TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

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CENTRIFUGATION

INTRODUCTION

WHAT IS CENTRIFUGE?

Centrifuge is a device for separating particles from a solution according to there size, shape, density, viscosity of the medium.

WHAT IS CENTRIFUGATION?

Centrifugation is a process which involves the use of the centrifugal force for the sedimentation of heterogeneous mixtures with a **centrifuge**.

Principle

- The centrifuge involves principle of centrifugation, where the acceleration at centripetal force causes denser substances to separate out along the radial direction at the bottom of the tube.
- 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.

Key Points

- the more dense a biological structure is, the faster it sediments in a centrifugal field;
- the more massive a biological particle is, the faster it moves in a centrifugal field;
- the denser the biological buffer system is, the slower the particle will move in a centrifugal field;
- the greater the frictional coefficient is, the slower a particle will move;
- the greater the centrifugal force is, the faster the particle sediments;
- the sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

- **Centrifugation** is a process which involves the application of the centripetal force for the sedimentation of heterogeneous mixtures with a centrifuge, and is used in industrial and laboratory settings.

- The particles' settling velocity in centrifugation is a function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity.

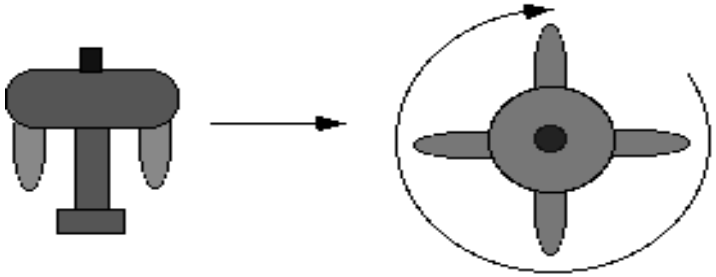
- This process is used to separate two immiscible substances. More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis.

- Chemists and biologists may increase the effective gravitational force on a test tube so as to more rapidly and completely cause the precipitate (pellet) to gather on the bottom of the tube.

- The remaining solution (supernatant) may be discarded with a pipette

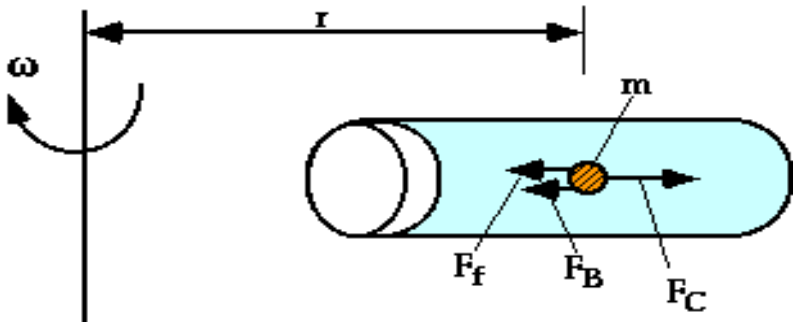
Force on Particle under centrifugation

Equation that describes the motion of this particle as follows:



$$F = ma$$

where m is the mass of the particle and a is the acceleration.

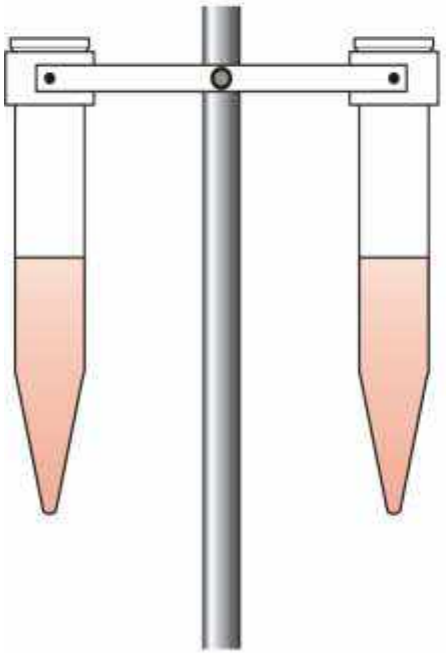


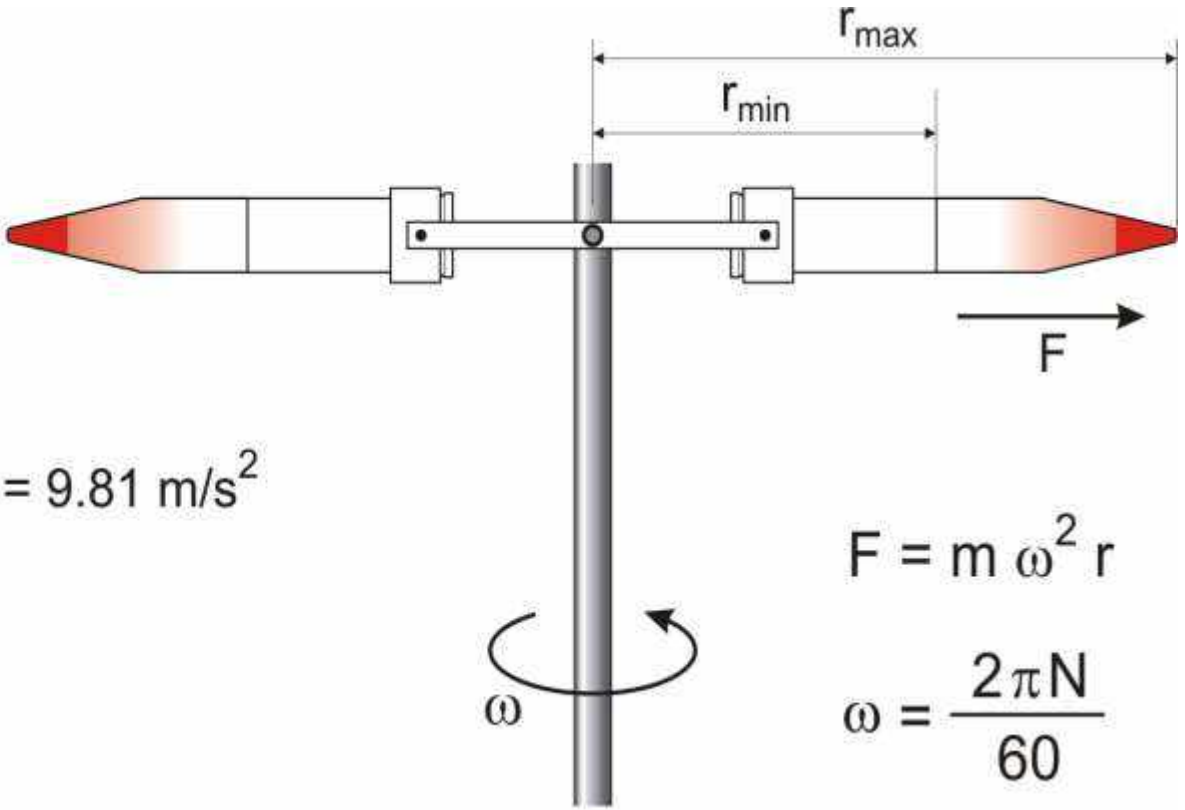
The particle (m) is acted on by three forces:

FC: the centrifugal force

FB: the buoyant force

Ff: the frictional force between the particle and the liquid





$g = 9.81\ m/s^2$

$$F = m\,\omega^2\,r$$

$$\omega = \frac{2\pi N}{60}$$

m = mass of particle

r = distance of particle from axis of rotation

ω = average angular velocity (rad/s)

N = revolution per minute, r.p.m.

Centrifugal force:- **The tube containing the suspension of particles is rotated at a high speed, which exerts a centrifugal force directed from the center of the rotor towards the bottom of the tube.**

Centrifugal Force:

$$F = M\omega^2 r$$

Where,

M: mass of particle

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

ω :Average angular velocity (radians/sec)

Centrifugal field :- **Depends on the radical distance of the particle from the rotation axis and the square of the angular velocity.**

$$G = r\omega^2$$

OR

$$G = \frac{4\pi^2 \left(\text{rev min}^{-1}\right)^2 r}{3600}$$

Angular Velocity:- **Detect to revolution per minute (r.p.m)**

$$\omega = \frac{2\pi \text{ rev min}^{-1}}{60}$$

The rate of centrifugation is specified by the angular velocity usually expressed as revolutions per minute (RPM), or acceleration expressed as *g*. The conversion factor between RPM and g depends on the radius of the centrifuge rotor.

Sedimentation rate:- **This force acts on the suspended particles pushing them towards the bottom of the tube at a rate determined by the velocity of the spinning rotor.**

Rate of Sedimentation:

$$\frac{dr}{dt} = \frac{M(1-\bar{\nu}\rho)}{N_A f}\omega^2 r$$

Where,

r = radius at which the organelle is located

t = time

M = molecular weight

ν = partial specific volume of the molecule; inverse of the density

ρ = density of the solvent

f = translational frictional coefficient

ω = angular velocity

NA = Avagadro's number

Sedimentation coefficient:- **Centrifugation separates particles in a suspension based on differences in size, shape and density that together define their sedimentation coefficient.**

Sedimentation Coefficient:

$$S = \frac{dr}{dt}\,(1/\,\omega^2 r)$$

- **This is know as the Svedberg equation and is usually expressed in Svedberg units,**

  S (= $\frac{\quad}{10^{-13}}$ second).

- **This equation indicates that 'S' is dependent upon the molecular weight, the density and the frictional coefficient.**

Relative Centrifugal Force (RCF)

- RCF, is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity.

Relative Centrifugal force is defined as $$f = M\omega^2 r$$

Where,

- F= intensity of centrifugal force
- M= mass of particle
- ω= angular velocity of rotation
- R= distance of migrating particles from central axis of rotation.

Classification of Centrifuge:

- Speed of sedimentation (Ultra Centrifuge or High Speed Centrifuge).
- Presence /absence of vacuum (ultra centrifuge or small bench top)
- Temperature control refrigeration.
- Volume of sample and capacity of centrifugation tubes

CENTRIFUGATION IN BIOLOGICAL RESEARCH

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. Microcentrifuges 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

High-speed centrifuges

High-speed or superspeed 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.

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

MICRO CENTRIFUGE (“MICROFUGE”, “EPPENDROF”)

- Sample volume is small in eppendrof tubes
- Refrigerated with or without
- Centrifuge maximum approx 10000 g
- Take tube of small volume up to 2ml.
- Commonly used of concentration protein

HIGH SPEED CENTRIFUGE

- Refrigerated
- Use for protein precipitates, large intact organelles cellular debris from tissue homogenization and microorganism
- They operate maximal centrifugal force of approx 50000g
- Use for research applications
- Differential separation of nucleus, mitochondrial, protein precipitate, etc.

ULTRACENTRIFUGE

- Refrigerated and evacuated
- The detail biochemistry analysis of subcellular structures and isolate biomolecules.
- Operate at upto 90000 g

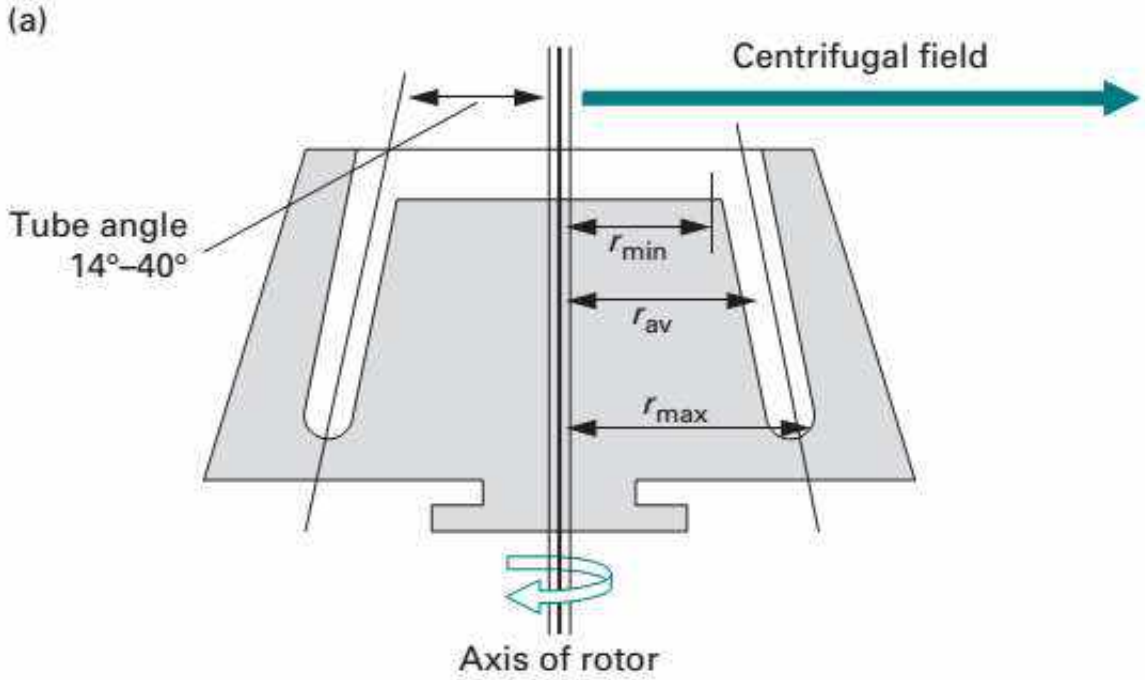
Type of rotor

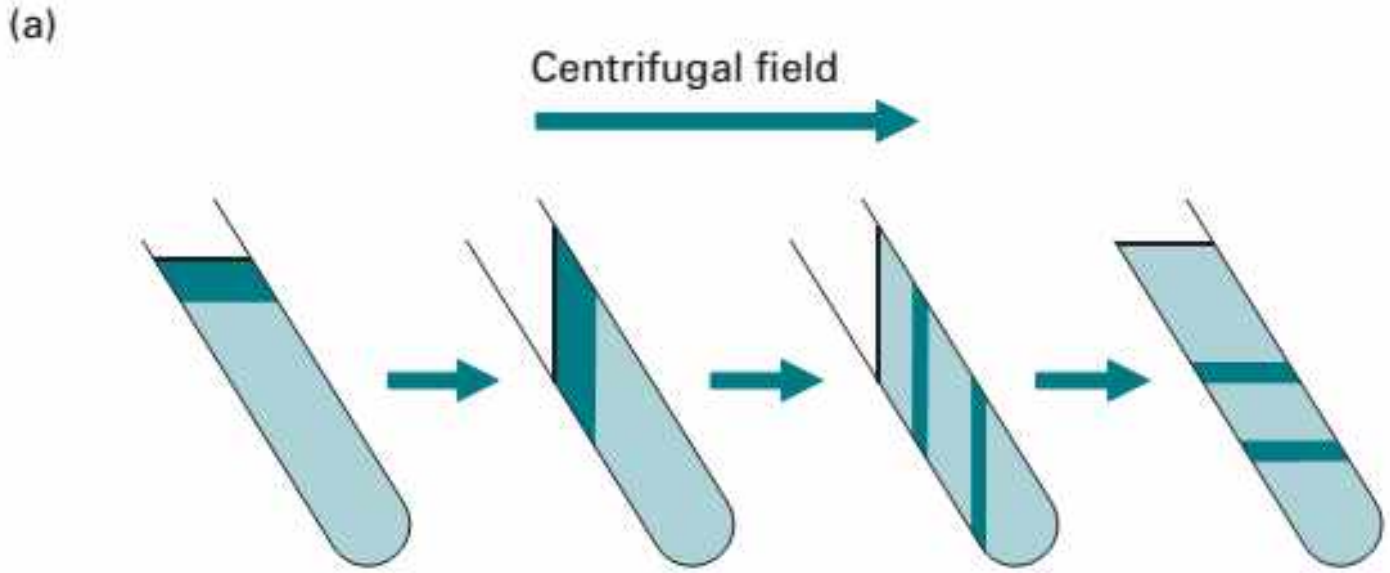
- Fixed angle rotor
- Swinging bucket rotor

Fixed angle rotor

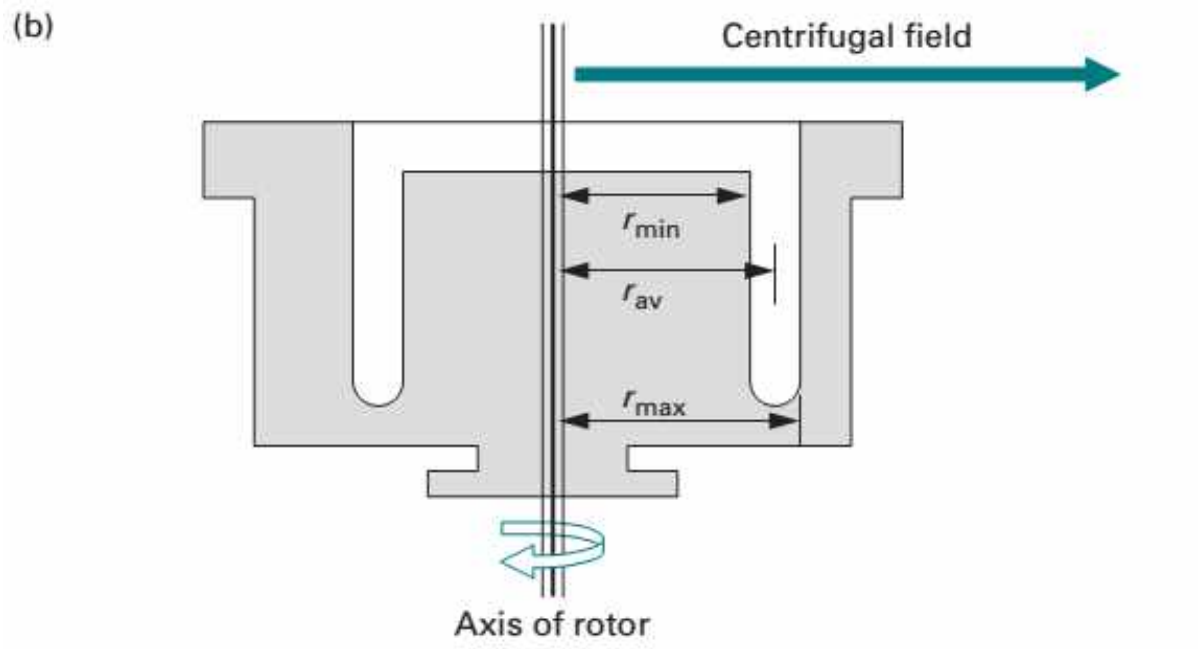
- Ideally tool for pelleting
- Isopycnic banding may form
- Centrifugation tubes are held at at fixed angle of between 14'-40' to vertical axis of rotation.

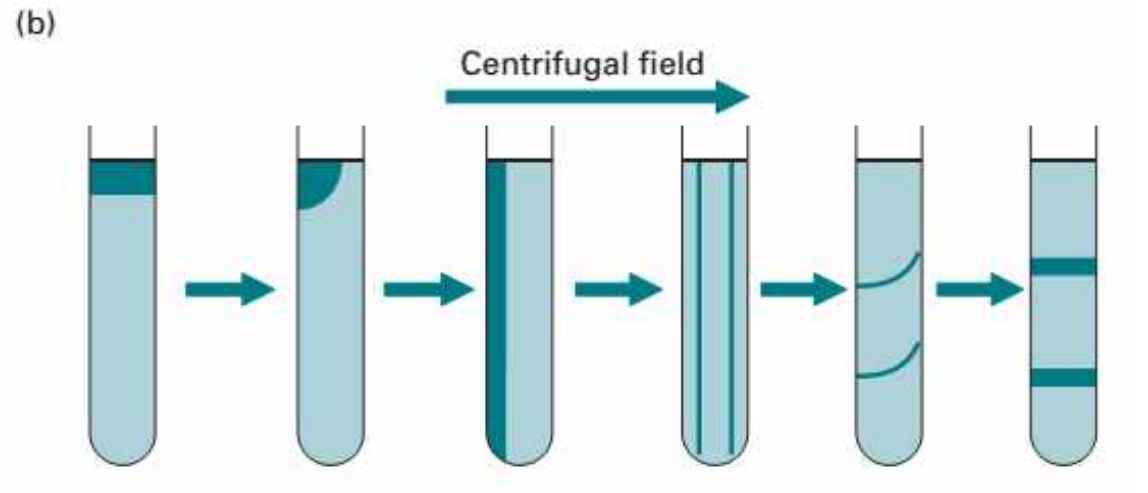
- Start of centrifugation particles are driven outward horizontally but strike side of the tube so that sediment pack against the side & bottom of the tube, with the surface of the sediment parallel to the shaft of centrifuge.


(a)
Centrifugal field
Tube angle
14°–40°
r_{min}
r_{av}
r_{max}
Axis of rotor


(a)
Centrifugal field

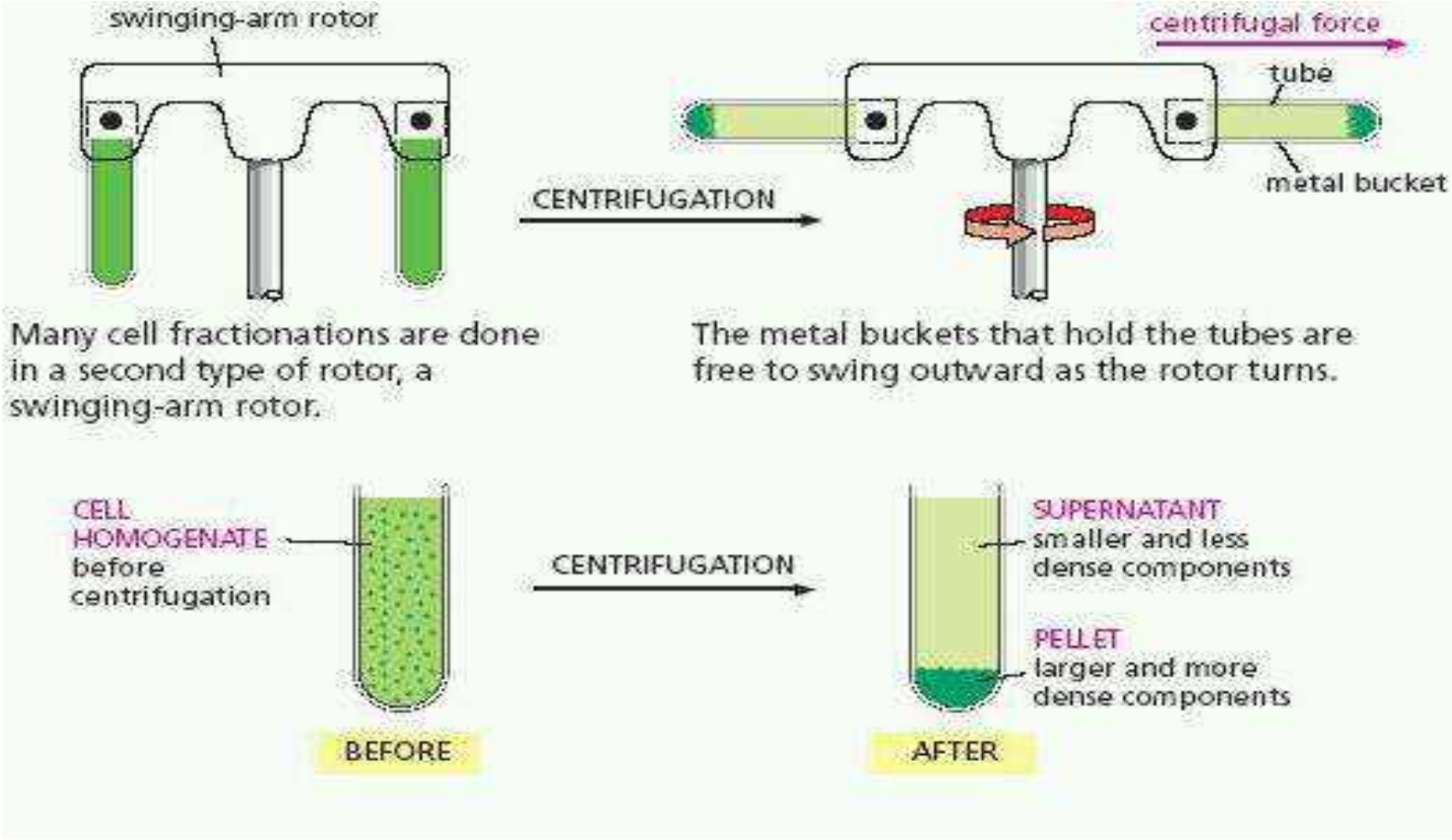
Vertical Tube Rotor



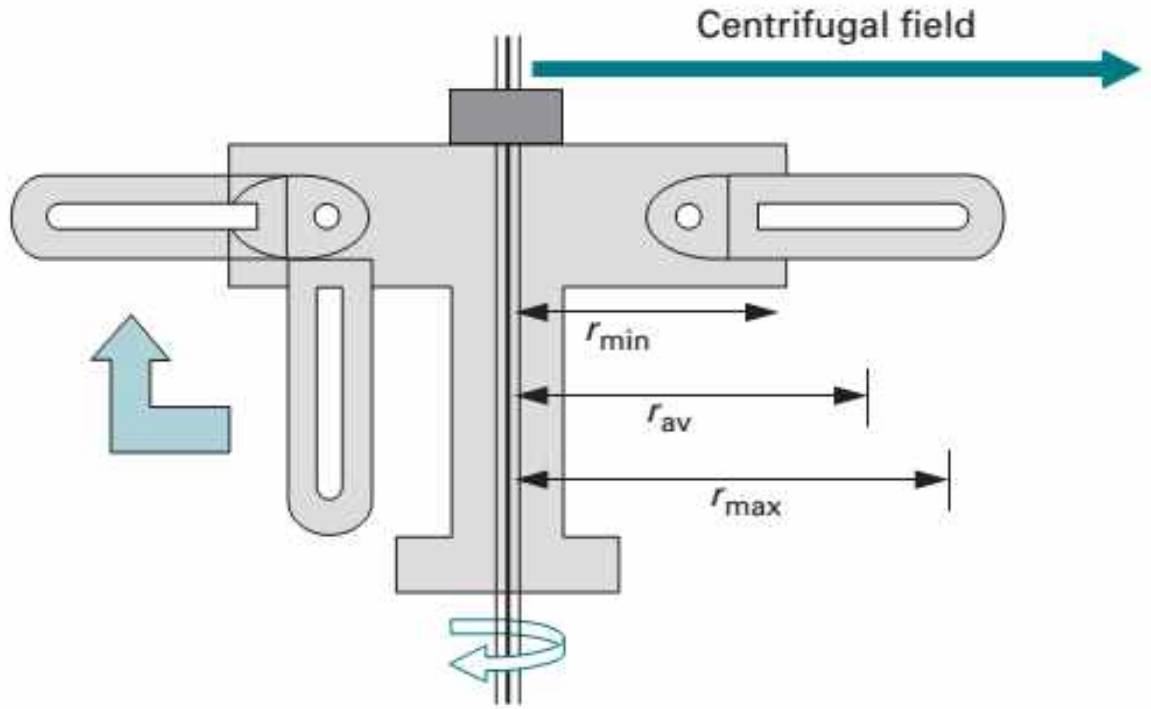


Swinging bucket rotors:

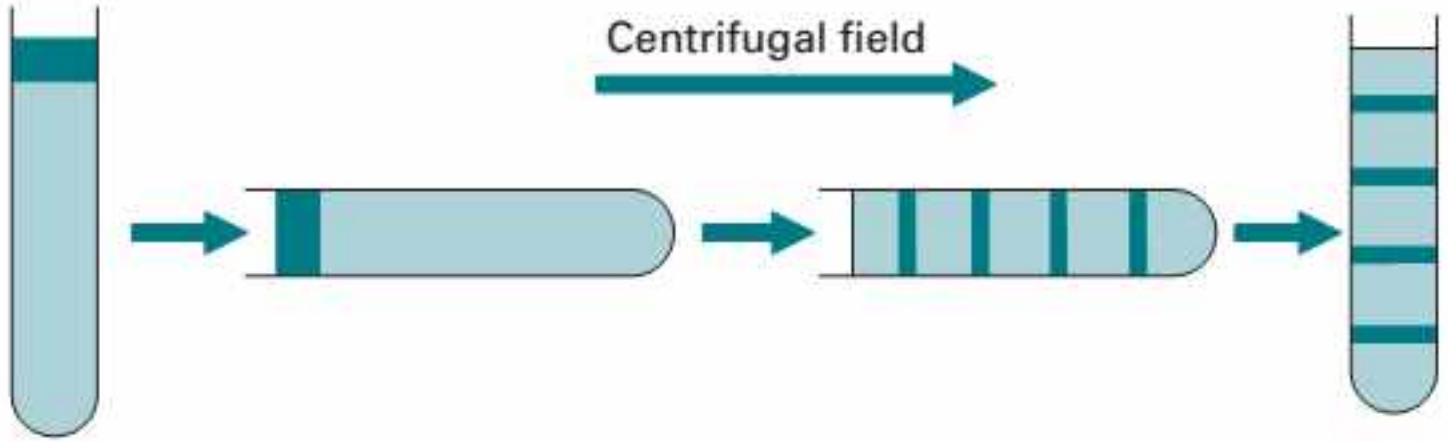
- Sample tubes are loaded into individual buckets that hang vertically while the rotor is at rest.
- When the rotor begins to rotate the buckets swing out to a horizontal position. Useful when samples are to be resolved in density gradients.
- The longer path length permits better separation of individual particle types from a mixture.
- This rotor is relatively inefficient for pelleting .


swinging-arm rotor
centrifugal force
tube
metal bucket
CENTRIFUGATION
Many cell fractionations are done in a second type of rotor, a swinging-arm rotor.
The metal buckets that hold the tubes are free to swing outward as the rotor turns.
CELL HOMOGENATE before centrifugation
CENTRIFUGATION
SUPERNATANT smaller and less dense components
PELLET larger and more dense components
BEFORE
AFTER

(c)


Centrifugal field
r_{min}
r_{av}
r_{max}
Axis of rotor

(c)


Centrifugal field

PREPARATIVE Vs ANALYTICAL CENTRIFUGATION

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.

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.

PREPARATIVE CENTRIFUGATION

Differential centrifugation

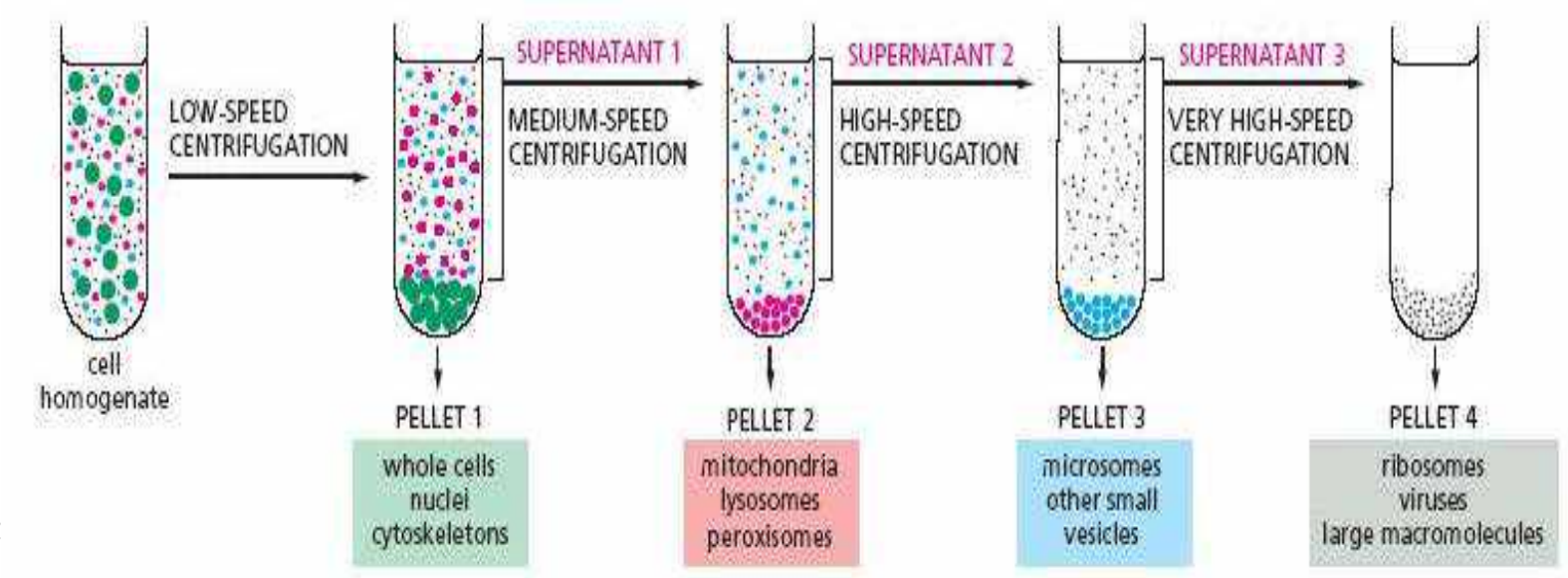
Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density. Crude tissue homogenates containing organelles, membrane vesicles and other structural fragments are divided into different fractions by the stepwise increase of the applied centrifugal field. Following the initial sedimentation of the largest particles of a homogenate (such as cellular debris) by centrifugation, various biological structures or aggregates are separated into pellet and supernatant fractions, depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles. To increase the yield of membrane structures and protein aggregates released, cellular debris pellets are often rehomogenised several times and then recentrifuged. This is especially important in the case of rigid biological structures such as muscular or connective tissues, or in the case of small tissue samples as is the case with human biopsy material or primary cell cultures.

- **Differential centrifugation** is a common procedure in microbiology and cytology used to separate certain organelles from whole cells for further analysis of specific parts of cells.

- In the process, a tissue sample is first homogenised to break the cell membranes and mix up the cell contents.

- The homogenate is then subjected to repeated centrifugations, each time removing the pellet and increasing the centrifugal force.

- Finally, purification may be done through equilibrium sedimentation, and the desired layer is extracted for further analysis



DENSITY GRADIENT CENTRIFUGATION

- It is a type of centrifugation procedure widely used in biochemistry to separate molecules based on their isopycnic point (their buoyant density).

- It is achieved by spinning biological (or other) preparations at high g-force over long periods of time, in buffers or solutions containing a varying amount of a viscous molecule (e.g. 0.8 M/1.2 M sucrose step-gradient used in postsynaptic density isolation or a 20–50% linear sucrose gradient used in the purification of clathrin coated vesicles (CCVs).

- Isopycnic means "of the same density."

Principle

In a suspension of biological particles, the rate of sedimentation is dependent not only upon the applied centrifugal field, but also on the nature of the particle, i.e. its density and radius, and also the viscosity of the surrounding medium. *Stokes' Law* describes these relationships for the sedimentation of a rigid spherical particle:

$$\nu = \frac{2}{9} \frac{r^2(\rho_p - \rho_m)}{\eta} \times g$$

where ν is the sedimentation rate of the sphere, 2/9 is the shape factor constant for a sphere, r is the radius of particle, ρ_p is the density of particle, ρ_m is the density of medium, g is the gravitational acceleration and η is the viscosity of the medium.

Key Point

When density of medium (Pm) became equal to density of particle (Pp), the sedimentation rate "V" become zero in the above given equation and hence so further sedimentation of particle occur and biomolecule get separated on the basis of their density.

Stokes' law

In 1851, George Gabriel Stokes derived an expression, now known as **Stokes' law**, for the frictional force – also called drag force – exerted on sphere/spherical objects.

The force of viscosity on a small sphere moving through a viscous fluid is given by:

$$F_d = 6\pi \mu R V$$

where F_d is the frictional force – known as **Stokes' drag** – acting on the interface between the fluid and the particle, μ is the dynamic viscosity, R is the radius of the spherical object, and V is the flow velocity relative to the object. In SI units, F_d is given in Newtons, μ in Pa·s, R in meters, and V in m/s.

EQUILIBRIUM (ISOPYCNIC) SEDIMENTATION

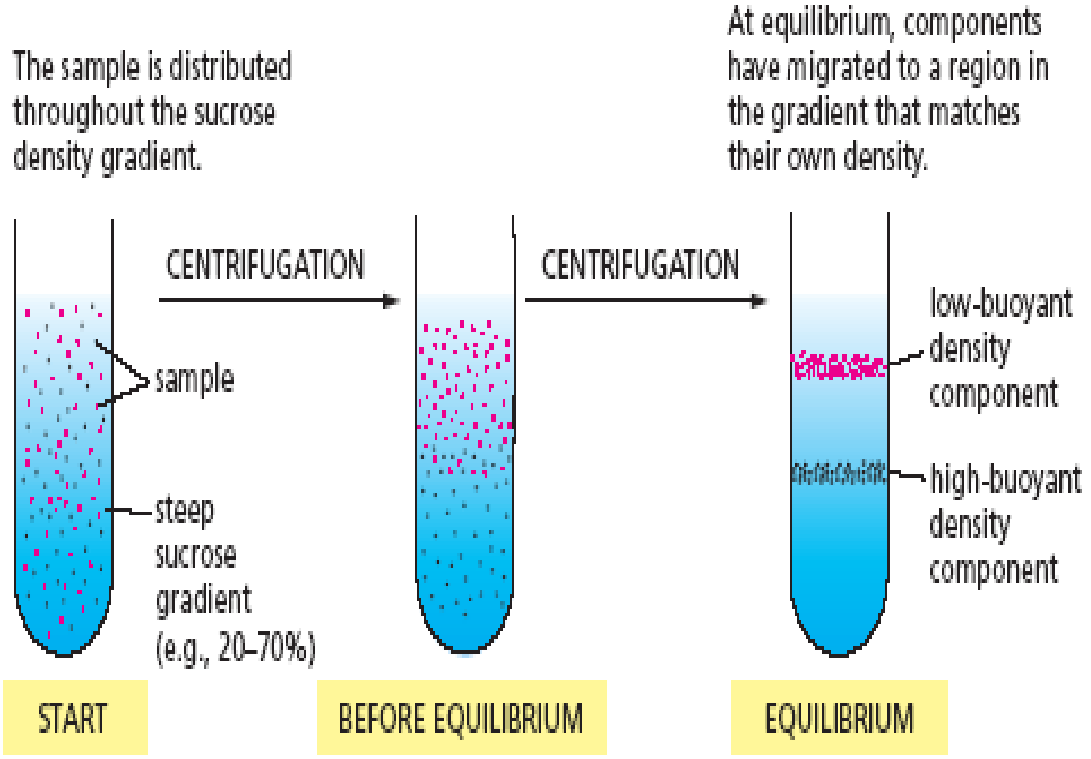
- **Isopycnic centrifugation**, also known as **density gradient centrifugation** or **equilibrium sedimentation** is a technique used to separate molecules on the basis of buoyant density.
- (The word "isopycnic" means "equal density".)
- Typically, a "self-generating" density gradient is established via equilibrium sedimentation, and then analyze molecules concentrated as bands where the molecule density matches that of the surrounding solution.
- A solution is prepared with the densest portion of the gradient at the bottom. Particles to be separated are then added to the gradient and centrifuged.

ISOPYCNIC CENTRIFUGATION

- It is also called as density gradient centrifugation.
- The solution of biological sample and cesium salt is uniformly distributed in a centrifuge tube and rotated in an ultra centrifuge.
- 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.

EQUILIBRIUM SEDIMENTATION

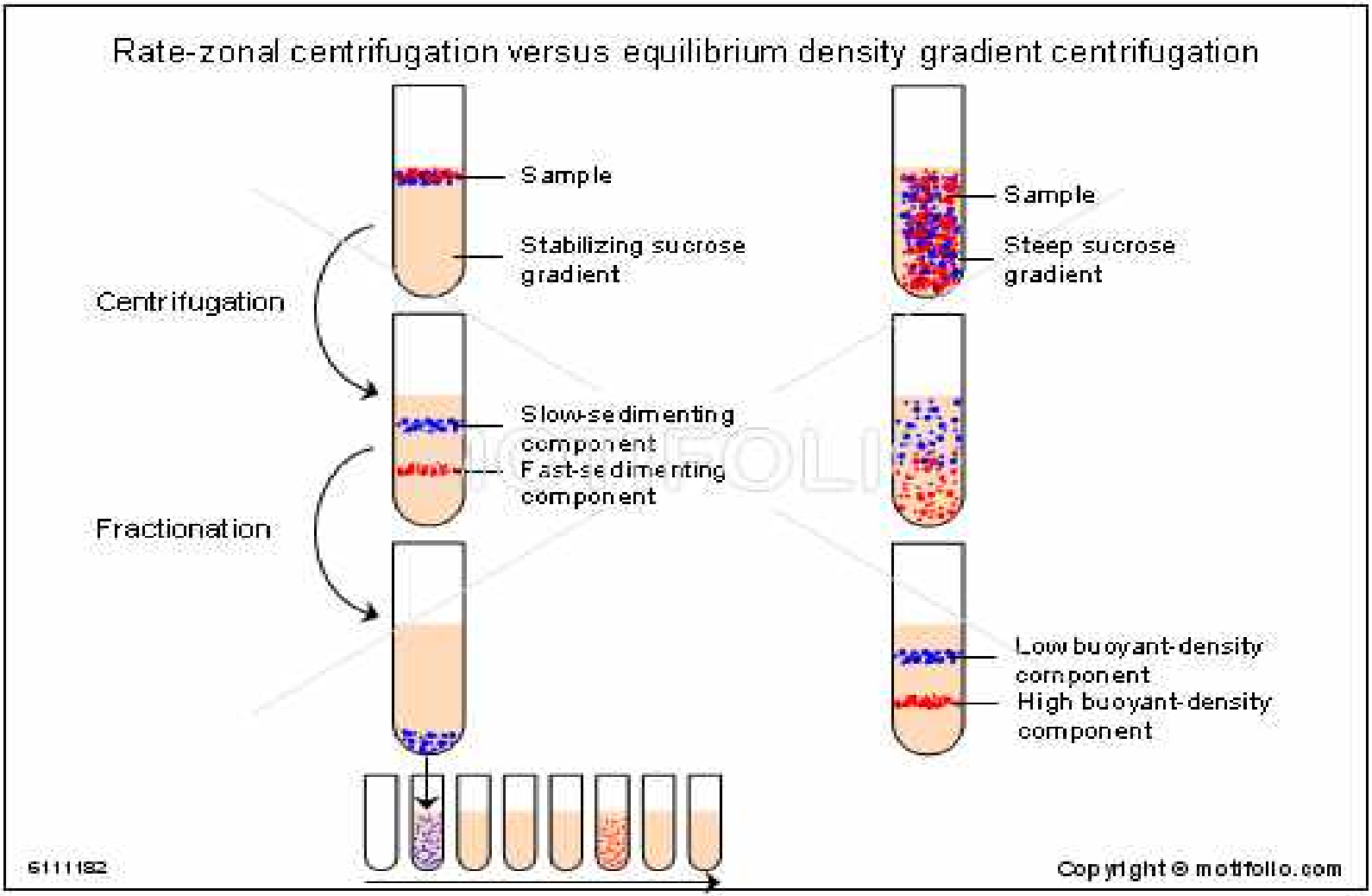
The ultracentrifuge can also be used to separate cell components on the basis of their buoyant density, independently of their size or shape. The sample is usually either layered on top of, or dispersed within, a steep density gradient that contains a very high concentration of sucrose or cesium chloride. Each subcellular component will move up or down when centrifuged until it reaches a position where its density matches its surroundings and then will move no further. A series of distinct bands will eventually be produced, with those nearest the bottom of the tube containing the components of highest buoyant density. The method is also called density gradient centrifugation.

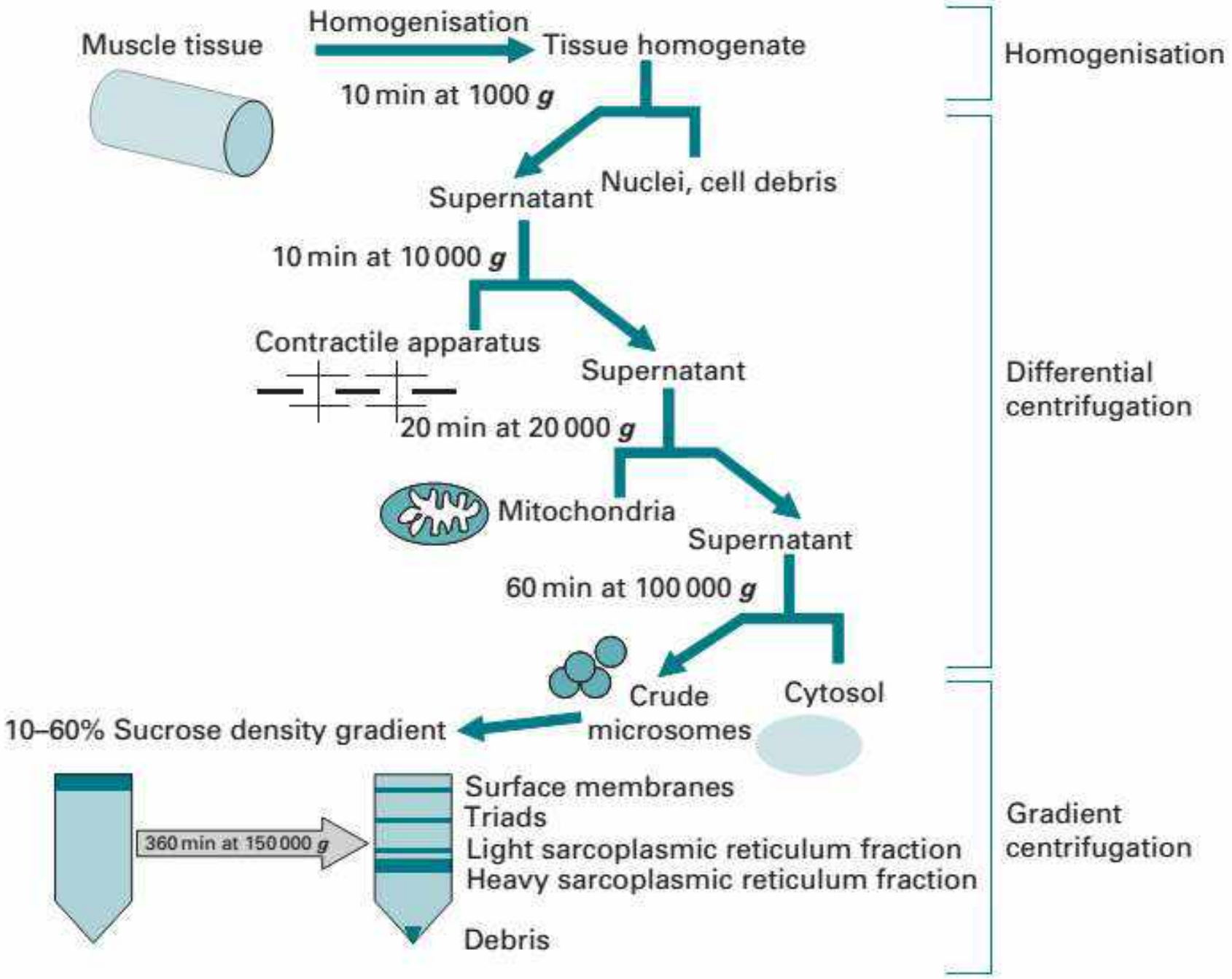


A sucrose gradient is shown here, but denser gradients can be formed with cesium chloride that are particularly useful for separating the nucleic acids (DNA and RNA).

The final bands can be collected from the base of the tube, as shown above.

ZONAL CENTRIFUGATION




Muscle tissue
Homogenisation
Tissue homogenate
10 min at 1000 g
Nuclei, cell debris
Supernatant
10 min at 10 000 g
Contractile apparatus
Supernatant
20 min at 20 000 g
Mitochondria
Supernatant
60 min at 100 000 g
Crude microsomes
Cytosol
10–60% Sucrose density gradient
360 min at 150 000 g
Surface membranes
Triads
Light sarcoplasmic reticulum fraction
Heavy sarcoplasmic reticulum fraction
Debris
Homogenisation
Differential centrifugation
Gradient centrifugation

ULTRACENTRIFUGATION

- The **ultracentrifuge** is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 1 000 000 g (approx. 9 800 km/s²).

- There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, andpolymer science.

- Theodor Svedberg invented the analytical ultracentrifuge in 1925,and won the Nobel Prize in Chemistry in 1926 for his research on colloids and proteins using the ultracentrifuge.

The ultracentrifuge was developed by a Swedish biochemist Svedberg (1923). The principle is based on the generation of centrifugal force to as high as 600,000 g (earth's gravity g = 9.81 m/s^2) that allows the sedimentation of particles or macromolecules. Ultracentrifugation is an indispensable tool for the ***isolation of subcellular organelles***, proteins and nucleic acids. In addition, this technique is also employed in the determination of molecular weights of macromolecules.

The rate at which the sedimentation occurs in ultracentrifugation primarily depends on the size and shape of the particles or macromolecules (i.e. on the molecular weight). It is expressed in terms of ***sedimentation coefficient***(s) and is given by the formula.

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

where v = Migration (sedimentation) of the molecule

ω = Rotation of the centrifuge rotor in radians/sec

r = Distance in cm from the centre of rotor

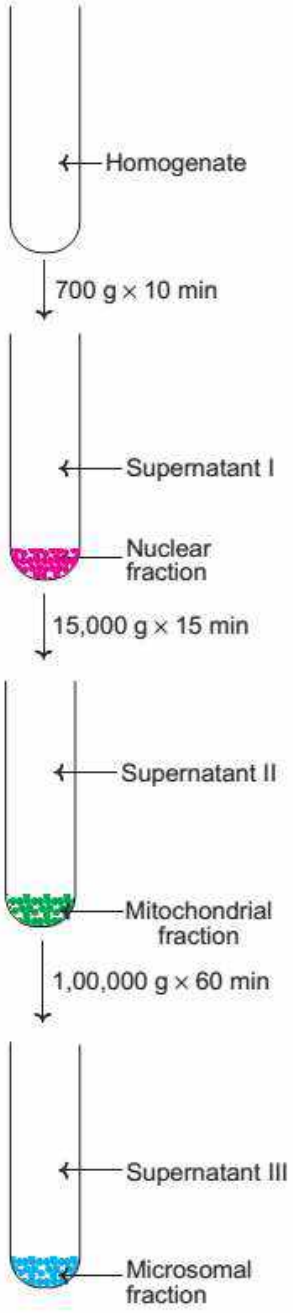
The sedimentation coefficient has the units of seconds. It was usually expressed in units of 10^{-13}s (since several biological macromolecules occur in this range), which is designated as one ***Svedberg unit.*** For instance, the sedimentation coefficient of hemoglobin is 4×10^{-13} s or 4S; ribonuclease is 2×10^{-13} s or 2S. Conventionally, the subcellular organelles are often referred to by their S value e.g. 70S ribosome.

Isolation of subcellular organelles by centrifugation

The cells are subjected to disruption by sonication or osmotic shock or by use of homogenizer. This is usually carried out in an isotonic (0.25 M) sucrose. The advantage with sucrose medium is that it does not cause the organelles to swell. The subcellular particles can be separated by differential centrifugation. The most commonly employed laboratory method separates subcellular organelles into 3 major fractions—nuclear, mitochondrial and microsomal (***Fig.41.10***).

When the homogenate is centrifuged at 700 g for about 10 min, the nuclear fraction (includes plasma membrane) gets sedimented. On centrifuging the supernatant (I) at 15,000 g for about 5 min mitochondrial fraction (that includes lysosomes, peroxisomes) is pelleted. Further centrifugation of the supernatant (II) at 100,000 g for about 60 min separates microsomal fraction (that includes ribosomes and endoplasmic reticulum). The supernatant (III) then obtained corresponds to the cytosol.

The ***purity*** (or contamination) of the subcellular fractionation can be ***checked by*** the use of ***marker enzymes.*** DNA polymerase is the marker enzyme for nucleus, while glutamate dehydrogenase and glucose 6-phosphatase are the markers for mitochondria and ribosomes, respectively. Hexokinase is the marker enzyme for cytosol.



TYPES

There are two types of ultracentrifugation:

1. **Analytical ultracentrifugation:- The aim of Analytical ultracentrifugation is use to study molecular interactions between macromolecules or to analyse the properties of sedimenting particles such as their apparent molecular weight.**

2. **Preparative ultracentrifugation:- The aim of Preparative ultracentrifugation to isolate and purify specific particles such as subcellular organells.**

Analytical ultracentrifugation

Two kinds of experiments are commonly performed on these instruments:

1. **Sedimentation velocity experiments:- Aim of SVEs to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their size distribution.**

2. **Sedimentation equilibrium experiments:- SEEs are concerned only with the final steady-state of the experiment, where sedimentation is balanced by diffusion opposing the concentration gradients, resulting in a time-independent concentration profile.**

Preparative ultracentrifugation

It is to isolate specific particles which can be reused

1. Differential ultracentrifugation:- Differential centrifugation is a common procedure in microbiology and cytology used to separate certain organelles from whole cells for further analysis of specific parts of cells.

2. Density gradient ultracentrifugation:- Based on denstiy difference. There are two types of density gradient ultracentrifugations under preparative ultracentrifugation such as.-

1. ZONAL or RATE and 2. ISOPYCNIC

1} ZONAL or RATE Centrifugation:

- Mixture to be separated is layered on top of a gradient (increasing concentration down the tube).
- Provides gravitational stability as different species.
- Move down tube at different rates.

2} ISOPYCNIC Centrifugation:

- Isopycnic means “of the same density”.
- Molecules separated on equilibrium position.
- Each molecule floats or sinks to position where density.

FUNCTIONS OF ANALYTICAL AND PREPARATIVE ULTRACENTRIFUGATION:

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.

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.

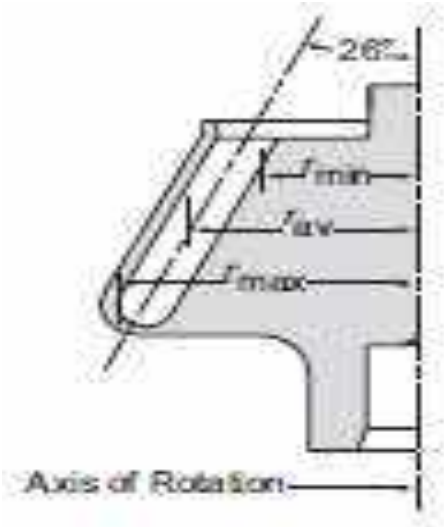
ROTORS FOR ULTRACENTRIFUGATION

1. Fixed-angle rotor,
2. Swinging-bucket rotor,
3. Vertical rotor and
4. Near-vertical rotor.

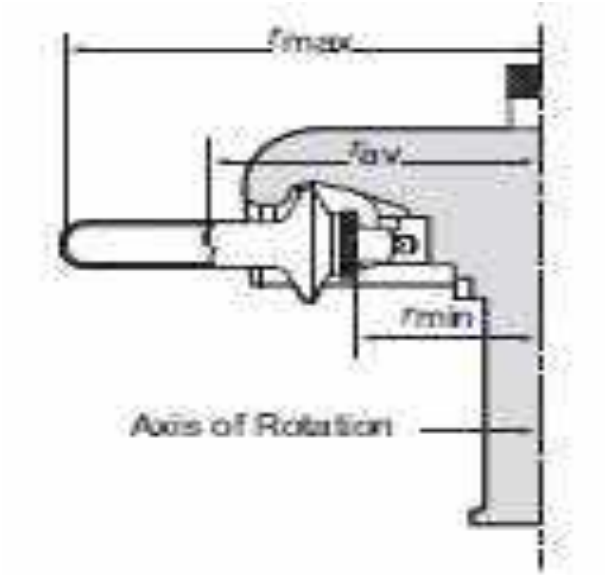
1.FIXED ANGLE ROTOR

- Fixed-angle rotors are general-purpose rotors that are especially useful for pelleting subcellular particles and in short column banding of viruses and subcellular organelles.

- Tubes are held at an angle (usually 20 to 45 degrees) to the axis of rotation in numbered tube cavities.



2.SWINGING BUCKET ROTOR

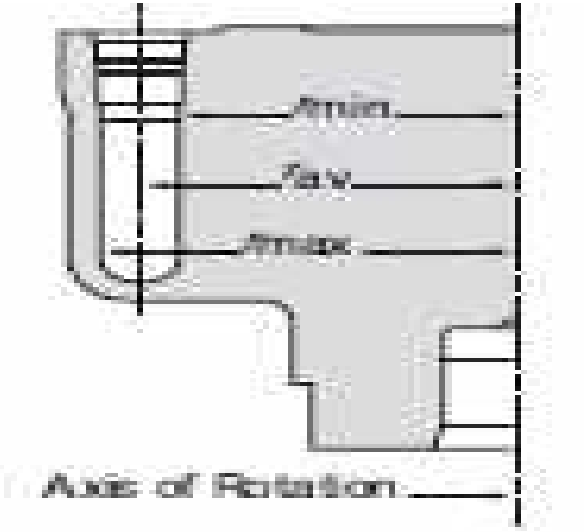


- Swinging-bucket rotor are used for pelleting, isopycnic studies and rate zonal studies.
- Tubes are attached to the rotor body by hinge pins or a crossbar. The buckets swing out to a horizontal position.

- Isopycnic studies (separation as a function of density).
- Rate zonal studies (separation as a function of sedimentation coefficient).

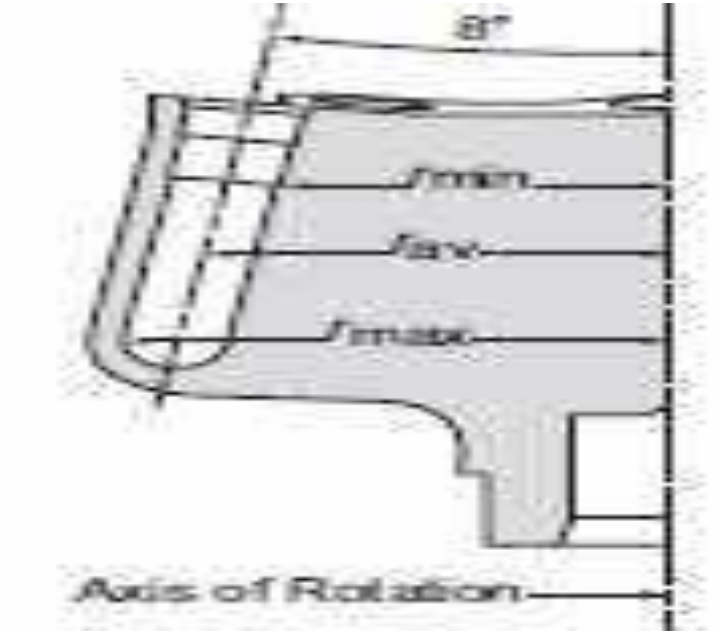
3.VERTICAL ROTOR

- Vertical rotors hold tubes parallel to the axis of rotation; therefore, bands separate across the diameter of the tube rather than down the length of the tube.
- Vertical rotors are useful for isopycnic and, in some cases, rate zonal separations when run time reduction is important.



4.NEAR VERTICAL ROTOR

- Near-vertical rotors are designed for gradient centrifugation when there are components in a sample mixture that do not participate in the gradient.
- Tubes are held at an angle (typically 7 to 10 degrees) to the axis of rotation in numbered tube cavities.
- In this rotor used only Quick-Seal and Opti-Seal tubes.



CENTRIFUGE CALIBRATION AND MAINTENANCE

This procedure provides accurate rotation speed, timer verification and centrifuges that are temperature controlled in a laboratory environment.

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 rebalancing the buckets and tubes.

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 0.5% sodium hypochlorite .

References

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