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Microbial Metabolism of *N*-Nitrosodiethanolamine in Lake Water and Sewage

JERRY R. YORDY AND MARTIN ALEXANDER*

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14853

The carcinogenic nitrosamine, *N*-nitrosodiethanolamine (NDElA), was degraded in samples of sewage and two lake waters, and microorganisms were responsible for the transformation. However, the rate of NDElA disappearance was slow. In the samples of lake water, the rate and extent of NDElA metabolism varied with the time of year, and no disappearance occurred in samples taken in winter. The products formed from NDElA were persistent in lake water. In sewage, no seasonal effect on the microbial conversion was evident, and the products of metabolism were slowly mineralized. NDElA is apparently converted to the same organic products in samples of all three environments. Although the products were not identified, the data suggest that they were modified dimers of NDElA.

N-Nitroso compounds have received a great deal of attention because of their carcinogenicity, mutagenicity, and teratogenicity (3, 9) and their presence in foods (13, 14), drugs (7), and pesticides (11). Nitrosamines have also been isolated from soils and water (5), and several investigators have shown the potential for nitrosamine formation in natural environments (1, 2, 6).

Because of the apparent widespread distribution of nitrosamines, it is important to have information on their stability. Rowland and Grasso (13) have shown that bacteria found in the human gut can break down certain nitrosamines to the parent amine, nitrite, and, in some instances, unidentified volatile products. Tate and Alexander (15) noted that *N*-nitrosodimethyl-, diethyl-, and dipropylamine were persistent in lake water and only slowly disappeared in sewage and soil. Oliver et al. (10) found the same nitrosamines to be mineralized in soils, although the reaction was slow.

N-Nitrosodiethanolamine (NDElA) is a proven carcinogen (8) which has been found in a variety of products, such as cutting fluids (16), shampoos, cosmetics, and lotions (4) which contain diethanolamine (DElA). It has also been shown that DElA can be nitrosated to form NDElA in samples of sewage and lake water, but in some instances the NDElA thus formed subsequently disappeared (Yordy and Alexander, submitted for publication). This paper describes the results of a study of the transformation of NDElA in these environments.

MATERIALS AND METHODS

Water samples were collected from North Lake (pH 5.2) in the Adirondack Preserve and from Cayuga Lake (pH 7.8), both in New York State. The samples were collected at the surface, approximately 1 m from shore. The North Lake samples were kept on ice until they were returned to the laboratory. Sewage (pH 7.8) was collected from the primary settling tank at the Ithaca, N.Y., sewage treatment plant. Experiments were initiated on the day that samples were collected, and the samples were filtered through Whatman no. 1 filter paper before starting experiments.

NDElA transformation was determined in sterile 50-ml Erlenmeyer flasks to which were added 5.0-ml samples of the environment being studied and radioactive NDElA. When sterile samples were needed, 0.24 ml of sample and radioactive NDElA contained in screw-capped test tubes were sterilized by autoclaving. Each test tube was sampled only once. For the isolation of products of NDElA metabolism for gas chromatographic-mass spectrometric analysis, 25 ml of Cayuga Lake water or sewage was incubated in a 250-ml Erlenmeyer flask. Flasks or tubes were incubated in the dark at approximately 22°C without agitation.

Tests of mineralization were conducted in 50-ml screw-capped Erlenmeyer flasks by the procedure described above except that a 16-mm hole was drilled in each cap and a 22-mm Teflon-silicone disk (Pierce Chemical Co., Rockford, Ill.) was used to seal the opening of the flask. To measure $^{14}CO_2$ production, the flasks were flushed with water-saturated air at a flow rate of 30 ml/min for 90 min. The effluent air was passed through a filter stick of 25- to 50-μm porosity (Ace Glass Co., Vineland, N.J.), and the CO_2 was trapped in 2.0 ml of a mixture of ethanolamine and methanol (1:1) in 15 ml of ACS scintillation cocktail (Amersham Corp., Arlington Heights, Ill.). After the flushing, 1.0 ml of methanol was added to the scintillation cocktail-ethanolamine mixture to replace the methanol lost during flushing.

NDElA breakdown was measured by a method involving thin-layer chromatography and ^{14}C-labeled NDElA. Radioactive NDElA was added to lake water or sewage, and at intervals thereafter, 40-μl portions

were removed from the flask or test tube and spotted on a thin-layer chromatography plate (plate LK5DF, Whatman Co., Clifton, N.J.). All samples were analyzed in triplicate. The stationary phase of the plates was silica gel containing an inorganic phosphor to enable location of the nitrosamine with ultraviolet light. A small amount of authentic, nonradioactive NDElA was then added as a marker to each preadsorbent area. When the plate was dry, the samples were chromatographed twice in a solution of 30% (vol/vol) acetonitrile in ethyl acetate. In this solvent system, all of the products formed from NDElA remained within the first 2 cm of the chromatogram. After the final chromatography, the NDElA spot and the area on the chromatogram containing the products were scraped from the plate into scintillation vials, 10 ml of ACS scintillation cocktail was added, and, after allowing the chemiluminescence to subside, the radioactivity in the vials was measured with a Beckman LS100C liquid scintillation spectrophotometer. The spots were removed quantitatively from the plates by first breaking the plate at the leading edge of the spot, adding 2 or 3 drops of water to the silica gel, and then pushing the silica gel off the plate into a scintillation vial. This procedure gave greater than 90% recovery of the compounds over a wide range of radioactivities.

Autoradiography was performed with Kodak X-ray film SB-5 (SMW X-Ray, East Syracuse, N.Y.). The film was exposed for 2 weeks and developed by procedures recommended by the manufacturer. The transformation products were separated by chromatographing them in a solvent system consisting of ethyl acetate, 95% ethanol, and acetic acid (4:5:1).

To produce a sufficient yield of products of NDElA metabolism for identification, nonradioactive NDElA was added to Cayuga Lake water at a concentration of 100 μg/ml, and the flasks were incubated for 48 days. The products were isolated by applying 10 2.0-ml portions of the lake water to preparative thin-layer chromatography plates (plate PLK5F, Whatman Co.). When dry, the plates were chromatographed twice in a solvent system consisting of 70% (vol/vol) ethyl acetate in 95% ethanol. The silica gel containing the products was scraped off the plate, and the products were eluted from the silica gel with methanol. After evaporating the methanol under N_2, the samples were derivatized for 16 h at 65°C with 0.1 ml of *bis*-trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane (Regis Chemical Co., Morton Grove, Ill.). The samples were analyzed on a gas chromatograph (model 3920, Perkin-Elmer Corp., Norwalk, Conn.) with a 1.8-m long, 2-mm-inner-diameter glass column packed with 3% OV-17 on 100 to 120 mesh Gas Chrom Q (Supelco, Inc., Bellefonte, Pa.) and a flame ionization detector. Helium was used as a carrier gas at a flow rate of 30 cm^3/min. The column was held at 120°C for 2 min and then programmed from 120 to 250°C at 4°C/min and held at 250°C for 8 min. The injector and detector temperatures were 200 and 270°C, respectively. Gas chromatographic-mass spectrometric analysis was performed with a Finnigan 3300 mass spectrometer and gas chromatograph (Finnigan Corp., Sunnyvale, Calif.). To ensure that the identified peaks were indeed products of NDElA metabolism, lake water containing added NDElA sampled at the start of the experiment and NDElA-free lake water incubated for 48 days were also analyzed.

NDElA was a gift of the Union Carbide Corp., Tarrytown, N.Y. [*U*-^{14}C]DElA and [*U*-^{14}C]NDElA were obtained from New England Nuclear Corp., Boston, Mass., and these compounds had specific activities of 20.8 and 6.38 mCi/mmol, respectively. The radiochemical purities of the labeled chemicals were greater than 98% as determined by thin-layer chromatography performed by the manufacturer.

RESULTS

The results of a typical experiment designed to examine mineralization of NDElA in samples of the three environments are shown in Fig. 1. The NDElA concentration in this instance was 1.1 μg/ml. Mineralization of NDElA was never observed in the Cayuga Lake and North Lake water samples, even though the process was examined with samples collected from these lakes at three different times of the year. The small amount of radioactive CO_2 which was formed (1.0 and 0.8% of the carbon from NDElA being converted to CO_2 in Cayuga Lake and North Lake water) probably results from the mineralization of radiochemical impurities present in the NDElA.

In sewage, NDElA was mineralized (Fig. 1), but CO_2 was formed from the carcinogen only after a relatively long period. Thus, at a concentration of 1.1 μg of NDElA per ml, CO_2 was not produced from the nitrosamine until about day 25, the small yield before this time being attributed to breakdown of radiochemical impurities. Over 40% of the carbon added as NDElA was recovered as CO_2 when the experiment shown in Fig. 1 was terminated.

Although NDElA was not mineralized in samples from the two lakes, the nitrosamine was metabolized. Breakdown of about 1.0 μg of NDElA per ml in North Lake water sampled at

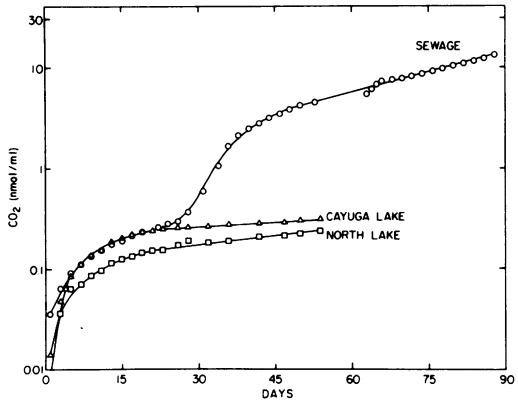

FIG. 1. *NDElA mineralization in samples of lake water and sewage.*

two different times is shown in Fig. 2. After a lag of several days, the nitrosamine in the July sample disappeared, accompanied by the appearance of stoichiometric amounts of products. To analyze the data and because the identities of the products were not known, it was assumed that the products contained four carbon atoms per molecule. This meant the products would have the same specific activities as the parent NDEIA, and the concentration of the products could therefore be determined from the amount of radioactivity that they contained. With water collected in September, a longer period of time elapsed before the NDEIA began to be metabolized, and even then the disappearance was only partial. The disappearance was still accompanied by the stoichiometric formation of products. In sterilized samples of water taken at both times, the nitrosamine persisted, and product formation was not detected. When tested at a concentration of 50 ng/ml, NDEIA persisted in nonsterile September water samples.

NDEIA was also metabolized in Cayuga Lake water (Fig. 3). The nitroso compound disappeared at concentrations of 50 ng and 1.0 μg of NDEIA per ml, and the disappearance was accompanied by a stoichiometric formation of products. In sterilized samples of Cayuga Lake water, the nitrosamine did not disappear, and no products were detected.

The time of year that the sample was taken from Cayuga Lake had a noticeable effect on NDEIA destruction (Fig. 4). As the seasons progressed from late summer through fall, the time required for the nitrosamine to disappear increased until in January no metabolism of the nitrosamine was observed up to 32 days. Once the loss became evident, the rates of NDEIA

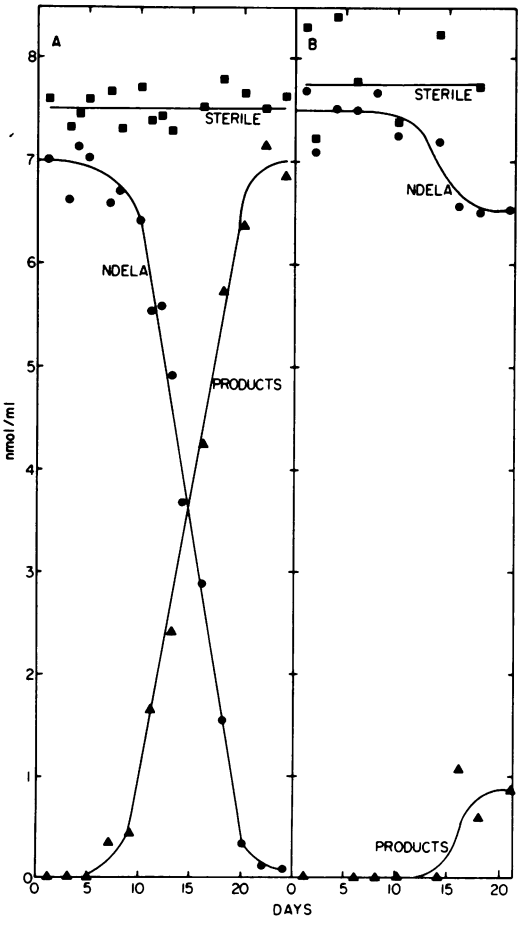

FIG. 2. *Transformation of NDEIA in North Lake water. The lines labeled "sterile" show the persistence of NDEIA, and the other lines represent nonsterile samples. (A) Water collected in July. (B) Water collected in September.*

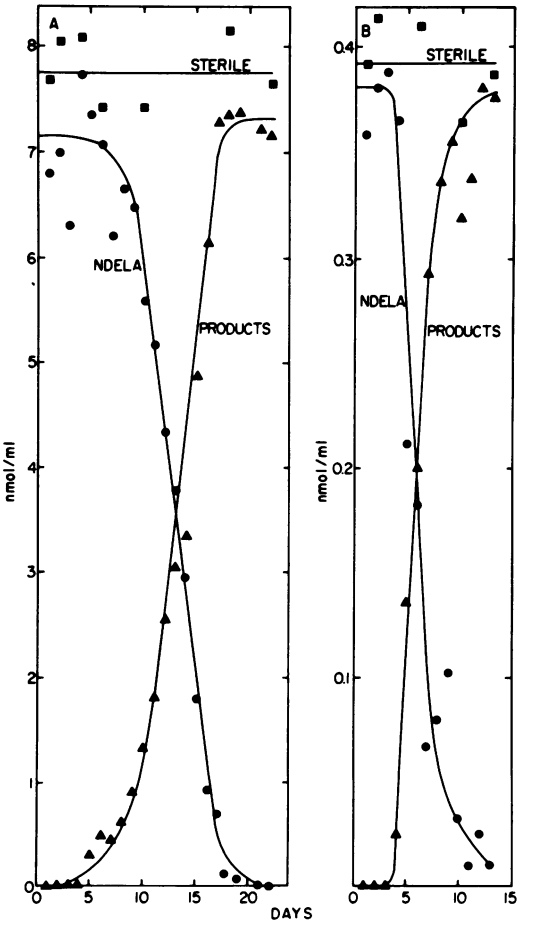

FIG. 3. *Breakdown of NDEIA in Cayuga Lake water. (A) 1.0 μg of NDEIA per ml. (B) 50 ng of NDEIA per ml.*

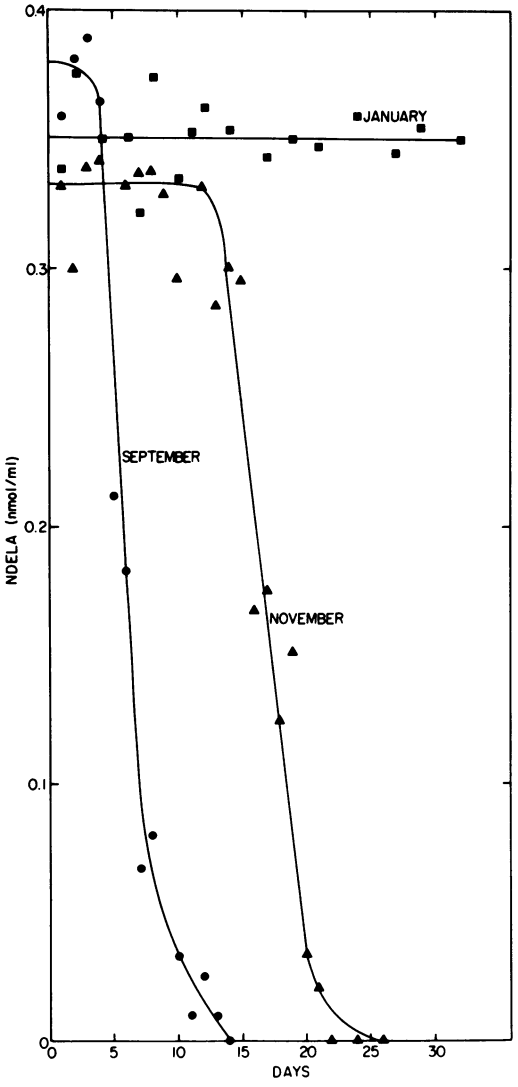

FIG. 4. *Effect of season on NDElA disappearance from Cayuga Lake water.*

disappearance were essentially the same in the September and November samples. The greatest rate of NDElA metabolism in Cayuga Lake water appeared to occur in the summer, because in an August water sample, NDElA at a concentration of 1.0 μg/ml was completely converted to soluble products in only 9 days.

In sewage, NDElA was eventually mineralized, but the nitrosamine was first converted to intermediate products which persisted for several weeks. The results of an experiment in which NDElA was added at a level of 1.1 μg/ml are depicted in Fig. 5. In this instance, the stoichiometric conversion of NDElA to the intermediates was not observed, suggesting that the organic products began to disappear before all of the nitrosamine was converted to these products. In tests with different samples of sewage, the nitrosamine was converted entirely to the organic products before the latter were mineralized. The data in Fig. 5 are derived from a study in which samples from the same batch of sewage were used, but one flask was used to measure the concentrations of NDElA and soluble organic products, whereas CO_2 evolution was determined with a second flask; hence, the fact that CO_2 evolution lagged somewhat behind the degradation of the organic products may have resulted from minor differences between the flasks caused by either the small sample size or differences in O_2 concentration. Although individual flasks were used, the same experiment was repeated with several batches of sewage, and the accumulation of organic intermediates and subsequent CO_2 evolution were always noted. At a concentration of 50 ng/ml, NDElA was also degraded in sewage. The nitrosamine did not disappear when added to sterilized sewage.

Because the nitrosamine was eventually mineralized in sewage but not in samples from the two lakes, it is possible that the indigenous microflora formed dissimilar organic products in samples of these environments. Hence, the possible similarities of the metabolites were compared in the three environments. Figure 6 is an autoradiogram of a thin-layer chromatogram of

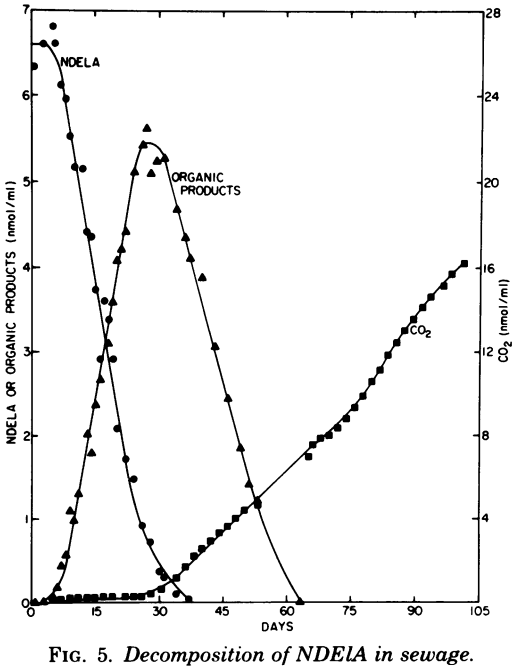

FIG. 5. *Decomposition of NDElA in sewage.*

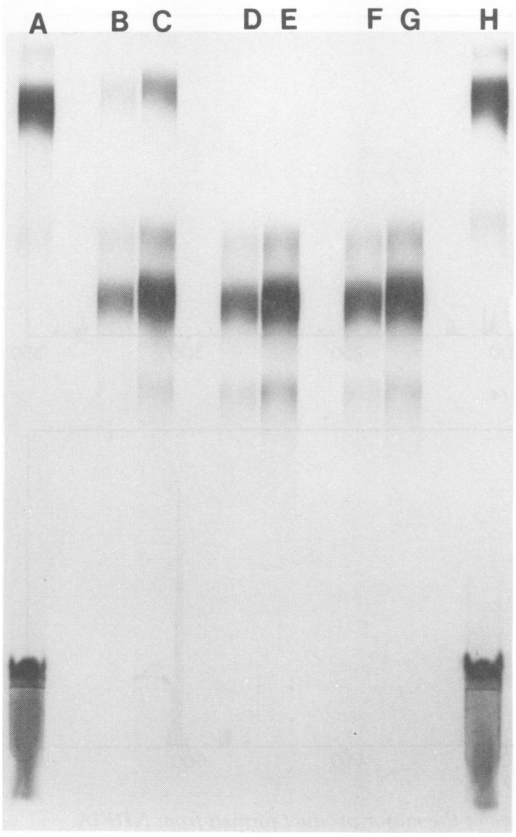

FIG. 6. *Autoradiogram of NDElA decomposition products. (A) Authentic DElA (at bottom of chromatogram) and NDElA (second from top of same chromatogram); (B and C) two samples of North Lake water; (D and E) two samples of Cayuga Lake water; (F and G) two samples of sewage; (H) single chromatogram with authentic DElA and NDElA.*

the products formed after 21 days from NDElA in samples of the three environments. Based on R_f values, it appears that the same degradation products were present in the three environments, and they were produced in approximately the same proportions relative to one another.

Attempts to identify these products by combined gas chromatography-mass spectrometry have been unsuccessful. The fragmentation pattern produced by electron impact of the most abundant NDElA degradation product is shown in Fig. 7. With electron impact, the trimethylsilyl derivative of this product yielded a peak at *m/e* 603, which results from the loss of 15 atomic mass units (CH_3) from the molecular ion. Chemical ionization, with methane as the reactant gas, was used to confirm that the mass of the original trimethylsilyl-derivatized product was 618. Computer analysis of the mass spectrum showed that there are three or four trimethylsilyl groups per molecule, and the molecule was probably symmetrical or a ring structure. One of the other trimethylsilyl-derivatized products also had a mass of 618, and a third product, when derivatized, had a mass of 632.

DISCUSSION

Based on the results observed for NDElA disappearance in the lake water and sewage samples, microorganisms are responsible for destruction of the nitrosamine. The seasonal variations in NDElA degradation appear to be caused by different factors in the two lakes. In North Lake, the fact that only partial degradation of the nitrosamine was observed in the September sample suggests that depletion of an essential nutrient occurred in the water sample. This might not occur under natural conditions because the lake receives additions of nutrients from the adjacent land areas. Incomplete destruction of another nitrosamine in sewage has been reported (15). The reason that NDElA at 50 pg/ml was not metabolized in the September water sample is unknown. In Cayuga Lake, a partial disappearance of the nitrosamine was never observed. Instead, increasingly long periods of time were required for the nitrosamine to disappear as the lake was subjected to the fall and winter seasons. Because the rates of decomposition were approximately the same in the September and November samples once active breakdown was initiated, a nutrient limitation does not seem to be the cause of the increased time for destruction of the nitroso compound. Presumably, the microorganisms responsible for NDElA metabolism were most abundant in the summer, after which the population declined through the fall until, in January, the population density was so low that none of the microorganisms was sampled. These experiments demonstrate that, although NDElA can be metabolized in freshwater, it may persist in the winter months.

Tate and Alexander (15) found that the dimethyl-, diethyl-, and dipropylnitrosamines were persistent for up to 108 days in Cayuga Lake water. On the other hand, the results of this work show that NDElA was destroyed readily under the proper conditions. The differences in findings may result from the fact that *N*-nitrosodialkanolamines are intrinsically more susceptible to microbial attack than *N*-nitrosodialkylamines, or the differences may result from the use by Tate and Alexander (15) of lake water collected in late winter or early spring, such waters not supporting rapid disappearance of the nitrosamines, as was here observed for NDElA persistence in the January water samples.

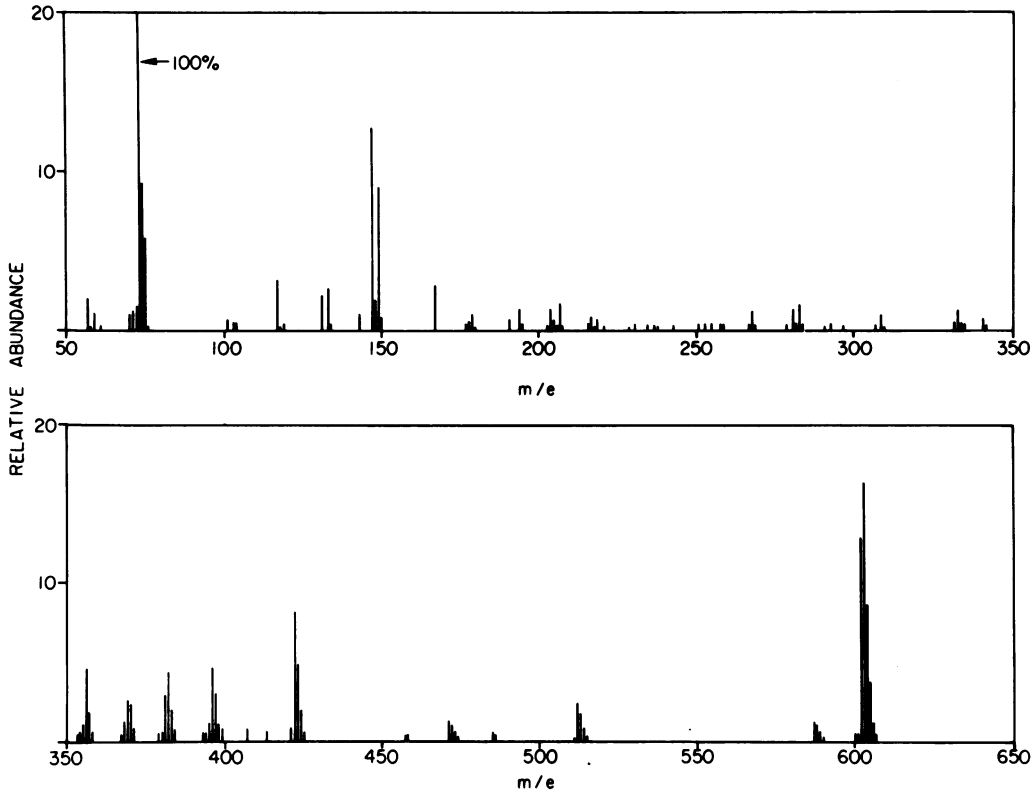

FIG. 7. *Mass spectrum of trimethylsilyl derivative of the major product formed from NDElA.*

The disappearance of NDElA and the appearance of products were apparently linear and not logarithmic processes. The linear rate of destruction may reflect the absence of microbial growth at the expense of NDElA. The lack of growth with the consequent absence of population increase may explain the relatively long period of time required for the destruction of only 1.1 μg of NDElA per ml. The reaction would thus appear to be cometabolic. However, the linear rate may have resulted from the responsible microorganisms being auxotrophs and the essential growth factors being made available to them at a linear rate.

The products formed from NDElA persisted in the lake water samples for relatively long times, and no CO_2 was produced from them for up to 55 days. The persistence of these organic products in lake water may be the result of the lack of a suitable organism or an essential nutrient in the lake water. In sewage, NDElA mineralization appears to involve two types of microbial populations, one converting NDElA to the organic products, a second oxidizing these compounds to CO_2. Mineralization of the products generated from NDElA also was a linear process. The length of time required for disappearance of these products suggests that, with the short residence time in a sewage treatment plant, they would be discharged into lakes and streams, where they would persist.

The identities of the organic products have not been established. Allowing for four trimethylsilyl groups per molecule, the molecular weight of the product is still more than twice the molecular weight of NDElA. This suggests the molecule may be a dimer of the NDElA and is probably in a more oxidized state than the original NDElA. It is not known whether the nitroso group is present in these products.

Because of the ease of formation of NDElA from DElA in freshwater and sewage (Yordy and Alexander, submitted for publication) and the enormous amounts of DElA that are used and undoubtedly discharged into inland waters and sewage, the present findings on the behavior of the carcinogen are of special significance. It is clear that NDElA can be metabolized in lake water and sewage; however, the lake waters show a definite seasonal effect, which suggests the nitrosamine might not be modified in the winter months in northern climates. Although NDElA can be mineralized, this process, when it occurs, will be slow in aquatic environments,

and hence the carcinogen or products of its incomplete breakdown—the toxicity of which are unknown—can pose a significant environmental problem.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Ayanaba, A., and M. Alexander.** 1974. Transformations of methylamines and formation of a hazardous product, dimethylnitrosamine, in samples of treated sewage and lake water. J. Environ. Qual. **3**:83–89.
2. **Ayanaba, A., W. Verstraete, and M. Alexander.** 1973. Formation of dimethylnitrosamine, a carcinogen and mutagen, in soils treated with nitrogen compounds. Soil Sci. Soc. Am. Proc. **37**:565–568.
3. **Barnes, J. M.** 1974. Nitrosamines. Essays Toxicol. **5**:1–15.
4. **Fan, T. Y., U. Goff, L. Song, D. H. Fine, G. P. Arsenault, and K. Biemann.** 1977. N-nitrosodiethanolamine in cosmetics, lotions and shampoos. Food Cosmet. Toxicol. **15**:423–430.
5. **Fine, D. H., D. P. Rounbehler, A. Bounbehler, A. Silvergleid, E. Sawicki, K. Krost, and A. DeMarrais.** 1977. Determination of dimethylnitrosamine in air, water, and soil by thermal energy analysis: measurements in Baltimore, Md. Environ. Sci. Technol. **11**: 581–584.
6. **Khan, S. U., and J. C. Young.** 1977. N-Nitrosamine formation in soil from the herbicide glyphosate. J. Agric. Food Chem. **25**:1430–1432.
7. **Lijinsky, W., E. Conrad, and R. Van de Bogart.** 1972. Carcinogenic nitrosamines formed by drug-nitrite interactions. Nature (London) **239**:165–167.
8. **Magee, P. N.** 1978. N-nitrosodiethanolamine, p. 77–82. *In* IARC Working Group, IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, vol. 17. International Agency for Research on Cancer, Lyon, France.
9. **Magee, P. N., and J. M. Barnes.** 1967. Carcinogenic nitroso compounds. Adv. Cancer Res. **10**:163–246.
10. **Oliver, J. E., P. C. Kearney, and A. Kontson.** 1979. Degradation of herbicide-related nitrosamines in aerobic soils. J. Agric. Food Chem. **27**:887–891.
11. **Ross, R. D., J. Morrison, D. P. Rounbehler, S. Fan, and D. H. Fine.** 1977. N-nitroso compound impurities in herbicide formulations. J. Agric. Food Chem. **25**: 1416–1418.
12. **Rowland, I. R., and P. Grasso.** 1975. Degradation of *N*-nitrosamines by intestinal bacteria. Appl. Microbiol. **29**: 7–12.
13. **Sen, N. P.** 1972. The evidence for the presence of dimethylnitrosamine in meat products. Food Cosmet. Toxicol. **10**:219–223.
14. **Sen, N. P., D. C. Smith, L. Schwinghamer, and J. J. Marleau.** 1969. Diethylnitrosamine and other *N*-nitrosamines in foods. J. Assoc. Off. Anal. Chem. **52**:47–52.
15. **Tate, R. L., and M. Alexander.** 1975. Stability of nitrosamines in samples of lake water, soil, and sewage. J. Natl. Cancer Inst. **54**:327–330.
16. **Williams, D. T., F. Benoit, and K. Muzika.** 1978. The determination of N-nitrosodiethanolamine in cutting fluids. Bull. Environ. Contam. Toxicol. **20**:206–211.