

GUIDELINES FOR SAFE AND HYGIENIC MEASURES WHILE WORKING IN BIOCHEMISTRY LABORATORY

While working in a biochemistry laboratory, one should follow the general guidelines for safe and hygienic use of laboratory chemicals and equipments. The general guidelines are:

1. **Protective Clothing:** Before entering the laboratory, always wear a neat and clean white apron or lab coat.
2. Before beginning the experiment, read the procedure carefully and make yourself, fully acquainted with all equipments / glass wares and biological material to be used. Since you will be handling biological material (blood, urine, body fluid etc.), proper handling is of paramount importance.
3. Handle with care while working with corrosive or toxic chemicals by adhering to right procedure.
4. A bottle should never be held by the neck but firmly held from the body.
5. Never add water in concentrated acid rather acids must be diluted by slowly adding them to water.
6. Acids, caustic materials, oxidizing materials must be mixed in the sink. This provides water for cooling as well as confinement of spillage.
7. While opening an ammonia bottle, keep your face away from bottle mouth.
8. Use rubber bulbs for pipetting reagents (Avoid mouth pipetting – use pasture pipette.)
9. Keep the work benches clean and discard the waste material in the dustbin only.
10. Don't spill the reagents or chemicals on the laboratory benches or your clothing or skin. If accidentally spills on the skin, immediately wash with tap water and inform the teacher.
11. Wash the test tubes and other glass wares immediately after use and keep it at the proper place. Clean your work table desk.
12. Cap the reagent bottles and keep them at the proper place just after use.
13. Clean all the equipments, switch them off properly and keep them at the proper place before leaving the laboratory.
14. Record your observation immediately after the experiment and submit the record book/manual for checking at the end of each class. You must get the record checked on the day of the experiment by your teacher.
15. Your efficiency in the laboratory is reflected how neatly, cleanly and methodologically you do your experiment.
16. Interpretation of the result is the most important component of the experiment. Make it a habit of discussing the result obtained with your teacher.
17. Before leaving the laboratory wash your hands properly.

SAMPLE COLLECTION

Venous blood is collected from the following site with the help of a hypodermic needle and is used for analysis.

The Sites of Collection

Jugular vein	-	Horse	20-gauge needle
	-	Cattle	16-gauge needle
External saphenous vein	-	Dog	21-gauge needle
		Sheep	21-gauge needle
Wing vein	-	Poultry	23-gauge needle

A small quantity of blood can be obtained by pricking the top of the ear (used in preparing a smear).

All types of equipment employed for collecting blood samples must be clean, dry and sterile. In general, plasma or serum should be prepared as soon as possible after the blood collection.

One of the following **anticoagulants** can be added to prevent coagulation:

1. Potassium or sodium oxalate	-	20 to 30 mg per 10 ml blood
2. Sodium citrate	-	30 mg
3. Heparin	-	2 mg
4. EDTA	-	10 to 20 mg
5. Heller- Paul mixture	-	Ammonium oxalate 3 parts Potassium oxalate 2 parts
6. NaF	-	6 – 10 mg

Separation of Plasma

Take the blood sample with an anticoagulant in a small test tube; centrifuge it at 3000 rpm for 10 minutes. Remove the tube and observe the contents.

The blood cells can be seen packed at the bottom of the tube below a clear plasma fluid. Siphon off the plasma carefully and preserve the same in the storage vial. In case of hemolysis, repeat the process right from blood collection.

Collection of Serum

Take 5 ml of freshly-drawn blood in a test tube without adding anticoagulants. Allow it to clot at room temperature and let the clotted blood stand for 15 minutes preferably in a refrigerator. The blood clot will retract and clear fluid will ooze out. Remove the supernatant serum carefully. For quicker results, the clotted blood may be centrifuged for 15 minutes at 2500 rpm.

Exercise No. 1

EFFECT OF TEMPERATURE AND pH ON ENZYME ACTIVITY

1. Effect of pH on Enzymes

pH affects the charge on the amino acid residues at the catalytic site of the enzyme and also that of the substrate. Both may lead to the alteration of binding of the substrate to the enzyme and subsequently catalysis itself. Additionally, enzymes, being proteins, are also denatured at extreme pH.

A fixed-time assay method is used for the following experiment. Enzyme activity is inversely proportional to the amount of unreacted substrate which is left out.

Material:

1. Sodium caseinate solution: Mix 4 g of casein with 90 ml of water, 2 ml 1N Sodium hydroxide. Warm to 35°C and then add 1 N HCl until the pH becomes 8. Make up to 100 ml.
2. Trypsin 4% w/v solution in water. Adjust pH to 7.
3. Trichloroacetic acid solution (TCA) 10% e/v in water, ice cold.
4. Hydrochloric acid 5% v/v in water.
5. Test tubes (4) on a stand.

Method:

1. Add

Tube	Sodium caseinate	Hydrochloric acid	Sodium hydroxide	Trypsin	Remarks
1.	2 ml	-----	-----	1 ml	
2.	2 ml	5 drops	-----	1 ml	
3.	2 ml	-----	5 drops	1 ml	
4.	2 ml	-----	-----	-----	

2. Mix and incubate at 37°C for 4 min (carefully note zero and final time).
3. Stop the reaction by adding 2 ml TCA.
4. Compare the amount of precipitated undigested casein.

2. Effect of Temperature on Enzymes

Like any other reaction, enzymes catalysis also proceeds at a faster rate at higher temperatures. Increased kinetic energy increases the number of collisions between the reactants resulting in faster reaction. The activity roughly doubles every 10°C. However, after a certain temperature (generally 40°C), the enzyme protein is denatured due to the breakage of various bonds involved in stabilizing the protein structure. Most enzymes are completely inactivated above 70°C.

A fixed-change (achromic point of starch) assay method is used for studying the effect of temperature on enzyme activity. In this, the enzyme activity is inversely proportional to the time taken for hydrolysis of a given amount of starch.

Material:

1. Discs of starch-impregnated paper (-20). Notebook/duplicating paper may be satisfactory. Check with iodine before use. Use a paper which gives the least blue color to complete the experiment quickly. Use paper punch for cutting discs.
2. Enzymes: α -Amylase present in your saliva. Collect by spitting in a small beaker.
3. Visualizer: Iodine solution 0.005 N in 3% w/v Potassium iodide.

Different temperature regimes:

1. Below ambient temperature; about 4°C, use refrigerator/BOD incubator/Ice bath.
2. Ambient.
3. Above ambient; about 37°C, use water bath/hot plate/oven forceps or even alpines. Spot Plate, Beakers 10 ml 3/Flat bottom vials.

Method:

1. Divide Saliva into 3 beakers.
2. Before mixing the enzyme with the substrate, incubate the two components, saliva and discs, separately for sufficient time to bring them to the appropriate temperature.
3. Deposit separate drops of iodine solution on the plate.
4. Add paper discs to the saliva at a low temperature at zero time. Avoid overlapping and see that each one is wetted uniformly.
5. At fixed-time intervals i.e. 2 minutes, pick up a disc with forceps and place it on iodine drops on the spot plate.
6. Note the fading blue color intensity with time.
7. Continue until no color change occurs (achromic point).
8. Repeat the experiment at the other two temperature regimes i.e. ambient and above ambient.

Reagent	Blank (1)	Standard (2)	Acid (3)	Alkali (4)	Enzyme Boiled (5)	Enzyme Cold (6)
Starch	5ml	5ml	5ml	5ml	5ml	5ml
HCl	-	-	1ml	-	-	-
NaOH	-	-	1ml	-	-	-
Saliva Boiled	-	-	-	-	0.1ml	-
Saliva Cold	-	-	-	-	-	0.1ml
Saliva	-	0.1ml	0.1ml	0.1ml	-	-

Observations	Test tubes	Benedict's reagent	Iodine
	1)	-	+
	2)	+	-
	3)	-	+
	4)	-	+
	5)	-	+
	6)	-	+

Observations

Exercise No. 2

ESTIMATION OF BLOOD/ SERUM GLUCOSE

Introduction

Carbohydrates are constituents of all living cells. Carbohydrates are defined as carbon compounds that contain hydrogen and oxygen in the ratio of 2:1, so that their empirical formula is $(CH_2O)_n$. There are, however, some exceptions to this rule. There occur trioses (CH_2O_3), tetroses (CH_2O_4), pentoses (CH_2O_5) and hexoses (CH_2O_6).

Animal as well as plant cells use carbohydrates as a source of energy. As per chemical nature, carbohydrates are aldehyde or ketone derivatives of polyhydric alcohols grouped as aldoses or ketoses, respectively. They are also classified as mono-, di-, oligo-, and polysaccharides.

The chemical properties of carbohydrates vary depending upon the number of hydroxyl groups and the presence or absence of $-CHO / >C=O$ groups. These variations are the basis for the development of color reactions for their identification. Under certain clinical circumstances, the estimation of carbohydrates becomes essential. Sugar levels (particularly glucose) in blood or urine are used as an important parameter for the diagnosis of diabetes mellitus. A few methods for their estimation are as follows:

ESTIMATION OF BLOOD GLUCOSE BY FOLIN-WU METHOD

Principle:

The proteins are precipitated by tungstic acid. The protein-free filtrate is boiled with an alkaline copper tartrate solution. The cupric ions are reduced to cuprous ions which, in turn, reduce phosphomolybdic acid. The intensity of the blue color of phosphomolybdate is measured.

Reagents Required:

1. 10% sodium tungstate
2. 2/3 N sulphuric acid
3. Alkaline copper solution
4. Phosphomolybdic acid
5. Stock standard glucose solution
6. Working standard solution

	Tube 1 (sample)	Tube 2 (standard)	Tube 3 (Blank)
Filtrate	2	-	-
standard soln	-	2	-
D.W.	-	-	2
Al-Copper sulfate.	2	2	2

Boiling 8 min → cool the tube in running water.

Phosphomolybdate 2 2 2
Acid

- * Fill the tube with distilled water upto 25 ml mark.
- * Read at 420 nm.

- * O.D. of Unknown = 0.04
- * O.D. of Standard = 0.06.

$$\text{Blood glucose (mg/100ml)} = \frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times 100$$

$$= \frac{0.04}{0.06} \times 100 = \frac{66.6 \text{ mg}}{100 \text{ ml}}$$

Interpretation : The blood glucose level of sample is higher than normal. So, it may be Hyperglycemia due to diabetes mellitus, Hyperpituitarism, Hyperadrenalinism, Hyperthyroidism, etc.

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Procedure:

1. Preparation of protein-free filtrate: In a test tube, take 7 ml of water. Add 1 ml of blood (or plasma or serum) and mix gently. Add 1 ml of 2/3 N sulphuric acid drop by drop with constant shaking. Allow to stand for 5 minutes and filter. This filtrate is known as protein-free filtrate.
2. Take 3 Folin-Wu tubes. Label them 'Unknown', 'Standard' and 'Blank'.
3. Pipette 2 ml of protein-free filtrate, 2 ml of working standard glucose solution and 2 ml of water into the tubes marked 'Unknown', 'Standard' and 'Blank', respectively.
4. Pipette 2 ml of alkaline copper Solution in each tube and mix.
5. Keep the tubes in a boiling water bath for 8 minutes. Remove the tubes from the bath, and cool them by immersing them in cold water.
6. Add 2 ml phosphomolybdic acid solution to each tube, and allow to stand for a few minutes.
7. Fill the tubes up to 25 ml mark with water and mix thoroughly by repeated inversion.
8. Measure the Optical Density (O.D.) of the Unknown solution at 420 nm wavelength using a blue filter.

Calculation:

$$\text{Blood glucose (mg/100ml)} = \frac{\text{OD of Unknown} \times 0.2 \times 100}{\text{OD of Standard} \times 0.2} = \frac{0.04}{0.06} \times 100 = \frac{200}{3} = 66.6$$

*Un = 0.04
St. = 0.06*

$$= \frac{\text{OD of Unknown (Test)} \times 100}{\text{OD of Standard}}$$

Result:

The concentration of blood glucose in a given sample is 66.6 mg/100ml.

ESTIMATION OF BLOOD GLUCOSE BY NELSON-SOMOGYI METHOD

Principle:

- The glucose, present in a protein-free filtrate being reducing in nature reduces the CuSO₄ of alkaline copper reagent to form copper which in turn reduces the arsenomolybdate reagent to form molybdenum blue.
- The blue color thus produced is measured for its O.D. to assay the concentration of glucose.

Materials Required:**Sample**

- Whole Blood

Reagents Required:

- Glucose stock standard; 100 mg% in water and saturated benzoic acid solution.

- Working standard: (20 times dilution) 5 mg% diluted stock solution.
- 0.3N Ba(OH)₂: 600 ml of water is taken in a liter volumetric flask and boiled for 3 minutes. 45 gm of Ba(OH)₂.8H₂O is added and boiling is continued until it dissolves. The content is filtered rapidly to remove the precipitates. Finally, dilute the volume up to 1 liter with water and store in a Pyrex bottle.
- 5% ZnSO₄: 50 gm of ZnSO₄.7H₂O is dissolved in water and volume is made up to 1 liter. The exact concentration can be assessed by titration.
- Alkaline-copper solution A: 25 gm of anhydrous Na₂CO₃, 25 gm of sodium potassium tartrate and 20 gm of NaHCO₃ along with 200 gm of Na₂SO₄ anhydrous are dissolved in 800 ml of water. After complete dissolution, the volume is made to 1 liter with water.
- Alkaline-copper solution B: 15 gm of CuSO₄.5H₂O is dissolved in 100 ml of water followed by the addition of drops of Concentrated Sulphuric acid.
- Alkaline copper solution: A and B are mixed in the proportion of 24:1 as per the requirement. The reagent is prepared very fresh.
- Arsenomolybdate reagent: 25 gm of ammonium molybdate.4H₂O is dissolved in 450 ml of water, 21 ml of Concentrated Sulphuric acid is added slowly with constant mixing. Then 3 gm of disodium orthoarsenate.7H₂O is dissolved in 25 ml of water in a separate container. Both the solutions are mixed thoroughly and placed in an incubator for 24-48 hours at 37°C.

Procedure:

Preparation of blood sample:

- To prepare 1:20 the protein free Somogyi's filtrate, 0.5 ml of blood is added to 7.5 ml of distilled water, followed by addition of barium hydroxide and zinc sulphate one ml each.
- The contents are mixed well, either centrifuged or filtered to collect the filtrate.

S. N.	Contents	Volume
1.	Blood	0.5 ml
2.	Distilled Water	7.5 ml
3.	Barium hydroxide	1.0 ml
4.	Zinc Sulphate	1.0 ml

Mix, stand for 10 minutes. Filter to collect, 1:20 protein free Somogyi filtrate

Color Development:

- Three Lewis Benedict's blood sugar tubes marked as blank, standard and test are taken. 0.5 ml of distilled water, 0.5 ml of working standard solution and 0.5 ml of test filtrate are taken in those tubes respectively.
- One ml of alkaline copper reagent is added to each of the tubes. The contents are mixed well. The tubes are stoppered and placed in a boiling water bath for exactly 20 minutes.
- After removal, the tubes are cooled under tap water to room temperature. Then 1 ml of arsenomolybdate reagent is added to all the tubes followed by the addition of 10 ml of distilled water to each.
- Finally, the water is mixed by inversion. The O.D. of the standard and test are read against the reagent blank at 540 nm of wavelength using a green filter.

S.N.	Reagents	B	S	T
1.	Distilled Water	0.5 ml	-	-
2.	Working standard	-	0.5 ml	-
3.	Test filtrate	-	-	0.5 ml
4.	Alkaline copper reagent	1.0 ml	1.0 ml	1.0 ml
Mix, stopper and place in boiling water bath for 20 minutes, followed by removal and cooling under tap water				
5.	Arsenomolybdate Reagent	1.0 ml	1.0 ml	1.0 ml
Mix well				
6.	Distilled water	10 ml	10 ml	10 ml
Take OD at 540 nm.				

Calculation

$$\text{Glucose in mg\%} = \frac{\text{OD (T)}}{\text{OD (S)}} \times \text{Concentration of Standard}$$

Concentration of standard = 5 mg/dl

Result

- The amount of glucose present in the given serum: ----- mg%

4.6.1 Normal values: (mg/dl)

Cattle	Buff.	Sheep	Goat	Pig	Horse	Dog & Cat	Chicken	Human
35-55	32-60	30-65	48.2-76.0	65-95	60-100	60 -100	167.8 (Av.)	65 - 110

Interpretation:

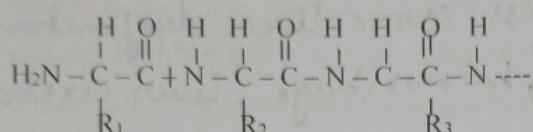
- The normal glucose of fasting blood is 80-120 mg/100ml (Folin-Wu method) & 70-100 mg% (Nelson and Somogyi).
- Hyperglycemia:** Plasma or serum glucose is about 10% higher as compared to blood glucose. The commonest causes of raised blood glucose levels are diabetes mellitus, hyperpituitarism, hyperadrenalinism, hyperthyroidism, administration of general anaesthesia, prolonged use of beta blockers; intracranial disease such as meningitis, encephalitis tumors, etc.
- Hypoglycemia:** The cause of decreased blood glucose is the overdose of insulin in diabetes, hypothyroidism, certain tumors of β -cells of islets of Langerhans and glycogen storage disorder etc. Dairy Cattle with ketosis and Sheep with pregnancy toxemia invariably have blood glucose values below 35 mg/ 100 ml.

Exercise No. 3

ESTIMATION OF PLASMA/ SERUM PROTEIN

Introduction

Protein forms the structural and functional basis of all the cells. Proteins are polymers of amino acid, which are linked to each other as shown below:



The backbone of a protein is made of peptide linkage and this is common to all the protein. The properties of the protein in a general sense are governed by the various R groups.

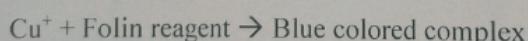
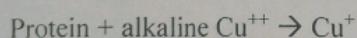
There are several methods of estimating protein, in food, agricultural and clinical samples, each of them varying in sensitivity and applicability. Lowry's Method of protein estimation based on the principles of colorimetry is given below;

LOWRY'S METHOD (PHENOL – FOLIN – CIOCALTEAU METHOD)

This method is based on the reaction of the aromatic groups of tryptophan and tyrosine of amino acids, with Folin's reagent to form a blue color. It is very sensitive method and can be applied on dilute solution.

Objective: Estimation of protein by Lowry Method.

Principle: Protein reacts with the **Folin-Ciocalteau reagent** to give a colored complex. The color formed is due to the reaction of the alkaline copper sulfate with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of color depends on the amount of these aromatic amino acids in the polypeptide chain.



Reagents:

1. Protein standard (5 mg/ml, Bovine serum albumin; BSA).
2. Alkaline copper reagent:
 - A. 2% sodium carbonate in 0.1 N NaOH
 - B. 0.5% copper sulfate solution in 1% sodium potassium tartrate solution (to be prepared fresh).
Mix 50 ml of reagent A with 1 ml of reagent B, just prior to use to prepare alkaline copper reagent.
3. Folin-Ciocalteau reagent: This is commercially available and has to be diluted with equal volume of water just before use. The reagent can also be prepared in the laboratory. Into a

2 litre flask, measure out 100 g sodium tungstate, 25 g sodium molybdate, 500 ml distilled water, 50 ml 85% phosphoric acid and 100 ml conc. HCl. The mixture is refluxed gently for about 10 hours with a condenser. After cooling, 150 g of lithium sulfate, 50 ml of distilled water and a few drops of bromine are added and boiled continuously for another 10 minutes without the condenser. This helps to remove excess bromine. After cooling, the volume is made upto 1000 ml and filtered if necessary. The filtrate should not have any greenish tint. If it has, it is again boiled with bromine. This is the stock reagent and is diluted with an equal volume of water just before use.

Procedure:

1. Take three test tubes and label them 'T' for Test, 'S' for Standard and 'B' for Blank.
2. Take 0.1 ml serum sample in T, 0.1 ml protein standard in S and 0.1 ml D. water in B tube.
3. Then mix 5.0ml alkaline copper reagent in each tube.
4. Mix thoroughly and allow to stand for 10 min at room temperature.
5. Add 0.5 ml of Folin reagent rapidly with immediate mixing.
6. The tubes are left as such for 30 minutes.
7. Read the absorbance (O.D.) of the blue color complex formed in standard and test at 650 nm against the blank using a red filter.

Calculation:

$$\frac{\text{Concentration of total Protein (g/dl)}}{\text{O.D. of Standard}} = \frac{\text{O.D. of Test}}{\text{Conc. of Standard}}$$

Result:

Protein concentration of the given plasma / serum is _____ g / dl.

Normal values:

Total Plasma Protein (g/dl)

Cattle	Buff.	Sheep	Goat	Pig	Horse	Dog	Cat	Poultry
6.6-7.8	6.8-7.7	6.0-7.5	6.0 -7.5	6.0-8.0	6.0-7.7	5-7	5.5 - 7.5	56.0

Albumin (g/dl)	2.0-3.5	2.3-4.1	2.2-3.0	1.9-2.4	2.9-3.8	2.9 -3.8	2.5-4	2.5 – 3.75	25.0
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Interpretation:

- Decreased level of total protein in serum is called hypoproteinemia and hypoproteinemia and hypoalbuminemia are observed in stress, severe liver dysfunction, renal disease, malnutrition, haemorrhage, liver cirrhosis etc.
- In contrast, an increased level of total protein is known as hyperproteinemia and is seen in severe dehydration, diarrhoea, Pregnancy, lactation, burns and in some cases of liver cancer.
- A/G ratio is quite important in the diagnosis of many diseases.
- Elevated levels of total proteins and albumin may be found in dehydration and multiple myeloma.

Exercise -4

ESTIMATION OF PLASMA/ SERUM INORGANIC PHOSPHATE

Principle:

The proteins of the blood are precipitated by Trichloro Acetic Acid. The Protein-free filtrate (P.F.F.) is treated with an acid molybdate solution and phosphomolybdate acid is formed. This phosphomolybdate acid is reduced by 1, 2, 4-Aminonaphthol sulphonic acid and blue color is formed. The intensity of the blue color varies with the concentration of phosphate present in the solution.

Reagents Required:

1. 10% TCA (Trichloro Acetic Acid): Dissolve 10gm trichloroacetic acid in water and make final volume upto 100ml with distilled water.
2. Molybdate solution: Dissolve 25gm Ammonium molybdate in 200ml distilled water in a volumetric flask. Add 300ml 10N H₂SO₄ and make the final volume upto 1 litre with distilled water.
3. 1,2,4- Aminonaphthol Sulphonic acid: Take 195 ml 15% Sodium bisulphite solution in a volumetric flask, add 0.5gm 1,2,4-Aminonaphthol sulphonic acid and add 5ml 20% Sodium sulfite solution. Transfer the solution into a brown glass bottle and store it in a cold place. The solution can be used for one month.
4. Stock Standard of Phosphorus: 0.351gm monopotassium phosphate dissolve in water and add 10ml 10N h₂so₄. Make the final volume upto 1L with distilled water. this solution contains 0.4 mg of phosphorous in 5ml.
5. Working standard of phosphorous take 5ml stock standard phosphorous solution and dilute to 50 ml with 10% trichloroacetic acid.

Procedure

1. Take 1 ml of serum and 9 ml of T.C.A. in a test tube. Mix properly. Keep it for 1-2 minutes and filter it, for protein-free filtrate (P.F.F.).
2. Take 3 test tubes and label them as sample standard and blank then add 5ml of protein-free filtrate (P.F.F),5ml of working standard solution and 5ml of distilled water in the sample, standard and blank test tube respectively.
3. Add 1 ml molybdate reagent, 0.4 ml Aminonaphthol sulphonic acid reagent and 3.5 ml distilled water in all the tubes.
4. Wait for 5 minutes and then read the optical density at 660 m μ wavelength using red filter.

Inorganic phosphorous mg/100ml =
$$\frac{\text{O.D. of Unknown} \times 0.04 \times 2 \times 100}{\text{O.D. of standard}}$$

Here

0.04-Concentration of standard means standard solution contains 0.04 mg of phosphorous

100-Dilution factor

Normal Values:

Species	Mg%
CATTLE, SHEEP& GOAT	3-7
PIG	4.8-7
HORSE	1.9-4.8
DOG	1.9-3.9
POULTRY	3.9-7

Phosphorous is present in the blood in the following forms:

1. Inorganic phosphorous
2. Organic phosphorous
3. Phospholipids

Excretion

Inorganic phosphorous is excreted in urine and faeces. The sources of urinary inorganic phosphorous are mainly plasma. The "renal Threshold" for phosphate excretion is about 2mg% of plasma. The reabsorption of phosphorous is inhibited by the parathyroid hormone

Interpretation

Nearly 80%phosphorous is present in bones and the remaining 20% is imported (present in body fluids) for carbohydrate metabolism, storage of energy synthesis of phospholipids and the acid-base balance. Organic phosphorous is present in RBCs while inorganic phosphorous in plasma serum. Plasma phosphorous levels are inversely related to plasma calcium levels.

Serum or heparinized plasma samples are preferred. The ratio between calcium and Phosphorous is 2:1. The normal values of phosphorous range from 4-7mg /100ml in young animals and 2.5-5 mg /100ml in adult animals

1. Low values of phosphorous (hypophosphatemia) may be observed in the following conditions
 - a) In rickets serum phosphorous level is 1-2 mg%.
 - b) There is a temporary decrease in serum phosphate during absorption of carbohydrates and fats.
 - c) Blood phosphorous levels are decreased in hyperparathyroidism.
 - d) Vitamin D deficiency causes low serum phosphorous and defects in the calcification of bones.
2. A high level of phosphorous (hyperphosphatemia) may be observed in the following conditions
 - a) Increase in acidosis
 - b) In severe renal disease example nephritis

- c) Increase in hypoparathyroidism
- d) Repair period of fracture healing
- e) A high concentration of organic phosphorus in serum has been estimated in diabetes mellitus.

Deficiency of Phosphorus:

- Rickets in young and Osteomalacia in adult animals. Animals with calcium and phosphorus deficiency exhibit a depraved appetite called Pica in cattle.
- Deficiency leads to reproductive disorders in cattle and buffaloes and stunted growth in young and reduced production of milk in adult buffaloes and cattle.
- Aphosphorosis (below normal serum inorganic phosphorus levels) causing severe Hypophosphataemia manifested by hind limb lameness occurs in high-yielding dairy cows (Milk-lameness)

Exercise -5

ESTIMATION OF PLASMA/ SERUM CALCIUM

Principle:

Calcium is precipitated from the serum as calcium oxalate and then with KMnO_4 solution.

Reagents Required:

1. 4% Ammonium Oxalate: 4 gm. Ammonium Oxalate in 100 ml distilled water.
2. 2% Solution of ammonia: 2 ml Ammonia solution and make final volume up to 100 ml with distilled water.
3. 0.01N KMnO_4 solution: 1ml of N/10 KMnO_4 is dissolved in 10 ml distilled water.
4. 1N sulphuric acid: Add 27.8 ml H_2SO_4 to 950 ml distilled water in a volumetric flask and make final volume up to 1lit with distilled water.

Procedure:

Take 1 ml of the serum, 2 ml distilled water and 1 ml Ammonium Oxalate solution into a centrifuge tube. Mix the content and stand for 30 minutes. Again, mix the content & centrifuge at 1500 RPM for 5 minutes. Take out the tube and pour off the supernatant and mix 3ml dilute NH_3 solution, centrifuge the tube at 1500 RPM for 5 minutes again take out the tube and pour off the supernatant liquid and add 2ml of 1N H_2SO_4 in the test tube in boiling water for about 1min. and then titrate with KMnO_4 solution. The end point of titration is represented by a permanent pink or purple color.

Preparation of Blank: take 2ml of 1N H_2SO_4 into a test tube. Place it in a boiling water bath for 1 minute and then titrate it with KMnO_4 solution.

Calculation:

$$\text{Mg. of Calcium}/100 \text{ ml of serum} = \frac{(x-b) \times 0.2 \times 100}{2}$$

Here,

x- ml. of KMnO_4 solution used in the titration for the sample solution.

b- ml of KMnO_4 used in Titration

0.2- 1 ml of 0.01N KMnO_4 solution is equal to 0.2 ml of the calcium.

100- Dilution factor.

Normal Values:

All animals: 9-12 mg%

Poultry: 17-39%

Excretion:

Calcium is excreted in the urine, bile and digestive secretions. Much of that is excreted in the faeces which has escaped absorption under optimum conditions. 75% of dietary calcium is absorbed and the remainder is the faecal calcium which is unabsorbed.

INTERPRETATION:

Blood calcium determination is preferred on serum. Normal blood calcium level in animals varies from 9 to 12 mg/100 ml. Young animals have higher values of calcium than adults. Calcium mobilization increases from skeletal reserves. This reduces the possibility of hypocalcemia leading to tetany.

a. Effects of Parathyroid hormone:

1. In Hyperparathyroidism: The following changes occur-

- 1) Hypercalcemia
- 2) Decrease in serum phosphate
- 3) Decrease renal tubular reabsorption of phosphate
- 4) Increased phosphate activity
- 5) Raised urinary Calcium & Phosphorous from bone decalcification and dehydration.
- 6) Loss of extra Calcium and Phosphate occurs from soft tissue and bones due to increased bone-destroying activity

2. In Hypoparathyroidism:

- 1) The concentration of serum calcium may drop below 7 mg/100ml
- 2) Increased serum phosphate and decreased urinary excretion of calcium and phosphorous
- 3) Normal or occasionally raised serum phosphate activity
- 4) Probably increased bone density

b. Tetany:

Decreased ionized fraction of serum calcium causes tetany due to –

1. An increase in the pH of blood
2. Poor absorption of calcium from the intestine
3. Decreased dietary intake of calcium
4. Increased renal excretion of calcium as in nephritis
5. Parathyroid deficiency
6. Increased retention of inorganic phosphorous as in renal tubular disease

c. Rickets:

This disease is caused by faulty calcification of bones, and shows phosphate values 1-2 mg% due to –

1. Vit-D deficiency
2. Deficiency of calcium and phosphorus in diet

3. Poor absorption of calcium from the intestine
4. Parathyroid deficiency
5. Increased serum alkaline phosphatase activity

d. Osteoporosis

The disease occurs in adults due to

1. Decalcification of bones as a result of calcium deficiency in diet
2. Hypoparathyroidism.
3. Low vitamin D contents in the body

Hypocalcaemia is observed in

1. Dietary deficiency of calcium
2. Severe nephritis
3. Rickets
4. Osteomalacia
5. Hypoparathyroidism
6. Smell fever
7. Reduction in plasma proteins
8. Pregnancy

Deficiency of Calcium

Insufficient intake of calcium, phosphorous and vitamin D or defect in deposition in bones results in rickets in young and demineralization of bone results in a condition known as osteomalacia in adult animals

Parturient paresis or milk fever in cows is the result of defective calcium metabolism in the body of animals. High yielding cows mostly in their third lactation suffer from fever

Puerperal tetany (Eclampsia) is most frequently encountered in small hyper excitable breeds of dogs. Functional disturbances associated with hypocalcemia in bitch are the result of neuromuscular tetany characterized by increased neuromuscular excitability

Hypocalcemic Syndromes in Animals

SPECIES	DISEASE
COW	Parturient hypocalcemia (milk fever)
BITCH	Puerperal tetany, eclampsia
QUEEN	Puerperal tetany, eclampsia
EWE	Pre and post parturient paresis ("Moss ill or staggers", "Lambing sickness")
GOAT	Hypocalcemia
SOW	Eclampsia

CHINCHILLA

Hypocalcemia

Hypercalcemia is observed in

1. After injection of PTH
2. In hyperparathyroidism
3. In hypervitaminosis -D
4. Polycythemia
5. Carcinoma

Exercise-6

DETERMINATION OF PLASMA/ SERUM MAGNESIUM

Principle

Serum proteins are precipitated by Sodium tungstate and Conc. H_2SO_4 . Magnesium in the protein-free filtrate (PFF) is complexed with Titan Yellow (a dye) in an alkaline medium and the resulting red color is measured by the colorimeter.

Reagents required:

1. **10% Sodium tungstate solution:** 10 gm Sodium tungstate in 100 ml distilled water
2. **2/3N H_2SO_4 :** 18.8 ml Conc H_2SO_4 and make final volume up to 1 litre with distilled water
3. **0.05% Polyvinyl alcohol:** 0.125 gm of polyvinyl alcohol in 250 ml distilled water
4. **0.05% Titan yellow:** 0.1gm titan yellow powder in 200 ml distilled water
5. **Calcium chloride solution:** 16.13 mg of Calcium chloride solution in 100 ml distilled water
6. **4N Sodium hydroxide:** 160 gms of Sodium hydroxide dissolve in water and make a final volume upto 1L with distilled water
7. **Stock standard of magnesium:** 8.458 gms of Magnesium Chloride dissolve in distilled water and make final volume upto 1 L with distilled water
8. **Working standard of magnesium:** Dilute 1ml of stock standard to 200 ml with distilled water

Procedure

1. Deproteinisation: Take 1ml serum 5ml distilled water, 2ml sodium tungstate and 2ml 2/3N H_2SO_4 into a test tube, mix the contents and filter it, filtrate is called PFF.
2. Take 3 test tubes and label them as Sample, Standard & Blank tube
3. Take 5ml of PFF in a sample tube 1ml working standard in the standard test tube and 1ml Calcium chloride in a blank tube
4. Then add 1ml distilled water in sample tube 5ml distilled water in standard tube and 5ml distilled water in blank tube.
5. Then add 1 ml Polyvinyl alcohol, 1 ml Titan yellow and 2 ml NaOH in all the 3 tubes. Wait for 1 minute & read the optical density of the sample and standard at 540 mu wavelength using a green filter

Calculation

$$Mg \text{ cf Mg/100 ml of serum} = \frac{\text{O.D of sample}}{\text{O.D of standard}} \times 0.02 \times 100$$

Here

0.02- working standard solution contains 0.02mg of Mg/ml of sample
100- Dilution factor

Normal Value:

All animals: 1-3 mg/100ml

Interpretation

Nearly 50% of magnesium in the body is present in bones. It activates the enzyme system and helps in the production and decomposition of acetylcholine. In cattle and sheep, muscular tetany results due to an imbalance of the magnesium-calcium ratio resulting in the release of acetylcholine.

1. Higher values of magnesium in the serum (**Hypermagnesemia**) may be observed in:

- a. Renal insufficiency
- b. CNS depression
- c. Low B.P

2. Low values of magnesium in the serum (**Hypomagnesemia**) may be observed

- a. Dietary deficiency of magnesium
- b. CNS irritability
- c. Grass tetany in ruminants
- d. Poor growth, skin lesions, poor bone formation typical titanic seizures and muscular contractions in calves

Deficiency of magnesium is related to poor doses in animals. Acute Magnesium deficiency causes vasodilatation, neuromuscular hyperirritability increases with the continuation of deficiency. A common form of magnesium deficiency tetany is called 'GRASS TETANY'. This occurs in animals grazing on rapidly growing young grasses or cereal crops. The high potassium content of young succulent plants creates an imbalance with Magnesium thereby causing a deficiency.

Exercise No. 7
ESTIMATION OF ASCORBIC ACID BY
DICHLOROPHENOLINDOPHENOL (DCPIP) METHOD

Principle

- Ascorbic acid being a strong reducing agent reduces the dye 2, 6 – dichlorophenol indophenol (a blue-colored compound) which attains a red color in acid solution to a colorless leucobase.
- Ascorbic acid in turn gets oxidized to dehydro ascorbic acid.

Reagents

- Standard 2, 6 – dichlorophenol indophenol dye solution (0.4 mg / mL).
- Test ascorbic acid solution.

Procedure

- Pipette out exactly 5 mL of the given ascorbic acid solution into a clean conical flask and titrate against the dye solution taken in the burette.
- The endpoint is the appearance of the light pink color, which persists for two minutes.
- Repeat the experiment for concordant values.

Observation

No.	Volume of Ascorbic acid Sample (mL)	Burette Reading (mL)		The volume of dye consumed (ML)	Concordant Value 'V' (mL)
		Initial	Final		

Calculation

- 1 mL of standard dye solution = 0.2 mg of ascorbic acid
- Titre Value = 'V' mL

- Concentration of ascorbic acid present in 5 mL of the given sample = $V \times 0.2$ mg
- The concentration of ascorbic acid present in 100 mL of the given sample = $V \times 0.2 \times 20$ mg.

Result

- The amount of ascorbic acid present in 100 mL of the given sample = _____ mg.

Exercise No. 8

**ESTIMATION OF MILK LACTOSE BY BENEDICT'S QUANTITATIVE
METHOD**

Principle

- The aldehyde group of lactose reduces the cupric ions present in Benedict's Quantitative Reagent (BQR) to insoluble cuprous hydroxide.
- Under mild alkaline conditions provided by sodium carbonate, cuprous hydroxide (yellow) on heating is converted into red cuprous oxide.
- Since, BQR contains potassium thiocyanate, a white precipitate of cuprous thiocyanate is formed in the reduction process, instead of the usual red precipitate of cuprous oxide.

Procedure

Step-I (Sample Preparation)

- Milk Proteins should be separated from milk before lactose estimation.
- Sodium tungstate is a potent protein-precipitating agent.
- Take 20 mL of milk in a 100 mL volumetric flask.
- Slowly add 10 mL of 10% sodium tungstate and 10 mL of 2/3 N H₂SO₄.
- Keep it for 15 minutes undisturbed for precipitate formation.
- Mix thoroughly; make the volume up to 100 mL with distilled water and filter.
- Fill a burette with this filtrate for lactose estimation.

Step-II

- Pipette out 10 mL of Benedict's Quantitative Reagent (BQR) into a 100 mL conical flask.
- Add 5g sodium carbonate and the mixer is boiled over a flame.
- While the solution is boiling, titrate rapidly against the filtrate from the burette.
- The endpoint is the appearance of a white-colored precipitate.
- Continue the titration till the last traces of the blue color of BQR disappear.
- Water may be added to make up for the loss due to evaporation.
- Repeat the experiment to get a concordant value.

Calculation

- Volume of filtrate consumed (V) = mL
- 10 mL of Benedict's quantitative reagent = 26.8 mg of lactose
- Lactose present in 100 mL of milk = $(26.8 / V) \times 100 \times (100 / 20)$
- Lactose present in 100 mL of milk = _____ mg.

Exercise No. 9
ESTIMATION OF SODIUM AND POTASSIUM BY FLAME
PHOTOMETER

Principle:

The sample solution is introduced in the form of a fine continuous spray into a non-luminous gas flame, using natural Acetylene, Propane, or Butane gas. Air or pure oxygen under pressure is used to maintain high burning temperatures and thereby keep the luminosity of the flame at a minimum. By the use of a color filter or diffraction grating the emitted light, of wavelength characteristic for the ion analyzed, is isolated and focused on a photoelectric cell.

Theory:

Flame photometry is also known as atomic emission spectrometry. It works based on heating the metal, in this case sodium, such that the atoms of the metal travel from the ground state to their excited state. The atoms then return to their ground state and release their energy as photons of ultraviolet radiation. The wavelength of the UV radiation is then measured. Relative precision and accuracy can be expected to be between $\pm 1\text{-}5\%$ because of the characteristic emission lines from the gas-phase atoms in the flame plasma, which eliminates interferences from most other elements. Since there are many experimental variables affecting the intensity of the light emitted from the flame, the result must be calibrated.

The purpose of this experiment is to determine the Na concentration in an unknown sample by comparing its emission intensity to corresponding emission intensity on the calibration curve which thereby provides a concentration of the unknowns based on the calibration curve of solutions of known concentrations.

Material required:

1. Serum Sample: 1 ml of serum is diluted to 100 ml with double glass distilled water.
2. Stock Standard of Sodium: 5.85 gm sodium chloride is dissolved in 1000 ml of double glass distilled water.

Procedure:

The serum dilution of 1:100 is prepared with double glass distilled water and is used for sodium analysis. The interference filter for sodium is fitted to the instrument. The highest concentration of sodium is taken (180 meq/liter) in a small beaker and fed to the atomizer through which it is sprayed to non-luminous gas flame and simultaneously the galvanometer light

is set at 100% emission. An emission of zero percent is set with a double glass of distilled water. The unknown serum sample (diluted 1:100) is sprayed into the flame and the percentage of emission is recorded. The quantum of sodium is calculated by referring to the standard graph prepared.

Standard Sodium:

Standard Sodium covering the range of 100 to 180 meq./litre of sodium is prepared by a stock standard having 100 meq./litre by dissolving 5.85 grams of sodium chloride in a 1000 ml of glass distilled water. The working standard is prepared from the stock standard by measuring 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8 ml aliquot into 100 ml of volumetric flask and the volume is made up by adding glass distilled water. These standards represent 100, 110, 120, 130, 140, 150, 160, 170 and 180 meq./litre of sodium, respectively at 1:100 dilution.

$$\text{SI Unit of Sodium} = \text{mmol/litre of serum} = \text{meq./litre of serum} \times 1$$

Normal Values (meq/litre):

Cattle	Sheep	Goat	Pig	Dog
132-152	139-152	142-155	135-150	141-155

Interpretation:

Normal ranges of Sodium are from 135-160 meq./litre. It is primarily responsible for the maintenance of osmotic pressure and acid-base balance.

1. Low values of Sodium in:

- a) Pregnancy
- b) Obstruction of the pylorus and other parts of gastrointestinal tract.
- c) Pneumonia
- d) Severe nephritis
- e) Addison's disease

2. High values of Sodium are not seen as often as the low level. They are found occasionally in:

- a) Acute nephritis
- b) Disease of the urinary tract when there is obstruction to the flow of urine i.e. enlarged prostate
- c) Water deprivation

Normal Values of Potassium (meq/litre):

Cattle	Sheep	Goat	Pig	Dog
3.9- 5.8	3.9- 5.4	3.5- 6.5	4.4- 6.7	3.7- 5.8

Interpretation:

1. Low values of Potassium may be observed in;

- a) Severe Vomiting and Diarrhoea

2. High values of Potassium may be observed in:

- a) Acute Bronchial Asthma
- b) Uremia
- c) Addison's Disease

Exercise No. 10

PAPER CHROMATOGRAPHY OF AMINO ACIDS

Introduction

6. Chromatography is the technique, which allows the resolution of mixtures, by effecting separation of some or all of their components in zones or in phases, which are different from those in which they are present.
7. Chromatography can also be defined as an analytical procedure for separating compounds based on differences in affinity for the stationary and mobile phases.
8. The substance will move with the mobile phase at a rate dependent upon their differential distributions between the two phases, which is called partition co-efficient.

There are three types of chromatography:

1. Partition
 2. Ion exchange
 3. Adsorption chromatography
9. Paper chromatography is a partition chromatographic technique
 10. Paper chromatography is the simplest and most widely used technique in Biochemistry, which was developed by Consden, Gordon, Martin and Synge in England in 1944, for which they were awarded the Nobel Prize.
 11. Paper chromatography is widely used in the field of Chemistry, Biology, Biochemistry and many other areas of science.

Principle

- Paper chromatography is the technique in which the analysis of an unknown substance is done by the flow of solvents on a special filter paper.
- One of the two solvents is immiscible or partially miscible, in the other solvent.
- The solvent rises up by the capillary action and by adsorption on the paper.
- The separation is effected by the differential migration of the mixture of substances. This occurs due to differences in partition coefficients.

Materials Required

Paper:

- Normally a rectangular sheet of Whatman filter paper No. 1 is used.
- According to need, the size of the paper is cut.
- Draw a line on the Whatman filter paper at a distance of about 2 cm from one end.
- Put the dots on this line, equal numbers to the test solutions, to be analysed, at equal distances, leaving 2 cm from each side.

For Separation of amino acids:

Chemicals:

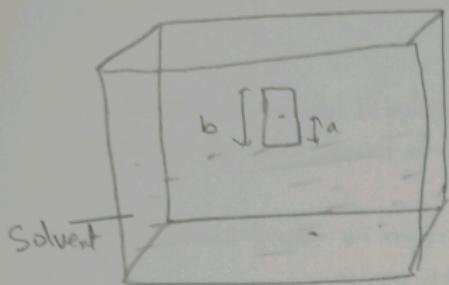
- Amino acid solution in water.
 - Lysine
 - Arginine
 - Aspartic acid
 - Glutamic acid
 - Mixture of all four amino acids.
- Solvent: Mix ethanol, water and ammonia solution in the ratio of 20:2.5:2.5 (v/v) or, mix n-butanol, acetic acid and distilled water in the proportion of 12:3:5 (v/v).
- Staining or locating reagent: Dissolve 300 mg of ninhydrin in 100 ml acetone.

Method:

A) Ascending technique:

- Take Whatman filter paper No. 1 for chromatography. Cut the paper to the size according to the need.
- A line is drawn with a pencil on the paper at a distance of about 2 cm from one end.
- Put 5 dots on this line 2 cm apart from each other. Put 1 drop (10 μ l) each of all the 5 solutions on the 5 dots.
- Apply small quantities at a time on the dot to allow it to dry and then put the next drop on the same dot. The spot should be within a diameter of 0.5 cm.
- Take the required quantities of the solvent in a jar and cover it with a lid. Curl the paper into cylindrical form and place in the jar in such a way that the paper should not touch the sides of the jar.
- Close the jar and run the chromatogram for about 4 hours. Remove the paper and mark the solvent front, with the help of a pencil. Dry the paper in hot air oven at 100 – 105 °C for 30 minutes. Remove the paper and spray with ninhydrin solution and again place the paper in oven at 100 – 105 °C for 5 minutes. The amino acids appear as purple spot on the paper.
- Calculate and compare R_f values with mixtures.

Ascending paper chromatography.



$b \Rightarrow$ Dist. travelled by solvent.
 $a \Rightarrow$ Dist. travelled by AA.

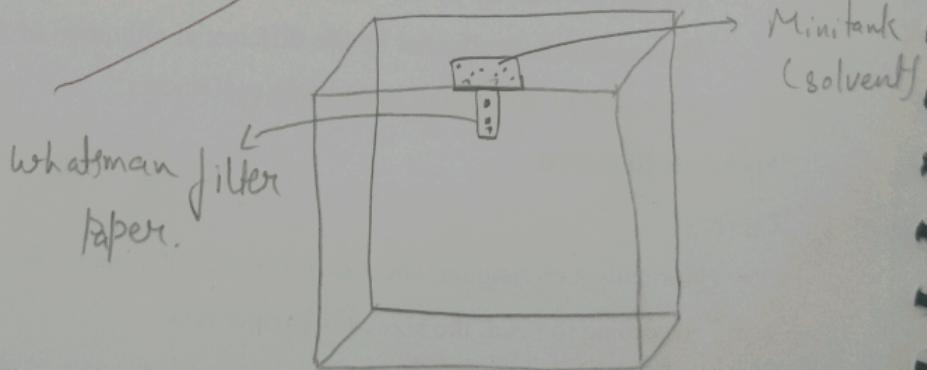
$$R \propto \frac{a}{b}$$

Retarding value.

Partition Co-efficient = $\frac{\text{conc of solute in mobile phase}}{\text{conc of solute in stationary Phase}}$

- # Principle - To know the unknown soln.
- Capillary action.
- Partition coefficient.

Descending chromatography



Whatman
filter
paper.

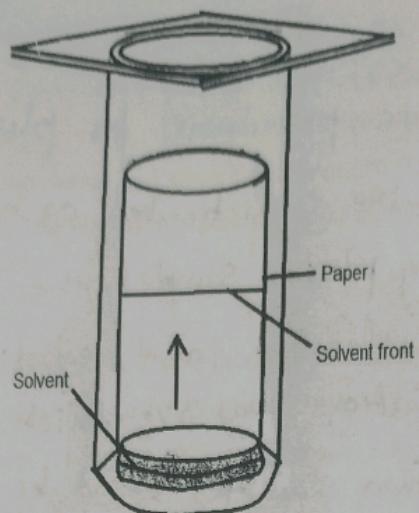
Calculation of Rf value:

- The distance of the solvent front is noted from the point of origin. Note the distance of the blue spot from the origin (this is called the distance traveled by amino acid).

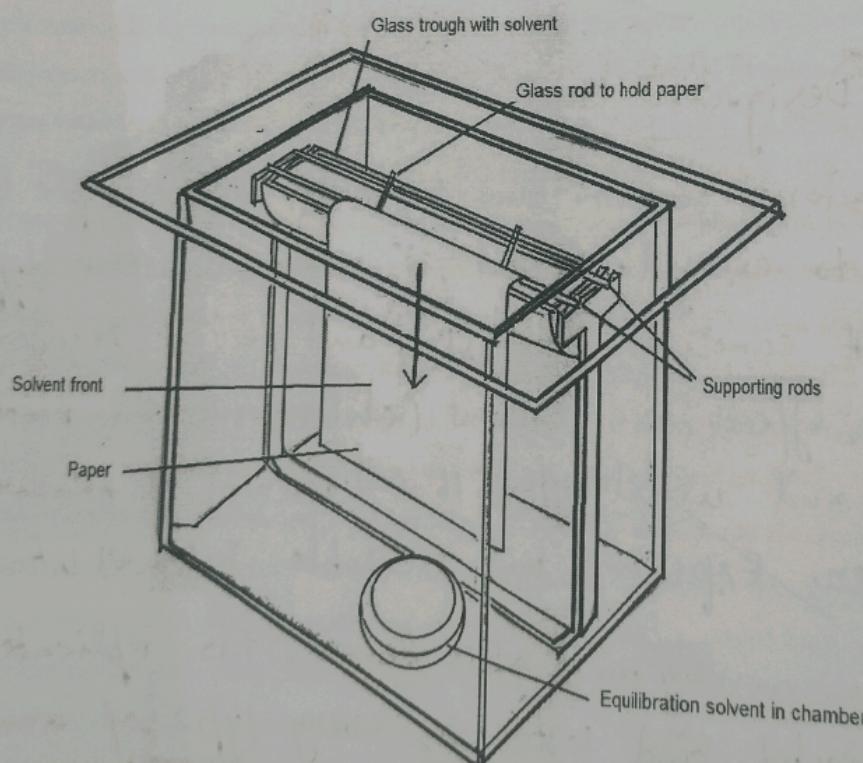
$$Rf = \text{Distance moved by the amino acid (X)} / \text{Distance moved by the solvent (Y)}$$

B) Descending technique:

- This type of chromatography is generally used. In this procedure, the solvent is kept in a jar at the top chamber and is allowed to flow down the paper.
- The liquid moves down by the capillary action as well as by the pull of gravity. In this case, the flow is more rapid as compared to the ascending method, so less time is required.
- Marking on the papers, loading of the samples and color development technique are all the same, as that described for the ascending technique.
- The solvent is placed in a trough at the top, which is usually made up of an inert material. The paper is suspended in the solvent and the lid is laid at the top. Two-dimensional development is rather impossible with this method.
- This is because the paper must be cut to fit into the trough.
- However, the descending technique is better than the ascending method for the compounds having low Rf values, which are not completely separated by ascending method, but can be done by descending method.



ASCENDING PAPER CHROMATOGRAPHY



DESCENDING PAPER CHROMATOGRAPHY

Result:

The R_f values of amino acids present in the given solution are:

Exercise No.11

ESTIMATION OF VITAMIN A BY COLORIMETRY

A simple Colorimetric procedure for plasma vitamin A is evaluated, which does not require sophisticated or expensive equipment. With widely varying plasma samples, the values showed a high degree correlation and good agreement. The intra-assay variation was 3% which is in the acceptable range. The plasma samples could be analysed within 4 weeks and the reagents, are found to be stable.

Procedure:

Experimental Design: A 2 X 2 factorial complete block design in which every sample is subjected to every treatment, is used to observe differences in vitamin A concentration in liver (pig) and percent recovery. The main effects are solvent (chloroform and methylene chloride) and wavelength (616 and 620 nm) as measured using Colorimeter. Experimental variables include vitamin A concentration, percent recovery and absorbance. Two replicate samples are selected and tested for every type of analysis.

Absorbance measurements are made using a Colorimeter. The slit width is selected automatically by switching to the "normal" position for higher solution work. Automatic repipet dispensers are utilized for micro-sampling. Standard Vitamin A Solution, about 4 to 5 mg of all-trans vitamin A acetate are weighed from a sealed vial containing 5 g of the vitamin and dissolved in 100 ml of chloroform and stored in an amber-colored bottle at -10°C. Trichloroacetic Acid Reagent A 30% TCA solution is prepared by dissolving in chloroform (CHCl_3) and methylene chloride (CH_2Cl_2) and stored in a glass stoppered amber bottle at refrigeration temperature. Before use, the reagent is warmed to room temperature ($24 \pm 1^\circ\text{G}$) and an appreciable amount is transferred to a repipet dispenser bottle. Dichloro-2-Propanol (1, 3-DCP) Two batches of 1, 3-DCP activated with 1-2% antimony trichloride (SbCl_3) are arranged. The reagent is stored at room temperature in an amber bottle. Before use the reagent is warmed to 25°C.

Colorimetric Determination: The wavelength (nm) of maximum absorption for the reagents 1, 3- DCP and (TCA) in either chloroform or methylene chloride is investigated over a period of 4 days using known concentrations of vitamin A acetate in chloroform stored at -20°C. The relationship between concentration and absorbance at 616 and 620 nm is noted and further studied during later quantitative and qualitative experiments.

Use of TCA Reagent: Serial dilutions of all-trans vitamin A acetate ranging from 2.5 to 10 mg/ml are prepared from stock solution on each day of analysis using chloroform (reagent grade). To 1 ml of known concentration of vitamin A acetate in a 1 cm quartz cell, 1 ml of TCA in either chloroform or methylene chloride is added from a fast delivery pipette. Absorbance at full-scale

deflection is recorded (within 5-7 seconds) at 620 or 616nm. This is determined over a period of 6 days.

Use of 1, 3-DCP Reagent: Known concentrations of all-trans vitamin A acetate in 0.5 ml chloroform (5-10 mg/ml) are pipetted into 5ml screw top glass test tube with the micro digital pipette. 1 ml of 1, 3-DGP is added to the tube stoppered, and mixed using a vertex mixer. The mixture is then warmed to 25°C in a water bath (within 2 minutes), poured into 1 cm quartz cell and absorption read at 550 nm.

Color Development and Stability with TCA-GHCl₃ and TGA-CH₂Gl₂, colorimetric determinations are carried out at room temperature with serial dilutions of all-trans vitamin A acetate respectively. To 1 ml of vitamin A in a cell, put 1 ml of TCA reagent from a fast delivery pipette. Absorbance at full-scale deflection and the stability of the blue-colored complex over a period of time (min) at 620 and 616nm are recorded. For the stability test, absorbance as a function of time (min) is plotted for each reagent-solvent complex stored from 0 to 6 days (0, 2, 4 and 6 days). 25 Sample analysis after separations, an aliquot (1.0 ml) of the solvent extract is carefully pipetted with an autopipette into a 1-cm quartz cell. To this is added 1 ml of TCA in either chloroform or methylene chloride from a fast delivery pipette with maximum absorbance recorded at full-scale deflection (usually within 5-7 seconds) at 620 and 616nm at different analysis times (within each day of analysis). All samples are run in duplicates within each day of analysis. Furthermore, 1, 3-dichlor-2- propanol (1, 3-DCP) activated with SbCl₃ practical grade is utilized in sample analysis. Recovery Tests Recoveries are carried out by adding a known concentration of vitamin A acetate standard from concentrates dissolved in chloroform, to 1 ml of liver tissue extract, analyzed before and after the addition of vitamin A. Samples are run in duplicates using TCA in chloroform or methylene chloride. The percent recovery is calculated as the ratio of actual to theoretical expressed as percent.