

# ON THE DISSOCIATION OF BOVINE $\beta$ -LACTOGLOBULINS A, B, AND C NEAR pH 7

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## Abstract

Sedimentation-equilibrium studies are made of the molecular weights of bovine  $\beta$ -lactoglobulins A, B, and C at pH 7.5. The order of dissociation of dimer to monomer is  $A > B \gg C$ . The dissociation constant ( $K_d$ ) for A and B is  $0.6 \times 10^{-4}$  and  $0.08 \times 10^{-4}$  mole  $l^{-1}$  at 20°C, respectively. The conditions chosen for these measurements are based on sedimentation velocity studies in the pH range 6–9. There is no change in sedimentation velocity behaviour following different times of standing at 20°C for pH  $\leq 7.5$ . The sedimentation patterns exhibit a single peak with some trailing on the solvent side. At low concentrations the plot of weight average sedimentation coefficient ( $\bar{s}$ ) versus concentration ( $C$ ) is in accord with that of a rapidly dissociating system of monomer–dimer type. There are time-dependent aggregations above pH 8. Effects of changes in ionic strength and addition of methanol are considered.

Using several values of  $K_d$ ,  $\bar{s}$  versus  $C$  curves are calculated by the method of Gilbert (1963) and compared with the present experimental curves and also those of Zimmerman, Barlow, and Klotz (1970). The agreement between theory and experiment is only moderate.

## I. INTRODUCTION

The work described in this paper is part of a program of comparative studies of the properties of genetic variants of proteins under a variety of environmental conditions. Such studies enable, *inter alia*, insight to be gained into those factors that determine the conformation and state of association of proteins and the nature of the intermolecular bonding involved in association. We have found it profitable to study the  $\beta$ -lactoglobulins because of the unusual array of such changes they exhibit as the pH, solvent, and temperature are varied (for reviews see Tilley 1960; Timasheff and Townend 1962; McKenzie 1967, 1971).

In the neighbourhood of the iso-ionic point (pH 5.2) bovine  $\beta$ -lactoglobulin has a molecular weight of 36,000 and consists of two monomer units (mol. wt. 18,000). Its conformation appears to be essentially the same from pH 2 to 6. However, Townend and Timasheff (1957) found that the protein shows increasing dissociation below pH 3.5 to the monomer. Tanford, Bunville, and Nozaki (1959) observed that the protein undergoes a reversible change in conformation near pH 7. Tanford and Taggart (1961) showed that the transition is ionization-linked, but concluded that no change in molecular weight (36,000) occurs. On the other hand, Georges and Guinand (1960) considered that dissociation occurs near pH 7.

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Considerable impetus was given to gaining an understanding of the variation in molecular size and conformation with pH of the  $\beta$ -lactoglobulins when genetic variants were discovered. We are concerned here with the molecular size at pH 7.5 of the three bovine variants (A, B, C) known at the time the work was undertaken. We also consider their behaviour in sedimentation velocity experiments in relation to the theories of Gilbert (1955, 1963) for rapidly associating-dissociating systems.

## II. EXPERIMENTAL AND THEORETICAL

### (a) *Materials and Glassware*

Because of the possibility of trace metal ion effects in the reactions being studied, precautions were taken throughout the present work to avoid contamination by heavy metals, particularly copper. Reagents and water of high purity were used.

Bovine  $\beta$ -lactoglobulins A and B were prepared by the method of Armstrong, McKenzie, and Sawyer (1967) and the C variant as described by Bell and McKenzie (1967). The proteins were isolated and stored as crystals, and not freeze-dried, in order to minimize the possibility of irreversible aggregation reactions.

### (b) *Methods*

#### (i) *Protein Concentrations*

These were determined by measuring the light absorption at 278 nm using the absorptivities determined for the A, B, and C variants by H. A. McKenzie, W. H. Sawyer, and G. B. Treacy (quoted in Bell and McKenzie 1967).

#### (ii) *pH Measurements*

pH measurements were made with a Leeds and Northrup pH-meter calibrated with phthalate and borate reference buffers as recommended by Bates (1964).

#### (iii) *Buffers*

The following buffers were used (ionic strength of each = 0.1):

pH 6.0: 0.02M cacodylic acid, 0.02M sodium cacodylate, 0.08M NaCl.

pH 7.5: 0.02M diethylbarbituric acid, 0.01M sodium diethylbarbiturate, 0.09M NaCl.

pH 8.2: 0.01M diethylbarbituric acid, 0.02M sodium diethylbarbiturate, 0.08M NaCl.

pH 8.8: 0.015M diethylbarbituric acid, 0.10M sodium diethylbarbiturate.

#### (iv) *Viscosity Measurements*

Viscosity measurements were carried out with Ostwald type capillary viscometers of small volume (2 ml) and of relatively large bore (c. 0.07 cm) and length. Two classes of viscometer were used, one giving a water flow time of c. 65 s at 25°C, the other a time of c. 60 s at 3°C. Results were expressed in terms of the reduced viscosity ( $\eta_{\text{red}}$ , in dl g<sup>-1</sup>).

#### (v) *Optical Rotatory Dispersion Measurements*

These were made with a Perkin Elmer model 141 photoelectric polarimeter. Specific rotations and the parameters  $a_0$  and  $b_0$  of the Moffitt and Yang (1956) equation were calculated from the experimental results as described by McKenzie, Sawyer, and Smith (1967).

#### (vi) *Sedimentation Experiments*

Sedimentation experiments were carried out with a Beckman Spinco analytical ultracentrifuge (model E) equipped with both Philpot-Svensson type schlieren and Raleigh-Philpot type interference optical systems, and with a rotor temperature indicator and control system. Cell centrepieces made of Kel-F polymer were used throughout. For *sedimentation velocity* experiments at pH 6.0, solutions were prepared immediately before each experiment by dissolving crystals of the protein directly into the buffer. For runs at pH 7.5, 8.2, and 8.8 a concentrated

solution of the protein was prepared in 0.1M NaCl and diluted with the appropriate buffer solution to give the desired protein concentration immediately before the commencement of the experiment. These procedures were employed in order to minimize the possible effects of time-dependent changes at the higher pH values. The experiments were performed at c. 20°C. Weight average sedimentation coefficients ( $\bar{s}$ ) were calculated from the schlieren patterns by the method of Goldberg (1953). Comparison of the sedimentation coefficients obtained in the various solvents was facilitated by converting them, by the method Svedberg and Pedersen (1940), to a standard basis corresponding to a weight average sedimentation coefficient ( $\bar{s}_{20,w}$ ) in water at 20°C. The partial specific volumes of the monomer and of the dimer of  $\beta$ -lactoglobulins A, B, and C were assumed to be the same and equal to the value determined for the A and B variants in 0.05M NaCl by McKenzie, Sawyer, and Smith (1967). *Sedimentation equilibrium* experiments were carried out at 20.5°C by the meniscus-depletion method of Yphantis (1964). The maximum time for attainment of equilibrium was calculated prior to the experiment by the Yphantis modifications of the equations of Svedberg and Pedersen (1940) and of Van Holde and Baldwin (1958). Making the extreme assumption that 100% monomer was present, the estimated maximum times of equilibrium for a speed of 40,000 r.p.m. were 10.5 and 10.4 hr by the respective equations. In practice, it was found that equilibrium was attained in less than 9 hr. Dilute solutions of the proteins (0.8–1.0 g l<sup>-1</sup>) were prepared in 0.1M NaCl and dialysed exhaustively against the pH 7.5 diethylbarbiturate buffer prior to the run. A six-channel cell with sapphire windows was filled in accordance with the procedure described by Yphantis (1964), except that Kel-F polymer oil was used as base fluid instead of FC-43 oil for the 0.3-cm column. Subsequent to these experiments Adams (1967) reported difficulty with precipitation of  $\beta$ -lactoglobulin in acetate buffer at pH 4.7 with FC-43 oil. We did not observe such an effect with Kel-F oil at pH 7.5. Adams and Fujita (1963) and Adams and Williams (1964) have shown that the following equation is valid for self-associating proteins at sedimentation equilibrium:

$$d \ln C / dr^2 = [(1 - \bar{v}\rho)\omega^2 \bar{M}_w^{app}]/2RT, \quad (1)$$

where  $C$  is the total concentration of all associating species,  $r$  is the radial distance,  $\omega$  is the angular velocity ( $2\pi \times$  r.p.s.),  $R$  is the universal gas constant,  $T$  is the Kelvin temperature,  $\rho$  is the density of solution,  $\bar{M}_w^{app}$  is the apparent weight average molecular weight at  $r$ , and  $\bar{v}$  is the partial specific volume.

In determining the weight average molecular weight ( $\bar{M}_w$ ) we have assumed that at the low concentrations used, the virial terms are negligible and that  $\bar{M}_w \approx \bar{M}_w^{app}$ . It was also assumed in treating the results that the refractive index of each species present is the same and that no volume change occurs in the association-dissociation reaction.

Two other parameters were calculated from the experimental results: the weight average apparent reduced molecular weight ( $\sigma_w^{app}$ ) and the number average apparent reduced molecular weight ( $\sigma_n^{app}$ ) (Yphantis 1964).

Making use of the procedure of McKenzie, Sawyer, and Smith (1967) the dissociation constant for the reaction was calculated from the  $\bar{M}_w$  values, by applying their equation (8). We justify the procedure and assumptions in Section IV.

#### (c) Calculation of Theoretical $\bar{s}$ versus $C$ Curves

Gilbert (1955) has made a theoretical treatment to predict the form of sedimentation patterns and the concentration dependence of sedimentation velocity for rapidly and reversibly associating systems. In order to simplify the treatment, effects of diffusion, cell shape, and concentration dependence of sedimentation coefficients of individual species were neglected. Subsequently Gilbert (1963) developed the theory to include the last effect. We have used this method (with minor modifications) to calculate  $\bar{s}$  versus  $C$  curves and compared them with our experimental curves. In the general case it is postulated that the interacting substance forms a series of polymers in reversible equilibrium, and which obey the general mass action equation

$$a_n = K_n a^n, \quad (2)$$

where  $a_n$  is the concentration of  $n$ -mer in moles l<sup>-1</sup> and  $K_n$  is the stoichiometric association constant for the reaction monomer  $\rightleftharpoons n$ -mer. It is necessary to express the sedimentation coefficient

( $s_n$ ) of the  $n$ -mer in terms of the total protein concentration ( $c$  in base moles  $l^{-1}$ ). Gilbert (1963) employed the equation

$$s_n = (s_n^0)(1 - gc),$$

but since the present measurements are concerned with pH values that are appreciably removed from the isoelectric point of the protein we have used the equation

$$s_n = s_n^0/(1 + gc), \quad (3)$$

where  $g$  is a constant. It can be shown that the weight average sedimentation coefficient of the whole boundary ( $\bar{s}$ ) is given by

$$\bar{s} = [1/(1 + gc)] \left[ \sum_{n=1}^{\infty} n a_n (s_n^0) \right] / c. \quad (4)$$

In the case of  $\beta$ -lactoglobulin near pH 7 we assume that only monomer and dimer are present; equations (2) and (4) then become:

$$a_2 = K_2 a_1^2. \quad (5)$$

$$\bar{s} = [1/(1 + gc)] [a_1 (s_1^0) + 2K_2 a_1^2 (s_2^0)] / c. \quad (6)$$

Making use of equation (2),  $a_1$ , the concentration of monomer in monomer moles  $l^{-1}$ , can be obtained from

$$2K_2 a_1^2 + a_1 - c = 0. \quad (7)$$

Hence  $\bar{s}$  can be calculated for given values of  $c$  and  $K$ .

It will be noted that  $g$  is in litres (base moles) $^{-1}$ . When we express the results in terms of the total weight concentration,  $C$ , in grams per litre, the constant,  $k$ , in the equivalent equation to (3) is expressed in the usual units of  $dl\ g^{-1}$ , where

$$g = kM_1/10. \quad (8)$$

### III. RESULTS

#### (a) Sedimentation Velocity Measurements, pH 6–9

If the dissociation of  $\beta$ -lactoglobulin were to be determined quantitatively it was first necessary for us to determine suitable experimental conditions. This was done by means of optical rotation and sedimentation velocity measurements. The optical rotatory dispersion (ORD) at 360–590 nm of the A, B, and C variants was determined over the pH range 2–9.2. The relevant curves for the pH dependence of the ORD parameters immediately after mixing have been presented in Figure 1 of McKenzie and Sawyer (1967). At first sight it might appear as if pH 8.5, where the ionization-linked transitions are complete, is a suitable pH for the sedimentation-equilibrium measurements. However, the time-dependent changes occurring at this pH precludes its use. On the other hand at pH values below 8, appreciable time-dependent changes in ORD do not occur at 20–25°C.

The effect of protein concentration on the sedimentation coefficients ( $s_{20,w}$ ) were determined at pH values of 6.0 and 7.5 at 20°C for the A variant and the relevant  $\bar{s}$  versus  $C$  curves are shown in Figure 1.

At all levels of concentration studied the sedimentation pattern exhibited a single peak with some asymmetry due to "trailing" on the solvent side. The same values of  $\bar{s}$  were obtained for a given protein concentration irrespective of whether the sedimentation experiment was commenced immediately after the protein was diluted with the relevant buffer, or the solution was mixed and then held for several hours before commencement of the run.

Measurements were also made immediately after mixing for the A variant at pH 8.2, and for the A, B, and C variants at pH 8.8. It can be seen that for the A

variant the  $\bar{s}$  versus  $C$  curves are displaced downwards with increasing pH. Also the decrease in  $\bar{s}$  is greater for A than for B and greater than B than for C at pH 8.8. When measurements were carried out following standing of the solutions it was found that there were slow increases with time in  $\bar{s}$ , the increase being greater at pH 8.8 than 8.2. Similar time-dependent effects were observed in electrophoresis (McKenzie and Sawyer 1966).

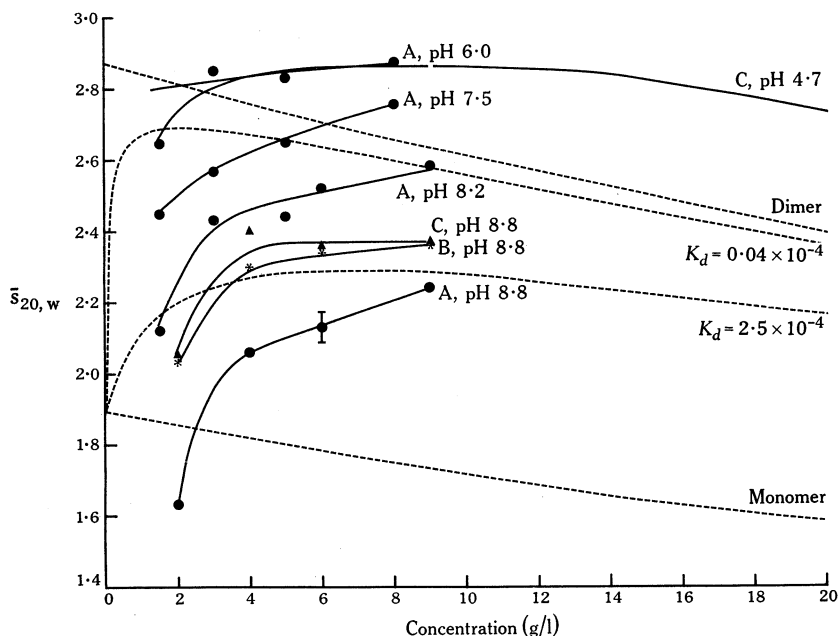


Fig. 1.—Relationship of weight average sedimentation coefficient  $\bar{s}_{20,w}$  (in Svedberg units) versus protein concentration for  $\beta$ -lactoglobulin variants. ● Experimental points for the A variant at pH 6.0, 7.5, 8.2, and 8.8. \*, ▲ Experimental points for the B and C variants at pH 8.8, respectively. For clarity an error bar, which has a typical value, is only shown for one experimental point. Likewise no experimental points are shown on the comparative curve (data of Bell and McKenzie 1967) for variant C at pH 4.7. --- Calculated curves for dimer, monomer, and dimer with dissociation constant  $K_d = 0.04 \times 10^{-4}$  and  $2.5 \times 10^{-4}$  mole  $l^{-1}$ .

On the basis of these observations and the theory of Gilbert (1963) it was concluded that bovine  $\beta$ -lactoglobulin exhibits some dissociation from dimer to monomer near pH 7, and that the level of dissociation appears to be pH-dependent and to differ for the A, B, and C variants. An appropriate pH to investigate the dissociation by sedimentation equilibrium is 7.5 and a protein concentration of  $1 \text{ g l}^{-1}$  should give a suitable concentration range for study by the high-speed method. Under these conditions dissociation should be appreciable and the time-dependent effects observed at higher pH absent.

#### (b) Sedimentation Equilibrium at pH 7.5

Some results for the sedimentation equilibrium of bovine  $\beta$ -lactoglobulin B at pH 7.5 are shown in Figures 2 and 3. A plot of fringe displacement ( $y - y_0$ ) versus radial distance ( $r$ ) is shown in Figure 2(a), and the points lie on a smooth curve. The

choice of  $y_0$  near the meniscus is critical in the meniscus-depletion method if precision is to be maintained for small net fringe displacements. The  $100\text{ }\mu\text{m}$  fringe displacement ( $\approx 0.3$  fringe or  $0.1\text{ g l}^{-1}$  protein), marked on Figure 2(a), is the minimum displacement at which Yphantis considers good precision can be attained. However, with considerable care in measurement we found that displacements as small as  $50\text{ }\mu\text{m}$  gave good precision. A typical plot of  $\ln(y - y_0)$  versus  $\frac{1}{2}r^2$  is shown in Figure 2(b). There is excellent agreement between the 9- and 10-hr measurements, indicating equilibrium had been attained. The upward curvature of the plot with increasing  $\frac{1}{2}r^2$ , is in agreement with the postulate that we are dealing with an associating system.

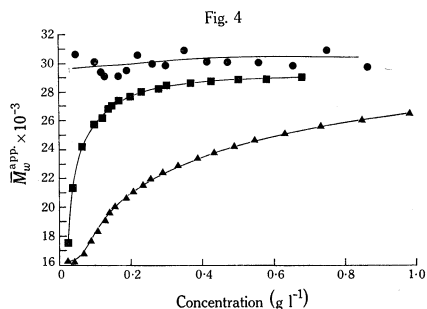
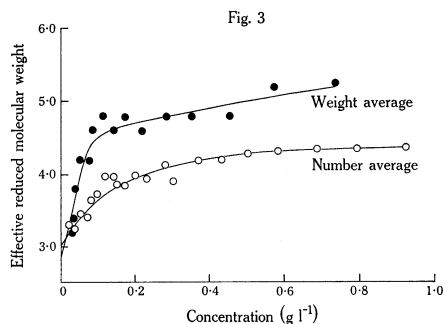
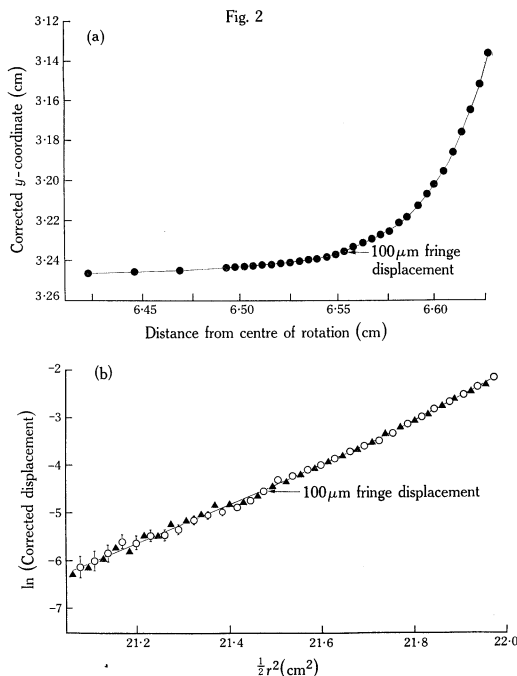


Fig. 2.—Sedimentation equilibrium of bovine  $\beta$ -lactoglobulin B at pH 7.5 (ionic strength =  $0.1$ , diethylbarbiturate buffer). The initial protein concentration was  $1.1\text{ g l}^{-1}$ . (a) Plot of the (blank corrected) interference fringe displacement versus the radial distance after attainment of equilibrium. (b) Natural logarithm of the fringe displacement versus  $\frac{1}{2}r^2$  (where  $r$  is the radial distance).  $\blacktriangle$  9-hr plate;  $\circ$  10-hr plate. The error bars represent an error in the fringe displacement of  $\pm 5\text{ }\mu\text{m}$ .

Fig. 3.—A plot of weight average apparent reduced molecular weight ( $\bullet$ ) and number average apparent reduced molecular weight ( $\circ$ ) versus concentration for the sedimentation equilibrium experiment on bovine  $\beta$ -lactoglobulin B of Figure 2.

Fig. 4.—Concentration dependence of the apparent weight average molecular weight  $\bar{M}_w^{\text{app}}$  of bovine  $\beta$ -lactoglobulins at pH 7.5.  $\blacktriangle$  A variant.  $\blacksquare$  B variant.  $\bullet$  C variant.

Values of  $\bar{M}_w^{\text{app}}$  versus  $C$  across the solution columns were calculated from the results and are shown in Figure 4, where they may be compared with results for similar experiments for the A and C variants. As the concentration decreased  $\bar{M}_w^{\text{app}}$  for the A and B variants decreased, approaching a value of  $17,000 \pm 1,500$  at zero

concentration. There appears to be no appreciable dissociation for the C variant over the range of concentration in which meaningful measurements could be made by the present method. The order of dissociation of the variants is  $A > B \gg C$ .

Values of  $\sigma_w^{\text{app.}}$  and  $\sigma_n^{\text{app.}}$  were obtained for the B variant and plotted against concentration to give an easier extrapolation to determine the apparent molecular weight at zero concentration (i.e. the molecular weight of the smallest species present). The results are shown in Figure 3, where the curves for  $\sigma_w^{\text{app.}}$  and  $\sigma_n^{\text{app.}}$  intersect near zero concentration ( $\sigma_w$  should equal  $\sigma_n$  at  $C = 0$ ) giving an extrapolated value for the molecular weight of 17,000. An estimate of the monomer molecular weight  $\sigma_m$  was made from the results at higher concentration using the approximation  $\sigma_m \approx 2\sigma_n - \sigma_w$ . A value of 17,500 was obtained at a concentration of  $0.3 \text{ g l}^{-1}$ .

TABLE 1  
COMPARISON OF DISSOCIATION CONSTANTS ( $K_d$ ) FOR BOVINE  $\beta$ -LACTOGLOBULINS AT pH 7.5 WITH VALUES AT OTHER pHs

Variant	pH	Ionic strength	Temp. (°C)	$10^4 K_d$ (mole $\text{l}^{-1}$ )	Method*	Reference
A	7.5	0.1	20.5	0.6	E	This paper
B	7.5	0.1	20.5	0.08	E	
C	7.5	0.1	20.5	<0.05	E	
A	6.9	0.1	20	0.21	E	Zimmerman, Barlow, and Klotz (1970)
B	6.9	0.1	20	0.07	E	
B	7.5	0.1	20	0.08	L	Georges, Guinand, and Tonnelat (1962)
AB	1.6	0.1	25	2.5	L	Townend, Weinberger, and Timasheff (1960)
AB	3.5	0.1	25	0.043	L	
A	2.7	0.1	25	1.3	L	Timasheff and Townend (1961)
B	2.7	0.1	25	0.5	L	
B	2.6	0.1	25	1.6	E	Albright and Williams (1968)

\* E, sedimentation equilibrium; L, light scattering.

Equilibrium constants for the association-dissociation of A and B variants were calculated from the experimental results. The method of McKenzie, Sawyer, and Smith (1967) was applied assuming the system was monomer-dimer ( $n = 2$ ), or monomer-trimer ( $n = 3$ , intermediates neglected). The plot of the right-hand side of their equation 8 gave a reasonably constant value (considering experimental error) over the concentration range  $0.2\text{--}0.9 \text{ g l}^{-1}$ , assuming  $n = 2$  for both A and B variants. There was a marked slope to the plots for  $n = 3$ . Thus it is considered that we are here dealing with a monomer-dimer reaction in the concentration range considered, and the values for the dissociation constants obtained are presented in Table 1. They are also compared with results of other workers for  $\beta$ -lactoglobulin dissociation near pH 7 and at low pH.

### (c) *Effects of Electrolyte and Methanol*

The effect of ionic strength and of methanol concentration on the behaviour of  $\beta$ -lactoglobulin at pH 7.5 was examined in the hope of gaining some insight into the forces binding the monomer units together. The A variant was chosen for this study.

The effect of addition of sodium chloride on the sedimentation coefficient is shown in Table 2. There is a small, but appreciable, increase in  $\bar{s}$  as the ionic strength is raised from 0.1 to 1.0 with sodium chloride.

TABLE 2  
EFFECT OF IONIC STRENGTH ON THE SEDIMENTATION COEFFICIENT OF  $\beta$ -LACTOGLOBULIN A AT pH 7.5

Ionic strength	Protein concn. (g l <sup>-1</sup> )	$\bar{s}_{20,w}$ (S)	$-\left[\alpha\right]_{578nm}$ (deg)
0.1	3.0	2.57	37.5
0.5	4.0	2.49	36.4
1.0	4.0	2.35	35.2

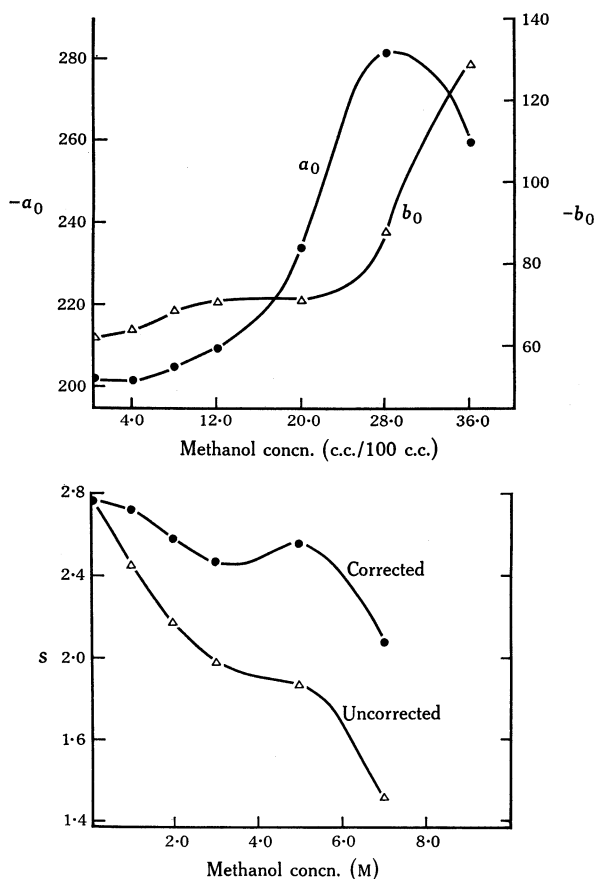


Fig. 5.—Effect of methanol on the optical rotatory dispersion parameters  $a_0$  and  $b_0$  of the Moffitt and Yang equation, and on the sedimentation coefficient  $\bar{s}$  (in Svedberg units) of bovine  $\beta$ -lactoglobulin A. The pH of the barbiturate buffer was 7.5 in the absence of added methanol. The values of  $s$  (corrected to 20°C) are shown both with and without correction for the viscosity and density of the solvent.

The effect of methanol concentration is shown in Figure 5 for a protein concentration of 8.0 g l<sup>-1</sup>. The values of  $s$ , with and without correction for viscosity and density of solvent, are shown to indicate the considerable correction necessary



to express the sedimentation coefficient correctly as  $s_{20,w}$ . Also shown are values of the  $a_0$  and  $b_0$  optical rotatory dispersion parameters. As the methanol concentration was increased to 2.0M,  $s_{\text{corr.}}$  decreased by 0.3 S,  $[\alpha]_{578\text{nm}}$  remaining virtually constant (within  $2^\circ$ ). Between 3.0 and 7.0M methanol there was a decrease of  $13^\circ$  in laevorotation. At 7.0M methanol a heavy component (sedimentation coefficient of 8 S) appeared in the sedimentation pattern. At 9.0M methanol all the protein was converted to the heavy form, the magnitude of  $[\alpha]_{578\text{nm}}$  decreasing by a further  $9^\circ$ . The values of  $a_0$  and  $b_0$  decreased with increasing methanol concentration above 3M. However,  $a_0$  reached a minimum at *c.* 7M methanol, thereafter increasing.

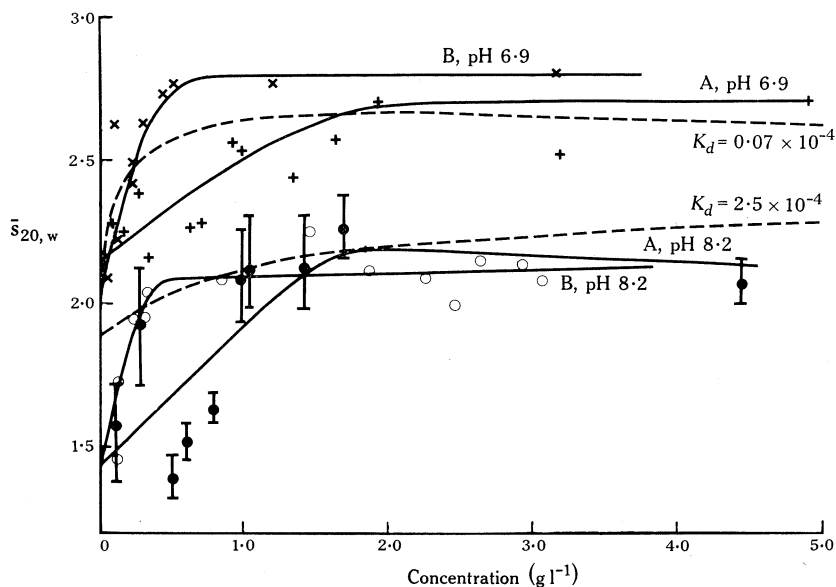


Fig. 6.—Comparison of the relationship of weight average sedimentation coefficient ( $\bar{s}_{20,w}$ ) and concentration of bovine  $\beta$ -lactoglobulins A and B at pH 6.9 and 8.2 as determined experimentally by Zimmerman, Barlow, and Klotz (1970) with theoretical curves calculated by us on the basis of Gilbert theory. For clarity, error bars for the experimental results are shown only on one set of data. Experimental points are identified as follows:  $\times$  B variant at pH 6.9;  $+$  A variant at pH 6.9;  $\circ$  B variant at pH 8.2;  $\bullet$  (with error bars) A variant at pH 8.2. --- Calculated for  $K_d = 0.07 \times 10^{-4}$  and  $2.5 \times 10^{-4}$  mole  $l^{-1}$ .

(d) *Theoretical Curves for Concentration Dependence of  $\bar{s}$*

We have calculated  $\bar{s}$  versus  $C$  curves by the procedure outlined in Section II(c) and compared the curves with our experimental results. In Figure 1, calculated curves are shown for the dimer, monomer, and for dimer-monomer systems for two values of the dissociation constant. We have assumed that

- (1)  $s_{20,w}^0$  for the monomer is 1.89 S [based on an extrapolated value from the data of Townend, Weinberger, and Timasheff (1960) for  $\beta$ -lactoglobulin and at pH 1.6];
- (2)  $s_{20,w}^0$  (dimer) =  $s_{20,w}^0$  (monomer)  $\times (2^3/1.044) = 1.52 s_{20,w}^0$ ;
- (3) the value of  $k$  is 0.1 dl  $g^{-1}$  [that of  $g$  then being given by equation (8)]—cf. Gilbert and Gilbert (1961).

The two values chosen for  $K_d$  were  $2.5 \times 10^{-4}$  and  $0.043 \times 10^{-4}$  mole l<sup>-1</sup>. These values were chosen as representing a probable range of values for  $K_d$  in the range pH 7.5–8.8.

Zimmerman, Barlow, and Klotz (1970) have recently published experimental curves of  $\bar{s}$  versus  $C$  for  $\beta$ -lactoglobulin A and B at pH 6.9 and 8.2. They supplemented schlieren measurements with ultraviolet absorption measurements, and hence were able to go to much lower concentrations than we have. Their results provide an unusually good opportunity to compare experimental with theoretical curves. We have calculated curves using their sedimentation equilibrium experimental value for  $K_d$  ( $= 0.07 \times 10^{-4}$  moles l<sup>-1</sup>) of the B variant at pH 6.9, and for  $K_d = 2.5 \times 10^{-4}$  moles l<sup>-1</sup> as representing an estimated value for pH 8.8 (where it is not possible to get sedimentation equilibrium data). These curves are shown in Figure 6.

#### IV. DISCUSSION

The meniscus-depletion method of Yphantis (1964) was chosen because it can be used at low concentrations, and absolute concentrations at any point in the solution column can be obtained directly (since  $C = 0$  near the meniscus) and difficult calibrations are avoided. However, in addition to the disadvantages considered by Yphantis it is necessary to give consideration to two potentially serious problems in the present application to associating-dissociating systems. While we have demonstrated that reasonable precision can be attained at low concentrations (fringe displacements) providing special care is taken in the experimental procedures and measurements, the overall precision is not high. This can be a serious disadvantage when one is trying to decide between definite and indefinite association for a given system since very high precision is then required (see also Van Holde, Rossetti, and Dyson 1969). A second serious problem may arise from the assumption made in treating the results that  $\bar{v}$  is the same for all forms of the protein present. If there is an appreciable change in volume ( $\Delta V$ ) in an association reaction, such as the monomer-dimer system considered here, there may be appreciable error in  $\bar{M}_w$  arising out of the high pressures developed in the cell at the high speeds characteristic of the meniscus-depletion method. This problem of pressure effects in ultracentrifugation has been long known (Svedberg and Pedersen 1940). From the good agreement between equilibrium constants determined by light scattering and sedimentation for  $\beta$ -lactoglobulin dissociation at low pH it seems that  $\Delta V$  is probably small for dissociation at higher pH. This is supported by the recent high and low speed equilibrium studies at pH 6.9 by Zimmerman, Barlow, and Klotz (1970).

In the treatment of our equilibrium data we have assumed that at pH 7.5 the second and higher virial coefficients are negligible for the low concentrations employed. Some justification for this comes from the reasonable constancy of the calculated  $K_d^{\text{app}}$  with concentration observed and the shape of the  $\bar{M}_w^{\text{app}}$  versus  $C$  curve observed (compare shape with theoretical curves for different values of  $B$  calculated for a hypothetical case of a monomer-dimer system by Adams 1967). After our work was carried out, a paper by Albright and Williams (1968) appeared in which a thorough analysis of the sedimentation equilibrium of bovine  $\beta$ -lacto-

globulin B at pH 2.6 was given. A surprising result from their work was that it was necessary to invoke both the second and third virial coefficient in order to account for the results over the wide concentration range studied (*c.* 0.5–20 g l<sup>-1</sup>). However, when one considers their results at the lower end of their concentration range which is still appreciably above ours, there is only need to invoke a single virial coefficient and even then the correction is not large. Considering the iso-ionic point of the protein is 5.2, it seems unlikely that the value of *B* would, at worst, be any greater at pH 7.5. Again this contention is supported by the recent studies of Zimmerman, Barlow, and Klotz (1970) who found *B* = 0 at pH 6.9.

Thus we consider that the values of  $\bar{M}_w$  and *K<sub>d</sub>* we have determined by the equilibrium method are reasonably accurate at low concentrations, and we make the following conclusions. The order of dissociation of the variants at pH 7.5 is *A* > *B*  $\gg$  *C*. The extent of dissociation of the *A* and *B* variants at pH 7.5 is comparable with these variants at pH 2.5 (see Table 1). The free energy changes ( $\Delta G$ ) for association of monomer to dimer for the *A* and *B* variants are of the order of 6 kcal mole<sup>-1</sup>. There is a small difference ( $\sim 0.5$  kcal mole<sup>-1</sup>) between  $\Delta G$  for the variants. This could arise from conformational differences between the variants, or because at least one of the amino acid residue substitutions is close to the point of contact between monomers. The relationship of this and the -SH group location are discussed elsewhere (McKenzie 1971).

Our experiments on the effect of electrolyte on the dissociation are in accord with the conclusion of Timasheff (1964) that the pH dependence of dissociation arises from electrostatic repulsion between the monomers and that the forces holding the units together may involve hydrophobic interactions. However, we could caution against their simple interpretation of the effects of methanol. The complications of aggregation and conformational change we observe in the presence of methanol clearly make difficult interpretation of the effect of organic solvents in relation to the nature of bonding between units. Before any measurements on solvent effects are interpreted, sedimentation measurements should be carried out to make sure that aggregation is not being induced by the solvent change.

On comparison of the theoretical and experimental  $\bar{s}$  versus *C* curves of Figures 1 and 6 it is concluded that, while there is qualitative agreement between calculated and theoretical curves, there is not particularly good quantitative agreement. As examples of this, the displacement of the experimental curves of Zimmerman, Barlow, and Klotz (1970) for the *A* and *B* variants at pH 6.9 with respect to one another and the fall in  $\bar{s}$  for *A* occurring at higher concentrations than that for *B* as the concentration is reduced, are as expected from the dissociation constants. However, there is not particularly satisfactory agreement in the contour or magnitude of the experimental *B* curve with the theoretical curve calculated for the relevant dissociation constant. The lack of agreement could arise from the inherent assumptions of the Gilbert method. However, there are other considerations that may be overriding. The precision of the experimental values is not high. Also the values assumed for  $\bar{s}$  for monomer and dimer in the theoretical treatment may not be valid. Furthermore, the value assumed for *k* of 0.1 dl g<sup>-1</sup> is possibly too high. The value calculated for the *C* variant from results (Bell and McKenzie 1967) at pH 4.7, running to higher concentration values than those shown in Figure 1, is 0.06 dl g<sup>-1</sup>.

While it can be argued that this value is due in part to association to  $n$ -mers  $> 2$ , it is of the same order as observed for comparable globular proteins (cf. Creeth and Knight 1965). It is obviously desirable to make further tests of this method for calculating  $\bar{s}$  versus  $C$  curves of dissociating systems, using more precise experimental results for both  $\beta$ -lactoglobulin and other proteins where dissociation occurs at low levels of protein concentration.

It will be noted from Figures 1 and 6 that there is considerable downward displacement of the  $\bar{s}$  versus  $C$  curves at higher pH values. The extrapolated values of  $\bar{s}$  at pH 8.2 and 8.8 both from our own results and those of Zimmerman, Barlow, and Klotz (1970) indicate values for  $\bar{s}^0$  (monomer) as low as 1.4 S as contrasted with values near pH 7 of 1.8–1.9 S which is near the value of 1.89 S for monomer used in the calculations and based on low pH data. This indicates a considerable change in frictional coefficient. That there is a change in hydrodynamic shape in this pH region (c.9) may be deduced from pH titration data of  $\beta$ -lactoglobulins (see the data of Ghosh *et al.* 1971; but cf. Tanford and Swanson 1957). On the other hand, we have measured the reduced viscosity of the A, B, and C variants in the concentration range 5–20 g l<sup>-1</sup> at pH 4.0 and 7.5 and observed little concentration or pH dependence,  $\eta_{\text{red}}$  being 0.035 dl g<sup>-1</sup> for each of the three variants. Unless there is a dramatic change in  $\eta_{\text{red}}$  at concentrations below 5 g l<sup>-1</sup>, it is difficult to reconcile the value of reduced viscosity with the extrapolated value for  $\bar{s}$  (monomer).

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