ANALYTICAL BIOCHEMISTRY 23, 72–83 (1968)

Analytical Techniques for Cell Fractions

VIII. Analytical Differential Centrifugation in Angle-Head Rotors[1]

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Received June 27, 1967

Angle-head centrifugation is widely used for the isolation of cells, subcellular particles, precipitates, and large molecules. These particles have sedimentation coefficients which may extend over six orders of magnitude (1). While zonal centrifuges now allow high-resolution separations to be made over a very wide range of particle sizes (2–5), no *single* zonal centrifuge experiment can extend over more than a fraction of this range. Angle-head precentrifugation is often a necessary prelude to high-resolution zonal separations, especially when a minor component is to be concentrated and resolved. Methods for optimizing such preparative separations are therefore of continuing interest. Specifically, we require methods for determining experimentally and with some precision the centrifugation time and speed required to sediment a given particle or activity. We would also like to know whether the behavior of particles sedimenting in angle-head centrifuge tubes can be predicted experimentally.

In this paper a general method for studying angle-head centrifugation is described and applied to three classes of particles which differ greatly in size—red blood cells, polystyrene latex, and bovine serum albumin (BSA). The work has been made possible by (*a*) the development of digital integrators for determining accurately the integral of $\omega^2 dt$ during the centrifugation cycle (2), (*b*) the availability of high-speed titanium rotors which allow particles as small as proteins to be sedimented in a reasonable period of time, and (*c*) the development of polycarbonate tubes which rarely deform in high centrifugal fields (6). The simple

[1] Research performed under the joint NIH-AEC Molecular Anatomy Program sponsored by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the U. S. Atomic Energy Commission.

[2] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

techniques described permit complex mixtures of particles to be characterized, provide data on which rational separation procedures may be based, and allow sedimentation coefficients of proteins (and possibly larger particles) to be approximated. The relationship between the studies reported in this paper and previous work by Pickels and Bauer (7–9) is also discussed. Although detailed theoretical studies on angle-head centrifugation are reserved for a later paper, one point should be stressed here. We are interested initially in how separations are usually made in practice, that is, in the separation of suspensions into only two fractions. The presence, absence, or behavior of moving boundaries is not considered initially. The theoretical value we are most interested in predicting is the value of $\omega^2 t$ for the complete sedimentation of a given particle species in a particular rotor configuration. Previous studies suggest that complete sedimentation may be approached asymptotically (7–9) and that complete sedimentation, at least of viruses, may not be obtained.

EXPERIMENTAL STUDIES

Centrifugal Procedures

In practice, a variety of centrifuge rotors are used to fractionate mixtures such as tissue extracts or homogenates. For this study, however, one rotor was employed so that data obtained with a wide range of particle sizes would be directly comparable. A Spinco No. 50 titanium angle-head rotor was spun in either a Spinco L-2 centrifuge or in an experimental zonal centrifuge equipped with temperature control and with an integrator which indicates the integral of $\omega^2 dt$ continuously in digital form (2). To ensure that the tube geometry was constant, the Oak Ridge type of polycarbonate tubes (obtained from International Equipment Company, Needham Heights, Massachusetts) with plastic closures (6) were used.

For particles at the large end of the spectrum, it was not possible to measure the centrifugal field accurately with this rotor and integrator. Some data points were therefore obtained at 1 g by mounting the tubes in a plastic block so that the tubes had the same angle with respect to the earth's gravitational field that the tubes have with respect to the centrifugal field in the rotor.

A 9 ml sample of each particle suspension was placed in each tube. After centrifugation, the tubes were rotated very slowly 180° while in place, until the pellet was flat in the bottom of the tube. With a long-stem Pasteur pipet, fluid was carefully removed along the upper wall, keeping the end of the pipet at, or just below, the surface (Fig. 1). The recovered liquid was placed in a 10 ml graduated centrifuge tube to

measure the volume removed. In this manner, 7 ml of the supernatant was recovered, thus avoiding the problem of deciding how much of a loose pellet to remove. When a very dilute sample such as polystyrene latex was used, a small cotton plug was also used in some experiments to help keep sedimented material at the bottom of the tube.

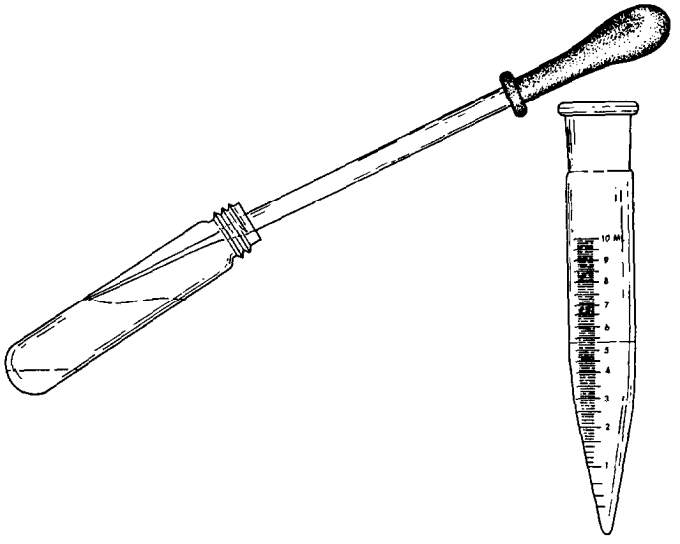

FIG. 1. Method used to remove fluid from angle-head tubes after they have been rotated *in situ* for 180°. See text for details.

For very long experiments (4 hr or more at 50,000 rpm), a digital integrator need not be employed, since the acceleration and deceleration times are small in proportion to the total time of centrifugation. The average speed may be accurately calculated from the odometer, and 7 min added to the time at speed to compensate for centrifugation during acceleration and deceleration. All experiments were conducted with the temperature control set on 5°C. At 50,000 rpm the actual rotor temperature was 10–11°C.

Calculations

Centrifugal treatment used for particle sedimentation has been expressed in a variety of ways including g minutes, speed, and time with either R_{av}, or R_{min} and R_{max} specified, or simply time, speed, and the manufacturer's rotor number. We have chosen the integral of $\omega^2 t$ because this value is used in further calculations of sedimentation rate and because the integrator used gives this number directly. The geometry of the sample in the rotor must also be known, however. Other numbers,

such as g minutes, are also characteristic not only of the time-speed profile, but of the rotor used, and the amount of sample placed in each tube.

The values of $\omega^2 t$ of interest range from 10^5 to 10^{13}. To accommodate this range, data are plotted on a nine-cycle log plot as shown in Figure 2. The radius of the menisci before and after removal of the 7 ml of supernatant are 4.88 and 6.8 cm, respectively, during rotation.

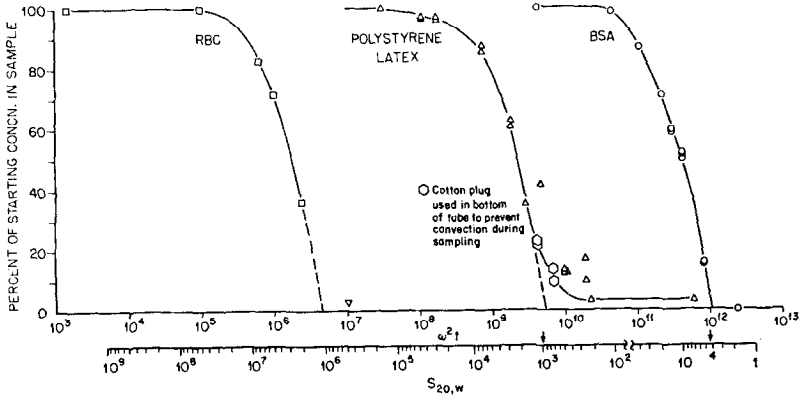

FIG. 2. Sedimentation of sheep red blood cells, polystyrene latex particles, and BSA. Points indicated by squares obtained at 1g using gravity; all other points obtained in Spinco No. 50 angle-head rotor. Points indicated with octagons obtained with small cotton plug in bottom of centrifuge tube. Along abscissa are plotted the integral of $\omega^2 t$ and the sedimentation coefficient in Svedberg units corrected to water at 20°C. The left-hand portion of this scale is for data taken at 5°C, the righthand segment for data taken at 10°C. Arrows indicate observed sedimentation coefficient for BSA and calculated sedimentation coefficient for polystyrene latex.

To plot both data obtained during sedimentation at rest and during rotation on the same plot it is necessary to consider the relation between the two. The value of $\omega^2 t$ equivalent to a given period of time at 1 g was calculated two ways. Form the equation

$$g = \frac{\omega^2 R_{av}}{980} = 1 \tag{1}$$

and by using the average radius, $R_{av} = 5.84$ cm, ω^2 was found to be 167.8. The second method of calculation uses the equations

$$s = \frac{R_2 - R_1}{t \times 980} \tag{2}$$

which applies to tubes at rest, and

$$s = \frac{1}{\omega^2 t} \ln \frac{R_2}{R_1} \tag{3}$$

which applies to sedimentation in the centrifuge. These may be equated if the same particle and geometry are used in both instances to give:

$$\omega^2 = \frac{980}{(R_2 - R_1)} \ln \frac{R_2}{R_1} = 169.2 \qquad (4)$$

Since the results agree within 1%, either method of calculation may be used; 1 hr at 1 g with this geometry is therefore equivalent to $\omega^2 t = 6.09 \times 10^5$.

In the separation cells used in the analytical ultracentrifuge, the sedimentation coefficient may be determined by analyzing the solution remaining in the upper compartment (10–12). These equations apply to ideal sedimentation in a sector-shaped compartment. The tubes used here have far from ideal geometry. Therefore, it has been considered of interest to ask: If the boundary-widening effect of diffusion is disregarded, how far does observed sedimentation in an angle-head rotor differ from that observed in sector cells?

By using equation 3 and the values of R_1 and R_2 corresponding to the menisci before and after removal of 7 ml of supernatant, values of $S_{\text{complete},t}$ were calculated for 20°C in water and are included in Figure 2. The scale for $S_{\text{complete},20}$ was calculated for data taken at 5°C for red blood cells and polystyrene latex, and at 10°C for BSA. These values are useful in that they indicate how closely an experimental system, which is subject to convective disturbances, may agree with theory. The sedimentation coefficient scale was aligned with the $\omega^2 t$ scale in Figure 2 by solving equation 3 for a series of values of $\omega^2 t$.

Sedimentation of Sheep Red Blood Cells

Sheep red blood cells were washed three times with the Veronal-buffered diluent (VBD) containing gelatin used for complement fixation studies (13). A total of 1.1 ml of packed cells was added to 32 ml of gelatin-free VBD; 7 ml of this suspension plus an additional 2 ml of VBD were added to each 10 ml polycarbonate tube. The tubes were either left in the angle holder in the refrigerator for sedimentation at 1 g or centrifuged briefly in the No. 50 rotor. Then 7 ml of supernatant was removed as described. A control tube was kept in the refrigerator and resuspended just before use. To effect complete hemolysis, 1 ml of each supernatant, and of the control, were added to 14 ml of buffered water (13) and read at 541 mμ in 1 cm light path cells in a Beckman DB spectrophotometer. The results are shown in Figure 2. The small optical density observed at $\omega^2 t = 10^7$ was due to a small amount of hemolysis. Results are given as percent of the starting sample found in the 7 ml of supernatant. The data plotted

in Figure 2 may be extrapolated to zero concentration of red cells in the supernatant as shown by the broken line in Figure 2, i.e., to a value of $\omega^2 t$ which would effect complete sedimentation. The extrapolated value of $\omega^2 t$ for complete sedimentation is 4×10^6, and the value for the sedimentation coefficient is approximately 10^6 S.

Polystyrene Latex Sedimentation

Polystyrene latex beads (Dow Chemical lot LS 055A) with a diameter of 0.188 μ (standard deviation 0.0076 μ in water) were diluted in distilled water and centrifuged to various values of $\omega^2 t$ as indicated in Figure 2. The absorbance of the 7 ml of supernatant removed was read against distilled water, and plotted as percent of absorbance of the starting sample. Data obtained with and without a small cotton plug in the bottom of the tube to prevent convection during sample recovery are given. A small residual absorbance was observed even after prolonged centrifugation. The slight skewness observed in the curve may relate both to a small amount of aggregation and to particle heterogeneity. A sedimentation coefficient of approximately 1000 is inferred from the plot using the data obtained with cotton plugs. By using a density of 1.0525 for polystyrene (14), a sedimentation coefficient of 1020 is calculated at 20°C in water for spherical particles having a diameter of 0.188 μ.

Sedimentation of BSA

A 1% solution of BSA in Veronal buffer (13) was centrifuged for periods up to 25 hr at 50,000 rpm. The absorbancies of the supernatants were read at 278 mμ with 1 cm light path cells after dilution with distilled water. Values are expressed as percent of the absorbance of the starting sample (Fig. 2). The curve, if extrapolated to the baseline (i.e., to zero concentration in the supernatant), intersects it at approximately 4 S, which is in fair agreement with published values (15).

It is of interest to see how closely the BSA data conformed to what would be expected theoretically in a sector-shaped separation cell such as is used in the analytical ultracentrifuge. By using the equation (refs. 10–12):

$$s = -\frac{1}{2\omega^2 t} \ln \left[\frac{R_0^2}{R_p^2} + \frac{C_t}{C_0} \left(1 - \frac{R_0^2}{R_p^2} \right) \right] \tag{5}$$

where R_0 = radius of the meniscus,

R_p = radius of the partition, or of the plane of the surface of the "pellet volume,"

C_0 = concentration of particles at the start of the run, and

C_t = concentration in supernatant at the conclusion of the run,

plots were made of $\omega^2 t$ as a function of concentration of particles remaining in the 7 ml of supernatant volume. Two values for the sedimentation coefficient were used that corresponded to the sedimentation coefficient for BSA at 10°C and a concentration of 1%, and the sedimentation coefficient as extrapolated to zero protein concentration at 10°C using equation 5. As shown in Figure 3, the experimental points fall between

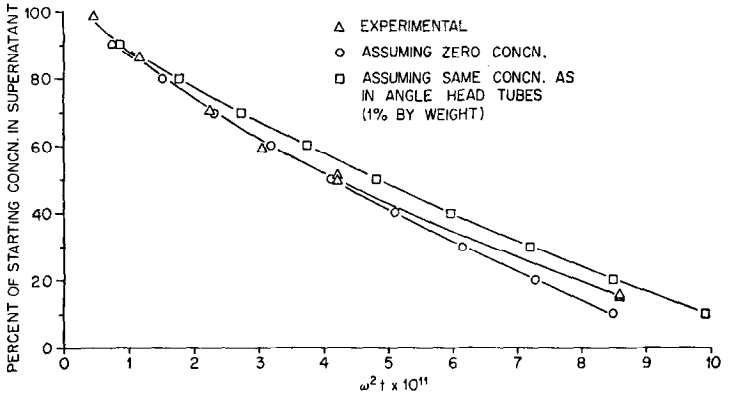

FIG. 3. Plot of theoretical and observed concentration of protein in supernatant in Spinco No. 50 angle-head polycarbonate centrifuge tubes as a function of $\omega^2 t$. The experimental values agree more closely with the theoretical plot for zero protein concentration than for 1%. Theoretical curves were calculated using the partition cell equation.

the two calculated curves for the most part and are closer to the curve calculated from equation 5 using the value for the sedimentation coefficient extrapolated to zero concentration.

The reproducibility of results is best indicated by considering the data for BSA. The results of two duplicate runs and one quadruplicate run are

TABLE 1
Reproducibility of BSA Concentration Measurements

$\omega^2 t$	Concentration in supernatant expressed as % starting concentration
3.07×10^{11}	58.1
	59.3
4.22×10^{11}	51.4
	51.4
	51.1
	49.8
8.61×10^{11}	15.2
	14.6

shown in Table 1 and indicate close agreement between different tubes run in the same rotor. The curve shown in Figure 3 indicates rather little scatter of points obtained in different experiments.

DISCUSSION

An extremely simple and reproducible method has been developed for measuring and describing sedimentation in angle-head centrifuges. The method is applicable over a very wide range of force-time values. The data obtained allow fractionation methods using centrifugation to be developed rationally. No indication has been found of anomalous non-reproducible sedimentation with the particle concentrations employed except with polystyrene latex. Insertion of a small cotton plug in the bottom of the centrifuge tube effectively eliminates this problem. The method used measures only transport to an arbitrarily chosen pellet volume and is not concerned with the measuring boundaries or with observing convective flow in the tube.

With BSA, transport to the pellet volume in an angle-head centrifuge occurs almost exactly as would be expected in a sector-shaped separation cell at zero protein concentration. Best agreement is observed when 50–70% of the protein has been sedimented. No evidence of an asymptotic approach to complete sedimentation was observed, as would be expected if extensive convection occurred during centrifugation. (It should be noted that somewhat different results would be obtained if an attempt were made to remove the entire supernatant solution, since a small amount of pellet material will slide off the packed pellet during the interval between the time the rotor comes to rest and that when the tubes are rotated 180° in position.)

The curve (Fig. 2) based on observations with polystyrene latex does not suggest a homogeneous particle species, and indeed the suspension is not perfectly homogeneous. Note, however, that when the center portion of the latex sedimentation is extrapolated to zero concentration in the supernatant as is done in Figure 2, the $\omega^2 t$ value obtained agrees very well with the calculated value for the average latex particle.

Measurements of red blood cells illustrate the upper size limits of the method used. Most points were obtained with the tubes subject to 1 *g*. For reasons discussed below, sedimentation under these two conditions may not be strictly comparable, and a centrifugal system for precisely controlled studies below 1000 rpm is required. This work suggests that the ordinary angle-head centrifuges are capable of yielding reproducible information on particle sedimentation over a very wide range of particle sizes.

The early theoretical and experimental studies of Pickeels (7–9) were

concerned chiefly with boundary movement, and the theory developed (7) neither predicts the fraction of particles in a given suspension sedimented as a function of integrated centrifugal force, nor agrees well with the experimental results obtained. Pickels concluded that the theory applied to relatively large particles (which were not studied) and only partially to the hemocyanin molecules examined, and that analytical ultracentrifuge data could not be used to predict accurately sedimentation in angle-head tubes. Quantitative studies on particle sedimentation in inclined tubes and a theory accurately describing such sedimentation therefore do not appear to be available.

The Pickels' theory proposes that particle enrichment takes place at the outer wall, and that depletion occurs at the inner wall. Enriched fluid, being denser, was thought to flow directly to the bottom, whereas depleted fluid was thought to flow centripetally to the top of the tube along the inner wall producing free convection. In the limiting case removal of particulate material would be logarithmic, with a constant fraction of the remaining suspended mass being removed per unit time at constant speed. Pickels' results may bee partly explained by the inability accurately to control or measure temperature, and by variations in the deceleration schedule (7). The puzzling results obtained with yellow fever virus (7, 8) may have been due to adsorption of a few virus particles on the wall of the centrifuge tube, to entrapment of virus in lipid-rich particles which floated, or to stir-back during deceleration.

The results obtained here with bovine serum albumin do not support the convective flow patterns proposed by Pickels (7). Rather they suggest that sedimentation occurs in an almost ideal fashion (in the sense that sedimentation in sector-shaped compartments is ideal) with very little free convection.

It is suggested that angle-head centrifugation theory should be modified to include the following considerations:

First, a clear distinction must be made between compact pellets and fluid volumes which are enriched or depleted. Compact pellets may form along the outer length of the centrifuge tube and may then slide to the bottom as the interplay of frictional and gravitational forces permits.

Particle enrichment of a fluid volume element which is free to move convectively occurs only when back-diffusion of particles sedimented to a surface takes place. Such enrichment resembles that occurring at the bottom of an analytical ultracentrifuge cell during an aproach to equilibrium. In such a case a denser fluid film may form at the outer sloping edge of an angle-head centrifuge tube. The thickness of the layer and the concentration are time dependent initially, in the absence of convection.

Regardless of the size or sedimentation rate of the suspended particles, a film of particle-depleted fluid will also form along the length of the inner (centripetal wall).

The question is, how will these enriched or depleted volumes move in a centrifugal field. As shown by Berman (16), Coriolis forces have negligible effects on particles of molecular dimensions but have marked effects on particles the size of whole cells and larger.

A fluid element sufficiently large to maintain an internal concentration difference during movement along the centrifuge tube must be accelerated (or decelerated) tangentially as it moves to a new radius. In the centrifuge used here a fluid element moving from the meniscus to the pellet volume increases its tangential velocity by 40%. To be thus accelerated, the fluid element must move laterally until it is in a portion of the tube which slopes with respects to the circumferential path of rotation. The net effect of this will be to rotate the fluid mass in the tube in a direction which would, if the tube were quickly moved to a vertical orientation, be opposite to the direction of rotor rotation. The movement of depleted fluid along the inner surface of the tube will tend to produce rotation of the fluid mass in the same direction. If fluid rotation does indeed occur as proposed here, it would tend to keep the concentration the same at all points having the same radius, and to make mass transport due almost entirely to sedimentation through the liquid, with convective movement playing a very minor part.

The results reported here agree with those of Charlwood (17) who showed that little anomalous sedimentation occurs when proteins sediment through preformed sucrose gradients in angle-head rotors.

Sedimentation in analytical ultracentrifuge tubes inclined so that the cell walls are at an angle to the radius is not a valid model for angle-head centrifugation, since Coriolis forces are directed *into* the impaction plane. In the angle-head centrifuge, in contrast, Coriolis forces are directed *along* the impaction plane and can produce fluid flow.

While these studies were initiated in an effort to devise better preconcentration steps for samples for subsequent zonal centrifugation, the technique is applicable to the solution of several other types of problems as well. By determining the enzyme activity, or virus or bacterial infectivity, information may be obtained on whether a single species of particle accounts for the activity. From the data obtained, both differential and zonal centrifugal experiments may be designed.[3]

[3] Digital $\omega^2 t$ integrators may be obtained from the International Equipment Co., Needham Heights, Mass., from MSE Ltd., London, England, and from the Spinco Division of Beckman Instruments, Palo Alto, Calif.

SUMMARY

A simple method for studying sedimentation in angle-head centrifuges has been developed. This method (made possible by the use of a titanium rotor, polycarbonate tubes, and an electronic $\omega^2 t$ integrator) is applicable to the study of particles having sedimentation coefficients ranging from 4 to 10^6 S. The range can be extended by including studies of $1g$.

Sheep red blood cells, polystyrene latex spheres, and BSA were employed as test particles. A 9 ml sample was used in each tube. At the end of a centrifuge cycle, the tubes were rotated *in situ* 180°, and then 7 ml of supernatant was carefully withdrawn. The remaining 2 ml was arbitrarily considered to be the pellet. When the concentration of particles remaining in the supernatant was plotted against the $\omega^2 t$ (on a log scale), very similar curves were obtained with each test particle.

The transport of BSA to the pellet volume followed closely that predicted from the separation cell equation.

It is proposed that the Pickels theory of angle-head centrifugation must be revised to include the effect of Coriolis forces on fluid elements moving radially in the centrifuge tube.

The results obtained suggest that the angle-head centrifuge may be used as a precision biophysical instrument.

REFERENCES

1. Anderson, N. G., Harris, W. W., Barber, A. A., Rankin, C. T., Jr., and Candler, E. L., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" (N. G. Anderson, ed), *J. Natl. Cancer Inst. Monograph* **21**, 253 (1966).
2. Anderson, N. G., Barringer, H. P., Babelay, E. F., Nunly, C. E., Bartkus, M. J., Fisher, W. D., and Rankin, C. T., Jr., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" (N. G. Anderson, ed.), *J. Natl. Cancer Inst. Monograph* **21**, 137 (1966).
3. Anderson, N. G., *Bull. Am. Phys. Soc.* **1** (Ser. II), 267 (1956).
4. Anderson, N. G., *J. Phys. Chem.* **66**, 1984 (1962).
5. Anderson, N. G., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" (N. G. Anderson, ed.), *J. Natl. Cancer Inst. Monograph* **21**, 9 (1966).
6. Cho, N., Barringer, H. P., Amburgey, J. W., Cline, G. B., Anderson, N. G. McCauley, L. L., Stevens, R. H., and Swartout, W. M., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis (N. G. Anderson, ed.) *J. Natl. Cancer Inst. Monograph* **21**, 485 (1966).
7. Pickels, E. G., *J. Gen. Physiol.* **26**, 341 (1943).
8. Bauer, J. H., and Pickels, E. G., *J. Exptl. Med.* **64**, 503 (1936).
9. Hughes, T. P., Pickels, E. G., and Horsfall, F. L., Jr., *J. Exptl. Med.* **67**, 941 (1938).
10. Tiselius, A., Pederson, K. O., and Svedberg, T., *Nature* **140**, 848 (1937).
11. Waugh, D. T., and Yphantis, D. A., *J. Phys. Chem.* **57**, 312 (1953).

12. YPHANTIS, D. A., "Separation Cells," *in* "Ultracentrifugal Analysis in Theory and in Experiment" (J. W. Williams, ed.), p. 227. Academic Press, New York, 1963.
13. CASEY, H. L., "Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test, Part II," *U. S. Public Health Monograph* **No. 74,** 1965.
14. CHENG, P. Y., AND SCHACHMAN, H. K., *J. Polymer Sci.* **16,** 19 (1955).
15. PEDERSON, K. O. *in* "The Ultracentrifuge" (T. Svedberg and K. O. Pederson), p. 355. Oxford, Univ. Press, 1940.
16. BERMAN, A. S., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" (N. G. Anderson, ed.), *J. Natl. Cancer Inst. Monograph* **21,** 41 (1966).
17. CHARLWOOD, P. A., *Anal. Biochem.* **5,** 226 (1963).