AP Biology Lab #4: Plant Pigments and Photosynthesis

OVERVIEW:

In this lab you will:

1) Separate plant pigments using chromatography.
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:

A) How chromatography separates two or more compounds that are initially present in a mixture.
B) The process of photosynthesis.
C) The function of plant pigments.
D) The relationship between light wavelength and photosynthetic rate.
E) The relationship between light intensity and photosynthetic rate.

After doing this lab you should be able to:

A) Separate pigments and calculate their R_f values.
B) Describe a technique to determine photosynthetic rates.
C) Compare photosynthetic rates at different light intensities or different wavelengths of light using controlled experiments.
D) Explain why the rate of photosynthesis varies under different environmental conditions.

INTRODUCTION: Exercise 4A – Plant Pigment Chromatography

When a substance absorbs visible light of certain wavelengths, while reflecting and/or transmitting light of other wavelengths, we see the substance as colored. The actual color depends on the wavelengths of light reaching our eyes. Such substances are called pigments.

When a pigment absorbs light energy, it momentarily forms an □excited□, or high energy, molecule. In most cases this energy is dissipated without doing any useful work. Plants, however, □trap□ the energy absorbed by their pigments, the chlorophylls, and couple this trapping process to the synthesis of carbohydrates. Other plant pigments, such as carotenoids, absorb wavelengths of light different from those absorbed by the chlorophylls. This energy is then transferred to the chlorophylls.

There are a number of different chlorophyll molecules and carotenoid molecules found in plants. In this laboratory activity you will separate these different photosynthetic pigments from a plant specimen using a technique known as chromatography.

Chromatography is a useful way for separating and identifying small quantities of related substances in a mixture. As the name implies, the technique was first used with pigment molecules, however, it is used for any substances that can be identified (often by staining) when the chromatography process is complete. Although there are many varieties of chromatography, it usually involves adding small quantities of the mixture being studied to a matrix (called the stationary phase) of cellulose, silica gel, alumina, or some other inert substance. A solvent (called the mobile phase) is then allowed to be absorbed by the matrix. As the solvent moves through the matrix by capillary action, it dissolves the substances in the mixture being studied and allows them to migrate through the matrix also. The solvent is formulated so that the substances are not completely soluble in it, preventing them from migrating through the matrix as quickly as the solvent. The greater the solubility, the faster the substance tends to move. As the substances being studied move through the matrix they also tend to adsorb (stick) to the matrix, with each substance adsorbing to a greater or lesser degree depending on their molecular structure. The movement through the matrix at different rates will separate the different substances. When the solvent nearly reaches the end of the matrix, the process is stopped. In our case, molecules separate both by their attraction to the paper AND their molecular size, since they have to travel against the force of gravity. Measurements are made from the origin to the solvent front and to each substance. Then a ratio of the distance traveled by the solvent to the distance traveled by each substance is calculated. This ratio, called the R_f, is constant for a given substance in a given solvent and matrix system. It is calculated as follows:

$$R_f = \frac{\text{distance the pigment migrated}}{}$$

distance the solvent front migrated

In this laboratory activity you will separate plant pigments using a variety of chromatography known as paper chromatography. In this case, therefore, the matrix will be cellulose. Attraction of the pigment molecules to the cellulose will be though the formation of hydrogen bonds.

Procedure: Exercise 4A

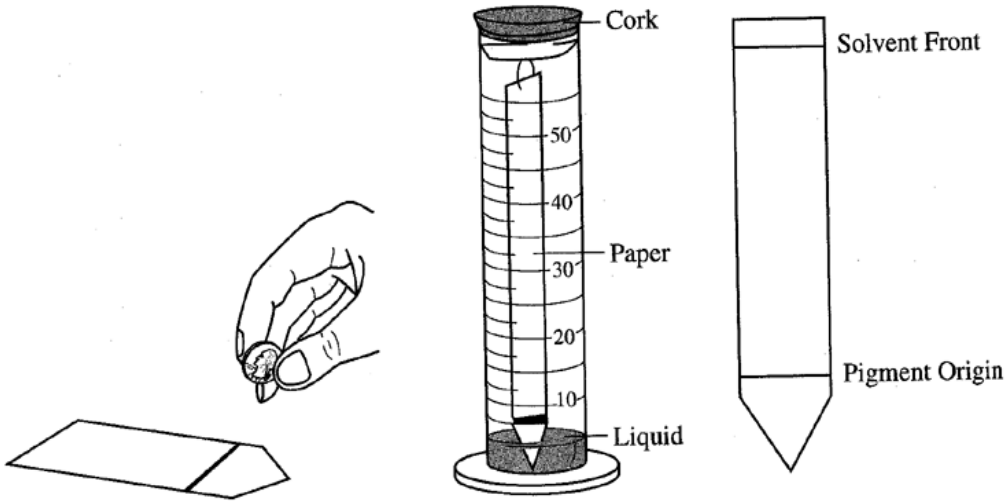
Making the Chromatogram

1. Wash a dry your hands thoroughly before performing the lab. Your fingerprints will leave oils on the chromatography paper that will adversely affect your results. Try also to handle the paper by the edges as much as possible.

2. Cut a piece of chromatography paper into a 1 cm x 13 cm strip. Punch a small hole at one end of the strip and make a light pencil line (not pen) 1.5 cm from the other end along the same horizontal plane as the bottom of the strip

3. Obtain a large test tube and a cork stopper with a J-hook attached to it. Hang the chromatography paper strip onto the J-hook and put the paper and stopper into the tube.

4. With a marker, draw a line on the outside of the tube between the tube bottom and the pencil line on the chromatography paper strip. Remove the stopper from the tube and take the chromatography paper strip off the hook. Fill the test tube to the marker mark with the chromatography solvent and stopper immediately.



This will saturate the atmosphere of the chamber insuring better separation. [**WARNING**: *The solvent is highly volatile, flammable, and poisonous*. **Do not inhale the solvent!**]

5. On your lab table, place a spinach leaf over the bottom of the chromatography paper and roll a quarter across the leaf, pressing strongly. This will deposit a great amount of pigment on the paper AND have the added benefits of not spreading the line too thinly. Move the leaf slightly and roll another pigment line on top of the first line. If necessary, roll a third time.

6. After producing a dark green line, carefully cut the corners of the chromatography paper strip at the green line end, to produce a V-shape at the bottom.

7. Quickly remove the stopper, attach the chromatography paper strip onto the J-hook and place the strip and the cork into the test tube. Try to place the strip upright in the chamber not touching the tube sides. Do not leave the test tube uncapped any longer than is necessary.

8. Allow the solvent to migrate up the chromatography paper strip until reaches approximately 1 cm from the top. Allow the tube to remain ***undisturbed*** during the process. Do not allow the solvent to reach the top of the paper.

9. When the solvent gets to the top of the paper, quickly remove the strip and recap the test tube. Gently mark the leading edge of the solvent with a pencil. Air dry the chromatography paper strip on a paper towel.

Part C. **Analyzing the Chromatogram**

1. The number of lines that you observe will depend on the extract and solvent you used. The major pigments you should be able to see are listed below.

Chlorophyll *a*	blue green	Carotene	orange yellow
Chlorophyll *b*	yellow green	Xanthophyll	yellow

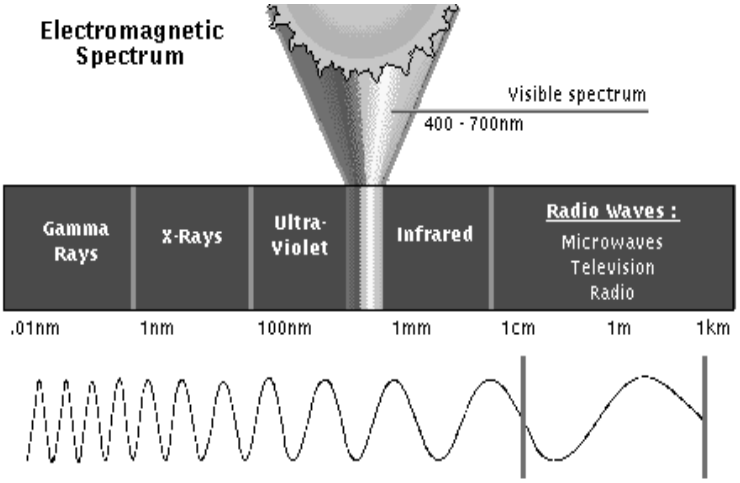
2. Lightly make a pencil line at the leading edge of <u>each</u> pigment line. Measure the distances traveled from the origin for the solvent and for each pigment. Calculate an R_f for each pigment. Organize your data in an appropriate data table, with a proper title, with data and location.

INTRODUCTION: Exercise 4B – Photosynthesis

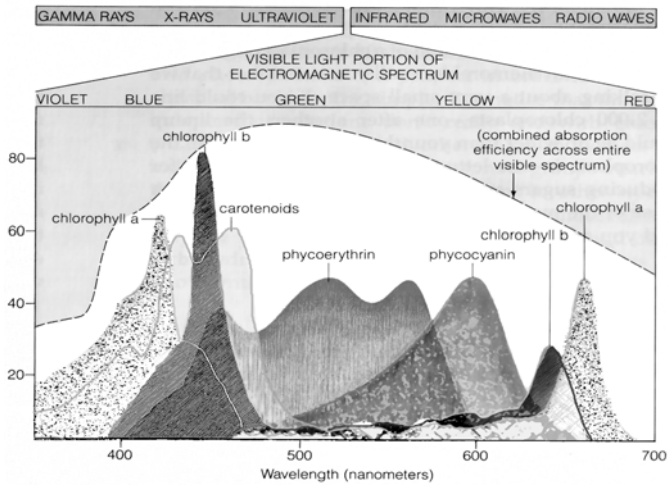
Introduction

Light is a part of a radiation continuum. Visible wavelengths of the light spectrum are absorbed by leaf pigments in the light reaction of photosynthesis. During this reaction, electrons are stripped from water and, after having passed through photosystems I and II, are used to reduce **$NADP^+$**. The photosystems found in chloroplasts of palisade mesophyll leaf cells, contain the pigments that absorb light; photosystem I contains mostly chlorophyll *a* and photosystem II contains mostly chlorophyll *b*. The products of the light reaction are O_2, ATP, and **NADPH**. The oxygen is released while the ATP and NADPH are used in the dark reaction to convert carbon dioxide to PGAL, then to glucose and other organic compounds.

Color, like the green of chlorophyll, provides us with both beauty and useful information. Color is a source of our pleasure in a sunset, in autumn leaves or in a bouquet of flowers. Color can also be an indicator of when our vegetables and fruits are ripe, when our coffee is strong enough or when a storm is coming. Chemical pigment molecules impart color in living organisms. These pigments may function as attractants (in flower petals), as light receptors for vision (in the rods and cones of the retina) or as energy transducers (in photosynthetic pigments of leaves).



The visible light spectrum like X-rays, radio waves and infrared rays are part of the spectrum of electromagnetic radiation. Types of electromagnetic radiation differ in both wavelength and energy level, but all types travel through space in waves. In the visible spectrum, the color of the light we see depends on its wavelength. Wavelength is measured in units called nanometers (1 x 10^{-9} m). Wavelengths of 400 to 700 nm comprise the visible light spectrum and the part of the electromagnetic spectrum that can excite photoreceptors within the human eye.



Molecules either absorb or transmit energy in the form of electromagnetic radiation in the visible spectrum. White light (normal daylight) is made up of all the wavelengths of electromagnetic radiation in the visible spectrum. How objects or chemical substances absorb and transmit the light that strikes them determines their color.

What we see as the color of an object, or a solution, is determined by what wavelengths of light are □left over□ to be transmitted or reflected by the object after its constituent molecules absorb certain wavelengths. For example, the pigment chlorophyll, present in the leaves of plants, absorbs a high percentage of the wavelengths of light in the red and violet to blue

ranges. Green light, not absorbed by chlorophyll molecules, is reflected from the surface of the leaf, the reason that most plants appear to be green.

In this lab activity you will study the Hill reaction, discovered in 1937 by Robert Hill. In the Hill reaction **$NADP^+$** (or any other electron acceptor) is reduced in the presence of light and chloroplasts. As did Hill, you will use an artificial electron acceptor molecule that will intercept electrons as soon as they are stripped from water. The artificial electron acceptor, **DPIP** (2, 6 dichlorophenolindophenol), is blue in its oxidized state and colorless in its reduced state. Thus, you can determine the Hill reaction rate by measuring the change in DPIP color over time.

In order to accurately measure the color change in DPIP, you will use an instrument called a spectrophotometer (see *Figure 2*). The spectrophotometer, which assesses the intensity of light transmitted through a liquid at specific wavelengths, is used extensively for bioanalytical determinations. Within the optical system of the Spectronic 20, rotation of a diffraction grating (which acts like a prism) allows the user to select specific wavelengths of light in a range from 375 nm to 625 nm. Light of a selected wavelength is passed through the sample solution and is picked up by a measuring phototube where the light energy is converted to a reading on a meter. Most spectrophotometers have two scales, one is a linear scale giving percent transmittance, and the other is a logarithmic scale giving absorbance.

The spectrophotometer that you will use emits varying visible wavelengths of light. When light is shined through a sample solution, such as DPIP, some light is absorbed and some is transmitted. The amount of transmitted light can be detected by a photocell and converted to electricity which, in turn, can be measured by a meter. As more light is transmitted, more electricity is produced, and, therefore, the meter needle will move a greater distance.

You will try to experimentally answer the following three questions during this laboratory investigation:

a. *Does the rate of the photosynthetic light reaction vary in the light and the dark?*
b. *Does the rate of the photosynthetic light reaction vary with different light intensities?*
c. *Does the rate of the photosynthesis light reaction vary with different wavelengths of light?*

Procedure: Exercise 4B

Part A. Setting Up The Apparatus

1. Your teacher makes sure the spectrophotometer is turned on 15 minutes before the lab; the wavelength set to 625 nm and will demonstrate its use. You may not personally be reading each cuvette measurement due to lack of sufficient machines but you should understand how the measurements are performed and how the *Spec 20* works.

2. Set up each experimental area with a floodlight and a flask full of water. Place the light on the table so that it will shine through the flask of water. Keep the light ***off*** until instructed to turn it on. (See Figure 2.)

3. Darken the room.

Part B. Collecting Data

1. Set up 4 spectrophotometer cuvettes according to the table below. Add the solutions in order, from left to right, on the table. Label the top rim of the cuvettes with the appropriate tube number. Using lens paper, wipe any fingerprints from the outside walls of each cuvette. ***Remember to handle cuvettes near the top only.*** Make aluminum sleeves for each cuvette so that they cover the walls and bottom and make a foil cap for the top. Don't make the sleeve so tight that it is difficult to pull your cuvettes out of them. **KEEP THE SLEEVES DRY - ADD ANY SOLUTION WHILE THE CUVETTES ARE OUT OF THE TUBES.** Remember to keep your tubes covered at all times EXCEPT when 3 of the tubes are being purposefully exposed to light.

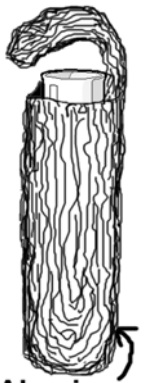


Table 1. Amount of solutions to be added to experimental cuvettes

SOLUTION TO BE ADDED	*CUVETTES*			
	(1) BLANK	**(2) DARK**	**(3) LIGHT**	**(4) BOILED**
Dist. H_2O	4.0 ml	3.0 ml	3.0 ml	3.0 ml
Buffer	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Chloroplasts	5 drops	5 drops	5 drops	5 drops **(boiled)**
DPIP	----	1.0 ml	1.0 ml	1.0 ml

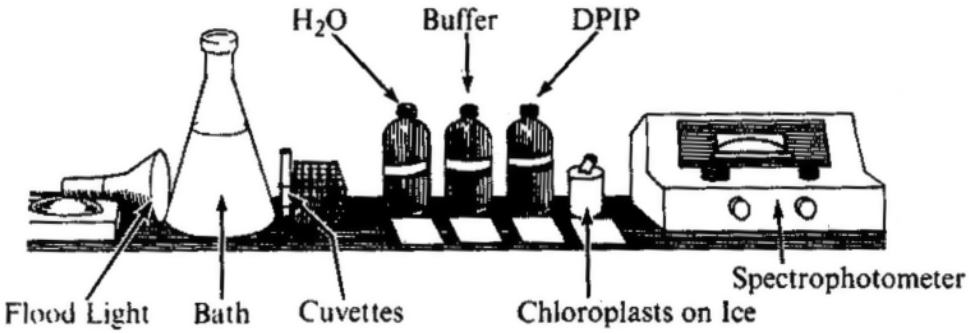
3. Add 5 drops of chloroplast suspension to tube 1, ***mix by agitating the tube swiftly while holding the cuvette at the top (be careful not to lose liquid!), wipe with lens paper***, and immediately re-insert the cuvette back into its aluminum sleeve. Place it in the test tube rack, in proper order.

4. Add 5 drops of chloroplast suspension to cuvette #2, mix, wipe, and immediately re-insert the cuvette back into its aluminum sleeve. Place it in the test tube rack, in proper order.

5. Add 5 drops of chloroplast suspension to cuvette #3, mix, wipe, and immediately re-insert the cuvette back into its aluminum sleeve. Place it in the test tube rack, in proper order.

6. Add 5 drops of BOILED chloroplast suspension (from the class stock of boiled chloroplasts) to cuvette #4, mix, wipe, and immediately re-insert the cuvette back into its aluminum sleeve. Place it in the test tube rack, in proper order.

7. When all the cuvettes have been filled, bring the entire rack of cuvettes, in proper order, to the spectrophotometry station. There may be another group making measurements so you have to wait your turn and make measurements swiftly and accurately so that you do not hold up the line. Check that the spectrophotometer sample holder is empty and close the cover. Adjust the spectrophotometer to zero by rotating the amplifier control (left) knob until the meter reads 0% transmittance.



Basic Set-up

8. Insert cuvette #1, the BLANK, into the sample holder and adjust the instrument to 100% transmittance by rotating the light-control (right) knob. Remove and retain cuvette #1 as a "**blank**" to recalibrate the instrument between readings. Even though this cuvette has a slight greenish color, we are telling the machine that it is perfectly clear, so that cuvettes with DPIP in them have reading reflecting a color change in DPIP *ONLY*, and are not influenced by the green from the chlorophyll inside them. In future readings, be certain that all cuvettes are inserted into the sample holder facing the same way.

9. Carefully remove cuvette #2 from its aluminum sheath and immediately insert it into the sample holder, read the % transmittance, and record the umber as the initial time (*time = 0*) reading. Immediately re-insert the cuvette back into its aluminum sleeve. **Light should not be permitted inside cuvette 2!** Place it in the test tube rack, in proper order. Repeat this procedure and record measurements for cuvettes 3 and 4, making sure to replace them in their sheath and in proper order.

10. Arrange the test tube rack so that all the cuvettes are equally exposed to the light. Remove cuvettes #3 and #4 from their aluminum sleeves, turn on the light and commence recording the time. Expose the

cuvettes to light for 5 minutes and immediately re-insert the cuvettes back into their aluminum sleeves. Bring the entire rack back to the spectrophotometry station and make another set of readings, remembering to use the blank FIRST to "zero" the machine before getting data for cuvettes 2-4. Return the entire rack of cuvettes (all in their sleeves) to your lab setup and make and record additional readings at the end of 10 and 15 minutes. (***Mix the solutions thoroughly*** just before each reading.)

11. Enter your individual data with classmates on a table similar to Table 2 below.

12. Clean up and put away all your materials. (Leave aluminum sleeves for the next class.)

Table 2. **Class data for rate of light reaction.**

TIME (min)	% Transmittance @ 640 *nm* of light							
	BLANK		**DARK**		**LIGHT**		**BOILED**	
	indiv group	***class***	***indiv group***	***class***	***indiv group***	***class***	***indiv group***	***class***
0	100	100						
5	100	100						
10	100	100						
15	100	100						

Procedure: Exercise 4B (Calculators):

1. Obtain two plastic Beral pipettes, two cuvettes with lids, and one aluminum foil covered cuvette with a lid. Mark one Beral pipette with a U (unboiled) and one with a B (boiled). Mark the lid for the cuvette with aluminum foil with a D (dark). For the remaining two cuvettes, mark one lid with a U (unboiled) and one with a B (boiled).

2. Plug the Colorimeter into Channel 1 of the LabPro CBL interface. Use the link cable to connect the TI Graphing Calculator to the interface. Firmly press in the cable ends.

3. Turn on the calculator and start the DATAMATE program. Press [CLEAR] to reset the program.

4. Prepare a *blank* by filling an empty cuvette ¾ full with distilled water. Seal the cuvette with a lid. To correctly use a Colorimeter cuvette, remember:
   - All cuvettes should be wiped clean and dry on the outside with a tissue.
   - Handle cuvettes only by the top edge of the ribbed sides.
   - All solutions should be free of bubbles.
   - Always position the cuvette with its reference mark facing toward the white reference mark at the right of the cuvette slot on the Colorimeter.

5. Set up the calculator and interface for the Colorimeter.
   a. Place the blank in the cuvette slot of the Colorimeter and close the lid.
   b. If the calculator displays COLORIMETER in CH 1, set the wavelength on the Colorimeter to 635 nm (Red). Then calibrate by pressing the AUTO CAL button on the Colorimeter and proceed directly to Step 7. If the calculator does not display COLORIMETER in CH1, continue with this step to set up your sensor manually.
   c. Select SETUP from the main screen.
   d. Press [ENTER] to select CH 1.
   e. Select COLORIMETER from the SELECT SENSOR menu.
   f. Select CALIBRATE from the SETUP menu.
   g. Select CALIBRATE NOW from the CALIBRATION menu.

First Calibration Point

h. Turn the wavelength knob of the Colorimeter to the 0% T position. When the voltage reading stabilizes, press ENTER. Enter “0” as the percent transmittance.

Second Calibration Point

i. Turn the wavelength knob of the Colorimeter to the Red LED position (635 nm). When the voltage reading stabilizes, press ENTER. Enter “100” as the percent transmittance.

j. Select OK to return to the setup screen.

k. Select OK to return to the main screen.

6. Obtain a 600-mL beaker filled with water and a flood lamp. Arrange the lamp and beaker as shown in Figure 2. The beaker will act as a heat shield, protecting the chloroplasts from warming by the flood lamp. Do not turn the lamp on until Step 11.

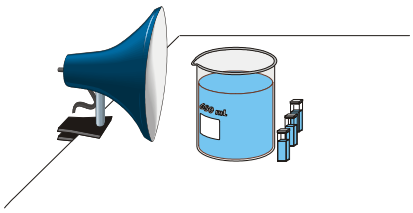


Figure 2

7. Locate the unboiled and boiled chloroplast suspension prepared by your instructor. Before removing any of the chloroplast suspension, gently swirl to resuspend any chloroplast which may have settled out. Using the pipette marked U, draw up ~1-mL of unboiled chloroplast suspension. Using the pipette marked B, draw up ~1-mL of boiled chloroplast suspension. Set both pipettes in the small beaker filled with ice at your lab station to keep the chloroplasts cooled.

8. Add 2.5 mL of DPIP/phosphate buffer solution to each of the cuvettes. **Important**: perform the following steps as quickly as possible and proceed directly to Step 10.

   a. Cuvette U: Add 3 drops of *unboiled* chloroplasts. Place the lid on the cuvette and gently mix; try not to introduce bubbles in the solution. Place the cuvette in front of the lamp as shown in Figure 2. Mark the cuvette’s position so that it can always be placed back in the same spot.

   b. Cuvette D: Add 3 drops of *unboiled* chloroplasts. Place the lid on the cuvette and gently mix; try not to introduce bubbles in the solution. Place the foil-covered cuvette in front of the lamp as shown in Figure 2 and mark its position. Make sure that no light can penetrate the cuvette.

   c. Cuvette B: Add 3 drops of *boiled* chloroplasts. Place the lid on the cuvette and gently mix; try not to introduce bubbles in the solution. Place the cuvette in front of the lamp as shown in Figure 2. Mark the cuvette’s position so it can always be returned to the same spot.

9. Take absorbance readings for each cuvette. Invert each cuvette two times to resuspend the chloroplast before taking a reading. If any air bubbles form, gently tap on the cuvette lid to knock them loose.

   a. Cuvette U: Place the cuvette in the cuvette slot of the Colorimeter and close the lid. Allow 10 seconds for the readings displayed on the calculator screen to stabilize, then record the absorbance value in Table 1. Remove the cuvette and place it in its original position in front of the lamp.

   b. Cuvette D: Remove the cuvette from the foil sleeve and place it in the cuvette slot of the Colorimeter. Close the Colorimeter lid and wait 10 seconds. Record the absorbance value displayed on the calculator screen in Table 1. Remove the cuvette and place it back into the foil sleeve. Place the cuvette in its original position in front of the lamp.

   c. Cuvette B: Place the cuvette in the cuvette slot of the Colorimeter and close the lid. Allow 10 seconds for the readings displayed on the calculator screen to stabilize, then record the absorbance value in Table 1. Remove the cuvette and place it in its original position in front of the lamp.

10. Turn on the lamp.

11. Repeat Step 10 when 5 minutes have elapsed.

12. Repeat Step 10 when 10 minutes have elapsed.

13. Repeat Step 10 when 15 minutes have elapsed.

14. Repeat Step 10 when 20 minutes have elapsed.

15. When all readings have been taken, select QUIT from the main screen.

DATA

Table 1			
Time (min)	Absorbance unboiled	Absorbance in dark	Absorbance boiled
0			
5			
10			
15			
20			

Table 2	
Chloroplast	Rate of photosynthesis
Unboiled	
Dark	
Boiled	

Results:

Plot all the transmittance data on the same line graph (*DO NOT PLOT THE DATA FOR THE BLANK*.) Put % transmittance on the y-axis and time on the x-axis. Label each line distinctively and make a key to tell the difference. Develop an appropriate tile, with data and location, placed at the bottom of the graph.

Discussion

1. What does the data tell you about the relative solubility, size and attraction to the paper of each of the chloroplast pigments?
2. Why was an organic solvent used for chlorophyll extraction instead of water?
3. How might one remove the individual pigments for further chemical analysis?
4. Does the chromatogram provide information about the relative concentrations of the pigments? Explain.
5. If you were to do identical chromatograms with pigment extracts from different algae specimens, would you get the same results? Explain.
6. It is sometimes said by biologists that leaves do not *turn* yellow in the autumn. What do you suppose is meant by that statement?
7. What is the function o f DPIP in this experiment?
8. What molecule found in chloroplasts does DPIP “replace” in this experiment?
9. What is the source of the electrons tat will reduce DPIP?
10. What was measured with the spectrophotometer in this experiment?
11. What is the effect on the reduction of DPIP? Explain.
12. What is the effect of boiling the chloroplasts on the subsequent reduction of DPIP? Explain.
13. 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?