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Identification of Nitroso Compounds from Biotransformation of 2,4-Dinitrotoluene

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The intermediates of microbial transformation of 2,4-dinitrotoluene by a mixed bacterial culture derived from activated sludge were identified as 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2-nitroso-4-nitrotoluene, and 4-nitroso-2-nitrotoluene. The biotransformation of 2,4-dinitrotoluene occurred only under anaerobic conditions with an exogenous carbon source. The two nitroso compounds were unstable and could be observed only at the early stage of 2,4-dinitrotoluene anaerobic biotransformation.

Disposal of ammunition wastewater is difficult and has caused environmental concern (4, 7). Compounds common in ammunition effluents, such as 2,4,6-trinitrotoluene, 2,4-dinitrotoluene (2,4-DNT), and related nitroaromatic compounds, are resistant to biological treatment processes such as the activated sludge system (1). Low levels of these compounds are also toxic to various forms of life, including fish (5).

Efforts have been made to investigate the metabolic fate of 2,4,6-trinitrotoluene (1, 4, 7). The literature suggests that 2,4,6-trinitrotoluene is transformed in biological systems into a variety of products without the cleavage of the aromatic ring. One of the major intermediates during the biotransformation of 2,4,6-trinitrotoluene and 2,4-DNT has been suggested as the nitroso compound (3, 4). However, the proposed intermediate nitroso compound has apparently eluded detection and identification. This paper describes the isolation and identification of the hypothetical nitroso intermediates during the biotransformation of 2,4-DNT. In addition, environmental factors affecting the formation of these nitroso compounds are discussed.

MATERIALS AND METHODS

Medium. The basal salts medium contained 0.5 g each of $NaNO_3$ and K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter of distilled water. The medium was not sterilized and was prepared immediately before initiation of the experiments. DNT was added separately at concentrations of 5 to 25 mg (in 1 ml of methanol) per liter as indicated.

Chemicals. 2,4-DNT, 2-amino-4-nitrotoluene (2-NH_2-4-NT), and 4-amino-2-nitrotoluene (4-NH_2-2-NT) were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. After repeated crystallization from acetone, the identity and purity of these compounds were confirmed by thin-layer chromatography, gas chromatography, and gas chromatography-mass spectroscopy. The nitroso compound, 2-nitroso-4-nitrotoluene (2-NO-4-NT), was synthesized via the oxidation of amines with Caro acid (6).

Inoculum. A fresh sample of municipal activated sludge was diluted 10-fold with distilled water (total volume, 1 liter) and maintained in a cyclone fermentor (2). One ml of benzene was added periodically as the carbon source, and this addition caused heavy bacterial growth as verified by phase microscopy. This inoculum was used in the initial experiments on 2,4-DNT transformation.

Culture conditions. The biotransformation experiments generally involved the use of six cyclone fermentors (2), three of which were operated under aerobic conditions and another three in an anaerobic environment. Two fermentors served as controls (aerobic and anaerobic), containing inoculum, DNT, and $HgCl_2$ (microbial inhibitor); another two fermentors contained only inoculum as a cell control; the two test fermentors included inoculum and DNT. Each fermentor contained 1 liter of basal salt medium, 100 ml of inoculum, with or without the spiked chemicals (5 to 25 mg of DNT $liter^{-1}$), or $HgCl_2$ (50 mg $liter^{-1}$). The fermentors were purged with a flow of air (aerobic) or nitrogen (anaerobic) 1 h before introducing the test compound. A gas flow rate of 20 ml min^{-1} was then maintained for each fermentor throughout the exposure time of 14 days.

Extraction and analytical procedures. A sample of 100 ml of culture broth was withdrawn from each fermentor at the start of the experiment and periodically thereafter. The broth was extracted with 4 × 10 ml of dichloromethane. If heavy growth was observed, the sample was first centrifuged at 15,000 × *g* for 20 min. The supernatant was extracted as above, and the cell pellet was extracted separately by using ultrasonication and several portions of dichloromethane. The extracts were transferred into hexane, evaporated to 2.0 ml, and analyzed on a Hewlett-Packard 5730A gas chromatograph fitted with a flame ionization detector and an electron captor detector. The column (2 mm by 180 cm) packing was Chromosorb W (AW-DMCS) coated with 3% OV-1, operating at 130 to 210°C (4°C min^{-1}). The mass spectra of both the metabolically produced and reference compounds were obtained on a Finnigan model 4000 or Carlo-Erbo gas chro-

TABLE 1. Anaerobic biotransformation of 5 mg of 2,4-DNT $liter^{-1}$

Day	% as 2,4-DNT control			
	2,4-DNT	2-NO-4-NT	4-NH_2-2-NT	2-NH_2-4-NT
0	81	0	0	0
1	82	0	0	0
2	28	20	24	3
3	8	7	27	20
7	6	0	8	10
10	5	0	3	3
14	0	0	1	1

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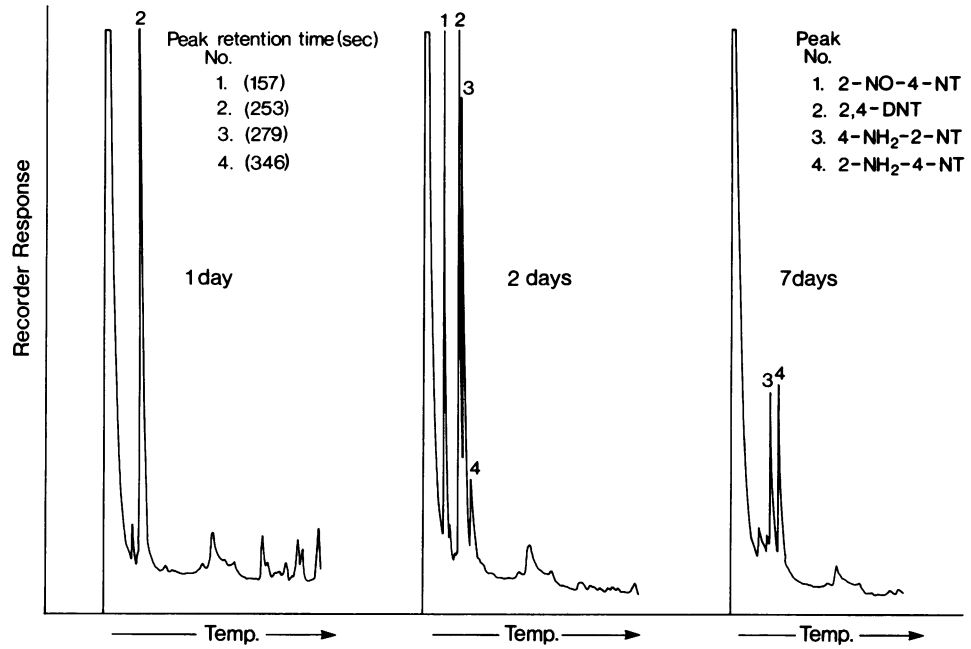

FIG. 1. Gas chromatograms of extracts from the anaerobic fermentor containing 5 mg of 2,4-DNT liter^{-1}.

matograph-mass spectrograph by using OV-1 or DB1710 capillary columns, respectively.

RESULTS

No breakdown of 2,4-DNT in the aerobic fermentors was observed, even after 14 days of incubation.

Table 1 and Fig. 1 show the results obtained with 2,4-DNT at 5 mg liter^{-1} after anaerobic incubation in the cyclone fermentors. 2,4-DNT was extensively biotransformed under the anaerobic conditions, with concurrent accumulation of three intermediates, i.e., 2-NO-4-NT, 2-NH_2-4-NT, and 4-NH_2-2-NT. However, these metabolic products disappeared with time, although their ultimate fates were not traced. The nitroso compound, 2-NO-4-NT, could only be detected at

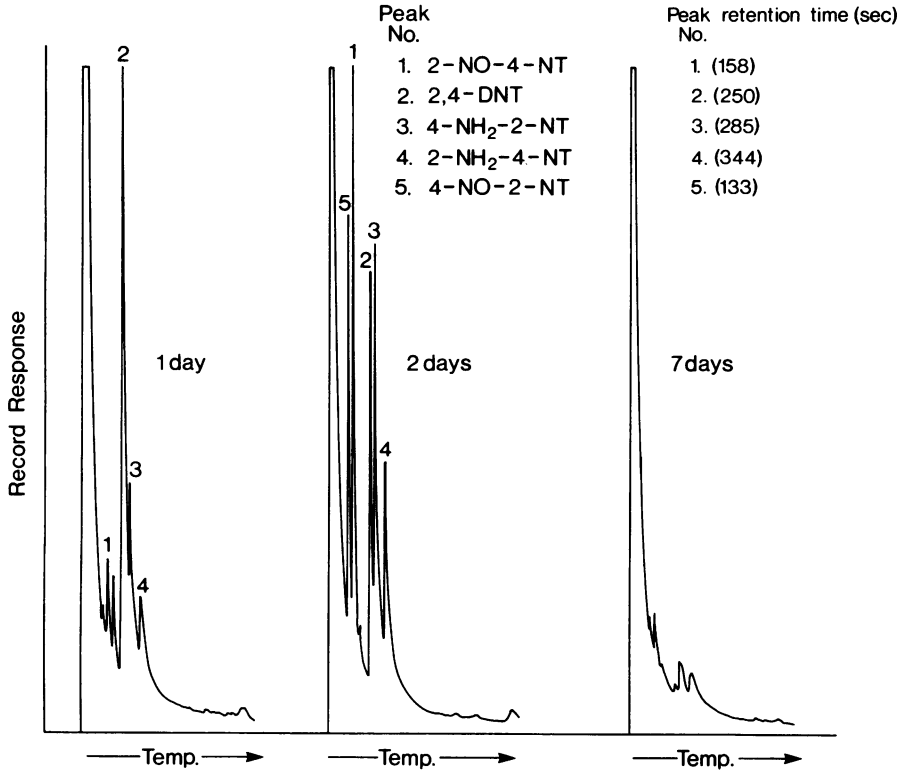

FIG. 2. Gas chromatograms of extracts from the anaerobic fermentor containing 25 mg of 2,4-DNT liter^{-1}.

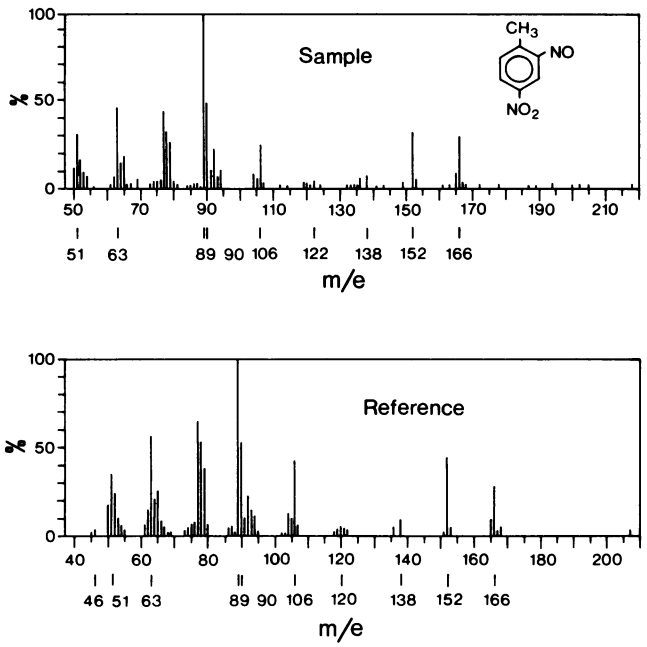

FIG. 3. Mass spectra of the nitroso intermediate 2-NO-4-NT.

the early stage of 2,4-DNT biotransformation, usually ca. 2 to 3 days after anaerobic incubation. The two amino intermediates (4-NH_2-2-NT, 2-NH_2-4-NT) were more stable and lasted almost throughout the entire 14 days of incubation. None of these intermediates were detected in any of the control fermentors.

To enhance the possibility of revealing the presence of additional 2,4-DNT biotransformation intermediates, which might have been present in very low concentrations, a higher concentration of 2,4-DNT (25 mg in 1 ml of methanol) was added to the fermentors. Again, no change was observed for 2,4-DNT in the aerobic fermentors. However, a new biotransformation product, which had not been observed before, was found in the anaerobic fermentor broth. This new intermediate was tentatively identified as 4-nitroso-2-nitrotoluene (4-NO-2-NT) as indicated by peak 5 in the gas chromatograms (Fig. 2).

Identities of the reference compounds and of the unknowns were confirmed by gas chromatography-mass spectroscopy. Analyses of peak 3 (Fig. 1 and 2) showed a parent ion at *m/e* 152(M^+). In addition, peak 3 exhibited two major fragment ions at *m/e* 135 and 107, which were also exhibited by the reference compound 4-NH_2-2-NT, thus confirming peak 3 as 4-NH_2-2-NT. The mass spectrum of peak 4 (Fig. 1 and 2) was found to display a parent ion at *m/e* 152(M^+) but with the absence of the ions at *m/e* 135 and 107, which is in agreement with the spectrum obtained from the reference compound 2-NH_2-4-NT, thus establishing peak 4 as 2-NH_2-4-NT. Peak 2 from the gas chromatograms (Fig. 1 and 2) was the remaining 2,4-DNT with a parent ion at *m/e* 182 (M^+).

Peak 1 (Fig. 1 and 2) was identified as 2-NO-4-NT, as the mass spectrum matched perfectly that of the reference standard 2-NO-4-NT prepared by Caro acid oxidation of 2-NH_2-4-NT (Fig. 3). Peak 5 of the gas chromatogram (Fig. 2) was probably 4-NO-2-NT (Fig. 4). Multiple attempts to synthesize this nitroso compound from 4-NH_2-2-NT failed. However, a comparison with the mass spectrum of 2-NO-4-NT revealed that 4-NO-2-NT has the same parent ions, with the addition to the spectrum of one OH loss. This same OH loss was also observed in the spectrum of 4-NH_2-2-NT and only occurred when there was a nitro group ortho to the methyl. From the agreement in the parent ion with the 2-nitroso isomer and the additional presence of the OH loss, it could be concluded tentatively that peak 5 was the nitroso compound 4-NO-2-NT.

DISCUSSION

Biological reduction of 2,4-DNT to 2-NH_2-4-NT and 4-NH_2-2-NT proceeds through the nitroso and hydroxylamino compounds. However, the proposed intermediate nitroso compounds have never been detected in culture broth (3). The apparent short life of these nitroso compounds under anaerobic conditions plus the aerobic stability of 2,4-DNT, as demonstrated in this study, may explain why these nitroso intermediates were not found in the aerobic cultures (3). For instance, 2-NO-4-NT could only be detected between 48 and 72 h in the anaerobic fermentor broth.

Biological reduction of 2,4-DNT by anaerobic cultures appears to be energy dependent. When dimethyl sulfoxide was substituted for methanol as the solvent carrier for 2,4-DNT, no breakdown of 2,4-DNT was noted in either aerobic or anaerobic fermentors. Methanol is a good substrate for anaerobic microorganisms and has been successfully utilized to stimulate microbial activity in the anaerobic digester from sewage treatment plants.

It should be noted that, with an abundance of cell growth, microorganisms sometimes are capable of producing a local meso-aerobic condition within an aerobic culture. Such a situation could also facilitate the biological reduction of nitroaromatic compounds. In one of these experiments, the air supply to the aerobic fermentor was accidentally low-

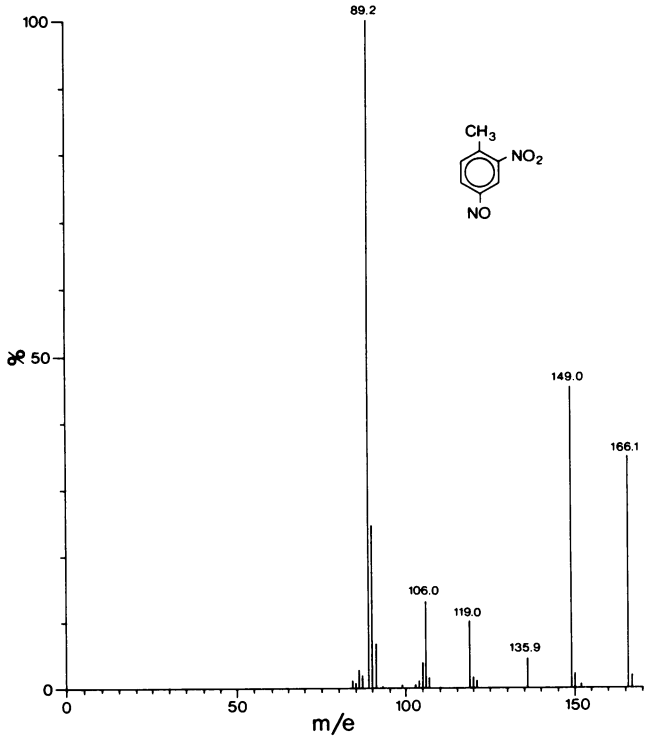

FIG. 4. Mass spectra of the nitroso intermediate 4-NO-2-NT.

ered, and reduction of 2,4-DNT with accumulation of 2-NH_2-4-NT and 4-NH_2-2-NT was observed. However, no trace amount of the nitroso compounds could be detected, implying that the nitroso intermediates could be formed only under anaerobic or meso-aerobic conditions, but they might be immediately transformed. Thus, in general, an anaerobic culture environment is a prerequisite for the formation of the nitroso intermediates during biological reduction of 2,4-DNT.

During biodegradation studies, much attention is often paid to the test medium and inoculum, but frequently there is a failure to appreciate the importance of the degradation environment under which the microorganisms carry out their work. These experiments indicate that for 2,4-DNT, anaerobic conditions are preferred for biotransformation. Under such conditions, a variety of biotransformation intermediates, including 2-NH_2-4-NT, 4-NH_2-2-NT, 2-NO-4-NT, and 4-NO-2-NT, was isolated and identified. No biotransformation products were detected under aerobic culture conditions.

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