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The Optimization of Density Gradients for Zonal Centrifugation

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Summary

By using established equations and techniques, it is possible to compute sucrose gradients which are optimized to minimize volume expansion of a zone during sedimentation in zonal rotors. These gradients have been tested experimentally, and it has been found that the greatest expansion of the sample zone occurred during the early stage of sedimentation. Following this expansion, the gradient maintained the volume within the limits set by the calculations.

INTRODUCTION

The introduction of zonal centrifuges as a tool for the separation of large biological particles is sufficiently new for the definition of the optimum experimental parameters to be still largely empirical. This means that the density gradients chosen for any experiment have been calculated on the basis of experiments rather than prediction. Hence, the resolution of the zone at the end of the experiment is dependent on a series of fortuitous circumstances. Martin and Ames (*1*) first pointed out that the superimposed density and viscosity gradients may be chosen to cancel the effects of the nonhomogeneous field on the zone and give it a constant velocity down the cell. Schumaker (*2*) has given exact relationships to describe the effect of the supporting gradient on the zone width, showing how a zone may sharpen or broaden according to circumstances as it sediments down the gradient. In an earlier publication (*3*), he suggested that the resolving power of zonal centrifugation could be enhanced if the gradient were chosen to sharpen the sedimenting zone. It would seem of interest to see if these gradient properties could be enlisted to improve the resolution of zones containing particles of known molecular parameters, and the present communication shows how such an optimum gradient may be computed.

EXPERIMENTAL

Equipment

The gradients were formed and introduced into the rotor by using a Beckman Instrument, Inc. gradient pump Model No. 131, at rotor speeds of approximately 3000 rpm. The sample was pumped in manually from a hypodermic syringe at a rate of approximately 2.5 ml/min. Much of the experimental work was carried out with B-XIV rotors running at 12°C, although some of the data were collected with B-XV rotors (*4*). The observations of zone spreading at low speeds were made at 22°C either visually or photographically using a B-XIV rotor equipped with a transparent Lucite lid.

At the end of a run, the gradient and sample were unloaded via a spectrophotometer, which recorded the light absorption at 250 mμ. Samples (20 ml) were collected until all the gradient had been pumped from the rotor; these volumes were then correlated with the spectrophotometer trace. The final sucrose concentrations were measured with a Bausch and Lomb refractometer calibrated to read percent sucrose. Previous tests showed that the presence of the sample or the buffer did not produce significant errors in the measurements.

Gradient Materials

The sucrose solutions contained 0.51 M sodium phosphate buffer (pH 7.5; $\mu = 0.2$).

Bovine Serum Albumin

A commercial preparation of crystalline bovine serum albumin (Armour Pharmaceuticals, Inc.) was dissolved in the appropriate sucrose buffer. It was then stained with Bromphenol Blue and the excess dye removed by extensive dialysis against the buffered sucrose.

NUMERICAL CALCULATIONS

Physically, the problem of maintaining a near-constant volume for the sedimenting zone resolves into one of decreasing the width of the zone as the molecule sediments. This will offset the volume increase produced by diffusion of the molecule and by radial dilution in the rotor. Berman (*5*) has related zone width to the gradient parameters, assuming negligible diffusion of a sedimenting particle, and although this is a limiting case, this equation has nevertheless been used for optimizing the gradient. The equality equation is given in (1a), together with the modified form used for the optimization (1b).

$$\int_{r_t^{(1)}}^{r_l^{(1)}} \frac{\eta(r)\,dr}{[\rho(p) - \rho(r)]r} = \int_{r_t^{(2)}}^{r_l^{(2)}} \frac{\eta(r)\,dr}{[\rho(p) - \rho(r)]r} \tag{1a}$$

$$f(\delta,r) = \int_{r_t^{(1)}}^{r_l^{(1)}} \frac{\eta(r)\,dr}{[\rho(p) - \rho(r)]r} - \int_{r_t^{(2)}}^{r_l^{(2)}} \frac{\eta(r)\,dr}{[\rho(p) - \rho(r)]r} \tag{1b}$$

Here the superscripts (1) and (2) refer to the radial positions in the cell, η is the viscosity at r cm, ρ is the density at r cm or of the particle (p), and δ is the zone width ($\delta = r_l - r_t$, l and t refer to the leading and trailing edges of the zone). The value of $f(\delta,r)$ can be reduced to a minimum by adjusting η and ρ within the two integrals. Thus, there are two functionally related parameters which can be adjusted to set δ to a minimum irrespective of the radial position of the zone. Bearing in mind that a curvilinear relationship exists between density and concentration of sucrose, while viscosity shows a marked nonlinear relationship (*6*), we decided to fix the density at each radial position and evaluate the viscosity.

The problem then reduced to finding the real roots of Eq. (1b) at each radial position. A Newton-Raphson procedure was adopted for this:

$$\eta_{(n)} = \eta_{(n+1)} - \frac{f(\delta,r)}{f'(\delta,r)} \tag{2}$$

The first derivative, $f'(\delta,r)$, of the function with respect to viscosity is not readily determined, and in the present work it was evaluated numerically as required. The procedure was as follows. The function $f(\delta,r)$ [Eq. (1b)] was first computed by setting equal viscosities in both integrals; this was followed by a second evaluation $[f_2(\delta,r)]$ in which the viscosity for $r^{(2)}$ was set equal to $\eta^{r^{(1)}} \times 1.2$, and the value of the derivative calculated from

$$f'(\delta,r) = \frac{f_2(\delta,r) - f_1(\delta,r)}{0.2 \times \eta^{r^1}} \tag{3}$$

The final Newton equation reduces to the form

$$\eta_{(n)} = \eta_{(n-1)} \left\{1 - \left[0.2 \frac{f_1(\delta,r)}{f_2(\delta,r)}\right]\right\} \tag{4}$$

The iterations were terminated when $|\eta_{(n)} - \eta_{(n-1)}| \leq 10^{-6}$ and the value of $\eta_{(n)}$ used. Each integral in Eq. (1b) was evaluated using Simpson's method. A test similar to the one shown above was employed to terminate the subdivision of the step lengths in the routine, using the difference between the ith and the $(i-1)$th evaluation to end the integration. The calculation was started at a set position determined by the radial position of the interface of the zone and the overlay $(r_{(st)})$. The sucrose concentration was specified at this position and from this the initial viscosity and density [ρ_1, Eq. (5)] was calculated to give a first evaluation of the integral [Eq. (1)]. The position of the zone was then moved an amount equal to half the initial zone width for the $r^{(2)}$ computation. The density at this position was calculated from the linear relationship.

$$\rho_{(2)} = \rho_{(1)} + (d\rho/dr)(r - r_{(st)}) \tag{5}$$

Initially a trial value for $d\rho/dr$ was taken for the first computation of the gradient, and the sucrose concentration was calculated from the computed densities (*6*).

So far the zone width has not been defined; if, however, this is assumed to be constant with respect to the cell dimensions, this would not lead to a constant volume of the zone in an experiment. To achieve a constant volume the theoretical zone width should be reduced by an amount equal to the broadening caused by radial dilution and diffusion of the sedimenting macromolecules. Thus a new δ must be computed at each radial dimension by using Eq. (6) to compute the new width.

$$\delta_{(k)} = \delta_{(k-1)} \left\{\frac{t_{(k-1)}}{t_{(k)}}\right\}^{1/2} \left\{\frac{r_{(k-1)}}{r_{(k)}}\right\}^{2} \tag{6}$$

Here t is the time required to move the zone to the kth from the $(k-1)$th position, and the correction for diffusion is based on the normal time dependence of translation diffusion. The second term allows for radial dilution and follows the well-known dilution law. In order that the diffusion effects may be incorporated, it is necessary to compute the true velocity of the zone from the molecule's sedimentation coefficient, making due allowance for viscosity and density. The new zone width ($\delta_{(k)}$) cannot be the one for which the viscosity is being calculated; thus, the present evaluations have this inherent error in the computed values. The magnitude of the error will be determined by the iterative step length along the cell. Tests showed that the values for the optimized viscosities were not improved by decreasing the step lengths below half the zone width. Furthermore, since the value for the width decreases along the cell, the error remains relatively constant irrespective of position.

As a result of these calculations, a density and viscosity profile was constructed along the radial dimensions of the cell by using the initial given density gradient [Eq. (5)] for computing the density. Hence the density will be related to sucrose concentration, whereas the initial optimized viscosity may not. In order that a practical gradient can be obtained which has both density and viscosity corresponding to a unique set of sucrose concentrations, it is necessary to repeat the calculation, varying the gradient until a compromise is reached between the optimized viscosity and that realizable with sucrose. The real viscosity is computed from the sucrose concentration (which is computed from the known density) at 0.3 cm intervals along the cell using Barber's relationship (*6*). The difference between this and the optimized value is calculated at each position and the squares of these differences are summed. The gradient value is lowered by 0.005 g/cm^4 using a first-order iterative procedure until the value of the summation, $\Sigma(\eta_{opt} - \eta_{cal})^2$, reaches a minimum. It follows from this approximation that the computed profile represents the gradient giving the best compromise between real and optimized viscosities. A computer program was written in EGTRAN (a local dialect of FORTRAN) to carry out the optimization; this requires the following parameters to start the computation; Initial sucrose concentration, from which the viscosity and density are computed; meniscus or overlay interface radius and peripheral radius (cm); initial width of loaded zone (cm); sedimentation constant and density of the zonal particle; and an initial value for the density gradient. The angular velocity, ω^2, must also be provided so that the accumulated $\omega^2 t$ can be computed at each radial position. Figure 1 shows that the differences between the

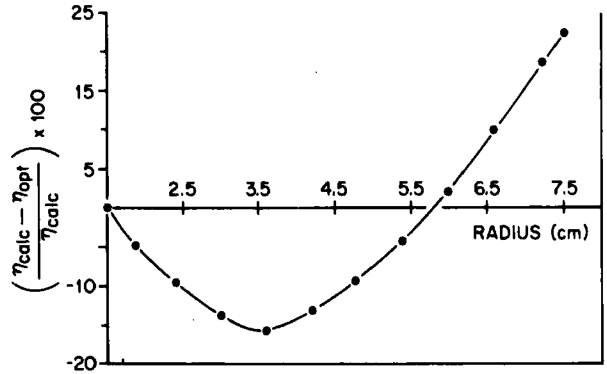

FIG. 1. The percentage deviation of the optimized from the experimental viscosity for a 19S particle density 1.33 g/cm.

computed and practically realizable viscosities have a marked bias; the greatest deviation occurs at the peripheral edge of the rotor. This was found to be consistent for all the calculations when using gradients which are linear with respect to radius. Possibly more elaborate gradient profiles can be produced, but we believe the present simple approach should be first tested experimentally, and then later other solutions can be sought.

The optimization took 5 min to calculate the profile shown in Fig. 2 on an English Electric KDF 9 computer, using the radial ordinates of the B-XIV zonal rotor and a gradient starting at 5% sucrose. The initial density gradient was set at 1.850×10^{-2} g/cm^4, and the resulting optimized gradient was 1.736×10^{-2} g/cm^4; the other parameters are as shown in the legend to Fig. 2.

The final results of the optimization are not practical as they stand since the values found at each radial step correspond to the moment in the run time when the zone crosses that radial position. In other words, it assumes that the supporting gradient is constructed from a substance which neither sediments nor diffuses, an unrealistic assumption. The next step, therefore, is to calculate the required sucrose profile to be loaded into the rotor at the start of the run. By using the accumulated $\omega^2 t$ and a proposed speed for the experiment, plus the sedimentation and diffusion coefficients for sucrose (0.26×10^{-13} sec^{-1} and 0.46×10^{-6} cm^2 sec^{-1}, respectively), it is possible to compute C/C_0 at each radial position with the Fujita-MacCosham analytical solution to the Lamm differential equation (7)

$$C/C_0 = \frac{e^{-\tau}}{2\epsilon}\left\{\left[1 - \Phi\left(\frac{\tau + Z}{2(\epsilon\tau)^{1/2}}\right)\right]\left(2 + \frac{Z + \tau}{\epsilon}\right)\exp(Z/\epsilon) - 2/\sqrt{\pi}\,(\tau/\epsilon)^{1/2}\exp\left[-\frac{\tau - Z}{4\epsilon\tau}\right]\right\} \quad (7)$$

$\tau = 2\omega^2 st$, where s is the sedimentation coefficient, t the time, and ω the radial velocity. $\epsilon = 2D/r_0^2\omega^2 s$, where D is the diffusion coefficient and r_0 is the starting position. $Z = 2LN(r/r_0)$, where r is the radial position to which C is referred. $\Phi(x)$ is the error function with x as the argument.

Examination of the equation parameters shows that during a centrifuge experiment the concentration of any radial location is a function of τ, assuming s and D for sucrose are independent of concentration

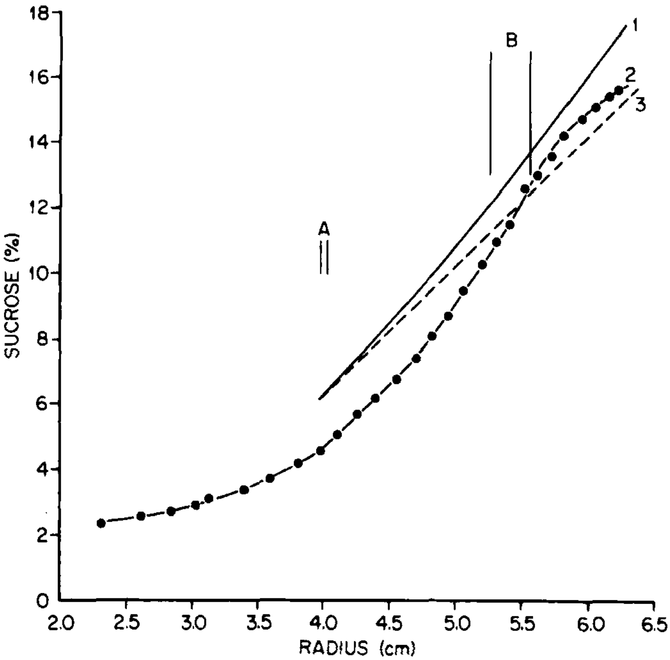

FIG. 2. The density profile for a 4S particle of density 1.33 g/cm³ and run temperature of 10°C. Inserted profile (1) (0.0189 g/cm⁻⁴); -- optimized profile (3), i.e., c in Eq. (7); -· experimental profile (2) obtained after sedimenting the albumin from A to B, taking 24 hr at 35 000 rpm ($\omega^2 t = 1.21 \times 10^{12}$ sec). The initial sample volume was 10 ml, and the final volume at two-thirds height of the zone was 60 ml (bandwidth 0.3 cm). The bandwidths for the start (A) and finish (B) are shown.

effects. Since the optimization gives sucrose concentrations (C) and time taken for the macromolecules to reach the radial location r, the inserted concentration (C_0) at zero time can be computed. The final result gives the C_0 vs radial position, and this is used to make the inserted gradient.

RESULTS

The computation of C_0 is approximate since the starting position is not a meniscus but a sandwich of finite thickness between the overlay and the gradient. It would be feasible to use more complex solutions to the Lamm equation which allow for this boundary condition, but in practice this was found to be unnecessary. From a practical viewpoint, two major limitations are imposed on the application of the results ırom these computations to the present requirements; namely, convection in the rotor due to thermal effects, and mechanical vibration. Neither of these appears to have any large influence on the redistribution of the sucrose during a zonal centrifuge experiment as shown in Fig. 2.

This illustrates a result from run 9.9.68 (Table 1). Data for the gradients loaded (curve 1), required from the optimization (curve 3) and found at the end of the experiment (curve 2), are given. The inserted gradient (curve 1) changed to give the experimental profile (curve 2) after 24 hr, and this crossed the predicted sucrose value (curve 3) between the center and leading edge of the unloaded zone. The predicted profile was computed by the optimization procedure for a 4S particle of density 1.33 g cm^{-1}. The calculated position for the zone coincided reasonably well with that found experimentally (pre-

TABLE 1
Sedimentation of Stained Bovine Serum Albumin in a B-XIV Rotor at a Speed of 35 000 rpm[a]

Run	Sample volume (ml)		Median radius (cm)		$\omega^2 t \times 10^{-12}$ (sec)	Run time (Hr)
	Start	Finish	Start	Finish		
9.12.68	10.	65.	4.1	5.45	1.26908	28
9.9.68	10.	60.	4.1	5.4	1.2088	24

[a] The gradients were as shown in Fig. 2. The introduced sample contained 25 mg albumin in 10 ml 4% sucrose. Final bandwidth in both experiments was approximately 0.3 cm.

dicted median zonal position for the particle was 5.25 cm, while the experimental position was found to be 5.45 cm).

Using the gradient for the 4S particle, experiments were made to test whether the sample zone retained its initial volume during sedimentation. Previous work (*8*) showed that with zonal rotors, gradients can be loaded with a sample which approached the maximum capacity for the zone as defined by Svensson et al. (*9*) and Berman (*5*). Hence, the quantity of albumin in the sample was maintained below this theoretical maximum for the present experiments. Calculations showed that a gradient of 0.0189 g cm^{-4}, the required gradient for bovine serum albumin, could hold 38 mg mass in a zone of 0.1 cm bandwidth, this being the bandwidth a 10-ml sample would produce at 4 cm for the center rotation for a B-XIV rotor.

Two experiments were carried out with this gradient, and the results are summarized in Table 1. Following sedimentation for 24 and 28 hr, the volumes of the unloaded zones were 60 and 65 ml, respectively. These volumes were evaluated from bandwidths of 0.3 and 0.32 cm, respectively, measured at two-thirds the height of the Gaussian protein concentration profile. This increase from the initial 10-ml sample to an approximate final volume of 60 ml was surprisingly large.

No explanation of the lack of conformity between the experimental results and those expected from theory was available until two experiments were made to measure the width of the starting zones immediately after insertion into the rotor. For these measurements bovine serum albumin samples were introduced into a rotor filled with gradient as before and then unloaded, either after 15 min or 4.5 hr at 35 000 rpm. The results of these experiments (Table 2) and measurements of the radial position of the zone show that the movement of the protein during these experimental periods was negligible. However, a large

TABLE 2

Tests of Zone Spreading under Normal Run Conditions[a]

Run No.	Sample radius (cm) Start	Finish	Sample volume unloaded	$\omega^2 t \times 10^{-12}$ (sec)	Run time (Hr)
9.17.68	4.1	4.15	35.	0.2260	4.5
9.19.68	4.1	4.1	25.	0.0087	0.25

[a] In both cases a 10-ml sample containing 25 mg of bovine serum albumin was introduced, and the centrifuge speed was 35 000 rpm.

increase in zone volume occurred during the first 15 min. Apparently the increase in volume was not complete in 15 min since the 4.5-hr run showed the final volume to be 3.5 times the loaded volume. This increase could not have been produced by such factors as changes in rotor geometry or translational diffusion of the albumin during this time.

These experiments showed that a normal experiment with zonal rotors was not going to answer our question concerning the effectiveness of the gradients in preventing volume expansion. To follow the expansion of the zone in more detail during the early stages of sedimentation, it was necessary to have a transparent end cap on the rotor. Fortunately, one made from Lucite was available, but this restricted the rotor speeds to less than 5000 rpm. With this modified B-XIV rotor, the sample and gradients were loaded as before, and the width of the stained bovine serum was recorded photographically. The rotor was photographed at intervals during the 5-hr run by using No. 52 Polaroid film (ASA 400) and a strobe light to illuminate the zone by reflected light. The width of the recorded band was measured on the prints with a travelling microscope.

This method of recording the bandwidths has the failing that the width was not recorded at two-thirds the concentration height. To achieve this, more elaborate equipment would be necessary. It was hoped, however, that preliminary data could be collected relating the increase in bandwidth to time in the rotor. The results given in Fig. 3 show that it took approximately 4 hr for the volume to reach a maximum value and thereafter the volume probably remained relatively constant. The data for the two experiments are consistent even though they are far from being precise. Hence, little further useful information can be abstracted from the graph except, say, that the width of 0.4 cm achieved after 4 hr at low speed corresponded approximately with the volume recorded in the 4.5 hr high-speed experiment (Table 2).

DISCUSSION

The initial premise in this study was that the increase in volume of the sedimenting zone was due solely to rotor geometry and diffusion of the macromolecule. The diffusion effects would be relatively small compared with geometrical factors, even when relatively lengthy experiments were carried out, as in the present study. Hence, it was hoped that by using the optimized gradients these volume changes

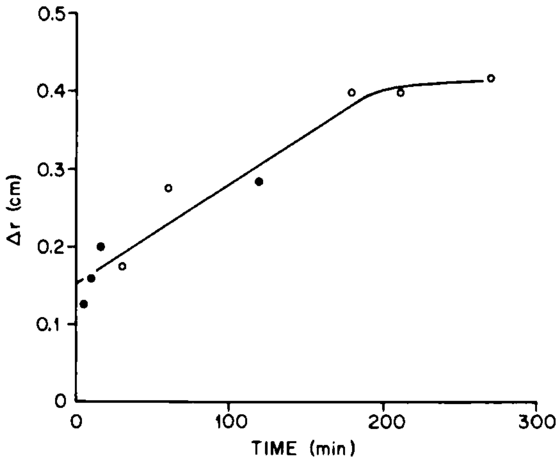

FIG. 3. The relationship between bandwidth (Δr) and time for stained bovine serum albumin in a B-XIV rotor running at 3500 rpm. Two experiments were made, and these are identified by the two symbols on the graph.

could be reduced by enlisting an increasing viscosity and density in the zone path. At the same time it was hoped to produce a profile which had minimum reduction in velocity of the sedimenting zone. The practical gradients did have a limited success since, if one assumes that the initial volume of 10 ml had reached its steady-state value of 40 ml within the first 4 hr, then the remaining 20–24 hr and 1.5 cm sedimentation produced a further increase of only 20 ml. Calculations show that in the absence of a sucrose gradient (a physical impossibility in practice), the volume would have increased to more than 100 ml during this period as a result of sectorial dilution and translational diffusion of the macromolecule.

The 20-ml expansion would be expected using the optimized gradients, since the viscosity and density increases were naturally towards the peripheral edge of the rotor and so do not reduce translational diffusion in the opposite direction towards the rotor center. Approximate calculations, made assuming no centrifuge field, showed that diffusion in this direction would increase the zonal volume by 27 ml 24 hr. Naturally this would be less in the presence of the centrifugal field, showing that the increases observed are close to the expected values. This suggests that after the first 4 hr the gradients are functioning in the manner expected.

The initial period after loading produces the greatest volume expansion, and no real explanation of the causes can be offered at present. Gradient overloading was not responsible since both the short-period experiments gave sharp Gaussian peaks after unloading. The possibility that the zone increased in volume during unloading was tested by comparing the bandwidths recorded on the photographs with those taken from the spectrophotometer profile. It was found that they corresponded closely (within 0.05 cm). The linear increase of the bandwidth with time is too imprecise to make it possible to compute an equivalent diffusion coefficient, but the speed of the expansion would infer a coefficient of at least ten times that recorded for bovine serum albumin.

The possibility that the stain is reversibly eluting from the protein has been considered and thought not to influence the answer greatly. The reasons for this are: (a) the correlations found between the photographic bandwidths and the bandwidths from the optical density profile recorded at 280 mμ, and (b) the lack of stain in the unloaded gradient, except at the fraction peak, even after 28 hr of sedimentation.

Stirring of the zone as a result of thermal effects or mechanical vibration from the centrifuge drive seemed to contribute little to the zonal expansion. If either factor had been important, then the sucrose in the gradient would diffuse quicker than expected, yet the predicted and experimentally determined sucrose profiles were in close agreement after 24 hr. Similarly, the rate of spreading would be related to the rotor speeds, yet essentially similar results were obtained with the short run at either low or high speeds.

Another possible cause of expansion is the so-called "streaming" effects described first by Anderson (*10*), Brakke (*11*) and further by Schumaker (*12*) and Sartory (*13*). Previous experience (*8*) suggested that this phenomenon did not occur in zonal rotors, and the present visual observations made on runs in the rotor fitted with the transparent end cap supported this. Droplet sedimentation was never observed in any of the experiments, and the isolation of Gaussian-shaped concentration profiles at the end of the experiments support the idea that droplet formation on a macroscopic scale does not occur. It is interesting to find a report of similar anomalous expansions (Price and Kovacs, *14*) where loss of resolution occurred during loading and running of small samples of blue dextran. This was especially noticeable when using step gradients.

Since the expansion during the beginning of the experiment is of

TABLE 3

Typical Gradient Parameters for a Selection of Particles

S (Svedbergs)	Particle density (g cm^{-3})	Run temp. (°C)	Gradient[d] (g cm^{-4}) Optimized	Gradient[d] (g cm^{-4}) Loaded	r start (cm)	Starting sucrose conc. (%)	Max.[d] capacity (mg)	Time to reach 5 cm (hr)
4[a]	1.33	10	0.01720	0.0189	0.1	5	35	28.0
7[a]	1.33	10	0.0159	0.0169	0.1	5	31	13.0
7[b]	1.33	10	0.01399	0.0368	0.1	25	58	20.0
19[b]	1.33	10	0.01678	0.0230	0.1	10	36	8.0
34[b]	1.53	5	0.01951	0.0293	0.3	5	78	5.5
50[b]	1.53	5	0.02045	0.0267	0.3	5	70	3.7
100[c]	1.50	10	0.01930	0.0270	0.1	5	25	33.0

[a] Rotor speed 35 000 rpm, starting at 4.0 cm.
[b] Rotor speed 35 000 rpm, starting at 2.8 cm.
[c] Rotor speed 10 000 rpm, starting at 2.8 cm.
[d] Computed assuming the inserted gradient is linear with radius.

such prime importance in determining the resolution of the zonal centrifuge, this stage is being studied further. However, for several years gradients equivalent to those computed by the optimization method have been used in The Oak Ridge National Laboratory for the preparation of macroglobulin from rat serum. The gradients were determined empirically, following a series of trial experiments in which the criterion for the best gradient was maximum resolution with minimum centrifuge time. In order that some appreciation of the type of gradients predicted for different particles might be gained, Table 3 was constructed. These results can only cover a few of the possible particle-vs-gradient combinations but serve to illustrate the type of gradients. It should be remembered that these are minimum gradients, and it is quite acceptable to raise the gradients in order to increase the loading capacity at the start.

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