PREPARATIVE CENTRIFUGATION

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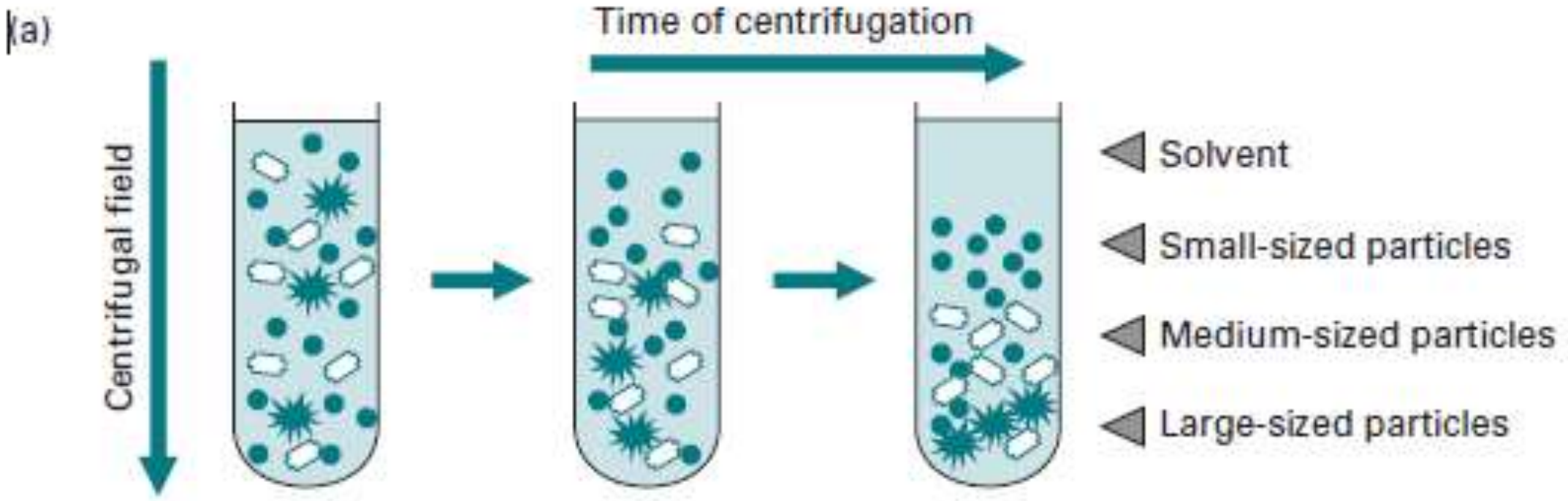
Differential centrifugation

- 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 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.

During differential sedimentation (a) of a particulate suspension in a centrifugal field, the movement of particles is dependent upon their density, shape and size.



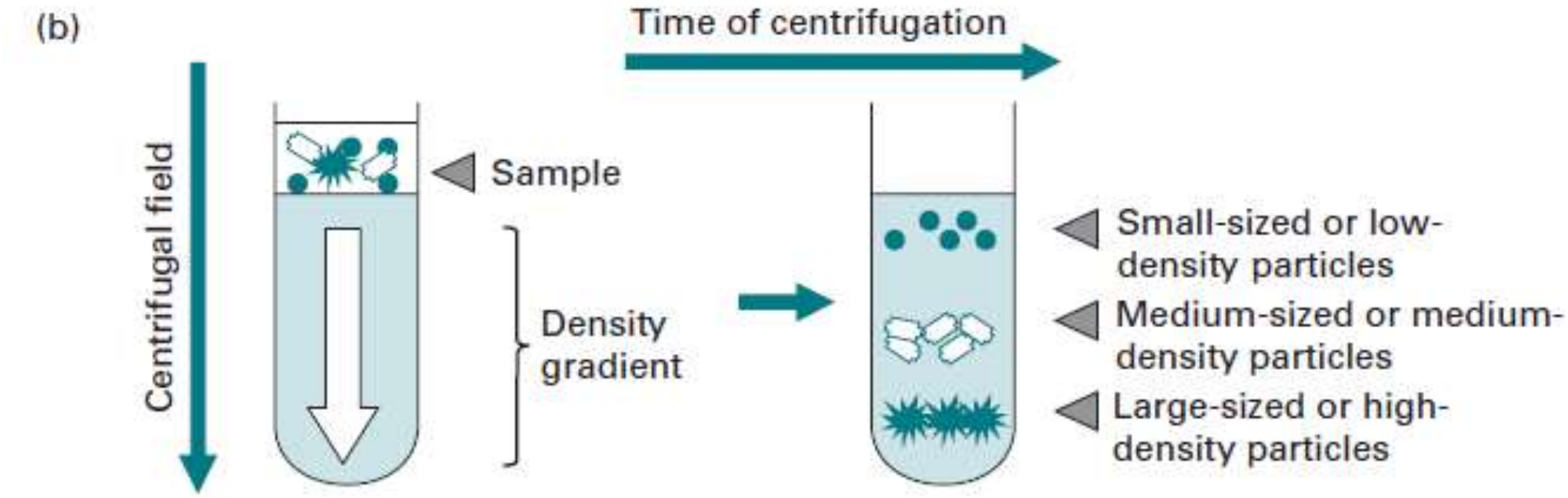
… Differential centrifugation

- 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.
- The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.
- However, during the initial centrifugation step smaller particles also become entrapped in the pellet causing a certain degree of contamination.
- At the end of each differential centrifugation step, the pellet and supernatant fraction are carefully separated from each other.
- To minimise cross-contamination, pellets are usually washed several times by resuspension in buffer and recentrifugation under the same conditions.
- However, repeated washing steps may considerably reduce the yield of the final pellet fraction, and are therefore omitted in preparations with limiting starting material.
- Resulting supernatant fractions are centrifuged at a higher speed and for a longer time to separate medium-sized and small-sized particles.
- With respect to the separation of organelles and membrane vesicles, crude differential centrifugation techniques can be conveniently employed to isolate intact mitochondria and microsomes.

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 or equilibrium methods can be used.
- In Fig. 3.4b, 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 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.



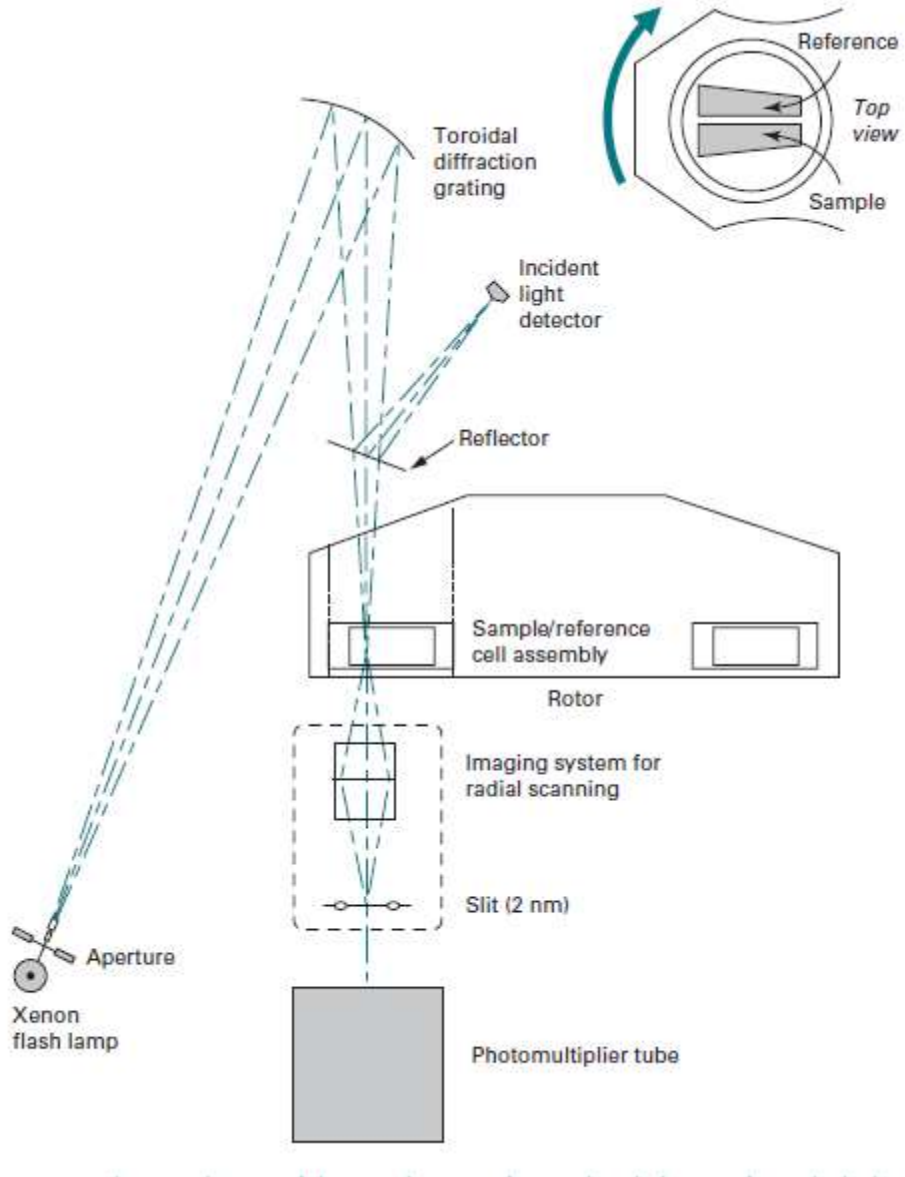
... Density-gradient centrifugation

- 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.

ANALYTICAL CENTRIFUGATION

- As biological macromolecules exhibit random thermal motion, their relative uniform distribution in an aqueous environment is not significantly affected by the Earth's gravitational field.
- 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.
- 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.
- Analytical ultracentrifugation is most often employed in
    - 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

Schematic diagram of the optical system of an analytical ultracentrifuge.



- The sedimentation velocity method can be employed to estimate sample purity.
- This method measures the refractive index gradient at each point in the ultracentrifugation cell at varying time intervals.
- **Relative molecular mass determination**
- For the accurate determination of the molecular mass of solutes in their native state, analytical ultracentrifugation represents an unrivalled technique.
- At the start of an experiment using the boundary sedimentation method, the biological particles are uniformly distributed throughout the solution in the analytical cell.
- The application of a centrifugal field then causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the centre of rotation.
- The solvent that has been cleared of particles and the solvent still containing the sedimenting material form a sharp boundary.
- The movement of the boundary with time is a measure of the rate of sedimentation of the biomolecules.
- The sedimentation coefficient depends directly on the mass of the biological particle.
- The movement of biomolecules in a centrifugal field can be determined and a plot of the natural logarithm of the solute concentration versus the squared radial distance from the centre of rotation yields a straight line with a slope proportional to the monomer molecular mass.

- Alternatively, the relative molecular mass of a biological macromolecule can be determined by the **band sedimentation technique.**
- In this case, the sample is layered on top of a denser solvent.
- During centrifugation, the solvent forms its own density gradient and the migration of the particle band is followed in the analytical cell.
- Molecular mass determination by analytical ultracentrifugation is applicable to values from a few hundred to several millions.
- It is therefore used for the analysis of small carbohydrates, proteins, nucleic acid macromolecules, viruses and subcellular particles such as mitochondria.