Absence of Nitroso Formation from [^{14}C]Methomyl and Sodium Nitrite under Simulated Stomach Conditions

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One part per million of radiolabeled methomyl (*S*-methyl *N*-[1-^{14}C][(methylcarbamoyl)oxy]thioacetimidate) was added to macerates of commercially purchased cured meats (ham and hot dog), which contained 16–20 ppm of residual sodium nitrite. These samples were then incubated under simulated stomach conditions (pH 2) to investigate the possible formation of nitrosomethomyl (certain nitroso compounds are suspect mutagens and carcinogens). No nitrosomethomyl (less than 1 ppb) was found in either meat macerate after 1 and 3 hr of incubation.

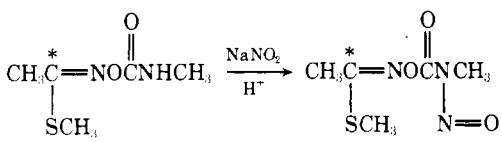
Methomyl (*S*-methyl *N*-[(methylcarbamoyl)oxy]thioacetimidate) is the active ingredient in Du Pont's Lannate methomyl insecticide. At the present time, Lannate is registered for insect control on several important crops, including broccoli, cabbage, cauliflower, head lettuce, sweet corn, tomato, and tobacco. Metabolism and degradation information on methomyl, furnished in support of registration, has been reported by Harvey et al. (Harvey et al., 1973; Harvey and Pease, 1973; Harvey and Reiser, 1973). A method for the determination of methomyl residues on crops using microcoulometric gas chromatography has been published by Pease and Kirkland (1968).

Recently, Elespuru et al. (1974) reported that a methylcarbamoyl pesticide, carbaryl (20 g), when combined with sodium nitrite (20 g) under acidic conditions for 2 days resulted in the formation of a mutagenic nitroso derivative. They further suggested that these nitroso derivatives may be formed in the stomach when sodium nitrite from cured meats and other sources and various foods containing pesticide residues are consumed at the same time, the stomach providing the acidic conditions for possible nitroso formation (Elespuru and Lijinsky, 1973; Lijinsky and Epstein, 1970).

This report describes an investigation into the possible formation of nitrosomethomyl in cured meat macerates under simulated stomach conditions, wherein both the methomyl and the sodium nitrite are at practical residue levels. No such nitroso formation was found (<1 ppb). This result might be expected from dilution effects plus the overriding presence of reactive, naturally occurring amino functions in all animal tissues. However, the studies were conducted for precautionary purposes.

EXPERIMENTAL DETAILS

Synthesis of Radiolabeled Nitrosomethomyl. The preparation of *S*-methyl *N*-[1-^{14}C][(methylcarbamoyl)-oxy]thioacetimidate ([^{14}C]methomyl) was described by Harvey et al. (1973). Radiolabeled nitrosomethomyl was synthesized by the following reaction.



Thirty milligrams of ^{14}C-labeled methomyl (137.1 μCi) was dissolved in a mixture of 45 μl of dimethyl sulfoxide and 15 μl of distilled water in a 2-ml reaction vial. After the solution had been cooled to 0° in an ice bath, 38 μl of glacial acetic acid, 18 μl of hydrochloric acid, and 38 mg of sodium nitrite were added. The reaction mixture was kept in the ice bath for 4 hr with occasional stirring. A yellow color slowly developed. After removal from the ice bath, 40 μl of distilled water and 500 μl of ether were added, the latter forming a yellow upper layer. The ether layer was pipetted into a small vial and partitioned twice with 100 μl of distilled water to remove unreacted methomyl, acids, and salts. Aliquots of the ether solution were analyzed for ^{14}C content by counting 10-μl aliquots in 15 ml of Aquafluor (New England Nuclear). Samples were counted on an Isocap/300 6868 scintillation system (Searle Analytic Inc.).

The ether solution was evaporated to dryness in a stream of nitrogen at room temperature. The total yield was 24 mg of [^{14}C]nitrosomethomyl (68% theoretical) and the specific radioactivity was 3.8 μCi/mg.

The product was dissolved in 2 ml of reagent grade methylene chloride. Aliquots of the resulting solution along with the reference materials, methomyl and *S*-methyl *N*-hydroxythioacetimidate in methylene chloride solution, were streaked onto four TLC plates (250-μ pre-coated silica gel 60F-254 TLC plates, Brinkmann Instruments Inc.) which were developed for 15 cm in four different solvent systems: EtOAc–$CHCl_3$ (1:1, v/v), $CHCl_3$–EtOAc (9:1, v/v), EtOAc, and CH_2Cl_2. The TLC R_f values for nitrosomethomyl, methomyl, and *S*-methyl *N*-hydroxythioacetimidate are given in Table I along with the chemical structures. Radioscans of the developed TLC plates were obtained with a Varian Berthold Model 6000-2 Automatic/Integrating TLC Radioscanner. The radiochemical purity of the nitrosomethomyl preparation was found to be 97% with 3% unreacted methomyl as an impurity.

Stability of Nitrosomethomyl. Nitrosomethomyl is an unstable compound. In the 100-ppm range, it has a half-life of about 1.5 hr in distilled water and in 0.01 *N* HCl (pH 2) at ambient temperatures. Methomyl and *S*-methyl *N*-hydroxythioacetimidate were found to be the only decomposition products, indicating the inherent instability of the nitroso derivative. In methylene chloride solution, nitrosomethomyl (1%) has a half-life of about 1 week in the freezer (−20°) and about 1 day at room temperature.

Because of the instability of this compound, all the following experiments were performed as rapidly as possible, and all extracts and solutions were kept refrigerated when possible. ^{14}C-Labeled nitrosomethomyl was repurified each time before use. Distilled water was used to partition nitrosomethomyl with methylene chloride in order to remove the decomposition products, methomyl and *S*-methyl *N*-hydroxythioacetimidate.

Structural Identification of Nitrosomethomyl. About 5 g of unlabeled nitrosomethomyl was also synthesized using the procedure described earlier. The calculated C, H, and N values for nitrosomethomyl ($C_5H_9N_3O_3S$) were (percent) C, 31.43; H, 4.72; N, 20.27; analysis of the preparation gave C, 31.43; H, 4.74; and N, 21.98. Mass spectroscopic

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Table I. TLC R_f Values of Nitrosomethomyl, Methomyl, and *S*-Methyl *N*-Hydroxythioacetimidate

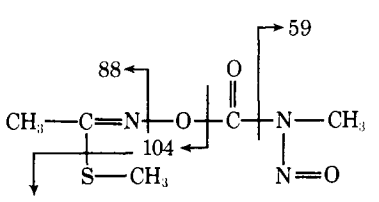
Solvent system	$CH_3C(SCH_3)=NOC(=O)NCH_3$ (N=O) *S*-Methyl *N*-[(*N*-nitrosomethylcarbamoyl)-oxy]thioacetimidate (nitrosomethomyl)	$CH_3C(SCH_3)=NOC(=O)NHCH_3$ *S*-Methyl *N*-[(methylcarbamoyl)-oxy]thioacetimidate (methomyl)	$CH_3C(SCH_3)=NOH$ *S*-Methyl *N*-hydroxythioacetimidate
EtOAc–$CHCl_3$ (1:9)	0.63	0.27	0.22
EtOAc–$CHCl_3$ (1:1)	0.85	0.54	0.64
EtOAc	0.78	0.56	0.75
CH_2Cl_2	0.23	0.06	0.05

Table II. Recoveries from Spiking Experiments

Experiment	Extraction percentage from meat, %	Total recovery in the final organic soln., %	Percentage detected from TLC plate: Nitrosomethomyl	*S*-Methyl *N*-hydroxythioacetimidate	Methomyl
Hot dog					
10 ppm of nitrosomethomyl spiked into meat extract		91	80	15	5
10 ppb of nitrosomethomyl spiked into meat extract		89	68	18	14
100 ppb of nitrosomethomyl spiked into macerated meat	72	56	48	35	17
10 ppb of nitrosomethomyl spiked into macerated meat	70	51	50	35	15
1 ppb of nitrosomethomyl spiked into macerated meat	69	51	43	57[a]	
1 ppm of methomyl spiked into meat, 1-hr incubation	94	89	<0.1	0.5	99.5
1 ppm of methomyl spiked into meat, 3-hr incubation	93	91	<0.1	0.5	99.5
Ham					
10 ppm of nitrosomethomyl spiked into meat extract		94	83	12	5
10 ppb of nitrosomethomyl spiked into meat extract		90	75	18	7
100 ppb of nitrosomethomyl spiked into macerated meat	75	55	39	19	31
10 ppb of nitrosomethomyl spiked into macerated meat	69	52	33	15	39
1 ppm of methomyl spiked into meat, 1-hr incubation	96	90	<0.1	0.5	99.5
1 ppm of methomyl spiked into meat, 3-hr incubation	95	93	<0.1	0.4	99.6

[a] Methomyl and *S*-methyl *N*-hydroxythioacetimidate were counted together.

analysis (15 eV) showed a parent molecular ion at 191 and major fragments as follows:



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NMR spectroscopic data showed that the doublet of the methyl protons on the carbamoyl group of methomyl has become a singlet peak. A broad band on the methomyl spectrum between 6.0 and 7.0 ppm was not observed in the nitrosomethomyl spectrum. These findings are consistent with the loss of an "NH" proton on the carbamoyl group of methomyl.

Evaluation of Analytical Methods for Incubation Tests. Two types of cured meats purchased in a local supermarket were used for the entire study. Direct analyses of the meats—ham and hot dog—by a certified USDA lab-

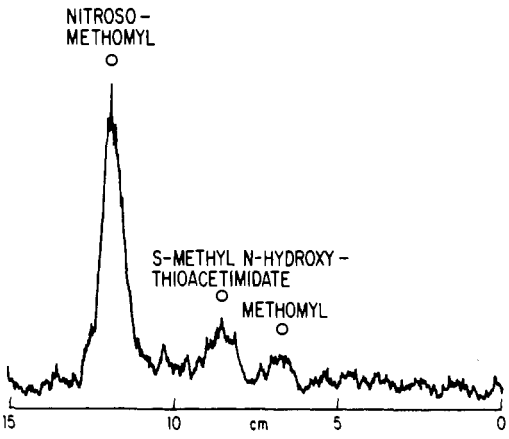

Figure 1. Nitrosomethomyl recovery studies in ham [10 ppb spiked into ham extract (0.01 *N* HCl) before methylene chloride partition]: TLC developing system, ethyl acetate–chloroform (1:1); scanner setting, measurement range 10; slit, 2 mm; found: 75% nitrosomethomyl, 18% *S*-methyl *N*-hydroxythioacetimidate, 7% methomyl.

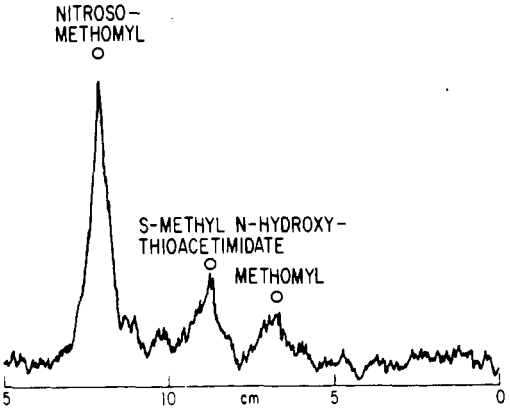

Figure 2. Nitrosomethomyl recovery studies in hot dog [10 ppb spiked into hot dog extract (0.01 *N* HCl) before methylene chloride partition]: TLC developing system, ethyl acetate–chloroform, (1:1); scanner setting, measurement range 10; slit, 2 mm; found: 68% nitrosomethomyl, 18% *S*-methyl *N*-hydroxythioacetimidate, 14% methomyl.

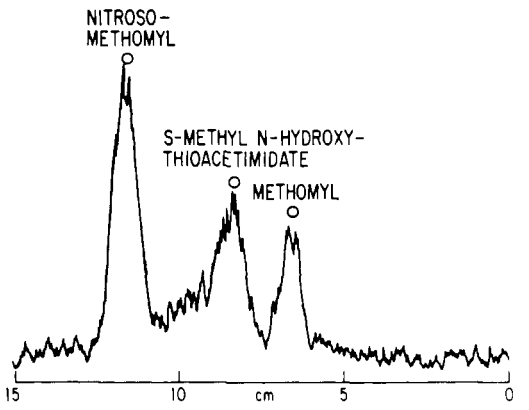

Figure 3. Nitrosomethomyl recovery studies in hot dog (10 ppb spiked into hot dog macerate): TLC developing system, ethyl acetate–chloroform (1:1); scanner setting, measurement range 10; slit, 4 mm; found: 50% nitrosomethomyl, 35% *S*-methyl *N*-hydroxythioacetimidate, 15% methomyl.

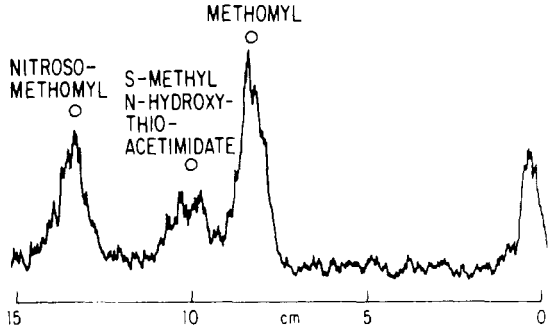

Figure 4. Nitrosomethomyl recovery studies in ham (10 ppb spiked into ham macerate): TLC developing system, ethyl acetate–chloroform (1:1); scanner setting, measurement range 10; slit 4 mm; found: 33% nitrosomethomyl, 15% *S*-methyl *N*-hydroxythioacetimidate, 39% methomyl, 13% at origin.

oratory gave 16 and 20 ppm of residual nitrite, as $NaNO_2$, respectively. These levels fall within the usual range (10–40 ppm), according to the same laboratory.

General Analytical Procedure. One hundred grams of each meat was mixed with 150 ml of 0.01 *N* HCl (pH 2) and macerated in a blender for 10 min. The mixture was centrifuged and the supernatant decanted. The solids were washed and centrifuged two more times with 100 ml of 0.01 *N* HCl. The combined aqueous solution was partitioned successively with 3 × 100 ml of methylene chloride. The organic layers were then combined, dried with sodium sulfate, and evaporated to dryness under a stream of nitrogen. The final residue was taken up with 2 ml of methylene chloride and subjected to TLC analysis.

Recovery of Nitrosomethomyl Added into Meat Extract. Two 100-g ham samples and two 100-g hot dog samples were extracted three times with 0.01 *N* HCl as described previously. One milligram (equivalent to 10 ppm based on 100 g of meat) and 1 μg (10 ppb based on 100 g of meat) of ^{14}C-labeled nitrosomethomyl were added into each of the meat extracts. The spiked aqueous solutions were immediately partitioned with 3 × 100 ml of methylene chloride and 91, 89, 94, and 90% of the total ^{14}C was recovered from the organic solvent phases of the 10-ppm hot dog, 10-ppb hot dog, 10-ppm ham, and 10-ppb ham samples, respectively. Table II shows that 68 to 83% of total radioactivity applied on the TLC plate is intact nitrosomethomyl. Methomyl and *S*-methyl *N*-hydroxythioacetimidate were the only decomposition products observed (Figures 1 and 2).

Recovery of Nitrosomethomyl Added into Macerated Meat. Three 100-g ground hot dog samples were spiked with 100, 10, and 1 ppb of nitrosomethomyl and stirred for 10 min in a blender. After extraction and partition into methylene chloride, samples were analyzed as before. Fifty-one to fifty-six percent of the total radioactivity was recovered in the final organic solution (Table II). Approximately one-half to one-third of the recovered radioactivity was intact nitrosomethomyl, the remainder being methomyl and *S*-methyl *N*-hydroxythioacetimidate (Figure 3).

A similar experiment using 100 and 10 ppb of nitrosomethomyl spiked into ham was also carried out. The recovery in the final methylene chloride solution was 52 and 55%, respectively (Table II), of the original spike. About one-third of the recovered radioactivity was intact nitrosomethomyl (Figure 4). In addition to the expected breakdown products in these two experiments, approximately 10% of the activity occasionally remained at the origin of the TLC plate. This is most likely due to purely mechanical reasons, i.e. holdup by "gummy" deposits (Figure 4).

In order to account for the total ^{14}C spiked into these samples, the meat residue after the extraction procedure was air dried, chopped into very small pieces, and combusted using a Packard Model 305 sample oxidizer, and the residual ^{14}C was determined by liquid scintillation counting.

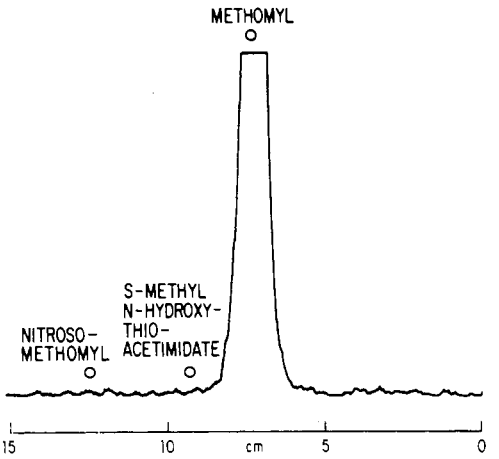

Figure 5. Hot dog spiked with 1 ppm of methomyl (1 hr incubation at 37°): TLC developing system, ethyl acetate–chloroform (1:1); scanner setting, measurement range 30; slit, 2 mm.

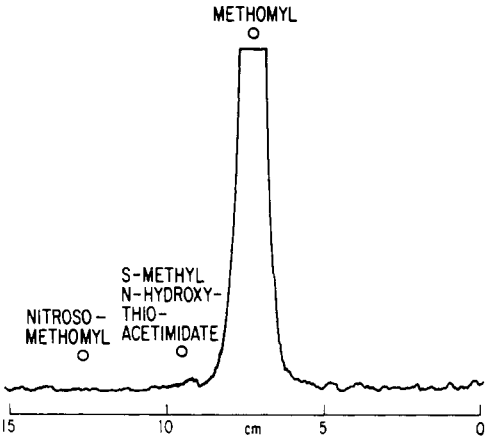

Figure 6. Ham spiked with 1 ppm of methomyl (3 hr incubation at 37°): TLC developing system, ethyl acetate–chloroform (1:1); scanner setting, measurement range 30; slit, 2 mm.

Approximately one-third of the added radioactivity remained on the meat after the initial (0.01 *N* HCl) extraction procedure. Additional losses of 15–20% of total ^{14}C also occurred in the 0.01 *N* HCl–methylene chloride partition step (^{14}C stayed in the aqueous phase), thus accounting for about 50% of the spiked radioactivity. The other 50% was recovered in the final extract (see Table II). Thus, every step of the analytical procedure was checked by scintillation counting, including the organic phase before and after solvent evaporation. No losses of radioactivity due to volatility of the compounds were observed.

Incubation Studies with Methomyl Added into Macerated Cured Meat. Two 100-g hot dog samples were ground and placed in 300-ml centrifuge bottles. Each sample was mixed in a blender for 10 min with 100 μg of ^{14}C-labeled methomyl (1.0 ppm) and 150 ml of 0.01 *N* HCl. The mixtures were incubated at 37° for 1 and 3 hr, respectively, before blending, extraction, partition, and TLC analyses. The analytical procedure was as described earlier. About 90% (Table II) of original spiked radioactivity was recovered in the organic solvent. Figure 5 shows a TLC radioscan of a hot dog sample incubated for 1 hr with 1 ppm of methomyl. Methomyl and trace amounts of *S*-methyl *N*-hydroxythioacetimidate were the only compounds detected by this technique (Table II, Figure 5).

Two ham samples were also ground, spiked with 1.0 ppm of ^{14}C-labeled methomyl, and incubated for 1 and 3 hr, respectively, with 150 ml of 0.01 *N* HCl. Again, about 90% of the spiked radioactivity was recovered in the final methylene chloride solution. Figure 6 shows a TLC radioscan of a ham sample which had been incubated for 3 hr with 1 ppm of methomyl. Methomyl and *S*-methyl *N*-hydroxythioacetimidate were the only radioactive compounds on the TLC plate.

The areas of silica gel on the TLC plates corresponding

Table III. Calculation of Maximum Possible Amount of Nitrosomethomyl Formed under Simulated Stomach Conditions

		1.0 ppm of methomyl spiked			
	1 ppb of nitrosomethomyl spiked into meat (hot dog)	Hot dog		Ham	
		1-hr incubation	3-hr incubation	1-hr incubation	3-hr incubation
dpm spiked in meat	843	1×10^6	1×10^6	1×10^6	1×10^6
dpm recovered in the final organic solution	430	9.0×10^5	9.3×10^5	9.1×10^5	9.5×10^5
dpm applied to TLC plate (20% of org. soln.)	86	1.8×10^5	1.86×10^5	1.82×10^5	1.89×10^5
Av background of silica gel on TLC plate (cpm)[a]	37	38	38	37	38
Methomyl band (cpm) (including background)		100,971	110,712	100,374	111,080
S-Methyl *N*-hydroxythioacetimidate band (cpm) (including background)	79[b]	474	588	488	479
Nitrosomethomyl band (cpm) (including background)	64	36	35	41	44
Net nitrosomethomyl (cpm) on TLC plate	27	−2	−3	5	6
Calculated nitrosomethomyl in meat (ppb)	*c*	<1	<1	<1	<1

[a] dpm = counts per minute/percent counting efficiency. Counting efficiency in these experiments ranged from 80 to 90%. [b] Methomyl band and *S*-methyl *N*-hydroxythioacetimidate band were counted together. [c] This column represents recovery data on 1 ppb of [^{14}C]nitrosomethomyl spike. This amount is detectable based on 27 cpm above background (37 cpm).

to nitrosomethomyl and *S*-methyl *N*-hydroxythioacetimidate were removed from the plates and the radioactivity of the silica gel was determined by liquid scintillation counting. The counts per minute (disintegrations per minute, dpm) for the 1.0-ppm methomyl spikes into ham and hot dog are given in Table III. Table III also shows the calculation of the maximum possible amount of nitrosomethomyl which could have been formed in these experiments. The maximum possible level of nitrosomethomyl in either meat is <1 ppb, which equals <0.1% of the applied methomyl.

DISCUSSION

For these tests aimed at examining the likelihood of pesticide interaction (specifically methomyl) with nitrites in cured meats, careful attempts were made to select realistic parameters. The temperature was body temperature. The incubation times (1 and 3 hr) bracketed normal residence times in the human stomach. The mass of solid matter (100 g of meat) to liquid (150 ml) was close to typical. The pH of the cured meat macerates was 2 both before and after the incubations. The cured meats were purchased commercially in local stores and were analyzed directly to ensure that they contained residual nitrite in the 16–20 ppm range. The amount of methomyl added (1 ppm based on solid content) represents a high level of consumed residue, since the highest tolerances for methomyl are in the 1–5 ppm range; most methomyl tolerances are <0.2 ppm. Also, there would be an immediate dilution of residue in the stomach with other foods containing no methomyl at all, e.g. a cured meat. Therefore, these studies, with their negative findings, indicate that there is very little likelihood that nitrites in the diet can combine with methomyl to form detectable amounts of a nitroso compound in the human stomach.

Extensive recovery studies of radiolabeled nitrosomethomyl added at various stages of the experimental procedure demonstrated the validity of the analytical method which is capable of detecting 1 ppb of nitrosomethomyl. The recovery studies also showed that nitrosomethomyl is inherently unstable and readily degrades to methomyl and *S*-methyl *N*-hydroxythioacetimidate under the experimental conditions.

ACKNOWLEDGMENTS

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N-Nitrosamines: Absence from Sauerkraut and Silage

Robert L. Tate III and Martin Alexander*

Volatile *N*-nitrosamines were not detected in corn silage and sauerkraut prepared in the laboratory. Nitrosamines were not formed if the corn was supplemented with 250 ppm of nitrate nitrogen and the cabbage was supplemented with 250 or 1250 ppm of nitrate nitrogen prior to the fermentation. Nitrosamine synthesis did occur in sauerkraut if both a secondary amine and nitrite were added. Thus, formation of volatile nitrosamines is unlikely even if the silage and sauerkraut are prepared from nitrate-rich plants.

The carcinogenicity and teratogenicity of *N*-nitrosamines assume particular importance in view of their isolation from foodstuffs such as cooked bacon (Fazio et al., 1973; Sen et al., 1973), frankfurters (Wasserman et al., 1972), and fish (Ender et al., 1964). They also may be formed in the mammalian intestine following ingestion of the secondary amine and nitrite precursors (Alam et al., 1971; Lane and Bailey, 1973; Lijinsky and Greenblatt, 1972). The synthesis of these toxicants requires the simultaneous occurrence of the two precursors, typically nitrite and a secondary amine, and usually an acid pH, although microorganisms may promote the synthesis at neutral pH values (Hawksworth and Hill, 1971; Ayanaba and Alexander, 1973).

Two characteristics of silage and sauerkraut suggest that nitrosamines might be formed in these fermented products. These properties are the acid pH of the final material and the occasionally high nitrate levels in the original crop used for silage formation or the cabbage. Nitrate levels of 313 to 2328 ppm have been noted in corn (Wilson, 1943), but the level varies with growth conditions and the rate of nitrate fertilization (Wright and Davison, 1964). Nitrate nitrogen levels of 0.2 to 0.3% (dry weight basis) are quite common in cabbage leaves and heads used for making sauerkraut (N. H. Peck, personal communication). The microbial conversion of a portion of this nitrate to nitrite during the fermentation might lead to spontaneous nitrosamine formation.

The present study was designed to determine whether *N*-nitrosamines might be formed in sauerkraut and silage and to establish factors affecting their production.

MATERIALS AND METHODS

Silage. Silage was prepared in the laboratory from corn chopped to about 9 mm length with a New Holland 770 chopper. Samples (100 g) were tightly packed in test tubes, 3.6 × 22 cm, as described by Wang and Burris (1960) and the tubes were sealed with parafilm. To simulate silage pre-

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