Centrifugation

THEORY

Basic Principles

Rotation of an object about a central axis generates a centrifugal force upon the object. If the object in question is a molecule or particle of molecular weight M, then

$$\text{centrifugal force} = M\omega^2 x \tag{5-1}$$

where ω is the angular velocity in radians/second (rad/sec) and x is the axis of rotation (i.e., radial distance from the center of rotation). Equation 5-1 dictates that the larger the molecule, or the faster the centrifugation, or the larger the axis of rotation, the greater the centrifugal force and the rate of molecular or particle sedimentation.

The considerations of Equation 5-1 hold throughout all media. However, biochemical experiments usually are conducted with soluble systems. Two forces significantly counteract the centrifugal forces on solubilized molecules or particles. First, molecules or particles must displace the solution media into which they sediment. Equation 5-2

$$\text{buoyant force} = M\omega^2 x\overline{V}\rho \tag{5-2}$$

describes this displacement or buoyant force. The symbol $\overline{V}$ is partial specific volume of the molecules or particles (i.e., cc of solution volume increase caused by addition of 1 g of solute), ρ is the density of the solution, and the other terms are as above. Equation 5-2 dictates that the higher the partial specific volume of the solute in question or the greater the density of the fluid being centrifuged, the slower the rate of molecular or particle sedimentation. Second, dissolved or suspended molecules or particles generate friction as they migrate through the solution. Equation 5-3

$$\text{frictional force} = f\left(\frac{dx}{dt}\right) \tag{5-3}$$

depicts this relationship where f is the frictional coefficient unique to the molecule or particle in question and dx/dt is the rate of sedimentation expressed as change in the axis of rotation with time.

Equations 5-1 to 5-3 can be used to derive a practical relationship between the rate of sedimentation (dx/dt) and the molecular or particle weight (M). A

sedimenting molecule or particle moves faster and faster in a centrifugal field until the centrifugal force equals the counteracting buoyant and frictional force (Equation 5-4).

$$\text{(5-4)} \qquad \begin{matrix}\text{centrifugal}\\ \text{force}\end{matrix} = \begin{matrix}\text{buoyant}\\ \text{force}\end{matrix} + \begin{matrix}\text{frictional}\\ \text{force}\end{matrix}$$

This occurs because the frictional force increases with increased rate of sedimentation, whereas centrifugal force and bouyant force are constant for any molecule and rotor speed. In practice, this balancing of forces occurs quickly with the result that a molecule sediments at a constant rate, (dx/dt).

Substituting Equations 5-1 to 5-3 into Equation 5-4 and rearranging, you obtain Equations 5-5 to 5-7.

$$\text{(5-5)} \qquad M\omega^2 x = M\omega^2 x\overline{V}\rho + f\left(\frac{dx}{dt}\right)$$

$$\text{(5-6)} \qquad M(1-\overline{V}\rho)\omega^2 x = f\left(\frac{dx}{dt}\right)$$

$$\text{(5-7)} \qquad M = \frac{f}{(1-\overline{V}\rho)}\left(\frac{\left(\frac{dx}{dt}\right)}{\omega^2 x}\right)$$

If you define a new term, a sedimentation coefficient, s, as $s = (dx/dt)/\omega^2 x$, substitution of this definition into Equation 5-7 yields Equation 5-8.

$$\text{(5-8)} \qquad M = \frac{(f)(s)}{(1-\overline{V}\rho)}$$

The frictional coefficient, f, can be evaluated through an experimentally determined diffusion constant, D, where:

$$\text{(5-9)} \qquad D = \frac{\left(\begin{matrix}\text{gas}\\ \text{constant}\end{matrix}\right)\left(\begin{matrix}\text{absolute}\\ \text{temperature}\end{matrix}\right)}{\text{frictional coefficient}} = \frac{RT}{f}$$

or

$$f = \frac{\mathrm{RT}}{D}$$

Substitution of Equation 5-9 into Equation 5-8 yields:

$$\text{(5-10)} \qquad M = \frac{RTs}{D(1-\overline{V}\rho)}$$

Equation 5-10 is the basis for *velocity sedimentation analysis,* in which the rate of sedimentation, expressed as the sedimentation coefficient, s, is used to evaluate the molecular weight, M, of the molecule or particle in question.

Sedimentation coefficient, s, units for biological macromolecules fall between 1 and 500 $\times$ 10^{-13} sec. Biochemists avoid the awkward unit of 10^{-13} seconds by defining one Svedberg unit, or Svedberg (depicted by an S) as 1 $\times$ 10^{-13} seconds. Thus single proteins have Svedberg values of 1–20 S, large nucleic acid molecules have Svedberg values of 4–100 S, and still larger subcellular particles have S values of 30–500 S.

Centrifuge Applications

Biochemists use two basic types of centrifuges: analytical centrifuges and preparative scale centrifuges. Analytical centrifuges or analytical ultracentrifuges (the prefix *ultra* implies faster speeds and higher centrifugal forces) only work with small (<1 ml) samples of dissolved or suspended solutes. Such centrifuges also employ elaborate optical systems to analyze the progress of solutes *during the centrifugation run.* These analytical centrifuge applications are very significant to biochemists; for example, the applications of Equation 5-10 to determine molecular weights usually employ analytical ultracentrifuges. However, such sophisticated centrifugal applications are beyond the mission or capacity of an introductory course.

In contrast, preparative scale centrifuges are capable of working with larger samples (10–2,000 ml). Preparative scale centrifuges also lack optical systems to analyze samples during the centrifugation run. Preparative scale centrifuges are necessary for many of the biochemical isolations or applications of this text; consequently, an understanding of their basic applications is fundamental to this course.

Preparative Scale Velocity Sedimentation Centrifugation. Velocity sedimentation is the centrifuge application used most frequently in biochemistry. Usually this procedure employs a fixed-angle rotor operating at a given speed for a defined time (Figure 5-1).

The practical considerations of fixed-angle rotor velocity sedimentation follow the principles of Equations 5-1 to 5-3. Each rotor type has a fixed potential axis of rotation dictated by the centrifuge tube holes in the rotor. Biochemists define the centrifugal force obtainable at any speed (i.e., rpm or ω^2) in terms of the axis of rotation (i.e., x) at the *center of the angled centrifuge tubes.* This leads to the "times gravity" convention in which the centrifugal force on a molecule or particle in the center of a tube in a given fixed-angle rotor rotating at a given rpm expressed as a relative centrifugal force in terms of gravity, such as 10,000 $\times$ gravity or 10,000 $\times$ g. Tables relating speeds of rotors with relative centrifugal forces or times gravity values

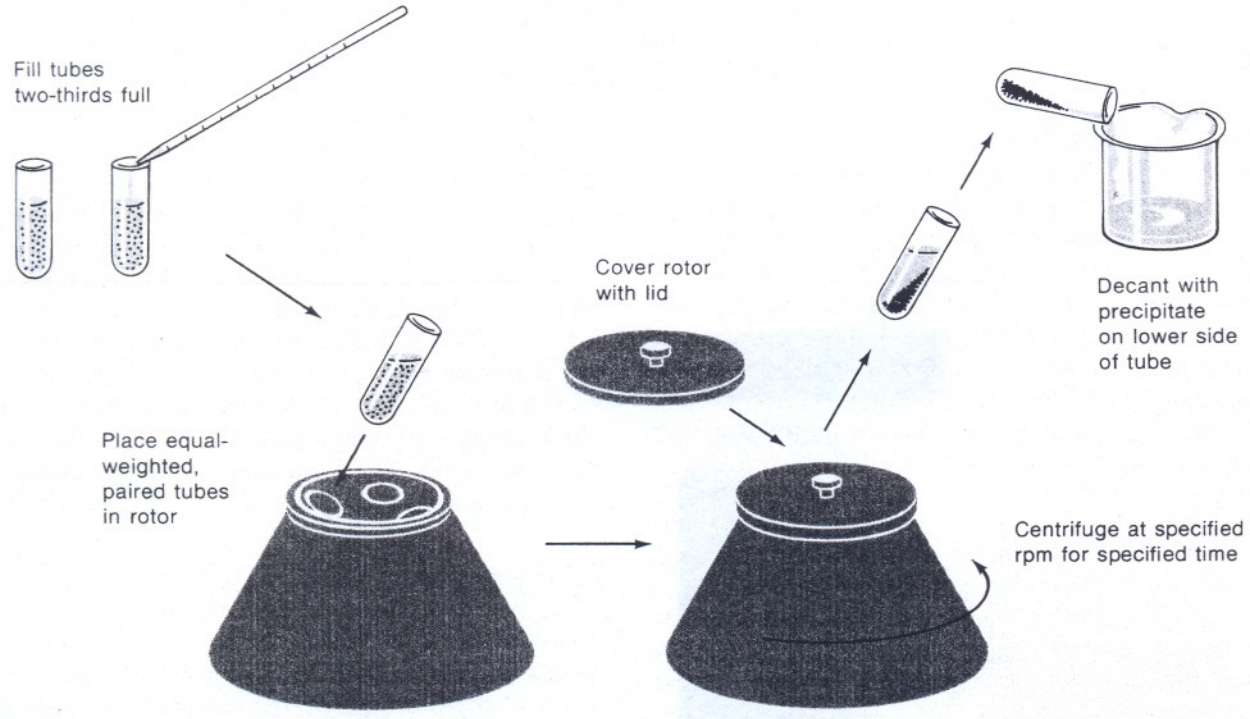

FIGURE 5-1
Velocity sedimentation in a preparative scale fixed angle rotor.

for the center of the centrifuge tubes are available for all centrifuges. Thus, use of the times gravity convention facilitates adaptation of rotor speeds and times to match the centrifugal or gravity forces used by others with different rotors or centrifuges.

All fixed-angle rotors also carry a designated maximum speed rating. *Operation of rotors at speeds in excess of these designated maxima can result in "rotor explosion" (i.e., disintegration) with possible great harm to the centrifuge and people nearby.* Last, preparative scale centrifuges may contain a constant temperature refrigeration system to minimize loss of biological activity and reduce disruptive convective effects caused by temperature differentials. The higher-speed preparative ultracentrifuges also usually employ vacuum systems that minimize air friction in the rotor chamber and resultant temperature fluctuations.

Fixed-angle rotors are frequently used in a kind of preparative scale velocity sedimentation called *differential centrifugation.* Figure 5-2 depicts the process on

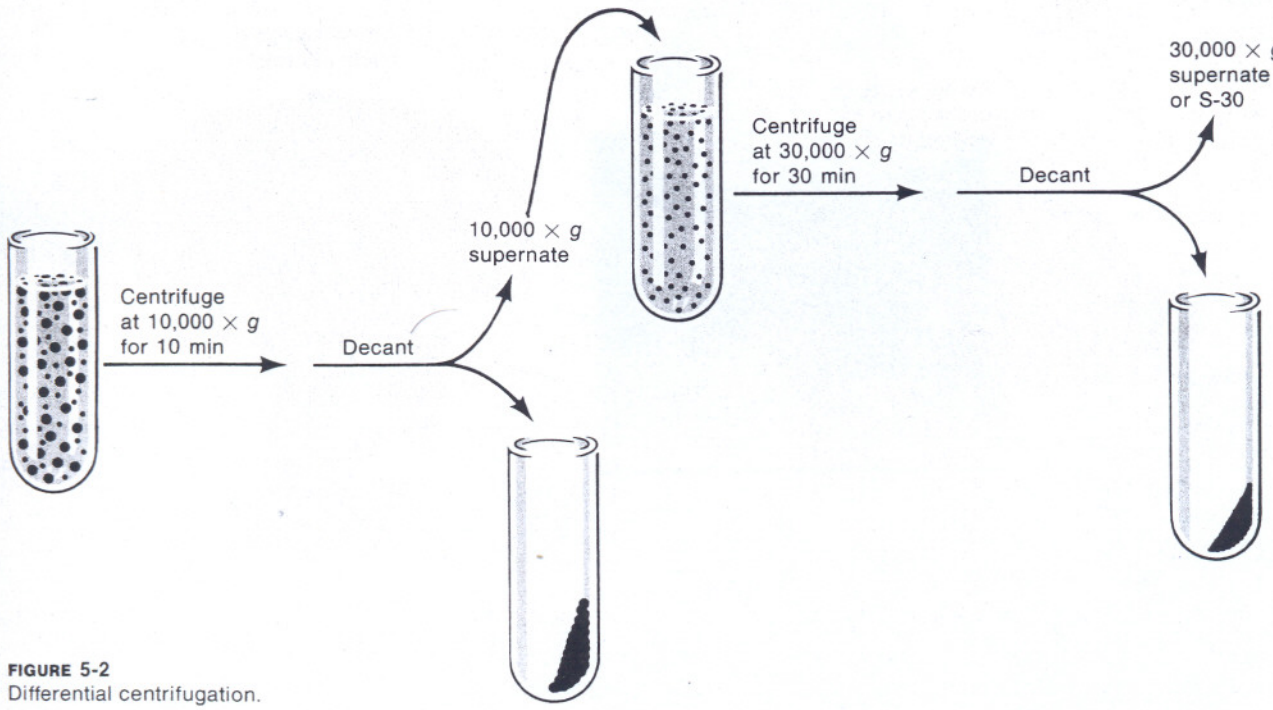

FIGURE 5-2
Differential centrifugation.

a sample containing suspended particles of two sizes. As can be seen, successive centrifugations at increasing speeds or gravity forces resolve different suspended materials or particles from each other and from a supernatant fraction. Supernatant fractions obtained after specific centrifugation are frequently abbreviated with the prefix S, which refers to the supernate obtained after a certain 1,000 times gravity centrifugation. Thus, the supernate obtained after the 30,000 $\times$ *g* centrifugation of Figure 5-2 is a S-30 fraction, and so forth. (Note: Do not confuse the prefix S symbol of supernate fractions with the S symbol of Svedberg values, which appears after a number.)

Differential centrifugation will readily fractionate or resolve different subcellular particles of cells from each other. Table 5-1 depicts a generalized pattern of differential centrifugation for the resolution of cellular and subcellular fractions from tissue or cellular homogenates.

The final 100,000 $\times$ *g* supernate (S-100) fraction therefore generally represents the truly soluble fraction containing soluble proteins and small molecules.

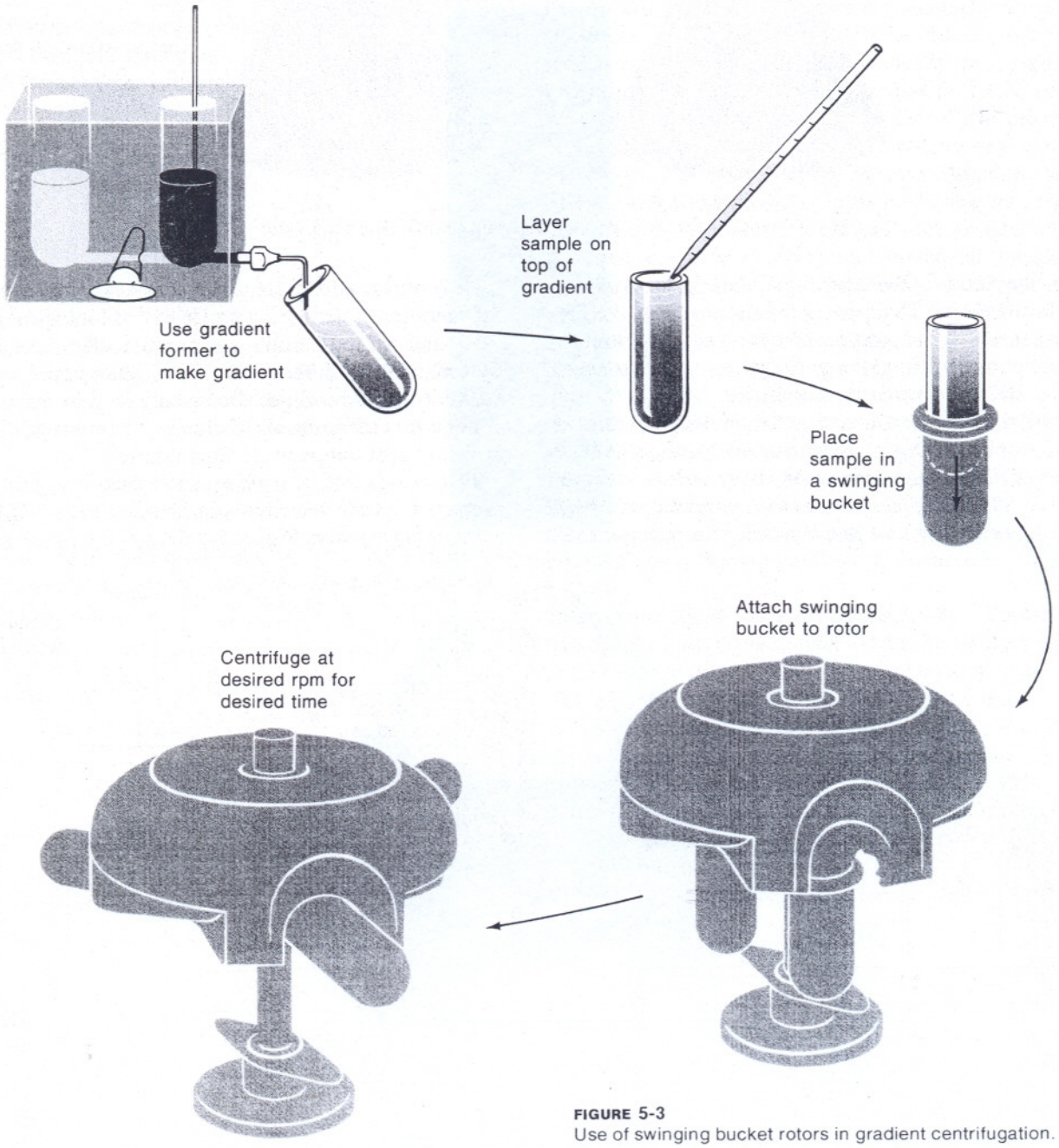

FIGURE 5-3
Use of swinging bucket rotors in gradient centrifugation.

ABLE 5-1
Generalized centrifugation conditions to sediment specific ells or particles (consider in terms of successive entrifugations at listed increasing *g* forces.)

Centrifugation conditions	Fractions sedimented
1000 × *g*, 5 min	Most eucaryotic cells
4000 × *g*, 10 min	Chloroplasts Most eucaryotic cell debris Most cell nuclei
15,000 × *g*, 20 min	Mitochondria Bacteria
30,000 × *g*, 30 min	Lysosomes Most bacterial cell debris
00,000 × *g*, 3 hrs	Ribosomes and polysomes

Table 5-1 lists both the relative centrifugal forces ınd the times required to sediment the various cellular components. That is, sedimentation involves an nterrelationship of both *g* force (i.e., rpm) and time. f you know the specific rpm for a given rotor to obtain ı required or specified *g* force for a specified time, you an use equation 5-11

5-11) $$\begin{pmatrix}\text{specified}\\ \text{time}\end{pmatrix}\begin{pmatrix}\text{specified}\\ \text{rpm}\end{pmatrix}^2 = \begin{pmatrix}\text{new}\\ \text{time}\end{pmatrix}\begin{pmatrix}\text{new}\\ \text{rpm}\end{pmatrix}^2$$

o determine an equivalent (yet possibly more convenient) longer time at a lower *g* force, or a shorter time at a higher *g* force. Note that Equation 5-11 holds for rotors with specified axes of rotation. Thus, Equation 5-11 may not be used to interrelate time and rpm between different rotor types.

Gradient Centrifugation. Most preparative centrifuges will accommodate one or more different swinging bucket rotors for use in gradient centrifugations. Figure 5-3 illustrates the basic principles of swinging bucket rotors.

Swinging bucket rotors can be used in two ways during gradient centrifugation: in density gradient sedimentation and in equilibrium density gradient centrifugation. Let's consider each application in turn.

Density gradient sedimentation is a form of velocity sedimentation. First the sample is layered on top of a linear or exponential gradient of dissolved inert organic material, such as sucrose or glycerol. The inert gradient agent both stabilizes the fluid environment of the centrifuge tube and facilitates sharp resolution of zones of the centrifuge fluid in the tubes after the centrifugation. Thus, as seen in Figure 5-4, a heterogeneous band of molecules or particles of different sizes sediments into the gradient following the rules of sedimentation velocity. After resolution within the gradient, the increasing density of the gradient fluid

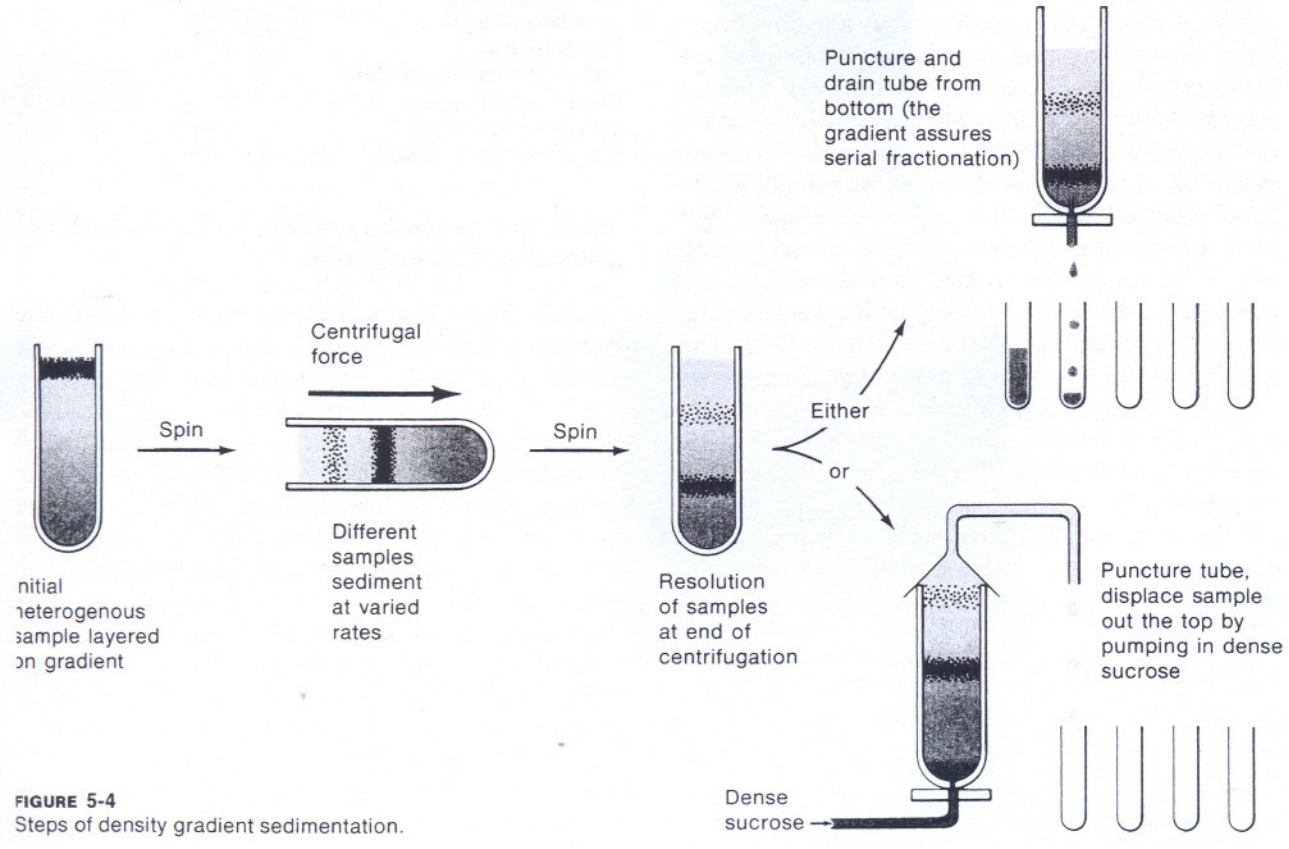

FIGURE 5-4
Steps of density gradient sedimentation.

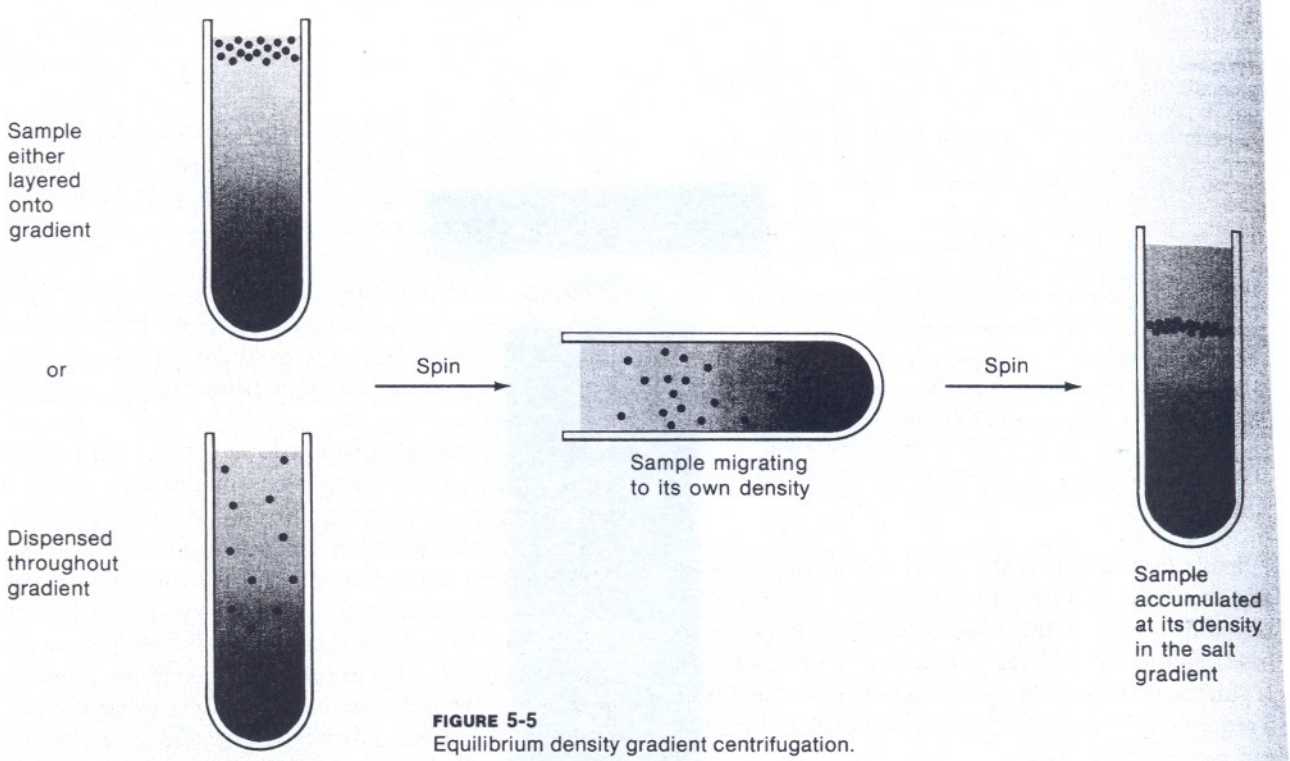

FIGURE 5-5
Equilibrium density gradient centrifugation.

facilitates the fractionation, so that each layer is resolved free of the less dense fluid above or the more dense fluid below. This method is very useful in preparative procedures, and it also can be used to determine molecular weights of soluble enzymes or proteins (see Martin and Ames, 1961).

Equilibrium density gradient centrifugation differs from density gradient sedimentation in two ways. First, equilibrium density gradient centrifugations employ denser salts solutions, (e.g., cesium chloride) that have densities spanning those of the biological molecules to be resolved. Second, the sample undergoes lengthy centrifugation so that the dissolved solutes accumulate at their own equilibrium densities within the salt gradient as a result of the sedimenting centrifugal force and the counteracting buoyant density of the higher salt solution (Figure 5-5). This process is called isopycnic centrifugation. Such a resolution can be an analytical tool: for example, DNAs accumulate at densities of ~1.7 g/ml, whereas RNAs accumulate at densities of ~1.9 g/ml. Further, such equilibrium density gradient centrifugation may be performed in a preparative scale centrifuge (as in Figure 5-5) or it may be performed in an analytical ultracentrifuge (see Meselson and Stahl, 1958).

EXPERIMENT ON CENTRIFUGATION

EXPERIMENTAL PROCEDURES

Materials

Fresh spinach (*Spinacia oleracea*) leaves
Cold isolation buffer (=0.3 M sorbitol; 0.1 M Tris-Cl, pH 7.8; 5 mM $MgCl_2$; 10 mM NaCl)
Blender
Beaker
Funnel
Glass rod
Cheesecloth
Centrifuge tubes (4 tubes of 50 mL; 4 tubes of 10 mL)
Balance
Centrifuge
Ice
Paint brush
Graduated cylinder of 10 mL
Parafilm
Acetone (90 %)
Spectrophotometer
Spectrophotometer cuvettes

Procedure for isolation and concentration measurement of chlorophyll

1) Weigh out 40 gr of fresh spinach (*Spinacia oleracea*) leaves from which the major veins have been removed.

2) Tear the leaves into small pieces and place them in a blender with 200 mL of cold isolation buffer.

3) Blend the mixture at low speed for 10 seconds. Use a glass rod to push any tissue pieces down the sides of the jar into the solution, and blend for 10 more seconds.

4) Filter the homogenate through 8 layers of cheesecloth into a beaker. Add approximately 40 mL of filtrate to each of four centrifuge tubes (50 mL), making sure they are balanced (∓ 0.1 gr).

5) Centrifuge at 4°C for 5 minutes at 1000xg.

6) Decant and discard the supernatant from all 4 tubes. Add 0.5 mL of cold isolation buffer each tube and gently resuspend the chloroplasts with a paint brush.

7) Store the centrifuge tube on ice, away from bright light.

8) Add 0.05 mL (=50 µL) of the chloroplast suspension to a clean glass conical centrifuge tube.

9) Use a graduated cylinder to measure out 7.5 mL of 90% acetone, add to the centrifuge tube, cover tightly with Parafilm and invert several times to dissolve the chlorophyll. A flocculent precipitate of protein should be visible.

10) Remove the protein by centrifuging for 2 minutes at about 500xg. The protein should form a pellet at the bottom of the tube. Pour the supernatant (acetone extract) into a clean tube and discard the tube containing the pellet.

11) Pour some of the supernatant into a clean spectrophotometer cuvette. Read absorbance of your sample at 652 nm using a blank of 90% acetone. If the absorbance reading is greater than 1.5, dilute your sample with 90% acetone until its absorbance below 1.5. Record how much additional acetone was added.

12) Use the equation below to determine the concentration of chlorophyll in your chloroplast sample:

$$\text{Chlorophyll (mg/mL)} = \text{absorbance} \times \frac{\text{total vol.(mL) of acetone used}}{\text{vol.(mL) of suspention used}} \times 0.029$$

IMPORTANT NOTE: Clean cuvettes and tubes and other lab items before leaving the laboratory.