Chapter 8

Organelle Isolation and Marker Enzyme Assay

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Student Outline

Introduction

Eukaryotic cells contain several types of intracellular membrane-bound structures known as organelles which perform a variety of specific functions. For example, mitochondria synthesize ATP, chloroplasts convert light energy to chemical energy, while the endoplasmic reticulum is involved in protein synthesis. The types and number of organelles in cells range from no organelle (in mature mammalian red blood cells) to many and diverse organelles (for example, in cells active in the synthesis of proteins for secretion).

Biochemical analysis of the structure and function of organelles requires a relatively pure sample. There are several methods for isolating subcellular organelles (Alberts et al., 1989; Darnell et al., 1990; Novikoff and Holtzman, 1976); in this laboratory you will employ one of the most widely-used methods, differential centrifugation.

Cells/Tissue Homogenization

An ubiquitous first step in the isolation of subcellular organelles is to homogenize the cells. This process involves breaking open the cell membrane (and the cell wall if present). Commonly used methods of homogenization include (1) dounce homogenization, where the cells are crushed between two revolving solid surfaces; (2) filtration, where cells are forced through smaller pores in a filter; (3) grinding, where cells are ground by swirling with glass beads; (4) sonication, where cells are bombarded with ultrasonic vibrations; and (5) solubilization, in which cell membranes are dissolved in detergents such as Triton X-100. Enzyme digestion is also used to remove cell-wall

constituents. The method of choice depends on the type of tissue to be homogenized and the specific purpose of the experiment.

Usually during the homogenization process isotonic sucrose is added to the homogenization buffer to prevent osmotic rupture of organelle membranes since it is usually important to purify intact organelles. Following homogenization, the homogenate is normally spun at low speed to remove any intact cells along with other large cellular debris. When this is done, the next step is subcellular fractionation.

Differential Centrifugation

Differential centrifugation is one of the widely-used techniques to separate cellular organelles. A slight modification of this technic known as rate zonal centrifugation is also used frequently in which organelles, after a single spin, band in a tube according to their sedimentation rate. The technique of differential centrifugation is shown schematically in Figure 8.1 and described below.

Size, density, and shape influence the movement of a subcellular particle in a centrifugal field. This movement (sedimentation) results from the interaction between a particle's weight and the resistance it encounters in moving through a suspension medium and the relative centrifugal force exerted on the particle. Under a given centrifugal force, particles that are relatively large or dense will sediment more rapidly than particles that are smaller and lighter. With respect to the major components found in cells, the order of sedimentation is typically (from most to least dense): nuclei, mitochondria, lysosomes, plasma membrane, endoplasmic reticulum, and contractile vacuoles. Depending on the specific cell type, however, this order can vary. Additionally, differences in the rate of sedimentation are sometimes not large enough to provide separation of one organelle from another.

Density-Equilibrium Centrifugation

A second widely-used procedure for separating organelles is known as density-equilibrium centrifugation (see Figure 8.2). In this procedure, subcellular particles are layered on a density gradient and subjected to a very high centrifugal force. Usually, the density gradient is formed by layering increasing concentrations of sucrose solutions in a centrifuge tube. However, other solutions can be used, such as Percoll (a colloid) and cesium chloride. These latter two solutions, when spun, will spontaneously set up a density gradient, thus alleviating the need to (as with sucrose) manually layer sucrose solutions of varied density (concentration).

During centrifugation, organelles initially layered on the density gradient will sediment until they arrive at the region of the gradient where the density of the suspension is equal to their own (Figure 8.2). At this point, an equilibrium condition is reached between the downward centrifugal force and the particle's tendency to float due to buoyancy, and sedimentation halts. Hence, this procedure is also known as isopycnic equilibrium centrifugation.

Used alone, neither differential nor density-gradient centrifugation normally provides preparations containing organelles of sufficient purity. A common practice is to use both types of centrifugation procedures in sequence. However, even with this approach, obtaining desired organelles free from contaminants can require additional steps. These steps can be many and varied, and often involve innovative approaches. For example, in one attempt to purify lysosomes, it was found that even after extensive purification, the lysosomes were contaminated with

mitochondria. Complete purification was finally achieved by feeding the cells Triton WR-1339, a very low-density compound that preferentially accumulated into the lysosomes. This, in effect, decreased the density of the lysosomes, allowing them to be easily separated from the mitochondria using density centrifugation.

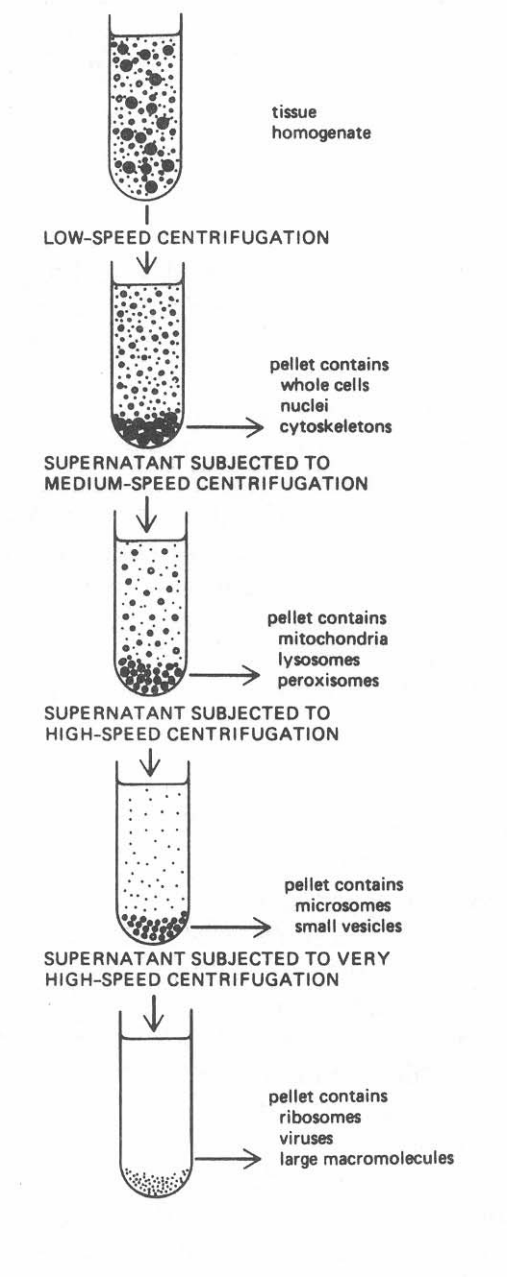

Figure 8.1. Schematic separation of organelles by differential centrifugation. See text for details.

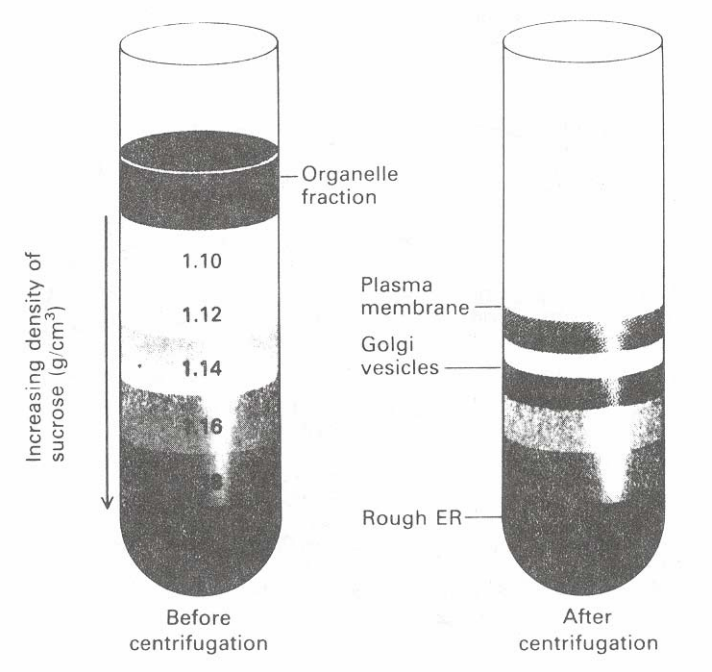

Figure 8.2. Schematic separation of organelles by density-equilibrium centrifugation. See text for details.

Marker Enzymes

Isolation of any organelle requires a reliable test for the presence of the organelle. Typically, this is done by following the activity of an enzyme that is known to be localized exclusively in the target organelle. Such enzymes are known as marker enzymes. For example, the enzyme acid phosphatase (that cleaves terminal phosphate group from substrates and has a pH optimum in the acidic range) is localized in lysosomes, while the enzyme succinate dehydrogenase is localized in mitochondria. By monitoring where each enzyme activity is found during a cell fractionation protocol, one can monitor the fractionation of lysosomes and mitochondria, respectively.

Marker enzymes also provide information on the biochemical purity of the fractionated organelles. The presence of unwanted marker enzyme activity in the preparation indicates the level of contamination by other organelles, while the degree of enrichment for the desired organelle is determined by the specific activity of the target marker enzyme. Although marker enzymes reveal much concerning the purity of the organelle preparation, electron microscopy is generally used as a final step to assess the preparation's purity and the morphology of the isolated organelle.

The Organism, *Dictyostelium discoideum*

Dictyostelium discoideum is a eukaryotic microorganism popularly known as social ameba or slime mold. These individual cells will grow so long as a proper food source is available. Under starvation conditions, however, 10^5 to 10^6 cells aggregate in an orderly manner to form a multicellular body which eventually differentiates into spores or stalk cells (Figure 8.3). Because of this unique life-cycle feature and the simplicity with which this organism is grown and handled, *D. discoideum* has become a model system for studying a variety of cellular processes. These include, but are not limited to, chemotaxis, signal transduction, pattern formation and differentiation, cell-cell communication, and endocytosis.

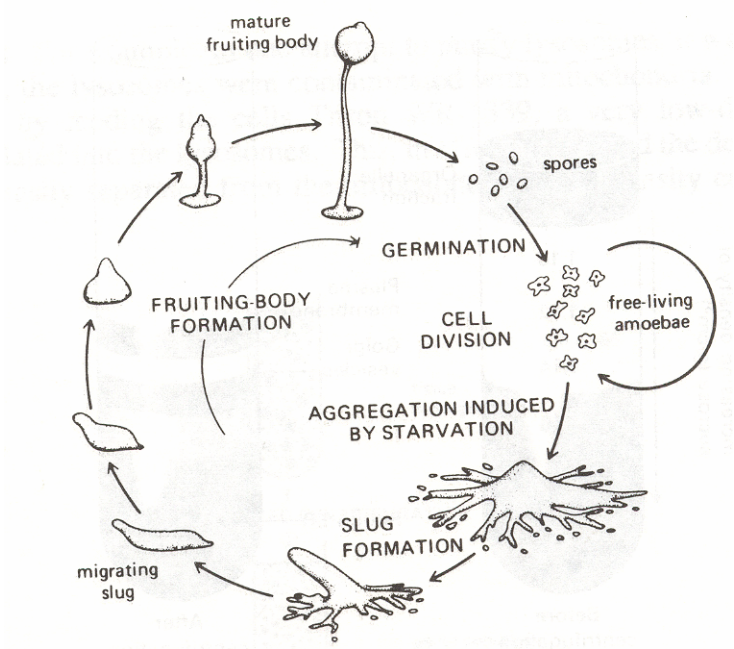

Figure 8.3. Life cycle of *Dictyostelium discoideum*. See text, Appendix B, and Loomis (1982) for details.

Synopsis of the Experiment

In this exercise, you will fractionate a homogenate of *D. discoideum* using differential centrifugation to separate mitochondria, lysosomes, and contractile vacuoles. The purified fractions will be assessed using marker enzymes specific to each of these cell organelles.

In this exercise, cells grown on nutrient broth will be harvested, washed, and homogenized by passing them through two filters having pores 5 μm in diameter. Aliquots of the homogenate will then be subjected to three different centrifugal forces and the pellets, as well as the supernatant fractions, will be assayed for marker enzymes specific to mitochondria, lysosomes, and contractile vacuoles. These enzymes and their associated organelles are given in Table 8.1. The ratio of these three markers in various fractions will give an indication about the relative enrichment of one organelle over another in a given fraction. The differential centrifugation steps used in the purification protocol make use of the fact that, in *D. discoideum,* mitochondria generally sediment faster than lysosomes, while contractile vacuoles sediment last.

Table 8.1. Organelles and associated marker enzymes used in this exercise.

Organelle	Marker enzyme
Mitochondria	Succinate dehydrogenase
Lysosomes	Acid phosphatase
Contractile vacuoles	Alkaline phosphodiesterase

Laboratory Protocol

Note: Steps 1 through 3 will be performed for you by a laboratory assistant. Students will therefore begin at step 4.

Preparation of Homogenate

1. Cells: Flask ____, cell density ____ × 10^6 cells/ml, centrifuge 60 ml cells at 500 RCF for 3 minutes (model GLC-2B or similar centrifuge), resuspend cells in 20 ml of Buffer A (5 mM Na-glycinate, pH 8.5 + 100 mM sucrose at room temperature) and centrifuge again. Resuspend in 1.5 ml of the same buffer (cell density approximately 2 × 10^8 cells/ml) at room temperature.
2. Homogenize cells by passing through 2 layers of 5 μm polycarbonate Nucleopore filters (diameter 25 mm) attached to a 3-ml plastic syringe (see Appendix B). Check homogenate under microscope for unlysed cells. Repeat homogenization if necessary.
3. Spin as in step 1 to remove unlysed cells and undispersed cell fragments. Discard pellet and save supernatant as homogenate. Dilute the homogenate 1:10 in ice-cold Buffer A (by adding 1 ml of homogenate + 9 ml of ice-cold Buffer A).

Centrifugation

4. Obtain 5 ml of diluted homogenate in a tube. Obtain four (4) microfuge tubes (1.5 ml capacity) and label them A through D. Next, pipet 1 ml of the diluted homogenate into each of the four labelled microfuge tubes. Keep microfuge tube A on ice; it is a control for total homogenate enzyme activity.
5. Using Table 8.2 as a guide, centrifuge tubes B through D in a Sorvall RC-5B containing an SS34 rotor precooled to 4°C. You will therefore need to perform three separate centrifugations. *Before starting any run, check that the rotor contains a tube from each group in the class. Also, make sure your tubes are in a balanced configuration in the rotor.*

Table 8.2. Centrifugation protocol.

Tube	Rpm	Minutes	RCF-min[1]
A	–	–	0
B	4,200	10	1.0×10^4
C	9,500	10	5.0×10^4
D	18,000	25	5.0×10^5

[1]RCF-min is a measure of centrifugal force (RCF × time of centrifugation in minutes) generated during the centrifugation. Relative Centrifugal Force (RCF) can be calculated from rpm by the following equation: $RCF = [(rpm \times 2\pi)/60]^2 \times (r/g)$, where r = radius of rotor (5.00 cm for SS34), g = gravitational constant (= 980 cm/sec^2). For example, for tube B, centrifugation at 4200 rpm generates RCF of 1×10^3, multiplied by 10 minutes, giving RCF-min of 1×10^4.

6. Label three microfuge tubes Bs, Cs, and Ds. Carefully separate (with eppendorf-type micropipets) the supernatants from tubes B through D as soon as they come out of centrifugation

and place each into the three tubes labelled Bs, Cs, and Ds. Resuspend the pellets remaining in tubes B, C, and D in 0.9 ml of ice-cold Buffer A, and label them Bp, Cp, and Dp.

7. Assay the contents of tubes A, Bs, Bp, Cs, Cp, Ds, and Dp according to the protocols for:
   (a) succinate dehydrogenase: use 0.5 ml/assay;
   (b) acid phosphatase: use 0.1 ml of 1:10 dilution (1:10 dilution prepared as follows: 0.1 ml of fractions + 0.9 ml of ice-cold Buffer A); and
   (c) alkaline phosphodiesterase: use 0.10 ml/assay.

Assay for Succinate Dehydrogenase

The enzyme succinate dehydrogenase is an integral protein of the mitochondrial inner membrane. The major function of mitochondria is to generate energy (ATP) via oxidative phosphorylation. Succinate dehydrogenase, an FAD-containing enzyme, is involved in converting succinate to fumarate. In this assay, succinate is used as a substrate and nitroblue tetrazolium (NBT) as an artificial electron acceptor which changes to purple color when it accepts electrons. Thus, the formation of purple color is directly proportional to enzyme activity.

1. Label eight (8) glass tubes (13 × 100 mm) A, Bs, Bp, Cs, Cp, Ds, Dp, and BLANK. Pipet into each of these tubes:
   0.2 ml Buffer B [200 mM Na-Phosphate buffer, pH 7.4]
   0.1 ml 2.5 mg/ml NBT
   0.1 ml 1% Triton WR-1339
   0.1 ml Substrate B [100 mM Na succinate, pH > 7]

2. When ready, add to each of the seven tubes (A, Bs, Bp, Cs, Cp, Ds, and Dp) 0.5 ml of the appropriate enzyme fraction. Add 0.5 ml Buffer A to the tube labelled BLANK. Note the starting time for each reaction.
3. Incubate all tubes at 37°C for 30 minutes.
4. Stop the reaction by adding 2.0 ml of 2% sodium dodecyl sulphate to each tube.
5. Read Absorbance at 630 nm in a spectrophotometer adjusted to zero with the blank.

Assay for Acid Phosphatase

Acid phosphatase is present in lysosomes. The enzyme cleaves terminal phosphate groups and like other lysosomal enzymes operates maximally in acidic conditions. In this assay we will use a colorless compound, para-nitrophenol phosphate (pNPP) as the substrate for acid phosphatase. When the phosphate group of pNPP is cleaved, para-nitrophenol is generated. Para-nitrophenol is a yellow compound that is easily measured in a spectrophotometer.

1. Label eight (8) glass tubes (13 × 100 mm) A, Bs, Bp, Cs, Cp, Ds, Dp, and BLANK. Pipet into each of these tubes:
   0.1 ml Buffer C [0.25 M glycine-HCl, pH 3.0, containing 0.5% Triton X-100]
   50 µl Substrate C [50 mM pNPP in water]

2. Make 1:10 dilution of A, Bs, Bp, Cs, Cp, Ds, and Dp [1:10 dilution prepared as follows: 0.1 ml of fractions + 0.9 ml of ice-cold Buffer A]. When ready, add to each of the seven tubes (A, Bs, Bp, Cs, Cp, Ds, and Dp) 0.1 ml of the 1:10 dilution of the appropriate enzyme fraction. Add 0.1 ml Buffer A to the tube labelled BLANK. Note the starting time for each reaction.
3. Incubate all tubes at 37°C for 30 minutes.
4. Stop the reaction by adding 2.75 ml of ice-cold 0.2 M Na_3PO_4 (pH 12) to each tube.
5. Read Absorbance at 410 nm in a spectrophotometer adjusted to zero with the blank.

Assay for Alkaline Phosphodiesterase

Alkaline phosphodiesterase (PDE) is present in contractile vacuoles. We will use a colorless substance, para-nitrophenol thymidine 5′ monophosphate, as the substrate for alkaline phosphodiesterase. Cleavage of thymidine 5′ monophosphate generates para-nitrophenol which is a yellow compound and easily measured in a spectrophotometer described above for the acid phosphatase assay.

1. Pipet in eight (8) glass tubes (13 × 100 mm):
   0.1 ml Buffer D [0.25 M Tris-borate, pH 8.5, containing 0.5% Triton X-100, 20 mM $MgCl_2$ and 20 μM $ZnSO_4$]
   50 μl Substrate D [50 mM pNPT in water]
2. When ready, add to each of the seven (7) tubes (A, Bs, Bp, Cs, Cp, Ds, and Dp) 0.1 ml of the appropriate enzyme fraction. Add 0.1 ml Buffer A to the tube labelled BLANK. Note the starting time for each reaction.
3. Incubate all tubes at 37°C for 30 minutes.
4. Stop the reaction by adding 2.75 ml of ice-cold 0.2 M Na_3PO_4 (pH 12) to each tube.
5. Read Absorbance at 410 nm in a spectrophotometer adjusted to zero with the blank.

Tabulation of Results

Enter the data from your enzymes assays into Table 8.3. In addition to completing this table, you will need to plot a bar chart; for each RCF-min, plot *f* for each marker.

Table 8.3. Tabulation of results.

	Succinate dehydrogenase		Acid phosphatase		Alkaline PDE	
Fraction	A_{630}	*f**	A_{410}	*f*	A_{410}	*f*
A		0		0		0
Bp						
Bs		–		–		–
Cp						
Cs		–		–		–
Dp						
Ds		–		–		–

*Calculation of *f*: Calculate the fraction (*f*) of total activity present in the respective pellets. For example, for tube Bp: fraction (*f*) = Bp/(Bp + Bs), where Bp is the enzyme activity in the pellet fraction and Bs is the enzyme activity in the supernatant fraction.

Notes for the Instructor

This exercise was developed for a biology major junior-level undergraduate class. Three hours time was sufficient when students worked in pairs. It is necessary that the students should have the outline at least 1 week in advance and that they study it before coming to the laboratory. The exercise described here could be shortened or elaborated depending on available time and need. For a shorter version, I suggest deleting the second spin (RCF-min of 5×10^4 for lysosomes) and the assay for acid phosphatase. To elaborate the exercise, protein and other marker enzymes could be assayed. In addition, density centrifugation or another suitable step can be added for even better purification of organelles.

This exercise has been developed using an axenic Ax-3 strain of *D. discoideum*. The same principle applies when fractionating organelles from other cell-types. However, conditions should be worked out for different cell-types. The clear advantage here is that contractile vacuoles shows clean separation from mitochondria and lysosomes (as shown in Appendix C). Many cells which do not contain contractile vacuoles will not have such clear separation of organelles. The enzyme assays described here should work well with other cell-types.

You will need about 10 ml of cell culture per student pair if cells are at 1×10^7/ml. I homogenize cells in a large volume (up to 20 ml derived from 400 ml culture) for an entire class. I homogenize cells by passing them through polycarbonate filters (see step 2 in the Laboratory Protocol and Appendix B). Other methods of homogenization should also work well. However, it is important to use Buffer A because alkaline pH and a low ionic-strength buffer help dismantle cytoskeletons and therefore facilitates homogenization. See Das and Henderson (1983) for homogenization procedure using polycarbonate filters.

Expected results and examples of assignment questions and answers are given in Appendix A. Instructions for growing and maintaining *D. discoideum* are given in Appendix B. A table of the reagents that are required, instructions for the preparation of reagents, and the instruments, chemicals, and supplies that are required are given in Appendix C.

Additional points to remember include the following:

1. Keep all fractions *on ice* at all times.

2. Use of centrifuge: Tubes should be balanced, rotor lid should be tightly screwed on. Laboratory assistant should run centrifuges. A microfuge could be used with the appropriate speed (rpm) equivalent to the desired RCF-min.
3. Assay buffers can be added to the assay tubes well in advance (while centrifugation is on). Add substrate to all tubes just before starting assay (less than 10 minutes before assay). Start reactions by adding enzyme solutions and stagger them as indicated below.
4. It is imperative that each tube for the enzyme assay should be incubated for the indicated time. This is generally achieved by staggering tubes by definite intervals (1 minute). This means start the reaction in one tube. After 1 minute start the reaction in the next tube and so on. After 30 minutes, terminate the reaction in the same sequence with 1-minute intervals between tubes.
5. Colorimeter/spectrophotometer: Spectronic 20 is widely used for this purpose. However, other models should work well. The assay tubes (13 × 100 mm) described in the protocol will directly fit into a Spectronic 20 for reading color, eliminating the need to transfer to a cuvette for each assay tube.

Acknowledgements

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APPENDIX A
Expected Results and Examples of Assignment Questions

Expected Results

The results presented in Table 8.4 and Figure 8.4 indicate that the first spin (Sample B) brings down in the pellet most of the mitochondria without any significant amount of contractile vacuoles, giving excellent separation between these two organelles. The second spin (Sample C) pellets most of the lysosomes in addition to mitochondria while contractile vacuoles are still in the supernatant. The third spin pellets all markers indicating that they are pelletable organelles. This simple experiment implies that these three spins sequentially brings down mitochondria, lysosomes, and contractile vacuoles, respectively.

Table 8.4. Expected results (an example from a student's notebook).

	Succinate dehydrogenase		Acid phosphatase		Alkaline PDE	
Fraction	A_{630}	*f*	A_{410}	*f*	A_{410}	*f*
A	0.7	0	0.58	0	0.83	0
Bp	0.6	0.8	0.195	0.3	0.072	0.08
Bs	0.15	–	0.455	–	0.83	–
Cp	0.78	0.98	0.426	0.71	0.2	0.23
Cs	0.02	–	0.174	–	0.67	–
Dp	0.81	0.98	0.59	0.87	0.713	0.8
Ds	0.02	–	0.09	–	0.167	–

Examples of Assignment Questions

1. Based on *your* results, how would you propose to separate organelles *X* and *Y* from mitochondria, lysosomes, and contractile vacuoles in one experiment employing not more than three steps of centrifugation? (Answer in 5–7 lines.)

   Organelle *X* sediments at 5.0×10^6 RCF-min
   Organelle *Y* sediments at 1.0×10^3 RCF-min

   Answer: Spin first at 1.0×10^3 RCF-min to pellet only organelle *Y*. The supernatant is then to be spun at 5×10^5 RCF-min to remove in the pellet mitochondria, lysosomes and contractile vacuoles (see Table 2). The supernatant which now contains only organelle *X* could be spun at 5×10^6 RCF-min to pellet organelle *X*.

2. As you know, materials ingested by cells are sent to lysosomes while mitochondria are involved in oxidative phosphorylation (ATP synthesis). How would you propose to separate them if they happen to sediment at the same RCF-min and have the same density on density gradients? (Answer in 3–4 lines.)

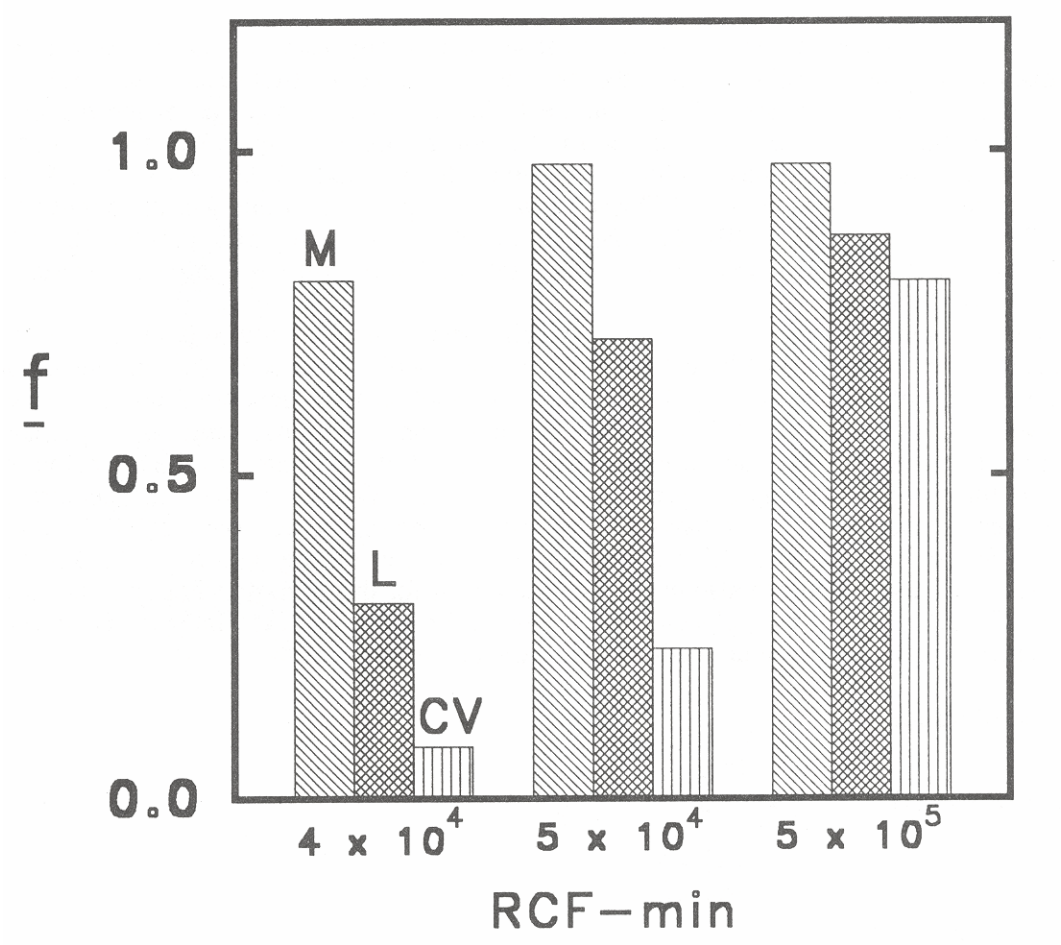

Figure 8.4. The data from Table 8.4 are plotted as *f* versus RCF-min for three markers.

Answer: Cells could be fed lighter compounds like Triton WR-1339 which accumulate in lysosomes and in turn make them more buoyant than mitochondria. Separation should then be possible on a density gradient.

3. A scientist using electron microscope has just observed a new organelle in *Paramecium*. This structure is 3 μm in size and has star-shaped morphology. The organelle resembles no other organelle and has no known marker enzyme activity. How would you proceed to characterize this organelle and what criteria would you use to monitor purification of this new organelle? (Answer in 5–7 lines.)

   Answer: Cell homogenate could be fractionated using a variety of conventional methods like differential, rate zonal, and density-equilibrium centrifugation. Since no marker enzyme is known for this organelle, unique morphology (star-shaped) under a microscope could be used to monitor the fractionation and purification of this organelle. An extra credit if student mentions light microscope since the organelle is large (3 μm) in size and should be visible in light microscope which is much faster and easier to operate than an electron microscope.

APPENDIX B
Growing Cells of Dictyostelium discoideum

Loomis (1982) provides a comprehensive treatise on this organism. *D. discoideum* is a eukaryotic microorganism which can be easily grown in the laboratory. The axenic strain Ax-3 used in this exercise can be obtained either from the American Type Culture Collection or from any one of the numerous research laboratories throughout the world who work on this organism. Cells are grown in axenic medium (known as HL-5) in sterile flasks (250-ml flasks with 50–100 ml medium or 500-ml flask with 100–200 ml medium).

Cells are grown on a rotary shaker (160 rpm) at room temperature. Cells will not survive temperatures higher than 25°C. Doubling time is 10–12 hours. Collect cells when density of cells is 5×10^6 to 1.5×10^7/ml. Cells can be harvested in clinical centrifuge at 500 g (about 1,500 rpm) for 5 minutes. Resuspend cells in Buffer A and centrifuge again.

Cell viability could be tested by mixing 1 part of cell suspension with 1 part of 0.4% Trypan blue in buffer. Observe under a microscope. Healthy cells remain colorless while dead cells take up blue color.

Homogenization of cells by passing them through polycarbonate nucleopore filters is a very convenient procedure (see Das and Henderson, 1983). A filter assembly using two filter membranes is prepared as shown in Figure 8.5. The assembly is attached to the end of a barrel of a syringe. Cell suspension is added into the barrel and the plunger is *gently but firmly* pressed to force cells through the filter membranes.

For long-term preservation, vegetative cells can be developed into spores (see Figure 8.3): 2×10^7 cells/ml in KK_2Mg buffer are plated onto a 5.5-cm Whatman No. 50 filter resting on a buffer-soaked Whatman AP pad in a sterile petri dish. You can also use non-nutrient agar for this purpose. Fruiting bodies will develop in 2–3 days. Collect spores in 10% glycerol in KK_2Mg buffer and store in the freezer. To germinate spores, thaw them quickly and add to axenic medium and grow as discussed above.

HL-5 Axenic Medium

D-glucose	10 g
Proteose peptone No. 3	10 g
Yeast extract	5 g
Na_2HPO_4	600 mg
KH_2PO_4	486 mg
Deionized water to make 1 liter	

1. Dispense into flasks and autoclave for 45 minutes.
2. Just before use add antibiotics to prevent bacterial growth: use a 100X mixture of penicillin (6 mg/ml) and streptomycin (10 mg/ml) available from GIBCO Laboratories (3175 Staley Rd., Grand Island, NY 14072 or 2260A Industrial St., Burlington, Ont. L7P 1A1). Filter sterilize. Add 1 ml per 100 ml of medium.

KK_2Mg Buffer

1. 10X of K-phosphate: Add 23 g of KH_2PO_4 and 10 g of K_2HPO_4 in 1 liter of water and autoclave.
2. Separately autoclave 5 g of $MgSO_4$ in 100 ml of water.
3. Add 100 ml of 10X K-phosphate solution + 10 ml of $MgSO_4$ solution and 890 ml of water to make 1X KK_2Mg buffer.

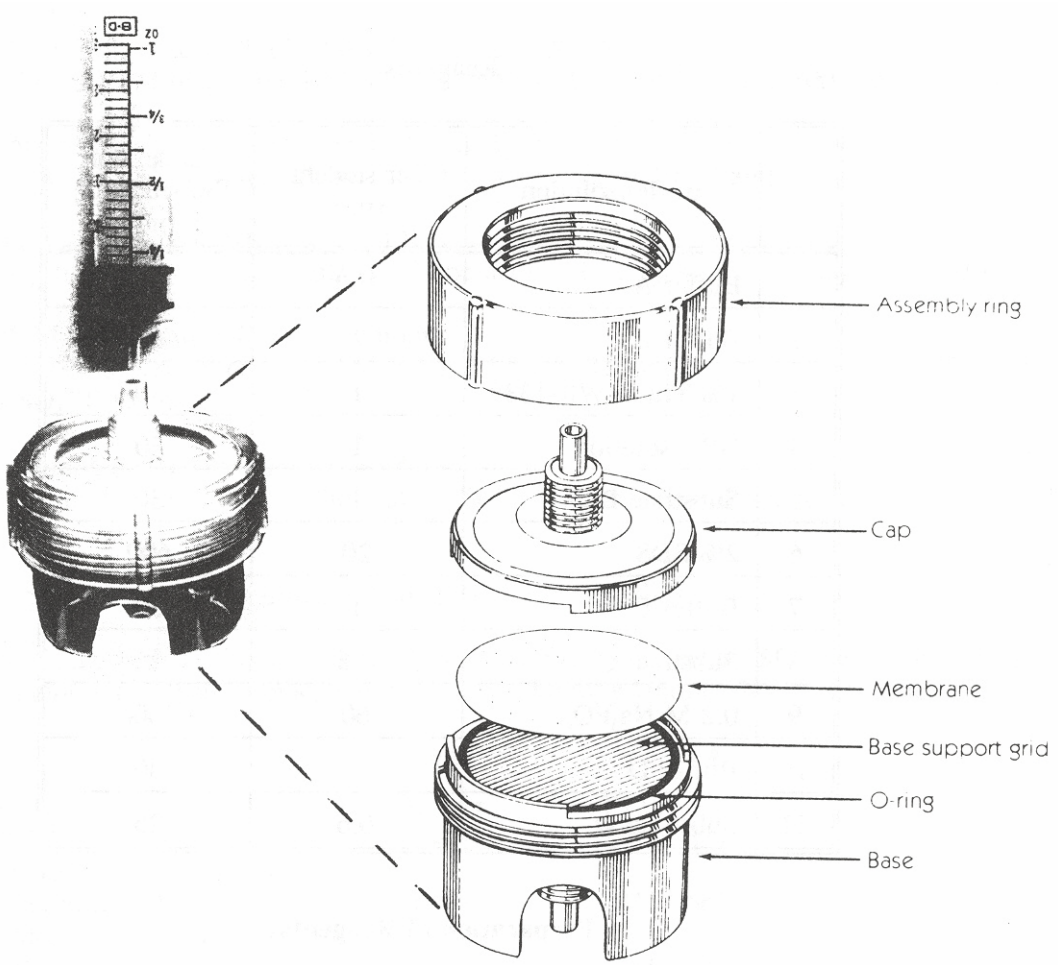

Figure 8.5. Schematic diagram of filtration assembly used to homogenize cells of *D. discoideum*.

APPENDIX C
Materials

Reagents

	Buffer/solution	Per student (ml)	Per session [30 students] (ml)
1	Buffer A	100	3000
2	Buffer B	2	65
3	1% Triton WR-1339	1	30
4	NBT solution	1	30
5	Substrate B	1	30
6	2% SDS	20	600
7	Buffer C	1	30
8	Substrate C	0.6	25
9	0.2 M Na_3PO_4	60	2000
10	Buffer D	1	30
11	Substrate D	0.6	25

Preparation of Reagents

Note: Except 2% SDS, store all reagents on ice during the exercise. Dispense them into aliquots as indicated and give them to students. Keep 2% SDS at room temperature.

1. **Buffer A**: 5 mM Na-glycinate, pH 8.5 + 100 mM sucrose
   As stock, make 100 ml of 1 M buffer (mol. wt. of glycine = 75); therefore take 7.5 g of glycine in about 70 ml water, adjust pH by dropwise addition of 10 N NaOH to pH 8.5. Make the volume to 100 ml. Freeze in 10 ml aliquots. Each day, thaw one tube. Dilute to 2 liter with water and add 68.4 g sucrose. This is Buffer A. Keep it ice-cold.

2. **Buffer B**: 200 mM Na-phosphate buffer, pH 7.4
   Make 500 ml each of 0.2 M of NaH_2PO_4 and 0.2 M Na_2HPO_4. Add NaH_2PO_4 solution to Na_2HPO_4 solution to get pH 7.4. Store at 4°C.

3. **1% Triton WR 1339**: 2 g in 200 ml water. Store at 4°C.

4. **NBT Solution:** Dissolve 50 mg of NBT in 20 ml of water. Prepare fresh *everyday*. Keep on ice.

5. **Substrate B**: 100 mM Na-succinate, pH > 7.
   Na-succinate (formula wt. = 270). Add 5.4 g in 200 ml water. Store frozen at -20°C.

6. **SDS 2%**: 2% sodium dodecyl sulphate.
   Add 60 g of SDS to 3 liters of water. Store at room temperature.

7. **Buffer C**: 0.25 M of glycine-HCl, pH 3.0 + 0.5% Triton X-100
   Prepare 300 ml of 0.25 M of glycine-HCl, pH 3.0 and add 1.5 g of Triton X-100. Store at 4°C.

8. **Substrate C**: 50 mM pNPP (mol. wt. 263 + 108 (6 H_2O) = 371)
   Dissolve 1.85 g in 100 ml of water. Store at -20°C in freezer in 10-ml aliquots.

9. **0.2 M Na_3PO_4, pH 12.0**: Mol. wt. Na_3PO_4, $12H_2O$ = 380.1
   Dissolve 608.1 g in 8 liters of water. pH should be about 12. Adjust if necessary. Store at 4°C.

10. **Buffer D**: 0.25 M Tris-borate, pH 8.5 + additions
    Make 300 ml of 0.25 M Tris-borate, pH 8.5, and add 1.5 g of Triton X-100, 1.218 g of $MgCl_2$,$6H_2O$ and 1.73 mg of $ZnSO_4$,$7H_2O$. Store at 4°C.

11. **Substrate D**: 50 mM pNPT (formula wt. = 443.3)
    Dissolve 2.217 g in 100 ml of water. Store at -20°C in freezer in 10-ml aliquots.

Instruments, Chemicals, and Supplies

Note: Common chemicals, such as Na_3PO_4, Na_2HPO_4, sodium dodecyl sulphate (SDS), and instruments, such as pH meters, commonly employed in biochemistry and cell biology laboratories are not listed below.

Items	*Supplier*	*Catalog #*
13 × 100-mm glass tubes	Baxter	T1269-6
1.5-ml microfuge tubes	VWR	20170-331
Polycarbonate membranes 25-mm diameter, 5-µm pores, Nucleopore (#110613)	VWR	28158-668
Swin-Lok filter holder, 25-mm diameter, Nucleopore #420210)	VWR	28163-045
Difco dextrose (Difco #0155-17-4)	VWR	DF0155-17
Proteose peptone No. 3 (Difco #0155-17-4)	VWR	DF0122-01
Yeast extract (Difco #0127-01-7)	VWR	DF0127-01
Sigma 104 phosphatase (pNPP)	Sigma	104-O
Thymidine 5-monophosphate PNP-ester ammonium	Sigma	T-5380
Nitroblue tetrazolium	Sigma	N-6876
Triton WR-1339 (tyloxapol)	Sigma	T-8761

Plastic or glass syringes: 5- or 10-ml capacity
Plastic or glass pipets: 5- and 10-ml capacity
Pipet-Aids (or rubber bulbs to use with the pipets)
200-µl and 1000-µl capacity eppendorf-type micropipets and pipet tips
Ice buckets and crushed ice

Baxter: Baxter Scientific Products, 1430 Waukegan Rd., McGraw Park, IL 60085-6787.
Sigma: Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178.
VWR: VWR Scientific, 800 East Fabyan Park, Batavia, IL 60510.

Instruments for the exercise:

Clinical bench-top centrifuge
Sorvall RC-5B/RC-2B-type or comparable refrigerated centrifuges which can use Sorvall SS34-type or similar rotors. (A microfuge could be used with appropriate speed [rpm] equivalent to the desired RCF-min.)
SS34, or similar, rotors
Water baths (37°C)
Refrigerator (1)
Spectronic 20 (or similar) spectrophotometers

Instruments for growing cells:

Gyratory shaker
Incubator at 22°C (if room temperature is not controlled)