

LABORATORY 9

The Light-dependent Reactions of Photosynthesis

I. Introduction

As you recall, plants are **autotrophs**, self-feeding organisms that capture randomizing light energy (photons) and store it for later use as fuel (carbohydrate) for cellular respiration. During the **light reactions** of photosynthesis, the **light energy** of photons is converted briefly to **electrical energy** (the flow of electrons through chlorophylls in the photosystems), and then into **chemical energy** (in the bonds of ATP and NADPH). The production of ATP from ADP by using light energy is known as **photophosphorylation**. Absorption of light energy creates an energy donor molecule with boosted energy relative to a nearby electron acceptor. Electrons move spontaneously from high-energy donors to receptors through an **electron transport chain**.

The chemical energy briefly stored as ATP (and NADPH) in the light reactions is transferred into the more stable bonds of **sugar** during the light-independent reactions of the **Calvin Cycle**, which takes place in the chloroplast stroma.

A. Photosystems

The light-dependent reactions take place on the membranes of the thylakoids, inside the chloroplasts. Chlorophyll and carotenoid pigments are embedded in the thylakoid membranes to form **photosystems**, designed to maximize the capture of photons. A photosystem consists of a few hundred molecules of **chlorophyll a**, **chlorophyll b**, and **carotenoid pigments**. As these pigments absorb photons, their electrons are boosted to a higher energy level (they become electron donors). Excited electrons pass from one pigment to another until they reach a specific chlorophyll a molecule positioned next to a **Primary Electron Acceptor** protein. At this **Reaction Center**, chlorophyll a passes its excited electron to the Primary Electron Acceptor, starting an electron transport chain that will result in the production of ATP (from ADP) and NADPH (from NADP^+). The energy that was once a photon is now briefly stored as chemical energy in the bonds of ATP and NADPH.

Note that the oxidation-reduction reaction that takes place between chlorophyll a and the Primary Electron Acceptor cannot occur unless the two molecules are in their proper positions in the thylakoid membrane. When you observed **fluorescence** in your isolated chlorophyll in the last lab, you were observing the re-release of light energy that would have been captured by the Primary Electron Acceptor if the chlorophyll was still normally embedded in a living thylakoid membrane.

Two different photosystems--**Photosystem I** and **Photosystem II**--are found on thylakoid membranes. The main difference between these two systems (named in order of their discovery) is the absorbance maximum of their respective chlorophyll a molecules. In Photosystem I, chlorophyll a (also known as **P700**) has a maximum absorbance at 700nm, and in Photosystem II, a chlorophyll a (also known as **P680**), associated with slightly different proteins, absorbs maximally at 680nm. As perceived by the human eye and brain, what "color" are these two wavelengths? _____.

B. Non-cyclic Photophosphorylation

In both photosystems, light drives the synthesis of ATP and NADPH by triggering a flow of electrons through the photosystem pigments and associated proteins. Two possible routes for electron flow--**cyclic** and **non-cyclic**--are known. Plants primarily use the non-cyclic route (Figure 1), and it is this pathway we will study today.

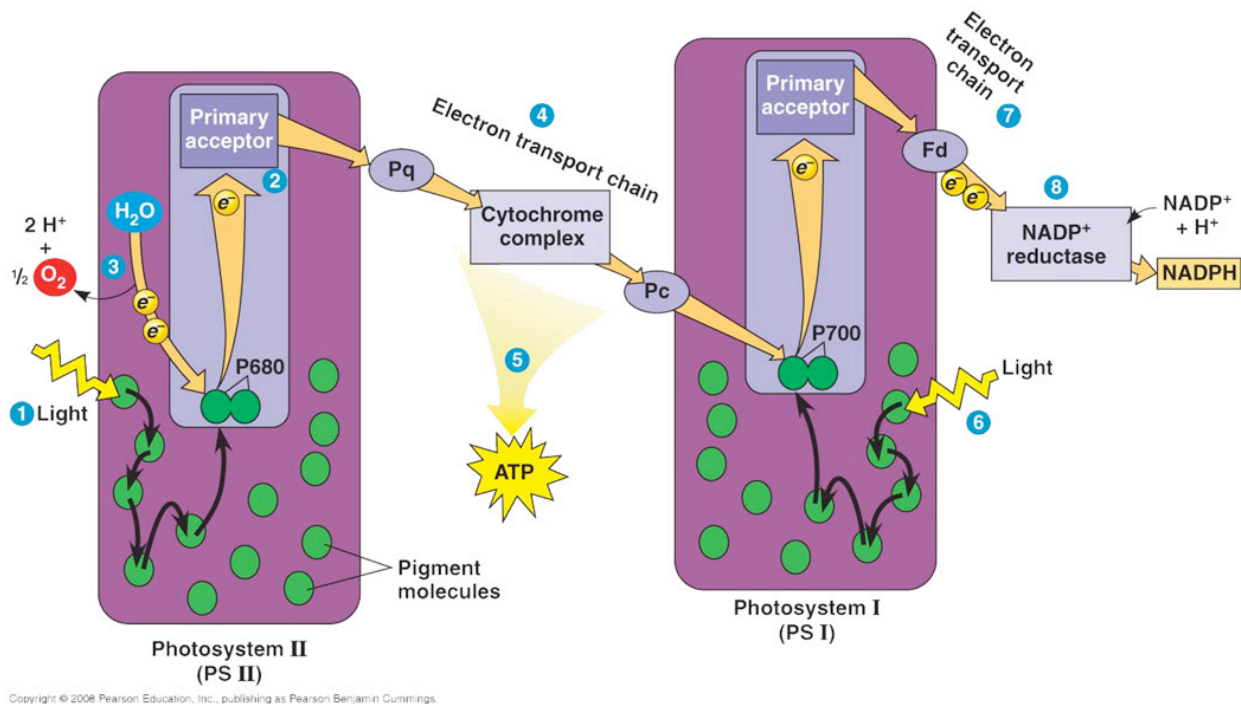


Figure 1. An overview of non-cyclic photophosphorylation.

When a photon is absorbed by Photosystem II and passed to the Primary Electron Acceptor, the chlorophyll a (P680) that has donated its excited electron is oxidized and highly reactive. The missing electron must be replaced. But how?

A specialized enzyme splits a water molecule to produce (1) two protons, (2) one oxygen atom, and (3) two free electrons. The oxygen atoms combine to produce molecular oxygen (O_2), and are eventually released via small gas-exchange pores in the leaves known as **stomates**. The hydrogen atoms will combine with NADP to form NADPH, and some eventually become components of sugar. The electrons are supplied to P680, replacing the ones lost to the Primary Electron Acceptor.

Excited electrons taken up by the Primary Electron Acceptor are passed along an electron transport chain similar that of cellular respiration. As electrons cascade exergonically "down" the electron transport chain, the energy they release is used to phosphorylate ADP into ATP. Because this reaction is driven by light, it is known as **photophosphorylation**.

An electron reaching the end of the electron transport chain "meets" P700, Photosystem I's Reaction Center. P700, also part of an active photosystem, has already donated its excited electron to the Primary Electron Acceptor. Highly reactive, P700 takes up the excited electron that has just cascaded down to it from Photosystem

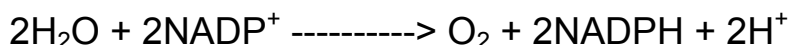
II and its associated electron transport chain. P700 is now re-supplied with an electron that can be boosted to a higher energy state and passed to the Primary Electron Acceptor when P700 once again absorbs a photon of the proper wavelength.

Finally, excited electrons are passed from the P700/Primary Electron Acceptor Reaction Center to a second electron transport chain. This chain transmits the electrons to an iron-containing protein known as **ferredoxin**. The excited electrons now in the possession of ferredoxin are transferred to **NADP⁺** by an enzyme known as **NADP reductase**. This final redox reaction stores the electrons' energy as chemical energy: the bonds of **NADPH**. This short-term energy storage molecule will donate its energy-carrying protons for sugar synthesis in the Calvin Cycle.

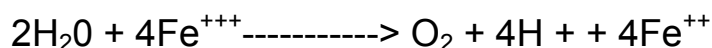
Because the electrons given up by P680 never cycle back and return to P680, this entire process is known as **non-cyclic photophosphorylation**.

C. The Hill Reaction

As described above, the first event in photosynthesis is the light-activated transfer of an electron from one molecule to another against an electro-chemical potential. This reduction results in the conversion of light energy into chemical energy.



In 1939, Dr. Robin Hill discovered that isolated, illuminated chloroplasts will produce oxygen when in the presence of a suitable electron acceptor. Hill used iron salts in the place of NADP, generating the following chemical reduction:



Hill's experiment was a landmark in the elucidation of photosynthetic processes, as it was the first to demonstrate that

- Oxygen evolution during photosynthesis occurs without carbon dioxide reduction.
- Oxygen evolved during photosynthesis comes from water, not carbon dioxide (as previously believed), since no CO₂ was used in Hill's experiment.
- Isolated (and in Hill's reaction, fragmented) chloroplasts could perform a significant partial reaction of photosynthesis.

In our last laboratory, you disrupted thylakoid membranes of spinach leaves to extract chlorophyll and carotenoid pigments and study their physical properties. This week, you will extract living chloroplasts from spinach cells *without* disrupting their membranes or their thylakoids. You will use these living, intact chloroplasts to perform a variation of Hill's reaction. Your isolated "photosynthesis factories" may be experimentally manipulated to study the effects of environmental variables on this first stage of photosynthesis, the **photolysis of water**.

You will use an artificial electron acceptor--a blue dye known as **indophenol**--to mimic the NADP found in a live cell. (In Hill's reaction, what is the chemical equivalent of indophenol? _____). As shown below, when indophenol (the R in the chemical formula indicates a variable functional group) accepts electrons and is reduced, it changes from blue to colorless (Figure 2).

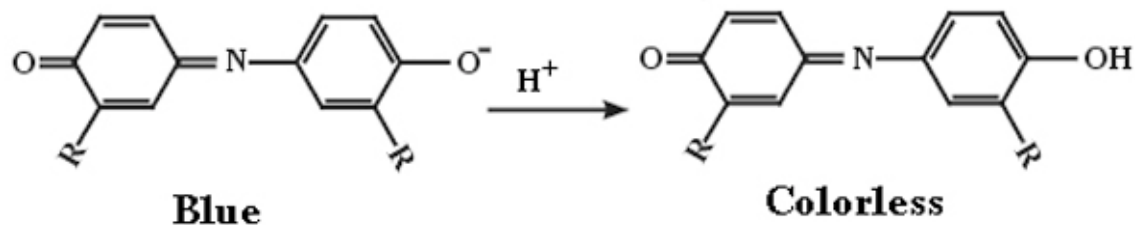


Figure 2. Reduction of indophenol dye results in a color change of this chemical, changing its absorption properties.

This property of our artificial electron acceptor makes it easy to monitor the rate of the light reactions via **colorimetry**. You will use a **spectrophotometer** to monitor the rate at which your indophenol changes from blue to colorless as it is reduced, mimicking the role of NADP in a live plant cell.

After you extract chloroplasts and suspend them in buffer, you will aliquot your sample into a series of small colorimetry tubes (test tubes, also known as **cuvettes**, made from optical quality glass). Be sure to have equal numbers of treatment and control tubes. As you will see, these must be kept on ice to prevent degeneration of the chloroplasts.

II. Experimental Approaches: Photolysis of Water

Your team should do as much background research as necessary to find an interesting, relevant problem to solve regarding the light reactions of photosynthesis. You will need to restrict your hypotheses to ones you can test using the techniques you will use in today's lab, but there are many possible aspects you might explore, from varying wavelengths of light to the effects of the chloroplast's fluid environment to the type of plants doing the photosynthesis.

As before, be sure you are not just using Science Fair Mentality. It is not very meaningful to test whether plants photosynthesize more rapidly with different wavelengths of light. Why? Because this has already been answered many times over, and is published in many textbooks. It is common knowledge, and your repeating this type of experiment is not really adding anything to our scientific knowledge about the light reactions. However, if you can find some interesting aspect of differing wavelengths that is not yet known, then feel free to propose your ideas to the class (and your instructor) for critique and suggestions.

Your hypotheses should be informed by your knowledge of photosynthesis and the photosynthetic pigments you studied in Part I of this lab. Remember that your team must present its hypotheses and proposed experiment to your colleagues and TA for feedback **the week before** you perform your experiment with the procedures outlined in this chapter.

A. Observations and Hypotheses

What is the observation your team will attempt to explain experimentally?

Observation:

List as many hypotheses as possible to explain your observation. If you were to test this hypothesis experimentally, what would your predicted outcome be?

1.

Prediction:

2.

Prediction:

3.

Prediction:

4.

Prediction:

If your work today is intended to be a pilot study, then state this clearly, and provide a logical rationale for why you are performing this pilot study. What do you hope to observe? What further hypotheses might your results generate?

If you are using a statistical hypothesis:

What is your **null** hypothesis regarding the system you are examining?

What is your **alternative** hypothesis?

B. Experimental Design

What **parameter** will you measure? _____.

Note that you will be alloquating equal amounts of chloroplast suspension into your treatment and control tubes. How might you ensure that there are equal numbers of chloroplasts in each tube? _____

What are the limitations of this method? _____

Describe your **control** group.

Describe your **treatment** group.

Describe your **experimental methods**.

III. Laboratory Techniques for Chloroplast Extraction

THIS EXPERIMENT WILL WORK ONLY IF YOUR EXTRACTED CHLOROPLASTS ARE KEPT COLD AND ARE PREPARED QUICKLY. READ AHEAD, AND BE SURE TO ICE DOWN OR REFRIGERATE ALL GLASSWARE YOU WILL USE. KEEP ALL PREPARATIONS ON ICE, AND AVOID CONTACT WITH YOUR WARM HANDS.

A. Preparation of Chloroplast suspension

1. In a blender, homogenize about 25g of healthy, dark spinach leaves in 100mL of ice cold 0.35M NaCl for about 30 seconds. (It should look like a watery spinach smoothie.)
2. Filter the homogenate through two layers of cheesecloth (on 8" x 8" piece folded in half will do) into a chilled 250ml beaker. You will get plenty of extract. There is no need to squeeze the cloth for more. (That just adds debris.) (**Don't waste cheesecloth! A section about 8" square should be more than sufficient for your needs.**)
3. Divide the filtrate between two centrifuge tubes. Fill each tube to 1cm from the top—no higher. Place any excess filtrate in a spare, chilled beaker until you are sure you don't need any more (e.g., if you have a centrifuge accident—which we hope you won't.). Both tubes should have about the same amount of liquid.
4. Insert each tube into a stainless steel centrifuge sleeve. Balance the tubes (in their sleeves) carefully before placing them opposite each other in the centrifuge to spin.

IMPORTANT NOTES FOR BALANCING THE CENTRIFUGE TUBES:

Tubes opposite each other in a centrifuge must weigh the same (i.e., be **balanced**). If this is not done properly, the centrifuge will roar obnoxiously and might even dance right off the table onto your foot. This can cause damage to the centrifuge. (Okay, your foot won't be too happy, either.) So balance your tubes carefully before centrifuging!

- a. Use a double-pan balance, available on the back table
- b. Place each plastic centrifuge tube into a stainless steel centrifuge sleeve.
- c. Place an empty 250mL beaker on each pan of the balance.
- d. ZERO THE BALANCE with the empty beakers on the pans. Adjust the sliding weight on the balance until the pointer on the small scale in the center of the balance rests exactly at the center of the scale.
- e. Place one *sleeved* centrifuge tube in each beaker.

- f. If the two tubes do not weigh the same, use the water droppers at the balance at the station to carefully **add water BETWEEN THE PLASTIC CENTRIFUGE TUBE AND THE STAINLESS STEEL SLEEVE**. *DO NOT add water to your chloroplast suspension. Add it only to the space between the tube and sleeve. (Why?)*
5. Once your two tubes are balanced, insert them into the centrifuge exactly 180° opposite each other in the centrifuge. (Please DO NOT run the centrifuge with only two tubes. Wait for another team to load additional tubes, and always try to run a full load. This will save time for everyone.) **IF THE CENTRIFUGE IS NOISY OR VIBRATES, YOUR TUBES ARE NOT BALANCED. STOP THE CENTRIFUGE AND RE-BALANCE YOUR TUBES.**
 6. Set the centrifuge at 1/2 speed (dial setting 6), and spin your filtrate for two minutes. Keep track of the time, as the centrifuge timers are not clock-accurate.
 7. Pour the **supernatant** (liquid) from all the tubes into a chilled 250mL beaker. Discard the **precipitate** (solid pellet at the bottom of the tube) by brushing it out with the test tube brushes provided. Throw it in the trash, **NOT THE SINK**.
 8. Rinse your centrifuge tube with distilled water. (Your TA will show you which tap.)
 9. Pour the supernatant from the beaker back into your rinsed centrifuge tubes. Re-balance the tubes, and centrifuge at full speed for 8 minutes. This time, pour off the supernatant and **SAVE THE PRECIPITATE**. *The pellet contains your chloroplasts.*
 10. To each of your centrifuge tubes, add 0.4mL of ice-cold 0.35M NaCl solution. Mix the contents with a clean, glass stirring rod to re-suspend the chloroplast pellet.
 11. Use a glass Pasteur pipet to gently draw up the pellet and expel it against the wall of the tube. Repeat this 5-6 times, or until the chloroplasts are very evenly suspended (no clumps visible).
 12. Combine the chloroplast suspensions from your tubes into a single, chilled 30 mL beaker. Keep on ice.
 13. Place 1 mL of this chloroplast suspension into a new chilled 30 mL beaker. Make a 1/10 dilution by adding 9 mL of chilled 0.35M NaCl. Keep on ice.
 14. Obtain the number of colorimetry tubes you decided to use (control, treatment or replicate). Be sure to label each tube carefully. **Place tape labels near the top of each tube, so as to not obstruct the spectrophotometer's light path.** Chill the tubes.
 15. Make sufficient buffer for your experiment, in a chilled 50 or 100 mL beaker. Make 10 mL of buffer for each tube you will use; multiply the recipe proportionally if you have more than two tubes. Do not make more buffer than you need. Unless you are manipulating the pH of your chloroplast environment, you will be using pH 7 phosphate buffer. But recipes for various pH buffers appear at the end of this chapter, in case your team intends to manipulate pH.
 16. Into each colorimetry tube, aliquot 8.0 mL of buffer solution and 0.2 mL of the 1/10 dilution chloroplast suspension. Show the first tube to your TA before preparing the other(s), to be sure things look fine before you proceed.

17. Make a mini ice water bath for each treatment by filling 100 mL beakers 2/3 with ice and water. Place each colorimetry tube into its own mini ice water bath.

Your samples are now ready for the experiment you designed in Part II. Set up your treatments so that you can keep the tubes in their mini ice-water baths during the experiment, and remove them only to take spectrophotometer readings.

B. Measuring Rate of Reaction via Colorimetry

1. Instructions for the use of the spectrophotometer can be found at each machine.
2. Be sure the spectrophotometer is set at 610 nm, as transmission of this wavelength (red) will increase as indophenol blue fades to colorless. Be sure you can explain why this makes sense (and for example, why we wouldn't use green or blue light).
3. Before you prepare your tubes for measurement, you must calibrate the spectrophotometer. There are detailed instructions on laminated cards at each spectrophotometer station that will explain how to do this. You will calibrate the machine with two standard spec tubes. These are located in a 250mL beaker next to the spectrophotometer (there's one set of two tubes for each team). Prepare the two spectrophotometer tubes with standard solutions, as follows:
 - a. Fill one tube with 10mL of 0.35M NaCl solution
 - b. Fill the other tube with 10mL of 0.35 NaCl solution + 0.15mL of indophenol blue dye. Cap both tubes securely with Parafilm.
 - c. Gently upend the indophenol tube to mix the dye into the solution until it a uniform, pale blue color.
 - d. At the end of lab, empty your two standard tubes, rinse them well with distilled water, and replace them in the 250mL beaker where you found them at the spectrophotometer station.
4. When you are ready to take readings, bring your iced sample tubes and bottle of indophenol blue to the spectrophotometer. Add 0.5mL of indophenol blue dye solution to each tube, mix well, and immediately take a colorimetry reading. This will be your "time = 0" data point. **IMPORTANT:** Invert each tube to mix its contents thoroughly, and wipe the tube dry before taking the reading. Standardize this procedure.
5. After taking readings, immediately put the tubes back in their treatment conditions (and mini ice water baths).
6. Measure the color change in your tubes at set intervals (two minutes works well), and record your data in the table grid provided. Label the grid appropriately.
7. For your presentation, make two graphs using Excel or similar software:
 - a. a raw data graph showing rate curves for each treatment. Obtain the rate of reaction for each treatment as the slope of the best-fit line *for the linear portion of the rate curve only*.
 - b. a results graph comparing the rates of reaction for all treatments.

C. Buffer Recipes

There are dozens of different types of buffer recipes, many of which are useful for biological applications. Different buffers can be titrated to a range of pH levels, with some having a narrow range, and others a very broad range. Here, we provide recipes for three different buffers useful in biological systems: **sodium phosphate buffer** (pH 5.8 – 8.0), **citrate buffer** (pH 2.5 - 5.6), and **glycine-sodium hydroxide buffer** (pH 8.6 - 10.6). These should provide a range of pH environments you can use to manipulate your experimental system.

Reagents for mixing pH buffer solutions can be found at stations on the window tables of the lab. Distilled water is available from the aluminum tap at the back of the lab. If you're not sure which tap to use, ask your Lab Instructor. (Don't use tap water!)

A. Sodium phosphate buffer (0.05 M, pH ~ 5.8 - 8.0)

1. Dissolve 0.69 grams of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (monobasic sodium phosphate *acid*) in 100mL of distilled water. This is the ACID component of your buffer.
2. Separately, dissolve 1.06 grams of Na_2PO_4 (dibasic sodium phosphate *base*) in 150mL of distilled water. This is the BASE component of your buffer.
3. Titrate to desired pH by slowly adding acid to base. (A pH of 7.0 will require approximately 2 volumes of acid to 3 volumes of base)
4. If you need a greater volume of buffer, adjust the quantities accordingly.

B. Sodium citrate buffer (0.05M, pH ~ 2.5 - 5.6)

1. Dissolve 0.8 grams of sodium citrate-2 H_2O in 100 ml H_2O (*base*)
2. Add 1.25 grams of citric acid (*acid*)
3. Dissolve 1.1g NaCl into 100mL of distilled water.
4. Add 1ml of this NaCl solution to your buffer
5. Check pH of solution.
6. If pH is greater than 4.5, titrate down with 1M HCl.
7. If pH is lower than 4.5, titrate up with 1M NaOH
8. When solution is the desired pH, add distilled H_2O to bring volume to the amount needed.

C. Glycine-sodium hydroxide buffer (0.05 M, pH ~ 8.6 - 10.6)

1. Dissolve 0.38 g of glycine (*acid*) in 100mL of distilled water
2. Separately, dissolve 0.2 g of NaOH (*base*) in 100mL of distilled water.
3. Titrate to desired pH.

