Laboratory 8
Succinate Dehydrogenase Activity in Cauliflower Mitochondria

I. Introduction

In eukaryotic cells, specific functions are localized to different types of organelles. The nucleus is the site of DNA storage and transcription, mitochondria are the sites of cellular respiration, chloroplasts are the sites of photosynthesis, lysosomes are the sites of degradative processes, and peroxisomes are the sites of reactions involving hydrogen peroxide. Each of these organelles can be differentiated from the others by the presence of specific marker enzymes that are uniquely required for its activities. In addition, each organelle can be characterized by its specific range of sizes and by its particular density. These latter characteristics can be used to separate the various cellular components in a crude homogenate from one another and to prepare a fraction enriched in a particular organelle. Most fractionation processes rely on centrifugation as the primary method of separation. The purpose of this experiment is to use differential centrifugation to isolate mitochondria from the white florets of cauliflower. Cauliflower will be used in this experiment because it is readily available in supermarkets and because it does not contain chloroplasts. It thus will be possible to prepare a sample of mitochondria without contamination with chloroplasts which are generally similar in size.

As part of today's lab, you will:

- make a crude homogenate of cauliflower
- prepare several fractions of the cauliflower homogenate by differential centrifugation
- measure the activity of succinate dehydrogenase in these fractions
- study the requirements for the succinate dehydrogenase reaction in the mitochondrial fraction.

In preparation for this laboratory session, please read the sections of your textbook that deals with cell organization, cell organelles, and cell fractionation. This experiment is based in part on an experiment described in Bregman, A. 1996. **Laboratory Investigations in Cell and Molecular Biology** (3/e). John Wiley and Sons, New York, NY.

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this laboratory session. Also, review the material in **Laboratory 3 (Spectrophotometric Analysis of Membrane Stability in Beet Root Cells)** about plant cell structure and in **Laboratory 5 (Measurement of β-Galactosidase Activity in Lactaid™ Tablets)** about enzymes. After preparing for the lab, you should be able to answer the following questions

A. What are the functions of the following eukaryotic organelles: nucleus, mitochondrion, chloroplast, peroxisome, lysosome, ribosome, and endoplasmic reticulum?

B. What is meant by differentiation centrifugation?

C. Which organelles sediment to the bottom of a centrifuge tube at the lowest centrifugal forces?

D. Which organelles sediment to the bottom of a centrifuge tube at the highest centrifugal forces?

E. Which organelles do you expect to find in a homogenate of cauliflower florets?

F. Which organelles should be missing from this homogenate?

G. How can you determine which fractions contain mitochondria?

H. Why might a particular fraction prepared by differential centrifugation contain more than one marker enzyme?

I. What is the reaction catalyzed by succinate dehydrogenase?

J. How will the activity of this enzyme be measured?

K. Why is sodium azide included in the assay?

III. Background Information

A. Differential Centrifugation

In differential centrifugation, disrupted cells are subjected to several sequential centrifugations at progressively greater speeds and forces (Figure 8.1). During the first centrifugation at about 600*g*, the largest organelles (usually the nuclei) collect at the bottom of the centrifuge tube. During the second centrifugation at about 10,000*g*, the next largest organelles (usually mitochondria, lysosomes, and peroxisomes) collect at the bottom of the tube. During the third centrifugation at about 100,000*g*, the smallest organelles (usually microsomes, ribosomes, and membrane vesicles) collect at the bottom of the tube. At the end of this process, only small molecules and macromolecules remain in supernatant fraction. Differential centrifugation can be done either in a fixed-angle centrifuge rotor or in a swinging-bucket centrifuge rotor.

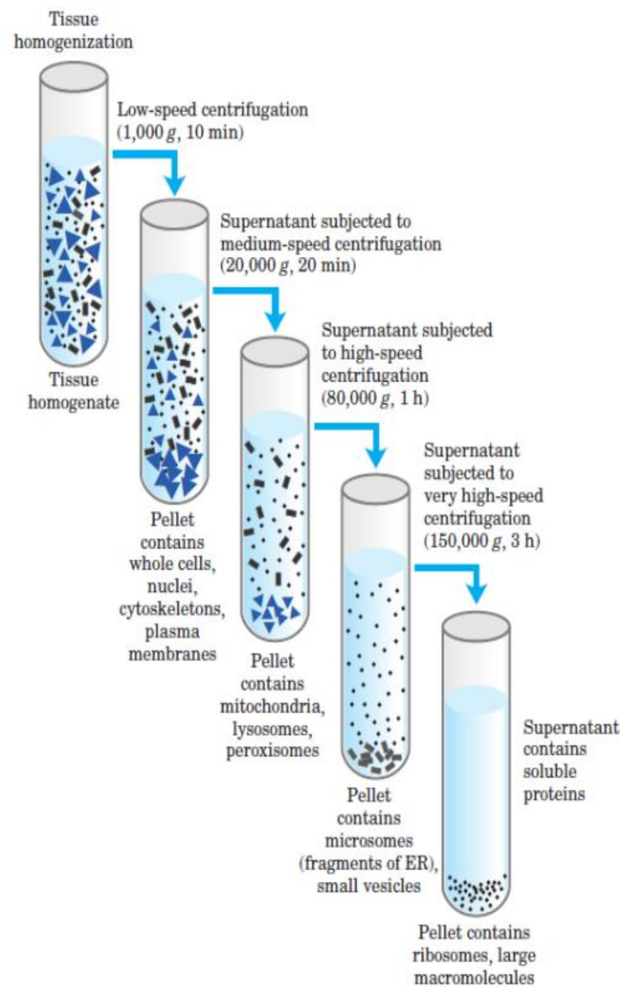

Figure 8.1 Cell fractionation procedure with differential centrifugation and a swinging bucket rotor

B. Marker Enzymes for Eukaryotic Organelles

Each of the membrane-bound organelles in a eukaryotic cell contains a specific set of proteins including various enzymes. The enzymes confined to a particular organelle can be used as markers to facilitate the identification of fractions obtained from a homogenate containing that organelle. Succinate dehydrogenase is often used as a marker enzyme for mitochondria because it is found exclusively in the inner mitochondrial membrane. In the same way, acid phosphatase is often used as a marker enzyme for lysosomes and peroxidase is used as a marker enzyme for peroxisomes.

1. Mitochondria

Mitochondria are the sites of cellular respiration in eukaryotic cells. The enzymes needed for the oxidation of pyruvate to form acetyl-CoA and for the catabolism of acetate through the Citric Acid Cycle (also called the Tricarboxylic Acid (TCA) Cycle or the Krebs Cycle) are located in the matrix of this organelle. The enzymes needed for the oxidation of fatty acids and some amino acids are also located in the mitochondrial matrix. The electron transport chains and the ATP synthases used to make ATP chemiosmotically are located in the extensions of the inner membranes called cristae.

One of the enzymes which functions in the Citric Acid Cycle is succinate dehydrogenase (SDH) (Figure 8.2)

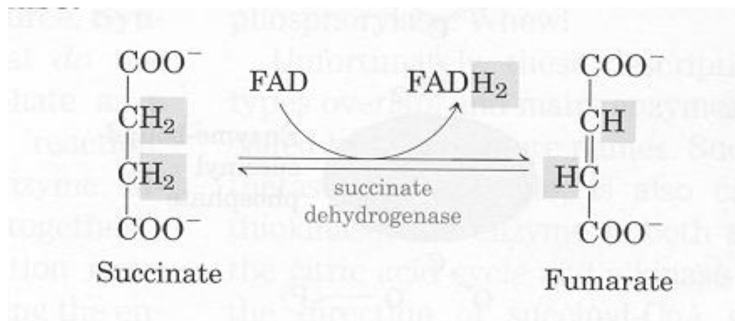

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Figure 8.2 Reaction in which Succinate dehydrogenase catalyzes the oxidation of succinate to form fumarate.

Unlike the other enzymes of the Citric Acid Cycle, which are found within the matrix of the mitochondrion, SDH is associated with the inner mitochondrial membrane in what is termed complex II. Electrons from succinate are transferred first to a tightly bound FAD coenzyme associated with the enzyme protein. They then enter the electron transport chain at the level of ubiquinone (Coenzyme Q). From there, they can be transferred along the chain to oxygen as the final acceptor (Figure 8.3). Because succinate dehydrogenase is only found in mitochondria, it is a suitable "marker enzyme" that can be used to identify cellular fractions containing these organelles.

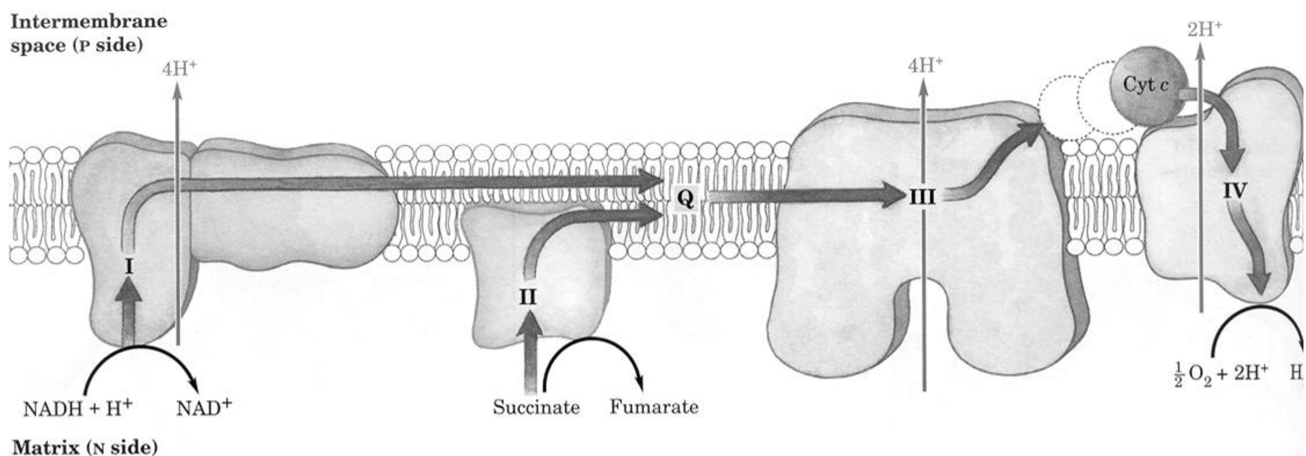


Figure 8.3. Electrons reach Q through Complexes I and II. QH2 serves as a mobile carrier of electrons and protons. It passes electrons to Complex III, which passes them to another mobile connecting link, cytochrome c. Complex IV then transfers electrons from reduced cytochrome c to O2. Electron flow through complexes I, III, and IV is accompanied by proton flow from the matrix to the intermembrane space. Recall that electrons from β oxidation of fatty acids can also enter the respiratory chain through Q.

2. Measurement of Succinate Dehydrogenase Activity

Succinate dehydrogenase activity in mitochondria can be measured in several ways. In this experiment, you will measure the enzyme's activity using 2,6-dichlorophenol-indolephenol, 2,6-dichloroindolephenol (DCIP or DCPIP) as an artificial electron acceptor. This dye is blue in its

oxidized form and absorbs light at 600 nm. The dye is colorless when it is reduced by accepting two protons and two electrons (Figure 8.4).

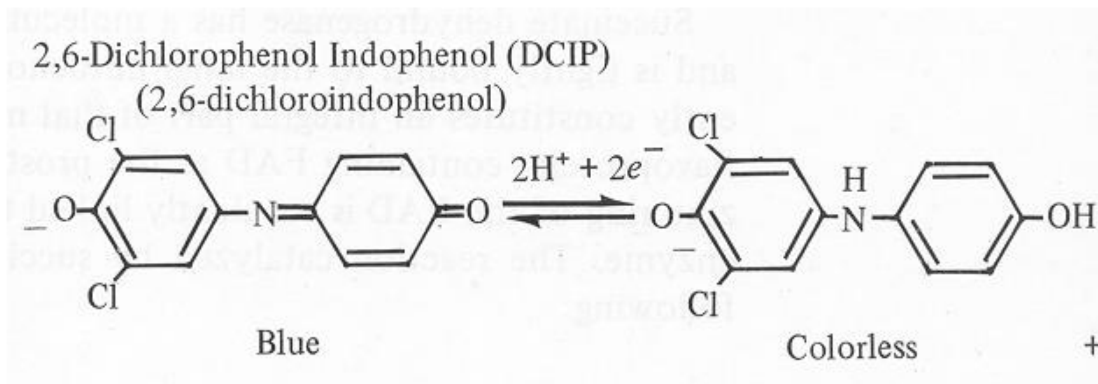


Figure 8.4 Reduction of DCIP.

To ensure that electrons coming from the oxidation of succinate go to the dye rather than to oxygen, sodium azide will be added to the reaction mixtures. Azide blocks the transfer of electrons from cytochrome **a** in Complex IV to oxygen. So as succinate is oxidized to fumarate in the presence of the enzyme, the color or absorbance of the solution will gradually decrease. This can be followed quantitatively in a spectrophotometer. The rate at which the absorbance decreases is a measure of the amount of enzyme activity.

Suppose you measure the activity of succinate dehydrogenase using DCIP as an electron acceptor and obtain the following data:

Time (minutes)	Absorbance (600 nm)
3	0.967
6	0.912
9	0.858
12	0.806
15	0.759
18	0.718
21	0.671
24	0.642
27	0.606
30	0.573

If you plot the absorbance at 600 nm on the Y axis as a function of time in minutes on the X axis, you can obtain a figure like figure 8.5.

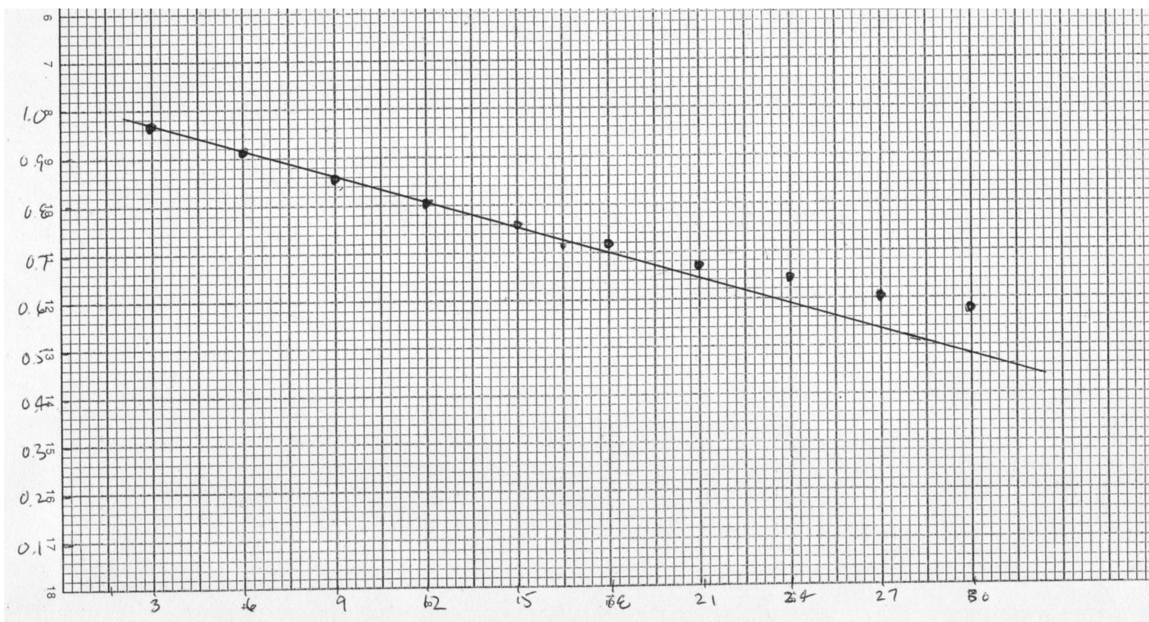


Figure 8.5 Plot of absorbance at 600 nm by time in minutes on the x axis

Notice that the absorbance decreases at a constant rate for about 15 minutes but then begins to slow down. That is, the data gradually deviate from a straight line. This is typical of many enzymatic reactions. To determine the rate of the reaction due to the succinate dehydrogenase, you should only use the initial rate of the reaction where the plot is linear. This initial rate can be determined from the graph by dividing the difference in Y by the difference in X for some interval. That is, the rate is given by the slope of the line and is [Y2 - Y1]/[T2 - T1]). In this case, the rate of the reaction can be estimated as ΔA_{600}/min = 0.0183.

The initial change in absorbance at 600 nm obtained graphically then can be used to calculate the activity of the enzyme in nmoles/min ml. The conversion factor for DCIP is 0.0215 A/nmole based on its molar extinction coefficient. (This coefficient assumes that the reaction volume is 1.0 mL) Refer back to **Laboratory 3 (Spectrophotometric Analysis of Membrane Stability in Beet Root Cells).** Suppose the reaction volume is actually 3.0 mL rather than 1.0 mL. we need to account for the total reaction volume in order to obtain a better estimate the enzyme's activity. So we would multiply by the volume used. In the example here we will assume that the reaction was actually carried out in a mixture with a total volume of 3.0 mL, so the relative activity would be three times higher.

Suppose that you find that 0.3 mL of enzyme give a decrease in A_{600}/min of 0.0183 as shown in the graph. From this, you can then calculate the relative activity as:

$$\frac{0.0183\ \Delta A}{\text{min x 0.3 mL enzyme}} \quad \text{x} \quad \frac{1\ \text{nmole}}{0.0215\ \text{A}} \quad = \quad \frac{2.83\ \text{nmole}}{\text{min mL enzyme}}$$

$$\frac{2.83\ \text{nmole}}{\text{min mL enzyme}} \quad \text{x} \quad \frac{3\ \text{mL}}{1\ \text{mL}} \quad = \quad \frac{8.49\ \text{nmole}}{\text{min mL enzyme}}$$

It is important to be careful to distinguish between the total volume of the reaction mixture (3.0 mL) and the volume of the enzyme used (0.3 mL).

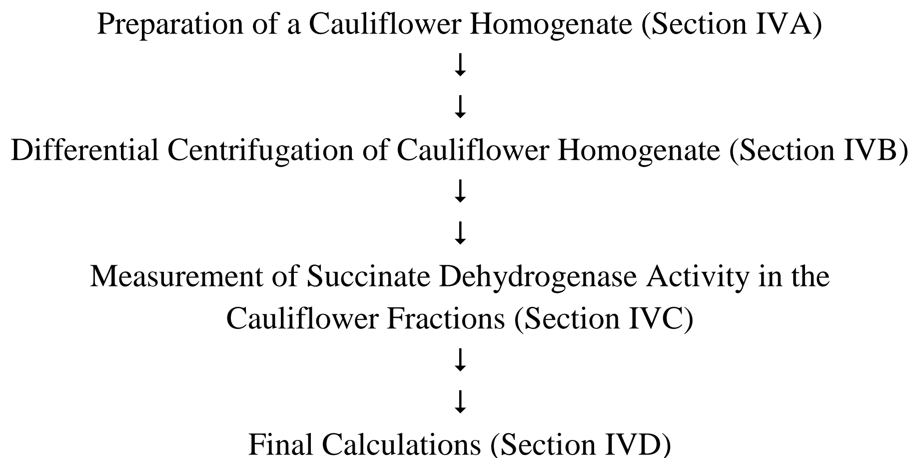
To determine total activity of the entire fraction you would use the volume measured before you removed any solution for testing. For instance your crude homogenate may be 40.5 mL so if your activity is 8.49 nmoles/min mL you would multiply these together to obtain total activity for that fraction or 343.8 nmoles/min.

It is often helpful to know the percentage of an enzyme's activity that was recovered in each fraction so you can determine where your enzyme activity is concentrated and the quality of your process. To do this for the current experiment you will divide the total activity in the nuclear, mitochondrial, or supernatant fractions by the total activity in the homogenate and multiply each by 100% to determine the % recovered in each fraction.

IV. Experimental Procedures

This experiment has several parts, which must be done in the order given. To isolate the mitochondria efficiently, the centrifugation steps will be coordinated so that all of the groups will centrifuge their samples at one time**. Because mitochondria are fragile and sensitive to heat, be sure to keep all of the materials that come in contact with your homogenate cold throughout the lab period.**

The following is a flow chart for the work that will be done during this lab session.



A. Preparation of Cauliflower Homogenate

The objective of this part of the experiment is to prepare a homogenate of cauliflower tissue. This homogenate will then be used to prepare four fractions by differential centrifugation.

1. Use a single-edge razor blade to remove about 20 g of the outer 2-3 mm of the cauliflower surface. Chop the tissue into very fine pieces with the razor blade.

2. Determine the weight of the plant material on a top loading balance and transfer it to a chilled mortar. Add 5 g of purified sand to the tissue and grind it for two (2) minutes. Gradually add 40 ml of ice cold mannitol grinding medium to the tissue and continue grinding for another four (4) minutes.

3. Cut off about 20 inches of double-thick cheesecloth and fold it over to make four layers. Place the cheesecloth over a chilled 250 ml beaker and filter the cauliflower homogenate through it. At the end of this process, pull up the cheesecloth around the plant debris and squeeze as much of the liquid into the beaker as possible.

4. Allow the cheesecloth filtrate to sit undisturbed for about 2 min to allow the sand and large particles to settle. Then transfer the liquid to a graduated cylinder and measure the volume and save it for the next part of the experiment.

Record the volume of the crude homogenate (H) here ________________________________

B. Differential Centrifugation of Cauliflower Homogenate

The objective of this part of the experiment is to fractionate the cauliflower homogenate and eventually obtain a mitochondrial fraction.

1. Transfer **5.0 ml** of the cauliflower homogenate (H) to a clean 15 ml plastic centrifuge tube and keep it in an ice bath for later analysis.

2. Transfer the rest of the cauliflower homogenate to a 50 ml plastic centrifuge tube. Centrifuge the material in the laboratory centrifuge in CLCC 367 for **5** minutes at about 600 *g* (2200 rpm). Make sure the centrifuge tubes are balanced. Because we have only one centrifuge, all of the groups will do this step at the same time.

3. At the end of the centrifugation, notice the pellet containing the unbroken cells, extra sand, and nuclei and the bottom of the tube. Decant the postnuclear supernatant fraction into a clean 50 ml plastic centrifuge tube.

4. Add **5.0 ml** of mannitol assay medium to the nuclear pellet. **Note that the assay medium is not the same as the grinding medium.** Carefully draw the liquid up and down to resuspend the organelles. Measure the volume of the nuclear fraction (N) in a small graduated cylinder. Transfer thus suspension to a clean 15 ml plastic centrifuge tube and keep it in an ice bath.

Record the volume of the nuclear fraction (N) including the assay medium here

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5. Centrifuge the postnuclear supernatant fraction for **15** minutes at 30,000*g* (16000 rpm) in the SS-34 rotor in the RC5B refrigerated centrifuge in CLCC329. Make sure the centrifuge tubes are balanced. Again, all of the groups will do this step at the same time.

6. At the end of the centrifugation, decant the post mitochondrial supernatant (S) into a graduated cylinder and measure its volume. Then transfer the supernatant to a flask and keep it on ice.

Record the volume of the post nuclear supernatant fraction (S) here

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7. Add **5.0 ml** of mannitol assay medium to the mitochondrial pellet. Carefully draw the liquid up and down to resuspend the organelles. Measure the volume of the mitochondrial fraction (M) in a small graduated cylinder. Transfer the mitochondrial suspension to a clean 15 ml plastic centrifuge tube and keep it in an ice bath.

Record the volume of the mitochondrial fraction including the assay medium here (M)

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C. Measurement of Succinate Dehydrogenase Activity in the Cauliflower Fractions

The objective of this part of the experiment is two-fold: 1) to determine the succinate dehydrogenase (SDH) activity in the four fractions: the crude homogenate (H), the nuclear fraction (N), the mitochondrial fraction (M), and post-mitochondrial supernatant (S); and 2) to determine the requirements for the reaction by the mitochondria. SDH activity will be measured as a decrease in absorbance due to the reduction in DCIP as succinate is oxidized to fumarate.

1. Turn on the Spectronic 20 Genesys spectrophotometer and allow it to warm up for 15 minutes. Set the wavelength to 600 nm.

2. Set up eight (8) reactions in 13 x 100 test tubes as shown in the following table. All of the volumes are given in **ml** and the total volume in each reaction mixture is 3.0 ml. Note also that reaction # 1 is a control reaction lacking DCPIP. **DO NOT ADD THE DCPIP TO THE OTHER CUVETTES UNTIL YOU ARE READY TO BEGIN THE REACTIONS.**

	Reaction Mixture							
Solution	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Mannitol assay medium	1.0	1.0	1.0	1.0	1.0	---	1.0	1.0
0.4 M Na azide	0.5	0.5	0.5	0.5	0.5	0.5	---	0.5
0.2 M Na succinate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	---
Crude homogenate (H)	---	0.5	---	---	---	---	---	---
Nuclear Fraction (N)	---	---	0.5	---	---	---	---	---
Mitochondrial Fraction (M)	---	---	---	0.5	---	0.5	0.5	0.5
Supernatant (S)	---	---	---	---	0.5	---	---	---
Water	1.0	---	---	---	---	1.0	0.5	0.5
5×10^{-4} DCPIP	---	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Note that reaction #1 is a "blank" that contains no homogenate and no DCIP. It will have no blue color and will be used to set the spectrophotometer to zero absorbance. Reactions #2 to #5 contain 0.5 ml of different fractions plus 0.5 ml of DCIP. These tubes will be used to measure the succinate dehydrogenase activity in the various fractions that were obtained in Section B by differential centrifugation. Reactions #6 to #8 contain 0.5 ml of mitochondria but are lacking either the assay medium, the Na azide, or the succinate. These reactions will be used to determine if these components are essential for the reaction.

3. Add the mannitol assay medium, sodium azide, organelle fractions, sodium succinate, and water to the tubes first and allow them to come to room temperature. **Be sure to carefully resuspend the various fractions by inversion of the tubes or swirling of the flask before removing the samples to add to the tubes.**

4. To start the reactions, add the DCIP at 30 second intervals to tubes 2 through 8. DO NOT ADD DCPIP TO CUVETTE 1.

5. As soon as the DCIP has been added, cover each tube with Parafilm and invert it several times to mix the solutions. After three (3) minutes have elapsed, begin to take readings. Place tube #1 into the instrument to set it to zero absorbance. Then read the absorbance of each of the other solutions at 600 nm. Continue to take readings at 3 minute intervals for up to 30 minutes. Fill in the following chart as you go along.

Table ____ Absorbance readings at 600 nm over a 30 minute period

	Tube							
Time	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
3								
6								
9								
12								
15								
18								
21								
24								
27								
30								

6. On linear graph paper, plot the A_{600} for each reaction as a function of time. Depending on the nature of your data you may want to use multiple graphs or just two graphs (Tubes 2-5), and (Tube 4, 6-7). You may find that the points do not form a straight line but there should be **an interval in which the absorbance drops at a consistent rate**. Draw a "best-fit" straight line through this region and calculate the rate of each succinate dehydrogenase reaction in ΔA_{600}/min. **(insert these graphs in your manual)** Also recall that we are looking at a change in absorbance so negative values are not used.

7. The conversion factor for DCPIP is 1 nmole/ 0.0215 A_{600} unit. Convert the rates into SDH activities in nmoles/min mL. Be sure to multiply by 3 since the total reaction volume was 3.0 mL rather than 1.0 mL. **Show those calculations here or in your lab notebook:**

D. Final Calculations

The objective of this part of the experiment is to summarize the data and begin to interpret it.

1. Once you have completed the succinate dehydrogenase activity calculations (step 7 in Section C), fill in the following chart using the values you found for tubes #2 through #5:

Fraction	**Volume (ml)**	**Activity (nmoles/min ml)**	**Total Activity (nmoles/min)**
Homogenate			
Nuclear			
Mitochondrial			
Supernatant			

2. Which of the fractions appears to have the most SDH activity? Is this logical? Explain.

3. Now calculate the percentage of the succinate dehydrogenase activity in the homogenate which was recovered in each of the other fractions. That is, **divide the total activity in each of the fractions, nuclear (N), mitochondrial (M) and supernatant (S) and by the total activity in the homogenate (H) and multiply by 100%.**

Fraction	**Percent of SDH Activity Recovered**
Nuclear	
Mitochondrial	
Supernatant	

4. Why might SDH activity appear in more than one fraction? Is this expected?

5. Now calculate the relative succinate dehydrogenase activity in the tubes from which various components of the reaction mixture with mitochondria were omitted. Fill in the following chart (Use tube #4 nuclear (N) part D #1, for comparison since it contained all components).

Reaction Mixture	**Component Omitted**	**Activity (nmoles/min ml)**	**Percentage**
4	None		100%
6	Assay Medium		
7	Na Azide		
8	Succinate		

6. What were the effects of omitting the assay medium, the azide, or the succinate from the reactions containing mitochondria? Why might there still be some activity if one of these components were omitted?