The Stability of Cysteine and Cystine during Acid Hydrolysis of Proteins and Peptides

A. S. Inglis‡ and Teh-Yung Liu
From the Biology Department, Brookhaven National Laboratory, Upton, New York 11773

SUMMARY
When solutions of cystine and cysteine are treated with dithiothreitol and then with sodium tetrathionate, quantitative yields of S-sulfocysteine can be obtained. This reaction has been applied to protein hydrolysates and the resulting S-sulfocysteine has been determined by ion exchange chromatography on an amino acid analyzer. With ribonuclease and reduced glutathione this procedure has given accurate values for the cystine or cysteine content of the protein or peptide. When tryptophan is also present, the recoveries of S-sulfocysteine can be obtained. This reaction with dithiothreitol and tetrathionate serves to convert any altered forms of the amino acids in the hydrolysates to the form of this derivative. This method for determining the S-sulfocysteine and to permit quantitative recovery in the protein (2, 3) or for X-carboxymethylcysteine after reduction by analyzing for cysteic acid after performic acid oxidation of the protein (4). Howev, there have been indications, both from colorimetric analysis (5) by Shinohara’s (6) method and from radiochemical analysis (7) of wool hydrolysates, that extensive degradation of cystine residues does not in fact occur but that cystine is probably converted to closely related derivatives. Such derivatives, with suitable modification, might be amenable to determination on the analyzer. One advantage of such an approach for the analysis of cysteine and cystine residues in proteins would be that a special hydrolysate would not be required for the analysis.

The reversible masking of reduced cystine residues in proteins with sodium tetrathionate (8) prompted tests on the applicability of the reagent to a study of the sulfur-containing amino acids in protein hydrolysates. An attempt has been made to identify both cysteine and cystine in acid hydrolysates as S-sulfocysteine by reduction of cystine with dithiothreitol (9) followed by treatment of the reaction solution with excess sodium tetrathionate (8, 10).

EXPERIMENTAL PROCEDURE
Materials—Bovine pancreatic ribonuclease A, Lot RAF 6508 was obtained from Worthington Biochemical Corporation. Egg white lysozyme, 3 times crystallized, Lot 28B-8120, was obtained from Sigma Chemical Company. Glutathione (reduced), Lot M 2535, was obtained from Mann Research Laboratory, and dithiothreitol (Cleland’s reagent) from Calbiochem. Sodium tetrathionate was prepared as described by Gilman et al. (11). Porcine pancreatic ribonuclease was a gift from Dr. C. H. W. Hirs.

Amino Acid Mixture—A standard solution containing 1.25 μM cystine and 2.5 μM aspartic acid was used to check the procedure and to establish the color factor for S-sulfocysteine on a Beckman-Spinco model 120C amino acid analyzer. The color values obtained for both amino acids were identical with those obtained when the derivitization procedure was applied to the calibration mixture provided with the analyzer. The presence of a dip in the base line just preceding the peak from S-sulfocysteine may make it desirable to integrate the peak by hand rather than by an electronic integrator. The aspartic acid was used as an internal standard. The dimethylsulfoxide ninhydrin reagent of Moore (12) was used.

Performic Acid Oxidation—The method of Moore (3) was used.

Hydrolysates—Hydrolysis was carried out in heavy-walled test tubes, cleaned first in H2SO4-HNO3 (3:1), rinsed in deionized water, and oven-dried. The protein, 2 to 5 mg, was hydrolyzed under vacuum (20 to 30 μ) at 110° for 22 hours with 1 ml of...
constant boiling HCl (twice redistilled from all glass apparatus). When tryptophan was present in the protein, sodium tetrathionate (0.5 to 1.0 ml, about 2-fold excess over tryptophan) was usually added to the hydrolysis tube before addition of HCl. Tetrathionate in the hydrolysate will react with dithiotheritol in the reduction step, and the quantities of reagents to be used subsequently should be selected accordingly.

The HCl hydrolysate was concentrated by rotary evaporation at 37°, and the residue was dissolved in 4 ml of deionized water. Since 0.1-μmole amounts of individual amino acids are optimal with a Spinco model 120C analyzer, the quantities of proteins and peptide taken generally allowed duplicate runs and the analysis of the untreated hydrolysate. The latter is desirable for establishing whether other ninhydrin-positive substances are present in the position of S-sulfocysteine.

Reduction—One milliliter of the hydrolysate was placed in a test tube (the heavy-walled hydrolysis tube may be used), followed by 3 ml of deionized water, 0.1 ml of pyridine, and 1 ml of dithioetheriol solution (approximately 4 μmoles per ml). The tube was flushed with N₂ for 1 min, sealed with a silicone rubber stopper, shaken on a Vortex shaker, and placed at 37° for 1 hour.

Oxidation—At the end of the reduction period about 200 μmoles (60 mg) of sodium tetrathionate (Na₂S₄O₆·2H₂O) were added to the test tube, and the mixture was allowed to stand for 16 hours at 25°. Oxygen was then bubbled through the solution for 7 min. After at least 1 hour, the solution (or a suitable aliquot thereof) was dried by rotary evaporation, diluted with pH 2.2 buffer (5 to 10 ml), and applied to the 36-cm column of the analyzer.

Preparation of S-Sulfocysteine Dihydrate—To 3.6 g (10 mmoles) of Na₂S₄O₆·2H₂O in 30 ml of water were added 1.21 g of cysteine (10 mmoles). The reaction mixture was stirred at 37°, and the residue was dissolved in 4 ml of deionized water. Constant boiling HCl (twice redistilled from all glass apparatus) was usually added to the hydrolysis tube before addition of HCl.

Confirmation as S-sulfocysteine was obtained by comparison with the infrared spectrum and the chromatographic behavior of the preparation with those of a sample of S-sulfocysteine prepared by the method of Clarke (13).

RESULTS

Preliminary experiments with mercaptoethanol as the reducing agent were unsuccessful because of the formation of the mixed disulfide of mercaptoethanol and cysteine. After treatment with dithioetheriol followed by sodium tetrathionate, a mixture of cysteine and aspartic acid gave only two peaks on the long column of the Spinco amino acid analyzer—the aspartic acid peak and one in the elution position of cysteic acid. The color yield of the new amino acid was much lower than that of cysteic acid. It was convenient throughout to relate the color factor to that of aspartic acid; S-sulfocysteine gave a color yield which was 0.745 ± 0.02 times that for aspartic acid under our conditions, but the color factor should be checked with the given analyzer. The product with ninhydrin also showed a ratio of these areas is 2.3:1 for S-sulfocysteine, whereas it is 6:1 for cysteic acid.

Analysis of hydrolysates for half-cystine residues

Results are expressed as percentage recovery and are averages of at least two analyses.

<table>
<thead>
<tr>
<th>Hydrolysate</th>
<th>Cysteine</th>
<th>Cysteic acid</th>
<th>S-Sulfocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine pancreatic ribonuclease A⁺</td>
<td>70</td>
<td>96</td>
<td>97.5</td>
</tr>
<tr>
<td>Potato acid phosphatase⁺</td>
<td>60</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Standard mixture of amino acids A</td>
<td>93</td>
<td>—</td>
<td>96</td>
</tr>
<tr>
<td>B⁻</td>
<td>91</td>
<td>—</td>
<td>98</td>
</tr>
<tr>
<td>Glutathione (reduced)⁶</td>
<td>—</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Pericne ribonuclease⁶</td>
<td>78</td>
<td>—</td>
<td>94</td>
</tr>
</tbody>
</table>

* Conditions: 22 hours under reduced pressure in twice distilled HCl at 110°.
* Calculations are based on the content of 8 half-cystine residues per mole of protein as 100% (2, 3).
* Cysteic acid value was taken as 100%.
* Not determined.
* Hydrolyzed for 72.5 hours.
* Calculated by using glutamic acid as an internal standard. (The peptide contains glutamic acid, cysteine, and glycine in a ratio of 1:1:1.)
* Calculations are based on the content of 8 half-cystine residues per mole of protein as 100% (16).

The results obtained after hydrolysis of cystine-containing samples are given in Table I. For ribonuclease A, a purified sample of potato acid phosphatase, and reduced glutathione the values obtained for the number of half-cystine residues are in good agreement with those obtained by performic acid oxidation (3) and higher than the chromatographic analyses of the untreated hydrolysates for cystine, which showed no unusual peaks. The results, therefore, establish that closely related derivatives (such as cysteine) which can be converted to S-sulfocysteine by dithioetheriol and tetrathionate are present in appreciable amounts in the hydrolysates; further, the results with the standard mixture of amino acids show that prolonged hydrolysis times do not substantially increase the formation of these products.

Successful results were also obtained with porcine ribonuclease (Table I), which is a glycoprotein (16) containing 30% carbohydrate and 70% protein. These results demonstrate that the present procedure is also applicable to a glycoprotein.

However, these proteins or the peptide do not contain tryptophan, which would be expected to react with cysteine (17). Streptococcal proteinase, which contains 5 tryptophan residues but only 1 half-cystine residue per mole of molecular weight

The results in Table II show that simple addition of tetrathionate to the hydrolysis mixture is sufficient to protect cysteine during hydrolysis. Whether this is due to interaction of tetrathionate with tryptophan or to the protection of cysteine by formation of the S-sulfocysteine has not been determined. However, the precipitation of sulfur in the hydrolysates of the proteins containing tryptophan compared to those without tryptophan and the disappearance of the tryptophan peak on the short column chromatogram are suggestive of interaction with tryptophan.

Accurate results are obtained with lysozyme, which is high in cysteine (Table II). A typical chromatogram obtained when a lysozyme hydrolysate was treated with dithiothreitol followed by tetrathionate is shown in Fig. 1. Results for the analysis of lysozyme after 72 hours of hydrolysis confirmed those obtained for the mixture of free amino acids in suggesting that the hydrolysis time is not critical for the analysis of cystine under the present conditions. The acceptable result obtained after the usual acid hydrolysis without tetrathionate was unexpected in this case but probably reflects the importance of structure and amino acid composition on the reactions that occur during hydrolysis of proteins.

To confirm the applicability of the method to proteins that are high in cysteine, the results with reduced wool were compared with those on the untreated wool. The results, when tetrathionate was present during hydrolysis, were in full agreement (Table II).

The chromatograms in these experiments with ribonuclease and lysozyme were carefully examined for the recoveries of the other amino acids in the hydrolysates. The presence of tetrathionate had no detectable effect on the values for any of the other constituents, including serine, threonine, and methionine, except for unexplained low values for tyrosine (80%) in ribonuclease.

**DISCUSSION**

Many reductants and oxidants have been used for studying the S—S and —SH groups in proteins (21). The quantitative nature of the reduction of disulfides by Cleland's reagent (dithiothreitol) (9) and the reversible protection of thiols in proteins with tetrathionate (8) suggest their use as analytical reagents.

![Fig. 1. Effluent curve obtained by ion exchange chromatography of a sample of egg white lysozyme hydrolysate treated with dithiothreitol and then with sodium tetrathionate.](http://www.jbc.org/)

**Table II**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percentage recovered as S-sulfocysteine&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCl hydrolysis</td>
</tr>
<tr>
<td>Bovine pancreatic ribonuclease A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98</td>
</tr>
<tr>
<td>Streptococcal proteinase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>78</td>
</tr>
<tr>
<td>Egg white lysozyme&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97</td>
</tr>
<tr>
<td>Egg white lysozyme&lt;sup&gt;f&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Wool I&lt;sup&gt;e&lt;/sup&gt;</td>
<td>90</td>
</tr>
<tr>
<td>Wool II&lt;sup&gt;e&lt;/sup&gt;</td>
<td>83</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of duplicate analyses of 22-hour hydrolysates.

<sup>b</sup> Calculations are based on the content of 8 half-cystine residues per mole of protein as 100% (2, 3).

<sup>c</sup> Calculations are based on the content of 1 half-cystine residue per mole of protein as 100% (18).

<sup>d</sup> Calculations are based on the content of 8 half-cystine residues per mole of protein as 100% (19).

<sup>e</sup> Hydrolyzed for 72 hours.

<sup>f</sup> Not determined.

<sup>g</sup> Merino wool which contains 23 μmoles of cysteine and 450 μmoles of cystine per g of protein. Calculations are based on amperometric determination with methylmercuric iodide as 100% (20).

<sup>h</sup> Reduced Merino wool which contains 845 μmoles of cysteine and 88 μmoles of cystine per g of protein. Calculations are based on amperometric determination with methylmercuric iodide as 100% (20).
The present work demonstrates that quantitative determinations of cystine or cysteine may be made along with the determination of other free amino acids with an amino acid analyzer by conversion of cysteine to S-sulfocysteine.

With respect to the specificity of the procedure, it should be stressed that any ninhydrin-positive substance eluted without retardation on the amino acid analyzer will interfere and produce high results. The possibility of interference can be checked by running the untreated sample as a blank determination. If this is not convenient, the area of the 440 nm peak should be calculated, and the ratio of this area to the area calculated for the 570 nm absorption, when compared with a standard analysis, should give an indication of the possible presence of interfering substances. In this connection, when serine and threonine (0.5 μmole) were hydrolyzed in the presence of tetrathionate (3 mg) and the resulting hydrolysate was treated with dithiothreitol followed by tetrathionate, as described under “Experimental Procedure,” sulfoserine and sulfothyreonine were not found to be present on the chromatogram. The conclusion is that under the experimental condition described, serine and threonine do not form substances which interfere in the analysis of S-sulfocysteine by this procedure.

The present procedure provides a new method for the determination of half-cystine residues in proteins. It gives results comparable with those of the performic acid method (3). Since the same hydrolysate can be used as a control on the procedure and the chemical reactions are carried out on the free amino acids in the same solution, conditions are optimal for reproducible results. If for some reason the reactions were not quantitative, this would be evident from the appearance of cysteine on the chromatogram. In routine practice, small differences occurred in the factor obtained for standard cystine solutions; for the most accurate results it is recommended that a control be run in parallel with the unknown solution.

For the analysis of protein that contains both 3-carboxymethylhistidine and cysteine, the present procedure is particularly advantageous. In the earlier studies of Crestfield, Stein, and Moore (22), the protein samples were reduced and carboxymethylated before amino acid analysis in order to convert cystine to S-sulfocysteine by this procedure and thus permits quantitative yields of S-sulfocysteine.

The experimental conditions recommended are not the only ones that will give satisfactory results. Reproducible color constants (aspartic acid × 0.008 ± 0.02) were obtained after only 1 hour with tetrathionate (10-fold excess over dithiothreitol) and use of a cystine-aspartic acid solution; ribonuclease A hydrolysates treated under identical conditions gave a peak in the S-sulfocysteine position which corresponded to 8 half-cystine residues per mole when the above constant was used for the calculations. Possibly, after the short reaction time with tetrathionate, other sulfonated species are present in addition to the S-sulfocysteine, but all the derivatives have the similar color constant. Pihl and Lange (10) suggested that the S-sulfosulfenyl derivative of a cysteinyl residue was stable in proteins because of steric factors. However, in the reaction of tetrathionate with free cysteine under the experimental conditions described, sulfuric derivatives were compared by paper chromatography and infrared spectrum with an authentic sample of S-sulfocysteine prepared by the method of Clarke (13). It has not been established whether substances other than S-sulfocysteine are determined when the procedure is applied to a protein hydrolysate, but, in view of the good recoveries obtained for five proteins and a peptide with markedly different cysteine and cystine content and general amino acid composition, this consideration would not be expected to invalidate the method as a means of determining the total number of half-cystine residues in proteins.

Apart from the small inconvenience of adding tetrathionate to the 6 N HCl in the hydrolysis tube, this addition may lead to low tyrosine values as exemplified by the result with ribonuclease. However, the results of experiments on proteins containing both thiol groups and tryptophan indicate that the addition of tetrathionate is essential for correct results. Streptococcal proteinase, with 1 half-cystine residue and 5 tryptophan residues per molecule, yielded only 78% of the expected amount of S-sulfocysteine when tetrathionate was not added to the hydrolysis tube, while similarly the two Merino wool samples, which contained 23 and 845 μmoles of thiole per gram of wool protein, respectively, yielded only 90 and 83% of the expected values. Tetrathionate and S-sulfocysteine (14) are unstable in hot acid, but there is evidence from the present work that during acid hydrolysis in 6 N HCl tetrathionate reacts with tryptophanyl residues, thereby preventing reaction of this amino acid residue with cysteine. That the cysteine may then be determined quantitatively after hydrolysis as S-sulfocysteine by the procedure described herein opens the possibility of determining the half-cystine residues in modified proteins, for example, S-sulfopeptides, after acid hydrolysis.

The results of our studies indicate that cystine is not so unstable during acid hydrolysis as was thought in 1954 (23). The main change in hydrolytic procedures in the intervening years has been the removal of dissolved oxygen before the sealing of the tubes (24). This step, which is known to be essential for quantitative yields of 3-carboxymethyl cysteine, probably is helpful for the preservation of cysteine and cystine and was employed in the present experiments. The action of dithiothreitol in our procedure may reduce small amounts of oxidized products which previously were not determinable as cysteine and thus permits quantitative yields of S-sulfocysteine.

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REFERENCES

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