On the Determination of Cystine as Cysteic Acid

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In the procedure of Schram, Moore, and Bigwood (1) for the determination of the cystine plus cysteine content of a protein, the directions for the concentration of the reaction mixture need to be followed explicitly in order to avoid overoxidation. The addition of a reducing agent to destroy the excess performic acid before the concentration step would seem appropriate if the oxidant would have no deleterious effect on the subsequent acid hydrolysis or the chromatographic determination of cysteic acid. We have tested a number of reducing agents for this purpose. The most generally useful results have been obtained with HBr. The bromine formed is volatile and can be rapidly removed from the reaction mixture under reduced pressure.

Bromine itself is an effective reagent for oxidizing cystine to cysteic acid, yet Thompson (2) found that with proteins he could not obtain high yields of cysteic acid by bromine oxidation. The data indicated, however, that if performic acid were used for the initial reaction, the subsequent brief exposure of cysteic acid residues to bromine would not be likely to be detrimental. The present experiments started from this premise.

Methionine sulfone is more sensitive to overoxidation by performic acid than is cysteic acid.1 The yield of methionine sulfone can be improved by removing the performic acid by lyophilization rather than by rotary evaporation. The use of HBr, however, has invariably permitted quantitative yields of methionine sulfone. The present procedure thus provides a method for determining both cystine plus cysteine and methionine as their oxidation products. The determination, as was the earlier one (1), is also fully applicable to carbohydrate-containing samples.

EXPERIMENTAL PROCEDURE

Test Solution of Amino Acids—Dissolve 20 mg of cystine, 30 mg of methionine, and 20 mg of alanine in 2 ml of 1 N NaOH, and make to a volume of 10 ml with water. The solution should be used within an hour or two.

Protein for Analysis The sample should contain approximately 0.1 mg of cystine (usually 2 to 5 mg of protein). The protein need be weighed only approximately if the results are to be calculated in terms of the molar ratios of the constituent amino acid residues.

Performic Acid—Add 1 ml of 30% H$_2$O$_2$ to 9 ml of 88% formic acid. Let the mixture stand for 1 hour at room temperature. Cool to 0°.

Oxidation—Add the protein, or 0.100 ml of the test solution of amino acids, to a Pyrex ignition tube (Corning No. 9680, 18 × 150 mm). Add 2 ml of performic acid solution. Let the reaction mixture stand at 0° for 4 hours for soluble proteins, or overnight for proteins that do not dissolve in the performic-acid mixture (cf. (1)). Then add 0.30 ml of 48% HBr, with swirling of the reaction tube in the ice bath.

Concentration—Attach the reaction tube to a Craig rotary evaporator (operated at a 15° angle) through an adapter of Tygon tubing, 1/4 or 5/8-inch in interior diameter. Approximately 20 ml of 1 N NaOH are added to the condenser to absorb the bromine which distills over. The concentration of the solution to dryness is complete in approximately 30 minutes at a bath temperature of 40°.

Constant boiling HBr has a tendency to condense in the stem of the rotary evaporator before reaching the condenser. To minimize this tendency, the level of the water in the 40° bath should reach to the top of the reaction tube. When the distillation from the tube is complete, the tube can be raised above the level of the condenser before the rotary evaporator is stopped, so that condensate in the stem will run into the condenser rather than back into the tube. Wipe the rim free from any condensate. Alternatively, the whole stem of the condenser may be warmed to 40°, or a high vacuum pump can be used with solid CO$_2$ in the condenser bath. The results are not sensitive to a small amount, e.g. 0.05 ml of residual HBr during the hydrolysis by HCl.

Hydrolysis—Add 3 ml of 6 N HCl to the residue. Seal the tube under reduced pressure (3, 4) and place it in an oven at 110 ± 1° for 18 hours.

Chromatography—Remove the HCl on the rotary evaporator. Dissolve the residue in 5 ml of pH 2 buffer and use a 2-ml aliquot for analysis on a 150-cm column of Amberlite IR-120 operated either manually (5) or automatically (6). In the latter method, the integration constant for cysteic acid is the same as that for aspartic acid.

If chromatography is to be performed on Dowex 1-X8 (cf. (1)), dissolve the residue in 5 ml of pH 3.4 sodium monochloroacetate buffer (0.20 N in NaOH, 0.25 N in monochloroacetic acid, containing 3 ml of BRJ-35 solution (7) per liter). Use a 2-ml aliquot on a column of Dowex 1-X8 (0.9 × 60 cm, minus 400 mesh, Bio-Rad Laboratories) equilibrated with the same buffer. Collect 1-ml fractions at a rate of approximately 4 ml per hour. Cysteic acid emerges in a peak at approximately 60 ml. The color yield from cysteic acid in the manual ninhydrin method (8) is 0.99 under these conditions. In standardizing the method it is important to ascertain whether the primary standard is the anhydrous acid or the monohydrate.

Calculations—The yield of methionine sulfone in the oxidation is 100 ± 2% and that of cysteic acid is 94 ± 2% (Table I). In practice, the amount of cysteic acid obtained chromatographically is divided by 0.94 to give a corrected value. The number of half-cystine + cysteine or methionine residues per

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1 E. Schram, personal communication. Memorandum by Ottilia Stephanek-Benedek to the Faculty of Medicine, University of Brussels, for Certificate in Biological Chemistry, 1958.
molecule of a purified protein is usually most conveniently calculated by reference to the molar quantity of a stable amino acid (alanine, leucine, glutamic acid, or aspartic acid) determined from the same effluent curve. The number of residues of the reference amino acid, or the amount if the results are being expressed on a percentage basis, is known from previous analyses of the unoxidized protein. This calculation makes it unnecessary to determine the moisture content of the sample of protein used for the cysteic acid analysis; the calculation by reference also makes it possible to take up the residue in 5 ml of buffer instead of making a quantitative transfer to a 5-ml volumetric flask. The latter procedure is followed if the analysis is to be calculated for the oxidized sample on a weight basis, which is essential if the Dowex 1 column is used.

RESULTS

Schram, Moore, and Bigwood (1) have shown that the performic acid oxidation can be applied to the determination of cystine-cysteine in soluble and insoluble proteins as well as in the analysis of foods. The present improvement increases the dependability of the method for cysteic acid and extends the method to permit the accurate determination of methionine as the sulfone.

As documented in Table I of their paper (1), Schram, Moore, and Bigwood obtained a reproducible 90 ± 2% yield of cysteic acid on oxidation of cystine or cysteine; when pure cysteic acid was put through the procedure as a control there was a 3 to 4% loss of the amino acid. The 94% yield of cysteic acid obtained in the present experiments is in confirmation of the originally reported results, with the elimination of the 3 to 4% loss by overoxidation during the removal of the excess performic acid.

The method in its present form has been applied in this laboratory to a variety of purified proteins and glycoproteins. The increased precision has been particularly helpful in the analysis of the streptococcal proteinase of Elliott (10) which is found to contain only 1 residue of half-cystine per molecule (11). The oxidation has also been employed to obtain the sulfonic acid derivative from the hydantoin of cystine in the NH₄-terminal residue method of Stark and Smyth (12).

### TABLE I

<table>
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<th>Sample</th>
<th>Column</th>
<th>Recovery</th>
<th>Cysteic acid</th>
<th>Methionine sulfone</th>
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* Theory, in residues per molecule: half-cystine, 8.00; methionine, 4.00 (4, 9).

Chromatography—The use of the column of Amberlite IR-120 for the measurement of cysteic acid is permissible only if the hydrolysate does not contain any other constituent which is eluted at the cysteic acid position. In all cases where we have used the method, the prior analysis of the unoxidized protein gave no peak at this position; the cysteic acid determination thus is preceded by a control or "blank" chromatogram. A peak at the cysteic acid position is prevented with greater certainty when the suspension of unoxidized protein in 6 N HCl is de-salted before hydrolysis as recommended by Crestfield, Moore, and Stein (4). If dissolved air is left in the solution, there may be a trace of oxidation of cystine to cysteic acid during the acid hydrolysis at 110°.

When the control analysis on the unoxidized protein indicates that there may be interference at the cysteic acid position, the analysis of the oxidized sample may be performed with the Dowex 1-X8 column. Analyses of a given sample by both methods gave the same result (Table I).

The modified procedure has not been applied in detail to food analysis. The successful results obtained with ribonuclease in a mixture containing 6% protein and 94% carbohydrate (Table I) indicate that the sealed-tube technique for hydrolysis will be applicable to food analysis in preference to hydrolysis under reflux (13). When 3 mg of protein are present in 3 ml of 6 N HCl, the ratio of solvent to protein (1000:1) is close to the range which Dustin et al. (14) found suitable for the hydrolysis of protein without interference from carbohydrate. This conclusion is supported by the finding that all of the performic acid-stable amino acids of ribonuclease were quantitatively recovered in the analysis performed in the presence of acid-washed starch (cf. Table I). In addition, successful analyses of unoxidized ribonuclease in the presence of starch have confirmed the probable suitability of the hydrolytic conditions for the general case.

Oxidation—The performic acid treatment results in the destruction of residues of tyrosine and histidine as well as tryptophan. Therefore, analysis of the oxidized protein does not offer a means for the complete analysis of the protein. Also, the present procedure is purely for analytical purposes and not for the preparation of intact oxidized proteins for structural study. The use of HBr causes the liberation of bromine which, from the experiments of Mueller, Pierce, and du Vigneaud (15) and Witkop (16), is known to cleave peptide chains at tyrosine and tryptophan residues. The use of a minimum of performic acid, as in the experiments of Hirs (17) with a tryptophan-free protein, or reduction and alkylation (4, 18) remain the methods of choice for the cleavage of the disulfide bonds for structural studies. The chromatographic determination of half-cystine as carboxymethylcysteine offers an alternative to performic acid oxidation for analytical purposes. The recovery of carboxymethylcysteine is 100% when the residual air is removed from the hydrolysis mixture (4).

Reduction by Sodium Sulfite—One of the reducing agents examined in this study was sodium sulfite. The reagent was fully effective for destroying performic acid in test runs with cystine. However, when sodium sulfite was employed with protein samples, there was sometimes observed a decrease in the recovery of serine and threonine coincident with appearance of compounds eluted at the cysteic acid position from Amberlite IR-120. This observation led to the following test: serine alone was submitted to the hydrolysis conditions in 2 ml of 6
n HCl to which 100 mg of Na₂SO₄ were added. There was a 25% conversion of serine to O-sulfoserine as evidenced by chromatographic determination of the derivative and the residual serine. This result means that appreciable quantities of sulfate (or phosphate) are to be avoided as a general rule in the hydrolysis of proteins for amino acid analysis.

The use of HBr as the reducing agent in the present procedure has the added practical advantage that there is no foaming of the solution after addition of HBr. The SO₃, or the Cl₂ liberated if HCl is tried as the reducing agent, causes a foaming of the protein solution which is troublesome during the removal of the excess reagents on the rotary evaporator.

**SUMMARY**

The use of HBr as a reducing agent to destroy excess performic acid before hydrolysis of oxidized proteins can increase the precision of the measurement of cystine plus cysteine as cysteic acid and of methionine as the sulfone. The yield of cysteic acid in the oxidation is reproducibly 94 ± 2% and the yield of methionine sulfone is 100 ± 2%. This modification of the procedure of Schram, Moore, and Bigwood has been tested with purified proteins and with mixtures containing up to 94% carbohydrate.

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**REFERENCES**

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