Factors Affecting Resolution in, and Interpretation of Data from, Zonal Rotors

By

G. D. BIRNIE

Beatson Institute for Cancer Research, 132 Hill Street, Glasgow, C. 3, Scotland

Since the technique was first introduced by BRAKKE (1951) density-gradient centrifugation has been used to separate the constituents of a variety of mixtures of biological particles with considerable success. The technique has yielded much useful information although serious limitations are imposed by the capacity and geometry of swing-out rotor tubes, particularly in high-speed rotors. In the zonal rotor (ANDERSON, 1966a, b) these limitations are virtually eliminated and density-gradient separations of relatively large quantities of material with significantly better resolution have now become possible. However, considerable care has still to be taken to ensure that the best possible resolution between zones of particles is obtained. While some of the factors affecting resolution in zonal rotors are common to all density-gradient centrifugation methods, others are peculiar to zonal rotors. It is the purpose of this article to discuss the major factors affecting resolution in, and interpretation of data from, density gradients, with particular reference to separations in zonal rotors. A rigorous mathematical treatment of these factors is beyond the scope of this article. However, it should be noted that, in calculating sedimentation coefficients, extensive use has been made of the tables published by MCEWEN (1967).

The separation of a mixture of particles into zones or bands of particles by centrifugation through a density gradient can be achieved in one of two ways. First, there is rate-zonal sedimentation in which each zone consists of particles which have sedimented at the same rate, that is, they have the same sedimentation coefficient. High-resolution rate-zonal separations require more care, both in operation and in interpretation; they are by far the most frequently used in zonal rotors. Second, there is isopycnic sedimentation, in which the particles in each zone have the same buoyant density. Such separations are the simplest, but have been least used, particularly in zonal rotors. In addition, a combination of rate-zonal and isopycnic sedimentation is possible. This method is rarely used deliberately, but is often encountered, too frequently without being recognised, particularly when whole tissue homogenates are being fractionated. No further discussion of this "method" is necessary, since each component of the combination can be analysed separately.

However, it is necessary to emphasise that the possibility of such separations occurring must be foreseen. Obviously, isopycnic banding of a zone during a rate-zonal separation, or *vice versa*, will lead to erroneous conclusions if it goes unrecognized.

Rate-Zonal Separations

Resolution

When the resolution of a rate-zonal separation is considered, the nature and shape of the density gradient receives most attention. However, other factors are involved which have an important bearing on the resolution achieved. First, the correct rotor must be chosen. Many zonal rotors are now available, ranging from the low-speed AXII rotor (maximum, 5000 rev/min) to the high-speed titanium BXIV rotor (maximum, 47 000 rev/min). In general, the faster rotors should give better resolution since, in them, separations take place more rapidly so that there is less time for zones of particles to widen by diffusion. However, these rotors are not suitable for use with larger particles, such as whole cells or nuclei. In such cases, the speed of rotation during loading (2000–5000 rev/min) is sufficient to cause significant sedimentation, and thus broadening, of the sample band while it is being introduced into the rotor. Significantly better resolution between zones of large particles is obtained in the AXII rotor which is rotating relatively slowly (500–600 rev/min) during the loading period.

The second factor is the sample itself. As in all rate-zonal separations the volume of the sample, and hence the width of the sample band, is critical. This is illustrated by Fig. 1, in which the fractionation of a 25 ml sample in a BXIV rotor (total capacity 670 ml) is compared with that of the same volume of sample in the BXV rotor (total capacity 1670 ml). It is clear that the resolution obtained in the smaller rotor (Fig. 1 a), in which the volume of sample is 6% of that of the gradient, is significantly poorer than in the larger rotor (Fig. 1 b), in which the sample volume is only 2% of the gradient volume. The same improvement in resolution can be achieved with the BXIV rotor if the sample volume is reduced to 8 ml.

The density of the sample zone is also of importance. Obviously, it must not exceed the density of the light end of the gradient. If it does, the local instability so introduced is spontaneously eliminated by diffusion with consequent broadening of the sample zone to an extent dependent on the amount by which the density of the sample exceeds that of the top of the gradient. In addition, the density of the sample zone should not be much greater than that of the overlay solution. Density discontinuities are rapidly smoothed out in a zonal rotor by diffusion of sucrose from the gradient (see Fig. 5), and this diffusion causes back-diffusion of the sample material which results again in broadening of the sample zone. The occurrence of such sample band diffusion is clearly seen in Fig. 1, in which there are relatively large differences in density between the top of the gradient, the sample and the overlay solutions. Ideally, there should be no discontinuities in the density gradient in these regions.

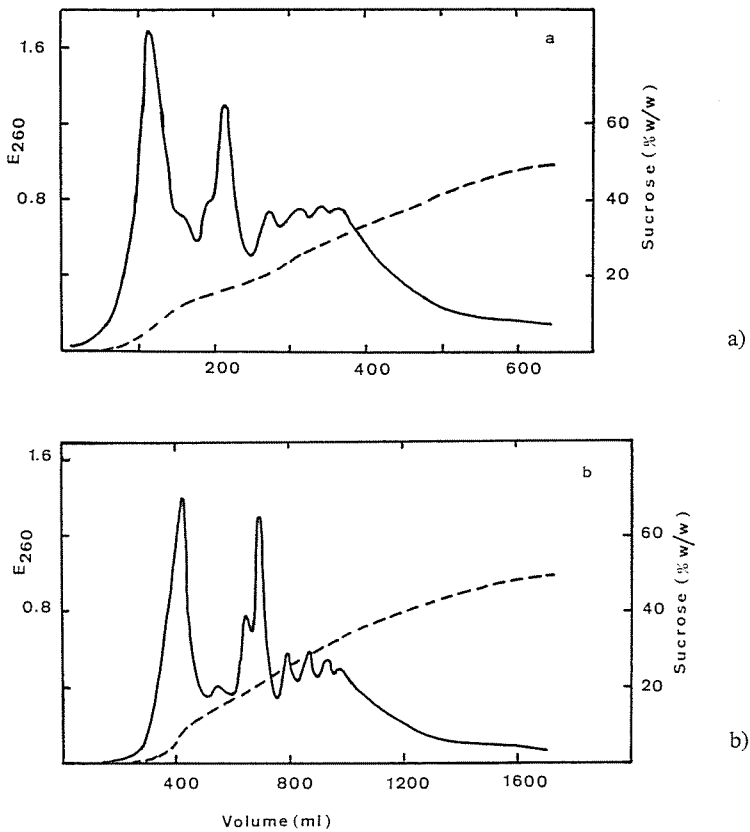

Fig. 1. Fractionation of a 25 ml sample of a post-mitochondrial extract of mouse embryos, (a) in a BXIV rotor (400 ml of gradient) and (b) in a BXV rotor (1200 ml of gradient). Further details: BIRNIE et al., 1969.

Considerable loss of resolution also occurs if the sample is not introduced into the rotor at a slow and even rate. At rates greater than 2 to 5 ml/min, sufficient mixing of the sample with the topmost layer of the gradient takes place to cause significant broadening of the sample zone (CLINE, 1971). For the same reason it is necessary to load the overlay solution slowly, particularly that part immediately overlying the sample zone. Since it is extremely difficult to load solutions into a rotor slowly and evenly with a hand-operated syringe, the use of a suitable pump is recommended, particularly when maximum resolution is being sought.

The third factor affecting resolution is the nature and shape of the density gradient itself. Density gradients which are linear with volume are popular since they are the simplest to prepare. In tubes, gradients linear with volume are also linear with radius (that is, distance from the axis of rotation) over at least 80% of the tube. However, in zonal rotors precisely that feature which is designed to eliminate the wall-effects encountered in swing-out rotors introduces a complication in that gradients linear

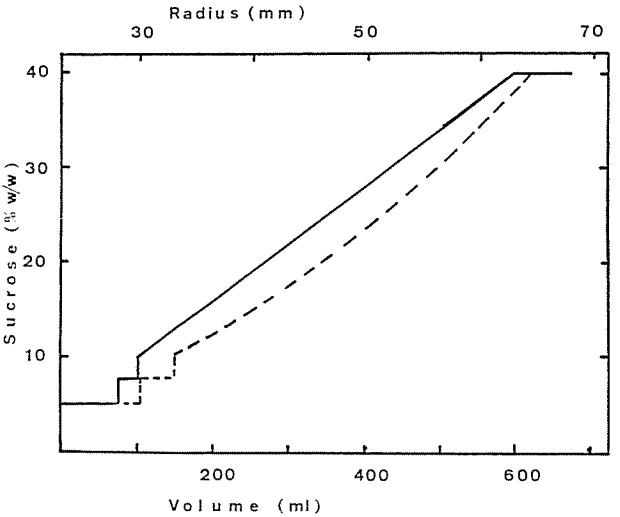

Fig. 2. Relationship of radius and volume of a BXIV rotor. A 500 ml sucrose gradient (10% to 40% w/w) linear with volume is plotted against (i), the volume (——) and (ii), the radius (– – – –) of the rotor. Volumes of overlay and sample are 75 ml and 25 ml, respectively.

with volume are not linear with radius. This is illustrated by Fig. 2, in which the contents of a BXIV rotor have been plotted as a function first of rotor volume and, second, of rotor radius. It is clear there is a significant deviation from linearity in the second case. Fortunately, calculation of gradients linear with radius is simple. Preparation of them is also simple, particularly with the programmable gradient engines now available commercially. Indeed, very good approximations can often be obtained using simple homemade gradient engines of the types described by ANDERSON and RUTENBERG (1967), BIRNIE and HARVEY (1968) and HINTON and DOBROTA (1969).

One major attraction of using gradients linear with radius is the ease with which sedimentation coefficients can be calculated. However, so far as resolution is concerned, these gradients are not always the most suitable. Why this is so is shown by Fig. 3, in which the sedimentation coefficients of particles in a gradient linear with radius (curve B) are plotted as a function of rotor radius (curve A). It is clear that in the first half of the gradient resolution is good while in the remainder it becomes increasingly poor. Such a gradient is useful for separating some mixtures of particles, for example when a rapidly-moving zone is sedimented into the bottom half of the gradient and so separated from two or more slowly-moving zones which are, at the same time, resolved in the top half. Unfortunately, not all mixtures of biological particles behave in this way (for example, polysomes) and other shapes of gradient can give better resolution with complex mixtures of particles such as tissue homogenates. In these cases, convex gradients (for example, curve C, Fig. 3) are often used. Convex gradients have one additional advantage, that is, the region of maximum capacity (ability to support a zone of particles) is at the top, exactly where it is required since the gradient must support a mixture of particles in one zone in that region. As

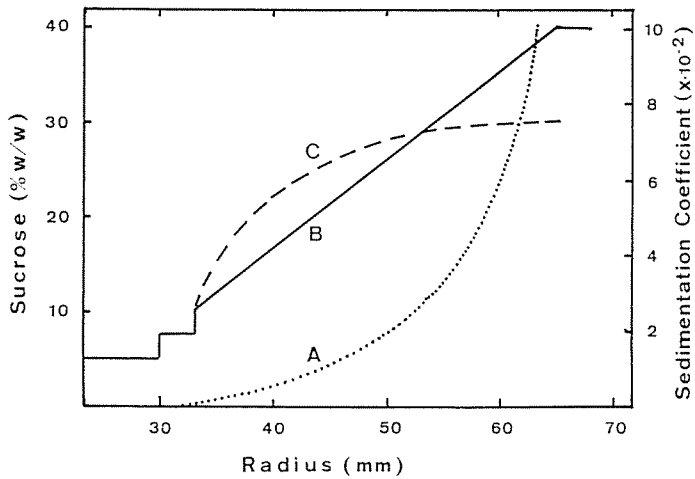

Fig. 3. Sedimentation coefficients (curve A) of particles in a 10% to 40% (w/w) sucrose gradient linear with radius (curve B) in a BXIV rotor. Conditions – overlay, 75 ml; sample, 25 ml; gradient, 500 ml; speed, 45,000 rev/min; time, 1 h.; temperature, 5°; particle density, 1.2 g/cm^3. Curve C is a convex 10% to 30% (w/w) gradient.

the mixture migrates and separates into a series of zones, less capacity is required so that the gradient can become more shallow and thus be capable of much greater resolution than if it had continued as steeply as it had begun. However, the latter end of the gradient must not be too shallow, particularly when large amounts of complex mixtures are being fractionated. The danger of overloading the shallow part of the gradient is most acute when the mixture contains particles which migrate rapidly as a relatively broad, and so dilute, zone. If these particles band isopycnically in the shallow region of the gradient they may form a narrow, and so concentrated, zone in which the capacity of the gradient is exceeded. Also, when complex mixtures like tissue homogenates are fractionated, it is found quite commonly that a zone of particles which has migrated rapidly to its isopycnic point is overtaken by a more slowly-moving zone of denser particles. If the gradient is too shallow at that point it will become overloaded, the local instability so caused resulting in loss of resolution.

In recent years, a number of complex gradients have been designed for use in zonal rotors. These include isometric gradients (SPRAGG et al., 1969), equilibrium gradients (SPRAGG, personal communication), isokinetic gradients (STEENSGAARD, 1970) and equivolumetric gradients (PRICE, *vide infra*). Each of these gradients has been designed with particular purposes in mind. While they may be applied directly to other separations, frequently it is found that, for a particular mixture of particles, a gradient must be "tailored" specifically to suit the mixture and the separation required, especially if the maximum resolution is desired. Designing any one such complex gradient from first principles is difficult and requires the assistance of a computer. Production of the gradients is simplest with a programmable gradient engine, though close approximations can usually be achieved with one of the home-made variety.

So far in this discussion it has been assumed that the gradient introduced into the rotor remains unchanged during the time taken for the mixture of particles to be separated. While it is true that shallow, light gradients alter very little and only slowly during centrifugation, heavy, steep gradients do change both rapidly and substantially. Fig. 4 shows the change which took place in a 10% to 60% (w/w) sucrose gradient in only 20 min. The rate at which a gradient changes is very rapid initially, but quickly diminishes. However, movement in the gradient does continue

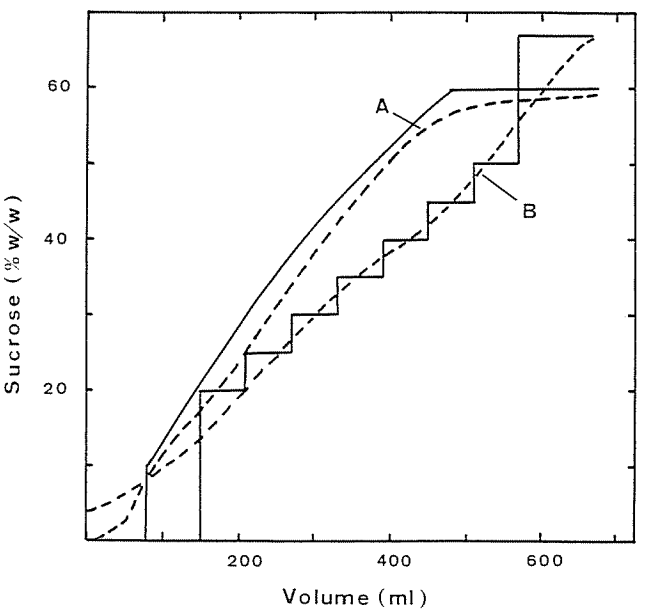

Fig. 4. Changes observed in shapes of gradients in a BXIV rotor with time: (A) in 20 min at 4000 rev/min; (B) in 17 h at 30,000 rev/min. In both (A) and (B) the solid line is the gradient introduced into the rotor, the dashed line is the gradient recovered from the rotor.

for a long period until, eventually, an equilibrium gradient is achieved, that is, a gradient in which the rate of back-diffusion is exactly balanced by the rate of sedimentation of the gradient material. Fig. 4 also shows how discontinuities in a gradient are eliminated. Although the change shown here was that found after 17 hours, 90% of the gradient movement had taken place within the first hour.

The effect of such *in situ* gradient movements is again to decrease resolution since the diffusion of the gradient material is accompanied by diffusion and broadening of the particle zones. This problem can, in theory, be overcome by loading the rotor initially with an equilibrium gradient. One such gradient, designed to separate 5 S, 9 S and 12 S RNA, is shown in Fig. 5. However, gradients of this type suffer from a number of disadvantages which limit their usefulness. First, an equilibrium gradient is only in equilibrium at one specified rotor speed and temperature, but it is in the rotor at a very much lower speed for a considerable time while it, and the

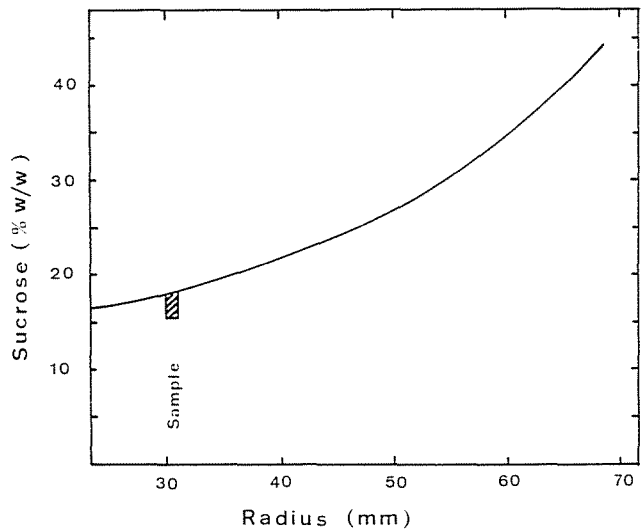

Fig. 5. An equilibrium gradient for separating 5 S, 9 S and 12 S RNA at 45,000 rev/min and 10° in a BXIV rotor.

sample and overlay solutions, are being loaded. Second, equilibrium gradients are shallow and thus have a low capacity. For example, the maximum capacity of the gradient shown in Fig. 5 is only 25 mg of RNA. Third, the density gradient is continuous throughout the rotor. This means that (a) the density of the sample zone must be adjusted with very great care to avoid introducing density inversions and (b) the sample must be inserted into the gradient itself. Both of these operations are tricky. Finally, computation of these specific equilibrium gradients is difficult and requires a sophisticated computer. It is technically possible to centrifuge a gradient until it does reach equilibrium before the sample is introduced into the rotor, but this is time-consuming. The use of self-forming equilibrium gradients has, until now, been confined to isopycnic banding separations, mainly with caesium salts. The formation of equilibrium gradients with most other gradient materials, for example sucrose, takes very much longer than with caesium salts.

Interpretation

Once the separation of a mixture of particles has been achieved and a series of zones obtained, the question arises of how to identify the particles within each zone. Experience with rate-zonal separations in swing-out rotors immediately suggests that the first step is calculation of the sedimentation coefficients. Just as with swing-out rotor gradients, in experiments with zonal rotors in which the gradient is linear with radius the calculation of S values is a simple manual operation (see MCEWEN, 1967). With more complex, non-linear gradients, these calculations can readily be done with a computer. However, it is necessary to ask how accurately the sedimenta-

tion coefficients can be calculated. A number of the factors which have already been shown to affect resolution in zonal rotors also influence the accuracy of such calculations.

The problem of the movement of the gradient while sedimentation is taking place is probably the most pertinent since, quite obviously, the more the gradient has changed during the experiment, the less accurate are the calculated sedimentation coefficients. In general, steep gradients deviate more than shallow gradients and gradients change more during long runs than during short ones, though it must be remembered that the initial rate of change is the most rapid. Unless an equilibrium gradient is used some degree of change must be expected. The only practical answer is to compare the gradient recovered from the rotor after the experiment with the initial gradient. The closer these are, the more accurate will be computed sedimentation coefficients. In special cases, it may be possible to add to the sample a material of known sedimentation coefficient close to that of the zone of particular interest, to provide an internal correction for gradient movement.

The accuracy with which the initial position of the sample zone is known is also important. If the mixture to be separated does not contain material which will not sediment during the experiment (for example, the soluble protein in a tissue homogenate) then it may be worthwhile to add a suitable material to the mixture. However, in all cases, care must be taken to adjust the density of the overlay and sample solutions to obviate back-diffusion of the material used as the marker of the initial position of the sample zone, since such back-diffusion leads to serious errors.

Variations in temperature and errors in determining temperature during the sedimentation also lead to erroneous computations, particularly with heavy sucrose gradients in which the viscosity of the solution varies substantially with the temperature. Fortunately, in recent years, methods of measuring and controlling the temperature of rotors have improved so that errors due to this factor have been minimised, though not eliminated. However, one problem in this area remains, that is, the existence of a temperature gradient from the centre to the periphery of the rotor. That such a gradient exists is known, but its magnitude, and hence the magnitude of the error so introduced, has not been measured.

Finally, inadvertent errors introduced by practical difficulties encountered in pumping large volumes of (frequently) dense and viscous solutions must be remembered. The possibility of introducing errors as a result of slight undetected cross-leaks, particularly with the older types of seal assembly, is very real. In such cases, the golden rule is "be patient and pump slowly".

The conclusion to be drawn from the foregoing discussion is that sedimentation coefficients calculated from the distribution of the bands in a zonal rotor are not always as accurate as may be thought, or hoped. Under specially-selected conditions, S values calculated from zonal experiments can be quite accurate. However, in general, the errors involved are such that sedimentation coefficients determined in this way should be used only to indicate the possible nature of the particles in particular zones and positive identification must depend on other criteria such as specific enzyme assays, chemical analyses and light or electron microscopic examination of the material.

Isopycnic Sedimentation

This method has been much less used than has rate-zonal sedimentation in zonal rotors. One drawback of zonal rotors, particularly of the B-series, is the difficulty of maintaining sharp density discontinuities, such as can be obtained in swing-out rotors. For example, zonal rotors do not give such sharp fractionations of microsomes as has been obtained by TATA and his co-workers (TATA, 1969), who have used swing-out density gradients to separate the submicrosomal fractions as sharp bands at the interfaces between layers of sucrose solution of different densities. However, zonal rotors do have the advantage of much greater capacity, and isopycnic sedimentation in zonal rotors has been used successfully to separate nuclei from avian reticulocytes and erythrocytes (MATHIAS et al., 1969), to isolate plasma membranes (HINTON et al., 1971), to purify mammalian DNA (WILLIAMSON, 1969) and to isolate and partly purify mammalian viruses (FOX et al., 1968).

One advantage of using isopycnic sedimentation in zonal rotors as compared to rate-zonal sedimentation is that the capacity of the rotor is extremely large. This is because the sample need not be introduced as a narrow zone but may occupy virtually the whole of the rotor. One application of this is illustrated by Fig. 6, which shows that a virus can be isolated, and partly purified, from litre volumes of culture fluid by isopycnic banding in a BXIV rotor (FOX et al., 1968). The use of this technique to purify mammalian DNA is illustrated by the experiment summarised in Fig. 7, in which 32 mg of DNA have been banded in 450 ml of a self-formed CsCl gradient (WILLIAMSON, 1969). Fig. 7 also shows that the degree of resolution achieved in CsCl gradients in a zonal rotor is comparable to that in a swing-out rotor, though less than that in a fixed-angle rotor (FLAMM et al., 1969). Two other important points with regard to CsCl gradients in zonal rotors should be noted. First, there is no

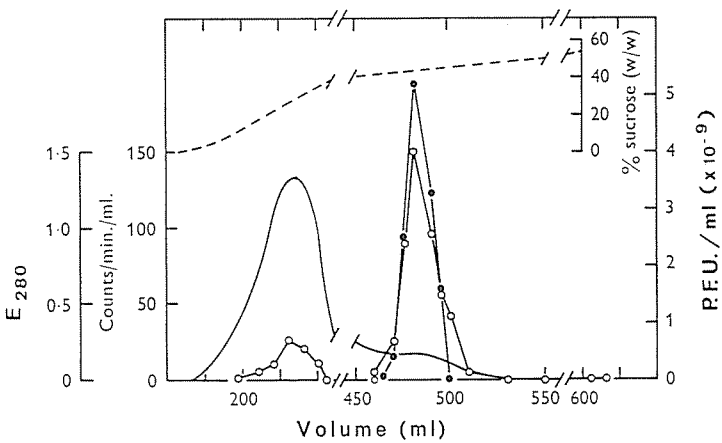

Fig. 6. Isopycnic banding of Semliki Forest virus in a 20% to 50% (w/w) sucrose gradient in a BXIV rotor. Centrifugation was for 16 h at 29,000 rev/min and 10°. ——, E_{280}; – – – –, sucrose gradient; ●——●, infectivity titre; ○——○, radioactivity (from FOX et al., 1968).

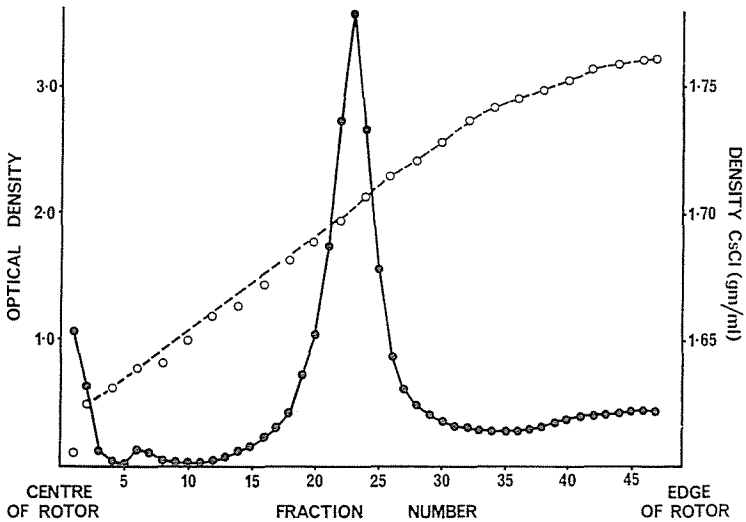

Fig. 7. Isopycnic banding of 32 mg of mouse DNA in a self-formed CsCl gradient in a BXIV rotor. The volume of CsCl was 450 ml, of initial density 1.695 g/cm³; centrifugation was for 72 h at 39,000 rev/min and 20°. ●—●, E_{260}; o---o, CsCl gradient (from WILLIAMSON, 1969).

need to use an immiscible overlay as is usual in CsCl gradients in tubes since the rotor can be spun while only partly filled (WILLIAMSON, 1969). Second, caesium salts rapidly and destructively attack aluminium and gradients of CsCl can only be used in titanium zonal rotors.

Isopycnic sedimentation has one other advantage over rate-zonal sedimentation, that is, the density gradient is much less critical. The only criteria to be observed are, first, that the range of the gradient must cover the range of the buoyant densities expected and, second, the gradient must be shallow enough to separate the particles. In fact, extremely dense but shallow sucrose gradients are quite feasible (see, for example, MATHIAS et al., 1969). Movement of the gradient during centrifugation is not critical so long as the period of centrifugation is long enough to ensure that all the particles have reached their isopycnic points. By the time this is achieved the period during which the gradient undergoes rapid change will have been passed and any changes which may still be taking place will be very slow, and the error introduced will not be significant. If the gradient which is recovered from the rotor is monitored, an accurate estimate of the buoyant density of the particles in each zone will be obtained.

Conclusion

Zonal rotors have enormous potential for fractionating large quantities of complex mixtures of biological particles, either by rate-zonal or by isopycnic sedimentation. In general, these rotors have much greater capacities and resolving power than swing-out rotors. Despite some pitfalls, quite respectable fractionations can be

achieved even when the simple precautions outlined in this article have not been taken. When care is taken, however, to circumvent these pitfalls, the increase in resolution obtained enables the capabilities of zonal rotors to be utilised to the full, and, in most cases, more than repays the extra effort required to design and obtain optimum conditions for the particular separation required.

Acknowledgments

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