Reaction of Ethylene Oxide with Histidine, Methionine, and Cysteine*

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(Received for publication, September 29, 1958)

Since it was shown (2) that the treatment of casein with ethylene oxide could diminish the biological availability of the histidine and methionine of this protein, it became of interest to determine whether other proteins were affected in the same way and to elucidate the nature of the chemical reactions involved. Particular interest in such reactions derives from the use of ethylene oxide as a fumigant in the food industry (3, 4) and from the inability of an earlier study (5) of proteins treated with this reagent to account for the interaction with histidine and methionine. The reactions of ethylene oxide with available sulphydryl, hydroxyl, carboxyl, and primary amino groups of proteins have been documented (5).

The destructive action of ethylene oxide on histidine and methionine has now been observed with two other proteins, and lysine appears to be another amino acid which is altered by ethylene oxide. Chemical justification for these observations was sought. Initially, model systems were investigated in which the individual amino acid or amino acid derivatives were treated with ethylene oxide in aqueous solution. In the case of histidine, the studies have been extended to intact protein. Evidence is presented that the nitrogen atoms of the imidazole ring and the sulfur of methionine undergo hydroxyethylation under mild conditions in the presence of ethylene oxide, imidazole yielding the 1,3bis-(2-hydroxyethyl)imidazolium ion and N-acetylmethionine being converted to S-(2-hydroxyethyl)-N-acetylthe tin. The double alkylation of the mercapto group of cysteine also can result in formation of a sulfonium derivative.

EXPERIMENTAL

Materials and Methods—The technique for treating solutions with gaseous ethylene oxide has been previously described (6). The process is conducted at room temperature and in the presence of a large excess of the gas.

The moisture content of commercial proteins was determined gravimetrically after the preparations had been dried for 48 hours in a forced-draft oven at 80°. The same treatment was used in preparing the moisture-free samples. The protein samples were treated as previously described (2). After exposure to ethylene oxide, the samples were hydrolyzed in 2.5 N HCl at 121° for 12 hours, and microbiological amino acid assays were conducted on the hydrolysates.

N-acetylmethionine was prepared as described by Kolb and Toennies (7). A Beckman model M2 pH meter with glass electrodes was used in preparing the titration curves. Primary amino groups were determined by the manometric nitrous acid method of Van Slyke (8).

Imidazole was purchased from Eastman Chemical Corporation and all amino acids and proteins from Nutritional Biochemicals. They were used without further purification. Quantitative elemental analyses were supplied by Galbraith Laboratories, Knoxville, Tennessee.

RESULTS

Amino Acids of Ethylene Oxide-treated Proteins—The diminution of biologically-available histidine, methionine, and lysine of three proteins after treatment with ethylene oxide is shown in Table I. That the decrease in amino acid content was not the result of a growth antagonist in the treated proteins was demonstrated by assaying each hydrolysate in duplicate at five different concentrations.

The inability of the treated protein samples to support the growth of weanling rats was demonstrated by incorporating them at a level of 9 per cent into a purified diet (2) as the sole source of amino acid nitrogen. The inhibition of growth was completely reversed in each case by dietary supplements of dl-histidine-HCl, dl-methionine, and L-lysine-HCl, or in some cases only one

<table>
<thead>
<tr>
<th>Protein</th>
<th>Moisture Treat.</th>
<th>Histidine*</th>
<th>Methionine*</th>
<th>Lysine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Labco)</td>
<td>%</td>
<td>26.1</td>
<td>26.5</td>
<td>72.4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>18.9</td>
<td>21.8</td>
<td>67.6</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>10</td>
<td>11.1</td>
<td>14.3</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>14.7</td>
<td>17.9</td>
<td>76.6</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>0</td>
<td>8.0</td>
<td>13.8</td>
<td>52.7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.5</td>
<td>8.0</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>19.7</td>
<td>29.2</td>
<td>50.7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>17.6</td>
<td>27.3</td>
<td>53.6</td>
</tr>
<tr>
<td></td>
<td>11.8</td>
<td>5.7</td>
<td>19.5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the per cent decrease after treatment with ethylene oxide.

† Taken from a dissertation presented to the faculty of Virginia Polytechnic Institute in partial fulfillment of the requirements of the degree of Doctor of Philosophy. Present address, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts.
or two of these amino acids, depending on the protein and the level of moisture content during the treatment. Thorough drying of the proteins before treatment reduced the damaging effect of the ethylene oxide.

Reaction of Ethylene Oxide with Imidazole—During exposure to ethylene oxide, an aqueous solution of imidazole became strongly alkaline, suggesting the production of a quaternary ammonium group in analogy with the behavior of pyridine derivatives when treated with ethylene oxide (6). The product was isolated as follows. Imidazole, 70 mmoles, and HCl, 140 mmoles, in 20 ml of H2O were treated with gaseous ethylene oxide for 72 hours. The final pH was 9.5. The solution was acidified with HCl, concentrated to a syrup under vacuum, diluted with 20 ml of hot absolute ethanol, and filtered. Acetone was added dropwise to the point of incipient cloudiness and crystallization allowed to proceed overnight at \(-2^\circ\). The product was recrystallized twice from ethanol-acetone to yield fine, white, highly deliquescent needles which were dried in a vacuum over H2SO4. Phosphorus pentoxide could not be used since the product sublimed somewhat under vacuum and was dehydrated and polymerized on the surface of this desiccant. The product was too deliquescent for convenient melting point determination. Elementary analysis was

\[
\text{C}_2\text{H}_5\text{N}_2\text{O}_2\text{Cl}
\]

Calculated: C 43.58, H 6.81, N 14.55, Cl 18.42

Found: C 43.36, H 6.99, N 14.26, Cl 18.18

From the empirical formula, it was evident that 2 moles of ethylene oxide had been added to the imidazole molecule. When no acidic hydrogen could be titrated with sodium methoxide (ruling out the possibility that the compound was a hydrochloride) the reaction was presumed to have occurred as shown in Scheme I.

![Scheme I. 1,3-bis-(2-hydroxyethyl)imidazolium hydroxide](image)

This product was converted to the 1,3-bis-(2-hydroxyethyl)-imidazolium chloride (Compound I) upon the addition of HCl.

Additional evidence for this structure was obtained by degrading Compound I in 25 per cent KOH (weight for weight). Pinner and Schwarz (9) have demonstrated that 1,3-dialkylimidazoles yield the corresponding primary alkylationamines by periodate oxidation to formaldehyde, which was colorimetrically determined with chromotropic acid. The only amine found was ethanolamine in 80 per cent of the theoretical yield of 2 mmoles.

![Diagram](image)

**Table II.** Decrease of chemically determined histidine of casein treated with ethylene oxide

<table>
<thead>
<tr>
<th>Sample</th>
<th>Moisture</th>
<th>Treatment</th>
<th>mg. nitrogen</th>
<th>Per cent decrease after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>312</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>12</td>
<td>215</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>9.5</td>
<td>12</td>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>9.5</td>
<td>24</td>
<td>94</td>
<td>70</td>
</tr>
</tbody>
</table>

* After treatment, the samples were hydrolyzed in 2.5 N HCl for 12 hours at 121°, neutralized with NaOH, decolorized with charcoal (Norit), and assayed for histidine by the colorimetric method of McPherson (11), standardized with mL-histidine-HCl. Nitrogen was determined by the standard Kjeldahl method.
There is some evidence that in native protein, imidazole groups may be protected from reaction with ethylene oxide. No diminution in histidine (chemical assay) could be detected in a sample of crystalline chymotrypsin (Nutritional Biochemicals) which had been treated in aqueous solution with an excess of ethylene oxide for 5 hours, although the proteolytic activity (14), as measured on a casein substrate, had been reduced by about 30 per cent. Denaturing the chymotrypsin in 8 M urea apparently permitted reaction with the imidazole groups; subsequent treatment with ethylene oxide reduced the color in the histidine assay by at least 60 per cent.

Reaction of Ethylene Oxide with Methionine—The reaction of methionine with ethylene oxide yields a product which has resisted crystallization; therefore all evidence concerning its structure was obtained by analysis of solutions of methionine after treatment with ethylene oxide for 24 hours, followed by removal of excess reagent under vacuum. The product gives no color in the standard McFarland-Sullivan nitroprusside assay (15) for methionine, suggesting alternation of the thioether group. Unlike methionine, the product forms a water-insoluble complex with the reineckate ion and with phosphotungstic acid. This behavior is typical of sulfonium derivatives of methionine (16). The solubility of the reineckate in dilute alkali is taken as evidence that the carbonyl group of the methionine remained unesterified. Primary amino groups had been completely alkylated as evidenced by the failure of the product to react with ninhydrin and the failure of a calculated 0.02 mmole of the product to yield nitrogen by the Van Slyke manometric technique.

Ethylene Oxide Treatment of N-Acetylmehtionine—To study more directly the reaction of ethylene oxide with the thioether group, N-acetylmehtionine was chosen as reactant. The pH of an aqueous solution of N-acetylmehtionine shifted from 2 to 9 and the reaction product obtained is soluble in water and ethanol, but insoluble (separates as an oil) in acetone and hexane. Repeated attempts at crystallization have failed. Like ethylene oxide-treated methionine, the product yields a water-insoluble, acetone-soluble phosphotungstate. Since the formula of the phosphotungstate would be difficult to establish, the ratio of C:N:S was calculated from the elementary analysis:

\[ \text{C}_4\text{H}_{11}\text{NO}_3\text{S}-\text{phosphotungstate} \]

Found: C 6.53, N 0.86, S 2.11%

The ratio of C:N:S was calculated to be 8.91:1:1.07 which reflects the addition of 1 mole of ethylene oxide to each mole of N-acetylmehtionine.

The titration curve of the product is shown in Fig. 1. The compound is slightly alkaline in solution and has two titratable groups of pK' approximately 2.9 and 12.1. It is apparent from their quenching of ultraviolet irradiation that a basic group has been merely warming a solution of Compound II in 0.5 N NaOH on a nitrogen was bubbled through the solution and traps during the gradual heating. At 90°, the nitrogen swept over a gas which formed a solid HgCl₂ adduct in the first trap. The gas was identified as CH₃SH by its odor and by the C:S:H ratio (1:1:3).
Table III

Paper chromatography of NaOH hydrolysate of S-(2-hydroxyethyl)-N-acetyl-methionine, thetin*

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Rf of ninhydrin-positive spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S-(2-hydroxyethyl)-N-acetyl-methionine, thetin</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>DL-methionine</td>
<td>0.51</td>
</tr>
<tr>
<td>3</td>
<td>NaOH hydrolysate of Compound II</td>
<td>0.22, 0.51</td>
</tr>
<tr>
<td>4</td>
<td>NaOH hydrolysate of Compound II + DL-methionine</td>
<td>0.24, 0.52</td>
</tr>
</tbody>
</table>

* Chromatographed on Whatman No. 1 paper, with a 1-butanol:acetic acid:H2O (100:21:50) solvent; developed papers sprayed with 0.4 per cent ninhydrin in 1-butanol.

The primary amino group of the cysteine had been completely alkylated by the ethylene oxide as indicated by the failure of the product to react with ninhydrin and the failure to yield nitrogen when analyzed (0.02 mmole of the product in solution) by the Van Slyke manometric procedure. Again, the final pH of the reaction solution is evidence that the carboxyl group of the cysteine had not been esterified. Esterification would have consumed another proton and a strongly alkaline solution would have been expected.

**Scheme III**

**Reaction of Ethylene Oxide with Cysteine-HCl**—The reaction of an aqueous solution of cysteine-HCl (pH 1.0) with ethylene oxide produces a clear yellow-colored solution and elevates the pH to 8.5. The product, which could not be crystallized, yields an insoluble precipitate with phosphotungstic acid. This property is destroyed by heating the product to 100° in dilute alkali for a few minutes. As a result of the studies with methionine, these data are taken as evidence that 2 moles of ethylene oxide converted the mercapto group into a sulphonyl group.

\[
R - SH + 2C_2H_4O \xrightarrow{H_2O} \xrightarrow{+} R - S\left(\text{CH}_2 - \text{CH}_2 - \text{OH}\right)_2 + OH^- \quad (3)
\]

The primary amino group of the cysteine had been completely alkylated by the ethylene oxide as indicated by the failure of the product to react with ninhydrin and the failure to yield nitrogen when analyzed (0.02 mmole of the product in solution) by the Van Slyke manometric procedure. Again, the final pH of the reaction solution is evidence that the carboxyl group of the cysteine had not been esterified. Esterification would have consumed another proton and a strongly alkaline solution would have been expected.

**DISCUSSION**

The applicability of the reactions reported in this paper to the safety of using ethylene oxide as a commercial fumigant in the food industry has been previously discussed (6, 27). The studies with fumigated proteins give qualitative evidence of the potential danger of fumigating proteinaceous foodstuffs with ethylene oxide. Reasons for the accelerating action of moisture in ethylene oxide-vitamin reactions were discussed previously (6). Similar reasoning would apply to the ethylene oxide-amino acid reactions.

The strong polarity of the ethylene oxide-amino acid products may help to explain the isoelectric and electrophoretic shifts of proteins after ethylene oxide treatment, as observed by Fraenkel-Conrat (5), whose methodology could not detect the specific reactions with the imidazole of histidine and the thioether group of methionine. From theoretical considerations, the involvement of imidazole group quaternization in these shifts has already been suggested by Alexander (23).
that the methylsulfonyl derivative will replace methionine for rats grown on a methionine-deficient diet.

The reaction product of ethylene oxide with lysine has not been identified. The decrease in microbiologically available lysine of ethylene oxide-treated Labco casein, lactalbumin, and egg albumin is influenced by the presence of water and possibly by the protein itself. These ethylene oxide-treated proteins were unable to support the growth of weanling rats when fed at a level of 9 per cent of the diet. By supplementation experiments, Labco casein and lactalbumin were found to be deficient in histidine and methionine, and egg albumin deficient in histidine and lysine. In analogy with the results with other amino acids, it is assumed that hydroxyethylation of one or both of the primary amino groups of lysine can account for the observed decrease in the availability of this amino acid.

The toxicity of subcutaneous injections of the ethylene oxide-cysteine-HCl reaction product in the rat was reported in an earlier paper (2). Now, with more information about the probable structure of the product, a relationship is suggested between this compound and a series of synthetic sulfoxoniums (30, 31) which act as spasmolytics, presumably by virtue of their antagonism to acetylcholine. The toxic effect of the cysteine derivative does appear to be mediated through the central nervous system. Death is preceded by a generalized flaccid paralysis and respiratory failure. The synthetic spasmolytics and the cysteine product have the following structure in common: (R)S═CH—CH—O═

The relationship to acetylcholine is apparent. The structure of the product, a relationship is suggested between the decrease in microbiologically available histidine and methionine, and egg albumin deficient in histidine and methionine, and egg albumin deficient in histidine and lysine. In analogy with the results with other amino acids, it is assumed that hydroxyethylation of one or both of the primary amino groups of lysine can account for the observed decrease in the availability of this amino acid.

The decrease in the microbiological availability of histidine and methionine in proteins treated with ethylene oxide appears to be correlated with the electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, particularly nitrogen and sulfur.

In model experiments conducted in aqueous solution at room temperature, ethylene oxide reacts readily with imidazole or histidine to yield the corresponding 1,3-bis-(2-hydroxyethyl)-imidazolium derivative. With methionine or N-acetylhistidine, ethylene oxide hydroxyethylates the sulfur to yield the corresponding sulfonium derivatives. Likewise, double alkyla
tion of the mercapto group of cysteine produces a sulfonium group. The primary amino groups of these amino acids also become alkylated, but esterification of carboxyl groups does not seem to be involved.

The liability in alkali of the ethylene oxide-produced “onium” groups in these amino acids has been studied and some of the degradation products identified.

Acknowledgments—The authors are indebted to the following members of the staff at the National Institutes of Health: to Mr. Howard Bakerman and Mrs. Marjorie Romine for performing the microbiological amino acid assays; and to Drs. Olaf Mickelsen and J. P. Greenstein for making available the acylase preparation.

REFERENCES


1 H. G. Windmuller, C. J. Ackerman, and R. W. Engel, unpublished data.