# A Difference Sedimentation Equilibrium Technique for Measuring Small Changes in Molecular Weight. I. Theory and Computer Simulation†

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ABSTRACT: Measurements of small changes in molecular weight are essential for the analysis of both strongly and weakly self-associating proteins and for the study of the effect of ligands on the association–dissociation equilibria. In addition, such measurements provide the basis for interpreting data from difference sedimentation velocity experiments since changes in sedimentation coefficients are due to either alterations in frictional coefficients or to shifts in association–dissociation equilibria involving monomers and oligomers. Since very small changes in molecular weight can be determined only indirectly from separate experiments and conventional methods have limited accuracy, we have developed a difference sedimentation equilibrium technique for the direct measurement of differences in effective molecular weight ($\Delta\sigma$). A theoretical treatment is presented which shows that $\Delta\sigma$ is obtained directly from the slope of a plot of the difference in concentration ($\Delta c$) divided by the average concentration ($\bar{c}$) *vs.* the square of the radial position. With double-sector ultracentrifuge cells, $\Delta c$ can be measured directly with interference optics and $\bar{c}$ can be determined by integration of the schlieren pattern. The slope of the plot is shown theoretically to be independent of cell imperfections, and mismatches in menisci, cell bottoms, and initial concentrations provided that there are no experimental errors in the data. Computer simulations were used to confirm these conclusions and to evaluate the effect of mismatches on $\Delta c$ and $\bar{c}$. When the data are subject to either random or systematic errors defects in the cell or experimental technique will affect the magnitude of both $\Delta c$ and $\bar{c}$; however, as shown by a theoretical treatment of the potential accuracy of the method, the determination of $\Delta\sigma$ is affected only slightly. Computer simulations of high-speed difference sedimentation equilibrium experiments indicate that accurate measurements can be made even for changes in effective molecular weight as small as 1%.

In recent years much interest has been focused on proteins which show concentration-dependent or ligand-mediated changes in their state of aggregation. These systems are most often studied with equilibrium or transport techniques which have provided much of our knowledge of the association–dissociation behavior of proteins.

Although the transport methods are exceedingly sensitive to the type of association–dissociation equilibria and the strength of the interactions, their applicability is limited by the lack of rigorous theory necessary to explain the complex behavior of self-associating systems undergoing transport in a centrifugal field or during chromatography (Gilbert, 1955, 1959; Cox, 1969, 1971; Cann, 1970; Nichol and Winzor, 1972; Zimmerman *et al.*, 1971). Hence, equilibrium techniques, and sedimentation equilibrium in particular, have been more extensively employed (Adams, 1964, 1965, 1967; Adams and Williams, 1964; Roark and Yphantis, 1969; Teller *et al.*, 1969). These techniques, however, do not possess the sensitivity of the transport procedures, and in addition do not always make a clear distinction between reversible self-associating and heterogeneous noninteracting systems; thus the two types of methods are complementary. Ideally, a transport technique is used to demonstrate (or confirm) the presence of an association–dissociation equilibrium and an equilibrium method then employed to analyze the system. The validity of the model proposed from the analysis should subsequently be tested by comparing the actual transport behavior with a computer simulation.

The use of either the two techniques together or sedimentation equilibrium alone is limited by the inability to measure small differences in molecular weight. Since, at present, molecular weights can be measured with a precision of only 2–3%, slight changes in the state of aggregation cannot be interpreted unambiguously in terms of association–dissociation equilibria. Hence there is a need for a method which is capable of measuring quantitatively small differences in molecular weights. Accordingly, we have developed a difference sedimentation equilibrium technique which provides directly accurate results for changes in molecular weight as small as 1%.

The protocol of the method is similar to that of a normal equilibrium experiment except that the second solution is used instead of the solvent to fill the reference sector. A subtractive optical system, such as the Rayleigh interferometer, produces a pattern which represents the difference between the concentration distributions of the macromolecules in the two compartments. These data when combined with knowledge of the average of the concentrations ($\bar{c}$) in the two sectors, as a function of radial position, yield the difference in effective molecular weight ($\Delta\sigma$). Mathematical manipulation of the expression relating the concentration distribution at equilibrium to molecular weight allows $\Delta\sigma$ to be computed from the slope of the plot of the difference in concentration ($\Delta c$) divided by the average concentration *vs.* the square of the radial distance ($r$). When perfect data are employed, theoretical considerations show that

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the slope of this plot is independent of defects in the cells, mismatches in menisci, cell bottoms, and initial concentrations. Computer simulations have been used to determine optimal experimental conditions and to evaluate the effects of imperfections in apparatus and technique on the observed parameters, $\Delta c$ and $\bar{c}$. An analysis of errors shows that with data subject to random or systematic inaccuracies these cell defects and mismatches cause only a relatively small effect on the determination of $\Delta\sigma$.

In this communication we describe the theoretical basis for the technique and in the following paper we present details of the experimental procedures and their applications to known and unknown systems.

## General Considerations

Difference techniques are often employed when it is necessary to measure quantitatively changes in the magnitude of a parameter which are small relative to the parameter itself. For example, perturbations in the ultraviolet spectrum of a protein resulting from the addition of a ligand are usually determined by comparing directly the spectrum of the macromolecule plus the ligand to that of the protein alone. Recently it has been shown in this laboratory (Kirschner and Schachman, 1971) that small changes in sedimentation coefficient can be measured far more accurately with a difference technique than by subtracting the values obtained in separate experiments. Consequently, it seemed likely that small differences in molecular weight could be determined in a similar manner.

The use of sedimentation equilibrium is based on the relationship between the molecular weight of a substance and its concentration as a function of radial position in a centrifugal field. Thus the difference in molecular weights between two substances will be related to the difference between their concentration distributions. The profile of the concentration difference can be obtained directly with a double-sector cell by using the second sample instead of the solvent to fill the reference compartment. Hence, unlike the conditions of a normal sedimentation equilibrium experiment in which one sector contains the sample and the other the solvent, each compartment contains one of the two substances for which the molecular weight difference is desired. An optical system which subtracts a physical parameter such as optical density or refractive index of one solution from that of the other will yield a pattern which represents the difference concentration distribution. The observed distribution will be due solely to the solutes of interest provided the buffers are identical or are not detected by the optical system employed.

The success of a difference sedimentation equilibrium technique, as measured by its ability to determine small changes in molecular weight more accurately than is possible with other procedures, depends as much on the method of analysis as on the reliability of the data. In particular, there are two criteria which should be satisfied if a significant improvement in accuracy relative to existing techniques is to be realized. First, the equation used to calculate $\Delta\sigma$ must not be a function of the size or shape of the liquid column. This requirement is necessary since frequently there are substantial geometric differences between the two compartments of a double-sector ultracentrifuge cell. Such variations can drastically alter the difference concentration distribution and lead to considerable difficulty in interpreting the data correctly unless the analysis is independent of these effects. Second, the technique should make use of data from the entire solution rather than relying on a single point. The use of all the information contained in the difference concentration distribution allows a more complete description of the system under investigation as well as giving a statistically more reliable value for the change in molecular weight. The derivation (Kirschner, 1971) which follows satisfactorily fulfills both requirements.

## Theory

The equilibrium distributions of two ideal solutions of single components can be expressed in terms of the effective molecular weights using the well-known thermodynamic relationships

$$\mathrm{d}\ln c_1/(\mathrm{d}r^2/2) = \sigma_1 \tag{1}$$

and

$$\mathrm{d}\ln c_2/(\mathrm{d}r^2/2) = \sigma_2 \tag{2}$$

where the subscripts 1 and 2 refer to solutions 1 and 2, $c$ is the concentration at the radial level, $r$, and $\sigma \equiv M(1 - \bar{V}\rho)\omega^2/rt$ (Yphantis and Waugh, 1956), where $M$ is the molecular weight, $\bar{V}$ is the partial specific volume, $\rho$ is the density of the solution, $\omega$ is the angular velocity of the rotor, $R$ is the gas constant, and $T$ is the absolute temperature. Subtracting eq 1 from eq 2 gives

$$\frac{\mathrm{d}\ln c_2 - \mathrm{d}\ln c_1}{\mathrm{d}r^2/2} = \frac{\mathrm{d}(\ln c_2 - \ln c_1)}{\mathrm{d}r^2/2} = \sigma_2 - \sigma_1 = \Delta\sigma \tag{3}$$

We now define two quantities, the average concentration, $\bar{c}$, and the difference in concentration, $\Delta c$

$$\bar{c} = (c_1 + c_2)/2 \tag{4}$$

$$\Delta c = c_2 - c_1 \tag{5}$$

With these definitions we obtain

$$c_1 = \bar{c} - \Delta c/2 \tag{6}$$

and

$$c_2 = \bar{c} + \Delta c/2 \tag{7}$$

as well as

$$\ln c_1 = \ln(\bar{c} - \Delta c/2) = \ln\bar{c} + \ln(1 - \Delta c/2\bar{c}) \tag{8}$$

and

$$\ln c_2 = \ln(\bar{c} + \Delta c/2) = \ln\bar{c} + \ln(1 + \Delta c/2\bar{c}) \tag{9}$$

Substituting eq 8 and 9 into eq 3 gives

$$\frac{\mathrm{d}[\ln(1 + \Delta c/2\bar{c}) - \ln(1 - \Delta c/2\bar{c})]}{\mathrm{d}r^2/2} = \Delta\sigma \tag{10}$$

Since $\Delta c/2\bar{c}$ is always 1 or less, we can expand the logarithmic terms to give

$$\ln(1 + \Delta c/2\bar{c}) = \frac{\Delta c}{2\bar{c}} - \frac{1}{2}\left(\frac{\Delta c}{2\bar{c}}\right)^2 + \frac{1}{3}\left(\frac{\Delta c}{2\bar{c}}\right)^3 + \ldots + \frac{-(-1)^n}{n}\left(\frac{\Delta c}{2\bar{c}}\right)^n \tag{11}$$

and

$$\ln(1 - \Delta c/2\bar{c}) = \frac{-\Delta c}{2\bar{c}} - \frac{1}{2}\left(\frac{\Delta c}{2\bar{c}}\right)^2 - \frac{1}{3}\left(\frac{\Delta c}{2\bar{c}}\right)^3 + \ldots + \frac{-1}{n}\left(\frac{\Delta c}{2\bar{c}}\right)^n \tag{12}$$

Substituting eq 11 and 12 into eq 10 yields

$$\frac{\mathrm{d}\sum_{n=0}^{\infty}\frac{(\Delta c/\bar{c})^{2n+1}}{(2^{2n})(2n+1)}}{\mathrm{d}r^2/2} = \Delta\sigma \tag{13}$$

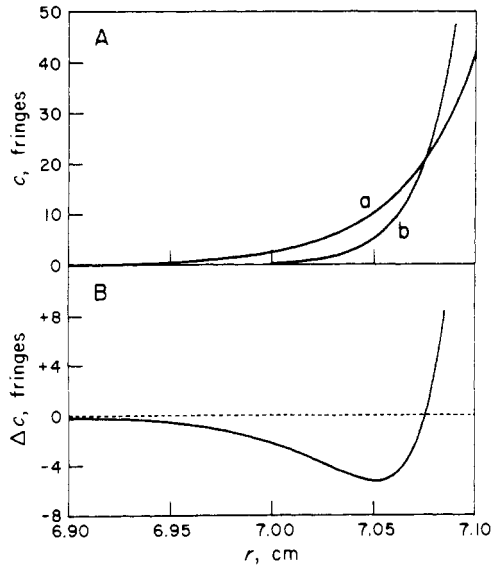


FIGURE 1: Concentration and difference concentration distributions in a high-speed sedimentation equilibrium experiment. (A) Curves a and b show the concentration distributions for two macromolecules whose molecular weights are in the ratio of 2:1. The experimental conditions were chosen so that the values of $\sigma$ for a and b are 4 and 8 $cm^{-2}$, respectively. Both initial concentrations are 4 fringes. (B) The figure shows the difference in concentration between the distributions (b − a) in (A) as a function of radial position.

which for small values of $\Delta c/\bar{c}$ reduces to

$$(d\Delta c/\bar{c})/(dr^2/2) = \Delta\sigma \qquad (14)$$

Equation 13 was derived from the differential form of the sedimentation equilibrium equation without assumptions regarding cell geometry. Hence the computation of $\Delta\sigma$ using eq 13 does not require that the liquid columns in the two compartments be geometrically identical. In addition, the series in eq 13 converges so quickly that normally only the first term need be considered. Thus the simplified expression given by eq 14 is generally sufficient. For example, if $\Delta c/\bar{c}$ were 0.5, a value higher than those typically encountered, the second term of the series would be only 0.01 or 2% of the first. For smaller values of $\Delta c/\bar{c}$ the higher terms become even less significant. Hence the slope of the plot of $\delta c/\bar{c}$ *vs.* $r^2/2$ gives directly the difference in effective molecular weight.[1]

## Computational Methods

The simulated experiments were performed with the use of either a Control Data 6400 computer or a Hewlett-Packard 9820a desk-top calculator equipped with a 9862a plotter. All simulations assumed sector-shaped cell compartments and the concentration distributions (Svedberg and Pedersen, 1940) were calculated from

$$c = \frac{c_0\sigma(b^2 - a^2)e^{\sigma r^2/2}}{e^{\sigma b^2/2} - e^{\sigma a^2/2}} \qquad (15)$$

where $c_0$ is the initial concentration, $a$ and $b$ are the positions of the meniscus and cell bottom, respectively, and $c$ is the concentration at radial position, $r$. Unless stated otherwise, the initial concentration employed in the calculations was 20 fringes. Concentrations are given in terms of fringes because the difference molecular weight technique was designed primarily for use with the Rayleigh optical system. Conversion from fringes to mg/ml is accomplished by dividing the value in fringes by the constant of proportionality which is normally four fringes/mg/ml for a cell of 12-mm path length (Richards and Schachman, 1959; Babul and Stellwagen, 1969). The positions of the meniscus and cell bottom were 6.8 and 7.1 cm, respectively, in all calculations.

## Simulated Experiments

For simplicity and to clarify the effects of imperfections in ultracentrifuge cells and the unavoidable errors in handling samples, we consider two types of simulated experiments. The first assumes the cells to be perfect and that no mismatches in menisci, cell bottoms, or initial concentration have occurred. This allows a simple assessment of the effects of rotor speed and the magnitude of $\Delta\sigma/\sigma_1$ on the observed patterns. The second treats the more realistic situation in which imperfections are present so that their effects on the difference concentration profiles can be evaluated. The results show that there is no influence on the measurement of $\Delta\sigma$ provided that the calculations are made with data which are free of experimental error.

*Perfect Cells.* Figure 1 shows the concentration distributions from a high-speed experiment in a double-sector cell using two substances, one in each compartment, whose molecular weights were in the ratio of 2:1. The concentration distribution in each sector is shown in Figure 1A, and the concentration difference as a function of radial position is presented in Figure 1B. As seen in Figure 1A, the two curves start essentially at zero, diverge, and then cross further down the cell. As a result the difference pattern cannot be a monotonic curve but must contain a relative extremum and crossover as shown in Figure 1B. Although this may not have been expected, it can be explained in a physical sense by considering the relationship between the effective molecular weight of a substance and its distribution in a centrifugal field. The heavier the species the greater its redistribution and the higher its concentration at the bottom of the cell. The concentration of the lighter species must, therefore, by conservation of mass, be greater in the centripetal region of the liquid column. Since the concentration of both species is essentially zero at the meniscus, an extremum as well as a crossover results. For perfect cells the crossover always occurs, but the extremum requires a $c_b/c_m > 7$ or a $\sigma_1 > 0.8$ $cm^{-2}$ (this value is only slightly dependent on $\Delta\sigma/\sigma_1$). While the extremum does exist mathematically at values of $\sigma_1 < 0.8$ $cm^{-2}$, it no longer has physical reality as it occurs at radial positions centripetal to the meniscus.

The positions of both these points are readily related to $\Delta\sigma$. If we represent $\Delta c$ by

$$\Delta c = A_2 e^{\sigma_2 r^2/2} - A_1 e^{\sigma_1 r^2/2} \qquad (16)$$

where

$$A = \frac{c_0\sigma(b^2 - a^2)}{e^{\sigma b^2/2} - e^{\sigma a^2/2}}$$

the position of the extremum can be computed by evaluating $r$ at $d\Delta c/dr$ equal to zero. The crossover position is obtained by setting eq 16 equal to zero and calculating $r$.

The algebraic manipulations are greatly simplified if one makes the assumption that $e^{\sigma b^2/2} > e^{\sigma a^2/2}$ (which is valid for $\sigma > 1$). The resulting equations relating the extremum and crossover to $\Delta\sigma$ are

[1] Dr. I. M. Klotz has kindly provided us with an alternative derivation for the evaluation of difference sedimentation equilibrium experiments. This treatment permits the calculation of $\Delta\sigma$ from the position of the crossover. His expression is not the same as that derived below and does not require knowledge of the geometry of either sector. We are indebted to Dr. Klotz for communicating his treatment.

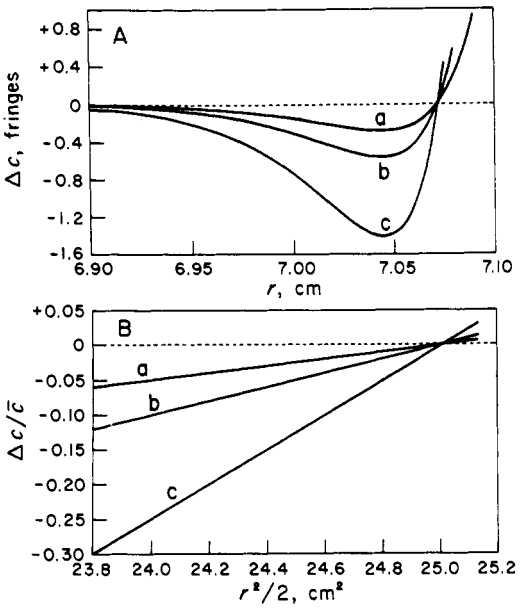


FIGURE 2: Difference sedimentation equilibrium as a function of $\Delta\sigma$. (A) Curves a, b and c show the $\Delta c$ distributions, in a cell with sector-shaped compartments for values of $\Delta\sigma/\sigma_1$ of 1, 2, and 5%, respectively. These curves were calculated for an initial concentration of 20 fringes, meniscus and bottom positions of 6.8 and 7.1 cm, and a $\sigma_1$ of 5 $cm^{-2}$. (B) Data from the above experiments were plotted as $\Delta c/\bar{c}$ *vs.* $r^2/2$. The slopes of lines a, b, and c are 0.05, 0.10, and 0.25, respectively.

$$r_e = \sqrt{b^2 - \frac{4}{\sigma_1} + \frac{2\Delta\sigma}{\sigma_1^2}} \qquad (17)$$

and

$$r_c = \sqrt{b^2 - \frac{2}{\sigma_1} + \frac{\Delta\sigma}{\sigma_1^2}} \qquad (18)$$

where $r_e$ and $r_c$ are the positions of the extremum and crossover points, respectively. These positions as shown by eq 19 and 20 are relatively insensitive to changes in $\Delta\sigma$

$$dr_e = -\frac{d\Delta\sigma}{r_e\sigma_1^2} \qquad (19)$$

$$dr_c = -\frac{d\Delta\sigma}{2r_c\sigma_1^2} \qquad (20)$$

Thus the values of $r_e$ and $r_c$ are of little value in measuring small differences in $\sigma$ (in fact, as will be shown later, neither the crossover nor the extremum is observed under normal experimental conditions).

The large inequality of molecular weights used in Figure 1 was necessary to illustrate the relevant features in the concentration and difference concentration distributions. In general we are interested in much smaller differences in molecular weights, usually less than 10%. Typical difference patterns for values of $\Delta\sigma/\sigma_1$ of 1, 2, and 5% are shown in Figure 2A. As expected, the magnitudes of the values of $\Delta c$ are very small and almost proportional to $\Delta\sigma/\sigma_1$. Although not obvious from Figure 2A, the crossover point moves very slightly down the cell as $\Delta\sigma/\sigma_1$ increases. Likewise the position of the extremum also changes as $\Delta\sigma/\sigma_1$ is altered, and again this movement is not easily detected by visual inspection of the curves. Figure 2B shows the plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$ for these particular experiments. Since $\sigma_1$ = 5 $cm^{-2}$, the calculated slopes of 0.050, 0.100, and 0.25 for the 1, 2, and 5% changes are correct.

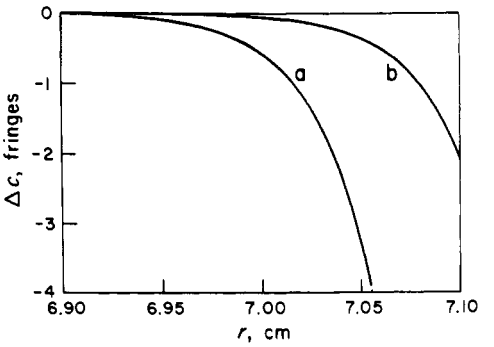


FIGURE 3: Effect of mismatches on the difference concentration distribution. Curve a shows the difference concentration distribution which results from a bottom mismatch of 30 $\mu$ when identical solutions are used to fill both cell compartments. The rotor speed was adjusted so that $\sigma_1$ = 5 $cm^{-2}$, the initial concentrations were 20 fringes, and the position of the meniscus in each sector was 6.8 cm. The cell bottoms are at 7.1000 and 7.1030 cm. Curve b represents the distribution that results from a meniscus mismatch of 30 $\mu$ or a difference in initial concentrations of 1%. In both cases the bottom positions of the two sectors are identical.

*Real Cells.* In actual experiments both ultracentrifuge cells and experimental techniques are subject to imperfections. The positions of the cell bottoms are usually mismatched by 30–50 $\mu$, the radial walls are often slightly bowed, and the path lengths of the two compartments are seldom identical. Similarly, the radial positions of the menisci generally differ slightly, the cell is rarely aligned perfectly in the rotor, and the initial concentrations of the two solutions may not be precisely equal.

Although errors in technique and defects in ultracentrifuge cells should, theoretically, have no effect on the determination of $\Delta\sigma$ from perfect data, they do affect the measured quantities, $\Delta c$ and $\bar{c}$. As shown by the theoretical treatment both $\Delta c$ and $\bar{c}$ are dependent on the exact geometric conditions of the experiment as well as on $\sigma_1$ and $\Delta\sigma$. We restrict this discussion to effects of mismatches in menisci, cell bottoms, and concentrations, and will not consider geometric distortions of the sectors because their effects will be similar to those of the other imperfections and are difficult to calculate. The different mismatches are caused either by errors in sample handling or defects in cells. For instance, discrepancies in the positions of the bottoms of the liquid columns can occur either from misaligning the cell in the rotor or from imperfections in the molded centerpieces. Differences in optical path length result from actual discrepancies in initial concentrations or from inequalities in the thicknesses of the sectors. Likewise, meniscus mismatches are due to errors in filling the cells or to imperfections in the centerpieces.

Inspection of the integration constant, $A$, shows that bottom mismatches will have the largest effect since $e^{\sigma b^2/2}$ is the dominant term. Figure 3 illustrates the effect of unequal bottom positions on the difference concentration distribution for a high-speed experiment in which $\Delta\sigma$ is zero. If the cell compartments were perfect and the solution columns identical, $\Delta c$ would be zero throughout the cell. When a bottom mismatch of 30 $\mu$ (an average value for a typical cell) is introduced a substantial difference concentration distribution results. As shown in Figure 3 similar, although smaller, changes are produced by discrepancies in initial concentrations and mismatches of menisci. The effect of a similar bottom offset on the $\Delta c$ pattern for an experiment in which $\Delta\sigma/\sigma_1$ = 1% is illustrated in Figure 4. This defect has completely masked the extremum and crossover points, producing a monotonic curve.

In actual experiments there may be a combination of mismatches. Figure 5A shows the calculated difference concentra-

tion distribution for a $\Delta\sigma/\sigma_1$ of 1% when the discrepancies in the bottoms, menisci, and initial concentrations are 30 $\mu$, 100 $\mu$, and 10%, respectively, as well as the distribution obtained from a perfect cell. The third curve represents the difference concentration distribution when the mismatches are reversed in the two compartments of the cell. Despite the striking discrepancies in the observed patterns, the slopes of the plots of $\Delta c/\bar{c}$ *vs.* $r^2/2$, as shown in Figure 5B, are not affected; hence the correct result of 0.050 was obtained in all the simulated experiments.

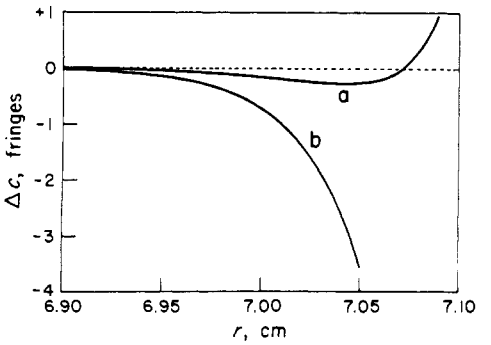


FIGURE 4: Effect of a bottom mismatch on a nonzero difference concentration distribution. Curve a shows the distribution in a perfect cell for a $\Delta\sigma/\sigma_1$ = 1%. In this simulated experiment $\sigma_1$ is 5 $cm^{-2}$ and all other parameters are as described in Figure 2. Curve b shows the distribution that results when the 30 $\mu$ bottom mismatch used in Figure 3 is superimposed on the above experiment.

## Analysis of Errors

In the previous section difference sedimentation equilibrium experiments were simulated using computer-generated data. However, these data, unlike the experimentally measured quantities, contained neither systematic nor random errors. The effect of such errors on the accuracy of the measured value of $\Delta\sigma$ is evaluated in the following analysis. We consider these effects as a function of concentration and discuss how they are influenced by the operational parameters of rotor speed and initial concentration. To separate these effects from those which arise from defects in equipment and technique we treat only ideal cells in this portion of the analysis. Finally we consider the consequences of cell imperfections on the accuracy of the method.

*Derivation of Error Functions.* The inaccuracy in the dependent variable of a function which results from errors in the independent variables is given by the total differential of that function. For difference sedimentation equilibrium experiments the value of $\Delta\sigma$ can be expressed in terms of $\Delta c$, $\bar{c}$, and $r^2$. The differential of this relationship is given by

$$\delta\Delta\sigma = \frac{\partial\Delta\sigma}{\partial\Delta c}\delta\Delta c + \frac{\partial\Delta\sigma}{\partial\bar{c}}\delta\bar{c} + \frac{\partial\Delta\sigma}{\partial r^2}\delta r^2 \quad (21)$$

It is usually assumed that measurements of $r^2$ are free of error so that eq 21 simplifies to

$$\delta\Delta\sigma = \frac{\partial\Delta\sigma}{\partial\Delta c}\delta\Delta c + \frac{\partial\Delta\sigma}{\partial\bar{c}}\delta\bar{c} \quad (22)$$

There are two expressions from which $\delta\Delta\sigma$ can be calculated depending on whether an average over the solution column or a point error is desired. The slope of the plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$ and hence $\Delta\sigma$ is given by the least-squares formula

$$\Delta\sigma = \frac{N\sum\frac{r^2}{2}\frac{\Delta c}{\bar{c}} - \sum\frac{r^2}{2}\sum\frac{\Delta c}{\bar{c}}}{N\sum\frac{r^4}{4} - \left(\sum r^2/2\right)^2} \quad (23)$$

The differential, $\delta\Delta\sigma$, assuming that $\delta\Delta c_i = \delta\Delta c_j = \delta\Delta c$ (where $i$ and $j$ refer to any two points) and $\delta\bar{c}_i = \delta\bar{c}_j = \delta\bar{c}$ can be written

$$\delta\Delta\sigma = \frac{\left|\left(N\sum\frac{r^2}{2\bar{c}} - \sum\frac{r^2}{2}\sum\frac{1}{\bar{c}}\right)\delta\Delta c\right| + \left|\left(\sum\frac{r^2}{2}\sum\frac{\Delta c}{\bar{c}}\frac{1}{\bar{c}} - N\sum\frac{r^2}{2\bar{c}}\frac{\Delta c}{\bar{c}}\right)\delta\bar{c}\right|}{N\sum\frac{r^4}{4} - \left(\sum\frac{r^2}{2}\right)^2} \quad (24)$$

Hence eq 24 is used to calculate the average error in an experiment. A point error can be calculated from eq 14. Upon differentiation we obtain

$$\delta\Delta\sigma = \left|\left(\frac{\sigma_1}{\bar{c}} + \frac{\Delta\sigma}{2\bar{c}}\right)\delta\Delta c\right| + \left|\left[\frac{\Delta c}{\bar{c}^2}\left(\sigma_1 + \frac{\Delta\sigma}{2}\right) - \frac{\Delta\sigma}{\bar{c}}\right]\delta\bar{c}\right| \quad (25)$$

The value of $\delta\Delta\sigma$ calculated from eq 24 is actually the result for systematic errors. An approximate value of $\delta\Delta\sigma$ due to random errors is obtained by dividing this quantity by $(N-1)^{1/2}$, where $N$ is the number of data points used.

*Limitations Due to Ultracentrifuge Optical Systems.* In evaluating potential errors we must first consider limitations of the ultracentrifuge optical systems. Although it has been calculated by Richards *et al.* (1972) that current optical systems are capable of resolving gradients as high as 500 fringes/cm, there may be another limitation imposed by the physical dimensions of components in the system. This occurs because the axial deviation of the light is proportional to the refractive index gradient so that at large values of $dc/dr$ the deviation is sufficiently great to cause the light to strike a nontransparent component, such as the optical tube or a lens mount. Such deviated light will fail to reach the detector. In the instruments used under our experimental conditions (concentrations, speeds, and column heights) this takes place between 350 and 400 fringes/cm as judged from the loss of the centrifugal portion of the $\Delta c$ pattern. If one is using Rayleigh optics the limit may be even lower due to rotor precession or the lack of resolving power of the photographic plate. These difficulties can be eliminated by the use of larger optical components and laser light sources (Williams, 1972; Paul and Yphantis, 1972a,b).

In the subsequent analysis all data corresponding to values of $dc/dr$ above 400 fringes/cm have been considered unusable. The radial position at which this gradient is reached can be determined from

$$dc/dr = \sigma c r \quad (26)$$

The magnitude of the errors that exist in the experimental data depends, in part, on the optical systems used to produce the data. The concentration difference, $\Delta c$, can be measured either interferometrically or with the split-beam photoelectric scanner. Since presently available scanners do not possess the necessary accuracy, we assume that $\Delta c$ is measured with Rayleigh optics. When the two compartments of a double-sector cell are filled with different solutions, the value of $\bar{c}$ can be de-

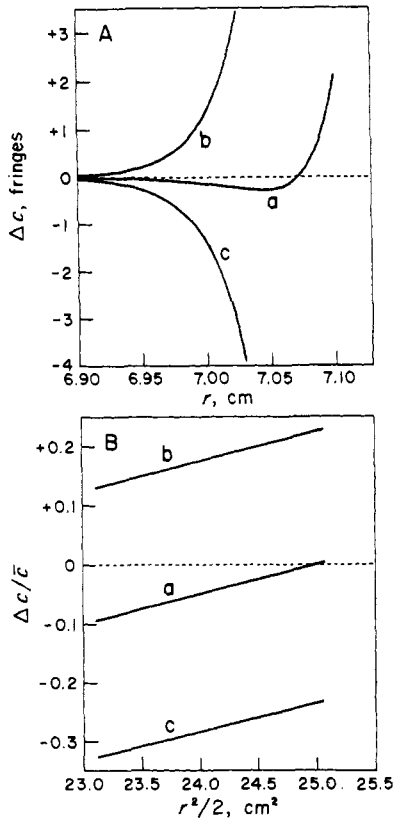


FIGURE 5: Difference sedimentation equilibrium experiments with a combination of cell imperfections. (A) Curves a, b, and c show the $\Delta c$ distributions for three experiments in which $\Delta\sigma/\sigma_1 = 1\%$ and $\sigma_1 = 5$ cm$^{-2}$. Curve a shows the resultant profile for a perfect cell. Curve b represents the distribution for an experiment with bottom, meniscus, and initial concentration mismatches of 30 $\mu$, 100 $\mu$, and 10%, respectively. Curve c shows the pattern which results from mismatches which are of the same magnitude but opposite in sign from those which give rise to b. (B) The data from the above experiments are replotted as $\Delta c/\bar{c}$ *vs.* $r^2/2$. The slopes of all three lines give the correct value for $\Delta\sigma$ of 0.050.

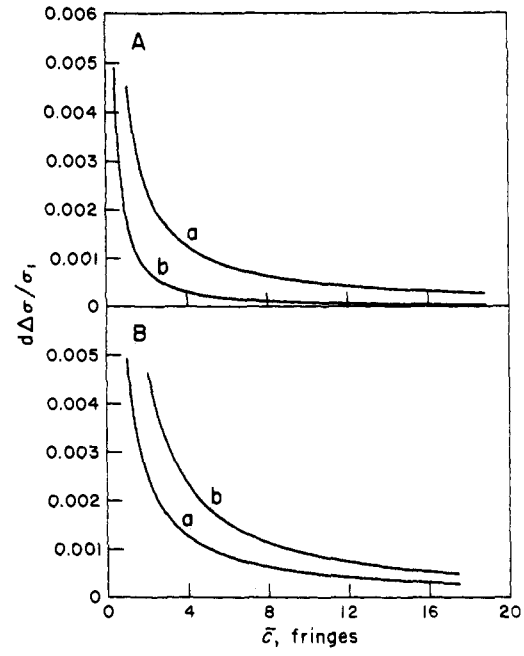


FIGURE 6: Point average error functions of a high-speed experiment in a perfect (A) and an imperfect cell (B). The effects of a systematic error of 0.005 fringes in $\Delta c$ on the measured value of $\Delta\sigma/\sigma_1$ as a function of $\bar{c}$ is shown by curve a. Curve b shows the corresponding error for a systematic inaccuracy of 0.05 fringe in $\bar{c}$. The total error at any average concentration is the sum of the two curves at that point. The error functions were calculated from eq 25 for an experiment in which $\sigma_1 = 5$ cm$^{-2}$ and $\Delta\sigma = 0.05$ cm$^{-2}$. The initial concentration is 4 fringes. Other conditions are as described in Figure 2. Curves a and b in (B) show the error function for an experiment in which a bottom mismatch of 50 $\mu$ is present. All other conditions are as described above.

termined only with the schlieren optical system since there is no reference compartment which contains the solvent. Hence the errors used in this analysis are those which would be present in data obtained from the Rayleigh and schlieren optical systems.

In all of the following calculations experimental errors corresponding to values of 0.005 and 0.05 fringe were assumed for $\delta\Delta c$ and $\delta\bar{c}$, respectively. These amounts were chosen because they represent reasonable *systematic* errors. This degree of accuracy can be obtained routinely by exercising care in the alignment of the optical systems and in reading the patterns on the photographic plates (Springer and Schachman, 1974).

*Error as a Function of $\bar{c}$ for Perfect Cells.* The dependencies of both the $\Delta c$ and $\delta\bar{c}$ terms of the error functions on $\bar{c}$ are shown in Figure 6A by curves *a* and *b*, respectively. These functions were calculated from eq 25 using values of $\sigma_1$, $\Delta\sigma$, and $c_0$ of 5 cm$^{-2}$, 0.05 cm$^{-2}$, and 4 fringes, respectively. Since the $\delta\Delta c$ term depends only on $\bar{c}$, curve a is accurate for all values of $\sigma_1$, $\Delta\sigma$, and $c_0$. Curve b, however, while strictly valid for the conditions listed above, only approximates the error that would exist under different circumstances as the $\delta\bar{c}$ term is dependent on $\Delta c$ as well as $\bar{c}$. It is clear that the effect of both errors decreases markedly with increasing concentration, so that both precision and accuracy can be improved by using data at higher concentrations. Conversely $\delta\Delta\sigma$ increases as $\bar{c}$ decreases so that there is considerable risk of substantial inaccuracy if the value of $\Delta\sigma$ is computed from data obtained at low concentrations. Hence for a given experiment there is a concentration below which the data will not be acceptable.

*Effect of Rotor Speed and Initial Concentration on Experiments in Perfect Cells.* Table I shows the errors due to each term in eq 24 for values of $\sigma$, of 3, 4, and 5 cm$^{-2}$. The errors were computed for simulated experiments by using data at 100-$\mu$ intervals from $\bar{c} = 2$ fringes to $dc/dr \approx 400$ fringes/cm. The values have been calculated for initial concentrations of 4 and 20 fringes. Looking first at the $\delta\Delta c$ term we see that the per cent error is essentially independent of both $c_0$ and $\sigma_1$. The lack of dependence on $\sigma_1$ is due to the fact that the average of $\bar{c}$ over the usable region decreases as $\sigma_1$ increases. Since the error is a function of $\Sigma(1/\bar{c})$ the $\delta\Delta\sigma$ increases. This effect approximately compensates for dividing by larger values of $\sigma_1$. More interesting is the lack of dependence of the $\delta\Delta c$ term on $c_0$, since as shown in the table the magnitude of this expression remains almost constant as $c_0$ is varied. This results from the restrictions on the usable regions of the cell imposed by the lower concentration limit on the one hand and the maximum gradient on the other. For instance, we can calculate from eq 26 that for $\sigma = 5$ cm$^{-2}$ and $c_0 = 20$ fringes the upper usable radial limit is 7.017 cm or more than 800 $\mu$ short of the cell bottom. Lowering $c_0$ to 4 fringes moves this point to 7.06 cm, but also moves the lower limit approximately the same distance in a centrifugal direction. Hence the concentration range over which the measurements can take place remains essentially unaltered and consequently so does the error. The only change that has occurred is a shift in the region used to obtain the data toward the meniscus.

The expression involving $\delta\bar{c}$ behaves in a slightly different

TABLE I: Average Errors in $\Delta\sigma/\sigma_1$ Caused by Uncertainties in $\Delta c$ and $\bar{c}$.[a]

| Error in Fringes | | $\delta\Delta\sigma/\sigma_1$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | $\sigma_1 = 3$ cm$^{-2}$ | | $\sigma_1 = 4$ cm$^{-2}$ | | $\sigma_1 = 5$ cm$^{-2}$ | |
| $\delta\Delta c$ | $\delta\bar{c}$ | $c_0 = 4$ | $c_0 = 20$ | $c_0 = 4$ | $c_0 = 20$ | $c_0 = 4$ | $c_0 = 20$ |
| 0.005 | 0 | 0.0012 | 0.0011 | 0.0012 | 0.0011 | 0.0011 | 0.0013 |
| 0 | 0.05 | 0.0002 | 0.0004 | 0.0003 | 0.0004 | 0.0003 | 0.0005 |

[a] These average errors were calculated from eq 24 including all data from $c = 2$ fringes to $dc/dr = 400$ fringes/cm. Calculations were based on meniscus and liquid bottom positions of 6.8 and 7.1 cm, respectively. The values assumed for $\delta\Delta c$ and $\delta\bar{c}$ were 0.005 and 0.05 fringe, and $\Delta\sigma/\sigma_1$ was 0.01 for all experiments. Initial concentrations are given in fringes.

manner, increasing slightly as both $c_0$ and $\sigma$ increase. Consequently this term can be minimized by performing the experiment at as low a rotor speed and with as low an initial concentration as possible. The importance of the $\delta\bar{c}$ term, however, is minimal when attempting to develop optimal conditions for an experiment with a perfect cell, since it accounts for only one-third of the total error.

*Effects of Cell Imperfections on* $\delta\Delta\sigma$. Since cell imperfections affect the concentration distributions of the species involved, and as both $\bar{c}$ and $\Delta c$ appear in the error function, defects in equipment and experimental technique will influence the accuracy with which $\Delta\sigma$ can be measured. Normally, however, the alteration in the distributions significantly influences only the difference concentration.

As can be seen by inspection $\bar{c}$ but not $\Delta c$ appears in the $\delta\Delta c$ terms of eq 24 and 25; hence imperfections will have virtually no effect on this portion of the error functions. A caution must be added, however, since mismatches will raise the concentration difference at the meniscus, and if they are large enough an error in labeling $\Delta c$ may result. This factor must be considered when a rotor speed is chosen. The term containing $\delta\bar{c}$, however, is dependent directly on the absolute magnitude of $\Delta c$, increasing as $\Sigma\Delta c$ increases. Unfortunately, mismatches and cell imperfections usually lead to such an increase which may, if care is not taken, be fivefold or more. Hence, for real cells, the $\delta\bar{c}$ term can become quite important especially in high-speed experiments. This is illustrated in Figure 6B which shows the point average error functions for an experiment in which $\sigma_1 = 5$ cm$^{-2}$, $\Delta\sigma = 0.05$ cm$^{-2}$, $c_0 = 4$ fringes, and $b_2 - b_1 = 50$ $\mu$. The values for $\delta\Delta c$ and $\delta\bar{c}$ were assumed to be 0.005 and 0.05 fringe, respectively. If we restrict ourselves to the data between $\bar{c} = 2$ and 10 fringes we find the following. First, the $\delta\bar{c}$ term is larger than the $\delta\Delta c$ term. Second, the sum of the two over this range of concentrations averages about 0.003, a value double that calculated for a perfect cell. This increase, although small in an absolute sense, can become quite significant if the change in molecular weight that is to be measured is small (~1%). For example, in this experiment the presence of a 50-$\mu$ bottom mismatch increased the error in the value of $\Delta\sigma/\sigma_1$ from 15 to 30%. Thus it is desirable to minimize the effects of imperfections. This can be accomplished by lowering $\delta\bar{c}$, reducing the size of the mismatches, or arranging them so their effects cancel one another. Normally the only mismatch of consequence, and the one most difficult to control, is that of the cell bottoms. Measurements on many centerpieces indicate that the positions of the bottoms often differ by about 30 $\mu$, although in some cases the difference may be considerably greater. Centerpieces with particularly large differences in the radial positions of the bottoms (>60 $\mu$) should not be used, but if there is no alternative the cell can be misaligned slightly in the rotor to compensate for the imperfection. Alignment is very critical. For instance, with a $c_0$ of 15 fringes a rotation of the cell only the width of the scribe line will change $\Delta c$ by 4 fringes at $dc/dr = 400$ fringes/cm. Hence it is important that the cell be aligned appropriately in terms of its physical dimensions. Menisci can be matched to 20 $\mu$ or better and the path lengths of most centerpieces do not differ by more than 0.1%. Since the $\Delta c$ distribution is not particularly sensitive to mismatches in either of these parameters, these small differences cause only a negligible effect.

## Discussion

The proper conditions for a high-speed experiment are determined by a number of partially conflicting requirements. First, there is a lower limit on the rotor speed that can be employed if meniscus depletion is used to establish the absolute values of $\Delta c$ and $\bar{c}$. The centrifugal field generated must be sufficient to ensure that both quantities, $\Delta c_m$ and $\bar{c}_m$, are reduced to negligible size at the meniscus or systematic errors in the measurement of the distributions will result.[2] Such errors may cause a sizable inaccuracy in the calculated value of $\Delta\sigma$. As shown in the following paper, the value of $\Delta c/\bar{c}$ at the meniscus is usually greater than 0.1. In addition, for an average cell (bottom mismatch of 30–50 $\mu$) the technique is about ten times more sensitive to errors in $\Delta c$ than in $\bar{c}$. As a result the lowest rotor speed at which a meniscus depletion experiment can be performed successfully is determined by the magnitude of $\Delta c_m$. Second, $c_0$ and $\sigma_1$ should be chosen so as to allow meaningful data to be obtained over as much of the solution column as possible. Such an arrangement maximizes the quantity of available data and hence minimizes statistical errors. Since the size of the usable region of the cell increases as $\sigma_1$ decreases, the rotor speed employed ought to be no greater than that required by meniscus depletion. However, as shown by the error analysis, the combination of $c_0$ and $\sigma_1$ should produce, at a radial level near the bottom of the cell, the largest concentration gradient which permits light to reach the photographic plate. Third, it is desirable to keep the effects of cell imperfections small, since if they are large a substantial decrease in precision and accuracy may occur. This results from increases in $\Delta c_m$ and the $\delta\bar{c}$ term of eq 24. Both effects can be lessened by filling each compartment with the proper sample so that the difference concentration distribution which is produced by the $\Delta\sigma$ is opposite in sign to that which is due to the imperfections. Alternatively the cell can be appropriately misaligned in the rotor. Fourth, the region of the

[2] The values of $\bar{c}$ are obtained by integration of the schlieren pattern which gives $d\bar{c}/dr$ as a function of $r$.

cell used to obtain the data should not have a large base-line correction as such a situation may increase the uncertainty in the measured values of $\Delta c$. Frequently these distortions are most severe near the bottom of the liquid column. Hence it may be desirable to shift the area from which the data are collected toward the meniscus. This can be accomplished by raising the initial concentration.

Although these requirements conflict to some degree, it is usually possible to reach a satisfactory compromise. Optimally $c_0$ should be 2–10 fringes and the rotor speed chosen so that $\sigma_1$ lies between 4.5 and 5 $cm^{-2}$. The high velocities need be employed only if cells containing large imperfections or high initial concentrations are used. Under these conditions, $\Delta c$ becomes insignificant (<0.001 fringe) for several hundred $\mu$ centrifugal to the meniscus. This permits the absolute values of $\Delta c$ to be accurately established by averaging multiple readings at several radial positions where the difference concentration is known to be essentially zero. In addition $\Delta\sigma$ can be calculated from sufficient data so as to keep the statistical error reasonably small while retaining some flexibility in shifting the region of the cell used to obtain those data.

The accuracy of the method, in actual practice, is difficult to assess from computer simulations because it depends on a variety of experimental factors, including the nature of the particular cell employed. It is possible, however, to make an estimation for a properly executed experiment with an "average" cell. Under such conditions one should be able to measure $\Delta\sigma/\sigma_1$ with a systematic error not exceeding 0.002–0.003 using data from $\bar{c}$ = 2–10 fringes. This value assumes a bottom mismatch of 30–50 $\mu$, minimal base-line deviations, and errors in labeling $\Delta c$ and $\bar{c}$ of 0.005 and 0.05 fringe, respectively. In the following paper we show that this degree of accuracy is readily obtainable.

The method described in this communication should be useful for problems which require the measurement of small changes or differences in molecular weight. One such application is the study of self-associating systems.[3] The use of the difference technique should allow the determination of equilibrium constants which are too small or too large to be measured with existing procedures. A second, and perhaps more important application, lies in the investigation of the effects of ligands on self-associating proteins. Often the addition of such effector molecules shifts the equilibrium in a biologically significant manner. However, these perturbations are frequently so small that they can be detected only by a sensitive technique which provides direct and accurate measurements of changes in molecular weight. There is, however, a complication which arises when the technique is applied to heterogeneous systems (interacting or not). This difficulty stems from the unequal effects that changes in the radial position of the cell bottom[4] have on the concentration distributions of macromolecules which differ in molecular weight. Hence there will be a $\Delta\sigma$ due to variations in the compositions of the two solutions at conjugate radial levels which arise solely from mismatching the cell bottoms. These effects, however, are small provided the solution consists mainly of one component and the offset is not large (<30–40 $\mu$). Since the technique will normally be employed under such conditions, the problem will usually not be serious and therefore should not interfere in most applications to interacting systems. If the method is used for solutions containing appreciable quantities of more than one species, the cell bottoms must be closely matched or some difficulty may be encountered. Further work is needed to clarify this point. The difference technique may also be applied to measure the change in buoyant molecular weight produced by the binding of a ligand to a protein. Accurate knowledge of this quantity is essential for correctly interpreting changes in sedimentation coefficient ($s$) produced by such binding. In the past, corrections for increases in buoyant molecular weight have been made by assuming additivity of volumes (Kirschner and Schachman, 1971; Smith and Schachman, 1973). This assumption is of critical importance since even small deviations from additivity can appreciably alter the size of the correction which in many cases is large relative to the experimentally observed change in $s$. Difference sedimentation equilibrium provides an ideal solution to this problem since it measures directly the change in buoyant molecular weight.

Although we have considered mainly high-speed experiments, the method is in principle equally applicable to low-speed procedures. The low-speed technique is of interest because it permits data to be obtained at high concentrations and therefore may provide increased accuracy and allow the measurement of smaller association constants than would be possible otherwise. The use of the technique at high concentrations raises the general question as to what effect nonideality has on the experimentally observed $\Delta\sigma$. Equation 13 was derived for ideal solutions and hence the plot of $\Delta c/\bar{c}$ *vs.* $r^2/2$ does not correct for nonideality. However, the method is normally used to compare directly similar materials at nearly equivalent concentrations. Hence at least partial cancellation should occur. Apparently this is the case in high-speed experiments since as can be seen from the results in the following paper the effects of nonideality were not detectable in the concentration range of 2–11 fringes. However, in low-speed experiments at much higher concentrations the degree of cancellation may not be adequate to reduce the effects sufficiently so that they can be safely neglected. Hence for such applications eq 13 may have to be reformulated to include terms for nonideality.

Some biological materials are too labile to allow the successful application of normal sedimentation equilibrium methods. In such cases the approach to equilibrium technique (Archibald, 1947) is often employed. Similarly there may be a need to determine rapidly small differences in molecular weight. Hence a difference Archibald method may be useful. The function which allows $\Delta\sigma$ to be computed from the approach to equilibrium technique is given by

$$\Delta\sigma = \frac{\bar{c}_m\left(\frac{\delta\Delta c}{\delta r}\right)_m - \Delta c_m\left(\frac{\delta\bar{c}}{\delta r}\right)_m}{[\bar{c}_m^2 - (\Delta c_m/2)^2]r_m} \qquad (27)$$

where the subscript m refers to the value of the quantity at the meniscus (Kirschner, 1971). The use of eq 27 requires extrapolation of the data to the meniscus and hence is bound to be less reliable than the difference sedimentation equilibrium method. Nonetheless the difference Archibald method may be useful for some systems.

[3] The measured value of $\Delta\sigma$ is a weight-average quantity and for heterogeneous systems it gives the difference in point-by-point weight-average molecular weights.

[4] Alteration of the meniscus position also has an effect but is small and can be safely neglected.

## References

Adams, E. T., Jr. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 509.
Adams, E. T., Jr. (1965), *Biochemistry 4*, 1646.
Adams, E. T., Jr. (1967), *Biochemistry 6*, 1864.
Adams, E. T., Jr., and Williams, J. W. (1964), *J. Amer. Chem. Soc. 86*, 3454.

Archibald, W. J. (1947), *J. Phys. Colloid Chem. 51,* 1204.
Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216.
Cann, J. R. (1970), Interacting Macromolecules, New York, N. Y., Academic Press.
Cox, D. J. (1969), *Arch. Biochem. Biophys. 129,* 106.
Cox, D. J. (1971), *Arch. Biochem. Biophys. 142,* 514.
Gilbert, G. A. (1955), *Discuss. Faraday Soc. No. 20,* 68.
Gilbert, G. A. (1959), *Proc. Roy. Soc., Ser. A 250,* 377.
Kirschner, M. W. (1971), Ph.D. Thesis, University of California, Berkeley.
Kirschner, M. W., and Schachman, H. K. (1971), *Biochemistry 10,* 1900.
Nichol, L. W., and Winzor, D. J. (1972), Migration of Interacting Systems, Oxford, London, Clarendon Press.
Paul, C. H., and Yphantis, D. A. (1972a), *Anal. Biochem. 48,* 588.
Paul, C. H., and Yphantis, D. A. (1972b), *Anal. Biochem. 48,* 605.
Richards, E. G., Bell-Clark, J., Kirschner, M., Rosenthal, A., and Schachman, H. K. (1972), *Anal. Biochem. 46,* 295.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63,* 1578.
Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci. 164,* 245.
Smith, G. D., and Schachman, H. K. (1973), *Biochemistry 12,* 3789.
Springer, M. S., and Schachman, H. K. (1974), *Biochemistry 13,* 3726.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University Press.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164,* 66.
Williams, R. C., Jr. (1972), *Anal. Biochem. 48,* 164.
Yphantis, D. A., and Waugh, D. F. (1956), *J. Phys. Chem. 60,* 623.
Zimmerman, J. K., Cox, D. J., and Ackers, G. K. (1971), *J. Biol. Chem. 246,* 4242.

# 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 ($\Delta c$) and the average concentration ($\bar{c}$), as functions of radial distance. With double-sector ultracentrifuge cells, $\Delta c$ 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 $\Delta c$. 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, $\Delta c$, 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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