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A Difference Sedimentation Equilibrium Technique for Measuring Small Changes in Molecular Weight. II. Experimental†

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ABSTRACT: Difference sedimentation equilibrium is a subtractive technique for measuring directly small differences in molecular weight. The method requires the simultaneous determination of two parameters, the difference in concentration (Δc) and the average concentration ($\bar{c}$), as functions of radial distance. With double-sector ultracentrifuge cells, Δc is measured interferometrically while $\bar{c}$, due to the lack of a reference compartment containing the solvent, is obtained from the integration of the schlieren pattern. The technique has been tested by measuring known changes in effective molecular weight ($\Delta\sigma$), of 1–10%, produced by the addition of various quantities of D_2O to one of each sample pair. The results show that small differences can be measured, despite the presence of imperfections in the ultracentrifuge cells, with an absolute error of less than 0.2% using data from $\bar{c}$ = 2–10 fringes. Although the combination of Rayleigh and schlieren optics proved satisfactory for the determination of $\Delta\sigma$ it appeared that both the accuracy of the method and the concentration range over which meaningful data could be collected were restricted by the measurement of $\bar{c}$. Hence we developed a special three-compartment cell and corresponding Rayleigh mask which permitted the interferometric determination of $\bar{c}$ as well as Δc. While experience with this equipment is limited and some of its advantages and disadvantages have not been fully assessed, it appears to be capable of substantially improving the sensitivity of the technique.

In the preceding paper we presented a theoretical treatment for evaluating directly small changes in the effective molecular weight of macromolecules by a difference sedimentation equilibrium technique (Springer *et al.,* 1974). Computer simulation was used to analyze the effect of various parameters such as mismatches of the menisci and bottoms of the two solutions. These calculations and a study of the potential sources of errors indicated that the technique could be used satisfactorily for the measurement of differences in effective molecular weight as small as 1%. In this communication we describe the implementation of the method and present experimental results along with developments aimed at increasing its sensitivity and accuracy.

Conventional double-sector ultracentrifuge cells are suitable for difference sedimentation equilibrium experiments since they permit the direct measurement of the difference in concentration, Δc, by means of interference optics. For this purpose each compartment would contain one of the two solutions. However, the absence of a solvent compartment precludes the measurement of the absolute concentration with the Rayleigh interferometer. Hence we have employed the schlieren optical system for the determination of the average concentration, $\bar{c}$. This procedure is shown to be practical and capable of provid-

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‡ This material was submitted in partial fulfillment of the Ph.D. requirements in Molecular Biology at the University of California, Berkeley, Calif.

ing reasonably accurate values of $\bar{c}$ as a function of radial position, r. Thus the combination of the two optical systems provides the required data for the calculation of the difference in effective molecular weight, $\Delta\sigma$. Since the schlieren optical system is not capable of accuracy approaching that of the Rayleigh interferometer, we have developed a method that permits $\bar{c}$ as well as Δc to be determined interferometrically. This was accomplished by constructing a three-compartment cell and a special Rayleigh mask which allows the comparison of the two solutions with the solvent as well as with each other. In this way both Δc and $\bar{c}$ can be measured directly.

Experimental Section

Materials. The catalytic subunit of aspartate transcarbamylase was used as a test material because it has no tendency either to aggregate or dissociate in normal buffers. In addition, its size, mol wt $1.0_2 \times 10^5$ (Cohlberg *et al.*, 1972), permitted the use of relatively low rotor speeds. The subunit was prepared from the native enzyme by the neohydrin procedure (Kirschner, 1971) followed by chromatography on a 2.5 × 90 cm Sephadex G-200 column. A variant of the enzyme,[1] r_4c_6, was used for some experiments. This protein was purified by repeated chromatography on DEAE-Sephadex (Yang *et al.*, 1974). The aspartate transcarbamylase used in the experiment with r_4c_6 was chromatographed on Sephadex G-200 to remove aggregated material. For studies with identical solutions of the catalytic subunit the solvent employed was either 0.1 M Tris-acetate (pH 7.5) containing 10^{-3} M EDTA or 0.02 M imidazole acetate (pH 7.3) containing 0.08 M triethylammonium acetate and 10^{-3} M EDTA. The latter buffer was also used for all experiments involving D_2O. The measurement of the difference in molecular weight between r_4c_6 and native aspartate transcarbamylase was performed in 0.04 M potassium phosphate (pH 7.5) buffer containing 2×10^{-3} M EDTA.

Methods. A Beckman Spinco Model E ultracentrifuge equipped with a Rayleigh interference optical system and a phase plate was used for all experiments. The optical system was aligned according to the procedure of Richards *et al.* (1971), with the camera lens focused at the two-thirds level in a cell assembled with sapphire windows. A precision Rayleigh mask (Kirschner and Schachman, 1971; Richards *et al.*, 1972) mounted on the lower lens was used for all but one experiment which will be described below. The mask was aligned to 10 μ translationally and 0.2° rotationally by 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). The cell assemblies were of the standard double-sector type with two modifications. First, the slits in the lower window holders were widened to 1 mm to shorten exposure times, and second, Teflon window liners (Horbett and Teller, 1972) were substituted for the usual bakelite ones. The liners were used routinely until they were so compressed and distorted that the windows were no longer tight in the holders. They proved to be satisfactory at 20,000 rpm for 6–10 experiments. Base lines were reproducible within the 3 μ reading error until distortion of the liners occurred. Subsequent replacement of the liners restored the base line to its original state.

Rotor speeds of 13,000 and 20,000 rpm were used for studies of r_4c_6 and the catalytic subunit, and all experiments were allowed to proceed for 24 hr before photographs were taken. This time was more than sufficient for the attainment of an equilibrium distribution for all samples (Yphantis, 1964). No attempt was made to determine experimentally when equilibrium was reached because the shallow nature of the difference concentration patterns does not afford sufficient sensitivity. At the conclusion of the equilibrium experiment the contents of the cell was mixed by vigorously shaking the rotor without removing the cell and a base line was obtained by accelerating the rotor rapidly to one-third the equilibrium speed. This low speed was used so as to prevent any redistribution of the protein near the meniscus that might cause an error in labeling fringes. No detectable change occurred in the cell base line between this and the equilibrium speed. Rayleigh patterns were photographed on Kodak Spectroscopic IIG plates both with and without a Wratten 77A filter. Metallographic plates were used to photograph the schlieren patterns.

All plates were measured on a Nikon 6C comparator equipped with a 50X objective and an L & W digital readout system. The Δc patterns were read by averaging 3 dark fringes at each radial distance, except for the reference position near the meniscus. The reference point was chosen as close to the meniscus as the pattern allowed, and read five times averaging 3 fringes for each reading. The average of these readings was used as $\Delta c = 0$ provided the value for a second point 200 μ away differed by less than 0.005 fringe. If not, the procedures were repeated using other radial positions, near the meniscus, and if agreement was still not reached the run was discarded. Lack of agreement is indicative of a failure to achieve meniscus depletion. In general this results from the presence of particularly large mismatches combined with too low a rotor speed and too high an initial concentration. Normally the variation between the two points was about 0.003 fringe. The base line was read in an identical manner and subtracted from the Δc pattern to obtain the net deviations. The base lines for the cells used in these experiments showed no measurable fringe shifts in the region of the meniscus and deviations of less than 0.03 fringe over the remainder of the liquid column.

The average concentration was calculated from the schlieren pattern by means of a trapezoidal integration. This procedure yields $\bar{c}$ directly provided the gradients formed in the two sectors are sufficiently similar so that the schlieren images overlap. If this occurs the pattern thickens somewhat and becomes a measure of $d\bar{c}/dr$. Since there was no base line an average value of $d\bar{c}/dr$ near the meniscus was used as a zero reference for the entire cell.[2] The Y positions in this region could be reproduced within 25 μ, at a phase plate angle of 75°, without difficulty. This corresponds to a concentration difference of less than 0.05 fringe, and was used as the estimated systematic error. Near the bottom of the cell the schlieren pattern was very steep and measurements of suitable precision were obtained by reading X positions at a given Y. This procedure necessitated an interpolation to find $\bar{c}$ at the radial positions for which Δc had been measured. Initially this was done graphically, but in later experiments an eight-point Lagrange interpolation was used. Overlapping regions were interpolated to minimize the effects of a bad point. In the single case where both

[1] Aspartate transcarbamylase is composed of six regulatory (r) and six catalytic (c) chains (Weber, 1968; Cohlberg *et al.*, 1972); hence in the nomenclature used here it would be called r_6c_6. The variant, r_4c_6, lacks two regulatory chains (one regulatory subunit).

[2] As a result of the lack of a solvent base line in this type of experiment, there are complications resulting from the redistribution of buffer ions. With most dilute buffers, *e.g.*, 0.1 M phosphate, there would be no difficulties. When higher concentrations of a third component are employed, a separate experiment with the solvent could be used to obtain the appropriate base line. Although additional reading errors would be introduced, this procedure should be satisfactory if special precautions are exercised so that the cell is cleaned without disassembly, the loading is similar to that for the solutions, and the cell is oriented reproducibly in the rotor.

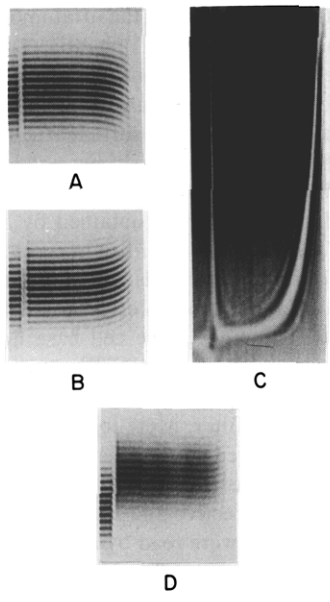

FIGURE 1: Difference concentration and schlieren patterns for catalytic subunit of aspartate transcarbamylase. (A) The difference concentration distribution for an experiment in which identical solutions of protein, at 5 mg/ml in 0.02 M imidazole acetate (pH 7.3) buffer, were added to both sectors. The rotor speed was 20,000 rpm (σ = 4.8) and the column height 3 mm. The photograph was taken 23 hr after the start of the experiment. (B) This photograph shows the difference concentration distribution for the same solutions after the experiment was terminated, the cell rotated in the rotor the width of the scribe line, and equilibrium reestablished. (C) Shows the schlieren pattern, at a phase plate angle of 75°, from which $\bar{c}$ was calculated. (D) The difference concentration profile for an experiment in which one of the solutions contained 50% D_2O. The cell was deliberately misaligned in the rotor to produce a bottom mismatch which moved the extremum and crossover points toward the meniscus and into an observable region. The mismatch is also responsible for decreasing the size of the fringe displacements.

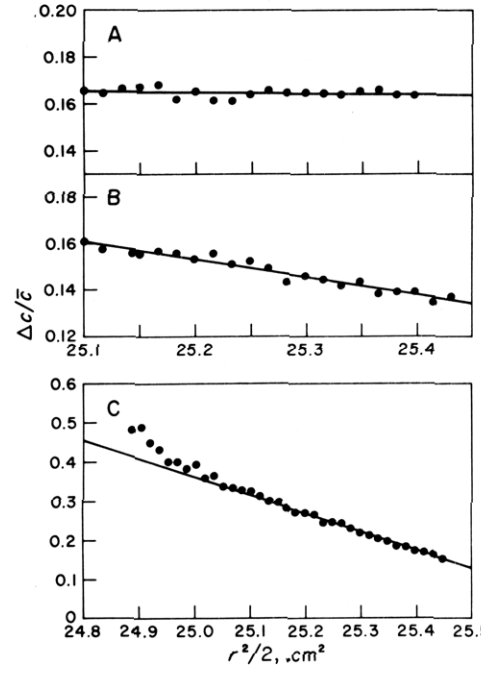

FIGURE 2: Analysis of difference sedimentation equilibrium experiments. (A) Results of experiment presented in Figure 1A and C. Plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$ using data from $\bar{c}$ = 2.3–9.7 fringes. The observed slope of −0.0045 corresponds to a value of −0.0009 for $\Delta\sigma_1$. (B) Data from $\bar{c}$ = 2.1–11 fringes for an experiment with catalytic subunit (4.5 mg/ml) in which one solution contained 6.25%, by volume, D_2O. (C) Plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$ with data from 0.56 to 9 fringes for an experiment in which one solution of catalytic subunit contained 37.5% D_2O.

procedures were used the agreement was excellent. The areas were converted to concentrations by use of the appropriate factors calculated from the prior calibration of the optical system at various phase plate angles (Schachman, 1957). The photograph at an angle of 75° was usually the one used in calculating $\bar{c}$. Values for $\Delta\sigma$ were obtained from a least-squares fit of a plot of $\Delta c/c$ *vs.* $r^2/2$.

Results

Three types of experiments were used to test the accuracy and precision of the method. First, measurements were performed on identical solutions. The difference concentration distributions which occur in these experiments are caused mainly by the bottom mismatches which result from cell imperfections and misalignment of the cell in the rotor. Since $\Delta\sigma$ must be zero despite the concentration differences, these studies permit a critical assessment of the validity of the conclusions drawn from theory and computer simulation. Second, varying amounts of D_2O were added to one sector to produce known $\Delta\sigma$'s. These experiments provide a check on the accuracy as well as the precision of the technique. Third, the method was used to determine the difference in molecular weight between aspartate transcarbamylase and r_4c_6. Since the latter lacks one regulatory subunit (Yang *et al.*, 1974) the difference in molecular weight should be 3.4×10^4 (Weber, 1968; Cohlberg *et al.*, 1972).

Identical Solutions of Catalytic Subunit of Aspartate Transcarbamylase. Figures 1A and C show the interference and schlieren patterns from a typical experiment in which aliquots of the same protein solution were placed in both sectors. If the two liquid columns were identical in terms of the menisci and bottom positions, and the optical path lengths were the same, the fringe pattern would have been flat; hence the shallow downward curvature indicates mismatches in any or all of these parameters. The magnitude of the difference concentration distribution can be attributed to a discrepancy in the positions of the cell bottoms of about 50 μ resulting from an error in casting the centerpiece, a misalignment of the cell in the rotor by 0.4°, or a combination of both. The small mismatch in menisci and the slight difference in thickness between the sectors exert only a very small influence on the difference concentration distribution. The concentration gradients in the two compartments differed so little that the composite schlieren pattern, shown in Figure 1C, resembled that resulting from a sedimentation equilibrium experiment involving redistribution of molecules in only one compartment. Figure 2A shows the plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$ for this experiment. These data represent a concentration range from 2.3 to 9.7 fringes over which Δc varied from 0.38 to 1.6 fringes. As seen in Figure 2A, the values of $\Delta c/\bar{c}$ fall on a straight line with a slope corresponding to a value of $\Delta\sigma/\sigma_1$ equal to -0.0009 ± 0.0011.

In order to assess the effect of mismatch in the cell bottoms on the difference concentration distribution and the accuracy of the technique the experiment was repeated with the cell rotated by the thickness of the scribe line. Since the scribe lines subtend an arc of approximately 1.4° the rotation corresponds to about a 160-μ change in the positions of the bottoms with respect to each other. As seen in Figure 1B, the difference concentration distribution is almost a mirror image of the previous one and the fringe shift between the two is about four fringes near the bottom of the cell. This change is in good agreement with that calculated from the sedimentation equilibrium equation. Despite the alteration in the Δc distribution the value of

$\Delta\sigma/\sigma_1$ of -0.0008 was in excellent agreement with that obtained in the previous experiment. These results show that $\Delta\sigma/\sigma_1$ can be measured precisely and accurately despite the presence of cell imperfections and mismatches. A separate experiment gave a value of -0.0007 ± 0.0011 for $\Delta\sigma/\sigma_1$.

Measurement of $\Delta\sigma$ Caused by the Addition of D_2O. The accuracy of the technique was tested further by performing experiments with pairs of solutions for which $\Delta\sigma$ could be evaluated independently with a high degree of precision. This was achieved by adding D_2O to one sample; in this way the density of the solution as well as the molecular weight and partial specific volume of the protein were altered in a known manner. Moreover, because of the low molecular weight and density of D_2O, any redistribution in H_2O is slight. Thus there is no appreciable density gradient or fringe shift along the cell due to the D_2O. The use of D_2O does have a disadvantage since high concentrations of the reagent cause a blurring of the fringes and consequently some increase in the experimental error in measuring the interference pattern. This loss of definition is due to the difference in refractive index between H_2O and D_2O which results in higher-order fringes of lower quality being located in the diffraction envelope.

The expected $\Delta\sigma$ produced by the addition of D_2O can be calculated from

$$\Delta\sigma = \frac{\omega^2}{RT}\left[(1 + 0.0155X)M\left(1 - \frac{\bar{V}\rho_2}{(1 + 0.0155X)}\right) - M(1 - \bar{V}\rho_1)\right] \quad (1)$$

where M is the molecular weight of the protein, ω is the angular velocity, R is the gas constant, T is the absolute temperature, X is the volume fraction of D_2O, 0.0155 is the fractional increment in molecular weight caused by deuteration in 100% D_2O (Edelstein and Schachman, 1967), $\bar{V}$ is the partial specific volume of the protein, and ρ_2 and ρ_1 are the densities of the solutions with and without D_2O. The densities were calculated assuming additivity of volumes.

For most experiments the difference concentration distributions were shallow curves similar to those shown in Figures 1A and 1B. However, when $\Delta\sigma$ was large and the mismatches small, both the extremum and crossover points were visible as shown in Figure 1D. The mismatches in this particular experiment were arranged to shift these features toward the meniscus and into the observable region of the cell. Since the solution in one compartment contained 50% D_2O the fringes were poorly defined.

Figure 2B shows the results of an experiment in which one solution contained D_2O at a concentration of 6.25%. The plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$, based on data from $\bar{c} = 2.5$ and $\Delta c = 0.3$ fringe to $\bar{c} = 11.0$ and $\Delta c = 1.5$ fringes, is a straight line with a slope corresponding to a value for $\Delta\sigma/\sigma_1$ of -0.0164 ± 0.0009. The value calculated from eq 1 is -0.0150. Similar excellent agreement was obtained between the measured and expected values of $\Delta\sigma/\sigma_1$ for a series of experiments over a large range of D_2O concentrations. These results are summarized in Figure 3 where the measured values are plotted against those calculated from eq 1. These findings demonstrate that with normal double-sector cells it is possible to measure changes in σ of 0–10% with an average accuracy and precision of 0.2%. The data used for these measurements encompassed a concentration range of 2–11 fringes.

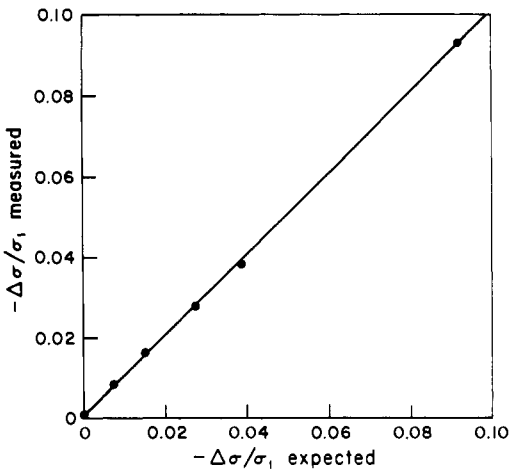

FIGURE 3: Comparison of the expected and measured values of $\Delta\sigma/\sigma_1$. The expected values of $\Delta\sigma/\sigma_1$ produced by the addition of D_2O to one sector were calculated from eq 1. The slope of the least-squares line through the points is 1.00 and the intercept is 0.0007.

Comparison of r_4c_6 with Native Aspartate Transcarbamylase. The discovery of r_4c_6 provided another experimental system suitable for testing the technique. Since the molecular weight of regulatory subunit is 3.4×10^4 the expected difference between aspartate transcarbamylase ($M = 3.1 \times 10^5$) and r_4c_6 is 3.4×10^4. However the sample of r_4c_6 used in the experiment was actually composed of 90% r_4c_6 and 10% aspartate transcarbamylase. Since little separation between these two components occurs under the influence of the centrifugal field used in the experiment, the mixture can be considered to be a single component with a molecular weight 3000 greater than that of r_4c_6. Thus the observed difference in molecular weight should have been 3.1×10^4 (3.4×10^4–0.3×10^4). The measured value, assuming no change in $\bar{V}$, of $3.1 \times 10^4 \pm 0.1 \times 10^4$ is in excellent agreement with the expected result.[3]

Despite the accuracy of the experiment, a problem arose which limited the precision of the data. The schlieren patterns from the two sectors, instead of superimposing over the entire visible solution column, diverged about half-way down just in the region where the data became useful. The occurrence of this phenomenon increased substantially the error in the values of $\bar{c}$. Data of reasonable precision were obtained by reading the center of the white area in the region of superimposition and the center of the lower pattern after divergence (the upper pattern could also have been used). The average concentration was calculated from the relation of $\bar{c} = c + |\Delta c|/2$ (if the upper pattern had been read the relation would have been $\bar{c} = c - |\Delta c|/2$). In principle, this procedure is sound, but in practice the uncertainty in the measurements in the region of the divergence can lead to both inaccuracy and imprecision.

Three-Compartment Cell and Rayleigh Mask. Although the combination of Rayleigh and schlieren optics proved satisfactory for the determination of $\Delta\sigma/\sigma_1$ it appeared that the accuracy of the method was limited by the measurement of $\bar{c}$. Estimations of the effects of systematic errors in the experimental data indicated that more than one-half could be attributed to inaccuracies in determining this quantity by integration of the schlieren patterns. Hence efforts were directed toward the construction of a cell and appropriate mask which would permit both $\bar{c}$ and Δc to be measured interferometrically. This was accomplished by designing a cell containing three separate compartments for the two solutions and the solvent along with a mask and shutter system which provided interference patterns for each of the three pairs of compartments. The resulting cen-

[3] This assumption should be valid since the partial specific volumes of the subunits are essentially identical (Cohlberg *et al.*, 1972).

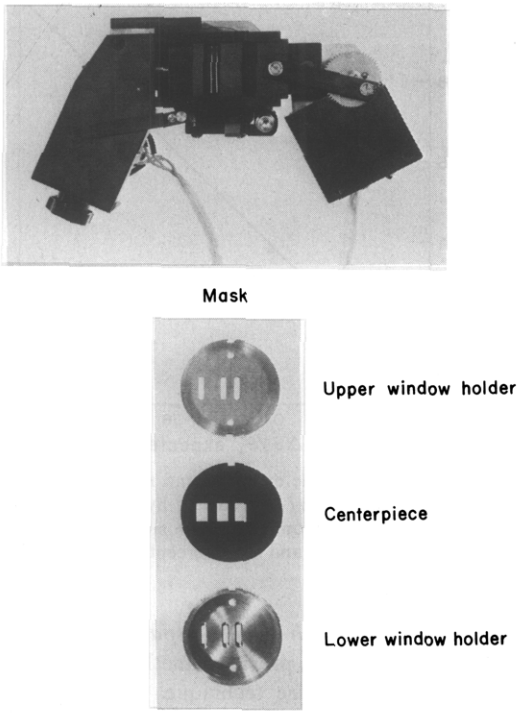

FIGURE 4: Photographs of the Rayleigh mask and ultracentrifuge cell used for experiments in which both $\bar{c}$ and Δc are determined interferometrically.

terpiece and Rayleigh mask are shown in Figure 4. Several features of the design should be noted.

First, the individual compartments (which are rectangular with width 2.5 mm and length 4.2 mm) and the corresponding slits in the Rayleigh mask are unevenly spaced. This makes it possible to obtain interference between any pair of compartments by positioning the shutter to expose the proper slits. Although light will still pass through all three compartments as the rotor turns, interference will occur only between the pair whose spacing is the same as that of the illuminated slits. Thus each of the solutions can be compared with the solvent as well as with each other. Second, the two compartments containing the solutions were arranged symmetrically with respect to a radius through the center of the cell; otherwise the measured concentration differences would not represent the true values as nonconjugate radial positions would be compared. Third, the cell was designed so that the inner compartments were used for the solutions. This arrangement was chosen because the fringe spacing is inversely proportional to the distance between the slits responsible for the interference pattern. Since the concentration differences are generally small, it is advantageous to have as large a fringe separation as possible in the Δc pattern. Fourth, the physical size of the rotor holes and the need for a reasonable wall thickness between openings limited both the spacing and the length of the compartments. The latter was not a problem since columns for sedimentation equilibrium experiments are short, but the former makes it impossible to design this cell to be compatible with Rayleigh masks used with conventional double-sector cells. Conversely, it makes the Rayleigh mask designed for the present application incompatible with other cells unless a fourth slit is added to provide a slit pair with the proper spacing. With this fourth slit, the conversion from one type of experiment to the other simply requires a translation of the mask and a movement of the shutter plate to expose the proper pair of slits.

This conversion, between types of sedimentation experiments, was made practical and the time necessary to align the mask shortened by using an externally controlled Ledex 216091-032 stepping motor to provide translational adjustment. The motor has 36 steps/revolution and drives a 32-pitch screw through a 5:1 gear train to provide a movement of about 4 μ/step. Limitations of space prevented motorizing the rotational adjustment as well. However, this adjustment is fixed by the position of the cylindrical lens and, provided the slits are parallel, there should be no need to realign the mask rotationally when converting between the two types of sedimentation experiments or after a drive has been changed. The rotational adjustment is similar to that of the previous design (Kirschner and Schachman, 1971; Richards *et al.*, 1972) with the exception that the scales were placed on the parts of the mask that rotate relative to one another. This allows the reproducible rotational positioning of the mask (to $\frac{1}{8}°$) regardless of any backlash that may be present. A second Ledex stepping motor (216100-032) is used to position the top plate which acts as the shutter. The entire mask is placed on the lower lens mount and fits in the vacuum chamber with a minor modification to the heater cup.

The use of the three-compartment cell presents a number of operational problems. First, the extra compartment contributes to the background fogging of the plate and consequently to the deterioration of the fringe quality. This significantly decreases the magnitude of the concentration gradient that can be resolved photographically. Although at present this is a limitation, it can be circumvented by using plates of higher contrast. Such photographic plates are very slow and reasonable exposure times can be obtained only if a high intensity light source, such as a laser, is used.

Second, the three-compartment cell gives rise to a series of partially overlapping images which combine to form rather complicated patterns. If the symmetrical slit pair is used (slits a and b in Figure 5A) the symmetrical sectors (1 and 2 in Figure 5A) will give a single image on the plate. This is true even though they are both imaged at different times by a and b as seen in Figure 5B–D, since when imaged these compartments are always at the same radial positions. Interference will occur when sectors 1 and 2 are simultaneously over slits a and b as in Figure 5C. As the rotor continues to turn, sector 3 is imaged first by slit b and they by a as in Figure 5E and F. However, since 3 is offset relative to 1 and 2, it is not at the same radial position as 1 and 2 and hence its image will not fully superimpose with theirs. Sector 3 does not give rise to interference fringes, and serves only to increase the background density and add an additional meniscus to the pattern. Figure 6 shows a photograph of a typical pattern. The image of sector 3 is shifted in a centrifugal direction so that the centripetal region of fringes has a lower density than the rest, and a light grayish area appears at radial positions beyond the fringes' right edge. The meniscus from sector 3 appears in the image of the solution column of sectors 1 and 2.

When slits b and c are used to allow interference between sectors 2 and 3, a similar situation occurs except this time four images are formed since slits b and c each image sectors 2 and 3 at different positions (sector 1 is imaged at the same positions as 2). The size of the overlaps and positions of the menisci can be quantitatively accounted for by simple geometric considerations of situation described in these paragraphs.

This multiple imaging can be beneficial as well as confusing because it provides a reference near the beginning of the solution which makes plate alignment less critical. This is important because a counterbalance that will allow interference with two slit pairs and still have the same exposure time as does the

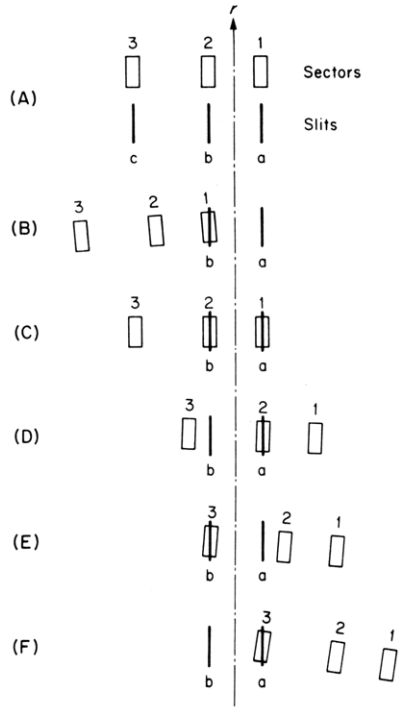

FIGURE 5: Factors which give rise to the complex image formed with the three-compartment cell and symmetric slit pair. The arrangement of the sectors and slits relative to the center line are shown in A. Sectors 1 and 2 and the corresponding slits a and b are the symmetrical pairs. The positions of the rotor at which image formation occurs are shown in B–F. Rotation is assumed to be clockwise and the center of rotation is located below the bottom of the diagram. Each sector is imaged first by slit b and then by a. Interference can occur only when the two sectors are over the slits simultaneously as in C. The asymmetric spacing prevents interference from any sector pair except 1–2 when slits a and b are used. Because they are at the same radial positions sectors 1 and 2 form only a single image, but 3 which is further from the axis of rotation has its image shifted in a centrifugal direction. Analogous patterns are obtained when the slit combination is either a and c or b and c. For such arrangements interference patterns are obtained for sector pairs 1–3 and 2–3.

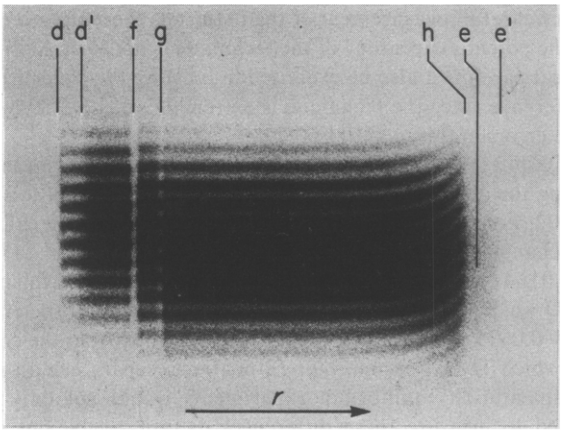

FIGURE 6: Typical fringe pattern obtained with slits a and b of Figure 5. The image formed by interference between sectors 1 and 2 (see Figure 5) is denoted by the interval dh. The fringe pattern does not extend to the cell bottom (e) because the concentration gradients in compartments 1 and 2 are so large that deviated light does not reach the photographic plate at radial levels beyond h. The image of sector 3 is shifted in a centrifugal direction and is indicated by d'e'. The plate density is greatest (except for the interval he) in the region where the images of all compartments superimpose. Areas of nonoverlap dd' and ee' have lower densities as does the interval he. In this particular experiment the compartments were equally filled; hence the menisci of 1 and 2 superimpose and are imaged at f while that of 3 is seen at position g.

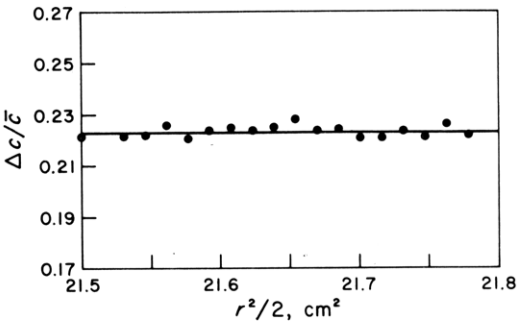

FIGURE 7: Results with the three-compartment cell. Identical solutions of catalytic subunit at 4.7 mg/ml in 0.1 M Tris-acetate buffer (pH 7.5) containing 10^{-3} M EDTA were placed in the two symmetrical compartments. Buffer was added to the offset sector. The plot used data from $\bar{c}$ = 1.2–6.6 fringes. The slope of the line gives a value for $\Delta\sigma/\sigma_1$ of 0.0004.

cell is not easily built. The reference used is the image of the meniscus from sector 3 formed by slit b which is the slit common to both pairs. Since the image of the meniscus appears in the liquid column, it can be unambiguously located.

Third, the nature of the asymmetric cell and the use of what is essentially an offset mask place a constraint on the buffer systems that can be used. The limitation occurs because the effective geometry of sector 3 is different from that of the other two and the offset mask compares nonconjugate radial positions. These differences are equivalent to about a 300-μ shift in radial position, so that buffer ions which redistribute significantly will cause an appreciable error in measuring $\bar{c}$. Normal buffers, however, can be used without any difficulty. For example, the error across the cell due to the redistribution of 0.1 M phosphate is only 0.004 fringe, but if 8 M guanidine hydrochloride is used an error of 0.4 fringe will be made, significantly altering the measured $\bar{c}$.

Our experience thus far with this cell and mask has been limited and some of its advantages and disadvantages have not yet been assessed. One such experiment with identical solutions of the catalytic subunit is illustrated by the data in Figure 7. The slope of the line gives a value for $\Delta\sigma/\sigma_1$ of 0.0004 ± 0.0011. This is similar in accuracy and precision to the results obtained earlier using schlieren optics to measure $\bar{c}$. However, the result was obtained using a lower average $\bar{c}$ (from 1.2 to 6.6 fringes). While this plot is not indicative of the increased accuracy and precision of the procedure, it does demonstrate that accurate measurements can be obtained at lower concentrations.

Discussion

As was shown in the experiments described above, the difference sedimentation equilibrium technique is capable of measuring very small changes in σ with a high degree of accuracy. The expression used to analyze difference sedimentation equilibrium experiments was derived in a manner that eliminated the direct effects of geometric differences between cell compartments on the determination of $\Delta\sigma$. As a consequence two quantities, the difference in concentration and the average concentration, must be measured as functions of radial position. The concentration difference between the sectors is obtained directly by employing the interferometer as the subtracting device. If standard ultracentrifuge cells are used the average concentration can only be determined by the integration of the schlieren pattern. This presents a problem because of the relative imprecision of schlieren optics and the lack of a base line.

Very careful measurement of the pattern is therefore necessary if the potential accuracy of the technique is not to be sacrificed. Great care must also be exercised in labeling the concentration difference since the technique is extremely sensitive to systematic errors in this quantity.

Despite these difficulties, $\Delta\sigma/\sigma_1$ was routinely measured, using the above procedures, with an absolute error of less than 0.002. For example, when sufficient D_2O to produce a calculated change of -0.0150 was used a measured value for $\Delta\sigma/\sigma_1$ of -0.0164 was obtained. A second experiment, in which less D_2O was added, gave a value of -0.0084 instead of the expected -0.0071. A result of -0.0009 was obtained from the control in which D_2O was omitted. In contrast, separate high-speed sedimentation equilibrium experiments would not only have failed to measure these differences quantitatively, but could not even have demonstrated their existence. The data used in the analyses of these experiments were restricted to concentrations greater than 2 fringes. Values of $\Delta c/\bar{c}$ obtained at lower concentrations are very sensitive to systematic errors and therefore may introduce substantial inaccuracy in the measured change in σ. This is illustrated in Figure 2C which shows the results of one of the D_2O experiments. The plot has been constructed using data from $\bar{c}$ = 0.56–9 fringes. Considerable curvature occurs at the lowest concentrations and a satisfactory value of $\Delta\sigma/\sigma_1$ [$\delta(\Delta\sigma/\sigma_1) < 0.002$] was not obtained unless all points below $\bar{c}$ = 2 fringes were ignored. The hyperbolic nature of the plot results from the errors which occurred in labeling Δc and $\bar{c}$ (see Figure 6; Springer *et al.*, 1974). Qualitatively similar results were found in other experiments although both the degree of curvature and the concentration at which it became negligible were somewhat variable. This variation reflects small differences in the magnitude of the systematic errors between separate experiments. In general, however, data above $\bar{c}$ = 2 fringes consistently yielded satisfactory values for $\Delta\sigma/\sigma_1$.

The difference sedimentation equilibrium method is remarkably insensitive to cell defects and many of the expected errors in experimental technique. Imperfections in ultracentrifuge cells, however, affect the measurements of $\Delta\sigma$ indirectly by altering the $\delta\bar{c}$ term of the error functions (eq 24 and 25, Springer *et al.*, 1974). These defects are unavoidable because current technology does not allow the manufacture of centerpieces with the precision necessary to prevent perturbations of the difference concentration distribution. Errors due to faulty sample handling are indistinguishable from those caused by cell imperfections. Mismatches of initial concentrations and misalignment of the cell in the rotor are identical in effect with differences in optical path length, bottom mismatches, and variances in sector geometry. Similarly discrepancies in the radial positions of the two menisci may arise either from incorrectly filling the compartments or from spatial differences between them. The loss of accuracy, however, is generally small, provided the defects are not unreasonably large. This is illustrated by the results from the D_2O titration in which measurements were made with cells containing effective bottom mismatches that sometimes exceeded 50 μ. The average absolute error in $\Delta\sigma/\sigma_1$ in these experiments was not significantly larger than that calculated for a hypothetical perfect cell. Likewise the failure to use optimal initial concentrations has little effect. This was also demonstrated by the results obtained with D_2O since these experiments were performed with initial concentrations 2–5 times higher than recommended without an appreciable decrease in accuracy.

Despite the insensitivity of the technique to imperfections in equipment and experimental procedure, these factors must be considered in the choice of a rotor speed if meniscus depletion is used to label Δc. Otherwise the various defects, which can increase the size of Δc at the meniscus by fivefold or more, may cause a sizable error in determining the absolute values of the concentration differences.

The accurate measurement of a change in molecular weight is dependent upon the proper alignment of the Rayleigh mask. The mask must be positioned so that the slits are symmetric about, and parallel to, a reference radius which is itself parallel to the axis of the cylindrical lens. If this orientation is not achieved a systematic error in measuring $\Delta\sigma$ will result. The tolerance for translational and rotational alignment depends upon the size of the error which is permissible. To reduce this inaccuracy to a negligible value the mask should be positioned within 10 μ and 0.1°. Although it is possible to achieve sufficient accuracy with the general utility mask (Rosenthal, 1969; Richards *et al.*, 1972), it is an extremely time-consuming and frustrating task. The mask described by Bowers and Haschemeyer (1969) affords some improvement as it permits external control of the rotational adjustment. The precision mask (Kirschner, 1971; Richards *et al.*, 1972) is more easily aligned than either of the preceding since the adjustments have greater sensitivity and allow the translational positions to be reproduced with a fair degree of accuracy. The mask described here is even more advantageous because of the externally adjustable translational control, and the ease of reproducing, at will, any desired translational or rotational position with a high degree of accuracy. The procedure for aligning the mask is sufficiently sensitive to position it within the required tolerances.

The use of Rayleigh optics to measure concentration distributions depends on the relation between fringe displacement and concentration (or concentration difference). If the slits in the mask are parallel this relation should be constant. However, it has been shown in velocity experiments that the fringe spacing and therefore the displacement increases with a lower mask (Kirschner, 1971; Richards *et al.*, 1972) acreases with an upper mask (Richards and Schachman, 1959) in regions associated with a high-concentration gradient. It is important to consider this phenomenon in the analysis of the difference concentration distributions because the regions which are most useful for determining $\Delta\sigma$ occur at high gradients. If fringe separation increased (bowing) over this region and was not taken into account, an error in the measured value of $\Delta\sigma$ would result. The shallow nature of difference concentration patterns lends itself nicely to measuring the bowing effect. However, the phenomenon does not seem to be present in these experiments as the fringe separation remained constant across the entire gradient in the three plates that were examined. While this does raise some question as to the nature of the effect, its absence removes concern over inaccuracies which bowing would have caused.

The analysis of a difference molecular weight experiment requires the comparison of two quantities as a function of radial position and is therefore more susceptible than other techniques to inaccuracies in plate alignment. While this is normally a very accurate procedure, precise alignment of the schlieren pattern, which lacks fringes in the counterbalance holes, can be accomplished only with great difficulty. Uncertainties in alignment frequently produce discrepancies in radial positions of 10–20 μ. However, the effect of errors of this magnitude on the measurement of $\Delta\sigma$ is slight. For example, if the radial positions of the patterns were out of register by 20 μ an error of 0.0002 would result in the measured $\Delta\sigma/\sigma_1$, for σ_1 = 5 cm^{-2}. At lower values of σ_1 the inaccuracy would be smaller still. Hence plate misalignment will not be a problem if reasonable care is exercised.

The use of schlieren optics to measure $\bar{c}$, although allowing the determination of $\Delta\sigma$ with conventional cells, limits both the accuracy and concentration range of the technique. For these reasons a special three-compartment cell and Rayleigh mask which permit the interferometric determination of both $\bar{c}$ and Δc were designed.[4] The spacing of the compartments and slits is asymmetric so that when a given slit pair is exposed only the sector pair with the corresponding separation can cause interference. The slits to be used can be chosen at will by positioning an externally adjustable top plate which serves as a shutter. Although the testing has not yet been completed, the new equipment seems capable of decreasing the absolute error in measuring $\Delta\sigma/\sigma_1$ to 0.001 if data from 2 to 10 fringes are employed. Alternatively, if an error of 0.002 is acceptable, usable data can be obtained from concentrations below 1 fringe (0.25 mg/ml). This concentration limit applies to 12-mm cells. If optical artifacts do not interfere, concentrations as low as 0.1 mg/ml may provide meaningful data when 30-mm cells are employed.

Although we have considered only the interferometer as the subtracting device, the split-beam photoelectric scanner is in principle equally applicable. In fact, it has been employed by Barlow *et al.* (1969) to measure a difference in molecular weight. However, no details of the experiment or the method used to analyze it were published. Absorption optics have not been used in the work described in this communication because current scanners do not possess the accuracy required by the technique, and the existing multiplexing systems are not sufficiently versatile to allow the measurement of both $\bar{c}$ and Δc in a single experiment. Despite the technological limitations the scanner has two inherent advantages that more than justify the effort required to build an instrument with the necessary accuracy and flexibility. First, and most important, absorption optics have the sensitivity necessary to lower the usable concentration range to the μg/ml region where many interesting biological phenomena occur. Second, the scanner has the ability to discriminate between various molecular species through the selective use of particular wavelengths.

[4] The authors are indebted to Dr. D. C. Teller for suggesting an alternative procedure for obtaining both Δc and $\bar{c}$ without requiring the three-compartment cell and special Rayleigh mask. This method employs a six-compartment Yphantis cell with sectorial openings so that the absolute values of the concentrations of each solution can be measured in the conventional manner from two pairs of compartments and Δc can be measured from the remaining pair. Use is then made of a transform which permits the concentration distribution of the solute molecules to be transformed from one coordinate system to another provided that the positions of the menisci and liquid bottoms are known precisely in both systems. In this way the concentration distributions of the two solutions in the upper and lower compartments can be related directly to the difference concentration distribution measured in the middle compartments. This procedure has the merit of being unaffected by the redistribution of buffer ions and provides independent evidence for the concentrations at the meniscus.

Acknowledgment

The authors would like to thank Mrs. Boihon Yee for her excellent technical assistance.

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