Chapter 1 | Centrifugation Technique

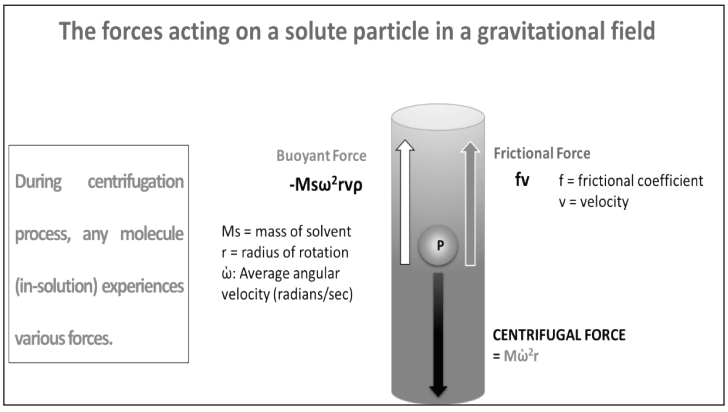
1.1. INTRODUCTION

Cell is made up of numerous biological molecules having different mass, shape and density. Molecules having more than 0.2mm diameter, remain suspended in biological fluid. If a solution containing heavier particles is left undisturbed then they tend to be sediment under the influence of gravity. Under physiological conditions, our cellular dynamics put check on such phenomenon. Svedberg (1920) proposed that sedimentation rate of a given particle is proportional to the force applied on it. In simple words, the particles will sediment rapidly, if an external force is applied. This applied force has to be in multiple of gravitational force (g). These observations finally became the core principle of centrifugation technique.

Centrifugation is one of the most applied research techniques in biochemistry, medicine, cellular and molecular biology for the research and clinical applications. It is often used for the isolation and analysis of various cell, organelles and different biomolecules including nucleic acids, proteins, lipids or carbohydrates dissolved or dispersed in cellular solvents.

1.2 PRINCIPLES OF CENTRIFUGATION

Behaviour of any suspended particle in a given solution is defined by its density. In a solution, particles whose density is higher than that of the solvent, will sink (sediment), and particles that are lighter, will float to the top. If there is no difference in density (isopycnic conditions), the particles hover. To take advantage of even tiny differences in density among biomolecules, a much more powerful "centrifugal force" is applied for separation in place of gravity. A centrifuge can provide such centrifugal force (g-force) to separate suspended particles from their surrounding medium.



▲ **Figure 1** Forces acting on a solute particle in gravitational field

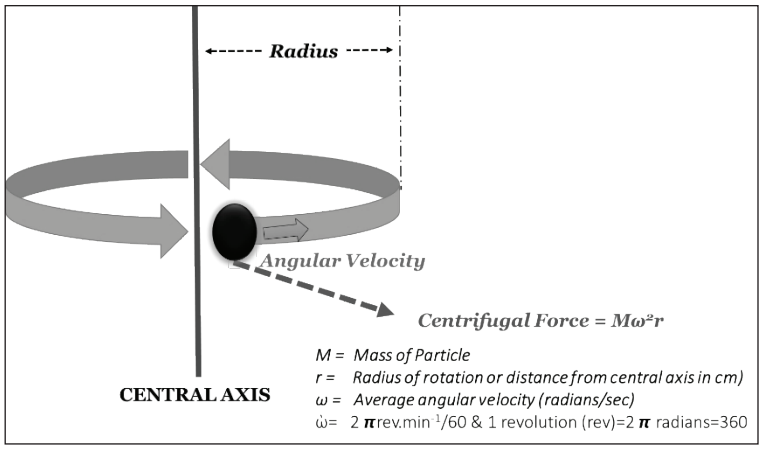
Any object moving in a circle at a steady angular velocity is subject to an outward directed force (F) which depends on the angular velocity in radians, w, and the radius of rotation, r, in centimeters. Centrifugal force is applied in order to counteract the combined force of buoyant force offered by particle in solution and frictional force offered by solution to particle.

Centrifugal force can be expressed in 'g' or 'rpm'. Since the geometry of the rotor has a significant influence on the absolute values of the centrifugal force. Hence it is better to express the centrifugal force in g (gravity) rather than rpm. The values of centrifugal force can easily be converted to rpm if the radius (r in cm) of rotor is known.

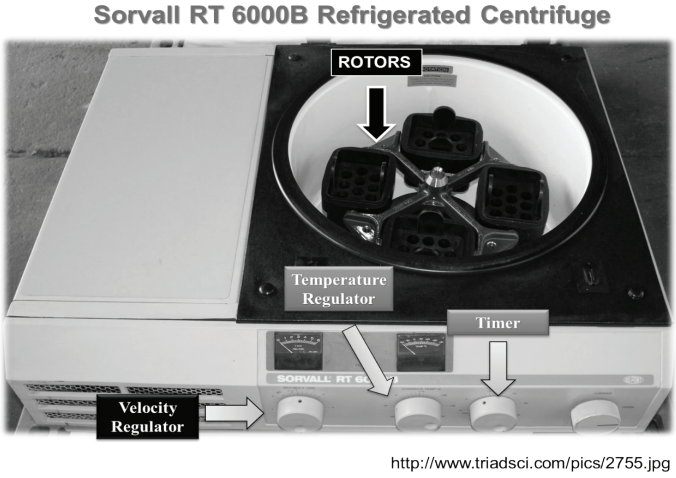
1.2.1 Instrumentation

Any centrifuge machine consists of the following components (Fig 3).

1. **Speed Regulator**: It is selected by rheostat and monitored with a tachometer. Rotor velocity is prevented by going above certain limits.
2. **Temperature Regulator**: A thermocouple/ IR radiometric sensor is placed in rotor chamber to monitor the temperature in rotor chamber.
3. **Vacuum System**: It is essentially required, when the centrifuge is used at > 4,000 rpm.
4. **Refrigeration facility**: Many centrifuges are available with (refrigerated device) or without cooling function.
5. **Rotors**: Rotor is the rotating unit of the centrifuge, which has fixed holes drilled at an angle. Centrifuge tubes are placed inside these holes and the rotor spins to aid in the separation of the materials. The performance of a rotor is often expressed as *k'-factor* which is a measure of the time taken for a particle to sediment in a sucrose gradient. The centrifugation times (t) and k'-factors for two different rotors (1 and 2) are related by: $t_1 = k_1 t_2 / k_2$. The most efficient rotors which operate at a high centrifugal force and have a low sedimentation path length contain lowest k'-factors.



▲ **Figure 2** *Angular* movement of a particle due to centrifugal force



▲ **Figure 3** Components of a centrifugation machine

In centrifuge, rotor spins upon an axis in central compartment. Rotors are generally described by their type, sample-volume capacity, number of tubes (of maximum volume) and maximum speed. There are three types of rotor used during biochemical purification i.e. Swinging-bucket, fixed-angle and vertical rotors.

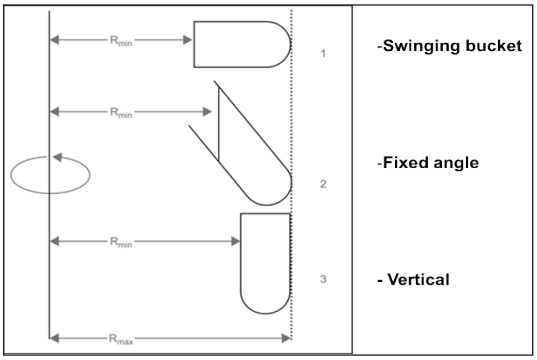
Fixed Angle Rotors

1. Most ubiquitous rotors used in centrifugation. Generally used for basic pelleting applications (differential separations), either to pellet particles from a suspension and discard the excess debris, or to collect the pellet.
2. Tubes in pocket at fixed angle in rotor. Angle 10 to 50 degrees from vertical – at rest and during spin.
3. Pellets always asymmetrically distributed toward the outer aspect of the bottom of the tube.
4. These rotors can accommodate volumes ranging from 0.2 mL to 1 L, with speeds ranging from single digits to 1,000,000 × g (relative centrifugal force, RCF). Use up to 600,000 x g Particles migrate to wall before moving towards bottom
5. The lower the K factor of a fixed-angle rotor, the higher the pelleting efficiency.

Swinging-bucket Rotors

1. In the swinging-bucket rotor, at rest, the tube and bucket are vertical and the meniscus of the liquid is at 90° to the earth's vertical centrifugal field. During acceleration of the rotor the bucket, tube and meniscus reorient through 90° in the spinning rotor's radial centrifugal field

2. There rotors are ideal for separating large-volume samples (up to 12 L) at low speeds.
3. A swinging-bucket rotor system consists of three parts: 1) the rotor body attaches to the centrifuge drive and has four or six arms to support the buckets, 2) the buckets are placed onto the arms of the rotor body, and 3) trunnion pins are used to hold the buckets in place.
4. Swing-buckets can support both rate-zonal and isopycnic types of separations. Swing-buckets are preferred for rate-zonal separations, because the distance between the outside of the meniscus and the outside of the bottom of the tube is long enough for separation to occur.



▲ **Figure 4** Influence of centrifugal on the sedimentation behaviour in different type of rotors

Vertical Rotors

1. Vertical rotors are specially introduced for high-speed analytical ultracentrifuges (1970). These are "near-vertical" rotors – tube angle = 8 degrees and not used for pelleting purposes.
2. These rotors are good for isopycnic separations, specifically for the banding of DNA in cesium chloride. In this type of separation, the density range of the solution contains the same density as the particle of interest; thus the particles will orient within this portion of the gradient. Isopycnic separations are not dependent on the path-length of the gradient but rather depend on centrifugation time, which must be sufficient for the particles to orient at the proper position within the gradient. Once it is determined that a vertical rotor is appropriate for the end-user application, volume and speed become the deciding factors for which rotor to use.
3. Vertical rotors have very low K factors (typically in the range of 5–25), indicating that the particle must only travel a short distance to pellet (or in this case form a band); therefore run time is minimized.

Table 1: Types of rotors and their applications

Type of rotor	Pelleting	Rate-zonal Sedimentation	Isopycnic
Fixed-angle	Excellent	Limited	Variable*
Swinging-Bucket	Inefficient	Good	Good**
Vertical	NS	Good	Excellent
Zonal	NS	Excellent	Good

NS = not suitable
*Good for macromolecules, poor for cells, and organelles
**Good for cells and organelles, caution needed if used with CsCl

1.3 CLASSIFICATION OF CENTRIFUGES

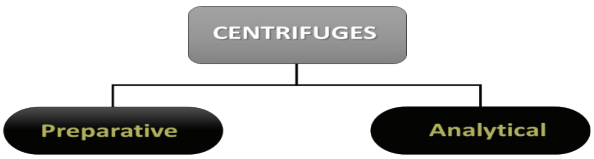
Different types of centrifuges are employed, defined by its objective of the experiment. Routinely it can be classified based upon

- The types of applications
- Its maximum operational speed
- Mechanism of separation

1.3.1 Classification based on their applications

Centrifuges are of two types i.e. Preparative and Analytical Centrifuges.

Preparative Centrifuge: These centrifuges are often used for fractionation/precipitation during purification process. In this technique large sample volume are used to separate organelles and molecules. e. g. Clinical Centrifuge.



Analytical Centrifuge: This technique is used only after purification process, for the analysis and characterization. Generally small sample volume is used for deriving hydrodynamic information. e. g. Analytical Ultracentrifuge.

1.3.2 Classification based on the mechanism of separation

Centrifuges can be of Differential centrifuges or Density gradient centrifuges. Subtypes of each are also described.

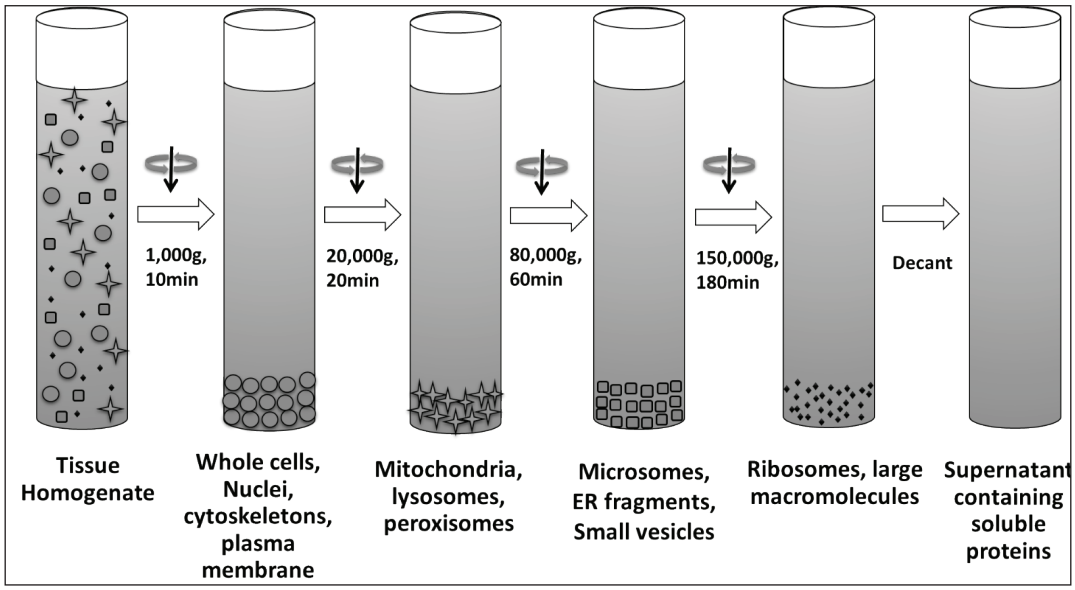
1.3.2.1 Differential centrifugation

Differential centrifugation is one of the most commonly used separation method for pelleting cellular organelles (Table 1). The rate of sedimentation is influenced by its size and the applied g-force. The density of the liquid is uniform and it is less than the particles constituting the homogenate (density of liquid << density of particles). The viscosity of the background liquid is generally low in magnitude.

Table 2: Separation of cellular components using Differential Centrifugation

Force	Time	Sediment
1000g	5min	Eukaryotic cell
4,000g	10min	Chl,cell debris, nuclei
15,000	20min	Bacteria, mito
30,000	30min	Small organelle
200,000	1hr	Small vesicle
100,000	3-10hrs	Ribosome
200,000	10-24hrs	Membrane sheets

In this method, tissue sample is first homogenized in some buffer. After sample homogenization, homogenate is placed in centrifuge and spun at constant centrifugal at constant temperature (Fig 5). After spin pellet is recovered from bottom of centrifuge tube by decanting supernatant. In case the supernatant is not transparent then sample is centrifuged at higher speed. High speed spinning is effective is sedimenting lighter cellular organelle. This process is repeated till the required organelle is not isolated as pellet.



▲ **Figure 5** Fractionation of cellular organelles using differential centrifugation

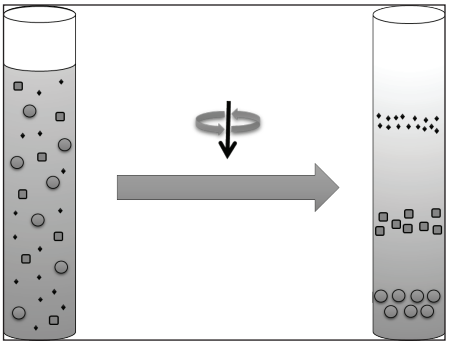
1.3.2.2 Density gradient centrifugation

Density gradient centrifuges are used to separate cellular components based on the minor differences between their densities. These centrifuges are mainly used for the purification of viruses, ribosomes, membranes etc. A gradient are prepared by using special chemical i.e. sucrose and cesium chloride. Based on the types of material used for making gradient, two subtypes of density gradient centrifugation exists i.e. Rate zonal & Isopycnic centrifugation (Fig 6).

1.3.2.2.1 Rate Zonal Centrifugation

Rate zonal centrifugation is also known as band or gradient centrifugation. It is based on the concept of sedimentation coefficient (movement of sediment through liquid medium). Generally sucrose is used to make discontinuous gradient. Sucrose gradient is created by gently overlaying variable concentrations in decreasing order (higher first followed by lower concentrations). Lowest concentration is on the top while higher sucrose concentration is present at the bottom of centrifuge tube.

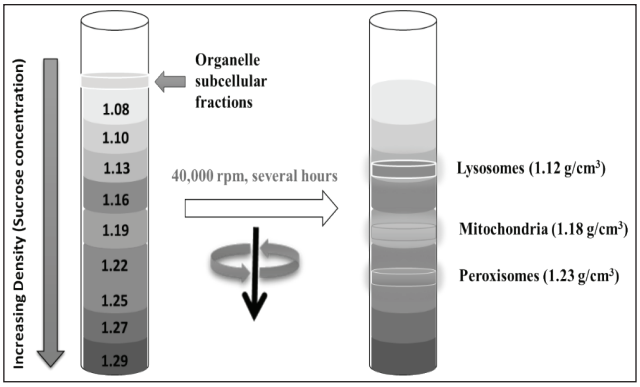
Before centrifugation, sample is placed on the top of this gradient and spun at higher speed. The particles travel through the gradient until their density matches with the density of sucrose. After this fractions are removed and further analyzed (Fig 7).



▲ **Figure 6** Different types of Density gradient centrifugation

1.3.2.2.2 Isopycnic centrifugation

Isopycnic centrifugation is a continuous type of density gradient centrifugation. Cesium chloride is used to make continuous gradient. The solution of biological sample and cesium salt is uniformly distributed in a centrifuge tube and rotated in an ultracentrifuge. Under the influence of centrifugal force the cesium salts redistributes to form a density gradient from top to bottom. The sample molecules move to the region where their density equals to the density of gradient (Fig 5). Isopycnic centrifugation is most commonly used to separate DNA fragments.



▲ **Figure 7** Cellular fractionation using rate zonal centrifugation

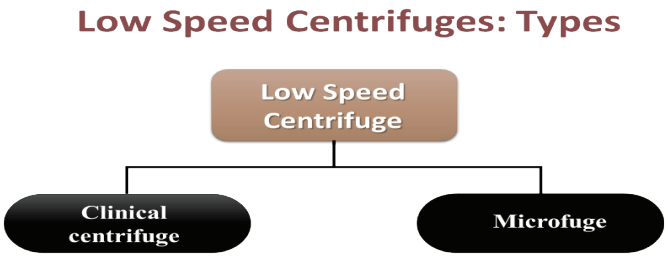
1.3.3 Classification based on their maximum operating speed

Based on the maximum velocity used during centrifugation experiment, different types of centrifuge machine are found starting from simple centrifuges (operate <5,000 rpm) to ultracentrifuges which often run at > 75,000 rpm (Table 2).

Table 2: Comparative account of different types of centrifuges based on their applications

		CENTRIFUGATION TYPES		
S.No.	**Parameters**	**Low Speed**	**High Speed**	**Ultra**
1	Maximum Speed (rpm)	10,000	28,000	100,000
2	Maximum RCF (g)	7,000	100,000	800,000
3	Refrigeration	Some	Yes	Yes
	APPLICATIONS			
1	Pelleting of cells	Yes	Yes	Yes*
2	Nuclei precipitation	Yes	Yes	Yes*
3	Pelleting of membranes fractions	Some	Some	Yes*
4	Pelleting of Ribosomes/Polysomes	---	---	Yes
5	Macromolecules	---	---	Yes
6	Viruses	---	Most	Yes
7	Small precipitates	Some	Most	Yes*

1.3.3.1 Desktop Centrifuges



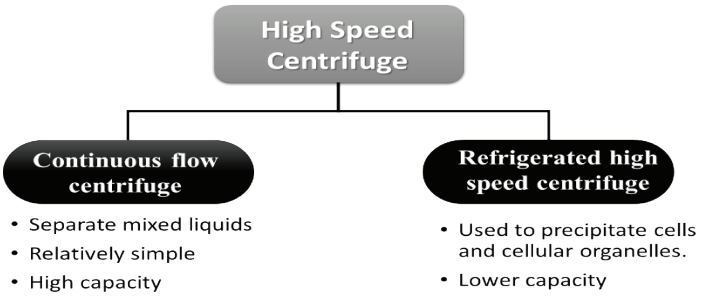
Desktop centrifuges are the low speed centrifuges, used for routine sedimentation purposes. These centrifuges may attain the maximum velocity of 4,000-5,000 rpm. Low speed centrifuges comes as two models. Clinical Centrifuge and Microfuge. Both of these instruments generally use fixed angle and swinging bucket rotors for various clinical applications e. g. sedimentation of RBCs.

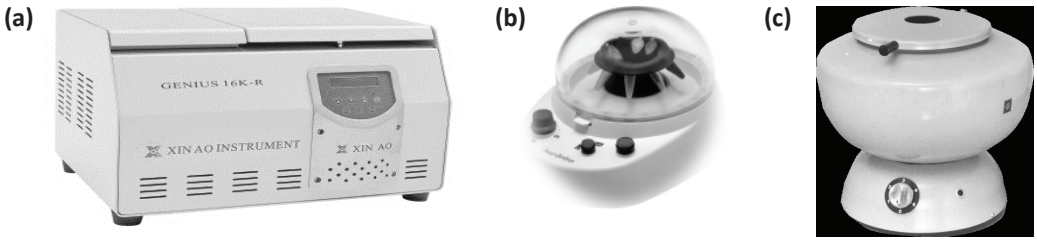
Clinical Centrifuges: These are the simplest and least expensive centrifuge which operate up to 4,000 rpm. Small bench-top centrifuges are equipped with or without refrigeration. These are common in clinical labs for the separation of blood/plasma or serum. Clinical centrifuges can handle approx. (up to) 100 mL of sample volume, depending on diameter of the centrifuge tubes (Fig 6a).

Micro-centrifuges: Micro-centrifuges are laboratory table top simplest centrifuge. These centrifuges can generate up to ~15,000 x g force. Microfuges are very common very common in biochemistry/molecular biology/biological labs and often used for pelleting purposes (Fig 6b). These centrifuge are of low capacity and used to accommodate 2mL/ tube volume.

1.3.3.2 High speed centrifugation

Any biochemical purification scheme essentially require uses of the large capacity centrifuges (Fig 6c). High speed centrifuges are used for the separation of nucleus, mitochondrial, protein precipitation, large organelle, cellular debris and other protein aggregates. They operate at speed of 20,000-25,000 rpm which is equivalent to the centrifugal field of >100,000 g. Most of these system are equipped with refrigeration facility. Both swing bucket and fixed angle rotors are compatible with these kind of centrifuges.



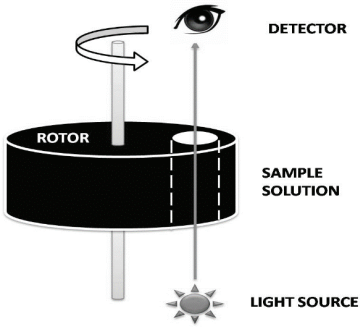


▲ **Figure 6** Types of Centrifuges. a) Clinical Centrifuge b) Microfuge c) High speed bench top centrifuge

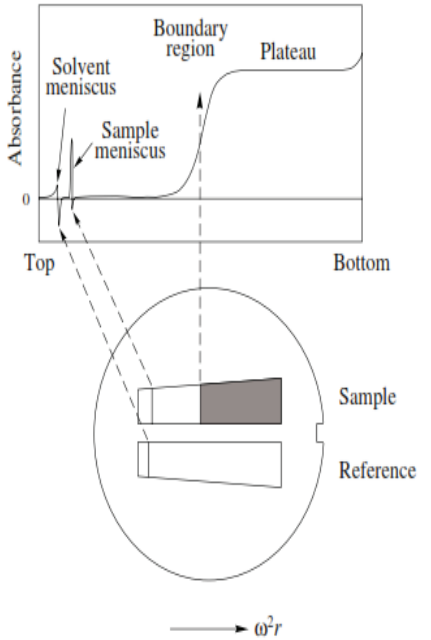
1.3.3.3 Analytical Ultracentrifuge

Theodor Svedberg (1925) invented the analytical ultracentrifuge for his research on colloids and proteins. In 1926, he was awarded Nobel Prize in Chemistry. It is the most advanced and sophisticated version of a centrifugation machines. Ultracentrifuge can be used both for analytical and preparative purposes. In preparative mode, it is used for the separation/purification of biological samples (DNA, RNA, proteins, lipoproteins, ribosomes, viruses etc). In analytical mode, it is used for physical measurement of sedimentation behavior and sedimentation constant of biomolecules. This system routinely attains 65,000-75,000rpm during research applications. Due to high speed, intense heat is generated, thus the spinning chambers must be refrigerated and kept at high vacuum to reduce friction. In ultracentrifuge, special head rotors (vertical rotors) are used. Balancing of these rotors is very critical and requires utmost care during centrifugation experiments. This instrument is very expensive and have limited lifetime.

Instrumentation: An analytical ultracentrifuge must spin a rotor at an accurately controlled speed and at an accurately controlled temperature and vacuum, and must allow the recording of the concentration distribution of the sample at known times. In order to achieve rapid sedimentation and to minimize diffusion, high angular velocities may be necessary (up to 60,000 rpm). At 60,000 rpm, a typical ultracentrifuge rotor generates a centrifugal field in the cell of about 250,000 × g. Under these conditions, a mass of 1 g experiences an apparent weight of 250 kg; i.e., 1⁄4 ton. The rotor must also allow the passage of light through the spinning sample, and some mechanism must be available for temperature measurement (Fig 7).



Ultracentrifuge is essentially contain instrumentation similar to any differential centrifugation machine, equipped with built in optical detection system. The concentration distribution of the sample inside the centrifuge cell is determined using absorbance measurements during or after sedimentation. Three types of optical systems are available for the analytical ultracentrifuge (absorbance, interference and fluorescence) that permit precise and selective observation of sedimentation in real time. These optical system measures the difference in absorbance between the sample and reference sectors as routinely performed in any double-beam spectrophotometer (Fig 7). Double-sector cells allow the user to take account of absorbing components in the solvent, and to correct for the redistribution of solvent components, particularly at high g values. The sample solution is placed in one sector, and a sample of the solvent in dialysis equilibrium with the sample is placed in the reference sector. The reference sector is usually filled slightly more than the sample sector, so that the reference meniscus does not obscure the sample profile. Absorbance data is recorded and replotted as a function of distance travelled by the molecule of interest, during experiment.



▲ **Figure 7** Sedimentation behaviour of a sample in ultracentrifuge

Analytical applications of ultracentrifuge

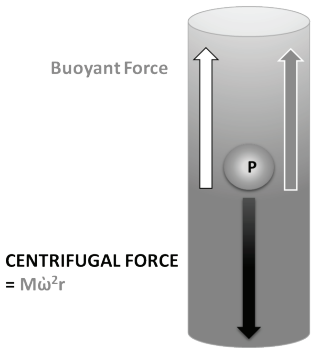
Analytical ultracentrifugation is a powerful method for the quantitative analysis of macromolecules in solution. It is still the most versatile, rigorous and accurate means for determining the molecular weight and other physico-chemical properties of a protein or other macromolecule. In fact no other instrument can provide this information as accurate and precise as ultracentrifuge can do. This level of accuracy and precision is derived from the fact that sedimentation analysis by ultracentrifuge is firmly based on thermodynamics principle and are experimentally determinable.

Analytical Ultracentrifuge is used to derive various thermodynamic and hydrodynamic information using sedimentation velocity and sedimentation equilibrium experiments. One easy way to differentiate between sedimentation velocity and sedimentation equilibrium experiment is to look for the kind of measurement done. If we monitor the rate at which boundaries of molecules move during their redistribution, then we are conducting a sedimentation velocity experiment. If we determine the concentration distribution after equilibrium is reached, then we are conducting an equilibrium sedimentation experiment.

1.4 SEDIMENTATION VELOCITY EXPERIMENTS

Sedimentation velocity experiments provide sedimentation and diffusion coefficients that contain information concerning the size and shape of macromolecules and the interactions between them. It can also be used to determine information about purity, molar mass, state of association, protein interactions, hydrodynamic shapes, conformational changes, size-distributions and other properties of proteins. Sedimentation coefficients are particularly useful for monitoring changes in conformation in proteins and in nucleic acids. Bending in nucleic acids induced by protein binding may also be amenable to study by difference sedimentation.

When a solute particle is suspended in a solvent and subjected to a gravitational field, it is being pulled by gravitational force. As discussed earlier, this particle will only sink when an external force will be applied to counteract the combined strength of two forces (buoyant force of particle and frictional force offered by water molecules). If the magnitude of combined forces is higher than particle will never sediment (Fig 8).



- Centrifugal Force (F) =Mω²r
  - M: Mass of particle
  - r: radius of rotation (distance of particle from axis of rotation; in cm)
  - ω: Average angular velocity (radians/sec)
- ω=2 $\pi . rpm/60$
- 1 revolution (rev)=2 π radians=360

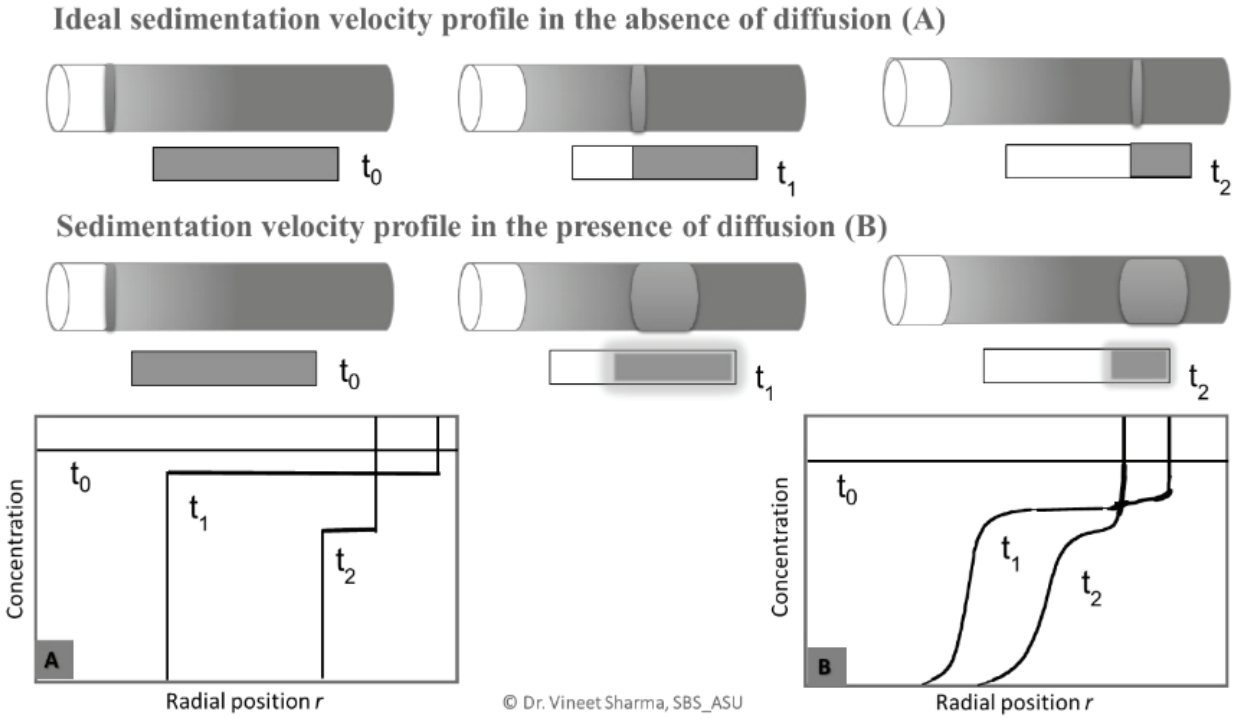
Very shortly these three forces (Centrifugal force & Buoyant + Frictional force) come in balance: Upon rearranging equation=

$$s \equiv \frac{v}{\omega^2 r} = \frac{M_b}{f}$$

SEDIMENTATION COEFFICIENT (S) = velocity of the particle per unit gravitational acceleration

In any sedimentation velocity experiment, a uniform sample is first loaded into the sample slots and subjected to high acceleration spinning (40,000 and 60,000 rpm's). Biomolecules differing in mass and shape, experience different forces and separate out in layers, forming boundaries in solution. These boundaries are basically a concentration gradient that forms as a result of the movement of the particles (Fig 9).

Although the velocity of the individual particles resulting from the centrifugal force cannot be determined, a series of scans (such as absorbance or refractive index detection) is performed on the sample as it spins to record the movement of particle boundaries over time (Fig 9). As the boundary progresses down the cell, the concentration in the plateau region decreases from radial dilution, and the boundary broadens from diffusion. The midpoint positions, r_{bnd}, of the boundaries are indicated. The diffusion coefficient (D) can be determined by measurement of the spreading of a boundary. The rate of movement of the boundary is very useful information and can be used to calculate the sedimentation coefficient (s).

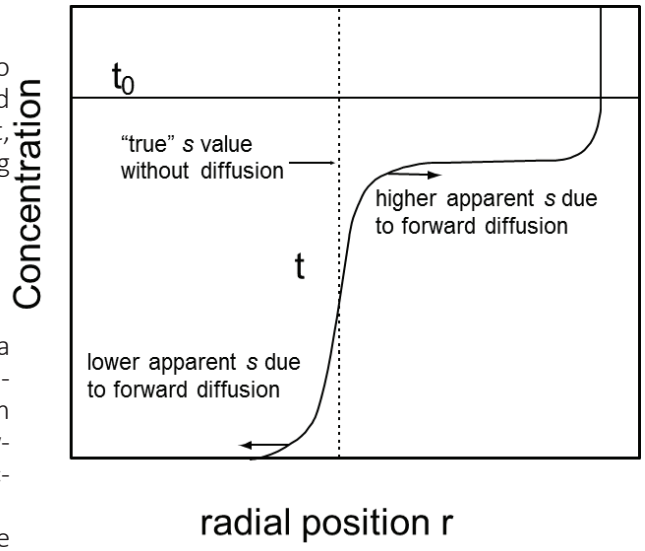


▲ **Figure 9** Sedimentation equilibrium experiment for determination of equilibrium constant

1.4.1 Determination of the diffusion coefficient from a distribution of apparent sedimentation coefficients g(s*)

The mobility of a particle at a certain time corresponds to sedimentation coefficient. Converting the distance traveled by the particle after time t into sedimentation coefficient, yields a distribution of apparent sedimentation coefficient g (s*) or c (s*).

$$s^* = \frac{1}{\omega^2 \cdot t} \cdot \ln\left(\frac{r}{r_m}\right)$$



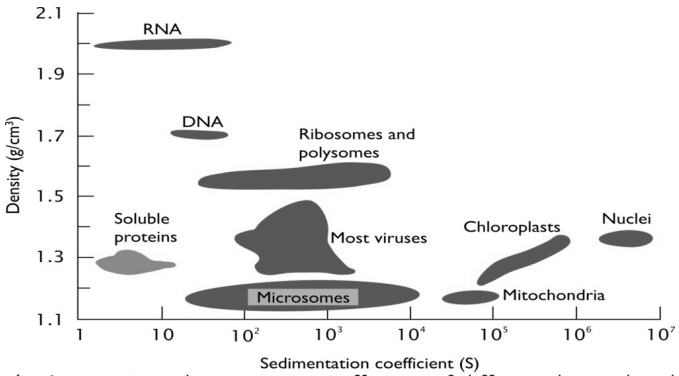
Diffusion constant can be used to decipher the purity of a sample. A homogeneous product will often produce a boundary that is sharper. In contrast, a heterogeneous sample can produce multiple boundaries or a very broad boundary. However, these are only general rules of thumb because characteristics of the sample can produce contradictory results.

A critical advantage of a sedimentation velocity procedure is that it can be performed in a relatively short amount of time (often as low as 3–5 hours), as opposed to sedimentation equilibrium (which can often take days). Another important advantage of sedimentation velocity is that it can be used to analyze samples over a broader range of pH, ionic strength, and temperature conditions. One critical disadvantage of sedimentation velocity experiment is that interacting systems (such as proteins that reversibly self-associate) can provide data that is difficult to interpret if those systems change during the course of the experiment.

Sedimentation Coefficient (S-Value)

The quantum shift in the use of centrifugal force to separate biologically important substances, was the coupling of mechanics, optics and mathematics by T. Svedberg and J.W. Williams in the 1920's. In honor of that work, the value for a molecule's (or organelle's) sedimentation velocity in a centrifugal field is known as its Svedberg constant or S value for short. Sedimentation coefficient has dimensions of seconds and one Svedberg unit is equivalent to 10^{-13} seconds.

S-value: It is a number that gives information about sedimentation behavior of the particle. Sedimentation of a particle is influenced by molecular properties (size, shape, density), solvent properties or the gradient material (density, viscosity, temperature) but independent of operating conditions.



▲ **Figure 10** Sedimentation coefficient of different biomolecules

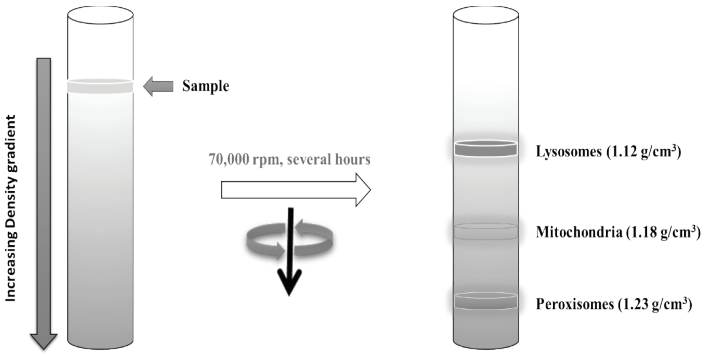
It is the ratio of the velocity to the centrifugal field. In terms of molecular parameters, it is proportional to the buoyant molar mass (Mb) i.e. molar weight corrected for the effects of buoyancy and inversely proportional to the frictional coefficient (f) which depend upon the shape and size of particle in solution (Fig 10). In a given centrifugal field, molecules with different molecular weights, or different shapes and sizes, generally move with different velocities; i.e., they will have different sedimentation coefficients. For many substances, the value of S lies between 1 and 100×10^{-13} seconds. The larger the S-value, the faster the particle separates. Serum albumin has a sedimentation coefficient of 4.5 × 10-13seconds or 4.5 S.

The sedimentation coefficient of biomolecule can be influenced by the molecular weight (heavier particles tend to sediment faster), density (dense particles tend to sediment faster), molecular shape (unfolded proteins or a more highly elongated shape will experience more friction from solvent, so will tend to sediment slower), solute

concentration (higher solute concentration tends to lower the rate of sedimentation), solvent concentration/viscosity (higher solvent concentration and viscosity will tend to increase friction and lead to a lower sedimentation coefficient) and charge of the protein and its interaction with solvent (charged particle will travel more quickly through a polar solvent).

1.5 SEDIMENTATION EQUILIBRIUM EXPERIMENT

It is a thermodynamic method where equilibrium concentration gradients at lower centrifugal fields are analyzed to define molecule mass, assembly stoichiometry, association constants and solution non-ideality. This analysis is sensitive only to the mass of a particle (not its shape), and is performed at slower velocities than those used during sedimentation velocity experiments.



▲ **Figure 8** Determination of Sedimentation Constant using sedimentation velocity experiment

As the sample spins, the components separate out under the influences of two opposite forces. One of these force is acceleration due to spinning while counter balance force is due to molecular diffusion. After some time, sedimentation and diffusion approach equilibrium in all parts of the solution column. At this equilibrium, both the processes are in balanced state and the concentration distribution from the top to the bottom no longer changes with time, and is a function of molecular weight. When the densities of the solute and solvent are equal, $(1- v\rho)=0$, then at this place with the help of density gradient, one can also determine the density of a macromolecule. Sedimentation is still regarded by many as the best method to determine the molecular weights of macromolecules in a sample. Analysis of sedimentation behavior (sedimentation coefficient) of a molecule in solution, may shed light upon

1. Same purity and homogeneity
2. Molecular mass of individual molecules
3. Size and shape of the macromolecule especially in solution
4. Distinguish between biomolecules based on difference in their densities.
5. Molecular interactions and aggregation behavior of biomolecules
6. Shape, Conformational change of protein structure e.g. ligand-binding study.

1.6 QUESTIONS AND ANSWERS

1. An effective way of purifying liquids containing suspensions is
   - **a)** crystallization
   - **b)** decanting
   - **c)** centrifugation
   - **d)** separating funnel

2. Which of the following separation method is suited for a protein sample with large differences in molecular mass?

a) dialysis

b) salting out

c) density gradient centrifugation

d) rate zonal centrifugation

3. In this type of rotors, the sample tubes are loaded into individual buckets that hang vertically while the rotor is at rest. When the rotor begins to rotate the buckets swing out to a horizontal position

a) swinging-bucket

b) fixed-angle

c) vertical

d) None of the above

4. After centrifugation of milk, the supernatant is

a) Fat

b) Whey

c) Casein

d) Water

5. This type of rotor is not suitable for pelleting applications but is most efficient for isopycnic (density) separations due to the short path length.

a) swinging-bucket

b) fixed-angle

c) vertical

d) None of the above

Answer Key: 1 = ⓒ; 2 = ⓓ; 3 = ⓐ; 4 = ⓑ; 5 = ⓒ.