Fixation of Nitrite Nitrogen during the Humification of α-Naphthol in Soil Suspensions

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The mechanism by which nitrite nitrogen is fixed on α-naphthol in buffered soil suspensions was examined and the role of microorganisms in these reactions verified. The infrared analysis of the humic matter extracted from normal and sterile soil suspensions suggested the formation of nitro and nitroso aromatic compounds. Nitro- and nitrosonaphthol were identified by gas chromatography and mass spectrometry. Upon prolonged incubation, reduction of nitro and nitroso aromatics was occurring in both types of soil suspensions. The GC–MS results indicated the formation of 1-azido-3- or -4-nitrobenzene and 1-amino-3- or -4-nitrobenzene. After 40 days, the formed nitro and nitroso aromatic compounds disappeared completely. In the case of microbially active soil suspensions, the nitro and nitroso compounds formed were more diverse and their transient levels were higher.

The reactions between nitrite and soil phenolic compounds have been studied before by various authors. Bremner (1957) and Kainz and Huber (1959) assumed formation of *p*-nitrosophenols between NO_2^- and phenolic compounds in the soil. Bremner and Nelson (1968) and Nelson and Bremner (1969, 1970) reported that phenolic compounds in solution, buffered at pH 5.0, reacted with NO_2^- at different rates depending on the nitrosation degree of the compounds considered. Bremner and Fuhr (1966) and Stevenson and Swaby (1964) have assumed that the lignin-derived fractions of soil organic matter are responsible for binding NO_2^- on the phenolic constituents of soil organic matter to form nitrosophenols. Fixation has also been investigated in several soils differing in pH and organic carbon with ^{15}N-labeled NO_2^- (Smith and Chalck, 1980).

Humification may be defined as a process in which various organic molecules during the process of decay interact via free radicals to form stable phenol heteropolymers (Flaig et al., 1975; Mathur and Schnitzer, 1978). There is little information about the mechanism of humification of simple phenols or about the yields and chemical structures of humic acids formed as a result of such reactions (Schnitzer et al., 1984).

Recently, Azhar et al. (1986a–c) have suggested that the nitrification intermediate nitrite reacts with phenolic compounds present in the soil humic matter to form organic nitrogen. In view of this, the mechanism by which nitrite nitrogen is fixed on α-naphthol in buffered soil suspensions was examined and the role of microorganisms in these reactions verified. The nitro and nitroso aromatic compounds produced from these primary products in both normal and sterile soil suspensions were analyzed.

MATERIALS AND METHODS

Laboratory Incubations. Model experiments for the nitrosation reactions were set up with 1-L Erlenmeyer flasks containing 100 g of grassland soil (location Gistel, Belgium) and 500 mL of phosphate buffer solution. The Gistel soil has the following characteristics: pH (H_2O) 6.5; sandy clay; CEC 14.4 mequiv/100 g. The soil suspension was dosed with 0.5 g of α-naphthol/L, and 10 mg of NO_2-N/L was added every other day. The treatments were (1) normal soil suspension and (2) sterile soil suspension. In the latter case the contents of the Erlenmeyer flasks were prior to the addition of nitrite autoclaved at 120 °C for 30 min. The NO_2^- solution was separately autoclaved. The final pH of the buffered soil suspension was 7.2. The sterility of the autoclaved soil suspension was verified by regular plate counting.

Table I. Valence Vibration Ranges of Nitro and Nitroso Groups in Different Positions (Pouchert, 1971)

peak	freq, cm^{-1}	nitro or nitroso group in different positions
a	1055–950	nitroso (N–O) stretching vibration of oxime
b	1110	para hydroxy nitroso group
c	1250	amino group situated ortho to the nitro function
d	1265	nitroso dimer in trans form
e	1315	amino group situated para to the nitro group function
f	1335	ortho hydroxy nitroso compound
g	1350	symmetric stretching aromatic nitro group
h	1410	nitroso dimer in cis form
i	1515	monomeric aromatic nitroso compound (N=O) stretching vibration
j	1540	asymmetric stretch aromatic nitro group
k	1615	C=N band of azo group
l	2050–2160	azido group (N_3)

Humic Matter Extraction. A 50-mL portion of soil suspension was taken from every flask. NaOH was added up to 0.5 N to extract the humic matter (Sequi et al., 1975). The samples were put in the shaker for 2 h at room temperature. Undissolved materials were removed by centrifugation. The supernatant was poured into a watch glass and dried at 40 °C. The samples were subsequently stored at 22 °C in a desiccator.

Infrared Spectra. A pellet suitable for infrared analysis was prepared by grinding 2.5 mg of the humic matter extract with 200–250 mg of dry potassium bromide. The IR spectra were recorded with a Perkin-Elmer 1310 IR spectrometer (4000–650 cm^{-1}). As a reference, the typical absorption bands for various nitro and nitroso groups configurations are given in Table I. From this it can be seen that in the presence of nitro and nitroso groups various absorbance peaks can show up, but a drop in transmittance should occur particularly in the 1300–1550-cm^{-1} range (Bellamy, 1960).

Gas Chromatography–Mass Spectroscopy. The humic matter extract was also analyzed by high-resolution capillary gas chromatography combined with mass spectroscopy. In the humic matter extracts, the phenolic

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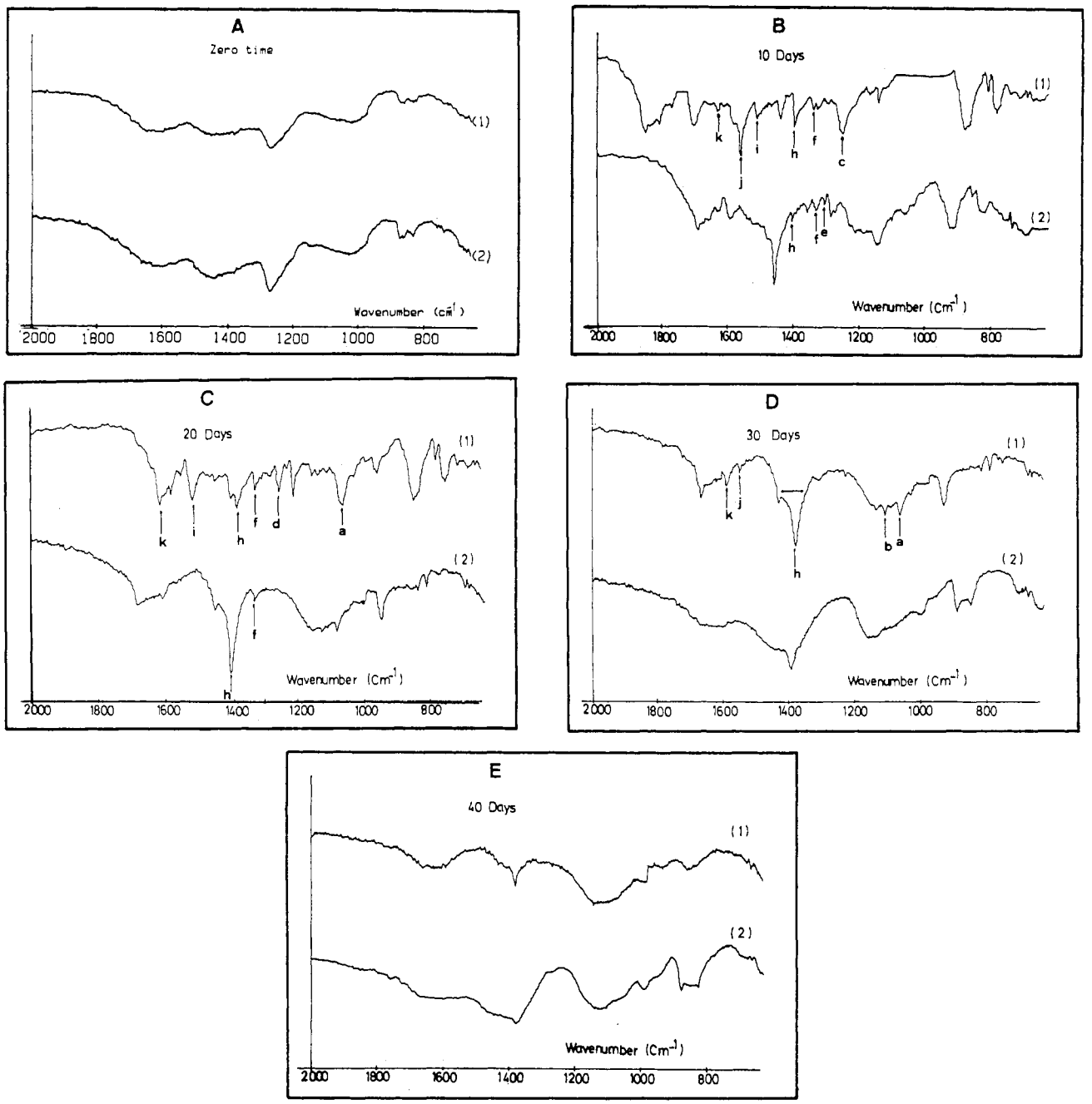

Figure 1. IR spectra of the humic matter extracted from the soil suspensions: A = zero time, B = 10 days, C = 20 days, D = 30 days, E = 40 days. Key: (1) normal soil buffer suspension amended with naphthol and nitrite; (2) sterile soil buffer suspension amended with naphthol and nitrite. For peak identification, see legend of Table I.

compounds are most probably present in their sodium form, as the extract was obtained by alkaline treatment (0.5 N NaOH) of the soil suspension. A 100-mg portion of the humic matter extracted from a treatment was placed in a binary mixture of 20 mL of hydrochloric acid (0.25 N) and 4.0 mL of dichloromethane. Hydrochloric acid was added to convert the salts of the phenolic compounds into the pure phenols. Two glass beads were added to the sample, and the mixture was rotated for 15 min. The vessel was then placed in an ultrasonic bath for 2 min. The dichloromethane layer was collected and concentrated to 1 mL under a stream of nitrogen. A 1-μL portion was injected in the gas chromatograph. The samples were analyzed on a 18 m × 0.32 mm capillary column coated with OV-1 (0.3-μm df). The column was installed in a Carlo Erba 4160 gas chromatograph equipped with a cold on-column injection system and FID detector. The samples were introduced at 35 °C. The column was ballistically heated to 100 °C and then temperature programmed to 200 °C at 3 °C/min.

The identity of the major compounds was obtained by introducing the capillary column directly into the source of a quadrupole mass spectrometer, working in the electron impact mode (Finnigan 4000). The instrument is equipped with a Nova 6000 data system.

RESULTS

Infrared Analysis. The analysis of the humic matter extracted from soil at zero time (Figure 1A) showed no distinguished frequency fall in the range of nitroso group absorbance. Only a weak vibration was observed at 1280 cm^{-1}. The latter could be due to the presence of C–O vibrations.

The humic matter extracted from both soil suspensions showed different spectra upon incubation. Clear vibrations were observed at the range of nitro or nitroso group vi-

Table II. m/z Values of the Compounds Found

peak	compound	m/z (%)
1	nitrobenzene	123 (72), 93 (15), 77 (100), 65 (15), 51 (60)
2	1-azido-3- or -4-nitrobenzene	164 (18), 136 (29), 90 (33), 63 (100), 51 (52), 44 (19)
3	α-naphthol	144 (100), 115 (53), 116 (12), 81 (15), 57 (15), 63 (12)
5	nitrosonaphthol	
	isomer 1	173 (65), 156 (90), 143 (12), 128 (40), 115 (100), 101, 102 (30), 89 (30), 76 (40), 63 (45), 51 (50)
	isomer 4	173 (100), 156 (75), 145 (20), 128 (75), 115 (95), 101 (30), 89 (30), 76 (34), 63 (35), 51 (30)
	isomer 5	173 (95), 156 (100), 143 (20), 128 (40), 115 (75), 101 (25), 89 (20), 76 (25), 63 (30), 51 (30)
6	nitronaphthol	
	isomer 1	189 (100), 173 (10), 157 (10), 143 (10), 128 (20), 114 (50), 103 (10), 99 (25), 75 (18), 63 (20), 50 (12), 44 (20)
	isomer 2	189 (70), 173 (100), 156 (70), 144 (15), 128 (40), 115 (90), 101 (15), 89 (20), 75 (18), 63 (20), 51 (18)
7 and 8	1-amino-3- or -4-nitrobenzene	
	isomer 1	138 (100), 108 (45), 92 (35), 80 (15), 65 (68), 52 (10)
	isomer 2	138 (100), 108 (38), 92 (30), 60 (10), 65 (75), 49 (10)

brations. It can be concluded that nitro compounds (1540, 1350 cm^{-1}) and nitroso compounds and quinone oximes (1615, 1514, 1410, 1335, 1265, 1050 cm^{-1}) are present (Figure 1B–E).

GC and MS Results. The results obtained by GC and MS analyses showed that no nitro or nitroso compounds were present at zero time (data not shown). Analysis of the humic matter extracted from the normal soil suspension after 10-days incubation revealed the formation of the following compounds (Figure 2A): nitrobenzene (peak 1), 1-azido-3-nitro- or 1-azido-4-nitrobenzene (peak 2), some residual α-naphthol (peak 3), unknown compound (peak 4), nitrosonaphthol isomers (peak 5), and nitronaphthol (peak 6). The mass spectra of 1-azido-3-nitrobenzene and 1-azido-4-nitrobenzene are identical. The m/z values of the compounds mentioned are summarized in Table II. After 20 and 30 days of incubation (Figure 2B,C) the normal soil suspension showed the presence of the same compounds but with different levels. Peak 3, which is α-naphthol, had become proportionally smaller. Two new compounds appeared, i.e., peaks 7 and 8, whose identities were elucidated as 1-amino-3-nitro- or -4-nitrobenzene (Table II).

The sterile soil suspension showed after 10-days incubation the formation of nitrosonaphthol (peak 5) and some nitronaphthol (peak 6) (Figure 2A). After 20 and 30 days, the nitro- and nitrosonaphthol disappeared and the analyses showed only residual α-naphthol (Figure 2B,C).

After 40 and 60 days both samples showed no nitro or nitroso compound.

DISCUSSION

In soils, phenolic compounds produced through microbial synthesis and lignin degradation are thought to be the predominant monomeric constituents that are oxidatively coupled by indigenous enzymes to form stable humic substances (Martin and Haider, 1971). If such predominant products react with nitrogen, the result could lead to the formation of humic substances rich in nitrogen.

The present study was conducted to give a better understanding of the mechanisms by which nitrite nitrogen is fixed into phenolic compounds and of the formation of stabile humic substances rich in nitrogen in the soil.

The results obtained from both IR spectra and GC analyses indicated that no nitro or nitroso aromatic compounds were present at zero time. After 10 days of incubation, the IR spectra showed different frequency drops at the range of nitro and nitroso compounds. These results indicate that the nitrosation occurred between the added nitrite and α-naphthol (or α-naphthol degradation products) in both sterile and normal soil suspensions. Recent work by Ralt et al. (1988) showed that axenic cultures of *Escherichia coli* catalyze nitrosation of amines.

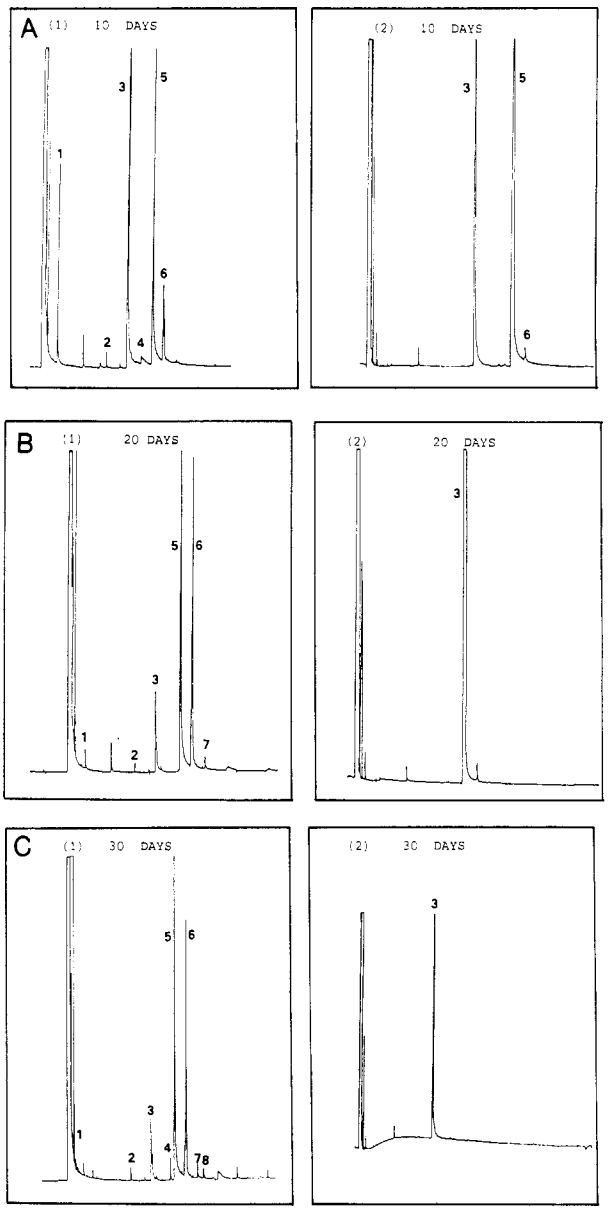

Figure 2. GC chromatogram of the humic matter after A = 10, B = 20, and C = 30 days of incubation. Key: (1) normal soil buffer suspension amended with naphthol; (2) sterile soil buffer suspension amended with naphthol. For peak identification, see legend of Table II.

Our results illustrate that the reactions between nitrite and phenolic compounds in the soil are not exclusively biochemical since the sterile incubation conditions did not prevent the formation of nitro and nitroso aromatic com-

Table III. Schematic Overview of the Phenomena Observed[a]

incubn time, days	normal soil suspension α	β	σ	sterile control α	β	σ
0	−	−	3	−	−	3
10	+	+	1–4 5, 6	+	−	3, 5, 6
20	+	+	1–3, 5 6, 7	−	−	3
30	+	+	1–8	−	−	3

[a] Key: α, nitrosation occurring; β, reduced nitrogen formation; σ, compound detected. Compounds: 1, nitrobenzene; 2, 1-azido-3-nitrobenzene or 1-azido-4-nitrobenzene; 3, α-naphthol; 4, unknown; 5, nitrosonaphthol; 6, nitronaphthol; 7 and 8, 1-amino-3- and/or -4-nitrobenzene.

pounds. The formed nitro and nitroso aromatics were identified by GC–MS as nitronaphthol and nitrosonaphthol isomers. However, in the case of microbially active soil suspensions, some additional compounds were detected. The GC–MS results indicated the formation of 1-azido-3-nitro- or 1-azido-4-nitrobenzene (Table II). These products must somehow result from microbial metabolism.

After 20 and 30 days of incubation the nitrosation reactions continue in the case of normal soil suspension, while they seem to have stopped in the sterile soil suspension. In the latter, only some nitrosonaphthol together with residual α-naphthol was present. No nitro- or nitrosonaphthol was detected in the sterile soil suspension (Table III). On the other hand, reactions continued to occur in the normal soil suspension, until 30 days of incubation. Naphthol had almost completely reacted at that time. In the case of normal soil suspension, the reduction of nitro and nitroso aromatic compounds was also occurring. An amino aromatic compound was isolated and identified as 1-amino-3-nitro- or -4-nitrobenzene. After 40 days, all the formed nitro and nitrosonaphthol, together with the biotransformation products, had disappeared.

The ability of biological systems to effect the disappearence of nitro and nitroso aromatic compounds has been investigated by several researchers. The initial reaction between a biological system and nitro aromatics is the stepwise reduction of the nitro groups to yield amino and hydroxylamino transformation products, which may then undergo additional reactions yielding azoxy compounds (Dale, 1921; Channon et al., 1944; Takahashi et al., 1963; Jensen and Lautrup-larson, 1967; McCormick et al., 1976). These reactions also occur in vitro with homogenates of beef heart (Westfall, 1954) or with cell-free extracts of *E. coli* (McCormick et al., 1976). It is worth mentioning that bacteria and fungi that catalyzed the disappearance of trinitrotoluene in an aerobic nutrient medium have been isolated from soil (Osmon and Klausmeier, 1972).

Carpenter et al. (1978) studied the fate of labeled trinitrotoluene in an activated sludge system. Their results indicated the disappearance of the aromatic nitro compound after 5 days from the aerated reactor. The major part of the ^{14}C was found in the precipitates. The solubility properties and infrared spectra of the precipitates suggested that they were macromolecular structures of the polyamide type formed by the reaction of the nitro aromatic biotransformation products with lipids, fatty acids, and protein constituents of the microbial biomass.

Sjoblad and Bollag (1981) and Martin and Haider (1980) reported that the oxidative enzymes present in soil act to link covalently phenolic compounds and aromatic amines and in the process form humic materials. These enzymes

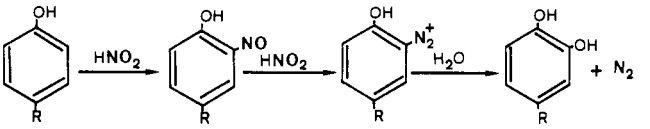

Figure 3. Reaction of phenols with nitrous acid (Nelson, 1982).

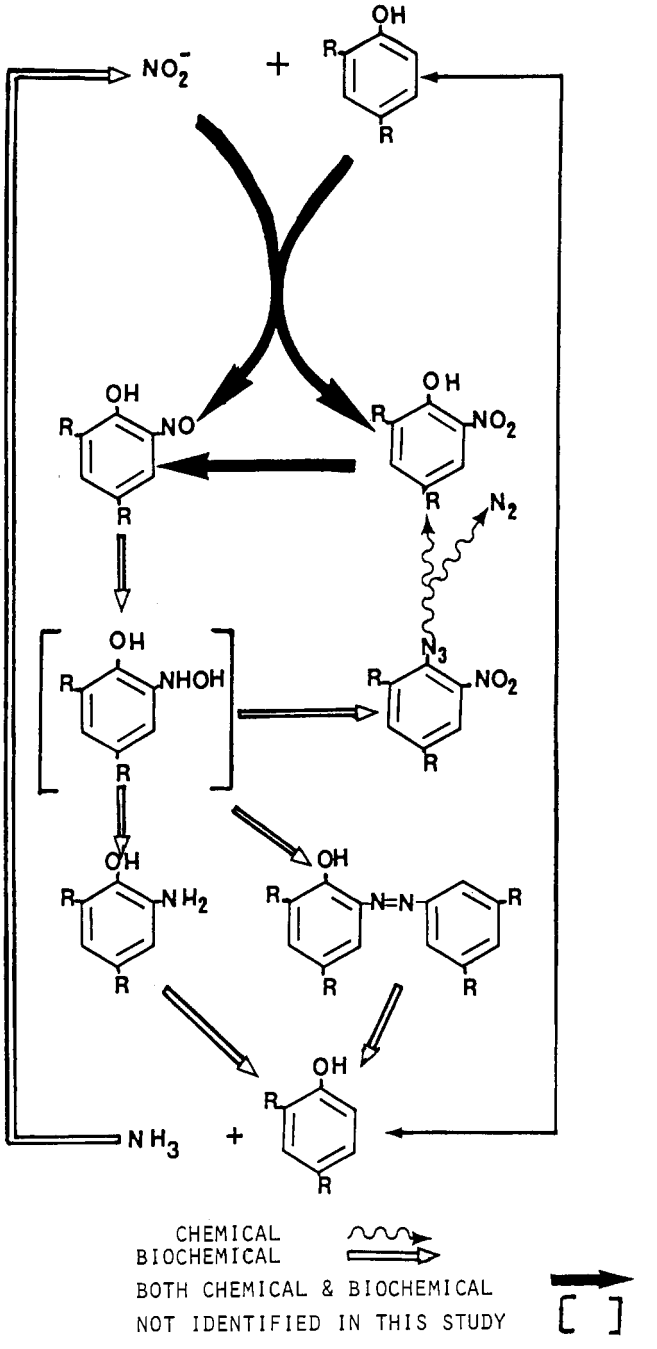

Figure 4. Proposed pathway for fixing nitrite on phenols and formation of humic heteropolymers.

belong to one of two groups, i.e., monophenol monooxygenases and peroxidases or lactases, which contain Cu and require O_2 for activity.

McCormick et al. (1976) found that a variety of nitro aromatic compounds were reduced by hydrogen in the presence of enzyme preparations from *Veillonella alkalescens*, consistent with the proposed reduction pathway $RNO_2 \rightarrow RNO \rightarrow RNHOH \rightarrow RNH_2$. The pattern of reduction depended on the species, on the atmosphere (air or H_2), and on the nitro reductase activity associated with the protein fractions, one having some ferredoxin-like

properties and the other possessing hydrogenase activity. *E. coli* and *Pseudomonas* FR_2, actively growing aerobically in the presence of 100 μg of TNT/mL, generated enough reducing potential to reduce two of the three nitro groups, but not the third. Resting cells of both organisms in air formed 2,2,6,6′-tetranitro-4,4′-azoxytoluene.

In the present study, nitrite is postulated first to be attached to the phenolic compound. Then the reduction of nitro or nitroso aromatic compounds can occur. Subsequently, oxidative enzymes present in the soil act to covalently link phenolic and aromatic amines, forming humic materials. The data of Berry and Boyd (1984) point in the same direction. These authors found that the enzymatic processes operative in soil act to couple phenols and aromatic amines. They determined the relative reaction rates of the various substrates by measuring their disappearance using high-performance liquid chromatography.

There has been, to our knowledge, no report before in the literature of an aromatic azido compound formed under natural conditions (Biffin et al., 1971). The isolation of an azidobenzene derivative from the normal soil suspension suggests that microorganisms somehow contribute to its formation. According to the height of the peaks (Figure 2A), the ratio of azido to nitro and nitroso aromatics was about 3%.

A mechanism was proposed by Bremner (1957) for the formation of N_2 during the treatment of lignin and humic acid with NO_2^- under strongly acidic conditions. Nelson (1982) reported that this mechanism involves formation of an α-nitrosophenol and the N_2 is apparently produced through decomposition of the diazo group in the diazonium compound formed by reaction of this α-nitrosophenol with HNO_2 (Figure 3).

Our data suggest that nitrite is a key compound with regard to the binding of mineral nitrogen into organic form. The experiments are to be considered as an approximation of the real world situation. Yet, they illustrate that nitrite is bound to phenolic compounds in the soil humic matter in the nitro and nitroso forms (Figure 4). Apparently these nitro and nitroso aromatic phenols serve as basic structural units forming the humic polymer in the soil and thus contribute to the formation of soil organic nitrogen.

Registry No. Nitrobenzene, 98-95-3; 1-azido-3-nitrobenzene, 1516-59-2; α-naphthol, 90-15-3; 1-azido-4-nitrobenzene, 1516-60-5; 1-amino-3-nitrobenzene, 99-09-2; 1-amino-4-nitrobenzene, 100-01-6; nitrite, 14797-65-0.

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