Experiment CM-4

Photosynthesis in Isolated Thylakoids

Note: The lab presented here is intended for evaluation purposes only. iWorx users should refer to the User Area on www.iworx.com for the most current versions of labs and LabScribe2 Software.
Experiment CM-4: Photosynthesis in Isolated Thylakoids

Background

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.

Figure CM-4-B1: Components of Photosystems I and II. Site of action for DCMU and DPIP are labeled.
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 (Figure CM-4-B1).

The electron is accepted by a modified, colorless chlorophyll a molecule known as pheophytin. Pheophytin donates an electron to a quinone molecule (Q\textsubscript{a}), the next component in the chain which is bound to a protein within the Photosystem II (PSII) complex. To receive this electron, Q\textsubscript{a} needs to be in its oxidized state. Once Q\textsubscript{a} receives the electron, it is able to donate the electron to the next component in the chain (Q\textsubscript{b}), which also must be in an oxidized state to receive the electron. And so, the process continues through the chain. The bound Q\textsubscript{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 is the cytochrome b\textsubscript{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\textsubscript{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, ferrodoxin. After the electron passes through ferrodoxin, 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 replace 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\textsubscript{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\textsubscript{1}-CF\textsubscript{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 photo-phosphorylation.
**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 stops.

**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 or consumption is an oxygen polarograph, which consists of a dissolved oxygen electrode placed in a reaction chamber, a current to voltage adapter, a stir motor, and a recorder.

In 1954, Dr. Leland Clark invented the first membrane-covered electrode designed to measure the concentration of oxygen in blood, solution, and gases. This electrode was innovative because both the anode and the cathode were in a housing that was covered by the same selectively permeable polyethylene membrane. The membrane allowed only a small amount of oxygen to diffuse across it, which reduced the amount of oxygen depleted from the sample. This electrode provided accurate measurements of the oxygen concentration in the sample because the electrons flowing between the cathode and anode were proportional to the concentration of oxygen in the sample.

The ISE-730 dissolved oxygen electrode used in this experiment has a Teflon (tm) membrane that permits a limited amount of oxygen to diffuse from the solution being measured to the electrolyte solution inside the removable membrane housing of the electrode (Figure CM-4-B2). The DO2-200 current to voltage adapter, to which the ISE-730 is connected, supplies the ISE-730 electrode with a polarizing voltage that generates a flow of electrons from the platinum cathode to the silver anode. The number of electrons flowing between the cathode and the anode is proportional to the concentration of oxygen inside the membrane housing, which is proportional to the concentration of oxygen in the solution in the reaction chamber.

Changes in the flow of electrons are converted to changes in voltage by the DO2-200 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.
Figure CM-4-B2: The ISE-730 dissolved oxygen electrode, shown with its membrane housing removed. The end of the housing is covered with a Teflon (tm) membrane secured in place by an O-ring. The platinum wire inside the glass tube is the cathode and the silver sleeve that surrounds the glass tube is the anode. Electrons are conducted between the two electrodes by an electrolyte solution that fills the housing.

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.

*Cellular Metabolism – Photosynthesis - Background*
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:

\[ 2H^+ + 2e^- + 2 \text{MV} \rightarrow 2 \text{MVH} \]

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:

\[ 2 \text{MVH} + O_2 \rightarrow 2 \text{MV} + H_2O_2 \]

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_2) 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, students will learn:

- how to calibrate and use an oxygen polarograph;
- how to measure the functionality of isolated thylakoids;
- how to measure electron transport in a complete photosystem;
- how to measure electron transport in a single photosystem (PS I).
Experiment CM-4: Photosynthesis in Isolated Thylakoids

Equipment Required
PC or Mac computer
iWorx/IXTA
USB cable
IXTA power supply
ISE-730 Dissolved oxygen electrode
RPC-100 respiration/photosynthesis chamber
Magnetic stir motor, stir bar, and motor controller
High intensity light source with full intensity control (The Dyna Lume Sun-Lite I is excellent)
Light meter
Vortex mixer
10µl micropipette with gel-loading tips.
Thylakoid suspension (See appendix)
Thylakoid reaction media (See appendix)
Methylamine uncoupler solution (See appendix)
Photosystem I reaction media (See appendix)
Concentrated O₂ depletion solution
Squirt bottle filled with deionized water
Pasteur pipet with plastic tip

IXTA Setup

1. Place the IXTA on the bench, close to the computer.
2. Check Figure T-1-1 in the Tutorial chapter for the location of the USB port and the power socket on the IXTA.
3. Check Figure T-1-2 in the Tutorial chapter for a picture of the IXTA power supply.
4. Use the USB cable to connect the computer to the USB port on the rear panel of the IXTA.
5. Plug the power supply for the IXTA into the electrical outlet. Insert the plug on the end of the power supply cable into the labeled socket on the rear of the IXTA. Use the power switch to turn on the unit. Confirm that the red power light is on.
Start the Software

1. Click on the LabScribe shortcut on the computer’s desktop to open the program. If a shortcut is not available, click on the Windows Start menu, move the cursor to All Programs and then to the listing for iWorx. Select LabScribe from the iWorx submenu. The LabScribe Main window will appear as the program opens.

2. On the Main window, pull down the Settings menu and select Load Group.

3. Locate the folder that contains the settings group, IPLMv4Complete.iwxgrp. Select this group and click Open.

4. Pull down the Settings menu again. Select the Photosynthesis-LS2 settings file from Cellular Metabolism.

5. After a short time, LabScribe will appear on the computer screen as configured by the Photosynthesis-LS2 settings.

6. For your information, the settings used to configure the LabScribe software and the IXTA unit for Experiment CM-4 are programmed on the Preferences Dialog window which can be viewed by selecting Preferences from the Edit menu on the LabScribe Main window.

7. Once the settings file has been loaded, click the Experiment button on the toolbar to open any of the following documents:
   - Appendix
   - Background
   - Labs
   - Setup (opens automatically)

Warning: *The ISE-730 dissolved oxygen electrode has been prepared by the laboratory staff. When you receive your electrode: 1) Handle it carefully. The tip of the electrode is covered by a delicate Teflon (tm) membrane which can tear easily. 2) Do not tighten or loosen the plastic housing holding the Teflon (tm) membrane. Tightening the housing will stretch or tear the membrane; loosening the housing will cause the electrolyte to leak out of the electrode and affect its responsiveness.*

Dissolved Oxygen Electrode Setup

1. Locate the ISE-730 dissolved oxygen electrode ([Figure CM-4-S1](#)) in the iWorx kit.

2. Plug the BNC connector on the ISE-730 dissolved oxygen electrode to the BNC connector on the channel labeled DO2 on the IXTA ([Figure CM-4-S3](#)).

3. Place the small magnetic stir bar in the bottom of the chamber.

4. Fill the chamber with room temperature deionized water.

5. Install the oxygen electrode into its port on the polarograph chamber.

6. 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.
Note: If the solution in the chamber is stirred, changes in oxygen concentration reach the electrode instantaneously. If a stirrer is not used, changes in the rate of oxygen production are limited by the rate of diffusion.

Figure CM-4-S1: The ISE-730 dissolved oxygen electrode.

Figure CM-4-S3: Dissolved oxygen electrode (ISE-730) connected to an IXTA with a male DIN-DIN cable.
Calibration of Dissolved Oxygen Electrode

Aim: To calibrate the dissolved oxygen electrode.

The standard used for calibrating the dissolved oxygen electrode is the known concentration of oxygen in air-saturated deionized water. The amount of oxygen that is dissolved in water is known as its solubility (S) and it is dependent upon the temperature, oxygen pressure in the air, and the concentrations of dissolved solutes in the water. Solubility (S) can be determined by using the following equation:

\[ S = \left( \frac{\alpha}{22.414} \right) \left( \frac{(P-p)}{P} \right) \left( \frac{r\%}{100} \right). \]

In the equation, \( \alpha \) is the absorption coefficient of \( \text{O}_2 \) at the temperature, \( p \) is the vapor pressure of water at the temperature, \( P \) is the barometric pressure, and \( r\% \) is the percent oxygen in the air. For example, at 26°C and 760mmHg and a concentration of oxygen in air of 21%, \( S \) equals:

\[ (0.02783/22.414\text{L/mole})(734.91\text{mmHg}/760\text{mmHg})(0.21) = 252\mu\text{MO}_2 \]

Procedure

1. Fill the RPC-100 respiration chamber with fresh deionized water.
   - Turn off the stirrer if it is on.
   - Remove the ISE-730 dissolved oxygen electrode from the RPC-100 respiration chamber.
• Remove the water from the chamber using a plastic-tipped Pasteur pipet fitted with a bulb.
• Fill the chamber with fresh room temperature deionized water.
• Replace the ISE-730 electrode in its port on the chamber. Make sure there is enough water in the chamber to submerge the tip of the oxygen electrode.
• Turn on the stirrer and adjust its speed so the stir bar is rotating quickly and evenly.

2. Type Saturation-DI Water in the Mark box to the right of the Mark button.

3. Click Record. The recording will eventually reach a stable level near the top of the recording channel. Press the Enter key on the keyboard to mark the recording when the output of the electrode is constant. At this point in the recording, the output of the oxygen electrode is equal to the saturation concentration of oxygen in deionized water at room temperature. Click Stop to halt the recording.

4. Follow the procedure described in Step 1 to replace the deionized water in the RPC-100 chamber with zero-percent oxygen calibration solution at room temperature. Make sure there is enough solution in the chamber to submerge the tip of the oxygen electrode.

5. Type No Oxygen in the Mark box to the right of the Mark button.

6. Click Record. The recording will eventually reach a stable level near the bottom of the recording channel. Press the Enter key on the keyboard to mark the recording when the output of the electrode is constant. At this point in the recording, the output of the oxygen electrode is equal to no oxygen being dissolved in deionized water at room temperature. Click Stop to halt the 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, like your lab group folder). Designate the file type as *.iwxdata. Click Save to save the data file.

8. Prepare the chamber for Exercise 1:
   • Turn off the stirrer, and then remove the ISE-730 electrode from the chamber.
   • Hold the electrode over the beaker used for collecting waste liquid, and rinse the electrode with deionized water from a wash bottle. Blot any drops of water from the electrode. Place the electrode in a beaker of deionized water.
   • Remove the oxygen-depleted water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber ten times with deionized water.
   • Fill the chamber with deionized water. Replace the electrode in the chamber.

Units Conversion

1. Measure the temperature (in °C) in the lab room. Assume the barometric pressure in the lab room is one atmosphere (760mmHg) and the concentration of oxygen in the air is 21%. From Table CM-4-S1, find the dissolved oxygen concentration ([O₂]) in deionized water at room temperature. This concentration will be used in Step 6 to calibrate the dissolved oxygen.
electrode.

2. Scroll to the beginning of the calibration data for the ISE-730 dissolved oxygen electrode.

Table CM-4-S1: Concentration of Oxygen \([\text{O}_2]\) in Air-Saturated Deionized Water at 1 Atmosphere

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>(\text{O}_2) Abs Coeff (a)</th>
<th>(\text{H}_2\text{O}) Vapor Press (p in mmHg)</th>
<th>([\text{O}_2]) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>284</td>
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<td>.03044</td>
<td>18.65</td>
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<td>23</td>
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<td>21.07</td>
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</tr>
<tr>
<td>24</td>
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<td>22.38</td>
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</tr>
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<tr>
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<td>.02608</td>
<td>31.82</td>
<td>234</td>
</tr>
</tbody>
</table>

Figure CM-4-S5: The LabScribe toolbar.

3. Use the Display Time icons on the LabScribe toolbar (Figure CM-4-S5) to adjust the Display Time of the Main window to show the data collected at both the 100% and 0% saturation levels of oxygen in water on the Main window at the same time (Figure CM-4-S6). The required data can also be selected by:
• Placing the cursors on either side of data required.
• Clicking the Zoom between Cursors button on the LabScribe toolbar to expand the data with both the 100% and 0% saturation levels of oxygen in water to the width of the Main window.

4. Click the Double Cursor icon so that two blue cursors appear on the Main window. Place one cursor on the flat section of data collected when the saturation of dissolved oxygen in water was 100% and the second cursor on the flat section of data collected when the saturation of dissolved oxygen in water was 0%.

5. To convert the output of the ISE-730 dissolved oxygen electrode from a voltage to the molarity of dissolved oxygen in a sample:
   • Click on the arrow next to the title of the [Oxygen] channel to open the channel menu.
   • Select Units from the channel menu and Simple from the Units submenu.

6. The Simple Units Calibration window will appear (Figure CM-4-S7). On this window:
   • Select 2 point calibration from the pull-down menu in the upper-left corner of the window.
   • Put a check mark in the box next to Apply units to all blocks.
   • Notice that the voltages from the positions of the cursors are automatically entered into the value equations.
   • From Table CM-4-S1, find the concentration of dissolved oxygen in water at the room temperature that is 100% saturated. Enter this concentration in the corresponding box to the right of the voltage at 100% oxygen. Enter zero in the corresponding box to the right of the voltage for 0% oxygen. Enter the name of the units, μMolar, in box below the concentration. Click on the OK button to activate the units conversion.
Precautions

1. 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.

2. The lights in the room will be turned off during the experiment. It is important to have 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 10µl), disposable gel-loading micropipette tips, a squirt bottle of deionized water, a squirt bottle of 70% ethanol, Pasteur pipettes with soft plastic tubes 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.

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 absorbance 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, where the dilution factor is 100 (2.0 ml/0.020 ml):

\[
\text{mg chl/ml} = (A_{663})(0.00802)+(A_{645})(0.0202)) \times \text{dilution factor}
\]
Experiment CM-4: Photosynthesis in Isolated Thylakoids

Exercise 1: 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 electrode from the chamber and place it in a beaker of deionized water. Remove the deionized water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber two times with deionized water.

2. Fill the chamber with the yellow thylakoid reaction media. Carefully replace the electrode in 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. Remove the electrode and the bubble should burst. Replace the electrode, turn on the stirrer, and check for bubbles, again.

4. Turn off the light source used to illuminate the reaction chamber:
   - If the light has a power switch which is separate from the intensity control, use this switch to turn off the light.
   - If the light does not have a separate power switch, plug the light source into a power strip that has a switch. Use the switch on the power strip to turn off the light.
   - While the light is off, set the intensity control of the illuminator to the maximum level (100%).

5. Make sure no light is reaching the polarograph chamber.

6. Type Thylakoids in Dark in the Mark box to the right of the Mark button. Position the trace near the bottom of the screen. Click the Record button.

7. While 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 µl aliquot of thylakoids from the tube.

8. Press the Enter key on the keyboard to mark the recording as a 10µl aliquot of thylakoids is added to the chamber through the reagent port. 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. Continue recording.

9. Type Thylakoids in Light at 100% in the Mark box. 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. Continue recording.
Note: Put the tip of the micropipette down the reagent port 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 chamber before releasing its plunger. If the plunger is released while the tip of the micropipette is still in the chamber, solutions could be siphoned from the chamber.

10. Type Methylamine Added in the Mark box. Press the Enter key on the keyboard to mark the recording as a 10µl aliquot of methylamine uncoupler solution is added to the chamber through the reagent port. Record the oxygen production of the thylakoids exposed to 100% light in the presence of an uncoupler for one minute. Continue recording.

11. Turn off the light to put the thylakoids in the dark. Record for another minute. Click Stop to halt recording.

12. Select Save in the File menu.

13. Remove the electrode from the chamber, and rinse the electrode with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.

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 µMO₂/sec is considered excellent. If your rates are significantly lower than that value, consult your instructor.

Exercise 2: 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 altered using the intensity control of the light source. As stated in Exercise 1, 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 replace the electrode in the chamber and turn on the stirrer.
5. Check the chamber for the presence of bubbles. Use the same technique described in Exercise 1 to remove any bubbles from the chamber.

6. Make sure no light is reaching the polarograph chamber.

7. Type Whole Chain in Dark in the Mark box to the right of the Mark button. Position the trace near the bottom of the screen. Click the Record button.

8. While 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 µl aliquot of thylakoids from the tube.

9. Press the Enter key on the keyboard to mark the recording as a 10µl aliquot of thylakoids is added to the chamber through the reagent port. 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. Continue recording.

10. Type Whole Chain in 20% Light in the Mark box. 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. Continue recording.

11. Repeat Step 10 for light intensities of 30, 40, 60, 80 and 100%.

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 electrode from the chamber, and rinse the electrode with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.

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. Between Steps 9 and 10:

   - Type Methylamine Added in the Mark box.
   - Press the Enter key on the keyboard to mark the recording as a 10µl aliquot of methylamine uncoupler solution is added to the chamber through the reagent port.
   - Record the oxygen production of the thylakoids exposed to 100% light in the presence of an uncoupler for one minute.
   - Continue recording.

**Exercise 3: 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 2 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 4: Retest the Functionality of the Thylakoids**

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

**Procedure**

1. Repeat Exercise 1 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 1 to that in Exercise 4.
3. Compare the rate of change in oxygen concentration with the uncoupler from Exercise 1 to that from Exercise 4.

**Data Analysis**

**Rate of Electron Transport**

By international consensus, the rate of electron transport in mitochondria is expressed as the rate of oxygen consumption. The units used to express these rates are: moles O₂/hr/mg protein. Oxygen consumption rates are standardized for comparison of experiments performed in different laboratories around the world. So, the size of the reaction chamber, the amount of mitochondrial protein in the chamber, and the time period for that change in oxygen concentration need to be included in the calculation of the rate.

Early in 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 μMolarO₂ and abbreviated μMO₂.
Changes in \( O_2 \) Concentrations in Exercises 1 and 4

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.

Follow these steps to measure the rates of change of the oxygen concentration during these exercises.

1. Scroll through the recording and find the section of data recorded during Exercise 1.

2. Use the Display Time icons to adjust the Display Time of the Main window to show the complete experimental run on the Main window. The complete experimental run can also be selected by:
   - Placing the cursors on either side of the complete run; and
   - Clicking the Zoom between Cursors button on the LabScribe toolbar to expand the complete experimental run to the width of the Main window.

3. Click on the Analysis window icon in the toolbar or select Analysis from the Windows menu to transfer the data displayed in the Main window to the Analysis window.

4. Look at the Function Table that is above the Oxygen Concentration channel displayed in the Analysis window. The functions, \( V2-V1 \), \( T2-T1 \), and Mean \( dv/dt \) should appear in the table.

5. Once the cursors are placed in the correct positions for determining the change in the oxygen concentration in the chamber, the values of the parameters in the Function Table can be recorded in the on-line notebook of LabScribe by typing their names and values directly into the Journal.

6. The functions in the channel pull-down menus of the Analysis window can also be used to enter the names and values of the parameters from the recording to the Journal. To use these functions:
   - Place the cursors at the locations used to measure the change in oxygen concentration.
   - Transfer the names of the mathematical functions used to determine the change in oxygen concentration to the Journal using the Add Title to Journal function in the Lung Volumes Channel pull-down menu.
   - Transfer the values for the change in oxygen concentration to the Journal using the Add Ch. Data to Journal function in the Oxygen concentration channel pull-down menu.

7. In the Analysis window, use the mouse to click on and drag the cursors to specific points on the recording to measure the following rates:
   - Dark rate, which is the rate of oxygen production by the thylakoids in the absence of light and uncoupler. Set the cursors ten seconds apart on a linear section of the recording of the dark rate. The value for the Mean \( dv/dt \) is the average rate of change of oxygen concentration (\( \mu \)Molar/sec) over that ten-second period.
   - 100% Light rate, which is the rate of oxygen production by the thylakoids in the presence of full light. Set the cursors ten seconds apart on a linear section of the recording of this rate. The value for the Mean \( dv/dt \) is the average rate of change of oxygen concentration (\( \mu \)Molar/sec) over that ten-second period.
- 100% Light with Uncoupler rate, which is the rate of oxygen production by the thylakoids in the presence of full light and methylamine. Set the cursors ten seconds apart on a linear section of the recording of this rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration (µMolar/sec) over that ten-second period.

- Dark and Uncoupler rate, which is the rate of oxygen production by the thylakoids in the presence of Methylamine, but with no light. Set the cursors ten seconds apart on a linear section of the recording of this rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration (µMolar/sec) over that ten-second period.

8. Record the values for these four rates in the Journal using the one of the techniques described in Steps 5 or 6.

9. Use the same technique described in the previous steps to measure and record these four rates from Exercise 4.

Figure CM-4-L1: 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 0.331010 µMolar O₂/sec.
Changes in $O_2$ Concentrations in Exercises 2 and 3

In Exercises 2 and 3, 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. Scroll through the recording and find the section of data recorded during the first experimental run in Exercise 2, the oxygen production rates with different light intensities in the absence of an uncoupler.

2. Use the same techniques described for the analysis of Exercises 1 and 4 to display the complete experimental run on the Main window, transfer the data to the Analysis window, selecting the correct positions of the cursors, and transferring the measurements to the Journal.

3. In the Analysis window, use the mouse to click on and drag the cursors to specific points on the recording to measure the following rates:
   - Whole Chain in Dark rate, which is the rate of oxygen production by the thylakoids in the absence of light. Set the cursors ten seconds apart on a linear section of the recording of the endogenous rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration ($\mu$Molar/sec) over that ten-second period.
   - Whole Chain in Different Light rates, which are the rates of oxygen production by the thylakoids in the presence of 20, 30, 40, 60, 80, and 100% of the full light intensity. Set the cursors ten seconds apart on each linear section of each recording of these rates, as performed in other rate measurements. The value for the Mean_dv/dt at each light intensity is the average rate of change of oxygen concentration ($\mu$Molar/sec) over that ten-second period at each light intensity.
   - Whole Chain Returned to Dark rate, which is the rate of oxygen production by the thylakoids in the absence of light at the end of the experimental run. Set the cursors ten seconds apart on a linear section of the recording of this rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration ($\mu$Molar/sec) over that ten-second period.

4. Repeat Steps 1 through 3 for the second experimental run of Exercise 2, which measured the effects of light intensity on whole-chain electron transport in the presence of an uncoupler.

5. Repeat Steps 1 through 3 for the two experimental runs of Exercise 3, which measured the effects of light intensity on PS I electron transport in the absence and the presence of an uncoupler.

Note: Remember that in Exercise 3, the rates of oxygen consumption are measured when Photosystem I is studied.

6. Make sure all the changes in oxygen concentration are recorded in the Journal:
   - At all the intensities of illumination;
   - With or without an uncoupler;
   - In the whole electron transport chain or in PS I.
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$_2$ ($\mu$MO$_2$ or $10^{-6}$ moles/liter) per second, and the polarograph chamber has a 1.2 milliliter (ml) capacity; then 0.396 nanomoles (nmoles or $10^{-9}$ moles) of oxygen are produced in one second:

   \[(0.333\mu$MO$_2$/sec)(1.2ml) = 0.396 nmoles O$_2$ produced in one second.\]

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$_2$ in one second, the hourly production rate is 1.44 micromoles O$_2$ ($\mu$moles O$_2$) per hour:

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

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.

4. 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.

5. 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 µl 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:

\[(1.44 \times 10^{-6} \text{ moles O}_2/\text{hr})/(0.025 \text{ mg chl}) = 57.6 \times 10^{-6} \text{ moles O}_2/\text{hr/mg chl} = 57.6 \mu\text{moles O}_2/\text{hr/mg chl}.\]
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 1 and 4 in Table CM-4-L1, for Exercise 2 in Table CM-4-L2, and for Exercise 3 in Table CM-4-L3. 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. The dark rate is adding itself to the actual rate.
   - 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. The dark rate is subtracting itself from 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 µmoles O₂/hr/mg chl in the dark and produced oxygen at the rate of 57.6 µmoles O₂/hr/mg chl in 100% light, their actual rate of light-induced oxygen production is:
   \[ (57.6 \text{ µmoles O}_2/\text{hr/mg chl}) - (-9.6\text{ µmoles O}_2/\text{hr/mg chl}) = 67.2 \text{ µmoles O}_2/\text{hr/mg chl} \]

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 data tables.

**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 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 CM-4-L13: Exercises 1 and 4: Thylakoid Functionality; Oxygen (O₂) Production Rates expressed as 10⁻⁶ moles O₂/hr/mg chlorophyll.

<table>
<thead>
<tr>
<th>Light Conditions</th>
<th>Exercise 1 (Beginning)</th>
<th>Exercise 4 (End)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thylakoids in Dark</td>
<td>Recorded</td>
<td>Actual</td>
</tr>
<tr>
<td>Thylakoids in 100% Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 100% Light with Uncoupler</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table CM-4-4: Exercise 2: Thylakoids with Whole Chain Electron Transport. Oxygen (O₂) Production Rates expressed as 10⁻⁶ moles O₂/hr/mg chlorophyll.

<table>
<thead>
<tr>
<th>Light Conditions</th>
<th>Without Uncoupler</th>
<th>With Uncoupler</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recorded</td>
<td>Actual</td>
</tr>
<tr>
<td>Thylakoids in Dark</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thylakoids in 20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 30%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 40%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 60%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table CM-4-5: Exercise 3: Thylakoids with Electron Transport through PS I only. Oxygen (O$_2$) Production Rates expressed as 10$^{-6}$ moles O$_2$/hr/mg chlorophyll.

<table>
<thead>
<tr>
<th>Light Conditions</th>
<th>Without Uncoupler</th>
<th>With Uncoupler</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recorded</td>
<td>Actual</td>
</tr>
<tr>
<td>Thylakoids in Dark</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thylakoids in 20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 30%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 40%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 60%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thylakoids in 100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Experiment CM-4: Photosynthesis in Isolated Thylakoids

Appendix I: Recipes for Solutions Used in the Experiment:

**Thylakoid Isolation Buffer**

<table>
<thead>
<tr>
<th>Concentration (mMolar)</th>
<th>Chemical</th>
<th>Grams/Liter DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Tricine</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>Sucrose</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>NaCl</td>
<td></td>
</tr>
</tbody>
</table>

Mix all components in 925 ml of deionized water until dissolved. Adjust pH to 7.4-7.5. Add deionized water to bring final volume to 1L. Store in 200ml aliquots in foil wrapped bottles at 4°C. Need about 500 mls per lab period.

**Thylakoid Reaction Buffer**

<table>
<thead>
<tr>
<th>Concentration (mMolar)</th>
<th>Chemical</th>
<th>Grams/Liter DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Tricine</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂·6H₂O</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>K₃Fe(CN)₆ KFerricyanide</td>
<td></td>
</tr>
</tbody>
</table>

Mix all components in 925 ml of deionized water until dissolved. Adjust pH to 7.5. Add deionized water to bring final volume to 1L. Store in 200ml aliquots in foil wrapped bottles at 4°C. Need about 250 mls per lab period.

**Methyamine Uncoupler**

<table>
<thead>
<tr>
<th>Concentration (Molar)</th>
<th>Chemical</th>
<th>Grams/100ml DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>Methylamine</td>
<td>20.26</td>
</tr>
</tbody>
</table>

Dissolve in 100 mls of deionized water. Dispense 0.1 ml per group in capped tubes.
**Photosystem I Reaction Buffer**

<table>
<thead>
<tr>
<th>Concentration (Molar)</th>
<th>Chemical</th>
<th>Mls of Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Tricine Stock Solution</td>
<td>10</td>
</tr>
<tr>
<td>0.01</td>
<td>Methyl Viologen Stock Sln.</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>KCN Stock Solution</td>
<td>1</td>
</tr>
<tr>
<td>0.0001</td>
<td>DCMU Stock Solution</td>
<td>1</td>
</tr>
<tr>
<td>0.001</td>
<td>DPIP Stock solution</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Deionized Water</td>
<td>64</td>
</tr>
</tbody>
</table>

Add 0.04g of sodium Ascorbate to the buffer. Make fresh just before each lab period. Use within 5 hours.

**Tricine Stock Solution**

<table>
<thead>
<tr>
<th>Concentration (Molar)</th>
<th>Chemical</th>
<th>Grams/100ml DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Tricine</td>
<td>8.96</td>
</tr>
</tbody>
</table>

Dissolve in 90 mls of deionized water. Adjust pH to 7.5, Add deionized water to bring to final volume of 100ml. Store at 4°C.

**Methyl Viologen Stock Solution**

<table>
<thead>
<tr>
<th>Concentration (mMolar)</th>
<th>Chemical</th>
<th>Grams/100ml DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Methyl Viologen</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Dissolve in 100 mls of deionized water. Store at 4°C.

**Potassium Cyanide (KCN) Stock Solution**

<table>
<thead>
<tr>
<th>Concentration (mMolar)</th>
<th>Chemical</th>
<th>Grams/50ml DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Potassium Cyanide</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Dissolve in 50 mls of deionized water. Store at 4°C.
DCMU Stock Solution

<table>
<thead>
<tr>
<th>Concentration (mMolar)</th>
<th>Chemical</th>
<th>Grams/50ml 70% EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>DCMU</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

Dissolve in 50 mls of 70% Ethanol. Freeze 25 mls in 1 ml aliquots in capped tubes. Store the remainder at 4°C.

DPIP Stock Solution

<table>
<thead>
<tr>
<th>Concentration (mMolar)</th>
<th>Chemical</th>
<th>Grams/50ml DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DPIP</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Dissolve in 50 mls of deionized water. Store at 4°C.

Zero-Percent Oxygen Calibration Solution

<table>
<thead>
<tr>
<th>Concentration (Molar)</th>
<th>Chemical</th>
<th>Grams/50ml DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>Sodium Hydrosulfite</td>
<td>13</td>
</tr>
</tbody>
</table>

Dissolve in 50 mls of deionized water. Store at 4°C.

Appendix II: DO2-200 Current to Voltage Adapter

Note: This is used with the IWX/214 only.

The DO2-200 is a current to voltage adapter designed to work with a Clark-style oxygen electrode. This adapter delivers a polarizing voltage of -0.8V 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 DO2-200 is 10mV 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.
1. If using the DO2-200 current to voltage adapter, plug a DIN-DIN extension cable into the back of the DO2-200. Plug the other end of this cable into channel 3 on the IWX-214.

2. Connect the BNC connector from the ISO-730 dissolved oxygen electrode into the front of the DO2-200.

3. Follow the procedures in the main body of the lab exercises.

Appendix III: ISE-730 Dissolved Oxygen Electrode

Electrode Assembly and Preparation

The ISE-730 dissolved oxygen electrode (Figure CM-4-A2) is shipped with a membrane housing covering the glass electrode tip. The end of the housing is covered with a Teflon™ membrane. If the electrode is unused, or has been stored for a long period according to the directions provided in this set of instructions, the membrane housing does not contain the electrolyte solution needed to make the electrode work.

Warning: When removing and replacing a membrane, calibrating the electrode, or measuring oxygen concentrations, do not apply pressure against the internal electrode. Any excessive pressure against the internal electrode can cause the electrode to crack rendering it useless and unrepairable.
1. To add electrolyte solution to the membrane housing, or to check on the level of electrolyte solution in the housing, carefully unscrew the membrane housing from the body of the oxygen electrode.

2. If the membrane housing contains electrolyte solution, check the level of the solution in this housing. The minimum height of electrolyte in the housing should be 6 mm, which will make the housing about one half full. When the housing is placed over glass element of the electrode, the solution will be high enough to make contact with the silver anode that surrounds the glass electrode tip.

3. If electrolyte solution needs to be added to the membrane housing, push the filling tube provided in the kit into the opening in the nipple of the bottle of electrolyte solution. Then, fill the housing with electrolyte to the minimum height. To fill the membrane housing without adding bubbles, squeeze the bottle of electrolyte until there is no air in the filling tube; then, gently place the bubble-free end of the filling tube near the inside of the Teflon™ membrane and add electrolyte until its level reaches the minimum height. If an air bubble is trapped near the inside Teflon membrane, gently tap the side of the membrane housing to loosen the bubble.

4. Carefully insert the electrode tip into the membrane housing. Do not trap any air bubbles near the electrode tip and inside the Teflon membrane. Slowly screw the membrane housing onto the body of the electrode to prevent the creation of pressure that could stretch the Teflon membrane. Stop screwing the membrane housing onto the body of the electrode when the housing makes contact with O-ring above the threads on the body of the electrode. The O-ring should be visible in the gap between the membrane housing and the body of the electrode (Figure CM-4-A3).

Warning: Screwing the membrane housing onto the body of the electrode too quickly or too far can stretch or break the membrane and prevent the electrode from working properly.

5. Check the end of the assembled electrode to make sure the membrane housing is seated properly against the tip of the electrode. If the housing is seated properly, the tip of the glass electrode protrudes only slightly beyond the end of the housing.
Handling
When necessary, the membrane of the electrode can be replaced by following the assembly and preparation procedure described above.

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.) for a couple of minutes, after each use, to remove the protein from the surface of the membrane. This cleaning will prolong the useful life of the membrane.

Storing
1. Always rinse or clean the electrode before storing.
2. For long-term storage (periods 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 dust off of the electrode tip.
3. For short-term storage, the electrode can be left in room air with the filled membrane housing still attached.

Problem Solving
1. When there is little or no response from the electrode:
   • Remove the membrane housing from the electrode and inspect the internal electrode for cracks. If the internal electrode is cracked, it cannot be repaired and the complete electrode must be replaced.
   • Examine the membrane housing and determine if it contains the appropriate amount of electrolyte solution. An insufficient amount of electrolyte solution in the housing can be caused by a hole in the Teflon membrane. Replace the membrane housing with a new one. Fill the housing with the appropriate amount of electrolyte and install the housing by following the assembly and preparation procedure described above.
2. When there is a sluggish response from electrode:
   • Clean the surface of the membrane.
   • Replace the membrane housing with a new one.
   • Polish the face of the internal electrode and replace the membrane housing with a new one.
3. To repolish the face of the internal electrode:
   - Remove the membrane housing and rinse the internal electrode with deionized water.
   - Place a piece of polishing paper, or 3200-grit wet/dry sandpaper, on a flat surface.
   - Place a couple of drops of deionized water on the polishing paper.
   - Hold the glass portion of the internal electrode firmly between the thumb and forefinger, with the tip of the glass electrode perpendicular to the polishing paper.
   - Polish the tip of the electrode in the wet area of the polishing paper using circular motions with very light pressure. About 10 circles are all that is required to polish the tip.
   - Rinse the electrode thoroughly with deionized water.
   - Assemble the electrode using a new membrane housing.