# Chapter 3

# Mitochondria and Metabolism in Honeybee Flight Muscle


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# Introduction

This exercise is intended for an introductory level course, although by going into greater detail in the study of respiration it could also be used for an intermediate level cell physiology course. The isolation of mitochondria and the first assay can be comfortably performed in 2 – 2.5 hours and forms a complete exercise. Performing the second assay and directly observing the mitochondria requires a total of 3 hours. The goals and objectives of the exercise are listed below.

## Goals and objectives

Conceptual objectives:

- Learn how enzymatic reactions can be isolated by (a) fractionating cell contents, and (b) manipulating a biochemical pathway.
- Understand how techniques for isolating an enzymatic reaction can then be used to study its subcellular location.

Content-specific goals:

- Determine experimentally the location of respiration reactions in a cell.
- Become familiar with some basic techniques and precautions for working with enzymes.
- Be able to interpret an experimental assay of enzyme activity; correctly identify and describe controls used in such an assay.

- Understand basic steps in breakdown and oxidation of glucose, especially the roles of the Krebs cycle and electron transport chain.
- Explain how oxidation reactions in the Kreb's cycle can be monitored indirectly by measuring bleaching of the dye 2,6-dichloroindophenol.
- Understand the role of oxygen in cellular respiration.
- Learn how cyanide acts as a metabolic poison.

## Student Outline

**Background**

The commonality of organisms is perhaps nowhere better illustrated than in the chemical activities of cells. Despite the wide variety of types of cells in an even wider variety of organisms, each cell carries out a large set of fundamentally similar chemical processes. Even the detailed sequences of reactions in these processes are almost identical in, for example, a bacterium like *E. coli* and a cell from the flight muscle of a pigeon. The differences in the chemical activities of different kinds of cells, although interesting and important, seem minor by comparison with their basic similarities.

Of particular significance for all living organisms are reactions which release energy by making small molecules from larger ones. With the one significant exception of photosynthesis, all the energy required for synthetic processes and the other activities of organisms is derived from the bond energy released by either the cleavage of large molecules or by the oxidation of the components of larger molecules. How then can we investigate these reactions out of the hundreds which take place simultaneously in every cell, and more specifically, how can we locate where they take place within an eukaryotic cell? Two alternative approaches seem feasible:

1. The molecules of a particular enzyme might be physically isolated and their activity assayed as it operates on a substrate in an otherwise enzyme free vessel.

2. An enzyme might be isolated chemically without actually separating it from the remainder of the cell. For example, the next enzyme in the sequence might be inactivated so we could measure the accumulation of the reaction product. Or the concentration of a particular substrate can be manipulated to favor a specific reaction.

The two schemes may be profitably combined to study the subcellular location of some particular enzyme or group of enzymes. Such combined approaches to location of specific enzymatic activity vary considerably in technique. For example, in a histological approach thin slices of cells can be treated with specific reagents then the colored products viewed with a microscope. Alternatively, masses of cells can be disrupted and then fractionated into different subcellular components by differential centrifugation.

In this lab we will adopt the latter path for physically localizing the enzyme. During the first part of the exercise you will grind up tissue to break the cells, filter to remove the coarsest parts, then centrifuge the remainder to fractionate further the debris. Following these procedures you will then measure the activity of a key respiratory enzyme in different cell fractions to infer

its location in an intact cell. At the end of the exercise you will use selective staining of a sample of dissected tissue to observe the site of respiration directly.

**Choice of tissue**

It is worth examining the steps leading to the choice of a tissue for studying respiration reactions. For the purposes of the lab an ideal tissue would (a) have a high glycolytic and respiratory metabolism, and (b) have large organelles easily separated from the soluble fraction of the cell by centrifugation. In general, small particles require much more vigorous centrifugation than large ones, so without long treatment in a very fancy machine we are limited to relatively large components. Fortunately the components of particular interest to us are the mitochondria; these large and discrete organelles are well endowed with enzymes and are also the site of the energy-releasing reactions we are concerned with.

Not surprisingly, the tissues containing the largest number of mitochondria are also those which are most active in oxidative, energy-yielding processes. Classic sources of mitochondria in animals are liver and flight muscle; plant sources include cauliflower heads (flower buds) and bean sprouts. Among the possible plant sources for mitochondria these latter two tissues have the advantage that they are actively growing and metabolizing yet do not contain chloroplasts, an organelle whose presence could interfere with our study of respiratory reactions. Unfortunately, neither of these tissues is fully suitable for our purposes; not only do they contain fewer mitochondria than liver or muscle tissue but their mitochondria are small and must be spun for 30 minutes or more in a high-speed centrifuge to be successfully separated from the cytoplasm. In addition, both of these plant tissues contain enzymes (such polyphenol oxidase) whose activity interferes with the assay procedures you will be using.

We are left then with animal tissues. Of the two tissues types we could choose, flight muscle has one major advantage over liver: whereas each liver cell performs a wide variety of biochemical reactions and is thus far more heterogeneous chemically and structurally, cells in flight muscle are specialized for a single task with fewer different kinds of components. Consequently, flight muscle is more easily fractionated than liver. But why restrict consideration to the muscle used in flight? One reason is that flying is a very energetically costly activity, and the concentration of enzymes in flight muscle is correspondingly great. But the difference between flight muscle and other muscle in most flying animals isn't just in the quantity of enzymes. Flight muscles perform the complete task of oxidizing fuel (carbohydrate or fat) to produce carbon dioxide and water. Most other muscles (the heart is a notable exception) have only a limited capability for performing the later, oxidative steps of this process, and therefore produce an intermediate compound, lactic acid, that is exported for oxidation elsewhere. As you will be determining, it is these later, oxidative steps which are carried out by mitochondria. In short, flight muscle is far richer in mitochondria than most other muscle.

If we wish to isolate the mitochondria of flight muscles, what creature shall we use? The only groups of flying animals presently in existence are bats, birds, and insects. Bats are scarce and unpropitious, so the choice lies between birds and insects. Insects have much larger mitochondria than birds and far less in the way of membranes, connective tissues, and other impediments to easy isolation. Of the kinds of insects that are raised commercially and hence are readily available in sufficient numbers, the most suitable choice is honeybees.

**Procedures and general precautions**

To minimize the possibility of being stung, use only well-anesthetized bees fresh from the carbon dioxide anesthetic. If a container of bees begins to get lively, cover it and re-anesthetize immediately. If a bee wanders groggily away, it should be summarily dispatched. Spill out only a few bees at a time. When handling the bits of bees, remember that the abdomen is the business (stinger) end and that isolated abdomens may be presumed off-ended!

Successful work on enzymes demands much greater attention to small matters like the cleanliness of test tubes and other glassware than does most other chemistry. Washing should be followed by two rinses in distilled water. And mitochondria follow the usual osmotic rules: water, if pure, is instantly disruptive, so all dilutions *must* be done with the buffer solution or isolation medium.

**Preparation of mitochondria**

The keynotes here are teamwork and synchronization. You will work in teams of four. Fill a large bowl halfway with ice and use this to chill a clean, dry mortar and pestle and a beaker containing about 25 mL of the isolation medium. Set out paper toweling, forceps, and scissors, razor blades, or scalpels. Each team will be supplied with a jar containing about 80-100 bees (~10 g), which you will anesthetize with $CO_2$ as directed by your TA. Once your bees are fully anesthetized, three members of the team should act as bee cutters with the fourth keeping them supplied, picking up the thoraxes, dispatching stragglers, etc. The cutters must separate the thorax from the head and the abdomen. Start by using the tips of a pair of scissors to clip off the abdomens of all of the bees spilled from the jar. This will immobilize them should they start to recover from the anesthetic. Then remove the heads from the thoraxes using the scissors or forceps and a scalpel or razor blade; wings and legs may be ignored. The important thing here is *speed*, not precision. The fourth student should transfer the thoraxes to the dry, chilled mortar as they are isolated until *all* bees have been operated on.

Wipe off the outside of the beaker containing the isolation medium, measure 10 mL into a graduated cylinder, and pour it into the mortar. Carefully mash the thoraxes with the pestle for two or three minutes, until you no longer hear the cracking of thoraxes. Since the mitochondria are not firmly attached within the muscles, the crushing and grinding need not be especially vigorous; indeed, excessive crushing liberates muscle fibrils, which tend to clog the filter. Keep ice from falling into the mortar.

While the thoraxes are being mashed, one member of the team should wash his or her hands very thoroughly, rinsing with distilled water. This “clean-hands” person should then take a 10 cm x 20 cm piece of percale cloth, fold it in two, wet the cloth with the isolation medium, and wring it out. He or she should arrange the cloth as a two-layer filter in the funnel, and put the funnel in a chilled flask. Your group is now ready to pour the already liquefied portion of the contents of the mortar into the filter. Next, grind the drained thoraxes a bit more with the pestle, and pour the entire contents of the mortar into the filter. The mortar and pestle should then be rinsed with an additional 5 mL of isolation medium and that fluid poured off into the filter also. Stir the homogenate in the filter with a glass rod to assist filtration. Finally, with great care, the “clean-hands” person should draw the ends of the cloth together and forcibly express the remainder of the fluid by hand, without getting any debris in the filtrate.

Carefully pour the filtrate into a clean centrifuge tube marked "SED." (for "sediment") and add fresh isolation medium if necessary to bring the volume to the 8 mL mark. Pour any remaining filtrate in excess of 8 mL into a clean test tube labeled "**homogenate**" and keep it on ice.

Mark the centrifuge tube of filtrate to identify your group then give it to your TA for centrifugation. If all went as expected, the filtrate (homogenate) should contain the soluble portion of the contents of the thorax (both intracellular and extracellular fluid) and the mitochondria, but should exclude muscle fibrils, cuticles, and any undisrupted tissue. Fortunately, there is practically nothing else in the thorax of a bee but flight muscle.

A true ultracentrifuge is unnecessary for separating the mitochondria of the flight muscle of insects, and you will use a standard clinical desk centrifuge. The power of a centrifuge is rated in G's or gravity units: one G is the attraction felt by a body at the surface of the earth. A load of ten to twenty G's, equivalent to increasing one's weight ten to twenty times, is tolerable for well-protected humans for brief periods (as in the acceleration of a rocket). A clinical centrifuge operates up to roughly 1000 G's. The effectiveness of centrifugation is approximately proportional to the force exerted and the time during which it is applied. You will use ~1000 G's for 15 minutes; balancing of tubes, loading, and unloading will be supervised by the TA. Since only one run will be made, all material must be ready on time: loading is scheduled for forty minutes after the start of the laboratory.

**Assaying enzymatic activity in the Krebs Cycle**

*Background*

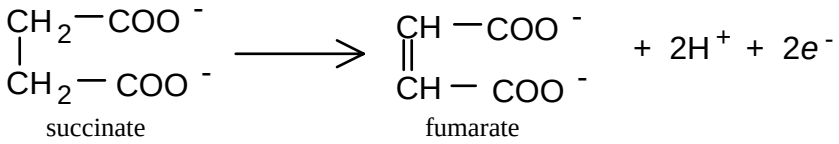
The conversion of simple sugars ($C_6H_{12}0_6$) to water and carbon dioxide involves two kinds of molecular alterations: cleavage and oxidation. In practice, cleavage precedes oxidation. Rather than oxygen being attached to the sugar, the sugar is broken up bit by bit and hydrogens removed for eventual combination with oxygen. Thus several enzymes which catalyze the removal of hydrogen are involved in the general sequence of reactions which oxidize sugar. These enzymes are termed **dehydrogenases**, with each designated in addition by the name of the substrate from which the hydrogen is removed. Under normal circumstances, the hydrogen is linked to oxygen only after the intervention of the so-called "cytochrome electron-transport chain," the primary site of generation of ATP. (Refer to the figure in the appendix of this exercise.)

To assay the activity of a dehydrogenase, it is most convenient to measure the rate at which hydrogens (i.e., hydrogen ions and electrons) are removed from the substrate. One very easy way to measure this is to use the hydrogens to reduce and decolor a reagent dye called 2,6-dichloroindophenol (2,6-D). The rate at which this dye is bleached is a measure of the level of dehydrogenase activity. But several complicating factors must be addressed.

First, the assay as described cannot distinguish between the various substrates and enzymes from which the hydrogen has been removed: the same result is obtained with, for example, succinic dehydrogenase as with lactic dehydrogenase. This problem is circumvented by comparing the rate of decoloration with and without the addition to the system of the specific substrate under scrutiny. A second problem is that the hydrogens do not normally accumulate, but instead are passed to the cytochromes; thus the 2,6-D must compete with the cytochromes and you may end up merely

assaying the results of the competition. This latter problem may be avoided by "poisoning" the cytochrome system; a little cyanide does the job nicely.

You will consider one particular dehydrogenase: **succinic dehydrogenase,** one of the enzymes of the Krebs (TCA) cycle. This enzyme catalyzes the oxidation, via dehydrogenation, of succinate to fumarate:




Enzymes, of course, catalyze the reaction in both directions; the actual direction under normal circumstances is determined by other factors.

*Assay procedures*

To carry out the assay protocol, you will need several reagents, all of which should be handy before you start:

| | | |
|---|---|---|
| Sodium succinate | (SUC) | in screw top bottle or small flask |
| 2,6-dichloroindophenol | (2,6-D) | in screw top bottle |
| Potassium cyanide | (CYN) | in dropper bottle |
| Buffer | (BUF) | in screw top bottle or 250 mL flask |

All of these should be at room temperature. You should also have at hand (a) one labeled 5-mL pipette for the buffer, (b) two labeled 1-mL pipettes, one for the succinate and one for 2,6-D, and (c) two labeled 2-mL pipettes, one for supernatant and one for sediment. Do not use pipettes except for their intended, labeled contents.

Set out a row of six clean test tubes in a rack, numbering them with a grease pencil. Each tube will eventually be filled with 6 mL. of liquid; a list of the contents of each tube is given below. Make up each tube as indicated, but leave out the samples of the supernatant ("Sup") and sediment ("Sed") from the bee thoraxes. ***Be careful with the cyanide - it doesn't discriminate between bees and biologists.***

**Table 3.1** Contents of experimental test tubes for first assay.

| Tube | Succinate | Cyanide | 2,6D | Buffer | Supernatant | Sediment |
|---|---|---|---|---|---|---|
| 1 | - | 1 drop | 1 mL | 4 mL | 1 mL | - |
| 2 | - | 1 drop | 1 mL | 4 mL | - | 1 mL |
| 3 | 1 mL | - | 1 mL | 3 mL | - | 1 mL |
| 4 | 1 mL | 1 drop | 1 mL | 3 mL | 1 mL | - |
| 5 | 1 mL | 1 drop | 1 mL | 3 mL | - | 1 mL |

Now prepare a dilute solution of 2,6-dichloroindophenol to define an arbitrary but constant endpoint for the decoloration; for this purpose, put one or two small drops of the stock dye

solution (from the dropper bottle) in a test tube containing 6 mL of water. This is the sixth and final tube.

Following centrifugation separate the material in your tube into two fractions: "**sediment**" and "**supernatant**." The former is the pellet from the bottom of the centrifuge tube, which will be resuspended in fresh, cold, isolation medium. The supernatant is the fraction that did not form a pellet. Supernatant should be carefully poured off into the plastic centrifuge tube marked "SUP." The volume of sediment can be estimated by marking the outline of the pellet with a grease pencil and later adding water to the tube to fill this outline. The sediment should be diluted to the same volume as the supernatant ( about 8 mL) by breaking up the pellet with a glass rod and re-suspending it in the tube. Record the dilution factor of the sediment (the ratio of final volume to original volume) below. Keep the supernatant and sediment samples in the ice bath.

dilution factor:

When all is ready, add 1 mL of supernatant to Tube #1, agitate it, and note the time. One minute later, add 1 mL of suspended sediment to Tube #2 and agitate it. Similarly, every minute add either supernatant or sediment, as indicated, to another tube in sequence. Leave the tubes in the test tube rack after the initial agitation, and do not handle them. As each tube decolorizes, note the time and determine the elapsed time since the addition of the supernatant or sediment.

**Table 3.2. Results for first assay, enzymatic activity in the Krebs cycle.**

| Tube # | Start time (Ses. or Sup. added) | End Time (2,6-D decolored) | Elapsed time to decoloration |
|---|---|---|---|
| 1 | | | |
| 2 | | | |
| 3 | | | |
| 4 | | | |
| 5 | | | |

*Data analysis*

If you obtained a clean fractionation of the muscle tissue into cytoplasmic and mitochondrial fractions you should see distinct color changes in some of your tubes within 5-10

minutes. These qualitative differences will probably be sufficient to assess enzymatic activity, but as a more quantitative measure you can use the time needed to decolor to the standard endpoint. Since rate is proportional to $time^{-1}$, convenient units for reaction rates are "reciprocal minutes," and the elapsed times should be converted to these units before further calculation.

One further step is necessary to make all of the data comparable. The sediment resulting from the centrifugation was diluted before any assay of enzymatic activity; the supernatant was not. The dilution factor for the sediment was previously recorded; the dilution factor for the supernatant is, of course, one. The rates measured (reciprocal minutes) should be multiplied by the appropriate dilution factor to get rates which refer to the original distribution of material between supernatant and sediment ($rate_1$ to $rate_5$).

An indication of the effectiveness of 2,6-D in competing with the cytochrome system for hydrogens is given by a comparison of the reaction rates measured in Tube #3 (without cyanide) and Tube #5 (same, with cyanide).

Further analysis requires that the background level of dehydrogenase activity be subtracted from the rates measured when a specific substrate was added:

$$\text{SUC dehydrogenase, supernatant} = rate_4 - rate_1 \quad \text{(SUC - Sup.)}$$

$$\text{SUC dehydrogenase, sediment} = rate_5 - rate_2 \quad \text{(SUC - Sed.)}$$

These rates, then, are the primary results of the assays. Comparisons between assays of different groups are facilitated by calculating the ratio: (SUC - Sed) (SUC - Sup.) This latter figure gives a convenient index of the relative activity of SUC dehydrogenase in sediment and supernatant.

*Questions*

Before continuing your investigation using the homogenate you reserved earlier, be sure you understand your work thus far by answering the following questions.

1. Which tubes do you compare to determine whether the reaction that converts succinate to fumarate takes place in the fraction containing the mitochondria? Why?
2. How do Tubes 1 and 2 act as controls?
3. Why might you observe some enzymatic activity in tubes containing the supernatant fraction?
4. Why is it necessary to add cyanide to the tubes?
5. Describe what happens in Tube 3. What causes this pattern?

**Assaying glycolysis and respiration combined**

Your observations with the supernatant and sediment portions of the bee flight muscle should have demonstrated that you were able to isolate a Krebs cycle reaction and subsequent electron transport chain reactions in the mitochondria-containing tissue fraction. With these same fractions and the homogenate you saved (containing both mitochondria and the cytoplasmic contents of the cells), you can now determine whether mitochondria alone are sufficient to carry

out the combined processes of glycolysis and respiration. You will thus be able to infer the location of the reactions of glycolysis.

Prepare three clean test tubes with buffer, dye, cyanide, and substrate as shown in the table below. Label each tube accordingly to the cell fraction that will be added. Since you want to study the whole sequence of reactions in the break down of glucose, you will use this compound as your starting substrate instead of succinate.

**Table 3.3. Experimental set up for assaying enzymatic activity in glycolysis and respiration combined.**

| Tube | Subtrates: Glucose | Cyanide (CYN) | 2,6-D | Buffer (BUF) | Supernatant (Sup) | Sediment (Sed) | Homogenate (Homog) |
|---|---|---|---|---|---|---|---|
| A | - | 1 drop | 1 mL | 3 mL | 1mL | | - |
| B | - | 1 drop | 1 mL | 3 mL | - | l mL | - |
| C | 1 mL | 1 drop | 1 mL | 3 mL | - | - | l mL |
| A’ | | | | | | | |
| B’ | | | | | | | |
| C’ | | | | | | | |

** Looking at the reactions in glycolysis summarized in the appendix, what additional compound do you think needs to be included with the glucose as a substrate, and why?

** What three additional tubes should you include as controls, and what should each contain?

Agitate the tubes of sediment and homogenate to resuspend the mitochondria. Next add 1 mL of the designated cell fraction to the appropriate tubes at 1 minute intervals, and as before, measure the time required for decolorization.

**Table 3.4. Results for second assay, enzymatic activity in glycolysis and respiration combined.**

| Tube # | Start time (Sed, Sup, or Homog added) | End time (2,6-D decolored) | Elapsed time to decoloration |
|---|---|---|---|
| A | | | |
| B | | | |
| C | | | |
| A’ | | | |
| B’ | | | |
| C’ | | | |

*Interpreting the experiment*

1. In which tube do you detect the greatest enzymatic activity? Why?

2. Why is this experiment an indirect test of the cytoplasmic localization of glycolysis?

3. In principle, how could you go about testing directly that glycolysis takes place in the cytoplasm? Why might the pyruvic acid to lactic acid reaction (or its reverse) be a good choice for the above project? Where does this reaction fit on the diagram in the appendix?

**Observing mitochondria directly**

*Background*

In the first part of the lab you inferred the presence of mitochondria in the sediment fraction by their biochemical action. However, with a suitable dye it is also possible to observe metabolically active mitochondria directly. In this part of the lab you will attempt to observe them more in less *in situ* in dissected specimens of muscle tissue.

The dye you will use to make the mitochondria recognizable is *tetrazolium*. Tetrazolium salts when they are oxidized are water soluble and colorless, but when they are reduced -- e.g. by accepting electrons being passed down the electron transport chain -- they become relatively insoluble and turn pink, blue, or purple depending on the particular salt. As a result, they selectively stain active mitochondria. Of course the staining will only occur if the mitochondria are (a) provided with a substrate (such as succinate) for the dehydrogenases to act on and (b) that the electron transport chain is blocked to prevent oxygen from accepting the electrons (as with cyanide).

****** Why must both of these conditions be met for the stain to work?

*Mitochondria in dissected flight muscle tissue*

You will do this work in pairs. Using the well-anesthetized bee provided by your TA, proceed as follows:

1. Place the bee on a paper towel and with a razor blade quickly cut off the head and abdomen, as before. Remove the wings and legs from the thorax.

2. Cut open the thorax along the dorsal midline. This will separate it into right and left halves and expose the flight muscle, which constitutes virtually the entire contents. With a dissecting needle or fine forceps remove a small bit of the brown-colored muscle and *immediately* transfer it to a very small drop of 0.32M mannitol solution on a clean microscope slide. (*Both* students in a team should *each* prepare a slide in this fashion.) Add a drop of mannitol to the opened thorax to prevent it from drying out.

3. Under your dissecting microscope, gently tease apart the muscle fibers with dissecting needles so that they are spread thinly and not clumped together.

4. Now add a small drop of the tetrazolium staining mixture (tetrazolium, cyanide, and succinate) from the dropper bottle. ***Be careful with this solution because both the cyanide and tetrazolium components are poisonous!*** Don't let it touch your skin; if you do get a drop on you, rinse it off promptly. After 2-3 min. carefully add a coverslip to your preparation so it doesn't dry out.

5. After a few more minutes the muscle preparation should gradually turn a pale purple color. Examine your slide under the highest power of your compound scope and look for regular, spherical bodies stained blue and lying in rows between the muscle fibers. These are the mitochondria. In the intact muscle the rows are arranged at regular intervals, although they may be dislodged from this orientation in your preparation. For best view, increase the light level (and open your condenser more than normal for high power), and look for a single layer of teased muscle fibers. Your TA will put the best preparations from the class on a demonstration microscope with an oil immersion lens to further increase the magnification.

Sketch some mitochondria and associated muscle fibers in the space to the right.

muscle fibers and mitochondria of bee flight muscle

****** Is the stain taken up significantly by the muscle fibers? Why or why not?

****** Notice the functional and structural relationship between the mitochondria and the muscle fibers that make up most of the remainder of the cell. The units that produce energy are conveniently located next to the units that utilize that energy. Can you think of another system (or cell type) where you might expect to find a similar relationship?

When you finish with the slides containing the tetrazolium solution, put them in the specially marked beaker for special cleaning. *Do not* wash them yourselves.

**Before leaving the lab, make sure the mortar, pestle, centrifuge tubes, and all glassware at your table are thoroughly scrubbed and rinsed with distilled water. Do *not* use soap.**

## Supplies and Materials

**For each group of four students:**

| Item | (Quantity) |
|---|---|
| 1) Large culture dish | (1) |
| 2) 100-mL beaker | (1) |
| 3) Small mortar and pestle | (1) |
| 4) 10 cm X 20 cm piece of percale cloth | (1) |
| 5) 25-mL graduated cylinder | (1) |
| 6) Small glass funnel | (1) |
| 7) 125-mL flask | (1) |
| 8) Glass stirring rod | (1) |
| 9) Grease pencils | (2) |
| 10) Forceps | (3) |
| 11) Scalpels | (3) |
| 12) Small scissors w/sharp points | (3) |
| 13) Razor blades | (3) |
| 14) Dissecting needles | (8) |
| 15) Plastic clinical centrifuge tubes, 10-mL, with mark at 8 mL: one tube labeled "SUP" the other labeled "SED" | (2) |
| 16) Test tube rack | (1) |
| 17) Glass test tubes, 16 x 150 mm<br>6 tubes labeled with numbers "1" through "6"<br>1 tube labeled "homogenate" | (13): |

3 tubes labeled “A,” “B,” and “C”
3 tubes labeled “A’,” “B’,” and “C’”

18) Pipettes (1 each:)
2-mL labeled “SUP”
2-mL labeled “SED”
1-mL labeled “SUC”
1-mL labeled “2,6-D”
5-mL labeled “buffer”
19) “Pipette Pumps™” (2 blue and 1 green)
20) Stop watch (1)
21) 0.1M potassium cyanide in 30-mL, square dropping bottle, labeled “Cyanide: **POISON!**” (1)
22) 2,6-D (dichloroindophenol) in labeled, 30-mL, square dropping bottle (1)
23) Tetrazolium staining mixture in 30-mL, square dropping bottle labeled “Tetrazolium staining mix: **POISON!**” (1)
24) 0.32M mannitol in labeled, 30-mL, square dropping bottle (1)
25) Screw top bottle of buffer, labeled, about 200 mL (1)
26) Screw top bottle of sodium succinate, labeled, about 200 mL (1)
27) Screw top bottle of 2,6-D, labeled, about 200 mL (1)
28) Screw top bottle of glucose, ~200 mL, labeled (1)
29) Paper towels (1 dz.)
30) Mason jar w/ cheesecloth cover held in place w/ ring or rubber band (1)
31) Live bees (in Mason jar) (about 10g per jar)
32) Compound microscopes, preferably with oil immersion lenses (2)
33) Glass slides (4)
34) Cover slips (1 box)
35) Dissecting microscopes (4)

**Materials for each lab room:**

Item (Quantity)

1) 1-L bottle isolation medium (stored in refrigerator or kept on ice) (1)
2) Large (e.g. 30 gallon) trash can, with lid, containing 5 cm distilled water (1)

| | | |
|---|---|---|
| 3) | Bricks, placed in bottom of trash can supporting metal rack or grid | (2) |
| 4) | Small insulted ice chest with 4-5 chunks dry ice | (1) |
| 5) | Larger ice chest with crushed ice | (1) |
| 6) | Hammer | (1) |
| 7) | Thick work gloves | (1 pr.) |
| 8) | Mason bee-jar with 8-10 live bees | (1) |
| 9) | "Sting-Kill™" kit (topical anaesthetic) | (1) |
| 10) | Small clinical desk centrifuge (to hold twelve 10-mL tubes) | (1) |
| 11) | Test tube racks | (2) |
| 12) | 1000-mL beaker, labeled "Tetrazolium Waste – **POISON**" (Beaker should contain 300-400 mL acetone or ethanol.) | (1) |

**Solutions (all in labeled bottles):**

1) Isolation medium:

0.25 M sucrose: 342.3 g/mol x 0.25 mol/L = 85.58 g/L
1.0 mM EDTA: 380.2 g/mol x 0.001 mol/L = 0.38 g/L
Mix the above with distilled water to make 1 liter. Refrigerate. Number of liters needed depends on class size (40 mL per student group).

2) Buffer:

15.0 mM KCl: 74.56 g/mol x 0.015 mol/L = 1.12 g/L
2.0 mM EDTA: 380.2 g/mol x 0.002 mol/L = 0.76 g/L
5.0 mM $MgCl_2$: 203.3 g/mol x 0.005 mol/L = 1.02 g/L
50.0 mM "Tris" (THAM): 121.1 g/mol x 0.05 mol/L = 6.06 g/L
Mix the above with distilled water to make 1 liter. Bring buffer solution to a pH of 7.5 with HCl. This buffer will also be used to make sodium succinate and glucose solutions. Number of liters needed depends on class size (50 mL per student group).

3) Sodium Succinate:

0.25 M sodium succinate: 270.2 g/mol x 0.25 mol/L = 67.55 g/L
Mix with 1 liter of buffer. One liter makes enough for 20-25 student groups.

4) 2,6-dichloroindophenol:

2.5 mM 2,6-dichloroindophenol: 290.8 g/mol x 0.0025 mol/L = 0.73 g/L
Make 500 mL: 0.37 g 2,6-D to 500 mL distilled water. Keeps indefinitely.
Dispense to glass screw top bottles and dropping bottles.

5) Potassium cyanide:

0.1 M KCN: 62.12 g/mol x 0.1 mol/L = 6.21 g/L
Mix with distilled water in small amounts: 1.24 g KCN in 200 mL distilled water is enough for 12 dropping bottles.
Make fresh each semester!
Label as "potassium cyanide, **POISON.**"
Dispense to dropping bottles.

6) Glucose solution:

0.015 M glucose: 2.7g/liter
Mix with 1 liter of buffer. Refrigerate. One liter makes enough for 20-25 student groups.

7) Tris (THAM) buffer, pH7.4:

Mix 43.0 mL 1 N HCl with 166.0 mL 0.4 M Tris (12.1 g in 250 mL distilled water).

8) Tetrazolium staining mixture:

Dissolve 50 mg. of tetrazolium blue in 2.5 mL of N-N dimethyl foramide (DMF).
Add 5 g sodium succinate and 100 mL distilled water.
Add 200 mL tris buffer.
Add 1.8 g KCN.
Label as **POISON.**
Dispense to dropping bottles.

9) Mannitol solution:

0.32 M mannitol: 182.8 g/mole x 0.32 mole/liter = 58.5 g/L
Make small amount by adding 11.7 g to 200 mL distilled water.
Dispense to dropping bottles.

## Notes for the Instructor

**Safety**

Safety is an obvious concern in this exercise. Students should be admonished to be careful with the cyanide and tetrazolium solutions, and it is best to put out only small quantities (15-20 mL) in bottles that are hard to knock over.

Regarding the bees, the most likely source of stings is not from intact, live bees but rather from careless handling of the clipped off abdomens. Students have been stung after putting their arm on an abdomen that was not kept on the paper towel after the cutting, stepping on an abdomen on the floor (student was wearing sandals), and wadding up the paper towel with the detached abdomens. (This has really happened!) Careless handling of the bee jars can also results in stings, most often from a student putting a hand over the cheesecloth at the top of the jar or dropping the jar, breaking it. The best solution in that extreme case is for the instructor to

open a window (if possible) and turn off the lights while everyone calmly leaves the room. The bees will usually fly out the window.

While cutting the bees, students should only spill out roughly 12-15 bees per group member, then firmly reseal the jar so that the remaining bees don't escape once the anaesthetic wears off. If a group has poured out too many bees to cut before some start waking up and walking around, it is important for the instructor to step in decisively and calmly help everyone cut off abdomens as quickly as possible. The bees can then no longer escape or sting. As a last resort, bees that are vibrating their wings and getting ready to fly should be squashed with any large flat object except an open hand. Because not all of the students may be familiar with bee anatomy, it may be a good idea to use a diagram illustrating the three body regions: head, thorax (has wings and legs), and abdomen (has stinger).

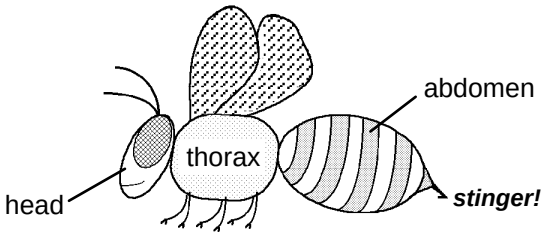



**Figure 3.1. Diagrammatic anatomy of the honeybee.**

Two minutes in the carbon dioxide anaesthetic is usually enough to knock the bees out sufficiently for cutting, but longer periods (e.g., 5 minutes) don't seem to cause any harm. However, repeated anesthetization, as may be necessary while prepping the lab, will adversely affect the bees over the long term and so should be kept to a minimum.

Students who know that they have a severe allergic reaction to bee stings should wait outside of the lab room until all of the bees have been cut up, the abdomens are properly disposed of, and the thoraxes are being ground.

**Assays and analyses**

Usually the color change in the first assay occurs fast enough in the expected tubes (see below) that it is not necessary to calculate decoloration rates in "reciprocal minutes" or to apply the dilution factor obtained by measuring the volume of supernatant and sediment. However, this information is very useful to have if the activity levels are low, or there seems to be little difference in the time to decoloration between tubes. The sediment fraction is typically only about a tenth that of the supernatant, so multiplying reciprocal minutes by the dilution factor to correct for the difference in volume will make it obvious that rate of change is indeed much greater with the sediment fraction.

Typically Tube 5 turns colorless the most quickly. Sometimes Tubes 1 and 4 also change but more slowly or not as much. Tube 3 also changes, usually from the bottom up and eventually has a thin blue layer on top. It is quite possible that some tubes will not change at all or so slowly that it is not feasible to measure a rate of decoloration. That means that a qualitative analysis of the results is quite sufficient.

Usually changes are noticed in the tubes within 5 minutes after the last tube is started. If there is no change at all in any tube after 6-7 minutes, tell the group to add an additional 1 mL of supernatant or sediment, as appropriate, in the same order as before. This "double-shotting" will usually compensate for low activity levels. If there is still no effect, supernatant or sediment from another, successful group can be used.

The most informative comparison is between Tube 4 (supernatant + substrate) and Tube 5 (sediment + substrate). Faster (or more complete) change in Tube 5 is the evidence that the machinery for the succinate to fumarate conversion and for the electron transport chain are located in the sediment fraction of the cell, the part that contains the mitochondria.

Tubes 1 and 2 in the first assay are the controls that indicate that we have in fact chemically isolated the specific succinate to fumarate reaction postulated for Tubes 4 and 5. Manipulating this reaction by adding excess substrate should make it go faster and result in faster decoloration of the dye. Measuring the rate of any decoloration that occurs in the *absence* of added substrate (Tubes 1 and 2) controls for the effect of succinate already present in the system and especially for other reactions in the cell that could also cause decoloration. Without the controls to measure background levels of activity, we could not be confident that a change in Tube 5 was indeed due to the succinate to fumarate conversion, and that the enzyme responsible (and the electron transport chain) were located in the mitochondria.

Some activity may occur in the supernatant fractions (Tubes 1 and 4) if they contain pieces of mitochondria. The enzyme responsible for the succinate to fumarate conversion, succinate dehydrogenase, is membrane bound in the mitochondria, as is the electron transport chain (see appendix diagram). Thus even small fragments of mitochondria that do not sediment out fully (e.g., ones produced by overly vigorous grinding) can still show activity. In addition, other, unknown redox reaction in the cytoplasmic fraction of the cell may be able to reduce the 2,6-D.

Tube 3 is especially interesting. It contains the sediment fraction with mitochondria but does not have cyanide to block the transfer of electrons to oxygen. However, once the oxygen is used up by the mitochondria, then the 2,6-D can act as an alternative electron acceptor. Decoloration starts at the bottom, farthest from a source of atmospheric oxygen and where the mitochondria tend to settle. Eventually, only a thin layer of oxidized, blue dye remains at the surface where oxygen can diffuse in from the air. The thickness of this layer indicates how far oxygen can move by diffusion to supply the metabolically active mitochondria in the test tube.

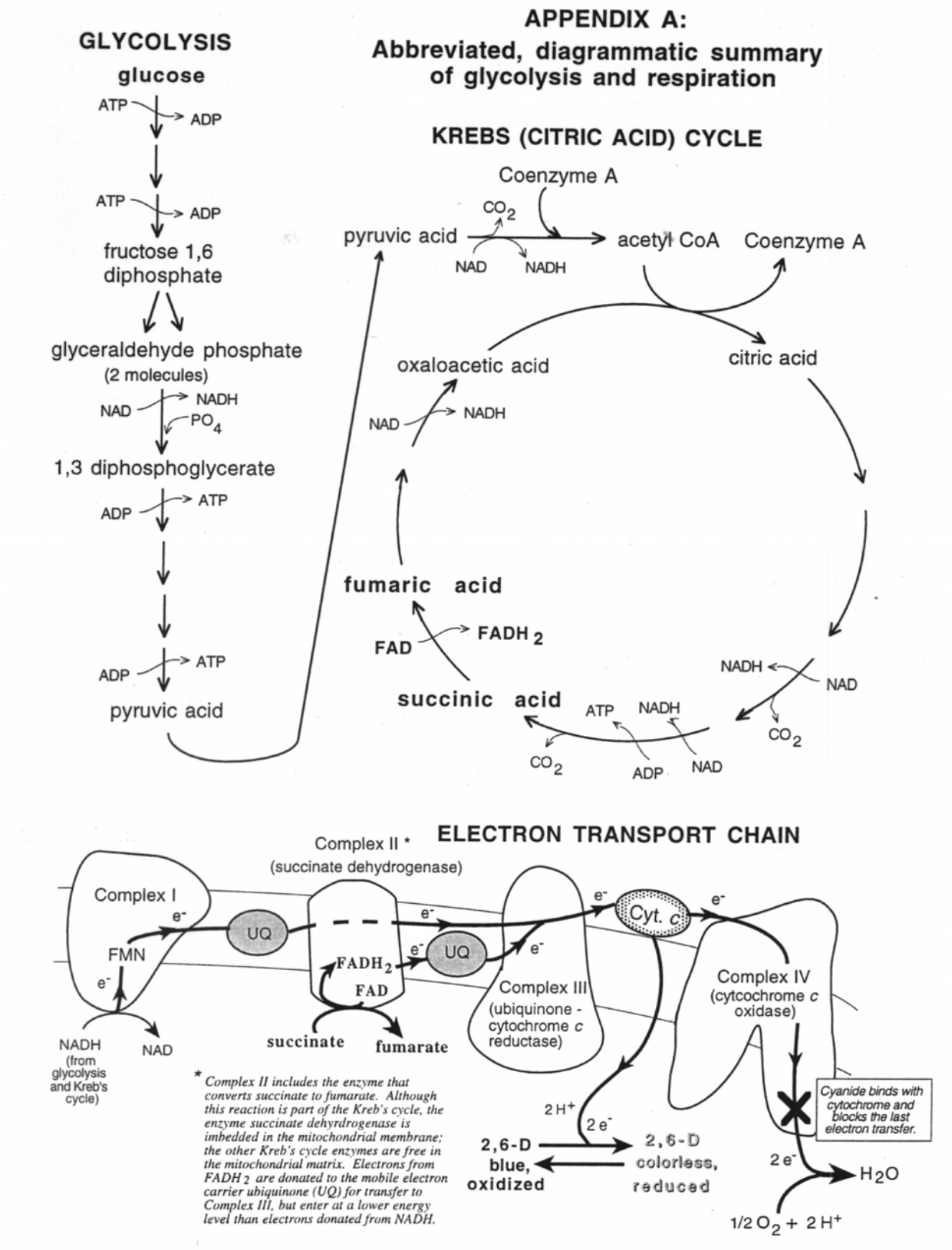
Notice that the diffusion distance is very short. If we think of Tube 3 with its active mitochondria as a model "organism," we can see that diffusion from the external environment alone can only sustain a very thin creature, like a flatworm. Larger animals require a circulatory system. You can demonstrate this by taking a Tube 3 from one of the students' assays and agitating the contents vigorously. This distributes oxygen in the air throughout the "organism." The oxygen is able to pull electrons off of the dye so the tube turns blue again. If it is left undisturbed, e.g., the circulation ceases, the oxygen will soon be consumed and the dye will again decolor as it accepts electrons. The cycle can be repeated as long as the mitochondria remain alive and active.

For the second assay, the substrates are glucose, and in principle also ATP to "prime the pump" at the start of glycolysis. In practice there appears to be enough ATP (and NAD) present in the homogenate that it is not necessary to add these compounds. The controls for this second assay are obviously tubes that contain sediment, supernatant, or homogenate but not glucose.

The greatest activity in the second assay is expected in Tube C. (If the bees are well fed and active it is not unusual for the control for Tube C to be nearly as active as Tube C itself; apparently there is already an excess of glucose in the tissues.) We can thus infer that the cytoplasmic fraction contains the enzymes for glycolysis because Tube C contains both the cytoplasm and the mitochodrial fractions of the cell whereas Tube B, which contains only mitochondria, shows little activity with glucose as a substrate. Tube A, which has only the cytoplasm, also typically shows little activity because our assay depends on the presence of the electron transport chain in the mitochondria to reduce the 2,6-D, and these are missing. If time and material permit, students can confirm the role of both cell fractions by measuring the activity of a mixture of the supernatant and sediment fractions, essentially "reconstituting" the cell contents.

## Acknowledgement

This lab is derived from an exercise originally developed by Professor Steve Vogel of the Department of Zoology, Duke University.


APPENDIX A:
Abbreviated, diagrammatic summary of glycolysis and respiration
GLYCOLYSIS
glucose
ATP
ADP
ATP
ADP
fructose 1,6 diphosphate
glyceraldehyde phosphate
(2 molecules)
NAD
NADH
PO4
1,3 diphosphoglycerate
ADP
ATP
ADP
ATP
pyruvic acid
KREBS (CITRIC ACID) CYCLE
Coenzyme A
CO2
pyruvic acid
NAD
NADH
acetyl CoA
Coenzyme A
citric acid
oxaloacetic acid
NAD
NADH
fumaric acid
FAD
FADH2
succinic acid
NADH
NAD
ATP
NADH
CO2
ADP
NAD
ELECTRON TRANSPORT CHAIN
Complex II *
(succinate dehydrogenase)
Complex I
e-
UQ
e-
FMN
e-
FADH2
FAD
e-
UQ
e-
Cyt. c
e-
Complex III
(ubiquinone - cytochrome c reductase)
Complex IV
(cytcochrome c oxidase)
NADH
(from glycolysis and Kreb's cycle)
NAD
succinate
fumarate
* Complex II includes the enzyme that converts succinate to fumarate. Although this reaction is part of the Kreb's cycle, the enzyme succinate dehyrdrogenase is imbedded in the mitochondrial membrane; the other Kreb's cycle enzymes are free in the mitochondrial matrix. Electrons from FADH2 are donated to the mobile electron carrier ubiquinone (UQ) for transfer to Complex III, but enter at a lower energy level than electrons donated from NADH.
2 H+
2 e-
2,6-D
blue, oxidized
2,6-D
colorless, reduced
Cyanide binds with cytochrome and blocks the last electron transfer.
2 e-
H2O
1/2 O2 + 2 H+

# APPENDIX B
## TA Flow Chart for Isolation and Assay of Bee Flight Muscle Mitochondria

1. Pre-rinse glassware twice with distilled water.
2. Chill a dry mortar and pestle and a beaker containing 25 mL of isolation medium.
3. Anesthetize bees.
4. Quickly cut up 10g of bees, in small batches at a time. (Cut *all* bees in the jar; *do not leave any bees uncut!*) Collect thoraxes in mortar. Add 10 mL isolation medium. Remove mortar from ice and place flask on ice to chill.
5. Grind bee thoraxes in mortar.
6. Filter liquefied mortar contents into the chilled flask. Grind thoraxes again briefly. Filter. Rinse mortar with 5 mL of isolation medium and filter entire contents.
7. Add filtrate to *pre-measured* 8 mL mark on centrifuge tube labeled SED. (for "sediment"). Save the remainder in the test tube marked HOMOG. (for "homogenate").
8. Mark group i.d. on centrifuge tube. Keep chilled until centrifuged.
9. TAs take tubes for a spin in centrifuge.
10. Prepare assay tubes as specified in lab manual.
11. When centrifuge tube returned, carefully pour off supernatant, and place in chilled tube marked SUP. (for "supernatant"). Mark sediment level on centrifuge tube. Re-suspend pellet in SED centrifuge tube filled to 8 mL mark.
12. Run assays. Discuss experimental designs and results.
13. CLEAN *ALL* GLASSWARE *SCRUPULOUSLY*. SCRUB AND RINSE WITH DISTILLED WATER, BUT *DO NOT USE SOAP*.