Experiment CM-3: Mitochondrial Respiration

Equipment Required

PC or Mac computer

IXTA, USB cable, power supply

ISE-730 Dissolved oxygen electrode

RPC-100 respiration/photosynthesis chamber

Magnetic stir motor, stir bar, and motor controller

10µl micropipette and gel-loading tips

Vortex mixer

See Appendix:

- Mitochondrial suspension
- Glutamate/Pyruvate, Succinate, Ascorbate electron donor solutions
- Uncoupler solution
- Rotenone, Antimycin A, Sodium Azide electron transport chain inhibitor solutions
- Concentrated O_2 depletion solution (1.5 M Sodium Dithionite)

Squirt bottle filled with deionized water

Pasteur pipet with plastic tip

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

Dissolved Oxygen Electrode Setup

1. Locate the dissolved oxygen electrode and plug it into channel A5 on the front of the TA.
2. Place the small magnetic stir bar in the bottom of the chamber.
3. Fill the chamber with room temperature deionized water.

Figure CM-3-S1: Dissolved oxygen electrode connected to an IXTA.

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.*

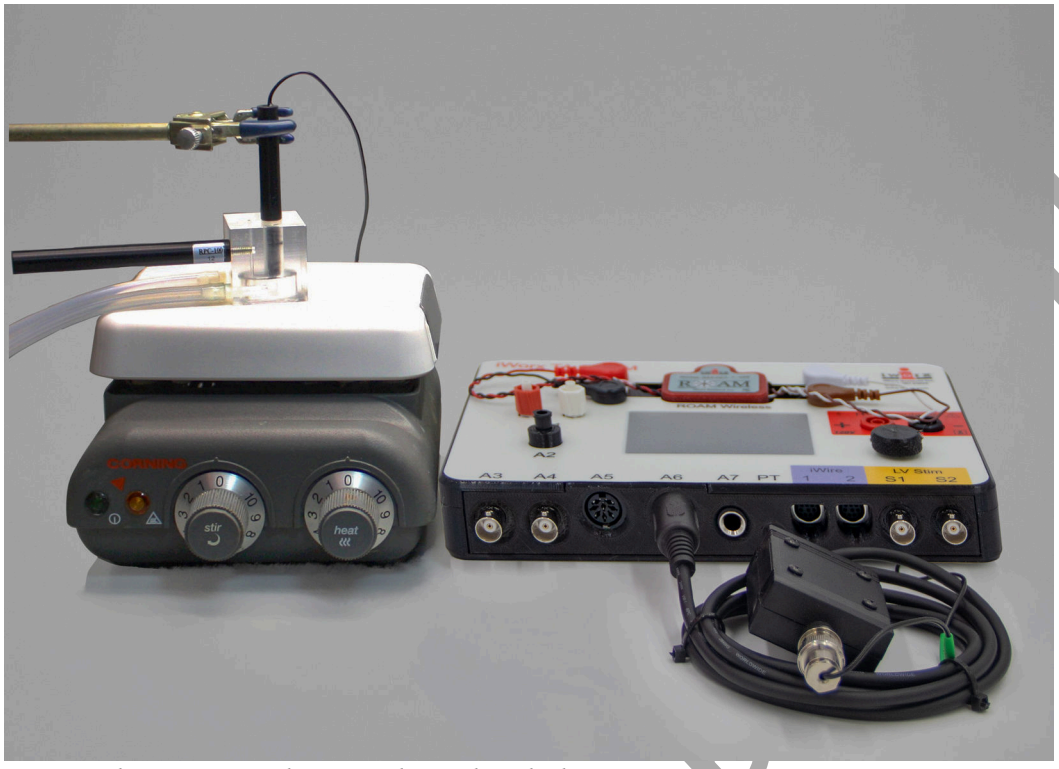

Figure CM-3-S2: The oxygen polarograph used with the IXTA.

Calibration of Dissolved Oxygen Electrode

Aim: To calibrate the dissolved oxygen electrode.

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

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

In the equation, α is the absorption coefficient of O_2 at the temperature, p is the vapor pressure of water at the temperature, P is the barometric pressure, and r% is the percent oxygen in the air. For example, at 26^{o}C and 760mmHg and a concentration of oxygen in air of 21%, S equals:

$$(0.02783/22.414\text{L/mole})(734.91\text{mmHg}/760\text{mmHg})(0.21) = 252\mu MO_2$$

Procedure

1. Fill the RPC-100 respiration chamber with fresh deionized water.
    - Turn off the stirrer if it is on.
    - Remove the dissolved oxygen electrode from the RPC-100 respiration chamber.

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

2. Type **Saturation-DI Water** in the Mark box.
3. Click Record on the Main window. The recording will eventually reach a stable level near the top of the recording channel. Click the mark button to mark the recording when the output of the electrode is constant. At this point in the recording, the output of the oxygen electrode is equal to the saturation concentration of oxygen in deionized water at room temperature.
4. Follow the procedure described in Step 1 to replace the deionized water in the RPC-100 chamber with zero-percent oxygen calibration solution at room temperature. Make sure there is enough solution in the chamber to submerge the tip of the oxygen electrode.
5. Type **No Oxygen** in the Mark box.
6. The recording will eventually reach a stable level near the bottom of the recording channel. Click the mark button to mark the recording when the output of the electrode is constant. At this point in the recording, the output of the oxygen electrode is equal to no oxygen being dissolved in deionized water at room temperature.
7. Click Stop to halt the recording.
8. Select Save As in the File menu, type a name for the file. Click on the Save button to save the data file.
9. Prepare the chamber for Exercise 1:
    - Turn off the stirrer, and then remove the electrode from the chamber.
    - Hold the electrode over the beaker used for collecting waste liquid, and rinse the electrode with deionized water from a wash bottle. Blot any drops of water from the electrode. Place ithe electrode in a beaker of deionized water.
    - Remove the oxygen-depleted water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber ten times with deionized water.
    - Fill the chamber with deionized water. Replace the electrode in the chamber.

Units Conversion

1. Measure the temperature (in ^{o}C) in the lab room. Assume the barometric pressure in the lab room is one atmosphere (760mmHg) and the concentration of oxygen in the air is 21%. From Table 1 find the dissolved oxygen concentration ($[O_2]$) in deionized water at room temperature. This concentration will be used in Step 6 to calibrate the dissolved oxygen electrode.
2. Scroll to the beginning of the calibration data for the dissolved oxygen electrode.

3. Use the Display Time icons on the LabScribe toolbar to adjust the Display Time of the Main window to show the data collected at both the 100% and 0% saturation levels of oxygen in water on the Main window at the same time.
4. Click the Double Cursor icon so that two cursors appear on the Main window. Place one cursor on the flat section of data collected when the saturation of dissolved oxygen in water was 100% and the second cursor on the flat section of data collected when the saturation of dissolved oxygen in water was 0%.

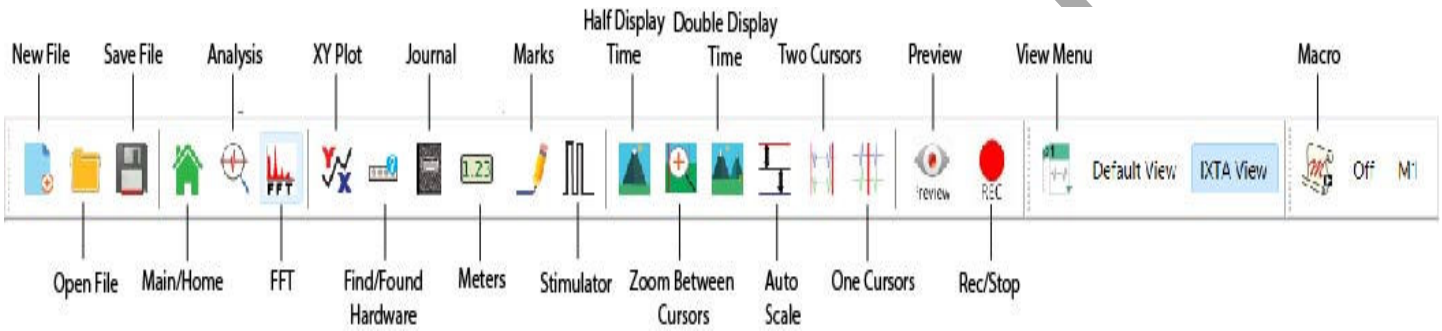

Figure CM-3-S3: The LabScribe toolbar.

Table CM-3-S1: Concentration of Oxygen [O2] in Air-Saturated Deionized Water at 1 Atmosphere.

Temp (°C)	O2 Abs Coeff (a)	H2O Vapor Press (p in mmHg)	[O2] (µM)
20	.03102	17.54	284
21	.03044	18.65	278
22	.02988	19.83	273
23	.02934	21.07	267
24	.02881	22.38	262
25	.02831	23.76	257
26	.02783	25.09	252
27	.02736	26.74	247
28	.02691	28.35	243
29	.02649	30.04	238
30	.02608	31.82	234

5. To convert the output of the dissolved oxygen electrode from a voltage to the molarity of dissolved oxygen in a sample:
    - Click on the arrow next to the title of the [Oxygen] channel to open the channel menu.
    - Select Units from the channel menu and Simple from the Units submenu.
6. The Simple Units Calibration window will appear. On this window:
    - Select 2 point calibration from the pull-down menu in the upper-left corner of the window.
    - Put a check mark in the box next to Apply units to all blocks.
    - Notice that the voltages from the positions of the cursors are automatically entered into the value equations.
    - From Table 1 find the concentration of dissolved oxygen in water at the room temperature that is 100% saturated. Enter this concentration in the corresponding box to the right of the voltage at 100% oxygen saturation. Enter zero in the corresponding box to the right of the voltage for 0% oxygen saturation.
7. Enter the name of the units, µMolar, in box below the concentration. Click OK.

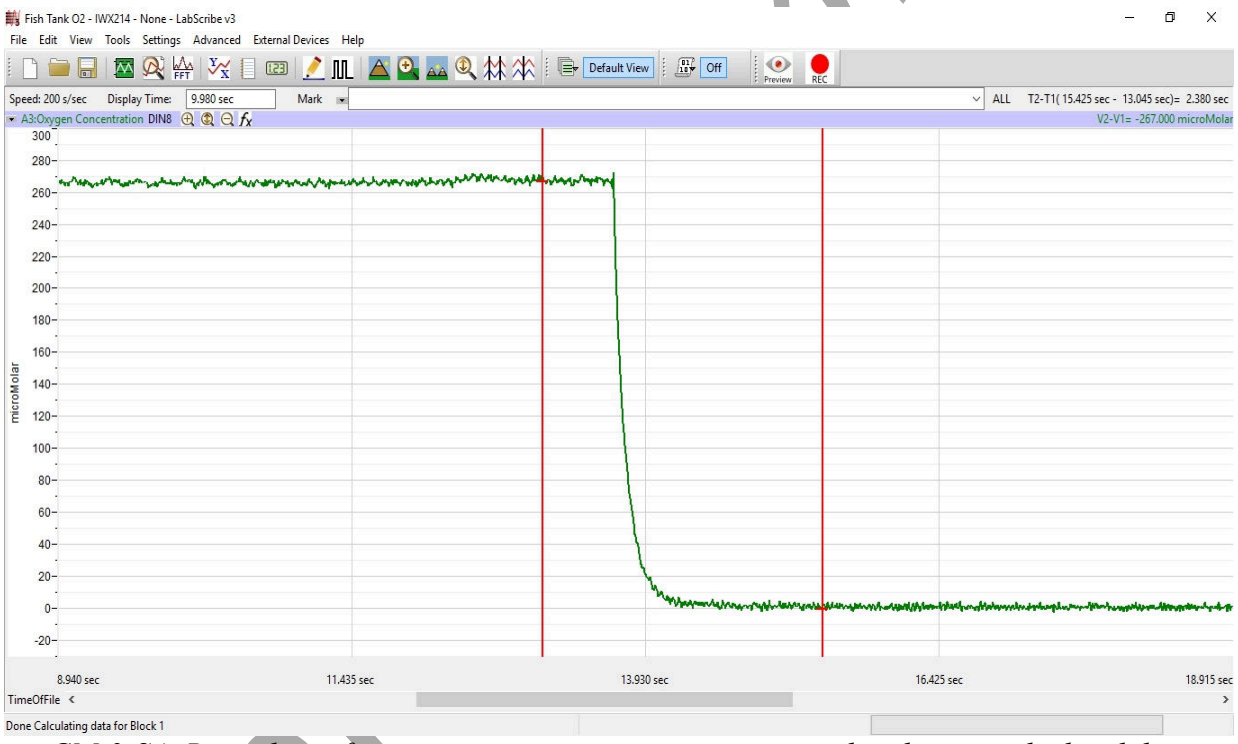

Figure CM-3-S4: Recording of oxygen concentrations in air saturated and oxygen depleted deionized waters used to convert the units of the Y-axis from voltage to O_2 concentration (µMolar).

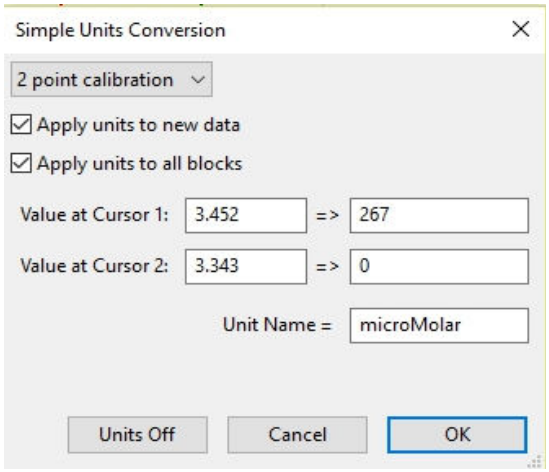

Figure CM-3-S5: The Simple Units Conversion dialogue window with the voltages at the cursors set to equal the dissolved oxygen concentrations used in calibration.

Experiment CM-3: Mitochondrial Respiration

Exercise 1: 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.

Approximate Time: 45 minutes

Procedure

1. Turn off the stirrer for the chamber. Remove the electrode from the chamber and place it in a beaker of deionized water. Remove the deionized water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber two times with deionized water.
2. 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.
3. Check the chamber for the presence of bubbles. If bubbles are present, turn off the stirrer, allow the bubble to rise to the top. Remove the electrode and the bubble should burst. Replace the electrode, turn on the stirrer, and check for bubbles, again.
4. Type **Endogenous Rate** in the Mark box.
5. Click Record and click the mark button to mark the recording. Record the endogenous rate of respiration of the mitochondria until the recording is a line, not a curve.

Note: *The line should not be sloping up (O_2 evolution); it should be flat (no O_2 usage) or sloping down slightly (a little O_2 consumption). It could take as long as five minutes for the trace to become a straight line.*

6. As the recording continues, type **Glutamate/Pyruvate** in the Mark box. Click the mark button 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 Glutamate/Pyruvate until the recording returns to a straight line. This usually occurs within a minute. Continue to record.
8. Type the words **ADP Added to Glutamate/Pyruvate** in the Mark box. Click the mark button to mark the recording as a 10µl aliquot of ADP solution is added to the chamber through the reagent port.

9. Since ADP causes an increase in the rate of oxygen consumption, the slope of the recording will be steeper after ADP is added. When the limited amount of ADP in the chamber is exhausted, the rate of oxygen consumption returns to a slope similar to the one recorded before the addition of ADP. Continue to record for one minute after all the ADP was consumed.
10. Type **Uncoupler** in the Mark box. Click the mark button to mark the recording as a 10µl 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 in the File menu.
13. Remove the electrode from the chamber and rinse it well with deionized water and place it in a beaker of deionized water. Remove the fluid from the chamber using a Pasteur pipet with a plastic tip. Rinse the chamber ten times with deionized water.
14. Repeat Steps 2 through 13 for one of the other electron donors (Succinate or Ascorbate). Then, repeat these steps for the third and final electron donor.

Exercise 2: Effects of Inhibitors

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

Approximate Time: 45 minutes

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:
    - Electron donors and inhibitors will be added to the chamber in 10µl 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 recording becomes linear after the addition of ADP, add the inhibitor. When the recording becomes linear, again, Add the next electron donor to the chamber, followed by ADP and another inhibitor.
    - 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 Exercise 1.
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. Fill the chamber with deionized water and put the ISE-730 electrode back in the chamber.

Exercise 3: 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.

Approximate Time: 45 minutes

Procedure

1. Turn off the stirrer for the chamber. Remove the electrode from the chamber and place it in a beaker of deionized water. Remove the deionized water from the chamber with a plastic-tipped Pasteur pipet. Rinse the chamber two times with deionized water.
2. Fill the chamber with mitochondrial suspension. Carefully replace the electrode in the chamber and turn on the stirrer.
3. Check the chamber for the presence of bubbles. If bubbles are present, remove them by following the instructions provided earlier.
4. Type **Mitochondria Only** in the Mark box..
5. Click Record and click the mark button to mark the recording. As in Exercise 1, record the endogenous rate of respiration of the mitochondria until the recording is linear. Continue recording.
6. Type **Succinate** in the Mark box. Click the mark button to mark the recording as a 10µl aliquot of Succinate is added to the chamber. Continue recording
7. Type **ADP** in the Mark box. Thirty seconds after the addition of succinate, click the mark button to mark the recording as a 10µl aliquot of ADP is added to the chamber. Continue recording.
8. Type **Oligomycin** in the Mark box. Thirty seconds after the addition of ADP, click the mark button to mark the recording as a 10µl aliquot of Oligomycin is added to the chamber. Continue recording.
9. Type **Uncoupler** in the Mark box. Thirty seconds after the addition of Oligomycin, click the mark button to mark the recording as a 10µl aliquot of uncoupler is added to the chamber. Continue recording for 30 seconds.
10. Click Stop to halt recording.
11. Select Save in the File menu.
12. Remove the electrode from the chamber and rinse the electrode well with deionized water.

13. 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 this exercise, but reverse the order in which Oligomycin and the uncoupler are added to the chamber.

Data Analysis

Rate of Electron Transport

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

Early in this experiment, the oxygen probe was calibrated using air-saturated, deionized water at room temperature. Through this calibration, the Y-axis of the recording channel was converted from voltage to oxygen concentration or [Oxygen], which is expressed as $\mu MolarO_2$ and abbreviated μMO_2.

Change in O_2 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. Scroll through the recording and find the section of data recorded during the first experimental run with Glutamate/Pyruvate in Exercise 1.
2. Use the Display Time icons to adjust the Display Time of the Main window to show the complete experimental run on the Main window. The complete experimental run can also be selected by:
    - Placing the cursors on either side of the complete run; and
    - Clicking the Zoom between Cursors button on the LabScribe toolbar to expand the four selected breathing cycles to the width of the Main window.
3. Click on the Analysis window icon in the toolbar or select Analysis from the Windows menu to transfer the data displayed in the Main window to the Analysis window.
4. Look at the Function Table that is above the Oxygen Concentration channel displayed in the Analysis window. The functions, V2-V1, T2-T1, and Mean_dv/dt should appear in the table.
5. Once the cursors are placed in the correct positions for determining the change in the oxygen concentration in the chamber, the values of the parameters in the Function Table can be recorded in the on-line notebook of LabScribe by typing their names and values directly into the Journal.

6. The functions in the channel pull-down menus of the Analysis window can also be used to enter the names and values of the parameters from the recording to the Journal. To use these functions:
    - Place the cursors at the locations used to measure the change in oxygen concentration.
    - Transfer the names of the mathematical functions used to determine the change in oxygen concentration to the Journal using the Add Title to Journal function in the Lung Volumes Channel pull-down menu.
    - Transfer the values for the change in oxygen concentration to the Journal using the Add Ch. Data to Journal function in the Oxygen concentration channel pull-down menu.
7. In the Analysis window, use the mouse to click on and drag the cursors to specific points on the recording to measure the following rates:
    - Endogenous rate, which is the rate of oxygen consumption by the mitochondrial mixture in the absence of electron donors, ADP, or uncouplers. Set the cursors ten seconds apart on a linear section of the recording of the endogenous rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration (µMolar/sec) over that ten-second period.
    - Glutamate/Pyruvate rate, which is the rate of oxygen consumption by the mitochondrial mixture in the presence of the first electron donor tested. Set the cursors ten seconds apart on a linear section of the recording of the Glutamate/Pyruvate rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration (µMolar/sec) over that ten-second period.
    - Glutamate/Pyruvate + ADP rate, which is the rate of oxygen consumption by the mitochondrial mixture in the presence of the first electron donor tested and ADP. Set the cursors ten seconds apart on a linear section of the recording of the Glutamate/Pyruvate + ADP rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration (µMolar/sec) over that ten-second period.
    - Glutamate/Pyruvate + Uncoupler rate, which is the rate of oxygen consumption by the mitochondrial mixture in the presence of the first electron donor tested and an uncoupler. Set the cursors ten seconds apart on a linear section of the recording of the Glutamate/Pyruvate + Uncoupler rate. The value for the Mean_dv/dt is the average rate of change of oxygen concentration (µMolar/sec) over that ten-second period.
8. Record the values for these four rates in the Journal using the one of the techniques described in Steps 5 or 6.
9. Measure and record the endogenous, donor, donor + ADP, and donor + uncoupler rates for the other electron donors.

Standardizing the Rate of O_2 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 μMO_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:

   $(0.666\mu MO_2/sec)(1.2ml) = 0.799$ nmoles O_2 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 (μmoles O_2) per hour:

   $(0.799 \times 10^{-9}$ moles O_2/sec)(60sec/min)(60min/hr) = 2880×10^{-9} moles O_2/hr =

   2.88×10^{-6} moles O_2/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:

   (2.5mg prot/ml)(1.2ml in chamber) = 3.0 mg protein in chamber.

4. 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:

   $(2.88 \times 10^{-6}$ moles O_2 consumed/hr)/(3mg protein) = 0.960×10^{-6} moles O_2/hr/mg protein = 0.96 μmoles O_2/hr/mg protein.

5. Calculate the rates of oxygen consumption (or production) for each section of each experimental run using the steps presented above.
6. Enter the oxygen consumption or production rates for each section of each experimental run in the appropriate tables:
   - Donors and uncouplers in Table 1.
   - Donors and inhibitors in Table 2.
   - Inhibitors and uncouplers in Table 3.

P to O Ratios

Calculate the P:O ratios for each of the three donors: glutamate/pyruvate, succinate, and ascorbate.

1. Determine the amount of oxygen consumed by the mitochondria due to the presence of ADP in the polarograph chamber:
    - Subtract the oxygen consumption rate for mitochondria in the presence of an electron donor from the oxygen consumption rate for mitochondria in the presence of an electron donor and ADP. The result is the net rate of oxygen consumption due to ADP being present. For example:

3.7×10^{-6} moles O2/hr/mg protein (e^- donor and ADP) - 0.6×10^{-6} moles O2/hr/mg protein (e^- donor) =

3.1×10^{-6} moles O2/hr/mg protein (ADP only)

    - 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:

$(3.1 \times 10^{-6}$ molesO_2/hr/mg protein)(3mg protein) = 9.3×10^{-6} moles O_2/hr

    - Multiply the moles of oxygen consumed per hour due to ADP by the time it took to consume all the ADP in the chamber. The product is the total amount of oxygen (O_2) consumed when ADP was available. In our example, if 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:

$(9.3 \times 10^{-6}$ moles O_2/hr)(30s)(hr/3600s) = 0.078×10^{-6} 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:

$(0.078 \times 10^{-6}$ moles $O_2)(2O/O_2) = 0.155 \times 10^{-6}$ 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 µl (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 µmoles (0.3×10^{-6} moles):

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

4. Divide the moles of ADP added to the chamber (Step 18) by the moles of atomic oxygen (O) consumed (Steps 16 and 17) 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.
6. Enter the P:O ratios for each electron donor in Table 4.

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 electron 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 electron 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 electron 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 CM-3-L1: Exercise 1: Oxygen (O_2) Consumption Rates for Mitochondria and Electron Donors, ADP, and Uncoupler. Rates are expressed as 10^{-6} moles O_2/hr/mg protein.

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

Table CM-3-L2: Exercise 2: Oxygen (O_2) Consumption Rates for Mitochondria with Electron Donors, ADP, and Inhibitors. Rates are expressed as 10^{-6} moles O_2/hr/mg protein.

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

Table CM-3-L3: Exercise 3: Oxygen (O_2) Consumption Rates from Mitochondria with an ATPase Inhibitor and an Uncoupler. Rates are expressed as 10^{-6} moles O_2/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 CM-3-L4: P:O Ratios for Three Electron Donors with ADP Present.

	Electron Donors		
	Glutamate/Pyruvate	Succinate	Ascorbate
P:O Ratio			