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Transformation of Nitrosamines in Soil and *in Vitro* By Soil Microorganisms*

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N-nitroso compounds have been found to be highly toxic and carcinogenic to laboratory animals at mg/kg level (WOLF and WASSERMAN 1972). Some of these compounds have been detected in fish (SEN et al. 1972), bacon (SEN et al. 1973), alcoholic beverage (MADUAGWU and BASSIR 1979) and polluted air (FINE et al. 1976).

The formation of nitrosamines in natural ecosystems in presence of high concentrations of secondary amines and nitrite has been demonstrated (PANCHOLY 1978). Secondary amines occur widely among plants (SMITH 1971) and ultimately find their way into soil. During decomposition, certain pesticides (thiram, eptam, vernam) are also known to produce secondary amines (TATE and ALEXANDER 1974). There exists the possibility that nitrite concentration may attain high levels at localized microsites in soil to effect nitrosation of secondary amines.

The formation and presence of nitrosamines in soil and water may not be hazardous if they are decomposed before entering into the water system, and are not accumulated. Although formation of nitrosamines in various ecosystems has been studied extensively, information on their decomposition is meagre.

This report is concerned with transformation of dimethylnitrosamine (DMNA), diphenylnitrosamine (DPNA), and nitrosopiperidine (NPYR) in soil, factors affecting transformation of DMNA, and ability of selected soil microorganisms to transform DMNA.

MATERIALS and METHODS

Three soil types used were a sandy loam, a silt loam, and a clay. The soil samples were air dried, sieved through a 2 mm screen and stored at 10-15 C until needed. Soil pH was determined by glass electrode using 1:1 ratio of soil and water, soil texture by the hydrometer method (BOUYOUCOS 1951), and organic matter content by the modified Scholenberger method (ALLISON 1965). The properties of the soil types used are given in Table 1.

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TABLE 1

Analyses of Soil Samples

Soil type	pH	Percent composition			Organic matter (%)
		Sand	Silt	Clay	
Sandy loam	4.8	70	20	10	2.16
Silt loam	7.2	32	42	55	4.50
Clay	6.8	22	25	52	7.40

A portion of the sandy loam soil was amended with pulverized wheat straw at ratio of 95:5 (soil:wheat straw, w/w). Ten grams of unamended or amended soil were dispensed into test tubes (20 X 150 mm). Each soil tube received 25 µg of nitroso-N/g of soil as one of the three nitrosamines--DMNA, DPNA or NPYR. Sufficient distilled water was added to the tubes to obtain moisture content at field capacity. The soil tubes were incubated in the dark at 30 C and analyzed for nitrite, residual nitrosamines and dimethylamine (DMA) (where DMNA was used) at 0, 5, 10, 15, 20 and 30 days of incubation.

The contents of the tube were transferred to a micro-Kjeldahl flask with addition of water to bring to a final volume of 20 ml. Two milliliters were removed for the determination of NO_2 by the method of MONTGOMERY and DYMOCK (1961). The remaining portion was steam distilled (HEATH and JARVIS 1955), and nitrosamine content was determined in the distillate by the photochemical method of DAIBER and PREUSSMANN (1964). DMA was measured by the colorimetric method of DOWDEN (1938).

The effects of temperature, water logging, and possible role of soil microflora in the transformation of DMNA were studied in sandy loam soil using ^{14}C-DMNA (obtained from New England Co.). To 10 g of soil were added 0.01 µCi ^{14}C-DMNA plus 100 µg unlabelled DMNA and 4 ml of distilled water. For the determination of temperature effect the soil tubes were incubated at 4, 25 or 37 C. In order to study the effect of soil texture, two additional soil types, e.g. a silt loam and a clay were used. To assess the role of soil microflora in decomposition of DMNA, ^{14}C-DMNA was added to the soil tubes previously sterilized by autoclaving three times, for 2 hr. each, and to non-autoclaved soil. The tubes were incubated in the dark and the contents were analyzed for residual ^{14}C-DMNA and ^{14}C-DMA produced as a result of breakdown of the nitrosamine at 0, 5, 10, 20 and 30 days of incubation.

The contents of the tubes were steam distilled as described earlier. The distillate was extracted with CH_2Cl_2 in a separatory funnel (TESFAI et al. 1977); the CH_2Cl_2 fraction was used for the determination of DMNA content, and the water fraction for DMA content. One milliliter of either CH_2Cl_2 or water fraction was added to 9 ml of "Ready-Solv EP" scintillation cocktail (Beckman) for counting of residual ^{14}C-DMNA and ^{14}C-DMA, produced as a result of breakdown of DMNA.

Escherichia coli, Enterobacter cloacae, Pseudomonas sp., Ps. fluorescens, Serratia marcescens, Staphylococcus aureus and Streptomyces sp. were tested under axenic culture condition for their ability to degrade DMNA. The inoculum grown on nutrient broth for 18-20 hr was harvested by centrifugation, washed twice in 0.1 M phosphate buffer (pH 7) to obtain a cell density of approximately 4 X 10^8/ml. Five milliliters of the bacterial suspension were introduced into a flask containing 15 ml of a mineral salts solution supplemented with glucose at 0.1%. The salt solution was composed of KH_2PO_4, 5.5 g; K_2HPO_4, 10.0 g; $MgSO_4$, 0.1 g; dist. water, 1000 ml. Filter sterilized DMNA solution was added at 25 µg nitroso-N/ml. Three replicates were used in each case. The flasks were incubated in the dark at 30 C on a rotary shaker at 100 rpm for 20 hr. and the contents were analyzed for residual DMNA and DMA formed as a result of transformation of DMNA by the methods described earlier.

In an effort to isolate microorganisms that can degrade and utilize the nitrosamine as source of nitrogen, enrichment culture was set up with 10 g soil in a flask amended with DMNA at 25 µg nitroso-N/g soil. The triplicate flasks thus prepared were covered with aluminum foil to exclude exposure to light and incubated aerobically at 30 C for 7-15 days. A small portion of the soil from the enrichment culture was suspended in water; a loopful of the suspension was streaked on agar plate containing DMNA and salt solution fortified with glucose at 0.2% and micronutrients ($CaCl_2$ (anhydrous), 15.0 mg; $Fe_2(SO_4)_3$, 0.6 mg; $ZnSO_4$, $7H_2O$, 0.2 mg; $CuSO_4$, $5H_2O$, 0.2 mg; $MnSO_4$, 0.2 mg; dist. water, 1000 ml). Filter sterilized DMNA was added at 25 µg nitroso-N/ml. Any colony that appeared on the plate was transferred to a fresh plate of the same composition. Cultures were maintained on the same medium.

RESULTS and DISCUSSION

In unamended sandy loam 17% of added DMNA was lost in 10 days incubation, thereafter, no further loss was noted during the next 30 days incubation period. Under similar conditions NPYR and DPNA continued to be degraded, and 33 and 68% respectively were lost at the end of 30 days incubation period (Fig. 1). The quantity of NO_2^- recorded during the incubation period did not correlate with quantity of the nitrosamines lost because of transformation of NO_2^- through ubiquitous nitrification and denitrification processes in soil. DMA did not accumulate because it is metabolized with relative ease as pointed out by TATE and ALEXANDER (1976), and MOSIER and TORBIT (1976), and PANCHOLY (1978).

In soil amended with wheat straw, which increased organic matter content from 2.16 to 17.5%, disappearance of the nitrosamines was accelerated substantially; by day 15 over 60% of added DMNA was lost, DPNA disappeared completely by day 10, and about 50% of NPYR was lost in 30 day incubation period (Fig. 1). Low recovery of NPYR at 0 day could not be explained.

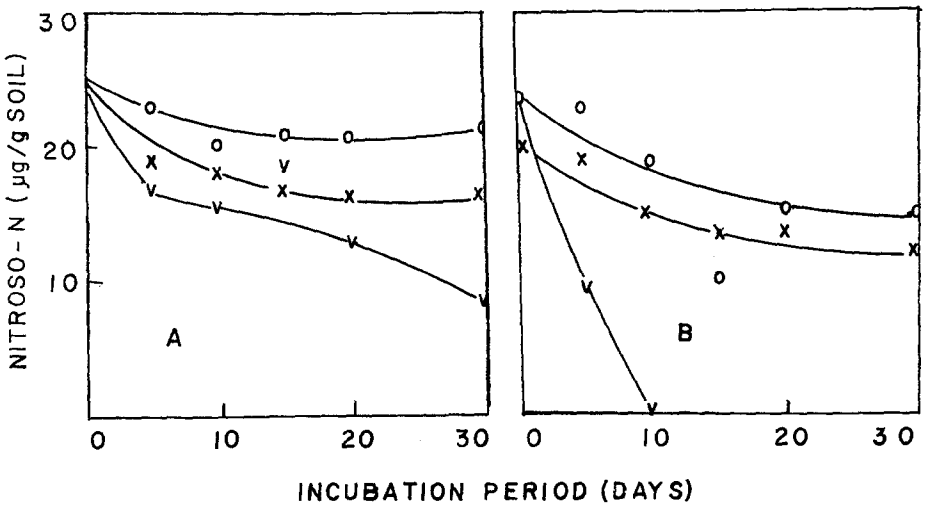

FIGURE 1. Disappearance of DMNA (o), NPYR (x), and DPNA (v) from sandy loam soil unamended (A) and amended (B) with wheat straw.

DPNA disappeared faster both in unamended and in amended soil than the other two nitrosamines. ROWLAND and GRASSO (1975) found that DPNA was more degradable than either DMNA or NPYR by intestinal bacteria, which conforms with our results in soil. Our data indicate that DMNA is relatively stable in sandy loam soil; TATE and ALEXANDER (1976) reached similar conclusions using silt loam. The higher rate of loss of all three nitrosamines in amended soil than in unamended soil was apparently due to higher microbial activity resulting from added organic matter. However, the possibility of adsorption of a part of the nitrosamines by the soil organic matter could not be ruled out.

Effects of temperature, waterlogging, and role of soil microflora in decomposition of DMNA was studied by scintillation counting methods; recovery of added ^{14}C-DMNA by this method was 90%. At all three incubation temperatures about 20-30% of added DMNA disappeared in first 20 days of incubation, but little loss was noted thereafter, and even after 30 days of incubation over 50% of the added DMNA was retained. The rate and quantity of DMNA lost appeared not to be influenced by incubation temperature (Fig. 2A). The disappearance of DMNA was slightly higher in sandy loam soil than in either clay or silt loam soil. In general the influence of soil texture in the rate of degradation of DMNA was minimal (Fig. 2B).

The yield of ^{14}C-DMA, initial product of degradation of ^{14}C-DMNA, steadily increased from 2-3% to 25-30% in 30 days incubation (Figs. 2 A,B).

The longevity of DMNA in waterlogged soil was compared with that in soil with moisture content at field capacity. To one set of soil tubes was added sufficient distilled water so that water level stood at least 2 cm above the soil level. This created

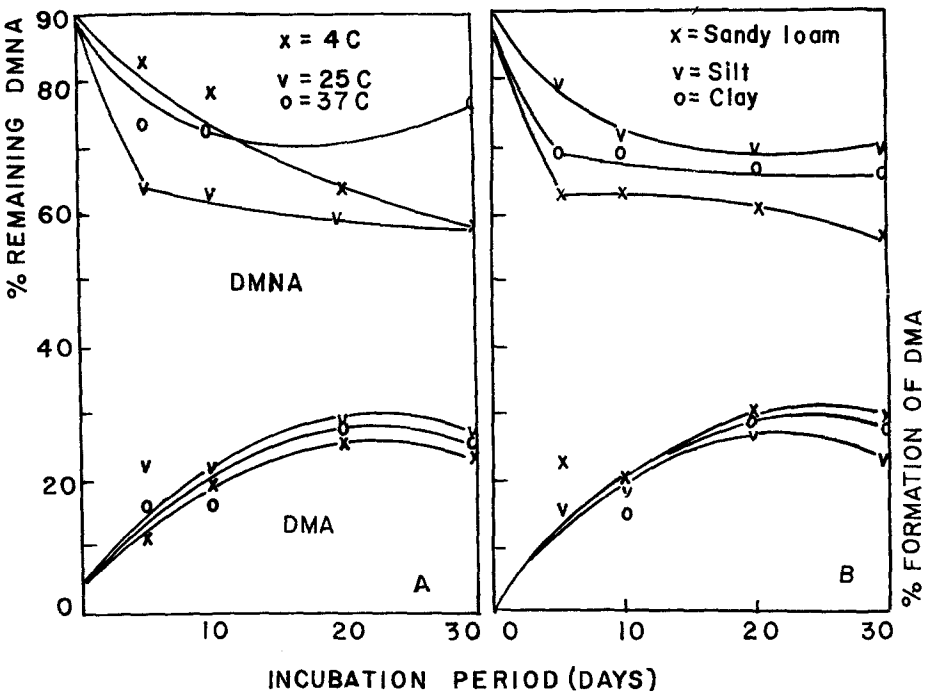

FIGURE 2. Disappearance of DMNA and formation of DMA in sandy loam soil incubated at different temperature (A), and in different soil types (B).

anaerobic condition. The other set of the soil tubes was maintained at field capacity. In both conditions about 60-70% of added DMNA were retained at the end of 30 days incubation period (Fig. 3A). However, the rate of loss was higher at field capacity than at waterlogging condition. The results indicated that the soil with better aeration favors the degradation of DMNA than soil under waterlogging condition. These findings are in agreement with MOSIER and TORBIT (1976) who found that in cattle manure 61% of added DMNA was lost after 28 days of incubation when the sample was kept continuously stirred (increased aeration), whereas under static condition that loss was only 28% during the same period. TATE and ALEXANDER (1976) also noted very little loss of DMNA from flooded soil.

The loss of DMNA was higher in non-autoclaved than in autoclaved soil (Fig. 3B). The disappearance of DMNA from autoclaved soil indicated the involvement of some abiological factors in the process. The adsorption of DMNA by soil micell and organic matter particles may play a role in this respect. MILLS and ALEXANDER (1976) noted little formation of DMNA in soil if organic matter is destroyed. Autoclaving of soil could have altered the soil structure resulting in some adsorption of DMNA.

Enrichment culture was set up with the soil amended with DMNA for isolation of microorganisms that could possibly utilize DMNA as nitrogen source. Although bacterial colonies developed

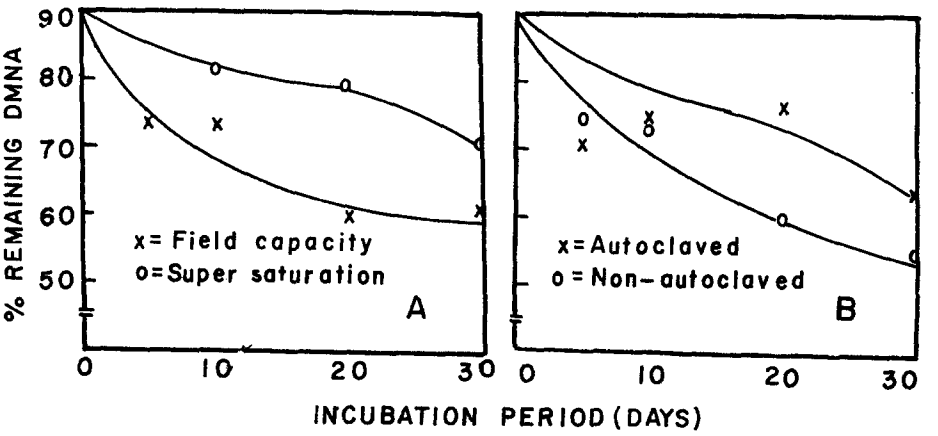

FIGURE 3. Disappearance of DMNA in sandy loam soil held at two different moisture content (A), and in autoclaved and non-autoclaved soil (B).

on agar plates containing salt solution supplemented with glucose and DMNA, growth in broth of the same composition was negligible; gram stained slide revealed that majority of these bacteria were gm - short rod, and few were gm + cocci.

Seven microbial cultures were tested for their ability to degrade DMNA. Formation of DMA, and loss of DMNA in the culture fluid were taken as criteria of DMNA degradation.

The recovery of DMNA was 91-94% from the culture fluid of all except E. coli and S. marcescens where the percent recoveries were 79 and 83 respectively (Table 2). It appears that soil microorganisms in general are capable of degrading DMNA with extreme sluggishness. Working with intestinal bacteria ROWLAND and GRASSO (1975) came to the conclusion that majority of intestinal bacteria can degrade nitrosamines.

TABLE 2

Degradation of DMNA by Selected Microorganisms

Microorganism	% DMNA recovered	DMA produced (g/ml)
Escherichia coli	79	1.6
Enterobacter cloacae	93	0
Pseudomonas fluorescens	94	0
Ps. fluorescens[1]	74	1.1
Pseudomonas sp.	91	0
Staphylococcus aureus	94	0
Serratia marcescens	83	0
Streptomyes sp.	92	0
Streptomyces sp.[1]	62	0

[1] Inoculum grown in presence of DMNA.

In one experiment Ps. fluorescens and Streptomyces sp. were acclimatized to DMNA by growing in salt solution supplemented with glucose, $(NH)_2SO_4$ and DMNA for seven days with three changes prior to inoculum preparation. Lower DMNA recovery from the culture fluids of these two acclimatized cultures compared with their parent cultures suggests involvement of an adaptive enzyme in the degradation of DMNA which is contrary to the findings of ROWLAND and GRASSO (1975).

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