



Each reaction mixture contains a very high concentration of substrate so that there will still be a relatively high substrate concentration during the first 5 or 10 minutes of the assay.

The velocity of the reaction will be monitored by measuring the change in absorbance at 600 nm ( $\Delta A_{600}$ ) with time. Recall that the change in  $A_{600}$  shows the extent to which the dye DCPIP is reduced by NADH or  $FADH_2$ . For each of three enzyme concentrations, you will plot the change in absorbance versus time and, from the initial slope of each curve, determine the initial velocity. The initial velocities can then be plotted against enzyme concentration.

The velocity of an enzymatic reaction can be altered by the presence of an inhibitor. You will study the effect of the inhibitor malonate ( $^-OOC-CH_2-COO^-$ ) on the succinate dehydrogenase reaction. A determination of the velocity in the presence of malonate may allow you to postulate the type of inhibition that it exhibits.

You will run three controls in these kinetic studies. In one control, the reaction mixture contains no azide. In a second control, there is no added succinate. The third control contains inactivated enzyme. The enzyme is inactivated by heating the mitochondrial suspension, thereby denaturing the proteins.

### Experimental Procedure

#### **Part I-** *Isolation of Mitochondria- Week 1*

You will isolate the mitochondrial fraction from cauliflower cells using the method of *differential centrifugation*. You will homogenize the cauliflower tissue in a mortar and pestle with a buffered, isotonic mannitol solution and a quantity of sand. After squeezing the homogenate through cheesecloth to remove the larger pieces, you will centrifuge the supernatant at a relatively low speed to pellet the nuclear fraction and whole cells. You will then decant the tube and centrifuge the supernatant at a relatively high speed to pellet the mitochondrial fraction. For all cell fractionation procedures, the solutions and containers must be kept ice-cold.

1. Using a single-edged razor blade, remove a total of 20 g of the outer 2-3 mm of the cauliflower surface.
2. Place the tissue in a chilled mortar with 20 ml of ice-cold mannitol buffer and 3 g of cold, purified sand. Grind the tissue with a chilled pestle for 3 minutes. Add 20 ml more buffer and grind for 1 additional minute.
3. Filter the suspension through four layers of cheesecloth into a chilled 50 ml centrifuge tube and also wring out the juice into the tube.
4. Centrifuge the filtrate at  $1200 \times g$  ( $\sim 3,300$  rpm in the JA-20 rotor) for 10 minutes at  $4^\circ C$ . Make sure that the centrifuge tubes are balanced. Proceed to **Parts II & III** while you wait.
5. Decant the supernatant into a clean, chilled centrifuge tube(s) and spin at  $26,000 \times g$  ( $\sim 14,500$  rpm in the JA-20 rotor) for 15 minutes at  $4^\circ C$ . Again, be sure that the tubes are balanced. The nuclear pellet may be discarded.
6. Decant and discard the postmitochondrial supernatant and add 10.0 ml of ice-cold mannitol buffer to the mitochondrial pellet.

7. With a rubber policeman, scrape the mitochondrial pellet from the wall of the centrifuge tube and then with a Pasteur pipette, thoroughly resuspend the sediment in the mannitol buffer. It is important that the clumps be completely dispersed.
8. Place the mitochondrial suspension on ice, where it should be kept for the entire experiment.

**Part II-** *Assay for Dehydrogenases- Week 1*

1. Set up a series of reaction mixtures based on the table below at room temperature in clear microcentrifuge tubes. The available substrates are citrate, isocitrate,  $\alpha$ -ketoglutarate, succinate, fumarate, malate, and oxaloacetate. Set up two reactions for each substrate corresponding to reactions 2 and 3 (a total of 15 tubes). Add all of the components, **except for the mitochondrial preparation**, in the order indicated. Check to make sure that the  $\text{NAD}^+$  is in solution.

Solution	Reaction (volumes in $\mu\text{L}$ )		
	1	2	3
mannitol buffer	400	225	160
0.1 M substrate	--	50	50
0.01% DCPIP	50	50	50
0.01 M $\text{NAD}^+$	--	--	65
0.04 M sodium azide	50	50	50
mitochondrial preparation	--	125	125

2. Add the mitochondrial preparation to each specified reaction and mix well by inversion. As soon as the preparation has been added to each tube, record your observations and note the time.
3. Incubate the reactions at room temperature for 60 min in the dark. Mix the solutions occasionally by inverting the tubes.

**NOTE:** In the meantime, proceed with the rest of the lab!

4. Examine the tubes visually. It will be most helpful to place them in a well-lit area. Note the intensity of the blue color of the mixtures, especially in comparison with the control tube (1). Rank the mixtures, to the best of your ability, by color intensity. Alternatively, group them in similar categories (e.g., no color, some color, lots of color).

**Part III- Kinetic Analysis of Succinate Dehydrogenase Activity- Week 1**

1. Label 10 cuvettes as shown in the table below. Except for the ice-cold mitochondrial suspension, all solutions should be kept at room temperature.
2. **Prepare the mitochondrial suspension for cuvette 7 by heating a 0.6 ml aliquot to 100 °C for 5 min and then cooling in an ice bath. Students often forget to do this!!!**
3. To all cuvettes, add the various solutions given across the top of the table, **except for the mitochondrial suspension**. First, add the correct volume of assay buffer to all tubes. Then, in the same manner, add the correct volumes of azide\*, DCPIP\*, malonate\*, and succinate as indicated in the table. Cover each cuvette with parafilm and invert to mix contents.

Cuvette	Mannitol Buffer	Azide* (0.04 M)	DCPIP* ( $3 \times 10^{-4}$ M)	Malonate* (0.2 M)	Succinate* (0.2M)	mitoch. suspension
Blank 1	2.2 ml	0.3 ml	--	--	0.3 ml	0.2 ml
1	1.9 ml	0.3 ml	0.3 ml	--	0.3 ml	0.2 ml
Blank 2	1.8 ml	0.3 ml	--	--	0.3 ml	0.6 ml
2	1.5 ml	0.3 ml	0.3 ml	--	0.3 ml	0.6 ml
Blank 3	2.0 ml	0.3 ml	--	--	0.3 ml	0.4 ml
3	1.7 ml	0.3 ml	0.3 ml	--	0.3 ml	0.4 ml
4	1.5 ml	0.3 ml	0.3 ml	0.2 ml	0.3 ml	0.4 ml
5	2.0 ml	--	0.3 ml	--	0.3 ml	0.4 ml
6	2.0 ml	0.3 ml	0.3 ml	--	--	0.4 ml
7 (heated)	1.7 ml	0.3 ml	0.3 ml	--	0.3 ml	0.4 ml **

\* Poisonous, do not allow contact with skin; wear gloves.

\*\* This aliquot should have been denatured by heating.

4. Thoroughly resuspend the mitochondrial suspension, then add the correct volume of mitochondrial suspension to each cuvette. As soon as the mitochondrial suspension has been added, cover the cuvette with parafilm, invert twice to mix the contents, and record the time.
5. Use blank 1 for the calibration blank just prior to recording the 0-minute  $A_{600}$  reading for cuvette 1 (note that you should read the actual  $\lambda_{max}$  for your particular spectrophotometer, which may not be exactly 600 nm; once you select an  $\lambda_{max}$ , use it for the entire experiment). Similarly, use blank 2 for the calibration blank for cuvette 2 and blank 3 for cuvettes 3-7, obtaining a 0-min absorbance reading for all cuvettes.
6. At 5-minute intervals, measure the absorbance of the seven cuvettes for at least 45 minutes (if necessary). **Always remember to blank with the correct reference cuvette as in step 5.**

### **Analysis**

- For Part II, identify the dehydrogenases active in the mitochondrial preparation and rank them according to their relative specific activities. Does the presence of NAD<sup>+</sup> stimulate the activity of any or all of the dehydrogenase enzymes? Explain.
- For Part III, calculate for each cuvette the total change in absorbance ( $\Delta A_{600}$ ) at each time interval. The total change in absorbance is the difference between the 0-min reading for each cuvette and the reading for that cuvette at the specified time.
- Determine the reaction rates for all cuvettes by plotting the total change in absorbance versus time. Draw the best-fit curve for each plot.
- What volume of mitochondrial suspension gives the highest velocity? With what volume of mitochondrial suspension does the velocity level off most rapidly? How do you explain these observations?
- How does the rate in the reaction mixture with malonate (cuvette 4) differ from the rate in the comparable mixture without malonate? Explain. From your data, can you determine what type of inhibitor malonate is? What would you predict based on its structure?
- What is the evidence that the electron transport system is functioning in the isolated mitochondria?
- What is the evidence that there is very little endogenous succinate present in the mitochondrial fraction?
- How much reduction of DCPIP is due to factors other than succinate dehydrogenase?
- Plot the initial velocity versus enzyme concentration for cuvettes 1, 2, and 3. For the initial velocity, take the total change in absorbance after 5 min and divide by 5 to obtain the  $\Delta A/\text{min}$ . For the different enzyme concentrations, use the 3 volumes of mitochondrial suspension added: 0.2, 0.4, and 0.6 ml. The origin should be a fourth point for the line. Draw the best-fit line, and then comment on what happens to the initial velocity when the enzyme concentration is doubled and tripled.

### **Part IV- Enzyme Activity Assays- Week 2**

Your data from Week 1 should provide useful information about dehydrogenases present in mitochondria, as well as their possible requirements for NAD<sup>+</sup>. Your goal this week is to examine quantitatively one aspect of the kinetics of one or more dehydrogenases. (You should pick dehydrogenases that were quite active in Part II.) Possible experiments to design include, but are not limited to, the following:

- ✓ How do the kinetic constants (in terms of the substrate) compare for different dehydrogenases? You may examine different enzymes from the same source or the same enzyme from different sources (e.g., white asparagus, strawberries, radish...).
- ✓ What type of inhibitor is malonate? What is its  $K_i$ ?

Once you have decided which kinetic aspect of the dehydrogenases you wish to examine, come up with an experimental plan. You will need to review basic enzyme kinetics from BC 367 in order to design this experiment. Specifically, what parameter will you vary in your set of experiments? What data will you collect? How will you analyze it? What controls might you need to run? Once you have a plan in hand, you and your lab partner will need to schedule a time to meet with your instructor *well before lab* to discuss it. Otherwise, you may not have the materials available in lab to run your experiment. When you come to lab to implement your experiment, you can follow the general guidelines below. Of course, you will need to adapt these steps for your particular experiment. Write up a complete experimental protocol in your lab notebook for your pre-lab this week.

1. Repeat the mitochondrial preparation done last week (as described in Part I), possibly with a source in addition to cauliflower, but scale it up by a factor of two to end up with 20 mL of mitochondria. Be sure to store the mitochondrial fraction on ice throughout the experiment.
2. Set up a test assay similar to that outlined in Sample 3 in the table of Part III. This will allow you to gauge how much mitochondrial suspension to use in subsequent trials. Note that if you add too much mitochondrial suspension, you run the risk of light-scattering, and poor data, because of the opacity of the sample. Note that you can use your test sample as your calibration blank in each case. Add all reagents but the mitochondria and DCPIP. You will need to calibrate quickly after adding the mitochondrial suspension (because the reaction will be proceeding as soon as you add it), then add the DCPIP and start acquiring data.
3. Monitor the reaction for 1 minute at the  $\lambda_{\text{max}}$ . If the rate is either too fast or too slow, adjust the amount of mitochondrial suspension and/or substrate and try again.
4. Once you have an observable rate, set up an analysis that varies the source of mitochondria, the substrate, or some other factor. For each trial, obtain the rate in terms of  $\Delta A/\text{min}$ .

### Analysis

- What interesting facts emerge from your data? Do your findings agree with what you have learned about the citric acid cycle and the enzymes present? Do they agree with the literature?
- Do any other experiments come to mind after carefully analyzing your data?

### *ASSIGNMENT*

Write a paper reporting your results for this experiment. Be sure to consider all the points and questions in the “Analysis” sections above. Please see the lab syllabus and the course web page for additional details of this assignment and for a link to the JACS Author Instructions web page.

### References

- A. Bregman (1990) Investigations in Cell Biology  
R. Boyer (2000) Modern Experimental Biochemistry, 3<sup>rd</sup> Edition