

The Optical Rotatory Dispersion of the β -Lactoglobulins

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SUMMARY

An optical rotatory dispersion study of the three genetic variants of β -lactoglobulin has been carried out in the wave length region of 195 to 300 $m\mu$. The results allow several conclusions. (a) The native secondary structures of all three genetic variants are close to being identical. (b) The structure of the native β -lactoglobulins is predominantly not α -helical. This is shown by the profile of the optical rotatory dispersion curve between 228 and 240 $m\mu$ which displays a small maximum instead of a minimum at 233 $m\mu$. (c) The optical rotatory dispersion data is consistent with a native structure composed of 10 to 15% α -helix, with the remainder being essentially equal amounts of random and (probably) β structures. (d) In the native protein, some aromatic residues are present in an ordered, optically active conformation, which is destroyed by denaturation leading either to unfolding or to α -helix formation. (e) Addition of methanol (to more than 30% by volume) causes a sharp transition of the β -structured regions to α -helices; this is followed by a similar slow transformation of the randomly folded regions.

β -Lactoglobulin has been recognized for a long time as a protein with rather unusual optical rotatory properties (1, 2). It has one of the lowest reported values of $[\alpha]_D$ for a native globular protein and shows an exceptionally large increase in levorotation upon denaturation. Recent investigations of the dispersion of optical rotation above 300 $m\mu$ (2-4), with application of the Drude and Moffitt-Yang equations, have shown that the native protein is characterized by a small negative value of b_0 , independent of pH between pH 1.5 and 12, as well as a small negative value of a_0 which, however, becomes large after alkaline denaturation. When the two-term Drude equation of Shechter and Blout (5) is used to calculate the amount of α -helix, disagreement arises between the amount calculated with each of the two A_λ parameters. While b_0 has been found to be rather insensitive to molecular associations and reversible conformational changes which take place at pH values below the zone of irreversible denaturation (pH > 9), a_0 is extremely sensitive both to changes in the degree of molecular associations (monomer, dimer, octamer) (4) and to the two reversible conformational transitions (pH 4.5 to 6.0 and 6.5 to 9.0) which occur (6, 7) in all three of the

genetic variants of β -lactoglobulin. It is thus of interest to examine the optical rotatory dispersion behavior of these proteins in the lower wave length region where Cotton effects should occur. These effects may well explain the anomalous a_0 , b_0 , and A_λ parameters measured at higher wave lengths, and give us information on the structural features responsible for them. That Cotton effects might be present in the region of absorption of aromatic chromophores has been suggested by the reproducible deviation of the points measured between 300 and 335 $m\mu$ from the Moffitt-Yang straight line (4). This paper presents the results of an optical rotatory dispersion study on the β -lactoglobulins below 350 $m\mu$. The three variants, A, B, and C (β -A, β -B, and β -C), were studied as a function of pH, and β -A was studied in aqueous methanol solutions of varying composition.

EXPERIMENTAL PROCEDURE

Native proteins were freshly recrystallized samples of the three genetic variants. The *S*-sulfo- β -lactoglobulins, previously prepared by the method of Pèchère *et al.* (8), were stored lyophilized at -10° . Aqueous stock solutions of the native proteins were prepared at approximately 0.5 g/100 ml in 0.01 M NaCl. Concentrations were determined spectrophotometrically on the clarified stock solutions with an absorptivity value of 9.6 deciliters per g. The solutions were then volumetrically diluted with 0.01 M NaCl and the pH adjusted by addition of 0.01 M HCl or NaOH.

Protein solutions in urea were made up with no salt other than the amount already present in the stock solution. Solutions for the methanol study were made by diluting the stock solutions with acidic methanol (100 ml of anhydrous methanol-0.01 ml of concentrated HCl) or mixtures of acidic methanol and 0.01 M NaCl adjusted to pH 2. All of the solutions were used as soon as possible after being prepared. Measurements were made on a Cary model 60 spectropolarimeter.^{1, 2} A few samples were also measured on a Rudolph model 200S instrument² at the wave lengths of a mercury arc.

Two individual parallel-faced quartz cells were used with the Cary instrument. These were, respectively, 1-cm and 1-mm light path ($\pm 0.1\%$) and were shown to be essentially strain-free by absence of significant base-line changes during the entire

¹ We wish to thank Applied Physics Corporation for their courtesy in lending us a model 60 instrument for a short period.

² It is not implied that the United States Department of Agriculture recommends the above companies or their products to the exclusion of others in the same business.

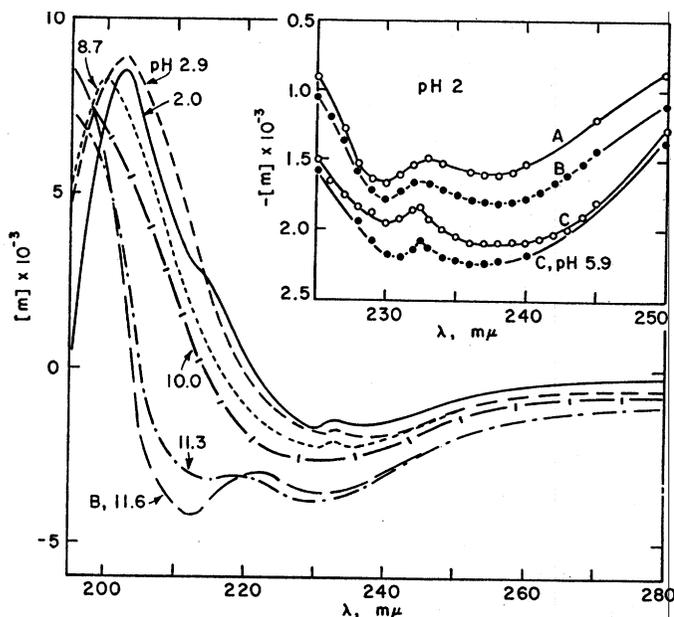


FIG. 1. Effect of pH variation between 2 and 11.6 on the low wave length Cotton effects present in the β -lactoglobulins. Inset: comparison of the three genetic variants.

series of experiments. The cells, positioned in the instrument in as reproducible a manner as possible, were never cleaned by touching the faces, but by washing with distilled water followed by reagent grade methanol and drying with air.

Lorentz factors were interpolated and extrapolated from the data of Foss and Schellman (9) and the residue rotations $[m]_{\lambda}$ were calculated by

$$[m]_{\lambda} = [\alpha]_{\lambda} M_0 \frac{3}{n^2 + 2} \quad (1)$$

where the mean residue weight, M_0 , for the β -lactoglobulins is taken to be 112, as previously reported (4). The rotatory dispersion parameters, a_0 and b_0 , of the Moffitt-Yang equation (10) are calculated from

$$[m]_{\lambda} = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (2)$$

where λ_0 is taken as 212 $m\mu$ (10).

The data on β -A as a function of pH, previously published in terms of the Moffitt-Yang dispersion parameters, were recalculated in terms of the Shechter and Blout two-term Drude equation (5) with the use of an electronic computer and the help of a Fortran program developed by T. F. Kumosinski of this laboratory.

RESULTS

195 to 270 $m\mu$ —The optical rotatory dispersion curves of β -A over this range are shown on Fig. 1 for pH values between 2.0 and 11.3. Curves obtained with the B and C variants are similar in character and magnitude of rotation and the one for β -B at pH 11.6 is shown as the broken line. Curves for β -B (pH 2) and β -C (pH 2 and 5.9) over a shorter wave length range have been included for comparison. Below pH 9 it can be seen that all of the curves are characterized by a shallow negative trough in the 228 to 240 $m\mu$ region and a positive peak at 200 to

203 $m\mu$. The 228 to 240 $m\mu$ troughs are reproducibly divided into bimodal patterns with minima at 236 to 238 and 230 to 231 $m\mu$ and small maxima at 233 $m\mu$. The values of the residue rotation $[m]$ in the troughs are of the order of -1500 to -2300° and the rise of the 233 $m\mu$ peak above the minima is 150 – 250° . While the shape of this portion of the optical rotatory dispersion curve is invariant for β -A, B, or C between pH 2 and 9, there is a distinct trend to more negative rotations as pH is increased or as one goes from β -A to β -B to β -C. This reflects exactly the previously reported changes in a_0 (4). In the same pH region, all of the variants have a positive maximum at 200 to 203 $m\mu$ with $[m]$ values of ~ 8000 to 9000 ; the peak is at 202 to 203 $m\mu$ below pH 6, shifting to lower wave lengths as the pH is raised (200 $m\mu$ at pH 8.7). As the pH is increased further into the zone of irreversible denaturation, the positive peak shifts to still lower wave lengths (below 195 $m\mu$ at pH 10), and the trough at 228 to 240 $m\mu$ also shifts to lower wave lengths as well as to more negative values of rotation. At the same time, its bimodal character disappears. At pH values above 11, the optical rotatory dispersion curve is characterized by a broad minimum at 230 $m\mu$ ($[m] \approx -3500$), a maximum at 220 to 222 $m\mu$, and another minimum at close to 210 $m\mu$. Further down, the curve rises sharply toward a positive maximum below 195 $m\mu$.

270 to 350 $m\mu$ —The β -A curves for pH 2 to 11.3 in this region of aromatic chromophore absorption are shown by the solid lines

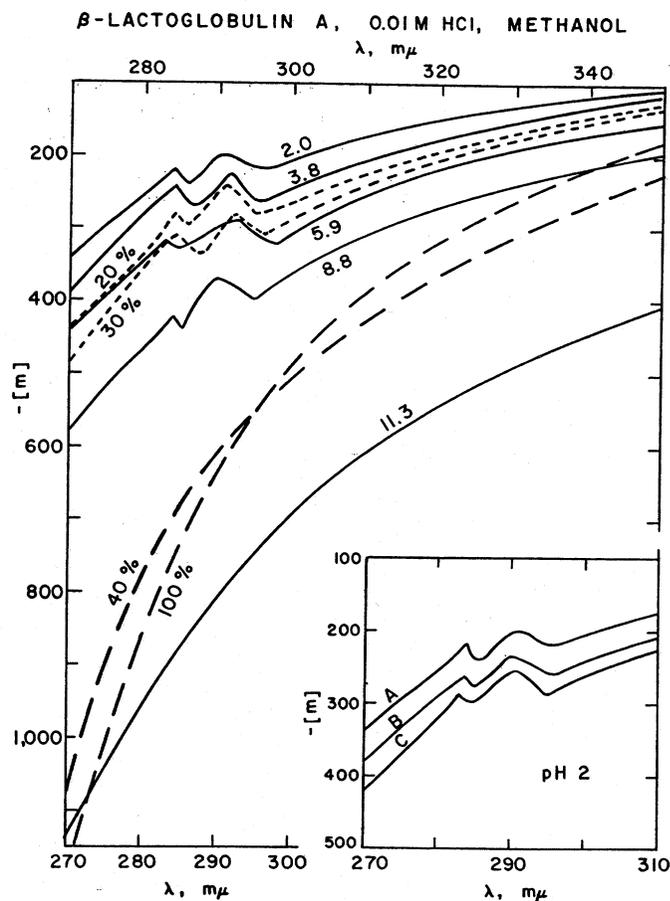


FIG. 2. Effect of variation of pH and methanol concentration on the aromatic residue absorption region Cotton effects found with β -lactoglobulin. Inset: comparison of the three genetic variants at pH 2.

of Fig. 2. In the *inset*, the three genetic variants are compared at pH 2. Above 300 m μ , all of the curves are smooth, and the rotation monotonely becomes less negative with increasing wave length. However, the region between 280 and 300 m μ is characterized by weak, but quite reproducible, aromatic Cotton effects present at pH values below 9. These have a peak at 283 to 284 m μ , a minimum at 285 to 287, a maximum at 290 to 292, and a second minimum at 295 to 296 m μ . The increase in $[m]$ from the 285 trough to the 290 peak is 50–70° for all three of the genetic variants in the pH zone of reversibility. When the protein is denatured by exposure to pH 11, all of these Cotton effects disappear, the rotation becomes highly negative, and approaches closely that observed in 8 M urea.

Effect of Methanol—The optical rotatory dispersion curves obtained with β -A in the presence of increasing amounts of methanol (0.01 M in HCl) are presented in Fig. 3. Details of the 225 to 250 m μ region (up to 40% methanol) are shown in the *inset* on an expanded scale. The 0 and 20% methanol curves are identical for all practical purposes. At 30% methanol, a small change occurs in the 225 to 240 m μ region. The rotation becomes more negative, with a broad minimum around 236 m μ , the maximum at 233 m μ vanishes, with only a point of inflection being still apparent at 229 m μ . As the methanol concentration is further increased to 40%, a dramatic change in the optical rotatory dispersion curve takes place. A well defined deep trough ($[m] = -5,800$) appears at 233 m μ , the low wave length maximum shifts down to 199 m μ , and more than doubles in

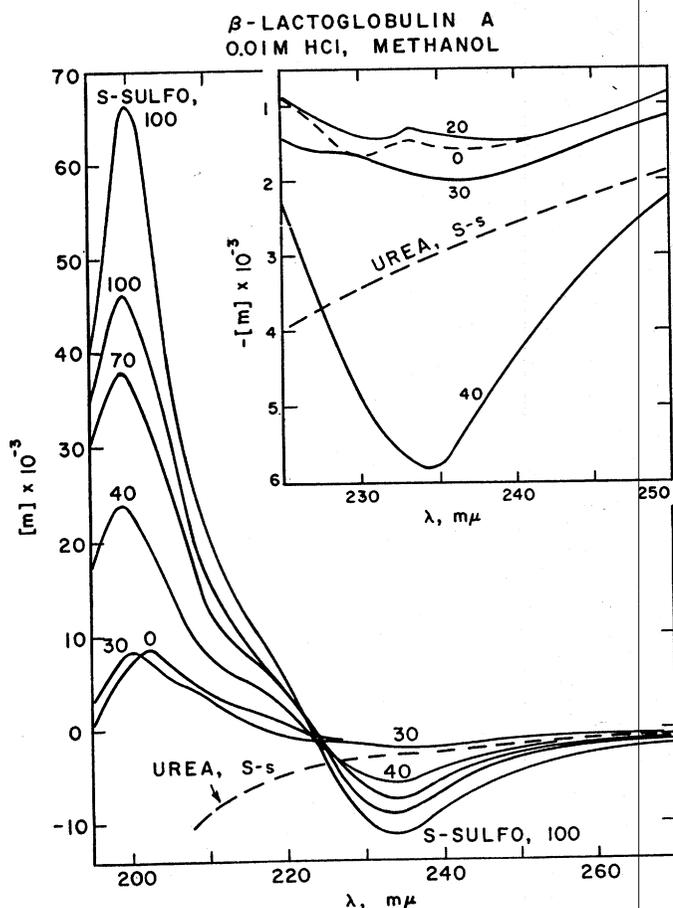


FIG. 3. Effect of methanol and urea on the optical rotatory dispersion of β -A below 270 m μ .

TABLE I

Optical rotatory dispersion parameters of β -lactoglobulin A in presence of methanol, 0.01 M in HCl

Methanol	$[m]_{233} \times 10^{-3}$	$[m]_{199} \times 10^{-3}$	a_0	b_0
%				
0	-1.6 (237) ^a	+8.5 (203) ^a	-140	-60
20	-1.5 (239) ^a	+9.0 (200) ^a	-140	-60
30	-2.0 (237) ^a	+8.2 (200) ^a	-176	-88
35			-209	-169
40	-5.8	+24.0	-225	-257
50			-171	-287
60			-122	-337
70	-7.5	+37.8		
80			-68	-403
100	-8.8	+46.0	+5	-465
100 (S-sulfonated)	-11.3	+66.0	-40	-600
8 M urea	-2.7		-710 ^b	-10 ^b
8 M urea (S-sulfo)	-3.4		-650	-20

^a The number in parentheses refers to the actual wave length at which the maximum or minimum is located and at which the reported rotation was measured.

^b Data from Reference 12.

height ($[m] = +24,000$). Here, the optical rotatory dispersion curve has assumed a form characteristic of a right-handed α -helix. Higher concentrations of methanol do not result in any additional changes in the shape of the curve, but the rotations at 199 m μ and 233 m μ progressively increase (the exact values are summarized in Table I). Rupture of the S—S bonds by S-sulfonation leads to a further increase in both positive and negative rotations in acidic methanol. The smooth curve obtained with the S-sulfonated material in 8 M urea (0.01 M HCl) is shown for comparison. It displays rotations which are continually more negative down to 210 m μ . Between 228 and 250 m μ , however, it runs between the curves for 30 and 40% methanol and is definitely more negative than the curve of the native protein.

In the higher wave length region shown in Fig. 2 by the *dashed lines*, the Cotton effects at 280 to 300 m μ are evident in 20 and 30% methanol, as in aqueous media. These Cotton effects, however, vanish completely when methanol is increased to 40%, the same concentration at which the sharp transition occurs at the lower wave lengths. This change is emphasized if the data are plotted in the Moffitt-Yang form as is shown on Fig. 4. The points obtained in 40 and 100% methanol, as well as in 8 M urea, fall on straight lines, confirming the total absence of Cotton effects in this region once the secondary structure has been altered. For comparison, data obtained in 20 and 30% methanol are also shown on this figure. It is evident that Moffitt-Yang plots are seriously perturbed even by relatively weak Cotton effects, such as those observed in the present case. Although there is no theoretical basis whatever for such a plot when Cotton effects are present in the 280 m μ region, its use can show the complete disappearance of such effects upon denaturation by the sudden change of the curves into straight lines.

The high wave length (315 to 590 m μ) data in methanol have been analyzed in terms of the Moffitt-Yang equation, and the a_0 and b_0 rotatory dispersion constants are given in Table I.

DISCUSSION

Secondary Structure of β -Lactoglobulins—From the shape of the optical rotatory dispersion curves of the three known genetic

β-LACTOGLOBULIN A
0.1M HCl

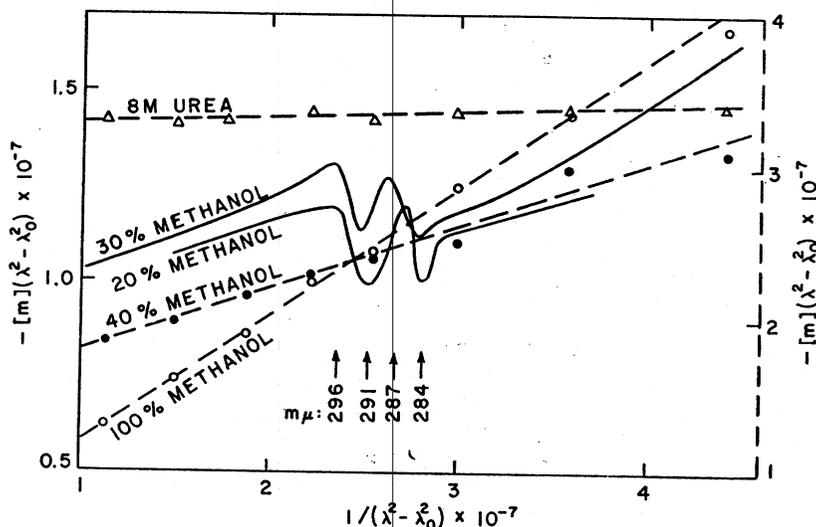


FIG. 4. Moffitt-Yang representation of the aromatic absorption region of optical rotatory dispersion data on β -A in the presence of methanol and urea.

variants of β -lactoglobulin (A, B, and C), it is evident that they have a very similar folding of their polypeptide chains and that, furthermore, changes which occur in this conformation with changing pH are essentially identical in all three.

The unusual optical rotatory behavior has been explained by three alternate possibilities (3, 11). (a) β -Lactoglobulin contains almost equal amounts of right-handed and left-handed α -helices; these unfold at the same rate during alkaline denaturation, while the left-handed ones are transformed to right-handed ones in organic solvents. (b) β -Lactoglobulin contains no α -helix at all. (c) β -Lactoglobulin contains some non- α -helical ordered structure, e.g. the pleated-sheet or β structure, whose contribution to the rotation causes the anomalous results in terms of the Moffitt-Yang and Shechter-Blout treatments. While the first alternative is discussed by Bell and McKenzie (13), Tanford, De, and Taggart (3) reject the first two on the basis of studies of this protein in organic solvents. Furthermore, the fortuitous simultaneous unfolding of both senses of α -helix appears most unlikely in view of the difference between their energies of stability (14). Such an unfolding could take place, however, if the two structures were closely linked, by S-S bonds for example, and were forced to undergo all of the transitions in a cooperative manner. The expected differences in behavior of such a structure in helix-forming and unfolding agents is hard to reconcile with that actually observed in β -lactoglobulin. The low wave length optical rotatory dispersion curves (Fig. 1) are a further argument for rejection of the first alternative, since the shape, with minima at 236 and 230 $m\mu$ and a maximum at 233 $m\mu$ are incompatible with a predominance of α -helical structure. Thus, β -lactoglobulin would seem to fall into the classification of Jirgensons (15) as an abnormally behaving globular protein.

We are now faced with identifying, if possible, the regular, nonhelical structure mentioned in the third alternative.

Litman and Schellman (16) have shown that many different conformations of peptide bonds can result in Cotton effects in the 220 to 240 $m\mu$ region. In the case of β -lactoglobulin, infrared absorption measurements on β -A between pH 1 and 12

have revealed (17) that in the native protein the predominant bands are those normally attributed to the β or pleated-sheet structure (18, 19). With this information, an attempt was made to analyze the optical rotatory dispersion properties of β -lactoglobulin in terms of a mixture of α -helix, β structure, and randomly folded chain (R structure).³

In the native state, at pH 4 and 25°, β -A has values of Moffitt-Yang parameters a_0 and b_0 equal to -140 and -60 ,⁴ respectively. From these experimental values and values of the parameters characteristic of the three pertinent types of structure, it is possible to calculate the relative amount of each present by using the equations (11, 20)

$$b_0 = f_\beta(b_0)_\beta + f_R(b_0)_R + f_\alpha(b_0)_\alpha \quad (3)$$

$$a_0 = f_\beta(a_0)_\beta + f_R(a_0)_R + f_\alpha(a_0)_\alpha \quad (4)$$

$$f_\beta + f_R + f_\alpha = 1 \quad (5)$$

where f is the fraction of each of the three structures and the subscripts β , R , and α refer to β structure, random chain folding, and α -helix, respectively. The values used for α -helix and random conformation are those normally assigned to these

³ In the present discussion of the internal structure of a globular protein, the term random structure does not imply a conformation in which neighboring residues are free to change their mutual positions and orientations, as is true in the random coil form of many synthetic polymers. It is meant rather that, in certain regions of the molecule, there is no continuous order, but the mutual positions and orientations of consecutive residues in the polypeptide chain change in an apparently arbitrary fashion. These positions and orientations, however, are fixed so that the three-dimensional space coordinates of the residues in question remain constant with time (except for small thermal fluctuations) and are essentially identical in all of the protein molecules present in the given conformation. Thus, such portions of the protein molecules give the appearance of being in a disordered, or random, structure, which, in fact, is composed of a number of well defined different conformations fixed in space.

⁴ The previously reported (4) higher values of b_0 were in error due to inclusion of points below 335 $m\mu$ in the calculation of b_0 . As is shown in the present paper, these are seriously affected by Cotton effects occurring below 300 $m\mu$.

structures ($(a_0)_\alpha = 0$, $(a_0)_R = -650$, $(b_0)_\alpha = -630$, $(b_0)_R = 0$) and are very close to those obtained with *S*-sulfonated β -lactoglobulin in acid methanol (α -helix) and 8 M urea (random) (see Table I). The a_0 and b_0 parameters used for the β conformation ($(a_0)_\beta = +400$, $(b_0)_\beta = 0$) are those reported by Ikeda, Maeda, and Isemura (21); these are much less certain than the corresponding values for the α and *R* structures. An average of the literature values (21-24) results in $(a_0)_\beta = +600$, $(b_0)_\beta = +100$. The use of these average values in the subsequent calculations, however, changes the derived amount of the β conformation by only 6% and does not alter significantly the conclusion that a considerable amount of β structure may be present in native β -lactoglobulin.

Before proceeding with the analysis, however, it was necessary to approximate the amount of α -helix present, since the value of $b_0 = -60$ can only give a minimal (25) helix content (9.5%) due to the presence of Cotton effects between 287 and 292 $m\mu$. α -Helix was therefore estimated from an examination of different indicators. (a) Comparison of height of the peak at 203 $m\mu$ with that of *S*-sulfo- β -lactoglobulin in acidic methanol results in 13% α -helix with the assumption that the 203 $m\mu$ peak is due primarily to this structure. (b) At alkaline pH values, where the aromatic Cotton effects disappear, b_0 attains values of approximately -80 , suggesting again 13% helix. (In this pH range, it would appear that b_0 actually does reflect the amount of α -helix present, since the two Shechter-Blout H_λ constants agree at about 18% helix. See below and Fig. 6.) In light of these facts, a value of 10 to 15% α -helix appears to be reasonable. By taking 13%, application of Equations 4 and 5, with $a_0 = -140$, results in 40% β structure and 47% randomly folded chain (use of the averaged parameters for β structure, gives 34% β and 53% *R*, which is certainly within the limits of this approximate analysis).

The shape of the optical rotatory dispersion curve of alkali-denatured β -lactoglobulin measured at pH 11.6 is typical for a polypeptide containing some α -helix but predominantly existing in a random structure; it is, in fact, strikingly similar to that of poly- α -L-glutamic acid at pH 6 (26). Since b_0 does not change with pH, the value of -76 at pH 11.6 (4) suggests again 13% α -helix. When this value and an a_0 value of -550 (4) are used with Equations 3 and 4, the result indicates no β structure remaining, the rest of the polypeptide chain being randomly folded at this pH. Since rotation at any wave length is the sum of rotations of all contributing structures, the portion of the dispersion curve due to the randomly folded component should be determinable by subtracting 13% of the $[m]_\lambda$ values of *S*-sulfonated β -lactoglobulin in acidic methanol (assumed 100% α -helix) from the experimental pH 11.6 curve of Fig. 1. The differences obtained are plotted as Curve A of Fig. 5. This curve has a shape typical of a random structure (27, 28) with a minimum at 208 $m\mu$, followed by a sharp rise on the low wave length side, crossing zero near 197 $m\mu$ and still rising at 195 $m\mu$. Furthermore, between 260 and 210 $m\mu$, this difference curve coincides with points obtained in 8 M urea and 0.01 M in HCl, conditions at which β -lactoglobulin is essentially completely denatured.

The presence of β structure in native lactoglobulin was further tested by subtracting the rotatory contributions of 13% α -helix and 47% random conformation from the experimental curves. The rotations subtracted were taken from the curve of *S*-sulfonated protein in acidic methanol (Fig. 3) and the curve of pH 11.6 corrected for residual α -helix (Curve A of Fig. 5). The resultant curve is given as B in Fig. 5. This dispersion curve is charac-

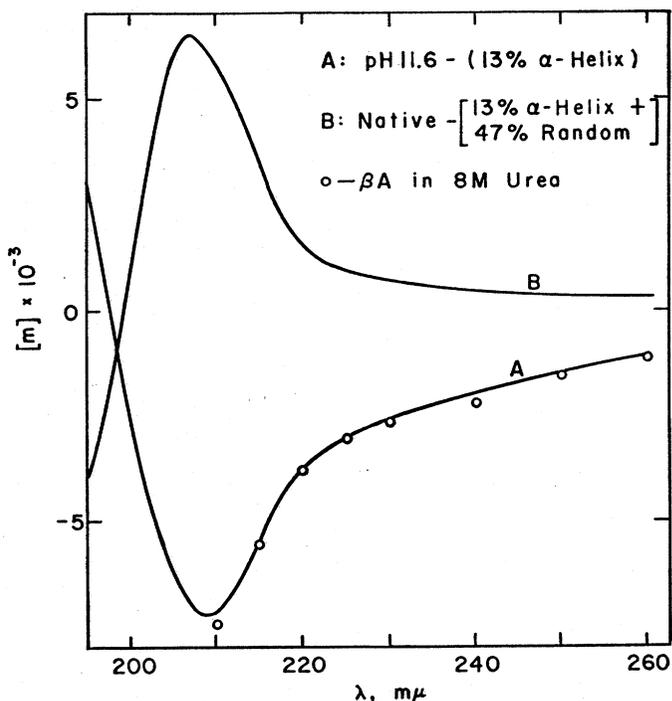


FIG. 5. Optical rotatory dispersion of the random portion of the β -A molecule at pH 11.3 (Curve A) and of the "third structure" of native β -lactoglobulin (Curve B). The circles are experimental points obtained in 8 M urea, pH 3.5.

terized by a positive maximum at 207 $m\mu$, negative rotations below 199 $m\mu$, and a very low positive rotation in the region 230 to 260 $m\mu$. This curve is quite similar to the one reported by Blout and Shechter (29) for an oriented β -structured film of polyisoleucine. Their curve also shows a maximum at 207 $m\mu$ and negative rotations below 197 $m\mu$; however, they report negative rotations with a broad trough around 230 $m\mu$, a feature not reproduced by the difference of optical rotatory dispersion curves of β -lactoglobulin. The similarities, on the other hand, permit the conclusion that 40% β structure is in no way incompatible with the rotatory dispersion of β -lactoglobulin. Furthermore, a ratio of the rotations at the 207 $m\mu$ peak of Fig. 5, B ($[m] = 6,500$), and the Shechter-Blout curve ($[m] = +20,000$) results in 33% β structure which agrees as well as could be expected with our estimated value of 40%.

An additional argument in favor of the presence of the three different structures in native β -lactoglobulin can be drawn from the Shechter-Blout (5) analysis of the previously published (4) optical rotatory dispersion data above 310 $m\mu$ over the pH range from 1.5 to 12.5. The results of this analysis are presented in Fig. 6, in which their A_λ and H_λ constants have been plotted as a function of pH, together with the best line through the a_0 points at the same pH values. Below pH 7, where there is little pH variation in any of the parameters, the A_λ constants yield highly inconsistent apparent α -helix contents (14 and 28%), suggesting presence of other structure or structures. Between pH 7 and 9.5, β -lactoglobulin undergoes a conformational transition which strongly affects a_0 as well as the Shechter-Blout parameters; at pH 9.5 the derived helical contents are 19 and 26%. As the pH is raised above 11, irreversible changes occur with rapid increases in $-a_0$ as well as in A_{193} . This latter change leads to agreement between the two derived H_λ values at an

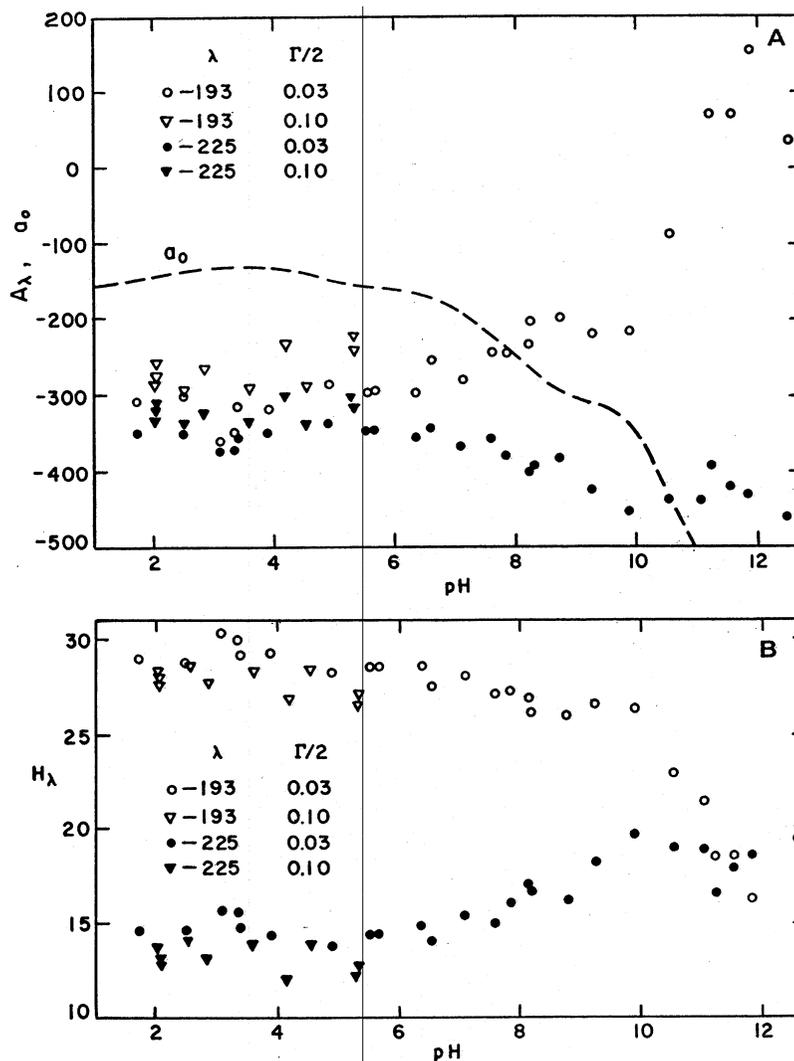


FIG. 6. Shechter-Blout representation of the optical rotatory dispersion data on β -A as a function of pH

apparent helical content of 17 or 18%. This is the pH region in which the three-structure analysis, described above, does not require the invoking of β structure. Below this pH, both the three-structure analysis and the Shechter-Blout method indicate that conformations other than α -helical and random are present in β -lactoglobulin. That this third conformation appears to be a quite plausible deduction.

Aromatic Cotton Effects—The Cotton effects between 280 and 300 $m\mu$ displayed below pH 9 by all three of the variants indicate that some aromatic side chains, located in an ordered region in the native proteins, have little freedom of motion. Comparison of the positions of maxima and minima with those reported in the literature suggest very strongly that the groups involved are tryptophans. For example, Myers and Edsall (30) have been able to associate part of the very striking aromatic Cotton effects given by carbonic anhydrase with tryptophan residues, in particular, the peak at 293 and trough at 289 $m\mu$. With β -lactoglobulin, the corresponding features lie at 292 and 287 $m\mu$. By analogy with carbonic anhydrase and the demonstration by Fasman *et al.* (25, 31) of the Cotton effects in polytryptophan and polytyrosine, the peak and trough at 292 and 287 $m\mu$ in β -lactoglobulin may be assigned to tryptophan residues, while the peak at

284 $m\mu$ may be due as well to tyrosine participation (32). The contribution of cystines must also be considered (33). In every case the maxima and minima given by β -lactoglobulin are 1 or 2 $m\mu$ lower than found by Fasman *et al.*, but it seems pertinent to point out that solvent perturbation difference spectroscopy has shown (12) that, in native β -lactoglobulin three out of four tryptophans per isoelectric molecule are buried.

The conformational transitions which occur in β -lactoglobulin below pH 9 do not seem to be reflected in the environment of the aromatic residues. No change is observed at all during the transition between pH 4 and 6. The more drastic transition between pH 6 and 9 seems to result in a slight enhancement of these effects. The curve obtained at pH 8.8 is strikingly similar to that reported by Kronman, Blum, and Holmes (34) for α -lactalbumin at pH 6. By comparison with spectroscopic data, these authors have attributed the entire set of Cotton effects in this region to tryptophan residues. In the case of β -lactoglobulin at pH 8.8, this question must remain open for the present.⁵ It should be noted that the change in pH from 6 to 8.8 results also

⁵ Unfortunately, it has not been possible to examine this transition by differential spectroscopy because of incipient tyrosine ionization at this pH.

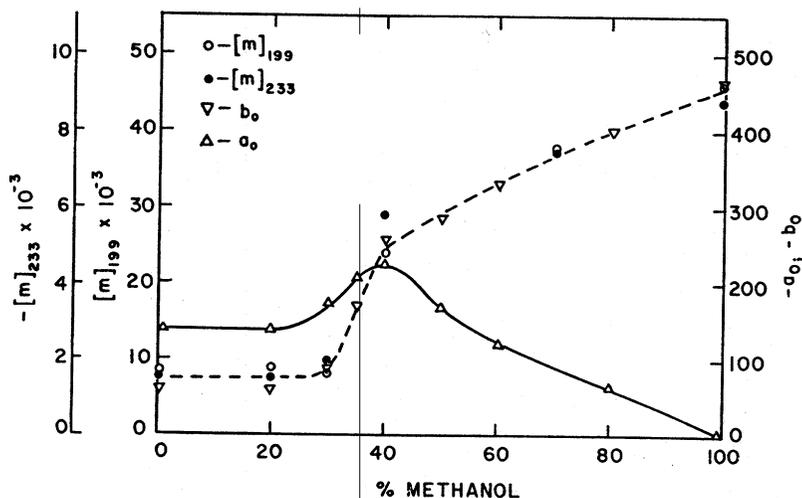
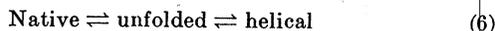


FIG. 7. Variation of a_0 , b_0 , $[m]_{199}$, and $[m]_{233}$ of β -A with increasing methanol concentration

in a shift of the positive low wave length peak from 203 to 200 $m\mu$ and a change in the Moffitt-Yang parameter, a_0 , from -150 to -270 . Both alkaline denaturation, which yields a random structure, and alcoholic, which causes α -helix formation, destroy the internal order responsible for the aromatic Cotton effects in a manner similar to the acid denaturation of carbonic anhydrase (30). Thus, in β -lactoglobulin, the ordered arrangement of the tryptophans, and possibly also the tyrosines, is intimately related to secondary structure, a situation which is not necessarily true in other proteins (34).

Methanol Denaturation—As has been shown above, addition of methanol to a β -lactoglobulin solution in 0.01 M HCl results in no changes below 30% by volume. By the time 40% is reached, the detailed features characteristic of the native structure have disappeared and a curve typical of predominance of α -helix is seen. Indeed, the curve obtained in 40% methanol is very like that obtained with poly-L-glutamic acid at pH 5.5 (26). A plot of the numerical values of the rotatory parameters $[m]_{199}$, $[m]_{233}$, b_0 , and a_0 as a function of methanol concentration (Fig. 7) shows the sharpness of the transition. While no changes are evident below 25% methanol, the first three rotatory parameters increase sharply in absolute value between 30 and 40%, the increase continuing at a much slower rate with further increase in methanol content. All three fall on the same transition curve, indicating that they reflect the conformational change in an identical manner. a_0 , on the other hand, rises steeply between 25 and 40% methanol, and then drops off in a monotone fashion. This behavior, in the case of β -lactoglobulin A, is quite similar to that of the b_0 and a_0 parameters observed by Tanford, De, and Taggart (3, 20) on mixed β -A- and β -B-lactoglobulin in helix-promoting organic solvents. By comparing their data to changes observed on addition of unfolding agents such as urea and formamide, they interpreted the observed changes in a_0 and b_0 in helix-promoting solvents in terms of a two-step transition.



An over-all transition from native to helical may proceed in two alternate ways. (a) The native structure may unfold to a random conformation which becomes predominant in a given range of solvent composition, or, (b) the unfolded (random) conformation may be present in only a small amount because it

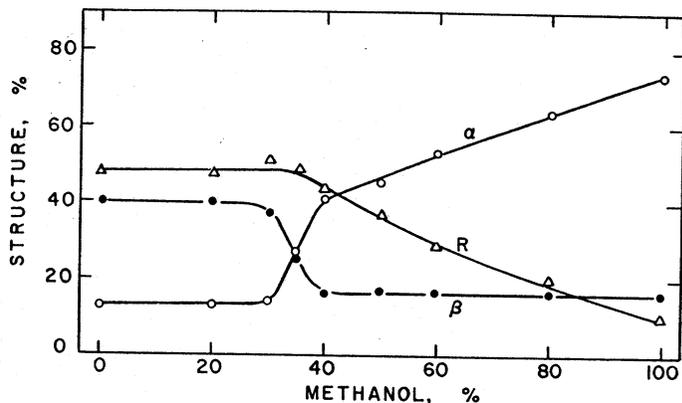


FIG. 8. Deduced structural composition (random, α -helix, and β structure) of β -A as a function of methanol concentration.

is rapidly refolded into an α -helical conformation. In their analysis of the data, Tanford *et al.* have taken as their three structural states the average native conformation, the α -helix, and the unfolded structure.

In analysing the methanol data, we have chosen to examine the data at each methanol concentration in terms of a mixture of α -helix, β structure, and randomly folded chain. By taking the values used above for b_0 and a_0 of the three conformations and applying Equations 3, 4, and 5 to the experimental a_0 and b_0 values at the various solvent compositions, we have obtained the results summarized on Fig. 8. The most noteworthy feature of the dependence of conformational composition on methanol content is the simultaneous sharp rise in the amount of α -helical structure and sharp decline in β structure in the interval of methanol composition from 30 to 40%;⁶ the first reflects the rapid change in b_0 to more negative values, the second the simultaneous change in a_0 in the same direction. It can be seen that the amount of random structure practically does not change over this range. Above 40% methanol, as the medium becomes less polar, the continued increase in the amount of α -helix occurs at

⁶ Again, use of other reported values of a_0 and b_0 for the β structure results in somewhat different percentages of the various structures without, however, altering the qualitative aspect of the transition in methanol.

the expense of random structure. The content of β structure, on the other hand, is unchanged up to 100% methanol, remaining at 14 to 16% depending on the value assigned to the Moffitt-Yang parameters. It is interesting to speculate whether this portion of the native molecule, which seems to be unaffected by solvent, might indeed be the large tryptic peptide which does not move on paper during high voltage electrophoresis (35).

While the present data indicate that at no time is the amount of random structure significantly increased by apolar solvents, it does not rule out the possibility that the $\beta \rightarrow \alpha$ transformation involves the formation of partially disordered intermediates of short lifetime. Such a pathway appears quite reasonable for both geometric and thermodynamic reasons. Nozaki and Tanford (36) have shown that in alcoholic medium unfolded protein conformations are not favored thermodynamically since the free energy of transfer of peptide linkages from water to alcohol is positive. If the $\beta \rightarrow \alpha$ transformation must go through such intermediates, these would be strongly driven to the lower energy α -helical conformation. Our experimental results are fully consistent with such a mechanism.

In addition, it is possible that some of the β structure is located on the surface of the protein molecule in the region of contact between the two identical chains in the native dimer. This dimer is dissociated into its subunits in acidic 40% methanol (37). Consistent with such a hypothesis is the fact that association of the monomeric subunits to the dimer or tetramerization of the β -A dimer to form a low temperature octamer are accompanied by a decrease in the negativity of a_0 but no change in b_0 (4). Although other explanations of this observation have been made, it is additionally possible that upon association, the amount of β structure present in the surface may be increased. From the a_0 value deduced for the octamer (4), one would obtain an increase in β structure from 40 to 47% with a concomitant decrease in the random portion of the molecule. Should the β structure, which goes over into α -helix in methanol, be located in or near the surface of the molecule (it would amount to only about 20% of the total protein structure), then the transition to α -helix could conceivably occur directly without the intermediate unfolding step. This would be followed then by the gradual ordering of the interior which had become more exposed to solvent as a result of the structural changes in the surface.

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