

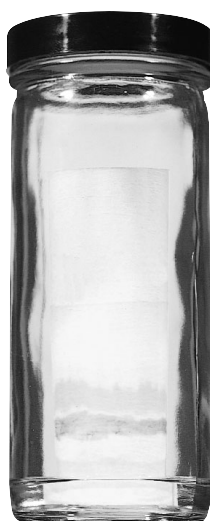
ADVANCED PLACEMENT[®] BIOLOGY

==== Laboratory 4 =====

Plant Pigments and Photosynthesis

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TEACHER'S MANUAL

CAROLINA

World-Class Support for Science & Math

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LABORATORY 4. PLANT PIGMENTS AND PHOTOSYNTHESIS

Objectives

In this laboratory, students will

- separate plant pigments using chromatography and calculate R_f values
- measure the rate of photosynthesis in isolated chloroplasts (The measurement technique involves the reduction of the dye DPIP. The transfer of electrons during the light-dependent reactions of photosynthesis reduces DPIP and changes its color from blue to colorless.)

Required Knowledge

Before beginning this laboratory, students should understand

- how to use a spectrophotometer

Expectations

At the completion of this laboratory, students should be able to

- understand the principles of chromatography
- calculate R_f (Reference front) values
- design an experiment in which chromatography is used as a separation technique
- describe a technique for determining photosynthetic rate
- understand the relationship between dependent and independent variables
- describe how light intensity, light wavelength, and temperature can affect photosynthesis
- design an experiment to measure how light intensity, light wavelength, and temperature can affect photosynthetic rates

Time Requirements

Exercise 4A (Plant Pigments) requires one 45-minute laboratory period. This period can be used to demonstrate paper chromatography and the apparatus for the measurement of photosynthesis when the DPIP-reduction technique is used.

Exercise 4B (Photosynthesis) requires one 45-minute laboratory period. This period can be used for the measurement of photosynthesis using the DPIP-reduction technique.

Preparation

Photocopy the Student Guide from this manual for your class.

Solutions

DPIP: Return the enclosed card for delivery of fresh DPIP solution. The solution has a shelf life of three months when stored in a refrigerator.

Chromatography solvent: Supplied ready to use. The solvent is 9 parts petroleum ether to 1 part acetone. **Caution:** Because of the volatility of the solvents, keep this and all solutions tightly capped.

Chlorophyll sample for chromatography: Students will place an ivy leaf or spinach leaf over a piece of chromatography paper. Then, they will roll the edge of a coin over the leaf, using a ruler as a guide, so that the pigments of the leaf are driven into the chromatography paper about 1.5 cm from the bottom. This will produce a straight line of pigment that can be chromatographed.

Student Materials and Equipment

Following is a list of the materials needed for one group of students to perform the exercises in this lab. Prepare as many setups as needed for your class. Prepare the chromatography solvent as near to lab time as possible.

Setup for Each Group

	Exercise 4A	Exercise 4B
Student Guides	4	4
No. 1 Whatman chromatography paper	1	
chromatography jar, tightly capped, with 10 mL of chromatography solvent	1	
*ivy or spinach leaf	1	
*coin	1	
stapler or paper clip	1	
*ruler	1	1
fish bowl filled with water		1
1-mL disposable plastic pipets		4
amber vial unboiled chloroplast suspension		1
amber vial boiled chloroplast suspension		1
lens tissue		1
*distilled water (13 mL)		1
vial 0.1 M phosphate buffer		1
vial DPIP solution		1
Parafilm® (for covering tops of cuvettes while mixing)		4 squares
*✓spectrophotometer (Spectronic® 20 or equivalent)		1
*cuvettes (spectrophotometer tubes)		5
*cuvette labels, numbered 1–5		1 set
*test tube rack		1
*100-watt floodlight		1
*clock or timer		1
*aluminum foil		1
*ice bucket and ice		1
*calculator		1

*Items not included in kit.

✓Item can be shared by more than one group.

Safety Equipment

Students should wear safety glasses, aprons, and gloves when working with the chromatography solvent. Avoid skin contact with the solvent. Students should work with a fume hood if there is one available.

Preparation

Exercise 4B: Photosynthesis

Solutions

0.5 M sucrose (for chloroplast suspension): Supplied ready to use.

0.1 M phosphate buffer: Supplied ready to use.

DPIP: **Return the enclosed card for delivery of fresh DPIP solution.** The solution has a shelf life of three months when stored in a refrigerator.

Chloroplast suspension

1. To prepare and prime the chloroplasts, incubate fresh spinach leaves under a light for a few hours. Do not allow the leaves to become hot.
2. Chill the bottle of 0.5 M sucrose.
3. Make sure the blender is unplugged.
4. Pour the cold sucrose solution into the blender.
5. *Loosely* pack fresh spinach leaves into the blender to a level one-inch above the blades.
6. Set up the empty sucrose bottle in a bucket of ice. Place the funnel in the mouth of the bottle and put two layers of cheesecloth in the funnel. The funnel and cheesecloth are supplied in the kit.
7. Blend the spinach leaves thoroughly (three 10-sec bursts).
8. Squeeze the blended material through the cheesecloth into the empty sucrose bottle.
9. Pour about 2 mL of the suspension into an amber vial labeled “unboiled.” Prepare one “unboiled” vial for each group of students. If the suspension coagulates, suspend it again before use. Maintain the chloroplast suspensions on ice.
10. For each group of students, prepare the boiled chloroplast suspension: Transfer about 2 mL of the suspension to a test tube. Place the test tube in a boiling water bath for five minutes.
11. Pour about 2 mL of the boiled suspension into an amber vial labeled “boiled.” Prepare one “boiled” vial for each group of students. If the suspension coagulates, suspend it again before use. Maintain the chloroplast suspensions on ice.

Note: Students must know how to use the spectrophotometer before beginning the exercise. Colorimeters and associated hardware/software can be substituted for the spectrophotometer.

Troubleshooting

Protocol Suggestions

If students have never used a spectrophotometer before, this can be a source of error. Consider having them practice with various dilutions of food coloring prior to doing this activity.

Make sure that your students do not contaminate tubes while mixing. Also, make sure that they do not add too much chloroplast suspension to the cuvettes, or the reduction of DPIP will occur too rapidly to be recorded. They must also begin the time course readings immediately after adding the chloroplast suspension to the cuvettes, or all DPIP will be reduced before the readings have begun. In the Student Guide, students are directed to add the chloroplast suspension to one cuvette at a time and take a reading immediately. Emphasize that they must follow these directions.

Erratic data can often be traced to students allowing more than five minutes to elapse between readings.

No reduction of DPIP

If the DPIP was not reduced by the unboiled chloroplast suspension in light, the suspension was not active. This could be because the spinach leaves were old and wilted, because too few leaves were used, or because the leaves were not ground sufficiently to release the chloroplasts. Small hand-held blenders may not grind leaves sufficiently. If you do not have access to fresh spinach or ivy, leaves from a living geranium plant work very well.

Too-rapid reduction of DPIP

In this case, the chloroplast suspension was so active that the DPIP was reduced before it could be measured. Have students repeat the procedure, adding only a single drop of the chloroplast suspension. If the reduction is still too rapid, dilute the chloroplast suspension in 0.5 M sucrose. Prepare 0.5 M sucrose solution by dissolving 17.1 g of sucrose in 80 mL of water, then bringing the final volume of the solution to 100 mL.

Erratic readings with the boiled chloroplast suspension

Boiling the chloroplast suspension causes the formation of debris in the suspension. If a bit of debris falls into the light beam of the spectrophotometer while a reading is being taken, this will disrupt the reading. To solve this problem, some teachers prefer to heat the chloroplasts in a 50°C water bath for several minutes rather than boil them. This deactivates the chloroplasts but produces little if any debris. Note that the chloroplasts may retain some slight activity after this treatment.

Alternate Exercises

Should a spectrophotometer be unavailable, use the following technique, but be aware that results may be less satisfactory.

- Gather four test tubes. Wrap one test tube with aluminum foil. Place all four in a test tube rack. To each tube add 6 mL of distilled H₂O, 2 mL of phosphate buffer, and 2 mL of DPIP. To test tubes 1 and 2, add 4 drops of chloroplast suspension. Immediately close the aluminum foil around the top of Tube 1. To Tube 3, add 4 drops of boiled chloroplast suspension. To Tube 4, add 4 drops of distilled water. Place all four tubes in front of floodlights for 10 minutes. Place a beaker of H₂O between the lights and tubes to protect the chloroplasts from excessive heat.
- Observe each tube and record the results on the data sheet.

Tube	Initial Color	Final Color	Reaction +/-
1. DPIP plus chloroplasts in dark			
2. DPIP plus chloroplasts in light			
3. DPIP plus boiled chloroplasts in light			
4. DPIP plus distilled water in light			

**Further
Investigations**

If time and equipment are available, the absorption spectrum of spinach pigments can be determined and plotted by using a chlorophyll extract and a spectrophotometer.

Prepare the extract by filling a mortar with fresh spinach. Grind the leaves thoroughly in acetone. **Caution:** *Do not use a blender. Acetone is highly flammable.* Filter through two layers of cheesecloth into a beaker on ice. Do not expose the solution to light for any longer than necessary. Put the chlorophyll extract in lightproof vials and store in a refrigerator or on ice.

Students should measure and plot the absorbance of the pigment extract from 400–700 nm at 50-nm wavelength intervals. Every 50 nm, reset the spectrophotometer to zero absorbance with the blank cuvette (containing acetone) before reading the absorbance of the sample cuvette.

Students can design other experiments using the DPIP reduction technique to investigate how factors such as light intensity or temperature might affect photosynthesis.

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- design an experiment to measure how light intensity, light wavelength, and temperature can affect photosynthetic rates

Exercise 4A: Plant Pigments

Introduction

Paper chromatography is a technique for separating and identifying pigments and other molecules from cell extracts that contain a complex mixture of molecules. In paper chromatography, the sample is applied to a piece of special paper, then one end of the paper is put into a solvent. The solvent moves up the paper by capillary action resulting from 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. In the procedure you are about to perform, the dissolved substances are pigments from a leaf. The pigments are carried along at different rates because they are not equally soluble in the solvent and also because they are attracted, in different degrees, to the cellulose in the paper (through the formation of hydrogen bonds).

Chlorophyll *a* is the primary photosynthetic pigment in all plants. A molecule of chlorophyll *a* is located at the reaction center of photosystems. Other chlorophyll *a* molecules, chlorophyll *b*, and the carotenoids (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 bright sunlight. Beta-carotene is the most abundant carotene in plants.

Procedure**Chromatography of Plant Pigments**

Your teacher will demonstrate the apparatus and techniques used in paper chromatography. Use the following steps as a guide.

1. Obtain an 8-cm square piece of chromatography paper and one fresh spinach (or other) leaf.
2. Make two pencil marks 1.5 cm from one edge of the chromatography paper as shown in Figure 4.1, below.

Figure 4.1 (not to scale)



3. Lay the leaf on the chromatography paper near one edge. Using the marks as a guide, lay a ruler (or other straight edge) on top of the leaf, so that the edge of the ruler is on the paper 1.5 cm from, and parallel to, the edge.
4. Using the ruler as a guide, roll a coin over the leaf so that you drive the leaf pigments into the paper in a straight line 1.5 cm from the edge of the paper. You should see a dark green stripe of pigment. If not, repeat this step using the same 1.5-cm line, but reposition the leaf so that you are rolling the coin over fresh leaf tissue. Use a pencil to mark the location of the bottom of the pigment line on the paper. Use this line as the origin.
5. Form a cylinder with the chromatography paper (see Figure 4.2) by stapling or paper-clipping each end so that the two edges do not overlap. Place the chromatography paper in the jar (**Caution:** Avoid breathing fumes from the solvent.) so that the pigment streak end of the paper is barely immersed in the solvent. The pigment stripe itself should not be in the solvent (see Figure 4.3).

Figure 4.2

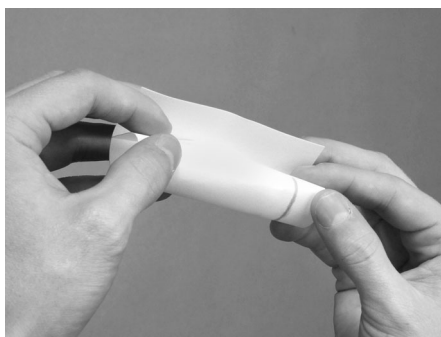


Figure 4.3



6. Cover the jar. Do not disturb the jar for several minutes. Check it often.
7. When the solvent is about 1 cm from the top margin of the paper, remove the paper and immediately mark the location of the solvent front before it evaporates.
8. Mark the bottom of each pigment band.
9. Beginning at the origin, measure the distance traveled by the solvent front and each of the pigment bands and record the results in Table 4.1. Band 1 is the pigment band nearest the origin, etc.

Table 4.1

Chromatography of Plant Pigments		
Band #	Distance (mm)	Band Color
1.		
2.		
3.		
4.		
Solvent Front		

Questions

1. Calculate the R_f (Reference front) values for each of the four pigments, using the formula:

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

Record the R_f values in Table 4.2, below.

Table 4.2

	= R_f for carotene (faint yellow to yellow-orange)
	= R_f for xanthophyll (yellow)
	= R_f for chlorophyll <i>a</i> (bright green to blue-green)
	= R_f for chlorophyll <i>b</i> (yellow-green to olive green)

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

3. If you repeated this activity using a different solvent, would you expect to get the same R_f values for the pigments? Explain your answer.

4. Which is the main photosynthetic pigment? What are the functions of the other three pigments?

Exercise 4B: Photosynthesis

Background

The human eye responds to a certain range of wavelengths of electromagnetic radiation. We call radiation within this range “visible light.” The shorter the wavelength, the greater the energy of the radiation. Ultraviolet radiation, X-rays, and gamma rays possess shorter wavelengths and more energy than visible light. These high-energy radiations can harm living tissues. Electromagnetic radiations with lower energy (longer wavelengths) than visible light are called infrared radiation and radio waves.

Plant cells contain pigments that absorb electromagnetic radiation of wavelengths within the visible range. The pigment molecules are part of complexes called photosystems, which are found in the chloroplasts of palisade mesophyll leaf cells.

When leaf pigments absorb light, electrons within the photosystems are boosted to a higher energy level and this energy is used to produce ATP and reduce NADP to NADPH. The high-energy products, ATP and NADPH, are then used to incorporate CO_2 into organic molecules, a process called carbon fixation.

Introduction

In this experiment, photosynthesis is detected by the donation of a high-energy electron to a dye molecule. The dye is 2,6-dichlorophenol-indophenol (DPIP). It will take the place of NADP as the electron acceptor in photosynthesis. Unreduced DPIP is blue. When it is reduced by a high-energy electron, DPIP changes from blue to colorless. Therefore, photosynthesis can be detected by following the color change of DPIP. In this activity, you will add an extract of chloroplasts from spinach leaves to a DPIP solution and incubate the mixture in the presence of light. As the DPIP is reduced and becomes colorless, the resultant increase in light transmittance is measured over time using a spectrophotometer.

This experiment is designed to test the hypothesis that light and active chloroplasts are required for the light reactions of photosynthesis to occur. The experimental design matrix is presented in Table 4.3.

Table 4.3 Experimental Design Matrix

Cuvettes					
Contents	#1 Blank (Unboiled)	#2 Dark/ Unboiled	#3 Light/ Unboiled	#4 Light/ Boiled	#5 No Chloroplasts
Phosphate Buffer	1 mL	1 mL	1 mL	1 mL	1 mL
Distilled H ₂ O	4 mL	3 mL	3 mL	3 mL	3 mL plus 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. 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 floodlight, a fishbowl full of water, and a test-tube rack (see Figure 4.4). The water in the fish bowl will act as a heat sink by absorbing most of the light's infrared radiation while having little effect on the light's visible radiation.

Figure 4.4



4. Your teacher will provide you with vials containing boiled and unboiled chloroplast suspensions. Keep these on ice.

Note: You have four plastic transfer pipets for setting up the experiment. Keep track of them carefully and use them as directed, so that you will not cross-contaminate reagents. The first is to be used for H₂O and then later for buffer solution, the second for DPIP, the third for unboiled chloroplasts suspension, and the fourth for boiled chloroplasts suspension. Be aware that the pipets have a 1-mL graduation mark at the top of the neck, near the bulb.

Note: When you add the chloroplasts to the dye solution, photosynthesis may reduce the dye very quickly. Add chloroplasts to only one cuvette at a time and measure each cuvette's transmittance immediately. Follow these instructions closely.

- At the top rim of the cuvettes, place labels numbered 1, 2, 3, 4, and 5, respectively. Using lens tissue, wipe the outside walls of each cuvette. Use the first of the plastic transfer pipets to add 4 mL of distilled H₂O to Cuvette 1. With the same pipet, add 3 mL of distilled H₂O to cuvettes 2, 3, 4, and 5. Add three additional drops of H₂O to Cuvette 5. Next, still using the first pipet, add 1 mL of phosphate buffer to all five cuvettes (1, 2, 3, 4, and 5). With a fresh (second) pipet, add 1 mL of DPIP dye to cuvettes 2, 3, 4, and 5.

Note: Handle cuvettes only near the top. Cover the walls and bottom of Cuvette 2 with foil and make a foil cap "cover" for the top. Light should not be permitted inside Cuvette 2 because it is the control for this experiment.

- Mix the *unboiled* chloroplast suspension, and transfer three drops of it to Cuvette 1 with a fresh (third) pipet. Set the pipet aside for reuse. Set the spectrophotometer to zero by adjusting the amplifier control knob until the meter reads 0% transmittance. Cover the top of Cuvette 1 with Parafilm® and invert it to mix. Insert Cuvette 1 into the sample holder and adjust the instrument to 100% transmittance by adjusting the light-control knob. Retain Cuvette 1 as a blank to be used to recalibrate the instrument between readings. 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.
- Mix the *unboiled* chloroplast suspension and, using the (third) pipet you used to add the unboiled chloroplasts to Cuvette 1, transfer three drops of the unboiled suspension to Cuvette 2. *Immediately* cover and mix Cuvette 2, remove it from the foil sleeve, insert it into the spectrophotometer's sample holder, and read the % transmittance. Record it as the "Time: 0 min" reading in Table 4.4. Rewrap Cuvette 2 in foil, turn on the incubation light, and place the cuvette in the incubation test-tube rack. Measure and record additional readings at 5, 10, and 15 minutes. Cover and mix the cuvette prior to taking each reading. Use Cuvette 1 occasionally to check and adjust the spectrophotometer to 100% transmittance.
- Mix the *unboiled* chloroplast suspension and, using the same (third) pipet, as before, transfer three drops of the unboiled suspension to Cuvette 3. *Immediately* cover and mix Cuvette 3, insert it into the sample holder, read the % transmittance, and record the results in Table 4.4. Place Cuvette 3 in the incubation test-tube rack beside Cuvette 2. Take and record additional readings at 5, 10, and 15 minutes. Cover and mix the cuvette prior to taking each reading.
- Mix the *boiled* chloroplast suspension and use the last (fourth) transfer pipet to transfer three drops of the boiled suspension to Cuvette 4. *Immediately* cover and mix Cuvette 4, insert it into the sample holder, read the % transmittance, and record the results in Table 4.4. Place Cuvette 4 in the incubation test-tube rack beside Cuvette 3. Take and record additional readings at 5, 10, and 15 minutes. Cover and mix the cuvette prior to taking each reading.
- Cover and mix Cuvette 5, insert it into the sample holder, read the % transmittance, and record the results in Table 4.4. Place Cuvette 5 in the incubation test-tube rack beside Cuvette 4. Take and record additional readings at 5, 10, and 15 minutes. Cover and mix the cuvette prior to taking each reading.

Table 4.4 Percent Transmittance of Chloroplast Suspension

Cuvette #	Time			
	0 min	5 min	10 min	15 min
2. Unboiled/Dark				
3. Unboiled/Light				
4. Boiled/Light				
5. No Chloroplasts				

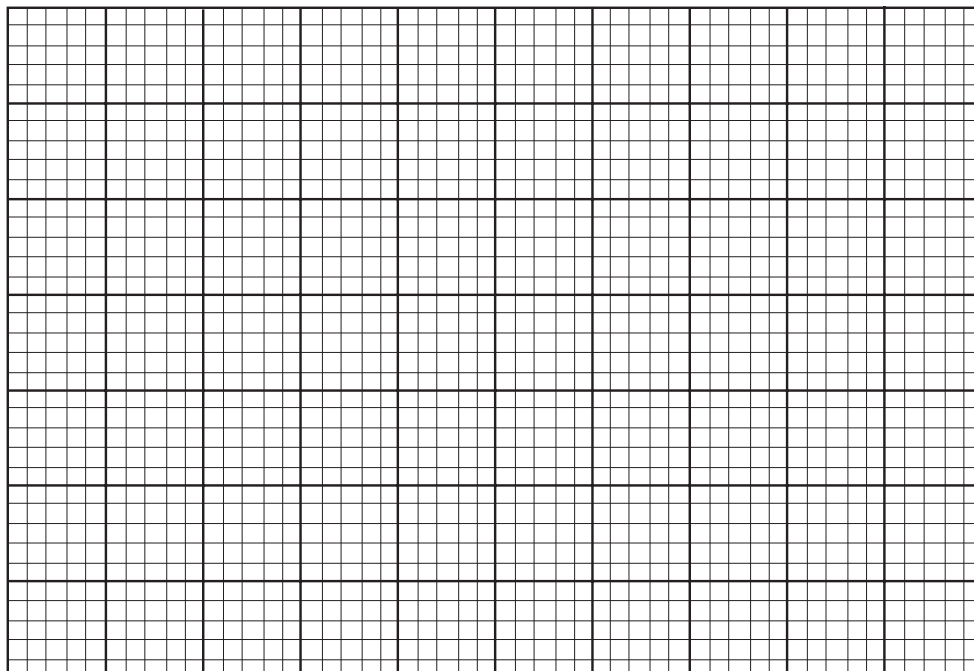
Plot the data from Table 4.4 on Graph 4.1, below. Label each plotted line.

- a. The independent variable is _____.
(Use to label the horizontal x-axis.)
- b. The dependent variable is _____.
(Use to label the vertical y-axis.)

Graph 4.1

Title: _____

Label (y-axis):



Label (x-axis): _____

Questions

1. What is the effect of darkness on the reduction of DPIP?

2. What is the effect of boiling the chloroplasts on the reduction of DPIP?

3. What is the difference in the percent transmittance between the unboiled chloroplasts that were incubated in light and those that were kept in the dark?

4. Based on the results you have recorded in Graph 4.1, what must be present for photosynthesis to occur?

5. Give the purpose of each cuvette.

#1.

#2.

#3.

#4. _____

#5. _____

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