University of Mysore
YUVARAJA'S COLLEGE
(Autonomous)
Mysuru - 570005

FIVE YEAR INTEGRATED M.Sc.
MOLECULAR BIOLOGY

A
E – BOOK
FOR

LAB - XI

Metabolism and Molecular Physiology

Editor

Dr. H.B. MAHESHA
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Experiment No. 1: Estimation of Keto Acid by DNPH.

Aim: To estimate the amount of keto acid by DNPH.

Principle: Ketoacids, when reacts with 2,4-DNPH gives a product hydrozone, which is estimated colorimetrically under alkaline condition. The concentration is directly proportional to intensity of colour, which can be read at 540 nm.

Requirements:

1. 2,4- Dinitrophenylhydrazine (DNPH) reagent: Dissolve 200 mg of DNPH in 85 mL of concentrated hydrochloric acid and make up to 1 litre with water.
2. 0.4 N NaOH:
3. Standard Pyruvic acid: Dissolve 12.5 mg sodium pyruvate in 100 mL of distilled water. Dilute this stock solution in the ratio of 1:10. The concentration of working standard is 10 µg / mL.

Observations and Calculations

Volume of standard pyruvic acid (10 µg/mL)	Volume of distilled water (mL)	Concentration of pyruvic acid (µg)	Volume of DNPH reagent (mL)	Incubate for 10 Min At room temperature	0.4 N NaOH	A540
0.0	1.0	00	0.5		10	0.00
0.2	0.8	2	0.5		10	
0.4	0.6	4	0.5		10	
0.6	0.4	6	0.5		10	
0.8	0.2	8	0.5		10	
1.0	0.0	10	0.5		10	
1.0 UK	0.0	To be estimated	0.5		10	

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.

4. Now add 0.5 mL of DNPH reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 20 min at room temperature.
6. Then add 10 mL of 0.4 N NaOH to each tube, mix the the contents and record the absorbance at 540 nm against blank.
7. Then plot the standard curve by taking concentration of pyruvic acid along X-axis and absorbance at 540nm along Y-axis.
8. Then from this standard curve calculate the concentration of keto acid in the given sample.

Result: The given unknown sample contains ----µg ketoacid/mL.

Experiment No. 2: Estimation of Urea by DAMO.

Aim: To estimate the amount of urea by DAMO.

Principle: In acidic medium DAMO is hydrolyzed in to diacetyl amine which reacts with urea during a short heating period forming a colored complex. The colour can be measured at 480 nm.

Clinical Importance: Normal blood urea level ranges from 20-40 mg per 100 mL of blood. The urea content is influenced by the amount of protein in diet. Blood urea is lower in pregnancy *i.e.,* 15-20 mg / 100 mL of blood. In some cases of severe liver diseases, the value is decreased. High urea level in blood serum is related to kidney diseases, enlarged prostate gland *etc.,*.

Requirements:

1. 2% DAMO: 2 gm of diacetylmonoxime in 100 mL of 2% acetic acid.
2. Acid mixture: Mix 25 mL of concentrated H_2SO_4 and 75 mL of 85% orthophosphoric acid and 70 mL of distilled water.
3. Standard urea solution: Dissolve 0.06 g of urea in 100 mL of distilled water. Dilute this stock solution in the ratio of 1:10. The concentration of working standard is 50 µg / mL.

Preparation of protein free filtrate: To 1 mL blood sample, add 8 mL distilled water, 0.5 mL of 2/3 N sulfuric acid and 0.5 mL of 10% sodium tungstate solution in a stoppered centrifuge tube and mix the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant as sample.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given serum sample (protein free filtrate) in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 0.4 mL of DAMO and 2.6 mL of acid mixture/colour reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 30 min in a boiling water bath and cool to room temperature.
6. Then record the absorbance at 480 nm against blank.
7. Plot the standard curve by taking concentration of urea along X-axis and absorbance at 480 nm along Y-axis.
8. Then from this standard curve calculate the concentration of urea in the given sample.

Observations and Calculations

Volume of standard urea (50 µg/mL)	Volume of distilled water (mL)	Concentration of urea (µg)	Volume of DAMO reagent (mL)	Acid Mixture (mL)	Incubate for 30 Min in a boiling water bath and cool to room temperature	A_{480}
0.0	1.0	00	0.4	2.6		0.00
0.2	0.8	10	0.4	2.6		
0.4	0.6	20	0.4	2.6		
0.6	0.4	30	0.4	2.6		
0.8	0.2	40	0.4	2.6		
1.0	0.0	50	0.4	2.6		
1.0 UK	0.0	?	0.4	2.6		

Result: The given unknown sample contains ----µg urea/mL.

Experiment No. 3: Estimation of Lactic Acid.

Aim: To estimate the amount of lactic acid.

Principle: The lactic acid is oxidized to acetaldehyde by sulphuric acid in the presence of copper sulphate, a purple colour is developed with *p* – hydroxydiphenyl. This colour can be measured at 650 nm.

Requirements:

1. 20 % Copper sulphate solution.

2. 4 % Copper sulphate solution.

3. Calcium hydroxide.

4. Sulphuric acid.

5. Parahydroxydiphenyl reagent: Dissolve 1.5 gm of PHDP in 1 mL of 5% NaOH by warming and stirring, and make up to 10 mL with water.

6. Standard lactic acid solution: Dissolve 34 mg of lithium lactate in 10 mL of distilled water in 100 mL volumetric flask. Add 0.1 mL of concentrated sulphuric acid and make up to the mark. This solution contains 0.2 mg per mL or 1mg in 5 mL.

For preparing the working standard solution, dilute this stock solution in the ratio of 1:10. This working solution contains 20µg/mL

Preparation of protein free filtrate: To 1 mL blood sample, add 8 mL distilled water, 0.5 mL of 2/3 N sulfuric acid and 0.5 mL of 10% sodium tungstate solution in a stoppered centrifuge tube and mix the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant as sample.

Procedure:

1. To 1 mL of protein free filtrate, add 1 mL of 20 % copper sulphate solution and make up the solution to 10 mL. Then add 1 gm of powdered calcium hydroxide, shake well until the contents dispersed uniformLy. Keep the test tubes at room temperature for 1 hr with interim shaking and centrifuge the contents.
2. Take 1 mL of supernatant in a clean test tube and add 0.25 mL of 4% copper sulphate solution followed by 6 mL of concentrated sulphuric acid. Mix the contents well by lateral shaking. Then keep the tube in a boiling water bath for 6.5 min. Then cool the

contents, add 0.1 mL of PHDP and mix well. Place the test tubes at room temperature for 30 min.

3. Now keep the tubes in a boiling for exactly 90 sec, cool to room temperature and read at 650 nm against the blank.
4. For standard curve take 0, 1, 2, 3, 4, 5 mL of working standard solution and treat as above.
5. Plot the standard curve by taking concentration of lactic acid along X-axis and absorbance at 650 nm along Y-axis.
6. Then from this standard curve calculate the concentration of lactic acid in the given sample.

Observations and Calculations

Volume of standard Lactic acid (20µg/mL)	Concentration of Lactic acid (µg)	Volume of 20% $CuSO_4$ solution (mL)	Makeup the volumeto 10 ml	Amount of CaOH (gm)	Keep at room temperature for 1 hr with shaking, centrifuge the content and collect the supernatant	Volume of superna tant (mL)	Volume of 4% $CuSO_4$ solution (mL))	Volume of H_2SO_4 (mL)	Incubate for 6.5 min in a boiling water bath	PHDP (mL)	Incubate at room temperature (RT) for 30 min followed by in boiling water bath for 90 seconds and cool to RT	A_{650}
0	00	1		1		1	0.25	6		0.1		0.00
1	20	1		1		1	0.25	6		0.1		
2	40	1		1		1	0.25	6		0.1		
3	60	1		1		1	0.25	6		0.1		
4	80	1		1		1	0.25	6		0.1		
5	100	1		1		1	0.25	6		0.1		
1.0 Unknown/ PFF	To be estimated	1		1		1	0.25	6		0.1		

Result: The given unknown sample contains ----µg lactic acid / mL sample.

Experiment No. 4: Estimation of Ammonia by Nitroprusside Method.

Aim: To estimate the amount of ammonia by nitroprusside.

Principle: NH_3 reacts with alkaline phosphate and hypochlorite to form indophenol blue catalyzed by nitroprusside. The colour can be measured at 600 nm.

Requirements:

1. Phenol Reagent: Dissolve 50 g of phenol and 2.5 g Na nitroprusside in 1 L water (Store in amber bottle).
2. Alkaline hypochlorite: Dissolve 25 g of NaOH in sufficient amount of water and dilute to 960 mL of water. Add 40 mL of Na hypochlorite solution.
3. 1 N NaOH: 2 g NaOH in 50 mL of water.
4. Standard: Dissolve 0.02 g $(NH_4)_2$ SO_4 in 100 mL of water. Dilute this stock solution in the ratio of 1:10 to get 20 µg/mL working standard solution.

Procedure:

7. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
8. Pipette out 1 mL of the given serum sample in another test tube.
9. Make up the volume to 2 mL with water in all the test tubes. A tube containing 2 mL of distilled water serves as the blank.
10. Now add 1 mL of phenol reagent, 1 mL of 1N NaOH and 1 mL of alkaline hypochlorite solution to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
11. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 15 min at room temperature.
12. Then record the absorbance at 600 nm against blank.
13. Plot the standard curve by taking concentration of urea along X-axis and absorbance at 600 nm along Y-axis.
14. Then from this standard curve calculate the concentration of ammonia in the given sample.

Observations and Calculations

Volume of standard ammonia (100 µg/mL)	Volume of distilled water (mL)	Concentration of urea (µg)	Volume of Phenol reagent (mL)	Volume of 1 N NaOH (mL))	Volume of Alkaline hypochlorite Solution (mL)	Incubate for 15 Min at room temperature	A_{600}
0.0	2.0	00	1	1	1		0.00
0.2	1.8	4	1	1	1		
0.4	1.6	8	1	1	1		
0.6	1.4	12	1	1	1		
0.8	1.2	16	1	1	1		
1.0	1.0	20	1	1	1		
1.0 UK	1.0	To be estimated	1	1	1		

Result: The given unknown sample contains ----µg ammonia/mL.

Experiment No. 5: Estimation of Uric Acid.

Aim: To estimate the amount of uric acid.

Principle: The uric acid in blood reduces phosphotungstic acid in the presence of sodium carbonate to blue coloured reduced phosphotungstate. The concentration is directly proportional to intensity of colour, which can be read at 700 nm.

Requirements:

1. **Tungstic acid**: Mix 25 mL of 10% sodium tungstate, 25 mL of 2/3 N H_2SO_4 and drop of phosphoric acid, make up to 400 mL with distilled water. Store in a brown bottle.
2. **Phosphotungstic acid (stock) solution:** Dissolve 50 g of sodium tungstate in about 400 mL of water. Add 40 mL of 85% phosphoric acid and reflux gently for 2 hours. Cool, transfer to 500 mL flask and make up to the mark with water. Keep this in a brown bottle
3. **Dilute solution for use:** Dilute 10 mL of the stock solution to 100 mL with water and store in a brown bottle.

4. **Sodium carbonate solution (10%).**
5. **Uric acid standard solution:** Dissolve 60 mg of lithium carbonate in 15-20 mL of water in a test tube. Heat the solution to 60 °C and pour on to 100 mg of uric acid taken in a small beaker. Stir until dissolved, heat further if necessary. Add 2 mL of 40% formalin and then slowly with shaking add 1 mL of 50% acetic acid. Make up to volume 100 mL and store in brown bottle.
6. **Uric acid working standard:** Dilute 1 mL of the stock to 20 mL with water. Store in a brown bottle. This contains 0.005 mg uric acid per mL.

Preparation of protein free filtrate: To 1 mL serum sample, add 9 dilute tugstic acid in a stoppered centrifuge tube and mix the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant as sample. 5 mL of supernatant is equivalent to 0.5 mL of serum.

Clinical Significance: The normal range of uric acid is 3-9 mg /100 mL of serum. Increased level indicates the diseases of joints.

Procedure:

1. Pipette out 0.0, 1, 2, 3, 4 and 5 mL of working uric acid standard in to the series of labeled test tubes.
2. Pipette out 5 mL of the given sample/ protein free filtrate in another test tube.
3. Make up the volume to 5 mL in all the test tubes. A tube with 5 mL of distilled water serves as the blank.
4. Now add 1 mL of sodium carbonate solution and 1 mL of dilute phosphotungstic acid solution to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 3 min at 25 °C in a water bath.
6. Then record the absorbance at 700 nm against blank.
7. Then plot the standard curve by taking concentration of uric acid along X-axis and absorbance at 700 nm along Y-axis.
8. Then from this standard curve calculate the concentration of uric acid in the given sample.

Observations and Calculations

Volume of standard Uric acid (5 µg/mL)	Volume of distilled water (mL)	Concentration of uric acid (µg)	Volume of Sodium carbonate solution (mL)	Volume of Phosphotungstic acid solution (mL)	A_{700}
0.0	5	00	1	1	0.00
1	4	5	1	1	
2	3	10	1	1	
3	2	15	1	1	
4	1	20	1	1	
5	0.0	25	1	1	
5 UK	0.0	To be estimated	1	1	

Result: The given unknown sample contains ----µg uric acid/mL.

Experiment No. 6: Extraction of Phospholipids.

Aim: To extract phospholipids and cholesterol from egg yolk.

Requirements:

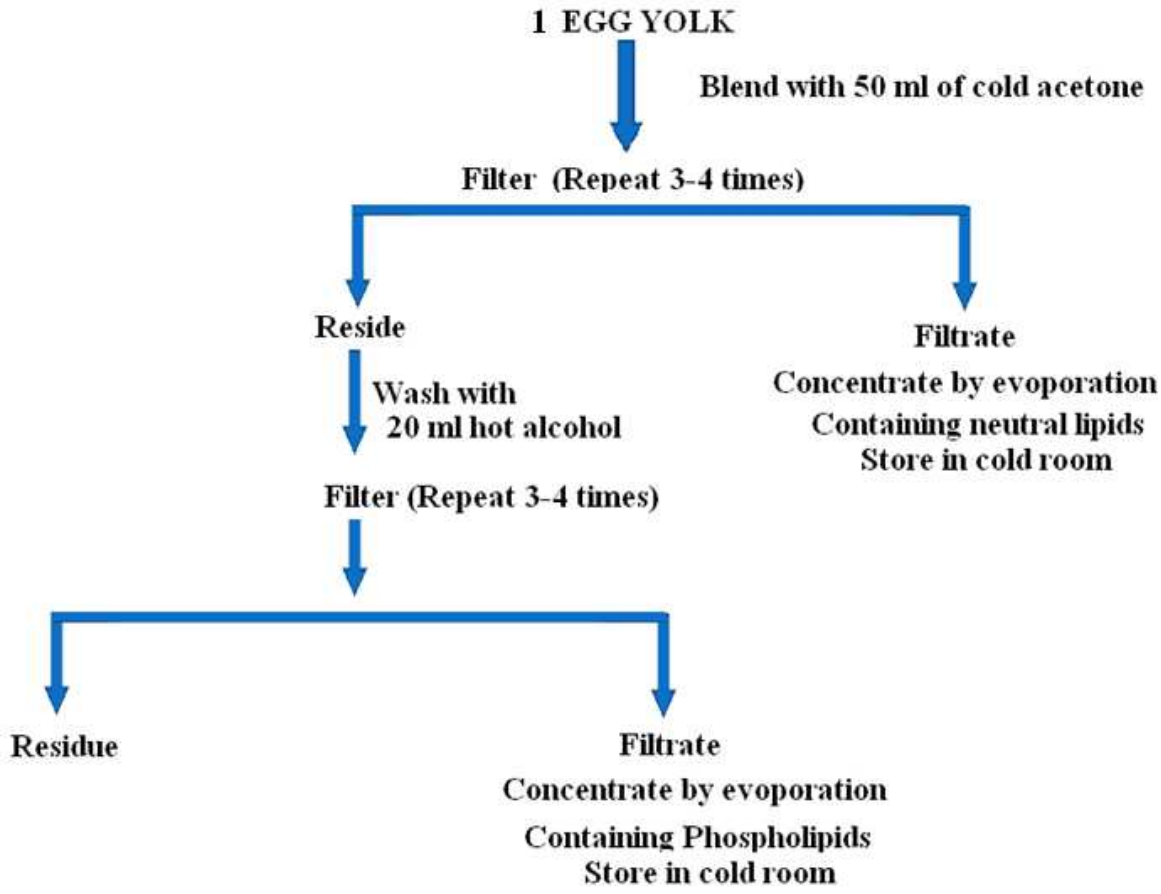
Eggs, acetone, alcohol, filter paper *etc*.,

Procedure:

1. Take a fresh chicken egg yolk in a beaker or blender and blend with 50 mL of cold acetone. Allow the mixture to stand for 10 min.
2. Filter through filter paper or muslin cloth and collect the filtrate in a separate container.
3. Repeat the steps 1 and 2 thrice or until the yolk become white in colour.
4. The acetone extracts containing most of the neutral lipids and pigments. This may be concentrated using flash evaporation or cold drying.

5. Then blend the solid residue with 20 mL of hot alcohol and filter to collect the supernatant.
6. Repeat the step 5 thrice and collect the filtrate in a separate container.
7. The alcohol extract contains phospholipids and concentrate using flash evaporation or cold drying.

   After evaporation calculate the yield of phospholipids as well as cholesterol. These compounds may be run on TLC for confirmation.



Experiment No. 7: Estimation of Phospholipids.

Aim: To estimate the amount of phospholipids by Bartlett method.

Principle: The organic phospholipid phosphorous is converted in to inorganic phosphorous which reacts with ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with ANSA forms a stable blue colour. This colour can be measured at 660 nm.

Requirements:

1. **Perchloric acid**.
2. **Ammonium molybdate reagent:** 2.5 % ammonium molybdate in 5 N sulphuric acid.
3. **Aminonaphthol sulphonic acid (ANSA):** Dissolve 500 mg ANSA in a mixture of 195 mL of 15% sodium bisulphate and 5 mL of 20 % sodium sulphite solution.
4. **Standard phosphorous solution:** Dissolve 35.1 mg of potassium dihydrogen phosphate in 100 mL of distilled water. Prepare the working standard by diluting this stock solution in the ratio of 1:10 to give a concentration of 80 µg phosphorous/mL

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given serum sample in another test tube.
3. Now add 0.5 mL of perchloric acid to all the test tubes including the test tubes labeled 'blank' and 'unknown', mix the contents and digest over a sand bath until they become colourless.
4. Then make up the volume to 4.3 mL with distilled water.
5. Now add 0.5 mL of ammonium molybdate solution. After 10 min add 0.2 mL of ANSA and incubate at room temperature for 20 min.
6. Then record the absorbance at 660 nm against blank.
7. Plot the standard curve by taking concentration of ip along X-axis and absorbance at 660 nm along Y-axis.
8. Then from this standard curve calculate the concentration of phospholipid in the given sample.

Note: The colour produced is proportional to the concentration of phosphorous upto 1.5 µ moles in the reaction mixture. These values may be expressed as the phospholipid (Lecithin) by multiplying by a factor of 25.

Observations and Calculations

Volume of standard phosphorous (500 µg/mL)	Concentration of phophorous (µg)	Volume of Perchloric acid (mL)	Digest over a sand bath until the mixture become colour less and clear	Volume of distilled water (mL)	Volume of Ammonium molybdate Solution (mL)	Incubate for 10 Min at room temperature	ANSA (mL)	Incubate for 20 Min at room temperature	A_{660}
0.0	00	0.5		4.3	0.5		0.2		0.00
0.2	16	0.5		4.3	0.5		0.2		0.17
0.4	32	0.5		4.3	0.5		0.2		0.34
0.6	48	0.5		4.3	0.5		0.2		0.51
0.8	64	0.5		4.3	0.5		0.2		0.68
1.0	80	0.5		4.3	0.5		0.2		0.9
1.0 UK	To be estimated	0.5		4.3	0.5		0.2		0.17

O.D. of the sample = ------- (A)

From the standard curve this O.D. gives ------- (B) µg of phosphorous.

i.e., $\frac{\text{B x 25}}{\text{Amount of sample taken in mg or mL (C)}} = \frac{\text{B x 25}}{\text{C}}$ = ------ (D) µg of phospholipid/ mg or mL sample.

Result: The given unknown sample contains ----µg phospholipid / mg or mL sample.

Experiment No. 8: TLC of Neutral Lipids and Phospholipids.

Aim: To identify the lipids in the given sample by thin layer chromatography.

Principle: This technique is similar to paper chromatography but is more convenient and less time consuming. Here instead of paper, the supporting material is either a glass plate or a plastic sheet or a piece of metal foil. A thin layer of stationary *i.e.*, silica gel (SiO_2) or alumina (Al_2O_3) is laid over this inert support. The solvent system is selected according to type of biomolecule under investigation.

Requirements:

1. **Activated TLC plates**: Place thoroughly cleaned and dried glass plates (20x20 cm) and spread a uniform layer of (0.2mm thickness) Silica Gel-G slurry with the help of a spreader, dry at room temperature and then activate at 110 °C for 30 min.
2. **Thin layer chromatographic tanks**:
3. **Developing Mixture/solvent system**: petroleum ether:diethyl ether:glacial acetic acid (80:20:1 v/v)
4. **Spraying reagents for location of spots on TLC plates:** 50% sulphuric acid.
5. **Lipid standards:** Cholesterol, Palmitate, Lecithin etc.,

Procedure:

1. Take an activated TLC plate and draw two straight lines. First one about 2 cm from the bottom and second one 1 cm from the top of the plate.
2. Subdivide the bottom line for spotting the samples with 2 cm between two samples.
3. Pipette 20 µl of all the standard and test samples, and spot in an order on the TLC plates. Air dry the plates for 5-10 min.
4. Meanwhile add solvent (mobile phase) to the TLC chamber and close it with the lid. Allow it to saturate the chamber for 10 min at room temperature.
5. Using forceps, pickup the TLC plate from the top; place the TLC plate in the TLC chamber vertically. Ensure that the solvent phase moves uniformLy along the plate.

6. Leave the plate in the chamber until the solvent has moved to the top pencil line of the TLC plate. When the solvent front has moved to the top line remove the plate with the help of forceps.
7. Place the TLC plate on a clean dry surface or on tissue and allow the mobile phase to evaporate completely for about 5-10 min.
8. Spray the detection reagent carefully and heat it in an oven at 110 °C for 5-10 min. areas containing lipids get charred and appear as black spots.
9. Locate the position of the lipid spots on the plate and measure the distance travelled by the individual lipid component.

Calculate the R_f value of each sample, identify and report the lipids present in the sample given.

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

Sample	R_f value
Standard (Cholestrol)	a
Standard (Lecithin)	a1
Sample No. 1	a

Report: The given standard No. 1 has the R_f value of ---- (a) and standard No. 2 has the R_f value of ----(a1).

The given sample No. 1 has the same R_f value of standard No. 1. Hence the sample No. 1 might be Cholesterol.

Experiment No. 9: Viability of Cells by Tryphan Blue Dye Exclusion.

Aim: To assay the viability of cells in the given sample using Tryphan blue.

Principle: Viability assay measures the percentage of cell suspension that is viable. Tryphan blue is a chemical dye can be used for this purpose. This is accomplished by dye exclusion, where the cells with intact membrane are able to exclude the dye, while the cells without an intact/viable membrane take-up the colouring agent.

Materials required: Cell sample, Tryphan blue (0.4% in distilled water), haemocytometer, compound microscope, *etc*.,

Procedure:

1. Prepare cell suspension of the given sample to be assayed using distilled water (approximately 10^6/mL).
2. Make 1:1 dilution of the suspension using 0.4 % tryphan blue solution.
3. Load the counting chamber of haemocytometer with the suspension.
4. Allow the preparation for few min (1-2) to settle. Count the number of unstained, stained and total number of cells using regular procedure of cell counting by haemocytometer.
5. Total number or percentage of viable cells *i.e*., unstained cells represents the percentage of viable cells.

Observations and Calculations:

No. of Stained Cells (A_1)	No. of Unstained Cells (A_2)	Total No. of Cells ($A_1+A_2=A_3$)	No. of Squares Counted (A_4)	Average No. of Cells		Average No. of Cells [Total] (a+b=c Or A_3/A_4)
				Stained (A_1/A_4=a)A	Unstained (A_2/A_4=b)	
100	50	150	5	20	10	30

Report: Based on the stain exclusion principle, the total number of viable cells in the given sample is -------

---

Experiment No. 10: Determination of Kinetics of Glucose uptake by Erythrocytes.

Aim: To determine the kinetics of glucose uptake by erythrocytes.

Introduction: RBCs are structurally and metabolically compared to other cells. Mature RBCs do not possess nuclear and cytoplasmic subcellular structures. RBCs are entirely dependent on glucose for its energy. Glucose is permeable in to erythrocytes. Glucose oxidation always ends in the formation of pyruvic acid. Due to absence of enzyme PDC, pyruvate is not converted to acetyl Co-A. In RBC glycolytic pathway takes diversion and forms 2,3 bisphosphoglycerate. This diversion is called Rapport Luebering Cycle. In this assay known amount of glucose is added to 50 µl of RBC and incubated. The glucose retained in the solution after the particular time intervals is determined using DNS method.

Isolation of RBC:

1. To 9 mL of freshly drawn blood add 1 mL of 3.2 % trisodium citrate, centrifuge at 1500 rpm for 15 min and separate supernatant plasma.
2. Then wash the packed erythrocytes with 10mM PBS, pH 7.4 thrice at 1500 rpm. After washing use erythrocytes for assay.

Requirements:

1. **Phosphate Buffered Saline (pH 7.4):** Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4.2H_20$ and 0.24 g KH_2PO_4 in 1 lt distilled water and set the pH 7.4.
2. **Glucose Solution:** Dissolve 90 mg glucose in 100 mL distilled water.
3. **Dinitrosalicylic acid reagent:** i. Suspend 1 gm of DNS powder in 10 mL distilled water, then add 10 mL of 4 N NaOH and make a clear solution. ii. Dissolve 30 g potassium sodium tartarate in sufficient water. Mix solutions i and ii and make up to 100 mL with water.

Procedure:

1. Pipette out 2 mL of glucose solution in to the series of labeled 6 test tubes.

2. Add 50 µl of erythrocytes to all test tubes.

3. Now centrifuge the first test tube as soon as the RBCs are added and collect 1 mL of supernatant. This tube serves as blank (otherwise 1 mL glucose solution may be used as blank).

4. Then incubate the remaining test tubes for 5, 10, 15, 20, 25 min at room temperature.

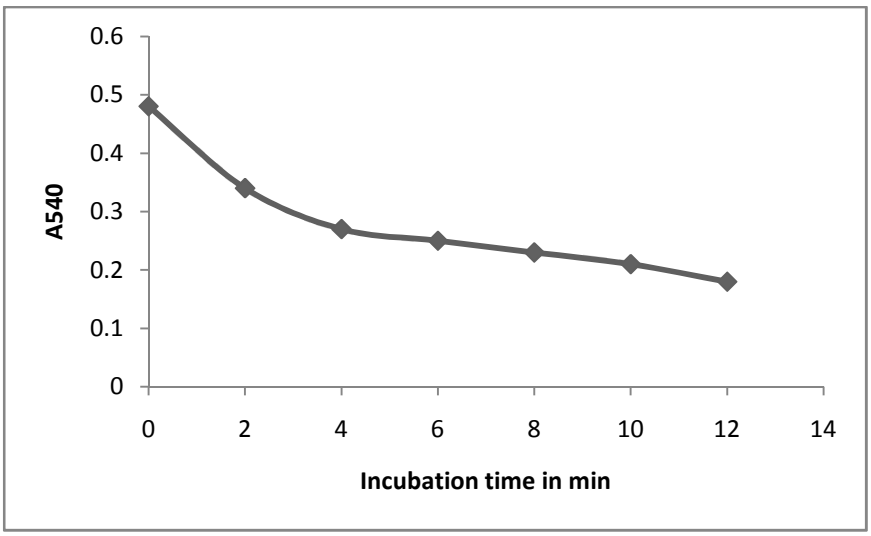
5. After incubation centrifuge the tubes and collect 1 mL of supernatant as samples.

5. Now add 0.5 mL of DNS reagent, incubate on a boiling water bath for 10 min, cool to room temperature, add 2.5 mL of water, mix and read at 540 nm.

7. Plot the curve by taking time of incubation along X-axis and absorbance at 540 nm along Y - axis.

Observations and Calculations:

Sl. No	Volume of Glucose (mL)	Volume of RBC (µl)	Incubation time (min)	Centrifuge at 3000 rpm for 5 min	Volume of supernatant (mL)	Volume of DNS (mL)	Keep in boiling water bath for 10 min	Volume of distilled water (mL)	A 540
1	2	50	0		1	0.5		2.5	0.48
2	2	50	2		1	0.5		2.5	0.34
3	2	50	4		1	0.5		2.5	0.27
4	2	50	6		1	0.5		2.5	0.25
5	2	50	8		1	0.5		2.5	0.23
6	2	50	10		1	0.5		2.5	0.21
7	2	50	12		1	0.5		2.5	0.18



Result: Reduction of glucose concentration indicates the gradual uptake of glucose by erythrocytes.

Experiment No. 11: Photosynthetic Reduction of 2, 6-Dichlorophenolindophenol.

Aim: To study the photosynthetic oxidation of 2, 6-dichlorophenolindophenol.

Principle: One of the first steps of photosynthesis is the splitting of water to donate two electrons to the reaction center, P680. This reaction is known as the Hill reaction. The electrons donated to P680 move through an electron transport chain to the reaction center P700, and eventually, to reduce NADP to NADPH. The complete reaction can be summarized as follows:

$$H_2O + NADP \xrightarrow[\text{Chloroplasts}]{\text{Light}} NADPH + 1/2\ O_2 + H^+$$

Other electron acceptors can be substituted for NADP, which allow the Hill reaction to be measured, and some of it components studied. The most commonly used electron acceptor is the dye 2,6 dichlorophenolindophenol (DCIP), which can accept electrons instead of P700. When DCIP is added to a chloroplast or thylakoid suspension, the following reaction occurs:

$$H_2O + DCIP \xrightarrow[\text{Photosystem II}]{\text{Light}} DCIP\ (\text{reduced}) + 1/2\ O_2$$

DCIP is a blue color in its oxidized form. When reduced, it is colorless. This property of DCIP allows the measurement of the rate of the Hill reaction.

Reagents: 1. Potassium phosphate buffer with 0.3M sucrose- 125mM pH 7.5.

2. 30 µg/mL DCIP in water.

Procedure: Prepare 25% homogenate of fresh Spinach leaves in potassium phosphate buffer with 0.3M sucrose, filter and centrifuge the filtrate at 1000g for 5 min. Resuspend

the chloroplast pellet in 10 mL of buffer and store on ice. In a test tube take 1.9 mL water, 3 mL buffer, 0.5 mL chloroplast suspension and 0. 1 mL DCIP, mix and keep in sunlight for 1 hour. A suitable control should also be prepared and keep in dark. Prepare a standard curve using different concentrations of DCIP on X-axis against absorption at 550 nm on Y-axis.

STANDARD CURVE:

Sl. No.	Water	DCIP	Concentration of DCIP (µg)	A_{550}
1	5.5	0.0	0	0.00
2	5.3	0.2	6	
3	5.1	0.4	12	
4	4.9	0.6	18	
5	4.7	0.8	24	
6	4.5	1.0	30	

Test:

Serial Number	Water	Buffer	Chloroplasts	DCIP	A_{550} against water
T-1 Incubated in dark	1.9	3	0.5	0.1	
T-2 Incubated in sunlight	1.9	3	0.5	0.1	

Calculation: Amount of DCIP reduced = O.D. of T1 - O.D. of T2 = ……..

From standard curve ……..µmole of DCIP reduced per hour.

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