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A Method for the Complete S Sulfonation of Cysteine Residues in Proteins*

William W.-C. Chan

ABSTRACT: A new method is described for the complete sulfonation of protein SH groups under mild conditions. The protein is treated with sodium sulfite and catalytic amounts of cysteine in the presence of oxygen and 8 M urea.

When applied to rabbit muscle aldolase, complete sulfonation was obtained within 1 hr. The reaction was shown to be specific for SH groups from studies of the extent of the reaction and the electrophoretic pattern of the product. S-Sulfonated aldolase was enzymatically inactive but after suitable treatment with β-mercaptoethanol was reconstituted to give the fully active enzyme. The 100% regeneration of enzyme activity suggests that the method might be suitable for studies where subsequent recovery of biological activity is desired. In contrast, the S-sulfonated aldolase prepared by two other methods gave little or no activity after similar treatment. The reaction requires the addition of cysteine which may be replaced by β-mercaptoethylamine but not by β-mercaptoethanol or dithiothreitol. Under the conditions studied complete sulfonation occurs in the pH range 7.0–8.5 but little reaction takes place at pH 9.5 or higher. These findings suggest a role for the protonated amino group of cysteine in the reaction mechanism. Lactate dehydrogenase and pepsinogen were also completely sulfonated by this method. It is therefore suggested that the method may be generally applicable to proteins containing cysteine or cystine residues.

Sulfitolysis has been frequently used for the cleavage of disulfide bonds in proteins (Cole, 1967). If the reaction is allowed to proceed in a dissociating medium (*e.g.*, at high concentrations of urea or guanidine hydrochloride) and in the presence of an oxidizing agent, all the half-cystine residues can be converted into the *S*-sulfonate cysteine derivative. The completely S-sulfonated proteins so obtained are useful in the separation of polypeptides since they are stable in neutral and acidic conditions (Swan, 1957). A distinct advantage of

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$$RSSR \xrightarrow{SO_3^{2-}} RSSO_3^- + RS^-$$
$$\uparrow \quad\quad\quad\quad\quad\quad \downarrow$$
$$[O]$$

(RSH = cysteine residues in proteins or other SH compound)

the *S*-sulfonate as a blocking group is its ready removal by excess thiol treatment. In this respect it is similar to disulfides.

The method has not been used to any extent on proteins which contain SH but not disulfide bonds. However, the same reactions are involved, regardless of whether disulfide bonds are present or not because in both cases, oxidation is required for complete sulfonation. For the purpose of modifying SH groups in sequence studies of proteins, the alternative methods of alkylation and performic acid oxidation are undoubtedly superior to sulfonation since the modified groups in these methods are stable to acid hydrolysis. Nevertheless, *S*-sulfonate or mixed disulfides must be used as blocking groups whenever it is desirable to remove them at a later stage.

The present work was undertaken in conjunction with studies on the subunits of rabbit muscle aldolase. It has been shown that aldolase can be reversibly dissociated into subunits (Stellwagen and Schachman, 1962; Deal *et al.*, 1963a). Under certain conditions, the subunits will reassociate to give the native enzyme with essentially complete recovery of activity. However, when conventional methods (Swan, 1957; Katsoyannis *et al.*, 1967) were used to prepare S-sulfonated aldolase and the *S*-sulfonate groups were subsequently removed, only little or no enzyme activity could be recovered by reassociation of the subunits. This suggested that these methods might have caused irreversible physical or chemical change to the protein. An attempt was therefore made to find alternative procedures for sulfonation. This paper describes a simple method which leads to complete S sulfonation under mild conditions. A striking property of the method is that a 100% recovery of aldolase activity is realized when the S-sulfonated derivative of aldolase is treated with excess mercaptoethanol. Although most of the results described above were obtained using aldolase, other evidence presented indicates that the method should be generally applicable to proteins containing cysteine or cystine residues.

Materials and Methods. Fructose diphosphate aldolase (4.1.2.13) was prepared from rabbit muscle by the method of Taylor *et al.* (1948) and recrystallized three times. Aldolase activity was assayed spectrophotometrically as described by Racker (1947) and the unit of activity was defined as the amount required to cleave 1 μmole of FDP/min. The molecular weight of aldolase was taken as 160,000 (Kawahara and Tanford, 1966; Sia and Horecker, 1968). Lactate dehydrogenase (1.1.1.27) from rabbit muscle (lot 76153, specific activity 425 units/mg) was supplied by Calbiochem. Pepsinogen was a product of Worthington Biochemical Corp. Urea (A.C.S. grade) was recrystallized from 95% ethanol, washed with ether, and dried in an oven at 40°. Cysteine hydrochloride, EDTA, and sodium sulfite were reagent grade and supplied by the Fisher Scientific Co. Tris base and *N*-ethylmaleimide were obtained from Sigma Chemical Corp. The reagent grade guanidine hydrochloride used was made by J. T. Baker and Co. and was found to be completely soluble in water. ^{35}S-Labeled sodium sulfite (lot 328–118) was purchased from New England Nuclear Corp. The initial specific activity was 10.5 mCi/mmole. It was diluted by adding carrier until the specific activity was close to 100,000 cpm/μmole as determined below.

Determination of Protein Concentration. Protein concentration was assayed either spectrophotometrically from the absorption at 280 mμ in 0.1 N NaOH or by the trichloroacetic acid method of Bücher (1947). The extinction coefficient, $E^{1\%}_{280\,m\mu}$, in 0.1 N NaOH was taken to be 9.1 for aldolase (Baranowski and Niederland, 1949) and 8.85 for lactate dehydrogenase (Pfleiderer and Jeckel, 1957). The trichloroacetic acid method was calibrated against the spectrophotometric method using solutions of aldolase. For S-sulfonated proteins which are insoluble in water the method was modified by adding urea to a 2 M final concentration. The modified method was similarly calibrated.

Determination of Radioactivity. Aliquots of solutions were dried on filter paper strips or Millipore filters and placed in scintillation vials. To each vial was added 15 ml of a toluene solution containing 4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis[2-(5-phenyloxazolyl)]-benzene per l. The vials were counted in a Nuclear-Chicago, Model Unilux, scintillation counter. Counting efficiency was approximately 60%.

Measurement of the Incorporation of ^{35}S-Labeled Sulfite into Proteins. Three different procedures were used and all gave identical results. In one method, after the reaction the mixture was dialyzed exhaustively against water. The protein was then dissolved in 0.1 N NaOH and protein concentration was determined by measuring the absorption at 280 mμ. Aliquots of the same solution were dried on filter paper strips for radioactivity counting.

Alternatively, after the reaction the protein was precipitated by adding trichloroacetic acid to a final concentration of 10% and centrifuged. The precipitate was washed by redissolving in 1 ml of 90% formic acid, then diluted to 3 ml, and reprecipitated by adding trichloroacetic acid and centrifuged. The washing procedure was repeated three times. The protein was finally washed with acetone and then with ether, dried, and dissolved in 0.1 N NaOH. Protein and radioactivity were determined as described above.

In the third method, a 0.1-ml aliquot of the reaction mixture was added to 3 ml of 10% trichloroacetic acid solution. After 5 min the trichloroacetic acid solution was filtered through Millipore HAWP 02400 filters (pore size 0.45 μ) and washed thoroughly with 10% trichloroacetic acid solution. The filters were dried and counted for radioactivity. The amount of protein was calculated from the concentration in the original reaction mixture assuming complete precipitation and transfer. The specific activity of the ^{35}S-labeled sulfite used was determined using both Millipore filters and filter paper strips and the appropriate value was used for each method.

Regeneration of Free SH Groups and Reassociation of the Subunits of Aldolase. Lyophilized S-sulfonated aldolase (0.5–1.0 mg/ml) was dissolved in 1 M Tris-HCl solution (pH 7.5) containing 4 M urea. β-Mercaptoethanol was added (final concentration 0.7 M) and the mixture was incubated for 2 hr at 25°. The protein subunits were then allowed to reassociate by diluting the mixture ten times with a 1 M Tris-HCl solution (pH 7.5) containing bovine serum albumin (2.5 mg/ml), EDTA (25 mM) and β-mercaptoethanol (10 mM). The enzyme activity was determined after approximately 15 min.

Disc gel electrophoresis was carried out with a Canalco Model 12 apparatus using the polyacrylamide system of Davis (1964). Recrystallized urea was added to all the solutions used (final concentration 8 M) except the buffer in the reservoirs.

Results

Incorporation of ^{35}S-Labeled Sulfite into Aldolase. The oxidation of SH groups by molecular oxygen is known to be catalyzed by copper and *o*-phenanthroline (Kobashi and Horecker, 1967). Initially this reaction was used to promote the formation of disulfide bonds. Cysteine was added to facilitate the formation of mixed disulfides since for steric reasons not all of the SH groups in aldolase could be expected to form disulfide bonds with one another. In this experiment, the reaction mixture contained 6.3×10^{-3} M aldolase, 0.1 M $Na_2{}^{35}SO_3$ (specific radioactivity 112,000 cpm/μmole), 2×10^{-7} M $CuSO_4$, 0.1 M Tris-HCl (pH 8.4), and 8 M urea. After incubation at 25° in an open vessel, the mixture was exhaustively dialyzed against water. The specific radioactivity of the dialyzed protein was found to be 19,300 cpm/mg which represents an incorporation of 27.5 sulfonate groups/mole of aldolase. Since aldolase has been shown (Swenson and Boyer, 1957) to contain approximately 28 SH groups/mole, the results, therefore, indicated that complete reaction had taken place. In a control experiment, aldolase was first treated with excess *N*-ethylmaleimide in 8 M urea, dialyzed, and finally treated with the above sulfonation procedure. The incorporation of radioactivity in this case represents less than 0.1 sulfonate group/mole showing that incorporation occurred specifically at cysteine residues.

Regeneration of free SH Groups in S-Sulfonated Aldolase. The ^{35}S-labeled protein prepared above was treated with β-mercaptoethanol in 4 M urea solution. A portion of the product was then dialyzed exhaustively against water and assayed for protein-bound radioactivity. The remaining portion was diluted to allow the subunits to reassociate and assayed for FDP aldolase activity. Details of these procedures are described in the Methods section.

The dialyzed, thiol-treated protein was found to contain less than 0.1 of ^{35}S-labeled sulfite group/mole of aldolase. The *S*-sulfonate groups were therefore completely removed from the protein. The enzyme activity of the reconstituted enzyme was 9.5 μmoles of FDP cleaved/min per mg of protein, which is identical with the activity of the native enzyme assayed under the same conditions. In control experiments where S-sulfonated aldolase was treated in exactly the same way except that β-mercaptoethanol was replaced by water, no aldolase activity was detectable even at ten times the usual protein concentration. The high recovery of aldolase activity is entirely reproducible and may be obtained from the S-sulfonated derivative even after several months of storage at 4° as a lyophilized powder. These results suggest that residues in the enzyme which are essential to activity have not been irreversibly modified by the above treatment.

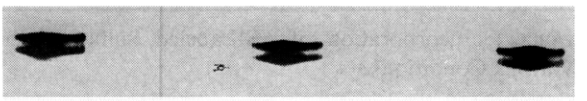

FIGURE 1: Disc gel electrophoretic patterns of S-sulfonated aldolase and CM-aldolase. Left: *S*-CM-aldolase; middle: S-sulfonated aldolase; right: mixture of previous two aldolase derivatives.

Electrophoretic Pattern of S-Sulfonated Aldolase. The specific nature and the extent of the above reactions was further shown by examination of the products in disc gel electrophoresis in 8 M urea. As shown in Figure 1, the electrophoretic pattern of S-sulfonated aldolase consists of two bands and is identical with that of *S*-CM-aldolase. The similarity in the electrophoretic migration of the two derivatives was confirmed when the same pattern was obtained by electrophoresis of a mixed sample containing equal amounts of each derivative. This indicates that the total net ionic charge is the same in both cases. At the alkaline pH of the electrophoresis, the CM and the sulfonate groups would both contribute one negative charge per group. This means that the number of sulfonate groups is equal to the number of CM groups and indicates that complete sulfonation has taken place.

The two components in CM-aldolase have been shown to correspond to two carboxymethylated subunits (α and β) which differ in primary structure (Chan *et al.*, 1967; Morse *et al.*, 1967). By analogy, the two components in S-sulfonated aldolase are presumably the sulfonated derivatives of these subunits. The fact that only two components were obtained indicates that side reactions which result in extensive change in the net charge of the protein did not occur.

The Nature of the Sulfonation Reaction and the Catalytic Requirements. The incorporation of ^{35}S-labeled sulfite into aldolase was used to study the various requirements of the reaction. The results are summarized in Table I. Surprisingly, it was found that the addition of either *o*-phenanthroline or copper or both did not affect the extent of the reaction. However, if cysteine was omitted, the amount of incorporation was less than 10%. The role of molecular oxygen was studied by performing the reaction under a nitrogen atmosphere. The extent of the reaction was greatly reduced indicating that oxygen was essential for the reaction. The limited amount of sulfite incorporated was probably due to residual traces of oxygen since in this experiment no attempt was made to remove dis-

TABLE I: Incorporation of ^{35}S-Labeled Sulfite under Various Conditions.[a]

Additions	Sulfite Groups Incorpd/Mole	% of Total Protein SH
None	27.3	98
$CuSO_4$	27.5	98
$CuSO_4$ + *o*-phenanthroline	26.5	95
Cysteine omitted	2.3	8
Cysteine omitted but with $CuSO_4$ + *o*-phenanthroline	3.1	11
N_2 atmosphere[b]	11.1	40

[a] The reaction mixture contained dialyzed aldolase (2 mg/ml), Tris-HCl (0.1 M) (pH 8.4), urea (8 M), cysteine (2×10^{-4} M), and $Na_2{}^{35}SO_3$ (0.05 M) (80,000 cpm/μmole) except where otherwise stated. After incubation at 25° for 1 hr in an open vessel the protein was precipitated with trichloroacetic acid, washed, and radioactivity was determined. [b] A closed Warburg flask was used, and after introducing a nitrogen atmosphere the reaction was started by tipping cysteine into the mixture containing the remaining reagents.

TABLE II: The Effect of Various SH Compounds on the Sulfonation of Aldolase.[a]

SH Compound Added	Sulfite Groups Incorpd/Mole	% of Total Protein SH
None	2.3	8
Cysteine (2×10^{-4} M)	27.8	99
β-Mercaptoethanol (2×10^{-4} M)	1.6	6
β-Mercaptoethanol (2×10^{-3} M)	0.8	3
Dithiothreitol (2×10^{-4} M)	8.2	30
β-Mercaptoethylamine (2×10^{-4} M)	27.8	99

[a] The reaction conditions used were identical with those described in Table I except that cysteine was omitted.

solved oxygen completely from the solutions of the reactants.

S-Sulfonated aldolase prepared in the absence of copper and *o*-phenanthroline was compared with the same derivative prepared in the presence of these reagents. The electrophoretic patterns of the two samples were indistinguishable and the recoveries of enzyme activity upon treatment with excess mercaptoethanol were identical. The only difference observed was that after dialysis the protein derivative prepared in the presence of copper still contained significant amounts of copper as shown by color reaction with sodium diethyldithiocarbamate. Since this may be an undesirable contamination in many cases, copper and *o*-phenanthroline were omitted in all subsequent experiments.

The Effect of Other Sulfhydryl Compounds. Since cysteine was shown to be necessary for the reaction, other SH compounds were tested for their ability to promote sulfonation. As shown in Table II, there appears to be some specificity in the requirement for a SH compound. β-Mercaptoethanol was completely ineffective at two different concentrations and dithiothreitol (Cleland's reagent) had only a slight effect on the incorporation. On the other hand, β-mercaptoethylamine was similar to cysteine in giving a complete reaction. The significance of these results is discussed in a subsequent section.

Other Properties of the Reaction. Figure 2 shows the time course of the sulfonation of aldolase. Under the conditions employed, the reaction was complete in 1 hr. The rate of the reaction was found to vary considerably with the extent to which the reaction mixture was in contact with air. For a typical, large-scale preparation using 200 mg of aldolase in 40 ml of 8 M urea solution, it was found necessary to direct a slow stream of air over the mixture with constant stirring.

The effect of varying the concentration of cysteine is presented in Figure 3. Complete incorporation was obtained at cysteine concentrations of 2×10^{-3} and 2×10^{-4} M. The amount of incorporation then decreased with either higher or lower cysteine concentration. These effects are discussed later in connection with the mechanism of the reaction.

Since the oxidation of SH groups by molecular oxygen is known to be catalyzed by traces of heavy metals, the effect of chelating agents was investigated. The results are given in Table III. EDTA was found to have a partially inhibitory effect at concentrations of 10^{-3} M or higher. The effect could be abolished by adding Cu^{2+} or Mg^{2+} ions. On the other hand, sodium diethyldithiocarbamate which is known to bind strongly to copper does not inhibit significantly even at 10^{-2} M concentration. Although the participation of trace metals is not entirely ruled out by these results, the fact that EDTA inhibits only at high concentrations suggests that it is of secondary importance.

Table IV shows the effect of pH on the sulfonation reaction. Complete sulfonation was still obtained when the pH was reduced to 7.0. At pH lower than 7.0 the extent of the reaction decreased in agreement with the suggestion that one of the reacting species is $SO_3{}^{2-}$ (Cecil and McPhee, 1955) and the pK_2 of sulfite is about 7. The decrease in incorporation at or above pH 9.5 is interesting in that it suggests that a protonated amino group in cysteine may be necessary for its effect on the reaction.

Comparison with Other Methods of Sulfonation. In

TABLE III: The Effect of EDTA and Sodium Diethyldithiocarbamate on the Sulfonation of Aldolase.[a]

Additions	Sulfite Groups Incorpd/ Mole	% of Total Protein SH
None	27.5	98
EDTA (10^{-4} M)	26.8	96
EDTA (10^{-3} M)	20.2	72
EDTA (10^{-2} M)	11.5	41
EDTA (10^{-2} M) + $CuSO_4$ (10^{-2} M)	27.0	96
EDTA (10^{-2} M) + $MgSO_4$ (10^{-2} M)	27.2	97
Sodium diethyldithiocarbamate (10^{-4} M)	27.2	97
Sodium diethyldithiocarbamate (10^{-2} M)	24.8	89

[a] The reaction conditions were identical with those described in Table I.

preliminary experiments, it was found that little or no aldolase activity could be recovered from S-sulfonated aldolase prepared by the cupric ammonium sulfite method (Swan, 1957) and by the use of tetrathionate in combination with sulfite (Bailey and Cole, 1959; Katsoyannis *et al.*, 1967). However, previous workers have used these methods successfully to prepare the S-sulfonated derivatives of the A and B chains of insulin (Dixon and Wardlaw, 1960; Katsoyannis *et al.*, 1967) and insulin activity was obtained from these derivatives after treatment with excess thiol and reoxidation. The extent of recovery of biological activity is important in many applications of the sulfonation reaction. It was, therefore, of interest to compare the above-mentioned methods using as a criterion the subsequent recovery of aldolase activity under identical conditions. The results are shown in Table V. In two separate experiments, no aldolase activity was recovered after using the tetrathionate–sulfite method. The failure to obtain activity was probably not due to the effect of substituting guanidine hydrochloride (8 M) for urea, since when the method described in this paper was applied in guanidine hydrochloride (8 M), 80% recovery was obtained. The cupric ammonium sulfite method was found to lead to the recovery of only a small fraction of the original activity. Thus, in the case of aldolase the method described in this paper is far superior in the extent of recovery of activity.

TABLE IV: The Effect of pH on Sulfonation.[a]

Buffer Used (pH)	% of Total Protein SH
Sodium acetate (5.0)	26
Sodium citrate (6.0)	47
Sodium phosphate (7.0)	98
Sodium phosphate (7.5)	97
Tris-HCl (7.5)	102
Tris-HCl (8.4)	102
$NaHCO_3$–CO_3 (9.5)	21
$NaHCO_3$–CO_3 (10.0)	15

[a] The reaction conditions were identical with those described in Table I except for changes in the buffer used. The buffer concentration was 0.1 M in all experiments.

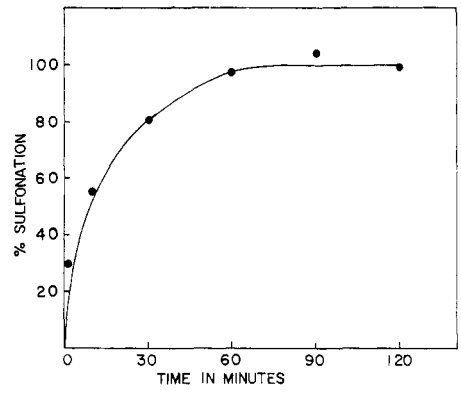

FIGURE 2: Time course of the sulfonation of aldolase. The incorporation of 28 sulfite groups/mole of aldolase is taken as 100%. The reaction conditions were as described in Table I. At various times indicated, aliquots were removed and added to 10% trichloroacetic acid to stop the reaction. Incorporation was determined by the Millipore method (see Materials and Methods).

Application of the Method to Other Proteins. In order to test if the above sulfonation method was generally applicable, two other purified proteins were similarly sulfonated. In one experiment lactate dehydrogenase (1.1.1.27) from rabbit muscle was used. The commercial preparation, which contained mainly the M_4 isozyme with small amounts of the M_3H hybrid, was passed through a Sephadex G-25 column to remove $(NH_4)_2SO_4$.

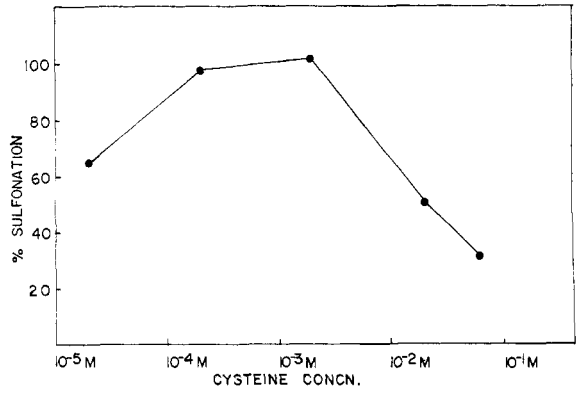

FIGURE 3: The effect of varying cysteine concentration on the extent of sulfonation of aldolase. The details described in the legend to Figure 2 also apply here except that cysteine concentration is varied.

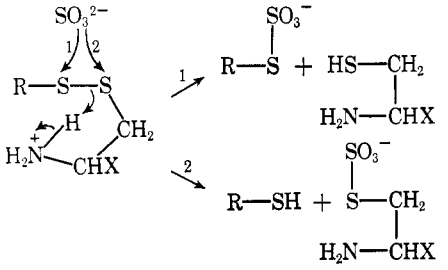

FIGURE 4: A possible explanation of the effect of cysteine and β-mercaptoethylamine. R represents protein and X = CO_2 or H for cysteine and β-mercaptoethylamine, respectively.

This sample was sulfonated with radioactive sulfite as described for aldolase.

The incorporation was 0.119 μmole of sulfite/mg of protein or 16.1 equiv for a molecular weight of 135,000 daltons. Other workers (Di Sabato *et al.*, 1963; Pfleiderer *et al.*, 1959) have reported the presence of 16 SH groups/mole of the enzyme and the absence of disulfide bonds. The above data therefore show that lactate dehydrogenase was completely sulfonated by this method.

Commercial pepsinogen was similarly treated with ^{35}S-labeled sulfite. The incorporation of 5.8 groups of sulfite/mole again indicates complete reaction since the molecule of pepsinogen is known to contain 3 disulfide bonds (Arnon and Perlmann, 1963). These results suggest that the method should be applicable to the sulfonation of proteins in general. In this method, the native configuration of the protein may be regarded as unimportant since the reaction takes place in dissociating medium.

Discussion

It is evident from the above results that the SH groups of aldolase are rapidly and completely sulfonated when treated with sodium sulfite and catalytic amounts of cysteine in 8 M urea solution. Three lines of evidence indicate that the reaction is specific for SH groups. (1) The number of sulfonate groups incorporated is equal to the number of SH groups known to be present in aldolase. (2) All of these sulfonate groups are removed upon treatment with excess thiol. (3) The electrophoretic pattern of the product is identical with that of CM-aldolase, as would be expected from a consideration of the net charge in these derivatives. Although modification of other residues under the reaction conditions cannot be ruled out, there appears to be no sulfonation of residues other than cysteine since *N*-ethylmaleimide-treated aldolase did not incorporate significant radioactivity when similarly treated with ^{35}S-labeled sulfite. Carbamylation is unlikely to occur to a significant extent since urea solutions freshly prepared from recrystallized urea are known to be relatively free from cyanate (Stark *et al.*, 1960). The complete recovery of enzyme activity suggests that residues which are essential for enzyme activity have remained intact.

The reaction requires the addition of certain SH compounds and the presence of molecular O_2 and thus indicates that the reaction takes place *via* the formation of disulfide bonds and subsequent sulfitolysis. Although the oxidation of SH compounds in aqueous solutions by molecular O_2 has been much studied, the mechanism of the reaction is far from clear (Cecil and McPhee, 1959). The system studied here is further complicated in that the formation of protein–protein disulfide bonds, protein–cysteine-mixed disulfide, and cysteine are all possible. At any moment, during the reaction, all these components react reversibly with sulfite, unoxidized cysteine, and protein SH. No attempt is therefore made to formulate precisely the course of the reaction on the basis of available data. However, since the initial presence of cysteine is required, the reaction clearly does not proceed mainly *via* protein–protein disulfides as intermediates. The extensive formation of these bonds in aldolase subunits which have a molecular weight of approximately 40,000 daltons (Kawahara and Tanford, 1966; Sia and Horecker, 1968) will encounter considerable steric hindrance.

The reaction is likely to involve the initial formation of cystine and the mixed disulfide between cysteine and protein SH. Subsequent sulfitolysis then gives cysteine sulfonate and the S-sulfonated protein. The observation that cysteine can be replaced by β-mercaptoethylamine but not by β-mercaptoethanol or dithiothreitol indicates a role for the neighboring amino group. The decrease in the extent of the reaction at pH 9.5 further suggests that the amino group must be protonated. It has been reported previously (McPhee, 1956) that disulfide molecules containing a positive charge undergo much faster sulfitolysis. In the case of the mixed disulfide between protein SH and cysteine, a possible explanation is presented in Figure 4. The reaction proceeds *via* the nucleophilic attack of the SO_3^{2-} ion on one of the sulfur atoms. This attack is facilitated by the ability of the displaced sulfur atom to pick up a proton immediately from the neighboring amino group. Since aldolase was shown to undergo rapid sulfonation, it is possible that in Figure 4

TABLE V: Recovery of Aldolase Activity from S-Sulfonated Aldolase.[a]

Method of Sulfonation	% Recov of Aldolase Act.
Tetrathionate+sulfite in guanidine hydrochloride[b]	0
Cupric ammonium sulfite in 8 M urea[c]	22
Cysteine+O_2+sulfite in 8 M urea[d]	100
Cysteine+O_2+sulfite in guanidine hydrochloride[d]	80

[a] Aldolase was sulfonated at 1 mg/ml in each case. The products were dialyzed and then treated as described in Methods for the regeneration of enzyme activity. [b] Katsoyannis *et al.* (1967). [c] Swan (1957). [d] As described in this paper.

reaction 1 takes place in preference to reaction 2. However, there are no obvious mechanistic reasons for such preference and an examination of molecular models has failed to reveal any steric reasons. Kinetic studies using structural analogs of cysteine are being conducted in this laboratory in order to clarify the reaction mechanism.

The proposed role of cysteine is in agreement with the effect of varying cysteine concentration shown in an earlier section. At low cysteine concentrations, the formation of mixed disulfide is limited and protein sulfonation is therefore incomplete. On the other hand at concentrations higher than the optimal range, a considerable amount of cysteine remains unoxidized at the end of the incubation period thus tending to reverse the reaction.

Previous work (Stellwagen and Schachman, 1962) has shown that aldolase subunits exist as "markedly disorganized" polypeptides in solutions containing $\geqslant 4$ M urea. Thus, during S sulfonation and the subsequent removal of sulfonate groups both of which take place in 8 M urea, the aldolase polypeptides assume configurations essentially different from its native structure. The introduction of 28 negative charges of considerable bulk must be expected to lead to a further departure from the native state especially since aldolase has been reported to contain 7–8 (Kowal *et al.*, 1965) or 16 (Stellwagen and Schachman, 1962) "buried" SH groups. That the native enzyme was obtained after reconstitution can be inferred from previous work using similar conditions (Stellwagen and Schachman, 1962) and is supported by the complete recovery of activity. Thus, there appears to be a spontaneous refolding of the aldolase polypeptides into the native configuration. This represents one of an increasing number of cases (Anfinsen and Haber, 1961; Deal *et al.*, 1963b) where proteins have been reconstituted from a denatured state. The result is consistent with the hypothesis that the native structure of a protein is determined only by its amino acid sequence (Crick, 1958).

The sulfonation reaction was applied by Dixon and Wardlaw (1960) in an elegant separation of the A and B chains of insulin. The use of the reaction is, however, not restricted to the cleavage of disulfide bonds but may include the reversible blocking of SH groups. An important consideration in many such applications is the recovery of biological activity. Hitherto complete sulfonation of proteins was attained by using either cupric ammonium hydroxide at pH 10 (Swan, 1957) or sodium tetrathionate (Bailey and Cole, 1959) as oxidizing agents. As shown in this paper, the recovery of aldolase activity after exposure to these sulfonation procedures is low. In contrast, the method described here gave a complete recovery of aldolase activity. It remains to be determined if such differences are shown when the methods are applied to other proteins. The reasons for these differences are not known. A previous report (Bailey and Cole, 1959) indicates that there may be some modification of tryptophan residues when protein is treated with tetrathionate. At the alkaline pH of the cupric ammonium hydroxide method, some deamidation or peptide hydrolysis may occur. The method described in this paper thus represents an alternative procedure which may have considerable advantages over existing methods.

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Spectral Studies of Iron Coordination in Oxidized Compounds of Hemoproteins. Difference Spectroscopy below 250 mμ*

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ABSTRACT: The feasibility of observing the spectral behavior of protein groups in the coordination sphere of the iron in hemoproteins has recently been demonstrated. Application of the method, difference spectroscopy below 250 mμ, to oxidized compounds of hemoproteins is reported in this paper. The absolute absorption spectrum (250–450 mμ) of bacterial catalase compound I is displayed. Ultraviolet difference spectra (210–280 mμ) of compounds III of metmyoglobin and methemoglobin and compounds I and II of bacterial catalase and horseradish peroxidase *vs.* the free hemoproteins are shown. Compounds III of metmyoglobin and methemoglobin, and compounds II of bacterial catalase and horseradish peroxidase have one difference band which peaks in the region 237–247 mμ. Absorption in this region is assigned, in part, to a transition involving histidine in the fifth coordination position, and in the other part to a charge transfer transition involving porphyrin. Compound I of bacterial catalase has two difference bands (235 and 224 mμ), while I of horseradish peroxidase has a broad band which is probably the sum of two bands. The spectral properties of compounds I are like those of ferric hemoprotein complexes. The spectral properties of compounds II and III support the other evidence that these are ferryl structures.

Several years ago, in the course of investigating possible involvement of tyrosine in the formation of compound I of catalase, we recorded difference spectra *vs.* free enzyme down to a wavelength of 250 mμ and noted that the absorbance rose just as this wavelength was approached. We were thereby prompted to look deeper into the ultraviolet region, and found the bands described in this paper. In checking possible explanations of these bands, we were led to try more fundamental experiments with hemoprotein complexes (Brill and Sandberg, 1968). In the latter research, the groups involved have been identified on the basis of data from simple ligands. In this paper, the difference spectra of peroxide compounds *vs.* free proteins are reported and used to describe with more assurance than previously the coordination spheres of the iron in the oxidized compounds of MetMb,[1] MetHb, BMC, and HRP.

Only one spectroscopically distinct compound (denoted in the literature as "compound III") has been observed upon reaction of MetMb with any of the substrates H_2O_2, methyl hydroperoxide, or ethyl hydroperoxide. The stoichiometry of reduction of compound III by ferrocyanide indicates that this compound has one oxidizing equivalent above free MetMb (George and Irvine, 1952, 1953). Since the peroxide substrate has two oxidizing equivalents, one of the equivalents is not retained by the heme group.

Similarly, only one spectroscopically distinct compound (also called "III") has been observed for the reaction of MetHb with H_2O_2 (Keilin and Hartree, 1951; Dalziel and O'Brien, 1954). The oxidation state of MetHb III has not yet been determined. It is unlikely to differ from that of MetMb III.

The reaction of HRP with either H_2O_2 (concentration less than 1 mM so that a third compound of peroxidase does not form) or alkyl hydroperoxides produces two spectroscopically distinct compounds, labeled "I" and "II" on the basis of order of appearance. The stoichiom-

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[1] Abbreviations used: MetMb, horse heart ferrimyoglobin; MetHb, horse ferrihemoglobin; BMC, bacterial micrococcus catalase; HRP, horseradish peroxidase; HBC, horse blood catalase; suffixes I, II, and III, compounds I, II, and III.