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# DENSITY GRADIENT CENTRIFUGATION

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Richard Hinton and Miloslav Dobrota

*Wolfson Bioanalytical Centre, University of Surrey, Guildford, Surrey GU2 5XH, U.K.*


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# Contents

# Introduction to zonal centrifugation

## *1.1. The first applications of centrifugation in biology*

A pleasing surprise given to writers of this type of article is the discovery of useless, but to them novel, facts, like the use of centrifuges for separating biological structures by Miescher in 1872. Little attention was paid at that time to the structure of cells, and for many years the use of centrifuges was restricted to applications such as the separation of milk, the collection of precipitates and (from about 1930 on) the separation of large particles such as nuclei. At that latter period most biochemists directed their attention to the separation of purified fractions – especially enzymes – rather than to analysis of the structure of the cell. Accordingly, sophisticated analytical ultracentrifuges were developed for testing the homogeneity of purified fractions, but preparative centrifuges were chiefly used for the collection of precipitates.

Two major advances paved the way for a more wide-spread use of centrifugal techniques. Firstly, the development of alloys with a high strength in relation to their density permitted centrifugation at high speed of much larger quantities of material. Secondly, the development of methods for examining biological specimens under the electron microscope (see Palade 1971) revealed the complexity of the internal structure of cells. Earlier workers had attempted to purify nuclei (Behrens 1932) and mitochondria (Bensley and Hoerr 1934) but light microscopy was the only method of assessing these preparations. Given the complexity of the structure of the cell and the essentially arbitrary nature of the fractions which were being

separated, what was needed was not 'pure' preparations, but a systematic and quantitative study of the distribution of subcellular particles and of enzymes between different fractions. De Duve (1971) has stated his belief that the insistence of workers such as Claude and Schneider and Hogeboom (1951) on the necessity for quantitative experiments ensured that the better understanding of the internal structure of the cell was rapiddly followed by an understanding of the biochemical role of the component parts.

These early studies were all performed using the technique which was later called differential centrifugation or, more properly, differential pelleting (Reid 1972b). This method was refined by De Duve and his colleagues, and the 'mitochondrial' fraction was resolved into a heavy fraction containing mainly mitochondria and a 'light' fraction enriched in lysosomes (de Duve and Berthet 1954; de Duve et al. 1955). The importance of these advances is seen in the vast volume of work on the fractionation of different types of cell which followed.

While differential pelleting has been an enormously useful technique for cell fractionation, a number of workers realised that only particles differing considerably in size could be separated in this way. Alternative techniques, rate and isopycnic zonal centrifugation were, in fact, proposed at about the same time as the differential pelleting scheme was developed, but the application of these methods was limited by the apparatus available (Anderson 1956; Allfrey 1959; de Duve et al. 1959). The full potentiality of density gradient centrifugation only began to be realised with the development of high capacity swing-out rotors and zonal rotors during the early 1960's.

## *1.2. Centrifugal techniques*

### *1.2.1. Analytical ultracentrifugation*

As we have mentioned, early high speed centrifuges were mainly used for the study of 'extracts' from cells. Both this use and metallurgical limitations meant that the volume of specimen had to be minimised. Rotors were designed with transparent windows so that the distri-

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bution of particles in the centrifugal cell could be examined during centrifugation. A typical example of such an analytical rotor is shown in Fig. 1.1. The cells in such a rotor are filled with a uniform suspension of the mixture to be analysed. The rotor is

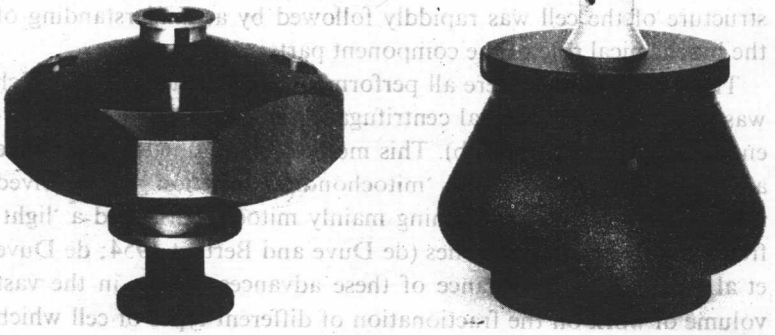

Fig. 1.1. (left) A rotor for an analytical ultracentrifuge. (right) A high-speed preparative centrifuge rotor (Beckman type 65) is shown for comparison.

accelerated to its operating speed. Particles move at a rate determined by their size and shape and by the centrifugal force. Thus, if the cell was initially filled with a uniform suspension (Fig. 1.2A1) containing only one type of particle, a clear zone will appear to move slowly down the cell as the particles that were in that region sediment away (Fig. 1.2B1). If a mixture of particles were initially present, each type of particle will sediment at its own speed, so that after centrifugation the distribution will be as shown in Fig. 1.2B2. If some generalised property such as refractive index or ultraviolet absorbtion is measured, then patterns similar to those shown in Fig. 1.2C will be obtained. If an optical system sensitive to changes of refractive index is used (Schlieren optics) then the output will show a series of peaks (Fig. 1.2D). The latter is the form of output normally chosen, but it is important to realize that these peaks do not represent a zone of particles moving down the cell through a clear supporting medium, but the 'back end' of a sedimenting block of

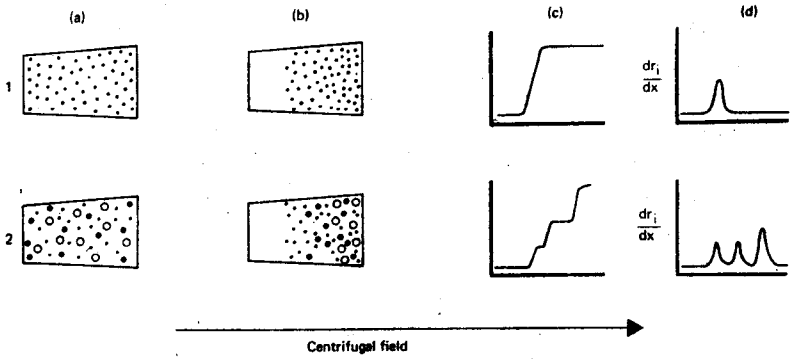


Fig. 1.2. Separations in an analytical rotor. The particles are initially distributed uniformly through the cell (A). As centrifugation proceeds, the particles sediment down the cell. Each type of particle will sediment at a distinctive rate. Thus as each group of particles sediment, a series of interfaces will form (B). These interfaces will appear to sediment through the cell at the same speed as the particles with which they are associated. The interfaces may be detected either by measuring the distribution of particles through the cell using some property common to all the particles such as ultraviolet absorbance (C) or by using an optical system (Schlieren optics) which provides an output which is related to the rate of change of refractive index (D). The latter system is less sensitive, but the output is more easily interpreted.

particles. As will be seen later, the use of an initially uniform suspension and the measurement of the clearance from the cell circumvents many problems.

Analytical ultracentrifugation has been greatly developed from this essentially simple basis, but not in such a way as to fall within the scope of this article. Readers who are interested will find more extended accounts in books and articles by Schachman (1959), Trautman (1964) and Bowen (1970).

### *1.2.2. Differential pelleting*

Differential pelleting is similar in principle to separations in an analytical ultracentrifuge. The centrifuge tube is filled with a uniform suspension. During centrifugation particles move down the centrifuge tube and pellet on the bottom. Ideally, centrifugation is continued

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for just long enough to pellet all of the largest class of particles (Fig. 1.3C). This will yield a supernatant free from one type of particle which may then be centrifuged at a higher speed to separate the next largest type of particle and so on. Unfortunately, the pellets are not homogeneous. Suppose that of the three particles shown in the illustration (Fig. 1.3) the middle and the smallest-sized sediment at respectively half and one tenth of the rate of the largest particles. As the particles are initially uniformly distributed, one half of the middle-sized particles and one tenth of the smallest sized particles will be found in the pellet if centrifugation is continued for just long enough to sediment all of the largest particles (see Fig. 1.3C).

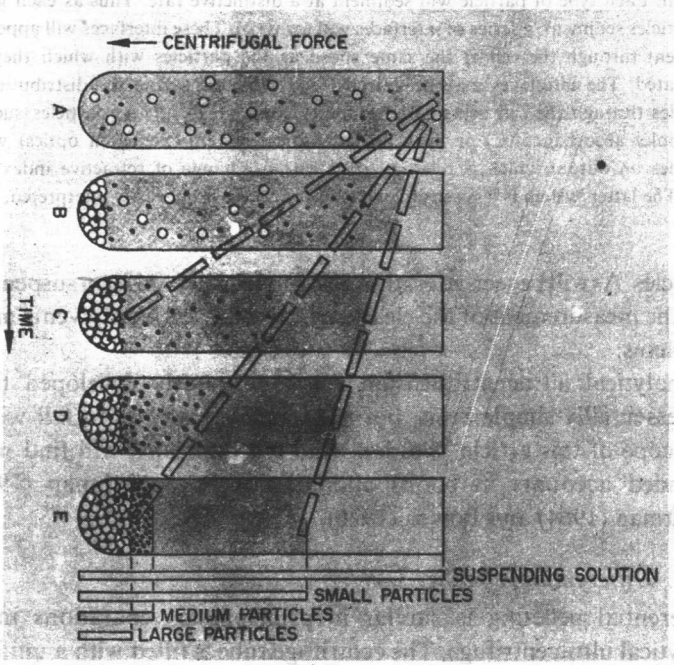


Fig. 1.3. Fractionation of particles by differential pelleting (from Anderson 1966a). For details see text.

More prolonged centrifugation would result in even greater contamination.

The separation achieved by differential centrifugation may be improved by 'washing' the pellets. The pellets are resuspended in the homogenisation medium and recentrifuged under the same conditions as in the original pelleting. In the example used above, the pellet C contained half of the middle sized particles and one tenth of the smallest sized particles. If this pellet were resuspended in the same volume of liquid as the original suspension and were recentrifuged in the same way as before, all the largest particles would be recovered in the pellet, but only one half of a half (i.e. 25%) of the middle sized particles and one tenth of a tenth (i.e. 1%) of the original amount of the smallest particles. Hence, differential pelleting with washing of the pellet is an efficient way of separating particles which differ greatly in size, but not for separating particles of similar size. It is for this reason that differential pelleting has been effectively limited to the separation of the five fractions described by de Duve and colleagues in 1955. If greater resolution is required, other techniques must be employed.

### *1.2.3. Rate-zonal centrifugation*

The technique of rate-zonal centrifugation was first proposed by Brakke (1951). In essence, the technique is very simple. A small volume of a suspension is layered over a shallow density gradient. The latter is required to stabilize the sedimentation of the particles (see § 2.1.3). On centrifugation, particles move away from the starting zone with velocities determined both by their size and shape and by the centrifugal force to which they are subjected. After centrifugation for a certain time, particles will be found in a series of zones spaced according to the relative velocities of the particles (Fig. 1.4). In this way particles differing in sedimentation rate by 20% or less can be separated without undue difficulty. Rate-zonal centrifugation thus complements differential centrifugation.

As with most simple-sounding techniques there were a number of problems which limited the usefulness of the technique especially

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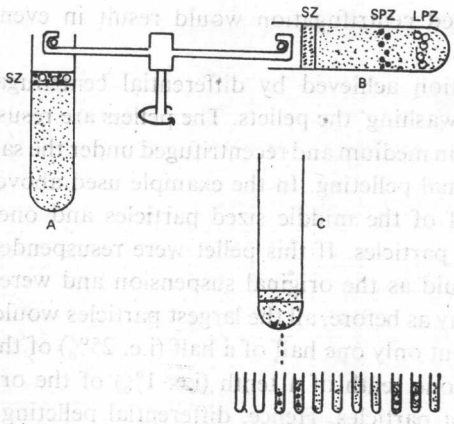


Fig. 1.4. Fractionation of particles by rate-zonal centrifugation in a swing-out rotor. The gradient and sample layer are introduced at rest. The tubes are attached to the rotor and accelerated, swinging out to a horizontal position (B). Zones of particles move down the tube at their characteristic rates. Centrifugation is continued until an adequate separation is obtained. The rotor is then decelerated to rest, and the gradient and separated zones: sample zone SZ, small particle zone (SPZ) and large particle zone (LPZ) recovered, for example by dripping out the gradient through a small hole punctured in the bottom of the tube (C) (redrawn from Anderson 1966a).

in the ten years following its introduction (see Brakke, 1960, for a survey of work carried out in this period). Rate-zonal separations cannot be carried out in angle head rotors as the sample mixes with the gradient during acceleration of the rotor (§ 3.2) and until recently, the capacity of swing-out rotors was severely limited. As the sedimenting zones are as broad as, or broader than, the starting zone, the volume of material which can be loaded onto a rotor is limited if any degree of separation is to be achieved. In addition, the concentration of material in the sample must not be too high, or the entire band will mix with the top part of the gradient (see § 2.1.2). Rate-zonal centrifugation was initially used mainly for analytical separations such as the analysis of the size distribution of samples of polysomes (McQuillen et al. 1959) or of RNA (Nomura et al. 1960) although very soon after the introduction of the technique

Thomson and his colleagues (Thomson and Mikuta 1954; Thomson and Klipfel 1958) used rate sedimentation to separate mitochondria and lysosomes. Improvement in the design of swing-out rotors and especially the introduction of zonal rotors has greatly extended the application of rate-zonal centrifugation and it is with this method that we shall be mainly concerned. Vertical tube rotors (see p. 261) may also be used.

### *1.2.4. Isopycnic zonal centrifugation*

Subcellular particles may differ not only in size but also in density, as was first demonstrated by Harvey (1931, 1932). A suspension containing living cells was layered over a solution of greater density than the cells. On centrifugation, the cells banded at the interface and, when they were examined in the microscope, the contents of the cell appeared to have separated into a number of layers. This separation was not lethal to the cells, but if the speed of centrifugation was increased, the cell broke in two, the nuclei being associated with the denser fragment. These density differences were exploited in order to purify nuclei by flotation in organic solvents (Behrens 1938) and, much later, by sedimentation through dense sucrose (Chauveau et al. 1956).

Most early separations were performed by suspending the particles in a liquid of defined density and centrifuging. Particles less dense than the medium floated, particles more dense pelleted. Fractionations by stepwise flotation are tedious, especially when more than one particle type is to be separated. Later investigators therefore developed the technique of isopycnic zonal centrifugation (Meselson et al. 1957; de Duve et al. 1959). A suspension of the particles to be separated is either layered over a density gradient or the particles are actually suspended in the solutions used to make the gradient. On centrifugation, particles either rise or sediment until they reach a liquid of their own density. Here they have no weight and do not move any further regardless of the time of centrifugation. The particles may be recovered as a series of zones, each particle at its own density.

This technique, first used in analytical ultracentrifuges, is free from

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many of the problems of rate zonal centrifugation. In 1959, Beaufay and colleagues, using this technique, showed that a group of enzymes involved in the formation and breakdown of hydrogen peroxide which sedimented like lysosomes on differential centrifugation were, in fact associated with quite distinct particles the microbodies (or peroxisomes).

Since that time, isopycnic zonal centrifugation has been widely applied to biological separations and will be discussed in some detail below (Chs. 2, 3 and 8). However, the technique has one major disadvantage as compared with rate-zonal centrifugation. During isopycnic separations, particles are inevitably exposed to high concentrations of the gradient solute. This may cause damage to the particles and the possible appearance of extra bands which correspond to the damaged particles. 'Separations' may thus be achieved which bear no relation to differences which actually exist in the living cell. These problems are considered in some detail in later sections.

## *1.3. The development of centrifuges and rotors*

### *1.3.1. The centrifuge*

Since we are primarily concerned with high-speed centrifugation, we will not discuss the relatively slow-speed bench-type centrifuges in any detail. These have been commercially available from the middle of the 19th century in the form of hand driven machines and (from 1911–1912) electrically driven. These machines were similar to present day bench centrifuges and capable of similar speeds, i.e. up to 3000 revs/min. The feature of most interest is the use of a swing-out four place head, a design which was not adopted for high-speed centrifugation until much later.

In order to appreciate the problems, let us examine the major design requirements for a modern high-speed centrifuge. 1) a reliable and accurately controllable high-speed drive system is needed; 2) since air friction at speeds above 20,000 revs/min causes excessive heating, a rotor operated above 20,000 revs/min must be spun in a partial vacuum; 3) the rotor must be made of a material strong enough