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THE DENATURATION OF PROTEINS

II. ULTRAVIOLET ABSORPTION SPECTRA OF BOVINE SERUM ALBUMIN AND OVALBUMIN IN UREA AND IN ACID SOLUTION

A. N. GLAZER*, H. A. McKENZIE** AND R. G. WAKE
Physico-Chemical Unit (C.S.I.R.O. Division of Food Preservation) and Biochemistry Department, University of Sydney (Australia)

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SUMMARY

The effect of acid and urea on bovine serum albumin and ovalbumin has been investigated by ultraviolet spectrophotometry. Acid and urea bring about very similar shifts in the ultraviolet spectrum of bovine serum albumin. These changes are very rapid. The difference spectrum of bovine serum albumin at 287 mμ was investigated as a function of pH over the range 0.5–4.2. The effect of pH on ΔA_{287} was found to be closely similar to the effect of pH in this region on the sedimentation coefficient, viscosity and optical rotation. The effect of acid on the ultraviolet spectrum of ovalbumin is variable. The spectrum of ovalbumin in urea solution changes immediately at low pH. In urea solution at higher pH values the spectrum of ovalbumin changes with time. The change in A_{287} follows apparent first-order kinetics at both 25° and 30° at pH 6.1 and 7.8. The apparent order of the reaction with respect to urea at pH 7.8 and 30° is 7.8 ± 0.2, and at pH 6.1 and 25°, 7.6 ± 0.5. The change with time in the optical rotation of ovalbumin in 7 M urea at pH 6 and 25° is not simple first order. The half-time is inversely proportional to the 13.5 power of the urea concentration. These results are discussed in relation to current concepts of the mechanism of denaturation of bovine serum albumin and ovalbumin.

INTRODUCTION

In the region 250–310 mμ, the ultraviolet absorption of proteins arises essentially from the absorption of the aromatic side chains of tyrosine, tryptophan and phenylalanine residues with very small contributions from cystine and cysteine residues[1]. The protein spectra are usually shifted towards longer wavelengths (bathochromic effect) when compared with the corresponding spectra of their constituent amino acids mixed in the correct proportions[2–5]. When proteins are titrated with alkali to high pH, the tyrosyl residues become ionized and the absorption maximum

* Present address: M. R. C. Laboratory of Molecular Biology, Hills Rd., Cambridge (Great Britain).
** Present address: Department of Physical Biochemistry, Australian National University, Canberra, A.C.T. (Australia).

shifts from about 279 mμ to approximately 295 mμ. This effect was first observed by CRAMMER AND NEUBERGER[3] in studies on ovalbumin and insulin and advantage has been taken of it to distinguish normal from abnormal tyrosyl residues[3,4,6].

Since the urea denaturation of bovine serum albumin and ovalbumin is usually associated with the production of more loosely coiled structures we investigated the effect of urea on the ultraviolet absorption spectra of these proteins. We observed a shift of the spectra to shorter wavelengths on denaturation. In a preliminary communication[7] we indicated that changes in the difference spectra broadly paralleled molecular expansion. LASKOWSKI *et al.*[8] observed a similar shift in the ultraviolet absorption spectrum of insulin on titration with acid and on tryptic hydrolysis and interpreted this change in terms of rupture of tyrosine–carboxylate hydrogen bonds. In the present paper we report in more detail the changes in the ultraviolet spectrum of bovine serum albumin and ovalbumin on denaturation, compare this change to the rate of change of certain other properties and discuss the earlier interpretation in the light of more recent publications on the ultraviolet spectra of proteins.

EXPERIMENTAL

Materials

The ovalbumin, bovine serum albumin and urea were obtained as in Part I (see ref. 9). Stock solutions were prepared by dissolving the required protein in water and exhaustively dialysing in the cold against several changes of distilled water. The protein concentration of the stock solution was determined by the Kjeldahl nitrogen method of MCKENZIE AND WALLACE[10], taking the values 15.7 g/100 g and 16.0 g/100 g for the nitrogen contents of ovalbumin and bovine serum albumin respectively, and by light absorption at 280 mμ of an appropriately diluted solution. The value of $A_{1\text{ cm}}^{1\text{ g/100 ml}}$ for bovine serum albumin was taken as 6.60 and for ovalbumin as 7.12. (The latter experimental value of ATKINSON AND MCKENZIE[11], differs from that of 6.6 obtained by CRAMMER AND NEUBERGER[3].)

Methods

The technique of pH measurement was that of Part I. For work in the pH range 5.8–8.0 the buffers were prepared by mixing stock solutions of Na_2HPO_4 and KH_2PO_4 in correct proportions. For pH values below 5, NaCl–HCl mixtures were used to give the desired pH and ionic strength (I).

Optical rotation measurements were carried out as in Part I. Most of the absorption measurements were carried out with a Beckman DU spectrophotometer (Beckman Instruments Inc., Fullerton, Calif. (U.S.A.)). Some later measurements were made with a Cary Model 14 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif. (U.S.A.)) in the Department of Physical Biochemistry, Australian National University. Both 10- and 2-mm cells were used. Each cell was calibrated with water and tyrosine solution. The temperature of the spectrophotometer cell compartment was maintained within ± 0.1° of the desired value by circulation of water around the cell compartment from an external temperature-controlled water bath. Difference spectra were obtained by two methods: (a) subtraction of separately determined spectra and (b) direct measurement.

Corrections were made for the absorption of the solvents used: these corrections

were small. In all cases the adherence to Beer's law was checked. The difference spectra results are reported as changes in molar absorptivity ($\Delta\varepsilon$). A positive value of $\Delta\varepsilon$ indicates that the solute has a higher absorbancy in the experimental environment than in the reference solvent. If the change in environment causes a bathochromic ("red") shift, then $\Delta\varepsilon$ is positive at those wavelengths where the spectrum has a negative slope and negative where the slope is positive. The difference spectrum shows maxima at those wavelengths where the spectra have maximum negative slopes. For a hypsochromic ("blue") shift the difference spectrum shows minima at those wavelengths where the spectra have maximum negative slopes.

The reaction mixtures were prepared in the following way: the protein solutions and the appropriate solvent mixture were brought to the reaction temperature and then aliquots were mixed. A sample was transferred to the spectrophotometer or the polarimeter tube and the first reading taken within approx. 2 min of mixing.

RESULTS

Effect of acid on the ultraviolet absorption spectrum of bovine serum albumin

In the pH range 4.4–7.6 the ultraviolet spectrum of bovine serum albumin was found to be virtually independent of pH. Below pH 4.4 a hypsochromic shift of the absorption maximum from 279 mμ was observed. At pH 3.2 the peak had moved to 277 mμ. This spectral change appeared to be immediate (*i.e.* it was complete before a reading could be taken). No further change with time in the difference spectrum was observed even after 24 h at pH 2.0. Below pH 1 there was a small shift back to higher wavelengths, the peak having moved to 277.5 mμ at pH 0.8.

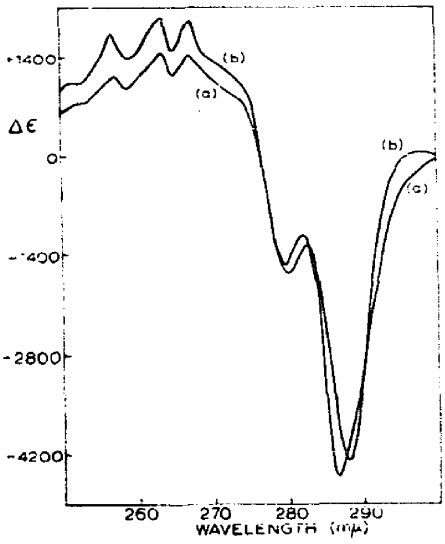

Fig. 1. Difference spectra (molar absorptivity difference, $\Delta\varepsilon$, *versus* wavelength in mμ) of bovine serum albumin (0.47 g/100 ml). 2-mm cells, at 25°. (a) Protein at pH 2.1 *versus* protein at pH 5.1, both 0.15 M in NaCl. (b) Protein at pH 5.1 in 7 M urea *versus* protein at pH 5.1, both 0.15 M in NaCl.

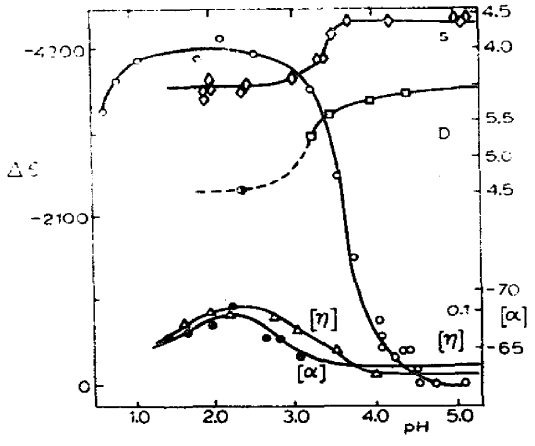

Fig. 2. Effect of acid on difference spectra, optical rotation, viscosity, sedimentation and diffusion of bovine serum albumin. (a) $\Delta\varepsilon_{287}$ *versus* pH (I 0.15, NaCl) at 25°, present work; (b) $[\alpha]_D$ *versus* pH (I 0.10, NaCl) at 25°, data from ref. 12; (c) $[\eta]$ *versus* pH (I 0.10, NaCl) at 25°, data from ref. 12; (d) $s_{20,w}$ in Svedbergs *versus* pH (I 0.1, 0.02–0.1), data from ref. 13; (e) D_{20} in Fick Units *versus* pH (I 0.15, 0.33), data from ref. 13, 14.

Difference spectra for the protein in acid *versus* the native protein near neutral pH showed a minimum near 287–288 mμ with a shoulder near 280 mμ, as shown in Fig. 1. It was found that Beer's law was obeyed for $\Delta\varepsilon$ at 287 mμ over the protein concentration range examined, namely 0.08–0.50 g/100 ml.

The effect of pH, in the acid region, on the absorbancy difference at 287 mμ was then examined. A plot of $\Delta\varepsilon$ *versus* pH is given in Fig. 2. Also shown in the same figure for comparison are plots of optical rotation, $[\alpha]_D$, intrinsic viscosity $[\eta]$, sedimentation coefficient (s) and diffusion coefficient (D) *versus* pH (data from refs. 12–14).

Effect of urea on the ultraviolet absorption spectrum of bovine serum albumin.

On exposure of native bovine serum albumin to urea concentrations above 2 M over the pH range 4–9, an immediate hypsochromic shift in the absorption maximum at 279 mμ occurred. The extent of this shift was dependent on the urea concentration. The spectrum of the protein in urea solutions resembled that of the protein in acid solution below pH 3. Likewise difference spectra of the protein in urea *versus* the native protein at neutral pH resembled those of the protein in acid *versus* the native protein at neutral pH. A minimum in $\Delta\varepsilon$ was also observed at 287–288 mμ with a shoulder at 280 mμ. The similarity of the changes is apparent from Fig. 1. A strong dependency of $\Delta\varepsilon_{287}$ on urea concentration is shown in Fig. 3.

Effect of acid on the ultraviolet specirum of ovalbumin

In our original note[7] we reported the effect of acid on the spectrum of ovalbumin at a concentration of 0.097 g/100 ml. There was virtually no shift (< 5 Å) in the wavelength of maximum absorption over the pH range 1.7–5.2, but there was a small diminution of the absorbancy at pH values below 3. The difference spectrum of 0.097 g/100 ml ovalbumin at pH 1.7 *versus* ovalbumin at pH 5.2 showed no minimum but a general small depression of absorbancy, there being no change with time over a

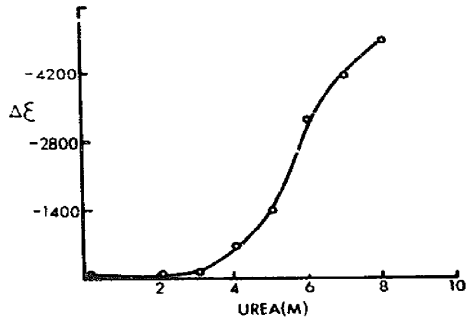

Fig. 3. Effect of urea concentration on $\Delta\varepsilon_{287}$ for bovine serum albumin at 25°. Protein concentration: 0.47 g/100 ml (pH 7.8) (0.035 M phosphate; 9 Na_2HPO_4: 1 KH_2PO_4).

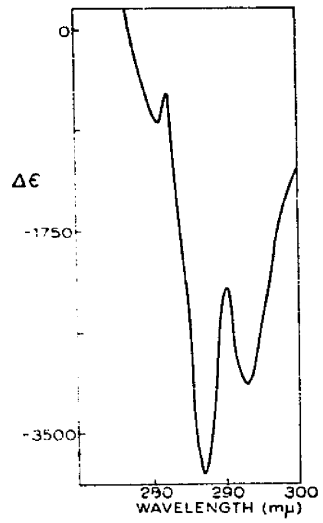

Fig. 4. Difference spectrum for ovalbumin in 7 M urea (pH 6.1) (0.012 M phosphate) *versus* ovalbumin at pH 5.9 (0.012 M phosphate). Protein concentration: 0.10 g/100 ml in 1-cm cells. After 3 h at 25°.

period of several hours. In a subsequent study in this laboratory, M. B. SMITH[15] observed the effect of acid (pH 3.0) at 25° and 60° on the difference spectrum of both 1 and 2 g/100 ml ovalbumin *versus* ovalbumin at pH 6.5 and found a minimum in $\Delta\varepsilon$ at 295 mμ. We have reexamined the effect of acid on ovalbumin difference spectra at 25°, for several protein concentrations. At 0.1 g/100 ml we confirmed our earlier observations. At 0.33, 0.40, 0.66 and 1.00 g/100 ml we obtained minima in the difference spectra. The results at each of the latter concentrations were variable: some spectra exhibited minima at 293.5 mμ, whereas others exhibited minima both at 287 and 293.5 mμ. It is obvious that our earlier results at low concentrations are valid for those concentrations but that at higher ovalbumin concentrations acid may cause more profound changes in the spectrum. This effect is being further investigated.

Effect of urea on the ultraviolet spectrum of ovalbumin

The effect of 7 M urea on the absorption spectra of ovalbumin at pH values of 3.1, 6.1, 7.8 and 9.3 was examined. It was found that at pH 3.1 there was an immediate hypsochromic shift in the ultraviolet absorption spectrum. At the other pH values there was a time-dependent hypsochromic shift. The shift was smallest at pH 7.8. Difference spectra (when reaction was complete) showed a pronounced minimum at 287 mμ with a numerically smaller minimum at 292.5 mμ and a shoulder at 280.7 mμ. A typical difference spectrum, that of ovalbumin in 7 M urea (pH 6.1) (0.012 M PO_4) *versus* ovalbumin at pH 5.9 (0.012 M PO_4) is shown in Fig. 4.

The time rate of change of the absorbancy difference at 287 mμ (ΔA_{287}) for ovalbumin in urea (pH 7.8) at 30° was determined. The reaction was considered to be over when there was no further change in absorbancy over a period of 45 min. In a few experiments readings were taken for a further 6 h and no more change took place. The final value of $\Delta\varepsilon_{287}$ was independent of the concentration over the range studied (0.077–0.154 g/100 ml). The rate of change of ΔA_{287} at 8.0 and 8.5 M urea is shown in Fig. 5. The change is apparent first order*.

When the logarithms of the first order velocity constants for the rate of change of ΔA_{287} are plotted against the logarithms of the urea concentrations, a straight line plot is obtained and the slope, obtained by the method of least squares, gives an apparent reaction order with respect to urea of 7.8 ± 0.2 (see Fig. 6).

A similar plot for pH 6.1 gives an apparent order with respect to urea of 7.6 ± 0.5.

The rate of change of ΔA_{287} for ovalbumin (0.5 g/100 ml) in 7 M urea at 30° (pH 6.1) was examined at ionic strengths of 0.01 (added NaCl) and 0.20 (added NaCl). There was no significant effect of variation of I with added NaCl. The rate of change in the presence of 0.01 M NaCl was compared with that in the presence of orthophosphate. It was found that orthophosphate (0.0014 M Na_2HPO_4, 0.0013 M KH_2PO_4) inhibited the reaction: the first order velocity constant falling from 0.062/min to

* The term "order with respect to time" is used by LAIDLER[16] to describe the apparent order obtained by plotting values of log $(-dc/dt)$ against log c at various times during the course of a reaction, it is distinguished from "order with respect to concentration" obtained by plotting initial values of log $-dc/dt$) against corresponding varying initial values of log c. The distinction is a useful one since, in a complex reaction, the orders obtained by the two methods may be different, owing to changes occurring in quantities other than the concentrations of reactants, such as the accumulation of inhibitors.

However, the term "order with respect to time" is not very satisfactory since it seems to imply $(-dc/dt) = kt^n$.

0.028/min. (Similar effects were observed by SIMPSON AND KAUZMANN[17] on the optical rotation change at pH 7.8.)

The effect of temperature on the reaction rate of ovalbumin (0.4 g/100 ml) in 8 M urea (pH 7.8) (0.04 M PO_4) was examined. The first order rate constant at 30° was 0.052/min compared with 0.032/min at 25°.

A comparison was made of the rate of change of ΔA_{287} with that of $[\alpha]_D$ for ovalbumin in 7 M urea at 25° (pH 6.1). A plot of log $(\Delta A_\infty - \Delta A_t)$ *versus* $t/t_{0.5}$ (where t is the half time) gave a straight line (see Fig. 7).

As discussed in Part I the rate of change of $[\alpha]_D$ was not simple first order at each urea concentration studied (6–9 M). A final specific rotation (α_f) and half time, $t_{0.5}$,

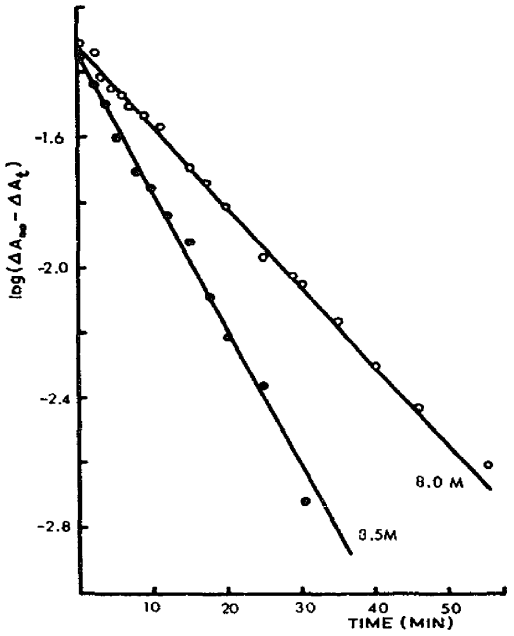

Fig. 5. Rate of change of ΔA_{287} with time for ovalbumin (0.096 g/100 ml, 1-cm cells) in 8.0 and 8.5 M urea (pH 7.8) (0.04 M PO_4) at 30°. Plot of log $(\Delta A_\infty - \Delta A_t)$ *versus* time (min) for each urea concentration. ΔA_t is the value of ΔA_{287} at a time "t" from the commencement of the reaction and ΔA_∞ is the final value of ΔA_{287}.

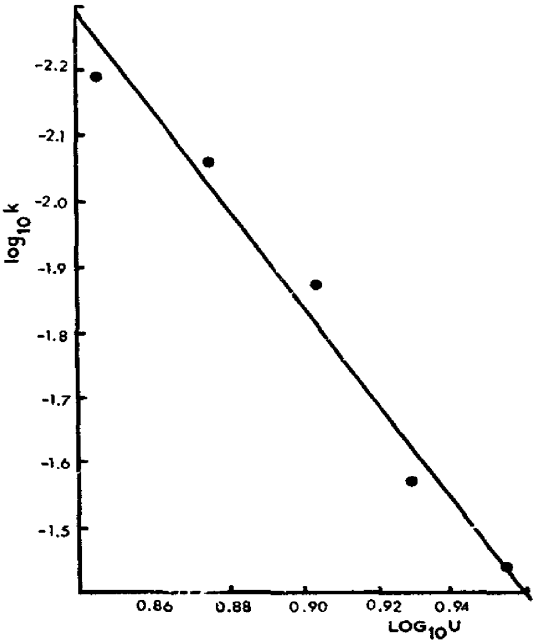

Fig. 6. Dependence of rate of change of ΔA_{287} for ovalbumin, (0.4 g/100 ml, 2-mm cells) on the urea concentration at pH 6.1 (0.012 M phosphate). Plot of log k (the velocity constant) *versus* log U (the urea concentration).

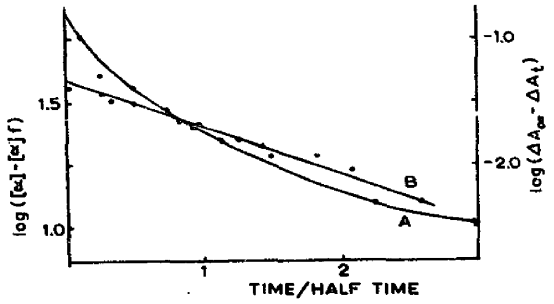

Fig. 7. Comparison of change of optical rotation and absorbancy with time for ovalbumin in 7 M urea. Curve A, optical rotation $[\alpha]_D$ (0.012 M phosphate buffer). Curve B, absorbancy difference at 287 mμ (ΔA_{287}) (0.2 M NaCl).

for the "primary" reaction were obtained for each urea concentration according to the method of SIMPSON AND KAUZMANN[17]. When log $(\alpha_f - \alpha)$ was plotted against $t/t_{0.5}$ (α being the specific rotation at a time t) for each urea concentration, straight lines were not obtained in agreement with SIMPSON AND KAUZMANN's observations (pH 7.9). A typical plot for 7 M urea (pH 6.1) at 25° is shown in Fig. 7 for comparison with the absorbancy plot. The half time for the primary $[\alpha]_D$ change was 40 min compared with 32 min for the ΔA change (both in phosphate). As discussed in Part I these half times vary somewhat from preparation to preparation.

A plot of log $t_{0.5}$ for the $[\alpha]$ change *versus* log[urea concentration] gave a slope of 13.5 ± 1.0 compared with 7.6 ± 0.5 for the equivalent A plot.

DISCUSSION

There is an immediate change of the ultraviolet spectrum of bovine serum albumin in acid and in urea (for the latter at all pH values in the range 4–9). The difference spectrum (*versus* the native protein) shows a minimum in $\Delta\varepsilon$ at 287 mμ. At low protein concentration the ultraviolet spectrum of ovalbumin undergoes little change in dilute acid. At higher protein concentrations more profound changes in the spectrum occur. At pH 3.1 in the presence of 7 M urea, there is an immediate change in the ultraviolet spectrum. The difference spectrum shows minima at 287, 292.5 mμ. At other pH values in urea solution there is a time-dependent change in the ovalbumin ultraviolet spectra with similar changes in the difference spectra. These observations enable certain conclusions to be drawn regarding the mechanisms of the denaturation of these proteins, their stability and their structure.

Minima at 287 mμ (also the 280 mμ shoulder) in difference spectra similar to those we have observed for bovine serum albumin in acid and urea have been obtained by LASKOWSKI *et al.*[8] for insulin in acid solution and on limited tryptic digestion and by SCHERAGA[18] for RNAase in acid solution. The additional minimum we observe at 293 mμ in urea solutions of ovalbumin is also present in acid and urea solutions of conalbumin (see Part IV, ref. 19), and in difference spectra of lysozyme[20]. Insulin and RNAase contain tyrosine but no tryptophan. Lysozyme contains 7 tryptophan and 3 tyrosine residues. Bovine serum albumin contains 19 tyrosine and 2 tryptophan residues per mole. Ovalbumin contains 9 tyrosine and 3 tryptophan residues, while conalbumin contains 18–21 tyrosine and 13 tryptophan residues. These observations on difference spectra for proteins and others on model compounds show that the 287-mμ minimum (including the 280-mμ shoulder) is due to differences in the environment of the tyrosine residues while that at 293–295 mμ is due to environmental differences for tryptophan residues[20,21].

In our first communication[7] we considered the possibility of spectral changes due to tyrosine residues arising from changes in tyrosine hydrogen bonds. LASKOWSKI *et al.*[8] interpreted spectral shifts for insulin in terms of rupture of tyrosyl–carboxylate hydrogen bonds. This conclusion has been questioned subsequently by a number of workers. WETLAUFER *et al.*[22] examined the effect of sodium acetate and urea in the spectra of tyrosine and phenol. Bathochromic shifts were observed in both media. Similar, but smaller, shifts were observed for *O*-methyl tyrosine. BIGELOW AND GESCHWIND[23] examined the effect of media of high refractive index on the spectra of the model compounds tryptophan, tyrosine, phenylalanine, indole, phenol and *O*-

methyl tyrosine. In nearly all cases there was a bathochromic spectral shift. The bathochromic shift was explained in terms of increase in refractive index of the solvent (see also BAYLISS AND MCRAE[24]). The two exceptions were explained in terms of specific interactions. Under non-denaturing conditions, salts and urea sometimes produce bathochromic shifts for normal tyrosine and tryptophan residues in proteins; except LiBr, NaBr and NaCl which produce hypsochromic shifts for normal tyrosine residues. Under denaturing concentrations of salts and urea hypsochromic shifts, both for tyrosine and tryptophan residues, are invariably produced. While this hypsochromic shift (the "denaturation blue shift") for abnormal tyrosyl residues may be due to changes in hydrogen bonding of tyrosine hydroxyl groups this explanation cannot be valid in the case of shifts for tryptophane residues. A number of workers[6,25,26] have suggested that some of the aromatic residues in proteins are involved in hydrophobic bonding, and are in an environment of high refractive index. On denaturation these chromophores are exposed to media of lower refractive index and hypsochromic shifts may be expected[21,23].

The hypsochromic spectral changes we have observed for bovine serum albumin in acid solution closely paralleled changes in other properties reflecting the molecular expansion of bovine serum albumin. The results are consistent with changes in the degree of hydrogen bonding. While these changes occur in the carboxyl titration region they do not parallel protonation of carboxylate groups. It is not possible to identify the acceptor groups. As WILLIAMS AND FOSTER[25] have pointed out the bovine serum albumin results are also consistent with changes in hydrophobic bonding. The additional change in $\Delta\varepsilon_{287}$ for bovine serum albumin in acid below pH 1 is similar to that of $[\alpha]_D$. The latter has been discussed by YANG AND FOSTER[12].

The spectral changes for bovine serum albumin in urea solution at all pH values are similar to those for bovine serum albumin in acid solution. The similarity of the changes in other properties for bovine serum albumin in urea and in acid are striking *e.g.* the laevorotation and viscosity increases, the decrease in sedimentation coefficient and volume. These observations point to the essentially similar nature of the conformation changes taking place in urea and in acid (see ref. 27). The immediate and generally reversible nature of all of these changes indicates the considerable configurational adaptability of bovine serum albumin.

For low concentrations of ovalbumin under moderately low pH conditions, there are no pronounced effects on the ultraviolet spectrum. This is in accord with the observations of YANG AND FOSTER[12] and KAUZMANN[27] that ovalbumin does not expand significantly in acid solution under these conditions (see also the titration curve[28] and absence of change of polarization of fluorescence[13]).

The time-dependent hypsochromic shifts we have observed for ovalbumin in urea solution, may reflect changes in tyrosine hydrogen bonding or hydrophobic bonding. If the type of mechanism for urea denaturation of ovalbumin proposed by SIMPSON AND KAUZMANN[17] is valid then the order of reaction with respect to urea should reflect the number of sites participating in the process being followed. The apparent order of the spectral change with respect to urea is approx. 8. This is of significance in view of CRAMMER AND NEUBERGER'S[3] finding that there are 7–8 non-ionizing tyrosine residues in ovalbumin. Furthermore HARRINGTON[29] has shown, by pH studies, that there are 8 acid and 8 base binding groups liberated during the guanidine hydrochloride denaturation of ovalbumin. These considerations suggest that tyrosine hydrogen

bonds are being broken in the urea denaturation and the difference spectra reflect these changes. However, the alternative of changes in hydrophobic bonding cannot be eliminated at present.

The kinetics of the spectral changes assume special significance in view of the fact that this property changes at a different rate from other properties so far studied (*e.g.* optical rotation and viscosity). The spectral change is simple first order with respect to time. The optical rotation change is not simple first order and is apparent 14th order with respect to urea concentration. The optical rotation criterion is not specific for a particular conformation change but reflects overall conformation changes. When ovalbumin has few, if any, S–S bonds the abnormal tyrosine residues may well impose some constraint on the molecule. The activation process may involve the rupture of a critical number of these side-chain bonds.

Clearly ultraviolet spectral measurements are useful in following environmental changes in proteins. However, caution is necessary in their interpretation and the results obtained should be judged only in conjunction with other criteria[30].

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