Experiment CM-4: Photosynthesis in Isolated Thylakoids

Equipment Required

PC or Mac computer

IXTA, USB cable, 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.

See Appendix:

- Thylakoid suspension
- Thylakoid reaction media
- Methylamine uncoupler solution
- Photosystem I reaction media
- Concentrated O_2 depletion solution

Squirt bottle filled with deionized water

Pasteur pipet with plastic tip

Warning: The 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 dissolved oxygen electrode and plug it into channel A5 on the IXTA.
2. Place the small magnetic stir bar in the bottom of the chamber and fill the chamber with room temperature deionized water.
3. Install the oxygen electrode into its port on the polarograph chamber.
4. 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: Dissolved oxygen electrode connected to an IXTA.

Figure CM-4-S2:The oxygen polarograph used to record photosynthetic electron transport. Black tape should cover the side of the polarograph block facing the light source, except on the area directly on the 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.

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:

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

In the equation, α is the absorption coefficient of 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 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 DO2 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 the its speed so the stir bar is rotating quickly and evenly.
2. Type **Saturation-DI Water** in the Mark box.
3. Click Record. The recording will eventually reach a stable level near the top of the recording channel. Click the mark button 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.
6. Click Record. The recording will eventually reach a stable level near the bottom of the recording channel. Click the mark button 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. Click Save to save the data file.
8. Prepare the chamber for Exercise 1:
    - Turn off the stirrer, and then remove the 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 ^{o}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 1 find the dissolved oxygen concentration ([O_2]) 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 dissolved oxygen electrode.

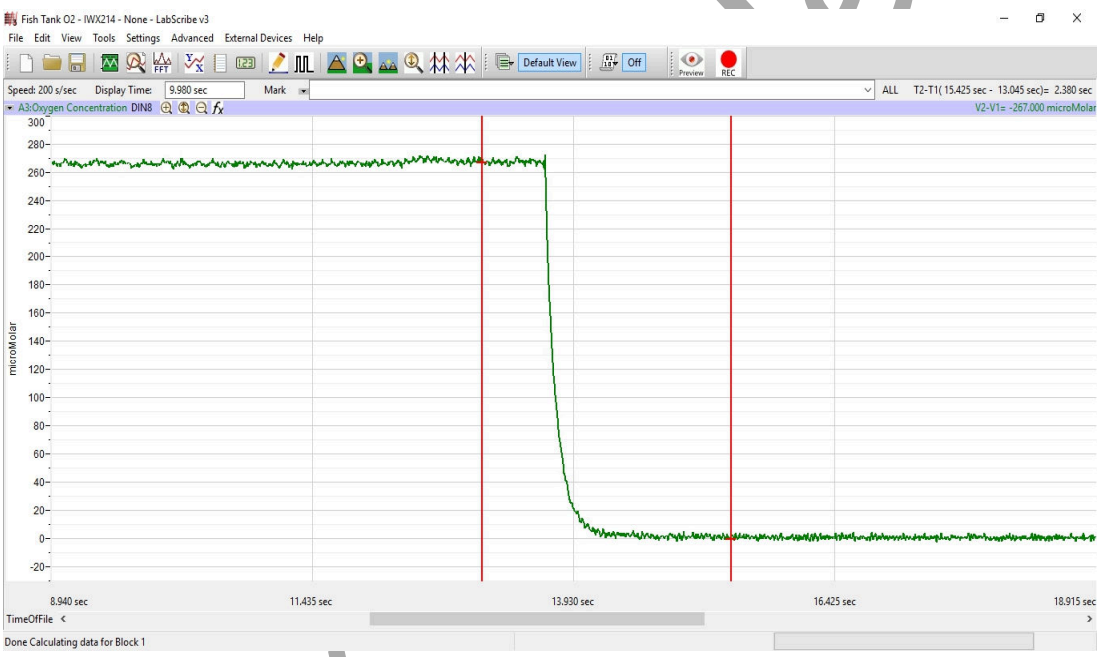

Figure CM-4-S3: Recording of oxygen concentrations in air saturated and oxygen depleted deionized waters used to convert the units of the Y-axis from voltage to O_2 concentration (µMolar).

3. Use the Display Time icons on the LabScribe toolbar 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. 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 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 dissolved oxygen electrode from a voltage to the molarity of dissolved oxygen in a sample:
    - Click V2-V1 on the right of the Oxygen channel.
    - Select Simple.
6. The Simple Units Calibration window will appear. 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 1, 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.

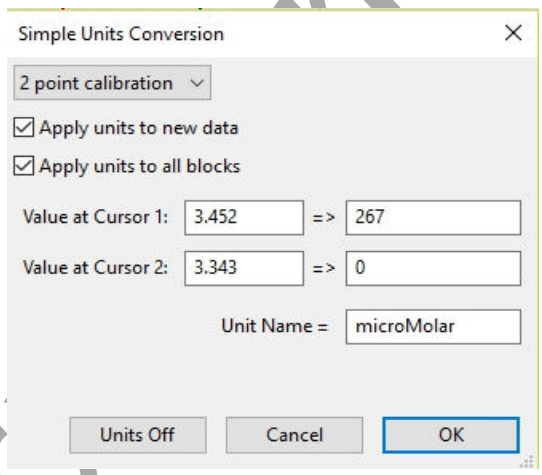

Figure CM-4-S4: The Simple Units Conversion dialogue window with the voltages at the cursors set to equal the dissolved oxygen concentrations used in calibration.

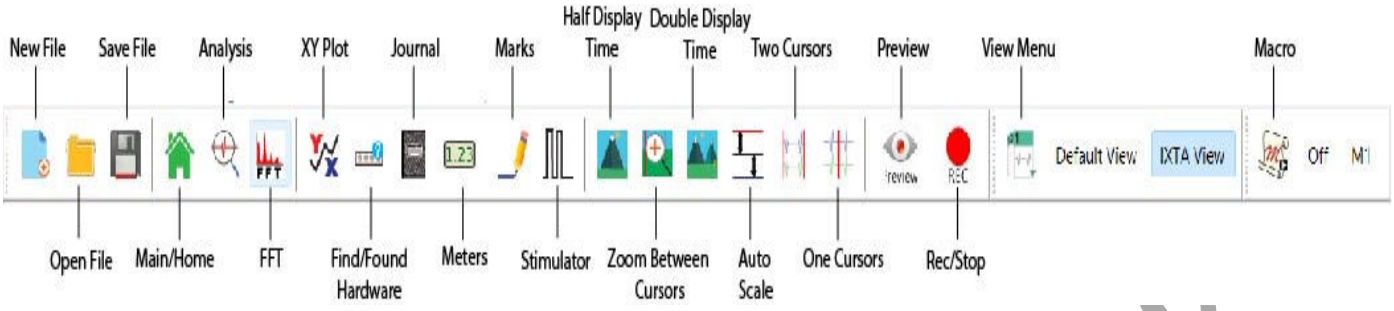

Figure CM-4-S5: The LabScribe toolbar.

Table CM-4-S1: Concentration of Oxygen [O2] in Air-Saturated Deionized Water at 1 Atmosphere

Temp (°C)	O_2 Abs Coeff (a)	H_2O Vapor Press (p in mmHg)	[O2] (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

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^{o}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.

Approximate Time: 15 minutes

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. 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. Click the mark button to mark the recording as a 10 µl 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. Click the mark button 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. Click the mark button 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 μMO_2/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.

Approximate Time: 20 minutes

The same reaction media used in Exercise 1 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 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. 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. Click the mark button 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. Click the mark button 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.
    - Click the mark button 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 for one minute
    - Click Stop and Save your data file.

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.

Approximate Time: 20 minutes

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.

Approximate Time: 20 minutes

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 O_2/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 $\mu MolarO_2$ and abbreviated μMO_2.

Changes in 0_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 (µMolar/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 (µMolar/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.

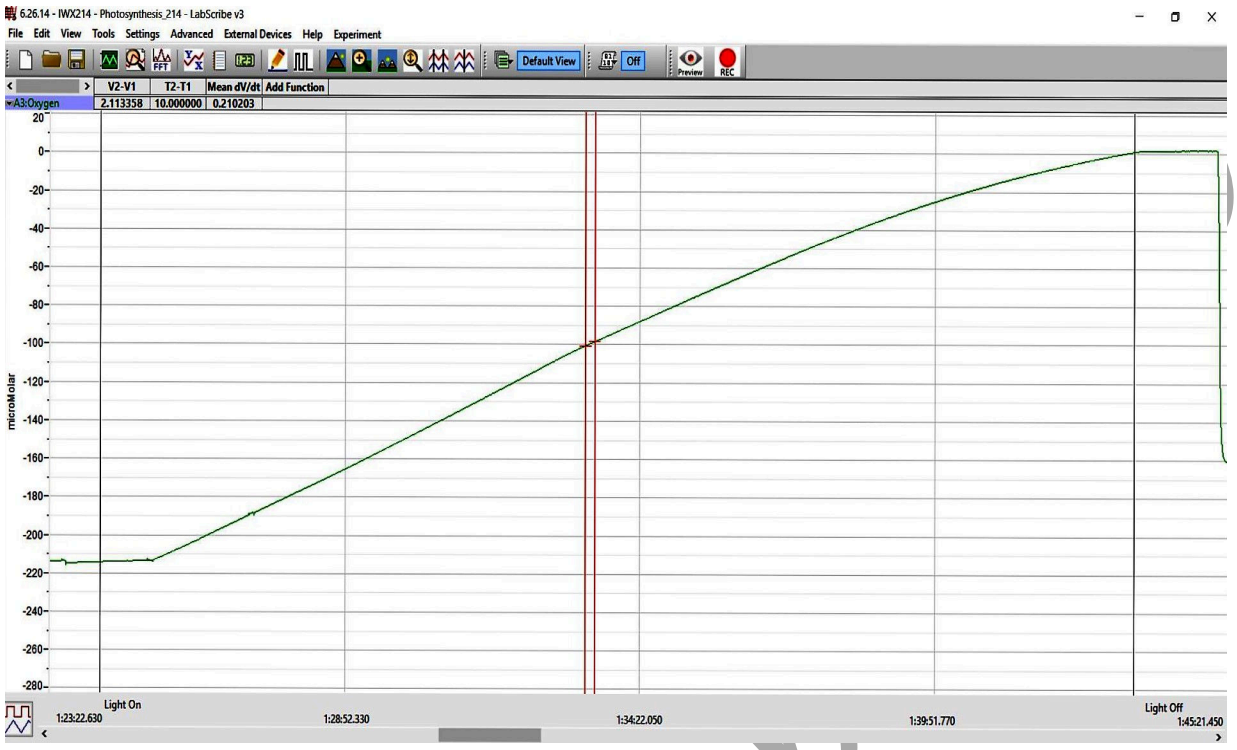

Figure CM-4-L1: Change in oxygen concentration in the polarograph chamber created by thylakoids with whole electron transport chains exposed to 80% light in the presence of an uncoupler. As displayed in the Analysis window, the marks indicate the times when the shutter controlling the light was opened and closed. Cursors are 10 seconds apart. The average rate of change (Mean_dv/dt) in the oxygen concentration over that period is the 0.210203 µMolar O_2/sec.

Changes in 0_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 (µMolar/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 (µMolar/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 (µMolar/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 0.210 microMolar O_2 (µMO_2 or 10^{-6} moles/liter) per second, and the polarograph chamber has a 1.2 milliliter (ml) capacity; then 0.252 nanomoles (nmoles or 10^{-9} moles) of oxygen are produced in one second:

    $(0.210\mu MO_2/sec)(1.2ml) = 0.252$ 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 0.252 nmoles O_2 in one second, the hourly production rate is 907 micromoles O_2 (µmoles O_2) per hour:

$(0.252 \times 10^{-9}$ molesO_2/sec)(60sec/min)(60min/hr) = 907×10^{-9}moles O_2/hr = 0.907×10^{-6} molesO_2/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 mg chl/ml)(0.010 ml) = 0.025 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:

$(0.907 \times 10^{-6}$ molesO_2/hr)/(0.025 mg chl) $=22.7 \times 10^{-6}$ molesO_2/hr/mg chl = 22.7 µmolesO_2/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 1, for Exercise 2 in Table 2, and for Exercise 3 in Table 3. 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 O_2/hr/mg chl in the dark and produced oxygen at the rate of 22.7 µmoles O_2/hr/mg chl in 100% light, their actual rate of light-induced oxygen production is:

   (22.7 µmoles O2/hr/mg chl) - (-9.6µmoles O2/hr/mg chl) = 32.3 µmoles O_2/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 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 CM-4-L13: Exercises 1 and 4: Thylakoid Functionality; Oxygen (O_2) Production Rates expressed as 10^{-6} moles O_2/hr/mg chlorophyll.

	Exercise 1 (Beginning)		Exercise 4 (End)	
Light Conditions	Recorded	Actual	Recorded	Actual
Thylakoids in Dark		0		0
Thylakoids in 100% Light				
Thylakoids in 100% Light with Uncoupler				

Table CM-4-4: Exercise 2: Thylakoids with Whole Chain Electron Transport. Oxygen (O_2) Production Rates expressed as 10^{-6} moles O_2/hr/mg chlorophyll.

	Without Uncoupler		With Uncoupler	
Light Conditions	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 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.

	Without Uncoupler		With Uncoupler	
Light Conditions	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%				

Isolation of Thylakoids – also shown in the Setup Document

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 (4ºC) 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):

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