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# Analytical Differential Centrifugation as a Precise Biophysical Tool

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## INTRODUCTION

Anderson (*1*) introduced a novel procedure to enable the ready acquisition of the complement of discrete sedimentation coefficients present in a mixture. This was developed as an aid in providing rational separations in zonal centrifuges, and has been applied over large molecular weight ranges (*2*). The ultimate utility of such a technique depends on the precision and reproducibility of the measured sedimentation coefficient values, particularly if these are close together. The work presented here discusses the limitations and errors inherent in the method, and offers practical suggestions for its optimization and most efficient application.

## EXPERIMENTAL

All experiments were conducted in a Model L Beckman preparative ultracentrifuge fitted with a temperature controller and an $\omega^2 t$ integrator. Runs were performed using a Ti50 titanium rotor and polycarbonate screwcap centrifuge tubes (Fisher Chemical Co.). The running temperature was set at a nominal 20°C for most experiments. Sample temperatures were measured using a thermometer and a centrifuge tube in the rotor containing all components but protein. Bovine serum albumin (Sigma Chemical Co.), crystallized, was checked for dimer content before use.

Experiments were carried out essentially according to Anderson (*1*) with some significant modifications, the reasons for which are discussed later. Thus centrifuge tubes, filled to a specified radius with the test solution, were placed in the rotor whose sample holes each contained about 0.5 mL of water. After spinning for varying values of $\int_{t=0}^{t=t} \omega^2 t$,

where $\omega$ is the angular velocity and $t$ the time in seconds, the tubes were rotated 180° *in situ* and carefully removed. The contents of the upper portion of the tube were gently extracted to constant radius using a syringe with a tip bent to 90°, mixed, and the concentration read spectrophotometrically.

## PRACTICAL ASPECTS

The first experiments were run according to Anderson (*1*), in that tubes were filled with a known volume of sample, and a known volume of the upper layer removed at particular $\omega^2 t$ values. From the geometries of the tubes and the rotor, these volumes were converted into radii from the center of rotation. This supposes that tubes are identical and do not undergo deformation during centrifugation. They do, in fact, deform, and the rather large errors this introduces may be minimized by putting a small amount (~0.5 mL) of water in each rotor hole to act as a support. Additionally, tubes should be filled and sampled to constant radius—readily done with a clamped syringe in a small stand. All data reported here were obtained with these modifications.

Bovine serum albumin was used as the test molecule because it is well characterized and has a sedimentation coefficient which probably represents the lower limit one would wish to assess by this method. Data obtained for larger particles should be more accurate.



## RESULTS AND DISCUSSION

The data obtained, if plotted as $C_t/C_0$ vs $\omega^2 t$, where $C_0$ is the starting concentration and $C_t$ the concentration at time $t$ of the uniformly mixed upper solution, yield a curve as in Fig. 1. The apparent sedimentation coefficient, $s$, is readily calculated when $C_t/C_0 = 0$ using Eq. (1) or (2) although this is not a necessary condition for the computation to be made. The condition $C_t/C_0 = 0$

$$s\omega^2 t = -\frac{1}{2}\ln\left\{\left(\frac{r_1}{r_2}\right)^2 + \frac{C_t}{C_0}\left(1 - \frac{{r_1}^2}{{r_2}^2}\right)\right\} \qquad (1)$$

exists when a "nondiffusing" macromolecular solute passes the sampling plane, $r_2$. However, if the macromolecule does have diffusive properties within the time scale of the experiment, then the greater the diffusion coefficient, $D$, of the macromolecule, the larger will be the trailing effect behind the boundary. This leads to an $\omega^2 t$ value at $C_t/C_0 = 0$ which is higher than would be expected, and hence the observed $s$ is low. However,

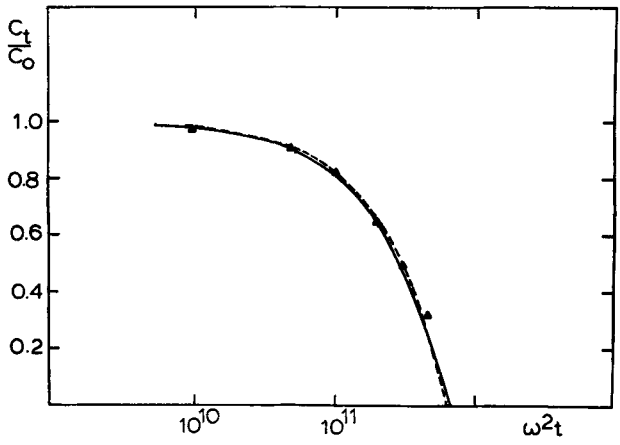


FIG. 1. Theoretical curves from Eq. (1) (——) and Eq. (2) (- -) fitted to analytical differential centrifugation run of bovine serum albumin (△). Starting radius, $r_1$, was 5.24 cm, and the sampling plane was 6.7 cm from the center of rotation. No correction has been made for viscosity effects. Each point represents the mean of four data points.



up to the point where $C_t/C_0 = 0.5$, this trailing does not affect the results for any value of $D$.

Equations derived to describe the depletion of macromolecule in the upper layer vary according to assumptions made concerning container shape, ideality of sedimentation, etc. For example, Eq. (1) is that derived by Svedberg and Pedersen (*3*) for the partition cell in the analytical ultracentrifuge. Such a cell is sector-shaped, and nonideal hydrodynamic effects are minimized. Equation (2), however, is readily derived for the parallel-sided tube case, if nonideal effects are ignored.

$$s\omega^2 t = -\ln\left\{\frac{r_1}{r_2} + \frac{C_t}{C_0}\left(1 - \frac{r_1}{r_2}\right)\right\} \tag{2}$$

If the correct equation describing the depletion process were known, then the problems of diffusion could be eliminated by using a standard curve fitting procedure for the experimental configuration used up to the $C_t/C_0 = 0.5$ point. The hydrodynamic events occurring in the anglehead rotor are not readily discerned however, particularly during acceleration and deceleration, although attempts have been made (*4*, *5*). The approach taken here was empirical, i.e., to take large numbers of data and determine which equation fitted better. Forty-two runs were made with several replicates of each data point. Even in experimental configurations where the differences between the curves describing Eqs. (1) and (2) were maximized

by making $r_1/r_2$ large, a simple choice between the two equations could not be made (Fig. 1). The values of $s$ obtained from both equations usually differed by less than 10%. There was, however, a marked tendency for $s$ values determined from Eq. (1) to be closer to the value expected than those from Eq. (2).

Examination of Eq. (1) shows that a constant percentage error in the value of $C_t/C_0$ has its greatest effect on the value of $s$ as $C_t/C_0$ approaches 1. Biological assays, and to a lesser extent spectrophotometric assays in mixed systems, often have large errors associated with them, perhaps up to 25%. The effect of these large errors on the $s$ values obtained is shown clearly in Fig. 2. These are theoretical curves, the $s$ values shown having been computed with Eq. (1) at each $C_t/C_0$ value with the appropriate error imposed. In practice, such a smooth curve is not obtained unless a systematic error dominates, and random errors are normally found. Although assessment of $s$ as $C_t/C_0$ approaches zero would be more favorable from the error point of view, therefore the diffusional problem again intrudes. A compromise, mentioned above, is to curve fit Eq. (1) to $C_t/C_0 = 0.5$ and extrapolate to $C_t/C_0 = 0$. This proves to be quite satisfactory. The reproducibility of measurement was determined in several experiments by using multiple replicates. The results, shown in Table 1, indicate a high reproducibility, with a standard deviation of 3% or less. Subsequent experiments were normally carried out in duplicate.

These considerations having been established, attempts were made to measure the concentration dependence of bovine serum albumin. These

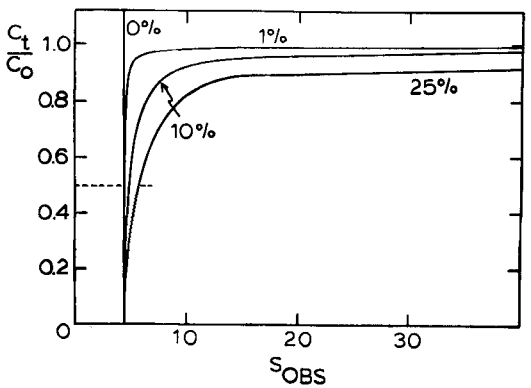


FIG. 2. A plot of observed sedimentation coefficient vs actual $C_t/C_0$ values with different percentage errors imposed on $C_t/C_0$ for Eq. (1). Only the negative halves of the error envelopes are shown. The dotted line denotes the envelope at $C_t/C_0 = 0.5$. The curves were computed using $r_1 = 5.24$ cm, $r_2 = 6.9$ cm, and $s = 4.4$ sveds.

TABLE 1[a]

| Tube no. | $\int\omega^2 t$ | $C_t/C_0$ |
|---|---|---|
| 1 | $4.6 \times 10^{11}$ | 0.274 |
| 2 | " | 0.268 |
| 3 | " | 0.279 |
| 4 | " | 0.270 |
| 5 | " | 0.278 |
| 6 | " | 0.287 |
| Mean 0.277, SD 0.007 | | |

[a]The initial radius was 5.24 cm and the sampling plane radius was 6.90 cm. With a BSA concentration of 2 mg/mL, the data yield $S^c_{25,\omega} = 4.2$.

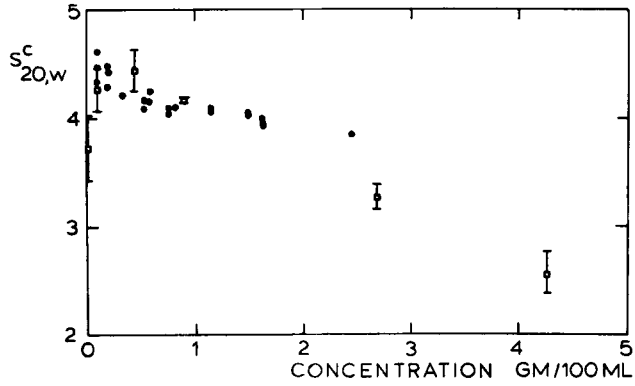


FIG. 3. The concentration dependence of the sedimentation coefficient of bovine serum albumin determined by analytical differential centrifugation. Open squares denote the means of four determinations with the bars representing standard deviations. The solid circles are the data of Kegeles and Gutter (*6*).

experiments (Fig. 3) showed that as the stabilizing effect due to the viscosity of the protein solution itself diminished, mixing of the tube contents became dominant. The addition of sucrose to 10% w/w reduced this considerably, but not completely. Further increases in the sucrose concentration increased stability but were not deemed desirable because of potential tonicity effects. Figure 3 shows the results in 10% w/w sucrose corrected for the viscosity and density of the sucrose. Also shown in Fig. 3 are the highly accurate data of Kegeles and Gutter (*6*). Considering the relative crudity of the experiment in the anglehead rotor, the agreement is remarkably good, and indicates the usefulness of analytical differential centrifugation, not as a means of determining the concentration dependence of $s$, but of $s$ itself. However, it is apparent that mixing does occur in the tube. This is minimized by the use of cotton plugs, as suggested

by Anderson (*1*), to prevent swirling from the bottom, and by the use of a stabilizing viscosity-increasing component added to the solution. If this component cannot be added, then the $s$ determination should be carried out at several concentrations. Mixing cannot indicate a higher $s$ value than the true one, thus the highest $s$ obtained (unless extrapolation to zero concentration can be made) should be taken as the best approximation to the correct value.

These experiments show the considerable utility of the analytical differential centrifugation method as an accurate tool in the design of zonal centrifuge separations. Furthermore, the method may be used with confidence for the determination of $s$ itself, and has a number of advantages over the commonly used density gradient sedimentation in a sucrose gradient in the swinging bucket rotor. Of these, one of the most important is that a very large number of points may be used to define the depletion curve, whereas in the gradient method a large number of fractions must be assessed to obtain a single point from which $s$ is extracted. Furthermore, droplet sedimentation (*7*, *8*) cannot occur in the method described here.



## REFERENCES


1. N. G. Anderson, *Anal. Biochem.*, *23*, 72–83 (1968).
2. C. Cotman, D. H. Brown, B. W. Harrell, and N. G. Anderson, *Arch. Bioch. Biophys.*, *136*, 436–447 (1970).
3. T. Svedberg and K. O. Pedersen, *The Ultracentrifuge*, Oxford University Press, London, 1940.
4. R. Trautman and S. S. Breese, Jr., *J. Phys. Chem.*, *63*, 1592–1603 (1959).
5. B. R. Ray and W. R. Deason, *Ibid.*, *59*, 956–962 (1955).
6. G. Kegeles and F. J. Gutter, *J. Am. Chem. Soc.*, *73*, 3770–3777 (1951).
7. C. J. Remenyik, G. W. Dombi, and H. B. Halsall, *Arch. Biochem. Biophys.*, *201*, 500–505 (1980).
8. H. B. Halsall and W. K. Sartory, *Anal. Biochem.*, *73*, 100–108 (1976).


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