# PRACTICAL MANUAL

# OF

# VETERINARY BIOCHEMISTRY

**VPB-Unit-II**

Course Title–Intermediary Metabolism

(MSVE-2016)

(B.V.Sc. & A.H. SECOND PROFESSIONAL YEAR-(2025-26)

**Volume II (Unit-II)**

**NAME:** ------------------------------------------------------------

**ROLL No.:** ---------------------------------------------------------

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**DEPARTMENT OF VETERINARY PHYSIOLOGY AND BIOCHEMISTRY**

**MJF COLLEGE OF VETERINARY & ANIMAL SCIENCES,**

**CHOMU, JAIPUR (RAJASTHAN**

# CERTIFICATE

This is to be certify that ..……………………………………………………

Mr./Ms. ..……….……………………………..……………………………

Roll No. …………………………………… of Second Year B.V.Sc. & A.H. has successfully completed all practicals in Veterinary Biochemistry during Second year of the academic year………………………………………………………...

Date:

Place:

| **Signature** | **Signature** |
|---|---|
| **(Head of Department)** | **(Course Teacher)** |

# PREFACE

This Laboratory Manual has been prepared for the under graduate students of B.V.Sc. & A.H. in accordance with the syllabus designed by the Veterinary Council of India. The efforts have been made to make the manuscript worthy, realisticand easily understandable for the students, teachers and Veterinary Practitioners. We hope this manual will servevery useful tool to the undergraduate and graduate students of Veterinary Science who are undergoing courses in Veterinary Biochemistry.

It’s our pleasure to thank Dean Sir, M.J.F College of veterinary and Animal Sciences, Chomu, Jaipur for providing necessary facilities and rendering all helps in preparing this course manual.

**Course Incharge**

Department of Veterinary Physiology
& Biochemistry

# FOREWORD

I am very happy to go through the Practical Manual entitled Veterinary Biochemistry" Department of Veterinary Physiology and Biochemistry, MJF College of Veterinary & Animal Sciences, Chomu, Jaipur (RAJ.) The Manual covers the practical syllabus of undergraduate course (Veterinary Biochemistry, Volume II) Prescribed by Veterinary Council of India (New VCI 2016) for B.V.Sc. & A.H. programme.

The Manual is a good attempt and is based on cumulative experience of teaching undergraduate courses. The language used in the manual is simple and lucid. the outline and description of practical exercises covering objectives, materials required, procedures and observations to be taken have been nicely presented which would be helpful in conducting practical's more effectively.

I hope this manual will make its own place in the libraries 'of Agricultural Universities, Veterinary and Animal Science Colleges and various Livestock Institutions in near future.

I congratulate the authors for the efforts put in bringing out this practical manual.

**Dean**
MJF College of Veterinary &
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# ACKNOWLEDGEMENT

Ever since the introduction of new course for professional B.V.Sc. & A.H. degree programme under Veterinary Council of India pattern in Veterinary Colleges/Universities in the country, there was a dire need to have a practical manual on Veterinary Biochemistry subject which covers the practical syllabus of undergraduate (Veterinary Biochemistry) These new course was not dependently developed in most of the Veterinary College/University before the introduction of Veterinary Council of India programme. The present manual covers the practical with objectives, material required, procedure, steps to follow precautions to be taken, observations to be recorded and exercise to be done by the students. We hope that users will find the manual immensely useful.

We look forward to receiving the valuable suggestions of readers for improvement of this manual.

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# Exercise:01
# Effect of temperature and pH on enzyme activity

**Effect of pH:**

The pH has a significant influence on the rate of enzymatic reaction. Many enzymes may be characterized by a certain pH value at which the rate is maximal. This value is called the pH optimum. The shift of pH to both sides from the optimum value leads to the decrease in the rate of reaction. Optimal pH is often close to the pH of the tissue where enzyme is located, e.g., pepsin secreted in the stomach has optimal pH of about 2.0 (gastric pH is 2.0 – 3.0); pancreatic α-amylase secreted in the duodenum has optimal pH of about 7.0(intestinal pH is 6.0-7.0).

**Procedure: Assay the pH optimum of salivary amylase.**

1. Take 8 test tubes and pour the volumes of 0.2 M $Na_2HPO_4$ and 0.1 M of citric acid in each, as indicated in the table below.
2. Add 5 ml of 0.5% starch solution and 2.5ml of 250-fold diluted saliva into each of the tubes. The diluted saliva is added at equal time intervals beginning from the first tube.
3. Set the tubes for 15 min. After that take a few drops from tube no. 5 into a separate clean tube and add 1 drop of iodine solution. The blue color indicates that the reaction is not completed. Repeat this test until a brown color appears.
4. Add 2 drops of iodine solution into each of the tubes and shake them. Observe in which of the tubes the starch is cleaved completely.

**Buffer solutions for the assay of amylase pH optimum**

| Tube No. | 0.2%$NaHPO_4$ solution(ml) | 0.1M citric acid solution(ml) | pH | Remarks |
|---|---|---|---|---|
| 1 | 1.29 | 1.21 | 5.0 | |
| 2 | 1.51 | 0.99 | 5.6 | |
| 3 | 1.65 | 0.85 | 6.2 | |
| 4 | 1.82 | 0.68 | 6.6 | |
| 5 | 1.93 | 0.57 | 6.8 | |
| 6 | 2.06 | 0.44 | 7.0 | |
| 7 | 2.27 | 0.23 | 7.4 | |
| 8 | 2.53 | 0.07 | 8.0 | |

**Effect of Temperature:**

The main feature of enzymes is their specificity, both from the standpoint of the type of reaction they catalyze, and the kind of substrates on which they act. It means that an enzyme can act on the appropriate substrate or substrate group, e.g. D-amino oxidase catalyses oxidation of many amino acids, whereas sucrose hydrolyses sucrose only. Enzymes usually act in a narrow range of temperature. Raising the temperature upto $40^{O}C$ increases the rate of reaction, because the higher kinetic energy of molecules determines more frequent collisions between the enzyme and the substrate. However, the subsequent rise of temperature leads to the inactivation of enzyme by denaturation. The range of temperature at which the enzyme exhibits maximal activity is called the optimal temperature.

**PROCEDURE:**

Take6 clean test tubes. Pour 5 ml of 2% sucrose solution into each of the following tubes: No. 1, No.2, No.3. Pour 3ml of 1% starch solution into each of the remaining tubes: No.4, No.5, No.6.

1. Add 0.5ml of sucrose solution into tubes No.1 and No.4
2. Add 0.5ml of 5-fold diluted saliva into test tubes No.2 and No.5.
3. Add 0.5ml of boiled sucrose solution into tube No.3.
4. Add 0.5ml of 5-fold diluted and boiled saliva into test tube No.6.
5. Shake the contents of each tube and place the tubes in water bath at $37^{0}C$ for 10 min.
6. Cool the tubes under a stream of running of water.
7. The enzymatic cleavage of sucrose is determined by Fehling's reaction. For this purpose take tubes No.1, No.2, No.3 and transfer some ml of the contents into separate clean tubes. Add 1ml of Fehling 1 reagent and 1ml of Fehling 2 reagent to each of the tubes. Heat the tubes over gas fire until precipitate appear.
8. The starch cleavage is determined by addition of 1 drop of iodine solution into the tubes No.4, No.5, No.6.
9. Indicate the color of reaction mixture in the respective column of the table shown below:

**Hydrolysis of sucrose and starch by enzymes:**

| Tube No. | Substrate | Enzyme | | Fehling,s | Iodine |
|---|---|---|---|---|---|
| | | No boiling | After boiling | | |
| 1 | sucrose | Sucrose | - | | |
| 2 | sucrose | amylase | - | | |
| 3 | sucrose | - | sucrase | | |
| 4 | starch | Sucrose | - | | |
| 5 | starch | amylase | - | | |
| 6 | starch | - | amylase | | |

# Exercise: 2
# Estimation of blood or plasma glucose

## ESTIMATION OF BLOOD GLUCOSE BY NELSON-SOMOGYI METHOD

**Principle:**

Blood is deproteinised by a zinc sulphate and barium hydroxide solution which gives a filtrate containing no reducing substances other than glucose. The filtrate is heated with an alkaline copper reagent and then treated with special arseno-molybdate colour reagent. The colour developed is compared with that of standard.

**Reagent required:**

1. Barium hydroxide – 45 gm $Ba(OH)_2$. Make final volume up to 1litre with D. water
2. Zinc sulphate solution – 50 gm $ZnSO_4$. Make final volume up to 1 litre with D. water
3. Alkaline copper A – 50 gm Sodium carbonate
   50 gm Rochella salt (Sod. pot. tartrate)
   40 gm Sodium bicarbonate
   400 gm Sodium sulphate
   Make final volume up to 2 litre with D. water
4. Alkaline Copper B – 150 gm $CuSO_4$. Make final volume up to 1 litre with D. water then add 0.5 ml $H_2SO_4$

   **Alkaline copper reagent – 4ml Alkaline copper B + 96 ml Alkaline copper A. Prepare freshly**
5. Arseno-molybdate colour reagent – 100 gm Amm. Molybdate +1800 ml D. water+
   84 ml Conc. $H_2SO_4$. Then add 12gm. Disodium ortho-arsenate in 100 ml D. water. Mix with stirring to acidify molybdate solution.
6. Standard glucose – 1gm glucose, 10-15 ml 0.2% benzoic acid. Make final volume up to 100 ml with D. water

7. Working Standard Glucose – 1 ml St. solution and make final volume 100 ml with benzoic acid.

**PROCEDURE:**

1. Deproteinization – Take 1 ml blood, 4.5 ml $ZnSO_4$ and 4.5 ml $Ba(OH)_2$ solution in a test tube. Mix and filter,filtrate is called protein free filtrate (PFF).
2. Then take 3 potato tubes or folinwu tube and label them as sample, standard and blank.
3. Then take 1 ml PFF into sample tube, 1 ml working standard glucose into standard tube and 1 ml D. water in blank tube.
4. Then add 1 ml alkaline copper reagent in all the three tubes.
5. Place the tubes in boiling water for 20 minutes.
6. After 20 minute take the tubes out and cool them under running tap water.
7. Mix 1 ml colour reagent in all the three tubes.
8. Make the final volume up to 25 ml with D. water in all the three tubes.
9. Record the optical densities of sample and standard against blank at 540 mμ wavelength using green filter.

**Calculation:**

$$Glucose\ (mg\%) = \frac{O.D. of unknown}{O.D. of standard} X\ 100$$

**Normal values:**

**Ruminants** – 35-70 mg%

**Non- ruminant and camel** – 60-100mg%

**Interpretation:**

1. **Hyperglycemia (Increased level of glucose):-** It may be observed under following conditions.
    a. Kidney diseases
    b. Hyperthyroidism
    c. Pancreatic disease (Diabetes mellitus)
    d. Hepatic disease
2. **Hypoglycemia (decreased level of glucose):-** It may be observed under following conditions.
    a. After insulin injection
    b. Hypoendocrine disturbances

## EXERCISE PRACTICE

**Observation:**

**Calculation:**

**Interpretation:**

# Exercise: 3

## ESTIMATION OF TOTAL SERUM PROTEINS BY LOWRY'S METHOD

**Principle:**

The principle is based on a coloured reaction of aminoacid tyrosine with Folin Ciocalteau's or Lowry's or phenol reagent. The amount of protein present and colour intensity has a linear relationship over a limited range which can be recorded on a colorimeter.

**Reagent Required:**

1. Folin Ciocalteau's reagent
2. 5N NaOH
   200 gm NaOH in 100 ml D. water
3. St. tyrosin solution
   0.2 gm tyrosin dissolve in 0.1N HCl and make the final volume to 1 litre with 0.1 N HCl

**PROCEDURE:**

**Preparation of sample solution:**

Take 0.2 ml serum, 25 ml D. water, 2 ml 5N NaOH and 1 ml Folin Ciocalteau's reagent in 50 ml volumetric flask. Then make the final volume up to 50 ml with D. water.

**Preparation of standard solution:**

Take 4 ml st.tyrosin in 50 ml volumetric flask, to this add 25 ml D. water, 2 ml 5N NaOH and 1 ml folin ciocalteau's reagent. Make the final volume to 50 ml with D. water.

**Preparation of blank solution:**

Take 25 ml D. water, 2 ml 5N NaOH and 1 ml folin ciocalteau's reagent in 50 ml volumetric flask. Make the final volume to 50 ml with distilled water.

**Reading of sample:**

Keep the flask at room temperature for 5 minute for colour development and after 5 minute set the colorimeter to zero by using blank solution. Then read the optical density of sample solution and standard solution at 660 mμ wave length using red filter.

**Calculation:**

$$Total\ Proteins\ (gm\%) = \frac{O.D.\,of\,unknown}{O.D.\,of\,standard} X 6.4$$

Here 6.4 is the average protein value

**Normal values:**

Cattle 7-8.5, Pig 6.5-8.5,

Sheep 6-8, Dog 6.0-7.5

Goat 6.5-7.5, Chicken 4.0-5.2

**Interpretation:**

1. **Hypoproteinemia** – Lower level of serum protein is known as hypoproteinemia. It can be observed in following conditions
    a. Dietary deficiency of protein
    b. Stress factor
    c. Haemorrhage or excessive bleeding
2. **Hyperproteinemia** – Higher level of serum protein can be observed in following conditions
    a. Haemoconcentration
    b. Dehydration
    c. Pregnancy
    d. Lactation

## EXERCISE PRACTICE

**Observation:**

**Calculation:**

**Interpretation:**

# Exercise: 4

## ESTIMATION OF SERUM INORGANIC PHOSPHORUS BY THE METHOD OF FISKE & SUBBAROW

**PRINCIPLE:**

The proteins of the blood are precipitated by Trichloro Acetic Acid. The Protein free filtrate (PFF) is treated with an acid molybdate solution and phosphor molybdic acid is formed. This phosphor molybdic acid is reduced by 1,2,4-Aminonaphthol sulphonic acid and blue colour is formed. The intensity of blue colour 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 1 lit. Volumetric flask. Add 300ml 10 N $H_2SO_4$ and make final volume upto 1 lit. with distilled water.

3. **1, 2, 4 Amino naphthol Sulphonic acid:** Take 195ml 15% Sodium bisulfite solution in a volumetric flask, add 0.5gm 1, 2, 4 Amino naphthol sulphonic acid and add 5ml 20% Sodium sulfite solution. Transfer the solution into brown glass bottle and store in cool place. This solution can be used for one month.

**4. Stock Standard of Phosphorus:** Dissolve 0.351gm monopotassium phosphate in water and add 10ml 10 N $H_2SO_4$. Make the final volume up to 1 litter with distilled water. This solution contains 0.4 mg of Phosphorus in 5ml.

**5. Working standard of phosphorus:** Take 5ml Stock Standard Phosphorus solution & dilute to 50ml 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 5 ml. of protein free filtrate (P.F.F.), 5 ml. of working standard solution and 5 ml. of distilled water in sample, standard and blank test tubes respectively.
3. Add 1 ml molybdate reagent, 0.4 ml Aminonaphthol sulfonic 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 mµ wavelength using red filter.

**CALCULATION:**

$$PhosphorusConc(mg\%) = \frac{O.D.\ of unknown sol.}{O.D. of standard sol.} X100X0.04X2$$

***Here-***

0.04- Concentration of standard means standard solution contains0.04 mg of Phosphorus

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 |

***Phosphorus present in the blood in following forms:***

1. Inorganic phosphorus
2. Organic phosphorus
3. Phospholipids

**EXCRETION:**

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

**INTERPRETATION:**

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

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

1. **Low values of phosphorus (Hypophosphatemia)** may be observed in the following conditions:

   a. In Rickets serum phosphorus level is low as 1-2 mg%

   b. There is temporary decrease in serum phosphate during absorption of Carbohydrates and fats.

c.Blood phosphorus level is decreased in hyperparathyroidism.

d. Vitamin D deficiency causes low serum phosphorus and defect in the calcification of the bones.

**2. High values of phosphorus (Hyperphosphatemia) may be observed in the following conditions:**

a. Increase in acidosis.

b. In severe renal disease eg. Nephritis.

c. Increased in hypoparathyrodism

d. Repair period of fracture healing.

e. A high concentration of inorganic Phosphorus in the serum has been estimated in diabetes mellitus, but there is a low concentration of organic phosphorus.

**DEFICIENCY OF PHOSPHORUS:**

Since calcium and phosphorus are necessary for bone formation their deficiency leads to rickets in young and osteomalacia in adult animals. Animals with calcium and phosphorus deficiency exhibit deprave appetite called pica in cattle. Deficiency leads to reproductive disorder in cattle and buffaloes and stunted growth in young and reduced production of milk in adult buffaloes and cattle.

In aphosphorosis it is found that affected animals have below normal serum inorganic phosphorus levels and normal calcium and magnesium values. Diets such as green growing cereals containing high levels of carotene which has an anti Vit. D effect has been recognized to cause osteomalaciahypophosphataemia in grazing sheep. Aphosphorosis causing severe hypophosphataemia manifested by hind leg lameness occurs in high yielding dairy cows (milk-lameness). Hypophosphataemia is considered to be

important in the development of post parturient haemoglobinurea in dairy cattle. It is also responsible for intra-vascular haemolysis.

**PRACTICE EXERCISE:**

# Exercise: 5

## ESTIMATION OF SERUM CALCIUM BY CLARK-COLLIP METHOD

**PRINCIPLE:**

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

**REAGENTS REQUIRED:**

1. **4% Ammonium Oxalate**: 4gm. Ammonium oxalate in 100ml distilled water.
2. **2% Solution of Ammonia**: 2ml Ammonia solution and make final volume upto 100ml with distilled water.
3. **0.01N $KMnO_4$ solution**: 1ml of N/10 $KMnO_4$ is dissolved in 10ml distilled water.
4. **1N sulphuric acid:** Add 27.8 ml $H_2SO_4$ to 950ml. distilled water in a volumetric flask and make final volume upto 1 lit. 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 keep it for two hrs. Again mix the content & centrifuge at 1500 RPM for 5 minutes. Take out the tube and pour off the supernatant liquid. Then add 3 ml. diluted $NH_3$ solution in the tube and again centrifuge the tube at 1500 RPM for 5 minutes. Again take out the tube and pour off the supernatant liquid and add 2 ml of 1

N $H_2SO_4$ in the test tube and place the tube in boiling water bath for about 1 min. and then titrate with 0.01N $KMnO_4$ solution. The end point of titration is represented by a permanent pink or purple colour.

**Preparation of Blank:** Take 2 ml. of 1 N $H_2SO_4$ into a test tube. Place it in boiling water bath for 1 minute and then titrate with 0.01N $KMnO_4$ solution.

**CALCULATION:**

***mg. of Calcium/100 ml of serum = (x-b) x 0.2 x 100/ 2***

*Here*

x- ml. of $KMNO_4$ solution used in the titration for sample solution.

b- ml. of $KMnO_4$ used in titration for blank solution.

0.2- 1 ml. of 0.01 N $KMnO_4$ 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 condition. The 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 in association with phosphorus is necessary for normal bone growth in animals.

In parturient animals especially in cow, ewe and bitch, calcium mobilization increases from skeletal reserves. This reduces the possibility of hypocalcaemia leading to tetany.

***a. Effects of Parathyroid hormone:***

**1. In Hyperparathyroidism:** The following changes occur-

i. Hypercalcemia
ii. Decrease in serum phosphate
iii. Decrease renal tubular reabsorption of phosphate
iv. Increased phosphatase activity.
v. (v) Raised urinary Calcium & Phosphorus from bone decalcification & dehydration.
vi. Loss of extra Calcium and Phosphorus occurs from soft
vii. tissues and bones due to increased bone destroying activity.

**2. Hypothyroidism:**

(i) The concentration of serum calcium may drop below 7 mg/100 ml.

(ii) Increased serum phosphate and decreased urinary excretion of calcium and phosphorus.

(iii) Normal or occasionally raised serum phosphatase activity.

(iv) Probably increased bone density.

***b. Tetany:***

Decreased ionized fraction of serum calcium causes tetany due to -

(i) An increased pH of the blood.

(ii) Poor absorption of calcium from the intestine.

(iii) Decreased dietary intake of calcium.

(iv) Increased renal excretion of calcium as in Nephritis.

(v) Parathyroid deficiency.

(vi) Increased retention of inorganic Phosphorus as in renal tubular disease.

***C. Rickets:***

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

(i) Vit-D deficiency

(ii) Deficiency of Calcium and Phosphorus in diet.

(iii) Poor absorption of Calcium from the intestine.

(iv) Parathyroid deficiency.

(v) Increased serum alkaline phosphatase activity.

***D. Osteoporosis:***

Disease occurs in adult, due to-

i. Decalcification of bones as a result of calcium deficiency in diet.
ii. Hypoparathyrodism
iii. Low Vit.-D contents in body.

**Hypocalcaemia is observed in:**

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

**DEFICIENCY OF CALCIUM:**

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

Parturient paresis or milk fever in cow 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 the small, hyperexcitable breeds of dogs. Functional disturbances associated with hypocalcemia in the bitch are the result of neuromuscular tetany, characterized by increased neuromuscular excitability.

**Hypocalcemic Syndromes in Animals**

| **Species** | **Disease** |
|---|---|
| Cow | Parturient hypocalcaemia (milk fever) |
| Bitch | Puerperal tetany, Eclampsia |
| Queen | Puerperal tetany, Eclampsia |
| Ewe | Pre and postparturient Paresis<br>("Moss ill or staggers", "lambing sickness") |
| Goat | Hypocalcaemia |
| Sow | Eclampsia |
| Chinchilla | Hypocalcaemia |

**Hypercalcemia is observed in:**

1. after injection of PTH
2. in hyperparathyroidism
3. in hypervitaminosis -D
4. Polycythemia
5. Carcinoma.

**PRACTICE EXERCISE:**

# Exercise: 6

## DETERMINATION OF SERUM MAGNESIUM BY TITAN YELLOW METHOD

**PRINCIPLE :**

Proteins of the serum are preceipittated by Sodium tungstate & $H_2SO_4$ combination. The Magnesium in a protein free filterate (PFF) is complexed with Titan yellow (a dye) in an alkaline medium and resulting red colour is measured by colorimeter.

**REAGENTS REQUIRED :**

1. **10% Sodium tungstate solution** : 10gm Sodium tungstate in 100ml distilled water.
2. **2/3 N $H_2SO_4$ :** 18.8 ml Conc. $H_2SO_4$& make final volume up to 1 litre with distilled water.
3. **0.05% Polyvinyl alcohol** : 0.125gm. of Polyvinyl alcohol in 250ml distilled water.
4. **0.05% Titan Yellow** : 0.1gm Titan Yellow Powder in 200ml distilled water.
5. **Calcium Chloride solution :** 16.13mg of Calcium Chloride in 100ml distilled water.
6. **4 N Sodium hydroxide:** Dissolve 160gm Sodium hydroxide dissolve in water & make final volume upto 1 litre with distilled water.
7. **Stock standard of Magnesium** :8.458gm Magnesium Chloride dissolve in distilled water & make final volume upto 1 litre with distilled water.
8. **Working standard of Magnesium** : Dilute 1ml of stock standard to 200ml with distilled water.

**PROCEDURE:**

1. Deproteinisation : Take 1ml serum, 5ml distilled water, 2ml Sodium tungstate and 2ml. 2/3 N $H_2SO_4$ 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 5 ml of PFF in a sample tube. 1ml working standard in standard tube and 1ml Calcium Chloride in a blank tube.
4. Then add 1 ml distilled water in the sample tube, 5ml distilled water in standard tube and 5ml distilled water in blank tube.
5. Then add 1 ml polyvinyl alcohol, 1ml Titan yellow and 2 ml NaOH in all the three tubes. Wait for 1 minute & read the optical density of sample and standard at 540 mμ wavelength using green filter.

**CALCULATION :**

$$MagnesiumConc(mg\%) = \frac{O.D.\ of unknown sol.}{O.D. of standard sol.} X 100 X 0.02$$

*Here*

0.02 - working standard solution contains 0.02 mg of Mg/ml of sample.

100- Dilution factor

**NORMAL VALUE :**

All animals: 1-3 mg/100 ml

**INTERPRETATION :**

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

a. Dietary deficiency of Magnesium

b. CNS irritablility

c. Grass tetany in ruminants.

d. Poor growth, skin lesions, poor bone formation.

e. Typical tetanic seizures and muscular contractions in calves.

Deficiency of Magnesium is related with poor dose of animals. Acute Magnesium deficiency causes vasodilation. Neuromuscular hyperirritability increases with the continuation of deficiency. A common form of Magnesium deficiency tetany is called as GRASS TETANY. This occurs in animals grazing on rapidly growing young grasses or cereal crops. The high Potassium content of young succulent plants create an imbalance with Magnesium thereby causing a deficiency.

**PRACTICE EXERCISE:**

# Exercise: 7
# Estimation of Ascorbic acid by Dicholorophenolindophenol (DCPIP) method

**Principle:**

Ascorbic acid is a water soluble and heat labile vitamin. It is also known as Vitamin C. It is abundantly present in citrus fruits and all the fresh vegetables. Ascorbic acid reduces 2,6-dichloro phenol indophenol to a colourless leucoform. The ascorbic acid gets oxidised to dehydro ascorbic acid. Though the dye is a blue coloured compound, the endpoint is appearance of pink colour. The dye is pink coloured in acidic medium. Oxalic acid is used as the titrating medium.

**Materials:**

1. Oxalic acid 4%
2. DCPIP:42gms of sodium bicarbonate is dissolved in a few ml of distilled water. The 2 solutions are mixed and volume is made upto 200ml with distilled water.
3. Stock Standard Solution: 100 mg of ascorbic acid is dissolved in 100ml of 4% oxalic acid solutoin in a titrating medium.
4. Working Standard Solution: 10ml of stock solution is diluted to 100ml with 4% oxalic acid. The concentration of working standard is100 µg/ml.

**Procedure :**

Standard: 5ml of standard solution is pipetted out into conical flask. 10 ml of 4% oxalic acid is added and titrated against the dye. The end point is appearance of pink colour which persists for a few minutes. The amount of dye consumed is equivalent to the amount of ascorbic acid.

Test: 5ml of test sample is pipetted out into a 100ml of conical flask. 10ml of 4% oxalic acid is added and titrated against the dye. The endpoint is appearance of pink colour which persists for sometime.

**Calculations :** The amount of ascorbic acid present in the given sample is

$$\frac{\text{Volume of dye required to titrate test}}{\text{Volume of dye required to titrate std.}} \times \text{conc. of std} \times \text{Dilution factor}$$

# Exercise : 8
# Estimation of milk lactose by Benedicts quantitative method

**Principle:** Lactose is determined on the protein free filtrate of milk by copper reduction method, followed by the method of Folin and Wu for blood sugar. The protein free filtrate (P.P.F.) of the milk is heated with alkaline copper solution using a **Folin-Wu** tube to prevent reoxidation. Cuprous oxide formed is treated with phsophomolybdic acid solution and a blue colour is obtained, which is then compared with a standard solution. This method is well suited for routine analysis.

## Reagents Required:

1. 10% sodium tungastate,
2. 2/3 N sulphuric acid,
3. Folin- Wu alkaline copper solution,
4. Phosphomolybdic Acid Solution (Acid molybdate solution),
5. Working standard of the lactose
6. Diluted acid molybdate solution (1 : 4)

## Procedure:

1. Take 1 ml milk into a 100 ml volumetric flask. To this add 2 ml of 10% sodium tungastate and 2 ml of 2/3 N sulphuric acid.
2. Mix well and let stand for five minutes at room temperature.
3. After five minutes dilute to the mark with distilled water.
4. Filter the solution and filtrate is known as Protein free filtrate (P.F.F.).
5. Then take 1 ml of the protein free filtrate and 1 ml of distilled water in folin-wu tube. This tube is marked as sample tube.
6. Take 2 ml of the working standard lactose solution in another folin-wu tube.Mark this tube as standard tube.
7. Then add 2 ml alkaline folin-wu copper solution to each tube and heat in boiling water bath for 8 min.
8. After 8 minutes take out the tubes cool them and add 4 ml of the acid molybdate reagent to each tube.
9. After 1minute add dilute acid molybdate solution to 25 ml mark and mix.

10. Read the optical density of the sample and standard at 420 mµ wavelength using a blue filter, setting the colorimeter at zero against distilled water blank.

**Normal Value for Cattle: 4.8 %**

**Calculation:**

$$Lactose(\%) = \frac{O.D. of unknown}{O.D. of standard} X \frac{0.6X100X1}{0.01X1000}$$

Or $$Lactose(\%) = \frac{O.D. of unknown}{O.D. of standard} X6$$

*Here o.6 denotes lactose in std. Sol, 0.01 denotes amount of sample taken and 100 and 1000 are dilution factors.*

# Exercise: 9

# Estimation of sodium by flame photometer

## Estimation of serum sodium

**Principle:**

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

Material Required :

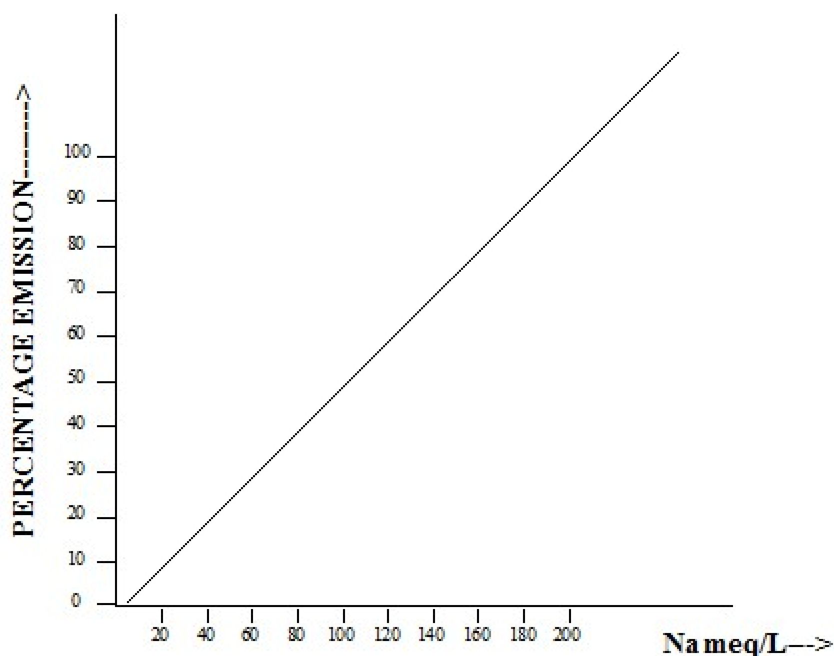
1. Serum Sample:1 ml. of serum isdiluted to 100ml with double glass distilled water.
2. Stock Standard of Sodium: 5.85 gm. sodium chloride isdissolved in 1000ml of double glass distilled water.

**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 isfitted to the instrument. The highest concentration of sodium is taken. (180 meq/litre) in a small beaker and fed to the atomiser through which it is sprayed to non-luminous gas flame and simultaneously the galvanometer light isset at 100%

emission. Emission of zero percentisset with double glass distilled water.

The unknown serum sample (diluted 1 : 100) issprayed into the flame and the percentage of emission isrecorded. The quantum of sodium iscalculated by referring the standard graph prepared.




**STANDARD SODIUM :**

Standard solution covering the range of 100 to 180 meq./L. of sodium isprepared by a stock standard having 100 meq./L. by dissolving 5.85 gm. of sodium chloride in a 1000 ml of glass distilled water. The working standard are prepared from this 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 ismade up by adding glass distilled water. These standards represent 100, 110, 120, 130, 140, 150, 160, 170 and 180 meq./L. of sodium, respectively at 1:100 dilution.

SI Unit for Sodium = m. mol/lit of serum = meq/lit of serum x 1

**NORMAL VALUES :**

| Species | meq/lit. |
|---|---|
| Cow | 132-152 |
| Sheep | 139-152 |
| Goat | 142-155 |
| Pig | 135-150 |
| Dog | 141-155 |

**INTERPRETATION :**

Normal range of sodium is from 135 to 160 meq./lit. It is primarily responsible for maintenance of osmotic pressure and acid-base balance.

**1. Low values of Sodium (Hyponatremia) 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(Hypernatremia)** are not seen as often as the low level. They are found occasionally in :

a. Acute nephritis.

b. Diseases of urinary tract when there is obstruction to the flow of urine for example in enlarged prostate.

c. Water deprivation.

**EXERCISE PRACTICE**

**Observation:**

**Interpretation:**

# Exercise: 10

## ESTIMATION OF SERUM POTASSIUM BY FLAME PHOTOMETRY

**PRINCIPLE:**

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

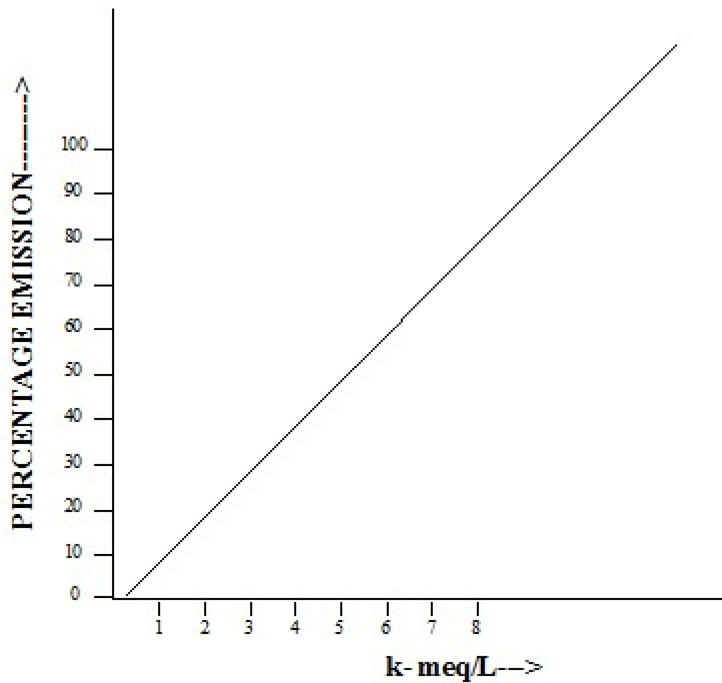
**Material required**

1. Serum Sample: 1 ml. serum isdiluted to 100 ml. by adding double glass distilled water (1 :100)
2. Stock Standard of Potassium: 0.746 gm. of potassium chloride isdissolved in enough double glass distilled water and made up final volume 1000 ml. with double glass distilled water.

**Procedure:**

The serum dilution of 1 : 100 isprepared with double glass distilled water and isused for potassium analysis. The interference filter for potassium isfitted to the instrument. The highest concentration of potassium istaken. (9 meq./litre) in a small beaker and fed to the atomiser through which it issprayed to non-luminous gas flame and simultaneously the galvanometer light isset at 100% emission. Emission of zero percentisset with double glass distilled water.

The unknown serum sample (diluted 1 : 100) issprayed into the flame and the percentage of emission isrecorded. The quantam of potassium iscalculated by referring the standard graph prepared.




**STANDARD POTASSIUM :**

The solution covering a range from 3 to 9 meq./Litre of potassium at 1: 100 are prepared. A stock standard containing 10 meq./Litre is prepared by dissolving 0.746 gm of potassium chloride in glass distilled water and diluted to 1000 ml. From this stock solution working standard 3, 4, 5, 6, 7, 8 and 9 meq./Litre of potassium at a dilution of 1 : 100 are prepared by diluting respectively 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 ml. of stock solution in 100 ml volumetric flask by adding distilled water.

SI Unit for Potassium: mmol./litre of serum = meq./litre of serum x 1

**NORMAL VALUES :**

| Species | meq/lit. |
|---|---|
| Cow | 3.9-5.8 |
| Sheep | 3.9-5.4 |
| Goat | 3.5-6.5 |
| Pig | 4.4-6.7 |
| Dog | 3.7-5.8 |

**INTERPRETATION :**

**1. Increased serum potassium (Hyperkalemia) may be observed in:**

a. Acute bronchial asthma

b. Uremia

c. Addison's disease

**2. Decreased serum potassium (Hypokalemia) may be observed in:**

a. Severe vomiting & diarrhoea.

b. Paralysis in patient with familiar periodic paralysis.

## EXERCISE PRACTICE

**Observation:**

**Calculation:**

**Interpretation:**

# Exercise: 11
# Paper or thin layer Chromatography of amino

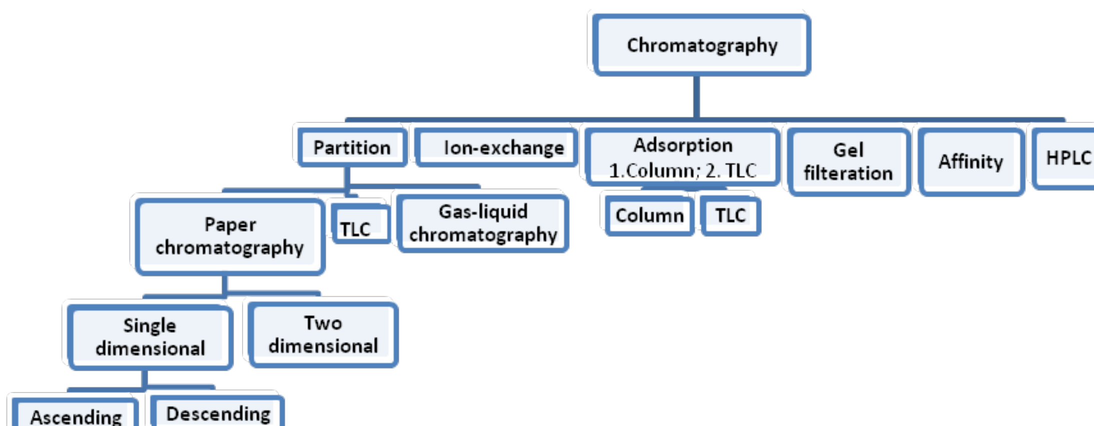
# acids

**Definition:**
Chromatography is one of the most useful and popular tools of biochemistry. It is an analytical technique dealing with the separation of closely related compounds from a mixture. These include proteins, peptides, amino acids, lipids, carbohydrates, vitamins and drugs.

**History-**
Discovery of chromatography goes to the Russian Botanist Mikhail Tswett. He coined the termed the chromatography (Greek: chroma-color, graphein- to write)

**Principles & classification-**
Chromatography usually consist of a mobile phase & stationary phase. The mobile phase refers to the mixture of substances dissolved in a liquid or a gas. The stationary phase is a porous solid matrix through which the sample contained in the mobile phase percolates. The interaction between the mobile and stationary phase results in the separation of the compounds from the mixtures. These interactions include the physiochemical principles such as adsorption, partition, ion-exchange, molecular sieving & affinity.

**Different methods of chromatography-**

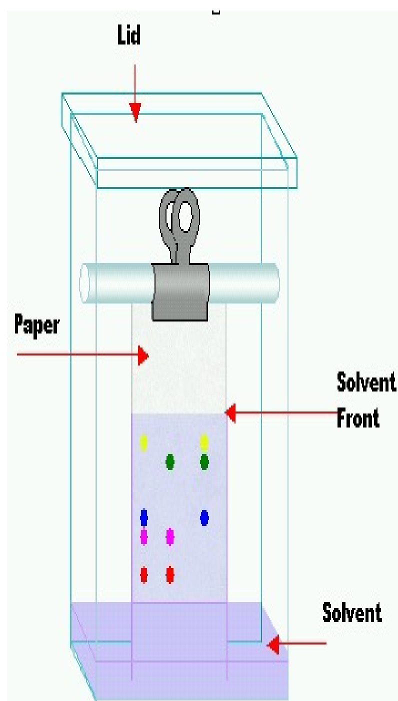


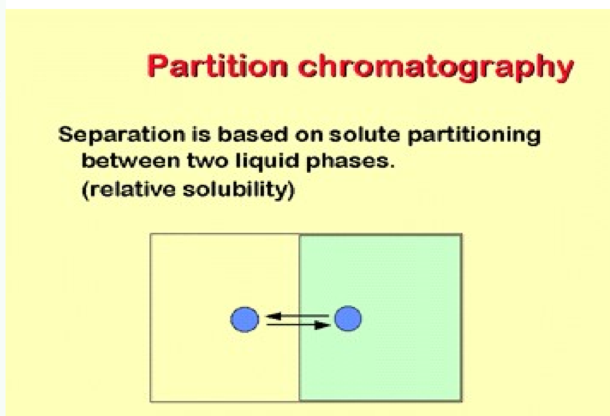

1) **Partition chromatography:**

The molecules of a mixture get partitioned between the stationary phase & mobile phase depending on their relative affinity to each one of the phases.

**a) Paper chromatography:**

Separation of amino acid, sugars, peptides. A few drops of solution containing a mixture of the compounds to be separated is applied at one end, usually 2 cm. above a strip of filter paper .The paper is dried and dipped in to a solvent mixture consisting of butanol, acetic acid & water in 4:1:5 ratio When the migration of the solvent is upward, it is referred to as ascending chromatography. In descending chromatography the solvent moves downward.




**Paper chromatography**

$$Rf\ Value = \frac{distance\ travelled\ by\ the\ substance}{distance\ travelled\ by\ solvent\ front}$$

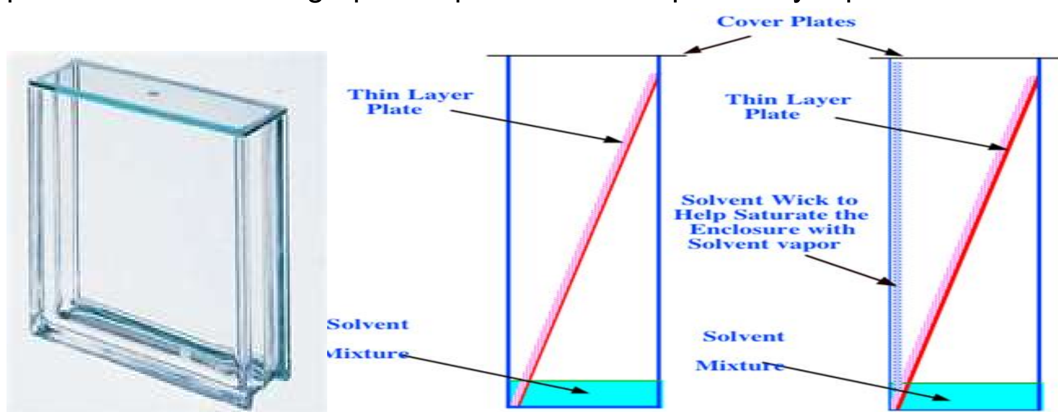
It is rather difficult to separate a complex mixture of substances by a single run with one solvent system. In such a case, a second run is carried out by a different solvent system, in a direction perpendicular to the first run. This is referred to as two dimensional chromatography.

- **PROCEDURE -**Whattman filter paper is cut into 35X15 cm sheet. A pencil line is drawn about 3cm. above the shorter edge of the paper. 5 points are marked at equal spacing leaving 2.5cm from the two edges. On the middle point a mixture of four amino acids is applied with the help of a fine capillary. It is dried with the help of hot air blown. This process is repeated 2 to 3 times. On the other points, the individual amino acid of the mixtures are similarly applied.
- If a chromatographic tank is not available, specimen jar(about40*15cm) with fitting lid can very well serve the purpose. In such a jar, put about 100 to 150ml of the solvent mixture and replace the lid so that the lid is air tight. Apply vaseline to avoid leakage. In an hour, the inside atmosphere will be saturated with the solvent vapour. Fold the paper in which sample has been applied in the shape of a cylinder and ties the opposing ends of the paper with thread. Open the lid & place this folded paper in upright position in the jar, the pencil line lower most & about a cm above the solvent.

Replace the lid. The paper should stand absolutely vertically. Leave the chromatogram to develop for 10 to 15 hours. Take out the paper, cut the stitches & let it dry in the air. The location reagent (0.2 % Ninhydrin in acetone) is sprayed uniformly on the paper with the help of an all glass sprayer. The paper is then allowed to dry first in the air & then in a hot air oven at $105^0$C for 3 min. Purple colored amino acid spots are seen on the chromatogram.

**REAGENTS -** 1) N–butanol(chromatographic grade)
2) Glacial acetic acid
3) Solvent mixture of butanol, acetic acid and DW.(4:1:5)
4)Ninhydrin:0.2 % solution of ninhydrin in acetone.

**b) Thin layer chromatography(TLC)-** The principle of TLC is the same as described for paper chromatography (partition ). In place of paper, an inert substance Cellulose is employed as supporting material. Cellulose is spread as a thin layer on glass or plastic plates. The chromatographic separation is comparatively rapid in TLC.

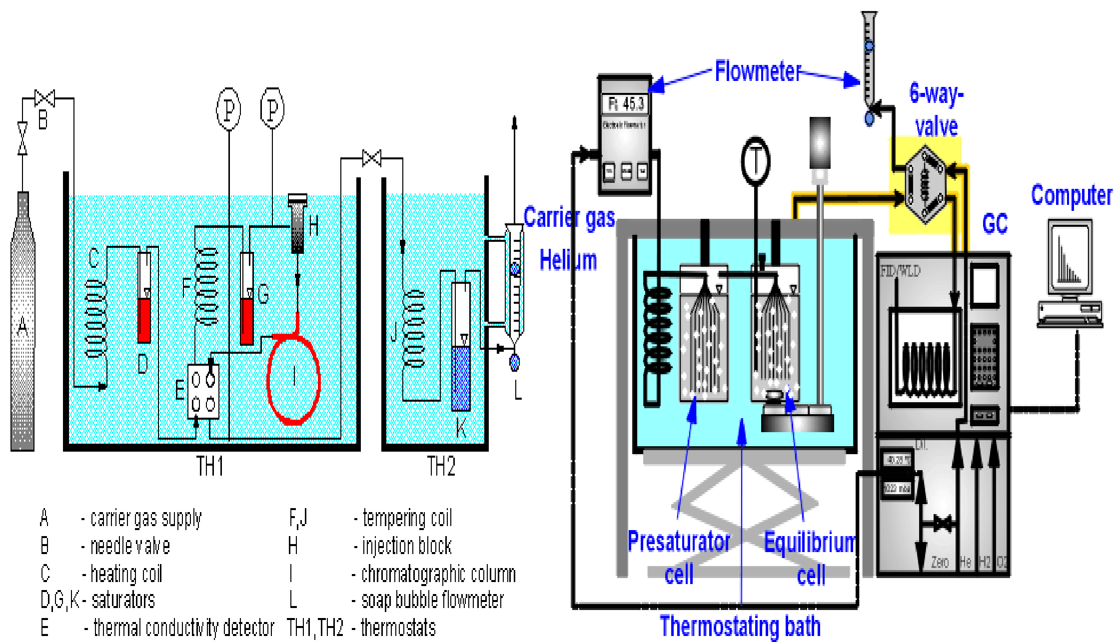



***TLC CHAMBER***

**Thin layer chromatography(TLC)**

*c)* ***Gas liquid chromatography (*GLC) –**

Method of choice for the separation of volatile substances. In gas liquid chromatography the stationary phase is an inert solid material impregnated with a non volatile liquid. This is packed in a narrow column and maintained at high temperature (around 200*C). A mixture of volatile material is injected into the column along with the mobile phase, which is an inert gas(argon, helium or nitrogen). The separation of the volatile mixture is based on the partition of the components between the mobile phase (gas) and stationary phase (liquid). The separated compounds can be identified and quantities by a detector. The detector works on the principles of ionization or thermal conductivity. GLC is sensitive, rapid and reliable, used for the quantitative estimation of biological materials such as lipids, drugs and vitamins.



***Gas liquid chromatography*** **(GLC)**

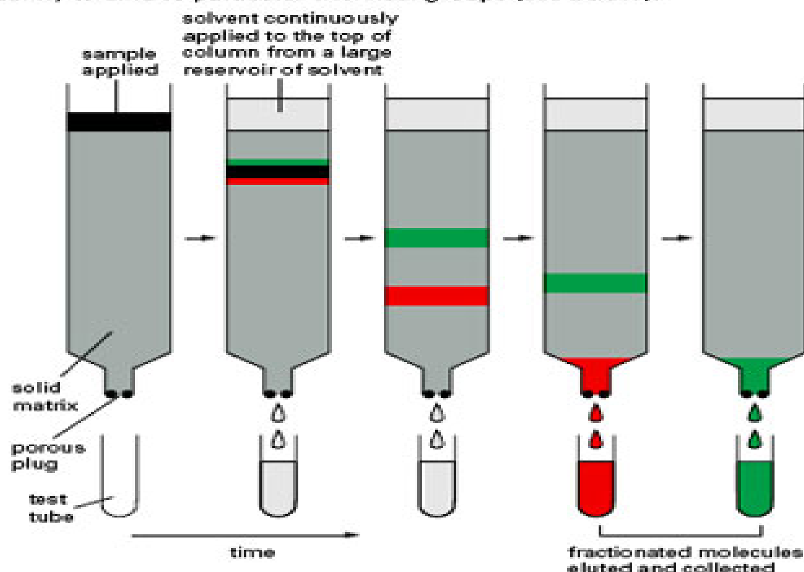
**PROCEDURE:**

Samples are applied as a spot and 5 mm diameter on the lower right corner of the plates under a stream of warm air. Plates are first developed in a standard brinkman developing chamber saturated with vapours of solvent mixture with chloroform: methanol: acetic acid: water (250:74:19:3:v/v). Plates are dried in air for 5 min. and exposed to iodine vapours in sealed chamber for 30-60seconds. The pale yellow areas are quickly outlined using a dental probe and the plates are exposed to air until the iodine has evaporated from the spots. Plates are sprayed lightly with 10N$H_2SO_4$and then heated at 110$^{\circ}$C for 15 min. The silica gel in each spot is scraped with the aid of a sharp edged polyethylene blade on paper. The weighing papers are then transferred to 12 ml conical centrifuged tube and eluted by different solvents for estimation by photoelectric colorimeter.

## 2) Adsorption column chromatography:

COLUMN CHROMATOGRAPHY

Proteins are often fractionated by column chromatography. A mixture of proteins in solution is applied to the top of a cylindrical column filled with a permeable solid matrix immersed in solvent. A large amount of solvent is then pumped through the column. Because different proteins are retarded to different extents by their interaction with the matrix, they can be collected separately as they flow out from the bottom. According to the choice of matrix, proteins can be separated according to their charge, hydrophobicity, size, or ability to bind to particular chemical groups (see *below*).



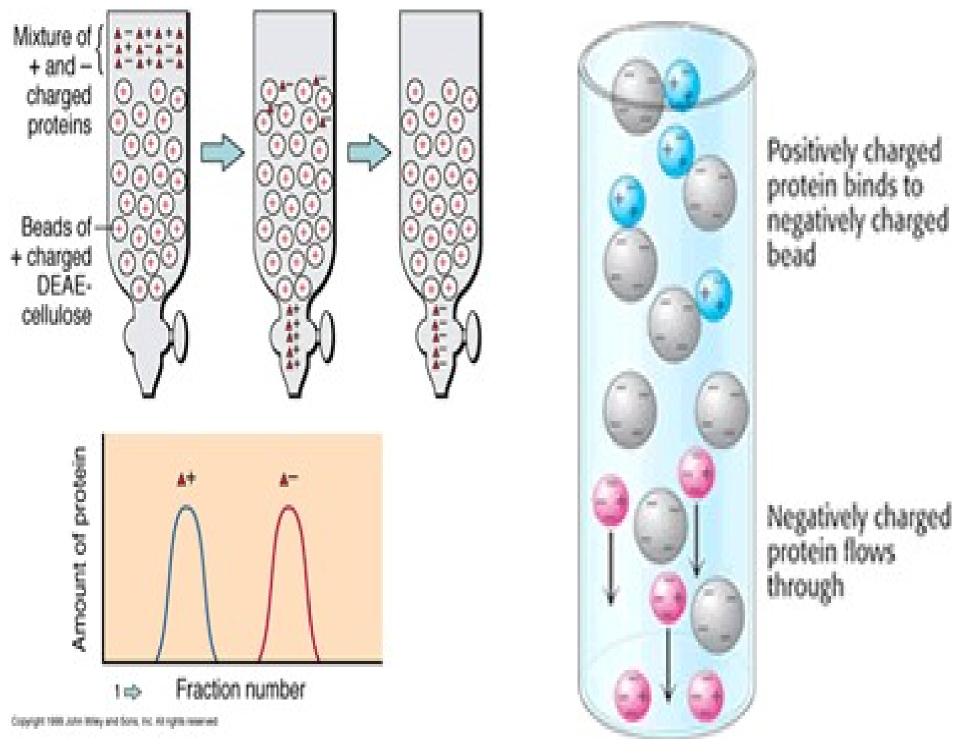

The adsorbents such as silica, gel, alumina, charcoal powder and calcium hydroxyapatite are packed into a column in a glass tube. The sample mixture in a solvent is loaded on this column. The individual components get differentially adsorbed on to the adsorbent. The elution is carried out by a buffer system (mobile phase). The individual compounds come out of the column at different rates which may be separately collected and identified.

3) **Ion exchange chromatography:**

***separation based upon the overall charge of the molecules**

***matrix retards passing proteins of opposite charge**

a. ***DEAE cellulose*** **[dimethylaminoethyl cellulose] (+)**
b. ***CM-cellulose*** **[carboxymethyl cellulose] (-)**




**Ion exchange chromatography**

The stationary phase has an ionically charged surface, opposite that of the eluents.

It involves the separation of molecules on the basis of their electrical charges. Ion exchange resins - cation exchangers and anion exchangers- are used for this purpose.

$\mathbf{R^+ A^- + B^- R^+ B \Leftrightarrow A^-}$

A cation exchanger ($H^+R^-$) its cation (H+) with another cation (C+) in solution.

$\mathbf{H^+ R^- + C^+ \Leftrightarrow C^+ R^- + H^+}$

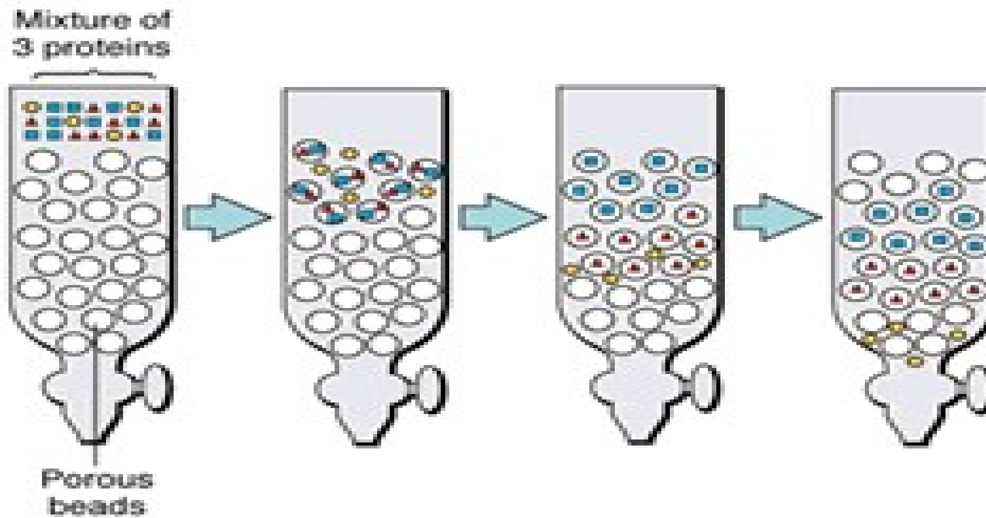
### 4) Gel filtration chromatography:

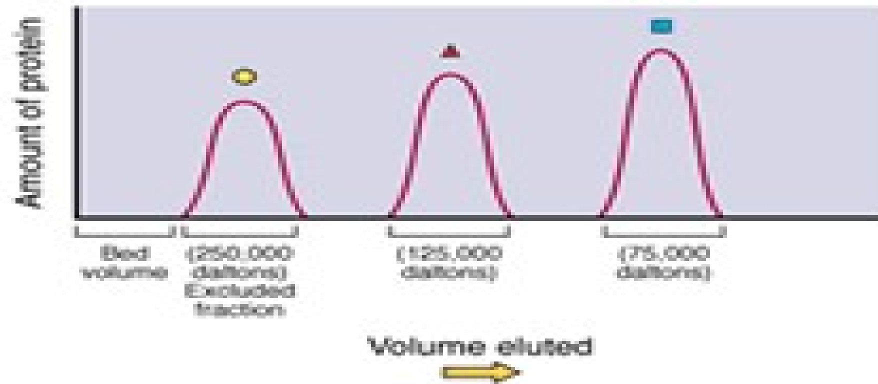
- Separation based upon molecular size
- Column is filled with semi-solid beads of a polymeric gel that will admit ions and small molecules into their interior but not large ones
- When a mixture of molecules and ions dissolved in a solvent is applied to the top

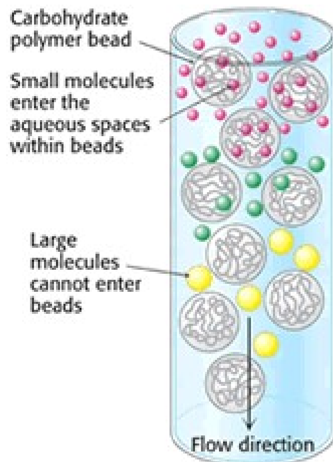
of the column, the **smaller molecules (and ions) are distributed through a larger volume of solvent** than is available to the large molecules

- Consequently, the **large molecules move more rapidly** through the column, and in this way the mixture can be separated (fractionated) into its components
- The **porosity of the gel can be adjusted** to exclude all molecules above a certain size
- *Sephadex*, *Sepharose* or *Sephacryl*, which are fine porous beads, are trade names for gels that are available commercially in a broad range of porosities

The separation of molecules is based on their size shape and molecular weight. This technique is also referred to as **molecular sieve** or **molecular exclusion chromatography**. The apparatus consists of a column packed with sponge like gel beads containing pores. The gels serve as molecular sieves for the separation of smaller and bigger molecules. The solution mixture containing molecules of different sizes (say proteins) is applied to column and eluted with a buffer. The larger molecules cannot pass through the pores of gel and therefore, move faster. On the other hand, the smaller molecules enter the gel beads and are left behind which come out slowly. The **gel-filtrationchromatography** can be used for an approximate determination of molecular weights.

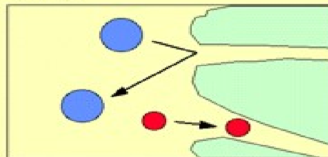


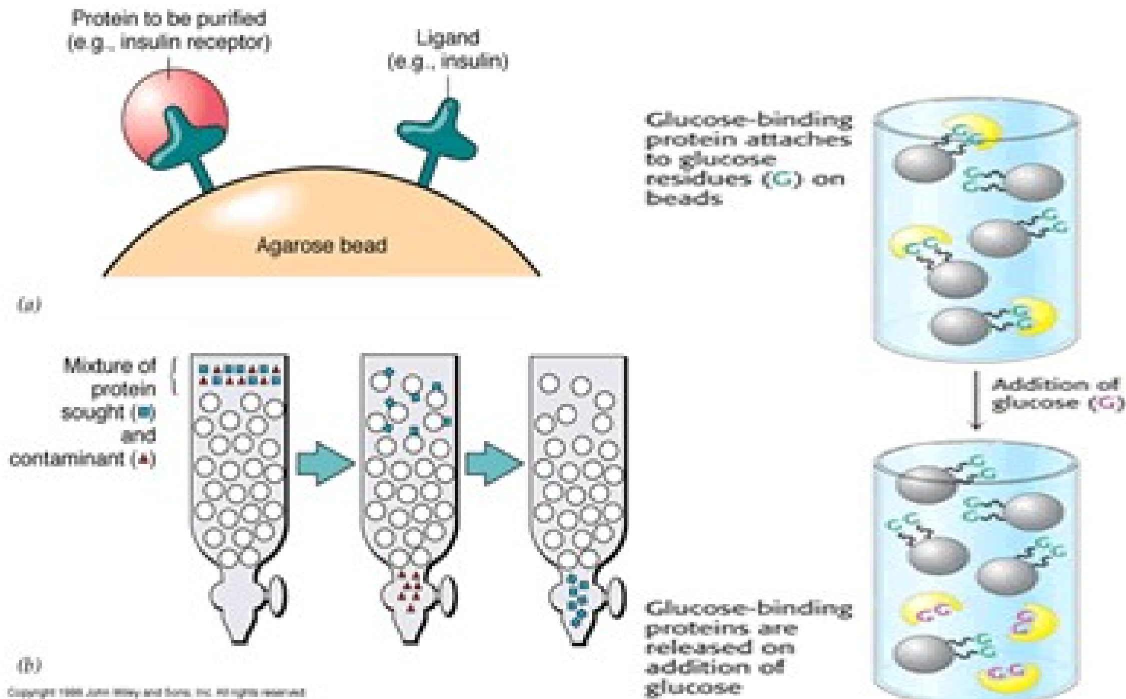

## Size exclusion chromatography

**Separation is based on molecular size. Stationary phase is a material of controlled pore size. Also called gel permeation.**



5) **Affinity chromatography:**

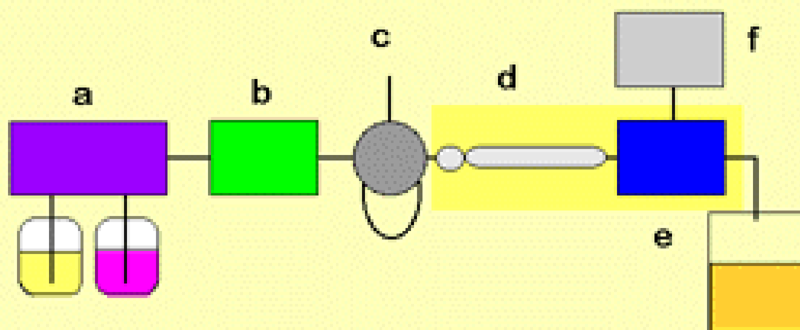
- **separation by specific binding interactions** between column matrix and target proteins




The principle of affinity chromatography is based on the property of specific and non covalent binding of proteins to other molecules referred as **ligands**. The technique involves the use of ligands covalently attached to an inert and porous matrix in a column. The immobilized ligands act as molecular fishhooks to selectively pick up the desired protein while the remaining protein passed through the column.

**6) High performance liquid chromatography (HPLC):** In general, the chromatographictechniques are slow and time consuming. The separation can be improved by applying high pressure in the range of 5000 - 10000 psi (pounds per square inch). This technique is also referred to as high pressure liquid chromatography. HPLC requires the use of non compressible resin materials and strong metal columns. The eluants of the column are detected by methods such as U.V. absorption and fluorescence.

## Basic HPLC equipment




a - gradient controller
b - pump/dampning system
c - sample introduction
d - column/precolumn
e - detector
f - data output

# HPLC

# Exercise: 12

## Estimation of vitamin A by colorimetry (Carr-Price reaction)

Vitamin A (also referred to as retinol, vitamin A1, antiopthalmic factor or axerophthol), plays an important role in maintaining integrity of mucus secereting cells of epithelial tissues. In vitamin A deficiency, the epithelial tissues are keratinised. The tissues affected are salivary glands, respiratory tract, eyes, skin and sex organs. It also plays a critical role in the process of vision.

**Principle**

Estimation of vitamin A is based on the reaction of preformed vitamin A with antimony trichloride (Carr-Price reaction). When a solution of antimony trichloride in chloroform is added to a dilute solution of a vitamin A containing sample, blue colour appears which soon reached a maximum intensity and then rapidly fades or changes to reddish brown or other colours, depending on nature of sample. Under carefully controlled conditions, the blue colour of the unknown, with colour formed by a standard solution of vitamin A, is used to determine vitamin A in unknown sample.

**Reagents**

Chloroform free from water (freshly distilled before use)

95% ethyl alcohol (peroxidise free)

Antimony trichloride-25% solution in dry chloroform. Discard if solution is turbid.

KOH solution-50% in distilled water.

Vitamin A standard-0.1g of vitamin A acetate.

Anhydrous sodium sulphate.

Diethyl ether.

0.5 N KOH in distilled water.

**Protocol**

Preparation of unsaponified fraction

Colorimeteric procedure (Carr-Price reaction)

**Preparation of unsaponified fraction**

In case of animal or dairy products containing fat, saponification and extraction is necessary, for vitamin A estimation. This is carried out as follows

Weigh 0.1 g of standard vitamin A acetate and 1 g of ghee. Transfer these separately to two saponification flasks. To each add 40 ml ethyl alcohol and 7 ml of 50 % KOH. Reflux in boiling water bath for 30 minutes. Cool, add 30 ml of distilled water. Mix thoroughly. Transfer into a separating funnel. Extract 3 times, with 50 ml portions of ether and discard aquous phase.

Combine the ether extracts (these contain vitamin A) in another separating flasks, wash with 100 ml water followed by 50 ml of 0.5 N KOH. Again wash with 100 ml portions of water till the washings give no color with phenolphthalein. Remove moisture from the ether extract by adding 5-10 g of anhydrous sodium sulphate allowing it to settle. Thereafter, decant the ether extract carefully into another flask. Rinse the first flask with ether to remove any traces and add this wash to ether extract. Evaporate the extract to dryness. Dissolve the residue obtained in 100 ml chloroform. Dilute 1 ml of this solution to 10 ml with chlotoform, to get fixed concentration of 0.1 mg/ml for standard. For sample, dilute may be carried out depending upon concentration.

b) Colorimeteric procedure (Carr-Price reaction)

Reagents are added as per protocol given below. To 1 ml of the sample/standard chloroform solution, 9 ml of antimony trichloride is added, and the blue color obtained is read at 620 nm, within 4 seconds. This can be done by adding antimony trichloride just when taking colorimetric reading.

Protocol

| Tube no. | B | 1 | 2 | 3 | 4 | 5 | 6 | 7 | S1 | S2 | S3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| StdVit A | - | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.5 | 1 | |
| µgVit A | - | 10 | 20 | 30 | 40 | 50 | 60 | 70 | - | - | - |
| Chloroform (ml) | 1 | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.3 | 0.5 | 0 | |
| Antimony trichloride (ml) | 9.0 ml in each tube | | | | | | | | | | |
| | Immediately take absorbance at 620 nm | | | | | | | | | | |

Observation

| Tube no. | B | 1 | 2 | 3 | 4 | 5 | 6 | 7 | S1 | S2 | S3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| µgVit A | - | 10 | 20 | 30 | 40 | 50 | 60 | 70 | - | - | - |
| Absorbance (620 nm) | | | | | | | | | | | |

**Calculations**

From the reading of standard, equate the values mathematically to determine concentration of vitamin A in ghee sample. Express results in terms of IU of vitamin A 100 g of ghee (1 IU=0.344 µg of vitamin A acetate).

**Notes:**

During extraction, sometimes the final chloroform solution obtained is turbid. This turbidity is due to moisture and the solution can be made clear using anhydrous sodium sulphate, as it absorbs moisture.

Antimony trichloride is toxic and corrosive to skin. Avoid contact with skin. Preferably take from sealed bottle and don't use if the crystals are moist of if any liquid or colored products are visible.

Saponification refers to conversion of an oil or fat into a soap, be reaction with an alkali (Soap is sodium or potassium salt of a long chain fatty acid e.g. sodium stearate) in this method of estimation of vitamin A, the need of saponification is to liberate the free vitamin A alcohol from synthetic vitamin A acetate of the natural form of vitamin A present in the unknown sample. The alkaline hydrolysis converts irreversibly vitmianA acetate in to free vitamin A alcohol and water soluble sodium acetate. It is the alcohol form which is estimated.

Vitamin A is slowly destroyed when its solution are exposed to light. Hence, analytical operations are conducted in amber or red glassware.

Vitamin A is characterised by an absorbtion maximum at 325 µm. This forms the basis of the spectrophotometric method of estimation performed vitamin A content.