A Study of the Combined Sedimentation and Chemical Equilibrium of β-Lactoglobulin B in Acid Solution*

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ABSTRACT: The monomer–dimer equilibrium of β-lactoglobulin B in acid solution has been studied by using the sedimentation equilibrium experiment. There is a quite general belief that, at combined chemical and sedimentation equilibrium, solution nonidealities cannot be taken into consideration, but in a recent series of papers Adams and collaborators have described a method by which such nonidealities are expressed and evaluated as activity coefficients, thus allowing for the computation of the equilibrium constants involved. Our experiments, designed to test this more comprehensive approach, required the determination of solute apparent weight-average molecular weights at a number of fixed concentrations and over wide concentration limits. The β-lactoglobulin B in solution at low pH values does provide a good test substance; it forms solutions in which monomer and its dimer are the predominant solute species in a solvent which under proper conditions can be considered as a single component. Pertinent theory is given in rather elementary form to describe the behavior of this simplest of associating systems. Using it for the interpretation of the data, numerical results for the activity coefficients and the equilibrium constant are obtained, tabulated, and discussed from the point of view of their uniqueness and meaning.

The sedimentation equilibrium experiment is a useful one for the characterization of macromolecular systems, for not only is it suitable for the determination of the molecular weight of a protein and of at least two of the more common average molecular weights in paucidisperse or polydisperse solute systems, but also it may provide activity or second virial coefficient data. The effects of nonideality in any real macromolecular system are often significant and sometimes overlooked.

It is important to note that in deriving the essential working equations for the interpretation of the experimental data it is not at all necessary to restrict them to idealized behaviors. Thus, while the purpose of the experiment is usually taken to be that of ascertaining solute molecular weights, the observed concentration distributions of the solution components at equilibrium may provide much additional and significant information regarding other thermodynamic properties of the macromolecular system. For solutions of isolated monomeric proteins the sedimentation equilibrium procedure has had great success since, by working at or near the isoelectric point and with adequate amounts of supporting electrolyte, the effects of solute–solvent interactions have been made small and many theroetical complexities have been removed.

Until the past few years the study of systems at combined sedimentation and chemical equilibrium, such as proteins which undergo association–dissociation reaction, likewise required the use of simpler equations which really describe apparent quantities. In fact the thermodynamic properties of such chemically reacting systems have been investigated largely by osmotic pressure and light-scattering techniques because the feeling was and still is widespread that solution nonidealities cannot be taken into consideration at sedimentation equilibrium. In a recent and very valuable review of the subject, "Interacting Protein Systems" by Nichol *et al.* (1964), one finds the following sentence. "For chemically reacting systems, both the sedimentation equilibrium method and the Archibald approach-to-equilibrium method can yield results permitting the evaluation of weight-average molecular weights and equilibrium constants, provided that the systems are thermodynamically ideal except for the occurrence of the reaction and that the assumption can be made that all products and reactants have the same partial specific volumes and specific refractive index increments."

But there are cases of great interest and significance in which the adjustment of solution media to produce pseudo-ideal conditions for the combined sedimentation and chemical equilibrium experiment is not possible. (Again, the term "ideal" is used without reference to the presence of the reaction.) Such a case is the monomer–dimer reaction of β-lactoglobulin B, a well-established reaction of the type we wished to study in detail. This mutual interaction is known to occur in the pH interval 2–3 (Townend *et al.*, 1960), a region far removed from the isoelectric point, so that even in the presence of a moderate amount of supporting electrolyte nonideality of solution behavior is to be expected. Fortunately, in a very recent series of papers, written

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after the appearance of the Nichol *et al.* survey, Adams (1965) has described a method by means of which the solution nonideality can be expressed as activity coefficients by using apparent weight-average molecular weight *vs.* concentration data, thus allowing computation of the true equilibrium constants for association–dissociation equilibria. Our experiments, then, were designed to test the validity of the Adams' disclosures for the simple monomer–dimer case before later extension to more involved reactions.

Theory

Basic Theory for the Analysis of the Nonideal Monomer–Dimer Reaction

Introduction and General Equations. As far as we are aware, the first treatment of *nonideal* self-associating systems as adapted to the equilibrium ultracentrifuge was that of Adams (1962). Then, the recognition by Adams and Fujita (1963) that, in principle, the apparent weight-average molecular weight of a reacting system at equilibrium must be a function only of the concentration at any given point in the cell led them to a different analysis of the problem. According to them, once this information (*i.e.*, M_{wc}^{app} as a function of concentration c) is available, the behavior of the reaction may be described in terms of nonideality coefficients for the system as a whole, so that the actual functional dependence of the concentration distribution on radial distance now plays a less important role in the analysis. Thus, one may perform a *series of experiments* at different initial concentrations and construct a continuous plot of apparent molecular weight *vs.* concentration over a much wider range of concentration than is available from a single experiment. In a sense, then, molecular weight averages, evaluated at each radial distance in the cell, may be regarded as equivalent to the information which is made available by a large number of light-scattering experiments over the same concentration range. This follows because the weight-average molecular weight evaluated at a given radial distance (r) is a function only of the concentration at that point, and is identical with that which would be obtained from a light-scattering experiment at that concentration.

The method for the numerical evaluation of these nonideality coefficients and subsequently the true equilibrium constants is given by Adams and Williams (1964) and Adams (1965). The self-association of monomers to form a dimer may be represented by the statement

$$2M_1 \rightleftharpoons M_2 \tag{1}$$

In the theory of equilibrium sedimentation it is convenient to assume that the partial specific volumes of the interacting species are equal. Further, it is necessary to regard the refractive index increment of each species as being the same. For our monomer–dimer reaction it would seem that neither of these approximations is too unrealistic, since all of the experiments were performed at relatively low rotor speeds.

We describe the equilibrium constant for the reaction on a gram basis, with the concentration being expressed in grams per unit volume of solution.

$$K_2 = \frac{c_2 y_2}{c_1^2 y_1^2} \tag{2}$$

The activity coefficients y_1 and y_2 are likewise expressed in terms of the corresponding concentration scale.

The total concentration of macromolecular component is

$$c = c_1 + c_2 = c_1 + K_2 c_1^2 \frac{y_1^2}{y_2} \tag{3}$$

where the subscripts 1 and 2 indicate the monomer and dimer, respectively. An important consequence of this relationship is that the reacting macromolecule is to be regarded as a single component in the description of the number of components of the system.

The weight- and number-average molecular weights for such a system are defined by the expressions

$$M_w = \frac{\Sigma c_i M_i}{\Sigma c_i} = \frac{M_1(1 + 2K_2 c_1(y_1^2/y_2))}{(1 + K_2 c_1(y_1^2/y_2)} \tag{4}$$

$$M_n = \frac{\Sigma c_i}{\Sigma(c_i/M_i)} = \frac{M_1(1 + K_2 c_1(y_1^2/y_2))}{\left(1 + \frac{K_2 c_1 y_1^2}{2y_2}\right)} \tag{5}$$

Application of the Sedimentation Equilibrium Experiment to the Monomer–Dimer Reaction. If the time required to come to sedimentation equilibrium is long compared to that required for the attainment of the chemical equilibrium, it may be possible to employ the equilibrium sedimentation experiment for a description of the chemical reaction. This fact was first recognized by Tiselius (1926) who considered only the ideal solution case.

The basic condition for sedimentation equilibrium is that the total potential be constant everywhere in the cell

$$\bar{\mu}_i = \text{constant} \tag{6}$$

where $\bar{\mu}_i = \mu_i - (\omega^2 r^2/2)$. The chemical equilibrium between monomer and dimer must satisfy the relation (written on a gram basis)

$$\mu_1 = \mu_2 \tag{7}$$

Equation 6 becomes, for each species

$$d\mu_i = M_i \omega^2 r dr \tag{8}$$

Consideration of the chemical potential as a function

only of pressure and concentration (at constant temperature) leads to the following basic equation for the sedimentation equilibrium of the monomer.

$$\left(\frac{\partial \mu}{\partial c_1}\right)_{p,T} \frac{dc_1}{dr} + \bar{v}_1 M_1 \frac{dp}{dr} - M_1 \omega^2 r = 0 \qquad (9)$$

The concentration-dependent part of the chemical potential is written in the usual way

$$\mu_i = (\mu_i^0)_{T,p,c} + RT \ln y_i c_i \qquad (10)$$

If it is assumed that $\ln y_i$ may be expanded as a power series in the concentration (c), valid at low concentrations, we may write

$$\begin{aligned} \ln y_1 &= B_{11} M_1 c + B_{12} M_1 c^2 + \ldots . \\ \ln y_2 &= B_{21} M_2 c + B_{22} M_2 c^2 + \ldots . \end{aligned} \qquad (11)$$

This functional dependence of the activity coefficient on concentration satisfies the convention that the activity coefficient approach the value unity in the limit of infinite dilution.

As a mathematical convenience, we now follow the suggestion of Adams and Fujita (1963) and assume that $B_{11} = B_{21}$ and $B_{12} = B_{22}$. Then, for the monomer–dimer equilibrium (since $M_2 = 2M_1$)

$$\begin{aligned} \ln y_1 &= B_1 M_1 c + B_2 M_1 c^2 + \ldots \\ \ln y_2 &= 2B_1 M_1 c + 2B_2 M_1 c^2 + \ldots . \end{aligned} \qquad (12)$$

This type of approximation has often been made. For example, Townend *et al.* (1960) used a single virial coefficient, $B_1 = B_2 = B$, in their study of the dissociation of β-lactoglobulin by light scattering. Indeed, Edelhoch *et al.* (1953) actually measured B for mercaptalbumin monomer and dimer separately by light-scattering techniques and found identical values for the two species. Undoubtedly the validity of such an approximation will vary with individual systems, but use of this assumption may in part be justified by the success with which the nonideality part of any particular system can be described. The extension to the case of two virial coefficients is obvious.

As a consequence of eq 12, $y_2/y_1^2 = 1$, so that eq 2 becomes

$$K_2 = \frac{a_2}{a_1^2} = \frac{c_2}{c_1} \qquad (13)$$

Utilizing this relation, the total concentration of the macromolecule may be expressed as

$$c = c_1 + K_2 c_1^2 \qquad (14)$$

and the expression for the weight-average molecular weight corresponding to the concentration c becomes simply

$$M_{wc} = \frac{M_1(1 + 2K_2 c_1)}{(1 + K_2 c_1)} \qquad (14a)$$

An apparent weight-average molecular weight, M_{wc}^{app}, corresponding to any fixed concentration (c) is defined by the equation

$$M_{wc}^{app} = \frac{1}{rc(r)} \frac{dc(r)}{dr} \frac{RT}{(1 - \bar{v}\rho)\omega^2} \qquad (15)$$

in which the several symbols have their usual significance. The symbols M_{wc} and M_{wc}^{app} are used throughout in order that they be not confused with weight-average molecular weights over the entire cell.

Equations 8, 12, 13, and a derivative of 14 now may be combined with the definition for M_w to give the following relationship for the monomer–dimer association at sedimentation equilibrium (Adams and Williams, 1964).

$$\frac{M_1}{M_{wc}^{app}} = \frac{M_1}{M_{wc}} + B_1 M_1 c + 2B_2 M_1 c^2 \qquad (16)$$

Determination of the Equilibrium Constant and Nonideality Terms. For the analysis of the nonideal monomer–dimer association we follow the most recent development by Adams (1965). The first step is to define an apparent weight fraction of monomer f_1^{app}, analogous to Steiner's equation for the weight fraction f_1 of monomer in an ideal solution. The Steiner (1952, 1954) equation is

$$\ln f_1 = \int_0^c \left(\frac{M_1}{M_{wc}} - 1\right) d \ln c \qquad (17)$$

Similarly we may define the apparent weight fraction of monomer

$$\ln f_1^{app} = \int_0^c \left(\frac{M_1}{M_{wc}^{app}} - 1\right) d \ln c \qquad (18)$$

and by substitution for M_1/M_{wc} from eq 16 we obtain

$$\int_0^c \left(\frac{M_1}{M_{wc}^{app}} - 1\right) d \ln c = \int_0^c \left(\frac{M_1}{M_{wc}} - 1\right) d \ln c + \int_0^c B_1 M_1 dc + 2 \int_0^c B_2 M_1 c dc \qquad (19)$$

or

$$\ln f_1^{app} = \ln f_1 + B_1 M_1 c + B_2 M_1 c^2 \qquad (19a)$$

$$f_1^{app} = f_1 e^{B_1 M_1 c + B_2 M_1 c^2} \qquad (19b)$$

The weight fraction of monomer is $f_1 = c_1/c$. In a similar

fashion we write $f_1^{app} = c_1^{app}/c$, where c_1^{app} is the apparent concentration of monomer. Using these relationships it is seen that

$$c_1^{app} = cf_1^{app} = c_1 e^{B_1M_1c + B_2M_1c^2} \quad (20)$$

The quantity c_1^{app} can be obtained as a function of c from the experimental data, since f_1^{app} can be evaluated through use of eq 18. This procedure requires the evaluation of the area of a plot of $((M_1/M_{wc}^{app}) - 1)/c$ *vs.* c. As Adams and Williams (1964) have shown, this plot has as its limit $-K_2 + B_1M_1$, a quantity which also may be obtained very roughly from the limiting slope of a plot M_1/M_{wc}^{app} *vs.* c. Although the integral in eq 18 may not be particularly sensitive to the limiting value of the integrand, it may be necessary to carry out a reiterative procedure to obtain a precise evaluation of c_1^{app}, since, in general, this limiting value will be only approximately known.

Now we move to show how the quantities defined in this section can be used with the experimental data, M_{wc}^{app} as a function of c, to provide values for the quantities B_1M_1, B_2M_1, K_2, and M_{wc}. Equations 4, 14, and 16 may be combined and rearranged to give

$$\left[\frac{M_1}{cM_{wc}^{app}} - (\sigma + B_2M_1c)\right]^{-1} = c_1 + 2K_2c_1^2 \quad (21)$$

where σ is defined by

$$\sigma = B_1M_1 + B_2M_1c \quad (22)$$

From eq 14 we have $2c = 2c_1 + 2K_2c_1^2$, hence subtracting eq 21 from the quantity $2c$ and applying eq 20 (*i.e.*, $c_1 = c_1^{app}e^{-\sigma c}$) we have

$$2c = \frac{1}{\dfrac{M_1}{cM_{wc}^{app}} - (\sigma + B_2M_1c)} + c_1^{app}e^{-\sigma c} \quad (23)$$

in which

$$B_2M_1c = \left(\frac{2cM_1}{M_{nc}^{app}} - c - c_1^{app}e^{-\sigma c} - c^2\sigma\right)\Big/(c^2/3) \quad (24)$$

Equation 24 is derived by making use of the definition of σ to eliminate B_1M_1 from the following equation (Adams, 1965).

$$\frac{cM_1}{M_{nc}^{app}} = \int_0^c \frac{M_1}{M_{wc}^{app}}dc = \frac{cM_1}{M_{nc}} + \frac{B_1M_1c^2}{2} + 2\frac{B_2M_1c^3}{3} = c_1 + \frac{K_2c_1^2}{2} + \frac{B_1M_1c^2}{2} + \frac{2B_2M_1c^3}{3} \quad (25)$$

The value of M_1/M_{nc}^{app} may be obtained by numerical evaluation of the integral in eq 25.

Substitution of the expression for B_2M_1c into eq 23 leads to an equation in one unknown, $\sigma = \sigma(c)$. Using the experimentally available values of M_1/M_{wc}^{app}, c_1^{app}, and c, one can then solve this combined equation for σ by successive approximation as a function of the total concentration. The values of B_2M_1 and B_1M_1 may be obtained from the slope and intercept, respectively, of a plot of σ against c. Once $\sigma = B_1M_1 + B_2M_1c$ is determined the concentration of monomer in the solution may be determined as $c_1 = c_1^{app}e^{-\sigma c}$ at various values of c. Finally, the equilibrium constant for the reaction is determined from the simple relation

$$K_2 = (c - c_1)/c_1^2 \quad (26)$$

and M_{wc} may be obtained from either of eq 4 or 16.

Materials

Preparation of β-Lactoglobulin B. The association–dissociation behaviors, below pH 3, of the A and B forms of β-lactoglobulin appear to be similar. However, in their light-scattering investigations, Timasheff and Townend (1961) found slight, but what they believed to be definite, differences. In order that the results be more definitive our experiments have been carried out with the separated form, β-lactoglobulin B. The separation of forms A and B from the protein containing approximately equal amounts of each was accomplished according to the chromatographic procedure described by Piez *et al.* (1961). Gradient elution arranged to provide a linear gradient in the ionic strength of the solvent at constant pH was employed. The isoelectric pH of β-lactoglobulin is very close to 5.2 at an ionic strength of 0.10 (Tanford and Nozaki, 1959), with a difference of about 0.10 pH unit between the two forms. The pH of the buffers used for elution in the separation was 5.8.

Selectacel type 40 DEAE resin (capacity, 1.1 mequiv/mg) was used as the stationary phase in the column. The resin was prepared for use by alternate washings with 0.1 N HCl and 0.1 N NaOH, ending with the acid, then it was washed with buffer. The 2.25 × 40 cm column and the three-times-recrystallized (Pentex, Inc.) β-lactoglobulin of concentration 7 g/dl were separately equilibrated with 0.05 M phosphate buffer (pH 5.8) at 4° before 0.75 g of protein was added to the column.

The chromatograph was obtained by measurement of the optical density at 278 mμ of every few fractions. Those from the center of each peak were pooled and dialyzed against distilled water for 6 hr to remove excess salt before lyophilization. The salt content of the lyophilized product, which was in the form of a flocculent, snow-white powder, was 20–30%. Almost equal amounts of the A and B forms were found in the Pentex protein which consisted of less than 10% minor components. A rechromatograph of 100 mg of one of the form B fractions failed to reveal the presence of a second peak. It was estimated that this form was contaminated with less than 1% of the A protein. An

examination of the form of the chromatographs reveals that the quality of the separation of form B from A is excellent, but that tailing of the B peaks, though slight, could lead to a contamination of the A fractions with this component. The chromatogram from a typical experiment is reproduced in Figure 1.

Buffers. The composition of each of the three buffers used in the present study is seen in Table I. These

TABLE I

pH	Ionic Strength	NaCl (N)	HCl (N)
2.58	0.10	0.05	0.05
2.20	0.15	0.10	0.05
2.58	0.15	0.10	0.05

solutions were prepared by weighing out the appropriate amounts of reagent grade NaCl and 0.5233 N HCl to give solutions of the desired ionic strength. Sufficient glycine, pK = 2.4, was used in each case to adjust the pH and to provide buffering capacity to the system. Measurements of pH were made on the Beckman Model G pH meter.

Experimental Methods

Preparation of the Solutions. Stock solutions prepared by adding protein to 0.1 N NaCl (pH 5) to give 5% concentrations were allowed to stand for 0.5 hr, then centrifuged. The supernatant solution was divided into two portions, one of which was diluted with an equal volume of buffer. Each portion was then dialyzed against six successive portions of fresh buffer for a total of 30 hr at 4°. A slight turbidity of solutions at pH 2.58 appeared during dialysis and an intermediate centrifugation treatment was required. All solutions of β-lactoglobulin B for experiments under a given set of conditions were prepared by diluting stock solutions with buffer which has been brought to dialysis equilibrium with the protein solutions.

Concentrations of solutions for each experiment were determined by ultraviolet absorption measurements at 278 mμ, using a Beckman DB spectrophotometer and an extinction coefficient of 9.6 dl/cm (Townend *et al.*, 1960). Dilutions were made by weighing and the difference between solution and solvent density was taken into account. An average of three concentration determinations, with a mean deviation of less than 0.5%, was used for each experiment. It should be noted that determination of concentrations by differential refractometry would be the method of choice if protein solutions were not limited in quantity, for this method avoids the introduction of dn/dc, the extinction coefficient, and the uncertainties concomitant with each.

Density Measurements. The buffer densities were

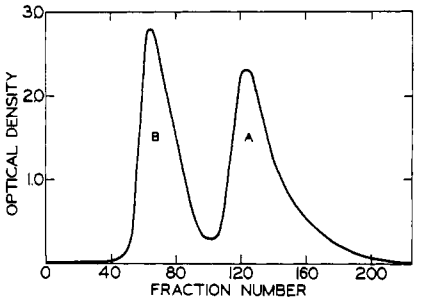

FIGURE 1: Typical chromatogram showing separation of β-lactoglobulin B from β-lactoglobulin A.

determined by pycnometry, using a 50-cc Weld-type pycnometer (Daniels *et al.*, 1962). The temperature of the equilibrating bath was maintained at 25 ± 0.01°. All mass values were taken from a graph of weight *vs.* time at the same elapsed time to eliminate error due to evaporation from the capillary. Weighings were made with a Mettler balance, with all readings being corrected for air buoyancy in the usual way. The density of protein solutions was calculated according to the equation $\rho = \rho_0 + (1 - \bar{\nu}\rho_0)/c$.

Equilibrium Ultracentrifugation. INSTRUMENTATION. All molecular weight data contained in this report were obtained from the photographic records of experiments performed at 25 ± 0.05°, using a Spinco Model "E" analytical ultracentrifuge, equipped with a standard RTIC unit. The actual value of the temperature was determined in terms of the RTIC reading from an independent calibration of the thermistor assembly.

The 22-lb Spinco An-J low-speed rotor was used. It was found to be almost essential for experiments at rotor speeds of less than 10,000 rpm for with the mechanical gear and speed-control assembly currently used on our instrument this heavier rotor provided much improved speed control and greatly reduced the precession of the rotor.

A new double-sector cell equipped with a 12-mm Epon centerpiece, interference double-slit window holders, and sapphire windows was used for all experiments. In principle, quartz windows would have been sufficient for these experiments, but the superior quality of sapphire windows seems to be sufficiently great to warrant their exclusive use whenever optimum results are desired.

Both the Rayleigh interference and schlieren optical systems were used. The schlieren diaphragm consisted of a phase plate upon which is deposited a metal line at the phase plate edge. A symmetrically centered double-slit interference mask was mounted on the upper collimating lens holder.

PREPARATION AND FILLING OF THE CELL. The cell was loaded using a syringe microburet. The amount 0.110 ml of solvent was placed in the left sector (viewed upright from screw ring end) and 0.095 ml of solution was placed in the right sector, giving a solution column height of about 0.27 cm. Following a suggestion

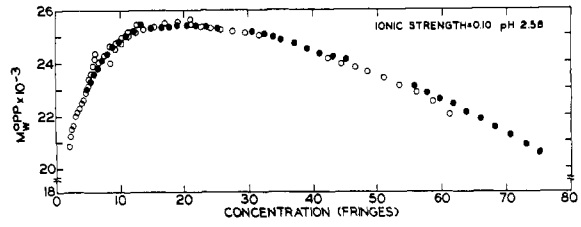

FIGURE 2: M_{wc}^{app} vs. concentration (in fringes). β-Lactoglobulin B in solution at pH 2.58, Γ/2 = 0.10.

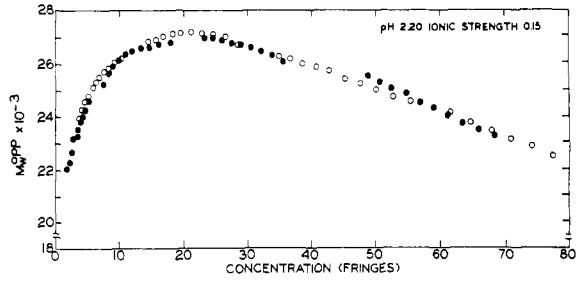

FIGURE 3: M_{wc}^{app} *vs.* concentration (in fringes). β-Lactoglobulin B in solution at pH 2.20, Γ 2 = 0.15.

by Dr. E. T. Adams, Jr., we have not used a liquid "false bottom" or layering fluid.

Prior to final loading of the cell the solution compartment was rinsed two or three times with small quantities of the protein solution to be characterized in order to minimize concentration changes due to adsorption of protein on the centerpiece walls. To further minimize adsorption, plastic rather than steel needles were used in filling the cell. At the conclusion of each experiment the cell was disassembled, washed, thoroughly rinsed, and dried with ethanol.

CHOICE OF SPEED, OVERSPEEDING, AND TIME TO EQUILIBRIUM. The initial solute concentrations were selected to cover a wide range, thus it was necessary to use different rotor speeds. The latter were chosen in order to obtain roughly 10–25 fringes on the interference pattern at equilibrium. They varied between 8225 and 16,220 rpm, with the lower speeds being used at the higher protein concentrations. To obtain a fixed number of fringes the speed varies approximately with $c_0^{1/2}$.

The time required for the attainment of equilibrium as determined by the equation of Van Holde and Baldwin (1958) was about 15 hr for a typical experiment. This time could be reduced by shortening the solution column, but not without concomitant loss of resolution and accuracy. However, Hexner *et al.* (1961) have described a technique of overspeeding by which the time to equilibrium can be reduced by a factor of approximately three. We have used their procedure with excellent success.

DETERMINATION OF c_r. Concentrations at the meniscus, c_m, were calculated in each case by using the conservation of mass statement. The distribution of solute concentration throughout the cell was then obtained from the relationship $c_r = c_m + \Delta c$, where Δc is computed from the fringe difference between the meniscus and the radial position (r), according to the statement

$$\Delta J = \frac{a}{\lambda}\left(\frac{\partial n}{\partial c}\right)\Delta c$$

DETERMINATION OF OPTICAL LEVER ARM CONSTANT. From a comparison of schlieren pattern area and fringe difference across the cell for the same system, whether at equilibrium or in a transient state, it is possible to compute the optical lever arm constant (b) provided the cylindrical lens magnification factor G is known. It was this procedure which was adopted. With θ representing the schlieren angle, it turns out that

$$b = \frac{\text{area of schlieren pattern (cm}^2\text{)}}{\lambda G \tan\theta\, \Delta J}$$

Results and Discussions

The experimental data required for the evaluation of the nonideality coefficients and the equilibrium constant for the association reaction are the values of M_{wc}^{app} as a function of solute concentration. These data for the β-lactoglobulin B in three buffer systems from sedimentation equilibrium experiments are presented in Figures 2–4. The concentration of macromolecule is expressed in Rayleigh fringe numbers for convenience, since the experimental record, as obtained from the photographic plates, is given in these terms. The concentration (grams per deciliters) is related to the fringe number (J) through the formula $J = 40.2c$. The value $1.82(10^{-3})$ dl/g at 546 mμ (Halwer *et al.*, 1951) was used for the refractive increment (dn/dc) of the β-lactoglobulin in solution in arriving at this conversion factor.

For all experiments except those at the very lowest concentrations, the schlieren and interference data were combined to calculate the apparent weight-average molecular weights at the several radial positions, M_{wc}^{app}, each corresponding to a definite c value. For solutions of less than 0.2 g/dl protein concentration, molecular weight averages were evaluated by differentiation of the data obtained exclusively from the fringe pattern. For each separate experiment, molecular weight averages, M_{wc}^{app}, were computed at constant intervals at 8–10 radial positions through the cell. However, values near the extremes of the cell were not generally used because of the lack of precision in the schlieren data in those regions. The data points in Figures 2–4 from successive experiments are marked alternately with filled and open circles. Before drawing these figures the molecular weight data for each experiment were plotted as a function of concentration in the cell and a smooth curve was drawn through the data

points. This procedure aided in smoothing the data, especially in the elimination of a few values which departed abnormally from the apparent molecular weight curve.

With reference to Figures 2–4, it will be noticed that the apparent molecular weights increase rapidly at low concentrations, a behavior which is indicative of self-associating systems. A unique aspect of these plots, however, is that the apparent molecular weights attain a maximum molecular weight which is not equal to any integral multiple of the monomer and subsequently decrease with increasing concentrations. This situation is unlike the more common one in which the molecular weight increases monotonically with concentration tending toward a maximum which corresponds, for example, to the dimer molecular weight in the case of dimerization. The observed behavior in the present case has to be explained by the existence of a pronounced solution nonideality, which becomes significant long before the weight-average molecular weight of the associating system approaches the value corresponding to the dimer molecular weight. For such a system, the advantage of conducting the investigation over a large concentration range, as opposed to attempting to extract information from only one or two experiments, is here clearly evident. In the central portions of this plot, M_{wc}^{app} is nearly independent of concentration, so that an experiment carried out by using a solution in this intermediate concentration range would not reveal the presence of a reaction of all; as a consequence one might be led to quite erroneous conclusions about the nature of the system.

Another interesting aspect of these plots is that the data from successive experiments merge quite well to form a smooth continuous curve. This is particularly true at low concentrations where experiments at different initial concentrations superimpose to a considerable extent. Although there is evidence of some discontinuity at the higher concentrations, the effect is not pronounced. A merger of the individual molecular weight *vs.* concentration curves is of course required by theory, as Adams and Fujita (1963) have pointed out. It is difficult to think of reasons why it is sometimes not observed in practice. Thus, Adams (1962), Squire and Li (1961), and Kakiuchi (1965) have all observed a very significant discontinuity of their molecular weight data for the association reactions of insulin, adrenocorticotropin, and *Bacillus subtilis* α-amylase, respectively. In each case it was found that solutions of different initial protein concentrations yielded somewhat different apparent molecular weights at positions in the cell which corresponded to the same total concentration, and this difference was seemingly greater than could be explained by a consideration of the experimental errors alone. Kakiuchi noted that the failure of his curves to superimpose could have been caused by the presence of zinc in the solution, while Adams was at the time unable to give a satisfactory explanation for the results he obtained. In a very extensive investigation of the behavior of bovine

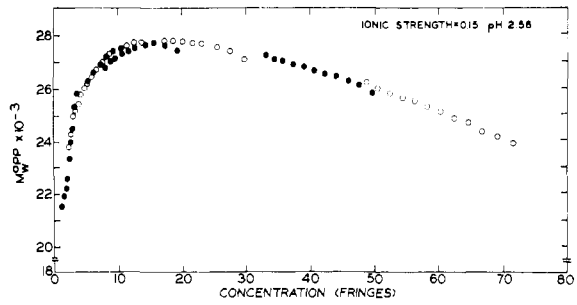

FIGURE 4: M_{wc}^{app} *vs.* concentration (in fringes). β-Lactoglobulin B in solution at pH 2.58, $\Gamma/2 = 0.15$.

insulin in acid solution, Jeffrey and Coates (1966) attributed the failure of the molecular weight data to form a continuous curve to the inadequacies of the prior curve-fitting procedures.

In a recent presentation, Adams (1966) reported that the anomalous results observed by sedimentation equilibrium methods could be explained by assuming that the layering fluids commonly used in the experiment are interacting with the protein in solution so as to precipitate it at the solution-layering fluid interface. For this case, methods which make use of initial concentration values obtained from measurements made before loading the solution into the cell will give molecular weights which are too low. He presented an apparent molecular weight *vs.* concentration plot for β-lactoglobulin A at pH 4.6 obtained in experiments performed without the use of any layering fluid in the cell. Now the molecular weight values from experiment to experiment are found to merge very well over an extensive range of concentration. In a recent contribution Albright and Williams (1967) found that molecular weights could be measured with sufficient and good accuracy by following this procedure.

Before the Adams theory can be applied to the calculation of the nonideality terms and the equilibrium constant, the curves of Figures 2–4 must be transformed into the reduced reciprocal apparent molecular weight function, M_1/M_{wc}^{app} *vs.* concentration curves since this is the significant plot for the subsequent computation. In order to calculate this reciprocal function from the apparent molecular weight values it is necessary to know the molecular weight of the monomer, which must correspond to the redefined component monomer weight according to the definition of Casassa and Eisenberg (1964). In practice this definition of neutral component is used whenever the solution is dialyzed against the solvent to constant chemical potential of the dialyzable components. The hypothetical addition of the redefined component to the supporting electrolyte inside the dialysis bag would now cause no flow of diffusible ions through the membrane, and any change in the chemical potential of the solution will be due only to changes in the amount of this component. The definition means that one can consider the apparent index of refraction difference between solution and

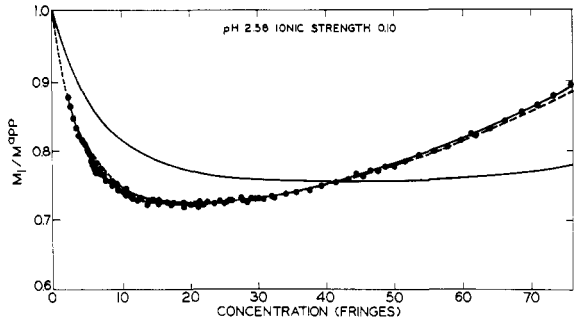

FIGURE 5: M_1/M_{wc}^{app} (and generated function, M_1/M_{nc}^{app}) *vs.* concentration (in fringes). β-Lactoglobulin B in solution at pH 2.58, Γ/2 = 0.10. Points: values of M_1/M_{wc}^{app} calculated from data of Figure 2. Dashed line: computed from B_1, B_2, and K values. Full line, without data points: from calculated values of M_1/M_{nc}^{app}.

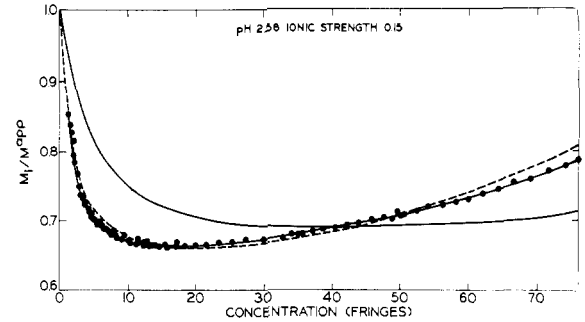

FIGURE 7: M_1/M_{wc}^{app} (and generated function, M_1/M_{nc}^{app}) *vs.* concentration (in fringes). β-Lactoglobulin B in solution at pH 2.58, Γ/2 = 0.15. Points: values of M_1/M_{wc}^{app} calculated from data of Figure 4. Dashed line: computed from B_1, B_2, and K values. Full line, without data points: from calculated values of M_1/M_{nc}^{app}.

solvent as due solely to the presence of the macromolecular component. Unfortunately, a calculation of the Casassa and Eisenberg monomer molecular weight requires the knowledge of a distribution parameter (Γ) available from equilibrium dialysis data.

In the absence of such information one is limited to obtaining the desired monomer molecular weight by direct experimental measurement. Using the curves of Figures 2–4 it is not possible to extrapolate the apparent molecular weight data to infinite dilution with any degree of accuracy. However, the curves, on rough extrapolation, do tend toward a limiting value which is crudely estimated to be about 18,000, the value usually given for the monomer molecular weight of β-lactoglobulin. For our purpose it was considered to be adequate to obtain the monomer molecular weight in another way. Under several sets of conditions of pH and ionic strength, for which the M_{wc}^{app} *vs.* concentration curve was nearly flat, an average value of about 18,400 was obtained by extrapolation, and this is the value that was used for subsequent calculations. In spite of the fact that the Casassa and Eisenberg component would vary slightly for each different set of pH and ionic strength conditions used in these experiments, it was observed that the extrapolated value of M_1 did not seem to vary significantly with the several sets of conditions. This was interpreted to mean that the differences in the limiting molecular weight defined according to Casassa and Eisenberg are not great enough to detect experimentally for the variations in ionic strength and pH which were employed. It should be also mentioned that Piez *et al.* (1961) obtained a value of 18,360 from an amino acid analysis of β-lactoglobulin A.

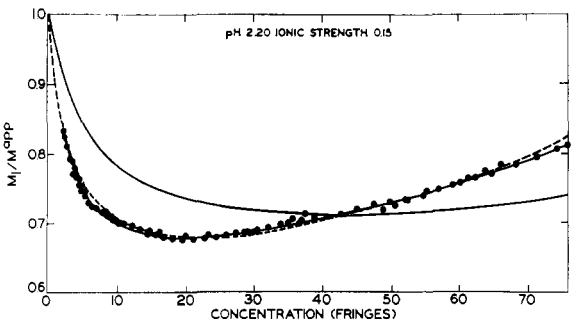

FIGURE 6: M_1/M_{wc}^{app} (and generated function, M_1/M_{nc}^{app}) *vs.* concentration (in fringes). β-Lactoglobulin B in solution at pH 2.20, Γ/2 = 0.15. Points: values of M_1/M_{wc}^{app} calculated from data of Figure 3. Dashed line: computed from B_1, B_2, and K values. Full line, without data points: from calculated values of M_1/M_{nc}^{app}.

The M_1/M_{wc}^{app} values calculated from the data of Figures 2–4 and the monomer molecular weight (M_1) are plotted as a function of concentration in Figures 5–7. The smooth curve drawn through the data points was used to provide the values for all subsequent calculations. The resultant values of M_1/M_{wc}^{app} were used to compute the apparent weight fraction of monomer (f_1^{app}) at several values of c, according to eq 18. The apparent concentration of monomer was subsequently obtained by application of the relationship $c_1^{app} = cf_1^{app}$. These values, along with those for f_1^{app} are tabulated as functions of concentration for the three systems in Table II.

In Figure 8 is presented a plot of the parameter $\sigma = B_1M_1 + B_2M_1c$ as a function of the concentration, expressed as fringes. At each concentration these data were obtained by application of eq 23 and 24. The quantity M_1/M_{nc}^{app} as a function of concentration, necessary for the computation of according to eq 24, is also shown (full line, without data points) in Figures 5–7, respectively. This function was generated from the apparent weight-average molecular weight curves according to eq 25, where the indicated integration

TABLE II: Values of c_1^{app} and f_1^{app} as a Function of Concentration.

J	$-\ln f_1^{app}$	f_1^{app}	c	$c_1^{app} = cf_1^{app}$
		pH 2.58, Γ/2 = 0.10		
10	0.4657	0.620	0.248	0.154
20	0.6540	0.519	0.497	0.258
30	0.7650	0.465	0.745	0.346
40	0.8293	0.432	0.993	0.428
50	0.8913	0.409	1.242	0.509
60	0.9281	0.395	1.490	0.589
70	0.9533	0.387	1.738	0.671
76	0.9637	0.381	1.887	0.719
		pH 2.20, Γ/2 = 0.15		
10	0.5861	0.556	0.248	0.138
20	0.6882	0.452	0.497	0.224
30	0.9352	0.398	0.745	0.296
40	1.0120	0.364	0.993	0.361
50	1.0728	0.342	1.242	0.425
60	1.1192	0.326	1.490	0.486
70	1.1538	0.316	1.738	0.548
76	1.1700	0.311	1.887	0.586
		pH 2.58, Γ/2 = 0.15		
10	0.6783	0.506	0.248	0.126
20	0.9098	0.403	0.497	0.200
30	1.0513	0.350	0.745	0.261
40	1.138	0.321	0.993	0.318
50	1.202	0.301	1.242	0.374
60	1.256	0.285	1.490	0.425
70	1.295	0.274	1.738	0.476
76	1.313	0.269	1.887	0.508

was performed numerically by using the trapezoidal rule. The slope and intercept of the plot of σ *vs.* fringe number were determined graphically to provide the values of the nonideality coefficients B_2 and B_1, respectively, according to eq 22. These values are provided in Table III for each system studied. It may be noticed that the intercept, *i.e.*, B_1, for the three systems was greatest for the protein–buffer system at pH 2.58, ionic strength 0.10, and least for the system at pH 2.58, ionic strength 0.15. The value of B_1 for the protein solution at pH 2.20, ionic strength 0.15, is intermediate, but its value lies closer to that corresponding to the

TABLE III: Summary of Data.

	B_1M_1	B_2M_1
pH 2.58, Γ/2 = 0.10	0.108	0.013
pH 2.20, Γ/2 = 0.15	0.070	0.018
pH 2.58, Γ/2 = 0.15	0.056	0.020

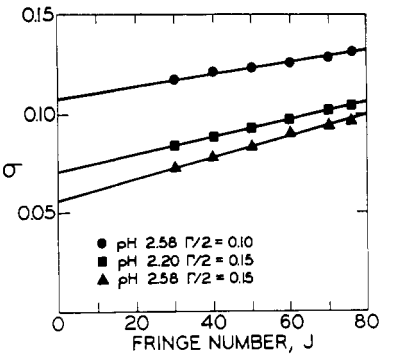

FIGURE 8: Parameter $\sigma = B_1M_1 + B_2M_1c$ as a function of fringe number.

other solution of ionic strength 0.15, illustrating the effective action of supporting electrolyte in suppressing charge nonidealities. These results can be best understood with reference to the titration curve for β-lactoglobulin of Tanford and Nozaki (1959) from which it can be seen that the charge on the protein does not depend much on the pH in this region of the acid side of the curve. A condition of nearly maximum charge (proton binding) has been reached at pH 2.5.

The values for B_2, obtained from the slopes of σ *vs.* fringe number curves, exhibit a trend which is apparently opposite to that for B_1 with respect to ionic strength and charge. Although the accuracy with which B_2 is known is not great, a consideration of the precision involved in the evaluation of σ suggests that this trend is a real one and that the observed relationship between these values as function of ionic strength and pH is the correct one. In the absence of more data of this kind, however, it may be difficult to decide unequivocally whether or not this relationship of the third virial coefficient actually has true physical significance. There is no question, however, that σ does indeed depend on the concentration, a fact which cannot be ignored if an accurate description of the apparent molecular weight behavior of the system is desired. It is not possible, for these systems, to solve another equation analogous to eq 23 which would yield a single virial coefficient independent of the value of the concentration used in the computation. Such a solution, if obtained, would not be truly representative of the system as exemplified by the apparent molecular weight behavior, especially at the higher concentrations.

Calculation of the Equilibrium Constant. Using values of σ obtained from Figure 8, the concentration of monomer was calculated as a function of the total protein concentration from the equation $c_1 = c_1^{app}e^{-\sigma c}$ where $c_1^{app} = cf_1^{app}$. The equilibrium constants were evaluated under each set of conditions from eq 26. Numerical values for them as functions of concentration are collected to form Table IV. It will be noticed that the equilibrium constants also display a trend with pH and ionic strength; however, this trend is opposite

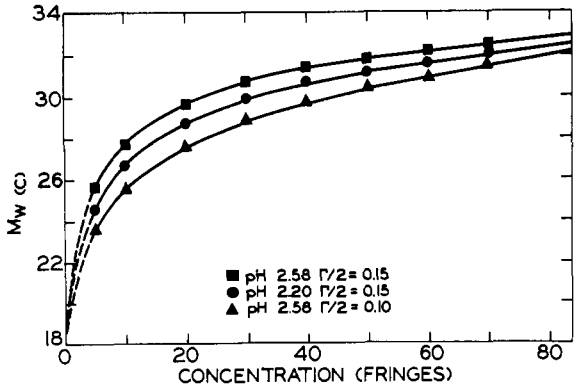

FIGURE 9: Idealized curves for M_{wc} as a function of concentration.

to that for B_1M_1 because of the nature of the reaction, that is, higher pH and ionic strengths favor stronger association and higher equilibrium constants. The constancy of the K values over the entire range of concentrations in each case is taken to be an indication that the values of σ are reliable. This must be true if eq 23 and 24 provide unique solutions for the nonideality coefficients. The true equilibrium constant must be independent of the nonideality terms.

It is of significance that it was not necessary to obtain the values for K at infinite dilution, rather they were calculated by using an analysis of the data at moderately high concentrations where the apparent molecular weight data are more precise.

The function M_{wc} *vs.* fringe number has been plotted to form Figure 9. For each of the three solvent systems the equilibrium constant given in Table IV has been utilized for its calculation. These plots illustrate the differences in the affinity of the association reaction which are caused by the environmental variations. Had there been no solution nonidealities ($B_1 = B_2 = 0$) these are the curves which would have been obtained directly by experiment.

It might be thought possible to obtain different numerical combinations of K and the nonideality coefficients B_1 and B_2 which would be equally successful in reproducing the experimental observations of apparent molecular weight dependence upon the several factors. Unfortunately there is no simple and direct proof of the uniqueness of these quantities as we have evaluated them, but one may resort to internal correlations and indirect comparisons to put matters upon a satisfactory basis.

For instance, it is possible to make a more conventional evaluation of the equilibrium constants by using equations already provided by Rao and Kegeles (1958). For the ideal monomer–dimer reaction, expressed as a dissociation

$$K = \frac{c(2M_1 - M_w)^2}{M_1(M_w - M_1)}$$

TABLE IV: Values of the Equilibrium Constants. β-Lactoglobulin B in Acid Solution.

c	c_1	K	(K)
	pH 2.58, Γ/2 = 0.10		
0.248	0.150	4.37	
0.497	0.244	4.25	
0.745	0.317	4.26	
0.993	0.380	4.24	
1.242	0.437	4.21	
1.490	0.489	4.19	
1.738	0.535	4.26	
		4.25 ± 0.10	(4.6)
	pH 2.20, Γ/2 = 0.15		
0.248	0.135	6.22	
0.497	0.215	6.10	
0.945	0.278	6.04	
0.993	0.331	6.04	
1.242	0.379	6.01	
1.490	0.417	6.17	
1.738	0.459	6.07	
1.887	0.481	6.08	
		6.10 ± 0.15	(6.7)
	pH 2.58, Γ/2 = 0.15		
0.248	0.124	8.08	
0.497	0.193	8.15	
0.945	0.247	8.16	
0.993	0.294	8.09	
1.242	0.337	7.97	
1.490	0.372	8.08	
1.738	0.407	8.02	
1.887	0.422	8.23	
		8.10 ± 0.20	(10.4)

At infinite solute concentrations the values for K computed according to this equation will be apparent ones, K^{app}, but Adams and Fujita (1963) have shown that a plot of K^{app} *vs.* concentration will give the quantity $K - B_1M_1$ as its intercept. Not unexpectedly, the apparent equilibrium constants are strongly dependent upon concentration, and this fact makes for difficulty in the extrapolation to infinite dilution. The values for K obtained in this way, included in parentheses in Table IV, are seen to be in quite good agreement with the ones already described. The K^{app} *vs.* fringe number curves, with their extrapolations, are presented in Figure 10.

In order further to test the correctness of our equilibrium constant data and less directly, the nonideality coefficients, eq 16 has been used to generate the function M_1/M_{wc}^{app} from the observed values for B_1, B_2, and K. The resulting plots are illustrated by the dashed lines in Figures 5–7. The agreement between the calculated curves and the best curves through the data (solid lines) is seen to be very good. Except for a slight

departure at the highest concentrations studied, the over-all shape of the curve is reproduced remarkably well by the calculated function of M_1/M_{wc}^{app}. A true appreciation of how well the calculated curves fit the experimental data can best be gained by realizing that the nonideality coefficients found for these systems are extraordinarily large, being of the order of 10–100 times greater than is generally found in buffered and supporting electrolyte–protein systems, and of the same order of magnitude as those often found for synthetic polymer–solvent solutions.

It is also appropriate to compare our results with those of Timasheff and Townend (1961) who studied this system by light scattering under similar conditions. For this comparison it is necessary to change the units we have used for virial coefficient and equilibrium constant into those used by them. After conversion of concentration scales from grams per deciliter to grams per liter, our second virial coefficient B may be compared with theirs, written as $2B_0/M_{1/2}$. The relationship between the equilibrium constants can be expressed as $K_d = 20/M_1K$, where K_d represents the dissociation constant of Timasheff and Townend and K is the constant we have calculated, considering the reaction as an association. The factor $20/M_1$ changes the equilibrium constant from the gram to the molar basis.

For the protein–buffer system at a pH of 2.7 and ionic strength 0.10 at 25° Timasheff and Townend obtained the value 6.9×10^{-7} mole l/g² for their second virial coefficient. When properly reduced in units our value becomes 5.9×10^{-7}, which is in reasonably good agreement. Although the difference noted may not be significant, a possible explanation for the slightly lower value is afforded by the fact that part of nonideality effect we observed takes the form of a third virial coefficient, which becomes significant above 1 g/l. concentration. Timasheff and Townend have not considered this possibility, so that perhaps if their measurements had been extended to concentrations much above this value, the existence of a finite value for the third virial coefficient would have been revealed.

A similar comparison of the equilibrium constant for this reaction under the same set of conditions is not as satisfactory. Again reduced to the units of Timasheff and Townend our data yield a value for K_d of 16×10^{-5} mole/l. as compared with their datum 5.08×10^{-5} mole/l. at 25°. (It might be noted that for β-lactoglobulin A, the light-scattering K_d is given as 13.0×10^{-5} mole/l., again at 25°.) It is always difficult to compare equilibrium constants of this nature quantitatively, but certainly this difference is greater than can be attributed to experimental uncertainty. It can be shown that it arises from the difference in apparent molecular weights as observed by the two experimental methods. The apparent molecular weights from both laboratories are in very good agreement at the higher concentrations (and perhaps hence the observed agreement in the virial coefficients) but they do differ rather significantly at the low concentrations where our data give somewhat (but not pronounced) lower values of M_{wc}^{app}.

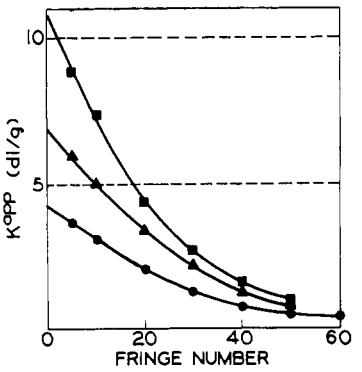

FIGURE 10: Plots of K^{app} *vs.* fringe number (concentration). (●) pH 2.58, Γ/2 = 0.10; (▲) pH 2.20, Γ/2 = 0.15; and (■) pH 2.58, Γ/2 = 0.15.

However, it is not believed that the differences in the equilibrium constant values, caused by the differences in the observed apparent molecular weights in the relatively low concentration regions by light scattering as compared to sedimentation equilibrium, should invalidate our judgment about the essential soundness of the Adams method in application to analysis of systems of the type in question. This approach has been shown to be suitable in dealing with a basic problem, namely, that of the separation and quantitative description of two distinct effects which cause an apparent dependence of molecular weight on concentration. They are the existence of an association–dissociation equilibrium and of solution nonideality, in this particular instance presumably charge effects.

Acknowledgments

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