Conformational Changes in Proteins as Measured by Difference Sedimentation Studies. I. A Technique for Measuring Small Changes in Sedimentation Coefficient*

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ABSTRACT: Increased interest in the detection and measurement of the conformational changes in macromolecules produced by specific ligands has prompted a reinvestigation of the applicability of hydrodynamic methods and, in particular, of a technique for the direct measurement of small differences in sedimentation coefficient. Difference sedimentation based on Rayleigh interference optics, as described by Richards and Schachman is a subtractive technique for measuring small differences in sedimentation coefficient directly.

This method was shown to be capable of great precision but at the time of its introduction it suffered from systematic errors. These inaccuracies have been found now to be due to deficiencies in ultracentrifuge cells, misalignment of the Rayleigh mask, and an optical anomaly produced by high concentration gradients. Suitable remedies have been devised and a detailed discussion is given of the experimental and theoretical aspects of the technique. The method was tested by measuring differences in sedimentation between aqueous solutions of bushy stunt virus and solutions containing small amounts of D_2O. The precision and accuracy of the difference sedimentation method were independent of the actual difference in sedimentation coefficients even for changes less than 0.2%. The technique has been applied to a number of proteins having molecular weights of 3×10^4–3×10^5 and changes in sedimentation coefficient of only 0.01 S were measured readily with an accuracy better than ±5% (corresponding to ±0.0005 S). Specific ligands produced significant changes in the sedimentation coefficients thereby indicating that a number of these proteins had undergone conformational changes. A discussion of other hydrodynamic methods indicates that difference sedimentation should have unique advantages for studying small conformational changes in macromolecules.

Early studies on protein denaturation showed that the size and shape of many protein molecules changed appreciably when they were exposed to the action of reagents such as urea, hydrogen or hydroxyl ions, and detergents (Neurath *et al.*, 1944). These changes were revealed clearly by hydrodynamic measurements such as intrinsic viscosity and sedimentation or diffusion coefficients (Edsall, 1953). From the alterations in these parameters it was inferred that there was a general disorientation (or "unfolding") of the polypeptide chains which is now described as a disruption of the secondary, tertiary, and quaternary structures (Linderstrøm-Lang, 1952; Kauzmann, 1959). Much valuable information was deduced from these hydrodynamic techniques about the structure of proteins and the attractive forces responsible for their three-dimensional architecture.

In more recent research on proteins such as studies on the mechanism of action of enzymes it has become necessary to detect and measure accurately much less drastic changes in the conformation of the macromolecules. These changes generally are so small that the conventional hydrodynamic methods are inadequate because of limitations in their precision. As a consequence these methods were abandoned in favor of more sensitive techniques such as difference spectroscopy (Wetlaufer, 1962; Donovan, 1969), optical rotatory dispersion, and circular dichroism (Beychok, 1966, 1968; Ulmer and Vallee, 1965; Yang, 1967), and spectrofluorimetry (Weber, 1953; Chen *et al.*, 1969; Stryer, 1968). The application of these optical methods has enriched greatly our knowledge of the conformational changes in proteins which accompany their interactions with specific ligands. In many instances it is difficult to distinguish among a very localized change in the conformation of the polypeptide chain at the site of the binding, a general alteration in the bulk structure of the protein, and a large perturbation in a physical property (such as the absorption at a specific wavelength) due to the binding of the ligand without a concomitant change in the conformation of the protein. For crystalline proteins this ambiguity disappears since X-ray diffraction studies provide detailed structural data and unequivocal evidence for conformational changes in the macromolecules (Muirhead and Perutz, 1963; Steitz *et al.*, 1967).

As pointed out by Kauzmann (1959) it is desirable to study both short range properties, *e.g.*, ultraviolet absorption spectra and optical rotatory dispersion, and shape properties such as intrinsic viscosity and sedimentation coefficient since the information from the two sets of data is complementary. Although short-range properties potentially can reveal detailed structural information, as yet our theoretical understanding of them is often deficient. In contrast the bulk properties and changes in them resulting from the interaction of a protein with a ligand yield only gross information which can be interpreted satisfactorily from theoretical treatments. Hence hydrodynamic techniques, especially when used in conjunction with other methods sensitive to short-range

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effects, should prove of value in studies of conformational changes in proteins. The potential utility of hydrodynamic methods for measuring conformational changes in proteins accompanying their interaction with ligands has been demonstrated recently with three different enzymes. Gerhart and Schachman (1968) observed a 3.6% decrease in the sedimentation coefficient of ATCase[1] upon its interaction with the substrate analog, succinate. With the relaxed form of glutamine synthetase Shapiro and Ginsburg (1968) detected a 3% increase in the sedimentation coefficient (and a much larger decrease in the intrinsic viscosity) upon the addition of Mn^{2+} ions. Similarly, Conway and Koshland (1968) found a large change in the intrinsic viscosity of GPDH on binding NAD. A more recent study of a purified antibody (Warner *et al.*, 1970) revealed an increase in the sedimentation coefficient as a result of interaction with a specific hapten and this increase was attributed to a conformational change in the protein. For many systems the change in hydrodynamic properties may be significantly less than those illustrated here and there is a need therefore for a more sensitive, accurate method which measures directly the change in the physical property. Accordingly we have turned to the difference sedimentation technique introduced by Richards and Schachman (1957, 1959).[2]

The difference sedimentation method described here is based on the Rayleigh interferometer which provides a direct subtraction of the concentration distribution curves for two solutions contained in a double-sector ultracentrifuge cell. The resulting interference pattern yields directly the difference in sedimentation coefficients for the two solutions. As developed by Richards and Schachman (1959) the difference technique was shown to be of great sensitivity, but it suffered from systematic inaccuracies. Accordingly further investigations were conducted on the method so as to discover the sources of the errors and to devise suitable remedies. This communication describes these efforts which resulted in the design and construction of a Rayleigh mask (Kirschner, 1971; Kirschner and Schachman, 1970; Richards *et al.*, 1971a), the clarification of some optical anomalies which affect fringe spacing (Kirschner, 1971; Richards *et al.*, 1971a), improvement in the quality of ultracentrifuge cells, and the systematization of working procedures for analyzing the ultracentrifuge patterns. Experimental tests are described which show the reliability and sensitivity of the technique with systems containing known differences in sedimentation coefficient. Preliminary results are given for a variety of proteins to illustrate the potential of the difference sedimentation technique for the study of conformational changes. As shown with these systems changes in sedimentation coefficients of only 0.2% (corresponding to 0.01 S for a protein of molecular weight 10^5) can be measured readily with an accuracy better than ±5% (corresponding to ±0.0005 S).

Terminology

It seems appropriate that we comment on terminology because of the introduction of the term, "difference sedimentation" to describe the technique previously called differential sedimentation by Richards and Schachman (1957). The latter term has already acquired a variety of specific meanings and further confusion might be avoided by the adoption of a consistent and plausible terminology.

In addition to its use for describing the series of cycles of alternate high- and low-speed centrifugation for preparative purposes, differential sedimentation has been employed in three different ways for analytical ultracentrifuge experiments. Historically it was used first to describe layering experiments which measure the change in sedimentation coefficient with concentration, ds/dc (Hersh and Schachman, 1955). This technique is analogous to the conceptually similar differential moving-boundary method for transference numbers (Longsworth, 1943); and though the term, differential sedimentation coefficient, is a misnomer and should be avoided, the description of the method as a differential sedimentation technique seems appropriate. The term differential sedimentation was used again to denote another technique (Richards and Schachman, 1957, 1959) by which small differences in sedimentation coefficients were measured directly through the use of interference optics to subtract the concentration–distance curves for two solutions contained in the separate compartments of a double-sector ultracentrifuge cell. Gerhart and Schachman (1968) avoided the use of any nomenclature when they used schlieren optics with two cells (one containing plane windows and the other a wedged window) for increasing the accuracy in measuring small differences in sedimentation coefficients. However, in a subsequent paper Schumaker and Adams (1968) used the term differential sedimentation for their use of essentially the same method.

We propose that differential sedimentation be reserved for the layering experiments with "concentration" boundaries (Kohlrausch, 1897), since it has precedence historically over the other uses for analytical ultracentrifuge studies and also because of its relationship to the earlier differential moving-boundary method for the concentration dependence of transference numbers. Moreover the term differential connotes the change of some quantity (sedimentation coefficient) with respect to a continuous variable such as distance, time or, in this case, concentration. The term "difference" is more appropriate for measuring the relationship of two separate solutions to each other in analogy with difference spectroscopy where the parameter which is being measured compares two distinct solutions and there is no continuous variation in the property. Finally, though the use of wedged windows permits the simultaneous examination of two solutions thereby substantially increasing the accuracy in comparing two samples, the experimental parameter which is measured is neither a differential nor a difference. Thus we feel that difference sedimentation should be reserved for the technique introduced by Richards and Schachman (1957) since the *experimentally* measured quantity is directly related to the difference between two real quantities.

General Considerations

The difference in refractive index between the solutions in the two compartments of a double-sector ultracentrifuge cell is readily measured at each level in the cell by means of the Rayleigh interferometer. For most ultracentrifuge experiments one of the two compartments is filled with solvent and thus the interferometer provides a direct measure of the change in refractive index (or concentration) in the other liquid (solution) as a function of distance from the axis of rotation. If both solutions are identical, despite the presence

[1] Abbreviations used are: ATCase, aspartate transcarbamylase; GPDH, glyceraldehyde 3-phosphate dehydrogenase; BSV, bushy stunt virus.

[2] Richards and Schachman (1957, 1969) used the terms "differential ultracentrifuge technique" and "differential method" for the experiments referred to here as "difference sedimentation." See the section on terminology for our attempt to justify this change in nomenclature.

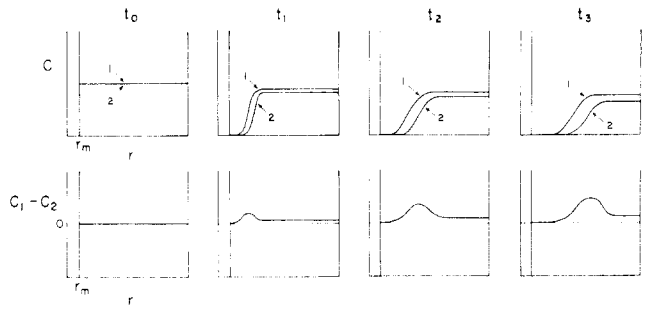

FIGURE 1: Concentration distributions for difference sedimentation. The upper series of drawings shows the integral concentration, c, *vs.* radial distance, r, distributions at different times, t, for solutions in the two sectors of a double-sector ultracentrifuge cell, each filled to the same meniscus level, r_m, and both at the same initial concentration. Solute molecules in solution labeled 2 sediment more rapidly than those in solution labeled 1. The bottom series of drawings shows the difference concentration, $c_1 - c_2$, *vs.* distance patterns at the corresponding times as measured by the interferometer.

of boundaries or concentration gradients the Rayleigh pattern consists of a series of parallel, straight interference fringes. In sedimentation velocity experiments these fringes would be straight only if the boundaries migrate and spread at the same rate so that the difference in refractive index is zero at all conjugate levels through the cell. When one boundary moves more rapidly than the other the difference in concentration varies with radial position and the fringe pattern in the boundary region is warped to produce curved fringes whose shape resembles the patterns observed with the schlieren optical system (Richards and Schachman, 1957, 1959).

Figure 1 illustrates the basic principle of the difference sedimentation technique. Assume that the two compartments of the double sector cell are filled to the same level, r_m, and that the concentrations of protein, for example, are identical in the two solutions. Initially the concentration *vs.* distance profiles for the two solutions are identical as shown in the pattern for t_0 and the difference, represented as $c_1 - c_2$, is zero throughout the cell. If the macromolecules in compartment 2 migrate more rapidly than those in compartment 1 the boundaries would separate progressively as shown for different times, t_1, t_2, and t_3. The corresponding difference patterns, shown in Figure 1, resemble bell-shaped curves. If the two boundaries have the shape of integral Gaussian curves and they migrate at almost equal sedimentation rates then the difference pattern would appear Gaussian as well. For the situation portrayed in Figure 1 the sedimentation coefficients for the two solutions are assumed to be significantly different; hence the radial dilutions in the two plateau regions differ and this is seen in the difference pattern as a progressive elevation of the "base line" ahead of the difference boundary. Had the sedimentation rates of the two solutions been reversed the difference pattern would curve the other way (toward negative values). Thus the curvature of the difference pattern (or fringes) shows directly which solution contains the more rapidly sedimenting molecules. In addition the area (more properly the first moment of the difference concentration) under the curve gives directly the difference between the two sedimentation coefficients.

For the hypothetical experiment illustrated by Figure 1, we assumed that the two compartments of the cell were filled to the same level. Under these circumstances identical solutions would give identical concentration distributions and zero difference in concentration throughout the experiment. If, with identical solutions in the two compartments, the menisci are not superimposed there will be a non-zero difference concentration distribution as the two boundaries traverse the cell; however, as shown by Richards and Schachman (1959) the first moment of this distribution will not change during sedimentation. To test the difference method they proposed that identical solutions be used with a slight displacement of the menisci so that the first moment of the resulting difference concentration pattern could be measured and shown to be constant during the course of the experiment. When the two solutions contain macromolecules varying slightly in sedimentation coefficient and the radial positions of the menisci differ, a difference boundary will be observed initially (unlike that in Figure 1) and the first moment of the difference concentration will vary during sedimentation.

Since the two samples are examined in the ultracentrifuge simultaneously, comparisons of relatively similar solutions are not affected by the usual sources of experimental error stemming from variations in rotor temperature and speed. This same rationale is employed in sedimentation velocity experiments with the schlieren optical system when a normal and wedged-window cell are used simultaneously to produce two schlieren patterns. This latter technique leads to an increase in the accuracy of measuring small changes in sedimentation coefficient (Gerhart and Schachman, 1968; Schumaker and Adams, 1968; Warner *et al.*, 1970; Warner and Schumaker, 1970a,b). With the schlieren method differences in sedimentation coefficient of 0.3–0.5% can be detected. However, for many purposes this level of precision is still inadequate since it is often necessary not only to detect such differences but to measure them quantitatively and accurately. With the difference sedimentation technique based on interference optics, as described here, it is possible to measure directly changes in sedimentation coefficient as small as 0.2% with an accuracy of ±5%. As shown in the following paper (Kirschner and Schachman, 1971) the difference sedimentation method can be used to obtain a conformational titration curve for the catalytic subunit of ATCase when the total change in the sedimentation coefficient is only 0.06 S.

As in most difference methods the technique is not devoid of "noise." Accordingly we have considered problems arising from misalignment of the optical system, optical artifacts resulting from the high concentration gradients produced by the sedimenting macromolecules, and deficiencies in the construction of the double-sector ultracentrifuge cells. Remedies for circumventing these difficulties have been devised and the difference sedimentation technique was tested by measuring the difference in sedimentation coefficient of bushy stunt virus (BSV) in buffer in one compartment *vs.* BSV in the same buffer containing D_2O in the other compartment. D_2O causes a decrease in the sedimentation coefficient through its contribution to the viscosity and density of the solution. The results obtained with the wedged-window method and schlieren optics at high D_2O concentrations and at zero D_2O concentration were interpolated to give values for comparison to experimental results obtained with the difference sedimentation method based on interference optics.

Theory

As shown by Richards and Schachman (1959) the difference in sedimentation coefficients can be related to parameters which are readily evaluated from the interference patterns.

The sedimentation process for each solution is treated in terms of the familiar transport equation (Baldwin, 1953; Schachman, 1959; Fujita, 1962) which is a statement of the conservation of mass. In eq 1 the first term expresses the

$$\frac{c_0}{2}(r_p^2 - r_m^2) - \int_{r_m}^{r_p} crdr = \frac{c_0 r_p^2}{2}(1 - e^{-2\omega^2 st}) \quad (1)$$

total mass of solute present initially at concentration, c_0, between the surface at the meniscus, r_m, and the plateau, r_p; the second term describes the mass between the two surfaces after sedimentation for a time, t; and the term on the right represents the total mass of solute transported across the surface in the plateau region at r_p during the time, t. In this formulation ω is the angular velocity and s is the sedimentation coefficient. For polydisperse or interacting systems, s is the weight-average sedimentation coefficient corresponding to the concentration in the plateau region. No allowance is made in the treatment presented here for the slight changes which may occur in s due to radial dilution during the experiment.

Similar equations can be written for the transport of macromolecules in each of the two sectors and subtraction can be performed after designating the appropriate terms by the subscripts, 1 and 2, for the different solutions. A common plateau level, r_p, is selected for both compartments of the double-sector cell and it is assumed that the initial concentrations of the solutions are identical and that at time, t, there is no material between r_{m_1} and r_{m_2}. After performing the subtraction, rearranging terms, and introducing Δc for $c_2 - c_1$, Δs for $s_2 - s_1$, $\bar{s}$ for $(s_1 + s_2)/2$, Δr_m for $r_{m_2} - r_{m_1}$, and $\bar{r}_m$ for $(r_{m_1} + r_{m_2})/2$, we obtain

$$\int_{\bar{r}_m}^{r_p} \Delta crdr + \bar{r}_m \Delta r_m c_0 = \frac{c_0 r_p^2}{2} e^{-2\omega^2 \bar{s} t}(e^{\Delta s \omega^2 t} - e^{-\Delta s \omega^2 t}) \quad (2)$$

This equation can be simplified further by the substitutions of $(\bar{r}_m/\bar{r})^2$ for $e^{-2\omega^2 \bar{s} t}$ and $(1/\bar{s}) \ln (\bar{r}/\bar{r}_m)$ for $\omega^2 t$. In this way we obtain

$$\int_{\bar{r}_m}^{r_p} \Delta crdr + \bar{r}_m \Delta r_m c_0 = \frac{c_0 r_p^2}{2}\left(\frac{\bar{r}_m}{\bar{r}}\right)^2 [e^{(\Delta s/\bar{s}) \ln (\bar{r}/\bar{r}_m)} - e^{-(\Delta s/\bar{s}) \ln (\bar{r}/\bar{r}_m)}] \quad (3)$$

where $\bar{r}$ is the average equivalent boundary position which for a small separation of symmetrical boundaries corresponds to the maximum ordinate of the difference pattern. In general, the exponent, $(\Delta s/\bar{s}) \ln (\bar{r}/\bar{r}_m)$, will be small and we can make the approximation that $e^z - e^{-z}$ is equal to $2z$ (even for $\Delta s/\bar{s}$ of 5% the error stemming from the neglect of higher terms in the expansion is negligible). Hence eq 3 can be rearranged to give

$$\frac{\int_{\bar{r}_m}^{r_p} \Delta crdr}{\bar{r}_m^2 c_0} = \left[\left(\frac{r_p}{\bar{r}}\right)^2 \ln \frac{\bar{r}}{\bar{r}_m}\right]\frac{\Delta s}{\bar{s}} - \frac{\Delta r_m}{\bar{r}_m} \quad (4)$$

Equation 4 shows that the fractional difference in the sedimentation coefficients for the two solutions, $\Delta s/\bar{s}$, is obtained directly as the slope of a plot of

$$(1/\bar{r}_m^2 c_0)\int_{\bar{r}_m}^{r_p} \Delta crdr \text{ vs. } (r_p/\bar{r})^2 \ln (\bar{r}/\bar{r}_m)$$

It is not necessary either to have the two compartments filled to the same level or to know the separation of the menisci, Δr_m, since this value affects only the intercept in the plot and not the slope. All the values needed for implementation of eq 4 are obtained readily.[3] The integration required for the evaluation of the first moment of the difference concentration is performed to an arbitrary level, r_p, in the plateau region common to both solutions.

In the above derivation which is very similar to that of Richards and Schachman (1959) we assumed that the concentrations of the two solutions were identical and that the sedimentation coefficients were independent of concentration. A concentration difference between the two solutions causes the fringes in the plateau region of the difference pattern to be displaced relative to those in the supernatant region. The required modification of the treatment is given later for those experiments in which there is a small difference in the concentrations of the two solutions.

Experimental Section

Materials

BSV was a gift kindly provided by C. A. Knight. Its sedimentation coefficient was 132 S and the partial specific volume was 0.712 ml/g (Cheng, 1953). ATCase from *Escherichia coli* was purified by the method of Gerhart and Holoubek (1967). The catalytic subunit of ATCase was prepared by dissociation of the enzyme by *p*-hydroxymercuribenzoate followed by separation of the subunits on DEAE-Sephadex according to the procedure of Gerhart and Holoubek (1967). Some of the studies on ATCase were conducted by V. Pigiet. GPDH from C. P. Boehringer was studied by G. D. Smith. The purified antibody, IgG, the hapten, arsanilic acid, and the analog, sulfanilic acid, were kindly supplied by M. E. Koshland. Bovine carboxypeptidase A was obtained from Worthington Biochemicals. Lysozyme from egg whites and D_2O, 99.8% pure, were obtained from Bio-Rad Laboratories. The latter had a specific gravity of 1.107 at 25°.

Methods

Sedimentation velocity experiments were performed with a Beckman-Spinco Model E ultracentrifuge equipped with a Rayleigh interference optical system (Svensson, 1950; Richards and Schachman, 1959). The optical system was aligned according to the procedure of Richards *et al.* (1971b) and the camera lens was focused at the two-thirds level in the cell assembled with sapphire windows. In all experiments the Rayleigh mask or beam splitter was placed over the collimating lens (Yphantis, 1960; Bowers and Haschemeyer, 1969; Rosenthal, 1969; Kirschner, 1971; Richards *et al.*, 1971a) rather than on the condensing lens mount as in customary practice.[4] For some of the experiments the Rayleigh mask having adjustable slit widths and separations was employed (Rosenthal, 1969; Richards *et al.*, 1971a).

[3] Neither the time of sedimentation nor the angular velocity are required for the determination of $\Delta s/s$. For measurement of s, of course these quantities must be known. It should be noted that c_0 must be determined independently with a synthetic boundary cell or in a conventional sedimentation velocity experiment.

[4] There is a hazard in mounting the Rayleigh mask on the collimating lens holder since there is a risk that the crucial optical component would lose its proper orientation after opening and closing the the vacuum chamber. Experience has shown, however, that in routine use, the chamber, after being closed and evacuated, apparently is seated reproducibly, hence the mask adjustment is not disturbed as a result of the movement of the chamber.

Although this proved superior to the commercial mask assembly on the condensing lens, we found it necessary to design a mask assembly which could be aligned both laterally and rotationally to very great precision and which could be positioned vertically as close to the cell as possible without endangering the movement of the rotor. The description of this mask is presented elsewhere (Kirschner, 1971; Richards *et al.*, 1971a).

The Rayleigh mask was aligned according to the procedure of Richards and Schachman (1959) with a modified, wide single-sector centerpiece made by removing the dividing rib from a conventional double-sector centerpiece (Yphantis, 1960). Preliminary alignment was performed with a lysozyme solution (20 mg/ml) and the rotor operating at 20,000 rpm. Under these conditions large refractive index gradients were produced at the top and bottom of the liquid column and the mask was adjusted to give straight interference fringes. Then the mask alignment was checked, and modified if necessary, in an experiment with BSV, 5 mg/ml, in which the speed was varied so that ample time elapsed for the boundary to spread by diffusion. The migration of the boundary through the cell was followed and appropriate adjustments of the mask were made so that the fringes were straight in the region of the large refractive index gradients. Since the sedimentation of BSV produces very large refractive index gradients the adjustment procedure was exceedingly sensitive and misalignment of the Rayleigh mask of only 10 μ or 0.05° was readily detected. In all adjustments of the mask, efforts were made to have the central fringes suffer no deviation in the region corresponding to the boundary. Fringes on either side of the zero-order fringe bowed slightly (Kirschner, 1971; Richards *et al.*, 1971a).

In the preparation of solutions for each difference sedimentation experiment, special efforts were made to obtain solutions of equal concentration. Hamilton syringes fitted with a Cheney adapter (The Hamilton Co., Inc., Whittier, Calif.) were employed for delivering liquids. In this way concentrations were matched to within less than 0.1%. Solutions of different concentrations of D_2O and H_2O were made by weight and additivity of volumes was assumed. The volume concentration of D_2O was calculated from the known densities of D_2O and H_2O.

Ultracentrifuge cells for difference sedimentation experiments contained double-sector, unfilled epoxy centerpieces and sapphire windows. Such centerpieces proved advantageous for these experiments because the two sectors have essentially the same shape and almost equal volumes. Centerpieces made from epoxy resin filled with either aluminum or charcoal were found to be less satisfactory probably because of uneven shrinkage during the molding process. (Special thanks are due Alfred Stern for molding and machining the special centerpieces.) The lower window holder had slits of 1 mm width and the upper window holder had 4-mm openings so that all four radial walls were clearly visible from above. With this cell assembly it was possible to orient the cells in the rotor with the microscope aligning tool (Spinco part 332308) so that the centerpiece walls were positioned along radii from the center of the rotor. Single-sector cells were employed for experiments with schlieren optics; one cell contained plane quartz windows and the other was assembled with an upper 1° wedged window.

The two compartments of the double-sector cells were filled so that there was a meniscus separation of 0.1 ± 0.05 mm. This separation with solutions of concentration about 5 mg/ml produced a convenient area (1 mm² on the photographic plate) for the difference pattern obtained with identical solutions. If the menisci are not separated sufficiently the difference concentration distribution has a small first moment which is difficult to measure accurately. Too large a separation of the menisci leads to a large area under the difference boundary with a consequent loss in sensitivity in measuring accurately the change in area produced by solutions which differ slightly in sedimentation coefficient. Since the volumes of the compartments in the double-sector cells differed slightly, each cell assembly prior to routine use was calibrated to obtain the menisci positions in terms of the amounts of solutions. This information and the use of Hamilton syringes permitted the filling of the cells readily with the desired separation of menisci.

Only with BSV was there any difficulty in resolving the fringe pattern. BSV solutions at 5 mg/ml produce such sharp boundaries that some of the deviated light is intercepted by lens holders on the optical track with the result that the boundaries initially did not produce resolvable difference patterns. This difficulty was circumvented by a sequence of speed changes which involved 17,000 rpm until the boundaries moved from the meniscus, 8000 rpm for 40 min to permit spreading of the boundaries by diffusion, and finally 17,000 rpm for the migration of the virus particles throughout the cell. With proteins like the catalytic subunit of ATCase speeds of 60,000 rpm were employed. Difference patterns of high quality were obtained even at 68,000 rpm in experiments on carboxypeptidase A. These sedimentation experiments were performed with a titanium rotor.

All ultracentrifuge experiments were performed at temperatures near 20°. Monochromatic light was produced with a Wratten 77A filter. Spectroscopic IIG plates were used to photograph the fringe patterns and metallographic plates were employed for the schlieren patterns.

Initial concentrations, c_0, in fringes were obtained in separate low-speed ultracentrifuge experiments with a double-sector synthetic boundary cell. Alternatively conventional sedimentation velocity experiments were conducted and the fringes across the boundaries were measured and then corrected for radial dilution to give the initial concentration (Richards and Schachman, 1959).

Calculation of $\Delta s/s$. A Nikon Model 6C microcomparator was used for measuring the x and y coordinates of the difference pattern. Each frame on the photographic plate was aligned so that the fringes corresponding to the images of the inner and outer reference holes in the counterbalance cell were parallel to the x motion of the microcomparator. A "white-light" pattern on each side was used to locate the zero-order fringe in the patterns obtained with monochromatic light; measurements were made of the x and y coordinates of the zero-order fringe. The first moment of the difference concentration distribution was calculated by summing the ordinates, Δc, measured at equal increments of the square of the radial distance from the axis of rotation (Richards, 1960). The rationale for this summation stems from the replacement of $\int \Delta c r dr$ by $\int \Delta c d(r^2/2)$
Accordingly we can write

$$\int_{\bar{r}_m}^{r_p} \Delta c r dr = \int_{\bar{r}_m^2}^{r_p^2} \Delta c d(r^2/2) \simeq \sum_{i=1}^{n} \Delta c_i \Delta(r_i^2/2) \qquad (5)$$

which for equal increments of Δr_i^2 becomes

$$\int_{\bar{r}_m}^{r_p} \Delta c r dr \simeq \Delta(r^2/2) \sum_{i=1}^{n} \Delta c_i \qquad (6)$$

Thus the first moment of the difference pattern is equal to the sum of the ordinates (Δc_i) measured at each increment of the square of the radial position multiplied by the magnitude of the increments, $\Delta(r^2/2)$.

Ordinates were measured at increments of $\Delta(r^2)$ of 15 mm^2 corresponding to intervals of 0.125 mm near the meniscus (r = 60 mm), 0.115 mm at the center of the cell (r = 65 mm), and 0.107 mm at the cell bottom (r = 70 mm). A program was written for the Olivetti Programma 101 to give a table of r positions as measured on the photographic plate in terms of distance from the reference wire; these positions corresponded to equal increments of the square of the radial distance from the axis of rotation. The fringe heights (for the zero order fringe) were measured over a range from 1 mm on the centripetal side of the difference boundary to a level 1 mm into the plateau region. Ordinates were summed by a trapezoidal summation.

A sample calculation of the first moment of a difference concentration distribution is given in Table I. Column 1 lists the radial positions as measured on the comparator in mm from the reference wire in the counterbalance cell. These values represent equally spaced increments of the square of the distance from the axis of rotation. In column 2 are listed the fringe heights corresponding to each position. The fringe heights are given directly in micrometer readings on the y axis. Column 3 gives y_0, the average ordinates for the supernatant and plateau regions. Subtracting the value of y_0 for the supernatant from the readings in column 2 gives the absolute value of the fringe height as shown in column 4. The interval numbers for the summation are given in column 5.

As shown in Table I the fringes in the plateau region were displaced relative to those in the supernatant. Some slight displacement is to be expected if there is a small difference in sedimentation coefficients; under these circumstances there would be differing extents of radial dilution of the two solutions and the concentrations in the plateau regions would differ slightly. This effect is illustrated schematically in Figure 1. For this situation the first moment of the difference pattern is evaluated by summation of the fringe displacements above (or below) a base line obtained in a separate experiment with buffer (or H_2O) in both compartments. Such base lines generally exhibit only slight, gradual vertical deviations as a function of radial position (less than 0.05 fringe across the width of an average difference boundary) and they are more or less parallel to the lines drawn from the fringes representing the reference holes in the counterbalance cell. However, in difference sedimentation experiments the fringe displacement across the boundary (from supernatant to plateau) is frequently many times greater in magnitude and even opposite in direction to that which is expected theoretically. When this occurs it is no longer appropriate to employ a base line obtained from an experiment with buffer in both compartments. Hence a base-line correction was devised to circumvent the difficulty arising from the fringe displacement. The various factors which contribute to this displacement are considered in a later section where it is shown that the following procedure accounts satisfactorily for the different effects.

It is assumed that corrections for the fringe displacement vary in a linear manner across the difference boundary. Instead of attempting to calculate the sum of each ordinate minus the corresponding base-line value (with buffer in each

TABLE I: Determination of the First Moment of a Difference Concentration Distribution.[a]

r[b] (mm) (1)	y[c] (mm) (2)	y_0[d] (mm) (3)	$(y - y_0)$[e] (mm) (4)	Interval No. (5)
13.762	4.791			
14.016	4.793			
14.269	4.793			
14.522	4.792			
14.774	4.792	4.792	0.000	1
15.026	4.773		−0.019	2
15.277	4.726		−0.066	3
15.528	4.626		−0.016	4
15.778	4.468		−0.324	5
16.028	4.308		−0.484	6
16.243[f]	4.325		−0.557	6.9
16.277	4.242		−0.550	7
16.526	4.362		−0.430	8
16.775	4.521		−0.271	9
17.023	4.681		−0.111	10
17.271	4.784		−0.008	11
17.518	4.859		+0.067	12
17.765	4.881		+0.089	13
18.011	4.894	4.894	−0.102	14
18.257	4.894			
18.502	4.896			
18.747	4.894			

Meniscus position ($\bar{r}_m$) 7.645 mm
Maximum ordinate position ($\bar{r}$) 16.243 mm
Position in plateau region 18.011 mm

The trapezoidal sum of the ordinates, $\sum_{i=1}^{n}\Delta c_i$, is 2.229 mm.

$$\int_{r_m}^{r_p} \Delta c r dr \simeq \Delta\left(\frac{r^2}{2}\right)\sum_{i=1}^{n}\Delta(c_i - c_i, \text{ base line})$$

$$\simeq \Delta\left(\frac{r^2}{2}\right)\left[\sum_{i=1}^{n}\Delta c_i - \frac{n}{2}\Delta c_p\right]$$

$$\simeq \frac{15}{2}\left[2.229 - \frac{14}{2}(-0.102)\right]$$

$$\simeq 22.07 \text{ mm}^3$$

[a] Both compartments of a double-sector cell were filled with BSV at a concentration of 5 mg/ml in 0.1 M potassium phosphate buffer, pH 6.80. One compartment contained 3 ml/ml of D_2O. [b] The radial position in mm is measured from the reference wire. The interval between each position is at equal increments of the square of the radial distance (15 mm^2). [c] The y ordinate represents the vertical position of the zero-order fringe as read directly on the microcomparator in mm. [d] y_0 is the average ordinate in millimeters for the supernatant and plateau regions. The readings at low r values correspond to the supernatant and those at high values of r represent the plateau region. [e] $y - y_0$, where y_0 represents the reading in the supernatant; the absolute value of $y - y_0$ provides a direct measure of Δc_i in millimeters of fringe displacement. [f] This position corresponds to the maximum ordinate of the difference pattern.

TABLE II: Calculation of $(\Delta s/s)$.[a]

Pattern (1)	$\Sigma\Delta c_i$[b] (2)	$\int_{\bar{r}_m}^{r_p}\Delta c r dr / c_0\bar{r}_m^2 \times 10^3$ (3)	r_p[c] (4)	$\bar{r}$[c] (5)	$\left(\frac{r_p}{\bar{r}}\right)^2 \ln \frac{\bar{r}}{\bar{r}_m} \times 10^2$ (6)
1	2.09	0.945	13.253	11.517	3.08
2	2.54	1.149	15.778	14.837	4.93
3	2.94	1.330	18.011	16.243	6.76
4	3.43	1.551	20.691	18.637	8.59
5	4.04	1.827	23.084	21.235	10.37

Slope $= 1.14 \times 10^{-2}$
$\Delta s/s = 0.0114$

Initial concentration (c_0) (mm) 18.02 fringes or 4.685
Average meniscus position ($\bar{r}_m$)[c] (mm) 7.624
Interval $-\Delta(r^2/2)$ (mm^2) 7.5
$(\Delta r^2/2/c_0\bar{r}_m^2)$ (mm^{-1}) 4.708×10^{-4}

[a] Data was obtained from Table I and similar determinations of the difference pattern at different times during centrifugation. [b] This sum includes the base-line correction (see Table V). [c] All readings represent distances, as read on the microcomparator, from the reference wire.

compartment), we write

$$\sum_{i=1}^{n}\Delta(c_i - c_i, \text{base line}) = \sum_{i=1}^{n}\Delta c_i - \frac{n}{2}\Delta c_p \qquad (7)$$

where Δc_p represents the displacement of the plateau fringe relative to the fringe in the supernatant and n is the number of intervals. The correction calculated in this manner is illustrated at the bottom of Table I along with the result of the trapezoidal summation of the ordinates, $\Sigma\Delta c_i$, from $i = 1$ to $i = n$, relative to the height of the fringe in the supernatant region. The magnitude of this fringe shift is usually about 0.1 fringe, where the total height of the difference boundary is 2–3 fringes. The shift shown in Table I is unusually large amounting to about 0.3 fringe.

In the calculation of the first moment we have used the y ordinate as a measure of the difference in concentrations between the two solutions at the various conjugate levels. These values of the y ordinates include parameters such as geometrical terms and the magnification factor of the cylindrical lens. However, c_0, also in millimeters of fringe displacement, appears in the denominator of eq 4; hence there is no need to evaluate the various optical constants.

As shown in eq 4 the fractional difference in sedimentation coefficients, $\Delta s/s$, is determined from the first moments of the difference concentration distribution at different times plus knowledge of the corresponding positions of the maximum ordinates of the difference patterns, the average of the menisci positions, and the initial concentration. Although the initial concentration is usually expressed as the number of fringes (including fractional fringes), it is readily converted into millimeters of fringe displacement simply by multiplying the number of fringes by the fringe separation. The fringe separation is a characteristic of the optical system and is determined from measurements in the supernatant region (or plateau) or in an experiment with H_2O in both compartments of the cell. Table II illustrates the calculation of $\Delta s/s$. The sum of the corrected ordinates for each pattern (column 2) is multiplied by $(1/c_0\bar{r}_m^2)\Delta(r^2/2)$ to give values of $(1/c_0\bar{r}_m^2)\int\Delta c r dr$, from $\bar{r}_m$ to r_p, shown in column 3. In column 4 are listed the positions, r_p (in millimeters from the reference wire), in the plateau region at which the summation was terminated. The values of the maximum ordinate of the difference pattern, $\bar{r}$, are given in column 5. From the values of r_p, $\bar{r}$, and the average of the menisci positions, $\bar{r}_m$, the appropriate values of $(r_p/\bar{r})^2 \ln (\bar{r}/\bar{r}_m)$ for each pattern are evaluated and listed in column 6. When the values in column 3 are plotted *vs.* those in column 6 a straight line is obtained with a slope, $\Delta s/s$, equal to 0.0114. In a later section this experimental value determined by the difference sedimentation technique is compared with that expected from other measurements with schlieren optics.

Pitfalls and Remedies

Since the introduction of the difference sedimentation technique (Richards and Schachman, 1959) there have been substantial improvements in ultracentrifuge cell centerpieces and windows, and sensitive tests have been devised for the alignment of the Rayleigh interference optical system. Despite these advances there are three types of problems encountered in difference sedimentation experiments. One stems from fringe displacements arising from differences between the two compartments of the double-sector cells such as unequal optical paths or imperfections in the walls of the sectors. A second arises from the difficulty in aligning the Rayleigh mask so that the optical system compares conjugate radial positions in the two solutions. Finally, in the region of large concentration gradients the deviation of light causes complications which affect the fringe spacing in the interference patterns.

Fringe Displacement. In all difference patterns there was a displacement of the fringes corresponding to the supernatant region compared to those representing the plateau region. This fringe shift, which was also observed by Richards (1960), could be as great as 0.3 fringe with some cells. If identical solutions are placed in each compartment of a double-sector cell and the volumes are unequal so that the menisci are at different levels, a difference pattern is produced during sedimentation. Since the concentrations and the sedimentation coefficients are the same the concentration difference should be zero in both the supernatant and plateau regions. Thus the fringe in the plateau region, after correction for any base-line distortions with buffer or H_2O in both compartments, should be at the same level as that for the fringe in the supernatant. Frequently, however, there was a displacement of the fringes even for experiments with BSV where the centrifugal field is relatively low and pressure effects are likely to be small. A similar anomaly was observed with solutions of macromolecules having slightly different sedimentation coefficients. For such systems, if the initial concentrations are equal, there will be a slight difference in the concentrations in the plateau regions due to variations in the amount of radial dilution. However, the observed displacement of the fringes on either side of the difference boundary was often significantly larger than that expected from the unequal dilutions accompanying the migration of the macromolecules.

In general the potential sources of a fringe displacement

in difference patterns are: (1) unequal initial concentrations for the two solutions; (2) unequal radial dilution of the two solutions caused by differences in sedimentation coefficients; (3) unequal dilution of the solutions due to convective disturbances resulting from nonsector-shaped compartments in the ultracentrifuge cell; and (4) unequal optical paths for the light traversing the two solutions at conjugate levels. These effects are discussed in turn.

UNEQUAL INITIAL CONCENTRATIONS. As already indicated, if the initial concentrations of the solutions are not equal, but the solutions are similar in other respects, there will be a concentration difference in the plateau region but not in the supernatant (where the concentration is zero, of course, for both solutions). This difference is revealed as a displacement of the fringe on the two sides of the difference boundary. Moreover, this displacement would decrease during an experiment as the concentration of the two solutions and hence the concentration difference decreased due to radial dilution. Since for many experiments displacements were found even when aliquots of the same solution were placed in the two compartments of the cell, we can conclude that a variation in the initial concentrations is not the sole cause for the fringe displacement. We must, therefore, look elsewhere for a satisfactory explanation of the displacement.

UNEQUAL RADIAL DILUTION DUE TO SEDIMENTATION. When the molecules in the two solutions differ in sedimentation coefficient there must be unequal radial dilution in the two compartments and a consequent fringe displacement. The magnitude of this fringe displacement will be larger the greater the difference in sedimentation coefficients and it is obviously invalid to assume that the fringe displacement across the boundary is zero and to draw an approximate baseline from the supernatant to the plateau region. However, it can be shown that despite this approximation the percentage error in the determination of $\Delta s/s$ is independent of the actual difference between the two sedimentation coefficients. Moreover, the error introduced in this manner depends only on the width of the difference boundary and is rarely as large as 10%. The reasoning which forms the basis for this conclusion is presented in the Appendix.

UNEQUAL DILUTION DUE TO CONVECTION. As shown in the following section, when identical solutions with offset menisci are placed in a double-sector cell a fringe displacement develops as soon as the difference boundary moves away from the menisci. This displacement seldom varied in amount as the solute molecules sedimented. If one of the two compartments had a nonsector shape or if the walls had imperfections some molecules would strike the walls leading to local accumulations of solute and convective flow. This process would lead to dilution of the contents beyond that expected from the radial dilution expression. A concentration difference between the two solutions would develop and a fringe displacement in the plateau region would result. But this displacement would increase progressively during the experiment. Only rarely was this effect observed and the few centerpieces which produced this type of fringe displacement were discarded.

UNEQUAL OPTICAL PATHS. Since fringe displacements were often found with identical solutions, it seemed likely that they arose from small differences in optical path length, Δa, caused either by imperfectly constructed centerpieces or by uneven compression of the centerpieces when the cell is assembled and tightened. Support for this hypothesis came from the observation that the displacement of the fringe varied with the centerpiece, the cell housing, and the window holders. If the optical paths between the two compartments are unequal, then the magnitude of the total refractive index difference between identical solutions in the two compartments will depend on the refractive indices of the solutions. Since the refractive index of the solution in the plateau region is greater than that in the supernatant region, a small difference in the total path length for light traversing the two sectors will lead to significantly larger difference in refractive index for light rays passing through the plateau region as compared to light through the supernatant region; hence fringes corresponding to the plateau region will be displaced relative to those representing the supernatant region. The base line, produced by the interferometer and representing the difference in refractive index when the same material is in both compartments of the cell, therefore will vary with concentration if the optical path length through one sector is larger than the other.

For a cell containing liquid columns of unequal height (optical path) the effective difference in the total optical path through the cell with buffer in the two compartments, Δl_B, is equal to the difference in path lengths, Δa, multiplied by the refractive index of the buffer, n_B, plus any difference in the optical paths through the windows, $\Delta(tn)_{\text{windows}}$ where t and n refer to the thickness and refractive index of the windows. Thus we can write

$$\Delta l_B = n_B \Delta a + \Delta(tn)_{\text{windows}} \tag{8}$$

In an analogous way we can write an equation for the effective difference in the optical paths for light rays passing through the solution, Δl_s. The refractive index of the solution, n_s,

$$\Delta l_s = n_s \Delta a + \Delta(tn)_{\text{windows}} \tag{9}$$

is readily expressed as $n_B + (dn/dc)c$, where dn/dc is the specific refractive increment and c is the solute concentration. If we assume that any heterogeneity in the windows (either in terms of thickness or actual refractive index) at conjugate levels is the same at all levels, *i.e.*, is independent of radial position, then incorporation of the value of n_s in eq 9 followed by subtraction of eq 8 gives

$$\Delta l_s - \Delta l_B = \Delta a \left(\frac{dn}{dc}\right) c \tag{10}$$

The fringe displacement across the boundary is thus proportional to the concentration of solute. If the cells are perfect, *i.e.*, $\Delta a = 0$, there would be no fringe displacement. For protein solutions at a concentration of 5 mg/ml, a fringe displacement of 0.1 fringe would be observed in a 12-mm cell if the two liquid columns differed by 0.06 mm.

Since some cells produced substantial fringe displacements even with identical solutions and since the calculation presented above illustrates that extremely small differences in the path length could produce a sizeable fringe shift we examined the positions of the zero-order fringe throughout an experiment with identical solutions of BSV in the two compartments. The menisci were displaced slightly and the vertical coordinates of the white-light fringe were measured relative to the reference white-light fringe corresponding to the air space above the liquid columns. The results of these measurements for various radial positions (corresponding to supernatant, boundary, and plateau regions) and for different times of sedimentation are given in Table

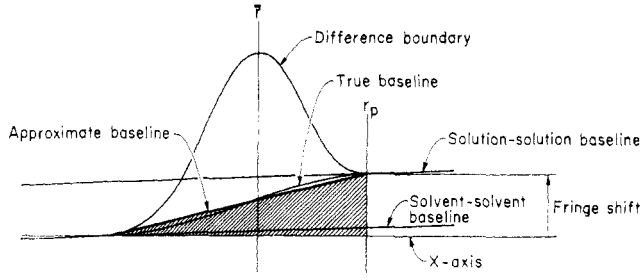

FIGURE 2: Base-line correction for the shift of the plateau fringes relative to the supernatant fringes for a symmetrical difference boundary with a maximum ordinate at a radial position of $\bar{r}$ and width $2(r_p - \bar{r})$, where r_p is at a level just into the plateau region. The x axis represents the x direction on the microcomparator. The solvent–solvent base line is shown sloped relative to the x axis. The approximate and true base lines are discussed in the text.

TABLE III: Displacement of Fringe across Difference Concentration Boundary.[a]

Radial Position[d] (r)	Displacement of Zero-Order Fringe above Ref Fringe[b,c]			
	A[e]	B[e]	C[e]	D[e]
15.5		0.088	0.088	0.091
19.0	0.128		0.093	0.090
24.0	0.137		0.095	0.092
27.0	0.140	0.136		
30.0	0.133	0.138	0.141	

Average fringe height (solution *vs.* solution), 0.135 mm
Average fringe height (solvent *vs.* solvent), 0.091 mm
Fringe displacement across boundary, 0.044 mm

[a] Identical solutions of BSV (5 mg/ml in 0.1 M phosphate buffer) were placed in double-sector centrifuge cell, so that menisci were staggered, and sedimented at 17,000 rpm. A base-line photograph was taken as soon as the centrifuge reached speed. Achromatic fringes were photographed with an optically flat glass serving as a white-light filter. [b] Fringe heights in millimeters corrected for base line. [c] Measurements to left of dotted line correspond to supernatant region. Those to right correspond to plateau region. [d] Distance on photographic plate in millimeters from reference wire. [e] *A*, *B*, *C*, and *D* corresponds to 8-, 40-, 60-, and 84-*min* sedimentation.

III. Early in the experiment, A, before the boundary had migrated from the meniscus, the fringe corresponding to the solution region was about 0.135 mm above the reference fringe and did not vary much with radial position. After 40-min sedimentation, B, the difference boundary had moved to a level about 22 mm from the reference wire and the fringe height in the supernatant was 0.088 mm above the reference. The fringe in the plateau region remained at the same height, 0.137 mm. At still later times, 60 and 84 min (C and D), when the difference boundary had migrated farther through the cell, the fringe height in the supernatant remained at a relatively constant level about 0.091 mm above the reference. Similarly the fringe height in the plateau region was relatively constant at about 0.138 mm. These results can be summarized in another way. At a radial position 30 mm from the reference wire, the fringe heights in the plateau region were almost invariant with time (0.133, 0.138, and 0.141 mm). Similarly they were at a constant height in the supernatant region (0.088, 0.088, and 0.091 mm). However at a radial position 24.0 from the reference wire the fringe shifted from a height of 0.137 at 8 min (A) when solute molecules were present to a height of 0.095 and 0.092 mm at 60 and 84 min (C and D), respectively, after the boundary had migrated past that radial position. The average shift between supernatant and plateau was 0.044 mm. These results show conclusively that the fringe heights in the plateau region did not change with time as would be expected for a radial dilution effect. Instead both in the supernatant and plateau regions the fringe heights were independent of time. Only across the boundary was there a displacement and this shift downward (toward the reference fringe in the air space) was consistent with the lowering of the refractive index as solute molecules migrated from regions in the cell. Thus we conclude that the fringe displacement can be attributed to a slight difference in the optical paths through the two compartments of the cell.

The effect described here should be observed as well during the alignment of the optical system when the modified single-isctor centerpiece (with the center rib removed from a conven-eonal double-sector centerpiece) is used for adjusting the translational and rotational orientation of the Rayleigh mask (Richards and Schachman, 1959; Yphantis, 1964; Richards *et al.*, 1971b). If, as is often the case, this adjustment is made when the cell is filled with a concentrated protein solution and the speed of the rotor is so low as to produce large gradients at the top and bottom of the solution column the fringe displacement could not be detected. Only when the alignment test is performed with a moving boundary could the displacement be observed. Generally the test patterns for various positions of the Rayleigh mask are examined visually, rather than by the microcomparator, and any fringe displacement, if present at all, may escape detection. But it should be emphasized that the fringe displacement demonstrated by the data in Table III may not be present in the alignment experiments since they are performed with cell centerpieces which have had the center rib removed. Further experiments are required with a variety of cell assemblies to determine whether the presence of a center rib in the centerpiece leads to a distortion of the assembled cell with a consequent slight difference in the optical paths through the two compartments.

Base-Line Correction. For those cell assemblies which, with identical solutions in the two compartments, produce a fringe displacement on the two sides of the boundary a base-line correction is required. Since most of the displacement is attributable to the unequal optical paths for light traversing the two solutions, the base-line correction should have the form of an S-shaped curve (Figure 2) from the supernatant to the plateau. A correction of this type would be complicated but an approximate base line, in the form of a straight line, suffices. All of the results presented here and in the following paper (Kirschner and Schachman, 1971) were obtained with this approximate base-line correction.

The approximate base-line correction shown in Figure 2 is intended to subtract contributions to the observed fringe displacement which arise from all sources except that arising from a difference in the concentrations of solute. These displacements may be divided into two categories, those which are observed when pure solvent is in both sectors and

those which depend on the redistribution of macromolecular solutes. The former, which include inhomogeneities in cell windows and differences in cell thickness have been found either to change negligibly across the width of the difference boundary or can be considered linear across that region. The latter which have been shown to arise from differences in initial concentration or differences in path length should be proportional to the concentration difference and therefore, after subtraction of the solvent–solvent base line, should have the form of the S-shaped curve which is denoted by the term "true base line" in Figure 2. Since this base line can be approximated by a straight line, a line drawn directly from the supernatant to the plateau region will incorporate both the concentration-dependent and concentration-independent base-line approximations. However, since the menisci are purposely mismatched there can be a contribution to the fringe displacement due to unequal redistribution of buffer salts or unequal compression of the solvent. In most experiments this has not been a problem. However, very high concentrations of salt do cause curvature of the fringes making the base-line approximation difficult. In experiments with carboxypeptidase we avoided this problem by using 1 M triethylamine hydrochloride which has a partial specific volume very close to one and as a consequence there was no observable redistribution.

The approximate base line shown as a solid line in Figure 2 has been found satisfactory for all experiments. The first moment of the difference concentration is the area between the true base line and the difference boundary when summed at equal increments of the square of the radial distance. To make the approximate base-line correction one sums the height of the difference boundary along the X axis from a point just into the supernatant region to a point just into the plateau region and then subtracts the crosshatched area between the X axis and the approximate base line from the total area.

This approximate base line also is of use for experiments in which inadvertently the two solutions differ in initial concentrations. For such situations, of course, the fringes in the plateau region would be displaced relative to those in the supernatant region. In addition, eq 4 requires modification in order to account for the variations in the initial concentrations. The final equation takes the form

$$\frac{\int_{\bar{r}_m}^{r_p} \Delta c r dr - \frac{\Delta c_p}{2}(r_p^2 - \bar{r}^2)}{\bar{r}_m^2 c_0} = \left[\left(\frac{r_p}{\bar{r}}\right)^2 \ln \frac{\bar{r}}{\bar{r}_m}\right]\frac{\Delta s}{s} - \frac{\Delta r_m}{\bar{r}_m} \quad (11)$$

where the term, $(\Delta c_p/2)(r_p^2 - \bar{r}^2)$, represents the first moment in the difference pattern stemming from the difference in the initial concentrations, Δc_0. Richards and Schachman (1959) also considered this effect and their version of eq 11 contained Δc_0 rather than Δc_p where the latter represents the difference in the concentrations of the two plateau regions at any time during the experiment. If there is a slight experimental error in the preparation of the two solutions so that the concentrations are not equal the excess first moment, $(\Delta c_p/2) \times (r_p^2 - \bar{r}^2)$, can be subtracted from the first moment of the difference pattern; in this way eq 11 reduces to eq 4. Such concentration differences should be avoided by appropriate experimental procedures since all macromolecules exhibit characteristic dependences of sedimentation coefficient on concentration and interpretations of the measured values of $\Delta s/s$ would be complicated unnecessarily.

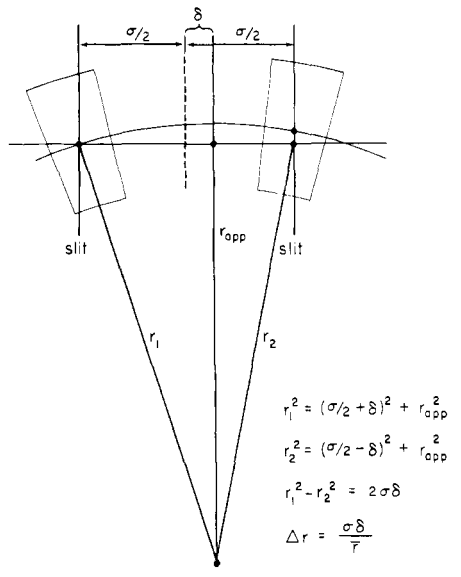

FIGURE 3: Effect of translational misalignment of the Rayleigh mask. The Rayleigh mask is aligned parallel to the axis of the cylindrical lens and a reference radius from the axis of rotation but is displaced a distance, δ, from that radius. The slit separation is σ. The common radial distance, r, gives projections r_1 for slit 1 and r_2 for slit 2 at the axis of the cylindrical lens. The difference in the projected distance, Δr, is given in terms of δ, σ, and the average projection, $\bar{r}$.

Alignment of the Rayleigh Mask. When the optical system is aligned, the Rayleigh mask and the cylindrical lens are adjusted relative to each other so that the two slits in the mask are symmetrical about and parallel to a reference radius from the axis of rotation; moreover this radius and the axis of the cylindrical lens must be parallel. If this arrangement is not achieved precisely the interference optical system compares nonconjugate levels in the two compartments in terms of their refractive indices. As a consequence, if identical solutions are placed in the two compartments of a double-sector cell with the menisci at the same level (as in the special cell which contains a communicating channel between the two compartments to allow equalization of hydrostatic pressure or in the cell with the center rib removed to produce a 7° single-sector cell) the boundaries during sedimentation, as viewed by the optical system, will appear displaced from each other. Thus a difference pattern would be observed. The first moment of the apparent concentration difference will increase or decrease during the experiment if there is a translational misalignment of the Rayleigh mask. If the mask is oriented incorrectly in a rotational sense the first moment of the apparent difference concentration will first decrease and then increase (or *vice versa*) during the migration of the solute molecules and only at one radial level will the interferometer compare truly conjugate positions.

The apparent $\Delta s/s$ produced from translational and rotational misalignment of the Rayleigh mask is calculated readily and these calculations serve to illustrate the stringency of the requirements imposed on the placement of the mask. As seen in Figure 3 a given radial position, r, in the two sectors actually appears as two distinct levels, r_1 and r_2, when the mask is translationally displaced from its correct position (symmetrical about the radius from the center of the rotor). Thus if the cell were filled to exactly the same

TABLE IV: $(\Delta s/\bar{s})_{app}$ for Translational Misalignment of the Rayleigh Mask.

Mask Displacement (μ)	$(\Delta s/\bar{s})_{app}$		
	$\bar{r}$ = 62 mm[a]	$\bar{r}$ = 68 mm[a]	Eq 14
5	1.9×10^{-4}	4.5×10^{-5}	1.1×10^{-4}
15	5.7×10^{-4}	1.4×10^{-4}	3.3×10^{-4}
40	1.5×10^{-3}	3.6×10^{-4}	8.7×10^{-4}
100	3.8×10^{-3}	9.1×10^{-4}	2.2×10^{-3}

[a] Values of $(\Delta s/\bar{s})_{app}$ were calculated from eq 12 for a slit separation of 4 mm and a meniscus position of 60 mm. In the calculations the boundary positions were taken as 62 and 68 mm, respectively.

level with identical solutions and the boundary had migrated to r, a misaligned optical system would register two boundaries at r_1 and r_2. The interferometer would give a difference pattern, of course, and an incorrect value of $\Delta s/\bar{s}$ would be calculated as if the samples were different.

For an infinitely sharp boundary at r the first moment of the concentration to a level r_p is $\int cr dr$, from r_p to r_m, or $(c_p/2)(r_p^2 - r^2)$. Similar expressions can be written for the two compartments with appropriate subscripts designating the apparent boundary positions, r_1 and r_2. In this way we obtain $(c_p/2)(r_1^2 - r_2^2)$ for the first moment of the difference concentration, $\int \Delta c r dr$, from r_p to r_m. But the term, $r_1^2 - r_2^2$, is readily shown to be equal to $2\sigma\delta$ (Figure 3), where σ is the slit separation and δ represents the displacement of the Rayleigh mask from its correct position relative to the radius which serves as the reference for the axis of the cylindrical lens. Thus for two identical, infinitely sharp boundaries starting from a common radial position, r_m, a difference boundary results if the mask is displaced laterally. A value of the apparent fractional difference in sedimentation coefficient, $(\Delta s/\bar{s})_{app}$, is readily calculated for various extents of misalignment of the mask. Substituting $c_p\sigma\delta$ in eq 4, replacing c_p/c_0 by $(r_m/\bar{r})^2$, and selecting r_p so that $r_p/\bar{r}$ is essentially unity leads to an expression for $(\Delta s/\bar{s})_{app}$

$$(\Delta s/\bar{s})_{app} = \frac{\sigma\delta}{\bar{r}^2 \ln (\bar{r}/r_m)} \tag{12}$$

Table IV shows the results of calculated values of $(\Delta s/\bar{s})_{app}$ for various values of δ (column 1). In column 2 are given the calculated values of $(\Delta s/\bar{s})_{app}$ for a radial position (62 mm) near the meniscus. Similar calculations were made for the difference boundary when it reached a level (68 mm) near the bottom of the cell; these values are tabulated in column 3. Comparison of the values in these two columns shows clearly that the effect of misalignment of the mask is less pronounced when the boundary is near the bottom of the cell. This result is due to the fact that the excess first moment decreases with radial distance since the radii make progressively smaller angles with the parallel slits as shown in Figure 3, and that this decreasing first moment is compared with a larger sedimentation time, $\ln (\bar{r}/\bar{r}_m)$, as the boundaries move in a centrifugal direction.

In the above treatment it was assumed that the menisci were at the same level and hence $(\Delta s/\bar{s})_{app}$ was calculated from a single difference pattern at a specified level in the cell. In practice $\Delta s/\bar{s}$ is obtained from the change in the first moment of the difference concentration as the solute molecules sediment. As already seen in Table II the first moment changes with position; hence we can calculate the change in the first moment of the difference concentration between a level near the top of the cell, r_t, and another near the cell bottom, r_b. In this way we evaluate $(\Delta s/\bar{s})_{app}$ without using the meniscus as one of the points. For this situation, infinitely sharp boundaries and identical solutions filled to the same level, eq 4 takes the form

$$\frac{\sigma\delta}{\bar{r}^2} = (\ln \bar{r}/\bar{r}_m)(\Delta s/\bar{s})_{app} - \left(\frac{\Delta r_{m,app}}{\bar{r}_m}\right) \tag{13}$$

where $\Delta r_{m,app}$ represents the apparent mismatching of the menisci. Rewriting eq 13 for two boundary positions and solving the two simultaneous equations for $(\Delta s/\bar{s})_{app}$ yields

$$(\Delta s/\bar{s})_{app} = \frac{\sigma\delta[(1/\bar{r}_t^2) - (1/\bar{r}_b^2)]}{\ln \bar{r}_t/\bar{r}_b} \tag{14}$$

Equation 14 can be used directly to evaluate $(\Delta s/\bar{s})_{app}$ for differing extents of misplacement of the Rayleigh mask and the results are presented in column 4 of Table IV for the boundary moving from 62 mm (r_t) to 68 mm (r_b). The calculations show that appreciable values of $(\Delta s/\bar{s})_{app}$ would be obtained for identical solutions if the mask is misplaced laterally by 40–100 μ. These sample calculations demonstrate clearly that it is mandatory to locate the mask correctly if the errors in $\Delta s/\bar{s}$ are to be avoided.

Similar considerations can be applied to estimate the effect of rotational misorientation of the Rayleigh mask since a rotation of the mask through an angle, θ, about the center of the cell leads to an apparent displacement of identical boundaries. Geometrical treatment of Figure 3 for small values of θ gives

$$r_2^2 - r_1^2 = 2\sigma \tan\theta\,(\bar{r} - r_c) \tag{15}$$

where r_c is the distance from the center of the cell to the axis of rotation (65 mm). With this result for the apparent boundary positions we obtain

$$(\Delta s/\bar{s})_{app} = \frac{(\bar{r} - r_c)\sigma \tan\theta}{\bar{r}^2 \ln \bar{r}/r_m} \tag{16}$$

Equation 16 shows that $(\Delta s/\bar{s})_{app}$ can be calculated for various degrees of misorientation of the Rayleigh mask. Sample calculations are summarized in Table V for difference boundaries at 62 mm (column 2) and 68 mm (column 3). In these calculations only a single difference pattern at a specified level in the cell was considered along with the position of the meniscus. As in the treatment for the laterally misplaced mask we can write equations for two boundary positions and combine them to solve for $(\Delta s/\bar{s})_{app}$. This procedure leads to

$$\left(\frac{\Delta s}{\bar{s}}\right)_{app} = \frac{\sigma \tan\theta\left[\frac{\bar{r}_t - r_c}{\bar{r}_t^2} - \frac{\bar{r}_b - r_c}{\bar{r}_b^2}\right]}{\ln \bar{r}_t/\bar{r}_b} \tag{17}$$

Substituting various values for θ and 62 mm for $\bar{r}_t$ and 68

TABLE V: $(\Delta s/\bar{s})_{app}$ for Rotational Misalignment of the Rayleigh Mask.

Mask Rotation (deg)	$(\Delta s/\bar{s})_{app}$		
	$\bar{r}$ = 62 mm[a]	$\bar{r}$ = 68 mm[a]	Eq 17
0.05	9.8×10^{-5}	-2.3×10^{-5}	5.1×10^{-5}
0.3	5.0×10^{-4}	-1.4×10^{-4}	3.1×10^{-4}
1	1.9×10^{-3}	-4.6×10^{-4}	1.0×10^{-3}
4	7.9×10^{-3}	-1.9×10^{-3}	4.1×10^{-3}

[a] Values of $(\Delta s/\bar{s})_{app}$ were calculated from eq 16 for a slit separation of 4 mm and a meniscus position of 60 mm. In the calculations the boundary positions were taken as 62 and 68 mm, respectively.

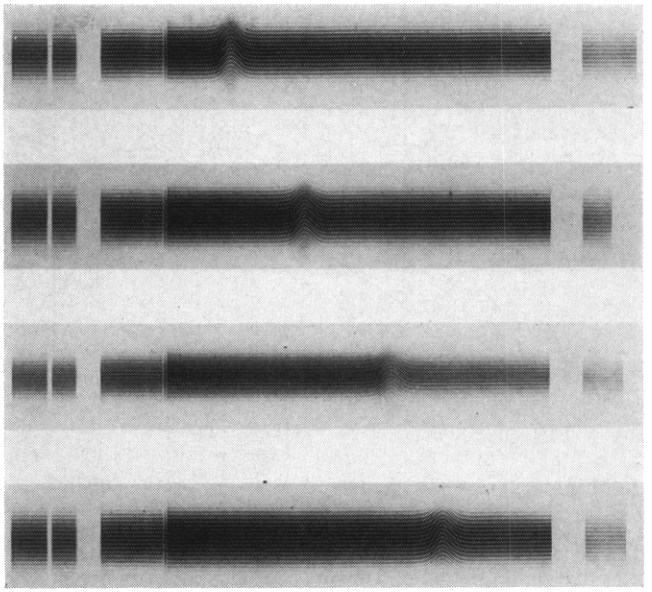

FIGURE 4: Difference sedimentation patterns for BSV. Both compartments were filled with BSV at a concentration of 5 mg/ml in 0.1 M potassium phosphate buffer at pH 6.8, and the rotor was spun at 17,000 rpm. One compartment contained 0.03 ml/ml of D_2O. The photographs were taken at 48, 64, 76, and 88 min after reaching speed. The third photograph was taken with achromatic light using flat glass in place of the Wratten 77A filter.

mm for $\bar{r}_b$ yields the values of $(\Delta s/\bar{s})_{app}$ shown in column 4 of Table V. The values listed here show that the magnitude of $(\Delta s/\bar{s})_{app}$ decreases as the distance of the difference boundary from the axis of rotation increases. It is also important to note that rotational misalignment of the Rayleigh mask causes $(\Delta s/\bar{s})_{app}$ to change sign as the difference boundary moves from a level near the meniscus (column 2) to the bottom of the cell (column 3). Only at the center of the cell (r_c = 65 mm) is $(\Delta s/\bar{s})_{app}$ equal to zero.

Tables IV and V show that misalignment of the Rayleigh mask will produce a non-zero value of $\Delta s/\bar{s}$ even when the solutions are identical. For solutions containing macromolecules differing slightly in sedimentation coefficient, the difference sedimentation technique will yield erroneous results for $\Delta s/\bar{s}$ if the mask is not aligned correctly. Errors due to this effect are systematic and corrections for them can be made but it is obviously preferable to avoid them. If the rotational adjustment of the mask is in error by 1° and the lateral positioning is incorrect by 0.04 mm a spurious $\Delta s/\bar{s}$ of 0.002 results. Thus alignment of the Rayleigh mask is critical if the full potential of the difference sedimentation technique is to be realized. With care the general utility Rayleigh mask (Rosenthal, 1969; Richards *et al.*, 1971a) can be aligned to within 0.3° and 0.015 mm. Under these circumstances there would be a systematic error in $\Delta s/\bar{s}$ of 0.0006. The precision mask (Kirschner, 1971; Richards *et al.*, 1971a) is more easily aligned since the adjustments for both rotation and translation are very sensitive and any desired positions can be reproduced at will. It can be aligned routinely to better than 0.05° and 0.005 mm. Hence, through its use systematic errors in $\Delta s/\bar{s}$ due to misalignment of the Rayleigh mask can be reduced below 0.0002.

Fringe Bowing. As shown by Richards and Schachman (1959) the fringe spacing decreases in the region of the image corresponding to large refractive index gradients. This "necking" of the fringe pattern was seen readily during alignment tests with a 7° single-sector cell and with the Rayleigh mask mounted above the rotor on the holder for the condensing lens. An analogous but opposite effect, bowing of the fringes, has since been observed when the Rayleigh mask is mounted just above the collimating lens (Kirschner, 1971; Richards *et al.*, 1971a). It is important to take this phenomenon into account in analyzing the difference concentration boundaries since the measurement of the first moment is based on the principle that the fringe displacement is proportional to the difference in concentration and independent of concentration gradients. Fringe bowing was found to decrease during a sedimentation experiment as the individual boundaries broaden due to diffusion; hence there could be an anomalous decrease (or increase) in the first moment of the difference concentration, and a consequent error in $\Delta s/\bar{s}$, if this effect is not circumvented. The effect is small, to be sure, and it can be reduced substantially by placing the Rayleigh mask close to the ultracentrifuge cell (Kirschner, 1971; Richards *et al.*, 1971a). Moreover, it can be eliminated by selecting for measurement the central (or zero order) fringe. This fringe can be identified in difference sedimentation experiments through the use of a "white-light" exposure. The precision mask described by Richards *et al.* (1971a) produces only a 2.2% change in fringe spacing even for large gradients; thus the error introduced by selecting the wrong fringe for measurement is likely to be small.

Results

Two types of experiments were performed on BSV in order to test the reliability of the difference sedimentation technique. In addition, preliminary studies were conducted on a variety of protein systems to illustrate the applicability of the method in studies of conformational changes in proteins. The first type of experiment involved identical solutions in the two compartments of the cell. Since for such systems $\Delta s/\bar{s}$ must equal zero, the experiment serves as a test of the theoretical equations and the various approximations as well as the various operational innovations such as the Rayleigh mask and its alignment. Data from these experiments were useful in assessing the complications stemming from fringe displacement and bowing. The second type of experiment was designed to test the accuracy as well as the precision of the technique; hence studies were performed on a system for which Δs could be evaluated independently

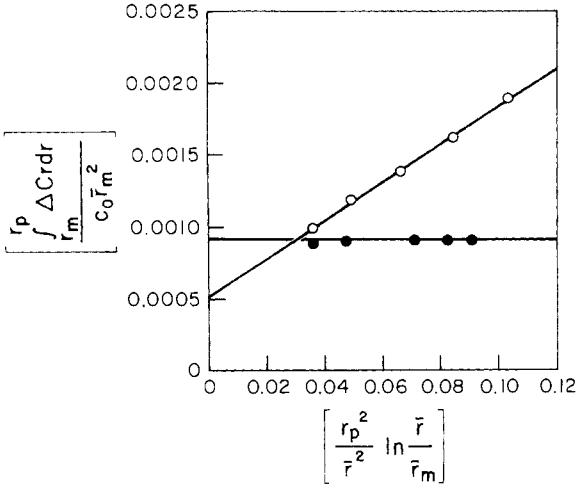

FIGURE 5: Difference sedimentation of BSV in H_2O–D_2O solutions. The filled circles represent experiments with identical solutions of BSV at a concentration of 5 mg/ml. The open circles represent data from the experiment shown in Figure 4 ($\Delta s/\bar{s} = 0.0114$) and given in Tables I and II. The data are plotted according to eq 4 and the slopes provide a direct measure of $\Delta s/\bar{s}$.

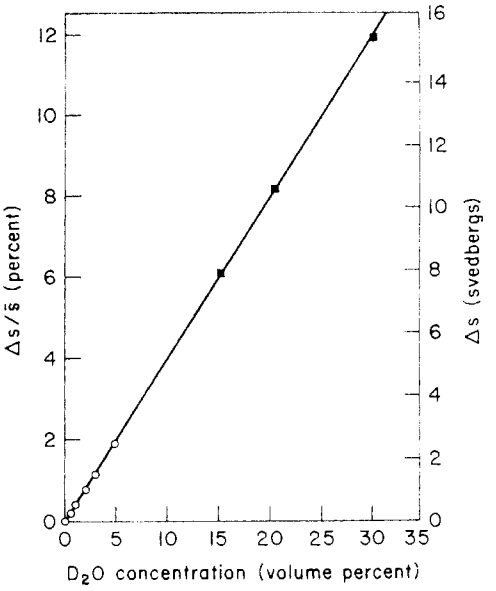

FIGURE 6: Difference in sedimentation coefficient as a function of D_2O concentration. Solutions contained identical concentrations of BSV (5 mg/ml) and varying concentrations of D_2O in 0.1 M potassium phosphate buffer (pH 6.8). The values of $\Delta s/\bar{s}$ were multiplied by the denominator of eq 18 to correct for the nonlinearity of $\Delta s/\bar{s}$ with the concentration of D_2O. Results obtained with simultaneous experiments with schlieren optics (plane and wedged windows) are shown as ■. Those obtained by the difference sedimentation technique with interference optics are shown as ○.

with a high degree of accuracy. Accordingly measurements were made on BSV in H_2O solutions *vs.* BSV in solutions containing various amounts of D_2O. Since D_2O increases the viscosity and density of the medium (while also increasing the solute molecular weight by H–D exchange), sizeable decreases in sedimentation coefficient are produced by the addition of D_2O. These changes in $\Delta s/\bar{s}$ at high concentrations of D_2O were measured by schlieren optics with plane and wedged-window cells and the resulting values were used for interpolation to much lower values of $\Delta s/\bar{s}$.

Bushy Stunt Virus. Figure 4 shows a series of difference patterns from an experiment with BSV. The two compartments of the cell were filled to slightly different levels; thus two menisci are evident. As a consequence even the first pattern after the fringes were resolved had a significant first moment. The third pattern in the series in Figure 4 was obtained with "white light" so as to illustrate the method employed in locating the zero-order fringe in those patterns produced by monochromatic light. In difference sedimentation experiments the fringes are of low order since the two solutions under comparison are very similar in refractive index and the change across the difference boundary is seldom greater than a few fringes. Hence the fringe patterns are generally of higher quality than those obtained in conventional sedimentation velocity experiments and many sedimentation equilibrium studies.

Data from two difference sedimentation experiments are plotted in Figure 5 as

$$(1/c_0\bar{r}_m^2)\int_{\bar{r}_m}^{r_p} \Delta c r dr \text{ vs. } (r_p/\bar{r})^2 \ln \bar{r}/\bar{r}_m$$

The points for both experiments fit straight lines and the precision is high. For the experiment indicated by ● the two compartments were filled with aliquots of the same BSV solution; hence $\Delta s/\bar{s}$ should be zero. As seen from the plot, the value of $\Delta s/\bar{s}$ evaluated from the slope was 0.00013, a result very close to zero. Similar experiments were performed at periodic intervals and for each the Rayleigh mask was repositioned by the method described in the experimental section. The observed values of $\Delta s/\bar{s}$ were 0.0006, 0.0004, and 0.0003. Thus the technique gave the theoretically predicted linear plots, and the precision and accuracy for identical solutions were excellent.

The plot indicated by ○ in Figure 5 was obtained in an experiment with BSV in buffer *vs.* BSV in the same buffer containing D_2O at a concentration of 3% (v/v). From the slope of the straight line $\Delta s/\bar{s}$ was calculated to be 0.0114 in excellent agreement with the expected value, 0.0119, obtained by interpolation of the results evaluated at higher D_2O concentrations. Thus, even though the difference in sedimentation coefficient was only about 1%, the error in measuring that small difference directly was less than 5%. Similar experiments for even smaller values of $\Delta s/\bar{s}$ gave comparable accuracy. For example, a 0.5% D_2O–H_2O solution and a 1.0% D_2O–H_2O solution (*vs.* H_2O solutions in both cases) produced $\Delta s/\bar{s}$ values of 0.0020 ± 0.0001 and 0.0038 ± 0.0001 as compared to the expected values of 0.0020 and 0.0040, respectively. Separate experiments on the same pair of solutions showed a precision of about 5% in measuring small differences in sedimentation coefficient. Values of $\Delta s/\bar{s}$ equal to 0.0179, 0.0173, and 0.0182 were obtained for three independent experiments of BSV in 5% D_2O *vs.* BSV in H_2O. These results taken together indicate that both the precision and the accuracy of the difference sedimentation technique are within 5% even for values of $\Delta s/\bar{s}$ as low as 0.002.

Further evidence for the accuracy of the difference sedimentation method came from studies of BSV at varying D_2O concentrations. The results in Figure 6 show that the data from experiments with Rayleigh optics using low concentrations of D_2O fit closely the line defined by the results with schlieren optics for the D_2O solutions at concen-

trations of 15, 20, and 30%. Small corrections were applied to the measured values of $\Delta s/s$ prior to plotting the results in Figure 6. These corrections were required since $\Delta s/s$ should not vary in a strictly linear fashion with D_2O concentration. From the definition of $\Delta s/s$ as $2(s_2 - s_1)/(s_1 + s_2)$ and the well-known dependence of sedimentation coefficient on molecular weight and density and viscosity of the solution (Svedberg and Pedersen, 1940) we can write

$$\frac{\Delta s}{s} = \frac{\left[(k_0 - k_2) + \frac{(k_1 - 1)\bar{V}\rho_0}{1 - \bar{V}\rho_0}\right]x}{1 + \left[k_0 + k_2 - 2 - \frac{(k_1 - 1)\bar{V}\rho_0}{1 - \bar{V}\rho_0}\right]\frac{x}{2}} \quad (18)$$

In eq 18, x is the volume fraction of D_2O, ρ_0 is the density of the aqueous solution (0% D_2O), $\bar{V}$ is the partial specific volume of the solute molecules, k_0 and k_1 are the ratios of the viscosity and density of D_2O to those of H_2O at 20°, and k_2 is the fractional change in molecular weight of the solute due to the exchange of deuterium for hydrogen. Equation 18 is valid for only dilute solutions of D_2O since higher order terms describing the viscosity and density of D_2O (relative to H_2O) were neglected. The nonlinearity in $\Delta s/s$ *vs.* D_2O concentration resulting from the denominator in eq 18 was circumvented by multiplying each measured value of $\Delta s/s$ by the term

$$1 + \left[k_0 + k_2 - 2 - \frac{(k_1 - 1)\bar{V}\rho_0}{1 - \bar{V}\rho_0}\right]\frac{x}{2}$$

The correction term was evaluated with 0.712 ml/g for $\bar{V}$ (Cheng, 1953), 1.230 for k_0, 1.108 for k_1 (Kirshenbaum, 1949), and 1.0155 for k_2 (Edelstein and Schachman, 1966). For the highest concentration of D_2O (30% v/v) the correction applied to $\Delta s/s$ was only 2% and it amounted to less than 1% for D_2O concentrations about 10%. With this correction the values for $\Delta s/s$ were found to be proportional to D_2O concentration (see Figure 6) and the measured values by the difference sedimentation technique were in excellent agreement with those obtained by interpolation of the data obtained with schlieren optics.

Proteins of Molecular Weight from 3 × 10⁴ to 3 × 10⁵. Since results of high precision and accuracy were obtained in measurements of small changes in the sedimentation coefficient of BSV, it seemed of interest to apply the difference sedimentation technique to studies of conformational changes in proteins. With macromolecules of low sedimentation rate and high diffusion coefficient, determinations of $\Delta s/s$ are more difficult than with BSV since larger centrifugal fields are required and the difference boundaries are broader. In one of the earliest applications of the difference sedimentation technique, Richards and Schachman (1957) found evidence for a conformation change in serum albumin since its sedimentation coefficient actually decreased (about 0.5%) in binding sodium dodecyl sulfate despite the increase (about 4%) in its effective molecular weight, $M(1 - \bar{V}\rho)$, where M and $\bar{V}$ are the molecular weight and partial specific volume of the solute, and ρ is the density of the solution. At the time of that study many of the problems inherent in the method had not been studied and the limitations imposed by mask misalignment and fringe displacement were not appreciated. Recently Godfrey and Harrington (1970) applied the technique, as originally described, for investigations of conformational changes in myosin. Only the preliminary results are given here for five different systems and more detailed application to one of these is the subject of the following paper (Kirschner and Schachman, 1971).

TABLE VI: Conformational changes in ATCase.[a]

Sample	$(\Delta s/s)$ (%)	$(\Delta s/s)_{cor}$[b] (%)
ATCase *vs.* ATCase	−0.02	−0.02
ATCase–carbamyl phosphate *vs.* ATCase–phosphate	−0.48	−0.58
ATCase–carbamyl phosphate succinate *vs.* ATCase–phosphate glutarate	−3.61	−3.92
ATCase–CTP *vs.* ATCase	−0.25	−2.61

[a] All experiments were performed at a temperature of 20 ± 0.5° in 0.04 M phosphate buffer, 2 mM mercaptoethanol, and 0.2 mM EDTA (pH 7.00). The concentration of all ligands was 2×10^{-3} M. [b] The $\Delta s/s$ was corrected for the binding of 6 moles carbamyl phosphate and 4 moles of succinate and 6 moles of CTP. The partial specific volumes were calculated for phosphate (0.35 ml/g), carbamyl phosphate (0.48 ml/g), succinate (0.61 ml/g), and CTP (0.34 ml/g) according to Traube (1899) and McMeekin *et al.* (1949).

Table VI shows results on ATCase a regulatory enzyme which in experiments with schlieren optics (Gerhart and Schachman, 1968) was found to undergo a 3.6% decrease in sedimentation coefficient upon the addition of the competitive inhibitor, succinate, in the presence of carbamyl phosphate. In that study it was shown that ATCase in the presence of carbamyl phosphate alone suffered only a small decrease in sedimentation coefficient ($\Delta s/s$ was about −0.6%). With the difference sedimentation technique $\Delta s/s$ for ATCase in the presence of carbamyl phosphate *vs.* phosphate was −0.48%. The accuracy of the measurements is illustrated by the control (Table VI) for which $\Delta s/s$ was −0.02%. This high degree of accuracy was obtained even though the ATCase preparation contained a small amount of aggregated material (16 S). Nonetheless the first moment of the difference concentration of the principal component (11.7 S) was measured accurately by integration to a level just beyond the main boundary. Some increase in $\Delta s/s$ is expected as a result of binding of the substrate and correction for this effect yields a value of −0.58% for $(\Delta s/s)_{cor}$ for ATCase in the presence of carbamyl phosphate. Upon the addition of succinate, an analog of the second substrate, aspartate, in the presence of carbamyl phosphate $\Delta s/s$ was −3.61%. Correction for the increase in $M(1 - \bar{V}\rho)$ due to the binding of succinate (Changeux *et al.*, 1968; Hammes *et al.*, 1970) leads to −3.92% for $(\Delta s/s)_{cor}$. This large decrease in sedimentation coefficient indicates that the enzyme molecules undergo a large conformational change such as an increase in volume or an alteration in shape. The value for $\Delta s/s$ upon the addition of CTP, an inhibitor of the enzyme, was −1.25%. The binding of CTP to ATCase (Winlund and Chamberlin, 1970; Hammes *et al.*, 1970) should cause a positive change in the sedimentation coefficient because of the increase in the effective molecular weight of the sedimenting species. This effect should yield a value of +2.36% for $\Delta s/s$. Since

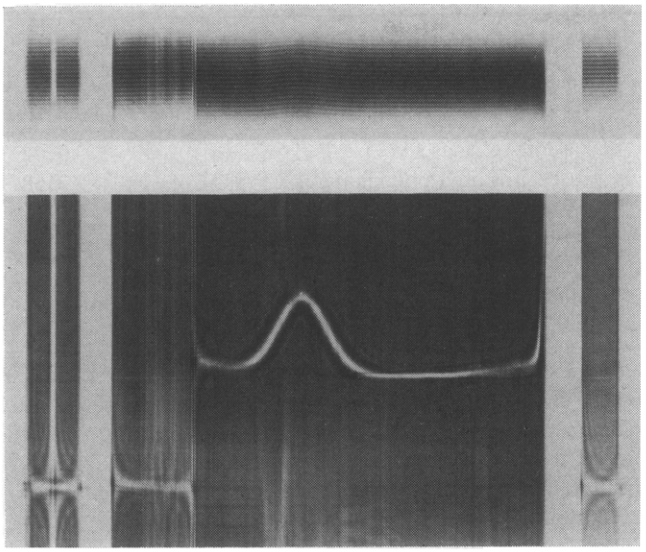

FIGURE 7: Difference sedimentation and schlieren pattern of carboxypeptidase A. The upper photograph shows a difference sedimentation experiment with each sector containing carboxypeptidase A at a concentration of 5 mg/ml in 0.05 M Veronal buffer–1 M triethylamine hydrochloride (pH 7.5) at 20° taken 48 min after the rotor had reached its operating speed of 68,000 rpm. The lower figure is a photograph with schlieren optics with the phase plate at an angle of 60° at about the same time. The concentration of β-phenylpropionate was 2×10^{-4} M.

the observed change in $\Delta s/\bar{s}$ was only -0.25% we can conclude that $(\Delta s/\bar{s})_{cor}$ is -2.61% which can be attributed to a conformational change in the enzyme molecules such as a swelling or a change in shape to a less symmetrical form. For the experiment sufficiently low concentrations of CTP were used so that the effect of the unbound CTP on the viscosity and density of the solution would not be significant.

Results of difference sedimentation experiments on smaller proteins are presented in Table VII. With apoGPDH the control gave a value of $+0.04\%$ for $\Delta s/\bar{s}$. Upon the addition of 3 moles of NAD/mole of enzyme, $\Delta s/\bar{s}$ was $+3.81\%$. Some of this increase can be attributed to the change in the effective molecular weight due to binding NAD and correction for this effect gives $+1.89\%$ for $(\Delta s/\bar{s})_{cor}$. This change may stem from either a modification of individual molecules or a shift in the association–dissociation equilibria (G. D. Smith and H. K. Schachman, unpublished observations); additional experiments are obviously required before these findings can be interpreted in terms of either of these alternatives.

Similar precision was obtained for IgG. With identical solutions $\Delta s/\bar{s}$ was -0.014% and the hapten, arsanilate, caused an increase of 0.82%. Some of the change in $\Delta s/\bar{s}$ was caused by binding and correction for this effect yielded a value of $+0.30\%$ for $(\Delta s/\bar{s})_{cor}$. Thus some conformational change occurred as a result of the interaction of the antibody and univalent hapten in agreement with the observation of Warner *et al.* (1970) on another system.

The difference sedimentation technique produced results of comparable accuracy with even smaller molecules as shown by the data for the catalytic subunit of ATCase (mol wt 1×10^5). For the control $\Delta s/\bar{s}$ was -0.013% and the stereospecific ligands caused an increase of 1.05% only a part of which can be attributed to binding. More extensive studies (Kirschner and Schachman, 1971) showed that this change in sedimentation coefficient is attributable to a conformational alteration in the protein molecules.

Even for small protein molecules such as carboxypeptidase A (3.3 S) results of high precision can be obtained. Figure

TABLE VII: Conformational Changes for Different Proteins.

Protein System	s (S)	$(\Delta s/\bar{s})$ (%)	$(\Delta s/\bar{s})_{cor}$[a] (%)
ApoGPDH[b] *vs.* ApoGPDH	7.8	+0.04	+0.04
ApoGPDH–NAD[b] *vs.* ApoGPDH		+3.81	+1.89
IgG *vs.* IgG	6.7	−0.014	−0.014
IgG–arsanilate[c] *vs.* IgG–sulfanilate		+0.82	+0.30
Catalytic subunit[d] *vs.* catalytic subunit	5.8	−0.013	−0.013
Catalytic subunit–carbamyl phosphate succinate[d] *vs.* catalytic subunit–phosphate–glutarate		+1.05	+0.62
CPase A[e] *vs.* CPase A	3.3	+0.02	−0.02
CPase A–phenylpropionate[e] *vs.* CPase A		+0.05	−0.01

[a] The values for $\Delta s/\bar{s}$ were corrected for the increase in buoyant weight due to the binding of the ligands. It was assumed that at the concentration of ligands added that GPDH bound 2.88 moles of NAD (Conway and Koshland, 1969) with a $\bar{V}$ of 0.62 ml/g, IgG bound completely 2 moles of arsanilic acid with a $\bar{V}$ of 0.51 ml/g, catalytic subunit bound 3 moles of carbamyl phosphate and phosphate and 2 moles of succinate (Hammes *et al.*, 1970; Changeux *et al.*, 1968; Vanaman and Stark, 1970) with partial specific volumes of 0.48, 0.35, and 0.61 ml per g, respectively, and carboxypeptidase A bound 1 mole of β-phenylpropionate (Coleman and Vallee, 1964) with a $\bar{V}$ of 0.96 ml/g. All partial specific volumes were calculated by the method of Traube (1899) and McMeekin *et al.* (1949). [b] ApoGPDH was prepared by the method of Murdock and Koeppe (1964). Difference sedimentation experiments were conducted at 4° in 0.1 M Tris-HCl buffer containing 0.002 M EDTA at pH 8.0 at a protein concentration of 7 mg/ml and at a rotor speed of 60,000 rpm. Where noted 3 moles of NAD/mole of protein was added to one sample. Experiments were conducted by G. D. Smith. [c] Difference sedimentation experiments of IgG antibody were conducted at 20° in 0.1 M NaCl–0.05 M sodium phosphate buffer (pH 7.5) at a protein concentration of 5.75 mg/ml and at a rotor speed of 60,000 rpm. The total concentration of arsanilic and sulfanilic acids was 1×10^{-4} M. [d] Difference sedimentation experiments of catalytic subunits from ATCase were conducted at 20° in 0.04 M potassium phosphate buffer (pH 7.0) containing 2 mM mercaptoethanol and 0.2 mM Na_2EDTA at a protein concentration of 7 mg/ml and at a rotor speed of 60,000 rpm. Carbamyl phosphate, succinate, glutarate, and additional phosphate were at 2×10^{-3} M. [e] Difference sedimentation experiments on carboxypeptidase A were conducted at 20° in 1 M triethylamine hydrochloride–0.05 M Veronal buffer (pH 7.5) at a protein concentration of 8.1 mg/ml, using a titanium rotor at a speed of 68,000 rpm.

7 shows a difference sedimentation boundary for this system. This pattern was obtained at 68,000 rpm with a titanium rotor and the fringes were of high quality. Although the difference boundary was readily detected and its first moment could be measured accurately from the interference patterns the schlieren pattern (see Figure 7) showed only a single broad boundary due to the similarity of the small sedimentation coefficients and the large diffusion coefficients of the molecules in the two compartments of the cell. The ligand, phenylpropionate, caused only a slight change, $+0.05\%$, in $\Delta s/s$ most of which is attributable to the binding and the consequent increase in the effective molecular weight of the protein.

Discussion

As seen from the experiments with proteins of various molecular weights the difference sedimentation technique with Rayleigh optics possesses great sensitivity and yields accurate results in measurements of very small differences in sedimentation coefficients. The quantities measured from the interference patterns in a difference sedimentation experiment give directly the fractional change in the sedimentation coefficient, $\Delta s/s$. The precision was essentially independent of the absolute difference in sedimentation coefficient even for values of $\Delta s/s$ less than 0.2%. For ATCase of molecular weight 3×10^5 a conformational change leading to a value of $\Delta s/s$ of -0.48% was detected readily and measured accurately. The control with aliquots of the same solution gave -0.02% for $\Delta s/s$. With smaller proteins the base-line correction is more hazardous due to the width of the difference boundary and the greater distortion of the cell windows at the higher rotor speeds. Nonetheless experiments with carboxypeptidase A showed that a difference in sedimentation coefficient of only 0.0016 S could be measured readily with a precision of about ± 0.0007 S.

Though the equations for $\Delta s/s$ are derived by a general transport treatment which provides a measure of the weight-average sedimentation coefficient for material in the plateau region, polydispersity or heterogeneity can introduce difficulties in difference sedimentation experiments. In a heterogeneous system when the components have different enough sedimentation coefficients that they separate early in an experiment one can integrate under each boundary independently and determine $\Delta s/s$ for each component. For ATCase, which contained about 5% aggregates and for the IgG antibody preparation containing about 10% protein sedimenting faster than the main component, the major boundaries were utilized to determine $\Delta s/s$ after the minor components had separated. The first moment of the second boundary could also be added to that of the major component to determine $\Delta s/s$ for the whole mixture. Care must be taken to apply the linear base-line correction to each boundary independently and use the concentration of each component for the calculation of $\Delta s/s$.

The most stringent requirement for accurate measurement of $\Delta s/s$ is that the Rayleigh mask be aligned so that it is oriented parallel to and symmetrical about the reference radius from the axis of rotation. Misalignment produces a systematic error in $\Delta s/s$, but this is readily detected in control experiments with identical solutions. The alignment procedure given by Richards *et al.* (1971b) is sufficiently sensitive to detect any errors due to mask misalignment but previous designs of the Rayleigh mask did not allow for the required, convenient, and sensitive rotational and translational adjustment. The Rayleigh mask described by Kirschner (1971) and by Richards *et al.* (1971a) possesses the necessary adjustments to give an accuracy better than $\pm 2 \times 10^{-4}$ for $\Delta s/s$.

All base-line corrections, fringe displacement corrections, and corrections due to small differences in initial concentrations may be incorporated in a simple approximate base-line correction. The error due to fringe bowing is difficult to estimate since it depends on the concentration gradient. However, under adverse circumstances in experiments with BSV measuring areas under either the $+3$ fringe or -3 fringe produces a $\Delta s/s$ of 0.05% instead of zero. This value can be considered as about the maximum error due to fringe bowing, since the concentration gradients for experiments with BSV were almost at the limit of resolvability. Moreover this error due to fringe bowing may be eliminated by using the zero-order fringe.

In principle, for small values of $\Delta s/s$, precision can be enhanced by increasing the concentration of material. For a $\Delta s/s$ of 0.005 the first moment of the difference boundary with an initial area of 1 mm^2 would change by 30% as the boundary moves from the meniscus to a point halfway down the cell for a sample with a concentration of 5 mg/ml. At a concentration of 10 mg/ml the change would be 60%. Since the precision of measuring the average change in the area is about 1%, one can therefore measure a $\Delta s/s$ as small as 0.0008 to a precision of better than 10%.

In practice there is a limit to the amount of sensitivity that can be achieved by increasing the concentration.[5] It is sometimes difficult to match the concentration of material in both sectors, and errors due to approximations in that portion of the base-line correction are increased. Fringe displacement is also proportional to concentration and errors in this correction will also be increased. For materials with low sedimentation coefficients and correspondingly broad boundaries, errors in any base-line correction generally increase in proportion to the concentration. With molecules having high sedimentation coefficients and sharp boundaries the limiting factor is that an increase in the concentration leads to large concentration gradients. If the gradient is too great the fringes will be unresolvable. High gradients also increase any errors due to fringe bowing. In experiments presented here maximum precision was obtained at concentrations between 5 and 10 mg per ml. With some sacrifice of precision it is also possible to measure small differences in sedimentation coefficients with solutions too dilute for accurate measurements by schlieren techniques. For example differences in sedimentation coefficient of $0.9 \pm 0.1\%$ were measured on an ATCase hybrid (V. P. Pigiet and H. K. Schachman, unpublished results) at a concentration of 1.8 mg/ml.

[5] The principle employed here whereby the interferometer is used to subtract the concentration–distance pattern of one sample from that for another to give directly a difference boundary can be used as well with absorptions optics. Indeed the split-beam photoelectric adsorption system has been used for this purpose (Lamers *et al.*, 1963; Schachman, 1963). With the photoelectric scanner some of the pitfalls described here for the Rayleigh optical system can be avoided. However, the absorption system as yet does not have the accuracy obtained readily with interference optics and stringent tests of the potential of the scanner for difference sedimentation experiments have not been made. Its inherent sensitivity and ability to discriminate among various molecular species through the use of light of various wavelengths commend it for difference sedimentation studies on dilute and even impure preparations. In its present form the scanner is restricted to solutions of low optical density since the switching circuit operates on the signal obtained from the first sample passing across the light beam; thus if the absorption of the sample is too great there will be insufficient light to operate the necessary switching circuit.

The addition of a component, such as a specific ligand, to a solution of macromolecules can cause a change in their sedimentation coefficient through an increase in molecular weight resulting from binding, an alteration in the partial specific volume and frictional coefficient stemming from the interaction, and a change in the viscosity and density of the solvent due to the contribution of the unbound ligand. For many systems the change in s may be attributed largely to an alteration in the conformation of the macromolecules which affects their hydrodynamic behavior. However, the sensitivity of the difference sedimentation technique is so great that the other factors can be significant and must be evaluated. Neglect of them can lead to erroneous interpretations. For example, Jaenicke and Gratzer (1969) concluded that a 3% increase in the sedimentation coefficient accompanying the binding of 4 moles of NAD to yeast glyceraldehyde 3-phosphate dehydrogenase at 20° constituted strong evidence for a conformational change in the enzyme. The binding of NAD not only increases the molecular weight (by 667 for each bound molecule) but it also affects the partial specific volume of the complex appreciably since NAD has a substantially higher density than most proteins. The fractional change in s due solely to binding a ligand of molecular weight, M_L, and partial specific volume, $\bar{V}_L$ to a protein with molecular weight, M_P, and partial specific volume, $\bar{V}_P$, can be expressed by

$$\frac{\Delta s}{s} = \frac{M_L(1 - \bar{V}_L\rho)}{M_P(1 - \bar{V}_P\rho) + \frac{M_L}{2}(1 - \bar{V}_L\rho)} \quad (19)$$

Substitution of the appropriate quantities (with $\bar{V}_L$ equal to 0.62 ml/g calculated by the method of Traube (1899)) for this system leads to a value of 2.7% for $\Delta s/s$ which is within the experimental error of (±0.5%) of the observed value. Thus the experimental results can be attributed to binding of the ligand.

The effect of ligands on the solution density and viscosity can be eliminated by placing compounds in the reference sector which mirror the contribution of the ligand to the solvent viscosity and density (Gerhart and Schachman, 1968). The extreme stereospecificity of most biochemical reactions allows one to place in the reference sector isomers or compounds differing only slightly in structure but which would be expected to have about the same effect on the viscosity and density of the solvent. Sulfanilic acid, a chemical similar in structure to arsanilic acid but which does not cross react with IgG directed against the arsanilate group was used to compensate for the effect of the latter on the solvent viscosity and density in experiments with IgG. Similarly, phosphate and glutarate, substances which have little effect on ATCase or catalytic subunit compensated for carbamyl phosphate and succinate. In the experiments on GPDH no additions were made to the reference sector to compensate for NAD since it had been shown (Conway and Koshland, 1968) that the first 3 moles bind almost stoichiometrically. Where no such compounds exist one can measure directly the effect either by separate viscosity and density experiments or by difference sedimentation experiments with macromolecules which do not interact with the ligands as shown by Gerhart and Schachman (1968) with aldolase and ligands specific for ATCase.

Even small changes in the partial specific volume of a protein, $\bar{V}_P$, could cause an appreciable change in $\Delta s/s$. For example, a partial specific volume change from 0.7400 to 0.7405 ml per g would produce a $\Delta s/s$ of almost 0.2%. The partial specific volumes of proteins are relatively insensitive to conformation, since good estimates of them can be made by summing the partial specific volumes of the constituent amino acid residues (McMeekin *et al.*, 1949). The change in the partial specific volume due to the complexing of a ligand can thus be accounted for by assuming additivities of weights and volumes.

As Schumaker concluded (1968) it is likely that changes in sedimentation coefficient accompanying the binding of small ligands primarily reflect a change in the frictional coefficient which is a function of the hydrodynamic size and shape of the molecule. He pointed out that hemoglobin undergoes an 8% contraction in volume upon oxygenation which would correspond to an increase of 3% in sedimentation coefficient for a spherical molecule (Schumaker, 1968). ATCase, which produces a corrected $\Delta s/s$ of 3.92% accompanying the binding of succinate and carbamyl phosphate, must increase its volume by 12% assuming that it were a spherical molecule (Gerhart and Schachman, 1968). The catalytic subunit of ATCase undergoes an increase in sedimentation coefficient of 0.62% (after correcting for the increase in molecular weight due to bound ligands) which correspond to a decrease in volume of about 2%. IgG antibody which undergoes a corrected increase in s of 0.30% would have undergone a 1% decrease in volume or corresponding change in shape. For all of these systems it is assumed that the binding of ligands is not accompanied by any change in association–dissociation equilibrium involving monomers and oligomers. The validity of this assumption must be assessed, however, by independent experiments (Kirschner and Schachman, 1971). With hemoglobin in particular the dissociation of tetramers to dimers is markedly different for the unliganded and liganded species; hence a change in s may be a reflection of shifts in the association–dissociation equilibrium rather than a change in the hydrodynamic volume of individual molecules. This uncertainty can be resolved frequently from studies of $\Delta s/s$ at different protein concentrations.

However, it would be valuable if there were a technique for measuring directly and accurately very small differences in molecular weight, $\Delta M/\bar{M}$. Such a technique would enable one to make a simple evaluation of the contribution to $\Delta s/s$ of changes in association–dissociation as well as changes in the effective molecular weight due to bound ligands. Although such a method employing the double-beam ultraviolet scanner has been reported (Barlow *et al.*, 1969), it is unlikely that it has sufficient precision for the magnitude of changes measured by difference sedimentation. Now that remedies have been devised to overcome some of the pitfalls inherent in the use of the Rayleigh interferometer for measuring $\Delta s/s$ it seems appropriate to investigate the possibility of using the same principle for measuring $\Delta M/\bar{M}$ in difference sedimentation equilibrium experiments.

In view of the importance of hydrodynamic methods for studying conformational changes in proteins it would be useful to consider the range of applicability of common techniques. Table VIII gives a comparison of sedimentation velocity using schlieren optics, sedimentation velocity using simultaneous experiments with plane and wedged-window cells, difference sedimentation, and viscometry for measuring isotropic volume changes and changes in axial ratio for an assumed ellipsoid of revolution with an axial ratio of 3, a sedimentation coefficient of 6 S, and an intrinsic viscosity of 0.04 dl/g. Although intrinsic viscosity is in principle more sensitive to changes in volume than the sedimentation

TABLE VIII: Accuracy in Measuring Hydrodynamic Changes.

Vol Change (%)	Error in Measurement (per cent)[a] Viscometry[b]	Sedimentation[c]	Sedimentation (Wedge Window)[d]	Difference Sedimentation[e]
30	8	15	4	5
5	50	90	25	5
0.3	∞	∞	∞	5
Axial ratio change (%)				
50	6	12	3	5
5	60	∞	30	5
0.5	∞	∞	∞	5

[a] The error in measurement for each method is calculated as its intrinsic experimental error divided by the calculated change in the experimental parameter caused by various changes in volume and shape. It is assumed that the protein is an impenetrable prolate ellipsoid of revolution with an axial ratio of 3, an intrinsic vicosity of 0.04 dl/g and a sedimentation coefficient of 6 S. Values for changes in intrinsic viscosity were calculated from Mehl *et al.* (1940) and for changes in frictional factor from Svedberg and Pedersen (1940). [b] The experimental error for viscometry is calculated by assuming that for a viscometer with an outflow time for the solvent of 100 sec, a 10-mg/ml solution of the protein would have an outflow time of 104 sec. If outflow time can be measured to approximately ±0.025 sec the specific viscosity can be measured to ±1.25%. [c] It is assumed that two separate velocity experiments are comparable to ±1.5%. [d] The experimental error for wedge-window schlieren experiments is assumed to be ±0.4%. It is given as ±0.5% by Gerhart and Schachman and ±0.3% for a protein of about 7 S by Schumaker and Adams (1968). [e] The error in difference sedimentation is ±5% down to differences in s of 0.1% for a protein at a concentration of 10 mg/ml as described in the text.

coefficient, it cannot be measured as precisely. Moreover for viscometry there is no readily available difference technique analogous to those used for sedimentation velocity studies. For measuring large changes in shape or size all four methods are applicable and other considerations should apply in choosing among them. However, for changes in shape or volume of less than 5% only difference sedimentation can be employed with reasonable accuracy. Difference sedimentation with interference optics is sensitive enough to measure to an accuracy of 5% a $\Delta s/s$ corresponding to a change in volume of 0.3% or in axial ratio of 0.5%.

It is likely that many macromolecules which have been shown to undergo changes in spectra accompanying ligand binding can be studied by means of difference sedimentation. Correlation of spectral changes with changes in a hydrodynamic parameter of conformation such as the sedimentation coefficient would yield valuable information as to which processes are due to local interactions between the ligands and the macromolecules and which involve bulk changes in the macromolecules.

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Appendix

When there is a difference between the sedimentation coefficients of the macromolecules in the two compartments of the cell, part of the fringe displacement is due to variations in radial dilution. Therefore there will be an error in $\Delta s/s$ in assuming that the fringe displacement should be zero and in drawing an approximate base line from the supernatant to the plateau region. The magnitude of the "real" fringe displacement will be larger the greater the difference in sedimentation coefficient, but the percentage error in evaluating $\Delta s/s$ is independent of this difference. If no correction is made to allow for a "real" fringe displacement the error in the value of $\Delta s/s$ could be as much as 10%. Thus a correction of the type described here is desirable.

The fringe displacement due solely to differences in radial dilution, $(\Delta c_p)^t$ is the difference between the concentration in the plateau region of one compartment (denoted by subscript 1) and that of the other compartment (denoted by subscript 2)

$$(\Delta c_p)^t = c_0(e^{-2\omega^2 s_2 t} - e^{-2\omega^2 s_1 t}) \quad \text{(i)}$$

Making the same substitutions used to obtain eq 4, we have

$$(\Delta c_p)^t = 2c_0\left(\frac{\bar{r}_m}{\bar{r}}\right)^2\left(\ln\frac{\bar{r}}{\bar{r}_m}\right)\left(\frac{\Delta s}{s}\right) \quad \text{(ii)}$$

The linear base-line correction decreases the true first moment of the concentration difference by $\int(\Delta c_p)^t r dr$, from r_p to $\bar{r}_m$. Accordingly, in practice eq 4 takes the form

$$\frac{\int_{\bar{r}_m}^{r_p}\Delta c r dr - \int_{\bar{r}_m}^{r_p}(\Delta c_p)^t r dr}{c_0\bar{r}_m^2} = \left(\frac{r_p}{\bar{r}}\right)^2\left(\ln\frac{\bar{r}}{\bar{r}_m}\right)\left[\frac{\Delta s}{s} - \left(\frac{\Delta s}{s}\right)^t\right] - \frac{\Delta r_m}{\bar{r}_m} \quad \text{(iii)}$$

where $(\Delta s/s)^t$ designates the error in $\Delta s/s$ made in assuming that the fringe displacement should be zero. The addition of $(\Delta s/s)^t$ to the calculated values of $\Delta s/s$ yields the true value for $\Delta s/s$. From eq iii and eq 4 we obtain

$$\left(\frac{\Delta s}{s}\right)^t = \frac{\int_{\bar{r}_m}^{r_p}(\Delta c_p)^t r dr}{c_0\bar{r}_m^2\left(\frac{r_p}{\bar{r}}\right)^2\left(\ln\frac{\bar{r}}{\bar{r}_m}\right)} \quad \text{(iv)}$$

The first moment of the excess concentration, summed in increments of the square of the radial distance is given by

$$\int_{\bar{r}_m}^{r_p}(\Delta c_p)^t r dr = \frac{(\Delta c_p)^t}{2}(r_p^2 - \bar{r}^2) \quad \text{(v)}$$

By substituting eq ii for $(\Delta c_p)^t$ on the right side of eq v we obtain an expression for the additional first moment in terms of the difference in sedimentation coefficients.

$$\int_{\bar{r}_m}^{r_p} (\Delta c_p)^t r dr = (r_p^2 - \bar{r}^2) c_0 \left(\frac{r_m}{\bar{r}}\right)^2 \left(\ln \frac{\bar{r}}{\bar{r}_m}\right) \left(\frac{\Delta s}{s}\right) \quad \text{(vi)}$$

To solve for $(\Delta s/s)^t$ we substitute eq vi for $\int (\Delta c_p)^t r dr$, from r_p to $\bar{r}_m$, into eq iv. Thus we obtain

$$\left(\frac{\Delta s}{s}\right)^t = \left[\frac{r_p^2 - \bar{r}^2}{r_p^2}\right] \frac{\Delta s}{s} \quad \text{(vii)}$$

In a single experiment the values for $\bar{r}/r_p$ are nearly the same in each pattern and hence $(\Delta s/s)^t$ is a constant correction to $\Delta s/s$. From eq vii the correction to $\Delta s/s$ is proportional to $\Delta s/s$ and the constant of proportionality depends only on the relative values of r_p and $\bar{r}$. Rewriting eq vii assuming that $r_p + \bar{r}$ is approximately equal to $2r_p$, we obtain

$$\frac{\left(\frac{\Delta s}{s}\right)^t}{\left(\frac{\Delta s}{s}\right)} = \frac{2(r_p - \bar{r})}{r_p} \quad \text{(viii)}$$

The percentage error due to neglecting the fringe displacement depends on the width of the difference boundary, $2(r_p - \bar{r})$. Moreover, it is small since $(r_p - \bar{r}) \ll r_p$. For experiments on BSV $\bar{r}/r_p$ was equal to 0.988 and the correction in $\Delta s/s$ was therefore about 2%. With diffuse boundaries such as those for carboxypeptidase, however, $\bar{s}/r_p$ was about 0.946 and the correction in $\Delta s/s$ was about 10%. If the boundaries are broad and one suspects a significant correction to $\Delta s/s$ because of differences in radial dilution, the measured values of $\Delta s/s$ can be simply corrected by dividing by $(\bar{r}/r_p)^2$. This correction is equivalent to removing the term $(r_p/\bar{r})^2$ from eq 4.

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Conformational Changes in Proteins as Measured by Difference Sedimentation Studies. II. Effect of Stereospecific Ligands on the Catalytic Subunit of Aspartate Transcarbamylase*

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ABSTRACT: The technique of difference sedimentation has been employed to measure small changes in the sedimentation coefficient of the catalytic subunit of aspartate transcarbamylase (ATCase) in the presence of specific ligands. Succinate or carbamyl phosphate alone produced small increases in the sedimentation coefficient of the catalytic subunit which stemmed principally from the added weight and density of the bound ligands. Both ligands together at concentrations of 2 mM, however, produced an increase in s of 1.05%, which is three times larger than the expected increase due merely to binding the ligands. Differential sedimentation experiments using a synthetic boundary cell showed that the concentration dependence of the sedimentation coefficient was nearly the same in the presence or absence of ligands. This result indicated that the increase in sedimentation coefficient could not be due to a small shift in the association–dissociation equilibrium promoted by the addition of ligands. The difference in sedimentation coefficient plotted as a function of succinate concentration in the presence of carbamyl phosphate gave a titration curve with a midpoint of 2×10^{-4} M. Comparison of these results to those for the native enzyme (Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538) showed that the conformational changes for the subunit and the native enzyme occurred at the same succinate concentration though they were opposite in direction. It appears that carbamyl phosphate and succinate cause changes in the catalytic subunit which result in a more contracted or isometric conformation, but that these are linked to changes in the conformation in the whole enzyme complex which result in a more swollen or anisometric form for ATCase.

Much functional significance has been attributed to conformational changes in enzyme molecules. Alterations in the three-dimensional structure of an enzyme on binding substrates have been considered to be important as part of the mechanism of enzyme catalysis (Koshland, 1958; Lumry, 1959; Jencks, 1969) and in particular in the regulation of enzyme activity (Monod *et al.*, 1965; Koshland *et al.*, 1966). Some experimental techniques used to study conformational changes in proteins suffer from ambiguity in that they cannot distinguish local effects from bulk changes in the three-dimensional structure of the protein. These local effects stemming from the interaction between the ligands and protein may produce a marked change in some physical–chemical parameter (such as absorption or optical rotation) without a gross change in the protein conformation. Hydrodynamic methods, since they are a reflection of the volume and shape of the protein molecules, can make important contributions in removing this ambiguity. Since difference sedimentation has been shown to be an extremely sensitive and accurate hydrodynamic method (Richards and Schachman, 1959; Kirschner and Schachman, 1971), we have applied it to the study of conformational changes in the catalytic subunit of aspartate transcarbamylase (ATCase).[1]

ATCase catalyzes the first step specific to pyrimidine biosynthesis in *Escherichia coli*. It shows two types of binding site interactions, cooperative kinetics with the substrates aspartate and carbamyl phosphate and feedback inhibition with the end product of the pathway, cytidine triphosphate (CTP) (Gerhart and Pardee, 1962; Bethell *et al.*, 1968). It has been shown that the native enzyme can be dissociated by *p*-hydroxymercuribenzoate into two types of subunits (Gerhart and Schachman, 1965) which are readily separated and obtained in pure form. The catalytic subunits catalyze the reactions leading to the formation of carbamyl aspartate

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[1] Abbreviation used is: ATCase, aspartate transcarbamylase.