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 for cellular respiration. During the **light reactions** of photosynthesis, photon energy 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). During the **Calvin Cycle** (sometimes known as the light-independent reactions, since it can proceed either in dark or light), the chemical energy briefly stored as ATP and NADPH is transferred for long-term storage into the bonds of **sugar**.

A. Photosystems

The light-dependent reactions take place on the membranes of the thylakoids, which are located 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. In this excited state, electrons pass from one pigment to another until they reach a specific chlorophyll a molecule positioned beside a **Primary Electron Acceptor** protein. At this **Reaction Center**, chlorophyll a passes the 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 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. Of these, plants use primarily the non-cyclic route, and it is this pathway we will study today. *Review the*

figure in your text illustrating non-cyclic electron flow, and be sure you understand the process before you continue.

1. Overview of Non-cyclic Photophosphorylation in a Live Plant Cell

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?

Meanwhile, a specialized enzyme splits water molecules to produce hydrogen atoms, oxygen atoms, and free electrons. The oxygen atoms combine to produce molecular oxygen (O2), and are eventually released via small gas-exchange pores in the leaves known as **stomates**. The hydrogen atoms are eventually used as 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 to the one seen in 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**.

2. 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.

$$2H_2O + 2NADP^+ \text{ ----------> } O_2 + 2NADPH + 2H^+$$

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:

$$2H_20 + 4Fe^{+++}\text{-----------> } O_2 + 4H + + 4Fe^{++}$$

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

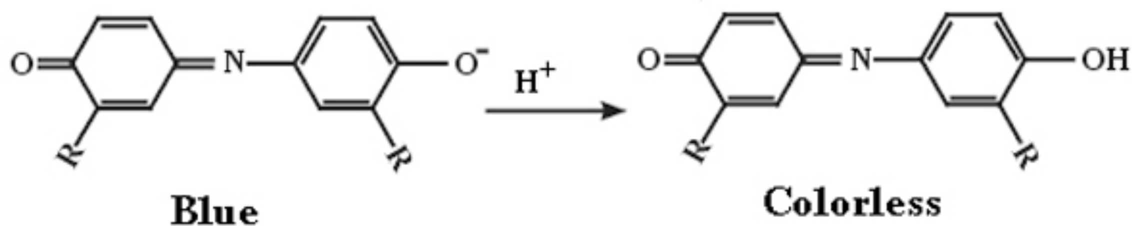
(1) Oxygen evolution during photosynthesis occurs without carbon dioxide reduction.

(2) Oxygen evolved during photosynthesis comes from water, not carbon dioxide (as previously believed), since no CO_2 was used in Hill's experiment. (In the 1940's, another experiment using water labeled with heavy oxygen (^{18}O) confirmed this result.)

(3) Isolated (and in Hill's reaction, fragmented) chloroplasts could perform a significant partial reaction of photosynthesis.

In our last laboratory, you disrupted the thylakoid membranes of spinach plants 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, and 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.



This property of our artificial electron acceptor makes it a simple matter to monitor the rate of the light reactions via **colorimetry**. You will use a spectrophotometer (Figure 9-1) 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.

You will follow a procedure that will enable you to extract living chloroplasts from plant cells, alloquating your sample into a series of small colorimetry tubes (test tubes made from optical quality glass, also known as **cuvettes**) which you should separate into equal numbers of treatment and control tubes. These must be kept on ice to prevent degeneration of the chloroplasts, but this should not interfere with your manipulating your choice of environmental variable that you suspect might have an effect on the light reactions.

II. The Effects of Environmental Variables on the Photolysis of Water: Experimental Design

A. Asking a Scientific Question

Work in groups of four. Before you begin your chloroplast extraction and manipulation, you must decide what scientific question you hope to answer by doing so. Consider what you know about chlorophyll absorption spectra, the activity of proteins and enzymes at different temperatures, pH levels, etc. Your laboratory instructor will tell you what supplies are available in the lab for your experiments. Make a short list of questions about how changes in one such environmental variable (temperature? light? pH? physical trauma, such as boiling?) might affect the rate (or other aspect) of reaction (as indicated by the color change of indophenol dye). Write your questions below.

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Once you have come up with some interesting questions, you and your partner should decide which of them might be answerable with an experiment you could perform in the laboratory today. Once you have selected your question, move to the next phase.

B. Null and Alternative Hypotheses

What is your **null** hypothesis regarding the question you have chosen?

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What is your **alternative** hypothesis?

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C. 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? ____

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Describe your **control** group.

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Describe your **treatment** group.

Describe your **experimental methods**.

What **statistical test** will you use to determine whether there is a significant difference between your treatment and control groups? ____________________

What is your **prediction**? ____________________

III. Laboratory Techniques for Chloroplast Fun

A. Preparation of Chloroplast suspension

THIS EXPERIMENT WILL NOT WORK PROPERLY IF YOUR EXTRACTED CHLOROPLASTS ARE NOT KEPT COOL. READ AHEAD, AND BE SURE TO ICE

DOWN OR REFRIGERATE ALL GLASSWARE YOU WILL BE USING IN YOUR EXTRACTION. KEEP ALL PREPARATIONS AS COLD AS POSSIBLE.

1. In a blender, macerate about 50g of spinach leaves in 200ml of ice cold 0.35M NaCl for about 30 seconds (or until obvious leaf fragments have disappeared, giving way to a delicious-looking spinach shake).
2. Filter the homogenate through two layers of cheesecloth into a cooled 250ml beaker. This is done most easily by making a little "bag" out of the cheesecloth and squeezing the homogenate to retrieve as much of the liquid as possible. Caution! Too much squeezing will introduce unwanted cellular debris, and may affect your results!
3. Divide the filtrate among an equal number of cooled centrifuge tubes (use as few tubes as possible). Balance the tubes carefully, making sure that they have the same mass, and then insert them on opposite sides of a centrifuge.
4. Set the centrifuge at 1/2 speed, and spin your filtrate for two minutes.
5. Pour the **supernatant** (liquid) from all the tubes into a beaker. Discard the **precipitate** (solid pellet at the bottom of the tube) and rinse your centrifuge tube with distilled water.
6. Pour the supernatant from the beaker back into your rinsed centrifuge tubes. Re-balance the tubes, and centrifuge at full speed for 8 minutes. Discard the supernatant. **SAVE THE PRECIPITATE**.
7. To your centrifuge tubes, add equal amounts (note the volume here: ____________) of ice-cold NaCl solution. Mix the contents with a clean, glass stirring rod until the chloroplasts are very evenly suspended in the solution.
8. Place your tubes of suspension in crushed ice. Obtain the number of colorimetry tubes you and your partner decided to use. Into each tube, alloquat 8.0ml of pH 6.9 phosphate buffer solution and 0.2ml of chloroplast suspension.
9. Be sure to label all of your colorimetry tubes carefully (treatment and control, etc.). Place tape labels near the top of each tube, being sure not to obstruct the spectrophotometer's light path.
10. Your samples are now ready for the experimental manipulation you designed in Part II.

B. Measuring Rate of Reaction via Colorimetry

1. Keep all tubes on ice during the course of this experiment, except when you are measuring the change in indophenol color.

2. An instruction booklet for use of the spectrophotometer is beside each machine. Absorbance will be set at 610nm, as transmission of this wavelength (red) will increase as indophenol fades to colorless.

3. When you are ready to begin taking readings, bring your iced sample tubes and

bottle of indophenol to the spectrophotometer. Add 0.15ml of the indophenol dye solution to each test tube in turn, mix very well, and immediately take a colorimetry reading. This will be your "zero" data point.
(IMPORTANT: Be sure to mix the contents of the tubes thoroughly, and wipe dry before each reading. Mixing procedure should be standardized.)

4. 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, and record all experimental methods. You will be graphing these data in your lab report, so be sure you write down data you can interpret later.

Your laboratory instructor will explain what will be required of your team in terms of a laboratory report. Good hunting!

Figure 9-1. The Spectronic 20D+ spectrophotometer.

Grid for data collection: