

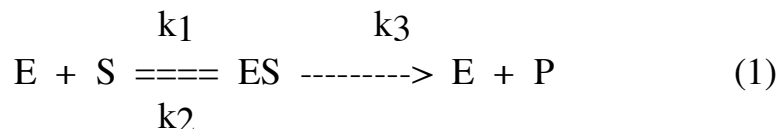
Exercise 3

Succinate Dehydrogenase Activity in Isolated Mitochondria

Introduction

Enzyme Kinetics

Enzymes are protein catalysts that accelerate the rates of biochemical reactions and regulate metabolic pathways. The study of the various factors that influence the rate of enzyme-catalyzed reactions is referred to as **enzyme kinetics**. Much of the pioneering work on enzyme kinetics was done in the early 1900's by German biochemists Leonor Michaelis and Maude L. Menten, who formulated a theory to explain the major events in enzyme catalysis. The Michaelis-Menten theory assumes that the enzyme (E) and substrate (S) combine reversibly to form an enzyme-substrate (ES) complex. The ES complex then breaks apart to form free enzyme and the product (P). These reactions are summarized by the equation:



where k_1 , k_2 , and k_3 are rate constants for the individual reactions. Although the derivation is not given here, it can be shown that the initial velocity (v_i or v_0) of an enzyme-catalyzed reaction is given by the equation:

$$v_i = \frac{k_3 [E] [S]}{K_m + [S]} \quad (2)$$

where $[E]$ is the enzyme concentration, $[S]$ is the substrate concentration at the start of the reaction, and K_m (the Michaelis constant) is the substrate concentration at which the initial velocity is 50% of the maximal reaction velocity (V_{max}). Note that in the numerator $k_3 \times [E]$ equals the V_{max} of the reaction; making this substitution gives the more familiar form of the **Michaelis-Menten equation**:

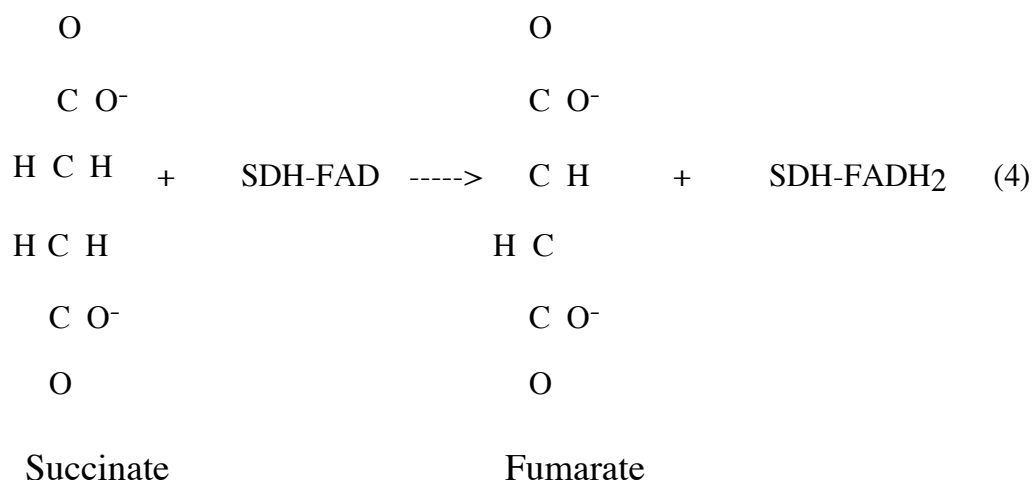
$$v_i = \frac{V_{max} [S]}{K_m + [S]} \quad (3)$$

According to equation 3, the initial velocity of a reaction is determined by three factors: the initial substrate concentration, the K_M for the reaction, and the V_{max} . Since V_{max} varies with the concentration of enzyme, it stands to reason that v_i will also vary with enzyme concentration (as was shown in equation 2).

In this exercise, you will examine several factors that influence enzyme activity. The enzyme you will study is **succinate dehydrogenase**, an important component of the Krebs cycle. In one set of reactions you will determine the effects of enzyme concentration on the initial velocity of the reaction. You will also study the effects of a **competitive inhibitor** on the reaction rate. As its name implies, a competitive inhibitor competes with the normal substrate for the active site on the enzyme, thereby interfering with enzyme activity. Other reactions will be run to assess the availability of substrate and the viability of the electron transport system in isolated mitochondria

Succinate Dehydrogenase

The mitochondria contain the biochemical machinery for aerobic cellular respiration, the process by which sugars, fatty acids, and amino acids are broken down to carbon dioxide and water, with some of their chemical energy captured as adenosine triphosphate (ATP). A key series of reactions in cell respiration is the Krebs (citric acid) cycle, a complex pathway involving some nine enzymes and numerous metabolic intermediates. One of the best studied enzymes in the Krebs cycle is succinate dehydrogenase (SDH; E.C. 1.3.99.1), which catalyzes the oxidation of succinate to fumarate as shown in the following equation:



In this reaction, two hydrogen atoms are removed from succinate by **flavin adenine dinucleotide (FAD)**, a **prosthetic group** that is tightly attached to succinate dehydrogenase. Two electrons from the reduced SDH-FADH₂ complex are then transferred to ubiquinone, a soluble component of the electron transport system Complex II. Succinate dehydrogenase is unique among the Krebs cycle enzymes in that it is tightly bound to the inner mitochondrial membrane; the other enzymes of the pathway are located in the mitochondrial matrix. Because SDH is bound to the inner membrane, it is easily isolated along with the mitochondria by the technique of differential centrifugation (figure 1).

ISOLATION OF MITOCHONDRIA

The *in vitro* analysis of succinate dehydrogenase activity will be carried out using the mitochondrial fraction isolated from the cells of cauliflower (*Brassica oleracea*) by the procedures shown in figure 1. You will first homogenize the cauliflower tissue in a buffered, isotonic mannitol solution by grinding with a mortar and pestle. After filtering the crude homogenate through cheesecloth to remove the larger tissue pieces, you will centrifuge the filtrate at 600 x gravity for 10 min to sediment the nuclear fraction. You will then centrifuge the supernatant at 10,000 x gravity for 30 min to sediment the mitochondrial fraction. The mitochondrial fraction will be the source of your enzyme. For all of the cell fractionation procedures, the solutions and containers should be ice-cold.

1. Sterilize glass to isolate medium using a autoclave cycle.



2. After enough sterility has been achieved.

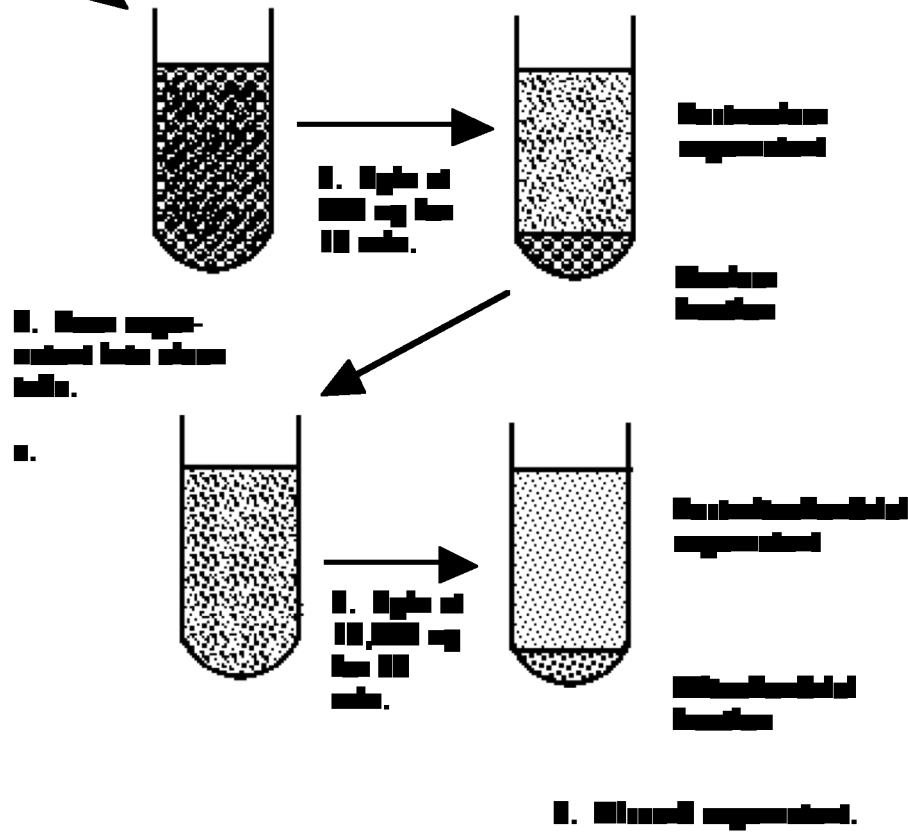


Figure 1. Soil Sterilization and Isolation of microorganisms from soil by differential centrifugation.

Measurement of Succinate Dehydrogenase Activity

Since none of the key components can be measured directly, the reaction succinate \rightarrow fumarate is measured by monitoring the reduction of an **artificial electron acceptor**. To use an artificial electron acceptor, the normal path of electrons through the mitochondrial electron transport system must be blocked. This is accomplished by adding either sodium azide or potassium cyanide to the reaction mixture. These poisons inhibit the transfer of electrons from cytochrome a_3 to the final electron acceptor, oxygen, thus electrons cannot be passed along by the preceding cytochromes and coenzyme Q. Instead, the electrons from SDH-FADH₂ can be picked up by an artificial electron acceptor, such as the dye **2,6dichlorophenolindophenol (DCIP)**. The reduction of DCIP can be followed spectrophotometrically, since the oxidized form of the dye is blue and the reduced form is colorless. This reaction can be summarized as



The change in absorbance, measured at 600 nm, can be used to follow the reaction over time.

To vary the concentration of the enzyme in the reaction mixture, you will add different volumes of the resuspended mitochondrial fraction. Since the total volume in each reaction mixture is the same, the concentration of enzyme in each mixture is directly proportional to the volume of mitochondrial suspension added. To avoid the influence of substrate concentration on v_i , [succinate] will be kept high enough that it does not change appreciably during the first 15-20 minutes of the reaction.

To study the effects of a competitive inhibitor on the activity of succinate dehydrogenase, you will add **malonate** to a reaction mixture. Malonate is a classic example of a competitive inhibitor; it has a molecular structure that is similar to succinate, so it will bind to the active site of succinate dehydrogenase. However, malonate is sufficiently different from succinate that it cannot be dehydrogenated.

In addition to the above reactions, three control reactions will be run. In one control, the reaction mixture will contain no mitochondrial

suspension. In the second control, no succinate will be added, while in the third, azide will be omitted.

Background Reading

You should read the appropriate sections of your textbook on the structure of enzymes, the kinetics of enzyme-catalyzed reactions, and the Krebs cycle. In addition, various cell biology and biochemistry texts and other books devoted specifically to enzymes are available in the library.

There are several questions that you should consider **before** coming to lab:

- * What is the botanical term for that portion of the cauliflower with which you will be working? That is, is it a flower? A fruit? A stem?
- * Why do the isolation and assay buffers contain compounds such as mannitol, potassium phosphate, KCl, *etc*? That is, what is the purpose of each?
- * Why is it important that these solutions be at pH 7.2?
- * Why are the homogenization and fractionation procedures carried out at low temperature?

PROCEDURES

Isolation of Mitochondrial Fraction

1. Use a single-edged razor blade to remove **20 g** of the outer 2-3 mm of the cauliflower surface. Each group will need 20 g of tissue.
2. Place the tissue in a chilled mortar with **40 ml of ice-cold mannitol grinding buffer**(0.3 M mannitol; 0.006 M KH_2PO_4 ; 0.014 M K_2HPO_4 ; pH 7.2). Grind the tissue vigorously with a chilled pestle for 4 min. Note: While it is not essential, 5 grams of cold, purified sand added to the grinding mixture will help to break down the cell walls.
3. Filter the suspension through four layers of cheesecloth into a chilled 50 ml centrifuge tube. Wring out the juice into the tube.
4. Centrifuge the filtrate at **1000 x gravity for 5 min** at 0-4°C. Make sure the centrifuge tubes are balanced (± 0.1 g) and placed opposite one another in the centrifuge.
5. Decant the postnuclear supernatant into a clean, chilled centrifuge tube(s) and spin at **10,000 x gravity** for 30 min at 0-4°C. Again, be sure the tubes are balanced. The nuclear pellet left over from step 4 can be discarded in the sink.
6. Decant and discard the postmitochondrial supernatant. Add 8.0 ml of ice-cold mannitol assay buffer (0.3 M mannitol; 0.006 M KH_2PO_4 ; 0.014 K_2HPO_4 ; 0.01 M KCl; 0.005 M MgCl_2 ; pH 7.2) to the mitochondrial pellet. The pellet should be brownish, due to the presence of cytochromes in the mitochondria.
7. With a Pasteur pipette, scrape the mitochondrial pellet from the wall of the centrifuge tube and then with a Pasteur pipette thoroughly resuspend the sediment in the assay buffer. It is important that the clumps be completely dispersed.
8. Store the mitochondrial suspension in an ice bath until needed.

Measurement of Succinate Dehydrogenase Activity

1. Allow the Spectronic 20 to warm up for at least 5 min. The wavelength should be set at 600 nm.
2. Label 11 cuvettes as shown in table 1. Except for the ice-cold mitochondrial suspension, all solutions should be at room temperature.
3. To all cuvettes add the various solutions listed across the top of table 1, **except for the mitochondrial suspension**. First, add the correct volume of assay buffer to all tubes. Then, in the same manner, add the volumes of azide, DCIP, malonate, and succinate indicated in the table. Cover each cuvette with Parafilm and invert twice to mix the contents.

Table 1. Reagents for the *in vitro* measurement of succinate dehydrogenase activity.

Tube	Assay Buffer	Azide* (0.04 M)	DCIP (5×10^{-4} M)	Malonate (0.2 M)	Succinate (0.05 M)	Mitochondrial suspension
Blank 1	4.0 ml	0.5 ml	---	---	0.5 ml	0.0 ml
1	3.5 ml	0.5 ml	0.5 ml	---	0.5 ml	0.0 ml
Blank 2	3.7 ml	0.5 ml	---	---	0.5 ml	0.3 ml
2	3.2 ml	0.5 ml	0.5 ml	---	0.5 ml	0.3 ml
Blank 3	3.4 ml	0.5 ml	---	---	0.5 ml	0.6 ml
3	2.9 ml	0.5 ml	0.5 ml	---	0.5 ml	0.6 ml
Blank 4	3.1 ml	0.5 ml	---	---	0.5 ml	0.9 ml
4	2.6 ml	0.5 ml	0.5 ml	---	0.5 ml	0.9 ml
5	2.7 ml	0.5 ml	0.5 ml	0.2 ml	0.5 ml	0.6 ml
6	3.4 ml	---	0.5 ml	---	0.5 ml	0.6 ml
7	3.4 ml	0.5 ml	0.5 ml	---	---	0.6 ml

*** Caution: Azide is poisonous. Do not pipette any solution by mouth!**

4. Thoroughly resuspend the mitochondrial suspension with a Pasteur pipette. Then add the appropriate volume of mitochondrial suspension to **blanks 1-4**, cover the tubes with Parafilm, and invert twice to mix the contents. Return the tube to the test tube rack.

5. Zero the Spec 20 using blank 1.

6. At precise time intervals, the appropriate volume of mitochondrial suspension will be added to tubes 1-4 and absorbance readings taken, as follows:

Time (min:sec)

0:00 Add suspension to tube 1 (vol. = 0 ml), cover with Parafilm, and invert to mix.

0:10 Read the absorbance of tube 1; re-zero the Spec 20 using blank 2.

1:00 Add suspension to tube 2, cover with Parafilm and invert to mix.

1:10 Read the absorbance of tube 2; re-zero the Spec 20 using blank 3.

2:00 Add suspension to tube 3, cover with Parafilm and invert to mix.

2:10 Read the absorbance of tube 3; re-zero the Spec 20 using blank 4.

3:00 Add suspension to tube 4, cover with Parafilm and invert to mix.

3:10 Read absorbance of tube 4; re-zero the Spec 20 using blank 1.

5:10 Read the absorbance of tube 1; re-zero the Spec 20 using blank 2.

6:10 Read the absorbance of tube 2; re-zero the Spec 20 using blank 3.

7:10 Read the absorbance of tube 3; re-zero the Spec 20 using blank 4.

8:10 Read the absorbance of tube 4; re-zero the Spec 20 using blank 1.

Continue reading the absorbance of tubes 1-4 at five minute intervals until 20 minutes have elapsed from the first reading. Be sure to re-zero the Spec 20 with the appropriate blank before each reading. Do not hold the tubes or leave them in the Spec 20 between readings (*Why not?*

7. Repeat the above procedures (step 6) using tubes 5-7, again taking absorbance readings for each tube at 5 minute intervals. Note that blank 3 from the first experiment can be used to zero the Spec 20 for tubes 5-7.

Analysis of Results

Start by determining the change in absorbance since time zero for each reading. For each reading, subtract the actual absorbance at each time point from the absorbance at time zero for that tube.

1. For tubes 1-4 , plot change in absorbance (y-axis) versus elapsed time (x-axis) for each reaction. Draw a smooth curve through the data points for each reaction. Note: The units for time should be the same for all readings (*i.e.*, use either seconds or minutes).
2. Calculate the initial velocity (v_i) for each reaction as the slope of a straight line drawn through the first two or three data points. Initial velocity should be expressed as: Δ absorbance / min (or Δ absorbance / sec).
3. On a separate graph, plot initial velocity versus enzyme concentration (expressed as volume of suspension added) for reactions 1-4. Draw a line connecting the four data points.
4. According to Fig. 2, how does initial reaction velocity vary with the concentration of enzyme? Is this consistent with the Michaelis-Menton equation?
5. For tubes 3 and 5-7, plot change in absorbance (y-axis) versus elapsed time (x-axis) for each reaction. Draw a smooth curve through the data points for each reaction. Note: The units for time should be the same for all readings (*i.e.*, use either seconds or minutes).

6. Are your results for tube 5 consistent with the characteristics of a competitive inhibitor? Do the results from tube 6 provide evidence that the electron transport system continues to function in isolated mitochondria?
7. What do the results from tube 7 indicate about the effects of substrate concentration on enzyme-catalyzed reactions? Was there any succinate present in your isolated mitochondria?
8. Based on your results, what can you conclude about the potential influence of enzyme concentration, inhibitors, and substrate concentration on enzyme activity *in vivo*? Under what circumstances might the concentrations of enzyme, substrate, and inhibitors vary in a cell?
9. What other factors influence enzyme activity *in vivo*? How important are these factors in regulating metabolic pathways?

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