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The Archibald Molecular Weight Determination. A General Theory for Data Extrapolation and Its Application*

Verner H. Paetkau†

ABSTRACT: Archibald's method of determining molecular weights by ultracentrifugation has several advantages over others. Its over-all effectiveness is impaired, however, by the necessity of extrapolation to obtain certain critical data. This necessity is created by optical artifacts occurring near the meniscus. Although various empirical extrapolation methods are known, no theoretically valid, completely general method has yet been described. Such a method, based on the Fujita–MacCosham solution to Lamm's differential equation [Fujita, H., and MacCosham, V. J. (1959), *J. Chem. Phys. 30*, 291], is described in this paper. Its application is effected by an iterative, self-correcting computer program. Data generated from the Fujita–MacCosham equation are used to test the influences of various parameters on the efficacy of the method, and β-lactoglobulin B is used as a "real" test system. A separate determination of the optical constant is made optional by a technique based on the principle described by R. Trautman and C. F. Crampton [(1959), *J. Am. Chem. Soc. 81*, 4036]. The molecular weight obtained is almost unaffected by another practical difficulty of the Archibald method, namely, the inability to measure the true meniscus position. The output data of the computer program appear to provide a very sensitive criterion for nonideal behavior.

Archibald's method of determining molecular weights in the ultracentrifuge has a unique complement of advantages: (i) it is faster than conventional equilibrium methods; (ii) it is based on equilibrium theory, making the interpretation of results more straightforward than in sedimentation–diffusion methods; and (iii) the fractionation occurring during a run facilitates a study of the composition of a paucidisperse system. Unfortunately, artifacts arising from optical phenomena invalidate data obtained at the ends of the liquid column, where the equilibrium condition exists. Thus it is difficult to obtain either the exact position of, or the concentration gradient at, the meniscus, using the schlieren optical system. This is the major drawback of the Archibald method. A technique for obtaining the meniscus position by using data obtained at different times of centrifugation was described some years ago (Trautman, 1958). There are several empirical methods for extrapolating the concentration gradient to the meniscus. Recently, conditions leading to linearity in $(\partial c/\partial r)$ *vs.* r have been derived (LaBar, 1966a,b) on the basis of the Fujita–MacCosham (1959) equation. These conditions are somewhat restrictive, especially in experiments designed to deplete markedly the solute concentration at the inner meniscus.

The major purpose of this study was to derive a generally valid procedure for extrapolating data to the menisci, based on the Fujita–MacCosham equation. By evaluating some of the terms in this equation numerically, it is possible to derive a simple function, which I call "recip" because it is directly related to $1/M$, correlating the measured variables. Recip is proportional to r^2, where r is the distance to the center of rotation. The numerical evaluations occurring in recip explicitly depend upon a value of the molecular weight, which is the

* From the Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin. *Received May 25, 1967.* Supported by grants from the National Science Foundation and the National Institutes of Health.

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property being determined. There are two reasons why this does not lead to any difficulty. First, even an approximately correct value of M, such as the one calculated from the input data by the usual Archibald method (*i.e.*, assuming the meniscus data are correct), serves to initiate an iterative, converging series of calculations leading to a self-consistent set of parameters. The second reason stems from the limiting properties of recip, namely

$$\lim_{r \to \text{meniscus}} \text{recip} = 1/M \qquad \text{(i)}$$

and

$$\lim_{r \to \text{meniscus}} \mathrm{d}(\text{recip})/\mathrm{d}r^2 = 1/Mr_j^2 \qquad \text{(ii)}$$

where M and r_j are the (true) molecular weight and meniscus position, respectively. That is, any error in recip incurred by initially using an incorrect value of M will vanish at the point of interest, the meniscus, and thus permit a more accurate determination of M. Condition ii predicts that recip will be a linear function of r^2, with a small slope. In theory, M will be almost unaffected by any error in r_j.

The calculations described require a high-speed computer, but once the data have been adapted to computer analysis as indicated here, two further advantages are realized. First, data can be read from the schlieren photographs at irregular intervals and the usual manual operations, apart from analyzing the photographs and punching cards, are avoided. Secondly, the principle described by Trautman and Crampton (1959) can be used to evaluate the optical constant from pictures taken at various times during the run.

By this method, the apparent molecular weight of β-lactoglobulin B was 35,700, $\pm 2\%$ using data from both the inner and outer menisci. To test the efficacy of the method under various conditions, ultracentrifuge data were generated from the Fujita–MacCosham equation and subsequently analyzed.

Theory

1. Definition of Symbols Used (units in parentheses). c = concentration of solute (mg/ml); c_0 = initial concentration; r = radial distance to center of rotation (cm); M = molecular weight, assumed constant; s = sedimentation coefficient, assumed constant (sec); t = time (sec); D = diffusion coefficient, assumed constant (cm^2/sec); $\bar{v}$ = partial specific volume of solute (cm^3/gm); ρ = solvent density ($\mathrm{gm/cm}^3$); $R = 8.315 \times 10^7$ (erg/(mole °K)); T = absolute temperature (°K); n = refractive index, occurring in $(\partial n/\partial r)$, the refractive index gradient; k = constant which converts refractive index units (n) to concentration units (c); ω = angular velocity of rotor (radians/sec). The subscripts (a, i, p, and b) used to denote radial position; a = innermost end of liquid column, which will be denoted inner meniscus; i = given data point anywhere in the cell; p = plateau region, where $(\partial n/\partial r) = 0$; b = outermost end of the liquid column, which will be denoted outer meniscus.

$$\Phi(x) = \frac{2}{\sqrt{\pi}} \int_0^x \exp(-q^2)\mathrm{d}q \ (\equiv \text{"erf } x\text{"})$$

σ is defined at point i by Klainer and Kegeles (1955) as

$$\sigma_i = \int_{r_a}^{r_p} \left(\frac{r}{r_a}\right)^2 \left(\frac{\partial n}{\partial r}\right) \mathrm{d}r - \int_{r_a}^{r_i} \left(\frac{\partial n}{\partial r}\right) \mathrm{d}r \ (r_a \leq r_i < r_p)$$

or

$$\sigma_i = \int_{r_p}^{r_b} \left(\frac{r}{r_b}\right)^2 \left(\frac{\partial n}{\partial r}\right) \mathrm{d}r - \int_{r_i}^{r_b} \left(\frac{\partial n}{\partial r}\right) \mathrm{d}r \ (r_p < r_i \leq r_b)$$

The following groupings of terms are used in the Fujita–MacCosham equations: $\epsilon = 2D/(r_j^2\omega^2 s)$, $T = 4Dt/(\epsilon^2 r_j^2)$, and $Z = (2/\epsilon) \ln (r/r_j)$.

At the inner meniscus, j = a; at the outer meniscus, j = b. Thus, these constants will have different values at the two ends of the liquid column.

2. Development of the Extrapolation Method. A. ARCHIBALD CONDITION. According to Klainer and Kegeles (1955)

$$c_a = c_0 - k\sigma_a \qquad \text{(1a)}$$

$$c_b = c_0 + k\sigma_b \qquad \text{(1b)}$$

Equations 1a and 1b give the top and bottom meniscus concentrations, respectively, as long as there is a plateau region in the cell (defined by $(\partial n/\partial r)_{r=r_p} = 0$). The definitions of σ_a and σ_b are given by the formulas in section 1.

At the ends of the liquid column, where the net solute flux is zero, the Archibald (1947) condition exists.

$$\frac{k}{r_a}\left(\frac{\partial n}{\partial r}\right)_{r=r_a} = \frac{M(1 - \bar{v}\rho)\omega^2}{RT}[c_0 - k\sigma_a] \qquad \text{(2a)}$$

$$\frac{k}{r_b}\left(\frac{\partial n}{\partial r}\right)_{r=r_b} = \frac{M(1 - \bar{v}\rho)\omega^2}{RT}[c_0 + k\sigma_b] \qquad \text{(2b)}$$

For convenience, the constant $((1 - \bar{v}\rho)\omega^2/RT)$ will be replaced by the symbol X.

B. FUJITA–MACCOSHAM EQUATION. Fujita and MacCosham (1959) introduced an innocuous approximation into Lamm's differential equation and obtained an analytical solution for the boundary condition of uniform initial concentration (c_0). The conditions of the solution were that the cell is semiinfinite and s is independent of c. I will not reproduce the Fujita–MacCosham formula as given in other references (Fujita and MacCosham, 1959; Fujita, 1962; LaBar, 1966a) but rather will express it by the various terms defined below. *If M and s are known*, all these terms can be evaluated numerically at any (r, t) coordinate.

$$S1 = \Phi\left(\frac{T - Z}{2\sqrt{T}}\right)$$

$$S2 = 2\sqrt{\frac{T}{\pi}}\exp\left[-\frac{(T - Z)^2}{4T}\right]$$

$$S3 = \Phi\left(\frac{T + Z}{2\sqrt{T}}\right)$$

The definitions for $S1$, $S2$, and $S3$ apply at either end of the liquid column, keeping in mind the differences in ϵ, T, and Z mentioned earlier. In addition, for r near r_a, let

$$S4 = [(2 + Z + T)(1 - S3)\exp(Z) - S2]\exp(-\epsilon T)/2$$

$$S5 = [1 - S1 - \exp(Z)(1 - S3)]\exp(-\epsilon T)/2$$

and for r near r_b, let

$$S6 = [S2 + (2 + Z + T)(1 + S3)\exp(Z)]\exp(-\epsilon T)/2$$

$$S7 = [1 + S1 - (1 + S3)\exp(Z)]\exp(-\epsilon T)/2$$

Using these terms, the Fujita–MacCosham equation becomes

$$c = c_0(S4 + S5) \quad (r \text{ near } r_a) \tag{3a}$$

$$c = c_0(S6 + S7) \quad (r \text{ near } r_b) \tag{3b}$$

The exact first derivatives are given by:

$$\frac{\partial c}{\partial r} = 2\frac{c_0}{r\epsilon}S4 \quad (r \text{ near } r_a) \tag{4a}$$

$$\frac{\partial c}{\partial r} = 2\frac{c_0}{r\epsilon}S6 \quad (r \text{ near } r_b) \tag{4b}$$

An approximate form of the first derivative, obtained when $\exp(-\epsilon Z/2)$ is set equal to 1, is quite similar (Fujita, 1962). The approximate and exact forms are identical at $r = r_a$ and $r = r_b$ (*i.e.*, $Z = 0$), therefore the extrapolation procedure described here probably would be unaffected if the approximate form were used.

C. EXTRAPOLATION PROCEDURE. The ratios defined in eq 5 are derived from eq 3 and 4.

$$\frac{rc}{(\partial c/\partial r)} = \frac{\epsilon r^2}{2}\left\{1 + \frac{S5}{S4}\right\} \quad (r \text{ near } r_a) \tag{5a}$$

$$\frac{rc}{(\partial c/\partial r)} = \frac{\epsilon r^2}{2}\left\{1 + \frac{S7}{S6}\right\} \quad (r \text{ near } r_b) \tag{5b}$$

Recip is a function defined by

$$\text{recip} = \frac{rc}{\partial c/\partial r}\left(\frac{S4}{S4 + S5}\right)\frac{(1 - \bar{v}\rho)\omega^2}{RT} \quad (r \text{ near } r_a) \tag{6a}$$

$$\text{recip} = \frac{rc}{\partial c/\partial r}\left(\frac{S6}{S6 + S7}\right)\frac{(1 - \bar{v}\rho)\omega^2}{RT} \quad (r \text{ near } r_b) \tag{6b}$$

Using eq 5 and the definition of ϵ, it follows that

$$\text{recip} = \frac{1}{Mr_a^2}r^2 \quad (r \text{ near } r_a) \tag{7a}$$

$$\text{recip} = \frac{1}{Mr_b^2}r^2 \quad (r \text{ near } r_b) \tag{7b}$$

A plot of recip *vs.* r^2 will yield $1/M$ at $r = r_a$ or $r = r_b$. $S5$ and $S7$, respectively, are zero at these two points, but $S4$ and $S6$ are never zero; thus there are no singular points or undefined regions for recip. In practice, recip is plotted as a linear function of r^2 for $0.988 < (r^2/r_b^2)$ or $(r^2/r_a^2) < 1.012$ and the extrapolated value at $r = r_a$ or $r = r_b$, as the case may be, is taken as $1/M$ for that particular photograph.

D. EVALUATION OF s. It is necessary to know s, D, and k to evaluate the various "S constants" of sections 2B and 2C (above). The method of obtaining k is described in section 2E.

For the first set of calculations, M is determined from the raw input data using an initial value of k. The estimated meniscus data are used only for this calculation; during the first round of computation, the meniscus data are corrected by making $(\partial n/\partial r)_{r=r_j}$, σ_j, and $\text{recip}_{r=r_j}$ consistent.

The sedimentation coefficient (s) is calculated by the transport method (Baldwin, 1953).

$$Q = \ln\left[1 + \frac{r_a^2\int_{r_a}^{r_p}\left(\frac{\partial c}{\partial r}\right)dr - \int_{r_a}^{r_p}r^2\left(\frac{\partial c}{\partial r}\right)dr}{c_0 r_a^2}\right] = -2\omega^2 ts \tag{8}$$

In the computer program, Q is calculated simultaneously with the value of σ_a, and s determined from the slope of Q *vs.* time. A time correction (d) is obtained as follows. The "equivalent time of centrifugation" (t) is defined as $\delta + d$, where δ is the time relative to an arbitrary zero, conveniently taken as the time when the rotor attained full speed. Then

$$Q = (-2\omega^2\delta)s - 2\omega^2 sd \tag{9}$$

A plot of Q *vs.* $(2\omega^2\delta)$ gives a value for d as well as s. This value of d is added to δ in all subsequent calculations. Since t enters the calculation of T and Z, this correction is important, especially at short times. D is obtained from s and M by the definition $M = sRT/D(1 - \bar{v}\rho)$.

E. Optical constant (k). It is possible to determine M without independently measuring k. Although the principle of the method has been described by Trautman and Crampton (1959), the approach and equations used here are somewhat different.

The procedure in sections 2C and 2D is used to calculate a value of M and c_a for each picture, using any value for k. This first set of parameters will be denoted by primed symbols: k', M', and c_a'. The procedure for the bottom meniscus is entirely parallel, and only the final result will be given (eq 10b). The correct values of k, c_a, and M will de denoted by unprimed symbols. From eq 2a

$$\left(\frac{\partial n}{\partial r}\right)_{r=r_a} = \frac{XM'c_a'r_a}{k'} = \frac{XMc_ar_a}{k}$$

thus $M'c_a' = Mc_ak'/k$. In addition, from eq 1a

$$\sigma_a = \frac{c_0 - c_a'}{k'} = \frac{c_0 - c_a}{k}$$

Therefore $c_a = c_0 - k(c_0 - c_a')/k'$ and

$$M'c_a' = Mk'c_0/k - M(c_0 - c_a') \qquad (10a)$$

At the bottom meniscus

$$M'c_b' = Mk'c_0/k - M(c_0 - c_b') \qquad (10b)$$

A plot of $M'c_j'$ *vs.* $(c_0 - c_j')$ (j = a, j = b) will yield k, the correct optical constant, and M, the "average" molecular weight. This type of plot will be referred to as a "Trautman–Crampton" plot. In practice, reiterations are carried out until k no longer changes.

3. Computer Program and Algorithms. A CDC 1604B computer (32K memory) has been used to run the FORTRAN 63 program outlined below. This particular installation has a library subroutine for the error function integral. A copy of the program, and its detailed description, will be furnished upon request. An outline of the various steps and the relevant algorithms are as follows.

(1) Read in data and relevant constants: an initial value of k, c_0, rotor speed in revolutions per minute, r_a, r_b, solvent density, and partial specific volume of solute.

(2) Calculate and store σ_j for each value of r_j sufficiently close to r_a (or r_b) to meet a predetermined criterion. (I have chosen to calculate σ_j only for $(r_j/r_a)^2 < 1.012$ and $(r_j/r_b)^2 > 0.988$.)

$$\sigma_j = \frac{1}{2r_a^2}\sum_{i=1}^{N-1}\left[\left(\frac{\partial n}{\partial r}\right)_i r_i^2 + \left(\frac{\partial n}{\partial r}\right)_{i+1} r_{i+1}^2\right][r_{i+1} - r_i] - \frac{1}{2}\sum_{i=1}^{j-1}\left[\left(\frac{\partial n}{\partial r}\right)_i + \left(\frac{\partial n}{\partial r}\right)_{i+1}\right][r_{i+1} - r_i] \qquad (11)$$

near the inner meniscus, with N the number of the last data point for which $(\partial n/\partial r)$ is greater than 0. Near the outer meniscus, $[r_{i+1} - r_i]$ will be replaced by $[r_i - r_{i+1}]$, since r decreases with increasing i.

(3) Calculate Q for each photograph, corresponding to a particular time of centrifugation, using the algorithm

$$Q = \ln\left[1 + \frac{k}{2c_0}\sum_{i=2}^{N}\left[\left(\frac{\partial n}{\partial r}\right)_i + \left(\frac{\partial n}{\partial r}\right)_{i-1}\right][r_i - r_{i-1}] - \frac{k\sigma_a}{c_0}\right] \qquad (12)$$

Calculate the linear least-squares fit of Q *vs.* $2\omega^2\delta$ to obtain s and the time correction (d). This calculation is limited to data from the inner meniscus.

(4) Calculate the various S terms used in the extrapolation procedure. Carry out a least-squares fit of eq 7, omitting the data obtained right at the menisci. The value of recip at the meniscus for a given data set will be the best value of $1/M$ for that set. Correct c_j' (j = a, j = b) by making c_j', $(\partial n/\partial r)_{r=r_j}$, and M consistent with each other.

(5) Fit the relevant data to eq 10, and obtain an improved value of k. Calculate an improved value of s, the various "S constants" and recip using this k value. Reiterate until k is stationary

Experimental Section

The centrifugation of β-lactoglobulin B (Pentex Incorporated, lot no. 2) was carried out in the laboratory of Dr. J. W. Williams by Mrs. D. Hancock. A filled-epon double-sector cell, an AnJ rotor, and the Model E ultracentrifuge (schlieren optical system) were used. The protein was dissolved in, and dialyzed against, 0.05 M Tris (phosphate) buffer at pH 7.1. Fluorocarbon FC-43 was used to form the outer meniscus (Ginsberg *et al.*, 1956). The preparation, properties, and ultracentrifugation of phosphofructokinase have been described (Paetkau and Lardy, 1967).

Results

1. Dependence of Recip on D. The values of s and D generated during the analysis of data pertaining to the centrifugation of β-lactoglobulin B were markedly different from the sedimentation and diffusion coefficients for that protein. Since D is calculated from s and M, where M is the correct molecular weight, this discrepancy can be traced to a difference between s calculated from the transport method (corrected for density and viscosity effects) and $s_{20,w}$. Some possible sources of this discrepancy will be considered in the Discussion.

To determine the effect of varying D on the final value of the molecular weight, data were generated from the Fujita-MacCosham equation and analyzed by the recip function method. D was then arbitrarily varied by ±10% and the data analysis was repeated.

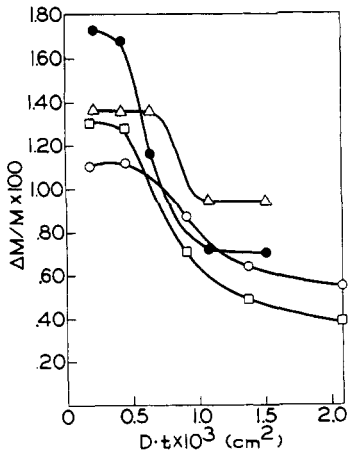

FIGURE 1: Effect of a 10% change in D on the calculated molecular weight at the inner meniscus. Data were generated by the Fujita–MacCosham equation and analyzed by the recip function method. D was then varied by $\pm 10\%$ and the molecular weight was recalculated. The ordinate is the average per cent change in M resulting from a $\pm 10\%$ change in D (all changes were averaged without regard to sign). The abscissa is D (the correct value) times t in seconds. Two different molecular weights and various rotor speeds were used in generating the data: (○) M = 36,000, speed = 2000 or 5000 rpm (data were identical); (□) M = 36,000, speed = 25,000 rpm; (△) M = 190,000, speed = 2000 or 5000 rpm (identical); (●) M = 190,000, speed = 12,459 rpm.

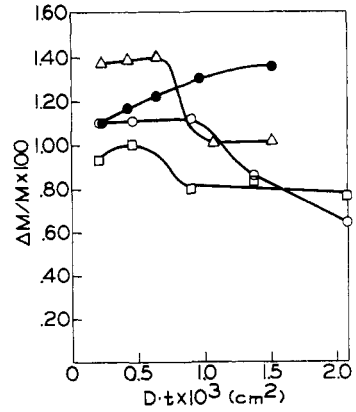

FIGURE 2: Effect of a 10% change in D on the calculated molecular weight at the outer meniscus. Conditions and symbols are identical with those in Figure 1.

Since s does not enter the calculations explicitly, the effect of varying D in this way is exactly the same as if s had been changed, and D was then calculated from the correct ratio s/D (*i.e.*, M). Figures 1 and 2 show the results of varying D by $\pm 10\%$, taking the average deviation produced by these two variations, without regard for sign.

Even if D is different from the correct value, it is possible to reduce the resulting error in M by taking the first photograph after a certain time has elapsed. It is interesting that (i) at the top meniscus, the relationship of M calculated to D used is about the same at widely varying rotor speeds and molecular weights; (ii) there are conditions under which the error in M does not decrease within a reasonable time (as in Figure 2) at a rotor speed of 12,459 rpm and molecular weight 190,000.

To examine the behavior of a real system, data taken during the ultracentrifugation of β-lactoglobulin B was subjected to the same procedure. The results (Figure 3) indicate a somewhat smaller dependence of M on D than predicted by theory. The D variation procedure is written into the program as an automatic check on any data being analyzed, to indicate the relative margin of confidence in the final answer.

2. Dependence of Recip on r_a and r_b. Another important problem of the Archibald method is measuring the exact position of the meniscus. The recip function method is highly insensitive to variations in r_a or r_b, as shown in Figures 4 and 5. Data taken during the ultracentrifugation of β-lactoglobulin B were computed by the recip function program and the values of recip plotted against r. Although recip is explicitly a linear function of r^2, it will also be essentially linear with respect to r over the short distances depicted in Figures 4 and 5. This parameter is used as the abscissa to show the nonlinearity of $\partial c/\partial r$ even

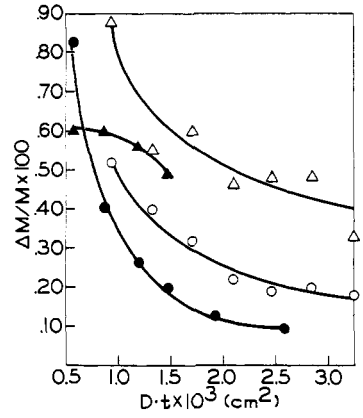

FIGURE 3: Effect of a 10% change in D on the calculated molecular weight of β-lactoglobulin B and 7S phosphofructokinase. A solution of 10 mg/ml of β-lactoglobulin B in 0.05 M Tris (phosphate) buffer (pH 7.1) was centrifuged at 12,459 rpm in a double-sector 12-mm cell. Photographs were taken of the schlieren optical pattern (80° phase-plate angle) at 10-min intervals between 15 and 105 min after the rotor reached top speed. The average per cent change in M resulting from a $\pm 10\%$ change in D is plotted for the inner (○) and outer (△) menisci. Phosphofructokinase from rabbit muscle was treated with 0.8 M urea at pH 5.8 and centrifuged at 8225 rpm. D was varied, and M recalculated as for β-lactoglobulin B. (●) Inner meniscus, (▲) outer meniscus.

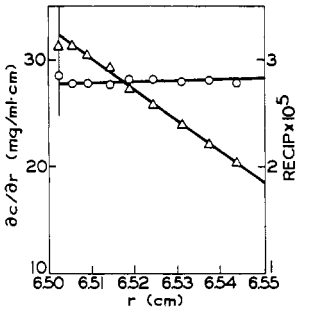

FIGURE 4: Comparative ease of extrapolating $\partial c/\partial r$ and the recip function for β-lactoglobulin B at the inner meniscus. Data were read from a photograph taken 55 min after the rotor reached full speed, and subjected to analysis. The values of $\partial c/\partial r$ (△, left-hand ordinate) and of recip (○, right-hand ordinate) are given on the same relative scale to permit a comparison. The recip values obtained from the analysis of theoretical data corresponding to β-lactoglobulin (M = 36,000) are superimposable on the recip line, and are not drawn in. The inner meniscus position is given by the line at r = 6.5025 cm.

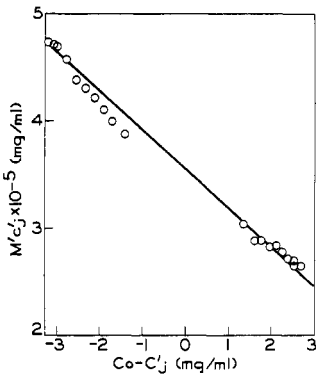

FIGURE 6: Trautman–Crampton plot for data obtained during the centrifugation of β-lactoglobulin B. The experimental conditions were as described in Figure 3. Data from the inner ($(c_o - c_j')$ positive, $c_j' = c_a'$) and outer ($(c_o - c_j')$ negative, $c_j' = c_b'$) menisci were fitted by a least-squares straight line. The initial concentration (c_o) was 10 mg/ml. The apparent concentrations at the menisci (c_j') are calculated on the basis of the value of k' (see text). The slope of the line is 35,700; the intercept is 355,000 (mg/ml).

over a short radial distance. In these figures, $\partial c/\partial r$ varies 7 and 18% per 0.01 cm at the inner and outer menisci, respectively. The average slope of recip in the ten photographs of the experiment was also calculated (there was no significant trend in the slope during the run). At the inner meniscus, an average change of 0.3% in recip per 0.01 cm was observed; at the outer meniscus, the average deviation in the same distance was 1.5%. Thus, inability to measure the meniscus position exactly will have only a small effect upon the molecular weight obtained by the recip function method.

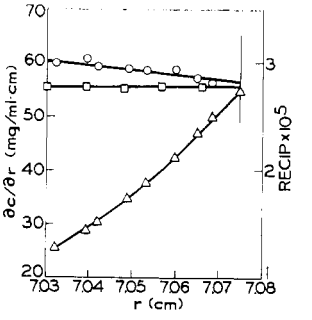

FIGURE 5: Real and synthetic data for β-lactoglobulin B at the outer meniscus. Data were read from a photograph taken 65 min after the rotor reached full speed. (○) Recip function values (right-hand ordinate), using real data from the experiment described in Figure 3; (△) $\partial c/\partial r$ (left-hand ordinate) from the same experiment; (□) recip function values (right-hand ordinate) obtained from the analysis of Fujita–MacCosham data corresponding to β-lactoglobulin B. The outer meniscus position is given by the line at r = 7.0746 cm.

The data of Figures 4 and 5 were compared to data generated from the Fujita–MacCosham equation for a protein of molecular weight 36,000 and a rotor speed of 12,459 rpm. The theoretical data from the inner meniscus region were superimposable upon the recip curve of Figure 4, indicating near-ideal behavior of the latter. The Fujita–MacCosham data, however, were somewhat different from the experimental points near the outer meniscus (see Figure 5). This nonideality in behavior near the outer meniscus is considered in the Discussion.

3. Efficacy of the Trautman–Crampton Plot in Providing the Optical Constant k. In the data analysis of β-lactoglobulin B, an input value of 10.0 was provided for k. After one cycle of calculations, the molecular weight indicated by the Trautman–Crampton plot (Theory, section 2E, eq 10a and b) was 35,500, and k had been changed to 13.41. A second cycle of calculations gave rise to a molecular weight of 35,700 and a k of 13.47. At this point, k no longer changed. These data are shown in Figure 6. In a separate high-speed experiment with the same sample and cell, k was determined to be 13.44. The molecular weight 35,700 compares favorably to the known value, 36,000. The same sample, centrifuged to over-all equilibrium (72 hr), had an apparent molecular weight of 34,200 between the concentrations of 5 and 13 mg/ml, and M increased to 37,000 at the bottom of the cell (concentration 17 mg/ml).

4. Molecular Weight of Phosphofructokinase. Crystallized rabbit muscle phosphofructokinase was converted to the 7S form by treatment with 0.8 M urea

at pH 5.8 (Paetkau and Lardy, 1967). A sample was centrifuged, and the data were analyzed by the recip function method. The average M value, based on both inner and outer meniscus data, was 1.91×10^5, standard error = 2.1×10^3. When the data were analyzed without the recip function extrapolation, the average M was 1.86×10^5, standard error = 4.6×10^3. The relationship of this form of phosphofructokinase to other forms, including the active one, has already been discussed (Paetkau and Lardy, 1967).

By varying D and recalculating recip, the dependence of M on D used was obtained and compared to β-lactoglobulin B in Figure 3. M was even less sensitive to D for 7S phosphofructokinase than it was for the lactoglobulin.

Discussion

In the Discussion, the terms "s coefficient" and "D coefficient" will be used to refer to the parameters calculated in the recip function program; the terms "sedimentation coefficient" and "diffusion coefficient" will be restricted to the literature values of these parameters.

The use of computers to analyze ultracentrifuge data is a comparatively recent development, but there are already many examples of computing programs relevant to molecular weight determination.[1] The existing programs applying to the Archibald method appear to be designed as substitutes for the usual hand calculations, not as attempts to improve the basic procedure by making fuller use of the data. Exact solutions of the infinite series type have long been known for the Lamm equation (see Schachman, 1959). The Fujita–MacCosham equation represents a closed approximate solution to Lamm's equation (Fujita and MacCosham, 1959; Fujita, 1962) but the approximation is of such an innocuous nature that Peterson and Mazo (1961) concluded that, at least for the Archibald experiment, it should now be unnecessary to resort to numerical solutions. These authors also suggested that an iterative application of the Fujita–MacCosham equation should determine the molecular parameters from preequilibrium centrifugation data.

Although the method described in this paper uses data from the outer meniscus to determine the optical constant, personal experience suggests that a more reliable procedure is to determine the optical constant separately. This can be done either by accelerating the rotor after the Archibald data are obtained to form a boundary near the inner meniscus, or by a separate synthetic boundary run in the same cell, as suggested by Ehrenberg (1957). β-Lactoglobulin B shows marked nonideality near the outer meniscus, as indicated by the difference between the theoretical and experimental slopes of recip in Figure 5. In Figure 6, the apparent molecular weight at the outer meniscus is lower than the average in early photographs, and increases during the run. At the inner meniscus, there is considerably less change in M (inner meniscus average M = 35.6, standard error = 0.5; outer meniscus average M = 35.2, standard error = 0.8). The behavior at the outer meniscus is consistent with a primary charge effect and concentration-dependent aggregation, acting oppositely on the apparent M. In this case, it is fortuitous that the optical constant from the Trautman–Crampton plot (Figure 6, k = 13.47) agreed as well as it did with the value obtained independently (13.44). In general, it is more reliable to determine k independently, and to use this information as a check on nonideal behavior.

Optical alignment has not been mentioned in this paper. This does not imply that fidelity of the optical system should be taken for granted; the two crucial steps for the Archibald method (Trautman, 1958; LaBar, 1966a), namely, positioning of the light source on the optic axis and checking the phase plate, still apply. Equilibrium centrifugation of sucrose (LaBar, 1966b) is a simple and effective check for the phase plate. The recip method probably reduces errors due to off-axis illumination, since the primary effect of this alignment is to change the apparent menisci positions (Trautman, 1958). Under "good" conditions (*e.g.*, the data of Figure 4), the recip method is highly insensitive to the choice of meniscus position. Other authors reporting on the importance of the "correct" choice of meniscus position in determining M indicate effects as large as a 10% change in apparent M per 0.01 cm in r (Cheng, 1957; Trautman, 1958).

In the case of theoretically generated data it is possible to reduce the dependence of apparent M on D essentially to 0 by making recip a second-order function of r^2 (unpublished observations). This suggests, and examination of the data confirms, that when D is incorrect, recip is a nonlinear function of r^2. (For the "correct" D, recip is apparently a linear function of r^2, in practice as well as in theory.) Thus, the reason for a dependence of apparent M (*i.e.*, the value of recip at $r = r_j$) on D is that a higher order curve is being fitted by a straight line. Higher order curve fitting has not been adopted in general because with real data nonideality effects often distort the shape of the recip curves, especially near the outer meniscus, and wild data points may throw off such a curve more easily than a linear fit.

A peculiar feature of the procedure, and one which necessitated the analysis of the effects of D on M, is the discrepancy ($\pm$10–15% in the case of β-lactoglobulin B) observed between the s and D coefficients and the literature values of the sedimentation and diffusion coefficients. A large part of this discrepancy can be traced directly to an imprecision in Q in these experiments. Since there was little net transport of solute, Q is very small, and the resulting error in the s coefficient is correspondingly large. In fact, the average deviation in Q from the straight line determining the s coefficient is of the same size as the apparent

[1] For a review, see R. Trautman, Fractions No. 2, Beckman Instruments Inc., Palo Alto, Calif.

discrepancy between the s coefficient and the known sedimentation coefficient. Certainly this method is not designed to measure the sedimentation and diffusion coefficients; these parameters are still obtained most accurately by experiments designed to this end. Fortunately, the recip function method is relatively insensitive to discrepancies in the s or D coefficients.

Since theoretically recip is directly proportional to r^2 with a proportionality constant (*i.e.*, a "true" slope) equal to $1/Mr_j^2$, it is possible to determine the value of the D coefficient which gives this "true" slope. From the slope of recip near the outer meniscus (β-lactoglobulin B), the D coefficient would be predicted to be considerably smaller than the generated one. However, under these conditions the recip function was nonlinear. The nonlinearity was qualitatively in agreement with a D coefficient decreasing with increasing concentration (*i.e.*, a negative second derivative of recip *vs.* r^2).

Under one set of conditions (M = 190,000, speed = 12,459 rpm) the error in M incurred by a 10% error in D (theoretical data) did not decrease below 1% in a reasonable time (Figure 2). This appears to limit the applicability of the method, but in practice it has been shown that there can be less dependence of M on D than in the theoretical case, primarily because of the peculiar behavior of recip near the outer meniscus. On the one hand, there is no straightforward theoretical treatment which will account for the anomalies near the outer meniscus, and the recip function method is rendered less meaningful thereby. On the other hand, the behavior of β-lactoglobulin B data indicates compensating effects, which make M less dependent on D than theoretically predicted (compare Figure 3 with Figures 1 and 2). Since the actual behavior of the system being studied will determine the reliability of the calculated M, the D variation calculations have been written into the program. Each set of data will then be characterized by its own relationship between D value used and M calculated. This relationship is useful as an indicator of the confidence level of that particular data set.

The apparent heterogeneity, or nonideality, or both, of β-lactoglobulin B near the outer meniscus has been mentioned. It is difficult to predict the effects of these situations on the method in general. It is apparent that the recip function method will still lead to a more accurate estimate of the molecular weight at the meniscus than existing, empirical methods, simply by producing a graph with a smaller slope (recip *vs.* r^2) than the usual relationship ($\partial c/\partial r$ *vs.* r).

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