
A. Introduction

The study of the structure, function and biochemistry of cellular compartments and organelles often requires the isolation and purification of these cellular structures. Cell fractionation is the process scientists use to produce fractions of functioning cellular components. The cell fractionation process involves two basic stages. First, the disruption of the tissue and gentle lysis of the cells, a process which creates a suspension of cell components, and second, the separation of the cellular components into purified fractions, a process usually accomplished through centrifugation.

For several reasons, mammalian red blood cell plasma membrane is the perfect cellular component on which to first learn basic fractionation techniques. One reason is that mature mammalian red blood cells are very easy to obtain. A second reason is that mammalian red blood cells are easy to lyse. And a third reason is that mammalian red blood cells have no internal membrane (erythrocytes have no nucleus and no organelles), and therefore there is no source of possible contamination of the plasma membrane fraction from other types of cellular membranes.

The scientific objective of today's laboratory activity is to produce a relatively pure fraction of mammalian red blood cell plasma membranes. Once purified, the membranes will be frozen for later analysis of their protein composition.
B. The Mammalian Red Blood Cell

Composition of Mammalian Whole Blood

Mammalian whole blood consists of two basic parts. Plasma, the non-cellular liquid part, makes up approximately 55% of total blood volume. It is approximately 90% water, 7% protein, and 3% other organic and inorganic molecules and ions. The cellular components (formed elements) of the blood make up approximately 45% of the total cell volume. Approximately 99% of the cells are red blood cells (erythrocytes). The remaining cells are white blood cells (leukocytes) and platelets (thrombocytes).

When mammalian whole blood is subjected to centrifugation (5000 x g, 5 min.), the cellular components collect at the bottom of the tube to form a pellet. Since the red blood cells are the most dense of all the cells, they collect at the very bottom. The white cells and platelets form a sometimes inconspicuous buffy coat on top of the red cells. The supernatant, that is, the liquid that sits on top of the pellet, is plasma.

The Mammalian Red Blood Cell

The normal mammalian red blood cell is a flexible biconcave disc, approximately 7 - 8 micrometers in diameter. This shape optimises the layered, or laminar, flow of blood through blood vessels.

Mature mammalian red blood cells have no nuclei or organelles. The nuclei and organelles are extruded from the cell during early phases of red cell production (erythropoiesis). The intracellular compartment of the mature mammalian red cell consists entirely of a cytoplasm containing nothing more than an aqueous solution of simple inorganic and organic molecules and macromolecules, with a very high concentration of the protein hemoglobin. This
lack of cellular structure enables mammalian red blood cells to efficiently squeeze through the smallest capillaries. Moreover, the lack of mitochondria eliminates red blood cell demand for oxygen and consumption of the oxygen they transport; mammalian red blood cells produce ATP by through glycolysis and lactic acid fermentation.

Since mature mammalian red blood cells lack nuclei, mitochondria and rough endoplasmic reticulum, they do not contain DNA and consequently cannot divide. They also cannot synthesize RNA nor synthesize any new proteins, and consequently have a limited life span. Mature red blood cells circulate for about 100–120 days in the body before they are removed by the spleen.

C. Cell Fractionation

Introduction

Cell fractionation is the process of producing relatively pure fractions of cellular components. The process involves two basic steps: disruption of the tissue and lysis of the cells, followed by centrifugation.

Tissue Disaggregation & Cell Lysis

The first step in cell fractionation is tissue disruption and cell lysis. The objective is to disaggregate the cells and gently break them open with minimum damage to the cellular components. This can be accomplished in several ways: 1) homogenization, 2) sonication, and 3) chemical / osmotic lysis. The particular method one chooses depends on the tissue and cell type and the particular cell fraction of interest.

Homogenization is probably the most widely used cell disruption technique. Homogenization is a process by which fluid shearing is used to break open plasma membranes. It involves the use of a mechanical homogenizer, like a blender or a motorized mortar and pestle. The process is a very sensitive one, and care must be taken to prevent mechanically damaging the very components one is trying to isolate. Therefore, to get desirable and reproducible results, care...
must be taken to control the homogenization conditions. Three different types of homogenizers are in common use. A **Dounce Homogenizer** consists of a glass pestle and a glass mortar. A **Potter-Elvehjem Homogenizer** consists of a teflon pestle and a glass pestle.

**Sonication** involves the use of high frequency sound waves to lyse cells. Sonication is often used when prokarytic cells are to be lysed. The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe initiates the formation of microscopic vapor bubbles that form momentarily and implode, causing shock waves to radiate through a sample.

**Osmotic lysis** is a method that can be used to disrupt some cells, such as mammalian red blood cells. In this method, a buffered hypotonic solution followed by simple mechanical agitation can effectively lyse the cells by osmotically swelling the cells to the point of lysis. Mechanical agitation is employed to break open swollen cells that have not lysed.

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**Centrifugation**

Most of the cellular components in a cell lysate will eventually, given time, settle to the bottom of a tube. To accelerate this process, the lysate can be subjected to **centrifugation**. In centrifugation, the lysate produced from tissue aggregation is rotated at a high speed, imposing a force on the particles perpendicular to the axis of rotation. The force is called a **relative centrifugal force (RCF)**, expressed as a multiple of the force of Earth's gravitational force (x g). For example, an RCF of 1000 x g is a force 1000 times greater than Earth's gravitational force. When a particle is subjected to centrifugal force, it will migrate away from the axis of rotation at a rate dependent on the particle's size and density.

Several centrifugation methods are used by biologists. **Differential centrifugation** is a centrifugation method whereby the sequential centrifugation of a cell lysate at progressively increasing centrifugation force is used to isolating cellular components of decreasing size and density. The separation of the cellular components is based solely on their sedimentation rate through the centrifugation medium, which, in turn, is dependent on the size and shape of the cellular components. Each centrifugation step results in the production of a **pellet**, usually containing a mixture of cellular components of the same size and/or density. The fluid resting above the pellet, the **supernatant**, can be removed and subjected to additional centrifugations to generate pellets containing other cellular components. The primary advantages of the technique are that it is relatively rapid and simple and it usually requires a high-speed centrifuge, which is commonly found in laboratories. The primary disadvantage of the process is that it only separates cellular components that differ significantly in size. Therefore the fractions are crude.
Each aquaporin protein is a 6-pass membrane protein. The six membrane spanning segments are connected by 5 loops, designed loops A through E. Loops A, C and E are extracellular loops; loops B and D are cytosolic loops. Loops B and E fold into the central pore of the protein to help form the lining of the pore. These two loops contain the signature amino acid sequence motif Asn-Pro-Ala (NPA). The amino acid sequences come together midway between the pore openings, creating the very narrow pore through which water molecules pass in single file.

To obtain satisfactory fractionation using any centrifugation scheme, four centrifugation conditions must be controlled:

- a) fluid density
- b) type of centrifugation rotor
- c) relative centrifugation force (RCF) and duration of its application
- d) centrifugation temperature.

The unit of RCF is the gravitational unit (g). The RCF produced by a centrifuge depends directly on the rotor size and the speed of rotation. Knowing the RCF that needs to be produced and the rotor size, one can calculate the rotation speed (RPM) using the following formula:

$$ RCF = 0.00001118 \times r \times RPM^2 $$

where \( r \) is the radius of the rotor in cm.
Centrifuges and Centrifuge Rotors

There are three basic types of centrifuges used routinely by biologists. They differ in, among other things, the rotational speed and relative centrifugal force that can be generated. **Microcentrifuges** are table-top centrifuges used to process small volumes. They can attain speeds up to approximately 12,000–13,000 rpm. They are typically used in cell culture, microbiology and molecular biology. **High-speed centrifuges** handle larger volumes and can attain higher speeds, up to approximately 30000 rpm. They come in both table-top and flow models. **Ultracentrifuges** are designed to process moderate volumes of sample at speeds in excess of 70,000 rpm. Ultracentrifuges are generally employed to isolate small particles, such as ribosomes and viruses and macromolecules, such as proteins. They are also used in cell fractionation techniques that require centrifugation of cellular components through relative high-density centrifugation media.

Centrifuge rotors are the highly-engineered devices that hold the centrifugation tubes as they are spun. There are two basic types of rotors routinely used by biologists: fixed angle rotors and swinging bucket rotors.

**Fixed angle rotors** hold the centrifugation tubes at a fixed angle (generally 20 - 40 degrees) as they are spun. These are the most commonly used rotors in the cell biology laboratory. In a fixed angle rotor, the materials are forced against the side of the centrifuge tube, and then slide down the wall of the tube, resulting in a faster separation of particles. They generally have no moving parts.

**Swinging bucket rotors** have buckets that are free to swing out on a pivot perpendicular to the axis of rotation. They are the rotor of choice when using a density gradient centrifugation medium. Moreover, if there is a danger or scraping off an outer shell of a particle (such as the outer membrane of a chloroplast), then the swinging bucket is the rotor of choice. Swinging bucket rotors have hinges that hold separate buckets, making this type of rotor more prone to mechanical failure.
D. This Week’s Experiment

Introduction and Specific Objective

For several reasons, mammalian red cell plasma membranes are the perfect cellular component on which to first learn basic fractionation techniques. First, mature mammalian red cells are very easy to obtain. Second, mammalian red cells are easy to lyse. And third, mammalian red cells have no internal membrane (erythrocytes have no nucleus and no organelles) and therefore have no source of possible contamination from other types of cellular membranes. For these reasons, the mammalian red blood cell has always been the preferred model for the study of membrane structure and biochemistry.

This week’s laboratory activity is not based on a particular scientific question or hypothesis. The specific objective of today's experiment is to produce a relatively pure fraction of sheep red blood cell plasma membrane using a simple differential centrifugation protocol. Once produced, the fraction will be frozen and stored for later in the semester, when it will be analyzed for its protein content using SDS-polyacrylamide gel electrophoresis.

Experimental Design

In today's lab, each pair of students will obtain a 20 ml sample of sheep whole blood and will prepare from that sample a relatively pure fraction of sheep red blood cell plasma membrane using a simple osmotic lysis techniques and a simple differential centrifugation protocol.

Equipment & Materials

- 2 35 ml polycarbonate centrifugation tubes
- 1 ice bucket
- 1 100 ml media bottle containing Isotonic Sodium Phosphate Buffer, pH 6.8
- 1 100 ml media bottle containing Hypotonic Sodium Phosphate Buffer, pH 6.8
- 5 ml & 10 ml pipets
- Pasteur pipets
- Sheep whole blood
- Sorvall SA-600 fixed-angle centrifuge rotor (maximum radius = 12.95 cm)
- Sorvall High-speed centrifuge
Experimental Protocol

1. Before you begin, you must precool the following by embedding them in a bucket of ice:

- Isotonic Sodium Phosphate Buffer
- Hypotonic Sodium Phosphate Buffer
- Sheep Whole Blood (10% hct.)
- Two 40 ml Centrifuge Tubes

2. Using a 10 ml plastic pipette, transfer 20 ml of whole blood to a 40 ml round bottom centrifuge tube. Balance the tube with that of another group by simply adding blood to the lighter tube. Place the balanced tubes directly across from one another in the centrifuge. Once the rotor is full, centrifuge the blood at 5,000 x g (5,900 RPM) for 5 minutes at 0-4 C to collect the red blood cells.

3. Using a pasteur pipet, carefully decant the supernatant (plasma) from the tube and discard. Add 20 ml ISOTONIC sodium phosphate buffer and resuspend the red cells by GENTLY inverting the tube five times. This will help wash the red cells of any residual plasma. (Make sure the entire pellet is suspended. You may need to knock the bottom of the tube against the heal of your hand to loosen the entire pellet.). Balance the tube with that of another group by adding some isotonic buffer to the lighter tube, place the balanced tubes directly across from one another in the centrifuge. Once the rotor is full, centrifuge the blood at 5,000 x g (5,900 RPM) for 5 minutes at 0-4 C to collect the red blood cells.

4. Now its time to lyse the cells. Decant and discard the supernatant. Add 25 ml HYPOTONIC sodium phosphate buffer. The red cells will begin to lyse due to the osmotic stress of the hypotonic buffer. Cap the tubes and vigorously agitate the tubes for 5 seconds every 30 seconds for a period of 5 minutes. The agitation will accelerate hemolysis. As the cells lyse, the hemoglobin-rich cytoplasm spills out, becoming a very deep purple color.

5. After 5 minutes of agitation, balance the tube with that of another group by adding hypotonic buffer to the lighter tube, then place the balanced tubes directly across from one another in the centrifuge. This is a high speed centrifugation, so the balance is particularly important in this step. Once the rotor is full, centrifuge the blood at 40,000 x g (16,600 RPM) for 5 minutes at 0-4 C to collect the red cell plasma membranes.

6. Following centrifugation, you can see that it is nearly impossible to distinguish the plasma membrane pellet from the supernatant. So, we need to "feel" our way as we decant the supernatant. With a 10 ml serological pipet, decant the top 25 ml of the deep red supernatants with a 10 ml pipet. As you decant, be careful not to overfill the pipet and contaminate the pipetting device. Also, make sure you keep track of the volume you are decanting. Then, add 25
ml HYPOTONIC buffer to the tube, seal the tubes and invert three times to wash the plasma membrane of hemoglobin.

7. Now, pour the contents of the centrifuge tube into the second centrifuge tube. Balance the tube with that of another group by adding hypotonic buffer to the lighter tube, then place the balanced tubes directly across from one another in the centrifuge. This is another high speed centrifugation, so the balance is particularly important in this step. Once the rotor is full, centrifuge the blood at 40,000 x g (16,600 RPM) for 5 minutes at 0-4 C to collect the red cell plasma membranes.

8. Once the centrifugation is over, you should be able to distinguish the pellet from the supernatant. Repeat steps 5 & 6 if time permits (No need to pour into a new tube). You can decant the supernatant with a pasteur pipet at this point.

9. Following the final decant, add 3 ml ISOTONIC buffer to each pellet, swirl to resuspend the pellets, transfer the suspension of plasma membranes to a freezing tube and freeze the suspension for future use.

E. Bibliography