[CONTRIBUTION FROM THE BEN MAY LABORATORY FOR CANCER RESEARCH AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO]

Sulfhydryl-Dependent Aggregation Accompanying the Denaturation of Bovine Plasma Albumin by Urea[1,2]

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RECEIVED DECEMBER 23, 1953

When a solution of bovine plasma albumin, in the *p*H range 7 to 9, is exposed to concentrated urea, two phenomena take place. The first process is extremely rapid and is accompanied by a large increase in the viscosity of the solution. The second process is much slower and manifests itself by a continued gradual rise in the viscosity, leading, if the protein concentration is sufficient, to the formation of a clear, firm gel. The slow reaction, but not the fast one, depends on the presence of free sulfhydryl groups; it is postulated to involve an aggregation of albumin molecules brought about by an intermolecular chain reaction of sulfhydryl with disulfide groups.

When a solution of a protein such as human or bovine plasma albumin is exposed to concentrated urea, a severe alteration of the protein structure takes place.[3,4] The viscosity of the solution is markedly increased, and, if the protein concentration is sufficient, a firm clear gel is formed after a period of time.[5] It was reported previously[6] that the ability of plasma albumin to form such a gel depends on the presence of the single sulfhydryl group which is present in about three-fourths of the molecules of albumin.[7] The present paper is concerned with the influence of this sulfhydryl group on the viscosity changes induced by urea in solutions of bovine plasma albumin too dilute to form solid gels.

Experimental

Materials.—The bovine plasma albumin employed in these experiments was obtained from Armour and Co.; amperometric titration with silver nitrate showed it to possess 0.78 of an equivalent of sulfhydryl per mole of anhydrous protein (mol. wt. 69,000). "Iodoacetamide-treated" albumin was prepared by allowing 1250 mg. of albumin to react for 24 hr. at 25° with 6.8 mg. of iodoacetamide (2 moles of iodoacetamide per mole of protein) in 25 ml. of 0.1 *M* phosphate buffer, *p*H 8.0. The solution was dialyzed at 2° against several portions of the buffer and then diluted with buffer to the desired protein concentration. Amperometric titration indicated the complete absence of sulfhydryl groups in iodoacetamide-treated albumin. Reagent grade urea was recrystallized once from ion-free water. Distilled water, deionized with Amberlite MB-1 resin, was used for all solutions.

Measurements.—Viscosity measurements were carried out in Ostwald–Cannon viscosimeters in a water-bath at 30 ± 0.01°. Prior to mixing, 4 ml. of a 2.5% solution of protein and 6 ml. of 10 *M* urea (each in 0.1 *M* phosphate buffer *p*H 8.0 unless otherwise noted) were allowed to reach temperature equilibrium at 30° in a 25-ml. round bottom flask possessing a crease in the bottom to separate the two solutions. When additional reagents such as silver nitrate were employed, these were incorporated in the albumin solution. At zero time the flask was swirled in the bath to ensure thorough mixing, and a 5-ml. aliquot was transferred to the viscosimeter by a pipet which had been previously equilibrated at 30°. Thus the final concentration was 1% (1.4×10^{-4} *M*) in albumin and 6 *M* in urea. The time of passage of the solution through the viscosimeter was the order of 70 seconds and was measured to ±0.02 second with a Cenco–Harrington timer. The results are expressed in terms of the reduced viscosity, *i.e.*

$$\eta_{red} = \frac{1}{c}\left[\frac{t_{(albumin + urea)}}{t_{(urea)}} - 1\right]$$

where *t* is the passage time and *c* is the protein concentration in g. per 100 ml. solution. In these experiments $c = 1$.

Results

When a 1% solution of bovine plasma albumin in phosphate buffer is exposed to 6 *M* urea, a marked increase in the viscosity of the medium occurs even before a measurement can be made (1 to 2 minutes). The rapid initial viscosity change is followed by a more gradual rise which continues for many hours (Fig. 1). This secondary viscosity change is not very pronounced unless buffer or

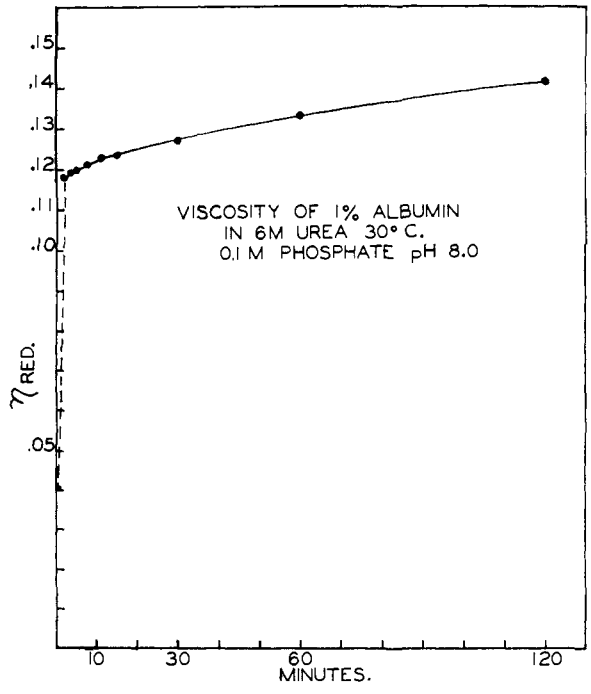

Fig. 1.—Change in viscosity of a bovine plasma albumin solution after exposure to urea.

(1) Presented before the Division of Biological Chemistry, 124th meeting of the American Chemical Society, Chicago, September, 1953.

(2) This investigation was supported in part by grants from the National Institutes of Health, Public Health Service (RG-3053) and from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

(3) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(4) Subsequent to the completion of this manuscript, an extensive study of urea-induced changes in the viscosity and optical rotation of plasma albumin solutions has been reported: (a) W. Kauzmann and R. B. Simpson, THIS JOURNAL, **75**, 5154 (1953); (b) H. K. Frensdorff, M. T. Watson and W. Kauzmann, *ibid.*, 5157, 5167 (1953).

(5) W. Ramsden, *J. Physiol.*, **28**, xxiii (1902); F. G. Hopkins, *Nature*, **126**, 328, 383 (1930).

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

(7) W. L. Hughes, Jr., *Cold Springs Harbor Symposium on Quantitative Biology*, **14**, 79 (1949); E. V. Jensen, V. D. Hospelhorn, D. F. Tapley and C. Huggins, *J. Biol. Chem.*, **185**, 411 (1950).

other salt is present (Fig. 2); the viscosity curves obtained with 0.19 *M* sodium chloride or potassium nitrate are quite similar to those observed with 0.1 *M* phosphate buffer. In the *p*H range studied (7 to 9), the rate of the secondary viscosity change is moderately enhanced by an increase in the *p*H.

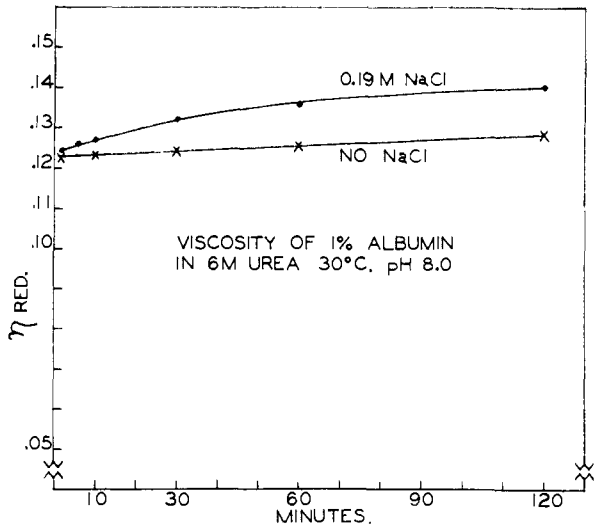

Fig. 2.—Effect of salt on the viscosity change of an albumin-urea solution (no buffer present).

If the albumin solution is first treated with one equivalent of silver nitrate per mole of protein in order to block the sulfhydryl group, the initial viscosity reading after mixing with urea is somewhat higher than in the absence of silver (Fig. 3). However, the viscosity of the silver-treated albumin solution does not increase further but remains constant for several hours.[8,9] If 2 equivalents of silver nitrate per mole of protein are added, the actual viscosity observed is significantly lower than when only one equivalent of silver is used. Addition of more than two moles of silver nitrate per mole of albumin causes precipitation of the protein under the conditions employed.

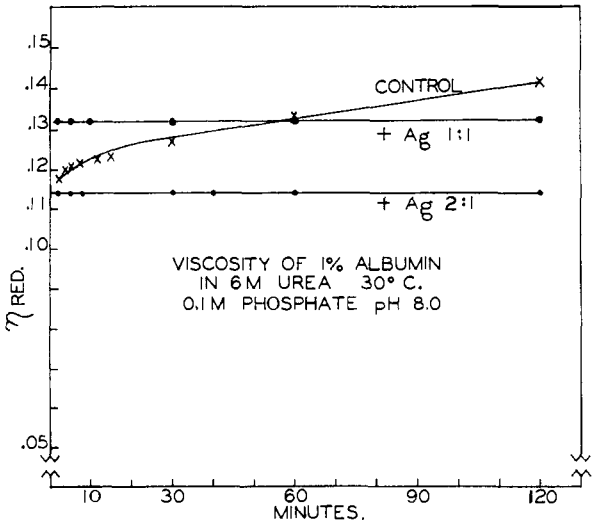

Fig. 3.—Effect of silver nitrate on the viscosity change of an albumin-urea solution.

(8) In some experiments the viscosity of the silver-treated albumin remained constant for 18 hours; in others there was a very slight increase in viscosity at 18 hours.

(9) This effect of silver ion and of iodoacetamide on the viscosity of bovine plasma albumin in urea at *p*H 7–9 is somewhat different from that observed by Kauzmann, *et al.*,[5b] with *p*-chloromercuribenzoate at *p*H 10, but it is similar to that of the latter reagent on ovalbumin at *p*H 7.5.

A similar elimination of the secondary slow viscosity change is observed if the sulfhydryl group of albumin is destroyed by other means. Solutions of albumin which have been treated with iodoacetamide reach their maximum viscosity immediately after mixing with urea, and this value does not change thereafter (Fig. 4). When one mole of silver nitrate is added to the iodoacetamide-treated albumin, the viscosity value is lowered in a manner similar to that observed when two moles of silver nitrate are added to ordinary albumin.

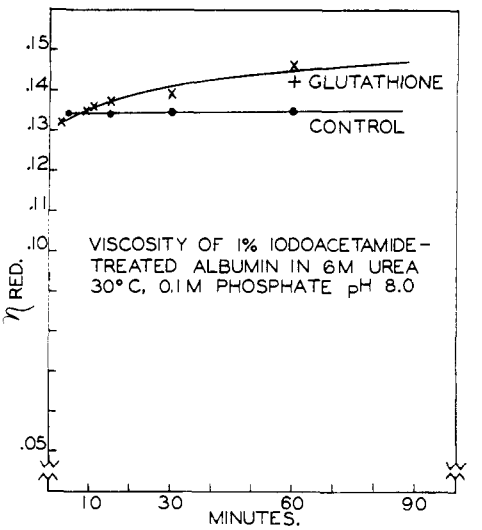

Fig. 4.—Restoration of the secondary viscosity change to iodoacetamide-treated albumin by glutathione (1 mole of GSH per mole of protein).

If a small amount of a simple compound containing a sulfhydryl group is added to the solution of iodoacetamide-treated albumin immediately prior to mixing with urea, the secondary continued viscosity increase reappears. The effect of one mole of glutathione per mole of protein is illustrated in Fig. 4; similar results were obtained with monothioglycol (mercaptoethanol). In order to restore the secondary viscosity change it is necessary that the sulfhydryl compound be added to the iodoacetamide-treated albumin immediately before mixing with urea; if the mercaptan is allowed to stand with the protein for 30 minutes before adding the urea, little restoration of the secondary viscosity change is observed.

Discussion

From the foregoing results it appears probable that there are two separate phenomena taking place when a solution of plasma albumin is treated with concentrated urea. The first process (reaction A) is extremely rapid and is accompanied by a large increase in the viscosity of the system; the second process (reaction B) is relatively slow and leads to a continued gradual increase in the viscosity. Occur-

rence of two distinct phenomena also is indicated by the results of Kauzmann[4] who measured changes both in the optical rotation and in the viscosity of solutions of bovine plasma albumin after exposure to urea. The rapid initial viscosity rise was found to be accompanied by a significant increase in levorotation, whereas no further optical change took place during the subsequent gradual viscosity rise. Reaction A has been considered[4] to involve an unfolding of the protein molecule. We believe that reaction B consists of an aggregation of unfolded protein molecules. This aggregation causes a continued increase in the viscosity of the solution and, if the protein concentration is sufficient, leads to the formation of a gel structure which binds urea and water molecules within its network.

Reaction B, but not reaction A, depends on the presence of free sulfhydryl groups either of the protein molecule or of simple organic substances. The ability of this single group on the albumin molecule to bring about aggregation and the formation of a cross-linked gel network suggests that a chain type of reaction is involved. The chain mechanism illustrated in Fig. 5, which was proposed previously to explain the formation of gels,[6,10] is in accord with the present observations in regard to viscosity changes. In the presence of concentrated urea, the disulfide groups of albumin become available for reaction with sulfhydryl groups. The sulfhydryl group of one albumin molecule is considered to react with a disulfide group in another molecule to form an intermolecular disulfide linkage while generating a new sulfhydryl group (equation I). This new sulfhydryl then repeats the process by reaction with another disulfide group either in the first albumin molecule or in a third, and so on. In this way a large number of protein molecules can become linked together.

The foregoing scheme explains the ability of simple sulfhydryl compounds to restore both the secondary viscosity change and the capacity for gelation to solutions of albumin in which the sulfhydryl group has been irreversibly blocked with iodoacetamide. Simple sulfhydryl compounds should be as effective as protein sulfhydryl groups in starting the reaction chain which, when once underway, proceeds independent of the manner in which it was initiated (equation II). Furthermore the observed

(10) The sulfhydryl–disulfide chain reaction recently has been utilized to explain exchange reactions observed with disulfide-containing peptides (F. Sanger, *Nature*, **171**, 1025 (1953)) and cross-linking phenomena in the denaturation of ovalbumin by heat or urea (M. Halwer, THIS JOURNAL, **76**, 183 (1954)).

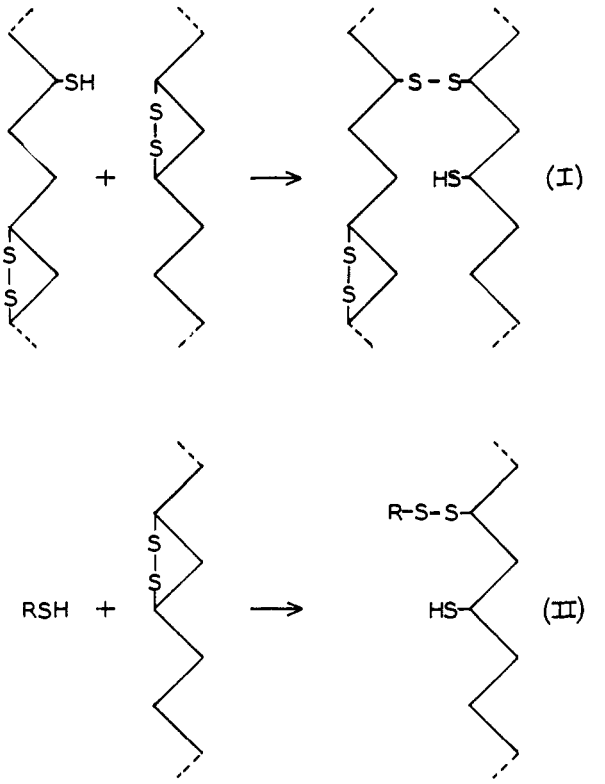

Fig. 5.—Postulated mechanism of sulfhydryl-induced aggregation.

enhancement of the viscosity change and gelation by an increase in the *p*H is to be expected, since the reaction of mercaptans with disulfide groups involves the mercaptide ion and takes place more rapidly as the *p*H is increased.[11]

At present we do not understand why the viscosity observed immediately after mixing with urea should be higher when the sulfhydryl group is blocked than when it is present. Nor is it clear why the further addition of silver nitrate to albumin in which the sulfhydryl group already is blocked should lead to a significant lowering of the viscosity in urea. The latter phenomenon would appear to involve reaction of silver ion with groups other than sulfhydryl.

The authors wish to express their appreciation to Professor C. Huggins for his interest and coöperation.

CHICAGO, ILLINOIS

(11) T. Bersin and J. Steudel, *Ber.*, **71**, 1015 (1938).