Molecular Interactions in β-Lactoglobulin. V. The Association of the Genetic Species of β-Lactoglobulin below the Isoelectric Point[2]

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The association reactions of the genetic species of β-lactoglobulins A and B have been studied between pH 3.7 and 5.2. Ultracentrifugal, electrophoretic and light scattering measurements have shown that 90% of β-A can tetramerize in this pH range, the maximum of the reaction being between pH 4.40–4.65, while β-B cannot form aggregates greater than a dimer. In mixtures of the two genetic species, mixed tetramers are formed. Previously reported equilibrium constants for this reaction were confirmed. It was shown by direct fractionation that 10% of β-A is inert toward tetramerization.

Introduction

In previous communications[3-6] it has been reported that β-lactoglobulin undergoes a reversible aggregation at close to 0° in the pH region between 3.7 and 5.2, with a maximum at pH 4.40–4.65. From an analysis of ultracentrifugal, electrophoretic and light scattering data obtained with preparations differing in their contents of the genetic species of this protein,[7] it was concluded that this association proceeds to the stage of tetramer.[6] These results were consistent furthermore with the concept that 90% of β-lactoglobulin A (β-A) can tetramerize, that β-lactoglobulin B (β-B) itself does not yield a tetramer and that in the presence of β-A, approximately 30% of β-B can enter into the tetramerization reaction forming mixed aggregates. It is the purpose of this paper to present a verification of these conclusions from experiments carried out with the separate genetic species of this protein.

Experimental

Material.—The genetically different β-lactoglobulins used in this study were samples kindly given us by Dr. Aschaffenburg, as well as material prepared by us from the milk of individual cows.

Before the preparation of β-A and β-B, the milk of a number of cows of various breeds was screened by the paper electrophoresis technique of Aschaffenburg.[7,8] The screening study was carried out in collaboration with members of the Dairy Cattle Research Branch, U. S. Department of Agriculture, and has been reported elsewhere.[9]

The β-A and β-B were prepared from the milk of individual cows mainly by the method of Aschaffenburg.[8] Since this method includes a step in which the protein is exposed to pH 2, at which it is known to dissociate into molecular subunits,[10] this method was compared with that of Palmer[11] which involves no such step. For this comparison milk from a single milking was divided into two portions and β-lactoglobulin was prepared from each portion by one of the techniques. The proteins obtained were found to crystallize with equal facility. Comparison of their ultracentrifu-

gal patterns at pH 2.0 (25°), 4.65 (2°) and 5.3 (25°) and their electrophoretic patterns at pH 4.65, 5.3 and 5.6 revealed no difference between the two preparations either in β-A or β-B. This was taken as evidence that protein prepared by the Aschaffenburg procedure is not altered.

Methods.—The ultracentrifugal experiments were carried out in a Spinco Model E[12] analytical ultracentrifuge at 59,780 r.p.m., using Kel-F cells. The ultracentrifuge was equipped with a temperature control unit and a phase plate. Most electrophoretic experiments were performed in a Perkin–Elmer Model 38-A[12] apparatus at ice temperature. In the study of the β-A subfractions, the electrophoretic experiments were performed on a Spinco Model H electrophoresis–diffusion apparatus[12] at 1.5°, using an 11 ml. cell. Area analyses were carried out planimetrically from enlarged projected tracings. Electrophoretic mobilities and sedimentation constants were measured with the help of a microcomparator.

Light scattering measurements were performed in the Brice photometer[13] equipped with 2 mm. slit optics; operations were carried out according to the modified Dintzis technique,[14] as described previously.[6]

All pH's were measured at 25°, using a Beckmann Model G pH meter.[12] Protein concentrations were determined spectrophotometrically at 278 mμ using a value of 0.96 liter g.⁻¹-cm.⁻¹ for the absorptivity[5] of both genetic species which are known to have identical absorptivity values.[15]

Fractionation of β-A.—β-Lactoglobulin A was dissolved in a 0.1 N NaCl solution at 25°. This solution was adjusted to pH 2.0 with N HCl and kept two hours at that pH. The pH was then adjusted to 5.10 with N NaOH and the solution dialyzed against several changes of distilled water according to the usual β-lactoglobulin recrystallization procedure. After two days, the heavy oil which came out of solution was separated by centrifugation and decantation of the supernatant. Both fractions were then seeded with a crystal of β-A. A heavy crystalline precipitate came rapidly out of the supernatant, while the oil slowly turned to a crystalline mass. Approximately two-thirds of the protein was found in the oil, the remainder in the supernatant fraction.

Analysis of the Data.—All ultracentrifugal, electrophoretic and light scattering data were analyzed by methods described in papers II[5] and III[6] of this series. Theoretical light scattering curves and "component" distributions under ultracentrifugal and electrophoretic "reaction boundaries"[16] were calculated using equilibrium constants[17] previously

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

(2) This work was presented in part at the 133rd National Meeting of the American Chemical Society, San Francisco, April 1958, and the 135th Meeting, Boston, April 1959.

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

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

(5) R. Townend, R. J. Winterbottom and S. N. Timasheff, *ibid.*, **82**, 3161 (1960).

(6) R. Townend and S. N. Timasheff, *ibid.*, **82**, 3168 (1960).

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

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

(9) R. D. Plowman, R. Townend, C. A. Kiddy and S. N. Timasheff, *J. Dairy Sci.*, **42**, 922 (1959) (Abst.).

(10) (a) R. Townend and S. N. Timasheff, THIS JOURNAL, **79**, 3613 (1957). (b) R. Townend, L. Weinberger and S. N. Timasheff, *ibid.*, **82**, 3175 (1960).

(11) A. H. Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(12) Mention of a specific commercial product does not constitute an endorsement by the United States Department of Agriculture.

(13) B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950).

(14) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, THIS JOURNAL, **79**, 782 (1957).

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

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

(17) For the sake of clarity, the previously deduced values of the equilibrium constants[5] for the reaction $4\beta \rightleftarrows \beta_4$ are presented:

$$2.0°: \begin{cases} pH: 3.90 & 4.14 & 4.40 \\ K_a: 1.6 \times 10^9 & 2.1 \times 10^{11} & 6.9 \times 10^{11} \end{cases}$$

$$2.0°: \begin{cases} pH: 4.65 & 4.90 & 5.10 \\ K_a: 5.5 \times 10^{11} & 2.2 \times 10^{10} & 3.4 \times 10^8 \end{cases}$$

$$pH\ 4.65: \begin{cases} T,\ °C.: 2.0 & 4.5 & 8.0 \\ K_a: 5.5 \times 10^{11} & 2.0 \times 10^{11} & 4.5 \times 10^{10} \end{cases}$$

$$pH\ 4.65: \begin{cases} T,\ °C.\ 15.0 & 25.0 \\ K_a: 6.0 \times 10^9 & 4.5 \times 10^8 \end{cases}$$

deduced from light scattering measurements on β-A and β-B mixtures of various compositions.[6,18] It was considered that only 90% of β-A can tetramerize. The second virial coefficient at pH 4.65 was taken as zero.[6]

Results and Discussion

Stoichiometry of the Aggregation.—Ultracentrifugal analyses of β-A and β-B were carried out at 2° between pH 3.7 and 5.2 in 0.1 ionic strength acetate buffers. In this pH range, β-A gives a bimodal sedimentation distribution similar to that previously reported for the preparation containing both species,[3,5,19] while β-B gives only a single peak over the entire pH range even at concentrations as high as 90 g./l. The pH dependence of the "component" distribution under the β-A "reaction boundary" is shown in Fig. 1. Like pooled β-lactoglobulin, β-A gives maximal aggregation at pH 4.40-4.65; however, β-A shows the greater percentage of "heavy component" over the entire pH range concerned.

The concentration dependence of the fraction of total area present under the rapidly sedimenting peak has been reported previously. It was found to be consistent with the concept that only 90% of the protein can aggregate. In a similar way, electrophoretic experiments on β-A were carried out as a function of protein concentration at pH 4.65. The results, presented in Table I, show that, as the protein concentration increases, the amount of "fast component" increases.

TABLE I

ASSOCIATION OF β-LACTOGLOBULIN A.[a] AREA DISTRIBUTION
IN ELECTROPHORESIS IN pH 4.65 ACETATE OF 0.1 IONIC
STRENGTH, 1°

Protein concn., g./l.	Rapid component, %	
	Obsd.	Calcd. acc. to Gilbert theory
5.2	71	74
10.0	79	82
16.0	86	85
16.3	85	85
20.4	87	86

[a] 90% aggregable material.

The ultracentrifugal and electrophoretic data were analyzed in terms of the Gilbert theory of moving boundaries in an aggregating system.[5,20] The calculated percentages of "rapid component" are shown as the filled circles of Fig. 1 for the pH dependence and by the last column of Table I for the concentration dependence. In both cases, good agreement is obtained between the predicted values and the experimental data. This is strong evidence for the correctness of the equilibrium constants previously reported[6] and underlines the validity of the method of component analysis described in paper I.[18]

Although β-B has been shown not to give aggregates this large[4] under conditions at which β-A tetramerizes, it was of interest to check the degree to which it is capable of forming mixed aggregates with β-A. For this purpose, ultracentrifugal and electrophoretic analyses were carried out at

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

(20) G. A. Gilbert, Discussions Faraday Soc., No. 2, 1955, p. 68; Proc. Roy. Soc. (London), A250, 377 (1959).
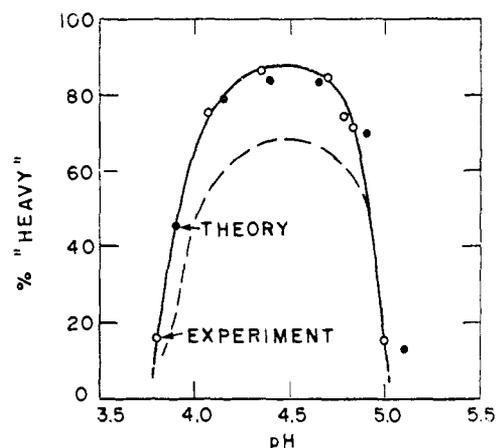
Fig. 1.—"Component" analysis of ultracentrifugal patterns of β-lactoglobulin A, ca. 20 g./l.; Γ/2 = 0.1 acetate buffers, 2°: ●, calcd. by Gilbert theory; O, experimental points; dashed line, curve obtained on pooled β-lactoglobulin at 30 g./l. (ref. 5, Fig. 1).

pH 4.65 on synthetic mixtures of β-A and β-B. In mixtures poor in β-A, it can be seen (Table II, Col. 4) that the fraction of area under the rapidly sedimenting peak is greater than the fraction of protein represented by β-A. If it is considered, as suggested previously,[5] that 30% of β-B is capable of entering into a mixed aggregation reaction with β-A, the Gilbert theory values for the fraction of protein expected to be present under the "fast boundary" are shown in Table II, column 3. The

TABLE II

ULTRACENTRIFUGAL AREA DISTRIBUTIONS IN SYNTHETIC
MIXTURES OF β-LACTOGLOBULINS A AND B

Ratio A:B	Prot. concn., g./l.	Rapid component, %	
		Calcd.	Obsd.
100% B	19.8	26.2	.....
15:85	16.4	30.4	20 ± 10
30:70	20.0	40.9	46 ± 5
50:50	20.0	53.0	56.7
60:40	22.8	59.7	63.9
70:30	15.0	62.0	59.8
80:20	17.1	69.6	69.2
87:13	20.0	75.0	74.5
100% A	20.4	82.9	84.6

agreement with experiment is found to be good. The experimental data at low degrees of aggregation are relatively imprecise, since under these conditions the rapid "component" appears only as a poorly resolved shoulder on the main slow peak and area analyses become extremely difficult. This is because the sedimentation constant of the rapid peak, s_R, decreases as the amount of aggregate decreases.[5,20] In the case of β-B alone, 26.2% tetramer was predicted on the basis of 30% aggregable material. No trace of heavy component was observed, however, since β-B can form tetramers only in mixture with β-A. The single peak in sedimentation indicates that β-B alone cannot form any aggregates greater than a dimer, which will not resolve into a bimodal peak.[20]

Results of similar experiments on synthetic mixtures, carried out electrophoretically, are shown in Fig. 2. In this case, the protein concentration is
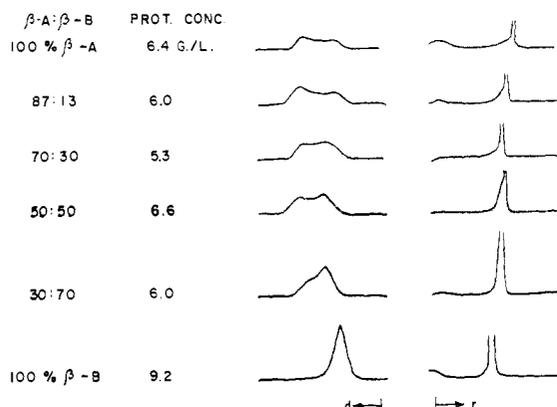
Fig. 2.—Electrophoretic patterns of βA–B mixtures; Γ/2 = 0.1 acetate buffer pH 4.65. Starting positions shown by vertical lines, 8000 sec. at 9.85 v./cm.

Fig. 3.—Analysis of light scattering data on β-lactoglobulin A at pH 4.65 in Γ/2 = 0.1 acetate buffer. Experimental points: O, 4.5°; ●, 8°; △, 15°; ▲, 25°; lines are theoretical curves calculated as described in the text.

much lower than in the ultracentrifugal experiments. It can be seen however, that on the descending side in β-A and all the mixtures, the boundaries are bimodal. As the relative amount of β-B increases, the fraction of the area under the rapidly migrating peak decreases. This indicates that the rapid peak is due to the formation of the tetramer, which is completely absent in 100% β-B. On the rising side, a hypersharp boundary appears in all cases. Quantitative analysis of these electrophoretic data is complicated by the fact that in this pH region β-B undergoes a type of transition similar to that observed by Cann for other proteins.[21] This gives rise to an anomalous appearance of the electrophoretic boundary and renders very difficult any analysis by such methods as the Gilbert theory. Qualitatively, however, the data of Fig. 2 are in good agreement with the concept that a fraction of β-B can enter into mixed association with β-A.

According to the Henry equation,[22] the mobility (u) is related to the charge Q (in e.s.u.) by

$$u = \frac{Q}{6\pi\eta r} \frac{1}{(1 + \kappa r)} f(\kappa r) \qquad (1)$$

where r is the radius of the spherical molecule, η is the viscosity of the medium, and κ is the Debye–Hückel parameter. Q can be replaced by $\bar{Z}e$ where $\bar{Z}$ is the mean net charge of the protein molecule and e the electronic charge. In the case of β—A, the electrophoretic mobilities of the peaks in the descending limb corresponding to monomer (mol. wt. 36,000) and tetramer (mol. wt. 144,000) have been found to be 1.2 × 10⁻⁵ and 4.1 × 10⁻⁵ cm.² sec.⁻¹ volt⁻¹, respectively, at pH 4.65.[5] If monomer and tetramer are represented by equivalent spheres, $r_T = 4^{1/3} r_M$. All parameters in eq. 1 save r, $\bar{Z}$ and u are identical for the two species, and the ratio of the radii is a constant. Taking the radius a_i of the supporting electrolyte ions into account, we get

$$\bar{Z}_T = 4^{1/3} \frac{u_T}{u_M} \frac{1 + \kappa (r + a_i)_T}{1 + \kappa (r + a_i)_M} \bar{Z}_M \qquad (2)$$

Substitution of values for the various parameters gives: $\bar{Z}_T = 7.53 \bar{Z}_M$. By titration under non-

aggregating conditions (room temperature) the $\bar{Z}_M$ of β-lactoglobulin at pH 4.65 has been found to be +7.[23] Using equation 2, this gives a $\bar{Z}_T$ for the tetramer of + 52, or + 12 per monomer unit. The charge difference of +5 per monomer unit is too great, however, to be explained in terms of the assumptions on the molecular shape and charge distributions which are inherent in the Henry equation. It is possible that some specialized configuration of charges exists so that when monomer units combine, sites with local attraction for protons or sodium ions may be established.[24]
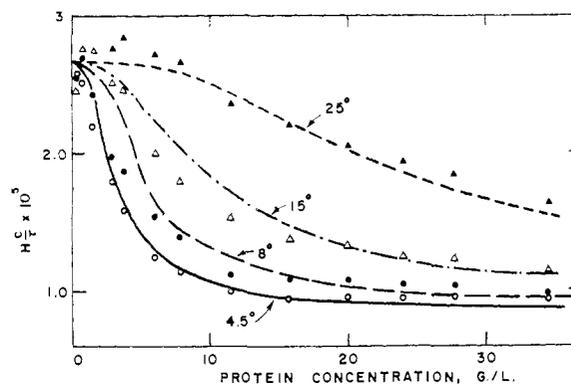
As a further check on the stoichiometry of the aggregation, light scattering measurements were carried out on β-A, β-B and a 1:1 mixture of the two as a function of temperature at pH 4.65.

The results obtained with β-A are presented in Fig. 3. The symbols are experimental points determined at 4.5, 8, 15 and 25°. The lines are theoretical curves calculated assuming 90% of the protein aggregable. The excellent agreement obtained bears out again the earlier analysis of the data from mixtures of various compositions.

The data at 4.5° on β-A, β-B and a 1:1 mixture are shown in Fig. 4. The dot-dash line is the best curve drawn through the points obtained with β-B at 25°. It is found to be a horizontal line with the points (not shown on the Fig.) equally distributed about it. Since at 25° β-B does not aggregate to any extent, the data can be taken as proof that under these conditions the second virial coefficient is equal to zero, confirming the reasoning used previously[6] in arriving at an assignment of zero to the slope. The open circles are the points obtained with β-A at 4.5° (also shown in Fig. 3), the triangles are points obtained with a 1:1 mixture of β-A and β-B. The dashed lines are curves calculated assuming that 90% of β-A can tetramerize, while 30% of β-B can also do so when mixed with β-A. The quantitative agreement obtained in two cases establishes the correctness of the previously proposed stoichiometry of the reaction, i.e., the aggregate is a tetramer (mol. wt. 144,000); 90% of β-A can aggregate; 30% of

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

(22) D. C. Henry, Proc. Roy. Soc. (London), A133, 106 (1931).

(23) Y. Nozaki, L. G. Bunville and C. Tanford, THIS JOURNAL, 81, 5523 (1959).

(24) C. Tanford, ibid., 79, 5340 (1957).
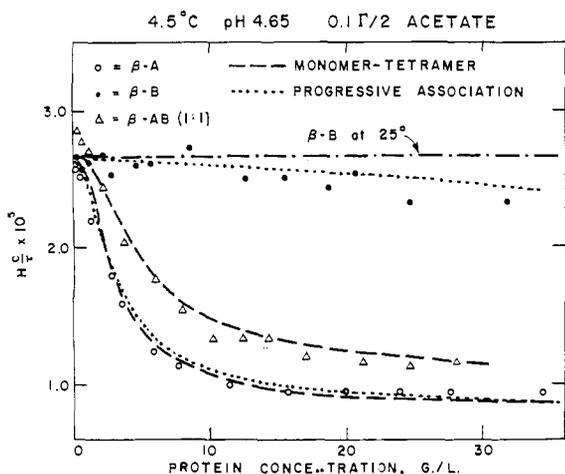
Fig. 4.—Analysis of light scattering data on β lactoglobulins at pH 4.65, Γ/2 = 0.1 acetate buffer, 4.5°. Experimental points: O, β-A; △, 1:1 β-A–β-B mixture; ●, β-B; dashed lines, theoretical curves for monomer-tetramer aggregation; dotted lines, theoretical curves for progressive association; dot-dash line best curve through experimental points on β-B at 25°.

the β-B molecules are capable of reacting with β-A, forming mixed tetramers. In the case of β-A, as will be shown below, this effect is due to molecular heterogeneity. With β-B no molecular heterogeneity has so far been demonstrated. However, β-B appears to undergo a molecular transformation in this pH range,[25] and it is possible that prior to entering into the association reaction, β-B must undergo an isomerization (β-B⇌β-B*, the equilibrium constant, K_{Isom}, of the unimolecular reaction being around 0.35–0.55). The overall reaction scheme is

$$4\beta A \xrightleftharpoons{\quad} (\beta A)_4 \quad (10\% \ \beta\text{-A not reactive})$$

$$2\beta B \begin{cases} \nearrow 2\beta B^* \xrightleftharpoons{\quad} (\beta B^*)_2 \\ \quad \text{or} \\ \searrow (\beta B)_2 \quad \text{(with part of } \beta B \text{ not reactive)} \end{cases} \quad (3)$$

$$n\beta A + m\beta B \xrightleftharpoons{\quad} (\beta A)_n(\beta B)_m; \quad n + m = 4; \quad m \leq 2$$
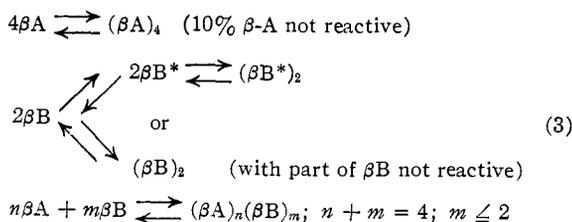
It is unreasonable to expect that the aggregation reaction can proceed by the simultaneous collision of four molecules. Therefore, calculations have been carried out for the case of progressive aggregation via the dimer and trimer stages. Since, as will be shown below, there are good reasons to believe that the tetramer is in the shape of a closed ring, it has been assumed that it results from the formation of four identical bonds; then, the addition of the last monomer molecule would have a much greater free energy change than the formation of either of the two bonds between the first three monomer units. This would result in a molecular distribution strongly favoring tetramer over other aggregated species. The dotted lines on Fig. 4 have been calculated for this case which can

(25) R. Townend and S. N. Timasheff, *Abstracts, 135th Meeting,* Am. Chem. Soc., Boston, 1959, p. 16-C.
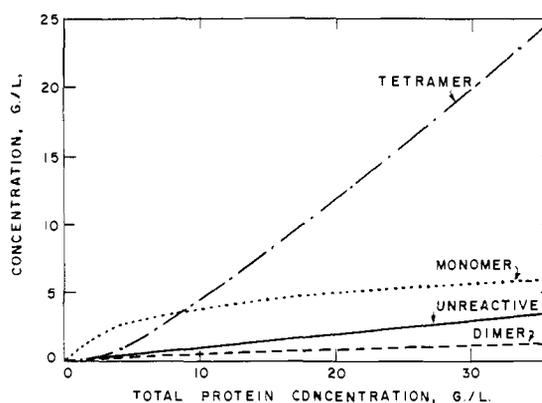
Fig. 5.—Species distribution for aggregation of β-lactoglobulin A, calculated by eq. 4.

be represented by

$$2\beta \underset{}{\overset{K_1}{\rightleftarrows}} \beta_2$$

$$\beta_2 + \beta \underset{}{\overset{K_1}{\rightleftarrows}} \beta_3 \quad (4)$$

$$\beta_3 + \beta \underset{}{\overset{K_2}{\rightleftarrows}} \beta_4$$

$$K_2 = K_1^{1/4}$$
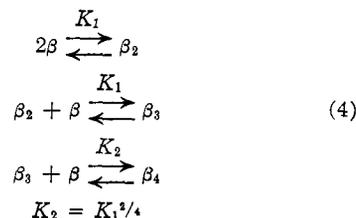
Expressing concentrations as mole fractions, the over-all equilibrium constant,[6] K_a (for the $4\beta \rightleftarrows \beta_4$ reaction), is 3.43×10^{16}; then, if the four bonds are considered to be identical as a first approximation, $K_2 = 9.26 \times 10^7$, $K_1 = 1.92 \times 10^4$. The filled circles in Fig. 4 are the light scattering points obtained with β-B at 4.5° and are in fair agreement with the theoretical curve for dimerization of 30% of the protein. In the case of β-A, the direct monomer–tetramer and progressive aggregation cases are indistinguishable. This is a result of the relation between the equilibrium constants in eq. 4. The calculation of species distribution for this case (Fig. 5) shows that dimer and trimer are never present in significant amount. The amount of trimer present is zero within the limits of accuracy of the present calculations. It is for this reason that tetramerization results in two ultracentrifugal peaks. If dimers and trimers were present in appreciable concentrations a single peak would be obtained.[20]

Light scattering and ultracentrifugal experiments have clearly demonstrated that the association of β-lactoglobulin attains a limit when the tetramer stage is reached. If all the bonds are considered to be equivalent and the bonding is taken as occurring at specific sites on the molecule, β-A must be at least divalent with respect to the aggregation reaction. However, since no large three-dimensional networks are formed, it is extremely unlikely that β-A could have more than two reactive sites per molecule. If the binding sites on a β-A molecule were at opposite ends of the molecule, large linear polymers of the protein might be possible. These, however, cannot be induced to form even at very high protein concentrations[6]; the sites must then be located on the molecules so that the formation of aggregates greater than tetramers is sterically hindered. The
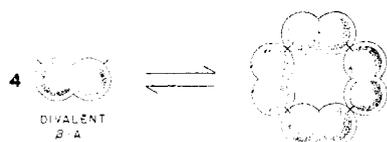
Fig. 6.—Schematic model of the tetramerization of β-lacto-globulin A.

Green and Aschaffenburg model[26] of the β-lacto-globulin molecule is composed of two identical spheres, with the 36,000 molecular weight species having an axial ratio of 2:1. It follows, then, that the reactive groups responsible for the aggregation must be symmetrically distributed on the double sphere molecule, and the cyclic tetramerization of β-A must result in a compact structure. If the bonds form, for example, at 90° angles, the end result would be a closed ring tetramer, as shown in Fig. 6. In mixtures of the two species, β-B can participate in mixed tetramers, although by itself it can form no aggregate greater than dimers. Such a situation could possibly be due to the presence of two types of interaction sites on each molecule of β-B, one being capable of interaction only with β-A. This is not too unrealistic if the structural difference between β-A and β-B should result in a steric effect hindering β-B bond formation at the second site. Work on this phase of the problem is currently in progress.

In Fig. 7 is presented the dependence of the sedimentation constant on concentration for β-A and β-B at 2°, pH 4.65. Analysis of the data by the previously described method[5] yields 6.80 S for the sedimentation constant of the tetramer at infinite dilution. This is slightly higher than 6.40 S obtained from the pooled milk protein.[5] In that case, however, the data analysis was complicated by the presence of 34% of non-aggregable protein. Thus, the present value can be considered as more accurate. 40% hydration[27–29] gives an f/f_0 value of 1.33. This corresponds to an axial ratio of 1:3.7 for an oblate ellipsoid of revolution and of 3.5:1 for a prolate ellipsoid, in agreement with the concept of a compact structure.

In the case of β-B, calculation of the average value of $s_{20,w}$ as a function of concentration was carried out[20,10] assuming that 30% of the protein can dimerize. As can be seen in Fig. 7 this curve falls very close to that for non-aggregating protein,[30] and it becomes impossible to detect ultracentrifugally such a degree of dimerization.

The pH dependence of the aggregation suggests very strongly that carboxyl groups play an important role in the binding mechanism, since the pH maximum of aggregation corresponds to the pK of carboxyl ionization. This is further supported by experiments which show that such carboxyl-blocking agents as lead ions can strongly

(26) D. W. Green and R. Aschaffenburg, J. Molecular Biology, 1, 54 (1959).

(27) T. L. McMeekin and R. C. Warner, THIS JOURNAL, 64, 2393 (1942).

(28) D. W. Green, A. C. T. North and R. Aschaffenburg, Biochem. Biophys. Acta, 21, 583 (1956).

(29) S. N. Timasheff and V. Luzzati, unpublished low angle X-ray scattering measurements.

(30) J. P. Johnston and A. G. Ogston, Trans. Faraday Soc., 42, 789 (1946).
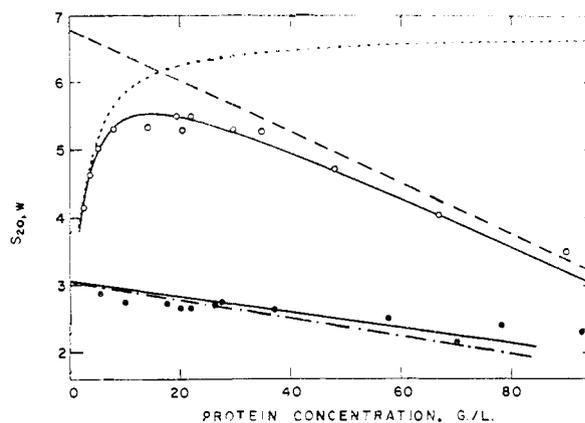
Fig. 7.—Dependence on concentration of the sedimentation constants of β-A and β-B at pH 4.65, 2°. Experimental points: O, β-A; ●, β-B; solid lines, theoretical curves calculated with the Gilbert theory for tetramerization of 90% of β-A and dimerization of 30% of β-B; dot-dash line, concentration dependence of the β-lactoglobulin monomer[30]; dotted line, theoretical curve for tetramerization of β-A (equilibrium only); dashed line, deduced concentration dependence of the sedimentation constant of the tetramer (hydrodynamic effect only) (the upper solid line represents the sum of the two effects).

interfere with the aggregation.[31] The only presently known chemical difference between the two species is the presence in β-A of two more carboxyls than in β-B.[15] It would seem desirable, however, to refrain from further speculations until more detailed information is available on the nature of the 18,000 mol. wt. subunits[9] of the two species and more definitive statements can be made concerning the structure of these molecules.

Fractionation of β-A.—In all the data analyses, it has been assumed that β-A contains 10% of a material which cannot aggregate. While this assumption seems to be in quantitative agreement with all the experimental data, it is based strictly on the observation by Tombs[32] that β-A can be resolved into two fractions by partial precipitation with salt. These differ in the amount of rapid "component" in electrophoresis at pH 4.65. Tombs, however, presented no direct evidence for the effect of the two fractions on the aggregation, and it is well known that in this pH range a number of proteins give complex electrophoretic patterns.[33] In the course of studies on β-lactoglobulin A at low pH's (1.5–2.0), it was observed by us that on subsequent recrystallization, two fractions are obtained, one coming out as an oil, the other remaining in solution and generally separating out only on seeding. (No such effect is observed in β-B, which crystallizes out normally after exposure to pH 2.). This procedure was applied to the sample of β-A given us by Dr. Aschaffenburg and the resulting materials were analyzed ultracentrifugally and electrophoretically at pH 4.65. The results of these measurements are presented in

(31) R. Townend and S. N. Timasheff, unpublished experiments.

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

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

TABLE III

PERCENTAGE OF RAPID COMPONENT IN β-LACTOGLOBULIN A pH 4.65, Γ/2 = 0.1 ACETATE BUFFER (ASCHAFFENBURG PREP.)

Fraction	Electrophoresis (1°)			Sedimentation (2°)		
	g./l.	Calcd.	Obsd.	g./l.	Calcd.	Obsd.
Original	16.3	85.0[a]	85.0	20.5	83.5[a]	84.5
Supernatant	12.3	...	67.0	21.6	...	62.5
Oily	12.8	93.9[b]	91.2	21.8	93.0[b]	89.2

[a] Assuming 90% aggregable material. [b] Assuming 100% aggregable.

Table III in which the percentage of the area found experimentally under the rapid component is compared with that calculated with the Gilbert theory.[20] In both sedimentation and electrophoresis an assumption that 90% of the starting material is aggregable yields quantitative agreement between the theoretical and experimental values. In the case of the oily fraction, the amount of aggregable material has increased although not to 100% as is shown in the last line of the table (ca. 96% seems to be a more realistic value). The supernatant has been enriched with the non-aggregable material. The lack of complete purification in the oil is probably due to occlusion of non-aggregable material in it. A second treatment of the oil did not result in further purification, as now all the protein precipitated out on dialysis against distilled water. This also can serve to show that the non-aggregable component is not a product of the acid treatment.

A second sample of β-A, prepared in our Laboratory by the Palmer procedure,[11] was subjected to an identical treatment. The descending electrophoretic patterns at pH 4.65 of the original β-A and the two fractions are shown in Fig. 8. The important point to note is the change in area under the slowly migrating portion of the boundary. This area is greatest in the supernatant and smallest in the oil. Since, according to Gilbert, this area should remain constant for a system in aggregating equilibrium, the excess area in the cases of the unfractionated and the supernatant materials must be due to the presence of non-aggregable material. The fact that the area under the slow "component" does not change with concentration for the oily fraction points to the absence of significant amounts of non-aggregable protein. These results establish beyond any doubt that β-A contains 5–10% of material which cannot aggregate and prove that the electrophoretically different components
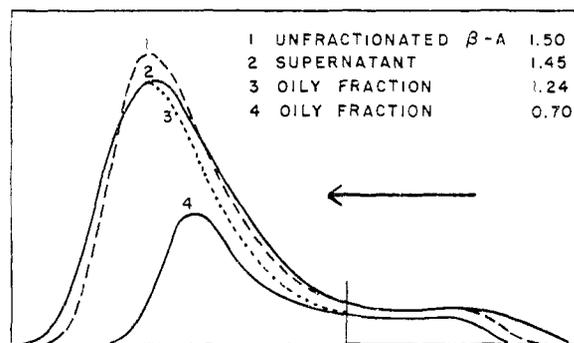
Fig. 8.—Superposition of descending electrophoretic patterns of β-lactoglobulin A fractions, Γ/2 = 0.1 acetate buffer pH 4.65, 21,600 sec. at 4.87 v./cm.; protein concentrations in g./100 ml. given in figure.

of Tombs[32] are related to the association reaction. This justifies, then, the last assumption made in the analysis of the aggregation data.

Conclusions.—The data and its analysis presented above establish definitely that β-lactoglobulin A is composed of two fractions, one (90%) which can aggregate to a tetramer, the other (10%) being unable to aggregate. β-Lactoglobulin B can form a dimer with 30% of the protein reacting. This may be the result either of molecular heterogeneity or of an isomerization which precedes aggregation. In mixtures of the two proteins, mixed tetramers can be formed with not more than two molecules of β-B participating. The equilibrium constants, previously deduced from studies on complex mixtures, describe quantitatively the aggregation of the individual genetic species of this protein. The tetramer appears to be in the shape of a closed ring with the bonds being formed at specific sites symmetrically located on the molecules. It is highly probable that carboxyl groups are involved in the bond formation, but a detailed analysis of the nature of the bonds requires further information on the structure of the subunits of the two β-lactoglobulins. Work to that effect is presently in progress in our Laboratory.