[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

The Kinetics of Protein Denaturation. V. The Viscosity of Urea Solutions of Serum Albumin[1]

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The behavior of the viscosity of solutions of bovine serum albumin in urea shows similarities to that of ovalbumin but there are also some significant differences. The much more rapid initial unfolding of serum albumin results in an instantaneous increase in viscosity on addition of urea. The subsequent reactions are influenced by the fact that serum albumin contains a much larger fraction of disulfide groups and a smaller fraction of sulfhydryl groups. As a result, the serum albumin molecule contains a large number of intramolecular cross links which have an important effect on the structure of the denatured molecule, and probably also on that of the native molecule. These cross links can be ruptured and rearranged by various reagents and the effects on the viscosity are described. The intrinsic viscosities of the unfolded and unaggregated ovalbumin and serum albumin molecules in strong urea solutions are similar to those of randomly coiled polymers of similar chain length. It is suggested that the ease of both the unfolding and "reversal" of denaturation of serum albumin are a result of the large number of disulfide cross links which it contains.

In Part IV[3] we have discussed the relationship of aggregation and unfolding to the viscosity of urea solutions of ovalbumin. In view of the much greater ease with which serum albumin unfolds in urea, it is interesting to compare the behavior of the viscosity of its solutions in urea with that of ovalbumin. This is particularly interesting because Huggins, Tapley and Jensen[4,5] have shown that sulfhydryl and disulfide groups play an important role in the aggregation of serum albumin. In this paper we shall investigate this role further and describe differences in the behavior of serum albumin and ovalbumin which may give a clue to the cause of some of the well-known differences between these two proteins.

Experimental

Crystalline bovine serum albumin was obtained from Armour and Company. In preparing stock solutions, the water content of the powder was first determined by drying a small weighed sample for approximately one hour at 100°. The stock solution was then made up to the desired weight of protein per unit volume of solution by mixing the calculated weights of water and protein, assuming a partial specific volume of 0.75 ml./g. for the protein. The concentration was checked by measuring the optical rotation. ($[\alpha]_D$ −61.4 ± 0.3° for solutions prepared in this way.) Reduced viscosities are expressed in (g. per 100 ml.)$^{-1}$. Further experimental details will be found in Part IV.[3]

Results and Discussion

1. Effect of Urea Concentration and *p*H.— The effect of the urea concentration on the reduced viscosity changes of 1% serum albumin solutions was studied at three *p*H values. Figure 1 shows the results of experiments in borate buffer (*p*H about 10), in which the change with time was large. At lower *p*H values the time dependence of the viscosity is much less marked: in acetate buffer (*p*H 4.8–5.8 depending on the urea concentration) the reduced viscosity jumps immediately to within 10% or less of its final value and the subsequent further increase is completed in 200 minutes. In 0.1 *M* HCl (*p*H 1 to 3) the viscosity change is instantaneous and no further change takes place for at least 200 minutes.

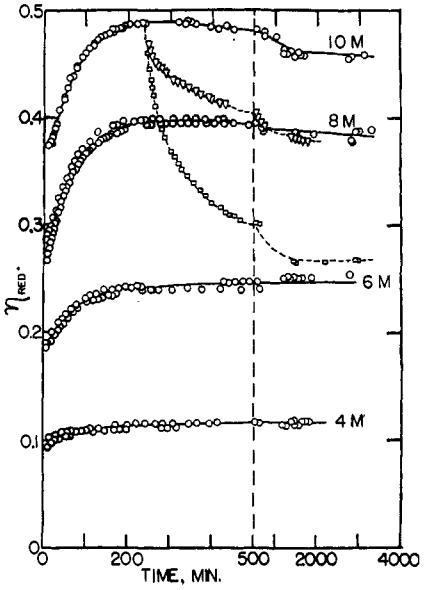

Fig. 1.—Effect of urea on the reduced viscosity (in g.$^{-1}$ 100 ml.) of serum albumin at 30°, *p*H 9.8–10.5 (0.025 *M* borate buffer), protein concentration 1%. Dashed curves show behavior on dilution of urea to 8 *M* (▽) and to 6 *M* (□) after 240 min. at 10 *M*. Time scale changes at 500 min.

The constant or maximum values of the reduced viscosities ultimately reached at a given *p*H increase strongly with the urea concentration (Fig. 2). The effect of urea on the reduced viscosity of 1% serum albumin is thus similar to its effect on the intrinsic viscosity of ovalbumin, presumably for the reasons already discussed in Part IV; the unfolded molecule is less compact at the higher urea concentrations because intramolecular hydrogen bonding is less extensive.

These observations are in agreement with those of Neurath and Saum,[6] who found increasing reduced viscosities and decreasing diffusion constants for horse serum albumin as the urea concentration was raised to 6.66 *M* with acetate buffers of *p*H 5.2.

With low urea concentrations (below about 3 *M*) at *p*H 5 and 10 the reduced viscosity is not very different from that of the native protein, but in 0.1

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(2) U. S. Public Health Service Predoctoral Research Fellow of the National Cancer Institute, 1951–1952.

(3) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5157 (1953).

(4) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).

(5) C. Huggins and E. V. Jensen, *Science*, **113**, 477 (1951).

(6) H. Neurath and A. M. Saum, *J. Biol. Chem.*, **128**, 347 (1939).

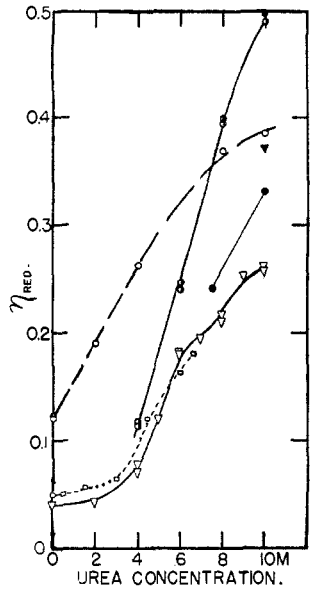


Fig. 2.—Dependence of final reduced viscosity of protein solutions on urea concentration at 30°: 1% bovine serum albumin: 0.025 *M* borate buffer, *p*H 9.8–10.5 (◑); 0.05 *M* acetate buffer, *p*H 4.8–5.8 (▽); 0.1 *M* HCl, *p*H 1.1–3.0 (○). 1% horse serum albumin (data of Neurath and Saum[6]): 0.2 *M* acetate buffer, *p*H 5.2, plus 0.2 *M* NaCl (□). 0% ovalbumin (intrinsic viscosities; data of Part IV[3]): 0.05 *M* phosphate buffer, *p*H 7.5 (●); 0.05 *M* borate buffer, *p*H 10.2 (▼).

M HCl, even in the absence of urea, the viscosity is considerably above the value for the native protein. This has also been observed by Pedersen[7] in acid solutions.

Both the magnitude and the rate of change of the time-dependent contribution to the reduced viscosity of 1% serum albumin increase with the *p*H between *p*H 5 and 10. Figure 3 shows that in 8 *M* urea there is an instantaneous increase in the reduced viscosity from the value of the native protein, 0.05[8] to about 0.2. The optical rotation[9] shows a similar jump without, however, any further time-dependent changes. Consequently it is not unreasonable to suppose that, as was also observed with ovalbumin, the initial change represents unfolding of the compact native protein structure whereas the subsequent changes may represent either aggregation or some further unfolding. These possibilities will be discussed in more detail below.

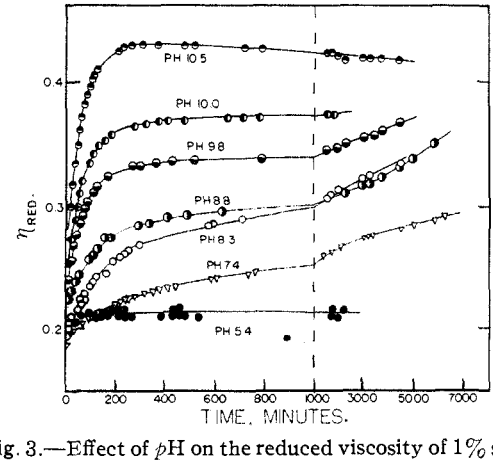


Fig. 3.—Effect of *p*H on the reduced viscosity of 1% serum albumin in 8 *M* urea at 30° (*p*H 5.4, acetate buffer; *p*H 7.4, 8.3, phosphate buffers; *p*H 8.8–10.5, 0.05 *M* borate buffers). Time scale changes at 1000 min.

(7) K. O. Pedersen, *Discussions Faraday Soc.*, **13**, 49 (1953).
(8) A. Polson, *Kolloid Z.*, **88**, 51 (1939).
(9) R. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5154 (1953).

In an experiment in 8 *M* urea with 0.05 *M* NaOH (*p*H 12.3) the reduced viscosity of 1% serum albumin increased with time, reached a maximum value of 1.20 after about one hour and then decreased at a rate of 0.04 per hour for at least six hours. This maximum value is far above the values found at lower *p*H and is also considerably above the value found with ovalbumin at the same *p*H. The subsequent decrease, undoubtedly caused by hydrolysis, is very similar to that found with ovalbumin under similar circumstances.

2. **Effect of Protein Concentration.**—Figure 4 shows the viscosity changes at various protein concentrations and Fig. 5A shows the concentration dependence of the viscosity after various times in 8 *M* urea at *p*H 10. These results are very similar to those found with ovalbumin in that they show that at higher protein concentrations extensive aggregation takes place under these conditions, as manifested by the positive curvature of the reduced viscosity *vs.* concentration curves (*cf.* Part IV).

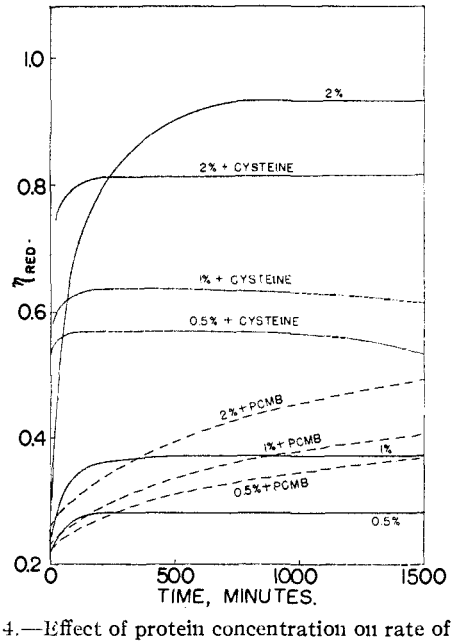


Fig. 4.—Effect of protein concentration on rate of change of reduced viscosity of serum albumin in 8 *M* urea at 30° and *p*H 9.9–10.0 (0.05 *M* borate buffer). Percentage protein as indicated along curves. Curves marked "PCMB" show effect of 0.005 *M* PCMB and curves marked "cysteine" show effect of 0.02 *M* cysteine plus 0.02 *M* NaCl.

It is significant that the reduced viscosity extrapolated to infinite dilution of protein does not change appreciably with the time. This means that no important change in the molecular shape occurs after the initial instantaneous reaction in 8 *M* urea at *p*H 10. The subsequent increase in viscosity at finite protein concentrations is caused

exclusively by aggregation. This is consistent with the behavior of the optical rotation and with the viscosity–diffusion constant measurements of Neurath and Saum.[6]

3. **Effect of Sulfhydryl Reagents.**—The results of Huggins, Tapley and Jensen,[4] who found that specific sulfhydryl reagents in small concentrations inhibit the gelation of serum albumin in urea, prompted us to investigate the effects of such reagents on the viscosity changes of serum albumin solutions during urea denaturation.

a. **Effect of *p*-Chlormercuribenzoate (PCMB).**—Figures 4 and 5B show that the effect of PCMB on serum albumin is rather different from its effect on ovalbumin. The initial rate of change of the viscosity is decreased by this reagent, but the viscosity continues to increase for a long time. At the lower protein concentrations the viscosity eventually exceeds that of the corresponding solutions without PCMB. The slight curvature of the plots in Figure 5B shows that aggregation is slight but that the molecular shape continues to change with time. This change must be a manifestation of an additional unfolding step in serum albumin. We believe that it results from the rupture by hydrolysis of disulfide bonds which cross link the denatured molecule and keep it from uncoiling completely (*cf.* Fig. 1 of Part IV).

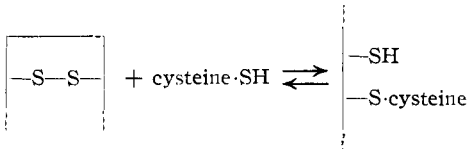
Many compounds containing disulfide groups are split rather readily by strong alkali, presumably according to the reaction[10]

$$RSSR + H_2O \longrightarrow RSH + RSOH$$

The very unstable sulfenic acid usually reacts further by dismutation. The SS groups of oxidized glutathione are completely hydrolyzed by 1.0 *N* NaOH in 2 hours at 30°, and those of insulin by 0.5 *N* NaOH in 15 minutes at 40°.[10] At *p*H 7 and 37° the SS groups of oxidized glutathione are completely split in one hour if an excess of silver ion is present, though they are unaffected in the absence of silver.[11] It is reasonable to suppose that the silver ion displaces the initial hydrolytic equilibrium by combining with the liberated sulfhydryl groups, and thereby promotes the hydrolysis. In the light of this evidence it is suggested that PCMB acts in a manner analogous to silver and thus promotes the hydrolytic splitting of disulfide.

The inhibition of aggregation by PCMB is in accord with the conclusions of Huggins, Tapley and Jensen: in the absence of PCMB aggregation takes place through intermolecular SS bonds which are formed by means of an exchange reaction between SH groups and intramolecular SS groups. Removal of the SH by reaction with PCMB prevents aggregation by stopping the exchange reaction.

b. **Effect of Cysteine.**—Intramolecular SS cross links can be ruptured very effectively by adding an excess of cysteine, giving the reaction



(10) A. Schöberl and P. Rambacher, *Ann.*, **538**, 84 (1939).
(11) R. Cecil, *Biochem. J.*, **47**, 572 (1950).

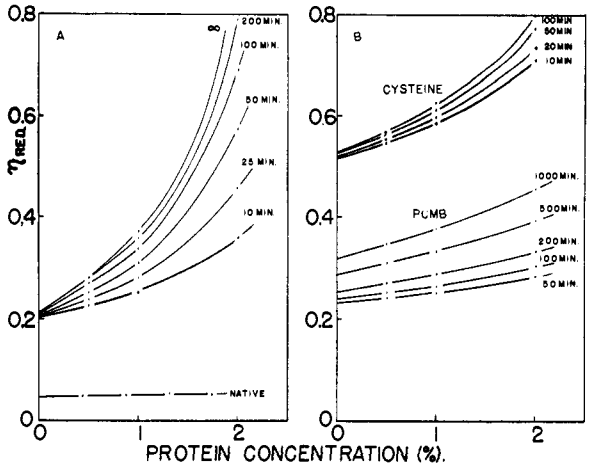


Fig. 5.—Effect of sulfhydryl reagents on concentration-dependence of reduced viscosity of serum albumin in 8 *M* urea at 30° and *p*H 9.9–10.0 at various times: A, no added reagents; B, lower curves, with 0.005 *M* PCMB; upper curves, with 0.02 *M* cysteine plus 0.02 *M* NaCl.

This reaction should be rapid in alkaline solutions and should have an equilibrium constant not very far from unity, at worst. Therefore the addition of a moderate excess of cysteine to serum albumin at *p*H 10 should rapidly throw the equilibrium well to the right and rupture most of the intramolecular disulfide cross links. If, as has been suggested in the previous section, these disulfide cross links prevent the complete uncoiling of the denatured molecule, then the addition of cysteine to serum albumin should increase its intrinsic viscosity in urea. If the excess is large enough it should also prevent aggregation.

The effect of 0.02 *M* cysteine on several concentrations of serum albumin in 8 *M* urea at *p*H 10 is shown in Figs. 4 and 5B. The behavior is exactly as expected; the intrinsic viscosities are about doubled and the concentration dependence of the reduced viscosity shows only a slight indication of aggregation.

c. **Effect of Sulfite.**—Another reagent which might be effective in splitting disulfide cross links is sulfite. This splitting is thought to proceed by the reaction[12]

$$RSSR + SO_3^{=} \rightleftarrows RS^- + RSSO_3^-$$

In a polarographic study of this reaction using cystine, the equilibrium was found to be rapidly attained and to lie quite far to the left (equilibrium constant of the order of 0.01).[13] Splitting by sulfite has been used extensively in the determination of SS in similar disulfides and in protein hydrolyzates.[14] No direct data are available for soluble proteins, but stress relaxation measurements on wool fibers[15] and hair[16] in sodium bisulfite solutions suggest that the disulfide of keratin is split by this reagent and that molecular rearrangement takes place subsequently in the stressed fiber. In view of

(12) H. T. Clarke, *J. Biol. Chem.*, **97**, 235 (1932).
(13) W. Stricks and I. M. Kolthoff, THIS JOURNAL, **73**, 4569 (1951).
(14) B. Kassel and E. Brand, *J. Biol. Chem.*, **125**, 131 (1938).
(15) S. M. Katz and A. V. Tobolsky, *Textile Res. J.*, **20**, 87 (1950).
(16) C. E. Reese and H. Eyring, *ibid.*, **20**, 743 (1950).

the great differences in the reactivity of the various disulfides, it is not possible to predict the behavior of serum albumin, though the splitting must be favored by high *p*H. The behavior of protein disulfide in other reactions gives the impression that it is more reactive than cystine disulfide, so splitting by sulfite might on chemical grounds be expected to be fairly extensive in the experiments to be described here.

The results obtained with sodium sulfite (Fig. 6) are more difficult to interpret than those for PCMB and cysteine. In the first place, these experiments did not show the usual degree of precision in that the experimental points did not fall on smooth curves and repeat runs did not reproduce the original results very closely. It appeared that the agitation of the solution in the viscosimeter during a viscosity measurement tended to accelerate the viscosity increase, especially during the final stages of the reaction.

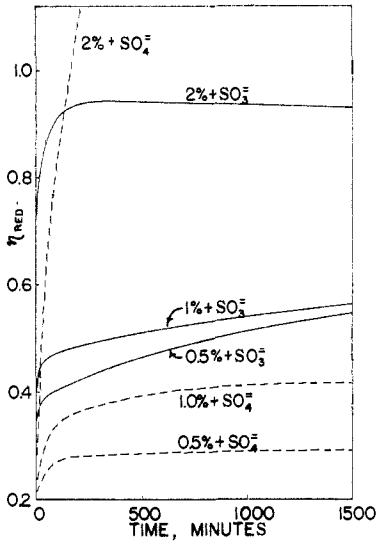


Fig. 6.—Effect of sulfite and sulfate on reduced viscosity at 0.5, 1 and 2% serum albumin in 8 *M* urea at 30° and *p*H 9.9–10.0 (0.05 *M* borate buffer). Solid curves, effect of 0.02 *M* Na_2SO_3; dashed curves, effect of 0.02 *M* Na_2SO_4. The reduced viscosity of the 2% serum albumin solution containing sulfate levelled off at a value of 2.0 after 900 min.

In the second place, sulfite may show two opposing effects: (a) it may act as an electrolyte similar to sulfate, and (b) it should act as a disulfide-splitting agent. Consequently a study was first made of the effect of sodium sulfate on the viscosity. The addition of 0.02 *M* sodium sulfate strongly increased the aggregation of 2% serum albumin in 8 *M* urea (see Fig. 6), a final reduced viscosity of 2.0 being reached in about 900 minutes as compared with 0.93 in the absence of sodium sulfate. As already suggested in connection with the enhancement of the gelling of ovalbumin by sulfate, this increased aggregation may be caused by the reduction of the electrostatic repulsions between protein molecules (or portions thereof).[17]

The effect of sulfite at a serum albumin concentration of 2% is different from that at the lower protein concentrations. The initial rate of the viscosity change is increased in both cases, but with 2% protein a steady viscosity is soon reached. This is approximately equal to that attained without added reagent, but much lower than the final value attained in the presence of sulfate. At the lower protein concentrations, on the other hand, there is no levelling off, and the viscosities become even higher than those attained in the presence of sulfate.

This complexity is not unexpected, since splitting of intermolecular disulfide bonds would cause a decrease in the viscosity, while rupture of intramolecular disulfide would lead to a more expanded structure, and hence an increase in the viscosity. With 2% protein much aggregation normally takes place, and the effect of sulfite on the intermolecular disulfide links here over-balances its effect on the intramolecular links. At the lower protein concentrations, on the other hand, there are fewer intermolecular linkages so that the effect of breaking the intramolecular bonds is more noticeable. The excess of sulfite is also larger at the lower protein concentration.

4. The Reversibility of the Viscosity Changes.—At *p*H 5 the viscosity changes in 1 to 1.5% serum albumin are apparently instantaneously and completely reversible when the urea concentration is changed from 10 to 6 *M* either early in the reaction (after 12 minutes) or after a considerably longer exposure (2400 minutes). With the earlier dilution at *p*H 5 even the slight time-dependent portion of the viscosity change normally observed in 6 *M* urea is reproduced in spite of the fact that the viscosity after 12 minutes in 10 *M* urea is well above the maximum value reached at *p*H 5 in 6 *M* urea. This is in accord with the supposition that no appreciable amount of aggregation occurs by means of disulfide–sulfhydryl exchange at *p*H 5. On reducing the urea concentration from 10 to 6 *M* the more "swollen" protein molecule immediately shrinks to the configuration normally obtaining at the lower urea concentration.

These observations are reminiscent of those made by Neurath, Cooper and Erickson,[19] who after treating serum albumin with 8 *M* urea at *p*H 5 were able to recover about 85% as "reversibly denatured" on removing the urea by dialysis. The recovered protein was very similar to the native material with respect to its intrinsic viscosity and diffusion constant, though it differed from it slightly in its solubility in ammonium sulfate solutions, crystallizability and electrophoretic mobility. These authors suggested that the unfolded molecule refolds on removal of the urea. It would not be surprising if the refolded molecule did not return exactly to the form of the native molecule and hence differed from it in some respects.

(17) An apparently contradictory effect has been reported by Boyer, Ballou and Luck,[18] who found that 0.50 *M* sodium sulfate prevented the viscosity increase in human serum albumin denatured by 2.5 *M* guanidinium chloride at *p*H 7.5. This, however, represents an entirely different case, since guanidinium chloride is itself a strong electrolyte and the addition of a moderate amount of sulfate does not change the ionic strength appreciably. Moreover, the effect of sodium sulfate at a concentration of 0.5 *M* could be quite different from that at 0.02 *M*. It will be recalled that large amounts of sodium sulfate strongly inhibit the unfolding of ovalbumin (Part I).[9]

(18) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **162**, 199 (1946).

(19) H. Neurath, G. R. Cooper and J. O. Erickson, *ibid.*, **142**, 249 (1942).

Figure 1 includes two curves showing the changes in the reduced viscosity of 1% serum albumin at *p*H 10 on dilution of the urea from 10 to 8 *M* and to 6 *M*. At this *p*H the reversibility with respect to the urea concentration is again nearly complete, but it is much slower than at the lower *p*H. This agrees with the supposition that aggregation through the disulfide groups has taken place at this *p*H, and the reversible but comparatively slow exchange reaction between mercaptide ion and disulfide is the rate-determining step.

Figure 7 illustrates the lack of reversibility with respect to changes in the *p*H. Serum albumin was exposed to 8 *M* urea and sodium hydroxide at *p*H 10 for 240 minutes and then brought to *p*H 5.5 by the addition of sodium acetate and acetic acid in 8 *M* urea. The small, instantaneous decrease in the viscosity probably represents a contraction of the loosely coiled molecule caused by the reduction of the net charge on the molecule. The viscosity remains, however, considerably above the value reached by serum albumin denatured at the lower *p*H from the start. Apparently the aggregation is not reversed since the sulfhydryl–disulfide exchange reaction is too slow at this low *p*H.

5. Shapes of the Urea-Denatured Ovalbumin and Serum Albumin Molecules.—The increase in the viscosity during the urea denaturation of serum albumin and ovalbumin has been interpreted in terms of an increase in the axial ratio of the molecule.[6,20] The ratio is said to increase from three or four to one in the native protein up to nine or fifteen to one in the denatured protein. According to this interpretation, denaturation results in a molecule having a highly elongated rod shape.

It is well known that denaturation is accompanied by a large increase in the entropy.[21–26] This has been explained[22,24,27] in terms of an increase in the number of configurations available to the protein molecule which is undoubtedly largely the result of the existence in the denatured protein of some degree of random coiling similar to that known to be important in most synthetic linear high polymers.[27,28] If this is the case, the interpretation of the increase in viscosity on denaturation in terms of a change in axial ratios is not necessarily relevant, nor is the use of the concept of an "equivalent hydrodynamic ellipsoid."[29] It would be better to discuss the intrinsic viscosities of denatured proteins by comparing them with the values found for high polymers of similar molecular weight and chain length. When such a comparison is made it is found that the intrinsic viscosities of ovalbumin and cysteine-treated serum albumin in strong urea have indeed nearly the values expected of a randomly coiled linear high polymer in a good solvent. This is discussed in more detail elsewhere.[27]

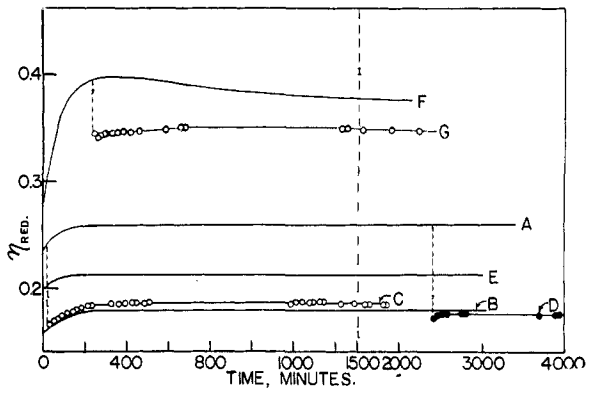


Fig. 7.—Reversibility of the change in reduced viscosity of serum albumin with respect to changes in *p*H and urea concentration: A, 10 *M* urea at *p*H 5.3; B, 6 *M* urea at *p*H 5.8; C, urea concentration reduced to 6 *M* after 12.5 min. at 10 *M*, *p*H 5.3; D, urea concentration reduced to 6 *M* after 2400 min. at 10 *M*, *p*H 5.3; E, 8 *M* urea at *p*H 5.4; F, 8 *M* urea at *p*H 10; G, 8 *M* urea, *p*H reduced from 9.8 to 5.5 after 240 min. (A-E, 0.05 *M* acetate buffer; F, 0.025 *M* borate buffer. Time scale changes after 1500 min.).

6. Comparison of the Results for Ovalbumin and for Serum Albumin.—Although there are many points of similarity in the behavior of the viscosities of serum albumin and of ovalbumin in urea solutions, there are two striking differences.

(1) There is an instantaneous initial increase in the viscosity at all urea concentrations with serum albumin whereas the corresponding increase with ovalbumin is much slower, especially at the lower urea concentrations. This is caused by the much greater ease of unfolding which is typical of serum albumin and which has already been noted and discussed in connection with the behavior of the optical rotations of the two proteins (Part III).

(2) The intramolecular disulfide cross links play a very much greater role in serum albumin than in ovalbumin. This might have been expected from the fact that serum albumin contains 18 SS (cystine) groups per molecule[30] whereas ovalbumin contains only two.[31] Our results show that a definite partially uncoiled state of serum albumin exists in which these cross links remain intact, and that they may be ruptured to give a completely uncoiled molecule having a considerably higher intrinsic viscosity. This means that the two halves of at least some of the 18 cystine residues in serum albumin must be incorporated into widely separated parts of the polypeptide backbone, since if the two halves of each cystine were neighbors or near neighbors along the polypeptide chain, the shape and hence the intrinsic viscosity would not change very

(20) (a) G. R. Cooper and H. Neurath, *J. Phys. Chem.*, **47**, 383 (1943); (b) F. W. Putnam, J. O. Erickson, E. Volkin and H. Neurath, *J. Gen. Physiol.*, **26**, 513 (1943); (c) H. Bull, *J. Biol. Chem.*, **133**, 39 (1940).

(21) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **17**, 393 (1934).

(22) L. Pauling and A. E. Mirsky, *Proc. Nat. Acad. Sci. U. S.*, **22**, 439 (1936).

(23) R. M. Herriott, *J. Gen. Physiol.*, **21**, 519 (1938).

(24) H. Eyring and A. E. Stearn, *Chem. Revs.*, **24**, 253 (1939).

(25) M. Kunitz, *J. Gen. Physiol.*, **32**, 241 (1948).

(26) M. A. Eisenberg and G. W. Schwert, *ibid.*, **34**, 583 (1951).

(27) W. Kauzmann, in "The Mechanism of Enzyme Action," McCollum–Pratt Symposium, June, 1953, to be published by Johns Hopkins University Press.

(28) H. Mark and A. V. Tobolsky, "Physical Chemistry of High Polymeric Systems," Interscience Publishers, Inc., New York, N. Y., 1950, pp. 324–331.

(29) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953). Note especially footnote 41.

(30) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, *ibid.*, **71**, 2479 (1949); reinterpretation of results of Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(31) H. L. Fevold, *Advances in Protein Chem.*, **6**, 188 (1951).

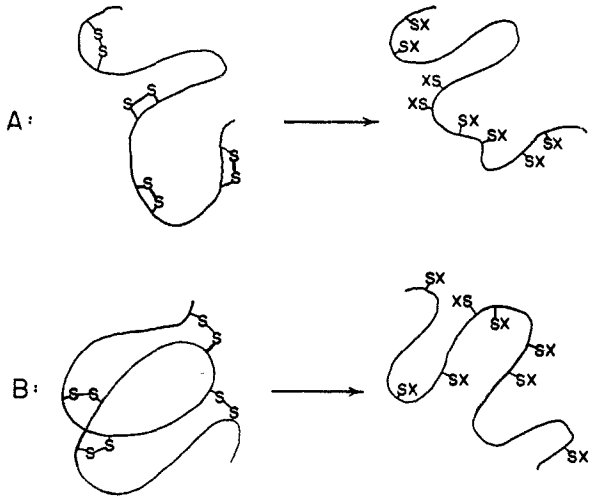


Fig. 8.—Effect of arrangement of cystine residues on shape of unfolded serum albumin molecule: A, two halves of each cystine residue are close neighbors along polypeptide chain. Rupture of —S—S— has slight effect on coiling. B, two halves of each cystine are far apart along chain. Rupture of —S—S— permits a more extended coil.

much when the disulfide links were ruptured (Fig. 8).

It is worth pointing out that the intramolecular disulfide cross links in serum albumin may be responsible for the ease with which the denaturation of serum albumin may be reversed. The cross links may restrict the uncoiling of the denatured molecule to such an extent that on removal of the denaturing agent the molecule is able to find its way back to a folded state similar to that in the native molecule. The well-known irreversibility of the denaturation of ovalbumin would then be accounted for by the absence of such a partially uncoiled state because there are so few SS links in ovalbumin. Furthermore, the denaturation of serum albumin should become irreversible when the intramolecular disulfide links are broken. Elsewhere we shall show that this is indeed probably the case.

The disulfide cross links may also be responsible for the comparative ease with which serum albumin is denatured because these relatively abundant cross links might place constraints on the polypeptide chains making it impossible for them to fold into a structure having the same high degree of stability as would otherwise be attainable. Ovalbumin, with only two disulfide cross links, is not subject to this constraint and might therefore be able to fold in a more stable configuration which can better resist the action of urea.

PRINCETON, NEW JERSEY

[CONTRIBUTION NO. 1176 FROM THE STERLING CHEMISTRY LABORATORY OF YALE UNIVERSITY]

Electrolyte–Solvent Interaction. III. Tetrabutylammonium Bromide in Methanol–Methyl Ethyl Ketone Mixtures[1]

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Tetrabutylammonium bromide shows slight ionic association ($K = 0.04$) in methanol, and considerably more ($K = 0.00159$) in methyl ethyl ketone. In mixtures of these solvents, the salt shows negligible association ($K > 1$). As the ketone content of the mixtures increases, the viscosity decreases, but the equivalent conductance of the salt decreases also, so that the Walden product $\Lambda_0\eta$ initially decreases. A minimum then appears, and in the ketone-rich mixtures, conductance increases much more rapidly than the viscosity decreases. Both the change of association and mobility with composition of the solvent can be accounted for by assuming (1) that methanol is depolymerized by addition of ketone and (2) that specific interaction ("solvation") then occurs between monomeric methanol and the solute.

Introduction

Recent work[3,4] on the conductance of electrolytes in mixed solvents has given experimental confirmation of the expectation that the conventional spheres-in-continuum model for the system electrolyte–solvent would fail in cases where specific interaction between solute and solvent occurs. The ultimate goal of the investigation is to clarify the rather vague concept of solvation. In a general sort of way, we may expect to find all gradations from stoichiometric complexes to loose aggregates in which an ion simply drags with itself an average (not necessarily integral) number of solvent molecules by ion–dipole attraction. The size of the sphere which is hydrodynamically equivalent to a given ion would then vary from solvent to solvent, depending on the nature of the bonding and the effective thickness of the solvate shell; the diagnostic symptom will obviously be a variation of the Walden product $\Lambda_0\eta$ with solvent. Likewise, the size of the electrostatically equivalent sphere (as calculated from the association constant) should vary from solvent to solvent if solvation is selective. Furthermore, the hydrodynamic and electrostatic radii need not agree numerically, and certainly should differ if solvate molecules are expelled on the formation of ion pairs.

Rather than merely comparing results in different solvents, studies of mixed solvents are preferred, because two additional pieces of information then become available: (1) any abrupt change in electrolyte properties on adding solvent A to B or *vice versa* will indicate a preferential interaction of cation or anion with A or B, and (2) interaction between the solvents themselves will appear as a

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(2) Results presented in this paper are abstracted from a dissertation presented by F. Muriel Sacks to the Graduate School of Yale University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, June, 1953.

(3) H. Sadek and R. M. Fuoss, THIS JOURNAL, **72**, 301 (1950); correction, *ibid.*, **72**, 5803 (1950).

(4) R. C. Miller and R. M. Fuoss, *ibid.*, **75**, 3076 (1953).