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

Reactivity of Sulfhydryl and Disulfide upon Denaturation of Proteins. I. Sulfhydryl in Native Serum Albumin and upon Denaturation in Guanidine Hydrochloride Solution

BY I. M. KOLTHOFF, ADA ANASTASI,[1] W. STRICKS, B. H. TAN AND G. S. DESHMUKH

RECEIVED MAY 11, 1957

The sulfhydryl content of native bovine serum albumin as determined by amperometric titration with silver nitrate or mercuric chloride was found to be 0.68 SH per mole of albumin (mol. wt. 69,000). Upon denaturation in 4 *M* guanidine hydrochloride (GHCl) at *p*H 7 or *p*H 9 in the absence of oxygen all the sulfhydryl was recovered upon titration with silver or mercury of the denaturation mixture or of this mixture after dilution to 0.8 *M* GHCl. After longer periods of denaturation in the absence of oxygen the "direct" amperometric titration method with silver and with mercury yielded less than 0.68 sulfhydryl per mole of albumin. However, by the "excess reagent method" all of the original sulfhydryl was found. The apparent decrease in sulfhydryl is attributed to a cross-linking reaction by which part of the sulfhydryl becomes less readily accessible to silver or mercury. The cross-linking reaction is much faster at *p*H 9 than at *p*H 7. Sulfhydryl in native albumin is not oxidized in 24 hours by oxygen in a buffer of *p*H 9 or 7, but it is oxidized in the denaturation mixture. Oxygen inhibits but does not prevent the cross-linking reaction in the denaturation mixture. Upon dilution of an oxygen-free denaturation mixture of *p*H 9 to 0.8 *M* GHCl and exposure to oxygen the oxidizable sulfhydryl increases with time of denaturation. This is explained by assuming that upon dilution of the denaturation mixture (*p*H 9), a rapid reversal occurs by which the sulfhydryl becomes non-oxidizable as in the native protein. Upon longer periods of denaturaton at *p*H 9 the exchange reactions make the reversal less and less complete. Traces of copper(II) greatly accelerate the oxidation of cysteine, but do not affect the rate of sulfhydryl oxidation in the denaturation mixture. Viscosity data substantiate the interpretation of the various results.

Recent investigations[2-5] indicate that the reactivity of sulfhydryl and disulfide groups plays an important role in the interpretation of changes in physical properties which occur during denaturation of proteins. The chemistry, biological importance, and the liberation and measurement of sulfhydryl and disulfide groups of denatured proteins has been treated in several reviews.[6-9] The literature about and the number of methods for the estimation of sulfhydryl and disulfide groups in proteins is vast,[9] and the great difficulties in the determination of these groups and the interpretation of the results have been realized by investigators working in this field.

During the last several years amperometric titration methods have been worked out in this Laboratory for the rapid, accurate and specific determination of sulfhydryl and disulfide groups in amino acids and proteins.[10] These methods have been applied successfully to a study of the sulfhydryl content of normal and pathological blood sera.[11] In the course of this work we found that these methods can be made use of in a study of the reactivity of sulfhydryl and disulfide groups in proteins after various types of denaturation. For a number of years we have been studying the change of reactivity of sulfhydryl and disulfide groups in crystalline bovine plasma albumin upon denaturation under various conditions in guanidine hydrochloride solutions. In the present paper we report on the reactivity of the sulfhydryl group in native and denatured albumin toward diammine silver ion ($Ag(NH_3)_2^+$) and mercuric chloride, $HgCl_2$, as determined by the amperometric titration technique and also on the oxidizability of albumin sulfhydryl by oxygen. A subsequent paper will deal with the reactivity of disulfide groups. The term reactivity as used in this paper is defined in the experimental part.

The progress of the work has been slow since many of the results could not be interpreted. It was necessary to vary the experimental conditions in a great variety of ways in order to avoid wrong conclusions which could have been drawn from the results obtained by a certain procedure. For example, we find in agreement with other workers 0.68 mole sulfhydryl per mole of native albumin. Upon denaturation in 4 *M* guanidine hydrochloride at *p*H 9 the amperometric titration of sulfhydryl with silver or mercuric chloride indicated that the sulfhydryl decreased rapidly and continuously. This was caused mainly by the presence of traces of oxygen. Although the sulfhydryl in the native albumin is perfectly stable toward oxygen, it becomes extremely sensitive to oxygen when the protein is denatured. However, even when oxygen was excluded completely, the amperometric titration after denaturation at *p*H 9 gave low sulfhydryl values. It appeared that part of the sulfhydryl reacted slowly with the reagent. In order to titrate all sulfhydryl it was necessary to add a slight excess of reagent and continue the titration after the solution was allowed to stand for a certain period of time.

The results obtained in the present paper refer to denaturation in 4 *M* guanidine hydrochloride. Further experiments with denaturing agents other than guanidine hydrochloride (*e.g.*, urea, detergents, heat) have been started.

Materials.—Crystalline bovine plasma albumin was an Armour product. During the course of these studies four

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

(2) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).

(3) (a) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5167 (1953); (b) V. D. Hospelhorn, B. Cross and E. V. Jensen, *ibid.*, **76**, 2827 (1954).

(4) H. Lindley, *ibid.*, **77**, 4927 (1955).

(5) G. Markus and F. Karush, *ibid.*, **79**, 134 (1957).

(6) E. S. G. Barron, *Advances in Enzymol.*, **11**, 201 (1951).

(7) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(8) M. L. Anson, *Advances in Protein Chem.*, **2**, 361 (1945).

(9) H. Neurath and K. Bailey, Editors, "The Proteins," Vol. IB, Academic Press, Inc., New York, N. Y., 1953.

(10) I. M. Kolthoff and W. Stricks, THIS JOURNAL, **72**, 1952 (1950); *Anal. Chem.*, **23**, 763 (1951); W. Stricks and I. M. Kolthoff, *ibid.*, **25**, 1050 (1953); W. Stricks, I. M. Kolthoff and N. Tanaka, *ibid.*, **26**, 299 (1954); I. M. Kolthoff, W. Stricks and L. Morren, *ibid.*, **26**, 366 (1954).

(11) W. Stricks, I. M. Kolthoff, D. G. Bush and P. K. Kuroda, THIS JOURNAL, **73**, 328 (1953); I. M. Kolthoff, W. Stricks, L. Morren and A. Heyndrickxs, *J. Natl. Cancer Soc.*, **14**, 1233 (1954).

batches have been used. All the samples had the same sulfhydryl- (0.68 SH per mole albumin, mol. wt. 69,000) and disulfide content (18 moles –S–S– per mole albumin) on a water-free basis. Sulfhydryl and disulfide were determined by methods described previously.[10] The different samples varied somewhat in their appearance. Some were extremely fluffy and developed a persistent froth upon dissolution in water, while others were rather dense powders which dissolved in water without appreciable formation of foam. Upon denaturation the behavior of the various batches was qualitatively the same but differed somewhat quantitatively. However, the differences were so small that the interpretation of the results is valid for all the samples.

The water content of the albumin was determined by heating a sample to constant weight at 110° and was found to vary from 3 to 6%. Stock solutions which were about 7 to 10% based on water-free albumin, were stored in a refrigerator at 4°. Solutions stored in this way for eight days did not show any change in properties. Albumin solutions used in this work were not older than eight days.

The guanidine hydrochloride (denoted in this paper as GHCl) was a reagent grade product from Fisher or Eastman. Considering the fact that the guanidine concentration in the denaturation experiments was of the order of 10,000 times greater than the molar albumin concentration, special efforts were made to purify the GHCl. The purification method suggested by Greenstein, *et al.*,[12] consists in recrystallization at room temperature from methanol in the presence of large quantities of ether. If ether is not carefully distilled over sodium before use, it is liable to leave traces of oxidizing substances in the guanidine hydrochloride. Evidence of this fact was found in several of our experiments. We therefore improved and simplified Greenstein's purification method by elimination of ether. The commercial GHCl was dissolved in hot methanol, the saturated hot solution was filtered through a hot water funnel and cooled in a freezing mixture at about −10° with vigorous stirring. The tiny GHCl crystals were filtered through fritted glass, washed with cold (−10°) methanol and dried at 50° *in vacuo* for about 5 hours. This method not only eliminated the use of large quantities of ether but also gave a product of better purity. When purified by this method the results became reproducible when different batches of GHCl were used. Guanidine hydrochloride of satisfactory purity also was prepared from commercial guanidine carbonate by Anson's method[13] and subsequent recrystallization of GHCl from hot methanol. Stock solutions of 6 and 8 *M* guanidine hydrochloride were prepared at room temperature. Using the glass electrode the apparent *p*H of GHCl solutions prepared with commercial as well as purified GHCl at concentrations of 1 to 8 *M* was found to vary between 5.8 to 5.4. All stock solutions of GHCl were adjusted with a Beckman Model G *p*H meter to *p*H 7 by the addition of an appropriate amount of ammonia or sodium hydroxide or by dissolving the GHCl in a phosphate or ammonia buffer of the proper *p*H. All other chemicals used were commercial C.P. reagent grade products. The *p*H of all denaturation mixtures was adjusted to the value at which the denaturation was studied.

Experimental

(1) **Denaturation.**—A guanidine hydrochloride–buffer mixture of the desired guanidine and buffer concentration was placed into a 100-ml. narrow-mouth bottle which could be sealed with a screw cap provided with a Buna-N rubber gasket. The solution was made air-free by bubbling nitrogen (Linde 99.9% pure) through a needle inserted in the rubber gasket while another needle permitted escape of gas. To the air-free solution was added by means of a syringe an appropriate volume of *air-free albumin stock solution*. The bottle was sealed and kept in a thermostat at the desired temperature, usually 25°. At various intervals (5 minutes to 30 hours) a sample of the denaturation mixture was withdrawn by means of a syringe which was filled with nitrogen and the sample titrated either without dilution or after dilution with a given volume of an air-free buffer. Buffer concentrations of 0.04 *M* phosphate (*p*H 7 to 9) or 0.01 *M* borax (*p*H 9.2), or 0.1 *M* ammonia plus 0.1 *M* ammonium nitrate (*p*H 9) in the denaturation mixture were found to be sufficient for a good *p*H control of the solutions. At a given *p*H the results were independent of the nature of the buffer. The *p*H remained unchanged over a denaturation time of at least 70 hours. Most of the experiments were carried out at the same GHCl concentration (4 *M*); the albumin concentration was 1% unless otherwise stated.

(2) **Oxidation of Sulfhydryl with Oxygen.**—Experiments on the oxidizability of sulfhydryl in denatured albumin were performed in two ways. Oxygen was passed from the beginning through the denaturation mixture for 15 minutes, the bottle was closed and an over-pressure of oxygen (*ca.* 1.5 atm. pressure) applied with the aid of a syringe. After a given time a portion of the oxidized mixture (usually 5 ml.) was added to 20 ml. of a buffer and after removal of air was titrated with silver nitrate or mercuric chloride. In another set of experiments denaturation was carried out in air-free mixtures and after various times of denaturation 5 ml. of the denaturation mixture was added to 20 or 40 ml. of a buffer of *p*H 9 (in some instances of *p*H 7) and the resulting solution treated with oxygen as described above. After two hours of exposure to oxygen no more sulfhydryl was further oxidized in 0.8 *M* GHCl. At the beginning the reaction was very rapid in 0.8 *M* GHCl; after 15 minutes of the order of 90% of oxidizable sulfhydryl had reacted. This was found with phosphate buffer of *p*H 7 as well as with ammonia buffers of *p*H 9. After completion of the oxidation the solution was made air-free and the unoxidizable sulfhydryl determined by the usual amperometric titration. After oxidation in an ammonia buffer (*p*H 9) less sulfhydryl was found than in phosphate or borate buffers of the same *p*H.

(3) **Amperometric Titrations.**—The apparatus and procedures used for the amperometric titrations were essentially the same as those described in previous papers.[10]

The platinum wire electrode was rotated by means of a synchronous motor at a speed of 600 or 900 r.p.m. The titration vessels were beakers of 125- to 250-ml. capacity. The beaker was kept filled with nitrogen and covered with a tight-fitting rubber stopper provided with holes for the platinum electrode, in- and outlet tube for nitrogen, salt bridge and buret. Reagent was administered from 5-, 2- and 1-ml. semi-microburets with 0.01-ml. divisions. Titrations were carried out at a potential of −0.2 to −0.3 volt *vs.* the saturated calomel electrode (S.C.E.). In most of the titrations the volume of the titration mixture was 20 to 30 ml. and the albumin concentration 0.2 to 1%. The air-free denaturation mixture was titrated either undiluted or after dilution (usually 5 times) with the proper buffer solution. The buffer either did not contain GHCl so that the concentration of the denaturing agent was decreased in proportion to the dilution before titration, or the buffer contained the same concentration of GHCl as the denaturation mixture. Oxygen was removed from the solutions with a stream of pure nitrogen which was bubbled through the solution during the entire titration.

Titrations were carried out with 10^{-3} to 10^{-2} *M* silver nitrate and mercuric chloride as titrating agents.

Most of the argentimetric titrations were performed in ammoniacal titration mixtures (0.1 *M* in NH_3 and NH_4NO_3). Non-ammoniacal solutions (phosphate or borate buffers) gave poorly defined end-points in argentimetric titrations. Appropriate blank experiments with ammoniacal solutions indicated that guanidine hydrochloride at concentrations of 0.8 to 1 *M* and potassium chloride up to concentrations of 4 *M* had no effect on the titration results with native albumin.

Mercurimetric titrations gave well-defined end-points in ammoniacal medium (0.1 *M* NH_3, 0.1 *M* NH_4NO_3, 0.8 to 4 *M* GHCl *p*H 9) as well as in phosphate (0.06 *M* Na_2HPO_4, 0.04 *M* NaH_2PO_4, 0.8 to 4 *M* GHCl, *p*H 7) buffers.

Figure 1 illustrates titration lines obtained under various conditions with native and denatured albumin solutions. Generally mercurimetric titrations give excess reagent lines with steeper slopes and hence better defined end-points than argentimetric titrations. Titration lines obtained with denatured albumin exhibit a flatter slope than those obtained with native albumin in the same medium (compare for instance lines 1 with 3 and 2 with 4 for both argentimetric and mercurimetric titrations). Apparently denatured albumin suppresses the diffusion current of the excess reagent ($Ag(NH_3)_2^+$ or $HgCl_4^-$) to a greater extent than the native albumin does under the same conditions, the effect being more pronounced with silver ammine than with mercuric chloride.

(12) J. P. Greenstein and W. V. Jeurette, *J. Biol. Chem.*, **142**, 176 (1942).

(13) M. L. Anson, *J. Gen. Physiol.*, **24**, 399 (1941).

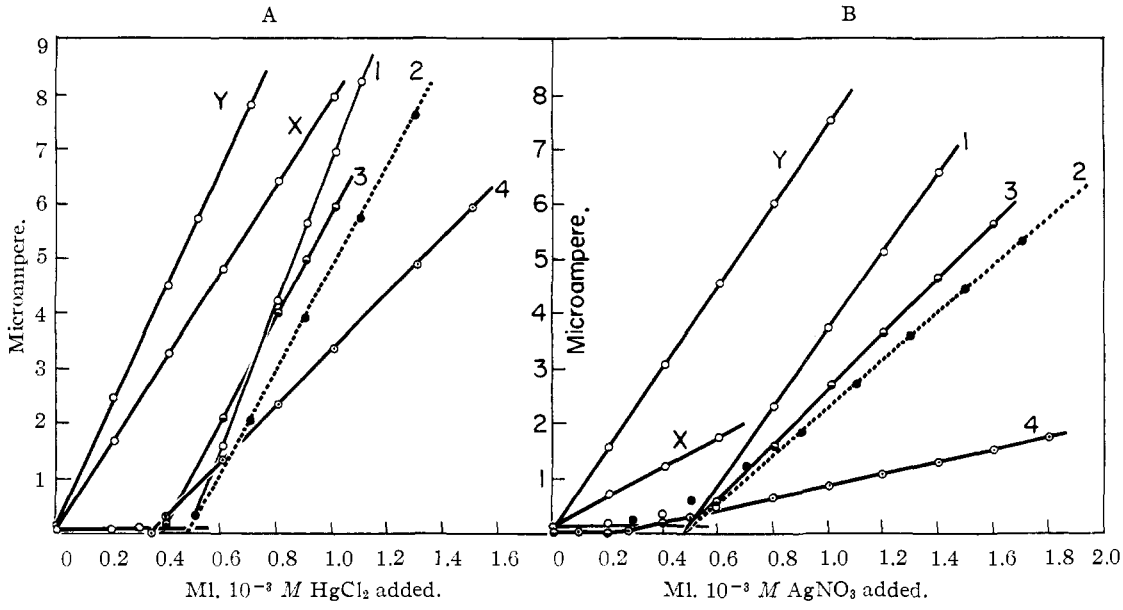


Fig. 1.—(A) mercurimetric; (B) argentimetric titrations of 5 ml. of 1% native and denatured albumin, added to 20 ml. of ammonia buffer (0.1 M NH_3, 0.1 M NH_4NO_3, pH 9.2) at various GHCl or KCl concentrations: 1, native albumin, titrated in 0.8 M GHCl; 2, native albumin, titrated in 4 M KCl; 3, albumin (denatured in 4 M GHCl, 0.04 M phosphate buffer, pH 7, 1% albumin, 2 hours denaturation time), titrated in 0.8 M GHCl; 4, same as 3, titrated in 4 M GHCl. Blanks: Y, titrating agent added to 25 ml. of 0.8 M GHCl, 0.1 M NH_3, 0.1 M NH_4NO_3; X, titrating agent added to 25 ml. of 4 M GHCl, 0.1 M NH_3, 0.1 M NH_4NO_3.

Comparison of lines 1 with 2 and 3 with 4 in Fig. 1A and B reveals that a large increase in the electrolyte concentration (from 0.8 to 4.0 M) of the titration mixture causes a depression of the diffusion current of the excess of reagents, the effect being stronger again in argentimetric than in mercurimetric titrations. A similar difference between the mercury and silver lines is also seen in the blank experiments (Fig. 1, X and Y) which were performed in the absence of albumin.

The end-point of a titration corresponds to the volume of the titrating agent at the intersection of the excess reagent line with the line presenting the residual current as measured in the air-free solution before addition of the titrating agent. The residual current varied from zero to 0.1 μa.

A series of experiments was conducted with denaturation mixtures in the presence of various excesses (over sulfhydryl) of silver nitrate or mercuric chloride initially added to the albumin. At the end of the denaturation period there was already an excess of silver or mercury in the solution. In order to find the amount of reagent bound to sulfhydryl, the current was measured upon successive additions of reagent. The current was plotted against the total amount of reagent added (including the amount added initially) and straight lines were obtained. The line was extended to zero current and its intersection with the zero current line was taken as the end-point of the titration.

Another procedure which has been applied quite generally was to add after a given time of denaturation approximately 50% excess of reagent to the titration mixture, either in 4 or 0.8 M GHCl. After various periods of standing successive amounts of reagent were added and the end-point was found graphically as described in the previous section. This method is called the "excess reagent titration." In all the titration experiments precautions were taken against contamination with oxygen from the time the denaturation sample was diluted.

The titration results are expressed as the number of gram atoms of silver, or mercury, bound per mole albumin (mol. wt. 69,000) at the end-point. Since the conditions of the titrations are chosen in a way (large excess of ammonia or chloride ion) as to exclude practically any binding of the titrating agent to groups other than the sulfhydryl group, the amount of reagent used up at the end-point is a specific measure of the reactivity of sulfhydryl toward silver or mercury under the given experimental conditions.

(4) **Viscosity.**—The viscosity of denaturation mixtures was determined in the conventional way by measuring the time of flow of the albumin solution and of the solvent in an Ostwald type viscometer at 25°. The results of the viscosity experiments were expressed in terms of reduced viscosity

$$\eta_{red} = 1/c \left(\frac{\eta}{\eta_0} - 1\right)$$

where c is concentration of albumin in per cent., η is viscosity of the denaturation mixture (protein-guanidine hydrochloride-buffer) and η_0 is the viscosity of the "solvent," a solution of the same composition as that of the denaturation mixture, except that distilled water was added instead of the albumin stock solution. The ratio of η/η_0 was assumed to be equal to the ratio of the time of flow of the protein solution to that of the solvent.

Results and Discussion

Amperometric Titration of Sulfhydryl.—Argentimetric and mercurimetric titrations of various batches of native crystallized albumin gave end-points which corresponded to a mole ratio of silver or mercury to albumin of 0.68 ± 0.02. This is in good agreement with results reported by Hughes[14] who showed that albumin consists of two fractions, one of which (mercaptalbumin) makes up two thirds of the total albumin and is found to contain one sulfhydryl group per mole. Our results also imply that under our experimental conditions the stoichiometric reaction ratio in the titration of native albumin is one silver or one mercuric chloride per sulfhydryl group. Apparently, PSAg and PSHgCl (P denotes albumin) are formed in the interaction of native albumin with diammine silver and mercuric chloride, respectively, and the sulfhydryl in native albumin appears to be readily accessible to

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

interaction with the titrating agents. Mercurimetric titrations gave the same results in ammoniacal (*p*H 9.2) and in non-ammoniacal (phosphate *p*H 7 or borate *p*H 9.2) titration mixtures. This was found with native and denatured albumin. Greenstein and Edsall[15] who used the porphyrindin titration for sulfhydryl in proteins reported that titratable sulfhydryl in myosin can be completely suppressed in the presence of ammonium chloride in the titration mixture, an effect which they could eliminate by the addition of concentrated guanidine hydrochloride. Our results indicate that the reactivity of albumin sulfhydryl (denatured or native) toward silver or mercury is not affected by the presence of ammonium ion in the titration mixture.

After many hundreds of denaturation experiments with four different batches of albumin and titrations with both silver and mercury had been carried out, it became apparent that the poor reproducibility in the sulfhydryl determinations was partly due to the extreme sensitivity to oxygen of sulfhydryl in denatured albumin at *p*H 9, even though this group is stable to oxygen in solutions of the native protein (*vide infra*). The sensitivity to oxygen in the denaturation mixture is particularly pronounced at *p*H 9, and is considerably less at *p*H 7 than at *p*H 9. Another difficulty in the interpretation of the sulfhydryl titrations is that when the denaturation mixture is allowed to stand part of the sulfhydryl becomes more difficultly accessible to silver or mercuric chloride.

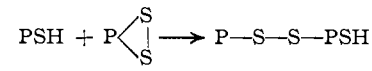
All the subsequent experiments were carried out in the complete absence of oxygen. The molar concentration of sulfhydryl in 1% albumin solutions is of the order of 10^{-4} *M* and oxidation of a considerable fraction of sulfhydryl can occur when the protein is exposed even to traces of oxygen in the denatured state at *p*H 9. The results of experiments carried out in the absence of oxygen are summarized below. When to the fresh air-free 1% albumin solution in the denaturation mixture (4 *M* GHCl) at *p*H 7 or 9 is added one mole of silver or mercuric chloride per sulfhydryl (0.68 per mole of albumin) all of the sulfhydryl is found even after 20 hours of denaturation. These titrations were carried out at *p*H 7 (mercuric) or 9 (silver and mercuric) in 0.2% albumin solutions, either in 4 *M* or in 0.8 *M* GHCl. At a concentration of 0.8 *M* the GHCl has no denaturing effect.

Upon denaturation of 1% albumin in a phosphate buffer at *p*H 7 and subsequent titration at *p*H 7 (mercury) or 9 (mercury and silver) after five times dilution either with buffer 4 *M* in GHCl or GHCl-free buffer, all sulfhydryl was found when the titrations were made within approximately 8 hours of standing of the denaturation mixture at 25°. After longer standing the titrations carried out according to the general procedure gave less than 0.68 sulfhydryl per mole of albumin. For example, after 24 hours of denaturation the mercuric chloride titration yielded as an average 0.57 SH in 4 *M* GHCl and 0.59 in 0.8 *M* GHCl. Similar results were found in the titration with silver; however, mercuric chloride gives a much sharper end-point when titrations are made in 4 *M* GHCl. The low values are attributed to a slow accessibility of part of the sulfhydryl to mercury or silver. When 1.3 to 1.5 moles of mercury or silver per mole of sulfhydryl was added to the titration mixture (excess titration method) and the titration finished after 15 minutes of standing, all sulfhydryl was recovered even after 40 hours of denaturation. When the denaturation of 1% albumin was carried out at *p*H 9 and the direct titration carried out after dilution to 0.2% albumin in 4 *M* GHCl, the sulfhydryl value was 0.55 after 30 minutes of denaturation and decreased very slowly upon further times of denaturation. For example, after 20 hours of denaturation a value of 0.48 was found. When the direct titrations were carried out in 0.8 *M* GHCl the sulfhydryl values were 0.60 after 30 minutes and 0.50 after 20 hours of denaturation. Again, when 1.5 moles of silver or mercuric chloride per mole of original sulfhydryl was added to the titration mixture and the titration finished after 15 to 30 minutes of standing, all of the sulfhydryl (0.68 per mole albumin) was titrated. The time necessary for completion of the reaction with all of the sulfhydryl was found to increase with time of denaturation. For example, after 5 minutes of denaturation at *p*H 9 all of the sulfhydryl was found after 3 minutes reaction time and the same result was obtained after one hour reaction time. After 6 hours of denaturation, when the mixture had become quite turbid, a reaction time of 15 to 30 minutes was required for complete recovery.

From these and other experiments it can be concluded that upon denaturation in 4 *M* GHCl at *p*H 7 and 9 all of the sulfhydryl can be titrated with mercuric chloride or silver, provided the titration is carried out under the proper conditions. The rate of reaction of part of the sulfhydryl with the reagent decreases with the time of denaturation, this effect being more pronounced at *p*H 9 than at *p*H 7. The direct amperometric titration to the endpoint gives misleading results under these conditions. Quite generally, when the direct titration gives low results, it is recommended that the titration be repeated by adding an excess of reagent and finishing the titration after various reaction periods.

From the above results it also may be concluded that the molar reaction ratio of sulfhydryl to mercuric chloride which is 1 to 1 in native albumin, remains the same in the denatured protein.

The slow decrease of the apparent reactivity of the sulfhydryl group upon denaturation of albumin is attributed to a cross-linking reaction as first suggested by Huggins, *et al.*[1]



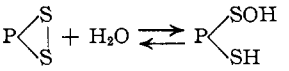
where P denotes interacting molecules of denatured protein. This type of cross-linking was also postulated by Kauzmann[16] to explain viscosity changes of protein solutions upon denaturation in urea or guanidine hydrochloride solutions. Hal-

(15) J. P. Greenstein and J. T. Edsall, *J. Biol. Chem.*, **133**, 397 (1940); J. T. Edsall, J. P. Greenstein and J. W. Mehl, THIS JOURNAL, **61**, 1613 (1939).

(16) W. Kauzmann, "The Mechanism of Enzyme Action," Ed. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 70.

wer[17] found evidence from light scattering for such a reaction in heat denatured ovalbumin. Our results are accounted for by assuming that in the denatured protein part of the sulfhydryl is present in the network and less readily accessible to reagents than in the unpolymerized protein. The rate of cross-linking increases with increasing *p*H and the above effect is observed after much shorter denaturation periods at *p*H 9 than at *p*H 7. Similarly a turbidity in the denaturation mixture is observed much sooner at *p*H 9 (about 3 hours at 25°) than at *p*H 7 (more than 60 hours). Even in turbid solutions at *p*H 9 all the sulfhydryl could be recovered by the excess reagent method.

Experiments at *p*H 7 and 9 also have been carried out at concentrations of 2, 1.5, 0.5 and 0.2% albumin in the denaturation mixture. The extent of cross-linking reaction increased with increasing protein concentration, but all of the sulfhydryl could be recovered by using the "excess reagent" titration method. Upon denaturation in 2 and 4 *M* GHCl at *p*H 10 for 2 hours or less the apparent sulfhydryl values by the direct titration method at *p*H 9 were slightly less than 0.68. By the excess reagent method more than 0.68 sulfhydryl per albumin molecule was found. For example, after 2 hours of denaturation (mixture quite turbid) the excess method yielded 0.76 sulfhydryl and after 3 hours of denaturation 0.90. In addition to a rapid cross linking reaction at *p*H 10 by which part of the sulfhydryl becomes less readily accessible, the actual sulfhydryl content increases by hydrolytic fission of some of the disulfide groups



Finally, as an illustration of the extreme sensitivity toward oxygen of the sulfhydryl in the denaturation mixture at *p*H 9 we report the results of one set of experiments in which the albumin concentration was 1% and the oxygen concentration

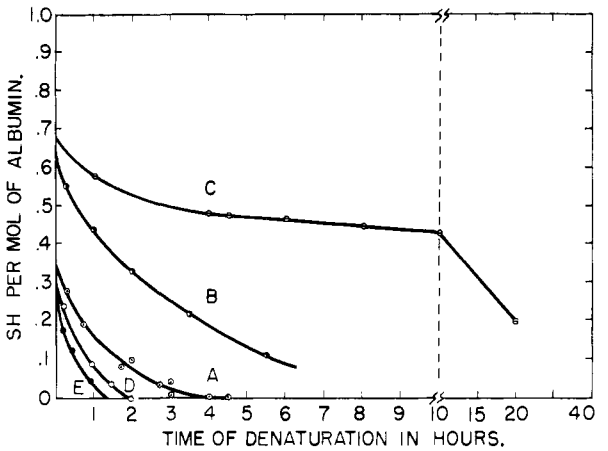


Fig. 2.—Sulfhydryl after treatment with oxygen of denaturation mixture in 4 *M* GHCl. All solutions 1% in albumin, except B which was 0.2% in albumin. Curves A, B, D and E at *p*H 9; C at *p*H 7; D denatured for 2 hours in nitrogen before treatment with oxygen; E denatured for six hours in nitrogen, then oxidized.

(17) M. Halwer, THIS JOURNAL, **76**, 183 (1954).

only 1.3×10^{-4} *M*. After 45 minutes of denaturation only 0.36 mole of sulfhydryl per mole of albumin was found by direct titration and 0.40 by the excess reagent method. After 2 hours of denaturation both methods yielded 0.20 and after 2.5 hours 0.17 sulfhydryl.

Oxidation of Sulfhydryl with Oxygen.—The sulfhydryl in native albumin is not oxidized by oxygen. Buffered solutions of native albumin at *p*H 7 and 9 were treated with oxygen and allowed to stand for 20 hours at room temperature. Upon removal of the oxygen and amperometric titration with either silver or mercuric chloride all the sulfhydryl was recovered (0.68 SH per mole). The same results were obtained when the protein–buffer solutions were 0.8 *M* in GHCl. The oxidizability of the sulfhydryl with oxygen upon denaturation in 4 *M* GHCl at 25° was investigated under varying conditions. The results of a few hundred experiments are summarized in Fig. 2 and 3. When a

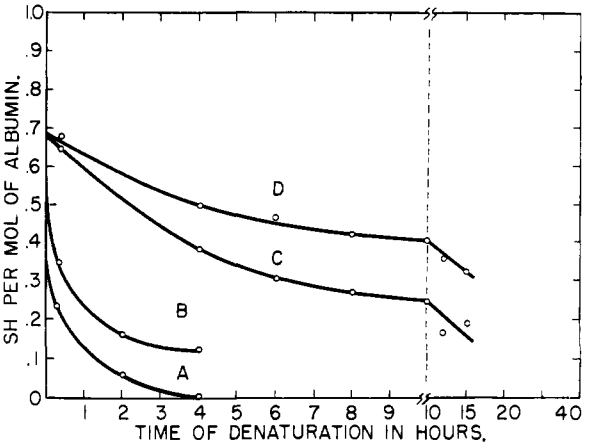


Fig. 3.—Denaturation mixtures 1% in albumin and 4 *M* in GHCl kept in nitrogen. After given time diluted to 0.8 *M* GHCl (0.2% albumin) in buffer *p*H 9 or *p*H 7 and treated with oxygen. Curves A and B denatured at *p*H 9. C and D denatured at *p*H 7. Curves B and D oxidized at *p*H 7 or 9 (ammonia-free). Curves A and C oxidized in ammonia buffer *p*H 9.

denaturation mixture at *p*H 9 which was 1% in albumin was kept exposed to oxygen for various periods of time before fivefold dilution and removal of oxygen with nitrogen, the titratable sulfhydryl was found to decrease with time as is illustrated by curve A in Fig. 2. In all instances one mole of silver and of mercuric chloride reacted per mole sulfhydryl. From Fig. 2 it is seen that the rate of decrease of sulfhydryl upon treatment with oxygen of the 1% albumin solution in the denaturation mixture is much smaller at *p*H 7 than at *p*H 9.

In the diluted mixture the rate of oxidation was the same at *p*H 7 and 9 in phosphate and borate buffers; however, lower sulfhydryl values were found when the exposure to oxygen was carried out in an ammonia buffer at *p*H 9. This has been found true both when the denaturation was carried out at *p*H 7 or 9 (Fig. 3). In the experiments in Fig. 3 the ammonia buffer was 0.1 *M* in ammonia; in buffers 0.5 *M* in ammonia the curve was slightly below that in 0.1 *M* base. No interpretation of

this small but reproducible effect of ammonia can be offered at this time.

It is well known that the unfolding process (not the cross-linking) of albumin is more or less reversible. In experiments on reactive disulfide, to be reported in a subsequent paper, it will be shown that all the disulfide groups in albumin can be titrated amperometrically with silver in the denaturation mixture composed of 4 *M* GHCl in the presence of 0.05 *M* sulfite. When the denaturation mixture is diluted to 0.8 *M* and the titration carried out immediately after dilution at *p*H 9, only a small fraction of the disulfide groups is titrated. As far as "reactive disulfide" is concerned, the reversal of the denaturation is very rapid.

In order to account for the results obtained on the oxidizability of the sulfhydryl group after a short period of denaturation at *p*H 7 in nitrogen and dilution to 0.8 *M* GHCl, we must assume a more or less complete reversal of the denaturation as far as oxidizability by oxygen is concerned. According to viscosity data (see next section) the unfolding is a very rapid process and is certainly complete within 15 minutes. However, when a denaturation mixture was kept at *p*H 7 in nitrogen for 15 minutes and then diluted to 0.8 *M* GHCl and exposed to oxygen, 0.67 sulfhydryl per mole of albumin was found. After one minute of denaturation at *p*H 9 and dilution (0.8 *M* GHCl) to *p*H 7 or 9 (not in ammonia) 0.62 sulfhydryl was found upon oxygen treatment for 2 hours When no secondary changes have occurred during denaturation, the reversal upon dilution appears to be complete as far as oxidizability with oxygen is concerned, regardless of whether the diluted solution has a *p*H of 7 or 9.

The oxidizable sulfhydryl increases with increasing period of denaturation in nitrogen. For example, after 6 hours of denaturation at *p*H 7 and subsequent dilution to 0.8 *M* GHCl and oxygen treatment, 0.47 sulfhydryl was found (in ammonia buffer 0.31; see Fig. 3). When the mixture was diluted and allowed to stand in nitrogen from 1 to 24 hours, the same values as above were found. This indicates that the reversal as far as oxidation of sulfhydryl with oxygen is concerned is an extremely rapid process, and, for an unknown reason, is less complete in ammonia buffers than in other buffers. Similar experiments on the rate of reversal after denaturation (for 24 hours at *p*H 7 or for 30 minutes and for 2 hours at *p*H 9) substantiated this conclusion.

The question arises now as to why the oxidizable sulfhydryl increases with time of denaturation or why the reversal becomes less and less complete. This must be attributed to secondary changes during denaturation. In the denatured state the sulfhydryl reacts with intramolecular and intermolecular disulfide groups, the latter reaction giving rise to cross-linking. Neither the intra- nor the intermolecular exchange is reversible. These secondary changes occur faster the higher the *p*H. In experiments with low molecular weight compounds it has been found[18] that the equilibrium between sulfhydryl and disulfide is established slowly, the rate of reaction increasing with increasing *p*H. In accordance with this interpretation it is seen (Fig. 3) that the oxidizable sulfhydryl increases much faster upon denaturation in nitrogen at *p*H 9 than at *p*H 7. After 4 hours of denaturation and subsequent dilution and oxidation in ammonia buffer at *p*H 9 all of the sulfhydryl was found oxidized. On the other hand, when the mixture was diluted and oxidized in the ammonia buffer after denaturation at *p*H 9 for only two minutes and then exposed to oxygen 0.59 sulfhydryl was found unoxidized. After the short time of denaturation at *p*H 9 relatively little irreversible change by exchange reactions had occurred and the reversal was relatively complete. The extent of cross-linking increases with increasing albumin concentration as is evidenced from the time after which a turbidity appears in the denaturation mixture. A larger extent of cross-linking probably accounts for the fact that upon exposure of the denaturation mixture to oxygen the oxidizable sulfhydryl is found to increase much faster in 1% than in 0.2% albumin solutions (Fig. 2). It would seem then that the unfolded cross-linked protein is more readily oxidized by oxygen than the uncross-linked albumin. In agreement with this postulate is the fact that sulfhydryl is oxidized faster when the denaturation mixture at *p*H 9 has been allowed to stand in nitrogen before treatment with oxygen than when the denaturation mixture is exposed to oxygen from the beginning. This is clearly illustrated by curves A, D and E in Fig. 2.

No systematic study has been made of the effect of oxygen concentration on the rate of oxidation. However, in all instances the same curves were obtained with oxygen and with air in denaturation mixtures as well as in the diluted denaturation mixtures (0.8 *M* in GHCl). Thus, in this concentration region the reaction is zero order with regard to oxygen. In all the oxidation experiments there always was a gas pressure distinctly greater than one atmosphere. Without this overpressure the rates of oxidation were always found less than with overpressure, but the differences were very small. Experiments with other oxidizing agents than oxygen are being planned; they may provide a further test of the above interpretation.

Cross-linking still occurs upon denaturation in oxygen, but it is slower in oxygen than in nitrogen. For example, upon denaturation of 1% albumin in 4 *M* GHCl at *p*H 9 in nitrogen the solution became opalescent after four to six hours at 25°, while in oxygen the turbidity was not observed until after 40 hours. Upon denaturation at *p*H 10 the turbidity also appeared much faster in nitrogen than in oxygen. Viscosity experiments (*vide infra*) also indicate that a slow cross-linking occurs upon denaturation in oxygen. We may conclude therefore that the intra- and intermolecular exchange reactions between sulfhydryl and disulfide also occur upon denaturation in oxygen, but to a lesser extent than in nitrogen because of dimer formation.

The oxidizability with oxygen of the sulfhydryl group also was investigated at *p*H 7 and *p*H 9 in the presence of one equivalent of silver (0.68 mole per

(18) I. M. Kolthoff, W. Stricks and R. C. Kapoor, THIS JOURNAL, **77**, 4733 (1955).

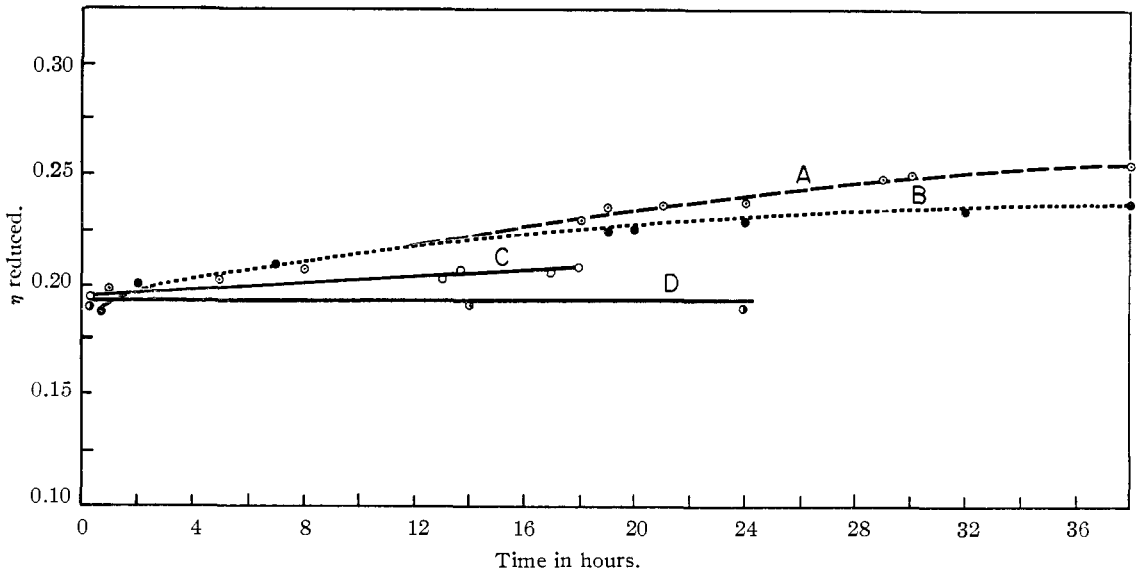


Fig. 4.—Reduced viscosities of 1% albumin solution in 4 *M* GHCl at *p*H 7, 25°: A, in nitrogen; B, in oxygen; C, with 2 eq. of $AgNO_3$ in O_2 or N_2; D with 2 moles of $HgCl_2$ per mole albumin, in O_2 or N_2.

mole albumin) in the denaturation mixture which was 1% in albumin, 4 *M* in GHCl and saturated to oxygen. Even after 20 hours of denaturation all the sulfhydryl was recovered, both at *p*H 7 and 9. When the albumin was denatured in nitrogen for 2 hours at *p*H 7, and one equivalent of silver was added after five times dilution, all the sulfhydryl (0.68) was recovered after treatment with oxygen at *p*H 9, while in the absence of silver during the oxidation only 0.52 sulfhydryl was found. This effect of silver may be attributed to the slight dissociation of the silver mercaptide. This conclusion is substantiated by the fact that one equivalent of silver has a strongly retarding effect upon the oxidation by oxygen of sulfhydryl in cysteine. Upon exposure of cysteine to oxygen in an ammonia buffer of *p*H 9 for 1, 2 and 48 hours, 12, 34 and 100% sulfhydryl was found oxidized. The same results were found when the solution was 0.001 *M* in ethylenediaminetetracetate. In the presence of one equivalent of silver only 2, 4 and 14% sulfhydryl was found oxidized after the above times. A similar retarding effect of silver upon the oxidation was found at *p*H 7.[19] On the other hand, as is well known, a trace of copper greatly accelerates the oxidation by oxygen of cysteine to cystine. Both at *p*H 7 and 9 all the cysteine was found oxidized by oxygen within two hours in the presence of 0.02 equivalent of copper. It is interesting that the presence of 0.02 equivalent of cupric copper at *p*H 7 and 9 did not have any effect upon the rate of oxidation of sulfhydryl of albumin in 4 *M* GHCl solution saturated to oxygen. This is attributed to the formation of a very stable copper(II)–albumin complex in a molar ratio of one to one.[20]

Viscosity.—Two viscometers with considerably different flow times were used. The reduced viscosities, determined in both viscometers, of denatured albumin solutions were found the same. After we had observed that oxygen in the denaturation mixture at *p*H 9 causes a rapid oxidation of sulfhydryl, precautions were taken to exclude oxygen during the denaturation and measurement of the viscosity in experiments which were carried out in nitrogen. It appeared that brief exposure of denaturation mixtures to the atmosphere had only a slight effect on the results. Some results on the change of the viscosity upon denaturation under various conditions of 1% albumin in 4 *M* GHCl at *p*H 7 and 9 are given in Figs. 4 and 5. The scale of the unit of reduced viscosity (ordinate) in Fig. 4 is twice as large as in Fig. 5. In agreement with other workers[1,21] an extremely rapid increase of the viscosity is found when the protein is mixed with the denaturing agent. The subsequent increase of viscosity with time is attributed to cross-linking and aggregation.[1,21] This subsequent increase of the viscosity is much faster at *p*H 9 than at *p*H 7 which substantiates the conclusion drawn from the reactivity experiments of the sulfhydryl group that the rate of aggregation increases rapidly with increasing *p*H.[21] Both at *p*H 7 and 9 the slow increase in viscosity is found when the denaturation mixture is kept saturated with oxygen; again the rate of change is greater at *p*H 9 than at *p*H 7, but less than in nitrogen. This substantiates the previous conclusion that the cross-linking reaction occurs to some extent upon denaturation in oxygen. The presence of two equivalents of silver nitrate (referred to sulfhydryl) in the denaturation mixture brings about a marked reduction in the rate of change of the viscosity with time of standing. The same viscosity data were found both in nitrogen and oxygen. The effect of silver was to be expected, since the rate of cross-linking is dependent on the mercaptide ion concentration and the silver mercaptide is dissociated slightly. However, even at *p*H 7, a slight increase of the viscosity with time

(19) See also L. Michaelis and E. S. G. Barron, *J. Biol. Chem.*, **81**, 29 (1929).

(20) I. M. Kolthoff and B. R. Willeford, THIS JOURNAL, **79**, 2656 (1957); see also I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, *ibid.*, **77**, 1919 (1955).

(21) See *e.g.*, H. K. Freusdorff, M. T. Watson and W. Kauzmann, *ibid.*, **75**, 5167 (1953).

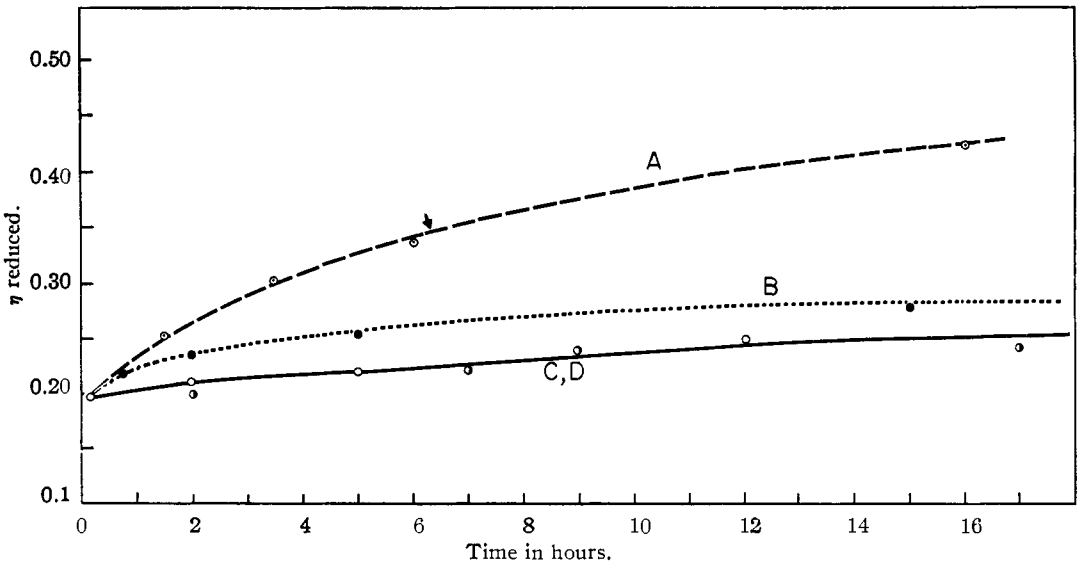


Fig. 5.—Reduced viscosities of 1% albumin solution in 4 *M* GHCl at *p*H 9, 25°: A, in nitrogen; B, in oxygen; C, with 2 eq. of $AgNO_3$, both in N_2 or O_2; D with 2 moles of $HgCl_2$ both in N_2 or O_2.

was observed in the presence of 2 equivalents of silver. In previous work[22] we found the mercuric mercaptide bond much more stable than the silver mercaptide bond. Consequently, the effect of mercuric chloride upon the change of the viscosity is found greater than that of silver. Actually, the presence of 2 moles of mercuric chloride per mole of albumin prevented any secondary change of the viscosity at *p*H 7. Fifteen minutes after the preparation of the denaturation mixture the reduced viscosity (25°) was 0.19, and the same value was measured after 24 hours of standing, either in nitrogen or oxygen. Mercuric chloride does not prevent a slight increase in the viscosity at *p*H 9 (see Fig. 5). It is noteworthy that the extrapolated value of the reduced viscosity in the presence of 2 moles of mercuric chloride per mole sulfhydryl at *p*H 9 is exactly the same as the value of 0.19 (with mercury) found at *p*H 7. This would indicate that the viscosity of denatured albumin is the same at *p*H 7 and 9. From extrapolated viscosity data upon denaturation in urea in the absence of reagents which inhibit cross-linking Kauzmann, *et al.*,[21] arrived at the same conclusion.

General Conclusions

The apparent decrease in sulfhydryl upon denaturation of albumin in 4 *M* GHCl which is attributed to cross-linking, as a result of which the sulfhydryl becomes less accessible to silver and mercury, has not been described in the literature. The opposite effect, namely, an apparent increase of the sulfhydryl content upon denaturation of a protein has been reported by various authors. Native egg albumin, for example, was found to contain no sulfhydryl upon titration with *p*-chloromercuric benzoate, *o*-iodobenzoate or ferricyanide,[23-25] while after denaturation with GHCl 1.07% (*p*-chloromercuric benzoate), 1.29% (iodobenzoate) and 0.96% (ferricyanide) sulfhydryl, expressed as cysteine, was found. Using the amperometric titration technique with silver nitrate and mercuric chloride as reagents, Ingram[26] found four sulfhydryl groups per mole of native human hemoglobin and eight sulfhydryl groups after denaturation with sodium dodecyl sulfate. In these cases the sulfhydryl is not or only partially accessible to the reagent in the native protein, but it becomes accessible upon unfolding during denaturation.

Native albumin cannot form a dimer (disulfide) by oxidation of the sulfhydryl, but it can be obtained from the mercury dimer PSHgSP,[14,27] as recently shown by Straessle.[28] The fact that sulfhydryl in native albumin cannot form a dimer upon oxidation with oxygen but does so upon denaturation in 4 *M* GHCl (or 6 *M* urea; not yet reported in this paper) accounts for several contradictory and confusing statements in the literature on the sulfhydryl content of albumin and other proteins. Methods which are based upon the oxidation of the sulfhydryl to disulfide should be interpreted with caution. For example, Todrick and Walker[29] could not detect sulfhydryl in native or heat-denatured serum albumin with phenol indo-2,6-dichlorophenol as reagent. Such methods may indicate the apparent absence of sulfhydryl in a native protein but its presence in a denatured protein. Greenstein,[30] using the porphyrindin method for the titration of sulfhydryl in serum albumin, did not detect sulfhydryl in the native protein, but found 0.34%—expressed as cysteine (1.97 SH per mole albumin)—upon denaturation in concentrated guanidinium salt solutions.

(22) I. M. Kolthoff and W. Stricks, THIS JOURNAL, **72**, 1950 (1951).

(23) L. R. MacDonnell, R. B. Silva and R. E. Feeney, *Arch. Biochem. Biophys.*, **32**, 278 (1951).

(24) L. Hellerman, F. P. Chinard and V. R. Deitz, *J. Biol. Chem.*, **147**, 443 (1943).

(25) A. E. Mirsky, *J. Gen. Physiol.*, **24**, 709 (1941).

(26) V. M. Ingram, *Biochem. J.*, **59**, 653 (1955).

(27) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(28) R. Straessle, *ibid.*, **76**, 3138 (1954).

(29) A. Todrick and E. Walker, *Biochem. J.*, **31**, 292 (1937).

(30) J. P. Greenstein, *J. Biol. Chem.*, **136**, 795 (1940).

Acknowledgment.—Financial support from the U. S. Public Health Service and from the Louis and Maud Hill Family Foundation is gratefully acknowledged. Preliminary work on the development of some of the procedures has been carried out by Dr. R. K. Neogy.

We are indebted to Dr. R. W. Lumry and Dr. B. R. Willeford for their interest in our work.

MINNEAPOLIS, MINN.

[CONTRIBUTION FROM THE RADIATION BRANCH NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

A Mechanism for the Reaction of Fe^{++}, H_2O_2 and Glycine in an Aerobic Aqueous Solution

BY CHARLES R. MAXWELL AND DOROTHY C. PETERSON

RECEIVED APRIL 11, 1957

The kinetics and stoichiometry of the action of Fenton's reagent in aerobic aqueous solutions of glycine are presented and a mechanism is proposed to describe the observations. The reaction is initiated and the rate controlled by the production of an OH free radical from a H_2O_2 molecule. The stoichiometry, which varies as the reaction proceeds, is the result of two pairs of competing steps: the first between Fe^{++} and glycine for the OH free radical and the second between Fe^{++} and Fe^{+++} for an intermediate organo-peroxy free radical. The Fe^{++}–peroxy reaction regenerates the H_2O_2 used in the initial step and thus accounts for the small $\Delta H_2O_2/\Delta Fe^{++}$ ratios observed in the early phase of the reaction. The Fe^{+++}–peroxy reaction regenerates the Fe^{++} used in the initial step and thus accounts for the large $\Delta H_2O_2/\Delta Fe^{++}$ ratios observed in the late phase of the reaction. HCOCOOH and NH_3 are formed by both reactions. The effects of H^+ concentration and of glycine concentration have been measured and are discussed. The ratios of the reaction rate constants of the competing steps have been evaluated for various conditions.

Introduction

A mixture of H_2O_2 and Fe^{++}, commonly referred to as Fenton's reagent, has been used since before the turn of the century to study the oxidation of organic compounds in aqueous solution. More recently, as the mechanism of the reaction began to be more fully understood[1-3] it has been used extensively to study the effect of OH free radicals. We became interested in the action of this reagent upon glycine as a result of a study[4] of the effect of ionizing radiation upon solutions of this amino acid. Since ionizing radiation in aqueous solution reacts indirectly[5] through a complex mixture of H and OH free radicals and H_2O_2, it was believed that an investigation of the Fenton reaction would provide a mechanism which would be useful in the interpretation of radiation data.

Dakin[6] has reported that the products of the oxidation of amino acids by Fenton's reagent are in general ammonia, a keto acid, an aldehyde and carbon dioxide with the yield of the aldehyde usually greater than the yield of the keto acid. However, with glycine he found the yield of HCOCOOH greater than the yield of HCHO. Merz and Waters,[7] on the other hand, have listed only HCHO as the product of the oxidation. The authors,[4] working with ionizing radiation rather than Fenton's reagent, found HCOCOOH, HCHO, NH_3, CO_2, and in addition observed that the ratios between the yields of HCOCOOH, HCHO and NH_3 varied with the dose of radiation. These observations were interpreted in terms of secondary reactions which oxidized the HCOCOOH and HCHO but did not affect the NH_3.

Merz and Waters[7] have proposed a general scheme for the action of OH radicals from Fenton's reagent upon organic substrates in which many compounds are oxidized by a chain reaction involving the substrate and H_2O_2. Glycine and alanine are listed among these compounds. The rationale for this chain, which is initiated by an OH radical formed from Fe^{++} and H_2O_2 and terminated by the reaction of an OH radical with a Fe^{++} ion, appears to be based solely upon large H_2O_2 consumption/Fe^{++} oxidation ratios. No quantitative measurements were reported for the yield of products or consumption of substrate nor of the rate of the reaction.

Kolthoff and Medalia[8] have suggested another scheme for the action of Fenton's reagent upon organic substrates. In the presence of dissolved oxygen (which Merz and Waters did not consider) this scheme includes a different chain reaction which is also initiated by the action of an OH radical upon the substrate. This chain which involves oxygen (not H_2O_2), substrate and Fe^{++} provides for the oxidation of many molecules of substrate and Fe^{++} ions for each OH radical formed (H_2O_2 consumed). However, here again the mechanism appears to be based solely upon H_2O_2 consumption/Fe^{++} oxidation ratios. No data were reported for substrate oxidation, product yield or rates of reaction.

From the above it is apparent that data as to the extent of secondary reactions, the actual substrate oxidation or product yield, and the rate of the reaction is needed. We, therefore, first studied the yields of products as a function of "OH exposure" and differentiated the initial and secondary products. The study of the reaction was then limited to conditions which eliminated or minimized the

(1) F. Haber and J. Weiss, *Proc. Roy. Soc.* (*London*), **A147**, 332 (1934).

(2) A. I. Medalia and I. M. Kolthoff, *J. Polymer Sci.*, **4**, 377 (1949).

(3) W. G. Barb, J. H. Baxendale, P. George and K. R. Hargrave, *Trans. Faraday Soc.*, **47**, 462, 591 (1951).

(4) C. R. Maxwell, D. C. Peterson and N. E. Sharpless, *Rad. Research*, **1**, 530 (1954).

(5) A. O. Allen, *ibid.*, **1**, 85 (1954).

(6) H. D. Dakin, *J. Biol. Chem.*, **1**, 171 (1905).

(7) J. H. Merz and W. A. Waters, *J. Chem. Soc.*, S15, 2427 (1949).

(8) I. M. Kolthoff and A. I. Medalia, THIS JOURNAL, **71**, 3777, 3784 (1949).