Chapter 5

Centrifugation

Centrifugation is a process used to separate or concentrate materials suspended in a liquid medium. It is a method to separate molecules based on their sedimentation rate under centrifugal field. It involves the use of the centrifugal force for the sedimentation of molecules. It is also used to measure physical properties (such as molecular weight, density and shape) of molecules. If centrifugation is used for separation of one type of material from others; it is termed as *preparative centrifugation*; whereas if it is used for measurement of physical properties of macromolecules then termed as *analytical centrifugation*.

Principle of centrifugation

Particles suspended in a solution are pulled downward by Earth's gravitational force. In a solution, particles whose mass or density is higher than that of the solvent *sink* or sediment, and particles that are lighter than it *float* to the top. The greater the difference in mass or density, the faster they sink. This sedimentation movement is partially offset by the buoyancy of the particle. Because the Earth's gravitational field is weak, a solution containing particles of very small masses usually remain suspended due to the random thermal motion. However, sedimentation of these particles can be enhanced by applying centrifugal forces. A centrifuge does the same thing. It increases the sedimentation by generating centrifugal forces as great as 1,000,000 times the force of gravity.

Let us consider a solution being spun in a centrifuge tube. The centrifugal force acting on a solute particle of mass m,

$$\text{Centrifugal force} = m\omega^2 r$$

where, ω is the angular velocity in radians per second,

r is the distance from the center of rotation to the particle, and

$\omega^2 r$ is the centrifugal acceleration.

A particle will move through a liquid medium when subjected to a centrifugal force. Hence, we must also consider the particle's buoyancy due to the displacement of the solvent molecules by the particle. This buoyancy reduces the force on the particle by $\omega^2 r$ times the mass of the displaced solvent.

Buoyant force = $m_0\omega^2r$

where, m_0 is the mass of fluid (solvent) displaced by the particle

$m_0 = m\bar{v}\rho$

Where, ρ is the density of the solution (g/mL). $\bar{v}$ is the *partial specific volume* of the particle. As measuring the volume of a very small particle is difficult, for convenience we use a term called partial specific volume. $\bar{v}$ is the volume in mL that each gram of the particle occupies in solution.

Thus, the net force acting on the particle downward is given by,

$$\text{Net force} = \text{Centrifugal force} - \text{Buoyant force}$$
$$= m\omega^2r - m_0\omega^2r$$
$$= m\omega^2r - m\bar{v}\rho\,\omega^2r$$

When particles move downward through the solution, the motion is also opposed by the frictional force. The frictional force is equal to the product of the frictional coefficient, f ($Nm^{-1}s$) and the sedimentation velocity, v. It acts in the opposite direction to the net force:

$$F_{friction} = v \cdot f$$

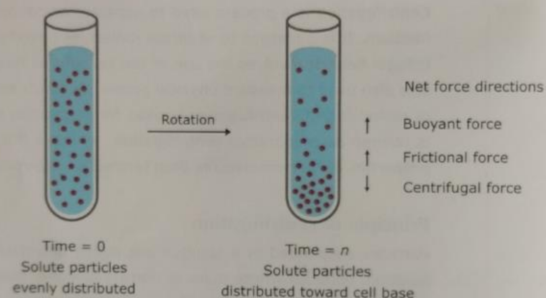

Figure 5.1 A schematic diagram of a centrifugation experiment.

At steady state, then, the *frictional force* is equal to the net force and the molecule moves with velocity v downward:

$$vf = m\,\omega^2r - m\bar{v}\rho\,\omega^2r$$

$$vf = m\,\omega^2r\,(1 - \bar{v}\rho)$$

Since, m (mass in grams of a single particle) = M/N

M is the molar weight of the solute in g/mol

N is Avogadro's number.

$$fv = \frac{M\omega^2r(1-\bar{v}\rho)}{N}$$

Now, the *sedimentation coefficient,*

$$s = \frac{v}{\omega^2r} = \frac{M}{N}\frac{(1-\bar{v}\rho)}{f}$$

The *sedimentation coefficient* is the ratio of a velocity to the centrifugal acceleration. The sedimentation coefficient has units of second. A sedimentation coefficient of 1×10^{-13} second is defined as one Svedberg, S, (1S = 10^{-13} second). This unit is named for The Svedberg, a pioneer in the field of centrifugation.

Important conclusions drawn from the equation:

- The sedimentation velocity of a particle is proportional to its mass.
- A dense particle moves more rapidly than a less dense one because the opposing buoyant force is smaller for a dense particle.
- Shape, too, is important because it affects the viscous drag. The frictional coefficient of a compact particle is smaller than that of an extended particle of the same mass.
- The sedimentation velocity depends also on the density of the solution (ρ). Particle sinks when $\bar{v}\rho < 1$, floats when $\bar{v}\rho > 1$.

Sedimentation coefficients of biological macromolecules are normally obtained in buffered solutions whose viscosity and density may differ from those of water. It is also measured at different temperatures. Hence, we standardize the sedimentation coefficient value in standard conditions i.e. pure water at 20°C (denoted by $s_{20,w}$).

$$s_{20,w} = s_{exp} \frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{exp}} \frac{\eta_{exp}}{\eta_{20,w}}$$

where, $s_{20,w}$ is the sedimentation coefficient of the molecule in pure water at 20°C,
s_{exp} is the experimentally measured sedimentation coefficient of the molecule,
η_{exp} is the viscosity of the solvent at the experimental temperature T (°C),
$\eta_{20,w}$ is the viscosity of water at 20°C,
$\rho_{20,w}$ is the density of water at 20°C,
ρ_{exp} is the density of the solvent at given temperature T (°C) and
$\bar{v}$ is the partial specific volume of the molecule.

Relative centrifugal fields are used because different centrifuge rotors have different geometries and the appropriate rpm with one rotor may not be correct if a different rotor is used. It is, therefore, important to know the value of r for the rotor being used. But r is not constant throughout a centrifuge tube, so different parts of the sample will be subjected to different RCFs. Usually the important figure is the r_{max}, which is the maximum radial distance for the rotor. If the rotor is angular or a swinging-bucket type, then r_{max} corresponds to the bottom of the centrifuge tube; if the rotor is an upright one, then r_{max} gives the distance to the outer wall of the tube.

Relative centrifugal field

Particles suspended in a fluid move, under the influence of gravity, towards the bottom of a vessel at a rate that depends, in general, on their size and density. Centrifugation utilizes centrifugal forces which are greater than the Earth's gravitational force to increase the sedimentation rate of particles. This is achieved by spinning the vessel containing the fluid and particles about an axis of rotation so that the particles experience a centrifugal force acting away from the axis. The force is measured in multiples of the Earth's gravitational force and is known as the relative centrifugal field (RCF) or more commonly, the *g* force. For example, an RCF of 500 ×g indicates that the centrifugal force applied is 500 times greater than Earth's gravitational force.

The RCF generated by a rotor depends on the speed of the rotor in revolutions per minute (rpm) and the radius of rotation (i.e. the distance from the axis of rotation). The equations that permit calculation of the RCF from a known rpm and radius of rotation is:

$$RCF = \frac{r\omega^2}{g}$$

where, r is the radius in centimeters,
g is the acceleration due to gravity (cm/sec^2) and
ω is the angular velocity.

Angular velocity can be defined as $\omega = \frac{2\pi \times rpm}{60}$ radians / sec

$$RCF = \frac{r}{g} \times \left(\frac{2\pi \times rpm}{60}\right)^2 = r \times (rpm)^2 \times \left(\frac{4 \times 3.14 \times 3.14}{60 \times 60 \times 980}\right) = 1.12 \times r \times (rpm)^2 \times 10^{-5}$$

The force applied to the samples varies according to the size of the centrifuge as a larger centrifuge will have a higher radius and a smaller centrifuge will have a shorter radius. For example, when revolving at 2000 rpm, a larger centrifuge with a longer radius length will spin samples at a higher *g* force than a smaller centrifuge with a shorter radius length.

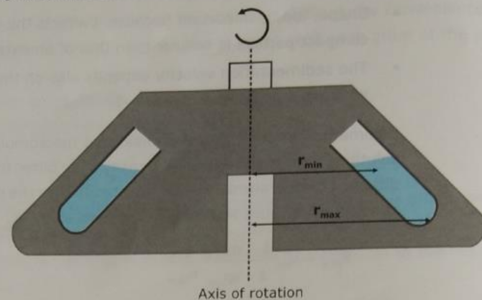

Figure 5.2 A diagram illustrating the variation of RCF with r, the distance of the sedimenting particles from the axis of rotation.

Differential centrifugation

Differential centrifugation separates particles based on differences in sedimentation rate, which reflect differences in sizes and densities. This centrifugation process is used mainly for the separation of sub-cellular components.

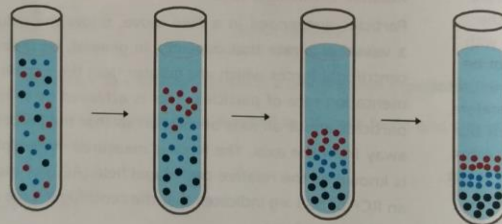

Figure 5.3 Differential centrifugation separates particles based on differences in sedimentation ra[illegible] which reflect differences in size and/or density. Particles which are large or dense sediment rapidly, tho[illegible] that are intermediate in size or density sediment less rapidly, and the smallest or least dense partic[illegible] sediment very slowly. Eventually, all of the particles reach the bottom of the tube.

In the process of separation of sub-cellular components, the preparation of broken cells [illegible] poured into a centrifuge tube and is initially centrifuged at low centrifugal force long enoug[illegible] to completely sediment the largest and heaviest sub-cellular component. The supernata[illegible] obtained is carefully decanted and is again centrifuged at a higher centrifugal force for sed[illegible] menting the next heavier entity in the extract. This process is continued and at each ensurin[illegible] step the centrifugal force as well as the centrifugal time is increased to successively sedime[illegible] the lighter components and particles. In this method, the separation occurs due to the d[illegible] ferential sedimentation rates of the sub-cellular organelles because of the differences in the sizes and densities.

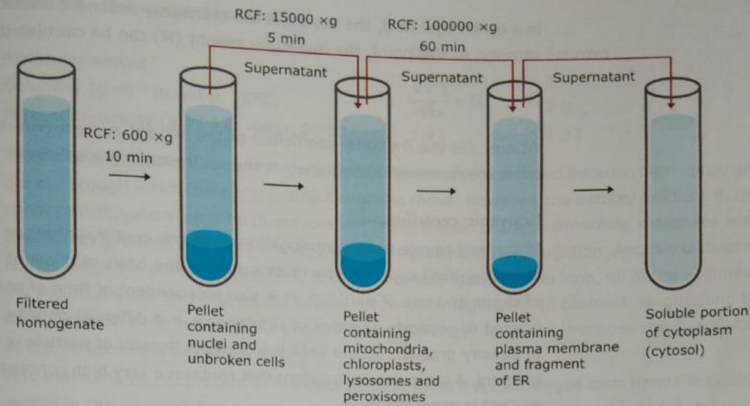

Figure 5.4 Differential centrifugation and separation of cell organelles. The different sedimentation rate of various cellular components make their separation possible. The tissue of interest is first homogenized. Subcellular fractions are then isolated by subjecting the homogenate and subsequent supernatant fractions to successively higher centrifugal forces and longer centrifugation times. During each step of the centrifugation process, particles of a given size and density are removed as a pellet from suspension. Each time, the supernatant from one step is decanted into a new centrifuge tube and subjected to greater centrifugal force to obtain the next pellet.

Density gradient centrifugation

In differential centrifugation, the particles about to be separated were uniformly distributed throughout the solution prior to centrifugation. Density gradient centrifugation is a variation of differential centrifugation in which the sample is centrifuged in a medium that gradually increases in density from top to bottom. The gradient consists of an increasing concentration of solute (and therefore density) from the top of the tube to the bottom. It is of two types: *Rate zonal centrifugation*, in which the sample is centrifuged in a preformed gradient and *isopycnic centrifugation*, in which a self-generating gradient forms during centrifugation.

Rate zonal centrifugation

Rate zonal centrifugation (also termed as *velocity centrifugation*) is used to separate particles on the basis of differences in their sedimentation rate. Separation of particles occurs according to their size and/or density. Although the rate of sedimentation is strongly influenced by the size (mass) and density of particles, even slight variation in shape also affects the rate of sedimentation. In zonal centrifugation method, materials used for the preparation of density gradients are sucrose, glycerol, ficoll etc. A 5–20% sucrose solution is commonly used to form density gradient. The density range is chosen so that the density of the particles is greater than the density of the medium at all points during the separation.

In this centrifugation process, samples are centrifuged just long enough to separate the molecules of interest into discrete zones. If a sample is centrifuged much longer than necessary, all the components of sample will end up in a pellet at the bottom of the tube.

In addition to the preparation and purification of macromolecules and cellular components, rate zonal centrifugation can be used to determine the sedimentation coefficients and molecular weights of biological macromolecules. If a purified molecule such as a protein is spun

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RCFs of over
×g.

in a centrifugal field, the molecule will eventually sediment towards the bottom at a constant velocity. At this point, the molecular weight (M) can be calculated as:

$$M = \frac{f \times v}{\omega^2 r}$$

Where, f is the *frictional coefficient* of the solvent system (which has been calculated from other measurements) and $v/\omega^2 r$ is the *sedimentation coefficient*.

Isopycnic centrifugation

In contrast to rate zonal centrifugation, isopycnic centrifugation (or *equilibrium density gradient centrifugation*) separates particles solely on the basis of buoyant density and is independent of shape and size of particles. It is also independent of time of centrifugation. This technique is used to separate particles of similar size, but different density. Sedimentation of particles in density gradient occurs until the buoyant density of particle is equal to the density of the gradient. A steep density gradient that contains a very high concentration of sucrose (20–70%) or CsCl is generally used.

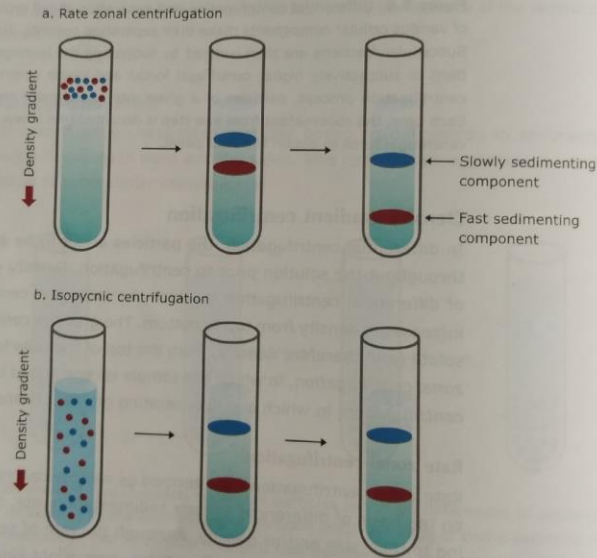


Figure 5.5 (a) Rate-zonal centrifugation is a variation of differential centrifugation in which the sample for fractionation is placed as a thin layer on top of a gradient of solute. The gradient consists of an increasing concentration of solute—and therefore density—from the top of the tube to the bottom. When subjected to a centrifugal force, particles differing in size and/or density move downward as discrete bands that migrate at different rates. Because of the gradient of solute in the tube, the particles at the leading edge of each zone continually encounter a slightly denser solution and are, therefore, slightly impeded. As a result, each zone remains very compact, maximizing the resolution of different particles.
(b) Isopycnic centrifugation also includes a gradient of solute that increases in concentration and density, but in this case the solute is concentrated so that the density gradient spans the range of densities of the particles about to be separated. During centrifugation, the particles move into the gradient until each reaches its equilibrium (or buoyant) density—the point in the gradient at which the density of the particle is exactly equal to the density of the gradient.

Table 5.1 Density gradient media

	CsCl	*Sucrose*
Molecular weight	168.36	342.30
Solubility (g ml^{-1} in water, 20°C)	1.2	2.0
Maximum density (g ml^{-1} in water, 20°C)	1.91	1.32

The zonal and equilibrium centrifugation methods are often confused because both utilize gradients through which the sedimenting molecules move. However, the density gradient in rate zonal centrifugation plays no direct role in the separation of the sedimenting molecules while the density gradient performs the separation in equilibrium centrifugation. Another distinction is that when the centrifugation time of a zonal centrifugation is too long, all of the sedimenting molecules will be found on the bottom of the centrifuge tube. In contrast, lengthening the centrifugation time in equilibrium centrifugation will have no effect, because the distribution of the molecules is at equilibrium.

Any macromolecules present in the CsCl solution when it is centrifuged form bands at distinct points in the gradient and exactly where a particular molecule forms band will depend on its buoyant density. DNA has a buoyant density of about 1.70 g/cm^3, and therefore it will migrate to the point in the gradient where the CsCl density is also 1.70 g/cm^3. In contrast, protein molecules have much lower buoyant densities and so float at the top of the tube, whereas RNA forms a pellet at the bottom. Density gradient centrifugation can therefore separate DNA, RNA and protein.

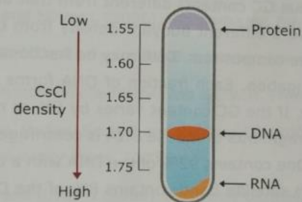


Figure 5.6 CsCl density gradient centrifugation. Separation of protein, DNA and RNA in a density gradient.

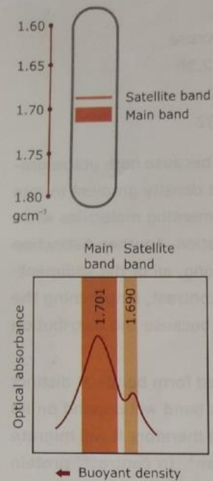
DNA separation by equilibrium density gradient centrifugation

Equilibrium density gradient centrifugation with caesium chloride (CsCl) is a useful tool for fractionating, quantitatively separating and characterizing DNA based on differences in their buoyant densities. CsCl is used because, at a concentration of 1.6 to 1.8 g/mL, it is similar to the density of DNA. During centrifugation, a gradient of the caesium ions is formed.

The DNA molecules having differences in the relative proportions of AT (adenine and thymine base pairs) to GC (guanine and cytosine base pairs) can be separated by CsCl density gradient centrifugation. An AT base pair has a lower molecular weight than a GC base pair and therefore, for two DNA molecules of equal length, the one with the greater proportion of AT base pairs will have a lower density, if all other factors being equal. The density (ρ) of DNA is related to the GC content. A linear relationship exists between the densities of DNA and their GC content.

$$\rho = 1.660 + 0.098\,(G+C)\ g/cm^3$$

where, G+C is the mole fraction of G+C in the dsDNA.
ρ is the Buoyant density of DNA.



Mouse DNA is separated into a main band and a satellite band by centrifugation through a density gradient of CsCl.

Figure 5.7 Density of DNA as a function of GC content.

The buoyant density of most double stranded linear DNA in CsCl solution is ~1.70 g/cm^3. In CsCl solution, the density of ssDNA is ~0.015-0.020 g/cm^3 greater than that of dsDNA of the same base composition. The buoyant density of ssRNA in CsCl solution is >1.8 g/cm^3.

Difference in density between highly repetitive satellite DNA from rest of DNA is used for separation of these two by CsCl density gradient centrifugation. DNA that consists of very large numbers of short tandem repeats (termed satellite DNA) may have a base composition (and thus GC content) different from that of the genome as a whole. If so, the satellite DNA will have a different buoyant density from the rest of the DNA, as this property depends on the base composition. DNA may be fractionated according to density by CsCl density gradient centrifugation. Each fraction of DNA forms a band at the position corresponding to its own density. If the GC content varies by 5% or more, separate bands are obtained. For example, when fragments of mouse DNA is centrifuged on a CsCl density gradient, two DNA bands are seen. One contains 92% of the DNA with a density of 1.701 gm/cm^3 (GC content ~42%) and the thin satellite band contains 8% of the DNA with a density of 1.690 gm/cm^3 (GC content ~30%). Satellite DNA was originally defined by this density separation. However, in cases where the average satellite DNA base composition is close to that of the genome as a whole, the satellite DNA cannot be physically separated using a density gradient.

EtBr is a planar compound which intercalates between base pairs in the DNA double helix. Each molecule of EtBr which intercalates causes the double helix to unwind, decreasing Tw and increasing Wr. When one molecule of EtBr intercalates in the DNA double helix, the helix untwists by 26°. Addition of 14 EtBr molecules to a DNA molecule results in the unwinding of one full turn. Thus, Tw decreases by one.

Separation on the basis of conformation

In laboratory, CsCl equilibrium density gradient centrifugation is also used for the separation of linear DNA (non-supercoiled) molecules from circular supercoiled DNA. It is carried out in the presence of DNA intercalating dye EtBr. DNA-EtBr intercalation causes the unwinding of the DNA helix, which reduces the buoyant density of DNA, by as much as 0.125 g/cm^3 for linear DNA. However, covalently closed circular supercoiled DNA, with no free ends, has very little freedom to unwind, and can only bind a limited amount of EtBr. The decrease in buoyant density of a circular supercoiled DNA is much less, only about 0.085 g/cm^3. In contrast, linear DNA molecules with free ends are not as topologically constrained and can, therefore, bind more EtBr molecules, resulting in a more decrease in buoyant density. As a consequence, covalently closed circular supercoiled DNA form a band in an EtBr-CsCl gradient at a different position to linear DNA.

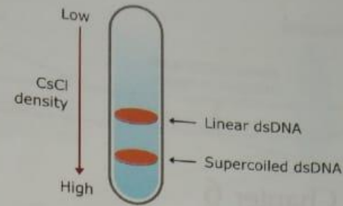


Figure 5.8 Separation of supercoiled DNA from non-supercoiled linear DNA molecules by density gradient centrifugation in the presence of ethidium bromide (EtBr) is shown here. EtBr binds to DNA molecules by intercalating between adjacent base pairs, causing partial unwinding of the double helix. This unwinding results in a decrease in the buoyant density. However, supercoiled circular DNA, with no free ends, has very little freedom to unwind, and can only bind a less amount of EtBr as compared to linear dsDNA. The decrease in buoyant density of a supercoiled molecule is, therefore, much less. As a consequence, supercoiled molecules form a band in an EtBr-CsCl gradient at a different position to linear DNA.

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