must occur at sites other than sulfhydryl and that Cu^{II} and Hg^{II} do not compete, in the early stages of the interaction process at least, for interaction with the same sites on the albumin molecule.

Acknowledgments.—The authors are grateful to Professor J. T. Edsall, Dr. F. R. N. Gurd and Dr. A. B. Biswas for helpful discussions.

POONA 8, INDIA

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

Reactivity of Sulfhydryl and Disulfide in Proteins. II. Reactive Disulfide as Related to Viscosity and Optical Rotation in Denatured Bovine Serum Albumin

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

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Simple methods are described for the amperometric titration with mercuric chloride of disulfide in native and denatured bovine serum albumin (BSA) in the presence of sulfite at a rotated mercury pool electrode. The mercury reacts with the reduced protein in a mole ratio of 1 $HgCl_2$ to 2SH. The total number of disulfide bonds per mole of BSA is 17. Procedures are given for the determination of the equilibrium concentration of disulfide groups in BSA in its reaction with sulfite at *p*H 6. No reactive disulfide is found in the native protein under the specified experimental conditions; in 4 *M* guanidine hydrochloride (GHCl) solution, the maximum value of the number of reacted disulfide groups is 11. The order of magnitude of the equilibrium constant of the reaction has been determined. Breaking these 11 bonds gives rise to an increase of the reduced viscosity from 0.19 to 0.48; rupture of the last 6 disulfide groups has no further effect on the viscosity. In 4 *M* GHCl $[\alpha]_D$ is $-101°$ and this value is not affected by reducing the disulfide groups with sulfite or 2-mercaptoethanol. This observation is in striking contrast to that in 0.2 *M* sodium decyl sulfate as denaturing agent, where rupture of the disulfide bonds gives rise to a large decrease of levorotation. The relation between experimental results and structural characteristics of native and denatured BSA is discussed.

In a recent paper, Markus and Karush[2] related the reactivity of disulfide groups to changes in the specific rotation and reduced viscosity in human serum albumin and bovine γ-globulin, both in the native and denatured state. They used sodium decyl sulfate as denaturing agent and β-mercaptoethylamine as reducing agent.

In the present paper we report on the reactivity of disulfide, with sulfite and with 2-mercaptoethanol (ESH), in bovine serum albumin (BSA), both native and denatured with guanidine hydrochloride (GHCl).

The amount of "reactive" disulfide determined by the sulfite method greatly depends upon experimental conditions, since the reaction with sulfite is an equilibrium reaction.

Denoting the protein disulfide by $\overset{P}{\diagdown}\!\!\left(\begin{matrix}S\\|\\S\end{matrix}\right)_n$, we can write

$$\overset{P}{\diagdown}\!\!\left(\begin{matrix}S\\|\\S\end{matrix}\right)_n + m\,SO_3^{=} \rightleftharpoons \overset{P}{\diagdown}\!\!\left(\begin{matrix}S^-\\|\\SSO^-_3\end{matrix}\right)_m\left(\begin{matrix}-S\\|\\-S\end{matrix}\right)_{n-m} \quad (1)$$

For low molecular weight disulfides (cystine) we had developed in this Laboratory a simple and rapid amperometric titration technique, using a rotated platinum electrode as indicator electrode and silver nitrate as reagent.[3] This titration must be carried out in an ammoniacal medium at *p*H 9, and gives the theoretical disulfide content for low molecular weight compounds. It was applied to the determination of disulfide bonds in BSA. In the native state no reactive disulfide was found, but in an ammoniacal buffer, 4 *M* in GHCl and 0.05 *M* in sodium sulfite, all disulfide groups could be titrated. Dr. Deshmukh, in this Laboratory, found a value of 17 to 18 disulfide bonds per mole, in the present paper an average value of 17 was found, while in an earlier paper[4] an average value of 18 was reported. There is some uncertainty in the literature about the exact disulfide content of BSA. A value of 18.6 is inferred from a statement by Haurowitz.[5] The low value of 14 disulfide and the value of 2 sulfhydryl groups per mole of BSA reported by Tristram[6] cannot be correct. The most accurate work on human serum albumin has been carried out by Brand,[7] who reported 18 disulfide groups.

Using the amperometric argentimetric titration method, an extensive study was made of the reactive disulfide in an ammoniacal denaturation medium at *p*H 9 at varying concentrations of BSA, GHCl and sulfite. The reactive disulfide was found to increase with the time elapsed between each addition of successive volumes of silver nitrate and the measurement of the current. The reason is that the equilibrium in eq. 1 is displaced to the right in the presence of the mercaptide binding agent. Therefore the results have no exact meaning and will not be reported.

The titration with silver nitrate of low molecular weight disulfides gives good results when carried out in an ammoniacal buffer at *p*H 9,[8] but cannot be used at lower *p*H's.

For amperometric titrations of the sulfhydryl formed in the reaction with sulfite (eq. 1) at *p*H lower than 9, we first used mercuric chloride as reagent and the dropping mercury electrode as indicator electrode. This method yielded good and

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

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(5) F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press, Inc., New York, N. Y., 1950, p. 32.

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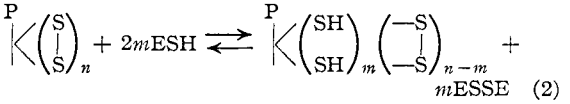
reproducible results but it is tedious and time-consuming and cannot be used for diluted protein solutions (less than 0.5%), because of the limited sensitivity of the dropping electrode.

We then developed a simple rotated mercury pool electrode (R.M.P.E.), which is described in the experimental section. This electrode is easy to use, exhibits no difficulties related to "conditioning" of the surface and gives reproducible results. Quite generally, we found it a good indicator electrode for sulfhydryl determinations instead of the rotated platinum electrode. Amperometric titrations with the R.M.P.E. were carried out at varying *p*H and varying protein and disulfide concentrations. In this paper, only the results obtained at *p*H 6 are reported.

As stated above, the equilibrium in eq. 1 is shifted to the right during the titration. In order to find the equilibrium concentration of sulfhydryl at a given protein and sulfite content and at a given *p*H, it was necessary to freeze the reaction. After several efforts, the principle applied by Cecil and McPhee[9] for low molecular weight compounds was tried. These authors changed the *p*H of their equilibrium mixture to a value of 2, at which *p*H the reaction in eq. 1 neither occurs to the right nor to the left, even after a considerable time. In the investigations here described, the equilibrium of reaction 1 was established in 4 *M* GHCl at *p*H 6 and the equilibrium concentration of sulfhydryl determined at *p*H 2. An extensive study of the *p*H effect on the equilibrium reaction will be reported in a subsequent paper.

Another method also was applied to study the reactivity of disulfide links. The reduction was effected at *p*H 6 with 2-mercapto-ethanol (ESH)



and the equilibrium conditions established by determining polarographically the low molecular weight disulfide formed (ESSE). At low ESH concentrations, the amount of unreacted low molecular weight mercaptan also can be measured. Because of uncertainties in the determination of diffusion current constants of ESH and ESSE in the highly viscous medium, the results by the ESH method are not as accurate as those by the sulfite method and only a few results are reported. These results substantiate the conclusions drawn from those obtained by the sulfite method.

In order to correlate "reactive disulfide" with structural properties, the reduced viscosity and the optical rotation of the protein in the denaturing medium were determined under the same conditions used in the determination of the reactivity. The relation between the number of disulfide bonds broken and the changes in the intrinsic viscosity and specific rotation is interpreted in terms of changes in the protein structure.

Experimental

Materials

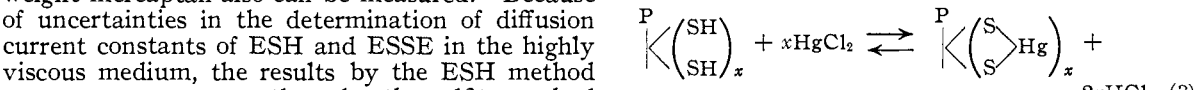
Crystalline bovine plasma albumin and guanidine hydrochloride and their stock solutions were the same as used or described in the first paper of this series.[10] Sodium sulfite was a C.P. reagent grade Merck product. Stock solutions, 1 *M* in sodium sulfite, were prepared in air-free water and analyzed for sulfite iodometrically. Pure, colorless 2-mercaptoethanol (ESH) was obtained by distillation under vacuum of a yellow technical product from Union Carbide and Carbon Co. The iodometric titer corresponded to a purity of 99%. Stock solutions 1 *M* and 0.2 *M* in ESH were prepared in air-free water and stored in a refrigerator. Pure bis-β-oxy-ethyl disulfide (ESSE) was obtained by oxidation of ESH with iodine and extraction with ether. The ether extract was dried over water-free magnesium sulfate and the ether evaporated. The residue of ESSE was dried in a vacuum desiccator for several days. All the other chemicals were C.P. reagent grade products.

Sulfite Methods

Amperometric Titrations.—The rotated mercury pool electrode is a glass cup about 5 mm. in height and 6 mm. in diameter and filled with mercury. The cup is fused to a glass tube with a U bend at the bottom. A sealed platinum wire makes contact with mercury in the glass tube. In order to get regular and reproducible results, the glass wall of the cup must be covered with a film of a water repellant, for which silicone was used. "Dri-Film SC 87" was supplied by General Electric, Waterford, N.Y. Electrical contact is established through a sealed-in platinum wire and mercury. The electrode is rotated at a speed of 75 r.p.m. Potentials were measured and are reported *versus* the saturated calomel electrode (SCE).

In Fig. 1 is given the current–potential curve at the R.M.P.E. of 5×10^{-5} *M* mercuric chloride in a solution 4 *M* in GHCl and 0.01 *M* in total sulfite at *p*H 2. The residual current is negligibly small until −0.25 volt, where reduction of sulfur dioxide occurs. The sensitivity of the electrode used was satisfactory for the purpose and corresponded to a current of 2.5 μa for a 10^{-5} *M* mercuric chloride solution in the indicated medium. The proportionality between diffusion current and concentration is indicated in the insert in Fig. 1. The line corresponds to the reagent line in a titration with mercuric chloride as reagent. The range of potentials to be used in titrations at *p*H 2 is small. From curves (1) and (2) in Fig. 1, it is evident that the titration must be carried out at a potential between −0.1 and −0.3 volt. At *p*H 2 we used a potential of −0.2 volt. The range becomes much greater with increasing *p*H; *e.g.*, at *p*H 7, the reduction wave of sulfite does not occur until −1.1 volt.

The end-point in the titration of sulfhydryl groups of denatured albumin at the mercury electrode corresponds to the ratio 1 $HgCl_2$ to 2SH. Denoting for simplicity the sulfhydryl in the reduced denatured protein by 



Procedures. 1. Equilibrium Concentration of Sulfhydryl and Rate of Reaction.—The concentration of BSA in the denaturation mixture was varied between 0.1 to 1%. A mixture 4 *M* in GHCl, 0.05 *M* in phosphate was adjusted to a *p*H of 6 to 6.5. The mixture was kept at 25 ± 0.1° and made air-free by passing through pure nitrogen, whereupon an air-free sodium sulfite solution with adjusted *p*H was added, to give a known total sulfite concentration in the final mixture and a *p*H of 6 to 6.5. After various times of standing, the *p*H was brought to 2. When the concentration of albumin in the denaturation mixture was 0.1%, an appropriate amount of 6 *M* hydrochloric acid was added with stirring to give a *p*H of 2. When the denaturation mixture contained 1% BSA, 5 ml. of the mixtures was withdrawn under air-free conditions and added to 45 ml. of air-free 4 *M* GHCl of *p*H 1.6. This gave a titration-mixture 0.1% in BSA and 4 *M* in GHCl at *p*H 2. In blank experiments the *p*H of the various solutions to be mixed as to give a denaturation mixture of *p*H 6 to 6.5 and a final titration mixture at *p*H 2 were predetermined. The *p*H was checked

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(10) I. M. Kolthoff, *et al.*, THIS JOURNAL, **79**, 5102 (1957).

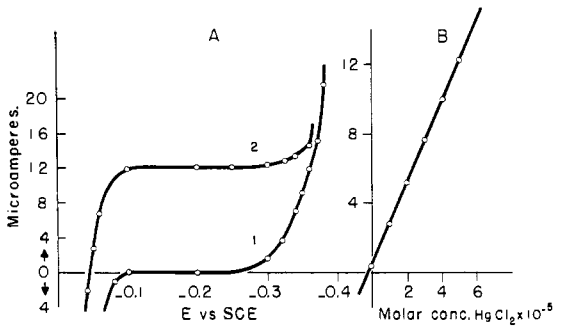

Fig. 1.—(A) Current-potential curve at R.M.P.E. of mercuric chloride in 4 *M* GHCl, *p*H 2, in the presence of 0.1 *M* sodium sulfite: (1) residual current; (2) 5×10^{-5} *M* $HgCl_2$. (B) Current-concentration line at R.M.P.E. at −0.2 volt of mercuric chloride in 4 *M* GHCl, *p*H 2 in the presence of 0.1 *M* sodium sulfite.

at the end of the titrations. Nitrogen was passed over the solution during the titration.

Notes. (a) **Blank Experiments.**—Mixtures of *p*H 2 being 4 *M* in GHCl, 0.1 to 1% in BSA and 0.01 to 0.1 *M* in total sulfite were prepared and titrated after 5 minutes to 2 hours standing at 25°. End-points were found corresponding to 0.68 to 0.74 mole of sulfhydryl per mole of BSA (which is the SH content of native unreduced BSA[10,11]) showing that at *p*H 2 the protein disulfide does not react with sulfite.

(b) In order to show that the reaction in eq. 1 is not reversed at *p*H 2, solutions equilibrated at *p*H 6 and adjusted to *p*H 2 were titrated immediately and after varying times of standing at *p*H 2. The same results were found when titrated immediately or after 24 hours of standing. Moreover, titrations were made at *p*H 3.5 instead of 2 and the same values were found. If there were any reversal, it would be different at *p*H 2 than at *p*H 3.5. Finally, the most convincing proof that no reversal of reaction 1 occurs at *p*H 2 was provided by experiments (to be reported in a subsequent paper) in which solutions equilibrated at *p*H 6 were titrated at *p*H 2 after complete removal of sulfur dioxide. In these experiments, the same value for sulfhydryl as in samples equilibrated at *p*H 6 and titrated at *p*H 3.5 or 2 without removing sulfur dioxide was found. Viscosity measurements at *p*H 2 substantiated the results of these experiments.

2. Titration of Disulfide at *p*H 6. (a) **Total Disulfide.**—The air-free mixture at *p*H 6 was made 0.05 *M* in total sulfite and 20 equivalents of standard mercuric chloride per mole of BSA were added. After 10 minutes of standing, the titration was continued with mercuric chloride under air-free conditions at a potential of −0.2 volt. The point of intersection of the reagent-line with the line corresponding to the small residual of the mixture before adding reagent gives the end-point. Unless stated otherwise, the above procedure was applied. It also was used in the determination of reactive disulfide in native albumin.

(b) **Direct Titration.**—The reaction mixture containing sulfite was titrated directly with mercuric chloride at *p*H 6, the current being measured at a specified time after each successive addition of reagent.

ESH Method

Current–voltage curves were measured manually[12] at 25 ± 0.1°. Oxygen was removed by passing pure nitrogen through the solution in the polarographic cell, an atmosphere of nitrogen was maintained over the solution during the measurements. One capillary was used with the following characteristics (open circuit): $m = 1.91$ mg. sec.$^{-1}$, $t = 3.98$ sec. (height of mercury = 55 cm.).

Polarography of ESH and ESSE.—Current-potential curves were determined in phosphate buffer (*p*H 7), the solutions being 4 *M* in GHCl. ESH gives well-defined anodic waves (see Fig. 2), the diffusion current at −0.15 volt being proportional to concentration. The diffusion current constant I_d was calculated to be 3.5 and the diffusion coefficient 8.8×10^{-6} cm.^{2} sec.$^{-1}$ (Ilkovic equation) all in 4 *M* GHCl at 25°. The half-wave potential was −0.21 volt at *p*H 6. ESSE also gives well-defined cathodic waves (Fig. 2). From the diffusion current constant of 6.2 a diffusion coefficient of 6.9×10^{-6} cm.^{2} sec.$^{-1}$ was calculated. The half-wave potential was −1.03 volt at *p*H 6, all in 4 *M* GHCl at 25°. The characteristics of the polarograms of ESH and ESSE were not affected by 1% BSA.

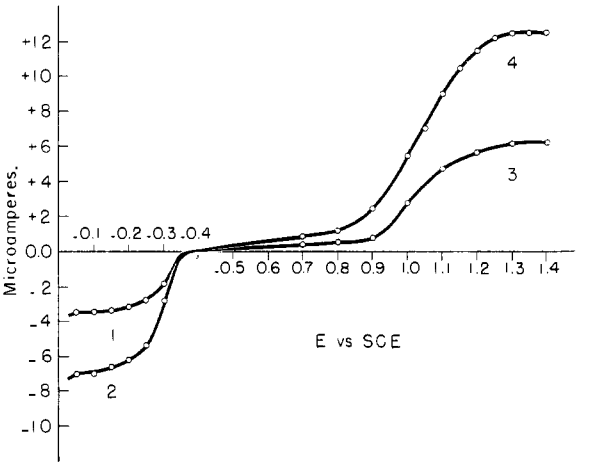

Fig. 2.—Polarograms of ESH and ESSE in 4 *M* GHCl, *p*H 6 (25°): (1) 1×10^{-3}; (2) 2×10^{-3} *M* ESH; (3) 1×10^{-3}; (4) 2×10^{-3} *M* in ESSE. Corrected for i_r.

Procedure.—The reaction of BSA disulfide with ESH was studied in a solution 1% in BSA, 4 *M* in GHCl and 0.1 *M* in ESH at *p*H 6. The number of disulfide bonds reduced was calculated by measuring the increase of the diffusion current of ESSE at −1.35 volt after different times of standing. It was found that even at low ESH concentrations, the number of moles of ESSE formed corresponds to one-half the number of moles of ESH which have disappeared. Thus the reaction proceeds according to the equation

$$P\begin{matrix}S\\|\\S\end{matrix} + 2ESH \rightleftharpoons P\begin{matrix}SH\\SH\end{matrix} + ESSE \qquad (4)$$

No indication of mixed disulfide formation (eq. 5) was obtained.

$$P\begin{matrix}S\\|\\S\end{matrix} + ESH \rightleftharpoons P\begin{matrix}SH\\SSE\end{matrix} \qquad (5)$$

Viscosity

Viscosity measurements were performed as previously described,[9] in solutions 1% in BSA, 4 *M* in GHCl at *p*H 6 and concentrations of (total) sulfite varying between 0.001 and 0.1 *M*, and also in solutions of the same composition in the presence of 20 equivalents of mercuric chloride. The viscosity was also determined in solutions 1% in BSA, 4 *M* in GHCl and 0.1 *M* in ESH at *p*H 6.

Optical Rotation

The optical rotation was measured with a Rudolph precision ultraviolet polarimeter using the sodium D line. The instrument was made available to us by Dr. J. A. Schellman, to whom we express our sincere appreciation. The temperature of the cuvette was controlled by circulating water through the trough of the polarimeter from a constant temperature bath at 25°. The albumin solutions were prepared in concentrations of 1 g./100 ml. and measured in a 1 dm. tube.

Results

Sulfite Method

(1) Total Disulfide. (a) **In Denatured BSA.**—Total reactive disulfide was determined according to the above procedure. In calculating the di-

(11) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947); Cold Spring Harbor *Symposia Quant. Biol.*, **14**, 79 (1949).

(12) J. J. Lingane and I. M. Kolthoff, THIS JOURNAL, **61**, 825 (1939).

sulfide content, the sulfhydryl content of the untreated BSA, equal to 0.68 per mole, was taken into account.

TABLE I

DETERMINATION OF TOTAL DISULFIDE IN DENATURATION MIXTURE AFTER ADDING 20 EQUIVALENTS OF MERCURIC CHLORIDE TO REACTION MIXTURE AT *p*H 6

Concn. of BSA, %	Concn. of total sulfite, *M*	Time of standing at *p*H 6, min.	*p*H of titr. mixture	No. of SS groups per mole of BSA
0.1	0.05	5	6	16.3–16.3
0.1	.05	10	6	17.0
1.0	.05	5	6	17.3
1.0	.05	5	4.8	17.5
1.0	.05	5	2.5	17.1
1.0	.05	5	1.9	16.5
1.0	.02	10	2.5	17.3
1.0	.01	45	1.7	16.3
1.0	.005	240	2.2	17.1
1.0	.003	120	1.8	(14.1)
1.0	.003	12 hours	2.0	(16.1)

The average value of the data reported in Table I (omitting the last two figures, when the reaction was not complete) corresponds to 17.0 disulfide groups per mole of BSA. The titration can be finished at any *p*H between 6 and 2.

Several experiments were carried out in which different amounts of mercuric chloride were added to the reaction mixture at *p*H 6 containing different (total) sulfite concentrations. After various periods of time varying from 5 minutes to 12 hours, the titrations were finished at *p*H 2. When the amount of mercuric chloride added corresponded to the equilibrium concentration of sulhydryl (see next section) the results at all sulfite concentrations were considerably greater than found upon direct titration, again indicating the displacement of the equilibrium in eq. 1.

Direct titrations (without adding first an excess of mercuric chloride) were made at *p*H of 6 at varying sulfite concentrations. When the readings were made within 30 seconds after each addition of reagent, the results found at sulfite concentrations of 0.002, 0.005 and 0.01 *M* were equal to the equilibrium concentrations given in the next section. At higher sulfite concentrations, higher values were obtained as a result of a displacement of the equilibrium in eq. 1. The direct titration method at *p*H 6 is not recommended for the determination of the equilibrium concentration. However, the results indicate that in eq. 1 the reaction from left to right is relatively slow at *p*H 6.

(b) **In Native BSA.**—An interesting observation is that no reduction wave of mercuric chloride at the R.M.P.E. was obtained in buffers containing native BSA and sulfite. As stated previously, a normal wave was observed in a denaturation mixture which was 4 *M* in GHCl. However, when GHCl was replaced by water or by 4 *M* potassium chloride, the native albumin completely eliminated the mercuric chloride wave. A difference in the structure of the absorbed film on mercury of native and denatured albumin is a subject of further study. Because of this masking of the reagent wave, it was necessary to carry out the titration in the presence of 4 *M* GHCl in the determination of reactive disulfide in native albumin. The reaction medium at *p*H 6 was free of GHCl, but after the indicated reaction time a measured volume of the reaction mixture was transferred to a GHCl solution of appropriate concentration and *p*H so that the final concentration of GHCl was 4 *M* and the *p*H was 2.

In a reaction mixture of *p*H 6 which was 1% in BSA, 0.1 *M* in sulfite and 0.05 *M* in phosphate, no reactive disulfide was found within a reaction period of 24 hours. When the reaction mixture also contained 20 equivalents of mercuric chloride, 17 disulfide groups had reacted within a reaction period of 5 hours. The same result was obtained when 25 equivalents of mercuric chloride was added and the reaction mixture allowed to stand for 24 hours. In the presence of mercuric chloride a pronounced turbidity was observed in all reaction mixtures. Upon standing, a gel settled in the bottom of the flask. This gel probably is composed of the mercury mercaptide of the protein.

(2) **Equilibrium Concentration and Rate of Reaction.**—Some results which were used for calculation of the equilibrium constant are summarized in Table II. When the (total) sulfite concentration is 0.02 to 0.1 *M*, equilibrium is established within 5 minutes at a BSA concentration of 1%. The rate decreases with decreasing sulfite concentration. In 0.003 *M* sulfite (molar ratio 1:1.25 with respect to total disulfide) equilibrium is established within one hour. Even at a very high concentration of sulfite (0.05 to 0.1 *M*), no more than 12 to 13 disulfide bonds can be reduced at a *p*H of 6:5.

TABLE II

CONCENTRATION EQUILIBRIUM CONSTANT IN EQUATION 7 AT 25° IN 4 *M* GHCl AT *p*H 6.5

BSA, %	P(S–S), Initial	$M \times 10^4$ Equilibrium	$\Sigma SO_3^=$, Initial	$M \times 10^3$ Equilibrium	–SH, $M \times 10^4$ Equilibrium	*K*
1.0	15.7	8.6	3	2.3	7.1	0.26
1.0	15.7	6.1	5	4.04	9.6	.37
1.0	15.7	3.1	10	8.75	12.6	.59
0.1	1.57	0.64	1	0.9	0.95	.15
0.1	1.57	0.31	3	2.75	1.26	.19

ESH Method

Considering that the polarographic method is considerably less accurate than the sulfite method, we only report one set of data obtained in a solution which was 1% in BSA, 4 *M* in GHCl and 0.1 *M* in ESH at *p*H 6. The equilibrium value was obtained after a 4-hour reaction period at 25° and 13 disulfide groups per mole of albumin reacted in this time. This number conceivably may be greater, it is only approximate because of the uncertainties involved in the method.

Viscosity

The solutions were 1% in BSA, 4 *M* in GHCl at *p*H 6 and contained varying concentrations of (total) sulfite. The results are presented in Fig. 3. The initial reduced viscosity in the absence of sulfite was 0.195 and increased to only 0.215 after 12 hours, indicating that almost no cross-linking occurs at this *p*H. A maximum value of 0.48 was attained when the concentration of sulfite was 0.02

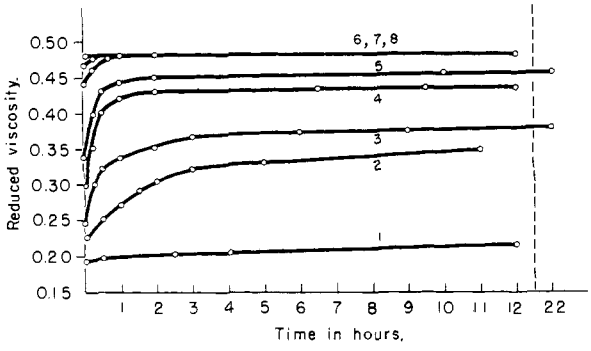

Fig. 3.—Viscosity as a function of time in 1% BSA, 4 *M* in GHCl, *p*H 6 in the presence of sulfite (no $HgCl_2$). Molarity of (total) sulfite: (1) 0; (2) 0.001; (3) 0.003; (4) 0.005; (5) 0.01; (6) 0.02; (7) 0.05; (8) 0.1.

to 0.1 *M*. This value remained constant with time, indicating again that practically no cross-linking takes place at *p*H 6, and corresponds to the cleavage of 11 disulfide bonds as determined by titration. At lower sulfite concentrations, when less disulfide bonds are ruptured, the final viscosities were lower. In Fig. 4 are presented the

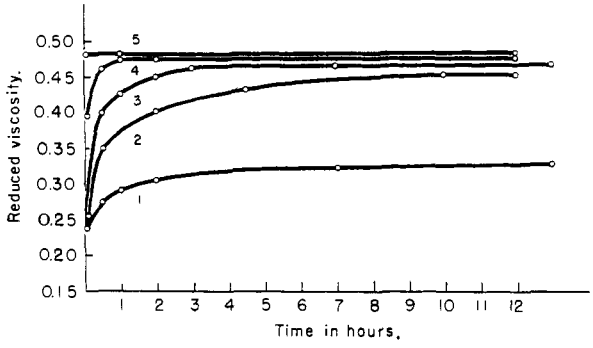

Fig. 4.—Viscosity as a function of time in 1% BSA, 4 *M* in GHCl, *p*H 6 in the presence of sulfite and 20 equivalents of $HgCl_2$. Molarity of (total) sulfite: (1) 0.001; (2) 0.003; (3) 0.005; (4) 0.01; (5) 0.1.

changes in viscosity as a function of time measured in the presence of sulfite and 20 equivalents of mercuric chloride. The final value of the reduced viscosity was almost the same at concentrations of sulfite varying between 0.003 and 0.1 *M* and practically equal to the maximum value in the absence of mercuric chloride. At a sulfite concentration of 0.001 *M*, the viscosity is lower, because the sulfite concentration is too small to break all the disulfide bonds (molar ratio of sulfite to disulfide was 0.4).

Optical Rotation

A solution 1% in BSA, 4 *M* in GHCl and 0.05 *M* in phosphate at *p*H 6 gave a value of $[\alpha]_D$ of $-101° \pm 1°$ (25°), while a value of $-98°$ was found when the mixture also contained 0.02 *M* sulfite. When, in addition, 20 equivalents of mercuric chloride also were present, $[\alpha]_D$ was $-89°$. Since the effect of the reduction of disulfide upon $[\alpha]_D$ differs so much from that reported by Markus and Karush[2] for human serum albumin, we also have made some experiments in which sodium decyl sulfate was used for the denaturation instead of 4 *M* GHCl. The following results were obtained. At *p*H 7, the specific rotation of native BSA was $-60°$, while a value of $-65 \pm 1°$ was found in 0.2 *M* sodium decyl sulfate. When the latter solution also was made 0.1 *M* in ESH, the specific rotation dropped to $-47°$.

Discussion

The results reported in the Experimental part permit a closer understanding of the structural characteristics of native and denatured BSA.

(1) Based upon the results obtained by the sulfite titration method (Table II) and viscosity measurements (Fig. 3), a curve has been plotted (Fig. 5) giving the relation between the reduced

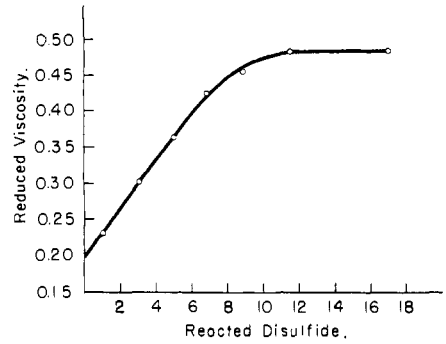

Fig. 5.—Relation between reacted disulfide per mole of BSA and reduced viscosity.

viscosity and the number of reacted disulfide groups per mole of BSA. Under specified conditions, the viscosity can be used as an indicator of the number of the reacted disulfide groups, *provided that no side reactions (cross-linking) occur*. At a *p*H considerably greater than 6, the viscosity is no longer a reliable indicator of the number of reacted disulfide groups because of the great effect of cross-linking upon the viscosity. For example, at a *p*H of 9, extremely large viscosities were obtained as a result of cross-linking.

In agreement with the above results, the maximum value of the reduced viscosity of 0.48 in a denaturation mixture 4 *M* in GHCl and 0.1 *M* in ESH, at *p*H 6, is the same as the maximum value found in sulfite solutions.

(2) One of the most interesting results of this study is the fact that the maximum value of the reduced viscosity is attained after 11 disulfide bonds have been broken. The increase in viscosity is practically linear until 8 disulfide bonds have been broken and becomes considerably less between 8 and 11 reacted disulfide groups. No further increase in the viscosity is observed when all the 17 disulfide bonds are broken. This can be safely concluded from the constancy of the viscosity in a denaturation mixture 0.1 *M* in sulfite as compared to that in a mixture which contains also 20 equivalents of mercuric chloride. In the absence of the mercuric chloride, 11, and in the presence of mercuric chloride, 17, disulfide groups have reacted. From some of the results in Table I, it is clear that the mercury mercaptide of the completely reduced protein (34 SH groups) is stable at *p*H 2.

The presence of mercuric chloride does not affect the viscosity of the reduced protein. There would be a possibility that one mercury could link together sulfhydryl groups from two different molecules. The viscosity data show conclusively that this is not the case.

The six disulfide groups which do not react with sulfite alone must be more stable to chemical attack than the first eleven. The great increase in viscosity which accompanies the breaking of the first 8 to 11 disulfide bonds tends to indicate that these bonds are present between helices of the native molecule; the other 6 may be distributed over the various helices.

(3) From the above it is evident that 11 disulfide bonds are much less stable than the remaining 6. We have tried to calculate the equilibrium constant corresponding to eq. 1. For simplicity, the reaction is written as

$$P(S\text{–}S) + SO_3^{=} + H^+ \rightleftharpoons P\begin{matrix}SH\\ SSO_3^-\end{matrix} \qquad (6)$$

The following assumptions were made: (1) the equilibrium constant of each of the 11 disulfide groups is the same: (2) the reaction is consecutive, *i.e.*, one disulfide group does not affect the reaction of another disulfide group. This assumption is naturally not correct, but it may be approximately so because of the relatively large distance of the various disulfide groups in the molecule. In the equilibrium reaction, the concentrations of both SH and the SSO_3^- groups must be considered. In the equation

$$K = \frac{[\text{–SH}][\text{–SSO}_3^-]}{[P(S\text{–}S)][\Sigma SO_3^{=}]} = \frac{[\text{–SH}]^2}{[P(S\text{–}S)][\Sigma SO_3^{=}]} \qquad (7)$$

(–SH) and ($-SSO_3^-$) represent the total molar concentration of these groups in the reacted protein molecule and $[\Sigma SO_3^{=}]$ the total molar concentration of sulfite at *p*H 6.5. Based upon the above assumption and considering that only 11 disulfide groups are reactive under the experimental conditions, we assume that in 1% unreacted BSA (1.43×10^{-4} *M*) the concentration of reactive disulfide is $11 \times (1.43 \times 10^{-4}) = 1.57 \times 10^{-3}$ *M*. The denaturation mixture was 4 *M* in GHCl and small fluctuations of ionic strength have no effect.

The results are given in Table II. The assumptions made at best are a rough approximation and the reported values of *K* may be considered to represent at least the order of magnitude. Further studies at varying *p*H are planned.

(4) Although native BSA gives zero reactive disulfide when titrated directly at *p*H 9 or any lower *p*H in the presence of much sulfite, all the disulfide groups can be made to react in the presence of an excess of mercuric chloride. This proves conclusively that *all the disulfide bonds in the native BSA are accessible to the reagent*, but all these groups are much more stable in the native than in the denatured state. This behavior is not surprising because in the native state the hydrogen bonds will have a strong stabilizing effect upon the disulfide bonds. On the other hand, in the denatured state all the hydrogen bonds are broken.

(5) No effect upon the specific optical rotation by the breaking of 11 disulfide bonds in the denaturation mixture with sulfite has been observed. On the other hand, Markus and Karush[2] reported with human serum albumin (HSA) at *p*H 7.4 a continuous decrease of the optical specific rotation from -72 to $-44°$, upon reduction of disulfide bonds in 0.1 *M* sodium decyl sulfate as denaturing agent. In the experimental part we have confirmed their observation. Markus and Karush attribute the change in optical rotation to a cleavage of the disulfide bonds which allows the molecule to form spontaneously additional intrahelical hydrogen bonds. We made some experiments with ESH as reducing agent under conditions (*p*H 7) that all disulfide groups were reduced. Again, this reduction did not affect the rotation in 4 *M* GHCl, but had a great effect in decyl sulfate.

The difference in results observed by Markus and Karush in detergent and by us in 4 *M* GHCl must be attributed to the different structure of albumin denatured with GHCl (or urea) and with detergent. The GHCl competes with the peptide hydrogen bonds that stabilize the secondary structure of protein, while the detergent does not affect the hydrogen bonds directly.[13] This effect on the hydrogen bonds undoubtedly accounts for the fact that the specific rotation in 4 *M* GHCl was found to be $-101°$, while in 0.2 *M* sodium decyl sulfate a value of $-65°$ was observed, as compared to $-60°$ with native BSA.

We intend to extend our studies on reactive disulfide in BSA as related to changes in physical properties to a variety of denaturing agents or combination of denaturing agents over a wide range of *p*H. Also, the reversibility of the reaction of sulfite with denatured albumin will be investigated.

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(13) G. Markus and F. Karush, THIS JOURNAL, **79**, 3264 (1957).