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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF ENGINEERING & TECHNOLOGY

LT27.Preparative and Analytical centrifuge

Content Outline

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2. Density gradient centrifuge
3. Rate Separation / Rate zonal centrifugation
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Preparative Centrifuge

Preparative centrifugation

Preparative ultracentrifugation is operated at relative centrifugal fields of up to 9,00,000 g. Preparative rotors are used for pelleting of cellular organelles such as mitochondria, microsomes, ribosomes and viruses. They are also be used for sucrose gradient separation of cellular organelles and caesium salt gradient separation of nucleic acids. After the centrifugation, the rotor is allowed to stop and the gradient is gently pumped out of each tube to isolate the separated components. There is no optical read-out to collect fractions and analyze after each run. The organelles and molecules are separated on the basis of their **sedimentation velocities** or **buoyant densities.**

Categories of preparative centrifugation

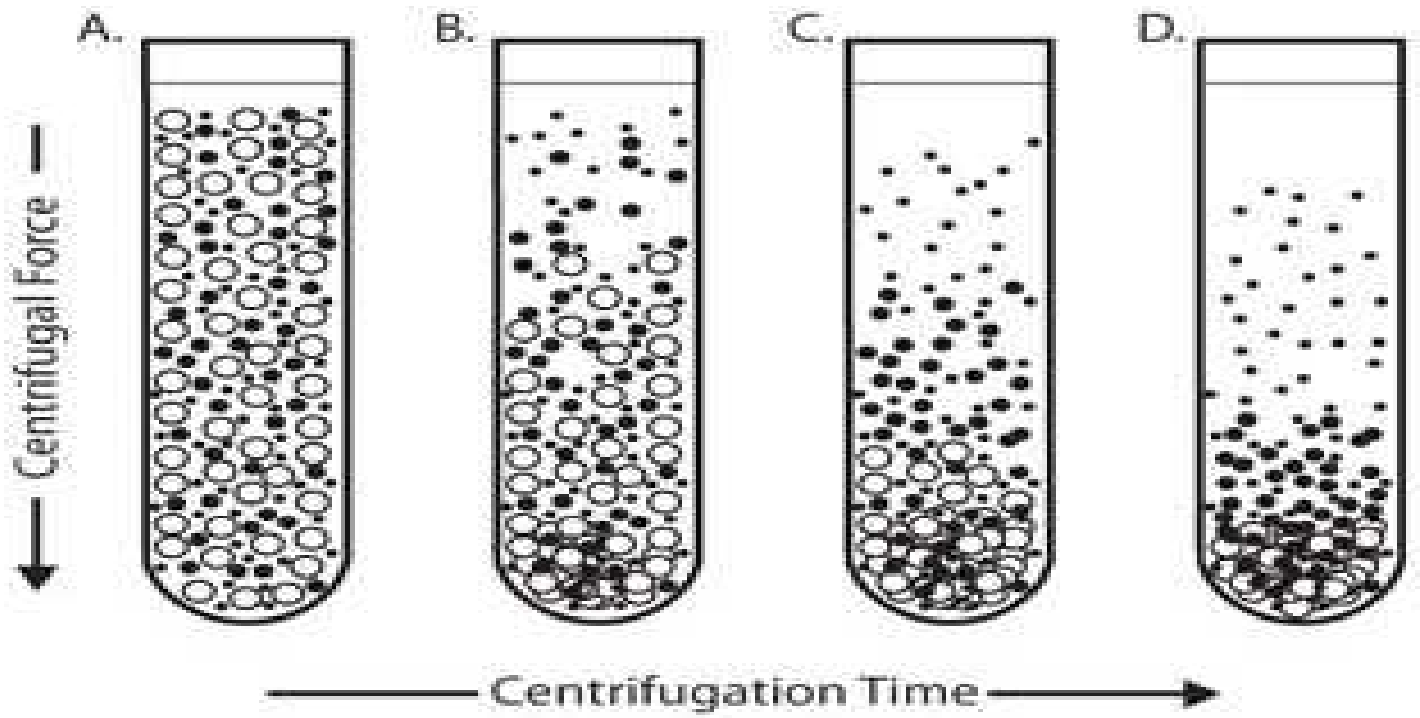
(a) Differential centrifugation

Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density. This mode is basically used for isolation of larger cellular structures, the nuclear fraction, mitochondria, chloroplasts or large protein precipitates.

•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. Initially all particles of a homogenate are evenly distributed throughout the centrifuge tube and then move down the tube at their respective sedimentation rate during centrifugation.

•Differential pelleting is commonly used for harvesting cells or producing crude subcellular fractions from tissue homogenate. For example, a rat liver homogenate containing nuclei, mitochondria, lysosomes, and membrane vesicles that is centrifuged at low speed for a short time will pellet mainly the larger and more dense nuclei. Subsequent centrifugation at a higher centrifugal force will pellet particles of the next lower order of size (e.g., mitochondria) and so on. It is unusual to use more than four differential centrifugation cycles for a normal tissue homogenate.

•Due to the heterogeneity in biological particles, differential centrifugation suffers from contamination and poor recoveries. Contamination by different particle types can be addressed by resuspension and repeating the centrifugation steps (i.e., washing the pellet)



Differential Centrifugation; Particles of different densities or size will sediment at different rates with the largest and most dense particles sedimenting the fastest followed by less dense and smaller particles

(b) Density gradient centrifugation

To 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.

Rate separation / Rate Zonal Centrifugation

As the name signifies, different-sized molecules will occupy different zones in a centrifuge tube after cen-trifugation. This separation of macromolecules at different zones is stabilized by using Sucrose gradient. So, this technique is also known as Sucrose Density Gradient Centrifugation. This is used for separating all types of particles and organelles.

•The speed at which particles sediment depends primarily on their size and mass instead of density. As the particles in the band move down through the density medium, zones containing particles of similar size form as the faster sedimenting particles move ahead of the slower ones. Because the density of the particles is greater than the density of the gradient, all the particles will eventually form a pellet if centrifuged long enough.

In analytical ultracentrifuge the rate of sedimentation can be measured by taking photographs of the moving boundaries of sedimenting particles. To separate particles of same densities, they are labelled and then centrifuged in Sucrose density gradient. Particles labelled with H^3 have the greater mass and sediment faster than those labelled with C^{14}

Equilibrium Density Gradient Centrifugation (Isopycnic density gradient)

The second type of density gradient cen-trifugation is known as Equilibrium Density Gradient Centrifugation where the density gradient is formed during centrifuga-tion. In isopycnic separation, also called buoyant or equilibrium separation, particles are separated solely on the basis of their density. Particle size only affects the rate at which particles move until their density is the same as the surrounding gradient medium. The density of the gradient medium must be greater than the density of the particles to be separated. By this method, the particles will never sediment to the bottom of the tube, no matter how long the centrifugation time.

•The material used in this process is the aqueous solution of Caesium chloride (CsCl). 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. Ficoll (copolymer of Sucrose and epichlorhydrin) is used for the separation of whole cells and cellular organelles.

•A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation,is layered on top of a preformed liquid density gradient. The densities of protein, DNA and RNA are 1.3, 1.6 to 1.7 and 1.75 to 1.8 g/ml, respec-tively. The same chemical CsCl can separate the different macromolecules like DNA and RNA due to the fact that Cs^+ binds to DNA at

phosphate groups, while it binds to RNA both at phosphates and at the hydroxyl groups of sugar thus increasing the density of RNA more than that of DNA.

Table 7.2: Use of some common gradient chemicals

Chemicals	Ionic strength	Density of aqueous solution $g.cm^{-3}$	Common uses
Caesium chloride	High	1.91	Separating DNA, nucleoproteins, viruses, isolation of plasmid
Caesium sulphate	High	2.01	Separation DNA, RNA, etc.
Sodium bromide	High	1.53	Fractionation of Lipoproteins
Glycerol	Non-ionic	1.26	Separation of membrane fragments, protein
Sucrose	Non-ionic	1.32	Separation of subcellular particles, proteins, nucleic acids etc.
Ficoll	Non-ionic	1.17	Separation of cells, nucleic acids, organelles etc.
Dextran	Non-ionic	1.13	Cells, microsomes etc.
Percoll	Non-ionic	1.30	Cells and organelles
Metrizamide	Non-ionic	1.46	Cells, organelles, nuclei membrane
Nycodenz	Non-ionic	1.42	Cells, organelles, membranes, nucleoproteins, viruses etc.

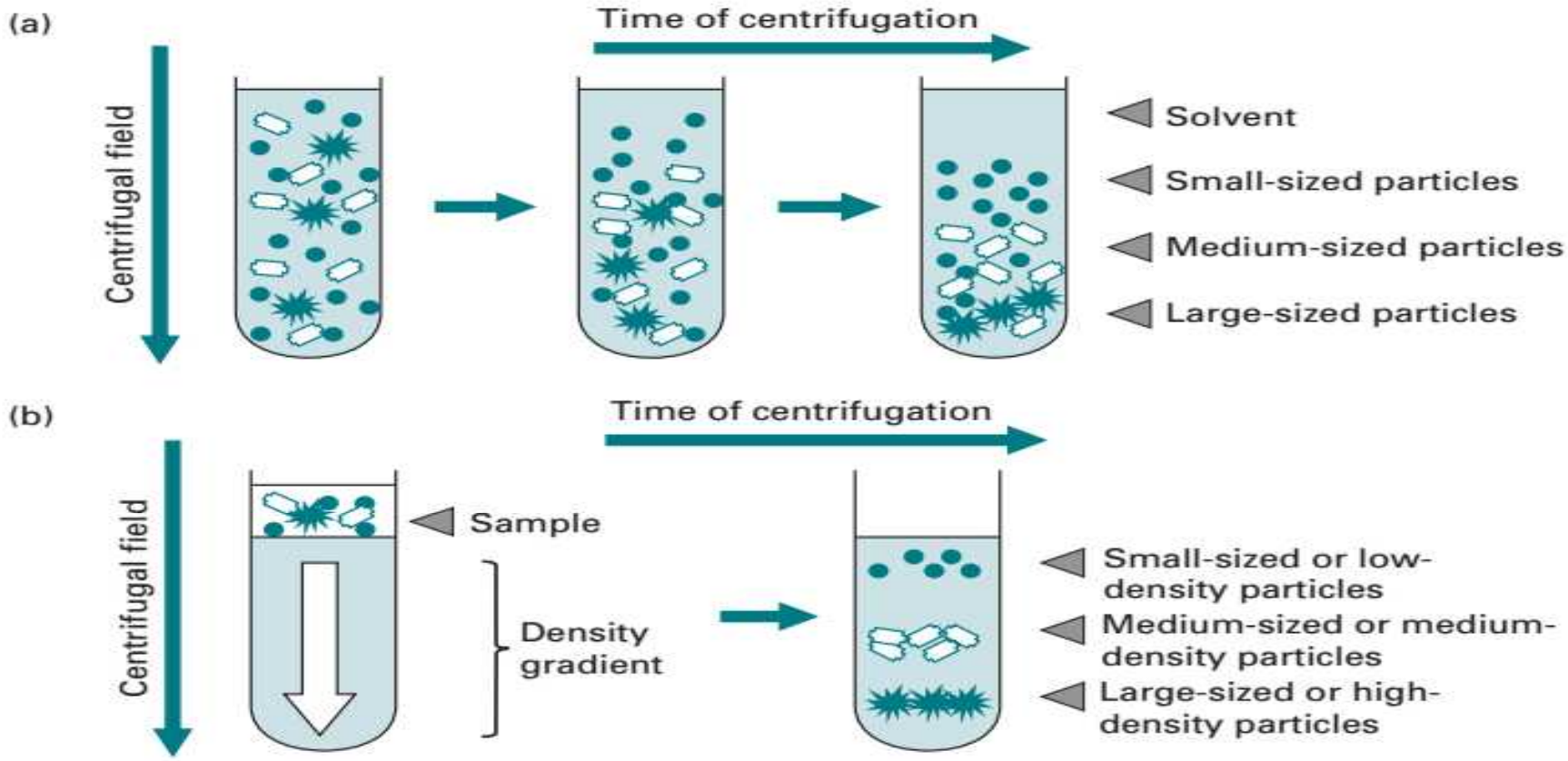


Diagram of particle behaviour during differential and isopycnic separation. During differential sedimentation (a) of a particulate suspension in a centrifugal field, the movement of particles is dependent upon their density, shape and size. For separation of biological particles. using a density gradient (b), samples are carefully layered on top of a preformed density gradient prior to centrifugation. For isopycnic separation, centrifugation is continued until the desired particles have reached their isopycnic position in the liquid density gradient. In contrast, during rate separation, the required fraction does not reach its isopycnic position during the centrifugation run.

Analytical centrifugation

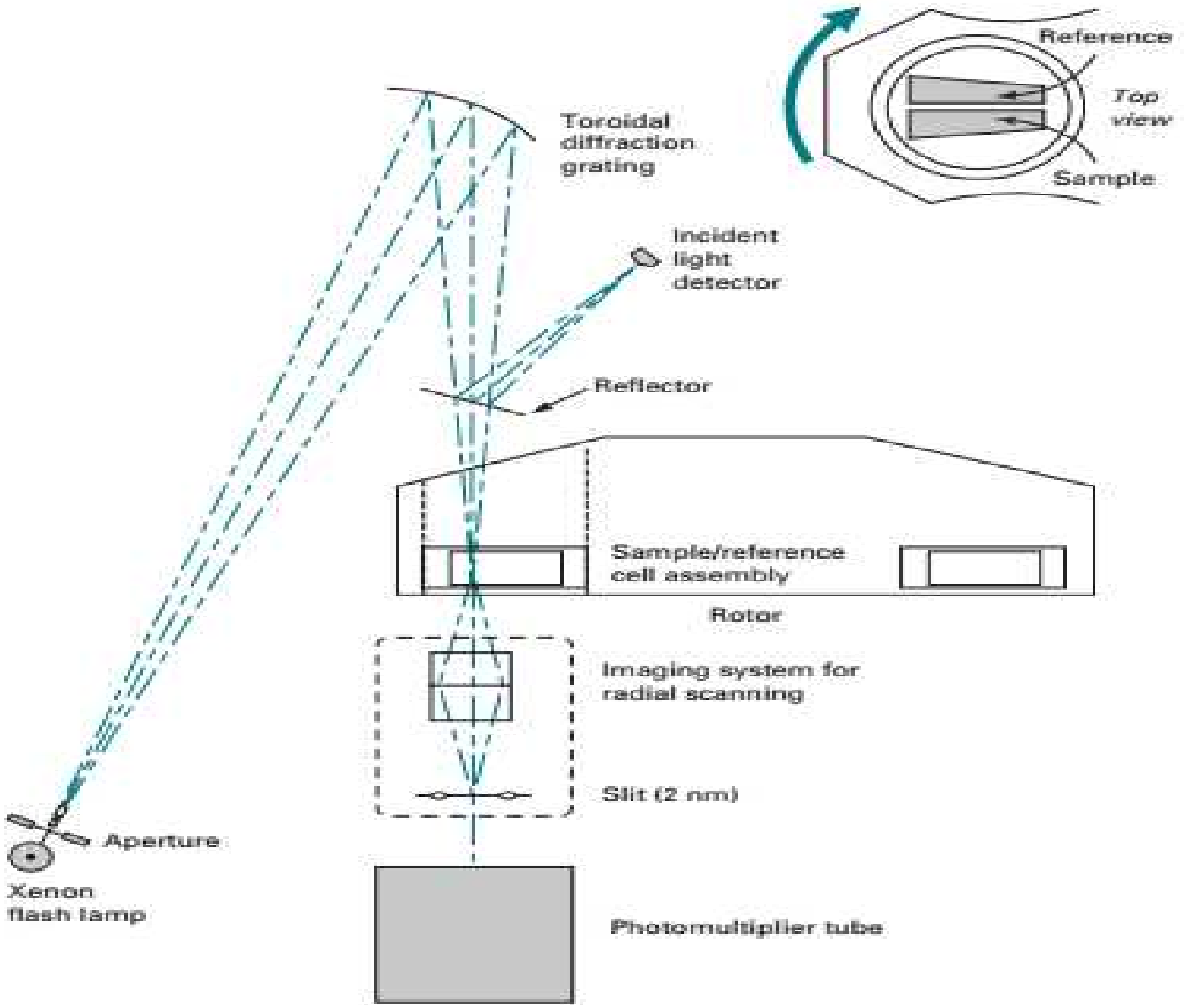
This instrument has many applications in the fundamental studies of macromolecules show-ing the molecular weight, purity and shape of the material. Isolated biomolecules in solution only exhibit distinguishable sedimentation when they undergo immense accelerations, e.g. in an ultracentrifugal field. A typical analytical ultracentrifuge can generate a centrifugal field of 250 000 g in its analytical cell. Within these extremely high gravitational fields, the ultracentrifuge cell has to allow light passage through the biological particles for proper measurement of the concentration distribution. The availability of high-intensity xenon flash lamps and the advance in instrumental sensitivity and wavelength range has made the accurate measurement of highly dilute protein samples below 230 nm possible.

Working

•There is an arrangement of a special optical system to determine the concentration distributions within the sample during centrifugation.

•There are two special optical cells on the rotor, known as the Ana-lytical cell and the Counterpoise cell. There are two holes (Reference holes) in the counterpoise cell for the calibration of distances in the analytical cell. The rotor chamber has an upper and lower lens and the upper lens is joined with a camera lens which emits lights on the photographic plate. Light from the light source comes through the bottom. The principle of monitoring in this system is done either through the ultraviolet absorption system or by noting the differences in the refrac-tive index. If the concentration is uniform, light passes through it without any deviation. But if the light passes through a solution of different density zones, it is refracted at the boundary between these zones.

•By measuring the re-fractive index between the reference solvent and the solution, the concentration of solute at any point can be measured. In recent models, the photographic plate system has been replaced by electronic scanning system which can directly measure and plot the concentration of the sample at all points in the analytical cell.


Reference
Top view
Sample
Toroidal diffraction grating
Incident light detector
Reflector
Sample/reference cell assembly
Rotor
Imaging system for radial scanning
Slit (2 nm)
Aperture
Xenon flash lamp
Photomultiplier tube

Usage of analytical centrifuge

Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a sedimentation velocity approach or sedimentation equilibrium methodology. The hydrodynamic properties of macromolecules are described by their sedimentation coefficients and can be determined from the rate that a concentration boundary of the particular biomolecules moves in the gravitational field. Such studies on the solution behaviour of macromolecules can give detailed insight into the properties of large aggregates and thereby confirm results from biochemical analyses on complex formation.

Further Analytical ultracentrifugation is most often employed in following applications

- the determination of the purity of macromolecules;
- the determination of the relative molecular mass of solutes in their native state;
- the examination of changes in the molecular mass of supramolecular complexes;
- the detection of conformational changes; and in ligand-binding studies

References & Further reading

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