption, vomiting, diarrhoea, malabsorption syndrome, metabolic alkalosis, renal tubular acidosis, drugs like diuretics and Toni-Fanconi syndrome.

The term diabetes mellitus describes a metabolic disorder of multiple aetiology, characterised by chronic hyperglycemia, with disturbance of carbohydrate, fat and protein metabolism; resulting from defects in insulin secretion, insulin action or both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and in the absence of effective treatment, even death. Often symptoms are not severe or may be absent and consequently hyperglycaemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure and/ or neuropathy with risk of foot ulcers, amputation, Charcot joints and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at an increased risk of cardiovascular, peripheral vascular and cerebrovascular diseases. Several pathogenic processes are involved in the development of diabetes. These include processes that destroy the beta cells of the pancreas with consequent insulin deficiency and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin.

The requirements for diagnostic confirmation for a person presenting with severe symptoms and gross hyperglycemia differ from those for the asymptomatic person, with blood glucose values found to be just above the diagnostic cut-off value. The diagnosis of diabetes in an asymptomatic subject should never be made on the basis of a single abnormal blood glucose value. Severe hyperglycaemia detected under conditions of acute infective, traumatic, circulatory or other stress may be transitory and should not in itself be regarded as diagnostic of diabetes.

At least, one additional plasma/blood glucose test result with a value in the diabetic range, either in fasting, random, or oral glucose tolerance test (OGTT), must be obtained. If the above fails to establish diagnosis, surveillance should be maintained with periodic retesting until diagnosis becomes clear. Factors such as ethnicity, family history, age, adiposity must be considered before deciding on diagnostic or therapeutic course of action.

In children symptoms include very high blood glucose levels, marked glycosuria and ketonuria.

The OGTT is principally used for diagnosis: (a) When blood glucose levels are equivocal i.e. between the levels that establish or exclude diabetes; (b) During pregnancy; (c) Epidemiological studies.

MATERIALS REQUIRED

Reagents

- a. Glucose powder
- b. $\frac{2}{3}$ N H_2SO_4
- c. 10% Na-tungstate
- d. Benedict's qualitative and quantitative reagent
- e. Phosphomolybdic acid reagent.

Procedure

Patient Preparation

The OGTT should be conducted in the morning after at least three days of unrestricted diet (> 150 g carbohydrate daily) and usual physical activity. Recent evidence suggests that a reasonable (30–50 g) carbohydrate containing meal should be consumed on the evening before the test. The test should be preceded by an overnight fast of 8–14 h, during which only water may be drunk. Smoking is not permitted during the test. The presence of factors that influence interpretation of the result must be recorded (e.g. medications, inactivity, infection, etc.).

Glucose Load

After collection of the fasting blood sample and urine, the subject should drink 75 g anhydrous glucose or 82.5 g glucose monohydrate (or equivalent carbohydrate containing food) in 250–300 ml of water, over the course of 5 minutes.

For children, the test load should be 1.75 g of glucose per kg body weight.

Sample Collection and Processing

Blood sample and urine must be collected again 2 hours after the glucose load. It is sufficient to measure blood glucose while fasting and at 2 hours post load. Timing of the test starts from the beginning of the drink. Blood sugar is estimated as described in Chapter 21. Unless the glucose concentration can be determined immediately, the blood sample should be collected in a tube containing sodium fluoride (6 mg/ml of whole blood).

Urine is analysed as Chapter 16, item no.2; while interpreted as Chapter 19.

Epidemiological Studies

For population studies of glucose intolerance and diabetes, individuals have been classified by their blood glucose concentration, measured after an overnight fast and/or 2 hours after a 75 g oral glucose load.

Gestational Diabetes

To determine if gestational diabetes is present in pregnant women, a standard OGTT should be performed. Pregnant women who meet WHO criteria for diabetes mellitus, or IGT are classified as having gestational diabetes mellitus (GDM). After the pregnancy ends, the women should be re-classified as having either diabetes mellitus or IGT.

Glucose concentration in mmol/L (mg/dl)

		Whole blood		Plasma venous
		Venous	Capillary	
Diabetes mellitus	Fasting	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥ 126)
	or 2 hours post glucose load Or both	≥ 10 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)
Impaired glucose tolerance	Fasting	< 6.1 (< 110)	< 6.1 (< 110)	< 7.0 (< 126)
	and 2 hours post glucose load	≥ 6.7 (≥ 120)	≥ 7.8 (≥ 140)	≥ 7.8 (≥ 140)
Impaired fasting glycaemia	Fasting	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 6.1 (≥ 110) and < 7.0 (< 126)
	or 2 hours post glucose load	≥ 10 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)
	Or both	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)

Glycated haemoglobin, reflects the average glycaemia over a period of weeks.

PRINCIPLE

Chromatography is a physical method of separation of the components of a mixture by a continuous redistribution between two phases, one stationary and the other moving. A variety of attractive forces between the stationary phase, and the substance to be separated lead to the selective retardation of the latter, relative to the moving phase. Under suitable conditions the resulting different rates of migration can bring about complete separation of the substances. Chromatographic procedures can be classified according to whether mobile phase is liquid or gaseous and the stationary phase solid or liquid.

If a substance in solution is shaken with an immiscible solvent, it will distribute itself so that at equilibrium the ratio of its concentration in two phases A and B $[A]/[B]$ is a constant. The ratio is termed the partition coefficient and is characteristic of a particular substance for a given pair of solvents. The components of a mixture of substances with different partition coefficients will distribute themselves in different proportions between two such immiscible solvents.

Several factors are of importance in the separation of substance by paper chromatography. These involve partition and a combination of partition and decomposition. The predominant factor is the partition between two immiscible phases. Theoretically, the paper functions as an inert support for the aqueous solvent. Partition takes place between stationary aqueous phase and the moving solvent phase. The solute moves in the direction of solvent flow at a velocity, which is governed by the differential attraction between stationary aqueous phase and moving organic phase. Several other factors are important for determining the rate of migration, molecular weight, type of paper used, size of the sampling chamber, amount of the sample, temperature, etc. The migration rate of the solute is characterised by the term R_f .

R_f is defined as distance traveled by the solute/distance traveled by the solvent.

It is always less than 1.

Materials

- i. Whatman no.1 chromatography paper (size 30 cm \times 10 cm).
- ii. Standard amino acids.
- iii. Solvent: n-butanol: acetic acid: water:: 4:1:5.
- iv. 1% ninhydrine in acetone.

Procedure

Draw a line with pencil on Whatmann no. 1 chromatography paper along the width, 3 cm above the lengthwise edge of the chromatography chamber. Mark points on the line 2 cm apart and 2 cm away from the edges. Apply 10 μl each of the amino acid on the spots. Apply small quantities at a time on the spot and allow the spot to dry before the next application is made on the same spot. Add solvent in the chromatography chamber and allow the chamber to become saturated with solvent vapour. Transfer the paper to the solvent trough in the chromatography chamber and anchor it. Close the chamber to avoid evaporation of the solvent. Allow the solvent to run about 15 cm or 1 hour. Remove the paper, the solvent front is marked and dry the paper in air. Spray the paper with the staining solution. The paper is placed above hot air oven or heated with dryer. The spots are marked and R_f is determined.

Paper Electrophoresis

Electrophoresis is the migration of charged particles through an electrolyte when subjected to an electric field. Negatively charged ions move toward the anode and positively charged ions move towards the cathode. In alkaline solution, proteins are negatively charged and hence move to the anode.

Migration in an electric field depends upon—charge of ions, size of charged particles, pH of the medium, strength of the electric field.

The migration is opposed by the viscous drag arising from the friction between the moving molecule and the support medium. The viscosity and shape along with the size of moving particle also influence this migration. Large size and high ionic strength of the buffer will retard the movement.

Electrophoresis could be of several types depending upon the support media used.

- Inert media: Support media is like paper, cellulose acetate or agar gel.
 - Media as molecular sieve: Substances like agarose and polyacrylamide gel (PAG) with varying molecular size are used. They act as molecular sieve restricting the movement of large particles.
- Paper electrophoresis uses Whatman no. 1 filter paper as the inert support medium.

Materials Required

- Paper electrophoresis apparatus.
- Power supply device.
- Filter paper Whatman no. 1.
- Borate buffer: 0.5 M boric acid and NaOH, pH 8.6.
- Bromophenol blue dye in alcohol.
- Dilute acetic acid.
- Sodium hydroxide 0.05 N.

Procedure

- Cut Whatman no.1 filter paper into strips of the required size (35 cm × 4 cm) and mark a line with pencil about 5–6 cm from one end.
- Fill both the wells of clean dry electrophoresis apparatus with buffer to equal levels.
- Wet the cut paper strips with buffer and remove the excess buffer by pressing between the layers of dry filter paper.
- Place the paper strip(s) on the bridge of the apparatus in a manner that its two ends are dipped in the buffer in two chambers.

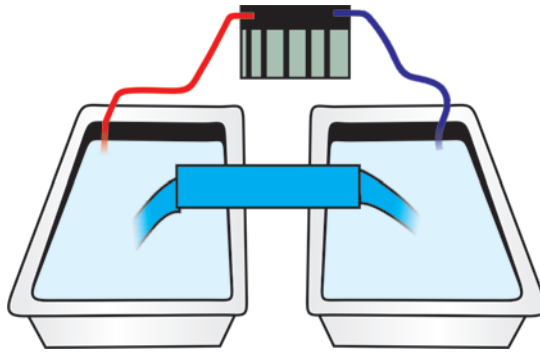


Fig. 38.1: Apparatus for electrophoresis

- e. Connect the chamber to power supply.
- f. Switch on the power supply and adjust the power supply by choosing either voltage at 3.2 V/cm length of paper or current of 1.5–2 ampere for each strip.
- g. Allow the run for 10 mins, and then switch off.
- h. Apply 5–10 μl of serum sample with a micropipette or capillary tube.
- i. Cover the lid, switch on the power supply and allow it to run for 4 hours undisturbed.
- j. Switch off the power supply and remove the strips.
- k. Dry the strips and fix the proteins on the strip.
- l. Stain the strips with bromophenol blue; remove excess stain with dilute acetic acid.
- m. Air dry the strips and note the different bands (Fig. 38.1).

Quantitation

This can be done using densitometer. It can also be done by elution i.e. cutting the each fraction and eluting the dye absorbed in 5 ml NaOH. This colour can be measured at 540 nm using NaOH solution as blank. The colour of NaOH will be proportional to dye eluted from the protein fractions.

In this experiment, serum proteins are separated on agarose gel. A few microlitres of serum is applied on an agarose gel plate and placed in electrophoretic chamber. The chamber consists of two compartments containing the buffer. Contact is made to the gel plate and buffer compartment by means of filter paper strips. A direct current is allowed to flow across the plate. After a definite period of “run”, the plate is removed. The proteins would have separated to distinct bands, all moving towards anode to different extents. The bands are visualised by staining with a dye. The intensities of the coloured bands give an idea about the nature and relative concentration of the resolved protein fractions. In normal serum five well-characterised bands namely: albumin, α -1, α -2, β , and γ -globulins are seen. Albumin moves fastest towards anode and γ -globulin has lowest mobility.

Since the electrophoretic pattern of serum proteins in certain diseases varies markedly from a normal pattern, it is of great diagnostic significance in several conditions like nephrosis, liver diseases and multiple myeloma, etc.

Direct scanning in a densitometer quantitates the bands.

Materials

- Borate buffer, pH 8.6: Containing 0.5 M boric acid and NaOH.
- Agarose: 1% agarose in borate buffer.
- Fixative: 30 ml glacial acetic acid mixed with 40 ml methanol, and volume is made up to 100 ml with distilled water.
- Destaining solution: 5% acetic acid solution.
- Staining solution: Coomassie brilliant blue in acetic acid.

Procedure

Agarose is heated in buffer till agarose is dissolved. When the prepared gel temperature comes down to about 60°–70°C, about 1.5 ml is pipetted out, and gently applies to one end of a clean slide and allowed to cover entire slide evenly.

Fill the two buffer compartments with equal volume of buffer. Keep the agarose plate in position inside the chamber. Place two filter paper (Whatman 3) strips having the same width as the agar plate on both ends of the plate and connect the two buffer compartments. Soak a thin strip of filter paper with the serum sample and place it carefully on the gel at one third of the distance from the cathode end of the plate. Close the chamber and the current is adjusted at 120V. After two hours run, turn off the current. Remove the agar plate and immerse in fixative for 15 minutes. Dry the plate and immerse in staining solution for 5 minutes. Wash with destaining solution to clear the background colour. Dry the slide and observe various bands.

Clinical Significance

In normal electrophoretogram, the proportions of various protein bands are as follows: albumin—56%; α -1 globulin—3%, α -2 globulin—13.5%, β globulin—15.5%, and γ -globulin—12%. In nephrosis, albumin level decreases and α -2 globulin level increases. In chronic liver disease albumin band is affected. In multiple myeloma an abnormal band (M band) appears between β - and γ -globulin (Figs 39.1 and 39.2).

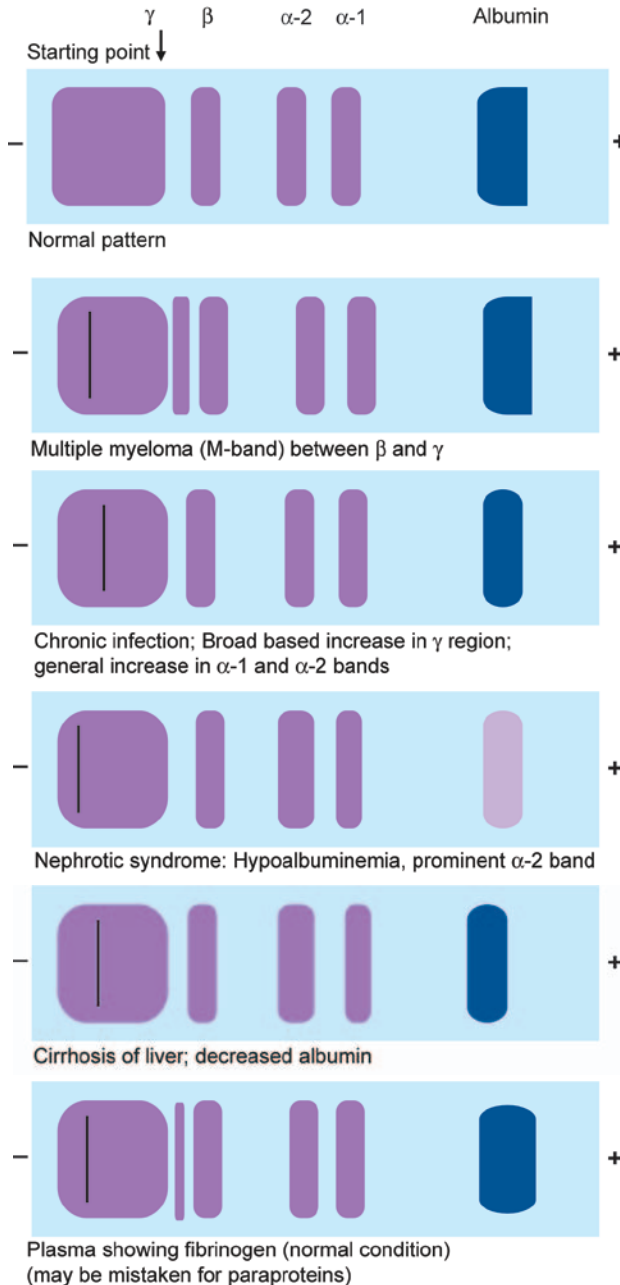


Fig. 39.1: Serum electrophoretic patterns

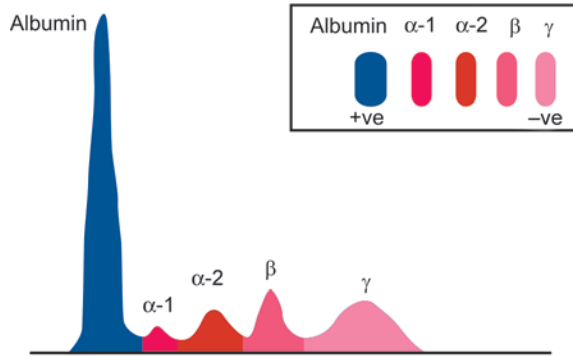


Fig. 39.2: Normal electrophoretic pattern

Gastric Function Tests

The stomach is a major organ of digestion and performs the following functions:

1. Stomach is a reservoir of ingested foodstuffs.
2. It has a great churning ability, which promotes digestion.
3. It elaborates HCl and proteases (pepsin) that are responsible for the initiation of digestive process.
4. The products obtained in the stomach (peptides, amino acids) stimulate the release of pancreatic juice and bile.

The parietal cells of gastric glands produce HCl. The pH in the gastric lumen is as low as 0.8. The protons are therefore transported against the concentration gradient by an active process. K^+ -activated ATPase is connected with the mechanism of HCl secretion. The process involves an exchange of H^+ ions for K^+ ions. This is coupled with the consumption of energy, supplied by ATP. The H^+ are continuously generated in the parietal cells from carbonic acid. The bicarbonate ion thus generated, enter the blood in exchange for Cl^- ions. The latter diffuse into the gastric lumen to form HCl. Gastrin stimulate HCl secretion.

Following a meal, there is a slight elevation in the plasma bicarbonate concentration that is linked to gastric HCl secretion. This is known as alkaline tide.

There are several tests for gastric function evaluation.

FRACTIONAL TEST MEAL

It involves the collection of stomach contents by Ryle's tube in fasting. This is followed by a gastric stimulation, giving a test meal. The stomach contents are aspirated by Ryle's tube at different time period (usually every 15 minutes for 2 hours). The samples are analysed for free and total acidity. Free acidity is due to free HCl only without stimulation. Presence of acidic salts and other organic acids, e.g. lactic acid, butyric acid and proteins bound H^+ ions, etc. gives combined acidity. Total acidity represents the sum total of free acidity and combined acidity.

Gastric juice is titrated with standard alkali N/10 NaOH using suitable indicator. Topfer's indicator (dimethyl amino benzene 0.5% in ethanol) is used for free acidity. It changes colour from red to yellow. Total acidity is usually 0–40 mmol/L.

Phenolphthalein is used for total acidity when colour changes from yellow to red (pH 8.3–10). The difference gives the combined acidity. As the free acid is titrated, addition of more alkali changes pH leading to ionisation and release of hydrogen ions from acids bound with proteins and other organic substances.

PENTAGASTRIN STIMULATION TEST

Pentagastrin is a synthetic peptide, which stimulates the gastric secretion. The stomach contents are aspirated by Ryle's tube in fasting condition. This is referred to as residual juice. The gastric juice elaborated for the next one hour is collected and pooled which represents the basal secretion. Pentagastrin (5 µg/kg body weight) is now given to stimulate gastric secretion. The gastric juice is collected at 15 minutes interval for one hour. This represents the maximum secretion. Each sample is measured for acidity.

Procedure

Take 5 ml of gastric juice in a conical flask and add a drop of phenolphthalein and titrate with N/10 NaOH to a light pink end point.

Basal acid output (BAO) refers to the acid output (millimol per hour) under the basal conditions i.e. basal secretion. Normal BAO is 1–2.5 mmol/h.

Maximal acid output (MAO) represents the acid output (millimol per hour) after the gastric stimulation by pentagastrin. Normal MAO is 20–40 mmol/h.

AUGMENTED HISTAMINE TEST MEAL

Histamine is a powerful stimulant of gastric secretion. The basal gastric secretion is collected for one hour. Histamine (0.04 mg/kg body weight) is administered subcutaneously and the gastric contents are aspirated for next one hour (at 15 minutes interval). The acid content is measured in all samples.

TUBELESS GASTRIC ANALYSIS

This involves administration of a cation exchange resin that gets quantitatively exchanged with the H⁺ ions of the gastric juice. The resin is then excreted into urine that can be estimated for an indirect measure of gastric acidity.

Clinical Significance

Increased gastric HCl secretion is found in Zollinger-Ellison syndrome (a tumor of gastrin secreting cells of pancreas), chronic duodenal ulcer, gastric cell hyperplasia, excessive histamine production, etc.

A decrease in gastric HCl is observed in gastritis, gastric carcinoma, pernicious anaemia, etc.

Liver Function Tests

Liver is the central organ of metabolism. It performs several functions.

1. Metabolic functions: It actively participates in carbohydrate, lipid, protein, vitamin, and mineral metabolism.
2. Excretory functions: Bile pigments, bile salts and cholesterol are excreted.
3. Protective functions and detoxification: Foreign compounds are eliminated by phagocytosis. Ammonia is detoxified to urea. Liver is responsible for detoxification.
4. Haematological functions: Liver is involved in the formation of blood, synthesis of plasma proteins and destruction of erythrocytes.
5. Storage functions: Glycogen, vitamin A, D and B₁₂, and iron are stored in liver.

The liver function tests (LFT) are the biochemical investigations to assess the capacity of liver to carry out any of the functions it performs. The major liver function tests may be classified as follows:

- i. Excretory functions: Bile pigment, bile salts, bromosulphthalein.
- ii. Serum enzymes: Transaminases, alkaline phosphatases, 5'-nucleotidase, γ -glutamyl transpeptidase.
- iii. Metabolic capacity: Galactose tolerance, antipyrine clearance.
- iv. Synthetic function: Serum albumin, prothrombin time.
- v. Detoxification: Hippuric acid synthesis.

ZINC SULPHATE TURBIDITY TESTS

This test is an indirect measure of γ - globulin in serum. Pipette 0.1 ml fresh serum into 6 ml ZnSO₄ solution (dissolved in barbitone buffer, pH 7.5). Mix, stand for 30 minutes, and read the turbidity in a colorimeter.

With normal serum very little turbidity is noticed (2–8 units). In chronic liver disease, turbidity increases. Persistence of high turbidity after viral hepatitis suggests the development of chronic hepatitis.

BILIRUBIN (See CHAPTER 30)

Bilirubin is the excretory end product of haeme degradation. It is conjugated in the liver to form bilirubin diglucuronide, and excreted in bile. Van den Bergh reaction is a specific reaction to identify the increase in serum bilirubin. Normal serum gives a negative Van den Bergh reaction.

Van den Bergh reagent is a mixture of equal volumes of sulphanilic acid (in dilute HCl) and sodium nitrite. Diazotized sulphanilic acid reacts with bilirubin to form a purple coloured azobilirubin.

Bilirubin as such is insoluble in water while the conjugated bilirubin is soluble. Van den Bergh reagent reacts with conjugated bilirubin and gives a purple colour immediately (within 30 seconds). This is referred to

as a direct positive Van den Bergh reaction. Addition of methanol dissolves the unconjugated bilirubin which then gives the Van den Bergh reaction and this is referred as indirect positive. The reaction is highly useful in the understanding the nature of jaundice. If the serum contains both conjugated and unconjugated bilirubin, the purple colour is produced immediately. This type of reaction is called biphasic. The response of Van den Bergh reaction can differentiate the jaundice as follows:

- Indirect positive: Haemolytic jaundice
- Direct positive: Obstructive jaundice
- Biphasic: Hepatic jaundice.

The conjugated bilirubin, being water soluble, is excreted in urine.

TRANSAMINASES (See CHAPTER 33)

The activities of two enzymes—namely serum glutamate pyruvate transaminase (ALT or alanine transaminase) and serum glutamate oxaloacetate transaminase (AST or aspartate transaminase)—are widely used to assess the liver function. ALT is a cytoplasmic enzyme while AST is found in both cytoplasm and mitochondria. Serum ALT and AST are increased in liver damage.

ALKALINE PHOSPHATES (See CHAPTER 32)

Alkaline phosphatase (ALP) is mainly derived from bone and liver. A rise in serum ALP level, associated with serum bilirubin is an indicator of biliary obstruction. ALP is also elevated in cirrhosis of liver and hepatic tumors.

γ -glutamyl Transpeptidase

This is a microsomal enzyme widely distributed in body tissue, including liver. Measurement of γ -glutamyl transpeptidase (GGT) activity provides a sensitive index to assess liver abnormality. It is highly elevated in biliary obstruction and alcoholism. Several drugs (e.g. phenytoin) induce (liver synthesis) and increase this enzyme in circulation.

5'-nucleotidase

The serum activity of 5'-nucleotidase (normal level: 2–15 U/L) is elevated in hepatobiliary disease and this parallels ALP.

SERUM ALBUMIN (See CHAPTER 26)

Albumin is solely synthesised in the liver. It has a half-life of about 20–25 days. Therefore, it is a good marker to assess chronic liver damage. Hypoalbuminemia is commonly observed in patients with severe liver damage. Functional impairment in liver is frequently associated with increased synthesis of globulins. Cirrhosis of the liver causes a reversal of albumin/globulin ratio.

Prothrombin Time

The liver synthesises all the factors concerned with blood clotting. A decrease in the concentration of plasma clotting factor is found in the impairment of liver function. This can be assessed in the laboratory by measuring prothrombin time, which is prolonged in patients with liver damage, compared to normal. The half-lives of clotting factors are relatively short (5–72 h), therefore, changes in prothrombin time occur quickly.

Hippuric Acid Synthesis

The liver is the major site for detoxification. Determination of hippuric acid synthesis is an ideal test for assessing the detoxification function of liver. Hippuric acid is produced in the liver when benzoic acid combines with glycine.

About 6 g of sodium benzoate (dissolved in 250 ml water), is orally given to the subject, after a light breakfast (usually 2 hours later) and after emptying the bladder. Urine collections are made for the next 4 hours and the amount of hippuric acid excreted is estimated. Theoretically, 6 g of sodium benzoate should yield 7.5 g of hippuric acid. In healthy persons, about 60% of sodium benzoate (equivalent to 4.5 g hippuric acid) is excreted in urine. A reduction in hippuric acid excretion (< 3 g) indicates hepatic damage.

Urine Urobilinogen

Since urobilinogen is formed in the intestine by the reduction of bilirubin. It is increased in haemolytic jaundice, low in obstructive jaundice. In hepatocellular jaundice, the value is low to normal.

Urine urobilinogen is derived from the part of the urobilinogen absorbed from the intestine, which is not re-excreted by the liver. The amount present is thus depends both on the amount of bilirubin entering the intestine and on the ability of liver to excrete the urobilinogen coming to it from intestine. The urobilinogen present in the normal sample of urine gives a positive test with Ehrlich reagent up to a dilution of 1 in 20. A positive test with higher dilution indicates increased excretion. Urobilinogen excretion is increased in haemolytic jaundice due to over production and in hepatocellular jaundice because of the reduced capacity of the damaged liver to excrete it into the bile. It is low in obstructive jaundice and practically absent if the obstruction is complete.

Biochemical changes for the differential diagnosis of three types of jaundice:

	Haemolytic jaundice	Obstructive jaundice	Hepatic jaundice
Serum bilirubin	Unconjugated ↑	Conjugated ↑	Both ↑
Van den Bergh reaction	Indirect +	Direct +	Biphasic
Serum enzymes	AST, ALT and ALP→	ALP ↑↑, ALT and AST marginal ↑	ALT and AST ↑↑, ALP marginal ↑
Bilirubin in urine	–	Excreted	Excreted
Urobilinogen in urine	Excretion ↑	→ or ↓	→ or ↓

↑ : Increase, ↓: Decrease, →: Normal

Kidney Function Tests

The kidneys are performing the following major functions:

- i. Maintenance of homoeostasis: Responsible for the regulation of water, electrolyte and acid-base balance in the body.
- ii. Excretion of metabolic waste products: The end products of protein and nucleic acid metabolism. These include urea, creatinine, creatine, uric acid, sulphate, and phosphate.
- iii. Retention of substance vital to body: Glucose, amino acid, etc.
- iv. Endocrine function: Producing hormones like erythropoietin, calcitriol and rennin.

Several tests are employed in the laboratory to assess kidney (renal) function. These include:

- a. Glomerular function tests: All the clearance tests (inulin, creatinine, urea) are included in the group.
- b. Tubular function tests: Urine concentration or dilution, urine acidification tests.
- c. Analysis of blood/serum: Estimation of blood urea, serum creatinine, protein and electrolyte are often useful.
- d. Urine examination: Routine examination of urine for volume, pH, specific gravity, osmolarity, and presence of certain abnormal constituents.

Clearance is defined as the volume of plasma that would be completely cleared of a substance per minute. In other words, clearance of a substance refers to the millilitres of plasma, which contains the amount of that substance excreted by kidney per minute. Clearance (C) expressed as ml/min, can be calculated by using the formula:

$$C = U \times \frac{V}{P}$$

Where, U = Concentration of the substance in urine

V = Volume of urine in ml excreted per minute

P = Concentration of the substance in plasma.

The maximum rate at which the plasma can be cleared of any substance is equal to the GFR. For creatinine estimation see Expt No 23.

Creatinine clearance test: In the traditional method, creatinine content of a 24 hours urine collection and the plasma concentration in this period are estimated. Instead of 24 hours urine collection, the procedure is modified to collect urine for 1 hour, after giving water.

The normal range is 120–145 ml/min.

A decrease in creatinine clearance value (< 75% normal) serves as sensitive indicator of a decreased GFR, due to renal damage.

UREA CLEARANCE TEST

Urea is the end product of protein metabolism. After being filtered, it is partially reabsorbed by the renal tubules. Hence, urea clearance is less than GFR and, further, the protein content of the diet influences it. Therefore, urea clearance is not as sensitive as creatinine clearance. It is calculated by the formula:

$$C_m = U \times \frac{V}{P}$$

Where, C_m = Maximum urea clearance

U = Urea concentration in urine

V = Volume of urine in ml excreted per minute

P = Urea concentration in plasma

The above calculation is applicable if the output of urine is more than 2 ml/ min. The normal value is around 75 ml/ min. For urea estimation *see* Chapter 22.

It is observed that the urea clearance drastically changes when the volume of urine is less than 2 ml/ min. This is known as standard urea clearance (C_s) and the normal value is around 54 ml/min. It is calculated by the modified formula:

$$C_s = U \times \frac{\sqrt{V}}{P}$$

A urea clearance value below 75% of the normal is an indicator of renal damage.

URINE CONCENTRATION TEST

A specific gravity of urine 1.020 in the early morning urine sample is considered to be normal. If the specific gravity of urine is above 1.020 for at least one of the samples collected, the tubular function is considered to be normal.

Osmolality and Specific Gravity

The osmolality of urine is variable. In normal individuals, it may range from 500 – 1200 milliosmoles/kg. The plasma osmolality is around 300 milliosmoles/kg. The normal ratio of the osmolality between urine and plasma is around 2–4. It is found that the urine (without any protein and high molecular weight substance) with an osmolality of 800 mOsm/kg has a specific gravity of 1.020.

ANALYSIS OF BLOOD (OR SERUM)

Estimation of serum creatinine and blood urea is often used to assess the overall kidney function, although these tests are less sensitive than the clearance test. Serum creatinine is a better indicator than urea in this regard.

URINE EXAMINATION

The routine urine examination is undoubtedly a guiding factor for renal function. The volume of urine excreted, its pH, specific gravity, osmolality, the concentration of abnormal constituents (such as protein, ketone bodies, glucose, and blood) may help to have some preliminary knowledge of kidney function.

See Chapter 15 and 16.

ELIMINATION OF FOREIGN SUBSTANCES: PHENOL SULPHONPHTHALEIN (PSP) TEST

The subject empties the bladder in the morning. He is given 600 ml water to drink. After 30 minutes 6 mg dye in 1 ml is injected intravenously and the subject is advised to take rest. Urine is collected at 15, 30, 60 and 120 minutes after injection. The concentration of the dye is estimated.

Normal kidney excretes at least 25% of the dye in 15 minutes and about 65% in 2 hours. Amount excreted in the first specimen is most significant. The mean normal clearance value for this dye is about 400 ml. This indicates that a large proportion of the dye is removed by tubular excretion and a small fraction by glomerular filtration. This test is valuable in assessing impairment of tubular excretory capacity.

Pancreatic Function Test

FUNCTIONS OF THE PANCREAS

1. It is an endocrine gland that synthesises hormones, i.e. glucagons, insulin, gastrin (see in Table)
2. It also serves as an exocrine gland by synthesising and conveying potent digestive enzymes in a bicarbonate fluid to facilitate the duodenal digestion of food.

Hormone	Cell of origin	Action
Glucagon	Alpha cell	Increases plasma glucose by stimulating hepatic glycogenolysis and gluconeogenesis and adipose tissue lipolysis
Pro-insulin	Beta cell	Insulin precursor
Insulin	Beta cell	Stimulates membrane transport, alters membrane bound enzymes stimulate protein synthesis, inhibits protein degradation, stimulates mRNA and DNA synthesis
Gastrin	Delta cell	Role in pancrease not understood
Somatostatin	Delta cell	Not fully understood
Pancreatic polypeptide	F cell	Not fully understood

Changes in the internal and external secretions of the pancreas may be found in pancreatic disease either separately or together. However, tests in pancreatic disease are mostly concerned with external secretions of the organ.

COMPOSITION OF PANCREATIC JUICE

Pancreatic specimen is obtained by duodenal drainage. The duodenum normally contains pancreatic juice, bile, saliva and gastric secretions with some components from duodenal mucosa. The flow of pancreatic juice is stimulated by injection of secretin, a hormone normally produced by upper intestinal mucosa in response to the presence of acid. Pancreatic juice flow begins five minutes after a meal and is at its peak in 2–3 hours and lasts for 6–8 hours.

CHARACTERISTICS OF PANCREATIC JUICE

- Volume : 500–800 ml/ day
- Sp. gravity : 1.007
- Total solids : 1.5–2.5 g%
- pH : 7–8.2

- Bicarbonate: 70–100 mEq/ L
- Chloride: 50–95 mEq/L
- Sodium: 100–150 mEq/L
- Potassium: 2–8 mEq/L

EXTERNAL SECRETION OF PANCREAS

The hormone cholecystokinin-pancreozymin (CCK-PZ) is the main stimulus for the release of digestive enzymes from the pancreatic acinar cells. Essential amino acids and long chain fatty acids are the most powerful stimulants of CCK-PZ.

Digestive enzymes of the pancreas include:

1. **α -amylase**: It randomly hydrolyses α -1,4-glycosidic linkages of starch to disaccharides.
2. **Lipase**: It partially hydrolyses neutral fats to diglycerides and monoglycerides at optimal pH 7 to 9. Emulsifying action of bile salts and bile acids is helpful for optimal enzyme action.
3. **Peptidase** (Trypsin and chymotrypsin): These are secreted in the inactive forms. Trypsinogen is initially activated by enterokinase, secreted by the small intestine but later trypsin activates both precursors.
4. Other pancreatic secretions are ribonuclease, deoxyribonuclease, cholesterol esterase and phospholipase.

TESTS IN PANCREATIC DISEASES

The pancreas can be studied directly by estimating the output of fluids, bicarbonate and enzymes obtained by duodenal intubation.

- a. Stimulation using exogenous hormones: Tubes are introduced into specific regions of the gastrointestinal tract. Although mixture of biliary and pancreatic secretions can be avoided, but contamination with salivary and gastric secretions can be minimized by using double lumen tube with one part 2.5 cm longer than the other. The shorter tube ends at pyloric antrum and other in third part of duodenum. The tube is positioned with fluoroscopic guidance. The basal collections of the endogenous duodenal contents are obtained 10 minutes apart. The patient is then given intravenously one unit of secretin per kg body weight. This will stimulate the pancreas to produce water and bicarbonate. Three samples of pancreatic secretion entering the duodenum are collected at the interval of 10 minutes. The aspirate is examined for volume, bicarbonate, bilirubin and one or more enzymes.

Normal post secretin response will be a significant increase in volume (1.5 ml/kg/30 min). A peak bicarbonate concentration will be at least 90 mmol/L.

For acute pancreatic necrosis patients, this test is hazardous, while chronic pancreatitis patients are unable to secrete juice of high bicarbonate content. This test may assist in diagnosis of pancreatic carcinoma. Cytological examination of aspirate may be helpful in the diagnosis of carcinoma. Patients with ductal obstructive lesions may exhibit elevation of serum amylase during and after the test.

- b. Pancreozymin stimulation test: Pancreozymin is given immediately after the collection of the samples for the secretin stimulation test. The enzymatic content of the stimulated pancreatic juice is used to assess pancreatic function test.
- c. Cholecystokinin stimulation test: Cholecystokinin is given along with secretin as a pancreatic function test.

DETERMINATION OF BICARBONATE

Add 5 ml of duodenal juice to 10 ml 100 mm HCl in a small beaker. Boil it to expel CO₂ and titrate with 100 mM NaOH solution using phenolphthalein as an indicator. The difference between volume of standard and alkali used in ml multiplied by 20 gives the bicarbonate concentration (mmol/L).

DETERMINATION OF BILIRUBIN (See CHAPTER 30)

Determination of Amylase

The amylase contents in duodenal juice may be 1000 times greater than serum, especially following hormonal stimulation. So considerable dilution is needed for this estimation (See Chapter 31 for the method).

Determination of Lipase

Discussed in serum lipase estimation.

Determination of Trypsin

Principle

Rate of formation of carboxyl group from N- α -benzoyl-L-arginine ethyl ester (BAEE) at pH 8.2 is measured at 405 nm.

Reagents

- Sodium hydroxide: 40 mm
- Sodium barbitone: 10 g/L.

BAEE substrate

To 10 ml sodium barbitone add 90 ml water and 0.5 g BAEE and adjust pH 9.0

Acetate buffer (50 mm, pH 5.8): Dissolve 6.8 g $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in water and add 0.5 g CaCl_2 and make this to one litre. Adjust pH 5.8 with strong acetic acid.

Procedure

Mix 1 ml duodenal content with 9 ml acetate buffer using magnetic stirrer at 25°C. Put 5 ml BAEE substrate to a new vial and connect to a pH meter. Mix 1 ml of above diluted duodenal juice and check pH of the solution. When pH reaches to 8, add 100 μl NAOH and start a stop watch. Note the time to reach the pH to 8.4.

Calculation

As 4 μmol of alkali is used to neutralize, so enzyme activity = 4 $\mu\text{mol}/$ time (in min).

Enzyme concentration = Activity \times dilution \times 1000.

In acute pancreatitis, bicarbonate and enzyme outputs are depressed

INDIRECT STIMULATION OF PANCREAS

Duodenal contents are studied after an oral test meal. The contents are removed using a single lumen tube. The test liberates secretin and CCK-PZ from the duodenal mucosa, and pancreatic stimulation results. Lundh used a standard meal to carry out the test and analysed trypsin from the sample.

Lundh Test

After overnight fast, duodenal tube is passed through the nose. In $\frac{1}{2}$ to 1 hour, it should reach to the third portion of the duodenum and the tip is then adjusted fluoroscopically. Drainage the content by syphonage, after gentle

suction at the start, into a cylinder kept in iced water at 8 cm below patient level. The resting juice is removed and the meal (8 g corn oil, 15 g casein, 40 g glucose in hot water to make total 300 ml) is then taken within 15 minutes. The tube is allowed to drain for 2 hours, volume is measured and hydrogen ion content is determined.

Secretion of hydrogen ion for normal subjects is 12–20 mmol/min/ml. In pancreatic insufficiency, secretion is less than 10 mmol/min/ml. The test has a merit of greater simplicity.

Indirect Study Methods

Tests based on determination of enzymes in serum and urine—such as amylase and lipase.

Serum Lipase

Lipase, an esterase is acting on ester linkage in triglycerides. Olive oil (50% emulsion) is incubated with serum overnight, and liberated fatty acid is titrated with 0.05 N NaOH using phenolphthalein as an indicator. Normal value is up to 1.5 U.

The method takes a long time. A rapid specific turbidimetric method is available. In acute pancreatitis, enzyme lipase can be detected in 90% cases. In chronic pancreatitis, serum lipase estimation is of relatively little importance. In carcinoma of pancreas serum lipase is elevated.

Methaemalbumin

Methaemalbumin in serum and ascitic fluid of patient is formed by the action of pancreatic digestive enzymes on haemoglobin.

Tests Based on Impaired Digestion

Decreased excretion of faecal enzyme especially chymotrypsin indicates impaired digestion.

Test Based on Disordered Carbohydrate Metabolism

Glucose tolerance test is done to identify whether pancreatic lesion is involved.

Laboratory Tests in Acute Pancreatitis

- Transient hyperglycemia may occur.
- In alcohol related pancreatitis, serum bilirubin level may elevate.
- Serum levels of trypsin, lecithinase and deoxyribonuclease are elevated.
- Low serum calcium level indicates to serious form of pancreatitis.
- Turbidity of serum indicates pancreatitis.
- Leucocytosis in acute pancreatitis.
- Haemoconcentration, so elevated haemoglobin.
- Some pancreatic disorders are associated with hyperlipidemia and hypercalcaemia.
- Some familiar types are associated with aminoacidurias.
- In fibrotic diseases affecting pancreas, high concentrations of sodium and chloride ions in the sweat is demonstrated.

Visual Procedures

These include radiographic methods such as ultrasonic scanning, computed tomography and endoscopic retrograde cholangiopancreatography (ERCP).

Noninvasive Test

The patient is fed orally with a substance which is specifically cleaved by the pancreatic enzyme action. PABA excretion index (PEI) is a simple test wherein a synthetic dipeptide N-benzoyl-L-tyrosyl-p-aminobenzoic acid (BT PABA or bentiromidel) is administered with light breakfast. Normally pancreatic chymotrypsin splits the peptide bond between trypsin and PABA residues. PABA is absorbed, metabolised and excreted in urine. The amount of PABA appearing in urine is estimated. Urine is collected after 6 hours and PABA excretion is measured. Excretion of less than 40% dose is pathological.

The patients should avoid sulphonyl urea, sulphonamide, laxative diuretics 24 hours prior to the test.

CSF (Cerebrospinal Fluid)

Domenico Cotugno described CSF in 1774. CSF is found within the subarachnoid space and ventricles of the brain, as well as around the spinal cord. The fluid originates in the choroids plexus and returns to the blood in the vessels of the lumbar region. The total volume of fluid is about 125 ml. It is a transudate or ultrafiltrate of plasma. CSF has the chloride concentration higher than in the plasma, while the P is much lower. Because the concentrations of non-diffusible anions like proteins are lower in CSF than in the plasma, as compensation, the chloride ions are increased.

The amount of Ca^{2+} present in the fluid is only about half that found in serum (4.1–5.9 mg/dl), while Na^+ is considerably higher and the K^+ a little lower than in serum. The bicarbonate content of the fluid is about the same as for plasma. The pH values of the fluid and plasma are about the same.

BIOCHEMICAL ANALYSIS

The protein concentration is usually 10–30 mg/dl, out of which about 20 mg/dl is albumin, and globulin is about 5–10 mg/dl. In all bacterial infections of the meninges, the protein concentration is increased. But in such cases, the neutrophil cell count is also increased. In viral infections, the protein concentration is not significantly increased, but mononuclear cells are abundant. In brain tumors, albumin level is raised, but cell count is normal; this is called albumino-cytological dissociation.

Normal CSF electrophoresis shows the following pattern: 60% albumin, 8% gamma-globulins and 32% other globulins. The electrophoretic pattern is abnormal when IgG synthesis in brain is increased. Oligoclonal bands are found in such conditions. In multiple sclerosis, the characteristic finding is an increase in globulin levels, especially IgG fraction. Serum protein concentration is also measured and the IgG index is calculated as:

$$\text{IgG index} = \frac{[\text{CSF IgG} \times \text{serum albumin}]}{[\text{Serum IgG} \times \text{CSC albumin}]}$$

In multiple sclerosis, the index is increased, showing an absolute increase in IgG level. The cause is believed to be the increased synthesis of IgG in CNS.

The glucose level in CSF (normal 50–70 mg/dl) is lower than the plasma level. Hence, estimation of plasma glucose along with CSF glucose is always done to avoid misinterpretation due to a change in the plasma glucose.

	Colour and appearance	Cell count	Protein	Sugar	Coagulation
Normal	Clear and colourless	0–4 x 10 ⁶ /L	10–30 mg%	50–70 mg%	Not seen
Bacterial (purulent) meningitis	Opalescent	↑↑ Polymorphs	↑↑	↓↓	May clot on standing
Tuberculous meningitis	May opalescent	Lymphocytes and mononuclear cells	↑	↓	Cobweb type coagulation
Viral infection	Clear and colourless	↑	↑	Normal	Nil
Brain tumour	Clear and colourless	Within normal range	↑	↓	Solidifies
Subarachnoid haemorrhage	Blood stained in fresh haemorrhage	RBCs and WBCs	↑	Not significant	Nil

Physical characteristics		Chemical composition	
Appearance	Clear, colourless, no coagulum, no sediments	Chloride	120–130 mEq/L
pH	7.4–7.6	Calcium	5.5–6 mg%
Sp. gr	1.006–1.007	Pi	1.5–2.1 mg%
		Urea	20–40 mg%

In a suspension of particles, particle sedimentation rate depends on the nature of the particles, nature of the medium in which the particles are suspended and the force applied to the particles. One important factor affecting the sedimentation of particles is viscosity. The rate at which a macromolecule sediments is characterised by its sedimentation coefficient. This has been defined by Svedberg and Pederson as the sedimentation velocity per unit of centrifugal field, commonly referred to as its sedimentation coefficient, S .

The rate of sedimentation is dependent on the applied centrifugal field (G) being directed radially outwards, determined by the square of the angular velocity of the rotor (ω , in radians s^{-1}) and the radial distance (r , in cms) of the particle from the axis of rotation. According to the equation, $G = \omega^2 r$

Since one revolution of the rotor is equal to 2π radians, its angular velocity, in radians s^{-1} , can be expressed in terms of revolutions per minute (revmin^{-1}).

$$\omega = \frac{2\pi \text{ revmin}^{-1}}{60}$$

The centrifugal field (G) is then:

$$G = \frac{4\pi^2 (\text{revmin}^{-1})^2 \cdot r}{3600}$$

It is generally expressed as a multiple of the earth's gravitational field ($g = 981/\text{cm}^2$), and is referred to as the relative centrifugal field (RCF)

$$\text{RCF} = \frac{4\pi^2 (\text{revmin}^{-1})^2 \cdot r}{3600 \times 981}$$

$$\text{RCF} = (1.118 \times 10^{-5}) \cdot 4\pi^2 (\text{revmin}^{-1})^2 \cdot r$$

TYPES OF CENTRIFUGE

Low-speed Centrifuge

These machines are used routinely for the initial processing of biological samples, have maximum speed 4000 to 6000 revmin^{-1} and have maximum relative centrifugal fields of 3000 to 7000 g . Applications include rapid sedimentation of blood samples, and of synaptosomes.

High-speed Refrigerated Centrifuge

Maximum speed $25,000 \text{ revmin}^{-1}$ and relative centrifugal field of about $60,000 \text{ g}$. The rotor can be of fixed angle or swinging bucket type. This type of centrifuge is used to collect microorganisms, cellular debris, large cellular organelles and proteins precipitated by ammonium sulphate.

Ultracentrifuge

They are subdivided into: analytical and preparative ultracentrifuge.

Preparative ultracentrifuge can spin rotors to a maximum speed of $80,000 \text{ rpm}$ and can produce a relative centrifugal field of about $6,00,000 \text{ g}$. The rotor chamber is refrigerated, sealed and evacuated to minimize excessive rotor temperature. Centrifuge tubes must be accurately balanced within 0.1 g of each other. Applications include study of macromolecule/ligand binding kinetics, steroid hormone receptor assays, separation of the major lipoprotein fractions from plasma, and deproteinization of physiological fluids for amino acid analysis.

Analytical ultracentrifuge can spin rotors to a maximum speed of $70,000 \text{ rpm}$ and can produce a relative centrifugal field of about $5,00,000 \text{ g}$, and consist of motor. The rotor contained in a protective armored chamber that is refrigerated and evacuated. An optical system enable observation of sedimenting material during centrifugation to determine concentration distribution in the sample at any time during centrifugation.

The optical system measures the difference in refractive index between the reference solvent and the solution by the displacement of interference fringes caused by slits placed behind the two liquid columns.

TYPES OF ROTOR

Preparative centrifuge rotors are four main types: swing-out (swing bucket), fixed angle, vertical and zonal.

Swing-Out Rotors

In these rotors, the sample solutions in tubes are in individual buckets, which move out perpendicular to the axis of rotation, as the rotor rotates. The centrifugal force is exerted along the axis of the tube in these rotors. Since the centrifugal force is axial, some particles are sedimented against the wall of the tubes.

Fixed Angle Rotors

In these rotors, the tubes are at fixed angle, varying from 14° to 20° . Rotors with shallow angles are more efficient in pelleting because the sedimentation path length is shorter. Moreover, reorientation of the solution in the tubes enhances the loading capacity of isopycnic gradients.

Vertical Rotors

The tubes are held in a vertical position. The diameter of the tube and their design enables them to generate very high centrifugal forces. Vertical rotors are not suitable for pelleting, but can be used for isopycnic centrifugation.

Zonal Rotors

Zonal rotors are of two types: batch type and continuous flow. Continuous flow type rotors are less frequently used.

Zonal rotors can be used for separation of human blood cells, fractionation of membranes from a rat liver, nuclear pellet, fractionation of tissue cultures cell, post-nuclear supernatant, and harvesting of virus from tissue culture fluid.

Various types of rotor, their characteristics and applications

Types of rotor	Types of separation		
	Pelleting	Rate-zonal	Isopycnic
Fixed angle	Excellent	Poor	Good
Vertical	Poor	Good	Excellent
Swing-out	Inefficient	Good	Adequate
Zonal	Poor	Excellent	Adequate

TYPES OF CENTRIFUGAL SEPARATION

Differential Pelleting

Differential centrifugation separates particles according to size and density. However, the centrifugal force to pellet the larger particles from the top of the solution is also often sufficient to pellet the smaller particles near the bottom of the tube. Hence, a pure preparation of the smallest particles cannot be obtained in a single step.

Rate-zonal Centrifugation

The sample is layered as a narrow zone on the top of a density gradient and thereby, minimizes the convection currents in the liquid column during centrifugation. This is ideal for the particles of defined size (e.g. proteins, ribosomes and RNA).

However, particles of the same type are often heterogeneous. In such cases, particles can be separated by some other means, such as density.

Isopycnic Centrifugation

Particles are separated on the basis of density. Prolonged centrifugation does not affect the separation as long as the density gradient remains stable.

Appendices

1

Appendix

Safety in the Clinical Biochemistry Laboratory

A worker in a clinical biochemistry laboratory is exposed to certain health risks. These risks arise due to mechanical, chemical and microbial hazards.

MECHANICAL AND ELECTRICAL HAZARDS

The increasing use of electrically operated instruments in laboratories, such as photometers, centrifuges, electrophoresis apparatus, water baths, hot plates, etc. requires trained personnel for installation and maintenance. Lid of electrophoresis equipment should not be open until it has been disconnected from the power supply. Unpleasant sensations are felt at as low a current as 1 mA AC or 5 mA DC. When 100 mA AC or DC current passes through the body, the victim's heart beat stops.

Power points should be located at places where the equipments are being used. It should be separate for each equipment, be visible and easily approachable. All instruments must be switched off from the wall socket after use. No inflammable liquid or gas should be kept near the electrical appliances.

There should be no loose connection inside the flame photometer. The improper use or poorly maintained centrifuges may be hazardous. Corrosive liquids should be centrifuged in sealed containers to avoid spillage. Liquids should not be poured into centrifuge tubes, which are kept inside the machine.

Special glasses should be wearing to protect eyes, while visualizing chromatograms under UV light that may cause corneal and conjunctival damage to eyes.

Injuries like cut by broken glass in the laboratory are prone to infection.

CHEMICAL HAZARDS

Contact with chemicals can injure skin, mouth and stomach. Toxic fumes from acids, volatile solvents and carcinogens may cause injury to the respiratory tract. Strong acids (sulphuric, hydrochloric, nitric, trichloric), alkalis (sodium and potassium hydroxides) and phenols are corrosive liquids that can cause severe chemical burns. Caustic liquids or liquids emitting harmful vapors should never be sucked by mouth. Pipetting should be done by autopipettes and dispensers.

Volatile solvents like carbon tetrachloride are very toxic to the liver, while benzene causes bone cancer. Toxic chemicals should be sparingly used and that also in a well-ventilated room or in a fume cupboard.

Carcinogenic agents like benzidine, o-toluidine, o-dianisidine, aromatic amines, azo dyes and N-nitroso compounds should be avoided as far as possible.

Hands should be washed with soap and water after working with chemicals. Glasswares contaminated with chemicals should be thoroughly washed.

EXPLOSIVE CHEMICALS

Organic solvents, such as ether, undergo spontaneous oxidation in clear, colorless bottles forming peroxides. This may cause explosion. Distillation of flammable solvents on naked flame must be avoided, and efficient condensation of vapors should be taken care. Concentrated nitric acid mopped with paper or cotton often ignites due to auto-oxidation. Reducing agent like metallic zinc powder may ignite spontaneously when drying on filter paper.

A laboratory should possess fire-extinguisher, sand and buckets full of water at convenient places. Fire-extinguishers should be periodically inspected for their operational capacity. Water should never be splashed over electrical lines as this may cause electrical shocks.

POISONS

Cyanides and barbiturates should be kept under lock and key.

INFECTION HAZARD

Laboratory personnel come directly in contact with blood, stool and urine of patients which carry disease organisms. The infecting organism can enter body through a cut in skin or through the oral route. Hands or fingers if accidentally injured by needle or broken glass, clean the wound and remove the broken glass piece. Use antiseptic lotion and apply a sterile dressing, before referring to a doctor.

Any spillage of infectious material on benches and racks should be cleaned and disinfected. Wear gloves during work and wash them thoroughly with soap and water; disinfect with disinfectant or autoclave.

If a pathological sample has to be transported from the place of collection to the laboratory, precaution should be taken to ensure that the containers reach the laboratory in an upright position.

DISINFECTANTS

Formalin, phenol, hypochlorite and glutaraldehyde are commonly used disinfectants.

Formalin

Formalin, 40% solution (W/V) of formaldehyde is used along with added methanol as stabilizer. 5% formalin (i.e. 2% formaldehyde) can be used for disinfecting contaminated surfaces and discarded containers. However, its vapor causes irritation to eyes.

Phenolic Solution

Lysol and xylenol mixed with water is used to disinfect instruments and surfaces.

Hypochlorite Solution

A 10% solution of hypochlorite is used as disinfectant by liberating chlorine. It is corrosive to metals. A more dilute solution (1%) can be used for instruments which are not soiled with protein material.

Glutaraldehyde solution

A 20% glutaraldehyde solution is effective against virus. It is non-corrosive to metals and less irritant to eyes.

PRECAUTIONS

1. Bottles containing chemicals and reagents should be clearly labeled and the hazard noted.
2. Do not carry large bottle by neck, but hold the bottles with both hands.
3. Keep bottle in use on shelves within eye level.
4. Take care while opening bottles or pouring from bottles containing corrosive chemicals including strong acids or alkalies.
5. Ground glass stoppers should not be used in acid or alkali containing bottles as they may get stuck.
6. Use small measuring cylinder for acid or alkali measurement. For more accurate precision, use pipette plugged with non-absorbent cotton wool or rubber tube.
7. Toxic chemicals including cyanide and barbiturate should be kept locked in cupboard and mouth pipetting is forbidden.
8. Organic solvents may have toxic properties. Benzene is toxic to bone marrow, while carbon tetrachloride or other halogenated hydrocarbons are toxic to liver. Therefore, exposure should be controlled to minimum, and fume cupboard should be used.
9. Carcinogenic chemicals such as aromatic amines (benzidine or orthotoluidine) should be kept in well closed bottles, labeled "carcinogenic" and avoid any contact with skin.
10. Rubber or plastic gloves should be used while handling carcinogens, which must be washed afterwards under cold running water.
11. In case of accidental skin contact, wash in cold running water for several minutes.
12. Work with oxidizing agent such as perchloric acid should be carried out in fume cupboard as it may explode.
13. Picric acid should be stored under water in a container closed by a cork or rubber stopper as it may explode on percussion when dry.
14. Ether must be kept in dark bottle, as it form peroxide when exposed to sunlight in clear bottle and may cause explosion in sufficient concentration.
15. While distilling a solvent, the container must never be heated over naked flame.
16. Flammable gas stored in cylinder should be kept outside of the laboratory when not in use.

FIRST AID MEASURES

In spite of best precautions, accidents do occur in the laboratory. Each laboratory, therefore, should have First Aid box with the following items:

1. Sterilized gauze and cotton
2. 2" width bandage; crepe bandage
3. Adhesive plaster
4. Sterilized eye pads
5. 500 ml washed bottle for eye washing
6. One bottle milk of magnesia
7. One bottle 1% acetic acid
8. Where there is risk of cyanide poisoning
 - a. Ferrous sulphate solution (158 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 3 g citric acid per litre distilled water
 - b. 6% Na_2CO_3
9. Adrenaline injection (1 in 1000)
10. Aminophylline
11. Dexona
12. Disposable syringe and needle.

Initial measures can be taken before sending to medical practitioner, in following accidents:

Whenever any irritant liquid splashes on eye, wash eye with plenty of cold water

If corrosive liquid is accidentally sucked, wash mouth with plenty of water. Swallowing is rare, but if strong acid is sucked, allow one or two table spoon of milk of magnesia to drink. For cyanide poisoning, a mixture of ferrous sulphate and sodium carbonate should be given.

Profuse bleeding can be stopped by applying pressure at the bleeding point.

2

Appendix

Case Reports

1. Laboratory analysis of a patient shows:

- Blood urea: 80 mg/dl
- Urinary urea: 2400 mg/dl
- Rate of urine flow: 1 ml/min.

Calculate urea clearance of the patient.

Hint: The rate of urine flow of the patient is less than 2 ml/min, hence, the modified urea clearance is calculated as $\frac{U \times \sqrt{V}}{P}$

$$\begin{aligned}\text{So, urea clearance} &= \frac{2400 \text{ ml/min}}{80} \\ &= 30 \text{ ml/min}\end{aligned}$$

Normal clearance in such case is 54 ml/min, hence urea clearance of the patient is 55.5%. Reduced urea clearance may be due to renal damage.

2. A patient has the following biochemical parameters

- Blood urea: 100 mg/dl
- Urinary urea: 2400 mg/dl
- Rate of urine flow: 4 ml/min.

Calculate urea clearance of the patient.

Hint: The urea clearance is calculated as $\frac{U \times V}{P}$

$$\begin{aligned}\text{So, urea clearance} &= \frac{2400 \times 4 \text{ ml/min}}{100} \\ &= 96 \text{ ml/min}\end{aligned}$$

Normal clearance in such case is around 75 ml/min. It is normal case.

3. One patient has the following biochemical parameters

- Serum creatinine: 2.5 mg/dl
- Urinary creatinine: 100 mg/dl
- Rate of urine flow: 2 ml/min.

Calculate urea clearance of the patient.

Hint: Creatinine clearance is calculated as $\frac{U \times V}{P}$

$$\begin{aligned}\text{So, urea clearance} &= \frac{100 \times 2 \text{ ml/min}}{2.5} \\ &= 80 \text{ ml/min}\end{aligned}$$

Normal creatinine clearance is 120-145 ml/min. A decreased creatinine clearance is a sensitive indicator of reduced glomerular filtration.

4. A patient has the following laboratory report:

- Blood sugar (Fasting): 180 mg/dl
- Urine: Rothera's test positive
- Serum creatinine: 2 mg/dl.

Discuss diagnosis.

Hint: A positive Rothera's test in urine indicates the presence of ketone bodies. Ketosis occurs either due to severe diabetes mellitus or starvation. Fasting blood sugar indicates diabetes mellitus. A chronic complication of diabetes mellitus is associated with nephropathy, which is indicated by the elevated serum creatinine level. The patient is suffering from Diabetic ketoacidosis.

5. A young person attended OPD with severe abdominal pain, looks pale, sweaty, sick and has shallow respiration and painful deep breathing. Laboratory investigations revealed:

- Urine: Reducing sugar +
- Blood sugar (Random): 181 mg/dl
- Serum calcium: 7 mg/dl
- Serum amylase: 210 Somogyi units/dl
- SGOT: 30 IU/L
- SGPT: 30 IU/L

Diagnose the case.

Hint: Hyperglycemia, low serum calcium, elevated amylase activity (Normal: 80–180 Somogyi Unit/dl), and upper side of normal transaminase level indicates a case of acute pancreatitis.

6. A 10-year-old girl with numerous xanthomas showed the following biochemical parameters:

- Blood glucose: 82 mg/dl
- Serum cholesterol: 272 mg/dl
- Serum triglyceride: 598 mg/dl
- Chylomicrons: ++++
- Apolipoprotein C-II: ↓↓

Diagnose the case.

Hint: The deposition of lipids in subcutaneous tissue leads to xanthomas. Apo C-II activates extrahepatic lipoprotein lipase in vessel walls; clearance of triglyceride from chylomicrons and VLDL. Therefore, reduced apo C-II activity associated with elevated chylomicrons, mild elevation of cholesterol level but increased triglyceride level due to lipoprotein lipase deficiency indicated Type I hyperlipoproteinemia.

7. One Undergraduate student had the following biochemical parameters:

- Serum bilirubin (conjugated): 0.5 mg/dl
- Serum bilirubin (unconjugated): 9.5 mg/dl
- Serum ALP: 8 KAU; SGOT: 30 IU/L; SGPT: 26 IU/L
- Urine, Bile salts: –ve; Bile pigments: –ve; Urobilinogen: +++
- Feces stercobilinogen: +++

Diagnose the case.

Hint: Unconjugated bilirubin level elevates in hemolytic jaundice without change in liver marker enzymes. Urobilinogen excretion is increased in this case.

8. One Postgraduate student had the following biochemical parameters:

- Serum bilirubin (conjugated): 5.5 mg/dl
- Serum bilirubin (unconjugated): 4.5 mg/dl
- Serum ALP: 20 KAU; SGOT: 230 IU/L; SGPT: 260 IU/L
- Urine—Bile salts: ++; Bile pigments: +; Urobilinogen: +
- Feces stercobilinogen: +

Diagnose the case.

Hint: There is elevation of both unconjugated and conjugated bilirubin level, along with transaminase activities. Bile salts, bile pigments and urobilinogen also excreted with marginal elevation of alkaline phosphatase activity. It is a case of hepatic jaundice.

9. Laboratory investigation of one young person showed the following biochemical parameters:

- Serum bilirubin (conjugated): 8.5 mg/dl
- Serum bilirubin (unconjugated): 1.5 mg/dl
- ALP: 45 KAU; SGOT: 70 IU/L; SGPT: 90 IU/L
- Urine—Bile salts: ++; Bile pigments: +; Urobilinogen: –ve
- Feces stercobilinogen: –ve

Diagnose the case.

Hint: Elevation of conjugated bilirubin level along with highly elevated ALP activity indicated Obstructive jaundice.

10. Laboratory investigation of a patient showed the following biochemical parameters:

- Blood urea: 140 mg/dl
- Serum creatinine: 5.6 mg/dl
- $[\text{Na}^+]$: 126 mmol/L
- $[\text{K}^+]$: 5.8 mmol/L
- $[\text{Ca}^{+2}]$: 6 mg/dl
- Hb%: 7 g%

Diagnose the case.

Hint: Elevated blood urea and serum creatinine indicates renal failure. Anemia, as indicated by low hemoglobin concentration is a factor for chronic renal failure. Failure of the kidneys to synthesise rennin, failure of the adrenal cortex to secrete aldosterone, and renal tubular resistance to aldosterone are the most common causes of hyperkalemia. Hyperkalemia occurs with $[\text{Na}^+]$ depletion because of diminished Na^+ reabsorption. Chronic renal failure results in hypocalcemia because of hypoproteinemia, hyperphosphatemia, low serum 1, 25 (OH)₂D, or skeletal resistance to PTH.

Therefore, it is a case of chronic renal failure.

11. Laboratory investigation of a middle aged person showed the following biochemical parameters:

- Serum bilirubin: 4 mg/dl
- Serum bilirubin (unconjugated): 1.5 mg/dl
- SGOT: 130 IU/L
- SGPT: 70 IU/L
- GGT 200 U/L (normal up to 40 U/L)

Diagnose the case.

Hint: Increased serum bilirubin level and transaminase activities indicate liver disease. GGT is a marker of alcoholism (recent). Alcoholic liver disease is further confirmed from $\text{SGOT} > \text{SGPT}$.

12. Laboratory investigation of a patient revealed:

- Serum bilirubin: 4 mg/dl
- Serum bilirubin (unconjugated): 1.5 mg/dl
- SGOT: 130 IU/L
- SGPT: 110 IU/L
- Serum HBSAg (Hepatitis B surface antigen): Positive

Diagnose the case.

Hint: Viral hepatitis.

13. One Executive person suddenly felt chest pain with severe sweating. Laboratory analysis showed:

- CPK-MB: 210 U/L (normal <24 U/L)
- LDH: 1540 U/L (normal 225–480 U/L)
- SGOT: 120 IU/L
- Troponin I: Strongly positive

Diagnose the case.

Hint: CPK-MB, LDH and SGOT are marker enzymes, whereas troponin I is a no-enzyme marker of myocardial infarction. After infarction, CPK-MB elevates first, followed by SGOT, which returns to normal level within 4 to 5 days, and then LDH. Troponin I is released to the blood within 4 hours after the onset of cardiac symptoms, peaks at 12–16 hours, and remain elevated for 5 to 9 days. It is a case of acute myocardial infarction.

14. One lady with hyperventilation having following laboratory findings:

- Blood pH: 7.5
- pCO₂: ↓ in blood
- Blood bicarbonate: within normal range

What is diagnosis?

Hint: Increase blood pH (normal: 7.35–7.45) indicated alkalosis and decreased pCO₂ with normal blood bicarbonate identifies as Respiratory alkalosis.

15. One patient with chronic renal failure showing following laboratory findings:

- Blood pH: <7.3
- Low blood bicarbonate
- Increase anion gap

What is diagnosis?

Hint: Low blood pH indicates acidosis, and low blood bicarbonate with increased anion gap is due to Metabolic acidosis.

16. Interpret the GTT result with 75 g oral glucose:

- Fasting glucose: 84 mg%
- 30 min: 96 mg%
- 1 h: 112 mg%
- 1 h 30 min: 120 mg%
- 2 h: 116 mg%

What is diagnosis?

Hint: Normal GTT curve.

17. A 30-year-old woman during her second pregnancy had a glucose tolerance test and the results are: Fasting glucose level: 125 mg/dl; 1 h glucose level: 210 mg/dl; 2 h value: 170 mg/dl.

- Comment on GTT results.

- What will be the result for Benedict's test?
- How will you rule out lactosuria in this case?

Hint: Gestational diabetes mellitus.

18. A comatose patient with tremors admitted in the hospital. His blood glucose level was 300 mg/dl. His urine for Rothera's and Benedict's tests was positive. What is the diagnosis?

Hint: Diabetic ketoacidosis.

19. An apparently healthy man has fasting blood sugar 80 mg/dl. After heavy breakfast of one-and-half hours, his blood sugar was 140 mg/dl and urine sample at that time was positive for Benedict's test. What is the diagnosis?

Hint: Renal glucosuria.

20. A boy was brought to the hospital with the complaint of mental retardation. Blood chemistry revealed that serum phenylalanine was abnormally high. Phenylpyruvate, phenylacetate and phenyllactate was present in appreciable amounts in urine. What is the probable diagnosis?

Hint: Phenylketonuria

21. A heavy smoker Executive person, weighing 90 kg felt crushing chest pain, which radiated to the left arm. He was sweating profusely. The ECG at the time of admission was normal limits. By 3 h, the ST segment elevated; by the 8th h, Q waves started to appear, and T wave inversion was noticeable by 10th h. The serum enzyme levels (in mU/ml) given below. What is the diagnosis?

Time	LDH	CK	CK2	AST	ALT
3 hours	140	10	0	30	20
6 hours	160	190	50	45	20
12 hours	280	330	70	60	30
24 hours	980	1300	220	100	40
2 days	1100	640	40	120	50
3 days	1500	150	5	80	30
5 days	860	30	0	60	20
Reference	< 200	< 50	0	< 40	< 40

Hint: Acute myocardial infarction.

22. A middle aged woman admitted with recurrent pain in the abdomen and jaundice. The pain often aggravated after intake of fatty food. The patient was also complaining of itching. A routine urine examination showed the presence of bile pigments and bile salts, but urobilinogen was absent.

- What is the most likely cause?
- Explain the findings in urine.

Hint: Obstructive jaundice due to cholelithiasis.

23. One executive entertained a party in which much food and alcohol had been consumed. In the next early morning, he woke up with excruciating pain in ankle. His ankle joint was swollen, and red, felt hot to touch, and was very tender and stiff. The laboratory data showed: blood glucose—130 mg/dl, blood urea—38 mg/dl, Serum creatinine—0.9 mg/dl, Serum uric acid—9.6 mg/dl. What is the most probable diagnosis?

Hint: Gout

24. A 50-year-old lady complains of hoarseness of voice and feeling of tiredness. Despite of loss of appetite she has started putting on weight and feeling comfortable in warm weather. She was referred to the Endocrinologist. What is the most probable cause?

Hint: Hypothyroidism.

25. A 10-year-old boy with certain complications showed following laboratory results. Blood urea: 75 mg/dl; serum creatinine: 3.2 mg/dl; serum sodium: 125 mmol/L; serum potassium: 5.2 mmol/L; urine protein: 4 g/L. Give probable diagnosis.

Hint: Nephrotic syndrome leading to renal failure.

26. Serum sample collected from a 10-year-old boy with stunted growth gave the following results. Serum calcium: 8.2 mg/dl; serum phosphorous: 2.8 mg/dl; serum alkaline phosphatase: 720 U/L. What is the possible cause?

Hint: Vitamin D deficiency.

27. A bedridden patient showed the following clinical laboratory data. Serum bilirubin: 8 mg%; direct bilirubin: 0.2 mg%; ALP: 60 U/L; AST: 30 U/L; ALT: 26 U/L. Urine bile pigments and bile salts are negative. Give the most probable diagnosis.

Hint: Pre-hepatic jaundice.

28. A 70-year-old woman showed the following laboratory results. Serum sodium: 124 mmol/L; serum potassium: 3.6 mmol/L; serum bicarbonate: 12 mmol/L; blood urea: 136 mg%; serum creatinine: 3.4 mg%; serum calcium: 6.8 mg%; serum phosphate: 7.6 mg%; serum albumin: 3.1 g%; ALP: 90 U/L.
A. What is the most probable diagnosis?
B. Interpret the calcium and phosphate level.

Hint: Renal failure with multiple complications.

29. A 25-year-old woman, with pregnancy was admitted to the hospital with acute abdomen pain of 4 h duration. The pain was of sudden onset. It radiated to the back. Patient was febrile, but not jaundiced. Her plasma electrolyte values were normal. Plasma amylase was 3110 U/L, AST and ALP were mildly elevated. What is the provisional diagnosis?

Hint: Acute pancreatitis.

30. A patient was admitted with acute abdominal pain. Laboratory tests indicated elevated serum amylase, serum lipase and urinary amylase. What is probable diagnosis?

Hint: Acute pancreatitis

31. A student was admitted to the hospital. He was ill looking and frankly jaundiced. On the day prior to the development of jaundice, he noticed that his urine was deep colored and frothy. The laboratory findings showed: Plasma total protein: 7.7 g%; albumin 4.2 g%; ALP-i 150 U/L; ALT-i 4000 U/L; serum bilirubin-4 mg%; urine bilirubin; ++. What is the most probable diagnosis? What further tests do you suggest?

Hint: Acute infective hepatitis.

32. A child with stunted growth, edema (particularly on legs and hands), discoloration of skin and hair, apathy and moon-face. The child had frequent respiratory infections and diarrhea. Past history revealed that the child was mostly breast-fed until 2-years age. Later the child was given diluted buffalo milk and small quantities of rice with butter and dal. The laboratory findings shows: Hemoglobin: 7 g%, serum protein: 4 g%, serum albumin: 2 g% and serum potassium: 2.8 mEq/L. What is probable diagnosis?

Hint: Kwashiorkor.

33. A 5-year-old girl had bone deformities such as bow legs and pigeon chest. She had delayed eruption of teeth. The girl was from a strict vegetarian family and she used to take very low amount of milk. The laboratory findings are: Calcium: 6.5 mg%; phosphate: 2.5 mg%; ALP: 210 IU/L; calcitriol: 10 pg/dl (normal: 25–60 pg/dl). What is the probable diagnosis?

Hint: Rickets.

34. A tuberculosis patient was given isoniazide (INH). After the completion of treatment regime, the patient developed neurological manifestations. His urine contained increased concentration of xanthurenic acid. What are the reasons?

Hint: Drug-induced vitamin B₆ deficiency

35. A 7-year-old boy with enlarged abdomen was irritable, lethargic and frequently hungry. History revealed that his childhood development was comparatively slow. Clinical examination showed enlarged liver. Blood parameters are: fasting glucose: 40 mg%; pH: 7.25; ketone bodies: 5 mg% (normal: 2–3 mg%). While serum lactate, triglyceride and uric acid levels were elevated. Comment.

Hint: von Gierke's disease.

36. A 30-year-old person was admitted in a hospital with severe chest pain. His clinical and biochemical investigations indicated that he suffered a mild myocardial infarction. His lipid profile data are given here. Total cholesterol: 416 mg%; triglyceride: 157 mg%; HDL- cholesterol: 42 mg%; VLDL-cholesterol: 31 mg%; LDL- cholesterol: 341 mg%. What is the most probable diagnosis?

Hint: Familial hypercholesterolemia.

37. A 52-year-old Industrialist had a routine medical checkup. His laboratory findings are given here. Fasting blood sugar: 84 mg%; serum creatinine: 0.9 mg%; serum total bilirubin: 0.8 mg%; ALT: 76 IU/L; AST: 124 IU/L; GGT: 84 IU/L. What is your probable diagnosis?

Hint: Hepatic damage due to alcoholism.

38. A 24-year-old man had generalized edema with puffiness of the face in the morning. His laboratory findings showed; serum total protein: 4.5 g%; albumin: 1.5 g%; Serum cholesterol: 326 mg%; blood urea: 36 mg%; serum creatinine: 1.3 mg% and urinary protein: 15 g%.

Hint: Nephrotic syndrome.

39. A 51-year-old post-menopausal woman had the symptoms of lethargy, depression, muscle weakness, loss of memory, loss of appetite, constipation, polyuria, and polydipsia. She had 2 episodes of renal stones in past 4 years. Chemical analysis of stone revealed that calcium phosphate was the major constituent. The following are the laboratory findings. Calcium: 12.8 mg%; phosphate: 2.5 mg%; chloride: 112 mEq/L; ALP: 224 IU/L. Comment.

Hint: Hyperparathyroidism.

40. A 58-year-old person was brought to the Hospital in a confused and semiconscious state. He had low BP and feeble pulse. His breath was fruity odor. Laboratory findings show that: Blood pH: 7.2; plasma bicarbonate: 12 mmol/L (normal: 24–30 mmol/L); plasma carbonic acid: 1.2 mmol/L; random blood glucose: 578 mg%; blood urea: 38 mg%; serum creatinine: 1.5 mg%; urine sugar and ketone bodies are strong positive. Identify the case.

Hint: Diabetic ketoacidosis.

41. A 5-year-old boy with retarded growth was brought to the Hospital with a complaint of diarrhea. On examination he was found to have cataract in the eye. Urine examination showed reduction with Benedict's reagent but not with glucose oxidase method. What is your comment?

Hint: Galactosemia.

42. The Laboratory information of a patient is: Blood pH: 7.6; pCO₂: 21 mm Hg; Plasma HCO₃⁻: 28 mEq/L; H₂CO₃: 0.7 mEq/L.

Hint: Respiratory alkalosis.

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Appendix

Reference Values

REFERENCE VALUES IN BLOOD

Sugar (F)	70–110 mg%	ALP	39–117 IU/L
Sugar (PP)	100–140 mg%	ACP	0–5.1 U/L
Glycated Hb	6% of total Hb	Total cholesterol	150–250 mg%
Urea	10–40 mg%	HDL-C	35–65 mg%
Urea clearance		LDL-C	90–150 mg%
Standard	54 ml/min	Triglyceride	50–190 mg%
Maximal	75 ml/min	LDL-C/HDL-C	2–2.5
Creatinine	0.7–1.4 mg%	Total calcium	8.5–11 mg%
Creatinine clearance	125 ml/min	Ionized calcium	4.5–5.5 mg%
Total protein	6–8 g%	Phosphorous	2.5–4.5 mg%
Albumin	3.5–5.5 g%	Amylase	35–135 U/L
Globulin	2–3.5 g%	Sodium	135–150 mEq/L
A:G ratio	1.5–2.5	Potassium	3.5–5.0 mEq/L
Uric acid	2–7 mg%	Chloride	95–106 mEq/L
Total bilirubin	0.2–1 mg%	pCO ₂	36–46 mm Hg
Conjugated bilirubin	0.1–0.4 mg%	pO ₂	90–110 mm Hg
Unconjugated bilirubin	0.2–0.8 mg%	pH	7.35–7.45
ALT	5–41 U/L	LDH	240–480 U/L
AST	5–38 U/L		

REFERENCE VALUES IN URINE

Urine	
Total protein	<150 mg/ day
Albumin	<30 mg/ day
Urea	2 g%
Creatinine	1–2 g/day
Amylase	1500–1800 Somogyi units/day
Chloride	10–15 g/day as NaCl

REFERENCE VALUES IN CSF

Protein	15–45 mg%
Sugar	50–100 mg%
Chloride	120–130 mEq/L

REFERENCE VALUES IN GASTRIC JUICE

BAO	1–2.5 mEq/L
MAO	20–40 mEq/L

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Appendix

Anticoagulants

The blood used may be in the form of serum, plasma or whole blood. Serum or plasma separation requires the use of anticoagulants. Many different anticoagulants are required in varying amounts and act by different mechanisms. Hence, their use is critical depending upon the nature of analysis.

Anticoagulants	Requirement	Mechanism
Heparin	2 mg%	Prevent thrombin formation
Oxalate salt of Na ⁺ , K ⁺ and NH ₄ ⁺	2–3 mg/ml	Ca ²⁺ precipitation
Sodium citrate	3–6 mg/ml	Forms calcium complex
EDTA	10–20 mg/ml	Ca ²⁺ chelation

Heparin is generally the most preferred anticoagulant. Samples for blood sugar also use sodium fluoride, an inhibitor of enolase enzyme of glycolysis, in addition to an oxalate (1:3) to prevent the glucose utilization by RBC.

Common changes that occur in blood sample after collection are:

- Glucose can be converted to lactate by glycolysis.
- Due to hemolysis or permeability through red cell membrane, plasma concentration of potassium, LDH or aminotransferase can increase.
- Exposure to sunlight can destroy bilirubin.
- Sample for arterial blood gas analysis if come in contact to air can alter results.
- Some labile enzymes may loose their activity.

PRESERVATION OF URINE

- 10 ml of concentrated HCl is put in container for 24 h urine.
- A few crystals of thymol are adequate.
- 3–4 drops of formalin can be used per 100 ml of urine.
- Toluene, acetic acid can also be used.

Commonly used samples for biochemical tests

Sample	Tests	Collection Method
Whole blood	Sugar, urea, blood gases	Arterial or venepuncture
Plasma	Enzymes, electrolytes, metabolites, e.g. creatinine, uric acid, cholesterol, etc.	Blood with anti-coagulant, centrifuged at 200 rpm
Serum	-do-	Blood with anti-coagulant, centrifuged at 200 rpm
CSF	Sugar, protein, chloride	Lumbar puncture
Gastric juice	Acid, blood	Aspiration by syringe through Ryle's tube

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Appendix

Solutions

Acidimetry and Alkalimetry: The two terms are complementary. They involve determination of strength of acid or an alkali solution by titration against a standard solution of an alkali or an acid as the case may be. If the strengths in normality of the alkali and the acid solutions are S_A and S_B respectively and V_A ml of the alkali exactly neutralizes V_B ml of the acid then, $V_A S_A = V_B S_B$.

If the strength of the one is known, the other can be calculated out.

Strength of a solution: It measures the amount of solute in a definite volume of the solvent. This can be expressed either in term of molarity or in term of normality.

Molar solution: Solution, which contains one g molecular weight of the solute in 1000 ml of solvent. Thus, a molar solution of oxalic acid ($C_2H_2O_4 \cdot 2H_2O$) contains:

$$2 \times 12 + 2 \times 1 + 4 \times 16 + 2 \times 18 = 126\text{g per litre.}$$

Normal solution: It is that solution, one litre of which contains one g equivalent weight of the substance. The g equivalent of a substance is that weight in g, which combines with or displaces from a substance 1.008g of hydrogen, 8g of oxygen or 35.457g of chlorine.

Mathematically, equivalent weight = Molecular weight/ valency.

Percent solution can be expressed in three ways (1) weight/unit weight (2) weight/unit volume (3) volume/unit volume.

The **standard solutions** are of two types (a) primary standard and (b) secondary standard solution.

Primary standard solutions are those solutions, which are prepared by accurately weighing a chemically pure substance in a chemical balance and then dissolving in a known volume. The substance should not change its composition either during weighing or in a solution during its preparation. The following compounds are generally used for primary standard solutions.

- For alkali solution use Na_2CO_3 (anhydrous) and $Na_2B_4O_7 \cdot 10H_2O$.
- For acid solution use oxalic acid, succinic acid, potassium hydrogen phosphate, etc.
- For Redox system use Potassium dichromate, Sodium oxalate, Potassium bromate, Potassium iodate.

Secondary standard solutions are those which cannot be prepared by direct weighing in a chemical balance. These are usually prepared by standardizing against some primary standard solution. Examples are solution of NaOH, KOH, HCl, H_2SO_4 , $KMnO_4$, etc.

Preparation of a Standard Solution of Hydrochloric Acid or Sulphuric Acid

Standard solutions of these acids cannot be prepared by direct weighing. An approximately 0.1N solution is first prepared and then its strength is accurately determined by titration against a standard solution of an alkali.

Concentrated hydrochloric acid is about 12N; Concentrated sulphuric acid is about 36N.

Buffer solution: Certain solutions possess the ability to resist an appreciable changes in H^+ ion concentration (i.e. pH) when acids or bases are added to these solutions. Buffer solutions usually consist of a weak acid or base together with its salt.

Preparation of phosphate buffer (0.1N)

pH value	ml	
	0.1N KH_2PO_4	0.1N Na_2HPO_4
5.8	92.0	8.0
6.2	81.4	18.6
6.5	68.2	31.8
7.0	38.9	61.1
7.4	19.2	80.8
7.9	6.8	93.2

Indicators: When an acid is titrated against an alkali solution or vice versa, it is necessary to determine the equivalence point or the end point very correctly. This is done with the help of an indicator. An indicator is a substance which show, one characteristic color with an acidic solution and a different color with an alkaline solution. Indicators must exist in unionized and ionized forms which are controlled by hydrogen ion concentration. The indicators mostly used are phenolphthalein, methyl orange and methyl red. Litmus is unsuitable for quantitative work. A broad range indicator like a pH paper indicates different color at different pH is known as Universal indicator.

Indicator	Acid color	Alkali color	pK	pH range
Thymol blue	Red	Yellow	2.0	1.2–2.8
Methyl orange	Red	Yellow	3.7	3.1–4.4
Bromocresol green	Yellow	Blue	4.7	3.8–5.4
Methyl red	Red	Yellow	5.1	4.2–6.3
Bromothymol blue	Yellow	Blue	6.8	6.0–7.6
Phenol red	Yellow	Red	7.6	6.8–8.4
Phenolphthalein	Colorless	Red	9.0	8.3–10.0

Choice of indicators

Nature of titration	pH range	Example
Strong acid- strong base	4.5–9.5	Bromocresol green, methyl red, phenolphthalein
Strong acid- weak base	3–7	Bromocresol green, methyl red, methyl orange
Weak acid- strong base	7–10.5	Phenolphthalein, thymol blue, thymol phtalein

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Appendix

Preparation of Reagents

Acetate buffer, 0.2 M, pH 4.7: Dissolve 27.2 g sodium acetate crystals in about 500 ml distilled water. Adjust pH to 4.7 with glacial acetic acid and make up to 1L with water.

Acetate buffer, 3 M, pH 5.0: Dissolve 40.8 g sodium acetate crystals in about 70ml distilled water. Add 8 ml glacial acetic acid and make up the volume to 100ml with water. This buffer is used for osazone preparation.

Alkaline copper reagent: Dissolve 40 g anhydrous sodium carbonate in about 400 ml distilled water and transfer it to a litre flask. Dissolve 7.5 g tartaric acid in this solution and add 4.5 g copper sulphate. Mix and make the volume to 1litre.

Ammonium molybdate [(NH₄)Mo₇O₂₄, 4H₂O] reagent: Dissolve 100 g molybdic acid in 144 ml of NH₄OH (sp. Gr. 0.9) and 271 ml water. Add this solution with constant stirring slowly into 489 ml conc. HNO₃ and 1148 ml water. Keep this mixture for several days and then filter.

Antimony trichloride (SbCl₃) reagent: 25% SbCl₃ in chloroform.

Arsenomolybdate reagent (Nelson): Dissolve 25 g ammonium molybdate in about 450 ml water. Add 21 ml conc. H₂SO₄, mix and add 3 g disodium hydrogen arsenate (Na₂HAsO₄, 7H₂O) dissolved in 25 ml water. Mix and keep at 37°C for 24 h for complete dissolution.

Barfoed's reagent: Dissolve 25 g copper acetate in 450 ml boiling water. A precipitate may be formed. Add immediately 4 ml glacial acetic acid to the hot solution, stir to mix. Cool and make up to 500 ml with water. Filter off any impurities sedimented.

Barium hydroxide, 0.3 N: Add 28 g of Ba(OH)₂, 8H₂O to 500 ml hot water. Boil for a few minutes. Cool and filter. 5 ml of this reagent should neutralize 5 ml of 5% zinc sulphate with phenolphthalein as indicator.

Baryta mixture: Mix 1 volume of saturated solution of barium nitrate and 2 volumes of a saturated solution of barium hydroxide.

Benedict's qualitative reagent: Dissolve with the aid of heat, 173 g of sodium citrate and 100 g of anhydrate sodium carbonate in about 100 ml water in a beaker. Separately dissolve 17.3 g CuSO₄, 5H₂O in about 100 ml water and transfer this to the first solution, slowly with stirring. Make up the volume to 1L.

Benedict's quantitative reagent: Dissolve 200 g of sodium citrate, 75 g anhydrate sodium carbonate, 125 g potassium thiocyanate in about 600 ml water with the aid of heating. Separately dissolve 18 gm CuSO₄, 5H₂O

in about 100 ml water and transfer this to the first solution, slowly with stirring. Add 5 ml of 5% potassium ferrocyanide. Make up the volume to 1L.

Benedict's uric acid reagent: Dissolve 100 g of pure sodium tungstate in a litre Pyrex flask with 600 ml water and add 50 g pure arsenic acid (As_2O_5) followed by 25 ml of 85% phosphoric acid and 20 ml of conc. HCl, boil for 20 min, cool and dilute to 1L.

Biuret reagent, stock: Dissolve 45 g Rochelle salt (sodium potassium tartrate) in about 400 ml 0.2 N NaOH. Add 15 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and stir to dissolve completely. Add 5 g potassium iodide and dissolve. Make up to 1L with 0.2 N NaOH.

Biuret reagent: Dilute 200 ml of stock Biuret reagent to 1L with 0.2 N NaOH containing 5 g potassium iodide per litre.

Bromocresol green indicator: Dissolve 40 mg in 97.7 ml water and add 2.3 ml of 0.1 N NaOH.

Chlorophenol red indicator: Dissolve 40 mg in 96.2 ml water and add 3.8 ml of 0.1N NaOH.

2,6-Dichlorophenol indophenol: Dissolve 50 mg of the sodium salt of the dye in 160 ml water by warming. Add 42 mg NaHCO_3 and make up the volume to 200 ml.

Ehrlich's diazo reagent: Dissolve 2 g p-dimethyl aminobenzaldehyde in 100 ml 20% HCl.

Esbach's reagent: Dissolve 10 g picric acid and 20 g citric acid in 1L water.

Fehling's Solution

Solution A: Dissolve 34.6 g pure $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water and dilute to 500 ml.

Solution B: Dissolve 170 g of Rochelle salt (Sodium-potassium tartrate) and 71 g of NaOH in water and dilute to 500 ml.

Folin's alkaline copper reagent: Dissolve 40 g anhydrate sodium carbonate in about 400 ml water. Add 7.5 g tartaric acid and dissolve. Separately dissolve 4.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in about 100 ml water and transfer this slowly to the first solution. Make up the volume to 1L.

Fouchet's reagent: Dissolve 25 g trichloroacetic acid in about 50 ml water. Add 10 ml 10% ferric chloride. Make up to 100 ml with water.

Foulger's reagent: Dissolve 40 g urea in 80 ml of 40% (w/v) H_2SO_4 . Add 2 g stannous chloride and boil till clear solution is obtained. Cool and make up to 100 ml with 40% H_2SO_4 .

Glucose standard (O-Toluidine method): Dissolve 10 mg glucose in about 50 ml distilled water. Add 30 ml 10% trichloroacetic acid. Make up the volume to 100 ml with distilled water.

Iodine, N/50 solution: Dilute 10 ml of 0.1 N iodine to 500 ml with 2% potassium iodide.

Lead acetate solution: Dissolve 180 g lead acetate in about 700 ml distilled water with boiling. Add this hot solution to the finely powdered lead oxide and boil for one and half hour with occasional stirring. Cool, filter and add distilled water to make the volume 1L.

Methyl red indicator: 0.05% in 50% alcohol.

Nessler's solution: Dissolve 115 g HgI_2 and 30g KI in 500 cc water, add 500 ml 6N NaOH; filter.

Ninhydrin reagent: 0.1 g% in acetone.

Nippe's fluid: 0.1 g each of KCl, KBr and KI dissolved in 100 ml of glacial acetic acid.

Osazone buffer: 100 g phenyl hydrazine HCl, 350 g sodium acetate and 125 ml glacial acetic acid in 200 ml water, warm to dissolve and make to 1.5 L.

O-Toluidine reagent: To 5 g thiourea, add 90 ml O-Toluidine and dilute to 1L with glacial acetic acid. Store in a brown amber bottle and keep the reagent in a refrigerator.

Phenol red indicator: 1% in 95 ml alcohol and 5 ml 0.1N NaOH.

Phenolphthalein indicator: 0.1% in 50% alcohol.

Phosphate buffer, 0.2 M, pH 6.6: Dissolve 27.2 g KH_2PO_4 in about 600 ml water. Adjust the pH using 1N NaOH. Make up the volume to 1L with water.

Phosphate buffer, 0.05 M, pH 7.0: Dissolve 6.8 g KH_2PO_4 in about 500 ml water. Adjust the pH using 1N NaOH. Make up the volume to 1L with water.

Phosphomolybdic acid reagent: Dissolve 35 g molybdic acid and 5 g sodium tungstate in 200 ml 10% NaOH. Add 200 ml water. Boil till all the ammonia from molybdic acid is removed. Cool and dilute to 350 ml with water. Add 125 ml 85% phosphoric acid. Make up to 500 ml and mix.

Phosphotungstic acid, stock: Dissolve 100 g sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) in 800 ml water in a round bottomed flask. Add 80 ml of 85% phosphoric acid, attach a reflux condenser and gently boil for 2 h. Cool. Dilute to 1L with water, mix and store in a brown bottle. Dilute 1 in 10 with water for use.

Seliwanoff's reagent: Dissolve 50 mg resorcinol in 33 ml conc. HCl and dilute to 100 ml.

Sodium hypobromite: Mix 25 ml liquid bromine with 250 ml 40% NaOH.

Topfer indicator: Dissolve 0.5 g dimethylamino azobenzene in 100 ml 95% alcohol.

Urease suspension: Grind 10 g horse gram powder (*Dolichos uniflorous*, a rich source of urease) with 100 ml 30% ethanol.

Vanden Bergh reagent A: Dissolve 1 g sulphanilic acid in 15 ml conc. HCl and make up to 1L with water.

Vanden Bergh reagent B: Dissolve 0.5 g sodium nitrite in water and make up to 1L.

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Appendix

Strength and Density of Reagents

STRENGTH OF SOME COMMON ACIDS AND AMMONIUM HYDROXIDE

	Approximate			Vol required to make 1L N soln.
	Sp.gr.	% by wt.	Normality	
Acetic acid, glacial	1.05	99.5	17.4	58cc
Hydrochloric acid	1.18	35.0	11.3	89cc
Nitric acid	1.42	69.8	16.0	63cc
Phosphoric acid	1.69	85.0	14.7	23cc
Sulphuric acid	1.84	96.0	36.0	28cc
Ammonium hydroxide	0.90	27(NH ₃)	14.3	71cc

DENSITY OF VARIOUS LIQUIDS

Liquid	g/cc	°C
Acetone	0.792	20
Alcohol, ethyl	0.791	20
Alcohol, methyl	0.810	0
Benzene	0.899	0
Carbon disulphide	1.293	0
Carbon tetrachloride	1.595	20
Chloroform	1.489	20
Ether	0.736	0
Glycerin	1.260	0
Mercury	13.6	-
Phenol	0.958	15
Water	1.00	4

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