

# Experiment 11: Photosynthesis in Isolated Thylakoids

## Introduction

Photosynthesis is the process by which light energy from the sun is used to produce sugars from carbon dioxide and water. In higher plants, this process takes place in chloroplasts, the subcellular organelles that occupy about half the volume of the plant cell. The reactions of photosynthesis can be divided into two categories, reactions that require light and those that do not. Light energy is required by photosynthetic electron transport to transfer electrons donated by water, at a low potential energy level, to NADPH at a higher potential energy level. NADPH is the source of energy for sugar production that takes place during a process that does not require light, the Calvin Cycle.

## Chloroplast Structure

The chloroplast is a large membrane-bound organelle about 2 to 10 microns long. The most obvious structures in a chloroplast are the many flattened sacs called thylakoids that are visible in photographs taken by electron microscopes. Thylakoids are often arranged in stacks called grana, but they can also be dispersed. Most of the components of the photosynthetic electron transport chain are embedded in the membranes of the thylakoids. The viscous, sap-like liquid that surrounds the thylakoids is the stroma, which contains the proteins and substrates of the Calvin Cycle. The stroma is contained by the double membrane that forms the outer envelope of an intact chloroplast.

## Photosynthetic Electron Transport

With a few subtle uncertainties, the components of the photosynthetic electron transport chain are well characterized. The distinguishing feature of this chain is that it contains two different photosystems, each has a different chlorophyll molecule. The chlorophyll molecules, P680 in Photosystem II and P700 in Photosystem I, receive light energy and use it to boost electrons to a higher energy level. These electrons are used to make NADPH which is needed for the Calvin Cycle.

The process begins when P680 (Photosystem II) uses energy from light to boost one of its electrons to a higher energy level (Table 2-1 on page 2). The electron is accepted by a modified, colorless *chlorophyll a* molecule known as pheophytin. Pheophytin donates an electron to a quinone molecule ( $Q_a$ ), the next component in the

chain which is bound to a protein within the Photosystem II (PSII) complex. To receive this electron,  $Q_a$  needs to be in its oxidized state. Once  $Q_a$  receives the electron, it is able to donate the electron to the next component in the chain ( $Q_b$ ), which also must be in an oxidized state to receive the electron. And so, the process continues through the chain.

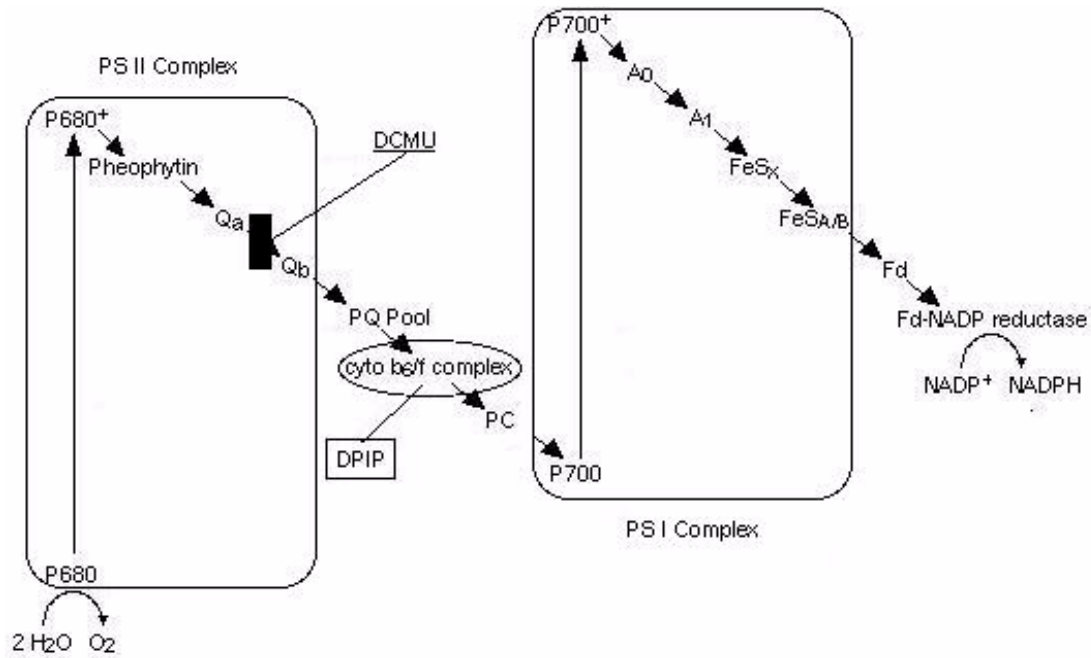


Figure 2-1: Components of Photosystems I and II. Site of action for DCMU and DPIP are labeled.

The bound  $Q_b$  molecule passes the electron to one of the plastoquinone (PQ) molecules in the large pool of these molecules that shuttle electrons from PSII to the next protein complex in the chain. PQ can perform this task because it is able to diffuse rapidly with the lipid bilayer.

The next recipient of the electron is the cytochrome  $b_6$ -f complex, that is composed of an iron-sulfur protein and three cytochromes. These compounds are bound to each other and transfer electrons internally from high to low energy levels, but the exact order of the transfer within the complex is uncertain.

The electron is then transferred to a plastocyanin molecule, which is another small diffusible protein that shuttles electrons between the cytochrome  $b_6$ -f complex and Photosystem I (PSI).

When the electron reaches P700 (PSI), more energy from light boosts the electron to a higher energy level where it is accepted by another bound molecule, ferredoxin. After the electron passes through ferredoxin, it is used to reduce NADP.

Back at PSII, P680 still needs to replace the electron it sent to a higher energy level. The electron is replaced by one extracted from water by the PSII complex; so, this reaction also yields protons and an oxygen atom.

### **Photo-phosphorylation**

As electrons are passed down the electron transport chain, some components, like cytochrome  $b_6-f$ , translocate a proton from the stroma to the intrathylakoid space. This creates a pH gradient across the thylakoid membrane, so that the area outside the thylakoid is basic (pH 8 in the light) and area inside is acidic (about pH 5). The energy stored in this pH gradient is used to make ATP according to the chemiosmotic hypothesis. The thylakoid membrane has knob-like structures called  $CF_1-CF_0$  ATPases, which are similar to the ATPase complexes in mitochondria. These ATPases have a channel in their middle through which protons move back to the stroma. As the protons flow, the rest of the complex captures the energy released and uses it to add a phosphate to ADP. This process is known as photophosphorylation.

### **Calvin Cycle**

The ATP and NADPH produced by the light-requiring reactions of photosynthetic electron transport are the high energy compounds that drive the Calvin Cycle. The Calvin Cycle is a series of enzymatically catalyzed reactions that take place in the stroma and do not require light. Six turns of this cycle are needed to fix six molecules of  $CO_2$  into a six-carbon sugar that will be used to supply energy to the cell. The critically important enzyme involved in this process is ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco). It is responsible for covalently bonding  $CO_2$  to a five carbon compound in the cycle.

Even though the Calvin Cycle does not require light, it requires products of the light reactions, ATP and NADPH. So, if the plant is in the dark, and all the ATP and NADPH is consumed, the Calvin Cycle comes to a halt.

### **Oxygen Polarograph**

A simple way of monitoring photosynthetic electron transport is to measure the oxygen produced in PS II. The PS II complex is replacing the electron sent from P680 to a higher energy level with an

electron extracted from water. A simple device that can be used to measure oxygen production is an oxygen polarograph. It can also be used to measure oxygen consumption in cells and mitochondria.

An oxygen polarograph consists of a reaction chamber, an oxygen electrode, a current to voltage adapter, a stirring device, and a recording device. The suspension and the solutions needed for the reactions are placed in a small plastic or glass chamber. The chamber has a port on the side or top for the placement of a Clark-type oxygen electrode, which uses a polarizing voltage to create a current or flow of electrons between the silver and platinum elements in the electrode. The output of the electrode is connected to a current to voltage adapter, and the output of this adapter is connected to the recording device. As the oxygen concentration in the chamber changes during the experiment, the current flowing between the two metals in the oxygen electrode changes in proportion to the oxygen concentration in the chamber. Changes in the current are converted to changes in voltage by the adapter, and the voltage output of the adapter is recorded by the data acquisition unit.

Since the volume in the chamber is small, the chamber has a flat bottom for the use of a stirbar. The stirbar assists the movement of the suspension across the membrane of the electrode and permits instantaneous recording of any changes in oxygen concentration.

The amount of oxygen produced is stoichiometrically related to the number of electrons passed down the photosynthetic electron transport chain. In this case, one oxygen atom is produced for every two electrons sent through the chain. Therefore, the production of NADPH is also stoichiometrically related to the amount of oxygen produced. However, due to the complexity of the reactions, the stoichiometry of ATP synthesis is variable and unknown.

### **Whole Chain Electron Transport**

In order for electron transport to occur through both photosystems, a compound that accepts electrons must be added to the reaction media in the polarograph chamber. This substance is ferricyanide, which will accept electrons from PS I, near the very end of the whole electron transport chain. The ferricyanide molecule can pull an electron off a component that is upstream in PS I; then, the component replaces its lost electron with one from an adjacent upstream component. This process continues all the way back through the chain to P680, that replaces its lost electron with one from water. Oxygen is then produced as the result of water being used as an electron donor.

## Uncoupling

When electron transport is uncoupled from the phosphorylation of ADP to ATP, the rate of electron transport increases. In this experiment, the uncoupler that will be added to the reaction mixture is methylamine. This substance functions by equilibrating the hydrogen ion concentrations across the thylakoid membrane and preventing the formation of the pH gradient used to drive phosphorylation. From experiments with methylamine and other substances, it has been discovered that the rate of electron transport is inversely proportional to the size of the pH gradient.

## PS I Electron Transport

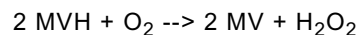
Electron transport through portions of the chain can be studied by using inhibitors that block electron flow, artificial donors that contribute electrons downstream of the block, and artificial electron acceptors that can be used in the assay of electron flow. Such an experiment will be done in this lab. Electron transport through PS I will be studied by blocking the flow of electrons from PS II and supplying PS I with an electron donor and an acceptor.

The flow of electrons from PS II is blocked by addition of the potent inhibitor, DCMU, that prevents the transfer of electrons between  $Q_a$  and  $Q_b$ . All the electron carriers downstream (on the PS I side of the block) become oxidized as the electrons remaining in the chain are drawn off by electron acceptors. Upstream (on the PS II side of the block), the electron carriers become reduced as electrons from P680 flow into them. Eventually, when all the carriers upstream of the block are filled with electrons, electrons from water are no longer needed and oxygen is no longer produced. If PS II is separated from PS I, the flow of electrons through PS I cannot be measured by oxygen production at PS II. To monitor the flow of electrons through PS I using an oxygen polarograph, another means of monitoring a change in oxygen concentration, that is stoichiometrically related to the rate of electron flow, must be used.

To study the flow of electrons through PS I, a new electron donor and acceptor are needed. In this experiment, DPIP and methyl viologen are added to the reaction media as the donor and the acceptor, respectively. DPIP donates electrons to cytochrome  $f_1$  downstream of the DCMU block. Methyl viologen accepts electrons from PS I according to the following reaction:



In an aerobic environment like water, a second reaction takes place after methyl viologen is reduced. Methyl viologen is easily auto-oxidized according to the following reaction:



Once it is cycled back to its oxidized state, methyl viologen is ready to pick up additional electrons to repeat the process.

When experimenting on PS I with these reagents, oxygen is consumed, not produced, as the electrons are being moved to a higher energy level. Since the stoichiometry of the reaction states that one molecule of oxygen (O<sub>2</sub>) is consumed for every pair of electrons transported through PS I to the acceptor, oxygen consumption can be used as the indicator of electron flow through this photosystem.

By comparing the rates of electron transport through Photosystem I with the rates through the whole-chain, it is possible to locate the rate-limiting step in photosynthetic electron transport.

In this experiment, you will:

- learn how to calibrate and use an oxygen polarograph;
- learn how to measure the functionality of isolated thylakoids;
- measure electron transport in a complete photosystem;
- measure electron transport in a single photosystem (PS I).

## Equipment Required

PC computer  
iWorx/214 and USB cable  
Oxygen electrode  
Current to voltage adapter  
Plexiglas™ respiration/photosynthesis chamber  
Magnetic stir motor, stir bar, and motor controller  
High intensity light source with a full range intensity control  
(The Dyna Lume Sun-Lite I is an excellent light source)  
Light meter  
Vortex mixer  
10µl micropipette  
Thylakoid suspension  
Thylakoid reaction media (See appendix)  
Methylamine uncoupler solution (See appendix)  
Photosystem I reaction media (See appendix)  
Concentrated O<sub>2</sub> depletion solution (1.5 M Sodium Dithionite)  
Squirt bottle filled with deionized water  
Pasteur pipet with plastic tip

## Equipment Setup

- 1 Connect the iWorx unit to the computer (described in Chapter 1).
- 2 Plug one end of the DIN-DIN cable into Channel 3 on the iWorx/214 unit. Plug the other end of this cable into the DIN connector on the DO2-100 current to voltage adapter (Figure 2-2 on page 7).
- 3 Attach the cable of the oxygen electrode to the BNC connector on the current to voltage adapter (Figure 2-2 on page 7).

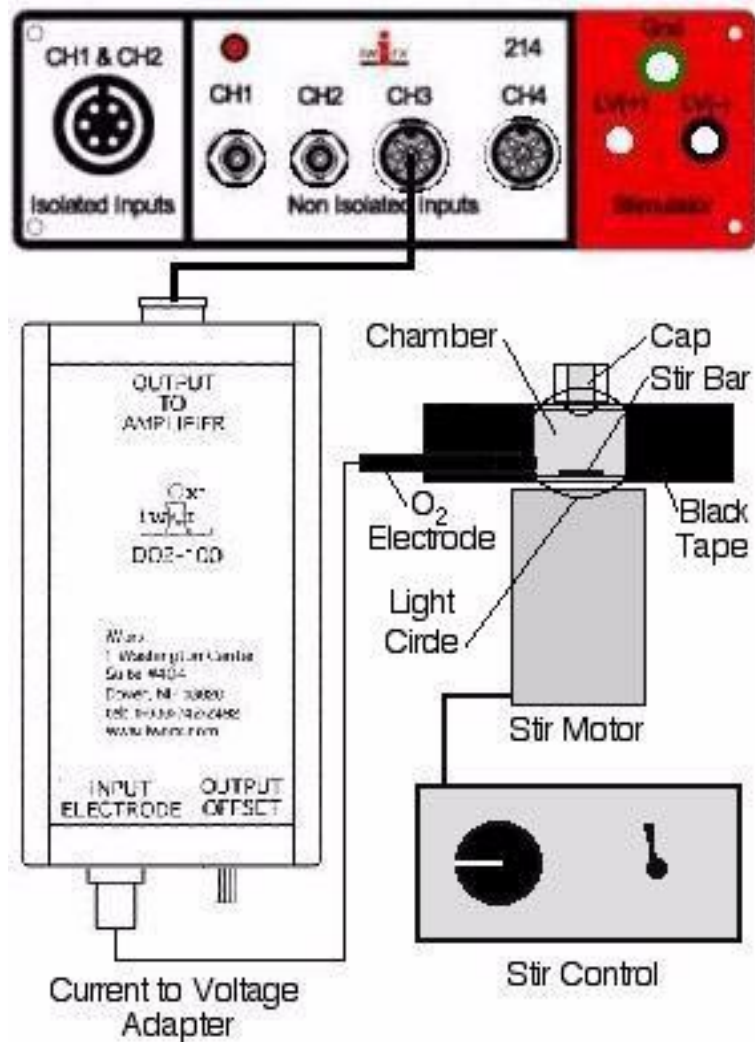


Figure 2-2: The oxygen polarograph used to record photosynthetic electron transport. Black tape covers the side of the polarograph block facing the light source, except on the area directly in front of the chamber. The light is focused on this open area so that the circle of light is only large enough to cover the chamber.

- 4 Install the oxygen electrode into its port on the polarograph chamber.
- 5 Place the small magnetic stir bar in the bottom of the chamber.

- 6 Plug the high intensity light source into the AC outlet. Align the light bulb so that it is about 2" from the side of the polarograph chamber.
- 7 Turn on the light and adjust its beam to cover the open area on the side of the polarograph chamber (Figure 2-2 on page 7).

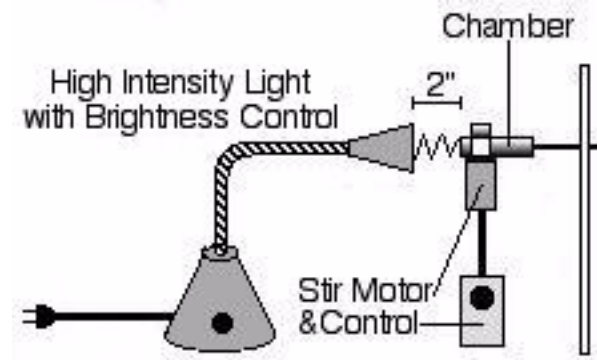


Figure 2-3; The high intensity illuminator (*Dyna Lume Sun-Lite I*) is 2" from the surface of the chamber. At this distance, the thylakoids or cells in the chamber are saturated with light, without a significant change in the temperature of the chamber or its contents.

- 8 Fill the chamber with room temperature deionized water. Check the chamber for leaks.
- 9 Position the chamber over the center of the magnetic stirrer. Turn on the stirrer, starting at a slow speed. Reposition the chamber over the stirrer so that the stir bar is centered in the chamber. Turn up the speed of the stirrer to the maximum rate that allows the stir bar to rotate evenly.

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**Note:** Changes in oxygen concentration in the chamber reach the electrode instantaneously if the solution in the chamber is stirred. If a stirrer was not used, recording changes in the rate of oxygen production would be limited by the rate of diffusion.

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## Start the Software

- 1 Click the Windows **Start** menu, move the cursor to **Programs** and then to the **iWorx** folder and select **LabScribe**; or click on the LabScribe icon on the Desktop
- 2 When the program opens, select **Load Group** from the **Settings** menu.
- 3 When the dialog box appears, select **ahkXXX.iws** (where XXX is the model of iWorx unit being used). Click **Load**.
- 4 Click on the **Settings** menu again and select the **Photosynthesis** settings file.

- 5 After a short time, LabScribe will appear on the computer screen as configured by the **Photosynthesis** settings.

## Exercise 1: Calibration

Aim: To calibrate the oxygen electrode.

The standard used for calibrating the oxygen electrode is the known concentration of oxygen in air-saturated deionized water. The amount of oxygen that is dissolved in water is dependent upon the temperature, oxygen pressure in the air, and the concentrations of dissolved solutes in the water. For example, the concentration of oxygen in deionized water at 26°C and 1 atmosphere is 252 micromolar ( $\mu\text{M}$ ), or 252 micromoles ( $10^{-6}$  moles) of  $\text{O}_2$  per liter of water.

## Procedure

- 1 Fill the polarograph chamber with deionized water before proceeding with the calibration procedure. Cap the chamber and turn up the speed of the stirrer to the maximum rate that allows the stir bar to rotate evenly.
- 2 Click **Start**.
- 3 Type the words "Saturation-DI Water" on the comment line to the right of the **Mark** button, and press the **Enter** key on the keyboard. This comment is used to indicate the water is saturated with as much oxygen as it can hold.
- 4 When the trace is stable (no vertical movements of the trace), record for an additional 10 seconds before going to the next step.
- 5 Type the words "No Oxygen" on the comment line. Use the micropipette to place 10 $\mu\text{l}$  (microliters) of 1.5M Sodium Dithionite  $\text{O}_2$  depletion solution into the chamber. Press the **Enter** key on the keyboard to mark the recording. In a few seconds, this small amount of solution will deplete all the oxygen from the deionized water stirring in the chamber. Record the drop in the oxygen concentration in the chamber until the trace is a flat line at a lower amplitude (Figure 2-4 on page 10).
- 6 Click **Stop** to halt recording.
- 7 Select **Save As** in the **File** menu, type a name for the file. choose a destination on the computer in which to save the file (e.g. the **iWorx** or class folder). Click the **Save** button to save the file (as an **\*.iwd** file).
- 8 Turn off the stirrer for the chamber. Remove the water and the oxygen depletion solution from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber 3 or 4 times with deionized water from a squirt bottle. Fill the chamber with deionized water and turn on the stirrer.

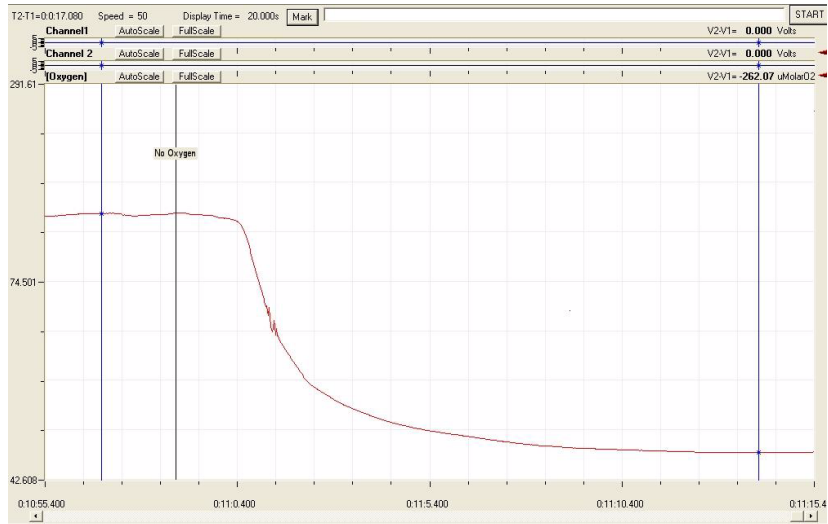


Figure 2-4: Change in concentration of oxygen in deionized water as the result of depletion.

## Units Conversion

- 1 Determine the temperature ( $^{\circ}\text{C}$ ) and the barometric pressure in the lab. The oxygen concentrations in deionized water, over a short range of temperatures at 760mmHg, have been calculated and are listed in Table 2-1 on page 11. The absorption coefficients of oxygen and the vapor pressures of water at these temperatures are also listed.
- 2 The concentration of oxygen dissolved in deionized water, or its solubility ( $S$ ), can be determined more accurately by using the following equation:

$$S = (a/22.414) ((P-p)/P) (r\%/100)$$

where  $a$  is the absorption coefficient of  $\text{O}_2$  at temperature,  $p$  is the vapor pressure of water at temperature,  $P$  is the barometric pressure, and  $r\%$  is the percent oxygen in the air. At  $26^{\circ}\text{C}$  and 760mmHg, assuming the concentration of oxygen in air is 21%,  $S = 252\mu\text{MO}_2$ :

$$(.02783/22.414\text{L/mole})(734.91\text{mmHg}/760\text{mmHg})(21/100) = 252\mu\text{MO}_2$$

- 3 Select the section of the recording before and after the oxygen is depleted from the chamber (Figure 2-4 on page 10). To view this section of the recording in its entirety on the same window, it may be necessary to click either of the **Display Time** icons in the toolbar (Figure 2-5 on page 11).
- 4 Click the **2-Cursor** icon (Figure 2-5 on page 11) so that two blue vertical lines appear over the recording window. Place one cursor on the plateau corresponding to the oxygen concentration in the fully oxygenated water. Place the other cursor on the lower amplitude plateau that corresponds to the absence of oxygen in the chamber.

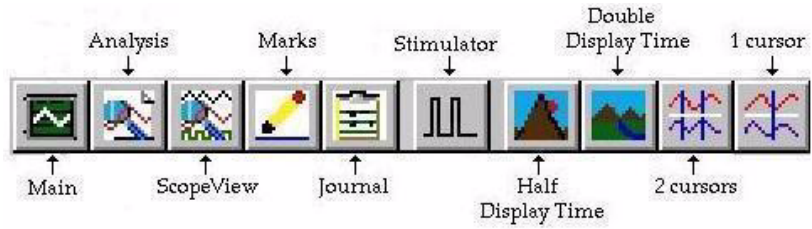


Figure 2-5: The LabScribe toolbar.

- Right-click on the Channel 3 window to open the **Right-click** menu. Select **Units** from the **Right-click** menu. Note that the voltage values for the positions of Cursors 1 and 2 are already entered in the units conversion window. Enter the concentration of oxygen dissolved in water at room temperature next to the voltage value for **Cursor1**. Enter “0” next to the voltage value for **Cursor2**. Enter “ $\mu\text{MolarO}_2$ ” for the unit name. Click **OK**. Now, the units on the Y-axis correspond to the oxygen concentration.

**Table 2-1: Concentration of Oxygen [O<sub>2</sub>] in Air-Saturated Deionized Water at a Barometric Pressure of 760mmHg.**

Temperature (°C)	O <sub>2</sub> Absorption Coefficient (a)	H <sub>2</sub> O Vapor Pressure (p) (mmHg)	[O <sub>2</sub> ] (μM)
20	.03102	17.54	284
21	.03044	18.65	278
22	.02988	19.83	273
23	.02934	21.07	267
24	.02881	22.38	262
25	.02831	23.76	257
26	.02783	25.09	252
27	.02736	26.74	247
28	.02691	28.35	243
29	.02649	30.04	238
30	.02608	31.82	234

## Precautions

- Your use of time in this experiment is critical, since thylakoids in suspension are useful for a limited period of time (3 to 4 hours). Complete all the exercises before analyzing the data.
- The lights in the room will be turned off during the experiment. It is important to gather all the supplies that you will need at your station

before the lights are turned off. Some of the items needed close to your work station include: a test tube rack, thylakoid reaction media, Photosystem I reaction media, methylamine uncoupler, a micropipette (set to 10 $\mu$ l), disposable micropipette tips, a squirt bottle filled with deionized water, a squirt bottle filled with 70% ethanol, Pasteur pipettes with a soft plastic tube on the their tips, and an insulated ice bucket. Small amounts of reagents will be dispensed in capped microfuge tubes. Larger amounts should be kept in capped test tubes.

- 3 Fill your ice bucket halfway. Place a 100ml beaker, for holding your tube of isolated thylakoids, in the ice. The microfuge tubes with the thylakoids will be dispensed when the room lights are turned off. Keep your ice bucket covered when the thylakoids are inside.

## Exercise 2: Functional Quality of the Thylakoid Preparation

**Aim:** To determine the rate of oxygen production by thylakoids and use it as a measure of the functional quality of the thylakoid preparation.

Oxygen production is a result of photosynthetic electron transport through both photosystems. This experiment will be performed with high light intensity, with and without an uncoupler.

### Procedure

- 1 Turn off the stirrer for the chamber. Remove the oxygen-depleted water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber ten times with deionized water.
- 2 Fill the chamber with the yellow thylakoid reaction media. Carefully fit the cap on the chamber and turn on the stirrer.
- 3 Check the chamber for the presence of bubbles. If bubbles are present, turn off the stirrer, allow the bubble to rise to the top. Take off the cap and the bubble should burst. Replace the cap, turn on the stirrer, and check for bubbles, again.
- 4 Open the **Windows** menu on the LabScribe software and select the **Preview** window. Change the channel displayed to **[Oxygen]** channel. Turn the offset knob on the DO2-100 adapter to position the trace near the bottom of the screen. As oxygen is produced, the trace will move up.

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**Note:** *Moving the trace with the offset knob does not affect the calibration.*

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- 5 If your light source has a power switch which is separate from the intensity control, make sure the light source is turned off. If your light source does not have a separate power switch, plug the light source into a power strip that has a switch, make sure the power strip is turned off. Set the intensity control of the illuminator to the maximum level (100%).

- 6 Make sure no light is reaching the polarograph chamber. Click **Start**. Type the words "Thylakoids in Dark" on the comment line to the right of the **Mark** button. Position the trace near the bottom of the screen.
- 7 Mix the tube with the thylakoid preparation on the Vortex mixer, so the suspension is even. Use the micropipette to collect a 10  $\mu$ l aliquot of thylakoids from the tube. Press the **Enter** key on the keyboard to mark the recording as a 10 $\mu$ l aliquot of thylakoids is added to the chamber through the cap. Be careful not to add an air bubble to the chamber. Record the rate of oxygen production of the thylakoids in darkness for one minute.

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**Note:** Put the tip of the micropipette through the hole in the cap of the chamber and push its plunger to discharge the donor solution into the chamber. Do this carefully so that no bubbles are introduced into the chamber. Remove the micropipette from the cap before releasing its plunger. If the plunger is released while the tip of the micropipette is still in the cap or the chamber, solutions could be siphoned from the chamber by the micropipette.

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- 8 Type the words "Thylakoids in Light at 100" on the comment line. Press the **Enter** key on the keyboard as the light is turned on and the thylakoid suspension in the chamber is illuminated at the highest intensity. Record oxygen production at this intensity for one minute.
- 9 As the recording continues, type the words "Methylamine added" on the comment line. Press the **Enter** key on the keyboard to mark the recording as a 10 $\mu$ l aliquot of methylamine uncoupler solution is added to the chamber through the cap.
- 10 Record oxygen production of the thylakoids exposed to 100% light in the presence of an uncoupler for another minute.
- 11 Turn off the illuminator to put the thylakoids in the dark. Record for another minute. Click **Stop** to halt recording.
- 12 Select **Save As** in the **File** menu, type a name for the file. choose a destination on the computer in which to save the file (e.g. the **iWorx** or class folder). Click the **Save** button to save the file (as an **\*.iwd** file).
- 13 Remove the cap from the chamber, and rinse the cap with deionized water. Dry the cap. Make sure there is no fluid in the hole of the cap.
- 14 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about ten times.
- 15 This exercise was designed to test the functionality of the thylakoids. They should demonstrate a rate of oxygen production that increases substantially when an uncoupler is added. A change in oxygen concentration, in the presence of an uncoupler, that is equal to or greater than 0.400  $\mu$ MO<sub>2</sub>/sec is considered excellent. If your rates are significantly lower than that value, consult your instructor.

### Exercise 3: Whole-Chain Electron Transport

Aim: To examine the effects of light intensity on the rate of electron transport through the complete chain of Photosystems I and II, with and without an uncoupler.

The same reaction media used in Exercise 2 is used in this exercise. The electron acceptor, ferricyanide, is present in the chamber; water is the ultimate electron donor; and, oxygen production will be used as a measure of electron transport.

#### *Procedure*

- 1 In this exercise, the intensity of the light illuminating the thylakoids in the chamber will be altered using the intensity control of the light source. As stated in Exercise 2, the full brightness of the light source is equal to 100% illumination. Use the light meter to determine the positions of the intensity control that correspond to 20, 30, 40, 60, and 80% illumination.
- 2 Set the intensity control of the light source to deliver 20% illumination. Turn the light off.
- 3 Turn off the stirrer for the chamber. Remove the water from the chamber with a plastic-tipped Pasteur pipet.
- 4 Fill the chamber with the yellow thylakoid reaction media. Carefully fit the cap on the chamber and turn on the stirrer.
- 5 Check the chamber for the presence of bubbles. If bubbles are present, turn off the stirrer, allow the bubble to rise to the top. Take off the cap and the bubble should burst. Replace the cap, turn on the stirrer, and check for bubbles, again.
- 6 Make sure no light is reaching the polarograph chamber. Click **Start**. Type the words "Whole Chain in Dark" on the comment line to the right of the **Mark** button. Position the trace near the bottom of the screen.
- 7 In darkness, mix the tube with the thylakoid preparation on the Vortex mixer, so the suspension is even. Use the micropipette to collect a 10  $\mu$ l aliquot of thylakoids from the tube. Press the **Enter** key on the keyboard to mark the recording as a 10  $\mu$ l aliquot of thylakoids is added to the chamber through the cap. Be careful not to add an air bubble to the chamber.
- 8 Record the rate of oxygen production of the thylakoids in darkness for one minute.
- 9 Type the words "Whole Chain in Light at 20" on the comment line. Press the **Enter** key on the keyboard as the light is turned on and the thylakoid suspension in the chamber is illuminated at 20% brightness. Record oxygen production in the light for one minute or until the slope of the trace is constant.
- 10 As the recording continues, type the words "Whole Chain in Light at 30" on the comment line. Press the **Enter** key on the keyboard as the intensity control on the light source is set to 30% brightness. Record the new rate of oxygen production for one minute or until the slope is constant.

- 11 Repeat Step 10 for light intensities of 40, 60, 80 and 100% brightness.
- 12 Turn off the light and record for one minute. Click **Stop** to halt recording.
- 13 Select **Save** in the **File** menu.
- 14 Remove the cap from the chamber, and rinse the cap with deionized water. Dry the cap. Make sure there is no fluid in the hole of the cap.
- 15 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about ten times.
- 16 Repeat Steps 2 through 15, with one exception. After Step 8 and before Step 9, add 10  $\mu$ l of the uncoupler, methylamine, to the chamber. Mark the recording at the appropriate times to indicate the presence of the uncoupler and the light intensities used.

### Exercise 4: PS I Electron Transport

**Aim:** To examine the effects of light intensity on the rate of electron transport through Photosystem I, with and without an uncoupler.

Since the rates of electron transport will be measured through PS I only, an alternate method of measuring electron transport is used. Because methyl viologen is used as the electron acceptor in this exercise, oxygen will be consumed rather than produced.

#### *Procedure*

- 1 Repeat Exercise 3 with Photosystem I reaction media, which contains: methyl viologen as the electron acceptor; DCMU as the electron transport inhibitor between PS II and PS I; and DPIP as the alternate electron donor to PS I.
- 2 Begin the recording at the top of the screen because the trace will move toward the bottom of the screen when the thylakoids are illuminated.
- 3 Perform a run without the uncoupler. Then, perform a run with the uncoupler, methylamine. Mark the recordings at the appropriate points to indicate the reagents and light intensities used.
- 4 This reaction media contains DCMU, which is a very potent inhibitor of electron transport. It is not very soluble in water and adheres to the walls of the plastic polarograph chamber. It is critically important to clean out the polarograph chamber thoroughly with 70% ethanol at least 10 times and with deionized water at least ten times after each of these runs.

### Exercise 5: Retest the Functionality of the Thylakoids

**Aim:** To determine if the ability of the thylakoids to perform electron transport remained constant or declined over the course of the experiment.

## Procedure

- 1 Repeat Exercise 2 to measure the functionality of the thylakoids after the other exercises were completed.
- 2 Compare the rate of change in oxygen concentration without the uncoupler from Exercise 2 to the same variable from Exercise 5.
- 3 Compare the rate of change in oxygen concentration with the uncoupler from Exercise 2 to the same variable from Exercise 5.

## Analysis: Rate of Electron Transport

By international consensus, the rate of electron transport in thylakoids is expressed as the rate of oxygen production. The units used to express these rates are: moles  $O_2$ /hr/mg chlorophyll. Oxygen production rates are standardized for comparison of experiments performed in different laboratories around the world. so, the size of the reaction chamber, the number of thylakoids in the chamber, and the time period for that change in oxygen concentration need to be included in the calculation of the rate,.

In Exercise 1 of this experiment, the oxygen probe was calibrated using air-saturated, deionized water at room temperature. Through this calibration, the Y-axis of the recording channel was converted from voltage to oxygen concentration or [Oxygen], which is expressed as  $\mu\text{Molar}O_2$  and abbreviated  $\mu\text{MO}_2$ .

## Changes in $O_2$ Concentrations in Exercises 2 and 5

The rate of change of the oxygen concentration in the polarograph chamber can be measured directly from the recordings by measuring the average slope (**mean\_dv/dt**) of the trace.

In Exercises 2 and 5, the functional quality of the thylakoid preparation was measured at the beginning and the end of the experiment. Follow these steps to measure the rates of change in the oxygen concentration in the polarograph chamber during these exercises:

- 1 Adjust the appropriate **Display Time** icon on the LabScribe toolbar (Figure 2-5 on page 11) so that the recording for Exercise 2 appears on the **Main** window.
- 2 Click the **2-Cursor** icon (Figure 2-5 on page 11), so that two blue vertical lines appear over the recording window. Place the two blue cursors on either side of the complete experimental run.
- 3 Click the **Analysis** icon (Figure 2-5 on page 11) to open the window. The selected data from the **Main** window will be present in the **Analysis** window.
- 4 Select the **[Oxygen]** channel (CH 3) in **Display Channel** list, on the left side of the **Analysis** window. From the **Table Functions** list, select **V2-V1** and **T2-T1** under **General**, and **mean\_dv/dt** under **Derivative** (Figure 2-6 on page 17).

- In the **Analysis** window, position the two blue cursors on the section of the experimental run that corresponds to oxygen production from thylakoids in the dark. Set the cursors on a linear portion of this data, and use the **T2-T1** value displayed in the **Table of Functions** to set the cursors ten seconds apart. The value for the variable **mean\_dv/dt** is the average rate of change in the oxygen concentration (in  $\mu\text{Molar}/\text{sec}$ ) in the chamber taken over ten seconds (Figure 2-6 on page 17).

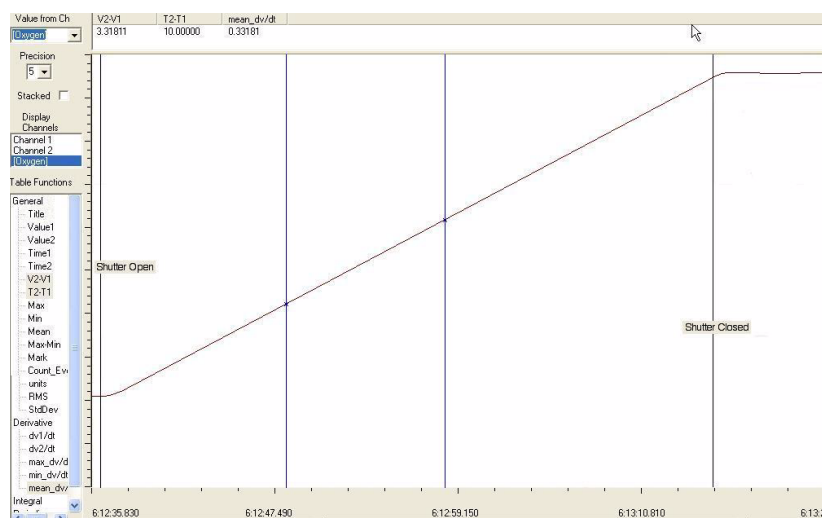


Figure 2-6: Change in oxygen concentration in the polarograph chamber created by thylakoids with whole electron transport chains exposed to 80% light in the presence of an uncoupler. As displayed in the **Analysis** window, the marks indicate the times when the shutter controlling the light was opened and closed. Cursors are 10 seconds apart. The average rate of change (**mean\_dv/dt**) in the oxygen concentration over that period is the  $0.33181 \mu\text{Molar O}_2/\text{sec}$ .

- Enter data into the **Journal** by either typing the titles and values directly or by using the **Right-click** menu. Place the cursors to take measurements; then, select **Add Title to Journal** or **Add Data to Journal** from the right-click menu to add the measurements to the **Journal**.
- Move the blue cursors to the next section of the experimental run when the thylakoids were illuminated by light at an intensity of 100% (setting of 100 on the variable transformer).
- Repeat Steps 5 and 6 to measure the rate of change in oxygen concentration for thylakoids that are illuminated. All the average rates of change in oxygen concentration should be measured across a ten second period.
- Move the blue cursors to the next section of the experimental run when the thylakoids were illuminated by light at an intensity of 100% with the uncoupler present.
- Repeat Steps 5 and 6 to measure the rate of change in oxygen concentration in thylakoids that are illuminated in the presence of an uncoupler.

- 11 Repeat Steps 1 through 10 for Exercise 5 which involved measurements from thylakoids remaining at the end of the lab period.
- 12 Make sure all the rates of change in oxygen concentration at all the intensities of illumination, with or without an uncoupler, are recorded in the **Journal**.

### *Changes in O<sub>2</sub> Concentrations in Exercises 3 and 4*

In Exercises 3 and 4, the effects of light intensity on electron transport in the whole electron transport chain and PS I were measured in the absence and presence of an uncoupler. Follow these steps to measure the rates of change in the oxygen concentration in the polarograph chamber during these exercises:

- 1 Adjust the appropriate **Display Time** icon on the LabScribe toolbar (Figure 2-5 on page 11) so that the recording of the first experimental run of Exercise 3 appears on the **Main** window. This is the portion of the exercise that measures the effects of light intensity on whole-chain electron transport in the absence of an uncoupler.
- 2 Click the **2-Cursor** icon (Figure 2-5 on page 11), so that two blue vertical lines appear over the recording window. Place the two blue cursors on either side of the complete experimental run.
- 3 Click the **Analysis** icon (Figure 2-5 on page 11) to open the window. The selected data from the **Main** window will be present in the **Analysis** window.
- 4 Select the **[Oxygen]** channel (CH 3) in **Display Channel** list, on the left side of the **Analysis** window. From the **Table Functions** list, select **T2-T1** under **General**, and **mean\_dv/dt** under **Derivative**.
- 5 In the **Analysis** window, position the two blue cursors on the section of the experimental run that corresponds to oxygen production from thylakoids in the dark. Set the cursors on a linear portion of this data, and use the **T2-T1** value displayed in the **Table of Functions** to set the cursors ten seconds apart. The value for the variable **mean\_dv/dt** is the average rate of change in the oxygen concentration (in  $\mu\text{Molar/sec}$ ) in the chamber taken over ten seconds.
- 6 Enter data into the **Journal** by either typing the titles and values directly or by using the **Right-click** menu. Place the cursors to take measurements; then, select **Add Title to Journal** or **Add Data to Journal** from the right-click menu to add the measurements to the **Journal**.
- 7 Move the blue cursors to the next section of the experimental run when the thylakoids were illuminated by light at an intensity of 20% (setting of 20 on the variable transformer).
- 8 Repeat Steps 5 and 6 to measure the rate of change in oxygen concentration for thylakoids that are illuminated at the intensity of 20%. All the average rates of change in oxygen concentration should be measured across a ten second period.

- 9 Move the blue cursors to the next sections of the experimental run when the thylakoids were illuminated by increasing intensities of light (30, 40, 60, 80, and 100). Repeat the directions of Steps 5 and 6 to measure the rates of change in oxygen concentration for each of the remaining intensities of light.
- 10 Repeat Steps 1 through 9 for the second experimental run of Exercise 3, which measured the effects of light intensity on whole-chain electron transport in the presence of an uncoupler.
- 11 Repeat Steps 1 through 9 for the two experimental runs of Exercise 4, which measured the effects of light intensity on PS I electron transport in the absence and the presence of an uncoupler.
- 12 Make sure all the rates of change in oxygen concentration at all the intensities of illumination, with or without an uncoupler, in the whole electron transport chain or PSI, are recorded in the **Journal**.

### **Standardizing the Rate of Oxygen Production**

To standardize the rates of oxygen production (or consumption) according to the adopted convention, the volume of the polarograph chamber, the measured rate of change in oxygen concentration, and the concentration of thylakoids in the chamber must be incorporated into the calculations.

- 1 Multiply the rate of change in the oxygen concentration by the volume of polarograph chamber. The product of this calculation is the number of moles of oxygen produced in one second. For example, if the change in oxygen concentration is 0.333 microMolar O<sub>2</sub> (μMO<sub>2</sub>) per second, and the polarograph chamber has a 1.2 milliliter (ml) capacity; then 0.396 nanomoles (nmoles) of oxygen are produced in one second:

$$(0.333\mu\text{MO}_2/\text{sec})(1.2\text{ml}) = 0.396 \text{ nmoles O}_2 \text{ produced in one second;}$$

or

$$(0.333 \times 10^{-6} \text{ moles O}_2/\text{L})(1.2 \times 10^{-3} \text{ L}) = 0.396 \times 10^{-9} \text{ moles O}_2 \text{ produced in one second.}$$

- 2 Next, the moles of oxygen produced in one second must be converted to an hourly rate. In our example, if the amount of oxygen produced is 0.396 nmoles O<sub>2</sub> in one second, the hourly production rate is 1.44 micromoles O<sub>2</sub> (μmoles O<sub>2</sub>) per hour:

$$(0.396 \times 10^{-9} \text{ moles O}_2/\text{sec})(60\text{sec}/\text{min})(60\text{min}/\text{hr}) = 1440 \times 10^{-9} \text{ moles O}_2/\text{hr} = 1.44 \times 10^{-6} \text{ moles O}_2/\text{hr.}$$

- 3 Finally, the moles of oxygen produced per hour must be standardized for the amount of thylakoids in the chamber. Clearly, if a chamber contains more thylakoids, the rate of oxygen production will be greater. After the

thylakoids are isolated, the laboratory staff determines the concentration of chlorophyll (chl) in the preparation using a spectrophotometer. The concentration of chlorophyll is proportional to the concentration of thylakoids. Then, the lab staff dilutes the preparation with the appropriate volume of buffer to create a stock suspension of thylakoids that has the same approximate concentration of thylakoids in each lab session. In our example, if the concentration of chlorophyll in the stock solution is 2.5 milligrams of chlorophyll per milliliter (mg chl/ml), and 10  $\mu$ l of thylakoid suspension is added to the polarograph chamber, the amount chlorophyll added to the chamber is 0.025 mg:

$$(2.5 \text{ mg chl/ml})(0.010 \text{ ml}) = 0.025 \text{ mg chlorophyll in the chamber.}$$

To express the rate of oxygen production properly, the hourly rate of oxygen production must be divided by the amount of chlorophyll in the chamber:

$$\begin{aligned} & (1.44 \times 10^{-6} \text{ moles O}_2/\text{hr}) / (0.025 \text{ mg chl}) = \\ & 57.6 \times 10^{-6} \text{ moles O}_2/\text{hr/mg chl} = 57.6 \text{ } \mu\text{moles O}_2/\text{hr/mg chl.} \end{aligned}$$

- 4 Calculate the rates of oxygen production (or consumption) for each section of each experimental run using the steps presented above.
- 5 Enter the rates for Exercises 2 and 5 in Table 2-2 on page 19, for Exercise 3 in Table 2-3 on page 20, and for Exercise 4 in Table 2-4 on page 20. These rates should be placed in the columns labeled "Recorded".

### **Correction for Non-Zero Dark Rates**

- 1 Sometimes, before the polarograph chamber is illuminated, thylakoids (or cells) will either produce or consume oxygen at a low rate. If the thylakoids are producing oxygen (+ rate) in the dark, then the recorded rate is greater than the actual light-induced rate of oxygen production because the dark rate is adding itself to the recorded rate. Likewise, If the thylakoids are consuming oxygen (- rate) in the dark, then the recorded rate is less than the actual light-induced rate of oxygen production because this dark rate is negating a portion of the actual rate.
- 2 When determining the actual rate of oxygen production (or consumption) attributable to light, the non-zero dark rate, whether positive or negative, must be subtracted from the recorded rates in the same experimental run. For example, if thylakoids consumed oxygen at a rate of -9.6  $\mu$ moles O<sub>2</sub>/hr/mg chl in the dark and produced oxygen at the rate of 57.6  $\mu$ moles O<sub>2</sub>/hr/mg chl in 100% light, their actual rate of light-induced oxygen production is:

$$\begin{aligned} & (57.6 \text{ } \mu\text{moles O}_2/\text{hr/mg chl}) - (-9.6 \text{ } \mu\text{moles O}_2/\text{hr/mg chl}) = \\ & 67.2 \text{ } \mu\text{moles O}_2/\text{hr/mg chl} \end{aligned}$$

- 3 For each experimental run in each exercise, subtract the non-zero dark rate from the recorded light-induced rates to yield the actual light-induced rates. Enter the actual rates in the appropriate columns on the tables (Table 2-2 on page 22, Table 2-3 on page 22, Table 2-4 on page 22).

## Questions

- 1 Do thylakoids consume or produce oxygen when they are in the dark? What happens to the oxygen production rate when the thylakoids and reagents in the chamber are exposed to light?
- 2 What effect does the uncoupler have on the oxygen production rates? Explain the effect in terms of phosphorylation, electron transport, and the chemiosmotic hypothesis.
- 3 What is the relationship between light intensity and the rate of the oxygen production in a whole electron transport chain? What is the relationship between light intensity and the rate of the oxygen production in a whole electron transport chain in the presence of an uncoupler? Plot a graph of the oxygen production rates from whole electron transport chains, with and without the uncoupler, as a function of light intensity.
- 4 What is the relationship between light intensity and the rate of the oxygen consumption in the PS I electron transport chain? What is the relationship between light intensity and the rate of the oxygen consumption in the PS I electron transport chain in the presence of an uncoupler? Plot a graph of the oxygen consumption rates from whole electron transport chains, with and without the uncoupler, as a function of light intensity.
- 5 What is the stoichiometry of oxygen produced to electrons transported in the whole electron transport chain? What is the stoichiometry of oxygen consumed to electrons transported in the PS I electron transport chain?
- 6 Which electron transport chain, whole or PS I only, has the higher rate of electron transport when coupled to phosphorylation? Which chain, whole or PS I only, has the higher rate of electron transport when uncoupled from phosphorylation?
- 7 How do the oxygen production rates (coupled and uncoupled) at the beginning of the experiment compare to the rates at the end of the experiment? For what reason might they be different?

**Table 2-2: Exercises 2 and 5: Thylakoid Functionality; Oxygen (O<sub>2</sub>) Production Rates expressed as 10<sup>-6</sup> moles O<sub>2</sub>/hr/mg chlorophyll.**

Light Conditions	Exercise 2 (Beginning)		Exercise 5 (End)	
	Recorded	Actual	Recorded	Actual
Thylakoids in Dark		0		0
Thylakoids in 100% Light				
Thylakoids in 100% Light with Uncoupler				

**Table 2-3: Exercise 3: Thylakoids with Whole Chain Electron Transport. Oxygen (O<sub>2</sub>) Production Rates expressed as 10<sup>-6</sup> moles O<sub>2</sub>/hr/mg chlorophyll.**

Light Conditions	Without Uncoupler		With Uncoupler	
	Recorded	Actual	Recorded	Actual
Thylakoids in Dark		0		0
Thylakoids in 20%				
Thylakoids in 30%				
Thylakoids in 40%				
Thylakoids in 60%				
Thylakoids in 80%				
Thylakoids in 100%				

**Table 2-4: Exercise 4: Thylakoids with Electron Transport through PS I only. Oxygen (O<sub>2</sub>) Production Rates expressed as 10<sup>-6</sup> moles O<sub>2</sub>/hr/mg chlorophyll.**

Light Conditions	Without Uncoupler		With Uncoupler	
	Recorded	Actual	Recorded	Actual
Thylakoids in Dark		0		0
Thylakoids in 20%				
Thylakoids in 30%				
Thylakoids in 40%				
Thylakoids in 60%				
Thylakoids in 80%				
Thylakoids in 100%				

## Appendices

### ***D02-100 Current to Voltage Adapter***

The D02-100 is a current to voltage adapter designed to work with a Clark-style oxygen electrode. This adapter delivers a polarizing voltage of  $-0.8\text{V}$  to the electrode to create a current, or flow of electrons, between the silver and platinum elements in the electrode. The flow of electrons between these elements increases and decreases as the concentration of oxygen in the polarograph chamber increases and decreases, respectively. The adapter then converts the changes in current to changes in voltage that can be recorded by a data acquisition unit. The output of the D02-100 is  $10\text{mV}$  for every nanoampere of current that is flowing. If a two-point calibration is performed, the voltage output of the adapter can be related to the oxygen concentration in the chamber. The adapter also has an offset control which allows the recording to be positioned on the screen without affecting the calibration of the electrode.

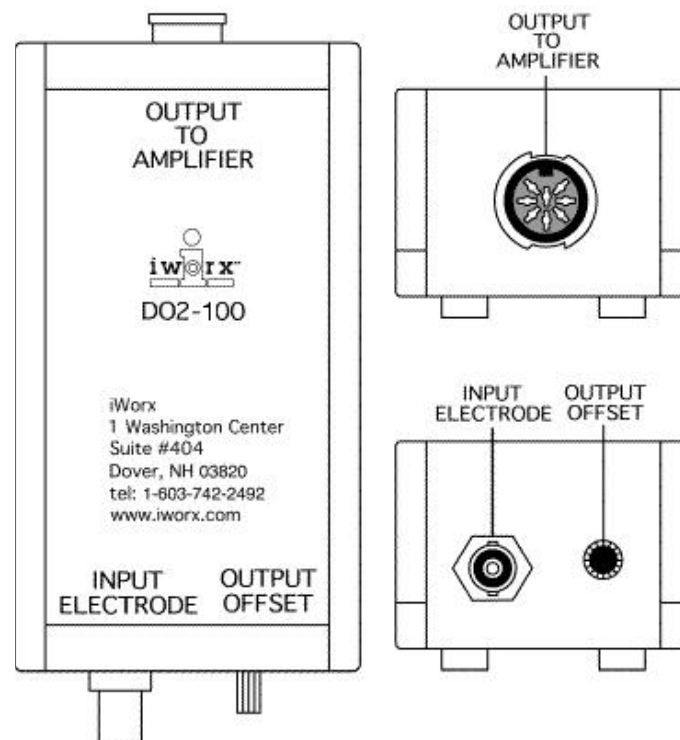


Figure 2-7: Top and side views of the DO2-100 current to voltage adapter.

## ISE-CB-730 Microelectrode

### Assembly and Preparation

- 1 Unscrew the acrylic housing with the affixed Teflon™ membrane from the oxygen electrode.
- 2 Check the level of electrolyte solution in this housing. The minimum height of electrolyte in the housing should be 6 mm.
- 3 If solution needs to be added to the housing, attach a filling tip to the bottle of electrolyte and fill the housing to the minimum height. To fill the housing without adding bubbles, gently place the bubble-free end of the filling tip against the inside of the Teflon™ membrane and add electrolyte until its level reaches the minimum height.
- 4 Insert the electrode tip into the housing. Be careful! Do not trap any air bubbles near the electrode tip. Screw the housing onto the body of the electrode until it stops.
- 5 Check the tip of the electrode for proper seating within the housing. If the electrode protrudes slightly beyond the end of the housing, it is seated correctly.

### Handling

When necessary, the membrane of the electrode can be replaced by following the assembly and preparation procedure above.

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**Note:** *When removing and replacing a membrane as well as when calibrating or making measurements, be careful not to apply pressure against the internal electrode. Any excessive pressure against the internal electrode can cause the electrode to crack rendering it useless and unrepairable.*

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### Cleaning

When using the electrode in solutions containing protein, the electrode should be soaked in an enzyme cleaning solution such as Terg-a-zyme (Alconox, Inc.) after each use for a couple of minutes to remove the protein from the membrane surface. This will prolong the useful life of the membrane.

### Storing

Always clean and rinse the electrode before storing. For long-term storage which is over 1 month:

- Remove the membrane housing from the electrode.
- Rinse the internal electrode with distilled water and pat dry.

- Place a new, unfilled membrane housing over the internal electrode and attach loosely (Do not seat completely). This membrane will serve to keep the dust off of the electrode tip.
- For short-term storage, the electrode can be left in room air with membrane housing still attached.

## Isolation of Thylakoids

- 1 Wash 16 fresh spinach leaves (*Spinacia oleracea*) in tap water and pat dry with paper towels.
- 2 Tear the leaves into small pieces (< 4 cm per side) and place the pieces in the chilled (4°C) jar of a kitchen blender. Pour 200 ml of cold thylakoid isolation buffer, containing 0.4 grams of sodium ascorbate, on top of the leaves.
- 3 Homogenize the leaves at low speed for 10 seconds until all the large leaf fragments are in a slurry. Then, homogenize the slurry at high speed for 20 seconds.
- 4 Filter the homogenate through 8 layers of cheese cloth into a 400 ml beaker. Place the collected filtrate in large centrifuge bottles that can be used in high capacity refrigerated centrifuge (IEC CU5000, for example)
- 5 Spin the filtrate for 2 minutes at 1000 rpm (~1200xG). Collect the supernatant, and place it in clean centrifuge bottles. Discard the pellet.
- 6 Spin the supernatant at 2100 rpm (~2500xG) for 10 minutes. Discard the supernatant. Resuspend the pellet in about 5 mls of cold thylakoid isolation buffer (without sodium ascorbate). Add what remains of the 200 ml of thylakoid suspension buffer to the suspension.
- 7 Spin the suspension at 1500 rpm (~1800xG) for one and a half minutes. Collect the supernatant, and place it in clean centrifuge bottles. Discard the pellet.
- 8 Spin the supernatant at 2100 rpm (~2500xG) for 10 minutes. Discard the supernatant. Resuspend the pellet in about 2 ml of cold thylakoid isolation buffer (without sodium ascorbate).
- 9 Dilute a 20 µl sample of the final thylakoid suspension in 2ml of 80% acetone. Filter the solution through Whatman #4 filter paper. Place the filtered solution in a cuvette. Use a cuvette filled with 80% acetone as the blank. Determine the absorbances of the green solution at 663 nm and at 645 nm. The concentration of chlorophyll in the original suspension is calculated from the following equation:

$$\text{mg chl/ml} = ((A_{663})(0.00802) + (A_{645})(0.0202)) \times \text{dilution factor};$$

the dilution factor, in this example, is 100 (2.0 ml/0.020 ml).

## Reagents

### 1 Thylakoid isolation buffer, pH 7.4-7.5 in deionized water:

50 mM Tricine  
400 mM Sucrose  
50mM NaCl

Store in 200ml aliquots in foil wrapped bottles at 4°C. Need about 500 mls per lab period.

### 2 Thylakoid reaction buffer, pH 7.5 in deionized water:

50 mM Tricine  
5 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O  
2 mM K<sub>3</sub>Fe(CN)<sub>6</sub> (Potassium Ferricyanide)

Store in 200ml aliquots in foil wrapped bottles at 4°C. Need about 250 mls per lab period.

### 3 3.0 M Methylamine

20.26g Methylamine in 100 mls deionized water. Need about 0.1 ml per lab group, each lab period.

### 4 Photosystem I reaction buffer. Make fresh just before each lab period and use within 5 hours.

0.5M Tricine stock solution	10 mls
0.01M Methyl viologen stock solution	1 ml
0.01M KCN (Potassium Cyanide) stock solution	1 ml
0.0001M DCMU stock solution	1 ml
0.001M DPIP stock solution	1 ml
deionized water	64 mls
Sodium ascorbate	0.04 g

### 5 0.5M Tricine stock solution:

Mix 8.96 grams of Tricine into 90 mls of deionized water. Adjust the pH to 7.5. Bring final volume to 100ml. Store at 4°C.

### 6 0.01M Methyl viologen stock solution:

Mix 0.26 grams of methyl viologen into 100 mls of deionized water. Store at 4°C.

**7** 0.01M KCN (Potassium cyanide) stock solution:

Mix 0.033 grams of KCN into 50 mls of deionized water. Store at 4°C.

**8** 0.0001M DCMU stock solution:

Mix 0.0012 grams of DCMU into 50 mls of 70% Ethanol. Freeze 25 mls, in 1 ml aliquots in capped tubes. Store the remainder at 4°C.

**9** 0.001M DPIP stock solution

Mix 0.12 grams of DPIP into 50 mls of deionized water. Store at 4°C.

**10** 1.5M Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (Sodium Dithionite) O<sub>2</sub> depletion solution

Mix 13 grams of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> into 50 mls of deionized water. Store at 4°C.

