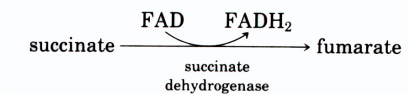
Krebs Cycle Oxidations in Mitochondria

Pyruvate, the product of glycolysis, is oxidized to acetyl in the mitochorndria. Acetyl enters the Krebs cycle where it is completely oxidized; the electron carriers, NAD^+ and FAD, are reduced to NADH and $FADH_2$. Although oxygen is not consumed during the reactions of the Krebs cycle, the Krebs cycle cannot occur in the absence of oxygen. (Why?)

This exercise will look at the oxidation of succinate to fumarate, one of the steps of the Krebs cycle. The oxidation of succinate is catalyzed by the enzyme, succinate dehydrogenase. The two hydrogen atoms removed in the oxidation are transferred to FAD, forming $FADH_2$.



During experimental conditions, the hydrogen atoms can be transferred to alternative electron acceptors, such as 2,6-dichlorophenol indophenol (DPIP). DPIP in its oxidized state is blue. It loses color when reduced. We can measure the loss of color using a spectrophotometer. The rate of decolorization provides a means of measuring the reactions of the Krebs cycle.

In this exercise you will measure absorbance. DPIP has maximum absorbance of light at 600nm wavelength. Review the procedure for operating the spectrophotometer before doing the exercise.

Materials Needed

Spectrophotometer
8 Spectrophotometer cuvettes
8 Test tubes
2 50-ml beakers
Mitochondrial mixture extract from fresh lima beans (BeanJuice Supernatant)
Mitochondrial mixture extract from boiled lima beans (Boiled Bean Supernatant)
Dropper bottles of
- Succinate
- Malonate
- DPIP ***(Note: Poison!)***
- Phosphate buffer
- $HgCl_2$ ***(Note: Poison!)***

Procedure

1. Turn on the spectrophotometer. After it warms up, set the absorbance to 100%. Transmittance should be 0%. Set the wavelength to 600nm.
2. Preparation of Mitochondria Mixture. (This will have been done by the Laboratory Staff)
   - Blend 50g of lima beans (fresh, thawed frozen, or soaked dry beans) with phosphate buffer.
   - Centrifuge the solution for 5 minutes.
   - Decant the supernatant. This is your mitochondria source.

   Boil a portion (about 1/6) of the supernatant mixture for 5 minutes.

3. Set up 8 test tubes according to the directions in Table 1 below, **except do not add the succinate** until you are ready to read the absorbance using the spectrophotometer. Note: Tube 8 will be your "Blank" solution for standardizing the spectrophotometer. You will also need 8 spectrophotometer tubes.

Table 1

****Do not add the succinate until you are ready to run the Spectrophotometer.**

Tube#	Bean Juice Supernatant	Boiled Bean Supernatant	DPIP	Phosphate Buffer	$HgCl_2$	Malonate	Succinate**
1	---	0.3ml	0.3ml	4.3ml	---	---	0.1ml
2	0.3ml	---	0.3ml	4.4ml	---	---	---
3	0.3ml	---	0.3ml	4.3ml	---	---	0.1ml
4	0.3ml	---	0.3ml	4.0ml	---	0.3ml	0.1ml
5	0.3ml	---	0.3ml	3.8ml		0.3ml	0.3ml
6	0.3ml	---	0.3ml	4.0ml	0.3ml	---	0.1ml
7	0.3ml	---	0.3ml	4.2ml	---	---	0.2ml
8 (Blank)	0.3ml	---	---	4.6ml	---	---	0.1ml

4. Recheck the spectrophotometer to be sure the absorbance reads 100% and the wavelength is set to 600nm. Fill a spectrophotometer tube with the Blank solution (Tube 8) and place it into the spectrophotometer. Set the spectrophotometer to 100% transmittance and 0% absorbance. You are now ready to read DPIP absorbance in the experimental tubes.

Ideally steps 5 – 11 will be completed in about a two-minute time frame.

5. Add the succinate to Tube 1, cover it with parafilm and invert the tube to mix in the succinate. Fill a spectrophotometer tube with the Tube 1 solution and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
6. Fill a spectrophotometer tube with the Tube 2 solution (this tube has no succinate) and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
7. Add the succinate to Tube 3, cover it with parafilm and invert the tube to mix in the succinate. Fill a spectrophotometer tube with the Tube 3 solution and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
8. Add the succinate to Tube 4, cover it with parafilm and invert the tube to mix in the succinate. Fill a spectrophotometer tube with the Tube 4 solution and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
9. Add the succinate to Tube 5, cover it with parafilm and invert the tube to mix in the succinate. Fill a spectrophotometer tube with the Tube 5 solution and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
10. Add the succinate to Tube 6, cover it with parafilm and invert the tube to mix in the succinate. Fill a spectrophotometer tube with the Tube 6 solution and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
11. Add the succinate to Tube 7, cover it with parafilm and invert the tube to mix in the succinate. Fill a spectrophotometer tube with the Tube 7 solution and immediately read the absorbance and record the absorbance in Table 2. Also record the time of the reading.
12. Repeat your readings for each of spectrophotometer tubes 1 → 7 at 2-minute intervals recording your data in Table 2 for 16 – 20 minutes.

Table 2: Oxidation of Succinate in the Krebs Cycle

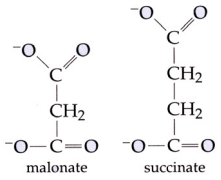
	Absorbance in Lima Bean Supernatant							
Time Started								
Reading	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
0								
2								
4								
6								
8								
10								
12								
14								
16								
18								
20								

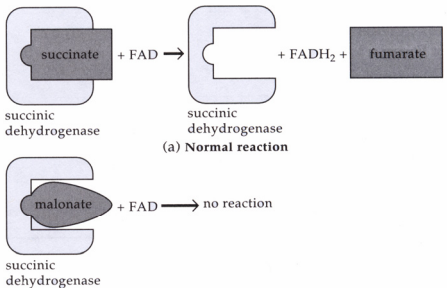
11. Graph your results. Use absorbance on the Y-axis and time on the X-axis. Use different colors or symbols for each tube, and include the legend.

Discussion Questions

1. What do your data indicate about the reaction of succinate –> fumarate in the mitochondria of lima bean seeds?

5. Malonate has a molecular shape similar to succinate, and can bind to the enzyme succinic acid dehydrogenase. Does malonate inhibit the reaction? If so is malonate a competitive or non-competitive inhibitor of succinate?





2. Does this reaction indicate that the lima beans were doing aerobic respiration? How do your results from tube 1, the boiled supernatant mixture, support your response?

3. Did you have a control tube? If so, which tube served as the control?

4. Mercury compounds often inhibit enzymatic activity. Did you find this to be so for this reaction?