MODULE V

CENTRIFUGATION

- **A centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed.**

- **It is composed of a metal rotor with holes in it to accommodate a vessel of liquid and a motor or other means of spinning the rotor at a selected speed.**

PRINCIPLES OF CENTRIFUGATION

- **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 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 surface.

- **The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady.**

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- **More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis.**

- **The rate of centrifugation is specified by the angular velocity measured in revolutions per minute (RPM), or acceleration expressed as *g*.**

- **The conversion factor between RPM and *g* depends on the radius of the sample in the centrifuge rotor.**

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.

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.

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

$v\rho$ = 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. i.e, 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.**

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.**

- **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.**

RELATIVE CENTRIFUGAL FORCE

Centrifugation is based on the fact that any object moving in a circle at a steady angular velocity is subject to an outward directed force, *F*. The magnitude of this force depends on the angular velocity in radians, ω, and the radius of rotation, *r*, in centimeters.

$$F = \omega^2 r \qquad (1)$$

F is frequently expressed in terms of the earth's gravitational force and is then referred to as the relative centrifugal force, RCF, or more commonly as the "number times g."

$$\text{RCF} = \frac{\omega^2 r}{980} \tag{2}$$

To be of use, however, these relationships must be expressed in terms of "revolutions per minute," rpm, the common way in which the operating speed of a centrifuge is expressed. Since rpm values may be converted to radians using the equation

$$\omega = \frac{\pi(\text{rpm})}{30} \tag{3}$$

then

$$\mathrm{RCF} = \frac{\frac{(\pi \mathrm{rpm})^2}{30^2}(r)}{980}$$

$$= (1.119 \times 10^{-5})(\mathrm{rpm})^2 r \qquad (4)$$

- **The considerations used to calculate the RCF exerted on a sample in a centrifuge rotor require that the sample be located at a fixed distance r, from the centre of rotation. Owing to rotor design, r varies from top to bottom of the sample holder.**

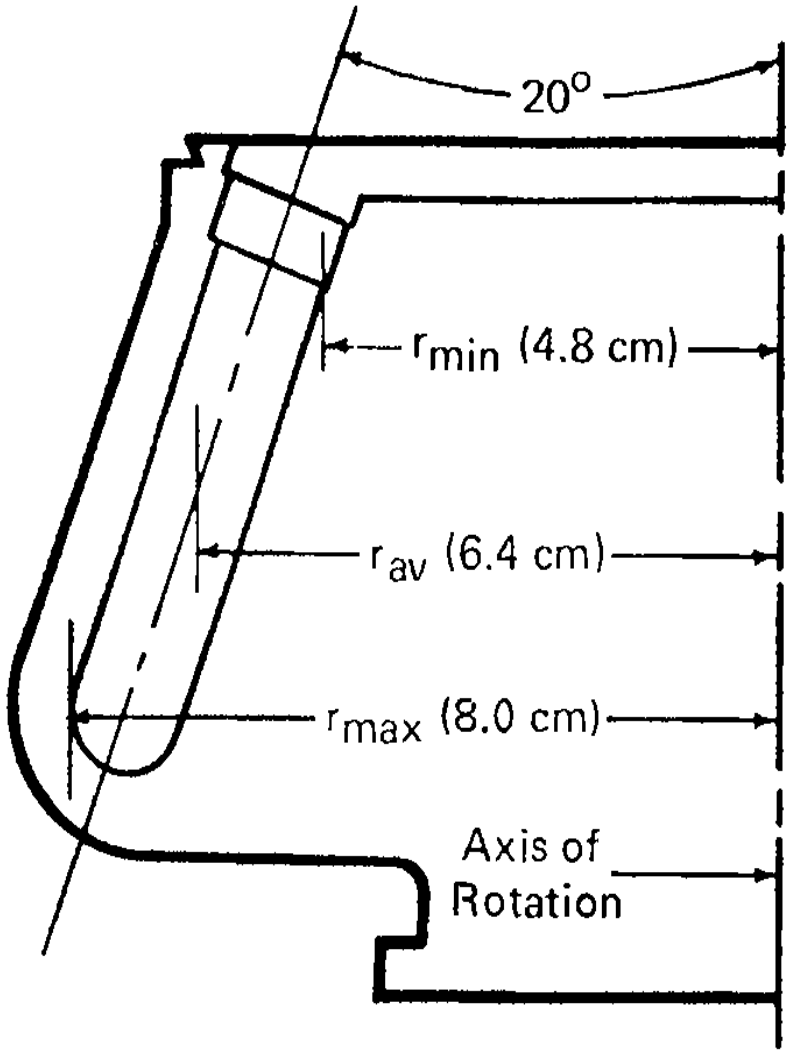


Figure 9-1. Cross-sectional diagram of an angle head rotor showing the distances from the axis of rotation to the top, middle, and bottom of the centrifuge tube. (Courtesy Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.)

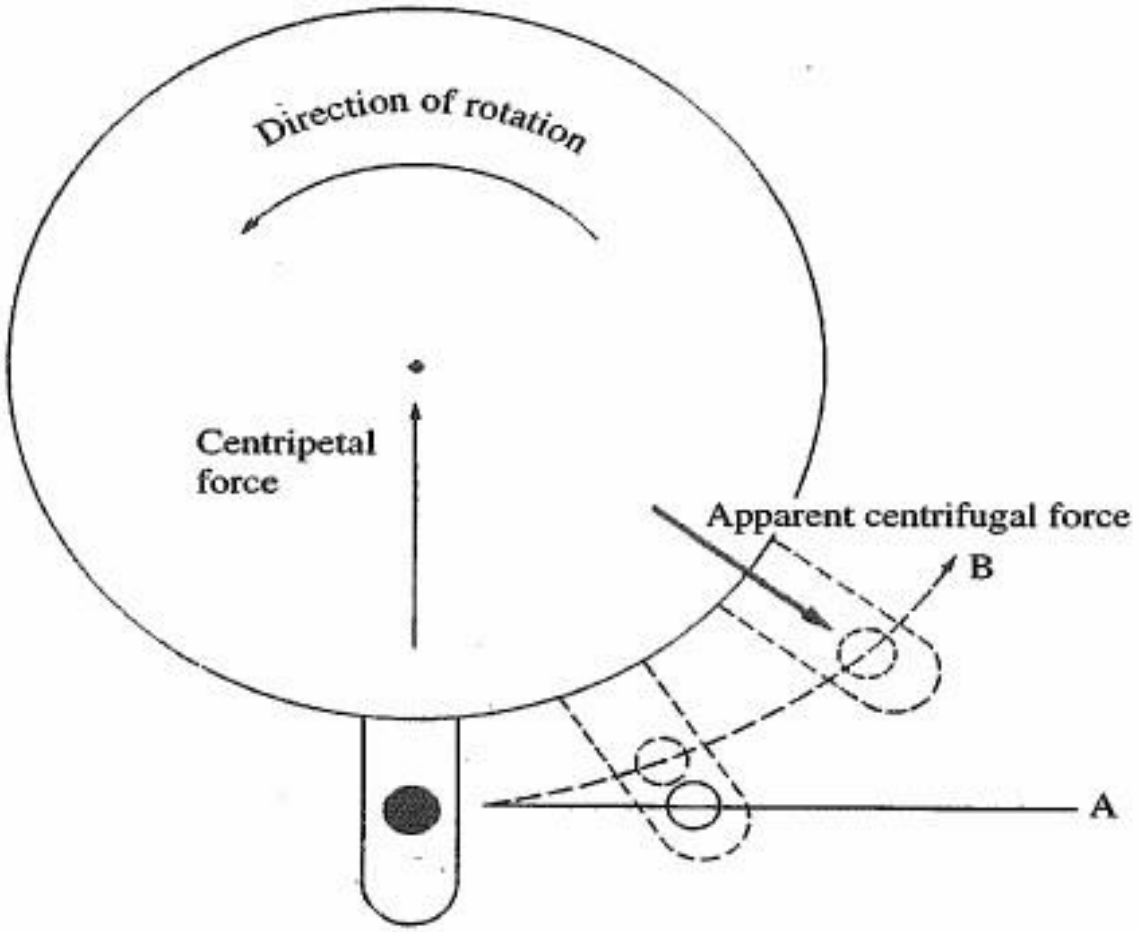


Figure 3.38 Centrifugal effects. 1

- **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.**

Basis of separation

1. **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.

2. **Shape:** the shape of particle ex- circular particles will settle down easily as compared to polygonal shape particles.

3. Density: this component is main play of centrifugation principle, denser the object, lower the settling.

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.**

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 seconds).**

- **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”).**

TYPES OF CENTRIFUGES

1. Microcentrifuges

- Microcentrifuges are used to process small volumes of biological molecules, cells, or nuclei.
- Microcentrifuge tubes generally hold 0.5 - 2 mL of liquid, and are spun at maximum angular speeds of 12000-13000 rpm

- **Micro centrifuges are small enough to fit on a table-top and have rotors that can quickly change speeds.**

- **They may or may not have a refrigeration function.**

DESK TOP CLINICAL CENTRIFUGES

Clinical or desk top centrifuges are the simplest and least expensive centrifuges available. These instruments are most often used to compact or collect small amounts of substances that sediment rapidly (red blood cells, coarse or bulky precipitates, and yeast cells). The maximum speed of most desk model centrifuges is below 3000 rpm and all of them operate at ambient temperature. Although their speed and temperature of operation cannot be closely regulated, they can be used for a wide variety of applications that would otherwise needlessly employ larger and more sophisticated instruments.

2. High-speed centrifuges

- High-speed or super speed centrifuges can handle larger sample volumes, from a few tens of millilitres to several litres.

- Additionally, larger centrifuges can also reach higher angular velocities (around 30000 rpm). The rotors may come with different adapters to hold various sizes of test tubes, bottles, or microtiter plates.

3. Ultracentrifuges

- Ultracentrifugation makes use of high centrifugal force for studying properties of biological particles.

- Compared to microcentrifuges or high-speed centrifuges, ultracentrifuges can isolate much smaller particles, including ribosomes, proteins, and viruses.

- **Ultracentrifuges can also be used in the study of membrane fractionation.**

- **This occurs because ultracentrifuges can reach maximum angular velocities in excess of 70000 rpm.**

- **Additionally, while microcentrifuges and supercentrifuges separate particles in batches (limited volumes of samples must be handled manually in test tubes or bottles),**

ultracentrifuges can separate molecules in batch or continuous flow systems.

- **Two types of ultracentrifuges developed:**

1. **Analytical**

2. **Preparative**

Ultracentrifugation

- Preparative ultracentrifugation
  - Differential centrifugation
  - Density gradient centrifugation
    - Rate zonal centrifugation
    - Isopycnic centrifugation
- Analytical ultracentrifugation

1. Analytical

- Uses small sample size (less than 1 ml)
- Built in optical system to analyze progress of molecules during centrifugation
- Uses relatively pure sample·
- Used to precisely determine sedimentation coefficient and MW of molecules

•Beckman Model E is an example of centrifuge used for these purposes.

2. Preparative

- **Larger sample size can be used**
- **No optical read-out – collect fractions and analyze them after the run**
- **Less pure sample can be used**
- **Can be used to estimate sedimentation coefficient and MW**

- **Generally used to separate organelles and molecules. Most centrifugation work done using preparative ultracentrifuge**

- **Several models available, including L5-65 and L5-75 used for preparative purposes.**

1. Density gradient centrifugation

- **is used to separate macromolecules that differ only slightly in size or density.**
- **A procedure for separating particles such as viruses or ribosomes or molecules such as DNA in which the sample is placed on a preformed gradient such as sucrose or cesium chloride.**

- **Upon centrifugation either by rate zonal or equilibrium procedures, the macromolecules are 'banded' in the gradient and can be collected as a pure fraction.**

- **Two techniques are commonly used.**

1. **zonal centrifugation**
2. **Isopycnic centrifugation**

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).**

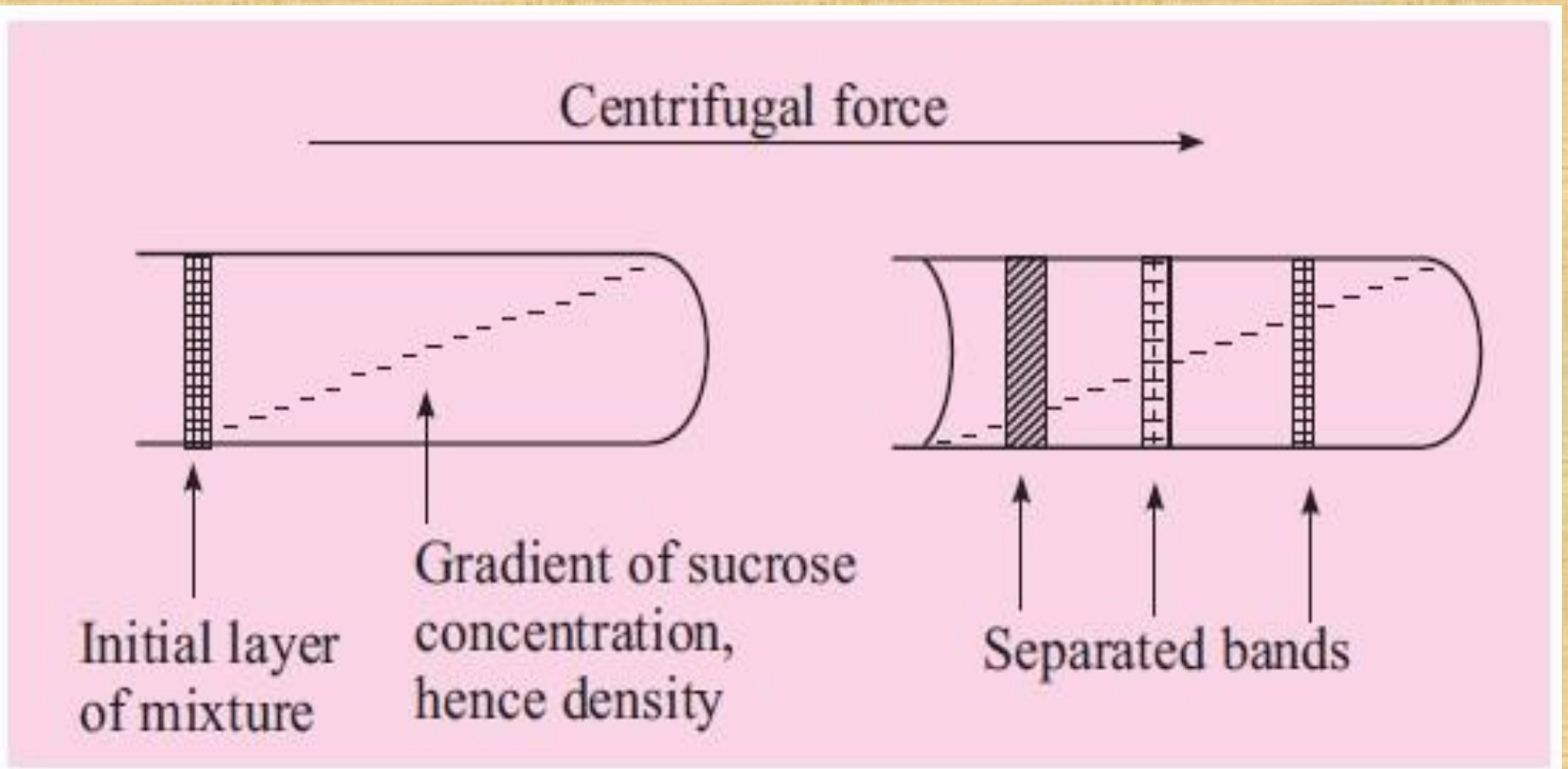


Fig. 28.3: Velocity Gradient Centrifugation

- In 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.

- 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.

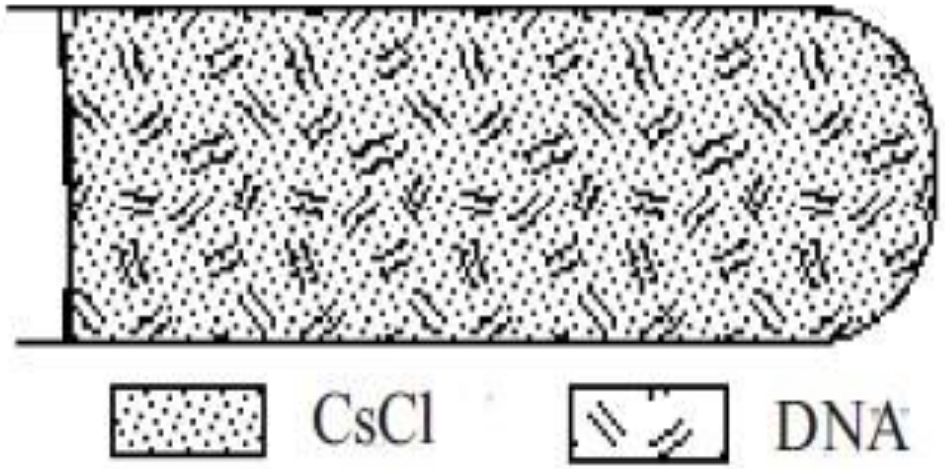
- **Under centrifugal force, the particles will begin sedimenting through the gradient in separate zones according to their size shape and density. Particles reach the bottom of the tube.**

- **The run must be terminated before any of the separated.**

- **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.**

(a) Before centrifugation
CsCl and sample uniformly distributed



(b) After centrifugation
CsCl redistributes giving density gradient
Nucleic acid species form bands at "equal density" levels

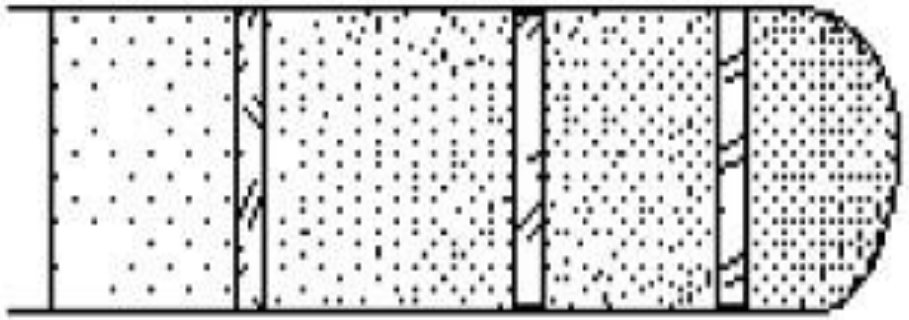


Fig.28.5: Before and After Centrifugation

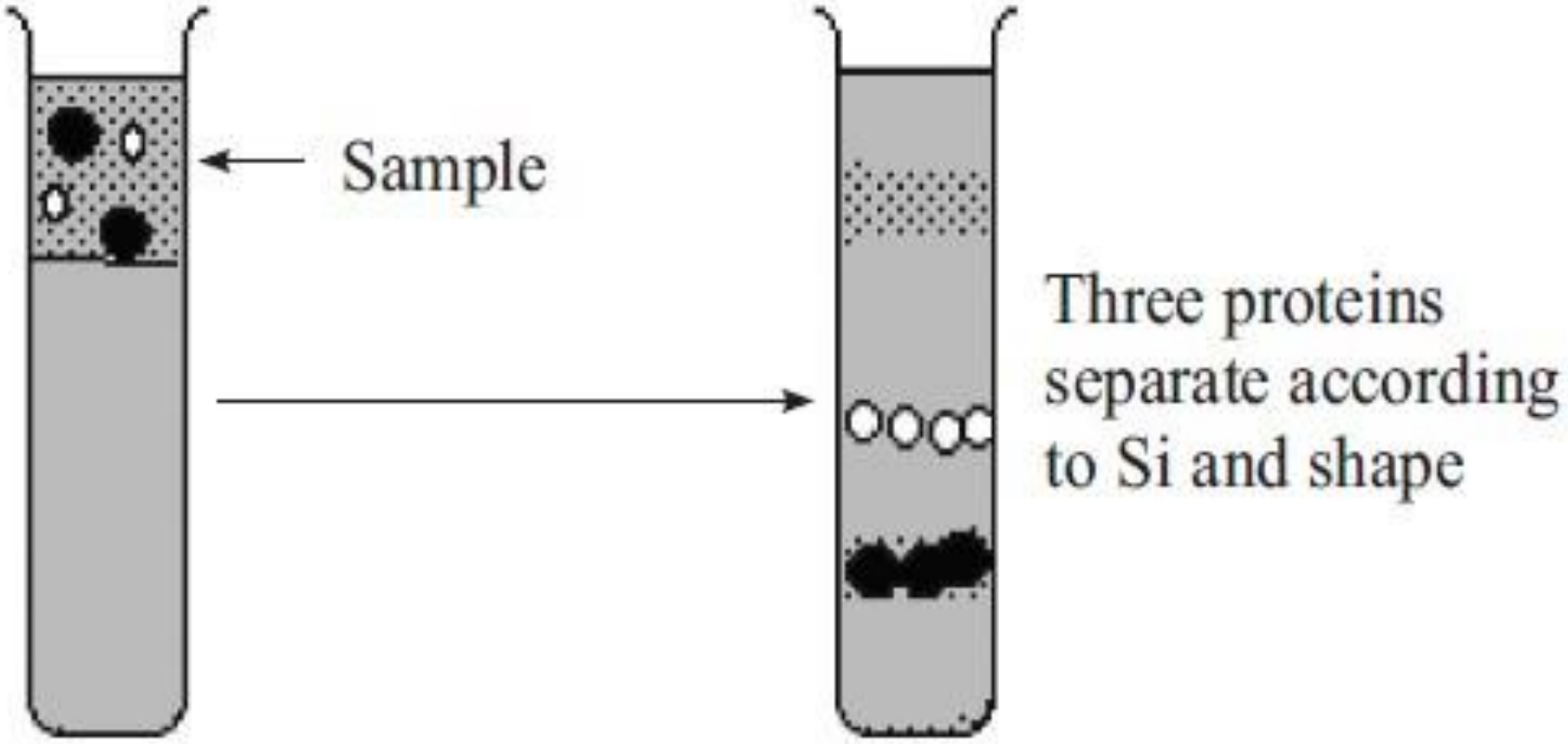


Fig. 28.4: Rate Zonal Centrifugation

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.**

- **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.**

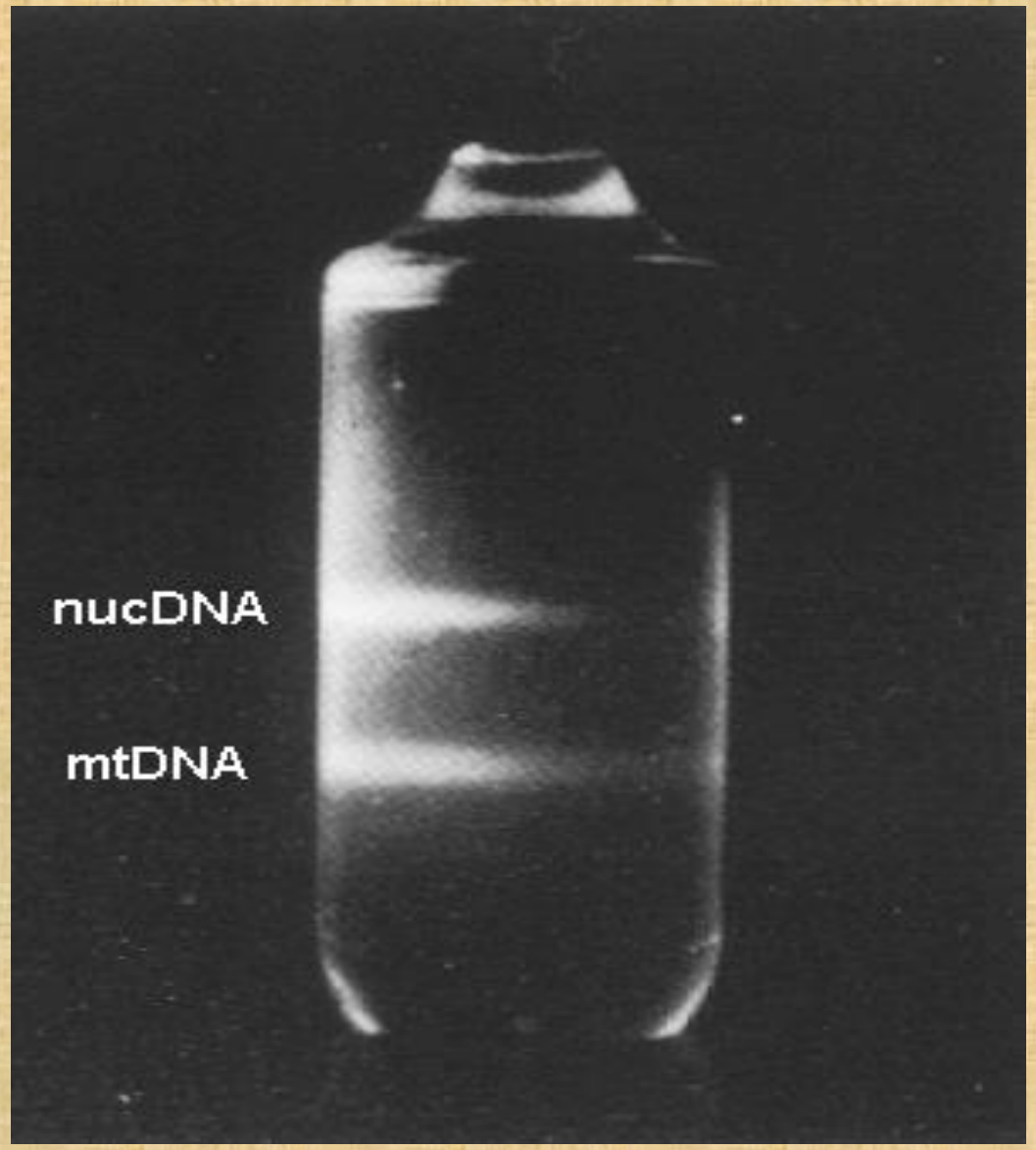
- **A density gradient only forms *during* centrifugation, as a result of sedimentation and diffusion processes.**

- **Each particle moves to the region corresponding to its own *buoyant density*.**

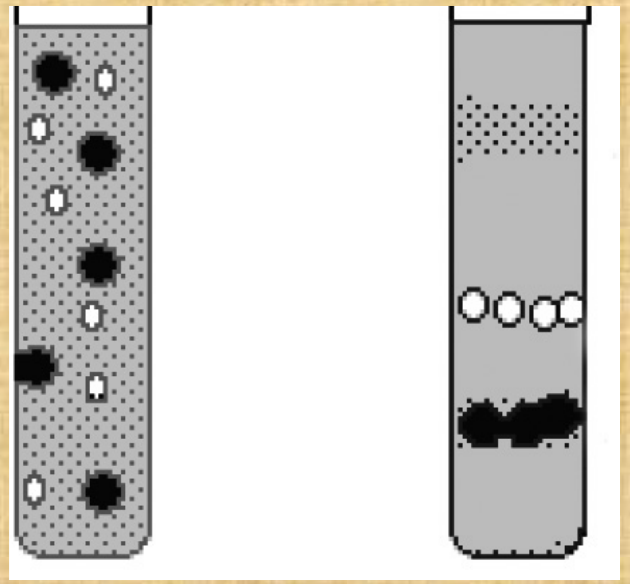
- **Each particle will sediment only to the position in the centrifuge tube at which the gradient density is equal to its own density, and there it will remain.**

- **The isopycnic technique, therefore, separate particles into separate zones solely on the basis of their density differences, independent of time.**

- Centrifugation stops once equilibrium has been reached.
- The samples are obtained by fractionation, and their concentration is measured using the appropriate methods.
- In many density gradient experiments, particles of both the rate zonal and the isopycnic principles may enter into the final separations.



Preparative density-gradient ultracentrifugation of DNA
(SM Carr & OM Griffiths.1987. Biochem Genet 25:385-390)



Low density

Medium density

High density

Isopycnic separation with self-generating gradient - the sample is evenly distributed throughout the centrifuge tube

Rate-Zonal Separation

- In rate-zonal separation, particles are separated based on their size and mass. This means that they migrate through the gradient according to these properties - which allows their separation into distinct zones or bands, if they were layered as a thin zone onto the top of the gradient.

- **This is useful in separating out particles with the same or very similar densities, but different sizes or masses: size is a much stronger discriminator than density in determining where particles migrate to in a given time, as sedimentation rate or velocity of a particle in a gravitational field is directly proportional to the density difference, but depends upon the square of the diameter.**

- **Many proteins and other macromolecules, such as antibodies and virus particles, are isolated in this way.**

- **It is generally the case that rate-zonal separations are dynamic rather than static: that is, if centrifugation is continued long enough, all the zones end up as one pellet at the bottom of the tube.**

Isopycnic Separation

In isopycnic separation the particles migrate through the solvent gradient until they reach the point where their buoyant density is equal to that of the gradient. This is known as the isopycnic point or isodense position. Once the particles have reached their isopycnic point they will no longer move in the gradient, regardless of how much longer the centrifuge is run for.

An example of this type of gradient is a caesium chloride concentration gradient. This is a salt that - for example - has a density of 1.08 g/cm^3 for a 10% (w/v) solution, increasing to 1.58 g/cm^3 for a 50% (w/v) solution. It and other heavy metal salts have the problems of being somewhat toxic and exerting a very strong osmotic pressure, as well as chemically affecting certain macromolecules.

MOVING BOUNDARY/ZONE CENTRIFUGATION/ DIFFERENTIAL CENTRIFUGATION

- In moving boundarycentrifugation, 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, 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.**