times for carboxyhemoglobin at the two temperatures is 1.21 (Table II). However even the approximate agreement here is misleading since the ratio of relaxation times at other degrees of carbon monoxide saturation is never this large and frequently less than 1. At zero saturation, for example, the ratio is 0.95. Thus the temperature dependencies of the other molecular processes involved hide the effect of temperature on viscosity if indeed the phenomena under consideration are viscosity dependent.

As in the case of oxygen control, the dielectric parameters undergo changes only in the range of pressures at which hemoglobin adsorbs the added gas. At higher pressures, above saturation, the parameters remain constant. The changes with both gases are thus linked directly to the compound-forming reactions. As in the case of oxygen, it would be attractive to relate the minima and maxima to the various intermediate compounds of carbon monoxide with hemoglobin. In the first paper of this series[2] Takashima was able to predict the oxygenation isotherm with some accuracy from dielectric increment changes. However, reasons have been given to doubt the validity of this treatment[3] and they apply with equal strength to carbon monoxide uptake.

The mechanism whereby carbon monoxide controls the dielectric properties of hemoglobin is no better understood than that through which oxygen exerts control. The several possibilities have been discussed for oxygen and may be extended to the case of carbon monoxide.[3]

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

Reactivity of Sulfhydryl and Disulfide in Proteins. III. Oxidation with Ferricyanide of Sulfhydryl in Native and Denatured Bovine Serum Albumin

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Sulfhydryl in native bovine serum albumin (BSA) is not oxidized by ferricyanide (Feic), but it is after denaturation with guanidine hydrochloride (GHCl) or urea. Since the oxidation of BSA with Feic is unspecific for sulfhydryl, other groups being also oxidized, the oxidation of sulfhydryl was followed by allowing the denatured BSA to react with an excess of Feic and then determining the remaining sulfhydryl by amperometric mercurimetric titration. The rate of oxidation is faster at *p*H 9 than 7 and at the same *p*H faster in 4 *M* GHCl than in 8 *M* urea. In the presence of these denaturing agents the reaction is greatly accelerated by copper(II) in a concentration equimolar to that of protein. Additional evidence of disulfide(dimer) formation is derived from viscosity measurements. Upon fivefold dilution of a freshly prepared denaturation mixture of *p*H 7 or 9, the sulfhydryl is no longer oxidizable with ferricyanide; in this respect the denaturation is reversible.

Several authors[2-5] have described changes in reactivity of sulfhydryl and disulfide[6] groups upon denaturation of proteins. In a previous study[7] with bovine serum albumin (BSA) it was pointed out that the reactivity of sulfhydryl groups may vary with the kind of reagent used. Upon denaturation of BSA in 4 *M* guanidine hydrochloride (GHCl) solution the reactivity of sulfhydryl with mercaptide binding agents (silver nitrate, mercuric chloride) remains unchanged. These experiments have now been repeated in 8 *M* urea solution and the same results were obtained. For example, after 5 hr. of denaturation at *p*H 7 and 9 at 25°, 0.68 mole of sulfhydryl per mole of albumin was titrated. On the other hand, sulfhydryl in solutions of native BSA was found stable toward oxygen, while in 4 *M* GHCl it was oxidized to disulfide, the rate of reaction being greater at *p*H 9 than at *p*H 7. In the present paper are reported results on the oxidizability with ferricyanide (Feic) of sulfhydryl in BSA in the native state and in the denaturing media which were 4 *M* in GHCl or 8 *M* in urea.

In preliminary work it was shown that sulfhydryl in low molecular weight compounds, like cysteine and β-mercaptoethanol, can be titrated amperometrically at the rotated platinum electrode (RPM) with ferricyanide. At *p*H 7 to 9 the reaction is rapid and the end-point is detected with satisfactory accuracy and precision. Upon application of this method to denatured BSA solutions, results were obtained which depended upon the speed of addition of Feic. The reason for the variation of the results, which has been recognized by previous workers,[8-10] is that Feic is not specific for sulfhydryl and can oxidize other groups in the protein molecule during its reaction with sulfhydryl. Efforts to improve the situation by accelerating the reaction by addition of one mole of copper(II) per mole of albumin were unsuccessful because the copper also acted as an accelerator of the side reactions.

Instead of determining the amount of Feic consumed, the amount of sulfhydryl which had not reacted with Feic was titrated successfully by the amperometric mercurimetric technique.[7] The final procedure which gives accurate and reproducible

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results is described in the Experimental part. The rate of reaction of Feic with sulfhydryl in denatured albumin was found to be greatly dependent on *p*H, and in all experiments the medium was carefully buffered. This effect of *p*H on the rate of oxidation was not mentioned by Anson[11] in his work on the reactivity with Feic of sulfhydryl in egg albumin denatured with GHCl. Anson[12] observed that traces of copper greatly accelerate the oxidation with Feic of sulfhydryl in denatured egg albumin at *p*H 7. In our work with BSA traces of copper in the denaturation medium did not affect the rate of oxidation. The reason is that copper(II) and albumin form a stable complex in a molar ratio of one to one.[13] When one mole of copper was added per mole of denatured albumin a pronounced acceleration of the oxidation was observed, in 4 *M* GHCl and in 8 *M* urea. On the other hand no oxidation of sulfhydryl was found in native albumin, even in the presence of one mole of copper.

Experiments also were carried out in which the oxidation of sulfhydryl by Feic was tested after a five-fold dilution of the Feic-free denaturation mixture. The results indicate reversibility of the denaturation as far as the oxidizability of sulfhydryl is concerned.

Experimental

Materials.—Stock solutions of crystalline bovine plasma albumin and guanidine hydrochloride were prepared as previously described.[7] Urea was a Mallinckrodt reagent grade product which was purified by recrystallization from 70% ethanol. Stock solutions 10 *M* in urea had a *p*H of 6.9 as measured with the glass electrode. All other chemicals were C.P. reagent grade products.

Procedures.—The denaturing mixtures were prepared by diluting the 10 *M* urea or 6 *M* GHCl stock solutions with appropriate volumes of phosphate buffer of the proper *p*H so as to obtain final mixtures 4 *M* in GHCl or 8 *M* in urea and 0.05 *M* in phosphate at *p*H 7 or 9. To these solutions was added an amount of stock BSA solution to give a concentration of 1% in protein in the final denaturation mixture. The studies of reversibility were carried out by diluting the denaturation mixtures with buffers 0.05 *M* in phosphate at *p*H 7 or 9, to a concentration of 0.2% in BSA and 0.8 *M* in GHCl or 1.6 *M* in urea. All experiments were carried out at 25°.

Oxidation in the Denaturation Mixtures.—The air-free stock BSA solution was added to an air-free denaturation mixture containing an amount of Feic corresponding to twice the concentration of sulfhydryl in BSA (2 × 0.68 mole per mole of albumin). Some experiments also were carried out by adding copper(II) to the denaturation mixture in a concentration corresponding to one mole of Cu(II) per mole of BSA. After varying periods of standing at 25°, a 5-ml. sample of the mixture was added to 20 ml. of air-free buffer and titrated with mercuric chloride.

Oxidation after Dilution of the Denaturation Mixture.—The denaturation mixture was the same as above but did not contain Feic. After a given time of denaturation 5 ml. of the mixture was added to 20 ml. of air-free buffer. Two moles (referred to sulfhydryl) of Feic was added either immediately or after various times of standing. In some experiments 1 mole of Cu(II) per mole of BSA also was added. After a reaction time of 5 minutes at *p*H 9 and of 30 minutes at *p*H 7 the sulfhydryl was titrated with mercuric chloride.

Titration of SH.—The amperometric mercurimetric titration technique was the same as described previously.[7] A solution 1×10^{-3} *M* in $HgCl_2$ was used as titrating agent and a mercury coated R.P.E. was used as indicator electrode at an applied potential of −0.2 volt *vs.* SCE. A small residual current due to Feic in solution did not affect the accuracy of the titrations. An example of a titration line is given in Fig. 1.

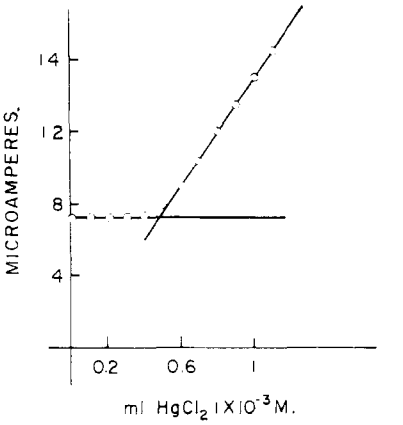

Fig. 1.—Titration of 0.2% BSA in 0.8 *M* GHCl at *p*H 9 in the presence of 2 moles Feic (per mole of SH), after previous denaturation in 1% BSA, 4 *M* GHCl at *p*H 7.

Results

Experiments on oxidation with two moles (per mole of SH) of Feic, of buffered solutions 1% in BSA at *p*H 7 or 9, either copper-free or containing one mole of Cu(II) per mole of BSA, showed that sulfhydryl is not oxidized when BSA is in the native state even after a long period of standing (24 hr. or more).

Oxidation in the Denaturation Medium.—From the results presented graphically in Fig. 2, it is clear that the rate of oxidation is considerably

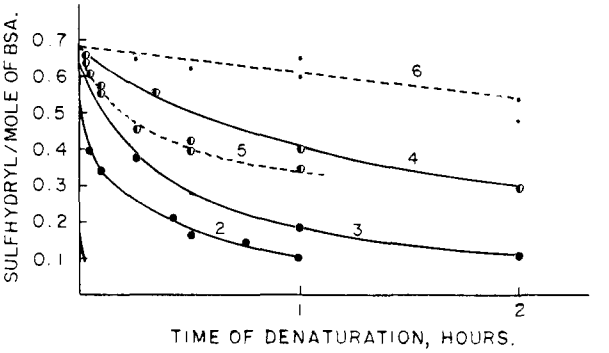

Fig. 2.—Unoxidized sulfhydryl: Curves 1, 2, 3, 4, denaturation mixtures 1% in BSA in the presence of 2 equivalents of Feic: (1) 4 *M* in GHCl at *p*H 9; (2) 8 *M* in urea at *p*H 9; (3) 4 *M* in GHCl at *p*H 7; (4) 8 *M* in urea at *p*H 7. Curves 5 and 6, denaturation mixtures 1% in BSA (Feic-free) after given time of denaturation diluted five times in buffers immediately treated with 2 equivalents of Feic; (5) denatured in 4 *M* GHCl or 8 *M* urea at *p*H 9, diluted in buffer of *p*H 9; (6) denatured in 4 *M* GHCl or 8 *M* urea at *p*H 7, diluted in buffer of *p*H 7.

greater at *p*H 9 than at *p*H 7 and greater in 4 *M* GHCl than in 8 *M* urea. For example, in 4 *M* GHCl at *p*H 9, all of the SH was oxidized within 5 minutes, while in 8 *M* urea at the same *p*H about 25% of the SH was still unoxidized after 30 minutes of reaction.

Some experiments in mixtures 8 *M* in urea and 2 *M* in KCl were run in order to see whether the

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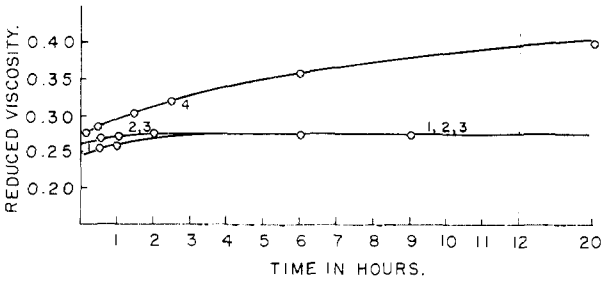

Fig. 3.—Reduced viscosity of 1% BSA in 4 *M* GHCl at various *p*H, effect of Feic: (1) *p*H 7, with 2 moles of Feic (per mole of SH); (2) *p*H 7 with 10 moles Feic; (3) with 3 and with 20 moles Feic at *p*H 9; (4) *p*H 10, with 10 moles Feic.

lower rate of oxidation in 8 *M* urea as compared to that of 4 *M* GHCl could be attributed to an ionic strength effect. The results given in Table I indicate that the oxidation in the presence of 2 *M* potassium chloride is even slower than in its absence.

TABLE I

UNOXIDIZED SH (MOLES PER MOLE OF ALBUMIN) IN A DENATURATION MIXTURE 8 *M* IN UREA AT *p*H 9, CONTAINING 2 MOLES OF FEIC PER MOLE OF SH, IN THE PRESENCE AND IN THE ABSENCE OF 2 *M* KCl

Time of oxidation →	5 min.	30 min.	1 hr.	2 hr.
With 2 *M* KCl	0.46	0.24	0.18	0.12
Without KCl	0.34	0.19	0.10	0

The experiments in Fig. 2 were repeated in the presence of copper(II) as a catalyst. The oxidation of sulfhydryl was found to be complete within one minute in the GHCl denaturation mixture at *p*H 7 and within 5 minutes in the urea denaturation mixtures both at *p*H 9 and 7.

Figure 3 shows the change with time in reduced viscosity of BSA in the denaturation mixtures (4 *M* GHCl) upon oxidation with ferricyanide. The initial reduced viscosity of BSA in the denaturation mixture at *p*H 7 or 9 was 0.195. It increased to 0.27 upon oxidation with an excess of ferricyanide and remained constant with time of standing. This same value was found with one mole of copper present. At *p*H 10 a slow increase with time of the reduced viscosity was observed in the presence of 10 equivalents of Feic.

Oxidation after Dilution of Denaturation Mixture.—Blank experiments were carried out with native albumin in 0.8 *M* GHCl or 1.6 *M* urea which showed that in these media no oxidation of sulfhydryl by Feic occurs at *p*H 9 or 7, even after a reaction period of 24 hr.

The average results of a great number of experiments on the oxidizability of sulfhydryl after dilution of the denaturation mixtures are represented by the dotted lines 5 and 6 in Fig. 2. The results were practically the same upon denaturation in GHCl and in urea.

When dilution was made after a brief period (up to 3 minutes) of denaturation at *p*H 9, none of the sulfhydryl was oxidizable. Upon longer time of denaturation a fraction of the sulfhydryl became oxidizable; this fraction increased with increasing time of denaturation. Similar results were observed after denaturation at *p*H 7, except that the increase of oxidizable sulfhydryl with time of denaturation was much smaller than at *p*H 9. For example, after 1 hr. of denaturation at *p*H 9, 50% of the sulfhydryl was found oxidizable, but less than 10% after denaturation for one hour at *p*H 7.

In one set of experiments in which denaturation was performed in a mixture 1% in BSA, 4 *M* in GHCl at *p*H 5.8 for a period of 4 hr. and oxidation carried out after 5 times dilution to final *p*H of 9, none of the sulfhydryl was found oxidizable either in the presence or absence of one mole of Cu(II) per mole of BSA.

Discussion

The results reported in the present paper supplement our previous work on oxygen oxidation.[6] The sulfhydryl group in native BSA is freely accessible to reagents which combine with it ($AgNO_3$,$HgCl_2$). However, the folded structure of the native molecule makes impossible an oxidation which involves the interaction of two protein molecules with the formation of a disulfide. This reaction readily occurs after unfolding the protein in the denaturation mixture. Even after a brief period of exposure of the albumin to the denaturing agent the oxidation of sulfhydryl with ferricyanide is quantitative. This result is in agreement with that of other authors who have reported that the primary denaturation reaction (unfolding, swelling) is a very rapid process. The above interpretation is substantiated by the results of viscosity measurements (Fig. 3). The reduced viscosity of the oxidized denatured albumin (dimer) was found equal to 0.27 as compared to 0.195 of the denatured protein monomer. No exchange reaction between sulfhydryl and disulfide can occur in the oxidized protein dimer; for this reason the viscosity remained constant with time, even at a *p*H of 9. However, at *p*H 10 (curve 4, Fig. 3) hydrolytic fission of disulfide groups probably accounts for the increase of the viscosity with time of the oxidized protein.

When the albumin containing denaturation mixture is diluted before secondary (cross-linking) reactions take place, none of the sulfhydryl is found oxidizable. In this respect the denaturation reaction appears to be perfectly reversible. Like the denaturation reaction proper, this reversal is found to be very rapid. Of course, after cross-linking reactions have occurred, it is impossible to obtain monomer protein upon dilution of the denaturation mixture. In such a cross-linked protein at least part of the sulfhydryl becomes oxidizable. The much faster cross-linking at *p*H 9 than at *p*H 7 accounts for the difference in shape of curves 5 and 6 in Fig. 2. At *p*H 5.5 no cross-linking was observed even after a denaturation time of four hours.

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