Experiment CM-2: Mitochondrial Metabolism

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 150 mm)

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 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.*

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Exercise 1: Calibrate the Spectrophotometer

Aim: To calibrate the spectrophotometer.

Approximate Time: 10 minutes

Procedure

1. With no cuvette in the holder, use the zero adjust to set the absorbance to infinity.
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 absorbance to zero (0).

Note: You will use this "blank", Tube 1, at the beginning of each set of future measurements—do not discard! You will need to "Blank" the spectrophotometer at the beginning of each exercise.

Exercise 2: The Reaction without Cyanide

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

Approximate Time: 15 minutes

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.

Approximate Time: 15 minutes

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.

Approximate Time: 15 minutes

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.