General Biology: Cells and Molecules

Lab 2: Isolation of Mitochondria from Beef Liver

Materials:

- Fresh beef liver (mmm...)
- Top loading balances
- 500 mL of 0.25 M sucrose, 10 mM HEPES buffer, pH 7.5 (Homogenization buffer)
- 500 mL of 0.25 M sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5 (Suspension buffer)
- Teflon homogenizers
- Refrigerated centrifuge
- 2 mL Eppendorf centrifuge tubes*
- Janus Green B stain
- 12 compound microscopes
- Slides and coverslips
- Lens paper; Q-tips; lens cleaner
- Plastic tubs for washing glassware
- Ice buckets (12)
- Centrifuge tube racks

Objectives:

1. Describe how organelles and cell structure can be studied by fractionating cells.
2. Determine the presence of mitochondria within a cell fraction using an assay.
3. Learn to use a compound microscope, make a wet mount.
4. Understand the relationship between rpm and rcf (to be explained in lab) and to calculate rcf from rpm.

Background

In order to study the parts of a cell (organelles), the cell has to be separated into its component parts by cell fractionation while still maintaining the properties of the individual organelles. A number of methods have been devised to "break open" (lyse) the cell. Among these are ultrasonic vibration, osmotic shock, and grinding. Here we use the grinding action of a tissue homogenizer to lyse the cells so that the parts can be separated without damage. The resulting material is referred to as **cell homogenate** or **cell extract**. Differences in physical characteristics of organelles, such as size, shape, and density, make it possible to separate them by spinning in a centrifuge at various speeds and for different periods of time. This process is appropriately called **differential centrifugation**. The larger components tend to settle first.

As a result of centrifugation, the larger material tends to settle at the bottom of the centrifuge tube. This material is called a **pellet**. The liquid that floats above the pellet is called the **supernatant**. In this lab, the first pellet will contain amyloplasts; as the major component, making it white with a tinge of green from the chloroplasts. The second pellet is most likely to contain nuclei. Most of the chloroplasts will settle into the second and third pellets as evidenced by the characteristic green color. The final supernatant will contain mitochondria and most of the cytosolic protein. This is useful to know, especially if you plan to do future labs involving assays for mitochondria and cytosolic protein.

In this lab, you will separate the organelles from the cells of cauliflower using differential centrifugation. You will use a colorimetric assay to indicate the presence of the mitochondria. You will also attempt to visualize them under the microscope (although they are quite small).

Procedure

1. Using a scalpel, cut a small piece of beef liver and weigh it on a top loading balance. Trim it to a 1 g piece (weigh it out to nearest 0.1g).
2. Add the liver to a mortar and pestle apparatus. Add 9.0 ml Homogenization Buffer (0.25 M sucrose, 10 mM HEPES pH 7.5). This will produce a 10% extract, (a term used to indicate a homogenized suspension).
3. Transfer your crude extract to a tissue homogenizer. Insert the Teflon plunger and begin to pump up and down. There is a tight fit between the Teflon plunger and the sides of the tube that creates a shearing action of the tissue. It will take approximately 4 'pumps' to completely homogenize the tissue.
4. Each student should then fill a 2mL Eppendorf centrifuge tube with 2 mL of the extract and centrifuge at 4,500 x g for 10 minutes at 4° C. We will centrifuge all samples together to save time.
5. Decant the supernatant into clean centrifuge tubes and place the tube with the pellet on ice.
6. Recentrifuge the supernatant at 16,000 x g for 25 minutes at 4° C.
7. Decant and discard the supernatant.
8. Resuspend the pelleted mitochondria in 2 ml of Suspension buffer (0.25 M sucrose, 10 mM HEPES, 1 mM EDTA pH 7.5) by pipetting up and down.
9. Recentrifuge the suspended pellet at 16,000 x g for 25 minutes at 4° C.
10. Decant and discard the supernatant. Resuspend the washed pellet (it should contain the mitochondria) in 20 ml of Homogenization Buffer (0.25 M sucrose, 10 mM HEPES pH 7.5) and place the suspension in an ice bucket until further use is required. The suspension will remain active for approximately 4-6 hours if kept on ice.
11. In a new tube, mix 3 drops of Janus Green B* solution with 100 μL of mitochondrial suspension. Observe the color of the tube.
12. Place one drop of this mixture onto a slide. Put a coverslip on and observe under the compound microscope. Nancy will demonstrate the use of the scopes and help you identify the mitochondria (if we can!).

*Janus Green B is a dye that is blue/green in color. It accepts H^+ (hydrogen ions) that are used in the making of ATP at the mitochondria. When the dye accepts the H^+, it casues the dye to become colorless (this is a REDOX reaction – we will learn more about them next week).