

**Department of Biochemistry**

**Shrimati Indira Gandhi College**

**I M.Sc Biochemistry**

**Practical –I ( Biochemical techniques and  
Enzymology)**

**Sub.Code : P16BC15P**

## **Objectives:**

1. To assay the activity of enzymes from different sources.
2. To stimulate their interest in learning the structure, function and kinetics of enzyme and their role as catalyst and regulator of cell metabolism and to serve as foundation for more advanced enzymology courses

## **Contents**

1. Estimation of proteins by Lowry / Bradford method
2. Estimation of phospholipids by phosphorous assay
3. Estimation of sodium and potassium by Flame photometry
4. Effect of pH, temperature and substrate concentration for amylase and urease and determination of  $V_{max}$  &  $K_m$
5. Effect of inhibitor on activity of any one enzyme
6. Effect of activator on activity of any one enzyme
7. Desalting of proteins by dialysis
8. Separation of polar and non polar lipids by TLC
9.  $R_f$  value calculation of various amino acids using TLC and PC
10. Separation of serum proteins by paper electrophoresis

## Estimation of Protein by Lowry's Method

### **Aim**

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

### **Principle**

This method is more sensitive than the biuret method since it determines the low concentration of protein. The reagent called Folin Ciocalteu is quite complex and contains phosphomolybdic acid and tungstate. The aromatic amino acid tyrosine and tryptophan present in protein reacts with these and produce a dark blue colour which can be read at 640nm (red filter).

### **Reagents Required**

#### **1) Preparation of stock solution:**

200mg of BSA was weighed accurately and dissolved in 100ml of water.

#### **2) Working standard:**

10ml of stock solution was taken and diluted to 100ml with distilled water.

#### **3) Alkaline copper reagents:**

**Solution A:** 2% of  $\text{NaCO}_3$  in 0.1 N NaOH.

**Solution B:** 0.5% of  $\text{CUSO}_4$  in 1% sodium potassium tartarate solution.

Mix 50 ml of solution A and 1 ml of solution B just before use.

#### **4) Folin Ciocalteu Reagent:**

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

Alternatively ready to use refluxed Folin Ciocalteu reagent (2N) was diluted with the equal volume of distilled water just before use (2ml of commercial reagent +2ml of DW).

### Preparation of Sample

5 gram of wheat flour was dissolved in 100 ml of water and filtered the filtrate was used as sample.

### Procedure:

0.2ml to 1ml of working standard was pipetted out into different labeled tubes. The volume was made upto 1ml with water. To this 4.5ml of alkaline copper reagent was added, mixed well. Allow it to stand for 10 minutes at room temperature. 0.5ml of folins phenol reagents was added and allowed it to stand for 20 minutes at room temperature. For test 0.1ml of sample was taken and 1ml of water was taken as a blank. They were treated as standard. The blue color developed was read at 640nm in a colorimeter. The amount of protein is calculated from the graph.

### Result:

The amount of Protein present in the given sample was -----.

#### Estimation of Protein by Lowry's Method

S.N	CONTENT	B	S1	S2	S3	S4	S5	T1	T2
0.									
1.	Volume of standard (ml)	---	0.2	0.4	0.6	0.8	1.0	---	---
2.	Concentration in $\mu\text{g}$	---	40	80	120	160	200	---	---
3.	Volume of test solution (ml)	---	---	---	---	---	---	0. 1	0.1
4.	Volume of distilled Water (ml)	1	0.8	0.6	0.4	0.2	---	0. 9	0.9

5.	Volume of alkaline copper reagent (ml)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Allow it to stand for 10 minutes at room temperature									
6.	Vol. of folin phenol reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Allow it to stand for 20 minutes at room temperature and blue color developed was read at 640nm.									
7.	Optical density at 640nm.								

**Calculation:**

-----O.D corresponds to -----  $\mu\text{g}$

0.1ml of sample contains----- $\mu\text{g}$

100ml of sample contain =  $\times 100 / 0.1 \mu\text{g}$

=  $\mu\text{g} / 1000 = \text{mg}$ .

100ml of sample was made from 5gm of wheat flour

$\therefore$  5g of wheat flour contain =  $\text{mg}$

100g of wheat flour contain =  $\times 100 / 5 \text{ mg}$

=  $\text{mg} / 1000 = \text{g/dl}$ .

**Concentration:**

**200mg = 100ml**

**? = 10ml      10ml = 20mg**

**100ml = 20ml**

**0.2ml = ?      0.2ml = 0.04mg = 0.04 $\times$ 1000 = 40 $\mu\text{g}$ .**

## Estimation of Phospholipids

### **Aim**

To estimate the amount of phospholipids present in the oil

### **Principle**

Phosphate reacts with molybdic acid to form phosphomolybdic acid on treatment with amino naphthol sulphonic acid (ANSA). Phosphomolybdic acid is reduced to produce a deep blue colour which is probably a mixture of oxides of molybdenum. The blue colour developed is read at 620nm.

### **Reagents Required**

#### **Stock solution**

35.1 mg of Potassium di hydrogen phosphate in 100ml of water.

#### **Working standard**

10 ml of stock is diluted to 100 ml

#### **Ammonium molybdate**

2.5g of ammonium molybdate in 100 ml of 3N sulphuric acid.

## ANSA

Add 0.5g of ANSA to 195 ml of 15% sodium bisulphite and 5 ml of 20% sodium sulphite. Stir until it dissolves. Store it in brown bottle.

### Test solution

1 ml of oil is treated with 5 drops of perchloric acid and heated in a boiling water bath to digest the mixture. The content in the tube should be just boiling. Initially the sample would get charred and latter get cleared. At this stage the digestion is complete. Allowed to cool and it is used as test solution.

### Procedure

The standard in the range of 0.5 -2.5 ml was taken in a series of test tubes. 1ml of test sample was also taken in "T" test tube. All the test tubes were made upto 9 ml with distilled water. A blank containing 9 ml of water alone was also taken. To all the test tubes 1 ml of ammonium molybdate and 0.4 ml of ANSA were added. The blue colour developed was read at 620nm after 10 minutes.

### Result

The amount of phospholipids present in the given sample was -----.

Estimation of Phospholipids									
S.No	Content	B	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>4</sub>	S <sub>5</sub>	T <sub>1</sub>	T <sub>2</sub>
1	Volume of standard solution (ml)	-	0.5	1	1.5	2	2.5		
2	Concentration of Solution (µg)		4	8	12	16	20		
3	Volume of Test solution (ml)							1	1
4	Volume of Distilled water (ml)	9	8.5	8	7.5	7	6.5	8	8
5	Volume of ammonium molybdate (ml)	1	1	1	1	1	1	1	1
6	Volume of ANSA (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
The blue developed was read at 620 nm									
7	Optical density at 620 nm								

### Calculation

----- OD corresponds to -----µg of Phosphorus

∴ 1 ml of test solution contain -----μg of Phosphorus

1 ml of test solution was prepared from 1ml of oil

1ml of oil contain ---μg of Phosphorus

∴ 100ml of oil contain = ---X100/1= -----μg of Phosphorus

Amount of Phospholipid = Amount of Phosphorus X 25

Amount of Phospholipid present in oil = -----X25=-----μg of Phospholipid

i.e = -----/1000=----- mg of Phospholipid

Laboratory Manual



## Estimation of Sodium and Potassium by Flame Photometry

### **Aim:**

To estimate the amount of Sodium and Potassium using flame photometry

### **Principle**

When a liquid sample containing a metallic salt solution is introduced into a flame, the solvent vaporizes leaving solid salt. The salt in turn is converted into gaseous state. All the parts of the gaseous molecule are dissociated to give free neutral atoms (or) radicals excited by thermal energy of the flame. The excited atoms which are unstable quickly emit photons and return to lower energy state. The measurement of the emitted photons forms the basis of flame photometry.

### **Instrumentation**

It consists of a burner in which fuel LPG and air used under controlled conditions to produce a blue cone shaped flame. The nebulizer is used to aspirates the sample. The emission in the flame is measured using filters specific for sodium and potassium.

### **Preparation of Stock Standard**

#### **Sodium (Na) – 1000 mmol/litre**

Dissolve 58.5 g of sodium chloride in 1 Litre of double distilled water.

#### **Potassium (K) – 100 mmol/litre**

Dissolve 7.46 g of potassium chloride in 1 litre of double distilled water.

### **Preparation of stock standards (Na-140/5 mmol/L)**

Take 70 ml from sodium stock standard solution and add 25 ml from potassium stock standard solution. Then it is made up to 500 ml with double distilled water.

### **Preparation of working standard**

Dilute the above solution to 1:100 with DDW

### **Sample preparation**

Dilute the clear serum with double distilled water 1:100. Avoid unusual turbidity as this more value.

Connect all the connection correctly and lighten the flame in blue cones, run the double distilled water as blank by adjusting the digital display to 000. Run the standard for sodium by adjusting to 140. Repeat the blank reading to ensure 000. Again read standard sodium at 140.

Repeat for several times. Then simultaneously run with test solution without disturbing the adjustment and record the results. Change the filter, in the same way run for potassium, adjust the standard for K to 5. Then simultaneously run the test solution without disturbing the adjustments and record the results.

**Filter used for Na-Orange**

**Filter used for K- Deep red**

**Results**

The amount of sodium in the given sample was -----

The amount of potassium in the given sample was -----

Laboratory Manual

## Effect of substrate concentration on salivary amylase activity

### **Aim:**

To determine the effect of substrate concentration on the activity of salivary amylase.

### **Principle:**

The starch forms a complex with iodine in acidic medium giving a blue colour which is read colorimetrically at 660nm upon treatment with enzyme hydrolysis of starch takes place, gradually losing its linkage which is used for the assessment of enzyme activity.

### **Reagent required:**

#### **Phosphate Buffer (pH-7)**

##### **Solution A:**

17.8g of disodium hydrogen phosphate in 1litre of water (0.1M).

##### **Solution B:**

15.6g of sodium dihydrogen phosphate in 1litre of water (0.1M).

Mix 69ml of solution A and 31ml of solution B to form 100ml.

### **Buffered substrate:**

#### **Starch: phosphate Buffer (4:5)**

100mg of starch was dissolved in 100ml of distilled water and warmed in a water bath to dissolve starch.40ml of starch solution was taken and mixed with 50ml of phosphate buffer

### **Stock iodine solution:**

3g of potassium iodide was dissolved in 50ml of water and added 1.3g of iodine. The solution was to be made upto 100ml with distilled water.

### **Working iodine solution:**

10ml of iodine solution was made upto 100ml with distilled water.

### **Saline:**

900mg of sodium chloride was dissolved in 100ml of water.

### **Enzyme:**

1ml of saliva was made up to 10ml with saline.

### **Procedure:**

Pipetted out various concentrations of buffered substrate into a series of test tubes and the volume was made up to 3 ml with water. 0.5 ml of enzyme was added to "T" test tubes alone. The test tubes were incubated at 37°C for 15 minutes. The reaction was stopped by the

addition of 0.4 ml of iodine. Then the enzyme was added to the control test tubes alone. The total volume was made up to 4.1ml with water in all the test tubes. The blank was prepared according to the table. The colour developed was read at 660nm.

**Result:**

The  $K_m$  and  $V_{max}$  for the effect of substrate concentration on the activity of salivary amylase by **Michaelis – Mentenplot** was

$K_m =$

$V_{max} =$

The  $K_m$  and  $V_{max}$  for the effect of substrate concentration on the activity of salivary amylase by **Line weaver Burk plot** was

$K_m =$

$V_{max} =$

Laboratory Manual



## Model calculation

Line weaver Burk plot

Let the difference in OD were 0.02, 0.04, 0.06, 0.08, 0.08, 0.08

[S] $\mu\text{g}$	1/[S]	V	1/V
250	$0.004=4 \times 10^{-3}$	0.02	50
500	$0.002=2 \times 10^{-3}$	0.04	25
750	$0.0013=1.3 \times 10^{-3}$	0.06	16.67
1000	$0.001=1 \times 10^{-3}$	0.08	12.5
1250	$0.0008=0.8 \times 10^{-3}$	0.08	12.5
1500	$0.000667=0.67 \times 10^{-3}$	0.08	12.5

From the graph

$$-1/km = -0.04 \times 10^{-3}$$

$$\therefore km = 1/0.04 \times 10^{-3} = 1/40 = 0.025 \mu\text{g}$$

## Effect of pH on salivary amylase activity

### **Aim:**

To determine the effect of pH on salivary amylase activity.

### **Principle:**

The starch forms a complex with iodine in acidic medium giving a blue colour which is read colorimetrically at 660nm upon treatment with enzyme hydrolysis of starch takes place, gradually losing its linkage which is used for the assessment of enzyme activity.

### **Reagent required:**

#### **Phosphate Buffer (pH - 6-7.6)**

##### **Solution A:**

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

##### **Solution B:**

26.8 g of disodium hydrogen phosphate .7H<sub>2</sub>O in 1litre of water (0.1M).

### **Buffered substrate:**

#### **Starch: phosphate Buffer (4:5)**

100mg of starch was dissolved in 100ml of distilled water and warmed in a water bath to dissolve starch.40ml of starch solution was taken and mixed with 50ml of phosphate buffer

### **Stock iodine solution:**

3g of potassium iodide was dissolved in 50ml of water and added 1.3g of iodine. The solution was to be made upto 100ml with distilled water.

### **Working iodine solution:**

10ml of iodine solution was made upto 100ml with distilled water.

### **Saline:**

900mg of sodium chloride was dissolved in 100ml of water.

### **Enzyme Source:**

1ml of saliva was made up to 10ml with saline.

**Phosphate buffer at various pH were prepared as follows (pH – 5.8-7.8)**

<b>pH</b>	<b>Solution A (ml)</b>	<b>Solution B (ml)</b>	<b>water in ml</b>
<b>5.8</b>	<b>92</b>	<b>8</b>	<b>100</b>
<b>6</b>	<b>87</b>	<b>13</b>	<b>100</b>
<b>6.2</b>	<b>81.5</b>	<b>18.5</b>	<b>100</b>
<b>6.4</b>	<b>73.5</b>	<b>26.5</b>	<b>100</b>
<b>6.6</b>	<b>62.5</b>	<b>37.5</b>	<b>100</b>
<b>6.8</b>	<b>51</b>	<b>49</b>	<b>100</b>
<b>7</b>	<b>39</b>	<b>61</b>	<b>100</b>
<b>7.2</b>	<b>28</b>	<b>72</b>	<b>100</b>
<b>7.4</b>	<b>19</b>	<b>81</b>	<b>100</b>
<b>7.6</b>	<b>13</b>	<b>87</b>	<b>100</b>
<b>7.8</b>	<b>8.5</b>	<b>91.5</b>	<b>100</b>

**Procedure:**

Pipetted out 1ml of buffered substrate of different pH (6, 6.4, 6.8, 7.2, 7.6) into a series of test tubes. 0.5 ml of enzyme was added to “T” test tubes alone. The test tubes were incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 0.4 ml of iodine. Then the enzyme was added to the control test tubes alone. The total volume was made up to 7.1 ml with water in all the test tubes. The blank consisted of 0.4ml of iodine and 7.6ml of water. The blue colour developed was read at 660nm.

**Result:**

The optimum pH of salivary amylase at 37°C was 6.8





## Effect of temperature on salivary amylase activity

### **Aim:**

To determine the effect of temperature on salivary amylase activity.

### **Principle:**

The starch forms a complex with iodine in acidic medium giving a blue colour which is read colorimetrically at 660nm upon treatment with enzyme hydrolysis of starch takes place, gradually losing its linkage which is used for the assessment of enzyme activity.

### **Reagent required:**

#### **Phosphate Buffer (pH-7)**

##### **Solution A:**

17.8g of disodium hydrogen phosphate in 1litre of water (0.1M).

##### **Solution B:**

15.6g of sodium dihydrogen phosphate in 1litre of water (0.1M).

Mix 69ml of solution A and 31ml of solution B to form 100ml.

### **Buffered substrate:**

#### **Starch: phosphate Buffer (4:5)**

100mg of starch was dissolved in 100ml of distilled water and warmed in a water bath to dissolve starch.40ml of starch solution was taken and mixed with 50ml of phosphate buffer.

### **Stock iodine solution:**

3g of potassium iodide was dissolved in 50ml of water and added 1.3g of iodine. The solution was to be made upto 100ml with distilled water.

### **Working iodine solution:**

10ml of iodine solution was made upto 100ml with distilled water.

### **Saline:**

900mg of sodium chloride was dissolved in 100ml of water.

### **Enzyme Source:**

1ml of saliva was made up to 10ml with saline.

### **Procedure:**

Pipetted out 1ml of buffered substrate was added into a series of test tubes. 0.5 ml of enzyme was added to "T" test tubes alone. The test tubes were incubated at different temperatures (15°C, 25°C, 37°C, 45°C, and 55°C) for 15 minutes. The reaction was stopped



## Effect of substrate concentration on Urease activity

### **Aim**

To study the effect of substrate concentration on urease activity.

### **Principle**

Depending on the affinity of the enzyme towards the substrate,  $K_m$  value changes. Thus greater the affinity of the enzyme for the substrate the lesser will be the  $K_m$  value. Lesser the affinity of the enzyme for the given substrate higher will be the  $K_m$  value. By applying MM equation and Line weaver burk plot it is possible to determine the  $K_m$  and  $V_{max}$  of any substrate. When Urea solution is incubated with urease, which converts urea into carbon dioxide and ammonia. Nessler's reacts with ammonia to give yellow colour which is read colourimetrically at 520 nm.

### **Reagents Required**

#### **Enzyme source**

Soak 5g of powdered horse gram in 100 ml of water. Leave it for half an hour and filtered and the filtrate was used as enzyme source.

#### **Phosphate Buffer (pH -7)**

##### **Solution A:**

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

##### **Solution B:**

26.8 g of disodium hydrogen phosphate  $\cdot 7H_2O$  in 1litre of water (0.1M).

#### **Phosphate buffer at pH – 7.0**

pH	Solution A (ml)	Solution B (ml)	water (ml)
7	39	61	100

#### **Buffered substrate of varying concentration (0.1- 0.5%)**

Prepare varying concentration (0.1- 0.5%) of urea in phosphate buffer at pH - 7

### 1N Sulphuric acid as enzyme inhibitor (100 ml)

$$N_1 V_1 = N_2 V_2$$

$$36x = 1 \times 100$$

$$\therefore X = 1 \times 100 / 36$$

### 10% sodium tungstate

10 g in 100 ml of DW

### Nessler's reagent

Dissolve 100 g mercuric iodide, 40g of potassium iodide in 400ml of DW. Dissolve 100g of Sodium hydroxide to the iodine solution with stirring. Make up this volume 1000ml and filter it.

### Procedure

Into a series of test tubes pipetted out 1ml of buffered substrate of varying concentration (0.1 – 0.5%) as “Controls” and “Tests” labeled test tubes. The tubes were incubated at 60°C for 2 minutes for acclimatization. Add 1ml of 1N sulphuric acid to all the “C” tubes alone as enzyme inhibitor. Add 1ml of enzyme to all the tubes and incubate at 60°C for 15 minutes. After incubation add 1ml of 1N sulphuric acid to all the “T” tubes alone as enzyme inhibitor. Then add 1 ml of 10% sodium tungstate to all the tubes to precipitate the protein. Allowed to stand for 5 minutes and centrifuged. Take 0.5 ml of supernatant and made up to 9.5 ml with distilled water. Add 0.5ml of nessler's reagent and the yellow colour developed was read at 520 nm. Plot the graph and calculate the  $K_m$  and  $V_{max}$  by MM equation and by LB plot

### Result

The  $K_m$  and  $V_{max}$  for the effect of substrate concentration on the activity of urease by

**Michaelis – Menten plot** was

$$K_m =$$

$$V_{max} =$$

The  $K_m$  and  $V_{max}$  for the effect of substrate concentration on the activity of urease by **Line weaver Burk plot** was

$$K_m =$$

$$V_{max} =$$



### Effect of pH on Urease activity

#### **Aim**

To study the effect of pH on urease activity.

#### **Principle**

Drastic changes in pH modify the activity of enzyme due to ionization and deionization of active site. When Urea solution is incubated with urease, which converts urea into carbon dioxide and ammonia. Nessler's reagent reacts with ammonia to give yellow colour which is read colourimetrically at 520 nm.

#### **Reagents Required**

##### **Enzyme source**

Soak 5g of powdered horse gram in 100 ml of water. Leave it for half an hour and filtered and the filtrate was used as enzyme source.

##### **Phosphate Buffer (pH - 6-7.6)**

##### **Solution A:**

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

##### **Solution B:**

26.8 g of disodium hydrogen phosphate  $\cdot 7H_2O$  in 1litre of water (0.1M).

##### **Phosphate buffer at various pH were prepared as follows (pH – 6-7.6)**

H	Solution A (ml)	Solution B (ml)	Water (ml)
5.8	92	8	100
6	87	13	100
6.2	81.5	18.5	100
6.4	73.5	26.5	100

6.6	62.5	37.5	100
6.8	51	49	100
7	39	61	100
7.2	28	72	100
7.4	19	81	100
7.6	13	87	100
7.8	8.5	91.5	100

### Buffered substrate

Prepare 1% urea in phosphate buffer of varying pH

### 1N Sulphuric acid as enzyme inhibitor (100 ml)

$$N_1 V_1 = N_2 V_2$$

$$36x = 1 \times 100$$

$$\therefore X = 1 \times 100 / 36$$

### 10% sodium tungstate

10 g in 100 ml of DW

### Nessler's reagent

Dissolve 100 g mercuric iodide, 40g of potassium iodide in 400ml of DW. Dissolve 100 g of Sodium hydroxide to the iodine solution with stirring. Make up this volume 1000ml and filter it.

### Procedure

Pipetted out 4ml of buffered substrate of different pH (6.4, 6.8, 7, 7.4, 7.8) into a series of test tubes. Add 1ml of 1N sulphuric acid to all the "C" tubes alone as enzyme inhibitor. Keep the tubes for inhibition for 2 minutes at 60°C. 1 ml of enzyme was added to all the test tubes and incubate at 60°C for 15 minutes. Add 1ml of 1N sulphuric acid to all the "T" tubes alone as enzyme inhibitor. Add 1 ml of 10% sodium tungstate to all the tubes to precipitate the protein. Allowed to stand for 5 minutes and centrifuged. Add 0.5ml of supernatant to 9 ml of water and 0.5ml of nessler's reagent. The blank consisted of 0.5ml of nessler's reagent and 9.5ml of water. The yellow colour developed was read at 520nm.

### Result





9	OD at 520 nm											
10	OD difference											

### Effect of Temperature on Urease activity

#### Aim

To study the effect of temperature on urease activity.

#### Principle

The velocity of the enzyme catalyzed reactions increases with increase in temperature. The temperature provides energy for the reaction. At optimum temperature the enzyme has maximum activity. But above optimum temperature the activity slows down and the reaction stops because the enzyme gets denatured. When Urea solution is incubated with urease, which converts urea into carbon dioxide and ammonia. Nessler's reagent reacts with ammonia to give yellow colour which is read colourimetrically at 520 nm.

#### Reagents Required

##### Enzyme source

Soak 5g of powdered horse gram in 100 ml of water. Leave it for half an hour and filtered and the filtrate was used as enzyme source.

##### Phosphate Buffer (pH -7)

###### Solution A:

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

###### Solution B:



Allowed to stand for 5 minutes and centrifuged												
6	Supernatant (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
7	Distilled water (ml)	9.5	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
8	Nessler's Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9	OD at 520 nm											
10	OD difference											

10 g in 100 ml of DW

**Nessler's reagent**

### Effect of Temperature on Urease activity

Dissolve 100 g mercuric iodide, 40g of potassium iodide in 400ml of DW. Dissolve 100 g of Sodium hydroxide to the iodine solution with stirring. Make up this volume 1000ml and filter it.

#### **Procedure**

Into a series of test tubes pipetted out 1ml of buffered substrate as "Controls" and "Tests" labeled test tubes. The tubes were incubated at varying temperature (30°C, 45°C, 60°C, 75°C, 90°C) for 2 minutes. Add 1ml of 1N sulphuric acid to all the "C" tubes alone as enzyme inhibitor. Add 1ml of enzyme to all the tubes and incubate at varying temperatures for 15 minutes. After incubation add 1ml of 1N sulphuric acid to all the "T" tubes alone as enzyme inhibitor. Then add 1 ml of 10% sodium tungstate to all the tubes to precipitate the protein. Allowed to stand for 5 minutes and centrifuged. Take 0.5 ml of supernatant and made upto 9.5 ml with distilled water. Add 0.5ml of nessler's reagent and the yellow colour developed was read at 520 nm.

#### **Result**

The optimum temperature for the activity of urease was found to be 60°C.

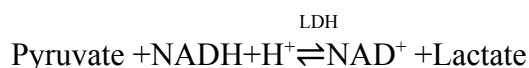
## Effect of Inhibitor on Lactate Dehydrogenase activity

### Aim:

To determine the effect of Inhibitor on Lactate Dehydrogenase activity.

### Principle:

The enzyme activity assessed according to the method described by king (1965) with slight modification. Enzyme catalyzes the following the reaction



Lactate dehydrogenase acts on pyruvate to give lactate. During this reaction the reducing equivalent (NADH+H<sup>+</sup>) gets oxidized. The yellow colour developed is read at 440 nm.

### Reagent required:

#### 1. Enzyme source:

Added 2 ml of heparin to 5ml of blood. The mixture was allowed to stand for 5 minutes. The reaction mixture was centrifuged for 10 minutes. The supernatant was taken for assay as enzyme source.

#### 2. Buffered substrate:

**Glycine Buffer:** 0.1 m glycine and 0.1m NaCl in 1L of distilled water

**Substrate (Sodium pyruvate):** 11 mg of sodium pyruvate is mixed with 50ml of glycine buffer.

#### 3. Dinitro phenyl hydrazine (DNPH) reagent

100 mg of DNPH is dissolved in 100 ml of 2N HCL

#### 4. Inhibitor (Acetone)

1 ml of acetone is made upto 50 ml of water.

#### 5. Co-factor

50 mg of NADH is dissolved in 100 ml of water.

#### 6. 0.4N sodium hydroxide

8 g of NaOH is dissolved in 500 ml of distilled water.

### Procedure:

Into a series of test tubes pipetted out 1 ml of buffered substrate and 0.1ml of enzyme to all the test tubes. 0.1ml was added to the “C” test tubes alone and all the test tubes were incubated in the water bath at 37°C. 0.2 ml of NADH was added to “T” test tubes alone. Increasing concentrations of inhibitor was added to all the test tubes and the test tubes were incubated at 37°C for 15 minutes.

1ml of DNPH was added to all the test tubes and again incubated at 37°C for 15 minutes. Then add 10ml of 0.4N NaOH with respect to inhibitor volume. A blank containing 1.2ml of water was treated same as control as test.

The yellow colour developed was read at 440 nm. A graph was plotted for OD values. From the graph the effect of inhibitor activity on LDH was determined.

### Result

From the graph, increase in inhibitor concentration is found to decrease the activity of Lactate dehydrogenase proportionally.

S.No	Contents	B	C <sub>1</sub>	T <sub>1</sub>	C <sub>2</sub>	T <sub>2</sub>	C <sub>3</sub>	T <sub>3</sub>	C <sub>4</sub>	T <sub>4</sub>	C <sub>5</sub>	T <sub>5</sub>
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
2	Enzyme (ml)	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
3	Volume of Distilled water (mL)	-	0.1	-	0.1	-	0.1	-	0.1	-	0.1	-
Incubated at 37°C for 15 minutes												
4	Volume of NADH	-	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
5	Volume of inhibitor (Acetone)	-	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.5	0.5
Incubated at 37°C for 15 minutes												
4	Volume of DNPH	1	1	1	1	1	1	1	1	1	1	1
5	Volume of NaOH (ml)	10	9.9	9.9	9.8	9.8	9.7	9.7	9.6	9.6	9.5	9.5
9	OD at 440 nm											
10	OD difference											

### Effect of Inhibitor on Lactate Dehydrogenase activity

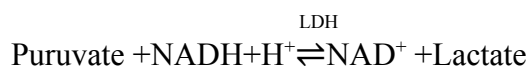
## Effect of Activator on Lactate Dehydrogenase activity

### Aim:

To determine the effect of activator on Lactate Dehydrogenase activity.

### Principle:

The enzyme activity assessed according to the method described by king (1965) with slight modification. The enzyme catalyses the following reaction



Lactate dehydrogenase acts on pyruvate to give lactate. During this reaction the reducing equivalent ( $\text{NADH} + \text{H}^+$ ) gets oxidized. The yellow colour developed is read at 440 nm

### Reagent required:

#### 1. Enzyme source:

Added 2 ml of heparin to 5ml of blood. The mixture was allowed to stand for 5 minutes. The reaction mixture was centrifuged for 10 minutes. The supernatant was taken for assay as enzyme source.

#### 2. Buffered substrate:

**Glycine Buffer** :0.1 m glycine and 0.1m NaCl in 1L of distilled water

**Substrate (Sodium pyruvate)**: 11 mg of sodium pyruvate is mixed with 50ml of glycine buffer.

#### 3. Dinitro phenyl hydrazine (DNPH) reagent

100 mg of DNPH is dissolved in 100 ml of 2N HCL

#### 4. Activator (Magnesium sulphate)

1 g of Magnesium sulphate is dissolved in 50 ml of water.

#### 5. Co-factor

50 mg of NADH is dissolved in 100 ml of water.

#### 6. 0.4N sodium hydroxide

8 g of NaOH is dissolved in 500 ml of distilled water.

**Procedure:**

Into a series of test tubes pipetted out 1ml of buffered substrate and 0.2ml of enzyme to all the test tubes. 0.2ml was added to the “C” test tubes alone and all the test tubes were incubated in the water bath at 37°C. 0.2 ml of NADH was added to “T” test tubes alone. Increasing concentrations of activator was added to all the test tubes and the test tubes were incubated at 37°C for 15 minutes.

1ml of DNPH was added to all the test tubes and again incubated at 37°C for 15 minutes. Then add 10ml of 0.4N NaOH with respect to activator volume. A blank containing 1.2ml of water was treated same as control as test.

The yellow colour developed was read at 440 nm. A graph was plotted for OD values. From the graph the effect of activator activity on LDH was determined.

**Result**

From the graph, increase in activator concentration is found to increase the activity of Lactate dehydrogenase proportionally.



S.No	Contents	B	C <sub>1</sub>	T <sub>1</sub>	C <sub>2</sub>	T <sub>2</sub>	C <sub>3</sub>	T <sub>3</sub>	C <sub>4</sub>	T <sub>4</sub>	C <sub>5</sub>	T <sub>5</sub>
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
2	Enzyme (ml)	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
3	Volume of Distilled water (mL)	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-
Incubated at 37°C for 15 minutes												
4	Volume of NADH	-	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
5	Volume of Activator [Magnesium sulphate]	-	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.5	0.5
Incubated at 37°C for 15 minutes												
4	Volume of DNPH	1	1	1	1	1	1	1	1	1	1	1
5	Volume of NaOH (ml)	10	9.9	9.9	9.8	9.8	9.7	9.7	9.6	9.6	9.5	9.5
9	OD at 440 nm											
10	OD difference											

**Effect of Activator on Lactate Dehydrogenase activity**

## Desalting of proteins by Dialysis

### Aim:

To purify and desalt protein by dialysis.

### Principle:

Dialysis is a process used to selectively remove small molecules from a sample containing mixture of both small and large molecules. It is commonly used for removing salt from protein. Dialysis is effectively achieved using a semipermeable membrane which allows only the small molecules to pass freely through the membrane and not the large colloidal protein molecules.

### Material required:

Dialysis membrane

Gelatin

0.1N HCl

Thymol blue: 0.1g in 100ml of ethanol (filter if needed).

### Procedure:

#### Preparation of the membrane:

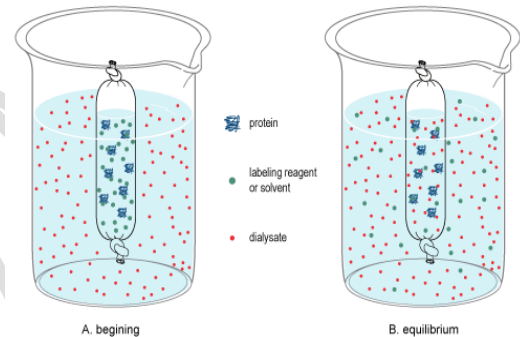
Cut dialysis membrane into required size. The dry dialysis membrane contains 10% glycerol which can be removed by soaking in water for 10 minutes.

#### Desalting of protein

Take 50ml 5% gelatin into a 100ml beaker and 50ml of DW into another beaker. Added 1ml of thymol blue indicator into each of these beakers. To water added continuously drop by drop 0.1N HCl. This is done until the indicator had a colour between yellow-pink (pH 1.2-2.8) and red orange. Add 0.1N HCl until a similar shade is obtained in gelatin solution. The two solutions then had the same hydrogen ion concentration. Then transfer the gelatin solution into a dialyzing bag and suspend it into the beaker containing water for 4 hrs at room temperature.

### Result

The gelatin solution gradually turned into yellow (decreased acidity). The outside solution turned pink (increased acidity) indicating the diffusion of hydrogen ion and small molecules through the membrane.



## Separation of lipids by Thin layer chromatography

### Aim

To separate the mixture of lipids by thin layer chromatography

### Principle

Thin layer chromatography consists of thin layer adsorbent such as silica gel, alumina or cellulose on a glass plate or aluminium foil. This layer of adsorbent acts as a stationary phase. The adsorbent like silica has hydroxyl groups which act as interacting groups. The sample partitions between the mobile and the stationary phase, will move to various distance based on charge solubility and adsorption. The retention factor or  $R_f$  is the characteristics of the substance. The  $R_f$  value is the distance moved by the compound to that moved by the solvent.

### Requirements

1. Thin layer chromatography plate 20X20cm
2. Silica gel
3. Thin layer chromatography apparatus
4. Petroleum ether-diethyl ether – acetone(90:10:1)/  
Toluene –ethyl acetate – formic acid (6:3:1)

### Procedure

The plates were coated with slurry of silica gel in 0.02M sodium acetate to a thickness of 250 $\mu$ m. After spreading the plates were activated by heating at 105°C for 15 minutes. 5 $\mu$ l sample of various lipids were spotted as in paper chromatography. The plates were developed in petroleum ether-diethyl ether–acetone(90:10:1). The chamber was saturated with the same solvent system. The plates was then kept in the chamber and allowed to run until the solvent front rises to 3/4<sup>th</sup> of the plate.

The plate was removed, dried and solvent front was marked. From this value lipid sample were calculated. Iodine vapour is extensively used as a universal reagent for organic compounds. The iodine spot disappears rapidly but can be made more permanent by spraying 0.5% benzidine solution in absolute ethanol. Iodine vapour is concentrated in the form of cloud over the region where the compounds have separated.

### Result

The given lipid was identified as -----

## Separation of amino acids by thin layer chromatography

### Aim

To separate the mixture of amino acids by thin layer chromatography

### Principle

Thin layer chromatography consists of thin layer adsorbent such as silica gel, alumina or cellulose on a glass plate or aluminium foil. This layer of adsorbent acts as a stationary phase. The adsorbent like silica has hydroxyl groups which act as interacting groups. The sample partitions between the mobile and the stationary phase, will move to various distance based on charge solubility and adsorption. The retention factor or  $R_f$  is the characteristics of the substance. The  $R_f$  value is the distance moved by the compound to that moved by the solvent.

### Requirements

1. Thin layer chromatography plate 20X20cm
2. Silica gel
3. Thin layer chromatography apparatus
4. N-butanol-acetic acid-water(80:20:20).
5. Ninhydrin in acetone

### Procedure

The plates were coated with slurry of silica gel in 0.02M sodium acetate to a thickness of 250 $\mu$ m. After spreading the plates were activated by heating at 105°C for 15 minutes. 5 $\mu$ l sample of various amino acids were spotted as in paper chromatography. The plates were developed in N-butanol-acetic acid-water (80:20:20). The chamber was saturated with the same solvent system. The plates was then kept in the chamber and allowed to run until the solvent front rises to 3/4<sup>th</sup> of the plate.

The plate was then removed; dried and solvent front was marked. The plates were sprayed with 0.3% solution of ninhydrin in acetone. The coloured spots developed by heating the plates at 110°C for 10 minutes. Once the colours were developed plates were exposed to vapours of concentrated ammonium hydroxide which helps in stabilization of colours.

### Result

The given amino acids identified as -----

## Separation of Amino Acids by Paper Chromatography

### Aim:

To separate and determine the R<sub>F</sub> value of amino acids by paper chromatography.

### Principle:

Chromatography is a method by which a mixture of compounds in small quantities can be separated qualitatively. There are two phases, the stationary and mobile phase. When the mobile phase moves along the stationary phase separation of substance takes place. In paper chromatography the paper acts as an inert supporting media and the substances are applied as a spot on the paper and substance are carried along with the solvent as the partition co-efficient differs for different amino acids. Therefore each amino acid are separated individually.

### Material Required

Whatmann No.1 circular chromatography paper.

Solvent system: n- butanol: glacial acetic acid: water (4:1:5 v/v)

Ninhydrin (Location reagent): 0.2% in acetone.

### Procedure:

Whatmann No.1 Circular chromatography paper was taken. From the center a circle was drawn with a radius of 1 cm. At equal distances positions were marked for the application of sample. Each amino acid solution was applied separately on each spot by using a capillary tube. On the last spot the unknown amino acid was applied. The size of the spot should be as small as possible. The spots were air dried before development. The chamber was completely saturated with aqueous phase in order to obtain good separation. Into a petriplate was taken organic butanolic phase, the paper was kept in the petriplate, the solvent raised up the paper with the help of paper wick by capillary action. This arrangement was kept undisturbed for 30 minutes. The solvent front moved in a circular (radial) fashion. When it reached 3/4<sup>th</sup> of the paper the run was stopped and the paper was removed the solvent front was marked and the paper was dried. Then 0.2% ninhydrin was sprayed on paper using sprayer. The paper was then dried for 10 minutes at 105°C. The amino acids appear as purple spots on white background and they were marked. The R<sub>f</sub> was calculated by

$$R_F = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

**Result:** The given unknown amino acids was identified as -----

## Separation of serum proteins by paper electrophoresis (cellulose acetate)

### Aim:

To separate serum proteins by using paper electrophoresis.

### Principle:

The principle is based on the migration of charged particle of any size in liquid medium under the influence of an electric field. Depending on kind of charge the molecule carry they move towards either to cathode (or) to an anode. An ampholyte become positively charged in acidic condition and migrate to cathode, in alkaline condition they become negatively charged and migrate to anode. The rate of migration of an ion in electric field depends on net charge of molecule, size and shape of particle.

### Reagents required

Buffer – pH- 8.3 - 8.6

Tris – 600mg (50 mM Tris)

Glycine – 750mg (100 mM)

DW – 100ml

### Cellulose acetate strip – 1 No

### Strip holding frame – 1

### Ponceau S Stain ( 0.2 % Ponceau S in 3% TCA)

Ponceau S – 50 mg

TCA – 750 mg

DW – 25 ml

### Destaining Solution (5% V/V acetic Acid, 7% V/V ethanol)

To prepare 50 ml

Acetic acid – 2.5 ml

Ethanol – 3.5 ml

DW 44 ml

## Sample preparation

Take a drop of serum on a slide and add a few crystals of bromophenol blue and mix.

## Procedure

Mark the cellulose paper strip +ve and -ve ends with pencil. At 1 cm away from the -ve end, mark the sample application line. Take about 10 ml of buffer in a petridish and place the cellulose acetate paper strip slowly using forceps. Allow 5-10 minutes for wetting. Place the wet cellulose acetate strip on the tissue paper and gently blot it. Apply the prepared sample on the sample application line. Place the cellulose strip on immediately onto the frame. Wet the whatmann filter paper wick in the buffer and blot it. Place the wick on the cellulose acetate strip at each end. Pour 25 ml of buffer to anode and cathode reservoir slowly. Close the lid and connect the tank to the power pack and switch on. Set the voltage knob to 100v and turn on the switch on the power pack. Continue the run for about 1 hour till the bromophenol blue dye reaches the +ve end. Switch off the power pack. Air-dry the strips for about 10 minutes till the strip become completely dry. Place the strip in a petridish and add about 20 ml of Ponceau S Stain. Gently stain for 10 minutes. Destain for 5 minutes by shaking intermittently. Repeat few times with fresh destaining solution. Till the cellulose acetate background becomes white. Remove the strip and dry. Protein bands appear as pink bands.

The albumin moves most rapidly and so is found at the greatest distance from the start line. It is followed in turn by the  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  and  $\gamma$  globulins. The  $\gamma$  globulin has the smallest -ve charge. Fibrinogen (when plasma is used) is found as a narrow band between the  $\beta$  and  $\gamma$  globulin.

## Result

In paper electrophoresis the serum can be separated into a number of fraction viz albumin, globulin ( $\alpha_1, \alpha_2, \beta$ , and  $\gamma$ ) depending on sensitivity of the method of  $\beta$  can further be resolved into  $\beta_1$  and  $\beta_2$ .

# Laboratory Manual