Principles of Centrifugation

Objectives:

After this class, student should be able to understand-

- the basic principles of centrifugation
- the different types of centrifugation
- applications of this technique in biological science
- the precautions to be taken while handling a centrifuge

Introduction

Eukaryotes contain nucleus and a variety of membrane bound organelles like the mitochondria, endoplasmic reticulum, golgi complexes, peroxisomes and lysosomes. In addition to these, plants contain vacuoles and chloroplasts. Cells also contain granules for storing nutrients such as starch and fat. In order to study the macromolecules and metabolic processes within the cells, it is helpful to isolate one type of sub-cellular organelle from the rest of the cell contents by sub-cellular fractionation. Centrifugation is one of the techniques used for sub-cellular fractionation.

Basic principles of Centrifugation

Centrifugation is a technique used for the separation of particles using a centrifugal field. It causes denser particles to settle to the bottom of tubes, while low density substances rise to the top. In this process, denser component of the mixture migrates away from the axis and lighter components migrate toward the axis.

The process of centrifugation is carried out in an instrument known as **centrifuge**.

Centrifuge has three basic components:

- A rotor

- A drive shaft
- A motor

The rotor holds the tube or bottles containing the liquids to be centrifuged. Different rotor types and sizes can be mounted on the drive shaft. The drive shaft is connected to the motor which provides the power to turn the rotor and thus the tube can be spun at definitive speed. Rotation of the rotor about the central axis generates a centrifugal force on the particles in the suspension.

Two forces counteract the centrifugal force acting on the suspended particles. They are the buoyant force and the frictional force. When the centrifugal force, which is exerted on the particle due to rotation, exceeds the counteracting buoyant and frictional forces, it results in sedimentation of the particles.

The rate of sedimentation depends on:

- The applied centrifugal field
- Size, density and radius of the particle
- Density and viscosity of the suspending medium

The rate of centrifugation is expressed as revolutions per minute (RPM), which is specified by the angular velocity (ω).

One revolution per minute (rpm) =

$$\text{One revolution/minute} = A = \text{rpm} = \frac{\omega \times 60}{2\pi}$$

$$\omega = \frac{2\pi \times \text{rpm}}{60}$$

Where ω= angular velocity (radians/second).

The centrifugal force $\mathbf{F = m\omega^2 r}$,

Where, F = intensity of the centrifugal force,

m = effective mass of the sedimenting particles,

ω = angular velocity of rotation in rad/sec,

r = distance of the migrating particles from the central axis of rotation.

Thus, the force on the sedimenting particles increases with the velocity of the rotation and the distance of the particle from the axis of rotation.

A more common measurement of the centrifugal force F, in terms of the earth's gravitational force, g, is Relative Centrifugal Force (RCF). **RCF = (1.119×10^{-5}) $(rpm)^2(r)$**

This equation relates RCF to revolutions per minute of the sample and the radius of the rotor.

For example, if the rotor with an average radius of 8 cm rotates at 20,000 rpm, then the relative centrifugal force (RCF) created is 35,800 g.

Types of rotor:

There are three types of rotors. They are Fixed angle rotor, Vertical tube rotors and Swinging bucket rotors.

1) Fixed angle rotors: The tubes are held at angle of 14 to 40^o to the vertical axis. Particles move radially outwards and travel a short distance. It is useful for differential centrifugation. Here, reorientation of the tube occurs during acceleration and deceleration of the rotor.

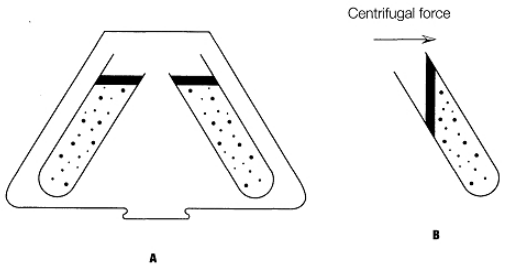


Fig: Fixed angle rotor

2) Vertical tube rotors: They are held vertical parallel to the rotor axis. The particles move short distance and the time of separation is shorter. The disadvantage is that the pellet may fall back into solution at the

end of centrifugation.

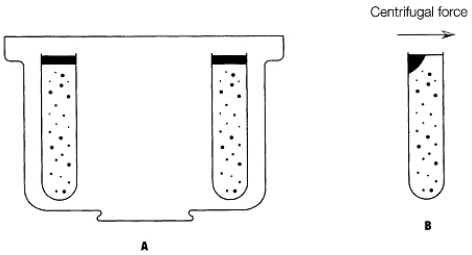


Fig: Vertical tube rotor

3) Swinging bucket rotors: The rotor swings out to the horizontal position when the rotor accelerates. The particle travels for a longer distance and thus it may allow better separation.

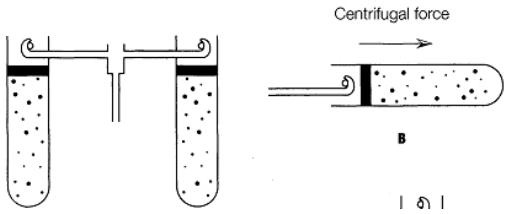


Fig: Swinging bucket rotor

Types of centrifugation:

On the basis of speed used,

It can be divided into three types: Low-speed centrifugation, High-speed centrifugation and Ultracentrifugation.

1) **Low-speed centrifugation**: This centrifugation has a speed in the range of 1-6000 rpm, with RCF values up to 6000 g. These instruments usually operate at room temperature with no means of temperature control of the sample. Two types of rotors, i.e., fixed angle and swinging bucket may be used. Low-speed centrifuges are especially useful for the rapid sedimentation of coarse precipitates or red blood cells as pellets at the bottom of the tube.

2) **High-speed centrifugation**: This is used when higher speeds and

temperature control of the rotor chamber are essential. Rotor chambers are maintained near 4^{o}C. All types of rotors can be used for high speed centrifugation. The speed range is 1000-25,000 rpm with 50,000 g. The preparation of biological samples always requires the use of high speed centrifuge. It is used to sediment cell debris after cell homogenization, ammonium sulfate precipitates of protein, microorganisms and cellular organelles such as chloroplasts, mitochondria and nuclei.

3) **Ultracentrifugation**: It is the most sophisticated of the centrifuges. Since the separation is a long process and require high speed, there is generation of heat and thus are provided with internal cooling system. The rotor chamber on all ultracentrifuges is covered with protective steel armor plate. The rotor chamber is sealed and evacuated by pump to attain vacuum in order to reduce friction.

The first analytical ultracentrifuge was developed by Svedberg in 1920.

As compared to high speed centrifuges or microcentrifuges, the ultracentrifuge can isolate much smaller particles like ribosomes, proteins and viruses. Ultracentrifuges can also be used to study membrane fractionation.

Depending on the purpose of use, centrifugation can be divided into two types:

(Preparative and analytical centrifugation. preparative is just for a preparative scale separation and in analytical centrifugation, analysis of certain parameters are performed.)

Preparative and Analytical centrifugation:

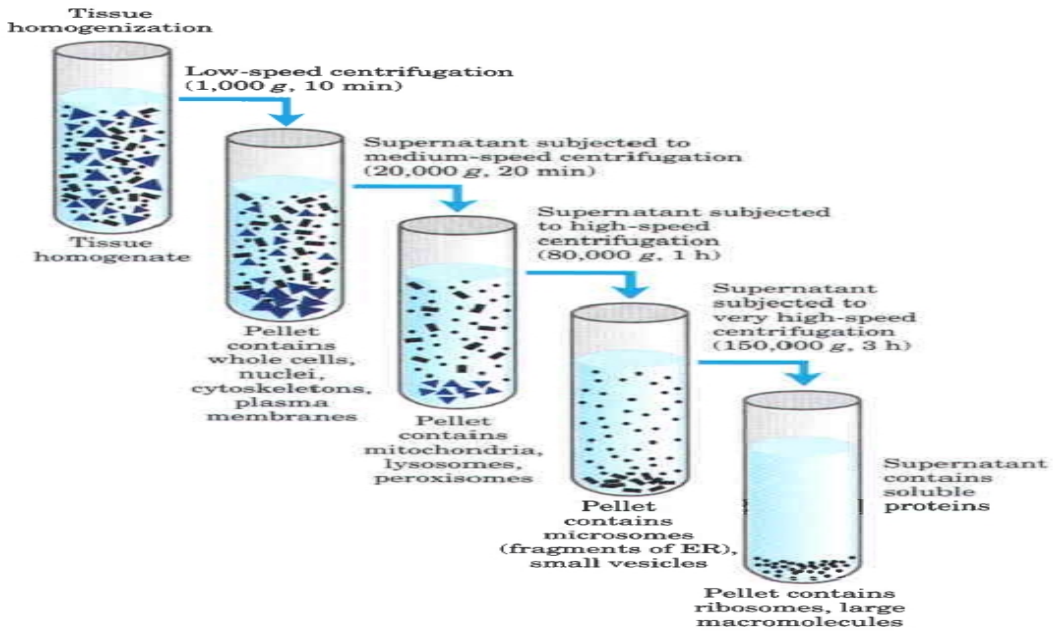
Preparative centrifugation: The preparative-scale separation procedure is simple as it requires the placing of the sample in the tube, inserting the tube in the rotor and spinning the sample for a fixed period. These results in two phases, pellet (the particles which is settled at the bottom of the tube) and the supernatant. This technique is also called Velocity sedimentation centrifugation. Relatively heavy precipitates are sedimented in low speed centrifugation, whereas lighter organelles such as ribosomes require the high

centrifugal forces of an ultracentrifuge. It is primarily used for separation and purification of sample for further analysis.

Preparative-scale separation makes use of specific method of separation, like the differential centrifugation and density gradient centrifugation.

Differential centrifugation consists of successive centrifugation at increasing rotor speeds. Differential centrifugation of a cell homogenate leads to the separation and isolation of the common cell organelles. The large and small particles in the suspension can be separated by centrifugation at different speeds.

For example, a tissue homogenate which contains the whole cells, nuclei, cytoskeletons, plasma membrane, mitochondria, lysosomes, peroxisomes, microsomes, endoplamic reticulum, small vesicles, large molecules like ribosomes and protein can be separated by differential centrifugation. After a centrifuge run at low speed at 1000 g for 10 min, the heavier particles like nuclei, whole cell, cell debris, plasma membrane will settle down at the bottom of the tube forming pellet. The supernatant thus obtained can be subjected to medium speed centrifugation at 20,000 g for 20 min. Sub-cellular organelles like mitochondria, lysosomes, peroxisomes will settle as pellet at the bottom of the tube. The supernatant again can be run on a high speed centrifugation at 80,000 g for 1 h, thus settling down miscrosomes and small vesicles as pellet. The supernatant can be further centrifuged to separate out organelles like ribosome from the soluble protein.



Thus differential centrifugation is used to fractionate cell homogenates into their components. The tissue homogenate contains many sub-cellular organelles which differ in size and therefore sediments at different rates. Each pellet is a mixture of different sub-cellular organelles. Therefore, the differential centrifugation is a rough fractionation of the cytoplasmic contents which can be further purified by density gradient centrifugation.

The density gradient centrifugation can be divided as Zonal centrifugation (separation is depending on size) and Isopycnic centrifugation (separation is depending on density).

The difference between zonal and isopycnic centrifugation is that in zonal centrifugation, a density gradient is already created in the tube with a suitable medium, having high density at the bottom. Whereas in Isopycnic centrifugation, the biological sample and the medium is uniformly distributed in the tube and then rotated in an ultracentrifuge. But under the influence of centrifugal force, selected medium redistributes to form density gradient from top to bottom.

In zonal centrifugation, the particles of interest are placed on top of the gradient medium and centrifuge in an ultracentrifuge. Solutions of sucrose or glycerol or cesium chloride or cesium sulfate are the high density solutions which are used to make the gradient medium. The particles travel through

the gradient until they reach a point at which their density matches with the density of the surrounding sucrose medium forming separate bands. The fraction can be removed and analyzed later. The particles are separated on the basis of their sedimentation coefficient. Particles with larger mass and more compact structure have high sedimentation coefficient and they form band at the bottom.

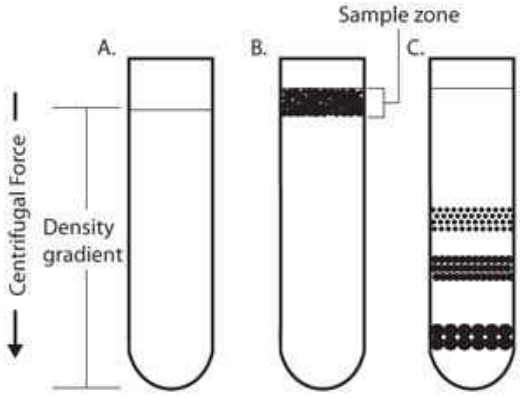


Fig: Rate-zonal centrifugation

Since each organelle has different ratios of lipid and protein content, there is a difference in their buoyant densities. These organelles are separated by centrifugation through a column of solvent with graded density of sucrose solution.

The sucrose solution is most concentrated at the bottom of the tubes and decrease in concentration or density towards the top of the tube. So, various organelles move down the tube to an equilibrium position where their density is equal to that of the sucrose at that position.

For example, mitochondria, lysosome, peroxisomes which are collected as a single pellet in differential centrifugation, can be effectively separated from one another by density- gradient centrifugation. After isolation, they can be biochemically characterized for their lipid, protein, nucleic acid and enzyme contents.

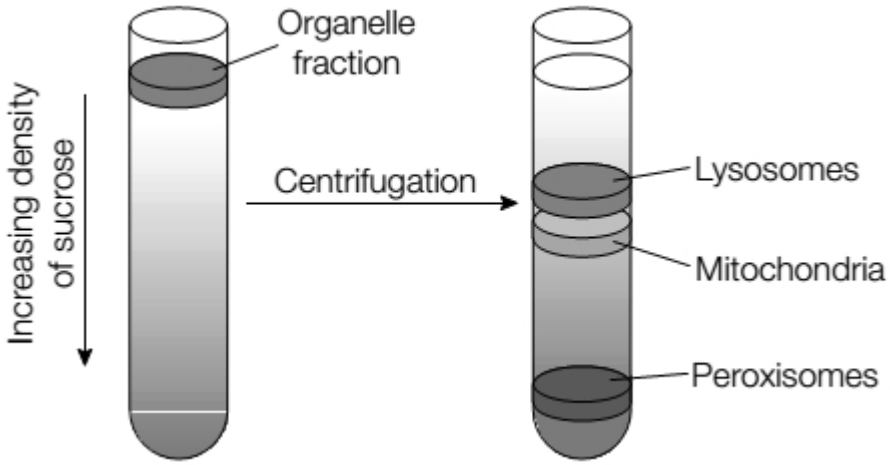


Fig: Separation of organelles by rate zonal centrifugation

In Isopycnic centrifugation, the sample is uniformly mixed with the gradient material before centrifugation. After centrifugation, the particles are separated into different zones depending only on their density difference and not on the size. Higher density particles settle down, less density particle settle at the top and medium density particle form band in the middle.

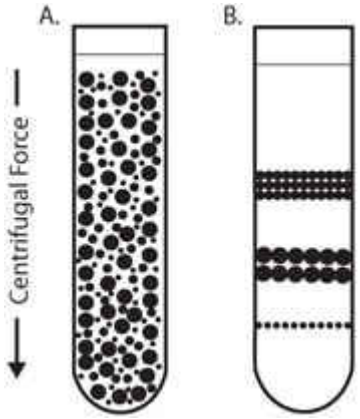


Fig: Isopycnic centrifugation

Thus the density gradient centrifugation is not just a separation technique but it is also a method of measuring densities of particles in a mixture.

Analytical Centrifugation: These are designed for performing physical measurements on the sample during sedimentation. These centrifuges are

equipped with optical systems to monitor directly the sedimentation of the sample during centrifugation. In an analytical centrifugation, a variety of analytical measurements like molecular weight, density, purity of the biological samples, conformational change of protein structure, ligand - binding study can be made. These analytical techniques require the use of an ultracentrifuge and again can be classified as differential or density gradient.

Precaution and care during centrifugation:

1) Filled centrifuge tubes should be weighed carefully and balanced before centrifugation.
2) Tubes should be properly capped and the lid of the centrifuge closed during centrifugation.
3) In case of any spillage, the centrifuge should be properly decontaminated and cleaned.
4) Select proper rotor for the centrifugation.
5) Carefully clean the rotor chamber and rotor after centrifugation.
6) Carefully read the manual before using centrifuges.

Applications of centrifugation:

There are many applications of centrifugation in biological science. Centrifugation has been used to

- Remove cellular debris from blood to prepare cell free plasma or serum
- Concentrate cellular elements and other components for microscopic analysis or chemical analysis
- Separate protein bound or antibody bound ligand from free ligand in immunological assay.
- Separate lipid components like chylomicrons from other components of plasma.
- Separation of sub cellular organelles, RNA, DNA.
- For the determination of purity and shape of the biomolecules. If there is any change in shape and size of the macromolecule during different

experimental conditions, it can be determined by studying the sedimentation coefficient.

- For determination of the relative molecular mass of the biological macromolecule by density gradient centrifugation in an analytical ultracentrifuge. Here, the sample is kept on top of the denser solvent and during centrifugation the solvent forms its own gradient along with the sample. During this, physical analysis like molecular mass determination of the migrated bands can be performed.
- Density gradient centrifugation technique was used by Meselson and Stahl to prove that DNA replication is semi-conservative by using different isotopes of nitrogen in cesium chloride gradient medium.

Conclusion:

Centrifugation is the process of using the centrifugal force to separate the lighter portion of solution, mixture or suspension from the heavier portions. It is a modern and an easy technique of separation. Due to centrifugation technique, it is easy to separate cellular and sub cellular components of cells for research or for any kind of biochemical analysis.

Summary:

Centrifugation is one of the sub-cellular fractionation techniques. This technique leads to the separation of various macromolecules, organelles from each other. Centrifugation is of various type, depending on the purpose of use. It has be a low speed, high speed or an ultracentrifuge. This technique can be again used as a preparative or analytical procedure. It can be as simple as separating the coarse granule of RBC from the serum, or as sophisticated as an analytical ultracentrifuge where measurement of molecular weight or purity of the biological sample can be done during the centrifugation process. Precautions should always be taken while using this technique. With a vast range of applications, centrifugation is widely used in

clinical laboratories or in research.

Glossary:

1. Centrifuge: It is the machine where centrifugation is carried out.

2. Centrifugation: Centrifugation is the process of using the centrifugal force to separate the lighter portion of solution, mixture or suspension from the heavier portions.

3. Preparative centrifugation: It is a type of centrifugation where the centrifugation procedure is done for a preparative scale separation of the sample.

4. Analytical centrifugation: It is a type of centrifugation where analysis of certain parameters is performed during the centrifugation procedure.

5. Tissue homogenate: The slurry of tissues and cells which results when cell structure has been mechanically disrupted.

FAQs:

1. What is centrifugation?

Ans: Centrifugation is a technique used for the separation of particles using a centrifugal field. It is a process used to separate or concentrate materials suspended in a liquid medium. Centrifugation uses gravity and centrifugal force to separate particles heavier than the liquid medium. It is a highly accelerated form of sedimentation. Centrifuges spin the material at high rotation speeds and separate the particulate from the liquid. Centrifugal force can reach many thousand times that of gravity, quickly separating the liquid/solid material, sometimes even to the nano-particle level.

2. How does a Centrifuge Work?

Ans: Centrifuges work by spinning a vessel at high speeds to create separation between materials inside the centrifuge operation. Centrifuges spin at high speeds to push material, usually of solid state, away from the center of the vessel and out of the liquid state.

3. Are all centrifuges the same?

Ans: No, definitely not. Although they work on the same principles, centrifuges are of various type which is according to what type of sample used; what type of conditions are required during centrifugation, or depending on whether it require high or low speed, etc.

4. Mention few the applications of centrifugation.

Ans: Few applications of centrifugation are- clarification of slurry, concentration, recovery of product, to separate lipid components like chylomicrons from other components of plasma, separation of sub cellular organelles, RNA, DNA and determination of purity and shape of the biomolecules.

5. What is differential centrifugation?

Ans: Differential centrifugation consists of successive centrifugation at increasing rotor speeds. The large and small particles in the suspension can be separated by centrifugation at different speeds. Thus differential centrifugation is used to fractionate cell homogenates into their components.

6. What is the difference between zonal and isopycnic centrifugation?

Ans: The difference between zonal and isopycnic centrifugation is that in zonal centrifugation, a density gradient is already created in the tube with a suitable medium, having high density at the bottom. Whereas in Isopycnic centrifugation, the biological sample and the medium is uniformly distributed in the tube and then rotated in an ultracentrifuge. But under the influence of centrifugal force, selected medium redistributes to form density gradient from top to bottom.

So, in Zonal centrifugation the separation is depending on size and in Isopycnic centrifugation the separation is depending on density.