Experiment CM-2: Mitochondrial Metabolism

Background

When sugars are metabolized, the initial stages of their breakdown take place in the cytoplasm. Here, sugars are converted to pyruvic acid in a process called glycolysis. In the presence of oxygen, pyruvic acid is directed through the Krebs cycle inside the mitochondria. Here water, carbon dioxide and ATP are formed.

In this experiment, you will examine one step within the Krebs cycle: the oxidation of succinic acid to fumaric acid. This reaction is catalyzed by the mitochondrial enzyme succinic dehydrogenase (SDH). SDH is covalently bonded to flavin adenine dinucleotide (FAD). FAD is reduced as succinic acid is oxidized. The reduced FAD passes its electrons through the electron transport system, where they are eventually passed to molecular oxygen to form water.

You will add a mouse liver extract, containing SDH, to a series of solutions. An artificial dye in the mixture will absorb some of the electrons produced by the reaction and will become lighter; you will monitor this color change using a spectrophotometer. You will make up a solution with cyanide, to poison the electron transport system and monitor the change in dye color. Finally, you will examine the effect of a competitive inhibitor (malonate) on the rate of electron production.

Equipment Required

Spectrophotometer and cuvettes

Balance

Refrigerated centrifuge or centrifuge in cold room

Two polypropylene centrifuge tubes

Homogenizer and tubes

100ml beaker

100ml Erlenmeyer flask

Pipettes

Ice bucket and ice

Test tubes (18 x 150mm)

Buffers and Reagents (See appendix)

Equipment Setup

1. Turn on the spectrophotometer. Set the wavelength to 600nm and allow the instrument to warm up for at least 15 minutes.
2. Place approximately 50ml of homogenizing solution in a beaker and place the beaker, the homogenizing fluid, the homogenizing tube, the centrifuge tubes and the test tubes on ice.

Tissue Preparation

The class will be presented with a fresh mouse liver.

1. Weigh out approximately one gram of liver on a balance.
2. Quickly transfer the liver to the beaker containing cold homogenizing solution.

Note: *You should make every effort to store the liver tissue and mitochondrial suspensions at zero degrees Celsius to prevent loss of enzymatic activity—i.e. store them on ice and remove to room temperature for as short a time as possible.*

3. Decant off (and discard) the discolored fluid and place the liver in the chilled homogenizing tube.
4. Add 20ml homogenizing fluid and homogenize for 30 seconds at top speed.
5. Place about half of the fluid in the homogenizing tube into each of two chilled centrifuge tubes and balance with homogenizing fluid.
6. Centrifuge at 600G for 10 minutes in the refrigerated centrifuge.
7. Pour the supernatant from each tube into a graduated cylinder and make up to 100ml with SPT buffer.
8. Store all solutions and liver extract on ice.

Tube Preparation

Use the recipes in Table CM-2-1 on page CM-2-1 to make up the solutions in Tubes 1 through 4. Store the tubes on ice.

Table CM-2-1: Recipes for the Solutions in Tubes 1 through 4.

Solutions	Volume (ml)			
Tube Number	1	2	3	4
200 mM Na Succinate	0.5	0.5	0.5	0.5
200 mM Na Malonate	0.0	0.0	0.0	0.5
50 mM KCN**	0.1	0.0	0.1	0.1
SPT Buffer	3.4	2.5	2.4	1.9

Note: *Do not pipette any solutions by mouth.* ***You are working with potassium cyanide (KCN)!*** *Use a bulb on all pipettes, or use dropper pipettes.*

Exercise 1: Calibrate the Spectrophotometer

Aim: To calibrate the spectrophotometer.

Procedure

1. With no cuvette in the holder, use the zero adjust to set the transmittance to zero.
2. Add 1.0ml of the liver extract to **Tube 1** and pour the contents into a clean cuvette—this is the blank, since it contains no dye.
3. Insert the cuvette into the holder and align the marks on the cuvette and the holder. Adjust the light control to set the transmittance to 100.

Note: *You will use this "blank",* ***Tube 1****, at the beginning of each set of future measurements—do not discard!*

Exercise 2: The Reaction without Cyanide

Aim: To measure the rate of the reaction, without cyanide.

Procedure

1. Add 1.0ml of the 2,6-dichlorophenolindophenol (the dye) to **Tube 2**.
2. Add 1.0ml of the liver extract to tube two, place a piece of parafilm over the mouth of the tube and shake a few times.
3. Quickly pour the contents into a clean cuvette and place it into the spectrophotometer and read (and write down) the absorbance immediately and every 30 seconds for 10 minutes.

Exercise 3: The Effect of Cyanide

Aim: To measure the rate of reaction in the presence of cyanide.

Procedure

Repeat Exercise 2 using **Tube 3**.

Exercise 4: The Effect of a Competitive Inhibitor

Aim: To measure the rate of reaction in the presence of malonate.

Procedure

Repeat Exercise 2 using **Tube 4**.

Data Analysis

1. Graph absorbance as a function of time for the data from Tubes 2, 3, and 4. Use linear regression analysis to find the best line for each reaction.
2. Make a histogram to compare the rate of color change of each tube to others.

Questions

1. Look at the histogram and compare the reaction rates of Tubes 2 and 3. Comment on the function of potassium cyanide in this experiment.
2. Look at the histogram and compare Tubes 3 and 4. Comment on the effectiveness of malonate as a competitive inhibitor.
3. Is the correlation coefficient for the line graph of Tube 4 as high as the values for Tubes 2 and 3? Look at the curve for Tube 4; explain the profile in terms of competitive inhibition.

Appendix

All solutions should be refrigerated or kept on ice.

Table CM-2-2: Recipe for Homogenizing Medium.

Concentration (mMolar)	Reagent	Grams/Liter in DI H_2O
250	Sucrose	85.57
0.01	EDTA	0.00292
15.0	Tris-HCl	2.36
200	Sodium Succinate	54.0
200	Sodium Malonate	29.6
Adjust the pH of the Medium to 7.4		

Table CM-2-3: Recipe for SPT Buffer.

Concentration (mMolar)	Reagent	Grams/Liter in DI H_2O
250	Sucrose	85.57
20.0	K_2HPO_4 Anhydrous	3.48
15.0	Tris-HCl	2.36
Adjust the pH of the Medium to 7.4		

Table CM-2-4: Concentrations of Reagents that Affect Mitochondrial Respiration.

Concentration (mMolar)	Reagent	Grams/Liter in DI H_20
200	Sodium Succinate	54.0
200	Sodium Malonate	29.6
50.0	Potassium cyanide	3.256
0.10	6-Dichlorophenolindophenol	0.0268