microorganisms in vitro at the 0.002*M* level to determine their effect on the rate of cellulose digestion and short-chain fatty acid formation.

Alanine, α-ketoglutarate, and lactate decreased the rate of cellulose digestion, whereas the other metabolic acids that were added had no effect on the rate of cellulose digestion.

Approximately 40% of the carbon in cellulose was recovered in the short-chain fatty acids, acetic, propionic, and butyric acid, whereas approximately 50% of the carbon of glucose was recovered in these fatty acids.

All the metabolic acids except alanine that were added to the fermentation medium were metabolized to some extent to short-chain fatty acids. Added pyruvate, malonate, α-ketoglutarate, and glutamate primarily increased the formation of acetic acid. Succinate and aspartate primarily increased the formation of propionic acid, whereas lactate and malate increased the formation of both acetic and propionic acid in approximately the same proportion.

The added metabolic intermediates affect major metabolic pathways involved in the metabolism of glucose to volatile fatty acids. The major pathway of propionic acid formation in rumen bacteria involves succinate decarboxylation.

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FOOD STERILIZATION

Stability of Certain B Vitamins Exposed to Ethylene Oxide in the Presence of Choline Chloride

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Exposure to ethylene oxide of crystalline B vitamins suspended in starch with choline chloride added resulted in the destruction of practically all the thiamine and large amounts of the riboflavin, pyridoxine, niacin, and folic acid. The effect of the ethylene oxide on thiamine may be due in part to the increase in pH that occurred in the presence of choline chloride. The alkalinity may explain the destruction of the thiamine but cannot explain the destruction of niacin, riboflavin, or folic acid. The mechanisms for the latter reactions are unknown. Cocarboxylase was also destroyed under the above conditions. About 40% of the thiamine in a stock diet was destroyed following exposure to ethylene oxide. There was no destruction of pantothenic acid, biotin, or vitamin B_{12} when the vitamin mixtures were exposed to ethylene oxide.

Ethylene oxide has been used for the chemical sterilization of a number of substances including foods. Although only a few foods are currently being treated with this compound, there are suggestions that the method might be valuable for many other foods (*3*).

The current interest in ethylene oxide prompted an extension of the previous observation which indicated on the basis of rat growth studies that thiamine in purified diets was destroyed on exposure to the gas (*7*). The poor growth of the rats on the diet treated with ethylene oxide could be partially overcome by feeding extra supplements of thiamine. Furthermore, a small percentage of the rats fed the treated diet showed neuromuscular changes indicative of a thiamine deficiency.

In order to evaluate the influence of ethylene oxide upon the B vitamins in the purified diet, starch-vitamin mixtures were exposed to the gas. The individual vitamins, as well as a combination of the individual vitamins, were dispersed in a choline chloride–starch mixture and exposed to ethylene oxide. Choline chloride was included in each mixture because earlier work (*7*) showed that this compound played an important role in the destruction of thiamine whereas choline citrate appeared to be much less active.

Experimental

The vitamin mixture which was used in the purified diet at a level of 2%

contained in each gram: 0.885 gram of corn, 200 γ of thiamine hydrochloride, 2000 γ of nicotinic acid, 250 γ of pyridoxine hydrochloride, 300 γ of riboflavin, 83 γ of folic acid (pteroylglutamic acid), 1840 γ of calcium pantothenate (expressed as pantothenic acid), 8.5 γ of biotin, 10 γ of vitamin B_{12} (cyanocobalamin), 100 γ of menadione, and 100 mg. of choline chloride. This mixture will be referred to as the choline chloride–starch-multivitamin mixture. It was prepared in batches of 1 or 2 kg. To ensure uniform mixing, the vitamins were first triturated with a small amount of the cornstarch and then the entire batch was thoroughly mixed in a mortar.

(Actually, enough folic acid had been added to produce a concentration of 100 γ per gram of mixture. However, on bioassay, the folic acid was only 83% active for *Streptococcus faecalis*. The value listed above is for the active folic acid. The bioassay for biotin indicated the above potency even though 10 γ per gram has been added.)

Besides the above vitamin mixture, various combinations of vitamins suspended in starch were tested. These are referred to as the choline chloride–starch–individual vitamin mixtures. In the initial experiment, the vitamins studied were folic acid and riboflavin. Mixtures of these substances contained in each gram, 450 γ of folic acid or 550 γ of riboflavin, together with 100 mg. of chloline chloride and 899.5 mg. of cornstarch. (Folic acid and riboflavin were chosen for the initial study since ethylene oxide treatment of the vitamin mixture produced a slight orange tinge in its original pale yellow color. These two compounds, as well as vitamin K, are yellow. It was shown later, however, that the vitamin K was primarily responsible for the color change.)

Other experiments were carried out with riboflavin and folic acid, as well as with thiamine, pantothenic acid, niacin, biotin, and pyridoxine. Fifty-gram samples were prepared which contained the individual vitamins dispersed in the choline-starch mixture at levels equal (except for the preliminary trial) to those in the vitamin mixture described above.

In one experiment, the ratio of thiamine to choline chloride was altered and in another cocarboxylase was substituted on a molar basis for thiamine in the individual vitamin–starch-choline mixture. The stock diet was also assayed for thiamine before and after exposure to ethylene oxide.

One half of each sample was left untreated as a control. The portions to be treated with ethylene oxide were put into large Petri dishes and spread out so that the depth of the material was approximately 1 cm. The Petri dishes were stacked in a 10-liter desiccator (without desiccant) with glass rods placed between the plates. The small vent at the top was connected to a 2-foot length of rubber tubing to minimize diffusion losses while providing for a pressure release. The stopcock was left open throughout the entire period of ethylene oxide exposure. Ten milliliters (8.84 grams) of liquid ethylene oxide were poured down the side of the desiccator and the lid was rapidly replaced. The desiccator was left in a forced draft hood at room temperature (24° C.) for 18 hours. At the end of that time the Petri dishes were removed and exposed to air overnight to dissipate any residual gas.

Since riboflavin is light sensitive, the control sample which was not treated with ethylene oxide was left in the hood under the same conditions as the ethylene oxide–treated sample. Both samples were left in a dark cabinet during the aeration period.

The samples were prepared for microbiological assay as follows: One gram of the sample was suspended in 500 ml. of water, the pH adjusted to 6.8, the suspension stirred with a magnetic stirrer for 20 minutes, and then filtered. As a check on the completeness of extraction, the residue was re-extracted and the extract assayed. To determine whether the observed values were truly representative of the mixtures, additional aliquots were extracted and assayed. The results of these assays showed that the procedure for preparation and extraction of the samples was satisfactory.

The microbiological procedures used for the determination of niacin, pantothenic acid, and folic acid were essentially those described by Dann and Satterfield (*4*). These procedures were modified by the addition of 0.4 mg. each of pyridoxal hydrochloride and pyridoxamine dihydrochloride per liter of double strength medium. The procedure used for the determination of riboflavin was that recommended by the Association of Vitamin Chemists (*1*). The medium used for the biotin assay was that reported by Teply and Elvehjem (*13*) for the assay of folic acid. It was modified by including folic acid and omitting biotin, peptone, and DL-alanine. The method for vitamin B_{12} was that described in the Pharmacopeia (*12*).

Lactobacillus arabinosis 17-5 ATCC 8014 was used as the test organism in the pantothenic acid, niacin, and biotin assays. *Lactobacillus casei* ATCC 7469 and *Streptococcus faecalis* R ATCC 8043 were used for the riboflavin and folic acid assays, respectively.

The microbiological procedure of Atkins and coworkers (*2*) was used for the assays for pyridoxine.

Thiamine was determined by the thiochrome method as modified by Mickelsen, Condiff, and Keys (*10*).

Vitamins Destroyed by Ethylene Oxide

The stability of the various vitamins when dispersed in starch and exposed to

Table I. Effect of Exposing Mixtures of Vitamins and Cornstarch to Gaseous Ethylene Oxide

		Choline Cl-Starch-Vitamin Mixture			Choline Cl-Starch-Multivitamin Mixture		
		Vitamin Concentration, γ/G.			Vitamin Concentration, γ/G.		
Vitamin Tested	Expt. No.	No EO[a]	EO	Destr., %	No EO	EO	Destr., %
Thiamine	1	198	0	100	192	0	100
	2	200	0	100	198	0	100
	5	100[b]	0	100			
	5	400	0	100			
Cocarboxylase[c]	6	142	0	100			
Niacin	2	1900	890	53	1960	360	82
	3	1900	950	50	1940	1010	48
	4	1880	357	81			
Pyridoxine HCl		222	65	71	187	55	71
Riboflavin	1	466	171	63			
	2	270	88	67	288	207	28
	3	277	102	63	276	135	51
Folic acid	1	505	315	38			
	2	79	51	35	66	32	52
	3	88	63	28	75	50	33
Pantothenic acid	2	1780	1670	6	1810	1700	6
	3	1760	1760	0	1800	1800	0
Biotin	2	8.7	8.4	3			
	3	7.8	7.8	0	8.0	8.0	0
Vitamin B_{12}	7	8[d]	7[d]	12	10	10	0

[a] Exposure to gaseous ethylene oxide, as described in text.
[b] Choline chloride concentration was one half that described in text.
[c] The cocarboxylase concentration was equivalent to 100 γ per gram of thiamine. The choline chloride concentration was twice that described in the text.
[d] This sample contained sucrose in place of starch.

gaseous ethylene oxide is shown in Table I. Thiamine was completely destroyed both in the choline chloride–starch-thiamine mixture and in the complete multivitamin mixture. There was a loss of one half to four fifths of the niacin, three quarters of the pyridoxine, one third to two thirds of the riboflavin, and one third to one half of the folic acid activity in both the single vitamin and the complete multivitamin mixtures. Practically no pantothenic acid, vitamin B_{12}, or biotin was destroyed in the choline-vitamin-starch mixture or in the multivitamin mixture.

Exposure of a stock diet (Hunt Club dog meal) to ethylene oxide under the above conditions reduced the thiamine content from 2.54 to 1.52 γ per gram. When the purified diet was exposed to the same conditions, all of the thiamine was destroyed. Since thiamine probably exists in foods in a form similar to cocarboxylase, the stability of the latter compound to ethylene oxide was tested. In the starch-choline chloride–cocarboxylase mixture all of the thiamine activity of the cocarboxylase was destroyed as a result of ethylene oxide exposure. There is a possibility that in natural products the vitamins are within cells where they are protected by cell walls and other cellular components, while in the starch-vitamin mixture the vitamins are distributed on the surface where they are easily accessible to ethylene oxide. Evidence for the greater stability of thiamine in meat compared to crystalline thiamine in aqueous solution was presented by Greenwood, Beadle, and Kraybill (*6*).

Influence of Choline on Destruction of Vitamins by Ethylene Oxide

The type of choline compound present in the diet influenced the destruction of vitamins. When the purified diet was prepared with choline citrate, the growth inhibition resulting from ethylene oxide treatment was much less than that seen when choline chloride was present (*14*). Further studies indicated that in a mixture of starch, choline, and thiamine greater destruction of thiamine occurred when choline was present as the chloride rather than the citrate (*7*).

Originally it appeared that the ratio between choline and thiamine in the diet influenced the degree of destruction of thiamine. This was based on the observation that only 60% of the thiamine in a purified chick diet was destroyed as a result of exposing the diet to ethylene oxide (*14*), whereas in the purified rat diet so treated all of the thiamine was destroyed (*7*). The concentration of choline chloride in the chick diet was the same as in the rat diet, while the thiamine concentration was doubled. For this reason a few tests were made with the choline chloride–starch-thiamine mixture to determine the effect of varying the thiamine concentration on its stability. The data in Table I show that in the above system all of the thiamine is destroyed even though the thiamine concentration is doubled. There is a possibility that substances other than choline chloride may either hasten or retard the destruction of nutrients when food or food products are exposed to ethylene oxide.

In one experiment the ratio of riboflavin to choline chloride was twice that in the other experiment. In all cases the destruction of riboflavin in the choline chloride–starch-riboflavin mixture appeared to be a constant fraction of the amount present (Table I). A similar situation occurred with folic acid where, in one case, its concentration in the starch-choline chloride mixture was five times that in the other case. Here, again, the destruction of folic acid was related to the concentration.

The marked destruction of thiamine, riboflavin, niacin, and folic acid in both the choline-starch–single vitamin mixture and the multivitamin mixture raised the question as to how choline influenced these reactions. Both the hygroscopic properties of choline chloride and the alkalinity resulting from ethylene oxide treatment of mixtures containing choline chloride may be involved in the phenomenon. Further work is being done on the role that choline plays in the destruction of the vitamins.

Table II. Influence of pH on Stability of Thiamine, Riboflavin, and Calcium Pantothenate

Substance	Buffer 0.1M	pH	Vitamin Concentration, γ/Ml. Original	Final
Thiamine	Phosphate	7	100	102
	Phosphate	8	100	98
	Pyrophosphate	9	100	82
	Borate	10	100	0
Riboflavin	Phosphate	7	2.50	2.55
	Phosphate	8	2.50	2.60
	Pyrophosphate	9	2.50	2.70
	Borate	10	2.50	2.40
Ca pantothenate	Phosphate	7	5.00	5.25
	Phosphate	8	5.00	5.25
	Pyrophosphate	9	5.00	5.25
	Borate	10	5.00	5.40

Effect of pH on Stability of the Vitamins

The pH of the suspension prepared by mixing 2 grams of the ethylene oxide–treated choline chloride-starch-multivitamin mixture with 10 ml. of water was usually from 9.0 to 9.3. The pH of the choline chloride–starch-thiamine mixture treated with ethylene oxide was also from 9.0 to 9.1 when tested in the same way. The pH values of the untreated vitamin mixtures were less than 6.0. The preceding pH values may not represent the actual situation since the compounds in the diet are not in solution and, furthermore, it is conceivable that values much higher than these may occur in localized parts of the mixture.

In an effort to evaluate the possible role that alkalinity had in the destruction of the vitamins and because further information was needed on the stability of vitamins exposed at room temperature to various hydrogen ion concentrations, the following study was carried out. Thiamine and riboflavin, both of which showed destruction on exposure to ethylene oxide, and calcium pantothenate, which was not affected thereby, were dissolved separately in solutions of different pH values. When thiamine was kept in solution at a pH of 9 or below for 48 hours, not more than 20% of the vitamin was destroyed (Table II). The crystalline vitamins in the indicated concentrations in each case were added to 10 ml. of buffer and left at room temperature (23° C.) for 48 hours. At pH 10, all of the thiamine was destroyed. These findings are in agreement with those of Melnick, Robinson, and Field (*9*), who found that when thiamine was kept at 37° C. for 16 hours, there was practically no destruction below pH 8.5. Practically no riboflavin or calcium pantothenate was destroyed at any of the pH values tested. The stability of pantothenic acid is in agreement with the report of Frost and McIntire (*5*), who found this vitamin to be more stable at the higher pH ranges when solutions thereof were heated.

The authors' findings suggest that much of the destruction of thiamine indicated in Table I may be associated with alkalinity (Table II). Further studies on this point are under way. The mechanism is unknown whereby riboflavin is destroyed as a result of exposure of vitamin mixtures to ethylene oxide. Under the conditions used in this study, riboflavin was stable in a borate buffer at pH 10 (Table II). Other work currently in progress indi-

cates that the stability of riboflavin in alkaline solutions may be influenced by the type of buffer and the concentration of the vitamin.

However, the destruction of nicotinic and folic acids cannot be attributed to the alkaline reaction. According to Hundley (*8*), nicotinic acid is stable on heating with 1 or 2*N* alkali. Folic acid has been kept in solution (protected from light) at pH 9 and 10 for 6 months in the authors' laboratory in the refrigerator without showing any change in potency. This further evidence indicates that alkalinity resulting from ethylene oxide exposure is not the sole factor responsible for the destruction of vitamins.

The influence of moisture in the diets on the destruction of vitamins is now under study. Early in this work it was observed that the presence in the desiccator of a wad of cotton saturated with water during ethylene oxide treatment had no influence on the growth inhibition resulting therefrom. Although the atmosphere may have been saturated with water vapor, there is a possibility that the diet itself had not come into equilibrium with the water.

Discussion

A recent report indicates that when yeast and a rat diet composed of natural products were exposed to ethylene oxide according to one of the methods used commercially, there was no significant destruction of thiamine, riboflavin, nicotinic acid, pantothenic acid, or choline (*11*). There was, however, an indication that some folic acid and pyridoxine were destroyed. The conditions used in the preceding study differ in a number of respects from those used in the present study. The commercial procedures for chemical sterilization with ethylene oxide vary considerably and in some cases approximate those used in the present study.

The work reported in this paper reemphasizes the importance of evaluating the nutritional effects resulting from the treatment of a food with any compound. It is not necessarily enough to study the toxicity of the compound itself, since it may react with substances in the food to produce alterations in nutritional value which would not be apparent in the results obtained from the application of classical toxicological procedures.

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TOXICITY IN OIL MEAL

Bioassay for Toxic Factor in Trichloroethylene-Extracted Soybean Oil Meal

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A bioassay with calves has been developed for estimation of the toxic factor in trichloroethylene-extracted soybean oil meal which is responsible for the production of a fatal aplastic anemia in the bovine. Using the thrombocyte count and the percentage of lymphocytes as the principal and the total leucocyte count and death as supplementary criteria of toxicity, it is possible to differentiate among the biological effects on calves of daily doses of 1/100, 1/40, 1/20, 1/8, 1/6, 1/4, or more pound per day per 100 pounds of body weight of a standard specimen of the toxic meal. Acute or prolonged aplastic anemia of different degrees of severity can be produced in calves by feeding different levels of toxic trichloroethylene-extracted soybean oil meal.

SINCE A SEVERE HEMORRHAGIC DISEASE in cattle was first associated with the feeding of trichloroethylene-extracted soybean oil meal (*17*), this disease has again been encountered in several countries where trichloroethylene was used for the extraction of oil from soybeans [for a review of the literature see (*5*, *11*, *14*)]. During a recent outbreak of this disease in Minnesota (*11*) it was characterized as an aplastic anemia (*11*, *15*) and under controlled experimental conditions the disease has been reproduced in calves (*6*, *9*), heifers (*12*), milch cows (*9*), and horses (*7*) with specimens of trichloroethylene-extracted soybean oil meal of known origin. It has also been clearly established (*8*, *16*, *17*) that the consumption of trichloroethylene is not responsible for the toxic effects of trichloroethylene-extracted soybean oil meal, but the nature of the toxic agent is not known. Until it is identified and can be detected

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