I. 1. CHEMICAL MODIFICATION OF THIOL AND DISULFIDE GROUPS IN PROTEINS AND PEPTIDES

J. M. Swan

Division of Protein Chemistry, Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia

I. Introduction

The recent striking advances in our knowledge of protein structure have been made possible in part by the application of methods for chemically modifying selected functional groups. Sanger's use of 2,4-dinitro-1-fluorobenzene to mark terminal and side-chain amino groups is an obvious example, and it is worth remembering that the numerous chemical methods for the detection or determination of amino acids residues in intact protein depend on specific chemical reactions of the residue in question. In this paper it is proposed to review briefly some of the more specific methods for effecting chemical changes in the thiol and disulfide groups, with special reference to any disadvantages or limitations, and to present some new results in regard to certain of these methods.

II. Some Present Methods for the Specific Modification of Thiol and Disulfide Groups

1. Oxidation with Peracids

Performic acid oxidation of disulfide and thiol sulfur to sulfonic acid groups has now been used in an impressive number of structural investi-

gations (see, for example, references *1–3*). Aqueous peracetic acid has been used similarly to obtain soluble keratin proteins (*4*).

The advantages of these peracid reagents are favorable solubility properties, ease of removal of excess reagent, and the special fact that introduction of the strongly acid sulfonic group often facilitates electrophoretic separation of peptides. The main disadvantages seem to be destruction of tryptophan and conversion of methionine to the *S,S*-dioxide (*5*), while partial destruction of lysine and arginine residues (*6*), chlorination of tyrosine residues (*7*), and an undesirable oxidation of lanthionine and other sulfides (*8*) can also occur.

2. Reduction of Disulfides by Thiols

The process of disulfide reduction by incubation with an excess of a simple thiol has been widely used in studies on wool (for reviews see references *9, 10*) and is now being used increasingly in studies on soluble proteins (*11–13*). Eldjarn and Pihl (*14*) and Kolthoff *et al.* (*15*) have shown that mixed disulfides are intermediates in this equilibrium reaction, and the concentration of mixed disulfides can often be surprisingly high even when the relative excess of thiol is fairly large. The extent of reduction is critically dependent on pH, and can increase greatly in the presence of urea or a detergent.

Favored reagents are cysteine, 2-aminoethane thiol, and thioglycollic acid. In alkaline solution the possibility of some simultaneous conversion of cystine to lanthionine would seem to exist, but in our experience this side reaction is of negligible importance. Surprisingly, however, we find that small amounts of lanthionine can be produced in wool during acid thiol reduction, the reaction being due to the thiol and not simply to the pH (*16*). A more important limitation may be the possibility of causing partial racemization of cystine residues when alkaline thiol solutions are used. It is well known that cystine and its derivatives are especially easily racemized in alkali (*17*) and we have found (*18*) that if wool is reduced with alkaline thioglycollate and subsequently alkylated with iodoacetate, the *S*-carboxymethylcysteine isolated after hydrolysis may be quite extensively racemized (e.g. 45%) unless special care be taken to keep the pH and temperature low.

3. Cleavage of the Disulfide Bond with Sulfite and with Cyanide

a. Sulfite

The cleavage of disulfide bonds by aqueous sulfite solutions is a highly specific equilibrium reaction, but the reaction rate and point of equilibrium

varies widely with different disulfides (*19, 20*). The reaction is one of oxidation-reduction, half the disulfide appearing as thiol and the other half as an *S*-alkyl thiosulfate, representing oxidation of the sulfite ion.

$$RSSR + SO_3^{--} \rightleftharpoons RS^- + RSSO_3^- \quad (1)$$

Cecil and McPhee (*19*) have concluded from a kinetic study of simple disulfides that the reaction is a reversible bimolecular displacement above pH 9, and that the rate of reaction with sulfite is dependent on the net charge in the vicinity of the disulfide bond, a negative charge causing a large decrease in the rate constant. The disulfides used reacted only with sulfite and not bisulfite ions, but Cecil and Loening (*21*) have now shown that bisulfite ions can apparently react with insulin.

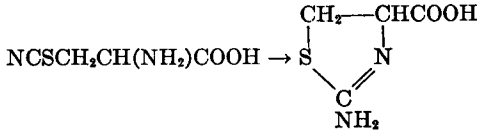
As a specific reaction for structural studies, the disadvantages of sulfite cleavage are the ready reversibility, the somewhat unstable nature of the alkyl thiosulfate group (see Section IV below), and the fact that the cleavage is unsymmetrical. It also follows from the reversibility of Eq. 1 that even small amounts of sulfite could cause extensive "rearrangement" of the disulfide bonds in a protein, analogous to thiol-catalyzed rearrangement, and such processes are probably important in the "setting" and "supercontraction" of wool fibers in sulfite solutions, and in the stoichiometric mercuric chloride titration of the disulfides in wool (see Eq. 10 below).

b. Cyanide

Cyanide ions react with the disulfide bond according to the equation (2)

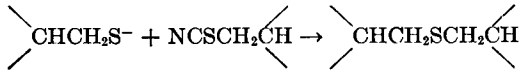
$$RSSR + CN^- \rightleftharpoons RS^- + RSCN \quad (2)$$

This again is an equilibrium reaction, and cyanide ions are liberated when thiols are incubated with alkyl thiocyanates (*22*). In the case of cystine, the first-formed β-thiocyanoalanine cyclizes to 2-amino-2-thiazoline-4-carboxylic acid (*23*).



(3)

With proteins, where the amino groups of the cystine residues are no longer available for this type of cyclization, decomposition of the β-thiocyanoalanyl ("*S*-cyanocysteinyl") residue supervenes and lanthionine is formed together with thiocyanate ion (*24*).



(4)

4. Reaction with Silver Ions, Mercuric Salts, and Organic Mercurials

Schiller and Otto (*25*) and later Fromm (*26*) found that aromatic disulfides are decomposed by boiling ethanolic potassium hydroxide according to the equation (5)

$$2RSSR + 2H_2O \rightarrow 3RSH + RSO_2H \quad (5)$$

The same over-all reaction occurs under milder conditions in the presence of mercuric ions, which form an insoluble mercaptide with the thiol (*27*). Cecil (*28*) has studied the reaction between silver nitrate and both cystine and oxidized glutathione and has found that between pH 4.5 and pH 9 the over-all equation (6) is

$$2RSSR + H_2O + 3Ag^+ \rightarrow 3RSAg + RSO_2H + 3H^+ \quad (6)$$

Nothing is known of the mechanism of these reactions and the equilibrium

$$RSSR + H_2O \rightleftharpoons RSH + RSOH \quad (7)$$

which is often postulated as an initial step, must be regarded only as a hypothesis. In fact Cecil and McPhee (*29*) have described results which favor the idea of a direct attack by Ag^+ ion as the initial step in the reaction between disulfides and aqueous silver nitrate.

The titration of thiol groups in proteins with mercury or silver ions may be complicated by the slower reaction of the metal ion with disulfide. This problem can be overcome by the use of organic mercurials which in the absence of sulfite react in a monofunctional and highly specific manner with thiols only (*30*).

$$RSH + R'HgCl \rightarrow RSHgR' + HCl \quad (8)$$

The use of sulfite is advantageous in the titration of disulfides with silver (*31*), mercury (*32*), or organic mercurials (*33, 34*); under appropriate conditions the reactions are

$$RSSR + Ag^+ + SO_3^{--} \rightarrow RSSO_3^- + RSAg \quad (9)$$

$$2RSSR + HgCl_2 + 2SO_3^{--} \rightarrow 2RSSO_3^- + (RS)_2Hg + 2Cl^- \quad (10)$$

$$RSSR + R'HgCl + SO_3^{--} \rightarrow RSSO_3^- + RSHgR' + Cl^- \quad (11)$$

In addition to their analytical value these reactions serve to produce in convenient manner the *S*-sulfocysteinyl ($RSSO_3^-$) residue for possible further modification (see Sections III and IV).

III. The Conversion of Thiols and Disulfides to Alkyl Thiosulfates

In the disulfide-sulfite equilibrium (Eq. 1) it is clear that if the thiol formed could be reoxidized to disulfide, the latter would eventually be

wholly converted to alkyl thiosulfate. In fact Clarke (*35*), and later Kolthoff and Stricks (*36*), prepared *S*-sulfocysteine ("cysteine sulfonic acid") by passing air through an ammoniacal solution of cystine containing excess sulfite. Such an oxidation also occurs in the Folin-Marenzi method of cystine analysis using phosphotungstic acid in sulfite at pH 5, and in the amperometric titration of cystine and cysteine with ammoniacal cupric sulfite (*36*):

$$RSSR + 2Cu^{++} + 2SO_3^{--} \rightarrow 2RSSO_3^- + 2Cu^+ \quad (12)$$

$$RSH + 2Cu^{++} + SO_3^{--} \rightarrow RSSO_3^- + 2Cu^+ + H^+ \quad (13)$$

The reactions shown in Eq. 12 and 13 have been made the basis of a method for dissolving keratin and may be employed generally for the modification of —SH and —S—S— (*37*). The reactions are extremely rapid and appear to be very highly specific; other advantages are symmetrical fission of the disulfide, and the possibility of marking the $—SSO_3^-$ group with radioactive sulfur by using sulfite containing sulfur-35. The chief disadvantage in protein studies is the rather prolonged dialysis necessary to remove cuprous and cupric ions, and the instability of the $RSSO_3^-$ group, especially to high alkalinity. Independently of this work Bailey (*38*) has effected the same over-all reaction using either sodium tetrathionate or iodosobenzoate as oxidant.

In general, the reactions shown in Eqs. 12 and 13 can be carried out as follows. Cupric ammonium hydroxide is prepared by adding concentrated ammonia to a copper sulfate solution until the precipitate just redissolves. Concentration is adjusted to some suitable value, e.g. 0.05 *M*, and the pH to about 10. A 10% excess of this solution is added all at once to a solution of the protein or other disulfide or thiol compound containing a two- to fourfold excess of sodium sulfite. A transitory black precipitate of the cuprous mercaptide may appear, which soon redissolves leaving a pale greenish solution. If allowed to stand in contact with air, the cuprous copper is reoxidized to cupric and the color returns to dark blue. In the case of protein solutions the copper is removed by dialysis against citrate or ethylenediaminetetraacetate, or the protein may be purified by repeated precipitation, e.g. with ammonium sulfate. If the $RSSO_3^-$ product is not retained during dialysis, and cannot be readily precipitated by salt, methods such as electrophoresis, chromatography, or counter-current distribution must be resorted to for separation of the product from cuprous, cupric, and other ions. It should be noted that the copper cannot be removed by complexing with cyanide or precipitating with hydrogen sulfide, since these reagents also react with $RSSO_3^-$ (see Section IV below). For a study of the stoichiometry of the reaction of cupric ammonium sulfite with protein thiols and disulfides, the techniques of Stricks and Kolthoff (*39*), as

described for cystine and oxidized glutathione, can be used, except that in some cases it is necessary to work in 8 *M* urea solution.

Kolthoff and Stricks have pointed out that the conversion of cystine to *S*-sulfocysteine by sulfite in the presence of oxygen is greatly catalyzed by small amounts of cupric copper. In our laboratory Dr. S. J. Leach has studied this catalytic reaction using hydroquinone, cobalt, iron, and other metals in addition to copper. Some of his results are given in Figs. 1 and 2. Figure 1 shows polarograms of a solution of cystine containing sulfite, buffer salts, and a trace of ferric iron. At time 0, waves due to RSH, RSSR, and $RSSO_3^-$ are all evident; at time ∞ (24 hours) only one large single wave due to $RSSO_3^-$ is found. The rate of reaction for different catalysts

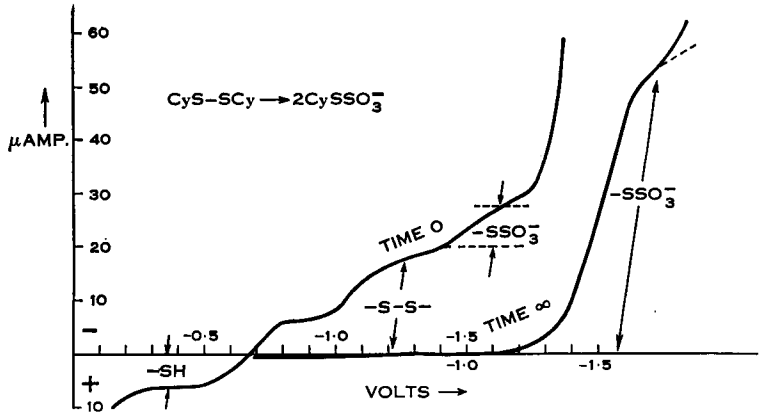


FIG. 1. Polarograms of a solution of cystine at pH 9.2 containing sulfite and a trace of Fe^{+++}, before and after prolonged aeration. (5×10^{-3} *M* RSSR, 0.1 *M* NH_4^+, pH 9.2, 1.1 equivalents $SO_3^=$, 1×10^{-5} *M* Fe^{+++}, air.)

is followed by measuring the unchanged cystine at increasing time intervals by amperometric mercuric chloride titration (Eq. 10). Figure 2 shows that under the conditions used (pH 9.2, 1.1 equivalents sulfite, 0.001 equivalents catalyst) ferric iron is the most efficient catalyst and hydroquinone the least efficient catalyst.

Leach has also extended the sulfite-air-catalyst method to the conversion of protein —S—S— and —SH groups to $—SSO_3^-$. Here again the reaction is followed by taking aliquots and estimating the amount of unchanged disulfide in the protein by amperometric mercuric chloride titration in the presence of sulfite at pH 9.8; in the case of proteins it is also necessary that the solution be 8 *M* in urea so that all the residual disulfide bonds react readily (*39a*). The formation of *S*-sulfocysteinyl residues can

also be followed by observing the height of the polarographic reduction wave of the $RSSO_3^-$ group, which in 8 *M* urea at pH 9.8 starts at −1.20 to −1.25 volts versus a saturated calomel electrode. It is important that all reagents be very pure, otherwise the wave merges with the hydrogen ion discharge current. Figure 3 illustrates the course of such an amperometric titration of intact insulin. Figure 4 shows some preliminary results

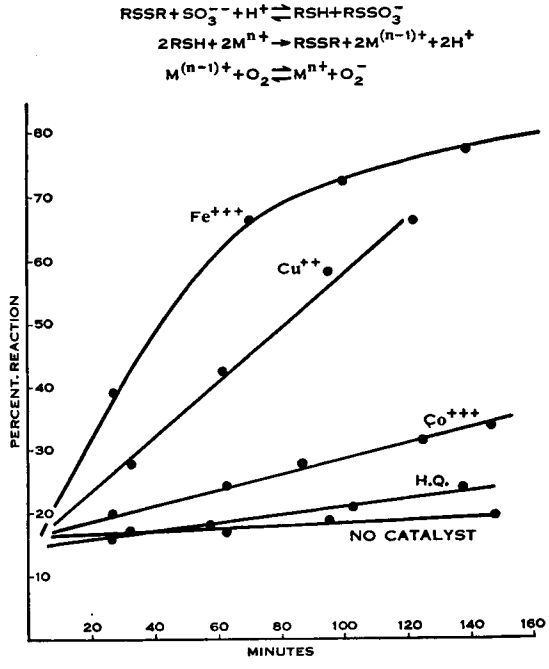


FIG. 2. The effect of different catalysts in converting cystine to *S*-sulfocysteine by the combined action of sulfite and oxygen. [5×10^{-3} *M* RSSR, 1×10^{-5} *M* metal (M), 1.1 equivalents $SO_3^=$, 0.1 *M* NH_4^+, pH 9.2.]

in the conversion of insulin to a mixture of "*S*-sulfo A chain" and "*S*-sulfo B chain" using Cu^{++} as catalyst.

The reaction of insulin with sulfite, air, and a trace of copper proceeds at any pH between 7 and 10.5, being faster at higher pH, and is very greatly accelerated in 8 *M* urea, when the reaction is complete in about an hour. Bovine serum albumin will react under the above conditions only if urea is present, and also requires slightly more than one atom of copper, the first being apparently strongly bound to the protein and not available for the catalytic process.

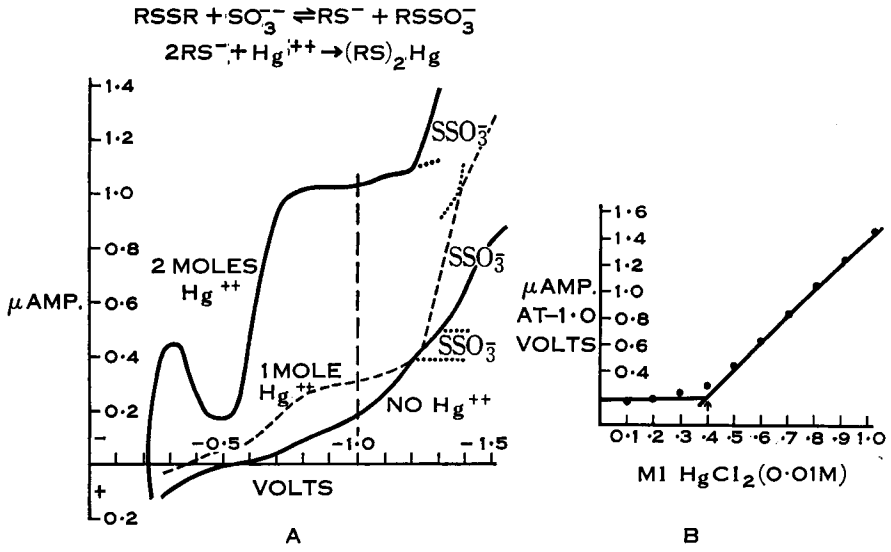


FIG. 3. Amperometric titration of insulin (18.4 mg.) with mercuric chloride (0.01 *M*) in the presence of sulfite and 8 *M* urea. (0.1 *M* $NH_4Cl - NH_4OH$, pH 9.2, 0.2 *M* Na_2SO_3, 0.5 *M* KCl.) B. 0.40 ml. $HgCl_2 \equiv 12.5\%$ cystine.

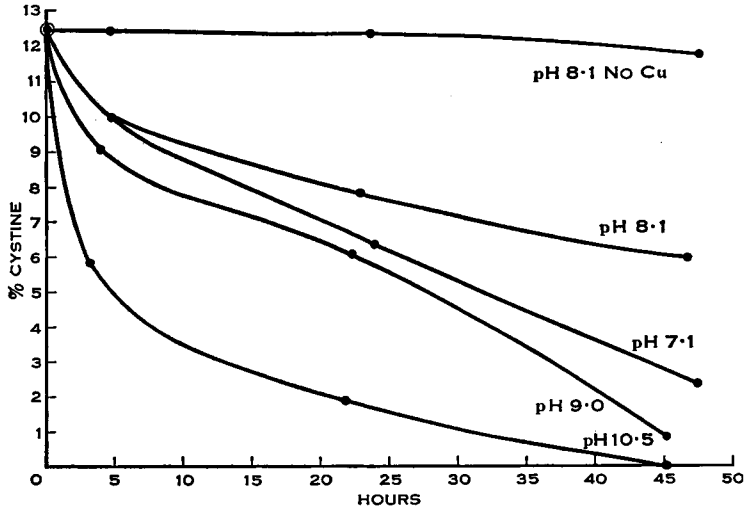


FIG. 4. Reaction of insulin (0.79%) with sulfite (10 × RSSR) in the presence of air and copper (RSSR/10) in tris buffer with 0.1 *M* Na_2SO_3 and 5×10^{-4} *M* $CuSO_4$ at 21°.

IV. Some Properties of *S*-Sulfocysteinyl and *S*-Cyanocysteinyl Residues

Since *S*-sulfocysteinyl residues are readily introduced into proteins (Eqs. 1, 9–13), it becomes of interest to know more about their reactivity.

Simple alkyl thiosulfates ("Bunte salts") are well known. With hot acid or reducing agents they yield thiols; with alkali they decompose to give disulfide and dithionate as the main products, although more complex side reactions are also possible (*39b*). Mild oxidation yields a disulfide, under more vigorous conditions the sulfonic acid is obtained. Reactions of particular interest in peptide and protein chemistry are the nucleophilic displacements with thiol anion, sulfite ion, and cyanide ion. All these involve cleavage of the thiosulfate —S—S— bond with liberation of sulfite ions:

$$RSSO_3^- + R'S^- \rightleftharpoons RSSR' + SO_3^{--} \quad (14)$$

$$RSSO_3^- + S^*O_3^{--} \rightleftharpoons RSS^*O_3^- + SO_3^{--} \quad (15)$$

$$RSSO_3^- + CN^- \rightleftharpoons RSCN + SO_3^{--} \quad (16)$$

Equation 14 is simply the reverse of equation 1, and under controlled conditions provides a synthesis of unsymmetrical disulfides (*37, 40*). The equilibrium displacement with sulfite proceeds quite rapidly at room temperature (*41*) and can be used for adding or removing a radioactive label from the thiosulfate group. We are currently investigating the cyanide displacement, especially by means of the polarograph, and find that this also is an equilibrium reaction. When *S*-sulfocysteine itself is treated with cyanide the $RSSO_3^-$ wave steadily decreases due to cyclization of the RSCN product to 2-amino-2-thiazoline-4-carboxylic acid (Eq. 3). Reaction of *S*-sulfocysteinyl proteins with cyanide has been demonstrated using *S*-sulfokerateine labeled with sulfur-35 (*37*) and formation of the —SCN group has been confirmed by polarography.

It has also been shown that these *S*-cyanocysteinyl residues can be introduced by reaction of —SH groups with cyanogen chloride at pH 7; (*22*) the assumption that this reaction leads directly to the formation of combined 2-amino-2-thiazoline-4-carboxylic acid in the chain (*42*) would seem to be unwarranted.

The *S*-cyanocysteinyl residue can be estimated by observing the liberation of cyanide on treatment with either thiols (reverse of Eq. 2) or sulfite (reverse of Eq. 16). In the reaction of protein —SH with cyanogen halides careful control may be necessary, since these compounds partake of some of the properties of halogens. At pH 8, cyanogen bromide reacts with cysteine to yield mainly 2-amino-2-thiazoline-4-carboxylic acid, together with 20–30% of cystine, but at pH 5 or lower it behaves merely as an oxidant and cystine is formed quantitatively.

It has been suggested that it may be possible to effect a specific fission of the protein chain by conversion of the *S*-cyanocysteinyl to a dehydroalanyl residue, which should then be easily hydrolyzed. The *S*-cyanocysteinyl residue is stable at pH 7 (*42*), but decomposition becomes apparent at pH 10.9 with the appearance of thiocyanate ion. This could arise either by the desired elimination or by a direct displacement by hydroxyl ion with formation of serine. In studies with *S*-cyanoglutathione no evidence has yet been obtained for this latter possibility or for the analogous formation of β-cyanoalanine in cyanide solution. On the other hand the amounts of thiocyanate so far found during treatment of *S*-cyanoglutathione and *S*-cyano-proteins in various alkaline solutions have been sometimes much less than the total of $—CH_2SCN$ groups which has disappeared. At pH values above 12 both SCN^- and CN^- ions are found and some disulfide is formed, presumably by partial hydrolysis to thiol followed by the reverse of Eq. 2 (cf. reference *43*). Further possibilities are hydrolysis of —SCN to $—SCONH_2$, and various cyclizations of the —SCN group onto either of the two adjacent peptide groups. Although liberation of thiocyanate sometimes occurs with great ease, as in the formation of lanthionine (Eq. 4), it is clear that further work will be necessary to establish the exact fate of the RSCN group in alkaline solution.

References

1. F. Sanger, *Biochem. J.* **44**, 126 (1949).
2. J. M. Mueller, J. G. Pierce, H. Davoll, and V. du Vigneaud, *J. Biol. Chem.* **191**, 309 (1951).
3. C. H. W. Hirs, *J. Biol. Chem.* **219**, 611 (1956).
4. P. Alexander, R. F. Hudson, and M. Fox, *Biochem. J.* **46**, 27 (1951).
5. G. Toennies, and R. P. Homiller, *J. Am. Chem. Soc.* **64**, 3054 (1942).
6. C. Earland and C. S. Knight, *Biochim. et Biophys. Acta* **22**, 405 (1952).
7. E. O. P. Thompson, *Biochim. et Biophys. Acta* **15**, 440 (1954).
8. J. M. Swan and E. F. Woods, *Biochim. et Biophys. Acta* **25**, 432 (1957).
9. P. Alexander and R. F. Hudson, "Wool, Its Chemistry and Physics," p. 247. Chapman and Hall, London, 1954.
10. D. M. Greenberg, "Amino Acids and Proteins," p. 567. C. C. Thomas, Springfield, Illinois, 1951.
11. H. Lindley, *J. Am. Chem. Soc.* **77**, 4927 (1955).
12. G. Markus and F. Karush, *J. Am. Chem. Soc.* **79**, 134 (1957).
13. E. Katchalski, G. S. Benjamin, and V. Gross, *J. Am. Chem. Soc.* **79**, 4096 (1957).
14. L. Eldjarn and A. Pihl, *J. Biol. Chem.* **225**, 499 (1957).
15. I. M. Kolthoff, W. Stricks, and R. C. Kapoor, *J. Am. Chem. Soc.* **77**, 4733 (1955).
16. W. E. Savige, I. W. Stapleton, and J. M. Swan, unpublished (1958).
17. A. Neuberger, *Advances in Protein Chem.* **4**, 344 (1948).
18. H. Lindley and J. M. Swan, unpublished (1958).
19. R. Cecil and J. R. McPhee, *Biochem. J.* **60**, 496 (1955).
20. W. Stricks, I. M. Kolthoff, and R. C. Kapoor, *J. Am. Chem. Soc.* **77**, 2057 (1955).

21. R. Cecil and U. E. Loening, *Biochem. J.* **66,** 18P (1957).
22. J. M. Swan, *in* "Current Trends in Heterocyclic Chemistry" (A. Albert, G. M. Badger, and C. W. Shoppee, eds.), p. 65. Butterworths, London, 1958.
23. A. Schöberl, M. Kawohl, and R. Hamm, *Chem. Ber.* **84,** 571 (1951).
24. W. R. Cuthbertson and H. Phillips, *Biochem. J.* **39,** 7 (1945).
25. R. Schiller and R. Otto, *Ber.* **9,** 1637 (1876).
26. E. Fromm, *Ber.* **41,** 3403 (1908).
27. S. Blackburn and F. Challenger, *J. Chem. Soc.* p. 1872 (1938).
28. R. Cecil, *Biochem. J.* **47,** 572 (1950).
29. R. Cecil and J. R. McPhee, *Biochem. J.* **66,** 538 (1957).
30. R. Benesch and R. E. Benesch, *Arch. Biochem. Biophys.* **38,** 425 (1952).
31. I. M. Kolthoff and W. Stricks, *J. Am. Chem. Soc.* **72,** 1952 (1950).
32. W. Stricks, I. M. Kolthoff and N. Tanaka, *Anal. Chem.* **26,** 299 (1954).
33. E. M. Gross and R. Egan, *Federation Proc.* **15,** 265 (1956).
34. J. P. E. Human, *Textile Research J.* **28,** 647 (1958).
35. H. T. Clarke, *J. Biol. Chem.* **97,** 235 (1952).
36. I. M. Kolthoff and W. Stricks, *J. Am. Chem. Soc.* **73,** 1728 (1951).
37. J. M. Swan, *Nature* **180,** 643 (1957).
38. J. L. Bailey, *Biochem. J.* **67,** 21P (1957).
39. W. Stricks and I. M. Kolthoff, *Anal. Chem.* **23,** 763 (1951).
39a. S. J. Leach, *Biochim. et Biophys. Acta* in press (1959).
39b. L. Rosnati, *Gazz. chim. ital.* **75,** 225 (1945).
40. A. Schöberl and G. Bauer, *Angew. Chem.* **69,** 478 (1957).
41. A. Fava and S. Pajaro, *J. Am. Chem. Soc.* **78,** 5203 (1956).
42. W. N. Aldridge, *Biochem. J.* **48,** 271 (1951).
43. A. Schöberl and A. Wagner, *in* "Methoden der organischen Chemie," (E. Müller, ed.), Vol. 9, p. 69. Thieme, Stuttgart, 1955.

Discussion

JENSEN: I will make a comment about your statement about the bovine serum albumin requiring extra copper because the first one may be bound very tightly to sulfhydryl, even though this anticipates what I am going to say tomorrow myself. We observed by spectrophotometric means, and Professor Kolthoff by an amperometric study, that one mole of copper is very tightly bound to the protein, but this binding does not involve the sulfhydryl group at all. This is rather interesting, for usually one thinks of heavy metals binding more tightly with the sulfhydryl group than with any other. If this happens with bovine serum albumin it may happen with other proteins as well, so if one is working with copper, he should be on the lookout for such a phenomenon.

SWAN: I am very pleased to hear this because if we assume that the copper is bound to —SH it is rather a puzzle why this should not allow either easy oxidation to a dimeric disulfide, with subsequent formation of *S*-sulfocysteine, or else act catalytically for the oxidation of thiol produced by sulfite fission of a nearby disulfide, or perhaps even react directly with sulfite and the oxidant (air) to give *S*-sulfocysteine without intervention of the disulfide, thus

$$RSCuOH + SO_3^{--} = RSSO_3^- + Cu^{++} + OH^- + 2(e)$$

If the copper is tightly bound elsewhere, then the necessity for more than one atom

of cupric copper can be understood. Incidentally, it may be worth pointing out that there is evidence for the intermediate formation of cuprous mercaptides in this reaction; here again it can be postulated that the mercaptide is converted directly to *S*-sulfocysteine,

$$RSCu + SO_3^{--} \rightarrow RSSO_3^- + Cu^+ + 2(e)$$

rather than by reoxidation to disulfide followed by further fission.

JENSEN: I think one can say definitely that the first mole of copper is not on the sulfhydryl group.

KOLTHOFF: Experimentally we confirmed that you need one more copper per mole of albumin before you get any catalytic effect. I mean catalytic effect in any oxidation.