BINDING OF AROMATIC AMINES

TO SOIL HUMIC SUBSTANCES

By

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ABSTRACT

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Model oxidative coupling systems were used in an effort to gain a better understanding of the processes which operate in soil to: (1) couple phenolic compounds together (humification), and (2) bind xenobiotic aromatic amines to humus constituents. The overall goal was to decrease the bioavailability of xenobiotics in soil through enhanced binding.

The relationship between molecular structure and reactivity for peroxidase mediated coupling of mono-substituted phenol and aniline compounds (electron donors) was investigated. The effects of structure on reactivity were treated quantitatively by use of the Hammett equation. The rho (ρ) values obtained from plots of electron donor rate constants versus their respective Hammett substituent sigma (σ) constants were negative for both phenols and anilines, demonstrating that reactivity was increased by the presence of electron-donating substituents on the aromatic ring. Furthermore, a relative comparison of reactivities for phenolic humus constituents demonstrated that phenolic compounds which possessed the 3-C acrylic group (e.g. ferulic acid) were significantly more reactive than other naturally occuring phenolic compounds tested (e.g. vanillic acid). These results suggested the preferential utilizaiton of lignin derived phenols possessing the acrylic group during peroxidase mediated synthesis of humic materials in soils.

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The peroxidase mediated cross-coupling of mono-substituted anilines with various phenolic compounds was also investigated. Reactivity of aniline, nitroaniline, and chloroaniline was greatly enhanced in the presence of a highly reactive electron donor such as ferulic acid. An investigation was also undertaken to demonstrate the feasability of using oxidative coupling enzymes and their highly reactive natural phenolic substrates to detoxify soil contaminated with aromatic amines. 3,3'-Dichlorobenzidine (DCB) served as the test compound and its binding fate in soil was manipulated by additions of peroxidase and ferulic acid. Addition of ferulic acid to DCB-amended soil significantly enhanced covalent binding of DCB to the soil humic fraction while addition of peroxidase alone failed to enhance DCB binding. The use of ferulic acid as an activating compound to enhance binding of DCB seemed reasonable based on the previous rate enhancement investigations. Compounds such as ferulic acid may be useful in detoxification of contaminated soils.

DEDICATION

The author wishes to dedicate this dissertation to my wife, Sandra, my brother, David and my parents, Iva and Vaughn.

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CHAPTER I

SOIL HUMUS: ITS FORMATION AND REACTIONS WITH AROMATIC AMINES

Soil organic matter, also commomly refered to as humus, consists of humic and non-humic substances. Humic substances are high-molecular weight black to brown colored materials, which form as a result of secondary synthesis reactions in soil (23). Humic substances are dissimilar to the high molecular weight biopolymers of plants and microorganisms and are distinctive to the soil environment. Non-humic substances are compounds that belong to the known classes of biochemicals, such as amino acids, peptides, carbohydrates, lipids and phenolic compounds (23).

The maintenance of soil humic substances results from a balanced equilibrium between synthesis and decomposition. The formation of soil humic substances is believed to result from one or more of three different pathways which are discussed below.

Through the years several pathways have been postulated to explain the formation of soil humic substances. An earlier classical theory (the lignin theory) proposed that humic substances originated from modified lignins (25). According to this theory lignin, partially modified by soil microorganisms, condenses with proteins by way of a Schiff base to yield soil humic substances. In this case protein is the source of nitrogen found in soil humic substances. The modifications of lignin thought to have taken place were: (1) the loss of methoxy groups, (2) generation of o-dihydroxyphenols, and (3) oxidation of aliphatic side chains to form carboxyl groups. An

important feature of the lignin theory is that the backbone of humic substances in soils are modified lignins. As such, humic substance formation is not a synthetic process.

Another early theory proposed that soil humic substances are formed from condensation reactions involving sugars and amino acids (14). According to this theory sugars and amino acids condense by way of amine addition to carbonyl groups of sugars to form imines. The resulting glycosylamine subsequently undergoes an Amadori rearrangement to form N-substituted -1-amino-deoxy-2-ketose (NADK). The product of this rearrangement (NADK) is highly unstable and is subject to numerous reactions which result in dark colored polymers.

The currently accepted theory of soil humic substance formation is the polyphenol theory. This theory states that the basic structural backbone of humic materials form as a result of polymerization of lignin and microbe derived quinone monomers (18,21). As such, this is a synthetic process unique to soils. One important source of the organic monomers are plants which contribute lignins to the soil environment. Once plant lignins reach the soil they undergo decomposition to simpler aromatic organic molecules such as ferulic acid which may then be either mineralized or stabilized via incorporation into the soil humic fraction. One important group of microorganisms for lignin decomposition are the basidiomycetes, more commonly refered to as the "white rot fungi". The "white rot fungi" are able to grow exclusively on lignin and in fact prefer lignin as a carbon and energy source to many other carbon sources (23).

Soil microorganisms are also thought to contribute significant amounts of phenolic compounds or polyphenols (from both aromatic and non-aromatic precursors), towards humic substance formation by way of their anabolic pathways. For example, Haider and Martin (11) have shown that the soil fungus Epicoccum nigrum synthesizes 2,4-dihydroxy-6-methylbenzoic acid (orsellinic acid) and 2-methyl-3,5-dihydroxybenzoic acid (cresorsellinic acid) in a glucose-aspargine medium. Futhermore, during growth both orsellinic acid and cresorsellinic acids undergo modification to yield the following phenolic compounds: pyrogallol, resorcinol, gallic acid, phloroglucinol, 3,5-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid. Thus, the combined processes of lignin degradation and microbial synthesis in soil result in the formation of a pool of polyphenols from which humic substances are synthesized. According to the polyphenol theory, phenolic compounds which originate either by way of lignin decomposition or microbial synthesis undergo oxidation reactions to form aryloxy radicals or quinones. The aryloxy radicals and quinones couple together or to the growing humic polymer to form stable humic substances. Other organic components of soils which are involved in the formation of humic substances are: proteins, amino acids, and carbohydrates etc. Thus, the polyphenol theory differs from the lignin theory in that the former is a synthetic process which builds a high molecular weight polymer from a low molecular weight monomer.

The formation of quinone and aryloxy radicals from phenols is a process believed to be mediated by polyphenoloxidases and peroxidases present in soil (22,23) and by inorganic catalysts and autooxidation

(21,26). The two major groups of oxidative coupling enzymes present in soil are peroxidases and phenoloxidases (e.g. laccases) (22,23). Peroxidases contain a heme prosthetic group and have an absolute requirement of H_2O_2 for activity. A generalized free radical mechanism has been well established for peroxidase and is presented in Figure 1. Guaiacol was chosen as a representative substrate (electron donor) to illustrate the peroxidase mechanism because phenolic compounds are generally considered to be natural electron donors. The overall reaction mechanism requires two equivalents of electron donor and one equivalent of H_2O_2 to produce a coupled product in a simplified three component system. Peroxidases are known to oxidize a wide variety of chemical compounds including aromatic amines, presumably by the mechanism shown in Figure 1.

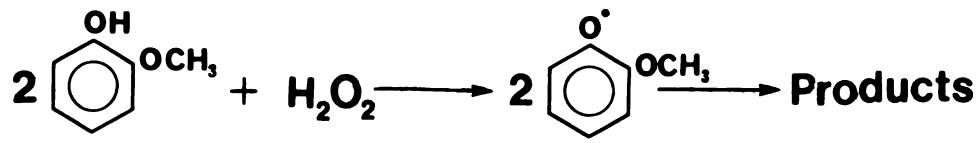


Figure 1. Reaction mechanism for peroxidase mediated coupling of phenols (22).

While peroxidases of different origins all appear to react according to the general mechanisms illustrated in Figure 1 (12), oxidases in general, appear to be more mechanistically diversified. Laccases are considered to be one of the most important classes of oxidases present in soil (22). Laccases are Cu containing enzymes and require O_2 as a co-substrate for activity. Phenols are generally considered to be the natural substrates for laccase enzyme as was the case for peroxidases. Substrate specificity for laccases, however, is somewhat more restricted compared to peroxidases, and normally do not utilize aromatic amines.

The fate of organic pollutants or xenobiotic chemicals in soil has become a major health concern in recent times due to the potential for surface and ground water contamination and for crop uptake and movement into the food chain. Certain xenobiotic chemicals pose serious health hazards due to their toxic and carcinogenic properties. One major environmental fate of hazardous organic chemicals is immobilization at the soil surface by association with humic substances. It is important to understand the nature of the xenobiotic-humic substance complex, for only by understanding this association can we begin to put together a comprehensive soil pollution indices that would relate relevant xenobiotic physiochemical properties with bioavailability and hazard assessment data.

One important class of persistant xenobiotic compounds found in soil are the alkyl- and halogen-substituted anilines. Aromatic amines serve as the basic structural matrix for many commonly used herbicides and pesticides and are also used in the manufacture of

plastics and fabric dyes. The appearance of aromatic amines in the soil environment may therefore be related to the intentional application of herbicides or the unintentional releases often associated with manufacturing processes and waste disposal. Because the ring carbons are not rapidly mineralized in soil, aromatic amines remain available for binding reactions to soil humic substances. This association with soil humus further stabilizes aromatic amines against microbial degradation. Even chlorocatechols (produced from degradation of 2,4-D) which are readily degradable in culture solutions are stabilized in soil against microbial degradation by attachment to soil humus (Stott et al., 24).

It has been well documented that aromatic amines, when added to soil, become strongly and irreversibly bound to soil humic materials. This binding results from the formation of covalent bonds between soil humus and the amine group of anilines. The significance of bound residues as potential sources of future contamination is essentially unknown. Several examples can be described which illustrate that for aromatic amines in soil, binding processes are the predominant fate. For example, an investigation by Chisaka and Kearney (8) demonstrated that the herbicide 3',4'-dichloropropionanilide (propanil) is readily transformed by soil microorganisms to 3,4-dichloroaniline (DCA) in soil.

Chisaka and Kearney (8) observed that $^{14}CO_2$ evolution from ring-labelled propanil was less than 3% after 25 days of incubation in soil. Hsu and Bartha (13) found that up to 90% of the 3,4-dichloroaniline released during biodegradation of phenylamide herbicides becomes solvent-unextractable due to binding to soil

organic matter. The differences in bond stabilities for hydrolyzable versus non-hydrolyzable chloroaniline residue complexed with soil humic substances suggested to Hsu and Bartha (13) that two distinctly different covalent binding mechanisms were operative. Hsu and Bartha (13) also demonstrated that with time physically adsorbed and hydrolyzable 3,4-dichloroaniline residues slowly shifted to the non-hydrolyzable form.

Other aromatic amines have been studied with respect to their fate in soil. For example, Boyd et al., (6) have investigated the fate of 3,3'-dichlorobenzidine (DCB) in soil and discovered that essentially 90% of the DCB added to soil was irreversibly bound to the soil humic fraction. Boyd et al., (6) observed that an initial sharp decrease in the amount of solvent-extractable DCB was accompanied by a sharp increase in NaOH-extractable DCB, which indicated that movement of DCB into the humic fraction was essentially complete since NaOH-extractable DCB plus solvent-extractable DCB remained constant.

Several investigators have studied the general phenomenon of aromatic amine binding to soil humic substances. It is generally thought that several different types of chemical or biochemical reactions are responsible for binding of aromatc amines to soil humic substances. The reaction mechanisms that have been postulated to delineate the nature of aromatic amine binding to soil humic components may be divided into two main categories. Category one employs the use of classical organic reaction mechanisms while the second category enlists the aid of biocatalysts (enzymes).

Hsu and Bartha (15,16) first proposed imine formation to explain the rapid binding of primary aromatic amines to soil humic substances (Figure 2).

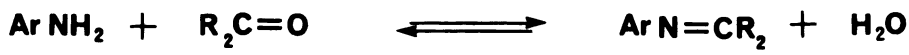
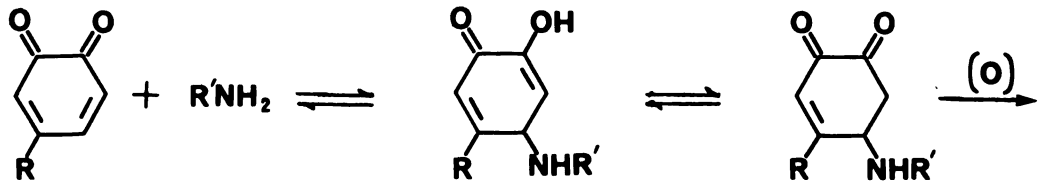
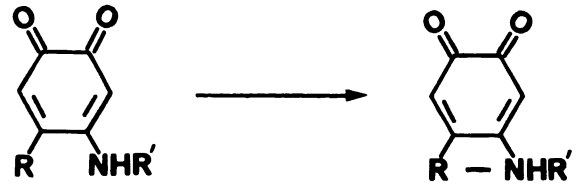


Figure 2. Imine formation reaction (15,16).

The reaction involving aromatic amines and the carbonyl group of aldehydes or ketones to produce imines is a reversible reaction. The equilibrium can be shifted towards the two starting reactants if excess water is present. In soils, it is not clear where the equilibrium lies under normal conditions (19). Imine formation was a mechanism suggested by Hsu and Bartha (15,16) to explain the readily exchangeable binding nature of a primary aromatic amine with soil humic substances.

There is, however, another type of binding involving aromatic amines with soil humic substances that is resistant to hydrolysis and not readily exchangeable. Cranwell and Haworth (9) proposed that primary or secondary (aromatic) amines might undergo a nucleophilic substitution reaction with a Michael acceptor. In this case the Michael acceptor would be a quinone-like residue which is attached to the soil humic matrix (Figure 3).





R' = Humic substance matrix

Figure 3. Michael addition of aromatic amine to humus matrix (19).

The nucleophilic addition of a primary or a seconary (aromatic) amine to the quinone moiety of the soil humic substance matrix is a slow and reversible reaction which is followed by a rapid tautomerization step. Oxidation of the quinone-amine complex results in stabilization of the (aromatic) amine. Continued internal reactions would likely take place to completely secure the (aromatic) amine into the humic substance matrix and thus produce a complex (aromatic) amine which resists acid or base hydrolysis and is not readily exchangeable (9,19).

The imine formation and Michael addition mechanisms discussed thus far require, in principle, the presence of two reactants; an aromatic amine and the carbonyl and quinone moieties of the humic component. The next group of mechanisms to be presented not only requires the presence of these two reactants but also a biocatalyst as well. In this process polyphenols are coupled together after being enzymatically oxidized to the aryloxy radical. This enzyme may also convert aromatic amines to radicals which then couple with soil humic materials. Alternatively, the aromatic amine may react chemically as described above with humic materials formed via

oxidative coupling reactions.

Although, several investigators have emphasized the importance of oxidative coupling enzymes in the binding processes involving aromatic amines and soil humic substances, there still exists a paucity of information regarding the role of these enzymes in a comprehensive binding mechanism. Berry and Boyd (4) have attempted to gain some insight into the specific coupling mechanism detailing aromatic amine binding to soil humic substances by investigating the relationship between chemical structure and reactivity for peroxidase mediated cross-coupling of mono-substituted anilines with various phenolic humus constituents. Because the reactivities of aniline, nitroaniline, and chloroaniline were so greatly enhanced in the presence of a highly reactive electron donor such as ferulic acid, Berry and Boyd (4) concluded aniline compounds were reacting by a different mechanism than occurs when they are present as the sole electron donors. They suggested that the most plausible mechanism is a secondary chemical reaction between anilines and intermediates or products produced during the enzymatic oxidations of phenolic electron donors. Similar results were obtained by Bollag et al., (5) when they investigated laccase mediated cross-coupling of phenolic compounds with various anilines. They determined that coupling of chloroanilines to phenolic compounds resulted from chemical reactions since chloroanilines alone with laccase did not produce oligomeric products (Bollag et al., 5). The results obtained by Bollag et al. (5) and Berry et al. (4) suggest that aromatic amine binding to soil humic components results from a chemical reaction which may be only indirectly controlled by the presence of oxidative coupling enzymes

in soil.

Although binding of aromatic amines to soil humic substances has been well established several investigators have demonstrated the existance of azobenzenes and triazenes in soil after additions of aniline. For example, Bartha et al. (3) observed formation of 3,3',4,4'-tetrachloroazobenzene (TCAB) in soils that had been incubated with DCA. Peroxidases were believed to be the responsible catalytic agents in the coupling processes. Bordeleau et al. (7) proposed the following pathway for formation of TCAB in DCA incubated soil (Figure 4).

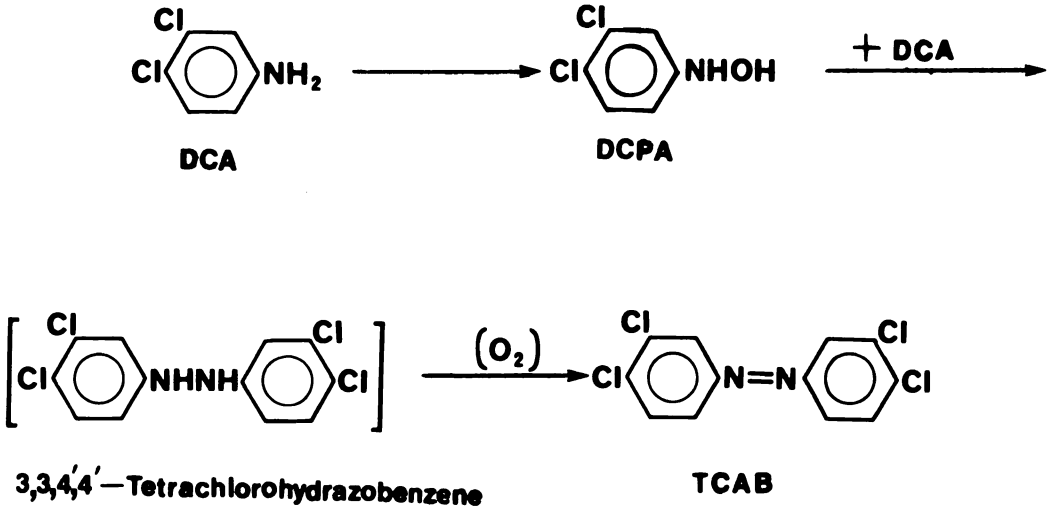


Figure 4. Proposed pathway for formation of TCAB in DCA-amended soil (3).

The initial step of the formation pathway is the peroxidase mediated formation of 3,4-dichlorophenylhydroxylamine (DCPA) from DCA. DCPA then reacts chemically with a molecule of DCA to form 3,3,4',4'-tetrachlorohydrazobenzene which subsequently undergoes oxidation to form TCAB. In another example, Plimmer et al. (20) demonstrated that 1,3-bis (3,4-dichlorophenyl) triazene could be formed in soil that had been incubated in the presence of high levels of the herbicide propanil. Plimmer et al., (20) suggested the following pathway for synthesis of triazene from propanil (Figure 5).

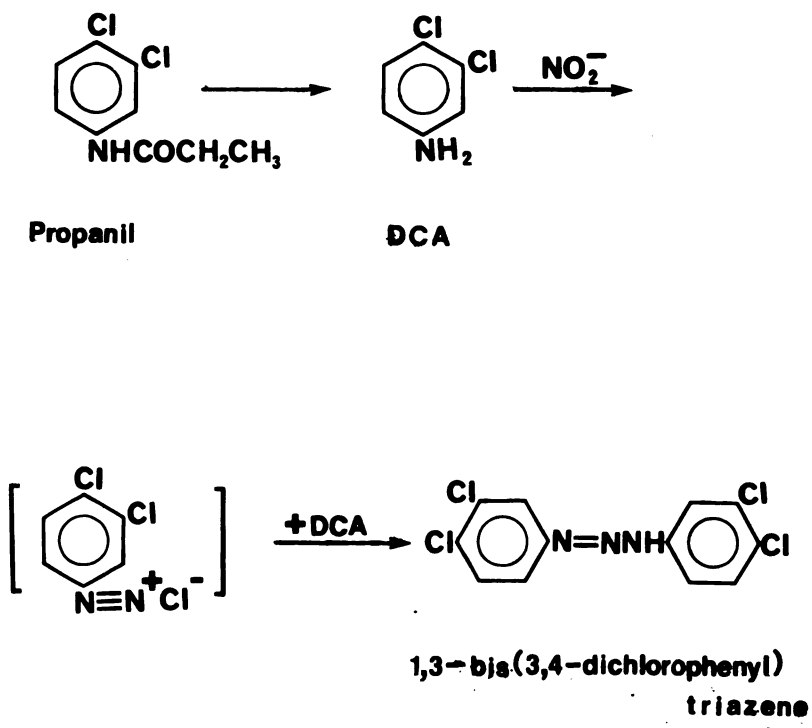


Figure 5. Proposed pathway for triazene formation via a diazotization mechanism (20).

The initial step of the formation of triazene from propanil is the transformation of propanil to DCA which is believed to be mediated by soil microorganisms. DCA is then postulated to undergo a chemical reaction with NO_2^- to produce a diazonium cation. The diazonium cation is then thought to react with a DCA molecule to produce the triazene. The formation of the triazene is therefore believed to be dependent on the concentration of NO_2^- in soil and does not require an oxidizing enzyme such as peroxidase.

It should be pointed out that in the preceding situations where azobenzenes and triazenes have been isolated from soil, investigators had to add artificially high levels of the starting reactant, chloroaniline. High levels of aniline are required to observe dimerization due to the low probability factor of an aniline "finding" and coupling to another aniline in soil. It is more likely that under normal circumstances, where there is 100 to 1000X less anilne present in soil, the more realistic scenario is that aniline will become bound to the humic components in soil (1,2,10,13,15,16,17).

The binding of xenobiotic chemicals to soil humic materials is a phenomenon which warrants an intense research effort both from the standpoint of pure and applied science. It is important to understand the physical nature of the binding process for only by understanding the nature of the xenobiotic-humic substance interaction can we be in a position to access the contamination potential of soil borne xenobiotics. It is also important to gain a fundamental understanding of these binding processes for only then can we begin to manipulate the binding of xenobiotic compounds in

soils. Enhancing the binding processes operative in soil which act to covalently couple xenobiotic and humic substances may offer great promise for detoxifying contaminated soils.

REFERENCES

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

2. Bartha, R. 1971. Fate of herbicide-derived chloroaniline in soil. J. Agr. Food Chem. 19:380-381.

3. Bartha, R., H.A. Blinke, and D. Pramer. 1968. Pesticide transformations: production of chloroazobenezenes from chloroanilines. Science. 161:582-583.

4. Berry, D.F. and S.A. Boyd. 1984. Reaction rates of phenolic humus constituents and anilines during cross-coupling. Soil Biol. Biochem. Submitted for publication.

5. Bollag, J.-M., R.D. Minard, and S.Y. Liu. 1983. Cross-linkage between anilines and phenolic humus constituents. Environ. Sci. Technol. 17:72-80.

6. Boyd, S.A., C.-W. Kao, and J.M. Suflita. 1984. Fate of 3,3'-dichlorobenzidine in soil: Persistence and binding. Environ. Tox. Chem. 3:0-0.

7. Bordeleau, L.M., J.D. Rosen, and R. Bartha. 1972. Herbicide-derived chloroazobenzene residues: pathway of formation. J. Agr. Food Chem. 20:573-577.

8. Chisaka, H., and P.C. Kearney. 1970. Metabolism of propanil in soils. J. Agr. Food Chem., 18:854-858.

9. Cranwell, P.A., and R.D. Haworth. 1971. Humic acid IV - The reaction of amino acid esters with quinones. Tetrahedron 27:1831-1837.

10. Freitag, D., I. Scheunert, W. Klein, and F. Korte. 1984. Long-term fate of 4-chloroaniline-^{14}C in soil and plants under outdoor conditions. A contribution to terrestrial ecotoxicology of chemicals. J. Agr. Food Chem. 32:203-207.

11. Haider, K., and J.P. Martin. 1967. Synthesis and transformation of phenolic compounds by Epicoccum nigrum in relation to humic acid formation. Soil Sci. Soc. Am. Proc. 31:766-772.

12. Hewson, W.D., and L.P. Hager. 1979. Peroxidases, catalases, and chloroperoxidase. In The Porphyrins, Vol. VII. Academic Press, New York.

13. Hsu, T.-S., and R. Bartha. 1976. Hydrolyzable and nonhydrolyzable 3,4-dichloroaniline-humus complexes and their respective rates of biodegradation. J. Agr. Food Chem. 24:118-122.

14. Hodge, J.E. 1953. Chemistry of browning reactions in model systems. J. Agr. Food. Chem. 1:928.

15. Hsu, T.-S. and R. Bartha. 1974. Biodegradation of chloroaniline humus complexes in soil and in culture solution. Soil Sci. 118:213-220.

16. Hsu, T.S., and R. Bartha. 1974. Interaction of pesticide-derived chloroaniline residues with soil organic matter. Soil Sci. 116:444-452.

17. Iwan, J., G.-A., Hoyer, D. Rosenberg, and D. Goller. 1976. Transformations of 4-chloro-o-toluidine in soil: Generation of coupling products by one-electron oxidation. Pest. Sci. 7:621-631.

18. Martin, J.P., and K. Haider. 1971. Microbial activity in relation to soil humus formation. Soil Sci. 111:54-63.

19. Paris, G.E. 1980. Covalent binding of aromatic amines to humates. 1. Reactions with carbonyls and quinones. Environ. Sci. Technol. 14:1099-1106.

20. Plimmer, J.R., P.C. Kearney, H. Chisaka, J.B. Yourt, and V. Klingebiel. 1970. 1,3-Bis(3,4-dichlorophenyl) triazene from propanil in soil. J. Agr. Food Chem. 18:859-861.

21. Shindo, H., and P.M. Huang. 1982. Role of Mn(IV) oxide in abiotic substances in the environment. Nature. 298-363-365.

22. Sjoblad, R.D., and J.-M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In Soil Biochemistry E.A. Paul and J.M. Ladd (eds.), Vol. 4. chapt. 3. Marcel Dekker, Inc. New York.

23. Stevenson, F.J. 1982. Humus Chemistry. Chapt. 1. John Wiley and Sons, New York.

24. Stott, D.E., J.P. Martin, D.D. Focht, and K. Haider. 1983. Biodegradation, stabilization in humus, and incorporation into soil biomass of 2,4-D and chlorocatechol carbons. Soil Sci. Soc. Am. J. 47:66-70.

25. Waksman, S.A. 1932. Humus. Williams and Wilkins, Baltimore.

26. Wang, T.S.C., and M.-C. Wang. 1983. Catalytic synthesis of humic substances by using aluminums as catalysts. Soil Sci. 136:226-230.

CHAPTER II

OXIDATIVE COUPLING OF PHENOLS AND ANILINES BY PEROXIDASE; STRUCTURE-ACTIVITY RELATIONSHIPS

INTRODUCTION

In soils, phenolic compounds produced through microbial synthesis and lignin degradation are thought to be the predominant monomeric constituents which are oxidatively coupled by indigenous enzymes to form stable humic substances (Martin and Haider, 1971). Aromatic amines present in soil naturally or as environmental pollutants may also be coupled together, or to the growing humic polymer during the enzymatic polymerization of phenolic monomers. The end result of these synthetic processes are large molecular weight polymers of remarkable stability (mean residence time >500 years) (Stevenson, 1982) which consist of coupled phenols, aromatic amines and other organic soil constituents such as amino acids and proteins.

The oxidative enzymes present in soil which act to covalently link phenolic compounds and aromatic amines in the process forming humic materials belong to one of two groups: monophenol monooxygenases or peroxidases. Laccases, which are the most important class of monooxygenases in soil, contain Cu and require O_2 for activity (Sjoblad and Bollag, 1981). Most peroxidases contain a heme prosthetic group and have an absolute requirement of H_2O_2 for activity. Laccases and peroxidases are thought to couple phenolic compounds by way of a free radical mechanism (Sjoblad and Bollag, 1981) and have been used to synthesize model humic polymers (Martin and Haider, 1980). Whereas laccases are inactive with many simple substrates, peroxidases have been shown to couple a wide variety of substituted anilines (Sjoblad and

Bollag, 1981).

Some of the more persistant xenobiotic chemicals found in soil are the alkyl- and halogen-substituted anilines which serve as the basic structural unit for many commonly used herbicides and pesticides. Because ring carbons of aniline compounds are generally resistant to mineralization in soil (Bartha et al., 1968), the aniline moiety is often the principal biodegradation intermediate of these herbicides. Aniline compounds are also used in a variety of industrial processes including the manufacture of dyes and pigments. Thus, the appearance of aniline and its compounds in soil may result from the intentional application of herbicides and from unintentional releases associated with manufacturing processes and waste disposal. Because many aniline based compounds are persistent in soil they often become chemically bound to soil humic substances. It is important to understand the nature of these reactions for many anilines are suspected carcinogens.

In an earlier investigation, Bordelleau and Bartha (1972), attempted to gain some insight into the relationship between molecular configuration and susceptibility to enzymatic coupling of substituted anilines by a fungal peroxidase and aniline oxidase. They concluded that, in general, reactivity was enhanced by electron donating substituents which apparently increased electron density at the reaction center, i.e. the $-NH_2$ group. These conclusions were based on a semi-quantitative measurement of the amount of products produced after a given reaction time.

The overall goal of our research was to gain a better understanding of enzymatic processes operative in soil which act to couple phenols and aromatic amines. In the present study we have investigated the

relationship between chemical structure and reactivity for the horseradish peroxidase mediated oxidative coupling of substituted anilines and phenols. Relative reaction rates of the various electron donor compounds were determined by measuring their disappearance using high performance liquid chromatography. The effects of structure on reactivity were treated quantitatively by use of the Hammett equation. We discuss implications concerning the nature of the transition state in the rate controlling step of these reactions.

EXPERIMENTAL

Materials. Horseradish peroxidase (HRP) Type II (Donor: hydrogen-peroxide oxidoreductase; E.C. No. 1.11.1.7) was purchased from Sigma Chemical Co. Two different batches were used for these studies: for phenols a HRP batch was used with a RZ value of 1.7 and a specific activity of 200 purpurogallin units per mg solid and for anilines the batch used had a RZ value of 1.52 with a specific activity of 152 purpurogallin units per mg solid. The anilines and phenols were obtained from Aldrich Chemcial Co. and Eastman Kodak Co. and used without further purification. All aniline, phenol and HRP solutions were prepared in a 0.1 M phosphate buffer (pH = 6.9) which was made from double glass distilled deionized water.

Kinetic Measurements. Aniline, phenol, and HRP solutions were prepared fresh before each rate study. Kinetic experiments were carried out at a constant temperature of 20 $\pm$ .01 ^{o}C in open beakers containing 100 mL of phosphate buffered solution, 21.5 umoles of aniline or phenol (electron donors), and 107.4 umoles of H_2O_2. Preliminary experiments revealed that the concentration levels of H_2O_2 used produced maximum rates of reaction at the aformentioned levels of aniline, phenol, and HRP. The concentration levels determined for the two electron donors, aniline and phenol, were used for all monosubstituted aniline and phenol compounds for cross comparison purposes.

To initiate the reaction 0.5 mL of HRP solution, containing 65 activity units for phenols or 152 activity units for aniline were added to the reaction mixture while stirring vigorously. Reaction rates were measured by monitoring disappearance of the aniline or phenol compounds. From 100 mL of the reaction solution 2.8 mL aliquots were periodically withdrawn and placed into glass vials containing 0.7 mL of a 50 mM KCN solution. Potassium cyanide was found to be effective in inhibiting HRP activity and was used to stop the reactions. The samples were placed on ice and stored in the dark until HPLC analysis which was carried out immediately after collection of the samples. Control experiments were performed using HRP that had been deactivated by boiling approximately 1.5 h.

Sampling times were adjusted for the various compounds according to their reaction rates. Sampling time intervals for the aniline and phenol compounds were as follows: (1) 5-s intervals: p-Cl-phenol, o-OCH_3-phenol, m-CH_3-phenol and o-Cl-phenol, (2) 10-s intervals: phenol, m-CH_3-aniline and o-CH_3-aniline, (3) 15-s intervals: aniline,

(4) 20-s intervals: m-OCH_3-phenol, p-Cl-aniline, and m-OCH_3-aniline, (5) 40-s intervals: o-CH_3-phenol, and m-Cl-phenol, and (6) 300-s intervals; m-Cl-aniline and o-Cl-aniline. A time zero sample and four additional samples were taken at the above specified intervals. In each case, the total amount of electron donor disappearance at the final sampling time was between 15 and 40% of the initial amount present. All experiments were performed in triplicate. Three individual rate constants and the standard deviation from the mean of these three values were calculated for each compound listed above. The mean k values were used in Figures 1 and 2.

Rates of reaction for p-OCH_3-aniline, p-OCH_3-phenol, p-CH_3-aniline, p-CH_3-phenol and o-OCH_3-aniline were too rapid to follow by our sampling procedure at the previously specified extent of reaction limits and HRP levels. Therefore, it was necessary to develop a dilution technique in order to ascertain the appropriate rate constants. The dilution technique consisted of determining rate constants for the compounds listed above at the following HRP dilution levels: p-CH_3-phenol 5,10,15,20 fold dilution, p-OCH_3-phenol and p-CH_3-aniline 10,20,30,40 fold dilution, o-OCH_3 aniline 20,30,40,50 fold dilution and p-OCH_3-aniline 50,100,150,200 fold dilution. The levels of electron donors and H_2O_2 were held constant at 21.5 umoles and 107.4 umoles, respectively. Duplicate experiments were run per HRP dilution level. In each case we observed a linear increase in the rate constants corresponding to increases in HRP concentration. The rate of reaction at the undiluted level of HRP was calculated from a linear regression of reaction rate and HRP activity (Table 1). To support the dilution technique as a valid method for estimating reaction rates at a higher

HRP level, we attempted to measure directly the reaction rate of p-CH_3-aniline and p-CH_3-phenol at the undiluted HRP level. The experiments were run in triplicate with our minimum sampling interval of 5-s. Only the first 3 sampling points could be evaluated due to the rapid rate of reaction. The extent of reaction for p-CH_3-aniline and p-CH_3-phenol were 70% and 44%, respectively. The rate constants for p-CH_3-aniline and p-CH_3-phenol estimated by direct measurement were 1.49 x 10^{-5} M s^{-1} and 9.42 x 10^{-6} M s^{-1}, respectively. The rate constants calculated for p-CH_3-aniline (2.31 x 10^{-5} M s^{-1}) and p-CH_3-phenol (1.0 x 10^{-5} M s^{-1}) by dilution techniques were in good agreement with these values.

Analytical procedures

Solution concentration of the various aniline and phenol compounds were measured using a Waters high-performance liquid chromatograph consisting of a Model 6000A pump, Model 45 pump, Model 720 systems controller, and coupled with a Model 480 Lambda Max variable wavelength UV absorbance detector. Detection wavelengths were 245 and 280 nm for the anilines and phenols, respectively. The sample injection valve (Rheodyne 7125) was fitted with a 20 uL loop. The analytical column was a Waters Radial-PAK C18 cartridge held in a RCM-100 Radial Compression Module. Peak areas were measured using a Hewlett Packard 3390A integrator. The mobile phase consisted of a 50% acetonitrile: 50% 0.1 M acetate buffer (pH 4.7) mixture with a flow rate of 2 mL min^{-1}. The mobile phase ratio was adjusted slightly to give a retention time of approximately 3.15 min.

Table 1. Reaction rates determined by enzyme dilution.

Compound	HRP level a.u.	k u Ms^{-1}	R^2	Projected k † $\mu M\ s^{-1}$
p-methoxyphenol	6.50	3.14	.99	34.5
	3.26	1.33		
	2.16	0.792		
	1.62	0.574		
p-methylphenol	13.0	1.93	.99	10.0
	6.50	0.975		
	4.34	0.593		
	3.26	0.395		
p-methoxyaniline	3.04	3.12	.99	185
	1.52	1.37		
	1.02	0.587		
	0.76	0.386		
p-methylaniline	15.2	1.96	.99	23.1
	7.60	0.711		
	5.07	0.370		
	3.80	0.211		
o-methoxyaniline	7.60	1.5	.99	32.7
	5.07	0.90		
	3.80	0.63		
	3.04	0.536		

†Projected value at 65 HRP activity units for the phenol compounds and at 152 activity units for the aniline compounds.

RESULTS AND DISCUSSION

The horseradish peroxidase (HRP) mediated coupling of aniline and phenol compounds is believed to result from a series of reactions which may be illustrated as follows (Benon, Bielsk, and Gebicki, 1977):

$HRP + H_2O_2 \longrightarrow$ Compound I — step 1

Compound I + $AH_2 \longrightarrow$ Compound II + AH• — step 2

Compound II + $AH_2 \longrightarrow$ HRP + AH• — step 3

$2AH• \longrightarrow$ products — step 4

The reaction rate for step 3, the rate controlling step, is dependent on the nature of the electron donor (Benon et al., 1977). In order to understand the relationship between chemical structure and reactivity in the sequence of reaction shown above, reaction rates were measured by following substrate disappearance. In this study, we have concerned ourselves with the initial stages of the overall coupling reactions to reduce the possibility of product interference through (1) enzyme inhibition, (2) enzyme destruction, or (3) secondary non-enzymatic reactions with the parent compounds. The kinetic data obtained during the initial stages of reaction could be described adequately by a straight line according to the equation:

$$\frac{-d[AH_2]}{dt} = k \qquad [1]$$

where $[AH_2]$ represents concentration of the electron donor. With the exception of m-chloroaniline ($R^2 = 0.90$), R^2 values of 0.96 to 0.99 were obtained. The rate constants (k) obtained for the monsubstituted anilines and phenols are listed in Tables 2 and 3, respectively.

To evaluate the relationship between molecular structure and reactivity, rate constants of the meta- and para-substituted electron donors were compared to their corresponding substituent sigma (σ) constants derived from the Hammett equation:

$$\log \frac{k_x}{k_o} = \sigma\rho \qquad [2]$$

were k represents the rate constant for the reaction in question. This equation quantitates the effects of substitution of a group X for H on the reaction rate k. The o subscript denotes the unsubstituted species under investigation (in this case aniline or phenol) and x denotes the meta- or para-substituted species.

Sigma constants are characteristic of a substituent group X and sum up the total electronic effects (resonance plus field) of X when attached to, in this case, aniline or phenol. The value of σ indicates the electron withdrawing or releasing effect of a substituent: electron-withdrawing substituents have positive σ values, and electron releasing substituents have negative σ values. Hammett σ constants are not generally used for the ortho position because groups in that position usually fail the treatment due to what has been termed the ortho effect (March, 1977). Brown and Okamoto (1958) have proposed an additional set of constants, the σ^+ values, for cases in which direct resonance interaction of the substituent and on electron-deficient reaction site is possible. These are modified σ values that reflect the increased resonance contribution to the substituent effect when direct conjugation is present. The σ and σ^+ constants for substituents in the meta and para positions are listed in Tables 2 and 3.

Table 2. Rate constants for monsubstituted anilines

Compound	σ	σ^+	$k^{\dagger}$ uM s^{-1}
p-NO_2	0.78	0.79	NR ‡
m-NO_2	0.71	0.67	NR
o-NO_2	-		NR
p-Cl	0.23	0.11	0.479 ± 0.040
m-Cl	0.37	0.40	0.0226 ± .0057
o-Cl	-	-	0.0341 ± 0.0003
aniline	0.0	0.0	0.724 ± 0.035
p-CH_3	-0.17	-0.31	23.1 §
m-CH_3	-0.07	-0.10	1.80 ± 0.02
o-CH_3	-	-	1.72 ± 0.05
p-OCH_3	-0.27	-0.78	185 §
m-OCH_3	0.12	0.05	0.638 ± 0.004
o-OCH_3	-	-	32.7 §

† Rate constant ± standard deviation.

‡ NR, no reaction

§ Rate constant k obtained using enzyme dilution technique.

Table 3. Rate constants for monosubstituted phenols

Compound	σ	σ^+	$k^{\dagger}$ uM s^{-1}
p-NO_2	0.78	0.79	NR‡
m-NO_2	0.71	0.67	NR
o-NO_2	-		NR
p-Cl	0.23	0.11	4.62 ± 0.07
m-Cl	0.37	0.40	0.331 ± 0.012
o-Cl	-	-	2.85 ± 0.20
phenol	0.0	0.0	1.42 ± 0.12
p-CH_3	-0.17	-0.31	10.0§
m-CH_3	-0.07	-0.10	3.22 ± 0.23
o-CH_3	-	-	0.347 ± 0.014
p-OCH_3	-0.27	-.78	34.5§
m-OCH_3	0.12	0.05	0.625 ± 0.021
o-OCH_3	-	-	2.76 ± 0.31

† Rate constants ± standard deviation.

‡ NR, no reaction

§ Rate constant obtained using enzyme dilution technique

The rho (ρ) value, which can be obtained graphically from a plot of log k versus σ , is a measure of reaction susceptibility to electrical effects. Positive values of ρ indicate that a particular reaction under study is enhanced by electron-withdrawing groups while negative values of ρ indicate that the reaction is enhanced by electron donating groups. A change in the slope of log k versus σ , i.e. ρ , indicates a change in the reaction mechanism (March, 1977).

The relationship between σ and log k values for the meta and para substituted anilines and phenols is illustrated in Figure 1. In both cases ρ is negative [ρ(anilines) = -5.30, ρ (phenol) = -2.51] showing that the overall reaction involving HRP was enhanced by electron-donating substituent groups and hindered by electron-withdrawing groups. Figure 2 illustrates the relationships between the σ^+ values versus the respective log k values, and again both plots give negative ρ values. The R^2 values obtained from linear regression analysis of σ and σ^+ versus log k values clearly show a better association with σ^+. The R^2 values obtained using σ^+ were 0.97 and 0.81 (Figure 2) for the anilines and phenols, respectively, as compared to 0.90 and 0.66 for log k versus σ (Figure 1).

A better association of log k values with σ^+ indicated the formation of a developing positive charge in the transition state for both anilines and phenols (March, 1977). A more strongly negative ρ value was obtained for the anilines (ρ = -3.33) as compared to the phenols (ρ = -1.67) indicating a larger electron demand at the reaction center. The following reaction between Compound II and aniline leading to the formation of a radical cation in the transition state seems to be

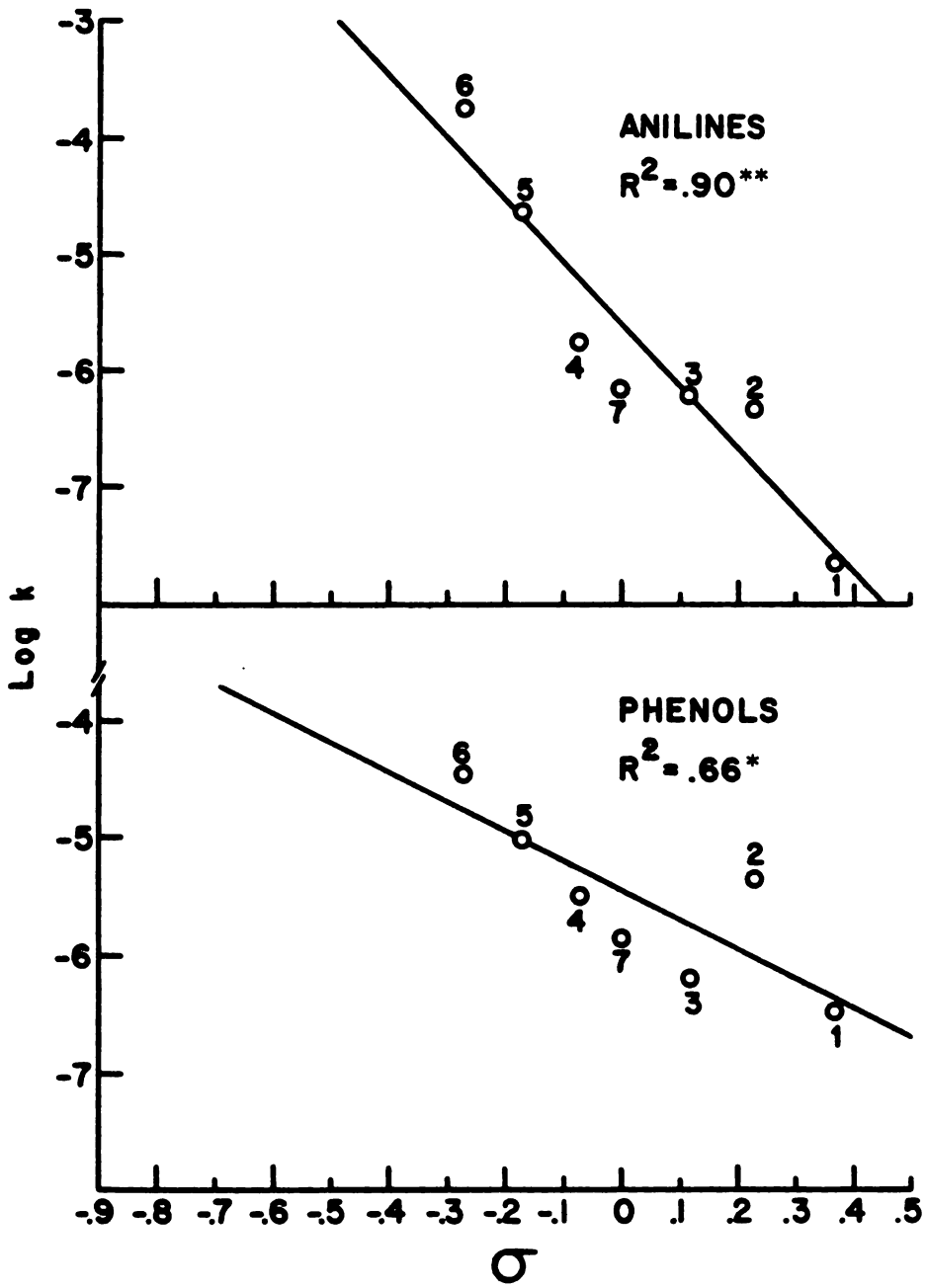


Figure 1. Correlation between log k values and substituent σ values. Numbers on the plots refer to the following substituents: (1) m-Cl; (2) p-Cl; (3) m-OCH_3; (4) m-CH_3; (5) p-CH_3; (6) p-OCH_3 (7) no substituent (aniline or phenol).

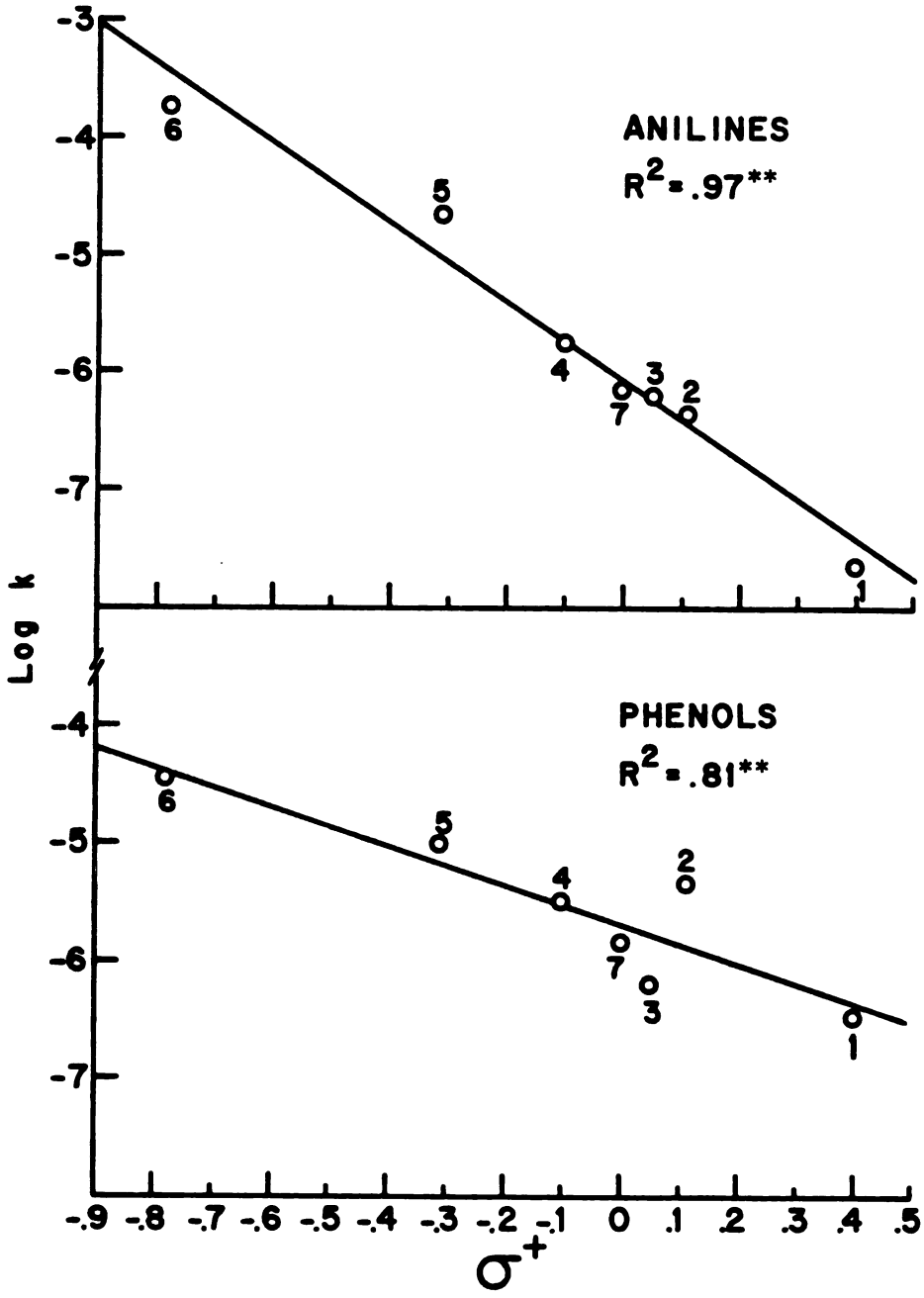
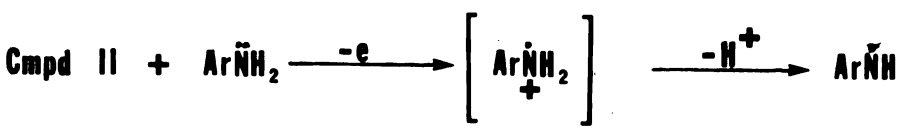
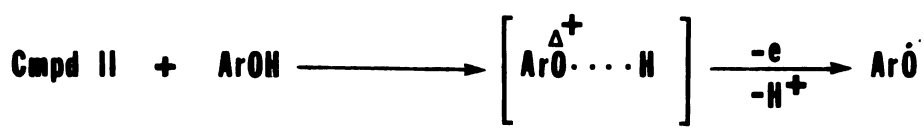


Figure 2. Correlation between log k values and substituent σ^+ values. Numbers on the plots refer to the following substituents: (1) m-Cl; (2) p-Cl; (3) m-OCH_3; (4) m-CH_3; (5) p-CH_3; (6) p-OCH_3 (7) no substituent (aniline or phenol).

plausible based on our data:



For the phenols, where the ρ value was less negative, a similar transition state could be applicable where the phenolic O-H bond becomes polarized to give at least a partial positive charge ($\Delta+$) on the phenolic oxygen:



In this transition state the electron has been partially transferred to compound II.

Critchlow and Dunford (1972) have suggested that the reaction with Compound II is dependent on the nucleophilicity of the electron donor towards iron. In their mechanism the rate determining step is proton transfer from a distal acid group to the imidazole group of histidine which occupies the fifth coordination site of the heme iron. The proton transfer facilitates electron transfer from the substrate, which is located on the opposite side of heme. The degree of protonation required for the electron transfer differs for individual substrates, with more reactive compounds like p-cresol only requiring hydrogen bond formation. The anilines are of intermediate reactivity and require a fraction of the imidazole groups to be protonated (Dunford and Cotton, 1975). Thus the degree of protonation required for reaction depends on

the nucleophilicity or electron donor strength of the substrate. This mechanism would explain why in general electron donating groups of aniline or phenol enhance the reaction rate since these groups would increase nucleophilicity. It does not appear to account for our observation that log k values associated best with σ^+ which indicates a favorable resonance interaction of electron donating substituents with a developing positive charge in the transition state. This interaction involving the substituent groups requires that the positive charge be located on the electron donor. The better fit with σ^+ as compared to σ would argue that the rate limiting step was an electron transfer process as we have illustrated in structure I and II above for aniline and phenol.

In summary, we have shown that the susceptibility to reaction of substituted anilines and phenols with HRP was enhanced by the presence of electron-donating groups and hindered by electron-withdrawing groups. Presence of the NO_2 group, which was the strongest electron-withdrawing group tested, on aniline or phenol rendered these compounds non-reactive with HRP. This overall result was consistent with earlier work (Bordelleau and Bartha, 1972) where a fungal peroxidase was used. In our study, we have also shown that the order of reactivity of the substituted anilines and phenols could be understood by reference to the σ^+ substituent values. This order was found to be $OCH_3 > CH_3 > Cl$ for *para* substituents and $CH_3 > OCH_3 > Cl$ for *meta* substituents, in agreement with that predicted using the σ^+ values. Bordelleau and Bartha (1972) have reported the order of reactivity for *meta*- and *para*-substituted anilines as $OCH_3 > CH_3 > Cl$. We attribute the observed

differences in reaction order to be an artifact of the methodology used by Bordelleau and Bartha (1972) and not an inherent difference in the two peroxidases. We have also shown that reaction rates were more highly correlated with σ^+ constants than σ constants. Therefore, the formation of a positive or partially positive transition state as the rate limiting step was indicated.

The results presented here would suggest that the degree to which anilines and phenols become bound to soil humic molecules through enzymatically mediated oxidative coupling reactions may be affected by substituent groups on the aromatic ring. For example, in the case of the dinitroaniline herbicides (e.g., Trifluralin) these reactions may be prohibited due to the presence of the strongly electron withdrawing NO_2 group. In contast, reactivity would be facilitated by electron donating groups such as OCH_3, which are common substituents on lignin derived polyphenols.

REFERENCES

1. Bartha, R., H. A. B. Linke, and D. Pramer. 1968. Pesticide transformations: Production of chloroazobenzenes from chloroanilines. Science 161:582-583.

2. Benon, H., J. Bielski, and J. M. Gebicki. 1977. Application of radiation chemistry to biology. In W. A. Pryor (ed.) Free Radicals in Biology, Vol. III. Chapt. 1. Academic Press, New York.

3. Bordeleau, L. M., and R. Bartha. 1972. Biochemical transformation of herbicide derived anilines: requirements of molecular configuration. Can. J. Microbiol. 18:1873-1882.

4. Brown, H. C., and Y. Okamoto. 1958. Electrophilic substituent constants. J. Am. Chem. Soc. 80:4979-4987.

5. Critchlow, J. E., and H. B. Dunford. 1972. Studies on Horseradish peroxidase: X. The Mechanism of the oxidation of p-cresol, ferrocyanide, and iodide by compound II. J. Biol. Chem. 247:3714-3725.

6. Dunford, H. B., and M. L. Cotton. 1975. Kinetics of the oxidation of p-aminobenzoic acid catalyzed by Horseradish peroxidase compounds I and II. J. Biol. Chem. 250:2920-2932.

7. March, J. 1977. Advanced Organic Chemistry. Chapt. 9. McGraw-Hill Book Co., New York.

8. Martin, J. P., and K. Haider. 1971. Microbial activity in relationship to soil humus formation. Soil Sci. 111:54-63.

9. Martin, J. P., and K. Haider. 1980. A comparison of the use of phenolase and peroxidase for synthesis of model humic acid-type polymers. Soil Sci. Soc. Am. J. 44:983-988.

10. Sjoblad, R. D. and J. M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In E. A. Paul and J. M. Ladd (eds.) Soil Biochemistry, Vol. 5. Chapt. 3. Marcel Dekker, Inc. New York.

11. Stevenson, F. J. 1982. Humus Chemistry. Chapt. 1. John Wiley and Sons, New York.

CHAPTER III

REACTION RATES OF PHENOLIC HUMUS CONSTITUENTS AND ANILINES DURING CROSS-COUPLING

INTRODUCTION

Oxidative coupling of phenols is an important synthetic process leading to the formation of a wide variety of natural products including humic substances (Taylor and Battersby, 1967). Phenolic compounds produced through microbial synthesis and lignin degradation are thought to be important monomeric constituents of humic materials in soils (Martin and Haider, 1971). Peroxidases and oxidases (e.g. laccases) serve as important biocatalysts in these oxidative coupling processes which ultimately yield high molecular weight humic materials consisting of coupled phenols and other organic soil constituents such as amino acids and proteins (Sjoblad and Bollag, 1981; Stevenson, 1982). Oxidative coupling reactions are not limited to naturally occurring phenolic compounds but also include a variety of xenobiotic compounds. One important class of xenobiotic compounds which are known to covalently bind to soil humic components during oxidative coupling reactions are the alkyl- and halogen-substituted anilines. Bartha (1971) has observed that the ring carbons of aniline compounds are generally resistant to mineralization and are therefore available for oxidative coupling reactions. Anilines find their way into the soil environment as a result of herbicide or pesticide degradation and from unintentional releases associated with manufacturing processes and waste disposal. It is important to understand the nature of the coupling reactions involving anilines for many of these compounds are suspected carcinogens.

In an earlier investigation, we were able to gain some insight into the relationship between molecular structure and reactivity of peroxidase mediated oxidative coupling of mono-substituted phenols and anilines (Berry and Boyd, 1984). We observed that reactivity of either mono-substituted phenol or aniline compounds was dependent on electron density at the reaction center (-OH or $-NH_2$). Reaction rates were enhanced with electron donating substituent groups (eg. $-CH_3$ or $-OCH_3$) on the aromatic ring. Electron withdrawing groups (e.g. -Cl or $-NO_2$) decreased reactivity relative to the unsubstituted species.

In the present investigation we have expanded our study of the relationship between chemical structure and reactivity for the peroxidase mediated oxidative coupling of substituted phenols to include naturally occurring phenols of both plant and microbial origins. As such, we determined rate constants for various potential humus constituents such as ferulic acid, vanillic acid, pyrogallol, and protocatechuic acid. In addition, we have investigated the peroxidase mediated cross-coupling of phenols and anilines in an effort to gain a better understanding of how anilines might bind to soil humic substances. In this regard it was of interest to determine if the reactivity of anilines as sole electron donors (Berry and Boyd, 1984) was changed by the presence of phenolic humus constiuents as additional reactants. Relative rate constants of the various substituted aniline and phenol compounds were determined by measuring their disappearance using high pressure liquid chromatography. We discuss implications of the oxidative coupling reactions with respect to the formation of (1) humic substances in

soil and (2) bound residues involving aromatic amines and soil organic matter.

MATERIALS AND METHODS

Materials. Horseradish peroxidase (HRP) Type II (Donor: hydrogen-peroxide oxidoreductase; E.C. 1.11.1.7) was purchased form Sigma Chemical Co. The HRP used had a specific activity of 152 purpurogallin units per mg solid and a RZ value of 1.52. The anilines and phenols were obtained form Aldrich Chemical Co. and Eastman Kodak Co. and used without further purification. All aniline-phenol, phenol, and HRP solutions were prepared in 0.1 M phosphate buffer (pH 6.9) which was made up from double glass distilled deionized water.

Kinetic Measurements. Aniline, phenol, and HRP solutions were prepared fresh before each rate study. Kinetic experiments were carried out at a constant temperature of 20 $\pm$ .01°C in open beakers containing 100 mL of phosphate buffered solution with the following reactants: aniline and or phenolic compounds HRP and 107.4 umoles of H_2O_2. Additional experimental details of this procedure have been published previously (Berry and Boyd, 1984). To initiate the reaction 0.5 mL of HRP solution was added to an open beaker containing the other aforementioned reactants while stirring vigorously.

Reaction rates were measured by monitoring disappearance of the aniline and phenol compounds. From 100 mL of the reaction solution

2.0 mL aliquots were periodically withdrawn and placed into glass vials containing 0.50 mL of a 50 mM KCN solution. Potassium cyanide was found to be effective in inhibiting HRP activity and was used to stop the reactions. The samples were placed on ice and stored in the dark until HPLC anlaysis which was carried out immediately after collection of the samples. Control experiments were performed using HRP that had been deactivated by boiling approximately 1.5 h.

HRP mediated oxidative coupling of various phenol and phenolcarboxylic acids. For these experiments 100 mL of phosphate buffered solution contained 21.5 umoles of phenol. To initiate the reaction 1.52 activity units (a.u.) of HRP was added to the reaction mixture. Sampling time intervals for the various phenolic compounds were as follows: (1) 5-s intervals: coniferyl alcohol, p-hydroxybenzoic acid, ferulic acid, and caffeic acid. (2) 30-s intervals: pyrogallol, (3) 2 min intervals; guaiacol and catechol, (4) 5 min intervals: gallic acid, (5) 10 min intervals: vanillic acid and resorcinol, (6) 15 min intervals: phloroglucinol and protocatechuic acid. A total of 5 samples were measured in order to determine the rate constants. The extent of the reactions was between 15 and 40%. All experiments for the phenol monomer study were performed in triplicate. The mean rate constant and the standard deviations are listed in table 1.

HRP mediated cross-coupling for ferulic acid with substituted aniline compounds (5:1 ratio): Simultaneous rate study 1. For these experiments 100 mL of phosphate buffered solution contained 53.7

umoles ferulic acid and 10.74 umoles of aniline. To initiate the reaction 0.608 (a.u.) of HRP was added to the reaction mixture. Sampling times were adjusted for the various reactant pairs according to their reaction rates.

Sampling time intervals for ferulic acid-aniline compounds cross-coupling reaction were as follows: (1) 20-s intervals: ferulic acid vs. p-methoxyaniline, ferulic acid vs. p-methylaniline, ferulic acid vs. aniline and ferulic acid alone (2) 30-s intervals: ferulic acid vs. m-chloroaniline, (3) 30-s intervals to 2 min then 2 min intervals to 6 min: ferulic acid vs. p-nitroaniline. In order to determine a rate constant a time zero sample and four additional samples were measured for the aniline compounds while only a time zero sample and three additional samples were measured for the ferulic acid. A rate constant for the ferulic acid alone was determined at the 53.7 umole level in order to serve as a means of comparison for the cross-coupling reactions. In each case, the total amount of reactant disappearance was between 15 and 40% of the initial amount present. All experiments in rate study (1) were performed in triplicate. Three individual rate constants (k) and the standard deviation from the mean of these three values were calculated for each compound. The rate constant values and their standard deviations are listed in Table 2.

HRP mediated cross-coupling of o-chloroaniline with substituted phenolic compounds (1:5 ratio): Simultaneous rate study 2. For these experiments 100 mL of phosphate buffered solution contained 10.74 umoles o-chloroaniline and 53.7 umoles of various substituted phenols. To initiate the reaction 15.2 a.u. of HRP was added to the reaction mixture. Sampling time intervals for the reactant pairs were as follows: 5-s intervals: o-Cl aniline vs. ferulic acid, (2) 30-s intervals: o-chloroaniline vs. guaiacol, (3) 120-s intervals: o-chloroaniline vs. vanillic acid. Five samples were measured for o-chloroaniline while 4 samples were measured for the phenols. The extent of reaction for the o-chloroalinine was between 15 and 40% while for the phenolic compounds it was between 50 and 80%. All experiments in rate study (2) were performed in duplicate. The mean rate constant and their respective standard deviations were calculated for each compounds and are listed in Table 3.

Analytical Procedures. Solution concentration of the various aniline and phenol compounds were measured using a Waters high-performance liquid chromatograph consisting of a Model 6000A pump, Model 45 pump, Model 720 systems controller, and coupled with a model 480 Lambda Max variable wavelength UV absorbance detector. Detection wavelengths were 245 and 280 nm for the anilines and phenols, respectively. The sample injection value (Rheodyne 7125) was fitted with a 20 uL loop. We used a waters uB and a Radial-pak C18 analytical column. Peak areas were measured using a Waters Data Module, model 730. The mobile phase for the cross-coupling studies consisted of acetonitrile: 0.1 M acetate buffer, pH 4.7 mixture with a flow rate of 1 mL min^{-1}. The mobile phase ratio had to be adjusted

to the extent that product peaks did not overlap with the reactant peaks. In a few cases where similar solubilities of the aniline-phenol reactants existed both reactants could be chromatrographed using the same mobile phase ratio. In these cases the wave length setting of 245 nn was used. For most other situations the mobile phase ratio had to be readjusted and required that two injections be made from the same sampling times in order to follow both phenol and aniline disapperance events. The mobile phase of the phenol monomer study consisted of acetonitrile: 5% acetic acid mixture with a flow rate of 1 mL min^{-1}. The mobile phase ratio was usually adjusted to give a retention time of 6 to 8 minutes.

RESULTS AND DISCUSSION

The horeseradish peroxidase (HRP) mediated coupling of aniline of phenol compounds (electron donors, AH_2) is believed to result from a series of reactions which may be illustrated as follows (Benon *et al.*, 1977):

$$HRP + H_2O_2 \longrightarrow \text{Compound I} \qquad \text{Step 1}$$

$$\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + AH\bullet \qquad \text{Step 2}$$

$$\text{Compound II} + AH_2 \longrightarrow HRP + AH\bullet \qquad \text{Step 3}$$

$$2AH\bullet \longrightarrow \text{products} \qquad \text{Step 4}$$

Compounds I and II represent different oxidized states of HRP with compound I representing the highest oxidized form. Reaction rates involving compounds I and II are dependent on the nature of the electron donors (Childs and Bradsley, 1975) and step 3 is the rate controlling step of the overall series of reactions (Benon *et al.*, 1977). Peroxidases of different origins all appear to react according to the general scheme shown above (Hewson and Hager, 1979).

In the present study we have concerned ourselves primarily with initial stages of the overall coupling reactions to reduce the possibility of enzyme destruction and inhibition. All kinetic data obtained from the reactions could be described adequately by the zero order rate expression:

$$-\frac{d[A]}{dt} = k$$

where [A] represented either the concentration of the phenol or

aniline compound. Generally, the extent of the reactions monitored was between 15 and 40% and R^2 values between 0.96 and 0.99 were obtained. In some cases the extent of the reactions monitored was between 50 and 80% and R^2 values decreased somewhat ranging from 0.89 to 0.98.

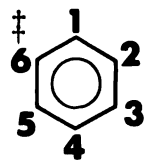
We compared the rate of oxidation of various phenolic compounds to advance our understanding of the relationship between chemical structure and reactivity for HRP mediated oxidative coupling of substituted phenols. The phenolic compounds used are thought to be natural soil components which are coupled together during the synthesis of humic substances. The compounds tested are listed in Table 1 along with their respective rate constants. Compounds containing the acrylic 3-C group, viz, coniferyl alcohol, p-hydroxycinnamic acid, ferulic acid, and caffeic acid are lignin derivatives whereas the remaining compounds may occur in soil as a result of lignin degradation or microbial synthesis. It is important to point out that we did not attempt to use a saturating electron donor concentration, which could in part account for the different reactivities observed. It was our intent to compare reactivity at environmentally relevant concentrations realizing that saturating concentrations probably never occur in soil.

Several definite trends with respect to structural effects on reactivity of the compounds studied could be discerned. Generally reactivity was enhanced by (1) addition of an acrylic group, (2) addition of OH groups to phenol, (3) OH groups positioned ortho with respect to one another as compared to meta OH groups, and (4) substitution of an OH group by an OCH_3 group ortho to an OH group.

Table 1. Rate constants for the reaction of potential phenolic humus constituents with HRP and H_2O_2.

Compound	Structure ‡	k (uM s^{-1})†
Coniferyl alcohol	1(CH_2OH-CH=CH-), 3(OCH_3), 4(OH)	4.67 ±0.15
p-Hydroxycinnamic acid	1(COOH-CH=CH-), 4(OH)	4.40 ±0.40
Ferulic acid	1(COOH-CH=CH-), 3(OCH_3), 4(OH)	3.60 ±0.10
Caffeic acid	1(COOH-CH=CH-), 3(OH), 4(OH)	3.47 ±0.21
Pyrogallol	1(OH), 2(OH), 3(OH)	0.44 ±0.02
Guaiacol	1(OH), 2(OCH_3)	0.13 ±0.08
Catechol	1(OH), 2(OH)	0.113 ±0.03
Gallic acid	1(COOH), 3(OH), 4(OH), 5(OH)	0.042 ±0.001
Vanillic acid	1(COOH), 3(OCH_3), 4(OH)	0.0280 ±0.0005
Resorcinol	1(OH), 3(OH)	0.026 ±0.004
Phloroglucinol	1(OH), 3(OH), 5(OH)	0.012 ±0.00_2
Protocatechuic acid	1(COOH), 3(OH), 4(OH)	0.007 ±0.001

†Rate constants ± standard deviation.

‡ 

Addition of a carboyxl (COOH) group directly on the phenol ring decreased reactivity.

One of the most striking effects of structure on reactivity was that the 3-C acrylic group strongly enhanced reactivity. For example, addition of the acrylic group to guaiacol (forming ferulic acid) resulted in a 28X increase in the rate constant (Table 1). Similarly, addition of the acrylic group to catechol (forming caffeic acid) resulted in a 31X increase in the rate constant (Table 1). Because the acrylic group is not strongly electron donating the enhancement effect is most likely due to increased resonsance stability of the free radical intermediate (AH•).

A rate enhancement effect was also observed when we increased the number of OH groups on the aromatic ring. For example, by adding an OH group to catechol (forming pyrogallol) we observed a 3.7X increase in the rate constant. It may be that by increasing the number of OH groups, the number of reaction centers increases and hence the probability of a reaction, increases. Hydroxyl substituent groups also contribute electron density to the reaction center, which would be expected to increase reactivity (Berry and Boyd, 1984).

Phenolic compounds possessing a carboyxl group directly on the aromatic ring exhibited a decrease in reactivity. For example, addition of the carboxyl group to pyrogallol (forming gallic acid) reduced the rate constant by a factor of 10 (Table 1). Similarily, the observed rate constant of protocatechuic acid was approximately 16X smaller than catechol. We attribute this decrease in reactivity to the electron withdrawing ability of the carboxyl substituent group (Berry and Boyd, 1984).

Humic substance maintenance in soil results from a balanced equilibrium of decomposition and synthesis. Phenolic compounds such as those listed in Table 1 are thought to play an important role in the synthetic process. In an earlier study Haider and Martin (1975) incubated several ^{14}C-labelled phenols in soil and found that lignin derived phenolic compounds containing the C-3 acrylic group, e.g. p-hydroxycinnamic acid and caffeic acid, were retained in soil whereas phenol-carboxylic acids such as vanillic acid and syringic acid were readily degraded. The observed stabilization of these lignin derived compounds containing the acrylic group is interesting in light of our findings involving HRP mediated oxidative coupling of phenolic compounds. We observed that phenolic compounds containing the acrylic group (viz. ferulic, caffeic, and p-hydroxycinnamic acids and coniferyl alcohol) reacted much more readily with HRP than did the phenol-carboxylic acids (viz. vanillic, gallic, and protocatechuic acids). These results suggested the possibility that in soils humic substances may be synthesized preferentially from lignin derived phenolic residues which possess the acrylic group. Conversely, phenolic residues not containing the acrylic group would be expected to be less important in peroxidase mediated synthesis of humic materials, and as a result, become more susceptable to microbial decomposition.

The incorporation of various aniline compounds during the oxidative coupling of model phenolic humic constituents was also studied. Ferulic acid was selected as the model phenolic compound based on the results described above, and we measured the simultaneous reaction rates of ferulic acid and various anilines. A

5:1 ratio of ferulic acid:aniline was used because the level of aniline compounds found in soil would normally be significantly lower than the level of phenolic humus constituents with which they are believed to react.

In the simultaneous rate study (denoted rate study 1) the rate of ferulic acid disappearance was observed to be independent of the presence of any of the substituted aniline compounds examined (Table 2). Rate constants of the substituted aniline compounds varied somewhat depending on the substituent group present and were 6 to 60X slower than the rate constants observed for ferulic acid. Generally, rate constants for anilines increased as electron donating ability of the substituent group increased (Table 2).

A comparison of rate constants obtained from our previous study (Berry and Boyd, 1984) dealing with HRP mediated coupling of mono-substituted anilines revealed that reactivity of the aniline compounds was generally enhanced in the presence of ferulic acid. The rate constant determined previously for aniline as sole electron donor was 0.724 uM s^{-1} with 152 a.u. HRP (Berry and Boyd, 1984). For aniline disappearance in simultaneous rate study 1, i.e. in the presence of ferulic acid, we observed a rate constant of 0.40 uM$\cdot s^{-1}$ with 0.608 a.u. of HRP. If we assume a linear relationship between reaction rate and enzyme level, as demonstrated in our previous study (Berry and Boyd, 1984), we can estimate a rate constant of 0.0029 uM s^{-1} for aniline as the electron donor based on 0.608 a.u. of HRP. Thus, at an equal level of HRP activity, reactivity of aniline was enhanced 138X in the presence of ferulic acid. An even more dramatic example of reactivity enhancement is provided by m-chloroaniline.

Table 2. Rate constants for the simultaneous reaction of various aniline compounds and ferulic acid with HRP and H_2O_2.

Aniline compound	k (uM s^{-1})† aniline	k (uM s^{-1}) † ferulic acid
p-OCH_3	0.37 ± .031	2.36 ± 0.49
p-CH_3	0.38 ± .020	3.04 ± 0.16
Aniline	0.40 ± .060	2.37 ± 0.35
m-Cl	0.17 ± .003	2.08 ± 0.09
p-NO_2	0.04 ± .007	2.30 ± 0.32
None	-	2.50 ± 0.09

†Rate constant ± standard deviation.

Table 3. Rate constants for the simultaneous reaction of various phenolic compounds and o-chloroaniline with HRP and H_2O_2.

Phenolic Compound	k (uM s^{-1}) † phenol	k(uM s^{-1})† chloroaniline
Ferulic acid	26.7 ± 0.009	2.00 ± 0.00
Guaiacol	3.49 ± 0.20	0.19 ± 0.02
Vanillic acid	0.77 ± 0.00	0.03 ± 0.004

†Rate constant ± standard deviation.

Again, if we adjust the rate constant obtained previously (Berry and Boyd, 1984) to a HRP level of 0.608 a.u., reactivity of m-chloroaniline was enhanced 1,880X in the presence of ferulic acid (Table 2). Reactivity of p-methylaniline was enhanced 4.1X in the presence of ferulic acid (Table 2).

Another very interesting observation was that in our previous study involving structure-activity relationships (Berry and Boyd, 1984) p-nitroaniline was found to be non-reactive in the presence of HRP and H_2O_2. In the simultaneous rate study, however, we found that p-nitroaniline readily disappeared in the presence of ferulic acid. The enhanced reactivity of aniline, nitroaniline and chloroaniline in the presence of ferulic acid suggests that these compounds are reacting by a different mechanism than occurs when they are present as sole electron donors. The most plausible explanation is that the anilines are reacting chemically with intermediates or products produced during the enzymatic oxidation of ferulic acid.

It should be pointed out that as we go from aniline compounds possessing electron withdrawing groups (e.g. p-NO_2, m-Cl) to those containing electron donating groups e.g. (p-CH_3, p-OCH_3) the reactivity enhancement effect produced by ferulic acid falls off and eventually reaches a point where there is in fact a reversal of the enhancement effect. For example, p-methoxyaniline has a rate constant that is 2 times smaller (0.37 uM s^{-1}) in the presence of ferulic acid as compared to the adjusted rate constant of 0.74 uM s^{-1} representing p-methoxyaniline as a sole electron donor (Table 2). In this case it appears that the aniline compound is reacting enzymatically with HRP. The decreased rate observed for

methoxyaniline in the presence of ferulic acid may be due to the competitive nature of the electron donors for the reactive site of HRP. Thus, for aniline compounds which are highly reactive with HRP (e.g. p-methoxyaniline) the rate of the enzymatic reaction exceeds the rate of secondary chemical reactions and the aniline compounds compete with ferulic acid for the reaction site of HRP. Because ferulic acid was present in 5 fold excess the rate of p-methoxyaniline was decreased.

The results presented above would suggest that in soil coupling of most aniline compounds to phenolic humus constituents would occur much more readily than coupling of aromatic amines together to form oligomeric products such as azobenzenes. Although azobenzenes have been isolated from soil, unrealistically high levels of chloroaniline were required (Bartha et al., 1968).

To determine if the rate of aniline disappearance was dependent on the reactivity of the specific phenolic compounds used, we examined o-chloroaniline disappearance in the presence of (1) ferulic acid, (2) guaiacol, and (3) vanillic acid (denoted simultaneous rate study 2.) The results (Table 3) demonstrated that the rate of disappearance of o-chloroaniline was directly related to the rate of the phenolic compounds used. Reactivities of the phenolic compound listed decreased in the order ferulic acid > guaiacol > vanillic acid, as did the rate of disappearance of o-chloroaniline (Table 3).

We observed that rates of disppearance of the o-chloroaniline were enhanced in the cross-coupling reaction as compared to the case where chloroaniline was present as sole electron donor. From our previous investigation (Berry and Boyd, 1984) we can estimate a rate

constant of 0.0034 uM s^{-1} at 15.2 a.u. of HRP for o-chloroaniline as sole electron donor. Based on this figure, reactivity was enhanced by 9X, 56X, and 588X in the presence of vanillic acid, guaiacol, and ferulic acid respectively. Figure 1 illustrates the simultaneous disappearance of o-chloroaniline and ferulic acid. The identical curve shapes clearly show that the rate of o-chloroaniline disappearance was directly dependent on the rate of disappearance of ferulic acid.

In summary, the results presented here clearly demonstrate that the reactivity of substituted anilines, especially nitro and chloroanilines can be greatly enhanced by including in the reaction mixture a highly reactive electron donor such as ferulic acid. We attribute the enhancement effect to result from secondary non-enzymatic chemical reactions involving anilines and the products of the peroxidase mediated coupling of phenolic electron donors. Bollag et al. (1983) have also proposed a secondary non-enzymatic chemical reaction mechanism for the laccase mediated cross-coupling of phenols and chloroanilines. Our observations along with those of Bollag et al. (1983) suggest that aniline binding to soil humic components results from a chemical reaction which may be only indirectly controlled by the presence of oxidative coupling enzymes in soil. An alternative mechanism for reactivity enhancement can be proposed in the case of peroxidase mediated cross-coupling. This alternative mechanism involves the two different oxidized forms of HRP, compounds I and II, and is based on their differential abilities to oxidize an electron donor. The possibility exists that a slow reacting aniline compound such as m-chloroaniline may react at a

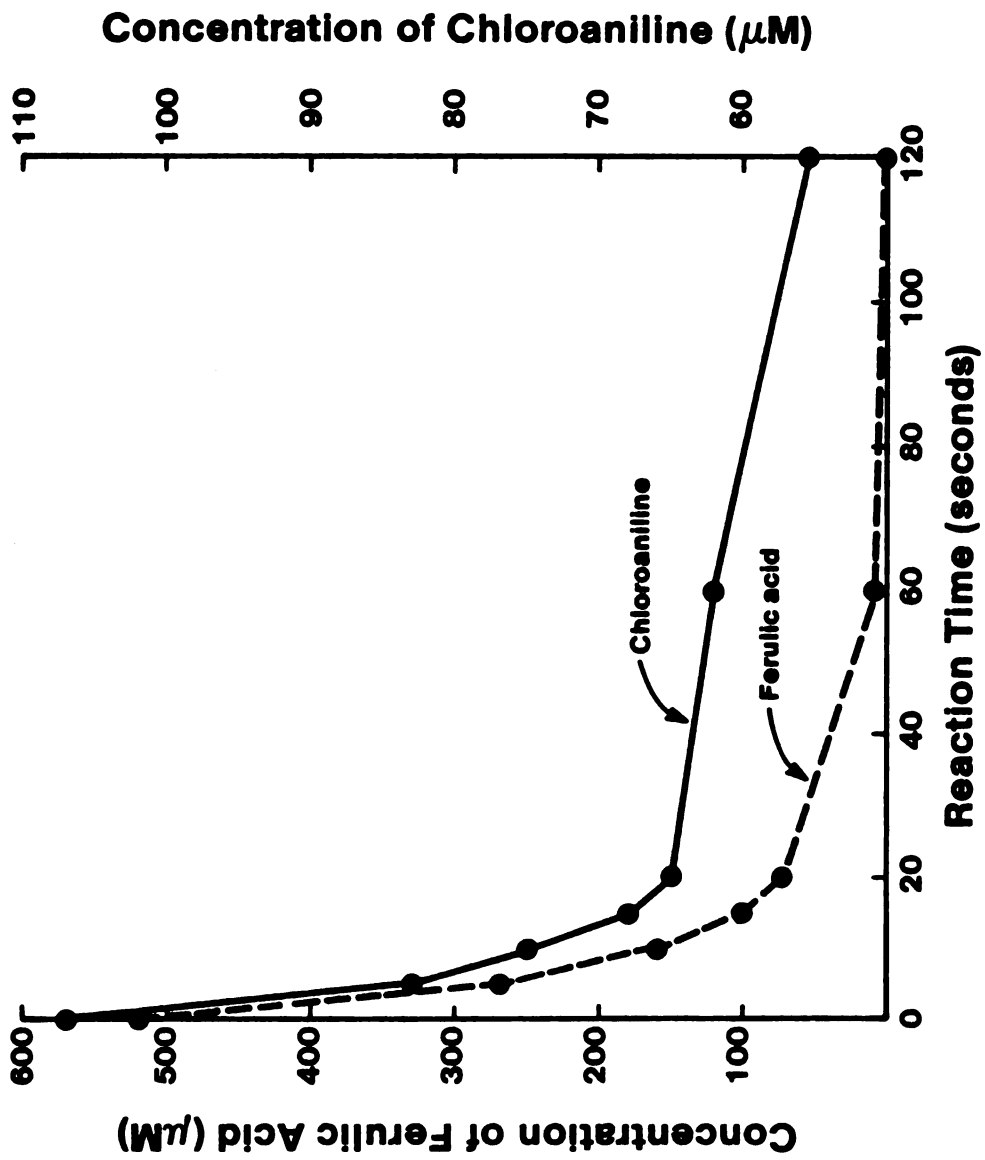


Figure 1. Simultaneous disappearance of ferulic acid and o-chloroaniline in the presence of HRP and H_2O_2.

reasonable rate with compound I but not with compound II. Thus, the reaction with compound II, the rate controlling step, results in a relatively slow observable reaction rate. However, a readily usable electron donor such as ferulic acid, added to the system containing the slow reacting aniline compound, could react preferentially with compound II thus completing the HRP reaction cycle and producing the enhancement effect for aniline disappearance.

REFERENCES

1. Bartha, R. 1971. Fate of herbicide-derived chloroanilines in soil. J. Agr. Food Chem. 19:385-387.

2. Bartha, R., H.B. Linke, and D. Pramer. 1968. Pesticide transformations: Production of chloroazobenzenes from chloroanilines. Science 161: 582-583.

3. Benon, H., J. Bielski, and J.M. Gebicki. 1977. Application of radiation chemistry to biology. In W.A. Pryor (ed.) Free Radicals in Biology, vol. III. Chapt. 1. Academic Press, New York.

4. Berry, D.F., and S.A. Boyd. 1984. Oxidative coupling of phenols and anilines by peroxidase: Structure-activity relationships. Soil Sci. Soc. Am. J. 48:565-569.

5. Bollag, J.-M., R.D. Minard, and S.Y. Liu. 1983. Cross-linkage between anilines and phenolic humus constituents. Environ. Sci. Technol. 17:72-80.

6. Childs, R.E., and W.G. Bardsley. 1975. The steady-state kinetics of peroxidase with 2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochm. J. 145:93-103.

7. Haider, K., and J.P. Martin. 1975. Decomposition of specifically carbon-14 labeled benzoic and cinnamic acid derivatives in soil. Soil Sci. Soc. Am. J. 39: 657-662.

8. Hewson, W.D., and L.P. Hager. 1979. Peroxidases, catalases, and chloroperoxidase. In The Porphyrins. Vol. VII. Academic Press, New York.

9. Martin, J.P., and K. Haider. 1971. Microbial activity in relationship to soil humus formation. Soil Sci. 111:54-63.

10. Sjoblad, R.D., and J.-M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In Soil Biochemistry E.A. Paul and J.M. Ladd (eds.), Vol., 4. Chapt. 3. Marcel Dekker, Inc. New York.

11. Stevenson, F.J. 1982. Humus Chemistry. Chapt. 1. John Wiley and Sons, New York.

12. Taylor, W.I., and A.R. Battersby (eds). 1967. Oxidative coupling of phenols. Marcel Dekker, Inc., New York.

CHAPTER IV

COUPLING OF PHENOLIC HUMUS CONSTITUENTS BY PHENOL OXIDASE: A KINETIC STUDY

INTRODUCTION

Phenolic compounds of either microbial or lignin origins are important constituent units in the formation of soil humic substances (Martin and Haider, 1971). The currently accepted theory regarding humic substance formation in soil holds that humic acid is a poly-condensate of C_6, C_6-C_1, C_6-C_3, and other phenolic compounds which are derived from microbial decomposition of lignins and from anabolic pathways of soil microorganisms (Stevenson, 1982). Polymerization of these phenolic monomer units presumably takes place either by autooxidation reactions or more likely by enzyme mediated oxidative coupling reactions (Sjoblad and Bollag, 1981). Soil humic substances are also known to contain other components such as peptides, amino acids, polysaccharides, and lipids (Stevenson, 1982).

The oxidative enzymes in soil which act to covalently couple phenolic compounds belong to one of two major groups: phenol oxidases or peroxidases (Sjoblad and Bollag, 1981). Most peroxidases contain a heme prosthetic group and have an absolute requirement for H_2O_2 (co-substrate) for activity. Laccases, which are presumably the most important class of phenol oxidases in soil, contain Cu and require O_2 (co-substrate) for activity (Sjoblad and Bollag, 1981). Both laccases and peroxidases are thought to couple phenolic compounds by way of a free radical mechanism (Sjoblad and Bollag, 1981) and have been used to synthesize model humic polymers (Martin and Haider, 1980). Generally speaking, peroxidases will oxidize a larger range of substrates when compared to laccases.

The overall goal of our research is to gain a better understanding of the enzymatic mediated processes which act to couple naturally occuring phenolic humus constituent units together. In a previous investigation (Berry and Boyd, 1984) we demonstrated that reactivity depended upon the number and type of substituent group(s) on the phenolic compounds. For example, reactivity was enhanced by addition of: hydroxyl groups (-OH), a 3-C acrylic group, and substition of an OH group by an OCH_3 group ortho to an OH group. Furthermore, addition of a carboxyl group (COOH) directly on the phenol ring decreased reactivity.

In the present study we have investigated the relationship between chemical structure and reactivity for laccase mediated oxidative coupling of phenolic humus constituents. For our investigations we used a partially purified laccase preparation that had been isolated from the culture filtrate of the soil fungus Rhizoctonia praticola. Relative rate constants for various substituted phenol compounds were determined by measuring their disappearance using high pressure liquid chromatography.

MATERIALS AND METHODS

A crude extracellular laccase preparation was isolated from the culture medium of the soil fungus Rhizoctonia praticola which had been cultured in a modified Czapek Dox medium (Sjoblad and Bollag, 1976). The laccase preparation was isolated from the culture medium (approximately 30 L) by filtration, then practically purified by dialysis and DEAE-cellulase column chromatography. For details on the isolation and purification procedures refer to Bollag et al., (1979). After DEAE-cellulose column chromatography the laccase preparation was concentrated by lyophilization and then rehydrated with 20 mL of glass double distilled deionized water, fractioned into 1.1 mL portions and then stored in the frozen state until use. The phenol compounds were obtained from Aldrich Chemcial Co. and Eastman Kodak Co. and used without further purification. All phenol and laccase preparation solutions were prepared in 0.1 M phosphate buffer (pH 6.8) which was made up from double glass distilled deionized water.

Kinetic measurements.

Phenol solutions were prepared fresh before each rate study. Kinetic experiments were carried out at a constant temperature of 30 $\pm$ .01°C in an open beaker containing 50 mL of air saturated phosphate buffered solution with 21.5 umole of phenol. Air was continuously supplied to the solution to insure saturation (approximately 240 uM O_2).

To initiate the reaction 0.5 mL of the laccase preparation was added to the reaction mixture while stirring vigorously. Reaction rates were measured by monitoring disappearance of the phenol compounds. From 50 mL of the reaction solution 1.4 mL aliquots were periodically withdrawn and placed into glass vials containing 0.35 mL of 50 uM KCN solution. Potassium cyanide was found to be effective in inhibiting the laccase activity and was used to stop the reactions. The samples were placed on ice and stored in the dark until HPLC analysis, which was carried out immediately after collection of the samples. Control experiments were preformed using laccase preparations that had been deactivated by boiling approximately 30 min.

Sampling times were adjusted for the various compounds according to their reaction rates. Sampling time intervals for the phenol compounds were as follows: (1) 30-s intervals, coniferyl alcohol and ferulic acid; (2) 1 min intervals, gallic acid and pyrogallol; (3) 2 min intervals, protocatechuic acid and catechol; (4) 5 min intervals, vanillic acid; (5) 6 min intervals, guaiacol; (6) 10 min intervals, resorcinol and phloroglucinol. A time zero sample and four additional samples were taken at the above specified intervals with the exception of catechol where a time zero sample and five additional samples were taken.

In each case, the total amount of substrate disappearance (phenolic compound) at the final sampling time was between 15 and 40% of the initial amount present. All experiments were performed in duplicate. Two individual rate constants and the standard deviation from the mean of these two values were calculated for each compound

listed above.

Analytical Procedures

Solution concentrations of the various phenol compounds were measured using a Waters high performance liquid chromatograph consisting of a model 6000A pump, model 45 pump, model 720-systems controller, and coupled with a model 480 Lambda Max variable wavelength UV absorbance detector. The detection wave length was set at 280 nm for the phenols. The sample injection valve (Rheodyne 7125) was fitted with a 20 uL loop. We used a Waters u Bondapak C_{18} analytical column. Peak areas were measured using a Waters model 730 data module. The mobile phase consisted of an acetonitrile-5% acetic acid mixture with a flow rate of 1 mL min^{-1}. The mobile phase ratio was adjusted to give a retention time of 5 to 6 minutes.

RESULTS AND DISCUSSION

In the present study we have concerned ourselves primarily with initial stages of the overall coupling reactions to reduce the possibility of enzyme destruction and inhibition. All kinetic data obtained from the reactions could be described adequately by the zero order rate expression:

$$-\frac{d[A]}{dt} = k$$

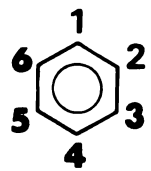
where [A] represents the concentration of the phenolic substrate. Generally, the extent of the reactions monitored was between 15 and 40% and R^2 values between .85 and .99 were obtained. We have compared rates of laccase mediated coupling of several phenolic compounds in an effort to advance our understanding of the structure-reactivity relationships. The phenolic compounds used in this study are thought to be natural soil components which, when coupled together, form the matrix of soil humic substances. The phenolic compounds studied are listed in Table 1 along with their respective rate constants. Phenolic compounds containing the 3-C acrylic group, e.g. ferulic acid are lignin derivatives whereas the remaining compounds are thought to find their way into the soil as a result of lignin decomposition and modification, or microbial synthesis.

We were able to discern several trends with respect to structural effects on reactivities. We generally observed that reactivity was enhanced by: (1) addition of a carboxyl group

Table 1. Rate constants for the reaction of phenolic humus constituents with a partically purfied laccase preparation.

Compound	Structure‡	K*(uM s^{-1})
Coniferyl alcohol	1(CH_2OH-CH=CH-), 3(OCH_3), 4(OH)	0.463 ±0.018
Ferulic acid	1(COOH-CH=CH-), 3(OCH_3), 4(OH)	0.339 ±0.035
Gallic acid	1(COOH), 3(OH), 4(OH), 5(OH)	0.200 ±0.001
Pyrogallol	1(OH), 2(OH), 3(OH)	0.125 ±0.035
Protocatechuic acid	1(COOH), 3(OH), 4(OH)	0.094 ±0.001
Catechol	1(OH), 2(OH)	0.074 ±0.01
Vanillic acid	1(COH), 3(OCH_3), 4(OH)	0.047 ±0.085
Guaiacol	1(OH), 2(OCH_3)	0.0_29 ±0.004
Resorcinol	1(OH), 3(OH)	-
Phloroglucinol	1(OH), 3(OH), 5(OH)	-

* Rate constant ± standard deviation

‡ 

directly on the ring, (2) addition of OH groups positioned ortho to one another as compared to meta OH groups, (3) substitution of a OCH_3 group by an OH group ortho to an OH group, and (4) addition of a 3-C acrylic group on the phenol ring.

We observed that placement of a carboxyl group directly on the phenolic ring increased reactivity. For example, addition of a carboxyl group to guaiacol (forming vanillic acid) increased the rate constant by a factor of 1.6 (Table 1). Similarly, the rate constant for gallic acid was 1.6X larger than the rate constant for pyrogallol.

These results are interesting in light of the fact that a carboxyl group is an electron withdrawing group (March, 1977) and as such would be expected to withdraw electron density away from the hydroxyl group which is presumed to be the reaction center for laccases (Sjoblad and Bollag, 1981). By constrast, Berry and Boyd (1984) have observed that peroxidase reaction rates with phenolic substrates are greatly reduced with the addition of an electron withdrawing group including COOH on the phenol ring. It has been well established that the reaction center for peroxidase mediated coupling of phenols is the hydroxyl group of the phenolic compound (Benon, 1977).

Our observations suggest that the laccase preparation used in this investigation does not depend on electron density at the reaction center (hydroxyl group) in exactly the same manner as does peroxidase. We also observed a rate enhancement effect when we increased the number of OH groups (ortho to one another) on the aromatic ring. For example, by adding an OH group to catechol

(forming pyrogallol) we observed a 1.7 x increase in the rate constant. It may be that by increasing the number of OH groups on the aromatic ring we have decreased the overall oxidation potential of the phenolic compound (pyrogallol is more easily oxidized than cataechol) and therefore the ability of the laccase preparation to oxidize its phenolic substrate.

One of the most interesting observations of the effects of phenolic substrates on reactivity was that the 3-C acrylic group enhanced reactivity. For example, addition of the acrylic group to guaiacol (forming ferulic acid) resulted in an 11.7X increase in the rate constant (Table 1). The results obtained in this study concerning the enhancement effects of the 3-C acrylic group addition were also observed by Berry and Boyd (1984) for peroxidase mediated coupling of various phenolic compounds including: ferulic, caffeic, and p-hydroxycinnamic acids. As a result of their investigation Berry and Boyd (1984) suggested that lignin derived phenols containing the acrylic group, e.g. ferulic acid, may be preferentially utilized during peroxidase mediated synthesis of humic substances in soil. The results presented in this study conerning laccase mediated coupling of phenolic compounds also seem to suggest the preferential utilization of phenolic compounds containing the 3-C acrylic group for laccase mediated formation of humic substances. It should be pointed out that the differences in reactivities from phenolic compound to compound was much greater relatively speaking for the peroxidase as compared to the laccase preparation.

REFERENCES

1. Benon, H., J. Beilski, and Gebicki. 1977. Application of radiation chemistry to biology. Chapt. 1. In W.A. Bryor (ed.) Free radicals in biology, Vol III. Academic Press, New York.

2. Berry, D.F., and S.A. Boyd. 1984. Reaction rates of phenolic humus constituents and anilines during cross-couping. Soil Biol. Biochem. Submitted for review.

3. Bollag, J.-M., R.D. Sjoblad, and S.-Y. Liu. 1979. Characterization of an enzyme from Rhizoctonia praticola which polymerizes phenolic compounds Can. J. Micro. 25:229-233.

4. March, J. 1977. Advanced organic chemistry, Chapt. 9. McGraw-Hill Book Co., New York.

5. Martin, J.P., and K. Haider. 1980. A comparison of the use of phenolase and peroxidase for synthesis of model humic acid-type polymners. Soil Sci. Soc. Am. J. 44:983-988.

6. Martin, J.P., and K. Haider. 1971. Microbial activity in relationship to soil humus formation. Soil Sci. 111:54-63.

7. Sjoblad, R.D., and J.-M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In E.A. Paul and J.M. Ladd (eds) Soil Biochemistry, Vol. 4, Chapt. 3. Marcel Dekker, Inc. New York.

8. Sjoblad, R.D., and J.-M. Bollag. 1976. Oxidative coupling of aromatic pesticide intermediates by a fungal phenol oxidase. Appl. Environ. Micro. 33:906-910.

9. Stevenson, F.J. 1982. Humus Chemistry. Chapt. 1. John Wiley and Sons, New York.

CHAPTER V

ENHANCED BINDING OF 3,3'-DICHLOROBENZIDINE TO SOIL HUMIC COMPONENTS UPON ADDITION OF FERULIC ACID TO SOIL

INTRODUCTION

Contamination of soils by improper disposal of industrial waste chemicals has now become recognized as one of our most serious pollution problems. Once the soil has become contaminated, the potential exists for surface and ground water contamination. This potential was realized to the fullest extent near a manufacturing site in Michigan where improper disposal of waste chemicals containing 3,3'-dichlorobenzidine (DCB) (Figure 1) resulted in contamination of soil, surface waters, and ground water (Kelly, 1978).

DCB, an aromatic amine, is primarily used in the manufacture of dyes and pigents. DCB is a demonstrated animal carcinogen and has been shown to give a positive test in the Ames Salmonella assay indicating that it possesses mutagenic properties (Jones, 1980). Furthermore, a postive correlation has been observed for exposure levels of benzidine (in the work place) and incidence of bladder cancer in humans (Jones, 1980).

Contamination of natural waters by soil-borne xenobiotic compounds is a function of that compound's persistance and mobility in soil. For persistant compounds such as DCB, mobility in the soil environment is especially important because the compound exists in soil for long periods of time thereby enhancing the potential movement and subsequent contamination of groundwaters.

The predominant fate of aromatic amines (such as DCB) in soil generally appears to be formation of stable covalent linkages with

soil humic substances (Boyd et al., 1984; Chisaka and Kearney, 1970; Hsu and Bartha, 1974). Because aromatic amines are not readily mineralized in soil they remain available for binding (Chisaka and Kearney, 1970; Hsu and Bartha, 1974; Boyd et al., 1984). Generally, it has been observed that after entry into the soil environment, aromatic amines exhibit a sharp decrease in solvent-extractability. Boyd et al., (1984) showed that for DCB, loss of solvent-extractability was accompanied by a simultaneous increase in NaOH-extractability. Boyd et al. (1984) concluded that DCB had formed covalent linkages with the soil humic fraction. The formation of bound (non-extractable) residues rendered DCB highly immobile (Boyd, 1984) although some movement of DCB through the soil profile was observed. In general, bound residues would be expected to be less mobile and bioavailable than the unbound form.

Peroxidases and phenol oxidases are believed to be the major biocatalysts operative in soil which act to couple aromatic amines to soil humic substances (Sjoblad and Bollag, 1981). In a previous investigation (Berry and Boyd, 1984) we studied the peroxidase mediated cross-coupling of phenolic humus constituents and anilines in an effort to gain a better understanding of how anilines might bind to soil humic substances. A significant result of that study was that reactivity of chloroaniline was greatly enhanced in the presence of certain phenolic humus constituents such as ferulic acid. These results suggested that peroxidase in combination with reactivity enhancing agents such as ferulic acid might be useful in enhancing the formation of bound residues involving aromatic amines and humic substances in soils. Peroxidase has been used to remove

pollutants from coal-conversion waste waters (Klibanov, 1983). It has been suggested that enzymes might also be used as biological decontamination agents to detoxify soils.

Our objective in the present investigation was to manipulate the binding of DCB in soil by additions of biocatalysts and activating compounds such as ferulic acid. The ultimate goal was to enhance the irreversible incorporation of toxic or carcinogenic residues into stable humic substances thus, reducing their mobility and essentially obliterating their chemical identity.

MATERIALS AND METHODS

Soil incubations

A Rubicon sandy loam was used for all the experiments in this study and was obtained from the Muskegon Wastewater Treatment Facility, Muskegon, MI. The samples were collected from the top 15 cm of the soil profile, air dried, ground and passed through a 2 mm sieve. The Michigan State University Soil Testing Laboratory carried out the soil analysis with respect to particle size analysis, organic matter content, and pH. The Rubicon soil was found to contain the following percentage of sand, silt, and clay; 82.4, 4.2, and 13.4 percent of organic matter was 2.45 and the pH determined to be 7.5.

The 3,3'-dichlorobenzidine (3,3'-dichloro-4,4'diaminobiphenyl) used in this study was purchased from Chem Service (West Chester, PA) as the free base and was used without further purification. 3,3'-dichlorobenzidine (ring-UL-^{14}C) was purchased from ICN Pharmaceuticals, Inc., having a radiochemical purity of >99% and a specific activity of 35 mCi/mmol.

Horseradish peroxidase (HRP) Type II (Donor: hydrogen-peroxidase oxidoreductase; E.D. 1.11.1.7) was purchased form Siga Chemical Co. The HRP had a specific activity (a.u.) of 155 purpurogallin units per mg solid and an RZ value of 1.52.

Experiment one. A two-kilogram batch of soil contained in an amber colored glass jar was moistened to approximately 37% field capactiy by addition of 110 mL of distilled water plus 20 mL of

ethanol containing ^{14}C-labelled and unlabelled DCB. The soil was continuously mixed while the water-ethanol mixture containing the DCB was added dropwise using a pasteur pipet. The soil was prepared in this fashion to give a total unlabelled DCB concentration of 5 ug/g air dried soil the glass jar was closed and the soil incubated at room temperature. The amount of radioactivity ([^{14}C]DCB) added to the soil was 21.3 nCi/g air dried soil. Soil samples were withdrawn at various intervals (0, 1, 2, 4, 7, 10, and 14 days) during the initial 14-days of incubation after which the DCB-amended soil was split into 5 portions and each treated as follows: (Portion 1) water control, 20 mL of distilled water was added to 421 g of DCB-amended soil; (Portion 2) 5 mL of a HRP solution (2.0 x 104 a.u.) was added to 210 g of DCB-amended soil followed by 5 mL of H_2O_2 solution (1.55 x 10^{-2} moles); (Portion 3) ferulic acid (0.12 g, powdered form) was first added to 421 g of DCB-amended soil, followed by 10 mL of HRP solution (4.0 x 10^3 a.u.), and then 10 mL of H_2O_2 solution (3.09 x 10^{-3} moles), and (Portion 4) ferulic acid (0.6 g, powdered form) was first added to 210 g of DCB-amended soil followed by 5 mL of an HRP solution (2.0 x 10^4 a.u.) and then 5 mL of H_2O_2 solution (1.55 x 10^{-2} moles).

Experiment two. A one-kilogram batch of soil was treated exactly the same as in experiment one but with one-half the amount of DCB additions. After a 14 day incubation period the DCB-amended soil was split up into three 210 g portions with each of the portions being treated in the following manner: (Portion 1) water control, addition of 10 mL of distilled water, (Portion 2) ferulic acid (0.6 g powdered form) was added first followed by 5 mL of an HRP solution (2

x 10^4 a.u.) and then 5 mL of H_2O_2 solution (1.55 x 10^{-2} moles); and (Portion 3) addition first of ferulic acid (0.6 g, powdered form) followed by addition of 5 mL of deactiviated HRP solution (2.0 x 10^4 a.u.) and then 5 mL of H_2O_2 solution (1.55 x 10^{-2}moles). HRP was deactivated by boiling for 1.5 h.

Analysis

Approximately 40 g soil samples were withdrawn at the appropriate time intervals and frozen until analysis could be carried out. From each of the samples (40 g) triplicate 10 g subsamples were placed in flasks along with 40 mL of a 60:40 (vol/vol) ethyl actetate/methanol solvent mixture and allowed to shake overnight. From each of subsamples, 5 mL of solvent were centrifuged (8,000 x g for 10 min) from which 2 mL was used for liquid scintillation counting. Figures 2 and 3 illustrate graphically the amount of solvent-extractable radioactivity and (expressed as a percentage of the added radioactivity) was calculated on a soil air dry weight basis.

Liquid-phase ^{14}C radioactivity was measured using a Beckman LS8100 liquid scintillation counter. Samples were counted in Aquasol-2 (New England Nuclear) LSC cocktail.

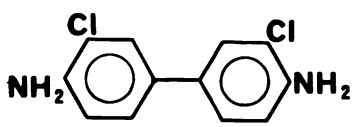


Figure 1. Chemical structure of 3,3'-dichlorobenzidine (DCB).

RESULTS AND DISCUSSION

In this investigation we have attempted to manipulate binding of DCB to soil humic components in an effort to decontaminate soil by enhancing the formation of bound (non-extractable) DCB residues. It is assumed that bound-DCB would be less mobile and bioavailable than the non-bound form. In order to demonstrate an altered DCB-soil binding curve we first allowed DCB to establish its normal unaltered binding curve over a 14 day time period. The normal binding pattern for DCB-amended soil is an initial decrease in solvent-extractable DCB residue followed by a period where levels of solvent-extractable DCB residue remains essentially constant (Boyd et al., 1984). After the 14 day time period we added ferulic acid, HRP, and H_2O_2 in various combinations in an attempt to alter the standard binding curve. Hydrogen peroxide was added to the DCB-amended soil, only after all the other reagents had been added. This sequence of additions was adopted because soils are known to contain catalase enzymes which utilize H_2O_2 as a substrate. Since we expected catalase to compete with peroxidase for H_2O_2 we therefore added H_2O_2 last in an effort to provide HRP with an opportunity to compete for the H_2O_2. It should be pointed out that HRP requires the presence of an electron donor (e.g. a phenolic compound) before it can complete its enzymatic cycle with H_2O_2. These compounds normally exist in soil as part of the soil humus. The additions of HRP and H_2O_2 were an attempt to rise the level of enzymatic activity in soil and thereby enhance the incorporation of DCB which occurs during the oxidative coupling process.

One reagent that was used in conjunction with HRP was ferulic acid.

In a previous study we were able to establish that ferulic acid, a very reactive substrate for HRP, enhanced the incorporation of aromatic amines into model humic substances (Berry and Boyd, 1984). Thus, ferulic acid was added in an attempt to facilitate the effectiveness of the enzyme treatment.

In Figure 2 (experiment 1) DCB binding curves 2, 3, and 4 represent reagent additions to DCB-amended soil and can be compared to DCB binding curve 1 which represents the control i.e. no reagent addition. Addition of the reagents HRP and H_2O_2 even at the higher concentration level (DCB binding curve 2) to DCB-amended soil only slightly decreased the amount of solvent-extractable radioactivity with respect to the control (curve 1). It is clear from the data obtained in experiment 1 for HRP plus H_2O_2 additions to DCB amended soil that we have not significantly altered the amount of solvent-extractable radioactivity. In contrast, relative to the control curve we did observe a sharp 38% decrease in the amount of solvent-extractable radioactivity in the [^{14}C]DCB-amended soil after addition of ferulic acid, HRP, and H_2O_2 at the lower concentration level (DCB binding curve 3). A follow up treatment, i.e. a second addition of the same reagents to the previously treated soil at 21 days produced a second deflection in binding curve (3) representing another decrease in the amount of solvent-extractable radioactivity. The follow up treatment produced about an 8% decrease in the amount of solvent-extractable radioactivity. Addition of ferulic acid, HRP and H_2O_2 at the higher (10X) concentration level to [^{14}C]DCB-amended soil produced a dramatic 64% reduction in the amount of solvent-extractable radioactivity (Figure 2, DCB binding curve 4).

The data obatined from experiment 1 demonstrated that the addition

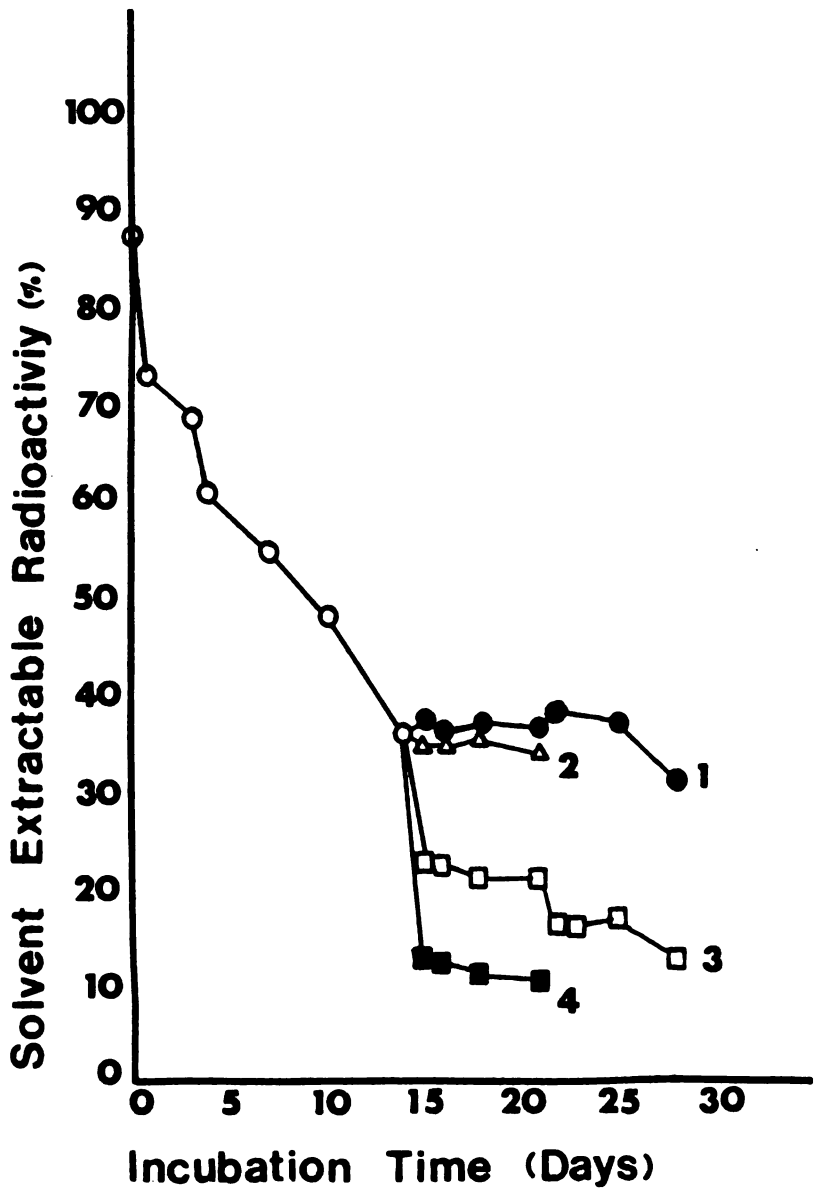


Figure 2. Experiment 1 DCB binding curve.

of ferulic acid was critical with respect to decreasing the amount of solvent-extractable radioactivity from [^{14}C]DCB-amended soil. Experiment 1 did not, however, demonstrate a clear requirement for HRP. Experiment 2 (Figure 3) was designed and carried out in order to obtain information regarding the role of HRP in the soil decontamination study. The evidence obtained from experiment 2 where we compared an active (curve 2) vs. deactivated (curve 3) HRP preparation suggested that HRP additions are not required for the enhanced binding phenomenon. We can see from Figure 3 that binding curves 2 and 3 are in fact identical signifying that there is no difference between deactivated or active HRP addition. Thus, ferulic acid alone appears to be responsible for the dramatic enhanced binding of DCB to soil.

Although, the mechanism by which the enhancement process takes place is still not well defined we can present a plausible explanation based on our previous studies. Earlier (Berry and Boyd, 1984) we examined peroxidase mediated coupling of phenolic humic constituents. The results of that study demonstrated that phenolic compounds which possess the 3-C acrylic side chain, e.g. ferulic acid were highly reactive with HRP and as such might preferentially be incorporated or bound to soil humic materials as opposed to other phenolic compunds which do not have the acrylic group. Furthermore, (Berry and Boyd, 1984) clearly established that the presence of ferulic acid enhanced the rate of aromatic amine binding to model humic materials during the peroxidase mediated coupling process. Thus, it may be possible that the presence of ferulic acid in DCB-amended soil stimulates the overall level of oxidative coupling reactions in soil thereby, increasing DCB binding. While it is clear that the addition of HRP did not stimulate

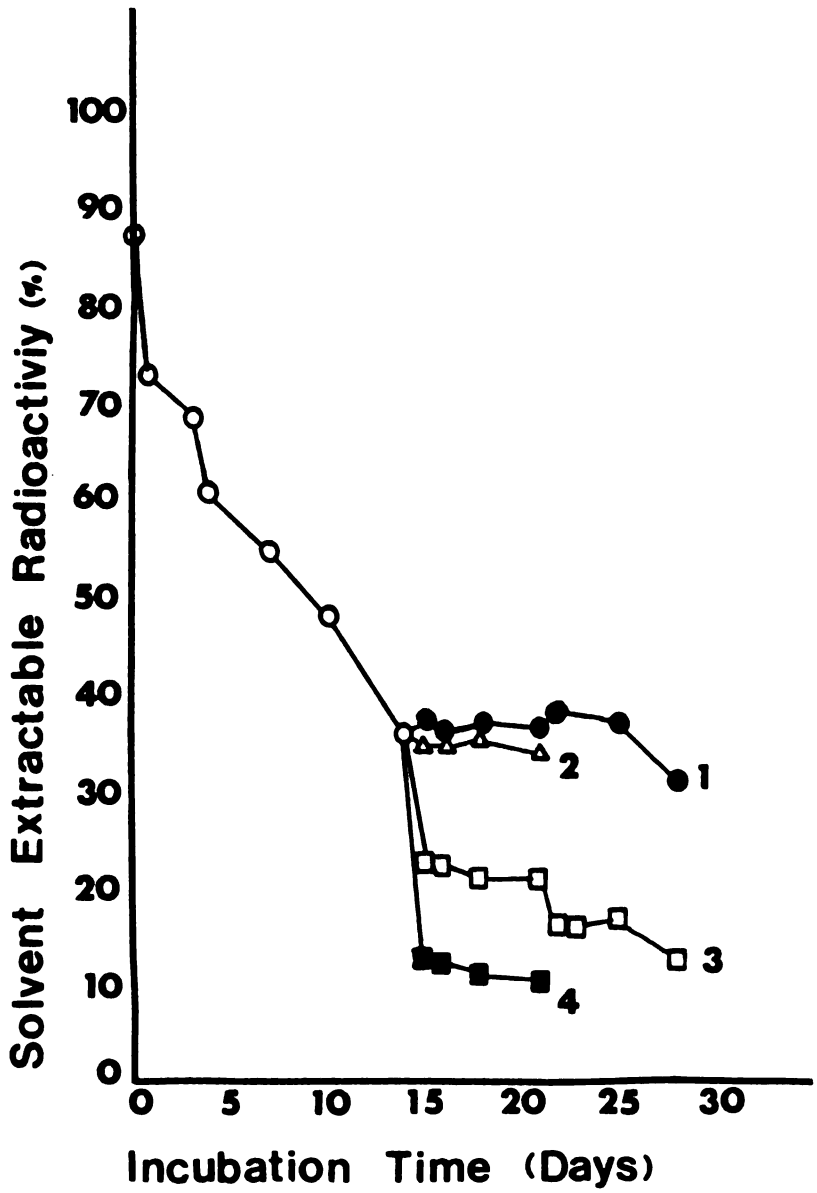


Figure 3. Experiment 2 DCB binding curve.

increased binding of DCB in DCB-amended soil it is plausible that natural soil oxidative coupling enyzmes, e.g peroxidases are functional in this enhancement process. It is also possible that addition of ferulic acid stimulated microbial activity which caused enhanced binding of DCB in DCB-amended soil. Just exactly how an increase in microbial activity could stimulate DCB binding is not clear. Additional experiments are underway to more clearly define the role of ferulic acid in the DCB enhanced binding process. It is clear, however, that addition of reagents such as ferulic acid could offer great promise as a means of detoxification of contaminated soil. Obviously, addition of a readily available compound such as ferulic acid is far more economically feasible than would be any type of enyzme addition.

REFERENCES

1. Berry, D.F., and S.A. Boyd. 1984. Reaction rates of phenolic humus constituents and anilines during cross-coupling. Submitted for publication in Soil Biol. Biochem.

2. Boyd, S.A., C.-W., Kao, and J.M. Suflita. 1984. Fate of 3,3'-dichlorobenzidine in soil. Persistence and binding. Environ. Tox. Chem. 3:000-000.

3. Boyd, S.A. 1984. Mobility of the sludge-borne carcinogen. 3,3'-Dichlorobenzidine in irrigated soils. Submitted for publication in J. Environ. Qual.

4. Chisaka, H., and P.C. Kearney. 1970. Metabolism of propanil in soils. J. Agri. Food Chem. 18:854-858.

5. Hsu, T.-S., and R. Bartha. 1974. Biodegradation of chloroaniline humus complexes in soil and in culture solution. Soil Sci. 118:213-220.

6. Jones, T.C. 1980. Benzidine, its congeners, and their derivative dyes and pigents. TSCA Chemical Assessment Series. EPA-440/9-76-018. U.S. Environmental Protection Agency, Washington, D.C.

7. Kelly, F.J. Attorney General for the State of Michigan. 1978. Complaint for injunctive relief, penalties, and damages. Bofors Lakeway, Inc., Defendant. Case no. 78-21380-CE. March 1, 1978, Lansing, MI.

8. Klibanov, A.M., T.-M., Tu, and K.P. Scott. 1983. Peroxidase-catalyzed removal of phenols from coal-conversion waste waters. Science. 221:259-260.

9. Sjoblad, R.D., and J.-M. Bollag. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In Soil Biochemistry E.A. Paul and J.M. Ladd (eds.) Soil Biochemistry, Vol. 4, Chapt. 3. Marcel Dekker, Inc., New York.