MARUDHAR KESARI JAIN COLLEGE FOR WOMEN VANIYAMBADI
PG AND RESEARCH DEPARTMENT OF BIOCHEMISTRY
E-NOTES

COLLEGE CODE: 318
SEMESTER : I
SUBJECT CODE: GOBC 15 A
CLASS :I M.Sc.,NFSM&D,CHEMISTRY
SUBJECT NAME: NME-BIOINSTRUMENTATION

SYLLABUS

UNIT-II **Centrifugation Techniques**

Centrifugation,Svedberg's Constant, Sedimentation velocity, Sedimentation equilibrium,Differential Density Gradient Centrifugation,Construction of Preparative and analytical ultra centrifuge

Ultracentrifugation

Introductory article

Article Contents

High-speed (ultra)centrifugation for fractionation of organelles and molecular components of tissues permits the elucidation of fundamental cellular processes such as oxidative phosphorylation, intracellular digestion, protein synthesis, bulk transport or biogenesis of organelles. Preparative centrifugation allows isolation of specific particles such as subcellular organelles, and analytical centrifugation differentiates the properties of particles and allows study of interactions between macromolecules.

Centrifugation, a Pivotal Method in Cell and Molecular Biology

Cell biology deals with the exploration of cells and tissues at both the morphological and biochemical levels, aiming to elicit the molecular mechanisms operating in any living organism. The biochemical analysis of biological samples by means of refined physical instruments and their examination using the plethora of microscopical techniques available are cornerstones for constructing a true cell biology. Yet tissue fractionation has to link the visible and the measurable to create a unified picture of the living cell. Miescher is supposed to be the first to use a centrifuge for the isolation of a cell organelle when he separated nuclei from human pus cells (Miescher, 1871). Another landmark in the history of tissue fractionation was the construction of the first high-speed centrifuge by Svedberg, and the elaboration of a detailed theory of the behaviour of macromolecules subjected to a centrifugal field (Svedberg and Pedersen, 1940). Claude initiated the quantitative fractionation procedure, which shifted the focus from preparative to analytical cell fractionation (Claude, 1946).

Refined since those times both technically as well as methodologically (De Duve, 1975), tissue fractionation has opened the way to the elucidation of such fundamental cellular processes as oxidative phosphorylation, intracellular digestion, protein synthesis, bulk transport in and out of the cell or organelle biogenesis. Highly sophisticated and versatile centrifuges, such as high-speed ultracentrifuges, are now among the most basic and valuable pieces of equipment available in biological laboratories of all kind and sizes. However, despite daily use by numerous workers, the potential of modern centrifuges is rarely realized in all its dimensions.

Ultracentrifuges

A centrifuge is a device for separating the constituents of an emulsion or the particles in a suspension. In cell fractionation experiments, cells (e.g. from a cell culture or a tissue) are collected by centrifugation in a benchtop or a high-

speed centrifuge, while subcellular organelles or macromolecules are obtained by subfractionating a homogenate in an ultracentrifuge.

Ultracentrifugation is basically carried out in two ways: 'Preparative' centrifugation aims to isolate and purify specific particles such as subcellular organelles, while the object of 'analytical' centrifugation is to study molecular interactions between macromolecules or to analyse the properties of sedimenting particles such as their apparent molecular weights. Preparative and analytical ultracentri- fuges can be distinguished accordingly. However, modern preparative ultracentrifuges (e.g. the Optima series of Beckman) are adapted to meet the requirements of both preparative and analytical centrifugation. To this end, the instruments are equipped with a scanning optical system needed for analytical studies in addition to the basic devices common to an updated preparative ultracentrifuge

– a frequency-controlled induction drive; a diffusion pump in tandem with a mechanical forepump to reduce chamber pressure to a low micron level; and a system to electro- nically control rotor speed and temperature. Other, more sophisticated features of such ultracentrifuges include microprocessor-based programmability; an acceleration/ deceleration accessory comprising variable profiles, which is advantageous when shallow density gradients are centrifuged; or the $\omega^2 t$-integrator, which indicates the accumulated centrifugal force and may be used to terminate a run when the desired centrifugal effect is reached.

Basic Concepts of Centrifugation

There are two modes of preparative centrifugation: differential centrifugation and density gradient centrifugation.

Differential centrifugation

Differential centrifugation, also termed pelleting, fractionates particles according to their size and shape. An

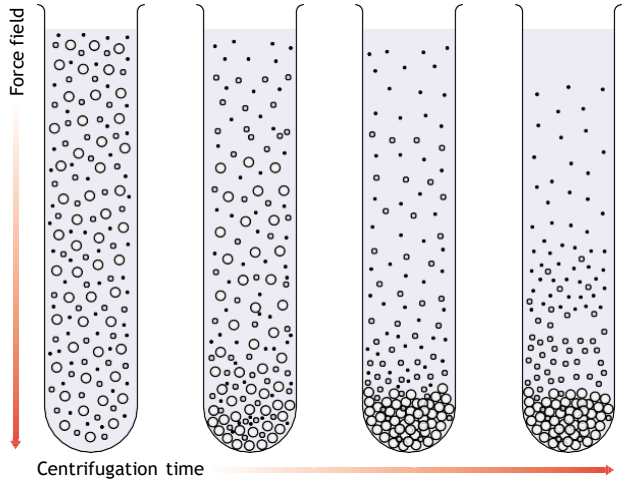

Figure 1 Differential pelleting.

initially uniform mixture of the particles in sample buffer (Figure 1) is separated by centrifugation into two fractions: a pellet containing the sedimented particles, and a supernatant comprising the unsedimented ones and the buffer.

As is illustrated in Figure 1, particular components will distribute differently to the pellet and the supernatant during centrifugation, depending on their size as well as the centrifugal conditions (e.g. centrifugation time). While the larger particles in the master mixture (open circles) will mostly be pelleted, the more slowly sedimenting ones (dark dots) will preferentially remain in the supernatant. For better separation, the supernatant may be subjected to another round of centrifugation at a higher speed, while the pellet can be resuspended and recentrifuged ('washed') to diminish the amount of contamination. Evidently, however, differential centrifugation leads only to an enrichment rather than a purification of particles, even after repeated recentrifugation – which, inevitably reduces its yield. Nevertheless, pelleting is an indispensable step in the purification of organellar fractions from a total tissue homogenate because of (a) the simplicity of sequentially enriching the cell organelles as shown by the following flow chart in Figure 2; (b) the advantage of using fixed-angle rotors with buckets of high capacity, which makes it attractive for fractionations on a preparative scale; and (c) the fact that the efficiency of gradient centrifugation, to be discussed next, is greatly enhanced by loading an enriched preparation of organelles on to a gradient rather than a crude homogenate that comprises all the components of the original tissue.

Density gradient centrifugation

According to the flow chart in Figure 2, the organelles in a total tissue homogenate can be enriched stepwise by differential fractionation. Yet, as is evident from Figure 1, their actual purification will not be accomplished in this way because of the cosedimentation of other components. To get a genuinely purified preparation of the organelle of interest, the particular contaminants have to be removed in a subsequent step by the more sophisticated technique of density gradient centrifugation. The rationale of this method is that particles already enriched in a fraction are subjected during centrifugation to a medium that varies in its density along the tube instead of being homogeneous as it is in differential pelleting. In doing this, even slight differences in the particles' physicochemical properties (size, sedimentation velocity, drag force) can be exploited for their resolution. Density gradient centrifugation is commonly used on a preparative scale but, in addition, has the advantage that analytical measurements can also be made.

Two modes of density gradient centrifugation can be distinguished: rate zonal and isopycnic centrifugation. In both methods a supporting column of fluid is used – the gradient – on top of which the sample is layered. This fluid, the gradient medium, consists of a suitable inert solute, usually of low molecular weight, dissolved in a solvent in which the sample particles can also be suspended. Basically, the density of a gradient may be increased continuously or discontinuously from top to bottom of the centrifuge tube (Figure 3). Discontinuous gradients, also called step gradients, are formed by adding consecutively less dense layers on top of the preceding more dense one (Figure 3). Continuous gradients are prepared by means of a so-called gradient mixer, which is commercially available. Continuous density gradients are applicable in more varied ways than step gradients. They are indispensable to rate zonal separations, and are also best suited to establishing the optimal conditions for isopycnic centrifugation. Moreover, they can be preformed or self-generating during centrifugation, whereas step gradients always have to be made freshly.

Methods of Density Gradient Centrifugation

Although both rate zonal and isopycnic centrifugation use density gradients as well as the same types of rotor, their principles are completely different, as may be inferred from eqn [2] below (see Basic Theory of Sedimentation).

Rate zonal centrifugation

In rate zonal centrifugation, particles are separated in terms of their distinct sedimentation velocities, reflected by their so-called sedimentation coefficients (see eqn [3] below). The sample solution is layered on top of the density gradient, and under centrifugal force the particles

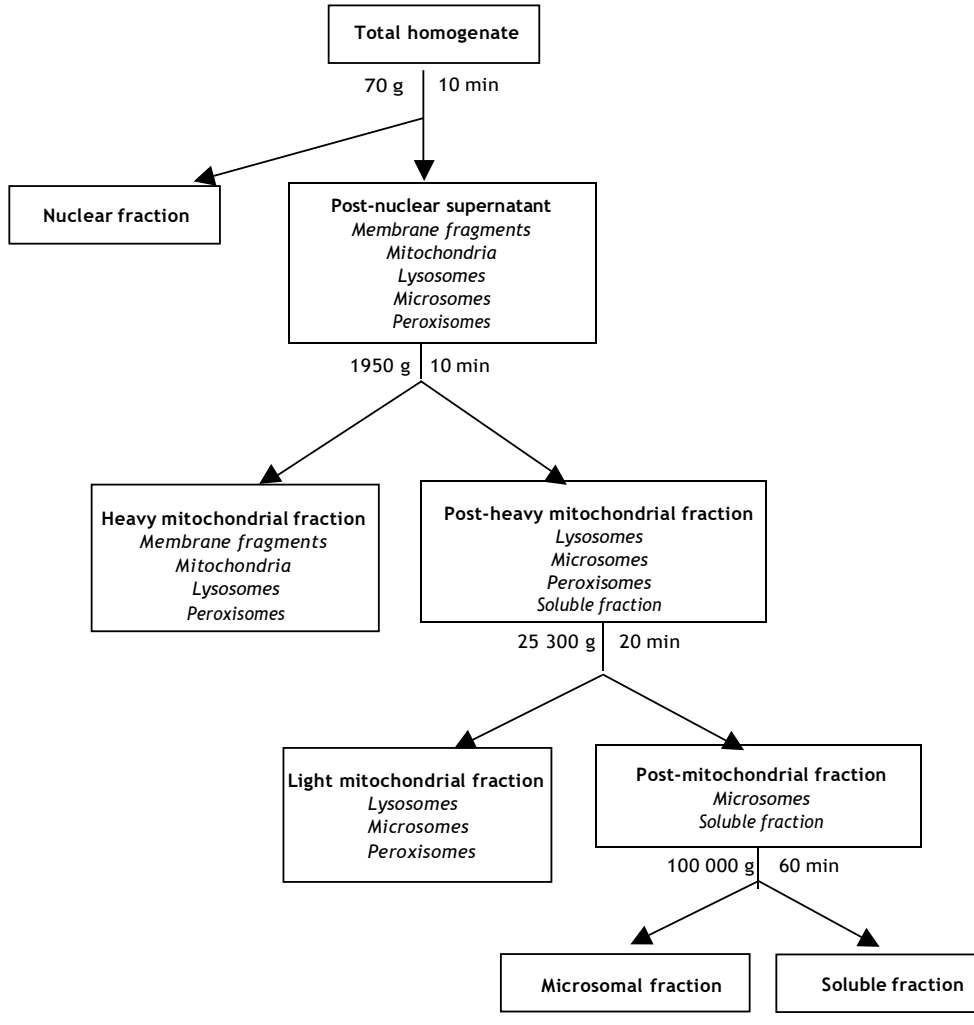

Figure 2 Flow chart summarizing the sequential steps in the subfractionation of a homogenate by differential centrifugation.

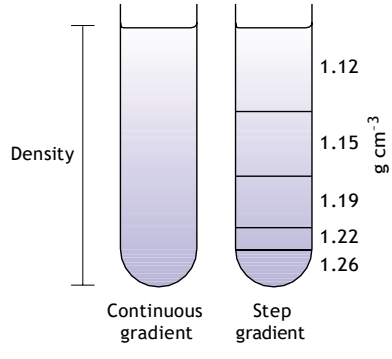

Figure 3 The density of a gradient may be increased continuously or discontinuously from top to bottom of the centrifuge tube.

will begin sedimenting through the gradient in distinct zones (Figure 4), each zone consisting of particles characterized by the same sedimentation rate (eqn [2]). For particles to migrate unimpeded, continuous density gradients are required for rate zonal centrifugation with the density along the gradient always being smaller than that of the particles. This implies that the run must be terminated before any of the separated zones reaches the bottom of the tube.

Figure 5 illustrates the subfractionation of a rat hepatic light mitochondrial fraction, resolved by rate zonal centrifugation. The enriched fraction was prepared according to the flow chart and loaded on to a continuous density gradient, which was spun in a vertical type rotor.

Isopycnic centrifugation

Isopycnic centrifugation takes advantage of differences in the effective buoyant densities of particles – that is, it does not rely on their sedimentation rates. The sizes and shapes of the particles do not affect separation; rather each

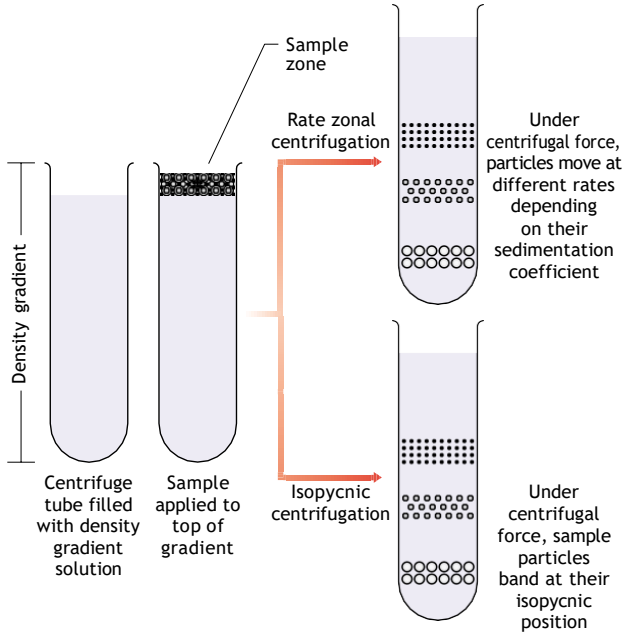

Figure 4 Modes of density gradient centrifugation: (upper) rate zonal centrifugation; (lower) isopycnic centrifugation.

particle sediments only to the position in the gradient at which its own effective buoyant density is equal to the actual gradient density: i.e. where $r_s = r_l$ (see eqn [2] below). It will remain there independently of the time of centrifugation (cf. Figure 4). Accordingly, the density gradient column has to encompass the whole range of densities of the sample particles, and its maximum density must exceed that of the densest particles. Although continuous density gradients are used for isopycnic centrifugation, step gradients are often better, provided that the appropriate conditions have been established.

Basic Theory of Sedimentation

As a rotor spins in the centrifuge, each particle in the sample effectively experiences centrifugal force, and will consequently sediment at a rate proportional to the force. However, other parameters also need to be considered that affect the sedimentation rate: namely the size and mass of the particle, the buoyancy it is exposed to in the gradient medium, and the frictional force reflected by the viscosity of the sample solution. The interaction of the various forces on the particle during centrifugation is schematically illustrated in Figure 6, indicating that buoyancy and frictional forces counteract the centrifugal force.

The basic principles of sedimentation originate from Stokes' law, which deals with the sedimentation of a sphere

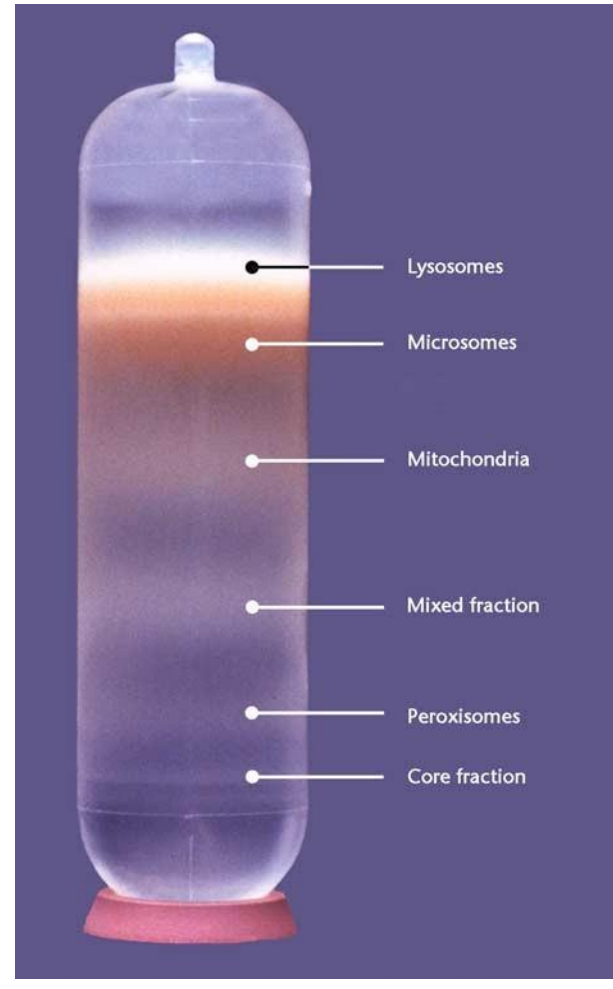

Figure 5 Photograph of an actual tissue separation: subfractionation of a rat hepatic light mitochondrial fraction by rate zonal centrifugation using a vertical rotor.

in a fluid and the force resisting its motion through it. This force, also called the frictional or drag force, is given by eqn [1], where v is the sedimentation rate of the sphere; r_s is the diameter of the sphere; η is the viscosity of liquid medium.

$$\text{Drag force} = 6\pi r_s \eta v \qquad [1]$$

Progressively increasing at first, the drag force approximates a maximum at which it is balanced by the net force on the spherical particle, which implies that the sphere is now sedimenting at a constant velocity. Deriving Stokes' law for the settling of a sphere at a given centrifugal force, F, the equilibrium between net and drag force may be expressed by eqn [2]

$$\frac{4}{3} r_s^3 \pi [\rho_s - \rho_l] F = 6\pi r_s \eta v \qquad [2]$$

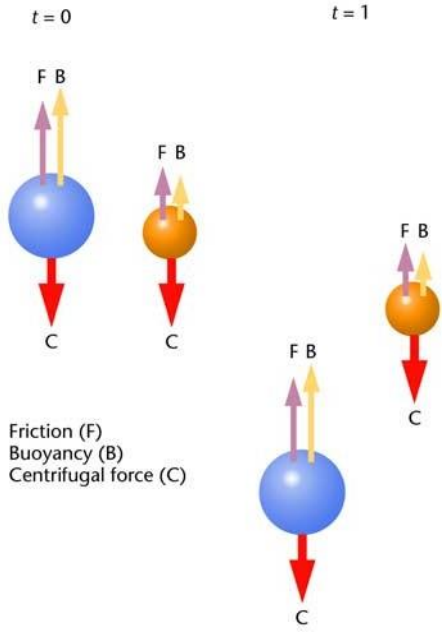

Figure 6 Buoyancy (B), friction (F) and centrifugal (C) forces acting on a particle during centrifugation.

In eqn [2], r_s is the sphere (particle) density and r_l is the liquid density; and $F = \omega^2 r$ where ω is the rotor speed (radians s^{-1}) and r is the distance between the particle and the centre of rotation (cm).

Equation [2] can be solved to give eqn [3] for sedimentation rate, n.

$$\nu = \frac{2r_s^2[\rho_s - \rho_l]}{9\eta} F \qquad [3]$$

The sedimentation rate is characteristic for a particle, and can be determined in an ultracentrifuge.

From eqn [3] and Figure 6 the following features of the sedimentation rate can be appreciated.

- For a given particle it is proportional to the particle's size.
- It is also proportional to the difference between the density of the particle and the density of the liquid medium.
- It is zero when the density of the particle is equal to the density of the liquid medium.
- It decreases as the viscosity of the liquid medium increases.
- It increases as the force field increases.

The sedimentation velocity per unit of centrifugal force is called the sedimentation coefficient, *s*, (Svedberg and Pedersen, 1940), given by eqn [4].

$$s = \frac{1}{\omega^2 r} \times \frac{\delta r}{\delta t} = \frac{2r_s^2[\rho_s - \rho_l]}{9\eta} \qquad [4]$$

Table 1 Normalized sedimentation coefficients $s_{w,20}{}^a$ of cellular organelles and constituents

Particle	Sedimentation coefficient [S]
Nuclei	$\sim 5 \times 10^5$
Mitochondria	$\sim 5 \times 10^4$
Lysosomes	$\sim 5 \times 10^3$
Smooth microsomes	$\sim 1 \times 10^3$
Rough microsomes	$\sim 5 \times 10^2$
RNA/proteins	$\sim 1 \times 10^1$

dr/dt is the rate of movement of the particle in centimetres per second. Sedimentation coefficients are usually expressed in Svedbergs [S], or 10^{-13} s. A particle whose sedimentation coefficient is measured as 10^{-12} s (10 S) may be described as a 10S particle.

Table 1 indicates sediment coefficients of various subcellular fractions.

Practical Aspects of Ultracentrifugation

Density gradients

Application of density gradients

Density gradients are widely used to separate and purify, on a preparative scale, a variety of cells, organelles and macromolecules such as nucleic acids or proteins (Schumaker, 1967; De Duve, 1971; Birnie, 1972). This is a prerequisite for analysis of the protein composition and enzymatic activities of the cellular fractions or the solvation and molecular interactions of the macromolecules. Gradients are also required for analytical experiments, for example to measure the apparent buoyant densities or sedimentation coefficients of particles; to estimate the size, the conformation or turnover rates of proteins and nucleic acids; and to investigate the effects of chemical, physical or biochemical treatment of the sample material.

Gradient shapes

While the density range of a gradient is basically determined by the mode of centrifugation (rate zonal or isopycnic centrifugation), its shape – as given by its concentration profile along the tube – can be modified and adapted to the conditions required. Thus, linear or exponential, steep or shallow continuous gradients can be prepared, and in a discontinuous gradient the number

and density of the layers can be varied to properly meet the required conditions.

Gradient material

The properties of an 'ideal' solute for gradients to be used in rate zonal or isopycnic centrifugation, as outlined, for example, by Hinton *et al*. (1974), can be summarized as follows.

- The solute should be stable in solution and its density range should be sufficient to span the bouyant densities of all particles to be separated.
- It should be neither hyperosmotic nor hyposmotic, and should cause minimal alterations in viscosity, ionic strength and pH.
- It should be totally inert towards biological materials: that is, it should have no detergent-like properties; it should not interact with the material, aggregate or disaggregrate the bioparticles or alter their biological activities that depend on conformation and composition; and it should not interfere with procedures for assaying of enzymatic activities, etc.
- It should not absorb in the UV or visible range.
- It should be easily and completely removable from the purified particles.
- It should be sterilizable.
- It should not be corrosive to the rotor and other devices, and should not be flammable or toxic.
- It should be inexpensive and readily available.

It has to be kept in mind, however, that there is no ideal all-purpose gradient material, and that in each case the solute has to be adapted to the desired type of separation. Frequently employed solutes for rate zonal and isopycnic density gradient centrifugation are simple sugars and analogous polyhydroxyl compounds (sucrose, sorbitol, glycerol); polysaccharides (Ficoll, dextran, glycogen); proteins (bovine serum albumin); iodinated organic compounds (metrizamide, nycodens); and inorganic salts (CsCl).

Rotors

When choosing a rotor for a cell fractionation experiment, the design of the experiment, i.e. analytical or preparative, the number of the individual samples to be centrifuged, their volume and composition, and how quickly it is desired to achieve the separation have to be taken into account. For this purpose, the rotor must be considered, in terms of the material it is made of, its geometry and capacity as well as its maximal speed, since these are the main variable parameters determining the procedure to be used for a particular separation.

Rotor material

Ultracentrifuge rotors are mostly manufactured from aluminium, titanium or fibre-reinforced composite materials. To achieve a uniform surface, which is particularly important for even transfer of heat from the bowl and for good temperature control, rotors are usually finished with an anodized (aluminium alloys) or a varnish coating.

Aluminium rotors are relatively light and easy to handle but are less durable and more susceptible to corrosion than other rotors. This is because the film of oxide protecting the surface is very brittle and tends to crack during centrifugation, thus giving aggressive chemicals such as salt solutions access to the quite reactive aluminium alloy.

Titanium rotors are generally stronger and more resistant to corrosion, making them the preferred rotor for rapid separations at high speeds or when corrosive chemicals are to be used. They are also less subject to fatigue than are aluminium rotors.

Composite rotors basically offer light weight and high strength, and hence faster acceleration/deceleration rates compared to metal rotors. However, there are severe technical problems concerning the correct alignment of the fibres throughout the rotor that have to be solved to make this kind of rotor attractive for daily use.

Types of rotor

Figures 7 to 9 illustrate the types of rotors most frequently used.

The vertical tube rotor (Figure 7) is often used for isopycnic and rate zonal separation, when short run times are required. Because the sample tubes are held parallel to

Figure 7 A vertical tube rotor.

Figure 8 A fixed-angle rotor.

Figure 9 A swing-out rotor.

the axis of rotation, particle path lengths are short, being limited to the diameter of the tube, which results in a reduction of the run time.

The fixed-angle rotors (Figure 8) are generally used for fractionating material on a preparative scale by differential pelleting, with the tube size of such rotors ranging from 0.2 to 500 mL. They also provide faster run times than swing-out rotors. Fixed-angle rotors have tubes that remain in one position, at an angle varying from 148 to 408 from the vertical, with a narrow angle favouring faster pelleting while a more compact pellet is obtained with a wide angle.

Swing-out rotors or swinging-bucket rotors (Figure 9) have hanging buckets that swing 908 upwards and are held in that horizontal position while spinning. Because of this position, particles can sediment along the full length of the tube, which is advantageous for rate zonal centrifugation with maximum separation between zones. Although run times in swing-out rotors are longer than with other rotor types, they are compensated by the excellent resolution of sample bands.

Zonal rotors are designed to fractionate large amounts of material by means of rate-zonal or isopycnic density gradient centrifugation. This is best evident in continuous-flow zonal rotors, which can process up to ten litres of liquid in relatively short times.

References

Anderson NG (1966) Zonal centrifuges and other separation systems. *Science (NY)* 154(745): 103–112.

Birnie GD (ed.) (1972) *Subcellular Components: Preparation and Fractionation*, 2nd edn. London: Butterworths.

Claude A (1946) Fractionation of mammalian liver cells by differential centrifugation. 1: Problems, methods, and preparation of extract. *Journal of Experimental Medicine* 84: 51–59.

De Duve C (1971) Tissue fractionation. Past and present. *Journal of Cell Biology* 50: 20D–55D.

De Duve C (1975) Exploring cells with a centrifuge. *Science (Washington DC)* 189: 186–194.

Hinton RH, Mullock BM and Gilhuus-Moe CC (1974) In: Reid E (ed.) *Methodological Developments in Biochemistry*, vol. 4, pp. 103. London: Longmans.

Miescher F (1871) In: Hirschwald A (ed.) *Hoppe-Seyler's Medizinisch-chemische Untersuchungen*, vol. 4, pp. 441–460. Berlin.

Schumaker VN (1967) Zone centrifugation. *Advances in Biological and Medical Physics* 11: 245–339.

Svedberg T and Pedersen KO (1940) *The Ultracentrifuge*. Oxford: Clarendon Press.

Further Reading

Birnie GD and Rickwood D (eds) (1978) *Centrifugal Separations in Molecular and Cell Biology*. London: Butterworths.

Price CA (ed.) (1982) *Centrifugation in Density Gradients*. New York: Academic Press.