A Sedimentation Equilibrium Method for Determining Molecular Weights of Proteins with a Tabletop High Speed Air Turbine Centrifuge

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SUMMARY

A technique has been developed for determining the molecular weights of proteins by sedimentation equilibrium in a tabletop, high speed, air turbine centrifuge. The small rotor containing six plastic tubes with a capacity of about 100 µl/tube can operate at 10^5 rpm corresponding to centrifugal fields up to 1.6 x 10^5 times that of gravity. Molecular weights are determined by measuring the depletion of protein from the upper 40% of solution. The addition of dextran or the use of relatively concentrated protein solutions (5 mg/ml) provided a sufficiently high density gradient at the bottom of the tubes to stabilize the concentration distribution of protein during the deceleration of the rotor. Experiments with known proteins showed that the fractional depletion in the upper 40% of the tubes varied linearly with the exponential of the reduced molecular weight as predicted by the theoretical treatment. The preliminary empirical plot can be used for determining the molecular weights of other macromolecules. Although knowledge of the partial specific volume is required, this value can be estimated readily from the chemical composition. Since the method does not depend on transport properties, the results are not affected by the shape of the molecules. Only small amounts of material are needed and impure samples can be used if a specific biological assay is available. Accuracies of about 10% were obtained and improvements in the technique should lead to an enhancement in the precision.

During the past 10 years, various empirical methods have been developed for determining the molecular weights of native proteins and the polypeptide chains produced by the addition of denaturants like guanidine hydrochloride or sodium dodecyl sulfate. Techniques such as gel chromatography (1–4), polyacrylamide gel electrophoresis (5), and electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (6–8) generally yield reliable results, exhibit great sensitivity, and encompass a broad range of molecular weights. Although these methods require calibration with proteins of known molecular weight and in some instances there are marked departures from the empirically determined relationships, the techniques have proved invaluable for studies of proteins which are available only in microgram amounts. Moreover, the equipment required is simple and inexpensive.

The availability of a tabletop high speed centrifuge, known as the Airfuge, afforded an opportunity to develop an additional method for determining molecular weights. This paper presents a preliminary account of a sedimentation equilibrium technique for measuring molecular weights ranging from about 10^4 to 1.5 x 10^5 with small quantities of dilute solutions which are centrifuged at fields about 30,000 times that of gravity. The method involves measurement of the fraction of protein remaining in the upper 40 µl of the solution (total volume of 100 µl) at the conclusion of the centrifuge experiment. Although presented here as an empirical technique, the method is based on the fundamental theoretical principles of sedimentation equilibrium (9) modified to account for the shape of the small tubes contained in the rotor and for the fractionation procedure invoked after equilibrium is attained.

A standard curve was obtained with proteins of known molecular weight and partial specific volume and it is seen that this empirical curve correlates approximately with calculated theoretical relationships.

GENERAL CONSIDERATIONS

The tabletop Airfuge is an air turbine centrifuge about 38 cm long, 28 cm wide, and 18 cm high and has an aluminum rotor which is only about 4 cm in diameter and contains six sample tubes. This rotor, which has turbine flutes machined into the bottom, is lifted and driven by ordinary laboratory compressed air so that it simulates a "spinning top" which rotates on a cushion of air. With readily available air pressures, the rotor attains its maximum operating speed of 100,000 rpm in about 30 s; at that speed the centrifugal field at the bottom of the tubes is slightly greater than 160,000 times that of gravity. For the sedimentation equilibrium experiments described here, special polyethylene tubes of 100-µl capacity were used instead of the standard cellulose propionate tubes which contain 175 µl. These thicker walled, slightly tapered tubes, 2 cm in length and approximately 4 mm in diameter (inner), were chosen both to reduce the path of sedimentation and to minimize convective stirring during the deceleration of the rotor. The tubes are oriented at an angle of 18° relative to the axis of rotation and the positions of the bottom of the tube and the meniscus for 100-µl samples are 1.42 cm and 0.95 cm, respectively. Because of the steep

* The theoretical treatment, along with other Supplementary Material and the References, is presented in miniprint following the text. The figures and tables in that section are identified by "S". Miniprint can be easily read by the aid of a large magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 960 Rockville Pike, Bethesda, Md. 20014. Request Document No. 195-3, cite authors, and include a check or money order for $1.65 per set of photocopies.
orientation of the tubes, their relatively narrow bore, and the convection which results from the shape of the tubes and the consequent wall effects, the transport of macromolecules to the bottom of the tubes is relatively rapid. Indeed, it is likely that convective flow down the inclined tube actually leads to a faster rate of transport than would occur due to sedimentation of the molecules in the absence of convection. After sufficiently large density gradients are established near the bottom of the tubes, generalized convection does not continue while the rotor is maintained at a constant high speed. Instead, an equilibrium state is probably attained in which the concentration varies with radial distance from the vertical meniscus according to the fundamental thermodynamic equations for sedimentation equilibrium (9-11).

At the termination of the experiment, some disturbance doubtless occurs because of the rapid deceleration and the large reduction of the centrifugal field. However, as shown below, this ‘stirring’ can be maintained at a relatively low level primarily because the tubes are so narrow and flow is reduced. As long as stirring of sedimented material into the upper part of the tube is prevented, the sample removed from each tube after the rotor is stopped is representative of fluid layers in the spinning rotor. As shown experimentally, the logarithm of the fraction of protein in the upper part of the tube varies in almost a linear fashion with the molecular weight (corrected for the buoyancy term). This empirical curve serves as a basis for determining molecular weights of proteins which have not as yet been well characterized. Moreover, as shown under ‘Supplementary Material,’ a preliminary theoretical treatment is available which accounts in a semiquantitative fashion for the experimental results in terms of the well established principles of sedimentation equilibrium.

**EXPERIMENTAL PROCEDURES**

All centrifuge experiments were performed with a Beckman Spinco Airfuge following the procedure described in the manual. Special, thick-walled, slightly tapered polyethylene tubes of 100-µl capacity were used for most experiments (these tubes are available from Spinco Division, Beckman Instruments, Inc.). Some determinations were also made with Beckman Microfuge tubes modified by removing the bottom 0.5 mm and cutting the top to give a total external length of 2 cm. For most experiments, the temperature of the solutions was approximately 27°C as measured at the termination of the experiment. Rotor speed was measured for each experiment with a stroboscope (General Radio Corp.) and found to be constant for a given setting of the air pressure (the variation in speed was less than 5%).

For a given setting of the air pressure (the variation in speed was less than 5%), the fraction of the initial amount of protein remaining in the upper 40 µl was independent of concentration when no density gradients were present at 60,000 rpm. At low concentrations of ovalbumin (0.1 and 1 mg/ml) and no dextran T40, the fraction remaining in the upper 40 µl was high (0.72) whereas at larger concentrations of protein, 8 and 16 mg/ml, this fraction decreased to 0.13 and 0.12, respectively.

When dextran T40 was present at 5 mg/ml, the fraction of peroxidase remaining in the upper 40 µl was independent of the protein concentration; at 0.05, 0.1, 0.2, 0.5, and 1.0 mg/ml, the values of F were 0.29, 0.30, 0.25, 0.27, and 0.34, respectively. For these experiments, the stabilizing density gradient is attributable to the dextran T40 and thus proteins even in very dilute solutions were sedimented efficiently. Deviations from expected equilibrium concentration distributions due to stirring at the end of the experiment would be more noticeable when the fraction of protein remaining in the upper 40 µl is very small. This hypothesis was tested by an experiment with aspartate transcarbamoylase at 5 mg/ml and dextran T40 at 5 mg/ml. After centrifugation of this enzyme (M = 3 x 10^6) for 18 h at 60,000, the fraction of the initial.

| Concentration of dextran T40/ mg/ml | Fraction of protein remaining |
|------------------------------------|-----------------------------|
| 0                                  | 0.91 (Peroxidase)           |
| 1                                  | 0.26                        |
| 2                                  | 0.20                        |
| 5                                  | 0.23                        |
| 10                                 | 0.30                        |
| 50                                 | 0.25                        |

*In this experiment, no dextran T40 was present but the solution instead contained serum albumin at 5 mg/ml.

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activity remaining in the upper 40 μl was $10^{-4}$. Based on extrapolation from results with smaller proteins, the fraction should have been $10^{-8}$. The concentration gradients, once established, were sufficiently stable that the time elapsed between the termination of the centrifugation and the sampling of the contents was not critical. In one experiment, alkaline phosphatase at 5 mg/ml was centrifuged for 8 h at 100,000 rpm in the presence of dextran T40 at 5 mg/ml. Samples (40 μl) removed from the tubes at 1 min, 20 min, and 60 min after the rotor was stopped all contained less than $10^{-8}$ of the original enzymic activity. Intentional mixing of the contents of the tubes yielded the initial activity.

Time Required to Attain Equilibrium—In order to test whether equilibrium distributions were obtained in the experiments described above (18 h), solutions of cytochrome c, ovalbumin, and serum albumin were centrifuged for various lengths of time at 40,000 rpm. Dextran T40 was present in each tube. The fraction for each protein and each time was plotted in Fig. 1A as log $F$ versus $σ$ where $σ$ is the reduced molecular weight (see "Supplementary Material" for mathematical description). As seen in these plots, the data for the three proteins for each time fitted approximately a linear relationship between log $F$ and $σ$. When the slopes of these lines (and others not shown) were plotted against time, as in Fig. 1B, it was found that a maximum slope was obtained at about 8 h. This evidence served as a criterion for the attainment of sedimentation equilibrium.

Sedimentation Equilibrium of 14 Proteins and the Dependence of Log $F$ on $M$—Various proteins of known molecular weight and partial specific volume (see "Supplementary Material") were centrifuged to sedimentation equilibrium and the fraction of protein remaining in the upper 40 μl of each solution was measured. The resulting values of log $F$ were plotted versus the calculated values of $σ$ as shown in Fig. 2. For convenience, a molecular weight scale was added at the top of the figure where the values of $M$ for all the proteins were normalized to the same partial specific volume, 0.73 ml/g, and the rotor speed was 42,000 rpm. The solid curve represents a best fit of the experimental results and the dashed curve corresponds to the stippled area in Fig. 2A-S.

**DISCUSSION**

As seen in Table I, the stabilizing density gradient formed from the sedimentation of dextran T40 (or proteins) at about 5 mg/ml was sufficient to prevent significant stirring during the deceleration of the rotor and the sampling procedure. Moreover, in accordance with theory (see "Supplementary Material"), the fraction of protein remaining in the upper 40 μl of the solutions was almost linearly dependent on the exponential of the reduced molecular weight (Fig. 2).
The accuracy of the molecular weight determinations depends on a number of factors. First is the assay for measuring the fraction of protein in the supernatant relative to the initial solution. Second is the use of appropriate experimental conditions so that the values of $F$ approach neither unity nor zero. This point is illustrated by the results in Fig. 2. For cytochrome $c$, the value of $F$ was about 0.7; hence for smaller molecules, $F$ would be closer to 1.0 and experimental errors in determining the concentrations of the initial solution and the supernatant would lead to much more uncertainty in the evaluation of $F$. Similarly, for proteins with molecular weights about 10$^8$, the values of $F$ are about 0.01 and errors in measuring the small amount of protein in the supernatant would lead to inaccurate values of $F$. A third factor influencing the accuracy of the molecular weight determinations is the calibration curve itself. This method like gel chromatography and polyacrylamide gel electrophoresis requires knowledge of the molecular weights of the proteins used as standards. The proteins should be homogeneous and have no tendency to aggregate or dissociate. Only one speed, 42,000 rpm, was employed for the experiments summarized in Fig. 2 and the results encompassed proteins with molecular weights ranging from 10$^4$ to 1.5 x 10$^6$. Greater precision could be obtained with two calibration curves, one for the smaller proteins at higher centrifugal fields so that the values of $F$ were lower and the other for larger proteins at lower fields and correspondingly higher values of $F$. A reasonable assessment of the accuracy obtainable is derived from the experiments with peroxidase; in these studies $F$ was 0.29 ± 0.05 for initial concentrations of enzyme varying from 0.05 to 1 mg/ml. According to the calibration curve in Fig. 2, the molecular weight of peroxidase is 41,000 ± 3,000; hence an uncertainty of about 17% in $F$ corresponds to an error in $M$ of less than 10%.

In the experiments described above, the 40-μl samples were removed manually without the use of any special device for lowering the pipette as liquid was being withdrawn. Some experimental error can be attributed to imprecision in the removal of the samples and this technique could be improved through the use of a vertical rack for adjusting the height of the pipette. The accuracy in measuring $F$ depends on the technique used for the determination of concentrations. Generally, enzyme assays are less precise than spectral determinations of concentration. But one of the principal advantages of the technique is its potential for molecular weight determinations of biologically active proteins even before they are purified. In this regard, it is analogous to gel chromatography and electrophoresis on polyacrylamide gels where the location of the component is based on assays for biological activity. The techniques differ, however, in that the transport methods require only the location of the species for the empirical determination of molecular weights, whereas the sedimentation equilibrium method requires quantitative assays of the amount of the active component in the supernatant and original solution. If the value of $F$ is in the correct range, the measurement of concentration need not be extremely accurate; for $F = 0.1$, an error of 30% would lead to only a 10% error in the estimation of the molecular weight.

According to theoretical treatments for sedimentation equilibrium (9-11), the depletion of macromolecules from the upper fraction of the solution depends on the partial specific volume as well as the molecular weight. For most proteins the partial specific volume, $V$, is about 0.725 ml/g (see range of values in Table I S). When the value of $V$ is not available experimentally, it can be calculated with excellent precision from the amino acid composition (12) or it can be assumed to be 0.725 ml/g. In an analogous way, the value of $V$ can be estimated satisfactorily from the chemical composition of nucleic acids, polysaccharides, glycoproteins, or lipoproteins.

In the experiments conducted thus far there appeared to be no complications resulting from the use of dextran T40 to create the stabilizing density gradient. Interactions between some macromolecules and dextran T40 may occur and in those cases misleading results would be obtained. For the experiments reported here the solutions of protein contained the dextran T40 throughout the entire tube. It is possible to layer the protein solution over a small volume of the more dense dextran T40 which is placed in the bottom of the tubes. In this way, problems stemming from interactions may be reduced. Alternatively, tubes containing some type of constriction at the bottom might help to reduce convective stirring during the deceleration of the rotor.

Just as in conventional sedimentation equilibrium experiments, the technique described here would provide useful information about the heterogeneity of the macromolecules through experiments at different speeds. Variations of $M$ with rotor speed would indicate heterogeneity or interacting systems.

With the existing rotor and stator, it is difficult to operate at speeds below 40,000 rpm. Hence, determinations of molecular weights greater than about 1.5 x 10$^9$ are difficult. Some modification in the Airfuge to circumvent this problem is needed. In contrast, it is possible to determine the molecular weights of very small proteins readily since the maximum centrifugal force available is so great. It should be emphasized that the molecular weights are not affected by the shape or hydration of the sedimenting material since the technique is based on the theory of sedimentation equilibrium. With improvements stemming from greater experience, the method promises to be a useful adjunct to the transport techniques used so widely for determination of molecular weights. The method is founded on well understood physical principles. Improvements in the theoretical treatment beyond the preliminary description presented under "Supplementary Material" should further enhance its value. In principle, the method presented here could be used for determining the molecular weights of unfolded polypeptide chains in guanidine HCl or sodium dodecyl sulfate solutions.

REFERENCES

See p. 2077.
Molecular Weight Determinations with a Tabletop Centrifuge

One method of determining the molecular weights of proteins involves the use of a tabletop centrifuge. The sample is placed in a small container, usually a microcentrifuge tube, and subjected to high centrifugal forces. The difference in the sedimentation rates of the sample and a reference compound provides a means to determine the molecular weight of the sample. The equations below describe the relationship between the sedimentation rate and the molecular weight.

**Equation 1**: 
\[ S = \frac{K \cdot d}{R^2} \]  
where \( S \) is the sedimentation coefficient, \( K \) is a constant, \( d \) is the density difference between the sample and the medium, and \( R^2 \) is the square of the radius of the sample.

**Equation 2**: 
\[ M = \frac{d^2 \cdot K \cdot V}{R^4 \cdot S} \]  
where \( M \) is the molecular weight, \( d \) is the density difference, \( V \) is the volume of the sample, \( R \) is the radius of the sample, and \( S \) is the sedimentation coefficient.

**Table 1**: Molecular Weights and Partial Specific Volumes of Reference Proteins

| Protein               | Molecular Weight | Partial Specific Volume |
|-----------------------|------------------|------------------------|
| Lysozyme              | 14300            | 0.72                    |
| Celulase              | 10460            | 0.75                    |
| Catalase              | 15180            | 0.71                    |
| Chymotrypsin          | 27200            | 0.73                    |
| Trypsin               | 23400            | 0.70                    |
| Bovine serum albumin  | 67000            | 0.76                    |
| Human serum albumin   | 69500            | 0.76                    |

**Figure 1**: Schematic drawing of a sample tube filled with the protein solution. The protein is at the bottom of the tube, and the spin is at the top. The tube is centrifuged at high speed to separate the protein from the solvent.

**Figure 2**: Schematic representation of the ultracentrifuge cell, showing the sample and reference components. The sample is at the bottom of the cell, and the spin is at the top. The cell is centrifuged at high speed to separate the sample from the reference.

Further work is clearly needed to determine the specific factors that influence the sedimentation rate. Further studies are needed to determine the extent to which the sedimentation rate is influenced by the concentration of the sample.

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