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S.Y.B.Sc. SEM-4

Subject: Biochemistry

Paper-401: BIOPHYSICAL & BIOCHEMICAL TECHNIQUES

Unit -2

HYDRODYNAMIC TECHNIQUES

Unit – 2: CENTRIFUGATION

- Small particles suspended in a liquid medium are almost insensitive to gravitation al settling. This is because for small particles the gravitational force is so minute that the random bombardment of the molecules of the surrounding medium far outweighs the direction force of gravity.
- A straight forward solution to this difficulty is to increase the gravitational potential energy by enclosing the particles in the medium will then sediment faster because a centrifugal force is acting upon them in addition to gravitational force.
- The application of the centrifugation technique range from collection and separation of cells, cell organelles and molecules and to study the molecular weight of macromolecules.

Relative Centrifugal Force (RCF):

Centrifugation is based on the fact that any object moving in a circle at a steady angular velocity is subjected 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.

$$F = \omega^2 r$$

F is frequently expressed in the term of the earth's gravitational force and is them referred to as the relative centrifugal force (RCF) or more commonly as the "number of times g".

$$RCF = \omega^2 r/980$$

For everyday use the relationship will be better if expressed in terms of resolution per minute (rpm)the common way in which the operative speed of a centrifuge rotor is expressed. Radians and rpm share the following relationship.

$$\omega = \pi rpm/30$$

Substituting ω, we can write

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

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

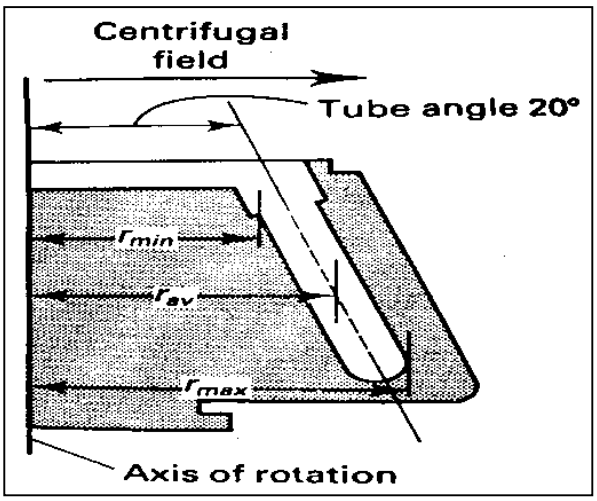


Figure 1:True radius of rotation

- Considering this relationship, since all other values are constants the RCF depends upon rpm and the radius of rotation (r). The value of r is constant for a given rotor; variations in the rpm alone will be a cause for variation in RCF.

- In true terms, r would be the distance between the particle in the sample holder and the rotor axis. As the time progresses the particle will sediment towards the bottom of the sample tube during the process of centrifugation and the radius (distance from the rotor axis) will also increases. Thus at every successive point of time during centrifugation the particle will experience a perpetual increase in RCF would be difficult to use true radius value.

- In actual practice the radius is calculated from the rotor axis to the middle of the liquid column in a centrifuge tube. This radius is known as average radius of rotor (r average).

$$r\ average = \frac{r\ minimum + r\ maximum}{2}$$

  - ♣ Depending on the radius (r), the RCF will vary. Different centrifuges use rotors of the different radius and hence would give different value of RCF at the same rpm. Therefore it is better to express the force acting upon a particle in the terms of RCF rather than the rpm.
  - ♣ Apart from RCF, the rate of the sedimentation of a given particle would also depend on the following factor:-
    1. Density and radius of the particle.
    2. Density and viscosity of the suspending medium.
    3. Shape of the particle.
    4. Concentration of suspension.

One Word Question

Sr. No.	Question	Answer
1	Centrifugation work on _____ principle	Sedimentation
2	Full form of RCF	Relative Centrifugal force
3	Full form of RPM	Rotation per minute
4	Particle Sedimentation is depends on	Density & Molecular weight
5	What is the unit of Sedimentation	Svedberg

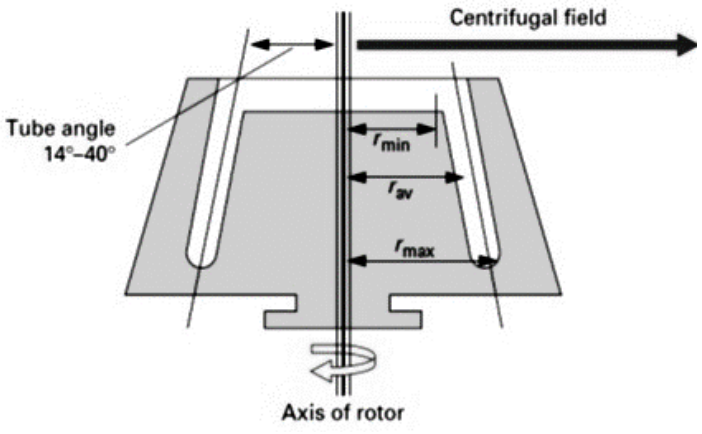
TYPES OF ROTORS:-

To illustrate the difference in design of rotors in various centrifugation methods.

1) Fixed-angle rotors,
2) Vertical tube rotors and
3) Swinging-bucket rotors

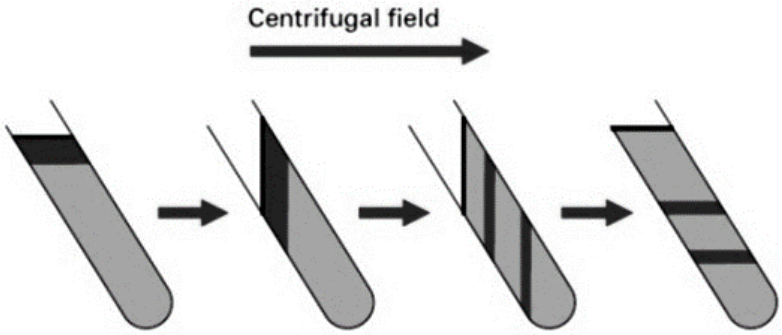
Fixed-angle rotors,

- Fixed-angle rotors are an ideal tool for pelleting during the differential separation of biological particles where sedimentation rates differ significantly, for example when separating nuclei, mitochondria and microsomes.
- In addition, isopycnic banding may also be routinely performed with fixed-angle rotors. For isopycnic separation, centrifugation is continued until the biological particles of interest have reached their isopycnic position in a gradient.



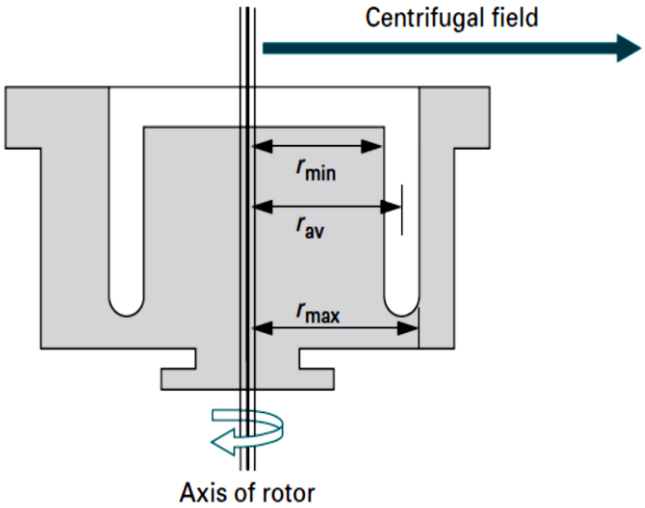
- This means that the particle has reached a position where the sedimentation rate is zero because the density of the biological particle and the surrounding medium are equal.

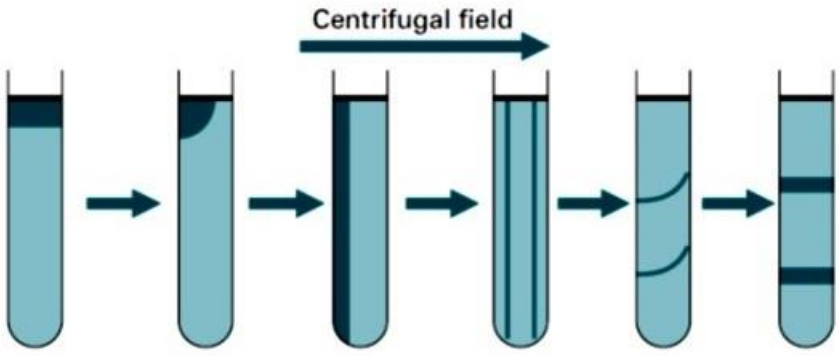
Centrifugation tubes are held at a fixed angle of between 14 ◦ and 40 ◦ to the vertical in this class of rotors.



2) Vertical tube rotors

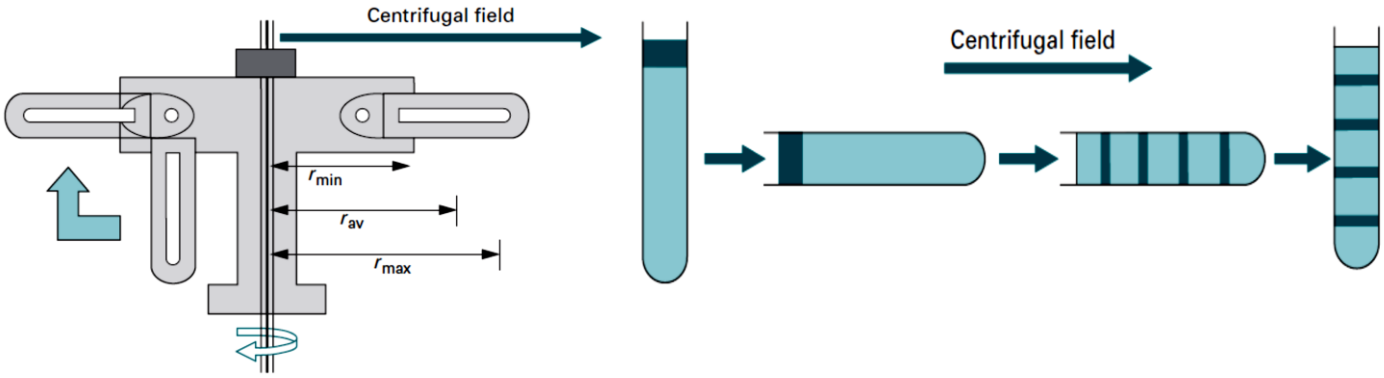
- Vertical rotors may be divided into true vertical rotors and near-vertical rotors.
- Sealed centrifuge tubes are held parallel to the axis of rotation in vertical rotors and are restrained in the rotor cavities by screws, special washers and plugs.
- Since samples are not separated down the length of the centrifuge tube, but across the diameter of the tube, isopycnic separation time is significantly shorter as compared to swinging bucket rotors.
- In contrast to fixed-angle rotors, near-vertical rotors exhibit a reduced tube angle of 7◦ to 10o and also employ quick-seal tubes.





3) Swinging-bucket rotors

Since a greater variety of gradients exhibiting different steepness can be used with swinging-bucket rotors, they are the method of choice when maximum resolution of banding zones is required (Fig.) such as in rate zonal studies based on the separation of biological particles as a function of sedimentation coefficient.



One Word Question

Sr. No.	Question	Answer
1	How many type of rotors	3 type
2	Fixed-angle rotors are an ideal tool for pelleting during the differential separation of	Biological sample
3	Fixed-angle rotors contain range of angle_____	14˚ to 40˚
4	Vertical angle rotors contain angle_____	90˚
5	Which type of rotor are best for separation	Swinging-bucket rotors

Centrifuges and their use:-

Centrifuge may be classified into three major groups-the small bench (table-top) centrifuge, high–speed centrifuge and centrifuge of two types, preparative and analytical.

Small Bench (Table-Top) Centrifuge:

- These are the simplest and least expensive centrifuge and exist in many types of design. They are often use to collect small amounts of material, and generally have a maximum small amount of material, and generally have a maximum speed of 4000 to 6000 rpm with maximum relative centrifugal fields of 3000 to 7000 X g.

- The maximum sample carrying capacity per tube is about 10-12ml. Most operate at room temperature but some of the latest designs incorporate a refrigeration system to keep rotor cool and thus prevent denaturation of proteins. Small microgram is available which provide instant acceleration to approximately 10000 X g. These centrifuges are extremely useful in sedimenting small volume (0.25 to1.5 ml) of material very quickly (1 to 2 minutes).

Uses:-

Typical application of table-top centrifuge includes:

1. Rapid sedimentation of blood samples for preparation of plasma or serum.
2. To sediment yeast or bacterial cells from culture.
3. To sediment coarse precipitates of chemical or biochemical reactions. E.g. Protein precipitates, Immune precipitates etc.

High-Speed Centrifuge:-

- ♣ High speed centrifuges can operate with the maximum speed of up to 25000 rpm providing about 90000 g centrifugation force in the process. They are usually equipped with refrigeration system to remove heat generated due to friction between the air and the spinning rotor.

- ♣ The temperature can easily be maintained in the range of 0-4°C by the means of a thermocouple. The highest carrying capacity may be 500-ml/tube. These instruments have a range of interchangeable fixed angle and swing out type rotors.

Uses:-

High speed refrigerated instruments are routinely used to collect microorganisms, cell debris, cells, large cellular organelles, precipitates of chemical reaction and immune precipitates. Although these centrifuges are use full in isolating sub-cellular organelles such as nuclei, mitochondria, lysosome etc., they cannot generate sufficient centrifugal force to effectively sediment smaller organelles such as ribosomes, microsomes and viruses.

Ultracentrifuge:-

The ultra-centrifuges are of two types: preparative and analytical ultracentrifuge. Preparative ultracentrifuge prepares various biological materials in larger quantities while the analytical ultra-centrifuge are used for studying the sedimentation properties of sedimentation of molecular weight.

Preparative Ultracentrifuge:-

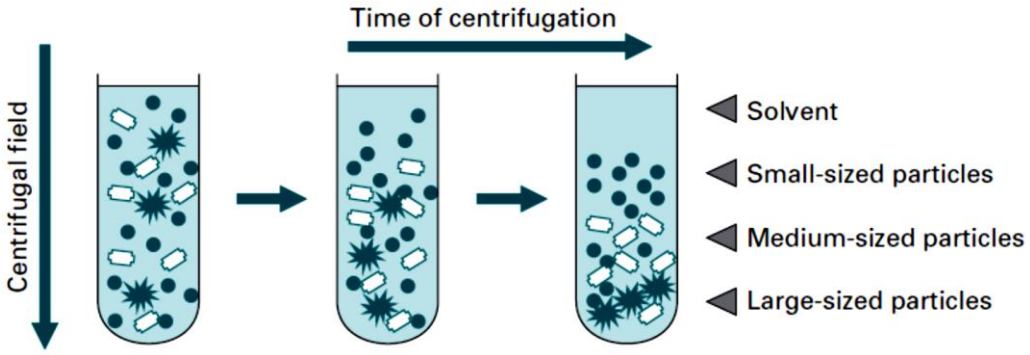
- Preparative ultracentrifuges are capable of spinning rotors to a maximum speed of 80000 rpm and can produce a relative centrifugal field up to 600000 g. The rotor chamber is refrigerated sealed and evacuated to minimized excessive rotor temperatures being generated by frictional resistance between the air and the spinning rotor. Infrared temperature sensor continuously monitors the rotor temperature and control the refrigeration system.
- An over speed control system is also incorporated in to these instruments to prevent operation of rotor above its maximum rated speed and electronic circuits to detect rotor imbalance and minimize the vibration caused by rotor imbalance.

Uses:

1. Isolation of viruses and sedimentation of smaller cellular organelles such as microsomes, ribosome, preparation of cytosol.
2. Purification of variety of sub cellular organelles e.g. mitochondria, liposome, nuclei, plasma membrane etc. by density gradient centrifugation.
3. Separation of nucleic acids DNA and RNA.
4. Tabletop preparative ultracentrifuges are used for samples of small volumes requiring high centrifugal forces. Examples include steroid hormone receptor assays, macromolecules-ligand binding studies, separation of lipoprotein fractions from plasma etc.

Differential centrifugation

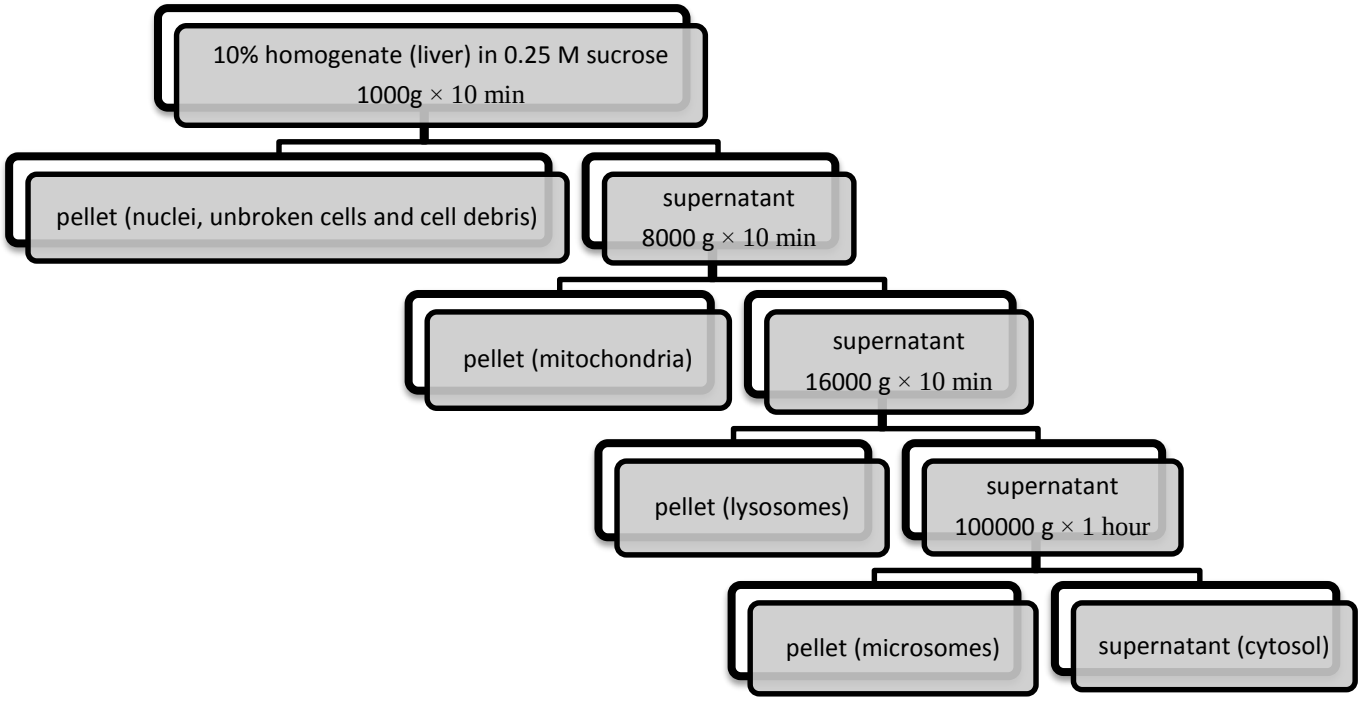
- Cellular and subcellular fractionation techniques are indispensable methods used in biochemical research. Although the proper separation of many subcellular structures is absolutely dependent on preparative ultracentrifugation, the isolation of large cellular structures, the nuclear fraction, mitochondria, chloroplasts or large protein precipitates can be achieved by conventional high-speed refrigerated 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 re-homogenised several times and then re-centrifuged. 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.



One Word Question

Sr. No.	Question	Answer
1	simplest and least expensive centrifuge	Small batch centrifuge
2	High speed centrifuges can operate with the maximum speed of up to_____?	25000 rpm
3	How many types of ultracentrifugation	Preparative and Analytical
4	Preparative ultracentrifuges are capable of spinning rotors to a maximum speed of _____?	80000 rpm
5	Differential Centrifugation Carried out at different	Rpm Value
6	Differential centrifugation is based upon the differences in	Sedimentation Rate

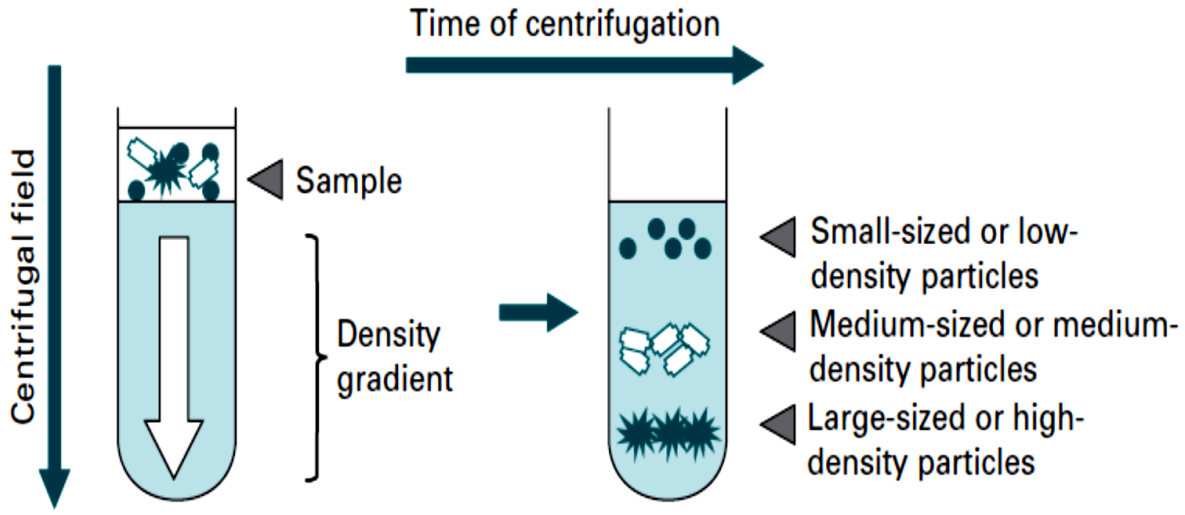
Isolation of sub cellular organelles from rat liver by differential centrifugation:



Density-gradient centrifugation

- To further separate biological particles of similar size but differing density, ultracentrifugation with preformed or self-establishing density gradients is the method of choice.
- Both rate separation and equilibrium methods can be used. In Fig. the preparative ultracentrifugation of low- to high-density particles is shown.
- A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation, is layered on top of a preformed liquid density gradient.
- Depending on the particular biological application, a great variety of gradient materials are available.
- Caesium chloride is widely used for the banding of DNA and the isolation of plasmids, nucleoproteins and viruses. Sodium bromide and sodium iodide are employed for the fractionation of lipoproteins and the banding of DNA or RNA molecules, respectively.
- Various companies offer a range of gradient material for the separation of whole cells and subcellular particles, e.g. Percoll, Ficoll, Dextran, Metrizamide and Nycodenz.
- For the separation of membrane vesicles derived from tissue homogenates, ultra-pure DNase-, RNase and protease-free sucrose represents a suitable and widely employed medium for the preparation of stable gradients.

- If one wants to separate all membrane species spanning the whole range of particle densities, the maximum density of the gradient must exceed the density of the most dense vesicle species. Both step gradient and continuous gradient systems are employed to achieve this.
- If automated gradient makers are not available, which is probably the case in most undergraduate practical classes, the manual pouring of a stepwise gradient with the help of a pipette is not so time-consuming or difficult.
- In contrast, the formation of a stable continuous gradient is much more challenging and requires a commercially available gradient maker.
- Following pouring, gradients are usually kept in a cold room for temperature equilibration and are moved extremely slowly in special holders so as to avoid mixing of different gradient layers.
- For rate separation of subcellular particles, the required fraction does not reach its isopycnic position within the gradient. For isopycnic separation, density centrifugation is continued until the buoyant density of the particle of interest and the density of the gradient are equal.



Density gradient centrifugation has variety of applications in biochemistry:

1. Separation of plasma lipoproteins i.e. chylomicrons, VLDL, LDL, IDL and HDL based on their densities.
2. Purification of sub cellular organelles i.e. nuclei, mitochondria, lysosome, peroxisomes, plasma membrane etc. from the fractions obtained by differential centrifugation.
3. Separation of ribosomal large and small subunits.
4. Separation of nucleic acids: RNA, DNA and RNA-DNA hybrids.
5. Purification of enzymes e.g. cytochrome oxidase from inner membrane of mitochondria.
6. Purification of viruses.

One word question

Sr. No.	Question	Answer
1	Sub cellular organelles can separated by	Differential centrifugation
2	Differential Centrifugation apply for	Biological sample
3	Which medium use for separation of Nucleic acid sample	Caesium chloride
4	Which gradient material use for the separation of whole cells and subcellular particles	Percoll, Ficoll, Dextran
5	Differential Centrifugation gradient developed from	Bottom to top level
6	Which Gradient material are common for sedimentation	Sucrose

Analytical Ultracentrifuge:

- Preparative ultracentrifuges concerns with the preparation of biological materials. Analytical ultracentrifuges are used to study sedimentation characteristics and structure of biological materials.

- Thus, preparative and analytical ultracentrifugation can be said to be mutually fulfilling as the first prepares and the other studies the characteristics of the preparation. Owing to this difference in function the analytical ultracentrifuge differs from a preparative ultracentrifuge as that it has a specially designed rotor and detector system, which helps in studying the progress of the sedimentation in a continuous manner.

- In other aspects the analytical ultracentrifuge is more or less same as the preparative ultracentrifuge. It operates at almost the same RCF; it also refrigerated and has an evacuated rotor chamber.

- The analytical ultracentrifuge has an elliptical rotor with two holes for holding the two centrifuge cells. One cell is known as the analytical cell while the other is known as counterpoise cell counter balance cell. The upper and lower planes of the analytical cell have quartz window for the passage of light to monitor progress of centrifugation.

- The optical used in an analytical ultracentrifuge might be either Schlieren (UV absorption) optical or Rayleigh interference optics. The Rayleigh interference operates on the basis that the region of the solution in the analytical cell harboring macromolecules will have a higher refractive index than the rest of the solution.

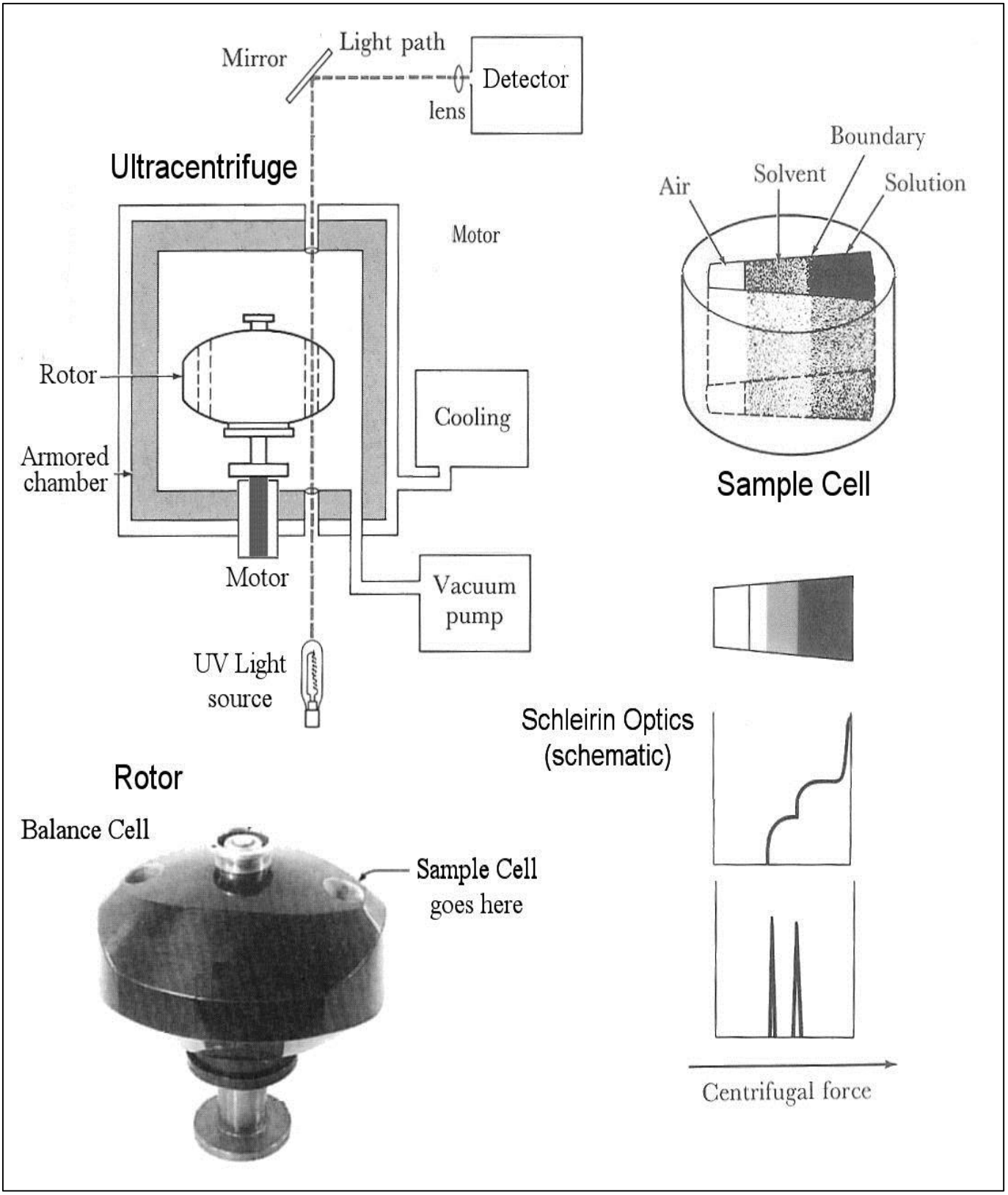
- With the progress in sedimentation, the macromolecules move down the cell and the peak also shift giving direct information about sedimentation characteristics of the macromolecules.

Uses:-

1. Determination of molecular weight of macromolecules could be done by the sedimentation velocity or sedimentation equilibrium method. The molecular weight of biology active species can be measured by using the analytical ultracentrifuge even when they are in a gross mixture.

2. Estimation of homogeneity of macromolecules. If a solution is homogenous i.e. it contain only one species of macromolecules, only one sedimenting peak will be observed when the ultracentrifuge is rotated at higher at higher speeds. If the solution is not homogenous and contain impurities, more than one peak will be observed. Purity of DNA preparations, proteins, enzymes and viruses has been tested using sedimentation velocity method.

3. Studies relating to macromolecular conformation. The rate of sedimentation will change in conformation of the macromolecules. This technique is used to study conformational change in macromolecules like proteins and DNA. E.g. denaturation of proteins, aggregation-dissociation of proteins, conformational change in allosteric proteins and conformational change in DNA etc. can be studied by analytical ultracentrifugation.



One word question

Sr. No.	Question	Answer
1	Analytical ultracentrifuges are used to study of	Sedimentation and structure
2	The analytical ultracentrifuge has an_____ rotor.	elliptical
3	The Sedimentation rate can be measured in Analytical centrifugation by_____?	UV Visible light
4	Analytical ultracentrifuges give the sedimentation Rate in_____?	Graphical
5	Analytical ultracentrifuges technique is used to study	conformational change in macromolecules