Studies on the Reaction of Sulfite with Proteins*

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Structural investigations on proteins frequently require as a first step the cleavage of the disulfide bonds that link half-cystine residues. Oxidation with performic acid has been used for this purpose first by Sanger (2) and later by Hirs (3), before a detailed investigation of the sequences of the amino acid residues in insulin and ribonuclease, two proteins that are devoid of tryptophan. Special studies (5-9) of this reaction have succeeded in rendering the yield of cystic acid and methionine sulfone virtually quantitative, and in minimizing undesirable side reactions with other amino acids, such as tyrosine. No conditions have been found, however, that will prevent the oxidative degradation of tryptophan. Since tryptophan would be expected to remain unaffected by reductive procedures capable of cleaving disulfide bonds, reductive methods have been investigated in several laboratories, including this one (1, 6-11). One of the procedures is based upon the well known reaction of sulfite with disulfide bonds in proteins noted in 1907 by Heffter (12) and studied later by Mirsky and Anson (13). The course of the reduction was described by Clarke (14) and by Lugg (15) (cf. also (16)), and the reaction has since been studied in detail by a number of investigators (17-20). Much of the work in peptide and protein chemistry in which sulfite has been used is reviewed by Cecil and McPhee (18).

In a recent paper in which the sulfite cleavage of disulfide bonds was considered in detail, Swan (9) proposed the use of cupric ions of 2 X 10-3 M in the disodium salt of ethylenediaminetetraacetic acid. Potassium-o-iodosobenzoate (0.5 M) solution was prepared daily from NaOH and o-iodosobenzoic acid obtained from A. D. Mackay, Inc. Sodium tetrathionate was prepared from sodium thiosulfate and iodine, as described by Gilman et al. (21), and 0.5 M solutions were adjusted to pH 7.0 with NaOH. The solid could be stored at 0°C for several days with only slight decomposition, but the solutions were considerably less stable. AgNO3 was Merck primary standard and the tris(hydroxymethyl)aminomethane, Sigma '121', was obtained from the Sigma Chemical Company. Urea was Merck analytical reagent which was purified by passing an 8 M solution over a bed of Amberlite MB-1 cation and anion exchange resin (such solutions were found to be stable for 10 to 15 days at 4°C). The need for this purification has been pointed out (22). Guanidine hydrochloride solutions were prepared from guanidine carbonate (Eastman) which had been recrystallized from water and ethanol. N-Ethylemaleimide was purchased from Mann Research Laboratories.

Oxidized and reduced glutathione were obtained from the Schwarz Laboratories. The L-cystine used was Merck reagent grade. β-Mercaptoethyamine hydrochloride was a sample supplied by Evans Chemetics, Inc. Beef insulin was the kind gift of Dr. Otto K. Behrens of the Lilly Research Laboratories. The ribonuclease was obtained from Armour (Lot 381-059). Trypsinogen (Lot TG 521) and chymotrypsinogen (Lots CG 52830 and CG 541) were obtained from the Worthington Biochemical Sales Company.

Amperometric Titration—Sulphydryl groups were determined by amperometric titrations which were carried out with the aid of a slight modification (23) of the apparatus and technique of Benesch et al. (22).

In the assay for disulfides, samples containing 1 to 2 micro-equivalents of disulfide groups in 0.2 to 0.5 ml of 8 M urea buffered at pH 7.4 with 0.2 M Tris-HNO3, were treated with 25 to 50 microequivalents of Na2S03 in 1.0 M or 0.5 M solution. After a given time, the sulphydryl groups liberated were titrated with freshly prepared 2 X 10-4 M AgNO3 added in 0.05 ml increments. Most of the titrations were performed in 20 ml of 8 M urea buffered in the apparent range pH 7.2 to 7.4 with a total buffer (Tris) concentration of 0.2 M and a KCl concentration of 0.01 M.

The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

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* A preliminary report of parts of this work has been presented at a meeting of the Biochemical Society (1).

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Preparation of S-sulfo-cysteine and S-sulfo-glutathione—To 10 µmoles of cystine or oxidized glutathione in 2 ml of water were added about 350 µmoles each of Na$_2$SO$_3$ and iodosobenzoate or tetrathionate (sulfite and oxidized glutathione each in 0.5 M solution). After 3 hours at room temperature, sul fate was removed from the reaction mixture by the addition of barium acetate, and cations were removed by passing the mixture through a bed of the acid form of Dowex 50-X8. The extent of the reaction was detected by chromatographing an aliquot of the reaction mixture on a column of Dowex 1-X8, and comparing the amount of the cystine or glutathione remaining (eluted at the column volume) with the amount of S-sulfonate formed. Either of the S-sulfonates may be determined individually on a 10 x 0.9-cm. column with 0.1 N HCl as eluent. When present together, the S-sulfonates may be separated on a 5 x 0.9-cm. column with 0.1 N sodium chloroacetate at pH 3.1 as eluent, a system that also has been used to determine cysteic acid. Waley's (24), in his recent characterization of S-sulfo-glutathione as a normal constituent of calf lens, has used columns of Dowex 1 and pyridine formate at pH 5.4 for the chromatography.

Preparation of S-sulfo-proteins—The procedure differs somewhat from the one described by Pechère et al. (11), notably by the use of a lower pH, a slightly higher temperature, and a different oxidizing agent. In a typical case, 100 mg of protein dissolved at 38°C in 5 ml of 8 M (or saturated) urea buffered at pH 7.4 with 0.2 M Tris buffer. The disulfide bonds were reduced initially with 0.62 ml of 1.0 M Na$_2$SO$_3$ (or 1.25 ml of 0.5 M Na$_2$SO$_3$ in 8 M urea) at pH 7.4 for 10 minutes, after which 1.25 ml of 0.5 M iodosobenzoate in 8 M urea were added in order to reoxidize the sulfhydryl groups that had been formed. After another interval of 10 minutes, the Na$_2$SO$_3$ treatment was repeated and further oxidation-reduction cycles were completed. The solution, which occasionally was turbid, was then dialyzed against three changes of distilled water with stirring of the reaction mixture by the addition of barium acetate, and cations were removed by passing the mixture through a bed of the acid form of Dowex 50-X8. The extent of the reaction was detected by chromatographing an aliquot of the reaction mixture on a column of Dowex 1-X8, and comparing the amount of the cystine or glutathione remaining (eluted at the column volume) with the amount of S-sulfonate formed. Either of the S-sulfonates may be determined individually on a 10 x 0.9-cm. column with 0.1 N HCl as eluent. When present together, the S-sulfonates may be separated on a 5 x 0.9-cm. column with 0.1 N sodium chloroacetate at pH 3.1 as eluent, a system that also has been used to determine cysteic acid. Waley's (24), in his recent characterization of S-sulfoglutathione as a normal constituent of calf lens, has used columns of Dowex 1 and pyridine formate at pH 5.4 for the chromatography.

Amino Acid Analyses—Proteins were hydrolyzed with 6 N HCl in evacuated sealed tubes at 110°C, and the hydrolysates were analyzed either by the procedure of Moore et al. (25) or with the recording equipment of Spackman et al. (26). Tryptophan was determined by the method of Drèze (27).

RESULTS

Reaction of Sulfite with Cystine and Oxidized Glutathione—As the first step in studying the reaction of sulfite with disulfides, the kinetics of the reduction of oxidized glutathione (26) at room temperature in 8 M urea with 0.2 M Na$_2$SO$_3$ were followed by amperometric titration. The results, given in Fig. 1, confirm the expected cleavage with the formation of one sulfhydryl group per molecule of disulfide. The reduced glutathione formed is relatively stable under these conditions unless oxidizing agents are present, but when cystine is reduced, the data in Fig. 1 show that the sulfhydryl groups formed in the cleaving of the disulfide bonds are so rapidly reoxidized, presumably to the disulfide, that a stoichiometric quantity of sulfhydryl groups is not observed.

To bring about quantitative conversion of cystine, cysteine, and reduced or oxidized glutathione to the corresponding S-sulfonates, all that is required, in addition to a 30- to 40-fold excess of sodium sulfite, is the presence of a similar excess of an oxidizing agent such as iodosobenzoate or tetrathionate. The extent of the reaction was followed by chromatography on columns of the anion exchange resin Dowex 1-X8. Cystine gave a 94 per cent yield of the S-sulfonate. The crystalline S-sulfonate of cysteine was described by Clarke (14) and the substance was synthesized in the present investigation and its ninhydrin color yield (29) determined. Values of 0.73 and 0.83, relative to leucine, were obtained, depending upon whether the substance was initially dissolved in 0.1 N HCl or in the sodium chloroacetate buffer at pH 3.1 used as eluent in the chromatogram shown in Fig. 2. Upon hydrolysis of cysteine-S-sulfonate with 6 N HCl at 110°C for 22 hours, cystine is recovered in a yield of 80 percent, which is the same as the recovery of 81 per cent obtained when cysteine itself is treated in this manner.

Attempts to prepare a crystalline S-sulfonate of glutathione were unsuccessful. When the ninhydrin color yield for glutathione itself is used for integration of the peak obtained upon chromatography of a sample of glutathione that had been completely converted to the S-sulfonate, the calculated recovery is about 70 per cent. Waley's (24) preparation of a pure Ba salt of S-sulfoglutathione should permit the determination of the precise color yield in the ninhydrin reaction and quantitative studies of different chromatographic procedures. The stability of the derivatives was examined in the present study, with the use of Dowex 1 chromatography for comparative analyses. About 95 per cent of the S-sulfonate was recovered unchanged after standing at pH 9 for 20 hours at room temperature, or after incubation with 3 equivalents of cysteine at pH 7 for 4 hours. If the quantity of cysteine was increased to 30 equivalents, the yield of S-sulfo-glutathione dropped to 77 per cent, while when cysteine was replaced by β-mercaptoethanolamine, the recovery of S-sulfo-glutathione was 63 per cent. Presumably, mixed disulfides are formed, as suggested by Swan (9).
S-sulfonate was unstable in 5 M guanidine hydrochloride, only about 55 per cent being recovered after 48 hours at 4° and pH 7. The nature of the decomposition in this case has not been investigated further.

Conversion to the S-sulfonates and chromatography on columns of Dowex 1-chloroacetate may prove useful in determining the cysteine and glutathione content of tissue extracts. An example of such an application is shown in Fig. 2. An extract of cat liver (2 ml., corresponding to 1.1 gm. wet weight of tissue) to which cysteine had been added was treated with 2 ml of 0.2 M phosphate buffer at pH 7, 2 ml of 0.1 M sodium sulfite at pH 7, and 0.4 ml of 0.5 M potassium-o-iodosobenzoate. After 30 minutes at room temperature, one-tenth of the mixture was chromatographed. The glutathione content, calculated on the basis of 70 per cent apparent yield of the S-sulfonate with a color factor of 0.93, was 136 mg per 100 gm. wet weight of tissue, in good agreement with the value of 138 mg per 100 gm. of tissue obtained by Dr. H. H. Tallan who chromatographed the same extract (without sulfite treatment) on a column of Dowex 50-X4 (30). The amount of cysteine or cystine present in the original tissue extract was too small to determine accurately. The positions of emergence of the S-sulfonates of cysteine and glutathione from the Dowex chloroacetate column were determined with the known compounds.

Reaction of Sulfite with Insulin—As has been indicated in a preliminary report (1), the S-sulfonates of the two insulin chains can be separated chromatographically. To convert the cystine residues in the protein to the S-sulfonates, three cycles of the oxidation-reduction procedure described in the experimental section were carried out at room temperature. The reaction mixture was dialyzed for 3 hours to remove most of the salts. When the clear solution was brought to pH 2.2 with N HCl, the phenylalanine chain partially precipitated, inasmuch as it is only sparingly soluble at low pH in the presence of low concentrations of salt. Enough solid urea (deionized) was added to redissolve the precipitate, and the solution was then transferred to a 0.9 X 5.0 cm column of Dowex 1-X8 in the chloroacetate form, with 0.1 M sodium chloride as eluent. Waley (24) has employed a similar chromatographic system for the isolation of naturally occurring S-sulfoglutathione from extracts of calf lens.

![Fig. 2. Chromatography of an extract of cat liver to which cysteine had been added and which had then been treated with sulfite and o-iodosobenzoate. The sample, which contained about 3 mg of glutathione and 0.3 mg of cysteine, was chromatographed on a 0.9 X 5.0 cm column of Dowex 1-X8 in the chloroacetate form, with 0.1 M sodium chloride as eluent. Waley (24) has employed a similar chromatographic system for the isolation of naturally occurring S-sulfoglutathione from extracts of calf lens.](attachment:fig2.png)

### Table I

Amino acid composition of S-sulfonates of peptide chains of insulin

Hydrolyses were performed for 22 hours unless otherwise indicated.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Glycyl chain</th>
<th>Phenylalanyl chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Theory</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.06</td>
<td>2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.97</td>
<td>4</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.00</td>
<td>1</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>Valine</td>
<td>1.81</td>
<td>2</td>
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<tr>
<td>Leucine</td>
<td>1.99</td>
<td>2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.00</td>
<td>1</td>
</tr>
<tr>
<td>Serine</td>
<td>2.02</td>
<td>2</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.10</td>
<td>0</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>4.50</td>
<td>4</td>
</tr>
<tr>
<td>Proline</td>
<td>0</td>
<td>1.10</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0</td>
<td>2.64</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.07</td>
<td>2</td>
</tr>
<tr>
<td>Lysine</td>
<td>0</td>
<td>0.97</td>
</tr>
<tr>
<td>Histidine</td>
<td>0</td>
<td>1.77</td>
</tr>
<tr>
<td>Arginine</td>
<td>0</td>
<td>1.09</td>
</tr>
</tbody>
</table>

* From formula of Ryle et al. (31).
* Value from 72-hour hydrolysate.
* Corrected for 80 per cent recovery of serine hydrolyzed for 22 hours in a known mixture of amino acids.
* Corrected for 85 per cent recovery of threonine hydrolyzed for 22 hours in a known mixture of amino acids.
* Corrected for 81 per cent recovery of cysteine from cysteine-S-sulfonate hydrolyzed for 22 hours.
* Corrected for 88 per cent recovery of tyrosine hydrolyzed for 22 hours in a known mixture of amino acids.

The purity of the two chains was established by amino acid analyses, the results of which are given in Table 1. As the formula of Ryle et al. (31) would require, no phenylalanine or proline was found in the glycyl chain. There was a trace of threonine in the glycyl chain and of isoleucine in the phenylalanine chain. All other amino acids were present in the expected quantities. As was to be expected from experiments with the pure substances, cysteine-S-sulfonate was converted to cystine in a yield of from 70 to 90 per cent. Isoleucine and leucine were present in a 22-hour hydrolysate of the glycyl chain to the extent of 0.95 and 1.99 residues respectively, but valine was very slow to appear. Values for valine in a 22- and a 72-hour hydrolysate were 1.37 and 1.81 residues, respectively.
As a further control on the specificity of the sulfite reaction, a synthetic mixture of amino acids was allowed to react with sulfite and then subjected to amino acid analysis. Cystine was converted to the S-sulfonate, but all of the other amino acids were recovered quantitatively, with the possible exception of tryptophan which showed a slight loss (about 5 per cent).

Addition of β-mercaptoethylamine to the S-sulfonate of the glycyl chain at pH 7, and subsequent addition of dilute HCl to pH 2, caused the separation of a precipitate, indicating that the S-sulfonate of the glycyl chain had been altered in some manner. Probably the altered material, which is soluble at pH 7 but insoluble at pH values more acid than pH 4, is either a mixed disulfide of the glycyl chain with β-mercaptoethylamine, or a simple polymer formed by reoxidation of the reduced chain. In any case, it seems likely that β-mercaptoethylamine has caused the elimination of the sulfonate group.

**Reaction of Sulfite with Ribonuclease**—The kinetics of the reaction of Na$_2$SO$_3$ with two proteins were studied, with the results shown in Fig. 3. Ribonuclease was selected for more detailed examination because it is a well characterized protein, and because, as may be seen from Fig. 3, its disulfide bonds were the most difficult to reduce and the sulfhydryl groups were also difficult to reoxidize.

The effect of urea concentration upon the rate of the sulfite reaction was determined by measuring the number of sulfhydryl groups formed after ribonuclease had been allowed to react with sulfite at 38° for 15 and for 60 minutes in various concentrations of urea. The results, given in Table II, indicate that, in 8 M urea, quantitative cleavage of the disulfide bonds occurs in less than 60 minutes, but that in lower concentrations of urea, the reaction is definitely slower.

The effect of temperature was next investigated, and it was found, as is shown in Fig. 4, that reduction in 8 M urea must be carried out at a temperature higher than 22° if quantitative cleavage of the disulfide bonds is to be effected in a convenient time.

The extent of S-sulfonation was also followed by chromatography on IRC-50 the products present after the reaction with sulfite had been carried out under various conditions. In addition, the extent of the reaction was measured by determining the apparent disulfide content of the S-sulfonated products. It was found that as the apparent disulfide content decreased, there was a concomitant decrease in the amount of material chromatographing at the ribonuclease A position, and an increase in the amount of material emerging at the column volume. Complete S-sulfonation, judged by the absence of determinable disulfide bonds and of material chromatographing at the ribonuclease A position was only obtained when the reaction was carried out at 38° through three oxidation-reduction cycles, the urea concentration being maintained at 8 M or greater by the addition of deionized urea (added as crystals). Completely S-sulfonated ribonuclease emerged unretarded on IRC-50 at pH 6.47 (32), and was quite soluble in water. Partially S-sulfonated products frequently contained some insoluble material. Upon paper electrophoresis, in 0.5 M borate at pH 9, the completely S-sulfonated product moved toward the anode, whereas the untreated ribonuclease migrated toward the cathode. The electrophoresis of both the treated and untreated material was somewhat unsatisfactory because both samples of protein gave streaks on the paper.

**Reaction of Sulfite with Chymotrypsinogen and Trypsinogen**—Having achieved complete S-sulfonation of insulin and ribonuclease, neither of which contains tryptophan, the same procedure was used in an attempt to prepare S-sulfonates of chymotrypsinogen and trypsinogen, two larger and less well characterized proteins that do contain tryptophan. From the results of the
kinetic studies (Fig. 3), chymotrypsinogen appears to be especially readily converted to the S-sulfonate. The disulfide bridges in the molecule are cleaved rapidly, and the sulfhydryl groups thus formed reoxidize readily in air even in the absence of an added oxidizing agent. Additional studies revealed that urea concentration had little effect upon either the cleavage or oxidation steps, but that temperature is important. From Fig. 5 it can be seen that the extent of the cleavage is slightly greater at 38° than at either 21 or 4°, but that the sulfhydryl groups persist longer at the lower temperatures. It would thus appear to be advantageous to carry out the S-sulfonation of chymotrypsinogen at 38°.

Although the presence of urea and an oxidizing agent were not required to convert chymotrypsinogen to the S-sulfonate, they were incorporated in the reaction mixture so as to study their effect and thereby learn whether the procedure employed with ribonuclease could be employed, if necessary, with tryptophan-containing proteins. The S-sulfonated derivative precipitated during dialysis and thereafter was insoluble in water in the range pH 3 to pH 9. Chromatographic examination by the technique of Piis et al. (32) was, therefore, impossible. The derivative was somewhat soluble in buffers containing urea, but upon paper electrophoresis with such buffers, the S-sulfonate precipitated at the origin. By varying the conditions of the reduction-oxidation procedure, products completely soluble or possessing varying degrees of solubility could be obtained; but the greater the solubility, the more unreacted chymotrypsinogen was found on chromatography or electrophoresis of the soluble portion of the reaction mixture, and the higher the amperometric assay for apparent disulfide content. Only in the insoluble material was no sulfhydryl or disulfide found after treatment in urea-Tris buffer with sulfite for 5 or for 30 minutes, conditions which revealed disulfide bonds in the insoluble portions of sulfite-treated ribonuclease that were occasionally encountered. Dialysis against 10-3 M N-ethylmaleimide, which would block free sulfhydryl groups and thus minimize polymerization, failed to prevent precipitation as the urea concentration was reduced.

Insoluble products were also obtained when chymotrypsinogen was oxidized with performic acid, or carboxymethylated with iodoacetate after reduction by NaBD₄ or thioglycollic acid (10). Apparently cleavage of the disulfide bonds in the molecule, followed by the introduction of anionic groups, confers insolubility.

To confirm the fact that the S-sulfonation procedure did not effect any amino acid residues except cystine, an amino acid analysis of an insoluble S-sulfonate derivative was performed, with the results shown in Table III. All of the amino acids were present in the expected quantities (33) with the possible exception of tryptophan, which yielded a figure about 7 per cent lower than that found for the untreated protein.

Tryptsinogen was also submitted to the same S-sulfonation procedure, and like chymotrypsinogen, yielded an insoluble derivative free from disulfide bonds, which precipitated during the dialysis procedure employed to remove the urea from the reaction mixture. It is of interest that trypsinogen carboxymethylated after reduction by thioglycollic acid or NaBH₄ was also insoluble (10).

**DISCUSSION**

It is apparent from the data presented in this communication that proteins vary considerably in the ease with which conversion to the S-sulfonate can be accomplished. Thus, ribonuclease requires the presence of both 8 M urea and an oxidizing agent, whereas the reaction of sulfite with chymotrypsinogen at pH 7.6 would probably proceed to completion without either of these reagents. The conditions outlined in “Experimental,” which suffice for the complete S-sulfonation of ribonuclease, should be adequate for many other proteins as well, but, in view of the different reactivities to be expected from different proteins, a kinetic investigation of each particular case would seem to be advisable.

One of the advantages of the sulfite method of cleaving disulfide bonds is the ease with which the reaction can be followed

![Graph showing the effect of temperature upon the action of sulfite (0.4 m) upon chymotrypsinogen (2 per cent) in 8 M urea.](http://www.jbc.org/)

**Table III**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>22.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>14.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>22.0</td>
</tr>
<tr>
<td>Valine</td>
<td>18.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.1</td>
</tr>
<tr>
<td>Serine</td>
<td>27.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>22</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>10.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.8</td>
</tr>
<tr>
<td>Proline</td>
<td>8.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.75</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.45</td>
</tr>
<tr>
<td>Lysine</td>
<td>12.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.95</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* From Wilcox et al. (33).
† Analysis lost.
‡ Corrected for losses on hydrolysis, cf. footnotes to Table I.
sulfonate linkage is relatively labile, there is no simple way of
not be so similar. Finally, Pechere et al. (11) showed that S-sul-
S-sulfonates and the corresponding untreated proteins would
fragments are liberated, or the amino acid composition of the
amino acid analyses of the S-sulfonated derivatives of insulin
occurs. An advantage of the sulfite method is its mildness and speci-
ficity. Experiments on known mixtures of amino acids and amino
acids. This is in contrast to the reduction-carboxymethylation
sequence of reactions that lead to the formation of S-carboxy-
hydrolyses (10). The extent of the sulfite re-
an amino acid composition of the
S-sulfonated and the corresponding untreated proteins would
not be so similar. Finally, Pechere et al. (11) showed that S-sul-
fonation in the presence of cupric ions at pH 10 did not lead to a
change in absorption spectrum, molecular weight, or the forma-
new end groups.
One disadvantage of the sulfite method is that, because the
S-sulfonate linkage is relatively labile, there is no simple way of
proving unequivocally that the reaction has gone to completion.
The S-sulfonates formed cannot be measured directly, because
they are not stable to hydrolytic procedures that liberate amino
acids. This is in contrast to the reduction-carboxymethylation
sequence of reactions that lead to the formation of S-carboxy-
methyleneysteine, a derivative that can be quantitatively deter-
mained in acid hydrolyses (10). The extent of the sulfite re-
action is currently determined in two general ways. In one way,
the reaction is judged to be complete when further vigorous re-
duction is no longer capable of revealing sulfhydryl groups, the
assumption being made that sulfhydryl groups are no longer
available because they have all been converted to the S-sulfonates.
This assumption, though probably valid, is difficult to prove.
The second method of determining the extent of the reaction is
to measure the number of SO₄⁻ residues that have been incorpo-
rated into the protein, either by the use of sulfite containing S₃⁸
as Pechere et al. (11) have done, or by a sulfate determination on
the sulfoprotein (1). A correlation between the amount of sul-
fite incorporated and the number of half-cystine residues in
the molecule is used as an indication of complete reaction; once more
a likely assumption but not a certainty.
The physical properties of the sulfoproteins seem to be similar
to those of other derivatives of the same proteins in which the
disulfide bonds have been ruptured and converted to anionic
groups. Thus, the S-sulfonate of the glycyl chain of insulin is
soluble, as is the derivative formed on performic acid oxidation,
whereas both the sulfonated and oxidized derivatives of the
phenylalanine chain are insoluble. In the case of ribonuclease,
S-sulfonation, performic acid oxidation, and reduction followed
by carboxymethylation of the sulfhydryl groups, all lead to solu-
ble products. With chymotrypsinogen and trypsinogen, on the
other hand, the same reactions lead in all instances to products
which have a strong tendency to aggregate and are, therefore,
insoluble under most conditions. Pechere et al. (11) by a graded
series of diazYPES, were able to obtain from these zymogens prod-
ucts that could be kept in aqueous solution under very restricted
conditions. The insoluble derivatives can be dissolved in urea
solutions or in sodium dodecylsulfate (11), but the necessity for
these additives limits to some extent the usefulness of the prod-
ucts.
The utility of the S-sulfoproteins may also be limited by their
lability. As Swan (9) has pointed out, the sulfonate residues
are also not eliminated fairly readily under certain conditions, but
whether this will be an advantage or a disadvantage will depend
upon the requirements of the given investigation.

SUMMARY

Conversion of a disulfide to two molecules of the corresponding
S-sulfonate can be brought about by the action of sodium sulfite
at pH 7.4 in the presence of an oxidizing agent such as o-iodoso-
benzoate or tetrathionate. The procedure has been investigated
with cysteine, oxidized glutathione, insulin, ribonuclease, chymo-
trypsinogen, and trypsinogen, amperometric titrations being used
to follow the reaction. The S-sulfonate derivatives of cysteine
and glutathione can be separated on columns of Dowex 1-X8.
The ease of conversion of the disulfide bonds in a protein varies,
ribonuclease requiring 8 M urea and an oxidizing agent, whereas
with chymotrypsinogen urea is not essential and air serves as a
fairly effective oxidizing agent. Reaction in 8 M urea with io-
odobenzoate at 38° and pH 7.4 converts all of the proteins stud-
ied to completely S-sulfonated products. The S-sulfonates of
the glycy1 and phenylalanine chains of insulin have been separated
on a column of Dowex 50-X2 and their purity determined by
amino acid analysis. Amino acid analysis of the S-sulfonate of
chymotrypsinogen has shown that the sulfite procedure does not
affect any amino acids other than cystine, with the possible ex-
ception of a 5 to 7 per cent loss of tryptophan.
The S-sulfonated derivative of the glycyl chain of insulin and
S-sulfonoribonuclease are freely soluble, whereas the derivatives of
the phenylalanine chain of insulin, of chymotrypsinogen, and of
trypsinogen are insoluble under most conditions.

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