BBA 3827

THE DENATURATION OF PROTEINS

I. SEDIMENTATION, DIFFUSION, OPTICAL ROTATION, VISCOSITY AND GELATION IN UREA SOLUTIONS OF OVALBUMIN AND BOVINE SERUM ALBUMIN

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(Received July 10th, 1962)

SUMMARY

A comparative study is made of the effect of pH on the urea denaturation of bovine serum albumin and ovalbumin. The denaturation process is followed by optical rotation, viscosity, gelation, sedimentation and diffusion measurements. In the pH range 3–10, bovine serum albumin shows a very rapid initial increase in laevorotation and viscosity in concentrated urea solution at 25°. Ovalbumin resembles bovine serum albumin in its behaviour in urea at low pH. Near neutrality and at alkaline pH values, ovalbumin undergoes a much slower change in these properties. The kinetics of the optical rotation change for ovalbumin generally resemble those reported by SIMPSON AND KAUZMANN, but there are some differences. Evidence is presented which indicates that these proteins behave in urea solution as random-coil polymers. The "unfolded" proteins show a tendency to aggregate slowly in 7 M urea. This is much more pronounced in neutral and alkaline solution and ovalbumin aggregates more readily than bovine serum albumin. The aggregation of "unfolded" bovine serum albumin appears to be due largely to a S-S–SH exchange reaction. This is only partly the case for ovalbumin. The effect of *p*-chloromercuribenzoate on S-S bonds is considered. The value of the techniques used is assessed. The importance of studies of the effect of pH in the classification of proteins with respect to thermodynamic and kinetic stability and in the elucidation of the mechanism of denaturation is stressed.

INTRODUCTION

Although the phenomenon of denaturation has been sporadically studied over the last sixty years its mechanism and the structural changes associated with it are still not completely understood[1,2]. The present series of papers describes a study of the denaturation of a variety of proteins by various agents under diverse conditions.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

Since the structural changes accompanying denaturation are probably complex, a number of experimental methods have been used to throw light on the denaturation process. The usefulness of these methods is also assessed.

Recent studies on the urea denaturation of bovine serum albumin and ovalbumin (mainly in the pH range 7–9) show that this process is accompanied by changes in optical rotation, indicating an extensive disorganization of the native protein structure[3,4]. It has generally been considered that aggregation does not take place as a result of the urea denaturation of these proteins. On the other hand recent viscosity and gelation experiments in neutral and alkaline urea solutions have indicated that gradual aggregation of these proteins may occur[5,6]. The present paper describes a comparative study of the denaturation process over a much wider pH range, using the techniques of optical rotation, viscosity, gelation, sedimentation and diffusion measurements. A preliminary report on this subject has appeared[7].

MATERIALS AND METHODS

Proteins

The ovalbumin was prepared from fresh hens' eggs and recrystallized at least three times by the method of KEKWICK AND CANNAN[8]. The moist crystals were stored in the presence of toluene at 4°. Bovine serum albumin was obtained from three sources: a laboratory preparation from fresh plasma by the method of COHN *et al.*[9] and commercial preparations from Armour and Co. and from Sigma Chemical Co. In our preparation decanol was not used in crystallization. Stock solutions (approx. 10 g/100 ml) of the proteins were made by dissolving in water and dialyzing exhaustively against distilled water in the cold (approx. 2–3°). The protein concentrations were determined either by drying samples to constant weight at 110°, by the Kjeldahl nitrogen method of MCKENZIE AND WALLACE[10], or by ultraviolet absorption at 278 mμ.

Urea

The urea (A.R. or C.P.) was recrystallized once by dissolution in 70% (v/v) aqueous ethanol at 50–70° and recovery at —10°. The crystals were filtered on a Buchner funnel and carefully washed with cold absolute ethanol. In order to minimise hydrolysis they were thoroughly dried at 30°. Stock urea solutions (10 M) were kept at 27° to prevent crystallization. Owing to the slow hydrolysis of urea in aqueous solution they were usually freshly prepared, but were never kept for more than three days.

pH measurements

pH measurements were carried out as described by MCKENZIE AND WAKE[11]. No theoretical meaning is attached to the measurements in concentrated urea systems. The following mixtures were used for pH adjustment: pH 3.3–4.1, appropriate 1 M HCl in 0.08 M NaCl; pH 5.6–6.4, appropriate ratio sodium acetate–acetic acid (total 0.05 M) in 0.05 M NaCl; pH 8.9–10.0, appropriate 1 M NaOH–0.05 M H_3BO_3 in 0.05–0.07 M NaCl. The pH values refer to those in 7 M urea; and the concentrations refer to the final mixture (total ionic strength 1). The pH in the protein–urea buffer was constant to 0.1–0.3 pH unit during the course of the reaction.

Preparation of reaction mixture

In earlier measurements the denaturation was brought about by adding the required quantity of stock urea solution at 25° to a known amount of protein in a salt–buffer solution at the same temperature. In later measurements the required quantity of protein solution (in water) was added to the urea–buffer mixture at 25°. After gently mixing, the reaction mixture could be transferred to either the viscometer or the polarimeter cell within a few minutes of starting the reaction.

Viscosity measurements

Ostwald-type viscometers were used for viscosity determinations. The internal diameter of the capillaries was approx. 0.9 mm. The working volumes were 11–12 ml and the flow times for water at 25° were 60–80 sec. The temperature of the viscometry bath was constant to ±0.02°. The viscosity procedure has been discussed by FRENSDORFF, WATSON AND KAUZMANN[5]. The correction of TANFORD[12] was applied where required but was usually negligible. The results of the viscosity measurements are expressed in terms of the reduced viscosity (dl/g).

$$\eta_{red} = \frac{\eta_{sp}}{c} = \frac{1}{c}\left(\frac{\eta - \eta_0}{\eta_0}\right)$$

where c is the concentration of protein in g/100 ml (dl) solution, η_{sp} is the specific viscosity, η is the viscosity of the protein–urea–salt mixture, η_0 is the viscosity of the "solvent" mixture. The intrinsic viscosity $[\eta]$ (dl/g) is the limiting value of the reduced viscosity as c approaches zero. The intrinsic viscosities in Table IV have been expressed in terms of ml solvent per ml protein ($[\eta] \times \bar{v}/100$).

Optical rotation measurements

These were made with a Schmidt and Haensch polarimeter. The angle of rotation could be read to 0.01°. Water from a constant temperature bath was circulated through the jacketed 1-dm polarimeter tube. Most of the measurements were made with an instrument fitted with a monochromator and the temperature of the reaction was constant to ±0.05°. The remainder were made with an instrument using a sodium lamp and the temperature was constant to ±0.1°.

Sedimentation measurements

These were carried out as described by MCKENZIE AND WAKE[12]. Sedimentation coefficients were corrected to water at 20° ($s_{20,w}$) using the following equation:

$$s_{20,w} = s_{t,s}\,\frac{\eta_{t,s}}{\eta_{20,w}}\,\frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)_{t,s}}$$

where the subscript t,s refers to the solvent at t°, and 20, w refers to water at 20°. η is the viscosity, $\bar{v}$ the partial specific volume and ρ the density. The density data of GUCKER, GAGE AND MOSER[13] were used. This correction was made to compare s for the denatured protein with that of the native protein and the $s_{20,w}$ value obtained does not refer to actual behaviour of the denatured protein in water. The latter comment applies also to $D_{20,w}$.

Diffusion measurements

The apparatus was similar to that of McKENZIE AND WAKE[9], but a modified Tiselius cell was used. In all cases a 0.5 g/100 ml protein solution was used above, and a 1.0 g/100 ml solution below the observed boundary. The two protein solutions were dialysed, in separate cellophane bags, against a larger volume of solvent for not less than 20 h at 25° while being rotated in the solvent at 60 rev./min. Precautions were taken while filling the cell to reduce evaporation, especially from the concentrated urea solutions. In some of the initial experiments it was found that a distortion in the diffusion pattern appeared after some time. This was thought to be due to an interaction between the petroleum jelly, used to grease the cell and the urea solution. The difficulty was overcome by using Dow Corning silicone stopcock grease as lubricant. Diffusion results are expressed in terms of D_{2m}, the reduced second moment of the refractive index gradient curve.

Partial specific volume ($\bar{v}$)

These measurements were made by the pycnometer method and by a modified magnetic float method.

Gelation

The stock protein solution was mixed with the relevant quantities of urea solution, sodium chloride solution and acid or alkali to give the required concentration of protein (3%), urea (7 M), sodium chloride (0.1 M) and initial pH. No buffer was added to these solutions. Gelling was arbitrarily considered to have taken place if the solution failed to flow on inverting the test tube (3 ml in a 1.0 × 7.5 cm tube).

RESULTS

Optical rotation

Bovine serum albumin: For urea concentrations above 3 M, over the pH range 3.2–11.0 the laevorotation of bovine serum albumin increased immediately after mixing (*i.e.* before a reading could be taken). All measurements refer to 25° unless otherwise stated. There was no further change in rotation over 24 h (except in neutral and alkaline solution where there was sometimes a small change of approx. 3° overnight). The extent of the increase in laevorotation was strongly dependent on the urea concentration. In 7 M urea the specific rotation $[\alpha]_D$ changed from −60° for the native protein to −100–110°. Typical plots of $[\alpha]_D$ against time are shown in Fig. 1. (It should be noted that at pH 3 in the absence of urea at I 0.1 there is little change in laevorotation from that of the native bovine serum albumin at pH 6, see ref. 14 for Part II.)

Ovalbumin: The effect of urea on the laevorotation of ovalbumin was strongly dependent on the urea concentration and the pH. At pH values near 3 in 7 M urea there was an immediate increase in laevorotation with no further change with time as shown in Fig. 2. Under these pH conditions the behaviour of ovalbumin in urea resembles that of bovine serum albumin. However, under neutral and alkaline conditions ovalbumin behaved differently, the reaction being much slower. Immediately after mixing $[\alpha]_D$ was similar to that of the native protein (−30.9°). This was followed by a time-dependent increase in laevorotation as shown in Fig. 2. The final

laevorotation and the half time of the change were strongly dependent on the urea concentration. For details of the kinetics of the change at pH 6.1 and pH 7.8 see ref. 11.

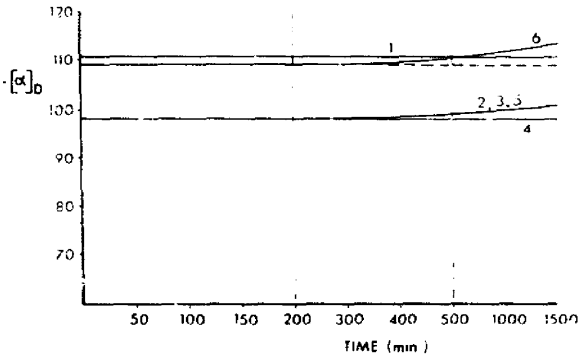


Fig. 1. Effect of time (min) on specific rotation $[\alpha]_D$ of bovine serum albumin in 7 M urea at 25°. Note change of scale at 200 min and at 500 min in this and in Figs. 2, 3, 5, 7. Protein concentration: 1 g/100 ml. Curve 1, pH 3.5; curve 2, pH 6.2; curve 3, pH 9.3; curve 4, pH 9.3, 0.0005 M PCMB present; curve 5, pH 9.3, 0.005 M PCMB present; curve 6, pH 9.3, 0.02 M cysteine present. Experimental points not shown to avoid confusion. For explanation of dashed line see text.

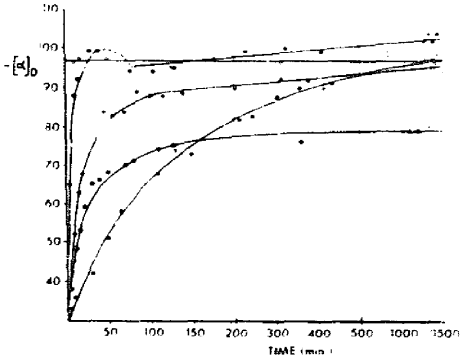
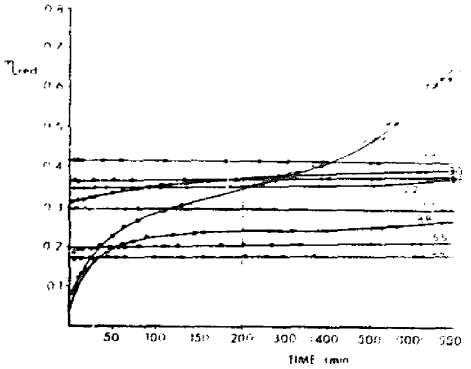


Fig. 2. Effect of time (min) on specific rotation $[\alpha]_D$ of ovalbumin (1 g/100 ml) in 7 M urea at 25°. Curve 1, pH 3.3; curve 2, pH 6.1; curve 3, pH 9.3; curve 4, pH 9.3, 0.005 M PCMB present; curve 5, pH 9.3, 0.02 M cysteine present. Dashed peak on curve 5 is not always reproducible.

Viscosity

Bovine serum albumin: The reduced viscosity (η_{red}) of bovine serum albumin (1 g/100 ml) increased immediately in 7 M urea at pH 6.3 from 0.04 for the native protein to 0.17 for the denatured protein. During the following 1–2 h it underwent a further slight increase and then remained constant, as shown in Fig. 3. The concentration dependence of the reduced viscosity at various times is shown in Fig. 4a.

Fig. 3. Effect of time on reduced viscosity (η_{red}) of bovine serum albumin and ovalbumin in 7 M urea solution at 25°. Curve 1, bovine serum albumin (0.93 g/100 ml) at pH 3.3; curve 2, bovine serum albumin (1.96 g/100 ml) at pH 3.3; curve 3, bovine serum albumin (1.87 g/100 ml) at pH 4.1; curve 4, bovine serum albumin (0.97 g/100 ml) at pH 6.3; curve 5, bovine serum albumin (1.96 g/100 ml) at pH 6.3; curve 6, ovalbumin (1.06 g/100 ml) at pH 3.3; curve 7, ovalbumin (2.12 g/100 ml) at pH 3.3; curve 8, ovalbumin (1.06 g/100 ml) at pH 6.3; curve 9, ovalbumin (2.12 g/100 ml) at pH 6.3.



At pH 3.2 there was an immediate increase in reduced viscosity. There was no appreciable change in viscosity with time at lower protein concentrations. There was a slow increase in viscosity with time at higher protein concentrations.

Near pH 4 there was an immediate increase in reduced viscosity (Fig. 3) followed, at higher concentrations, by a much more marked change with time compared with pH 3.3 (see DISCUSSION).

At pH 9.3 there was an immediate increase in reduced viscosity followed by a slow change with time as shown in Fig. 5. The latter change is strongly concentration dependent, as can be seen in Fig. 6a.

Ovalbumin: The reduced viscosity of ovalbumin in 7 M urea at pH 6.3 was close to that of the native protein (0.04), immediately after mixing, but increased with time as shown in Fig. 3. The marked concentration dependence of this increase is shown in Figs. 3 and 4b.

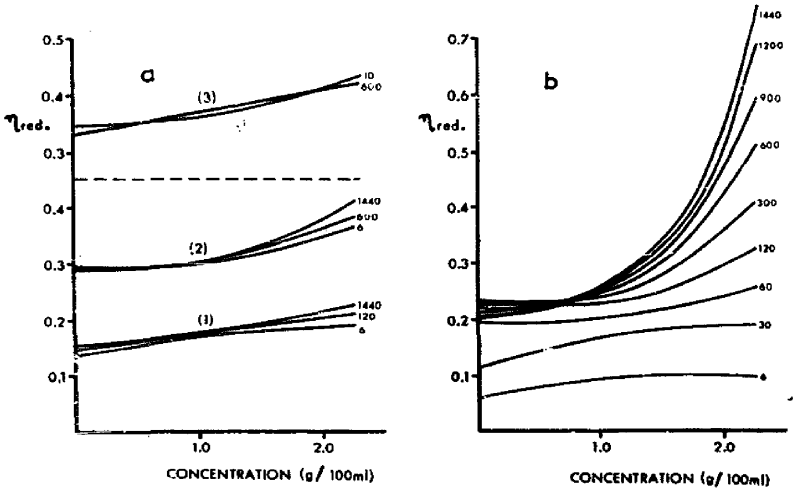


Fig. 4. Effect of protein concentration (g/100 ml) on reduced viscosity (η_{red}) at various times for bovine serum albumin and ovalbumin in 7 M urea at 25°. Times (min) shown at end of curves. In order to avoid confusion due to overlapping of curves only a limited number of curves is shown. (a) Set 1, bovine serum albumin at pH 6.4; set 2, bovine serum albumin at pH 3.3; set 3, ovalbumin at pH 3.3. (b) Ovalbumin at pH 6.4.

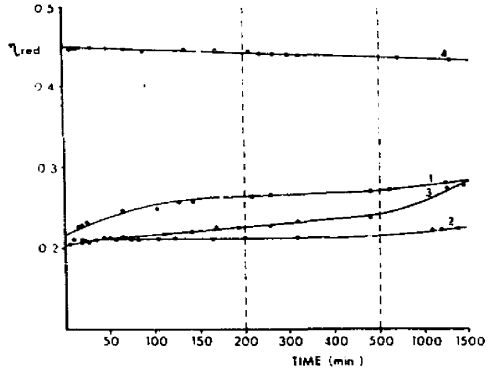


Fig. 5. Effect of time (min) on reduced viscosity (η_{red}) of bovine serum albumin (1.05 g/100 ml in 7 M urea at 25°. Curve 1, at pH 9.3; curve 2, at pH 9.3, 0.0005 M PCMB present; curve 3, at pH 9.2, 0.005 M PCMB present; curve 4, at pH 9.2, 0.02 M cysteine present.

At pH 3.2 the reduced viscosity underwent an immediate increase similar to that of bovine serum albumin. During the next 10 h there was no appreciable change. Typical results are shown in Fig. 3. There is only a small concentration dependence of reduced viscosity at various times (Fig. 4a).

At pH 9.2 and 7 M urea the reduced viscosity was initially similar to that of the native protein. There was a time-dependent increase in viscosity which was strongly dependent at later times on concentration as shown in Figs. 7 and 8a.

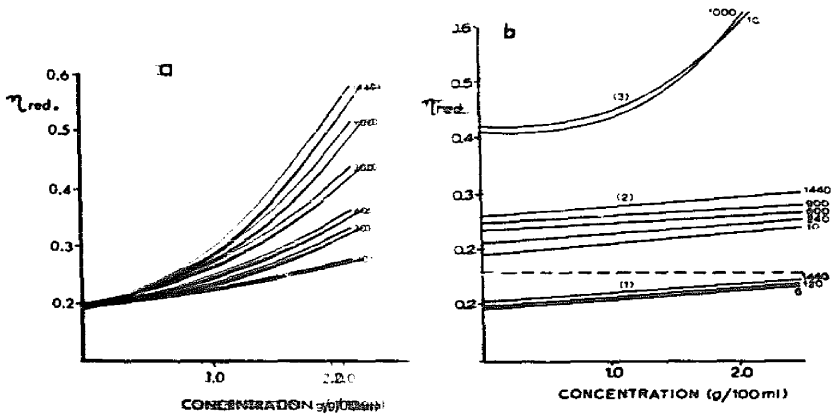


Fig. 6. Effect of protein concentration (g/100 ml) on reduced viscosity (η_{red}) of bovine serum albumin at various times in 7 M urea at 25°. Times (min) shown at end of curves. To avoid confusion only a limited number of curves is shown. (a) At pH 9.3. (b) Set 1, at pH 9.3, 0.0005 M PCMB present; set 2, at pH 9.2, 0.005 M PCMB present; set 3, at pH 9.2, 0.02 M cysteine present.

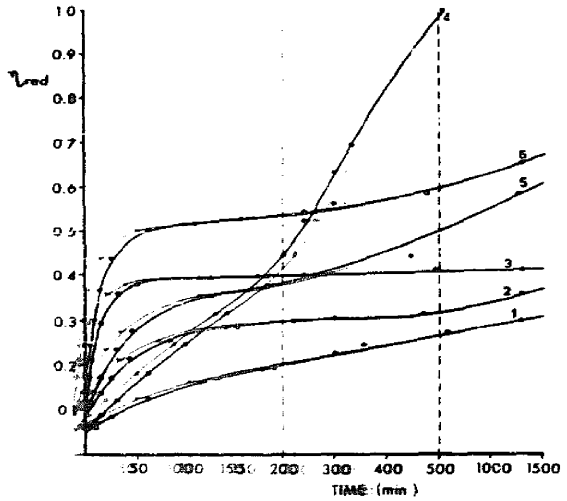


Fig. 7. Effect of time (min) on reduced viscosity of ovalbumin in 7 M urea at 25°. Curve 1, 0.5 g/100 ml ovalbumin at pH 9.5; curve 2, 0.5 g/100 ml at pH 9.5, 0.005 M PCMB present; curve 3, 0.5 g/100 ml at pH 9.6, 0.02 M cysteine present; curve 4, 2.0 g/100 ml at pH 9.7; curve 5, 2.0 g/100 ml at pH 9.5, 0.005 M PCMB present; curve 6, 2.0 g/100 ml at pH 9.6, 0.02 M cysteine present.

Effect of PCMB and cysteine

The viscosity measurements described above indicated a strong tendency for ovalbumin to aggregate slowly in neutral and alkaline urea solution and for bovine

serum albumin to do so in alkaline urea solution. In view of this it was decided to examine the effects of PCMB and cysteine on the urea denaturation.

The laevorotation of bovine serum albumin in 7 M urea at pH 9.3 still showed an immediate increase in the presence of these reagents. The extent of the change was little effected by the presence of PCMB. The laevorotation was enhanced in the presence of 0.02 M cysteine (Fig. 1). It appeared to show a further increase of approx. 4° overnight. It was difficult to determine whether this small increase was real as the cysteine blank did not change reproducibly with time.

In the presence of PCMB and 7 M urea at pH 9.3 there was an immediate increase in viscosity of bovine serum albumin. The concentration dependence of the reduced viscosity was small (Figs. 5 and 6b). When 0.005 M PCMB was present the intrinsic viscosity increased appreciably with time (Fig. 6b), but not when only 0.005 M PCMB was present.

The immediate increase in viscosity was much greater when 0.02 M cysteine was present. There was some concentration dependence (Figs. 5 and 6b).

The rate and extent of change of the optical rotation of ovalbumin in alkaline urea solution (approx. pH 9–9.5) both in the presence and absence of cysteine and PCMB, were not strictly quantitatively reproducible (see DISCUSSION). In the presence of 0.005 M PCMB the optical rotation changed more rapidly with time than in its absence (see Fig. 2), but the extent of the final change was little different from that in the absence of PCMB. When 0.02 M cysteine was present the rate and extent of the change were considerably greater (Fig. 2).

In the presence of 0.005 M PCMB the extent and rate of change of the reduced viscosity were greater for ovalbumin than in the absence of PCMB (Fig. 7). The concentration dependence was smaller than in the absence of PCMB but still appreciable at later times (Fig. 8b). When 0.02 M cysteine was added the rate and extent of the change was greater at the lower protein concentrations (Fig. 7). The concentration dependence of the reduced viscosity was less than in the absence of cysteine but was still appreciable at later times (Fig. 8c).

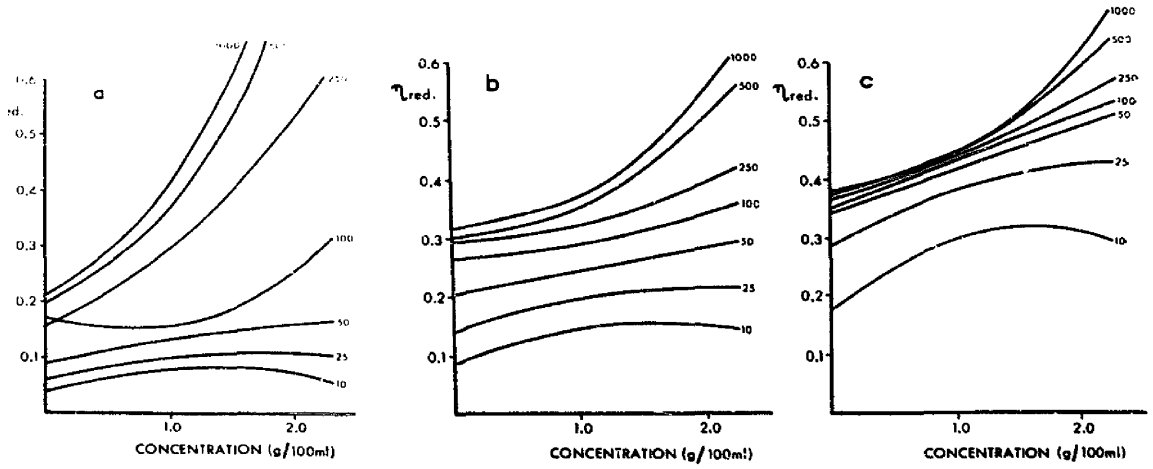


Fig. 8. Effect of protein concentration (g/100 ml) on reduced viscosity (η_{red}) of ovalbumin at various times in 7 M urea at 25°. Times (min) shown at end of curves. (a) At pH 9.5. (b) At pH 9.5, 0.005 M PCMB present. (c) At pH 9.6, 0.02 M cysteine present.

Gelation

Approximate gelling times for ovalbumin and bovine serum albumin in 7 M urea, 0.1 M NaCl and various pH values are shown in Table I. Also shown for comparison are gelling times reported by FRENSDORFF *et al.*[5] for ovalbumin in 10 M urea in 0.1 M NaCl at 30°.

TABLE I

EFFECT OF pH ON THE GELLING OF BOVINE SERUM ALBUMIN AND OVALBUMIN IN UREA

Protein concentration: 3 g/100 ml.

pH	Time for gelling (days)		
	Bovine serum albumin in 7 M urea at 25°	Ovalbumin in 7 M urea at 25°	Ovalbumin* in 10 M urea at 30°
2.6	—	No gel**	—
2.8	No gel	—	—
3.0	No gel	No gel	No gel
3.6	No gel	—	No gel
3.8	—	14	—
4.2	No gel		
4.7	—	0.5***	—
4.9	No gel	—	6
5.5	No gel	—	5.5
5.7	—	6	5
6.3	No gel§	3	4
7.1	No gel		2
8.0	No gel	0.6	1
9.1	0.6	—	—
9.6	Approx. 1	Approx. 0.6	0.5
10.5	—	Approx. 0.6	—
10.8	Approx. 1	Approx. 0.6	No gel
12.1	No gel	No gel	—

* Results of FRENSDORFF *et al.*[5].
** No gel means failed to gel in 14 days.
*** Slight flow in this gel up to 6 days.
§ Bovine serum albumin at 6 g/100 ml gelled.

Sedimentation and diffusion

The optical rotation and viscosity measurements indicated considerable disorganization of the native protein structure in urea. This change was rapid and, under appropriate pH conditions, was followed by a slow aggregation. These features made the reaction favourable to examination in the ultracentrifuge.

In Table II values are shown for the sedimentation coefficient (s) and diffusion coefficient (D_{2m}) for bovine serum albumin and ovalbumin under a variety of conditions. Values of s and D_{2m} for the native protein are shown at zero protein concentration as well as at finite concentration. An indication of the number of peaks obtained in the ultracentrifuge and of the dispersity is given. Approximate values of the particle weights of the denatured protein at finite concentration in urea are given in a number of cases. At the high protein concentration at the bottom of the ultracentrifuge cell gelation sometimes took place. A clear firm gel, about 0.1 mm thick was then observed at the bottom of the cell at the end of the experiment. The presence or absence of this gel is indicated in the table.

At pH 3.5 and a protein concentration of 1 g/100 ml, bovine serum albumin in 7 M urea showed a single peak in the ultracentrifuge (Fig. 9a). Under these conditions the viscosity measurements indicated little aggregation. There was a considerable decrease in $s_{20,w}$ and D_{2m}. Similar results are observed at pH 6.4 (Fig. 9b).

Viscosity measurements indicated a slow aggregation at higher pH values. There was little aggregation seen in the ultracentrifuge pattern (Fig. 9c) after denaturation in 7 M urea (pH 9–10) for 4 h, but after 26 h reaction considerable heterogeneity was evident in the pattern (Fig. 9d). Only a single peak (Fig. 9e) was observed at pH 9 in the presence of 0.02 M cysteine. The refractive index gradient curve for diffusion in the absence of cysteine at pH 9 showed considerable departure from the normal curve; but, when cysteine was present, there was no evidence of heterogeneity within the limitations of this test.

At pH 3.3 in 7 M urea at 25° ovalbumin (1 g/100 ml) showed a single peak in the ultracentrifuge. The pattern in Fig. 10a was obtained after 44 h reaction and a similar pattern was obtained after 20 h reaction. Under these conditions viscosity measurements indicated little or no aggregation. When this reaction mixture was adjusted to pH 5.7 and examined in the centrifuge extensive aggregation was evident in the pattern.

The viscosity measurements for ovalbumin (1 g/100 ml) in 7 M urea (pH 6.2) at 25° indicated extensive aggregation. This was also evident in the sedimentation pattern (Fig. 10b, c). These patterns are for 4 h and 27 h respectively. More extensive aggregation, accompanied by increased heterogeneity, was evident in patterns obtained at 88 and 147 h. Similar aggregation occurred at pH 5.2.

Extensive aggregation was also evident in sedimentation patterns at pH 9.4 (Fig. 10d). There appeared to be more aggregation at pH 9.9 than for pH 9.4 and pH 11 (see Table I). At pH 9.1 in the presence of 0.005 M PCMB aggregation was still evident but there was considerable sharpening of the peaks (Fig. 10e). The same effects were observed in the presence of 0.02 M cysteine (pH 8.6) (Fig. 10f). At lower pH (6.7) 0.02 M cysteine had little effect on the aggregation as expected from the pH dependence of the S-S splitting reaction. An approximate estimate of the particle weight of the fast component in the presence of PCMB or cysteine, near pH 9, indicated a value of 180000–270000.

Partial specific volume

The partial specific volume of bovine serum albumin and ovalbumin was found not to be changed significantly in the presence of urea within the accuracy of the pycnometer method used (approx. ±0.01). KAUZMANN[15] observed the expected small decrease in $\bar{v}$ for ovalbumin in 6–8 M urea, pH 7–8, at 30° using dilatometers. Similar small decreases (approx. 0.005) were observed for both proteins by CHARLWOOD[16] using the magnetic float method. In the present work it was considered to be sufficiently accurate to use the values of the native protein in the calculation of approximate particle weights in urea solution. The value for bovine serum albumin was taken as 0.73_0 and that for ovalbumin as 0.74_9 at 20°.

DISCUSSION

The laevorotation and intrinsic viscosity of bovine serum albumin and ovalbumin increase considerably in concentrated urea solution, indicating extensive "unfolding"

TABLE II

SEDIMENTATION AND DIFFUSION OF BOVINE SERUM ALBUMIN

S, Svedberg units; $1S = 10^{-13}$ c.g.s. unit, F, Fick units; $1F = 10^{-7}$ c.g.s. unit.

Conditions	pH	Sedimentation			Diffusion		$f \times 10^7 / \eta$	Approx. particle weight	Gel U.C. cell
		No. of peaks	$s_{25,s}$ (S)	$s_{20,w}$ (S)	$D_{2m,20,w}$ (F)	$D_{2m,25,s}$ (F)			
Native, 0.1 M NaCl	5.3	One	—	4.02	5.4_9 (0.5)	—		67 000	No
Native, 0.1 M NaCl	5.3	One	—	4.42 (0)					
7 M urea	3.5	One	0.95	1.9_2	3.9_1	2.8_1	1.04	See text	No
7 M urea	6.4	One	1.2_2	2.4_8	3.1_0	2.2_1	1.34	72 000	Gel
7 M urea	9.8	One spread 4 h	Approx. 1.4	Approx. 2.9					Gel
		One skew spread 26 h	Approx. 1.3	Approx. 2.6					Gel
7 M urea, 0.02 M cysteine	9.0	One	1.0_1	2.0_4	3.0_2	2.1_6	1.37	61 000	No

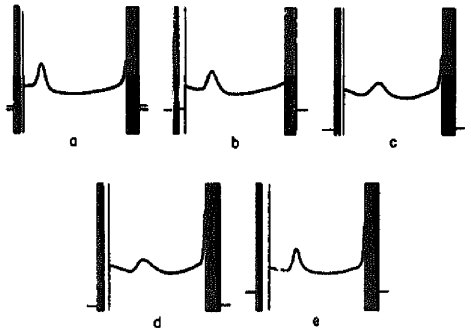


Fig. 9. Sedimentation patterns for bovine serum albumin (1 g/100 ml) in 7 M urea. The measurements were made at a speed of 59780 rev./min. (a) At pH 3.5, reaction time 20 h, time after reaching speed 185 min, $\theta = 75°$. (b) At pH 6.4, reaction time 100 min, time after reaching speed 215 min, $\theta = 75°$. (c) At pH 10, reaction time 4 h, time after reaching speed 248 min, $\theta = 65°$. (d) At pH 10, reaction time 26 h, time after reaching speed 255 min, $\theta = 65°$. (e) At pH 9, 0.02 M cysteine, reaction time 24 h, time after reaching speed 293 min, $\theta = 75°$. Direction of sedimentation: left to right.

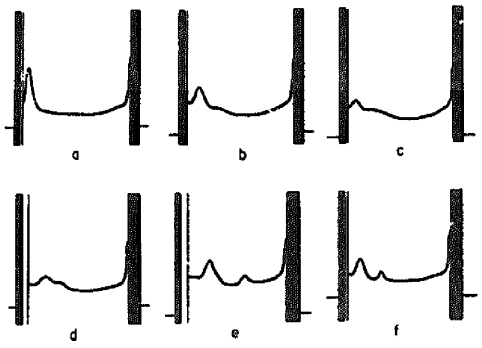


Fig. 10. Sedimentation patterns for ovalbumin (1 g/100 ml) in 7 M urea. The measurements were made at a speed of 59780 rev./min. (a) At pH 3.3, reaction time 4 h, time after reaching speed 124 min, $\theta = 75°$. (b) At pH 6.3, reaction time 4 h, time after reaching speed 135 min, $\theta = 70°$. (c) At pH 6.3, reaction time 27 h, time after reaching speed 136 min, $\theta = 70°$ (d) At pH 9.4, reaction time 20 h, time after reaching speed 185 min, $\theta = 75°$. (e) At pH 9.1 in 0.005 M PCMB, reaction time 20 h, time after reaching speed 245 min, $\theta = 65°$. (f) At pH 8.6 in 0.02 M cysteine, reaction time 20 h, time after reaching speed 206 min, $\theta = 75°$. Direction of sedimentation: left to right.

of the protein molecule. This is supported by sedimentation and diffusion measurements. In the case of bovine serum albumin this change takes place very rapidly over the pH range 3–11.0. Ovalbumin resembles bovine serum albumin in this respect at acid pH. At higher pH values the change is much slower. Viscosity, gelation and sedimentation measurements indicate a tendency for both proteins to aggregate slowly in urea solution. This tendency is more pronounced in alkaline urea, and ovalbumin tends to aggregate more readily than bovine serum albumin.

We now consider in more detail the various items of information which this study has provided and assess the utility of the methods used.

Optical rotation and viscosity measurements enable the process of denaturation to be followed simply, without disturbance. However the physical principles underlying optical rotation are complex[2,17], and it is not possible to interpret changes in it in precise structural terms. Increases in laevorotation on denaturation similar to ours, have been reported for a variety of proteins and enzymes[17]. These results together with measurements (particularly rotatory dispersion[19]) on synthetic polypeptides may be interpreted in terms of extensive disorganization of the protein molecule ("transconformation"[18]). This may not be the only interpretation of optical rotation changes. Usually it is assumed that the laevorotation increase is due to the "transconformation" reaction only and not to aggregation (if present). On the other hand optical rotation may increase or decrease appreciably during aggregation[20] and gelation[21]. However, the main laevorotation increase for bovine serum albumin and ovalbumin in urea, considered along with the increase in $[\eta]$ is almost certain due to unfolding.

The extent and kinetics of the optical rotation change for ovalbumin in urea solution for pH 6.2 and 7.8 at 25°, are generally similar to those observed by SIMPSON AND KAUZMANN[3] for ovalbumin in 7.5 M urea at pH 7.9 and 30° (for details see ref. 14). The strong dependence of the half time on the urea concentration (approx. α 14th power) is also similar. SIMPSON AND KAUZMANN noticed a mean deviation in the half times for runs made from the same protein stock solution of about $\pm 3\%$, though for runs using different stock solutions the variation was as great as 30%. A small variation in α_{final} was also observed. We have noticed differences in the final rotation and half times for different preparations but have also noticed differences in the kinetics. This lack of reproducibility was greater at alkaline pH values. ATKINSON AND MCKENZIE[22] have found some dependence of the half time on the protein concentration. These effects do not appear to be explicable in terms of the interesting "ovalbumin-x" of SMITH AND BACK[23]. They do not alter the general conclusions of this paper, and are being further investigated.

The reduced viscosity increases when the polypeptide chain becomes more "loosely" coiled. It is also sensitive to aggregation or disaggregation. These effects may be separated by the plots of reduced viscosity against concentration at various times. HUGGINS[24] has presented an equation relating the reduced viscosity and concentration of polymers.

$$\eta_{\text{red}} = [\eta] + k'[\eta]^2 c$$

The coefficient k' varies from system to system, but is usually in the range 0.3–0.8. This equation is obeyed for linear polymers in good solvents only over a limited concentration range (approx. 0–1.5%). HUGGINS has shown that the equation of MARTIN

$$\log \eta_{\text{red}} = \log [\eta] + k''[\eta]c$$

TABLE III

SEDIMENTATION AND DIFFUSION FOR OVALBUMIN

S, Svedberg units; 1S = 10^{-13} c.g.s. unit; F, Fick units; 1F = 10^{-7} c.g.s. unit.

Conditions*	pH	Sedimentation			Diffusion		$f \times 10^5 / \eta$	Approx. particle weight	Gel U.C. cell
		No. of peaks	$s_{25,s}$ (S)	$s_{20,w}$ (S)	$D_{2m,20,w}$ (F)	$D_{2m\ 25,s}$ (F)			
Native, 0.1 M NaCl	5	One	—	3.2_7***	7.6_7 (0.5)§			41 000	No
Native**	7	One	—	3.5_3 (0)					
7 M urea	3.3	One	0.6_7	1.3_9	3.2_4	2.3_3	1.27	43 000	No
7 M urea	5.1	Several							Gel
7 M urea	6.2	Several	Slow 0.8	1.6					Gel
			Fast 2.1–4.6	4.2–9.2					
7 M urea	9.4	Two diffuse	Slow 0.8	1.6					Gel
		peaks	Fast 1.5	3.0					
7 M urea	9.9	Two peaks	Slow 0.9	1.8					Gel
			Fast 1.5	3.0					
7 M urea	11.0	Two peaks	Slow 0.7	1.4					Gel
			Fast 1.5	3.0					
7 M urea, 0.005 M PCMB	9.1	Two peaks	Slow 0.8	1.7					Gel
		(sharp)	Fast 2.0	4.1					
7 M urea, 0.02 M cysteine	8.6	Two peaks	Slow 0.7	1.4					Gel
		(sharp)	Fast 1.8	3.7					
7 M urea, 0.02 cysteine	6.7	Two diffuse	Slow 0.9	1.9					Gel
		peaks	Fast 2.3	4.8					

* For ionic strength etc. see text.
** Data of BACK AND SMITH in *I* 0.05 PO_4, 0.1 M NaCl.
*** *s* values for 1 g/100 ml protein, unless indicated in parentheses.
§ *D* values for conditions in text, unless indicated in parentheses.

holds over a wider concentration range[24] (0–5 %). The results of an examination of the viscosity data of the present paper in terms of these equations are presented in Table IV. It will be noted that under certain conditions there is appreciable departure from the equations of HUGGINS AND MARTIN (0–2 % protein). Evidently in these cases there is an effect present which is greater than that due to the usual hydrodynamic interactions in normal polymer solutions. (Under the ionic strength conditions of the present work the electrostatic protein–protein interaction is negligible[25].) The ultracentrifuge patterns confirm that this effect is aggregation which is also responsible for the gelling of the solutions at higher protein concentration and for the gels found in the bottom of the ultracentrifuge cells at the completion of the sedimentation experiments.

In Table IV values of the intrinsic viscosity of the denatured proteins in urea (along with the data of KAUZMANN[26]) are given and may be compared with values for random coil polymers in good solvents (see Table I of ref. 20). It is apparent that there is a similarity in magnitude of the intrinsic viscosities of the two classes.

Changes in the hydrodynamic properties may also be observed in the ultracentrifuge, although compared with viscosity measurements the method is slow and there is a danger of disturbing the reaction. It has the advantage of distinguishing monomer and aggregation products and giving an indication of the change in frictional coefficient in those cases (as in the present measurements) where it can be assumed that one peak represents the unfolded protein. Diffusion is much less sensitive to

TABLE IV

INTRINSIC VISCOSITIES OF DENATURED PROTEINS

*Solvent**	*pH*	$[\eta]$ *ml/ml*	*Variation* η_{red} *versus concentration*
Bovine serum albumin (mol. wt. 67000, extended chain length 2200 Å)			
Native protein	5.3	5	
7 M urea	3.3	38	Some departure MARTIN**
7 M urea	6.4	24	HUGGINS obeyed, $k' = 1.4$
7 M urea	9.4	25	Departure MARTIN
7 M urea, 0.0005 M PCMB	9.3	25	HUGGINS obeyed, $k' = 0.6$
7 M urea, 0.005 M PCMB	9.2	36	HUGGINS obeyed, $k' = 0.3$
7 M urea, 0.02 M cysteine	9.2	55	Departure MARTIN c 1.2
8 M urea, 30°***	10.0	29	
8 M urea, 0.02 M cysteine, 30°***	10.0	71	
Ovalbumin (mol. wt. 45000 extended chain length 1500 Å)			
Native protein	5.3	6	
7 M urea	3.3	45	MARTIN obeyed
7 M urea	6.4	27	Departure MARTIN
7 M urea	9.5	28	Departure MARTIN
7 M urea, 0.005 M PCMB	9.5	41	Departure MARTIN
7 M urea, 0.02 M cysteine	9.6	51	Departure MARTIN
10 M urea, 30°***	10.0	51	
10 M urea, 30°***	7.6	45	
7.5 M urea, 30°***	7.3	33	

* For details of added salts see text.

** "Departure Martin" indicates that MARTIN AND HUGGINS equations not obeyed over protein concentration range, 0–2 %. "HUGGINS obeyed" indicates HUGGINS equation obeyed. "MARTIN obeyed" indicates MARTIN equation obeyed.

*** Data of KAUZMANN[26].

heterogeneity and can be applied usefully only to those systems which are essentially monodisperse.

The decreased values of s and D (compared with the native proteins) shown in Tables II and III indicate proportionate increases in frictional coefficients for the "slow" components, in agreement with the general interpretation. The ratio of the frictional coefficients to the solvent viscosities (in the absence of aggregation) are of similar magnitude to those of comparable random coil polymers[25].

Aggregation can be detected by viscosity measurements at varying protein concentration, but the intrinsic viscosity *per se* does not enable one to determine whether the denatured protein at zero concentration has the same particle weight as the native protein or is split into smaller subunits. It is desirable to know the particle weight at zero protein concentration as well as at finite concentration. To achieve this, sedimentation velocity measurements must be combined with measurements of low or zero resolving power with respect to heterogeneity. Accurate results can be obtained only for single "homogeneous" solutes in two component systems. If the measurements are to be of value they must be made in the denaturing solvent (to avoid reversible changes on dilution of the solvent). This involves using a multicomponent solvent in which the concentrations of components other than water (*e.g.* urea) are relatively large. The error in such measurements may be high if there is appreciable selective solvation or if there is appreciable dependence of sedimentation rate on concentration[27].

In the present work apparent particle weights in urea solution have been estimated where there was evidence from viscosity and sedimentation measurements that aggregation was virtually absent. The calculations have been made from s and D at finite concentrations. The results (with one possible exception, see below) along with those of others (see refs. 1, 5, 26)* indicate that at low protein concentration the unfolded protein has the same particle weight as the native protein. Our results are liable to error if there are appreciable selective solvation and interaction effects (concentration dependence) and if D_{2m} differs appreciably from D_{11} (see ref. 27).

In general it would appear from sedimentation velocity, equilibrium and Archibald method studies, at present in progress, that the magnitude of concentration dependence of sedimentation and diffusion does not significantly affect our conclusions on particle weights[29]. It is not possible to assess accurately the flow interaction effects (see EXPERIMENTAL). With regard to selective solvation in urea, measurements of KLOTZ, TRIWUSH AND WALKER[28] indicate low binding of urea by bovine serum albumin (< 0.01 g/g at neutral pH). Recent measurements[29] also indicate that binding of urea by bovine serum albumin is very small. (Bovine serum albumin generally binds small organic molecules to a greater extent than ovalbumin[1].)

The present measurements at finite concentration indicate an apparent particle

* The measurements quoted in refs. 1, 5 and 26 are those of BURK on the osmotic pressure of ovalbumin in 6.66 M urea (pH 4.8) at 0° and of horse serum albumin at 6.66 M urea (pH 5.8); the viscosity and diffusion measurements of NEURATH *et al.* for horse serum albumin in 8 M urea, pH 5 at 25°, and s and D measurements of ROTHEN on ovalbumin (no details). It should be pointed out that the method of extrapolation to zero concentration used is not entirely satisfactory. These measurements have usually been quoted to show that serum albumin and ovalbumin in urea have the same particle weight as the native protein. This appears to be true at zero or low protein concentration, but the present work shows that, under appropriate conditions of concentration and pH, aggregation of the unfolded molecules may occur.

weight of bovine serum albumin in urea at pH 3.5 which is less than that for the native protein. Studies to clarify this are in progress (ARMSTRONG AND MCKENZIE).

The effect of PCMB and cysteine

Bovine serum albumin has 17 S-S bonds and 1 SH group per molecule[30,31]. These intramolecular disulphide linkages are ruptured rapidly by cysteine in alkaline urea solution. Under these conditions there is an increase in the laevorotation and intrinsic viscosity for bovine serum albumin which is greater than that when cysteine is absent. (The interpretation of laevorotation changes when S-S bonds are broken is somewhat complex[32,33].) In this solvent the molecule probably resembles more closely a random coil.

If the aggregation of bovine serum albumin in alkaline urea is due to the S-S–SH exchange reaction of HUGGINS, TAPLEY AND JENSEN[34] it should be prevented by blocking the SH groups by reagents such as PCMB. FRENSDORFF *et al.*[6] showed, by viscosity measurements, that the aggregation was prevented in 8 M urea at 30° and pH 10 when considerable excess PCMB was present, and they observed an increase in viscosity with time. Our viscosity and sedimentation measurements show similar results at pH 9–10 in 7 M urea at 25° in the presence of 0.005 M PCMB (15–60 fold excess, according to protein concentration, 0.5–2.0 g/100 ml). Reduction of the PCMB concentration to 0.0005 M (1–5 fold excess) also eliminates aggregation but there is no slow unfolding as with 0.005 M PCMB. The additional unfolding step in the presence of 0.005 M PCMB is probably due to cleavage of S-S linkages in the bovine serum albumin. Two mechanisms for the cleavage are possible: (a) The disulphide groups are split according to the equation:

$$RSSR + OH^- \rightleftharpoons RS^- + RSOH,$$

the excess PCMB reacts with RS^- and drives the reaction to the right[6]; (b) PCMB reacts directly with the disulphide groups, the reaction being greater when the PCMB is present in considerable excess[35].

The present work does not enable a distinction to be made between these mechanisms. However, CECIL AND MCPHEE[36] obtained good evidence for a direct reaction of Ag^+ with simple disulphides.

In the estimation of SH groups in proteins, containing S-S groups, those methods using prolonged reaction time of mercurial, especially in the presence of denaturant, may lead to erroneous results.

The work of LEACH[31] indicates that ovalbumin has 4 or 5 SH groups of varying reactivity and possibly 1 S-S group per molecule. The increase in the rate and extent of the "unfolding" of ovalbumin in the presence of PCMB and cysteine may involve rupture of intra-chain S-S bonds and/or intra-chain bonds involving SH groups[37]. Both types of links (if present) would appear to join widely separated parts of the ovalbumin molecule. Oxidation of SH groups in alkaline urea in the absence of cysteine or PCMB and intramolecular disulphide exchange would further complicate the position.

The sedimentation and viscosity measurements show that PCMB and cysteine do not eliminate the aggregation of ovalbumin at pH 9–10. The sharpening of the sedimentation patterns in this pH range (compare lack of effect at pH 6) indicate that there is some reduction in aggregation in their presence. The S-S–SH exchange

reaction does not assume the importance for ovalbumin that it does for bovine serum albumin and a considerable part of the aggregation would appear to be due to other causes, *e.g.* intermolecular hydrophobic bonding or hydrogen bonding.

From measurements of the solubility of unfolded bovine serum albumin in salt solutions KAUZMANN AND DOUGLAS[38] have concluded that in acid urea solution there is little intra- or intermolecular exchange of the disulphide cross linkages. However, GUTTER, PETERSON AND SOBER[39] found aggregation of bovine serum albumin in 3.5 M urea at pH 4.5. Our present and unpublished work indicates a considerable slow aggregation of bovine serum albumin in 7 M urea near pH 4. If the conclusions of KAUZMANN AND DOUGLAS are correct, this aggregation must be due to some other cause such as hydrogen or hydrophobic bonding.

General

KAUZMANN[40] has classified proteins broadly into four classes on the basis of their thermodynamic and kinetic stability. He classifies bovine serum albumin as having low thermodynamic and kinetic stability and ovalbumin as having low thermodynamic stability but high kinetic stability. Such a classification seems to be of limited value unless it takes into consideration behaviour over an extensive range of pH and of temperature; thus bovine serum albumin and ovalbumin behave very similarly in 7 M urea at low pH.

It is of interest to note that we have found that β-lactoglobulin resembles bovine serum albumin in its ease of unfolding in urea at all pH values. On the other hand it shows no tendency to aggregate in urea. Under no conditions was any gel found for β-lactoglobulin at the bottom of an ultracentrifuge cell.

If, as seems likely, unfolding of proteins by urea involves two main stages, the first being rupture of pH-dependent side-chain bonds (*e.g.* hydrogen bonds) followed by transformation from a more or less helical structure to a random coil structure, the investigation of these will require detailed kinetic measurements over a range of pH values. Such measurements are in progress.

ACKNOWLEDGEMENTS

This work was initiated while one of us (H.McK.) was leader of the C.S.I.R.O. Physico-Chemical Unit. Grateful acknowledgement is due to Professors W. KAUZMANN, A. G. OGSTON and J. L. STILL and Dr. J. R. VICKERY for their interest and advice and to Miss J. BACK and Miss L. ATKINSON for their technical assistance. Part of the work was done during the tenure by R.G.W. of a C.S.I.R.O. Australian Studentship.

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