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

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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 sulfhydryl, 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 1,3-bis-(2-hydroxyethyl)imidazolium ion and N-acetyl-methionine being converted to S-(2-hydroxyethyl)-N-acetyl-methionine, thetin. 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.

*Acetyl-methionine 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**

*Table I 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.*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Moisture</th>
<th>Treatment</th>
<th>Histidine*</th>
<th>Methionine*</th>
<th>Lysine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Labco)</td>
<td>0</td>
<td>26.1</td>
<td>26.5</td>
<td>72.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>18.9 (28)</td>
<td>21.8 (15)</td>
<td>67.6 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.1 (37)</td>
<td>14.3 (40)</td>
<td>55.0 (29)</td>
<td></td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>0</td>
<td>14.7</td>
<td>17.9</td>
<td>76.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>8.0 (46)</td>
<td>13.8 (23)</td>
<td>52.7 (31)</td>
<td></td>
</tr>
<tr>
<td>Egg albumin</td>
<td>10</td>
<td>2.5 (83)</td>
<td>8.0 (55)</td>
<td>55.0 (28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>19.7</td>
<td>29.2</td>
<td>50.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.8</td>
<td>17.6 (11)</td>
<td>27.3 (6)</td>
<td>53.6 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.7 (71)</td>
<td>19.5 (33)</td>
<td>4.8 (92)</td>
<td></td>
</tr>
</tbody>
</table>

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

† Determined microbiologically, with *Leuconostoc mesenteroides* P-60.
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°C. 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

\[
\text{C}_6\text{H}_5\text{N}_2\text{O}_2\text{Cl}
\]

**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 (ruuling out the possibility that the compound was a hydrochloride) the reaction was presumed to have occurred as shown in Scheme I.

![Scheme I](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-dialkyl imidazolium chlorides yield the corresponding primary alkyl amines 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.

Unlike imidazole, Compound I gives no color when coupled with diazotized sulfanilic acid in the quantitative histidine assay method of McPherson (11), thereby making this a suitable method for following the reaction of ethylene oxide with imidazole and some of its derivatives, e.g. histidine.

**Reaction of Ethylene Oxide with Histidine**—The treatment of a solution of histidine-HCl for 24 hours with ethylene oxide yielded a product which resisted all attempts at crystallization. The following data were obtained by analysis of the product in solution.

The product formed no colored complex with diazotized sulfanilic acid, implying hydroxyethylation of the imidazole nitrogens as in the case of ethylene oxide-treated imidazole. This idea was supported by the recovery of ethanolamine (63 per cent of theory) from a 25 per cent KOH (weight for weight) hydrolysate of the product. The product did not react with ninhydrin and a calculated 0.02 mmoles of the product in solution was analyzed for primary amino nitrogen by the Van Slyke manometric method. Within the experimental error of the method (±2 per cent), no nitrogen could be measured. This is taken as evidence that the primary amino group has been alkylated as would be expected (5, 12).

Unlike histidine, the product formed an immediate water-insoluble precipitate with ammonium reineckate at acid pH, further support for the presence of the quaternary ammonium group.

**Chemical Detection of Ethylene Oxide-Imidazole Reaction in Proteins**—Table II shows the decrease in apparent histidine of casein samples of various moisture content treated with ethylene oxide. Amino acids other than histidine may contribute a small amount of color in this assay (13), so the values listed in the table represent the apparent histidine content. Samples 1 and 4 were also assayed for histidine microbiologically; the ethylene oxide-treated sample had 71 per cent less histidine than the untreated control.

**Table II**

<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°C, neutralized with NaOH, deoxygenated with charcoal (Norit), and assayed for histidine by the colorimetric method of McPherson (11), standardized with dl-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 McCarthy-Sullivan nitroprusside assay (15) for methionine, suggesting alteration 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 carboxyl group of the methionine remained unesterified. Primary amino groups had been completely alkylated as indicated 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-acetylmethionine**—To study more directly the reaction of ethylene oxide with the thioether group, N-acetylmethionine was chosen as reactant. The pH of an aqueous solution of N-acetylmethionine shifted from 2 to 9 in 50 ml. of H2O. Before treatment, pH 2.0 (——). After treatment with ethylene oxide for 24 hours, pH 9.0 (----).

According to Toennies and Kolb (17), methionine sulfonium salts often are more easily crystallized than the corresponding N-acetyl derivatives. Therefore, in an effort to obtain a crystallizable product, the thetin was subjected to the hydrolytic activity of a preparation of acylase (18). Very little or no hydrolysis occurred, even when the enzyme was used at a concentration 10 times that which hydrolyzed almost completely a sample of N-acetyl-L-methionine.

**Acid and Alkaline Hydrolysis of S-(2-hydroxyethyl)-N-acetylmethionine**—To gain additional information about the structure and stability of this thetin, which to the authors' knowledge has not been previously described, acid and alkaline hydrolyses were studied in some detail, particularly with respect to the stability of the sulfonium group.

Even after autoclaving a solution of Compound II in 6 N HCl for 14 hours at 121°, a large proportion of the sulfonium groups was still intact, as indicated by the voluminous precipitate which resulted upon the addition of phosphotungstic acid. However, paper chromatography of the autoclaved mixture in 1-butanol: acetic acid:H2O (100:21:50) revealed five ninhydrin-positive spots, only one of which appeared to be a sulfonium compound. Sulfonium compounds were detected by spraying the chromatograms with a methanolic solution of ammonium reineckate, followed by water to remove excess reineckate, and, after drying, detecting the sulfonium-reineckate complexes by their quenching of ultraviolet irradiation.

As expected (19), the sulfonium group is labile to alkali. Merely warming a solution of Compound II in 0.5 N NaOH on a steam bath for 4 hours completely destroyed any ability to form a precipitate with phosphotungstic acid. A disagreeable cabbage-like odor was evolved during the hydrolysis.

Subsequently, a solution containing 1 mmole per ml. of Compound II in 0.5 N NaOH was slowly heated in a three-necked flask fitted with a nitrogen inlet, a thermometer, and a water condenser. The top of the condenser was connected to a series of three traps containing saturated aqueous HgCl2 (19). 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-acetylmethionine, 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-acetylmethionine, thetin (Compound II)</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>0.23, 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.

and the high melting point (greater than 300°F) of the HgCl₂ adduct (20). After a few minutes, no more CH₂SH was evolved, and the first HgCl₂ trap was replaced by a trap containing saturated lead acetate to collect volatile mercaptans but not sulfides. The NaOH concentration of the thetin solution was increased to 1 N and the thetin was refluxed and aerated at 105°C for 15 hours. No lead precipitate formed, but a compound accumulated in the second trap (saturated aqueous HgCl₂) and is thought to have been CH₃-S--S--CH₂ according to the C:S ratio (1:1) and melting point of its HgCl₂ adduct (found, 142-144°C, uncorrected; literature, 147-148°C (16)). The CH₂SH and CH₃-S--S--CH₂ accounted for only 7 per cent of the sulfur of the thetin although the sulfonium group was completely destroyed during the hydrolysis.

Analysis of the thetin solution before and after alkaline hydrolysis revealed that the alkali had liberated a compound which was oxidized to formaldehyde by alkaline peroxide. The formaldehyde was determined colorimetrically with chromotropic acid (21). If this compound were ethylene glycol, a likely hydrolytic product of this thetin (19, 22) or any other compound which yields 2 moles of formaldehyde when oxidized with periodate, the hydrolyzed thetin solution was chromatographed, with the other ninhydrin-positive compound has not been identified.

The alkaline hydrolysis of Compound II may be tentatively formulated as shown in Scheme III.

![Scheme III](image)

**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°C 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 sulfonium group.

R—SH + 2C₂H₄O→R—S(CH₂—CH₂—OH)₂ + OH⁻

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 reactions of ethylene oxide with histidine, methionine, cysteine, and with the vitamins of the pyridine family (6) all have certain features in common. In each case, the reaction involves electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, either nitrogen or sulfur. The products are all produced under mild conditions, and in each case they are highly polar compounds containing an “onium” group. Tertiary heterocyclic nitrogen appears to be especially easily hydroxyethylated, so that one might expect purines and pyrimidines to be likely reactants. The alkylation potential of ethylene oxide is in many ways similar to that of the sulfur and nitrogen mustards (23, 24), which can also alkylate the nitrogen atoms of thioethers and the sulfur of methionine (23, 26).

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 methylsulfonium 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 hydroxyethylolation 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 the decomposition of this compound and a series of synthetic sulfoniums (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 derivative will replace methionine for inducing any toxic symptoms when injected into two rats at 2.5 times the LDr,0 of the cysteine-HCl product.

The decrease in the microbiological availability of histidine and methionine in proteins treated with ethylene oxide appears to be correlated with the electrophilic hydroxyethylolation 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-acetyl-methionine, ethylene oxide hydroxyethylates the sulfur to yield the corresponding sulfonium derivatives. Likewise, double alkylation 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.

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