Self-Association of β-Lactoglobulin B in Acid Solution and Its Variation with Temperature†

J. Visser,‡ Richard C. Deonier, E. T. Adams, Jr., and J. W. Williams*

ABSTRACT: Low-speed sedimentation equilibrium experiments have yielded apparent weight-average molecular weight data ($M_{w(c)^a}$) at 5, 10, 15, 25, and 35.5° as a function of concentration for β-lactoglobulin B in pH 2.64 NaCl–glycine buffer, ionic strength 0.16. The concentration dependence of $M_{w(c)^a}$ is ascribed to self-association under nonideal conditions. The data are analyzed by using three different theoretical approaches, with emphasis being given to the monomer–dimer scheme. The magnitude of the association constant K_2 is sufficiently large that the results from the different formulations are subject to appreciable variation, depending upon the mathematical expression and the concentration range involved. These variations are discussed, and the results are compared to those of previous workers.

There is presently much interest in the physical and mathematical methods by which protein self-associations in solution are studied. Certain basic principles have been established, but there remains the reservation that there are serious difficulties to be overcome in their application to specific cases. Basic to progress is the acquisition of accurate association reaction equilibrium constants, with a fundamental prerequisite for their evaluation being the availability of highly accurate weight-average molecular weight *vs.* protein concentration data.

The present study of β-lactoglobulin B was undertaken for several reasons: (a) to test the adequacy of several proper theoretical formulations for analyzing the self-associations; (b) to obtain thermodynamic data for comparison with later studies of its genetic variants; and (c) to provide a broader thermodynamic understanding of protein self-associations in general, in the hope that this will aid in the elucidation of the forces exerted between regions of the protein molecules, and perhaps in this case, provide an insight into the still unknown function of this protein.

The β-lactoglobulins have been the subject of extensive study from many points of view. The literature has been reviewed on several occasions, notably by McKenzie (1967, 1970). While β-lactoglobulin is absent from milk of the human, the guinea pig, and the camel, it has been isolated from the milk whey of such ruminants as the cow (Ogston and Tilley, 1955; Bell and McKenzie, 1967), the goat (Phillips and Jenness, 1965), and the sheep (Maubois *et al.*, 1965). Milk from one nonruminant (swine) has been found to contain β-lactoglobulin (Kessler and Brew, 1970). The bovine lactoglobulins have been isolated as four genetic variants (A, B, C, D), which are identical except for one or a few amino acid substitutions. The association behavior of these variants is largely influenced by pH changes: β-lactoglobulins A and B generally undergo at least a monomer–dimer equilibrium (Timasheff and Townend, 1961; Albright and Williams, 1968), and above pH 3.5, association beyond the dimer level takes place (Kumosinski and Timasheff, 1966). Even though at this time the amino acid sequence of β-lactoglobulin has been only partially elucidated (Frank and Braunitzer, 1967) and the X-ray crystallography is incomplete, one may nevertheless strive toward a relationship between the structure and the association behavior.

Some of the earlier data for the self-association of this protein at low pH (2–3) have been collected by use of light scattering (Timasheff and Townend, 1961) and of low-speed sedimentation equilibrium (Albright and Williams, 1968). Under these conditions, equilibrium constants could be obtained with reasonable accuracy; moreover, the appreciable thermodynamic nonideality could be successfully taken into account. There was a noticeable discrepancy in the absolute molecular weights as determined by Timasheff and Townend and by Albright and Williams, but there was complete accord on one important point: under the low pH conditions, the monomer–dimer equilibrium prevails.

It is the prevalence of the monomer–dimer equilibrium which makes this system particularly suited for testing calculation procedures. To begin with, it seems appropriate to consider first those systems in which only two or, at most, three parameters may be required, before attempting to study those systems which require many-parameter representations. Secondly, problems with ambiguous models are largely avoided, thereby allowing for greater certainty in the thermodynamic interpretation of the association constants.

Theory

Preliminary Considerations. Sedimentation equilibrium experiments provide us with information from which apparent molecular weight data as a function of concentration can be made available. The basic equation, in conventional symbols, is

$$M_{w(c)^a} = \frac{2RT}{\omega^2(1 - \bar{v}_2\rho_0)} \frac{\mathrm{d} \ln c(r)}{\mathrm{d}(r^2)} \quad (1)$$

The use here of the factor ρ_0, the density of the solvent, has been explained in a footnote of the Deonier–Williams (1970)

† From the Departments of Chemistry of The University of Wisconsin, Madison, Wisconsin 53706, and Texas A & M University, College Station, Texas 77843. *Received January 31, 1972.* This investigation was supported by NSF Grants GB-8239, GB-29100, and GU-3159, by NIH Grants GM-14963 and GM-17611, and by the Graduate School of the University of Wisconsin. Presented in part at the 62nd Annual Meeting of the American Society of Biological Chemists, San Francisco, Calif., June 13–18, 1971 (Visser *et al.*, 1971).

‡ On leave from the Agricultural University, Department of Biochemistry, Wageningen, The Netherlands.

article. Also some other assumptions are included, for instance that $\bar{v}_2$ is pressure independent, that it is the same for all of the several species in the equilibrium mixture, and that their refractive index increments are identical and invariant with concentration. The activity coefficients are expanded in a power series in concentration, and for each power of c, the virial coefficients of the different species are assumed to be equal (Adams and Fujita, 1963). In the present case we will utilize either one or two virial coefficients in the interpretation of the data, with their number being selected so that primary data are fitted within the experimental precision.

The apparent molecular weight may be expressed in terms of weight-average molecular weights (M_w) and nonideality contributions (eq 2).[1] Adams (1965) also developed the rela-

$$\frac{M_1}{M_{w(c)^a}} = \frac{M_1}{M_{w(c)}} + B_1M_1c + 2B_2M_1c^2 + \ldots \quad (2)$$

tion between apparent number-average molecular weight ($M_{n(c)^a}$) and $M_{w(c)^a}$.

$$\frac{M_1}{M_{n(c)^a}} = \frac{1}{c}\int_0^c \frac{M_1}{M_{w(c')^a}}\,dc' = \frac{M_1}{M_{n(c)}} + \frac{B_1M_1c}{2} + \frac{2B_2M_1c^2}{3} \quad (3)$$

The method developed by Steiner (1952) for evaluating the weight fraction of monomer present in ideal self-associating systems at a given concentration has been extended to nonideal systems by Adams and Williams (1964), who wrote

$$\ln f_1^a = \int_0^c [(M_1/M_{w(c')^a} - 1)/c']dc' = \ln f_1 + B_1M_1c + B_2M_1c^2 \quad (4)$$

in which f_1^a and f_1 are the apparent and true weight fractions of monomer, respectively.

When the data indicate that an associating system is involved, the above equations may be combined in various ways to permit the evaluation of the association constant(s) K_i and the virial coefficients. To do this one requires n (or $n + 1$) equations if n species and one (or two) virial coefficients are used for the description of the self-association. Other necessary equations are the definition of M_w and the statement of the conservation of mass. (See Adams, 1967, for more complete discussion.) The order in which the parameters are to be eliminated by use of these equations to give the most reliable results is difficult to judge. In most instances there has been a preference to solve first for the nonideality terms (Adams and Lewis, 1968; Albright and Williams, 1968; Hancock and Williams, 1969), and to emphasize the use of data at the higher concentrations. More recent developments tend to use graphical analyses not only for parameter choice but also for the establishment of the model (T'so and Chan, 1964; Roark and Yphantis, 1969; Chun and Kim, 1970; Tang *et al.*, 1971; Tang and Adams, 1971b; Chun *et al.*, 1972).

One of the primary purposes of the present work is the testing and evaluation of three analytical procedures for analyzing the monomer–dimer self-association: some recent developments by Tang *et al.* (1971) and by Chun *et al.* (1972); a general least-squares procedure; and a modification of the Van Holde–Rossetti method (Van Holde and Rossetti, 1967) which allows its application to the monomer–dimer system (Deonier and Williams, 1970).

The Method of Tang et al. (1971). Tang *et al.* (1971) have noted that when only one virial coefficient is necessary, the combination of eq 2 and 3 will lead to a quantity Z which is identical for both ideal and nonideal solutions, an interesting result in itself[2]

$$Z = \frac{2M_1}{M_{n(c)^a}} - \frac{M_1}{M_{w(c)^a}} = \frac{2M_1}{M_n} - \frac{M_1}{M_w} \quad (5)$$

In monomer–n-mer associations this quantity can be described as

$$\frac{2M_1}{M_n} - \frac{M_1}{M_w} = 2f_1\left(1 - \frac{1}{n}\right) + \frac{2}{n} - \frac{1}{n - f_1(n-1)} \quad (5a)$$

(*cf.* Chun and Kim, 1970).

In eq 5a f_1 is again the weight fraction of monomer, and n is the degree of aggregation. It is to be noted that eq 5a is quadratic in f_1. One can readily obtain f_1 by solving this quadratic equation; here the negative root is used. Once f_1 is obtained it is easy to obtain the equilibrium constant, K_n, from the definition of the total concentration, c, since $c = c_1 + K_nc_1^n$. Division of c by c_1, followed by rearrangement leads to

$$(1 - f_1)/f_1 = K_nc_1^{n-1} = K_n(cf_1)^{n-1} \quad (5b)$$

Thus, a plot of $(1 - f_1)/f_1$ *vs.* $(cf_1)^{n-1}$ will give a straight line whose slope is K_n.

Similarly one can use the definition of $M_1/M_{w(c)^a}$ to obtain B_1M_1. Here one notes that

$$\frac{M_1}{M_{w(c)^a}} = \frac{M_1}{M_{w(c)}} + B_1M_1c = \frac{1}{n - f_1(n-1)} + B_1M_1c$$

Simple rearrangement leads to

$$\frac{M_1}{M_{w(c)^a}} - \frac{1}{n - f_1(n-1)} = B_1M_1c \quad (5c)$$

Here a plot of $(M_1/M_{w(c)^a}) - \{1/[n - f_1(n-1)]\}$ *vs.* c has a slope of B_1M_1.

Extension of the same procedure to the case in which both a second and a third virial coefficient are involved can be achieved by combining eq 4 with 2 and 3 to eliminate B_1M_1 and B_2M_1 which gives (Tang and Adams, 1971a)

$$\frac{6M_1}{M_{n(c)^a}} - \frac{M_1}{M_{w(c)^a}} - 2\ln f_1^a = \frac{6M_1}{M_n} - \frac{M_1}{M_w} - 2\ln f_1 \quad (6)$$

[1] The nonideality has been represented by the following relation between the activity coefficient y_i and the concentration c: $\ln y_i = iB_1M_1c + iB_2M_1c^2$. This form has been adopted for computational convenience (*cf.* eq 41 in Adams and Williams, 1964).

[2] The quantity Z is the same as Roark and Yphantis' quantity $M_1/M_{y1(c)}$. However, for the case in which only one virial coefficient is required to describe the nonideality, they obtained Z from the relation, $M_1/M_{y1(c)} = M_1d(1/cM_n)/d(1/c) = Z$, instead of using direct combination of eq 2 and 3.

For the monomer–dimer case this equation reduces to the expression

$$\frac{6M_1}{M_{n(c)^a}} - \frac{M_1}{M_{w(c)^a}} - 2\ln f_1{}^a = 3 + 3f_1 - \frac{1}{2 - f_1} - 2\ln f_1 \tag{6a}$$

Obviously f_1 is the key quantity to be sought from the experimental data.

A Least-Squares Procedure. In general, model testing and estimation of parameters are possible by use of expressions for $M_1/M_{w(c)^a}$ in terms of the appropriate equilibrium constants and physically reasonable nonideality terms. Choice of the model to be tested will be based on the form of the $M_{w(c)^a}$ *vs.* c curve or from information derived from other techniques such as sedimentation velocity, low-angle X-ray scattering, electron microscopy, etc.

The data obtained were thus analyzed by means of a Wang desk-computer program which was based on a nonlinear least-squares fit. A measure of the "goodness of fit" can be defined as

$$\chi^2 \equiv \sum \frac{1}{\sigma_i{}^2}[y_i - y(x_i)]^2 \tag{7}$$

This least-squares analysis has been described in detail by Bevington (1969). In it the σ_i are the uncertainties in the data points y_i. The optimum value of the parameters can be obtained by minimization of χ^2 with respect to each of the parameters simultaneously. There are several routes which one can follow to find this set of parameters. In essence a grid search technique was used in which $M_1/M_{w(c)^a}$ values were calculated with a set of parameters and tested against the observed values by means of the χ^2 test. The parameters were then systematically changed, one at a time, to minimize χ^2 until a final minimum was reached.

AFVR Equation. A simple and direct method for the indefinite self-association mechanism has been developed by Van Holde and Rossetti (1967) and Van Holde *et al.* (1969) who used an expression in which the equilibrium constant is written as an explicit form dependent on $(M_{w(c)^a}/M_1)$, c, and B_1M_1. A similar procedure can also be applied to the monomer–dimer case (Deonier and Williams, 1970), by using the formula

$$\frac{R_a{}^2}{c[2(1 - B_1M_1R_ac) - R_a]^2} - \frac{1}{c} = 4K_2 \tag{8}$$

where $R_a = M_{w(c)^a}/M_1$. This equation is a form of an expression given by Adams and Fujita (1963), their eq 22, hence the designation AFVR (Adams, Fujita, Van Holde, Rossetti). The dimerization constant can be calculated pointwise, and by successive approximations of B_1M_1 the best choice for K_2 will be a least-squares fit to a straight line with slope zero. Changing the term $(1 - B_1M_1R_ac)$ into $(1 - B_1M_1R_ac - 2B_2M_1R_ac^2)$ expands this method to the case where a third virial coefficient is seemingly required, but this expression involves tedious calculation unless computer facilities are available.

Methods

Buffer Solutions. The protein samples were dissolved in pH 2.64 NaCl–glycine buffer of ionic strength 0.16. It was prepared by adding NaCl and HCl to appropriate volumes of deionized water (which had been passed over a column containing Bio-Rad AG501-X8(D) 20–50 mesh resin) to yield 0.10 M NaCl and 0.06 M HCl, after adjustment of the pH to the desired value by the addition of glycine. The pH meter (Beckman, Model G) was standardized against the Beckman pH 4 standard solution. The buffer solution was then made up to volume and stored in the cold. The number of buffer stocks was reduced to a minimum. The pH of additional stocks was not only checked against the standard buffer but also against the previous buffer solution.

Buffer densities were determined at 25° by using both Ostwald-Sprengel and capillary pycnometers. The density of water was taken to be 0.997044 g/cm^3 at this temperature for the pycnometer calibration. Corrections for air buoyancy were applied in the usual way. The density of the buffer at 25° was found to be 1.0064 g/cm^3. The densities at other temperatures were estimated by using this value and the temperature-dependence coefficient of water density.

Protein Solutions. Experiments were performed with two different protein samples, one which had been prepared by D. A. Albright for a previous study of this type (Albright and Williams, 1968), and another one which was kindly supplied by Dr. C. N. Pace, Texas A & M University. During the purification procedures in each case, toluene had been used as a preservative. The Pace protein was used for the majority of the experiments. It had been prepared from the milk of homozygous β-lactoglobulin B Holstein cows. Two of the 15 experiments were performed by using the Albright protein. The results agreed closely with experiments in which the protein supplied by Pace formed the test substance. The two samples are thus seen to possess indistinguishable molecular weight–concentration behaviors, at least under the conditions of our experiments. Furthermore since the result of a preliminary experiment with the Albright protein at pH 4.65 in acetate buffer and at ionic strength 0.1 agreed well with results of earlier investigators (Timasheff and Townend, 1961), the proteins seem comparable to those used in other researches.

The lyophilized protein was dissolved in the buffer. In preparation for dialysis, the 0.375-in. Visking casing was boiled for 5 min in water containing EDTA and then rinsed thoroughly with water and then with buffer. Dialysis at 4° was continued for at least 36 hr, with the dialysate being replenished four to five times. In all, three protein stock solutions were prepared, with concentrations ranging from 12 to 24 mg per ml. Then, each protein system was passed through a 0.45-μ Millipore filter and stored in the refrigerator as a stock solution. Lower solution concentrations for experiment were obtained by dilution with the final dialysate immediately before use. In most cases the solution was passed through a 0.45-μ Millipore filter again after dilution. All dilutions were performed gravimetrically.

Concentration Determinations. Protein concentrations were determined spectrophotometrically by measuring the optical absorbance, with the aid of a Gilford Model 222 photometer coupled with a Beckman Model DUR quartz monochromator. The extinction coefficient $\epsilon_{278}^{1\%}$ was originally taken to be 9.6 dl/g cm (Townend *et al.*, 1960a,b), but later on good reasons were found to believe this value to be too high. This is a subject of subsequent consideration. If the absorbance of the sample was less than 2.5 in a 1-cm cuvet, observations were made of the solution directly and without dilution. Otherwise, the sample was diluted with dialysate by weighing (with no bouyancy corrections being applied). The dilutions were made so that the resulting absorbance in a 1-cm cuvet

was less than one. Usually, measurements were made in triplicate on samples with an absorbance ranging from 0.6 to 0.8. The relative precision for these determinations was typically 0.2%. We emphasize that after stock dilution and before the absorbance measurement, it is important that the samples be passed through Millipore filters.

Concentration determinations based on refractive index measurements were performed with a Brice-Phoenix differential refractometer. The value employed for (dn/dc), the specific refraction increment at 546 nm, was 0.00182 dl/g (Halwer *et al.*, 1951) which is in essential agreement with that of Pedersen (1936), 0.00181 dl/g. Others have reported higher values. For example, the Perlmann and Longsworth (1948) data for a series of proteins, among them β-lactoglobulin, are consistently 1.9% higher than the data of Halwer *et al.* This fact suggests that a calibration factor is somewhere involved. Ghose *et al.* (1968) report 0.001883 dl/g for goat β-lactoglobulin.

Ultracentrifugation. The sedimentation equilibrium experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with the standard RTIC unit and Rayleigh/schlieren optical system. The condensing lens mask was fitted with a symmetrically placed double slit. The optical system had been aligned and focused by using the procedure of Gropper (1964). The camera lens was focused on the midplane of the cell. Under the conditions of the experiments performed here, all at low speeds, Wiener skewness is not significant. The ultracentrifuge was equipped only with mechanical gear-box speed control assembly, but with the use of the 22-lb AN-J rotor, constancy of speed was achieved to within satisfactory limits. Speeds were measured by using odometer readings and elapsed times; values were obtained at each temperature and the average of these values was used in the calculations. For these experiments the speeds ranged from 6160 rpm to 16,200 rpm, which resulted in a maximum of 20 fringes across the 12-mm cell. In two experiments an ultracentrifuge with electronic speed-control was used.

The RTIC unit was calibrated before starting the series of experiments by using a mercury-in-glass thermometer which had been calibrated for 25°. The experiments at 5° were performed first, and the temperatures were changed by switching the refrigerator off and changing the RTIC setting. When approaching 10° and 15° the refrigeration was turned on before reaching the required temperature. At 25° no refrigeration was required. To approach 35.5° it was necessary to turn on the refrigerator for the first hour after changing the RTIC setting and again to use refrigeration 0.75 to 1 hr before photographing the fringes. Without this procedure, it was impossible to obtain satisfactory pictures at 35.5°, which were otherwise obscurred because of condensation of oil on the lower collimating lens.

The times required to reach equilibrium at 5° were estimated from a simplified equation given by Van Holde and Baldwin (1958). For subsequent temperature changes, the time which elapsed between the change in temperature and the taking of the photograph of the fringes ranged from 15 to 19 hr. That this time was sufficient was verified by taking two Rayleigh photographs at different times late in the experiment. In some cases at 5°, the overspeed technique advocated by Hexner *et al.* (1961) was employed.

A 12-mm double-sector, aluminum-filled epon centerpiece and sapphire windows were used for all work. The cell thickness "a" was measured with a comparator, but the effect of compression arising from tightening of the cell was neglected. The cell-filling procedure was that described by Adams (1967). In the earlier procedures the cell was dismantled and washed after each experiment. Later on, the cell was not dismantled but was simply rinsed with each new protein sample before the final filling. The new sample was always of higher protein concentration than the preceeding one. The cell was filled up to approximately 3 mm; the solvent channel had a slightly higher meniscus. The blank corrections for windows and centerpiece were taken from Deonier and Williams (1970) since the same cell was used throughout.

Photographs were taken using Kodak Type II-G backed spectroscopic plates. A schlieren photograph was taken at the start of every experiment to check for cell leaks while at equilibrium at every temperature, and another schlieren picture was taken at the end in order to locate the bottom and meniscus positions of the cell. There was a tendency for the meniscus position at 35.5° to be slightly lower than at other temperatures, but otherwise no specific dependence of meniscus position on temperature was detected. Accordingly, the differences were ignored and the meniscus position was taken to be the average value.

The plates were measured in the usual way with a Gaertner Model 2001 RS, toolmakers' microscope. Depending on the quality of the plate, one to three fringe readings were made at each horizontal comparator setting, taking the average in the latter case.

Subsidiary Parameters. The partial specific volume $\bar{v}_2$ of the β-lactoglobulin B in solution was taken to be 0.751 cm³/g at 25° (Svedberg and Pedersen, 1940). Correction of $\bar{v}$ for the various temperatures employed in this study was effected by using the temperature coefficient of $\bar{v}$ for equine hemoglobin as determined by Svedberg and Pedersen, (1940). This agrees to a reasonable extent with the temperature-dependence behavior of $\bar{v}$ reported by Bull and Breese (1968) for a series of different globular proteins and for α-casein as reported by Schmidt (1969).

The wavelength λ of the light supplied by the AH6 lamp and Wratten 77A filter assembly was assumed to be 550 nm (Gropper, 1964). Together with the value of 0.00182 dl/g for dn/dc and with the cell thickness, a, the following relation between the initial concentration c_0 and the initial fringe number J_0 can be given: $J_0 = (a/\lambda)(dn/dc)c_0 = 39.62c_0$ where c_0 is in grams per deciliter.

The dependence of buffer pH on the temperature has been ignored throughout this study because of the assumption that its influence will be less than experimental error. From the data of Timasheff and Townend (1961) and Albright and Williams (1968), it is obvious that under the present pH conditions, variation in the ionic strength is much more important than the pH change. The protein is here nearly completely charged according to titration data (Tanford and Nozaki, 1959).

Results

General. There are presented in Figure 1 the apparent protein weight-average molecular weights, $M_{w(c)}{}^a$, at 15 and 25°, plotted as a function of concentration in fringes. The solid lines are interpolation curves used for obtaining the quantities required for the subsequent analyses. The two curves were constructed by using averaged values at concentration points where sufficient data were available. The various groups of symbols denote data derived from single experiments at a given initial protein concentration. The $M_{w(c)}{}^a$ data were calculated from the slopes of ln c *vs.* r^2 plots. The procedure described by Hancock and Williams (1969) to deter-

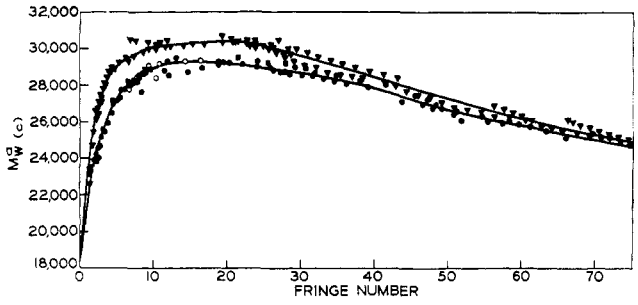

FIGURE 1: The apparent weight-average molecular weight of β-lactoglobulin B in solution at 15° (upper curve) and at 25° (lower curve *vs.* concentration in fringes. Both curves are for pH 2.64, $I = 0.16$, and $\epsilon_{278}^{1\%}$ 9.2 dl/g cm.

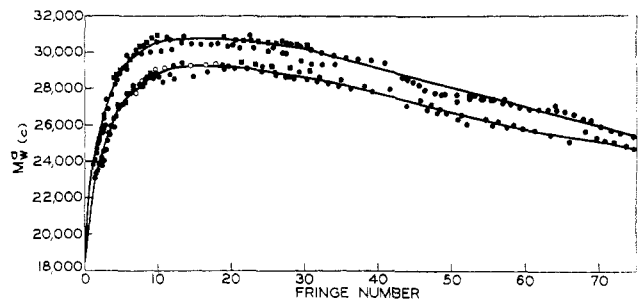

FIGURE 2: Apparent weight-average molecular weight of β-lactoglobulin B in solution *vs.* concentration in fringes, with $\epsilon_{278}^{1\%}$ 9.6 dl/g cm (upper curve) or $\epsilon_{278}^{1\%}$ 9.2 dl/g cm (lower curve). The open circles are values for which the protein concentration was measured by differential refractometry. Solution conditions are: pH 2.64, $I = 0.16$, and $T = 25°$

mine these slopes was rigorously applied. The average deviation in $M_{w(c)}{}^a$ at the different temperatures was in the range of 350–400 g/mole. An acceptable overlap between the results for different individual experiments existed throughout, indicating that the protein was pure, that pressure effects are not significant, and that the association reaction is reversible.

The $M_{w(c)}{}^a$ data were taken from the corrected interpolation curve at different fringe intervals, ranging from 0.1 to 0.2 of a fringe at the very low concentrations up to 1 fringe above 0.25 g/dl. These data at the five temperatures of observation are assembled in Table I.

Below 5 fringes, the data from "low-speed" sedimentation equilibrium experiments are less reliable than at higher concentrations; consequently, an extrapolation to obtain the monomer molecular weight, M_1, was not attempted. Instead, M_1 was taken to be 18,336 g/mole on the basis of the amino acid composition, reported by Frank and Braunitzer (1967).

TABLE I: Apparent Weight-Average Molecular Weight Data, $M_{w(c)}{}^a$, at the Five Temperatures, Corresponding to Every Fourth Fringe.[a]

Fringe Number	$M_{w(c)}{}^a$ (obsd)				
	5°	10°	15°	25°	35.5°
3	27940	27900	27575	25080	*b*
7	30400	30150	29635	28240	26435
11	31340	30630	30080	29160	27410
15	31600	30825	30300	29295	27870
19	31410	30840	30335	29285	27880
23	31050	30660	30200	29200	27740
27	30625	30300	29850	28975	27520
31	30150	29875	29375	28660	27245
35	29660	29380	28910	28260	26880
39	29145	28845	28450	27870	26540
43	28590	28350	27990	27460	26245
47	27980	27850	27520	27060	25970
51	27370	27440	27060	26650	25740
55	26735	27050	26600	26250	25475
59	26070	26665	26170	25850	25180
63	25380	26280	25835	25500	24870
67	24700	25900	25510	25220	24570
71	24045	25510	25200	24885	24270
75	23350	25130	24890	24570	23970

[a] At 3 fringes the following values are obtained for $M_{n(c)}{}^a$: 5°, 23775; 10°, 25420; 15°, 23339; and 25°, 22053. [b] $M_{w(c)}{}^a$ at 4 fringes is 24375; $M_{n(c)}{}^a$ is 22427.

Discrepancies. While the results of Albright and Williams (1968) exhibited features similar to the data of Timasheff and Townend (1961), there exist noticeable differences in the magnitudes of the molecular weights as reported from the two laboratories. The more recent data were some 6–8% lower than those of Timasheff and Townend, after due allowance had been made for the different solution conditions. In the present work, the protein concentrations also were first determined with the spectrophotometer, using $\epsilon_{278}^{1\%}$ 9.6 dl/g cm, a factor determined by Townend *et al.* (1960a,b). Using this value, the present results are in better agreement with the molecular weight data of Timasheff and associates than they are with the prior data from this laboratory (Albright and Williams, 1968).

In order to understand better the reason for the differences in data, differential refractometry was employed in addition to spectrophotometry for the determination of protein concentration. Agreement between the light scattering and the present sedimentation equilibrium molecular weight data by using the two quantities $\epsilon_{278}^{1\%}$ and dn/dc for assignment of concentration could be obtained only if $\epsilon_{278}^{1\%}$ was taken to be 9.1; incidentally this is the exact value which was independently determined experimentally by Dr. C. N. Pace (personal communication). Making allowance for residual light scattering, $\epsilon_{278}^{1\%}$ 9.2 was ultimately adopted for our computations of protein concentration. A lack of closer agreement with the Albright and Williams data may arise from perturbation of their spectrophotometry by light scattering, a problem which was largely avoided in the present study by careful Millipore filtration of the protein solutions immediately preceding the optical measurement.

There is thus a propagation of errors which arises from failure to obtain accurate protein concentration data. In Figure 2 there are shown apparent weight-average molecular weight *vs.* concentration curves at 25°, calculated by using $\epsilon_{278}^{1\%}$ 9.6 in the case of the upper curve and $\epsilon_{278}^{1\%}$ 9.2 for the lower curve. The open circles shown at the maximum region of the lower curve correspond to an experiment in which the concentration was determined by differential refractometry. It appears that an error of about 4% in the extinction coefficient causes an error of about 10% in the molecular weight; in agreement with Teller and associates (1969) we found that such a discrepancy can produce an error of roughly 100% in the association constant.

TABLE II: Several Data for K_2 at 10° and at 25°, Calculated by Tang *et al.* (1971) Methods I and II.

	K_2(dl/g), 10°	K_2(dl/g), 25°
Eq 5a, method I	42.3	21.8
Eq 6a, method II	31	12.8
Eq 6a, second approximation	41.1	21.4
Value ultimately used	44 (2B) 50 (1B)	21.0

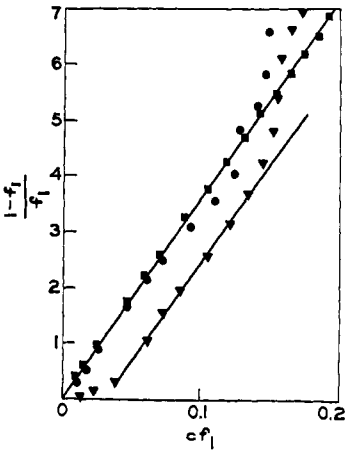

FIGURE 3: Plots of the quantity $(1 - f_1)/f_1$ *vs.* cf_1 for β-lactoglobulin B solution at 15°: experimental data, one virial coefficient (▼); same quantities, following adjustment of $M_{w(c)}{}^a$ data over the range 0–3 fringes (●); experimental data, two virial coefficients, following adjustment of $M_{w(c)}{}^a$ data over the range from 0 to 3 fringes (■).

Data Analysis for Monomer–Dimer Association by Various Methods. TANG *et al.* (1971). The association reaction is an exothermic process as shown by Figures 1 and 2 where $M_{w(c)}{}^a$ *vs.* c data from experiments at 15° and 25° are presented. From such data values of $M_{n(c)}{}^a$ and ln $f_1{}^a$ are computed. By substitution of these values in eq 5a and 6a, the true monomer fraction f_1 was calculated in two different ways. The association constants are calculated from the slopes of $(1 - f_1)/f_1$ *vs.* (cf_1) plots. When we sought to estimate the fraction of monomer, f_1 by using eq 5a, method I, we found that the data provide a linear relationship only over a very limited concentration range; in addition the curves do not pass through the origin as predicted. At the higher temperatures 25, 35.5°, the linear portion is reasonably extended, but with the 5 and 10° data, linear behavior is hardly to be found.

In Figure 3 the route to the evaluation of the parameters at the temperature 15° is indicated. Application of eq 6a, method II, provides $[(1 - f_1)/f_1]$ on cf_1 plots which are linear over an extended range, but they also do not pass through the origin. The method which involves the use of a second and third virial coefficient leads to 20–30% smaller values for B_1M_1 as compared to those cases in which the B_2 value is zero. The magnitude of the B_1M_1 terms at 15, 25, and 35° is nearly temperature independent, and the two methods of evaluation based upon eq 5a and 6a lead to concordant results. This situation indicates that one virial coefficient is here sufficient to describe the nonideality of the solution. For the 5 and 10° data, however, a single nonideality term seems not to be sufficient for the purpose.

From a reconstruction of the $M_{w(c)}{}^a$ *vs.* c curve, using the equilibrium constant and B_1M_1 values which had been estimated by means of eq 5a, it became clear that the low concentration data (<5 fringes) and particularly the ultimate extrapolation from 3 fringes to the monomer molecular weight at zero concentration were quite unreliable. Inserting estimates for the correct $M_{w(c)}{}^a$ data from zero to three fringes, reevaluating the quantities $M_{n(c)}{}^a$ and ln $f_1{}^a$, and subsequently reapplying both procedures for f_1 improve matters. The $[(1 - f_1)/f_1]$ *vs.* (cf_1) plot is now almost strictly linear over the whole concentration range and the curve passes very nearly through the origin. Its slope agrees with the one obtained using a single nonideality parameter. A few of these results are summarized in Table II. In cases where the equilibrium constant is small, application of the two virial coefficient approach is more successful than in the situation in which large K_2 values are involved.

SIMULTANEOUS SOLUTIONS OF THE PARAMETERS. As set down in the theory section, approximate values for the set of parameters (K_2, B_1M_1, and, where involved, B_2M_1) were taken to calculate $M_{w(c)}{}^a$ as a function of protein concentration. These figures were then judged by comparison with the observed values as given by a χ^2 test. Although in the low concentration range the experimental error may exceed 350 g/mole, nevertheless the quantity σ_i of eq 7 was taken to be constant over the whole concentration range ($\sigma_i = 350$). The number of data points at any given concentration is too small to convert σ_i into a variable with any degree of confidence.

By minimizing χ^2 as described, sets of parameters were obtained which give the best fit to the experimental data. The deviations of the calculated $M_{w(c)}{}^a$ values from the interpolation curve are less than the experimental error. Within experimental error the model still permits different combinations of parameters. However, to superimpose an additional criterion on the least-squares procedure by estimating the scatter around the generated model curve to be random or to have certain trends is not very useful since all members of each set of data points are obtained from one sedimentation equilibrium experiment. Such a series will tend to have more correlation than data points between different experiments. The final choice of model, then, involves selecting the most realistic of them. The results are summarized in the right-hand columns of Table III.[3]

[3] A reexamination of the data from Albright and Williams (1968) based on the χ^2 test reveals several interesting points. Under all their ionic strength and pH conditions one is able to find a set of two parameters (K_2, B_1M_1) which fits the experimental data. Whether or not the fit is considered to be sufficient depends of course on the criterion used. It is difficult to obtain a good estimate of the experimental error from their printed interpolation curve. Although the $M_{w(c)}{}^a$ values at pH 2.58, $I = 0.10$, can be reproduced with a two parameter fit, a deviation is found, especially at high fringe numbers. This may be a real trend or it may be caused by a single experiment of lesser precision. As expected, the three-parameter model provides a better fit. Albright and Williams obtained a less suitable set of parameters for one of their three conditions, namely at pH 2.58 and $I = 0.15$. The revised set, undoubtedly an improvement, accounts for two unexplained earlier inconsistencies. (1) The original B_2M_1 term was higher than that computed for the pH 2.58 and $I = 0.10$ solution. The revised value while somewhat imprecise is certainly less than 0.010. (2) The new value for K_2 of 11.4 is consistent with the plot, Figure 10 of the Albright–Williams report, which requires that $K_2 \simeq 11$ from the graph of $K_2{}^a$ *vs.* fringe number, an evaluation procedure described in detail by Adams and Fujita (1963).

TABLE III: Equilibrium Constants at Different Temperatures and Corresponding Best B_1M_1 and B_2M_1 Choices as Determined by the AFVR Equation (eq 8) and by the χ^2 Test.[a]

Temperature (°C)	AFVR Method			χ^2 Test		
	B_1M_1	B_2M_1	K_2	B_1M_1	B_2M_1	K_2
5	0.055	0.021	50.4 ± 5.5	0.055	0.021	50
10	0.105		53.5 ± 15.1	0.105		52
	0.095	0.0065	45.4 ± 7.5	0.085	0.0065	44
15	0.109		38.9 ± 10.1	0.1095		40
	0.093	0.0055	35.2 ± 6.8	0.094	0.005	35.5
25	0.110		23.1 ± 2.5	0.108		22.0
35.5	0.111		10.4 ± 0.8	0.111		10.2

[a] All association equilibrium constants, K_2, are expressed in dl/g, and the virial coefficients also correspond to the g/dl concentration scale.

AFVR METHOD. This method, based upon eq 8, was applied to the data, using both one virial coefficient and two virial coefficients. When one virial coefficient was used, no semiempirical modifications were applied; however, when two virial coefficients were required, estimates of these quantities (from Tang *et al.*, method II or the χ^2 test) were used for the initial stages of the analysis. The data, which were taken from the interpolation curves, were subjected to a least-squares analysis as described in the theory section. A plot of the estimates of K_2 so obtained as a function of concentration is shown in Figure 4, which gives the results of experiments at 25°. The deviations seen in this plot are taken to be reflections of errors in the data, rather than of incorrect model choice. Values for K_2 and B_1M_1 (and of B_2M_1, when appropriate) which have been obtained in this way are also shown in the left-hand columns of Table III. A comparison of the parameters obtained by use of eq 8 with the parameters which give the best fit to the experimental data shows that K_2 approaches the statistically best value typically within 1–2%, with an occasional exception of 5%. The error limits as written are standard deviations from the mean. The B_1M_1 term agrees with the best fit to within 1%.

OTHER MODELS. In addition to the monomer–dimer equilibrium mechanism, the use of other monomer–*n*-mer association models was considered, thereby to test for uniqueness. If the monomer–dimer system be correct, then a plot based on eq 5b will give a straight line; this was indeed observed and is shown in Figure 3. In addition the values of B_1M_1 calculated from eq 5c were relatively constant. Now if other values of n, such as $n = 3$ or 4, are used in eq 5a and f_1 is calculated for each choice of n, then the plot based on eq 5b will give pronounced deviations from a straight line. This behavior was observed. In addition values of B_1M_1 calculated from eq 5c when $n = 3$ or 4 were not constant. So, it appears that the monomer–dimer association is the proper choice for the monomer–*n*-mer representation.

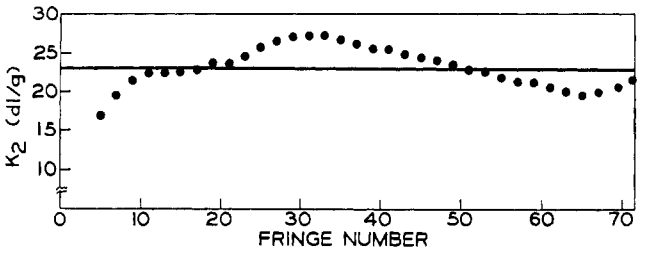

FIGURE 4: The equilibrium constant K_2 as a function of concentration. These values were calculated from the interpolation curve at 25° by use of the AFVR method (eq 8).

The Thermodynamic Quantities $\Delta G°$, $\Delta H°$, *and* $\Delta S°$. With good assurance of the essential purity of the protein and of the reaction mechanism (monomer–dimer) numerical values for the several fundamental basic thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$, can now be computed. They derive from the single equilibrium constant for the formation of the intermolecular bond and its temperature variation by use of conventional definitions and the Gibbs–Helmholtz equation.

From a standard thermodynamic description of chemical equilibrium, it follows that

$$-\Delta G° = -\sum_i^r \nu_i \mu_i°(T,P) = RT \ln \prod_i^r a_i^{\nu} \qquad (9)$$

where $\Delta G°$ is the standard Gibbs free-energy change of the reaction for the specified concentration scale, the ν_i are the stoichiometric mole numbers (positive for products and negative for reactants), the a_i are the activities of the species participating in the reaction, and r is the number of species in equilibrium. Since the approximation of Adams and Fujita (1963) has been introduced for the activity coefficients, they divide out in the product above when the gram per deciliter scale is chosen, so that

$$\prod_i^r a_i^{\nu_i} = \prod_i^r c_i^{\nu_i} = K \qquad (10)$$

This defines a reaction equilibrium constant K, which is as yet unspecified as to association or dissociation, and which does not depend on the solution nonideality. Thus we obtain the expression

$$-\Delta G° = RT \ln K \qquad (11)$$

in which the equilibrium constant K has been written in terms of concentration, as has been the case for all previous usage.

The effect of temperature on K is described by the Gibbs–Helmholz equation to give $\Delta H°$, the standard change in enthalpy, thus

$$-\Delta H° = \frac{R\,\mathrm{d} \ln K}{\mathrm{d}(1/T)} \qquad (12)$$

With data for $\Delta G°$ and $\Delta H°$, the standard entropy change, $\Delta S°$, is computed from the statement

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T} \qquad (13)$$

For the arithmetical operations involved in the estimation of these quantities, a conversion of concentration scales from grams per 100 ml to moles per liter is required. The equilibrium constants, K_2, of Table III were computed for the association reaction, with concentrations on the g/100-ml scale. In addition to the change over in the concentration scale to

TABLE IV: Values of K_d, ln K_D, and ΔG° (cal/mole) at the Several Temperatures.

	One Virial Coefficient			Two Virial Coefficients		
Temperature (°C)	$K_d \times 10^5$	ln K_D	$\Delta G^\circ \times 10^{-3}$	$K_d \times 10^5$	ln K_D	$\Delta G^\circ \times 10^{-3}$
5				2.2	−10.73	5.93
10	2.1	−10.77	6.06	2.5	−10.61	5.97
15	2.7	−10.51	6.02	3.1	−10.39	5.95
25	5.2	−9.87	5.84			
35.5	10.3	−9.17	5.63			

moles per liter, we have elected to consider the reaction as being a dissociation in order that direct comparison may be made of the quantities ΔG°, ΔH°, and ΔS° with earlier and corresponding data of Timasheff and Townend (1961) from light-scattering experiments. The equilibrium constant, K_D, is then a characteristic of the reaction $M_2 \rightleftarrows 2M_1$.

In Table IV there have been collected values at the several temperatures for the quantities K_d (g/ml), ln K_D and ΔG°. The degree of constancy of the equilibrium constant data for the association reaction at a given temperature is made evident by Figure 4.

Then from eq 12, and with the two slopes of the ln K_D *vs.* $1/T$ curves from Figure 5 (one virial coefficient, two virial coefficients) the value for ΔH° is computed. The two slopes are, respectively, 5528 and 4915; they correspond to values of ΔH° of 11,000 and 9800 cal. Then, from eq 13 one obtains as average values of ΔS° the values +17.5 and +13.4 eu, with small deviations from the mean.

It will be noted that in the preparation of Figure 5 two sets of association constant data have been used. They are delineated by the situations where one and two virial coefficients have been involved in the description of the reaction system. For the two-parameter fit, ln K_D data at four temperatures, 10, 15, 25, and 35.5° were utilized; in the other case data taken at 5, 10, and 15° were involved. The deviation from linearity of the plot which appears by using the association constant data for the three-parameter situation, especially at temperatures 5 and 10°, gives cause for concern because the van't Hoff plot is not a sensitive one.

In Table V we present a brief comparison of the data computed from the linear portion of our ln K_D *vs.* $1/T$ plot with those of Timasheff and Townend (1961). The comparison cannot be all that one might desire because the value of ΔG° at a given temperature is dependent upon the ionic strength of the solution; this situation is discussed in a footnote to the table.

TABLE V: Comparison of Data for Characteristic Functions of State at 25°.

Sedimentation Equilibrium (This Research), $I = 0.16$		Light Scattering (Timasheff and Townend, 1961)	
		$I = 0.16$[a]	$I = 0.10$
ΔG° (kcal/mole[b])	5.84	6.5	5.9
ΔH° (kcal/mole)	10.4		12.8
ΔS° (eu)	15.0		23.2

[a] Precise comparison cannot be made of our data with information already in the literature. However, and especially in view of a recent contribution by Nagasawa and Holtzer (1971), it is of interest to develop the subject in somewhat more detail. In doing so we make use of the idea that the standard free-energy change can be divided into two parts (Townend *et al.*, 1960a; Nagasawa and Holtzer, 1971), an attractive force term and an electrostatic one. $\Delta G^\circ = \Delta G^\circ_a + \Delta G^\circ_e$. Nagasawa and Holtzer have made use of their own titration data and data for ΔG° which had been obtained by Townend *et al.* (1960a) for β-lactoglobulin. In the former case the relative concentrations of the A and B variants were not stated; in the latter instance the composition was given as 69% A and 31% B. Townend and Timasheff (1961) had already computed ΔG°_e for the pooled sample by using a theory of Verwey and Overbeek. (For β-A and β-B values of ΔG°_e at $I = 0.1$ were given as −3.9 and −4.0 kcal/mole, respectively.) The newer and corresponding results of Nagasawa and Holtzer, taken from numerical solutions of the nonlinearized Poisson–Boltzmann equation, are recorded below, together with the earlier data, all in kcal/mole.

I	ΔG°_e(TT)	ΔG°_e(NH)
0.03	−6.1	−6.9
0.10	−4.0 (β-lactoglobulin B)	−4.2
0.30	−2.5	−4.0

Of the two differing values of ΔG°_e at $I = 0.30$ we suspect that the Nagasawa–Holtzer value is less worthy of trust. The different authors agree that the ΔG°_a values are independent of pH and ionic strength; the value $\Delta G^\circ_e = -4.0$ is inconsistent with this condition. It appears, too, that values of ΔG°_e are substantially the same for both the A and the B genetic variants. So, the electrostatic contribution at $I = 0.16$, the solution condition used by us, was estimated by interpolation on a graph which was constructed by using all the data given in the table, except for the value in question. Plotted was log $(-\Delta G^\circ_e)$ *vs.* $(I)^{1/2}$; the value found for ΔG°_e is −3.36 kcal/mole. Then with $\Delta G^\circ_a = 9.9$ kcal/mole, ΔG° (pH 2.7, $I = 0.16$, $T = 25°$) = 6.54 kcal/mole. [b] One mole of dimer is taken to be 36,672 g.

Discussion

Through the performance of sedimentation equilibrium studies with the β-lactoglobulin B solutions under well-defined conditions, we have sought to study the efficacy of certain of the theoretical formulations by which the $M_{w(c)}$[a] *vs.* c data may be interpreted in terms of a monomer–dimer association and to contribute to the knowledge of the thermodynamics of such behavior. For the overall consideration of the pros and cons of the problem, we shall first make some remarks about the data themselves and their interpretation and then consider the evaluation of some of the characteristic thermodynamic functions.

Any analysis of the forces which exist between protein monomers in solution is strictly dependent upon the accuracy of the experimental data, followed by an unambiguous analysis of these data in terms of a well-defined self-association mechanism. The sources of experimental error in a sedimentation

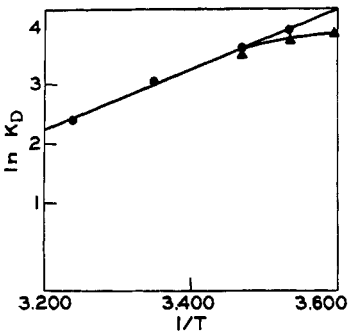

FIGURE 5: Van't Hoff plot (ln K_D *vs.* $1/T$) for the β-lactoglobulin B solutions at five temperatures. Values of K_D were calculated with allowance being made for either one (●) or two (▲) virial coefficients.

equilibrium molecular weight determination have been set down in many places; they begin with the errors in the optics and the error which is inherent in the reading of the photographic plates which record the redistribution of the solution components at equilibrium. In our report, special attention has been paid to the necessity for proper protein concentration assignments over the several solution cell distances. This will be particularly important if the self-association tendency is strong. Because of the precision available at high concentrations, we have selected the low-speed, short-column technique. This is not to deny the usefulness of the high-speed or meniscus depletion method in application to reactions of the kind being discussed, for it too might have been applied to this system with advantage.

In testing and evaluating the three selected analytical procedures for analyzing a monomer–dimer self-association, certain comparisons may be made. First, with reference to the methods of Tang *et al.* (1971), we note that there are several points to be considered in understanding the $[(1 - f_1)/f_1]$ on cf_1 plots. The quantity $Z = [(2M_1/M_{n(c)}{}^a) - (M_1/M_{w(c)}{}^a)]$ will overestimate f_1 if the monomer–dimer system cannot be described by two parameters: an equilibrium constant and a second virial coefficient.

The procedures used here to analyze the data do to some extent weigh the data points differently at the different ends of the curve. By taking a linear part of the $[(1 - f_1)/f_1]$ *vs.* cf_1 plot to evaluate K_2 one really ignores not only the data at very low concentration but also those in the high concentration regions if these points happen to deviate much from a smooth curve. Two virial coefficients are better evaluated in the higher concentration region of the $M_{w(c)}{}^a$ *vs.* c curve. As already pointed out, the parameters are not obtained independently of each other as both result from a solution for the quantity f_1, the true weight fraction of monomer.

The difficulties encountered in the application of eq 5a and 6a arose, in part, because the magnitudes of the K's are large. Teller *et al.* (1969) have presented model calculations which show that when the equilibrium constants are large, curves at different values of K are relatively insensitive to large changes in K. Consequently, high precision at low concentrations becomes essential.

Fluctuations in the $M_{w(c)}{}^a$ data will presumably be larger in the low concentration range. Thus the relative error in the integral function $M_1/M_{n(c)}{}^a$ will become greater and greater as the concentration decreases. The integral

$$\int_0^c \left(\frac{M_1}{M_{w(c')}{}^a} - 1\right)\frac{dc'}{c'} = \ln f_1{}^a$$

is sensitive to error particularly when the integral changes rapidly, that is, when the equilibrium constant is large. In the present instance, the graphical procedure which makes use of both $M_{n(c)}{}^a$ and $\ln f_1{}^a$ is almost unusable for correct estimation of the desired parameters without modification because of cumulative errors which enter into both numerical integrations, particularly the latter. Similar considerations obtain for the method based upon eq 6a, even though the integral

$$\frac{1}{c}\int_0^c \frac{M_1 dc'}{M_{w(c')}{}^a}$$

has a limit of unity at $c = 0$, and thus is less susceptible to error. It was because the experimental technique was to some extent ill suited to the system (*i.e.*, lack of sufficient precision at low concentrations) that it was necessary to employ adjusted low-concentration data.

The values obtained for the association constant, K_2, by using the grid-search technique together with a χ^2 test, and those calculated by using the AFVR equation are in excellent agreement. Both approaches are direct and they seem to produce satisfactory results, both for K_2 and for either a second virial coefficient or, when necessary, a second and third such coefficient.

For those situations in which the two parameter fit is satisfactory, the nonideality term, B_1M_1, is substantially constant over the temperature range investigated. Furthermore, the average result is but slightly lower than that of Timasheff and Townend (1961) who reported a value $B_1M_1 = 0.128$ (dl/g). These investigators made use of solutions of somewhat lower ionic strength, thus there is no qualitative inconsistency.

It is also worth noting that Timasheff and Townend find that the β-lactoglobulins A and B differ in their nonideality behavior. Unpublished B_1M_1 results of Tang and Adams (1971a,b) for β-lactoglobulin A and these data for the "B" variant are consistent with this disclosure. This is an unexpected situation, since at such low pH values the total charge is nearly the same in the two cases (Tanford and Nozaki, 1959).

The dissociation constants for β-lactoglobulin B at 15 and at 25° are in good agreement with those of Timasheff and Townend (1961). The higher association constant for the A-form reported by Tang and Adams (1971a,b) is consistent with their lower B_1M_1 data; this might be due to a difference in surface charge or to its distribution. A recent discussion by Nagasawa and Holtzer (1971) is perhaps pertinent, *cf.* footnote *a*, Table V.

With regard to the choice of reaction model, the monomer–dimer mechanism seems satisfactorily to account for the experimental information in the temperature interval 5–35.5°. There remains the complication that a fit of the data with two parameters becomes increasingly difficult as the temperature is lowered in this interval. The third parameter which eventually was required at 5° is in all probability a virial coefficient in as much as other models which utilize more than one equilibrium constant were tested, and they fail to serve the purpose. Presumably there will be uncertainty about the employment of the additional virial coefficient because one cannot easily predict at what protein concentration it will begin to make a relevant contribution in modifying the $M_{w(c)}{}^a$ *vs.* c behavior. In general, the question remains as to just how wide the concentration span for the $M_{w(c)}{}^a$ data ought to be for the safe assignment of a proper model, with a significant assignment of descriptive parameters.

The relative contributions of the different types of forces which are involved in protein self-association reactions are difficult to separate and to evaluate. In addition to the use of model compounds, there remain other approaches for exploitation: changes in solution conditions, such as pH, ionic strength, temperature, and dielectric constant, and local chemical modifications of the protein itself. This latter approach, difficult as it may be, has much to recommend it. The use of conditions under which the β-lactoglobulin molecules become highly charged are disadvantageous in some respects as the analysis for association becomes complicated because of strong electrostatic repulsions between the protein molecules. We elected to use solutions at low pH, but the added salt did serve to make relatively small the effects of solution nonideality. Both Timasheff and coworkers and we used the temperature as a variable.

As an overall conclusion it may be said that one may be satisfied with the assignment, monomer–dimer, as being descriptive of the reaction mechanism for the β-lactoglobulin B in the supporting solutions that we have used. During the progress of the research we were again and again confronted with the necessity for improvement in both the precision and the accuracy of the experiments by which the apparent weight- and number-average molecular weight data as a function of concentration can be acquired. Because of this situation, thermodynamic interpretations and an exact description of the nature of the forces which cause protein self-association reactions remain in early stages of quantitation.

Acknowledgment

We take this opportunity to thank Mr. P. J. Wan for his assistance with the computational procedures used in this work.

References

Adams, E. T., Jr. (1965), *Biochemistry 4*, 1646.
Adams, E. T., Jr. (1967), Fractions No. 3, Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.
Adams, E. T., Jr., and Fujita, H. (1963), *in* Ultracentrifugal Analysis in Theory and Experiment, Williams, J. W., Ed., New York, N. Y., Academic Press.
Adams, E. T., Jr., and Lewis, M. S. (1968), *Biochemistry 7*, 1044.
Adams, E. T., Jr., and Williams, J. W. (1964), *J. Amer. Chem. Soc. 86*, 3454.
Albright, D. A., and Williams, J. W. (1968), *Biochemistry 7*, 67.
Bell, K., and McKenzie, H. A. (1967), *Nature (London) 204*, 1275.
Bevington, P. R. (1969), Data Reduction and Error Analysis for the Physical Sciences, New York, N. Y., McGraw-Hill.
Bull, H. B., and Breese, K. (1968), *J. Phys. Chem. 72*, 1817.
Chun, P. W., and Kim, S. J. (1970), *Biochemistry 9*, 1957.
Chun, P. W., Kim, S. J., Williams, J. D., Cope, W. T., Tang, L-H., and Adams, E. T., Jr. (1972), *Biopolymers 11*, 197.
Deonier, R. C., and Williams, J. W. (1970), *Biochemistry 9*, 4260.
Frank, G., and Braunitzer, G. (1967), *J. Physiol. Chem. 348*, 1691.
Ghose, A. C., Chaudhuri, S., and Sen, A. (1968), *Arch. Biochem. Biophys. 126*, 232.
Gropper, L. (1964), *Anal. Biochem. 7*, 401.
Halwer, M., Nutting, G. C., and Brice, B. A. (1951), *J. Amer. Chem. Soc. 73*, 2786.
Hancock, D. K., and Williams, J. W. (1969), *Biochemistry 8*, 2598.
Hexner, P. E., Radford, L. E., and Beams, J. W. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1848.
Kessler, E., and Brew, K. (1970), *Biochim. Biophys. Acta 200*, 449.
Kumosinski, T. F., and Timasheff, S. N. (1966), *J. Amer. Chem. Soc. 88*, 5635.
Maubois, J. L., Pion, R., and Dumas, B. R. (1965), *Biochim. Biophys. Acta 107*, 501.
McKenzie, H. A. (1967), *Advan. Protein Chem. 22*, 55.
McKenzie, H. A. (1970), Milk Proteins, Vol. I, New York, N. Y., Academic Press.
Ogston, A. G., and Tilley, J. M. A. (1955), *Biochem. J. 59*, 644.
Nagasawa, M., and Holtzer, A. M. (1971), *J. Amer. Chem. Soc. 93*, 606.
Pedersen, K. O. (1936), *Biochem. J. 30*, 961.
Perlmann, G., and Longsworth, L. G. (1948), *J. Amer. Chem. Soc. 70*, 2719.
Phillips, N., and Jenness, R. (1965), *Biochem. Biophys. Res. Commun. 21*, 16.
Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci. 164*, 279.
Schmidt, D. G. (1969), Dissertation, Utrecht.
Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, Claredon Press.
Tanford, C., and Nozaki, Y. (1959), *J. Biol. Chem. 234*, 2875.
Tang, L-H., and Adams, E. T., Jr. (1971a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1303.
Tang, L-H., and Adams, E. T., Jr. (1971b), Paper Presented at the Southwestern Regional Meeting of the American Chemical Society, San Antonio, Texas, Dec 1–3.
Tang, L-H., Chun, P. W., and Adams, E. T., Jr. (1971), Paper Presented at the Meeting of the Biophysical Society, New Orleans, La., Feb 16–18.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164*, 66.
Timasheff, S. N., and Townend, R. (1961), *J. Amer. Chem. Soc. 83*, 470.
Townend, R., Weinberger, L., and Timasheff, S. N. (1960a), *J. Amer. Chem. Soc. 82*, 3175.
Townend, R., Winterbottom, R. J., and Timasheff, S. N. (1960b), *J. Amer. Chem. Soc. 82*, 3161.
Ts'o, P. O. P., and Chan, S. I. (1964), *J. Amer. Chem. Soc. 86*, 4176.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Van Holde, K. E., and Rossetti, G. P. (1967), *Biochemistry 6*, 2189.
Van Holde, K. E., Rossetti, G. P., and Dyson, R. D. (1969), *Ann. N. Y. Acad. Sci. 164*, 279.
Visser, J., Williams, J. W., Adams, E. T., Jr., Wan, P. J., and Deonier, R. C. (1971), 162nd Annual Meeting of the American Society of Biological Chemists, San Francisco, Calif., June 13–18.