An Approach to the Isolation of Biological Particles using Sedimentation Analysis*

STANLEY B. PRUSINER

From the Departments of Neurology and of Biochemistry and Biophysics, University of California, San Francisco, California 94143

A systematic, general approach for the design of an initial purification procedure for any biological particle is described in this communication. A series of centrifugations in fixed angle rotors has been used to obtain information on the sedimentation behavior of particles of interest (Anderson, N. G. (1967) Anal. Biochem. 23, 72–83). Refinements of this technique have facilitated the determination of sedimentation profiles of subcellular organelle markers in suspensions of murine spleen and brain. The degree of homogeneity of several particles with respect to size can be ascertained from the sedimentation profiles. Alterations in these profiles after mechanical disruption and treatment with detergents are readily measurable and have been found to be useful in both the characterization and isolation of subcellular particles. Because fixed angle rotors are used in these studies, the data obtained can be directly applied to the development of a preparatory scheme for purification of a desired particle. These methods for sedimentation analysis are readily applicable to subcellular organelles, macromolecular complexes, viruses, viral-like agents, and a variety of macromolecules.

Centrifugation methods for the separation and analysis of biological particles have contributed significantly to advances in many areas of molecular biology (1, 2). These methods not only provide a means of analyzing the size and density of specific particles, such as subcellular organelles. macromolecular complexes, viruses, and a variety of macromolecules, but also for separating the particles of interest from other subcellular structures.

In this communication, the efficacy of a technique using a series of centrifugations in fixed angle rotors is illustrated in studies on the sedimentation behavior of several subcellular particles. The application of this technique to the study of particles over a wide range of sizes has been described by Anderson and termed analytical differential centrifugation (3, 4). Several refinements of the technique are reported here, including the use of detergents and sonication to characterize the sedimentation behavior of particular particles. The sedimentation profiles derived from these studies have been used to ascertain the sizes of the particles, as well as their degree of homogeneity. Since these sedimentation profiles have been obtained using fixed angle rotors, the information gathered can be applied directly to designing the optimal conditions for a series of differential centrifugations which would comprise the initial steps in a purification scheme.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were the purest grade commercially available.

Preparation of Homogenates—Female, 2-month-old Swiss mice were obtained from Charles River Laboratories. Spleens and brains from 50 mice were removed immediately after sacrifice by cervical dislocation and washed in ice-cold isotonic saline. Cleared spleens were then minced with scissors and disrupted in 20 mM Tris/HCl, pH 7.6, and 250 mM sucrose using a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle, while the brains were homogenized in 320 mM sucrose. Four to five strokes were used for all homogenization procedures. Unless otherwise noted, all of the procedures described herein were performed at 4°. After homogenization, the tissue suspensions were centrifuged in a Sorvall SS 34 rotor at 1000 rpm (121 g) for 10 min at 4°. The pellets contained unbroken cellular debris and nuclei. These pellets were rehomogenized, centrifuged, and the second supernatant fluid combined with the first. The remaining pellets were then washed and centrifuged again. The three supernatant fractions were combined and used for the centrifugation studies described below.

Subcellular Marker Assays—For convenience and rapidity, enzymatic markers were used to identify subcellular organelles in these studies. Succinate dehydrogenase (EC 1.3.99.1) was used as a mitochondrial marker and measured according to the method of Singer and co-workers (5). Acid phosphatase (EC 3.1.3.2) was used as a lysosomal marker and was measured using the colorimetric substrate p-nitrophenylphosphate (6). Lactic dehydrogenase (EC 1.1.1.27) was used as a marker for "soluble" proteins and was measured by following the reduction of NADH spectrophotometrically (7). Protein was measured by the method of Lowry et al. (8) with bovine serum albumin as a standard.

RESULTS

Sedimentation studies were performed in a Spinco L2-65B ultracentrifuge with a 40-AI or 50-Ti fixed angle rotor. Two-milliliter cellulose nitrate tubes (8 mm diameter × 50 mm length) were filled with 121-g supernatant suspensions and sealed with aluminum caps. The tubes were then placed in Teflon adapters within the fixed angle rotor. The samples
were centrifuged at the desired speed for periods of 30 min or greater. The time required for the rotor to accelerate to the desired speed and the time of deceleration with the centrifuge brake on were measured with a stopwatch.

After centrifugation, the tubes were taken from the rotor, the aluminum sealing caps removed, and the tubes stored vertically for a short time until the supernatant could be aspirated as shown in Fig. 1. Removal of the tube from the centrifuge rotor and the adapter housing was done gently and slowly, as were all subsequent manipulations in order to minimize the disturbance of the pellet-supernatant fluid interface. For convenience and ease of manipulation, centrifuge tubes were tilted approximately 50° as shown in Fig. 1. A pipette was introduced along the wall of the centrifuge tube which had been positioned toward the interior of the rotor during centrifugation. This procedure minimizes contamination of the supernatant fraction by pelleted material, which remains attached to the wall of the centrifuge tube facing the exterior of the rotor during centrifugation. Approximately 1.6 to 1.8 ml of supernatant fluid were then slowly aspirated into the pipette. The remaining 0.2 ml of fluid overlying the pellet was left undisturbed. Whenever the fluid interface was disturbed considerable contamination of the supernatant fraction with pellet markers occurred.

In the experiments described below, the activities of subcellular markers in the supernatant fraction of samples centrifuged have been plotted as a function of $\omega t$, where $\omega$ is the angular velocity of the centrifuge rotor in rad/s and $t$ is the time of centrifugation in seconds.

In Fig. 2, succinate dehydrogenase and lactic dehydrogenase activities in the supernatant fractions are plotted as a function of the log of $\omega t$. The $\omega t$ values range from $7 \times 10^5$ to $3 \times 10^6$ rad/s. As shown, the mitochondrial marker, succinate dehydrogenase, rapidly disappears from the supernatant fractions as the $\omega t$ values are increased, i.e. the homogenates are exposed to progressively greater centrifugal forces. In contrast, lactic dehydrogenase, a soluble protein marker, is unaffected by increasing $\omega t$ values until high speeds of centrifugation for long periods of time are reached. When the homogenate is centrifuged at 50,000 rpm for 30 h, all of the lactic dehydrogenase activity disappears from the supernatant. It is noteworthy that the disappearance of succinate dehydrogenase and lactic dehydrogenase activities from the supernatant fractions occurs over a relatively narrow range of $\omega t$ values, indicating that the particles are quite homogeneous with respect to size.

In Fig. 3, the succinate dehydrogenase sedimentation profile is replotted with a profile for succinate dehydrogenase in a sample treated with 0.5% sodium deoxycholate, a detergent which is known to release many proteins which are bound to membranes (9). The sample treated with 0.5% deoxycholate exhibits a sedimentation profile for succinate dehydrogenase similar to that for lactic dehydrogenase as shown in Fig. 2. The data indicate that treatment with 0.5% deoxycholate releases all of the succinate dehydrogenase which was bound to mitochondria and thus allows the enzyme to behave as a solubilized protein which is rather homogeneous in size. In contrast to the large shift in the sedimentation profile of succinate dehydrogenase after detergent treatment, the profile of lactic dehydrogenase does not change substantially.

![Fig. 1](image1.png)

**Fig. 1.** Method used for removal of supernatants in analytical differential centrifugation. After centrifugation was completed, (A) tubes were carefully removed from rotor and (B) tilted through an arc of approximately 50° to provide convenient removal of the supernatant. C, a long tip Pasteur pipette was used to slowly remove the supernatant without disturbing the interface layer.

![Fig. 2](image2.png)

**Fig. 2 (left).** Sedimentation profiles of succinate dehydrogenase ($\times$—$\times$) and lactic dehydrogenase ($\triangle$—$\triangle$) activities in the 121-g supernatant fraction of murine spleen homogenate. Centrifugations in a Spinco Ti-50 rotor at 4° varied from 1,000 rpm for 10 min to 50,000 rpm for 30 h as shown. A relative activity of 100 for succinate dehydrogenase and lactic dehydrogenase represents 0.32 and 2.70 enzyme units, respectively. One unit of enzyme is the amount that catalyzes conversion of 1.0 mmol of substrate to product per min under standard assay conditions.

**Fig. 3 (center).** Sedimentation profiles of succinate dehydrogenase activities in the absence ($\times$—$\times$) and presence (O—O) of 0.5% deoxycholate (w/v) in the 121-g supernatant fraction of a murine spleen homogenate. Centrifugation conditions and enzyme activities as in Fig. 2.

![Fig. 4](image3.png)

**Fig. 4 (right).** Sedimentation profiles of acid phosphatase activities in the 121-g supernatant fraction of a murine spleen homogenate. Profiles of samples with no prior treatment ($\triangle$—$\triangle$), sonication for three 15-s bursts at 4° (O—O), and treatment with 0.5% deoxycholate (O—O) are depicted. A relative activity of 100 for acid phosphatase equals 0.20 unit of enzyme activity. Centrifugation conditions and enzyme activities as in Fig. 2.
with detergent treatment.

Fig. 4 examines the sedimentation behavior of acid phosphatase, a lysosomal marker, as a function of $\omega t$. The activity of acid phosphatase continues to disappear from the supernatant fractions over the entire range of $\omega t$ values. The slow decline in acid phosphatase activity indicates that the enzyme is associated with particles which are heterogeneous with respect to size. Prior sonication of the material prepared for analytical differential centrifugation shifts the sedimentation profile to the right as shown, whereas treatment with 0.5% deoxycholate creates a pattern similar to that observed for lactic dehydrogenase. The deoxycholate-treated acid phosphatase exhibits a pattern in which the activity falls over a narrow range of $\omega t$ values, thus indicating that the solubilized enzyme is homogeneous in size, as would be expected for protein released from lysosomes. In Fig. 5, the actual data from three separate experiments are plotted to demonstrate

Fig. 5. The reproducibility of acid phosphatase sedimentation profiles from three separate experiments is illustrated for three different conditions: A, no prior treatment; B, sonication for three 15-s bursts at 4°C; and C, treatment with 0.5% deoxycholate. Centrifugation conditions and enzyme activities as in Fig. 4.

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**FIG. 6.** Comparison of sedimentation profiles for total protein in 121-g supernatants from murine spleen (A) and brain homogenates (B). Untreated samples (××××), samples treated with deoxycholate (DOC) (○○○○) or lyssolecithin (△△△△), and sonicated samples (□□□□) are plotted.
the excellent reproducibility of the method under the conditions used. The sedimentation profiles of acid phosphatase for untreated (A), sonicated (B), and deoxycholate-treated (C) samples are shown. The close correlation among these experi-
mements for a given set of conditions is striking and statistical analysis of the data did not prove advantageous.

Fig. 6 illustrates the differences in sedimentation profiles between homogenates of mouse spleen and brain. The total protein profiles for the untreated, the sonicated, the lysosom-
thin-treated, and the deoxycholate-treated spleen preparations show small differences in their sedimentation behavior. To sediment the bulk of cellular protein, \(\omega t\) values of \(10^2\) rads/s are required. In contrast, sedimentation profiles of total protein for mouse brain indicate many large structures containing significant amounts of protein are preserved during homogenization in untreated samples and thus sedimented at relatively low \(\omega t\) values (Fig. 6B). Sonication shifts the total protein curve to the right, while lysolecithin and deoxycholate treatments produce an even larger shift for the brain preparations.

**DISCUSSION**

The studies described here demonstrate that a series of centrifugations with fixed angle rotors can be effectively used to evaluate the sedimentation behavior of biological particles varying in size over a wide range. Sedimentation profiles obtained by this procedure can provide an assessment of the degree of homogeneity of a given particle with respect to size. A sedimentation profile with a steep slope indicates that the particles of interest are quite homogeneous with respect to size, and thus all of these particles sediment over a narrow range of \(\omega t\) values. A sedimentation profile with a gradual slope indicates that the particles are rather heterogeneous with respect to size and thus these particles sediment over a wide range of \(\omega t\) values.

The sedimentation profiles of mitochondria, as marked by succinate dehydrogenase, and soluble proteins, as marked by lactic dehydrogenase, demonstrate that these particles are composed of a rather homogeneous population with respect to size. In contrast, several other subcellular particles, such as lysosomes, microsomes, and plasma membranes, exhibit sedimentation profiles which indicate that they are composed of several populations of particles of varying sizes. These observations are consistent with those reported using other methodologies (2, 10, 11). Thus, no single sedimentation coefficient can be derived to describe their behavior in a centrifugal field. In the case of acid phosphatase, which is a lysosomal marker, at least two populations of particles appear to be described by the sedimentation profile shown in Fig. 4 for the untreated sample.

The sedimentation coefficient of a particle can be estimated if the slope of the sedimentation profile is reasonably steep. Anderson (3, 4) has shown that a good approximation of the sedimentation coefficient can be calculated from data obtained with fixed angle rotors using Equations 1 and 2. The \(\omega t\) value at which 90% of the particles of interest have been sedimented can be determined directly from the sedimentation profile and used to calculate the \(S_{obs}\) value according to Equation 1:

\[
S_{obs} = \frac{1}{2} \ln \left( \frac{r_2}{r_1} \right)
\]

\[
S_{20,\omega} = \frac{n_{T,\omega}}{n_{20,\omega}} = \frac{n_{T,\omega}}{n_{20,\omega}} \times \frac{(\rho_p - \rho_{T,\omega})}{(\rho_p - \rho_{T,\omega})}
\]

\(S_{obs}\) is the sedimentation coefficient observed under the conditions chosen, \(r_1\) is the radial distance from the centrifugal axis to the center of the meniscus, and \(r_2\) is the radial distance from the centrifugal axis to the partition or plane of the pellet and interface (Fig. 1). An \(S_{obs}\) can then be calculated, if the density of the particle is known, according to Equation (2):

\(\rho_{T,\omega}\) and \(\rho_p\) are the viscosity and density, respectively, of the centrifugation media at the chosen temperature and \(\rho_p\) is the density of the particle.

From the sedimentation profile for succinate dehydrogenase presented in Fig. 2, an \(S_{20,\omega}\) of \(1.0 \times 10^3\) S can be calculated assuming a density of 1.16 g/cm³ for mouse spleen mitochondria. Corrections for the viscosity and density of the centrifugation media consider only the 8.5% sucrose in the homogenization buffer. Similarly, an \(S_{20,\omega}\) of 7.0 S for lactic dehydrogenase can be calculated assuming a density of 1.30 g/cm³. These sedimentation coefficients for lactic dehydrogenase and mito-
ochondrial succinate dehydrogenase agree reasonably well with values in the literature (11).

The use of a series of centrifugations in fixed angle rotors is particularly suitable for studying the sedimentation behavior of membranous particles. The technique allows an iso-osmotic medium of any desired composition to be employed which, in the case of membranous particles, can represent an important advantage over more commonly used gradient methods. Membranous particles often tend to form vesicles that behave as osmometers, i.e. their size and shape is dependent upon their osmotic environment (9). Hence, the use of an iso-osmotic medium allows these osmotically responsive particles to sedi-
ment in a constant environment. This is not achieved when gradients are used and the sedimentation behavior of the vesicles can be influenced by the changing osmolarity of a gradient. The size and diffusion characteristics of the mole-
cules comprising the gradient determine in large part the extent to which osmotic-dependent distortions of the vesicles will occur during sedimentation.

Considerable information about the physical properties of a particle can be obtained by analyzing the sedimentation behavior of the particle after mechanical disruption or deter-
gen treatment procedures. A shift in the sedimentation profile of a particle to the right indicates that the unit size of the particle has been reduced. Either removal of nonessential molecules from the particle or dissociation of a multimeric structure into oligomers or monomers can be responsible for such a shift in the sedimentation profile. The succinate dehydrogenase and acid phosphatase profiles exhibit a shift in the sedimentation profile after detergent treatment in contrast to lactic dehydrogenase, the profile of which does not change under these conditions. In general, membrane-bound particles show a dramatic shift to the right in their sediment-
ation profiles when procedures such as detergent treatment, which disrupt membranes, are employed.

Comparative studies of the sedimentation behavior of particles from different tissues or organisms (or both) are readily accomplished using analytical differential centrifugation. The influence of tissue source on the sedimentation behavior of subcellular structures is demonstrated in Fig. 6, where the total protein profiles for mouse spleen and brain homogenates under several conditions exhibit distinct differences. Even

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1 S. Prusiner, unpublished observation.
when different tissues are morphologically and functionally quite similar, a systematic exploration of the sedimentation behavior of subcellular organelles may prove useful. Recent studies on the comparative fractionation of kidney cortex and choroid plexus indicate that although the two tissues possess many similarities, the distributions of marker enzymes for brush border membranes differ substantially (12).

When the sedimentation behavior of an unknown particle is to be examined, analytical differential centrifugation is especially useful. Although the resolution of this technique appears to be considerably less than that achieved with gradients, numerous fractions from many gradients must be analyzed to obtain the equivalent information gathered by a single analytical differential centrifugation experiment. For example, the initial ascertainment of whether a given enzyme activity is associated with a particular subcellular organelle is more readily accomplished using analytical differential centrifugation. Gradient centrifugation, which can effectively separate particles differing relatively little in size, may then be used to refine and extend these determinations.

The procedures described in this communication may be used to systematically design an initial purification scheme for virtually any biological particle. Analysis of the sedimentation behavior of the particle using fixed angle rotors provides a rational basis for designing an initial isolation protocol as shown in Fig. 7. When the particles of interest are rather homogeneous with respect to size as described by Curve a (Fig. 7A), a pair of differential centrifugations of \( \omega \) values \( a_1 \) and \( a_2 \) rad/s (Fig. 7B) will prove useful in their isolation. Further purification of these particles may be possible if a perturbation (\( p \)), such as detergent treatment, shifts the sedimentation profile from Curve a to Curve b and a pair of centrifugations with \( \omega \) values of \( b_1 \) and \( b_2 \) rad/s are used as illustrated in Fig. 7, A and B. If no shift in the sedimentation profile described by Curve a occurs after perturbation (\( p \)), such an observation may also be valuable, for the sedimentation profiles of many other particles not of interest may be shifted by the perturbation (\( p \)) and thus removed simply by repeating the centrifugation series with \( \omega \) values of \( a_1 \) and \( a_2 \) rad/s. Alternatively, when the particles of interest have a profile like Curve c, purification procedures based on particle size will not be advantageous, and other procedures based on particle density or charge need to be explored (Fig. 7C). However, if perturbation (\( p \)) of these particles shifts their sedimentation profile from Curve c to Curve d, then a pair of differential centrifugations with \( \omega \) values of \( d_1 \) and \( d_2 \) rad/s may provide a purification (Fig. 7, C and D).

Complex, multicomponent sedimentation profiles as shown in Fig. 8 also provide valuable information for the design of purification schemes. Curve a is composed of two sedimentable components \( a' \) and \( a'' \) as illustrated in Fig. 8A. The application of a perturbant (\( p \)) causes a shift in the sedimentation profile to yield Curve b, but only the sedimentation of component \( a' \) is altered by \( p \). Using these profiles, a scheme for the purification of components \( a' \) and \( a'' \) can be developed as depicted in Fig. 8B. In contrast, as shown in Fig. 8C, one component (\( c' \)) of the sedimentation profile \( c' \) may represent an array of particles quite heterogeneous with respect to size, while a second component (\( c'' \)) is relatively homogeneous. In this case, the perturbant (\( p \)) gives a sedimentation profile described by Curve d. Similarly, these profiles can be used to create procedures for the purification of components \( c' \) and \( c'' \) (Fig. 8D). It is conceivable that a variety of even more complex sedimentation profiles may be observed, but the principles outlined above should remain the same with respect to analysis and application.

Finally, the techniques described in this communication for generating sedimentation profiles from a series of centrifugations in fixed angle rotors may be applied to virtually any biological particle, including viruses and viral-like agents. In the case of infectious particles, the infectivity remaining in each supernatant fraction after centrifugation must be determined. We have recently employed these procedures to study the sedimentation properties of the scrapie agent and to develop an initial purification scheme (13).

Acknowledgments - I am indebted to Dr. N. Anderson who first suggested the application of analytical differential centrifugation. Ms. P. Byrd, P. Cochran, and C. Hooper are thanked for expert technical assistance, and Ms. F. Elvin for expert editorial help. Many discussions with Drs. D. Garfin and A. Gordon are gratefully acknowledged.

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An approach to the isolation of biological particles using sedimentation analysis.
S B Prusiner

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