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## INTER- AND INTRAMOLECULAR INTERACTIONS OF $\alpha$ -LACTALBUMIN

### VIII. THE ALKALINE CONFORMATIONAL CHANGE

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(Received June 6th, 1966)

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#### SUMMARY

1. Previous investigations have shown that  $\alpha$ -lactalbumin undergoes a structural change at acid pH which involves molecular swelling, alteration of the environment of tryptophan residues and increased susceptibility to association and aggregation. The purpose of this investigation was to determine if a comparable molecular change could be induced in  $\alpha$ -lactalbumin at alkaline pH values.

2. Ultracentrifuge measurements demonstrated that  $\alpha$ -lactalbumin exists in a somewhat expanded state above pH 9.5, although the degree of expansion is somewhat less than that observed at low pH. The reversible time-dependent aggregation seen at low pH, however, is absent. Association to low molecular weight species was demonstrated as occurring above pH 9.5.

3. Measurement of ultraviolet difference spectra showed that a tryptophan blue shift underlies the usual tyrosine ionization red shift above pH 10. The emission maximum for tryptophan fluorescence shifted by about 10 m $\mu$  toward the red above pH 10 and an apparent increase in quantum yield was observed, reflected as an absence of alkaline quenching. These spectral changes are comparable to those observed during the acid transition.

4. The absence of a time-dependent aggregation, at alkaline pH, as well as a comparison of the quantitative aspects of the acid and alkaline conformational changes, leads to the conclusion that although association and aggregation result from structural changes, the molecular sites for these processes are not identical.

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#### INTRODUCTION

In previous papers<sup>1-8</sup> in this series, we have been examining the characteristics of the several conformational changes that  $\alpha$ -lactalbumin undergoes. The more drastic of these, which we have called acid denaturation, occurs below pH 4 and involves alteration of the environment of tryptophan residues reflected as changes in emission<sup>7</sup> and absorption<sup>2,3</sup> spectra. Both ultracentrifuge<sup>2</sup> and titration curve experiments (see preceding paper<sup>8</sup>) indicate that acid denaturation involves molecular swelling, although this does not result in enhanced exposure of tryptophan groups<sup>4</sup>. Acid-denatured  $\alpha$ -lactalbumin shows marked tendency to associate and aggregate<sup>1,2</sup>.

Titration curve<sup>8</sup> and optical rotation dispersion<sup>6</sup> measurements both indicate the presence of a comparable structural change above pH 10. We shall describe in this publication the results of spectral and ultracentrifuge measurements which provide further evidence for the similarity of acid and alkaline conformational changes.

#### MATERIALS AND METHODS

$\alpha$ -Lactalbumin (preparation No. R49) was prepared by the method of ROBBINS AND KRONMAN<sup>9</sup>. The characteristics of this particular preparation have been previously described<sup>3,8</sup>. The experimental procedures have been described in detail elsewhere<sup>1-8</sup>.

#### RESULTS

##### *Ultraviolet spectra at alkaline pH*

Difference spectra of  $\alpha$ -lactalbumin at high pH relative to pH 6 exhibited somewhat different characteristics than that anticipated for ionized tyrosine groups. This is illustrated in the dotted curve of Fig. 1 for a  $\alpha$ -lactalbumin solution at pH 12.2. Shown also for comparison is a spectrum determined in our laboratory for the tryptophan-free protein ribonuclease. All spectra of Fig. 1 have been normalized to unity at 295 m $\mu$  to facilitate comparison.  $\alpha$ -Lactalbumin solutions at pH 12.2 exhibited a shoulder at about 291 m $\mu$  and a maximum at 298-299 m $\mu$  in contrast with ribonuclease which showed no shoulder and yielded a maximum at about 295 m $\mu$ . The latter value has been observed for tyrosine in a variety of proteins having zero or low tryptophan contents such as insulin<sup>10</sup> and actin<sup>11</sup> (see also the review by WETLAUFER<sup>12</sup>).

All spectra obtained for  $\alpha$ -lactalbumin between pH 10 and 12 exhibited the above characteristics. The shoulder and shifted maxima observed with  $\alpha$ -lactalbumin are due to the superposition of a tryptophan blue shift on the normal tyrosine red

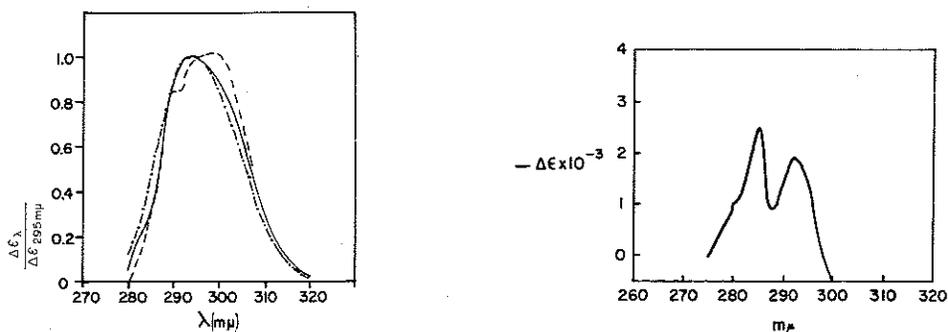


Fig. 1. Alkaline difference spectra of  $\alpha$ -lactalbumin and ribonuclease at 25°. ---,  $\alpha$ -lactalbumin, reference pH 6; -.-.-,  $\alpha$ -lactalbumin, reference pH 2; —, ribonuclease, reference pH 6. All solutions were 0.15 M in KCl. The alkaline values were about pH 12. All curves have been normalized to unity at 295 m $\mu$ .

Fig. 2. Tryptophan difference spectra of  $\alpha$ -lactalbumin at pH 12.5 at 25°. See text for procedure by which curve was obtained.

shift observed during the ionization of the latter groups. This tryptophan blue shift appears to correspond rather closely to that observed at acid pH values<sup>3</sup>. This can be demonstrated by measurement of a difference spectrum at high pH relative to that at pH 2 (dot-dashed curve, Fig. 1). The resulting spectrum had a maximum at approx. 295 m $\mu$  and exhibited no indication of the 291-m $\mu$  shoulder. It corresponds rather closely to that anticipated for ionization of tyrosine residues (solid curve, Fig. 1). The tryptophan difference spectrum can be obtained numerically from the two high pH difference spectra (dashed and dot-dashed curves, Fig. 1) obtained with the two reference states (pH 2 and pH 6). The resulting spectrum shown in Fig. 2 is a characteristic tryptophan difference spectrum having maxima at about 285 m $\mu$  and 292 m $\mu$ . The difference extinction coefficient  $\Delta\epsilon$  was about 2000, comparable to the value of 2080 observed at low pH (ref. 3). This agreement is not unexpected in view of the assumptions involved in the calculation. The pH dependence of the tryptophan spectral shift can be obtained from the relationship

$$(\Delta\epsilon)^{\lambda}_{\text{obs}} = f(\Delta\epsilon)^{\lambda}_{\text{tyr}} + \gamma(\Delta\epsilon)^{\lambda}_{\text{trp}} \quad (1)$$

where  $f$  represents the fraction of ionization of tyrosine at a given pH,  $(\Delta\epsilon)^{\lambda}_{\text{obs}}$  is the observed extinction coefficient using pH 6 as a reference solution and  $\gamma$  is the fractional change in the tryptophan spectrum at that pH.  $(\Delta\epsilon)^{\lambda}_{\text{tyr}}$  is the intrinsic difference extinction coefficient for  $f$  equal to unity and is obtained from the difference spectrum observed above pH 12 with a pH-2 reference solution.  $(\Delta\epsilon)^{\lambda}_{\text{trp}}$ , the extinction coefficient for  $\gamma$  equal to unity, was obtained from Fig. 2. Since  $f$  can be obtained by spectral measurements at 298 m $\mu$  where tryptophan interference is absent (see Fig. 2 and preceding paper<sup>8</sup>), the pH dependence of  $\gamma$  is readily determined. This is illustrated in Fig. 3. Since the absorbances of the tyrosine spectral shift are much larger in magnitude than those for tryptophan, the precision of the data shown in this figure is low and the curves should be regarded as semi-quantitative. The tryptophan spectral change occurs rather gradually, beginning somewhat below pH 10 with the

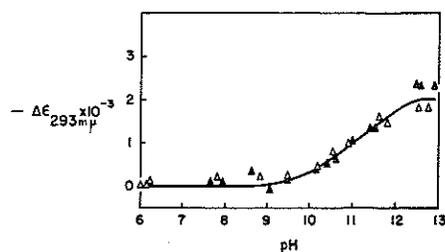


Fig. 3. pH dependence of  $\Delta\epsilon_{293\text{ m}\mu}$  for  $\alpha$ -lactalbumin. All solutions were 0.15 M in KCl.  $\blacktriangle$ , 10 $^{\circ}$ ;  $\triangle$ , 25 $^{\circ}$ .

bulk of the shift occurring over 2 to 2.5 units. This is in contrast with the observations at acid pH where the spectral shift occurred sharply in about one unit. The data below pH 12 show little, if any, temperature dependence (compare filled and unfilled symbols). The relative magnitudes of  $\Delta\epsilon_{293\text{ m}\mu}$  at 10 and 25 $^{\circ}$  above pH 12 are the reverse of what might have been anticipated on the basis of the acid transition<sup>3</sup>, but these differences are probably within experimental error.

*Fluorescence properties at alkaline pH*

Shown in Fig. 4B is a plot of the pH dependence of relative fluorescent intensity,  $(RI)_{340\text{m}\mu}$  of  $\alpha$ -lactalbumin at 340 m $\mu$ . These were calculated relative to a pH-6 solution taken as unity. The intensity remains constant over a rather wide range (5.8–10). Above pH 10 a decrease of the order of 15 % is observed. At still more alkaline pH, the intensity decreases until at pH 13.25 the relative intensity is only 65 % of that at pH 6. This relative constancy of tryptophan fluorescence in the alkaline pH region is particularly striking as compared to the marked quenching observed above pH 10 for tryptophan-containing proteins<sup>13,14</sup> and above pH 11 for tryptophan and tryptophan derivatives<sup>15,16</sup> and for poly-L-tryptophan<sup>17</sup>. The relative insensitivity of the fluorescence intensity to increases of pH above pH 10 appears to be a reflection of the conformational change detected by optical rotation dispersion<sup>6</sup> and by sedimentation velocity and absorption spectral measurements (see other sections of this paper). A shift in fluorescence spectrum above pH 10 provides further evidence for this conformational change. These emission spectra were typical of those observed with tryptophan-containing proteins (see ref. 7, for example). From pH 6 to about 10, the emission maximum occurred at 340–343 m $\mu$  (Fig. 4A). At higher pH, a relatively abrupt long-wavelength shift of about 10 m $\mu$  was observed which was complete by about pH 11. Decreasing the temperature at a given pH shifted the emission maximum to lower wavelengths (compare filled and unfilled symbols, Fig. 4A). These observations are comparable to those made for the acid transition<sup>7</sup>. The fluorescence characteristics of the alkaline conformational change further resemble those occurring during the acid process in that: (a) fluorescence changes are independent of time for intervals as short as 1 min after pH adjustment; (b) the changes in emission maximum and fluorescent intensity are fully reversible when a solution of  $\alpha$ -lactalbumin is brought from higher pH (*e.g.* pH 12) back to pH 6; and (c) the shape of excitation spectra is insensitive to changes in pH.

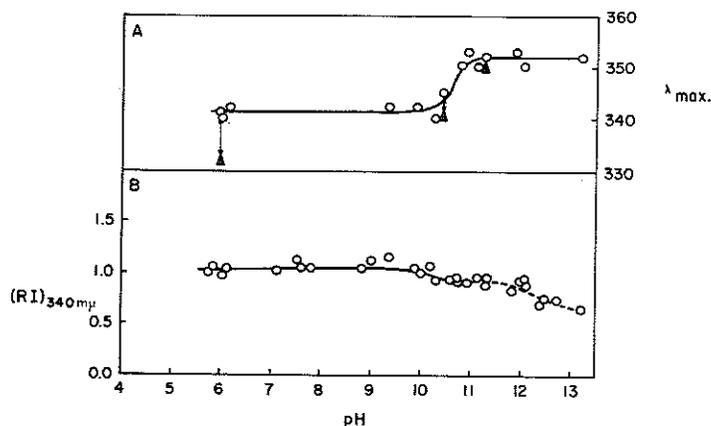


Fig. 4. pH dependence of tryptophan fluorescence for  $\alpha$ -lactalbumin. All solutions 0.15 M in KCl. O, 25°;  $\blacktriangle$ , 0–2°. A. Wavelength maximum of emission spectrum. B. Relative intensity of fluorescence at 340 m $\mu$ ,  $(RI)_{340\text{m}\mu}$ , assuming a value of unity at pH 6.

*Sedimentation properties*

Ultracentrifuge observations demonstrate that acid-denatured  $\alpha$ -lactalbumin shows increased tendency to aggregate and associate and that the monomeric molecule

exists in a somewhat swollen state. The sedimentation properties of  $\alpha$ -lactalbumin above pH 8.5 were therefore examined with the view of determining if  $\alpha$ -lactalbumin undergoes, (a) time-dependent aggregation or association and, (b) changes in frictional coefficient.

Exposure of  $\alpha$ -lactalbumin to pH 10 or 12 for periods of 1 h gave single symmetrical boundaries with no signs of aggregation over the concentration range 0.5 to 4 g per 100 ml. However, after 24 h, an unresolved heavy component was observed; the amount of which increased with time. Unlike the observations in the acid range<sup>1,2</sup>, adjustment of such a solution to pH 8.55 did not result in reversal of the aggregation. Urea had no disaggregating effect on "polymers" formed at high pH; for example, a solution of  $\alpha$ -lactalbumin of concentration about 3 g per 100 ml was allowed to stand at pH 10 for 48 h in 0.15 M KCl. The solution, which now contained a relatively large amount of aggregate, was adjusted to pH 8.55 and brought to a urea concentration of 4 M. Examination of the solution in the ultracentrifuge demonstrated the presence of a substantial amount of aggregate which sedimented rapidly to the bottom of the cell. No aggregation was noted with the control solution held at pH 8.55 for 48 h prior to addition of urea. An increase in ionic strength from 0.15 to 0.75 at pH 12 did not result in aggregation, contrary to what might have been anticipated on the basis of the characteristics of the acid aggregation process<sup>1,2</sup>. It would appear that the aggregation described above does not correspond to the reversible process seen below pH 2 but probably involves some destruction of disulphide bonds as well as formation of intermolecular disulphide bonds. The detection of an odor of  $H_2S$  from pH-12 solutions of  $\alpha$ -lactalbumin is in accord with the view that the disulphide bridges in  $\alpha$ -lactalbumin are subject to attack at high pH.

While the time-dependent aggregation observed below pH 4 appears to be absent above pH 8.5, association as defined earlier<sup>1,2</sup> and expansion of the  $\alpha$ -lact-

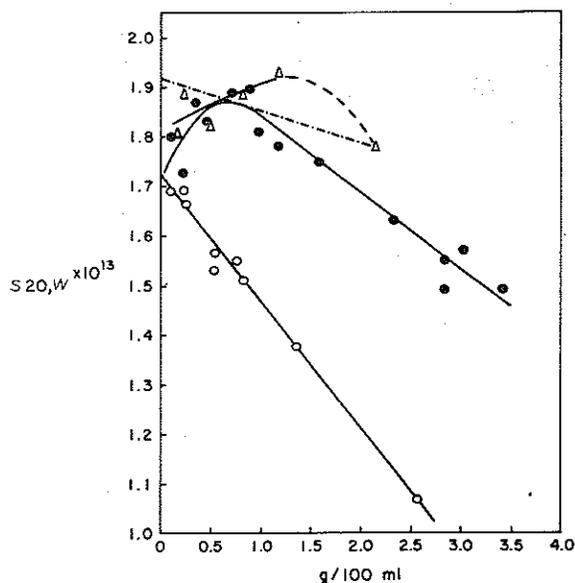


Fig. 5. Concentration dependence of the sedimentation constant of  $\alpha$ -lactalbumin at 25°. All solutions 0.15 M in KCl.  $\Delta$ , pH 9.5;  $\bullet$ , pH 10.0;  $\circ$ , pH 12.0. ---, pH 8.55.

albumin molecule do appear to occur. Shown in Fig. 5 are the concentration dependencies of the sedimentation constant of  $\alpha$ -lactalbumin at pH 9.5, 10 and 12 at 25° in 0.15 M KCl, together with that obtained at pH 8.5 (ref. 1). At pH 12 the sedimentation constant shows a monotonic negative dependence on concentration, extrapolating to a value of about 1.73 S, significantly lower than 1.93 found with the native protein at pH 8.55 (ref. 1). At pH 10, the *s* versus *c* curve goes through a maximum similar to that observed at pH 2 (ref. 2). The presence of such a maximum indicates that association occurs at pH 10 (see ref. 2 for detailed discussion of this point). The sedimentation constant at infinite dilution tends toward the value seen at pH 12, i.e., 1.73 S. The presence of a maximum at pH 9.5 likewise is indicative of association. The value of the sedimentation constant at infinite dilution appears lower than that observed at pH 8.5 but is somewhat higher than seen at pH 10 or 12. The data at low concentrations, however, may not be sufficiently precise to establish this with certainty.

The frictional ratios and molecular radii calculated from the sedimentation constants at pH 9.50, 10.00 and 12.00 are given in Table I together with values reported previously for  $\alpha$ -lactalbumin at pH 8.55 and 2.00 (refs. 1, 2). Although the alkaline-denatured  $\alpha$ -lactalbumin molecule exists in a somewhat swollen state, it is somewhat less expanded than the acid-denatured molecule.

TABLE I  
FRICTIONAL PROPERTIES OF NATIVE AND DENATURED  $\alpha$ -LACTALBUMIN

pH	$s_{20} \times 10^{13}$	Frictional ratio	Radius ( $\text{\AA}$ ) Einstein-Stokes sphere
8.55*	1.93	1.02-1.06	19
9.50	1.8	1.1	20
10.00, 12.00	1.73	1.1-1.2	21
2.00*	1.6	1.2-1.3	23

\* Data taken from ref. 2.

## DISCUSSION

### *Comparison of the acid and alkaline conformational changes*

The evidence cited in this paper, together with that reported earlier<sup>6,8</sup>, indicates that the structure of  $\alpha$ -lactalbumin becomes altered above pH 10. The denaturation occurring under these conditions and that observed at acid pH (refs. 1-8) have qualitatively similar characteristics. These together with the salient differences are summarized and discussed below: (a) Tryptophan difference absorption spectra are generated by both processes (see Fig. 2, for example). The difference molar extinction coefficient at 292 m $\mu$  to 293 m $\mu$  are 2000 for both processes. Solvent perturbation measurements have shown that the acid process involves no increase in the "exposure" of tryptophan groups, 3 groups remaining buried before and after denaturation<sup>4</sup>. Such measurements of tryptophan exposure cannot be extended into the region of the alkaline transition due to the interference by the spectrum of ionized tyrosines. However, the near identical values of  $\Delta\epsilon_{292-293 \text{ m}\mu}$  for acid and alkaline

denaturation makes it unlikely that the latter process could involve enhanced exposure. Furthermore, the relatively high fluorescent intensity at pH values above 12 (Fig. 4B) where the conformational change is essentially complete, indicates that the tryptophan groups must remain relatively shielded from the solvent and thus insulated to quenching by  $\text{OH}^-$ . (b) A long-wavelength shift of about  $10 \text{ m}\mu$  in the tryptophan emission spectrum is observed during both processes (see Fig. 4A and ref. 7). An increase in quantum yield was observed for the acid process while a similar change can be inferred for the alkaline process from the absence of any appreciable quenching at high pH (Fig. 4B). (c) Acid and alkaline denaturation both result in a swollen molecule, but the process appears to be somewhat less complete at alkaline pH values. This swelling is reflected as increased frictional ratios (Table I) and decreases in the magnitude of  $w$ , the titration curve electrostatic factor (preceding paper<sup>8</sup>). (d) The optical rotation dispersion parameter  $b_0$  decreases during denaturation. The change is  $75$  to  $100^\circ$  for the process occurring at acid pH (refs. 6, 18), while about  $50^\circ$  for the alkaline one<sup>6</sup>. These changes in  $b_0$  are a reflection of abolition of Cotton effects arising most likely from uniquely located tryptophan groups. While these effects are largely eliminated at pH 2, they still persist at pH 11.5 but are markedly reduced as compared to pH 7.4 or 5.6 (ref. 6). (e) The acid-denatured molecule shows characteristically low solubility, exhibits time-dependent aggregation and enhanced tendency to form lower molecular weight associated species<sup>1,2</sup>.  $\alpha$ -Lactalbumin at alkaline pH by contrast shows only enhanced tendency to associate (Fig. 5).

The similarity of the alterations of the spectral and optical rotation dispersion properties at acid and alkaline pH makes a detailed discussion of their molecular origins somewhat redundant here. Refs. 3, 4, 6 and 7 should be consulted for a more complete discussion of the origin of these changes. The comparative association-aggregation properties will be considered below.

#### *Aggregation and association of $\alpha$ -lactalbumin*

The quantitative differences in the association-aggregation behavior of  $\alpha$ -lactalbumin at acid and alkaline pH, *i.e.*, the absence of aggregation at alkaline pH is most likely due to differences in hydrophobic and hydrophilic groups at the molecular surface for both forms of the denatured protein. (See refs. 1 and 2, definition of the terms "association" and "aggregation".) The more restricted molecular swelling of  $\alpha$ -lactalbumin on alkaline as compared to acid denaturation, (Table I and ref. 2) as well as the differences in optical rotation dispersion cited in the previous section, indicates that the molecule is somewhat less disrupted at high pH than at acid pH values. We have suggested that both association and aggregation of the acid-denatured protein might be the result of changes in the density of hydrophilic and hydrophobic moieties at the molecular surface<sup>2</sup>. At that time, however, we could not determine if association and aggregation were consecutive or independent parallel processes. A consecutive series of reactions implies that the conformational change produces sites through which monomeric units interact to form low molecular weight polymers with polymerization of these units yielding high molecular weight species. If aggregation and association were independent parallel processes, the sites of interaction for association and aggregation would not be identical. This in turn suggests that a more limited conformational change might generate one type of site without

necessarily producing the other. The absence of aggregation, as well as the more restricted conformational change at alkaline pH would therefore suggest that association and aggregation of  $\alpha$ -lactalbumin are independent parallel processes.

An alternative explanation of the absence of aggregation at high pH might be a difference in molecular charge for the acid- and alkaline-denatured protein. While the aggregation *per se* has been shown to have electrostatic determinants at acid pH, the total absence of aggregation at high pH makes the latter explanation less likely.

#### *The pH dependencies of the conformational changes*

The absence of ionic strength dependencies for the acid and the alkaline conformational changes indicates that mutual electrostatic repulsion of charged side-chains are not of primary importance in promoting these processes. This is further demonstrated on comparison of the protonic charges in the regions of both transitions. For the acid process, the spectral transition begins near pH 4 and is complete by pH 3 (ref. 3) corresponding respectively to protonic charges of +5 and +14 (ref. 8). By contrast, the protonic charges corresponding to the range of the alkaline transition (pH 10–12, Fig. 3) are –13 and –26, respectively (ref. 8). Thus, the alkaline transition begins at more than twice the absolute value of the protonic charge than that for the acid transition and extends over a wider range of charge. It would thus appear that the pH dependencies of both conformational changes correspond to the titration of certain key groups involved in maintaining the native structure.

Since the acid conformational change occurs in the carboxyl titration region, and no evidence exists for abnormally titrating histidines<sup>8</sup>, it seems that the former groups are involved. The alkaline conformational change occurs in the pH region 10 to 12.5 (Fig. 3), where the 5 tyrosines and 12 lysines are titrated<sup>8</sup>. The pH for 50% change of  $(\Delta\epsilon)_{293\text{ m}\mu}$  is about pH 11 (Fig. 3). As stated previously, the curve of Fig. 3 is an average of data obtained at 10 and 25°. Lysines have a  $pK_{\text{app}}$  (pH for 50% ionization) of 10.6 and 11.3 at 25 and 10°, respectively, while the values for tyrosine are 11.2 and 11.5 at the same temperatures<sup>8</sup>. The spectral transition curve (Fig. 3) lies between the two lysine ionization curves. However, the differences may be too small to clearly differentiate between the involvement of tyrosines or lysines. The two types of interactions that might be responsible for stabilization of the native structure would be carboxylate-tyrosine hydrogen bonds and carboxylate- $\epsilon$ -amino salt-like linkages\*.

The involvement of the  $\epsilon$ -amino groups would be a particularly attractive explanation since there are 12 such groups, just sufficient to form bridges with the 12 carboxylate ions remaining untitrated at pH 4 where the low pH transition begins<sup>8</sup>. Changes in the optical rotation dispersion parameters on amidination of the  $\epsilon$ -amino groups of  $\alpha$ -lactalbumin suggest that these groups may be involved in maintaining

\* The importance of salt bridges in maintaining the structure of proteins has often been minimized. A recent determination of the X-ray crystallographic structure of the enzyme lysozyme at a resolution of 2 Å, however, clearly shows the pairing of a number of cationic and anionic groups<sup>19</sup>. While the structural importance of such salt-like linkages in stabilizing a protein structure cannot be evaluated at present, inspection of the lysozyme molecular model leaves little doubt of their existence. † (M.J.K.) am especially indebted to Dr. D. C. PHILLIPS for an opportunity to inspect his 2 Å resolution model of the lysozyme molecule.

the native structure<sup>5,\*</sup>. The evidence for the involvement of lysines is admittedly rather scanty.

*Tryptophan spectral shifts and tyrosine ionization*

Finally, some remarks are in order concerning the influence of tryptophan spectral shifts on determination of the ionization of tyrosyl groups. In the case of  $\alpha$ -lactalbumin, the use of uncorrected difference spectra could have led to serious underestimation of the maximum number of tyrosines ionizable at high pH, e.g., if a pH-6 reference solution had been used, the molar extinction coefficients for maximum ionization at 290 m $\mu$  and 295 m $\mu$  would have led to the erroneous conclusion that 4 rather than 5 tyrosines were ionized. The latter value, however, was obtained either by using a pH-2 reference solution or by calculation of maximum groups ionized from the extinction coefficient at 298 m $\mu$  or 300 m $\mu$  where the spectral contribution of the tryptophan blue shift was essentially zero.

TANFORD AND WAGNER<sup>21</sup> have shown that the tryptophan red shift in lysozyme is reflected as an apparent dependence of the tyrosine ionization curve on the wavelength of observation. No such dependence was demonstrable for  $\alpha$ -lactalbumin. The fraction of tyrosine ionized as obtained from data at 290, 295 and 298 m $\mu$  are in good agreement except, as might be anticipated, at very low and very high degrees of ionization. It can be shown that this absence of a wavelength-dependence of the ionization curve is only fortuitous and arises from the fact that the tyrosine ionization and the tryptophan spectral shift for  $\alpha$ -lactalbumin (Fig. 3) have comparable pH dependencies. This can be demonstrated using Eqn. 1 and appropriate values of  $f$ ,  $\gamma$ , and the molar extinction coefficients for the tyrosine ionization and tryptophan blue shift.

These observations with  $\alpha$ -lactalbumin appear to be of general interest in that they illustrate that an apparent independence of tyrosine ionization on the wavelength of observation is not sufficient to insure the absence of interference by other chromophores. Inspection of difference spectra for distortion from a normal shape (see Fig. 1, for example) and subsequent calculation of the extent of ionization above 298 m $\mu$  appears to be a more reliable procedure.

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\* Subsequent to the time of submission of the paper describing the amidination experiments, more detailed optical rotation dispersion information was obtained with  $\alpha$ -lactalbumin. We had originally assumed that the small decrease in  $b_0$  occurring on amidination of  $\alpha$ -lactalbumin was due to small alterations of helix content. It now seems more probable that these changes are a reflection of the partial abolition of the side-chain Cotton effects. Our original conclusion<sup>5</sup> that association at pH 6 of the amidinated protein was due solely to the increased hydrophobic character of the molecular surface does not seem completely justified at present.

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