Microbiology

US05EMIC26

Bioinstrumentation and Biotechniques

Unit- II Centrifugation:

(a) Principle of centrifugation.

(b) Separation methods in preparative centrifuge , differential and density gradient centrifugation.

(c) Ultracentrifugation-introduction and applications.

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Centrifugation

Introduction

- A Centrifuge is an instrument which produces centrifugal forces.
- It is containers fixed in such a way that they can be rotated around the central axis with the help of motors.
- Centrifugation is the technique used for separation of particles.
- The separation is based upon the behavior of particles in an applied centrifugal field.
- The particles which differ in density ,shape, size can be separated.

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- They sediment at different rates in centrifugal field.
- Each particles sediments at a rate which is proportional to applied field.
- The basis of centrifugation separation techniques is to exert a larger forces than the earth's gravitational field, this increase the rate at which the particles sediments

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Basic principle of centrifugation

- The rate of sedimentation or velocity (v) of a particle can be expressed in terms of its sedimentation rate per unit of centrifugal field. i.e. sedimentation coefficient(S).
- The S of most biological particles are very small and for convenience its basic unit is taken as 10^{-13} seconds which is termed as Svedberg unit (S) in memory of "Svedberg".
- The rate of sedimentation depends on the applied centrifugal field(G).
- G is determine by the square of the angular velocity (ω)of the rotor and the redial distance(r) of the particle from the axis of rotation.

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hence,

$$G=\omega^2 r \text{----------(1)}$$

Since one revolution of the rotor is equal to 2π radians ,its angular velocity ,in radians per second ,can be expressed in terms of revolutions per minute.

Therefore , common way of expressing rotor speed is

$$\omega = \frac{2\pi \text{ rev/min}}{} \text{----------(2)}$$

Therefore, the centrifugal field (G) in terms of rev/min is

$$G=\left(\frac{2\pi \text{ rev/min}}{3600}\right)^2 x\ r \text{ -------(3)}$$

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- **It is generally expressed as a multiple of the earth's gravitational force**

i.e. g = 980cm/sec ^{2}

i.e. Weight of the particle : Weight of particle in earth'
in centrifugal field gravitational force alone

- **This ratio is referred as the relative centrifugation field(RCF)**

$$\text{Therefore , RCF} = \frac{4\pi^2(\text{rev/min})^2 \times r}{3600 \times 980}$$

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- **Since biochemical experiments are usually conducted with particles dissolved or suspended in solution.**

- **The rate of sedimentation of a particle is dependent not only upon the applied centrifugal field but also upon the density and size of the particle, the density and viscosity of the medium in which it is sedimenting and the extent to which its shape deviates from spherical.**

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CENTRIFUGES AND THEIR USES :

Centrifuges may be classified into four major groups :

[a] Small Bench Centrifuges
[b] Large Capacity Refrigerated Centrifuges
[c] High Speed Refrigerated Centrifuges
[d] Continuous Flow Centrifuges

And ultracentrifuges are of two types :

[a] Preparative Ultracentrifuge
[b] Analytical Ultracentrifuge

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[a] Small Bench Centrifuges :

- These are the simplest and least expensive centrifuges.
- They are often used to collect small amounts of material that rapidly sediment (yeast cells, erythrocytes, coarse precipitates)

- Generally have a maximum speed of 4000 to 6000 rev min^{-1} with maximum relative centrifugal fields of 3000 to 7000 g.
- Most operate at ambient tem-perature.

- These centrifuges have proved extremely useful for sedimenting small volumes (250 mm^3 to 1.5 cm^3) of material very quickly (one or two minutes).
- Typical applications include the rapid sedimentation of blood samples.

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[b] Large Capacity Refrigerated Centrifuges :

- These have a maximum speed of 6000 rev min^{-1} and produce a maximum relative centrifugal field approaching 6500 g.
- They have refrigerated rotor chambers and vary only in their maximum carrying capacity, fixed angle rotors enabling separation to be achieved in 10, 50 and 100 cm^3 tubes.
- Large total capacity (4 to 6 dm^3) centrifuges are also available.
- These instruments are most often used to compact or collect substances that sediment rapidly, for example, erythrocytes, coarse or bulky precipitates, yeast cells, nuclei and chloroplasts.

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[c] High Speed Refrigerated Centrifuges :

- These instruments are available with maximum rotor speeds in the region of 25,000 rev min^{-1} generating a relative centrifugal field of about 60,OOO g.
- They generally have a total capacity of up to 1.5 dm^3 and a range of interchangeable fixed angle and swinging bucket rotors.

- These instruments are most often used to collect microorganisms, cellular debris, larger cellular organelles and ammonium sulphate protein precipitates.
- They cannot generate sufficient centrifugal force to effectively sediment viruses or smaller organelles such as ribosomes.

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[d] Continuous Flow Centrifuges :

- The continuous flow centrifuge is a relatively simple high–speed centrifuge.
- The rotor, through which particles suspended in medium flow continuously (usually 1 to 1.5 dm^3 min^{-1}), is, however, long, tubular and non–inter-changeable.
- As medium enters the rotating rotor, particles are sedimented against its wall and excess clarified medium overflows through an outlet port.
- The major application of this type of centrifuge is in the harvesting of bacteria or yeast cells from large volumes of culture medium (10 to 500 dm^3).

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Ultracentrifugation-introduction and applications

- **Ultracentrifugation can operate at speeds up to 75,000 rpm,which provide centrifugal force in excess of 5,00,000 g**
- **At these speed the friction between air & the spinning rotor generates significant amount of heat.**
- **To eliminate this source of heating rotor chamber evacuated & maintain vacuum 1 to 2 μ.**
- **Along with this ultracentrifugation has a refrigeration system which can maintain the temp. between 0 to 4 ℃.**
- **The drive shaft on which the rotor is mounted is merely 1/16 inches in diameter.**

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- **It is made up of aluminum or titanium alloy of high strength to withstand the great forces.**

- **In Ultracentrifugation rotor at excessive speeds can result in an explosion ,to prevent explosion the rotor chamber is always enclosed in a heavy armored plate.**

Ultracentrifuges are of two types :

[a] Analytical Ultracentrifuge
[b] Preparative Ultracentrifuge

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[a] Analytical Ultracentrifuge:

Centrifugation is carried out with desire for characterization known as analytical centrifugation.

- Speed- 70,000 rpm. & -RCF – 5,00,000 g.
- It consist of rotor ,motor & optical system.
- Rotor is suspended on a wire coming from the drive shaft of high speed motor.
- Rotor enclosed in protective armored chamber which is refrigeration & Evacuated.
- There is thermistor at the tip of rotor which measures the temperature.

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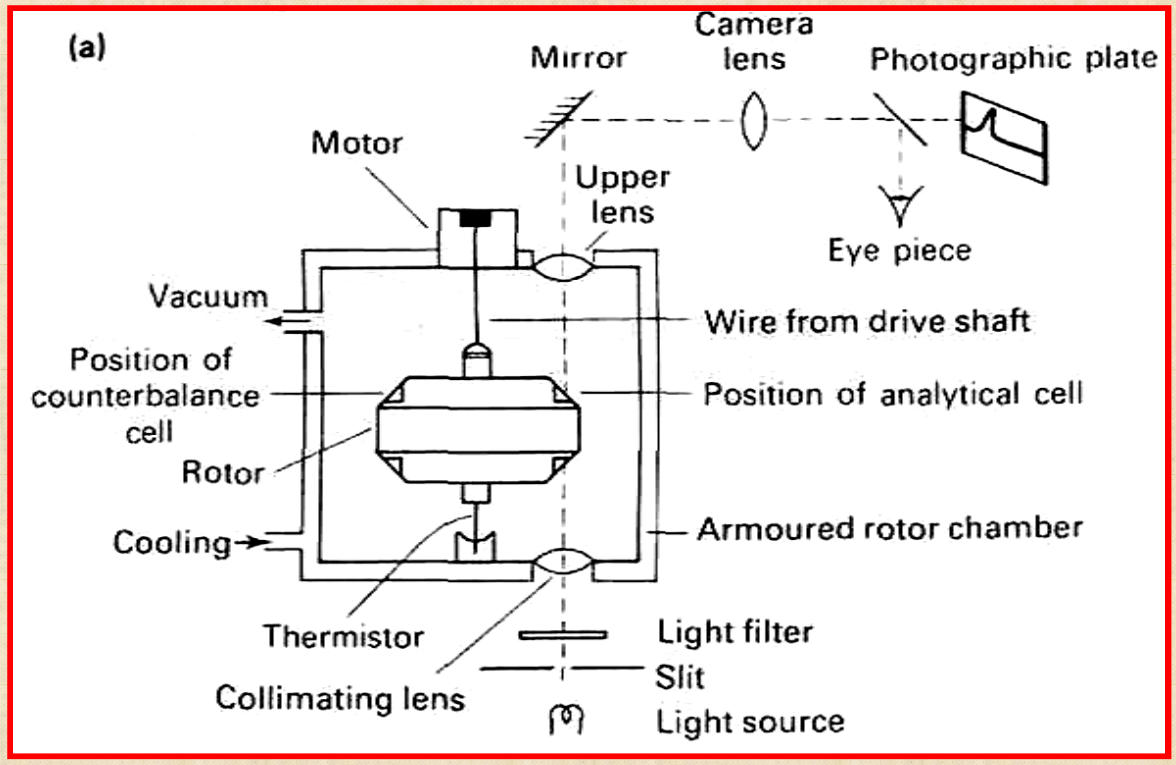
- **Optical system enables the sedimentation material to be observed at any time during centrifugation.**
- **The rotor chamber contains upper lens which focuses light out on photographic lenses and lower lens which collimates the light so that sample cell is illuminated by parallel beam.**



Diagrammatic representation of an analytical ultracentrifuge system

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[b] Preparative Ultracentrifuge

Centrifugation carried out for isolation and purification of components is known as preparative ultracentrifugation.

- Max speed- 80,00 rpm
- RCF- 6,00,000 g.
- Rotor chamber is refrigerated ,evacuated & sealed in heavy armored chamber.
- Three system available in preparative ultracentrifugation:

(1) **Temperature monitoring system**:

- It is simple, sophisticated.
- It utilizes infrared temp. sensors which continuously monitor rotor temp. & control refrigeration system.

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(2) Over speed system control:

- **It prevents operation of the rotor above its maximum speed.**

(3) Flexible drive shaft system:

- **It is electronic circuits system to detect rotor imbalance.**

- **A table top preparative ultracentrifugation called Air fuse centrifugation.**
- **It works in non vaccuated,friction free flow of air.**

Speed: 1,00,000 rpm

RCF: 16,00,000 g

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Application:

- Air fuse preparative ultracentrifugation is used in biochemical & clinical research where sample volume is very less & requiring high centrifugal forces.
  e.g. - Steroid, hormons ,receptors assays.
  - macromolecules –ligand binding studies
  - Separation of major lipoprotein fraction from plasma.
- Analytical ultracentrifugation have many application in biology especially in proteins and nucleic acid chemistry.
- It is used to determine molecular weight and purity of protein & nucleic acid.
- It is used in detection of conformational changes in macromolecules.

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Separation methods in preparative centrifugation:

- **Preparative Centrifugation is divided into Two types:**

  (1)Differential centrifugation

  (2)Density gradient centrifugation

- **Density gradient centrifugation is divided into two types:**

  (a)Rate Zonal Centrifugation

  (b)Isopycnic Centrifugation

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(1)Differential centrifugation

- **In differential centrifugation, separation of various components is achieved primarily based on the size of the particles.**
- **It is commonly used in simple pelleting and in obtaining partially pure preparation of sub cellular organelles and macromolecules.**
- **In this process, tissue sample is first homogenized to break cell membrane called *homogenate*.**
- **The homogenate is then subjected to repeated centrifugation. In each step, pellet is removed and centrifugal force is increased.**

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- **Finally, purification is done through equilibrium sedimentation, and desired layer is extracted for further analysis.**

- **Obtaining partially purified organelles by differential centrifugation serves as preliminary step for analysis and further purification using other types of centrifugal separation methods.**

- **Separation process is based on size and density of the particles. Larger and denser particles get pelleted out at lower centrifugal forces.**
  For example, unbroken whole cells will pellet at low speeds and short intervals such as 1,000g for 5 minutes.

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- **Smaller cell fragments and organelles remain in the supernatant and require more force and time to get pelleted out.**

- **The order in which cells get separated is shown below.**

  (a) Whole cell and Nuclei
  (b) Mitochondria, lysosomes and peroxisomes
  (c) Microsomes
  (d) Ribosomes and cytosol

- **Sedimentation of various components depends upon solvent density, mass, shape and partial specific volume of molecule, and rotor size and rate of rotation of the centrifuge.**

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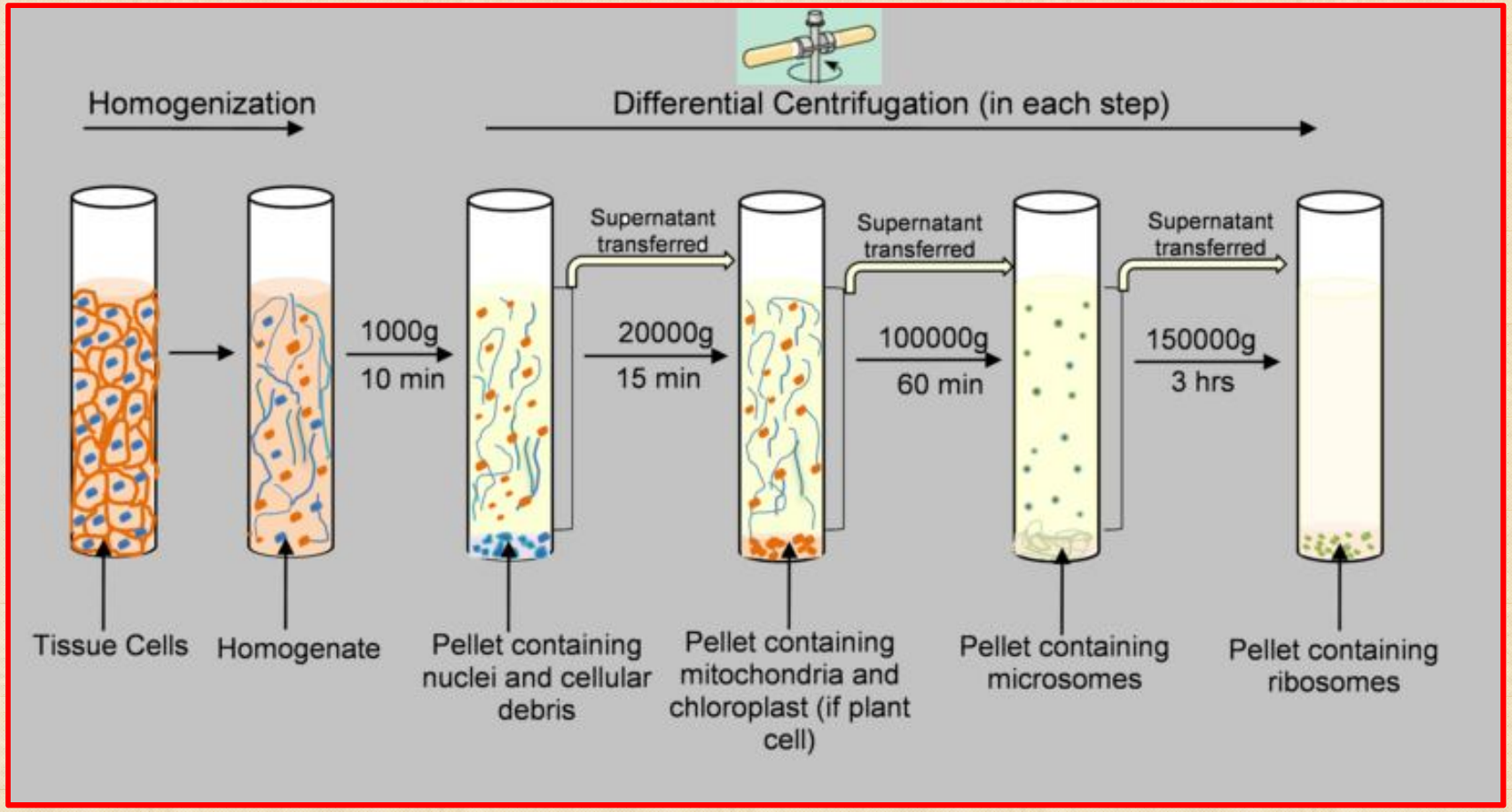
Diagram of Differential Centrifugation



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(2)Density gradient centrifugation

- **In differential centrifugation homogenous medium is used for separation.**

- **As opposed to that density gradient centrifugation the medium used has gradients, so here separation under the centrifugal field is therefore dependent upon the buoyant densities of the particles.**

- **A measure of the tendency of a substance to float in some other substance is known as buoyant density (ρ)of the particle.**
- **Density gradient centrifugation are of two types:**
  (a)Rate Zonal Centrifugation
  (b)Isopycnic Centrifugation

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Density gradient centrifugation

Important factor in density gradient centrifugation:

[1] Formation and choice of density gradients

[2] Sample application to the gradient

[3] Recovery and monitoring of gradients from centrifuge tubes

[4] Nature of gradient materials and their use

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[1] Formation and choice of density gradients:

- **All density gradient methods involve a supporting column of liquid, the density of which increases towards the bottom of the centrifuge tube. The gradient stabilizes the column of liquid in the centrifuge tube, preventing mixing of the separated particles.**

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- **Density gradients may be produced by techniques which fall into two major groups,**
  - **(i) the discontinuous or step gradient technique and**
  - **(ii) the con-tinuous density gradient technique**

(i) The Discontinuous Technique :

- **In this technique, solutions of decreasing density are care-fully layered over each other in the centrifuge tube, by means of a pipette.**
- **This discontinuous gradient can be used directly by layering the sample to be separated as a narrow zone on the top (lowest density) layer. The tube is then centrifuged under the appropriate experimental conditions.**
- **Discontinuous gradients have been found to be the most suitable for the separation of whole cells or sub-cellular organelles from plant or animal tissue homogenates.**

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(ii) The Continuous Technique :

- The continuous density gradient technique, which is probably the most common of the two, It requires the use of a special piece of apparatus known as a gradient former, many varieties of which are commercially available.
- It consists of two precision–bored cylindrical chambers of identical diameter which are interconnected at their base by a tube containing a control valve which allows the mixing of the contents of the two chambers to be regulated.
- One chamber (the mixing chamber), which is filled with a dense solution, contains a stirrer and possesses an outlet to the centrifuge tubes.
- The second chamber contains an equal amount (by weight) of a less dense solution.

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- **The hydrostatic pressures of the two liquid columns need to be equal otherwise liquid will flow through the connecting tube as soon as the control valve is opened.**

- **The dense liquid is then allowed to run through a filling pipe from the mixing chamber into the centrifuge tube and is immediately replaced in the mixing chamber by an equivalent amount of less dense solution via the control valve. This re–establishes hydrostatic equilibrium.**

- **The concentration of the gradient in the centrifuge tube will therefore decrease in a linear manner as the tube is filled.**
- **For most purposes, linear gradients are used since the gradual change in density along the gradient has been found to yield a much higher resolution of components such as ribosomal subunits and certain viruses.**

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[2] Sample application to the gradient:

- **Before the sample is applied to the density gradient its optimum volume and concentration should be determined.**
- **Sample volumes in the range of 0.2 to 0.5 cm^3 may be added to tubes of 1.0 to 1.6 cm diameter and sample volumes of up to 1 cm^3 to tubes having a diameter of approximately 2.5 cm.**
- **If the sample concentration on the gradient is either too high or too low then; the gradient may either become overloaded, resulting in a broadening of the separated zones and loss of resolution, or difficulty may be encountered in the iden-tification of the separated bands.**
- **Application of the sample to the gradient is generally made using a syringe, or in the case of fragile samples like DNA, by pipette.**

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[3] Recovery and monitoring of gradients from centrifuge tubes:

- After particle separation has been achieved, it is necessary to remove the gradient solution in order to isolate the bands of separated material.

- Removal of gradients from centrifuge tubes can be achieved by a number of tech-niques. If the bands can be visually detected, recovery can be achieved using a hypodermic needle or syringe. A common method, however, is that of dis-placement.

- Alternatively, the centrifuge tube may be punctured at its base using a fine hollow needle. As the drops of gradient pass from the tube through the needle they may be collected using a fraction collector and further analyzed.

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- **Analysis of the contents of the displaced gradient can be achieved by ultraviolet spectrophotometry, refractive index measurements, scintillation counting, enzymatic or chemical analysis.**

[4] Nature of gradient materials and their use:

- **There is no ideal all–purpose gradient material, the choice of solute depending upon the nature of the particles to be fractionated.**

- **The gradient material should permit the desired type of separation, be stable in solution, inert towards biological materials, should not absorb light at wavelengths appropriate for spectrophotometric monitoring (visible or ultraviolet range), be sterilizable, non-toxic and non-flammable.**

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- **Have negligible osmotic pressure and cause minimum changes in ionic strength, pH and viscosity, be inexpensive and readily available in pure form and capable of forming a solution covering the density range needed for a particular application without overstressing the rotor.**
- **Gradient–forming materials which provide the densities required for the separation of sub-cellular particles include salts of alkali metals (e.g. caesium and rubidium chloride), small neutral hydrophilic organic molecules (e.g. sucrose), hydrophilic macromolecules (e.g. proteins and polysaccharides) and a number of miscellaneous compounds more recently introduced and not included in the above group, such as colloidal silica (e.g. Percoll and Ludox) and non-ionic iodinated aromatic compounds (e.g. Metrizamide, Nycodenz and Renografin).**

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- **Ficoll (a copolymer of sucrose and epichlorhydrin) has been, successfully used instead of sucrose for the separation of whole cells and sub-cellular organelles by rate zonal and isopycnic centrifugation. Preparative centrifugation**

- **Density gradient centrifugation are of two types:**
  (a)Rate Zonal Centrifugation
  (b)Isopycnic Centrifugation

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(a)Rate Zonal Centrifugation

- **Particle separation by the rate zonal technique is based upon differences in size or sedimentation rates.**
- **The technique involves carefully layering a sample solution on top of a preformed liquid density gradient, the highest density of which does not exceed that of the densest particles to be separated.**
- **The sample is then centrifuged until the desired degree of separation is effected, i.e. for sufficient time for the particles to travel through the gradient to form discrete zones or bands which are spaced according to the relative velocities of the particles.**

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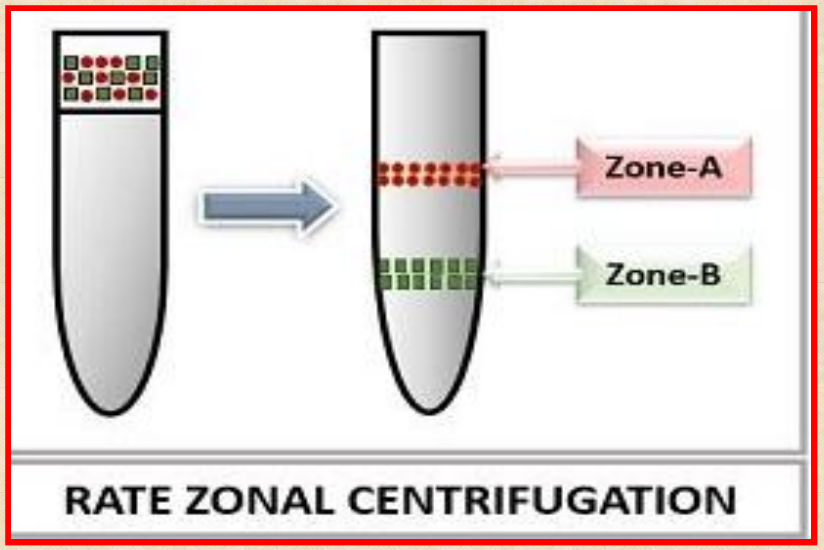
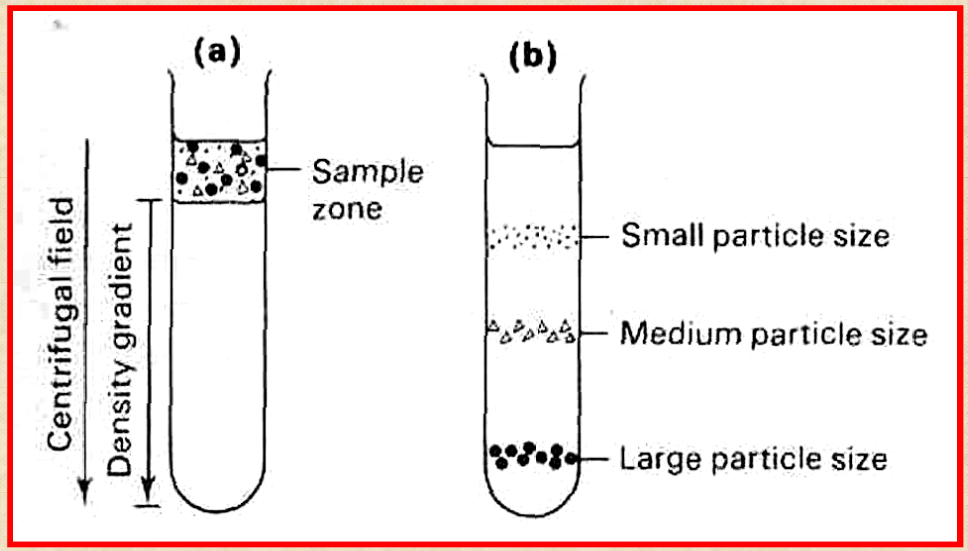
- **Since the technique is time dependent, centrifugation must be terminated before any of the separated zones pellet at the bottom of the tube.**
- **The method has been used for the separation of enzymes, hormones, RNA–DNA hybrids, ribosomal subunits, sub-cellular organelles, for the analysis of size distribution of samples of polysomes and for lipoprotein fractionations.**





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(b) Isopycnic Centrifugation

- **Isopycnic centrifugal ion depends solely upon the buoyant density of the particle and not its shape or size and is independent of time.**

- **Hence soluble proteins, which have a very similar density (e.g. $\rho = 1.3\ g\ cm^{-3}$ in sucrose solution), cannot usually be separated by this method, whereas sub-cellular organelles (e.g. Golgi apparatus, $\rho = 1.11\ g\ cm^{-3}$, mitochondria, $\rho = 1.19\ g\ cm^{-3}$ and peroxisomes, $\rho = 1.23\ g\ cm^{-3}$ in sucrose solution) can be effectively separated.**

- **The sample is layered on top of a continuous density gradient which cover the whole range of the particle densities which are to be separated.**

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- **The maximum density of the gradient, therefore, must always exceed the density of the most dense particle.**

- **During centrifugation, sedimentation of the particles occurs until the buoyant density of the particle and the density of the gradient are equal (i.e. where $\rho_p = \rho_m$).**

- **At this point no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on a cushion of material that has a density greater than their own.**

- **Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particles banding to form zones each at their own characteristic buoyant density.**

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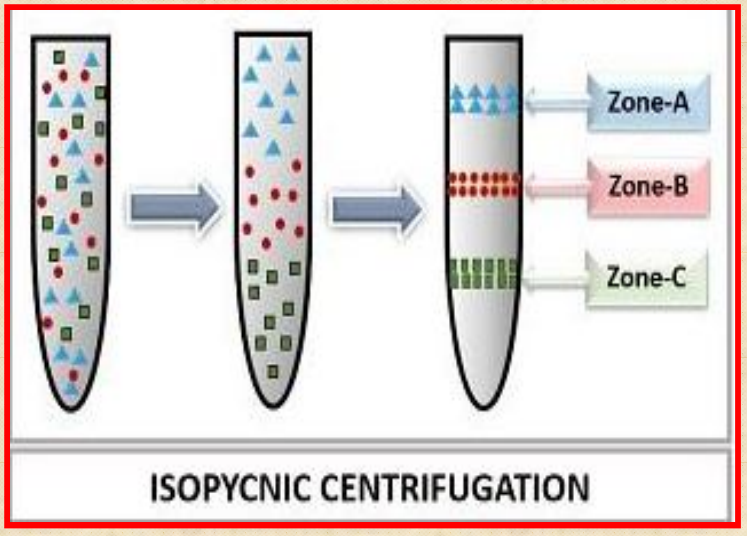

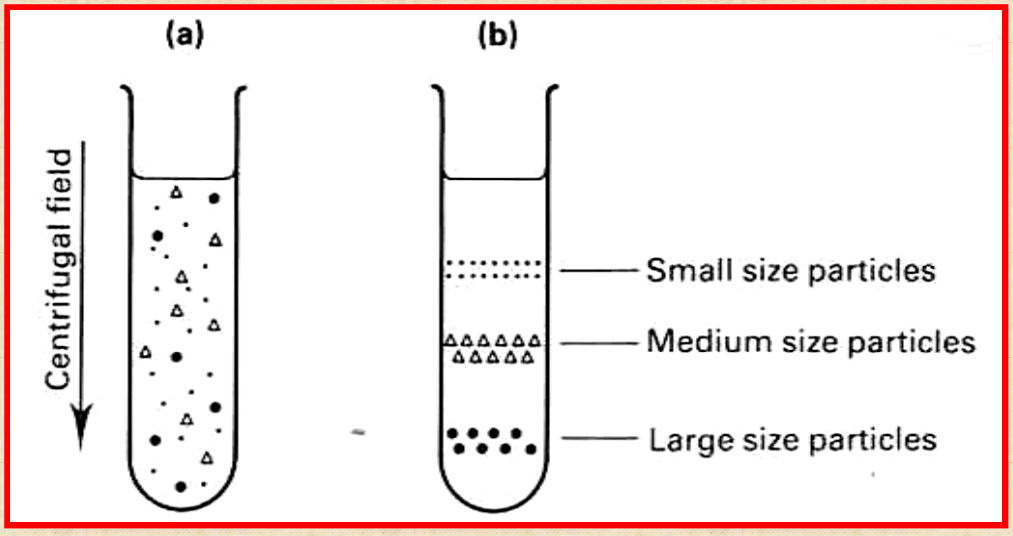

- **Isopycnic gradients have also been used to separate and purify viruses and analyze human plasma lipoproteins.**

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Significant difference between Rate Zonal and Isopycnic Centrifugation:

	Rate Zonal	Isopycnic
Synonym	s-zonal, sedimentation velocity.	Density equilibration, Sedimentation equilibrium
Gradient	Shallow, maximum gradient density less than that of the least dense sedimenting specie, gradient continuous.	Steep, maximum gradient density greater that that of the most dense sedimenting specie, continuous of discontinuous gradients.
Centrifugation	Incomplete sedimentation, low speed, Short time.	Complete sedimentation Till equilibrium is achieved, high speed, long time
Separations	RNA-DNA hybrids, Ribosomal subunits etc.	DNA ,plasma lipoprotein, Lysosomes, mitochondria Peroxisomes etc.

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