AN EXAMINATION OF THE QUANTITATIVE AMINO ACID CHROMATOGRAPHY OF S-CYSTEINOSUCCINIC ACID AS A MEANS OF DETERMINING THE EXTENT OF THE REACTION OF N-ETHYLMALEIMIDE WITH PROTEIN THIOL GROUPS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

Hernan Nunez-Arellano

1966

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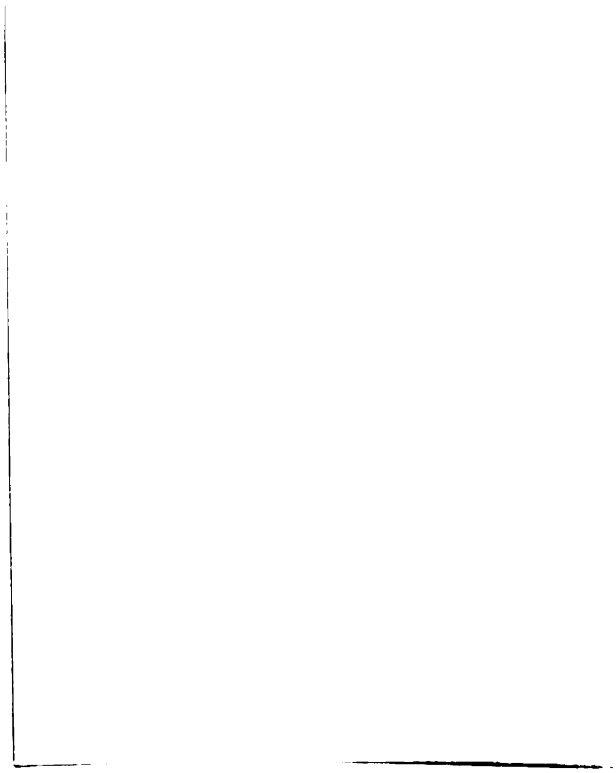

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By

Hernan Nunez-Arellano

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1966

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. J. C. Speck, Jr. for his guidance and constructive criticism throughout the course of this investigation. I am also very grateful to Mr. D. L. Schnider for being readily available for assistance and discussion. The assistance obtained in the laboratory from Messrs. D. J. Rynbrandt, J. LaRue, and G. Stone is greatly appreciated. The co-operation and the interest of Miss D. Bauer, who performed the chromatographic analysis, is also greatly appreciated.

Acknowledgment is also due to the University of California-University of Chile program for the fellowship which made possible this work.

I am especially grateful to my wife, Betty, for her love, understanding and encouragement throughout these studies.

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INTRODUCTION

The search for suitable reagents and procedures for quantitative determination of thiol groups in proteins, enzymes, food and tissues has attracted the attention of numerous workers during recent years. As a result, there are now available a large number of thiol determination methods (1,2). Most of them, however, have limitations of one kind or another (1). Benesch and Benesch (1) in reviewing this subject concluded that the most prudent approach for determining thiol groups in proteins is one involving the use of several different methods, based on different chemical reactions. Since the appearance of this review the situation does not seem to have changed appreciably. It seems, therefore, that a simultaneous effort for both the development of new analytical techniques and a better evaluation of those potentially useful ones would be appropriate.

N-ethylmaleimide appears to be one of the most frequently applied among the thiol reagents recently proposed (2). A reported disadvantage (affecting the accuracy of thiol determination based on the N-ethylmaleimide reaction) is its reaction with other nucleophilic groups (20,21,26); furthermore, N-ethylmaleimide has sometimes appeared to react incompletely with thiol groups in proteins (19). It turns out that N-ethylmaleimide does react with other nucleophilic groups; however, the lack of reactivity toward thiol groups

seems to be nonexistent provided that the protein is adequately denatured (19).

This situation leaves both the spectrophotometric methods (16,17) and the chromatographic method (21) in a critical position since the first ones are based on the consumption of the reagent, and the possible interferences of the side reaction products in the second have not been studied, except for ovoalbumin (21).

The accuracy of the results obtained with the chromatographic method is based on the assumption that the extent of hydrolysis of a model compound, S-cysteino-N-ethylsuccinimide is, under similar conditions, the same as that of the N-ethylmaleimide-modified protein with respect to the yield of S-cysteinosuccinic acid (21). From the agreement in the results of comparative studies with other thiol determination methods, the assumption has been presumed to be correct (21,26), but differences in reactivity toward thiol groups between N-ethylmaleimide and the other thiol reagents have been supposed when discrepancies in the results appear (25). Thus, no direct evidence for the applicability of the conversion factor has been given.

The present work was undertaken (a) to evaluate the chromatographic procedure as a method for estimating thiol groups by unequivocally characterizing and measuring the 2-S-cysteinosuccinic acid in a mixture of N-ethylmaleimide-side reaction products, and (b) to determine the validity of the assumption about the conversion factor so far made.

These studies were carried out on proteins with amino acid compositions completely characterized in order to have a fixed, known number of cysteine residues to react with N-ethylmaleimide, and a fixed, known number of stable amino acid residues to use as an internal standard. In this way, an agreement between the corrected S-cysteinosuccinic acid value recovered and the known amount of cysteine present in the protein, provided that the N-ethylmaleimide reaction with the cysteine residues occurs quantitatively, furnishes direct evidence for the applicability of the conversion factor to the protein.

With proteins of known sequence it was possible also to examine:

(1) the effect of adjacent residues on the reactivity of a cysteine residue with N-ethylmaleimide, especially if the adjacent residue has already undergone reaction with N-ethylmaleimide (this would apply especially to adjacent cysteine);

(2) the effect of adjacent residues, modified or not by N-ethylmaleimide, on the hydrolysis of N-ethylmaleimide-modified cysteine residue.

Since SH-containing proteins lacking disulfide bonds (*) with the properties above mentioned were not available, the capability and the conditions under which a recently proposed compound (3), dithiathreitol, could quantitatively reduce disulfide-containing proteins was also investigated. It was thought that this investigation would be useful from

the standpoint of determining which are the best reducing agents for disulfide bonds in proteins.

(*) Quantitative determination of cystine is not accurate.

LITERATURE REVIEW

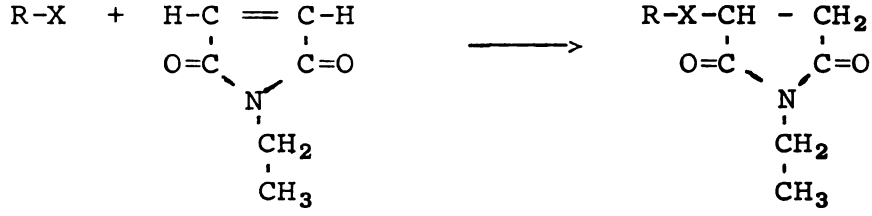
A. N-ethylmaleimide as thiol reagent.

Three different techniques for using the reactivity of N-ethylmaleimide towards thiol groups of proteins were proposed prior to the development of the chromatographic method studied in the present work.

1. - The first indication that N-ethylmaleimide could be useful as thiol reagent came from the observation of Friedman et al. (10) concerning the fast and quantitative reaction with glutathione and thiolacetic acid. Four years later, in 1953, Tseo and Bailey (11) estimated the thiol content of actin, myosin and ovoalbumin by using nitroprusside as an external indicator to determine the extent to which N-ethylmaleimide reacted with the proteins.

2. - In 1956, Benesch and Benesch (12), proposed a method for measuring the extent of the reaction based on the formation of a red color of the adduct of N-ethylmaleimide and the thiol groups. However, it was shown later that the coloration was due to both a radical-initiated and base-catalyzed polymerization of N-ethylmaleimide (13), and it has been reported that imidazole, histidine and cysteine catalyze this reaction in alkaline media (14). In the present work it has been found that dithiathreitol also appears to catalyze the reaction at pH 8.2.

3. - In 1958, Alexander (16) and Roberts and Rouser (17) introduced the spectrophotometric method. Since then, N-ethylmaleimide has been widely used for determination of reactive thiol groups in proteins, even when most of the time confidence in the values obtained has been based on the agreement between these values and those obtained by other methods. The spectrophotometric method is based on the decrease in absorbance at 300 mμ that occurs when the double bond of N-ethylmaleimide is destroyed by the addition of a nucleophilic group to the N-ethylmaleimide molecule (15).

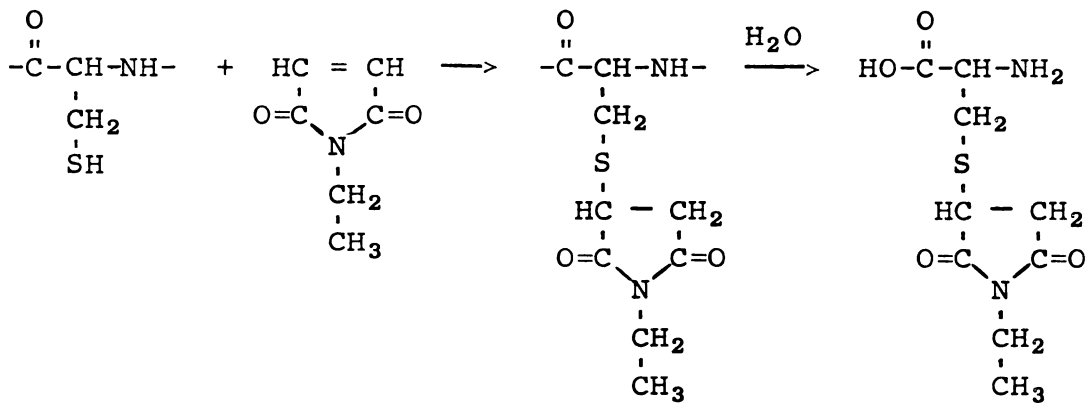


X = a nucleophile such as -SH or $-NH_2$.

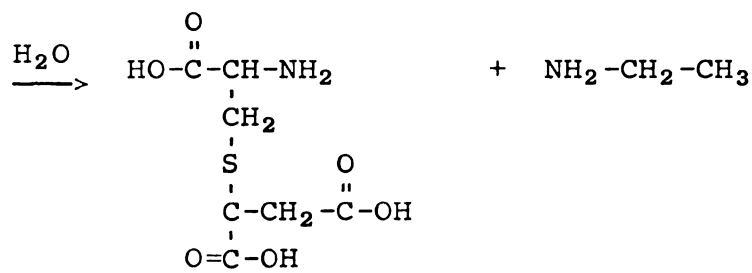
The conditions under which the method was developed (16,17) have been proved to be very appropriate for the exclusive reaction of N-ethylmaleimide with thiol groups of several proteins (18,19 . Nevertheless, a method which involves the measurement of the disappearance of the reagent, in contradistinction to the measurement of the formation of a derivative, provides inadequate information about the nature of the reaction (20).

4. - In 1961, Riehm and Speck (21), developed the chromatographic method with which this work is concerned. The

quantitative determination of the reaction between N-ethylmaleimide and thiol groups of proteins depends on the measurement of the S-cysteinosuccinic acid produced during acid hydrolysis of the N-ethylmaleimide-treated proteins. The reactions involved are indicated in the following scheme.



Cysteine residue + N-ethylmaleimide ⟶ N-ethylmaleimide-modified protein ⟶ S-cysteino-N-dethylsuccinimide



⟶ S-cysteinosuccinic acid + ethylamine

The S-cysteinosuccinic acid is quantitatively determined in the elution pattern of the chromatographic amino acid analysis (22).

In the preliminary communications (21) it was shown that S-cysteino-N-ethylsuccinimide is also formed, since the hydrolysis of the peptide bonds which link the cysteine

residue to the polypeptide chain is a considerable faster reaction than is the hydrolysis of the imide ring. The truth of this statement derives from observing the presence of both S-cysteinosuccinic acid and S-cysteino-N-ethylsuccinimide at the end of a period of 120 hours of hydrolysis of the latter substance in constant-boiling hydrochloric acid at 105°C. S-Cysteinosuccinic acid is not destroyed under these conditions (20,21).

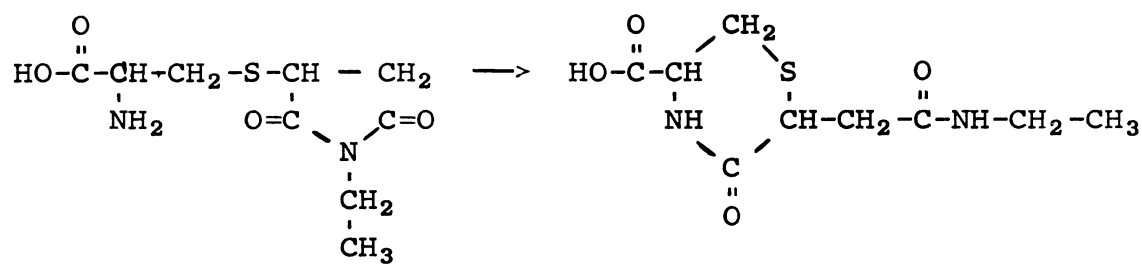
The introduction of a second center of symmetry during the reaction of either cysteine or a cysteine residue on reaction with N-ethylmaleimide implies that two new diastereoisomers may be produced that will show different chromatographic behavior. In fact, this has been found. The diastereoisomeric mixture of S-cysteino-N-ethylsuccinimide appears as two peaks, one overlapping proline (20) or glutamic acid (21) and the other preceding glycine (20, 21). A marked elevation of the base line between the two peaks was observed, the amount of ninhydrin-positive material present in this region depending on the length of the column (20). If one of the diastereoisomers is isolated and then chromatographed again, the equilibrium between the two species is re-established and two peaks appear in the same position of the original ones (20).

The amount of material present in the two diastereoisomeric forms have been measured either by column chromatography (20) or by paper chromatography using labeled N-ethylmaleimide (18,23). In the latter case, however, the

separation of the diastereoisomers was not achieved.

Racemic S-cysteinosuccinic acid is eluted from a 150-cm column as a single peak at about 15 ml ahead of the methionine sulfoxides (20,21) in chromatography by the method of Spackman, et. al. (22). Further studies have shown that the single S-cysteinosuccinic acid peak either is made asymmetric or is split into two peaks when the S-cysteinosuccinic acid stands in neutral or slightly basic solution. Presumably the formation of a different chemical entity occurs. The modification is reversed at acidic pH (24).

Prior to the introduction of the chromatographic method it was reported that when S-cysteino-N-ethylsuccinimide is exposed to mild alkaline conditions (pH 9) an intramolecular transamidation reaction involving the attack of the amino group on the imide carbonyl group occurs (14), forming a ninhydrin-negative product.



S-cysteino-N-ethyl-succinimide

2-(N-ethylcarboxymethyl)-3-oxo-2H,4H,5H,6H-1,4-thiazine-3-carboxylic acid

When this compound was hydrolyzed in 6M hydrochloric acid, ethylamine was released more rapidly than S-cysteinosuccinic

acid (20). Since in all acid hydrolyzates of S-cysteino-N-ethylsuccinimide the sum of the amounts of S-cysteinosuccinic acid and the residual S-cysteino-N-ethylsuccinimide account for the total amount of S-cysteino-N-ethylsuccinimide subjected to hydrolysis and since the release of ethylamine during acid hydrolysis of S-cysteino-N-ethylsuccinimide occurred at the same rate of the formation of S-cysteinosuccinic acid, it could be concluded that such an intramolecular transamidation reaction does not take place under the normal conditions of the method (20).

a) Variations of the chromatographic method:- Lee and Samuels (23), have measured the amount of S-cysteino-N-ethylsuccinimide itself rather than the formation of S-cysteinosuccinic acid produced from the acid hydrolyzates of N-ethylmaleimide-reacted proteins. The proteins were reacted with N-ethylmaleimide-^{14}C, hydrolyzed at 100-103^{0}C for 18 hours and the hydrolysates chromatographed on paper. The apparently very poor separation of the diastereoisomeric mixture of S-cysteino-N-ethylsuccinimide, compared with the separation of S-cysteinosuccinic acid and its N-ethylimide, permitted an estimation of the thiol groups that had reacted. In a similar experiment, involving two-dimensional paper chromatography, Morrell, et. al. (18), recovered 95 percent of the total radioactivity as the N-ethylmaleimide adduct.

Another variation of the method has been introduced by Smyth, et. al.(20) for a quantitative measurement of the

specificity of the N-ethylmaleimide towards thiol groups. The technique is based on a comparison between the amount of ethylamine and S-cysteinosuccinic acid liberated in an acid hydrolysis of N-ethylmaleimide-treated proteins. Since N-ethylmaleimide may react with nucleophilic groups other than thiol groups of cysteine residues (14,20,21,26), and liberate ethylamine during the acid hydrolysis, the finding of an equivalent amount of ethylamine and S-cysteinosuccinic acid indicates both the extent of the reaction of N-ethylmaleimide with thiol groups and the specificity of the reaction. Because ethylamine can also arise on acid hydrolysis of the reagent, the protein must be completely separated from the excess of N-ethylmaleimide before being submitted to hydrolysis. To favor specificity of N-ethylmaleimide towards thiol groups, the reaction mixture should be maintained below neutrality and excess of reagent should be avoided. Under these conditions, the reagent exhibits a high degree of specificity for thiol groups (20).

b) Conversion of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid:- The chromatographic method itself and all the possible variations have in common the fact that they use the extent of the hydrolytic process in a model compound, S-cysteino-N-ethylsuccinimide, to measure the formation of the hydrolysis products, S-cysteinosuccinic acid and/or ethylamine, of the originally formed protein - SH-N-ethylmaleimide adduct or the remainder of

S-cysteino-N ethylsuccinimide present after the hydrolysis process.

Two variables have been studied for this model: temperature and time of hydrolysis in either 6M or constant-boiling hydrochloric acid. The results of these studies are shown in Table 1.

Other variables, however, must have been involved, as can be inferred from discrepancies between results from different laboratories and from the same workers at different times (Table I). From the survey of the literature it is difficult to find out what these unknown variables might be because data concerning the standard employed has not been published except by Riehm and Speck (21). Apparently, however, the starting material employed for evaluating the extent of conversion of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid and the control of temperature are determining factors.

There is no case in which the conversion factor of the model compound, S-cysteino-N-ethylsuccinimide, had been directly proved to be the same as of the N-ethylmaleimide-modified proteins. All the evidence given for the agreement between the conversion factor of the N-ethylmaleimide-reacted proteins and that of the model are based on comparative studies. That is to say, the conversion factor has been assumed to be correct if the number of thiol groups found when the protein reacts with N-ethylmaleimide is the same as the number of thiol groups found by other methods (21,26).

Table I. Conversion of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid

Temperature (°C)	Hydrochloric acid Concentration	Hydrolysis time (Hours)	Percent of unhydrolyzed S-cysteino-N-ethyl-succinimide	Percent of ethylamine formed	Percent of S-cysteino-succinic acid formed	References
110	6M	72	7.6	84.8	88	Smyth, Blumenfeld & Konigsberg(20)
110	6M	72	-	-	85	Smyth, Battaglia & Meschi (4)
110	6M	72	-	-	94-95	Guidotti & Konigsberg (26)
110	6M	72	-	-	87	Morrell, Ayers, Greenwalt & Hoffman (18)
110	6M	66	-	-	84	Blumenfeld & Perlam (5)
105	6M	72	17.4	83	83	Smyth, Blumenfeld & Konigsberg(20)
105	CB*	72	-	-	87	Riehm & Speck (21)
105	CB*	120	-	-	94	Riehm & Speck (21)
105	6M	22	57.5	39	38	Smyth, Blumenfeld & Konigsberg(20)
105	CB*	24	-	-	44	Riehm & Speck (21)
100-103	6M	18	81	-	-	Lee & Samuels (23)
120	6M	60	-	-	100	Tkachuk & Hlynka (25)
120	6M	22	-	-	70	Tkachuk & Hlynka (25)

*CB: constant-boiling hydrochloric acid.

If a different thiol group content is found, a difference of reactivity between N-ethylmaleimide and the reagent used for comparison has been thought to occur (25). Also in some cases no comparison at all has been carried out (20,25).

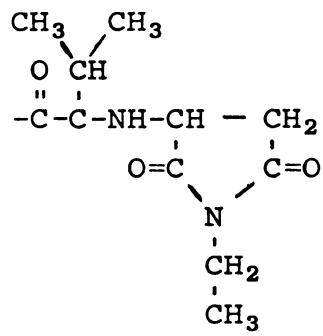
In general no large discrepancies in the comparative studies have been found. Therefore the over-all conclusion is that the conversion of S-cysteino-N-ethylsuccinimide and that of N-ethylmaleimide-modified cysteine residues to S-cysteinosuccinic acid occurs at the same rate during the acid hydrolysis. The only serious difference in hydrolysis rate has been reported with glutathione and wheat proteins reacted with N-ethylmaleimide-^{14}C. While the S-cysteino-N-ethylsuccinimide model is completely hydrolyzed in 60 hours at 120^{0}C in 6N hydrochloric acid, the peptide and proteins are completely hydrolyzed in 22 hours (25). Repetition of the experiment with glutathione reacted with unlabeled N-ethylmaleimide and hydrolyzed at 110^{0} (20) and 105^{0} (the present work) gave no confirmation of the abnormal finding.

B. Nonspecificity of the N-ethylmaleimide Reaction.

It has been adequately established that N-ethylmaleimide may react with thiol groups, amino-terminal groups of proteins and peptides, ε-amino group of lysine residues, and hidtidine residues in proteins and peptides.

When carbon monoxide hemoglobin reacts with N-ethylmaleimide under the conditions used in the spectrophotometric

method (16), a discrepancy has been found between the decrease in absorbance of N-ethylmaleimide at 300 mμ and the appearance of S-cysteinosuccinic acid and ethylamine (26). It is quite possible that the extra disappearance of N-ethylmaleimide is due either to some unspecified destruction of N-ethylmaleimide catalyzed by the protein solution or to some reaction which could not be detected (26). The same workers showed that larger concentrations of N-ethylmaleimide produce alkylation on the amino-terminal valine residue of the β-chain, presumably resulting in a product which yields ethylamine and N-(1,2-dicarboxyethyl)-valine on acid hydrolysis. Additional sites of alkylation in the



β-chain which give rise to an increased amount of ethylamine on acid hydrolysis was not identified, but histidine would be the most probable site of alkylation (26).

Later investigations have shown that treating the peptides Val —> His —> Leu —> Thr —> Pro —> Glu—> Glu —> Lys and Val —> Leu —> Ser —> Pro —> Ala —> Asp —> Lys —> with N-ethylmaleimide for 8 to 10 hours prior to hydrolysis decreased the recovery of valine, lysine and histidine on amino acid analysis (20). Studies on the rate of the N-ethylmaleimide

reaction with the N-terminal residue of peptide, glycyl-L-alanine, (20) have demonstrated that when N-ethylmaleimide is in 10-fold excess, the glycine peaks, obtained on regular column chromatography, completely disappeared after 16 hours of hydrolysis in 6N hydrochloric acid at 110^0C. Instead, a small amount of a product moving ahead of glycine was obtained. It seems probable that the reaction in this instance involves addition of the N-terminal-amino group to the olefinic double bond of N-ethylmaleimide, without opening the imide ring, the product being N-(1-ethyl-2,5-dioxopyrrolidin-3-yl) glycyl-L-alanine.

In treating ovoalbumin, β-lactoglobulin and lysozyme with 0.1M N-ethylmaleimide for 24 hours a decrease in the lysine recovered occurred concurrently with the increase of a peak eluted in the same position of the hydrolyzed product of N-ethylmaleimide-treated poly-L-lysine (21).

METHODS AND MATERIALS

A. Reaction of N-ethylmaleimide with reduced proteins

Insulin:- Seven to fifteen mg of protein, placed in a small beaker, were dissolved in 5 ml of 8M urea solution, freshly prepared using deionized urea (8) and 0.1M. pH 6.8 phosphate buffer. To the protein solution were added 0.16 ml of ethylenediaminotetraacetic acid solution (50 mg of EDTA per ml) and 90 to 100 mg of dithiathreitol. The final concentration of dithiathreitol thus was about 0.1M. This reaction mixture was allowed to stand at room temperature for 4 to 5 hours. During this time (and also after the addition of N-ethylmaleimide) the beaker was covered with Parafilm. At the end of this period, N-ethylmaleimide was added in a quantity sufficient to make the final N-ethylmaleimide concentration about 0.35M. This mixture was stirred until almost all the N-ethylmaleimide crystals went into solution. One hour later at which time all of the N-ethylmaleimide had dissolved, the solution was poured into a dialysis tube and dialyzed at 3-4^{0}C against several changes of distilled water for 48-72 hours. After freeze drying, 5 mg of the reacted protein was hydrolyzed in 3 ml of constant-boiling hydrochloric acid. The air was rigorously excluded from the tubes as described by Moore and Stein (7) except that the pressure was about 0.5 mm (mercury) instead of 50μ described by Moore and Stein. Moreover, the samples

were flushed three times with nitrogen during the evacuation. After 72 hours of hydrolysis at 105^0C the hydrolysates were cooled to room temperature, the ampoules opened and the hydrochloric acid evaporated in a rotary evaporator by maintaining the ampoule in a bath at 35^0C. The dry residue was taken up in 0.5 ml of 0.3 M, pH 6.05 citrate buffer in order to allow any cysteine present to undergo oxidation to cystine, and the resulting solution was allowed to stand at room temperature for 5 hours. This mixture then was dried and dissolved in an appropriate volume of pH 2.2 sample buffer for analysis in the amino acid analyzer.

Lysozyme:- The reductive reaction was carried out in 8M deionized urea solution at pH 8.2 in 0.1M phosphate buffer. After 5 hours at room temperature the pH was lowered to 6.8 by adding small amounts of 20 percent hydrochloric acid with a stirring rod while maintaining the reduced mixture under the pH electrode. Immediately after adjusting the pH, sufficient N-ethylmaleimide was added to make its final concentration 0.38M. Other conditions and procedures were the same as those described for insulin.

Trypsin:- The same conditions, amounts of protein and procedures described for insulin were used, except that half of the dithiathreitol concentration used for insulin was employed. In some cases ethylenediaminetetraacetic acid was not added.

B. Reaction of N-ethylmaleimide with reduced glutathione

Reduced glutathione (16 mg) was dissolved in 5 ml of 0.1M, pH 6.8 phosphate buffer. N-ethylmaleimide added to make the final concentration about 0.05M. The reaction mixture was allowed to stand at room temperature for 1 hour. After freeze drying, the residue was dissolved in 5 ml of constant-boiling hydrochloric acid. A 1-ml aliquot of this solution was placed in a hydrolysis ampoule and 2 ml of constant-boiling hydrochloric acid were added. Then it was treated as described for proteins reacted with N-ethylmaleimide.

C. Reaction of iodoacetate with reduced proteins

Fifteen to 20 mg of insulin placed in a small beaker were dissolved in 5 ml of 8M urea solution, freshly prepared using deionized urea (8) and 0.2M, pH 8.6 tris buffer. To the dissolved proteins 0.15 ml of ethylenediaminetetraacetic acid solution (50 mg per ml) and 90 mg of dithiathreitol were added, giving a final concentration of 0.11M in reducing agent. The further treatment was the same as that described for proteins reacted with N-ethylmaleimide, except that instead of N-ethylmaleimide, iodoacetic acid dissolved in an equivalent amount of 1M sodium hydroxide was added. Five to 10 percent excess, on molar basis, of iodoacetic acid relative to the total amount of thiol groups present in the sample was employed.

Lysozyme, was treated in exactly the same way except that half of the dithiathreitol concentration was used.

D. Conversion of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid

1. Hydrolysis of crystalline S-cysteino-N-ethylsuccinimide:

The starting material had been previously prepared in this laboratory by Dr. J. P. Riehm (21) by a method which differed somewhat from those previously described (6,14).

S-Cysteino-N-ethylsuccinimide (24.6 mg) was dissolved in 10 ml of constant-boiling hydrochloric acid. One ml of this solution, representing 10 μmoles of S-cysteino-N-ethylsuccinimide, was placed in a hydrolysis ampoule. To this were added 2 ml of constant-boiling hydrochloric acid. The evacuation and sealing of the ampoules was the same as described for the proteins. After 72 hours hydrolysis at 105°C, the sample was evaporated to dryness and the residue taken up in 10 ml of pH 2.2 citrate buffer (sample buffer for the amino acid chromatography) and 1 ml, representing 1 μmole of the original cysteine, was analyzed in the amino acid analyzer.

2. Hydrolysis of freshly prepared, unisolated S-cysteino-N-ethylsuccinimide:

This procedure had been previously devised in this laboratory by Dr. J. C. Speck, Jr.

L-Cysteine hydrochloride monohydrate (87.8 mg, 0.5 μmole) was dissolved in 0.1M, pH 6.0 phosphate buffer and

diluted to 50 ml. Ten ml of this solution was added to 1 mmole (125 mg) of N-ethylmaleimide. After dissolution of the N-ethylmaleimide (this required about 25 minutes) the solution was diluted to 25 ml with water. Ten ml of this solution was evaporated to dryness in a vacuum desiccator by pumping with a high vacuum pump. The dry residue was dissolved in 4 ml of constant boiling hydrochloric acid and 1-ml aliquot was taken for hydrolysis. The necks of the ampoules used for hydrolysis were rinsed with 2 ml of constant boiling hydrochloric acid. After hydrolysis, the samples were treated as described above.

E. Preparation of S-cysteinosuccinic acid for determination of its ninhydrin color value

The procedure had been previously divised in this laboratory by Dr. J. C. Speck, Jr.: L-cysteine hydrochloride monohydrate (175.6 mg, 1.00 mmole) was dissolved in 20 ml of 1M sodium maleate. A little water was added to help dissolution. The reaction was allowed to proceed for 30 minutes at room temperature. Then the volume was brought to 100 ml with water. This solution had a pH of 7.0*. Twenty-five ml of the above solution were adjusted to pH 1.0 with concentrated hydrochloric acid; then it was diluted to 50 ml and the resulting solution allowed to stand at room temperature for 1 hour. For analysis in the amino

*At this time nitroprusside reaction was negative.

acid analyzer 2 ml of this solution were diluted to 10 ml with pH 2.2 sample buffer.

Preparation of 1M sodium maleate:- Maleic acid (11.6 g, 0.1 mole) was partially dissolved in 10 ml of water. Then 10.6 ml of 50 percent sodium hydroxide were added slowly (heat is produced). Then the solution was diluted to 100 ml with water. The pH of several solutions prepared in the same manner was 9.5 to 10.1.

Proteins:- Crystalline insulin from bovine pancreas was obtained from Sigma Chemical Co., lot 55B-1820. Crystalline lysozyme (egg white) from Pentex Incorporated. Crystalline trypsin from Worthington Biochemical Corporation, Lot 680-681B. These materials were used without further purification.

Other reagents:- N-ethylmaleimide (lot number 114B-1610) was purchased from Sigma Chemical Co., and iodoacetic acid from Eastman Organic Chemicals. Dithiathreitol (lot numbers 60156 and 60167), L-cysteine hydrochloride monohydrate, and reduced glutathione (lot number 53578) were obtained from California Corporation for Biochemical Research.

Amino acid analysis:- All the analyses were carried out with the Spinco Model 120 Amino Acid Analyzer.

RESULTS

A. Ninhydrin color value of S-cysteinosuccinic acid

The color value of freshly prepared S-cysteinosuccinic acid was measured with the amino acid analyzer. The elution conditions were similar to those employed for the hydrolysates of N-ethylmaleimide-modified proteins. Table II shows that the average ninhydrin color value for 1 μmole of S-cysteinosuccinic acid is 85 percent of that of 1 μmole of leucine. The values of leucine were taken from those obtained in the analysis of the standard calibration mixture.

B. Conversion of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid

The extent of this conversion was measured with the amino acid analyzer after a known amount of S-cysteino-N-ethylsuccinimide was hydrolyzed in constant-boiling hydrochloric acid at 105°C for 72 hours. Table III shows that the average value of S-cysteinosuccinic acid found after the hydrolysis process is 81.5 percent of the total amount of S-cysteino-N-ethylsuccinimide present in the sample. The same value is obtained whether the starting material is the unisolated, freshly prepared substance or that in the pure, crystalline form.

Table II. Color value of S-cysteinosuccinic acid compared with the color value of leucine.

Experiment	Integration Constant		Percent of color
	Leucine	S-cysteino-succinic acid	
1	28.00	24.35	87
2	28.00	24.01	86
3	27.61	23.03	83
4	27.61	23.41	85

Table III. Conversion of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid.

Experiment	Origin of S-cysteinosuccinic acid	Percent Conversion
1	cysteine + N-ethylmaleimide	82
2	cysteine + N-ethylmaleimide	80
3	cysteine + N-ethylmaleimide	83
4	cysteine + N-ethylmaleimide	81
5	crystalline form	81
6	crystalline form	82

C. Reaction of N-ethylmaleimide with proteins

1. Calculation of thiol groups present.-- Calculating the true amount of S-cysteinosuccinic acid present in the hydrolysates of N-ethylmaleimide-modified proteins requires two steps:

a) The integration constant is taken as 85 percent of the integration constant value of leucine, since 1 μmole of S-cysteinosuccinic acid gave this fraction of the ninhydrin color value of 1 μmole of leucine (Table II).

b) Since 81.5 percent of the total amount of the protein-SH-N-ethylmaleimide adduct in the sample is hydrolyzed (assuming that its hydrolysis rate is the same as that of S-cysteino-N-ethylsuccinimide itself), the actual value is found by dividing the figure determined in step a) by 81.5.

2. Amino acid analysis of N-ethylmaleimide-modified proteins.-- Tables IV to VI give the relative number of amino acid residues of the unmodified and N-ethylmaleimide-reacted proteins after 72 hours hydrolysis at 105°C in constant-boiling hydrochloric acid. These figures represent the values obtained by using alanine as an internal standard. No corrections of the values obtained for amino acids that are partially destroyed on hydrolysis were made for either the unmodified or the N-ethylmaleimide-treated proteins. In every table the number of amino acid residues per mole of protein is included. The same criterion was

applied to make Table VII, where the results obtained for reduced glutathione reacted with N-ethylmaleimide are indicated. For this peptide, however, no internal standard was used. Instead the average number of μmoles of each one of the three species found in the samples is given.

The results indicate that almost all the expected modifications in the amino acid elution pattern when the proteins are reacted with a large excess of N-ethylmaleimide did occur. N-Ethylmaleimide reacts much faster and quantitatively with cysteine residues than do histidine residues and the ε-amino group of lysine residues (20,21, 26) under these conditions. Moreover, it was expected that the amino-terminal residues would react with N-ethylmaleimide. At least, it was known that amino-terminal glycine does (20).

Since the present work was intended to determine the effects of the modified amino acid residues above mentioned in the quantitative recovery of S-cysteinosuccinic acid, no attempt has been made to analyze on a quantitative basis other modifications than those observed in the cysteine residue values and its N-ethylmaleimide derivative, S-cysteinosuccinic acid.

In determining how the cysteine residue values were affected when the proteins were reacted with a large excess of N-ethylmaleimide, the following points were kept in mind:

1. If N-ethylmaleimide reacts quantitatively with cysteine residues they should not appear in the analysis of

the N-ethylmaleimide-modified protein hydrolysates. (To detect the cysteine residues which could have been left unreacted, they are, prior to the analysis process, oxidized to cystine.)

2. If all cysteine residues do react with N-ethylmaleimide and if the N-ethylmaleimide-modified amino acid residues, other than cysteine, do not interfere with the S-cysteinosuccinic acid peak, the equivalent amount of S-cysteinosuccinic acid relative to the theoretical amount of cysteine residues should be found. To find the actual amount of S-cysteinosuccinic acid, the conversion factor already known from the hydrolysis of the model compound, S-cysteino-N-ethylsuccinimide, must be applied.

3. On the other hand, if no cysteine residues were present, but the amount of S-cysteinosuccinic acid, after having been corrected, were not equivalent to that of cysteine, either the conversion factor was not applicable or some of the N-ethylmaleimide-modified amino acid residues were interfering.

Tables IV to VI show that the corrected S-cysteinosuccinic acid values are in good agreement with the theoretical value of the cysteine residues present in the molecules of insulin, lysozyme, and trypsin.

The values of the N-ethylmaleimide-modified amino acid residues, other than cysteine, cannot be described quantitatively, since there is not enough information available. However, the following points of reference can be taken in

account for a qualitative description of their recovery.

1. The N-ethylmaleimide-modified amino acid probably has a different chromatographic behavior than the unmodified one. Therefore, a lower recovery of the reacted amino acid is expected in the hydrolysates of the N-ethylmaleimide-modified protein than in those of the unmodified one. This is known to be the case of N-ethylmaleimide-modified lysine (21) and N-ethylmaleimide-modified glycine (20).

2. The N-ethylmaleimide-modified amino acids are expected to be ninhydrin positive, as those mentioned in the above paragraph are. Therefore, if they are eluted from the column within the volume used for the normal amino acids, a new peak for every N-ethylmaleimide-modified amino acid will appear.

3. The new peak may or may not overlap with the peak of a normal amino acid. Thus, it may not introduce a second modification in the elution pattern.

4. The modifications introduced by at least one of the two peaks of the unhydrolyzed S-cysteino-N-ethylsuccinimide diastereoisomers must be taken in account (20,21).

In the case of insulin (Table IV) there are four modifications which could decrease the recovery of lysine (20, 21), histidine (20,26), amino-terminal glycine (20) and (by generalizing what is known for amino-terminal glycine) amino-terminal phenylalanine. In fact this was found. The four mentioned amino acids are recovered in lesser amounts than the corresponding amino acids in the hydrolysates of

the unmodified protein. In the case of lysozyme (Table V), which contains lysine as the amino-terminal residue also, a lower recovery of lysine and histidine is found, as expected. For trypsin (Table VI) low recoveries of lysine and histidine were also found. However, the recovery of isoleucine, the amino-terminal residue of trypsin, appears to be normal. It was expected that the glutamic acid and proline values in all of the N-ethylmaleimide-modified proteins would be higher since the first of the two peaks formed by the unhydrolyzed S-cysteino-N-ethylsuccinimide is eluted simultaneously with glutamic acid (21) and N-ethylmaleimide-modified lysine is eluted at the same volume as is proline (24). Again this was observed.

In the case of insulin and lysozyme the recoveries of all of the other amino acid residues are in good agreement with the values found in the analysis of the untreated proteins, after being hydrolyzed under the same conditions. One extra peak appears between aspartic acid and threonine. It does not appear either in the chromatograms of the unmodified proteins or in that of those proteins reduced with dithiathreitol and reacted with iodoacetic acid. Therefore, it seems to correspond to an N-ethylmaleimide-amino acid derivative, probably N-ethylmaleimide-modified histidine. In the case of N-ethylmaleimide-modified trypsin the other amino acid values, except those for arginine, aspartic acid, phenylalanine, and glycine, are in good agreement with the values found in the hydrolyzates of the untreated trypsin.

Table IV. Insulin

Amino Acids	Theoretical number of amino acid residues per mole	Relative number of amino acid residues per mole of insulin recovered after 72 hours hydrolysis in constant-boiling hydrochloric acid at 105°C.					
		Unmodified protein	N-ethylmaleimide-modified protein				
			Experiment Number				Average
			1	2	3	4	
Lysine	1.0	1.0	0.9	0.8	0.8	0.8	0.8
Histidine	2.0	1.9	1.5	1.2	1.6	1.5	1.4
Arginine	1.0	1.1		1.1	1.1	1.1	1.1
Aspartic Acid	3.0	2.9	2.9	3.0	3.1		3.0
Threonine	1.0	0.9	0.9	0.9	0.9		0.9
Serine	3.0	2.4	2.4	2.4	2.4		2.4
Glutamic acid	7.0	7.4	7.8	7.9	7.4		7.7
Proline	1.0						
Glycine	4.0	4.2	3.5	3.4	3.5		3.5
Alanine	3.0	3.0	3.0	3.0	3.0		3.0
Valine	5.0	4.6	4.7	4.6	4.6		4.6
Isoleucine	1.0	0.9	0.9	0.9	0.8		0.9
Leucine	6.0	6.0	5.9	6.0	5.8		5.9
Tyrosine	4.0	3.7	3.6	3.7	3.6		3.6
Phenylalanine	3.0	2.9	2.4	2.3	2.2		2.3
S-cysteino-succinic acid			5.6	5.8	5.6		5.7
$\frac{1}{2}$ Cystine	6.0						

Table V. Lysozyme

Amino Acids	Theoretical number of amino acid residues per mole	Relative number of amino acid residues per mole of lysozyme recovered after 72 hours hydrolysis in constant-boiling hydrochloric acid at 105°C					
		Unmodified protein	N-ethylmaleimide-modified protein				
			Experiment Number				Average
			1	2	3	4	
Lysine	6.0	5.8	2.7	2.6	-	4.3	3.2
Histidine	1.0	1.0	1.0	0.9	-	0.8	0.9
Arginine	11.0	11.0	10.6	12.11	11.3	10.9	11.2
Aspartic Acid	21.0	21.3	19.6	19.6	22.2	20.2	20.4
Threonine	7.0	6.6	6.5	6.4	6.9	6.4	6.5
Serine	10.0	8.2	8.3	7.8	8.3	8.2	8.1
Glutamic acid	5.0	5.3	5.8	5.9	6.3	5.7	5.9
Proline	2.0	-	-	-	-	-	-
Glycine	12.0	12.0	12.1	12.0	12.0	12.1	12.0
Alanine	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Valine	6.0	6.0	5.9	5.9	6.0	5.9	5.9
Methionine	2.0	1.6	1.4	1.6	1.9	2.0	1.7
Isoleucine	6.0	6.0	5.9	5.9	6.0	5.8	5.9
Leucine	8.0	8.0	8.2	8.3	8.2	7.9	8.1
Tyrosine	3.0	3.3	-	2.9	3.0	3.0	3.0
Phenylalanine	3.0	3.1	-	2.9	3.0	3.0	3.0
S-cysteino-succinic acid			7.4	7.3	8.4	7.7	7.7
$\frac{1}{2}$ cystine	8.0	-	-	-	-	-	-

Table VI. Trypsin

Amino Acids	Theoretical number of amino acid residues per mole	Relative number of amino acid residues per mole of trypsin recovered after 72 hours hydrolysis in constant-boiling hydrochloric acid at 105°C				
		Unmodified protein	N-ethylmaleimide-modified protein			
			Experiment Number			Average
			1	2	3	
Lysine	14.0	13.0	9.5	10.2	10.2	10.0
Histidine	3.0	2.6	1.8	0.9	1.8	1.5
Arginine	2.0	2.0	1.0	-	0.9	1.0
Aspartic Acid	22.0	22.2	28.8	28.4	25.4	27.7
Threonine	10.0	9.4	9.7	9.0	9.7	9.5
Serine	35.0	26.4	28.6	27.5	26.7	27.6
Glutamic acid	14.0	14.8	19.2	23.0	18.4	20.2
Proline	9.0		-	-	-	-
Glycine	26.0	23.8	29.0	31.5	27.4	29.0
Alanine	14.0	14,0	14.0	14.0	14.0	14.0
Valine	16.0	15.5	16.1	17.0	15.6	16.2
Methionine	2.0	1.7	1.7	2.3	2.6	2.2
Isoleucine	15.0	14.0	14.3	14.6	13.9	14.2
Leucine	15.0	13.8	15.1	13.7	14.8	14.5
Tyrosine	10.0	9.2	8.9	8.1	9.2	8.7
Phenylalanine	3.0	3.4	3.9	4.5	3.8	4.1
S-cysteino-succinic acid			12.2	12.5	12.4	12.4
$\frac{1}{2}$ cystine	12.0	-	-	-	-	-

There is no simple explanation for the finding of these abnormal values. It is possible that the trypsin had undergone autolysis since the sample used in this study was one of crystalline material several years old. In this way, the extra amino-terminal residues formed on autolysis could, after reacting with N-ethylmaleimide, have modified the chromatographic pattern.

D. Reaction of N-ethylmaleimide with reduced glutathione

The experiments with glutathione were performed in order to determine whether the phenomenon observed by Tkachuk and Hlynka (25) could be observed under the present conditions. These investigators reported that the N-ethylmaleimide-modified cysteine residue formed when N-ethylmaleimide is allowed to react with glutathione is hydrolyzed faster than the same adduct formed with cysteine
The results obtained in the present work, however, show that the hydrolysis rate of the N-ethylmaleimide-modified glutathione is the same as that of N-ethylmaleimide-modified proteins and S-cysteino-N-ethylsuccinimide itself. This conclusion is based on two observations: (1) the finding of an equivalent amount of S-cysteinosuccinic acid and glycine, and (2) the presence of what appeared to be normal peaks corresponding to the unhydrolyzed S-cysteino-N-ethylsuccinimide after 72 hours of hydrolysis.

The average number of μmoles of glutamic acid, glycine and S-cysteinosuccinic acid found in the hydrolysates of the

peptide reacted with N-ethylmaleimide are given in Table VII.

The apparently abnormally high value of glutamic acid is explained from the simultaneous elution of one of the S-cysteino-N-ethylsuccinimide diastereoisomers. In one of the experiments it was observed that the amount of glutamic acid was equivalent to that of S-cysteinosuccinic acid and glycine. Such a result could be explained only if the absolute amount of glutamic acid present in the sample had been decreased. Therefore, a reaction between the amino-terminal glutamic acid and N-ethylmaleimide seems to occur.

Table VII. Glutathione

Amino Acids	Average number of μmoles of glutamic acid, glycine and S-cysteinosuccinic acid recovered from glutathione reacted with N-ethylmaleimide, after 72 hours hydrolysis in constant-boiling hydrochloric acid at 105°C
Glutamic acid	1.7
Glycine	1.4
S-cysteino-succinic acid	1.3

E. Reaction of iodoacetate with reduced proteins

The alkylation of insulin and lysozyme with iodoacetate was carried out under conditions very similar to that described by Crestfield, Moore and Stein (30). The main variations were that dithiathreitol was used instead of

β-mercapto-ethanol, the air was not excluded from the reaction mixture and the alkylation reaction time was longer.

The reason for carrying out these reactions was primarily to investigate the ability of dithisthreitol to reduce these well known proteins. The results show that the accomplishment of this purpose was complete since cystine is not present in the hydrolysates of the reacted proteins. Moreover, neither cysteine nor cysteic acid are present, which indicates the occurrence of a quantitative reaction of iodoacetate with the cysteine residues. Confirming this interpretation is the finding of the appropriate amount of carboxymethylcysteine (Table VIII).

In calculating the amount of carboxymethylcysteine represented for the respective peak on the chromatogram, the integration constant was taken as 88 percent of the average value of the integration constant of the other amino acids excluding proline and $\frac{1}{2}$ cystine. The recovery of carboxymethylcysteine under these conditions is 100 percent (30). Alanine was used as the internal standard.

F. Some general properties of dithiathreitol

The following tests were carried out in order to determine some of the general properties of this reagent. In all the experiments appropriate blanks were prepared.

(1) Ninhydrin test. The reduced form gave a positive test.

Table VIII. Proteins reacted with iodoacetate

Protein	Number of $\frac{1}{2}$ cystine residues per mole of protein	Relative number of moles of carboxymethylcysteine recovered after 22 hours hydrolysis in constant-boiling hydrochloric acid at 105°C.		
		Exp. 1	Exp. 2	Average
Lysozyme	8.0	8.3	8.1	8.2
Insulin	6.0	6.4	6.0	6.2

(2) N-ethylmaleimide polymerization. A solution of dithiathreitol at a concentration comparable to those used to reduce the analyzed proteins produced the red color, which is a characteristic of polymerized N-ethylmaleimide (13,14). The test was carried out at pH 8.2 at room temperature (22-23°C). The color appeared at about 10 minutes. This did not occur at pH 7.2 or lower.

(3) Stability. The reducing reaction mixtures containing the proteins and dithiathreitol, as reported elsewhere in the present work, were maintained at room temperature for 4 to 5 hours. During this time a very small percentage of the dithiathreitol underwent oxidation to the cyclic disulfide, 4,5-dihydroxy-o-dithiane (3). This fact was determined by diluting these solutions to appropriate concentrations and measuring thiol groups present by the spectrophotometric method (16).

(4) Amino Acid analysis of hydrolyzed N-ethylmaleimide-modified dithiathreitol. On analyzing hydrolyzed N-ethylmaleimide-modified dithiathreitol in the amino acid analyzer, the only ninhydrin positive material was one present in the same elution volume as that of ethylamine.

DISCUSSION

A. Chromatographic method

The results obtained with the method for quantitative determination of thiol groups in proteins employed here, show that N-ethylmaleimide is not absolutely specific for thiol groups (that is, side reactions do occur). However, the reaction of N-ethylmaleimide with the protein cysteine residues is complete and, furthermore, it is possible to carry out an adequate identification and measurement of the S-cysteinosuccinic acid after acid hydrolysis of the N-ethylmaleimide-modified proteins. That is to say, whatever is the behavior of the N-ethylmaleimide-modified amino acid residues other than cysteine, they do not interfere with the quantitative determination of S-cysteinosuccinic acid. This is true for N-ethylmaleimide-modified lysine (21), amino-terminal glycine (20) and histidine. The same can be said for N-ethylmaleimide-modified amino-terminal phenylalanine, amino-terminal lysine, and amino-terminal glutamic acid, since phenylalanine and glycine, lysine and glutamic acid are amino-terminal residues for insulin, lysozyme and glutathione respectively.

Therefore, at the present stage of the knowledge of the chromatographic method, under conditions which side reactions occur, it is anticipated that it will be successful at least when applied to proteins containing the amino-terminal residues involved in the present work.

It, of course, is possible that some of the N-ethylmaleimide-modified amino-terminal residues overlap with the peak of alanine which has been used as the internal standard. In the cases reported here this does not seem to be true since a good correlation has been found between the alanine values and those of most of the other amino acids. It should be added that modification of proteins produced by reaction of N-ethylmaleimide with amino acid residues other than cysteine cannot be quantitatively studied because not all of these derivatives have been prepared. The only partially characterized N-ethylmaleimide-modified amino acids are lysine (21) and glycine (20).

The different values reported for the factor for partial hydrolysis of S-cysteino-N-ethylsuccinimide to S-cysteinosuccinic acid (Table I) have made it difficult to apply any of the literature values. The strongest objection to these values is the lack of direct evidence to show that the model employed behaves during the hydrolytic process in a fashion similar to that of a N-ethylmaleimide-modified protein, as shown in the Literature Review.. The final direct evidence for the validity of any such conversion factor should come from a protein with known amino acid composition in which all the cysteine residues have reacted with N-ethylmaleimide. An agreement between the S-cysteinosuccinic acid recovered from such a modified protein and the S-cysteino-N-ethylsuccinimide itself, after both have been

hydrolyzed under the same conditions, would indicate that the assumption so far made is correct.* In the present work the conversion factor value of a model has been applied to N-ethylmaleimide-modified proteins, but at the same time the results obtained from them provide, from the above point of view, direct evidence for its validity. Thus, the easily prepared S-cysteino-N-ethylsuccinimide standard appears to be appropriate.

The application of this method to proteins containing a known amino acid sequence has allowed a direct measurement of the fact that N-ethylmaleimide can react quantitatively with all the cysteine residues present in polypeptide chains since no cystine or cysteine is left. This has been found even when the cysteine residue is linked to amino acid residues which may react with N-ethylmaleimide also. (For example, lysine, histidine or cysteine itself.) Therefore, the problem of partial reactivity of N-ethylmaleimide towards thiol groups in proteins seems to be caused by incomplete denaturation of the protein rather than inability to react with the cysteine residues.

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*The closest observation for testing this point, even when it was not reported with this purpose, comes from the analysis of N-ethylmaleimide reacted with ribonuclease (20). Unfortunately it suffers from the lack of agreement between the position of the two peaks corresponding to the diastereoisomers of the unhydrolyzed S-cysteino-N-ethylsuccinimide coming from the N-ethylmaleimide-modified protein and that of the peaks normally found when the hydrolyzed standard is analyzed. Moreover some cysteine seems to remain unreacted.

B. Dithiathreitol as reducing agent for disulfides

The results obtained in the reaction of reduced proteins with either N-ethylmaleimide or iodoacetate show that, neither cysteine nor cystine occurs among the hydrolysis products, demonstrating that cleavage of all of the disulfide bonds present in the reported proteins had been quantitatively achieved. The suspected cleavage of disulfied bonds when they are exposed to an excess of N-ethylmaleimide (27) can be discarded, since lysozyme hydrolysates showed no presence of S-cysteinosuccinic acid after reaction with N-ethylmaleimide for a long period (21,24). Further, the finding of the appropriate amounts of S-cysteinosuccinic acid and carboxymethylcysteine in these hydrolysates indicates that dithiathreitol can be successfully used for quantitatively determining cystine residues.

It should be mentioned that the presence of oxygen in the reaction mixtures did not appear to affect this dithiathreitol reduction. Moreover, this reagent appeared to be a better reducing agent for protein disulfide groups at higher pH since at pH 6.8 lysozyme underwent only 80 percent reduction, but at pH 8.2 this reduction was complete. This finding, however, is not a conclusive evidence for a pH effect in the reduction reaction because the pH can also affect the folding of the protein.

C. Urea as denaturating agent

Although this work has not been directly concerned with the properties of urea as a denaturating agent, it seems to be pertinent to state what has been observed while using it, since the cyanate ion formed in its aqueous solutions can react with sulfhydryl and amino groups (28,29). The alkylations with N-ethylmaleimide and iodoacetic acid reported here show that, whatever the concentration of cyanate is and whatever the extent of the reaction of this cyanate with SH groups is, at the end of the reducing reaction period, there is no interference with the reaction of the added sulfhydryl reagent.

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