AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY
OMR, PAIYANOOR, CHENNAI - 603104

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

ADVANCED BIOCHEMISTRY-LABORATORY MANUAL

LAB CODE: 481171L1

DEPARTMENT OF BIOTECHNOLOGY

AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY

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Paiyanoor, Chennai

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Register No:

Certificate that this is a bonafied record of the work done by _____ of _____ in the _____ laboratory of_____ department.

Staff In- Charge **Head of the Department**

Submitted for the University Practical Examinations held on_____

Internal Examiner **External Examiner**

CONTENTS

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.

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" reagent and heat in boiling water bath.	A reddish brown precipitate is formed	This shows the presence 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.
6.	**SELIWANOFF'S TEST :** To 2.5 mL of the reagent add 5 drops of sugar solution boiled and cooled.	Cherry red colour was formed.	
7.	**OZAZONE TEST:** 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.	This shows the presence of keto sugars (fructose). Presence of glucose or fructose. Presence of lactose. Presence of maltose.
8.	**HYDROLYSIS :** To 5mL of the test solution add 1 mL of hydrochloric acid boiled for 2 min and cooled , neutralized with 20% sodium	Yellow precipitate was formed.	

	carbonate. To this add phenyl hydrazine hydrochloride and sodium acetate and acidified with drops of glacial acetic acid, boiled and cooled.		Presence of sucrose, which is hydrolyzed to glucose and fructose.
9.	**<u>IODINE TEST:</u>** 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 CARBOHYDRATES - I

S. NO	EXPERIMENT	OBSERVATION	INFERENCE
1.	**SOLUBILITY TEST :** : Test the solution in • Acid • Water • Alcohol • Alkali		

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.		
3.	**BENEDICT'S TEST :** To 5mL of the test solution add 5mL of Benedict's reagent and heat in boiling water bath.		
4.	**FEHLING'S TEST :** To 5mL of the test solution add equal volume of Fehling's "A" and "B" reagent and heat in boiling water bath.		
5.	**BARFOED'S TEST :** To 5mL of the test solution add 5mL of Barfoed's reagent and heat in boiling water bath.		
6.	**SELIWANOFF'S TEST :** To 2.5 mL of the reagent add 5 drops of sugar solution boiled and cooled.		
7.	**OZAZONE TEST:**		

	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.		
8.	**HYDROLYSIS :** 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.		
9.	**IODINE TEST:** To 2mL of sugar add few drops of N/50		

	iodine solution.		

QUALITATIVE ANALYSIS OF CARBOHYDRATES - II

S. NO	EXPERIMENT	OBSERVATION	INFERENCE
1.	**SOLUBILITY TEST :** : Test the solution in • Acid • Water • Alcohol • Alkali		

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.		
3.	**BENEDICT'S TEST :** To 5mL of the test solution add 5mL of Benedict's reagent and heat in boiling water bath.		
4.	**FEHLING'S TEST :** To 5mL of the test solution add equal volume of Fehling's "A" and "B" reagent and heat in boiling water bath.		
5.	**BARFOED'S TEST :** To 5mL of the test solution add 5mL of Barfoed's reagent and heat in boiling water bath.		
6.	**SELIWANOFF'S TEST :** To 2.5 mL of the reagent add 5 drops of sugar solution boiled and cooled.		

7.	**OZAZONE TEST:** 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. **HYDROLYSIS :** 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.		
8.	**IODINE TEST:** To 2mL of sugar add few drops of N/50 iodine solution.		

9.			

QUALITATIVE TEST FOR PROTEINS

INTRODUCTION

Amino acids are organic compounds that contain amino and carboxyl groups. The R- in the above formula stands for different chemical groups (may be aliphatic, aromatic or heterocycylic) and this determines the characteristics of the amino acids.The color tests have frequently been used for qualitative detection of amino acids. Not all amino acids contain the same reactive groups. For this reason the various color tests yield reactions varying in intensity and type of color according to the nature of groups contained in the particular amino

acid under examination.

1.MILLON'SREACTION:

Principle

The reaction is due to the presence of the hydroxyphenyl group, C_6H_5OH in the amino acid molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol and thymol will give the reaction. Solutions of nitric acid containing mercuric nitrate reacts with phenols, producing red colors or yellow precipitates which react with nitric acid to form red solution. The reaction probably depends on the formation of a nitro compound; which then reacts with phenol.

Materials

Millon's reagent

Method

Add 3 to 4 drops of Millon's reagent to 5 ml of test solution. Mix and bring the mixture gradually to a boiling point by heating over a small flame. Development of red color is due to the presence of protein. Excess of reagent should however be avoided since it may produce a yellow color which is not a positive reaction.

Materials

1.Millon-Nasse reagent

2. 1% $NaNO_2$

Method

Add 1 ml of Millon-Nasse reagent to 5 ml of test solution. Place the tube in a boiling water bath for 10 mins. and cool the contents in water bath for 5 to 10 mins. and add 1 ml of 1% $NaNO_2$. A deep red color indicates tyrosine or other 3,5 unsubstitued phenol.

2. XANTHOPROTEIC REACTION:

Principle

This reaction is due to the presence in the amino acid molecule of the phenyl group $–C_6H_5$, with which the nitric acid forms certain nitro modifications. The particular amino acids which are of especial importance in this connection are those of tyrosine and tryptophan.

Phenylalanine does not respond to this test as it is ordinarily preformed.

Materials

1. Conc. HNO_3
2. Ammonium hydroxide
3. Sodium hydroxide

Method

Add 1 ml of conc. Nitric acid to 2 to 3 ml of test solution in a test tube. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color, cool the solution and carefully add ammonium hydroxide or sodium hydroxide in excess. Note that the yellow color deepens into an orange.

3. HOPKINS-COLE REACTION:

Principle

The formation in this test color is due to the presence of indoyl group. Gelatin does not respond to this test due to lack of amino acid tryptophan. Violet to blue colors develop when a mixture of protein and an aldehyde is layered over conc. sulphuric acid. A number of tests based on this principle have been suggested; all depends on the presence of the indoly group of tryptophan which reacts as follows (using glyoxylic acid as an example of an aldehyde).

This is called Hopkin-Cole test- A similar test was at one time recommended for detection of formaldehyde that had been as a preservative to milk, the formaldehyde reacting with indolyl groups of milk proteins to give a color.

Materials

1. Hopkin-Cole reagent
2. Conc. H_2SO_4

Method

Place 2 to 3 ml of test solution and an equal volume of Hopkins-Cole reagent in a test tube and mix thoroughly. Incline the tube and permit 5 to 6 ml of conc. sulphuric acid to flow slowly down the side of the tube, thus forming a sharp layer of acid beneath the amino acid solution. When stratified in this manner a reddish-violet color forms at the zone of contact of

the two fluids. If the color does not appear after starting for a few minutes, the tube may be rocked gently to cause a slight mixing of the liquids are mixed by gentle stirring the precipitate of protein dissolves and the violet color spread throughout the solution.

4. BIURET TEST:

Principle

The Biuret test is given by those substances whose molecules contain two cabamyl (-$CONH_2$) groups joined either directly or through a single atom of nitrogen or carbon. Similar substances which contain- $CSNH_2$,
-$C(NH)NH_2$, or – CH_2NH_2 in place of the –$CONH_2$ group also respond to the test. It follows from this fact that substance which are non-protein in character but which contain the necessary groups will respond to the biuret test.
Protein responds positively since there are pairs of CONH groups in the molecule. A copper coordination complex with the ring structure is probably produced. Short chain polypeptides give a pinkish violet color, longer one including proteins a more purple blue. The amino acid histidine gives a pink color, which depends on the peptide linkage, does not vary greatly in intensity from protein to protein. Several procedure based on this method have been suggested for quantitative determination of milk proteins, but it is not in general use in dairy research.

Materials

1. 10% NaOH
2. 0.5% $CuSO_4$

Method

To 2 to 3 ml of test solution in a test tube add an equal volume of 10% sodium hydroxide solution, mix thoroughly, and add a 0.5% copper sulphate solution drop by drop, mixing between drops, until a purplish-violet or pinkish-violet color is produced. The color depends upon the nature of the protein, proteoses and peptones give a decided pink; the color produced with gelatin is not far removed from a blue.

5. NINHYDRIN REACTION:

Principle

This test gives positive results with proteins, peptones, peptides, amino acids and other

primary amines, including ammonia. Proline and hydroxyproline give yellow color with ninhydrin, while other acids give blue to purple color.

Materials

1. 0.1% Ninhydrin
2. pH paper

Method

To 5 ml of dilute test solution, which must be approximately between pH 5 and pH 7 (a few drops of pyridine or a few crystals of sodium acetate may be used to adjust the pH), add 0.5 ml of 0.1 % ninhydrin, heat to boiling for one to two minutes, and allow to cool. A blue color develops if the test is positive.

6. FOLIN TEST:

A phosphomolybdotungstic acid reagent designed by Folin for phenol has been widely used for detection and analysis of indolyl and phenol groups in amino acids. A characteristic blue color is formed when amino acid solution is warmed with this reagent. The color so formed is due to the reaction of alkaline copper with the amino acid and the reduction of phosphomolybdate by tyrosine and tryptophan present.

Materials

1. Alkaline Na_2CO_3 solution (2% in 0.1 N NaOH)
2. $CuSO_4$-Na; K tartarate solution (0.5 % $CuSO_4$) in 1 % Na, K tartarate) prepared fresh by mixing stock solutions.
3. “Alkaline solution” (prepared by mixing 50 ml of the reagent (1) and 1 ml of the reagent (2)).
4. Folin-Ciocalteau reagent

Method

Add 5ml of the alkaline solution to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 mins. Add 0.5 ml diluted Folin-Ciocalteau reagent rapidly with immediate mixing. Observe for development of color after 30 mins. Development of characteristic blue color indicates presence of indolyl or phenol group.

7. SAKAGUCHI TEST:

Principle

Arginine and other guanidyl derivatives (glycocyamine, methylgyanidine etc) react with hypo bromide and alpha napthol to give a red colored product.

Materials

1. Sodium hydroxide solution (40%)
2. Alpha napthol solution (1% in alcohol)
3. Bromine water (a few drops of bromine in 100 ml distilled water)

Method

Mix 1 ml of sodium hydroxide with 3 ml of test solution and add 2 drops of alpha napthol. Mix thoroughly and add 4 to 5 drops of bromine water. Note the color formed. Formation of a red color indicates presence of guanidine group. This is a very sensitive and specific test.

8. NITROPRUSSIDE TEST:

Principle

Sodium nitroprusside reacts with compounds containing sulphahydryl groups produce an intensely red but somewhat unstable color.

Materials

1. Sulphur amino acids (1.0% cystine, cysteine and methionine)
2. Sodium nitroprusside (2% prepared fresh)
3. Ammonium hydroxide

Method

Mix 0.5 ml of a fresh solution of sodium nitropruside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide

QUALITATIVE TEST FOR PROTEINS- I

1.MILLON'S REACTION:

Principle

The reaction is due to the presence of the hydroxyphenyl group, C_6H_5OH in the amino acid molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol and thymol will give the reaction. Solutions of nitric acid containing mercuric nitrate reacts with phenols, producing red colors or yellow precipitates which react

with nitric acid to form red solution. The reaction probably depends on the formation of a nitro compound; which then reacts with phenol.

Materials

Millon's reagent

Method

Add 3 to 4 drops of Millon's reagent to 5 ml of test solution. Mix and bring the mixture gradually to a boiling point by heating over a small flame.

Materials

1.Millon-Nasse reagent

2. 1% $NaNO_2$

Method

Add 1 ml of Millon-Nasse reagent to 5 ml of test solution. Place the tube in a boiling water bath for 10 mins. and cool the contents in water bath for 5 to 10 mins. and add 1 ml of 1% $NaNO_2$.

2. XANTHOPROTEIC REACTION:

Principle

This reaction is due to the presence in the amino acid molecule of the phenyl group $–C_6H_5$, with which the nitric acid forms certain nitro modifications. The particular amino acids which are of especial importance in this connection are those of tyrosine and tryptophan.
Phenylalanine does not respond to this test as it is ordinarily preformed.

Materials

1. Conc. HNO_3
2. Ammonium hydroxide
3. Sodium hydroxide

Method

Add 1 ml of conc. Nitric acid to 2 to 3 ml of test solution in a test tube. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a

yellow color, cool the solution and carefully add ammonium hydroxide or sodium hydroxide in excess.

3. HOPKINS-COLE REACTION:

Principle

The formation in this test color is due to the presence of indoyl group. Gelatin does not respond to this test due to lack of amino acid tryptophan. Violet to blue colors develop when a mixture of protein and an aldehyde is layered over conc. sulphuric acid. A number of tests based on this principle have been suggested; all depends on the presence of the indoly group of tryptophan which reacts as follows (using glyoxylic acid as an example of an aldehyde).

This is called Hopkin-Cole test- A similar test was at one time recommended for detection of formaldehyde that had been as a preservative to milk, the formaldehyde reacting with indolyl groups of milk proteins to give a color.

Materials

1. Hopkin-Cole reagent
2. Conc. H_2SO_4

Method

Place 2 to 3 ml of test solution and an equal volume of Hopkins-Cole reagent in a test tube and mix thoroughly. Incline the tube and permit 5 to 6 ml of conc. sulphuric acid to flow slowly down the side of the tube, thus forming a sharp layer of acid beneath the amino acid solution.

4. BIURET TEST:

Principle

The Biuret test is given by those substances whose molecules contain two cabamyl (-$CONH_2$) groups joined either directly or through a single atom of nitrogen or carbon. Similar substances which contain- $CSNH_2$,

-$C(NH)NH_2$, or – CH_2NH_2 in place of the –$CONH_2$ group also respond to the test. It follows from this fact that substance which are non-protein in character but which contain the necessary groups will respond to the biuret test.

Protein responds positively since there are pairs of CONH groups in the molecule. A copper coordination complex with the ring structure is probably produced. Short chain polypeptides give a pinkish violet color, longer one including proteins a more purple blue. The amino acid histidine gives a pink color, which depends on the peptide linkage, does not vary greatly in intensity from protein to protein. Several procedure based on this method have been suggested for quantitative determination of milk proteins, but it is not in general use in dairy research.

Materials

1. 10% NaOH
2. 0.5% $CuSO_4$

Method

To 2 to 3 ml of test solution in a test tube add an equal volume of 10% sodium hydroxide solution, mix thoroughly, and add a 0.5% copper sulphate solution drop by drop, mixing between drops, until a purplish-violet or pinkish-violet color is produced.

5. NINHYDRIN REACTION:

Principle

This test gives positive results with proteins, peptones, peptides, amino acids and other primary amines, including ammonia. Proline and hydroxyproline give yellow color with ninhydrin, while other acids give blue to purple color.

Materials

1. 0.1% Ninhydrin
2. pH paper

Method

To 5 ml of dilute test solution, which must be approximately between pH 5 and pH 7 (a few

drops of pyridine or a few crystals of sodium acetate may be used to adjust the pH), add 0.5 ml of 0.1 % ninhydrin, heat to boiling for one to two minutes, and allow to cool.

6. FOLIN TEST:

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Materials

1. Alkaline Na_2CO_3 solution (2% in 0.1 N NaOH)
2. $CuSO_4$-Na; K tartarate solution (0.5 % $CuSO_4$) in 1 % Na, K tartarate) prepared fresh by mixing stock solutions.
3. "Alkaline solution" (prepared by mixing 50 ml of the reagent (1) and 1 ml of the reagent (2)).
4. Folin-Ciocalteau reagent

Method

Add 5ml of the alkaline solution to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 mins. Add 0.5 ml diluted Folin-Ciocalteau reagent rapidly with immediate mixing. Observe for development of color after 30 mins.

7. SAKAGUCHI TEST:

Principle

Arginine and other guanidyl derivatives (glycocyamine, methylgyanidine etc) react with hypo bromide and alpha napthol to give a red colored product.

Materials

1. Sodium hydroxide solution (40%)
2. Alpha napthol solution (1% in alcohol)
3. Bromine water (a few drops of bromine in 100 ml distilled water)

Method

Mix 1 ml of sodium hydroxide with 3 ml of test solution and add 2 drops of alpha napthol. Mix thoroughly and add 4 to 5 drops of bromine water. Note the color formed.

8. NITROPRUSSIDE TEST:

Principle

Sodium nitroprusside reacts with compounds containing sulphahydryl groups produce an intensely red but somewhat unstable color.

Materials

1. Sulphur amino acids (1.0% cystine, cysteine and methionine)
2. Sodium nitroprusside (2% prepared fresh)
3. Ammonium hydroxide

Method

Mix 0.5 ml of a fresh solution of sodium nitropruside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide

QUALITATIVE TEST FOR PROTEINS- II

1.MILLON'S REACTION:

Principle

The reaction is due to the presence of the hydroxyphenyl group, C_6H_5OH in the amino acid molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol and thymol will give the reaction. Solutions of nitric acid containing mercuric nitrate reacts with phenols, producing red colors or yellow precipitates which react

with nitric acid to form red solution. The reaction probably depends on the formation of a nitro compound; which then reacts with phenol.

Materials

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Method

Add 3 to 4 drops of Millon's reagent to 5 ml of test solution. Mix and bring the mixture gradually to a boiling point by heating over a small flame.

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Add 1 ml of Millon-Nasse reagent to 5 ml of test solution. Place the tube in a boiling water bath for 10 mins. and cool the contents in water bath for 5 to 10 mins. and add 1 ml of 1% $NaNO_2$.

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This reaction is due to the presence in the amino acid molecule of the phenyl group $–C_6H_5$, with which the nitric acid forms certain nitro modifications. The particular amino acids which are of especial importance in this connection are those of tyrosine and tryptophan.
Phenylalanine does not respond to this test as it is ordinarily preformed.

Materials

1. Conc. HNO_3
2. Ammonium hydroxide
3. Sodium hydroxide

Method

Add 1 ml of conc. Nitric acid to 2 to 3 ml of test solution in a test tube. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a

yellow color, cool the solution and carefully add ammonium hydroxide or sodium hydroxide in excess.

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Principle

This test gives positive results with proteins, peptones, peptides, amino acids and other primary amines, including ammonia. Proline and hydroxyproline give yellow color with ninhydrin, while other acids give blue to purple color.

Materials

1. 0.1% Ninhydrin
2. pH paper

Method

To 5 ml of dilute test solution, which must be approximately between pH 5 and pH 7 (a few

drops of pyridine or a few crystals of sodium acetate may be used to adjust the pH), add 0.5 ml of 0.1 % ninhydrin, heat to boiling for one to two minutes, and allow to cool.

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Materials

1. Alkaline Na_2CO_3 solution (2% in 0.1 N NaOH)
2. $CuSO_4$-Na; K tartarate solution (0.5 % $CuSO_4$) in 1 % Na, K tartarate) prepared fresh by mixing stock solutions.
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4. Folin-Ciocalteau reagent

Method

Add 5ml of the alkaline solution to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 mins. Add 0.5 ml diluted Folin-Ciocalteau reagent rapidly with immediate mixing. Observe for development of color after 30 mins.

7. SAKAGUCHI TEST:

Principle

Arginine and other guanidyl derivatives (glycocyamine, methylgyanidine etc) react with hypo bromide and alpha napthol to give a red colored product.

Materials

1. Sodium hydroxide solution (40%)
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3. Bromine water (a few drops of bromine in 100 ml distilled water)

Method

Mix 1 ml of sodium hydroxide with 3 ml of test solution and add 2 drops of alpha napthol. Mix thoroughly and add 4 to 5 drops of bromine water. Note the color formed.

8. NITROPRUSSIDE TEST:

Principle

Sodium nitroprusside reacts with compounds containing sulphahydryl groups produce an intensely red but somewhat unstable color.

Materials

1. Sulphur amino acids (1.0% cystine, cysteine and methionine)
2. Sodium nitroprusside (2% prepared fresh)
3. Ammonium hydroxide

Method

Mix 0.5 ml of a fresh solution of sodium nitropruside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide

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 LIPIDS -I

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QUALITATIVE ANALYSIS OF URINE FOR NORMAL CONSTITUENTS

Composition of Normal Urine

- ✓ **Water 96%**
- ✓ **Urea 2%**
- ✓ **Uric acid, Creatinine, Ammonia ,Sodium,Potassium , Chloride, Phosphate, Sulphate , oxalate – 2%**

Urine is an excretory product of the body. It is formed in the kidney. Urine examination helps in the diagnosis of various renal as well as systemic diseases.

URINE COLLECTION • Urine is usually collected in a sterile wide mouthed container.

Different method of urine collection are

FIRST MORNING SAMPLE (conc urine for biochemical analysis, casts and crystals)

RANDOM SAMPLE (chemical screening, microscopic examination)

24 HOUR URINE SAMPLE (quantitative estimation of proteins, sugars, electrolytes, hormones)

MID STREAM URINE – CLEAN CATCH URINE

SAMPLE PRESERVATION :

For determination of urea, ammonia, nitrogen and calcium – Hydrochloric acid is used. • For determination of sodium, potassium, chloride, bicarbonate, calcium, phosphorus, urea, ammonia, amino acids, creatinine, proteins, reducing substances and ketone bodies - Thymol is used • For determination of Ascorbic acid – Acetic acid is used • Toluene may also be used as a preservative

COMPOSITION OF NORMAL URINE

1. VOLUME • Normal volume of urine excreted per day by normal subjects is 1000-2000 ml/day.

   Night urine output < 400ml.

   Factors which influence the volume excreted : – Intake of fluid, proteins and salt. – Excessive perspiration and strenuous exercise decrease the volume of urine.

2. COLOR • Normal urine is pale yellow (due to presence of pigment urochrome) • When the output of urine is low it appears deep yellow. • Freshly voided urine is clear and transparent. • Long standing urine may become turbid. (Due to precipitation of phosphates)

3. ODOUR : Normal odour of urine is aromatic in nature. • Long standing urine may have an ammoniacal odour (due to decomposition of urea to ammonia).
4. PH : Normal PH of urine ranges from 5.5 – 6.5. Factors which influence PH : – High protein diet makes the urine more acidic – Diet rich in vegetables and fruits make the urine more alkaline. Measured using Dipsticks, Litmus Paper or Ph paper.
5. SPECIFIC GRAVITY :

Specific gravity indicates the concentrating ability of the kidneys.Normal range : 1.012 – 1.024 • Measured using urinometer, refractometer, dipsticks.

Sp. Gravity is affected by –Volume of Urine excreted, amount of solids present in the urine.

6. Urinometer/ Hydrometer consist of a thin stem graduated from 1000 to 1060 corresponding to specific gravities of 1.0 to 1.06. The bulb at the bottom is suitably weighed. Urinometer is calibrated at 60 deg F (15 deg C)
7. METHOD OF DETERMINATION OF SPECIFIC GRAVITY:
   1. Take sufficient urine in a container.
   2. Allow the urinometer to float in urine without touching the sides.
   3. Observe the specific gravity reading corresponding to the meniscus of urine.
   4. Note the temperature of urine using a thermometer.
   5. Temperature correction is done for specific gravity.
8. TEMPERATURE CORRECTION Observed sp. Gravity = 1010 Observed Room Temperature = 37 deg C Temp of Urine = 15 deg C Corrected Temperature = 37-15/3 = 7 Calibrated Sp. Gravity = 1010 + 7 = 1017 = 1.017

CHEMICAL CONSTITUENTS

NORMAL INORGANIC CONSTITUENTS

9. CHLORIDE • Normal chloride excreted in 24 hr urine sample is 8 – 15 g/ day(NaCl). Increased levels decreased levels polydipsia excessive sweating, fasting, diarrhoea, vomiting use of diuretics diabetes insipidus addison's disease infections cushing's syndrome

TEST FOR CHLORIDE METHOD – Take 2 ml of urine + 0.5 ml of conc. HNO_3 + 1ml $AgNo_3$.

OBSERVATION – Curdy white precipitate

INFERENCE – Chloride is precipitated as AgCl with $Ag\ No_3$ in presence of HNO_3.

10. SULPHATE :

    Daily excretion : 1g/ day • Sulphur is derived from catabolism of sulphur containing amino acids. Increased levels decreased levels homocysteinuria renal impairment high protein diet cystinuria

METHOD – 2ml urine + 2ml Barium Chloride OBSERVATION – White precipitate

INFERENCE – Sulphate is precipitated as barium sulphate with barium chloride.

11. CALCIUM :

Daily excretion – 200mg/day INCREASED LEVELS DECREASED LEVELS Hyper parathyroidism tetany renal stones hypervitaminosis d multiple myeloma

METHOD 5ml urine + 5 drops of 1% acetic acid + 5ml of potassium oxalate.

OBSERVATION: Trace amount of white precipitate

INFERENCE : Calcium is precipitated as calcium oxalate in acid medium.

12. INORGANIC PHOSPHATE • Daily excretion : 1gm/ day

    Increased levels decreased levels rickets / osteomalacia diarrhoea hyperparathyroidism nephritis acidosis parathyroid hypofunction pregnancy

    METHOD 5ml urine + few drops of conc HNO3 + a pinch of ammonium molybdate. Warm.

    OBSERVATION : Canary yellow precipitate.

    INFERENCE : Inorganic phosphate is precipitated as canary yellow ammonium phosphomolybdate.

13. AMMONIA :

    Daily excretion : 0.5-0.8gm/ day • Urinary ammonia is derived from glutamine and other amino acids by kidney. Increased levels decreased levels ingestion of acid forming foods alkalosis diabetic ketoacidosis nephritis urinary tract infection

    METHOD 5ml urine + 2% sodium carbonate (till the red litmus turns blue). Boil. During boiling, hold a piece of moistened red litmus paper at the mouth of the test tube.

    OBSERVATION: Red litmus turns blue

    INFERENCE: Ammonia liberated turns red litmus blue.

NORMAL ORGANIC CONSTITUENTS

14. UREA :

    Daily excretion : 20-30 gm/day • Urea is formed in the liver. • End product of protein metabolism. Increased levels decreased levels fever liver disease diabetes mellitus metabolic / respiratory acidosis increased intake of protein nephritis

    SODIUM HYPOBROMITE TEST

    METHOD 2ml urine + Few drops of sodium hypobromite solution.

    OBSERVATION : Brisk effervescence

INFERENCE : Hypobromite decomposes urea to give nitrogen gas.

SPECIFIC UREASE TEST METHOD

Label two test tubes as ‘TEST’ and ‘CONTROL’ Into TEST : 5ml urea solution + 2ml well mixed urease suspension. Into CONTROL : 5ml urea solution + 2ml urease suspension which is heated strongly. Incubate both TEST and CONTROL at room temperature for 15- 20mins. At the end, add 2 drops of phenolpthalein to both the tubes.

OBSERVATION In TEST : Pink Color In CONTROL : No pink color

INFERENCE Urease converts urea into ammonia and carbonic acid. Under the Ph of the reaction condition, ammonia and carbonic acid are converted to ammonium carbonate, Ph goes above 9.5. Since the content are alkaline, phenolphthalein gives pink color.

15. URIC ACID :

Daily excretion : 0.6-1gm/ day • End product of purine metabolism. • Increased levels of uric acid in urine is Uricosuria increased levels decreased levels leukemia purine free diet cancers gout

16. PHOSPHOTUNGSTIC ACID REDUCTION

TEST METHOD : 2ml urine + few drops of phosphotungstic acid + few drops of 20% sodium carbonate.

OBSERVATION Blue color is formed

INFERENCE Uric acid is a reducing agent in strong alkaline condition. It reduces colorless phosphotungstic acid to tungsten blue.

SCHIFF’S TEST METHOD Wet a piece of filter paper with a few drops of ammoniacal silver nitrate solution. Add 1 or 2 drops of uric acid solution on the same paper.

OBSERVATION: Black color is formed

INFERENCE: Uric acid reduces ammoniacal silver nitrate to metallic silver which is black in color.

17. CREATININE :

Daily excretion : 2gm/day in males, 1gm/day in females. • Urinary creatinine is formed from muscle creatine. Increased levels decreased levels muscular disorders renal failure myasthenia gravis thyrotoxicosis starvation uncontrolled diabetes mellitus

METHOD : Label 2 test tubes as ‘test’ and ‘control’. Into Test : 2ml urine + 2ml saturated picric acid+ few drops of 10% NaOH Into Control : 2ml water + 2ml saturated picric acid+ few drops of 10% NaOH OBSERVATION ‘TEST’ – Orange Color, ‘CONTROL’ – Yellow Color

INFERENCE :Creatinine reacts with alkaline picrate solution to form orange creatinine picrate.

ETHEREAL SULPHATE : 100mg of organic sulphate is excreted per day.

TEST FOR DETECTING ETHEREAL SULPHATE METHOD 1ml urine + 2ml BaCl2 + 2ml concentrated HCl. Mix and filter. Divide the filtrate into two portions. Boil one and compare with the other (control)

OBSERVATION : Trace turbidity compared to that in control (red color may also be seen)

INFERENCE : This test is done after removing the inorganic sulphate. Conc Hcl hydrolyses ethereal sulphate to inorganic sulphate which then gives precipitate with BaCl2.

18. UROBILINOGEN: Increased urobilinogen concentration in urine is a sensitive index of liver dysfunction or hemolytic disorder. • Urobilinogen is present in excessive amount in prehepatic and hepatic jaundice. • Urobilinogen is absent in urine in post hepatic jaundice

    METHOD 5ml of freshly voided urine + 1ml Ehrlich reagent, mix. Let stand for 5 mins.

    OBSERVATION Red color is seen when viewed through the mouth of the test tube.

    INFERENCE Urobilinogen reacts with p-dimethyl amino benzaldehyde of the reagent to give red color

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	I
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 = Test O.D x Concentration of standard x 100
Standard O.D 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 = _µg x100
Dilution factor

RESULT:

The amount of Glucose 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:

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 = $\frac{\text{mg} \times 100}{0.05}$

RESULT:

The amount of Cholesterol present in the given sample

i. . By calculation:
ii. By graph:

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

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

RESULT:

The amount of protein present in the given sample

i. By calculation:
ii. By graph:

ESTIMATION OF DNA BY DIPHENYLAMINE METHOD

AIM:

To estimate the amount of DNA present in the given sample

PRINCIPLE:

When DNA is treated with diphenylamine in acid condition a blue compound is formed at 595nm.This reaction is given by 2 deoxypentose in general and is not specific for DNA. In acid solution the straight chain form of deoxy pentose is converted to highly reactive β hydroxy levulaldehyde, which react with diphenylamine to give blue complex. In DNA only the deoxy ribose of the purine nucleotide react, so that the value obtain represent the half of the total deoxy ribose present.

MATERIALS REQUIRED:

1. Test tubes
2. Standard flask
3. Pipette
4. Spectrophotometer
5. Water bath

REAGENTS REQUIRED:
:

1. Buffered saline:

0.15M/litre of sodium chloride and 0.015M / litre or sodium citrate, pH 7

2. Diphenylamine reagent:

Dissolve 10gm of pure diphenylamine in 1litre of glacial acetic acid and add 25mL of concentrated sulphuric acid. This is prepared freshly.

2. DNA Stock Standard:

100mg of DNA is made up to 100mL with buffered saline.

1. Working Standard:

10 mL of the stock was made up to 100 mL.

PROCEDURE:

- For standard curve take 0.2 – 1mL of DNA in a series of test tubes and make up to 1mL with saline.
- Take 1mL of buffered saline as blank.
- Take 1mL of the test solution in 'T' tube.
- To all the tubes (standard, blank and test) add 2mL of diphenylamine reagent, heat in a boiling water bath for 10 min & cool.
- Read the optical density at 595nm.

TABULATION:

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (mL)	-	0.2	0.4	0.6	0.8	1.0	1.0
Concentration (µg)		200	400	600	800	1000	

Volume of saline (mL)	1.0	0.8	0.6	0.4	0.2	-	-
Diphenyl amine reagent (mL)	←---------- 2mL----------→						
Keep in boiling water bath for 10 min							
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 DNA

1.0mL the test sample contain ___ µg of DNA

100mL of the test sample = $\frac{\text{\_ µg x100}}{1.0}$

RESULT:

The amount of the DNA present in 100mL of the given solution

i. By calculation:

ii. By graph:

ESTIMATION OF RNA BY ORCINOL METHOD

AIM :

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

PRINCIPLE :

When pentose is heated with concentrated hydrochloric acid furfuryl is formed. Orcinol reacts with furfuryl in the presence of ferric chloride to give green colour is measured at 650 nm against an orcinol blank.

MATERIALS REQUIRED:

6. Test tubes
7. Standard flask
8. Pipette
9. Spectrophotometer
10. Water bath

REAGENTS REQUIRED

1. Stock standard:

100 mg of RNA was taken and made upto 100 ml saline.

2. Working standard:

10 ml of the stock was diluted to 100 ml with saline.

3. Orcinol reagent:

Dissolve 1g of $FeCl_3$.6H20 in 1 litre of concentrated sulphuric acid and 35 ml of 6 % orcinol in alcohol.

PROCEDURE:

- Pipette out aliquots of BSA standard (0.5-2.5 ml) in different test tubes and make up to 2.5 ml with saline.
- Take 2.5 ml of saline as blank.
- Take 2.5 ml of test solution
- Add 3 ml orcinol reagent to all the test tube
- Heat test tubes for 30 minutes and cool.
- Read the optical density at 665 nm.

TABULATION

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (ml)	-	0.5	1.0	1.5	2.0	2.5	2.5
Concentration (µg)							-
Volume of Saline (ml)	2.5	2.0	1.5	1.0	0.5	-	-
Volume of orcinol reagent (ml)	3	3	3	3	3	3	3
Heat in a boiling water bath for 30min and cooled							
O.D at 665 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 RNA

2.5 ml the test sample contain ___ µg of RNA

$$100 \text{ ml of the test sample} = \frac{\text{\_ µg x100}}{2.5}$$

RESULT:

The amount of RNA 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:

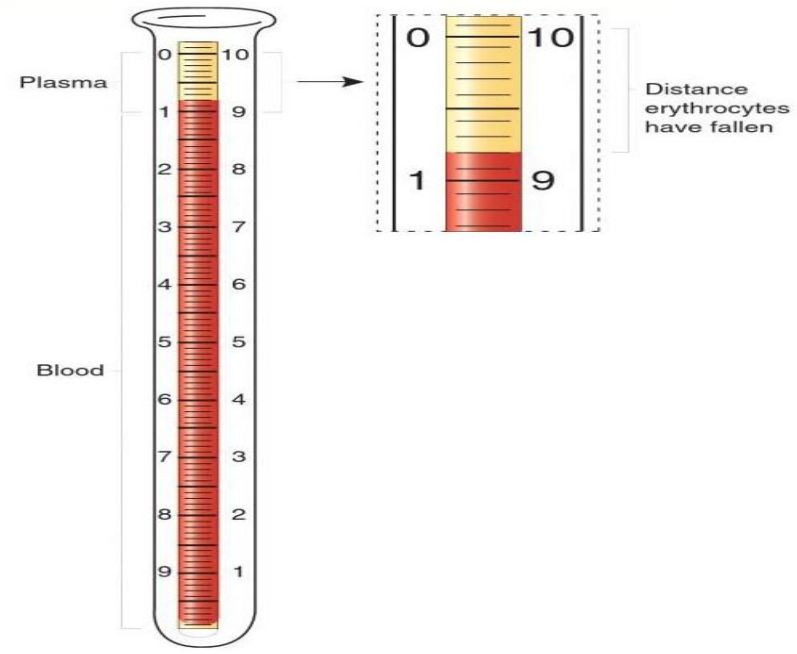
There are two main methods to determine ESR :

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.

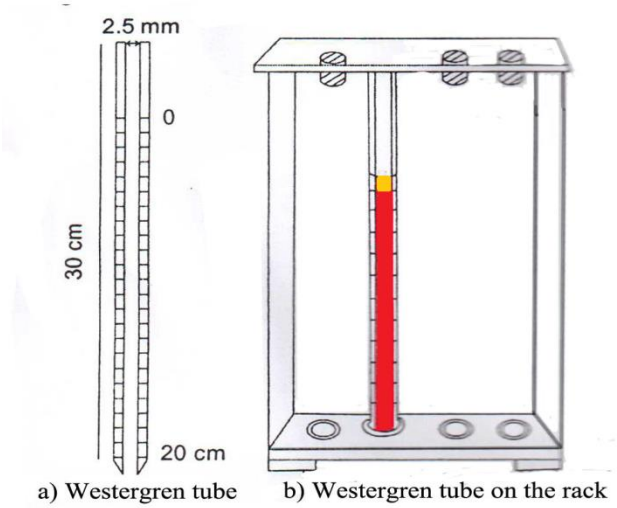
NORMAL VALUE:

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.

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%.

CHROMATOGRAPHIC SEPARATIONS OF SUGARS

The principle of chromatography involves separation of a mixture on the basis of specific differences in physical and chemical properties, which result from the structural differences of the chemically related groups of compounds which are under investigation. They therefore have differential affinity for both the mobile and stationary phases of the chromatographic systems. This chromatographic separation is the resultant of propelling (mobile phase) and retarding forces (stationary phase). The stationary phase in strict sense includes the medium (paper) together with the polar solvent (water). The mobile phase or propelling force includes both polar and non-polar solvent.

The separation is brought about by continuous partition between the mobile phase (solvent flowing along the paper) and the water held in the paper and paper *per se*. Paper together with water acts as an adsorbent ; it has a strong affinity for polar molecules which are held by hydrogen bonding and vander Waals' forces (Smith & Seakins, 1976).

16.2. Reagents

1. 5% *TCA* : Prepare by dissolving 5 gm of TCA in 100 ml of distilled water.
2. **Pyridine undiluted.**
3. **10% Iso-Propyl alcohol :** Prepare by diluting 10 ml of isopropyl alcohol in to 100 ml with distilled water.
4. **Solvent system (Butanol : Pyridine : water) :** Prepare by mixing Butanol : Pyridine : water in the ratio of 2 : 2 : 1

5. Alkaline silver oxide.

(*a*) *Saturated Silver nitrate in distilled water*—0.1 vol.

(*b*) *Sodium hydroxide :* Dissolve 0.5 gm NaOH in 5 ml of distilled water and dilute to 100 ml with ethanol—100 vol.

16.3. PROCEDURE

16.3.1. Preparation of sample for separation of sugars :

1. Take 0.1 ml of blood in 1 ml of 5% TCA and centrifuge at 2500 rpm for 5 minutes.
2. With 1 ml of supernatant, add 3 ml of pyridine and heat it over a boiling water bath, till the solution gets evaporated completely.
3. Dissolve the residue again in 3 ml of pyridine and evaporate it over a boiling water bath. Repeat this procedure for 4-5 times.
4. Dissolve the salt-free residue in 1 ml of 10% isopropyl alcohol.

SEPARATION

1. Take a Whatman No. 1 chromatogram paper (23 × 18 cm) and note down the flow direction.
2. Draw a line two inches above the lower margin and make two spots.
3. Spot the sample and the standard on the points separately and dry it using a hair dryer.
4. Fold the paper into a hollow cylinder and join the ends with cellophane tape.
5. Take 40 ml of solvent in a glass container (1500 ml) and keep the paper inside, (care should be taken to avoid any contamination with paper and the paper should not touch the sides of the glass container) and allow it to run.
6. After the completion of the run, take out the paper carefully and dry it in air.

LOCALISATION AND IDENTIFICATION OF SPOT

1. Dip the dried paper in silver nitrate.
2. When dried dip it in Sodium hydroxide.
3. Excess reagents to be removed by dipping in 2 M Ammonia.
4. Make out the spot and determine the Rg values and identify the different sugars.

THIN LAYER CHROMATOGRAPHY

AIM: To separate lipid

APPARATUS

- Nebulizer spray bottles
- Hot plate @ 200°C
- (TLC) plates (Whatman K6)
- Glass pasteur pipette
- Spray chamber
- TLC tanks
- Iodine vapor tank

REAGENTS

- Chloroform (B&J)
- Methanol (B&J)
- Deionized Water
- Ammonium Hydroxide
- Phosphorus spray
- Ninhydrin spray

PROCEDURE

1. **Prepare TLC Solvent Mixtures.**
    1. **65:25:4 (v/v/v) chloroform : methanol : water** – Mix 650 ml chloroform, 250 ml methanol, and 40 ml deionized water in a 1 liter graduated cylinder.
    2. **65:25:4 (v/v/v) chloroform : methanol: ammonium hydroxide** – Mix 650 ml chloroform, 250 ml methanol, and 40 ml ammonium hydroxide in another 1 liter graduated cylinder.
2. **Prepare TLC Migration Tanks.**
    1. Line the sides of the TLC tank with filter paper.
    2. Wet the filter paper and cover the bottom of one TLC tank with approximately 1cm of the chloroform:methanol: water solvent mixture.
    3. Prepare another TLC tank as described above and cover the bottom of it with approximately 1 cm chloroform:methanol:ammonium hydroxide solvent mixture.

3. **Spotting and Migration of TLC Plates.**
    1. Place two clean TLC plates on the hot plate silica side up for approximately three minutes to reactivate silica. Remove TLC plates from heat.
    2. Collect a sample of product in appropriate solvent.(no more than 50 mg/ml) with a glass pasteur pipette. Spot sample 1/4 inch from the bottom of each TLC plate.
    3. Place a TLC plate in each solvent tank. Make sure the solvent does not touch product sample. Replace lids on top of tanks.
    4. Remove TLC plates from tanks when 1/4 in from top. Place TLC plates on hot plate to remove excess solvent.
4. **Spraying and Interpreting TLC Plates.**
    1. Place TLC plates in iodine vapor1 tank for a minimum of 5 minutes. Place on hot plate to remove excess iodine.
    2. Place TLC plates in spray chamber. Completely spray TLC plates with ninhydrin2 spray. Place TLC plates on hot plate for one minute. Read TLC plates for any ninhydrin positive material/product.
    3. Place TLC plates in spray chamber and spray with phosphorus3 spray. Place TLC plates on hot plate for 30 seconds. Read TLC plates for phosphorus positive material.
    4. Dip plates in water and place them on the hot plate to determine if any contaminants are present as a visible wet spot. Repeat dip once.
    5. Allow the plates to remain on the hot plate until the sample disappears or shows a charred appearance.

ESTIMATION OF SERUM LACTATE DEHYDROGENASE (LDH)

AIM :

To determine the activity of Lactate dehydrogenase

Principle:

Serum is incubated with pyruvate and NADH at 37°C . The pH is maintained by a buffer. Initial and final concentrations of pyruvate in the assay system are measured by reacting pyruvate with 2,4-Dinitrophenylhydrazine to form a coloured hydrazone. Serum LDH is calculated from the decrease in pyruvate concentration**.**

Reagents:

1.Buffer -10gm of K_2HPO_4 .3H2O (7.72 gm of K_2HPO_4 dissolved in 500 ml of water. To this 200mg of Pyruvic acid (or 253 mg of sodium pyruvate) is added. With mixing 8ml of 0.3M of Phosphoric acid (made by diluting 2ml of 85% phosphoric acid to 100ml with water) is added. The pH is checked and adjusted to 7.6 . The volume is made upto 1L with water, a drop of chloroform is added and the solution is stored in refrigerator.

2. Buffered substrate – 10 mg of NADH is dissolved in 10ml of buffer.

3. DNPH – 200 mg of 2,4-Dinitrophenylhydrazineand 85ml of Con.HCl are added to water and mixed . The volume is made upto 1L with water.

4. Sodium Hydroxide (0.4N) – 16 g of NaOH is dissolved and made upto 1L with water(Kept in stoppered bottle).

PROCEDURE:

- ✓ Aliquotes of standard (0.2 - 1) were taken and made upto 1ml with water.
- ✓ 0.5ml of serum were used as test.
- ✓ 1ml of water were taken as blank.
- ✓ 1ml of buffered substrate were added to all the tubes and incubated at 37°c for 45 minutes.

- ✓ 1ml of DNPH were added to all the tubes mixed well and incubated at room temperature for 20 minutes.
- ✓ 10 ml of .4N NaOH were added to all the tubes mixed well and allowed to stand for 30 minutes for room temperature.
- ✓ The absorbents were read at 510 nm

TABULATION

Particulars	Blank	S1	S2	S3	S4	S5	T
Volume of standard (ml)	-	0.2	0.4	0.6	0.8	1.0	0.5
Concentration (µg)							-
Volume of water (ml)	1	0.8	0.6	0.4	0.2	-	-
Volume of buffered substrate (ml)	1	1	1	1	1	1	1
Incubated at 37°c for 45 minutes							
Volume of DNPH (ml)	1	1	1	1	1	1	1
Incubated at room temperature for 20 minutes							
Volume of 0.4N NaOH (ml)	1	1	1	1	1	1	1
Incubated at room temperature for 30 minutes							
Absorbance at 510 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 LDH

2.5 ml the test sample contain ___ µg of LDH

$$100 \text{ ml of the test sample} = \frac{\_\ \mu g \times 100}{2.5}$$

RESULT:

The amount of LDH present in the given sample

i. By calculation:

ii. By graph: