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Distribution, Movement, Persistence, and Metabolism of *N*-Nitrosoatrazine in Soils and a Model Aquatic Ecosystem

Philip C. Kearney,* James E. Oliver, Charles S. Helling, Allan R. Isensee, and Arnold Kontson

[*ring*-^{14}C]-*N*-Nitrosoatrazine (II) [2-chloro-4-(*N*-nitroso-*N*-ethylamino)-6-(isopropylamino)-*s*-triazine] was prepared by nitrosating [*ring*-^{14}C]atrazine (I) [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] at 0 °C with dinitrogen tetroxide and purified by silica gel column chromatography. The mobility of II, as measured by soil TLC, was only slightly less than that of I in five soils of differing texture and organic matter content. When II was held in the dark, it degraded to I in ecosystem water (33% degraded after 9 days and 62% after 18 days). Based on ^{14}C, bioaccumulation ratios (BR) for II in fish were 22 after 9 days and 31 after 18 days. For I, BR values were 17 and 16 after 9 and 18 days, respectively. No formation of II from I was detected (a) after 1, 2, or 3 months in either of two soils treated with 2 ppm I and 0, 100, or 1000 ppm N (as NH_4NO_3), or (b) after 1 month in a soil treated with 2 ppm I and 100 ppm N (NH_4NO_3) maintained at pH's 2.5, 3.5, or 4.5. Transient formation of II from I was observed in an acidic soil amended with 100 ppm N as sodium nitrite. The limit of detection was about 10 ppb for nitrosoatrazine under the experimental conditions described.

The possible formation of *N*-nitroso derivatives of pesticides or their metabolites in the environment is a subject of current interest. Pesticides N-nitrosated under in vitro and in vivo conditions in the laboratory have included the fungicide ziram (zinc dimethyldithiocarbamate), the insecticides carbaryl (1-naphthyl methylcarbamate) and propoxur (*o*-isopropoxyphenyl methylcarbamate), and the herbicides benzthiazuron [*N*-(2-benzothiazolyl)-*N'*-methylurea], simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine], and atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] (Eisenbrand et al., 1975). Wolfe et al. (1976) prepared *N*-nitrosoatrazine [2-chloro-4-(*N*-nitroso-*N*-ethylamino)-6-(isopropylamino)-*s*-triazine] by nitrosating atrazine with aqueous nitrous acid. Little is known about the environmental formation or stability of *N*-nitrosoatrazine. The extensive use of atrazine in crop production programs utilizing heavy application of N fertilizers has raised questions about the possibility of its N-nitrosation in soils.

This paper describes the synthesis of [*ring*-^{14}C]-*N*-nitrosoatrazine, the extent of *N*-nitrosoatrazine formation from [*ring*-^{14}C]atrazine in soils under various N and pH regimes, and the stability and distribution of [*ring*-^{14}C]-*N*-nitrosoatrazine and atrazine in soils and in a model aquatic ecosystem.

METHODS AND MATERIALS

Synthesis of [^{14}C]-*N*-Nitrosoatrazine. Anhydrous sodium acetate (50 mg) and a solution of dinitrogen tetroxide (N_2O_4, 0.25 mmol) in methylene chloride (0.4 mL) were combined at −78 °C, then a solution of atrazine (4.9 mg) plus [*ring*-^{14}C]atrazine (45 μCi, ~0.7 mg) in methylene chloride (4 mL) was added. The mixture was stirred and warmed to 0 °C, maintained at 0 °C for 0.5 h, then filtered through a column of Florisil in a Pasteur pipet. The solvent was evaporated and was replaced with a solution of acetic acid (6 μL) in benzene (2 mL). The resulting solution was warmed gently 15 min, then was concentrated somewhat and added to a column of silica gel (5 g). The column was eluted with benzene (15 mL), then with benzene–ethyl acetate (95:5, v/v). The first 18 mL of the latter was discarded, then the bulk of *N*-nitrosoatrazine was collected in the next 15 mL. This fraction was evaporated to dryness to give 3 mg of [*ring*-^{14}C]-nitrosoatrazine (22 μCi) as a yellow solid. Since *N*-nitrosoatrazine is very sensitive to light (Wolfe et al., 1976), all operations, including analyses, were carried out in a darkened room.

Soils Studies. *Nitrosoatrazine Formation in the Presence of Nitrate.* Two soils, Matapeake loam and Monmouth fine sandy loam, were utilized to study the possible formation of *N*-nitrosoatrazine. Ammonium nitrate was added to two 1-kg portions of each air-dried soil at rates equivalent to 100 and 1000 ppm N (i.e., 0.286 and 2.86 g/kg soil); a third portion of each soil received no NH_4NO_3. [*ring*-^{14}C]Atrazine (1 μCi) was also added to give a final concentration of 2 ppm. The soils were wetted to 75% field moisture capacity and stored in the dark. Core samples of the soil (~100 g) were taken after 1, 2, and 3 months, shake extracted with benzene–ethyl acetate (1:3) overnight, and again with methanol (2 h). Extracts were filtered, an aliquot removed for liquid scintillation

Agricultural Environmental Quality Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705.

counting, and the remainder of the extract concentrated under nitrogen to 0.1 mL for further analysis. Atrazine (R_f 0.52) was separated from *N*-nitrosoatrazine (R_f 0.71) by TLC on silica gel 60 F-254 plates developed with benzene–ethyl acetate (3:1). No-screen medical x-ray film was used for autoradiography of the TLC plates. For counting purposes 20 dpm above background were considered significant for the detection of nitrosoatrazine in the range of 10 ppb using the 0.1-mL aliquot.

A related soil experiment was initiated in which the pH was adjusted to 2.5, 3.5, and 4.5 with H_3PO_4 in both soils. The N (NH_4NO_3) concentration was 100 ppm and atrazine, 2 ppm. The soils were sampled and analyzed as described above.

Nitrosoatrazine Formation in the Presence of Nitrite. Duplicate Matapeake soil (pH 5.3) samples were acidified with H_3PO_4 to pH's of 2.5, 3.5, and 4.5, then were treated with $NaNO_2$ (100 ppm N) and [^{14}C]atrazine (2 ppm). The samples were wetted to 75% field moisture capacity, then stored and sampled (at 1, 4, and 10 weeks) as described previously.

The formation of *N*-nitrosoatrazine in soil/water suspensions in the presence of $NaNO_2$ was measured by a procedure similar to that described by Eisenbrand et al. (1975). The pH of suspensions of Matapeake soil (10 g) in water (50 mL) was adjusted to 2, 3, 4, and 5 with 1 N H_3PO_4. Then $NaNO_2$ (20 μmol, in 10 mL of water) and atrazine (1 μmol, in 10 mL of methyl Cellosolve) were added, the final volume adjusted with water to 100 mL, and the mixture maintained at 37 °C. Because $NaNO_2$ increases the suspension pH, preliminary studies were necessary to determine the additional H_3PO_4 needed to achieve the desired acidity in the final solution. Samples (10 mL) were removed after 5, 10, 15, 20, and 30 min, added to 5% K_2CO_3 (5 mL), and then extracted twice with methylene chloride (20 mL each). Duplicate 1-mL samples of the organic phase were analyzed by liquid scintillation counting to determine recovery; the remaining organic extract was reduced to 0.1 mL and separated by TLC. Radiocarbon in the atrazine and *N*-nitrosoatrazine spots was measured after scraping into scintillation vials.

Persistence of Nitrosoatrazine. The persistence of *N*-nitrosoatrazine was determined in Matapeake loam receiving 1 ppm *N*-nitrosoatrazine (plus 1 μCi/kg soil). Samples were removed after 1, 2, 3, 4, and 5 months, and analyzed as above.

Nitrosoatrazine Mobility. Movement of nitrosoatrazine in soils was assessed using soil thin-layer chromatography (soil TLC; Helling and Turner, 1968; U.S. Environmental Protection Agency, 1975). Five soils, broadly ranging in texture and organic matter content, were used; their properties and use in other pesticide mobility investigations are described elsewhere (Ambrosi and Helling, 1977). Most soil TLC plates were 10 × 20 cm, with four plates/soil. To these were added single applications of [*ring*-^{14}C]nitrosoatrazine (~6.6 μg, 0.024 μCi) and [*ring*-^{14}C]atrazine (0.80 μg, 0.020 μCi). The three Lakeland sandy loam soil plates were 20 × 20 cm and contained nitrosoatrazine and atrazine at rates ranging from ~3.3 to 16.5 μg and 0.4 to 2 μg, respectively. All operations were conducted in near darkness. Visualization of movement was by autoradiography.

Ecosystem Study. Five recirculating aquatic ecosystems were used as described elsewhere (Isensee, 1976) to measure the distribution, accumulation, and degradation of atrazine and *N*-nitrosoatrazine. Matapeake silt loam (400 g) was amended with [^{14}C]atrazine (two tanks) and [^{14}C]-*N*-nitrosoatrazine (two tanks) to give final concentration of 0.82 ppm (1 μCi); one untreated soil served as a control. The soils were flooded with 16 L of water. Two snails (*Helisoma* sp.), several hundred daphnids (*Daphnia magna*), 1 g of filamentous algae (*Oedogonium cardiacum*), and seven fish (*Gambusia affinis*) were added to each ecosystem. All tanks were maintained in the dark to eliminate photodecomposition. Water samples were taken every 2 to 3 days, and fish samples after 9 and 18 days. After 19 days, all organisms were harvested and analyzed.

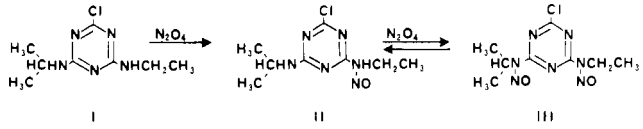

Figure 1. Synthesis of *N*-nitrosoatrazine from atrazine and dinitrogen tetroxide.

Table I. Degradation of Atrazine in Two Soils Containing 0, 100, and 1000 ppm N Added as NH_4NO_3

N treatment (ppm)/ incubation time (months)	^{14}C recovery, % of initially added			Total recovery, %
	Benzene–EtOAc	Methanol	Non-extractable	
	Matapeake loam			
0/1	36	12		
0/2	29	28		
0/3	17	33		
100/1	36	11		
100/2	27	29	35[a]	91
100/3	15	36		
1000/1	40	11		
1000/2	29	22	36	87
1000/3	17	34		
	Monmouth sandy loam			
0/1	50	9		
0/2	50	14		
0/3	34	17		
100/1	48	10		
100/2	47	16	29	92
100/3	35	16		
1000/1	47	10		
1000/2	41	17	29	87
1000/3	27	21		

[a] Since a large number of samples were examined, periodic samples were removed for combustion analysis, after extraction, to determine bound residues.

RESULTS AND DISCUSSION

Nitrosoatrazine Synthesis. Wolfe et al. (1976) and Eisenbrand et al. (1975) synthesized *N*-nitrosoatrazine by nitrosating atrazine with aqueous nitrous acid. We found this method produced rather low yields, and, therefore, employed more vigorous nitrosating conditions, N_2O_4 in methylene chloride. The N_2O_4 reagent, if used in excess, is capable of nitrosating both of the exocyclic N atoms of atrazine. *N,N'*-Dinitrosoatrazine (III in Figure 1) could thus be prepared and purified by column chromatography; however, it was unstable and rapidly denitrosated to the same mononitrosoatrazine (II) formed by nitrosation under milder conditions. For small scale preparations of *N*-nitrosoatrazine, it was convenient to nitrosate atrazine with N_2O_4 to a mixture of di- and mononitrosoatrazines, then briefly warm the mixture with a trace of acetic acid to degrade the dinitrosoatrazine to nitrosoatrazine.

Soil Studies. *Nitrosoatrazine Formation in the Presence of Nitrate.* Nitrosation with NO_3^- is not an anticipated process; however, NO_3^- is a form in which N is added to soils, and NO_3^- is converted to NO_2^- by soil organisms. The degradation and binding of atrazine in two

Table II. Effect of pH on Atrazine Degradation in Matapeake Loam Containing 100 ppm N Added as NH_4NO_3

Soil pH/incubation time (months)	^{14}C recovery, % of initially added: Benzene-EtOAc	Methanol
5.5/1	36	11
5.5/2	27	29
5.5/3	15	36
4.5/1	29	12
4.5/2	18	18
3.5/1	24	10
3.5/2	13	17
2.5/1	14	11
2.5/2	9	20

Table III. Nitrosoatrazine Formation from Atrazine and $NaNO_2$ (100 ppm N) and Binding in a Soil/Water Slurry as a Function of pH

pH	Nitrosoatrazine formed, %	Bound, %
5.0		30.3
4.0	0.7	40.7
3.0	19.3	45.4
2.0	35.7	51.9

soils amended with 0, 100, and 1000 ppm N (as NH_4NO_3) is shown in Table I. Two features were common to all treatments, i.e., extensive binding of atrazine (or its metabolites) to soil components and a gradual increase in methanol-soluble products. The formation of *N*-nitrosodimethylamine (NDMA) from ziram was reported to increase from 1.6% to about 8% of the theoretical yield when the *nitrite* concentration was increased from equimolar to a 20-fold molar excess (Eisenbrand et al., 1975). Our molar excesses of NH_4NO_3 to atrazine in soils were 385 (at 100 ppm N) and 3850 (at 1000 ppm N), yet no [^{14}C]nitrosoatrazine was detectable by TLC during the 3-month experiment. If nitrosoatrazine formation can only take place with unbound atrazine, and the concentration of NH_4NO_3 remains constant, then the possibility of nitrosoatrazine formation is reduced.

As the soil pH was decreased from 5.3 to 2.5, the extractable ^{14}C after atrazine treatment was decreased (Table II), but again no nitrosoatrazine was detected. The lower limits of detection of nitrosoatrazine using [*ring*-^{14}C]-atrazine was about 10 ppb under the experimental conditions described. Additional soil studies may be needed to clarify the relationship of the forms of N (i.e., NO_3^- vs. NO_2^-) and edaphic factors (pH, anaerobic vs. aerobic conditions) on nitrosoatrazine formation before any definitive statement can be made as to its significance in the environment. Although we examined a limited number of variables and the limit of detection was 10 ppb, our current study suggests that nitrosoatrazine is not formed in amounts that are significant at normal rates of field application of atrazine (2 ppm or 2 lb/acre in the surface 7.5 cm of soil) and 100 ppm NH_4NO_3.

Nitrosoatrazine Formation in the Presence of Nitrite. The chemical conversion of atrazine to nitrosoatrazine in soil slurries containing $NaNO_2$ (100 ppm N) at various pH values is shown in Table III. As the pH decreases, both binding and conversion of atrazine to nitrosoatrazine increase. The decreased desorption of atrazine from soil at low pH values has been reported previously (McGlamery and Slife, 1966). Although the significance of chemical formation of nitrosoatrazine in extremely acidic soil slurries is probably academic, since major crops cannot grow in acid soils, the data in Table III suggests that competing reactions, such as binding, may be important in further reducing or eliminating nitrosoamine formation. In soils receiving 100 ppm $NaNO_2$ plus 2 ppm atrazine, trace amounts of nitrosoatrazine were detected after 1 week (Table IV). After 4 and 10 weeks in these same soils, no nitrosoatrazine could be detected.

Table IV. Nitrosation of Atrazine (1 μmol) in Matapeake Loam with Sodium Nitrite (20 μmol)

Soil pH/incubation time, weeks	^{14}C recovery, % of initially added (yield of nitrosoatrazine, %): Benzene-EtOAc	Methanol
5.3/1	47 (0.38)	10 (0)
5.3/4	21 (0)	8 (0)
5.3/10	9 (0)	6 (0)
4.5/1	48 (0.75)	11 (0)
4.5/4	15 (0)	6 (0)
4.5/10	7 (0)	7 (0)
3.5/1	35 (0.68)	10 (0)
3.5/4	10 (0)	7 (0)
3.5/10	4 (0)	4 (0)
2.5/1	29 (0.45)	7 (0)
2.5/4	6 (0)	8 (0)
2.5/10	3 (0)	3 (0)

Table V. Degradation and Product Distribution of *N*-Nitrosoatrazine Added to Matapeake Loam

Incubation time, months	^{14}C recovery, % of initially added	Relative product distribution, %: Atrazine	*N*-Nitrosoatrazine	Polar[a]
	Benzene-EtOAc extract			
1	34	21.1	8.5	4.4
2	14	8.8	2.2	2.9
3	11	7.2	0.6	3.3
4	7	4.1	0.9	2.0
5	5	3.1	0.6	1.4
	Methanol extract			
1	22	4.0	3.5	14.5
2	20	3.0		17.0
3	21			
4	22	0.9		21.1
5	7	0.3		6.7

[a] "Polar" is that extractable material remaining at the origin of TLC.

Table VI. Mobility of Atrazine and Nitrosoatrazine in Soil TLC Systems

Soil type	Average R_f (± SD) of: Atrazine	Nitrosoatrazine
Norfolk sandy loam	0.72 ± 0.03	0.65 ± 0.08
Lakeland sandy loam	0.81 ± 0.09	0.73 ± 0.08
Sterling loam	0.65 ± 0.01	0.31 ± 0.07
Hagerstown silty clay loam	0.54 ± 0.01	0.49 ± 0.05
Benevola silty clay	0.56 ± 0.02	0.47 ± 0.01

Persistence of Nitrosoatrazine. If nitrosoatrazine were to be formed in an environment, and if it were to reach soil, the results in Table V show that the compound would be degraded. Denitrosation was one of the primary reactions of nitrosoatrazine, since atrazine was the major product identified in the soil extracts.

Nitrosoatrazine Mobility. Atrazine was the logical internal standard for mobility investigation since it is both precursor and product of nitrosoatrazine, its field and soil column leaching behavior(s) are well documented (Helling, 1970), and it has been studied in soil TLC for all the soils included in this study except Benevola silty clay loam (Helling, 1971b). Nitrosoatrazine was slightly less mobile than atrazine (Table VI). As is frequently the case, water

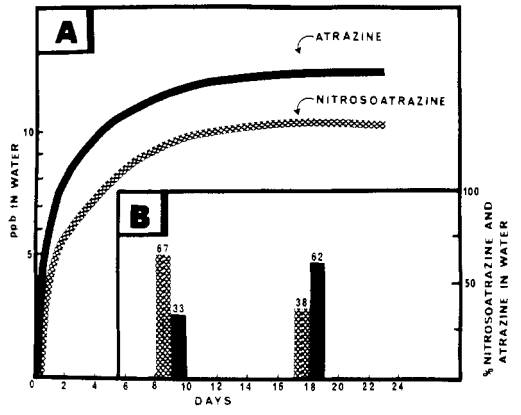

Figure 2. Atrazine and nitrosoatrazine distribution in water over treated soil, in a model aquatic ecosystem: (A) concentration as ppb, based on ^{14}C and assuming only parent exists; (B) relative distribution of atrazine and nitrosoatrazine sampled 9 and 18 days after introducing nitrosoatrazine-treated soil.

solubility differences [atrazine is 33 ppm; nitrosoatrazine is 227 ppm (Wolfe et al., 1976)] are not clearly related to relative mobility values. In Sterling loam, nitrosoatrazine was distinctly less mobile than atrazine. Sterling (pH 7.7) is the most alkaline soil that was tested. The first autoradiograms of Sterling showed faint streaking distinct from the main dark zone. This component moved to R_f ~0.60 and was thought to be atrazine. Atrazine was probably present in all samples, but was distinguishable from nitrosoatrazine only when R_f's differed substantially, as in Sterling soil.

To test whether high soil pH itself caused differential movement of these two triazines, or accelerated breakdown of nitrosoatrazine to atrazine, we purified samples of both compounds by conventional TLC, then applied them promptly to soil TLC plates of normal and modified Hagerstown and Sterling soils. Hagerstown silty clay loam had been treated with $CaCO_3$ and KOH during a 2-day period; its pH changed from 6.7 to 7.25. Similarly, Sterling loam had been treated incrementally with H_2SO_4 until its pH changed from 7.7 to 6.55. This approach had been effective in demonstrating, in these same soils, how soil acidity affects mobility of acidic herbicides (Helling, 1971a).

Movement of the compounds was individually the same in "limed" as in normal Hagerstown, and in acidified as in normal Sterling. Thus, soil pH did not account for the major decrease in nitrosoatrazine mobility in Sterling loam. Furthermore, the additional streaking originally seen above nitrosoatrazine was now absent; therefore, this compound did not break down during soil TLC leaching.

There was a tendency for reduced transport of the triazines as clay and/or organic matter content increased (Table VI). Sample size did not affect results, since the average R_f of atrazine in Lakeland sandy loam was 0.81 for both 0.8 μg and 0.4 to 2 μg samples. Nitrosoatrazine was also insignificantly affected.

To summarize the soil mobility studies, nitrosoatrazine was slightly less mobile than atrazine in four soils, but substantially less mobile in Sterling loam. Soil pH cannot account for this difference. Thus, nitrosoatrazine would not move away from the parent herbicide in the soil profile. The kinetics of nitrosoatrazine formation and degradation seem more significant in assessing residue distribution than does physical transport.

Ecosystem Studies. Figure 2A shows the rates of appearance of atrazine and nitrosoatrazine in water, based on ^{14}C from initial soil incorporation of both chemicals. Analysis of water samples after 9 and 18 days from tanks initially receiving only nitrosoatrazine showed the actual concentrations of nitrosoatrazine were 6.0 and 3.8 ppb, respectively. It was not possible to determine whether nitrosoatrazine was breaking down in water or while sorbed to soil. As in soils, nitrosoatrazine was unstable in water (Figure 2B), with denitrosation being a major degradation reaction. Wolfe et al. (1976) reported rapid photolysis of nitrosoatrazine in sunlight. Since our ecosystem studies were conducted in the dark, two reactions may occur, one in surface waters receiving direct UV radiation, and a slower denitrosation in dark water.

Table VII. Concentrations and Bioaccumulation Ratios of Atrazine and *N*-Nitrosoatrazine in Fish, Snails, and Algae[a]

	Atrazine		*N*-Nitrosoatrazine	
Day	ppb	BR	ppb	BR
		Fish		
9	197	17	188	22
18	186	16	301	31
		Snails		
18	98	8	888	91
		Algae		
18	104	9	154	16

[a] Determined in a model aquatic ecosystem. Soil was initially treated with 0.82 ppm of atrazine or nitrosoatrazine.

Bioaccumulation ratios (BR) in fish, snails, and algae are shown in Table VII. As compared with the large BR values reported for the chlorinated hydrocarbon insecticides, ^{14}C derived from nitrosoatrazine does not accumulate in fish. Nitrosoatrazine could not be identified in the fish extracts due to the low recovery of ^{14}C. The BR values for atrazine in fish (Table VII) would be in the same range (3–10) as those previously reported by Isensee (1976). BR values for algae harvested on day 18 for atrazine and nitrosoatrazine, and snails on the same date for atrazine were also comparably low. Concentrations and BR values for nitrosoatrazine were an order of magnitude higher in snails. At this time we offer no explanation for the tendency of snails to accumulate ^{14}C associated with nitrosoatrazine from water.

The possible formation and stability of nitrosoatrazine were examined in natural and artificial soil systems. Our results indicate that the possibility of nitrosoatrazine formation seems extremely remote in good agricultural soils (pH 5.0–7.0) receiving normal applications of atrazine (2 ppm) and even high rates of N fertilizer (100 ppm N). In both soil and water, synthetic nitrosoatrazine is unstable and is degraded, usually by denitrosation, to atrazine and polar products. High concentrations of NO_2^- produce transient amounts of nitrosoatrazine in acid soil. Based on past failures to detect nitrosoatrazine in a number of systems (Marco et al., 1976) and the noncarcinogenic response observed for the structurally related nitrosated herbicide nitrososimazine (Eisenbrand, 1977), nitrosoatrazine seems to pose no environmental threat.

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Nonenzymatic Microbial Acceleration of Nitrosamine Formation

Ho Seung Yang, James D. Okun, and Michael C. Archer*

Rate enhancements of from 12- to 49-fold occurred when dihexylamine was nitrosated at pH 3.5 in the presence of bacteria and yeast cells at a concentration of 12 mg/mL. Rates were similar in the presence of either boiled or unheated cells. The magnitude of the rate enhancement for nitrosation of other amines depended on the alkyl chain length. A nonenzymatic mechanism involving hydrophobic interactions of the precursor amines and cellular constituents is proposed.

Several authors have described the formation of carcinogenic nitrosamines in the presence of microorganisms (Sander, 1968; Klubes and Jondorf, 1971; Hawksworth and Hill, 1971, 1974; Collins-Thompson et al., 1972; Klubes et al., 1972; Ayanaba and Alexander, 1973; Ayanaba et al., 1973; Thacker and Brooks, 1974; Mills and Alexander, 1976). These reports have primarily concerned the nitrosation of dimethylamine and diethylamine at pH values above 5.0. In several microbial cultures, nitrosamine formation is apparently nonenzymatic (Klubes and Jondorf, 1971; Collins-Thompson et al., 1972; Mills and Alexander, 1976), and some have proposed that the reaction is catalyzed by one or more unidentified metabolic products. Another possibility is the enzymatic catalysis of nitrosamine formation in the presence of microorganisms (Hawksworth and Hill, 1971; Klubes and Jondorf, 1971; Klubes et al., 1972; Ayanaba and Alexander, 1973; Ayanaba et al., 1973; Sherbet and Lakshmi, 1973), but the evidence for such involvement of an enzyme is far from clear.

We have recently reported large rate enhancements for the nitrosation of several amines in the presence of micelle-forming surfactants (Okun and Archer, 1977). The present investigation, as an extension of these findings, was designed to examine the effects of microorganisms on nitrosamine formation at acidic pH. These conditions are of potential relevance to nitrosation of amines in the environment of the stomach, in which microorganisms derived both from food and the oral cavity may be present.

MATERIALS AND METHODS

Dimethylamine, di-*n*-butylamine, di-*n*-pentylamine, di-*n*-hexylamine, and piperidine were purchased from Eastman Organic Chemicals (Rochester, N.Y.), morpholine from Fisher Scientific Co. (Pittsburgh, Pa.), and diethylamine from J. T. Baker Chemical Co. (Phillipsburg, N.J.). All amines were purified by distillation prior to use.

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

Di-*n*-butylnitrosamine, diethylnitrosamine, nitrosopiperdine, and nitrosomorpholine were purchased from Eastman Organic Chemicals. Di-*n*-hexylnitrosamine was a generous gift from Dr. Harold Röper of the University of Hamburg. Di-*n*-pentylnitrosamine was synthesized from the purified parent amine according to the method of Druckrey et al. (1967).

Escherichia coli B (ATCC 11303) was purchased from Sigma (St. Louis, Mo.) and *Saccharomyces cerevisiae* was from Standard Brands Inc. (New York, N.Y.). *Saccharomycopsis lipolytica*, a generous gift from L. J. Wickerham (Northern Regional Research Laboratory, Peoria, Ill.), was grown in a medium containing 1% yeast extract, 2% peptone, 3% glycerol, and 0.1% dextrose. *Bacillus brevis* (ATCC 9999) was grown in a medium containing 5% yeast extract, 5% peptone, and 5% glucose. Cells were harvested in the stationary phase by centrifugation. Cell concentrations are in all cases reported as dry weight measurements.

Kinetic runs were performed in stoppered centrifuge tubes. Reaction mixtures (5-mL volumes) contained 20 mM amine and 20 mM sodium nitrite (unless otherwise specified) in citrate–phosphate buffer, pH 3.5, at 25 °C. The buffer was prepared by mixing 0.1 M citric acid with 0.2 M Na_2HPO_4. The microbial cell suspension was first added to a buffered solution of the amine and incubated for about 30 min. Reactions were initiated by addition of nitrite; at various times (0, 10, 20, 30, and 40 min), the nitrosation reactions were stopped by adding an excess of solid ammonium sulfamate. After incubation for about 20 min to insure destruction of nitrite, the contents of the tubes were extracted with an equal volume of methylene chloride. The nitrosamine concentration was then determined by a combined gas chromatography/thermal energy analysis system, as described by Fine and Roubehler (1975). Chromatography was performed on a column ($^1/_8$ in. × 10 ft) packed with 3% OV-17 or 10% FFAP on Chromosorb G. Control reactions in the absence of amine, nitrite, or cells were run in a similar manner.

In order to investigate the distribution of the rate-enhancement effect within the cells, we suspended 1 g of *E.*