CHAPTER

2

Centrifugation

Centrifugation is a technique for the separation of the components of an analyte based on differences in the rate of migration under the influence of a centrifugal field. Centrifugation techniques are generally classified into preparative and analytical techniques. The analytical centrifugation techniques are used for determination of molecular weight, determination of number of components, assessment of purity, etc. The preparative centrifugation techniques are primarily used for fractionation. However, the preparative centrifugation techniques are often used in the preliminary steps of many other analytical protocols.

2.1 PRINCIPLES

The centrifugal force acting on a particle in solution depends upon the density difference between the particle and the surrounding medium. The centrifugal force also depends on the distance of the particle from the center, r, and the rate of rotation ω.

$$F_{cen} = (m - m_s)\omega^2 r = (\rho - \rho_s)V\omega^2 r \qquad (2.1)$$

m Mass of the particle
V Volume of the particle
m_s Mass of the solvent displaced by the particle
ρ Density of the particle
ρ_s Density of the solvent

2.1.1 Sedimentation Velocity

The centrifugal force acting on the particle produces an acceleration and a transient increase in the velocity of the particle. The particle is

determination of the distribution of molecular weights in inhomogeneous samples. Accurate determination of relative molecular mass requires precise control of rotor speed and an optics system for monitoring the progress of the experiment. Analytical centrifugation requires much higher rotor speeds than preparative centrifugation. This combination of high rotor speeds, precise control of speed and optical monitoring system is available in an ultracentrifuge.

2.2.1 Sedimentation Coefficient

The sedimentation velocity of a particle depends upon the dimensions of the centrifuge and its rate of rotation and the viscosity of the solution. Therefore, for quantitative comparisons, the standard sedimentation coefficient is used, because it depends only on the properties of the analyte.

The sedimentation coefficient of a particle is defined as its velocity per unit centrifugal field. Therefore, the sedimentation coefficient s is:

$$s = \frac{v}{\omega^2 r} = \frac{(\rho - \rho_s)V}{f} = \frac{m(1 - \overline{V}\rho_s)}{f} \tag{2.7}$$

$\overline{V}$ is the partial specific volume of the solute of interest.

The standard sedimentation coefficient is estimated from the measured value of the sedimentation coefficient as follows. First the temperature and solution effects are estimated:

$$s_{20,w} = s\left(\frac{(1 - \overline{V}_{20,w}\ \rho_{20,w})}{(1 - \overline{V}\rho)}\right)\left(\frac{\eta_{T,w}}{\eta_{20,w}}\right)\left(\frac{\eta_{T,soln}}{\eta_{T,w}}\right) \tag{2.8}$$

s	Measured value of the sedimentation coefficient
$s_{20,w}$	Sedimentation coefficient estimated for temperature of 20°C and water as solvent
$V_{20,w}$	Partial specific volume of analyte at 20°C in water
$\rho_{20,w}$	Density of water at 20°C
$\eta_{20,w}$	Viscosity of water at 20°C
$\eta_{T,w}$	Viscosity of water at temperature T
$\eta_{T,soln}$	Viscosity of solution at temperature T

The sedimentation coefficient depends on the concentration of the analyte. Therefore, a series of measurements are made with different concentrations of the analyte. Then, the sedimentation coefficient at

zero concentration, $s^0_{20,w}$ is obtained by linear extrapolation of the experimental data.

The standard sedimentation coefficient, $s^0_{20,w}$ has units of s^{-1}. Typical values of sedimentation coefficient for macromolecules are of the order of $10^{-13}\ s^{-1}$. $10^{-13}\ s^{-1}$ is called a Svedberg, denoted by the symbol S. E.g., Ribosome small subunit has a standard sedimentation coefficient, $s^0_{20,w}$, of $30 \times 10^{-13}\ s^{-1}$ or 30S.

2.2.1.1 Sedimentation diffusion method : Svedberg equation

The Einstein-Sutherland equation gives the relationship between the frictional coefficient, f, and the diffusion coefficient, D.

$$D = \frac{kT}{f} \quad \Rightarrow \quad f = \frac{kT}{D} \tag{2.9}$$

k Boltzmann's constant
T Temperature

Equation (2.7) can be combined with the Einstein-Sutherland equation (2.9).

$$s = \frac{m(1 - \overline{V}\rho_s)D}{kT} \tag{2.10}$$

Multiplying, the numerator and denominator of Equation (2.10) by the Avogadro's number, we obtain the Svedberg equation:

$$M = \frac{RTs}{(1 - \overline{V}\rho_s)D} \tag{2.11}$$

The Svedberg equation allows us to determine the relative molecular mass (in Daltons) of the analyte from the experimentally determined sedimentation coefficient and diffusion coefficients. Knowledge of the partial specific volume is also necessary for the application of this method. The partial specific volumes of most proteins are similar. Therefore, the partial specific volume can be estimated with reasonable accuracy to avoid making an additional measurement of the partial specific volume.

2.2.1.2 Scheraga-Mandelkern equation

Einstein described a relationship between specific viscosity and volume fraction of spherical molecules. This equation can be extended for molecules that can be modeled as ellipsoids of revolution and combined with Equation (2.7) to obtain the Scheraga-Mandelkern equation. According to the Scheraga-Mandelkern equation:

$$\beta' = \frac{N_0 s[\eta]^{1/3}\eta_s}{(1-\bar{V}\rho_s)M^{2/3}} \quad => \quad M = \left(\frac{N_0 s[\eta]^{1/3}\eta_s}{\beta'(1-\bar{V}\rho_s)}\right)^{3/2} \tag{2.12}$$

β' Scheraga-Mandelkern parameter.[1]
η_s Viscosity of the solvent
N_0 Avogadro's number

$[\eta]$ Intrinsic viscosity

Relative molecular mass estimation from Equation (2.12) requires experimental determination of the sedimentation coefficient and the intrinsic viscosity. The Scheraga-Mandelkern parameter depends upon the shape of the molecule; however, the variation is very small. Therefore, approximate values of β' calculated for model systems can be used. Although the relative molecular mass is being determined by using hydrodynamic measurements, the relative molecular mass calculated by this method does not depend on the degree of hydration. In addition, experimental measurements of viscosity are easier than experimental measurements of diffusion coefficients for large biomolecules.

2.2.2 Sedimentation Equilibrium Method for Determination of Relative Molecular Mass

The sedimentation equilibrium method is based on the establishment of a concentration gradient of the analyte due to the combined effects of sedimentation and diffusion. The application of a mild centrifugal force initiates sedimentation of the analyte from a homogeneous solution. This produces a concentration difference. Diffusive effects act to oppose the establishment of concentration differences. The balance of the centrifugal and diffusive effects results in the establishment of

[1]The Scheraga-Mandelkern parameter is proportional to the cube root of the Simha factor that describes the shape dependence of viscosity; and inversely proportional to the Perrin factor that describes the shape dependence of the frictional coefficient (See Section 3.1.1).

described above. Therefore, although ultracentrifuges are expensive and difficult to use, they are useful for specialized applications such as characterization of very large macromolecular complexes, e.g., the mass of the ribosome, cytoskeletal components of the cell, nanogold particles, etc.

2.3 PREPARATIVE CENTRIFUGATION

The benchtop centrifuges, used for preparative centrifugation, are part of the standard equipment of most bioanalytical laboratories, unlike the ultracentrifuges used for analytical centrifugation.

2.3.1 Differential Centrifugation

Differential centrifugation separates the components of the analyte based on differences in their sedimentation velocity. According to Equation (2.5), the sedimentation velocity of a particle is linearly proportional to the density difference with the solvent and proportional to the square of the particle diameter (for spherical particles). The range of variation of density in the biological systems is quite small. However, there is a very large variation in the size, starting from small molecules, to complexes such as ribosomes, to cellular organelles such as mitochondria.

2.3.1.1 Subcellular fractionation

Differential centrifugation is used to separate the components of the cell homogenate. Separation is based primarily on differences in the size of the particles. Initially, low rotor speeds are used to sediment the largest particles. Subsequently, the rotor speed is increased in a step-wise manner, to sediment particles of other size ranges.

2.3.2 Density Gradient Centrifugation

Density gradient centrifugation is based on the use of density gradients to obtain much higher resolution than that possible with differential centrifugation. The density gradients may be preformed *in situ* during the course of the experiment. The two basic types of density gradient centrifugation experiments are: rate zonal centrifugation and isopycnic centrifugation.