BIO 303

Biochemistry – II

LABORATORY MANUAL

DEPARTMENT OF BIOLOGY

VIRTUAL UNIVERSITY OF PAKISTAN

LAHORE

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Contents

Experiment No. 1

Preparation of stock and working solutions in Laboratory

Apparatus Required

Weighing balance, volumetric flask, Cylinder, beaker, stirrer, pH meter or litmus paper

Chemicals required

Sodium chloride, Hydrochloric acid, sulfuric acid

Theory

Solution:

"A solution is a homogenous mixture of atoms, ions or molecules of two or more substances."
A homogenous mixture is that which has uniform position throughout its body.

Solvent and Solute

The solvent is the component of a solution that is visualized as dissolving another component called absolute. Usually the component present in the larger quantity is called the solvent, and the component present in the smaller quantity is called the solute.

Types of solution

Unsaturated solution

A solution that is capable of dissolving more solute at a given temperature than it already contains, is known as unsaturated solution.

Saturated solution

A saturated solution is the solution which can dissolve no more amount of the solute, at a given temperature.

Super saturated solution

A solution that contains more dissolved solute than a saturated solution is called super saturated solution.

Concentration and its units

Concentration means the relative amounts of the components of a solution. It tells the ratio of the quantity of one component to the quantity of the other or to the total quantity of solution. It has many units. Some common units are discussed below.

Mass Percentage

The ratio of the mass of the solute to the mass of the solution multiplied by 100 is called mass percentage.

$$\text{Mass Percentage of a solute} = \frac{\text{Mass of the solute} \times 100}{\text{Mass of solution}}$$

For liquid-liquid solutions, it is sometimes more convenient to express the concentration in the units of percentage by volume:

Volume Percentage of a liquid = $\frac{\text{Volume of the liquid x100}}{\text{Total volume}}$

Parts per Million (ppm)

This is used to express very dilute concentrations of a substance. One ppm is equal to 1mg of solute dissolved per litre of solvent or 1mg of solute dissolved per kg of solvent.

Molarity (M)

Molarity or the molar concentration is the number of moles of solute dissolved per dm^3 of solution. (1 dm^3 is equal to 1Liter)

Molarity = $\frac{\text{Number of moles of solute}}{\text{Volume of solution in liters}}$

Molality (m)

Molality is the number of moles of solute dissolved per kilogram of solvent.

Normality (N)

Normality is the number of equivalents dissolved per liter of solution.

$$\text{Normality} = \frac{\text{No. of equivalents}}{\text{Volume of solution in Litres}}$$

pH

The pH is defined as follows:

"The pH of a solution is the negative logarithm to the base 10 of the hydrogen ion (or Hydronium ion) concentration".

$pH = -\log_{10}[H^+]$

$pH = -\log_{10}[H3O^+]$

The pH of a neutral solution is 7, the pH of acids is less than 7 while that of bases is higher than 7.

Procedure

i. Preparation of 5% NaCl solution

1. Weigh 5grams of sodium chloride.
2. Dissolve the sodium chloride (NaCl) in 25 ml of distilled water in a beaker with the help of stirrer.
3. Transfer the contents into a volumetric flask. Add some more water (10-20ml) to the beaker and rinse the walls of beaker and add that to the same flask.
4. Raise the volume to 100 ml in the volumetric flask to prepare 5% NaCl solution.
5. Check the pH of the solution with pH meter.

Preparation of 5% hydrochloric acid solution (50ml) using 37% HCL.

Take 25 ml of distilled water in a volumetric flask.

Calculate the volume of HCL required by using the formula:

$C_1V_1=C_2V_2$

$37 \times V = 5 \times 50$

$V_1 = 250/37$

$= 6.7$ ml

Add 6.7ml of HCl to the volumetric flask slowly and raise the volume to 50ml with distilled water to prepare 50 ml of 5%HCl solution.

Record the pH of the Solution using pH meter or litmus paper.

Experiment No. 2

Basic biochemical methods such as iodine test for polysaccharides

Theory:

The iodine test is mainly performed to check the presence of carbohydrates among which starch and sugar are the main carbohydrates found in our food products.

Principles:

To produce the triiodide anion (I_3^-), elemental iodine is dissolved in an aqueous solution of potassium iodide. The resulting complex with starch produces an intense "blue-black" colour. The intensity of the colour decreases with increasing temperature and with the presence of water-miscible organic solvents such as ethanol. The test cannot be performed at very low pH due to the hydrolysis of the starch under these conditions. It is now thought that the iodine-iodide mixture combines with the starch to form an infinite polyiodide homopolymer. This was rationalized through single crystal x-ray crystallography and comparative Raman spectroscopy.

Material Required:

Knife, Spatula, Porcelain tile, Iodine solution and Food sample like potato or any other vegetable or fruit.

Procedure:

- Take a fresh potato which is washed, cleaned and dried. Peel off the skin of the potato as they are impermeable.
- Cut the potato into small cubes
- With the help of clean and dried spatula, place the potato sample on the clean and dried porcelain tile.
- Add 2 to 3 drops of dilute iodine solution on the potato samples.
- Keep the slides undisturbed and observe the change.

Observations:

There will be a change in color. A blue black color develops on the slice or cubes of the potato samples.

Results:

According to the observation the food sample or the potato slice turned to blue-black on adding the iodine solution. This proves the presence of starch in the given sample.

Experiment No. 03

Fermentation of sugars by Baker's yeast

Theory:

Yeasts are tiny, microscopic organisms—or microorganisms—that are actually a type of fungus. This means that they are more closely related to a mushroom than to plants and animals or bacteria (the latter of which are also microorganisms). These little critters might sound strange and different, but people have been using them for thousands of years to make bread rise. They turn this food into energy and release carbon dioxide gas as a result. This process is known as fermentation. When yeasts eat sugar and turn it into energy, they also produce carbon dioxide. This process is known as fermentation. In this activity, the balloons on the bottles should have captured carbon dioxide produced by the yeasts during fermentation.

Apparatus Required:

Beaker 500ml, 4 flasks of 250 ml, burner, tripod stand.

Reagents Required:

Yeast powder, water, Sugar.

Procedure:

- Take 5 flask and mark them A, B, C, D and E.
- Take 2.5g of yeast and put it in flask B, C and D.
- After that add 5g of sucrose in flask A, C and E.
- Add 10 gram of sucrose in flask D.
- Now add 100ml of distilled water to all flasks.
- Now boil only flask C for 5 minutes.
- Now close the mouth of all flasks with balloons.
- Leave the whole assembly for 24 hours.

Results:

Balloons of flask A,B and C doesn't blown but the balloons of flask D and E will blow. This shows that fermentation occur in flask D and E during which CO_2 evolved and become reason of blowing of balloons.

Experiment No. 04

Isolation of amylose and amylopectin from starch

Theory:

Amylose is an un-branched chain polymer of D-glucose units. Amylopectin is a branched chain polymer of D-glucose units. It gives a dark blue/black color when iodine solution is added. It gives a reddish brown color when iodine solution is added.

Material Required:

Sweet potato, Knife, mortar & pastel, blender, blue capped tubes, 20mM sodium phosphate buffer at pH 7 and vortex

Procedure:

- Peel off sweet potatoes and note the weight.
- Beet sweet potato with the help of mortar and pastel
- Now blend sweet potato in blende by adding 40ml of cold sodium phosphate buffer in it.
- Blend until it converts into slurry.
- Transfer the slurry in blue capped tubes and mix with the help of vortex.
- Filter the extract with the help of cotton cloth or syringe filter.
- Centrifuge the filtrate at 12000rpm for 5min at 4 degree centigrade.
- After centrifugation discard the pallet and store the supernatant at 4 degree centigrade.

Results:

The supernatant after centrifugation is consist of amylose and can be crystalized with the help of spray dryer in order to convert into powder form.

Experiment No. 05

Extraction of glycogen from liver

Theory:

Glycogen is a multibranched polysaccharide of glucose that serves as a form of energy storage in animals, fungi, and bacteria. The polysaccharide structure represents the main storage form of glucose in the body. Glycogen functions as one of two forms of energy reserves, glycogen being for short-term and the other form being triglyceride stores in adipose tissue (i.e., body fat) for long-term storage. In humans, glycogen is made and stored primarily in the cells of the liver and skeletal muscle. In the liver, glycogen can make up 5–6% of the organ's fresh weight, and the liver of an adult weighing 1.5 kg can store roughly 100–120 grams of glycogen. In skeletal muscle, glycogen is found in a low concentration (1–2% of the muscle mass) and the skeletal muscle of an adult weighing 70 kg stores roughly 400 grams of glycogen.

Material Required:

TCA, Centrifuge, glass cylinder, centrifuge tubes, 95% ethanol, water bath, Nacl

Procedure:

- Weight the liver sample and record the weight.
- Cut liver into small pieces and grind with about 0.5g of cold cold sand 10% TCA (1ml per g tissue).
- Centrifuge homogenate at 3000rpm for 5 minutes
- Pour off supernatant into ml graduated cylinder.
- Rinse out mortar with 5% TCA (using same volume as for 10% TCA already used)
- Add this rinsing fluid to the centrifuge tubes containing residue from first centrifugation
- Re centrifuge for another 5 min at 3000rpm
- Discard pallet. Add supernatant already collected.

- Record the total volume; add the double of the supernatant volume of 95% ethanol, slowly with stirring to supernatant
- Allow to stand while precipitate settles, if not add a little Nacl and warm cylinder in water bath at 37 degree centigrade.
- Centrifuge supernatant at 3000rpm for 3 min. Discard supernatant.
- Now add 3ml diethyl ether, stir up pellet, recentrifuge and discard supernatant. This final pellet contains glycogen from liver.
- Air- dry the glycogen in the tube and weight it.

Results:

Weight the centrifuge tube that contains the glycogen = gm

Weight the empty centrifuge tubes used in experiment = gm

So Glycogen content (g) =

Centrifuge tubes contains pallet – empty centrifuge tube

Record total glycogen yield in 100g liver.

Experiment No. 06

Acid and enzymatic hydrolysis of glycogen

Glycogen is released from the tissue by heating with strong alkali and precipitated on the addition of ethanol. Sodium sulphate is added as a co-precipitant to give a quantitative yield of glycogen. The polysaccharide is then hydrolyzed in acid and the glucose released is estimated.

Requirements:

1. Heart, liver, and muscle from a freshly killed rat.
2. potassium hydroxide (300 g/l)
3. Calibrated centrifuge tubes (10 ml). 30
4. Boiling water bath. 24
5. Saturated $NaS0_4$. 20 ml
6. Ethanol (95% v/v). 250 ml
7. Volumetric flasks (100 ml). 24
8. Test tubes calibrated at 10 ml. 100 ml
9. HCl (1.2 mol/l.). 100 ml
10. Marbles.
11. Phenol red indicator solution. 12 ml
12. NaOH (0.5 mol/l). 250 ml
13. Reagents for the estimation of glucose

Isolation of glycogen:

Accurately weigh the complete heart and muscle and about 1.5 g of liver. Place the tissues into a calibrated centrifuge tube containing 2 ml of KOH (300 g/l) and heat in a boiling water bath for 20 min with occasional shaking. Cool the tubes in ice, add 0.2 ml of saturated Na_2SO_4, and mix thoroughly. Precipitate the glycogen by adding 5 ml of ethanol (95% v/v), stand on ice for 5 min, and remove the precipitate by centrifugation. Discard the supernatant and dissolve the precipitated glycogen in about 5 ml of water with gentle warming, then dilute with distilled water to the 10 ml calibration mark and mix thoroughly. In the case of

the fed animals, transfer the liver sample quantitatively to a 100 ml volumetric flask and make up to the mark with water.

Enzymatic hydrolysis of Glycogen:

Objective:

To examine the polysaccharide nature of glycogen and show that hydrolysis increases the number of reducing groups.

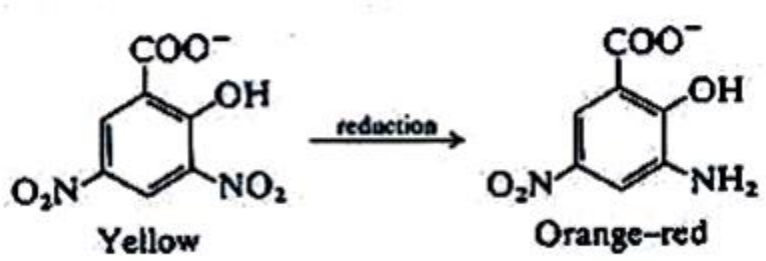
Introduction:

The structure of the glycogen molecule is fan-like; with long chains of glucose residues linked by 1, 4-glycosidic bonds, with 1, 6- links at the branch points. So the whole glycogen molecule has only one free reducing end, where the C1 of a glucose residue is free (exposed). Thus the glycogen molecule is essentially non-reducing. Hydrolysis converts glycogen from a non-reducing substance into reducing substances. Hydrolysis of the glycogen molecule with acid results in splitting of all its glycosidic bonds giving only glucose molecules as the product. Enzymes are more specific in the bond type they split. Thus salivary amylase (α-amylase) will randomly split only 1, 4- glycosidic bonds and produce a mixture of products consisting of glucose, maltose and maltotriose molecules. The increase in the number of reducing groups is determined using 3, 5-dinitrosalicylic acid (DNS) in alkaline solution. The oxidation of carbohydrate or related compounds is the main source of energy for many organisms. The readily digestible carbohydrates of the mammalian diet include the starches, amylase and amylopectin, as well as glycogen. Amylose is a linear polysaccharides consisting of glucose units linked to one another in sequence by α-1-4 bonds. Amylopectin and glycogen are branching polysaccharides, in addition to α-1, 4 bonds, they have α-1, 6- glycosidic bonds at the branch points. The hydrolysis of these glycosidic bonds is catalyzed by either acids or enzymes, in the acid-catalyzed hydrolysis there is a random cleavage of bonds, with the intermediates formation of all the various possible oligosaccharides and with the final conversion of these oligosaccharides to glucose. In the presence of amylases, which have been classified into two main groups, α and β according to the mode of their attack on the polysaccharide. The amylases of animal origin are all α-amylases and in the digestive system are found in saliva and in pancreatic juice. A-amylases catalyze the rapid, random hydrolysis of internal α-1, 4 bonds. They do not hydrolyze α-1, 6 linkages, regardless of molecular size nor do they hydrolyze maltose. Thus glycogen is initially split by α-amylase action into branched dextrins of medium molecular weight and only small amounts of maltose are formed. Further action of α-amylase decreases the molecular weight of these dextrins yielding oligosaccharides. The final degradation products of the action of α-amylase on

glycogen are glucose, maltose and isomaltose. A second enzyme β-amylases which is widely distributed in plants and microorganisms, also catalyze the hydrolysis of glycogen. They catalyze the successive hydrolysis of the second α-1, 4 glycosidic bond from the free nonreducing ands of glucose chains, releasing maltose units. But β-amylases do not hydrolyze α-1, 6 bonds, nor do they hydrolyze α-1, 4 bonds of glucose chains beyond an α-1, 6 branch residues. Thus, after all the nonreducing end glucose chains have been trimmed back to the branch residues, the final products of the action of β-amylase on glycogen are maltose and the remaining limit dextrin. There are many methods for measuring the hydrolysis of glycogen and other polysaccharides, such as measurement of reducing sugars, or change of decreasing viscosity and the loss of capacity to give a blue color with iodine and finally the formation of split products.

Principle:

Several reagents can be used to assay reducing sugars such as 3, 5 dinitrosalicylic acid in one of the compounds. In alkaline solution it is reduced to 3-amino-5- nitro salicylic acid, which is orange-red. Absorbance is determined at 540 nm.



Yellow Orange-red

Requirements:

Glycogen• Sodium dihydrogen phosphate (NaH2PO4) • Sodium hydroxide • Sodium chloride • Sodium potassium tartrate • 3,5-Dinitrosalicyclic acid (DNS) • HCl • Boiling water bath • Spectrophotometer • Small beaker • Big test tubes (25 ml) • Glass cuvettes.

Preparation of solutions:

0.02 M Na phosphate buffer, pH 6.9, containing 0.005 M NaCl (PS buffer):

1. Prepare 500 ml 0.04 M NaH2PO4 (MW 120). Dissolve 2.4 g in water and make up to 500 ml.

2. Prepare 250 ml of 0.04 M NaOH (MW 40). Dissolve 0.4g NaOH in water and make up to 250 ml.

3. 0.005 M NaCl(MW 58.5). Dissolve 0.2925g NaCl in a little water.

To 500ml of the NaH2PO4 solution add 224 ml of the NaOH solution. Mix and measure the pH.If is less than 6.9 adjust by adding more NaOH solution. Add the NaCl solution and make up to 1 liter.

Preparation of 3, 5 -dinitrosalicyclic acid reagent (DNS):

2M NaOH. Prepare 250 ml. Dissolve 20g in water and make up to 250 ml.

a) 3, 5- dinitrosalicyclic acid. Dissolve 10g in 200 ml 2M NaOH.(warm)

b) Sodium potassium tartrate. Dissolve 300g in 500ml water.

Prepare DNS reagent fresh by mixing A and B and making up to 1 liter with water

2M HCl: Take 16.7 conc. HCl in 100ml volumetric flask and make up to 100 ml with water.

1.2 M NaOH: Dissolve 4.8 g in water and make up to 100 ml.

Glycogen solution: Dissolve 32 mg glycogen in 4 ml phosphate buffer/ NaCl (8mg/ ml).

Saliva: Collect about 2 ml saliva in a small beaker. Immediately before use dilute 1: 20 with the buffer (Take 1ml saliva and add 19.0 ml buffer).

Procedure:

Label nine tubes 1 -9. Pipette 0.4 ml PS buffer in tube 1 and use as blank. Pipette 0.4 ml glycogen solution into each of the remaining tubes (2-9). Add 0.6 ml 2 M HCl to tube 9 and incubate in a boiling water bath for 30 min. Add 0.6 ml diluted saliva to each of the remaining tubes (1-8) and stand at room temperature. Immediately after adding diluted saliva to tube 2 stop action of the enzyme by adding 1 ml of DNS reagent. Stop the reaction in tubes 3-7 at 2 min. intervals and in tube 8 after 30 min in the same manner. After tube 9 has been in water bath for 30 min. add 1 ml 1.2 M NaOH to neutralize HCl, then add 1ml DNS reagent. Heat all the tubes for 5 min. in a boiling water bath. Cool by immersing in cold water. Add 8 ml water to each tube except tube 9. Add 7 ml water to tube 9. Read absorbance at 540 nm against the blank. NB. Since the amylase content of saliva varies between individuals, in some cases it may be necessary to make different dilutions of saliva to get reasonable results. You may therefore have to repeat the experiment.

Tube No.	PS Buffer (ml)	Glycogen Solution (ml)	Dilute saliva (ml)	2 M HCl (ml)	Time of Hydrolysis (min)	1.2.M NaOH (ml)	DNS Reagent (ml)	Water (ml)
1	0.4	-	0.6	-	30	-	1	8
2	-	0.4	0.6	-	0	-	1	8
3	-	0.4	0.6	-	2	-	1	8
4	-	0.4	0.6	-	4	-	1	8
5	-	0.4	0.6	-	6	-	1	8
6	-	0.4	0.6	-	8	-	1	8
7	-	0.4	0.6	-	10	-	1	8
8	-	0.4	0.6	-	30	-	1	8
9	-	0.4	-	0.6	30	1	1	7

Results:

Tube 9 contained the total glucose yield from complete hydrolysis of glycogen. Taking this as 100% conversion of glycogen to glucose, plot the percentage hydrolysis against time.

Tube No.	Time of hydrolysis(min)	Absorbance (540 nm)	percentage hydrolysis
2	0		
3	2		
4	4		
5	6		
6	8		
7	10		
8	30		
9	30		100

Hydrolysis and estimation of glycogen:

Pipette duplicate 1 ml samples of the glycogen solutions into test tubes calibrated at 10 ml, add 1 ml of HCl (1.2 mol/l), place a marble on top of each tube, and heat in a boiling water bath for 2 h. At the end of this period, add 1 drop of phenol red indicator and neutralize carefully with NaOH (0.5 mol/l) until the indicator changes from yellow through orange to a pink color. Dilute to 5 ml with distilled water and determine the glucose content by the 3.5 dinitrosalisylic acid method. Then use the standard curve you obtained to estimate the concentration of glucose per100 g sample.

Experiment No. 07

Extraction and estimation of lipids from plant tissue/seed and lipid separation from different tissues

Introduction:

Lipids play an important role in various cellular and physiological functions. Biochemical analysis of lipids requires their isolation. Depending on the type of the sample, extraction protocols vary considering the tissue structure, texture and lipid contents. The high sensitivity of some analytical methods employed to measure lipids requires the use of very pure solvents and clean glassware. Furthermore, all lipids must be protected against degradation through oxidation by solvent, oxygen, and enzymes in combination with temperature and light. Lipids can be broadly classified as polar and non polar lipids based on the head group present.

(Folch Extraction)

Requirements:

(1) Chloroform: Methanol (2:1, v/v), (2) 0.9% NaCl in water, (3) Homogeniser, (4) Lyophiliser

Procedure:

- The tissue is homogenised with Chloroform: Methanol (2:1, v/v) to a final volume 20 times the volume of the tissue sample (1 g in 20 ml of solvent mixture).
- Agitate the mixture for 15 min in an orbital shaker at RT.
- Centrifuge at 14,000 rpm for 10 min to get the clarified supernatant.
- Measure the volume of the supernatant and transfer to a fresh tube.
- Break phase by adding 0.2 volumes of 0.9% NaCl.
- Vortex the tube hard for 1 min.
- Centrifuge at a low speed of 2000 rpm to separate the two phases.
- Transfer the upper aqueous phase for analysis of ganglio- sides and polar molecules.
- Collect the lower organic phase containing lipids in a fresh tube.
- Evaporate in a lyophilizer or under a nitrogen stream and store below −80◦C.

Experiment No. 08

Thin Layer Chromatography

Thin layer chromatography (TLC) consists of a thin layer adsorbent such as silica gel, alumina or cellulose on a flat carrier like a glass plate, a thick aluminum foil, or a plastic sheet. This layer of adsorbent acts as a stationary phase. This method is widely used in lipid analysis or is the standard method in organic chemistry for qualitative analysis of organic reactions. The adsorbent like silica has hydroxyl groups which act as interacting groups. The sample partitions between the mobile and the stationary phase. Individual components in the sample will interact with the stationary phase based on charge, solubility and adsorption. The components could then be visualised by iodine vapors or fluorescent dyes. The retention factor or the Rf value is a characteristic of the substance. This is a constant for a particular substance for that specific solvent and plate system. The Rf value is the ratio of the distance moved by the compound to that moved by the solvent.

Requirements:

(1) TLC plate
(2) Mobile phase solvent
(3) Glass jar

Procedure:

1) Bake the TLC plate in an oven set at 100◦C for 1 hr before use.

(2) Pour the appropriate developing solvent into a glass jar at least one hr before use. This is to saturate the jar with the running solvent vapors.

(3) Mark the TLC plate with pencil.

(4) Spot the sample and the respective standards onto the plate.

(5) Dip the plate in the running solvent just below the sample load.

(6) Allow the solvent to run due to capillary action till it reaches nearly the end of the plate.

(7) Remove the plate from the jar and let it dry.

(8) Stain the plate for visualization of the compound.

(9) Measure the distance of the solvent and the compound travelled to obtain the Rf values.

Results:

Calculate the R_f by below given formula.

R_f = Distance travelled by the compound/ Distance travelled by the solvent front

For example, if a compound travels 2.1 cm and the solvent front travels 2.8 cm, the Rf is 0.75:

R_f = 2.1/2.8 = 0.75

Experiment No. 9

Macroscopic analysis of Urine

Key recommendations for urine sample collection and handling:

- Patients should be well at baseline. They should have no urinary tract infection, no acute febrile illness, no intense exercise within the previous 24 hours.
- Recommended urine collection is a fresh, first morning void. A minimum of 5ml should be collected.
- If a first morning void is not practicable, random spot samples are acceptable.
- Samples not able to be delivered to the laboratory within 8 hours, should be refrigerated.
- Analysis should be performed on the day of receipt but samples can be stored for up to 7 days at 2-8 °C if necessary.
- Cloudy or particulate samples should be centrifuged prior to analysis.
- Positive ACR results must be confirmed, ideally on a fresh, first morning void, by repeat measurement on 1-2 occasions within 3 months.
- Prolonged storage should be at -70 °C; samples should not be stored at -20 °C.

Apparatus Required:

Plastic cup with a lid for sample collection, test tubes, test tube holder, dropper, beaker, litmus paper

Macroscopic Examination

The physical appearance of a urine sample can often tell a great deal about a patient's condition. A change in color or clarity may indicate the presence of a disease and the need for additional testing.

COLOR

Color is usually some shade of yellow and often varies with the concentration of the sample. The most common color descriptions of normal urine are straw, light yellow, yellow and dark yellow. Amber colored urine is seen in patients with increased bilirubin levels and may indicate hepatitis.

CLARITY

Clarity is an indication of the transparency of a specimen. It is best to judge clarity by observing light through a recently mixed sample. Terms used to describe clarity include clear,

hazy, cloudy and turbid. Freshly voided urine that is properly collected is normally clear or slightly hazy, while contaminated urine is more likely to be hazy. Fresh urine that is cloudy is often the result of a bacterial urinary tract infection due to white blood cells in the urine. Turbid urine contains salt crystals that precipitate out as the specimen cooled.

pH

The pH is a measure of the degree of acidity or alkalinity of the urine. A pH below 7 indicates acidic urine; pH above 7 indicates alkaline urine. Normal, freshly-voided urine may have a pH range of 5.5 - 8.0. The pH of urine may change with diet, medications, kidney disease, and metabolic diseases such as diabetes mellitus. Colors on the pH reagent pad usually range from yellow-orange for acidic pH to green-blue when pH is alkaline.

Experiment No. 10

Estimation of glucose levels in urine

Apparatus Required:

Plastic cup with a lid, test tubes, test tube holder, dropper, beaker, water bath

Theory:

A urine glucose test measures the level of glucose, or sugar, in urine. It is less invasive than a blood glucose test, but it also tends to be less accurate. High glucose levels often indicate diabetes, a group of diseases that affects the way the body handles glucose.

A urine glucose test is a quick and simple way to check for abnormally high levels of glucose in the urine. The most common cause of elevated glucose levels is diabetes, a condition that affects the ability to manage glucose levels. The symptoms of diabetes include excessive thirst, blurred vision, and fatigue. When left untreated, diabetes can lead to long-term complications, including kidney failure and nerve damage. Other diseases with elevated glucose levels can be renal glycosuria, diabetes, or gestational diabetes.

The normal amount of glucose in urine is 0 to 0.8 mmol/L (millimoles per liter).

Benedict's Test:

Benedict's test is used for the detection of reducing sugars(sugar shaving free reactive carbonyl group) in urine.

Principle:

Glucose is a simple aldehyde sugarwith the molecular formula C6H12O6.

Benedict's test utilizes a mixture of sodium citrate,copper(II) sulfate and sodium carbonate in a slightly basic solution. Reducing sugars reduce the copper(II)ionstocopper(I)oxide(Red ppt).

$$\mathbf{R\text{-}CHO + 2Cu^{2+} + 5OH^{-} \longrightarrow R\text{-}CO_2^{-} + Cu_2O\ (redppt) + 3H_2O}$$

R-CHO =Reducingcarbohydrates $R\text{-}CO^{2-}$ =Carbohydrate ion

Reagents Required

i. Benedict's Reagent**:** Dissolve Sodium Citrate(173g) and anhydrous Sodium Carbonate(100 g) in about 700ml of distilled water by gently heating the contents.

Dissolve Copper Sulfate (17.3g) in about 100mL of distilled water in a separate beaker.Transfer this solution gradually in to the Carbonate-Citrate mixture with constant stirring and raise the volume to1L with distilled water.

ii. Sample: Urine collected from diabetic and healthy subjects.

Procedure:

1. Add 1 mlof samplein a test tube.

2. Then add 2 mlof Benedict's reagent.

3. Heat the solution in a boiling waterbath for3minutes.

4. Formation of red precipitate indicates positive test.

Important notes:

Benedict's test is a semi quantitative test. The color of the precipitate gives a rough estimate of the reducing sugars present in the given sample.

Green color - Up to 0.5 g%

Green precipitate - 0.5-1.0 g%(+)

Yellow precipitate -1.0-1.5 g% (++)

Orange precipitate- 1.5-2.0 g% (+++)

Experiment 11

Fehlings Test for glucose estimation

Apparatus Required:

Plastic cup with a lid, glass test tubes, holders, droppers, bottles for sample collection, test tube stand

Reagents Required

Fehling's solution A and B

Sample: Urine

Procedure:

In a test tube, add 2 ml of the test solution and add equal volumes of Fehling A & Fehling B and place it in a boiling water bath for few minutes.. When the contents of the test tube comes to boiling, mix them together and observe any change in color or precipitate formation. The production of yellow 'or brownish-red precipitate of cuprous oxide indicates the presence of reducing sugars in the given sample.

READINGS:

http://www.healthline.com/health/glucose-test-urine

Experiment No. 12

Estimation of albumin levels in urine

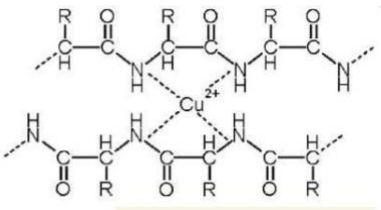
An albumin test checks urine for a protein called albumin. Albumin is normally found in the blood and filtered by the kidneys. If the amount of albumin is very small, but still abnormal, it is called microalbuminuria. Albuminuria is most often caused by kidney damage from diabetes (nephropathy) or chronic kidney disease. Screening for proteinuria is preferably done by measurement of urine albumin on a fresh, first morning void.

Biuret Test

This test is used for the detection of proteins containing at least two peptide bonds.

Principle

In an alkaline medium, protein reacts with copper (Cu^{2+})in the Biuret reagent leading to the formation of violet colored complex. This increases the absorbance at 540nm due to that is directly proportional to the concentration of protein.



Biuret complex

Reagents Required

1. 10% sodium hydroxide solution
2. 1% copper sufate solution or Biuret reagent
3. Sample: Urine

Procedure

1. Add 1 ml of urine in a test tube.
2. Add 1 ml of 10% sodium hydroxide solution and 2-3 drops of 1% copper sulfate solution.
3. Mix well
4. Appearance of violet color will indicate presence of proteins in the sample.

Pre-analytical factors affecting urine albumin concentration

Patient	Sample
Hydration status	Sample clarity
Exercise	Collection type
Fever	Adsorption to plastic
Posture	Storage temperature

CALCULATION:

Total protein concentration (gm/ dL) = $\frac{\text{O.D. of Test}}{\text{OD of Standard}} \times 5$

PRECAUTIONS:

1. Protect eyes fromsplash of the reagent.
2. Use automated pipettes and dispensing devices.

Heat and Acetic Acid Test (Protein)

Principle: based on precipitation by heat and coagulation by acids.

Procedure:

i. Fill test tube with urine (2/3 full) centrifuge.
ii. Heat the upper 2cm of the urine and observe the cloudiness. (Due to phosphates not albumin).
iii. Add 2 to 3 drops of 10% acetic acid .
iv. Cloudiness due to phosphates will disappear.
v. Repeat the heating. Persistent cloudiness indicates albumin. (Proteinuria)

Results:
If cloudiness developed at 40-60° C and disappears upon boiling but reappears on cooling, the protein present is called Bence-Jones protein. This protein is encountered in: Hyperglobulinemia - A condition characterized by abnormally large amounts of globulins in the blood. And in Multiple myeloma - also known as plasma cell myeloma, is the second-most common cancer of the blood.

Test For Bilirubin (Foam Test)

Principle: Bilirubin if present colors the foam yellow to green.

Procedure:

i. Place 5ml urine in a test tube. Place cover.
ii. Shake the urine vigorously for 3 mins.
iii. If Bilirubin is present, the foam produced will have a yellow to light green color.
iv. In patients with proteinuria, bilirubin bound to albumin can also appear in urine.

READINGS:

- http://www.healthline.com/health/glucose-test-urine

- Martin H., 2011. Laboratory Measurement of Urine Albumin and Urine Total Protein in Screening for Proteinuria in Chronic Kidney Disease.Clin.Biochem. Rev. Vol 32

Experiment No. 13

Estimation of chloride content in urine

1. **For Chlorides (Fantus Test)**

Principle:

$AgNO_3$ reacts with the chloride in urine to precipitate AgCl. Any excess $AgNO_3$ reacts with Potassium Chromate to form reddish ppt. of Ag_2CrO_4 (Silver chromate). The appearance of which indicates end point of reaction.

Procedure:

i. Place 10 drops of urine to a test tube and one drop CrO_4 solution as indicator.
ii. Add drop by drop 2.9% $AgNO_3$ solution until a permanent red brown color (end point) is developed.
iii. Number of drops consumed represent amount of chloride present. Normally 6 to12 drops.
iv. May indicate Hyperchloremia if it exceed 12.

Experiment 14

Test for Urinary phosphates Ammonium molybdate test (Test for Urinary phosphates)

Principle:

Upon warming with ammonium molybdate in the presence of nitric acid, inorganic phosphates are precipitated as canary yellow ammonium phosphomolybdate.

Procedure:

i. To 3 ml of urine, add a few drops of concentrated nitric acid and a pinch of ammonium molybdate.
ii. Warm it.
iii. Observe the yellow color of the precipitate.

Test for Urinary phosphates Interpretation:

a) Increased urinary phosphates: Rickets, osteomalacia, hyperparathyroidism, acidosis.

b) Decreased urinary phosphates: Diarrhea, nephritis, parathyroid hypofunction, pregnancy, hereditary fructose intolerance and galactosemia.

Experiment No. 15

Study of abnormal urine content bile pigment and salts.

REAGENT USED:

Sample: Urine

Reagents:

Nitric acid (HNO_3), Silver nitrate ($AgNO_3$), Sodium hydroxide(NaOH), Aceti cacid(CH_3COOH), $BaCl_2$, Sulphur particles

APPARATUS USED:

Test tubes, Test tube stand,Pipettes, Measuring cylinder.

CLINICALSIGNIFICANCE:

Abnormal urine conditions shows dysfunctionin kidney's filtrationprocess that may occur due to various disease conditions. Mainly presence of bile pigments and biles altsin urine samples are in dication of jaundice.

PROCEDURE:

A)Test of Inorganic constituents:

i) **Test for chloride ion:**

Took 1ml of urine and added 3 drops of diluted HNO_3 and 4 drops of dilute $AgNO_3$. Appearance of white precipitate indicate chloride ion.

ii) **Test for phosphateion:** Took 1ml of urine and added 4drops of dilute NaOH. Heated and appearance of white precipitate indicate the presence of phosphate ion.

iii) **Test for sulphateion:** Took 1ml of urine and added 3 drops of dilute CH_3COOH and 4drops of $BaCl_2$. Appearance of white precipitate indicate sulphate ion.

B) Test for organic constituents:

Test for bile Pigment: Took 5ml of fuming HNO_3in test tube and added 3ml of urine from side of tube. Appearance of green or blue of reddish yellow colour indicate the presence of bile pigment in the urine.

ii) **Test for Bile Salts:** To 1ml of urine added sulphur particles,if sulphur immerse in urine than Bile salts are present and if it floats then bile salts are absent.

OBSERVATION TABLE:

S. No.	EXPERIMENT	OBSERVATION	INFERENCE
1			
2			
3			
4			
5			

PRECAUTIONS:

1. Use clean and dry glassware.
2. Avoid regents to be splashed in eyes.

Experiment No. 16

Liver function tests (LFTs)

AIM: To estimate level of Blood SGPT (Liver function test)

REAGENT USED:

Sample: Venous Blood in plain bulb: (0.05 mL Serumis required)

Reagents: Reagent 1: Buffered Alanine -- KG Substrate (pH7.4) Reagent 2: DNPH colour Reagent

Reagent 3: Sodium Hydroxide, 4N

Reagent 4: Working Pyruvate Standard, 2mM

Preparation of working solutions:

Solution 1: Dilute 1 mL of Reagent 3 to 10 mL with Purified Water. Reagent 1, 2 and 4 are ready - to - use.

APPARATUS USED:

Test tubes, Test tube stand, Pipettes, Micropipettes, Spectrophotometer

CLINICAL SIGNIFICANCE:

ElevatedlevelsofGPTsarefoundinliverdiseases.ValueofSGPTisverymuchhigherthanthenormalin case of infective hepatitis and the rise begin with the prodermal period, helping early diagnosis.

PRINCIPLE:

Analysis of SGPT is carried out using 2, 4-DNPH as colouring reagent.

SGPT (ALT) Catalyses the followingreaction:

α-Ketoglutarate + L-Alanine $\longrightarrow$ L-Glutamate + Pyruvate

Pyruvates of or med is coupled with2,4-Dinitrophenylhydrazine(2,4-DNPH)to give the corresponding hydrazone, which gives Brown color in alkaline medium and this can be measured colorimetrically.

PROCEDURE:

Take 0.25mL reagent1 in a test tube and incubate at 37ºC for 5minutes. Now add 0.05mL serum and mix it well and again incubate it 37ºC for 60 minutes. Add 2.5mL of solution 1 in this mixture (also given in table).

Pipette into tube marked	Test (T)
Reagent 1	0.25 ML
Incubate at 37 ºC for 5 minutes	
Serum	0.05mL
Mix well and incubate at 37ºC for 60 minutes	

Mix well and allow to stand at Room Temperature (15 -30 ºC) for 10 minutes and take the reading (OD) aginst purified water on a colorimeter using a Green filter or on Photometer at 505 nm.

PROCEDURE FOR STANDARD CURVE

As the reaction proceeds with time, more amounts of products are formed. Since the end products inhibit the enzyme, there is more of Inhibition. This Is the major problem with colorimetric methods for the estimation of this enzyme. On the other hand In Kinetic methods, since the enzyme activity is measured during the Initial few minutes, the amount of products formed during that short time are negligible to cause any Inhibition. Because of the above problem, it is necessary to standardize any colorimetric method against a Standard kinetic method. So this standardization is done against the Standard Karmen Unit Assay (Kinetic) and this is extrapolated to different amounts of Pyruvate and this has been thoroughly rechecked. At this point it is important to not ethat the Standard graph of Enzyme activity (lU) on x-axis vs. Odon Y-axis is not alinearone, which shows that O.D. increases with increases in enzyme activity at a decreasing rate. A Sample Curve is given in Fig.
It is not necessary to plot Standard curve every time at estis performed. It should be plotted initially when the first test is performed.
Subsequently, periodic checking can be done by running only a couple of tubes viz.tubes,1 and 3 of the following table and their OD can be compared with the original curve. Each laboratory should establish its own standard curve as per the below mentioned procedure table.

Tube No.	1	2	3	4	5
Assigned Enzyme activity (IU/L)	0	24	61	114	190
Reagent to be pipette	Volume in mL				
Reagent 1	0.5	0.45	0.4	0.35	0.3
Reagent 4	-	0.05	0.1	0.15	0.2
Purified Water	0.1	0.1	0.1	0.1	0.1
Reagent 2	0.5	0.5	0.5	0.5	0.5
Mix well and allow to stand at Room Temperature (15 - 30· C) for 20					
Solution I	5.0	5.0	5.0	5.0	5. 5.0

Mix well by inversion. Allow to stand at Room Temperature (15-30ºC) for 10 minutes and measure the O.Do fall the five tubes against purified water on a colorimeter with a green

filter or on photometer at 505 nm. Plot a standard graph by taking enzyme activity on X-axis and O.D. on Y-axis

OBSERVATION TABLE:

S. No.	Reading for standard	Optical density (OD) at 505 nm
1.	1	
2.	2	
3.	3	
4.	4	
5.	5	

S. No.	Reading for tubes	Optical density (OD) at 505 nm
1.	Control	
2.	Test	

CALCULATIONS:

Mark the reading (OD) of Test (T) on the Y axis of the Standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

PRECAUTIONS:

1) Serum Samples must be completely free from haemolysis, since RBCs are very rich in this enzyme.

Haemolysis leads to the release of large amount of the enzyme and hence gives erroneous results.

2) Avoid the use of detergents to clean glassware.

3) Use clean and dry glassware.

4) Reagent 3 is corrosive; avoid contact with skin.

NOTES:

1)Serum samples can be store dat2-8·C (Refrigerator) for 5 days without any substantial loss of the enzyme activity.

2) A separate Blank is not necessary, since tube No.1 of Standard curve substitutes the Blank

3) If the enzyme activity is more than 190 units, repeat the Test using the Serum diluted1:5(or more if necessary) with Normal Saline and multiply the final result so obtained with an appropriate factor.

NORMAL VALUES:

- The normal range of values for AST (SGOT) is from 5 to 40 units per liter of serum (5-40 IU/L)
- The normal range of values for ALT (SGPT) is from 7 to 56 units per liter of serum.