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REACTIVITY OF TNT & TNT - MICROBIAL REDUCTION PRODUCTS WITH SOIL COMPONENTS

BY
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AND
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CONTAMINATION	MICROBIOLOGY	

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The results of preliminary studies with TNT and TNT-reduction products and soil components are presented. The findings indicate the potential for binding reactions between some of these compounds and humic acid, as well as for peroxidase catalyzed reactions with one TNT-reduction product. There is a need for continued effort in these areas to fully understand the implications of these types of reactions in regards to the fate of munitions compounds in soils.

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Preface

TNT and TNT-reduction products have been studied extensively in the past in order to resolve the problem of how to handle environmental hazards associated with these compounds. Previous microbiological investigations in this laboratory have focused on aqueous, sewage sludge, and composting systems. The fate of these same compounds in soils is also a concern due to the hazardous nature of the compounds, their resistence to mineralization by microorganisms, and their leaching into groundwaters.

This work was performed for the US Army Toxic and Hazardous Materials Agency (USATHAMA) under work unit 23214139000. The results will improve the basic understanding of the fate of these compounds in soils.

We wish to thank Jamie Rowe and Scott Cowburn for their technical assistance.

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INTRODUCTION

Contamination of soils by hazardous wastes (toxic, mutagenic, carcinogenic, teratogenic or explosive) is a problem of growing concern. The soil biochemistry and microbiology at a contaminated site must be evaluated in order to predict the consequences of such hazards with a degree of certainty. The basic reactions in soils (biological, chemical, and physical) must be elucidated for hazardous wastes on a compound by compound basis. These research activities are complicated by the heterogeneous nature of soils, including such variables as moisture, percent organic matter, hydrogen ion concentration, nutrient supplements, oxygen levels, and the presence of other organic chemicals.

Soil Components

Soils may be grossly divided into organic and inorganic components: the inorganic clays and minerals and the organic humus and nonhumus materials.[1] The nonhumic organic matter includes basic biomacromolecules, such as carbohydrates, proteins, and fats. These materials are generally of short half-lives, due to rapid turnover in soils as a result of microbial enzymatic activity. Humus matter consists of structurally heterogeneous compounds of mostly higher molecular weight and of long half-lives. Humus materials may be defined as complex hydrophilic polymers consisting of a heterogenic array of mostly aromatic, proteinaceous, and carbohydrate components and fragments,

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[1] Stevenson, F.J. Gross chemical fractionation of organic matter. In Methods of Soil Analysis, Part 2, C.A. Black, ed., Am. Soc. of Agronomy, Inc., Madison, WI. 1965. 1409-1421.

derived from decomposition processes.[2,3]

Humus is often divided into three main components, based on solubility characteristics: 1) humic acid, soluble in alkali and insoluble in acid; 2) fulvic acid, soluble in alkali and acid; and 3) humin, insoluble in both acid and alkali. Humic-type polymers of fungal and bacterial origin have also been described.[4] No exact structural assignments have been ascribed to humic acid due to its heterogeneous nature, although functional groups such as hydroxyl, carboxyl, keto, and phenolic hydroxyls are often implicated.

Importance of Humic Acids

The importance of humic acids in the soil environment is in their structural stability and resistance to biodegradation, thereby stabilizing soil organic matter. Humic acids also have the following characteristics: They 1) bind metal ions, having a high cation exchange capacity, 2) serve as a nitrogen sink, 3) impart texture, tilth and pore space to soils, 4) serve as plant hormones and 5) influence rates of decomposition in soils.[5]

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[2] Christman, R.F. and E.T. Gjessing (eds.). Aquatic and Terrestrial Humic Materials. Ann. Arbor Sci., Ann Arbor, MI (1983).

[3] Flaig, W., H. Beutelspacher, and E. Rietz. Chemical composition and physical properties of humic substances. In. Soil Components, Vol. 1, Organic Components, J.T. Gieseking, ed. Springer, NY, 1975, pp. 1-211.

[4] Kosinkiewica, B. Humic-like substances of bacterial origin. III. Production of humic-like substances by Pseudomonas acidororans in media containing certain benzene derivatives. Act. Microbiol. Pol. 26(4): 393-401 (1977).

[5] Stevenson, F.J. Role and function of humus in soil with emphasis on adsorption of herbicides and chelation of micronutrients. Biosci. 22(11): 643-650 (1972).

Most importantly for this research, humic acids exhibit an ability to bind organic residues.[6-17]

These binding reactions influence the rates of decomposition of the bound residues, the bioactivity and persistence in soils of these compounds and their ultimate fate in soils. The binding reactions are not very well understood; however, ion-exchange, Vander Waals forces, chelation, hydrogen bonding, hydrophobic interactions, and covalent bonding have been implicated in these interactions. According to the literature, the basis for these reactions comes from studies on the biotransformation of insecticides, pesticides, and

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[6] Ambrosi, D., P.C. Kearney, and J.A. Macchia. Persistence and metabolism of phosaline in soil. J. Agric. Food Chem. 26(6): 1302-1306 (1977).

[7] Bollag, J.M., P. Blattman, and T. Laanio. Adsorption and transformation of four substituted anilines in soil. J. Agric. Food Chem. 26(6): 1302-1306 (1978).

[8] Carter, C.W. and I.H. Suffet. Binding of DDT to dissolved humic materials. Env. Sci. Technol. 16(11): 735-740 (1982).

[9] Dunigan, E.P. and T.H. McIntosh. Atrazine-soil organic matter interactions. Weed Sci. 19(3): 279-282 (1971).

[10] Felsot, A. and P.A. Dahn. Sorption of organophosphorus and carbamate insecticides by soil. J. Agric. Food Chem. 27(3): 557-563 (1979).

[11] Hsu, T.S. and R. Bartha. Interaction of pesticide-derived chloroaniline residues with soil organic matter. Soil Sci. 116(6): 444-452 (1974).

[12] Kaufman, D.D., Still, G.G., Paulson, G.D. and S.K. Bandel. (eds) Bound and conjugated pesticide residues. ACS Symposium Series No. 29. Am. Chem. Soc., Washington, DC., 1976, pp. 1-396.

[13] Khan, S.V. Adsorption of dyfonate (O-ethyl-s-phenyl ethylphosphonodithionate) on humic acid. Can. J. Soil Sci. 57(1): 9-14 (1977).

[14] Khan, S.V. Distribution and characteristics of bound residues of Prometryn in an organic soil. J. Agric. Food Chem. 30: 175-179 (1982).

[15] Khan, S.V. and H.A. Hamilton. Extractable and bound (nonextractable) residues of Prometryn and its metabolites in an organic soil. J. Agric. Food Chem. 28: 126-132 (1980).

[16] Wahid, P.A. and N. Sethunathan. Soption-desorption of lindane by anaerobic and aerobic soils. J. Agric. Food Chem. 28: 623-625 (1980).

[17] Weber, J.F., S.B. Weed, and T.M. Ward. Adsorption of s-triazines by soil organic matter. Weed Sci. 17(4): 417-421 (1969).

herbicides to polar intermediates in soils, with subsequent binding to soil fractions. These binding reactions are instrumental in determining application rates of these compounds to farmland in order to assure sufficiently active residual concentrations. These reactions can have significant impact on the environmental fate of a variety of xenobiotic compounds, influencing rates of biological transformation, volatilization, photodegradation and physical/chemical instability.

2,4,6-Trinitrotoluene

2,4,6-trinitrotoluene (TNT), the most abundantly produced munition compound over the past decades, is responsible for groundwater, lagoon, and soil contamination problems.[18,19] The hazardous nature of TNT derives from its explosive, toxic and mutagenic nature.[20] As part of the Army's program to evaluate the potential for biological treatment of munition wastes, an assessment of the fate of TNT in soils and water is essential. Because binding reactions to components in soil organic matter play an important role in the fate of some pesticides, herbicides and insecticide residues, it should be determined whether or not these reactions also occur with munition wastes such as TNT. Similarly, enzymatic coupling reactions in soils mediated by polyphenoloxidase enzymes have been reported for a number of

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[18] Klausmeier, R.E., J.L. Osmon, and D.R. Walls. The effect of trinitrotoluene on microorganisms. Dev. Ind. Microbiol. 15: 309-317 (1978).

[19] Pereira, W.E., D.L. Short, D.B. Manigold, and P.K. Roscio. 1979. Isolation and characterization of TNT and its metabolites in groundwater by gas chromatography by mass spectrometer-computer techniques. Bull. Environ. Contam. Toxicol. 21: 554-562 (1979).

[20] Kaplan, D.L. and A.M. Kaplan. Mutagenicity of TNT-surfactant complexes. Bull. Environ. Contam. Toxicol. 28: 33-38 (1982).

phenolic, naphtholic and substituted aromatic residues.[21-28] The role these types of reactions play in the production of insoluble conjugates from TNT must also be addressed.

Previous studies on the biodegradation of TNT in simulated soil composting systems identified a significant percentage of TNT (as ^{14}C-labelled material) bound (not extractable with nonpolar organic and polar solvents) into humus fractions.[28] The percentage of bound material increased with compost age and stabilization (Table 1). Additionally, the aromatic ring of TNT is not cleaved by microbial enzymatic activity,[28,29] TNT-surfactant

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[21] Bollag, J.M., R.D. Minard, and S.Y. Liv. Cross-linkage between anilines and phenolic humus constituents. Environ. Sci. Technol. 17: 72-80 (1983).

[22] Bollag, J.M., S.Y. Liv, and R.D. Minard. Cross-coupling of phenolic humus constituents and 2,4-dichlorophenol. Soil Sci. Soc. Am. J. 44(1): 52-56 (1980).

[23] Liv, S.Y., R.D. Minard, and J.M. Bollag. Oligomerization of syringic acid, a lignin derivative, by a phenoloxidase. Soil Sci. Soc. Am. J. (1981).

[24] Sjoblad, R.D. and J.M. Bollag Oxidative coupling of aromatic pesticide intermediates by a fungal phenol oxidase. Appl. Environ. Microbiol. 33(4): 906-910 (1977).

[25] Sjoblad, R.D. and J.M.Bollag. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In Soil Biochemistry Vol. 5. E.A. Paul and J.N. Ladd (eds). Marcel Dekker, Inc., NY 113-152 (1981).

[26] Sjoblad, R.D., R.D. Minard, and J.M. Bollag. Polymerization of 1-naphthol and related phenolic compounds by an extracellular fungal enzyme. Pest. Biochem. Physiol. 6: 457-463 (1976).

[27] Suflita, J.M. and J.M. Bollag. Polymerization of phenolic compounds by a soil-enzyme complex. Soil Sci. Soc. Am. J. 45(2): 297-302 (1981).

[28] Kaplan, D.L. and A.M. Kaplan. Thermophilic biotransformations of 2,4,6-trinitrotoluene under simulated composting conditions. Appl Environ. Microbiol. 44(3): 757-760 (1982).

[29] McCormick, N.G., F.E. Feeherry, and H.S. Levinson. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Microbiol. 31: 949-958 (1976).

Table 1. Percentage of bound material in composts cured 24 and 91 days

	Percent of Total[1]	
	24 days	91 days
Solvent extractable[2]	86.6	61.5
Bound material[3]	5.7	22.1

[1] 3.75×10^6 disintegrations per min initial

[2] ether, ethanol, water and acetone combined

[3] humic acid, fulvic acid and humin combined

complexes have been found strongly associated with soil,[30] and evidence for higher molecular weight insoluble TNT-type conjugates has been found during the decomposition of TNT in water systems.[31] For all the above reasons, it is important to evaluate the potential for binding reactions of TNT and soil in order to determine the significance of these types of reactions in influencing the fate of TNT or its metabolites in soil.

[30] Kaplan, D.L. and A.M. Kaplan. 2,4,6-trinitrotoluene-surfactant complexes: decomposition, mutagenicity, and soil leaching studies. Environ. Sci. Technol. 16(9): 566-571 (1982).

[31] Carpenter, D.F., N.G. McCormick, J.H. Cornell, and A.M. Kaplan. Microbial transformation of ^{14}C-labelled 2,4,6-trinitrotoluene in an activated-sludge system. Appl. Environ. Microbiol. 35(5): 949-954 (1978).

MATERIALS AND METHODS

Chemicals

TNT was purchased from Eastman Kodak (Rochester, NY) and recrystallized. The 2-amino-4,6-dinitrotoluene (2A), 4-hydroxyl-2,6-dinitrotoluene (4OH), 2,4-diamino-6-nitrotoluene (2,4DA), and 2,6-diamino-4-nitrotoluene (2,6DA) were synthesized according to Sitzmann[32] by Dr. John H. Cornell.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was accomplished on a Waters system (Milford, MA) equipped with two Model 6000A solvent pumps, a Model 441 variable wavelength detector set at 229 nm, a Model 730 data module, and a Model 720 system controller. All analyses of TNT and TNT-reduction products were performed on a μBondapak C-18 reverse phase stainless steel column; 30 cm by 3.9 mm (Waters Associates) with methanol and water as solvent. The conditions for the analysis of the different compounds were previously described.[33]

Preparation of Humic Acid

Humic acid was prepared using modifications of published procedures[1,34] and as illustrated in Figure 1. Garden soil was sieved through a 2 mm-pore

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[32] Sitzmann, M.E. Chemical reduction of 2,4,6-trinitrotoluene (TNT initial products. National Technical Information Service, publication No. AD-764070, National Technical Information Service, Springfield, VA, pp. 1-9 (1973).

[33] Kaplan, D.L. and A.M. Kaplan. Separation of mixtures of 2,4,6-trinitrotoluene reduction products with liquid chromatography. Anal. Chim. Acta. 136: 425-428 (1982).

[34] Kononova, M.M. and N.P. Bel'chikova. Quick methods of determining the humus composition of mineral soils. Soviet Soil Sci. (Pochvovedeniye) 11: 1149 (1960).

screen, acid washed with 0.1 N hydrochloric acid and filtered. The acid-washed soil was extracted with 0.5 N sodium hydroxide and 0.1 M sodium pyrophosphate (200 mL of extractant per 40 g soil) overnight on a reciprocal shaker set at 225 rpm in filled centrifuge bottles. The samples were centrifuged at 8,000 rpm for 15 min and decanted through glass wool.

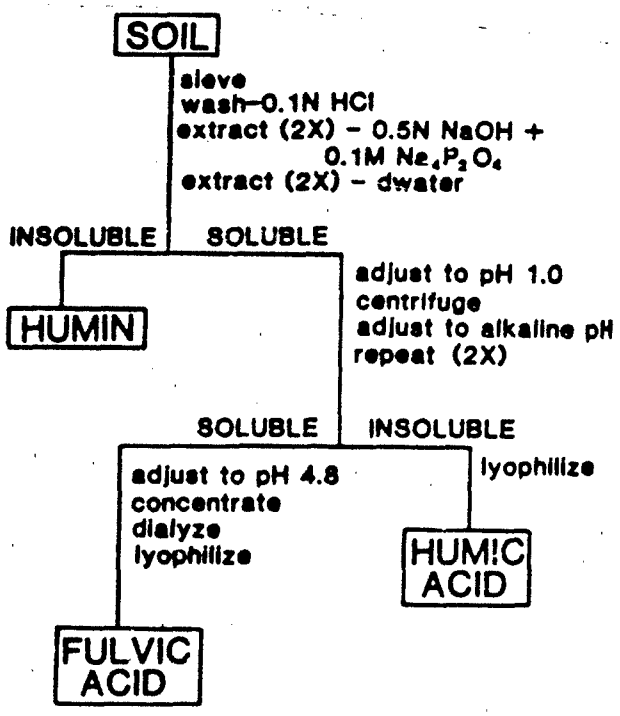

Figure 1. Extraction scheme for isolation of soil fractions.

The soil pellet was re-extracted as before, but only for one hour and the supernatants combined. The soil pellet was resuspended in distilled water and then centrifuged as before. The procedure was repeated and these extracts were combined with the previous two extracts. The soil pellet was oven dried at 45°C and represented the humin fraction.

The pH of the combined extracts was adjusted with concentrated hydrochloric acid to a pH of 1.0. The humic acids that precipitated were centrifuged out of the solution at 10,000 rpm for 10 min. After decanting, the humic acid pellet was redissolved in 0.5N sodium hydroxide and then reprecipitated with concentrated hydrochloric acid. The solution was centrifuged as before to collect the humic acid. The isolated humic acids were lyophilized.

The remaining acidified supernatant was adjusted to pH 4.8 with sodium hydroxide and then rotory evaporated to concentrate the solution. Salt contamination required dialysis of the concentrate against distilled water followed by lyophilization. The resulting product represents the fulvic acid fraction.

Acid Precipitation

The first procedure used to evaluate binding reactions was an acid precipitation method (Figure 2). This procedure was later dropped in preference to the dialysis method described below. A solution of humic acid at pH 5.5 was combined with the compound under study. After incremental reaction times, 1 mL aliquots were removed, acidified to low pH with concentrated hydrochloric acid, and centrifuged at 15,000 rpm for 10 min. The supernatant was decanted, filtered through 0.45 μm membrane filters and quantified by HPLC. The humic acids were removed during the acid precipitation and centrifugation process to prevent blockage of screens and subsequent pressure build-up on the HPLC. All reactions were protected from light with foil.

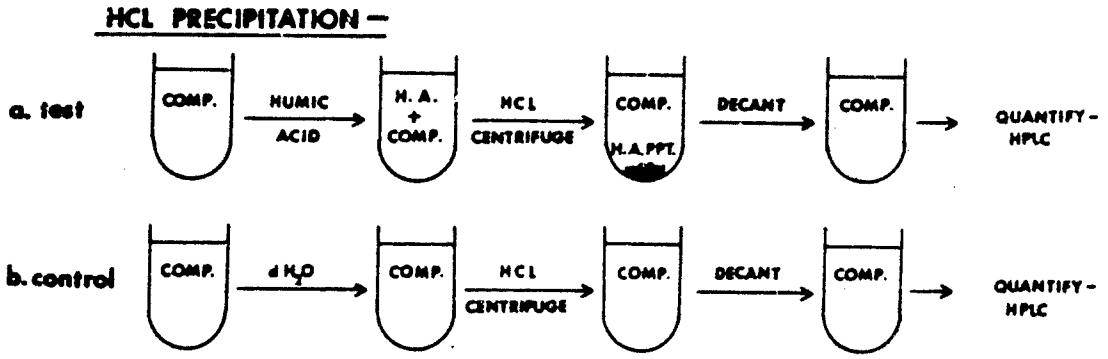

Figure 2. Acid precipitation technique for reactions with humic acid and TNT-reduction products.

Dialysis Method

Dialysis experiments were first run with 3,500 molecular weight cut-off (MWCO) dialysis tubing and later with 1,000 MWCO dialysis tubing, 25 mm x 10 m (Spectropor, Los Angeles, CA). Appropriate controls were run concurrently with each experiment as described below. The solutions of humic acid (2,500 ppm, 2,000 ppm, 1,500 ppm, or 1,000 ppm) were first dialyzed against two changes, 15 L each, of distilled water to remove lower molecular weight compounds that might interfere during the HPLC analysis. The dialysis systems were run in beakers set up as follows (Figure 3).

A) a 75 mL buffered solution of the compound under study into which is placed a dialysis tube containing 10 mL of humic acid at one of the concentrations listed earlier.

B) the same as system A except a lower concentration of humic acid in the tubing.

C) a control system with the same set-up as system A except the dialysis tube contains 10 mL distilled water instead of humic acid. The solution outside of the dialysis tubing is monitored.

D) a control system similar to system A except the solution in the beaker is the buffer without the compound under study.

E) a control system similar to system A except the dialysis tube contains 10 mL of distilled water. The solution inside of the dialysis tube is monitored.

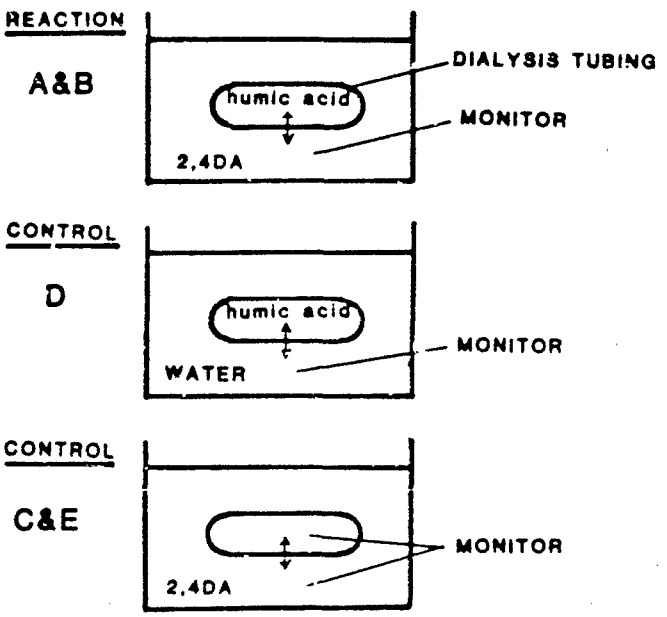

Figure 3. Dialysis technique for studying binding reactions.

Systems A to E were stirred at room temperature and covered with foil to prevent photodegradation. The 0.05 M buffered solutions consisted of sodium acetate, potassium phosphate and sodium carbonate at pH 4.0, 7.0, and 10.0, respectively.

The solutions on the exterior of the dialysis tubes were periodically sampled for HPLC analysis. The only exception was system E, where the internal solution in the dialysis tube was monitored in order to evaluate breakthrough of the compound into the inside of the tubing. The System C control accounts for any losses of the compound under study due to factors other than binding reactions with the humic acid. These losses could include adherence to glassware or to the dialysis tubing or instability of the compound. System D accounts for any interferences resulting from low molecular weight components of the humic acid, which pass into the exterior solution and are analyzed by HPLC.

The dialysis experimental set-up is the choice of methods to evaluate binding reactions because the humic acid, for the most part, is retained inside the tubing while the compound under study can freely diffuse through the tubing to reach equilibrium. With time there is a gradual diffusion of some lower molecular weight fractions of humic acid to the outside of the tubing, as indicated by the light amber color. The compound inside the tubing is represented by both bound and unbound fractions while outside only unbound materials are present. Once equilibrium is reached, the concentration of the unbound portion is the same on the inside and outside of the tubing.

TNT-like Synthetic Polymer-Ames Test

A synthetic polymer was produced by reaction of glycerol, 2,4-diamino-toluene, sebacyl chloride, and palmitoyl chloride. This product was evaluated in an Ames screening test for mutagenicity according to standard procedures.[35] The compound was tested over a range of concentrations with five strains of Salmonella typhimurium (TA98, TA100, TA1535, TA1537, TA1538) with and without metabolic activation. The tests were run in triplicate and a three-fold increase in back mutations was considered as the criterion for a positive test for mutagenicity.

Peroxidase Reactions

Peroxidase (3,339 units per mg) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Reactions were run with enzyme, 100 µL, 0.114 M hydrogen peroxide, the compound, and 5 mL of 0.05 M potassium phosphate buffer at pH 7.0. Control reactions were run without the enzyme or without the hydrogen peroxide oxidizing agent. Reactions were run with TNT, 4CHA, 2,4DA and

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[35] Ames, B.N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella mammalian-microsome mutagenicity test. Mut. Res. 31: 347-364 (1975).

guaiacol and results were monitored by HPLC. Peroxidase was chosen as the enzyme to study because it is produced extracellularly by a wide variety of microorganisms, it exhibits general specificity, and it is responsible for coupling and polymerization-type reactions.

RESULTS

The preliminary experimental runs to assess the potential for binding to humic acid were run using the hydrochloric acid precipitation reaction. Figure 4 with TNT and Figure 5 with 2,4DA illustrate the results from two binding studies. In Figure 4, the reactions of different concentrations of TNT (20 ppm to 0.1 ppm) with 2,000 ppm humic acid at different incubation times is plotted. A nonreaction result would be represented by a 45-degree line passing through the origin. The zero control (no humic acid) results show no significant change in concentration of TNT while at progressively longer incubation periods there is a decreasing amount of free TNT (unbound). The loss of free TNT increased with increasing initial concentration of TNT.

In Figure 4, the reactions of 1 ppm TNT with different concentrations of humic acid (0 to 2,000 ppm) are also illustrated. In general, there is an increasing reduction of free-TNT with increasing concentration of humic acid during the five days. Figure 5 presents similar reactions and results to those presented in Figure 4, but with 2,4DA instead of TNT.

Control reactions were run in all experiments to assure stability of the compound to the acid precipitation treatment. The results of one of these experiments are illustrated in Figure 6. The treatment of various concentrations of TNT (0.1 ppm to 20 ppm) without humic acid (the upper three lines),

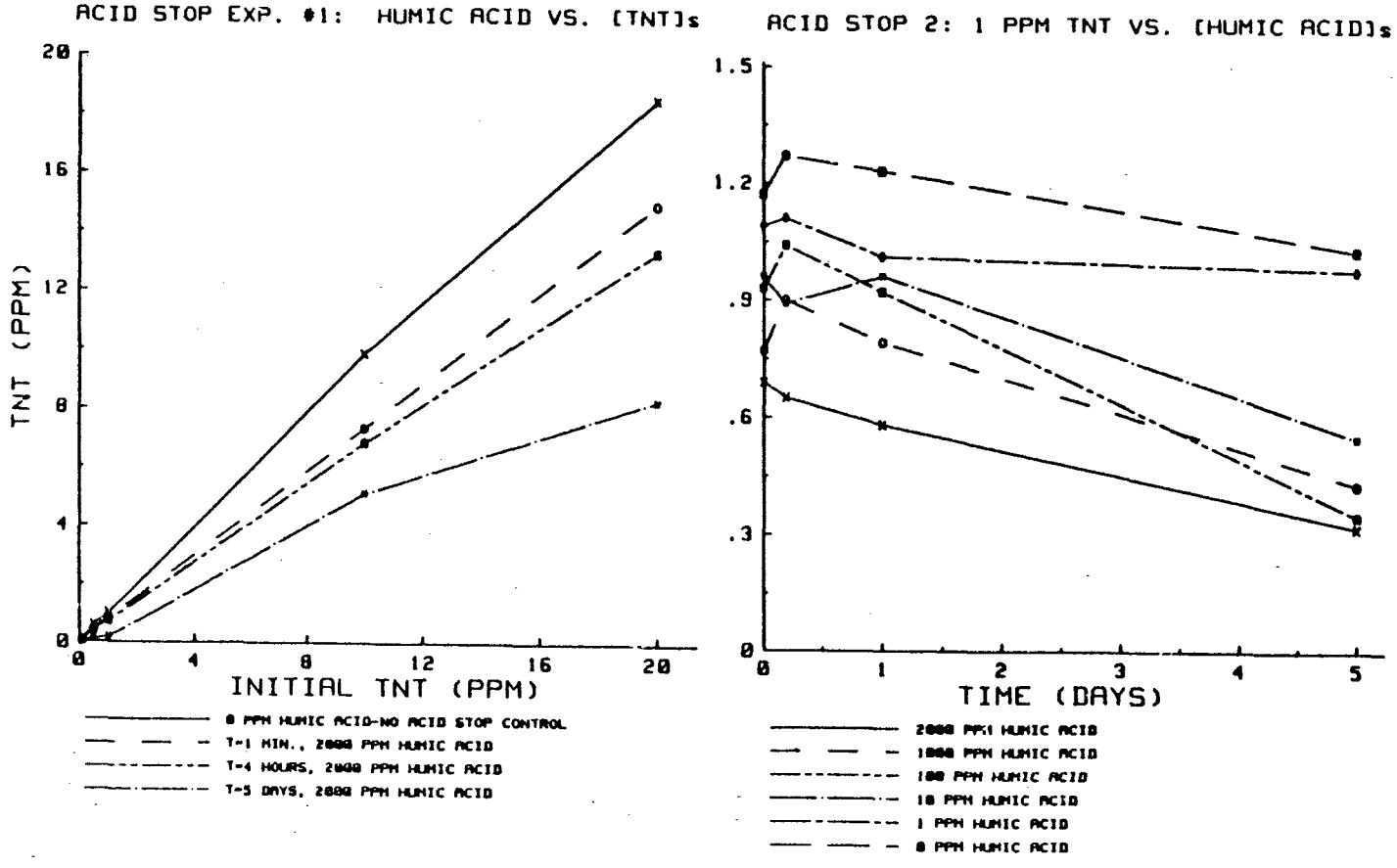

Figure 4. Acid precipitation experiment with various concentrations of TNT (left) and various concentrations of humic acid (right).

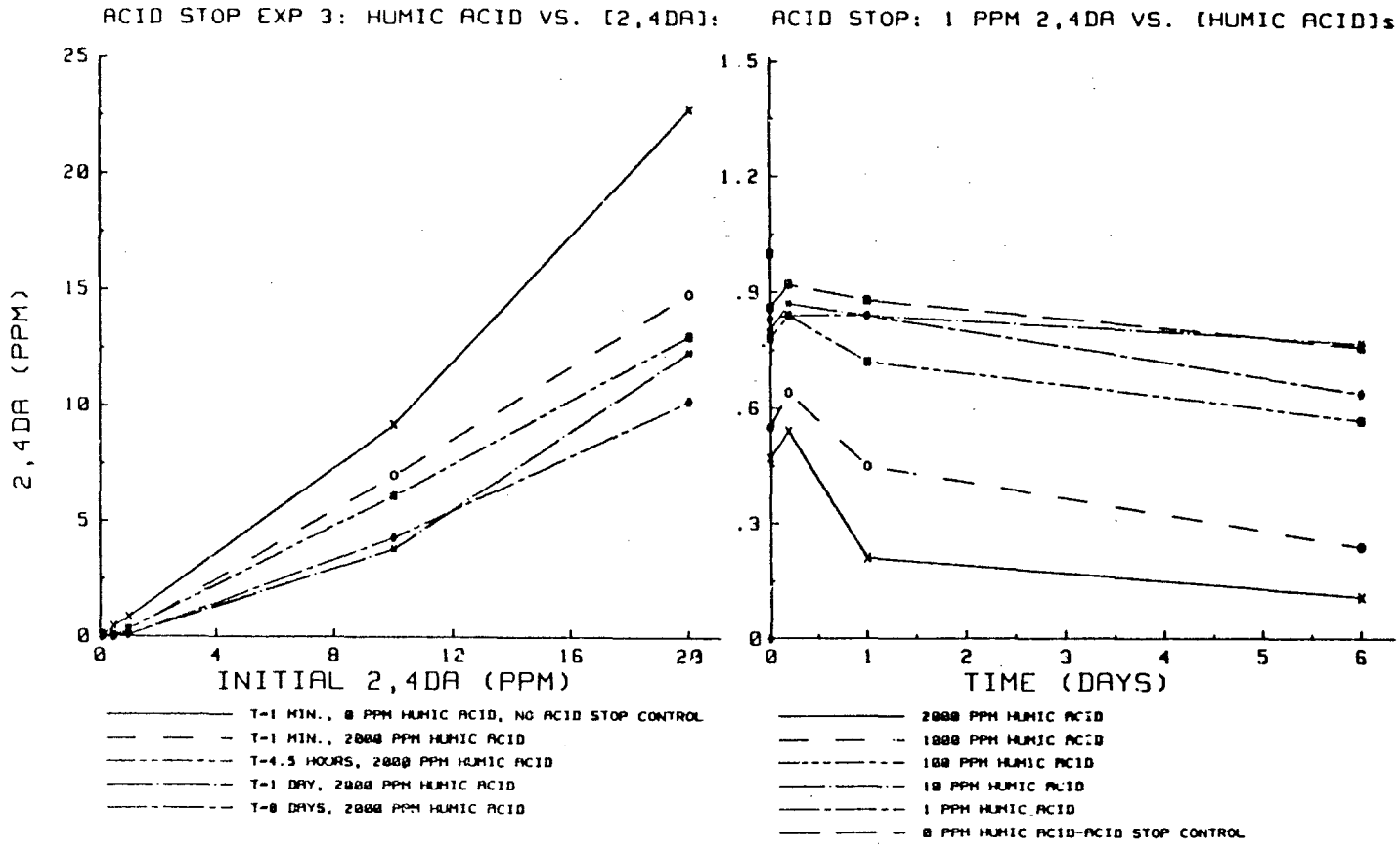

Figure 5. Acid precipitation experiment with various concentrations of 2,4DA (left) and various concentrations of humic acid (right).

but with the hydrochloric acid under these experimental test conditions shows that TNT was stable. When humic acid was also present in the test tube there was a significant reduction in concentration of TNT (lower line).

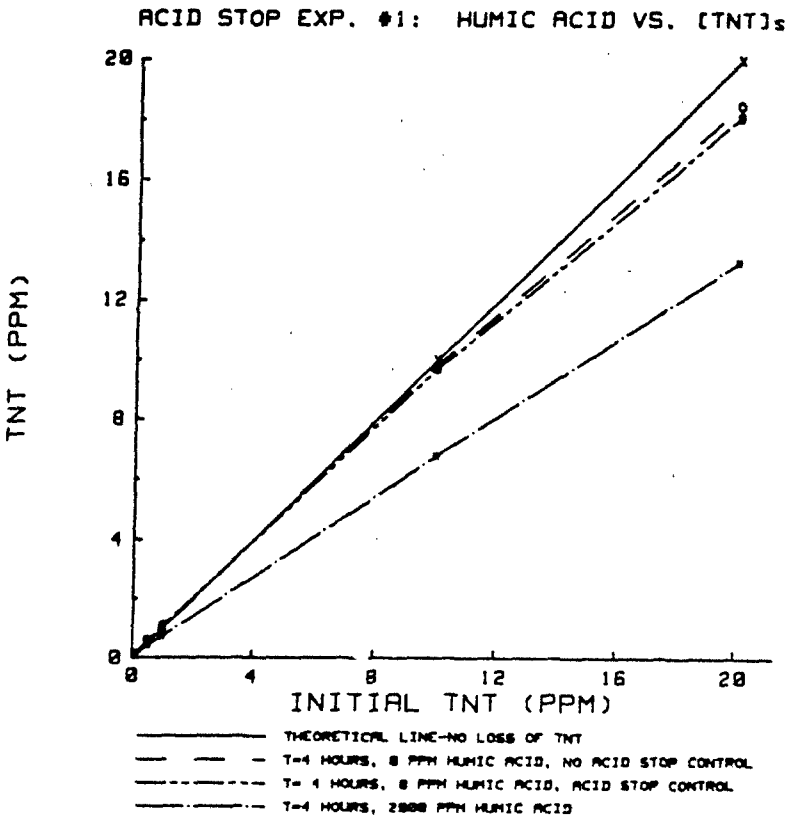

Figure 6. Acid Precipitation experiment with various concentrations of TNT, response with humic acid over time.

Despite the control runs and the apparent suitability of this reaction method, it became apparent that the pattern of results was very similar for every compound studied using acid precipitation. It was concluded that the results obtained were not representative of binding rates or binding capacity but in fact represented entrapment of the compounds during the acid precipitation and subsequent centrifugation.

As a result, dialysis experiments were chosen as a new experimental approach. The first set of experiments was run with 3,500 MWCO dialysis tubing and these results are presented in Figures 7 to 9. In Figure 7, the equilibration reactions with two TNT reduction products are illustrated (Figure 3, C and E). The equilibration period is the time required for the test compound present on the outside of the dialysis tube to diffuse through the tubing and equilibrate with the water on the inside of the tubing. Because of the volume of the test compound on the outside of the tubing is much larger than the volume of water within the tubing, there is only slight reduction in the concentration of the compound in the outside solution.

In Figure 8, binding reactions with TNT and 2,4DA are presented with 2,500 ppm and 1,250 ppm humic acid. The control curve with no humic acid in the dialysis tube is also illustrated. The results with TNT indicate little difference between the three curves up to about 50 min and inconsistent results with no overall reduciton in concentration at the last sampling time. The results with 2,4DA indicate a more consistent pattern of reactivity. There were larger reductions in concentration of 2,4DA with higher concentrations of 2,4DA.

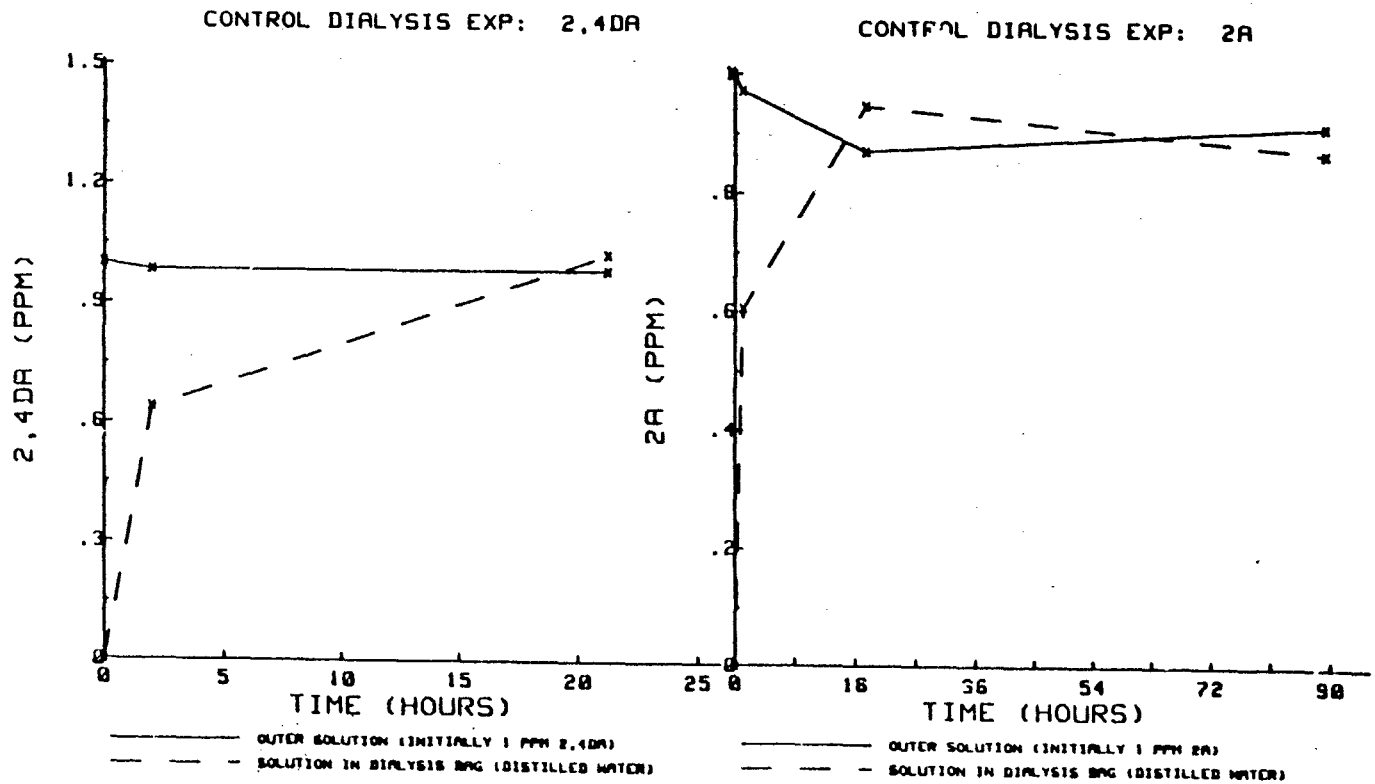

Figure 7. Dialysis experiment illustrating equilibration reactions with 2,4DA (left) and 2A (right).

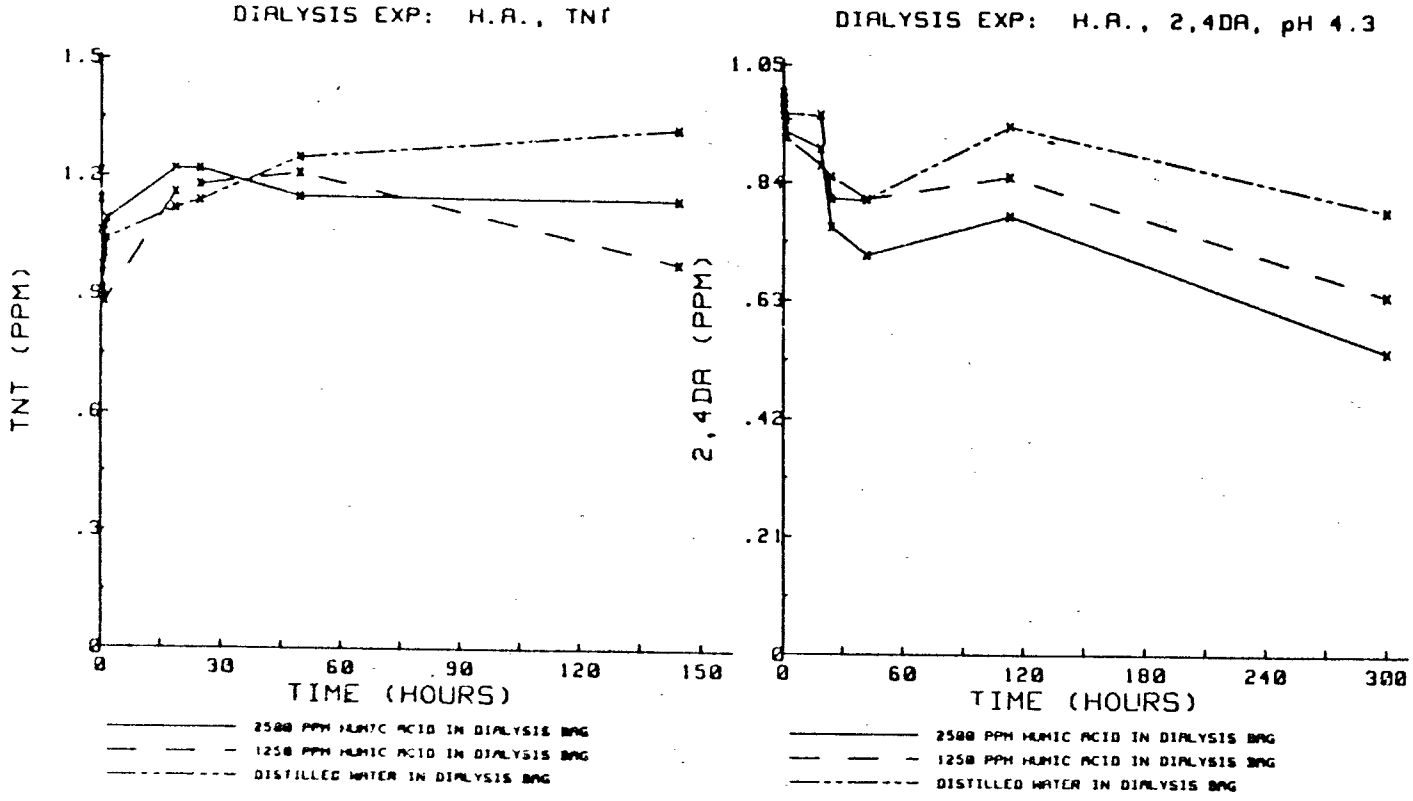

Figure 4. Dialysis experiment illustrating binding reaction results with TNT (left) and 2,4DA (right).

Experimentation was shifted to the 1,000 MWCO dialysis tubing in an effort to reduce the diffusion of some of the lower molecular weight fractions of humic acid through the tubing into the exterior solution. After extended periods of incubation, the exterior solutions turned a light amber color due to the diffusion of these compounds through the 3,500 MWCO tubing. This effect was less noticeable with the 1,000 MWCO tubing.

The stability of two of the compounds in solutions of different pH is illustrated in Figure 9. It appears that at pH 4.0 both diamino derivatives are unstable, while at neutral and alkaline pH no significant instability was noted over the two weeks.

Figures 10 to 12 illustrate binding reactions of 2,4DA with humic acid at three different hydrogen ion concentrations. For each figure there are two humic acid reactions 2,000 ppm and 1,000 ppm and two control reactions (no humic acid, inside and outside monitoring).

The results shown in Figure 10 at pH 4.0 indicate increased binding with time and with higher concentrations of humic acid. Even the control curves at this pH indicate compound instability as noted previously in Figure 6. At pH 4.0, the 2,4DA equilibration on the inside and outside of the tubing occurs after four to five hours. In Figures 11 and 12, similar results are found at pH 7.0 and pH 10.0 when compared with pH 4.0, with the exception of the longer equilibration time at 7.0 and the lack of instability of the controls over time except at pH 4.0.

In Figure 13 results are presented for binding between 2,6DA and humic acid at 7.0. Equilibration time is four to five hours and there is some indication of binding. Figure 14 illustrates similar reactions, but with 2A.

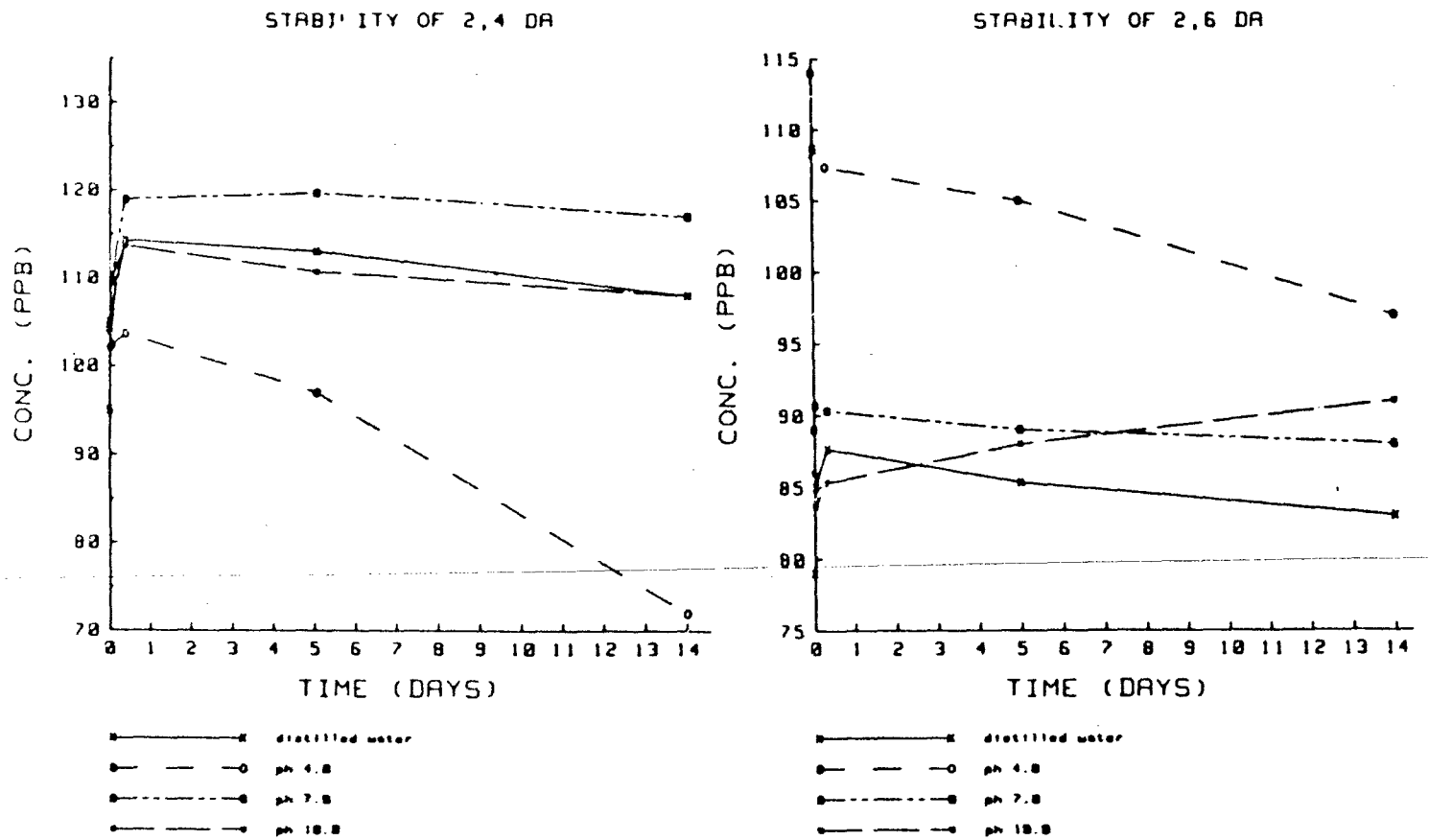

Figure 9. Stability of 2,4DA (left) and 2,6DA (right) at different hydrogen ion concentrations.

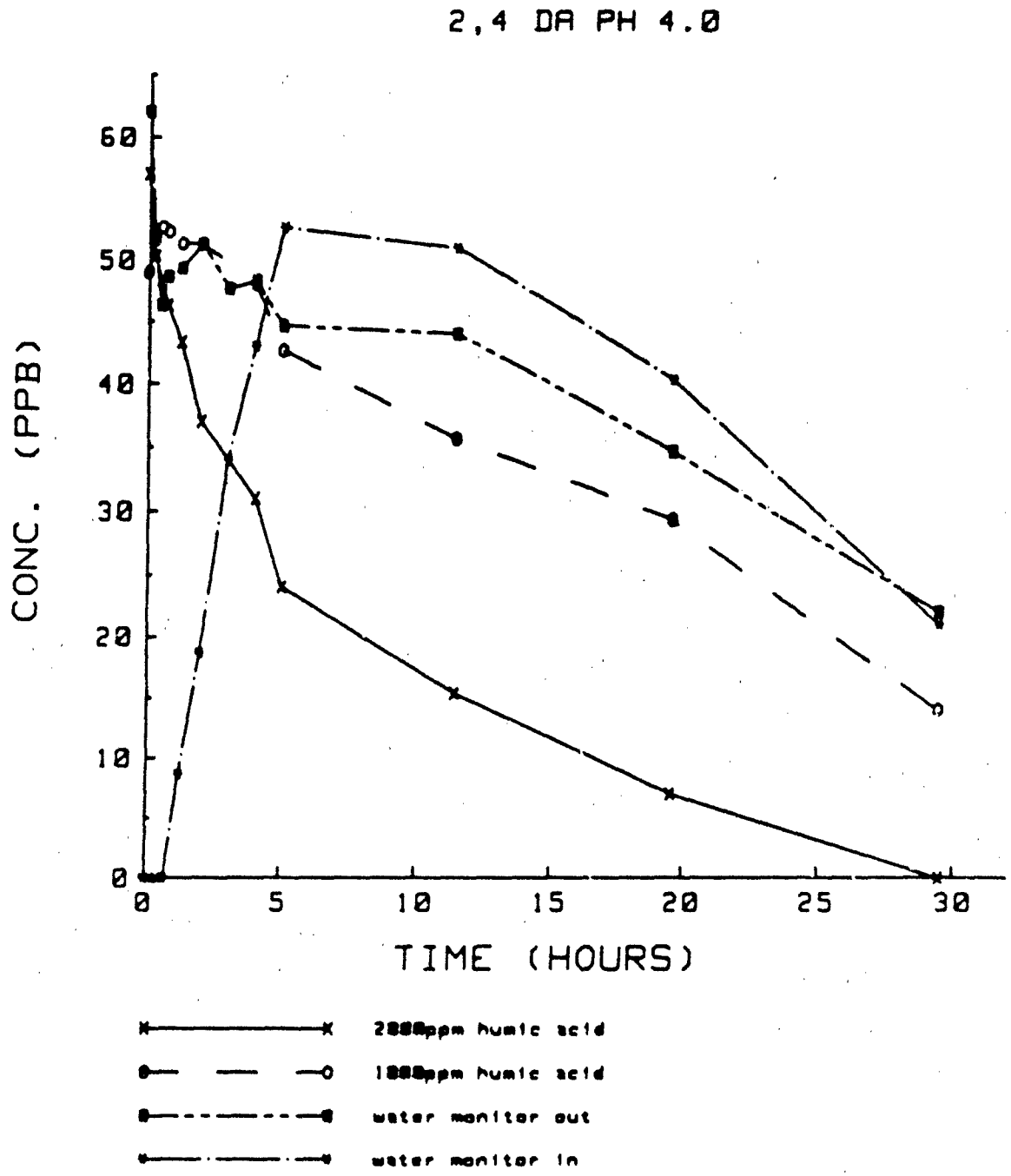

Figure 10. Dialysis experiment with 2,4DA at pH 4.0.

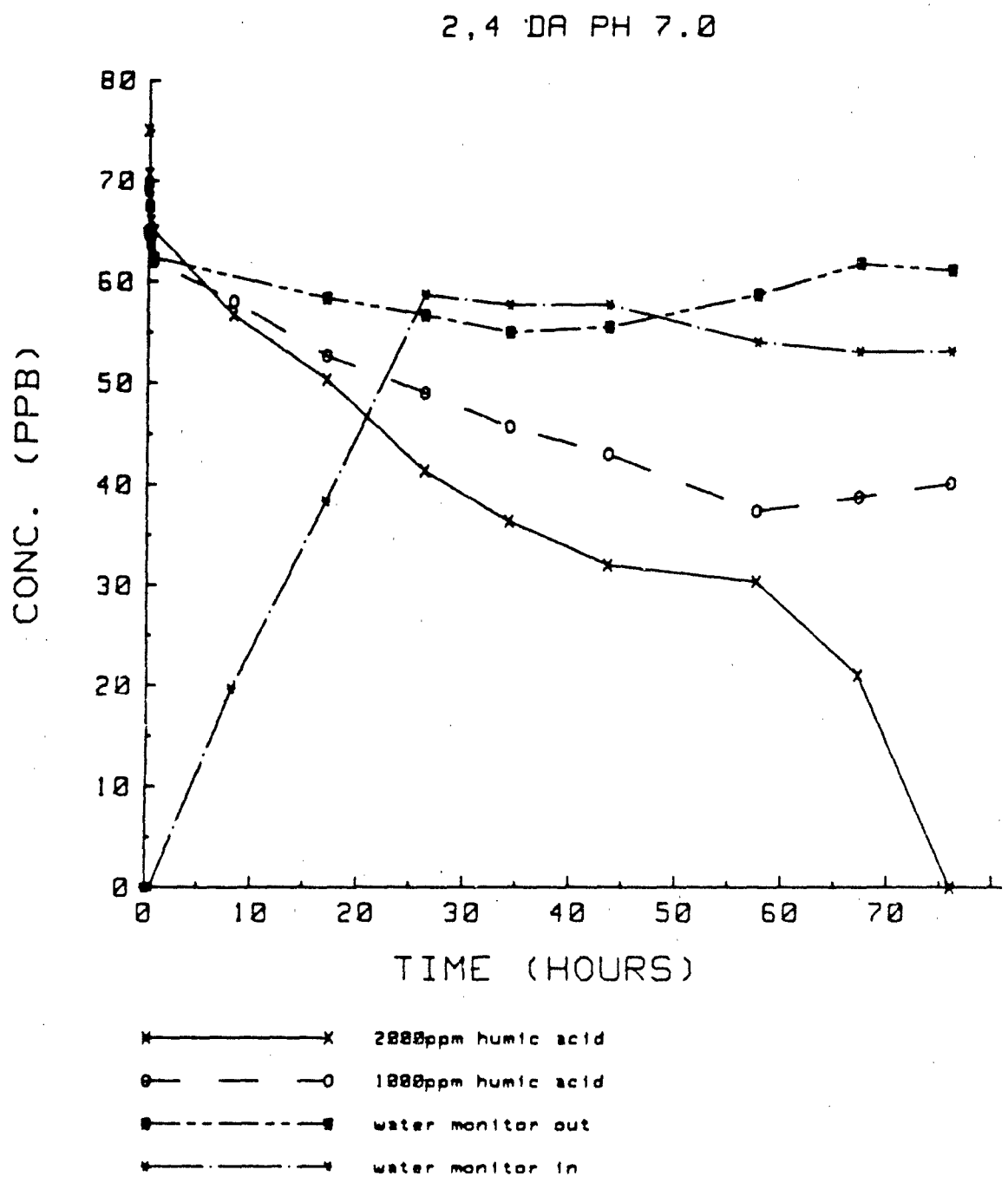

Figure 11. Dialysis experiment with 2,4DA at pH 7.0.

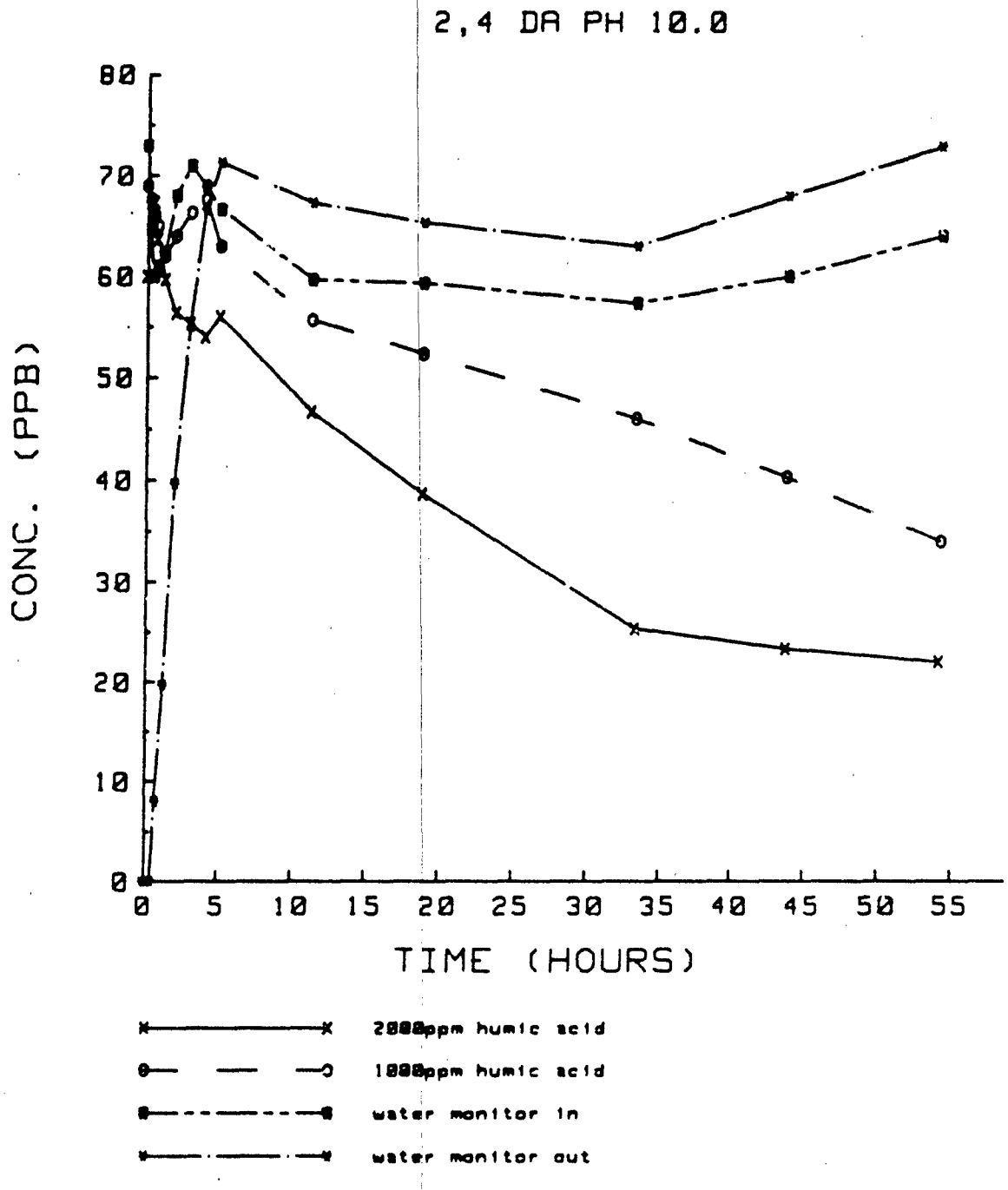

Figure 12. Dialysis experiment with 2,4DA at pH 10.

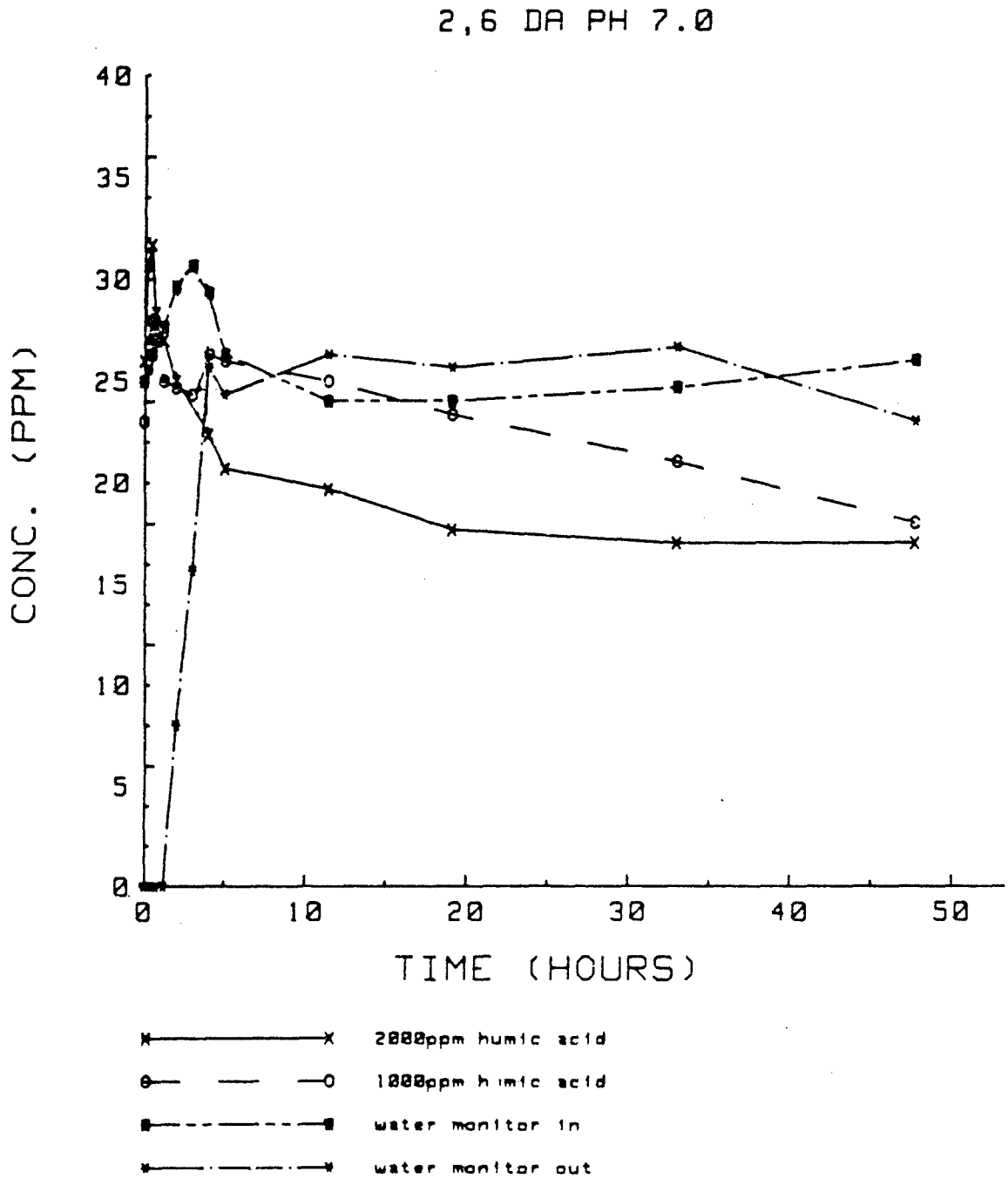

Figure 13. Dialysis experiment with 2,6DA at pH 7.0.

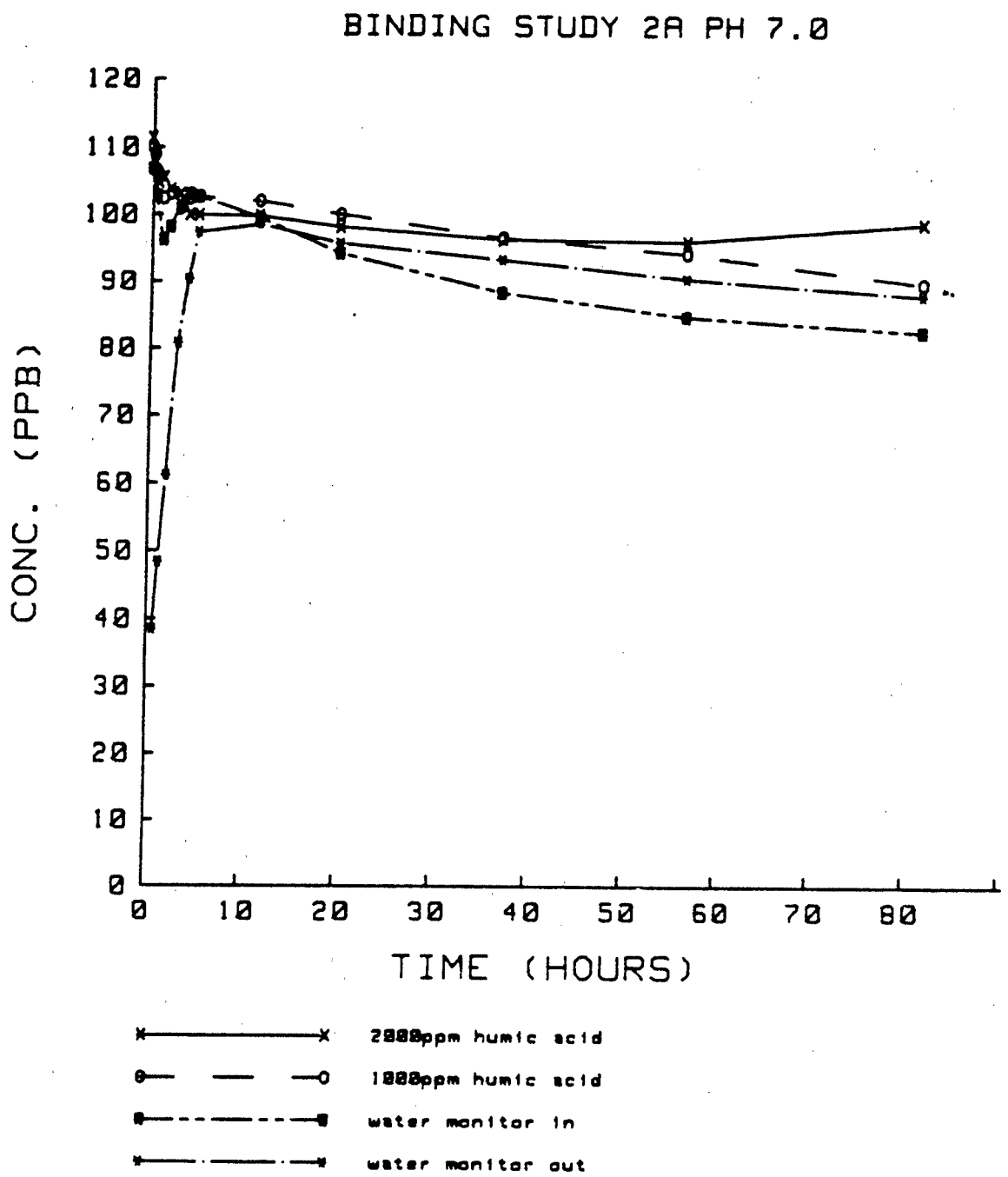

Figure 14. Dialysis experiment with 2A at pH 7.0.

In this case, no binding reactions are evident under these experimental conditions. This fact illustrates the discriminatory test methodology, unlike the results found for the acid-precipitation method.

The results of the reaction between peroxidase and 2,4DA are illustrated in Figure 15. A reaction occurred at pH 7.0 that did not occur in either control reaction (hydrogen peroxide without enzyme and enzyme without hydrogen peroxide). The yellow reaction product formed was isolated, but gas chromatography/mass spectrometry characterization was unsuccessful. Larger quantities of the products need to be synthesized in order to attempt further characterization. No peroxidase catalyzed reactions were observed with TNT or 4OHA.

The results from the Ames screening test for mutagenicity are presented in Table 2. The results indicate that TNT, as previously shown, is mutagenic both with and without metabolic activation, while the insoluble polymer is not. There is one exception with the polymer at 5 µg per plate with activation and strain TA1538, however, this is a borderline positive and it only occurred at the lowest concentration tested. Since no toxicity due to this polymer was noted at the higher concentrations, and these concentrations did not produce a positive test, it is doubtful that this one positive result is representative.

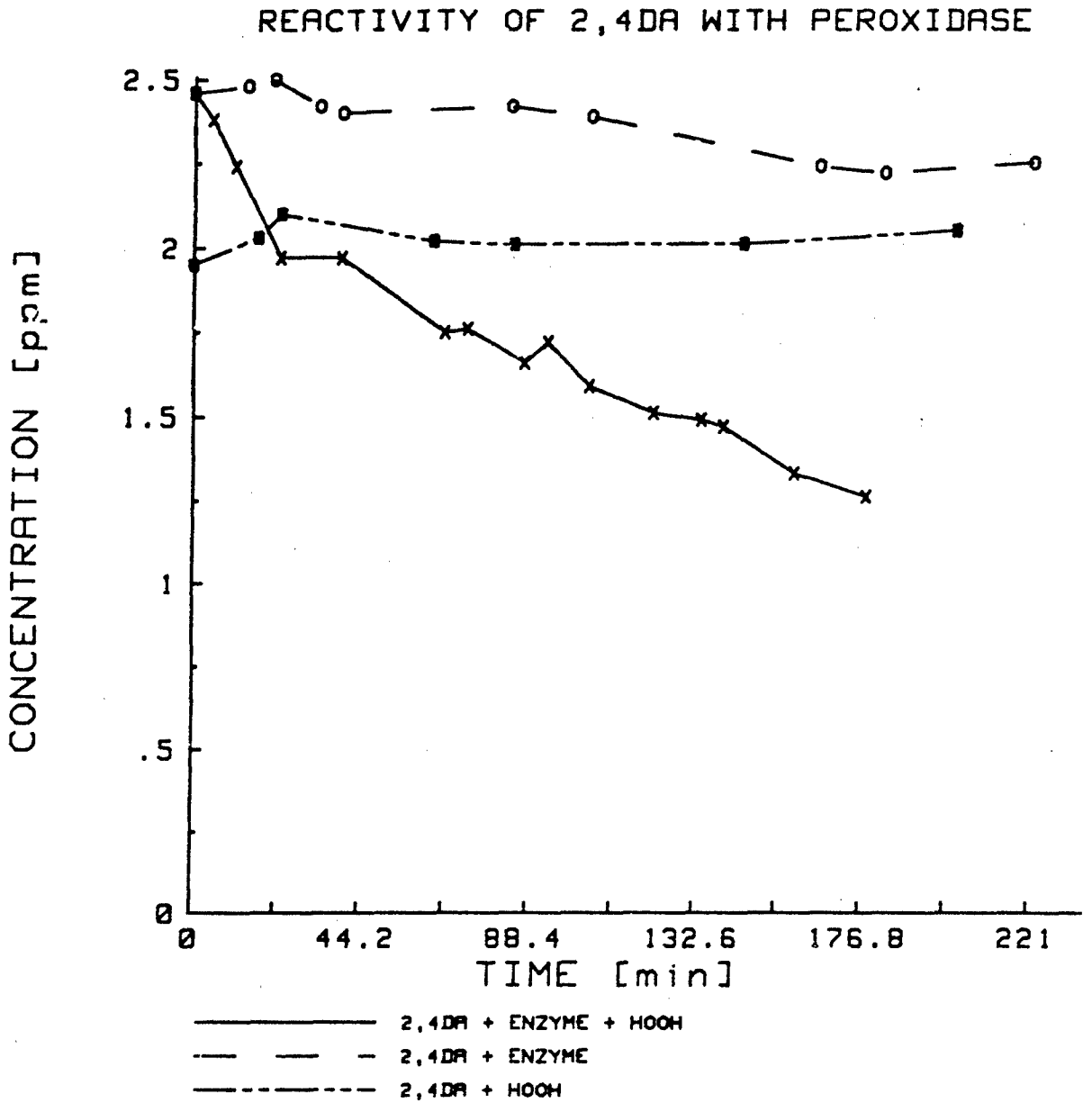

Figure 15. Enzymatic reaction between 2,4DA and peroxidase.

Table 2. Ames screening test results with the TNT insoluble polymer.

Compounds	Metabolic Activity	Micrograms per Plate	ppm (Approx)	Histidine Revertants Per Plate ($\bar{x} \pm 1SD$) TA1535	TA1537	TA1538	TA98	TA100
1) Negative Controls	+	0	0	15±8	9±2	6±3	13±3	117±16
	−	0	0	11±7	7±1	8±2	21±10	97±24
2) Positive Controls:								
sodium azide	−	1	0.04	103±19				108±28
9-aminoacridine	−	150	6	1522±486				
4-nitrophenylenediamine	−	10	0.4			796±65		
2-nitrofluorene	−	50	2				728±78	
2-anthramine	+	2	0.08	78±25	199±9	608±190	1014±54	1140±604
3) 2,4,6-trinitrotoluene	+	1,000	40	7±3	33±6*	7±6	19±6	T
	+	500	20	18±4	57±16*	6±1	39±2*	815±184
	+	50	2	12±2	12±4	7±1	27±16	131±13
	+	5	0.2	13±7	7±1	6±5	18±2	91±11
	−	1,000	40	T	T	T	13±4	T
	−	500	20	T	T	12±6	147±12*	T
	−	50	2	12±5	5±3	11±7	34±9	154±22
	−	5	0.2	11±5	5±3	6±3	26±10	91±6
4) Insoluble polymer	+	1,000	40	13±4	3±1	9±2	17±2	96±12
	+	500	20	13±2	3±3	13±7	19±1	99±6
	+	50	2	12±6	6±2	10±4	14±6	119±12
	+	5	0.2	11±1	3±2	24±5*	10±6	94±24
	−	1,000	40	9±1	6±1	7±2	8±6	96±5
	−	500	20	8±2	5±3	5±5	6±5	100±44
	−	50	2	16±1	5±1	7±4	22±12	89±14
	−	5	0.2	9±5	5±1	11±3	13±7	89±14

DISCUSSION

The preliminary results in this report require supplementation and an extension of effort in order to understand more fully the scope and impact of these types of soil reactions. Extended studies will permit a more complete assessment of the influence these reactions exert on the environmental fate of TNT and TNT-reduction products.

The results of these preliminary studies prompt the following observations.

1. The first evidence for binding reactions between munition waste compounds and soil organic matter fractions has been presented.

2. Some microbial reduction products (2,4DA and 2,6DA) but not necessarily TNT itself or other TNT-reduction products appear to be the active, binding species. It may be that soil binding reactions occur only after biotransformation of the parent TNT (Figure 16). The unreactive aryl-nitro groups become more reactive once microbially reduced to the corresponding arylamine.

3. We have demonstrated soil enzymatic reactions catalyzed by peroxidase with 2,4DA as the substrate. This reaction did not occur with TNT or some of the other TNT-reduction products. The demonstration of this reaction may indicate another route of transformation of TNT-reduction products in soils. Potential products from this peroxidase catalyzed reaction with 2,4DA are shown in Figure 17.

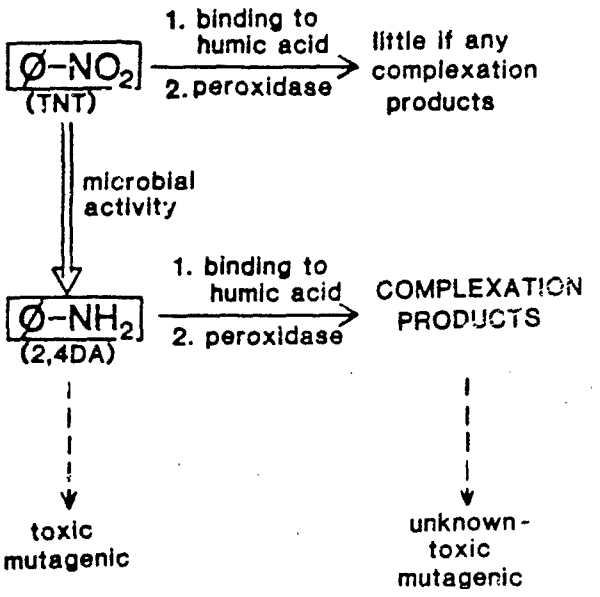

Figure 16. Scheme for TNT and TNT-reduction products which illustrates the active species involved in binding reactions.

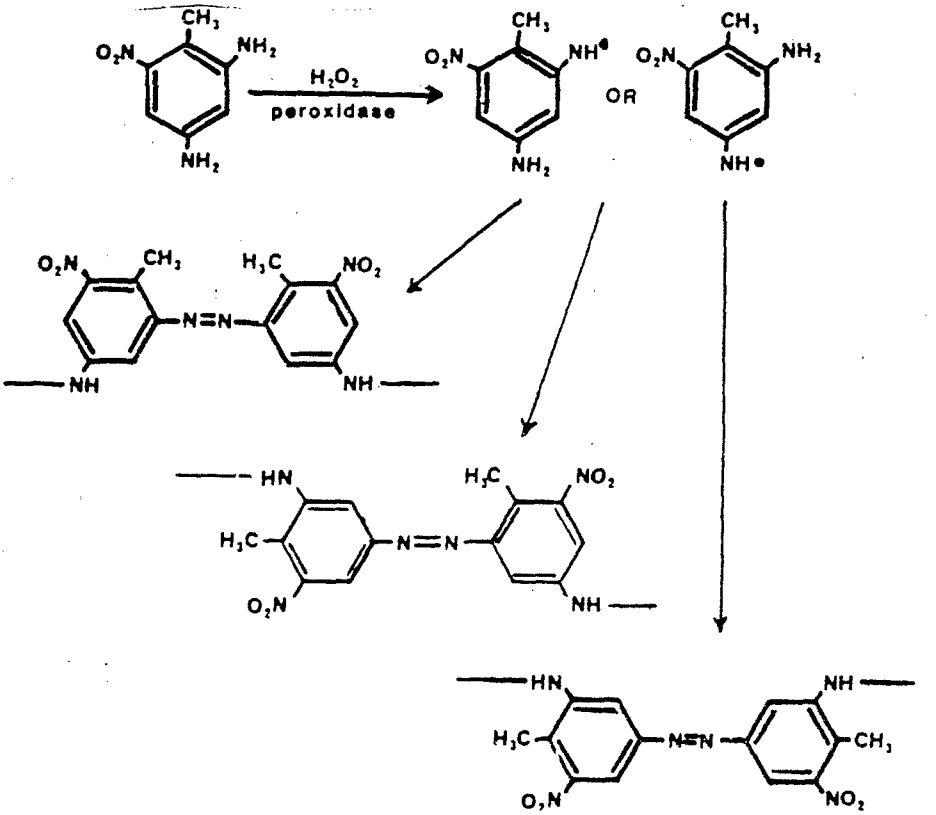

Figure 17. Postulated products formed during the peroxidase catalyzed reaction with 2,4DA.

Theoretically, these condensation products of diamines could continue to polymerize due to the availability of aryl-amines for further enzymatic oxidation. The azo compounds illustrated in Figure 18 could potentially form as azoxy compounds as well.[36]

There are a number of areas where further research efforts are required. Some of these areas are currently under investigation in ongoing efforts.

1. Studies should continue on binding reactions with TNT-reduction products and should address the variables of concentration of compound, concentration of humic acid, pH, and reaction time.

2. Binding reactions with other soil organic and inorganic fractions (fulvic, humin, clays[37]) should be evaluated.

3. Studies with peroxidase catalyzed reactions with TNT-reduction products, including isolation and characterization of the products from these reactions should continue.

4. Synthesis of sufficient bound product (TNT-reduction product and humic acid) will be accomplished in order to perform Ames screening tests for mutagenicity and to perform stability (chemical, physical, and biological) tests on the bound residues.

The microbial reduction products from TNT as well as TNT itself are toxic and mutagenic. It is unknown whether or not these characteristics are altered upon binding in soils since no research has been undertaken in this area.

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[36] Terpugova, M.P., V.G. Kostrovskii, V.G. Mazur, and I.L. Kotlyarevski. Determination of azoxy groups in polyazopolyarenes. Trans. from Isvestiya Akademii Nauk SSSR, Seriya Khimicheskaya, No. 11, pp. 2623-2625 (1970).

[37] Wang, T.S., S.W. Li, and Y.L. Ferng. Catalytic polymerization of phenol compounds by clay minerals. Soil Sci. 126: 15-21 (1978).

However, TNT-conjugates, such as the synthetic polymer (nonmutagenic) studied in this report, and TNT-surfactant complexes (mutagenic) have been assessed for mutagenic activity.

In regard to stability of the complexes, the turnover time of the bound residues is unknown. Instability could potentially result in a recontamination problem and therefore the effects of all environmental stresses on the stability of the bound residues must be addressed (i.e., moisture, temperature, pH).[38-41] Some research has been undertaken to address the microbial stability of soil-bound residues from insecticides, pesticides, and herbicides.[42-44]

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[38] Helling, C.S. and A.E. Krivonak. 1978. Physicochemical characteristics of bound dinitroaniline herbicides in soils. J. Agric. Food Chem. 26(5): 1156-1163.

[39] Hsu, T.S. and R. Bartha. 1974. Biodegradation of chloroaniline-humus complexes in soil and in culture solution. Soil Sci. 118(3): 213-220.

[40] Hsu, T.S. and R. Bartha. 1976. Hydrolyzable and nonhydrolyzable 3,4-dichloroaniline-humus complexes and their respective rates of biodegradation. J. Agric. Food Chem. 30: 161-164.

[41] Worobey, B.L. and G.R. Webster. 1982. Hydrolytic release of tightly complexed 4-chloroaniline from soil humic acids: an analytical method. J. Agric. Food Chem. 30: 161-164.

[42] Helling, C.S. and A.E. Krivonak. Biological characteristics of bound dinitroaniline herbicides insoils. J. Agric Food Chem. 26(5): 1164-1172 (1978).

[43] Khan, S.V. and K.D. Ivarson. 1981. Microbiological release of unextracted (bound) residues from an organic soil treated with prometryn. J. Agric. Food Chem. 29: 1301-1303.

[44] Saxena, A. and R. Bartha. 1983. Microbial mineralization of humic acid-3,4-dichloroaniline complexes. Soil Biol. Biochem. 15(1): 59-62.

5. Study and synthesis of insoluble residues of TNT or TNT-reduction products should continue. Some efforts in this area with other compounds have been reported in the literature. A significant production of nonextractable residues has been reported during the biotransformation of the fungicide pentachloronitrobenzene (aryl-nitro).[45,46]

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[45] Lamoureux, G.L. and D.G. Rusness. 1980. Pentachloronitrobenzene metabolism in peanut. 1. Mass spectral characterization of seven glutathione-related conjugates produced in vivo or in vitro. J. Agric. Food Chem. 28: 1502-1070.

[46] Rusness, D.G. and G.L. Lamoureux. 1980. Pentachloronitrobenzene metabolism in peanut. 2. Characterization of chloroform-soluble metabolites produced in vivo. J. Agric. Food Chem. 28: 1070-1077.

CONCLUSION

Preliminary results of reactions between TNT and TNT-microbial reduction products with soil components are presented. Evidence is presented for binding reactions between some of these compounds and humic acid. Evidence is also presented for peroxidase catalyzed reactions with at least one of these compounds. The need for continued effort in this area of soil - chemistry, biochemistry, and microbiology with munitions compounds is emphasized. Only with this understanding can we fully comprehend the various fates of these compounds in soils.

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