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THE DENATURATION OF PROTEINS

IV. CONALBUMIN AND IRON(III)-CONALBUMIN IN UREA SOLUTION

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SUMMARY

Extensive optical rotation, viscosity, sedimentation, and spectroscopic studies of the urea denaturation of conalbumin and its iron complex are presented and considered in relation to the structure of these molecules. At low pH (< 4.2), conalbumin undergoes reversible changes in optical rotation, sedimentation and viscosity. The extent of these changes is dependent on the ionic strength and is greatly increased in the presence of urea. Over the pH range 5.8–7.8, urea brings about extensive disorganization of both conalbumin and iron-conalbumin. The optical rotation and viscosity changes in urea (> 5 M) solutions of conalbumin are very rapid, those of urea solutions of iron-conalbumin are much slower. In the pH range 8.8–10. iron-conalbumin is also considerably more stable towards urea than conalbumin. Experiments with cysteine present indicate that disulphide bonds are of importance in stabilizing the native configuration of both conalbumin and iron-conalbumin. Both acid and urea bring about similar rapid changes in the region of tyrosine and tryptophan absorption of the ultraviolet spectrum of conalbumin. The analogous spectral changes in urea solutions of iron-conalbumin are much slower. The change of absorbancy of iron-conalbumin at 292 mμ in concentrated urea solutions is first order with respect to time. The apparent order of reaction with respect to urea at pH 5.9 and 30° is 14. The spectroscopic changes accompanying urea denaturation of iron-conalbumin in the pH range 9.6–10.2 are complex and irreversible.

Evidence is presented to show that at near-neutral pH, urea denaturation of conalbumin modifies, but does not destroy, the iron complex. Some preliminary experiments on the "renaturation" of conalbumin are reported.

INTRODUCTION

Few studies have been carried out on the denaturation of conalbumin, the "iron-binding protein" of egg white. Conalbumin was originally separated by precipitation

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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at pH values less than 4 and in the presence of salt[1,2]. The product differed in solubility and electrophoretic properties from the native protein. FRAENKEL-CONRAT AND FEENEY[3], using conalbumin prepared by this "acid modification reaction", showed that the protein lost its iron(III)-binding capacity on treatment with heat, alkalis, detergents and amides. WARNER AND WEBER[4] isolated crystalline iron–conalbumin and conalbumin by ethanol fractionation without exposure to low pH. They showed that iron(III) combined with two sites on each molecule of protein and bound one bicarbonate ion per metal ion[5]. WARNER[6] proposed a structure for the complex and reported that iron–conalbumin exhibited a higher stability towards alkali denaturation than the iron-free protein. AZARI AND FEENEY[7] found that conalbumin, but not the iron complex, was readily digested by chymotrypsin.

An extensive study of the urea denaturation of iron–conalbumin and iron-free conalbumin is reported here. The changes in a number of properties which depend in different ways on the structure of the protein molecule are compared under a variety of pH conditions. The importance of this approach has been stressed in Part I.

EXPERIMENTAL

Proteins

Iron–conalbumin was prepared by the method of WARNER AND WEBER[4]. The protein was recrystallised three times from ethanol–water (15–20%, v/v) and stored at 0°. Iron-free conalbumin was prepared by the addition of Dowex-1X8 resin in the chloride form to a concentrated solution of iron–conalbumin, according to WARNER AND WEBER[4]. The absorbancy at 470 mμ of a 1 g/100 ml solution in a 1-cm cell was less than 0.010, indicating the absence of the iron complex[4]. Great care was taken to avoid contaminating metal ions from reagents, glassware or dialysis tubing in the course of experiments. The protein concentration of stock solutions was determined by the Kjeldahl nitrogen method of MCKENZIE AND WALLACE[8], taking the value 16.4 g/100 g for the nitrogen content of conalbumin (this value was obtained from several parallel dry weight and nitrogen estimations), and by light absorption at 280 mμ. Iron–conalbumin was also estimated by light absorption at 470 mμ. The values of $A_{1\,cm}^{1\,g/100\,ml}$ used are given in Table II. One preparation of iron-free conalbumin was freeze-dried and stored in the lyophilized state at 0° for three months. No change in any of the physical properties or in behaviour towards urea was observed.

Methods

Spectrophotometric measurements were carried out as described in Part II (see ref. 10).

The procedure for sedimentation, viscosity and optical rotation measurements has been described in Part I (see ref. 11). The partial specific volume[12] of conalbumin and iron conalbumin was taken as 0.756 at 20°.

Preparation of the reaction mixture

The protein solution and the appropriate buffer–urea mixtures were brought to 30.0°. The solutions were then gently, but thoroughly mixed and an aliquot transferred by pipetting into the spectrophotometer cell or polarimeter tube or viscometer.

Using a Leeds and Northrup type 7666 pH meter pH measurements were carried

out on the reaction mixture at the beginning and the end of each experiment. The pH was found to be constant to within 0.2 units under all of the conditions used. The pH measurement procedure has been described[11].

RESULTS

Physical properties of conalbumin and iron–conalbumin

Values of intrinsic viscosity, $[\eta]$, optical rotation, $[\alpha]_D$, and sedimentation coefficient, $(s_{20,w})$ for conalbumin and iron–conalbumin are given in Table I. Data for ultraviolet and visible absorption spectra are given in Table II.

TABLE I

VISCOSITY, OPTICAL ROTATION AND SEDIMENTATION OF CONALBUMIN

Protein	$[\eta]$ (dl/g), pH 6.5, 0.1 M NaCl	$[\alpha]_D$ pH 6.5 0.1 M NaCl	$s_{20,w}$(S) for 1 g/100 ml						
			pH 6.8 0.1 M NaCl	pH 7.8 0.1 M NaCl	pH 9.4 0.05 M $Na_2B_4O_7$ 0.05 M NaCl	8 M Urea			
						pH 5.9 0.1 M NaCl	pH 7.8 0.1 M PO_4	pH 10.1 borate–KCl	pH 9.1 borate–NaCl 0.02 M cysteine
Conalbumin	0.040	−37°	—	4.7*	4.6	—	1.9	2.8	2.3
Iron–Conalbumin	0.040	−44°	4.8	—	4.7	3.1	—	2.8	2.8

* Data of PHELPS AND CANN[12].

TABLE II

SPECTRAL PROPERTIES OF CONALBUMIN

Protein	pH	λmax (mμ)	$A^{1\%}_{1\,cm}$ g/100 ml	
			Present work	Ehrenpreis and Warner[9]
Conalbumin	1.5	280	11.0	11.0
Conalbumin	6.0	280	11.3	—
Iron–Conalbumin	6.0	280	14.8	—
Iron–Conalbumin	6.0	470	0.621	0.620

The effect of acid on the viscosity and optical rotation of conalbumin

The effect of lowering the pH on the viscosity and optical rotation of conalbumin was examined. This was only done for conalbumin since iron–conalbumin is not stable below pH 5. There was a marked increase in reduced viscosity (η_{red}) and $[\alpha]_D$ for conalbumin below pH 4 as shown in Fig. 1.

If the pH were readjusted to neutrality after only a short exposure of the protein to the acid medium, both the viscosity and optical rotation values returned to those of the native protein. In solutions of the same ionic strength (*i.e.* I = 0.1, 0.1 M NaCl), PHELPS AND CANN[12] have observed that the sedimentation coefficient of conalbumin decreases continuously from a value of about 4.7 S at pH 4.2 to 3.0 S at pH 2.0. This decrease in sedimentation coefficient was also reversible.

Several experiments were carried out on the dependence of the reduced viscosity for conalbumin in acid solution on the ionic strength (I). On varying I over the range 0.07–0.15 with added sodium chloride no evidence of aggregation was obtained from the reduced viscosity measurements. There was evidence for aggregation in solutions of ionic strength outside these limits. This aggregation is a secondary effect, super-

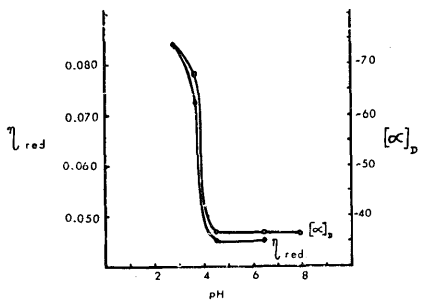

Fig. 1. Effect of pH on the reduced viscosity, (η_{red}), and optical rotation, $[\alpha]_D$, of conalbumin in 0.1 M NaCl. Protein concentration: 0.5 g/100 ml. Temperature, 30°.

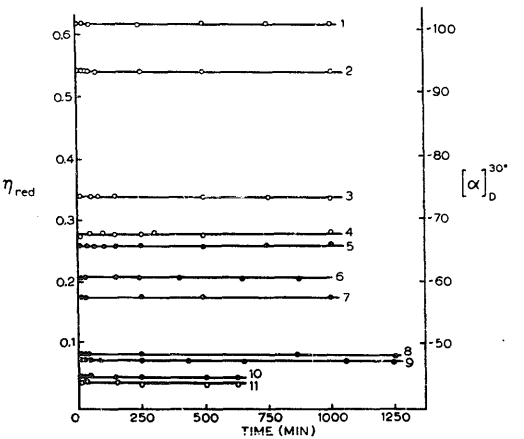

Fig. 2. The reduced viscosity (η_{red}) and optical rotation $[\alpha]_D$ of conalbumin in acid solution in the presence and absence of urea at 30°. O—O, optical rotation; ●—●, viscosity. Protein concentration: 0.5 g/100 ml and 0.1 M NaCl present, in all cases except where stated. 1, α, 7 M urea (pH 3.0) 0.25 g/100 ml of protein; 2, α, 4 M urea (pH 4.0); 3, α (pH 2.9); 4, α (pH 3.7); 5, η, 7 M urea (pH 3.0) no NaCl, 0.25 g/100 ml of protein; 6, η, 7 M urea (pH 3.0) 0.25 g/100 ml of protein; 7, η (pH 3.0) no NaCl, 1.0 g/100 ml of protein; 8, η (pH 2.9); 9, η (pH 3.7); 10, η (pH 5.2); 11, α (pH 5.2).

imposed on the structural modification induced by the acid medium. In view of these observations the experiments in acid solution involving urea were conducted at 0.1 *I*.

The effect of urea on the viscosity and optical rotation of conalbumin at pH 3–4

The above changes in the viscosity and optical rotation of conalbumin on lowering the pH below 4.2 take place immediately (*i.e.* before a reading can be taken) as shown in Fig. 2.

Similar, but greatly increased, changes in reduced viscosity and laevorotation take place in the acid pH region in the presence of urea (Fig. 2). Even urea concentrations as low as 2 M cause an increase in laevorotation.

The effect of urea on the viscosity and optical rotation in the pH range 5.9–8.2

Conalbumin: There was no change in the reduced viscosity and optical rotation of conalbumin in 3 M urea solution. In 4 and 5 M urea solutions, time-dependent changes in optical rotation and viscosity took place. In 6 M urea solutions (and in those of higher urea concentration) these changes were rapid (occurring before a reading could be taken). The results are shown in Fig. 3.

Since the same values for the final reduced viscosity were obtained with both 0.5 and 1 g/100 ml protein solutions, no indication of aggregation was obtained from the viscosity data.

The effect of pH in this range was examined by comparing the rate of change of optical rotation of conalbumin at pH 5.8 and 7.8 in 5 M urea. It was found that the rate of change of optical rotation is higher at the lower pH, but that the final value of the laevorotation reached was the same.

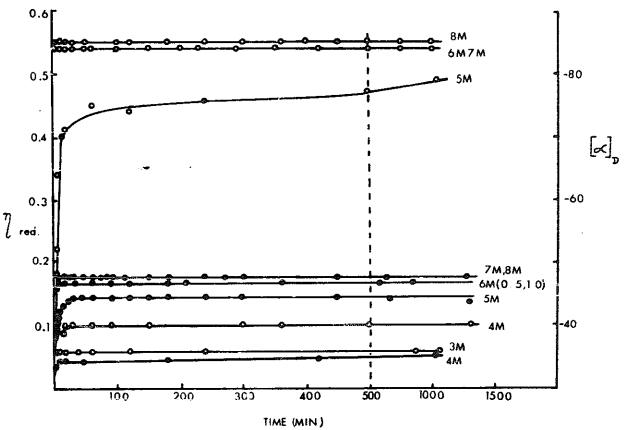

Fig. 3. Effect of urea on the reduced viscosity (η_{red}) and optical rotation $[\alpha]_D$ of conalbumin at pH 7.9–8.0 (0.1 M phosphate buffer) and 30°. Protein concentration: 0.5 g/100 ml except otherwise indicated. ○—○, optical rotation; ●—●, viscosity. Note change of scale at dashed line on this and other figures.

Iron–conalbumin: There was no change in reduced viscosity and optical rotation of iron–conalbumin in urea solutions up to and including 5 M for the pH range 5.9–8.2. At higher concentrations, time-dependent changes in optical rotation and viscosity took place as shown in Fig. 4. Over the range 0.25–1 g/100 ml, protein concentration appeared to have very little influence on the reduced viscosity.

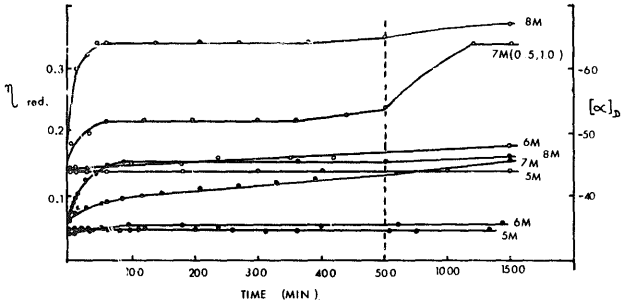

Fig. 4. Effect of urea on the reduced viscosity (η_{red}) and optical rotation ($[\alpha]_D$) of iron–conalbumin in 0.1 M NaCl, pH 5.9–6.3, at 30°. Protein concentration: 0.5 g/100 ml except where otherwise indicated. ○—○, optical rotation; ●—●, viscosity.

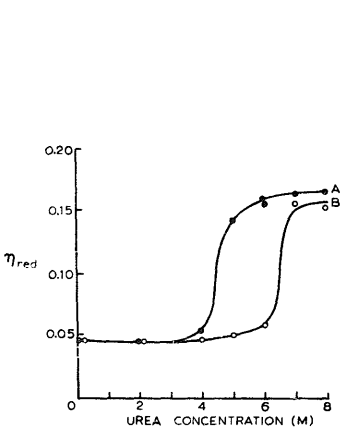

Fig. 5. Comparison of the effects of urea concentration on the viscosity of conalbumin and iron–conalbumin after 24 h at 30°. A. Conalbumin in 0.1 M phosphate buffer (9 parts of Na_2HPO_4 to 1 part of KH_2PO_4) (pH 7.9–8.0). B. Iron–conalbumin in 0.1 M NaCl (pH 5.0–6.3). Protein concentration: 0.5 g/100 ml.

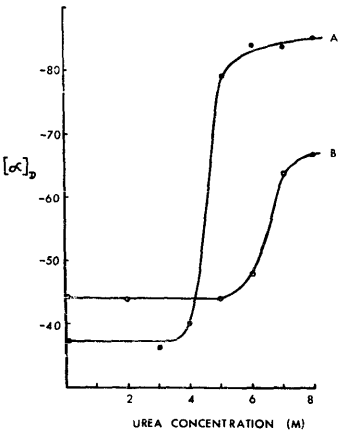

Fig. 6. Comparison of the effects of urea concentration on the optical rotation of conalbumin and iron–conalbumin. A, Conalbumin. B, iron–conalbumin. All conditions as in Fig. 5.

In each case, both the final viscosity and optical rotation value were lower than the corresponding values obtained with the iron-free protein at the same urea concentration. This is shown in Figs. 5 and 6.

The effect of urea on the viscosity and optical rotation in the pH range 8.8–10

Conalbumin: There was no change in the reduced viscosity and optical rotation for conalbumin in 3 M urea in this pH range. Time-dependent viscosity and optical rotation changes were observed in 4 M and 5 M urea solutions; these changes being more rapid than at pH 5.9 or 7.9. In solutions of higher urea concentration the changes were complete before readings could be taken. Typical results are shown in Figs. 7 and 8.

The final values of laevorotation and reduced viscosity were lower than those attained at the same urea concentration at pH 5.9 or 7.9. The ionic strength in these experiments was kept in the range 0.1–0.15 and under these conditions no evidence of aggregation was obtained over the protein concentration range 0.25–1.0 g/100 ml.

Cysteine was found to increase the extent of the laevorotation and viscosity change. PCMB also did so but to a much smaller extent than cysteine (Figs. 7 and 8).

Iron–conalbumin: The dependence of the rate of change of optical rotation and reduced viscosity of iron–conalbumin on urea concentration in this pH range was

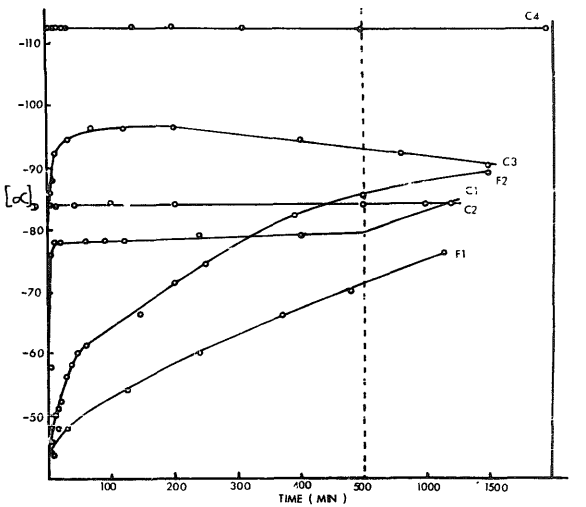

Fig. 7. Effect of 7M urea with and without cysteine and PCMB on the optical rotation of conalbumin and iron–conalbumin in alkaline solution at 30°. Borate–KCl buffer ($I = 0.12$). C1, Conalbumin (1.0 g/100 ml) in 5M urea (pH 9.3); C2, Conalbumin (0.50 g/100 ml) in 7M urea (pH 9.3); C3, Conalbumin (0.50 g/100 ml) in 7M urea and 0.005M PCMB (pH 9.3); C4, Conalbumin (0.75 g/100 ml) in 7M urea and 0.02M cysteine (pH 9.0); F1, Iron–conalbumin (0.50 g/100 ml) in 7M urea (pH 9.4); F2, Iron–conalbumin (0.5 g/100 ml) in 7M urea and 0.02M cysteine (pH 8.9).

found to be similar to that of pH 6.0. The final values reached are higher than those attained at the same urea concentration at the lower pH. This could be due to the slow hydrolytic fission of some of the disulphide linkages which has been reported to take place in this pH range[13].

Cysteine was found to accelerate the denaturation rate considerably. PCMB, however, had virtually no influence on the denaturation rate. From a comparison of the effect of cysteine on the optical rotation and viscosity of iron- and iron-free conalbumin in concentrated urea solutions (Figs. 7 and 8), it is clear that the disulphide bonds in iron–conalbumin are less readily split than those in conalbumin.

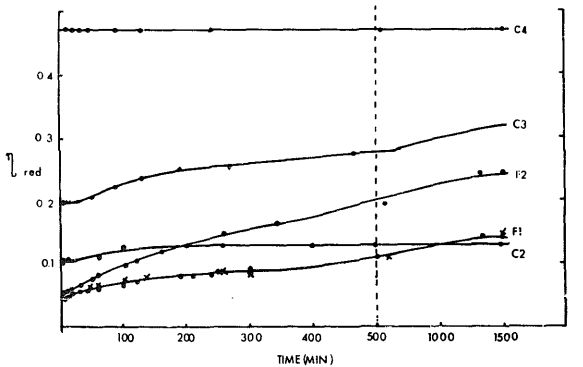

Fig. 8. Effect of 7M urea with and without cysteine and PCMB on the reduced viscosity of conalbumin and iron–conalbumin in alkaline solution at 30°. Effect of cysteine and PCMB. Buffer: borate–KCl ($I = 0.12$).C2, Conalbumin (0.25 g/100 ml) in 7M urea (pH 9.9); C3, Conalbumin (0.50 g/100 ml) in 7M urea and 0.005M PCMB (pH 9.3); C4, Conalbumin (0.75 g/100 ml) in 7M urea and 0.02M cysteine (pH 9.0). F1, Iron–conalbumin (0.5 g/100 ml) in 7M urea (pH 9.3) without PCMB (O—O) and with 0.005 M PCMB (×—×); F2, Iron–conalbumin (0.5 g/100 ml) in 7 M urea and 0.02 M cysteine (pH 9.0).

Sedimentation in urea solution

Sedimentation measurements for conalbumin and iron–conalbumin in 8 M urea solution were carried out after reaction was completed, at several pH values. Measurements were made at a speed of 59780 rev./min for 1 g/100 ml of protein. In all cases a single boundary was observed. Values for $s_{20,w}$ under these conditions are shown in Table I where they may be compared with those of the native protein. In no instance was there any gelation observed at the bottom of the ultracentrifuge cell. The red colour of iron-conalbumin in all cases sedimented with the protein schlieren boundary.

The decrease of $s_{20,w}$, together with the greatly increased viscosity and laevorotation, may be interpreted as showing extensive uncoiling and disorganization of the native conalbumin structure under the conditions studied.

Ultraviolet spectra of conalbumin and iron–conalbumin

Effect of acid on the ultraviolet spectrum of conalbumin: In solutions of pH 4.2 the absorption maximum of conalbumin at 280 mμ is shifted immediately towards the shorter wavelengths. The extent of this hypsochromic shift is dependent on the pH. At pH 3.1 the peak had shifted to 277 mμ. No further shift occurred on decreasing the pH below 3 until pH 1.0 was reached when the peak shifted back to 278 mμ.

Difference spectra were obtained by comparing the spectrum of the protein at pH values below 4.2 with that of the protein in neutral solution. At any given pH below 4.2, the difference in absorbancy was found to be greatest at 291–292 mμ, another slightly smaller peak being situated at 286 mμ (Fig. 9).

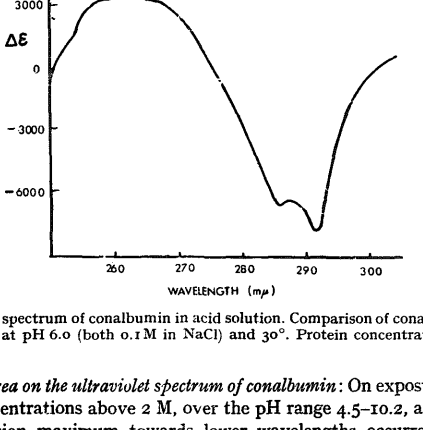

Fig. 9. Difference spectrum of conalbumin in acid solution. Comparison of conalbubumin at pH 0.9 with conalbumin at pH 6.0 (both 0.1 M in NaCl) and 30°. Protein concentration: 0.05 g/100 ml.

Effect of urea on the ultraviolet spectrum of conalbumin: On exposure of conalbumin to urea at concentrations above 2 M, over the pH range 4.5–10.2, an immediate shift of the absorption maximum towards lower wavelengths occurred. The spectrum resembled that of the protein in acid solution at pH 3.1. The absorption maximum in solutions of urea concentration 5 M or greater was shifted to 276 mμ.

Examination of the difference absorption spectra, obtained by comparing the spectrum of the protein in urea with that of the native protein revealed the same characteristics as those described above for acid solutions of conalbumin, namely absorption difference peaks at 291–292 mμ and 286 mμ (Fig. 10).

Effect of urea on the ultraviolet absorption spectrum of iron–conalbumin: The ultraviolet absorption spectrum of iron–conalbumin changes with time in the presence of urea, the rate of change being strongly dependent on the urea concentration. This behaviour contrasts sharply with that of iron-free conalbumin where spectroscopic changes in urea solutions are immediate. Urea brought about a general lowering in absorption intensity over the wavelength range 250–325 mμ. Over the urea concentration range 1–9 M and pH range 5.8–10.2, the greatest shift of the absorption

maximum was attained with 9 M urea and was to 277 mμ (as compared with 276 mμ for iron-free conalbumin).

Difference spectra were obtained by comparing the absorption of iron-conalbumin in urea with that of the native protein under the same conditions. These spectra all have in common the maximum difference peaks at 286 mμ and 291–292 mμ;

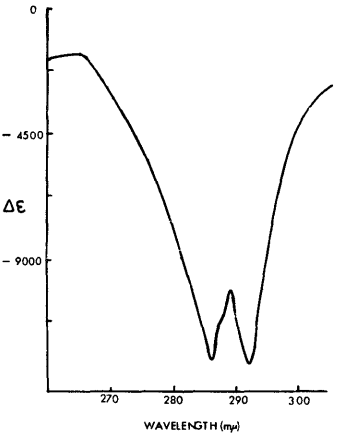

Fig. 10. Difference spectrum for conalbumin in urea solution. Conalbumin in 9M urea (pH 6.0) compared with conalbumin (pH 6.0) (both 0.1 M in NaCl) at 30°. Protein concentration: 0.033 g/100 ml.

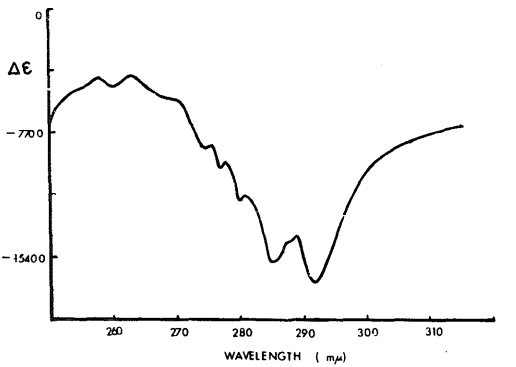

Fig. 11. Difference spectrum of iron–conalbumin in urea solution. Comparison of iron–conalbumin in 8M urea (pH 7.3) with iron–conalbumin at pH 7.3 (both 0.1 M in NaCl) after 24 h at 30°. Protein concentration: 0.088 g/100 ml.

i.e. the same positions as the difference spectra for conalbumin in acid or urea solutions (Fig. 11). There are also a number of smaller peaks detectable (*e.g.* 278 mμ).

Kinetics of the ultraviolet spectral changes for iron–conalbumin in urea solution: A preliminary study indicates that the rate of change of the ultraviolet absorption of iron–conalbumin in urea solution is apparent first order. The rate of change of absorbancy is greater than that of optical rotation or viscosity; *e.g.* the half time of the absorbancy change in 8 M urea (0.1 M NaCl) at pH 5.9 is 12.5 min while that of the optical rotation is 17 min. The rate of change of ultraviolet absorption is strongly dependent on the urea concentration: the change appears to be fourteenth order with respect to urea concentration at pH 5.9.

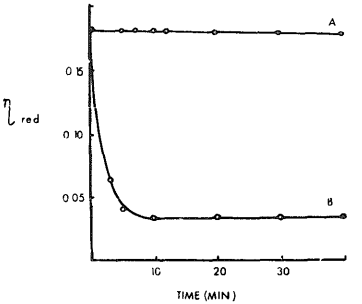

Fig. 12. Viscosity changes accompanying the denaturation and "renaturation" of conalbumin at pH 6.0. A, Conalbumin (0.5 g/100 ml) in 6 M urea (0.05 M NaCl); B, Solution A diluted with an equal volume of 0.05 M NaCl.

Effect of urea on the visible spectrum of iron–conalbumin

The visible spectrum of iron–conalbumin changes with time in urea solutions (6 M or higher), the rate of change being dependent on the urea concentration, and pH. In the pH range 6.0–9.5, the absorbancy at 470 mμ after 28 h at 30°, in urea solutions more concentrated than 7.5 M, was 54–58% of that of the native protein and the spectral band remained similar in shape to that of the native protein. In solutions of pH higher than 10, spectroscopic changes were complex, the visible spectrum being completely altered by urea denaturation.

The change at 470 mμ was found to continue as long as the optical rotation and viscosity continued to change. Under the same conditions, the half-times of the changes in these three properties were the same.

Some conclusions as to the effect of urea denaturation on the iron complex may be gained from the following observations: (a) If the urea-denatured protein was salted out with 0.6 saturated ammonium sulphate no colour was left in the supernatant. On dilution of the ammonium sulphate solution, the precipitate redissolved, complete recovery of colour being obtained. (b) In all of the ultracentrifuge measurements for iron–conalbumin in 8 M urea, it was observed that the colour sedimented with the protein. (c) From examination of the visible spectra, it was found that 55–60% of

the colour remained after 48 h denaturation with 8 M urea, the peak remaining at 470 mμ in the denatured protein in the pH range 6.0–9.5.

These observations suggest that the iron complex is in some way modified by the urea denaturation, but that the iron is not released.

A preliminary study was made of the kinetics of the absorbancy change at 470 mμ of solutions of iron–conalbumin in urea, both in the presence and absence of salts in the pH range 6.0–10. The reaction was not simple first order. It was apparent second order with respect to time for about 75 % of the total change. In view of the complex kinetics, no quantitative treatment is attempted here.

Renaturation of conalbumin

A few experiments were carried out on the renaturation of conalbumin. A solution of iron-free conalbumin was mixed with a urea–NaCl solution (both at 30°), the final mixture being 0.05 M NaCl, 6 M urea and 0.5 g/100 ml in protein (pH 5.9–6.1). An immediate change in viscosity was observed, the value of η_{red} being 0.182. The above reaction mixture (10 ml) was then pipetted into a flask containing 0.05 M NaCl (10 ml) and viscosity of the resultant mixture followed for 24 h. At the end of 7 min the reduced viscosity had decreased to 0.034, no further change being observed (Fig. 12).

A parallel experiment was carried out, optical rotation being the property followed.

Iron-binding capacity was determined by adding a 50 % excess of iron in the form of a ferric citrate (in pH 6.5 phosphate), and measuring the resultant colour produced at 470 mμ. The results are shown in Table III.

TABLE III

PROPERTIES OF NATIVE, DENATURED AND "RENATURED" CONALBUMIN IN 0.05 M NaCl, AT THE pH INDICATED

Property	*pH*	*Native protein*	*Denatured protein (6 M urea)*	*"Renatured" protein*
Optical rotation	6.0	−37°	−92°	−35° (3 M urea)
Viscosity	6.0	0.040	0.182	0.034 (3 M urea)
Iron-binding capacity	6.5	100 %	0	85 % (1.5 M urea)

An experiment was carried out along the same lines as the one described above to "renature" conalbumin exposed to 5 M urea in acid solution. The protein (0.5 g/100 ml) was exposed to 5 M urea in 0.05 M acetate buffer (pH 4.5) at 30°. After 24 h, the mixture was diluted with an equal volume of 0.05 M phosphate buffer (containing 9 parts of Na_2HPO_4 to one part KH_2PO_4) the final pH being 7.2 and allowed to stand for 24 h at 30°. After 24 h, the viscosity, optical rotation and iron-binding capacity were determined (Table IV).

No attempt was made to renature conalbumin from alkaline solution as the changes in spectroscopic properties in the pH region 9.6–10.2 were found to be irreversible. This was possibly due to hydrolytic splitting of disulphide groups.

TABLE IV

RENATURATION OF CONALBUMIN FROM 5M UREA IN ACID SOLUTION

Property	*Native protein*	*Denatured protein (5 M urea)*	*"Renatured" protein*
Optical rotation	-37°	-94°	-34° (2.5 M urea)
Viscosity	0.040	0.214	0.037 (2.5 M urea)
Iron-binding capacity	100%	0	80% (1.25 M urea)

DISCUSSION

A number of conclusions as to the secondary and tertiary structure of conalbumin may be drawn from the present study. In general iron–conalbumin shows a far greater stability towards urea (in the pH range 5.5–10.2) than does the iron-free protein.

The behaviour of conalbumin in acid solution is very similar to that of bovine serum albumin[11,14] and bovine-pseudoglobulin[15]. When the pH is less than 4, these proteins undergo a rapid "reversible" unfolding, which is accompanied by an increase in reduced viscosity and laevorotation, and a decrease in sedimentation coefficient. The unfolding also has a pronounced effect on the shape of the titration curve. The change in the above properties of conalbumin observed at acid pH may be caused by the liberation of prototropic side-chains near pH 4 resulting in a high degree of repulsion within the molecule. The recent experiments of WISHNIA, WEBER AND WARNER[22] (see also ref. 16) on the behaviour of conalbumin at low pH indicate that instability is introduced when 10 and 18 protons have been bound by a set of 18 basic groups, of which 16 are carboxylate ions.

The ultraviolet spectroscopic studies demonstrate that at low pH (< 4) in the absence of urea, and over the whole pH range in the presence of urea, closely similar immediate changes in the ultraviolet spectrum of conalbumin take place. In all cases there is a hypsochromic shift. The difference spectra show pronounced minima at 286 and 292 mμ. These minima correspond to the wavelengths of maximum negative slope in the spectrum. As discussed in Part II (see ref. 10) minima at 287 and 292 mμ arise out of changes in the environment of tyrosine and tryptophane residues respectively. The magnitude of $\Delta\varepsilon_{292}$ for conalbumin is not surprising since there are 13 tryptophane along with the 18 tyrosine residues per mole of conalbumin.

Similar hypsochromic shifts take place in the ultraviolet spectrum of iron–conalbumin in urea solution and the difference spectra exhibit similar minima. The changes for iron–conalbumin in urea takes place more slowly than those for conalbumin in acid or urea. The preliminary kinetic studies indicate that the ultraviolet changes for iron–conalbumin are more rapid than those of optical rotation, viscosity and visible spectra.

All of these ultraviolet spectral shifts for the conalbumin are of the "denaturation blue shift" type[10,17,18]. Changes in hydrophobic bonding on the addition of acid or urea could result in the tyrosine and tryptophane residues being exposed to media of lower refractive index causing the hypsochromic shift in the ultraviolet spectra. On

the other hand the changes in the tyrosine spectra could be due to breakage of hydrogen bonds in acid and urea. The recent work of WARNER *et al.*[5,16,21,22] on pH titration indicates that 7 of the 18 tyrosine residues in conalbumin may be buried in hydrophobic regions of the protein, and that a large proportion of the rest, possibly the 6 involved in chelating Fe^{3+}, are hydrogen-bonded.

The spectroscopy, viscosity and sedimentation studies of the urea denaturation of conalbumin and iron-conalbumin clearly show that urea brings about extensive disorganization of the native protein structure, iron-conalbumin showing a considerably higher stability. The iron-free protein showed its highest stability towards urea at about pH 8.0. From the experiments involving cysteine it is clear that the disulphide bonds are of importance in maintaining conalbumin in its native configuration. There was no evidence in the present work of any aggregation of the conalbumins following denaturation in urea under the conditions used. In this respect and in the effect of pH on the denaturation rate conalbumin resembles β-lactoglobulin.

The changes in chain configuration of conalbumin brought about by urea are accompanied by changes in the properties studied. The fact that the "swelling" of conalbumin in acid solution is accompanied by similar changes lends support to the idea that the changes in acid and urea may be essentially similar[19].

From the preliminary experiments on the "renaturation" of conalbumin from neutral and acid urea solutions, it would appear that conalbumin, in common with bovine serum albumin, possesses a high degree of "configurational" adaptability, the high number of disulphide bonds permitting it to return to the native configuration on removal or dilution of the denaturing agent.

From a number of experimental observations, it would appear that urea denaturation does not cause the liberation of iron(III) from iron-conalbumin. Such a reaction might, however, take place at a strongly alkaline pH, where the complexity of the spectroscopic results did not permit clear conclusions to be drawn. The situation is further complicated by possible competitive effects of buffer ions for binding sites.

The effect of urea denaturation is to disturb the configuration of the protein in the vicinity of the iron-complexing sites with resultant alteration in the visible spectrum. Somewhat similar effects have been reported by MARGOLIASH, FROHWIRT AND WIENER[20] for cytochrome *c*.

The difference in the observed resistance of conalbumin and its iron complex to urea denaturation may be explained if it is assessed that the chelate sites in conalbumin are made up of ligand groups which are attached to widely separated sections of the peptide chain. The higher stability of iron-conalbumin (see also the recent work of AZARI AND FEENEY[23]) may then be due to the stabilizing influence of the iron complex which provides two crosslinks between widely separated sections of the peptide chains, thus restricting unfolding.

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