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

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

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The changes in viscosity and sedimentation characteristics which accompany the thermal denaturation of dilute solutions of bovine plasma albumin differ significantly depending on whether or not the albumin sulfhydryl group is present in the free state. On heating, sulfhydryl-containing albumin aggregates more rapidly than does sulfhydryl-free albumin, but solutions of the latter become much more viscous. The addition of trace amounts of mercaptoethanol to solutions of iodoacetamide-treated albumin partially restores the coagulation characteristics typical of untreated albumin. The results support the concept that, during thermal denaturation, sulfhydryl groups promote lateral association of protein molecules through a chain reaction with disulfide groups.

When a solution of human or bovine plasma albumin is heated at 100° in the *p*H region near neutrality, the protein becomes denatured; if the protein concentration is sufficient, coagulation takes place to form a solid clot or gel. It was reported previously[3] that the physical properties of such coagula are markedly influenced by the single sulfhydryl group which is present in about three-fourths of the molecules of albumin. The clot formed by heating sulfhydryl-containing albumin is soft, opaque and synerizing, whereas albumin in which the sulfhydryl group has been destroyed, either by oxidation, alkylation or conversion to a heavy metal mercaptide, gives rise to clots which are firm, clear and non-synerizing. Moreover, the concentration of sulfhydryl-free albumin required to form a solid gel is less than one-half that required in the case of sulfhydryl-containing albumin.

The foregoing phenomena suggest that the molecules of thermally denatured albumin can aggregate by at least three different mechanisms.[3] These processes are: A. A rather slow reaction of unknown nature which causes the linking of protein chains at relatively few points in the molecule to form a regular, three-dimensional gel network resulting in a clear firm clot.[4]

B. A lateral or side-by-side type of aggregation involving the protein sulfhydryl group which imparts the properties of turbidity and syneresis to the coagulum.

C. Hydrogen bond formation between denatured protein molecules[5] which likewise leads to turbidity. When albumin is heated at or near its isoelectric point (*p*H 4.7) coagulation occurs chiefly through hydrogen bond formation,[6] but aggregation of this type takes place to a lesser extent at higher or lower *p*H conditions when electrostatic repulsion between protein molecules becomes significant due to the net charge.

(1) Presented before the Division of Biological Chemistry, 118th Meeting of the American Chemical Society, Chicago, September, 1950.

(2) This investigation was aided by a grant from the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

(3) E. V. Jensen, V. D. Hospelhorn, D. F. Tapley and C. Huggins, *J. Biol. Chem.*, **185**, 411 (1950).

(4) *Cf.* J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 388 (1947).

(5) A. E. Mirsky and L. Pauling, *Proc. Nat. Acad. Science*, **22**, 439 (1936).

(6) The curdy coagula which precipitate rapidly upon heating either sulfhydryl-containing or sulfhydryl-free albumin at its isoelectric point are largely dispersed by 8 *M* urea, whereas the gels which form more slowly at *p*H 7.4 are insoluble in urea.

The present paper describes the influence of the albumin sulfhydryl group on aggregation phenomena in solutions of thermally denatured bovine plasma albumin too dilute to form solid coagula. The viscosities of solutions of sulfhydryl-containing albumin after various periods of heating were compared with those of sulfhydryl-free albumin. Then the solutions of thermally denatured protein were examined in the analytical ultracentrifuge for differences in their sedimentation characteristics.

Experimental

The bovine plasma albumin employed in these experiments was obtained from Armour and Co.; amperometric titrations with silver nitrate showed it to contain 0.75 of an equivalent of sulfhydryl per mole of anhydrous protein (mol. wt. 69,000). The albumin was dissolved in $M/15$ phosphate buffer of *p*H 7.4 containing sufficient sodium chloride to make the total ionic strength 0.2. Albumin concentrations of 0.44% (6.4×10^{-5} *M*) and 0.27% (4.0×10^{-5} *M*) were employed.[7] Sulfhydryl-free albumin was obtained by incorporating in such solutions prior to heating one equivalent of the desired sulfhydryl reagent. "Iodoacetamide-treated" albumin was prepared as described in the preceding paper,[8] except that, following dialysis against ion-free water, the preparation was lyophilized; amperometric titration showed this protein to be sulfhydryl-free.

At zero time, Pyrex test-tubes containing 10 ml. each of the various albumin solutions were immersed in a boiling water-bath. After appropriate time intervals tubes were withdrawn and plunged into ice-water to halt the denaturation process; aliquot portions were then removed for viscosity and sedimentation studies. Viscosity measurements were carried out in Ostwald–Cannon viscosimeters of 5-ml. capacity immersed in a water-bath at 30 ± 0.02°. The time required for passage of a protein solution through the viscosimeter was measured to ±0.02 second with a Cenco-Harrington timer; this time was compared with that required for the buffer solution alone which was of the order of 260 seconds. Sedimentation patterns were obtained in a Spinco analytical ultracentrifuge; the patterns illustrated in Fig. 3 were obtained after five minutes spinning at 60,000 r.p.m. Because of the broadness of the peaks, the sedimentation constants reported must be regarded as only approximate.

Results

As illustrated in Fig. 1, heating to 100° of a 0.44% solution of bovine plasma albumin causes a small increase in its viscosity. The addition of one equivalent of a sulfhydryl reagent per mole of protein (*i.e.*, final concentration 6.4×10^{-5} *N*) has no effect on the viscosity of an unheated albumin solu-

(7) The 0.44% solutions of albumin remained perfectly clear after 5 minutes at 100°, but after ten minutes heating a slight turbidity was present. 0.27% solutions of albumin remained clear on prolonged heating.

(8) V. D. Hospelhorn, B. Cross and E. V. Jensen, THIS JOURNAL, **76**, 2827 (1954).

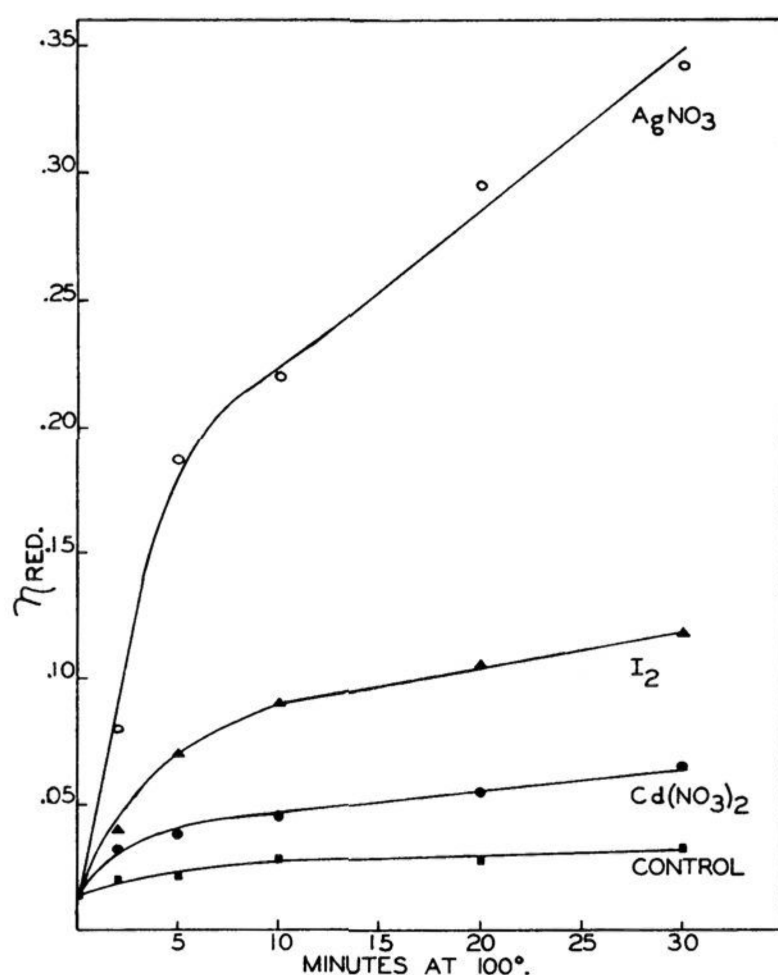

Fig. 1.—Viscosity of 0.44% solutions of bovine plasma albumin heated in the presence of sulfhydryl reagents (1 eq. of reagent per mole of protein).

tion, but it results in a marked elevation of the viscosity of the solution after heating. Silver nitrate produces a greater effect than the other sulfhydryl reagents tested.[9] Silver nitrate causes a similar though less pronounced elevation of the viscosity in 0.44% solutions of bovine plasma albumin denatured at 70° (Fig. 2, curves I and II) and in 0.27% albumin solutions denatured at 100° (Fig. 2, curves III and IV). In contrast to sulfhydryl reagents, an equivalent quantity of sodium nitrate has no effect on the viscosity.

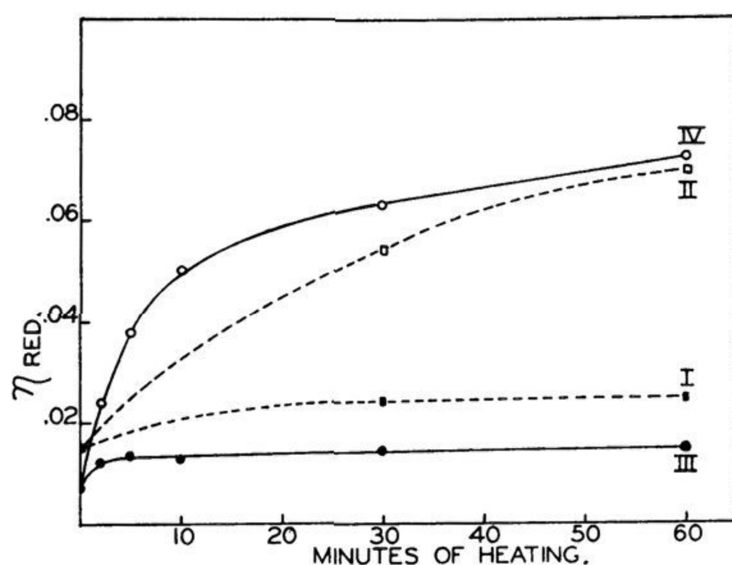

Fig. 2.—Viscosity of heated solutions of bovine plasma albumin: I, 0.44% albumin heated at 70°; II, same with $AgNO_3$ added; III, 0.27% albumin heated at 100°; IV, same with $AgNO_3$ added.

(9) In contrast to other sulfhydryl reagents, cupric nitrate, in either 1:1 or $^1/_2$:1 molar proportions, has no significant effect on the viscosity of heated albumin solutions, and its effect on the sedimentation pattern is unique. In coagulation studies it was observed[8] that cupric nitrate differs from other reagents in that it promotes the formation of clear clots without lowering the least coagulable concentration. It appears that cupric ion inhibits both process A and process B; this phenomenon will be discussed in a separate publication.

Native albumin previously has been formed to have a sedimentation constant of 4.3,[10] and the addition of silver nitrate was found to result in no effect on the sedimentation characteristics of unheated albumin. However, the sedimentation pattern of a solution of thermally denatured bovine plasma albumin depends markedly on whether or not the sulfhydryl group is free during heating (Fig. 3). After two minutes heating at 100°, sulfhydryl-containing albumin is practically all in a polymerized form (S_{20} = 11), whereas, in the presence of one equivalent of silver nitrate, there are two distinct components, one moving quite rapidly (S_{20} = 24) and the other consisting of apparently unaggregated albumin (S_{20} = 5). After ten minutes heating, sulfhydryl-containing albumin is further aggregated (S_{20} = 19); sulfhydryl-free albumin now is mostly aggregated (S_{20} = 32), but there still is some slower moving material (S_{20} = 4). After 30 minutes heating both solutions are entirely aggregated and quite heterogeneous as indicated by the broad humps.

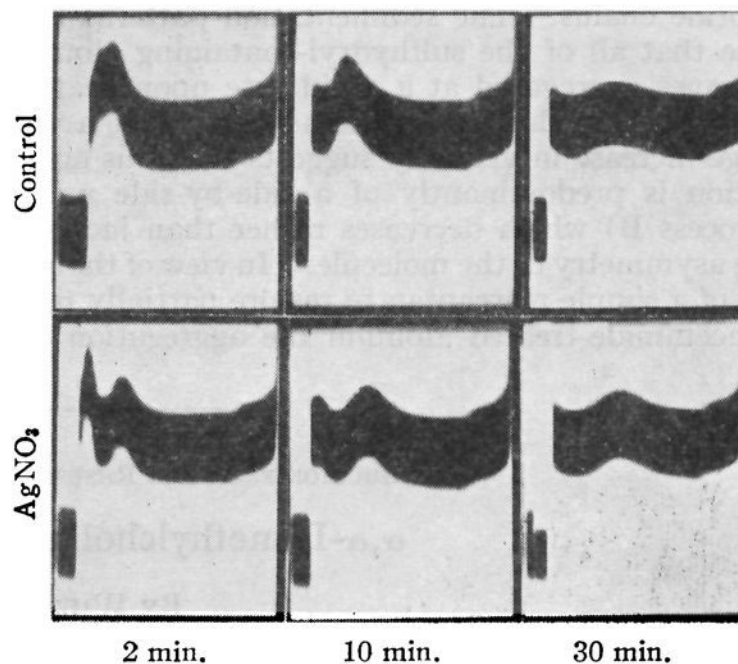

Fig. 3.—Sedimentation patterns of 0.27% solutions of bovine plasma albumin heated at 100° for various periods of time.

Sedimentation patterns similar to those observed with silver nitrate are obtained in the presence of mercuric chloride (molar ratio of either 1:1 or $^1/_2$:1), or with albumin previously treated with iodoacetamide.

When one mole of mercaptoethanol per mole of protein is added to the solution of iodoacetamide-treated albumin immediately prior to heating, the sedimentation pattern characteristic of heated sulfhydryl-containing albumin is not entirely restored, but more of the faster and less of the slower moving components are present. The ability of simple sulfhydryl compounds to restore, at least partially, to sulfhydryl-free albumin the aggregation characteristics of sulfhydryl-containing albumin is further illustrated by coagulation studies of more concentrated solutions of iodoacetamide-treated albumin

(10) S. Shulman, *Arch. Biochem. Biophys.*, **44**, 230 (1953).

carried out by the method described previously.[3] In contrast to sulfhydryl-containing albumin, which requires a concentration of 1.3% to form a solid coagulum on heating 30 minutes at 100° in 0.1 *M* phosphate buffer of *p*H 7.4, iodoacetamide-treated albumin forms a solid gel under these conditions in a 0.6% concentration. Although the gels formed from iodoacetamide-treated albumin are not entirely clear, they are markedly less turbid and much firmer than those obtained with sulfhydryl-containing albumin. Addition of one mole of mercaptoethanol per mole of protein raises the "least coagulable concentration" to greater than 0.9% and causes the coagula to be opaque, synerizing and typical of the clots formed from sulfhydryl-containing albumin. Even 0.1 mole of mercaptoethanol per mole of iodoacetamide-treated albumin will induce the foregoing effect on the gel characteristics.

Discussion

The foregoing viscosity and sedimentation data support the concept suggested by previous coagulation studies, namely, that during thermal denaturation of plasma albumin aggregation can take place by several mechanisms, and that free sulfhydryl groups promote lateral association of polypeptide chains. The sedimentation patterns indicate that all of the sulfhydryl-containing albumin becomes aggregated at a rapid rate upon heating. The fact that this process does not produce a very large increase in viscosity suggests that this aggregation is predominantly of a side-by-side nature (process B) which decreases rather than increases the asymmetry of the molecule. In view of the ability of a simple mercaptan to restore partially to iodoacetamide-treated albumin the aggregation and coagulation characteristics typical of sulfhydryl-containing albumin, it appears that process B involves a sulfhydryl-disulfide chain reaction similar to that described in the preceding paper[8] for the case of denaturation by urea.

The aggregation of sulfhydryl-free albumin at 100° takes place more slowly than that of sulfhydryl-containing albumin, but that portion which does aggregate forms a larger, or at least a more rapidly sedimenting, unit. This type of aggregation (process A) is accompanied by a large increase in viscosity in dilute solutions and an increased capacity for gel-formation in more concentrated solutions. That the reaction or reactions of process A possess a higher temperature coefficient than the other mechanisms of aggregation is indicated by the data in Fig. 2. Whereas the viscosity of a heated solution of sulfhydryl-containing albumin is influenced more by the protein concentration than by the temperature at which thermal denaturation is effected, the viscosity of silver-treated albumin depends more on the denaturation temperature than on the concentration. The chemical nature of process A as yet is obscure, but apparently it does not involve free amino groups.[3]

Apart from hypotheses, one definite conclusion is evident from the foregoing experimental results. The changes in viscosity and sedimentation characteristics which accompany the thermal denaturation of bovine plasma albumin differ significantly depending on whether or not the protein has been treated first with extremely small amounts of reagents believed to react selectively with sulfhydryl groups.

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[CONTRIBUTION FROM THE RESEARCH DIVISION, BRISTOL LABORATORIES, INC.]

α,α-Dimethylcholine: Esters and Carbamates[1]

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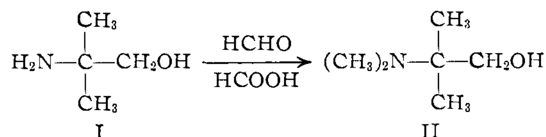
2-Dimethylamino-2-methyl-1-propanol, prepared easily by methylation of 2-amino-2-methyl-1-propanol, provides a convenient route to acetyl-α,α-dimethylcholine and analogs. A number of esters and carbamates of this type are reported herein. A cyclic α,α-disubstituted acetylcholine (III) also has been prepared.

In an attempt to obtain compounds which would act as acetylcholine but be less susceptible to hydrolysis by cholinesterase, we have synthesized a series of esters and carbamates of α,α-dimethylcholine. At the time that this investigation was started, no acyl α,α-dimethylcholines had been reported, although α,α-dimethylcholine itself had been described by Moyer and du Vigneaud[2] and by Alexander.[3] The commercial availability of 2-amino-2-methyl-1-propanol (I) appeared to offer a convenient starting material for the preparation of α,α-dimethylcholine and derivatives thereof.

Methylation of I with methyl iodide and potassium hydroxide gives α,α-dimethylcholine iodide directly[2]; methylation with formaldehyde and formic acid[4] gives 2-dimethylamino-2-methyl-1-propanol (II) in good yield.[5]



α,α-Dimethylcholine iodide also may be prepared by the Cannizzaro reaction on the methiodide of α-

(1) Presented before the Division of Medicinal Chemistry of the American Chemical Society, Kansas City, Mo., March, 1954.

(2) A. W. Moyer and V. du Vigneaud, *J. Biol. Chem.*, **143**, 373 (1942).

(3) E. R. Alexander, THIS JOURNAL, **70**, 2592 (1948).

(4) H. T. Clarke, H. B. Gillespie and S. Z. Weisshaus, THIS JOURNAL, **55**, 4571 (1933).

(5) V. Rosnati (*Gazz. chim. ital.*, **80**, 663 (1950); *C. A.*, **46**, 429 (1952)) has recently described this same preparation. He quaternized II with methyl iodide, then acetylated to obtain acetyl-α,α-dimethylcholine iodide (*cf.* compound V, Table I).