Sedimentation Equilibrium in Reacting Systems. VI. Some Applications to Indefinite Self-Associations. Studies with β-Lactoglobulin A*

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ABSTRACT: If it be assumed that all molar equilibrium constants are equal, then it is possible to analyze indefinite self-associations. The equations for the second positive moment ψ and the second negative moment $M_1^2\Sigma(C_i/M_i^2)$ and its apparent value are developed here for indefinite self-associations. It is shown how these quantities may be used with other available quantities (C, $\hat{\alpha}$, $M_1/M_{w\,app}$, and $M_1/M_{n\,app}$) in the analysis of indefinite self-associations.

Some sedimentation equilibrium experiments on β-lactoglobulin A in 0.2 M acetate buffer at 16° and pH 4.6 are reported. It appears that an indefinite self-association best describes the experimental data; the details of the analysis are reported.

In addition attention has been called to some experimental details which we believe aided us in these experiments. In an appendix to this manuscript it has been shown how the second negative moment $M_1^2\Sigma(c_i/M_i^2)$ or its apparent value can be developed from sedimentation equilibrium or osmometry of self-associating systems. Besides extending the analysis of discrete self-associations and being used for possible checks on the analysis, it can also be applied to indefinite self-associations.

Self-associating systems described by the equations

$$nP_1 \rightleftharpoons P_n, \qquad n = 2, 3, \ldots \tag{1}$$

or

$$nP_1 \rightleftharpoons qP_2 + mP_3 + \ldots \tag{2}$$

are widely encountered. In the first two equations P represents a solute, often a macromolecule, undergoing the self-association reaction. These associations have been reported for many proteins (Reithel, 1963; Nichol *et al.*, 1964; Squire and Li, 1961; Jeffrey and Coates, 1963, 1966), soaps and detergents (Annacker *et al.*, 1964; Ikeda and Kakiuchi, 1967), as well as with other compounds (Lamm and Neville, 1965; Broom *et al.*, 1967; Elias and Bareiss, 1967; Van Holde and Rossetti, 1967).

There are three areas that we wish to consider in this manuscript. The first is directed indefinite self-associations, *i.e.*, those which proceed without limit as described by eq 2. If it be assumed that the molar equilibrium constants are equal, then these systems can be analyzed. Besides showing another way in which $M_{n\,app}$ and $M_{w\,app}$ can be used to evaluate the intrinsic equilibrium constant (k) and the nonideal term ($\hat{B}M_1$), we will show how the second positive moment,[1] ψ (Adams, 1967), as well as the second negative moment, $M_1^2\Sigma(C_i/M_i^2)$, or its apparent value can be applied to the analysis. These new quantities can be used for the evaluation of k and $\hat{B}M_1$, or they can be used to check the analysis.

The second area to be discussed concerns sedimentation equilibrium experiments with β-lactoglobulin A in 0.2 M acetate buffer (pH 4.6) at 16°. Here it appears that an indefinite self-association best describes the data. The reasons for this choice are discussed, and we have emphasized some experimental precautions and techniques which we believe aided us in these experiments.

The last to be considered is the evaluation of the second negative moment, $M_1^2\Sigma(c_i/M_i^2)$, or its apparent value from sedimentation equilibrium or osmometry of self-associating systems. This is discussed in an appendix to the manuscript. We will assume here, as has been done previously (Adams and Fujita, 1963; Adams and Williams, 1964; Adams, 1965a, 1967), that the partial specific volumes ($\bar{v}$) for the associating species are equal

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[1] A moment is defined here as $\nu_k = \Sigma f_i M_i^k$, where ν_k is the kth moment, f_i is the weight fraction of species i, and M_i is the molecular weight of species i. Division of the quantity $\Sigma(c_i/M_i^2)$ by c converts it into the second negative moment, since $f_i = (c_i/c)$. It can also be noted that $M_{w(c)}$ leads to the first positive moment, c to the zeroth moment, and $c/M_{n(c)}$ to the first negative moment.

and that the refractive index increments $(\partial n/\partial c)$ for the associating species are equal.

Indefinite Self-Association

One of the types of self-associations that have been studied recently is the self-association that proceeds from monomer to dimer to trimer and so on indefinitely. The association can be described by the unlimited form of eq 2, and it can be considered to be made up of several simultaneous associations, as can any association represented by eq 2. On a molar basis these simultaneous associations can be represented as

$$\begin{aligned} 2P_1 &\rightleftharpoons P_2, \qquad K_{12} = [P_2]/[P_1]^2 \\ P_2 + P_1 &\rightleftharpoons P_3, \qquad K_{23} = P_3/[P_2][P_1] \end{aligned} \tag{3}$$

and so on for ideal solutions. Elias and Lys (1966), Elias and Bareiss (1967), and Van Holde and Rossetti (1967) have assumed that all molar equilibrium constants are equal; thus they obtain

$$\begin{aligned} [P_2] &= K[P_1]^2 \\ [P_3] &= K[P_2][P_1] = K^2[P_1]^3 \end{aligned} \tag{4}$$

and so on. If these concentrations are converted from moles per liter $[P_i]$ to grams per milliliter C_i, then one notes that

$$\begin{aligned} C_2 &= \frac{2KC_1^2}{1000M_1} = 2kC_1^2 \\ C_3 &= \frac{3K^2C_1^3}{1000^2M_1^2} = 3k^2C_1^3 \end{aligned} \tag{5}$$

and so forth. The quantity $k = K/1000M_1$ is called the intrinsic equilibrium constant. From the work of Elias and Lys (1966), Elias and Bareiss (1967), and Van Holde and Rossetti (1967) the following relations obtain.

$$\begin{aligned} C &= C_1 + 2kC_1^2 + 3k^2C_1^3 + \ldots \\ &= C_1(1 + 2kC_1 + 3k^2C_1^2 + \ldots) \\ &= C_1/(1 - kC_1)^2, \qquad \text{if } kC_1 < 1 \end{aligned} \tag{6}$$

This equation is also valid for nonideal solutions if one uses the convention that the logarithm of the activity coefficient $(\hat{y}_i)$ on the C concentration scale may be represented as

$$\ln \hat{y}_i = i\hat{B}M_1C + 0(C^2) \tag{7}$$

In addition, for $k\,C_1 < 1$, one obtains[2]

$$\frac{M_1}{M_{w\,app}} = \frac{1 - kC_1}{1 + kC_1} + \hat{B}M_1C \tag{8}$$

and

$$\frac{M_1}{M_{n\,app}} = 1 - kC_1 + \frac{\hat{B}M_1C}{2} \tag{9}$$

It is quite apparent that these equations can be used to evaluate both k and the nonideal term $\hat{B}M_1$. For example, a combination of eq 8 and 9 for $M_1/M_{w\,app}$ and $M_1/M_{n\,app}$ gives

$$\frac{M_1}{M_{w\,app}} = \frac{\dfrac{M_1}{M_{n\,app}} - \dfrac{\hat{B}M_1C}{2}}{2 - \left(\dfrac{M_1}{M_{n\,app}} - \dfrac{\hat{B}M_1C}{2}\right)} + \hat{B}M_1C \tag{10}$$

This equation in one unknown can be solved for $\hat{B}M_1$ by successive approximations.

The main purpose of this section is to show how $\Sigma C_iM_i^2$ and $\Sigma C_i/M_i^2$ can be applied to this association and be used in the analysis. The quantity ψ, which is given by eq 39 of Adams[1] (1967), becomes

$$\begin{aligned} \psi &= -(C_1 + 8kC_1^2 + 27k^2C_1^3 + 64k^3C_1^4 + \ldots) \\ &= \frac{-C_1(1 + 4kC_1 + k^2C_1^2)}{(1 - kC_1)^4} \end{aligned} \tag{11}$$

The substitution of eq 6 into eq 11 gives

$$\psi = \frac{-C(1 + 4kC_1 + k^2C_1^2)}{(1 - kC_1)^2} \tag{12}$$

Equations 11 and 12 are valid only if $kC_1 < 1$. It is appropriate now to demonstrate the derivation for the summation of the series for ψ. Let $x \equiv kC_1$ subject to the condition that $kC_1 < 1$; then

$$\frac{(1 + x)}{(1 - x)^3} = 1 + 4x + 9x^2 + 16x^3 + 25x^4 + \ldots \tag{13}$$

This equation is given by Tanford (1961) and also by Dwight (1961). Now let

$$y \equiv x\,\frac{(1 + x)}{(1 - x)^3} = x + 4x^2 + 9x^3 + 16x^4 + \ldots \tag{14}$$

The series given by y can be differentiated term by term to give a new convergent series when $x < 1$. For the mathematical theorems relating to differentiation and integration of such series, the reader should consult textbooks on advanced calculus (see, for example, Kaplan, 1952). Thus

$$y' = \frac{dy}{dx} = \frac{(1 + 2x)}{(1 - x)^3} + \frac{(x + x^2)(-3)(-1)}{(1 - x)^4} = \frac{1 + 4x + x^2}{(1 - x)^4} \tag{15}$$

[2] Since $M_1/M_{w\,app}$ or $M_1/M_{n\,app}$ is the same on the c (grams per deciliter) scale or the C (grams per milliliter) scale, it follows that $\hat{B}M_1c = BM_1C$; and since $C = c/100$, then it follows that $\hat{B}M_1 = 100BM_1$.

and also

$$y' = 1 + 8x + 27x^2 + 64x^3 + 125x^4 + \ldots$$

Since these derivatives of y must be equal, it follows that[3]

$$\frac{1 + 4x + x^2}{(1 - x)^4} = 1 + 8x + 27x^2 + 64x^3 + \ldots \quad (16)$$

when $x < 1$. It can be shown by the ratio test (Kaplan, 1952) that this series converges when $x < 1$.

It may be shown that for the ideal case, the quantity $M_1^2\Sigma C_i/M_i^2$ is given by the equation

$$M_1^2\sum C_i/M_i^2 = \int_0^C \frac{M_1^2 dC}{M_{n(c)}M_{w(c)}} = C_1 + \frac{kC_1^2}{2} + \frac{k^2C_1^3}{3} + \frac{k^3C_1^4}{4} + \ldots \quad (17)$$

Multiplying both sides of eq 17 by k gives

$$kM_1^2\sum C_i/M_i^2 = kC_1 + \frac{k^2C_1^2}{2} + \frac{k^3C_1^3}{3} + \frac{k^4C_1^4}{4} + \ldots = \ln\frac{1}{(1 - kC_1)} \quad (18)$$

when $kC_1 < 1$. Thus it is noted that

$$M_1^2\sum C_i/M_i^2 = \frac{1}{k}\ln\frac{1}{(1 - kC_1)} \quad (19)$$

For the nonideal case one obtains

$$\int_0^C \frac{M_1^2 dC}{M_{w\,app}M_{n\,app}} = M_1^2\sum(C_i/M_i^2)_{app} = \frac{1}{k}\ln\frac{1}{(1 - kC_1)} + \frac{\hat{B}M_1}{2}\int_0^C \frac{CM_1}{M_{w\,app}}dC + \hat{B}M_1\int_0^C \frac{CM_1}{M_{n\,app}}dC - \frac{(\hat{B}M_1)^2C^3}{6} \quad (20)$$

Thus, for the indefinite self-association, expressions have been obtained for all of the moments that are available for the discrete self-association; these additional equations may prove useful in checking the calculations. With indefinite self-associations the apparent weight fraction of monomer (f_a) is obtained from

$$\ln f_a = \int_0^C \left(\frac{M_1}{M_{w\,app}} - 1\right)\frac{dC}{C} \quad (21)$$

The equation for the apparent monomer concentration ($\hat{\alpha}$) is

$$\hat{\alpha} = Cf_a = C_1e^{\hat{B}M_1C} \quad (22)$$

[3] T. H. Donnelly, in connection with another problem, has also derived this equation (personal communication to E. T. Adams, Jr.).

One of the ways to check the calculations would be to calculate C from eq 6; here eq 22 would be used in the form

$$C_1 = \hat{\alpha}e^{-\hat{B}M_1C}$$

and eq 9 would be used to give

$$(1 - kC_1)^2 = \left(\frac{M_1}{M_{n\,app}} - \frac{\hat{B}M_1C}{2}\right)^2$$

Thus eq 6 would become

$$C = \frac{\hat{\alpha}e^{\hat{B}M_1C}}{\left[\frac{M_1}{M_{n\,app}} - \frac{\hat{B}M_1C}{2}\right]^2} \quad (23)$$

The calculated value of C would be compared with the observed value. Similarly the equation for ψ (eq 12) could be used to calculate C, since

$$-C = \psi(1 - kC_1)^2/(1 + 4kC_1 + k^2C_1^2) = \frac{\psi\left(\frac{M_1}{M_{n\,app}} - \frac{\hat{B}M_1C}{2}\right)^2}{\left\{1 + 4\left[1 - \left(\frac{M_1}{M_{n\,app}} - \frac{\hat{B}M_1C}{2}\right)\right] + \left[1 - \left(\frac{M_1}{M_{n\,app}} - \frac{\hat{B}M_1C}{2}\right)\right]^2\right\}} \quad (24)$$

Finally, the quantity $M_1^2\Sigma(C_i/M_i^2)_{app}$ could be calculated from eq 20 and compared with its observed value. In addition one can apply eq 9 together with the Van Holde and Rossetti (1967) eq 12 to our eq 20; the result of this procedure is

$$M_1^2\sum(C_i/M_i^2)_{app} = \frac{-4C\ln\left[\frac{M_1}{M_{n\,app}} - \frac{\hat{B}M_1C}{2}\right]}{\left\{\frac{(M_{w\,app}/M_1)^2}{(1 - \hat{B}M_1[M_{w\,app}/M_1]C)^2} - 1\right\}} + \hat{B}M_1\int_0^C \frac{CM_1}{M_{n\,app}}dC + \frac{\hat{B}M_1}{2}\int_0^C \frac{CM_1}{M_{w\,app}}dC - \frac{(\hat{B}M_1)^2C^3}{6} \quad (25)$$

Equation 25 can be solved for $\hat{B}M_1$ by successive approximations, or it can be used as a check on the value of $\hat{B}M_1$ obtained from eq 10 or from the procedure advocated by Van Holde and Rossetti (1967).

Experiments with β-Lactoglobulin A

β-Lactoglobulin A has been reported to undergo a monomer–tetramer (M_1 = 36,300) association at temperatures of 25° or less in the pH range of 3.7–5.2 (Townend and Timasheff, 1960; Timasheff and Townend, 1961; Kumosinski and Timasheff, 1966). At pH values below 3.5, β-lactoglobulin A has been reported to undergo a monomer–dimer (M_1 = 18,150) association

(Townend *et al.*, 1960; Kumosinski and Timasheff, 1966). We were interested in the association of β-lactoglobulin A in the pH 3.7–5.2 region for several reasons: (1) we wanted to study a monomer–tetramer equilibrium by sedimentation equilibrium and also to know if the 18,150 mol wt unit[4] was indeed absent; (2) we wished to compare sedimentation equilibrium experiments with light scattering experiments on the same material; and (3) in addition, we hoped to solve some vexing experimental problems peculiar to sedimentation equilibrium studies on interacting systems. The results of our experiments are reported in the following paragraphs.

Materials and Methods

β-Lactoglobulin A was graciously furnished to us by Drs. S. N. Timasheff and R. Townend. The protein was recrystallized to remove denatured material according to their instructions. All solutions used in these experiments were made up from recrystallized protein. The buffer, which was made with deionized distilled water, was sodium acetate and acetic acid to an ionic strength of 0.10 and a pH of 4.61 at 22.5°. pH determinations were done on a Beckman Research pH meter using glass and calomel electrodes, which were standardized against Harleco standard solution 4 (pH 4.01 at 25°). All stock protein solutions were dialyzed at least 24 hr in the cold (4–6°) with three changes of buffer. The stock solution was made up to be close to the highest concentration used in a series of experiments. For example, a stock solution was made up to a concentration of approximately 1 g/dl. Other stock solutions whose initial concentrations were about 0.75 and 0.50 g/dl were prepared by weight dilution using the buffer that was in dialysis equilibrium with the stock solution. Initial concentrations were determined in a Brice-Phoenix differential refractometer at a wavelength of 5460 Å at room temperature. These readings were corrected to 16°, the temperature at which the sedimentation equilibrium experiments were performed by previously described methods (Adams and Filmer, 1966).

Whenever possible the glassware, syringes, and ultracentrifuge cell parts were cleaned in an ultrasonic cleaner. The glassware and other equipment were rinsed with deionized water after cleaning; glassware was dried in a convection oven at 105–110°. In order to avoid problems of adsorption a Kel F Yphantis (1964) six-hole centerpiece was filled and emptied three times before the final filling. The protein solution was allowed to remain in the centerpiece about 5 min each time; the final filling was done as rapidly as possible to avoid problems of evaporation. The protocol for filling the cell was as follows: the solvent side was always filled first; then the most concentrated solution was placed in the centripetal or inner hole, middle concentration in the middle hole, and least concentrated solution in the centrifugal or outer hole. The solutions were placed in this order so that the most concentrated solution would be in the weakest centrifugal field and the most dilute solution would be in the strongest field. No layering oil was used in these experiments; the reasons for this will be discussed later. The Yphantis (1964) centerpiece we used had been lapped so that the faces were flat and parallel to ±0.0001 in.; this was done to prevent leakage from the centerpiece or between the holes. The cross-sectional width of the solution holes, f(r), was determined as a function of radial distance, r, from the center of rotation from measurements on a two-dimensional (Nikon Model 6) comparator. The same side of the centerpiece was always used for the solution or the buffer. This choice was made on the basis of the comparator measurements, and the centerpiece was marked to indicate this. In order to account for any shift of the centerpiece by the centrifugal field, the radial positions of the inner and outer edges of the middle hole, as well as the outer edge of the inner (centripetal) hole and the inner edge of the outer (centrifugal) hole, were calculated in each experiment; the average shift was then determined and used in these calculations.

The experiments reported here were performed at 16° on a Spinco Model E analytical ultracentrifuge, which was equipped with Rayleigh and schlieren optics and which had an electronic speed control. The basic sedimentation equation used was

$$\frac{\mathrm{d} \ln J}{\mathrm{d}(r^2)} = AM_{\mathrm{w\,app}} \tag{26}$$

Here, J, the number of interference fringes, is given by the equation

$$J = \frac{h}{\lambda}\frac{\mathrm{d}n}{\mathrm{d}c}c = \frac{h}{\lambda}(n - n_0) = \frac{h}{\lambda}\tilde{n} \tag{27}$$

where h is the thickness of the ultracentrifuge centerpiece (11.97 mm in these experiments), λ is the wavelength of the monochromatic light (5462 Å), dn/dc is the refractive index increment (1.822×10^{-3} dl/g: Halwer *et al.* (1951)), c is the protein concentration in grams per deciliter, and $n - n_0$ is the refractive index difference between the protein solution and the buffer in dialysis equilibrium with the protein solution. For these experiments $J = 39.94c$. The absolute fringe value was determined from the application of the conservation of mass; the meniscus concentration J_a was calculated from

$$\int_a^b (J_r - J_a)\mathrm{f}(r)\mathrm{d}r = (J_0 - J_a)\int_a^b \mathrm{f}(r)\mathrm{d}r \tag{28}$$

This is a modification of an equation used by Svedberg and Pedersen (1940). In eq 28, J_r is the absolute fringe value in the solution column of the ultracentrifuge cell at any radial position r from the center of rotation; the radial positions include the position of the air–solution meniscus ($r = a$) and the cell bottom ($r = b$) as well as any radial positions between them. The quantity f(r) is

[4] The molecular weight of 18,150 for the β-lactoglobulin A monomer is based on the amino acid analyses by Gordon *et al.* (1961).

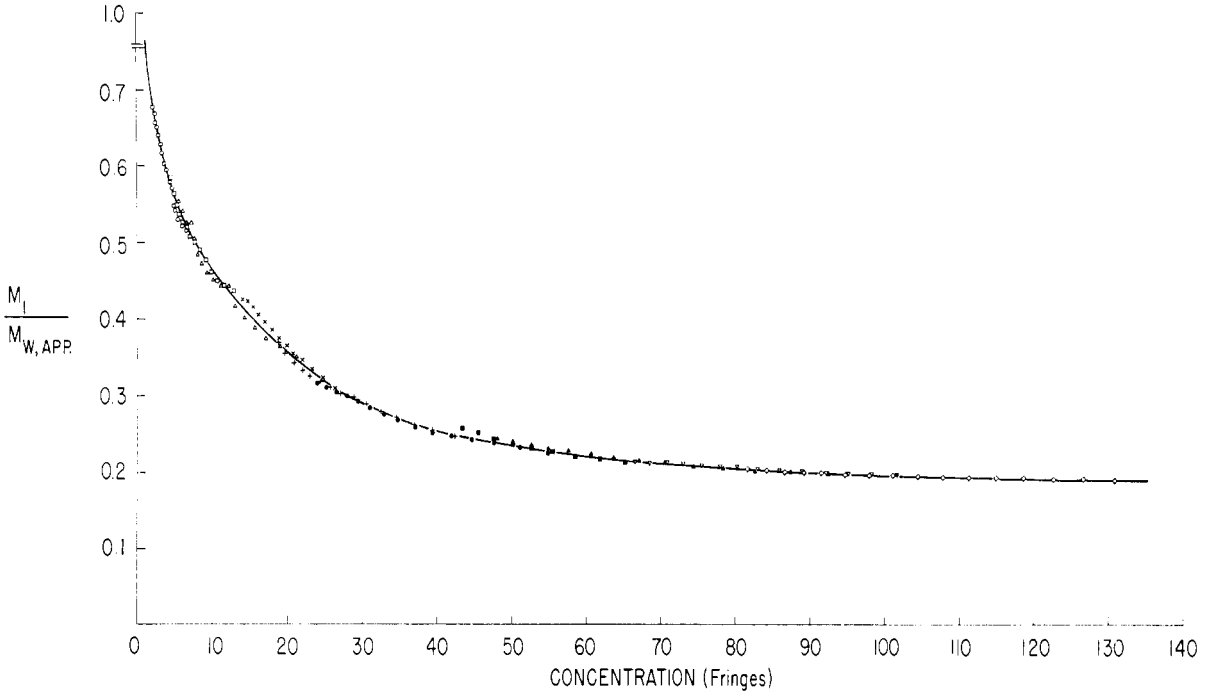

FIGURE 1: Plot of $M_1/M_{w\,app}$ against J, the fringe number, for the sedimentation equilibrium of β-lactoglobulin A at 16°. Ten different initial concentrations of β-lactoglobulin in 0.2 ionic strength acetate buffer (pH 4.61) are shown. The curve represents the best fit that may be drawn through the points.

the cross-sectional width of the centerpiece hole, and J_0 is the original concentration, which was determined by differential refractometry. From the schlieren data one observes

$$Z - Z_0 = \hat{Z} = K\mathrm{d}(n - n_0)/\mathrm{d}r \qquad (29)$$

The quantity $Z - Z_0 = \hat{Z}$ is the difference in height between the schlieren patterns for the solution (Z) and for the buffer (Z_0). The constant K is a product of several optical constants and is given by $K = Ghb \tan\theta$ where the angle θ is usually a 90° phase-plate angle (strictly speaking, θ is the angular difference between the position of zero vertical magnification and the phase-plate angle). Application of eq 27 leads to the result that

$$\frac{\mathrm{d}J}{\mathrm{d}r} = \frac{h\mathrm{d}(n - n_0)}{\lambda \mathrm{d}r} = \frac{\hat{Z}}{\lambda Gb \tan\theta} \qquad (30)$$

It is not necessary to evaluate the two optical constants G, the cylinder lens magnification factor, and b, the optical lever arm, since the magnified fringe spacing $\hat{H}$ (the fringe spacing observed on the photographic plate) is related to λGb by the equation

$$\hat{H} = \lambda Gb/S \qquad (31)$$

where S is the spacing between the pair of slits of the Rayleigh interferometer (usually in the lower window holder). Optical theory (Halliday and Resnicl., 1962) indicates that H, the spacing between interference fringes produced by this optical configuration, is given by

$$H = \frac{\lambda b}{S} \qquad (32)$$

Since the cylinder lens magnifies the spacing between fringes, the observed spacing is $\hat{H} = GH$; thus, multiplication of eq 32 by G leads to eq 31.

The AN-J rotor was used in these experiments to give maximum stability. Rotor temperature control was maintained by a refrigeration unit and by a heater controlled by the rotor temperature indicator control unit; a calibration curve based on the rotor temperature indicator control unit readings of the thermistor embedded in the rotor was prepared for an appropriate range of temperature. A calibrated (National Bureau of Standards) thermometer was used as a standard. The density of the acetate buffer was calculated to be 1.003 at 16° from tables in the appendices to Svedberg and Pedersen's (1940) monograph. The value of the partial specific volume used was $\bar{v} = 0.747$ (Svedberg and Pedersen, 1940); this value was obtained by interpolation of the reported value to 16° according to the methods described in the appendices to the Svedberg and Pedersen (1940) monograph. The speeds used in these experiments ranged from 4800 rpm for the highest initial concentration ($J_0 = 101.37$) to 12,000 rpm for the lowest initial concentration ($J_0 = 3.89$).

Results

The results of our experiments on β-lactoglobulin A solutions at 16° are shown in Figure 1 as a plot of $M_1/$

TABLE I: β-Lactoglobulin A Sedimentation Equilibrium Data at 16°.[a]

J (fringes)	c (g/dl)	$M_1/M_{n\ app}$	α[b]	$\int_0^c (cM_1/M_{w\ app})dc$[c]	$\int_0^c (cM_1/M_{n\ app})dc$[c]
125	3.130	0.271	0.1741	1.066	1.589
115	2.879	0.278	0.1704	0.922	1.378
105	2.629	0.286	0.1675	0.788	1.188
90	2.253	0.301	0.1624	0.607	0.920
75	1.878	0.320	0.1564	0.448	0.679
65	1.627	0.336	0.1518	0.355	0.536

[a] Values of $M_1/M_{w\ app}$ and $M_1^2(\Sigma c_i/M_i^2)_{app}$ are recorded in Tables II and III, respectively. [b] Calculated using values of $(M_1/M_{w\ app} - 1)/J$ obtained from the smooth curve drawn through Figure 1. [c] Calculated using c in grams per deciliter.

$M_{w\ app}$ against J, the fringe member. It is quite gratifying to note the reasonably good fit of the data at low concentrations, where previously much trouble had been experienced (Adams and Filmer, 1966; Adams and Fujita, 1963; Jeffrey and Coates, 1966; Squire and Li, 1961). Some of the valus of $M_1/M_{w\ app}$, $M_1/M_{n\ app}$, c, α, $M_1^2(\Sigma c_i/M_i^2)_{app}$, and other experimentally available quantities are displayed in Tables I–III. It appears that the association represented by Figure 1 is best described as an indefinite polymerization of monomer (M_1 = 18,150) to dimer to trimer and so on; the reasons for this choice will be elaborated in the discussion that follows.

The lowest apparent weight-average molecular weight ($M_{w\ app}$) observed was 26.8×10^3 at J = 2.12 fringes, and the highest value of $M_{w\ app}$ was 94.8×10^3 at J = 130.7 fringes. The trend of the $M_1/M_{w\ app}$ data at low concentrations is quite steeply upward, which gives support for the presence of the 18,150 mol wt unit. At higher concentrations from J = 80 fringes onward there is a leveling off in the plot of $M_1/M_{w\ app}$ *vs.* J. If the minimum molecular weight were 36,300 and a monomer–tetramer (M_1 = 36,300) equilibrium were present then the following observations could occur: (1) the curve of $M_1/M_{w\ app}$ *vs.* J would go directly to 0.5 (in Figure 1, M_1 has been chosen as 18,150) without any minimum in the plot at higher values of J or any maximum in the plot near J = 0 if the virial coefficient (BM_1) were zero (ideal) or negative; (2) on the other hand, if BM_1 were greater than zero, a molecular weight below 36,300 (below 0.5 on the $M_1/M_{w\ app}$ scale) could occur, but it would also require a maximum in the plot of $M_1/M_{w\ app}$ *vs.* J near zero protein concentration, as well as a minimum in the same plot at higher protein concentrations (Adams and Filmer, 1966). Inasmuch as this behavior is not observed, and since elaborate precautions have been taken to prevent or minimize the possibility of adsorption, we do feel that the 18,150 unit is present. This could probably be verified by some experiments with absorption optics and a scanner (Schachman, 1963; Schachman and Edelstein, 1966). Thus, it appears that the association is more complicated than the monomer–tetramer (M_1 = 36,300) association advocated by Townend and Timasheff (1960). There remain then two possibilities: the association is discrete or it is indefinite. As a result of several calculations on discrete association, a good choice seemed to be a monomer–dimer–octamer association. Among the equations used for this analysis were

$$\frac{16cM_1}{M_{n\ app}} - 10c = 72e^{-BM_1c} + 8BM_1c^2 - \frac{1}{\dfrac{M_1}{cM_{w\ app}} - BM_1} \quad (33)$$

and

$$64M_1^2\sum\left(\frac{c_i}{M_i^2}\right)_{app} - 21c = 45.5\alpha e^{-BM_1c} + 32BM_1\int_0^c \frac{cM_1}{M_{w\ app}}\,dc + 64BM_1\int_0^c \frac{cM_1}{M_{w\ app}}\,dc - \frac{2.5}{\dfrac{M_1}{cM_{w\ app}} - BM_1} - 64(BM_1)^2c^3 \quad (34)$$

TABLE II: Analysis of the Association of β-Lactoglobulin A at 16° as an Indefinite Association.[a]

		$M_1/M_{w\ app}$		
J	$\hat{B}M_1$	Calcd	Obsd	k
125	1.7	0.192	0.191	4.06×10^2
115	1.7	0.194	0.193	4.05
105	1.7	0.196	0.195	4.02
90	1.6	0.201	0.200	3.97
75	1.5	0.209	0.209	3.95
65	1.4	0.217	0.217	3.93

[a] $\hat{B}M_1$(av) = 1.6 ± 0.1. [b] k(av) = 4.00 ± 0.05 × 10^2.

TABLE III: Check on the Analysis of β-Lactoglobulin A at 16° as an Indefinite Association.

	$M_1^2(\Sigma C_i/M_i^2)_{app} \times 10^3$		$C_1 \times 10^3$		$C \times 10^2$	
J (fringes)	Calcd	Obsd	From Eq 21	From C, $\hat{B}M_1$, and $M_1/M_{w\,app}$	Calcd	Obsd
125	3.85 (3.74)[a]	3.79	1.91[b]	1.86[c]	3.18[d]	3.13
115	3.72 (3.64)	3.66	1.89	1.84	2.90	2.88
105	3.55 (3.55)	3.52	1.87	1.83	2.66	2.63
90	3.35 (3.33)	3.30	1.82	1.80	2.21	2.25
75	3.10 (3.15)	3.07	1.76	1.76	1.82	1.88
65	2.92 (2.98)	2.89	1.72	1.72	1.59	1.63

[a] The quantities in parentheses were calculated from eq 25. The quantities without parentheses were calculated using deduced values of $\hat{B}M_1$ (from eq 10), k and C_1 (from eq 9 and 6) in the right-hand side of eq 20. The differences arise from the fact that our procedures were used to evaluate k for the numbers without parentheses, whereas the values of k obtained from eq 25 are based on the use of Van Holde and Rossetti's (1967) eq 12. [b] C_1 was calculated from eq 21 as described in the text; the values of $((M_1/M_{w\,app}) - 1)/J$ obtained from the indefinite polymerization were used in the range J = 0–10 fringes. [c] C_1 was calculated from the use of eq 10, 9, and 6. [d] The concentration was calculated from eq 24; the values of $1 - kC_1$ and kC_1 used in these calculations were obtained from eq 9.

Equation 34, which is obtained by the appropriate combination of eq A7 with the equation for c, $M_{w\,app}$, and α in a manner similar to previous procedures (Adams and Filmer, 1966; Adams, 1967) with discrete associations, gave $BM_1(av) = 0.017 \pm 0.004$. Equation 33, obtained by a procedure analogous to that used in obtaining Adams' (1965a) eq 14b, gave $BM_1(av) = 0.014 \pm 0.003$. The values of c_1 and BM_1 obtained from eq 33 were used to calculate $K_2(av) = 24.1 \pm 0.3$ and K_8 (av) $= 4.0 \pm 0.3 \times 10^6$. A curve of $M_1/M_{w\,app}$ against J was generated from these constants; this is shown in Figure 2 as the dashed curve. It can be seen that the monomer–dimer–octamer calculations fit the experimental data poorly; thus we turned to the indefinite association.

A much better description of the observed association is given by an indefinite association with $K = 4.00 \pm 0.05 \times 10^2$ and $\hat{B}M_1 = 1.6 \pm 0.1$; the $M_1/M_{w\,app}$ *vs.* J curve for this model is given by the solid curve in Figure 2. Some of the data used in the calculations for the indefinite association as well as some checks on th calculations are shown in Tables II and III. For the indefinite association $\hat{B}M_1$ was calculated from eq 10 then kC_1 was obtained from eq 9, C_1 was obtained from eq 6 using the value of $(1 - kC_1)$ obtained from eq 9, and k was obtained by dividing kC_1 by C_1.

Originally, we were somewhat suspicious about the indefinite association, as our values for $\hat{\alpha}$, obtained from $M_1/M_{w\,app}$ values in Figure 1, would be lower than those required by the indefinite association. From Figure 2 it can be seen that the curve for the indefinite polymerization gives a good fit to the data in the range J = 0–40 fringes. When Figure 1 was drawn, it was assumed that all data were equally good and what appeared to be the best curve was drawn through the data points. The upper scatter observed in the 10–20 fringe range in Figure 1 may be the result of unintentional contamination from flasks, syringes, or other sources. The quantity $\ln f_a$ is calculated from eq 21, and the greatest contribution to the area under the curve of $((M_1/M_{w\,app}) - 1)/J$ *vs.* J occurs for these experiments in the range J = 0–40 fringes. Thus the integral in eq 21 appears to be more susceptible to error for very strong association. This viewpoint is supported by the following procedure: in calculating $\ln f_a$ from eq 21 the integrand, $((M_1/M_{w\,app}) - 1)/J$, was obtained from the indefinite polymerization curve in the range 0–10 fringes; in the range 10–40 fringes the integrand was obtained from a plot through the lower part of the observed data, and above 40 fringes, the integrand was obtained from the curve given in Figure 1. The results of this procedure are shown in Table III, and they support our viewpoint regarding the sensitivity of eq 21 to strong associations. In Table III another check on the indefinite association is given by the very good agreement between the values of $M_1^2(\Sigma C_i/M_i^2)_{app}$ calculated from eq 20 and 25 and the observed values (calculated from Figure 1). In addition, a comparison between the observed concentration and the concentration calculated from eq 24 has been shown. The derivative required for eq 24 was calculated from finite difference methods (Adams, 1967). A plot was made of these approximations to the derivative over the concentration range used (65–130 fringes), and the final value of the derivative was obtained from this plot.

Discussion

Our conclusion that an indefinite polymerization appears to give the best fit to the data is somewhat surprising; it certainly is quite different from the conclusions of Townend and Timasheff (1960) and the more recent experiments under similar conditions by Kumosinski and Timasheff (1966). We were quite frankly sur-

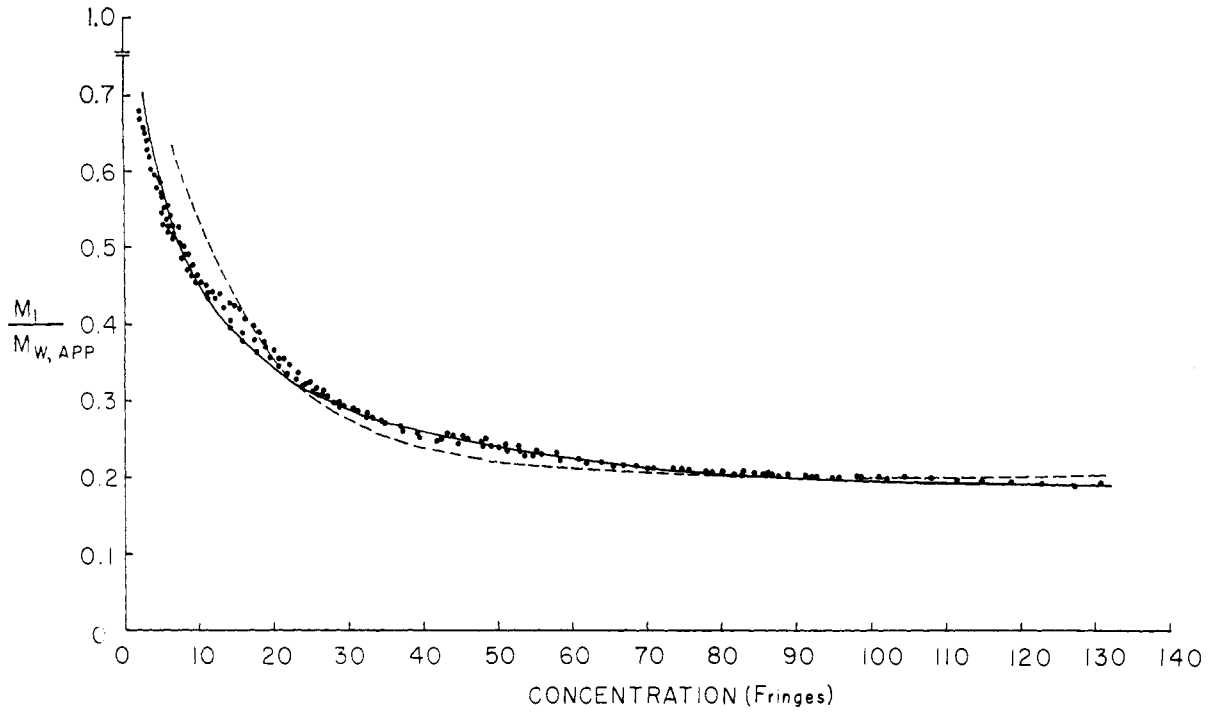

FIGURE 2: Comparison plots of $M_1/M_{w\,app}$ against J for the monomer–dimer–octomer (dashed line) association having average values of $K_2 = 24.1$, $K_8 = 4.0 \times 10^6$, and $BM_1 = 0.014$ with the indefinite association (solid line) having average values of $k = 4.00 \times 10^2$ and $\hat{B}M_1 = 1.6$. The points indicate the observed values. The indefinite association gives a much better description of the experimental data.

prised that a discrete association of the monomer–dimer–octamer type did not appear to fit the data. Although our values of $M_{w\,app}$ and the recent data of Kumosinski and Timasheff (1966) are similar at high concentrations (above 1.5 g/dl), they definitely disagree in the lower concentration range. The difference between the experiments reported here and the light-scattering experiments (Townend and Timasheff, 1960; Kumosinski and Timasheff, 1966) is certainly more than just experimental error. Reference to the figures in the paper by Kumosinski and Timasheff (1966) shows that there is a paucity of $M_{w\,app}$ data in the low concentration region, which is where significant changes appear to be occurring. It may be possible that the light-scattering experiment becomes very difficult to do at low concentrations (0.3 g/dl or less), or that dust may still be present in their solutions, or Steiner's (1953a,b) association–fluctuation coupling may be present, or all three of these effects may be present. It would appear from our experiments that we have an extremely powerful method for studying self-associating systems and resolving differences in the type of association.

As we mentioned earlier no layering oil was used in the experiments reported here. We discovered to our surprise that β-lactoglobulin A is precipitated by the supposedly inert fluorocarbon oil FC-43; this is shown in Figure 3. Both tubes in this figure have a 2% solution of β-lactoglobulin A in acetate buffer which is layered over the fluorocarbon oil. In the tube at the right, the solution has just been layered over the oil and some slight precipitate (probably surface denaturation) has formed. When the tube is gently inverted five or six times to simulate handling of the centerpiece, an emulsion appears to form; this was clarified by centrifugation in a clinical centrifuge. The results obtained with the clarified solution show a significant band of precipitate at the interface between the FC-43 oil and the protein solution; this is shown in Figure 3 left. The removal of this protein by different layering oils (FC-43, Dow Corning Silicone Oil No. 555, and Kel F Polymer Oil No. 1) was also observed. When layering oils were used it was not possible to obtain overlap of the data from different experiments at the low concentrations. By recrystallizing their protein to remove denatured material and by following our suggestion to avoid layering oils, Albright and Williams (1968) have obtained excellent curves of $M_1/M_{w\,app}$ *vs.* c for their sedimentation equilibrium studies on the association of β-lactoglobulin B. Thus, it appears that successful sedimentation equilibrium experiments on these associating systems are dependent upon particularly careful laboratory techniques and upon taking precautions previously not thought necessary.

The theory and analysis presented here were written for a two-component system. They can be extended to multicomponent systems (protein in buffer solution) by the use of the Casassa and Eisenberg (Casassa and Eisenberg, 1960, 1964; Eisenberg and Casassa, 1960) or the Scatchard and Bregman (1959) conventions. The use of these conventions requires the dialysis of the solution containing the macromolecule against the buffer solution, which we did. It is the values of $M_{w\,app}{}^*$, k^*, $B^*M_1{}^*$, etc. of the redefined component (by the Casassa and Eisenberg convention) that are measured and reported here. More complete details on the application of the Casassa and Eisenberg (1960, 1964) definition of com-

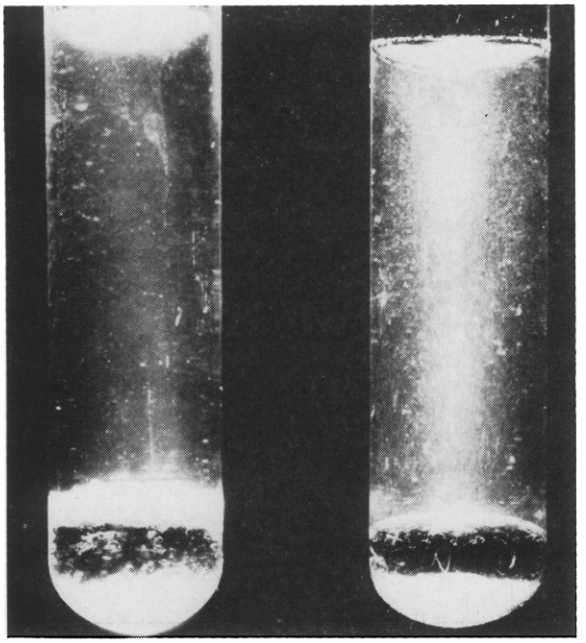

FIGURE 3: Illustration showing the precipitation of β-lactoglobulin A by the FC-43 fluorocarbon layering oil (lower phase). In the tube at the right, the solution has just been layered over the FC-43 oil. In the tube at the left, the tube has been inverted gently five or six times to simulate the handling of the ultracentrifuge cell, and then the mixture was centrifuged in a clinical centrifuge to clarify the solution. Note the significant amount of precipitate (left tube) at the interface of the two liquids. A 2-g/dl solution of β-lactoglobulin A in 0.2 ionic strength acetate buffer (pH 4.61) was used here.

ponents and its application to self-associating systems will be found in previous publications (Adams, 1965a,b).

Appendix

Evaluation of $M_1^2\Sigma(c_i/M_i^2)$ from Sedimentation Equilibrium or Osmometry. In the derivation to be presented a monomer–dimer–trimer association will be used, but it must be noted that this derivation can be extended to any self-association described by eq 1 or 2. Previous assumptions (Adams, 1965a, 1967; Adams and Filmer, 1966) regarding the partial specific volume ($\bar{v}$), the refractive index increment ($\partial n/\partial c$), and the natural logarithm of the activity coefficient (y_i) will be used. Furthermore, it will be assumed that several sedimentation equilibrium experiments have been performed, and that a smooth plot of $M_1/M_{w\ app}$ *vs.* c, such as is shown in Figure 1, has been constructed.

For the monomer–dimer–trimer association the expression for the desired quantity, $M_1^2\Sigma(c_i/M_i^2)$, is

$$M_1^2\sum(c_i/M_i^2) = c_1 + \frac{K_2c_1^2}{4} + \frac{K_3c_1^3}{9} \qquad \text{(A1)}$$

With the monomer–dimer–trimer association, the following equations have been shown to apply (Adams, 1965a, 1967; Adams and Filmer, 1966):

$$c = c_1 + K_2c_1^2 + K_3c_1^3 \qquad \text{(A2)}$$

$$cM_{w(c)} = M_1(c_1 + 2K_2c_1^2 + 3K_3c_1^3) \qquad \text{(A3)}$$

and

$$\frac{cM_1}{M_{n(c)}} = c_1 + \frac{K_2c_1^2}{2} + \frac{K_3c_1^3}{3} \qquad \text{(A4)}$$

Now if eq A4 is integrated in the following manner, then $M_1^2\Sigma(c_i/M_i^2)$ is obtained.

$$\int_0^{c_1} \frac{cM_1}{M_{n(c)}}\frac{dc_1}{c_1} = c_1 + \frac{K_2c_1^2}{4} + \frac{K_3c_1^3}{9} = M_1^2\sum(c_i/M_i^2) \qquad \text{(A5)}$$

Note that c is used here to represent concentrations in grams per deciliter. It can be shown by the use of eq A2 and A3 that the quantity dc_1/c_1 can be represented as

$$\frac{dc_1}{c_1} = \frac{M_1dc}{cM_{w(c)}} \qquad \text{(A6)}$$

The insertion of eq A6 into eq A4 leads to

$$M_1^2\sum(c_i/M_i^2) = \int_0^c \frac{M_1^2dc}{M_{n(c)}M_{w(c)}} \qquad \text{(A7)}$$

Equation A7 could be used as written for osmometry of self-associating systems, since both $M_{n(c)}$ and $M_{w(c)}$ are available from osmometry (Adams, 1965b); however, there is a more convenient form of eq A7 for osmometry. This form is

$$M_1^2\sum(c_i/M_i^2) = \frac{cM_1^2}{M_{n(c)}^2} - \int_1^{M_1/M_{n(c)}} \frac{cM_1}{M_{n(c)}}\,d(M_1/M_{n(c)}) \qquad \text{(A8)}$$

Equation A7 is evaluated by numerical integration from a plot of $M_1^2/M_{n(c)}M_{w(c)}$ against c. To evaluate eq A8 a plot is made of $cM_1/M_{n(c)}$ against $M_1/M_{n(c)}$, and the required integral is evaluated numerically. It should be noted that $M_1/M_{n(c)} = 1$, when $c = 0$.

The availability of $M_1^2\Sigma(c_i/M_i^2)$ is quite useful, as it extends the ability to analyze discrete self-associations to the presence of four (in osmometry) or five species (in sedimentation equilibrium) in chemical equilibrium with each other, and it can also be applied to the analysis of indefinite self-associations. It is possible in principle to develop lower moments of the type $M_1^{q+1}\Sigma(c_i/M_i^{q+1})$ from eq A7 or A8. First eq A7 or A8 must be multiplied by dc_1/c_1 (from eq A6) and then integrated from $c = 0$ to $c = c$ to give $M_1^3\Sigma(c_i/M_i^3)$. Successive repetition of this procedure leads to the other lower moments. Thus it is possible in principle to analyze any discrete self-association; however, the precision of the data (replotting of the data is required for each integration) will most likely limit this process to the evaluation of $M_1^3\Sigma(c_i/M_i^3)$ at best.

For nonideal solutions the analog of eq A7 is

$$M_1^2\sum(c_i/M_i^2)_{app} = \int_0^c \frac{M_1^2 dc}{M_{n\,app}M_{w\,app}} = M_1^2\sum(c_i/M_i^2) + \frac{BM_1}{2}\int_0^c \frac{cM_1}{M_{w\,app}} dc + BM_1\int_0^c \frac{cM_1}{M_{n\,app}} dc - (BM_1)^2c^3/6 \quad (A9)$$

The nonideal analog of eq A8 is obtained by replacing $M_{n(c)}$ with $M_{n\,app}$. Some applications of $M_1^2\Sigma(c_i/M_1^2)$ or its apparent value will be discussed in future publications.

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