JHARKHAND
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PRACTICAL LAB MANUAL

BIOCHEMISTRY

BACHELOR IN PHARMACY

SEMESTER- II

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Test For Carbohydrates

- Carbohydrates are widely prevalent in the plant kingdom, comprising the mono-, di-, oligo-, and polysaccharides. The common monosaccharides are the glucose, fructose, galactose, ribose etc. the disaccharides, i.e., the combination of two monosaccharides include sucrose, lactose and maltose. Starch and cellulose are polysaccharides consisting of many monosaccharide residues. Cellulose is most abundant organic compound on this planet since it forms part of cell wall in plants.
- Aldehydes (—CHO) and ketones (=CO) are active groups in carbohydrates. Carbohydrates contain many hydroxyl groups as well. The number of hydroxyl groups varies with the number of carbon atoms. Monosaccharides contain the free aldehyde group (maltose) and some do not have the free ones (sucrose). The polysaccharides, starch and cellulose, are polymers of monosaccharides linked through the active groups.
- The chemical properties of the saccharides vary depending upon the number of hydroxyl groups and the presence or absence of –CHO/=CO groups. These variations are the basis in the development of colour reactions to identify the saccharides.

REAGENTS

- ***Iodine Solution:*** Add a few crystals of iodine to 2% potassium iodide solution till the colour becomes deep yellow.
- ***Fehling's Reagent A:*** Dissolve 34.65 g copper sulphate in distilled water and make up to 500 mL.
- ***Fehling's Reagent B:*** Dissolve 125 g potassium hydroxide and 173 g Rochelle salt (potassium sodium tartrate) in distilled water and make up to 500 mL.
- ***Benedict's Qualitative Reagent:*** Dissolve 173 g sodium citrate and 100 g sodium carbonate in about 800 mL water. Heat to dissolve the salts and filter, if necessary. Dissolve 17.3 g copper sulphate in about 100 mL water and add it to the above solution with stirring and make up to volume to 1 L with water.
- ***Barfoed's Reagent:*** Dissolve 24 g copper acetate in 450 mL boiling water. Immediately add 25 mL of 8.5% lactic acid to the hot solution. Mix well. Cool and dilute to 500 mL.

EXPERIMENT NO - 1

Aim:

- Qualitative analysis of carbohydrates by the **Fehling's test**.

Requirements:

(a) Glasswares: Test tube

(b) Chemicals: Fehling's solution.

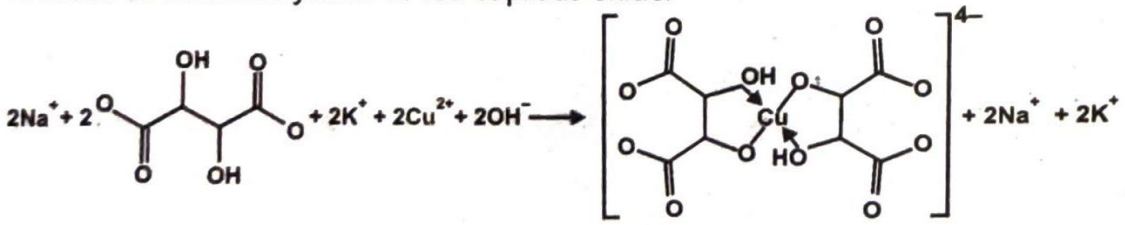
- **Fehling solution A:** It contains copper sulphate solution. It is prepared by dissolving 34.65 gm of $CuSO_4 \cdot 5H_2O$ in 500 mL of distilled water.
- **Fehling solution B:** It contains potassium hydroxide and Rochelle salt (Sodium potassium tartarate). It is prepared by dissolving 125 gm of KOH and 173 g of Rochelle salt in 500 mL of distilled water.
- Mix equal volume of Fehling A and Fehling B before use.

Principle:

- Sugars possessing a free, or potentially free, aldehyde or ketone group act as reducing agents and this fact becomes the basis of the tests performed for distinguishing them from the non-reducing sugars. This test is based on reducing property of carbohydrates. Such sugars have the property of readily reducing alkaline solutions of the metals like bismuth, mercury, iron and silver. The aldo sugars are oxidized to the corresponding aldonic acids whereas the keto sugars give rise to short chain acids. If the alkaline copper solution is heated in the absence of reducing sugar, it forms black precipitate of cupric oxide:

$$Cu(OH)_{2(aq)} \longrightarrow CuO(s) + H_2O_{(aq)}$$

- In the presence of a reducing sugar, however, the alkaline solution of copper is reduced to insoluble yellow or red cuprous oxide.



$$RCHO + 2Cu^{+2} + 4OH^- \longrightarrow RCOOH + Cu_2O + H_2O$$

- Fehling's solution is always prepared fresh in the laboratory. It is made from two separate solutions viz., Fehling's A and Fehling's B. The former is a solution of $CuSO_4$.

while the latter is a clear and colourless solution of aqueous potassium sodium tartrate (Rochelle salt) and a strong alkali (NaOH). In the final mixture, aqueous tartrate ions chelate to Cu^{+2} ions in bidentate ligands giving the bistartratocuprate (II)4-complex as shown in structure. The tartarate ions, by complexing with copper prevent the formation of $Cu(OH)_2$ from the reaction of $CuSO_4.5H_2O$ and NaOH present in the solution.

Procedure:

- To 2 mL of Fehling solution (1 mL of Fehling A + 1 mL of Fehling B), add 2 mL of carbohydrate solution.
- Mix and boil.
- Appearance of yellow or red precipitate of cupric oxide indicates the presence of reducing sugars.

Observation :

- Formation of yellow or brownish-red precipitate.

Remarks:

- The blue alkaline cupric hydroxide present in solution, when heated in the presence of reducing sugars, gets reduced to yellow or red cuprous oxide and it gets precipitated. Hence, formation of the colour precipitate indicates the presence of reducing sugars in the test solution.

Note:

- In case of mild reduction, leave the solution to stand for 10-15 minutes, and then decant the supernatant. A small amount of red or yellow precipitates may then be seen adhering to the inner side of the tube. Cuprous oxide is dissolved by ammonia. Hence, it is not possible to detect small quantities of reducing sugars in fluids saturated with ammonium salts e.g. urine.

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EXPERIMENT NO - 2

Aim:

- Qualitative analysis of carbohydrates by the **Seliwanoff's Test.**

Requirements:

(a) Glasswares: Test tube.

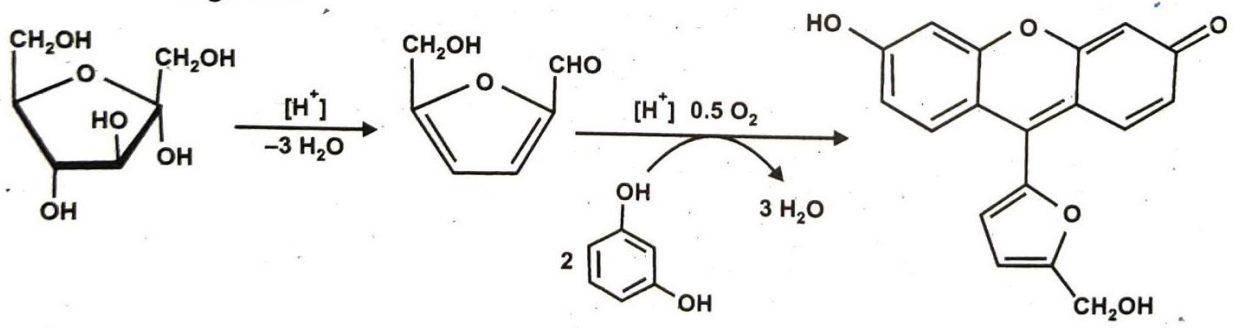
(b) Chemicals: Hydrochloric Acid, Resorcinol.

Reagent:

- Resorcinol in concentrated hydrochloric acid (diluted 1 : 1 with water).

Principle:

- This test is positive for ketohexoses only and hence is used in the detection of fructose. Ketohexoses, i.e. fructose on treatment with hydrochloric acid form 5 hydroxymethyl furfural which on condensation with resorcinol gives a cherry red coloured complex.
- Seliwanoff's test distinguishes between fructose and glucose. Overheating of the solution is avoided because on continuous boiling, aldoses will also give this test positive because of their conversion to ketoses by hydrochloric acid.
- Sucrose will also give Seliwanoff's test positive because the acidity of reagent is sufficient enough to hydrolyse sucrose to glucose and fructose but Benedict's test will be negative.



Procedure:

- To 3 mL of Seliwanoff's reagent in a test tube add 3 drops of carbohydrate solution.
- Heat over a flame for 30 seconds only.
- Cool the solution. An appearance of cherry red colour indicates the presence of fructose.

Observation:

- Appearance of deep red colour.

EXPERIMENT NO - 3

Aim:

- Qualitative analysis of carbohydrates by the **Osazone Test.**

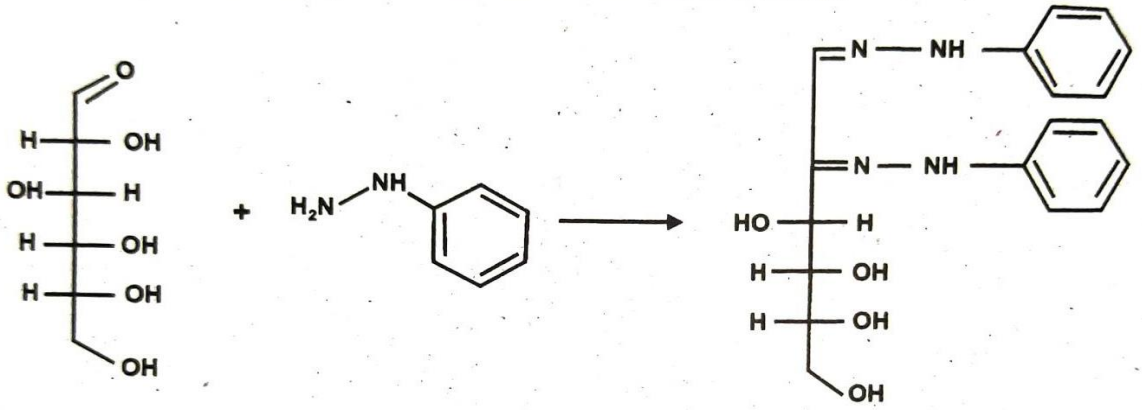
Requirements:

(a) Glasswares: Test tube, Stir rod.

(b) Chemicals: 10% HCl, 10% NaOH, red litmus paper, iodine reagent, Benedict's reagent, 1% starch and 2% sucrose solutions.

Principle:

- A solution of reducing sugar when heated with phenyl hydrazine, characteristic yellow crystalline compounds called Osazone are formed.



- These crystals have definite crystalline structure, precipitation time and melting point for different reducing sugars.

Procedure:

- In a clean and dry test tube, take roughly 0.5 g of phenylhydrazine mixture.
- Add 5 mL of disaccharide solution and 2 drops of glacial acetic acid. Mix.
- Place the test tube in boiling water bath for 30 minutes.
- After 30 minutes, take out the test tube from the boiling water bath and allow it to cool by itself in a test tube rack (Do not disturb the test tube in between as the osazones of disaccharides separates out on slow cooling).
- Appearance of yellow crystals takes place.
- Observe the shape of crystals under low power microscope.

Observation:

- Glucose, Fructose and Mannose: Needle-shaped yellow osazone crystals.
- Malatose: Sunflower shape

EXPERIMENT NO - 4

Aim:

- Qualitative analysis of carbohydrates by the **Iodine Test.**

Requirements:

(a) **Glasswares:** Test tube, Stir rod.

(b) **Chemicals:** Iodine solution, Polysaccharides.

Principle:

- Iodine forms a co-ordinate complex between the helically coiled polysaccharide chain and iodine centrally located within the helix due to adsorption.
- The colour obtained depends upon the length of the unbranched or linear chain available for complex formation.

Procedure:

- Add a few drops of iodine solution to about 1 mL of the test solution.

Observation:

- **Amylose:** A linear chain component of starch, gives a deep blue colour.
- **Amylopectin:** A branched chain component of starch, gives a purple colour.
- **Glycogen:** Gives a reddish brown colour.
- **Dextrins-Amylo, Eryhthro and Achrodextrins:** Formed as intermediates during hydrolysis of starch gives violet, red and no colour with iodine respectively.

Remarks:

- This indicates the presence of starch in the solution.
- The blue colour is due to formation of starch-iodine complex.

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EXPERIMENT NO - 5

Aim:

- Quantitative analysis of reducing sugars by dinitrosalicylic (DNSA) method.

Requirements:

(a) Equipment required:

- Spectrophotometer capable of measuring absorbance in the 540 nm region.
- Cuvettes for spectrophotometer.
- Water bath [100°C].

(b) Chemicals:

- Glucose standard solutions at the concentration ranging from 0.6 µmol/mL to 4.00 µmol/mL.
- 0.05M acetate buffer (pH 4.8).
- DNS reagent.

Reagents Preparation:

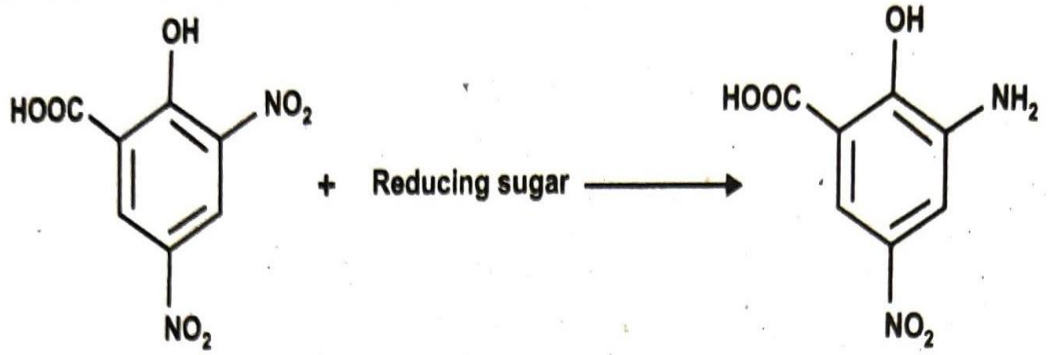
- In 1000 mL beaker dissolve 10 g of 3, 4-dinitrosalicyclic acid in 200 mL H_2O. Followed by continuous stirring slowly add a solution of NaOH dissolved in 150 mL distilled water.
- Incubate mixture at 50°C with stirring to obtain a clear solution.
- In small portions add 403 g of potassium sodium tartrate tetrahydrate.
- Filter mixture using filter paper and make up the volume to 1000 mL with water.
- Store in dark glass bottle at temperature below 20°C.

Execution time:

- From 20 – 30 min.

Principle:

- Reducing sugars have the property to reduce many of the reagents. A reducing sugar is one that in a basic solution forms an aldehyde or ketone. The aldehyde group of glucose converts 3,5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid, which is the reduced form of DNS.
- Water is used up as a reactant and oxygen gas is released during the reaction. The formation of 3-amino-5-nitrosalicylic acid results in a change in the amount of light absorbed, at wavelength 540 nm.



3, 5 -dinitrosaliyclic acid (DNS) **3-amino-5-nitro salicylic acid**

- Intensity of the colour is an index of reducing sugar.

Procedure:

- Prepare glucose standard solutions in 0.05 M acetate buffer (pH 4.8) ranging from 0.6-4.00 µmol/mL.
- Add 1 mL of each standard to separate tubes. To the tubes used as the blanks, add 1 mL of 0.05 M acetate buffer (pH 4.8).
- Prepare the unknown samples in an appropriate dilution.
- To each tubes, add 1 mL of 0.05 M acetate buffer (pH 4.8) and mix.
- Add 3 mL DNS reagent to all the test tubes and mix well. Place the tubes in a boiling water for 5 minutes.
- Cool the tubes to room temperature and measure the absorbance at 540 nm.

Calculation:

- Dilution factor (D.F.) = Final volume/Aliquot volume

Carbohydrate in 1 gram of sample = ----------mg/dl × dilution factor × 100

Normal Range = 4-5 gm

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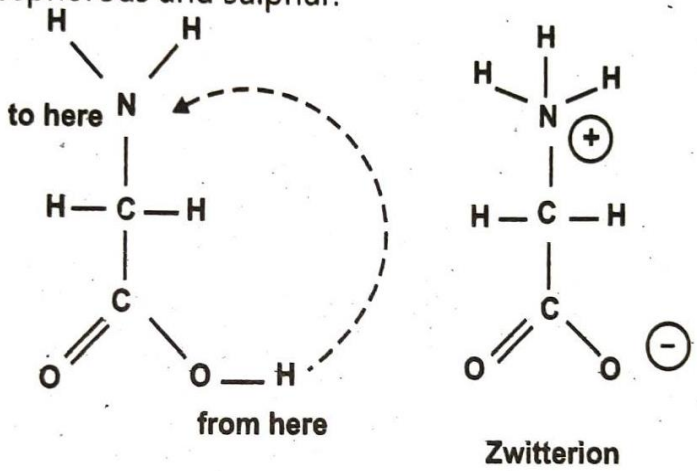
Test For Proteins and Amino Acids

- Protein is an important macronutrient essential for survival. They are constituent of cells and hence are present in all living bodies. 10-35% of calories should come from protein. Protein is found in meats, poultry, fish, meat substitutes, cheeses, milk etc.
- Proteins are large biological molecules composed of α-amino acids (Amino acid in which amino group is attached to α-carbon, which exist as zwitterions and are crystalline in nature). They contain carbon, hydrogen, oxygen, nitrogen and sometimes phosphorous and sulphur.



Zwitterion

- Amino acids are molecules contain both amino (NH_2) and carboxylic (COOH) group. Amino acid molecules undergo condensation reaction to form a specific type of linkage known as **peptide linkage.**
- Depending on the number of amino acid molecules involved in the condensation reaction, the products formed are classified as;

(a) Dipeptide: They are the products formed by the condensation of two α-amino acid molecules.

(b) Tripeptide: They are formed by the condensation of three α-amino acid molecules. If large number of amino acid molecules combine, the product formed is called polypeptide. A polypeptide having molecular mass greater than 10000 is called a protein. Proteins differ from one another primarily in their sequence of amino acid. There are about more than 20 amino acids. Some amino acids are not made by the body and are supplied through diet. They are called essential amino acids.

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EXPERIMENT NO - 6

Aim:

- Estimation of Protein by the **Biuret Method.**

Requirements:

(a) Glasswares: Test tube, Stir rod.

(b) Chemicals: Biuret reagent, NaOH, Protein standard solution.

Reagents Preparation:

- **Biuret reagent:**

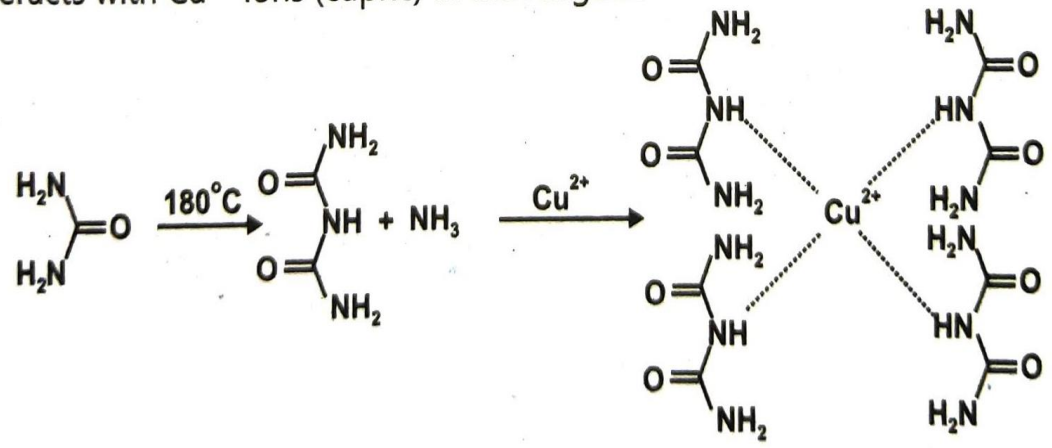
  Dissolve 1.5 gm of $CuSO_4$ and 4.5 gm of Na-K tartrate in 250 mL 0.2 N NaOH solution. Add 2.5 gm of KI and make up the volume to 500 mL with 0.2 N NaOH.

- **Protein standard solution:**

  Dissolve 500 mg of egg albumin in 50 mL of H_2O. Make up the volume to 100 mL to get the final concentration of 5 mg/mL.

Principle:

- This is the most commonly used method based on the fact that the — CO — NH (peptide) group of proteins form a purple complex with copper ions in an alkaline medium. Since, all proteins contain the peptide bond, the method is fairly specific and there is little interference from other compounds.
- Some substances like urea and biuret interfere because they possess the — CO — NH - group. Other interfering materials are reducing sugar like glucose, which interacts with Cu^{+3} ions (cupric) in the reagent.



Urea **Biuret** **Copper Complex**

Procedure:

- Pipette out standard protein solution into a series of tubes — 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL and make up the total volume to 4 mL by adding water.
- The blank tube will have only 4 mL of water.
- Add 6 mL of biuret reagent to each tube and mix well.
- Keep the tubes at 37°C for 10 minutes during which a purple colour will develop.
- The optical density of each tube is measured at 520 nm (green filter) using the blank reagent.
- Draw the graph to the known concentration of a protein in an unknown solution.

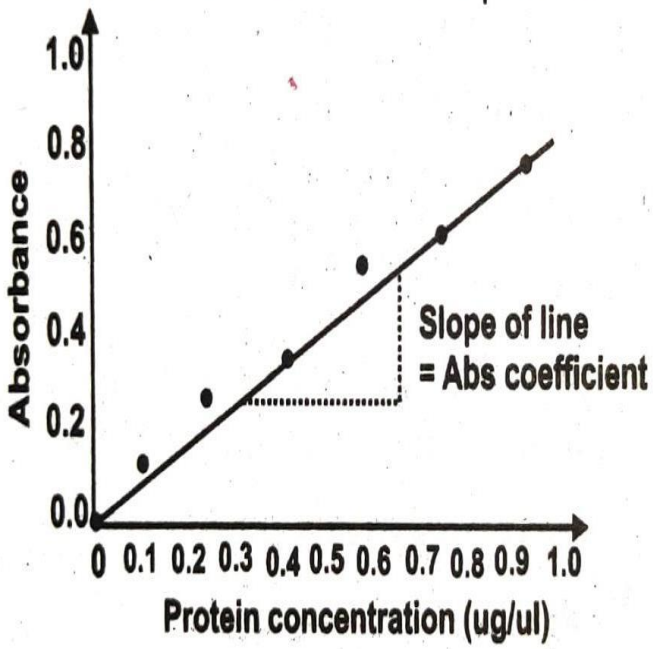


Fig.

Result:

- The given sample contains ..... mg of protein.

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Urine Analysis

- The average volume of urine excreted daily is about 1.5 litres. It contains nitrogeneous organic compounds such as urea, uric acid, creatinine, hippuric acid, indican, purines and amino acids as well as organic compounds which do not contain nitrogen. The average composition of urine excreted in 24 hours is as follows:

Urea	20-30 gm
Uric acid	0.7 gm
Amino acid	0.5-1 gm
Ammonia	0.7 gm
Creatinine	1.4 gm
Ascorbic acid	15-20 gm
Guanidine	3-16 gm
Hippuric acid	0.6 gm
Indican	4-20 mg
Iodine	50-250 mg
Lactic acid	50-200 mg
Chloride as NaCl	10-15 mg
Inorganic sulphate as S	60-120 mg
Neutral sulphate as S	80-160 mg
Sodium	3-5 gm
Calcium	0.1-0.3 gm
Phosphate as inorganic phosphates	1.0-1.5 gm

- The ordinary urine is examined under the following heads:

1. Physical examination.
2. Chemical examination.
3. Microscopic examination.

- The physical examination of urine includes appearance, colour, pH, specific gravity, etc. These tests are carried out before the microscopic or chemical examination.

EXPERIMENT NO - 7

Aim:

- To perform the physical examination of Urine.

1. Colour:

- The normal urine is yellow in colour. The intensity of normal urine is dependent on the concentration of urine. The yellow or amber colour of a normal urine is due to presence of a yellow pigment urochrome.
- The colour of urine changes in many disease conditions because of the presence of pigments that do not normally occur.

Colour	Possible cause
Orange	Concentrated urine
Almost colourless	Dilute urine
Yellow to yellow brown or greenish colour	Bile pigments
Reddish brown colour	Hemoglobin
Milky	Presence of fats
Black upon standing	Alkaptonuria
Brown-black colour on standing	Melanin or homogentisic acid
Orange brown	Urobilinogen
Cloudy	Presence of insoluble calcium and magnesium phosphates

- The urine may also assume many different colours following ingestion of various dyes, foods and drugs.

2. Odour:

- Freshly passed urine has got a characteristic aromatic odour due to the presence of some volatile aromatic acid.
- Ammonia odour may occur in certain infections of urinary tract.
- A fruity odour of acetone is typical of **Ketosis.**
- Certain foods and drugs may also produce abnormal odours.

3. Volume:

- The normal volume of urine voided by an adult per day ranges from 750 to 2000 mL. The average volume is 1500 mL.
- The amount of urine excreted is directly related to fluid intake, the temperature, climate and the amount of sweating that occurs.
- **Polyuria:** It is the increased excretion of urine. Polyuria may indicate the loss of concentrating ability by the kidney. Polyuria occurs in physiological conditions.
  - Excessive fluid intake
  - Ingestions of diurates
- **Pathological conditions**
  - Diabetes mellitus
  - Diabetes insipidous
  - Chronic renal damage
- **Oliguria:** It is decreased in which the urinary output is 500 mL per 24 hours. Oliguria occurs in
  - Less fluid intake
  - Excessive fluid loss due to vomiting, diarrhoea and sweating
  - Fever
  - Shock
  - Acute nephritis
  - Cardiac failure
- **Anuria:** It is a total loss of urine. The ratio of day urine (i.e. 8 AM to 8 PM) to night urine (i.e. 8 PM to 8 AM) should be at least 2 : 1 and sometimes 3 : 1 or more in healthier individual. In renal disease, this ratio is reduced and may even be reversed.

4. pH:

- The pH of the normal urine is on an acidic side, i.e. 5.5-6.5. The acidity of the urine is mainly due to acid phosphates.
- In general protein rich diets give rise to acidic urine attributable to the sulphur of the amino acids which is oxidised to sulphuric acid. Similarly phospholipids and nucleic acids all yield phosphoric acid. Alkaline urines are excreted where there is a predominance of vegetables and fruits in the diet. However, the production of ammonia by the kidney also plays an important part in influencing the pH of the urine since it forms salts with acids and can excreted as such.

Acid Urine:

- On a high protein diet.

- Uncontrolled diabetes
- Acidosis
- Fever.

Alkaline Urine:

- After meals
- Diet high in vegetables, citrus fruits, milk, etc.
- Renal tubular acidosis.

5. Specific Gravity:

- Normal specific gravity of urine varies from 1.005 to 1.030.
- Specific gravity is highest in the first morning specimen and is generally greater than 1.020. A specific gravity of 1.025 or above in a random normal urine sample indicates normal ability.
- The specific gravity of urine varies according to kidney function. Concentrated urine has a high specific gravity while diluted urine has a low specific gravity.
- Specific gravity of urine indicates the relative proportions of dissolved components to the total volume of the urine. It also reflects the relative degree of concentration or dilution of the urine sample.
- Low specific gravity is observed in the following:
  - High fluid intake
  - Diabetes insipidous
  - Glomerulonephritis
  - Pyelonephritis
- High specific gravity urine is observed in
  - Excessive loss of water due to sweating fever, vomiting and diarrhoea.
  - Diabetes mellitus
  - Nephrosis
  - Hepatic diseases
  - Congestive heart failure.
- Whereas fixed specific gravity of urine is an indication of several renal damages with disturbance of both concentrating and diluting abilities of kidney.

Determination of Specific Gravity:

- The specific gravity of urine is determined by urinometer.
- Urinometer is a glass made instrument having a cylindrical stem containing a scale.

- The urinometer is floated in a cylinder containing urine. Care should be taken that it should not touch the sides or bottom of the cylinder.
- The depth to which it sinks in the urine, indicates the specific gravity of urine.
- The urinometer is calibrated with respect to distilled water at 1.000 at specific temperature, indicated on the instrument itself. If the temperature of urine is above or below that temperature, a correction of + 0.001 for each 3°C should be made.

6. Total Solids:

- Total solids can be calculated approximately by multiplying the second and third decimal figures of specific gravity by 2.6 (Longe's coefficient).
- The product represents the number of gm of solids in 1 litre of urine, e.g. if the specific gravity of urine is 1.025, then 25 × 2.6 = 65 gm totals solids per litre. From this the output during 24 hours can be calculated.

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EXPERIMENT NO - 8

Aim:

- Qualitative examination for normal organic constituents in urine.

Requirements:

(a) Glasswares: Test tube, Stir rod.

(b) Chemicals: NaOH, Na_2CO_3, Phosphotungstic acid, Obermayer's reagent, Chloroform etc.

Test for Urea:

- 0.5 mL of 70% NaOH and 4 drops of bromine water was added to 1 mL of urine sample in a medium-sized test tube.
- The evolution of Nitrogen gas was observed and recorded.

Test for Uric Acid:

- 5 mL of 20% Na_2CO_3 added to 1 mL urine sample in a medium-sized test tube.
- The solution was mixed well using a stirring rod.
- After mixing, 5 drop of phosphotungstic acid reagent was added.
- Again, the solution was mixed well using a stirring rod.
- The formation of a blue solution was observed.

Indican Test:

- 5 mL of Obermayer's reagent was added to 5 mL urine sample in a large-sized test tube.
- The solution was mixed well using a stirring rod.
- 3 mL of chloroform was added to the solution. The solution was shaked well and the chloroform layer was allowed to settle.
- The formation of a blue colour in the lower (chloroform) layer was recorded.

Test for Creatinine:

- 1 mL of alkaline picrate solution composed of 5 : 1 saturated picric acid- 10% NaOH to 2 mL urine sample in a medium-sized test tube.
- The formation of an orange-coloured solution was noted.

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EXPERIMENT NO - 9

Aim:

- Qualitative Examination for Pathologic Organic Constituents in Urine.

Requirements:

Glasswares: Test tube, Stir rod.

Gunning's Test:

- Two large-sized test tubes were labeled at positive control and urine sample.
- 5 mL urine sample was basified with 5 drops of concentrated ammonium hydroxide.
- Lugol's solution was added to produce a black cloud which does not appear immediately.
- The solution was let stand for 5 minutes. Crystals of iodoform was formed.
- For the positive control, 2 mL of acetone was added to 5 mL acetone and the same procedures with the urine sample were followed.

Benedict's Test:

- Two large-sized test tubes were labelled at positive control and urine sample.
- For the urine sample, 5 mL of Benedict's reagent was added to 1 mL urine sample.
- The solution was mixed well using a stirring rod.
- It was heated in a boiling water bath for two to three minutes.
- After heating, it was allowed to let stand and be cooled.
- The formation of the precipitate was noted.
- For the positive control, 1 mL of urine sample was mixed with 1 mL 1% glucose solution.
- The same procedure with the urine sample was followed. The results obtained were recorded.

Exton's Test:

- Two large-sized test tubes were labelled at positive control and urine sample.
- For the urine sample, 3 mL of urine sample and 3 mL of Exton's reagent were mixed.
- The solution was warmed if there was a cloudiness that took place.
- When cloudiness persisted or increased upon heating, albumin was present.
- For the positive control, 3 mL of urine was mixed with a pinch of albumin. The same procedure with the urine sample was followed. The results obtained were recorded.

Smith's Test:

- Two large-sized test tubes were labelled at positive control and urine sample.
- For the urine sample, 5 mL of urine sample was placed in the test tube and the test tube was inclined and overlayed with 3 mL of tincture of alcoholic iodine mixture.
- At the point of contact, an emrald green colour was observed.
- For the positive control, 5 mL of urine sample was mixed with 10 drops of bile pigment from chicken or pig gall bladder. Succeeding and same steps from the urine sample were followed.
- The results obtained were recorded.

Test for Occult Blood:

- Two large-sized test tubes were labelled at positive control and urine sample.
- For the urine sample, 5 mL of guaiac powder in 95% ethanol was mixed with hydrogen peroxide.
- This solution was added to 3 mL acidified urine, urine with five drops of glacial acetic acid.
- A blue ring was formed and a presence of blood was determined.
- For the positive control, 3 mL of acidified urine, urine with five drops of glacial acetic acid was mixed with 10 drops of blood. The same procedure as with the urine sample was followed. The results obtained were recorded.

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EXPERIMENT NO - 10

Aim:

- Qualitative chemical examination of urine.

Requirements:

Glasswares: Test tube, Stir rod.

1. Reducing Sugars (Usually Glucose):

- Normally very small amounts of reducing sugars are excreted in urine, which are not detected by the reagents used. However, under abnormal conditions following reducing sugars are excreted in urine.

Reducing sugars	Conditions
Glucose	Renal glucosuria, Diabetes mellitus
Galactose	Galactosemia
Lactose	Pregnancy and lactation
Fructose and Pentoses	After ingestion of large amounts of vegetables or fruits

Causes of Glycosuria:

- Diabetes mellitus
- Non-diabetic glycosuria

(A) Glycosuria with hyperglycemia:

- Renal glycosuria, due to low renal threshold
- Alimentary glycosuria, after ingestion of lot of carbohydrates
- Glycosuria of pregnancy, in 10-15% of normal pregnancies

(B) Non-diabetic glycosuria with hyperglycemia:

- Hyperthyroidisim
- Emotional disturbances
- Ether anesthesia
- Increased intracranial pressure: tumours, fractures, encephalitis.

Benedict's Qualitative Test:

- To 5 mL of Benedict's qualitative reagent in a test tube, add 8 drops of urine.
- Boil for 2 minutes and cool.
- A change in blue colour to green-yellow-red precipitate indicates the presence of reducing sugars in urine.
- The colour of the precipitate gives an appropriate, i.e. rough amount of sugar in the urine.

Colour	Precipitate Result
Blue colour remains	No reducing sugar
Green colour	Less than 0.5 gm%
Green precipitate	0.5 to 1 gm%
Green to yellow precipitate	1.0 to 1.5 gm%
Yellow to red precipitate	1.5 to 2.0 gm%
Brick red precipitate	More than 2 gm%

Fehling Test:

- Mix 1 mL each of Fehling A solution and Fehling B solution.
- Then add 2 mL of urine. Boil for 2 min.
- Appearance of either green-yellow-red precipitate indicates the presence of reducing sugars.

Tests for Specific Reducing Sugar:

- The following tests will differentiate the various reducing substances which respond to Fehling and Benedict's test. Usually, the object in testing for reducing substances in urine is to determine the presence or absence of glucose.

(a) Fermentation test:

- Glucose and fructose are fermented by the enzyme present in the yeast and the liberation of carbon-dioxide confirms the test.

(b) Mucic acid test for lactose and galactose:

- Evaporate urine in a china dish containing a mixture of 100 mL of urine and 20 mL of concentrated HNO_3. Let the volume is reduced to 20 mL. Allow it to cool. Lactose or galactose yields mucic acid through the oxidising action of HNO_3 and this form a white precipitate.

(c) Seliwanoff's test for fructose

(d) Pentoses by orcinol test.

(e) Identification of carbohydrates by osazones formation.

2. Proteins:

- There is no single test capable of detecting the presence of proteins under all conditions. The proteins which appears in urine however are usually albumin and globulins of which albumin almost greatly predominate.

Causes of albuminuria:

(i) Functional: Orthostatic, severe muscular exertion, prolonged exposure to cold, pregnancy.

(ii) Organic:

(a) Pre-renal causes:

- No primary kidney disease
- Passive congestion of kidney
- Fever and toxaemia
- Intra-abdominal tumours
- Drugs and chemical poisoning

(b) Renal:

Primary disease of kidney

- Nephritis
- Nephrosis

(c) Post-renal causes:

- Pyetitis
- Cystitis
- Urethritis, prostatitis

Note: Normally proteins are not excreted in urine.

Test for Proteins:

(i) Heat Coagulation Test:

- Fill a test tube with two-third (2/3) of urine.
- Gently heat the upper half of the urine to boiling without disturbing the lower portion which serves as a control.
- A white turbidity indicates the presence of proteins or phosphates.
- Add few drops of glacial acetic acid. If precipitate dissolves, it is due to phosphates.

(ii) Sulphosalicyclic Acid Test:

- Take 5 mL urine in a test tube, add to it 1 mL of 25% sulphosalicyclic acid.
- Appearance of white precipitate indicates the presence of proteins in urine.

(iii) Nitric Acid Test (Heller's Test):

- In a clean dry test tube, take 3 mL of concentrated nitric acid.
- Put 2 mL of urine over the acid to form a separate layer.
- Appearance of white ring at the junction indicates the presence of proteins.
- Distinction between albumin and globulin when urine is mixed with an equal volume of saturated ammonium sulphate, the globulin is precipitated and albumin which remains can then be precipitated by boiling the filtrate or by addition of ammonium sulphate.

Bence Jones Proteins:

- Bence Jones proteins are found in urine in cases of multiple myeloma, i.e. cases of malignant disease involving the bone marrow and of leukemia. Its occurrence is very uncommon in urine.

Test for Detection of Bence Jones Proteins:

Heat coagulation test:

- The urine must be faintly acidic. Acetic acid being added if necessary. It is then gently heated in a test tube.
- Bence Jones protein gives a precipitate which appears about 40°C, it is maximal about 60°C, and then disappears as the temperature rises.
- The disappearance may be incomplete since albumin is often present as well. In that case, the boiling urine is filtered rapidly to remove albumin. Bence Jones proteins reappears as the filtrate cools. The precipitate flocculates and sticks to the side of test tube.

Quantitative analysis of proteins:

- Approximation to the quality of proteins present in the urine can be obtained by the use of Esbach proteinometer.
- The graduated tube is filled to the mark U by urine to be tested.
- Esbach reagent is added to the mark R. The tube is stoppered and after mixing or inverting once or twice without shaking is allowed to stand undisturbed overnight.
- The height of the precipitate in the tube is then read and the reading divided by ten gives the amount in gm of proteins in 100 mL of urine.

Esbach reagent:

- 20 gm citric acid, 10 gm picric acid dissolved in 1 litre of water.

3. Test for Ketone Bodies:

- Acetone, acetoacetic acid and β-hydroxybutyric acids are collectively known as ketone bodies. Ketone bodies are obtained as the intermediate products in the oxidation of fatty acids and are oxidised to carbon dioxide and water under normal conditions. In abnormal conditions, ketone bodies accumulate in the blood and pass out in urine. This condition is called ketosis. When the carbohydrate metabolism is defective, increased fat is oxidised for energy purposes giving rise to increased formation of ketone bodies. Such happens in diabetes mellitus, when glucose present is not metabolised hence ketone bodies appear in urine. Also during starvation when glucose supply to the body is restricted, ketone bodies appear in urine.

Causes of ketonuria:

- Diabetes mellitus
- Starvation.

Rothera's test:

- Saturate 5 mL of urine with Rothera's mixture (ammonium sulphate and sodium nitroprusside).
- Add 2 mL of liquor ammonia by the side of the test tube. Appearance of permanganate ring at the junction indicates the presence of ketone bodies in the urine.

4. Test for Bile Pigments:

- Bile pigments are billirubin and biliverdin. Most of the tests for bile pigments depend on the oxidation of bilirubin to coloured compounds of blue, green, violet, red and yellow. Normalurine does not contain bilirubin as bilirubin is bound to albumin. Bilirubin is present in the urine of obstructive jaundice and in some cases of hepatic jaundice due to high level of conjugated bilirubin.

Fouchet's Test:

- Take 5 mL of urine in a test tube. Add to it an equal volume of 10% $BaCl_2$ solution. Filter, dry the filter paper and add a drop of Fouchet's reagent.
- Green colour indicates the presence of bilirubin.
- Fouchet's reagent contains trichloroacetic acid and ferric chloride.

Gmelin's Test:

- Place 5 mL of fuming nitric acid in a test tube. Add 5 mL of urine.
- Appearance of blue, green and violet rings are seen at the junction if bilirubin is present.

Rosenbach Test:

- Filter some urine through a filter paper. Unfold the filter paper.
- Dry it. Add a drop of concentrated HNO_3 in the centre of filter paper.
- Green, blue, violet, and red colours shows the presence of bile pigments.

5. Bile Salts:

- Bile salts are sodium and potassium salts of glycocholates and taurocholates. Bile salts are formed in the liver from where they are excreted in the bile. They are absorbed by the intestine and passed back to the liver through portal circulation. Bile salts are present in urine in obstructive jaundice.

Hay Test:

- Sprinkle some sulphur powder in test tube containing urine.

- Sulphur remains on the surface in normal urine but sinks down in the presence of bile salts.

6. Blood:

- Blood appears in urine in hematuria and hemoglobinuria. Hematuria consists of haemoglobin pigment and unruptured corpusules. It is due to passing of blood through the kidney into urine because of lession of the kidney. Hematuria occurs in polynephritis and hemoglobinuria occurs in enteric fever, malaria, wrong blood transfusion and hemolytic poisoning.

Benzidine Test:

- The peroxidase activity of hemoglobin decomposes hydrogen peroxide and the liberated oxygen oxidises the benzidine to give a blue solution.
- In a test tube, take 3 mL of saturated solution of benzidine in glacial acetic acid.
- Add 3 mL of urine followed by 1 mL of hydrogen peroxide.
- Appearance of green or blue colour within few minutes indicates the presence of blood.

7. For Urobilinogen:

- Urobilinogen is normally present in the urine in very small amount. The excretion of urobilinogen is increased in:

  Hemolytic jaundice: In hemolytic jaundice, liver is not able to excrete the increased amount of urobilinogen absorbed from the intestine completely.

  Infective hepatitis: The liver cell is less able to excrete so more of bilirubin passes into general circulation and is excreted in urine. However it is absent in obstructive jaundice.

Ehrlich's Test:

- In a test tube, take 5 mL of urine. Add to an equal volume of Ehrlich reagent. Allow it to wait for five minutes.
- Appearance of faint pink or brown colour indicates the normal amount of urobilinogen, whereas deep red colour suggests increased amount of urobilinogen.
- The test is done in undiluted urine sample and also in serial dilution of the urine. Normally urobilinogen is present in urine up to a dilution of 1 : 10.

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Colorimetry

- Colorimetric procedures are widely used in the hospital laboratory because they are easy to perform, require small amount of blood and give results of high order accuracy.
- In principle, these methods depend upon the measurements of the amount of colour, i.e. intensity of colour produced during a chemical reaction in which the substance being estimated takes part quantitatively. Within certain limits the colour intensity of a solution is proportional to the concentration of the reacting substances and it is possible to obtain a measure of the concentration of the substance by determining the depth of the colour.
- The basic laws governing the absorption of light are formulated by Lambert's and Beer's which are as follows.

(a) **Lambert's (Bouguer) Law:** It states that the proportion of light absorbed by a substance is independent of the intensity of the incident light.

(b) **Beer's Law:** It states that the absorption depends only on the number of absorbing molecules through which the light passes.

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EXPERIMENT NO - 11

Aim : To estimate creatinine in given sample of blood.

Apparatus : Flasks and Graduated pipettes and photoelectric colorimeter.

Chemical reagents : Picric acid ... 1%
Sodium hydroxide ... 10%
Stock creatinine
Standard creatinine

Method used : Folin method

Principle : Creatinine in blood is estimated by folin modified method using photoelectric colorimeter. In this folin Wu i.e. protein free blood filtrate is used.

This creatinine (unknown) contain in filtrate is treated with picric acid in alkaline medium to obtain red coloured creatinine picrate.

Optical density of this red coloured creatinine thus obtained is compared with that of standard solution, similarly converted by picric acid to creatinine picrate.

By using colorimetry principle concentration of creatinine in given blood sample can be calculated.

Clinical significance : Creatinine represents the waste products of creatine metabolism and it arises in the body from the spontaneous breakdown of creatine phosphate. It is a non threshold substance. It is normally filtered by the glomerulli. As its excretion is not related with food protein so its variations in the excretion indicate some of the metabolic disorders.

Appearance of urine in urine is known as creatinuria.

Its excretion increases in fevers, starvation, on a carbohydrate free diet and in diabetes mellitus.

It may increases due to excessive tissue destruction releasing creatine or due to failure of creatine being properly phosphorylated.

So creatinine excretion is independent of food proteins and is to be considered as an index of endogenous protein metabolism.

Endogenous creatinine clearance is a rough measure of the glomerular filtration rate and is normally 100 – 130 ml/minute an adult of normal size.

Values below 90 ml/minute are indicative of diminished glomerular filtration rate.

Normal value 0.7 to 2.0 mg/100 ml blood.

Procedure :

[A] Preparation of unknown sample

1. In a flask labelled as "U", pipette out 5 ml of folin wu filtrate.
2. Add 2 ml of 1 % picric acid mix well.
3. Add 0.5 ml of sodium hydroxide solution 10%
4. Allow to keep for 15 minutes and obtain the optical density by using green filter (530 M μ). Note it as "Eu"

[B] Preparation of standard sample :

1. In a flask labelled as "S", pipette out 5 ml of standard creatinine solution.
2. Add 2 ml of 1 % picric acid. mix well
3. Add 0.5 ml of sodium hydroxide solution 10%
4. Allow to keep for 15 minutes and obtain the optical density by using photoelectric colorimeter using green filter (530 m μ). Note it as "Es"

[C] Preparation of blank sample :

1. In a flask labelled as "B" pipette out 5 ml of distill water.
2. To it add 2 ml of 1 % picric acid and 0.5 ml of 10 % sodium hydroxide solution.
3. Allow to keep for 15 minutes and compare in a calorimeter by using green filter. (530 m μ).

Calculations :

Note : Optical density of blank must be(minus) substracted from optical density obtained that of unknown and standard. By using photoelectric calorimetry principle

$$\text{Concentration of creatinine in unknown blood sample CU} = \frac{\text{Optical density of (Eu) unknown sample}}{\text{Optical density of standard sample "Es"}} \times \text{Concentration of standard creatinine (Cs)}$$

Standard creatinine solution is prepared by diluting 1 ml of stock solution (1 mg) to 500 ml.

i.e. 1 ml of standard creatinine solution ≡ 0.002 mg creatinine.

We have used 5 ml of standard creatinine ≡ 0.01 mg creatinine.

Now we have used 5 ml of folin wu blood filtrate.

5 ml filtrate ≡ 0.5 ml blood

Therefore

$$\text{Concentration of creatinine in unknown blood sample CU} = \frac{\text{Optical density of unknown sample "Eu"}}{\text{Optical density of standard sample "Es"}} \times 0.01 \text{ mg creatinine}$$

$$= \text{"x" (suppose)}$$

Now "x" mg of creatinine is present in 5 ml of folin wu filtrate.

But 1 ml of folin wu filtrate ≡ 0.1 ml of blood

∴ 5 ml of folin wu filtrate ≡ 0.5 ml of blood

∴ "x" mg of creatinine is present in 0.5 ml of blood.

To estimate milligram percentage of creatinine

If ∵ 0.5 ml ≡ "x" mg creatinine

$$\therefore \quad 100 \text{ ml} \equiv \frac{x \times 100}{0.5} \text{ mg creatinine}$$

Substitute value of 'x'

$$\text{We get "cu"} = \frac{Eu}{Es} \times \frac{1}{100} \times \frac{100}{1} \times \frac{10}{5}$$

$$= \frac{Eu}{Es} \times 2 \text{ mg \% creatinine.}$$

$$\therefore \quad \boxed{CU = \frac{Eu}{Es} \times 2 \text{ mg \% creatinine}}$$

EXPERIMENT NO - 12

Aim : To estimate blood sugar level in a given blood sample.

Folins sugar tube

Apparatus : Folins sugar tube
Pipettes graduated ... 2 ml, 5 ml.
Flasks.
Photoelectric calorimeter

Chemical reagents : Alkaline copper sulphate solution
Phosphomolybadic acid
Sodium tungstate 10%
Sulphuric acid 2/3 N
Benzoic acid solution
Stock glucose solution
Standard glucose solution No. 1
Standard glucose solution No. 2
Fluoride oxalate solution.

Principle : In this method protein free filtrate is obtained [folin-Wu filtrate] so that 10 ml of filtrate corresponds to 1 ml of blood sample. Protein free filtrate is obtained by precipitating the proteins of blood by tungstic acid. Then this protein free filtrate containing glucose is heated with alkaline copper sulphate solution. Thus glucose reduces copper sulphate to form equivalent quantity of cuprous oxide.

This cuprous oxide formed is reduced with phosphomlybdic acid to produce corresponding equivalent quantity of molybdenum blue. The molybdenum blue gives intense blue colour, the intensity of which is directly proportional to cuprous oxide which corresponds to the amount of glucose present in given sample of "folin wu" filtrate.

Reaction : $C_6H_{12}O_6 + CU^{++} \longrightarrow CU^{+} +$ Oxidation products of glucose.

glucose cupric

$CU^{+} Na_2 PO4 : 12\ MoO_3 \longrightarrow CU^{++} + 12\ MoO$

cuprous cupric molubdenum blue

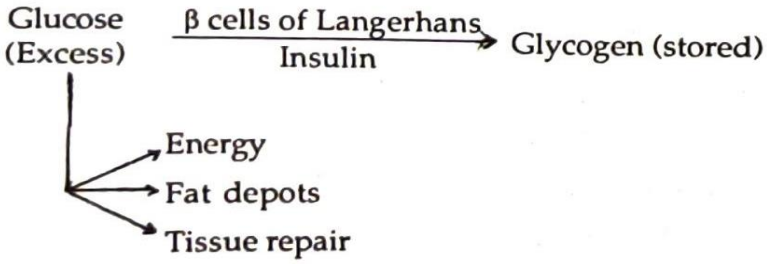
The blue colour obtained with test blood sample is compared with standard solution by similar procedure and by using photoelectric calorimeter. The optical density of test and standard is measured and concentration of glucose in blood can be calculated using colorimetric principle.

Clinical significance : The chief end product of carbohydrate digestion absorbed in the blood is monosaccharide like glucose and others like fructose galactose. etc. glucose metabolism supplies ...

Major amount of energy for body activities
Reserve fat depots
Tissue glycolipids
Amino acids

Thus it appears that glucose metabolism plays a central role in carbohydrate metabolism which is closely associated with metabolism of protein and fat.

Availibility of glucose from various diatery sources and its management/ utilisation is regulated by hormones of islets of langerhans. This can be explained schematically as follows ...



Thus blood sugar level is maintained and it is normally and expressed as Fasting. Post meal.

Hyperglycemia i.e. increase in blood sugar level is a real characteristic sign of "diabetes mellitus" Excess free glucose appears in blood due to lack of insulin or in functioning of β cells to secrete insulin.

In diabetes mellitus high values for fasting blood sugar are obtained and vary from normal to 500 mg/100 ml and over according to severity of condition.

Increase in blood sugar level above 500 mg/100 ml indicates increasing possibility of coma.

Hyperactivity of the thyroid, pituitary aderenal glands which includes sates of emotion stress increases blood sugar level about 150 mg%.

Similar increase in blood sugar level is observed in convulsions and in terminal stages of many diseases.

A moderate increase in blood sugar level i.e. hyperglycemia can occur in sepsis and number of infectious diseases.

Increase in blood sugar level is also found in some intracranial diseases such as meningitis, encephalities, tumours and haemorrhages.

Method used : Folin Wu (modified)

Procedure :

1. Wash clean, label three folin-wu tubes as ...
   Unknown ... 'U'
   Standard I – Std I
   Standard II – Std II
2. To the folin wu tube labelled as "U" take 2 ml of "Folin wu filtrate"
3. In a folin wu tube labelled as "Std I" take 1 ml of standard sugar solution I (0.1 mg sugar)
4. In a folin wu tube labelled as "Std II" take 1 ml of standard sugar solution II (0.2 mg sugar)
5. To all above tubes add 1 ml of alkaline copper sulphate solution.
6. Keep the tubes in boiling water bath for 6 to 8 minutes.
7. Remove from the water bath and add 1 ml of phosphommolyddic acid to all tubes.
8. Keep the tubes again in boiling water bath for 2 minutes and after 2 minutes cool to room temperature.
9. Add 25 ml of distill water to each tube mix well and record. Compare the optical densities by using photoelectric calorimeter by using tube filter 420 mμ.

Calculations : By using photoelectric calorimetry principle

$$\text{Concentration of glucose in given blood sample Cu} = \frac{\text{Optical density of unknown blood glucose sampe Eu}}{\text{Optical density of standard glucose Es}} \times \text{Concentration of std glucose (CS}$$

Now as 1 ml of standard sugar solution is used for experiment it is fact that

1 ml of standard sugar solution ≡ 0.1 mg of sugar

i.e. Cs concentration of standard glucose = 0.1 mg of sugar.

Therefore,

$$\text{Concentration of glucose in given blood sample CU} = \frac{Eu}{Es} \times Cs$$

$$= \frac{Eu}{Es} \times .1 \text{ mg}$$

$$= \text{"x" (suppose)}$$

Now "x" mg of sugar is present in 1 ml of folin wu filtrate of given blood sample i.e. in 0.1 ml blood.

Note : (1 ml o folin wu filtrate ≡ .1 ml of blood)

∴ To determine mg percentage of sugar

If ∵ 0.1 ml blood ≡ "x" mg of sugar

$$\therefore \quad 100 \text{ ml blood} \equiv \frac{100 \times x \text{ mg}}{0.1 \text{ ml blood}}$$

Substitute value of "x" ...

We get

$$\text{Cu} = \frac{100}{0.1} \times \frac{Eu}{Es} \times 0.1$$

$$\text{Concentration of unknown glucose in given blood sample} = \frac{Eu}{Es} \times 100 \text{ mg percent sugar.}$$

$$\text{Concentration of glucose in given blood sample CU} = \frac{\text{Optical density of unknown blood sample Eu}}{\text{Optical density of standard sugar solution}} \times 100$$

Report :
Patients name
Blood sugar (estimated)
Blood sugar (Normal)
Date :
Signature :

Preparation of reagents :

1. Sodium tungstate : 10 % w/v (Na_2 WO4 $2H_2O$) dissolve 10 gm of sodium tungstate in 100 ml of distill water.

2. Sulphuric acid : 2/3 N of sulphuric acid dissolve 3.5 gm in 100 ml of distill water.

3. Alkaline copper sulphate solution :

[A] (a) Anhydrous sodium carbonate ...40 gms
(b) Tartaric acid ...7.5 gms
(c) Dissolve in distill water ...400 ml

[B] (a) Copper sulphate crystalline ...4.5 gm
(b) Dissolve in distill water ...200 ml

Add solution 'B' to solution "A" with constant stirring and make the final volume with distill water to 1000 ml (1 litre)

4. Phosphomolybdic acid reagent :

(a) Molybdic acid —70 gm
(b) Sodium tungstate AR —10 gm
(c) Sodium hydroxide 10 % —400 ml
(d) Distill water —400ml

Boil for 30 minutes to remove ammonia. Add phosphoric acid (specific gravity – 1.75) – 250 ml. Add distill water to make 1 litre final volume.

5. Benzoic and solution :

Benzoic acid ...2.5 gms
Distill water ... 1000 ml (1 litre)
Boil to dissolve

6. Stock glucose solution (1 gm/100 ml)

Glucose (AR) ...1 gm
Benzoic acid solution ... 100 ml

7. Standard glucose solution No. 1 (10 mg/100 ml)

Stock glucose solution ... 1 ml
Benzoic acid solution ... 100 ml

8. Standard glucose solution No. 2 (20 mg/100 ml)

Stock glucose solution ... 2 ml
Benzoic acid solution ... 100 ml

EXPERIMENT NO - 13

Aim:

- Determination of serum total cholesterol.

Requirements:

(a) **Glasswares:** Test tube, Stir rod.

(b) **Chemicals:** Absolute alcohol, Diethyl ether, Chloroform, Acetic anhydride, Standard solution of cholesterol (100 mg%).

Reagent Preparation:

- Dissolve 100 mg of cholesterol in some amount of alcohol. Slightly warm (in water bath), if required. Make the volume to 100 mL with alcohol.

Principle:

- Cholesterol is present in blood both as free and as esters. Free cholesterol normally forms about 30% of the total cholesterol and the ester fraction forms 70%.
- Cholesterol and cholesterol esters from the serum are extracted into an alcohol-ether mixture (Alcohol precipitates the proteins; either solubilises the cholesterol part). The contents are centrifuged. The protein free extract is evaporated to dryness. The cholesterol residue is dissolved in chloroform and is measured colourimetrically by Liebermann-Burchard reaction.

Procedure:

Test:

- In a centrifuge tube, take 8 mL of alcohol, 2 mL of ether. Mix it.
- Add 0.2 mL of blood. Mix overall. Keep it in slanting position for half an hour. Centrifuge it.

- The supernatant is collected (which contains cholesterol) in another tube. This test tube is kept in boiling water for the evaporation of solvent and the residue, i.e. cholesterol sticks to the bottom of the flask is left behind.
- Chloroform, acetic anhydride and sulphuric acid must be of highest quality. It is particularly important that chloroform be especially anhydrous. Ordinary chloroform or old stock will lead to weak and uncertain colour.

Standard:

- In different tubes marked as S_1, S_2, S_3, and S_4, add 0.2, 0.4, 0.6 and 0.8 mL of standard cholesterol solution respectively.
- All tubes should be kept in boiling water bath for evaporation of solvent till the solvent evaporates and residue is left behind.
- Blank: Clean dry test tube.
- Now add the following reagents in test, different standard, and blank.

Reagent	Blank	Standard				Test
		S_1	S_2	S_3	S_4	
Standard cholesterol solution.	–	0.2 mL	0.4 mL	0.6 mL	0.8 mL	Test sample
$CHCl_3$	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL
Acetic anhydride	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL
Concentrated H_2SO_4	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL

- Mix well; keep it in dark for 10 minutes.
- Read optical density at 610 nm.

Observation:

- Optical density of test =
- Optical density of standard S_1 = concentration = 0.02 mg
- Optical density of standard S_2 = concentration = 0.04 mg
- Optical density of standard S_3 = concentration = 0.06 mg
- Optical density of standard S_4 = concentration = 0.08 mg

Calculations:

$$\text{The concentration of cholesterol} = \frac{\text{Optical density of test}}{\text{Optical density of std.}} \times \frac{\text{Conc. of std.}}{\text{Vol. of blood/ serum used}} \times 100$$

Interpretation:

- Normal serum cholesterol level is 150-250 mg/dl.
- Hypercholesterolemia is observed in the following conditions:

- Diabetes mellitus.
- Obstructive jaundice.
- Nephrotic syndrome.
- Cirrhosis of liver.
- Hypoparathyroidism.
- Xanthomatosis.

- Hypocholesterolemia is observed in the following:
  - Hyperthyroidism
  - Pernicious anaemia
  - Haemolytic anaemia
  - Malabsorption syndrome.

Buffers

Buffer Solutions:

- A buffer solution is one in which the pH of the solution is "resistant" to small additions of either a strong acid or strong base.
- Buffers usually consist of a weak acid and its conjugate base, in relatively equal and "large" quantities.
- Calculations are based on the equation for the ionization of the weak acid in water forming the hydronium ion and the conjugate base of the acid.

EXPERIMENT NO - 14

Aim:

- Preparation of carbonate-bicarbonate buffer of pH 10.2.

Requirements:

(a) Glasswares: Flask, Pipette, Stir rod.

(b) Chemicals: Sodium carbonate, Sodium bicarbonate, Distilled water.

Reagent Preparation:

- **Sodium carbonate solution 0.2M:** Dissolve 2.12 gm of anhydrous sodium carbonate in 100 mL distilled water.
- **Sodium bicarbonate solution:** Dissolve 1.68 gm of sodium bicarbonate in 100 mL of distilled water.

Principle:

- Buffer is a solution that resists the change in its pH due to slight dilution or due to addition of small amounts of acids or bases.
- There are two main types of buffers:
  - Acidic buffer that is a mixture of weak acid and its salt with strong base, for example, acetic acid-sodium acetate buffer.
  - Basic buffer that is a mixture of weak base and its salt with strong acid, for example, ammonium hydroxide-ammonium chloride buffer.
- The pH of the buffer solution can be calculated using Henderson's equation

$$pH = pKa + \log \frac{[Salt]}{[Acid]}$$

- Here Ka is the dissociation constant of the acid and pKa = −log Ka.

Procedure:

- Pipette out exactly 27.5 mL of sodium carbonate (Na_2CO_3) solution.
- To this add 22.5 mL of sodium bicarbonate solution and made upto 100 mL with distilled water which corresponds to 0.2 M sodium carbonate and bicarbonate buffer.
- Standardise pH meter and measure the pH of required buffer.
- This gives the Carbonate-bicarbonate buffer pH 10.2.

Result:

- Carbonate bicarbonate buffer was prepared and pH observed was 7.5 which was adjusted to 10.2 using 1N HCl and 5N NaOH.

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Salivery Amylase

- Human salivary alpha amylase (HSAmy) is an important enzyme found in the oral cavity. It belongs to the glycoside hydrolase family and exists in various isoforms in salivary secretions. Humans produce two kinds of alpha amylase (salivary and pancreatic amylase) that overall share about 97% homology.
- HSAmy is a type of hydrolase that breaks down complex carbohydrates into simple sugars, like glucose or maltose, by cleaving α-1,4-glucosidic bonds. Further breakdown of the starch is completed by pancreatic amylase later in digestion.
- This enzyme has distinct components including a calcium ion, chloride ion, and various amino acid residues that play critical roles in hydrolytic activity and substrate binding.
- HSAmy can also bind to oral *streptococci* bacteria. This results in the bacteria either being removed from or stored in the oral cavity. Storage and accumulation of the bacteria in the oral cavity causes dental complications like plaque and tooth decay.

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EXPERIMENT NO - 15

Aim:

- Study of enzymatic hydrolysis of starch.

Requirements:

(a) Glasswares: Test tube, Flask, Pipette, Stir rod.

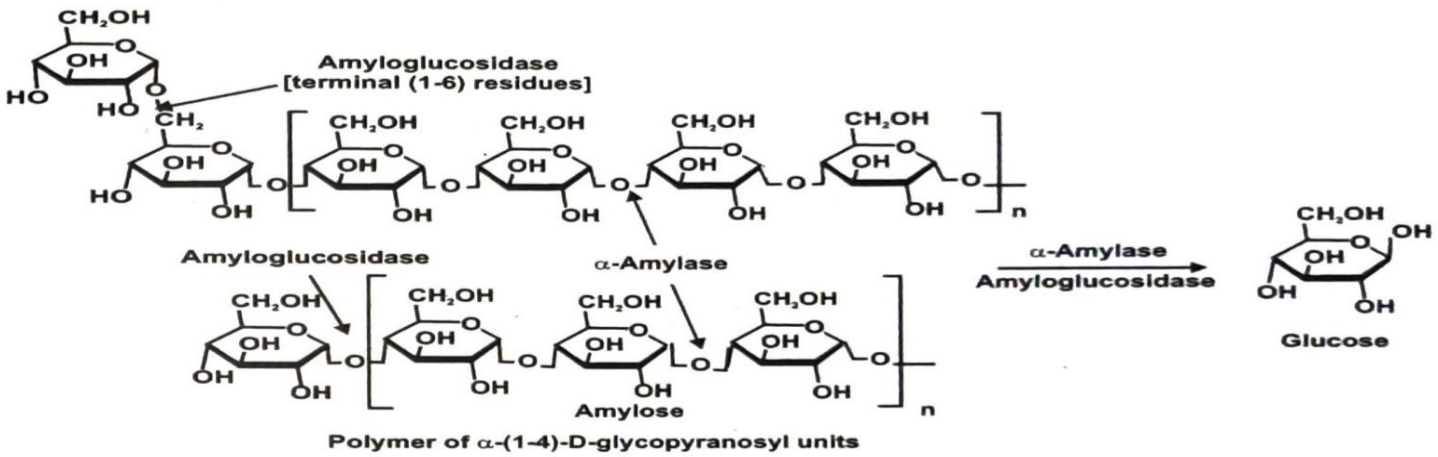
(b) Chemicals: Starch, phosphate buffer solution, Iodine solution.

Principle:

Starch is mainly composed of two parts:

- **Amylose:** a linear polymer made up of α-1-4 bound D-glucose units. This polysaccharide is making up approximately 20-30% of the structure.
- **Amylopectin:** a polysaccharide with highly branched polymer of α-1-6 bound glucose units.
- Amylase is an enzyme that hydrolysis alpha-bonds of large alpha-linked polysaccharides such as starch and glycogen, yielding maltose and dextrin. It is the major form of amylase found in humans and other mammals.

- Amylase is found in saliva. This form of amylase is also called "ptyalin". The enzyme randomly affects α-1-4 bonds, belonging to the amylose structure of starch, and maltose units form. Amylase does not affect the α-1-6 bonds that belong to the amylopectin structure of starch. As a result of hydrolysis carried out with α-amylase, besides the maltose and glucose units, a large branched dextrin structure is formed in the media.



Polymer of α-(1-4)-D-glycopyranosyl units

The optimum conditions for α-amylase:

- Optimum pH: 5.6-6.9
- Human body temperature: 37°C.
- Presence of certain anions and activators:
  - ➢ Chloride and bromide- most effective
  - ➢ Iodide- less effective
  - ➢ Sulphate and phosphate- least effective

Procedure: Part-A

- Add 1 mL of 1% starch solution and 1 mL phosphate buffer solution (pH = 6.8) into 4 test tubes.
- Place each test tube into water baths at 0°C, 25°C, 37°C and 95°C, respectively.
- Wait for a few minutes and add 1 mL diluted (1/10) saliva into each test tube.
- Place 1 drop of iodine solution on 4 watch glasses and take 1 drop from each test tube to observe if there is any starch left (Starch gives blue colour with iodine solution).
- Record the time for the hydrolysis of 1 mL starch in each test tube.

Procedure: Part-B

- Take 7 test tubes and prepare the following solutions

Reagent	T_1	T_2	T_3	T_4	T_5	T_6	T_7
1% Starch Solution	0.2 mL	0.5 mL	1 mL	2 mL	3 mL	4 mL	5 mL
Distilled Water	4.8 mL	4.5 mL	4 mL	3 mL	2 mL	1 mL	0 mL
Phosphate Buffer	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Saliva (1/20 diluted)	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL

- Shake each test tube thoroughly.
- Check each mixture for colour with iodine per minute.
- Record colour changes.

Result: .

- Time for the hydrolysis and the colour change of starch by α-amylase was recorded.

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EXPERIMENT NO - 16

Aim:

- Determination of Salivary amylase activity.

Requirements:

(a) Glasswares: Test tube, Flask, Pipette, Stir rod.

(b) Chemicals: Starch, Sodium Chloride, NaOH, Sodium potassium tartrate, Maltose, Distilled Water.

Reagent Preparation:

- **Substrate (Starch):** Mix 1 gm of soluble starch in 200 mL of 0.1M Phosphate buffer (pH 6.8) boil for 3 minutes and cool to room temperature and filter if necessary.
- **Enzyme:** Saliva is the best and easily available source of amylase. Collect some saliva in a beaker and dilute it to 1 : 20 dilution with distilled water.
- **1% Sodium chloride:** It is necessary for enzyme activity
- **DNS (Dinitro Salicylic acid):** Dissolve 1.6 gm of NaOH in 20 mL of distilled water. Take 1 gm of 3, 5 DNS in NaOH solution. In other beaker take 30 gm of Sodium potassium tartrate. Dissolve in 50 mL of distilled water. Mix this DNS solution and finally make the volume up to 100 mL with distilled water.
- **Standard solution of Maltose:** It is prepared by dissolving 200 mg Maltose in 100 mL of water (2 mg / 1 mL).

Principle:

- Amylase is the hydrolytic enzyme which breaks down many polysaccharides like Starch, Amylose, dextrins and yields a disaccharide i.e., Maltose.

Procedure:

- Take 0.5 mL of substrate and 0.2 mL of 1% NaCl in a test tube and pre-incubated at 37°C for 10 minutes then add 0.3 mL of diluted saliva and incubate for 15 minutes at 37°C.
- Stop the reaction by addition of 1 mL of DNS reagent mix well and keep the test tubes in boiling water bath for 10 minutes.
- Cool and dilute with 10 mL of distilled water.
- Read the colour developed at 520 nm. Simultaneously setup the colour developed at 520 nm.
- Simultaneously setup the blank as per the test by adding DNS prior to the addition of enzyme simultaneously.
- Set up the standards of different test tubes and repeat the experiment as per the test and measure the colour developed at 520 nm absorbance.

Sr. No.	Volume of Std. (ml)	Volume of Distilled water (ml)	Concentration of Standard (ml)	Volume of DNS (ml)	Boiling water bath for 10 minutes and cool it	Volume of Distilled water (ml)	OD at 520 nm
1	Blank	1.0	0.0	1.0		10	
2.	0.2	0.8	0.4	1.0		10	
3.	0.4	0.6	0.8	1.0		10	
4.	0.6	0.4	1.2	1.0		10	
5.	0.8	0.2	1.6	1.0		10	
6.	1.0	0.0	2.0	1.0		10	

Calculation:

- 1.5 mg of Maltose formed / 0.3. mL / 15 minutes
- 1.5×4 mg of Maltose formed / 0.3 mL of Enzyme / 1 hour
- $1.5 \times 4 \times 3.3$ mg of Maltose formed / 1 mL of Enzyme / 1 hour
- $1.5 \times 4 \times 3.3 \times 100$ mg of Maltose formed / 100 mL of Enzyme / 1 hour

Result:

- The amount of Maltose in the given unknown sample is _____ grams of Maltose formed per 100 mL of enzyme per one hour.

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