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BBCCL-112
MEMBRANE BIOLOGY AND BIOENERGETICS (LABORATORY)

MEMBRANE BIOLOGY AND BIOENERGETICS

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BBCCL-112 MEMBRANE BIOLOGY AND BIOENERGETICS

Dear learners welcome to the practical sessions of Membrane Biology and Bioenergetics. The lab exercises and experiments provided in this manual are based on the syllabus that you have studied in BBCCT-111. The concepts that you have studied in theory course of membrane biology and bioenergetics will be experienced during performing these lab experiments.

This lab course is worth 2 Credits and consists of seven laboratory experiments. The experiments are designed in such a way that you shall be able to experience and connect the theoretical concepts explained so far. Here also the basic concepts on which the experimental procedures are based have been discussed as required.

Expected Learning Outcomes

The broad objective of this lab course is to enable you to:

- Isolate RBC from blood sample;
- Evaluate the disrupting effect of detergent like molecules on biomembranes by dissolving the phospholipid component;
- Prove that during photosynthesis oxygen is evolved;
- Isolate and estimate the photosynthetic pigments;
- Isolation of mitochondria from rat liver; and
- Assay of mitochondrial marker enzyme succinate dehydrogenase.

Study Guide

We advise you to go through respective units of BBCCT-111 before you come to attend the practical sessions. This will enable you to easily understand the purpose of doing experiments and their applications. You should also read the principles of each experiment of this course along with procedure before you start performing the experiment. It is always good to prepare all the reagents freshly and store them under prearranged storage conditions. Adhere to all the safety measures and follow the safety instructions while handling the reagents. One of the good laboratory practices is to maintain your log books up-to-date i.e., enter the observations made while performing the experiments. Carry this laboratory manual and your log book during lab sessions.

Like all other IGNOU laboratory courses this is an intensive residential exercise requiring one week for completing 2 credits. Everyday there will be two laboratory sessions of 4 hours each. So there will be a total of 14 sessions. The first session will be introductory and the remaining 2ndto 12thsessions will be based on the exercises given in the course. A schedule for laboratory exercises will be given to you in the first session. Sessions 1 to 12 shall have guided exercises under the supervision of the Academic Counsellor. The last two sessions i.e., 13thand 14th will be unguided sessions and that shall be the term end examination. In each

session you shall perform exercises for 3 hours and in the remaining 1 hour you are advised to complete your practical note book. The laboratory notebook must be submitted to the counsellor for corrections and grading. 70% marks have been allocated for doing the experiments and for recording it properly. You are aware that there is a time constraint as you will have limited access to laboratory work; therefore, you are required not to miss any of the laboratory sessions.

Assessment of the experiments will be graded and you will have to appear for the *viva-voce* at the end of the practical session. At the end of the laboratory session you should perform the assigned experiment, which will be graded and final assessment will be made based on the continuous performance during the laboratory sessions, maintenance of log books and records followed by *viva-voce*. 30% marks are reserved for the assigned experiments.
For the better understanding of how to use laboratory apparatus few video links shall be provided where ever available. There might be a slight difference in the steps or procedure being explained in the video when compared to the procedure provided in this self-instructional material. However, the principles and reagents remain same. Hence, there is no need to worry about slight modifications adopted in the procedure.

We wish you best in this endeavour!!

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IMPORTANT INFORMATION

Attendance is compulsory in the Laboratory Course work held generally at the Study Centre.

The Laboratory Course is worth **2 credits** to be completed over **7 days** duration.

- **6 days** of **Guided** Laboratory work
- **1 day** for the **Unguided** Laboratory work

To successfully complete the laboratory course you will have to pass (at least **35% marks**) in the Guided and Unguided components separately.

EXPERIMENT 1

PREPARATION OF RBC GHOST CELL

Structure

1.1 Introduction

Expected Learning Outcomes

1.2 Principle

1.3 Materials Required

1.4 Procedure

1.5 Observations and Results

1.6 Precautions

1.7 Self-Assessment Questions

1.1 INTRODUCTION

In this laboratory exercise you will be learn how to prepare the RBC ghost cell. Membranes which are present in biological system are also known as biomembranes. These biomembranes share many features and structural components; however, they differ in composition and properties depending on activity of the cell or organelle. As you know, biological membranes are made up of lipids, proteins and carbohydrates and act as barrier to flow of molecules (Fig. 1.1).

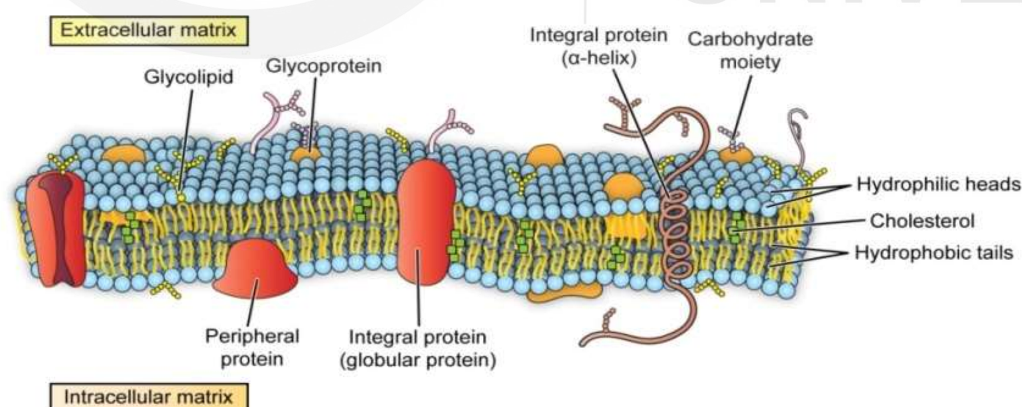

Fig. 1.1: Membrane structure: lipid bilayer, integrated protein and attached carbohydrate in the form of receptor with protein.

Proteins are present at the surface or embedded in the lipid bilayers and act as channels, transporters, pumps, enzymes and receptors. Carbohydrates are associated with lipids and/or proteins as oligosaccharides at the outer surface and act as cellular markers and help in recognition. Lipids form a bilayer of thickness ranging from 6-10 nm in most of the membranes and act as barrier

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to flow of polar molecules. In general, five types of lipids are present in the membranes: glycerophospholipids, sphingolipids, galactolipids, sulfolipids and sterols. All these are amphiphilic in nature.

Red blood cell (RBC) membrane is an extraordinarily valuable model for studying membrane structure and function. During its 4-month life span, human red blood cell membrane undergoes large reversible deformations still maintaining its structural integrity. It rapidly responds to applied fluid stresses, and is stronger than steel in terms of structural resistance. The red blood cell membrane consists of three basic components: a lipid bilayer, transmembrane (integral) proteins and a cytoskeletal network. A diagrammatic representation of the RBC membrane is given in Fig. 1.2. Membrane asymmetry is said to be present if the two individual layers differ in lipid composition.RBC membrane exhibit membrane asymmetry as sphingomyelin and phosphatidyl choline are preferentially located in the outer leaflet of the bilayer, whereas phosphatidyl ethanolamine and phosphatidyl serine are present mainly in the inner leaflet. A significant amount of cholesterol is present in both the leaflets. The major transmembrane proteins are **glycoproteins**, **band 3** and **glycophorin**.

Band 3 is a multispanning ion transport channel and exists in dimer / tetramer equilibrium. Band 3 tetramers link/tie the bilayer to the RBC skeleton. This linking is via an interaction between Band 3 cytoplasmic domain and ankyrin which is associated with spectrin.

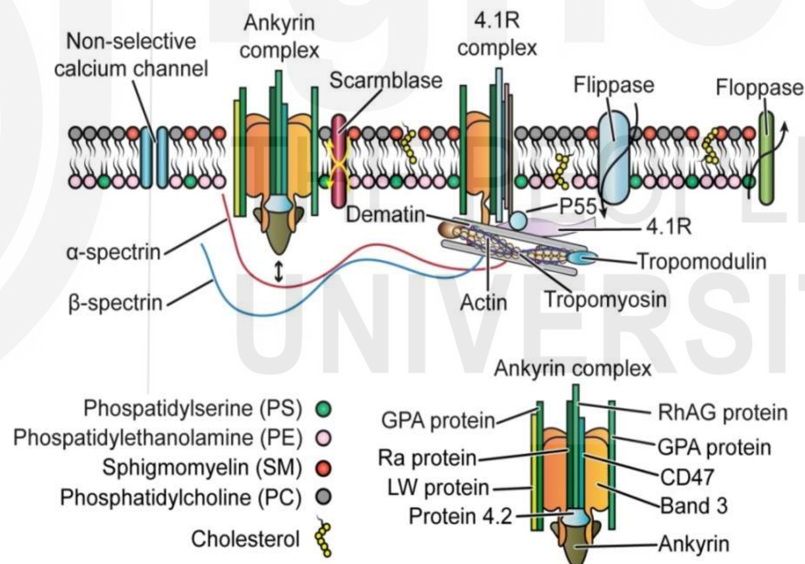


Fig. 1.2: Diagrammatic representation of the RBC membrane.

Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- explain the composition of a membrane;
- describe the principle behind RBC ghost preparation;
- explain the significance of RBC ghosts in membrane studies; and
- prepare RBC ghosts;

1.2 PRINCIPLE

The RBC ghosts are post-hemolytic residues of red blood cells. This means RBC ghosts are devoid of any intracellular structures and consist primarily of the cell membrane. When RBC membrane is treated with isotonic solution it loses its hemoglobin, so that only the membranes and free hemoglobin remains. These empty membranes are known as RBC "ghost" cells. Ghost cells are widely used in the study of structure, function and composition of red blood cells.

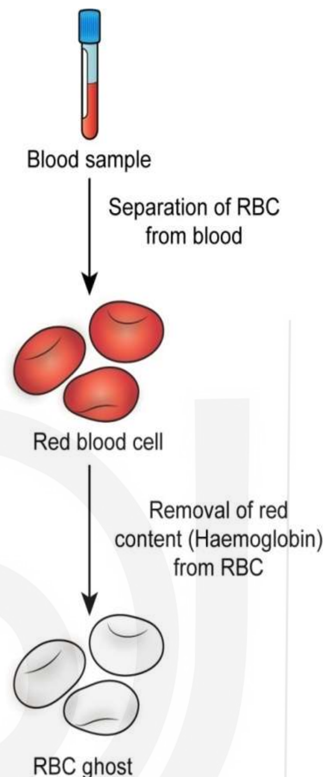


Fig. 1.3: Steps involved in the preparation of RBC ghost membrane.

1.3 MATERIALS REQUIRED

1. Fresh rat blood or time-expired human blood used for transfusion (2-3 ml)
2. Isotonic saline (8.9 g/liter) solution
3. Trisodium citrate, an anticoagulant (4 g/ 100 ml)
4. Centrifuge tubes (10 ml)
5. Incubator
6. Centrifuge machine
7. Colorimeter or spectrophotometer
8. Distilled water, and
9. Reagents for protein estimation by Lowry's method (optional)

Finger stick method

https://youtu.be/mmC5tDCviBc

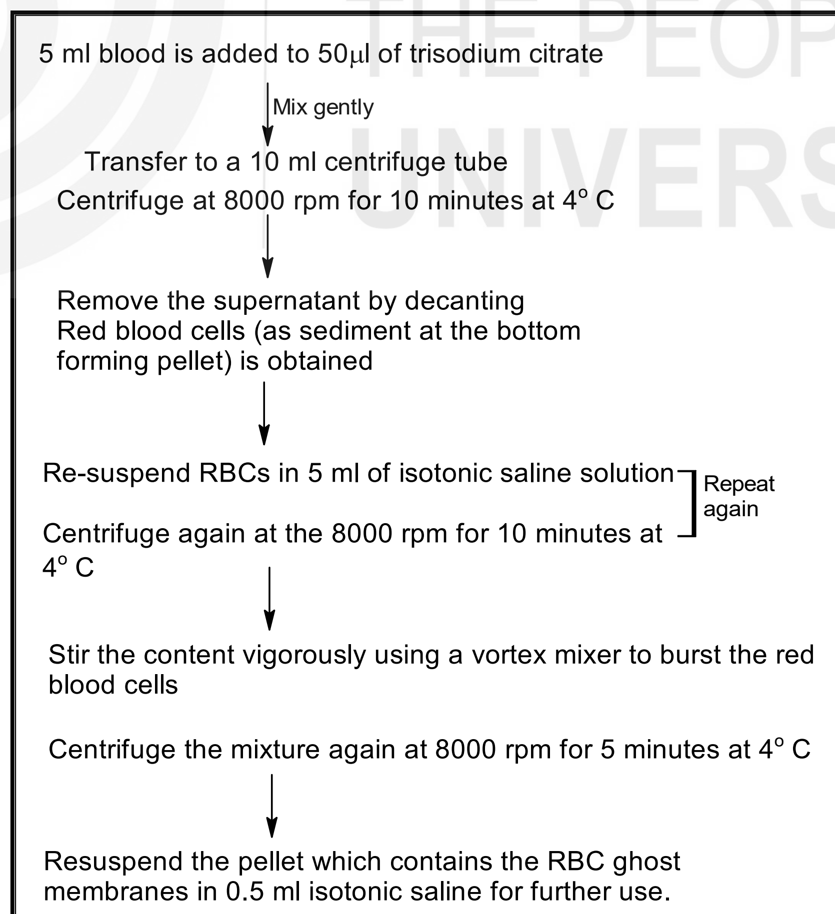
1.4 PROCEDURE

Plasma is a clear, straw coloured fluid portion of blood that remains after RBC, WBC, platelets and other cellular components are removed. It contains water, salts, enzymes, antibodies and other proteins.

Serum may be defined as blood plasma without fibrinogens. It includes all proteins not used in blood clotting such as electrolytes, antibodies, antigens, hormones, and any exogenous substances.

1. The blood sample is collected in a tube containing an anticoagulant trisodium citrate (5 ml blood is added to 50 μl of trisodium citrate) with the **finger stick method**. Gently mix this blood sample.
2. Transfer it to a 10 ml centrifuge tube and centrifuge at 8000 rpm for 10 minutes at 4°C.
3. After centrifugation, the plasma would appear as the upper layer as supernatant, while red blood cells would sediment at the bottom of the tube forming pellet.
4. Remove the supernatant by decanting and re-suspend the RBCs sedimented at the bottom of the tube, in 5 ml of isotonic saline solution. Centrifuge the tube again at the 8000 rpm for 10 minutes at 4°C. Repeat this washing step once again.
5. Gently re-suspend the washed RBCs in 5 ml distilled water. Stir the content of the tubes vigorously using a vortex mixer to burst the red blood cells.
6. Centrifuge the mixture again at 8000 rpm for 5 minutes at 4°C. Recover the pellet which contains the RBC ghost membranes, and re-suspend in 0.5 ml isotonic saline for further use.

Flow chart of the work process is given below.



1.5 OBSERVATIONS AND RESULTS

Take the suspended solution and measure the absorbance at 540 nm. Isotonic solution can be used as blank.

You can also measure the concentration of membrane protein in this sample by **Lowry method (for detail refer Experiment 3 in BBCCL 106)** using a colorimeter or a spectrophotometer and measuring the absorbance at 660 nm.

1.6 PRECAUTIONS

1. Any possibility of hemolysis must be avoided during collection of blood.
2. The anticoagulant should be mixed gently with the blood sample.
3. Mixing of the pellet before centrifugation should be gentle.
4. When working with tissues and body fluids infection can occur. So, while handling these materials precaution should be taken.
5. Waste should be sterilised properly in incubator kept specifically for that purpose and then should be disposed of.

1.7 SELF-ASSESSMENT QUESTIONS

1. Define serum.
2. Differentiate between serum and plasma.
3. What is isotonic solution
4. What is anticoagulant? Give example.
5. Explain the principle behind RBC ghost formation.
6. Explain the composition of a biomembrane.

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EXPERIMENT 2

STUDY THE EFFECT OF DETERGENTS ON MEMBRANE

Structure

2.1 Introduction

Expected Learning Outcomes

2.2 Principle

2.3 Materials Required

2.4 Procedure

2.5 Observation and Result

2.6 Precautions

2.7 Self-Assessment Questions

2.1 INTRODUCTION

Detergents cause the disruption of membrane by dissolving the phospholipid bilayer. The importance of detergents as tools for the study of membrane proteins cannot be underestimated. They are extensively used in the isolation and purification of the protein. In the case of RBC ghost cells, the effect of detergent is measured by measuring the absorbance of hemoglobin which is released from disrupted membrane. In this laboratory exercise you will observe the effect of different concentration of detergents on the RBC ghost cell which you have prepared in the previous experiment. You can also study the effect of various different detergents in similar manner.

Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- calculate percent lysis;
- explain the effect of detergents on the RBC ghost cell;
- describe the effect of detergent on membrane; and
- measure the effects of detergents on membrane in terms of absorbance.

2.2 PRINCIPLE

Most of the detergents disrupt the membrane by dissolving the lipid bilayer. In this experiment the effect of detergents on ghost cell membrane is monitored by measuring the absorbance of light by hemoglobin released from the disrupted RBC membrane.

2.3 MATERIALS REQUIRED

1. RBC ghost membrane suspended in 0.5 ml isotonic saline (from Experiment No 1)
2. Isotonic saline (8.9 g/liter) solution
3. Detergent solutions:

a) Triton X-100 (1 g / 100 ml)

b) Sodium dodecyl sulfate (1 g / 100 ml)

c) Lysophosphatidyl choline (10 mmol/ liter)

4. Centrifuge tubes (10 ml)
5. Incubator
6. Centrifuge machine
7. Colorimeter or spectrophotometer
8. Distilled water

2.4 PROCEDURE

1. Take 0.5 ml of the RBC ghost membranes and make up the volume to 5 ml by adding 4.5 ml of isotonic saline. Mix thoroughly.
2. Add 50 µl of the detergent solution (Triton X-100 or sodium dodecyl sulfate orlysophosphatidylcholine) and mix gently by swirling.
3. Place in an incubator at 37°C for 20 minutes.
4. After completion of incubation, centrifuge the mixture at 8000 rpm for 5 minutes at 4°C.
5. Take the supernatant and measure the absorbance at 540 nm using a colorimeter or a spectrophotometer.
6. **For measuring absorbance after "100 % Lysis" of membrane**

i) Take 0.5 ml of RBC ghost membranes (suspended in isotonic saline) and add 4.5 ml of Triton X-100 solution.

ii) Mix it thoroughly and incubate at 37°C for 20 minutes.

iii) Centrifuge at 8000 rpm for 5 minutes at 4°C.

iv) Take the supernatant and measure the absorbance at 540 nm. This represents "100 % Lysis" mentioned in the table.

2.5 OBSERVATION AND RESULTS

The value of absorbance is converted to represent lysis in percentage. First, there is a need to set the limits of maximum and minimum membrane lysis. The minimum limit is set by blank (in this case it is isotonic saline) and maximum by the detergents with 100% lysis.

$$\% \, \boldsymbol{lysis} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of 100\% lysis}} \text{ X } 100$$

The observation can be recorded in following manner:

SN	Volume of suspended RBC ghost membrane (ml)	Volume of isotonic solution (ml)	Volume (ml) of Detergent (Triton X-100) solution added	Mix and incubate at 37^0C for 20 min. Centrifuge and measure the absorbance of the supernatant.	Absorbance at 540 nm	% Lysis	Mean of the duplicates
Test sample	0.5	4.5	0.05				
	0.5	4.5	0.05				
Blank (isotonic saline)	-	4.5	0.02 (distilled water)				
100% Lysis	0.5	0	4.5				
	0.5	0	4.5				

You may examine the effects of as many detergents as you like in the same manner. First, it is advised to examine a range of concentrations of the detergent Triton X-100 (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, and 0.40 ml) and prepare a graph of percent membrane lysis (hemolysis) against the concentration of the detergents.

Repeat the experiment with the other detergents like lysolecithin, progesterone, etc and comment on the relative effectiveness of these detergents on the membrane lysis.

2.6 PRECAUTIONS

1. Mixing of the pellet before centrifugation should be gentle.
2. While using different detergents use clean pipettes and test tubes to avoid contamination and faulty results.

2.7 SELF-ASSESSMENT QUESTIONS

1. Explain the role of detergent on membrane.
2. What are detergents?
3. What is meant by 100 percent lysis.

EXPERIMENT 3

STUDY THE PHOTOSYNTHETIC OXYGEN (O_2) EVOLUTION IN *HYDRILLA* PLANT

Structure

3.1 Introduction

Expected Learning Outcomes

3.2 Principle

3.3 Materials Required

3.4 Procedure

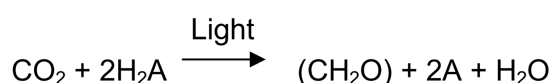
3.5 Observations and Results

3.6 Precautions

3.7 Self-Assessment Questions

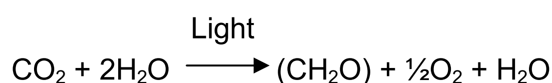
3.1 INTRODUCTION

Photosynthesis is a biological oxidation-reduction process in which light energy is converted into stable chemical products as described by the following general equation:



$$CO_2 + 2H_2A \xrightarrow{\text{Light}} (CH_2O) + 2A + H_2O$$

Here CO_2 is the electron acceptor and H_2A is a reduced compound that can serve as an electron donor. In this process, CO_2 is reduced into carbohydrate (CH_2O), while H_2A is oxidized into A. Plants, algae and cyanobacteria exhibit oxygenic photosynthesis in which water (H_2O) is used in place of H_2A, and its oxidation produces molecular oxygen which has been sustaining life on our planet.



$$CO_2 + 2H_2O \xrightarrow{\text{Light}} (CH_2O) + \frac{1}{2}O_2 + H_2O$$

Photosynthesis takes place in chloroplasts of green plants. It utilizes solar energy, carbon dioxide (from atmosphere) and water. Water undergoes photolysis during this process resulting in oxygen evolution.

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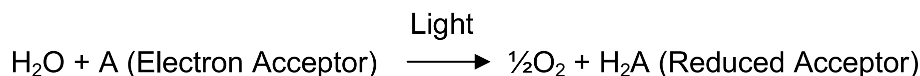
Robert Hill, a plant physiologist during 1930s, carried out simple and elegant experiments on isolated chloroplasts. He demonstrated that isolated chloroplasts when illuminated in the presence of an artificial electron acceptor (such as ferroxalate or ferricyanide, later benzoquinones and 2,6 - dichlorophenol indophenols, DCPIP were also used) could evolve molecular O_2 by splitting water even when CO_2 fixation was not taking place. This experiment is widely known as the Hill reaction in the honour of its discoverer. This marked the beginning of understanding the relationship between light induced electron transfer (light reaction) and CO_2 fixation (dark reaction). By radiolabelling studies, it was later demonstrated that the oxygen evolved during the Hill reaction was derived from oxidation of water molecules.



$$H_2O + A \text{ (Electron Acceptor)} \xrightarrow{\text{Light}} \tfrac{1}{2}O_2 + H_2A \text{ (Reduced Acceptor)}$$

In this exercise, you will learn to demonstrate evolution of oxygen during photosynthesis in Hydrilla plant.

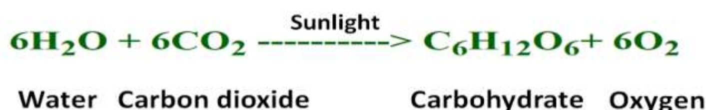
Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- explain photosynthesis;
- prove that oxygen is evolved during photosynthesis in green plants; and
- describe photolysis of water and explain evolution of oxygen.

3.2 PRINCIPLE

Atmospheric carbon dioxide is combined with water in oxygenic photosynthesis to produce carbohydrate while oxygen is evolved in gaseous form. This process occurs in thylakoid membranes of chloroplasts which have photosynthetic pigments such as chlorophylls and proteins embedded in it. Thylakoid membrane is an energy transducing membrane which converts solar energy into chemical energy. The overall reaction of photosynthesis is as follows:



3.3 MATERIALS REQUIRED

1. Hydrilla twigs
2. Beaker
3. Glass funnel
4. Test tube
5. Pond water
6. Glowing incense stick

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3.4 PROCEDURE

1. Take a beaker and fill three fourth of it with pond water.
2. Take some freshly cut Hydrilla twigs and dip them in the beaker.
3. Take a glass funnel and place it in the beaker in inverted position so that the wider part of the funnel covers the Hydrilla twigs and the tip of the funnel remains completely dipped in water as shown in Fig. 3.1
4. A beehive arrangement is used in between the base of the funnel and bottom of the beaker.
5. Fill a test tube with pond water and place it in inverted position on stem of the funnel.
6. Keep this assembly in open under the sun for 2-3 hours.

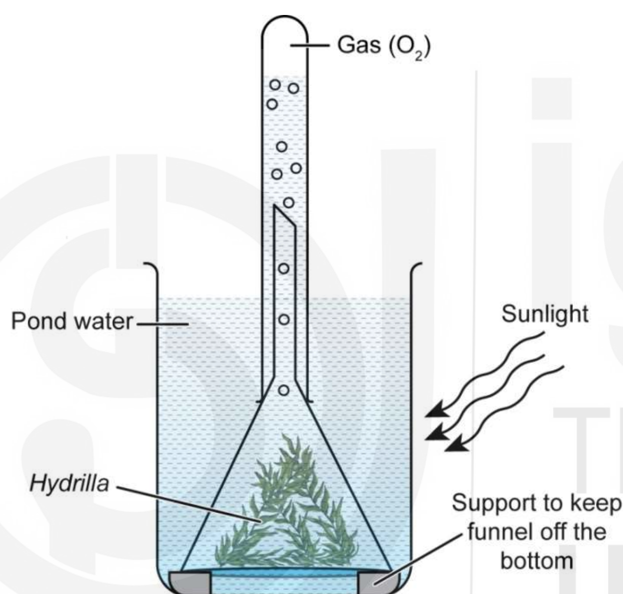

Fig. 3.1: A beehive arrangement for demonstrating the evolution of oxygen during photosynthesis.

3.5 OBSERVATIONS AND RESULTS

1. Gas bubbles i.e. oxygen produced during photosynthesis will be collected on top of the test tube in this arrangement.
2. Gently remove the test tube while keeping it inverted. Take proper care so that the gas collected from photosynthesis does not escape from the test tube.
3. Test the gas in the test tube by inserting a glowing incense stick which would burst into flames.
4. This would prove that the gas collected during photosynthesis was oxygen.
5. By this experiment, it is proved that oxygen is produced during photosynthesis in the presence of sunlight.

3.6 PRECAUTIONS

1. The Hydrilla twigs must be as fresh as possible.
2. The assembly should be exposed to sunlight for sufficient period of time.
3. The level of water in the beaker should be higher than the level of stem of the inverted funnel.
4. While removing test tube, place your thumb near mouth of test tube to prevent escape of gas evolved and remove gently.

3.7 SELF-ASSESSMENT QUESTIONS

1. Define photosynthesis.
2. What is the role of chloroplast in photosynthesis?
3. Explain Hills reaction.
4. What is photolysis?
5. Describe the process of photosynthesis in plants.
6. Explain why water and carbon dioxide are needed in photosynthesis?

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EXPERIMENT 4

ISOLATION OF CHLOROPLAST FROM SPINACH LEAVES AND ESTIMATION OF TOTAL CHLOROPHYLL

Structure

4.1 Introduction

Expected Learning Outcomes

4.2 Principle

4.3 Materials Required

4.4 Procedure

Isolation of Chloroplasts

Estimation of Chlorophyll

4.5 Observation and Result

4.6 Precautions

4.7 Self- assessment Questions

4.1 INTRODUCTION

In this laboratory exercise, you shall earn about the isolation of chloroplast and estimation of chlorophyll. Chloroplasts are specialized sub-cellular organelles present mostly in the leaves which are the major sites of photosynthesis. They are 5-10 µm in size, and have thylakoid membranes which contain photosynthetic pigments and proteins which carry out photosynthesis. Chlorophylls are the major photosynthetic pigments which make the leaves appear green in colour.

Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- prepare the extract (of leaves) for the experiment;
- isolate the chloroplast from leaves; and
- learn the estimation of chlorophyll.

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4.2 PRINCIPLE

Chloroplasts are surrounded by double membrane envelope. The interior of the chloroplasts has thylakoid membrane which is organized in appressed and non-appressed regions known as the grana and stroma lamellae. This thylakoid membrane system is embedded in a matrix which is rich in proteins. The thylakoid membrane system also encloses space in the interior which is known as thylakoid lumen (Fig. 4.1). Chloroplasts are isolated by homogenization of the leaf tissue in a buffered system followed by centrifugation.

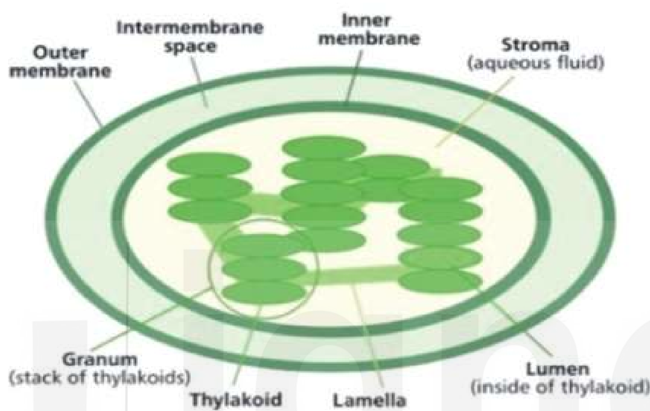

Fig. 4.1: Structural organisation of chloroplast.

Mikhail Tswett, a Russian botanist, separated the chloroplast pigments by chromatography, and showed that leaves have four types of photosynthetic pigments (Table 4.1).

Table 4.1: Photosynthetic pigments and their active absorption spectra.

Pigment	Color	Light absorption wavelength range
Chlorophyll a	Green	Around 430 nm and 662 nm
Chlorophyll b	Green	Around 453 nm and 642 nm
Carotenoids (Carotenes) and Xanthophylls	Yellow-Orange	From 460 nm to 550 nm

4.3 MATERIALS REQUIRED

1. Spinach leaves (approximately 30 grams)
2. Sharp knife
3. Chopping board

4. Kitchen blender
5. Cheese cloth or Muslin cloth
6. Glass beaker
7. Centrifuge tubes (50 ml)
8. Centrifuge tubes (1.5 ml)
9. Micropipette
10. Glass pipette
11. Centrifuges
12. Spectrophotometer

Reagents:

1. **Chloroplast isolation buffer (CIB) without BSA (Bovine serum albumin)**: 0.33 M sorbitol, 0.1M tris-chloride (pH = 7.8), 5mM $MgCl_2$, 10mM NaCl, 2mM EDTA.
2. **Chloroplast isolation buffer (CIB) with BSA (0.1% w/v)**: 0.33M sorbitol, 0.1M tris-Chloride (pH = 7.8), 5mM $MgCl_2$, 10mM NaCl, 2mM EDTA, 0.1% BSA
3. **40% (v/v) percoll**: 4 ml percoll and 6 ml CIB buffer with BSA to make 10 ml of 40% percoll (use 10 ml of 40% percoll for 6 ml of chloroplast suspension).Percoll consists of colloidal silica particles which are coated with polyvinylpyrrolidone (PVP). It is commercially available.
4. 80% (v/v) aqueous acetone

4.4 PROCEDURE

First we will learn how to isolate chloroplasts and then do the chlorophyll estimation.

4.4.1 Isolation of Chloroplasts

1. Wash fresh spinach leaves thoroughly first with tap water and then with distilled water. Remove the midrib and take 30 grams of leaves.
2. Quickly chop into small pieces with a sharp knife or blade.
3. Add 120 ml of CIB with BSA to it and homogenize in a kitchen blender. The jar of blender should be pre-chilled. Homogenization should ideally be done by intermittently turning the knob on and off in short strokes.
4. Filter the homogenate through 8 layers of cheese cloth.
5. The filtrate is then evenly divided into pairs of pre-chilled centrifuge tubes.
6. Centrifuge the homogenate at 250xg for 5 minutes at 4°C. The pellet which contains cell debris and starch is discarded.

7. Transfer the supernatant into pre-chilled centrifuge tubes, and centrifuge at 1000xg for 7 minutes at 4°C.

8. Discard the supernatant and re-suspend the green pellet with 2ml of CIB with BSA very gently with a glass rod or small paint brush.

9. Pool the suspended pellet from all centrifuge tubes.

10. Mix 4 ml of the 40% percoll suspension with 6 ml of CIB buffer with BSA. Gently overlay 6 ml of the chloroplast preparation over the percoll layer.

11. Centrifuge at 1700 x g for 6 minutes at 4°C.

12. The intact chloroplast will sediment to the bottom of the tube as a green pellet and the broken chloroplast will remain in the supernatant.

13. Carefully remove the upper layer of the chloroplast suspension leaving only the pellet containing the intact chloroplast.

14. Mix the pellet with 0.5 ml of CIB buffer without BSA. This is your isolated chloroplast preparation.

This method estimates the total chlorophyll which contains both Chl a and Chl b since absorption is measured at 650 nm which is the mean of absorption maxima of Chl a and Chl b. Estimation of individual chlorophylls and other photosynthetic pigments such as caroteniods involves more elaborate measurements.

4.4.2 Estimation of Chlorophyll

1. Add 10 µl of chloroplast suspension to 990 µl of 80% acetone, and mix gently. Centrifuge at 3000xg for 2 minutes.

2. Decant the supernatant in a separate tube. This extract contains total chlorophyll in solution, which shall be used for chlorophyll estimation.

3. Take sufficient volume of the 80% acetone extract in a glass cuvette, and measure the absorbance at 650 nm using a spectrophotometer.

4. 80% acetone is used to measure absorbance as reference or blank.

5. It's always better to measure the absorbance two to three times using fresh supernatant each time.

4.5 OBSERVATION AND RESULTS

Use the mean of two/three readings (A_{650}) for calculation using the following formula:

Total Chl = (A_{650} x 100)/36 mg/ml

The amount of total chlorophyll obtained from...................g of spinach leaves = ...g

4.6 PRECAUTIONS

1. All preparatory steps are to be carried out at 4°C and in the dark.

2. All the preparatory steps should be performed in quick succession to minimize the inactivation of chloroplasts.

3. Care is to be exercised during separation of supernatant to avoid any mixing with the pellet.

4. The acetone extract should preferably be centrifuged in glass tubes.

5. Acetone is volatile, so the acetone extract should be efficiently handled to avoid evaporation. Evaporation of acetone would alter the absorbance and the estimation would be erroneous.

6. Face mask should be used to avoid inhaling acetone.

4.7 SELF-ASSESSMENT QUESTIONS

1. Explain the role of chloroplasts in photosynthesis.

2. Name different photosynthetic pigments.

3. What is centrifugation?

EXPERIMENT 5

EXTRACTION AND SEPARATION OF PHOTOSYNTHETIC PIGMENTS BY PARTITION CHROMATOGRAPHY

Structure

5.1 Introduction

Expected Learning Outcomes

5.2 Principle

5.3 Materials Required

5.4 Procedure

5.5 Observation and Result

5.6 Precautions

5.7 Self-assessment Questions

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5.1 INTRODUCTION

Photosynthetic pigments are present in chloroplasts and they absorb solar radiation which is used in photosynthesis. The details of photosynthetic pigments are discussed in Unit 10 (Section 10.5) of the theory course BBCCT-111. Photosynthetic pigments are soluble in organic solvents, and many of them could be used for their extraction. Separation of these pigments form plant extract was first achieved by Mikhail Tswett who used a laboratory technique known as chromatography. Several forms of chromatographic techniques have been developed since then, and one of them is known as partition chromatography which is commonly used for this purpose.

5.2 PRINCIPLE

As discussed earlier several kinds of photosynthetic pigments are present in green plants which include chlorophyll a, chlorophyll b, carotenoids, and xanthophylls. The chemical nature of these pigments makes them soluble in non polar solvents. A number of non polar organic solvents such as acetone, methanol, diethyl ether, petroleum ether, and their combinations have been used for pigment extraction from plant tissues. All these methods extract by dissolving all the photosynthetic pigments in plants.

Partition chromatography is the most commonly used method for separation of photosynthetic pigments from crude extracts. It uses a solvent system which is a mixture of several organic solvents and water which differ in polarity. Due to the differences in chemical composition, the photosynthetic pigments have small differences in their polarities. As a result, each pigment prefers to stay in a particular component of the solvent system which has polarity close to that of the pigment. This is called **partitioning**. This effect is exaggerated in a chromatographic system which uses a solid support such as thin layer of silica gel coated on a glass plate (as in TLC), or simply a filter paper. The important feature here is the presence of a meshwork of pores present between particles of silica gel (in TLC) or between the cellulose fibers (in paper chromatography). A small part of the TLC plate or the paper used in chromatography, at an end, is dipped in the solvent system which allows the solvent to rise vertically by capillary action along the pores present in the TLC plate or paper used for chromatography. Another important point is that the individual components of the solvents rise to different heights and have different rates of mobility because of the differences in their physical properties such as surface tension. Now, since each pigment would like to stay with a particular component of the solvent system, and as each component will be rising up the plate or paper with different mobility, a separation of photosynthetic pigments will be easily achieved. In technical terms, the component which has the minimal relative mobility is known as the **stationary phase**, while the others are considered as the **mobile phase**. It should be clearly understood that the silica gel layer on the glass plate (used in TLC), or the paper (used in paper chromatography) is the solid support, and not the stationary phase. So a parameter known as **R_f value** is used as a characteristic of individual analyte molecules (pigments) in partition chromatography which is a measure of the movement of a particular analyte with respect to the overall movement of the solvent front (determined by the fastest moving component of the solvent system). It may also be noted that the R_f value may vary within a narrow range depending upon factors such as solvent system, extent of vapour saturation in the chromatographic chamber, temperature humidity etc. However the R_f value remains constant in a particular chromatographic setup, and relative differences among R_f values of different analytes will always be apparent. The details of chromatographic techniques may be found in Unit 4 of BBCCT-105 on Proteins.

Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- ❖ extract the photosynthetic pigments from biological samples such as leaves;
- ❖ understand the principle underlying partition chromatography;
- ❖ relate thin layer chromatography (TLC) and paper chromatography with partition chromatography; and
- ❖ separate photosynthetic pigments by chromatography.

5.3 MATERIALS REQUIRED

1. Fresh leaves (spinach)
2. Mortar and pestle
3. Silica gel-G
4. Whatman No.1 filter paper
5. Acetone
6. Petroleum ether
7. Isopropanol
8. Anhydrous magnesium sulphate
9. Distilled water
10. Capillary tube
11. Glass chromatographic tank/Jar with a glass plate to cover
12. Glass plates (length 20cm, breadth 20 or 10 cm, thickness 3 mm)

5.4 PROCEDURE

We shall perform this experiment in five steps. Lets learn each one by one.

5.4.1 Extraction of Photosynthetic Pigments

1. Take fresh spinach leaves and remove the midrib. Wash the leaves thoroughly first with tap water and then with distilled water.
2. Quickly chop them into small pieces with a sharp knife or razor blade.
3. Take 5 g of chopped leaves and add 5 g of anhydrous magnesium sulfate and 10 g of sand (silica).
4. Grind the mixture until a fine dry powder is obtained. The anhydrous magnesium sulfate would act as a dehydrating agent and help in the elimination of water from the leaves.
5. Transfer this powder to a small test tube and add sufficient volume of 90 % (v/v) aqueous acetone. Cover the test tube with a stopper and shake vigorously for about a minute to ensure that the photosynthetic pigments are dissolved in the solvent.
6. Allow this mixture to stand for 10 minutes, and then transfer the solvent present on top of the sediment that would have settled. Alternatively the mixture may be centrifuged at low speed and the green supernatant may be collected.
7. This is the extract of photosynthetic pigments. Keep it covered until used for separation by TLC or paper chromatography.

5.4.2 Preparation and Activation of TLC plate

8. Take 12 g silica gel G in a beaker and add 30 ml distilled water. Mix with the help of a glass rod to prepare homogeneous slurry.

9. Take a clean and dry TLC plate (glass plate 20 cm in length and breadth and 2-3 mm in thickness). Keep the plate horizontal and pour the slurry on to it.

10. Spread it gently either with a glass rod, or with a commercially available applicator, to cover the plate completely. Take care to have the thickness of slurry layer uniform (approximately 1 mm).

11. Leave the plate in a ventilated place to air dry.

12. Activate the air dried plate by placing in an oven at 80-100ºC for 30 minutes before use. The water present in the silica gel layer is removed in this step leaving a meshwork of pores through which the solvent rises during separation.

5.4.3 Sample Application

13. Take the pigment extract that was prepared in a suitable solvent (90 % v/v acetone).

14. Place the activated TLC plate horizontally on a white paper marked with a base line such that the line remains about 1 cm from one side of the plate. This will help in applying the sample on to the TLC plate in a straight horizontal line.

15. For sample application, take a glass capillary tube and touch the pigment extract with one end so that a small volume of extract rises in the capillary.

16. Gently touch the end of capillary at a point on the line on the plate. Wait for a few second for the solvent to evaporate and apply more sample on the same spot to make it concentrated which will help detection.

17. More samples may be applied by evenly spacing the spots along the horizontal line (Fig. 5.1).

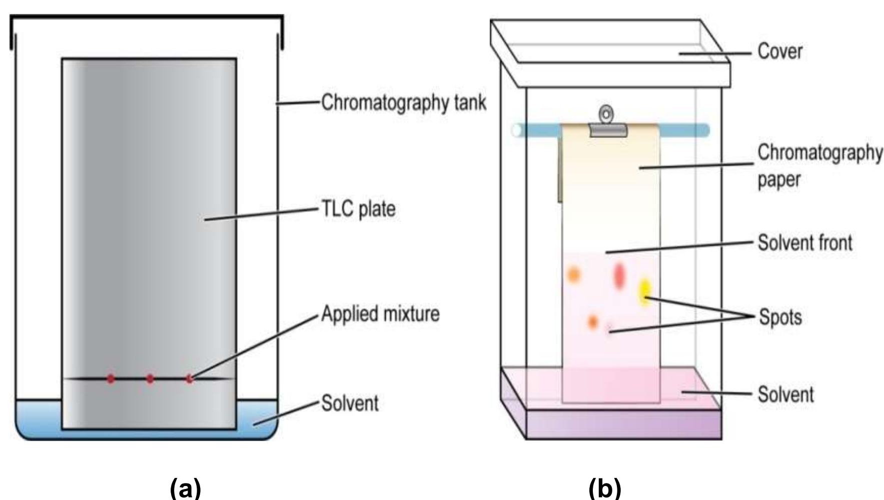

Fig. 5.1: Experimental setup for (a) thin layer chromatography and (b) paper chromatography.

18. Similarly, take the chromatographic paper and make a base line with light pencil across it, 1 cm above the bottom of the paper and put a short mark the line where sample is to be applied. Repeat step nos. 15-17.

5.4.4 Preparation of Solvent System

19. Make the solvent system by taking petroleum ether, isopropanol and water in 100:10:0.25 (v/v/v) proportions and mixing them by gentle swirling motion.

20. Take a TLC tank/jar and pour enough volume of this solvent system so as to cover a height of about 1 cm from the bottom.

21. Cover the jar with a glass plate and let it saturate with vapour of the solvent system.

5.4.5 Development of Chromatogram

22. Take the TLC plate to which sample was applied and place in the TLC tank vertically so that the lower part is dipped in the solvent system and the line along which sample was applied remains above the surface of the solvent system. Let the plate rest in this position with support of the wall of the TLC tank. Cover the TLC tank with the glass plate again and leave it for development.

23. The solvent will rise vertically along the activated plate by capillary action. Different components of the solvent system will have different mobility. Depending on the physicochemical properties each pigment would partition (would like to stay) in a particular component of the solvent system, and therefore will rise to different heights during the plate development. This is the basis of separation in TLC, and for this reason TLC is also called Partition chromatography (Fig. 5.2).

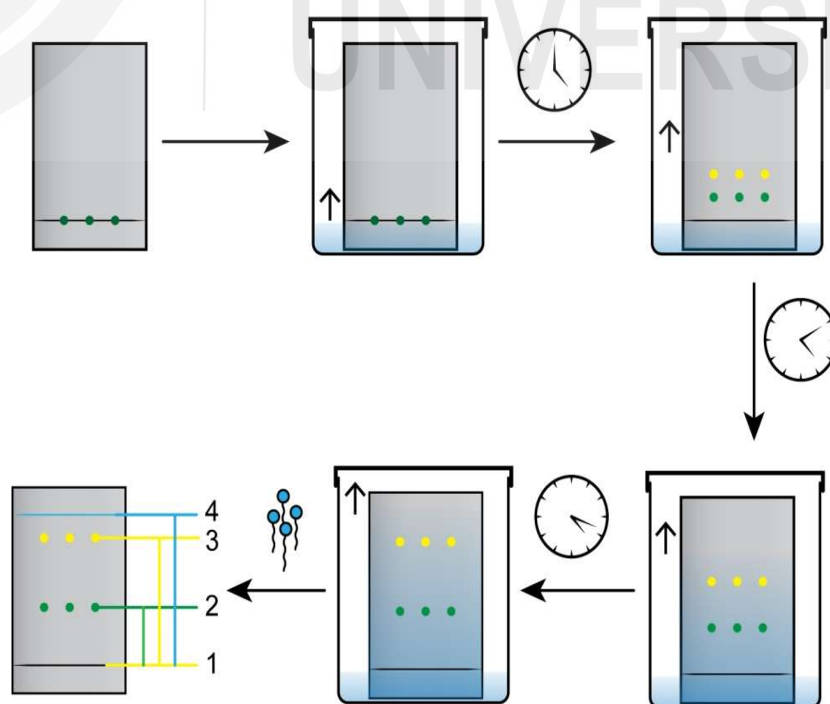

Fig. 5.2: Course of development of chromatogram during thin layer chromatography.

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24. Remove the TLC plate from the tank when the solvent front is approximately 1.0 cm away from the top of the TLC plate.

25. With a pencil, mark the level of the solvent front (highest level the solvent moves up the TLC plate) before the solvent evaporates and becomes invisible.

26. You will be able to see the spots of individual pigments along the direction of solvent rise. Mark the centre of each spot so that distance of each spot from the spot where sample was initially applied (the origin) could be measured (Fig. 5.3).

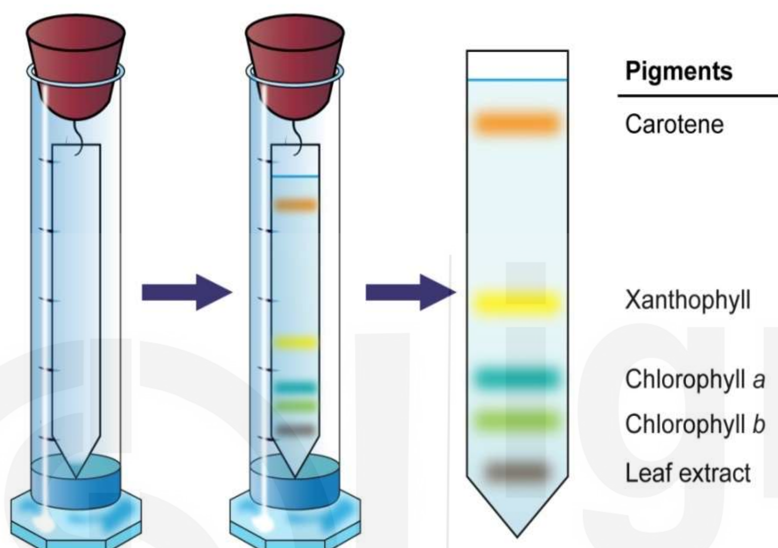

Fig. 5.3: Course of development of chromatogram during paper chromatography.

27. Now measure the distance of the solvent front and center of each spot from the origin. Your paper chromatogram will somewhat look like this (Fig. 5.4).

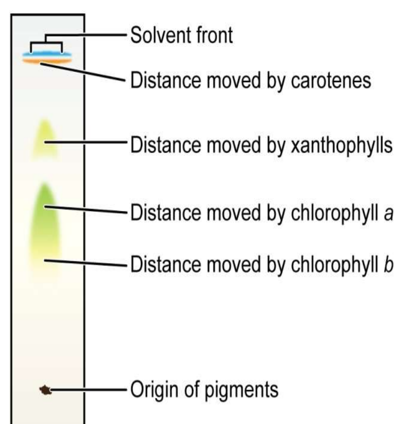

Fig. 5.4: Pictogram of a chromatograph.

Also, you can click picture of your TLC plate and paste here.

28. Calculate the R_f value.

5.5 OBSERVATION AND RESULT

The separated pigments are identified by comparing their R_f values to that of reference standards. Using the formula given below calculate the R_f values of the separated photosynthetic pigments and record your observation.

$$Rf = \frac{\text{The distance moved by the photosynthetic pigments from origin}}{\text{The distance moved by the solvent from the origin}}$$

Pigment	Colour	Standard R_f value	Obtained R_f value
Chlorophyll *a*	Blue-green	0.59	
Chlorophyll *b*	Yellow-green	0.42	
β-carotene	Orange	0.98	
Xanthophylls	Yellow	0.35	

5.6 PRECAUTIONS

1. Cap the tubes to prevent solvent evaporation.
2. Keep the TLC jar covered to prevent solvent evaporation which could alter the composition of the solvent system.
3. The developed spots may have a fair size. Consider the centre of spot for measurement of the distance travelled by pigments.
4. Encircle the spot so that the area could be calculated. The area of a spot is directly proportional to the amount of pigment. This may be required if relative abundance of individual pigments is to be determined.

5.7 SELF-ASSESSMENT QUESTIONS

1. Based on your knowledge on plant pigments, which pigments are likely present in the sample?
2. Which pigment travelled farthest and why?
3. Based on R_f values can we conclude that pigments present in two different samples might be same. Explain.
4. Describe the stationary and mobile phases.
5. Differentiate between paper and thin layer chromatography.

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EXPERIMENT 6

ISOLATION OF MITOCHONDRIA FROM RAT LIVER

Structure

6.1 Introduction

Expected Learning Outcomes

6.2 Principle

6.3 Materials Required

6.4 Procedure

6.5 Observations and Results

6.6 Precautions

6.7 Self-assessment Questions

6.1 INTRODUCTION

In a eukaryotic cell each sub-cellular compartment/organelle performs a specific biochemical function. Elucidation of role of the sub-cellular organelles is important for understanding the structure and function of a cell as a whole. One way to determine the biochemical function of an organelle is to isolate the intact organelle and study its function. The process of isolating sub-cellular organelles in their intact form is known as **sub-cellular fractionation**. The method for isolation of sub-cellular organelles was first developed by Albert Claude, Christian de Duve, and their colleagues in 1940s. The procedure comprises essentially of two distinct steps. In the first step, cells are lysed/ ruptured to release their content, and in the second step different sub-cellular organelles and particles are separated by fractionation. The organelles isolated by such methods are often contaminated due to presence of other organelles. The contaminations are detected by assaying the activities of enzymes which are characteristically present in organelles other than that which is being isolated.

Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- ❖ explain the concept of compartmentalization;
- ❖ understand the process of sub-cellular fractionation; and
- ❖ isolate mitochondria from rat liver tissue.

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6.2 PRINCIPLE

Isolation of mitochondria from cells is based on the principle of differential centrifugation which involves spinning a homogenate containing a heterogeneous mixture of sub-cellular components and organelles. The centrifugation is performed in a stepwise manner while increasing the centrifugal field in every successive centrifugation step. This leads to sedimentation of the largest organelle/component in the first centrifugation step. The subsequent centrifugation steps would sediment smaller and smaller components/organelles. Hence, the technique of differential centrifugation allows us to fractionate the crude homogenates on the basis of sizes of their components. The identity of sediments (pellets) obtained from each centrifugation step is established by assaying the activities of marker enzymes of different sub-cellular organelles. Purity of preparation may also be ascertained by assaying marker enzyme activities. For example, a pure mitochondrial preparation should exhibit high SDH activity, but it should not exhibit activities of enzymes which are markers of other sub-cellular organelles. You will assay SDH activity in next experiment.

6.3 MATERIALS REQUIRED

1. Fresh rat liver
2. Potter-Elvehjemhomoginizer
3. Centrifuge machine
4. Spectrophotometer/colorimeter
5. Reagents:
   a) Saline: 0.9% (w/v) NaCl solution
   b) Isolation buffer: 0.25 M sucrose, 5 mM HEPES buffer, pH 7.2, and 1 mM EDTA.

6.4 PROCEDURE

1. Dissect out liver from a rat, wash it with 0.9% cold saline. Weigh the liver and take approximately 4 g of liver tissue.
2. Chop it into small pieces with the help of a sharp blade. Perform it in ice cold conditions.
3. Add 20 ml isolation buffer to the chopped liver, and homogenize it using a prechilled Potter-Elvehjem homogenizer.
4. Centrifuge the homogenate in a refrigerated centrifuge at 700*g* for 10 minutes at 4°C (Fig. 6.1).
5. Transfer the supernatant in to another clean centrifuge tube and discard the pellet which has large tissue fragments, cell debris and nuclei.
6. Take the supernatant and re-centrifuge it at 700*g* for 10 minutes at 4°C. This step is to ensure that all the unwanted material, such as large tissue fragments, cell debris and nuclei, are completely removed.

7. Gently transfer the supernatant into a clean prechilled centrifuge tube. Centrifuge it at 9000*g* for 10 minutes at 4°C.

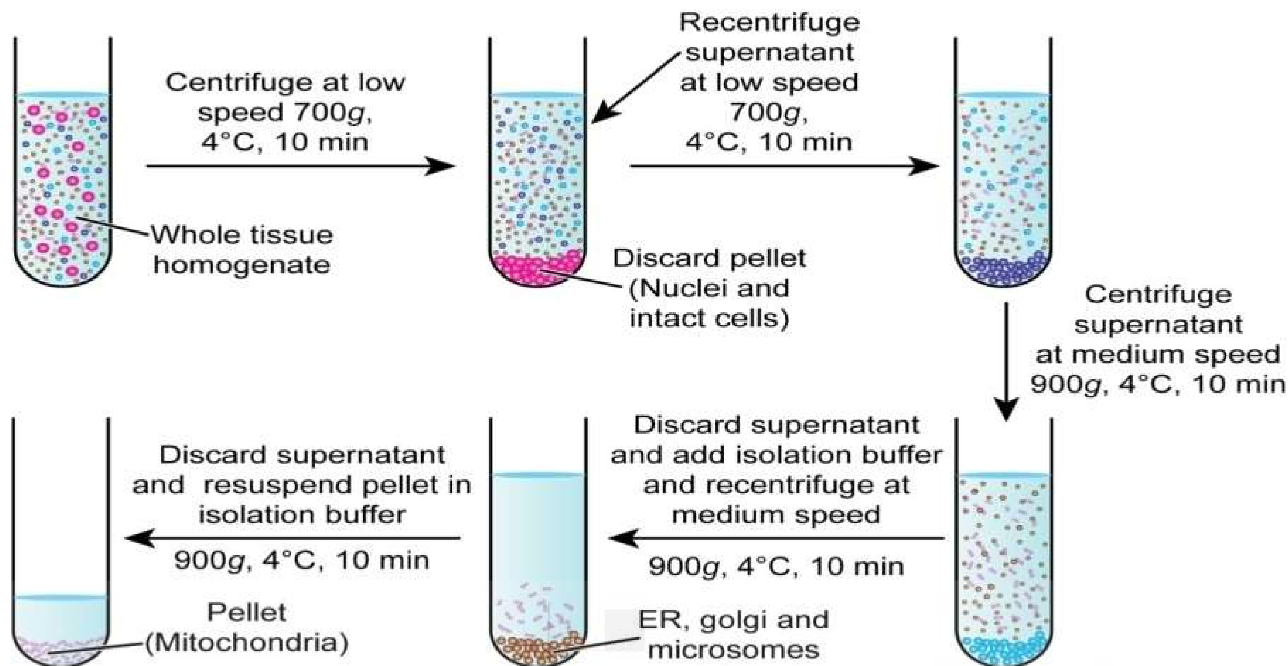


Fig. 6.1: Major steps in isolation of mitochondria.

8. Discard the supernatant and the loose upper layer of the pellet by gently pouring off the supernatant.

9. Gently re-suspend the pellet in 15 ml isolation buffer and centrifuge at 9000*g* for 10 minutes at 4°C. This is the washing step. Discard the supernatant and the loose upper layer of the pellet by gently pouring off the liquid.

10. Take the pellet and re-suspend the pellet in minimum possible volume of isolation buffer. This is your mitochondrial preparation to be used for assay of succinate dehydrogenase activity.

6.5 OBSERVATION AND RESULTS

Pellet suspended in isolation buffer is obtained which shall be used to assay succinate dehydrogenase activity.

6.6 PRECAUTIONS

1. Fresh rat liver tissue must be used for the experiment.
2. The homogenization of tissue should be performed properly.
3. During the isolation of mitochondria, the temperature should be maintained at 4°C.
4. The pH of extraction buffer should be fixed at 7.4.
5. Mixing of the pellet before centrifugation should be gentle.
6. Re-suspend the pellet in minimum possible isolation buffer for assay.

6.7 SELF-ASSESSMENT QUESTIONS

1. Define centrifugation.
2. What is differential centrifugation?
3. What is homogenization?
4. What is significance of compartmentalization in a cell?

EXPERIMENT 7

ASSAY OF THE ACTIVITY OF MARKER ENZYME SUCCINATE DEHYDROGENASE

Structure

7.1 Introduction

Expected Learning Outcomes

7.2 Principle

7.3 Materials Required

7.4 Procedure

7.5 Observations and Results

7.6 Precautions

7.7 Self-assessment Questions

7.1 INTRODUCTION

A molecular marker (biological markers) is a molecule present in the sample taken from an organism. Two types of markers have been used for determining the purity of isolated fractions: **morphological** and **biochemical**. The morphological markers are checked by microscopic examinations of the isolated fraction. Biochemical markers, such as marker enzyme, are tested by assay of enzyme activities of such enzymes in the isolated fraction. In other words, marker enzymes are uniquely and exclusively located in or associated with a particular intracellular fraction, and can be used to detect the presence of that specific sub-cellular component. Some marker enzymes of some sub-cellular organelles of plants and animal cells are given in Table 7.1.

Succinate Dehydrogenase (SDH) or succinate-coenzyme Q reductase or respiratory complex II is an enzyme complex, found in many bacterial cells and eukaryotes. It is the only enzyme that participates in both the citric acid cycle and the electron transport chain and is the marker enzyme for mitochondria. It is located in the inner mitochondrial membrane, and participates in TCA cycle. This is why it is also known as mitochondrial marker enzyme. It is tightly associated with the cofactor FAD and catalyses the following reaction:

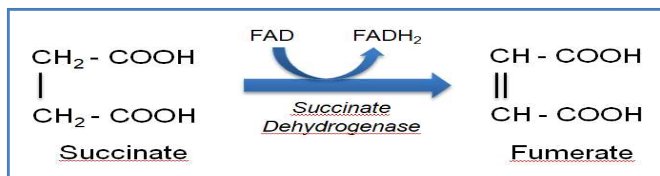


Table 7.1: Biochemical Markers used for various organelles

Organelle	Marker Enzyme	
	Plant Cell	**Animal Cell**
Nuclei	NADP pyrophosphorylase	NADP pyrophosphorylase
Chloroplast	Ribulose-1,5-bisphosphate carboxylase	--
Mitochondria	Succinate dehydrogenase, Cytochrome *c* oxidase	Succinate dehydrogenase, Cytochrome *c* oxidase
Peroxisomes	Catalase, D-amino acid oxidase	Catalase, D-amino acid oxidase
Vacuoles	Ribonuclease, Phosphodiesterase	--
Lysosomes	--	Acid phosphatase

Expected Learning Outcomes

After studying this unit, you should be able to:

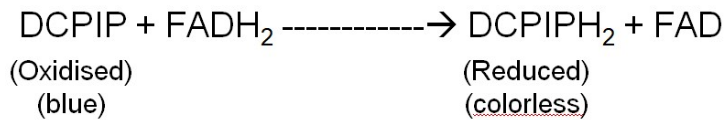
- ❖ explain molecular marker and its significance; and
- ❖ assay the activity of succinate dehydrogenase (SDH).

7.1 PRINCIPLE

The technique of differential centrifugation allows us to fractionate the crude homogenates on the basis of sizes of their components. However, the identity of sediments (pellets) obtained from each centrifugation step is established by assaying the activities of marker enzymes of different sub-cellular organelles. Purity of preparation may also be ascertained by assaying marker enzyme activities. For example, a pure mitochondrial preparation should exhibit high SDH activity, but it should not exhibit activities of enzymes which are markers of other sub-cellular organelles.

The objective of measuring enzyme activity is normally to check the presence or absence of an enzyme or to determine the amount of enzyme present under defined conditions, so that activity can be compared between samples.

Assay of SDH activity is performed in the presence of a blue colored dye dichlorophenol indophenol (DCPIP). If succinate dehydrogenase is active, $FADH_2$ will be produced, which will in turn reduce DCPIP. Reduced DCPIP is colorless, and the rate of DCPIP reduction, which would be proportional to SDH activity, can be followed spectrophotometrically.



(Oxidised) (blue) (Reduced) (colorless)

This forms the basis of the present colorimetric assay. Azide is added to block flow of electrons to the remainder of the electron transport chain rather than to DCPIP.

7.3 MATERIALS REQUIRED

1. Isolated Mitochondrial Fraction
2. Centrifuge machine
3. Test tubes
4. Spectrophotometer/colorimeter
5. Reagents:

a) Isolation buffer: 0.25 M sucrose, 5 mM HEPES buffer, pH 7.2, and 1 mM EDTA.

b) 120 mM sodium succinate solution adjusted to pH 7.2 with NaOH

c) 200 mM sodium azide solution made in isolation buffer

d) 1 mg/ml DCPIP made in distilled water

7.4 PROCEDURE

To assay the activity of Succinate Dehydrogenase

1. Set the spectrophotometer/colorimeter to a wavelength of 600 nm.
2. Adjust zero using the isolation buffer as the reference (also called blank).
3. Take a test tube and add 2.5 ml isolation buffer, 0.1 ml sodium azide solution, 0.1 ml of DCPIP solution and mix well.
4. Add 0.2 ml homogenate, mix quickly, transfer it into a cuvette, and record the OD every 30 second for endogenous activity. This represents the basal activity which is in the absence of exogenously added substrate.
5. Now add 0.1 ml sodium succinate (substrate) solution, mix quickly and measure absorbance every 30 seconds for 10 minutes.
6. Plot the absorbance against time to determine the average slope.

> The SI unit of enzyme activity is *katal* (kat) which represents the transformation of 1 mole of substrate per second.
>
> 1 U = 1 μmol/min
>
> 1 katal = 1 mol/sec

7.5 OBSERVATION AND RESULTS

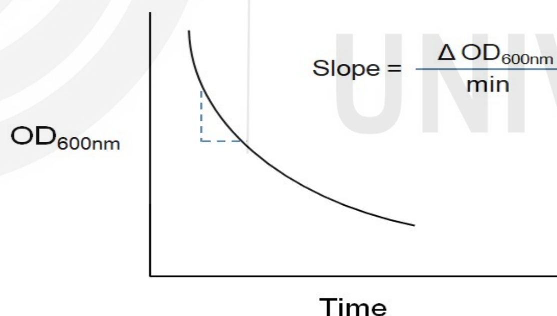
Record the absorbance every 30 second in the given table below.

Reagent	OD at 600 nm (time in min)									
Reaction mixture (2.5 ml isolation buffer + 0.1 ml sodium azide solution +0.1 ml of DCPIP) + **0.2ml homogenate**	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	1.0
Reaction mixture + 0.2ml homogenate + **0.1 ml sodium scuccinate (substrate)**	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	1.0

Calculations:

You need to correlate the absorbance of the product released in your assay with standard product curve. By using y= mx+c, from your (Standard curve) you need to check the concentration of product formed (reduced DCPIP which is colorless in this case), or substrate left in term of micro grams.

Plot the OD versus time and determine the average slope.



To calculate the units in any spectrophotometric based assay, Beer's law is used: A = ε l c

Where A = absorbance ($M^{-1} cm^{-1}$), b = pathlength of the cell (1 cm), c = concentration of the absorbing species (M) and ε = the molar extinction coefficient.

If succinate dehydrogenase is active, $FADH_2$ will be produced, which in turn will reduce DCPIP. Reduced DCPIP is colorless, whereas DCPIP (dichlorophenol indophenol) is a blue colored dye. As mentioned, the rate of DCPIP reduction would be proportional to SDH activity which you have recorded spectrophotometrically. Now, Calculate activity using the formula

Enzyme activity (units/ml extract) =

$$\frac{\Delta OD\ X\ Total\ vol\ of\ assay\ (ml) X\ dilution\ factor}{Time\ of\ incubation\ (min) X\ extinction\ coefficient\ X\ Vol\ of\ enzyme\ extract\ (ml)}$$

Here, ΔOD = slope from the absorbance versus time plot at 600nm

Molar extinction coefficient (ε) or molar absorptivity of DCPIP at 600 nm = 19.1 $mM^{-1}cm^{-1}$

Total reaction volume is 3ml

Dilution factor = Sum of all the volume/ volume of homogenate

= 3/0.2 =15 times

Thus, SDH Activity = $\frac{\Delta OD \times 3 \times 15}{10 \times 19.1 \times 0.2}$ units/ml extract

Molar extinction coefficient (ε) or molar absorptivity is is a measure of how strongly a chemical species or substance absorbs light at a particular wavelength. It is an intrinsic property of chemical species that is dependent upon their chemical composition and structure.

7.6 PRECAUTIONS

1. The material should be resuspended with gentle swirling or rolling on the laboratory bench.
2. Enzyme should be stored at - 20°C
3. Switch on the spectrophotometer to avoid any delay in monitoring absorption.
4. Do NOT add enzyme until ready to start your timer.

7.7 SELF-ASSESSMENT QUESTIONS

1. Define enzyme activity.
2. What is extinction coefficient?
3. What is the significance of marker enzyme?

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SOME SUGGESTED READINGS

1. An Introduction to Practical Biochemistry by David T. Plummer (Third Edition).

2. Laboratory Manual of Microbiology, Biochemistry and Molecular Biology By Jyoti Saxena, Mamta Baunthiyal and Indu Devi (Scientific Publishers India).

3. Mohandas, N. & Gallagher, P. G. Red Cell Membrane: Past, Present, and Future. Blood. 112, 3939–3948 (2008).

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