Biology 196 Laboratory
Photosynthesis

Laboratory Objectives
After completing this lab you should be able to:

- Write the chemical equation for photosynthesis; describe the origin and destination of the reactants and products.
- Identify the major pigments found within a spinach leaf and discriminate their chemical differences.
- Quantitatively measure the light absorbing properties of the pigments found within a spinach leaf.
- Use control groups and a colorimetric assay to reveal the role of carbon dioxide in photosynthesis

Photosynthesis[1]

Background
What would life on Earth be like without photosynthesis? It might resemble the surface of Mars, where no photosynthesis is known to occur. A world without photosynthesis would probably have more carbon dioxide and less oxygen in its atmosphere, as is the case on Mars. A vast array of life on Earth depends upon oxygen, and green plants are a primary food source for many living things, including humans. Without photosynthesis, the diversity of life on our planet would be very different—and potentially, nonexistent.

Photosynthesis is not just a simple process of oxygen production. It is an elegantly complex process that combines carbon dioxide, water, and energy from sunlight to produce a single-sugar molecule called glucose. The molecule required for photosynthesis to occur is **chlorophyll**, a green pigment contained in plant cell organelles called **chloroplasts**. Chlorophyll absorbs light energy and ultimately converts this radiant energy into chemical energy. The simple **redox** equation for photosynthesis is:

$$6CO_2 + 6H_2O \xrightarrow[chlorophyll]{light} C_6H_{12}O_6 + 6O_2$$

Photosynthesis involves a series of complex, interrelated reactions that can be grouped into two major categories. The first set of reactions is termed the **light-dependent reactions**. Here, light energy excites a chlorophyll molecule housed within a chloroplast. Through various interactions with water and other molecules during the light reactions, oxygen gas (O_2) is released. Other products of the light reaction move on to the next set of reactions, termed the **Calvin Cycle**. Here, these products and carbon dioxide (CO_2) from the environment interact to form **monosaccharides**, single-sugar molecules. The monosaccharides produced are glucose molecules and are utilized in other important cellular activities, such as cellular respiration.

Some parts of plants lack the ability to photosynthesize and are unable to produce glucose. These areas receive glucose from photosynthetic parts of the plant in the form of sucrose, a **disaccharide** formed from the joining of a glucose and fructose molecule. If there is an ample supply of glucose or sucrose in plant cells, they may form **polysaccharides** (made up of many bonded sugar molecules) in the form of **starch**. Starch can then be stored where needed and can be later utilized by the plant. For example, when photosynthesis is halted, such as when light or carbon dioxide is unavailable, the plant cells can break down the starch molecules into glucose monomers that can then act as fuel for cellular reactions. Starch may also be broken down into glucose molecules that can be transported to other plant cells.

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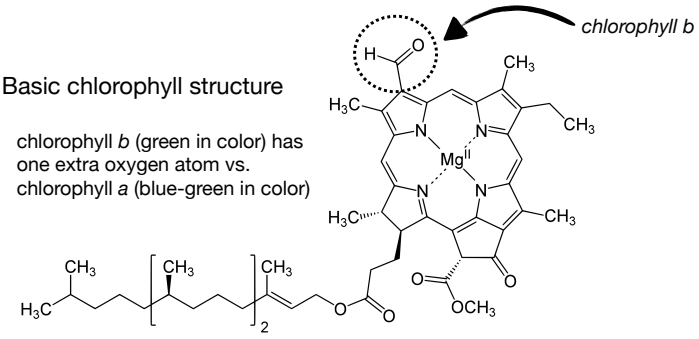
The first exercise will demonstrate the use of CO_2 as the carbon source for photosynthetic plants. We will utilize a pH indicator and the **bicarbonate buffer system** equation below. Predict how pH will be affected when plants remove CO_2 from solution:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Next, you will conduct two experiments demonstrating the involvement of multiple **light absorbing pigments** (including **chlorophyll**) in leaves. Some of the molecular structures and properties are included below.

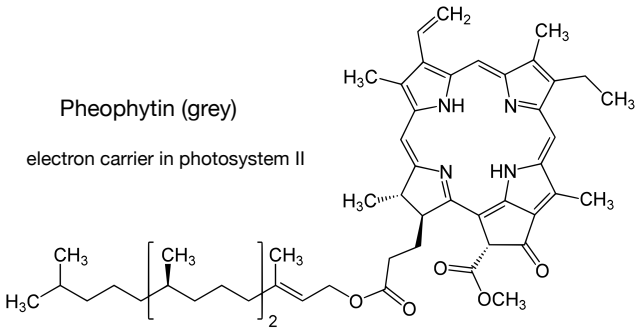
Basic chlorophyll structure

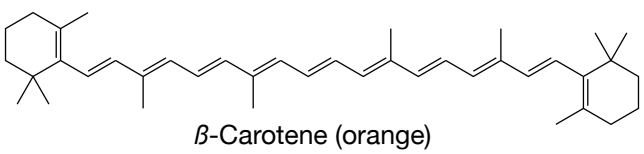
chlorophyll *b* (green in color) has one extra oxygen atom vs. chlorophyll *a* (blue-green in color)



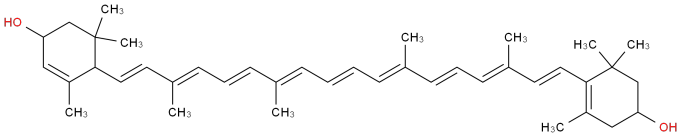
Pheophytin (grey)

electron carrier in photosystem II





ß-Carotene (orange)



Xanthophyll (bright yellows)

Notice that chlorophyll b has one more oxygen atom than chlorophyll a. How would this affect its **polarity**?

Activity #1: Carbon Dioxide Consumption

Materials:

- *Chara*
- 2 vials with caps
- marker
- bromothymol blue solution
- light source
- paper towels
- timer

Background and Procedure:

The chemical **bromothymol blue** is an indicator that appears blue in an alkaline (base) medium and yellow-green in an acid medium. CO_2 has been added to the bromothymol blue. Carbon dioxide (CO_2) in solution forms **carbonic acid** (H_2CO_3), turning the bromothymol yellow-green.

If carbon dioxide is removed from the solution, the solution will change to dark green and then to blue. Bromothymol blue will stain your hands and clothing. Practice safe laboratory procedures when performing this activity.

1. With a permanent marker, write your initials (or group symbol) on both of the capped vials.

2. Obtain an 8-cm sprig of *Chara* and place it in one of the vials provided.

3. Over a paper towel, fill both vials to overflowing with the yellow-green bromothymol blue solution and cap tightly. Take care not to spill on your hands and thoroughly clean up any spills.

4. Place both vials in sunlight or strong light source.

5. Wait one hour, and then answer the questions for Activity 1.

Activity #2: Thin Layer Chromatography of Leaf Pigments

Materials:

- Spinach
- Mortar and pestle
- 1/2 TLC plate (it will be cut in half, share with another table)
- 1 glass capillary tube
- scissors
- Isopropyl alcohol
- test tube rack
- electronic balance
- weigh boats or weigh paper
- stirring rods
- 10 mL graduated cylinder
- ruler
- permanent marker
- pencil
- water
- timer

Background and Procedure:

Photosynthetic plants utilize a variety of light-absorbing pigments. Due to the large quantity and intensely colorful nature of the chlorophylls (green), under many circumstances it is not possible to notice the presence of the other pigments.

Differences in chemical structure of these pigments result in differing amounts of polarity, allowing us to separate them from one another using Thin Layer Chromatography (TLC). Most of these pigments are found in membranes of the thylakoid and are predominantly hydrophobic. Therefore, we will use organic solvents, such as isopropyl alcohol ("rubbing alcohol"), as the solvent and petroleum ethers for the mobile phase or "developing solution" for the chromatography.

1. Weigh approximately 3-4 grams of fresh, dry spinach leaves with the stems completely removed.

2. Using a pair of scissors cut the leaves into very small pieces into the mortar. Using the pestle grind and pound the leaves into a mash. This will take several minutes and is an essential step, so don't rush it.

3. Add 10 mL of anhydrous isopropyl alcohol to the mortar and carefully, without splashing, continue to mix and crush the slurry for several more minutes. If some of the alcohol has evaporated you can add a couple more milliliters to maintain the original volume. Then place plastic wrap, a beaker, or other object over the top of the mortar to prevent evaporation of the alcohol. Wait five minutes.

4. Stir and crush the mixture for another thirty seconds. The liquid should be a dark green color. Use a transfer pipet to move the isopropyl-pigment solution into a glass test tube. Try to avoid the spinach solids. **Keep in mind that this tube of pigment/alcohol solution will also be used for *Activity #3*.**

5. Carefully cut the TLC plate lengthwise, to create two, more narrow strips. One half of the plate will be used per group. Using a ruler draw a thin line 1.5 cm from one end (this will be the bottom) using a standard pencil.

6. Using the glass capillary tube aspirate a small amount of the isopropyl solvent containing the leaf pigments. Carefully make a small dot on the center of the pencil line on the TLC plate. Allow approximately 1 minute to dry.

7. Repeat step 6 at least five times. You want a very dark and concentrated spot of pigments at one place on the thin pencil line. Be certain the spot is dry before continuing to the next step.

8. In a clean glass test tube add 0.75 mL of chromatography developing solution. ***Caution: this is a mixture of petroleum ethers.*** This solvent is highly flammable and mildly toxic. Be sure to dispose of this mixture properly (as outlined by your instructor) and take care with handling.

9. Carefully place your TLC strip into the test tube containing the developing solution. The dot containing the pigments should NOT come in direct contact with the solution. Using a piece of parafilm seal the top of the test tube and place in a test tube rack.

10. Observe the TLC plate for the next few minutes to watch the developing solution front move up the plate while the pigments separate.

11. When the developing solution is within 1 cm of the top of the plate remove it from the tube with a pair of forceps and place it on a paper towel to dry. Properly dispose of the remaining developing solution in the organic waste receptacle and dispose of your test tube in the glass waste.

Documentation and Retardation Factor (R_f):

Since the developing solution is extremely nonpolar, more hydrophobic pigments will travel easier with the solvent front nearer the top. The more polar (less hydrophobic) pigments will stick to the silica further down on the plate.

1. Draw a simple sketch of your TLC plate to the right, indicating the location of the bands you see and which pigments you think they are.

The Retardation Factor (R_f) is a way to normalize the separation of substances among different TLC plates using the same developing solution, and gives the user a relative difference in the separation of the substances. It is calculated as:

$$R_f = \frac{\text{distance from line to spot}}{\text{distance from line to developing solution front}}$$

2. Using a ruler calculate the R_f values for your separated pigments and log them into the table below, starting with the pigment closest to the solvent front (top).

Pigment Rf values

Pigments	R_f value	Suspected name of pigment

Activity #3: Light Absorption of Leaf Pigments

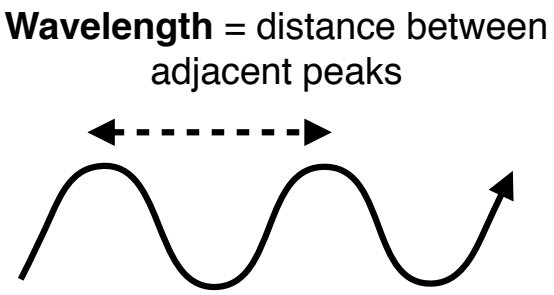
Materials:

- Spinach isopropyl-pigment solution from Activity #2
- spectrophotometric cuvettes
- test tubes
- test tube rack
- Isopropyl alcohol
- deionized water
- transfer pipets

Background and Procedure:

The visible spectrum of light consists of a complex mixture of electromagnetic radiation (EM). EM itself consists of photons, often described as "packets of energy", that can travel in waves. The different colors of light correspond to different wavelengths, a function of the energy of the photons (shorter wavelengths = more energy).

Color	Wavelength (nm)
Infrared	750
Red	700
Orange	650
Yellow	600
Green	550
Blue	500
Indigo	450
Ultraviolet	400



Molecules, including leaf pigments, absorb or reflect light of different wavelengths. Our perception of substances that are the color "blue" is due to the substance reflecting light in the 500 nm wavelength range, and that light is picked up by the retinas of our eyes.

We are going to use a spectrophotometer again (recall using one for the enzyme kinetics lab) to measure absorption of our isopropyl-pigment solution from Activity #2 at different wavelengths of light. Because it is a mixture, it will not tell us which pigment molecule accounts for the absorption. It will only tell us how much light of a given wavelength is being absorbed.

Prepare a dilution series:

1. Label 4 clean test tubes: "100%", "50%", "25%", and "12.5%". Add 2mL of Isopropyl alcohol to all of the tubes *except* the tube labeled 100%.

2. Remove 4 mLs of the isopropyl pigment solution from Activity #2 and place in the test tube labeled "100%".

3. Make a 50% dilution: Remove 2 mL from the "100%" tube and place in the tube labeled "50%" and swirl to mix.

4. Make a 25% dilution: Remove 2 mL from the "50%" tube and place in the tube labeled "25%" and swirl to mix.

3. Make a 12.5% dilution: Remove 2 mL from the "25%" tube and place in the tube labeled "12.5%" and swirl to mix.

Measure the light-absorbing properties of your extract solution :

1. Label a cuvette as "blank" and add one mL of isopropyl alcohol to it.

2. Remove a milliliter of solution from the undiluted mixture (tube labeled "100%") and place in a cuvette also labeled 100%.

3. Set the wavelength on the spectrophotometer to 450nm.

4. Place the blank cuvette containing only alcohol into the chamber and hit the zero absorbance button.

5. Next place your cuvette labeled 100% into the spectrophotometer and note the absorbance displayed.

   If the number is > 2.0 it is too concentrated. Add your 50% dilution to a new cuvette and test that dilution. Use your most concentrated dilution that gives you an absorption < 2.0 for the remainder of the activity.

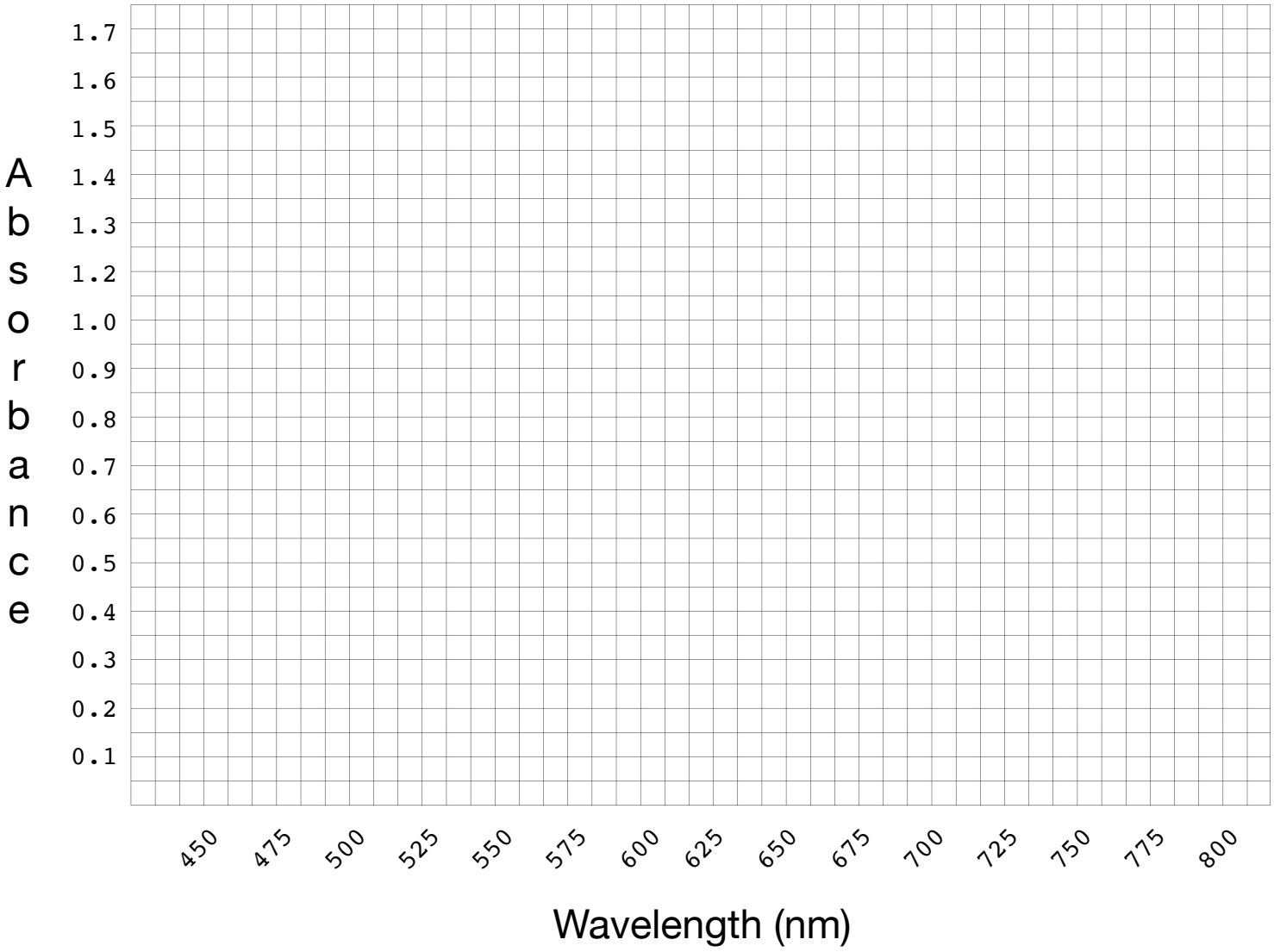
6. After recording the absorbance at 450nm, increase the wavelength to 475. Re-zero the absorbance using your "blank" cuvette and read the absorbance of the pigment mixture sample and record it in the table below.

7. Record the absorbance of your sample at 25nm increments from 450 - 800nm and record the results in the table below. It is important that you re-zero the absorbance using your blank cuvette each time you change the wavelength on the spectrophotometer.

Leaf Pigment Absorbances

Wavelength	Absorbance
450	
475	
500	
525	
550	
575	
600	
625	
650	
675	
700	
725	
750	
775	
800	

7. Plot the absorbances from the table above into a line graph below.



Photosynthesis Laboratory Questions

Questions for Activity 1: Carbon Dioxide

1. Compare the color of the two solutions. What happened?

2. What does this reveal about the role of carbon dioxide in photosynthesis?

3. What would you have expected to happen if the vials were instead placed in a dark room at the beginning of the experiment? Explain your answer.

4. What is the purpose of the vial that does not contain *Chara*?

Questions for Activity 2: Thin Layer Chromatography of Leaf Pigments

1. Comparing chlorophyll *a* and chlorophyll *b*, which of the two traveled furthest up the TLC plate? Why is that?

2. What is the purpose of having multiple chlorophylls, and what purpose do the other pigments serve?

3. If the pigments were found in the stroma, rather than in the thylakoid membrane, how would this affect their movement on the TLC plate?

Questions for Activity 3: Light Absorption of Leaf Pigments

1. Why do you need to re-blank the spectrophotometer every time you change the wavelength setting?

2. What wavelengths were most absorbed by the pigment mixture?

3. What wavelengths were least absorbed by the pigment mixture? Why do you think that is?

Study Checklist

To perform well on the quiz, you need to have a thorough knowledge of the following:

- You should know and understand all of the terms which appear in **boldface** type.
- Use your textbook to provide a more full understanding of the photosynthetic light reactions and Calvin Cycle.
- What are redox reactions, and which components of the photosynthetic reaction are oxidized/reduced?
- What are the major structures involved in photosynthesis in the plant tissue, and within the cells?
- Describe the origin and destination of the molecules of O_2 and CO_2.
- What happens to CO_2 when dissolved in water that allows the use of bromothymol blue as an indicator of its presence?
- Why do you need to assay negative control samples as well as your experimental samples?
- Why do leaves have multiple pigments? What roles do they perform in the leaf?
- What causes the pigments to move at different rates on the TLC plate?