ion. In systems containing amylopectin or glycogen, iodide would compete with polysaccharide for the available "molecular" iodine (I_2 or I_4). Although amylopectin or glycogen do not bind significant amounts of iodine from iodide-free solutions, it is conceivable that the changes in configuration of the polysaccharide resulting from triiodide complex formation may condition the polysaccharide for adsorption of molecular iodine. Based on experience with the Schardinger dextrins,[12] it was our expectation that the absorption of I_2 by starch should be accompanied by a measurable shift of the I_2 spectrum. Manners[7] has reported that a number of highly branched glycogens had visible absorption maxima ranging from 420 to 470 mμ.

In the absence of HIO_3, the spectrum of the glycogen-I_2 solution (Fig. 4, curve B) was similar to that of the αI_2 solution (Fig. 3, curve D). Although there is a significant amount of glycogen I_3^- complex formed, the small shift in the I_2 spectrum (3 to 5 mμ) was not significant. This visible maximum when corrected for absorption by glycogen I_3^- (assuming $\epsilon_{I_3^-}$ equal to that of glycogen I_3^-) produced a peak identical to that produced by I_2 within experimental error (ϵ_{max} I_2 = 460 mμ) and suggested that I_2 was not bound by glycogen, at least not as a helical complex. When HIO_3 was added to a glycogen–I_2 solution (Fig. 4, curve A), no spectral shift of I_2 was encountered. Therefore, spectral shifts reported to accompany the addition of glycogen to I_2 solutions may most likely be attributed to the formation of glycogen I_3^- or polyiodide complexes.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA]

Reactivity of Sulfhydryl and Disulfide in Proteins. V. Reversal of Denaturation of Bovine Serum Albumin in 4 *M* Guanidine Hydrochloride or 8 *M* Urea and of Splitting of Disulfide Groups in 4 *M* GHCl

BY I. M. KOLTHOFF, ADA ANASTASI[1] AND B. H. TAN

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The protein obtained after denaturation of bovine serum albumin (BSA) in 4 *M* guanidine hydrochloride or 8 *M* urea at *p*H 5 at 25° and appropriate dilution has the same viscosity, specific optical rotation and reactive disulfide (zero) as native protein, provided the dilutions are made within one hour of standing of the denaturation mixtures. This reversibility is instantaneous upon dilution of the denaturation mixture in 4 *M* GHCl but is attained only after 15 minutes upon dilution of a denaturation mixture in urea. After longer times of standing exchange reactions between sulfhydryl and disulfide make the denaturation irreversible with regard to the above properties. In 4 *M* GHCl at *p*H 5 the reaction between BSA and sulfite

$$P\left\langle\left(\begin{matrix}S\\S\end{matrix}\right)_m + n\ SO_3^= \rightleftharpoons P\left\langle\left(\begin{matrix}S^-\\SSO_3^-\end{matrix}\right)_n\right\langle\left(\begin{matrix}S\\S\end{matrix}\right)_{m-n}$$

was allowed to proceed to the right and then from right to left by removal of sulfite (or bisulfite). The reversal was found quantitative, all disulfide groups were reformed and by extrapolation the intrinsic viscosity was found the same as that of untreated BSA in 4 *M* GHCl, provided the protein concentration was less than 0.25%. At higher concentrations crosslinking reactions occur during the reversal.

Part of the work described in this paper deals with the reversal to the native state of bovine serum albumin (BSA) after denaturation in 4 *M* guanidine hydrochloride (GHCl) or 8 *M* urea and dilution to such a concentration that the GHCl or urea has no measurable denaturing effect. In addition to viscosity and optical rotation the reactivity of disulfide groups with sulfite served as indicators for the degree of reversal from the denatured to the native state. In previous papers[2,3] it has been shown that after denaturation of BSA in 4 *M* GHCl or 8 *M* urea and appropriate dilution the sulfhydryl group is not oxidized by oxygen or ferricyanide while it is oxidized readily in the denatured state. Since sulfhydryl is not oxidized in the native state, the denaturation can be reverted as far as this property is concerned.

A more extensive study has been made of the reversal of the splitting of disulfide groups with sulfite in BSA at *p*H 5 in 4 *M* GHCl solution. For low molecular weight disulfides (RSSR) the reaction $RSSR + SO_3^= \rightleftarrows RSSO_3^- + RS^-$ is reversible.[4,5] The breaking of all 17 or part of the disulfide bonds in denatured BSA with sulfite is accompanied by a very marked structural change as evidenced by a large increase of the intrinsic viscosity.[6,7] If the reaction could be forced back

$$P\left\langle\left(\begin{matrix}S\\S\end{matrix}\right)_m + n\ SO_3^= \rightleftharpoons P\left\langle\left(\begin{matrix}S^-\\SSO_3^-\end{matrix}\right)_n\right\langle\left(\begin{matrix}S\\S\end{matrix}\right)_{m-n} \quad (1)$$

quantitatively from right to left, it is hardly to be expected that the S–S bonds can be reformed in the original position. In the present study disulfide bonds were broken at *p*H 5 and the reaction reversed quantitatively by removing sulfite (for convenience sulfite refers to all charge forms of sulfurous acid), using a procedure described in the experimental part. The intrinsic viscosity and the optical rotation of the reverted protein were com-

(1) On leave from S. A. Farmitalia, Milano, Italy.

(2) I. M. Kolthoff, Ada Anastasi, W. Stricks, B. H. Tan and G. S. Deshmukh, THIS JOURNAL, **79**, 5102 (1957).

(3) I. M. Kolthoff and Ada Anastasi, *ibid.*, **80**, 4248 (1958).

(4) W. Stricks and I. M. Kolthoff, *ibid.*, **73**, 4569 (1951).

(5) C. Cecil and J. R. McPhee, *Biochem. J.*, **59**, 234 (1955); **60**, 496 (1955); McPhee, *ibid.*, **64**, 22 (1956).

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pared with those of the original protein in 4 *M* GHCl.

Experimental

Materials used and experimental procedures were the same as described in previous papers.

Reversal of Denaturation in 4 *M* GHCl or 8 *M* Urea.—Mixtures which were 1% in BSA, 4 *M* in GHCl (or 8 *M* in urea), 0.05 to 0.2 *M* in acetate buffer of *p*H 5 were allowed to stand at 25°. After given periods of time a sample was taken and diluted in an acetate buffer of *p*H 5 to give a concentration of 0.5% in BSA and 2 *M* GHCl (or 4 *M* in urea), 0.25% in BSA and 1 *M* in GHCl (or 2 *M* in urea) and 0.1% in BSA and 0.4 *M* in GHCl (or 0.8 *M* in urea), respectively. In a previous paper[5] it was shown that GHCl in a concentration of 2 *M* or less and urea in a concentration of 4 *M* or less do not exert a measurable denaturing effect on BSA at *p*H 5. On the other hand, the denaturation seems to approach completeness in 4 *M* GHCl or 8 *M* urea. If the denaturation is reversible, the properties of the native albumin should be found after two times or more dilution of the denaturation mixture. The intrinsic viscosity was determined in the dilution which was 0.5% and the specific rotation $[\alpha]_D$ in the dilution which was 0.25% in albumin, while "reactive disulfide" was determined in dilutions which were 0.1% in albumin. This reactive disulfide was determined by making the dilution 0.05 *M* in sulfite at *p*H 5 and allowing it to stand at 25° for 30 minutes. The sulfhydryl formed was titrated at *p*H 2 as described in previous papers.[4,5] At *p*H 5 all of the 17 disulfide groups are reactive in 8 *M* urea while 14 are found reactive in 4 *M* GHCl. On the other hand, reactive disulfide (as defined above) was found to vary between 0 and 1 S–S in native BSA. Some denaturation experiments were carried out at *p*H 9 in a 0.05 *M* borax solution. As shown in Fig. 1 the reduced viscosity increases only very slightly with time of standing at *p*H 5 (curve 2), after 10 hr. of standing the value in 1% BSA had increased from 0.19 to 0.205 while at *p*H 9 (curve 6) it increased in the same time from 0.19 to 0.375. In the presence of 2 millimoles of mercuric chloride per mole of BSA the viscosity at *p*H 5 remained 0.19 even after a week of standing (curve 5, Fig. 1).

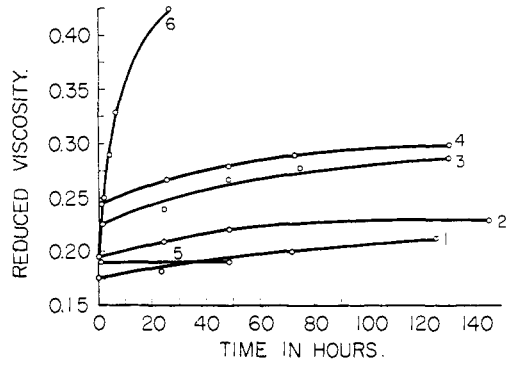


Fig. 1.—Viscosity of BSA denatured in 4 *M* GHCl at *p*H 5 as a function of time—concentration of BSA: 1, 0.5%; 2, 1%; 3, 1.5%; 4, 2%; 5, 1% BSA and 2 millimoles of mercury chloride per mole of BSA; 6, 1% BSA at *p*H 9.

The results after denaturation of 1% BSA in 4 *M* GHCl can be summarized as follows. When the denaturing medium was diluted twice within one hour of standing, the intrinsic viscosity was 0.06 while the same value was found for 0.5% native albumin in 2 *M* GHCl in the acetate buffer of *p*H 5. When the solution was diluted four times within one hour of standing, the viscosity was too small to be measured with the required accuracy. At this dilution the specific rotation was measured. Before dilution $[\alpha]_D$ was 100° and in the diluted mixture −60°, the same value as of native albumin in 1 *M* GHCl in the acetate buffer. Reactive disulfide after 10 times dilution as determined under conditions described above was 0.6 per mole of BSA, the same value (0 to 1) was found for native albumin in 0.4 *M* GHCl in the acetate buffer. When the dilutions were made after the denaturation mixture was allowed to stand for more than one hour, the intrinsic viscosity after dilution became slightly and increasingly greater than 0.06. After 2, 24, 48 and 96 hr. of standing of the denaturation mixture the intrinsic viscosities after dilution were 0.067, 0.075, 0.085 and 0.094, respectively. After these times of standing the following values for $[\alpha]_D$ were found: −61°, −63°, −64° and −66°, respectively. When diluted within 2 hr. of standing, reactive disulfide was the same as in native albumin, but this value increased to 1.5 after 24 hr., 2.7 after 48 and 2.9 after 96 hr. When the denaturing medium contained also 2 millimoles of mercuric chloride per mole of albumin, the same values of intrinsic viscosity, specific rotation and reactive disulfide were found as for native albumin in solutions of the same composition as the dilution, even when the denaturation mixture was allowed to stand for more than 24 hr. The changes observed after standing in the denaturation mixture and subsequent dilution were much greater at *p*H 9 than at *p*H 5. For example, when the denaturation mixture of *p*H 9 was diluted after standing for one hour, the intrinsic viscosity was 0.13 (0.06 for native albumin), $[\alpha]_D$ was −74.4 (−60° for native albumin) and reactive disulfide was 7. Presence of 2 moles of mercuric chloride per mole of BSA greatly reduced the differences between the values found after dilution and those in native albumin solutions of the same composition as the dilutions. For example, when diluted after 2 hr. the viscosity was 0.07, $[\alpha]_D$ −54° and reactive disulfide 2, while native albumin in the presence of 2 moles of mercuric chloride per mole of BSA had an $[\alpha]_D$ of −52°.

All the measurements in the diluted mixtures were made one minute after dilution. Longer standing after the dilutions were made did not affect the values reported above. This was not found when the denaturation was carried out in 8 *M* urea at *p*H 5. When diluted after 5 minutes of standing of the denaturation mixture, the intrinsic viscosity after 5 minutes of standing was 0.103, 0.080 after 30 minutes and 0.075 after 1 hr. or 6 hr. Similarly $[\alpha]_D$ was −68.8° when measured 5 minutes after dilution and −60.5° after 1 hr., after which time it did not change. On the other hand, reactive disulfide immediately after dilution did not change on further standing and was equal to 0.5, the same value as in native BSA. When the denaturation mixture was allowed to stand for 1 hr., 24, 48 and 72 hr. and then diluted, similar changes in viscosity and rotation were observed on standing of the dilutions as observed when the dilution was made after 5 minutes of denaturation. The final values observed after 1 hr. of standing of the dilutions were of the same order of magnitude as those measured after denaturation in 4 *M* GHCl and dilution within 1 hr.

Reversal of splitting of disulfide bonds with sulfite in 4 *M* GHCl at 24°: The BSA solution in 4 *M* GHCl in an acetate buffer of *p*H 5 was first equilibrated at a given sulfite concentration. In previous work[6,7] it has been shown that the equilibrium concentration of $P\begin{matrix}S^-\\ \diagdown\\ SSO_3^-\end{matrix}$ formed in reaction 1 can be determined by amperometric titration with mercuric chloride at *p*H 2 using a rotated mercury pool electrode (r.m.p.e.) as indicator electrode. At this *p*H the rates of the forward and backward reactions 1 are negligibly small.[6] After equilibration at *p*H 5 the air-free mixture at 25° was acidified with 4 *N* hydrochloric acid to a *p*H of 1.5.

Keeping the *p*H 1.5 and the temperature 25°, air-free nitrogen was bubbled through until all free sulfur dioxide had been removed. Sulfur dioxide gives a reduction wave at the dropping mercury electrode and also at the r.m.p.e. at *p*H 1.5, a limiting current at the r.m.p.e. being measured at −0.7 volt (*vs.* saturated calomel electrode). The disappearance of the sulfur dioxide wave served as an indicator for the complete removal of this compound from the solution. The same amount of sulfhydryl should be and was found as before removal of the sulfur dioxide. This titration was usually made and served as a test that no oxidation of sulfhydryl had occurred and that the equilibrium had not shifted. After removal of the excess sulfur dioxide the *p*H was readjusted to 5 with 4 *N* sodium hydroxide under oxygen-free conditions. A new equilibrium was allowed to establish, which after the first removal of excess sulfite is much more to the left (in reaction 1) than at the start of the experiment. When the reaction proceeds from right to left, some sulfite is formed again. This was removed again at *p*H 2, the *p*H readjusted to 5 and the process repeated until the reversal was quantitative and no sulfhydryl in excess of 0.68 mole per mole of BSA was found in amperometric titrations. After complete reversal the total number of disulfide groups was

found to be the same as in the original protein. The described procedure is not only very time-consuming but also very tedious. Much time was spent before the results could be duplicated; a technician not acquainted with the procedure should acquire such experience lest wrong results are obtained. The time involved in the complete reversal depends upon the initial concentration of sulfite in the original solution at *p*H 5. When this concentration was 0.05 *M*, 14 to 15 disulfide bonds were broken at *p*H 5. After adjustment of the *p*H to 2, from 12 to 15 hr. of nitrogen bubbling was required to remove the sulfur dioxide. This time was reduced to 4 hr. when the initial sulfite concentration was 0.005 *M* when 10 disulfide bonds were broken. Reëstablishment of the new equilibrium at *p*H 5 took 3 to 4 hr. With an initial sulfite concentration of 0.05 *M* using 1% BSA, the number of disulfide bonds broken after reëstablishment of equilibrium at *p*H 5 was 5 and equal to the number broken when a fresh 1% BSA solution in 4 *M* GHCl was equilibrated at the same total sulfite concentration at *p*H 5.[7] The removal of sulfur dioxide after the second readjustment to *p*H 1.5 took 2 hr. and establishment of the next equilibrium at *p*H 5 again 3 hr. Removal of sulfur dioxide after the third readjustment to *p*H 1.5 took 1 hr., the reversal was found complete after adjusting the *p*H to 5.

In order to reduce the time required in the above procedure for complete reversal, experiments have been made in which the excess of sulfite was removed with formaldehyde. To the equilibrated solutions of 1% BSA in 4 *M* GHCl and 0.005 *M* sulfite at *p*H 5 at 25° were added varying amounts of formaldehyde in excess over the total sulfite concentration and the *p*H was maintained at 5. After varying periods of standing at 25° the number of sulfhydryl groups was determined by amperometric titration and also the viscosity. It was found desirable to keep the initial concentration of formaldehyde between 0.006 and 0.01 *M*. When the formaldehyde concentration was greater than 0.01 *M*, difficulties were experienced in the amperometric titration, the reaction rate with mercuric chloride was much slower than in the absence of formaldehyde, and the slope of the excess reagent line became less steep. Moreover, the reversal reaction was considerably slower when the formaldehyde concentration was 0.05 *M* than when it was less than 0.01 *M*. The interfering effect of a large excess of formaldehyde is attributed to the reversible reaction

$$PSH + HCOH \rightleftharpoons PS{-}CH_2OH$$

Blank experiments were carried out in order to show that a concentration of 0.006 *M* formaldehyde neither affects the sulfhydryl titration of 1% BSA in 4 *M* GHCl nor the viscosity. When sulfite to a concentration of 0.005 *M* was added to the protein mixture which was 0.006 *M* in formaldehyde, no effect on the sulfhydryl titration or the viscosity was observed.

Procedure for Reversal by Volatilization of Sulfur Dioxide.—Forty-five ml. of a denaturation mixture in an acetate buffer of *p*H 5 which was 4 *M* in GHCl, about 0.5 to 1.5% in BSA, and of varying concentration in sulfite, was placed in a 125 ml. beaker. The container was covered with a tight-fitting rubber stopper provided with holes for the electrode, salt bridge and in- and outlet tubes for nitrogen. Adjustment of the *p*H was made by adding with constant stirring 4 *N* hydrochloric acid or 4 *N* sodium hydroxide. These high concentrations were used in order to keep volume changes small. During the addition of acid or base the *p*H was measured with a glass electrode. Air was carefully excluded during the entire operation which was carried out in a water bath of 25.0°. The reversal was brought about by following directions in the preceding section. After complete reversal the volume was brought to 50 ml. The initial concentration of BSA was such that after this adjustment of the volume the concentration was 0.5, 1 or 1.5%.

Reversal by formaldehyde was accomplished by simply adding to the sulfite-containing equilibrium mixture a specified amount of formaldehyde in slight excess over the amount of sulfite. The viscosity was measured at various periods of time after addition of the formaldehyde.

Results

By using either the volatilization or the formaldehyde procedure, the reversal could be made completely reversible, no $P\langle^{S^-}_{SSO_3^-}$ being found after the reversal (titration) while 17 disulfide groups were found in the protein after reversal. The optical rotation does not provide information regarding the reversal; in a 1% BSA solution in 4 *M* GHCl $[\alpha_D]$ is $-100°$ and this value remains the same on breaking all disulfide bonds and upon reforming all these bonds.

Viscosity appears to be the most sensitive indicator of structural differences between the denatured protein (in 4 *M* GHCl) before breaking disulfide bonds and after breaking these bonds and reforming them. Upon breaking all or more than 10 of the disulfide bonds, the reduced viscosity of 1% BSA in 4 *M* GHCl increases from 0.195 to 0.48. Much difficulty has been experienced in reproducing the viscosity data after the reversal. The reason appeared to be that the protein concentration was not kept exactly the same in these experiments. When this concentration was maintained constant, the viscosity data after reversion were reproducible. From curve 2 in Fig. 2 it is seen that the viscosity of the reverted protein increases very much when the BSA concentration becomes greater than 0.5%. Above 1.5% BSA this increase is very much greater than in denatured protein before splitting of disulfide groups (curve 3, Fig. 2) and also greater than after breaking of all

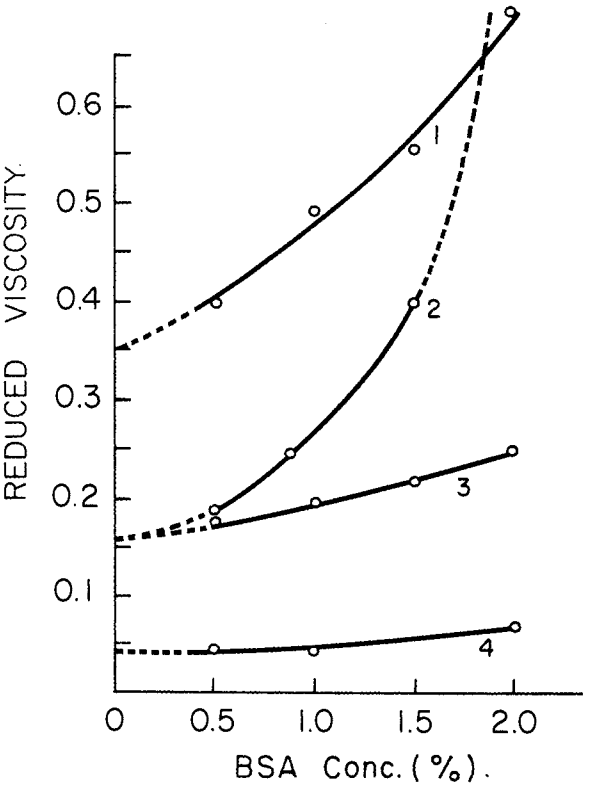


Fig. 2.—Viscosity as a function of BSA concentration in denaturation mixtures 4 *M* in GHCl at *p*H 5: 1, all disulfide broken by sulfite in the presence of excess of mercury chloride; 2, after breaking of disulfide and quantitative reversal by the volatilization or formaldehyde procedures; 3, no splitting of disulfide bonds; 4, native BSA.

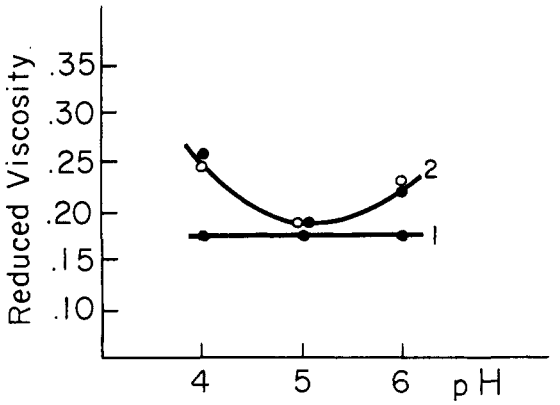
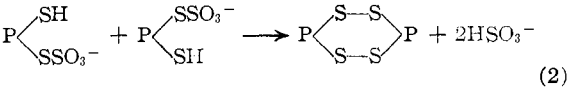


Fig. 3.—Viscosity as a function of *p*H in denaturation mixtures 0.5% in BSA, 4 *M* in GHCl: 1, untreated; 2, reverted after reacting with 0.005 *M* sulfite; ●, HCOH method; o, acidification method.

the disulfide bonds (curve 1, Fig. 2). Another interesting difference between the viscosity on curves 1 and 3 on the one hand and on curve 2 is that upon dilution of, for example, a 1.5% BSA solution, the values of the viscosity given by curves 1 and 3, respectively, were found; no such reversibility was found upon dilution of solutions the composition of which is given by curve 2; here dilution had little effect on the reduced viscosity. This difference in behavior is attributed to the formation of intermolecular disulfide bridges during the reversal process



(2)

and the polymer concentration does not change on dilution. The extent of this crosslinking reaction greatly increases with increasing BSA concentration and becomes almost negligible when this concentration becomes less than 0.5%. At this concentration the intrinsic viscosity of the reformed protein is only slightly greater (0.19) than before breaking of all the disulfide bonds (0.17). By extrapolation to lower concentrations curves 2 and 3 are found to coincide at 0.25% BSA, indicating that the reversal is complete as indicated by the viscosity.

Working with 0.5% BSA solutions, the same viscosity was found after reversal using either the volatilization or the formaldehyde procedure for the removal of sulfite (see Fig. 3). Results presented in Fig. 3 in which 10 disulfide bonds were broken 0.5% BSA at *p*H 5 but in which the reversal reaction was carried out at *p*H 4, 5 and 6, respectively, show that the crosslinking reaction 2 occurs to a minimum extent at *p*H 5 and is much more pronounced both at *p*H 4 and 6. Undoubtedly, the charge of the protein, the degree of ionization of the sulfhydryl groups and the charge form of the sulfite have an effect on the extent to which the crosslinking reaction 2 occurs, and no quantitative interpretation of the results in Fig. 3 can be given yet.

From the results in Fig. 2 it was concluded that reaction 2 is favored by a high concentration of

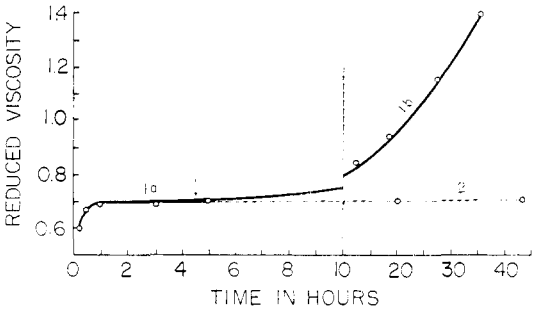


Fig. 4.—Viscosity as a function of time in 2% BSA, 4 *M* GHCl at *p*H 5 in the presence of 0.02 *M* sulfite: 1a, before addition of HCOH; 1b, after addition of 0.025 *M* HCOH (indicated by arrow); 2, no addition of HCOH.

protein. This is clearly illustrated by experiments in Fig. 4 in which the disulfide bonds were broken in a 2% BSA solution at *p*H 5 with 0.02 *M* sulfite. The reduced viscosity after 1 hr. became equal to 0.7 and remained constant thereafter. After formaldehyde in slight excess over sulfite was added (curve 1b, Fig. 4) the viscosity started to increase continuously, a value of 1.4 being measured 30 hr. after addition of the formaldehyde.

In the work reported so far no indication has been obtained of the occurrence of the crosslinking reaction 2 during the breaking of the disulfide bonds (forward reaction 1). As a matter of fact the following experiments show that reaction 2 did not occur under the specified conditions. Reaction mixtures 4 *M* in GHCl at *p*H 5 which were 0.5, 1 and 1.5% in BSA and 0.005 and 0.05 *M*, respectively, in sulfite were allowed to stand for 24 hr. at 25°. At both concentrations of sulfite the reduced viscosities were 0.40, 0.46 and 0.55 in 0.5, 1.0 and 1.5% BSA solutions, respectively. The mixtures which were 1 and 1.5% in BSA were then diluted to a concentration of 0.5% BSA keeping the GHCl concentration 4 *M*. The viscosity of both dilutions was 0.40, the same as found in the solution with an initial BSA concentration of 0.5%. If any crosslinking had occurred, the dilutions should have shown a higher viscosity than the solution with the initial BSA concentration of 0.5%; the dilution of the 1.5% BSA mixture should have a higher viscosity than that of the mixture originally 1% in BSA. The reason that no crosslinking has been observed during the breaking of the disulfide bonds is that the concentration of sulfite in all the experiments was large enough to break all the interhelix disulfide groups and to make the rate of backward reaction 1 and also of reaction 2 negligibly small. It would be expected that reaction 2 would be favored during the splitting (forward) reaction 1 by a small sulfite and a high protein concentration. Indeed, this was found to be true as shown by results in Fig. 5. The viscosity continued to increase with time even though the state of equilibrium in reaction 1 was attained fairly rapidly. In such an equilibrium mixture the conditions for the occurrence of reaction 2 are favorable.

At a *p*H of 9 only two disulfide groups are broken in a denaturation mixture 4 *M* in GHCl, 1% in BSA and 0.05 *M* in sulfite at 25°.[7] Thus conditions

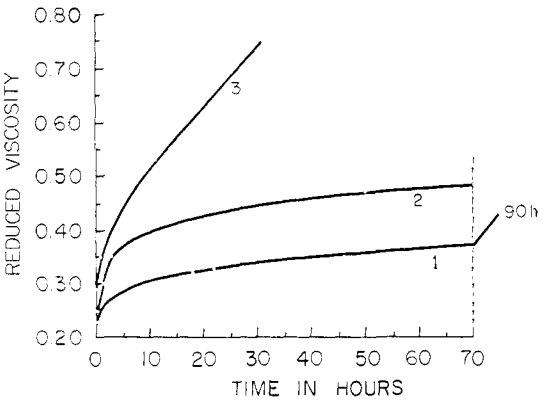


Fig. 5.—Viscosity as a function of time in BSA denaturation mixtures 4 *M* in GHCl at *p*H 5 with varying concentrations of sulfite: 1, 2% BSA, 0.0005 *M* sulfite; 2, 1% BSA, 0.001 *M* sulfite; 3, 2% BSA, 0.002 *M* sulfite.

for crosslinking according to reaction 2 should be very favorable at this *p*H, even at high sulfite concentrations. From Fig. 6 it appears that the crosslinking reaction at this *p*H is most pronounced when the sulfite concentration is 0.01 *M* (curve 3 in Fig. 6). After 2 hr. the viscosity was greater than in a mixture in which all the disulfide bonds were broken (curve 5) and it continued to increase with time of standing.

Discussion

The results reported in the section on reversal of the denaturation in 4 *M* GHCl or 8 *M* urea allow the conclusion that the BSA after proper dilution of a fresh denaturation mixture 1% in BSA and with a *p*H of 5 has the same properties as native albumin as far as intrinsic viscosity, optical rotation and reactive disulfide are concerned. This statement is true as long as no secondary changes by crosslinking occur. This crosslinking reaction in 4 *M* GHCl is very slow at *p*H 5 (see Fig. 1) and can be prevented by blocking the sulfhydryl group with mercuric chloride or methylmercuric chloride or similar agents. The crosslinking reaction is much faster at *p*H 9 than at *p*H 5; upon dilution even after only 10 minutes of standing of the denaturation mixture at *p*H 9 the properties of the native albumin are no longer found. The reversal of denatured to native albumin as far as the specified properties are concerned is also found after denaturation in 8 *M* urea before secondary changes occur. The reversal is practically immediate upon dilution of the 4 *M* GHCl but is relatively slow upon dilution of 8 *M* urea.

The ease of reversal of denatured BSA to the native state has been accounted for by Frensdorff[8] and Kauzmann[9] by assuming that the protein contains many interhelix disulfide bonds which are

(8) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5167 (1953).

(9) W. Kauzmann, "Denaturation of Proteins and Enzymes," Johns Hopkins Press, Baltimore, Md., 1954, p. 70.

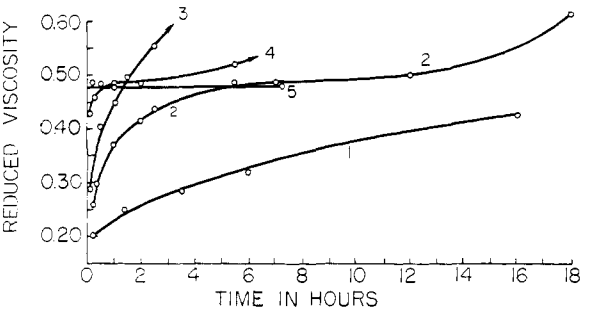


Fig. 6.—Viscosity as a function of time in denaturation mixtures 1% in BSA, 4 *M* in GHCl at *p*H 9: 1, no sulfite; 2, 0.005 *M* sulfite; 3, 0.01 *M* sulfite; 4, 0.1 *M* sulfite; 5, 0.1 *M* sulfite and 20 equivalents of mercury chloride.

responsible for the compact structure of the native albumin. These bonds are not broken upon denaturation and the compact structure of the native form is regained easily upon removal of the denaturing agent. Naturally, upon intermolecular crosslinking the denaturation becomes irreversible.

The most remarkable and intriguing result of the present study is that after breaking of the disulfide bonds in BSA with sulfite in 4 *M* GHCl and quantitative reversal of reaction 1 from right to left, the same intrinsic viscosity is found as before breaking of the disulfide bonds when the BSA concentration is no greater than 0.25%. This tends to indicate that all the disulfide bonds are reformed in the original position. If the view is accepted that denaturation in 4 *M* GHCl brings about partial uncoiling of the native BSA and that breaking of the disulfide bonds brings about further uncoiling by fission of interhelix chemical bonds, one would not expect *a priori* that the $\begin{matrix}S^- \\ \diagdown \\ \diagup \\ SSO_3^-\end{matrix}$ groups derived from a given disulfide bond would react together in the backward reaction 1 to reform the original disulfide bond. When interhelix disulfide bonds are broken and the protein molecule uncoils further (accompanied by a large increase in viscosity), the sulfhydryl and $-SSO_3^-$ group derived from the same disulfide group become separated over a relatively large distance, and it would seem improbable that the backward reaction would occur between two groups which are so far away from each other.

It is realized that no conclusive proof is given in this paper that after reversal of the breaking of the disulfide bonds the original structure is regained. It would be desirable to remove by dialysis the GHCl after reversal and to compare the properties (including immunological) of BSA obtained in this way with those of native BSA.

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