THE REACTION OF ETHYLENE OXIDE WITH SOME PROTEINS, AMINO ACIDS AND VITAMINS

by

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Chairman, Advisory Committee

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TO MY BELOVED PARENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

A report by Hawk and Mickelsen (48) that animal diets fumigated with ethylene oxide no longer supported the growth of weanling albino rats has prompted a series of investigations into the reactivity of this fumigant. The literature contains a number of reports which give evidence to the severe toxicity of ethylene oxide in biological systems but this activity has never been adequately assigned a chemical basis. The present study is the continuation of an investigation initiated to elucidate the nature and scope of ethylene oxide-nutrient interactions and to project this information to other possible reactions in biological material. The reactions of ethylene oxide with protein, essential amino acids and vitamins are primarily considered.

Ethylene oxide, also known as oxirane and officially as 1,2-epoxyethane, is the simplest member of the family of epoxy compounds and has the molecular formula, C_2H_4O. It is a gas at room temperature with a boiling point of 10.7° C. Ethylene oxide is colorless, has a sweetish odor, is highly flammable, and forms explosive mixtures with air in all proportions exceeding 20% of the gas. It is miscible in all proportions with water, ethanol and ether.

Ethylene oxide is recognized as a highly reactive organic compound capable of combining with most chemical groups containing an active hydrogen and with anions in aqueous solution to release hydroxide ions (20). The ability of ethylene oxide to react with groups containing an

available hydrogen is the basis of its importance in the manufacture of synthetic detergents, ethylene glycol, polyethylene glycols, acrylonitrile, ethanolamine, starch derivatives, petroleum emulsifiers and choline, and in its extensive use in the textile industry where it serves as a cross-linkage agent (2).

The insecticidal and germicidal properties of ethylene oxide were first reported in 1928 by Cotton and Roark (91), who noted that two pounds of the chemical per 1000 cubic feet of air-space was sufficient for complete sterilization. There was no apparent damage to food products so sterilized, but nutritional studies were not conducted. The earliest patent on the use of ethylene oxide in a sterilization procedure was issued to Schrader and Bossert (96) in 1936. In the interval 1938-1943 a series of patents was issued to Griffith and Hall for a method of food sterilization with ethylene oxide. The abstract of the latest patent re-issue (41) is quoted below to illustrate the nature of their process:

"The process of sterilizing normally dry material which comprises heating the material for at least one hour at a temperature not lower than 110° F., freeing the heated material of removable gases and moisture by applying a vacuum there-to, exposing the dry evacuated material at a heat of from 110° F. to 240° F. in a chamber to substantially undiluted ethylene oxide gas at a concentration of at least three-fourth pound to thirty-five cubic feet of chamber volume for at least two and one-half hours."

The ability of ethylene oxide to diffuse rapidly and to penetrate

porous materials quickly permits the fumigation of foods packed in containers of many types. Ethylene oxide has been recommended as a fumigant to improve the keeping qualities of flour (73) by protecting the gluten which in unsterilized samples is destroyed by proteolytic enzymes of microbial origin. Its use in fumigating spices, flavors, cocoa, and sugar has been reported along with recommendations for more widespread commercial adaptation (6)(110). Cellophane-wrapped cornmeal treated with one atmosphere of undiluted ethylene oxide kept well after 10 weeks of storage (106). The amount of ethylene glycol formed in the cornmeal during the fumigation was considered insignificant on a toxicological basis. Baerwald (8) has patented a fumigation process whereby ethylene oxide is added to packages of food either as a chilled liquid or as a snow after freezing it together with a diluant. No trace of ethylene oxide could be detected in such food packages three days after the addition of the chemical. A portion of the extensive literature that has accumulated on the employment of ethylene oxide as a cold sterilizing agent has been reviewed by Phillips and Kaye (86). To reduce the explosion hazard of handling pure ethylene oxide, published procedures often recommend its dilution with an inert gas such as SO_2 (22) or CO_2, the latter mixture being sold under the trade name, "Carboxide." (5)

On the basis of the high volatility of the fumigant and the inability to detect residues in fumigated products, ethylene oxide has been condoned for use in commerce. However, evidence of its reactivity with compounds of biological importance is widespread. The toxicity of the gas to microorganisms and insects is, of course, one clue. It is also

toxic to animals when inhaled or applied to the skin as an aqueous solution (50). The toxicity involves irritation of the respiratory passages and eyes, depression of the central nervous system and injury to the skin and various organs, particularly the lungs. Less direct data is also of interest. When ethylene oxide was applied to the fruitfly, Drosophila melanogaster, either through the diet, by injection, or as an aerosol, it proved strongly mutagenic (15). Mutation rate was measured by the emergence of lethal genes. Mutant strains of Saccharomyces cerevisiae have also resulted from treatment of the yeast with ethylene oxide (111). Ethylene oxide has been successfully used to inactivate viruses and rickettsiae in the production of stable soluble antigens for use in immunization (116)(88). Under carefully controlled conditions the viral preparations can be made non-infective without seriously altering their antigenicity. The deleterious effect of ethylene oxide on seed germination is well documented (91)(52). Wilson and Bruno (112) have reported that sterilization of a tryptose-phosphate broth with 0.5% liquid ethylene oxide caused a slow rise in pH. The broth proved toxic to mice when injected within six hours after the addition of 1% ethylene oxide. Ethylene oxide has been used successfully, however, to sterilize various carbohydrates for bacteriological media (55), a liver brei preparation for rearing the dipterous parasite Pseudosaccharophaga afinis (11), and a meat infusion-neopeptone broth which served as a growth medium for hemolytic streptococci (112).

The resistance of spores to ethylene oxide treatment appears to be related to their fat content (26). The spores of several species of

bacilli could be divided into two classes on the basis of their resistance to ethylene oxide, fat content, and electrophoretic mobility. Extraction of fat, amounting to about 8% of the spore dry weight, rendered all spores more sensitive to ethylene oxide and reduced inter-spore differences in sensitivity. The extract offered increased protection from ethylene oxide when added to spores, even those of a different species.

Until recently references to specific reactions of ethylene oxide with compounds of biological importance were few. The occurrence of such reactions during fumigation might have been implied from a study by El Khishen (34), who was able to recover only 92% of the ethylene oxide to which grain samples had been exposed. More specifically, Fraenkal-Conrat (35) demonstrated that the treatment of egg albumin and beta-lactoglobulin in solution with ethylene oxide resulted in a decrease in detectable carboxyl, sulfhydryl, phenolic, and primary amino groups. Hydroxyethylation was postulated in each case. The ethylene oxide treatment also caused changes in the isoelectric point, solubility, and electrophoretic mobility of the proteins. The conditions for amino acid and fatty acid esterification have been studied in some detail by the same author (36). Esterification is slow unless catalyzed by alkali metal ions, hydroxyl ions or halides. The hydroxyethylation of the primary amino groups of proteins has been confirmed in a study of ethylene oxide-treated lysozyme (72) and alanine (71). The lysozyme retained its lytic ability after the treatment. Further confirmation of amine hydroxyethylation comes from the work of Kiprianov (60) and Kovalenko (63). On the theoretical basis of its reactivity ethylene oxide has been classified by Ross

(92) and Alexander (3) as a cytotoxic alkylating agent, in the same category as the sulfur and nitrogen mustards. And a patent has been issued for the synthesis of a basic, high-molecular weight product by the treatment of casein in aqueous suspension with ethylene oxide (12). The nature of the reactions involved are not discussed.

The report by Hawk and Mickelsen (48) was the first published evidence that the ethylene oxide fumigation of dietary constituents could be nutritionally deleterious. Stock and purified diets that were exposed to 1.8 gm. of the fumigant per 100 gm. of diet for 18 hours failed to support the growth of weanling rats and usually resulted in death of the animals after about five weeks. Thiamine had been almost completely destroyed in these fumigated diets. However, the addition of neither thiamine nor a complete vitamin mix to the treated diets improved animal growth, and the above workers were led to suspect the destruction of other essential nutrients. More recent data by Bakerman et al (9) has demonstrated the lability of five of the B-vitamins to ethylene oxide. Fumigation of starch-vitamin mixtures for 18 hours in a 10 l. desiccator containing 8.8 gm. of ethylene oxide resulted in the following percent destruction, as determined chemically (thiamine and riboflavin) or microbiologically: thiamine, 100; cocarboxylase, 100; niacin, 50-80; pyridoxine-HCl, 71; riboflavin, 30-67; folic acid, 30-50; pantothenic acid, 0-6; biotin, 0; and vitamin B_{12}, 0-10. The presence of choline-Cl in the starch-vitamin mixtures was found to enhance the destruction. Less severe but significant destruction of the same vitamins was reported in yeast samples and a natural rat diet fumigated by a

commercial process (84). Yet weanling rats fed the fumigated natural diet grew almost as well as controls. The commercial process when compared to the procedure used by Bakerman and co-workers employs a lower fumigant concentration, shorter contact time, but higher temperature.

On the basis of the foregoing results, Mickelsen (78) has compared ethylene oxide with trichloroethylene and nitrogen trichloride in that all three compounds leave severe nutritional imprints on treated foods although the trace of residual chemical in the foods is not toxic per se. (78). The importance of nutritionally, as well as toxicologically, testing new food-treating chemicals is emphasized.

Hawk and Mickelsen's initial observation prompted a series of investigations in our laboratory which may be summarized as follows (113) (114):

1. The feeding to weanling rats of a stock diet which had been fumigated with high concentrations of ethylene oxide resulted in rapid weight loss, convulsive seizures, and death in about 20 days. Thiamine supplements offered only temporary improvement. Older rats fed the same diet exhibited severe growth inhibition but no unusual neural syndrome. Equally poor growth followed the feeding of a fumigated purified diet to weanling rats. Supplementing this diet with thiamine improved performance only slightly.

2. Weanling rats failed to grow when fed a purified diet containing 9% or 18% casein as the only protein source when this casein had been previously fumigated with ethylene oxide. Rats on such a diet often

exhibited a severe diarrhea but no specific lesions when examined grossly or histologically. The growth inhibition was immediately reversed when non-fumigated casein was substituted in the diet or when histidine and methionine were added to the diet at the levels that would be supplied by untreated casein. No other essential amino acids were active in reversing the growth inhibition. Only 29% of the histidine and 44% of the methionine of casein were available to the bacterium Lactobacillus mesentroides after fumigating the protein for 24 hours with ethylene oxide.

3. Growth inhibition on fumigated casein was a function of the duration of fumigation and became more severe as fumigation was extended to 24 hours. Progressive fumigation over this period also resulted in a continuous dilution of the nitrogen. The dilution after 24 hours indicated a 10% increase in mass of the casein. A progressive decrease in the electrophoretic mobility of casein on paper was evident as fumigation was continued over a 24 hour period.

4. Crystalline cysteine-HCl reacted readily with ethylene oxide to yield a brown viscous liquid which produced a flaccid paralysis, respiratory failure, and death in rats when injected subcutaneously. The LD_{50} was about 13 mg. of fumigated cysteine-HCl per 50 gm. of body weight.

5. Little or no alteration was observed in the ultraviolet absorption spectrum of casein following fumigation. Long-term gas uptake measurements on consecutive days indicated that much of the uptake observed when casein is exposed to ethylene oxide is caused by physical absorption of the gas by the protein. The fumigation of casein did not

alter its rate of hydrolysis by pepsin or trypsin.

It should be emphasized that the fumigation procedures and ethylene oxide concentrations employed in obtaining the above results, as well as in the subsequent work to be reported here, were not patterned after those reportedly used in industry. The objective has not been to justify or condemn existing ethylene oxide cold sterilization techniques but to elucidate the nature of the destructive action of the gas on food or feed constituents. It was felt that this goal could be reached more quickly by employing drastic fumigation conditions which, it was hoped, would have a maximally deleterious effect on the nutrients so treated.

The observed lability of histidine and methionine to fumigation was of particular interest since none of the reported ethylene oxide-protein reactions (35)(72) could account for this observation. Hydroxyethylation of the mercapto group was a likely explanation for the cysteine-ethylene oxide reaction, but neither alkylation of carboxyl nor amino groups could account for extensive histidine and methionine destruction in intact protein, where these groups are largely unavailable for reaction.

II. OBJECTIVES OF THIS WORK

As a consequence of the above results the immediate objectives of the present study were set forth as follows:

1. To fumigate proteins other than casein, and by means of rat feeding experiments, to assess fumigation damage to protein quality and to determine which amino acids are destroyed by the fumigant in each case;

also to determine the importance of moisture to protein damage during fumigation.

2. To elucidate as far as possible the reactions responsible for the lability of histidine and methionine and of niacin, pyridoxine, thiamine, riboflavin and folic acid to treatment with ethylene oxide.

3. To study in some detail the fumigation of an undenatured enzyme protein with respect to the lability of enzymatic activity to fumigation and the accessibility of amino acids to reaction.

In accordance with the stated objectives the experimental results of this study will be reported in three sections.

Chapter III is devoted to rat feeding experiments by which the protein quality of fumigated lactalbumin, egg albumin, alpha protein of soybeans and several commercial brands of casein have been assessed. The importance of moisture in determining the extent of fumigation damage has been evaluated in each case, as well as the minimum amino acid supplement required to restore the protein quality. Microbiological amino acid assays of some of the fumigated proteins are included. Histidine and methionine are labile to fumigation in at least three of these proteins, and lysine has emerged as another essential amino acid susceptible to reaction with ethylene oxide in intact protein.

Chapter IV contains the results of model reactions in which individual amino acids, vitamins and related compounds where fumigated in aqueous solution with ethylene oxide. In some cases the products were isolated and the structure analyzed. When the products could not be isolated, the fumigated solutions were studied to gain information about the course of

the reaction. The significant conclusion gained from these experiments is that ethylene oxide can, under very mild conditions, hydroxyethylate nitrogen and sulfur atoms possessing one or more lone pair of electrons, and that this reaction can involve the imidazole nitrogens of histidine, the thioether group of methionine, the mercapto group of cysteine, and the tertiary amine ring nitrogen of nicotinic acid, nicotinamide, and pyridoxine.

The final experimental section, chapter V, presents preliminary findings with respect to the fumigation of the proteolytic enzyme, chymotrypsin, the activity of which is diminished with ethylene oxide treatment.

As always, many of the experiments performed in the laboratory failed to yield conclusive results. However, they will be included in this presentation, though often without detail, for the sake of completeness and future reference. Likewise, the author recognizes the preliminary nature of many of the studies to be reported. This reflects the limitations in time and imagination of the investigator, and the desire to gain some information on a broad coverage of ethylene oxide reactions.

III. THE BIOLOGICAL VALUE OF SOME ETHYLENE OXIDE FUMIGATED PROTEINS

In this laboratory the fumigation of casein with ethylene oxide has resulted in a product incapable of supporting the growth of weanling rats when fed as 9% of a complete purified diet and as the sole source

of amino acid nitrogen (114). From similar experiments (78) Mickelsen has reported that only 10-15% inhibition of growth resulted from feeding fumigated casein, and only 22% and 17% of the histidine and methionine, respectively, of the protein had been destroyed, as compared with 71% and 56%, respectively in the experiments reported from this laboratory. The studies in question differed with respect to the source of the casein, the concentration of fumigant, and duration of fumigation. Mickelsen and co-workers used less fumigant and a shorter duration of fumigation in their experiments, conducted on Labco casein. In our experiments vitamin-free test casein, purchased from General Biochemicals, Inc., was employed.

To resolve the discrepancy an experiment was conducted in which casein samples from the two sources were nutritionally compared after having been fumigated under identical conditions. This was followed by experiments in which other proteins were fumigated with ethylene oxide and their nutritional value determined in rat-feeding trials. The proteins were selected on the basis of their availability, and their unlikeness with respect to source and quality, i.e. two high-quality milk proteins, an egg protein and a low-quality protein of plant origin. Also reported are the results of microbiological assays for selected amino acids in the fumigated and control samples.

Material and Methods

The rats used in these studies were 40-50 gm. weanlings purchased from Holtzman Co. and Sprague-Dawley, Inc., Madison, Wis. They were

randomized among the various experimental groups with respect to weight and sex, the number of males and females in each experiment being always equal. The animals were individually fed, ad libitum in wire-bottomed raised cages. Feed, in weighed portions, and water were replenished daily. The rats, as well as unconsumed feed, were weighed weekly.

The basal diet used throughout was composed as follows:

	gm./kg. diet
Sucrose	720
Hydrogenated vegetable oil[1]	100
Protein	90
B-vitamins in sucrose[2]	50
Mineral salts (95)	40
	1000

Fat-soluble vitamins were added as petroleum ether solutions, to provide in mg. per kg. of diet, alpha-tocopherol, 50; beta-carotene, 5; and calciferol, 0.125. Amino acid supplements were added at the expense of the whole diet. A 100% replacement supplement of an amino acid represents that quantity as was supplied by the protein of the diet before it was fumigated (17).

The moisture content of protein samples was determined gravimetrically after drying for 24 hours in a forced-draft oven at 80° C. Moisture-free samples were similarly prepared. To obtain protein samples of varying

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[1]Crisco

[2]To provide in mg. per kg. of diet, Vitamin B_{12}, 0.02; biotin, 0.2; folic acid, 0.4; thiamine-HCl, 5; pyridoxine-HCl, 5; menadione, 8; riboflavin, 10; calcium pantothenate, 20; niacin, 40; inositol, 200; and choline chloride, 2000.

moisture content, a calculated quantity of water was thoroughly mixed with dried samples of protein or with protein samples of predetermined moisture content. The reliability of this procedure was established by comparing the calculated with the experimentally determined moisture of some of these prepared samples.

After moisture adjustment, 200-300 gm. aliquots of the proteins in brown paper bags were placed into a large desiccator for fumigation. The fumigation procedure is an adaptation of that described by Allison (4) for soil sterilization, and a diagram of the equipment is shown in figure I. The desiccator was evacuated to 40 mm Hg and sufficient ethylene oxide gas then admitted to restore the pressure to ambient and, occasionally during the course of the treatment, to maintain ambient pressure as ethylene oxide absorbed on and reacted with the protein (113). The fumigation, conducted at room temperature for 24 hours, was terminated by withdrawing the excess ethylene oxide under vacuum and flushing the desiccator several times with air filtered through cotton. To standardize moisture content, all protein samples, fumigated as well as control, were heated 24 hours at 80° C. in a forced-draft oven prior to incorporation into diets.

For microbiological amino acid analyses, aliquots of the proteins used in the various rat feeding experiments were hydrolyzed in 2.5 N HCl by autoclaving 12 hours at 121° C. The hydrolysates were buffered by adding sodium acetate to 0.1 M and the pH adjusted to 4.5 with 3.5 N NaOH. Appropriately diluted aliquots were assayed microbiologically.[1]

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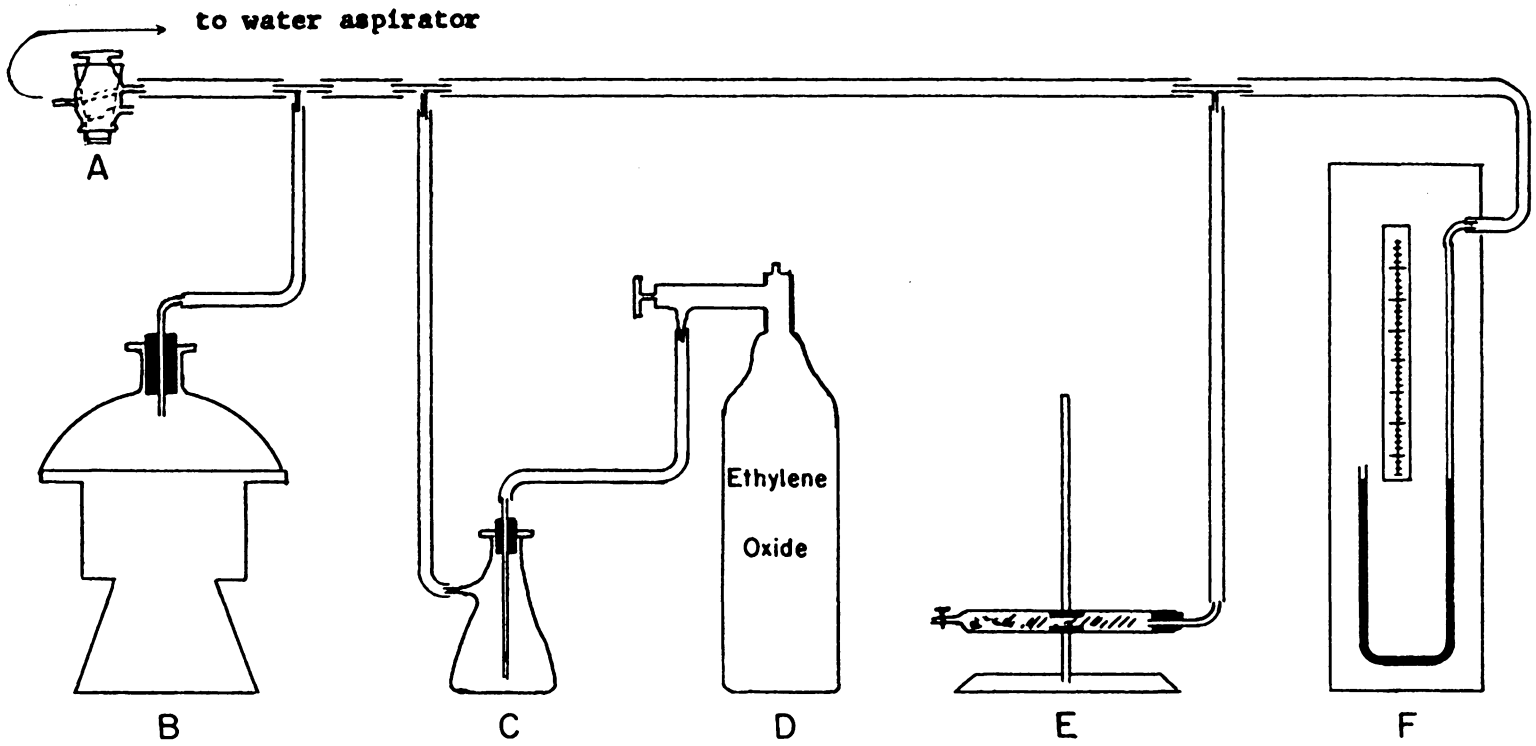
[1]The author is indebted to Howard Bakerman and Mrs. Marjorie Romine of the National Institutes of Health for kindly conducting these assays.



B - fumigation desiccator

C - trap (for liquid ethylene oxide)

D - ethylene oxide cylinder

E - air inlet, cotton-filled

F - mercury manometer

A - 3-way stop-cock

ETHYLENE OXIDE FUMIGATION APPARATUS, DIAGRAMMATIC SKETCH

Figure I

Unless otherwise indicated all proteins, protein hydrolysates and amino acids used were from Nutritional Biochemicals Corp., Cleveland, Ohio. Ethylene oxide gas (99.15% purity) was from Matheson Co., Inc. East Rutherford, N. J.

Results and Discussion[1]

Casein:

The growth inhibition resulting from feeding fumigated casein from two sources, fumigated casein hydrolysate (enzymatic), and casein fumigated after being fortified with a 100% replacement supplement of histidine and methionine is seen from Experiment No. 15 (figure 2). The commercial protein samples were fumigated without altering their moisture content. Even when fumigated under the same conditions, the greater lability of G.B.I.[2] casein as compared to Labco casein[3] is evident. Fumigated G.B.I. casein supported almost no growth, while weight gain with the fumigated Labco product was about 50% as great as with the untreated protein. It was suspected that a difference in moisture content might explain this result. Analysis revealed that commercial Labco casein contains 4.6% moisture while the G.B.I. casein contains 9.3%.

When the histidine and methionine content of casein are doubled by supplementation no reduction in protein quality occurred as a result of fumigation with ethylene oxide. Either the crystalline supplemented amino

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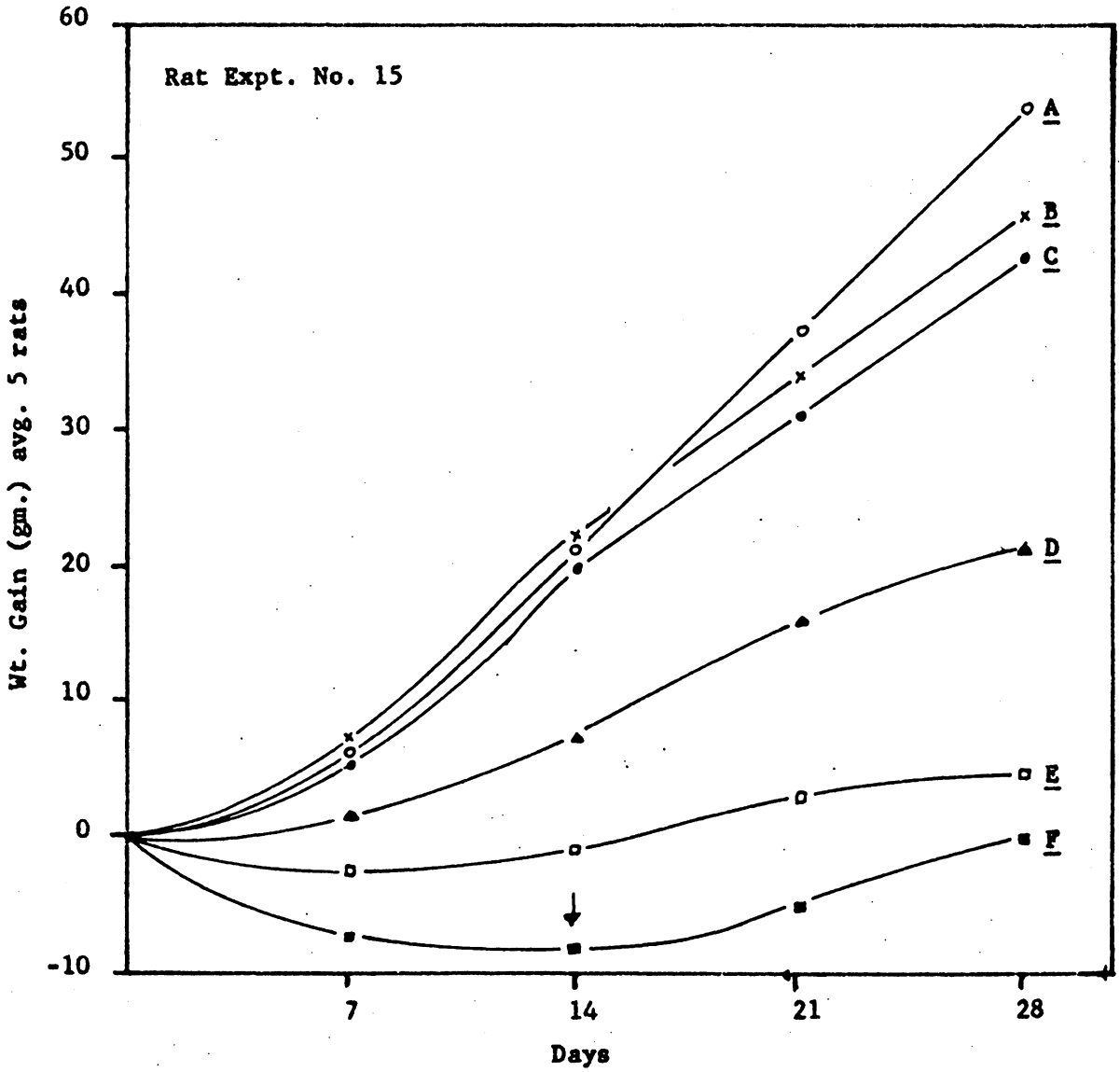
[1]Preliminary report: "Alterations in the Biological Properties of Some Ethylene Oxide Fumigated Proteins", by H. G. Windmueller and C. J. Ackerman, presented before a regional meeting of the Society of Experimental Biology and Medicine, Richmond, Va., October 11, 1957.

[2]General Biochemicals, Inc.

[3]Kindly supplied by Dr. Olaf Mickelsen, National Institutes of Health, Bethesda, Md.



A - 9% casein (G.B.I.), 0.279% L-histidine-HCl, 0.315% DL-methionine, all fumigated

B - 10.5% casein hydrolysate

C - 9% casein (G.B.I.)

D - 9% casein (Labco), fumigated

E - 9% casein (G.B.I.), fumigated

F - 10.5% casein hydrolysate, fumigated

↓ - supplemented with 0.279% L-histidine-HCl and 0.315% DL-methionine

GROWTH OF WEANLING RATS FED A PURIFIED DIET CONTAINING VARIOUS SOURCES OF CASEIN NITROGEN FUMIGATED WITH ETHYLENE OXIDE

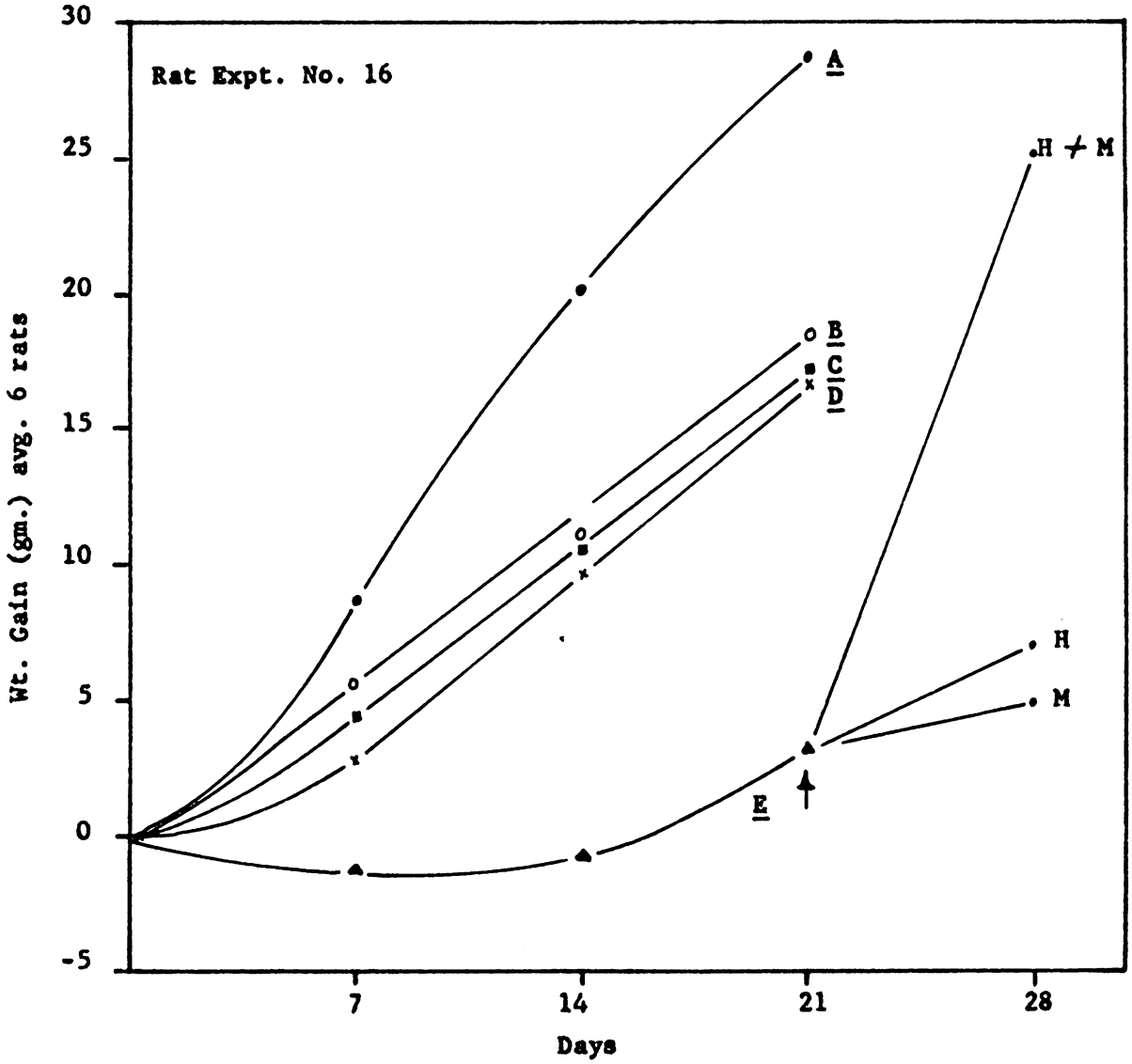
Figure 2

acids were not labile to the fumigation, an hypothesis supported by previous findings (114), or, if some destruction occurred, the net amount of these two essential amino acids remaining after the treatment was still sufficient to prevent either from being limiting to growth. The slightly superior growth on the supplemented fumigated casein as compared with the control can be explained by the added methionine, the most limiting essential amino acid in this protein.

Rats fed an ethylene oxide fumigated hydrolysate of casein were not able to maintain their body weight; and a 100% replacement supplement of histidine and methionine did not significantly reverse this severe growth inhibition. Hydrolysis of proteins frees the primary amine groups of all the constituent amino acids for reaction with the fumigant, so that this result is hardly surprising. The hydrolysate was incorporated at a 10.5% level in diets in order to make them equivalent in nitrogen to the 9% casein diets. The extra 1.5% was added at the expense of sucrose.

The capacity of moisture to increase the lability of casein to ethylene oxide fumigation is seen more directly from the results of the next experiment (figure 3). The feeding of Labco casein fumigated in the presence of 9.9% moisture produces the same extent of growth inhibition as the feeding of fumigated G.B.I. casein which has approximately the same moisture content (see figure 2). With less moisture fumigation damage is considerably reduced, there being no appreciable difference between results at 0.5, 1.7 or 5.4% water, all samples resulting in about a 40% reduction in growth. The capacity of a histidine plus methionine supplement, but neither amino acid alone, to effect a dramatic renewal of growth is again demonstrated in this experiment.[1]

[1] In this and the following experiments, branching of the growth curves, as in group E at 21 days, indicates the division of the experimental group into two or more sub-groups, each supplemented as indicated.



Casein:

A - unfumigated

fumigated after moisture adjusted to:

B - 1.7%
C - 0.5%
D - 5.4%
E - 9.9%

↓ - diet supplemented (2 rats/treatment)
H - 0.210% L-histidine-HCl
M - 0.236% DL-methionine

GROWTH OF WEANLING RATS FED A PURIFIED DIET CONTAINING 9% LABCO CASEIN FUMIGATED WITH ETHYLENE OXIDE IN THE PRESENCE OF VARIABLE AMOUNTS OF MOISTURE

Figure 3

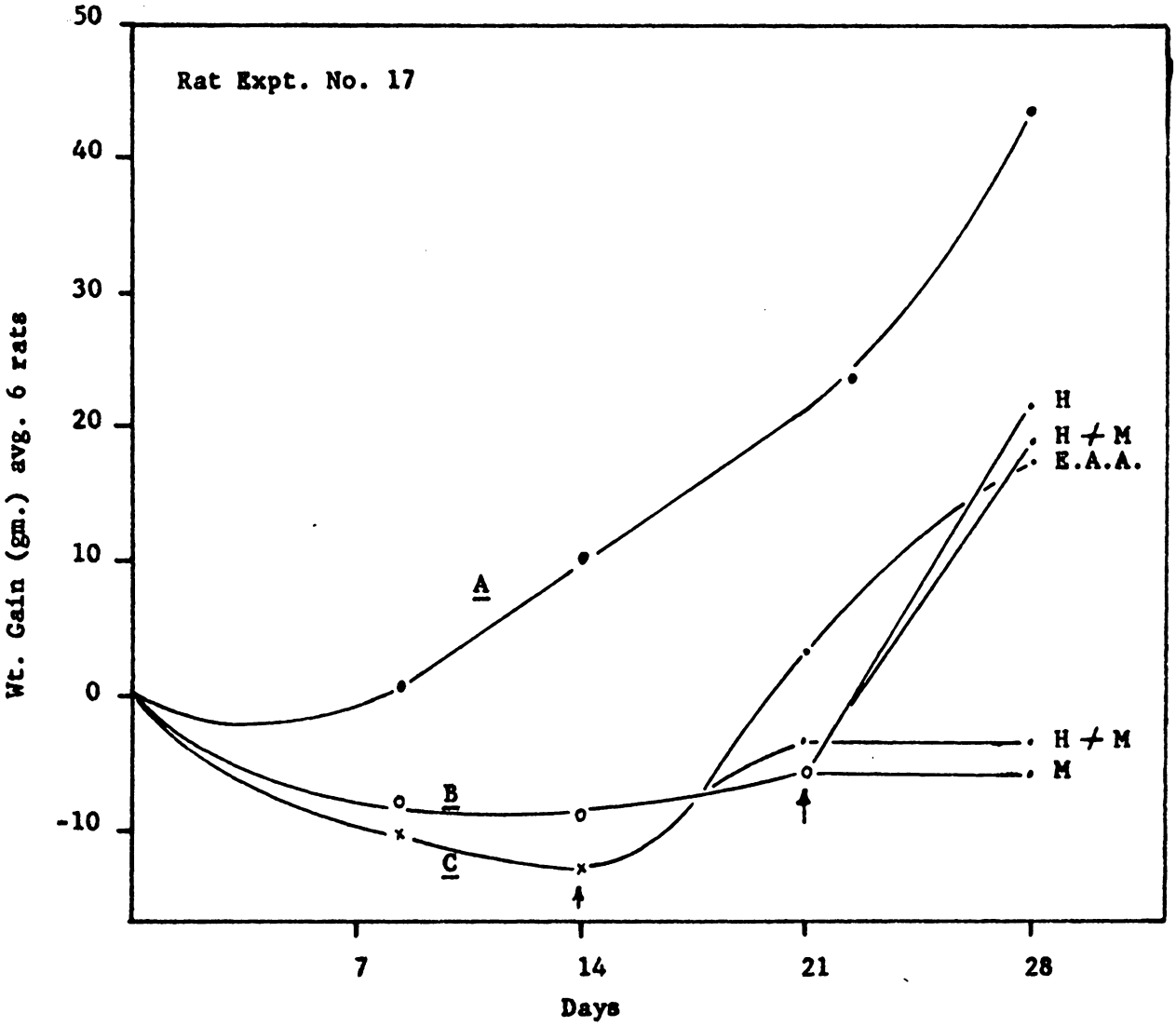
The quantity of the two amino acids used represents a 75% replacement supplement. On the basis of these studies the small reduction in casein quality after fumigation as reported by Mickelsen (78) may be assigned two causes: 1. the relatively low moisture content of his sample; and 2. as he has already suggested, the relatively low concentration of fumigant to which the casein was exposed.

Lactalbumin:

The results of feeding ethylene oxide-fumigated lactalbumin (Experiment No. 17, figure 4) reveal that this protein is very severely damaged when fumigated in the presence or absence of moisture. However, the extent of destruction is not equivalent in the two cases, as indicated during the amino acid supplementation phase of this trial. The fumigated dry lactalbumin supported growth when supplemented with histidine alone. Methionine alone was ineffective. On the other hand the fumigated wet lactalbumin was only slightly improved in quality by adding both histidine and methionine. Adding a 100% replacement supplement of all 10 essential amino acids was much more effective.[1] It was implied from this experiment that only histidine became limiting for growth when dry lactalbumin was fumigated with ethylene oxide but that histidine, methionine and possible other essential amino acids became limiting when moist lactalbumin was exposed to the fumigant.

Experiment No. 18 (figure 5) lends further support to this implication.

[1] 0.236% L-histidine-HCl, 0.243% DL-methionine, 0.486% DL-threonine, 0.576% DL-valine, 0.378% L-arginine-HCl, 0.432% DL-phenylalanine, 0.657% DL-isoleucine, 1.100% L-leucine, 1.041% L-lysine-HCl, and 0.207% DL-tryptophan.



Lactalbumin:

A - unfumigated

fumigated after moisture adjusted to:

B - 0.0%

C - 10.0%

↓ - diet supplemented (2 rats/treatment)

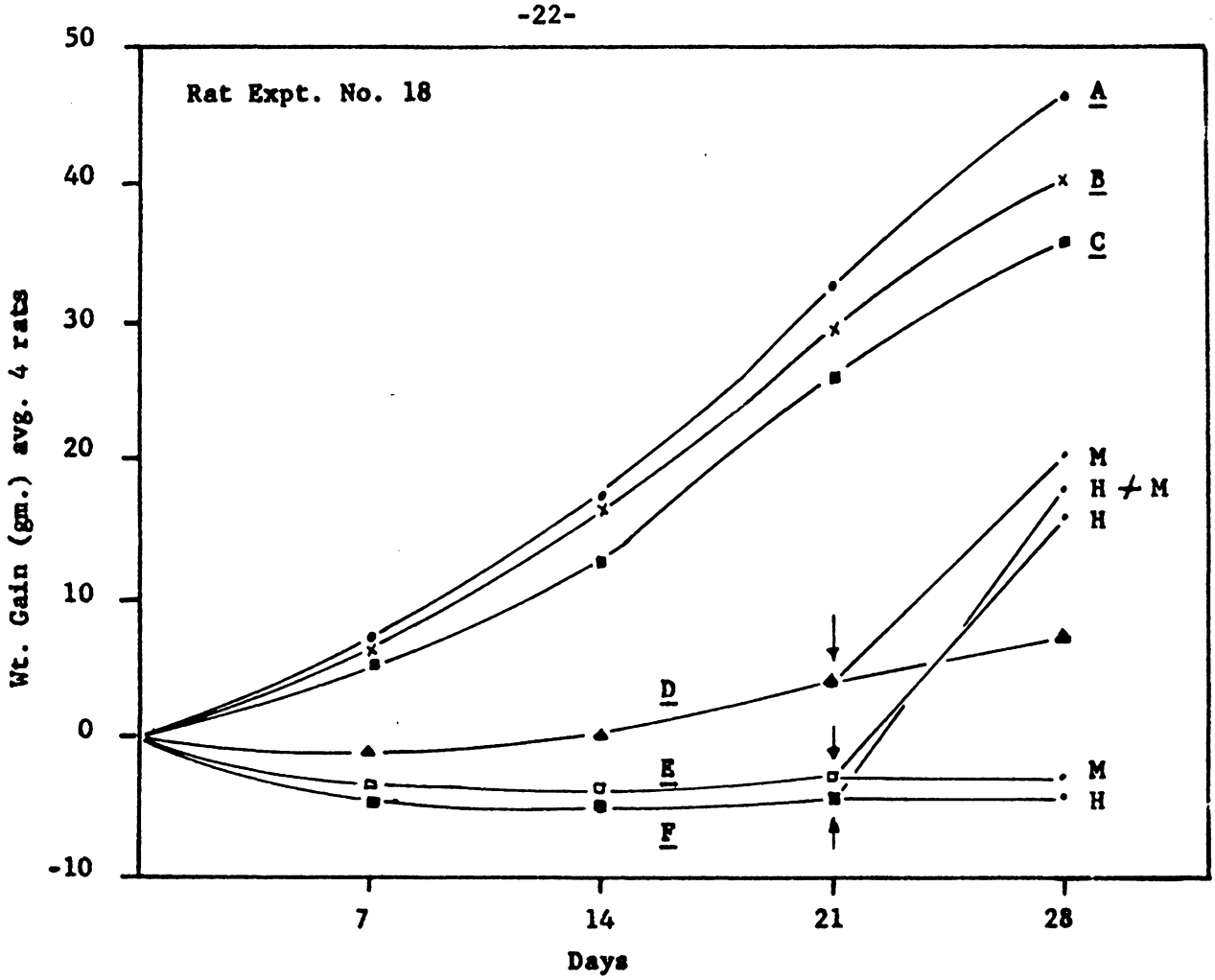
H - 0.236% L-histidine-HCl

M - 0.243% DL-methionine

E.A.A. - mixture of 10 essential amino acids

GROWTH OF WEANLING RATS FED A PURIFIED DIET CONTAINING 9% LACTALBUMIN FUMIGATED WITH ETHYLENE OXIDE IN THE PRESENCE AND ABSENCE OF MOISTURE - EFFECT OF AMINO ACID SUPPLEMENTATION (I)

Figure 4



	Lactalbumin: fumigated	W/moisture (%)	initial amino acid supplement
A	—		none
B	+	10	0.236% L-histidine-HCl + 0.234% DL-methionine
C	+	0	0.236% L-histidine-HCl
D	+	10	0.236% L-histidine-HCl
E	+	0	none
F	+	10	none

↓ - additional dietary supplement (2 rats/treatment)
H - 0.236% L-histidine-HCl
M - 0.234% DL-methionine

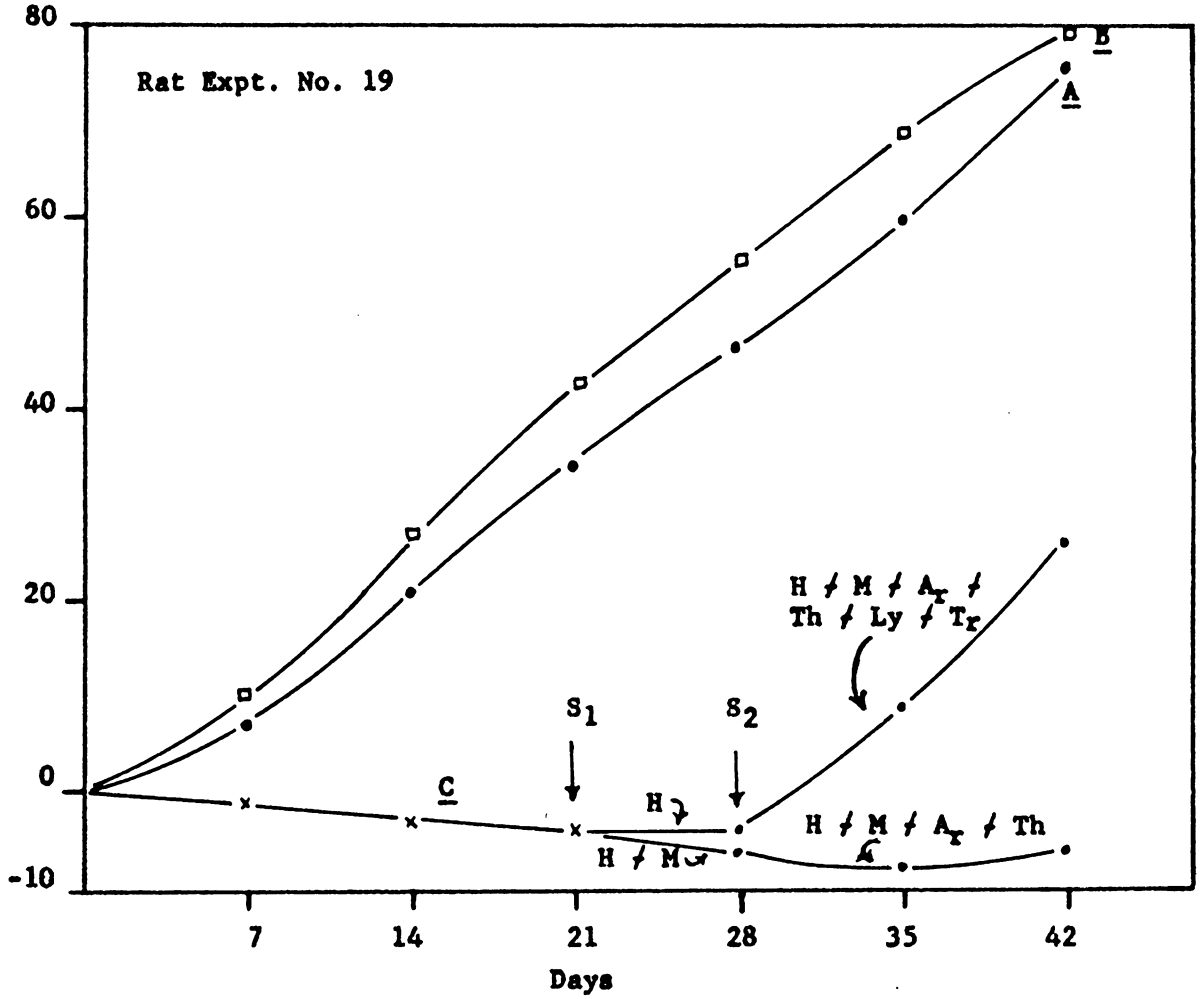
GROWTH OF WEANLING RATS FED A PURIFIED DIET CONTAINING 9% LACTALBUMIN FUMIGATED WITH ETHYLENE OXIDE IN THE PRESENCE AND ABSENCE OF MOISTURE - EFFECT OF AMINO ACID SUPPLEMENTATION (II)

Figure 5

The table below the figure lists the protein source and amino acid supplement of each diet at the beginning of the experiment. Three of the diets were further supplemented at 21 days. Excluding the control, A, the diets which supported growth contained fumigated wet lactalbumin plus histidine and methionine, B, or fumigated dry lactalbumin plus histidine, C. The fumigated wet lactalbumin plus histidine diet, D, was poor but greatly improved by adding methionine. Likewise, both the fumigated dry and wet unsupplemented lactalbumin diets (E and F respectively) failed to support growth. Histidine alone, but not methionine, improved the former, and histidine plus methionine, but not histidine alone, improved the latter.

Egg Albumin:

The results of feeding ethylene oxide-fumigated egg albumin (Experiment No. 19, figure 6) revealed the clearest picture of the importance of moisture in determining the extent of fumigation damage to proteins. There was no apparent damage to egg albumin fumigated after oven drying (0.0% moisture) but the sample concurrently fumigated without drying (11.8% moisture) was completely incapable of supporting growth of the rats. One hundred percent replacement supplements of histidine, histidine plus methionine, or histidine, methionine, arginine and threonine failed to initiate growth. It was only after lysine and tryptophan were added to this 4-amino acid supplement that dramatic improvement in animal response was observed. It was felt that the arginine and threonine were probably unimportant to this supplement and that growth had resulted from



Egg albumin:

A - unfumigated

fumigated after moisture adjusted to:

B, 0.0% C, 11.8%

S_1 = 1st dietary supplement (2 rats/treatment)

S_2 = 2nd dietary supplement (2 rats/treatment)

H = 0.270% L-histidine-HCl M = 0.432% DL-methionine

A_r = 0.664% L-arginine-HCl Th = 0.396% DL-threonine

Ly = 0.387% L-lysine-HCl Tr = 0.135% DL-tryptophan

GROWTH OF WEANLING RATS FED A PURIFIED DIET CONTAINING 9% EGG ALBUMIN FUMIGATED WITH ETHYLENE OXIDE IN THE PRESENCE AND ABSENCE OF MOISTURE - EFFECT OF AMINO ACID SUPPLEMENTATION

Figure 6

the presence of some combination of histidine, methionine, lysine, and tryptophan. Experiment 20 (table 1) was designed to reduce this combination to its simplest terms. Rats were fed a diet containing 9% egg albumin which had been fumigated with ethylene oxide in the

Table 1

Effect of Amino Acid Supplements On the Growth and Feed Efficiency of Weanling Rats Fed a Purified Diet Containing 9% Egg Albumin (11.8% moisture) Fumigated With Ethylene Oxide[1]

Group No. (3 rats/group)	Dietary Supplement[2] Hist	Meth	Tryp	Lys	Avg. wt. gain, 28 days (gm.)	Feed intake (gm.) / Wt. gain (gm.)
1					-3.0	-
2	+	+	+	+	37.0	4.19
3	+	+	+		13.7	9.42
4	+	+		+	37.0	4.18
5	+		+		13.0	10.1
6		+	+		-1.6	-
7	+			+	44.7	3.94
8		+		+	-2.6	-
9			+	+	-1.3	-
10	(unfumigated control)				43.7	4.27

[1]Rat Experiment No. 20

[2]Hist, 0.270% L-histidine-HCl; Meth, 0.432% DL-methionine; Tryp, 0.135% DL-tryptophan, and Lys, 0.387% L-lysine-HCl

presence of 11.8% moisture and supplemented with various combinations of the four indicated amino acids. Lysine was supplemented at a 54% replacement level, the other three amino acids at 100%. It can be seen from the tabulated data that weight gain and feed efficiency were good

(as compared with the unfumigated egg albumin control diet) when both histidine and lysine appeared in the supplement; that weight gain was reduced to about 30% and feed efficiency poor when histidine was present but not lysine; and that the absence of histidine completely prevented growth. So that in this protein sample, fumigated separately from the one used in Experiment No. 19, histidine was the most limiting amino acid, and it was followed by lysine.

Microbiological Amino Acid Assays of Ethylene Oxide - Fumigated Proteins:

The complete inability of histidine to stimulate growth when added to fumigated moist egg albumin in Experiment No. 19 but its capacity to evoke a partial response under similar conditions in Experiment No. 20 suggested a difference in the extent of lysine destruction in the protein samples used in the two experiments. That this was indeed the case is revealed by the results of microbiological amino acid assays for histidine, methionine, lysine and phenylalanine in these two samples, as well as in aliquots of the protein samples from the other rat feeding experiments (table 2). More than 2.5 times as much lysine was destroyed by fumigating the sample used in Experiment No. 19 as was destroyed in Experiment No. 20 although the difference in the extent of histidine and methionine destruction between these samples is comparatively small. At present no adequate explanation can be offered for this result. The two samples of egg albumin in question were fumigated under identical conditions. It is possible that failure to securely seal the fumigation desiccator during treatment of the lesser damaged of the two samples permitted the escape of ethylene oxide and its replacement by air during

Table 2

Biological Availability of Four Amino Acids From Proteins Fumigated with Ethylene oxide in the Presence of Variable Amounts of Moisture

Protein	Rat Expt. No.	Fumigated	% Moisture	mg./gm. dry protein[1]			
				Histidine	Methionine	Lysine	Phenylalanine
Casein	16	-		26.1	26.5	72.4	43.5
(Labco)	16	+	0.5	18.9 (28)[2]	21.8 (18)	67.6 (7)	44.1 (0)
	16	+	9.9	11.1 (57)	14.3 (46)	55.6 (23)	43.5 (0)
Lactalbumin	17	-		14.7	17.9	76.6	28.2
	17	+	0.0	8.0 (46)	13.8 (23)	52.7 (31)	27.3 (3)
	17	+	10.0	2.5 (83)	8.0 (55)	55.0 (28)	28.2 (0)
Egg albumin	19	-		19.7	29.2	56.7	44.7
	19	+	0.0	17.6 (11)	27.3 (6)	53.6 (5)	47.3 (0)
	19	+	11.8	5.7 (71)	19.5 (33)	4.8 (92)	41.6 (7)
	20	+	11.8	8.4 (57)	21.0 (28)	36.3 (36)	44.7 (0)

[1] determined microbiologically; each value represents the average of duplicate assays at five concentrations of the hydrolysate; assay organism, Leuconostoc mesentroides P-60

[2] nos. in ( ) indicate the percent decrease in availability after fumigation.

the course of the 24-hour treatment.

The microbiological amino acid assays, which are in complete agreement with the results of the rat feeding studies, may be further summarized as follows:

1. Ethylene oxide fumigation of casein, lactalbumin and egg albumin can reduce the biological availability of histidine, methionine, and lysine in each case. The availability of phenylalanine is not affected.

2. The presence of moisture during the fumigation enhances the extent of amino acid inavailability.

3. Generally, histidine is the most ethylene oxide-labile of these three amino acids and lysine the least labile. However, the relative inavailability of the three amino acids in various fumigated proteins, or in the same protein fumigated under different conditions, is not always the same.

The consistent assay results (within experimental error) when the protein hydrolysates were assayed at five widely varying concentrations rules out the possibility that microbial growth inhibition was caused by the presence of a toxic factor in the hydrolysates of the fumigated proteins. Rather the results suggest that ethylene oxide can convert at least three of the essential amino acids of intact protein into compounds which have little or no nutritive value for the rat or for certain bacteria. Thus there appears a striking resemblance between the action of ethylene oxide and sulfur mustard[1], which can also react with and make unavailable to the rat the histidine, lysine and methionine of casein treated with this war gas (59)(49).

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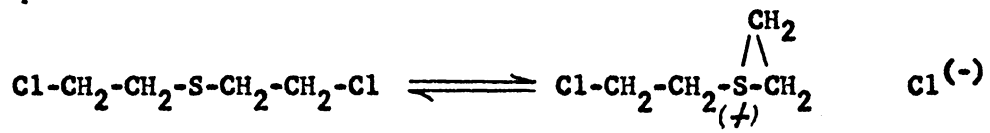
[1]bis-(2-chloroethyl)sulfide

The analogy is further extended to the stability of the new bonds to acid hydrolysis in both cases, and it gains chemical support from the following equilibrium.



Alpha Protein of Soybeans:

The final protein that was fumigated with ethylene oxide and fed to weanling rats was the alpha protein fraction of soybeans. The results of two separate experiments are shown in table 3.

Table 3

Growth of Weanling Rats Fed A Purified Diet Containing 9% Alpha Protein Fumigated With Ethylene Oxide in the Presence and Absence of Moisture

Rat Expt. No.	No. of Rats/ Treatment	Avg. Weight Gain after 42 Days			
		Unfumigated		Fumigated at % moisture	
		Not heated	Heated[1]	0.0%	10.0%
22[2]	6	-3.7		14.5	6.2
23	6	-2.0[3]	12.3[3]	43.0	28.7

[1]Heated 24 hours at 80° C.

[2]There was a high incidence of respiratory infection in rats of all groups during this experiment.

[3]3 rats per treatment

In both experiments fumigation, particularly in the absence of moisture, appeared to significantly improve the quality of this protein, which was incapable of supporting growth when fed per se. In the second experiment, a portion of the unfumigated sample was exposed to the identical heat treatment as was received by the fumigated wet sample[1], and some improvement in the subsequent performance of the rats was observed. It is noteworthy that in concurrent experiments, rats fed alpha protein exposed to three and six million r.e.p.[2] of gamma-irradiation were also able to grow (115), an average of 16.5 gm. in Experiment No. 22 and 41.8 gm. in Experiment No. 23. Severe respiratory infections among the animals of Experiment No. 22 account for the generally lower weight gains during this trial.

A statement from the manufacturer of the alpha protein revealed that during processing the highest temperature to which the product is exposed is 180-200°F. To destroy the heat-labile growth inhibitor of soybean protein, first observed by Osborne and Mendel (83), autoclaving at 250°F. is usually practiced. The inhibitor has been isolated by Kunitz (64) and shown to be a heat-labile, globin-type protein of molecular weight about 24,000 which stoichiometrically combines with and inhibits the intestinal proteolytic enzyme trypsin. This protein inhibits growth, however, even when fed together with predigested soy protein (108), so that trypsin inhibition is apparently not its only

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[1]the fumigated dry sample was heated for 24 hours both before and after fumigation.

[2]r.e.p. = roentgens - equivalent - physical; 1 r.e.p. = 83 ergs per gm. of irradiated material, as described in "Radioactivity Units and Standards", by R. B. Evans, Nucleonics 1 (2), 32 (1947).

mode of action. Recently Borchers et al (18) have reported that the feeding of certain antibiotics with unheated soy protein was as effective as autoclaving the protein in reversing the growth inhibition.

Thus the ability of the rats in experiments No. 22 and 23 to grow on the ethylene oxide-treated alpha protein suggests that this treatment, as well as the gamma-irradiation, was capable of destroying the heat-labile growth inhibitor. That this inhibitor was in fact present in the alpha protein was further indicated in another experiment in which three rats that had failed to grow after three weeks on a 9% alpha protein diet gained on the average 7 grams over the next 21 days during which period autoclaved[1] alpha protein was substituted for the unheated protein in their diet.

If ethylene oxide treatment is capable of destroying the action of the growth inhibitor then the fumigation of the sample containing 10% moisture may have also resulted in some amino acid destruction since less growth was achieved than when the fumigated dry alpha protein was fed. No microbial amino acid analyses of these protein samples were obtained.

The possibility that ethylene oxide could destroy the proteinaceous growth inhibitor became more reasonable when it was shown later (see chapter V) that the fumigation of crystalline chymotrypsin destroyed its proteolytic activity.

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[1]autoclaved 30 minutes at 121° C.

IV. REACTION OF ETHYLENE OXIDE WITH SOME AMINO ACIDS, AMINO ACID DERIVATIVES AND VITAMINS IN AQUEOUS SOLUTION

To obtain chemical justification for the observed reduction in the biological availability of histidine, methionine and some of the B-vitamins following exposure to ethylene oxide, model compounds were treated with the fumigant in aqueous solution and efforts made to isolate and identify the products. Several of the fumigated products yielded ethanolamine when degraded in alkali, and a convenient method for determination of this amine was developed.

Materials and Methods

The ethylene oxide fumigation of aqueous solutions was accomplished as described previously for the treatment of protein samples. The solutions, in 100 ml. or 250 ml. beakers, were fumigated at room temperature in a four-litre desiccator with continuous stirring by means of a magnetic stirrer. Projected into the desiccator through a rubber stopper in the top were the glass electrodes of a Beckman Model M2 pH meter and the extended tip of a burette. This allowed continuous observation of the pH of the fumigated solutions and adjustment of the pH as desired. As before, fumigations were terminated by removal of excess ethylene oxide under vacuum, a vacuum of 40 mm Hg being applied until bubbling of the gas from the solutions had ceased.

Ethanolamine was determined by oxidizing it to formaldehyde with alkaline periodate (7) and determining the formaldehyde with chromotropic acid[1] (70) by a modification of the method of Critchfield and Johnson (29). A sample containing 10 to 100 µg. ethanolamine (c.a. pH 7) was pipetted into a 25 ml. volumetric flask and the volume was adjusted to 10 ml. with water. To this were added successively 5 ml. 0.05 M borate buffer, pH 7.8 and 2 ml. 0.1 N $NaIO_4$. The flask was shaken and the reaction allowed to proceed 15 minutes at room temperature. Then 2 ml. of 5% aqueous Na_2SO_3 (w/w) was added to reduce the excess periodate. The flask was filled to the mark with water and the contents thoroughly mixed. A 2.5 ml. aliquot was pipetted into an Evelyn colorimeter tube. Two ml. of a 1% (w/w) aqueous solution of sodium chromotropate was added, followed by 8 ml. of concentrated H_2SO_4, which was allowed to slowly run down the inside of the colorimeter tube from the delivery tip of a burette. The tube was stoppered, swirled to mix, and allowed to cool for 2 to 12 hours, when the color was read against a reagent blank in an Evelyn colorimeter at 580 mµ. Several standards, usually containing 50 and 100 µg. of ethanolamine were run with each set of unknowns.

Unless other wise indicated all paper chromatography was done on 1" by 18" Whatman No. 1 paper strips in a 15" by 22" covered glass battery jar using the desending technique.

Imidazole, nicotinamide and nicotinic acid were from Eastman Organic Chemicals Corp., histidine, methionine and pyridoxine from Nutritional

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[1] 4, 5-dihydroxy-2,7-naphthalenedisulfonic acid (sodium salt) (Eastman Organic Chemicals Corp., Rochester, N. Y.)

Biochemicals Corp.; thiamine-HCl, riboflavin and folic acid from Merck and Co.; and ethanolamine from Fisher Scientific Co. N-acetylmethionine was prepared from methionine and acetic anhydride according to Kolb and Toennies (62). Titration curves were determined with a Beckman Model M2 pH meter with glass electrodes, and quantitative elemental analyses were supplied by Galbraith Laboratories, Knoxville, Tenn.

All water used in these studies had been once distilled from a Barnstead still.

Results and Discussion[1]

Imidazole:

In a preliminary experiment, the exposure of an aqueous solution of imidazole to ethylene oxide caused a rise in pH from 9.5 to 12.1, suggesting the alkylation of the tertiary nitrogen and the presence of a quaternary ammonium group. The product was isolated as follows:

70 mmoles of imidazole and 140 mmoles of HCl in 20 ml. H_2O were fumigated with 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 to the filtrate to the point of incipient cloudiness and crystallization effected overnight at -2° C. The product was twice recrystallized from ethanol and acetone to yield fine, white, highly

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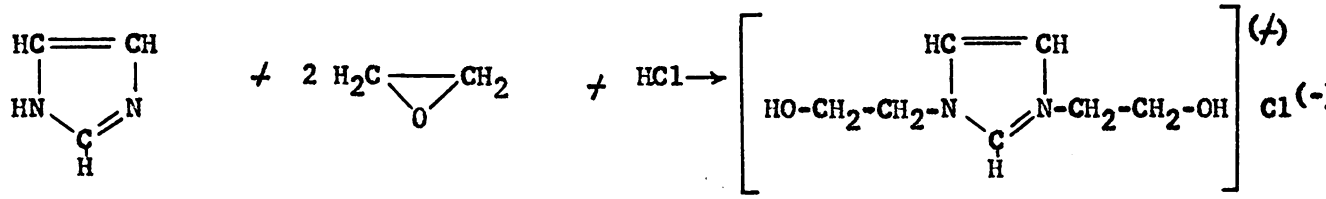
[1]Preliminary reports:

1. Reaction of Ethylene Oxide With Histidine, Methionine and Nicotinic Acid: by H. G. Windmueller and Howard Bakerman; Federation Proc. 17, 338 (1958).
2. Reaction of Ethylene Oxide With Histidine, Methionine and Nicotinic Acid: by H. G. Windmueller and C. J. Ackerman; Va. J. Sci. (in press)

deliquescant needles which were best dried in vacuo over concentrated H_2SO_4. Phosphorus pentoxide could not be used because the product had a tendency to sublime under vacuum, and it was rapidly dehydrated and polymerized to a black, insoluble mass on the surface of this desiccant. The product was too deliquescant for convenient melting point determination. Found, %C, 43.36; H, 6.99; N, 14.26; Cl, 18.18. Calculated for $C_7H_{13}N_2O_2Cl$, %C, 43.58; H, 6.81; N, 14.55; Cl, 18.42. The addition of $AgNO_3$ to a solution of the product gave an immediate precipitate of AgCl, indicating that the chlorine was active and probably ionic (97).

From the empirical formula it was evident that two 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 following reaction was visualized.



Imidazole

1,3-bis-(2-hydroxyethyl)imidazolium chloride

(I)

Additional evidence of structure was obtained by degrading I in 25% KOH (w/w). Pinner and Schwarz (87) have demonstrated that 1,3-dialkyl imidazoles yield the corresponding primary alkyl amines by such treatment, e.g. ethanolamine from the proposed ethylene oxide-imidazole product, I.

One mmole of I (quantitatively determined in solution by ionic chloride analysis (93)) was refluxed 24 hours in 25% KOH (w/w) and then distilled under 15 mm Hg vacuum into 10 ml. of 6 N HCl. The clear distillate contained a single primary amine which had the same R_f[1] on paper chromatograms as authentic ethanolamine-HCl, as determined in four solvent systems (table 4). The amine was detected by spraying with 0.4% ninhydrin [2] in 1-butanol.

Table 4

R_f Values of Ethanolamine hydrochloride and an Unknown Amine From the Alkaline Hydrolysate of 1,3-bis-(2-hydroxyethyl)imidazolium chloride (I)

Solvent	R_f		
	(a) ethanolamine-HCl	(b) alkaline hydrolysate[1] of (I)	(a) + (b)
1. 1-butanol saturated with H_2O	0.11	0.11	0.11
2. 1-butanol saturated with 3% aqueous NH_3	0.28	0.28	0.28
3. 1-butanol: acetic acid: H_2O (100 : 21 : 50)	0.36	0.36	0.36
4. phenol saturated with H_2O	0.55	0.55	0.55

[1] refluxed 24 hours in 25% KOH (w/w) and distilled under vacuum. (b) is the clear distillate.

[1] R_f was obtained by dividing the distance traveled by the compound on paper by the distance traveled by the solvent front.

[2] 1,2,3-indantrione hydrate

The amine in the distillate was quantitatively determined first by the manometric nitrous acid method of Van Slyke (105) and then by oxidation with slightly alkaline periodate, with determination of the resulting formaldehyde by the chromotropic acid procedure of Critchfield and Johnson (29). Both methods were standardized with authentic ethanolamine. A typical standard curve for the periodate-chromotropate method is shown in figure 7. The Van Slyke method, which determines all primary amines, gave nitrogen recoveries of from 97% to 103% of theory for ethanolamine. Both methods revealed that the KOH distillate of I contained 1.6 mmoles of amine per mmole of I (80% of theory). Oxidation by alkaline periodate implies that the amine is a 1,2-hydroxyamine, or a related compound (81). This fact plus the chromatographic data in table 4 strongly suggests that alkaline hydrolysis of I yields two equivalents of ethanolamine[1] and that the structure postulated for I is correct.

Unlike imidazole, I gives no color when coupled with diazotized sulfanilic acid in the quantitative histidine assay method of McPhereson (75), thereby making this a suitable procedure for following the reaction of ethylene oxide with imidazole and some of its derivatives, e.g. histidine. Also unlike imidazole, I gives an immediate water-insoluble precipitate with ammonium reineckate[2] at pH 7, additional support for the presence of a quaternary ammonium group.

To the author's knowledge 1,3-bis-(2-hydroxyethyl)imidazolium chloride has not been previously described.

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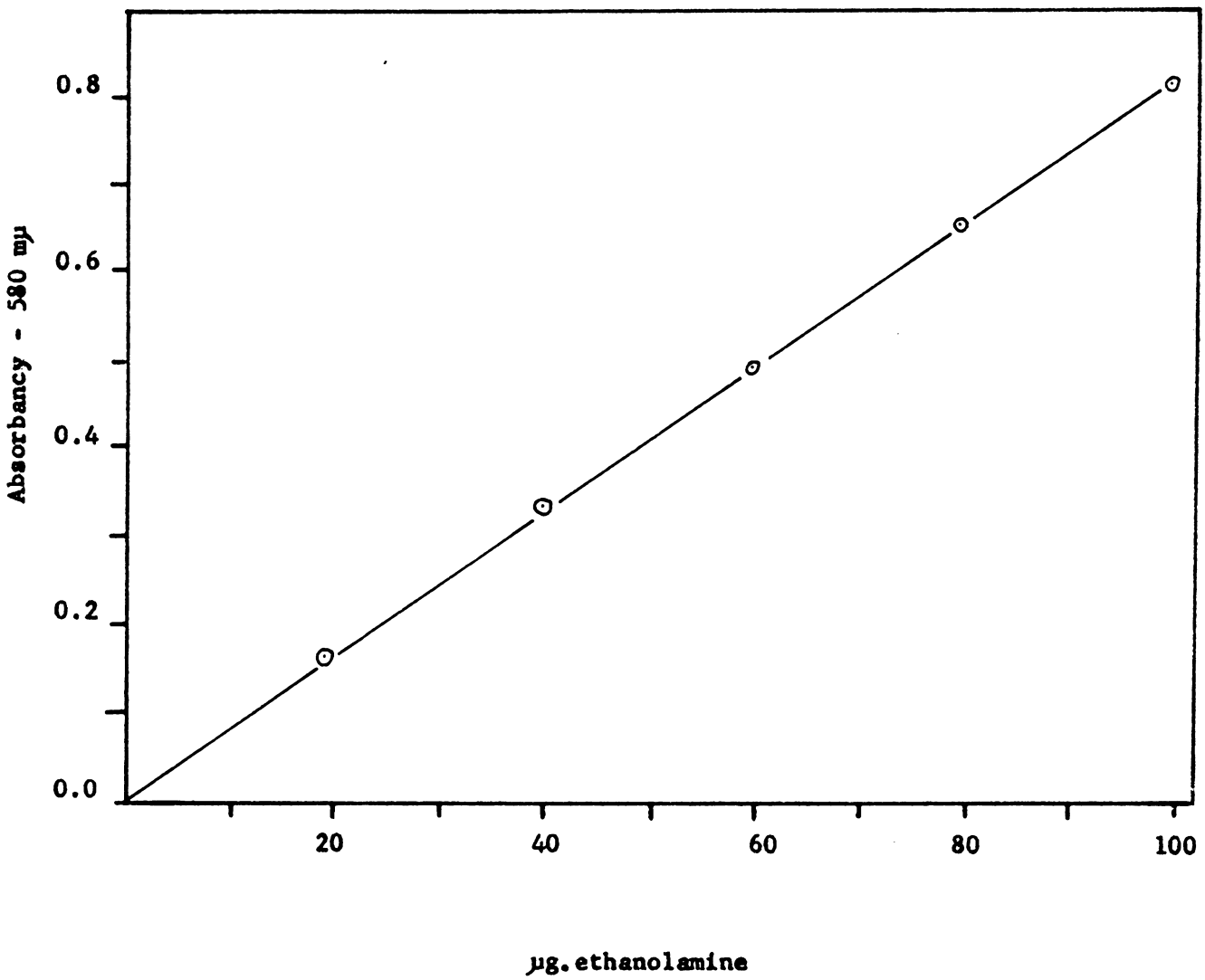
[1]in control experiments, only about 85% of added portions of authentic ethanolamine could be recovered in the distillation step.

[2]ammonium tetrathiocyanodiammono-chromate



STANDARD CURVE FOR COLORIMETRIC ETHANOLAMINE DETERMINATION

Figure 7

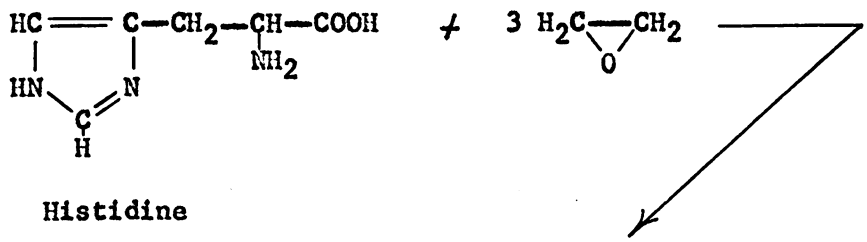
Histidine hydrochloride:

The fumigation of an aqueous solution of histidine-HCl for 24 hours caused a rise in alkalinity from pH 3.8 to c.a. pH 7. The solution was concentrated to a viscous pale-yellow syrup and taken up in hot 95% ethanol. When either acetone or dioxane were added dropwise to incipient cloudiness, subsequent cooling at 5° C. produced only a white, viscous syrup which showed no tendency to crystallize on standing. Repeated efforts to crystallize the product failed.

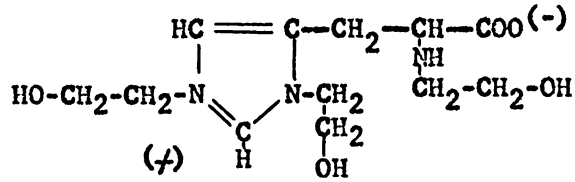
However, by analysis of an aqueous solution of ethylene oxide fumigated histidine-HCl, the following information was obtained concerning the course of the reaction.

The product formed no colored complex with diazotized sulfanilic acid in the quantitative histidine assay of McPhereson (75), implying hydroxyethylation of the imidazole nitrogens as in the case of ethylene oxide-treated imidazole. This implication was supported by the recovery of ethanolamine (63% of theory) from a 25% KOH (w/w) hydrolysate of the product. The hydrolysis and the recovery, identification, and determination of the ethanolamine were conducted as with the imidazole-ethylene oxide reaction product (see page 36-37). By Van Slyke amino nitrogen analysis (105) it was determined that the primary amino group of the histidine had been 98% alkylated. Unlike histidine but like the 1,3-bis-(2-hydroxyethyl)imidazolium chloride the ethylene oxide-histidine product formed an immediate water-insoluble precipitate with ammonium reineckate at acid pH, further support for the presence of a quaternary ammonium group.

From the above information, the following reaction is indicated:



Histidine



N,N',N''-tris-(2-hydroxyethyl)histidine, betaine

Measuring the reduction in primary amino groups by the Van Slyke method does not distinguish between those which have been singly hydroxyethylated and those which have been doubly hydroxyethylated, so that the possibility of double hydroxyethylation must be considered. The neutral pH of the fumigated histidine solution considered with the evidence for the presence of the quaternary ammonium hydroxide group makes it seem very unlikely that the carboxyl group of the histidine has been esterified by the ethylene oxide. Esterification would consume another proton and a strongly alkaline solution would be expected. The failure of ethylene oxide to esterify the carboxyl groups of alanine, tyrosine, benzoic acid and adipic acid under similar reaction conditions has been reported by Maekawa and Tsumura (71).

Chemical evidence for an ethylene oxide-histidine reaction in the fumigation of intact protein:

Table 5 shows the decrease in the apparent histidine content of casein samples of various moisture content fumigated with ethylene oxide. After fumigation, the samples were hydrolyzed with 2.5 N HCl at 121° C. for 12 hours, neutralized with NaOH, decolorized with charcoal (Norite) and assayed chemically. Amino acids other than histidine may contribute a small amount of color in this assay (51) so the values listed in the table represent the apparent rather than the true histidine content. Samples 1 and 4 were also assayed for histidine microbiologically (see footnote, page 14); the fumigated sample yielded 71% less histidine than the non-fumigated control.

The ability of fumigated histidine but not untreated histidine to form a water-insoluble reineckate at acid pH prompted a measurement of reineckate precipitable compounds from hydrolysates of fumigated and untreated lactalbumin (table 6). The protein samples have been previously described (see figure 4, samples A and C, page 21). Two gm. aliquots were hydrolyzed in 50 ml. of 3 N HCl at 121° C. for 12 hours. The hydrolysates were filtered, diluted to 100 ml. with H_2O and 10 ml. aliquots adjusted to the pH indicated in the table, using NaOH. After pH adjustment the volume of each aliquot was made to 25 ml. with H_2O and 2.5 ml. of 5% ammonium reineckate (w/w) in methanol was added. Precipitation was completed at 5° C. for three hours. The precipitates were filtered on sintered glass funnels coated with a filter aid[1], washed successively with four 2 ml. portions of 1-propanol and then solubilized in small portions of acetone, the acetone solutions being collected in 10 ml. volumetric

[1] "Super-cel"

Table 5

Decrease in Chemically Determinable Histidine of Casein after Fumigation with Ethylene Oxide

Sample	Moisture during fumigation (%)	Fumigation (hrs)	µg. histidine[1] / mg. N	% reduction after fumigation
1	0	0	312	-
2	0	12	215	31
3	9.5	12	120	60
4	9.5	24	94	70

[1] histidine determined by the colorimetric method of McPhereson (75), standardized with DL-histidine-HCl; nitrogen determined by the standard Kjeldahl procedure.

Table 6

Increase in Reineckate Precipitate from Lactalbumin Hydrolysate after 24 Hour-Fumigation of the Intact Protein with Ethylene Oxide

Protein	pH of reineckate precipitation	Absorbancy (520 mµ) of reineckate from 200 mg. protein
Lactalbumin	1.0	0.060
	7.0	0.040
	12.2	0.033
Ethylene oxide-fumigated lactalbumin	1.0	0.482
	7.0	0.040
	12.2	0.036

flasks which were subsequently filled to the mark with acetone. The quantity of the red acetone-soluble reineckate was determined at 500 mμ in a Beckman Model DU spectrophotometer using 1 cm. cells. The method was adapted from a published method for choline analysis (1) which involves the precipitation of choline with ammonium reineckate.

As expected from the properties of ethylene oxide fumigated histidine, the fumigation of a protein increases the amount of material which can be precipitated as a reineckate from the hydrolysate at acid pH. In the case of lactalbumin an eight-fold increase was observed. If the reineckate complex of the fumigated histidine had the same molar absorbancy index as the reineckate of choline, the value for which is 111 (1) then the increase in absorbance of 0.422 (0.482 minus 0.060) after fumigation would represent, by calculation, an hydroxyethylated-histidine content of the protein about 2.95%.[1] By another calculation, microbial assay of this sample (see table 2, page 27) revealed 83% destruction of the 2.1% histidine (17) in this protein, and from these data a 1.7% hydroxyethylated-histidine content would be anticipated. Lack of similar data on other fumigated protein samples prevents any

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[1]

$$a_M = \frac{A\,M}{b\,c}$$

a_M = molar absorbancy index (l. cm.$^{-1}$ M.$^{-1}$)
A = absorbancy
M = molecular weight (gm. M^{-1})
b = length of light path (cell width) (cm.)
c = concentration (gm. l.$^{-1}$)

$$c = \frac{A\,M}{b\,a_M} = \frac{(0.422)\ (155.16 \text{ gm. } M^{-1})}{(1.0 \text{ cm.})\ (111 \text{ l. cm.}^{-1} M^{-1})} = 0.59 \text{ gm. } l.^{-1} = 5.9 \text{ mg./10 ml.}$$

$$\frac{5.9 \text{ mg. (histidine)}}{200 \text{ mg. (protein)}} \times 100 = 2.95\%$$

general interpretation of these values which suggest that hydroxyethylated histidine is not the only component of the reineckate precipitate. Later studies with ethylene oxide-fumigated methionine and cysteine offered an explanation for this discrepancy; the products of the fumigation of both these amino acids also yield acid-insoluble reineckates.

Several attempts to isolate or identify the reineckate precipitated compound(s) from the hydrolysate of the fumigated protein failed. The compound(s) could be freed in solution by dissolving the complex in acetone followed by precipitation of the reineckate ion as the acetone-insoluble silver salt by the addition of saturated aqueous $AgNO_3$. Filtering the silver reineckate and chromatographing an aliquot of the filtrate on paper strips developed with an 1-butanol : acetic acid : H_2O (100 : 21 : 50) solvent revealed one major ninhydrin-sensitive amine spot (R_f 0.17) and four additional very faint spots (R_f 0.05, 0.09, 0.21, 0.41).

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 fumigation with ethylene oxide for 24 hours, followed by the removal of excess fumigant under vacuum.

A solution of fumigated methionine (pH 10) no longer exhibits any color in the standard McCarthy-Sullivan nitroprusside assay for methionine (74), strongly suggesting alteration of the thioether group. Unlike methionine, the fumigation product forms an acid-insoluble complex with

the reineckate ion and with phosphotungstic acid. This behavior is typical for sulfonium derivatives of methionine (67). The solubility of the reineckate in dilute alkali is taken as evidence that the carboxyl group of the methionine remains unesterified. Determination of primary amine groups by the manometric method (105) revealed virtually complete alkylation during the 24 hour fumigation with ethylene oxide.

The persistent emission of a rather strong sulfurous odor by solutions of fumigated methionine gives evidence that the methionine-ethylene oxide reaction product is unstable. Attempts to crystallize the product involved the concentration of an aqueous solution to a thick syrup, taking it up in absolute ethanol and attempting to effect crystallization by the drop-wise addition of acetone, ether, or hexane. A white, viscous syrup was deposited in each case either immediately or upon subsequent cooling to 5° C., even with vigorous rubbing. Strongly acidifying the product with HCl or HBr before concentration, followed by a similar solvent treatment as before also resulted in deposition of a syrup on the walls of the containing vessel. The difficulty of obtaining crystalline products of some sulfonium derivatives of methionine has been reported (104).

N-acetylmethionine:

In order to study more directly the reaction of ethylene oxide with the thioether group N-acetylmethionine was chosen as reactant. During fumigation an aqueous solution of N-acetylmethionine shifts from pH 2 to pH 9. The reaction product is soluble in water and ethanol but insoluble

(oils out) in acetone and hexane. Repeated attempts at crystallization failed. In addition to the methods described for the attempted crystallization of the ethylene oxide-methionine product the following methods were employed in an effort to purify this product: 1. precipitation from 1-propanol, 2-butanol, or n-amyl alcohol by the addition of a less polar solvent; 2. precipitation of the product as the phosphotungstate followed by the removal of the phosphotungstic acid by the addition of tetraethylammonium bromide, according to the methylmethionine sulfonium isolation procedure of Lavine and Floyd (68); 3. dissolving a concentrate of the product in glacial acetic acid, then adding acetic anhydride to remove the last trace of water, as recommended by Toennies and Kolb (104); 4. drastic cooling of a 1-propanol solution of the product in a mixture of solid CO_2 and acetone; (this procedure yielded a white amorphous solid which redissolved at room temperature) 5. removing the N-acetyl group from the molecule in the hope of obtaining a more easily crystallizable product (104). The fumigated N-acetylmethionine was subjected to the hydrolytic activity of a preparation of acylase[1] (16). Very little or no hydrolysis[2] was effected, however, even when the enzyme was used at a concentration ten times that which hydrolyzed almost completely a sample of N-acetylmethionine (L-isomer).

The fumigated N-acetylmethionine yields an acid-insoluble, alkali-soluble precipitate with ammonium reineckate. The reineckate complex is likewise very soluble in acetone and quite soluble in methanol and 1-propanol. And, as already indicated, with phosphotungstic acid (Fisher

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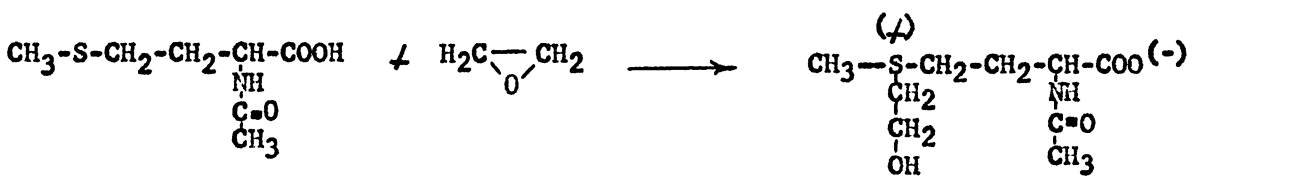
[1]The author is indebted to Dr. J. P. Greenstein, National Institutes of Health, for supplying the acylase preparation, through the courtesy of Dr. Olaf Mickelsen, of the same institute.

[2]Determined by manometric Van Slyke amino nitrogen determination (105).

Scientific Co.) the product yields a complex which is precipitated from water (97% yield based on sulfur analysis) and is soluble in acetone. Analysis of the phosphotungstate complex was as follows: %C, 6.53; N, 0.86; and S, 2.11. The analysis reflects a C:N:S ratio of 9:1:1, or the addition of one mole of ethylene oxide to each mole of N-acetylmethionine.

The titration curve of the product is shown in figure 8. The compound is slightly alkaline in solution and has two titrable groups with pKs at approximately 2.9 and 12.1. It is apparent from the titration curve that the weak alkalinity of the product is not the result of carboxyl group esterification but that a basic group has been produced as a result of the ethylene oxide fumigation.

All the data are consistent with the following formulation:



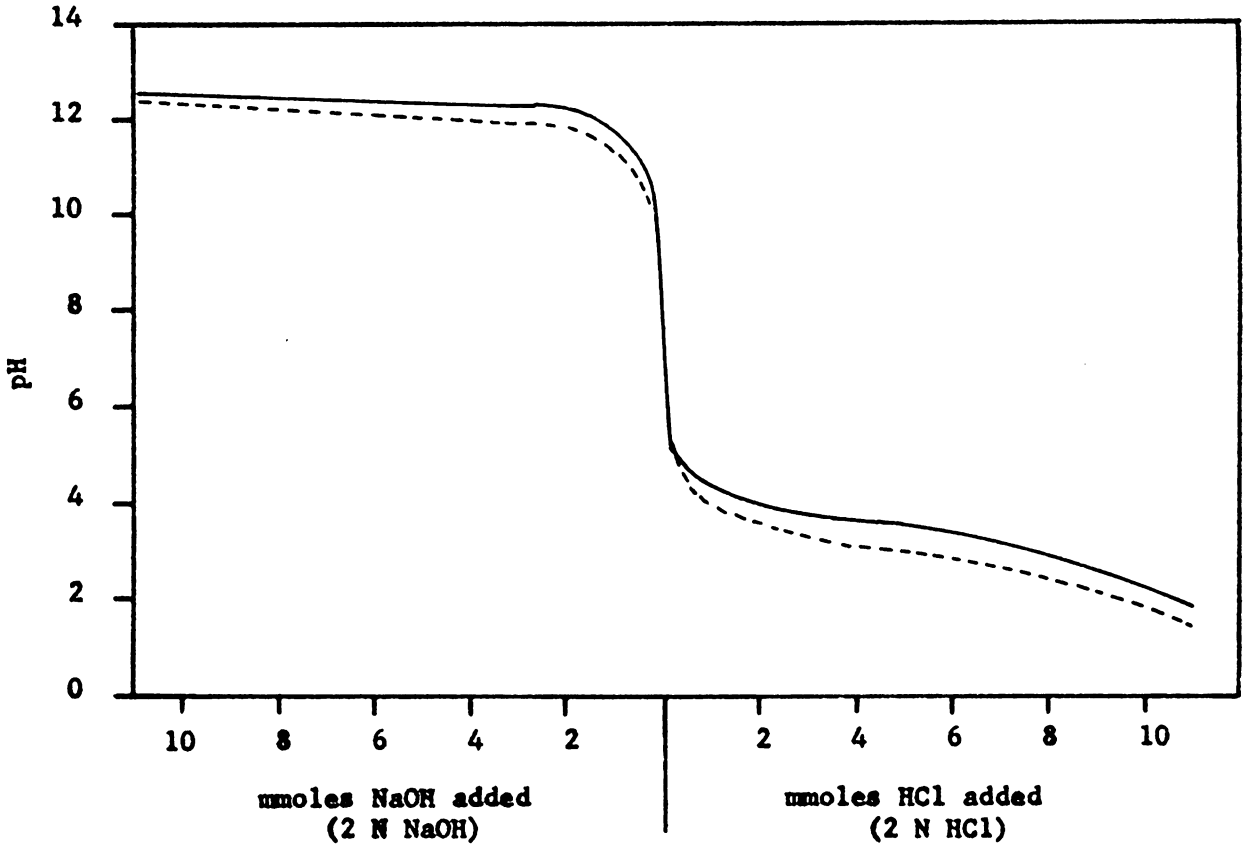
N-acetylmethionine

S-(2-hydroxyethyl)-N-acetylmethionine, thetin

(II)

Acid and alkaline hydrolysis of S-(2-hydroxyethyl)-N-acetylmethionine, thetin

To gain additional information about the structure and stability of the thetin resulting from the fumigation of N-acetylmethionine, which to the author's knowledge has not been previously described, acid and alkaline hydrolysis were studied in some detail, particularly with respect to the



——— N — acetylmethionine, 10.5 mmoles in 50 ml. H_2O

------- N — acetylmethionine, 10.5 mmoles in 50 ml. H_2O after fumigation for 24 hours with ethylene oxide

TITRATION CURVE OF N-ACETYLMETHIONINE BEFORE AND AFTER FUMIGATION WITH ETHYLENE OXIDE

Figure 8

stability of the sulfonium pole.

After autoclaving a solution of II in 6 N HCl for 14 hours at 121° C. most of the sulfonium groups were still intact, as indicated by the voluminous precipitate which resulted upon the addition of phosphotungstic acid. However paper chromatographic analysis of the autoclaved mixture revealed that some degradation had occurred. Duplicate chromatogams developed with 1-butanol : acetic acid : H_2O (100 : 21 : 50) revealed five ninhydrin positive spots (R_f 0.12, 0.18, 0.30, 0.41, 0.56), only one of which (R_f 0.18) appeared to be a sulfonium compound. Sulfonium compounds were detected by spraying the chromatograms with a methanolic solution of ammonium reineckate, flushing the papers with water to remove excess reineckate, and, after drying, detecting the sulfonium-reineckate complexes by their quenching of ultraviolet irradiation.

As expected (54) the sulfonium group is labile to alkali. Merely warming a solution of II in 0.5 N NaOH on a steam bath for four hours completely destroyed any ability to precipitate with phosphotungstic acid. A foul-cabbage odor was evolved during the hydrolysis.

Subsequently, a solution containing 1 mmole per ml. of 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 by tubing to a series of three traps containing saturated aqueous $HgCl_2$ (54). Nitrogen was bubbled through the solution and the traps during the gradual heating. At 90° C. the nitrogen swept over a gas which formed a solid $HgCl_2$ adduct in the first trap. The gas was

identified as CH_3SH by its foul-cabbage odor (Beilstein) and by the C:S:H ratio (1:1:3)[1] and the very high melting point (higher than 300° C.) of the $HgCl_2$ adduct (24). After a few minutes no more CH_3SH was evolved and the first $HgCl_2$ 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 refluxed and swept with nitrogen at 105° C. for 15 hours. No lead precipitate formed, but a compound accumulated in the second trap (saturated aqueous $HgCl_2$) and is thought to have been CH_3-S-S-CH_3, according to the C:S ratio (1:1)[2] and melting point of its $HgCl_2$ adduct (found, 142-144° C., uncorr.; literature, 147-148° C. (67)). The CH_3SH and CH_3-S-S-CH_3 accounted for only 7% of the sulfur of the thetin although the sulfonium group was completely destroyed during the hydrolysis.

Analysis of the thetin before and after the alkaline hydrolysis revealed that the alkali had liberated a compound which was oxidized to formaldehyde by alkaline periodate. The formaldehyde was determined colorimetrically with chromotropic acid (29). If this compound were ethylene glycol, a likely hydrolytic product of the thetin (54)(25), or any other compound which yielded two moles of formaldehyde after periodate oxidation, then, from the quantitative data, a yield of 0.9 moles per mole of thetin was obtained[3]; and methionine should be present as another major hydrolytic product. On this basis, the hydrolyzed thetin solution was chromatographed, with the results shown in table 7. Methionine does appear to be a product. The other ninhydrin-positive

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[1]%C, 3.20; H, 0.79; S, 8.01

[2]%C, 1.93; H, 0.69; S, 4.83

[3]The method was standardized with ethanolamine, which yields two moles of formaldehyde upon periodate oxidation just as does ethylene glycol (81).

compound has not been identified.

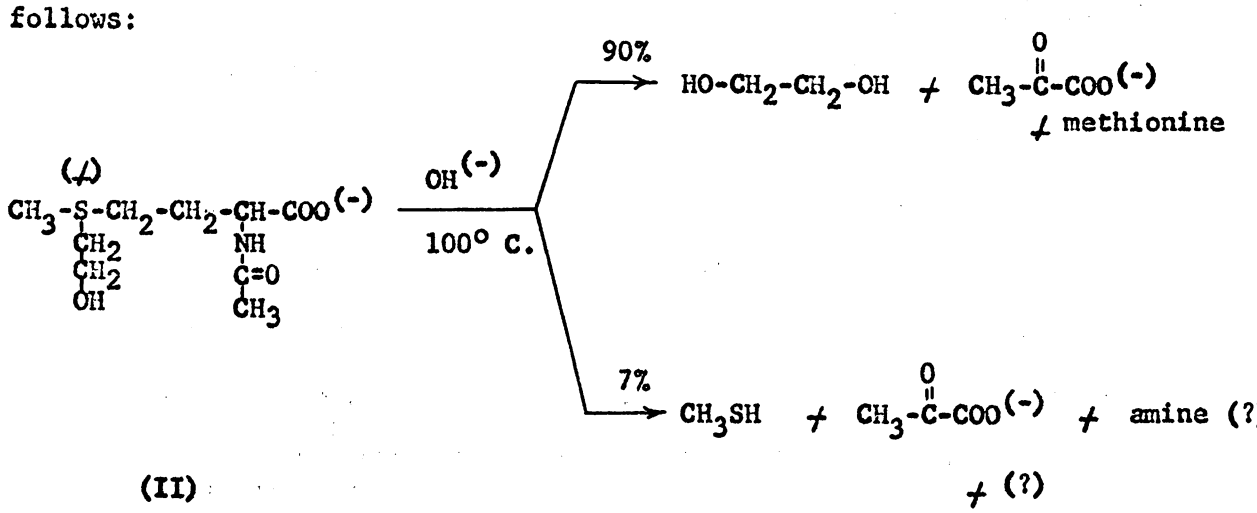
Table 7

Paper Chromatography of a NaOH Hydrolysate of S-(2-hydroxyethyl)-N-acetylmethionine, thetin

Sample	R_f of ninhydrin-positive spots[1]
S-(2-hydroxyethyl)-N-acetylmethionine, thetin (II)	(none)
DL-methionine	0.51
NaOH hydrolysate of II	0.23, 0.51
NaOH hydrolysate of II + DL-methionine	0.24, 0.52

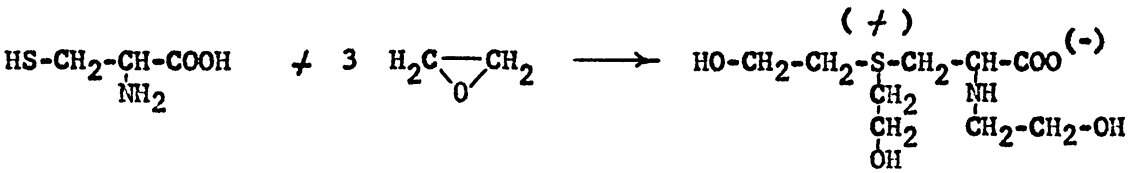
[1]Solvent, 1-butanol : acetic acid : H_2O (100 : 21 : 50); developed papers sprayed with 0.4% ninhydrin in 1-butanol and dried at 90° C. for five minutes.

The alkaline hydrolysis of II may be tentatively visualized as follows:



Cysteine hydrochloride:

The fumigation of an aqueous solution of cysteine-HCl (pH 1.0) for 24 hours produces a clear yellow color 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 90° C. in dilute NaOH. On the basis of the studies with fumigated methionine these data are taken as evidence that two moles of ethylene oxide have converted the mercapto group to a sulfonium group. The pH of the product solution suggests that the carboxyl group has not been esterified. Hydroxyethylation of amino groups was virtually complete. The following reaction is indicated, though double hydroxyethylation of the amino group is not unlikely.



Cysteine

S,S,N-tris-(2-hydroxyethyl) cysteine, thetin

A phosphotungstate adduct of the product was prepared by adding an excess of phosphotungstic acid to a solution of fumigated cysteine-HCl which had been stored under refrigeration for several months (pH 8.9). The water-insoluble adduct first precipitated as a greenish-yellow, highly-viscous syrup but it solidified on standing. Analysis gave 6.41% C, 1.13% N and 2.08% S, which indicates a N:S:C ratio of 1.00 : 0.81 : 6.64. Partial decomposition of the cysteine-ethylene oxide product with loss of a sulfur containing fragment is suggested by the low N:S ratio,

which, in the absence of decomposition would be 1:1. With the probability of some decomposition, the carbon analysis fails to provide reliable information as to the number of moles of ethylene oxide which reacted with each mole of cysteine.

Pyridine:

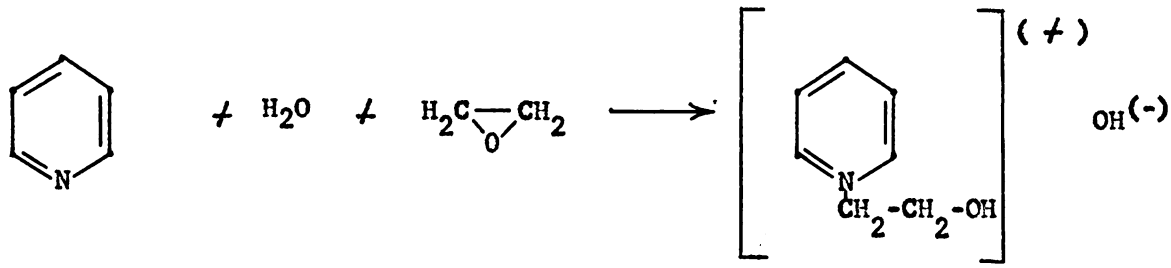
A study of the ethylene oxide-pyridine reaction was prompted by the nature of the imidazole-ethylene oxide reaction (tertiary amine group reaction) and by the realization that all of the B-vitamins found labile to ethylene oxide (9) contained in their structure one or more tertiary heterocyclic nitrogens. Pyridine was chosen as a simple molecule containing such a nitrogen and one which could easily be studied under the conditions of fumigation employed.

Reaction of ethylene oxide with pyridine has been observed by others. Lohmann (69) in 1939 reported the formation of highly colored soluble products of undetermined structure when he added ethylene oxide to pyridine or quinoline. Eastham et al (31)(32)(33) demonstrated that ethylene oxide reacts as readily with tertiary as with primary and secondary amines, and that the reaction with pyridine, in the presence of an acid, yields the N-(2-hydroxyethyl)pyridinium salt. The rate of reaction in excess pyridine was shown to be second order, being proportional to the concentration of acid and epoxide.

The following results were achieved using the fumigation technique previously described. When pyridine (110 mmoles in a 1:1 aqueous solution, pH 9.5) was exposed to ethylene oxide the clear solution became light brown after one hour, and at the end of two hours it was almost black and

quite viscous. Reaction in the vapor phase had deposited some of the dark oily product on the walls of the containing desiccator. After removal of excess ethylene oxide from the solution the pH was found to be greater than 13. Eighty mmoles of HCl were required to return the pH to 9.5. Upon acidification, the color of the pyridine product became deep yellow, the color change being freely reversible by alkali addition. A deep-red, highly-viscous syrup was deposited when the solvent was removed from the acidified reaction mixture under vacuum. It was highly soluble in water and 95% ethanol but insoluble in ethyl ether and acetone.

When pyridine (reagent grade, containing a trace of moisture) was fumigated without the addition of water similar products were obtained but the rate of reaction was observed to be much slower; no noticeable discoloration of the pyridine was seen until after four hours of fumigation. This is consistent with the observation of Knorr (61), who reported the failure of ethylene oxide to react with amines in the absence of water or ethanol as solvents. According to Eastham's formulation (32) water serves as a proton donor in the pyridine reaction.



N-(2-hydroxyethyl)pyridinium hydroxide

The high alkalinity of the product solutions resulting from the ethylene oxide fumigation of pyridine strongly suggests that formation of a quaternary pyridinium hydroxide had occurred. The instability of these compounds has been recognized (99) and no further attempt was made to purify or characterize the products of the reaction.

Nicotinamide:[1]

When an aqueous solution of nicotinamide was first fumigated with ethylene oxide no effort was made to curb the rising pH of the reaction mixture. As a result, the product isolated was not the anticipated N^1-(2-hydroxyethyl)nicotinamide, the alkali instability of which was not realized until later. Rather the product of a secondary reaction was isolated, as described below.

The ethylene oxide fumigation of 40.9 mmoles of nicotinamide in 30 ml. distilled water (pH, 7.4) for 20 hours resulted in a deep-red, strongly-alkaline solution which fluoresced under ultraviolet irradiation and evolved ammonia. The pH was adjusted back to 7.4 (no change in color observed) with 29.6 mmoles of HCl, and the solution was concentrated under vacuum to a very deep-red, highly-viscous syrup which was taken up in a minimum quantity of hot 50% ethanol. Acetone was added to the point of incipient cloudiness. A mass of fine red needles precipitated from this solution after cooling a few hours at 5° C. The product was twice recrystallized from 50% ethanol and acetone yielding 1.4 gm. of rust-colored needles, melting point 176-178° C. (uncorr.). The melting point (13) and analysis coincide closely with those of triethanolamine hydrochloride[2].

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[1] 3-carbamoylpyridine

[2] tris-(2-hydroxyethyl)amine hydrochloride

Found, %C, 39.32; H, 8.07; N, 7.60; Cl, 19.19. Calculated for $C_6H_{16}NO_3Cl$, %C, 38.80; H, 8.70; N, 7.55; Cl, 19.10. It was suspected that the product was contaminated with a trace of a red-colored impurity. This hypothesis was supported by paper chromatographic separation, with detection of the triethanolamine by spraying the developed papers with slightly acidic brom-phenol blue in 95% ethanol. The red impurity was detected by its fluorescence under ultraviolet irradiation. Authentic triethanolamine (Eastman Organic Chemicals) was chromatographed singly and together with the reaction products. The results are shown in table 8.

Table 8

Paper Chromatography of Products Isolated From Ethylene Oxide-Fumigated Nicotinamide

Solvent	Spotted at origin	R_f	
		Brom-phenol blue	Fluorescence under U.V. irradiation
1-butanol saturated with H_2O	Reaction products	0.58	0.00
	Triethanolamine	0.58	(none)
	Reaction products + triethanolamine	0.58	0.00
1-propanol : H_2O (7 : 3)	Reaction products	0.74	0.15 (streak)
	Triethanolamine	0.73	(none)
	Reaction products + triethanolamine	0.73	0.15 (streak)

Two reaction sequences could account for the presence of triethanolamine: 1. the reaction of three moles of ethylene oxide per mole of NH_3, released from the amide group of nicotinamide under the strongly alkaline conditions; 2. the reaction of two moles of ethylene oxide with ethanolamine, released by the alkaline degradation of the initially formed, N^1-(2-hydroxyethyl)nicotinamide. The reaction of ethylene oxide with ammonia is well documented in the literature. And the feasibility of the second hypothesis was supported by later work.

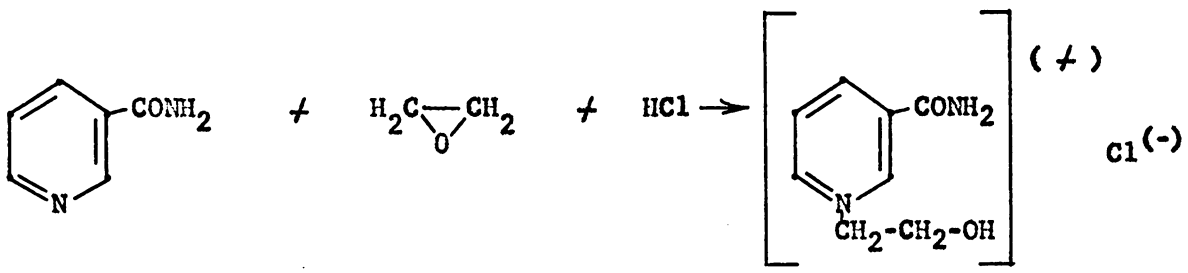
The fumigation of nicotinamide in 95% ethanol also yielded a deep-red fluorescent solution.

To eliminate the accumulation of the suspected unstable quaternary pyridinium hydroxide the pH of the reaction mixture in the next experiment was controlled by the continuous addition of HCl.

pH-controlled fumigation of nicotinamide:

A 30 ml. aqueous solution of 40.9 mmoles of nicotinamide was fumigated with ethylene oxide and the pH maintained between 7 and 10.7 by the dropwise addition of HCl. Initially the rise in pH was very rapid and acid addition was almost continuous. Inadverdent lowering of the pH to below pH 7 greatly reduced the reaction rate, as evidenced by the very slow rise in alkalinity. Above pH 7 further pH rise was again more rapid, suggesting that it is the tertiary amine base and not its acid cation which participates in the reaction. After six hours of pH-controlled fumigation further increase in alkalinity was slow and the reaction was terminated. The pale-yellow solution was decolorized with charcoal and attaclay and concentrated to a white crystalline mass, which

was twice recrystallized by dissolving in a convenient quantity of hot 95% ethanol with the dropwise addition of hot water, followed by the addition of acetone to incipient cloudiness and subsequent cooling at 5° C. Yield, 3.3 gm. (40% of theory) of white glistening platelets, melting point 195.5-196.0° C. (corr.). Found, %C, 47.76; H, 5.76; N, 14.01; Cl, 17.48. Expected for the monohydroxyethylated product, $C_8H_{11}O_2N_2Cl$ (III), %C, 47.40; H, 5.44; N, 13.81; Cl, 17.50. The following reaction was visualized:



Nicotinamide

N^1-(2-hydroxyethyl)nicotinamide chloride

(III)

An aqueous solution of III made strongly alkaline with NaOH exhibits the properties of a solution of ethylene oxide-fumigated nicotinamide in which pH was not controlled, i.e. it becomes deep-red in color, fluoresces under ultraviolet irradiation and evolves ammonia.

The compound III appears pure when chromatographed on paper and is easily detected by its strong quenching of ultraviolet irradiation. When chromatographed on Whatman No. 1 paper using the descending technique its R_f in two solvent systems was as follows: water saturated 1-butanol, 0.05; 1-propanol : H_2O (7:3), 0.39. Spots of III on paper chromatograms fluoresce strongly under ultraviolet irradiation ("Mineralite") after

having been sprayed with 1 M aqueous KCN (28)(21) or exposed to vapors of ethylmethyl ketone and ammonia (90), as is typical for quaternary nicotinyl compounds, such as the familiar N^1-methylnicotinamide.

Ethanolamine is a product of the alkaline degradation of III. Refluxing 1 mmole in 25 ml. of 25% aqueous KOH (w/w) for 14 hours produced a deep-orange solution. Vacuum distillation yielded a clear distillate which contained 0.55 mmoles of ethanolamine. Ethanolamine, the only amine detected, was identified and quantitated as previously described for the alkaline degradation of 1,3-bis-(2-hydroxyethyl)-imidazolium chloride (see page 36). The capacity of N-alkylpyridinium salts to yield the corresponding primary alkyl amines upon refluxing in strong base has been reported (79).

The complexity of products resulting from the dilute alkaline degradation of III was demonstrated by dissolving 20 mg. in 0.5 ml. of 1 N NaOH at room temperature followed by paper chromatography of the resulting red solution (1-propanol : H_2O, 7 : 3, ascending technique). Chromatograms revealed only a trace of the unchanged compound (R_f 0.22) but at least five new components; two yellow spots which fluoresced under ultraviolet irradiation, R_f 0.20, 0.36; a colorless fluorescent spot, R_f 0.61; and two spots which strongly quenched fluorescence, R_f 0.48 and 0.75. Only the R_f 0.48 spot fluoresced after exposure to vapors of ethylmethyl ketone and ammonia. A very similar array of spots was obtained by chromatographing a sample of N^1-methylnicotinamide dissolved in 1 N NaOH.

The compound N^1-(2-hydroxyethyl)nicotinamide chloride has been

previously reported in the literature (30). The compound was prepared from nicotinamide and ethylene chlorohydrin at 80-90° C. and assigned a melting point of 182° C., which is 14 degrees lower than the value found in this study. No other properties of the compound were discussed in this reference.

Concurrent studies with N^1-(2-hydroxyethyl)nicotinamide by Bakerman and co-workers:

Concurrent with the studies in our laboratory, Bakerman, Mickelsen and co-workers at the National Institutes of Health, Bethesda, Maryland have likewise studied the reaction of ethylene oxide with nicotinamide. Some important conclusions drawn from their work are summarized in the following statements (10):

1. N^1-(2-hydroxyethyl)nicotinamide chloride (III) has no nicotinamide (vitamin) activity for the chick or for the nicotinamide assay bacterium Lactobacillus arabinosus. And neither in the case of the chick nor the bacterium does the compound appear antagonistic toward active forms of the vitamin.

2. III, as well as N^1-methylnicotinamide chloride, has an ultraviolet absorption maximum at 265 mμ in water at pH 7.0. Nicotinamide absorbs maximally at 262 mμ, with a molar absorbancy index about 0.67 times as great as that of the two quaternary nicotinyl compounds above.

3. III can be assayed in solution by the fluorometric N^1-methylnicotinamide determination of Huff and Perlzweig (53), its molar fluorescence being 2.08 times that of N^1-methylnicotinamide chloride in this assay. Treatment of III with 1 N NaOH completely

destroyed its activity in this assay but did not alter its ultraviolet absorption spectrum appreciably.

4. Treatment of III with strong alkali permitted recovery of the amide nitrogen as ammonia (99% of theory).

5. III was recovered from a solution of ethylene oxide-fumigated nicotinamide by first absorbing the unreacted nicotinamide on charcoal in 2% acetic acid, followed by evaporation of the solution to dryness. At pH less than 10.5 III accumulated in amounts equivalent on a molar basis to the nicotinamide destroyed, as determined by microbial assay. III was determined fluorometrically. The same was true when nicotinamide was fumigated in a suspension of powdered cornstarch. At higher pH, III was degraded and failed to accumulate. Nicotinamide destruction was exponential up to the point of 80% destruction when plotted against time of reaction.

6. As would be expected, the nicotinamide of the pyridine nucleotides, e.g. DPN[1], is not subject to the destructive action of ethylene oxide. This was determined by microbial assay of a preparation of DPN after ethylene oxide treatment.

Nicotinic acid:

Initially nicotinic acid was solubilized as its hydrochloride and fumigated with ethylene oxide. There was no evidence of reaction with the nicotinic acid, which soon precipitated unchanged as ethylene oxide reacted with the HCl to yield ethylene chlorohydrin.

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[1]Diphosphopyridine nucleotide, or Coenzyme I

However, the fumigation of nicotinic acid as the sodium salt yielded the anticipated product. Eighty-one mmoles of nicotinic acid (sodium salt) in 40 ml. aqueous solution were fumigated with ethylene oxide, with drop-wise addition of 2 N HCl to maintain the pH between 7 and 11. At the end of seven hours 100 mmoles of acid had been consumed and further pH change was slow, apparently limited to the reaction of ethylene oxide with the chloride ion (20).

$$Cl^{(-)} + H_2C\!-\!O\!-\!CH_2 + H_2O \longrightarrow Cl\text{-}CH_2\text{-}CH_2\text{-}OH + OH^{(-)}$$

The pale-yellow solution (pH 7.0) was concentrated under vacuum and the viscous residue was taken up in absolute methanol. The NaCl which quickly precipitated was removed by filtration and a white crystalline product precipitated from the filtrate by the slow addition of acetone followed by cooling overnight at 5° C. The product was crystallized once from absolute methanol and once from 95% ethanol to yield 5.6 gm. (46% yield) of white platelets, melting point, 173° C. (d.). Found %C, 57.49; H, 5.37; N, 8.22. Expected for $C_8H_9NO_2$, %C, 57.50; H, 5.43; N, 8.38. The product gives a pH-neutral reaction in aqueous solution, decomposes in alkali to ethanolamine[1] and a deep-red colored product(s), and contains no active chloride ($AgNO_3$ test). All the data is consistent with its proposed structure as the betaine of N-(2-hydroxyethyl)nicotinic acid.

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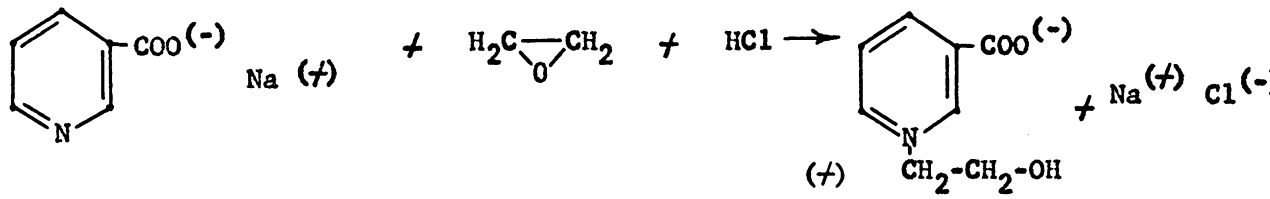
[1]alkaline hydrolysis and identification of ethanolamine as described previously, page 36 .



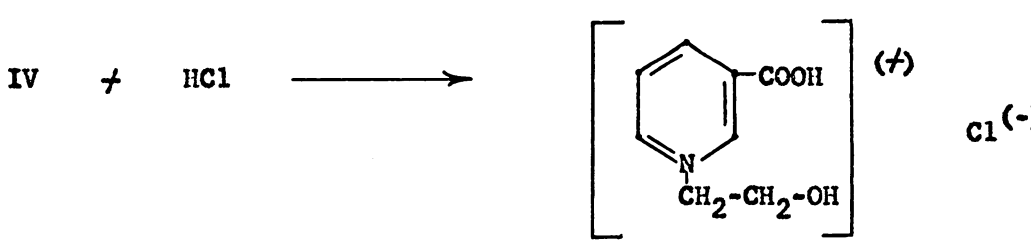
Nicotinic acid (sodium salt)

N-(2-hydroxyethyl)nicotinic acid, betaine

(IV)

A solution IV made strongly alkaline with NaOH reduces silver ions, depositing a mirror of metallic silver and indicating the presence of an aldehyde among its degradation products (98).

When an ethylene oxide-nicotinic acid reaction solution was adjusted to pH 2.5 with HCl at the end of the fumigation but before product isolation was begun, the product achieved after three recrystallizations, as described for IV, was an approximately 1:1 molar mixture of IV and its hydrochloride, V, as indicated by elemental analysis and equivalent weight determinations.



N-(2-hydroxyethyl)nicotinic acid chloride

(V)

Found[1], %C, 51.49; H, 5.28; N, 7.47; Cl, 9.77. Calculated for a 1:1

[1]The author is grateful to Dr. William C. Alford of the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, for performing this analysis.

mixture of IV and V, %C, 51.83; H, 5.16; N, 7.56; Cl, 9.56. Equivalent weight determinations, per mole of ionic chloride (93) and per mole titrable acidity, gave values of 367 and 365 respectively. The expected value of a 1:1 molar mixture of IV and V would be 371. If the mixture were assumed to contain IV and V in the ratio 0.98 : 1 then the anticipated equivalent weight per chloride or titrable acid group would be 367 and the anticipated analysis would be %C, 51.75; H, 5.16; N, 7.54; Cl, 9.69, values very close to those found. It is interesting that four crystallizations failed to separate these compounds. A crystalline structure involving both species could be visualized, particularly in light of their presence in equimolar quantities.

The following two solvent systems were found incapable of resolving a mixture of IV and V on paper chromatograms using ultraviolet quenching to detect the spots: 1-butanol saturated with water, R_f 0.05; 1-propanol : H_2O (7:3), R_f 0.37.

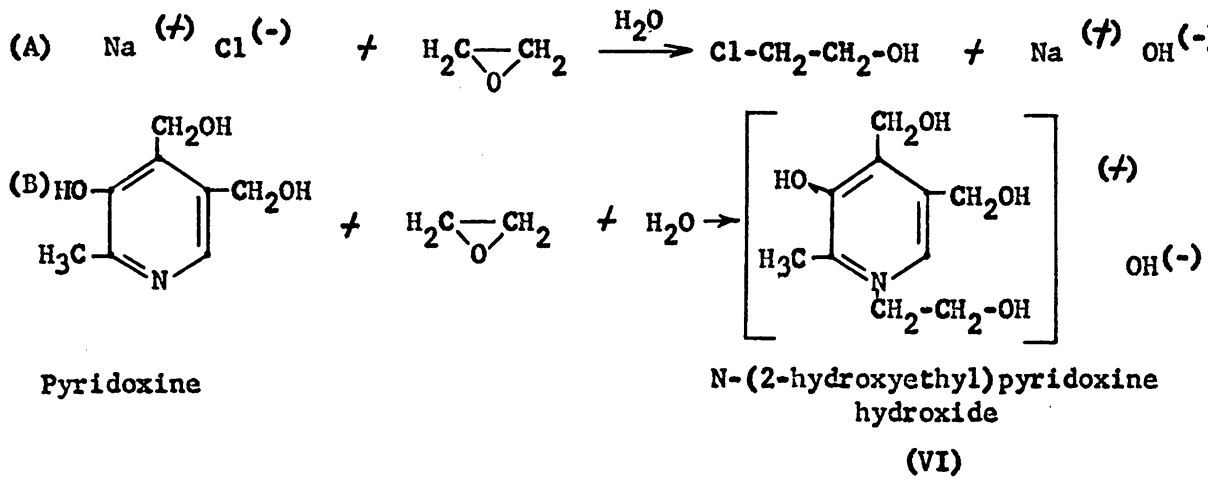
Bakerman (10), in a private communication, has reported that under most conditions of ethylene oxide treatment the vitamin activity of nicotinic acid is more rapidly destroyed than that of nicotinamide.

Pyridoxine:

The lability of vitamin B_6 (pyridoxine) to ethylene oxide fumigation (9) and the structural similarity of pyridoxine with nicotinamide prompted a more detailed investigation of the reaction of this vitamin with the fumigant.

After a solution of pyridoxine-HCl (pH 6) had been fumigated with ethylene oxide for 12 hours, the final pH was still 6 and there was no

visible evidence of any reaction. Had hydroxyethylation of the nitrogen of pyridoxine occurred, as with nicotinamide, a rise in pH would have been anticipated. From the studies with nicotinamide it was apparent that the fumigant reacts with free nicotinamide and not with its amine hydrochloride. Therefore, in the next experiment with pyridoxine, the vitamin solution was adjusted to pH 9.0 with 2 N NaOH, the solution now containing free pyridoxine and $Na^{(+)}$ and $Cl^{(-)}$ ions. This solution was then fumigated with ethylene oxide. To maintain the pH at exactly 9.0 the electrodes and delivery tip of a Beckman Model K automatic titrator were projected through the top of the fumigation desiccator and into the pyridoxine solution, and any rise in alkalinity was automatically titrated with 3 N HCl. To maintain the pH at 9.0 twelve ml. of acid (36 mmoles) were consumed during the first two hours of fumigation and 16 ml (48 mmoles) during the subsequent ten hours, at which time fumigation was terminated. This change in the rate of hydroxide ion production can be explained by assuming that initially two alkali-producing reactions were concurrently in progress, but that one of them was complete within two hours after fumigation was begun.



Pyridoxine

N-(2-hydroxyethyl)pyridoxine hydroxide

(VI)

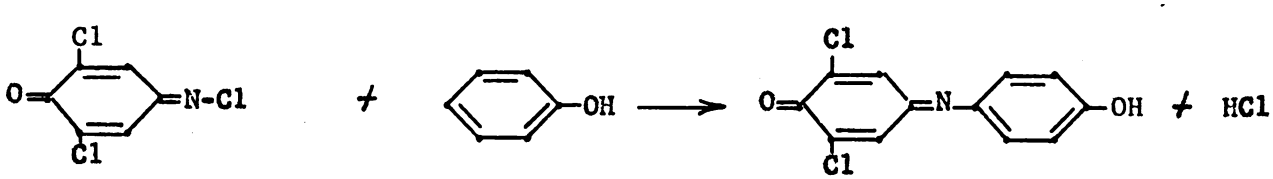
At constant pH the concentration of $Cl^{(-)}$ would be constant and therefore the rate of reaction (A) should be constant over the entire 12 hour period. Assuming that reaction (B) is complete after two hours the rate of (A) would be 48/10 or 4.8 mmoles of alkali produced per hour. So during the first two hours 2 X 4.8 or 9.6 mmoles of alkali resulted from reaction (A) leaving 36 minus 9.6 or 26.4 mmoles to come from reaction (B). This is in quite good agreement with the quantity of pyridoxine (24.4 mmoles) present, and lends support to the hypothesis that ethylene oxide combines with pyridoxine in H_2O in a reaction which consumes an equimolar quantity of protons, as in reaction (B).

After the 12 hours of fumigation the pale green reaction solution was concentrated to a viscous syrup and taken up in a small quantity of absolute ethanol. The NaCl which was precipitated (0.850 gm., 21.1 mmoles) in the ethanol was filtered and acetone added drop-wise to the pale green filtrate to the point of incipient cloudiness. On cooling a white syrup separated from this mixture. An excess of acetone was added to recover, by decanting, more of the syrup. The syrup was further purified by redissolving in absolute ethanol and reprecipitating, first with skellysolve ("Skelly B") and finally with ethyl ether. The properties of the pyridoxine-ethylene oxide product, as described below, were deduced by studying the reactions of the three-times precipitated syrup in aqueous solution.

Making an aliquot of the product solution strongly alkaline with NaOH produced no visible change. But upon boiling this alkaline solution

a deep-red color developed which became pale yellow when acidified with HCl. Adding NaOH and boiling returned the deep-red color. A solution of the product made alkaline without heat slowly became a deep green. Unfumigated pyridoxine shows none of these color changes. Refluxing the product in 25% KOH (w/w) for a few hours failed to degrade it to ethanolamine, as might have been expected on the basis of the behavior of ethylene oxide-fumigated nicotinamide and nicotinic acid. Paper chromatography of the KOH hydrolysate of the product failed to reveal any ninhydrin-positive (amine) component. The absence of ethanolamine was confirmed by the periodate-chromotropate method, described previously.

Like pyridoxine (57), the product in solution gave a positive phenol test with $FeCl_3$, evidence that none or only part of the phenolic hydroxyl groups had been alkylated by ethylene oxide. However, unlike pyridoxine (103)(45), the product failed to give a positive test with Gibb's reagent, N,2,6-trichloro-p-quinoneimine (Eastman Organic Chemicals). The test was conducted by adding a few drops of alcoholic Gibb's reagent to two ml. of the solution to be tested, then making alkaline with several drops of 2 N NaOH. Pyridoxine immediately gives a blue-green color which soon fades to a reddish-brown. According to Gibbs (39) the color is given by the coupling of the N,2,6-trichloro-p-quinoneimine and p-unsubstituted phenols.



Thus the behavior of the ethylene oxide-pyridoxine product parallels that of N-methylpyridoxine iodide which also gives a positive $FeCl_3$ test but a negative test with Gibb's reagent (58). The Gibbs test probably fails because the inductive effect of the quaternary nitrogen deactivates the adjacent ring position (para to the phenolic hydroxyl) toward electrophilic attack.

The phosphotungstate of the product was obtained by adding a solution of phosphotungstic acid to an aqueous solution of the syrupy product. The phosphotungstate was recrystallized by dissolving in a small volume of acetone and adding about 50 volumes of water. Analysis of the dried phosphotungstate revealed 11.22% C and 1.24% N, which reflects a C:N ratio of 10.6 : 1. Had only one mole of ethylene oxide reacted per mole of pyridoxine a ratio of 10:1 would have been expected. The analysis suggests that some of the pyridoxine may have been doubly hydroxyethylated.

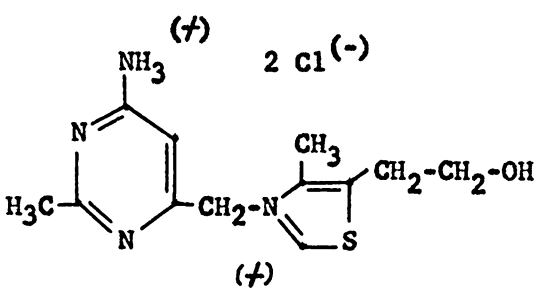
All data on the ethylene oxide-pyridoxine reaction is consistent if the major product is assumed to be VI, N-(2-hydroxyethyl)pyridoxine. Final proof, however, awaits isolation of the product in purer form.

Another equally unsuccessful attempt to purify or crystallize the product involved its precipitation from the reaction solution (pH 9.0) with methanolic ammonium reineckate. The reineckate was filtered, washed and dissolved in a small volume of acetone. The reineckate ion was precipitated by the addition of aqueous $AgNO_3$, removed by filtration, and the excess Ag precipitated from the filtrate by bubbling through H_2S for 30 minutes. The Ag_2S was filtered and the filtrate concentrated to

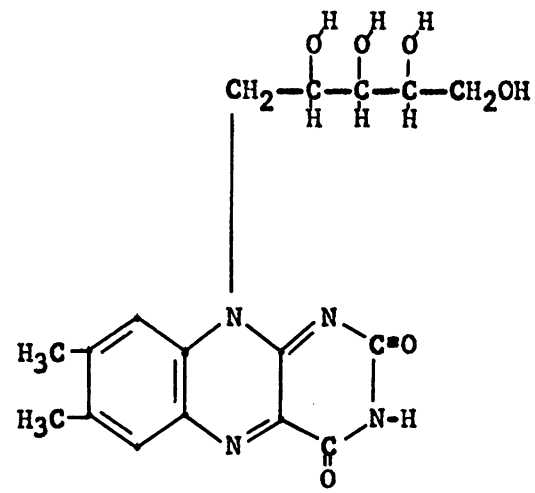
a pale green syrup. The syrup was taken up in absolute ethanol, but subsequent treatment with a variety of less polar solvents, with and without cooling, failed to induce crystallization.

Thiamine hydrochloride, riboflavin, and folic acid:

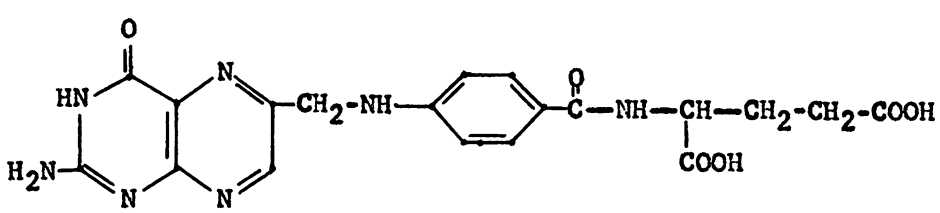
Aqueous solutions of thiamine-HCl, riboflavin and folic acid, the other three ethylene oxide-labile vitamins (9), were fumigated in a preliminary experiment to gain visual and titrimetric evidence concerning the nature of the reaction with the fumigant.



Thiamine hydrochloride



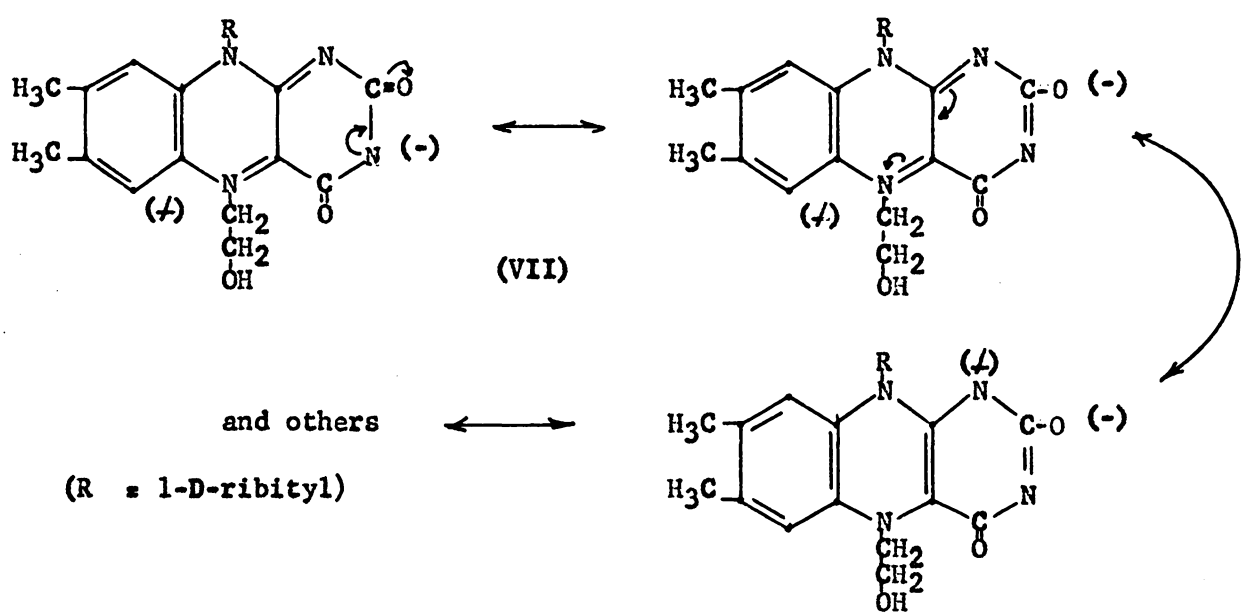
Riboflavin (6,7-dimethyl-9-(1-D-ribityl)-isoalloxazine)



Folic acid (pteroylglutamic acid)

In each case 0.5 gm. of the vitamin was dissolved in 25 ml. of water and the solution fumigated for 20 hours. The riboflavin and folic acid were brought into solution by the addition of a minimum quantity of dilute NaOH.

The ethylene oxide treatment of the riboflavin (1.33 mmoles, orange solution) yielded, after a few minutes of fumigation, a very dark brownish-red solution. No further color change was noted after 20 hours of fumigation but a small quantity of fluffy brown precipitate accumulated. The pH of the solution was 10.9 and was adjusted to pH 7 with 0.25 mmoles of HCl. The ethylene oxide reaction apparently involved the chromophoric isoalloxazine portion of the molecule. If it is assumed that one of the tertiary nitrogens were hydroxyethylated, forming a quaternary ammonium group, then failure of the reaction solution to become more alkaline could be explained by the capacity of the weakly acidic imide group to supply a proton to the reaction. A product such as the following (VII) could be visualized, this speculation admittedly being based on extremely meagre evidence and the product being only one of several hydroxyethylated structures which might be proposed.



A large number of resonance stabilized contributing structures could be drawn for such a compound and from the high degree of conjugation one might expect it to be highly colored. In any case, the nitrogens seem the most probable site of reaction with ethylene oxide.

The folic acid solution (1.13 mmoles, pH 7.0, yellow-orange in color) showed no visible change after fumigation, which raised the pH to 11.1. The pH was adjusted to 7.0 with 0.3 mmoles of HCl, at which point the solution became a very deep red. The yellow-to-red color change was freely reversible by the addition of acid or alkali, respectively, the sharp change in color occurring very near pH 7. Untreated folic acid does not exhibit any red color in acid solution. The folic acid molecule contains seven nitrogens, at least five of which, by analogy to simpler compounds, might become hydroxyethylated in the presence of ethylene oxide: the primary amino group and three tertiary heterocyclic nitrogens of the pteridine nucleus and the substituted amine nitrogen of the para-aminobenzoic acid moiety. Failure of the fumigated folic acid solution to become more alkaline can again be rationalized on the basis of the proton-donating capacity of the carboxyl groups of the molecule, which would result in betaines rather than quaternary ammonium hydroxides should the tertiary amine groups have become alkylated.

The fumigation of a thiamine-HCl solution (1.48 mmoles) produced no visible color change. The pH after the treatment was 10.5 but was adjustable to pH 7.0 with 0.1 mmoles of HCl. Adjustment of a solution of thiamine to pH 11 with NaOH before fumigation did not alter its subsequent behavior during the treatment with ethylene oxide, i.e. no highly-colored

products were observed.

Reactive sites of thiamine include the pyrimidine ring nitrogens, the primary amino group, the hydroxyl group and possibly the sulfur of the thiazole ring. Ethylene oxide-treated thiamine fails to give the thiochrome test (48); hydroxyethylation of the amino group alone could account for this since on a structural basis the formation of thiochrome would then be precluded. The importance of an ethylene oxide reaction with the hydroxyl group seems minimized for two reasons: 1. the phosphorylated ester of thiamine (cocarboxylase) is as labile to ethylene oxide fumigation as is thiamine (9); and 2. if ethylene oxide-hydroxyl group reactions were prevalent under the fumigation conditions employed, then ethylene oxide polymers of the hydroxyethylated derivatives of compounds like imidazole and nicotinamide would be expected, and they were not observed.

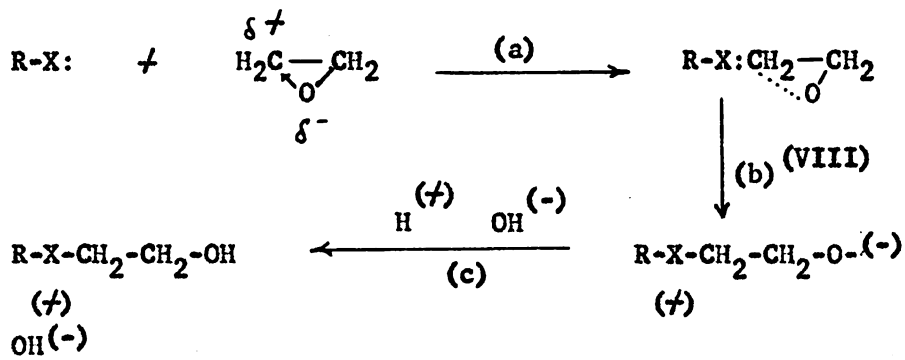
So the observed reactions of ethylene oxide with riboflavin, folic acid and thiamine are all best explained if hydroxyethylation of nitrogens is assumed in each case.

General discussion

The one conspicuous common feature in all of the ethylene-oxide-nutrient reactions studied is the capacity of this fumigant to bring about electrophilic hydroxyethylation under very mild conditions, i.e. 25° C. and atmospheric pressure. Destruction of all of the nutrients can be accounted for by hydroxyethylation of primary amino groups, heterocyclic tertiary nitrogens, mercapto groups or sulfide groups.

Direct evidence for such reactions was obtained in the case of histidine, cysteine, methionine, nicotinamide, nicotinic acid, and pyridoxine, and such reactions are implied from studies with fumigated riboflavin and folic acid. In each case hydroxyethylation occurs on an atom with one or more lone pair of electrons, either a nitrogen or a sulfur. And the products are, in most cases, strongly polar due to the formation of an "onium" pole.

In brief, the reaction is visualized as follows, where X is a sulfide-sulfur or a tertiary heterocyclic nitrogen:



(X) (IX)

The relative electronegativity of the oxygen (85) of ethylene oxide results in a partial polarization of the C-O bonds, leaving the carbons with a partial positive charge and rendering them electrophilic. Nitrogen and sulfur with their lone pair(s) of electrons are nucleophilic. The strain of the three-membered ring and the consequent lower energy level of the linear structures IX and X as compared with VIII lends driving force to the hydroxyethylation reaction.

The alkylating potential of ethylene oxide is in many ways similar to that of the nitrogen and sulfur mustards. Sulfur mustard, bis-(2-chloroethyl)sulfide, for example, also reacts with the thioether group of methionine to create a sulfonium pole (102), and the reaction of a series of nitrogen mustards[1] with the thioether group of methionine and the imidazole group of histidine has been reported (38).

The apparent ease of hydroxyethylation of heterocyclic tertiary nitrogens, e.g. in pyridine, makes the reaction of ethylene oxide with purines and pyrimidines seem very likely. Such reactions involving nucleic acids could explain, possibly, the mutagenic activity of ethylene oxide (15)(111), as mentioned earlier. That sulfur and nitrogen mustards can alkylate purines and pyrimidines by reaction with the ring nitrogens is indicated by the work of Wheeler and co-workers (109). The reaction of ethylene oxide with tertiary heterocyclic nitrogen requires the presence of an available proton, which may help to explain the stimulating effect of moisture on the effectiveness of ethylene oxide fumigation procedures (86) and on the rate of destruction of nutrients in the fumigation of animal diets (78) and proteins (see results, chapter III). As Phillips and Kaye have already proposed (86), the solvating action of the moisture is also probably a factor.

That the 1,3-bis(2-hydroxyethyl)histidine has no biological value for the rat, as shown by the rat-feeding experiments and microbiological assays, would have been anticipated from the report by Sakami and Wilson (94) that 1-methylhistidine is inert as a histidine substitute in this

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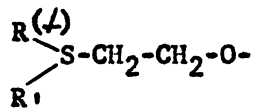
[1]methyl-[bis-(2-chloroethyl)]-amine; ethyl-[bis-(2-chloroethyl)]-amine; and tris-(2-chloroethyl)amine

species. The apparent inertness of the methyl-(2-hydroxyethyl)sulfonium of methionine is somewhat more novel considering the report by Bennett (14) that the dimethylsulfonium derivative will replace methionine for rats grown on a methionine deficient diet. Also in certain strains of Eschericia coli, Lactobacillus arabinosus, and Lactobacillus casei the methylsulfonium derivative of methionine was more effective than methionine in reversing the toxicity of sulfonamides and in promoting growth (76). The ethylsulfonium derivative of ethionine, however, competitively prevented the formation of methionine from its methylsulfonium derivative in E. coli (76). The methylsulfonium of methionine was also found capable of replacing methionine as a source of sulfur for bacteria of the genus Proteus (77) and as a methyl donor in the biosynthesis of creatine in rat liver slices (44) but not in rat liver homogenates (27). Interest in sulfonium forms of methionine has been stimulated by the finding that a sulfonium addition product of this amino acid and adenosine is the biologically active intermediate in transmethylations in which the methionine is involved (23).

The toxicity of subcutaneous injections of ethylene oxide-treated cysteine-HCl in the rat has been previously reported by the author (114). Now, with more information about the probable structure of the product, a relationship is suggested between this compound and a series of synthetic sulfoniums (89)(101) which act as spasmolytics, presumably by virtue of their antagonism to acetylcholine. This relationship receives some support from the observation that the toxic effect of the cysteine derivative does appear to be mediated through the central nervous system.

Death was preceded by a generalized flaccid paralysis and respiratory failure. The ethylene oxide-cysteine product and the toxic sulfoniums (89)(101) have the following structure in common:



A relationship to acetylcholine is readily apparent. The product of the ethylene oxide treatment of methionine did not induce any observed toxic symptoms when injected into two rats at 2.5 times the LD_{50} of the cysteine-ethylene oxide product.

The strong polarity of the ethylene oxide-amino acid products may help to explain the isoelectric and electrophoretic shifts of ethylene oxide-treated proteins, as observed by Fraenkal-Conrat (35), whose methodology could not detect the specific reactions with the imidazole of histidine or the thioether group of methionine. From theoretical considerations, the involvement of imidazole group quaternization in this shift has already been suggested by Alexander (3).

Little has been said about lysine, the third essential amino acid that is partially destroyed (from the biological point of view) in ethylene oxide-fumigated proteins. On the basis of the work of Fraenkal-Conrat (35) and others (72)(71), as well as from the results reported here, it is assumed that hydroxyethylation of one or both of its available amino groups can account for the observed destruction.

In establishing the conditions of reaction in these studies no effort was made to duplicate any of the varied ethylene oxide fumigation

procedures used by the food industry, except in so far as only low temperatures were employed. It is felt, however, that qualitatively the same reactions could occur during commercial fumigations. In the case of nicotinamide, the identical product is achieved whether the vitamin is fumigated in aqueous solution or in a powered cornstarch suspension (10). Thus far, the only report of nutrient destruction as a result of a commercial fumigation procedure has been that of Oser and Hall (84), who found only slight destruction of a group of B-vitamins. The low level of nicotinamide destruction in their samples (yeast and a natural rat diet) might be accounted for by the large proportion of this vitamin bound in the chemically-protected pyridine nucleotide form. In any case, the effect of existing ethylene oxide fumigation procedures on the nutritive value of food products would seem to bear further investigation.

V. REACTION OF NATIVE CHYMOTRYPSIN WITH ETHYLENE OXIDE

All the previous ethylene oxide-protein experiments reported in this study have involved the use of denatured, feed-grade, non-crystalline preparations of proteins. These experiments have revealed for the first time that the imidazole group of histidine and the thioether group of methionine may become hydroxyethylated in the presence of ethylene oxide. Consequently, evidence was sought that these same reactions could occur when highly purified native (undenatured) proteins were exposed to this reagent. The use of an enzyme protein in which either histidine imidazole or methionine thioether are directly involved in the enzymatic reaction

presented itself as one approach to this problem.

Only two literature reports were found which gave data on the ethylene oxide treatment of enzymatic proteins. Maekawa and Tsumura (71) treated trypsin in 14 ml. of pH 8.0 buffer containing 64 mg. crystalline enzyme and 148 mg. ethylene oxide. After 12 hours at 5° C. recovery of primary amino groups was 99.5% and recovery of proteolytic activity was 100%. After four days, recovery values were 96.0% and 49.3% respectively and after 12 days 94.7% and 41.2% respectively. Adding 50 mg. of magnesium ions (as $MgCl_2$) to the ethylene oxide-trypsin solution enhanced the reaction with the amino groups but had a questionable effect on destruction of proteolytic activity; after 12 days only 8.6% of the amino groups could be measured (ninhydrin) and proteolytic activity was reduced to 50.5% of the untreated control. On the basis of these data the authors felt unable to establish any relationship between ethylene oxide treatment and proteolytic activity of this enzyme.

Maekawa and Kushibe (72) have treated lysozyme in 16 ml. pH 8.7 buffer containing 64.2 mg. crystalline enzyme and 300 mg. ethylene oxide. After 12 hours at 5° C. recovery of primary amino groups was 100% and recovery of lytic activity was 80%. After 7 days these values were reduced to 76% and 7% respectively. However, adding 100 mg. of magnesium ions to the lysozyme-ethylene oxide solution enhanced the disappearance of the amino groups (none recovered after 7 days) but protected lytic activity (100% recovered after 7 days). After the 7 days of treatment 12 moles of ethylene oxide had been bound by the enzyme. A two-fold role for Mg^{++} was postulated: 1. protection of the enzyme

from denaturation, and 2. catalysis of amine hydroxyethylation by ethylene oxide.

For the studies reported herein chymotrypsin was the enzyme of choice because of its availability in crystalline form and because data from a number of sources suggests that the imidazole group of one or both of the histidine residues of this enzyme participate in its proteolytic as well as esterolytic activity. Fortunately, for the purpose of these studies, neither free primary amino groups (100) nor carboxyl groups (40) on the enzyme are paramount for enzymatic activity, so the reaction of ethylene oxide with these would not be expected to interfere. Besides imidazole, the phenolic group of tyrosine has been implicated in chymotryptic activity (100).

The available evidence for imidazole involvement in activity is indirect but quite conclusive. The photooxidation of chymotrypsin in the presence of methylene blue destroyed all enzymatic activity after four moles of oxygen had been consumed per mole chymotrypsin with concomitant oxidation of one of the two moles of histidine and three of the seven moles of tryptophan per mole of enzyme (107). The histidine oxidation was believed to be responsible for destruction of the enzymatic activity. The activity of chymotrypsin can be likewise destroyed by treatment with certain organic phosphorus compounds, such as DFP[1]. Photooxidized chymotrypsin was, however, incapable of reacting with DFP, suggesting a similarity in sites of reaction by the two treatments (107).

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[1]Abbreviation used in this chapter; DFP, diisopropylfluorophosphate; DP-, diisopropylphosphoryl radical; NPA, p-nitrophenyl acetate; PNP, p-nitrophenol; TCA, trichloroacetic acid

The optimum pH for chymotrypsin activity as well as for its reaction with DFP is about pH 8.0, with half maximal activity and DFP reactivity at pH 6.6 (80). The pK for dissociation of the imidazole group but no other protein functional group is also at pH 6.6. Similar pH optimum data for the chymotryptic hydrolysis of acetyl-L-phenylalanine ethyl ester also implicate imidazole as a functional enzyme site (43).

The reaction of chymotrypsin with DFP can be completely prevented by previously treating the enzyme with the fluorescent dye, 1-dimethylamino naphthalene-5-sulfonyl chloride (47). Chymotrypsin combines stoichiometrically with this dye in equimolar quantities. When dye-labeled amino acids were prepared and their properties studied compounds containing the dye-labeled imidazole ring bore the closest resemblance to the dye-labeled chymotrypsin (47).

An artificial substrate that has been found useful for studying the mechanism of action of chymotrypsin is p-nitrophenyl acetate (NPA), a colorless ester that is hydrolyzed to the orange-colored p-nitrophenol (PNP). The hydrolysis of NPA by chymotrypsin occurs in two steps (46). Upon mixing the enzyme and substrate there is an initial immediate release of one mole of PNP per mole of chymotrypsin. This is followed by a slower linear release of PNP, the rate of release being proportional to the concentration of chymotrypsin. Hartley and Kilby (46) visualize the sequence as follows:

$$\text{Chymotrypsin-H} + \underset{\text{(NPA)}}{\text{p-NO}_2\text{-C}_6\text{H}_4\text{-O-CO-CH}_3} \xrightarrow{K_1} \text{Chymotrypsin-CO-CH}_3 + \underset{\text{(PNP)}}{\text{p-NO}_2\text{-C}_6\text{H}_4\text{-OH}}$$

$$\text{Chymotrypsin-CO-CH}_3 \xrightarrow[H_2O]{K_2} \text{Chymotrypsin-H} + \text{CH}_3\text{COOH}$$

K_2 is very small as compared with K_1, where K represents the reaction rate constant. DP-chymotrypsin (chymotrypsin inhibited by reaction with an equimolar amount of DFP) hydrolyzes NPA only slowly, without any initial stoichiometric burst of PNP release. The slow rate is about 5% as rapid as the linear release of PNP by uninhibited chymotrypsin. The non-enzymatic hydrolysis of NPA by histidine and related compounds has been reported by Brecher and Balls (19). When NPA was hydrolyzed by N-benzoylhistidine methyl ester the products of the reaction were PNP and N-benzoyl-1 (or 3)-acetylhistidine methyl ester, indicating acetylation of one of the imidazole nitrogens.

Gutfreund and Sturtevant (42) have obtained kinetic data on the chymotryptic hydrolysis of NPA. Theyconcluded that the reaction occurs in three steps: 1. rapid absorption of substrate by enzyme; 2. rapid acylation of the enzyme, probably at the serine hydroxyl, with release of PNP; 3. slow release of the acetyl group and regeneration of the free enzyme, the step which, on the basis of pH dependence, appears to involve the imidazole group of histidine.

Support for the theory of Gutfreund and Sturtevant that the serine hydroxyl group is the initial recipient for the acetyl group came when Oosterban et al (82) were able to isolate from partially hydrolyzed DP-chymotrypsin two peptides containing DP-serine (O-phosphorylated).

And so it is not yet clear what role the one active imidazole group of chymotrypsin plays in the hydrolysis of NPA or in other esterolytic

and proteolytic reactions, whether it is the initial receiver of one of the cleavage products or whether it functions at some later stage of the hydrolysis. That it plays a vital role seems unquestionable and that the nitrogens of the imidazole are involved appears hardly a matter of conjecture. On this basis if the treatment of chymotrypsin with ethylene oxide were to reduce either its capacity to rapidly liberate equimolar amounts of PNP in the presence of NPA, or if the subsequent rate of PNP release were diminished this would constitute some evidence for an ethylene oxide-imidazole reaction in the native protein. If the proteolytic activity of the enzyme were diminished but not the capacity to hydrolyze NPA then this would be evidence for protein reaction but of a less specific nature, since the requirements for protein hydrolysis have not been as rigidly established.

Materials and Methods

Proteolytic activity was determined by a modification of the method of Kunitz (64). A casein substrate was prepared as follows: 25 gm. of vitamin-free test casein (Nutritional Biochemicals Corp.) was stirred into N/10 K_2HPO_4 and solution effected by adding a few ml. of 5 N NaOH. The solution was heated in a boiling water bath for 30 minutes, adjusted to pH 8.6 with 50% HCl, filtered, and stored at 2.5° C. until used. For assay of proteolytic activity, 1 ml. of appropriately diluted chymotrypsin solution was added to a series of tubes containing 5 ml. of casein substrate, previously warmed to incubation temperature (30° or 37° C.). Incubation was continued in a constant temperature water bath and at

various time intervals the reactions were stopped by the addition of 5 ml. of 10% aqueous (w/w) trichloroacetic acid (TCA). The TCA-insoluble protein was filtered, one to three ml. of the filtrate adjusted to 25 ml. with water and the absorbancy determined at 280 mμ in a Beckman Model DU spectrophotometer against a blank prepared by adding the chymotrypsin and TCA in rapid succession to a 5 ml. portion of substrate. In essence this procedure measures the rate of enzymatic release of tyrosine and tryptophan and TCA-soluble peptides containing these amino acids.

The rate of NPA hydrolysis was determined by slight modifications of the method of Hartley and Kilby (46). Details of the method are listed in table 9.

Histidine determinations of chymotrypsin hydrolysates were conducted by the method of McPhereson (75). The chymotrypsin samples were hydrolyzed in 2.5 N HCl for 12 hours at 121° C., the pH of the hydrolysates adjusted to about pH 7 with NaOH and the volume adjusted to 25 ml. with H_2O. One to five ml. aliquots containing 0.1 to 0.7 μmoles of histidine were used in the determination, standardized with DL-histidine-HCl.

Treatment of the chymotrypsin with ethylene oxide was conducted by one of two methods: 1. fumigation of the crystalline enzyme at room temperature in a desiccator, as described previously for the treatment of other proteins (aliquots of less than 100 mg. of the crystalline enzyme were placed in the desiccator in a small open beaker) (page 14); 2. addition of chilled liquid ethylene oxide to an aqueous or buffer solution of chymotrypsin. The additions of ethylene oxide were made from

a chilled 10 ml. burette. The temperature of the ethylene oxide was about -5° C. The ethylene oxide-chymotrypsin solutions were kept tightly stoppered and maintained at the temperatures indicated, either in a refrigerator or in a constant temperature water bath.

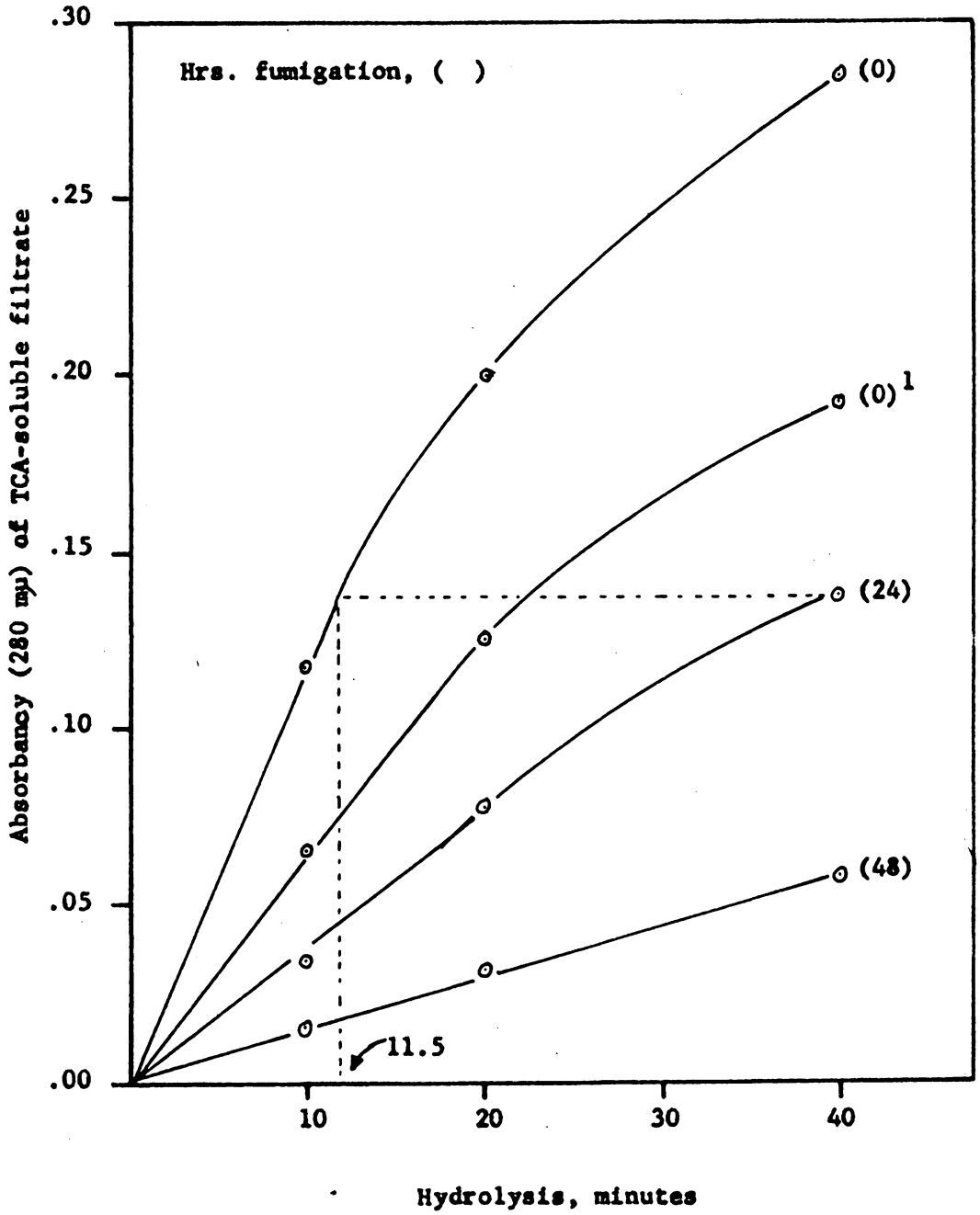
NPA was prepared from PNP and acetic anhydride by the method of Kaufmann (56). PNP was prepared from its sodium salt (Eastman Organic Chemicals) by neutralizing an aqueous solution with HCl, extracting into ethyl ether, drying the ether solution with Na_2SO_4 and evaporating the solvent. Melting point of NPA, found 80°-81° C. (uncorr.); literature (Beilstein), 81°-82° C.

Chymotrypsin (salt-free from ethanol, 6700 units per ml., prepared as described by Kunitz (65)) was purchased from Nutritional Biochemicals Corp.. Liquid ethylene oxide was from Eastman Organic Chemicals.

Results and Discussion

Effect of ethylene oxide fumigation of crystalline chymotrypsin on its proteolytic activity:

Figure 9 shows the relative rate of casein hydrolysis by chymotrypsin before and after fumigation of the crystalline enzyme with ethylene oxide vapor at room temperature for 24 and 48 hours. 6.5 mg. aliquots of chymotrypsin were fumigated simultaneously and removed from the fumigation desiccator to terminate the treatment. They were solubilized in 10 ml. of water and 1 ml. aliquots used for determination of the proteolytic activity, as previously described. The unfumigated sample was exposed to the same temperature and solvent treatment, only in the



[1]Using one-half the enzyme concentration of the other three determinations

EFFECT OF ETHYLENE OXIDE FUMIGATION OF CRYSTALLINE CHYMOTRYPSIN ON ITS RATE OF CASEIN HYDROLYSIS

Figure 9

absence of ethylene oxide.

The activity of a one-half concentration of the non-fumigated enzyme was determined to insure that enzyme concentration was a limiting factor in determining the rate of hydrolysis and to check the validity of the $[E] \times t = K$ relationship for this enzyme, i.e. whether for a given amount of hydrolysis the product of enzyme concentration, $[E]$, and hydrolysis time, t, was a constant. As indicated by the curves in figure 9 both conditions were met. Using one-half the enzyme concentration, approximately twice the hydrolysis time was required to achieve the same absorbancy value.[1] This relationship was then used to estimate the chymotrypsin activity of the ethylene oxide fumigated samples, $[E]_{ETO}$. Considering the absorbancy after 40 minutes hydrolysis by the fumigated chymotrypsin, $[E]_{ETO} \times 40 = [E] \times t$, where t is the time required for the unfumigated sample to achieve the same extent of hydrolysis. Setting [E] at 100, the percent activity remaining in the fumigated sample could be calculated.[2]

After 24 hours of fumigation 28.8% of the chymotrypsin activity remained and after 48 hours only 12.5% remained. Fumigation with the ethylene oxide produced no change in the gross appearance of the chymotrypsin crystals nor any visible change in their subsequent solubility. No other chemical or physical measurements were made with the fumigated crystals.

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[1]The one-half enzyme concentration curve shown in figure 9 is one of three obtained in different experiments to confirm the $[E] \times t = K$ relationship.

[2]Sample calculation: from figure 9, the absorbancy after 40 minutes hydrolysis by 24 hour-fumigated chymotrypsin was 0.1375, the same absorbancy achieved by the unfumigated sample in 11.5 minutes.

$[E] \times t = [E]_{ETO} \times t_{ETO}$; 100×11.5 min. $= [E]_{ETO} \times 40$ min.

$[E]_{ETO} = 28.8\%$ (remaining activity)

Effect of ethylene oxide treatment of aqueous chymotrypsin on its capacity to hydrolyze p-nitrophenyl acetate and casein:

To permit the conversion of absorbancy values to PNP concentration under the experimental conditions used, the absorbancy of a series of solutions containing from 1.013×10^{-5} M to 1.012×10^{-4} M PNP in 0.066 M phosphate buffer, pH 7.6, containing 5% 2-propanol was determined. Within this concentration range, which brackets all the values determined in this study, PNP concentration was found to be proportional to absorbancy according to the relationship, mmoles PNP/ml. = absorbancy $\times 8.05 \times 10^{-5}$.

Table 9 summarizes the results when 40 mg. samples of chymotrypsin were treated with varying amounts of ethylene oxide in two different solvents and at two temperatures. Only the most drastic of the treatments, i.e. the use of a 9:1 (v/v) water:ethylene oxide solvent for 24 hours at 30° C. altered the capacity of chymotrypsin to hydrolyze NPA. And under these conditions, and none of the others, the chymotrypsin solution became turbid, suggesting protein denaturation. Denaturation alone, rather than any specific reaction by the ethylene oxide, may have caused the diminution in NPA-hydrolysis activity. It is interesting that the extent of initial PNP release and subsequent slow release were not inhibited proportionally. However, the turbidity of this chymotrypsin solution interfered with accurate spectrophotometric results and therefore drawing further inferences from the lack of proportionality would be dangerous. The values 64.7 and 35.7 for percent remaining activity are conservative, since turbidity tends to increase the absorbance of light.

Table 9

Effect of Ethylene Oxide Treatment of Aqueous Chymotrypsin on Its Capacity to Hydrolyze p-Nitrophenyl Acetate and Casein

Sample[1] No.	Solvent (ml.)		Ethylene oxide (ml.)	Reaction temp. (°C.)	Reaction time (hrs.)	% Original Activity[3]		
						NPA hydrolysis		Casein hydrolysis[7]
	H_2O	$PO_4^{\equiv}$ Buffer[2]				initial PNP release[4,6]	slow, linear PNP release[4,5]	
1a		9.9	0.1	2.5	2	100	100	
b		9.9	0.1	2.5	25	100	100	
2a		9.9	0.1	30	2	100	100	
b		9.9	0.1	30	10	100	100	
3a	9.9		0.1	30	2	100	100	
b	9.9		0.1	30	10	100	100	83.8
4a	9.0		1.0	30	5	100	100	57.5
b	9.0		1.0	30	24	64.7	35.7	13.8

1. 40 mg. chymotrypsin per sample; final concentration during assay was 0.8 mg. chymotrypsin per ml.

2. 0.066 M phosphate buffer, pH 7.6

3. compared with chymotrypsin sample handled similarly, only the ethylene oxide was replaced by solvent

4. 1 ml. enzyme solution added to 4 ml. of substrate (room temperature) and the absorbancy read at 400 mμ in a 1 cm. cell in a Beckman Model DU spectrophotometer against a blank containing 4 ml. substrate + 1 ml. H_2O (or buffer). Substrate prepared by dissolving 23.12 mg. NPA in 10 ml. 2-propanol and just prior to assay adding 1 ml. of this 2-propanol solution to 19 ml. of 0.066 M phosphate buffer at pH 7.6. Final concentration of NPA during assay was 5.1×10^{-4} M.

5. calculated from slope of PNP-release plotted v.s. time (see figure 10)

6. by extrapolation of PNP-release v.s. time plot to zero time (see figure 10)

7. chymotrypsin samples diluted 1 to 5 with H_2O and activity of 1 ml. aliquots assayed as previously described. Three ml. of TCA-soluble filtrate diluted to 25 ml. for absorbancy determination (see figure 11)

The extent of initial PNP release and the slower linear PNP release rate of the untreated chymotrypsin and of samples 1 through 4a were remarkably consistent and are represented by curve A, figure 10. The activity of sample 4b is shown by curve B. Extrapolation of curve A to zero time indicates an initial burst of PNP release amounting to 3.4×10^{-5} mmoles per mg. chymotrypsin. If, as Hartley and Kilby (46) have indicated, chymotrypsin rapidly releases equimolar amounts to PNP then the molecular weight of chymotrypsin calculated from these data would be 29,400[1], which is in fair agreement with literature values which range from 17,000 to 40,000, with most estimates being near 22,500 (66).

The capacity to hydrolyze casein was impaired by less drastic ethylene oxide treatment of the chymotrypsin. Dissolving the enzyme in a 1% (v/v) aqueous ethylene oxide solution for 10 hours at 30° C. reduced activity to 83.8%. Using 10% (v/v) aqueous ethylene oxide the destruction of activity was much more extensive. The hydrolysis curves of samples 4a and 4b as compared to the control are shown in figure 11. The percent activity remaining after the ethylene oxide treatment was calculated as before.

From these studies it can be concluded that the requirements for casein hydrolysis are more rigid on the part of the chymotrypsin molecule than are the requirements for NPA hydrolysis. The capacity of chymotrypsin to hydrolyze NPA appears not to be labile, or only slightly labile, to the alkylating action of ethylene oxide. If imidazole groups are indeed functional in NPA hydrolysis then these groups would appear to be protected

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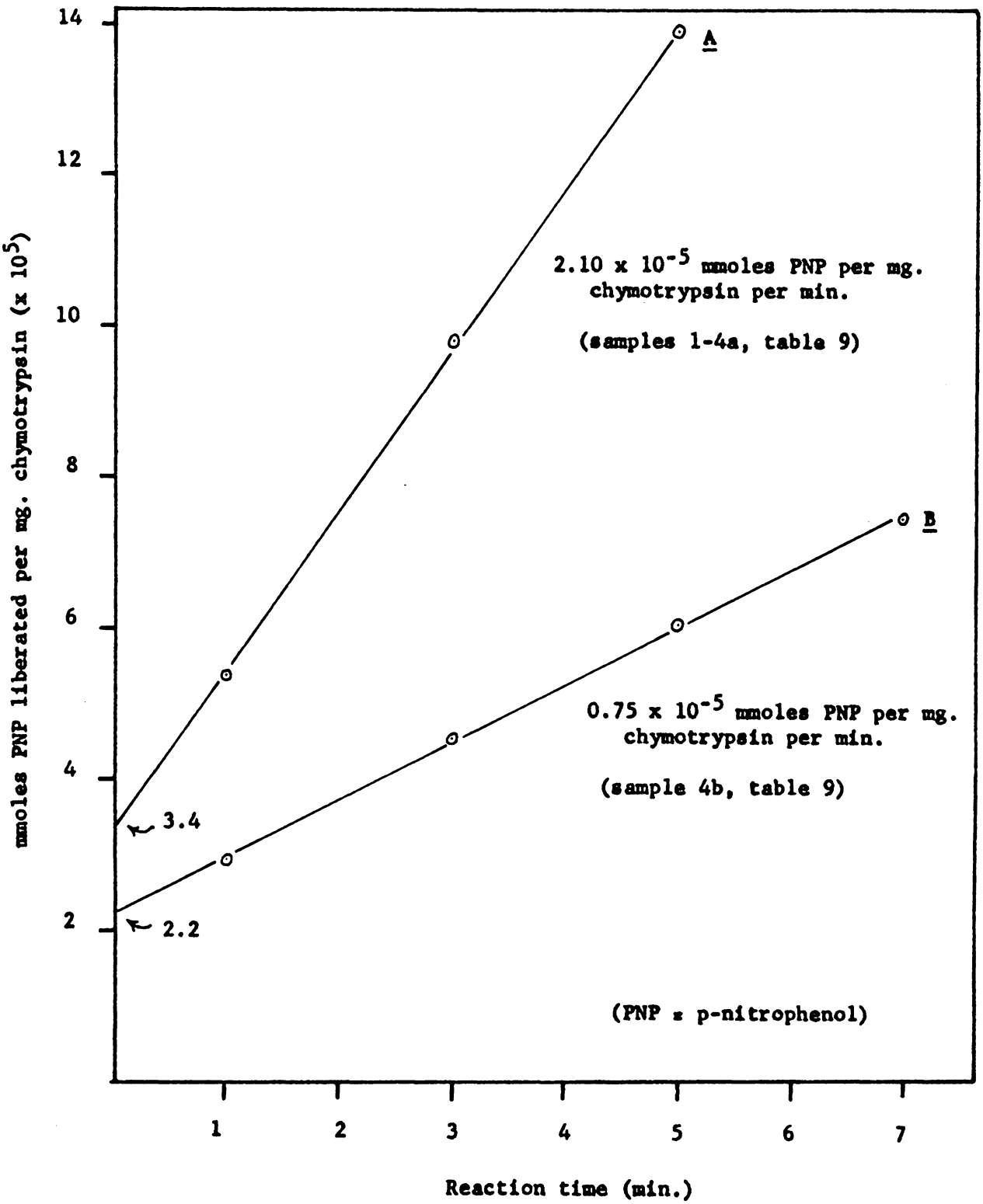
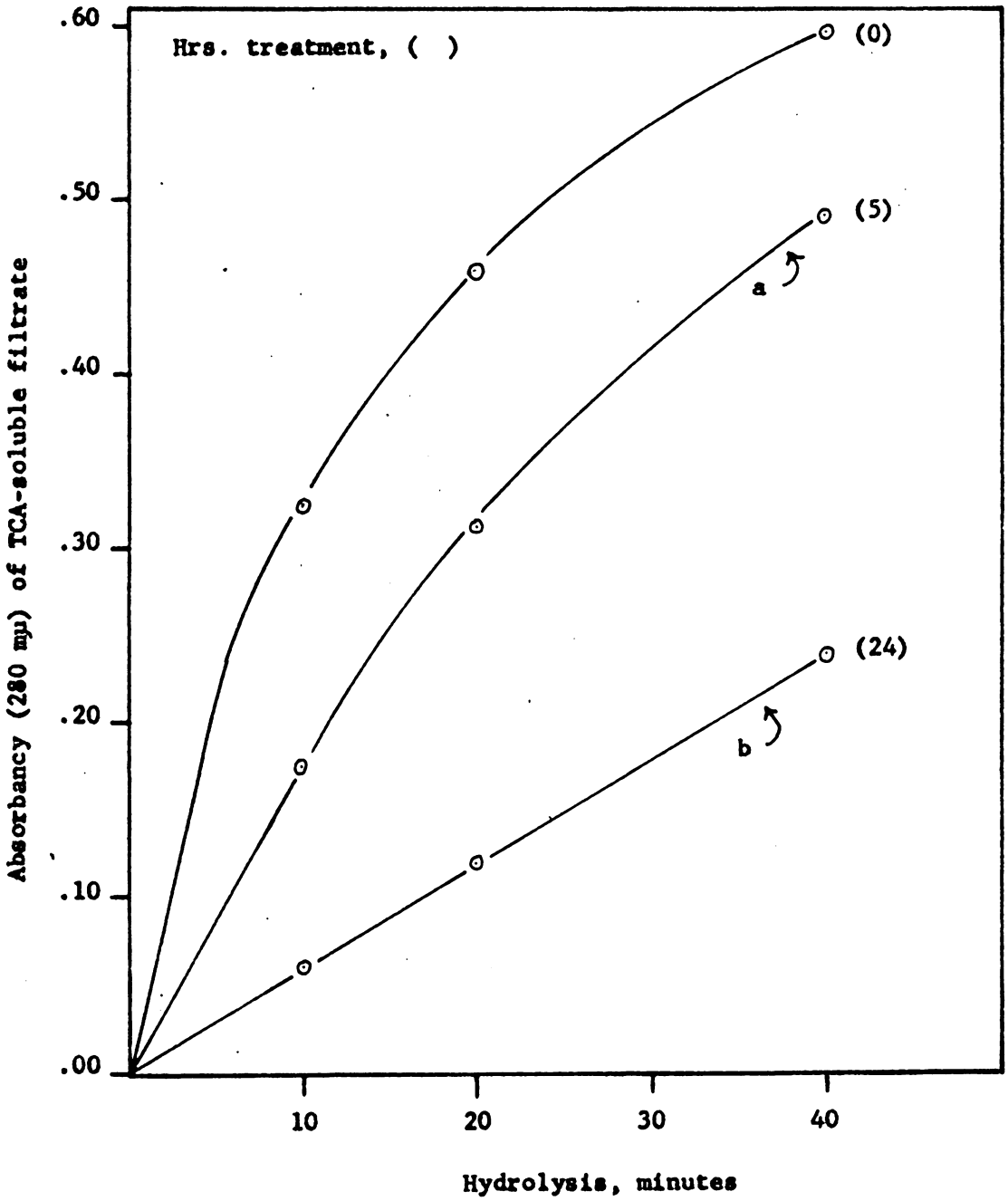
[1] $\frac{1 \text{ mg. chymotrypsin}}{3.4 \times 10^{-5} \text{ mmoles PNP}} = \frac{x \text{ mg.}}{1 \text{ mmole}}$ $\qquad x = 29,400$



A - chymotrypsin
B - chymotrypsin in ethylene oxide : H_2O (1:9 v/v), 24 hrs. at 30° C.

HYDROLYSIS OF p-NITROPHENYL ACETATE BY CHYMOTRYPSIN TREATED WITH ETHYLENE OXIDE

Figure 10



a - sample 4a, see table 9

b - sample 4b, see table 9

HYDROLYSIS OF CASEIN BY CHYMOTRYPSIN TREATED IN ETHYLENE OXIDE: WATER (1:9 v/v) at 30° C.

Figure 11

from hydroxyethylation in native chymotrypsin. The apparent correctness of this postulate was demonstrated in the following experiments.

Histidine determination of ethylene oxide-treated chymotrypsin:

The results of colorimetric histidine determinations on samples of chymotrypsin hydrolyzed with acid after various treatments with ethylene oxide are shown in table 10. Discussing the samples in order, untreated chymotrypsin (sample 1) yielded nearly three moles of histidine per mole of enzyme. From other reports (107) only two would be expected. The high value obtained can be explained by the lack of specificity of McPhereson colorimetric method in which amino acids other than histidine (imidazole) may contribute some color (51). The method is capable, however, of detecting hydroxyethylation of imidazole nitrogen by ethylene oxide, should it occur, since the altered imidazole would give no color in this assay (see page 37). The amount of color given by the assay is expressed as moles of histidine per mole of chymotrypsin for convenient comparison of the treatments.

Treating chymotrypsin in 9:1 (v/v) water:ethylene oxide for 5 hours at 30° C. (sample 2), a treatment which reduced its ability to hydrolyze casein but not NPA (see table 9, sample 4a) apparently did not result in imidazole hydroxyethylation. When chymotrypsin in 9 ml. of water was first denatured by heating, as described, the subsequent addition of 1 ml. of ethylene oxide appeared to reduce the imidazole content somewhat (sample 3). Denaturing the enzyme by autoclaving in a solution of 8 M urea, a standard denaturing solvent (37), was apparently much more effective in uncovering imidazole groups. Comparing samples 4 and 5,

Table 10

Apparent Histidine Content of Chymotrypsin Treated With Ethylene Oxide for Five Hours at 30° C. Before and After Denaturation

Sample No.	Sample	mg.	μmoles[1]	Solvent (ml.)			Histidine assayed[3]	
				H_2O	Ethylene oxide	8M urea	μmoles	moles/mole chymotrypsin
1	Chymotrypsin	40.0	1.78	10			5.21	2.93
2	"	40.0	1.78	9	1		5.58	3.14
3	" (A)[2]	40.0	1.78	9	1		4.64	2.61
4	" (A)	32.0	1.42	1		9	2.01	1.42
5	" (A)	32.0	1.42		1	9	0.66	0.46
6	L-histidine-HCl (A)	0.681	3.56	10			3.82	
7	" (A)	0.681	3.56	9	1		1.51	

[1]assuming molecular weight for chymotrypsin of 22,500

[2](A) indicates that sample was autoclaved for 30 min. at 121° C. before ethylene oxide (or 1 ml. H_2O in the case of controls) was added to the sample solution.

[3]by the colorimetric method of McPhereson (75) after treating the samples in the indicated solvents for 5 hours at 30° C., then adding conc. HCl to 2.5 N, autoclaving 12 hours at 121° C., and then adjusting to c.a. pH 7 with NaOH

approximately a 68% reduction in measurable imidazoles resulted when such denatured chymotrypsin was treated with the ethylene oxide. The histidine content of both the treated and untreated samples (4 and 5) were low because of the buffering action of the urea toward the HCl which was added to effect hydrolysis. The pH of these two solutions during "hydrolysis" was 8.5, so that these samples actually underwent little or no hydrolysis at all. The accuracy of the histidine assays of samples 4 and 5 was further reduced by the yellowish color imparted by the urea during the colorimetric histidine determination. Though inaccurate in the absolute sense, these assays strongly suggest that imidazole-ethylene oxide reaction did occur in the denatured chymotrypsin. Substantiation of this conclusion by using enzyme denatured by an agent other than urea seems advisable.

The ability of ethylene oxide to react with the imidazole of histidine-HCl under the conditions of this experiment can be seen by comparing histidine recovery from samples 6 and 7; a 58% reduction in measurable histidine resulted from the ethylene oxide treatment.

And so it seems that the question of an ethylene oxide-imidazole reaction in native chymotrypsin was answered more conclusively by direct chemical determination of the treated protein than by the somewhat more elegeant enzymatic approach, the further usefulness of which must await more detailed information on the mechanism of action of this enzyme. The results of the imidazole analyses fully corroborate the data obtained by the enzymatic approach. Ethylene oxide treatment of native chymotrypsin produces no hydroxyethylation of imidazole nitrogen nor does it reduce

the ability of the enzyme to hydrolyze NPA. The reduction in NPA hydrolysis after extended treatment with ethylene oxide, which caused visual evidence of denaturation, could have been due to the denaturation *per se*, to the eventual reaction of ethylene oxide with the denatured enzyme, or both. The accumulating amounts of ethylene glycol in the ethylene oxide-chymotrypsin solution may have participated in bringing about the denaturation.

VI. GENERAL SUMMARY AND CONCLUSIONS

The ethylene oxide-fumigation of commercial casein, egg albumin, and lactalbumin greatly reduced their ability to support the growth of weanling rats when these proteins were fed as 9% of a purified diet as the only source of amino acid nitrogen. Damage to nutritional quality was more severe when the proteins were fumigated in the presence of about 10% by weight of moisture than when they were previously dried. The nutritional quality of all three proteins could be completely restored by supplementary feeding of histidine, methionine and lysine, or, in some cases, only one or two of these amino acids. Microbiological assay of the fumigated proteins confirmed the reduction in the biological availability of these three amino acids, histidine being generally the most labile to reaction and lysine the least labile. However, neither the relative extent of destruction of the three amino acids nor the degree of apparent moisture catalysis was the same in the three proteins. Biological inavailability after fumigation amounted to 11% to 83% of the histidine, 6% to 55% of the methionine and 5% to 92% of the lysine, depending upon the protein and the percent moisture present during the fumigation. Amino acids other than histidine, methionine, and lysine appeared to be affected when an hydrolysate of casein was treated with ethylene oxide.

The ethylene oxide fumigation of soybean alpha protein improves its growth-promoting quality for the rat. Evidence is presented that fumigation inactivates the heat-labile proteinaceous growth inhibitor, long recognized as a component of soy protein.

Chemical justification for the destructive action of ethylene oxide on the biological value of histidine and methionine and certain B-vitamins was sought by reacting model compounds with the fumigant in aqueous solution and identifying the products. These studies have revealed that ethylene oxide at 25° C. and at atmospheric pressure can hydroxyethylate tertiary as well as primary and secondary amine nitrogens and can also hydroxyethylate the sulfur of thioethers. Imidazole and histidine yield the 1,3-bis-(2-hydroxyethyl)imidazolium derivatives; nicotinamide, nicotinic acid, and pyridine yield the corresponding N-(2-hydroxyethyl)pyridinium compounds; and N-acetylmethionine is converted to S-(2-hydroxyethyl)-N-acetylmethionine, thetin. The quaternary ammonium products of the imidazole and pyridine derivatives are unstable in alkali; ethanolamine is generally found as a product of degradation. The thetin derivative of methionine is also rapidly degraded in alkali. The reaction of ethylene oxide with amino acids results in hydroxyethylation of the primary amino group but no esterification of carboxyl groups was observed. Hydroxyethylated imidazole no longer forms a colored complex with diazotized sulfanilic acid. Therefore the extent of ethylene oxide-imidazole reaction in fumigated proteins can be rapidly determined by colorimetric analysis.

The mercapto group of cysteine appears to become doubly hydroxyethylated and converted into a sulfonium pole. The cysteine product causes a flaccid paralysis, respiratory failure and death when subcutaneously injected into weanling rats. Its structure bears certain similarities to other synthetic spasmolytics.

Evidence is presented that the reaction of ethylene oxide with pyridoxine, riboflavin and folic acid also involves the hydroxyethylation of tertiary heterocyclic nitrogen.

New compounds isolated and identified during these studies were 1,3-bis-(2-hydroxyethyl)imidazolium chloride; N-(2-hydroxyethyl)nicotinic acid, betaine; and N-(2-hydroxyethyl)nicotinic acid chloride. N^1-(2-hydroxyethyl)nicotinamide chloride was also obtained in crystalline form and its melting point found to be 14 degrees centigrade higher than previously reported in the literature. Data on the stability, chromatographic mobility, and detection techniques with respect to these new compounds are presented. Other products were studied only in solution or as solid adducts of reinecke's salt or phosphotungstic acid.

The protein and amino acid reactions of ethylene oxide were found to bear a striking resemblance to those of the sulfur and nitrogen mustards, a fact which may help explain the reported mutagenic action of ethylene oxide. The nature of the ethylene oxide-nutrient reactions also helps to explain the reported shift in electrophoretic mobility of ethylene oxide-fumigated proteins and the accelerating action of moisture on nutrient destruction during fumigation of food and feed constituents.

It is felt that the observed ethylene oxide-nutrient reactions could occur during commercial fumigation of food products and that the extent of nutrient destruction resulting from such processes bears further investigation.

The proteolytic activity of crystalline chymotrypsin was progressively

reduced by exposure of the crystalline enzyme to ethylene oxide vapor or by treatment of the enzyme in aqueous or buffer solution with the fumigant. However, treatment of chymotrypsin in aqueous solution with ethylene oxide did not impair its ability to hydrolyze p-nitrophenyl acetate. Likewise, the imidazole groups of native chymotrypsin appear to be protected from the hydroxyethylating capacity of ethylene oxide. Denaturing the protein in 8 M urea uncovered the imidazole groups and permitted reaction with the fumigant.

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ABSTRACT

THE REACTION OF ETHYLENE OXIDE WITH SOME PROTEINS, AMINO ACIDS AND VITAMINS

by

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A report that the ethylene oxide fumigation of animal diets reduced their nutritive value has prompted a more detailed study of the reactions of this commercially-used fumigant. It has been demonstrated that the 24-hour fumigation of commercial casein, egg albumin and lactalbumin with ethylene oxide greatly reduced the ability of these proteins to support the growth of weanling rats when the proteins were fed as 9% of a purified diet as the sole source of amino acid nitrogen. Thorough drying of the proteins reduced the extent of subsequent fumigation damage. The nutritional quality of the three proteins was completely restored by the supplementary feeding of L-histidine-HCl, DL-methionine, and L-lysine-HCl, or, in some cases, only one or two of these amino acids. Microbiological assay confirmed a reduction in the biological availability of 11% to 83% of the histidine, 6% to 55% of the methionine and 5% to 92% of the lysine, depending on the protein and the amount of moisture present during fumigation. Histidine was generally the most labile amino acid and lysine the least labile. Additional amino acids appeared to be affected when an hydrolysate of casein was fumigated.

The ethylene oxide fumigation of soybean alpha protein improves its growth-promoting quality for the rat. Evidence is presented that fumigation inactivates the heat-labile proteinaceous growth inhibitor, long recognized as a component of soybean protein.

Chemical justification for the destructive action of ethylene oxide on the biological value of histidine, methionine, and certain B-vitamins was sought by reacting model compounds in aqueous solution with the

fumigant and identifying the products. At 25° C. and atmospheric pressure ethylene oxide readily hydroxyethylates tertiary as well as primary and secondary amine nitrogen and also the sulfur of thioethers. Imidazole and histidine yield the 1,3-bis-(2-hydroxyethyl)imidazolium derivatives; nicotinamide, nicotinic acid, and pyridine yield the corresponding N-(2-hydroxyethyl)pyridinium compounds; and N-acetylmethionine is converted to S-(2-hydroxyethyl)-N-acetylmethionine, thetin. The reaction of ethylene oxide with amino acids results in hydroxyethylation of the primary amino groups but no esterification of carboxyl groups was observed. The extent of ethylene oxide-imidazole reaction in fumigated proteins could be determined colorimetrically.

In the presence of ethylene oxide the mercapto group of cysteine appears to become doubly alkylated to produce a sulfonium compound which causes a flaccid paralysis, respiratory failure and death when injected subcutaneously into weanling rats. The structure of the compound bears certain similarities to other synthetic spasmolytics. Evidence is presented that the reaction of ethylene oxide with pyridoxine, riboflavin and folic acid involves the hydroxyethylation of tertiary heterocyclic nitrogens. A striking resemblance was observed between the alkylating capacity of ethylene oxide and the sulfur and nitrogen mustards.

New compounds described are 1,3-bis-(2-hydroxyethyl)imidazolium chloride; N-(2-hydroxyethyl)nicotinic acid, betaine; and N-(2-hydroxyethyl) nicotinic acid chloride. N^1-(2-hydroxyethyl)nicotinamide chloride was also crystallized and its melting point found to be 14 degrees centigrade higher than previously reported in the literature. Other reaction products

were studied in solution or as solid adducts of reinecke's salt or phosphotungstic acid.

The proteolytic activity of chymotrypsin was progressively reduced by exposure of the cystalline enzyme to ethylene oxide vapor or by treatment of the enzyme in aqueous solution with the fumigant. However, treatment of chymotrypsin in aqueous solution with ethylene oxide did not impair its ability to hydrolyze p-nitrophenyl acetate. Likewise, the imidazole groups of native chymotrypsin appear to be protected from the hydroxyethylating action of ethylene oxide. Denaturing the protein in 8 M urea uncovered the imidazole groups and permitted reaction with the fumigant.

On the basis of these studies it is felt that the effect of existing commercial ethylene oxide fumigating processes on the nutritive value of foods and feedstuffs bears closer investigation.