EXPERIMENT 2

Cell Fractionation and DNA Isolation

Background Information

A. The Eukaryotic Cell

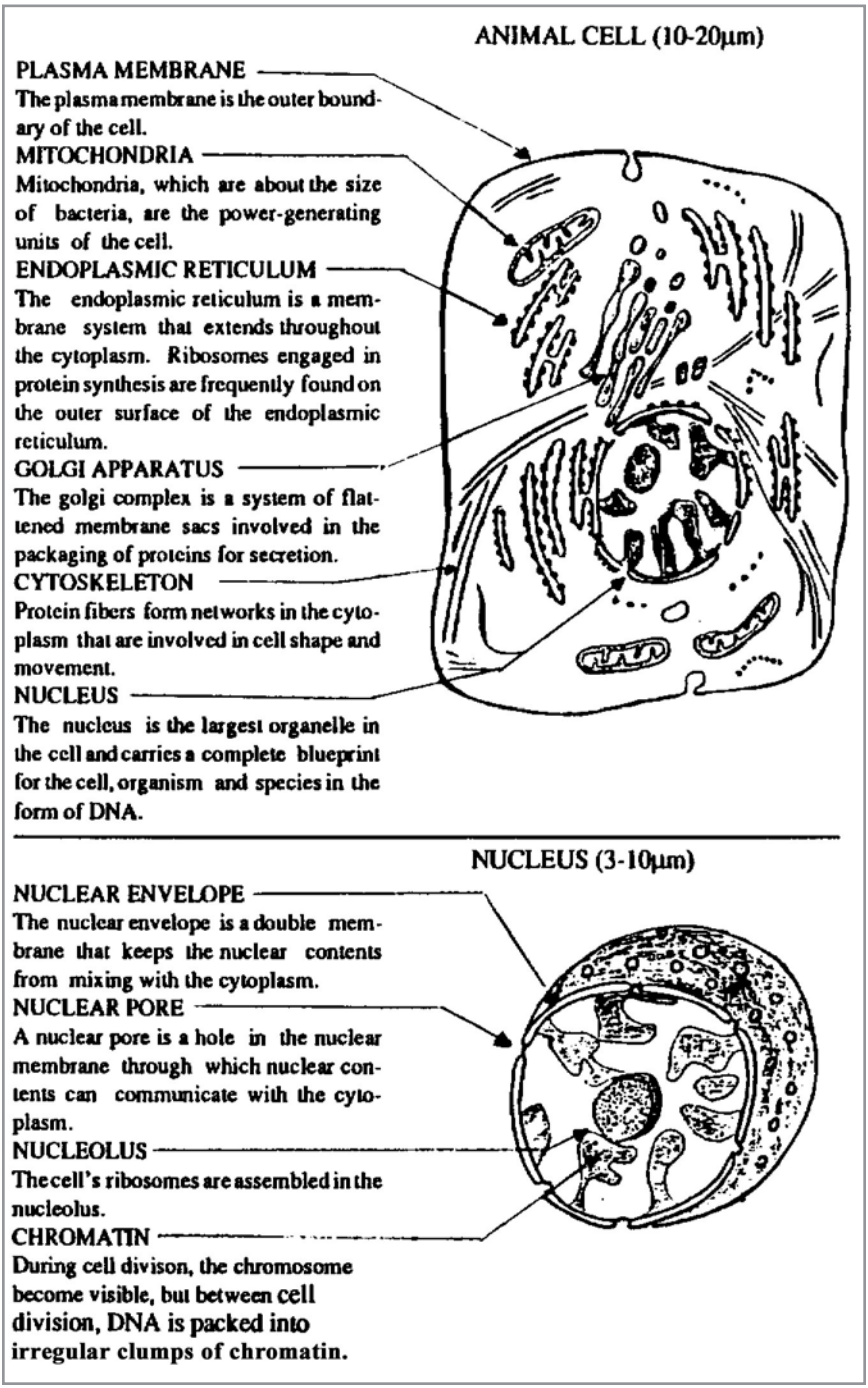
Cells are frequently classified into two basic types: prokaryotic and eukaryotic. The prokaryotic cell, so named because it lacks a discrete nucleus, is found in bacteria and blue-green algae. Prokaryotic cells have relatively simple internal structures, although their metabolic activities are as complex as those in humans. The eukarytoic plan is observed in true algae, fungi, protozoans, and the cells of higher animals and plants. The eukaryotic cell, by definition, has a nucleus that contains most of the cell's DNA. The nucleus is enclosed by a double membrane, the nuclear envelope, that separates nuclear components from the cytoplasm. The eukaryotic cell also contains a number of other specialized internal structures not found in prokaryotes including mitochondria, chloroplasts, and a rich array of internal fibers and membranes. A diagram of a typical animal cell is given in Figure 2-1.

The genetic material in a prokaryotic cell usually consists of a single circular molecule of DNA that is compacted within the cell interior. In contrast, DNA in the eukaryotic nucleus is partitioned into a number of chromosomes, and the DNA of an entire chromosome is thought to consist of a single linear molecule. Human cells contain 46 chromosomes and the average extended length of each chromosomal DNA molecule is about 4 centimeters. Therefore, the length of DNA molecules in length of DNA in an individual composed of 10^{14} cells is $2x10^{14}$ meters. This length is about 200 times greater than the distance from the earth to the sun! Clearly the DNA must be condensed or folded in some manner to fit within the confines of the nucleus.

In the nucleus of a typical eukaryotic cell, individual chromosomes can be identified only during cell division. However, a eukarytoic cell spends most of the life cycle in interphase, which is the stage between cell divisions. In interphase, the chromosomes are diffuse and the chromosomal material is called chromatin.

In addition to DNA, chromatin and chromosomes contain proteins called histones, which are present in essentially all cell types in both plants and animals. Histones are basic in nature because they have large amounts of the basic amino acids arginine and lysine. These basic proteins associate with the acidic DNA backbone, folding the long DNA molecules into chromatin and compact chromosomes. When these proteins are dissociated (removed) from chromatin, the DNA molecules unfold, resulting in an increase in the viscosity of the solution.

Figure 2-1. A Generalized Animal Cell and Nucleus

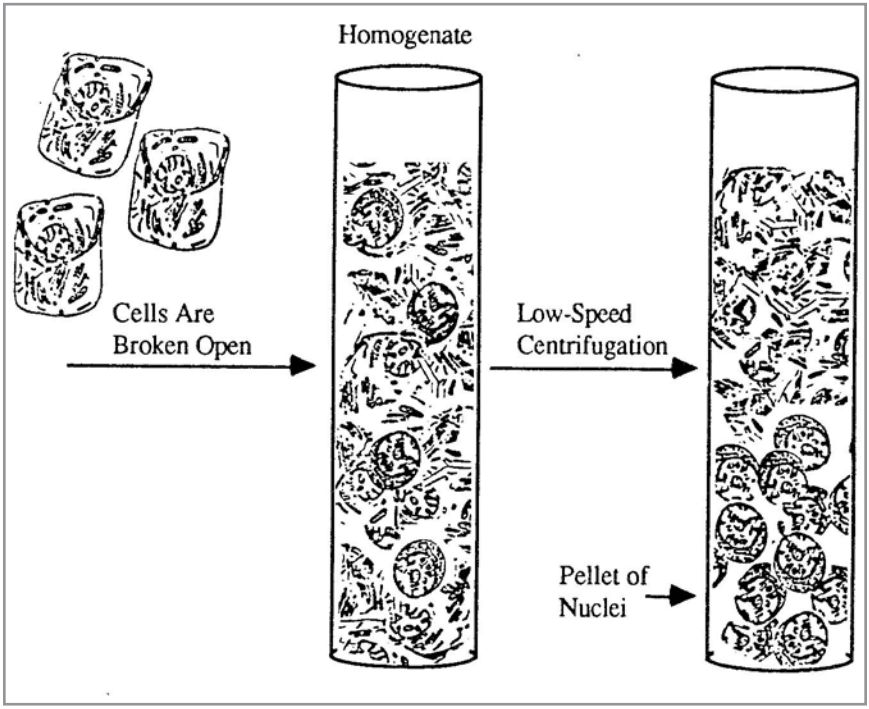


B. Cell Fractionation

An understanding of the eukaryotic cell at the molecular level requires isolating intact organelles. To accomplish this goal, techniques are required for disrupting cells in a controlled fashion. Cells can be broken open in various ways: by mechanical action, by osmotic shock or by addition of detergents that dissolve the plasma membrane. If carefully performed, disruption procedures reduce cells to an extract (called a homogenate) containing soluble components, plasma membrane fragments and intact organelles.

Nuclei are the largest organelles in a homogenate and can be separated from the other components by relatively low speed centrifugation. Following centrifugation, nuclei can be recovered as a pellet on the bottom of the centrifugation tube. These nuclei should retain the characteristic structure of those found in intact cells. The basic steps for the preparation of nuclei are shown in Figure 2-2.

Figure 2-2. Isolation of Nuclei



Objective
To isolate DNA from the nuclei of eukaryotic cells.

Materials Provided

Calf Thymus (15g) - The thymus should be thawed at the beginning of the class period by placing the bag containing the tissue in warm water.
Denatured alcohol- The alcohol should be placed in a freezer at least 3 hours before the laboratory period.
8 glass vials
8 rods for DNA spooling
Transfer pipets
Sodium Dodecyl Sulfate (SDS)
*Nuclear stain
Cheese cloth
*Nuclear buffer - This buffer should be placed in the refrigerator at least 3 hours before the laboratory period.

*Prepared as described in Appendix 2 of the Instructor's Manual

Materials Not Provided

Ice bath - ice chips in a large beaker are suitable
Microscope slides (16) and microscopes
Refrigerated centrifuge and centrifuge tubes (10ml-30ml capacity) - A small clinical centrifuge placed in the refrigerator is suitable for this exercise. A device for tissue disruption such as a food blender (Waring or similar types), a commercial glass homogenizer or a mortar with pestle. The device should be precooled before the laboratory session.
Funnel
Beakers
Toothpicks
Scissors

Procedure
There are three basic parts to this exercise: (I) Isolating nuclei from calf thymus cells. (II) Preparing DNA from these nuclei and (III) Comparing the isolated nuclei to the nuclei in intact thymus cells by microscopic analysis. Part I should be performed by the instructor or by the class as a group during the laboratory session. Eight students or eight teams of students should then perform parts II and III.

I. Isolation of Nuclei

1. Place the 15 grams of thymus into a 500ml beaker and cut the tissue into small (~$lcm^{3)}$ sections with scissors.

2. Place a few of the tissue sections into a precooled beaker. These sections will be used for the microscopic analysis described in Section III.

3. To the remainder of the tissue, add 100ml of cold nuclear buffer.

4. Pour the tissue-buffer mixture into a chilled blender and add about $10cm^3$ of ice chips. Turn on the blender and homogenize for 60 seconds. The mechanical action of the blender as well as the chemical action of a detergent that is present in the nuclear buffer should disrupt the plasma membrane leaving the nuclear envelope intact. If a blender is not available, pour the tissue-buffer mixture into a precooled mortar in small portions and grind the tissue sections with the pestle until a homogeneous suspension is formed.

5. Filter the homogenate through 2 layers of cheese cloth and discard the material that adheres to the cloth. Pour the homogenate into centrifuge tubes and centrifuge for 5 minutes at 2000x gravity. Increase the time and/or speed of centrifugation if pellets are not formed. If a centrifuge is not available, proceed to steps II and III (below).

6. Carefully pour off and discard the supernatant (top liquid layer). The pellets at the bottom of the centrifuge tubes contain nuclei.

7. Transfer the nuclear pellets to the chilled blender and add 100ml of nuclear buffer.

8. Blend the pellets for 10 seconds, filter the nuclear suspension through 2 layers of cheese cloth and store it in a chilled state.

II. DNA Isolation

In the nucleus, DNA molecules are tightly bound to histone proteins that fold these gigantic molecules into compacted chromatin. In order to liberate the DNA from chromatin, these proteins must be removed. One method to dissociate the histones from DNA uses the protein denaturant sodium dodecyl sulfate (SDS). This denaturant alters the histones, thereby disrupting the noncovalent bonds that link these proteins to DNA.

1. One member from each group should place 2ml of the nuclear suspension into a glass vial. Note the appearance and consistency of this suspension.

2. Using a large transfer pipet, add 1ml of the SDS solution to the vial and mix the contents of the vial carefully. Note the appearance and consistency of this solution.

3. After 5 minutes, **carefully** pour the cold alcohol into the vial so that the alcohol forms a layer on top of the solution. Add enough alcohol so that the vial is nearly full. Dip one end of the rod into the vial through the cold alcohol, into the DNA. Slowly rotate the rod and then raise it back into the alcohol. Fine DNA fibers should form on the end of the rod. Produce more of these DNA fibers by turning the rod and moving it up and down between the DNA and the alcohol.

4. After no more fibers can be formed, remove the rod and examine the DNA fibers wound around it. The fibers are the alcohol-insoluble form of DNA, contaminated with some proteins.

III. Microscopic Analysis

In this section, you will compare the nuclei in intact thymus cells to the nuclei that were isolated in Section I.

1. Obtain two clean microscope slides from your instructor and label one slide A and one slide B.

2. Using the blunt end of a toothpick, scrape the surface of one of the sections of thymus from Section I to remove a few cells. Smear these cells onto the center of the slide labeled A and allow the preparation to dry.

3. Using a transfer pipet, apply a small drop of the nuclear suspension to the center of the slide labeled B. Smear the nuclei on the slide using a clean toothpick and allow the preparation to dry.

4. In general, cells and organelles must be fixed before they can be stained. Fixation makes cells permeable to stains and causes them to adhere to the surface of the slide so they will not be washed away during the staining procedures. Place three drops of alcohol onto the slides in order to fix the preparations. After two minutes, rinse the slides in water.

5. Cells and cell organelles are almost invisible by standard light microscopy unless they are stained. To stain your preparations, place 3 drops of the nuclear stain onto your slides and stain for 5-10 minutes. Remove excess dye by rinsing the slides in water and then permit the slides to air dry for at least 10 minutes before microscopic examination. The slides can be viewed at this time or stored until the next laboratory session.

6. Locate the cells and nuclei under the low power of your microscope and then add a drop of oil and examine them with the oil immersion lens. The nuclei should stain purple and the cytoplasm pale blue. Identify the plasma membrane, cytoplasm and nuclei on slide A and compare the nuclei on slide A to those on slide B. Also note the nucleoli, which will appear as small dark blue dots in the nuclei.

Study Questions and Analysis

1. Compare the morphology of the isolated nuclei to the nuclei in intact thymus cells.

2. Describe the changes in the clarity and viscosity of the nuclear suspension that occurred upon addition of SDS. Why did these changes occur?

3. Thymus cells have a relatively small amount of cytoplasm and this feature makes thymus tissue ideal for DNA extraction. Why?