Molecular Interactions in β-Lactoglobulin. IX. Optical Rotatory Dispersion of the Genetic Variants in Different States of Association[2]

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An optical rotatory dispersion study of β-lactoglobulins A, B, and C has been carried out. The b_0 values of the three genetic variants have been found to be essentially identical. For β-A, b_0 maintains a value of 88 ± 6 between pH 1.7 and 11.9. a_0 undergoes large changes with changes in the states of aggregation or molecular conformation of the proteins. In the pH zones of intermolecular association, a_0 is related quantitatively to the degree of association and reflects the known changes in the geometric structure of the kinetic units.

Introduction

Bovine β-lactoglobulin has been known since 1955 to exist in two genetically controlled variants called β-lactoglobulin A (β-A) and β-lactoglobulin B (β-B).[3] Recently a third genetic variant has been discovered by Bell.[4] This isomer has been shown to be a β-lactoglobulin,[5,6] and is here designated β-lactoglobulin C (β-C); its chemical composition and physicochemical properties are the subjects of other communications from this laboratory.[7,8]

Studies carried out over the last few years[9–12] have shown that in the isoelectric region β-A undergoes a rapidly re-equilibrating tetramerization process. In contrast, β-B (which differs from β-A only by the replacement of two amino acids[13–15]) associates to a much lesser extent. The tetramer has a compact closed structure, best described by a cubic array of eight spheres.[16] In the acid region all three variants dissociate into subunits.[8,17–19] The dissociation is driven by nonspecific, largely electrostatic repulsion, and the reassociation is species specific.[8,20] In the present phase of our work we have addressed ourselves to the physical (structural) and chemical basis of the specificity of the association of the three genetic isomers of β-lactoglobulin. This paper deals with a systematic study of the optical rotatory properties which accompany the various states of association of these proteins. We have also studied the difference spectral behavior of these three isomers, which will be published separately.

Experimental

Materials and Auxiliary Measurements.—The preparation and properties of the β-A and β-B samples employed[21] have been described previously.[9–12] β-Lactoglobulin C was prepared from the milk of a homozygous cow of the Jersey breed, using the Aschaffenburg procedure,[22] and crystallized three times. The β-C milk was kindly supplied to us through the courtesy of Dr. Murray Brown of the Department of Dairy Science, Texas A and M College, Texas.

The desired pH and ionic strength were obtained by adjustment of stock protein solutions with 0.5 *M* NaCl, HCl, and NaOH or by the addition of solid NaCl. For the pH-dependent tetramerization study, 0.1 *M* sodium acetate–acetic acid buffers were used. Protein stock solutions were clarified by centrifugation and their concentrations were determined spectrophotometrically at 278 mμ using an absorptivity value of 9.6 dl./cm.-g.[10] Since the tyrosine and tryptophan contents of β-C do not differ from β-A or β-B,[7] its absorptivity was assumed to be the same. pH measurements were carried out with a glass electrode at room temperature.

Optical Rotation Measurements.—These measurements were made in a Rudolph Model 200S spectropolarimeter.[23] They were taken over the range of 312 to 578 mμ, employing both mercury and xenon arc lamps as sources of radiation. Most of the work was carried out with the aid of a specially designed 1.0 dm. all-quartz jacketed cell. The desired temperature of the solutions was maintained by circulating water from a thermoregulated bath through both the instrument compartment and the cell jacket. Temperature was measured to ±0.1° by means of a calibrated thermistor probe, which was attached to the stopper of the cell and came in direct contact with the solution during the experiment. Some of the room temperature measurements were performed in conventional quartz window demountable cells of 2-dm. light path, with 25° water circulated through the instrument jacket only.

The rotatory dispersion parameters, a_0 and b_0, were obtained from the intercepts and slopes of plots of $m'(\lambda^2 - \lambda_0^2)$ *vs.* $1/(\lambda^2 - \lambda_0^2)$. These plots are based on the Moffitt–Yang empirical equation[24,25]

$$[m']_\lambda = [\alpha]_\lambda M_0 \frac{3}{n^2+2} = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \quad (1)$$

where $[\alpha]$ is the specific rotation at wave length λ, n is the refractive index of the solution, and λ_0, a_0, and b_0 are the Moffitt–Yang parameters. λ_0 was taken to be 212 mμ. The mean residue

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

(2) This work was presented in part at the 147th National Meeting of the American Chemical Society, Denver, Colo., Jan., 1964.

(3) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955); *ibid.*, **180**, 376 (1957).

(4) K. Bell, *ibid.*, **195**, 705 (1962).

(5) R. Aschaffenburg, private communications.

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(7) E. B. Kalan, R. Greenberg, M. Walter, and W. G. Gordon, *Biochem. Biophys. Res. Commun.*, **16**, 199 (1964).

(8) R. Townend, T. T. Herskovits, and S. N. Timasheff, *J. Biol. Chem.*, in press.

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

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

(12) S. N. Timasheff and R. Townend, *ibid.*, **83**, 464 (1961).

(13) E. B. Kalan, W. G. Gordon, J. J. Basch, and R. Townend, *Arch. Biochem. Biophys.*, **96**, 376 (1962).

(14) W. G. Gordon, J. J. Basch, and E. B. Kalan, *J. Biol. Chem.*, **236**, 2908 (1961).

(15) K. A. Piez, E. W. Davie, J. E. Folk, and J. A. Gladner, *ibid.*, **236**, 2912 (1961).

(16) J. Witz, S. N. Timasheff, and V. Luzzati, *J. Am. Chem. Soc.*, **86**, 168 (1964).

(17) R. Townend and S. N. Timasheff, *ibid.*, **79**, 3613 (1957).

(18) R. Townend, L. Weinberger, and S. N. Timasheff, *ibid.*, **82**, 3175 (1960).

(19) S. N. Timasheff and R. Townend, *ibid.*, **83**, 470 (1961).

(20) R. Townend, C. A. Kiddy, and S. N. Timasheff, *ibid.*, **83**, 1419 (1961).

(21) Most of the data on a specific variant were taken on a lyophilized crystalline preparation from a single cow. A few experiments were done with samples prepared from the pooled milk of a number of homozygous animals. Small differences in the values of the rotatory parameters were regularly found between the samples.

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

(23) It is not implied that the U. S. Department of Agriculture recommends the above company or its products to the possible exclusion of others in the same business.

(24) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).

(25) W. Moffitt, D. D. Fitts, and J. G. Kirkwood, *ibid.*, **43**, 723 (1957).

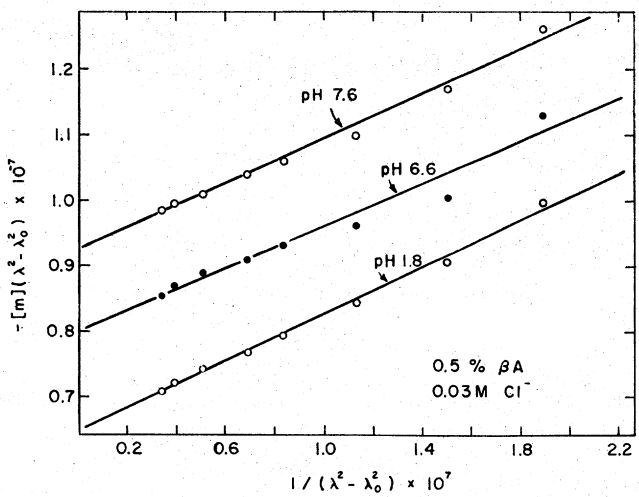

Fig. 1.—Typical optical rotatory dispersion data obtained with the β-lactoglobulins (β-A at three pH values, 25°).

weight, M_0, was calculated to be 112, using a molecular weight of 36,000[26] and the known amino acid composition of β-A and β-B.[14,15]

Typical dispersion plots of data obtained are shown in Fig. 1. The points fall passably well on straight lines in a Moffitt–Yang plot, although a slight upward curvature at high wave length was observed at all conditions studied. This curvature is of similar magnitude to that found in the data of Schellman[27] and Tanford and co-workers.[28] To minimize random error of plotting, the lines determining a_0 and b_0 were drawn in a consistent manner. Furthermore, in any series of measurements, all points were obtained by dilution from a single stock solution of protein, minimizing random error due to concentration measurements. Thus, while the absolute values of a_0 and b_0 reported here may be slightly different from those found in the literature under similar conditions, and may not correspond exactly to the absolute intrinsic values of these parameters, they are internally consistent, and their use in a comparative study, such as the present one, is perfectly valid.

Results

Data showing the pH dependence of the rotatory dispersion parameters of β-lactoglobulin A in the range between pH 1.7 and 11.9 are presented in Fig. 2. In this and the following figures, b_0 is plotted in the upper portion, while a_0 is plotted in the lower part. Protein concentrations were 5 g./l. in NaCl–HCl and NaCl–NaOH media. The half-filled circles represent data obtained at 0.1 ionic strength, the open and crossed circles are points obtained on two different protein preparations[21] at $\Gamma/2 = 0.03$; this lower ionic strength was chosen since the dissociations below pH 3.5 and above pH 9 are known to be the result of nonspecific electrostatic repulsion and as such are accentuated by lowered concentration of the supporting electrolyte. All points were obtained within the first 15 min. after adjusting to experimental conditions, except the filled circles, which represent measurements taken 1 hr. after the initial measurement. In the insert of Fig. 2, the a_0 data between pH 2 and 7 are shown with a fivefold magnification along the ordinate.

As a first gross observation, it can be seen that the value of b_0 remains essentially constant at 88 ± 6 over the entire pH range examined. On the other hand, a_0 varies considerably. Below pH 4.5, a_0 remains constant at a value close to −145, independent of ionic

(26) F. R. Senti and R. C. Warner, *J. Am. Chem. Soc.*, **70**, 3318 (1948).

(27) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30**, 395 (1958).

(28) C. Tanford, P. K. De, and V. G. Taggart, *J. Am. Chem. Soc.*, **82**, 6028 (1960).

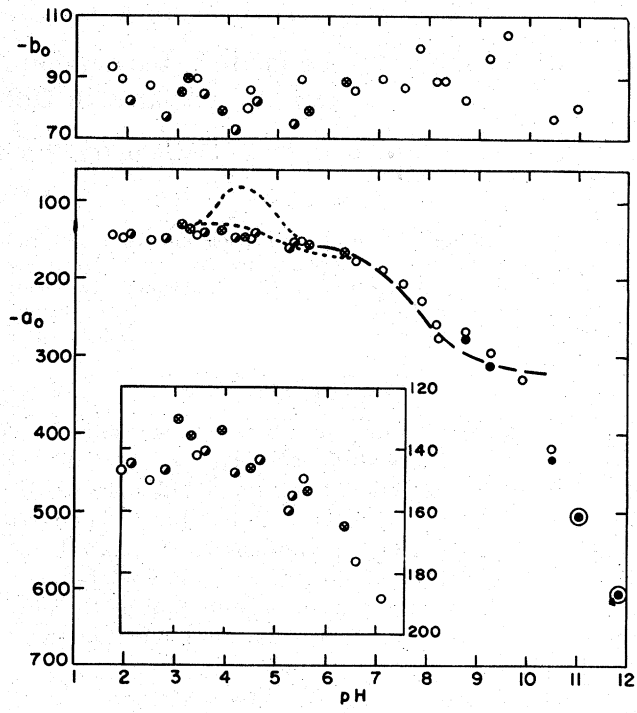

Fig. 2.—Dependence of a_0 and b_0 of β-A on pH. All points taken at 25°, 5 g./l. of protein concentration. Open circles: NaCl–HCl, $\Gamma/2 = 0.03$, single cow; crossed circles: same conditions, pooled β-A milk; filled circles: after standing 1 hr.; half-filled circles: $\Gamma/2 = 0.10$. Dashed line: pH dependence of conformational transformation [C. Tanford, L. G. Bunville, and Y. Nozaki, *J. Am. Chem. Soc.*, **81**, 4032 (1959)]; dotted lines: data of Fig. 5 (2°, β-A and β-B). Insert: pH 2–7.5 data with ordinate magnified five times.

strength. In fact, the variations between two samples of protein were somewhat larger than the difference in the a_0 values between the two ionic strengths. Above pH 5, a_0 begins to assume progressively more negative values; it passes through an inflection point between pH 8 and 9 (at $a_0 = -280$), above which an increase in pH leads to a more rapid increase in a_0 to a value of −610 at pH 11.8. Furthermore, except for a narrow zone around pH 9, the final values are attained in less than 15 min. At pH 9.3, 1 hr. of standing resulted in a shift of a_0 from −295 to −315, which did not change any more with time.

The pH dependence of a_0 can be discussed most conveniently in terms of four fairly discernible zones. These are: (1) the region acid to pH 3.5, in which the dissociation into 18,000 molecular weight subunits takes place; (2) pH 3.5 to 5.1, in which the reversible tetramerization occurs; (3) pH 5.3 to 8.5, in which β-A and β-B have been shown by Tanford and co-workers to undergo a reversible conformational change and two abnormal carboxyls become titratable,[29,30] and dissociation into subunits starts to occur; and (4) above pH 8.5, where β-lactoglobulin is largely in a dissociated state[31] and irreversible breakdown of the secondary structure starts to occur.[32,33]

While the present study deals primarily with the

(29) C. Tanford, L. G. Bunville, and Y. Nozaki, *ibid.*, **81**, 4032 (1959).

(30) H. Susi, T. Zell, and S. N. Timasheff, *Arch. Biochem. Biophys.*, **85**, 437 (1959).

(31) C. Georges, S. Guinand, and J. Tonnelat, *Biochim. Biophys. Acta*, **59**, 737 (1962).

(32) M. L. Groves, N. J. Hipp, and T. L. McMeekin, *J. Am. Chem. Soc.*, **73**, 2790 (1951).

(33) L. K. Christensen, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **28**, 37 (1952).

first two zones, *i.e.*, pH 1.5 to 5.5, all four will be discussed in turn.

Region of Tetramerization, pH 3.5 to 5.1.—Optical rotatory dispersion measurements were carried out on β-A, β-B, and β-C at 2 and 30° as a function of concentration (between 1.5 g./l. and 42 g./l.) in a 0.1 ionic strength, pH 4.57 acetate buffer. Under these conditions the tetramerization is maximal at 2°,[9] while at 30° the association cannot be detected by the criteria of light scattering[11] and sedimentation.[10] The results are shown in Fig. 3, where (as in subsequent figures) the data on β-A are represented by the open circles, β-B by filled circles, and β-C by half-filled circles. As can be seen, the values of b_0, summarized in Table I,

TABLE I

OPTICAL ROTATORY PARAMETERS OF THE β-LACTOGLOBULINS AT pH 4.57

Temp., °C.	Genetic variant	b_0	a_0
2	A	−76 ± 5	−132[a]
	B	−75 ± 2	−143[a]
	C	−73 ± 2	−156 ± 3
30	A	−75 ± 4	−148 ± 5
	B	−73 ± 4	−157 ± 3
	C	−72 ± 6	−177 ± 3

[a] Value extrapolated to zero protein concentration where no tetramer exists.

are essentially identical for the three proteins, as well as being independent of temperature or concentration. The values of a_0, on the contrary, are found to be different for the three proteins at both temperatures studied. Furthermore, at 2°, a_0 of β-A is a strong function of concentration, as it assumes progressively less negative values with an increase in concentration (from −129 at 1.34 g./l. to −86 at 36.5 g./l.). While β-B also exhibits a concentration dependence of a_0, it is much weaker than for β-A (from −146 at 1.8 g./l. to −129 at 35.9 g./l.). The a_0 values of β-C are seen to remain constant at −156 ± 3 over the entire concentration range studied. At 30°, no concentration dependence is exhibited by a_0 of any of the three variants.

The conditions of these experiments correspond exactly to those of maximal tetramerization. Since β-A tetramerizes very strongly in the cold, β-B very weakly, and β-C even more weakly, and this reaction is essentially suppressed for all variants at 30°, the observed results on the concentration and temperature dependence of a_0 suggest very strongly that these observed variations are a direct consequence of the tetramerization reaction, the tetramer having a less negative a_0 than the monomer.

Verification of this hypothesis was approached by establishing the direct correspondence of a_0 to the degree of tetramerization by quantitative examination of the results. This was done in the following manner. If it is assumed that the a_0 value observed at any concentration is an average of the intrinsic a_0 values of monomer and tetramer, and that each makes a contribution proportional to its weight concentration, C_i, then

$$a_0^{\text{exp}} = (a_0^{\text{m}}C^{\text{m}} + a_0^{\text{t}}C^{\text{t}})/(C^{\text{m}} + C^{\text{t}}) = \bar{a}_{0,\text{w}} \quad (2)$$

where the superscripts m, t, and exp refer to monomer, tetramer, and experimental value, respectively; a_0^{exp} then is a weight average quantity and is related at each

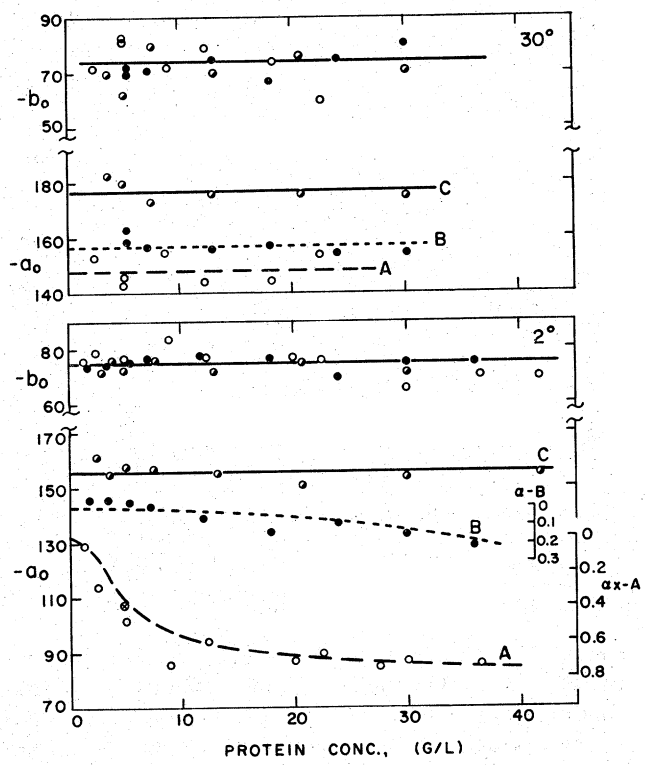

Fig. 3.—Concentration dependence of optical rotation parameter of β-A, β-B, and β-C in 0.1 Γ/2 acetate buffer, pH 4.57. Upper portion: 30°; lower portion: 2°. In this and all subsequent figures open circles represent data on β-A, filled circles β-B, half-filled circles β-C; dashed lines are calculated curves for β-A, dotted lines for β-B, solid lines for β-C. The crossed circle represents an independently measured point in 0.03 Γ/2 NaCl–HCl, pH 4.57.

concentration to the fraction of protein aggregated, α, and the fraction in each system capable of entering into the reaction, x, by

$$x\alpha = (a_0^{\text{exp}} - a_0^{\text{m}})/(a_0^{\text{t}} - a_0^{\text{m}}) \quad (3)$$

The denominator, assumed to be concentration independent, is hereafter referred to as $\Delta a_0^{\text{t}-\text{m}}$. α is related to the aggregation equilibrium constant, K_a,[11] by

$$K_a = \alpha M_m^3/[4(1 - \alpha)^4 x^3 C_2^3] \quad (4)$$

where M_m is the monomer molecular weight and C_2 is the total protein concentration in g./l., *i.e.*, $C^{\text{m}} + C^{\text{t}}$.

Using K_a and x from light scattering,[34] eq. 3 is solved simultaneously for the experimental concentrations, with corresponding a_0^{exp} values, yielding the a_0^{m} and $\Delta a_0^{\text{t}-\text{m}}$ parameters. For β-A these values are $a_0^{\text{m}} = -132$ and $\Delta a_0^{\text{t}-\text{m}} = 63$, *i.e.*, $a_0^{\text{t}} = -69$.

The concentration dependence curve of a_0 calculated in this way from the light-scattering information is shown by the dashed line in the lowest part of Fig. 3.

(34) For the sake of clarity the values of the equilibrium constants previously deduced[12] for the 4β-A ⇄ β-A₄ reaction are presented:

2.0°	pH	3.90	4.14	4.40	4.65
	K_a	1.6×10^9	2.1×10^{11}	6.9×10^{11}	5.5×10^{11}
2.0°	pH	4.90	5.10		
	K_a	2.2×10^{11}	3.4×10^8		
pH 4.6	T, °C.	2.2	6.1	10.5	15.5
	K_a	4.7×10^{11}	1.2×10^{11}	2.9×10^{10}	6.6×10^9
pH 4.6	T, °C.	20.1	25.0	30.0	
	K_a	1.5×10^9	3.5×10^8	8.3×10^7	

For the 4β-B ⇄ β-B₄ reaction, the pertinent values are (S. N. Timasheff and T. Kumosinski, unpublished data):

pH 4.6	T, °C.	2.0	10.0
	K_a	1.2×10^8	8.9×10^6

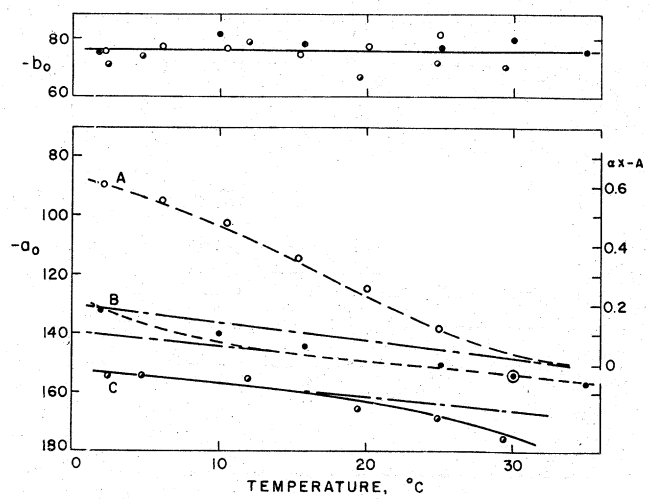

Fig. 4.—Temperature dependence of optical rotation of β-A, β-B, and β-C in 0.1 Γ/2 acetate buffer of pH 4.57. Protein concentrations: 22.4 g./l. of β-A, 30.0 g./l. of β-B, 30.0 g./l. of β-C. All symbols have same meaning as in Fig. 3. Dot-dash lines are derived base lines, as explained in text.

It is evident that this curve describes well the experimental points. On the right-hand ordinate, the corresponding values of the product $x\alpha$ are plotted, indicating the extent of tetramerization at any given concentration.

In the analysis of the β-B data, due to the small dependence of concentration it was assumed that the difference Δa_0^{t-m} is the same as for β-A, *i.e.*, 63. In this way the tetramerization constant of 1.2×10^8 l.^{3} mole^{-3} leads to values of a_0^m and a_0^t of −143 and −80 for β-B. The corresponding calculated curve is shown by the dotted line of the bottom part of Fig. 3. Again good agreement with the experimental points is obtained. The corresponding values of α are plotted on the right-hand ordinate.

For the general case of polymerization of degree n, eq. 3 and 4 may be combined into

$$K_a = \frac{(\Delta a_0^{p-m})^{n-1}(\bar{a}_{0,w} - a_0^m)M_m^{n-1}}{n[x\Delta a_0^{p-m} - (\bar{a}_{0,w} - a_0^m)]^n C_2^{n-1}} \quad (5)$$

Here a_0^p represents the rotatory parameter of the polymer rather than the specific tetramer. This equation is similar in form to eq. 5 of ref. 11, where α was obtained from light-scattering information alone.

The concentration dependence of a_0 for both β-A and β-B indicates strongly that a direct relation exists between optical rotation and the extent of association. As a further test of this hypothesis, the rotatory parameters of the three lactoglobulins were determined as a function of temperature at a pH in the maximal tetramerization zone, *i.e.*, at pH 4.57. The results are shown in Fig. 4. The protein concentrations used were: 22.4 g./l. of β-A, 30.0 g./l. of β-B, and 30.0 g./l. of β-C. Again b_0 is found to be independent either of temperature or of genetic variant, maintaining a constant value of −76 ± 4.[35] On the other hand, a_0 displays a strong temperature dependence with all three proteins. In the case of β-A and β-B, the monotone increase of a_0 with temperature reflects a superposition of the effect of tetramerization, strongest at low temperatures, on the intrinsic da_0/dt of the monomer. These data were analyzed in terms of the tetramerization equilibrium in a manner similar to that described above. The 2 and 30° values of a_0^m are known for the two proteins from the data of Fig. 3 and Table I (see Table I, column 4). Making the assumption that all effects other than the aggregation result in a linear dependence of a_0 on temperature within the range investigated, as indeed was found by Schellman for a mixture of these proteins[27] under conditions at which no molecular association occurs, straight lines were drawn between the a_0^m values at 2 and 30°. These are shown by the dot–dash lines of Fig. 4. Using this for a_0^m at the various temperatures of interest and assuming that Δa_0^{t-m} maintains a constant value of 63, independent of temperature, the expected values of a_0^{exp} were calculated by applying the light-scattering equilibrium constants, given in ref. 34, to eq. 3 and 4. The results are shown by the dashed (β-A) and dotted (β-B) lines of Fig. 4. The corresponding values of $x\alpha$ for β-A are plotted on the right-hand ordinate of this figure. Agreement between the experimental points and the calculated a_0 curve is excellent.

In the case of β-C, no upward deviation of a_0 is found at low temperature, indicating little or no aggregation. The downward curvature at the high-temperature end cannot be ascribed to the tetramerization reaction and must be the reflection of some other, as yet unknown, change in state of aggregation or molecular conformation.

The quantitative correlation of a_0 with tetramerization as a function of temperature and concentration suggested a detailed examination of the pH dependence of a_0 in the cold. Measurements were taken, therefore, on 30 g./l. solutions of the three genetic variants in 0.1 ionic strength acetate buffers at 2°. The results are shown in Fig. 5. With β-A and β-B, b_0 is once again essentially independent of pH, averaging −68 ± 7 for β-A and −70 ± 3 for β-B. With β-C, the b_0 value of −72 ± 7, found below pH 5.2, drops to −53 ± 2 above that pH.

The behavior of a_0 is quite complicated for all three variants. With β-A, as pH is increased above 3.5, a_0 first decreases from −125 until it reaches a minimum of −85 between pH 4.0 and 4.6; then, it increases rapidly to −140 at pH 5.2 and continues to increase slowly as pH is raised further. β-B and β-C a_0 values remain essentially constant (−129 and −150, respectively) below pH 4.6, but increase above this pH. For β-B the a_0 *vs.* pH curve appears sigmoid with a point of inflection around pH 5.1, while in β-C, the curve parallels the β-B curve up to pH 5.2, and continues to increase above that pH.

The β-A behavior of a_0 between pH 3.6 and 5.2 can be ascribed directly to the tetramerization reaction. Above pH 5.2, the slow increase must be assumed to be similar to that observed in β-B. In this connection, it is interesting to note that Tiselius electrophoresis experiments under these same conditions on β-B indicate a possible isomerization of structure in this pH region. In β-A this electrophoretic effect is much smaller and occurs at a slightly higher pH.

In order to correlate a_0 with equilibrium constants as a function of pH, it is necessary to know the pH dependence of a_0^m. This was obtained as follows: it

(35) It is true that, just as in the concentration dependence data of Table I, β-C has a slightly lower average value of b_0 (−72 ± 4) than either β-A (−77.5 ± 2) or β-B (−78 ± 2); this is well within experimental error, and no significance can be attached to it at this time.

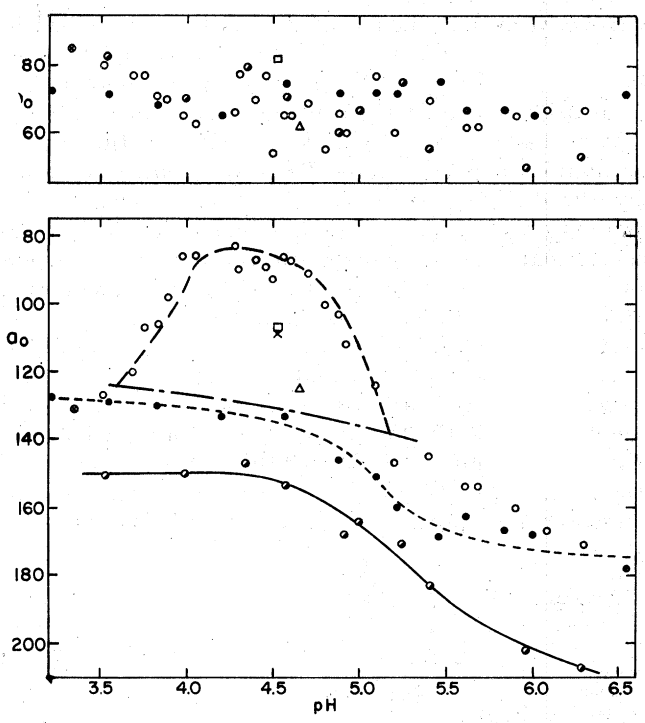

Fig. 5.—pH dependence of optical rotation of β-A, β-B, and β-C at 2° in 0.1 Γ/2 acetate buffers. Protein concentrations: 30.0 g./l. Symbols have same meaning as in Fig. 3. Triangle represents experimental point in Γ/2 = 1.0 (0.28 NaAc, 0.72 NaCl); square represents experimental point in Γ/2 = 0.03 NaCl–HCl, at 4.9 g./l.; X represents corresponding theoretical value; crossed circle was obtained in 0.03 Γ/2 NaCl–HCl.

is known that at pH 4.57, a_0^m is −132 at 2° (Table I); at 25° it is −145 (see Fig. 4). Comparison of the 25° data at pH 3.5 and 5.4 (Fig. 2) with those at 2° (Fig. 5) results again in a difference of 13. Assuming, therefore, that this difference is independent of pH, the best line drawn through the 25° points (Fig. 2) was translated by +13. The resulting curve, shown by the dot–dash line of Fig. 5, is the derived pH dependence of a_0^m at 2°. Prediction of the a_0^{exp} values was made, using eq. 3 and 4 as previously and the known equilibrium constants,[34] the value of $\Delta a_0^{t-m} = 63$, and this derived dependence of a_0^m. The resultant bell-shaped curve is shown as the dashed line in Fig. 5. It is seen to describe well the experimental points over the entire zone of tetramerization, again demonstrating that the observed variation of a_0 is a direct function of intermolecular association. To indicate the magnitude of the effects treated here, the β-A and β-B data of Fig. 5 have been drawn in as dotted lines on Fig. 2. It can be seen that the changes in a_0 due to tetramerization are rather small on the over-all a_0 scale, but nevertheless they are amenable to analysis by the present methods.

Two further experimental points have been included in Fig. 5. These are in 0.03 ionic strength NaCl–HCl (pH 4.52) and 1.0 ionic strength NaCl–HCl–NaOAc (pH 4.65). Since the tetramerization is essentially ionic-strength independent below Γ/2 = 0.3[36] and proceeds identically in chloride and acetate media,[10] a_0 could be calculated for the first point. The value obtained (−109) is in excellent agreement with the experimental one (−107). The point at Γ/2 = 1.0 is displaced slightly from the monomer base line. While at present no calculation could be made due to ignorance

(36) S. N. Timasheff and R. Townend, paper in preparation.

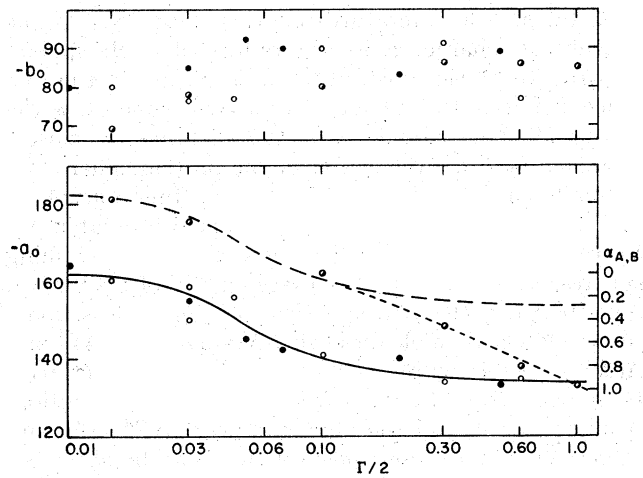

Fig. 6.—Ionic strength dependence of optical rotation of β-A, β-B, and β-C at pH 2.70, at 25°. Protein concentration: 4.2 g./l. of β-A, 5.1 g./l. of β-B, and 5.2 g./l. of β-C. Symbols have same meaning as in Fig. 3. Solid line: theoretical curve; dashed line: solid line translated by $-20a_0$.

of the equilibrium constant at this ionic strength, this higher value of a_0 is qualitatively in agreement with recent ultracentrifugal observations that at such high salt concentrations the tetramerization at pH 4.65 (2°) is highly suppressed.[37]

Acid Dissociation Region (Below pH 3.5).—Successful interpretation of the optical rotatory parameter a_0 in terms of tetramerization between pH 3.7 and 5.2 prompted us to examine similarly the dissociation reaction below pH 3.5. Measurements were carried out on 4–5 g./l. protein solutions at pH 2.7, 25°, as a function of ionic strength in NaCl–HCl solvents. The results are shown on Fig. 6 and the numerical values of the parameters are listed in Table II. Again b_0 is found

TABLE II

OPTICAL ROTATORY PARAMETERS OF THE β-LACTOGLOBULINS AT pH 2.7

Genetic variant	b_0	a_0 18,000 mol. wt.	a_0 36,000 mol. wt.
A	−83 ± 7	−162	−132
B	−87 ± 4	−162	−132
C	−81 ± 6	−182	...

to have very similar values for the three genetic variants and to be independent as well of ionic strength. On the other hand, a_0 decreases with an increase in ionic strength. Since the dissociation of β-A and β-B at this pH is known to be ionic-strength dependent and to be the result of nonspecific electrostatic repulsion, the following treatment could be used. The electrostatic free energy of repulsion, $\Delta(\Delta F^e)$, was calculated as a function of ionic strength using the Verwey–Overbeek equation[38] with a Debye–Hückel surface potential of the protein.[39]

$$-\Delta(\Delta F^e) = \frac{\bar{Z}^2 e^2 b^2}{DR}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)^2 e^{-\kappa(R-2b)}\gamma \quad (6)$$

where $\bar{Z}$ is the average charge of each spherical sub-

(37) R. Townend, unpublished data.

(38) E. J. W. Verwey and J. Th. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier Publishing Co., New York, N. Y., 1948.

(39) See, for example, J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press, Inc., New York, N. Y., 1958, Chapter 5.

unit, taken from literature titration data,[40,41] e is the elementary charge, b is the radius of each sphere, D is the dielectric constant of the medium, R the distance between the centers of the two spheres, κ the Debye–Hückel parameter, a the distance of closest approach between the center of the protein sphere and a small ion, and γ is a complicated function of κb and R/b, values of which have been tabulated by Verwey and Overbeek.[38] In the case of two touching spheres, $R = 2b$. The total free energy of attraction, ΔF^a, between the two spherical subunits[42] was taken to be -9.8 kcal./mole for both β-A and β-B.[19] ΔF^a and $\Delta(\Delta F^e)$ are related by $\Delta F^a = \Delta(\Delta F^e) - \Delta F^\circ$, the latter term being the standard free energy of dissociation. Applying eq. 6 and the above value of ΔF^a, ΔF° and thence the dissociation constant, K_d, may be obtained. Since K_d is related to α, $\bar{a}_{0,w}$ and Δa_0^{p-m} by equations similar to 3, 4, and 5, the ionic-strength dependence of the degree of association, α, could be compared to that of a_0. At low ionic strength (0.01) both proteins are essentially completely dissociated ($\alpha = 0.005$); therefore, a_0^m for both was taken as the plateau value of -162. Use of eq. 3 results then in a value of 30 for Δa_0^{p-m}. The resulting curve is drawn by the solid line of the lower part of Fig. 6; the corresponding values of the degree of association, α, are given on the right-hand ordinate of that figure. The shape of the calculated pH dependence of the a_0 curve is found to be identical with that of the experimental points, indicating that in this reaction as well a_0 is a direct function of molecular association.

In the case of β-C no thermodynamic dissociation data are available, although sedimentation data indicate that it dissociates in a manner similar to the other two variants in this pH range.[8] Translation of the α curve, calculated for β-A and β-B (shown by the dashed line of Fig. 6) agrees well with the β-C experimental points below an ionic strength of 0.1. At higher salt concentrations, however, a_0 continues to become increasingly more negative, once again indicating the existence of phenomena other than dissociation.

Results obtained at two concentrations for each of the three variants at pH 2.7, $\Gamma/2 = 0.03$, are presented in Table III, along with the a_0 values calculated from the equilibrium constant, using the above deduced values of a_0^m and Δa_0^{p-m}. The agreement with experiment is once again found to be satisfactory.

TABLE III

CONCENTRATION DEPENDENCE OF ROTATORY DISPERSION PARAMETERS OF THE β-LACTOGLOBULINS AT pH 2.7, $\Gamma/2 = 0.03$, 25°

Genetic variant	Concn., g./l.	b_0	a_0 Exptl.	a_0 Calcd.
A	5.0	−77	−159	−156
	27.7	−76	−154	−148
B	5.1	−85	−155	−156
	28.6	−73	−144	−148
C	5.2	−78	−175	−176
	35.6	−82	−167	−167

pH Alkaline to 5.2.—The last two pH zones will be treated together. The increase of a_0 with increasing pH between 5.2 and 6.5 at 2° has been described in conjunction with Fig. 5. If pH is increased further, a_0 continues to increase. Between pH 6 and 9, β-A and β-B are known to undergo a conformational transformation. The pH dependence of this transformation, determined by Tanford, *et al.*,[29] is shown by the dashed line of Fig. 2. The agreement with present data is good. It should be mentioned, furthermore, that our values of optical rotation in the region of this transformation are in excellent agreement with those of Tanford, *et al.*,[29] and Pantaloni.[43]

Above pH 9, irreversible changes are known to occur. Between pH 8.5 and 10.5, these changes are slow, since a_0 is time dependent, as shown in Fig. 2; above pH 10.5, the final value of a_0 is obtained within the first 15 min. after mixing the solution. Data on the reversibility of changes resulting from exposure to various pH are presented in Table IV. It is found that exposure to pH 7.6 does not affect b_0 and results in no irreversible changes in a_0. Exposure to higher pH or to 8 M urea, however, leads to changes in both parameters. It is remarkable that adjustment to pH 10.58 does not change b_0, while a large increase in a_0 is obtained. Reversal to acid pH from 10.6 produces partial reversal of a_0 (from -420 to -325), accompanied now by a change in b_0 to a more negative value (-120 from *ca.* -80). Standing for 1 hr. in 8 M urea at pH 3.5 produces a similar but smaller effect, the final values of a_0 and b_0 being slightly more negative than those obtained with the native isoelectric protein.

TABLE IV

EFFECTS OF HIGH pH AND 8 M UREA ON β-LACTOGLOBULIN

Genetic variant	Treatment	Rotatory dispersion parameters at pH 2.7, $\Gamma/2 = 0.03$: a_0	b_0
A[a]	None	−137	−85
B	None	−155	−85
C	None	−175	−78
A[a,b]	Exposed to pH 7.6, 25°, 30 min.	−140	−85
B	Exposed to pH 7.6, 25°, 30 min.	−163	−89
C	Exposed to pH 7.6, 25°, 30 min.	−177	−70
A[a,c]	Exposed to pH 10.6, 25°, 30 min.	−325	−120
A[a,d]	Exposed to 8 M urea, pH 3.5, $\Gamma/2 = 0.03$, 25°, 1 hr.	−256	−10

[a] Sample β-A from pooled milk. [b] The parameters at pH 7.6 for β-A are: $a_0 = -205$; $b_0 = -87$. [c] The parameters at pH 10.6 are: $a_0 = -420$; $b_0 = -77$. [d] The parameters of β-A in 8 M urea (pH 3.2, $\Gamma/2 = 0.03$) are: $a_0 = -700$; $b_0 = -20$. The urea was removed by dialysis in the cold against pH 2.7, $\Gamma/2 = 0.03$ NaCl–HCl.

Discussion

Intermolecular Associations.—The results described above show that intermolecular associations may have a strong influence on the optical rotatory behavior of proteins (as has been observed by Schellman with insulin[44]) which may be completely accounted for in terms of such interactions. Of particular interest is the magnitude and sign of the a_0 change during association, especially since it is accompanied by no change in b_0. The constancy of b_0 indicates that the 10–12% of ordered ("helical?") structure present in the native protein does not change either during association, conformational transformation, or even alka-

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

(41) Y. Nozaki, L. G. Bunville, and C. Tanford, *J. Am. Chem. Soc.*, **81**, 5523 (1959).

(42) D. W. Green and R. Aschaffenburg, *J. Mol. Biol.*, **1**, 54 (1959).

(43) D. Pantaloni, *Compt. rend.*, **252**, 2459 (1961).

(44) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30**, 415 (1958).

line denaturation. The observed b_0 value may, in fact, have nothing to do with the presence of ordered structure in the protein,[28] but may simply reflect the contribution of the side chains. The low a_0 values can be attributed to the compact structure of β-lactoglobulin (the 18,000 molecular weight subunit is found to be an impenetrable sphere by hydrodynamic,[18] small-angle X-ray scattering,[16] and X-ray diffraction[42] techniques). In such a structure the fraction of peptide bonds present in the low dielectric constant interior of the molecule would be at a maximum. This should result in a low negative value of a_0.[28,45,46]

When the two 18,000 molecular weight subunit spheres associate to a dimer, a_0 becomes less negative by 30. In this reaction a surface of contact is formed, *i.e.*, one additional locus in each subunit becomes buried in a region of low dielectric constant. This should result in a decrease in a_0. By the same token, formation of the 144,000 molecular weight "tetramer" should further decrease a_0. This aggregate has been shown to be best described by a closely packed array of eight spheres.[16] It contains an additional eight surfaces of intermolecular contact, which mean eight new regions of low dielectric constant, *i.e.*, two additional buried loci per sphere. The resulting further change in a_0 is found to be +63. It is quite striking that this value is, within experimental error, twice that obtained on the transfer of a single area unit per sphere from the outside to the solvent-free interior of the molecule, observed at low pH.

This interpretation of the direction of change in a_0 follows amazingly well the formation of new "interior" regions.[28] An alternate explanation is afforded, however, by the progressive introduction of symmetry into the structures of the various aggregated species. Association of two spheres, each of which possesses no center of symmetry as far as the interior folding of the polypeptide chain is concerned, results in a two-sphere object possessing a dyad axis of symmetry.[42] While on an atomic scale the various groups in the associated species are far removed from each other, nevertheless a small decrease in optical rotation due to the symmetrical arrangement about the axis of all atoms within this structure would not be surprising. Further association to the eight-sphere structure introduces new elements of symmetry, shown schematically in Fig. 7, which could further decrease the optical rotation. Thus, it appears that the observed decrease in a_0 during the two stages of association can be due either to the formation of new "interior" regions or to the introduction of additional symmetry into the molecular aggregates. Quite probably both effects contribute to the observed decrease in rotation.

Denaturation.—In examining β-lactoglobulin, workers in the past have always been struck by the apparently "abnormal" optical rotatory behavior of this protein. It is characterized by a low b_0 value (*ca.* −80), indicating little or no helical content, and a low a_0 value (*ca.* −130 to −160), which would suggest a great deal of order or symmetry.[47] Denaturation results in little change in b_0, but a very large change in

(45) C. Tanford, *J. Am. Chem. Soc.*, **84**, 1747 (1962).

(46) C. Tanford, C. E. Buckley, P. K. De, and E. P. Lively, *J. Biol. Chem.*, **237**, 1168 (1962). In footnote 1 of this paper, observations similar to ours have been reported on β-lactoglobulin.

(47) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).

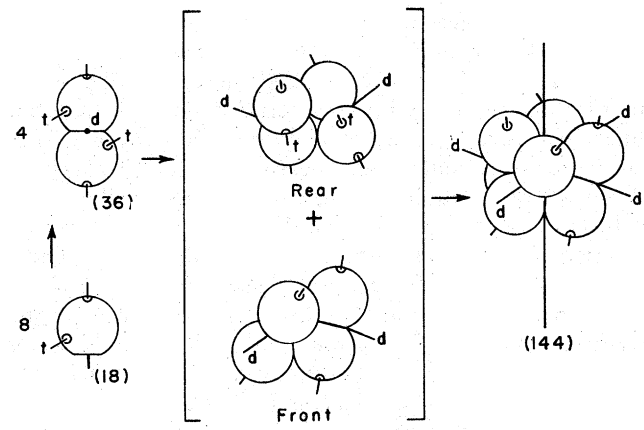

Fig. 7.—Models of progressive association of β-lactoglobulin from 18,000 (18) molecular weight to 144,000 (144). Dyad axes of symmetry are marked d, tetramerization sites t. The vertical line in the eight sphere (144) aggregate is a tetrad axis of symmetry (this structure has 422 symmetry)[S.N. Timasheff and R. Townend, *Nature*, **203**, 517 (1964)]. The identical tetramer structure has been deduced independently by Dr. D. W. Green (private communication).

$[\alpha]_D$ (to −120°), and in a_0 (to −630°). This can be seen in Fig. 2. These values suggest again very little change in helical content (which initially was almost nil) accompanied by a major conformational change. This has been interpreted in terms of changes in the dielectric constant of the medium surrounding the peptide bonds as they become exposed to the aqueous surroundings.[28] In the present study it has been found that the increase of a_0 during urea or alkaline denaturation can be partly reversed. The resulting final values of b_0 and a_0 obtained turn out to be much more "normal" than those of the native protein. The value of b_0 is more negative, indicating the appearance of some ordered regions, and a_0 is considerably more negative (by a factor of about two) than originally, bringing it into the realm of values compatible with the degree of helicity deducible from b_0.

Temperature Coefficient of a_0.—Another interesting feature of the rotation of all three types of β-lactoglobulin is their negative temperature coefficient of a_0 at pH 4.57 (Fig. 4). This agrees with the observation of Schellman[27] who found a similar change in $[\alpha]_D$ with temperature for the native pooled protein at pH 5.5; he further observed that the sign of da_0/dt changed upon urea or alkaline denaturation of the protein. Qualitative application of the rules of Kauzmann and Eyring[48] suggests that increase of temperature induces a decrease in the freedom of orientation of the groups within the native β-lactoglobulin molecules, and an increase if the protein is denatured. Tanford and co-workers[28] have argued convincingly that the primary stabilizing force of β-lactoglobulin in its native state is the hydrophobic effect, *i.e.*, the pressure of the water molecules on the nonpolar residues of the polypeptide chain tending to keep them "interior" and out of contact with the water.[49–52] This effect is characterized by a positive enthalpy, *i.e.*, it

(48) W. Kauzmann and H. Eyring, *J. Chem. Phys.*, **9**, 41 (1941).

(49) J. G. Kirkwood in "A Symposium on the Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1954, p. 4.

(50) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).

(51) G. Némethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).

(52) S. N. Timasheff in "Proteins and Their Interactions," Avi Publishing Co., Westport, Conn., 1964, in press.

becomes stronger with an increase in temperature. Thus, if this argument is valid, an increase in temperature (within the range studied here) should result in a tightening of the internal structure of β-lactoglobulin, a greater restriction of motion of the groups within the native molecule, and a greater negative rotation. A denatured protein, on the other hand, behaves in solution essentially like a random coil; an increase in temperature should increase the freedom of orientation of its groups through increased internal rotation[53] and Brownian motion and lead to a decrease in the optical rotation. In connection with this, it is of interest to note that ribonuclease, in which Tanford has found that the stabilization due to the hydrophobic effect is not sufficient to counteract the structure disruptive force of the configurational entropy, has a complicated temperature dependence of $[\alpha]_D$ in the native state, while this change is in the expected direction for the oxidized and denatured species.[54] An examination of Schellman's rotation data on some other proteins reveals that ovalbumin[55] and chymotrypsin[54] have a temperature dependence similar to that of β-lactoglobulin, although the native chymotrypsin case is complicated by a temperature-dependent transition. Ovalbumin has few if any internal disulfide bonds and therefore may owe most of its stabilization to noncovalent factors. In all cases, Schellman finds a positive temperature coefficient with denatured proteins. In some cases, such as insulin[44] and serum albumin,[55] the picture becomes complicated by aggregations and large conformational changes. It seems significant that nowhere can contradictions of the correlation with hydrophobic effect be found.

Comparison of the Three Genetic Variants.—The present optical rotation experiments have failed to show any vast differences between the gross native structures of the three genetic variants. The values of b_0 are identical within experimental error, and indicate little "helix" content in any of the three. Changes of a_0 follow quantitatively known association effects. In the pH region between 4.5 and 5.5 all three undergo a small transition in a_0, which corresponds to Tiselius electrophoretic behavior, at least with β-B.[36] In some experiments it was found that β-C is more susceptible to structural changes than either of the other two variants. At low pH, high salt concentrations lead to a continuous increase in a_0 (Fig. 6). Thus, while optical rotatory dispersion suggests great similarity of the three native isoelectric structures, in some respects it also indicates that β-C is less stable than the other two. A detailed study, aimed at elucidating further the differences between β-C and the other two better known variants, as well as the various structural changes found with changes in media, is presently under way in our laboratory.

Conclusions

The quantitative agreement between the rotatory dispersion parameters and the associations of the well characterized β-lactoglobulin system suggests the use of this technique in the study of other protein–protein interactions (*e.g.*, enzyme–substrate complex formation, antigen–antibody reaction, and the functional association of proteins made up of subunits). It should be possible to use the a_0 parameter in conjunction with other criteria of association which by themselves are insufficient to give quantitative answers. For example, while in the present case light-scattering equilibrium constants were available, the entire analysis could have been carried out by a combination of the changes in a_0 with Gilbert analysis[10,56] of sedimentation data when the system is in a state of rapid re-equilibration. Moreover, as in the case of the association of β-lactoglobulin, the magnitude and sign of the change in a_0 can lead to useful information about the geometry of the association.

Acknowledgment.—The authors wish to thank Miss Linda Mescanti for her very able performance of the spectropolarimetry measurements.

(53) See, for example, M. V. Volkenstein, "Configurational Statistics of Polymeric Chains," Interscience Publishing Corp., New York, N. Y., 1963.

(54) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30**, 439, 450 (1958).

(55) J. A. Schellman, *ibid.*, **30**, 429 (1958).

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