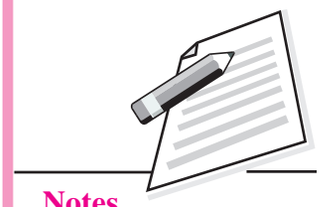


28

CENTRIFUGATION

28.1 INTRODUCTION

A **centrifuge** is the equipment generally driven by an electric motor that puts an object to rotate around fixed axis, and a perpendicular force is applied to axis. The particles get separated according to their size, shape, density, viscosity of the medium and rotor speed.

OBJECIVES

After reading this lesson, you will be able to

- describe centrifugation and its principle
- describe centrifugal force
- practice safety measures in the laboratory

28.2 PRINCIPLE

The centrifuge involves principle of sedimentation, where the acceleration at centripetal force causes denser substances to separate out along the radial direction at the bottom of the tube. By the same concept lighter objects will tend to move to the top of the tube; in the rotating picture, move to the center. In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top. The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady. To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful "centrifugal force" provided by a centrifuge.

What happens to a particle (macromolecule) in a centrifugal field?

Consider a particle m in a centrifuge tube filled with a liquid.

The particle (m) is acted on by three forces:

F_C: the centrifugal force

F_B: the buoyant force

F_f: the frictional force between the particle and the liquid

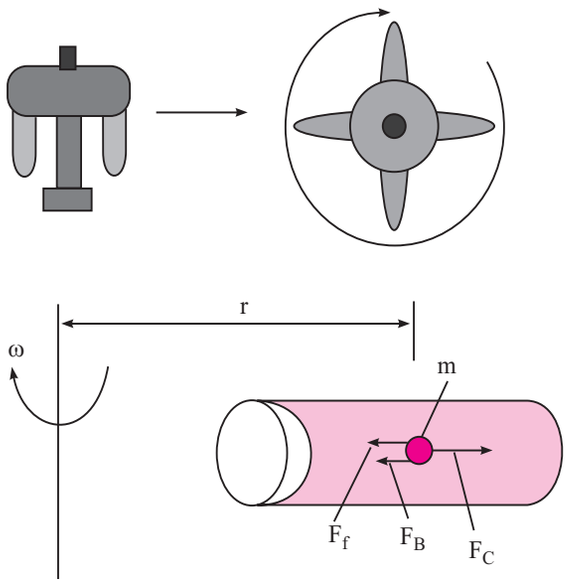


Fig. 28.1: Centrifugal field

28.3 SEDIMENTATION PRINCIPLE

Sedimentation is the tendency for particles in suspension to settle out of the fluid in which they are entrained, and come to rest against a barrier. This is due to their motion through the fluid in response to the forces acting on them: these forces can be due to gravity, centrifugal acceleration or electromagnetism. **Settling** is the falling of suspended particles through the liquid, whereas sedimentation is the termination of the settling process.

28.3.1 Svedberg Equation

The single most important advance 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. They initiated the mathematics and advanced the instrumentation to a point where it was possible to prove that proteins were large molecules that could be weighed in a centrifuge. 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.

The instrumentation has progressed quite far since the early work of Svedberg and Williams. Today, any technique employing the quantitative application of centrifugal force is known as ultracentrifugation. The design of the basic instruments, the rotors and the optical systems for measurement are too extensive to cover in this volume. For our purposes, we will concentrate on two types of rotor, and a few selected parameters to be measured.

Calculation of S:

$$S = \frac{v}{\omega^2 r} = \frac{M(1-\bar{v}\rho_{sol})}{N_{AV} f}$$

M = molecular weight ($m \times N_{AV}$)

s = svedberg coefficient

vρ = partial specific volume of the molecule

N = Avogadro's number

f = frictional coefficient

s = sedimentation coefficient (units: 1 Svedberg = 10-13 sec)

The above equation depends on the size of the molecule (M), however the shape of the molecule plays an important role in its behavior under centrifugal force so it is appropriate to take this (f) into account.

This is the Svedberg equation and is used to describe the motion of the particle in terms of molecular weight (a size term) and frictional coefficient (a shape term). The equation also relates the motion to the solvent density.

The Svedberg coefficients are not additive. That is, 40S plus 60S does not equal 100S. This is the case for the ribosomal subunits, where the combination of a 40S small subunit and a 60S large subunit produces an 80S complete ribosome.

28.4 CENTRIFUGAL FORCE

Centrifugal force, word from Latin ***centrum***, meaning "center", and ***fugere***, means "to flee", is the apparent force that draws a rotating body away from the center of rotation. It is caused by the inertia of the body as the body's path is continually redirected. In Newtonian mechanics, the term *centrifugal force* is used to refer to one of two distinct concepts: an inertial force (also called a "fictitious" force) observed in a non-inertial reference frame, and a reaction force corresponding to a centripetal force.

Notes

The term is also sometimes used in Lagrangian mechanics to describe certain terms in the generalized force that depend on the choice of generalized coordinates.

The concept of centrifugal force is applied in rotating devices such as centrifuges, centrifugal pumps. The two different forces are equal in magnitude, but centrifugal forces is opposite in direction to the centripetal force.

A centrifuge is used to separate particles or macromolecules:

1. Cells- biological components in tissues and cells are separated by centrifugation and this principle is widely used in biological laboratory, in fact it is one of the most essential instrumentation in design of a laboratory.
2. Sub-cellular components- substances like cytoplasmic fluid, nucleus, mitochondria, golgi bodies are separated by this principle.
   (a) Proteins- based on density protein in cells and tissues is separated using high speed centrifugation.
   (b) Nucleic acids- DNA, RNA, snRNA, etc., are separated by this method.

28.4.1 Basis of separation

- **Size:** size of the particle matters a lot while application of this principle. It has the basis that as much lesser the size will be, more the particle will be towards the base.
- **Shape**: the shape of particle ex- circular particles will settle down easily as compared to polygonal shape particles.
- **Density**: this component is main play of centrifugation principle, denser the object, lower the settling.

INTEXT QUESTIONS 28.1

1. The centrifuge works on the simple working principle of .....
2. What are the two distinct forces that come under the “centrifugal force”?
3. The ....., ... is used to describe the motion of a particle on the basis of molecular weight and frictional coefficient.
4. 1 Svedberg = ... unit .....
5. What is the difference between settling and sedimentation?

28.4.2 Methodology

- Utilizes density difference between the particles/macromolecules and the medium in which these are dispersed
- Dispersed systems are subjected to artificially induced gravitational fields.

28.4.2.1 Principle

Equipment

The acceleration achieved by centrifugation is expressed as a multiple of the earth's gravitational force (g = 9.81 m s^{-2}). Bench-top centrifuges can reach acceleration values of up to 15000 g, while high speed refrigerated centrifuges can reach 50000 g and ultra-centrifuges, which operate with refrigeration and in a vacuum, can reach 500000 g. Two types of rotor are available in high-powered centrifuges: *fixed angle rotors* and *swing-out rotors* that have movable bucket containers. The tubes or buckets used for centrifugation are made of plastic and have to be very precisely adjusted to avoid any imbalances that could lead to accidents.

$$\text{Relative centrifugal force } F = M\ r\ \omega^2$$

M: mass of particle

r: radius of rotation (cm) (ie.distance of particle from axis of rotation)

ω: Average angular velocity (radians/sec), ω = 2π revolutions/60 minutes

Theory

The velocity (v) of particle sedimentation during centrifugation depends on the angular velocity ω of the rotor, its effective radius (ref, the distance from the axis of rotation), and the particle's sedimentation properties. These properties are expressed as the Sedimentation

Coefficient S (1 Svedberg, = 10–13 s). The sedimentation coefficient depends on the mass M of the particle, its shape (expressed as the coefficient of friction, f), and its density (expressed as the reciprocal density v, "partial specific volume"). At the top right, the diagram shows the densities and sedimentation coefficients for biomolecules, cell organelles, and viruses. Proteins and protein-rich structures have densities of around 1.3 g cm^{-3}, while nucleic acids show densities of up to 2 g cm^{-3}. Equilibrium sedimentation of nucleic acids therefore requires high-density media – e.g., concentrated solutions of cesium chloride (CsCl). To allow comparison of S values measured in different media, they are usually corrected to values for water at 20°C ("S20W").

28.5 DENSITY GRADIENT CENTRIFUGATION

Density gradient centrifugation is used to separate macromolecules that differ only slightly in size or density. Two techniques are commonly used. In **zonal centrifugation,** the sample being separated (e. g., a cell extract or cells) is placed on top of the centrifugation solution as a thin layer. During centrifugation, the particles move through the solution due to their greater density.

The rate of movement basically depends on their molecular mass. Centrifugation stops before the particles reach the bottom of the tube. Drilling a hole into the centrifugation tube and allowing the contents to drip out makes it possible to collect the different particles in separate fractions.

During centrifugation, the solution tube is stabilized in the tube by a **density gradient**. This consists of solutions of carbohydrates or colloidal silica gel, the concentration of which increases from the surface of the tube to the bottom. Density gradients prevent the formation of convection currents, which would impair the separation of the particles. **Isopycnic centrifugation**, which takes much longer, starts with a CsCl solution in which the sample material (e. g., DNA, RNA, or viruses) is homogeneously distributed. A density gradient only forms *during* centrifugation, as a result of sedimentation and diffusion processes. Each particle moves to the region corresponding to its own *buoyant density*. Centrifugation stops once equilibrium has been reached. The samples are obtained by fractionation, and their concentration is measured using the appropriate methods.

28.6 MOVING BOUNDARY/ZONE CENTRIFUGATION

In moving boundary (or differential centrifugation), the entire tube is filled with sample and centrifuged. Through centrifugation, one obtains a separation of two particles but any particle in the mixture may end up in the supernatant or in the pellet or it may be distributed in both fractions, depending upon it size, shape,

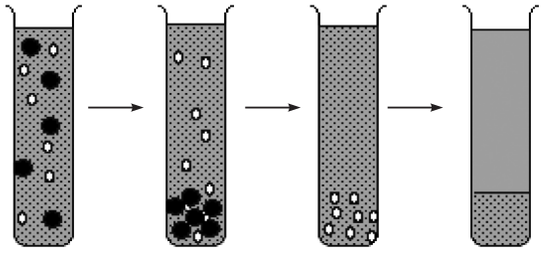


Fig. 28.2: Differential Centrifugation

density, and conditions of centrifugation. The pellet is a mixture of all of the sedimented components, and it is contaminated with whatever unsedimented particles were in the bottom of the tube initially. The only component which is purified is the slowest sedimenting one, but its yield is often very low. The two fractions are recovered by decanting the supernatant solution from the pellet. The supernatant can be recentrifuged at higher speed to obtain further purification, with the formation of a new pellet and supernatant.

28.7 RATE ZONAL CENTRIFUGATION

Particles of the same size (M) but different shapes (e.g., linear versus globular) will separate - the particle with the greater frictional coefficient (f) will move slower (rod shaped moves slower than globular). This technique is called velocity gradient centrifugation (a gradient of sucrose is used to linearize the motion of the particles).

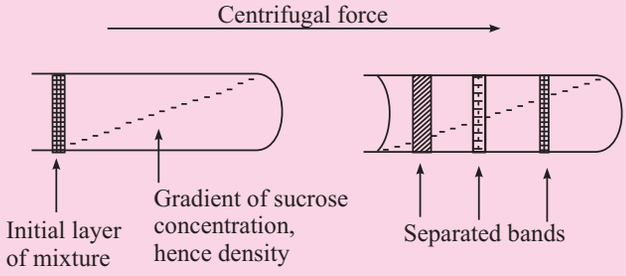


Fig. 28.3: Velocity Gradient Centrifugation

In rate zonal centrifugation, the sample is applied in a thin zone at the top of the centrifuge tube on a density gradient. Under centrifugal force, the particles will begin sedimenting through the gradient in separate zones according to their size shape and density. The run must be terminated before any of the separated particles reach the bottom of the tube.

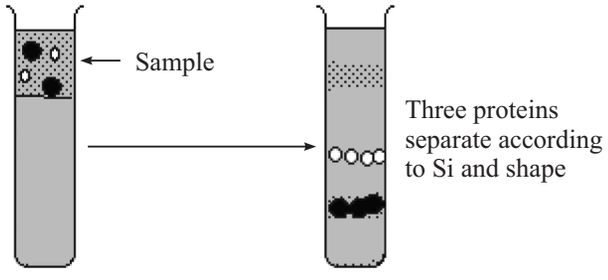


Fig. 28.4: Rate Zonal Centrifugation

Particles can be separated by density. When the density in the solvent equals the density of the particle, the denominator of the equation equals zero and therefore

velocity equals zero - the particle reaches its equilibrium density in the solvent this is called equilibrium density gradient centrifugation or isopycnic banding.

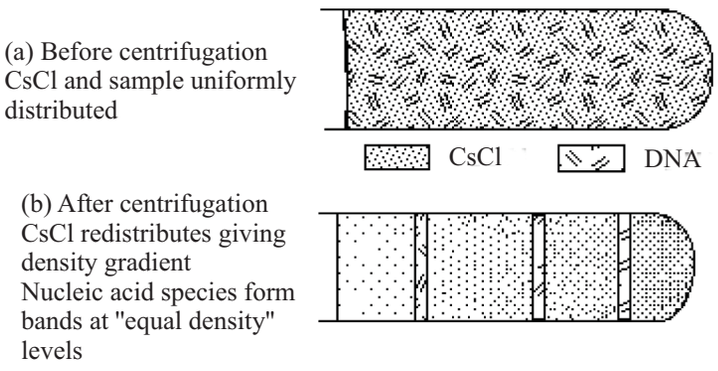


Fig.28.5: Before and After Centrifugation

28.8 ISOPYCNIC CENTRIFUGATION

In isopycnic technique, the density gradient column encompasses the whole range of densities of the sample particles. The sample is uniformly mixed with the gradient material. Each particle will sediment only to the position in the centrifuge tube at which the gradient density is equal to its own density, and there it will remain. The isopycnic technique, therefore, separate particles into separate zones solely on the basis of their density differences, independent of time. In many density gradient experiments, particles of both the rate zonal and the isopycnic principles may enter into the final separations. For example, the gradient may be of such a density range that one component sediments to its density in the tube and remains there, while another component sediments to the bottom of the tube. The self generating gradient technique often requires long hours of centrifugation.

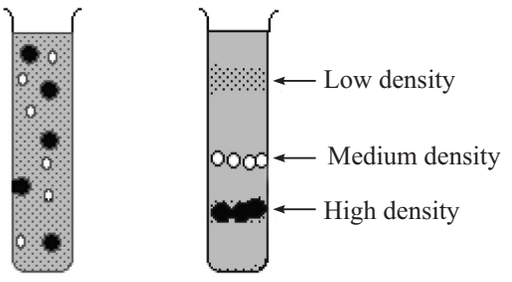
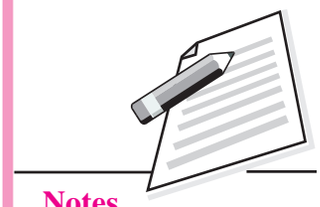


Fig. 28.6: Isopycnic separation with self-generating gradient - the sample is evenly distributed throughout the centrifuge tube

Isopycnically banding DNA, for example, takes 36 to 48 hours in a self-generating cesium chloride gradient. It is important to note that the run time



cannot be shortened by increasing the rotor speed; this only results in changing the position of the zones in the tube since the gradient material will redistribute farther down the tube under greater centrifugal force.

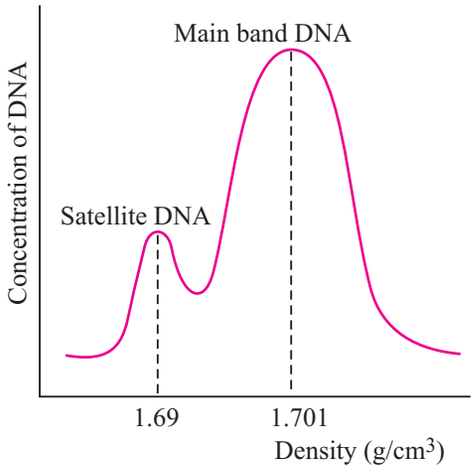


Fig. 28.7: Isopycnic banding of DNA

INTEXT QUESTIONS 28.2

1. The relative centrifugal force, F = ...
2. What are the two types of rotors found in high-powered centrifuges?
3. Write the type of centrifugation based on the given characteristics:
   - separate particles into separate zones solely on the basis of their density differences, independent of time .....
   - particles with same size but different shapes separate .....
   - separate macromolecules that differ only slightly in size or density .....
4. True/False.
   - Moving boundary centrifugation is also called differential centrifugation
5. Equilibrium density gradient centrifugation is also called as .....

28.9 SAFETY MEASURES

28.9.1 Safety while using centrifuge

1. The work surface must be level and firm. Do not use the centrifuge on an uneven or slanted work surface.

Notes

2. Balance the tubes in the rotor! If you want to run a tube with 10 ml of liquid, put another tube with 10 ml of water in the opposing hole on the rotor. If the liquid has a higher or lower density than water, you must balance the tubes by mass, not volume.
3. Do not open the lid while the rotor is moving. Even though many centrifuges have a "safety shutoff" if the lid is opened, the only thing this does is stop powering the rotor. The rotor will still spin due to its own inertia for a while until friction slows and eventually stops it.
4. If you see it wobbling or shaking, turn it off or pull the plug. A little vibration is normal, but excessive amounts can mean danger. FIRST, double check that you have correctly balanced the tubes. If the answer is yes and the wobbling still happens, contact the manufacturer or dealer and get the unit serviced. Do NOT continue to run a centrifuge that wobbles visibly when the rotor is spinning.
5. Wear a face shield and / or safety goggles if you have to work anywhere near a centrifuge that's in use.
6. Do not bump, jar, or move the centrifuge while the rotor is spinning. Make sure you don't have the cord dangling from a table edge where someone could catch their foot in it and pull down the centrifuge.

28.9.2 Precautions – working with bio-hazardous materials

The following procedures for centrifugation shall be used when working with bio-hazardous materials:

- Examine tubes and bottles for cracks or stress marks before using them. Discard any centrifuge tubes that have cracks in them.
- When working with bio-hazardous materials, wipe outside of tubes with disinfectant prior to removal from the biological safety cabinet and before placing in safety cups or rotors.
- Place all tubes in safety buckets or sealed rotors when centrifuging infectious materials.
- Inspect the "O" ring seal of the safety bucket and the inside of safety buckets or rotors. Open safety buckets or rotors in a biological safety cabinet.
- If any spills or leakage are apparent in the centrifuge rotor should be cleaned with a mild detergent, rinsed thoroughly with distilled water, and allowed to air dry completely (while in bio-safety cabinet).
- Clean the rotor and centrifuge well after each use.

28.9.3 Maintenance of Centrifuge

- Quality control
- Initial installation
- Initial calibration should be performed only by a qualified service technician.

28.9.3.1 Daily maintenance

- Wipe the inside of the bowl with disinfectant solution and rinse thoroughly.
- The centrifuge must not be used if the interior is hot, if unusual vibrations or noises occur, or if deterioration (corrosion of parts) is detected. A qualified service technician should be contacted.
- Most vibrations are due to improper balancing and can be corrected by re-balancing the buckets and tubes.

28.9.3.2 Monthly maintenance

- Clean the centrifuge housing, rotor chamber, rotors and rotor accessories with a neutral cleaning agent.
- Clean plastic and non-metal parts with a fresh solution of 5% sodium hypochlorite (bleach) mixed 1:10 with water (one part bleach plus nine parts water).

28.9.3.3 Annual maintenance

- The centrifuge must be serviced annually by a qualified service technician who must ensure that the unit operates safely and properly.
- The service should include cleaning condenser coils, fans, screens and filters, checking the centrifuge brushes, bearings, timer, temperature and speed, and checking for electrical integrity.
- The service technician must issue an inspection certificate indicating compliance with safety and proper operation. The most recent inspection certificate must be displayed close to the centrifuge.

28.9.3.4 Rotor safety

- It is an unavoidable fact that rotors age and become fatigued with repeated use. For the highest speed centrifuges, ultracentrifuges, rotors must be "**derated**" (their maximum rpm reduced gradually over time) and then eventually retired after a period of continuous use. This is due to the high stress on the titanium or aluminum from the ultra high gravitational forces, which will eventually lead to failure. Further, equipment that lasts 10–15 years can have multiple users, and rotors are often passed down over the life of the centrifuge. It is therefore important to manage all the rotors;

Notes

modern centrifuges have many built-in features that assist in this process, even for multiple user environments:

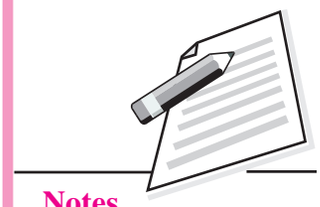
1. Rotor-life management allows end users to manage ultracentrifuge usage by rotor serial number, total number of hour's used or total number of cycles. This enables the centrifuge to alert the end user when it is time to derate or retire a rotor. Furthermore, once a serial number is denoted as derated, the centrifuge will not allow the rotor to be used in excess of the new reduced speed.
2. Automatic rotor ID prevents the running of non specified rotors in a given centrifuge. Rotor ID is achieved in a variety of ways, such as a magnet configuration on the bottom of the rotor, which is read by a sensor in the bottom of the chamber. Sometimes rotors are identified by inertia readings taken during acceleration, which compare the amount of energy being used to turn the rotor by the motor to the on-board database, which is programmed with the correct amount of energy required for all rotors usable in that unit.
3. Over speed protection and rotor ID are closely related. During an inertia check, the on-board system confirms that the programmed speed does not exceed the maximum rpm for the specified rotor. In the case of ultracentrifugation, speed disks on the bottom of the rotor are read by an optical eye, which limits the maximum speed by the number of black segments on the disk.
4. Rotor inspection/clinics can be conducted in the laboratory by most service technicians. Trained service technicians can inspect and educate laboratory staff on warning signs to look for that indicates imminent rotor failure.

INTEXT QUESTIONS 28.3

1. What is the meaning of the word "derated"?
2. True/False.
   - The work surface must not be level and firm.
   - Automatic rotor ID prevents the running of non specified rotors in a given centrifuge.
3. Match the following.

1. Daily maintenance	(a) Qualified service technician
2. Monthly maintenance	(b) Derated
3. Annual maintenance	(c) Disinfectant solution
4. Rotor safety	(d) Nueral cleaning agent

4. ..... and ..... are closely related in rotor safety



WHAT YOU HAVE LEARNT

- Centrifuge plays a vital role in the separation of biomolecules
- The techniques are very simple but has its role in advanced studies
- Different types of centrifuges are available for different purposes

ANSWERS TO INTEXT QUESTIONS

28.1

1. Sedimentation
2. • Inertial force
   • Reaction force
3. Svedberg equation
4. 10 -13 ; Unit – seconds
5. Settling is the falling of suspended particles through the liquid, whereas sedimentation is the termination of the settling process.

28.2

1. $F = Mr^2$
2. Fixed angle rotors and Swing-out rotors
3. • Isopycnic centrifugation
   • Rate zonal centrifugation
   • Density gradient centrifugation
4. True
5. Isopycnic banding

28.3

1. The meaning of the word "derated" is the rotor's maximum rpm reduces gradually over time.
2. • False • True
3. 1. (c) 2. (d) 3. (a) 4. (b)
4. Over speed protection and rotor ID