

Experiment 13: Carbon Dioxide Fixation in Intact Cells

Introduction

The photosynthetic reactions, that place in cells or organelles capable of the process, are grouped into two categories:

- The light-driven reactions of the photosynthetic electron transport chain (Figure 2-1 on page 2) that transform light energy into chemical energy in the form of the compounds, ATP and NADPH, which are utilized in the second category of reactions.
- The reactions of the Calvin Cycle (Figure 2-2 on page 2) that utilize the high energy compounds produced by the light-driven reactions to produce the sugars used as the energy source for other reactions in the plant cells.

The light-driven reactions of photosynthesis can be studied in another experimental protocol available from iWorx, Photosynthesis in Isolated Thylakoids. In that protocol, oxygen production and consumption are used to measure the flow of electrons through the molecules imbedded in the thylakoid membrane. These molecules are responsible for producing the high energy compounds, ATP and NADPH, which are used in the reactions of the Calvin Cycle

In today's experimental protocol, students will also use an oxygen polarograph to measure oxygen production of the green algae, Chlorella vulgaris. The amount of oxygen produced by these intact cells will be used as the measure of the amount of carbon dioxide consumed, or fixed, during the production of sugars in the Calvin Cycle.

Calvin Cycle

The ATP and NADPH produced during the light-driven reactions of photosynthesis are utilized in the series of enzymatic reactions, known as the Calvin Cycle, which are responsible for the formation of sugars from the fixation of carbon dioxide. In this cycle, ATP is the source of energy used to drive the reactions, and NADPH provides the reducing power for the attachment of electrons to carbon molecules that become sugars. In chloroplasts, the reactions of the Calvin Cycle take place in the stroma.

To produce a six-carbon sugar molecule, like glucose, six turns of the Calvin Cycle must occur. During each turn of the cycle, one molecule of carbon dioxide proceeds through a three-phase process that consumes 3 molecules of ATP and 2 molecules of NADPH. Ultimately the cost of producing a six-carbon sugar is 18 molecules of ATP, 12 molecules of NADPH, and 6 molecules of CO₂.

The three phases in the Calvin Cycle are:

- Phase 1: Carbon fixation. Carbon dioxide (CO₂) bonds to a five-carbon sugar named ribulose biphosphate (RuBP) through the actions of the enzyme, ribulose biphosphate carboxylase/oxygenase (Rubisco). Rubisco is the most abundant protein in chloroplasts and probably the most abundant protein on Earth. The product of this reaction is a six-carbon intermediate sugar, which immediately splits in half to form two molecules of 3-phosphoglycerate (3-PG).
- Phase 2: Reduction. ATP phosphorylates 3-phosphoglycerate (3-PG) to 1,3-biphosphoglycerate. NADPH₂ reduces 1,3-biphosphoglycerate to glyceraldehyde 3-phosphate (G3P), which is a simple three-carbon sugar that is eventually assembled into glucose and other sugars. For every three CO₂ molecules fixed, one molecule of G3P is incorporated into glucose, and the five remaining molecules of G3P are used in Phase 3 of the Calvin Cycle.
- Phase 3: Regeneration. Additional ATP molecules provide energy to convert glyceraldehyde 3-phosphate (G3P) molecules into ribulose biphosphate (RuBP). For every five molecules of glyceraldehyde 3-phosphate generated by Phase 2, three molecules of RuBP are formed. Each RuBP is ready to receive a new CO₂ molecule and begin another rotation of the Calvin Cycle.

Although the reactions of the Calvin Cycle have been referred to as the "dark reactions", they can take place in either the dark or the light. The Calvin Cycle is not directly dependent on light, but the cycle is dependent on the products of the "light reactions", ATP and NADPH. If a photosynthetic cell is in the dark, the Calvin Cycle in the cell will continue until the ATP and NADPH in the cell is exhausted. The Calvin Cycle in the cell cannot resume until more ATP and NADPH are generated by the light reactions in the cell.

Stoichiometry

During photosynthesis in intact cells, water is the ultimate source of the electrons that are driven through the photosynthetic electron transport chain by light energy (Figure 2-1 on page 2). The molecules in the chain use the energy released during the transfer of electrons down the chain to produce ATP and NADPH. During the release of electrons (e⁻) and protons (H⁺) from water, oxygen is also produced. The production of oxygen, as measured by a device like an oxygen polarograph, can be used to a measure of the production of electrons, ATP, and NADPH.

In intact photosynthetic cells, oxygen production can also be used to measure the carbon dioxide fixed into sugars because a 1:1 stoichiometric relationship exists between the amount of oxygen produced at one end of the photosynthetic process and the amount of carbon dioxide consumed at the other end. To insure the process occurs, the suspension of algae cells is supplemented with carbon dioxide, in the form of bicarbonate (HCO₃⁻), at the beginning of each experiment.

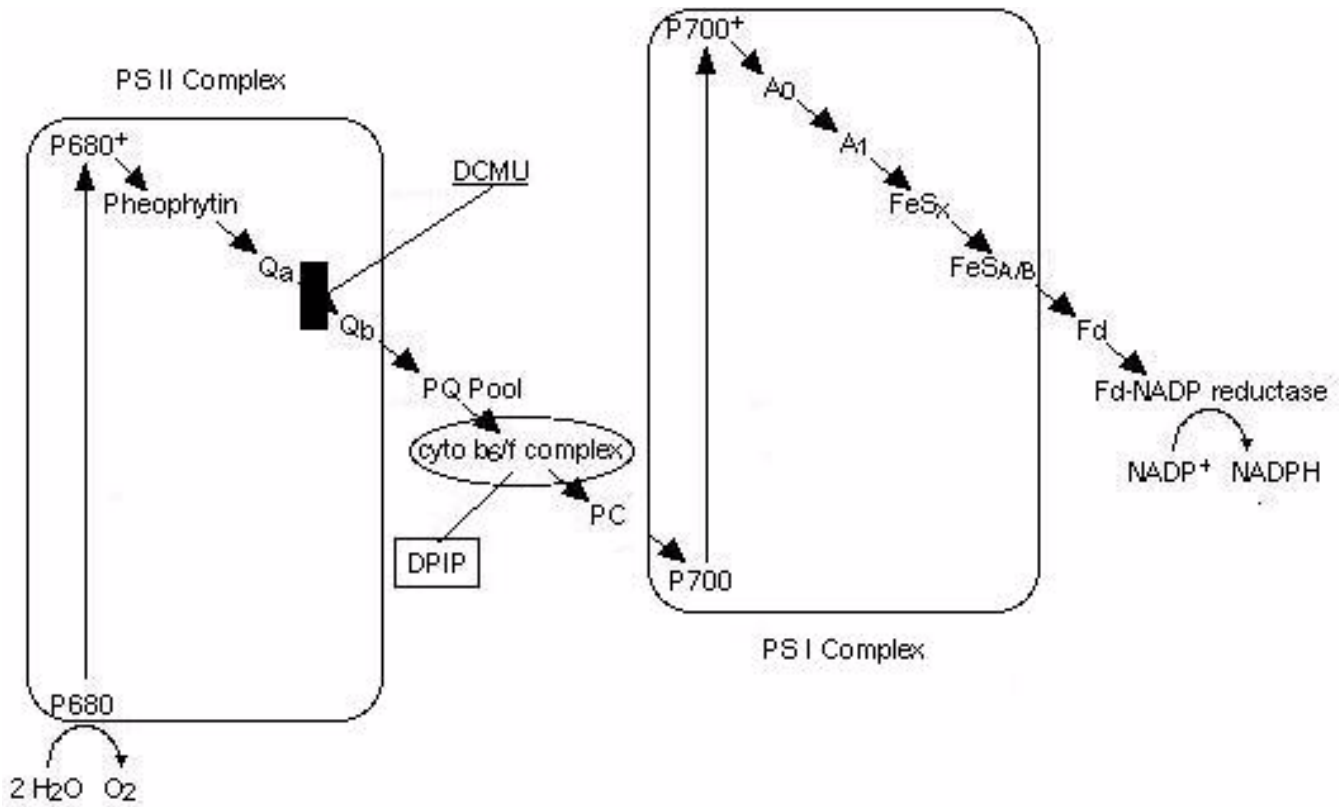


Figure 2-1: The electron transport chain used in the light reactions of photosynthesis.

The Calvin Cycle

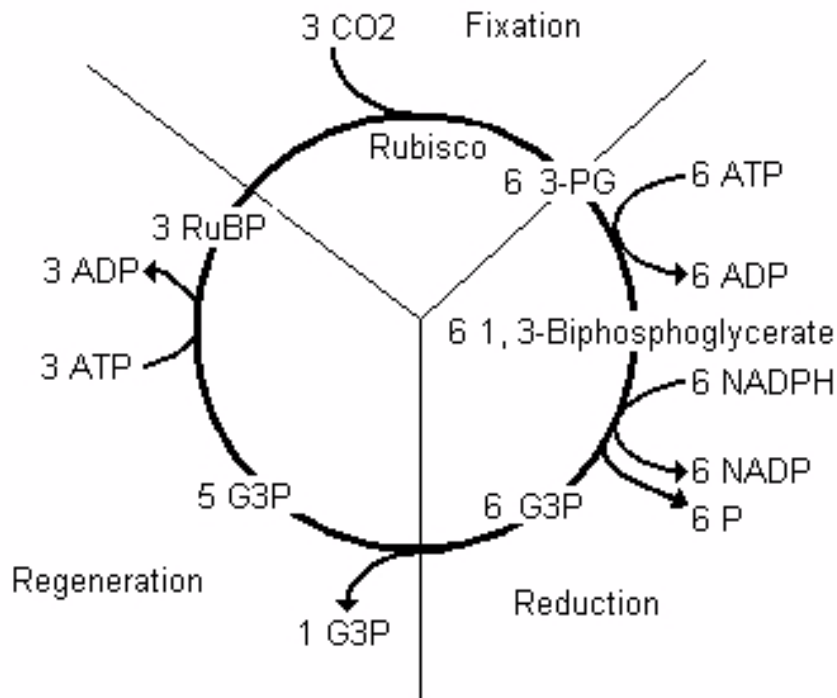


Figure 2-2: The Calvin Cycle used for the fixation of carbon dioxide.

Oxygen Polarograph

A simple device that can be used to measure oxygen production or consumption, and in this experiment, carbon dioxide fixation, is an oxygen polarograph.

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

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

Oxygen Measurements

In this experiment, you will perform 3 exercises that examine how three compounds affect the rate of carbon dioxide fixation in intact cells. The compounds are:

- Iodoacetamide (IAA), which inhibits certain enzymes of the Calvin cycle, but should have no effect on photosynthetic electron transport.
- 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks electron transport between the Q_a and Q_b quinones in the chain.
- Methylamine (MA), which should increase the rate of electron transport by uncoupling ATP synthesis from electron transport.

This experiment also contains three exercises that measure photosynthetic electron transport in thylakoids with the same three compounds. This permits the results from intact cells to be compared to the results obtained from thylakoids that do not have the enzymes of the Calvin Cycle.

Equipment Required

- PC computer
- iWorx/214 and USB cable
- Oxygen electrode
- Current to voltage adapter
- Plexiglas™ respiration/photosynthesis chamber
- Magnetic stir motor, stir bar, and motor controller
- High intensity light source with full intensity control (The Dyna Lume Sun-Lite I is excellent)
- Light meter
- Vortex mixer
- 10µl micropipette with gel-loading tips.
- Algal cells in suspension (See appendix)
- HCO₃⁻ solution (See appendix)
- MA (methylamine) uncoupler solution (See appendix)
- IAA (iodoacetamide) solution (See appendix)
- DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) solution (See appendix)
- Thylakoid suspension (See appendix)
- Thylakoid reaction media (See appendix)
- Concentrated O₂ depletion solution (1.5 M Sodium Dithionite)
- Squirt bottle filled with deionized water
- Pasteur pipet with plastic tip

Equipment Setup

- 1 Connect the iWorx unit to the computer (described in Chapter 1).
- 2 Plug one end of the DIN-DIN cable into Channel 3 on the iWorx unit. Plug the other end of this cable into the DIN connector on the DO2-100 current to voltage adapter (Figure 2-3 on page 4).
- 3 Attach the cable of the oxygen electrode to the BNC connector on the current to voltage adapter.
- 4 Place the small magnetic stir bar in the bottom of the chamber.
- 5 Fill the chamber with room temperature deionized water.
- 6 Install the oxygen electrode into its port on the polarograph chamber.
- 7 Plug the high intensity light source into the AC outlet. Align the light bulb so that it is about 2" from the side of the polarograph chamber (Figure 2-4 on page 4).
- 8 Turn on the light and adjust its beam to cover the open area on the side of the polarograph chamber.

- 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.

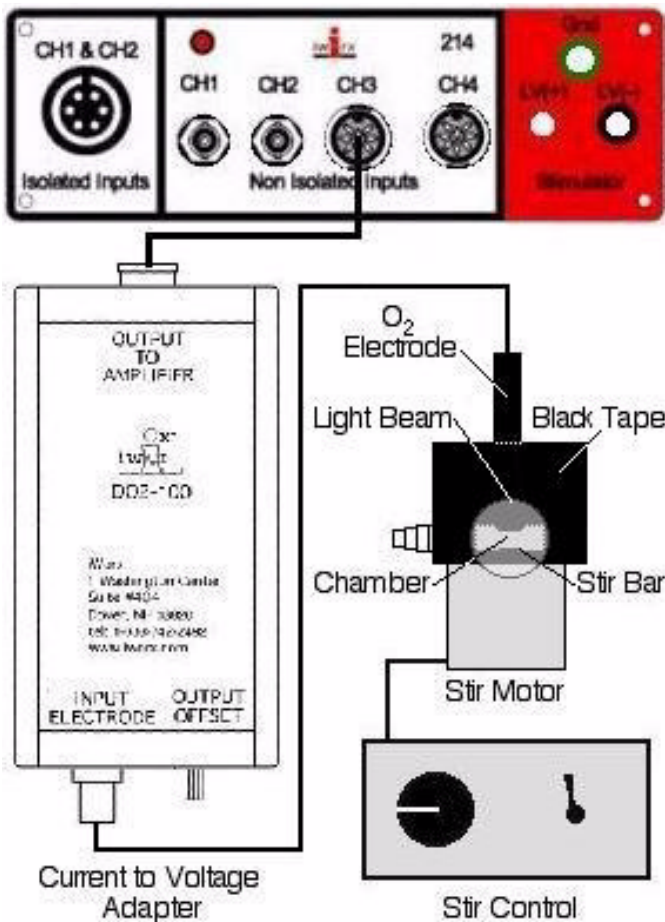


Figure 2-3: The oxygen polarograph used to record photosynthetic electron transport. Black tape covers the side of the polarograph block facing the light source, except on the area directly in front of the chamber. The light is focused on this open area so that the circle of light is only large enough to cover the chamber.

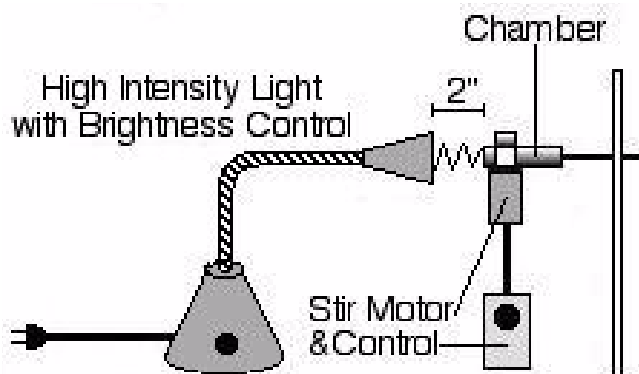


Figure 2-4: The high intensity illuminator, which is 2" from the surface of the chamber, saturates the thylakoids or cells in the chamber without a significant change in the temperature of the contents in the chamber.

Start the Software

- Click the **Windows Start** menu, move the cursor to **Programs** and then to the **iWorx** folder and select **LabScribe**; or click on the **LabScribe** icon on the Desktop
- When the program opens, select **Load Group** from the **Settings** menu.
- When the dialog box appears, select **AddedLabs.iws**. Click **Load**.
- Click on the **Settings** menu again and select the **CO2Fixation** settings file.
- After a short time, **LabScribe** will appear on the computer screen as configured by the **CO2Fixation** settings.

Exercise 1: Calibration

Aim: To calibrate the oxygen electrode.

The standard used for calibrating the oxygen electrode is the known concentration of oxygen in air-saturated deionized water. The amount of oxygen that is dissolved in water is dependent upon the temperature, oxygen pressure in the air, and the concentrations of dissolved solutes in the water. For example, the concentration of oxygen in deionized water at 26°C and 1 atmosphere is 252 micromolar (μM), or 252 micromoles (10^{-6} moles) of O_2 per liter of water.

Procedure

- Fill the polarograph chamber with fresh deionized water before proceeding with the calibration procedure. Place the electrode in the chamber and 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.

- Click **Start**.
- Type the words "Saturation-DI Water" on the comment line to the right of the **Mark** button, and press the **Enter** key on the keyboard. This comment is used to indicate the water is saturated with as much oxygen as it can hold.
- When the trace is stable (no vertical movements of the trace), record for an additional 10 seconds before going to the next step.
- Type the words "No Oxygen" on the comment line. Use a micropipette with a gel-loading tip to place 10 μl (microliters) of 1.5M Sodium Dithionite- O_2 depletion solution into the chamber through the reagent port. Press the **Enter** key on the keyboard to mark the recording. In a few seconds, this small amount of solution will deplete all the oxygen from the deionized water stirring in the chamber. Record the drop in

the oxygen concentration in the chamber until the trace is a flat line at a lower amplitude (Figure 2-5 on page 5).

- Click **Stop** to halt recording.
- Select **Save As** in the **File** menu, type a name for the file. choose a destination on the computer in which to save the file (e.g. the **iWorx** or class folder). Click the **Save** button to save the file (as an ***.iwd** file).
- Turn off the stirrer for the chamber. Remove the water and the oxygen depletion solution from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber 3 or 4 times with deionized water from a squirt bottle. Fill the chamber with deionized water and turn on the stirrer.

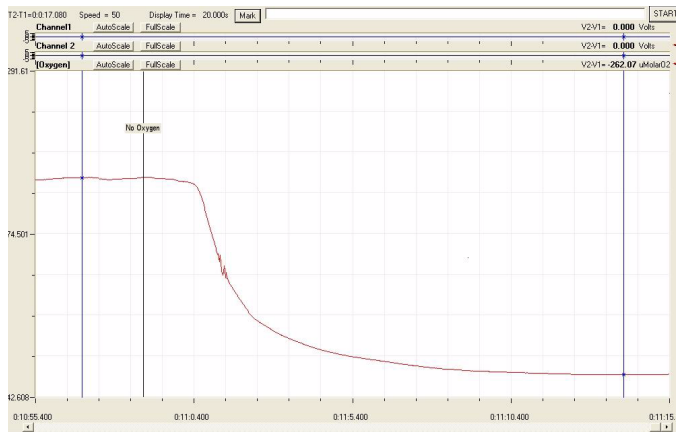


Figure 2-5: Change in concentration of oxygen in deionized water as the result of depletion.

Units Conversion

- Determine the temperature (°C) and the barometric pressure in the lab. The oxygen concentrations in deionized water, over a short range of temperatures at 760mmHg, have been calculated and are listed in Table 2-1 on page 5. The absorption coefficients of oxygen and the vapor pressures of water at these temperatures are also listed.
- The concentration of oxygen dissolved in deionized water, or its solubility (S), can be determined more accurately by using the following equation:

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

where *a* is the absorption coefficient of O₂ at temperature, *p* is the vapor pressure of water at temperature, *P* is the barometric pressure, and *r%* is the percent oxygen in the air. At 26°C and 760mmHg, assuming the concentration of oxygen in air is 21%, S = 252μMO₂:

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

- Select the section of the recording before and after the oxygen is depleted from the chamber (Figure 2-5 on page 5). To view this section of the recording in its entirety on the same window, it may be necessary to click either of the **Display Time** icons in the toolbar (Figure 2-6 on page 5).

- Click the **2-Cursor** icon (Figure 2-6 on page 5) so that two blue vertical lines appear over the recording window. Place one cursor on the plateau corresponding to the oxygen concentration in the fully oxygenated water. Place the other cursor on the lower amplitude plateau that corresponds to the absence of oxygen in the chamber.

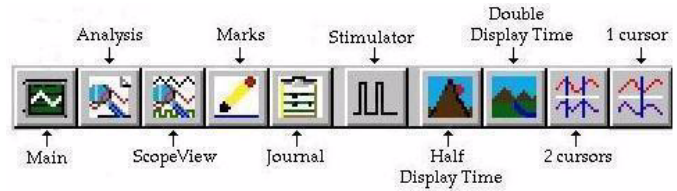


Figure 2-6: The LabScribe toolbar.

- Right-click on the Channel 3 window to open the **Right-click** menu. Select **Units** from the **Right-click** menu. Note that the voltage values for the positions of Cursors 1 and 2 are already entered in the units conversion window. Enter the concentration of oxygen dissolved in water at room temperature next to the voltage value for **Cursor1**. Enter “0” next to the voltage value for **Cursor2**. Enter “μMolarO₂” for the unit name. Click **OK**. Now, the units on the Y-axis correspond to the oxygen concentration.

Table 2-1: Oxygen Concentration [O₂] in Air-Saturated Deionized Water at 760mmHg.

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

Precautions

- Your use of time in this experiment is critical, since algal cells in suspension are useful for a limited period of time (3 to 4 hours). Complete all the exercises before analyzing the data.
- The lights in the room will be turned off during the experiment. It is important to gather all the supplies that you will need at your station before the lights are turned off. Some of the items needed close to your work station include: a test tube rack, a micropipette (set to 10μl), disposable gel-

loading micropipette tips, a squirt bottle filled with deionized water, a squirt bottle filled with 70% ethanol, Pasteur pipettes with soft plastic tubes on the their tips, a light-tight container for storing the suspension of algal cells, an insulated ice bucket for storing reagents, and reagents.

- 3 Small amounts of reagents will be dispensed in capped microfuge tubes. Larger amounts should be kept in capped test tubes.
- 4 Fill your ice bucket halfway. Place a 100ml beaker, for holding your tubes of reagents, in the ice.
- 5 The capped test tubes with the algal cells will be dispensed when the room lights are turned off. Keep the container with algal cells covered when the cells are inside.

Exercise 2: Carbon Dioxide Fixation in the Presence of an Uncoupler

Aim: To determine the rate of oxygen production by algal cells as an indicator of carbon dioxide fixation. This experiment will be performed with and without ATP synthesis being coupled to the electron transport chain.

Procedure

- 1 If your light source has a power switch which is separate from the intensity control, make sure the light source is turned off. If your light source does not have a separate power switch, plug the light source into a power strip that has a switch, make sure the power strip is turned off. Set the intensity control of the illuminator to the maximum level (100%).
- 2 Turn off the stirrer for the chamber. Carefully remove the electrode from the chamber. Remove the oxygen-depleted water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber five times with deionized water.
- 3 Swirl the tube of algae to suspend the cells homogeneously. Fill the chamber with algal cells. Carefully replace the electrode in the chamber and turn on the stirrer.
- 4 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.
- 5 Add a 10 μ l aliquot of HCO₃⁻ solution to the chamber through the reagent port. Be careful not to add an air bubble to the chamber.

Note: Put the tip of the micropipette down the reagent port and push its plunger to discharge the 10 μ l of the HCO₃⁻ 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.

- 6 Click **Start** and begin recording. Click the **Full Scale** button on the **[Oxygen]** channel. Turn the offset knob on the DO2-100 adapter to position the trace near the bottom of the screen. Moving the trace with the offset knob does not affect the calibration. As oxygen is produced, the trace will move up.
- 7 Type the words "Algal Cells in Dark" on the comment line. Press the **Enter** key on the keyboard. Record the rate of oxygen production of the algal cells in darkness for one minute.
- 8 Type the words "Algal Cells in Light at 100" on the comment line. Press the **Enter** key on the keyboard as the light is turned on and the algal cell suspension in the chamber is illuminated at the highest intensity. Record oxygen production at this intensity for two minutes.
- 9 As the recording continues, type the words "MA Added" on the comment line. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of methylamine (MA) uncoupler solution is added to the chamber through the reagent port.
- 10 Record oxygen production of the algal cells exposed to 100% light in the presence of methylamine for five minutes. Click **Stop** to halt the recording.
- 11 Select **Save As** in the **File** menu, type a name for the file. choose a destination on the computer in which to save the file (e.g. the **iWorx** or class folder). Click the **Save** button to save the file (as an *.iwd file).
- 12 Remove the electrode from the chamber, and rinse the electrode five times with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.
- 13 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about five times.

Exercise 3: Carbon Dioxide Fixation in the Presence of a Calvin Cycle Enzyme Inhibitor

Aim: To determine the rate of carbon dioxide fixation (oxygen production) with and without the Calvin Cycle taking place in the algal cells.

Procedure

- 1 Repeat Exercise 2 using Iodoacetamide (IAA) solution in place of Methylamine (MA) solution. Mark the recording at the appropriate times to indicate the presence of light or the inhibitor.
- 2 Select **Save** in the **File** menu.
- 3 Remove the electrode from the chamber, and rinse the electrode five times with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.
- 4 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water five times.

Exercise 4: Carbon Dioxide Fixation in the Presence of an Electron Transport Chain Blocker

Aim: To determine the rate of carbon dioxide fixation (oxygen production) with and without a block across the electron transport chains of the algal cells.

Procedure

- 1 Repeat Exercise 2 using DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) solution in place of MA (methylamine) solution. Mark the recording at the appropriate times to indicate the presence of light or the inhibitor.
- 2 Select **Save** in the **File** menu.
- 3 Remove the electrode from the chamber, and rinse the electrode ten times with 70% ethanol and five times with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.
- 4 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber ten times with 70% ethanol and five times with deionized water.

Exercise 5: Oxygen Production by Thylakoids in the Presence of an Uncoupler

Aim: To determine the rate of oxygen production by thylakoids, with and without ATP synthesis being coupled to the electron transport chain.

Procedure

- 1 In this exercise, use thylakoids and thylakoid reaction mixture in place of suspended algal cells.
- 2 Before adding thylakoids to the chamber, fill the chamber with the yellow thylakoid reaction media.
- 3 Make sure no light is reaching the polarograph chamber.
- 4 Click **Start**. Type the words "Thylakoids in Dark" on the comment line to the right of the **Mark** button. Position the trace near the bottom of the screen.
- 5 Mix the tube with the thylakoid preparation on the Vortex mixer, so the suspension is even. Use the micropipette to collect a 10 μ l aliquot of thylakoids from the tube. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l 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.
- 6 Type the words "Thylakoids in Light at 100" on the comment line. Press the **Enter** key on the keyboard as the light is

turned on and the thylakoid suspension in the chamber is illuminated at the highest intensity. Record oxygen production at this intensity for one minute.

- 7 As the recording continues, type the words "MA Added" on the comment line. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of Methylamine (MA) uncoupler solution is added to the chamber through the reagent port.
- 8 Record oxygen production of the thylakoids exposed to 100% light in the presence of methylamine for another minute.
- 9 Turn off the illuminator to put the thylakoids in the dark. Record for another minute. Click **Stop** to halt recording.
- 10 Select **Save** in the **File** menu.
- 11 Remove the electrode from the chamber, and rinse the electrode five times with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.
- 12 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about five times.

Exercise 6: Oxygen Production by Thylakoids in the Presence of a Calvin Cycle Enzyme Inhibitor

Aim: To determine the rate of oxygen production by thylakoids, with and without the Calvin Cycle taking place in the thylakoid suspension.

- 1 Repeat Steps 1 through 8 of Exercise 5. Mark the recording at the appropriate times to indicate the presence of light and the uncoupler, methylamine.
- 2 After the recording of the effect of methylamine in Step 8, type the words "IAA Added" on the comment line. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of Iodoacetamide (IAA) inhibitor solution is added to the chamber through the reagent port.
- 3 Record oxygen production of the thylakoids exposed to 100% light in the presence of iodoacetamide for another minute.
- 4 Turn off the illuminator to put the thylakoids in the dark. Record for another minute. Click **Stop** to halt recording.
- 5 Select **Save** in the **File** menu.
- 6 Remove the electrode from the chamber, and rinse the electrode five times with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.
- 7 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about five times.

Exercise 7: Oxygen Production by Thylakoids in the Presence of an Electron Transport Chain Blocker

Aim: To determine the rate of oxygen production by thylakoids, with and without a block of the electron transport chain.

- 1 Repeat Steps 1 through 8 of Exercise 5. Mark the recording at the appropriate times to indicate the presence of light, or the blocker and the uncoupler.
- 2 After the recording of the effect of methyamine in Step 8, type the words "DCMU Added" on the comment line. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of DCMU blocker solution is added to the chamber through the reagent port.
- 3 Record oxygen production of the thylakoids exposed to 100% light in the presence of DCMU for another minute.
- 4 Turn off the illuminator to put the thylakoids in the dark. Record for another minute. Click **Stop** to halt recording.
- 5 Select **Save** in the **File** menu.
- 6 Remove the electrode from the chamber, and rinse the electrode ten times with 70% ethanol and five times with deionized water. Make sure there is no water on the electrode before it is replaced in the chamber.
- 7 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. rinse the chamber ten times with 70% ethanol and five times with deionized water.

Analysis: Rate of Electron Transport

By international consensus, the rate of electron transport in thylakoids or cells is expressed as the rate of oxygen production. The units used to express these rates are: moles O₂/hr/mg chl (chlorophyll). Oxygen production rates are standardized for comparison of experiments performed in different laboratories around the world. So, the size of the reaction chamber, the number of thylakoids or cells in the chamber, and the time period for that change in oxygen concentration need to be included in the calculation of the rate,.

In Exercise 1 of this experiment, the oxygen probe was calibrated using air-saturated, deionized water at room temperature. Through this calibration, the Y-axis of the recording channel was converted from voltage to oxygen concentration or [Oxygen], which is expressed as μ MolarO₂ and abbreviated μ MO₂.

Changes in Oxygen Concentrations

The rate of change of the O₂ concentration in the 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 in the oxygen concentration in the polarograph chamber during these exercises:

- 1 Adjust the appropriate **Display Time** icon on the **LabScribe** toolbar (Figure 2-6 on page 5) so that the recording for Exercise 2 appears on the **Main** window.
- 2 Click the **2-Cursor** icon (Figure 2-6 on page 5), so that two blue vertical lines appear over the recording window. Place the two blue cursors on either side of the complete experimental run.
- 3 Click the **Analysis** icon (Figure 2-6 on page 5) to open the window. The selected data from the **Main** window will be present in the **Analysis** window.
- 4 Select the **[Oxygen]** channel (CH 3) in **Display Channel** list, on the left side of the **Analysis** window. From the **Table Functions** list, select **V2-V1** and **T2-T1** under **General**, and **mean_dv/dt** under **Derivative** (Figure 2-7 on page 8).
- 5 In the **Analysis** window, position the two blue cursors on the section of the exercise that corresponds to oxygen production from thylakoids or cells in the dark. Set the cursors on a linear portion of this data, and use the **T2-T1** value to set the cursors ten seconds apart. The value for the variable **mean_dv/dt** is the average rate of change in the oxygen concentration (in μ Molar/sec) in the chamber taken over ten seconds (Figure 2-7 on page 8).

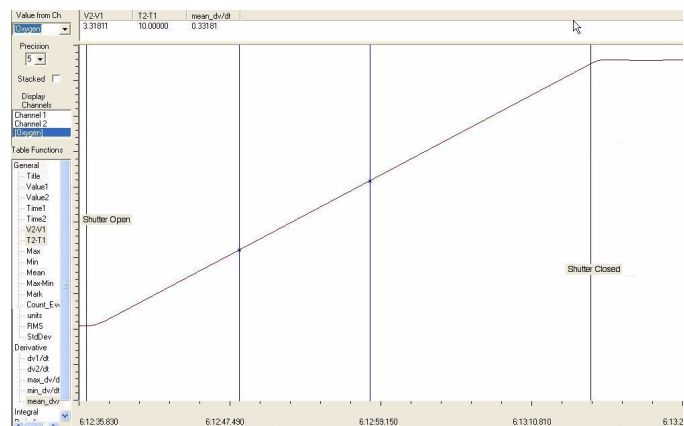


Figure 2-7: Change in oxygen concentration in the polarograph chamber created by thylakoids with whole electron transport chains exposed to 80% light in the presence of an uncoupler. As displayed in the **Analysis** window, the marks indicate the times when the shutter controlling the light was opened and closed. Cursors are 10 seconds apart. The average rate of change (**mean_dv/dt**) in the oxygen concentration over that period is the 0.33181 μ Molar O₂/sec.

- 6 Enter data into the **Journal** by either typing the titles and values directly or by using the **Right-click** menu. Place the cursors to take measurements; then, select **Add Title to Journal** or **Add Data to Journal** from the right-click menu to add the measurements to the **Journal**.
- 7 Move the blue cursors to the next section of the exercise when the thylakoids or cells were illuminated by light.

- 8 Repeat Steps 5 and 6 to measure the rate of change in oxygen concentration for thylakoids or cells that are illuminated. All the average rates of change in oxygen concentration should be measured across a ten second period.
- 9 Move the blue cursors to the remaining sections of the exercise when the thylakoids or cells were illuminated by light in the presence of an uncoupler, an inhibitor, a blocker, or a combination of these reagents.
- 10 Repeat Steps 5 and 6 on the remaining sections of the exercise to measure the rate of change in oxygen concentration during the presence of light and reagents.
- 11 Make sure all the rates of change in oxygen concentration are recorded in the **Journal**.

Standardizing the Rate of Oxygen Production

To standardize the rates of oxygen production according to the adopted convention, the volume of the polarograph chamber, the measured rate of change in oxygen concentration, and the concentration of thylakoids or cells 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 O₂ (μM_{O₂} 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:

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

- 2 Next, the moles of oxygen produced in one second must be converted to an hourly rate. In our example, if the amount of oxygen produced is 0.396 nmoles O₂ in one second, the hourly production rate is 1.44 micromoles O₂ (μmoles O₂) per hour:

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

- 3 Finally, the moles of oxygen produced per hour must be standardized for the amount of cells or thylakoids in the chamber. If a chamber contains more cells or thylakoids, the rate of oxygen production will be greater. After the thylakoids are isolated or the cells are grown, 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 or cells. The isolated thylakoids or cell suspension is diluted by buffer to create a stock suspension that has the same approximate concentration of chlorophyll for each lab sessions. For 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 for each exercise using the steps presented above. Enter the rates in Table 2-2 on page 10.

Correction for Non-Zero Dark Rates

Sometimes, before the polarograph chamber is illuminated, thylakoids or cells will either produce or consume oxygen at a low rate. If the thylakoids or cells are producing oxygen in the dark, then the recorded rate is greater than the actual light-induced rate of oxygen production. Likewise, if the thylakoids are consuming oxygen in the dark, then the recorded rate is less than the actual light-induced rate of oxygen production.

- 1 When determining the actual rate of oxygen production attributable to light, the dark rate must be subtracted from the recorded light rate in the same experimental run. When negative dark rates are subtracted from positive recorded rates, the actual rate is greater than the recorded rate. 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:

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

- 2 For each exercise, subtract the dark rate from the recorded light-induced rates to yield the actual light-induced rates. Enter the actual rates in the "light-dark" columns on the table (Table 2-2 on page 10).

Questions

- 1 Do algal cells consume or produce oxygen when they are in the dark? What happens to the oxygen production rate when the algal cells and reagents in the chamber are exposed to light?
- 2 What effect does MA (methylamine) have on the oxygen production rates of algal cells?
- 3 What effect does IAA (iodoacetamide) have on the oxygen production rates of algal cells?
- 4 What effect does DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) have on the oxygen production rates of algal cells?
- 5 What effect does MA have on the oxygen production rates of thylakoids?

- 6 What effect does IAA have on the oxygen production rates of thylakoids?
- 7 What effect does DCMU have on the oxygen production rates of thylakoids?
- 8 How do the oxygen production rates of algal cells in the dark, in the light, and in the light with MA compare to the rates from thylakoids under the same conditions?
- 9 How do the oxygen production rates of algal cells in the dark, in the light, and in the light with IAA compare to the rates from thylakoids under the same conditions?
Remember that MA was added to the chamber before IAA was added.

- 10 How do the oxygen production rates of algal cells in the dark, in the light, and in the light with DCMU compare to the rates from thylakoids under the same conditions?
Remember that MA was added to the chamber before DCMU was added.

Reference

Behrens P W, Bingham S E, Hoeksema S D, Cohoon, D L, Cox J C (1989). Studies on the incorporation of CO₂ into starch by *Chlorella vulgaris*. Journal Applied Phycology 1: 123-130

Table 2-2: Rates of Oxygen Production in Cells and Thylakoids Treated with an Uncoupler, an Enzyme Inhibitor, and an Electron Blocker.

Exercise	Rate of Oxygen Production (moles O ₂ /hr/mg chl)								
	No Reagent			Reagent 1			Reagents 1 & 2		
	Dark	Light	Light -Dark	Reagent	Light	Light -Dark	Reagent	Light	Light -Dark
2-Algae & Uncoupler				MA					
3-Algae & Calvin Cycle Inhibitor				IAA					
4-Algae & Electron Blocker				DCMU					
5-Thylakoids & Uncoupler				MA					
6-Thylakoids & Calvin Cycle Inhibitor				MA			IAA		
7-Thylakoids & Electron Blocker				MA			DCMU		

Appendices

D02-100 Current to Voltage Adapter

The D02-100 is a current to voltage adapter designed to work with a Clark-style oxygen electrode. This adapter delivers a polarizing voltage of -0.8V 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 DO2-100 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.

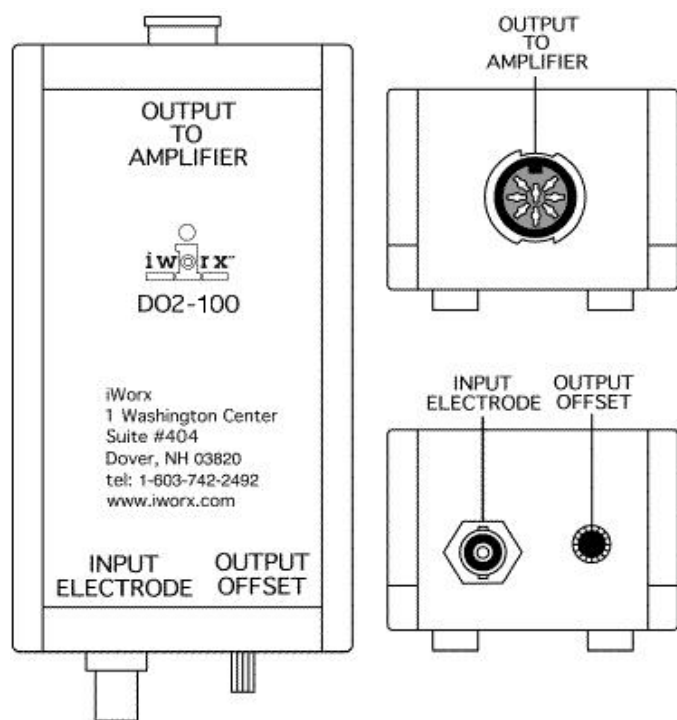


Figure 2-8: Top and side views of the DO2-100 current to voltage adapter.

ISE-730 Microelectrode

Assembly and Preparation

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

Handling

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

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

Cleaning

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

Storing

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

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

Culturing *Chlorella vulgaris*

- 1 Obtain a culture of *Chlorella vulgaris* (Beijernick's or similar strain) from a biological supply house or a culture collection. Follow the directions included with the culture or use the following steps.
- 2 Raise the algae in a 1 liter flask filled with 500ml of mineral salts medium.
- 3 The flask is kept in a lighted environmental room or chamber with an illumination level of 40-90 $\mu\text{mol photon/square meter/second}$ at 25°C.
- 4 Agitation of the culture and the carbon source is provided by bubbling CO₂ rich air (2% v/v CO₂) through the medium.
- 5 The KNO₃ concentration of the culture medium is set to support a cell density of about 1.7 grams/liter. Measure the rate of oxygen production of the stock culture in an oxygen polarograph illuminated with 100% light intensity. If the slope of the oxygen production line is steeper than 45 degrees, dilute the stock culture with deionized water if the cells will be used within a day.

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):

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

Reagents

- 1 Chlorella mineral salts medium:
 - 0.1g K₂HPO₄
 - 0.075g KH₂PO₄
 - 0.5g MgSO₄·7H₂O
 - 0.625g Ca(NO₃)₂·4H₂O
 - 3.0g KNO₃
 - 10.0mg FeSO₄·7H₂O
 - 8.0mg disodium EDTA
 - 2.86mg H₃BO₃
 - 1.81mg MnCl₂·4H₂O
 - 0.22mg ZnSO₄·7H₂O
 - 0.39mg Na₂MoO₄·2H₂O
 - 0.08mg CuSO₄·5H₂O
 - 0.05mg Co(NO₃)₂·6H₂ODissolved in deionized and Qs to 1 liter.
- 2 1.0 M NaHCO₃ solution
 - 8.40g of NaHCO₃ (Sodium Bicarbonate, Baking Soda) in 100 mls deionized water. Need about 0.1 ml per lab group, each lab period.
- 3 3.0 M Methylamine (MA)
 - 20.26g Methylamine in 100 mls deionized water. Need about 0.1 ml per lab group, each lab period.
- 4 0.50 M Iodoacetamide (IAA)
 - 4.624 g of Iodoacetamide in 50 mls deionized water. Need about 0.05 ml per lab group, each lab period.
- 5 0.0001M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) stock solution:
 - Mix 0.0012 grams of DCMU into 50 mls of 70% Ethanol. Freeze in 1 ml aliquots in capped tubes.
- 6 1.5M Na₂S₂O₄ (Sodium Dithionite) O₂ depletion solution
 - Mix 13 grams of Na₂S₂O₄ into 50 mls of deionized water. Store at 4°C.

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

50 mM Tricine
400 mM Sucrose
50mM NaCl

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

8 Thylakoid reaction buffer, pH 7.5 in deionized water:

50 mM Tricine
5 mM MgCl₂ · 6H₂O
2 mM K₃Fe(CN)₆ (Potassium Ferricyanide)

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

9 0.5M Tricine stock solution:

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

