East Point College of Pharmacy

East Point Campus, Jnana Prabha, Virgo
Nagar PostBengaluru – 560049, Karnataka

Approved
by
Pharmacy Council of India, New Delhi

Affiliated
to
Rajiv Gandhi University of Health Sciences
Karnataka
Bengaluru – 560041
India

LAB MANUAL

BIOCHEMISTRY

B. PHARM 2nd SEMESTER

EAST POINT COLLEGE OF PHARMACY
East Point Campus, Jnana Prabha, Virgo Nagar Post,
Bengaluru – 560049, Karnataka

B Pharmacy

Program Outcomes (PO's)

PO 1- Pharmacy Knowledge

Possess knowledge and comprehension of the core and basic knowledge associated with theprofession of pharmacy, including biomedical sciences; pharmaceutical sciences; behavioral, social, and administrative pharmacy sciences; and manufacturing practices.

PO 2- Planning Abilities

Demonstrate effective planning abilities including time management, resource management,delegation skills and organizational skills. Develop and implement plans and organize workto meet deadlines.

PO 3- Problem analysis

Utilize the principles of scientific enquiry, thinking analytically, clearly and critically, whilesolving problems and making decisions during daily practice. Find, analyze, evaluate and apply information systematically and shall make defensible decisions

PO 4- Modern tool usage

Learn, select, and apply appropriate methods and procedures, resources, and modernpharmacy-related computing tools with an understanding of the limitations.

PO 5- Leadership skills

Understand and consider the human reaction to change, motivation issues, leadership and team-building when planning changes required for fulfillment of practice, professional and societal responsibilities. Assume participatory roles as responsible citizens or leadership roles when appropriate to facilitate improvement in health and wellbeing.

PO 6- Professional Identity

Understand, analyse and communicate the value of their professional roles in society (e.g.health care professionals, promoters of health, educators, managers, employers, employees).

PO 7- Pharmaceutical Ethics

Honor personal values and apply ethical principles in professional and social contexts. Demonstrate behaviour that recognizes cultural and personal variability in values,communication and lifestyles. Use ethical frameworks; apply ethical principles while making decisions and take responsibility for the outcomes associated with the decisions

PO 8- Communication

Communicate effectively with the pharmacy community and with society at large, such as,being able to comprehend and write effective reports, make effective presentations and documentation, and give and receive clear instructions

PO 9- The Pharmacist and society

Apply reasoning informed by the contextual knowledge to assess societal, health, safety andlegal issues and the consequent responsibilities relevant to the professional pharmacy practice.

PO 10- Environment and sustainability

Understand the impact of the professional pharmacy solutions in societal and environmentalcontexts, and demonstrate the knowledge of, and need for sustainable development.

PO 11- Life-long learning

Recognize the need for, and have the preparation and ability to engage in independent and life-long learning in the broadest context of technological change. Self-access and use feedback effectively from others to identify learning needs and to satisfy these needs on an ongoing basis.

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Programme Specific Outcomes (PSO's)	
PSO 1	Acquire a thorough foundational knowledge in pharmaceutical sciences, including pharmacology, pharmaceutics, medicinal chemistry, and pharmacognosy, to excel in further academic pursuits
PSO 2	Gain expertise in the application of contemporary pharmaceutical techniques and technologies, enhancing employability across various sectors including the pharmaceutical industry, academia, and research institutions.
PSO 3	Equip with entrepreneurial skills and knowledge of pharmaceutical business management, including market analysis, product development, regulatory affairs, and financial planning, to initiate and run successful ventures in the pharmacy sector

Course Outcomes (CO's)	
Code: BP209P Biochemistry	
CO 1	Perform and identify the carbohydrates
CO 2	Perform and identify the proteins
CO 3	Identify normal and abnormal constituents of urine.
CO 4	Estimate the quantity of reducing sugars by DNSA methods and proteins by the biuret method.
CO 5	Determine the factors affecting enzyme activity and report it

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EAST POINT COLLEGE OF PHARMACY
East Point Campus, Jnana Prabha, Virgo Nagar Post,
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QUALTITATIVE ANALYSIS OF CARBOHYDRATES

IDENTIFICATION OF CARBOHYDRATES

CARBOHYDRATES:

Carbohydrates are naturally occurring organic compounds containing carbon, hydrogen and oxygen elements. Chemically they are "polyhydroxyaldehydes or polyhydroxyketones".

CLASSIFICATION:

1. **Based on chemical composition:**

Monosaccharide: Monosaccharide are those carbohydrates that cannot be hydrolysed further to give simpler units of polyhydroxy aldehyde or ketone. If a monosaccharide contains an aldehyde group then it is called aldose and on the other hand if it contains keto group then it is called as ketose. Glucose is also called aldohexose and dextrose and is abundant on earth.

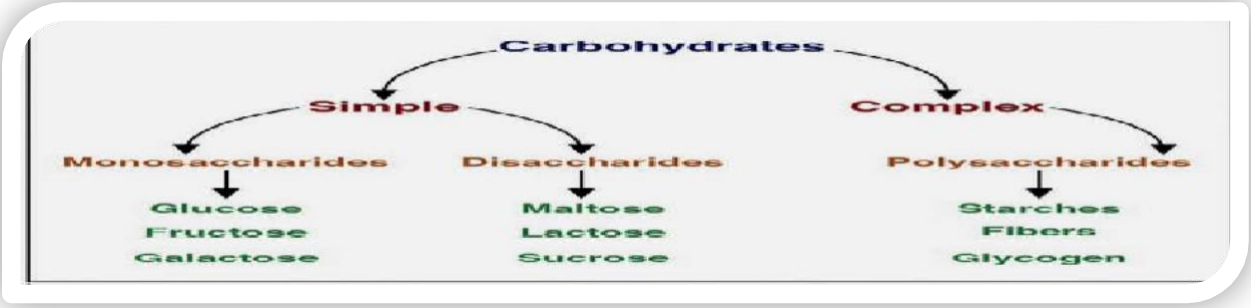
One of the most important monosaccharide is glucose. The two commonly used methods for the preparation of glucose are-

From Sucrose: If sucrose is boiled with dilute acid in an alcoholic solution then we obtain glucose and fructose.

$C_{12}H_{22}O_{11}$ (sucrose) $\longrightarrow$ $C_6H_{12}O_6 + C_6H_{12}O_6$

From Starch: We can obtain glucose by hydrolysis of starch and by boiling it with dilute H2SO4 at393K under elevated pressure.

Ex: Glucose ($C_6H_{12}O_6$), glucose(dextrose), fructose (levulose) and galactose.



2. **Disaccharides:** On hydrolysis, dissacharides yield two molecules of either same or different monosaccharide. The two monosaccharide units are joined by oxide linkage which is formed by the loss of water molecule and this linkage is called glycosidic linkage. Sucrose is one of the mostcommon disaccharides which on hydrolysis gives glucose and fructose.

Maltose and Lactose (also known as milk sugar) are other two important dissacharides. In maltose there are two α-D-glucose and in lactose there are two β-D-glucose which are connected by oxide bond.

3.Polysaccharides: contain long monosaccharide units joined together by glycosidic linkage. Most of them act as food storage for e.g. Starch. Starch is the main storage polysaccharide for plants.It is a polymer of α glucose and consists of two components-Amylose and Amylopectin.

Cellulose is also one of the polysaccharides that are mostly found in plants. It is composed of β-D- glucose units joined by glycosidic linkage between C1 of one glucose unit and C4 of the next glucose unit.

Glycogen: These carbohydrates are stored mainly in animal body. It is present in liver, muscles and brain. When the body needs glucose, enzymes break the glycogen into

Ex: Starch $\{C_{12}H_{22}O_{11}\}\longrightarrow$ $(C_6H_{12}O_6)$ n

A) Based on physical and chemical properties:

SUGARS: SWEET AND SOLUBLE IN WATER

1. **Reducing sugars**: Capable of reducing Fehling's solution and benedicts reagent.
   Ex: Glucose, fructose, maltose, lactose.
2. **Non-reducing sugars**: not capable of reducing Fehling's solution and benedicts reagent.
   Ex: Sucrose

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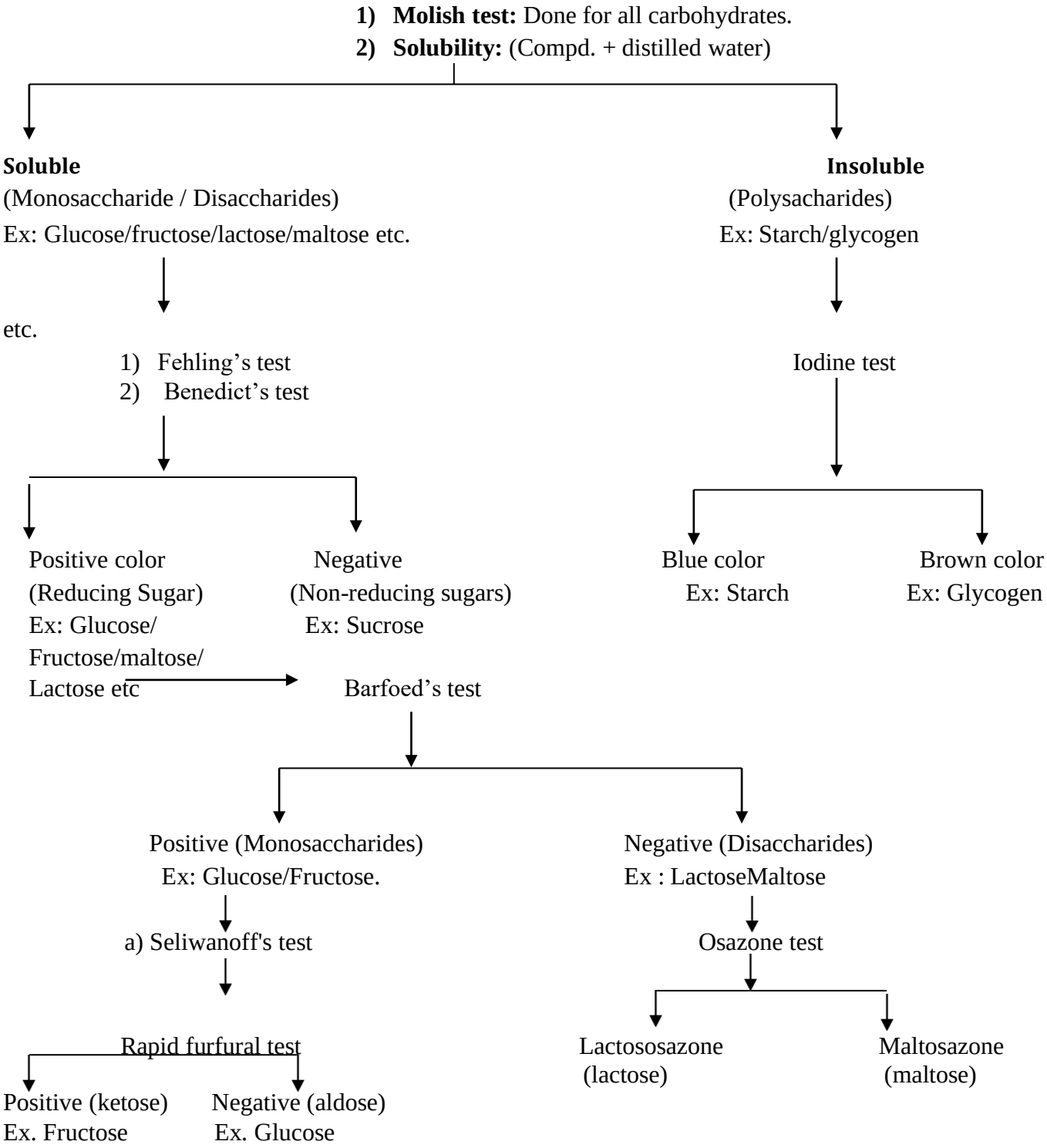
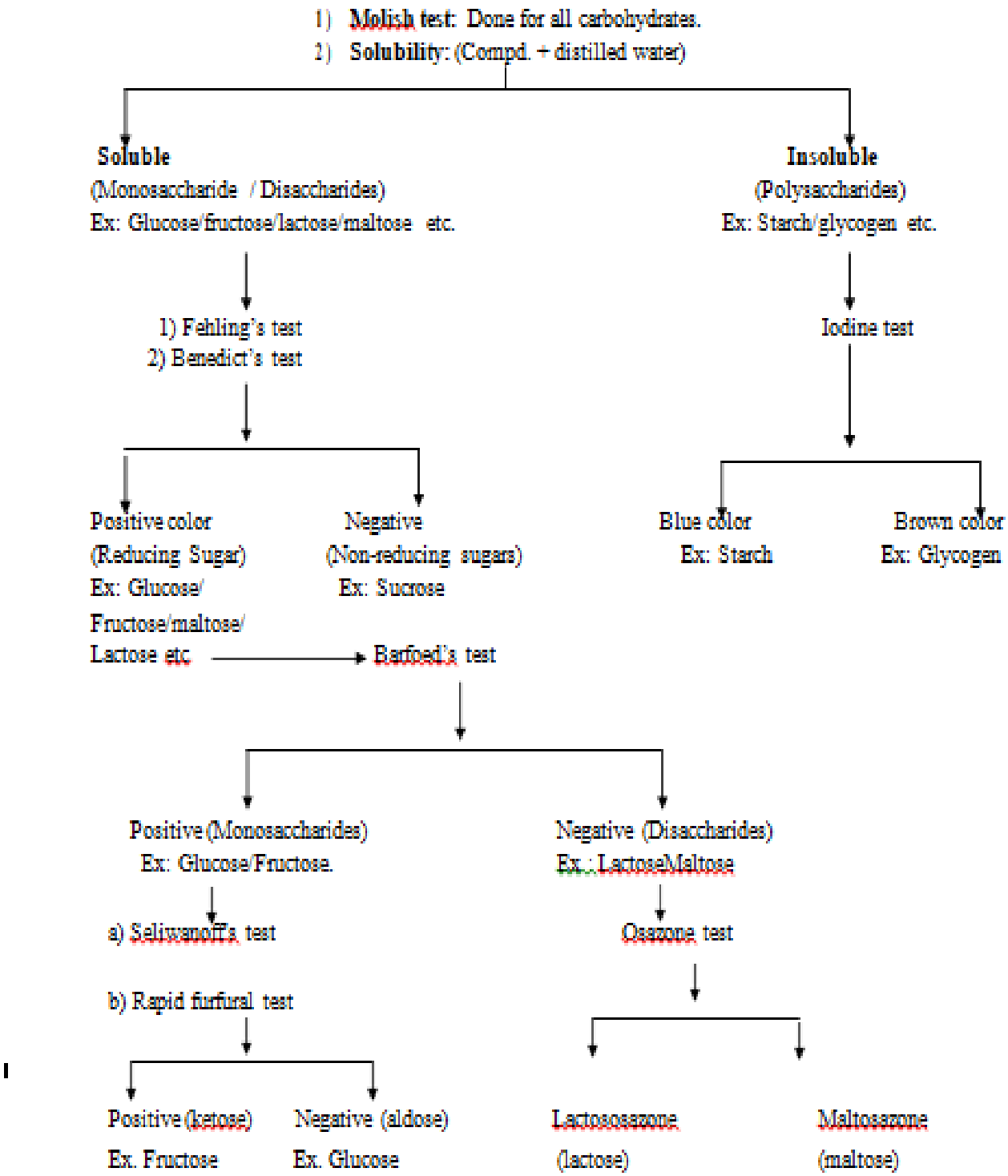
SCHEME FOR IDENTIFICATION UNKNOWN CARBOHYDRATE SAMPLE



SCHEME FOR IDENTIFICATION UNKNOWN CARBOHYDRATE SAMPLE



IDENTIFICATION TESTS FOR CARBOHYDRATES

Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of **α**-naphthol, shake +conc. H_2SO_4 along the sides of the test tube.	Violet ring at the junction of two liquid layers.	Carbohydrate is present.
Solubility: Compd.+ water	Soluble. Insoluble.	Mono / Disaccharides. Polysaccharides.
Fehling's test: 2ml fehling's solnA+2ml of fehling's soln B, heat & cool.	Yellow or brick red ppt.	Reducing sugars present.
Benedict's test: 5ml of benedict's reagent + 3 ml of sugar soln boil for 2 min.	Green, yellow or red ppt.	Reducing sugars present.
Barfoed's test: 2ml of test soln + 2 ml of Barfoed's reagent. Boil on water bath.	Brick red ppt. at the bottom of the test tube.	Monosaccharides present.
Seliwanoffs test: 1 ml of sugar soln + 3 ml ofSeliwanoffs reagent, boil for 2 min.	Cherry red colour.	Ketoses like fructose and sucrose present.

Osazone test: 0.2 gms sugar + 0.4 gms of phenyl hydrazine hydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min.	a) Greenish yellow needle shaped crystals arranged in fan-shape. b)Thin small needle shaped crystals appear like ball of prickles. c) Plate like crystals appears like sunflower.	a) Glucose present. b) Lactose present. c) Maltose present.

SIGNIFICANCE OF VARIOUS TESTS:

Molish test: This is the generalized test for carbohydrates. The reaction depends on the formation of furfural compounds from carbohydrates. Furfural compounds combine with α - naphthol to form violet colour.

Barfoed's test: This test is useful to differentiate between monosaccharide and disaccharides. Barfoed's reagent consists of copper acetate and acetic acid. This test is based on reduction in acidic medium.

Fehling's test: This test based on reduction of $CuSO_4$ in alkaline solution. In this test fehlings A and fehling's B solutions are used separately black ppt of cupric oxide. Fehlings A solution containssoln. of $CuSO_4$ and Fehling's B solution contains NaOH and sod. pot. tararate. Sod. pot.tararate isused to dissolve the ppt of cupric hydroxide.

Benedicts test: This test also based on reduction of $CuSO_4$ in alkaline solution. Benedict's reagent contains sod.citrate, sod. carbonate and copper sulphate. Sod. citrate is used to avoid black ppt of CuO.

Seliwanoffs test: This test is useful to differentiate between ketoses and aldoses. Seliwanoff's reagent contains resorcinol in conc. HCl.

Osazone test: This test is useful to differentiate among the reducing sugars, because yellow crystalline structures of the osazones formed are characteristic of particular sugar.

In this test phenyl hydrazine is reduced to phenyl hydrazone by sugar solution. Phenyl hydrazone with more amount of phenyl hydrazine forms osazone, which are yellow coloured crystalline form.

Glucosazone and fructosazone are insoluble in hot water [fructosazone forms within 2 to 3 min.

and glucosazone forms within 5 min.].

Lactosazone and maltosazone are soluble in hot water. Thus ppt is not obtained in hot condition. So solution is heated on a water bath for 30-45 min. when yellow soln. is obtained cool naturally slowly.

Inversion test:

This test is specified for the confirmation of sucrose. On hydrolysis with conc. HCl, sucrose forms the reducing monosaccharides, glucose and fructose and gives tests for reducing sugars.

$C_{12}H_{22}O_{11}$		$C_6H_{12}O_6$	+	$C_6H_{12}O_6$
Sucrose		Glucose		Fructose

Experiment No: 1

IDENTIFICATION OF CARBOHYDRATES-S#1

AIM: Identify the carbohydrate present in the given sample.

Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of α-naphthol, shake +conc. H_2SO_4 along the sides of the test tube. **Solubility:** Compd.+ water **Fehling's test:** 2ml fehling's solnA+2ml of fehling's soln B & heat & cool. **Benedict's test**: 5ml of benedict's reagent + 3 ml of sugar soln boil for 2 min. **Barfoed's test:** 2ml of test soln + 2 ml of Barfoed's reagent. Boil on water bath. **Seliwanoff's test:** 1 ml of sugar soln + 3 ml of Seliwanoff's reagent, boil for 2 min. **Osazone test:** 0.2 gms sugar + 0.4 gms of phenyl hydrazine hydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min.		

REPORT:

Experiment No: 2

IDENTIFICATION OF CARBOHYDRATES-S#2

AIM: Identify the carbohydrate present in the given sample.

Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of **α**-naphthol, shake +conc. H_2SO_4 along the sides of the test tube. **Solubility:** Compd.+ water **Fehling's test:** 2ml fehling's solnA+2ml of fehling's soln.B & heat & cool. **Benedict's test**: 5ml of benedict's reagent + 3 ml of sugar soln. boil for 2 min. **Barfoed's test:** 2ml of test soln. + 2 ml of Barfoed's reagent. Boil on water bath. **Seliwanoff's test:** 1 ml of sugar soln. + 3 ml of Seliwanoff's reagent, boil for 2 min. **Osazone test:** 0.2 gms sugar + 0.4 gms of phenyl hydrazine hydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min		

REPORT:

Experiment No: 3

IDENTIFICATION OF CARBOHYDRATES-S#3

AIM: Identify the carbohydrate present in the given sample.

Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of α-naphthol, shake +conc. H2SO4 along the sides of the test tube. **Solubility:** Compd.+ water **Fehling's test:** 2ml fehling's solnA+2ml of fehling's soln.B & heat& cool. **Benedict's test**: 5ml of benedict's reagent + 3 ml of sugar soln. boil for2 min. **Barfoed's test:** 2ml of test soln. + 2 ml of Barfoed's reagent. Boil on water bath. **Seliwanoff's test:** 1 ml of sugar soln. + 3 ml of Seliwanoff's reagent, boil for 2 min. **Osazone test:** 0.2 gms sugar + 0.4 gms of phenyl hydrazine hydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min.		

REPORT:

Experiment No: 4

IDENTIFICATION OF CARBOHYDRATES-S#4

AIM: Identify the carbohydrate present in the given sample.

Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of **α**-naphthol, shake +conc. H_2SO_4 along the sides of the test tube. **Solubility:** Compd.+ water **Fehling's test:** 2ml fehling's solnA+2ml of fehling's soln.B & heat & cool. **Benedict's test**: 5ml of benedict's reagent + 3 ml of sugar soln. boil for 2 min. **Barfoed's test:** 2ml of test soln. + 2 ml of Barfoed's reagent. Boil on water bath. **Seliwanoff's test:** 1 ml of sugar soln. + 3 ml of Seliwanoff's reagent, boil for 2 min. **Osazone test:** 0.2 gms sugar + 0.4 gms of phenyl hydrazine hydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min.		

REPORT:

Experiment No: 5

IDENTIFICATION OF CARBOHYDRATES- S#5

AIM: Identify the carbohydrate present in the given sample.

Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of **α**-naphthol, shake +conc. H_2SO_4 along the sides of the test tube. **Solubility:** Compd.+ water **Fehling's test:** 2ml fehling's solnA+2ml of fehling's soln.B & heat& cool. **Benedict's test**: 5ml of benedict's reagent + 3 ml of sugar soln. boil for2 min. **Barfoed's test:** 2ml of test soln. + 2 ml of Barfoed's reagent. Boil on water bath. **Seliwanoff's test:** 1 ml of sugar soln. + 3 ml of Seliwanoff's reagent, boil for 2 min. **Osazone test:** 0.2 gms sugar + 0.4 gms of phenyl hydrazinehydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min.		

REPORT:

Experiment No: 6

IDENTIFICATION OF CARBOHYDRATES-S#6

AIM: Identify the carbohydrate present in the given sample.

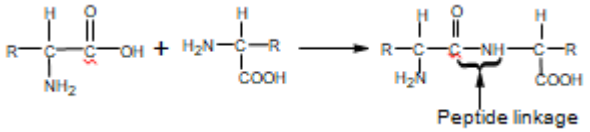
Test	Observation	Inference
Molish test: Aq or Alc.soln of subs. + 10% alc. soln of α-naphthol, shake +conc. H2SO4 along the sides of the test tube. **Solubility:** Compd.+ water **Fehling's test:** 2ml fehling's solnA+2ml of fehling's soln.B & heat & cool. **Benedict's test:** 5ml of benedict's reagent + 3 ml of sugar soln. boil for 2 min. **Barfoed's test:** 2ml of test soln. + 2 ml of Barfoed's reagent. Boil on water bath. **Seliwanoff's test:** 1 ml of sugar soln. + 3 ml of Seliwanoff's reagent, boil for 2 min. **Osazone test:** 0.2 gms sugar + 0.4 gms of phenyl hydrazine hydrochloride + 0.6 gms of sod. acetate + 4 ml of water. Heat on a water bath for 20 min.		

REPORT:

QUALTITATIVE ANALYSIS OF PROTEINS

IDENTIFICATION OF PROTEINS

PROTEINS: chemically are polymers of α-amino acids, where α-amino acids are polymerized through formation of no. of peptide linkages.



BIOCHEMICAL AND PHYSIOLOGICAL FUNCTIONS:

- **Enzymes:** Enzymes are highly specialized proteins, which catalyzes most of the chemical reactions of organic biomolecules in cells, tissue and systems.

Ex: Pepsin, trypsin.

- **Transport proteins:** Involved in carrying specific molecules or ions from one organ toanother.

Ex: Haemoglobin carrier oxygen and carbon dioxide.

- **Nutrient and storage proteins: Ex:** Casein and albumin of egg white.
- **Regulatory proteins:** They help to regulate cellular or physiological activity.

Ex: Insulin, growth hormone.

- **Contractile proteins: Ex:** Myosin.
- **Structural proteins:** They give the biological structure, strength or protection to the body tissue system. **Ex:** keratin.
- **Defense proteins:** Many proteins act as defense proteins and protect the host tissue against invading bacteria or virus. **Ex:** antibodies, α-globulins.

CLASSIFICATION OF PROTEINS:

1) According to physical nature and function:

a) Fibrous proteins: **Ex:** keratin, myosin, and collagen.

b) Globular proteins: **Ex:** Enzymes, hormones, haemoglobin.

2) According to chemical nature and composition:

a) Simple proteins: Formed from α-amino acids only. **Ex:** Albumin, globulin, gelatin.

b) Conjugated proteins: Formed from α-amino acids and non protein co-factor or prosthetic group.

i. Glycoprotein: Prosthetic group is carbohydrate. **Ex:** Mucin (saliva)

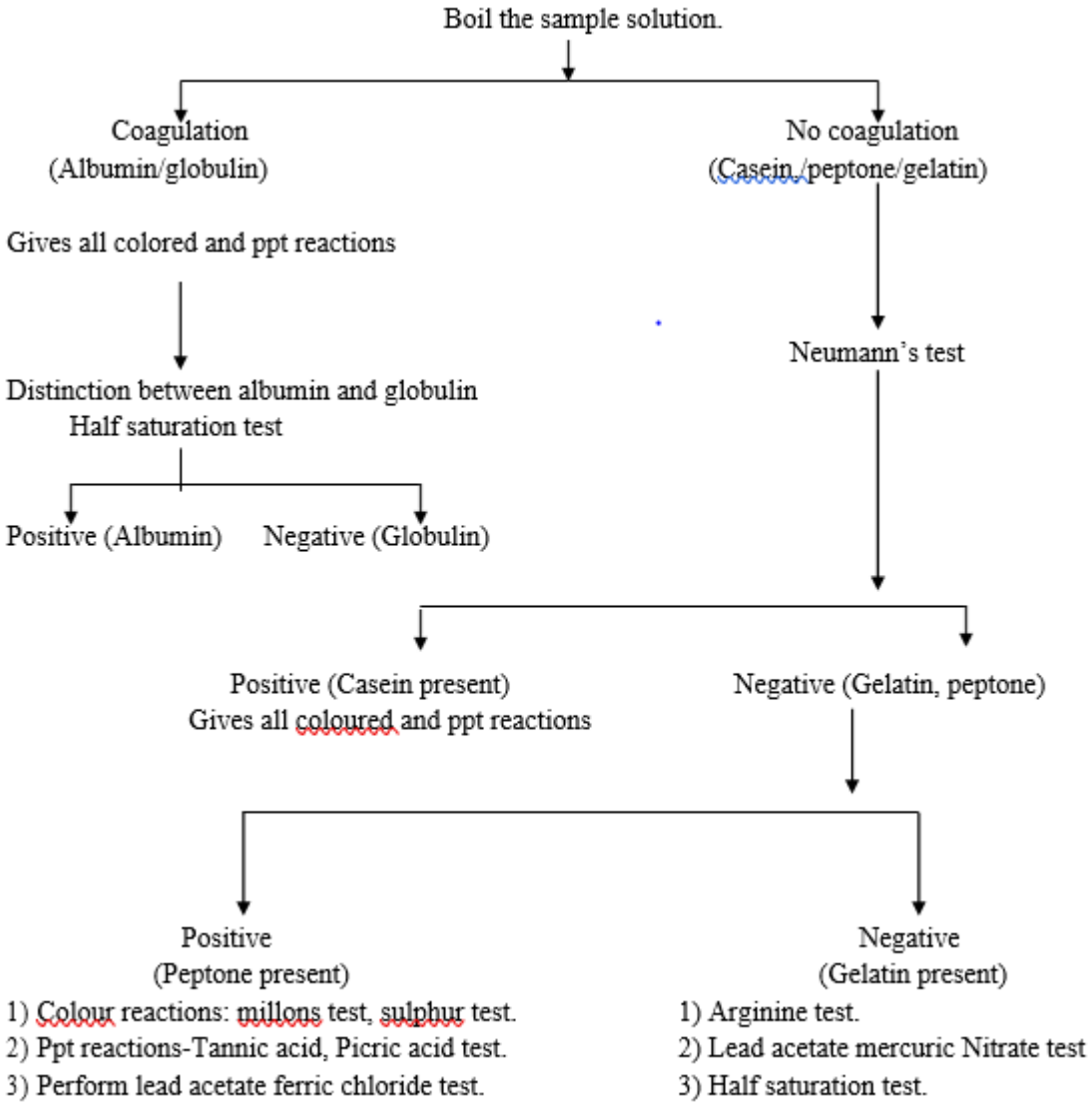
ii. Phosphoproteins: Prosthetic group is phosphoric acid. **Ex:** Casein.

iii. Chromoproteins: Prosthetic group is iron pigment. **Ex:** Haemoglobin.

c) Derived proteins: Intermediate degradation products of protein hydrolysis.

Ex: Peptone, proteases.

SCHEME FOR DETECTION OF UNKNOWN PROTEIN SAMPLE:



Test	Observation	Inference
Coagulation test:sample+water--boil	Coagulation	Proteins present. (albumin/globulin)
Biuret test:2 ml sample soln. + 2 ml 10% NaOH +2-3 drops of 1% $CuSO_4$soln.	Violet colour.	Proteins present.
Ninhydrin test: 2 ml sample soln. + 0.5 ml ninhydrin soln. Boil for 2 min.cool.	Blue colour.	Proteins present.
Xanthoproteic test: 2 ml sample soln.+1 ml conc. HNO_3 boil, cool + 40% NaOH drop by drop.	Orange coloured soln.	Proteins present.
Millon's test: 2 ml sample soln. + 2 ml millons reagent, boil, cool+ fewdrops of $NaNO_2$	Red ppt. and soln. becomes red.	Proteins present.
Aldehyde test: 2 ml sample soln. + 5 drops of millon's reagent + 5drops of formalin + 2 ml conc.H_2SO_4 from the side of test tube.	Violet ring at the junction	Proteins present. (peptone)
Arginine test: 2 ml sample soln. + 1 ml of 10% NaOH soln.+5 drops of 1% α-naphthol mix + 5 drops of freshly prepared sod.hypobromite soln.	Intense red coloured is obtained.	Proteins present. (gelatin)
Sulphur test: 3 ml of sample sol, n. +2 ml NaOH. Boil, cool + few drops oflead acetate, soln.	Black ppt.	Proteins present. (peptone)
Neumann's test: a) 5ml sample sol, n. + 3 drops of chlorophenol red indicator. Sol, n. turns pink + 1 % acetic acid drop wise till colour	Precipitate.	Casein present.

changes to yellow. b) above ppt + 3 to 4 drops of conc. H_2SO_4 10 to 12 drops of conc. HNO_3, heat until mix is colourless. Cool + few ml of amm. molybdate sol, n.	Shining yellow or cannery yellow colour is obtained.	Casein present.
Haller's test: 2 ml of conc. HNO3 + 2 ml of sample soln. from the side of the test tube.	A white ring at junction of two fluids.	Albumin present.
Half saturation test: 5 ml of sample soln. + 5 ml saturated Amm.sulphate soln. Keep for 5 min, ppt. obtained. Filter the above filtrate + 2 ml of 40% NaOH + 5 drops of 1% $CuSO_4$ soln.	Violet colour. No violet or pink colour.No rosy pink colour.	Albumin present. Globulin,gelatin, Casein present.
Full saturation test: 5 ml sample soln. + Amm.sulphate powder till the soln. saturated. Ppt is obtained, filter and perform biuret test with filtrate.	No violet or purple or Rosy pink colour.	Peptone present. Albumin present.

SIGNIFICANCE OF VARIOUS TESTS:

Biuret test:

The characteristic linkage in biuret is the - $CONH_2$-linkage or peptide linkage. The protein is warmed gently with 10% NaOH solution and then a drop of 5% copper Sulphate solution is added, the formation of a coloured co-ordination complex between the cupric ions, the nitrogen of the –CONH- and the oxygen of water. It is positive with all proteins.

Ninhydrin test:

Ninhydrin is a powerful oxidizing agent which causes oxidation decarboxycation of alpha - amino acids yielding Co_2, NH_3 & an aldehyde.

Millon's test:

Containing mercuric nitrite – dissolved in nitric acid HNO_3 (CONC) and then adding less free amount of nitrous acid. Phenolic compound, when heated with mercuric nitrate in nitric acid and trace of nitrous acid develop a red colour, tyrosine containing protein gives positive result.

Xanthoproteic test

Proteoses & peptones do not form precipitate with HNO_3 but their solutions become yellow & then orange when made alkaline. The white precipitate of protein is found by the addition of HNO_3, due to formation of metaproteins insoluble in HNO_3. The yellow colour is due to the formation of nitro – compounds from the protein molecule containing benzene ring. These nitro compounds in alkaline medium ionize freely & produce deep yellow or orange colour.

Sulphur Test:

Sulphur of the sulphur – containing amino acids reacts with the Sodium hydroxide forming sodium sulphide. A black or brown precipitate of lead sulphide is formed as a result of the reaction between sodium sulphide & lead acetate. This lead sulphide is insoluble in dilute Hcl.

Arginine test:

Arginine in presence of alcoholic α-naphthol forms a complex with sodium hypochlorite developing a bright – red colour.

Experiment No: 7

IDENTIFICATION OF PROTEINS-S#1

AIM: Identify the Protein present in the given sample.

Test	Observation	Inference
Heat Coagulation test: **Biuret test:** **Ninhydrin test:** **Xanthoproteic test:** **Millon's test:** **Aldehyde test:** **Arginine test:** **Sulphur test:** **Neumann's test:** **Haller's test:** **Half saturation test:Full** **saturation test:**		

REPORT:

Experiment No: 8

IDENTIFICATION OF PROTEINS- S#2

AIM: Identify the Protein present in the given sample.

Test	Observation	Inference
Heat Coagulation test: **Biuret test:** **Ninhydrin test:** **Xanthoproteic test:** **Millon's test:** **Aldehyde test:** **Arginine test:** **Sulphur test:** **Neumann's test:** **Haller's test:** **Half saturation test:** **Full saturation test:**		

REPORT:

QUANTITATIVE ESTIMATIONOF SUGARS & PROTEINS

Experiment No: 9

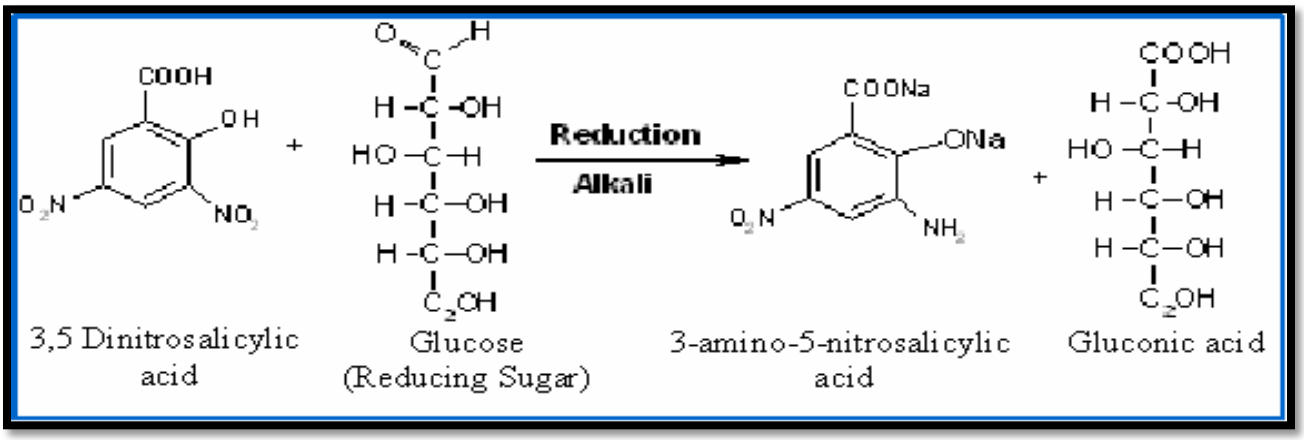
QUANTITATIVE ANALYSIS OF REDUCING SUGAR(GLUCOSE) BY DNS REAGENT METHOD

AIM: To estimate glucose in a given sample by DNS reagent method.

APPARATUS: Conical flask, Burette, Pipette**. Chemicals & Reagents:** DNS reagent, distilled water.

PRINCIPLE:

Several reagents have been employed which assay sugars by using their reducing properties. This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-



amino-5-nitrosalicylic acid under alkaline conditions, as illustrated in the equation below:

The chemistry of the reaction is complicated since standard curves do not always go through the origin and different sugars give different color yields. The method is therefore not suitable for the determination of a complex mixture of reducing sugar.

Reaction dynamics:

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

oxidation

aldehyde group----------> carboxyl group

reduction

3,5-dinitrosalicylic acid----------> 3-amino,5-nitrosalicylic acid

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color.

Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds are not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar is added to this sample. The increase in the absorbance upon the second color development is equivalent to the incremental amount of sugar added.

PROCEDURE:

- Preparation of DNS reagent:-

1. Preparation of sodium pot.tartarate:

Dissolve 45gm of sodium pot.tartarate in 75ml of water.

2. Preparation of 3,5-DNS solution:

Dissolve 1.5gm of DNS reagent in 3ml of molar NaOH.

3. Preparation of DNS solution:

4. Mix (1) and (2) ---> Makeup volume by 150ml by using water.

- Preparation of standard stock solution:

1. 0.1gm(100mg) in 100ml distilled water gives 1mg/ml solution.(1000mcg/ml)

- Preparation of aliquots of standards:

STANDARD STOCK SOLUTION						BLANK	SAMPLE
GLUCOSE SOL.	0.2ml	0.4 ml	0.6 ml	0.8 ml	1 ml	-	-
WATER	0.8 ml	0.6 ml	0.4 ml	0.2 ml	0 ml	1 ml	-
DNS REAGENT	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
HEAT IN WATER BATH & COOL DOWN TO RT							
Makeup with water (10ml)	7ml	7ml	7ml	7ml	7ml	7ml	7ml

CALCULATION:

CONCENTRATION (mcg/ml) x-axis	ABSORBANCE (y-axis)

(graph to be attached)

REPORT:

Experiment No: 10

QUANTITATIVE ANALYSIS OF PROTEINS BY BIURET REAGENT METHOD

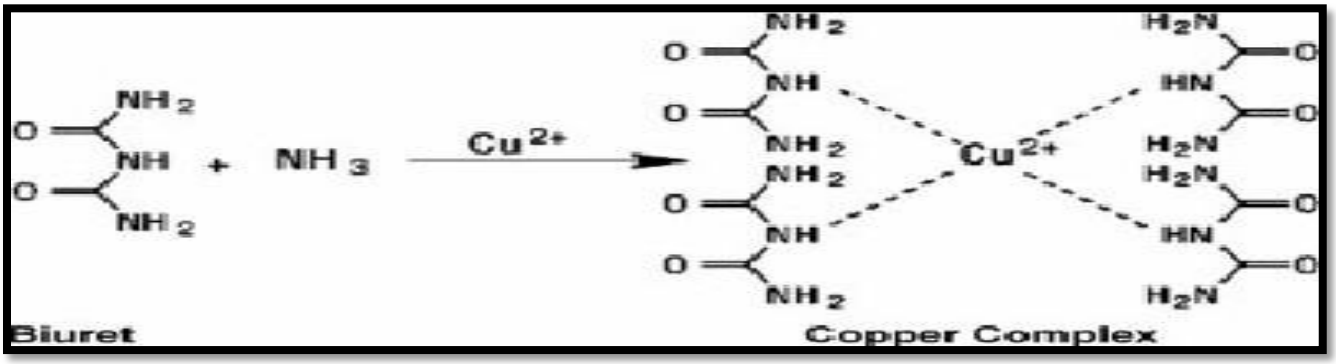
AIM: To estimate the amount of protein by using Biuret reagent method.

APPARATUS AND GLASS WARES REQUIRED: Test tubes, Pipettes, Colorimeter, etc.,

CHEMICALS & REAGENTS: Biuret reagent, distilled water, protein standard(Albumin)

PRINCIPLE:

The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a purple colour which can be measured at 540 nm.



Reagents Required:

- PREPARATION OF BIURET REAGENT-
- Dissolve 45gm of sodium pot.tartarate in 400ml of 0.2M NaOH. Add 15gm CuSO4 in powder to this & stir well. After dissolving the salt, add 5gms Potassium Iodide & make up the sol. to 1 litre with 0.2M NaOH, filter if necessary.
- Protein Standard: 5 mg BSA/ml.

PROCEDURE:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test tubes.
2. Pipette out 1 ml of the given sample in another test tube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
4. Now add 3 ml of Biuret reagent to all the test tubes including the test tubes labeled 'blank' and

'unknown'.

5. Mix the contents of the tubes by vortexing / shaking the tubes and warm at 37 ºC for 10 min.
6. Now cool the contents to room temperature and record the absorbance at 540 nm against blank.
7. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis.
8. Then from this standard curve calculate the concentration of protein in the given sample.

OBSERVATION:

Volume of Standard BSA (ml)	Volume of Distilled water (ml)	Conc. of protein (inmg)	Vol. of Biuret reagent (ml)		Absorbance@ 540nm
				INCUBATE AT 37ºC FOR 10MINS & COOL	

(graph need to be attached)

Notes:

Bovine serum albumin (also known as BSA or "Fraction V") is a Serum albumin protein derived from cows. It is often used as a protein concentration standard in lab experiments.

The nickname "Fraction V" refers to albumin being the fifth fraction of the original Edwin Cohn purification methodology that made use of differential solubility characteristics of plasma proteins. By manipulating solvent concentrations, pH, salt levels, and temperature, Cohn was able to pull out successive "fractions" of blood plasma. The process was first commercialized with human albumin for medical use and later adopted for production of BSA.

REPORT:

The given unknown sample contains ---------- mg protein/ml.

QUALITATIVE ANALYSIS OF URINE

INTRODUCTION

The major function of the urinary system is to remove waste products from our bodies and help regulate the volume and composition of blood by the production and excretion of urine. The urinary system consists of two kidneys, two ureters, one bladder, and one urethra. The composition of urine can vary greatly and constantly fluctuates with dietary intake (food and water) and metabolic activity. Urine consists mostly of water with various organic and inorganic substances such as urea, uric acid, creatine, sodium chloride, ammonia, sulfates, and phosphates as its principal ingredients. A clinical examination of urine can provide a convenient, cost effective and non-invasive means of assessing kidney function and providing an overall assessment of our body's health.

Typically, a complete urinalysis involves an examination of the physical characteristics of urine, a chemical analysis and a microscopic examination of urine sediment. Urine should be collected in a clean container, stored in a cool place, and tested as soon as possible.

Routine Urinalysis (Routine-UA):

- It consists of a group of tests performed as part of physical examination. It involvesmacroscopic and microscopic analysis.

Type of analysis:

- macroscopic analysis: **physical** characteristics and **chemical** analysis.
- microscopic examination: urine sediment is examined under microscope to identify the components of the urinary sediments.

A. PHYSICAL CHARACTERISTICS OF URINE - The physical characteristics of urine include observations and measurements of color, turbidity, odour, specific gravity, pH and volume. Visual observation of a urine sample can give important clues as to evidence of pathology.

1. COLOR

The color of normal urine is usually light yellow to amber. Generally the greater the solute volume the deeper the color. The yellow color of urine is due to the presence of a yellow pigment, **urochrome**. Deviations from normal color can be caused by certain drugs and various vegetables such as carrots, beets, and rhubarb.

2. ODOUR

Slightly aromatic, characteristic of freshly voided urine. Urine becomes more ammonia-like upon standing due to bacterial activity.

3. TURBIDITY

Normal urine is transparent or clear; becomes cloudy upon standing. Cloudy urine may be evidence of phosphates, urates, mucus, bacteria, epithelial cells, or leukocytes.

4. pH

Ranges from 4.5 - 8.0. Average is 6.0, slightly acidic. High protein diets increase acidity. Vegetarian diets increase alkalinity. Bacterial infections also increase alkalinity.

5. SPECIFIC GRAVITY

The specific gravity of urine is a measurement of the density of urine - the relative proportions of dissolved solids in relationship to the total volume of the specimen. It reflects how concentrated or dilute a sample may be. Water has a specific gravity of 1.000. Urine will always have a value greater than 1.000 depending upon the amount of dissolved substances (salts, minerals, etc.) that may be present. Very dilute urine has a low specific gravity value and very concentrated urine has a high value. Specific gravity measures the ability of the kidneys to concentrate or dilute urine depending on fluctuating conditions. Normal range 1.005 - 1.035, average range 1.010 - 1.025.

Low specific gravity is associated with conditions like Diabetes insipidus, excessive water intake, diuretic use or chronic renal failure.

High specific gravity levels are associated with diabetes mellitus, adrenal abnormalities or excessive water loss due to vomiting, diarrhoea or kidney inflammation. A specific gravity that never varies is indicative of severe renal failure.

Specific gravity can be determined by either of two methods using a refractometer or a urinometer.

a. **Refractometer -** measures the refractive index of urine which parallels the specific gravity. Procedure:

1. Collect mid-stream sample of urine in collection cup.
2. Pipette 1-2 drops of urine into the plastic chamber located on the top of the refractometer. Be

sure that the plastic is pressed firmly down in place on the refractometer.

3. Determine the specific gravity of the urine by looking through the refractometer and determining the value on the scale on the left hand side. The specific gravity value is where the light and dark intersect on the scale.

4. Clean the refractometer with kim wipes.

b. **Urinometer -** Is a weighted, bulb shaped device that has a specific gravity scale on the stem end.

Procedure:

1. Fill the cylinder with enough urine so that the urinometer will float in the urine and not touchthe bottom.

2. Be careful not to drop the urinometer in the cylinder! Gently release it in order not to breakor burst the cylinder. It should NOT touch the sides or bottom of cylinder.

3. The specific gravity can be read on the scale on the stern of the urinometer at the meniscus.

4. The specific gravity of water is 1.000 with respect to temperature. The urinometer can be checked periodically against this standard to ensure quality control at that temperature.

B. CHEMICAL ANALYSIS: - Includes various tests to identify normal organic/inorganic ad abnormal constituents which are present in urine.

ABNORMAL CONSTITUENT	ASSOCIATED CAUSES
Protein (albumin) –	Albumin is normally too large to pass through glomerulus. Indicates abnormal increased permeability of the glomerulus membrane. Non- pathological causes are: pregnancy, physical exertion, increased proteinconsumption. Pathological causes are: glomerulonephritis bacterial toxins, chemical poisons.
Glucose –	Glycosuria is the condition of glucose in urine. Normally the filtered glucoseis reabsorbed by the renal tubules and returned to the blood by carriermolecules. If blood glucose levels exceed renal threshold levels, the untransported glucose will spill over into the urine. Main cause: diabetes mellitus

Ketones –	Ketone bodies such as acetoacetic acid, beta-hydroxybutyric acid, and acetone can appear in urine in small amounts. These intermediate by- products are associated with the breakdown of fat. Causes: diabetes mellitus,starvation, diarrhea
Bilirubin –	Bilirubin comes from the breakdown of hemoglobin in red blood cells. The globin portion of hemoglobin is split off and the heme groups of hemoglobinis converted into the pigment bilirubin. Bilirubin is secreted in blood and carried to the liver where it is conjugated with glucuronic acid. Some is secreted in blood and some is excreted in the bile as bile pigments into the small intestines. Causes: liver disorders, cirrhosis, hepatitis, obstruction of bile duct
Urobilinogen –	Bile pigment derived from breakdown of hemoglobin. The majority of this substance is excreted in the stool, but small amounts are reabsorbed into the blood from the intestines and then excreted into the urine. Causes: hemolytic anemias, liver diseases
Hemoglobin –	Hemoglobinuria is the presence of hemoglobin in the urine. Causes: hemolytic anemia, blood transfusion reactions, massive bums, renal disease
Red blood cells –	Hematuria is the presence of intact erythrocytes. Almost always pathological. Causes: kidney stones, tumors, glomerulonephritis, physicaltrauma
White blood cells –	The presence of leukocytes in urine is referred to as pyuria (pus in the urine). Causes: urinary tract infection
Nitrite –	Presence of bacteria. *Causes: urinary tract infection*

C. MICROSCOPIC EXAMINATION

Examination of urine sediment may reveal the presence of different types of cells such as epithelial cells, leukocytes, erythrocytes, or renal cells. Different types of crystals, yeast, bacteria, or casts may also be present. Casts are cylindrical structures created by protein precipitation in the renal tubules.

Procedure:

1. Transfer urine sample to a conical centrifuge tube.
2. Centrifuge your sample at a **moderate speed for 5 minutes**.
3. Discard the supernatant (fluid off the top) by quickly pouring off fluid.
4. Tap tube with index finger to mix sediment with remaining fluid.
5. Make a wet mount of sample by transferring 1 drop of material to a slide and covering with acoverslip.
6. Examine the sample under the microscope under low and high power.
7. Identify what you see by comparing to charts. Draw a few of your observations.

CLINICAL SIGNIFICANCE: INORGANIC CONSTITUENTS

INORGANIC CONSTITUENT	NORMAL VALUE	CLINICAL SIGNIFICANCE
CHLORIDES (expressed as NaCl)	9.0 gm/lit	Reduced in fever, nephritis, diarrhoea , vomiting etc.
PHOSPHATES (expressed as P_2O_4)	2.5 gm/lit	Increased in osteomalacia, rickets, periosteosis, and hyperthyroidism.Decreased in pregnancy and hypothyroidism.
SULPHATES (expressed as SO_4)	1.5 gm/lit	Increased fever and diseases associated with increased metabolism. Decreased in diseases associated with loss of appetite and diminishedmetabolism.
AMMONIA	0.7 gm/lit	Increased in abnormal protein metabolism, diabetes ,ketosis and cystitis. Decreased in nephritis.
BICARBONATES	In traces...	----------

QUALITATIVE ANALYSIS OF NORMAL INORGANICCONSTITUENTS OF URINE

TEST	OBSERVATION	INFERENCE
Test for bicarbonate Take 3ml sample add dil.HCl or dilH_2So_4.	Effervescence of Co_2 gas is produced**.**	Presence of bicarbonates
Test for chloride Take 3 ml urine; add 1ml of HNO_3 (to prevent ppt.of urates by $AgNO_3$ and 1 ml of $AgNo_3$.	White curdy precipitate of silver chloride soluble in ammonium hydroxide solution.	Presence of chlorides
Test for phosphate Take 3 ml of urine, add 2ml of conc. HNO_3 and pinch of Ammonium molybdate. Warm on water bath if green colour is observed add few drops of HNO_3 and heat.	Canary yellow coloured ppt of ammonium phosphomolybdate is formed.	Presence of phosphate
Test for sulphate 5 ml. of urine + 1 ml. Of conc. HCl (to prevent ppt. Of phosphates) + 2 ml. of Barium chloride($BaCl_2$).	An opaque milkiness or a thick white ppt. of $BaSO_4$, insoluble in conc. Hcl.	Presence of sulphates
Test for ammonia i) 10 ml. of urine + 4 drops of phenolphthalein + drop by drop NaOH till a faint pink colour is obtained. Boil the urine, and hold a red litmus paper in vapour. Expose the vapours to a glass rod dipped in con.Hcl	The red litmus turns toblue. Dense white fumes are observed.	Presence of ammonia
Test for calcium 10 ml. of urine + few drops of NaOH + 1 % acetic acid + 2-3 ml of ammonium oxalate solution.	White ppt. of calcium oxalate.	Presence of calcium

QUALITATIVE ANALYSIS OF ABNORMAL CONSTITUENTS OF URINE SAMPLE

TEST	OBSERVATION	INFERENCE
Test for proteins (a) **Haller's nitric acid ring test:** (Principle: Nitric acid causes precipitationof protein) To 3 ml of nitric acid in a tube add 3 ml of urine by the wall of the tube in such a way that the two liquids do not mix.	White ring appears at the junction of the two fluids	Indicates the presence of albumin
Sulphosalicylic acid test: (Principle: Albumin, the protein, is denatured by sulphosalicylic acid a coagulation.) Add a few drops of Sulphosalicylic acid to 2ml of urine. Let it stand for 5mins & observe turbidity.	Turbidity appears	Indicates the presence of albumin
c) **Heat coagulation test**: (Principle: Thealbumin is coagulated after being heated.) Fill 3/4th of the test tube by urine. Heat theupper 1/3rd of the test tube by a smallflame.	Turbidity appears on the heated portion of the tube.	Indicates the presence of albumin
Tests for glucose a) **Bendict's Test:** (Principle: Copper sulphate of Benedict's qualitative solution is reduced by reducing substances on boiling to form the coloured precipitate of cuprous oxide.)	Blue color appears Light green precipitateappears Green precipitate appears Yellow precipitate appears Brick red precipitate appears	Sugar Absent 0.1-0.5 % of reducing sugar present 0.5 to 1.0 % of reducing sugar present 1-2 % reducing sugarpresent Above 2 % reducing sugarPresent
b) **Fehling's test:** 2ml of Urine and add 2.5ml of Fehling's A& B each. Heat & cool.	Red ppt. is seen.	Glucose is present.

Test for ketone bodies **Rothera's test:** (Principle: Acetoacetic acid forms a complex with nitroprusside in alkaline solution developing a permanganate colour) Saturate 5 ml of urine with ammonium sulphate by shaking vigorously. Then add 2 drops of freshly prepared 5% solution of sodium nitroprusside and 1 ml of ammonium hydroxide/strong ammonia along the sides of test tube. Allow it to stand in a rack for a while without disturbing. **Gerhard's test:** To 3ml of urine add 0.5ml of ferric chloridde drop by drop.	A permanganate colour develops just above the layer of un dissolved ammonium crystals. Violet colour is formed.	Indicates the presence of Ketone bodies like acetoacetic acid. (Acetone is absent) Indicates the presence of Ketone bodies like acetoacetic acid.
Tests for Bile salts **Hay's test:** To 5ml of urine in beaker, sprinkle little sulphur powder & observe without shaking.	Sulphur sinks to bottom	Bile salts are present.
Tests for Bile Pigments To 5ml of urine add 2ml of 10% Bacl2 and a pinch of Mag.sulphate. Mix well & allow to stand for 5mins. Filter the solution, unfold the filter paper & add few drops of fouchets reagent on ppt.	Blue or light green colour is observed.	Presence of bile pigments.
Tests for Blood **Benzidine test:** To 3ml of urine add mixture of 2 drops ofbenzidine & 2 drops of Hydrogen peroxide.	Blue/green which is stable is absent.	Absence of blood.

ABNORMAL CONSTITUENTS OF URINE

Proteinuria (protein in urine)

Small molecular weight proteins (such as Peptide Hormones, Insulin, Glucagon, Growth Hormone)can appear in the GF, these filtered proteins are largely reabsorbed by the proximal tubules via pinocytosis. Thus normally only trace amount of proteins are present in the urine that are not readilydetected. Albumin is the major protein present in **PROTEINURIA.**

Causes of Proteinuria:

a) Physiological proteinuria (0.5% of Protein Present)

Examples of such cases include severe exercise, standing for a long time (due to temporaryimpairment of renal circulation),Pregnancy.

b) Pathological Proteinuria

1. Pre-Renal : when thePrimary causes are factors operating before the kidney, such as a heart problem causing an impaired renal circulation, multiple myeloma.
2. Renal : when the lesion is in the kidney as in glomerulonephritis.
3. Post-Renal : as inflammation of the lower urinary tract.

GLYCOSURIA : It is a term used to refer to the presence of an unusual amount of reducing sugar in urine,once the specific sugar is identified it is given a more specific description glucosuria, fructosuria. This term is used to indicate an abnormal detection of glucose in urine. Normally not more than 10-20mg/dl,unusual amounts of glucose can be found in urine after anesthesia or emotional stress. It can be due to hyperthyroidism.Renal glucosuria which is caused by a disorder of the renal tubular function.

KETONURIA(KETONE BODIES) : It refers to the presence of unusual amount of ketone bodies in urine.Normally only 3-15 mg are excreted per day.Ketonuria can occur in cases such as ketosis and in starvation,excess fat metabolism.

Bilirubin : it is not present normally in significant amount since it is mainly excreted through bile, unusual amounts of bilirubin is detected in urine in cases of obstructive jaundice and unusual amounts of uribilinogen seen in cases of hemolytic jaundice.

Experiment No: 11

QUALITATIVE ANALYSIS OF ABNORMAL CONSTITUENTS PRESENT IN UNKNOWN SAMPLE

AIM: To identify any presence of abnormal constituents which can be found in unknown urine sample.

TEST	OBSERVATION	INFERENCE
Test for proteins (a) **Haller's nitric acid ring test:** (Principle: Nitric acid causes precipitationof protein) To 3 ml of nitric acid in a tube add 3 ml of urine by the wall of the tube in such a way that the two liquids do not mix. **Sulphosalicylic acid test:** (Principle: Albumin, the protein, is denatured by sulphosalicylic acid a coagulation.) Add a few drops of Sulphosalicylic acid to 2 ml of urine. Let it stand for 5mins & observe turbidity. (c) **Heat coagulation test**: (Principle: The albumin is coagulated after being heated.) Fill 3/4th of the test tube by urine. Heat the upper 1/3rd of the test tube by a small flame.		
Tests for glucose a) **Bendict's Test:** (Principle: Copper sulphate of Benedict's qualitative solution is reduced by reducing substances on boiling to form the coloured precipitate of cuprous oxide.)		
b) **Fehling's test:** 2ml of Urine and add 2.5ml of Fehling's A & B each. Heat & cool.		

Test for ketone bodies • **Rothera's test:** (Principle: Acetoacetic acid forms a complex with nitroprusside in alkaline solution developing a permanganate colour) Saturate 5 ml of urine with ammonium sulphate by shaking vigorously. Then add 2 drops of freshly prepared 5% solution of sodium nitroprusside and 1 ml of ammonium hydroxide/strong ammonia along the sides of test tube. Allow it to stand in a rack for a while without disturbing. • **Gerhard's test:** To 3ml of urine add 0.5ml of ferric chloridde drop by drop.		
Tests for Bile salts **Hay's test:** To 5ml of urine in beaker, sprinkle little sulphur powder & observe without shaking.		
Tests for Bile Pigments To 5ml of urine add 2ml of 10% Bacl2 anda pinch of Mag.sulphate. Mix well & allow to stand for 5mins. Filter the solution, unfold the filter paper & add few drops of fouchets reagent on ppt.		
Tests for Blood **Benzidine test:** To 3ml of urine add mixture of 2 drops of benzidine & 2 drops of Hydrogen peroxide.		

QUALITATIVE ANALYSIS OF NORMAL ORGANIC CONSTITUENTS OFURINE

TEST	OBSERVATION	INFERENCE
Test for urea **Effervescence test:** 3ml of urine+5 drops of sodium hypobromite. **Specific urease test:** 3ml urine + 2 drops ofphenolphthalein + pinch of urease powder. Mix & allow to stand for 5mins.	Brisk effervescence is seen	Urea is present.
Test for uric acid a) **Phosphotungstinic reduction test:** 3ml urine + 0.5ml of Phosphotungstinic acid + few drops of sodium carbonate.	Blue colour develops	Uric acid is present.
Test for creatinine **Wayne's test:** 3ml urine+ 3ml sod.nitroprusside + 10% NaOH. **Jaffe's test** 3ml urine + 1ml saturated picric acid + 1ml 10% NaOH.	Ruby red colour which changes to yellow Orange red colour is formed. Deep orange colour is seen	Creatinine is present.
Test for Ethereal sulphate: 3ml urine sample+ 1ml of conc. Hcl + 2ml $BaCl_2$. Mix & filter. Divide the filtrate into 2 parts. Boil one test tube & compare control.	Turbidity is not seen	Ethereal sulphate is absent.
Test for Urobilinogen: 3ml urine + 1ml of elhrich reagent. Mix & allow to stand for 5mins.	No reaction	Urobilinogen is absent.

CONSTITUENTS	NORMAL	SIGNIFICANCE
Urea	25-30g/lit	Major product of protein metabolism. Increased in fever, diabetes and excessive adrenal cortex activity.
Uric acid	0.7g/lit	Increased in leukaemia, severe liver disease, and certain stages of gout.
Creatine and creatinine	1.2-1.7 g/lit	Increased in dystrophy and starvation.
oxalates	10-30 mg/lit	Increased in diabetes and liver disorder.
Hippuric acid	0.7 g/lit	Increased after vegetable diet.
Urinary pigments	In traces	Increased in lead poisoning

QUANTITATIVE ANALYSIS OF BLOOD

Experiment No: 12

ESTIMATION OF CREATININE IN SERUM

AIM: To determine the amount of creatinine in given serum sample

APPARATUS REQUIRED: Pipette, volumetric flask, beaker and cuvette,

CHEMICALS REQUIRED: 1% picric acid, 10% NaOH, standard creatinine solution, urine sample,distilled water.

PRINCIPLE:

Creatinine is the breakdown product of creatine, which is an important part of muscles. Creatinine is removed by the body entirely by the kidneys. Creatinine can also be measured by blood test. Creatinine in urine is estimated by modified follin - wu method using photoelectric method. Urine is allowed to react with picric acid in alkaline medium to form a orange coloured complex, creatinine picrate (Jaffe's reactions). The optical density or absorbance of this complex is measured at a wavelength of 520nm. The O.D of sample is compared to that of standard solution having known amount of creatinine.

PROCEDURE:

SAMPLE NAME	TEST	STANDARD	BLANK
Distilled water	-	-	2.5 ml
Urine sample	2.5 ml	-	-
Standard creatinine		2.5 ml	-
10% NaOH	1 ml	1 ml	1 ml
1% picric acid	1 ml	1 ml	1 ml

Mix well and allow to stand for 5 minutes. Measure the optical density of each solution at 520 nm.

Clinical Significance:

Any structure that impairs the functions of kidney is likely to raise the creatinine level in blood. It is important to recognize whether the process leading to kidney dysfunction is longstanding or recent. Recent elevation may be more easily treated and reversed.

The most common cause of longstanding kidney disease in adults are high blood pressure and diabetes

mellitus. Certain drugs can sometimes cause abnormally elevated creatinine level. Serum creatinine can also transiently increase after injection of a large amount of dietary meat, thus nutrition can play a role in creatinine measurements.

Data collection:

Optical density of standard =

Optical density of blank =

Optical density of test=

CALCULATIONS:

Concentration of standard creatinine = 1 mg/ ml

= 1ml of stock + 99 ml of H_2O

Working stock = $\frac{1\ mg}{100ml}$

Amount of creatinine = $\frac{\text{0.D of test} - \text{0.D of blank}}{\text{0.D of standard} - \text{0.D of blank}} \times \frac{\text{conc.of std} \times \text{vol.of std} \times 100}{\text{vol.of std} \times \text{dilution factor of sample}}$

REPORT: The total amount of creatinine is found to be_____mg/dl.

Experiment No: 13

PREPARATION OF FOLIN-WU FILTRATE FROM BLOOD

AIM: To prepare follin - wu filtrate.

APPARATUS REQUIRED: Pipette, volumetric flask, beaker and funnel.

CHEMICALS REQUIRED: 0.65N H_2SO_4, 10% sodium tungstate solution.

PRINCIPLE:

Since the blood is coloured , it cannot be used directly for calorimetric reactions. A follin - wu filtrate is prepared by precipitating the protein in the blood by the use of sodium tungstenic acid. In order to complete the precipitation , H_2SO_4 is added.

PROCEDURE:

In a centrifuge tube, add 7ml of distilled water and 1ml of blood. The contents are mixed well, 1 ml of sodium tungstate solution is added along with 1 ml of 0.66N H_2SO_4 and is added to above mixture in portions by using a graduated pipette with constant shaking. The colour of precipitate gradually turns from red to brown. The solution is kept for 10 minutes and filtered. The filtrate is cleaned and colorless. The obtained filtrate is known as follin - wu filtrate.

REPORT:

Experiment No: 14

ESTIMATION OF GLUCOSE IN BLOOD (FOLLIN - WU METHOD)

INTRODUCTION AND PURPOSE OF THE EXPERIMENT:

The importance of testing the blood glucose level comes from the fact that the brain cells are very dependent on the extracellular glucose concentration for their energy supply. Hypoglycemia is likely to impair cerebral functions as well as do the hyperglycemia especially of rapid onset, which can cause cerebral dysfunction by affecting extracellular osmolarity.

AIM: ***To estimate the amount of glucose in blood.***

APPARATUS: Pipette, volumetric flask, beaker, spectrophotometer, cuvette, water bath and follin-wu.

CHEMICALS: Alkaline $CuSO_4$, phosphomolybdic acid, 0.66 N H_2SO_4, sodium tungstate.

PRINCIPLE:

The proteins in the blood are precipitated by addition of tungstenic acid and H_2SO_4. The protein free filtrate containing glucose is then made to react with alkaline $CuSO_4$ to form cuprous oxide. The cuprous oxide formed is treated with phosphomolybdic acid and is reduced to phosphomolybdus acid which gives blue solution. The intensity of blue solution is measured using colorimeter and compared with standard and blank. O.D are used to estimate amount of blood glucose in 100 ml of blood.

PROCEDURE:

In a 50ml beaker, 7 ml of distilled water and 1 ml of blood is added. The contents are mixed well, 1 ml of sodium tungstate solution is also added along with 1 ml of 0.66 N H_2SO_4 and is added to above mixture in portions by using a graduated pipette with constant shaking. The colour of the precipitate gradually turns from red to brown. The solution is kept for 10 minutes and then filtered. The filtrate obtained is clean and colorless. The obtained filtrate is known as follin - wu filtrate.

	TEST	STANDARD	BLANK
Distilled Water	-	-	0.2 ml
Serum (Follin-wu)	0.2 ml	-	-
Std. glucose	-	0.2 ml	-
Alkaline $CuSO_4$	2 ml	2 ml	2 ml
Phosphomolybdic acid	2 ml	2 ml	2 ml

Boil the test tubes for 15 - 20 minutes on a boiling water bath and cool for 2 minutes.

Mix well and allow to stand for 5 minutes. Make up the volume of the solution to 25 ml with distilled water. Measure the optical density of each solution at 530nm.

REPORT:

The amount of glucose present in 100 ml of blood =_____mg.

Experiment No: 15

QUANTITATIVE ESTIMATION OF SERUM CHOLESTEROL BYLIBERMANN BURCHARD'S METHOD

AIM: To estimate the amount of serum cholesterol present in the given sample by Libermann Burchard's method.

APPARATUS REQUIRED: Pipette, centrifuge tube, beaker, cuvette, colorimeter.

REAGENTS REQUIRED:

a) Alcohol & ether mixture - 90% ethyl alcohol and ether mixed in ratio of 3 : 1

b) Chloroform

c) Acetic anhydride & sulphuric acid mixture - mixed in the ratio of 20 : 1 before use

d) Stock standard solution - 200 mg of chemically pure cholesterol is dissolved in chloroformand made upto 1000ml with the same.

e) Working standard cholesterol solution - 1 ml of stock standard cholesterol solution is dilutedto 25 ml using chloroform. 5 ml of resultant solution consists of 0.4 mg of cholesterol.

PRINCIPLE:

Cholesterol and cholesteryl esters from serum is extracted into alcohol and ether mixture. The contents in centrifuge tube and protein free extract is evaporated to dryness. The cholesterol residue is then dissolved in chloroform and measured calorimetrically by Libermann Burchard's method. In this method, cholesterol reacts with acetic anhydride and H_2SO_4 mixture to give green colour, whose colour intensity can be measured using calorimeter at a wavelength of 680 nm.

PROCEDURE:

Pipette 12 ml of alcohol ether mixture in dry centrifuge tube. Add 0.1 ml of serum and shake vigorously for 1 min. Keep tube in vertical position for 30 minutes, then centrifuge at 1800 rpm for 5 minutes. Pour the supernatant fluid into a beaker and evaporate it on steam bath. Extract the residue twice with 2 ml portion of chloroform into a dry test tube. Make then volume to 5ml using chloroform and label it as test. Label the other two test tubes as standard and blank. Pipette 5 ml of standard cholesterol solution and 5 ml of chloroform in to blank respectively. Add 2ml of acetic anhydride -

H_2SO_4 mixture into all the 3 test tubes and place it in dark for 15 minutes. Measure the absorbance at 680 nm.

DATA COLLECTION:

Optical density of blank =

Optical density of standard =

Optical density of sample =

CALCULATION:

Cholesterol stock = 20 mg in 100 ml of $CHCl_3$ = 0.2 mg

Cholesterol working standard = 1 ml to 25 ml $CHCl_3$ (0.2 mg in 25 ml)

5ml = $\frac{0.2\times5}{25}$= 0.04 mg

Cholesterol in serum= $\frac{\text{0.D of test}-\text{0.D of blank}}{\text{0.D of standard}-\text{0.D of blank}} \times \frac{\text{conc.of std} \times 5}{\text{vol.of serum } (0.1\times5)} \times 100$

CLINICAL SIGNIFICANCE:

The normal total serum cholesterol in adults ranges from 130- 200 mg/dl. Hypercholestremia ia associated with conditions like hypothyroidism, uncontrolled diabetes, cholestasis, nephroticsyndrome, hypolipoproteinemia etc. Cholesterol level increases with age and also in third trimester of pregnancy. It also increases in those individuals who consume food rich with saturated fatty acids. Increased levels of cholesterol predisposing factor for atherosclerosis, coronary artery disease, myocardial infarction and other disorders of the heart. Hypercholestremia is seen in hyperthyroidism.

REPORT:

The amount of cholesterol present in given serum sample was found to be_____mg/dl.

BUFFERS

Experiment No: 16

PREPARATION OF BUFFER AND THEIR pH

INTRODUCTION AND PURPOSE OF EXPERIMENT:

Buffer solutions are defined as those solutions which changes the pH on addition of small quantity of acid or alkali solution from Henderson - Hasselback equation. The pH of buffer solution depends upon pKa constant and ratio of salt and acid. The ratio is considered to be same as the amount of salt and acid mixed together over the pH range 4 - 10 when the concentration of hydrogen and hydroxyl ions are very low and can be ignored.

AIM: To prepare buffer solutions and measure their pH.

EXPERIMENTAL SETUP:

Volumetric flask, burette, pipette, beaker.

PRINCIPLE:

The solutions that are able to resist the change in pH are termed as buffer solutions. A buffer solution consists of a mixture of weak acid and its salt or a weak base and its salt. To such solutions, when a small amount od acid or alkali is added, no significant change in pH takes place. Types of buffer solutions

These are mainly 2 types of buffer solutions:

Acid buffer solution - The solution having a mixture of weak acid and its salt. Basic buffer solution - The solution having a mixture of weak base and its salt.

SIGNIFICANCE OF BUFFER SOLUTIONS:

Buffer solutions help to maintain acid - base balance in our body. The maintenance of normalpH range within the body fluids becomes essential since the biochemical reactions that takes placein the living systems are very sensitive to even small changes in acidity and alkalinity. Saliva hasa pH between 6 and 7.5 which is necessary for functioning of ptylin , an enzyme in saliva which digests the carbohydrates.

Buffer systems of the body-

In our body, there are many buffer systems which prevent rapid changes in pH of a body fluid by converting strong acids and bases into weak acuds and bases. Thus, buffers are able to remove excess H^+ions from body fluids.

Following are the major buffer systems of the body fluids:

1) Carbonic acid - bicarbonate buffer system: It is found in plasma and kidney, an important regulator of blood pH. If there are excess of H^+ ions, the HCO_3^- ions act as a weak base and accepts H^+ to form carbonic acid. The carbonic acid dissociates further to yield CO_2 and H_2O molecules. If there is shortage of H^+, then the carbonic acid ionises to release H^+ ions and maintains pH.

2) Phosphate buffer system : The buffer system is able to maintain physiological pH at 7.4. K^+ ion is high in intracellular fluid. Therefore it is an important regulator of pH in the cytosol. This system occurs in the cells and kidneys. The system consists of monohydrogen or dihydrogen phosphate anion (HPO_4^- or $H_2PO_4^-$). When there is excess of H^+, HPO_4^- ion acts as a weak base by accepting a proton.

3) Protein (haemoglobin) buffer system: The system is most abundant buffer in body cell and plasma. The plasma protein and haemoglobin together constitute protein buffer of the blood. Proteins are composed of amino acids having carboxyl group and NH_2 group. When there occurs an excess of H^+ ions, the amino group acts as a base and accepts proton. The free carboxyl group can liberate proton to neutralize an alkaline condition.

Biological buffers-

Blood is an example of biological buffer where pH is maintained at 7.4 due to plasma and erythrocytes. Plasma contains carbonic groups and sodium salt of phosphoric acid as buffer. Erythrocytes contain hemoglobin or oxyhemoglobin and potassium salts of phosphoric acid as buffers.

Pharmaceutical Buffers-

These are important in ophthalmic solutions and parenteral preparations. Deviation from normal pH causes irritation. The buffering agents used in parenterals are acetate phosphate, citrate and glutamate buffers.

PROCEDURE:

Preparation of acid phosphate buffer-

Preparation of reagent-

1) Preparation of potassium dihydrogen phosphate:

2) Dissolve 27.218 g of potassium dihydrogen phosphate in little amount of H_2O and make upto 1000 ml.

3) Preparation of 0.2 N NaOH: Dissolve 8 g NaOH in small amount of water and make upto 1000 ml.

Preparation of alkaline - borate buffer-

Dissolve 12.36 g of boric acid and 19.91 g of potassium chloride in little amount of water and make upto 1000 ml.

Preparation of 0.2 N NaOH: Dissolve 8 g in little water and make upto 1000 ml.

REPORT:

ENZYMES

Experiment No: 17

STUDY OF ENZYMATIC HYDROLYSIS OF STARCH.

AIM: To carry out the experiment on enzymatic hydrolysis of starch by amylase.

PRINCIPLE:

Starch is hydrolysis during the process of digestion to dextrin and ultimately to glucose by the successive action of the enzyme α-amylase, maltose, iso-maltose. In this experiment the formation of maltose during the hydrolysis of starch by salivary α-amylase will be studied.

The salivary α-amylase is allowed to act on starch at intervals of 3,6,9,12 and 15 minutes and incubated at 37oc at a definite pH 6.9. The enzyme action is arrested by the addition of dinitro salicylic acid (DNSA). All the tubes are heated in boiling water bath for 10 min. The colour intensity of all the test tubes are measure at 540 nm. Variation in the colour intensity of all the test tubes are measured at 540 nm and optical density values are used to measure the degree of hydrolysis of starch.

PROCEDURE:

1. Prepare 1 % starch solution as substrate.
2. Pipette out and transfer 1 ml substrate solution into a clear test tubes labeled as p-6.
3. Add 1ml of phosphate buffer (pH 6.9) for all the test tubes.
4. Add 1ml of dilute salivary α-amylase to all the test tubes except test tube labelled as no.1
5. Incubate all the test tubes at 37^{o}C .
6. At interval of 3,6,9,12 and 15 min. Remove the 1 test tube after another immediately add1ml of DNSA reagent each.
7. Heat all the test tubes on boiling water bath for exactly 10 min remove and cool.
8. Note down the colour intensity and OD values of all the test tubes at 540 nm.
9. Variation in the colour intensity and OD values are used to measure the degree of starchhydrolysis by α-amylase enzyme.

REAGENTS:

Preparation of salivary α-amylase-

Wash the mouth thoroughly with distilled water. Take 20 ml of water into the mouth and gargle it for 15min,collect the diluted saliva into the beaker as used for estimation.

Preparation of phosphate buffer-

Dissolve 2.9g of di-sodium hydrogen phosphate and 0.5g of benzoic acid and diluted to 100ml with distilled water.

Preparation of 1% starch solution-

Dissolve 1 g of starch in 50ml distilled water and warm for 5 min, diluted to 100ml with distilled water.

REPORT:

Experiment No: 18

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

AIM: To study the effect of temperature on enzyme activity of a sample of salivary amylase.

APPARATUS REQUIRED: Pipette, volumetric flask, beaker, test tube, thermometer and funnel.

CHEMICALS REQUIRED: Saliva sample, DNS reagent and buffer reagent.

PRINCIPLE: The enzyme activity increases with increase in temperature upto a certain point, normally 37-40^{0}C, after the enzyme gets inactivated as a result of denaturation. At elevated temperature, the enzyme protein undergoes thermal decomposition, that decrease the effective concentration of enzyme. Therefore the effect of temp on activity of amylase is studied at pH 6.9 with varying the temp. The amylase is incubated with the starch substrate and varying temp in pH buffer for a specified period of time and the reaction is arrested with addition of DNSA reagent. The amylase activity is determined by measuring the amount of maltose released, for each temp with the help of Standard maltose curve.

The optimum temp of amylase enzyme is noted by plotting the graph of temperature on X-axis and enzyme activity on y-axis.

PROCEDURE:

Preparation of salivary α-amylase: Wash the mouth thoroughly with distilled water. Take 20 ml of distilled water into the mouth and gargle it for 15 minutes, Collect the diluted saliva into the beaker and used for estimation.

1. Setup 8 test tubes into which add 0.5ml starch and 1.5 ml of distilled water.
2. Maintain the temperature for 5 minutes at the following temperatures 0°C, 10°C, 25°C, 37°C, 48°C, 60°C, 75°C, 95°C with occasional shaking.
3. Then add 5 ml of salivary amylase mix and incubate for respective temperature for 5 minutes.
4. Prepare a blank and eliminate the saliva sample.
5. After 15 minutes add 1 ml DNS reagent and 5ml of distilled water into each test tube. Cool andmeasure the optical density at 540 nm.
6. Plot a graph of optical density *vs* temperature and prepare optimum temperature of salivary amylase.

Clinical Significance:

The velocity of enzyme activity increases with temperature and reaches a maximum the declines. The optimum temperature is the temperature at which the enzyme shows maximum activity. The temperature at which maximum amount of reactant is converted into product in a unit time is called optimum temperature. Temperature coefficient is defined as the increase in enzyme activity when the temperature is increased by 10°C. Increase in temperature increases the activation energy of reactants and collision increases and more amount of reactant is converted to product. The optimum temperature for most of the enzymes are 40 - 45 °C. Above that the enzyme denaturation occurs in lower temperatures, the enzyme becomes inactive and rate of enzyme reaction is also diminished. However, a few enzymes are active in low and higher temperatures. This may be due to the stable confirmation or rigid structure.

REPORT:

Experiment No: 19

EFFECT OF SUBSTRATE CONCENTRATION ON SALIVARY AMYLASE ACTIVITY

AIM: To study the effect of substrate concentration on salivary α-amylase activity.

PRINCIPLE:

At low concentration of substrate, the rate of the reaction is very slow, but it will increase with an increase in the substrate concentration ie there is small increase in the rate of the reaction, with an increase in the rate of the substrate concentration. Finally a point will be reached beyond which there are only a small increase in the reaction rate. If after increase in the substrate concentration,no more increase in the reaction rate and the reaction never reaches maximum, when we plot the graph of the substance con on X-axis and reaction rate on y-axis yields hyperbolic curve. The hyperbolic curve obtained due to the saturation of the enzyme active sites at high concentration of the substrate. Once a stage is reached, substrate is required only to replace the sites when the products are required and cannot increase the rate.

PROCEDURE:

1. Prepare 1% starch substrate solution.
2. Pipette out and transfer 0,0.2,0.4,0.6,0.8,1 and 1.2 ml of the above solution into a clean test tube\ numbered as 1-7 respectively.
3. Add sufficient distilled water to make 3ml in each case.
4. Pipette out and transfer 1ml of phosphate buffer solution into each test tube.
5. Pipette out and transfer 1ml of salivary α-amylase solution into each test tubes.
6. Incubate all test tubes at 37^{o}c for 5 minutes.
7. Soon after incubation add 1ml of DNSA reagent into each test tubes.
8. Boil all the test tubes on boiling water bath for 10 min, cool the test tubes.
9. Read the OD of all test solution, calorimetrically at 540nm against blank.
10. Calculate the amount of maltose released in each case with the help of standard maltose curve.
11. Determine the effect of substrate concentration on enzyme activity by plotting graph of substrate concentration on X-axis and enzyme activity on y-axis.

Reagents-

Preparation of salivary α-amylase

Wash the mouth thoroughly with water. Take 20 ml of water into the mouth and gargle it for15min, collect the diluted saliva into the breaker as used for estimation.

Preparation of phosphate buffer.

Dissolve 2.6g of di-sodium hydrogen phosphate and 0.5g of benzoic acid and diluted to 100ml with water.

Preparation of starch solution.

Dissolve 2 g of starch in 50ml distilled water and warm for 5min, diluted to 100ml with water.

Preparation of standard maltose

Dissolve 200mg of maltose diluted to 100ml with distilled water.

REPORT:

Vision and Mission of the Institution

Vision

The East Point College of Pharmacy aspires to be a globally acclaimed institution, **recognized** for **excellence in** pharmaceutical education, research and nurturing students for **holistic development.**

Mission

M1 Create pharmacy graduates through **quality education**

M2 Promote innovation, **creativity**, and excellence **in teaching**, learning, and **research**

M3 **Inspire** integrity, teamwork, critical thinking**, personal** development, and ethics in **students** and lay **the** foundation for lifelong learning

M4 **Serve** the **healthcare, technological, scientific**, and **economic** needs of then **society**.