

Experiment 12: Mitochondrial Respiration

Introduction

The mitochondria are small organelles, found in eucaryotic cells, with the primary function of providing most of the ATP needed for cellular function. Structurally, mitochondria have two membranes and a central space known as the matrix. The outer membrane is permeable to small molecules needed in the cell and the mitochondria, but large molecules are excluded. The inner membrane is highly invaginated into folds called *cristae* which extend into the matrix. The components of the electron transport chain (ETC) are localized in the inner membrane. The most common source of electrons for the ETC is the NADH molecule. NADH is produced by the Krebs (Citric Acid) Cycle, whose substrates and enzymes are found in the matrix.

Electron Transport Chain

The components of the mitochondrial electron transport chain (Figure 2-1 on page 2) exist in either an oxidized or a reduced form. A component is in the oxidized form when it is capable of receiving a pair of electrons from another component that already has a pair of electrons. The component holding the pair of electrons is in a reduced form and is capable of donating its electrons to a component that is oxidized and at a lower potential energy level. When mitochondria are isolated from cells the components of the ETC are all in their oxidized forms.

NADH can donate a pair of electrons to the first component of the electron transport chain, an oxidized flavoprotein in the NADH Dehydrogenase complex. As the flavoprotein accepts the pair of electrons, it becomes reduced and the NADH is oxidized to NAD. The pair of electrons is passed down all the subsequent components of the ETC; each component becomes reduced as it accepts the electrons and oxidized as it donates them. The ultimate acceptor of these electrons is oxygen which also picks up hydrogen atoms to yield water.

Chemiosmotic Hypothesis

As the process in the mitochondrial electron transport chain takes place, energy is released that can be used to make ATP. Though the details regarding the conservation of this released energy are still being debated, most scientists accept the *chemiosmotic hypothesis* as the general mechanism for the energy transfer. This hypothesis states that hydrogen ions are transferred to the space between the inner

and outer mitochondrial membranes as electron transport occurs. This results in a difference between the hydrogen ion concentration on either side of the inner mitochondrial membrane. The energy inherent in this difference in pH between the matrix and the inter-membrane space is used to make ATP.

The inner mitochondrial membrane has specialized regions with knob-like protein complexes called coupling factors or ATPases. These complexes use the energy conserved in electron transport to synthesize ATP from ADP and phosphate. Each pair of electrons passing through the entire ETC releases enough energy to generate three ATP's. If each NADH releases a pair of electrons which are accepted by an oxygen atom to make a molecule of H₂O, then three molecules of ATP are generated for each atom of oxygen consumed. This ratio of 3:1 is termed the P:O ratio. Some natural and artificial substrates do not yield a P:O ratio of 3:1. For example, FADH₂ from succinate dehydrogenase donates electrons at the level of the ubiquinone component of the ETC. The passage of the pair of electrons to oxygen releases only enough energy to make two ATP; so the P:O ratio is 2:1. If ascorbate is the electron donor to cytochrome C₁ the P:O ratio of 1:0.

Support for the chemiosmotic hypothesis is provided by experiments that use a class of compounds that uncouple the process of ATP synthesis from the process of electron transport. In the presence of an *uncoupler*, electron transport continues, but ATP synthesis is prevented. Many uncouplers simply equilibrate the hydrogen ions across the membrane; and, since there is no pH gradient across the membrane, there is no energy to drive ATP synthesis. The rate of electron transport, measured as the rate of oxygen consumption, increases when uncouplers are present and is affected only by the ability of the proteins in the ETC to pass electrons on to the next component. With a large pH gradient, the rate of electron transport is slow.

When mitochondria are isolated from animals or plants, they retain the ability to perform electron transport. The process can now be manipulated by experimenters, who can add substrates that donate electrons at different levels in the chain, or uncouplers, or inhibitors. Since the ultimate electron acceptor at the end of the chain is oxygen which is converted to water, the flow of electrons can be measured by monitoring oxygen consumption in the isolated mitochondrial suspension.

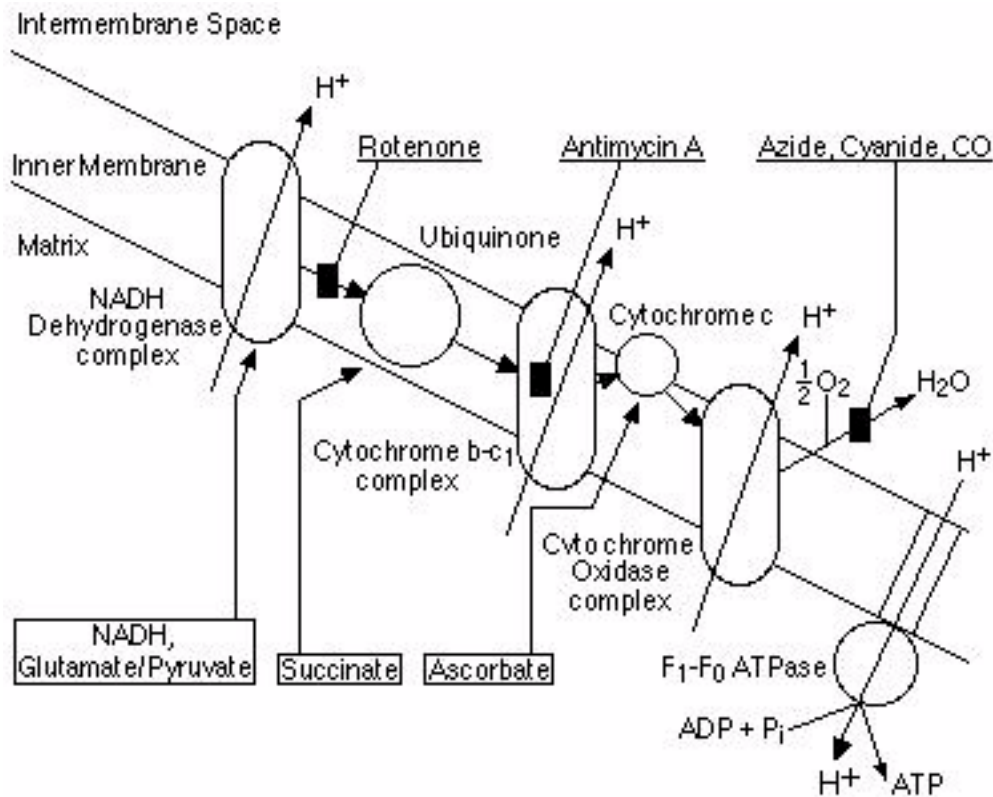


Figure 2-1: Components of the Mitochondrial electron transport chain with donors shown in boxes and the electron transport inhibitors underlined. Protons are transported into the intermembrane space at three components and flow back into the matrix at the ATPase, providing energy for phosphorylation.

Oxygen Polarograph

An oxygen polarograph is a device that is often used to measure oxygen consumption or production in cells and subcellular suspensions. 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. Also, since the volume in the chamber is small, the chamber has a flat bottom for the use of a stir bar. The stir bar assists the movement of the suspension across the membrane of the electrode and permits instantaneous recording of any changes in oxygen concentration.

Electron Donors and Inhibitors

A variety of electron donors (glutamate/pyruvate, succinate, ascorbate) can be added to isolated mitochondria to initiate electron transport at various points on the ETC. For example, glutamate and pyruvate (used together) donate electrons indirectly to the NADH dehydrogenase complex at the head of the ETC. These two molecules are actually substrates in the Krebs Cycle which generates NADH for use by the NADH dehydrogenase complex. Succinate is another substrate, whose reaction with succinate dehydrogenase yields FADH. FADH donates electrons farther down the ETC to the component known as ubiquinone. Ascorbate donates electrons directly to a component in the ETC, Cytochrome c_1 .

The electron transport can also be studied by adding blocking agents that act at different points in the chain. Then, substrates that act below the block can be added to start the flow of electrons again. Some of the popular blocking agents are: Rotenone, a fish poison, which blocks electron transport between the NADH dehydrogenase complex and ubiquinone; Antimycin A which blocks the ETC between Cytochrome b and Cytochrome c_1 ; azide and cyanide which block the ETC between cytochrome oxidase and oxygen.

If NADH is added to a suspension of mitochondria, electron transport begins. If rotenone is added to this suspension, electron transport stops. All components in the ETC that are downstream of the block become oxidized due to the lack of electrons flowing to them. The components upstream of the block remain reduced. Since there are no electrons flowing toward cytochrome oxidase, oxygen consumption also stops. If succinate is added to the suspension, the block created by rotenone is by-passed, and the flow of electrons and the consumption of oxygen will begin again. Other blocks and donors can be used to inhibit and restart the flow of electrons.

In this experiment, you will learn:

- how calibrate and use an oxygen polarograph;
- how various substrates, inhibitors, and uncouplers affect the rate of electron transport by monitoring changes in the rate of oxygen consumption;
- how various donors affect P:O ratios. P:O ratios indicate the amount of ATP produced and the number of protons moved across the inner mitochondrial membrane for the number of electrons flowing through the electron transport chain and the amount of oxygen consumed.

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
- 10µl micropipette
- Vortex mixer
- Mitochondrial suspension (See appendix)
- Glutamate/Pyruvate, Succinate, Ascorbate electron donor solutions (See appendix)
- Uncoupler solution (See appendix)
- Rotenone, Antimycin A, Sodium Azide electron transport chain inhibitor solutions (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 2-2 on page 3).

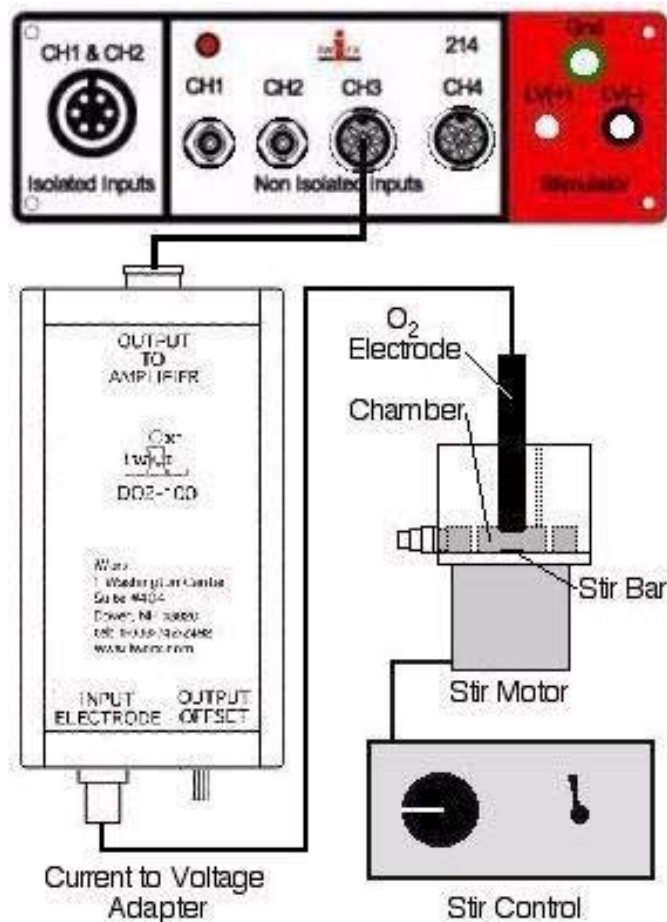


Figure 2-2: The oxygen polarograph used with the iWorx/214.

- 3 Attach the cable of the oxygen electrode to the BNC connector on the current to voltage adapter (Figure 2-2 on page 3).
- 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 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.

Start the Software

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

- 1 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.
- 2 Click **Start**.
- 3 Type the words "Saturation-DI Water" on the comment line to the right of the **Mark** button, and press the **Enter** key on the keyboard. This comment is used to indicate the water is saturated with as much oxygen as it can hold.
- 4 When the trace is stable (no vertical movements of the trace), record for an additional 10 seconds before going to the next step.
- 5 Type the words "No Oxygen" on the comment line. Use the micropipette to place 10 μl (microliters) of the oxygen depletion solution into the chamber. Press the **Enter** key on the keyboard to mark the recording. In a few seconds, this small amount of solution will deplete all the oxygen from the deionized water stirring in the chamber. Record the drop in the oxygen concentration in the chamber until the trace is a flat line at a lower amplitude (Figure 2-3 on page 4).
- 6 Click **Stop** to halt recording.
- 7 Select **Save As** in the **File** menu, type a name for the file. choose a destination on the computer in which to save the file (e.g. the **iWorx** or class folder). Click the **Save** button to save the file (as an *.iwd file).

- 8 Turn off the stirrer for the chamber. Remove the water and the oxygen depletion solution from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber 3 or 4 times with deionized water from a squirt bottle. Fill the chamber with deionized water and turn on the stirrer.

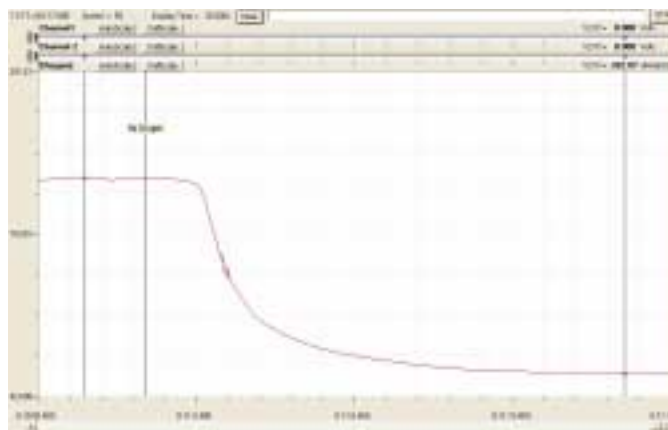


Figure 2-3: Change in concentration of oxygen in deionized water as the result of depletion.

Units Conversion

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

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

where a is the absorption coefficient of O_2 at temperature, p is the vapor pressure of water at temperature, P is the barometric pressure, and $r\%$ is the percent oxygen in the air. At 26°C and 760mmHg, assuming the concentration of oxygen in air is 21%, $S = 252\mu\text{M}\text{O}_2$:

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

- 3 Select the section of the recording before and after the oxygen is depleted from the chamber (Figure 2-3 on page 4). 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 2-4 on page 5).
- 4 Click the **2-Cursor** icon (Figure 2-4 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.

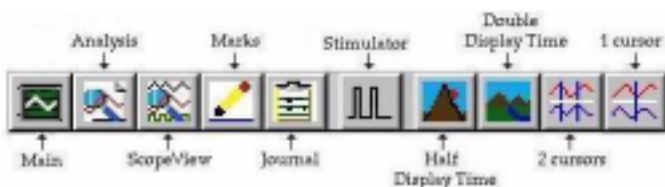


Figure 2-4: The LabScribe toolbar.

- 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 " μMolarO_2 " for the unit name. Click **OK**. Now, the units on the Y-axis correspond to the oxygen concentration.

Precautions

- Your use of time in this experiment is critical, since mitochondria in suspension are useful for a limited period of time (3 to 4 hours at the most). Complete all the exercises before analyzing the data.

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

- It is important to gather all the supplies that you will need at your station before beginning the experiments. These items include small capped tubes containing each of the electron donors, the uncoupler, ADP, and the mitochondrial suspension. A micropipette (set to 10μl), disposable micropipette tips, a squirt bottle filled with deionized water, and Pasteur pipettes with soft plastic tubes on their tips will also be needed.
- Fill your ice bucket halfway. Place a 100ml beaker, for holding your tube of isolated mitochondria, in the ice.

Exercise 2: Rate of Electron Transport

Aim: To determine the effects of various electron donors on the rate of electron transport in the absence of ADP, in the presence of ADP, and in the presence of an uncoupler.

Procedure

- Turn off the stirrer for the chamber. Remove the oxygen-depleted water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber ten times with deionized water.
- Mix the tube containing the mitochondrial suspension on the Vortex mixer. Fill the chamber with mitochondrial suspension. Carefully replace the electrode in the chamber and turn on the stirrer.
- 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 on the stirrer, and check for bubbles, again.
- Open the **Windows** menu on the LabScribe software and select the **Preview** window. Change the channel displayed to **[Oxygen]** channel. Turn the offset knob on the DO2-100 adapter to position the trace near the top of the screen. As oxygen is consumed, the trace will move down.

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

- Click **Start**. Type the words "Endogenous Rate" on the comment line to the right of the **Mark** button. Press the **Enter** key on the keyboard to mark the recording. Record the endogenous rate of respiration of the mitochondria until the trace is a line, not a curve. The line should not be sloping up (O₂ evolution); it should be flat (no O₂ usage) or sloping down slightly (a little O₂ consumption). It could take as long as five minutes for the trace to become a straight line.
- As the recording continues, type the words "Donor 1" on the comment line to the right of the **Mark** button. Press the **Enter** key on the keyboard to mark the recording as a 10μl aliquot of any electron donor is added to the chamber through the reagent port.

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.

- 7 Record the rate of respiration from the mitochondria in the presence of the donor, until the trace is a straight line (usually not more than one minute).
- 8 As the recording continues, type the words "ADP added to Donor 1" on the comment line to the right of the **Mark** button. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of ADP solution is added to the chamber through the reagent port.
- 9 Since ADP causes the rate of oxygen consumption to increase, the slope of the recording will be steeper after its addition. When the limited amount of ADP in the chamber is exhausted, the rate of oxygen consumption decreases to a slope that is similar to the slope before ADP was added. Continue to record for one minute past the point when all the ADP was consumed.
- 10 As the recording continues, type the word "Uncoupler" on the comment line to the right of the **Mark** button. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of the uncoupler is added to the chamber through the reagent port. Record the new oxygen consumption rate for one minute.
- 11 Click **Stop** to halt recording.
- 12 Select **Save As** in the **File** menu, type a name for the file, choose a destination on the computer in which to save the file (e.g. the **iWorx** or class folder). Click the **Save** button to save the file (as an *.iwd file).
- 13 Remove the electrode from the chamber and rinse it well with deionized water. Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about ten times.
- 14 Repeat Steps 4 through 14 for one of the other electron donors. Then, repeat these steps for the third and final electron donor.

Exercise 3: Effects of Inhibitors

Aim: To examine the effects of electron transport inhibitors on the rate of electron transport.

Before Coming to Lab

- 1 Using information in this laboratory protocol, design an experiment that will demonstrate the effects of each of the three inhibitors (Antimycin A, Azide, and Rotenone) that will be provided. If the inhibitors are added to the mitochondrial suspension in the proper sequence with one or more of the electron donors present, it is possible to complete the exercise in a single run. If an inhibitor is added in the wrong sequence, the effect of the inhibitor will not be demonstrated.
- 2 Construct a flow chart outlining the order in which reagents are added to the chamber. Also include the times between each addition.
- 3 Follow these tips:
 - Donors and inhibitors will be added to the chamber in 10 μ l aliquots through its reagent port.

- When a reagent is added to the chamber, wait 30 seconds before adding another compound. Thirty seconds is enough time to see a change in the slope of the line.
- Thirty seconds after an electron donor is added to the chamber, add ADP. ADP increases the slope of the line making inhibition easier to see.
- When the experimental run is finished, rinse the chamber with deionized water at least ten times.

In Lab

- 1 Present the flow chart of your experimental design to your instructor for approval.
- 2 Once approved, carry out your experiment using the techniques learned in Exercises 1 and 2.
- 3 At the end of this exercise, remove the electrode from the chamber and rinse it well with deionized water. Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about ten times.

Exercise 4: Effects of ATPase Inhibitor

Aim: To examine the effects of the ATPase inhibitor, oligomycin, on the flow of electrons.

Oligomycin binds to ATPase, and prevents it from making ATP and passing hydrogen ions across the membrane.

Procedure

- 1 Fill the chamber with mitochondrial suspension. Carefully replace the electrode in the chamber and turn on the stirrer.
- 2 Check the chamber for the presence of bubbles. If bubbles are present, remove them by following the instructions provided earlier.
- 3 Click **Start**. Type the words "Mitochondria Only" on the comment line to the right of the **Mark** button. Press the **Enter** key on the keyboard to mark the recording. As in Exercise 2, record the endogenous rate of respiration of the mitochondria until the trace is a line. Continue recording.
- 4 Type the word "Succinate" on the comment line to the right of the **Mark** button. Press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of Succinate is added to the chamber. Continue recording.
- 5 Type the word "ADP" on the comment line to the right of the **Mark** button. Thirty seconds after the addition of succinate, press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of ADP is added to the chamber. Continue recording.
- 6 Type the word "Oligomycin" on the comment line to the right of the **Mark** button. Thirty seconds after the addition of ADP, press the **Enter** key on the keyboard to mark the recording as a 10 μ l aliquot of Oligomycin is added to the chamber. Continue recording.

- 7 Type the word “Uncoupler” on the comment line to the right of the **Mark** button. Thirty seconds after the addition of Oligomycin, press the **Enter** key on the keyboard to mark the recording as a 10µl aliquot of uncoupler is added to the chamber. Continue recording for 30 seconds.
- 8 Click **Stop** to halt recording.
- 9 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).
- 10 Remove the electrode from the chamber and rinse it well with deionized water.
- 11 Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber with deionized water about ten times.
- 12 Repeat this exercise (Steps 1 through 11), but reverse the order in which Oligomycin and the uncoupler are added to the chamber.

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.

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 µMolarO₂ and abbreviated µMO₂.

Change in O₂ Concentration in the Chamber

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 Adjust the appropriate **Display Time** icon on the **LabScribe** toolbar (Figure 2-4 on page 5) so that the recording of the first experimental run in Exercise 2 appears on the **Main** window.
- 2 Click the **2-Cursor** icon (Figure 2-4 on page 5), so that two blue vertical lines appear over the recording window. Place one cursor about 30 seconds to the left of the mark

indicating the addition of the first electron donor. Place the other cursor at the end of the first experimental run.

- 3 Click the **Analysis** icon (Figure 2-4 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**.
- 5 In the **Analysis** window, position the two blue cursors on a linear section of the recording before the addition of the first electron donor. Oxygen consumption in this section of the recording occurred at the endogenous rate. Use the **T2-T1** value displayed in the **Table of Functions** to set the cursors ten seconds apart. The value for the variable **mean_dv/dt** is the average rate of change of the oxygen concentration (in µMolar/sec) in the chamber taken over ten seconds.
- 6 Enter data into the **Journal** by either typing the titles and values directly or by using the **Right-click** menu. Place the cursors to take measurements; then, select **Add Title to Journal** or **Add Data to Journal** from the right-click menu to add the measurements to the **Journal**.
- 7 Move the blue cursors to the next section of the experimental run when the first electron donor was present in the polarograph chamber.
- 8 Repeat Steps 5 and 6 to measure the rate of change of the oxygen concentration in the presence of the donor. This and all the average rates of change of the oxygen concentration should be measured across a ten second period.
- 9 Continue to move the cursors to the other sections of the experimental run when other reagents were added. Repeat Steps 5 and 6 for each section. Determine the rates of change of the oxygen concentration in the chamber when mitochondria were: alone; with an electron donor; with the same electron donor and ADP; and, with the same electron donor, ADP, and an uncoupler.
- 10 Measure the rates of change of the oxygen concentration for all sections of the experimental runs with other donors.
- 11 Measure the rates of change of the oxygen concentration for all sections of the experimental runs in Exercises 3 and 4 that were performed with donors and inhibitors in defined sequences.
- 12 Make sure all the rates of change of the oxygen concentration from each section of all the experimental runs are recorded in the **Journal**.

Standardizing the Rate of O₂ Consumption

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

- 1 Multiply the rate of change of the oxygen concentration by the volume of polarograph chamber. The product of this calculation is the number of moles of oxygen consumed in one second. For example, if the change in oxygen concentration is $0.666 \mu\text{M O}_2$ (micromolar or 10^{-6} moles/liter) per second, and the polarograph chamber has a 1.2 milliliter (ml) capacity; then, 0.799 nanomoles (nmoles or 10^{-9} moles) of O_2 are consumed in one second:

$$(0.666 \mu\text{M O}_2/\text{sec})(1.2\text{ml}) = \\ 0.799 \text{ nmoles O}_2 \text{ consumed in one second.}$$

- 2 Next, the moles of oxygen consumed in one second must be converted to an hourly rate. In our example, if the amount of O_2 consumed is 0.799 nmoles O_2 in one second, the hourly consumption rate is 2.88 micromoles O_2 ($\mu\text{moles O}_2$) per hour:

$$(0.799 \times 10^{-9} \text{ moles O}_2/\text{sec})(60\text{sec}/\text{min})(60\text{min}/\text{hr}) = \\ 2880 \times 10^{-9} \text{ moles O}_2/\text{hr} = \\ 2.88 \times 10^{-6} \text{ moles O}_2/\text{hr.}$$

- 3 Finally, the moles of oxygen consumed per hour must be standardized for the amount of mitochondria in the chamber. Clearly, if a chamber contains more mitochondria, the rate of oxygen consumption will be greater. After the mitochondria are isolated, the laboratory staff determines the concentration of mitochondrial protein in the preparation by performing a protein assay. The concentration of protein is proportional to the concentration of mitochondria. Then, the lab staff dilutes the preparation with the appropriate volume of buffer to create a stock suspension of mitochondria that has the same approximate concentration of mitochondria in each lab session. In our example, if the concentration of protein is 2.5 milligrams of protein per milliliter (mg prot/ml), and the polarograph chamber contains 1.2 ml of suspension, the amount mitochondrial protein in the chamber is 3.0 mg:

$$(2.5\text{mg prot}/\text{ml})(1.2\text{ml in chamber}) = \\ 3.0 \text{ mg protein in chamber.}$$

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

$$(2.88 \times 10^{-6} \text{ moles O}_2 \text{ consumed}/\text{hr})/(3\text{mg protein}) = \\ 0.960 \times 10^{-6} \text{ moles O}_2/\text{hr}/\text{mg protein} = \\ 0.96 \mu\text{moles O}_2/\text{hr}/\text{mg protein.}$$

- 4 Calculate the rates of oxygen consumption (or production) for each section of each experimental run using the steps presented above.
- 5 Enter the rates for all three exercises into tables. These rates should be placed in the columns labeled "Recorded".

Analysis: P to O Ratios

Calculate the P:O ratios for each of the three donors: glutamate/pyruvate, succinate, and ascorbate. Calculate the amount of oxygen consumed from the sections of data recorded in Exercise 2, where mitochondria are in the presence of a donor and ADP.

- 1 Determine the amount of oxygen consumed due to the presence of ADP in the polarograph chamber.

• Subtract the oxygen consumption rate for mitochondria with an electron donor from the oxygen consumption rate for mitochondria with an electron donor and ADP. This results in the net rate of oxygen consumption due to ADP being present. For example:

$$3.7 \times 10^{-6} \text{ moles O}_2/\text{hr}/\text{mg protein (e}^- \text{ donor and ADP)} \\ - 0.6 \times 10^{-6} \text{ moles O}_2/\text{hr}/\text{mg protein (e}^- \text{ donor)} = \\ 3.1 \times 10^{-6} \text{ moles O}_2/\text{hr}/\text{mg protein, with ADP}$$

• Multiply the rate of oxygen consumption due to ADP by the amount of mitochondria in the chamber to determine the amount of oxygen consumed per hour in the chamber:

$$(3.1 \times 10^{-6} \text{ moles O}_2/\text{hr}/\text{mg protein})(3\text{mg protein}) = \\ 9.3 \times 10^{-6} \text{ moles O}_2/\text{hr}$$

• Multiply the amount (moles) of oxygen consumed per hour by the time it took to consume all the ADP that was added to the chamber. The product is the amount of oxygen (O_2) consumed when ADP was available for reactions. In our example, all the ADP added to the chamber is consumed in 30 seconds, the amount of O_2 consumed is 0.078×10^{-6} moles of O_2 :

$$(9.3 \times 10^{-6} \text{ moles O}_2/\text{hr})(30\text{s})(\text{hr}/3600\text{s}) = \\ 0.078 \times 10^{-6} \text{ moles O}_2.$$

- 2 Multiply the moles of molecular oxygen (O_2) consumed by 2 to yield the moles of atomic oxygen (O) consumed. In our example:

$$(0.078 \times 10^{-6} \text{ moles O}_2)(2\text{O}/\text{O}_2) = \\ 0.155 \times 10^{-6} \text{ moles of O.}$$

- 3 Determine the amount of ADP added to the polarograph chamber during the experiment. Remember each ADP combines with a P to produce ATP. Multiply the volume of ADP solution added to the polarograph chamber by its concentration. In our example, $10 \mu\text{l}$ (10×10^{-6} liter) of ADP solution at a concentration of 0.03M (0.03 moles/liter) was added to the chamber, the amount of ADP added is equal $0.3 \mu\text{moles}$ (0.3×10^{-6} moles):

$$(10 \times 10^{-6} \text{ liter})(0.03 \text{ moles ADP}/\text{liter}) = \\ 0.3 \times 10^{-6} \text{ moles ADP.}$$

- 4 Divide the moles of ADP (or P) added to the chamber by the moles of atomic oxygen (O) consumed to yield the P:O ratio for the electron donor provided to the mitochondria. In our example, 0.3×10^{-6} moles of ADP are divided by 0.155×10^{-6} moles of O (atomic oxygen) to yield the P:O ratio of 1.935 to 1:

$$(0.3 \times 10^{-6} \text{ moles ADP}) / (0.155 \times 10^{-6} \text{ moles O}) = 1.935.$$

- 5 The conclusion from our example is that the electron donor must have been succinate.

Questions

- 1 What happens to the oxygen consumption rate when an electron donor (substrate) is added to the mitochondrial suspension? Why does this occur?
- 2 Are the oxygen consumption rates for each donor, in the absence of ADP, the same or different? Why would they be different or the same?
- 3 What happens to the oxygen consumption rate when ADP is added to a mixture of mitochondria and an electron donor? How does phosphorylation affect electron transport?
- 4 Why does the oxygen consumption rate for ADP in the presence of an electron donor revert to the rate that existed before the addition of the ADP?
- 5 Is the oxygen consumption rate for glutamate/pyruvate in the presence of ADP the same as the rate for succinate and ADP? Is the oxygen consumption rate for succinate with ADP the same as the rate for ascorbate with ADP? Is there a trend in the rates for the three donors? Why are the rates the same or different?
- 6 What effect does the uncoupler have on the oxygen consumption rates? Explain the effect in terms of phosphorylation, electron transport, and the chemiosmotic hypothesis.
- 7 Were the oxygen consumption rates with the uncoupler the same or different for each donor? What are the reasons for the similarity or the difference?
- 8 What happens to the oxygen consumption rate when oligomycin is added to the chamber after ADP? What happens to the rate when an uncoupler is added after oligomycin? What happens to the rate when the uncoupler is added after ADP and then oligomycin is added after the uncoupler?

Table 2-2: Exercise 2: Oxygen (O₂) Consumption Rates for Mitochondria and Donors, ADP, and Uncoupler. Rates are expressed as 10⁻⁶ moles O₂/hr/mg protein.

Mixture in Chamber	Donors		
	Glutamate/Pyruvate	Succinate	Ascorbate
Mitochondria Only			
Mitochondria & Donor			
Mitochondria, Donor, & ADP			
Mitochondria, Donor, Uncoupler			

Table 2-3: Exercise 3: Oxygen (O₂) Consumption Rates for Mitochondria with Donors, ADP, and Inhibitors. Rates are expressed as 10⁻⁶ moles O₂/hr/mg protein.

Mixture in Chamber	Donors		
	Glutamate/Pyruvate	Succinate	Ascorbate
Mitochondria Only			
Mitochondria & Donor			
Mitochondria, Donor, and ADP			
Mitochondria, Donor, ADP, and Antimycin A			
Mitochondria, Donor, ADP, and Azide			
Mitochondria, Donor, ADP, and Rotenone			

Table 2-4: Exercise 4: Oxygen (O₂) Consumption Rates from Mitochondria with an ATPase Inhibitor and an Uncoupler. Rates are expressed as 10⁻⁶ moles O₂/hr/mg protein.

ATP Inhibitor before Uncoupler		Uncoupler before ATP Inhibitor	
Mixture in Chamber	Rate	Mixture in chamber	Rate
Mitochondria Only		Mitochondria Only	
Mitochondria & Succinate		Mitochondria & Succinate	
Mitochondria, Succinate, & ADP		Mitochondria, Succinate, & ADP	
Mitochondria, Succinate, ADP, & Oligomycin		Mitochondria, Succinate, ADP, & Uncoupler	
Mitochondria, Succinate, ADP, Oligomycin, Uncoupler		Mitochondria, Succinate, ADP, Uncoupler, Oligomycin	

Table 2-5: P:O Ratios for Three Electron Donors with ADP Present.

	Donors		
	Glutamate/Pyruvate	Succinate	Ascorbate
P:O Ratio			

Appendices

DO2-100 Current to Voltage Adapter

The DO2-100 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.

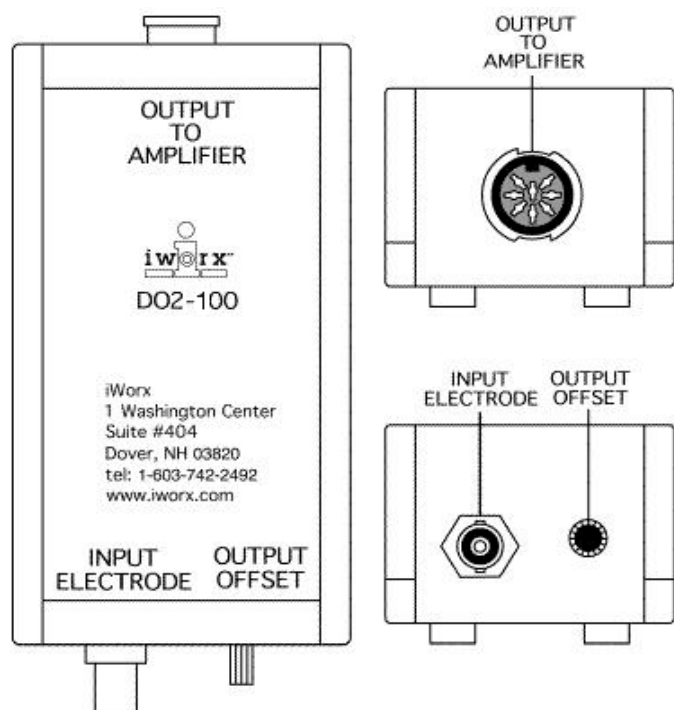


Figure 2-5: Top and side views of the DO2-100 current to voltage adapter.

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.
- 6 For short-term storage, the electrode can be left in room air with membrane housing still attached.

Isolation of Mitochondria

A variety of techniques for isolating mitochondria from the different tissues have been published and are available from sources on the Internet. Liver tissue has a high concentration of mitochondria and homogeneous cells that make it relatively easy to prepare isolated mitochondria. Some of the published techniques for isolating mitochondria from liver tissue are less time consuming. However, these quicker methods usually yield a lower concentration of mitochondria in an isolate that also contains more impurities which can dramati-

cally reduce the *viability* of the mitochondria. For example, a mitochondrial suspension containing a high concentration of lipids is functional for a shorter period of time than one that has a lower concentration of lipids. Lipids actually cause the mitochondria to lose their ability to maintain the chemiosmotic gradient needed for ATP synthesis. Good isolation techniques have steps which separate the lipids from the mitochondria using centrifugation.

A very successful technique for isolating mitochondria from liver tissue follows:

- 1 All materials that will contact liver tissue, homogenate, or mitochondria should be ice-cold throughout the procedure. All items that come into contact with tissues, homogenates, or organelles should be rinsed with homogenization or reaction medium before using. Wash glassware with water only; **no soap**.
- 2 For a lab section that has 12 work stations, a total of three rat livers will be needed to provide enough mitochondrial suspension for the exercises in this experiment. Select three healthy male Sprague-Dawley rats (250-300g body weight). Remove their food 24 hours before isolating the mitochondria from their livers. Continue to provide the rats with water.
- 3 After the 24 hour starvation period, anesthetize the rats with an appropriate anesthesia that is approved by your institution's animal use committee. Suitable mitochondria can be prepared from rats anesthetized in a CO₂ chamber until they stop breathing. Remove the rats from the chamber and decapitate them immediately. Allow as much blood to drain from the animal as possible.
- 4 Work quickly to isolate and remove the liver tissue from the peritoneal cavity. Rinse the tissue with ice-cold homogenization medium to remove excess blood, and store the tissue in ice-cold homogenization medium.
- 5 Remove the liver tissue from the medium. Blot the tissue dry with lab wipes (Kim Wipes) and weigh the tissue. Place the tissue in a clean Petri plate. Cut the tissue into small pieces (0.5 cm on a side). Then, mince the pieces for about one minute with a razor blade.
- 6 Place the tissue in a 50 ml homogenizer filled with about 35 ml of ice-cold homogenizing medium. If the homogenizer is too full, split the tissue into two aliquots.
- 7 If you are using a glass homogenizer with a Teflon pestle, homogenize the tissue with six up and down strokes with the speed set at 500 rpm. Pour the homogenate into 50 ml centrifuge tubes and store on ice.
- 8 Repeat Steps 4 through 6 for the two other rat livers.
- 9 Balance the pooled homogenate between four 50ml centrifuge tubes and centrifuge at 4°C and 800X G for 10 minutes.
- 10 As quickly as possible, collect the supernatants and pool them together in a rinsed, ice-cold beaker. Distribute the pooled supernatants into 8 centrifuge tubes, balance the tubes, and bring each tube to full volume with ice-cold homogenization medium.
- 11 Centrifuge this supernatant at 4°C and 10,000X G for 10 minutes.
- 12 After centrifugation, carefully remove the fat coat floating on top of the supernatant with a lab wipe (Kim Wipe). Then, carefully decant the supernatant. Save the pellets.
- 13 Manually resuspend the pellets with ice-cold homogenization medium. Use a small glass pestle to gently dislodge the pellet from the walls and bottom of the tube. Resuspend the pellet completely. Make sure there are no aggregated pellet pieces floating in the resuspended solution.
- 14 Bring the tubes back up to volume with homogenization medium and centrifuge the tubes at 4°C and 3,000X G for 5 minutes.
- 15 Carefully decant the supernatant (containing the mitochondria) from each tube into a new centrifuge tube. Take care to leave any solids behind.
- 16 Centrifuge the supernatant at 4°C and 10,000X G for 10 minutes.
- 17 Discard the supernatant. Occasionally, a "fluffy white" layer overlays the mitochondrial pellet. Add a few milliliters of homogenization medium to the tube, gently slosh it around, and pour off the "fluffy layer"
- 18 Resuspend the pellet as done in Step 13.
- 19 Centrifuge the supernatant at 4°C and 9,000X G for 10 minutes.
- 20 The supernatant should be clear. Pour it off. The pellet contains the mitochondria.
- 21 To extend their period of functionality, mitochondria can be kept cold in pellet form. This form minimizes the exposure of the mitochondria to oxygen and maintains their *dormancy* until they are mixed with the oxygen-rich reaction mixture.
- 22 When you are ready to use the mitochondria, resuspend each mitochondrial pellet with a few milliliters of mitochondrial reaction mixture and a small glass pestle. Do this lightly, but thoroughly.
- 23 Pour the resuspended mitochondria from all centrifuge tubes into a clean, rinsed, and ice-cold flask. Add mitochondrial reaction mixture until the total volume in the flask is 200 milliliters.
- 24 Perform a protein analysis to determine the concentration of mitochondrial protein in the mitochondria-reaction mixture suspension. This preparation usually yields a final suspension with 1.5 to 2.0 mg protein for each milliliter.

Reagents

- 1 Mitochondrial homogenization medium, pH 7.4, in deionized water:

200 mM Mannitol
70 mM Sucrose
2 mM HEPES
925 ml deionized water

Mix until all components dissolved. Add 0.5g of BSA (0.5g/L) by sprinkling on the surface of the medium and allow to dissolve. Do not stir! Adjust pH to 7.4. Add deionized water to bring final volume to 1000 ml. Make fresh daily; cool to 4°C before use.

- 2 Mitochondrial reaction mixture, 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.

Substrates

- 1 0.50 M Pyruvate/ 0.50 M Glutamate

4.12 g of Pyruvic acid, sodium salt and 6.34 g of Glutamic acid, sodium salt in 75 mls of mitochondrial reaction mixture (pH7.5). Mix. Dispense 1ml/tube in color-labeled tubes. Cap and store frozen.

- 2 1.0 M Sodium Succinate

20.25 g of Sodium succinate in 75 mls of mitochondrial reaction mixture (pH7.5). Mix. Dispense 1ml/tube in color-labeled tubes. Cap and store frozen.

- 3 Ascorbate/ TMPD, make fresh before each lab.

1.76 g of Ascorbic acid, sodium salt and 0.06 g TMPD in 10 mls of mitochondrial reaction mixture (pH7.5). Mix. Dispense 1ml/tube in color-labeled tubes. Cap and store on ice.

Energy Acceptor

- 1 0.03 M ADP

1.05 g of ADP in 75 mls of mitochondrial reaction mixture (pH7.5). Mix. Dispense 1ml/tube in color-labeled tubes. Cap and store frozen.

Uncoupler

- 1 0.01M Dinitrophenol (Uncoupler)

0.075 g of Dinitrophenol in 75 mls of mitochondrial reaction mixture (pH7.5). Mix. If it does not go into solution, add 1 pellet of NaOH. Dispense 1ml/tube in color-labeled tubes. Cap and store frozen.

Electron Transport Chain Inhibitor

- 1 0.0001M Rotenone

0.003 g of Rotenone in 75 mls of 95% Ethanol. Mix. Dispense 1ml/tube in color-labeled tubes. Cap and store frozen.

- 2 Antimycin A

10 mg of Antimycin A in 50 mls of 70% Ethanol. Mix. Dispense 1.66ml/tube in color-labeled tubes. Cap and store frozen.

- 3 1.0 M Azide

4.88 g of Sodium azide in 75 mls of mitochondrial reaction mixture (pH7.5). Mix. Dispense 1ml/tube in color-labeled tubes. Cap and store frozen.

Phosphorylation Inhibitor

Oligomycin

5 mg of Oligomycin in 10 mls of 70% Ethanol (very soluble). Mix. Dispense 0.33ml/tube in color-labeled tubes. Cap and store frozen.

