where the ligand L is in direct contact with the metal M, and an ion-pair M–S–L, where a molecule of solvent is interposed between the metal and the ligand. These authors argue that in the latter case one cannot expect any change of the visible spectrum but that the positions of the ultraviolet bands would be affected because of the "charge-transfer" nature of the ultraviolet spectra. It seems to the present authors, however, that if a "charge-transfer" process occurs between the metal ion and the ligand, we have no longer a simple ion-pair formation by purely coulombic interaction.

It also should be pointed out that in instances where ion-pair formations and the absorption spectra of resulting solutions were studied on nonmetallic systems,[7-9] in all cases the data indicated that ion-pair formation has very little influence on the absorption spectra. In this connection a recent series of papers by Kolthoff and Bruckenstein[26] on acid–base dissociations in glacial acetic acid should be mentioned also. These authors postulated that any dissociation in solvents of low dielectric constant occurs in two steps, $AX \rightarrow A^+X^- \rightarrow A^+ + X^-$, where the first step is ionization into ion-pairs and the second, the dissociation of the ion-pairs. They found that the second step does not influence the absorption spectra of solutions and, on this basis, were able to calculate dissociation constants of acids from spectrophotometric measurements which were in agreement with values obtained potentiometrically.

In conclusion, it seems that ion-pair dissociation or association does not seem to influence the absorption spectra of solutions to any significant degree, provided that the attraction is purely electrostatic. Where such changes have been observed, either an alternate explanation of a complex formation can be postulated, or, as Robinson and Stokes[27] suggest, we may have an interaction between the cation and an induced dipole in the anion. The argument may be rather semantic than factual and one may agree with the recent statement of Cohen[28] that the definition of an ion-pair is largely determined by the experimental method used in such studies.

(26) I. M. Kolthoff and S. Bruckenstein, THIS JOURNAL, **78**, 1 10, 2974 (1956); **79**, 1 (1957).

(27) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Butterworths Scientific Publications, London, 1955, p. 413.

(28) S. R. Cohen, *J. Phys. Chem.*, **61**, 1670 (1957).

IOWA CITY, IOWA

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

Reactivity of Sulfhydryl and Disulfide in Proteins. IV. Reaction between Disulfide and Sulfite in Bovine Serum Albumin Denatured in Guanidine Hydrochloride and Urea Solutions

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

RECEIVED OCTOBER 8, 1958

Four molar guanidine hydrochloride and 8 *M* urea have approximately the same denaturing properties toward bovine serum albumin as far as reduced viscosity, optical rotation and reactivity toward sulfite are concerned. The equilibrium concentration of reacted disulfide in 0.05 *M* and some other concentrations of sodium sulfite has been determined in these two denaturing media in the *p*H range between 2 and 9. A flat maximum is found between *p*H 4 and 6; at larger and smaller *p*H, the reacted disulfide decreases. The general appearance of the reacted disulfide *p*H curves is the same in both denaturing media. In 4 *M* GHCl 14 to 14.5 and in 8 *M* urea 16 to 16.5 react with 0.05 *M* sulfite at *p*H 5, the total number of disulfide groups in BSA being equal to 17. The value of 14.2 in 4 *M* GHCl does not change when the sulfite concentration is increased from 0.02 to 0.2 *M*. Experiments in solutions of denaturing agents over a wide range of concentrations revealed that the reactivity of disulfide groups does not increase when the GHCl concentration is made greater than 3 *N* or the urea concentration greater than 6 *N*. The reactivity of disulfide groups is already considerable at low concentrations of denaturant, where the extent of denaturation is small.

In a previous paper,[2] procedures have been given for the determination of the equilibrium concentrations of disulfide and sulfhydryl groups in bovine serum albumin (BSA) in its reaction with sulfite at *p*H 6 to 6.5. In the absence of mercuric chloride, no disulfide groups were found to react in the native state; but when denatured in 4 *M* guanidine hydrochloride (GHCl), the maximum value of the number of disulfide groups reacted was eleven. In the present paper, equilibrium values of reacted disulfide have been determined in BSA denatured in 4 *M* GHCl and 8 *M* urea in the *p*H range between 2 and 9. The reactivity of disulfide was also determined in more dilute and more concentrated solutions of the denaturing agents.

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

(2) I. M. Kolthoff, A. Anastasi and B. H. Tan, THIS JOURNAL, **80**, 3235 (1958).

Experimental

Materials.—The same materials were used as in the previous papers.[2] Urea was a Mallinckrodt product which was purified as previously described.[3]

Methods.—Air-free solutions of known composition were allowed to react with sodium sulfite at the specified *p*H. For convenience the sulfite concentration is expressed as molarity of total sulfite, irrespective of whether it was present mainly as sulfite, bisulfite or sulfurous acid. After given periods of reaction time, the *p*H was adjusted to 2 and the sulfhydryl groups titrated with mercuric chloride using the rotated mercury pool (RMPE) as indicator electrode.[2] When the concentration of the albumin was 1% in the denaturation mixture, it was diluted to about 0.1% with a solution of the denaturing agent of the same concentration as used in the reaction mixture. Quite generally the dilution was made by running the reaction mixture into an acid solution of the denaturing agent, keeping the *p*H during the dilution equal to 2. When the original BSA concentration was 0.1% in the reaction mixture, the dilution was kept at a

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

minimum during the adjustment of the *p*H to a value of 2. All experiments were carried out at 25° in the absence of oxygen. A concentration of (total) sulfite of 0.05 *M* at *p*H 2 did not interfere with the titration. In several experiments the concentration of total sulfite in the reaction mixture was 0.2 to 0.5 *M*. When the concentration of albumin in the reaction mixture was 1% and a ten fold dilution was made before titration, normal values for reacted disulfide were found when the reaction mixture had a *p*H between 4 and 7. However, low values were found after reaction of 0.1% BSA solutions with 0.2 to 0.5 *M* sulfite and subsequent adjustment of the *p*H. From several experiments it appeared that partially reduced BSA in the denaturation mixture at *p*H 2 reacts with sulfur dioxide when its concentration is 0.2 *M* or higher. Also, the larger excess of sulfur dioxide makes the end-point considerably less distinct. Even after removal of sulfur dioxide, keeping the *p*H at 2 during the removal, low values were found. No interference was observed when the sulfur dioxide concentration was 0.1 *M* or less.

Results

Experiments in 4 *M* GHCl and in 8 *M* Urea.—Equilibrium values of the number of disulfide bonds broken in the *p*H range between 2 and 9 at a total sulfite concentration of 0.05 are plotted in Fig. 1. Both in 4 *M* GHCl and 8 *M* urea, a flat

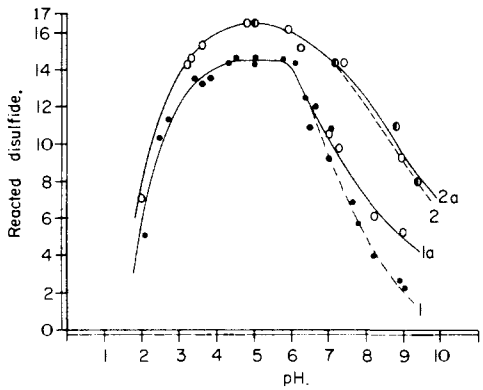

Fig. 1.—Reacted disulfide (moles per mole of BSA) at equilibrium as a function of *p*H. Solutions 0.05 *M* in sulfite: 1 and 1a in 4 *M* GHCl, 2 and 2a in 8 *M* urea; 1(●) and 2(○) in 1% BSA, 1a(○) and 2a(◐) in 0.1% BSA.

maximum is found between *p*H 4 and 6, the average maximum number of broken disulfide groups found in 20 determinations being between 14 and 14.5 in 4 *M* GHCl and between 16 and 16.5 in 8 *M* urea. In 8 *M* urea, the maximum at *p*H 5 is not as flat as it is in 4 *M* GHCl. In 8 *M* urea the same values are found in 1 and 0.1% BSA solutions (curves 2 and 2a in Fig. 1). Also, in 4 *M* GHCl, the BSA concentration does not affect the equilibrium value at *p*H smaller than 7. However, at *p*H between 7 and 9 in 4 *M* GHCl, higher values are found in 0.1% than in 1% BSA. Apparently cross-linking affects the reactivity of the disulfide groups with sulfite. Turbidity appeared a few minutes and gelation a few hours after preparation of a reaction mixture 1% in albumin, 4 *M* in GHCl and 0.05 *M* in sulfite. The rate of the cross-linking reaction increases with protein concentration and is considerably smaller in 0.1 than in 1% solution. The number of reacted disulfide groups at *p*H 9 was 2 in 1% albumin and 5 in 0.1%. Probably the true equilibrium value would be greater if no cross-linking occurred. At a *p*H of 9 the final value in 0.1% BSA was attained after a reaction time of 5 minutes and remained the same after a reaction time of 24 hr.

The following experiments show conclusively that the number of reacted disulfide groups decreases after cross-linking has taken place. A solution of 1% albumin in 4 *M* GHCl at *p*H 9 was allowed to stand for 12 hr. at 25° and then diluted ten times in a 4 *M* GHCl solution of such a *p*H as to yield a *p*H of 5 of the mixture after dilution. The solution was made 0.05 *M* in sulfite and the reacted disulfide determined by the standard procedure. After a reaction time of 1 hr. at *p*H 5, the titration value remained constant and the number of reacted disulfide groups was equal to 9.2, as compared to 14.2 in the absence of cross-linking. In order to decide whether all of the disulfide was accessible to sulfite in the cross-linked protein, an excess of mercuric chloride (20 moles per mole of BSA) was added to the reaction mixture at *p*H 5 before titrating at *p*H 2. After a reaction time of 1 hr., the number of reacted disulfide groups remained unchanged and was found equal to 16.7.

Since the total number of disulfide groups in BSA is 17, it is evident that all these groups are accessible for reaction in the cross-linked protein. Thus it may be concluded that cross-linking decreases the reactivity of disulfide groups with sulfite in the absence of excess of mercuric chloride. More excessive cross-linking at *p*H 9 was obtained by repeating the above denaturation experiments for 12 hr. at *p*H 9 in the presence of 0.001 *M* sulfite. The number of reacted disulfide groups found after dilution in 4 *M* GHCl at *p*H 5 was 7.6 as compared with 14.2 in the absence of cross-linking.

In 8 *M* urea solutions, the rate of cross-linking at *p*H 9 is much smaller than in 4 *M* GHCl (*vide infra*). Therefore the values in 8 *M* urea represent equilibrium values even at *p*H 9. No turbidity, even after 24 hr., was observed in this medium containing 1% BSA and 0.05 *M* sulfite.

The reduced viscosity as a function of the number of disulfide groups broken is plotted in Fig. 2. The data again show the similarity between 4

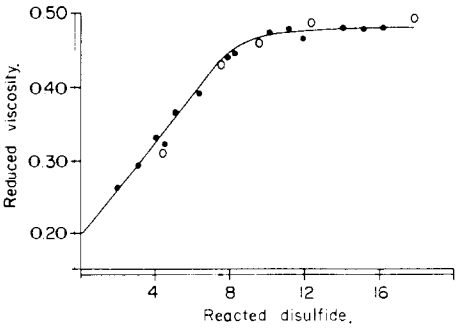

Fig. 2.—Relation between reacted disulfide (moles per mole of BSA) and reduced viscosity at *p*H 5: (1) ● in 4 *M* GHCl; (2) ○ in 8 *M* urea.

M GHCl and 8 *M* urea solutions as media for the reaction.

At a sulfite concentration of 0.5 *M* (1% BSA), the equilibrium value in 4 *M* GHCl is 14 to 14.5 in the range of *p*H from 4 to 7; in 8 *M* urea it is

16 to 16.5 at *p*H from 4 to 5 and decreases to a value of 15 to 15.5 at *p*H 7.

In Fig. 3 is plotted the equilibrium number of reacted disulfide groups at *p*H 5, as a function of the final (total) sulfite concentration. The same curves are obtained in 1 and 0.1% BSA. It is of interest to note that the curves in 4 *M* GHCl (1) and 8 *M* urea (2) almost overlap until 13 disulfide groups have reacted.

It is of particular interest that the maximum number of reacted disulfide groups in 8 *M* urea at *p*H 5 (16 to 16.5) is very close to the total number of disulfide groups in BSA (17). The lower maximum value of 14 to 14.5 in 4 *M* GHCl is not easily accounted for. As is evident from Fig. 3, the reacted disulfide in 4 *M* GHCl does not increase when the sulfite concentration is increased from 0.02 to 0.2 *M*. It is possible that the high electrolyte concentration has some effect on the sulfhydryl titration in BSA at *p*H 2. In order to test for such an effect, we carried out an experiment in 8 *M* urea and made the solution 4 *M* in lithium chloride after adjusting the *p*H to 2. Under such a condition a precipitate occurred during titration, indicating the effect of the salt. Other experiments were carried out in 8 *M* urea at *p*H 5 (1% BSA) at a sulfite concentration of 0.05 *M*, in the presence of 4 *M* lithium chloride. After ten times dilution in 8 *M* urea at *p*H 2, an average value of 14.8 reacted disulfide groups was found instead of 16.5. These results would tend to indicate that the equilibrium value of reacted disulfide is smaller in the presence of 4 *M* lithium chloride than in its absence. This effect was not found when the equilibrium value of reacted disulfide was less than 12 (experiments at sulfite concentrations of 0.01 or less). In this connection it is of interest to note that at equilibrium disulfide values less than 12, the reacted disulfide is the same in 4 *M* GHCl and 8 *M* urea.

All the titrations were reproducible within 3% when the number of reacted disulfide groups was 14 or less. However, the number of reacted disulfide groups fluctuated between 15.8 and 16.8 in some 15 experiments in 8 *M* urea with 0.05 *M* sulfite. When the experiments were carried out in 4 *M* lithium chloride, the values varied between 14 and 16.

It is of interest to mention that at a sulfite concentration of 0.05 *M*, both in 4 *M* GHCl and 8 *M* urea, equilibrium is established within 2 hr. at a *p*H of 5. At higher *p*H the equilibrium is established more rapidly; on the other hand, at *p*H smaller than 5 the rate decreases with decreasing *p*H. For example, in both denaturing media, equilibrium was established in 3 hr. at *p*H 4 and in 6 to 7 hours at *p*H 3. At *p*H 2 the rate of establishment of the equilibrium is very slow. The values reported in Fig. 1 refer to results obtained after a reaction time of 24 hr. at *p*H 2.

In separate experiments it was found that the rate of establishment of equilibrium is not affected by the presence of 4 *M* lithium chloride in the 8 *M* urea solution. All the rate curves obtained by plotting reacted disulfide *versus* time

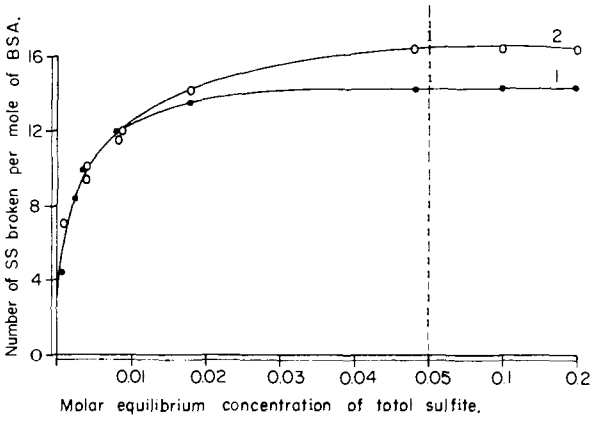

Fig. 3.—Relation between reacted disulfide (moles per mole of BSA) and equilibrium concentration of (total) sulfite in solutions at *p*H 5: (1) in 4 *M* GHCl 0.1 and 1% in BSA; (2) in 8 *M* urea, 0.1 to 1% in BSA.

were very similar to those giving the changes of reduced viscosity *versus* time.

Reactivity in Varying Concentrations of GHCl and Urea.—Reactive disulfide was determined in solutions of varying concentrations of GHCl and urea at a *p*H of 5 at 25° in the presence of 0.05 *M* sulfite. Viscosities were also determined. In the absence of sulfite the values of the viscosity in GHCl solutions increased slightly immediately after preparation of the solutions and continuously with standing. For example, in 4 *M* GHCl the reduced viscosity of 1% BSA solution at 25° was 0.195 within 15 minutes after preparation of the mixture. Values of 0.21 and 0.22, respectively, were found after 24 and 48 hr. of standing at 25°. This slight increase could be prevented by the addition of 2 moles of mercuric chloride per mole of albumin and thus must be due to a slow cross-linking reaction between sulfhydryl and disulfide even at a *p*H as low as 5. On the other hand, in 8 *M* urea solutions at *p*H 5 the viscosity increased slightly after preparation of the mixture until after 2 hr. it remained constant. For example, in 8 *M* urea (1% BSA) the reduced viscosity was 0.195 when measured within 15 minutes after preparation and 0.225 after 2 hr. Frensdorff, *et al.*,[4] found a similar appearance of time–viscosity curves in urea. Mercuric chloride does not affect the shape of the viscosity–time curve in urea, indicating that the initial slow increase cannot be attributed to an interaction between sulfhydryl and disulfide. The slow increase of the viscosity and the attainment of a constant value approximately 2 hr. after preparation of the mixture was observed at urea concentrations between 2 and 9 *M* at *p*H 5. In the first section of this paper evidence was given that the cross-linking reaction at *p*H 9 is much faster in 4 *M* GHCl than in 8 *M* urea. At *p*H 5 the cross-linking reaction is not noticeable in 8 *M* urea while it is observed in 4 *M* GHCl.

In Fig. 4 are plotted the initial reduced viscosity data of 1% BSA in GHCl solutions measured immediately after preparation and in urea solutions

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

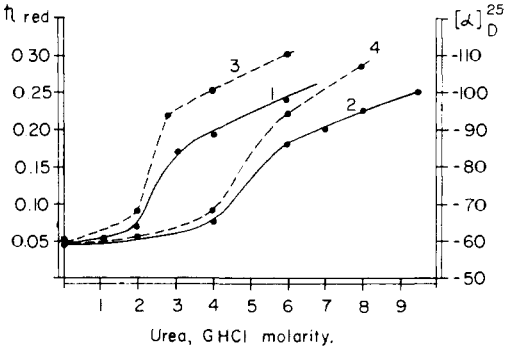

Fig. 4.—Reduced viscosity and optical rotation of 1% BSA solutions at *p*H 5 as a function of GHCl and urea concentrations: (1) reduced viscosity, GHCl; (2) reduced viscosity, urea; (3) optical rotation, GHCl; (4) optical rotation, urea.

after attainment of the constant value. It is seen that the effect of a given concentration of GHCl on the viscosity is equal to that of double the concentration of urea. Neurath, *et al.*,[5] found that the amount of irreversibly denatured horse serum albumin is the same in 2 *M* GHCl as in 6 *M* urea. In a study of the denaturation of ovalbumin, Schellman, *et al.*,[6] found that the order of the reaction is about the same for GHCl (13.5) and urea (15) but that GHCl has a threefold greater potency over urea as a denaturant. Curves 3 and 4 (dotted) in Fig. 4 give the changes of the specific optical rotation [α], as a function of the GHCl and urea concentrations. The curves are similar to those representing the viscosity changes and both properties provide a measure of the extent of denaturation.

Figure 5 gives a plot of the number of reacted disulfide groups with 0.05 *M* sulfite at *p*H 5 in 1% BSA solutions containing varying concentrations of GHCl or urea. Curves 1 and 2 represent equilibrium values. The equilibrium values at concentrations smaller than 3 *M* GHCl or 6 *M* urea could not be determined because precipitation of the reduced BSA occurs before equilibrium is established. The values in curves 3 (GHCl) and 4 (urea) were obtained after reaction times of 30 and 10 minutes, respectively. These values are considerably smaller than the equilibrium values. For example, in 3 *M* GHCl the number of reacted disulfide groups was 8.4 after 30 minutes, as compared to the equilibrium value of 14.4. Comparison of the curves in Figs. 4 and 5 reveals that, even though the extent of denaturation in 2 *M* GHCl or 4 *M* urea is very small, the reactivity of disulfide in these media is already quite pronounced.

Discussion

The largest number of disulfide bonds which can be broken in the optimum *p*H range is about 14 in 4 *M* GHCl and about 16 in 8 *M* urea, the total number of disulfide groups present being 17. In all instances all 17 disulfide bonds are broken by addition of a slight excess of mercuric chloride to the reaction mixture. Even in native albumin, all disulfide bonds can be broken in this manner.

(5) H. Neurath, G. R. Cooper and J. O. Erickson, *J. Biol. Chem.*, **142**, 249 (1942).

(6) J. Schellman, R. B. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5152 (1953).

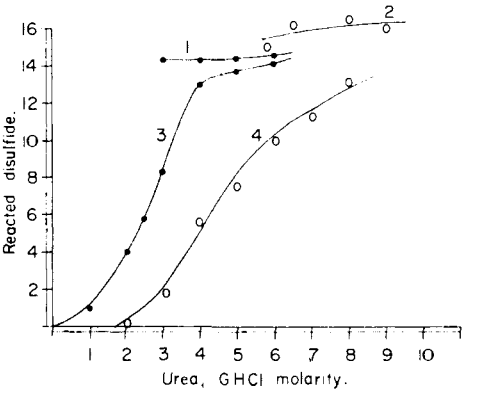

Fig. 5.—Reacted disulfide in 1% BSA solutions at *p*H 5 as a function of GHCl and urea concentrations: (1) and (3) GHCl; (2) and (4) urea: (1) and (2) equilibrium values; (3) 30 minutes reaction; (4) 10 minutes reaction.

Considering that in 4 *M* GHCl the maximum number of reacted disulfide (14) does not change when the sulfite concentration is increased from 0.02 to 0.2 *M*, it may be concluded that it is much easier to break the first 14 than the last 3 disulfide bonds (see Fig. 3). An equilibrium value of 14 is also obtained at *p*H 7 in 4 *M* GHCl when the sulfite concentration is raised to 0.5 *M*.

In a previous paper[2] an effort was made to calculate an equilibrium constant at *p*H 6.5 for the reaction

$$P\left(\begin{matrix}S\\|\\S\end{matrix}\right)_n + mSO_3^{=} \rightleftharpoons P\left(\begin{matrix}S^-\\SSO_3^-\end{matrix}\right)_m \left(\begin{matrix}-S\\|\\-S\end{matrix}\right)_{n-m} \quad (1)$$

It was assumed that at this *p*H only 11 of the disulfide groups are reactive, that they have the same reactivity and that the reactivity of an unreacted disulfide group is not affected by that of a reacted disulfide group. Thus in the expression for equilibrium conditions, *m* was taken equal to 1. At a given *p*H, the ratio of sulfhydryl to mercaptide ion is constant and the concentration of total sulfhydryl was expressed as [Σ-SH] in the equation for the equilibrium constant

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

In this equation, [Σ-SH] and [-SSO_3^-] denote the total molar concentrations of these groups in the protein molecule in the reaction mixture; [P-(S-S)], that of the unreacted disulfide; and [Σ-$SO_3^=$], the equilibrium concentration of total sulfite. The value of *K* was found to increase with the concentration of protein. From experiments of the type described in the experimental part, we have calculated values of *K* at a *p*H of 5. At this *p*H the maximum number of reactive disulfide groups is 14 in GHCl and very close to the total number of 17 in urea. Using equation 2, values of *K* were calculated from experimental results in 4 *M* GHCl, using a value of 14 for the maximum number of reactive disulfide groups. The results are given in Table I. Similar calculations for results in 8 *M* urea solutions are presented in Table II. In this medium the maximum number of disulfide groups is taken equal to 17. From the data in the last columns in Tables I and II it is seen

TABLE I

CONCENTRATION EQUILIBRIUM CONSTANT IN 4 *M* GHCl AT 25° AND *p*H 5 (REACTIVE DISULFIDE-14)

BSA concn. %	BSA concn. Molarity	[SS], $M \times 10^3$ Init.	[SS], $M \times 10^3$ Equil.	Sulfite, $M \times 10^3$ Init.	Sulfite, $M \times 10^3$ Equil.	[SH], $M \times 10^3$	$K' \times 10^{-2}$ (eq. 3)	K (eq. 2)
0.1	1.43×10^{-5}	0.2	0.175	0.4	0.375	0.025	4.4	0.0095
.1	1.43×10^{-5}	.2	.08	3	2.88	.12	5.2	.063
.1	1.43×10^{-5}	.2	.06	5	4.86	.14	4.8	.067
.1	1.43×10^{-5}	.2	.03	10	9.83	.17	5.7	.098
.5	0.715×10^{-4}	1.0	.4	3	2.4	0.6	6.2	.375
.5	$.715 \times 10^{-4}$	1.0	.3	5	4.3	.7	5.4	.38
.5	$.715 \times 10^{-4}$	1.0	.15	10	9.15	.85	6.2	.52
1.0	1.43×10^{-4}	2.0	0.8	3	1.8	1.2	8.5	1.00
1.0	1.43×10^{-4}	2.0	.6	5	3.6	1.4	6.5	0.91
1.0	1.43×10^{-4}	2.0	.3	10	8.3	1.7	6.8	1.16

TABLE II

CONCENTRATION EQUILIBRIUM CONSTANT IN 8 *M* UREA AT 25° AND *p*H 5 (REACTIVE DISULFIDE-17)

BSA concn. %	BSA concn. Molarity	SS, $M \times 10^3$ Init.	SS, $M \times 10^3$ Equil.	Sulfite, $M \times 10^3$ Init.	Sulfite, $M \times 10^3$ Equil.	SH, $M \times 10^3$	$K' \times 10^{-2}$ (eq. 3)	K (eq. 2)
0.1	1.43×10^{-5}	0.244	0.13	2	1.89	0.114	4.5	0.053
.1	1.43×10^{-5}	.244	.114	5	4.87	.13	2.4	.032
.1	1.43×10^{-5}	.244	.074	10	9.83	.17	3.2	.039
1	1.43×10^{-4}	2.44	1.44	2	1	1	7	0.7
1	1.43×10^{-4}	2.44	1.08	5	3.64	1.36	3.5	.48
1	1.43×10^{-4}	2.44	0.74	10	8.3	1.7	2.7	.46

that at a given protein concentration, the value of K is reasonably constant when from 8 to 12 disulfide bonds are broken; but that it increases strongly with the protein concentration. From the fact that the equilibrium is not affected by the protein concentration (Fig. 3) it is evident that equation 2 cannot be valid. A reasonably constant value for K' covering all the experiments in Tables I and II was found using the expression

$$K' = \frac{[\Sigma\text{-SH}]}{[\text{P(S-S)}][\Sigma\cdot SO_3^-]} \qquad (3)$$

The physical significance of the constancy of K' is not clear; from a practical viewpoint, the value of K' in the empirical expression 3 may serve to indicate the extent of the reaction between BSA and sulfite at a given *p*H. The expression for K is quite different for BSA than for a low molecular weight compound like cystine (RSSR), where two molecules are formed as a result of the splitting of the disulfide bond

$$\text{RSSR} + SO_3^- \rightleftarrows \text{RS}^- + \text{RSSO}_3^- \qquad (4)$$

As shown by Cecil and McPhee[7,8] and also in this Laboratory,[9] the mass action law written in a form similar to equation 2 holds for the cystine reaction with sulfite.

No definite conclusion can be drawn whether $SO_3^=$, HSO_3^- or both are the reacting species with denatured BSA. A maximum reactivity is found in a *p*H range close to the isoelectric point, where most of the sulfite is present in the form of HSO_3^-. The decrease in reactive disulfide in the *p*H range at the alkaline side of the isoelectric point might be attributed to an increase of the number of negatively charged groups near the disulfide groups and an increase in ionization of the sulfhydryl groups. The combined effect of these two factors appears to be greater than that of the increase of the sulfite ion concentration at the same total sodium sulfite concentration. Even though positively charged groups at the acid side of the isoelectric point may favor reaction with bisulfite or sulfite, the reactive disulfide is found to decrease when the *p*H becomes smaller than 4. Decrease of the concentration of the active species (HSO_3^- and /or $SO_3^=$) can only partly account for the decrease of the reactivity, because this effect is more or less compensated by decrease of dissociation of sulfhydryl groups. A detailed study of reaction 4 with several low molecular weight substances is planned in order to find a quantitative relation between *p*H and disulfide reactivity for various types of compounds. Also, in order to account for the validity of equation 3 for denatured BSA, studies are planned with model low molecular weight disulfide compounds which yield a sulfhydryl and $-SSO_3^-$ group in the same molecule upon breaking the disulfide bond.

For a discussion of the change of reactivity of disulfide with the concentrations of denaturing agents, it is desirable to make some statements concerning the extent of denaturation at various concentrations of GHCl or urea in the absence of sulfite. The extent of denaturation changes from almost zero to virtually completion at concentrations between 2 and 3 *M* GHCl or between 4 and 6 *M* urea (Fig. 4). At concentrations greater than 3 *M* GHCl or 6 *M* urea, the number of reactive disulfide groups does not increase with further increase of concentration of denaturant (Fig. 5). At low concentration of denaturant the reactivity of disulfide groups is greater than would be expected from the value of the reduced viscosity. For example, the reduced viscosity of native al-

(7) R. Cecil and J. R. McPhee, *Biochem. J.*, **58**, XIII (1954); **60**, (1955).

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bumin in 1% solution at *p*H 5 is increased from 0.045 to only 0.075 in 4 *M* urea, while the number of reacted disulfide groups increases from zero (reaction time of 24 hr.) to 5 in 4 *M* urea. This was after a reaction period of 10 minutes and the equilibrium value is undoubtedly considerably greater. It seems reasonable to conclude that unfolding or swelling, even to a slight extent as revealed by the slight increase of the viscosity, makes disulfide groups between helices much more reactive toward sulfite than they are in the native state.

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Lignin. X. Moment Relationship Derivation for the Distribution of Diffusion Coefficients in Polymers

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A derivation is given of an equation by which the distribution of diffusion coefficients can be evaluated in certain cases from concentration–position observations taken after a known time of diffusion of a polymer solute under "semi-infinite solid" geometry conditions.

Introduction

In the course of our studies of the distribution in molecular weights of lignin preparations,[2] it became desirable to be able to ascertain, at least approximately, the distribution of molecular weights or of diffusion coefficients within a separated polymer fraction. This can be done by repeated refractionations,[2b,3] but such procedures are tedious to carry out and they leave unresolved the question of what is the distribution in a particular fraction as finally obtained.

In certain cases the desired distribution can be estimated directly from diffusion data as previously recognized by Gralén[4] and by Daune and Freund.[5] In this Laboratory a solution-to-gel diffusion procedure[6] involving "semi-infinite solid" geometry[7] has been used to obtain estimates of mean diffusion coefficients. The data obtained in the course of carrying out this procedure, comprising measurements of solute concentrations at several distances from the boundary after a known time of diffusion, also can be used to obtain estimates of distribution of diffusion coefficients.

In Fig. 1A, the concentration–position data obtained from our diffusion experiments with vanillin and with a lignin sulfonate preparation are represented on a linear scale. In Fig. 1B, the same data are shown but with a "probability scale" for the ordinate, and it can be seen that a straight line is now obtained with the pure substance, vanillin. However, a curved line still is produced with the lignin sulfonate preparation and this curvature reflects the polydisperse nature of the preparation. The purpose of this paper is to set forth the derivation of an equation by which the statistical moments of the distribution of diffusion coefficients in polymers can be evaluated from data such as are illustrated in Fig. 1.

Moment Relationships

In general, the moment of integer order n, about a point b, for a continuous variable x, is defined[8–12] as

$$\alpha_n = \int_{-\infty}^{+\infty} (x - b)^n f(x)\, dx \qquad (1a)$$

where $f(x)$ satisfies the condition

$$\int_{-\infty}^{+\infty} f(x)\, dx = 1$$

For a discrete variable, x_k, the moment is

$$\alpha_n = \sum_k (x_k - b)^n f(x_k) \qquad (1b)$$

where $f(x_k)$ satisfies the condition

$$\sum_k f(x_k) = 1$$

When b is zero, equation 1 provides the "nth moment about the origin," μ'_n, and when b is the mean, there results the "nth moment about the mean," μ_n. A particular curve can thus yield any number of moments and in general a curve can be described in terms of an appropriate number of moments.[10]

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