Steiner, R. F. (1966), *Arch. Biochem. Biophys.* (in press).
Walsh, K. A., Kauffman, D. L., Kumar, K. S. V., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 301.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.
Wu, Y., and Scheraga, H. (1962), *Biochemistry 1*, 905.

Inter- and Intramolecular Interactions of α-Lactalbumin. VI. Optical Rotation Dispersion Properties*

Martin J. Kronman, Robert Blum, and Leo G. Holmes

ABSTRACT: Acid denaturation of α-lactalbumin occurring below pH 4 is accompanied by a decrease in the value of the optical rotation dispersion parameter, b_0, of the order of 75°. Moffitt–Yang plots of the native protein (*ca.* pH 6) in the visible and near-ultraviolet regions exhibited systematic deviations at the lower wavelengths (Hg lines 334 and 313 mμ). Plots for the acid-denatured protein were linear for the entire wavelength range considered. The amplitudes of troughs of the 225-mμ Cotton effects were virtually identical for the pH 2 and 6 protein, indicating that the change in b_0 was not due to "melting out" of helical regions of the α-lactalbumin molecule. Measurement of rotations in the range 250–300 mμ revealed a system of Cotton effects for the native protein (pH 6 and 7.48) with peaks at *ca.* 300 and 280–290 mμ, a trough at 290–295 mμ, and a broad plateau region extending from *ca.* 255 to 275 mμ. The rotatory dispersion spectrum for the acid-denatured protein was devoid of such Cotton effects. The alkaline-denatured protein (pH 11.4) showed a smaller decrease in b_0 with the Cotton effects system (250–300 mμ) being still evident but significantly reduced in amplitude. The Cotton effects observed for the native protein are probably due to the three "buried" tryptophans which are frozen in particularly favorable conformations.

Denaturation, which involves swelling of the α-lactalbumin molecule, permits greater free rotation of these tryptophans, thereby eliminating these special conformations. Since alkaline denaturation involves a lesser degree of swelling, these Cotton effects persist to some degree. Both the changes in b_0 and the systematic deviations in the Moffitt–Yang plots appear to be a consequence of the side chain Cotton effects. These observations illustrate the caution that must be exercised in interpreting optical rotation dispersion measurements for proteins with high contents of aromatic amino acids.

In this series of papers we have been considering various aspects of the several conformational changes that α-lactalbumin is capable of undergoing. The most subtle of these occurs when α-lactalbumin is brought from 25 to 0–2° (Kronman and Holmes, 1965). It is seen most distinctly at pH 6 where on lowering the temperature the two "exposed" tryptophan groups become inaccessible to the larger perturbants, sucrose and glycerol, but are "seen" by the smallest perturbant, heavy water (Kronman and Holmes, 1965). The two tryptophan groups involved appear to lie in "crevices" which "contract" as the temperature is lowered. Below pH 4 a more drastic conformational change occurs which, although accompanied by a relatively large "denaturation blue shift" of the tryptophan absorption spectrum (Kronman *et al.*, 1965b), does not involve an increased "exposure" (solvent perturbation) of tryptophan groups (Kronman and Holmes, 1965). Above pH 10 a process similar to that occurring below pH 4 takes place: swelling of the molecule occurs (F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation; M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation) accompanied by a short wavelength shift of the tryptophan absorption spectrum (M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation).

Preliminary measurements of the optical rotation of α-lactalbumin carried out in collaboration with the late L. Weil at the Eastern Regional Research Laboratory suggested that the pH 4 conformational change might involve some "melting out" of helical regions of the molecule. Subsequent determination of the optical rotation dispersion of α-lactalbumin carried out in the Natick Laboratories and tentative interpretation of the changes in the parameter b_0 had indicated that as much as one-third of the helical content had been lost.

* From the Pioneering Research Division, U. S. Army Natick Laboratories, Natick, Massachusetts. *Received January 31, 1966; revised April 4, 1966.* Presented in part before the Division of Colloid Chemistry at the 144th National Meeting of the American Chemical Society, Los Angeles, Calif., April 1963.

Certain abnormalities in the Moffitt–Yang plots obtained with the data for native protein, however, lead to the suspicion that this interpretation might not be correct. This paper presents the results of a more detailed study of the optical rotation properties of α-lactalbumin which has led to the conclusion that the changes observed in the parameter b_0 are probably due to alterations in the freedom of rotation of side chains such as tryptophans rather than to "melting out" of helical regions.

The results to be reported are a striking illustration of the necessity of critical interpretation of optical rotation dispersion data for proteins containing large amounts of aromatic amino acids. This conclusion has been anticipated by Hooker and Tanford (1964) and Fasman *et al.* (1964) from their study of the Cotton effects of tyrosine and tyrosine polymers. A preliminary account of this work has been previously made (Kronman *et al.*, 1965a).

Experimental Section

Materials. Preparations R49 and 45G of α-lactalbumin used in this study have been previously characterized (Kronman *et al.*, 1964, 1965b; Kronman and Holmes, 1965; Robbins *et al.*, 1965). Polyglutamic acid (PGA)[1] was obtained from Mann Laboratories (Lot No. 2036, intrinsic viscosity at pH 7.1 given as 1.31). Other chemicals were of reagent grade. Glass-distilled water was used throughout.

Preparation of Solutions. Solutions of α-lactalbumin free of ammonium sulfate were prepared as previously (Kronman *et al.*, 1965b) and then centrifuged to remove a small amount of haze commonly present. Adjustment to the desired pH was made by addition of small increments of acid or base with a Gilmont ultramicropipet to a known volume of protein solution. The adjustment was made in a water-jacketed vessel at the temperature at which ORD measurements were to be made. As pointed out previously (Kronman *et al.*, 1965b) the manner of pH adjustment and the temperature at which it is carried out is particularly important if precipitation of protein is to be avoided. Measurements of pH were made with a Beckman Model G pH meter using Leeds and Northrup miniature external electrodes. In a few earlier experiments at 25°, adjustment was at ambient temperature (22–28°) using the Model G internal electrodes. Concentrations of α-lactalbumin were obtained by ultraviolet analysis (Kronman and Andreotti, 1964) of the original dialysed solution, making appropriate correction for the dilution incurred on addition of acid or base. In order to eliminate possible effects of stray light, concentrations were chosen to maintain an optical density of ≤ 2 at the wavelength and the path length employed.

Solutions of PGA were prepared by dissolving the solid polymer in pH 4.6, 0.2 M acetate, or pH 7.3, 0.2 M phosphate buffer, the concentration being calculated from the nitrogen content determined for the solid, thereby avoiding the problem of residual water of hydration (Yang and Semejima, 1963).

ORD Measurements. ORD measurements were made with a Rudolph MSP-4 double monochromator, manual spectropolarimeter (see also Yang and Semejima, 1963). We have found a tendency of the wavelength calibration to "drift" in the visible region to be inherent in the construction of the instrument. This was minimized by maintaining the polarimeter room temperature at 22 ± 1.0°. In order to further circumvent this difficulty, measurements in the visible and near-ultraviolet regions were confined to the lines from a low-pressure mercury lamp (313, 334, 365, 405, 436, 547, and 578 mμ). In practice the monochromator was adjusted for maximum intensity in the region of a line and the wavelength was taken to be the nominal value rather than the monochromator reading. Wavelength drift was small for the near ultraviolet, *e.g.*, ±1 mμ, and was quite unimportant at still lower wavelengths.

Measurements in the ultraviolet were made with an Osram XBO 450-w xenon lamp. Our experience with this lamp polarimeter configuration demonstrated the necessity for careful monochromator alignment. Symptoms of relative misalignment of the two monochromators were: (a) at fixed setting of the entrance slits (1st monochromator) and exist slits (2nd monochromator), the wavelength of the exiting light (observed visually) was dependent on the position of the intermediate slit. (b) For fixed wavelength and fixed entrance and exit slits (*e.g.*, 0.10 and 0.05 mm) the intensity of light decreased rapidly as the intermediate slit was closed, falling virtually to 0 well before complete closure. Alignment of monochromators was effected by focusing a mercury lamp on the rear of the xenon lamp (with the rear plate of the xenon lamp housing removed) with the monochromator set at the 302.2-mμ Hg line. The mirror screws of both monochromators were alternately adjusted until maximum intensity was restored after closure of the intermediate slit. This process was repeated for successively smaller exit and entrance slits. This procedure was found to eliminate the symptoms described above and to bring the wavelength scale into good correspondence with the Hg line values, *i.e.*, with the exception of the 365-mμ line (error +1.3 mμ) the agreement between 302.3 and 546.1 mμ ranged from − 0.5 to 0.3 mμ.

Measurements were made in 10-, 1-, and 0.1-cm jacketed cells; the first of these was an all-quartz fused cell, the latter two were brass-jacketed Teflon cells with removable quartz window (Rudolph Instruments Engineering). In all cases blanks were determined immediately before or after (in some cases both before and after) measurements of a solution prior to disassembly or cleaning. Polarimeter slit widths for α-lactalbumin were ≤ 0.5 in the region 320–260 mμ and ≤ 0.3 mm in the region 260–225 mμ, corresponding to band widths of <1 and 2 mμ, respectively.

The 225-mμ Cotton Effect for PGA. In order to provide a basis for comparison of ultraviolet ORD data

[1] Abbreviations used in this paper are: PGA, polyglutamic acid; ORD, optical rotation dispersion.

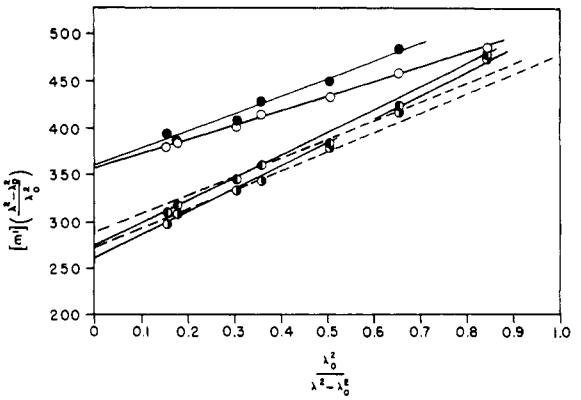

FIGURE 1: Moffitt–Yang plots for native and denatured α-lactalbumin, all data obtained with a single protein concentration for the entire wavelength range, path length, 10 cm. Solvent, 0.15 M KCl; temperature, 25°; protein concentration 0.7–1.2 g/100 ml; ●, pH 11.5; ○, pH 2.05; ◐, pH 5.63; ◑, pH 7.37. See text for explanation of dotted and solid curves. λ_0 taken to be 212 mμ.

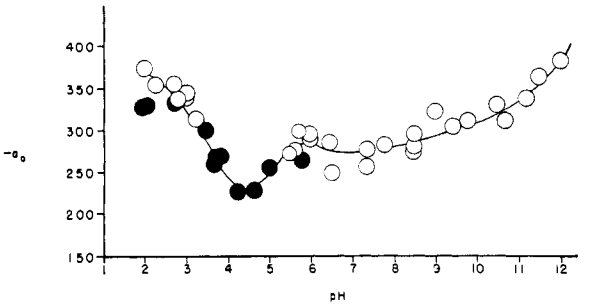

FIGURE 2: pH dependence of a_0 for α-lactalbumin. See Figure 1 for experimental details. ○, 25°; ●, 0–2°.

with those of other investigators, the ORD properties of PGA were examined. The position of the trough of the peptide Cotton effect for the α-helical form (pH 4.6, concentration *ca.* 0.1 g/100 ml) was found to be 233 mμ and $[m']_{233}$ was 15.4×10^3, in good agreement with currently reported values [see Yang and McCabe (1965) for a summary of these measurements]. A value of 1.88×10^3 was obtained for $[m']_{233}$ for the random form which is of comparable magnitude with values reported previously (Simmons *et al.*, 1961; Yang and Semejima, 1963). In contrast with the observations of Yang and Semejima (1963), who also used an MSP-4 spectropolarimeter, we found no significant slit dependence to $[m']_{233}$ over a range of band widths of 0.5–1.5 *m* (the change in rotation was *ca.* 0.7%). The difference in behavior of the two instruments may be the result of our alignment procedure (see above).

Results

ORD in the Visible and Near-Ultraviolet Regions. Shown in Figure 1 are representative ORD data plotted in the form of the Moffitt-Yang equation (Moffitt and Yang, 1956) for α-lactalbumin at pH 2.05, 5.63, 7.37, and 11.50 in 0.15 M KCl. The development of a small amount of turbidity at pH 11.5 prevented accurate determination of the rotation at 313 mμ. This point consequently has been omitted from the pH 11.5 curve. The data at pH 1.05 are well described by a straight line, in contrast with those obtained at pH 5.63 and 7.37. The solid lines in Figure 1 have been drawn through all seven points, assuming all wavelengths to have equal weight. The dotted lines have been drawn through the points corresponding to the five highest wavelengths. This deviation from the Moffitt–Yang equation at lower wavelengths occurs, as we shall see subsequently, from *ca.* pH 4 to >11. As we have pointed out in a preliminary communication (Kronman *et al.*, 1965a), regression lines computed for data obtained near pH 6 invariably lead to higher values of b_0 and of the average deviation of b_0 when the data obtained at 313 and 334 mμ were included. A similar treatment for data obtained near pH 2 showed b_0 to be insensitive to the inclusion or rejection of data at the two lowest wavelengths. At pH values between 7 and 11, for reasons which are not clear, the precision of the data is somewhat poorer, such that the regression lines, while yielding higher values of b_0 for the complete wavelength range, did not demonstrate a higher average deviation for this parameter as was observed at pH 6.

TABLE 1: Moffitt–Yang Parameters for Native and Denatured α-Lactalbumin at 25°.[a]

Protein (pH)	$-a_0$	$-b_0$
Native (7.37)	260 (268)[b]	250 (220)[b]
Native (5.63)	275 (280)[b]	230 (205)[b]
Alkaline denatured (11.5)	362	182
Acid denatured (2.05)	358	154
Urea (8 M) denatured (6.41)	602	45

[a] Parameters obtained from the equation

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

λ_0 was taken to be 212 mμ. Values of $[m']$ were computed using a mean residue weight of 123 for α-lactalbumin and values of *n*, the refractive index, interpolated from International Critical Table (1933) values for water, using the Sellmeir formula. [b] Values obtained from solid lines of Figure 1.

Shown in Table I are the ORD parameters derived from the curves of Figure 1, together with values obtained after denaturation in 8 M urea. Values of a_0 and b_0 obtained in the wavelength range 578–365 mμ are

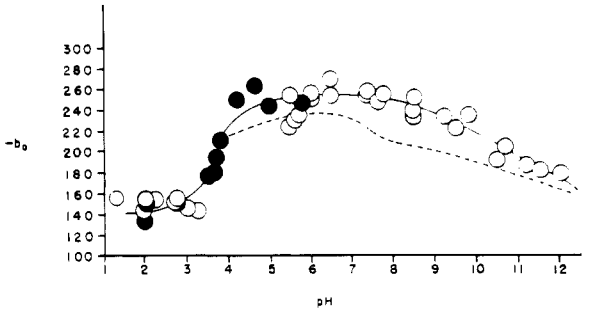

FIGURE 3: pH dependence of b_0 for α-lactalbumin. See Figure 2 for explanation of symbols. See text for explanation of solid and dashed curves.

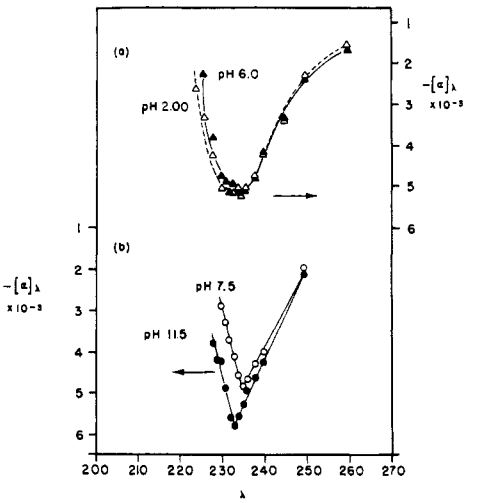

FIGURE 4: Wavelength dependence of $[\alpha]$ for α-lactalbumin in the region 225–260 mμ. Solvent, 0.15 M KCl; temperature, 25°; protein concentration, *ca.* 0.01 g/100 ml; path length, 0.1 cm.

underlined, in distinction from those obtained over the range 578–313 mμ. Where a single value is given, the fit was insensitive to the wavelength range used. Acid denaturation of α-lactalbumin is seen to involve a decrease in b_0 of 50–100°, depending upon the choice of values for that of the native state. Alkaline denaturation on the other hand involved a smaller change (*ca.* 20–70°). Changes in a_0 are of the order of 90° for both the acid and alkaline denaturation. Changes in a_0 and b_0 on urea denaturation of α-lactalbumin are more drastic; b_0 increased in levorotation by *ca.* 350°.

pH Dependence of a_0 and b_0. Shown in Figures 2 and 3 are values of a_0 and b_0 obtained over the pH range 2–12 from plots similar to those shown in Figure 1. The filled circles represent data obtained at 0–2°. The characteristically low solubility of α-lactalbumin at 25° in the pH range 3.5–5 necessitated carrying out ORD measurements at low temperature in this pH range (see also Kronman *et al.*, 1965b). The points shown in Figures 2 and 3 represent parameters obtained over the wavelength range 313–578 mμ (see previous section) while the dashed curves represent a smoothed curve for values obtained from the same set of data for the truncated range, 365–578 mμ.

Below pH 4–4.25, b_0 shows a monotonic decrease to attain a relatively constant value of *ca.* −150 at *ca.* pH 3 (Figure 3). With this decrease in b_0, the systematic deviation of the low wavelength points from the Moffitt–Yang equation disappears, reflected in Figure 3 as the coincidence of the dashed and solid curves (see also the curve at pH 2, Figure 1). The decrease in b_0 occurs in the same pH range where the alteration of a variety of physical properties indicate the occurrence of a conformational change (Kronman and Andreotti, 1964; Kronman, *et al.*, 1964, 1965b; F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation). The pH dependence of b_0 approximately parallels that of the difference extinction coefficient at 293 mμ (Kronman *et al.*, 1965b) (see also Discussion).

a_0 shows a small decrease in levorotation (*ca.* 60°) between pH 5.5 and 4.5 and then increases monotonically to attain a value of *ca.* −325 to −375 at pH 2 (Figure 2).

The tendency of α-lactalbumin to associate or aggregate at low pH (Kronman and Andreotti, 1964; Kronman *et al.*, 1964) raises the question as to the effect of these processes on the ORD properties. Measurement of $[\alpha]_{313}$ as a function of concentration showed increases in levorotation of *ca.* 5% (pH 3.00) and 2% (pH 2.00) in going from 0.5 to 1.5 g/100 ml. Thus, unlike insulin (Schellman, 1956), which shows a change of *ca.* 15% in rotation ($[\alpha]_D$) for a comparable concentration change, association of α-lactalbumin has only a minimal effect on the rotation.

In accordance with our observations of difference spectra (Kronman *et al.*, 1965b; M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation), changes in ionic strength have little, if any, effect on ORD properties; *e.g.*, in experiments carried out at 25° in salt-free solutions of α-lactalbumin, a_0 and b_0 were, respectively, −337 and −145 at pH 1.99 and −337 and −156 at pH 1.50. These values are comparable with those observed in 0.15 M KCl (see Table I and Figures 2 and 3).

Because of problems with turbidity (see Experimental Section) it was not possible to determine the temperature dependence of the ORD parameters in the chief area of interest, the transition region just below pH 4. The temperature dependence of the rotation is, however, very much of interest near pH 6 and at low pH because of the "crevice contraction" phenomena (see Kronman and Holmes, 1965; and Discussion). Measurements made with a single solution at 0–2 and 25° revealed changes of the order of 20–30° for a_0 and *ca.* 10° for b_0 at pH 2.00 and 6.00.

At alkaline pH values, b_0 decreases with increasing pH but rather more gradually than was observed in the acid region (Figure 3). The values attained at the highest pH examined (*ca.* pH 12) appear to be some-

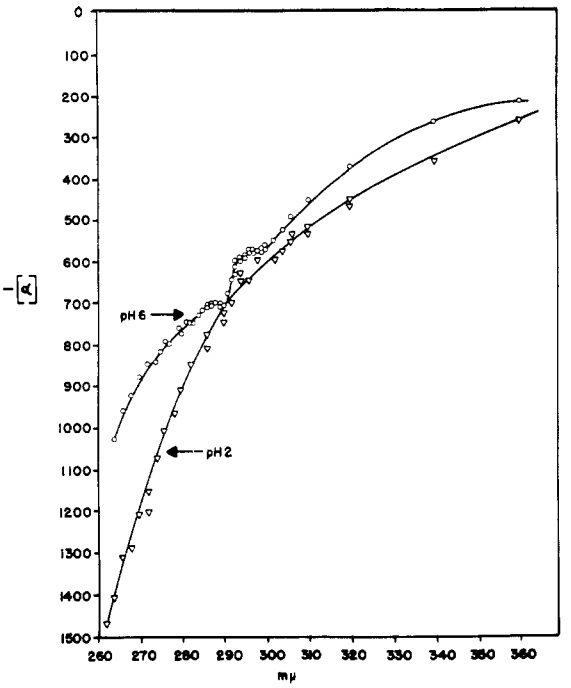

FIGURE 5: Wavelength dependence of [α] for α-lactalbumin >260 mμ. Solvent, 0.15 M KCl; temperature, 25°. Δ, pH 2.00; protein concentration, 0.0993 g/100 ml; path length, 1 cm. O, pH 6.00; protein concentration, 0.190 g/100 ml; path length, 1 cm.

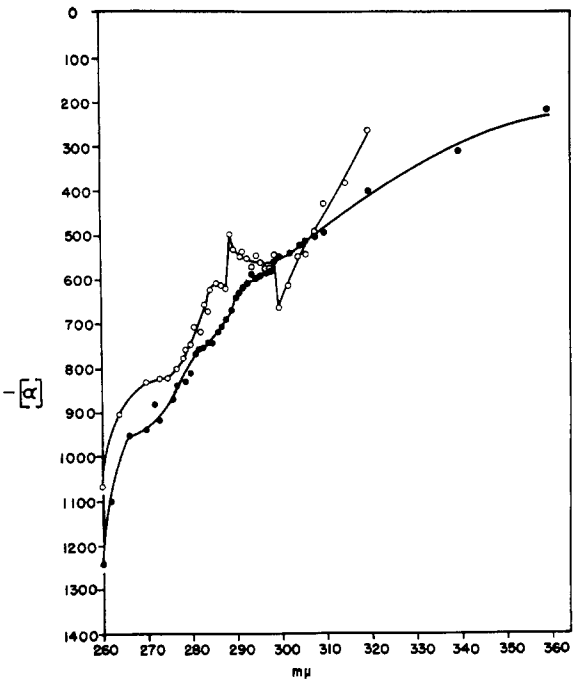

FIGURE 6: Wavelength dependence of [α] for α-lactalbumin >260 mμ. Solvent, 0.15 M KCl; temperature, 25°. O, pH 11.5; protein concentration, 0.0947 g/100 ml; path length, 1.0 cm. O, pH 7.48; protein concentration, 0.102 g/100 ml; path length, 1.0 cm.

what higher than the lowest values attained at the extreme of the acid range (−180 as compared to −150°). The decrease in b_0 was also accompanied by a decrease in the magnitude of the deviation of the data from the Moffitt–Yang equation (*cf.* dotted and solid curves of Figure 2). Experiments were not carried out above pH 12 since slow development of turbidity prevented accurate measurements, particularly in the ultraviolet region. A gradual increase in a_0 was also observed at alkaline pH (Figure 2) attaining a value of *ca.* −380 around *ca.* pH 12, comparable in magnitude to the change in a_0 seen at acid pH.

The 225-mμ Cotton Effect. An unsophisticated interpretation of the changes in a_0 and b_0 would lead to the conclusion that the acid and alkaline conformational changes involve "melting out" of certain helical regions of the α-lactalbumin molecule. Such a process should be accompanied by a decrease in the amplitude of the trough of the 225-mμ Cotton effect. The latter has been identified with the α-helical conformation (Simmons *et al.*, 1961; Blout *et al.*, 1962).

Shown in Figures 4a and b are the ORD curves obtained in the 225–260-mμ region for native (pH 6.0 and 7.5) and for pH 2.00 and 11.5 α-lactalbumin. Because of the high absorbance of α-lactalbumin solutions, ORD measurements could not be extended readily to lower wavelengths to observe the 190-mμ helix Cotton effect (Blout *et al.*, 1962). The ORD curves at pH 2 and 6 are quite similar in shape and in depth of the 233-mμ trough. This is entirely unexpected in view of the difference in corresponding values of b_0 (Figure 1 and Table I). The Cotton effects at pH 7.5 and 11.5 (Figure 4b) appear to come to sharper minima than those observed at pH 2 and 6 (*cf.* Figures 4a and b). The trough at pH 7.5 occurs at 235 mμ in contrast with the value of 233 mμ observed at the other pH values. This shift to 235 mμ appears to be real since it was observed in replicate experiments which were interspersed with measurements at other pH values where the 233-mμ trough was consistently observed. The amplitudes at pH 7.5 and 11.5 differ by *ca.* 1000°. However, contrary to what might have been anticipated from the relative magnitudes of b_0 at these two pH values (see Figure 2), the amplitude of the 233-mμ trough is greater at pH 11.5. It would thus appear that the changes in b_0 occurring during the acid and alkaline conformational changes are not a reflection of changes in the amplitudes of the troughs of the 235-mμ Cotton effects.

The Near-Ultraviolet Region. The parameter b_0 may reflect any optically active electronic transitions at wavelengths below the range of measurement; thus the absence of appropriate differences in the amplitude of the 233-mμ trough, together with the deviations observed in the Moffitt–Yang equation for native protein, suggest that Cotton effects might be present in the 260–300-mμ region, the region of absorption of side chain chromophores including tyrosine and tryptophan. Observations with α-lactalbumin at pH 2.00, 6.00.

TABLE II: Apparent Helix Content of Native and Denatured α-Lactalbumin.

pH	$[m']_{233}$[c] (1)	$-b_0$[a] (low) (2)	$-b_0$[b] (high) (3)	f_{233}[c,e]	f_{b_0}[f] (low) (5)	f_{b_0}[f] (high) (6)
2.00	4910 ± 240	140	140	0.23 ± 0.01	0.22	0.22
6.00	4730 ± 170	207	254	0.21 ± 0.01	0.33	0.40
7.45	3960 ± 530	220	255	0.15 ± 0.04	0.35	0.41
	(4940 ± 190)[d]			(0.23 ± 0.01)		
11.5	5545 ± 800	170	180	0.27 ± 0.06	0.27	0.29

[a] Interpolated from dashed curve, Figure 3. [b] Interpolated from solid curve, Figure 3. [c] Calculated from data of Figure 4 and similar experiments. [d] Value at trough of Cotton effect (see Figure 4). [e] Calculated from eq 1. [f] Calculated from eq 2.

7.45, and 11.5 substantiated this hypothesis (Figures 5 and 6). Each of these curves was obtained with a single protein concentration for the entire wavelength range. While the absolute values of [α] for a given wavelength varied somewhat in replicate experiments, the essential features of these curves were maintained.

Native α-lactalbumin at pH 7.45 gave a rather complex curve (Figure 6) having the following features: a distinct maximum at 300 mμ, a flat region extending to a second maximum at *ca.* 289 mμ, a small shoulder at *ca.* 287 mμ, and an inflection point in the region of 275 mμ. Native α-lactalbumin at pH 6.00 (Figure 5) gave a less complex curve; distinct plateaus are seen <300 and 290 mμ. Alkaline (pH 11.5) α-lactalbumin exhibits still less complexity (Figure 6); several inflections are observed near 295, 285, and 265 mμ. α-Lactalbumin at pH 2.00 gave only a smooth curve from 360 to 260 mμ (Figure 5).

Discussion

Cotton Effects in the 250–300-mμ Region. The maxima, minima, and plateaus observed for native and alkaline-denatured α-lactalbumin (Figures 5 and 6) are clearly the result of several Cotton effects occurring in the 250–300-mμ region. Since these are superimposed on a relatively large "background" rotation, their exact position is difficult to ascertain. Comparison of positions of these side-chain Cotton effects with those reported by other investigators must be made with caution, for the same reason. Myers and Edsall (1965) have observed ORD spectra for carbonic anhydrase B which are at least as complex as those we have obtained for α-lactalbumin. They find maxima at 297, 289, and 280 mμ and minima at 293, 285, and 265 mμ. The positions of these maxima are comparable to those observed with α-lactalbumin (see Figures 5 and 6). In β-lactoglobulin only two minima (286 and 284 mμ) and two maxima (296 and 291 mμ) are observed (Timasheff and Townend, 1965). Urry and Doty (1965) have recently reported similar Cotton effects which they attribute to tyrosine and tryptophan transitions. Beychok (1965) has reported circular dichroism bands for insulin, ribonuclease, and lysozyme in the region 240–280 mμ. Glazer and Simmons (1965) have shown that native lysozyme has a Cotton effect at 280–290 mμ and an inflection at 260–270 mμ. Treatment with sodium dodecyl sulfate abolished the Cotton effect and inactivated the enzyme without affecting the 233-mμ trough. As Glazer and Simmons point out, the fact that tryptophans are known to be present at the active site of lysozyme indicates that the Cotton effect observed in the native enzyme is likely due to tryptophan residues.

ORD and circular dichroism studies indicate that the amino acids cystine, tyrosine, and tryptophan exhibit significant Cotton effects above 250 mμ. The acid form of tyrosine shows a positive Cotton effect with a peak at 280 and a trough at 265 mμ (Iizuka and Yang, 1964), while for neutral tyrosine the peak is shifted to 286 and the trough to 259 mμ (Hooker and Tanford, 1964; Fasman *et al.*, 1964). Cystine exhibits a circular dichroism band centered at 248 mμ in 1 N sulfuric acid and at 255 mμ in water (Beychok, 1965). Fasman *et al.* (1965) have determined the ORD for L-tryptophan and poly-L-tryptophan. The amino acid has a small positive peak at *ca.* 297 mμ and a larger negative peak at 276 mμ. The polymer, presumably in helical form, has peaks at 296 and 288 mμ and troughs at 290 and <280 mμ. The fact that additional peaks appear in the polymer as compared to the amino acid has been taken to indicate the existence of either indole–indole interactions in a stacked helical array of tryptophans or interaction of indoles with the backbone peptide bonds. Since the long wavelength absorption band of tryptophan corresponds to two electronic transitions (Zimmermann and Joop, 1961) it appears possible that interactions of the type occurring in poly-L-tryptophan may have different effects on the rotational strength of the two transitions, *i.e.*, in the amino acid only one of the transitions would be optically active while in the polymer, they both would be. Similar interactions in proteins, particularly those involving the peptide backbone, might result in a more complex type of tryptophan–Cotton effect system (like poly-L-tryptophan) rather than that corresponding to the free amino acid.

Abolition of the Side-Chain Cotton Effects. Acid

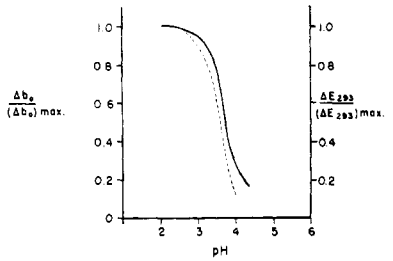

FIGURE 7: Comparison of spectral and ORD data for α-lactalbumin. See text for explanation. ——— $\Delta b_0/(\Delta b_0)_{max}$, – – – – $\Delta E_{293}/(\Delta E_{253})_{max}$.

denaturation of α-lactalbumin appears to essentially eliminate the side-chain Cotton effects observed at higher pH values (*cf.* curves of Figures 5 and 6). Since the amplitude of the trough of the 225-mμ Cotton effects are virtually the same for native (pH 6 and 7.45) and acid-denatured protein, the 50–100° difference in b_0 is most probably a reflection of the abolition of the side chain Cotton effects (see columns 1–3, Table II, and subsequent discussion). It likewise seems virtually certain that the systematic low wavelength deviations observed in Moffitt–Yang plots (see Figure 1 and dotted curves of Figure 3) are due to rotational contributions from these Cotton effects. The good fits of the low pH data at lower wavelengths is in accord with this conclusion (see Figure 1).

Acid-denatured α-lactalbumin molecule exists in a swollen state as compared to the native protein molecule (Kronman and Andreotti, 1964; Kronman *et al.*, 1964; F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation). The conformational change is accompanied by a blue shift of the tryptophan absorption spectrum (Kronman *et al.*, 1965b).

Shown in Figure 7 is a comparison of the changes in absorption at 293 mμ and in b_0, plotted as fractional change to facilitate comparison. Values of $\Delta b_0/(\Delta b_0)_{max}$ were computed from values taken from the solid curve of Figure 3. Values of ΔE_{293} were those observed at 25° (Kronman *et al.*, 1965b). Use of the low temperature curves (2–4°) would shift the $(\Delta E_{293}/\Delta E_{293})_{max}$ to slightly lower pH. It is apparent from the curves of Figure 7 that changes in b_0 roughly parallel the shifts in absorption spectrum occurring below pH 4. As we shall see subsequently, it seems reasonable to assign the change in b_0 (at least in part), as well as the spectral change, to alterations of the environment of three out of the five tryptophan residues in α-lactalbumin.

Solvent perturbation measurements have demonstrated that of the five tryptophans in α-lactalbumin, three are "buried" with respect to perturbant molecules as small as heavy water (effective diameter, 2 A) in *both* the native and denatured states (Kronman and Holmes, 1965). The latter observations lead to the conclusion that the "denaturation blue shift" of α-lactalbumin is not the consequence of enhanced "exposure" of tryptophan groups, but must correspond to changes in the environment of the three "buried" groups. The fact that "exposure" of these groups does not occur during molecular swelling at acid pH indicates that they are in rather inflexible regions of the α-lactalbumin molecule, perhaps within disulfide-linked loops. Further evidence for the rigidity of the structure surrounding the three "buried" groups can be cited. (a) Only 80% "exposure" of tryptophans results from treatment of α-lactalbumin with 8 M urea (Kronman and Holmes, 1965). (b) Treatment of the protein with chymotrypsin followed by carboxypeptidase liberates the five tryosines and the four phenylalanines but only two of the tryptophans (the "exposed" groups) (Weil *et al.*, 1959). Thus, in spite of the fact that 11 peptide bonds had been cut prior to carboxypeptidase treatment, sufficient structural integrity is maintained to continue to insulate the three "buried" groups from enzymatic attack. Swelling of the α-lactalbumin molecule should permit greater freedom of rotation of the three "buried" tryptophans in the denatured state as compared to that possible in the more rigid native state. This increased freedom would alter interactions with groups which are capable of perturbing the absorption spectrum.

The hypothesis that one of the significant differences in the environment of tryptophans in native and acid-denatured molecules is the ease of rotation of the three "buried" groups might explain equally well the observed side chain Cotton effects and the changes in b_0. The high rigidity of the native molecule could "freeze in" particular conformations of the tryptophan groups having special symmetry properties with concommitant enhancements of their rotational strengths and generation of Cotton effects in their characteristic absorption bands. In the swollen denatured molecule, enhanced freedom of rotation of these groups would destroy these special group conformations, thereby reducing or eliminating the side-chain Cotton effects.

The maxima, minima, and inflection points observed for native α-lactalbumin (pH 7.45, Figure 6) lie close to the peaks (296 and 288 mμ) and the troughs (290, 275–280 mμ) found for poly-L-tryptophan (Fasman *et al.*, 1965). As pointed out in the previous section, indole–peptide bond interactions in a protein might produce Cotton effects more characteristic of poly-L-tryptophan rather than the more simple one observed with the free amino acid. If this were the case, it would appear that the Cotton effects observed throughout α-lactalbumin could be accounted for solely on the basis of the tryptophan side chains. However, it is possible that more than one type of chromophore is involved.

The cystines forming the four disulfide bridges of α-lactalbumin (Gordon and Ziegler, 1955) may conceivably be involved. As Beychok (1965) has pointed out, restricted rotation about an S–S bridge can produce optical activity in the absorption band associated with sulfur electrons. Since the presence of nearby asymmetric centers will affect the magnitude of the rotation, expansion of disulfide-linked loops of the α-lactalbumin molecule might be expected to remove the bridges from the proximity of such groups, thereby reducing their

rotational contribution. Likewise, tyrosine residues might be involved, at least on the basis of the position of their characteristic Cotton effects (see previous section). No information is available, however, on the freedom of rotation of such groups within the α-lactalbumin molecule. Unfortunately it is virtually impossible to determine the degree of exposure of these groups by means of solvent perturbation because of the high tryptophan:tyrosine ratio (1:1) (Kronman and Holmes, 1965). The fact that tyrosine groups are freely titratable at alkaline pH (F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation) would seem to militate against their being "buried" in rigid regions of the molecule. Since, however, the titration of these groups is accomplished in a pH region where there is evidence of a conformational change (consider, *e.g.*, the decrease in b_0 at high pH, Figure 3), we cannot be certain that tyrosines exist in a "free" state at pH values closer to neutrality.

The conformational change at alkaline pH, like that occurring below pH 4, involves a swelling of the molecule, together with changes in the absorption spectrum characteristic of tryptophan groups (F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation; M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation). A comparison of the magnitudes of the changes in the Linderstrøm–Lang electrostatic factor, w, leads to the conclusion that swelling is somewhat less at the extreme of the alkaline transition (F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation). A similar conclusion is arrived at in comparing frictional coefficients calculated from sedimentation constants (Kronman *et al.*, 1964; M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation). The relative magnitudes of b_0 at pH 2 and 12 (Figure 3), as well as the relative persistence of the side chain Cotton effects at high pH (compare curves for pH 2 and 12 in Figures 5 and 6), would indicate that some restriction to rotation of side chain still persists in the less swollen molecule at pH 12.

The spectral changes occurring at alkaline pH values do not parallel the changes in b_0 as closely as seen at acid pH (Figure 7); *e.g.*, while changes in b_0 are quite evident by pH 9 (Figure 3), the short wavelength shift of the tryptophan absorption spectrum is not evident until pH 10. These differences will be considered at greater length (M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation).

Helix Content of α-Lactalbumin. Implicit in the use of the parameter b_0 or the quantity $[m']_{233}$ for the estimation of helix content is the assumption of the absence of rotational contributions from structures other than helix and coil forms.[2] As we have pointed out in a previous section of this paper, the decrease in b_0 observed on acid or alkaline denaturation appears to be the consequence of the elimination of a side-chain Cotton effect. If we assume for the moment that the amplitude of the 225-mμ Cotton effect is not influenced by side chain Cotton effects (see below for further consideration of this point), the magnitude of the error incurred in using b_0 for helix estimation can be estimated. Fractions of helix, f, were calculated from the amplitudes of the 233-mμ trough and from b_0 from the equations

$$f_{233} = -\frac{[m']_{233} + 1.88 \times 10^3}{(15.4 - 1.88) \times 10^3} \quad (1)$$

$$f_{b_0} = -\frac{-b_0}{630} \quad (2)$$

where 15.4 and 1.88×10^3 are the values of $[m']_{233}$ for the helical and coil forms of PGA, respectively (see Experimental Section). At pH 2.00 where side chain Cotton effects are virtually absent (Figure 5), f_{b_0} and f_{233} are in good agreement (Table II).[3] This also appears to be true within experimental error at pH 11.5 where the Cotton effects are markedly reduced (Figure 6, Table II) as compared to pH values closer to neutrality.

At pH 6.00 the helix content estimated from b_0 is 1.5–2 times as large as that obtained from $[m']_{233}$, depending upon the manner in which the line is drawn in the Moffitt–Yang plots (dashed and solid curves of Figure 2). α-Lactalbumin at pH 7.45 represents a less clear-cut case because of the small shift of the 233-mμ trough (see Figure 4 and below), but even in this case the difference between f_{b_0} and f_{233} is large (Table II).[3] It is quite apparent from these observations that the uncritical use of b_0 in estimation of helical content can lead to rather serious errors. An unsophisticated interpretation of the change in b_0 on acid denaturation of α-lactalbumin would lead to the conclusion that one-third to one-half of the helical content of the protein had "melted out" during the process. A more critical evaluation of the ORD properties over a wider wavelength range clearly does not substantiate this conclusion.

Finally, some remarks are in order concerning the use of $[m']_{233}$ as a measure of helix content. We are less concerned here with the problem of calculation of absolute helix contents from $[m']_{233}$; the question con-

[2] Schecter and Blout (1964) have proposed a relationship which appears to be useful in the detection of conformations other than helix and coil. Application of this relationship to data obtained with α-lactalbumin at pH values near 6 resulted in curves showing the same type of low wavelength deviated as seen with Moffitt–Yang plots (Figure 1). The helical contents calculated from the two parameters of the Schecter–Blout treatment were 0.38 and 0.33 at pH 5.63 and 0.39 and 0.35 at pH 5.82. The difference in helical contents as calculated from these two parameters, which should be an indication of structures other than helix and coil, are of comparable magnitude with those reported by Schecter and Blout for a number of proteins classified as helical.

[3] Use of a value of -12×10^3 for $[m']_{233}$ for 100% helix (Simmons *et al.*, 1961) would alter the apparent helix content, f_{233}, somewhat but the discrepancy between f_{233} and f_{b_0} at pH 6.00 and 7.45 would still remain. What the appropriate model substance for use as a standard for 100% helix should be is rather uncertain at present. The problem has been considered recently by Yang and McCabe (1965) who raise the question as to whether a *single* substance including PGA may be suitable in relating $[m']_{233}$ to helix content.

sidered is whether changes in $[m']_{233}$ may be used as a measure of changes in helix content, free of interference by adjacent Cotton effects (see also footnote 3).

Side chain Cotton effects occurring at wavelengths >233 mμ might affect both the magnitude of $[m']_{233}$ and the position of the trough. The latter seems to have occurred for α-lactalbumin at pH 7.45 (Figure 4) where the trough occurs at *ca.* 235 mμ. An alteration of the amplitude of the 225-mμ Cotton effect will be reflected as an "abnormal" background rotation, *i.e.*, the residue rotation for the coil form may be considerably different from the value obtained with PGA, 1.88×10^3 (see eq 1). This uncertainty will be particularly evident if one is interested in determining changes in helical content induced by alterations in pH or temperature, for example. Here, the magnitude of the background rotation in itself may be pH or temperature dependent if the conformational change involves alteration of the environment of side chains.

The above remarks, of course, are not restricted to the higher wavelength absorption bands of chromophores. Optical activity that might be associated with the low wavelength absorption bands (λ <225 mμ) of these chromophores would be expected to have similar influence. To our knowledge no one has detected such low-wavelength side chain Cotton effects in proteins. The high absorption of α-lactalbumin solutions precludes making such measurements for this protein.

References

Beychok, S. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 999.

Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.

Fasman, G. D., Bodenheimer, E., and Lindblow, C. (1964), *Biochemistry 3*, 1665.

Fasman, G. D., Landsberg, M., and Buchwald, M. (1965), *Can. J. Chem. 43*, 1588.

Glazer, A. N., and Simmons, N. S. (1965), *J. Am. Chem. Soc. 87*, 2287.

Gordon, W. G., and Ziegler, J. (1955), *Arch. Biochem. Biophys. 57*, 80.

Hooker, T. M., and Tanford, C. (1964), *J. Am. Chem. Soc. 86*, 4989.

Iizuka, E., and Yang, J. T. (1964), *Biochemistry 3*, 1519.

International Critical Tables (1933), Vol. 1, McGraw-Hill, New York, N. Y., p 106.

Kronman, M. J., and Andreotti, R. E. (1964), *Biochemistry 3*, 1145.

Kronman, M. J., Andreotti, R. E., and Vitols, R. (1964), *Biochemistry 3*, 1152.

Kronman, M. J., Blum, R., and Holmes, L. G. (1965a), *Biochem. Biophys. Res. Commun. 19*, 227.

Kronman, M. J., Cerankowski, L., and Holmes, L. G. (1965b), *Biochemistry 4*, 518.

Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 526.

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

Myers, D. V., and Edsall, J. T. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 169.

Robbins, F. M., Kronman, M. J., and Andreotti, R. E. (1965), *Biochim. Biophys. Acta 109*, 223.

Schecter, E., and Blout, E. R. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 695.

Schellman, J. A. (1956), *Compt. Rend. Lab. Carlsberg 30*, 415.

Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.

Timasheff, S. N., and Townend, R. (1965), *Biochem. Biophys. Res. Commun. 20*, 360.

Urry, P. W., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 2757.

Weil, L., Seibles, T. S., and Telka, M. (1959), *Arch. Biochem. Biophys. 79*, 44.

Yang, J. T., and McCabe, W. Y. (1965), *Biopolymers 3*, 209.

Yang, J. T., and Semejima, T. (1963), *J. Biol. Chem. 238*, 3262.

Zimmermann, H., and Joop (1961), *Elektrochem. Ber. Bunsebges. Physik. Chem. 65*, 61.