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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY,¹ PHILADELPHIA, PENNA.]**Molecular Interactions in β -Lactoglobulin. II. Ultracentrifugal and Electrophoretic Studies of the Association of β -Lactoglobulin below its Isoelectric Point²**

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An ultracentrifugal and electrophoretic study of the association of β -lactoglobulin between pH 3.5 and 5.2 has been carried out. Analysis of the data in terms of the Gilbert theory shows the aggregate to be greater than a dimer; trimer, tetramer and pentamer formations are compatible with the sedimentation data, with little or no intermediate components present. 90% of β -lactoglobulin A can associate, while β -lactoglobulin B does not form heavy aggregates when present by itself. 30% of β -lactoglobulin B can, however, form mixed aggregates with β -lactoglobulin A.

Introduction

In the previous paper³ it has been shown that the electrophoretic heterogeneity of β -lactoglobulin between pH 5.3 and 6.0 can be, to a large extent, explained in terms of the true molecular heterogeneity of that protein. Below this pH region, however, the observed heterogeneity has been attributed, at least in part, to the presence of intermolecular interactions,⁴ although part of the heterogeneity is the result of the presence of more than one protein species in β -lactoglobulin.⁵ As the result of electrophoretic and ultracentrifugal experiments, Ogston and Tilley⁴ concluded that, around pH 4.65, β -lactoglobulin can undergo a reversible temperature dependent association which is favored by low pH . It has been shown by the present authors⁶

that this association of β -lactoglobulin below its isoelectric point is restricted to the pH region between 3.5 and 5.2, with maximal association between pH 4.40 and 4.65. Furthermore, below this pH region, β -lactoglobulin is known to dissociate into smaller molecular units,⁷ this latter reaction becoming very prominent below pH 3.5. It is the purpose of this paper to present the results of a systematic ultracentrifugal and electrophoretic investigation of the association of β -lactoglobulin between pH 3.5 and 5.2. In order to avoid confusion, the term monomer will be used to designate the 36,000 molecular weight species. Heavier species will be referred to as aggregates.

Experimental

Materials.—The proteins used were preparations of β -lactoglobulin from pooled milk, given to us by Dr. W. G. Gordon, referred to as Preparation II,³ a sample of Polis " β_2 -enriched" protein^{3,8} given to us by Dr. T. L. McMeekin and samples of β -lactoglobulin A (β -A) and β -lactoglobulin B (β B)^{9,10} given to us by Dr. R. Aschaffenburg, as well as

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) This work was presented in part at the First Delaware Valley regional meeting of the American Chemical Society, Philadelphia, February 1956, at the 131st National Meeting of the American Chemical Society, Miami, April 1957, at the 132nd Meeting, New York, September 1957 and at the 133rd Meeting, San Francisco, April 1958.

(3) S. N. Timasheff and R. Townend, *THIS JOURNAL*, **82**, 3157 (1960).

(4) A. G. Ogston and J. M. A. Tilley, *Biochem. J.*, **59**, 644 (1955).

(5) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955).

(6) R. Townend and S. N. Timasheff, *Arch. Biochem. Biophys.*, **63**, 482 (1956).

(7) R. Townend and S. N. Timasheff, *THIS JOURNAL*, **79**, 3613 (1957).

(8) B. D. Polis, H. W. Schmukler, J. H. Custer and T. L. McMeekin, *ibid.*, **72**, 4965 (1950).

(9) R. Aschaffenburg and J. Drewry, *Nature*, **180**, 376 (1957).

(10) R. Aschaffenburg and J. Drewry, *Biochem. J.*, **65**, 273 (1957).

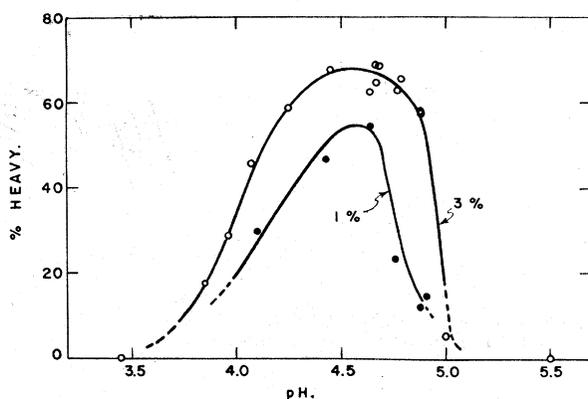


Fig. 1.—Ultracentrifugal composition of pooled β -lactoglobulin in the pH region below the isoelectric point, at 1–3° in 0.1 ionic strength acetate buffer.

several fractions¹¹ prepared by Dr. R. F. Peterson by the filter paper curtain electrophoresis technique.

Methods.—All ultracentrifuge experiments were carried out in a Spinco Model E analytical ultracentrifuge¹² at 59,780 r.p.m. using Kel-F cells. The ultracentrifuge was equipped with a phase-shift plate and in the later experiments, a temperature control unit. Electrophoretic measurements were done in a Perkin-Elmer electrophoresis apparatus, Model 38A¹³ at 0°. The ultracentrifugal and electrophoretic patterns were analyzed from enlarged projected tracings. In the case of ultracentrifugal patterns, the slowly sedimenting component was drawn by projecting the trailing edge of the boundary over the mid-point of the peak, the rapid component being obtained by subtraction of the area under the slow peak from the total area. In every case, two essentially symmetrical boundaries were obtained. The apparent compositions were not corrected for the Johnston and Ogston¹³ anomaly, since the theoretical treatment of a system in rapid equilibrium takes concentration effects into account directly.¹⁴ Sedimentation constants were measured with the aid of a micro-comparator. Concentrations were measured by ultraviolet absorption at 278 $m\mu$, using a value of 0.96 l./cm.g for the absorptivity. This was measured in pH 5.3 acetate buffer of 0.1 ionic strength. This value was determined on a sample whose concentration was established by drying at 105° to constant weight and checked by micro Kjeldahl nitrogen analysis. The absorptivities were found to be identical¹⁵ for the two genetic species of β -lactoglobulin. All pH 's were measured in a Beckman Model G¹² pH meter at 25°.

Results

The pH dependence of the amount of rapidly sedimenting material present in pooled milk β -lactoglobulin solutions (preparation II) is shown in Fig. 1. The experiments were carried out at 2–3° in 0.1 ionic strength acetate buffers. The protein concentrations were 10 and 25–32 g./l. As can be seen, little heavy component is present at pH 5.1, but as the pH is decreased, the degree of association increases rapidly down to pH 4.7. Below pH 4.40, the amount of rapidly sedimenting material decreases again until at pH 3.50 no more heavy material is observed in the ultracentrifuge. Between pH 4.40 and 4.65 the degree of association seems to be at a constant maximal value. Although above

(11) R. F. Peterson, unpublished experiments.

(12) Mention of specific firms and products does not imply endorsement by the Department to the possible detriment of others not mentioned.

(13) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 779 (1946).

(14) G. A. Gilbert, private communication.

(15) C. Tanford and Y. Nozaki, *J. Biol. Chem.*, **234**, 2874 (1959).

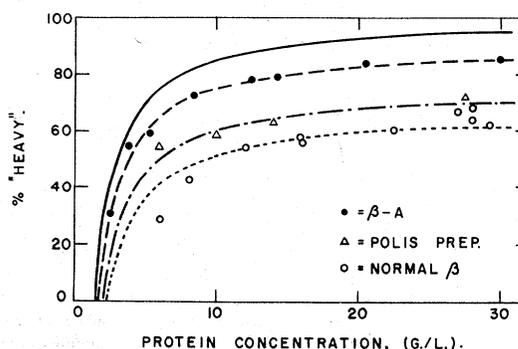


Fig. 2.—Ultracentrifugal composition of various preparations of β -lactoglobulin as a function of protein concentration at pH 4.65 in 0.1 ionic strength acetate buffer, 2°. Experimental points: ●, β -A; triangles, Polis " β_2 -enriched" prep.; ○, pooled β . Calculated curves; —, 100% aggregable material; - - -, 90% aggregable material; - · - ·, 76% aggregable material; · · · ·, 66% aggregable material.

pH 5.0, no analysis into components can be made, the ultracentrifugal patterns are more or less skewed and the sedimentation constant is above that of the unassociated protein until pH 5.30, as shown in Table I. Above pH 5.30, no association can be detected in the ultracentrifugal measurements, as the sedimentation constant remains normal even at high protein concentrations.

TABLE I
 pH DEPENDENCE OF SEDIMENTATION CONSTANTS OF POOLED β -LACTOGLOBULIN

pH	Temp., °C.	Prot. concn., g./l.	$s_{20,w} \times 10^{12}$	
			Slow	Fast
3.85	2.5	30.5	2.62, skewed forward	
4.07	2.6	28.8	2.5	4.29
4.40	3.0	25.8	3.3	4.83
4.64	3.1	29.1	3.1	5.01
4.88	2.5	29.4	2.9	4.29
5.02	2.0	30.0	3.12, skewed forward	
5.10	2.0	30.0	2.99	
5.20	2.0	30.0	2.83	
5.30	2.0	30.8	2.78	
5.49	2.0	30.0	2.80	

The concentration dependence of the amount of associated material present at pH 4.65 (1–3°) for pooled β -lactoglobulin, the Polis " β_2 -enriched" sample and β -lactoglobulin A is shown in Fig. 2. In all three cases, an initial rapid increase in the relative amounts of rapidly sedimenting material is observed up to a concentration of *ca.* 10 g./l., at which point more than 50% of the area in the ultracentrifugal diagrams is found under the rapidly sedimenting peak. At higher concentrations, a slow monotonic increase in heavy component can be observed with an increase in protein concentration. Typical ultracentrifugal patterns obtained at pH 4.65 and a series of concentrations are shown in Fig. 3.

The association of pooled β -lactoglobulin is strongly temperature dependent as is indicated in Fig. 4 for a protein concentration of 28–30 g./l. at pH 's 4.65, 4.90 and 4.10. The experiments were carried out in 0.1 ionic strength acetate buffers. At pH 4.65, there is no measurable heavy component

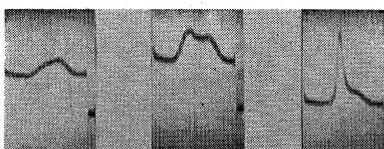


Fig. 3.—Sedimentation patterns of pooled β -lactoglobulin at various concentrations. pH 4.65, 0.1 ionic strength acetate buffer, 2.5°, 9,600 sec. after reading full speed. Sedimentation proceeds from right to left.

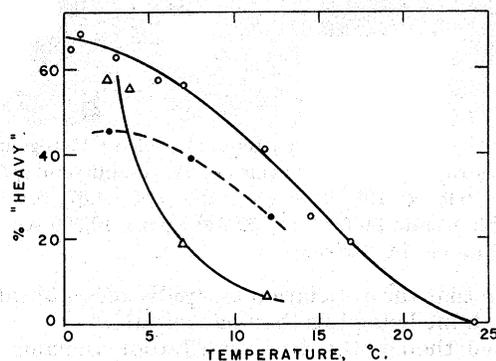


Fig. 4.—Ultracentrifugal composition of pooled β -lactoglobulin as a function of temperature. 28–30 g./l., 0.1 ionic strength acetate buffers. O, pH 4.65; ●, pH 4.10; Δ , pH 4.90.

present at 25°, but as the temperature is lowered, the degree of association increases rapidly, leveling off to a plateau below 4°. The data at pH 4.90 are subject to strong error, since, as shown on Fig. 1, this is in the region of a very steep dependence of the degree of association on the pH . In this pH region a deviation of pH of 0.02 units can result in a very serious difference in component distribution. Typical ultracentrifugal patterns obtained at pH 4.65 and a series of temperatures are shown in Fig. 5, where the gradual increase in heavy component with decrease in temperature can be seen. Above 20°, no heavy component can be seen in the ultracentrifugal patterns, nor is the sedimentation constant higher than that of the normal non-associated protein (Table II), indicating that any residual association could be only extremely weak at room temperature and above.

TABLE II

TEMPERATURE DEPENDENCE AT pH 4.65 OF SEDIMENTATION CONSTANTS OF POOLED β -LACTOGLOBULIN

Temp., °C.	Prot. concn., g./l.	$s_{20,w} \times 10^{13}$	
		Slow	Fast
1.0	27.7	3.3	5.00
3.1	29.1	3.1	5.01
11.9	29.6	3.2	5.10
14.4	29.6	2.4	3.92
16.9	29.6	2.80, highly skewed forward	
24.1	29.6	2.65, symmetrical	
30.0	31.4	2.80, symmetrical	
34.7	31.4	2.87, symmetrical	

The dependence of the sedimentation constant of each component on concentration is shown in Fig. 6. In the case of the slowly sedimenting material, the best line through the points obeys the equation

$$s_{20,w} = (3.04 - 0.0073C) \times 10^{-13}$$

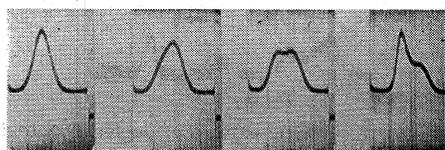


Fig. 5.—Sedimentation patterns of pooled β -lactoglobulin at various temperatures. pH 4.65, 0.1 ionic strength acetate buffer, 30 g./l.; 9,600 sec. after reaching full speed. Sedimentation proceeds from right to left: a, 25°; b, 15°; c, 8°; d, 2°.

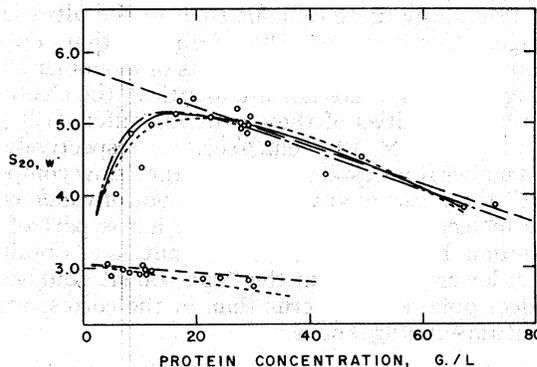


Fig. 6.—Concentration dependence of the sedimentation constants of the ultracentrifugal peaks of β -lactoglobulin in its zone of association. Circles represent the experimental points. ---, best lines of regression through experimental points (eq. 1 and 2). - - - -, calculated for pentamer; ———, calculated for tetramer; ······ (upper), calculated for trimer; ······ (lower), concentration dependence of $s_{20,w}$ of monomer when there is no aggregation.¹³

The slope is less than that reported by Johnston and Ogston¹³ for β -lactoglobulin under non-associating conditions. This results from the fact that, when aggregation is possible, the amount of slow-moving aggregable material is independent of total concentration (see Discussion, below). The observed decrease in $s_{20,w}$ of slow component with increasing total concentration is due only to increase in concentration of species incapable of aggregation.

The sedimentation constant of the rapidly sedimenting component first rises rapidly with an increase in concentration, then, above a concentration of 15 g./l., it is found to decrease in the usual manner of non-aggregating protein systems. Extrapolation to zero concentration from the high concentration portion of the curve yields a value of 5.77S for $s_{20,w}$. The best straight line of regression follows the equation

$$s_{20,w} = (5.77 - 0.027C) \times 10^{-13} \quad (2)$$

The ultracentrifugal composition of this system was compared with that obtained in electrophoresis, since Ogston and Tilley⁴ concluded that the two are related. For this purpose, a series of electrophoretic experiments on pooled β -lactoglobulin were carried out at pH 4.65 in 0.1 ionic strength acetate buffer. A typical pattern is shown in Fig. 7. On the descending side, the pattern can be divided into three components, having mobilities of 4.1, 1.8 and 1.2×10^{-5} cm.²/sec. v., at a protein concentration of 16 g./l., while on the rising side there is a hypersharp leading peak with a trailing shoulder of slightly lower apparent mobility. Anal-

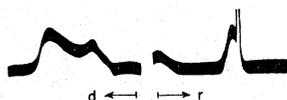


Fig. 7.—Electrophoretic pattern of pooled β -lactoglobulin in pH 4.65, 0.1 ionic strength acetate buffer. 12.8 g./l. protein, 8,000 sec. at 9.7 v./cm.

ysis of the descending peak into two components has given results in qualitative agreement with the ultracentrifugal data. In the case of electrophoresis, resolution into a slow and a rapid "component" is considerably more difficult than in the ultracentrifuge. Contrary to the sedimentation data, where the sedimentation constants of monomer and nonaggregating materials are identical, the electrophoretic mobilities of these proteins differ, (1.2×10^{-5} and 1.8×10^{-5} cm.²/sec. v., respectively). This makes it necessary to pool in the "slow component" these two migrating species, one of which is a true molecular entity, while the other is part of a "reaction boundary".¹⁶ As a result, one obtains much lower accuracy in the "component analysis" of electrophoretic patterns than in the corresponding ultracentrifugal ones.

Discussion

The results presented above show that β -lactoglobulin undergoes an extensive association in the pH region immediately below its isoelectric point. This association is strongly temperature dependent, being maximal at close to 0°, but still detectable at room temperature. In the isoelectric region little association occurs, although a weak association can be detected up to pH 5.2 from the value of the sedimentation constant. Above 5.2, no association can be detected in the ultracentrifuge. The association is also strongly pH dependent, with the maximal point being at pH 4.4–4.65, *i.e.*, away from the point of neutrality. Actually, in this pH range a 36,000 molecular weight unit of β -lactoglobulin carries a mean net charge of +7 to +10.¹⁷

Analysis of ultracentrifugal patterns into two components resulted in all cases in two symmetrical boundaries. No fraction of the total area was left between the two. The sedimentation constant of the slowly sedimenting material was found to be in agreement with values obtained at conditions of no association. This shows that the slow component truly represents non-associated β -lactoglobulin molecules. Analyses of patterns obtained at different times during a run revealed no significant change in the area distribution under the two peaks during the course of the run. Furthermore, runs carried out at different speeds to a constant value of $\omega^2 t$ resulted in identical relative area distributions between the two peaks independently of the run length, as shown in Fig. 8. Thus, the "component distribution" is stable with time and little area re-equilibration takes place during the course of a run. Therefore, area analyses taken after any duration of the run can be used in the comparative studies. Light scattering measurements, however, have

(16) L. G. Longworth, in M. Bier "Electrophoresis," Academic Press, Inc., New York, N. Y., 1959, p. 91.

(17) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

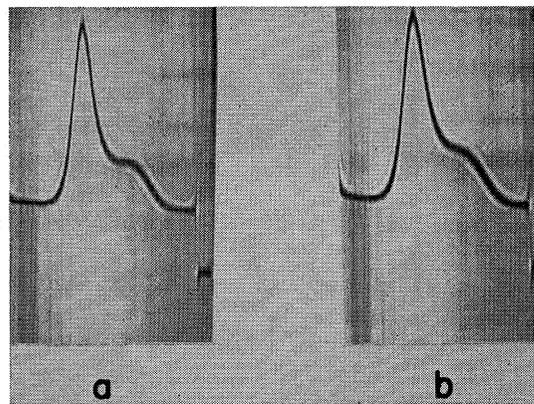


Fig. 8.—Sedimentation patterns of pooled β -lactoglobulin samples run for identical values of $\omega^2 t$ at different speeds. $\omega^2 t = 6.9 \times 10^9$, 27.8 g./l., 0.6°, pH 4.67, 0.1 ionic strength acetate buffer. (a) 59,780 r.p.m., 10,560 sec., (b) 52,340 r.p.m., 14,880 sec.

shown that the association is rapidly re-equilibrated, the half life being less than one minute.¹⁸

Even though the area distribution remains unchanged with time in the course of an ultracentrifugal run, the observed re-equilibration is rapid and can be considered as instantaneous with respect to the total duration of an ultracentrifuge run. Therefore, the theory of Gilbert^{19,20} is applicable to these data.

Although this theory was first presented in 1955, this study, to the best knowledge of the present authors, is its first quantitative application in a detailed investigation of an associating protein system. It appears desirable, therefore, to give a detailed presentation of the calculations involved and of the possible conclusions that can be reached using this theory.

Gilbert¹⁹ has analyzed the area distribution under a moving boundary for the case of the association of macromolecules when the rate of re-equilibration is very rapid with respect to the rate of boundary displacement. Diffusion and any effects due to pH and buffer component gradients in the system are neglected. For the reaction $nM \rightleftharpoons A$, if the aggregate moves faster than the monomer, the area distribution under the peak is found to be bimodal when the aggregated species is larger than a dimer. However, the area distribution under the peaks does *not* correspond to the stoichiometric proportions of monomer and aggregate. In the case when the aggregate is a dimer, the system should migrate as a single peak with a velocity intermediate between those of the monomer and dimer, the exact value of the velocity being determined by the equilibrium constant and the protein concentration. According to Gilbert, the dissociation equilibrium constant, K_G , is given by

$$K_G = \frac{M^n}{M} = \frac{n(1-\delta)}{\delta} \frac{C^{n-1}}{\left(1 + \frac{1}{n} \frac{\delta}{1-\delta}\right)^{n-1}} \quad (3)$$

where C is the protein concentration by weight (grams per liter in the present study) at any level

(18) A description of these results will be presented in the next paper of this series.

(19) G. A. Gilbert, *Discussions Faraday Soc.*, No. 2, 68 (1955).

(20) G. A. Gilbert, *Proc. Roy. Soc. (London)*, **A250**, 377 (1959).

x in the boundary. For a system with given values of K_G , n and C , the parameter δ is defined uniquely by equation 3. In Gilbert's treatment the aggregate is considered to be displaced with respect to a stationary monomer. In the usual procedures of ultracentrifugal and electrophoretic analyses, the species velocities are related by

$$v_A = [x/t + v_M(\delta - 1)]/\delta \quad (4)$$

where v_A and v_M are the actual velocities of the aggregate and monomer, respectively, and x is the distance of displacement of a given amount of protein in time t . Rearranging equation 4 and introducing the definition of the sedimentation constant s , we obtain

$$s_R = s_M + (s_A - s_M)\delta \quad (5)$$

where s_A and s_M are the sedimentation constants of aggregate and monomer, respectively, extrapolated to zero protein concentration. The sedimentation constant of the observed rapid peak, s_R ,²¹ for a system of given n and K_G is then defined by giving either δ or the corresponding value of C (eq. 3). δ varies between zero at $s_R = s_M$ (zero concentration) and unity at $s_R = s_A$ (infinite concentration). For a given degree of aggregation n , the minimum in the valley between the two maxima lies at

$$\delta_{\min} = \frac{n - 2}{3n - 1} \quad (6)$$

and has the same value independently of changes in protein concentration. Since δ at any given concentration is determined by eq. 3, it can be seen that below a total protein concentration corresponding to $\delta = \delta_{\min}$, only one peak will appear in the moving boundary system. As the concentration is increased, a second more rapid peak will appear, the area under the slow peak remaining constant. At twice the protein concentration which corresponds to δ_{\min} , in eq. 3, half of the area will lie on either side of the minimum. With a knowledge of the concentration at which the areas under the two peaks are identical, it becomes possible to calculate the area distribution over the entire concentration range, using the equation

$$\% \text{ Rapid component} = \frac{\text{Total area} - \frac{1}{2} \text{ area having 50:50 distribution}}{\text{Total area}} \times 100 \quad (7)$$

This equation is general and is independent both of n and of K_G .

When the data on β -lactoglobulin were examined in terms of this theory, it became obvious from the presence of the bimodal area distribution in the ultracentrifuge that the aggregate was larger than the dimer which had been postulated previously.^{4,6,22} The value of the sedimentation constant of the aggregate species, however, does not make it very likely that it is any greater than a pentamer.

Using eq. 7, the theoretical area distributions as a function of protein concentration were calculated

(21) An equation identical in form with equation 5 can be obtained for electrophoretic mobilities in the descending limb. This applies only in cases where the mobility of the aggregate is greater than that of the monomer.

(22) H. Klostergaard and R. A. Pasternak, *THIS JOURNAL*, **79**, 5671 (1957)

for the various β -lactoglobulins used in this study. In these calculations it was considered that β -A consists of two components, a major one which associates (90% of the total) and a minor one (10%) which does not. This is in agreement with Tombs²³ who has demonstrated the heterogeneity of β -A by solubility methods and has shown that electrophoretic distribution of areas of β -A at pH 4.65 is related to the presence of two such components. Calculations for β -A are shown in Table III.

TABLE III
GILBERT THEORY CALCULATION OF AREA UNDER SLOW PEAK FOR AGGREGABLE SPECIES OF β -LACTOGLOBULIN

Concn. aggregable, g./l.	% Rapid, obsd.	Concn. of slow, reactive, g./l.
2.25	31.0	1.47
3.37	54.5	1.33
4.77	59.0	1.64
7.65	72.0	1.53
11.2	78.0	1.53
12.9	79.0	1.6
18.4	84.0	1.2
27.0	85.0	1.5

Av. 1.48 \pm 0.14

Protein concentrations ranged from 2.5 to 30.3 g. per liter. The first column gives the concentration of material which can aggregate (90% of total concn.) and second column is the percentage of the area under the rapid "component." The product of the total concn. and the value in this column is then subtracted from the concn. of aggregable material (col. 1) and the difference, shown in the third column, is the concentration of protein on the slow side of the minimum in the pattern, *i.e.*, above δ_{\min} in the ultracentrifuge cell. The fact that this value is constant, 1.48 \pm 0.14 g./l. over the concentration range used, can be used as a further criterion for establishing that this is an associating system in rapid equilibrium.¹⁹ Using then 2.96 g./l. as the concentration at which the areas under the two peaks are identical, the theoretical curve for a 100% associable protein was calculated from eq. 7. This is shown by the solid line of Fig. 2. The dashed line is the theoretical curve for β -A derived from Table III and the solid line. This is done by adding 10% of the total concentration to the value of column 3 and considering this to be the area under the slow "component." As can be seen, the agreement with the experimental points is very good.

Considering that the concentration dependence of the area distribution of the aggregable species is identical for all preparations and is given by the solid line of Fig. 2, the data obtained with the Polis " β_2 -enriched" preparation and the pooled β -lactoglobulin (Prep. II) were found to be fitted best by composition distributions of 76% aggregable component and 66% aggregable component, respectively. Electrophoretic analysis at pH 5.3 and 5.6 has shown that the Polis " β_2 -enriched" preparation contains 77% of β -A, while Prep. II contains 59% of β -A.³ However, 10% of β -A cannot associate, and in each case the calculated amount of material capable of aggregating exceeds the amount of asso-

(23) M. P. Tombs, *Biochem. J.* **67**, 517 (1957).

ciable protein available from β -A. Thus, part of the protein found under the rapidly sedimenting boundary must originate from β -B. If it is assumed that the difference between the total aggregation observed and the aggregable (90%) amount of β -A present is due to association of β -B, it can be calculated that, in the case of the Prep. II protein, 32% of β -B can enter into the association, while in the Polis " β_2 -enriched" protein, 28% of the β -B should be able to react. These values would seem to indicate that *ca.* 30% of β -B can participate in the association reaction. It has to be pointed out that the uncertainty of this last number is rather large, since it is a derived quantity, two stages removed from the experimental measurements.

As a further check on the above area and composition analyses and the assumptions on the participation of portions of β -A and β -B in the association, ultracentrifugal measurements were carried out at pH 4.65 ($\Gamma/2 = 0.1$ acetate, 2°) on synthetic mixtures of β -A and β -B, as well as on some fractions enriched with respect to either one of the two components, which were prepared by the paper curtain electrophoresis technique.^{11,24} Theoretical area distributions were calculated for these systems and are compared with the experimental data in Table IV. In these calculations it was

TABLE IV
AREA DISTRIBUTIONS OF VARIOUS PREPARATIONS OF β -LACTOGLOBULIN

Preparation	% β -B	Prot. concn., g./l.	% Rapid component Calcd.	Obsd.
Synthetic mixture 1	50	20.0	53.0	56.7
Synthetic mixture 2	85	16.4	30.4	20 \pm 10
Pooled- β	41	12.0	54.0	54.4
" β_2 -enriched"	23	12.0	62.5	62.0
Fraction 1	85	11.9	26.3	29.0
Fraction 2	45	8.4	52.4	48.2
Fraction 3	27	12.1	61.2	57.5
Fraction 4	18	11.5	64.4	60.7

considered that 90% of β -A and 30% of β -B can associate, with the concentration dependence of the area distribution for the pure aggregable material shown in Table III and by the solid line of Fig. 2. The composition analysis of the fractions was carried out electrophoretically as described in the previous paper.³ The area distributions, calculated by eq. 7, are given in column 4 of Table IV. Comparison with the experimental data given in column 5 of Table IV shows reasonable agreement suggesting that the considerations on the aggregability of β -A and β -B are correct. In the mixtures high in β -B content, the "composition analysis" becomes quite difficult due to the fact that the rapid component is evidenced only by a strong skewing of the sedimenting boundary, with a resulting low precision of area analysis. In the case of pure β -B, no resolution into ultracentrifugal components takes place,²⁶ nor does its sedimentation constant deviate significantly from that observed at conditions where no association takes place. It would seem

then that β -B by itself either cannot associate or undergoes only a weak dimerization but can interact with β -A to form higher aggregates.

A Gilbert theory analysis of the electrophoretic data at pH 4.65 on pooled β -lactoglobulin resulted in reasonable agreement with the sedimentation experiments. This would again indicate a close correspondence between the electrophoretic and ultracentrifugal "components" at this pH . Thus, the electrophoretically rapidly migrating material on the descending side represents the aggregated species while the slow component is the monomer. The presence of a hypersharp leading boundary, followed by a small shoulder on the rising side, shows the presence of one aggregating component and of some slower inert protein.¹⁴

These composition analyses are in agreement with Ogston and Tombs²⁶ who, in analyzing their electrophoretic data, came to the conclusion that β -A can associate to a much stronger extent than β -B. These authors, however, had no direct evidence for changes in mass. It should be pointed out that both species are heterogeneous in electrophoresis at pH 4.65 but that the shapes of the patterns are completely different,²⁶ so that it is difficult to mutually identify the electrophoretic components of the two proteins at that pH , in 0.1 ionic strength acetate buffer. Furthermore, conclusions on aggregation reached from electrophoretic data alone are somewhat risky, since similar appearing anomalous patterns can be obtained also from other types of interactions,²⁷ *e.g.*, that of protein with buffer components.²⁸ Quantitative agreement of electrophoretic and ultracentrifugal measurements, on the other hand, is difficult to regard as being altogether fortuitous.

Klostergaard and Pasternak²² have reported on some ultracentrifugal and electrophoretic experiments on β -lactoglobulins A and B in a pH 4.8, 0.1 ionic strength acetate buffer. These authors have reported that β -A does not associate at all, while β -B associates strongly. Their electrophoretic patterns for the two β -lactoglobulins are identical with those presented by Ogston and Tombs²⁶ and by us.²⁶ They have concluded that the pattern obtained with β -A is the result of some phenomena other than association. However, their observation that the area under the slow peak in the descending boundary does not change with concentration is strong evidence in favor of association¹⁹ and suggests that the conclusion of these authors is erroneous. Their lack of observation of association with β -A in the ultracentrifuge is in full disagreement with our findings and with the suggestion of Ogston and Tombs.²⁶ This is probably attributable to the fact that Klostergaard and Pasternak worked at 8° , a temperature above the optimum for association (see Fig. 4) and in the pH region where the pH dependence of association is very steep (see Fig. 1). Their report of strong association of β -B is puzzling in view of the above-described results, our findings with this protein obtained from the milk of twelve

(24) W. Grassmann and K. Hannig, *Naturwissenschaften*, **37**, 397 (1950).

(25) R. Townend and S. N. Timasheff, *THIS JOURNAL*, **80**, 4433 (1958).

(26) A. G. Ogston and M. P. Tombs, *Biochem. J.*, **66**, 399 (1957).

(27) See, for example, R. A. Brown and S. N. Timasheff, in M. Bier "Electrophoresis," Academic Press, Inc., New York, N. Y., 1959, p. 317.

(28) J. R. Cann, *THIS JOURNAL*, **80**, 4263 (1958).

different cows,²⁵ and the conclusions of Ogston and Tombs.²⁶ Furthermore, ultracentrifuge experiments carried out by us with β -B between pH 4.1 and 4.8 at 2° have failed to show any appearance of heavy component. These will be described in detail later.

Having established the extent to which the different β -lactoglobulin species participate in the association, an attempt was made to obtain information on the stoichiometry of the reaction.

From the data of Fig. 2 and Table III, it is found that the point of equal area distribution in the curve for 100% aggregable material is at a concentration of 2.96 g./l. Using this information, dissociation constants were calculated for $n = 3, 4$ and 5, with the help of equations 3 and 6. The values of K_G obtained were $30 \text{ g.}^2 \text{ l.}^{-2}$, $40 \text{ g.}^3 \text{ l.}^{-3}$ and $60 \text{ g.}^4 \text{ l.}^{-4}$, respectively.

From these values of the equilibrium constants, the extrapolated values of $s_{20,w}$ for the monomer and polymer and eq. 3 and 5, the concentration dependence of the sedimentation constant of the aggregate could be calculated, assuming the aggregate to be in turn a trimer, tetramer or pentamer.

In the Gilbert theory, the value of δ and therefore of s is calculated for the leading edge of a peak which does not diffuse. In order to compare with experimental data where $s_{20,w}$ is measured at the median of a diffusing rapidly sedimenting peak, the calculation must be made for the concentration of protein present in the ultracentrifuge cell above the median. Thus, δ must be calculated for a concentration which corresponds to the sum of the area of the slow peak plus half the area of the rapid peak. This is compared then directly with the experimental $s_{20,w}$ of the rapid peak at a total protein concentration equal to the sum of the slow and the rapid peaks.²⁹

The calculation is carried out by successive approximations. First, using the experimental values of s_M (in the present case 3.04 S) and the extrapolated value of the linear portion of the experimental $s_{20,w}$ vs. concentration curve for the rapid peak, taken as s_A apparent (in this case 5.77 S), s_R apparent is calculated according to eq. 5 for various values of δ , related to the concentration by the given values of n and K_G (eq. 3). This curve, which describes only the equilibrium, is corrected then for the usual hydrodynamic effect by adding to its $s_{20,w}$ values the product of the total protein concentration at any given point with the slope of the linear portion of the experimental curve (in the present case $-0.027C \times 10^{-13}$). This gives a first approximation curve which falls below the experimental points. The difference between this curve and the experimental points at the maximum value of $s_{20,w}$ is the amount by which s_A apparent must be increased. The resulting difference in the linear portion at higher concentrations indicates the necessary change in the value of the slope. Repetition of the calculations, each time using the new values of s_A apparent and of the slope, yields final values of s_A and the slope which, together with equations 3 and 5, fully describe the data.

(29) We wish to thank Dr. G. A. Gilbert for pointing out this relation between calculated δ and the actual protein concentration.

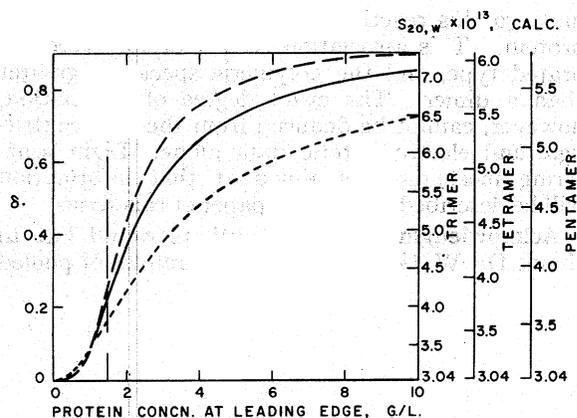


Fig. 9.—Dependence of sedimentation constant and the parameter δ on protein concentration for the association of the aggregable component of β -lactoglobulin. — — —, pentamer; ———, tetramer; ·····, trimer.

In this way it was possible to obtain the theoretical curves for $n = 3, 4$ and 5 and the corresponding K_G 's. The resulting sedimentation constant distribution with protein concentration, describing equilibrium only, is given in Fig. 9. In all three cases, a point of inflection appears at 1.5 g./l., indicating the first appearance of the faster peak at that protein concentration. From these curves, the area distribution under the ultracentrifugal pattern can be calculated for any protein concentration, by plotting the slope of the concentration vs. δ curve as a function of δ . It can be seen from Fig. 9 that the patterns are additive, *i.e.* increasing the protein concentration results in the addition of the new area to the rapid end of the sedimenting pattern. In practice, this is found to be the case within the approximation of neglect of diffusion and salt and pH gradient effects.

The hydrodynamic effect in the three cases, which is superimposed on the $s_{20,w}$ values of Fig. 9 is given by the calculated lines of regression

$$\begin{aligned} n = 3 \quad s_R &= (7.80 - 0.50C) \times 10^{-13} \\ n = 4 \quad s_R &= (6.40 - 0.34C) \times 10^{-13} \\ n = 5 \quad s_R &= (6.10 - 0.032C) \times 10^{-13} \end{aligned} \quad (2a)$$

Using the data of Fig. 9, and eq. 2a, the curves shown on Fig. 6 were calculated. All three curves are in good agreement with the experimentally obtained concentration dependence of the sedimentation constant of the rapid peak, and thus it is impossible to select between the three values of n according to this criterion.

The values of s_A given in eq. 2a result in f/f_0 values of 1.1, 1.4 and 1.8 for $n = 3, 4$ and 5, respectively. These would correspond to aggregates best described as a very compact unit of low hydration for the trimer, a linear end to end aggregate of high hydration for the pentamer and an intermediate case for the tetramer. From these considerations alone, however, it is not possible to establish the stoichiometry of the reaction.

The data and considerations presented above yield a complex picture of the association of β -lactoglobulin in the pH region below its isoelectric point. It appears that *ca.* 90% of β -lactoglobulin A and 30% of β -lactoglobulin B can associate; however, it is not clear whether β -lactoglobulin B can

undergo this reaction in the absence of the other protein. This association is of a rapidly reëquibrated type, and the polymeric species is greater than a dimer. The exact degree of association, however, cannot be deduced from the ultracentrifugal and electrophoretic data alone. Light scattering measurements aimed at that information will be described in the next paper of this series.

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