Centrifugation

Biochemical Techniques

SEC PAPER: SEM III

References

1. Boyer, R. F. (2012) Biochemistry Laboratory: Modern Theory and Techniques, (6 th ed.), Boston, Mass: Prentice Hall; ISBN-13: 978-0136043027.
2. Plummer, D. T. (1998) An Introduction to Practical Biochemistry (3rd ed.), Tata McGraw Hill Education Pvt. Ltd. (New Delhi); ISBN: 13: 978-0-07-099487-4 / ISBN:10: 0-07-099487-0.
3. Wilson, K. & Walker J (2010) Principles and Techniques of Biochemistry and Molecular Biology, (7 th ed.), Cambridge University Press; ISBN 978-0-521-51635-8.

Additional Reading

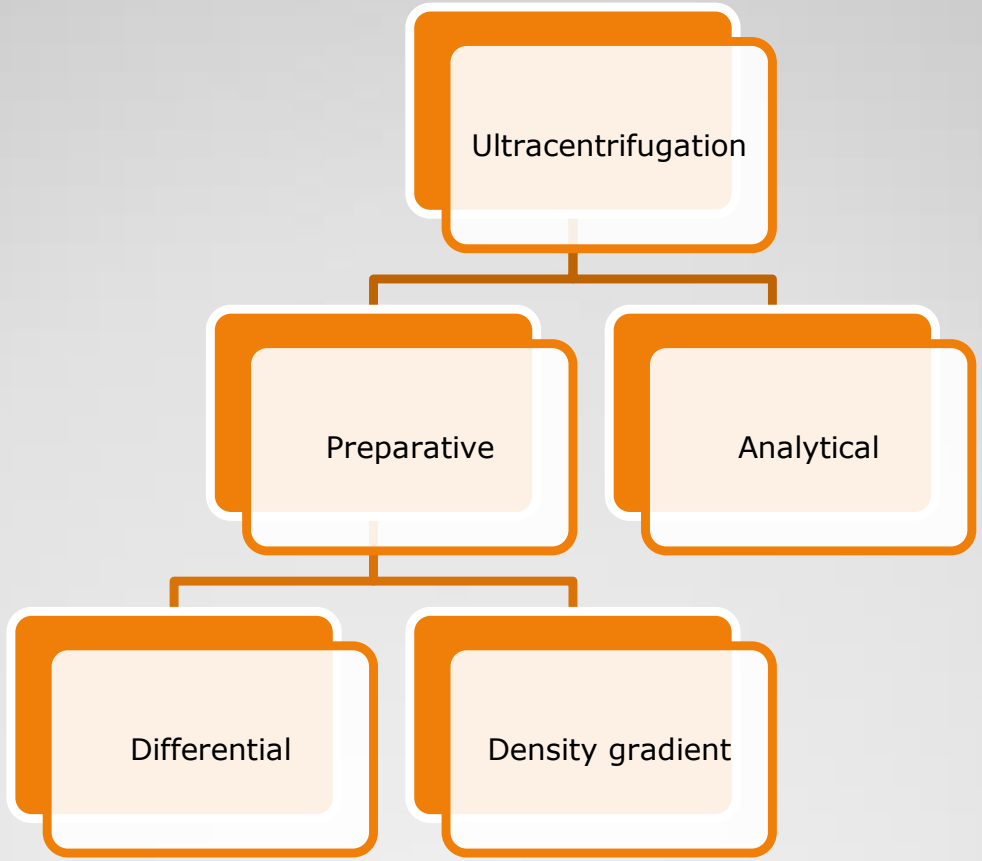
1. Cooper, T. G. (2011) The Tools of Biochemistry (2nd ed.), Wiley-Interscience Publication (New Delhi); ISBN: 13:9788126530168.
2. Freifelder, D. (1982) Physical Biochemistry: Applications to Biochemistry and Molecular Biology, (2 nd ed.), W.H. Freeman and Company (New York); ISBN:0-7167-1315-2 / ISBN:0-7167-1444-2.

- It is a process used to separate of concentrate materials suspended in a liquid medium based on their sedimentation rate under centrifugal field.
- Sample is placed in a vessel and under the influence of centrifugal force these particles sediment.
- The greater the mass of the substance faster it sediments under the influence of centrifugal force.
- In ultracentrifugation rotors rotate at very high speed ranging from 60,000 RPM and 200,000 x g to 150,000 RPM and 1,000,000 x g generating very high centrifugal force.

CentrifugationFactors determining sedimentation rate

- Rate at which rotor spins
- Time for which the force is applied
- Solvent density
- System temperature

Ultracentrifugation



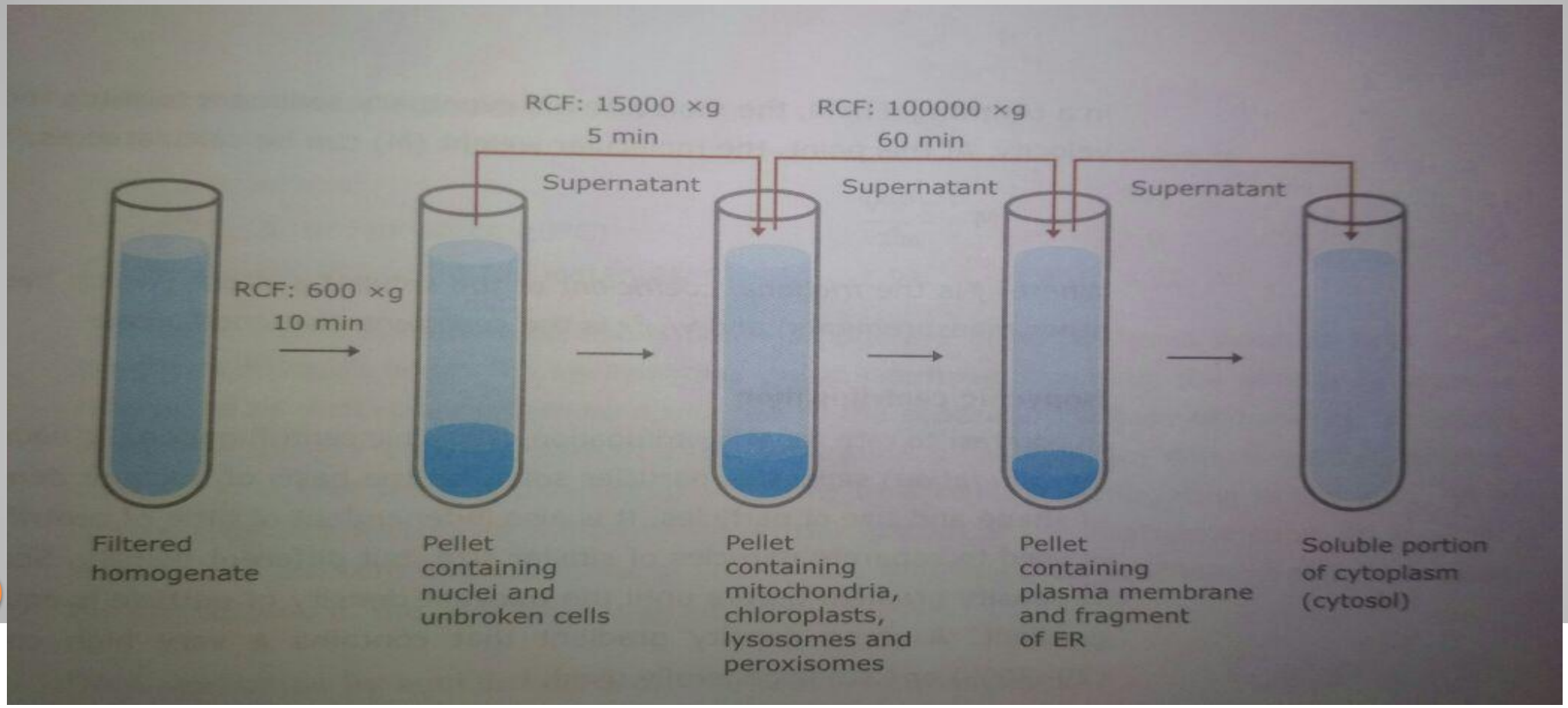
Preparative Ultracentrifugation

- Separation of one type of particles from other particles. For example: cellular organelles
- It is commonly used to purify the substances so that further studies can be performed upon them.
- Divided into two subtypes – differential and density gradient.
- Depends on centrifugal force, shape and size of particles and their density.

Differential centrifugation

- The separation is based upon the sizes and densities of particles.
- Mainly used for sub cellular fractionation.
- Denser and larger organelles sediment first in the pellet at comparatively low RCF and less time applied, supernatant contains other lighter organelles which are further centrifuged.
- With increasing RCF and time organelles separate according to their density and size.

D


RCF: 15000 ×g
5 min
RCF: 100000 ×g
60 min
Supernatant
Supernatant
Supernatant
RCF: 600 ×g
10 min
Filtered homogenate
Pellet containing nuclei and unbroken cells
Pellet containing mitochondria, chloroplasts, lysosomes and peroxisomes
Pellet containing plasma membrane and fragment of ER
Soluble portion of cytoplasm (cytosol)

Density gradient Centrifugation

- It is a variation of differential centrifugation in which the sample is centrifuged in a medium that gradually increases in density from top to bottom.
- Gradient consists of increasing concentration of solute from top to bottom.
- It is further divided into two types:
  - Rate zonal centrifugation (gradient is used)
  - Isopycnic (gradient forms itself)

Gradient material for DGC*

- It should be stable in solution
- Totally inert
- Not absorb UV or visible light.
- Easily removed/separated
- Inexpensive and readily available.

Rate zonal Centrifugation

- Particles are separated in terms of their molecular masses but shape also contributes to the difference in sedimentation rate.
- The sample solution is layered on top, as the force is applied the particles sediment in distinct zones matching their sedimentation rate.
- Variety of materials are used as the gradient material for example ficol (PBMC), glycerol, sucrose etc.
- This method can be used to determine molecular weight of particles.
- It is also called velocity centrifugation

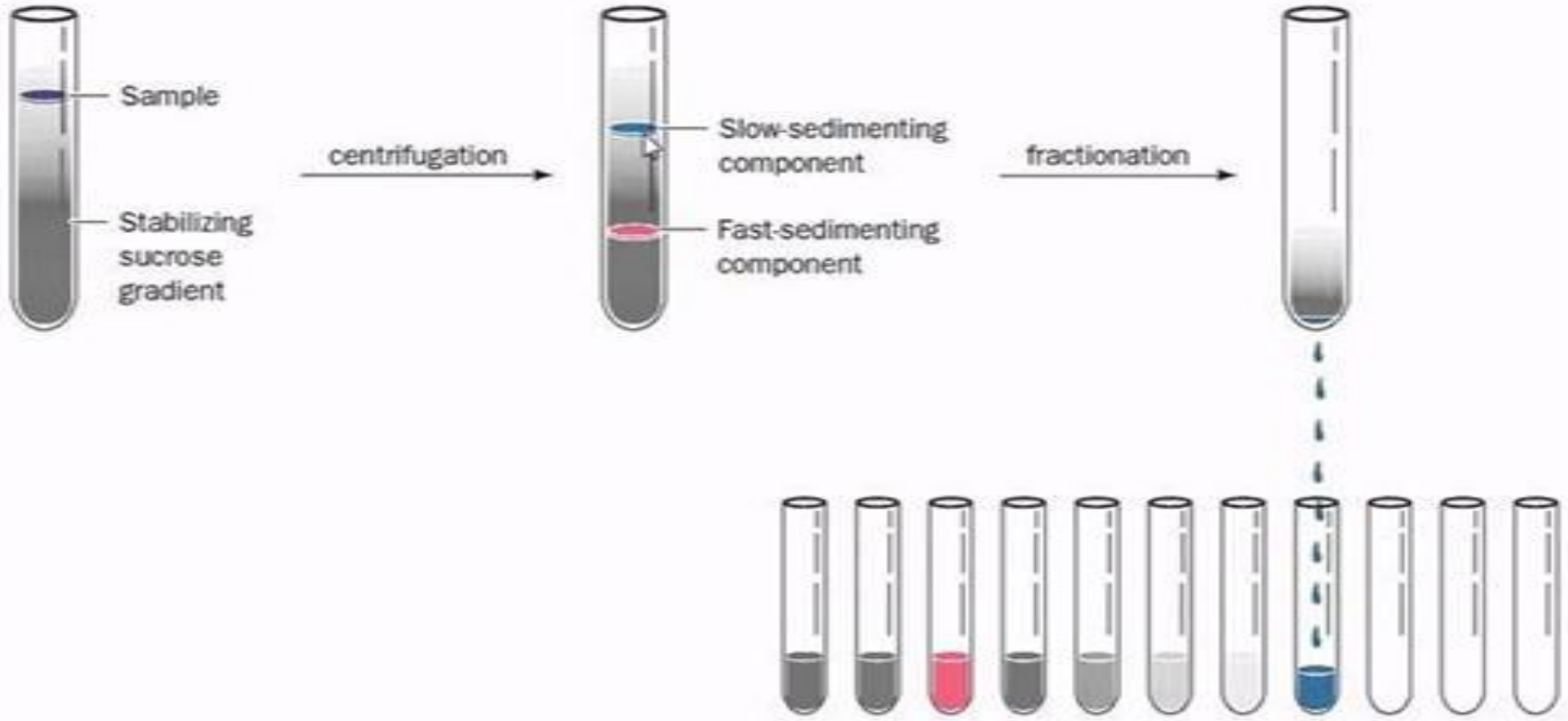


Figure 6-30 **Zonal ultracentrifugation.** The sample is layered onto a sucrose gradient (*left*). During centrifugation (*middle*), each particle sediments at a rate that depends largely on its mass. After the end of the run, the centrifugation tube is punctured and the separated particles (zones) are collected (*right*).

RNA isolation using Manual Method

1. Homogenize the tissue thoroughly in 500 µl of Trizol (phenol + Guanidinium chloride).
2. Keep at room temperature for 5-10 mins.
3. Add 200 µl of chloroform and mixed thoroughly; leave at room temperature for 10 mins.
4. Centrifuge at 13k at 4^{o}C for 15 mins and transfer the (aqueous phase) supernatant to a fresh tube.
5. Add 500 µl of isopropanol, mix well and store in -20^{o}C for 2 hrs to precipitate the RNA.
6. Centrifuge at 13k for 10 minutes at 4^{o}C. Discard the supernatant.
7. Add 500 µl of 70% ethanol to the pellet and mix by inverting.
8. Centrifuge at 13k for 10 minutes at 4^{o}C. Discard the supernatant and air dry the pellet.
9. The pellet can be dissolved in nuclease free water.
10. RNA can be checked on agarose gel electrophoresis for quality. If DNA contamination is present treat the RNA with DNase at 37 ^{o}C for 30 minutes.
11. RNA can be quantified using nanodrop and nuclease free water as blank.

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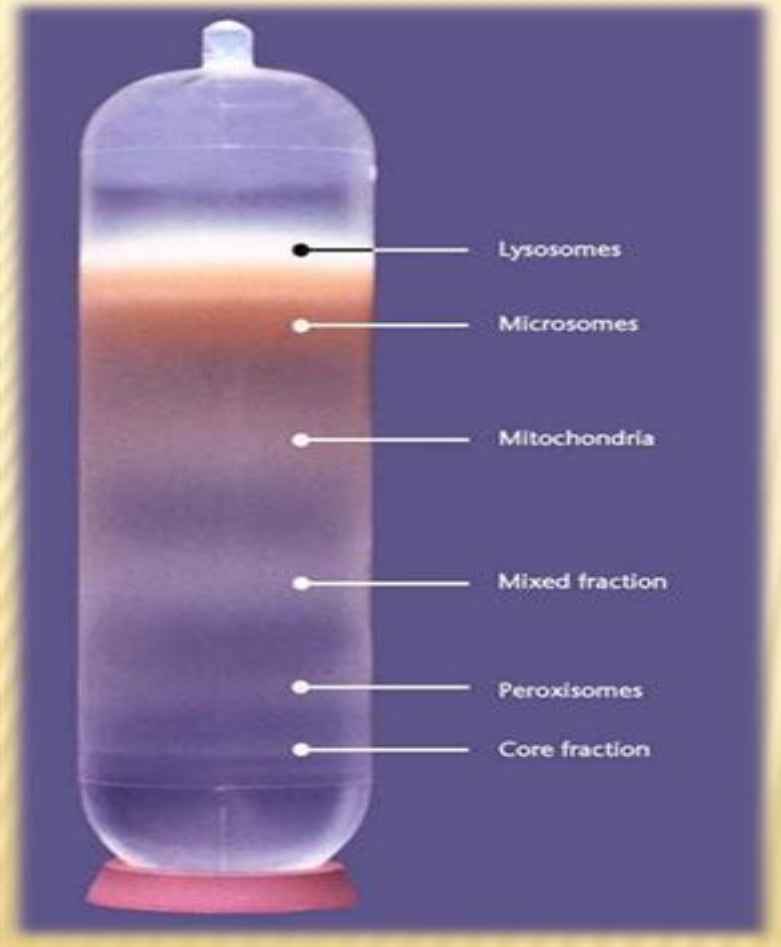
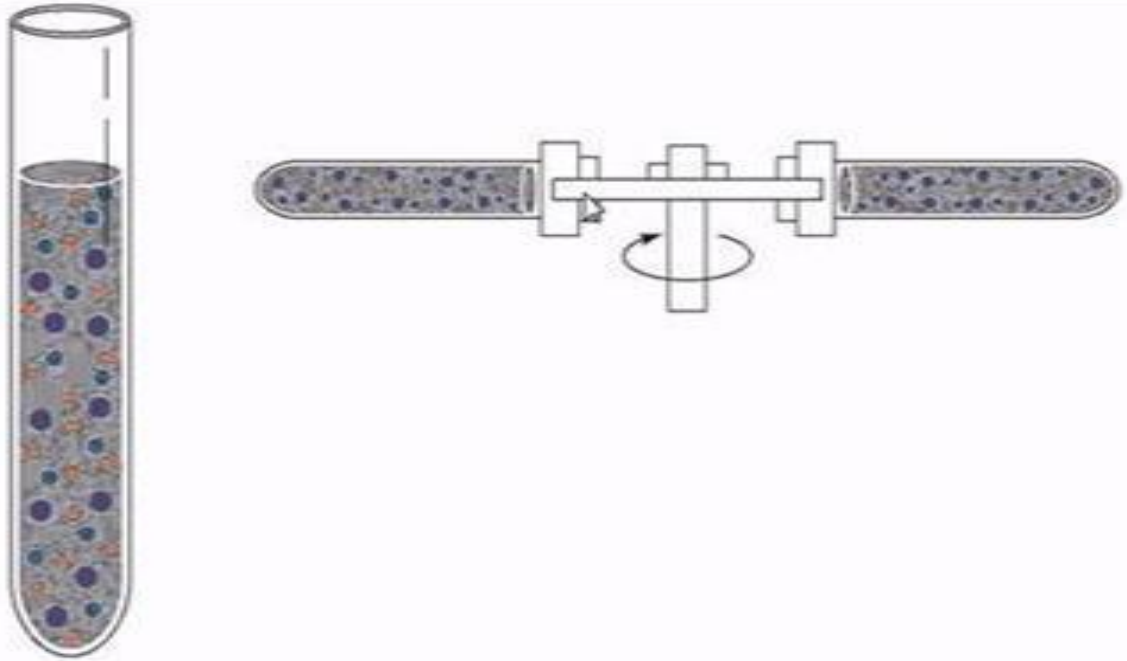


Photograph of an actual tissue separation: sub fractionation of a rat hepatic light mitochondrial fraction by rate zonal centrifugation using a vertical rotor.

Isopycnic centrifugation

- Isopycnic means of the same density
- Independent of shape and size of particle solely depends on its buoyant density.
- It is also independent of time.
- Sedimentation of particles occurs only when the buoyant density of that particle is exactly same to the density of the gradient at that zone.
- Used to separate molecules of similar sizes but different densities.
- A very high density solute is used as the medium. (CsCl)



1. Uniform mixture of sample and gradient-forming substance

2. Centrifugation

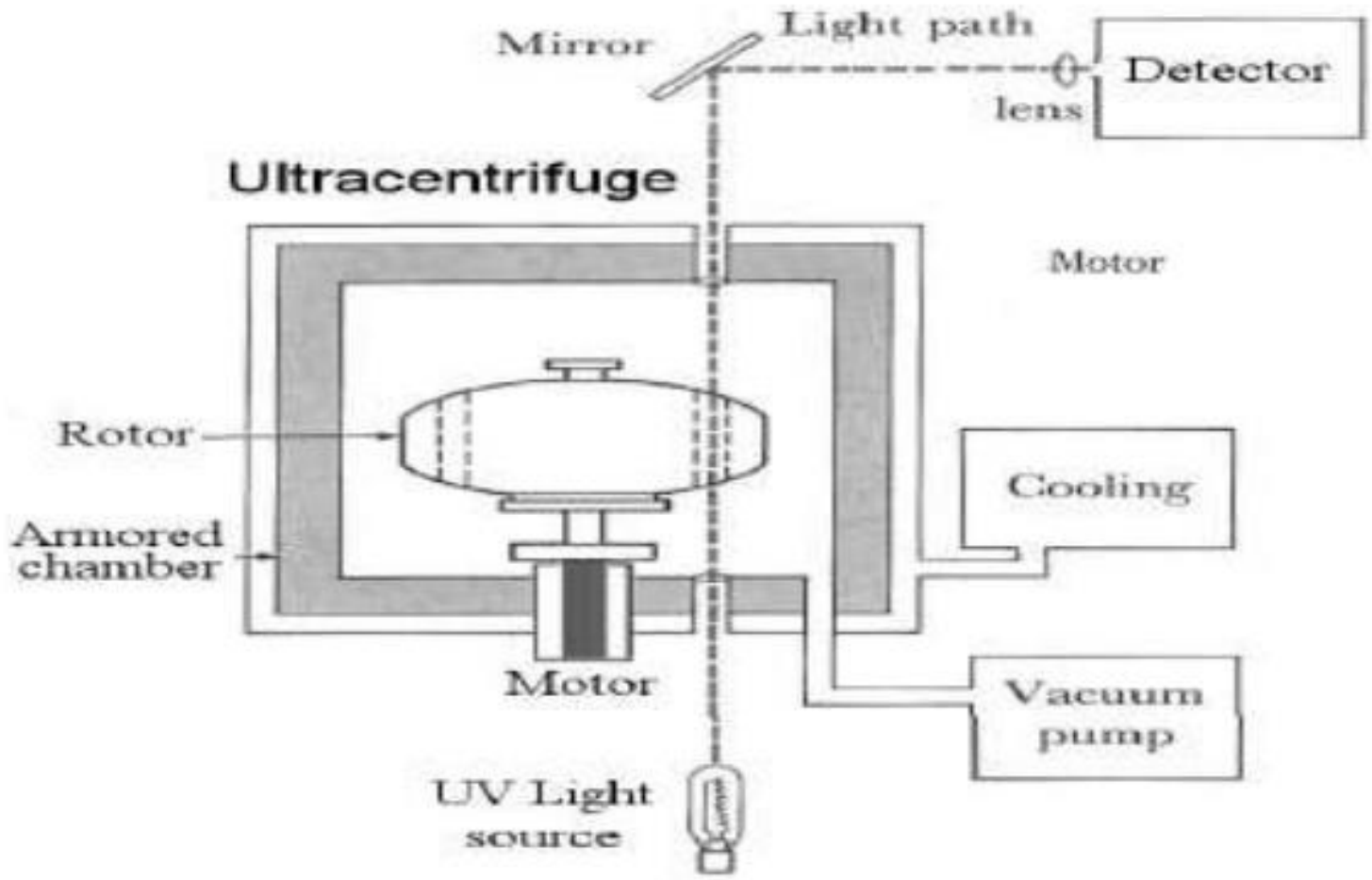
3. Gradient is formed and samples band at their isopycnic positions

Figure 6-32 **Isopycnic ultracentrifugation.** The centrifugation starts with a uniform mixture of a macromolecular sample dissolved in a solution of a dense, fast-diffusing solute such as CsCl (*left*). At equilibrium in a centrifugal field, the solute forms a density gradient in which the macromolecules migrate to their positions of buoyant density (*right*).

Analytical ultracentrifugation

- Basically used to study the behavior of macromolecules in a solution under the influence of RCF and interpret about size, shape, and molecular mass of the molecule.
- It is a centrifuge with one or several optic system to study the macromolecules while its being centrifuged.
- Commercially used AUC is Beckman- coulter AUC.

Schematic presentation of a Ultracentrifuge:



Fig; A Beckman Ultracentrifugation.

Applications of AUC

- Determine number of components and number of species; detection of impurities
- Molar mass of each species
- Kinds and stoichiometry of chemical reactions present in solution, including association with ligands, self-association.
- Shape and charge of the molecules, as inferred from their sedimentation frictional behavior.

Kind of experiments of AUC

- 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.
- 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 con centration profile. Used for determination of equilibrium constant for reversible interacting system.

Rotor

- Four types of rotors are available for ultracentrifugation
  - Fixed-angle rotor
  - Swinging-bucket rotor
  - Vertical rotor
  - Near-vertical rotor

  Rotors are made from either aluminum or titanium, or from fiber-reinforced composites.

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