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Amidase Activity in Soils: IV. Effects of Trace Elements and Pesticides[1]

W. T. FRANKENBERGER, JR., AND M. A. TABATABAI[2]

ABSTRACT

Amidase was recently detected in soils, and this study was carried out to assess the effects of 21 trace elements, 12 herbicides, 2 fungicides, and 2 insecticides on the activity of this enzyme. Results showed that most of the trace elements and pesticides studied inhibited amidase activity in soils. The degree of inhibition varied among the soils used. When the trace elements were compared by using 5 μmol/g of soil, the average inhibition of amidase in three soils showed that Ag(I), Hg(II), As(III), and Se(IV) were the most effective inhibitors, but only Ag(I) and As(III) showed average inhibition > 50%. The least effective inhibitors (average inhibition < 3%) included Cs(I), Ba(II), Ca(II), Fe(II), Ni(II), Al(III), Fe(III), Tl(IV), V(IV), As(V), Mo(VI), and W(VI). Other elements that inhibited amidase activity in soils were Cd(II), Co(II), Mn(II), Pb(II), Sn(II), Zn(II), B(III), and Cr(III). Enzyme kinetic studies showed that As(III) was a competitive inhibitor of amidase, whereas Ag(I), Hg(II), and Se(IV) were noncompetitive inhibitors.

When the pesticides studied were compared by using 10μg of active ingredient per gram of soil, the average inhibition of amidase in three soils ranged from 2% with dinitroamine, EPTC plus R-25788, and captan to 10% with butylate. Other pesticides that inhibited amidase activity in soils were atrazine, naptalam, chloramben, dicamba, cyanazine, 2,4-D, alachlor, paraquat, trifluralin, maneb, diazinon, and malathion. The inhibition of amidase by diazinon, alachlor, and butylate followed noncompetitive kinetics.

Additional Index Words: N fertilizers, heavy metals, herbicides, fungicides, insecticides, enzyme kinetics.

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RECENTLY, we detected amidase activity in soils (Frankenberger and Tabatabai, 1980a). This enzyme catalyzes the hydrolysis of aliphatic amides, producing NH_3 and the corresponding carboxylic acids ($R\text{-}CONH_2 + H_2O \rightarrow NH_3 + R\text{-}COOH$). The activity of this enzyme in soils deserves special attention because its substrates, especially formamide and oxamide, are potential N fertilizers (Brown and Reid, 1937; Rehling and Taylor, 1937; Terman et al., 1968; Hunter, 1974; Cantarella and Tabatabai, 1980[3]). Recent developments in fertilizer technology indicate that the CO produced in coal gasification can be used as a feedstock for the production of formamide and oxamide (Worthy, 1980).

Recent concerns about pollution of soils by trace elements[4] and pesticides have led several research centers to evaluate the effects of such materials on the biochemical processes in soils. This is because (i) several trace elements are added to soils as fertilizers, as impurities in fertilizers, or as components of municipal and industrial wastes (Berrow and Webber, 1972), and (ii) pesticides are common agricultural chemicals used in modern soil and crop management systems. Also, elements such as Pb and V are present in fuel oils and gasoline emitted into the atmosphere upon burning and deposited on soils, especially near urban areas (Lagerwerff, 1972; Tyler, 1974, 1976).

Recent studies have shown that enzymatic activity in soils is inhibited by most heavy metals, trace elements, or by both including urease (Tabatabai, 1977), phosphatases (Juma and Tabatabai, 1977), and sulfatase (Al-Khafaji and Tabatabai, 1979). Also, the inhibition of N mineralization and nitrification by trace elements and pesticides is well documented (Liang and Tabatabai, 1977, 1978; Hale et al., 1957). Application of pesticides to soils under laboratory conditions has been shown to significantly decrease soil microbial populations and reduce activities of several soil enzymes such as phosphatase, saccharase, *B*-glucosidase, and urease (Voets et al., 1974; Lethbridge and Burns, 1976; Cervelli et al., 1976). Generally, the degree of inhibition observed is related to the type and concentration of trace element or pesticide added and on the chemical properties and texture of the soil used. To our knowledge no studies have been conducted on the effects of trace elements and pesticides on amidase activity in soils. Because the substrates of amidase, such as formamide and oxamide, are potential N fertilizers, this study was conducted to evaluate the effects of trace elements and pesticides on amidase activity in soils. An additional objective was to study the mode of inhibition of this enzyme by selected trace elements and pesticides.

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[2] Graduate Research Assistant and Professor of Soil Biochemistry, respectively. Dep. of Agron., Iowa State Univ., Ames, IA 50011. Dr. Frankenberger currently is Assistant Professor, Dep. of Soil and Environ. Sciences, Univ. of Calif., Riverside, CA 92521.

[3] H. Cantarella and M. A. Tabatabai. 1980. Potential of some amides as nitrogen fertilizers. p. 166. Agron. Abstr.

[4] The term "trace element" is used here to refer to elements that are, when present in sufficient concentrations, toxic to living systems.

Table 1—Properties of soils used.

Soil Series	Soil Family	pH	Organic C	Total N	$CaCO_3$ equivalent	Clay	Sand	Amidase activity†
			%					
Muscatine	Fine-silty, mixed, mesic Aquic Hapludolls	5.6	2.63	0.227	0	28	3	220
Harps	Fine-loamy, mesic Typic Calciaquolls	7.6	3.24	0.274	2.96	30	31	195
Okoboji	Fine, montmorillonitic, mesic Cumulic Haplaquolls	7.0	4.70	0.418	0.57	34	31	335

† μg of NH_4-N released • g^{-1} soil • 2 hours^{-1} by using formamide as a substrate.

Table 2—Common name, trade name, and active ingredients of the pesticides used.

Common name	Trade name	Active ingredient
		Herbicides
Atrazine	AAtrex	2-Chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine
Naptalam	Alanap	Sodium N-1-naphthylphthalamate
Chloramben	Amiben	3-Amino-2,5-dichlorobenzoic acid
Dicamba	Banvel	3,6-Dichloro-o-anisic acid
Cyanazine	Bladex	2-[[4-chloro-6-(ethylamino)-s-triazin-2-yl] amino]-2-methylpropionitrile
2,4-D	2,4-D	(2,4-Dichlorophenoxy) acetic acid
Dinitroamine	Dinitramine	N^a, N^b-Diethyl-α,α,α-trifluoro-3,5-dinitrotoluene,2,4-diamine
EPTC plus R-25788	Eradicane	S-Ethyl-N,N-dipropylthiocarbamate plus N,N-diallyl-1,1-dichloroacetamide
Alachlor	Lasso	2-Chloro-2′-6′-diethyl-N-(methoxymethyl) acetanilide
Paraquat	Paraquat	1,1′-Dimethyl-4,4′-bipyridinium dichloride
Butylate	Sutan	S-Ethyl diisobutylthiocarbamate
Trifluralin	Treflan	α,α,α-Trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine
		Fungicides
Maneb	Menesan	Manganese ethylene bisdithiocarbamate
Captan	Merpan	cis-N-(Trichloromethylthio)-4-cyclohexene-1,2-dicarboximide
		Insecticides
Diazinon	Diazinon	O,O-Diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorothioate
Malathion	Malaspray	O,O-Dimethyl phosphorodithioate of diethylmercaptosuccinate

Table 3—Effects of trace elements on amidase activity in soils.

Trace element: Element	Oxidation state	Muscatine	Harps	Okoboji	Average
		Percentage inhibition of amidase activity in soil specified†			
Ag	I	62 (18)	53	60	55
Cu		1 (0)	2	2	2
Ba	II	1 (0)	3	2	2
Cd		6 (2)	6	5	6
Co		4 (1)	8	5	6
Cu		2 (0)	2	4	3
Fe		1 (0)	1	1	1
Hg		46 (13)	30	27	34
Mn		3 (1)	4	8	5
Ni		2 (0)	2	6	3
Pb		5 (1)	2	5	4
Sn		4 (1)	5	5	5
Zn		5 (2)	13	6	8
Al	III	1 (0)	0	5	2
As		99 (32)	98	97	98
B		9 (2)	5	9	8
Cr		4 (1)	4	4	4
Fe		2 (0)	0	5	2
Se	IV	27 (6)	18	16	20
Ti		1 (0)	0	1	1
V		2 (0)	1	5	3
As	V	2 (0)	1	3	2
Mo	VI	2 (0)	2	4	3
W		3 (0)	1	2	2
L.S.D. ($P < 0.05$)		2.9 (2.1)	2.7	2.9	
($P < 0.01$)		3.9 (2.8)	3.7	3.9	

† 5 μmol trace element/g of soil. Figures in parentheses indicate percentage inhibition of amidase activity by using 0.5 μmol trace element/g of soil.

MATERIALS AND METHODS

The soils used were surface samples (0 to 15 cm) selected to obtain a range in pH (5.6 to 7.6), organic matter content (2.63 to 4.70% organic C), texture (28 to 34% clay and 3 to 31% sand), and amidase activity (195 to 335 μg NH_4-N released • g^{-1} of soil • 2 hours with formamide as a substrate). Before use, each sample was air-dried and crushed to pass through a 2-mm screen. In the analyses reported in Table 1, pH was determined by a glass electrode (soil/water ratio, 1:2.5), organic C by wet oxidation (Mebius, 1960), particle-size distribution by pipette analysis (Kilmer and Alexander, 1949), and amidase activity by the method of Frankenberger and Tabatabai (1980a).

The trace elements used were Fisher-certified reagent-grade chemicals (Fisher Scientific Co., Chicago, Ill). Of these, Ag(I), Cd(II), Co(II), Cu(II), Fe(II), Zn(II), and V(IV) were added as the sulfate; Cu(I), Ba(II), Hg(II), Mn(II), Ni(II), Sn(II), Al(III), Cr(III), and Fe(III) as the chloride; Pb as the nitrate; and As(III), B(III), Se(IV), As(V), Mo(VI), and W(VI) as $NaAsO_2$, $Na_2B_4O_7$, H_2SeO_3, Na_2HAsO_4, H_2MoO_4, and Na_2WO_4, respectively.

The pesticides used included herbicides (atrazine, naptalam, chloramben, dicamba, cyanazine, 2,4-D, dinitroamine, EPTC plus R-25788, alachlor, paraquat, butylate, and trifluralin), fungicides (maneb and captan), and insecticides (diazinon and malathion). The active ingredients and trade and common names of the pesticides studied are listed in Table 2.

To determine the effects of a trace element or pesticide on amidase activity, 5 g of soil in a 50-ml volumetric flask were treated with 1 ml of solution containing either 2.5 or 25 μmol of trace element or 5 or 50 μg of active ingredient of pesticide. This solution was added dropwise to moisten the whole soil sample. After 30 min of equilibration, amidase activity was assayed as described previously (Frankenberger and Tabatabai, 1980a). This method involves determination by steam distillation of the NH_4-N produced by amidase activity when 5 g of soil is incubated with 0.2 ml of toluene, 9 ml of 0.1*M* THAM [Tris(hydroxymethyl)aminomethane][$H_2NC(CH_2OH)_3$], buffer pH 8.5 (we used 8 ml of this buffer because the soil sample was treated with 1 ml of trace element or pesticide solution), and 1 ml of 0.5*M* buffered formamide solution at 37°C for 2 hours.

The results of amidase activity from trace element- or pesticide-treated soils were compared with those obtained with 5 g of soil treated with 1 ml of water. Percentage inhibition of amidase activity by each trace element and pesticide was calculated from $(1-B/A)100$, where A is amidase activity of untreated soil, and B is amidase activity of trace element- or pesticide-treated soil.

In the study of determination of the mode of inhibition of amidase by Ag(I), Hg(II), As(III), Se(IV), diazinon, alachlor, and butylate, the 5 g of soil was treated with 1 ml of solution containing either 10 μmol of the trace element (2 μmol of trace element per g of soil) or 100 μg of active ingredient (a.i.) of pesticide (20 μg of a.i. pesticide per g of soil) as described, but amidase activity was assayed by adding 1 ml of buffered 0.10, 0.13, 0.20, 0.35, or 0.80*M* formamide (10, 13, 20, 35, or 80 m*M* on soil solution basis). The results obtained were plotted in the form of the Lineweaver–Burk plot: plotting 1/V vs. 1/S (V = velocity and S = substrate concentration). All results reported are averages of duplicate determinations calculated on an oven-dry basis, moisture being determined from loss in weight after drying at 105°C for 24 hours.

RESULTS AND DISCUSSION

Effects of Trace Elements

The effects of trace elements on amidase activity in soils varied considerably among soils; however, Ag(I), Hg(II), As(III), and Se(IV) were the most effective inhibitors (Table 3). The average inhibition observed with three soils by using 5 μmol of one of these four trace elements per gram of soil ranged from 20% with Se(IV) to 98% with As(III), but only Ag(I) and As(III) showed an average inhibition > 50%. Also, As(III) was a much stronger inhibitor than As(V). This is important because As(V) is readily reduced to As(III) in soils under anaerobic conditions. Reduction of the amount of trace element added per gram of soil by tenfold (from 5 μmol to 0.5 μmol decreased the degree of inhibition of amidase activity (Table 3). Other trace elements that inhibited amidase activity in soils were; Cu(I), Ba(II), Cd(II), Co(II),Cu(II), Fe(II), Mn(II), Ni(II), Pb(II), Sn(II), Zn(II), Al(III), B(III), Cr(III), Fe(III), Ti(IV), V(IV), As(V), Mo(VI), and W(VI). The average inhibition observed for the three soils studied by using 5 μmol of one of these elements per gram of soil ranged from 1% with Fe(II) and Ti(IV) to 8% with Zn(II) and B(III). The degree of inhibition of soil amidase by most of the trace elements studied is much less than that reported for inhibition of urease, sulfatase, and phosphatase in soils (Tabatabai, 1977; Juma and Tabatabai, 1977; Al-Khafaji and Tabatabai, 1979).

The pH values of the trace element solutions varied considerably. They ranged from 2.1 for Sn(II) to 9.6 for As(III) and B(III) solutions. Tests indicated, however, that, with the use of 0.1*M* THAM buffer (pH 8.5), the pH of the soil–buffer mixture ranged from 8.4 with the Muscatine soil to 8.6 with the Harps soil. The deviation in pH values resulting from the addition of trace elements in the presence of THAM buffer did not exceed ±0.1 pH unit. Therefore, the inhibition of amidase activity in soils observed in the presence of each of the trace elements studied was not due to changes in pH of the incubation mixture but was due to the reaction of the trace element with amidase functional groups.

Metal ions may inhibit enzyme reactions by complexing the substrate, by combining with the protein-active groups of the enzymes, or by reacting with the enzyme–substrate complex. The mode of inhibition is dependent on the type of substrate used. The information available about inhibition of other enzymes by metal ions, however, indicates that the inhibition usually is noncompetitive in nature. Metal ions are assumed

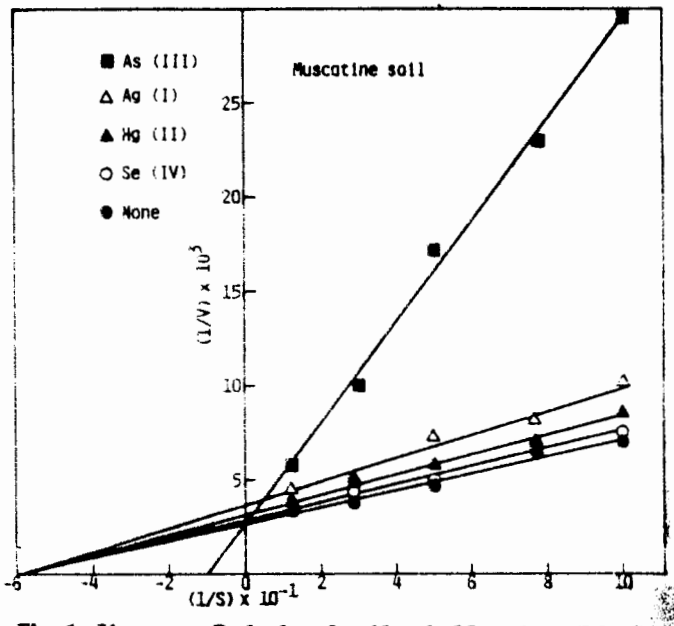

Fig. 1—Lineweaver–Burk plot of amidase in Muscatine soil in the presence and absence of selected trace elements (2μmol trace element/g of soil). Substrate concentration (S) and velocity (V) are expressed in *M* and μg of NH_4-N released g^{-1} · of soil · 2 hours^{-1} at 37°C, respectively.

to inactivate enzymes by reacting with sulfhydryl groups, a reaction analogous to the formation of a metal sulfide (Shaw and Raval, 1961). Sulfhydryl groups in enzymes may serve as integral parts of the catalytic active sites or as groups involved in maintaining the correct structural relationship of the enzyme protein. Studies of kinetics of the reaction catalyzed by amidase in soils suggested that the prototropic groups involved at the active sites of this enzyme include sulfhydryl of cysteine and/or α-amino groups (Frankenberger and Tabatabai, 1980b). The lack of strong inhibition of amidase by metal ions (Table 3) supports the finding of Woods and Orsi (1974) that thiol groups are not directly involved in the catalytic sites of amidase, even though thiol groups seem necessary for stabilization of the active amidase conformation. The α-amino groups at the end of polypeptide chain, which do not react with metal ions, seem to be one of the groups involved in the catalytic center of amidase (Frankenberger and Tabatabai, 1980b).

Experiments with 5 μmol of NaCl and K_2SO_4/g of soil indicated that the K^+, Na^+, Cl^-, and SO_4^{2-} associated with the trace elements studied did not have any effect on amidase activity in soils. Related anions, such as NO_2^- and NO_3^-, were not inhibitory.

The mode of inhibition of amidase activity by Ag(I), Hg(II), As(III), and Se(IV) was studied by determining the effect of the substrate concentration on the initial velocity of the enzyme reaction in soils in the presence and absence of the trace elements indicated. The mode of inhibition of amidase by these elements was studied because they showed the strongest inhibition. Our results indicate that As(III) is a competitive inhibitor while Ag(I), Hg(II), and Se(IV) are noncompetitive inhibitors of amidase activity in soils (Fig. 1). Apparently, arsenite (AsO_2^-) has a similar ionic structure to that portion of formamide that binds to the active site of

Table 4—Effects of pesticides on amidase activity in soils.

Pesticide	Percentage inhibition of amidase activity in soil specified†			
	Muscatine	Harps	Okoboji	Average
Herbicide				
Atrazine	4 (1)	5	3	4
Naptalam	6 (2)	6	5	6
Chloramben	6 (2)	4	2	4
Dicamba	8 (3)	0	1	3
Cyanazine	2 (0)	3	3	3
2,4-D	5 (2)	3	2	3
Dinitroamine	6 (2)	0	0	2
EPTC plus R-25788	6 (2)	1	0	2
Alachlor	13 (4)	7	5	8
Paraquat	7 (3)	4	0	4
Butylate	9 (3)	12	8	10
Trifluralin	3 (1)	2	8	4
Fungicide				
Maneb	8 (3)	0	0	3
Captan	7 (2)	0	0	2
Insecticide				
Diazinon	11 (4)	5	8	8
Malathion	13 (4)	5	4	7
L.S.D. ($P < 0.05$)	3.0 (2.9)	2.6	2.5	
($P < 0.01$)	4.1 (4.0)	3.6	3.4	

† 10 μg of active ingredient of pesticide/g of soil. Figures in parentheses indicate percentage inhibition of amidase activity using 1 μg of active ingredient of pesticide/g of soil.

amidase, altering the apparent K_m value but not the V_{max}. The other three elements decreased the V_{max} without altering the apparent K_m value (Table 5).

Effects of Pesticides

Similar to the effects of trace elements, the effects of pesticides on inhibition of amidase activity in soils varied among the three soils used (Table 4). The average inhibition observed for the three soils by using 10 μg of active ingredient of pesticide per gram of soil ranged from 2% with dinitroamine, EPTC plus R-25788, and captan to 10% with butylate. Reduction of the amount of active ingredient of the pesticide added per gram of soil by tenfold (from 50 μg to 5 μg per gram of soil) decreased the degree of inhibition of amidase activity. Other pesticides that inhibited amidase activity in soils were atrazine, naptalam, chloramben, dicamba, cyanazine, 2,4-D, alachlor, paraquat, trifluralin, maneb, diazinon, and malathion (Table 4). But the degree of inhibition of amidase by these pesticides was not large enough to inhibit the amidase activity in soils receiving normal rates of pesticide applications.

The pH values of the pesticide solutions ranged from 3.2 with captan to 9.9 with cyanazine. Most of the other pesticides solution had pH values ranging from 7 to 9. Tests showed that the pH of the reaction mixture containing THAM buffer at pH 8.5 in the assay of amidase activity of soils in the presence of pesticides was not significantly altered (±0.1 pH unit).

The mode of inhibition of amidase activity by alachlor, butylate, and diazinon was studied by determining the effect of the substrate concentration on the initial velocity of the enzyme reaction in soils in the presence and absence of these pesticides. These pesticides were specifically selected because alachlor contains a branched amide, butylate contains a linear amide, and diazinon contains no amide group (Table 2). These pesticides also were the most effective inhibitors of all the pesticides studied. The results obtained are plotted in Fig. 2, in the form of Lineweaver–Burk plot showing that the three pesticides act as noncompetitive inhibitors of amidase activity in soils, decreasing the V_{max} value without altering the apparent K_m value (Table 5). We originally expected butylate to behave as a competitive inhibitor because of the similarity in its chemical structure to that of formamide (both contain a linear amide group), but apparently, the amide group must be at the terminal end of the molecular chain to compete for the active site of amidase. Alachlor and diazinon were expected to be noncompetitive inhibitors of amidase because neither contains a linear amide group.

Table 5—Effect of some trace elements and pesticides on the apparent K_m and V_{max} values of amidase in soils.

Trace element or pesticide	Muscatine soil		Okoboji soil	
	K_m mM	V_{max}†	K_m mM	V_{max}†
None	17.3	385	17.6	475
Ag(I)	17.0	275	17.3	349
Hg(II)	17.2	322	17.7	393
As(III)	95.7	382	116.8	474
Se(IV)	17.2	346	17.6	448
Alachlor	17.2	365	17.5	456
Butylate	17.5	316	17.1	404
Diazinon	17.6	337	17.5	423

† μg NH_4-N released·g^{-1} of soil·2 $hours^{-1}$ by using formamide as a substrate.

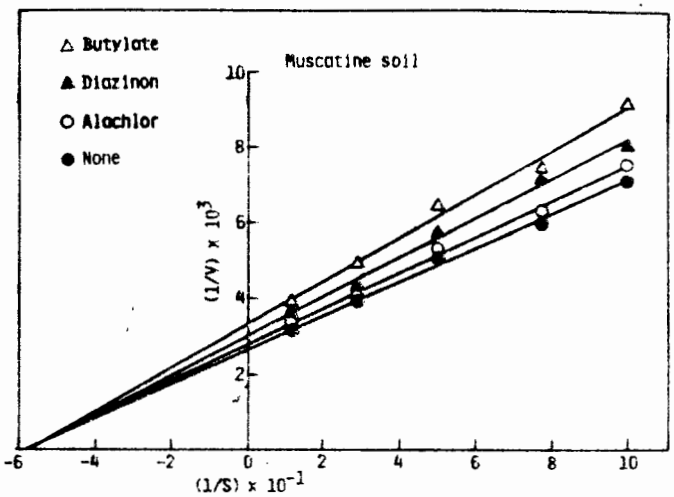

Fig. 2—Lineweaver–Burk plot of amidase in Muscatine soil in the presence and absence of selected pesticides (20 μg of active ingredient/g of soil). Substrate concentration (S) and velocity (V) are expressed in M and μg of NH_4-N released · g^{-1} of soil · 2 $hours^{-1}$ at 37°C, respectively.

CONCLUSIONS

Studies on the effects of trace elements and pesticides on amidase activity showed that certain trace elements and pesticides inhibit the reaction catalyzed by this enzyme in soil. The degree of inhibition observed for most trace elements and pesticides, however, was too small to be of concern under normal soil management practices. The degree of inhibition of amidase activity by heavy metals is much smaller than those reported for urease,

sulfatase, and phosphatase activities in soils. It is apparent that the functional groups of the active sites of amidase are different from those of other soil enzymes. Among the 21 trace elements studied, As(III) (arsenite) showed competitive kinetics with respect to the substrate (formamide) for amidase activity in soils. The results suggest that trace elements and pesticides added to soils under current management practices should have little or no effect on hydrolysis of amide fertilizers in soils.

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Comparison of Effects of Nitrate, Nitrite, and Nitric Oxide on Reduction of Nitrous Oxide to Dinitrogen by Soil Microorganisms[1]

J. F. GASKELL, A. M. BLACKMER, AND J. M. BREMNER[2]

ABSTRACT

Recent work has shown that nitrate inhibits reduction of nitrous oxide (N_2O) to dinitrogen (N_2) by soil microorganisms under anaerobic conditions. Studies to investigate the possibility that this inhibition is caused by nitrite or nitric oxide (NO) formed through microbial reduction of nitrate showed that both NO and nitrite inhibit reduction of N_2O to N_2 by soil microorganisms under anaerobic conditions (helium atmosphere) but that the inhibitory effect of NO on N_2O reduction is much smaller than the effects of nitrate or nitrite. Comparison of the effects of nitrate, nitrite, and mixtures of nitrate and nitrite on reduction of N_2O to N_2 by soils incubated under He showed that nitrate per se inhibits N_2O reduction and did not confirm a recent suggestion that the inhibitory effect of nitrate on N_2O reduction is due to nitrite formed through microbial reduction of nitrate.

Additional Index Words: **denitrification, inhibition of N_2O reduction.**

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THERE IS CONCERN that nitrogen (N) fertilization of soils to increase crop production may affect our climate by increasing the amounts of nitrous oxide (N_2O) evolved from soils to the atmosphere through denitrification of fertilizer-derived nitrate (Council for Agricultural Science and Technology, 1976; Yung et al., 1976; Crutzen and Ehhalt, 1977; Liu et al., 1977; McElroy et al., 1977). This concern has stimulated research to identify factors affecting reduction of N_2O to dinitrogen (N_2) by denitrifying microorganisms because there is good reason to believe that N_2O is a free, obligatory intermediate in reduction of nitrate to N_2 by denitrifying microorganisms (St. John and Hollocher, 1977; Firestone et al., 1979) and that the amounts of N_2O evolved from soils via denitrification of nitrate accordingly will be influenced by factors affecting reduction of N_2O to N_2 by soil microorganisms.

Blackmer and Bremner (1978) found that nitrate inhibited reduction of N_2O to N_2 by soil microorganisms and that its inhibitory effect on N_2O reduction increased with decrease in soil pH. These observations provided

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[2] Research Associate, Assistant Professor, and Professor, respectively, Dep. of Agron., Iowa State Univ., Ames, IA 50011.