AVIT
AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY

VINAYAKA MISSION'S RESEARCH FOUNDATION
(Deemed to be University under section 3 of the UGC Act 1956)

Accredited by NAAC

Approved by AICTE

DEPARTMENT OF BIOTECHNOLOGY

NAME OF THE LAB

17BTCC84- ADVANCED BIOCHEMISTRY LAB

HOD

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17BTCC84-ADVANCED BIOCHEMISTRY LAB

LIST OF EXPERIMENTS

1. Qualitative Analysis of Carbohydrates.
2. Qualitative Analysis of Amino acids.
3. Qualitative Analysis of Lipids.
4. Qualitatively analysis of Normal and abnormal constituents of Urine
5. Estimation of Glucose by O-toludine method.
6. Protein estimation by Biuret,
7. Estimation of Cholesterol by Zak's method.
8. Estimation of urea DAM method.
9. Estimation of Hemoglobin.
10. Separation of plant pigments by column chromatography (Demo).

HOD

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LABORATORY RULES AND REGULATIONS

1. **Always wear lab Coat while performing the experiment**
2. **Be careful with hot plates, Bunsen burner and other heat source**
3. **Wear disposable gloves when handling blood and other body fluids, mucus membranes non-intact skin or items and surfaces soiled with blood or body fluids.**
4. **Highly flammable chemicals must be handled in the fume hood.**
5. **Wash hands with soap and water before leaving the laboratory and before eating or drinking**
6. **Use mechanical pipetting devices; mouth pipetting is prohibited.**
7. **Report all spills , accidents or injuries to the instructor immediately**
8. **Properly dispose the broken glassware and other sharp objects (syringe needles) immediately in designated container.**
9. **Wear breathing mask as and when appropriate**
10. **Thoroughly clean the laboratory work space at the end of the laboratory session**
11. **Do not taste or smell hazardous chemicals**
12. **Never add water to acid. Always add acid to water**
13. **Never point the open end of the glassware containing the solution to be heated toward anyone.**
14. **Avoid distracting or startling others when handling hazardous chemicals**
15. **While heating a solution make sure not to overheat it; therefore, vigorously mix the solution by shaking or stirring.**
16. **Flasks with flat-bottoms or thin walls should not be dessicated**
17. **Do not use any machine that smoke or sparks or appear defective anyway.**

Molar Solution:

One gram molecular weight of the substance dissolved in one litre of solution.

$$1\ M = \frac{\text{Amount per litre in g}}{\text{Mol.wt}}$$

Normal Solution:

One gram equivalent weight of the substance in one litre of solution. Equivalent weight of a substance may be obtained by dividing the molecular weight by its valency.

$$1\ N = \frac{\text{Amount per litre in g}}{\text{Eq.wt}}$$

Percentage Solution:

1% solution for solid substance: 1g / 100mL
1% solution for liquids : 1mL / 100mL

Stock Reagent:

Stock reagent is a solution with higher concentration than its working concentration. A stock solution is necessary as its dilute solution may be unstable by simple dilution or its modification by addition of other chemicals.

Working Reagent:

These are prepared from the stock when required by dilution.

UNITS AND MEASUREMENTS

MASS:

1g	=	1000 mg (milligram)	= 10^{-3} kg
1mg	=	1000 µg (microgram)	= 10^{-3} g
1µg	=	1000 ng (nanogram)	= 10^{-6} g
1ng	=	1000 pg (pictogram)	= 10^{-9} g
1pg	=	1000 fg (femtogram)	= 10^{-12} g

Parts per million (ppm) = µg per gram or mg per kg = 10^{-6} g
Parts per billion (ppb) = ng per gram or µg per kg = 10^{-9} g

VOLUME:

1 L	= 1000 mL
1 dL (deciliter)	= 100 mL
1 mL	= 1000 µL (micro liter)
1 µL	= 1000 nL (nano liter)

QUALITATIVE ANALYSIS OF CARBOHYDRATES
GENERAL PROCEDURE

A positive observation should have positive inference and negative observation should have negative inference.Eg: When Benedict's Test is positive and Barfoed's is negative, the inference against Barfoed's test should be absence of monosaccharides and presence of reducing disaccharides.A good observer is a good scientist .The observation Should be done carefully .careful observation could help in new discoveries.Van den Berg forgot to add methanol in his reaction with bilurubin in serum ,he got a colour which he did not expect .It was then called direct reacting bilurubin.The student should record his own observation and not his neighbour's.

S. NO	EXPERIMENT	OBSERVATION	INFERENCE
1.	**SOLUBILITY TEST :** : Test the solution in • Acid • Water • Alcohol • Alkali	Carbohydrates are found to be soluble.	This shows the presence of carbohydrates.
2.	**MOLISCH'S TEST :** To 5mL of the test solution add 2 drops of molisch's reagent and 2mL of concentration sulphuric acid along the sides of the test tube.	A purple colour ring is formed at the junction of two layers, which spreads on standing.	This shows the presence of carbohydrates. The purple ring is due to furfural and its derivative.
3.	**BENEDICT'S TEST :** To 5mL of the test solution add 5mL of Benedict's reagent and heat in boiling water bath.	A reddish brown precipitate is formed.	This shows the presence of reducing sugar.
4.	**FEHLING'S TEST :** To 5mL of the test solution add equal volume of Fehling's "A" and "B"	A reddish brown precipitate is formed	This shows the presence

	reagent and heat in boiling water bath.		of reducing sugar.
	BARFOED'S TEST :		
5.	To 5mL of the test solution add 5mL of Barfoed's reagent and heat in boiling water bath.	A reddish brown precipitate is formed	This shows the presence of monosaccharides.
	SELIWANOFF'S TEST :		
6.	To 2.5 mL of the reagent add 5 drops of sugar solution boiled and cooled.	Cherry red colour was formed.	
7.	**OZAZONE TEST:**		This shows the presence of keto sugars (fructose).
	Dissolve phenyl hydrazine hydrochloride and sodium acetate in the ratio 1: 2.To these add 5mL of the test solution and acidify with 2 drops of glacial acetic and heat in boiling water bath.	Yellow precipitate is formed .The crystals were observed under microscope. • Needle shaped crystals were seen. • Powder puff shaped crystals were seen. • Flower petals shaped crystals were seen.	Presence of glucose or fructose. Presence of lactose. Presence of maltose.
	HYDROLYSIS :		
8.	To 5mL of the test solution add 1 mL of hydrochloric acid boiled for 2 min and cooled , neutralized with 20% sodium carbonate. To this add phenyl hydrazine hydrochloride and sodium acetate and acidified with drops of glacial acetic acid, boiled and cooled.	Yellow precipitate was formed.	Presence of sucrose, which is hydrolyzed to glucose and fructose.

9.	**IODINE TEST:** To 2mL of sugar add few drops of N/50 iodine solution.	Blue colour was formed	Presence of starch. Blue colour disappears on heating and reappears on cooling.

QUALITATIVE ANALYSIS OF AMINO ACIDS

The qualitative analysis of amino acids is a **qualitative measure**, where a chemical change brings some changes like the colour change, precipitation, ring formation etc. on the basis of which the amino acids can be detected and classified. The colour change is due to the change in the moiety or the structural configuration where the functional groups of amino acid react with the specific reagent to give specific results. Amino acids are the basic **building blocks** of protein. The structure of amino acid consisting of amine group (**-NH_2**), carboxylic group (**-COOH**) and **R-group** or side chain.

The side chain differs among all 20 different naturally occurring amino acids. These 20 naturally occurring amino acids are the essential amino acids, which broadly classified into three types:

- *Polar amino acids*
- *Non-polar amino acids*
- *Aromatic amino acids*

The melting point of an amino acid is about 200 degrees Celsius. The net charge of all amino acids becomes zero at neutral pH or at isoelectric point (pI), where they occur as "**Zwitter ions**".

At **isoelectric point**, the amino acid carries both positive and negative charge or it will neither move to cathode nor anode, even under the influence of the electric field. Isoelectric point or pI is different for different amino acids.

Accept glycine, all the amino acids are having an asymmetric C-atom (Carbon linked to 4 different groups), which shows **optical activity** to rotate a polarized light either to left or right.

NINHYDRIN TEST:

This test is used for the detection of all α-L- amino acids.

Principle:

Ninhydrin test is based upon the principle which makes the use of reagent "Ninhydrin". The amino acid reacts with the chemical reagent Ninhydrin to form an intermediate "**Hydrindantin**". Hydrindantin further reacts with Ninhydrin and ammonia to form a blue-purple pigment or **Ruhemann's purple** compound refers as "Diketohydrin". Therefore, the amino acid undergoes **degradation** through the series of chemical reaction to give specific results.

Other than amino acids, imino acids like proline, hydroxyproline also reacts with the Ninhydrin and gives a yellow coloured compound. Amines also react positively by reacting with the Ninhydrin reagent and gives a blue colour.

Method:

1. Prepare a 1ml solution of the given sample.
2. Then, add a few drops of Ninhydrin solution.
3. Boil the solution for 2 minutes and then cool the content.

Observation:

Observe the tubes for the appearance of any colour change.

Inference:

The appearance of purple colour indicates the presence of α -amino acids and yellow colour indicate the presence of imino acids.

1. XANTHOPROTEIC TEST

This test is specific for the detection of aromatic amino acids containing active benzene ring or aromatic nucleus.

Principle:

The Xanthoproteic test is based upon the principle of "**Nitrification**" reaction. In this test, the active aromatic amino acids undergo nitrification in the presence of concentrated nitric acid which forms a yellow coloured **nitro-derivatives**. The yellow colour turns into orange by the ionization of the phenolic group at alkaline pH.

Method:

1. Prepare a 1ml solution of the given sample.
2. Then, add a few drops of nitric acid.
3. Boil the solution for 2minutes and then cool the content.

Observation:

Observe the tubes for the appearance of yellow colour.

Inference:

If a solution gives a yellow colour then it indicates the presence of aromatic amino acids.

2. PAULY'S DIAZO TEST

This test is specific for the detection of amino acids like histidine and tyrosine.

Principle:

The principle of Pauly's diazo test is based upon the "**Diazotation**" reaction. Pauly's diazo test makes the use of chemical reagent (Sulphanilic acid) which undergoes Diazotation reaction to form a "**Diazonium salt**" in the presence of sodium nitrite and hydrochloric acid. The diazonium salt then couples with the amino acids (either tyrosine or histidine) by giving a red colour to the solution in the alkaline medium.

Method:

1. Take Sulphanilic acid reagent in a test tube and place in an ice bucket to cool.
2. Then add prechilled sodium nitrite solution and few drops of chilled amino acid solution.
3. At last, add sodium carbonate until the colour appears.

Observation:

Observe the test tube for the appearance of a red colour by the addition of sodium bicarbonate.

Inference:

The appearance of the red colour will indicate the presence of tyrosine and histidine.

4. MILLON'S TEST

This is the specific test for the detection of phenolic amino acids like tyrosine.

Principle:

The principle of Millon's test is based upon the "**Nitration**" reaction. Millon's test makes the use of nitrifying agent i.e. concentrated nitric acid, by which the phenolic amino acids are converted into nitrated amino acid by giving a red colour to the solution. The nitrated compound further forms a deeper yellow coloured salt of amino acids.

Method:

1. First, take the sample of amino acid.
2. Then, add Millon's reagent to the above and mix all the contents.
3. After that, boil the solution in a water bath for 5 minutes and then cool for a while.
4. At last, add sodium nitrite solution until the colour appears.

Observation:

Observe the test tube for the appearance of a red colour.

Inference:

The appearance of a red colour indicates the presence of tyrosine.

5. HISTIDINE TEST

This test is specifically used to detect the presence of Histidine. This test was discovered by **Knoop**.

Principle:

The principle of Histidine test is based upon the principle of "**Bromination**" reaction. In Histidine test, Bromination of amino acid occurs to give a yellow coloured compound in the presence of bromine in acid solution.

Method:

1. Prepare 1 ml sample of given amino acid.
2. Then add an acid solution containing 5% bromine in 33% acetic acid.
3. After 10 minutes, add 2 ml of 5% sodium carbonate solution.
4. Boil the solution in a water bath for 10 minutes.

Observation:

Observe the test tube for the appearance of a blue colour in a solution.

Inference:

The appearance of a blue colour indicates the presence of histidine.

6. **HOPKIN'S COLE TEST**

This test is specific for the detection of tryptophan.

Principle:

The principle of Hopkin's Cole test is based upon the "**Dehydration**" reaction. In Hopkin's Cole test, the amino acid reacts with the reagent glyoxylic acid in the presence of concentrated sulphuric acid. This reaction causes the dehydration of tryptophan which forms a purple coloured ring between the junctions of two solutions.

Method:

1. Prepare 1 ml sample of given amino acid.
2. Then add 1 ml of glyoxylic acid.
3. Mix the contents and add concentrated sulphuric acid from the side of the tube in an inclined position.

Observation:

Observe the test tube for the appearance of a violet ring in between the solution.

Inference:

Appearance of a violet coloured ring will indicate the presence of tryptophan.

7. **SAKAGUCHI TEST**

This test is specific for the detection of arginine.

Principle:

The Sakaguchi test is based on the principle of "**Oxidation**" reaction. Sakaguchi test makes the use of oxidising agent i.e. sodium hydroxide and α- naphthol reagent. Both sodium hydroxide and α- naphthol reacts with the arginine to give a characteristic red colour on the treatment with hypochlorite.

Method:

1. Take 1 ml of given amino acid sample.
2. Then, add 2 drops of sodium hydroxide.
3. After that, add 2 drops of α- naphthol and mix all the contents.
4. At last, add a few drops of hypochlorite solution.

Observation:

Observe the test tube for the appearance of the red colour in the solution.

Inference:

The appearance of the red colour indicates the presence of Arginine.

8. LEAD SULPHIDE TEST

This test is useful for the detection of amino acids like cysteine which contains the – SH or Sulfhydryl group.

Principle:

The principle of Lead sulphide test is based upon the "**Precipitation**" reaction. In Lead sulphide test, the cysteine reacts with sodium hydroxide and after boiling, it gets converted into sodium sulphide. Then, sodium sulphide reacts with the lead acetate and undergo precipitation reaction by forming black precipitates as lead sulphide.

Method:

1. Prepare 1 ml solution of given amino acid.
2. Then add a few drops of 40% sodium hydroxide.
3. Boil the solution in a water bath for about 5-10 minutes and then cool.
4. At last, add 10% of lead acetate solution.

Observation:

Observe the test tube for the formation of black precipitate in a solution.

Inference:

Formation of black precipitate indicates the presence of cysteine.

QUALITATIVE ANALYSIS OF LIPIDS

1. SOLUBILITY TEST:

The test is based on the property of solubility of lipids in organic solvents and insolubility in water.

PRINCIPLE:

The oil will float on water because of lesser specific gravity.

TEST:

Take 3ml of solvents in each test tube and add 5 drops of sample. For water and ethanol, it is insoluble and for chloroform and ether, it is soluble and hence the given sample is lipid.

2. TRANSPARENCY TEST:

All the lipids are greasy in nature. Therefore the test may be taken as group test for lipids.

PRINCIPLE:

The oil does not wet the paper.

TEST:

Take 3ml of ether in a test tube and dissolve 5 drops of oil in tit. Put a drop of the solution on the filter paper and let it dry. A translucent spot on the filter paper was observed and this indicates the greasy character of the lipid.

3. EMULSIFICATION TEST:

When oil and water, which are immiscible, are shaken together, the oil is broken up into very tiny droplets which are dispersed in water. This is known as oil in water emulsion. The water molecule due to the high surface tensions has a tendency to come together and form a separate layer. This is why the oil and water emulsion is unstable in the presence of substances that lower the surface tension of water. Eg: Sodium carbonate, soap, bile salts etc. The tendency of the water molecule to coalesce is decreased and the emulsion becomes stable. Since bile salts cause the greatest decrease in surface tension they are best emulsifying agents.

TEST: Take 3ml of water and add 5 drops of sample. In another test tube 10ml of water is added to ethanolic solution of lipid contents and are mixed and two layers of are observed and this confirms the presence of lipids.

4. TEST FOR UNSATURATION:

The unsaturated fatty acids absorb iodine at the double bonds until all the double bonds are saturated with iodine. Hence the amount of iodine required to impart its color to the solution is a measure of the degree of the fatty acids.

TEST: Take 1ml of of chloroform and add a methanol and one drop of oil. to this add 1drop of iodine. Chloroform dissolve sample give red color which decolorizes the iodine giving brown color. This indicates the presence of fatty acids

5. TESTS FOR GLYCEROL:

I. Acrolein test:

Take pure glycerol in a dry test tube; add to it a few crystals of potassium hydrogen sulphate. Warm gently to mix and then heat strongly. A very pungent odour of acrolein is produced. Acrolein is formed due to removal of water from glycerol by potassium hydrogen sulphate.

II. Dichromate Test:

Take in a dry test tube 3 or 4 ml of glycerol solution, to it add a few drops of 5% potassium dichromate solution and 5 ml of conc. HNO_3, mix well and note that the brown colour is changed to blue. This test is given by the substances containing primary and secondary alcohol groups. The chromic ions oxidize the glycerol and in this process they are reduced to chromous ions which give the blue colour. This test is also given by reducing sugars, so before confirming glycerol be sure that the reducing sugars are not present.

QUALITATIVE ANALYSIS OF URINE FOR ABNORMAL CONSTITUENTS

Substances which are not present in easily detectable amounts in urine of normal healthy individuals but are present in the urine under certain diseased conditions are said to be Abnormal constituents of urine.

1. Glucose:

Benedict's Test:

Principle: Copper sulphate of Benedict's qualitative solution is reduced by reducing substances

on boiling to form the coloured precipitate of cuprous oxide.

Test for Glucose:

Procedure	Observation	Inference
To about 5 ml of Benedict's reagent add 0.5 ml of urine and boil for 2 min	Blue colour appear	Sugar absent
	Light green precipitate appear	0.1-0.5 % 0f reducing sugar present
	Green precipitate appears	0.5 to 1.0 % of reducing sugar present
	Yellow precipitate appears	1-2 % reducing sugar present
	Brick red precipitate appears	Above 2 % reducing sugar present

Normal urine also contains a trace of glucose and glucuronates, but their amount is too small to cause reduction in Benedict's test. In Diabetes mellitus and in renal glycosuria, glucose is found in urine. This gives a Benedict's test positive.

2. Albumin:

a. Sulphosalicylic acid test:

Principle: Albumin, the protein, is denatured by sulphosalicylic acid a coagulation.

Procedure	Observation	Inference
Add a few drops of Sulphosalicylic acid to 2 ml of urine	Turbidity appears	Indicates the presence of albumin

b. Heat coagulation test:

Principle: The albumin is coagulated after being heated.

Procedure	Observation	Inference
Fill 3/4th of the test tube by urine. Heat the upper 1/3rd of the test tube by a small flame.	Turbidity appears on the heated portion of the tube	Indicates the presence of albumin

c. Heller's Nitric acid test:

Principle: Nitric acid causes precipitation of protein.

Procedure	Observation	Inference
To 3 ml of nitric acid in a tube add 3 ml of urine by the wall of the tube in such a way that the two liquids do not mix	White ring appears at the junction of the two fluids	Indicates the presence of albumin

3. Ketone bodies:

Rothera's Test:

Principle: Acetoacetic acid forms a complex with nitroprusside in alkaline solution developing a permanganate colour.

Procedure	Observation	Inference
Saturate 5 ml of urine with ammonium sulphate by shaking vigorously. Then add 2 drops of freshly prepared 5% solution of sodium nitroprusside and 1 ml of ammonium hydroxide. Allow it to stand in a rack for a while	A permanganate colour develops just above the layer of un dissolved ammonium crystals	Indicates the presence of Ketone bodies

ESTIMATION OF BLOOD GLUCOSE BY ORTHO TOLUIDINE METHOD:

AIM:

To estimate the amount of glucose present in the given sample.

PRINCIPLE:

When glucose is heated with Ortho toluidine in strong acidic condition, the aldehydic group in glucose condenses with aromatic amine to form glucosamine, which rearranges to form chromophoric schiff's base .The bluish green color obtained was read at 620 nm.

Glucose + O-Toluidine ----------► Glucosylamine + Schiff base

REAGENTS:

1. Ortho - toluidine:

1.5 gm of thiourea is taken and to this 950 mL of glacial acetic acid and 500mL of Ortho Toluidine are added mixed and stored in brown bottle at room temperature.

2. 3 % TCA

3. Stock Standard:

1g of glucose is dissolved in 100mL of water.

4. Working standard:

10 mL of the stock is diluted to 100mL with water.

PROCEDURE:

- To 0.2 mL of blood in a centrifuge tube, add1.8 mL of TCA, mix well and keep for 5 min, centrifuge& take0.5 mL of supernatant as test.
- Take aliquots of standard (0.1-0.5 mL) in a series of test tube and make up to 0.5 mL with distilled water.
- Take 0.5mL of water as blank.
- Add 3.5 mL of O-Toluidine to all the test tubes, mix well and keep in boiling waterbath for 10 min and cool.
- Read the colour developed at 620 nm .

TABULATION

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (mL)	-	0.1	0.2	0.3	0.4	0.5	0.5
Concentration (µg)							
Volume of distilled water (mL)	0.5	0.4	0.3	0.2	0.1	-	-
Volume of Protein free Filtrate (mL)	-	-	-	-	-	-	0.5
Volume of O-Toluidine (mL)	←---------- 3.5mL ----------→						
	Kept in boiling water bath for 10 min						
O.D at 620 nm							

CALCULATION:

Test concentration = $\frac{\text{Test O.D}}{\text{Standard O.D}}$ x Concentration of standard x $\frac{100}{\text{Dilution factor}}$

GRAPHICAL CALCULATION:

X O.D corresponds to ___ µg of Glucose

0.5mL of the test sample contain ___ µg of Glucose

100mL of the test sample = $\frac{\text{µg x 100}}{\text{Dilution factor}}$

RESULT:

The amount of Glucose present in the given sample

i. By calculation:

ii. By graph:

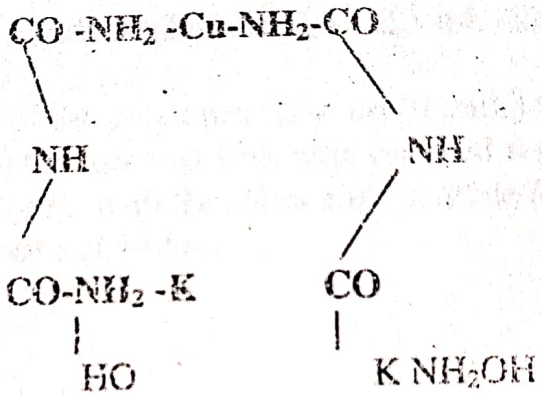
ESTIMATION OF PROTEIN BY BIURET METHOD

AIM:

To estimate the amount of protein present in the given sample by Biuret method.

PRINCIPLE:

Protein react with the Biuet reagent to give a purple colour,that can be measured at 540 nm. The colour is due the formation of cupric-potassium biuret or potassium cupric hydroxide.



The reaction is give by the substance containing the groups joined either directly or through carbon or nitrogen atom or substance containing Co - NH_2 ,CH_2 - NH_2 ,CH - NH_2 respond to the test. Protein suspend positively due to the presence of – CONH in the protein molecule.

MATERIALS REQUIRED :

1. Standard flask
2. Test tubes
3. Pipette
4. Distilled water

REAGENTS:

1. Protein stock standard:
   500mg of Egg albumin was dissolved in 100mL 0f 0.9% Saline.

2. Working standard:

   50 mL 0f the stock was made upto 100 mL with water.

1. Biuret reagent:

3g of copper sulphate & 9g of sodium potassium tartarate are dissolved in 0.2N sodium hydroxide. To this 5 g of potassium Iodide was added & made up to 1 litre with 0.2N sodium hydroxide.

PROCEDURE:

- Pipette out aliquots of standard albumin (0.5 – 2.5 mL) into a series of test tubes.
- Take a known volume of the given test solution (0.2mL) for estimation.
- Make the volume in all the tubes to 3mL with distilled water.
- Add 5mL of Biuret reagent to all the tubes and incubate for 15 min.
- Read the colour developed at 540nm.

TABULATION

Particulars	Blank	S_1	S_2	S_3	S_4	S_5	T_1	T_2
Volume of standard (mL)	-	0.5	1.0	1.5	2.0	2.5	0.2	0.2
Concentration (µg)								
Volume of Distilled water(mL)	3	2.5	2.0	1.5	1.0	0.5	2.8	2.8
Biuret reagent (mL)	5mL							
Incubate for 15 minutes								
O.D at 540 nm								

CALCULATION:

100mL of standard solution contain 500mg of Albumin

Amount of protein present in Egg Yolk(T1)

$$= \frac{\text{Test O.D}}{\text{Standard O.D}} \times \text{Standard concentration} \times \frac{100}{\text{Amount of Sample taken}}$$

Amount of protein present in Egg white(T2)

$$= \frac{\text{Test O.D}}{\text{Standard O.D}} \times \text{Standard concentration} \times \frac{100}{\text{Amount of Sample taken}}$$

GRAPHICAL CALCULATION:

Egg white T1 = Test concentration $\times \frac{100}{0.2}$

Egg yolk T2 = Test concentration $\times \frac{100}{0.2}$

RESULT:

The amount of protein present in 100mL of the given sample

i. .By calculation:
   i. Egg white solution =
   ii. Egg yolk solution

ii. .By graph
   a. Egg white solution =
   b. Egg yolk solution =

ESTIMATION OF PROTEIN BY FOLIN LOWRY'S METHOD

AIM:

To estimate the amount of protein present in the given sample.

PRINCIPLE:

Lowry's method of protein estimation is a most widely accepted method for accurate determination of protein concentration. This method is based on the combination of biuret method and Folin-Ciocalteau reaction. In the first step of the reaction the protein binds to copper in alkali medium and produces copper ions. In the step cupric peptide complex along with aromatic amino acids reduce phosphomolybdotungstate to heteropolymolybdenum blue. This reaction produces strong blue colour, which is read at 660nm.The colour yield predominantly depends upon tyrosine and tryptophan content of protein and to a lesser extent cysteine and other residues in protein.

MATERIALS REQUIRED:

1. Test tubes
2. Standard flask
3. Pipette
4. Spectrophotometer

REAGENTS REQUIRED:

1. Stock standard : Dissolve 100mg of Bovine serum albumin in 100mL of water , add few drops of 0.1N NaOH.

2. Working Standard: 20 mL 0f the stock is diluted to 100mLwith water.

3. Alkaline copper reagent:

Prepare on day of use by mixing 50mL of solution A and 1mL of solution B.

- Solution A:

Alkaline sodium carbonate solution: 2gm of sodium carbonate dissolved in 100mL of 0.1N sodium hydroxide

- Solution B:

Copper sulphate sodium potassium tartarate solution: 1gm of $CuSO_4.5H_2O$ in 200mL of solution (2gm of sodium potassium tartarate). Prepare fresh by mixing stock solution.

4. Folins ciocalteau reagent:

Dilute the commercial reagent with equal volume of water on the day of use. It is a solution of sodium tungstate and sodium molybdate in phosphoric acid and hydrochloric acid.

PROCEDURE:

- Pipette out aliquot's of BSA standard (0.1-0.5 mL) in different test tubes and make up to 1mL with water.
- Take 1mL of water as blank.
- Add 5mL of alkaline copper sulphate and 0.5 mL of Folin ciocalteau reagent to all the test tubes including 1mL of test solution with immediate mixing.
- After 30 minutes read the optical density at 620nm.

TABULATION

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (mL)	-	0.1	0.2	0.3	0.4	0.5	1.0
Concentration (µg)	-						-
Distilled water (mL)	1	0.9	0.8	0.7	0.6	0.5	-
Volume of alkaline copper reagent (mL)	5mL						
Incubate for 30 minutes							
Folin ciocalteau reagent(mL)	0.5mL						
O.D at 620 nm							

CALCULATION:

$$\text{Test concentration} = \frac{\text{Test O.D}}{\text{Standard O.D}} \times \text{Concentration of Standard} \times \frac{100}{\text{Amount of Sample}}$$

GRAPHICAL CALCULATION:

X O.D corresponds to ____ µg of proteins

0.1 mL of the test sample contain ____ µg of proteins

100 mL of the test sample = $\frac{\text{µg x 100}}{0.1}$

RESULT:

The amount of protein present in the given sample

i. By calculation:

ii. By graph:

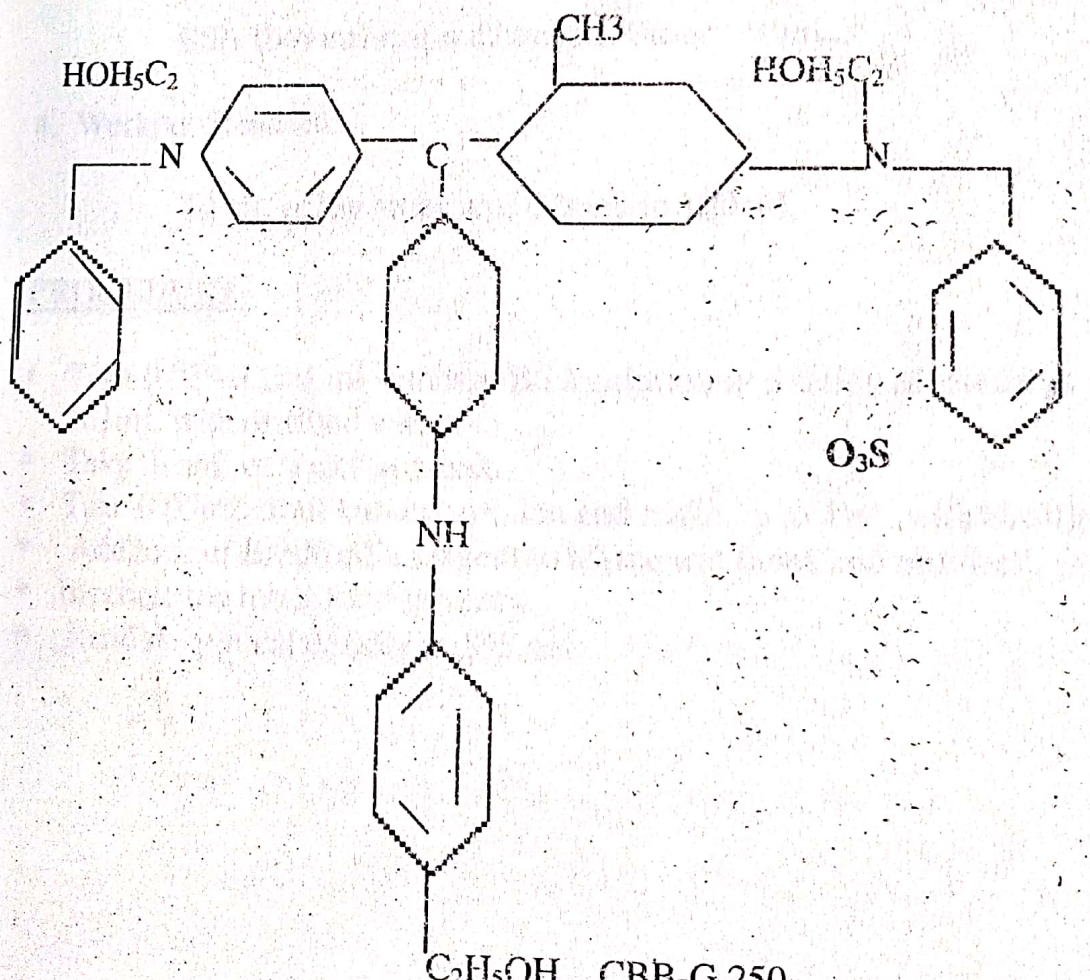
PROTEIN ESTIMATION BY BRADFORD'S METHOD

AIM:

To estimate the amount of protein present in the given sample

PRINCIPLE:

Protein purification often requires a rapid and sensitive method for the quantification of protein. Bradford introduced a protein determination method, which involves the binding of coomassie brilliant blue G-250 to the protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm and it is the increase in absorption at 595 nm, which is monitored. This assay is responsible and rapid with the dye binding process virtually complete in approximately 2 minutes with good colour stability for 1 hour.



MATERIALS REQUIRED:

1. Test tubes
2. Standard flask
3. Pipette
4. Spectrophotometer

REAGENTS REQUIRED:

2. Brad ford's reagent:

Dissolve 20mg of comassie blue in 10mL of 95% ethanol. 20 mL of 85% phosphoric is added gradually with frequent stirring .At this stage the solution will be dark brown. This is then made up to a volume of 200mLwith-distilled water. Filter it with whatmann No.1 filter paper. (The colour of the reagent should be greenish brown and it should be stored in amber colour bottles).

3. Protein stock standard:

BSA (bovine serum albumin) 50mg / 100mL.

4. Working Standard:

10 mL of the stock was diluted to 100 mL

PROCEDURE:

- Take 0.02 –0.1mL of standard BSA solution in a series of test tubes and make up to1mL with distilled water.
- Take 0.2mL of water as blank.
- Take 0.05mL of unknown solution and make up to 1mL with distilled water.
- Add3mL of Bradford's reagent to all the test tubes and mix well.
- Incubate the tubes for 5 minutes.
- Read the optical density at 595 nm.

TABULATION

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (mL)	-	0.02	0.04	0.06	0.08	0.10	0.05
Concentration (ug)	-						-
Distilled water (mL)	1	0.18	0.16	0.14	0.12	0.10	0.05
Volume of Brad ford's reagent (mL)	3 mL						
Incubate for 5 minutes							
O.D at 595 nm							

CALCULATION:

$$\text{Test Concentration} = \frac{\text{Test O.D}}{\text{Standard O.D}} \times \text{Concentration of Standard} \times \frac{100}{\text{Amount of Sample taken}}$$

GRAPHICAL CALCULATION:

X O.D corresponds to ____ µg of proteins

0.2 mL of the test sample contain ____ µg of proteins

$$100\text{mL of the test sample} = \frac{\text{µg} \times 100}{0.05}$$

RESULT:

The amount of protein present in the given sample

i. By calculation:
ii. By graph:

ESTIMATION OF CHOLESTEROL BY ZAK'S METHOD

AIM:

To estimate the amount of cholesterol present in the serum sample by ZAK's method.

PRINCIPLE:

Cholesterol is a steroid lipid, amphipathic in nature. It consistes of basic cyclopentano perhydro phenothrene nucleus. It is synthesized in liver from Acetyle CoA. It acts as a precursor for steroid hormones and vitamin D. The serum cholesterol exists in 2 forms.

REAGENTS:

1. FeCl3-CH3COOH reagent(0.05%)-0.05gms of FeCl3 is dissolved in 100ml of aldehyde free CH3COOH.

2. conc. H2SO4

3. Cholesterol standard

4. Stock Solution-100mg of cholesterol is dissolved in 100ml of acetic acid.

5. Working standard Solution–4ml of stock solution is dissolved in (or) diluted to 100ml with FeCl3-CH3COOH solution. The concentration of standard is 0.04 mg/ml.

PROCEDURE :

STANDARDS :

- Pipette 1-5 ml of standard solution in a series of testtubes.
- The volume in each test tube is made upto 5ml with FeCl3-CH3COOH reagent.
- 3ml of conc. H2SO4 is added to all the testtubes and mix well.
- Standards are incubated for about 20-30 minutes at room temperature.
- The intensity of standards is measured at 560 nm against blank.

BLANK:

- **5 ml of FeCl3-CH3COOH reagent, 3ml of H2SO4 are taken in a testtube, mixed well and used as a blank.**

TEST:

- **In the centrifuged tube 0.1ml of serum and 10ml of FeCl3-CH3COOH reagents are taken, mixed well for 5 minutes and then centrifuged.**
- **5 ml of supernatant is collected and added with 3ml of H2SO4.**
- **Test is incubated at room temperature to 20-30 Intensity is measured at 560nm against blank.**

TABULATION

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (mL)	-	1	2	3	4	5	5
Concentration (µg)	-						-
FeCl3-CH3COOH reagent. (mL)	5	4	3	2	1	-	-
Volume of Sulphuric acid (mL)	3 mL						
Incubate for 30 minutes							
O.D at 560 nm							

CALCULATION:

$$\text{Test Concentration} = \frac{\text{Test O.D}}{\text{Standard O.D}} \times \text{Concentration of Standard} \times \frac{100}{\text{Amount of Sample taken}}$$

GRAPHICAL CALCULATION:

X O.D corresponds to ___ mg of Cholesterol

5mL of the test sample contain ___ mg of Cholesterol

100mL of the test sample = $\frac{\text{mg x100}}{0.05}$

RESULT:

The amount of Cholesterol present in the given sample

iii. . By calculation:
iv. By graph:

ESTIMATION OF UREA BY DIACETYL MONOXIME METHOD

AIM: To determine the amount of Urea present in the given blood sample.

PRINCIPLE:

A pink colour complex is formed when urea is treated with di-acetyl monoxime and thiosemicarbazide in the presence of sulphuric acid, phosphoric acid and ferric chloride which is read at 525 nm in spectrophotometer.

REAGENTS:

1. Reagent A: 300 ml concentrated H_2SO_4(95-98%) 2 4 50 ml distilled water 100 ml concentrated H PO (85%) 100 mg ferric chloride (FeCl) Mix well and volume it to 1 litre with distilled water.
2. Reagent B: 500 g diacetyl monoxime (DAMO) 10 mg thiosemicarbazide (TSC) Mix well with distilled water to make 100 ml solution
3. Reagent C: Prepare immediately before use with Reagents A & B at 2:1 proportion.
4. Trichloro acetic acid (TCA) 5% (w/v) with distilled water.
5. Standard urea solution (0-150 nmol)

Working standard (50 µg/ml): Dissolve 50 mg urea in 1 litre of distilled water.

PROCEDURE:

- Protein is precipitated out from serum/plasma 0.2 ml mixed with 1.8 ml of 5 per cent TCA.
- Centrifuge at 2000 rpm for 10 min (Fig. 7.13) and collect supernatant for determination using the protocol given below.
- Boil in water bath for 5 minutes.
- Cool to room temperature and read absorbance at 525 nm against reagent blank.

- Plot a standard curve and read concentration of the sample against absorbance.

TABULATION:

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (mL)	-	.02	.04	.06	.08	.10	0.2
Concentration (µg)	-						-
Distilled Water (mL)	0.2	.18	.16	.14	.12	.10	-
Reagent C (mL)	3 mL						
Boil in waterbath for 5 minutes							
O.D at 525 nm							

CALCULATION:

$$\text{Test Concentration} = \frac{\text{Test O.D}}{\text{Standard O.D}} \times \text{Concentration of Standard} \times \frac{100}{\text{Amount of Sample taken}}$$

GRAPHICAL CALCULATION:

X O.D corresponds to ___ mg of Urea

0.2mL of the test sample contain ___ mg of Urea

$$\text{100mL of the test sample} = \frac{\text{mg} \times 100}{0.05}$$

RESULT:

The amount of Urea present in the given sample

i. . By calculation:
ii. By graph:

ERYTHROCYTE SEDIMENTATION RATE

The **erythrocyte sedimentation rate (ESR)** is a common hematological test for nonspecific detection of inflammation that may be caused by infection, some cancers and certain autoimmune diseases. It can be defined as the rate at which Red Blood Cells (RBCs) **sediment in a period of one hour.**

PRINCIPLE

When anticoagulated blood is allowed to stand in a narrow vertical glass tube, undisturbed for a period of time, the RBCs – under the influence of gravity- settle out from the plasma. The rate at which they settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour(mm/hr). This mechanism involves three stages:

- **Stage of aggregation:** It is the initial stage in which piling up of RBCs takes place. The phenomenon is known as Rouleaux formation. It occurs in the first 10-15 minutes.
- **Stage of sedimentation:** It is the stage of actual falling of RBCs in which sedimentation occurs at constant rate. This occurs in 30-40 minutes out of 1 hour, depending upon the length of the tube used.
- **Stage of packing:** This is the final stage and is also known as stationary phase. In this, there is a slower rate of falling during which packing of sedimented RBCs in column occurs due to overcrowding. It occurs in final 10 minutes in 1 hour.

METHODS:

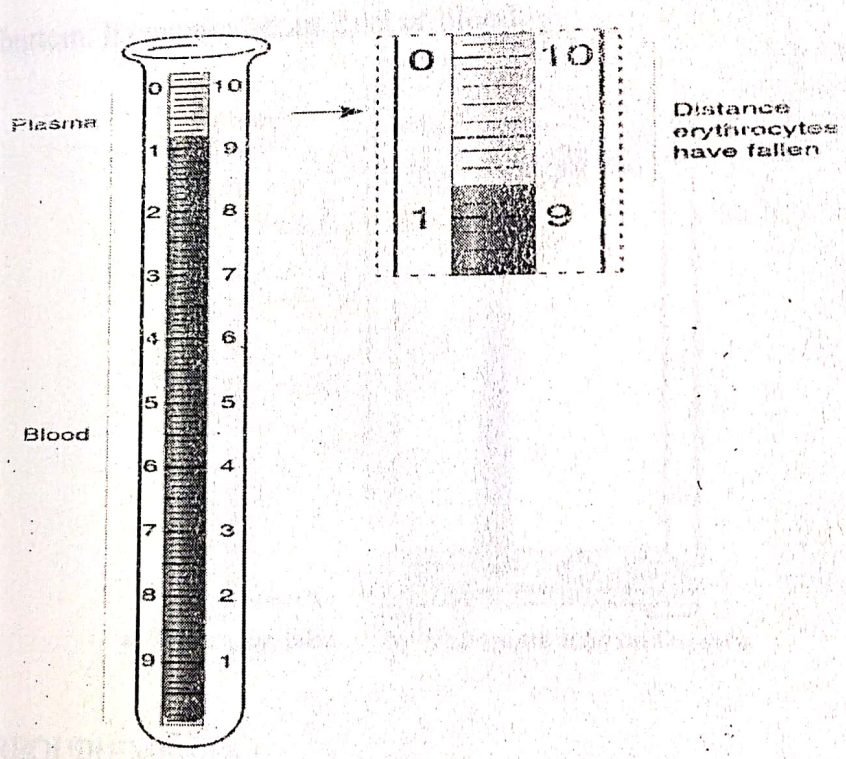
There are two main methods to determine ESR :

1. Wintrobe's method
2. Westergren's method

Each method produces slightly different results. Mosely and Bull (1991) concluded that Wintrobe's method is more sensitive when the ESR is low, whereas, when the ESR is high, the Westergren's method is preferably an indication of patient's clinical state.

WINTROBE'S METHOD:

This method uses Wintrobe's tube, a narrow glass tube closed at the lower end only. The Wintrobe's tube has a **length of 11 cm** and **internal diameter of 2.5 mm**. It contains 0.7-1 ml of blood. The lower 10 cm are in cm and mm. The marking is 0 at the top and 10 at the bottom for ESR. This tube can also be used for PCV. The marking is 10 at the top and 0 at the bottom for PCV.



REQUIREMENTS:

1. Anticoagulated blood (EDTA, double oxalate)
2. Pasteur pipette

PROCEDURE:

- Mix the anticoagulated blood thoroughly.

- By using Pasteur pipette, fill the Wintrobe's tube upto '0' mark. There should be no bubbles in the blood.
- Place the tube vertically in ESR stand and leave undisturbed for 1 hour.
- At the end of 1 hour, read the result.

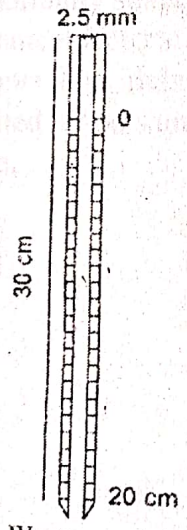
NORMAL VALUE:

For males : 0-9 mm/hr

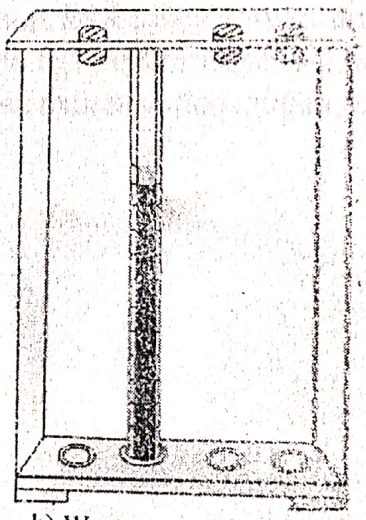
For females 0-20 mm/hr

WESTERGREN'S METHOD:

It is better method than Wintrobe's method. The reading obtain is magnified as the column is lengtheir. The Westregren tube is open at both ends. It is **30 cm in length** and **2.5 mm in diameter**. The lower 20 cm are marked with 0 at the top and 200 at the buttom. It contains about 2 ml of blood.



a) Westergren tube



b) Westergren tube on the rack

REQUIREMENTS :

1. Anticoagulated blood (0.4 ml of 3.13% trisodium citrate solution + 1.6 ml blood)
2. Westergren tube
3. Westergren stand
4. Rubber bulb (sucker)

PROCEDURE :

- Mix the anticoagulated blood thoroughly.
- Draw the blood into the tube upto 0 mark with the help of rubber bulb.
- Wipe out blood from bottom of the tube with cotton.

- Set the tube upright in stand. Make sure the pipette fits snugly to eliminate possible leakage and that the pipette is in vertical position.
- Leave the tube undisturbed for 1 hour.
- At the end of 1 hour, read the result.

NORMAL VALUE:

For males: 0-10 mm/hr

For females: 0-15 mm/hr

Some interferences which increase ESR:

- increased level of fibrinogen, gamma globulins.
- technical factors: tilted ESR tube, high room temperature.

Some interferences which decrease ESR:

- abnormally shaped RBC (sickle cells, spherocytosis).
- technical factors: short ESR tubes, low room temperature, delay in test performance (>2 hours), clotted blood sample, excess anticoagulant, bubbles in tube.

PACKED CELL VOLUME

When anti-coagulated blood is centrifuged at a standard speed, erythrocytes, which are heavier than white cells and plasma, will settle down at bottom. This red cells volume is known as Haematocrit or Packed Cell Volume (PCV).

Haematocrit or PCV is the volume of red cells expressed as a percentage of whole blood.

Methods:

There are two methods, used for the determination of haematocrit:

1. Macrohaematocrit

2. Microhaematocrit

Macrohaematocrit:

A large volume of blood is required in this method. Approximately 2 to 4 ml is required.

Principle:

Anticoagulated blood is taken in a Wintrobe tube. Fill upto the uppermost mark and then rotate for desired length of time.

The packed cell volume (PCV) of red cells is directly read from the graduated tube as %.

Requirement:

1. Blood specimen:

EDTA or double oxalated anti-coagulated blood is used in this method. Determine P.C.V. within six hr. of blood collection.

2. Wintrobe Tube:

It is 110 mm in length and 2.5 mm in diameter. The lower 100 mm are graduated or marked, from 100 at top and 0 (zero) at bottom for PCV.

3. Long necked pasture pipette or a special type of syringes is used for filling Wintrobe tube.

4. Centrifuge machine with known speed.

Procedure:

Mix 0.4 ml of EDTA with 2 ml blood. Fill the Wintrobe tube upto upper most mark with the help of pasture pipette or syringe. Fill the another Wintrobe tube to balance first one. If the blood sample is not available, fill the tube with water.

Place the Wintrobe tube in opposite side in centrifuge. Turn the centrifuge to slow speed, then slowly increase the speed to 3,000 rpm. Centrifuge for 30 min. at 3,000 rpm. After 30 min. switch off the centrifuge and allow it to stop by itself. Take out the Wintrobe tube and read PCV directly with the help of graduation mark given on the tube.

Normal Value:

i. In male – 42 to 50%

ii. In female – 36 to 38%

Microhaematocrit:

This method requires small amount of blood, 2 to 3 drops only. The blood can be obtained by finger puncture.

Principle:

Anti-coagulated blood is centrifuged in a sealed capillary tube, and then PCV is determined by a special haematocrit reader

Requirement

1. Blood Specimen:

Blood from finger puncture may be used or EDTA or double oxalate venous blood can also be used.

2. Capillary Tube:

Use plain capillary tube for anti-coagulated venous blood and use heparinised capillary tube (Coated with heparin internally) for blood obtained from finger puncture. The capillary tube is approximately 75 mm in length.

3. Microhaematocrit Centrifuge:

This is a special type of centrifuge. It has speed about 15,000 r.p.m. The top of centrifuge is flat with grooves. The centrifuge also has timer, which is usually set for 5 min.

4. Haematocrit Reader:

There are several types of readers used for reading hct. The simplest method is use of card reader, which can be made by hand.

5. Clay:

This is used to seal the end of capillary tube.

Procedure:

Draw the blood sample into appropriate capillary tube with capillary action. Use plain tube for anti-coagulated blood and heparinised tube for plain blood. In case of finger puncture, the blood should flow freely with little pressure. Now wipe off the first drop and then collect the blood specimen.

Fill the tube about 3/4th length with blood. Seal the another end of the tube with clay or wax or ultimately by heating. The sealing should be about 2 mm deep. Place two hct tubes in the groove of centrifuge exactly opposite to each other.

It is not necessary that the capillary tube have exact amount of blood level. In case, if there is no filled capillary tube to balance we can use an empty capillary tube. Centrifuge at 13,000 ± 2000 rpm.

Remove capillary tube from centrifuge. It will show three layers. Top layer is of plasma or serum; the middle layer is thin creamy white in colour and is known as Buffy coat. It is a layer of WBC; the last layer is the column of RBC. Use the hct reader for finding the value of hct.

Card Reader:

The reader is used as, hold the tube against Scale so that the bottom of red cell is matched with 0 (zero) line of the card. Move the tube across the card until the uppermost line of plasma is matched to 100% line of card.

Check to make sure that bottom of red cell column is still in the line of zero and the tube should be straight and vertical. The line that passes through the top of the column of RBC gives the hct. Value.

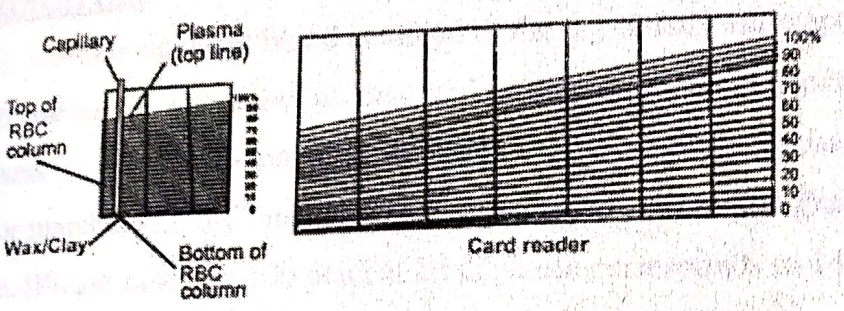


Fig. 10.1: Card reader

Importance:

Decrease in PCV is suitable measure for anaemia. The fall in hct may be seen in decreased oxygen supply to cells, heart disease or malignant condition, hct also rise in case of dehydration.

ESTIMATION OF HAEMOGLOBIN

AIM: To determine the haemoglobin in the blood

PRINCIPLE:

Anticoagulated blood is added to the 0.1 N HCl and kept for 5-7 minutes to form acid haematin. The color of this acid haematin should be matched with the solution, present in the calibration tube. Distilled water is added to the acid haematin until the color matches and the final reading is directly noted from the graduation in the calibration tube. [Please note that 100 percent on the scale corresponds to 14.5gm % to 15gm %].

REQUIREMENTS: Sahli's haemoglobinometer, Hydrochloric acid, distilled water.

PROCEDURE:

Place N/10 HCL in diluting tube up to the mark 20. Take blood in the haemoglobin pipette up to 20-cubic-mm-mark and blow it into diluting tube and rinse well. After 10 minutes add distilled water in drops and mix the tube until it has exactly the same color as the comparison standards. Note the reading, which indicates the percentage of haemoglobin.

RESULT:

The Hb estimation of the given sample is ..... g/100 ml of blood/.....g/dl of blood/.....G%.

EXTRACTION.SEPARATION AND DETERMINATION OF ABSORPTION SPECTRA OF PLANT PIGMENT

PURPOSE

In this experiment, the photosynthetic pigments common to all flowering plants will be extracted by liquidliquid extraction. The four main pigment components of plant leaves are chlorophyll a, chlorophyll b, carotene, and xanthophyll. The solvents needed for the extraction and separation will be petroleum ether, diethyl ether, methanol, and water. Finally, these pigments will be analyzed by measuring the absorption spectra of the plant pigments.

BACKGROUND

The four main pigment components of plant leaves are chlorophyll a, chlorophyll b, carotene, and xanthophyll. While most plant leaves appear green to our eyes, several different photosynthetic pigments of various colors are usually present in the chloroplast of green plants. The colors of these pigments are due to the numerous double bonds in their structure. The green color of leaves is provided by chlorophyll a and b, which absorb most of the light energy needed for photosynthesis to occur. The greatest absorption of light by chlorophyll is in the red, blue and violet wavelengths. Associated with these green pigments are several other pigments known as carotenoids. These are commonly yellow to red in color, and are also involved in harvesting light energy for photosynthesis.

A plant pigment mixture has been prepared in the stockroom from spinach leaves that have been pulverized in a blender with 80% acetone. Take care not to spill this solution. It is a concentrated chlorophyll solution and will produce a very nasty green stain on anything it comes in contact with. Please was all glassware with soap as soon as possible to avoid permanent green strains.

0. Work in pairs. Only two individuals to a group. Start with one pigment mixture sample and begin by sharing one 125 mL separatory funnel. Take turns extracting as outlined in the flow chart of procedures up to juncture "A" and "B." At the A" and "B" juncture, the mixture is split between partners. One partner will complete the extraction

in his/her own 125 mL separatory funnel for the isolation of chlorophyll a and xanthophyll in "A", and the other partner will complete the isolation of chlorophyll b and carotene in "B" in their separatory funnel. Finally, individuals will run one visible absorption analyses for each pigment isolated for a total of two spectra per individual. Partners will be responsible for exchanging and sharing graphical data gathered in the spectroscopic analysis.

If there is an odd number of students in the laboratory, a single individual will complete all work up to the juncture "A" and "B" shown in the flow chart scheme of the procedures. At this juncture, he or she will make a choice; complete the isolation and analysis for part A, or for part B. Finally, the individual will need to acquire the missing graphical data for the part he/she did not complete from someone in the classroom.

1. Obtain a 1 mL plant pigment mixture sample in a 10 mL beaker.
2. Transfer the 1 mL plant pigment mixture sample to a 125 mL separatory funnel.
3. Measure 20 mL of acetone in an Erlenmeyer flask. Rinse the 10 mL beaker of residual pigment with small portions of this acetone until all 20 mL of acetone have been transferred to the separatory funnel.
4. Carefully follow the directions outlined in the flow chart for the separation of chlorophyll a, chlorophyll b, carotene, and xanthophyll.
5. At juncture "A" and "B," split the mixture. One partner completes the isolation and runs the absorption spectrum of chlorophyll a and carotene, the other will complete the same for chlorophyll b and xanthophyll.

SEPARATION OF PLANT PIGMENT BY COLUMN CHROMATOGRAPHY (DEMO)

Column chromatography is a technique which can be applied to the separation of many complex mixtures. The sample solution is applied to the top of the column. The mobile phase flows down through the column filled with the stationary phase material.

The green leaves of plants contain a number of pigments viz: chlorophyll-a, chlorophyll-b, xanthophylls and carotenes. You will learn the separation of these pigments by column chromatography in this experiment.

PRINCIPLE:

The success of a separation by column chromatography depends on the choice of the stationary and mobile phases. The stationary phase material is filled in a column. Any of the three possible mechanisms: partition, adsorption or ion exchange can be employed by the use of a particular type of the stationary phase inside the column. For example, for the separation based on adsorption an adsorbent is packed in the column.

The choice of the mobile phase depends on the nature of the substance and how strongly it is adsorbed. In a number of cases such as alumina and silica gel as the adsorbent, the mobile phase is generally a non-polar solvent such as petrol and benzene because polar groups such as hydroxyl-(OH) group in water and in ethanol would cause desorption. Eluents containing two or more solvents may be used for better results. In such cases the polarity is increased by adding a polar solvent with a non-polar one.

REQUIREMENTS

Apparatus		Chemicals
Chromatography column	1	Calcium carbonate
Glass wool		Anhydrous Sodium Sulphate anhydrous
Cotton wool		
Beaker (100 cm^3)	2	Benzene
Conical flask (250 cm^3)	1	Petroleum ether
Mortar	1	Ethyl alcohol
China dish	1	
Separatory funnel	1	
Graduated cylinder (100 cm^3)	1	
Wash bottle	1	

1) **Preparation of the Extract:** Take 5-10 g of fresh grass (or leaves of a green plant), cut it up into fine pieces in a mortar, grind for about 30 seconds, add 10 cm^3 of ethyl alcohol and 20 cm^3 of petroleum ether, grind again. Decant the liquid into a separatory funnel after filtering through glass wool placed in an ordinary funnel. Add 10 cm^3 alcohol and 20 cm^3 petroleum ether again to the mortar containing grass, grind and transfer the liquid after decantation to the separatory funnel containing the first fraction. Shake gently. A light green emulsion may form, if shaken vigorously. Allow to settle the layers. The bottom layer is water-ethanol layer and the upper layer of petroleum-ether contains grass extract. Remove the bottom layer and wash the petroleum layer with water for 3 or 4 times until the layer is clear. Remove the aqueous layer. The extract is now free from alcohol but contains water in very small amount.

Transfer the upper layer containing the extract to a dry conical flask. To this, add anhydrous sodium sulphate (dried by heating in an oven/hot plate before use), shake the flask and leave it over for about 15 minutes to remove any water present with the extract. Transfer the extract to a clean and dry test tube, cover it and take it for chromatography.

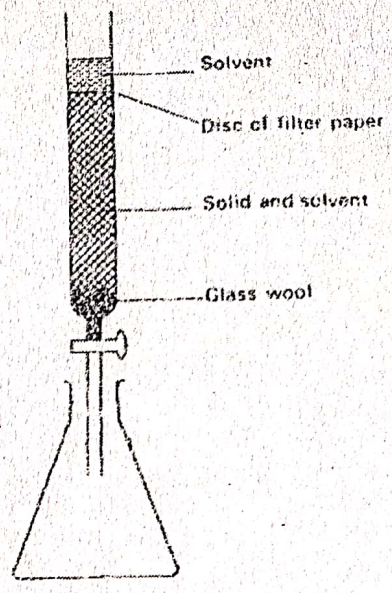
2) **Preparation of column:** Take a glass column or a burette of about 20 cm in length and 7-8 mm diameter tube. Place a small wad of cotton wool as the column support. Pack the column with anhydrous calcium carbonate (dried by heating in a china dish over a burner), tap it regularly with a glass rod. Add the adsorbent in small portions and gently press down until a column of 8-10 cm has been uniformly packed. Place a small wad of cotton wool at the top of the calcium carbonate column and use it for chromatography.

3. Take the uniformly packed column containing calcium carbonate and fix it in a stand vertically.

4. Take 1-2 cm^3 of dried extract of leaves, drip into the column in the form of a thin layer of solution, allow to run evenly into the adsorbent until a green zone 3-4 mm deep is formed at the top of the column. This is known as the loading of the sample.

5. Add the developer (benzene) to the column and allow the developer through the column packing till separate bands are observed.

6. Note the colour of different bands and their order.



Chromatographic column

The physical state of the column packing material should be such that it allows uniform packing of the column and a free flow of the solvent through it.

The extract from green leaves should be completely free from water since the presence of a polar substance can alter the course of development.

7. **If extra time is available, continue the passage of developer and collect the different coloured substances in fractions, noting the volume eluted by a measuring cylinder.**

RESULT

The bands observed on the column are of different colours. The uppermost thin yellowish green zone is chlorophyll-b, below this the bluish green zone of chlorphyll-a, next orange-yellow zone contains xanthopylls and the lowest orange zone contains carotenes. The carotenes are least adsorbed by the adsorbent and can be easily washed out of the column.