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The Construction and Analysis of Sucrose Gradients for Use with Zonal Rotors

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The rate of sedimentation of a particle in a sucrose solution depends on the viscosity and density of the medium. These two variables are related to the sucrose concentration and the temperature of the medium by new simple equations. These equations were used in a rapid iterative procedure that relates the distance moved by a zone in a continuous sucrose gradient to its sedimentation coefficient. It is shown by comparison with experiment that this iterative method allows the distance moved by a zone to be calculated rapidly. The method may therefore be used to optimize the separation of particles in a sucrose-gradient-centrifugation experiment. The method also allows the unknown sedimentation coefficients of several zones to be measured from a single sucrose-gradient-centrifugation experiment.

Centrifugation has been widely used to separate, purify and characterize many systems, from simple molecules to subcellular organelles [for reviews see, e.g., Cline & Ryel (1971) and Freifelder (1973)]. The introduction of large-scale preparative rotors and the use of sucrose gradients to stabilize the zones has allowed the several components of a complex mixture to be separated. The sucrose gradient not only stabilizes the separating zones, but it also affects the rate at which the particles sediment, depending on the viscosity and density of the sucrose solution.

Over the last decade, attempts have been made to predict the behaviour of a sedimenting particle in a sucrose gradient [see Funding & Steensgaard (1973), Schumaker (1967) and Steensgaard & Funding (1974)]. In previous methods, the viscosity and density were related to sucrose concentration and temperature either by using tabulated data or by use of polynomial equations. The method described in the present paper uses an iterative procedure with new equations to calculate the viscosity and density of the suspending medium at any point in either the B XIV or B XV zonal rotors.

Theoretical Analysis

The basic equation relating the rate of sedimentation of a particle to the centrifugal field is:

$$\frac{dr}{dt} = s'\omega^2 r \qquad (1)$$

where s' is the measured sedimentation coefficient of the particle; it is related to a hypothetical constant $s^0_{20,w}$ by:

$$\frac{s'\eta}{(\rho_p-\rho)} = \frac{s^0_{20,w}\eta_{20,w}}{(\rho_p-\rho_{20,w})} \qquad (2)$$

where η and ρ are the viscosity and density of the ambient medium and $\eta_{20,w}$ and $\rho_{20,w}$ are the values of the same variables for water at 20°C. The particle is assumed to behave ideally so its density, ρ_p, is a constant, which is not influenced by the density or composition of the surrounding medium nor is it dependent on the hydrostatic pressure. Similarly, $s^0_{20,w}$, the sedimentation coefficient of the particle extrapolated to zero solute concentration in water at 20°C, is assumed to be independent of both sucrose and solute in the surrounding fluid.

Combining eqns. (1) and (2) leads to:

$$\frac{dr}{dt} = s^0_{20,w}\omega^2 r\frac{(\rho_p-\rho)}{\eta}\cdot\frac{\eta_{20,w}}{(\rho_p-\rho_{20,w})} \qquad (3)$$

which may be rewritten as:

$$\ln r = \frac{s^0_{20,w}}{\phi_{20,w}}\int_0^t \omega^2\phi\,dt \qquad (4)$$

where ϕ is the buoyancy/viscosity ratio (Eikenberry, 1973) and eqn. (4) is one form of the sedimentation equation. This equation is easy to solve for homogeneous media in which the viscosity and density are constants and may be obtained from standard tables (e.g. Anderson, 1968). Eqn. (4) is more difficult to solve for zonal-centrifugation experiments, because ϕ depends on radius, since the sucrose concentration varies with distance from the centre of the rotor.

We used an iterative method of solving eqn. (4) in which the integration is replaced with a summation procedure in which the particle is assumed to sediment through a sucrose solution whose concentration changes in discrete steps. The buoyancy/viscosity ratio is calculated at r_0, the starting position of the sedimenting species, allowing dr, the distance sedimented in a time dt, to be calculated. The buoyancy/viscosity ratio at r_0+dr, or r, is then calculated so that the distance sedimented in a further interval, dt, may be computed. The

reiterative procedure is continued until dt is equal to the total time at speed.

This procedure is similar to that used by Steensgaard *et al.* (1973), who obtained the viscosity and density of the sucrose solution at any point in the rotor from sets of standard tables. In our procedure, the search routine is replaced by functions relating the viscosity and density of the sucrose solution to its concentration and temperature.

Two new equations were developed by using the data by Anderson (1968) from Barber's (1966) derived equations:

$$\rho = 1.0004 \exp\{10^{-4} \times [(43.52 - 0.039\theta)c - 0.0612(\theta - 4)^2]\} \quad (5)$$

$$\eta = 6581/\{[(61.5 + \theta) - (1 + 0.011\theta)c]^2\} \quad (6)$$

The sucrose concentration, c, is in g/100ml (%) and θ is the temperature in °C; the viscosity is measured in centipoise and the density in g/cm³. The maximum deviation between the values calculated by these equations and those obtained from the standard tables is 0.01 g/cm³ in density and 2% in viscosity, over the range 0–30°C and 0–50% sucrose, the range of values usually used in sucrose-gradient experiments.

The usual methods of preparing sucrose gradients is to either mix or layer specific volumes of differing concentrations of sucrose solutions. The sedimentation equation (eqn. 4), however, is defined in terms of the radius and so the volume of liquid used must be related to the distance from the centre of rotation. Both the B XIV and B XV rotors approximate to cylinders (van der Zeyst & Bult, 1972), so a quadratic equation should adequately relate radius to volume. The experimental data of Funding & Steensgaard (1973) were used in a least-squares analysis that gave for the B XIV rotor:

$$V + 38.42 = 17.78(r - 0.47)^2 \quad (7)$$

and for the B XV rotor:

$$V + 48.05 = 24.86(r - 0.484)^2 \quad (8)$$

The equations are given in this form to facilitate rapid transformation between radius and volume. Within the range normally used for sucrose-gradient experiments (i.e. an overlay volume greater than 175ml for the B XIV rotor and 300ml for the B XV rotor and more than 50ml of cushion), the greatest deviation in radius for a given volume between the tabulated data of Funding & Steensgaard (1973) and that given by eqns. (7) or (8) is 0.01 cm. This difference could reflect the precision of the original calibration. If the volume of overlay is less than the values quoted above, then the radii obtained by means of the equations differs from the original data (Funding & Steensgaard, 1973), because the presence of the pyramidical core means that the geometry of the rotors no longer approximates to a cylinder.

The coefficients in eqns. (7) and (8) differ from those of Eikenberry (1973), because he corrected the volume to zero at the bottom of the core pyramid; in our treatment, the need for this correction is avoided. Similarly, the equation that van der Zeyst & Bult (1972) developed from a geometrical analysis included a term to describe the volume of overlay used; for this reason, a numerical solution to their equation leads to coefficients that differ from both those given in eqns. (7) and (8) and those given by the equations of Eikenberry (1973).

Factors limiting the accuracy of the method

The iterative method of analysing the sedimentation behaviour of a particle allows the resolution of a sucrose gradient to be predicted. To be of value the performance of a gradient predicted by theory must match the behaviour observed in practice.

Factors that lead to uncertainty in the calculations of the distance moved by the sedimenting particle include: (*a*) limitations of the theoretical analysis; (*b*) insufficiently precise data for the particle's sedimentation coefficient and density; (*c*) limitations of the ultracentrifuge; (*d*) deviations from the assumed ideal behaviour of the particle; (*e*) diffusion of the suspending medium or the sedimenting solute. These sources of error are discussed, in order, below.

(*a*) *Limitations of the theoretical analysis.* Most zonal-centrifugation experiments are run between 0° and 30°C by using a gradient with a sucrose concentration that does not exceed 50%. Within this range the values of viscosity and density calculated by eqns. (5) and (6) do not differ from the tabulated values (Anderson, 1968) by more than 2% for viscosity or by 0.01 g/cm³ for density. In other words, over the restricted ranges given above, the use of the simple equations (eqns. 5 and 6) does not lead to an appreciable error.

The criterion used in choosing the time-interval dt in the iterative procedure was that the increase in sucrose concentration per iteration for the fastest moving particle did not exceed 0.1%. Changing the increase in sucrose concentration per iteration to values between 0.01 and 1% led to the calculated distance moved by the 50S L-sRS particles* to vary by not more than 3ml of the mean value.

(*b*) *Data for the sedimenting particles.* The value assumed for the density of the particle is that found for subribosomal particles ($\rho = 1.4$ g/cm³) and RNA (1.5 g/cm³) in sucrose (Petermann, 1964). An error as great as 0.05 g/cm³ in the density leads to a change of

* Abbreviations: S-sRS and L-sRS particles, the smaller and larger subribosomal particles respectively; S-rRNA, the RNA species of S-sRS particles; L-rRNA, the major RNA species (23S–28S) of L-sRS particles.

5% in the value of $(\rho_p-\rho)/(\rho_p-\rho_{20,w})$ at 0°C and 40% sucrose; in practice, at higher temperatures and lower sucrose concentration, the loss of accuracy, caused by an imprecise value for the density of the particle, should not exceed 2%. It is only as isopycnic conditions are approached that a very accurate value of particle density is required.

The rate of sedimentation of any particle is directly proportional to the sedimentation coefficient (eqn. 4) so the accuracy of the calculation of the distance moved depends directly on the accuracy of the value of the sedimentation coefficient. The latter may be readily measured in the analytical ultracentrifuge and it is then assumed that the particle behaves ideally so the sedimentation coefficient is not affected by temperature, pressure or interaction with either the ambient surroundings or any other solute particles that may be dispersed in the sample.

(*c*) *Shortcomings of the ultracentrifuge.* The problems that arise from using the ultracentrifuge are (i) lack of adequate temperature control, (ii) imprecise speed control and (iii) the need to take the acceleration and deceleration periods into account when computing the duration of the run.

(i) Inaccuracies of temperature control. The largest degree of uncertainty is that caused by inadequate temperature control; a change in temperature from 20° to 21°C causes a change in the viscosity of 30% sucrose of 3%. Assuming that the temperature gauge is accurate, the precision of the temperature is governed by the limits of the thermostat; these may be ±1°C of the defined temperature, leading to an uncertainty of 5% in the buoyancy/viscosity ratio. In addition there may exist a temperature gradient across the rotor, if the rotor and bowl were initially at the same temperature and the vacuum in the chamber is good, there should be little frictional heating and hence only a shallow temperature gradient between the edge and centre of the rotor.

(ii) Uncertainties of speed control. The speed is estimated from a dial and not from a digital read-out; there may be a slight error in setting the required speed. The selected speed is not kept constant, but varies between the lower and upper 'cut-out' values. The slight error in the speed ω is magnified in the sedimentation equation where ω^2 is used; an inaccuracy of 1% in the speed leads to a loss in accuracy of 2% in ω^2 and a similar error in the distance a particle is calculated to have moved. This error is not diminished simply by the use of a speed integration device; if the rotor speed varies then the integral in eqn. (4) cannot be separated into a constant ω^2 part and a changing ϕ, but the product of the two must be considered when the integral is evaluated. The loss of accuracy caused by deviations from the set rotor speed should be sufficiently small to be ignored.

(iii) Inaccuracies in timing the run. Because of the finite acceleration and deceleration periods of the ultracentrifuge, the time the rotor actually spends at speed differs from the theoretical duration of the run. It is straightforward to modify the programme based on the iterative procedure to include an allowance for the periods of acceleration and deceleration.

The necessity for an accurate knowledge of the speed of rotation of the rotor and the duration of the run is avoided if particles of known sedimentation coefficients are mixed with the sample and run as internal markers in the gradient. The amount of computation necessary to calculate the unknown sedimentation coefficient of a zone is decreased if a rate linear gradient is run with markers of known sedimentation coefficients [see, also, Schumaker (1967)].

(*d*) *Deviations from ideal behaviour of the particle.* It has been assumed that the sedimentation equation (eqn. 4), which was derived for particles that behaved ideally ($s_{20,w}=s^2_{20,w}$; ρ_p is a constant), also applies to real particles sedimenting through a sucrose gradient. At low concentrations, subribosomal particles do behave ideally and the separated zones are symmetrical. The behaviour becomes non-ideal and the zones become skewed when large loads are used [see, e.g., Eikenberry *et al.* (1970) and Steensgaard *et al.* (1975)], because the sedimentation coefficient, $s_{20,w}$, differs from $s^2_{20,w}$. The analysis does not accurately describe the sedimentation behaviour of any particle that exhibits non-ideal behaviour because the initial assumptions are no longer valid.

(*e*) *Diffusion of the suspending medium or the sedimenting solute.* (i) Medium. The medium that is used to stabilize the sedimenting zones is usually an aqueous solution of a low-molecular-weight substance, such as sucrose, glycerol or metrizamide. The diffusion coefficient of these substances is high, so that a sharp boundary is rapidly blurred. The loss of a step in sucrose gradient will be seen in the results (Figs. 1 and 2); the sucrose step that was introduced with the sample soon disappears. In the programme used to analyse the sedimentation behaviour of the particles, the step was assumed to be a permanent feature. This assumption did not lead to any great errors (see the Results section), but it may become more important for a step that is near the edge of the rotor and hence has more time to diffuse.

(ii) Solute. The peak-width depends directly on the diffusion of the sedimenting species; this is affected by the concentration and temperature of the sucrose solution (see below under 'Optimization of the separation of the components of a mixture'). The diffusion of high-molecular-weight solutes is sufficiently small for the effect on peak-width to be ignored if only short run times are involved.

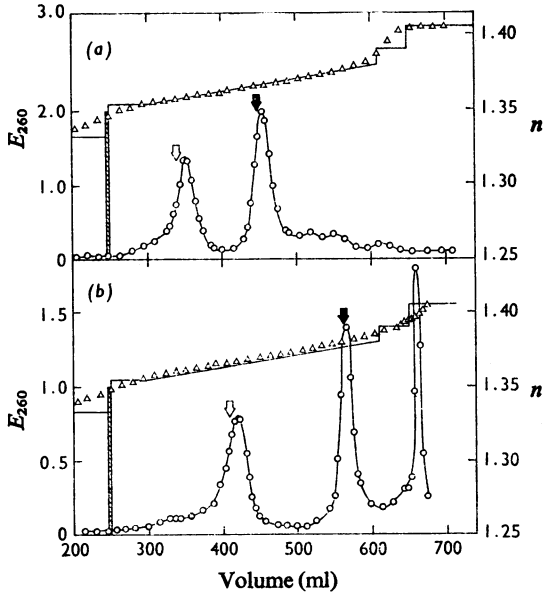

Fig. 1. *Effect of time on the separation of E. coli S-sRS particles and rabbit reticulocyte L-sRS particles*

The subparticles were isolated as described previously (Cox *et al.*, 1973) and mixed with sucrose to give 7mg of ribonucleoprotein/ml of 10% sucrose. The sample (3ml) (shaded area in the Figure) was layered on to a 15–40% sucrose gradient, overlaid with 250ml of sucrose-free A4 buffer (see the Methods section) and centrifuged at 43000rev./min in a B XIV rotor for 1h (*a*) or 2h (*b*) at 12°C. The rotor was unloaded at 20ml/min, 15s fractions were taken and the E_{260} was measured in a Pye–Unicam SP.500 spectrometer. The theoretical sedimentation rate of the two subparticles was calculated by the iteration procedure given in the text and assuming a density of 1.4g/cm³ for both particles and sedimentation coefficients of 30S for *E. coli* S-sRS particles (⇩) and 60S for rabbit reticulocyte L-sRS particles (⇩) (Cox *et al.*, 1973). △, Measured refractive index of the effluent; ——, expected distribution of the refractive index assuming that no diffusion occurred and eqn. (9) is valid; ○, measured E_{260}.

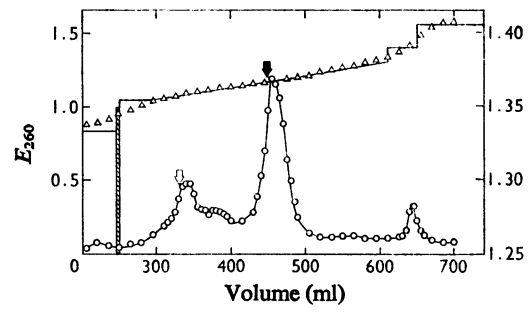

Fig. 2. *Separation of E. coli S-rRNA and L-sRS particles*

The subparticles were isolated by using the method of Cox *et al.* (1973); the RNA was prepared from the isolated small subparticles by precipitation of the guanidinium salt with ethanol (Cox, 1968). The gradient conditions used and the meaning of the symbols are the same as given in Fig. 1, except the run duration time was 1h and the temperature was 20°C. The density and sedimentation coefficient of the S-rRNA (⇩) were assumed to be 1.5g/cm³ and 17S and those of the L-sRS particles (⇩) to be 1.4g/cm³ and 50S (Cox *et al.*, 1973).

Scope of the method

Solution of the sedimentation equation for single and multi-component systems. The iterative method can be used to calculate the distance moved by a particle of defined sedimentation coefficient in a sucrose gradient. The method is based on the assumption that the particle behaves ideally, so the sedimentation coefficient is affected neither by the concentration of the solute nor by interaction with the gradient material, except for the effects of viscosity and density.

The lack of a concentration effect means that radial dilution can be ignored and there is no need to consider the diffusion of the sedimenting zone; the analysis is applied only to the centre of the zone (see above for a discussion of zone broadening). If the particle behaves ideally, the expected sedimentation behaviour is not changed by the suspending medium, so the method can be extended to analysing the sedimentation behaviour of samples containing several components. The iteration method is easily modified to find the distribution of sedimentation coefficients after an experiment by reiterating the main loop several times, each time with a new value for the sedimentation coefficient.

Separation of two-component mixtures. The iterative method has been described and the factors that could affect accuracy have been outlined; it remains to validate the analysis by comparison of the observed separation of different mixtures with the calculated separation.

The mixtures used were: (*a*) S-sRS particles of *Escherichia coli* ($s_{20,w} = 30.0$S, $\rho = 1.4$g/cm³) and L-sRS particles of rabbit reticulocyte ($s_{20,w} = 60.0$S, $\rho = 1.4$g/cm³); (*b*) *E. coli* S-rRNA ($s_{20,w} = 17.0$S, $\rho = 1.5$g/cm³) and L-sRS particles of *E. coli* ($s_{20,w} = 50.0$S, $\rho = 1.4$g/cm³). The mixtures were chosen to give a wider range of sedimentation coefficients (17.0–60.0S) and density (1.4 or 1.5g/cm³) than could be achieved by the separation of dissociated ribosomes. In addition, each component could be isolated easily in a preparative sucrose gradient (see the Methods section).

The accuracy of the theoretical analysis can be tested by running several experiments under different conditions. The gradient chosen was

15–40% sucrose, linear with volume; this is a steep gradient so that any particle that sediments, even as little as half-way into the gradient, still encounters a wide range of viscosity and density. By centrifuging the above mixtures in this gradient for different times (1 or 2h) and at different temperatures (12° or 20°C), the theoretical analysis is tested for accuracy under a wide range of conditions.

Methods

Ribosomal subparticles were isolated from *E. coli* and rabbit reticulocytes by using the methods described previously (Cox *et al.*, 1973). RNA was prepared from the subparticles by precipitation of the guanidinium salt with ethanol (Cox, 1968). All sucrose-zonal-centrifugation experiments were performed with an MSE super-speed 65 Mk II ultracentrifuge and a B XIV titanium rotor. The gradient was formed by using an MSE gradient engine with a cam cut to the desired shape (see Fig. 1). The sucrose solutions were made from AnalaR sucrose (BDH Chemicals, Poole, Dorset, U.K.) dissolved in sucrose-free A4 buffer (25mM-KCl/1mM-$MgCl_2$/50mM-Tris/HCl, pH6.8). The amount of overlay, the duration of the run and the temperature of the run were all variables that were defined for individual experiments. The acceleration and deceleration periods of the rotor were 15 and 45min respectively and both were approximately linear so the actual run time at speed was 20min less than the theoretical duration of the run.

The gradient was pumped out at 20ml/min, and 15s fractions were collected after discarding all but the final 50ml of overlay. The E_{260} of each of the fractions was measured in a 1cm-path-length cell in a Pye–Unicam SP.500 spectrophotometer against a blank containing water. The sucrose concentration was calculated from the refractive index measured in an Abbé refractometer (Bellingham and Stanley, London N.15, U.K.); experiment showed that the refractive index, n, of a sucrose solution was linearly related to the sucrose concentration (c in g/100ml) by the equation:

$$n = 1.3337 + 0.001422c \quad (9)$$

All calculations were performed with the N.I.M.R. Hewlett–Packard 3000 computer system.

Results

Separation of S-sRS particles of E. coli and L-sRS particles of rabbit

The accuracy of the interative method was checked by comparing the results of calculation with the experimental results for a wide range of conditions. This particular mixture of subparticles was centrifuged at 12°C for 1h (Fig. 1*a*) and 2h (Fig. 1*b*). Increasing the length of the run ensured that the full range of the gradient was tested. Moreover, the longer run required more iterations and is a more demanding test of the computational procedures. The observed positions of the zones of the components agreed well with the values calculated on the basis of the known $s_{20,w}$ values of the two subparticles (Cox *et al.*, 1973). No better agreement between theory and experiment could be expected because it is within the precision of the experimental method.

Separation of the S-rRNA and L-sRS particles of E. coli

The separation of S-RNA ($s_{20,w} = 16S$; $\rho = 1.5g/cm^3$) and *E. coli* L-sRS particles ($s_{20,w} = 50S$; $\rho = 1.4g/cm^3$) that was achieved after centrifuging for 2h at 43000rev./min at 20°C is given in Fig. 2. The final positions of the zones were a good agreement with the values of the distance moved calculated by means of the iterative method.

Discussion

Optimization of the separation of the components of a mixture

The number of trial-and-error experiments required to achieve the best separation of the components of a mixture is decreased if a rapid method is used to calculate the distribution of the components in the rotor after using a particular set of conditions. The iterative method, outlined in the Theoretical Analysis section, may be used to reveal the distribution of the components, but optimization of separation requires a knowledge of zone width as well as the distance between zones. A measure of how the zone width is affected by diffusion is achieved by assuming that the initial sample layer is infinitely thin; according to Crank (1956), the zone width Δr, at any time, t, is then given by:

$$\Delta r = \rho_{20,w}\eta_{20,w}\int_0^t \frac{1}{\eta}\,dt \quad (10)$$

or:

$$\Delta r \propto \int_0^t \frac{1}{\eta}\,dt \quad (11)$$

An approximate solution to eqn. (11) is obtained if the integral is replaced by a summation:

$$\sum_{t=0}^{t}(1/\eta)$$

which can be solved by modification of the interative procedure, to give a rapid calculation of the effect of changing run conditions on zone width. Factors other than simple diffusion influence zone width [see, for example, Sartory (1969) and Schumaker & Halsall (1971)].

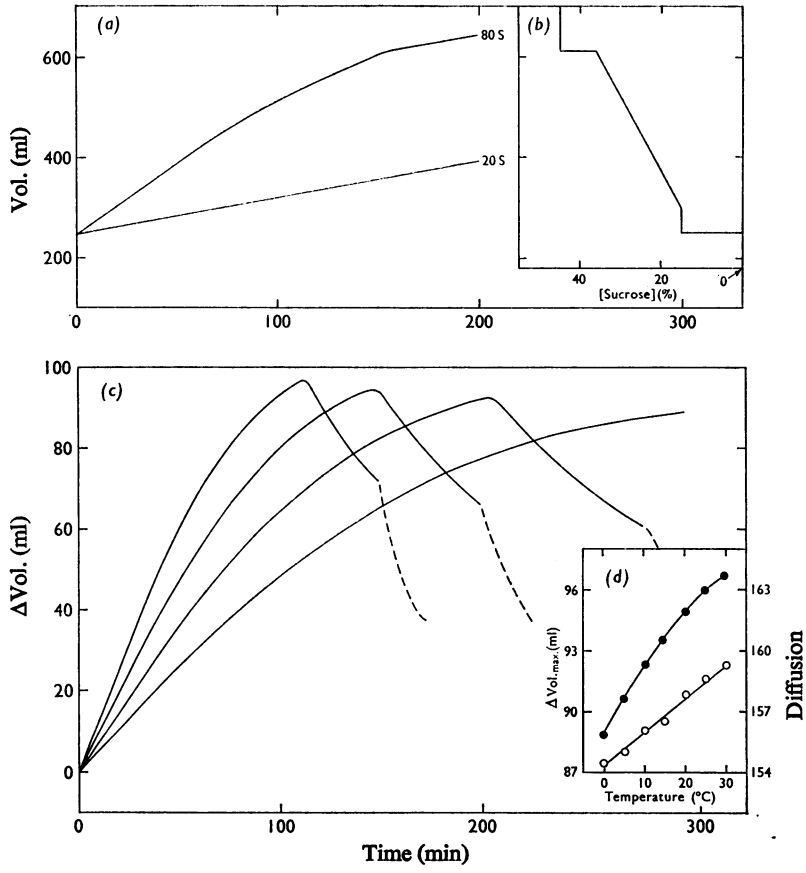

Fig. 3. *Optimization of separation of a 20S and an 80S particle*

The iteration procedure was modified to allow the distance moved by a 20S and an 80S particle (ρ_p of each = 1.4 g/cm^3) on a 15–38% sucrose gradient to be calculated in parallel. (*a*) Shows the distance moved by the two particles at 10°C on the gradient shown in (*b*). The separation of the two particles (Δvol.) was calculated for temperatures from 0° to 30°C; this difference is shown in (*c*) for 0°C (at left), 10°, 20° and 30°C (at right). The decrease after the maximum is due to the 80S particle reaching the cushion; ----, indicates that it was precipitated against the wall of the rotor. The maximum separation ($\Delta vol._{max.}$) (●) at each temperature is indicated in (*d*) together with an estimate of the diffusion (○) of the faster moving particle, made by summing $1/\eta$ at each cycle until the time of maximum separation was reached.

Fig. 3 shows the separation achieved in a 15–38% sucrose gradient run at different temperatures. Fig. 3(*d*) shows that an increase in the temperature of the run from 0° to 30°C increases the maximum separation of two peaks from 89 to 94 ml, but that the diffusion, and hence the zone width, also increases. The overall effect is that the advantage of increased separation of the peaks, as the temperature is raised. is offset by wider zones. Different conditions may be quickly compared in order to optimize the resolution of the gradient.

Conclusions

An iterative method of calculating the behaviour of a sedimenting zone in a sucrose gradient has been described. This procedure is simpler than those previously published, as new equations have been used to relate viscosity and density to sucrose concentration and temperature and others that relate volume and radius in the B XIV and B XV rotors.

The several factors that lead to either a loss of precision in the experiment or to a loss of accuracy in the calculation are described. Comparison of the calculated distribution of the sedimentation coefficients with that found by experiment demonstrates that calculation and experiment have much the same accuracy.

The method is sufficiently flexible that not only can other types of gradients, such as the inverted viscosity gradient of Churchill *et al.* (1973), be analysed but

also an allowance made for the extent of diffusion. This means that the resolution of a gradient may be measured rapidly so that the gradient that gives optimal separation may be identified.

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