

BARRETT LEWIS  
ALLEN - (6)

96

# LAB FOUR PLANT PIGMENTS AND PHOTOSYNTHESIS

## OVERVIEW

In this lab you will:

1. separate plant pigments using chromatography, and
2. measure the rate of photosynthesis in isolated chloroplasts using the dye DPIP.

The transfer of electrons during the light-dependent reactions of photosynthesis reduces DPIP, changing it from blue to colorless.

## OBJECTIVES

Before doing this lab you should understand:

- how chromatography separates two or more compounds that are initially present in a mixture;
- the process of photosynthesis;
- the function of plant pigments;
- the relationship between light wavelength and photosynthetic rate; and
- the relationship between light intensity and photosynthetic rate.

After doing this lab you should be able to:

- separate pigments and calculate their  $R_f$  values;
- describe a technique to determine photosynthetic rates;
- compare photosynthetic rates at different light intensities or different wavelengths of light using controlled experiments; and
- explain why the rate of photosynthesis varies under different environmental conditions.

## EXERCISE 4A: Plant Pigment Chromatography

Paper chromatography is a useful technique for separating and identifying pigments and other molecules from cell extracts that contain a complex mixture of molecules. The solvent moves up the paper by capillary action, which occurs as a result of the attraction of solvent molecules to the paper and the attraction of solvent molecules to one another. As the solvent moves up the paper, it carries along any substances dissolved in it. The pigments are carried along at different rates because they are not equally soluble in the solvent and because they are attracted, to different degrees, to the fibers in the paper through the formation of intermolecular bonds, such as hydrogen bonds.

Beta carotene, the most abundant carotene in plants, is carried along near the solvent front because it is very soluble in the solvent being used and because it forms no hydrogen bonds with cellulose. Another pigment, xanthophyll, differs from carotene in that it contains oxygen. Xanthophyll is found further from the solvent front because it is less soluble in the solvent and has been slowed down by hydrogen bonding to the cellulose. Chlorophylls contain oxygen and nitrogen and are bound more tightly to the paper than are the other pigments.

Chlorophyll *a* is the primary photosynthetic pigment in plants. A molecule of chlorophyll *a* is located at the reaction center of photosystems. Other chlorophyll *a* molecules, chlorophyll *b*, and the carotenoids (that is, carotenes and xanthophylls) capture light energy and transfer it to the chlorophyll *a* at the reaction center. Carotenoids also protect the photosynthetic system from the damaging effects of ultraviolet light.

### Procedure

Your teacher will demonstrate the apparatus and techniques used in paper chromatography. Here is a suggested procedure, illustrated in Figure 4.1:

1. Obtain a 50-ml graduated cylinder that has 1 cm of solvent in the bottom. The cylinder is tightly stoppered because this solvent is volatile, and you should be careful to keep the stopper on as much as possible.
2. Cut a piece of filter paper that will be long enough to reach the solvent. Cut one end of this filter paper into a point. Draw a pencil line 1.5 cm above the point.
3. Use a coin to extract the pigments from spinach leaf cells. Place a small section of leaf on the top of the pencil line. Use the ribbed edge of the coin to crush the cells. Be sure that the pigment line is on top of the pencil line. You should repeat this procedure 8 to 10 times, being sure to use a new portion of the leaf each time.
4. Place the chromatography paper in the cylinder so that the pointed end is barely immersed in the solvent. *Do not allow the pigment to be in the solvent.*
5. Stopper the cylinder. When the solvent is about 1 cm from the top of the paper, remove the paper and *immediately* mark the location of the solvent front before it evaporates.
6. Mark the bottom of each pigment band. Measure the distance each pigment migrated from the bottom of the pigment origin to the bottom of the separated pigment band. In Table 4.1 record the distance that each front, including the solvent front, moved. Depending on the species of plant used, you may be able to observe 4 or 5 pigment bands.

Figure 4.1

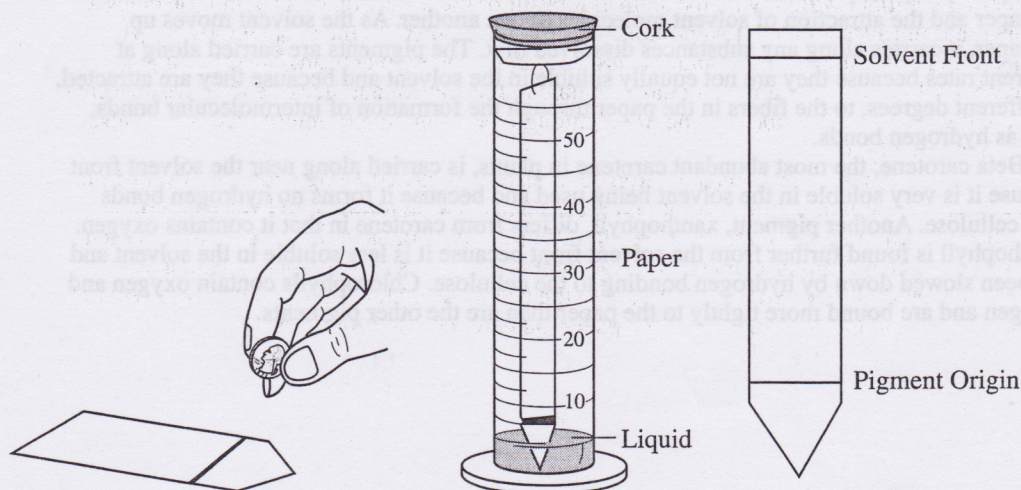


Table 4.1

Distance Moved by Pigment Band (millimeters)

Band Number	Distance (mm)	Band Color
1.	6	OLIVE
2.	11	BLUE/G
3.	16	YELLOW
4.	24	EGGSHELL
5.	145	YELLOW/O

Distance Solvent Front Moved 145 (mm)

**Analysis of Results**

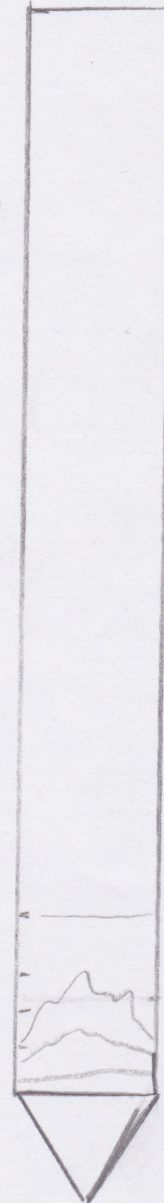
The relationship of the distance moved by a pigment to the distance moved by the solvent is a constant called  $R_f$ . It can be calculated for each of the four pigments using the following formula:

$$R_f = \frac{\text{distance pigment migrated (mm)}}{\text{distance solvent front migrated (mm)}}$$

Record your  $R_f$  values in Table 4.2.

Table 4.2

<u>1</u>	= $R_f$ for Carotene (yellow to yellow orange)
<u>.110</u>	= $R_f$ for Xanthophyll (yellow)
<u>.075</u>	= $R_f$ for Chlorophyll a (bright green to blue green)
<u>.041</u>	= $R_f$ for Chlorophyll b (yellow green to olive green)



**Topics for Discussion**

1. What factors are involved in the separation of the pigments?

THE SOLUBILITY, PARTICLE SIZES AND  
ADHESION TO THE PAPER ALL  
CONTRIBUTE TO THE SEPARATION OF  
PIGMENTS.

2. Would you expect the  $R_f$  value of a pigment to be the same if a different solvent were used? Explain.

NO. THE SOLUBILITY OF THE PIGMENTS IN THE SOLVENT GREATLY AFFECTS HOW FAR AND LONG THE SOLVENT IS ABLE TO CARRY A GIVEN PIGMENT.

3. What type of chlorophyll does the reaction center contain? What are the roles of the other pigments?

THE REACTION CENTER CONTAINS CHLOROPHYLL A. OTHER PIGMENTS ABSORB OTHER WAVELENGTHS CAUSING THEM TO REFLECT DIFFERENT SHADES OR EVEN DIFFERENT COLORS.

**EXERCISE 4B: Photosynthesis/The Light Reaction**

Light is a part of a continuum of radiation, or energy waves. Shorter wavelengths of energy have greater amounts of energy. For example, high-energy ultraviolet rays can harm living tissues. Wavelengths of light within the visible part of the light spectrum power photosynthesis.

When light is absorbed by leaf pigments, electrons within each photosystem are boosted to a higher energy level, and this energy is used to produce ATP and to reduce NADP to NADPH. ATP and NADPH are then used to incorporate CO<sub>2</sub> into organic molecules, a process called **carbon fixation**.

**Design of the Exercise**

Photosynthesis may be studied in a number of ways. For this experiment a dye-reduction technique will be used. The dye-reduction experiment tests the hypothesis that light and chloroplasts are required for the light reactions to occur. In place of the electron acceptor, NADP, the compound DPIP (2,6-dichlorophenol-indophenol), will be substituted. When light strikes the chloroplasts, electrons boosted to high energy levels will reduce DPIP. It will change from blue to colorless.

In this experiment chloroplasts are extracted from spinach leaves and incubated with DPIP in the presence of light. As the DPIP is reduced and becomes colorless, the resultant increase in light transmittance is measured over a period of time using a spectrophotometer. The experimental design matrix is presented in Table 4.3.

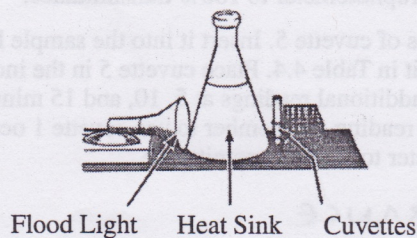
Table 4.3: Photosynthesis Setup

	Cuvettes				
	1 Blank (no DPIP)	2 Unboiled Chloroplasts Dark	3 Unboiled Chloroplasts Light	4 Boiled Chloroplasts Light	5 No Chloroplasts Light
Phosphate Buffer	1 mL	1 mL	1 mL	1 mL	1 mL
Distilled H <sub>2</sub> O	4 mL	3 mL	3 mL	3 mL	3 mL + 3 drops
DPIP	—	1 mL	1 mL	1 mL	1 mL
Unboiled Chloroplasts	3 drops	3 drops	3 drops	—	—
Boiled Chloroplasts	—	—	—	3 drops	—

### Procedure

1. Turn on the spectrophotometer to warm up the instrument and set the wavelength to 605 nm by adjusting the wavelength control knob.
2. While the spectrophotometer is warming up, your teacher may demonstrate how to prepare a chloroplast suspension from spinach leaves.
3. Set up an incubation area that includes a light, water flask, and test tube rack (see Figure 4.2). The water in the flask acts as a heat sink by absorbing most of the light's infrared radiation while having little effect on the light's visible radiation.

Figure 4.2: Incubation Setup



4. Your teacher will provide you with two beakers, one containing a solution of boiled chloroplasts and the other one containing unboiled chloroplasts. Be sure to keep both beakers on ice at all times.
5. At the top rim, label the cuvettes 1, 2, 3, 4, and 5, respectively. Be sure to follow your teacher's directions on how to label cuvettes. Using lens tissue, wipe the outside walls of each cuvette (*Remember: handle cuvettes only near the top*). Cover the walls and bottom of cuvette 2 with foil and make a foil cap to cover the top. Light should not be permitted inside cuvette 2 because it is a control for this experiment.
6. Refer to Table 4.3 to prepare each cuvette. *Do not add unboiled or boiled chloroplasts yet.* To each cuvette, add 1 mL of phosphate buffer. To cuvette 1, add 4 mL of distilled H<sub>2</sub>O. To cuvettes 2, 3, and 4, add 3 mL of distilled H<sub>2</sub>O and 1 mL of DPIP. To cuvette 5, add 3 mL plus 3 drops of distilled water, and 1 mL of DPIP.

7. Bring the spectrophotometer to zero by adjusting the amplifier control knob until the meter reads 0% transmittance. Add 3 drops of unboiled chloroplasts to cuvette 1. Cover the top with Parafilm® and invert to mix. Insert cuvette 1 into the sample holder and adjust the instrument to 100% transmittance by adjusting the light-control knob. *Cuvette 1 is the blank to be used to recalibrate the instrument between readings.* In other words, you will measure the light transmitted through each of the other tubes as a percentage of the light transmitted through this tube. For each reading, make sure that the cuvettes are inserted into the sample holder so that they face the same way as in the previous reading.
8. Obtain the unboiled chloroplast suspension, stir to mix, and transfer 3 drops to cuvette 2. *Immediately* cover and mix cuvette 2. Then remove it from the foil sleeve and insert it into the spectrophotometer's sample holder, read the % transmittance, and record it as the time 0 reading in Table 4.4. Replace cuvette 2 in the foil sleeve and place it in the incubation test tube rack. Turn on the flood light. Take and record additional readings at 5, 10, and 15 minutes. Mix the cuvette's contents just prior to each reading. Remember to use cuvette 1 occasionally to check and adjust the spectrophotometer to 100% transmittance.
9. Obtain the unboiled chloroplast suspension, mix, and transfer 3 drops to cuvette 3. *Immediately* cover and mix cuvette 3. Insert into the sample holder, read the % transmittance, and record it in Table 4.4. Place cuvette 3 in the incubation test tube rack next to cuvette 2. Take and record additional readings at 5, 10, and 15 minutes. Mix the cuvette's contents just prior to each reading. Remember to use cuvette 1 occasionally to check and adjust the spectrophotometer to 100% transmittance.
10. Obtain the *boiled* chloroplast suspension, mix, and transfer 3 drops to cuvette 4. *Immediately* cover and mix cuvette 4. Insert into the sample holder, read the % transmittance, and record it in Table 4.4. Place cuvette 4 in the incubation test tube rack next to cuvettes 2 and 3. Take and record additional readings at 5, 10, and 15 minutes. Mix the cuvette's contents just prior to each reading. Remember to use cuvette 1 occasionally to check and adjust the spectrophotometer to 100% transmittance.
11. Cover and mix the contents of cuvette 5. Insert it into the sample holder, read the % transmittance, and record it in Table 4.4. Place cuvette 5 in the incubation test tube rack next to tubes 2, 3, and 4. Take additional readings at 5, 10, and 15 minutes. Mix the cuvette's contents just prior to each reading. Remember to use cuvette 1 occasionally to check and adjust the spectrophotometer to 100% transmittance.

ABSORBANCE

Table 4.4: Transmittance (%)

Cuvette	Time (minutes)			
	0	5	10	15
2 Unboiled/ Dark	.958	.930	.95	.58
3 Unboiled/ Light	.94	.512	.105	.055
4 Boiled/ Light	.788	.816	.812	.809
5 No Chloroplasts/ Light	.941	.963	.675	.675

**Analysis of Results**

Plot the percentage of transmittance from the four cuvettes on Graph 4.1.  
Label each plotted line.

For this graph you will need to determine the following:

- a. The *independent* variable: TIME (min)  
Use this to label the horizontal (x) axis.
- b. The *dependent* variable: ABSORBANCE  
Use this to label the vertical (y) axis.

**Graph 4.1** Title: ABSORBANCE OVER TIME OF VARIOUS CHLOROPLASTS



**Topics for Discussion**

1. What is the function of DPIP in this experiment?

DPIP ACTS AS THE ELECTRON ACCEPTOR, TAKING THE PLACE OF NADP. IT ACCEPTED ELECTRONS FROM WATER WHICH WERE EXCITED BY PHOTONS AND CARRIED THROUGH PHOTOSYNTHESIS. COLOR CHANGE?

(-2)

2. What molecule found in chloroplasts does DPIP "replace" in this experiment?

NADP ✓

3. What is the source of the electrons that will reduce DPIP?

WATER (PHOTOLYSIS)

4. What was measured with the spectrophotometer in this experiment?

IT MEASURES THE PERCENT LIGHT TRANSMITTANCE THROUGH THE CUVETTE DUE TO DPIP REDUCTION.

5. What is the effect of darkness on the reduction of DPIP? Explain.

IF DPIP IS NOT EXPOSED TO LIGHT, IT DOES NOT (REDUCED) REACT AND THEREFORE ~~PHOTOSYNTHESIS~~ DOES NOT OCCUR. (NO COLOR CHANGE)

(-2)



6. What is the effect of boiling the chloroplasts on the subsequent reduction of DPIP? Explain.

BOILING THE CHLOROPLASTS  
CAUSES PROTEINS TO DENATURE ✓  
TO A STATE OF DISFUNCTION.  
THEY WILL NOT SYNTHESIZE  
AT SUCH A RATE THAT THE  
UNBOILED WILL.

(DISRUPTS CHLOROPLAST MEMBRANES)

7. What reasons can you give for the difference in the percentage of transmittance between the live chloroplasts that were incubated in the light and those that were kept in the dark?

IN THE DARK CUVETTE  
ELECTRONS DID NOT FLOW  
AND PHOTOSYNTHESIS DID NOT  
OCCUR. IN LIGHT CUVETTES  
ELECTRONS ARE FLOWING AND  
PROCESSES ARE OCCURRING ✓

8. Identify the function of each of the cuvettes.

Cuvette 1: CONTROL; TEST AFFECT OF  
DPIP

Cuvette 2: CONTRAST TO UNBOILED  
LIGHT; TEST AFFECT OF LIGHT

Cuvette 3: COMPARE TO BOILED  
LIGHT; "REGULAR" CHLOROPLASTS

Cuvette 4: COMPARE TO UNBOILED  
LIGHT; TEST AFFECTS OF BOILING

Cuvette 5: PROVE THAT CHLOROPLASTS  
ABSORB LIGHT ✓