

QUANTITATIVE DETERMINATION OF CYSTEINE AND CYSTINE IN PEPTIDES AND PROTEINS USING HYDRIODIC ACID

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Abstract

Determination of the composition of proteins by amino acid analysis after hydrochloric acid hydrolysis is unsatisfactory for cystine which is not all recoverable. When proteins were hydrolysed in hydriodic acid, half-cystine was released quickly and without measurable loss as cystine with freshly distilled acid and as cystine plus cysteine with hydriodic acid containing hypophosphorous acid. The necessary alterations to the normal operating conditions of an amino acid analyser to enable the resolution of cysteine from proline are discussed. This procedure for cystine analysis provides a simple alternative to determinations involving chemical modification of the amino acid.

I. INTRODUCTION

It has been shown (Inglis, Nicholls, and Roxburgh 1971) that hydriodic acid effectively hydrolyses peptide bonds and also converts threonine, serine, and methionine residues into α -aminobutyric acid, alanine, and homocysteine thiolactone respectively. The property of reduction is of particular interest for the determination of cysteine and cystine since cysteine is likely to be stable in this medium and cystine would be expected to be partially reduced to cysteine. This raised the possibility of determining these amino acids in proteins by automatic amino acid analysis after hydriodic acid hydrolysis of the unmodified protein; that is, without either oxidative (Moore 1963) or reductive (Crestfield, Moore, and Stein 1963) treatments of cystinyl residues prior to hydrolysis. A step in this direction was made recently (Inglis and Liu 1970) when it was shown that cystine and cysteine in proteins could be determined after hydrolysis in hydrochloric acid providing the hydrolysate was treated with dithiothreitol and then sodium tetrathionate to convert all cysteinyl compounds to the *S*-sulpho derivatives.

Because cysteine elutes with proline under the usual conditions of amino acid analysis (Moore and Stein 1963) the determination of this amino acid posed a problem. Moore and Stein (1963) recommended oxidation of cysteine to cystine—primarily to prevent high results for proline—but this does not lead to quantitative recoveries of cystine after hydrochloric acid hydrolysis, results usually being around 10% low. Mondino and Bongiovanni (1970) recently studied the production of cysteine from the interaction of cystine and tryptophan and recommended lithium citrate buffers for elution of cysteine from the amino acid analyser. We have also used a lithium system for the determination of cystine and cysteine after hydriodic acid hydrolysis.

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II. MATERIALS AND METHODS

Glutathione (oxidized) was from Schwarz Bioresearch Inc. The proteins lysozyme, ribonuclease, insulin, and myoglobin were as described previously (Inglis, Nicholls, and Roxburgh 1971). The hydrolytic conditions with hydriodic acid were also similar. Colourless hydriodic acid is recommended for cystine analysis. Small amounts of iodine can be tolerated in this determination, but larger amounts lead to variable results.

Amino acid analyses were made on a Beckman Spinco amino acid analyser, model 120B, using lithium citrate buffers, *c.* 0.3*N*. A column (55 by 0.9 cm) of Aminex A4 spherical resin was used, with a buffer flow rate of 68 ml/hr and ninhydrin flow rate of 40 ml/hr. A buffer change from pH 2.96 to pH 4.15 was made at 150 min, and a temperature change from 38 to 57°C was commenced at 90 min; back pressure was 190 lb/in². The analysis for acidic and neutral amino acids was completed with the elution of phenylalanine in 230 min.

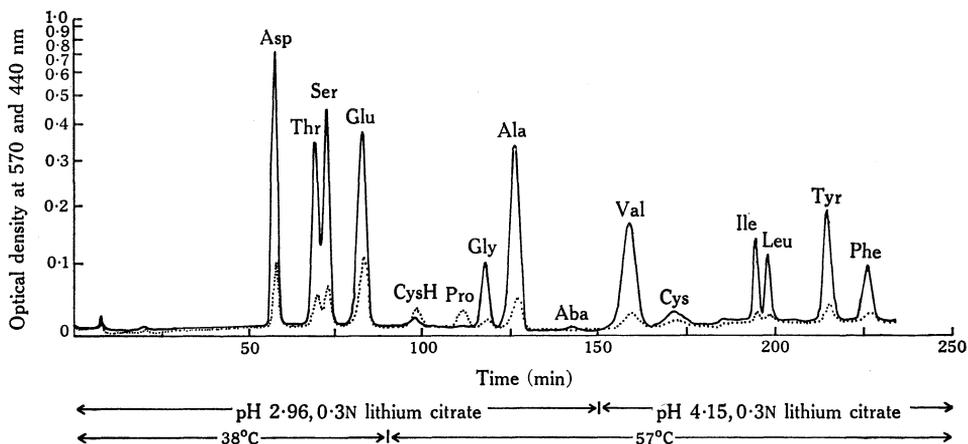


Fig. 1.—Elution curve for ribonuclease (0.035 μ mole) after hydrolysis with hydriodic acid. Aba, α -amino-n-butyric acid.

III. RESULTS

Figure 1 illustrates the separations obtained on the amino acid analyser for a ribonuclease hydrolysate. It has been pointed out previously (Benson, Gordon, and Patterson 1967) that the pH of the lithium buffer, the concentration of lithium and citrate ions, and the temperature of the column can all markedly affect the resolutions of amino acids in this system. This point is emphasized here. The buffer as prepared and adjusted to pH 2.96 with hydrochloric acid may not give optimal separations in the critical region asparagine–glutamic acid–glutamine and the most convenient further adjustment is usually a small alteration to the initial temperature of the column. A small drop in temperature will cause glutamic acid to be eluted more slowly relative to asparagine–glutamine. Conditions are less critical for other amino acids, including cysteine, and the resolution is excellent, as is shown in Figure 1. Cysteine is well resolved from glutamic acid and proline, α -amino-n-butyric acid appears midway between alanine and valine, and cystine is eluted between valine and the buffer change peak. The cysteine and proline peaks correspond to 6 and 4 residues respectively and reflect the low colour factor of cysteine (leucine factor \times 0.15).

However, it has certainly been possible to reproduce results for this amino acid to within $\pm 3\%$ at this level of loading.

TABLE 1
RECOVERIES OF CYSTINE AND CYSTEINE AFTER HYDROLYSIS OF SUBSTANCES IN CONSTANT-BOILING HYDRIODIC ACID

Substance	Hydrolysis Conditions		No. of $\frac{1}{2}$ -Cystine Residues	No. of Cysteine Residues	Total No. of Residues	Yield (%)
	Temp. (°C)	Time (hr)				
Glutathione (ox.)	120	3.5	—	1.9	1.9	95
Myoglobin (control)	140	4	—	—	—	—
Lysozyme	140	2	2.1	5.9	8.0	100
	140	2*	7.8	—	7.8	97
Ribonuclease	110	4.5	2.7	4.9	7.6	95
	140	6*	7.5	—	7.5	94
	140	4†	5.9	2.1	8.0	100
	110	22‡	1.2	7.1	8.3	104
Insulin	140	6	0.6	5.2	5.8	97
	120	6‡	1.4	4.2	5.6	93

* Freshly distilled acid. † Two parts hydriodic acid with one part water.

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Table 1 shows the values for cysteine and cystine obtained for various proteins and the peptide glutathione after hydrolysis with hydriodic acid. The acid used was either the colourless reagent containing hypophosphorous acid or the freshly distilled acid. After 4 hr at the boiling point, virtually all of the cystine in the proteins and peptides was recovered as cystine plus cysteine. Oxidized glutathione yielded stoichiometric amounts of cysteine, glutamic acid, and glycine. With the proteins the yields of cysteine were variable and obviously dependent on the presence of iodine in the hydriodic acid and the time and treatment prior to analysis. Freshly distilled hydriodic acid gave quantitative recovery of cystine and no cysteine but yields sometimes dropped off for older reagents or for hydriodic acid diluted with water. Nevertheless, yields averaged 97% for cystine determinations of lysozyme, ribonuclease, and insulin.

IV. DISCUSSION

(a) Chromatography

The chromatographic system used here is another modification of a system originally proposed by Benson, Gordon, and Patterson (1967) and subsequently recommended by other workers (Mondino 1969; Atkin and Ferdinand 1970; Kedenburg 1971) with appropriate changes to compensate for the effects of parameters such as the types of ion-exchange resin, increased flow rates, and single-column analyses. It has been used for analysing enzyme digests of wool for asparagine and glutamine (Holt, Milligan, and Roxburgh 1971). The conditions are closest to those

of Atkin and Ferdinand (1970) who used slightly faster flow rates with Aminex A4 resin. One advantage of this resin is that the desired resolutions can be obtained without developing high pressures in the chromatographic system. Unlike Atkin and Ferdinand (1970), we did not find it necessary to use wider and shorter columns than the usual 55 by 0.9 cm ones or to add propanol to the buffer for adequate resolution of the amino acids. Both systems require around 230 min for elution of phenylalanine but there are differences in the elution patterns. With our system the relative times of elution for amino acids eluted between alanine and methionine are greater and those after methionine are smaller than with that of Atkin and Ferdinand (1970). This facilitates resolution of amino acids such as α -amino-n-butyric acid and cystine without adversely affecting the separation of amino acids eluted with the second buffer. As indicated in Section III, small changes in temperature and pH affect the separations. Lanthionine is one of the more difficult amino acids to resolve in this system; DL-lanthionine is eluted before proline but *meso*-lanthionine overlaps proline and glycine and cannot be quantitated. If much *meso*-lanthionine is present, it will interfere with these peaks unless the conditions of chromatography are strictly correct. If these diastereoisomers are to be determined accurately, a separate run using the conditions established by Inglis and Nicholls (1968) would be preferable. *S*-Carboxymethylcysteine would normally elute with aspartic acid but can be resolved in some cases by reducing the temperature of the column to 30°C initially.

(b) *Hydrolysis*

This work demonstrates for the first time the complete recovery of cystine, as either cystine or cystine plus cysteine, from proteins by amino acid analysis of the hydrolysate. Inglis and Liu (1970) showed that cysteine and cystine were not usually extensively degraded during hydrochloric acid hydrolysis and developed a method for quantitative determination of these substances in the hydrolysate. With the present procedure no such after-treatment is required if the iodine content of the hydriodic acid is low. Accordingly we prefer to use a colourless (M.A.R.) hydriodic acid containing hypophosphorous acid to ensure a reducing situation, although with freshly distilled hydriodic acid cystine itself was obtained in quantitative yield. In a previous paper (Inglis, Nicholls, and Roxburgh 1971) it was shown that hydrolysis with 2.5N acid gave similar results to those obtained with constant-boiling hydrochloric acid. The results for cystine analysis with the diluted reagent are on the whole not quite as good as those with the concentrated reagent and so the latter is recommended where the best results for cystine content are desired.

The cysteine analysis is less accurate and less sensitive than other amino acid analyses because of the very low colour yield. If improved sensitivity is desired, flow cells larger than the 2.2 mm diameter of the 120B analyser could be easily incorporated in the system. Mondino and Bongiovanni (1970), who used a 10 mm flow cell, additionally increased the sensitivity of the 440 nm channel by a factor of 4 for cysteine determinations; alternatively, the cysteine could be oxidized to cystine (Moore and Stein 1963) or the amino acids could be converted to *S*-sulphocysteine (Inglis and Liu 1970). Another approach, attractive because it would eliminate these extra steps, is to convert the cysteine to a more stable derivative during the hydrolysis reaction. Preliminary attempts to do this by adding benzyl alcohol

(Iwamoto *et al.* 1961) to the hydriodic acid have been unsuccessful in that only 65% of cystine was converted to *S*-benzylcysteine. However, in this paper we have been concerned primarily with establishing that only cystine-cysteine interconversions occur on hydrolysis of proteins with fresh hydriodic acid, and so showing that a direct determination is possible in the course of a routine run on the amino acid analyser.

The time involved for complete liberation of cystine in constant-boiling hydriodic acid need not be longer than 6 hr since the very resistant Ile-Val bonds are quantitatively cleaved in this period (Inglis, Nicholls, and Roxburgh 1971). With lysozyme we found that hydrolysis for 2 hr at 140°C gave quantitative cystine recoveries (see Table 1) but longer hydrolysis times of up to 6 hr were used for other proteins without unduly affecting cystine recoveries. For proteins of unknown composition therefore, it should be safe to adopt similar hydrolysis times and ensure complete hydrolysis of the protein. Low results were obtained after hydrolysis in aged acid containing high iodine concentrations which suggests that the enhanced reducing action of hydriodic acid over hydrochloric acid is the probable reason for the observed improvement in cystine recovery with hydriodic acid hydrolysis.

The hydriodic acid hydrolysis procedure for determination of cystine is comparable in accuracy to and is much more convenient than alternative procedures which require chemical modification of the residue either prior to (Moore 1963; Crestfield, Moore, and Stein 1963) or after (Inglis and Liu 1970) hydrolysis in hydrochloric acid. The excellent recovery of the cystinyl residues as cystine after hydrolysis in freshly distilled hydriodic acid raises the interesting question of whether cysteine and cystine can both be determined specifically using a simple hydrolytic technique. This question is presently under examination.

V. ACKNOWLEDGMENT

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VI. REFERENCES

- ATKIN, G. E., and FERDINAND, W. (1970).—*Analyt. Biochem.* **38**, 313.
BENSON, J. V. JR., GORDON, M. J., and PATTERSON, J. A. (1967).—*Analyt. Biochem.* **18**, 228.
CRESTFIELD, A. M., MOORE, S., and STEIN, W. H. (1963).—*J. biol. Chem.* **238**, 622.
HIRS, C. H. W., STEIN, W. H., and MOORE, S. (1954).—*J. biol. Chem.* **211**, 941.
HOLT, L. MILLIGAN, B., and ROXBURGH, C. M. (1971).—*Aust. J. biol. Sci.* **24**, 509.
INGLIS, A. S., and LIU, T. Y. (1970).—*J. biol. Chem.* **245**, 112.
INGLIS, A. S., and NICHOLLS, P. W. (1968).—*Analyt. Biochem.* **24**, 209.
INGLIS, A. S., NICHOLLS, P. W., and ROXBURGH, C. M. (1971).—*Aust. J. biol. Sci.* **24**, 1235.
IWAMOTO, R. H., ACTON, E. M., BAKER, B. R., and GOODMAN, L. (1961).—*Chemistry Ind.* **1961**, 1404.
KEDENBURG, C. P. (1971).—*Analyt. Biochem.* **40**, 35.
MONDINO, A. (1969).—*J. Chromat.* **39**, 262.
MONDINO, A., and BONGIOVANNI, G. (1970).—*J. Chromat.* **52**, 405.
MOORE, S. (1963).—*J. biol. Chem.* **238**, 235.
MOORE, S., and STEIN, W. H. (1963).—"Methods in Enzymology." (Eds. S. P. Colowick and N. O. Kaplan.) Vol. 6. p. 819. (Academic Press: New York.)

