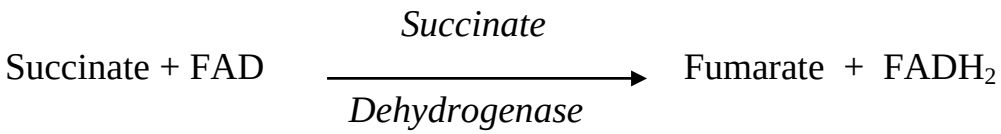
Assay of Mitochondrial Enzyme Activity in Mitochondrial Preparations

In this experiment you will assay the activity of the mitochondrial enzyme succinate dehydrogenase which catalyzes the following reaction:



The reduced form of the coenzyme ($FADH_2$) carries the protons and electrons to the electron transport chain, where ubiquinone accepts them and subsequently passes them down the chain to oxygen. The fumerate generated will continue to participate in the TCA cycle.

The activity of succinate dehydrogenase can be assayed by measuring the $FADH_2$ produced using an artificial electron acceptor; in this case, the dye 2,6-dichlorophenolindophenol (DCIP). The oxidized form of the dye will be reduced by accepting electrons from $FADH_2$ (instead of ubiquinone), with a corresponding change in color from blue to colorless. This change can be measured spectrophotometrically. To ensure that the electrons are passed to the dye and are not funneled into the electron transport chain, the poison sodium azide is added to the reaction to block the final transfer of electrons from cytochrome a_3 to oxygen. This effectively bottlenecks the electron transport chain, preventing $FADH_2$ from passing along its electrons to ubiquinone.

In this experiment, you will perform an enzyme assay on the mitochondrial prep and the post mitochondrial supernatant prepared in the previous experiment. You will determine the activity of the enzyme in each fraction by relating the oxidation of succinate by succinate deydrogenase to a decrease in absorbance resulting from the reduction of DCIP.

Be sure to refer to the "Cell Biology Lab Report Guidelines" found in the appendix of your lab manual.

Important note: Sodium azide is a poison, so please wear gloves and handle according to appropriate lab guidelines. Solutions must be discarded into marked waste receptacles. Do not pour it into the sink!

A. DCIP Standard Curve

Note that the mitochondrial and supernatant preps (MS & MP from last week) should be taken out of the freezer, thawed and kept on ice at all times. Just before use in the enzyme assay, it should be thoroughly mixed by gently pipetting up and down several times with a chilled Pasteur pipette.

1. Label and create three series of the six tubes listed in the table below (except for #1 which is the blank – you will not need 3 replicates of that tube).
2. Add the various components to each test tube as shown below. Cover each tube with a cap and invert several times to mix.
3. Set the wavelength on the Spec 20 to 610nm. Read and record the absorbance (A_{610}) of each tube, using tube #1 as a blank.
4. Repeat with the second and third sets of tubes 2-6 so that you have three sets of data for the standard curve.
5. Create a DCIP standard curve by plotting the A_{610} of each tube against the DCIP concentration.

DCIP Standard Curve Table

Tube	**Assay Buffer (ml)**	**200 µM DCIP (µl)**	**DCIP End Concentration (µM)**	**Absorbance at 610 nm** (A_{610}) **Series one**	**Absorbance at 610 nm** (A_{610}) **Series two**	**Absorbance at 610 nm** (A_{610}) **Series three**
1	5	0	0	0	0	0
2	4.9	100	4			
3	4.75	250	10			
4	4.6	400	16			
5	4.5	500	20			
6	4.25	750	30			

B. Succinate Dehydrogenase Assay (Carry out one set at a time)

1. Label and prepare sixteen experimental test tubes according to the chart below. Wait to add the succinate, DCIP and inhibitor to reaction tubes until you start measurements at step 4b. *Gently mix the mitochondrial suspension and the post mitochondrial supernatant using a pipette before addition to any experimental tubes.*
2. Cap each tube and slowly invert several times to gently mix the contents. Keep them all incubating on ice as you proceed, until you are ready to start the measurements of each set.

Reaction Set	Tube label	Assay Buffer (ml)	Azide (ml)	Mito-chondria (ml)	Super-natant (ml)	Assay Buffer with Glycerol (ml)	Inhibitor (µl)	Succinate (ml)	DCIP (ml)
	SET 1: Mitochondria								
Blank	MB	3.0	0.5	0.5	0	0	0	1.0	0
Reaction 1	MR1	2.5	0.5	0.5	0	0	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 2	MR2	2.5	0.5	0.5	0	0	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 3	MR3	2.5	0.5	0.5	0	0	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
	SET 2: Supernatant								
Blank	SB	3.0	0.5	0	0.5	0	0	1.0	0
Reaction 1	SR1	2.5	0.5	0	0.5	0	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 2	SR2	2.5	0.5	0	0.5	0	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 3	SR3	2.5	0.5	0	0.5	0	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
	SET 3: Control								
Blank	CB	3.0	0.5	0	0	0.5	0	1.0	0
Reaction 1	CR1	2.5	0.5	0	0	0.5	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 2	CR2	2.5	0.5	0	0	0.5	0	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 3	CR3	2.5	0.5	0	0	0.5	0	1.0 ***Add at step4b***	0.5 ***Add at step4b***
	SET 4: Inhibitor								
Blank	IB	2.9	0.5	0.5	0	0	100µl	1.0	0
Reaction 1	IR1	2.4	0.5	0.5	0	0	100µl	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 2	IR2	2.4	0.5	0.5	0	0	100µl	1.0 ***Add at step 4b***	0.5 ***Add at step4b***
Reaction 3	IR3	2.4	0.5	0.5	0	0	100µl	1.0 ***Add at step 4b***	0.5 ***Add at step4b***

3. Use the Blank to zero the Spec 20 for Set 1:
    a. **Equilibrate the test tube** blank of the first reaction set (MB, the blank of the mitochondrial set) to room temperature by immersing it in the beaker of tap water for a few seconds. This step should eliminate condensation on the tube during the assay. While it sits in the beaker of water zero the spec:
    b. **Zero the spectrophotometer:** Without a tube in the sample slot, use the front-left knob to place the needle exactly at 0% transmittance/infinite absorbance. Now you are ready to blank it with the tube soaking in water, remove it, wipe it dry.
    c. **Blank the spec**: Place the dried test tube blank (MB) in the Spec-20, close the cover, use the right knob to place the needle at 100% transmittance/0 absorbance. Remove the blank and keep at room temperature.

4. Now set up the three reaction tubes for Set 1 (READ THESE DIRECTIONS CAREFULLY AND PLAN YOUR ACTIONS WITH YOUR GROUP MEMBERS):
    a. Equilibrate the three reaction test tubes of the set (tubes MR1, MR2, and MR3 for the mitochondrial set) to room temperature by placing them in the beaker of tap water for at least 30 seconds to eliminate condensation on the tubes.
    b. While the tubes sit, carefully remove the caps and prepare to **add 1ml of succinate and 0.5ml of DCIP to each of the three reaction tubes.**
    c. Add succinate and DCIP to tube MR1, immediately start timing, cap the tube and gently invert to mix, wipe it with a kimwipe, place it in the spec and record the absorbance at 610 nm in the appropriate table below. Add succinate and DCIP to tube MR2 15 seconds after adding them to tube MR1, and measure tube MR2. After 15 more seconds, add succinate and DCIP to tube MR3 and measure tube MR3. The idea is to *stagger* your three reaction tubes by 15 seconds to give you time to measure them one after another. This way by the time you are done measuring a time point for tube MR3, you are [almost] ready to measure the next time point for tube MR1, and so on.
    d. Take a reading of all three tubes, every 2 minutes (15 seconds between the three replicates) and record in the data table for 20 minutes. Remember to invert and wipe the tube between each reading; do not leave it in the machine.
    e. When you have finished measuring the three reactions of the set, place those four tubes aside and set up the measurements for the next set. Continue measuring one set at a time until all four sets are measured.

    ***Note** that when you get to set four, the inhibitor set, add the inhibitor along with the succinate and DCIP, just prior to starting the measurements.

	Data table 1					
Time, minutes	**Set 1** Mitochondria			**Set 2** Supernatant		
	Reaction 1	Reaction 2	Reaction 3	Reaction 1	Reaction 2	Reaction 3
0						
2						
4						
6						
8						
10						
12						
14						
16						
18						
20						

	Data table 2					
Time, minutes	**Set 3** Control			**Set 4** Inhibitor		
	Reaction 1	Reaction 2	Reaction 3	Reaction 1	Reaction 2	Reaction 3
0						
2						
4						
6						
8						
10						
12						
14						
16						
18						
20						

Processing data and lab report write-up:

These graphs should allow you to infer the activity of the enzyme succinate dehydrogenase by assaying it using the colorometric changes that DCIP undergoes (for each succinate oxidized, one DCIP is reduced).

1. Graph 1: On the same graph prepare a plot with A_{610} against time for:
    a. Mitochondrial suspension reactions
    b. Post-mitochondrial supernatant reactions
    c. Control tube reactions
    d. Inhibitor tube reactions

    Include error bars using standard deviations of each set.

2. Graph 2: To further quantitate enzyme activity, use the DCIP standard curve to determine the concentration of DCIP present at each time point for the mitochondrial suspension, the post-mitochondrial supernatant, and the control tubes. On the same graph prepare a plot of DCIP concentration against time for:
    a. Mitochondrial suspension
    b. Post-mitochondrial supernatant
    c. Control tube
    d. Inhibitor tube

3. Discuss how the results from these fractions compare with each other and with what you might expect.

4. Use your mitochondrial count from last lab to calculate an estimate of mitochondria per ml of the mitochondrial suspension assay tube of graph 2.

5. What is the enzyme activity of 100 mitochondria per minute? Use the slope of the equation from graph 2a and your estimate from number 4 above. Show your work.

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