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Effect of Humic Constituents on the Transformation of Chlorinated Phenols and Anilines in the Presence of Oxidoreductive Enzymes or Birnessite

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Chlorinated phenols and anilines are transformed and detoxified in soil through oxidative coupling reactions mediated by enzymes or metal oxides. The reactions may be influenced by humic constituents, such as syringaldehyde or catechol, that originate from lignin decomposition and are also subject to oxidative coupling. In this study, the effect of humic constituents on xenobiotic transformation was evaluated in vitro based on the determination of unreacted chlorophenols and chloroanilines. In experiments with peroxidase, laccase, and birnessite (δ-MnO_2), the transformation of most chlorophenols was considerably enhanced by the addition of syringaldehyde. Less enhancement was observed using 4-hydroxybenzoic acid, and the addition of catechol resulted in a reduction of most transformations. The opposite was observed in experiments with tyrosinase, in which case catechol caused considerable enhancement of chlorophenol transformation. The varying effect of catechol can be explained by different transformation mechanisms involving either *o*-quinone coupling (with tyrosinase) or free radical coupling (with peroxidase, laccase, or birnessite). Regardless of the agent used to mediate the reactions, chloroanilines seemed to undergo nucleophilic addition to quinone oligomers, which resulted from coupling of the humic constituents. Catechol, which readily forms quinones and quinone oligomers, was most efficient in enhancing these reactions.

Introduction

Chlorinated phenols and anilines may be introduced into the environment by accidental spills, illegal release of industrial and municipal wastewater, and excessive use of pesticides. As analogues of humic constituents, these pollutants can be incorporated into the soil organic matter or dissolved humic materials. The incorporation relies largely on the oxidative coupling reaction mediated by extracellular phenoloxidases or metal oxides present in soil or sediments (*1*−*9*). The reaction of oxidative coupling is of great environmental significance because in most cases it leads to the detoxification of xenobiotic substrates (*4*).

In the presence of phenoloxidases or metal oxides, chlorinated phenols and anilines are first oxidized to free radicals or quinones. In the second stage, the oxidation products are subject to chemical coupling. If the reaction takes place in polluted aqueous environments, the oxidation products couple primarily to each other and precipitate out of solution in the form of nontoxic polymers (*9, 10*). In polluted soils, they couple mainly to humus, resulting in the formation of covalent linkages (*4*).

Both the aqueous and soil environments contain large amounts of natural phenols (e.g., ferulic acid, syringaldehyde, pyrogallol, hydroxybenzoic acid, or catechol) that originate from lignin decomposition and are major substrates for oxidative coupling reactions leading to the formation of humus (*11*). These humic constituents may influence the transformation of xenobiotics during oxidative coupling by competing for active sites on enzyme molecules or mineral surfaces or by cross-coupling with the xenobiotic molecules (*4*).

The use of enzymes and metal oxides that can stimulate binding or polymerization of the xenobiotic substrates is currently considered an alternative means of soil cleanup and water treatment (*1*−*4, 12*−*15*). In view of this idea, knowledge related to the effect of humic constituents on the transformation of the pollutants during oxidative coupling is of great significance. There are indications that enhanced transformation may result from high reactivity of humic constituents incubated with less reactive pollutants (*10, 14*). Preliminary experiments for the present investigation indicated, however, that high reactivity alone does not guarantee enhancement unless combined with other factors. A reactive lignin derivative such as catechol, for instance, was found to greatly reduce the transformation of 2,4-dichlorophenol in the presence of peroxidase. In the presence of tyrosinase, however, the transformation of 2,4-dichlorophenol was considerably enhanced by the addition of catechol. These observations indicated that, beside the reactivity of humic constituents, the transformation patterns of chlorinated phenols and anilines may depend on the mechanism of oxidative coupling, which may vary with the type of oxidizing agent and the chemical structure of compounds under investigation. To evaluate this hypothesis, it was deemed necessary to test in different combinations a wide variety of reaction components (chlorinated pollutants, humic constituents, and oxidizing agents). It was expected that by generating a sufficient amount of data, it should be possible to identify different transformation patterns and assign them to specific mechanisms of oxidative coupling.

Xenobiotic phenols and anilines are represented in this study by six compounds from each group, substituted analogously with chlorine atoms. The investigation includes 16 randomly selected humic constituents that vary in the type and the number of substituents. Enzymes used in this study represent monophenol monooxygenases (i.e., laccases and tyrosinases) and peroxidases. To oxidize phenols or anilines, these enzymes require either molecular oxygen (laccases and tyrosinases) or hydrogen peroxide (peroxidases) as electron acceptors. In the presence of laccases and peroxidases, the pollutants are oxidized to free radicals (*4, 10*). Free radicals are also generated in the presence of metal oxides (*2*), such as birnessite (δ-MnO_2), which was included in this investigation. Reactions with tyrosinases, on the other hand, lead to ortho-hydroxylation of monophenols and oxidation of the resultant *o*-diphenols to *o*-quinones (*16*).

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Using the selected enzymes and birnessite, the investigated compounds could be tested for all known mechanisms of oxidative coupling.

Materials and Methods

Chemicals. The compounds 2-, 3-, and 4-chlorophenol (2-CP, 3-CP, and 4-CP), 2,4- and 2,5-dichlorophenol (2,4- and 2,5-DCP), gallic acid, ferulic acid, protocatechuic acid, and guaiacol were purchased from Sigma Chemical Co. (St. Louis, MO). 2-, 3,- and 4-chloroaniline (2-, 3-, and 4-CA), 2,4-dichloroaniline (2,4-DCA), 2,4,5-trichloroaniline (2,4,5-TCA), 4-hydroxybenzoic acid (4-HBA), vanillic acid, and caffeic acid were bought from Aldrich Chemical Co. (Milwaukee, WI). 2,4,5-Trichlorophenol (2,4,5-TCP), syringaldehyde, vanillin, and syringic acid were purchased from Fluka AG (Buchs, Switzerland). Catechol, pyrogallol, and salicylic acid were obtained from Fisher Scientific Co. (Fair Lawn, NJ), and hydroquinone was bought from J. T. Baker (Phillipsburg, NJ).

Oxidoreductive Agents. The laccase (EC 1.10.3.2 *p*-phenol oxidase) isolated from the fungus *Polyporus pinsitus* was obtained from Novo Nordisk (Danbury, CT). One unit of laccase activity is defined as the amount of enzyme that causes a change in absorbance at 468 nm of 1.0/min in 3.4 mL of a 1 mM solution of 2,6-dimethoxyphenol in citrate phosphate buffer (pH 3.8).

Horseradish peroxidase with an RZ (Reinheitszahl) of 1.2 and an activity of 87 unit/mg of solid was purchased from Sigma Chemical Co. (St. Louis, MO). The RZ value, which represents peroxidase purity, is the ratio of absorbency at 403 nm due to the hemin group to that at 275 nm caused by the protein. The activity is expressed in units defined as the amount of peroxidase needed to form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 and 20 °C.

Mushroom tyrosinase, with an activity of 4400 unit/mg of solid, was obtained from Sigma Chemical Co. (St. Louis, MO). One unit of tyrosinase activity is defined as the amount of enzyme that causes an increase in absorbance at 280 nm of 0.001/min at pH 6.5 and 25 °C in a 3-mL reaction mixture containing L-tyrosine. Birnessite (manganous manganite, δ-MnO_2) was prepared according to the method of McKenzie (*17*).

Transformation Reactions. Because of the large number of assays to be carried out simultaneously, the determination of reaction kinetics for each combination was not feasible. Therefore, the effect of humic constituents on the extent of transformation was evaluated mostly on the basis of the overall disappearance of xenobiotics in the supernatant as determined by high-performance liquid chromatography (HPLC).

Unless specified otherwise, chlorinated phenols and anilines (0.3 mM) were dissolved in 0.2 M acetate buffer at pH 5.6, and 10-mL samples were incubated in triplicate with various enzymes and birnessite in the presence or absence of different humic constituents (0.3 mM). The incubations without humic constituents served as reference controls for calculating changes in transformation of the pollutants. Laccase, peroxidase, tyrosinase, and birnessite were applied at concentrations of 1.5 unit/mL, 0.15 unit/mL, 40 unit/mL, and 0.5 mg/mL, respectively. Peroxidase was used along with hydrogen peroxide (0.5 mM H_2O_2) as an electron acceptor. The remaining enzymes and birnessite utilized the molecular oxygen present in the solution. After a 2-h incubation with peroxidase and a 24-h incubation with laccase, tyrosinase, or birnessite, 0.17 mL of concentrated nitric acid was added to stop the reaction, and after centrifugation at 12000*g*, the reaction mixtures were analyzed by HPLC.

The kinetics experiments were carried out with laccase. Selected xenobiotics (4-CP and 4-CA) and humic constituents (syringaldehyde, catechol, or 4-HBA) were incubated with the enzyme separately or in combinations with each other under the above specified conditions except that the reactions were stopped after 10, 20, 30, 60, 180, 600, or 1440 min. In these experiments, the disappearance of humic constituents was also monitored.

The dependence of substrate transformation on the concentration of humic constituents was also investigated using laccase, which was incubated with 4-CP or 4-CA (0.3 mM) in the presence of 0.01, 0.03, 0.05, 0.1, 0.3, 1.0, or 3.0 mM syringaldehyde, 4-HBA, or catechol.

High-Performance Liquid Chromatography. Before HPLC analysis, the supernatant was filtered through a 0.45-μm membrane filter (Millipore Corp., Milford, MA) and washed with water and then with methanol to a specific volume. The analysis was conducted on a Waters Associates (Milford, MA) HPLC system equipped with two model 510 solvent delivery systems, a model 717plus autosampler, a model 440 UV absorbance detector operated at 280 nm (chlorophenols and humic constituents) or 254 nm (chloroanilines), and a Supelcosil 15 cm × 4.6 mm LC-18DB column of 5-μm particle size with a LC-18DB guard column (Supelco, Bellefonte, PA). As in previous studies (*13*, *15*, *20*), the mobile phases for analysis of chlorophenols and humic substituents were composed of an aqueous component A and a methanol component B, each containing 1% acetic acid (*13*). For chloroanilines and 4-HBA, the aqueous component A and the methanol component B each contained 2% acetic acid and 0.018 M ammonium acetate (*9*). The ratio of A to B ranged from 15/85 to 75/25 depending on the compound under investigation. Retention times for all analyzed compounds ranged from 4.5 to 7.5 min.

Results

Screening of Humic Constituents. The transformation of 4-CP in experiments utilizing peroxidase varied with the type of humic constituent. As shown in Table 1, humic constituents incubated with peroxidase may be divided into three groups: (a) those that enhanced the transformation of 4-CP relative to control samples, which did not involve humic constituents, (b) those that had minimal effect, and (c) those that reduced the transformation. Experiments with other enzymes and birnessite showed somewhat different transformation patterns (data not shown). With laccase, for instance, humic constituents from the first group enhanced the transformation of 4-CP. Enhanced transformation, although to a lesser extent, was also observed for the second group of humic constituents, and essentially no effect or reduced transformation was shown with the third group of humic constituents. When tyrosinase was employed, 4-CP was readily transformed in the presence or absence of the cosubstrates. With birnessite, only two cosubstrates from the first group (syringaldehyde and 2,6-dimethoxyphenol) enhanced the transformation; the remaining cosubstrates from this group reduced transformation. Cosubstrates from the second group were of minimal effect, and those from the third group reduced transformation by birnessite. Three humic constituents (syringaldehyde, 4-HBA, and catechol) were selected as representatives of the above listed groups of humic constituents for further investigation into their effects on the transformation of different chlorinated phenols and chloroanilines. Syringaldehyde represented humic constituents that enhanced 4-CP transformation; 4-HBA represented those that had minimal effect; and catechol was one of the humic constituents that reduced the transformation of 4-CP.

Reaction Kinetics. Figure 1 presents time courses for the laccase-mediated transformation of 4-CP and 4-CA in the presence of the three selected humic constituents. The transformation of 4-CP in the reaction with syringaldehyde (100%) was not only greater than in reactions without humic constituents (34%), with HBA (49%), and with catechol (16%),

TABLE 1. Transformation of 4-Chlorophenol Incubated with Peroxidase in the Presence of Various Humic Constituents

humic constituent	molecular structure[a]	transformation of 4-chlorophenol (%)
none (control)		32.3 ± 2.5
	Enhanced Transformation	
syringaldehyde	2(OCH_3), 4(CHO), 6(OCH_3)	82.0 ± 3.1
ferulic acid	2(OCH_3), 4(COOH−CH═CH−)	71.8 ± 8.7
guaiacol	2(OCH_3)	56.1 ± 0.9
vanillic acid	2(OCH_3), 4(COOH)	62.8 ± 3.8
2,6-dimethoxyphenol	2(OCH_3), 6(OCH_3)	73.0 ± 4.9
vanillin	2(OCH_3), 4(CHO)	74.2 ± 6.6
phloroglucinol	3(OH), 5(OH)	47.9 ± 2.9
	No Effect on Transformation	
4-hydroxybenzoic acid	4(COOH)	36.5 ± 2.2
salicylic acid	2(COOH)	34.8 ± 2.1
syringic acid	2(OCH_3), 4(COOH), 6(OCH_3)	32.0 ± 2.1
protocatechuic acid	2(OH), 4(COOH)	30.1 ± 7.3
caffeic acid	2(OH), 4(COOH−CH═CH−)	29.8 ± 0.3
	Reduced Transformation	
catechol	2(OH)	11.1 ± 0.7
gallic acid	2(OH), 4(COOH), 6(OH)	16.3 ± 1.7
hydroquinone	4(OH)	21.9 ± 0.8
4-methoxyphenol	4(OCH_3)	7.2 ± 1.2

[a] The numbers preceding the substituents refer to the position on the aromatic ring of phenol.

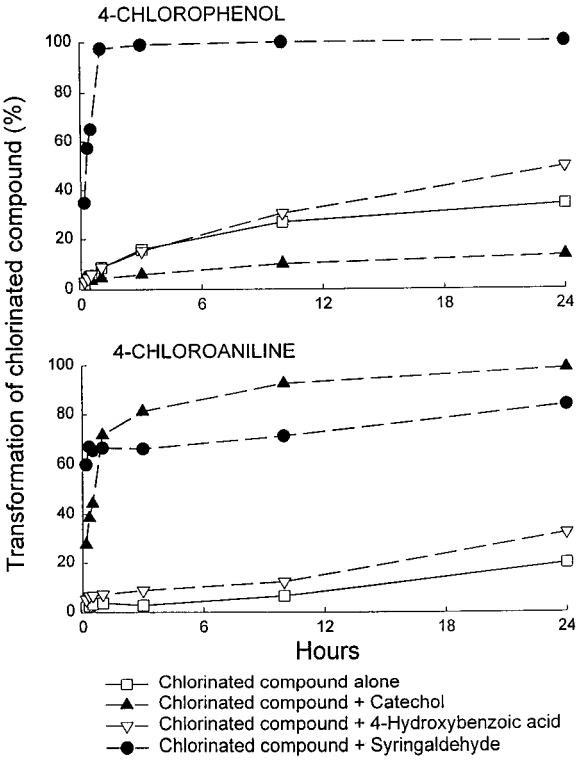

FIGURE 1. Kinetics of the transformation of 4-chlorophenol (0.3 mM) and 4-chloroaniline (0.3 mM) incubated at pH 5.6 with humic constituents (0.3 mM) in the presence of laccase (1.5 unit/mL).

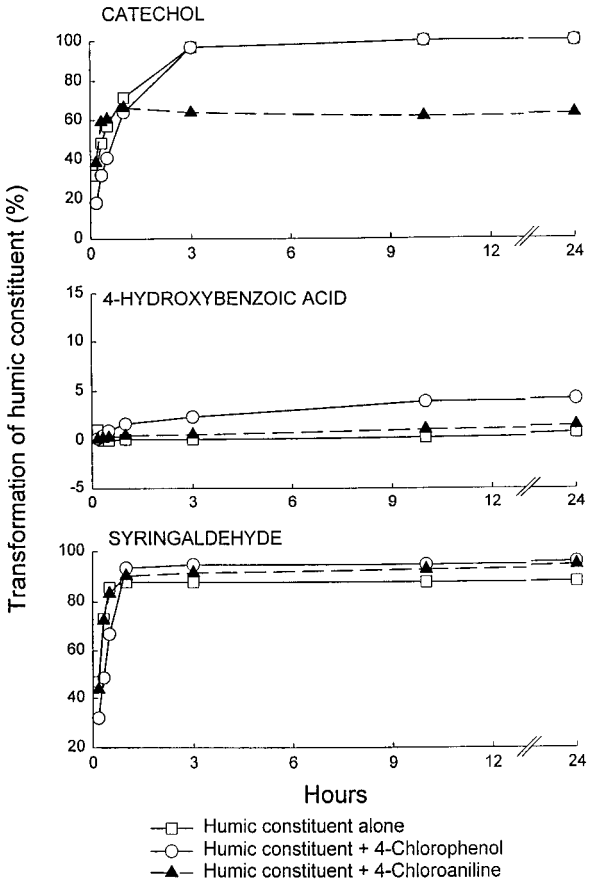

FIGURE 2. Kinetics of the transformation of humic constituents (0.3 mM) incubated at pH 5.6 with 4-chlorophenol (0.3 mM) or 4-chloroaniline (0.3 mM) in the presence of laccase (1.5 unit/mL).

but also much faster, approximating the maximum after only 1 h (Figure 1). The slopes of the time courses for 4-CP alone and 4-CP combined with 4-HBA or catechol indicated that maximum transformation had not been achieved within the 24-h incubation period.

The transformation of 4-CA (Figure 1) was rapid during the initial phase of the reactions with syringaldehyde and catechol, reaching 69% and 72%, respectively, after 1 h. Afterward, 4-CA transformation steadily increased to reach 82% with syringaldehyde and 100% with catechol within 24 h. The transformation of 4-CA alone and with 4-HBA was relatively slow, ranging from about 2% after 10 min to 20% and 32%, respectively, after 24 h.

Figure 2 illustrates how 4-CP and 4-CA were affecting the transformation of humic constituents during the 24-h incubations discussed above. The transformations of syringaldehyde and catechol incubated alone were very fast, approximating 90% and 100%, respectively, within 1−3 h.

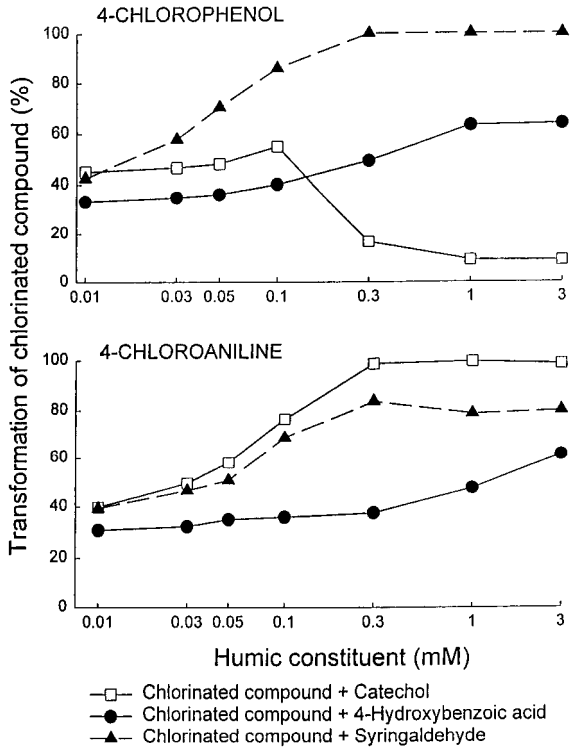

FIGURE 3. Effect of increased concentrations of humic constituents on the transformation of 4-chlorophenol (0.3 mM) and 4-chloroaniline (0.3 mM) incubated for 24 h with laccase (1.5 unit/mL, pH 5.6).

Upon the addition of 4-CP or 4-CA, transformation of syringaldehyde increased slightly during the first hour (to about 97%). The time course for catechol incubated with 4-CP was practically unchanged as compared to that for catechol incubated alone, while in the presence of 4-CA, catechol transformation was reduced to about 60% after a 3-h incubation and remained unchanged thereafter. Unlike the case for syringaldehyde and catechol, laccase-mediated transformation of 4-HBA was negligible (1% within 24 h) and increased only slightly in the presence of 4-CP and 4-CA (to 4% and 2%, respectively).

Effect of Humic Constituent Concentration. With one exception, the transformation of 4-CP and 4-CA was enhanced by increasing concentrations of humic constituents (Figure 3). The exception was the decreased transformation of 4-CP (from about 50 to 8%) when the concentration of catechol increased from 0.1 to 3 mM. At lower concentrations, ranging from 0.01 to 0.1 mM, a slight increase in 4-CP transformation was observed (from about 45 to 50%).

Transformation of both 4-CP and 4-CA increased quickly from about 40% to a maximum (99 and 82%, respectively) when the concentration of syringaldehyde was increased from 0.01 to 0.3 mM. In the same concentration range, a rapid increase was also observed in the transformation of 4-CA with catechol (from about 40 to 100%). No appreciable changes in the transformation rates were observed at syringaldehyde and catechol concentrations higher than 0.3 mM. Increasing the concentration of 4-HBA from 0.01 to 0.1 or 0.3 caused negligible increases (less than 10%) in the transformation of 4-CP and 4-CA. Faster transformation was observed at higher 4-HBA concentrations (0.3−3 mM), reaching about 65 and 60% for 4-CP and 4-CA, respectively.

Transformation of Chlorophenols. As expected based on Figures 1−3, the peroxidase-, laccase-, or birnessite-mediated transformation of chlorophenols determined after incubation was in most cases greater in the presence of syringaldehyde than in its absence (Table 2). Exceptions call for the reduced transformation of 3-CP with peroxidase and the unchanged transformation of 2,4-DCP with laccase. Considerably fewer enhanced and more reduced or unchanged chlorophenol transformations were observed in the presence of 4-HBA. In the presence of catechol, reduced transformation was observed in most of the reaction mixtures. The only exceptions were the enhanced transformation of 2-CP and the unchanged transformations of 3-CP and 2,4-DCP, all with laccase.

Catechol combined with tyrosinase caused a greater transformation of chlorophenols than did other humic constituents. Syringaldehyde combined with tyrosinase showed either negligible effect or decreased the transformation of the substrates. The addition of 4-HBA had practically no effect on tyrosinase-mediated reactions. The tyrosinase-mediated transformation of 4-CP was 100% regardless of humic constituent used.

Transformation of Chloroanilines. The transformation of chloroanilines was in most cases enhanced by the addition of humic constituents (Table 3). This outcome is consistent with the results presented in Figures 1−3. The greatest enhancement was caused by catechol, not only when it was applied in combination with tyrosinase, as was the case with chlorophenols, but also in the presence of other enzymes and birnessite. The only exception was the reduced transformation of 2-CA in the reaction mediated by birnessite.

Syringaldehyde caused a considerable increase in the transformation of chloroanilines (by more than 10%) only in reactions involving laccase and in the birnessite-mediated reaction with 2,4,5-TCA. The enhancements, however, were less than those observed for catechol. In the case of 2,4-DCA incubated with peroxidase, 4-CA incubated with tyrosinase, and 2-CA and 4-CA incubated with birnessite, the addition of syringaldehyde resulted in reduced transformation. In the remaining reactions, syringaldehyde either slightly enhanced transformation (by less than 10%) or had no effect (transformation of 2-CA, 3-CA, and 4-CA by peroxidase).

Most reactions involving 4-HBA resulted in a slight enhancement of chloroaniline transformation (by less than 10%). Only the transformations of 3-CA catalyzed by peroxidase and 4-CA catalyzed by laccase or tyrosinase were enhanced by more than 10%. In one reaction, 4-HBA caused a decrease in chloroaniline transformation (when combined with 2,4,5-TCA and birnessite), and in five other reactions, it had no effect (when combined with 2-CA and laccase or birnessite, with 4-CA and peroxidase or birnessite, and with 2,4,5-TCA and peroxidase).

Discussion

Results obtained in this study strongly confirm the hypothesis that the impact of humic constituents on the transformation of chlorophenols and chloroanilines depends primarily on which of the known mechanisms of oxidative coupling are involved in a specific reaction. Previous research indicated that substrates that are readily transformed by oxidoreductive enzymes and birnessite should enhance the transformation of less reactive chemicals (*10, 14*). For this reason, syringaldehyde and catechol, which are very reactive (Figure 2) due to the presence of the electron-donating methoxy group or another hydroxy group, were both expected to enhance the transformation of chlorophenols. In experiments with peroxidase, laccase, and birnessite, syringaldehyde increased transformation; catechol, however, had an adverse effect on the transformation (Figure 1, Tables 1 and 2). On the other hand, in the presence of tyrosinase, catechol caused a considerable enhancement of chlorophenol transformation, whereas syringaldehyde had negligible effect. This and other differences cannot be explained without considering the mechanism of transformation.

TABLE 2. Transformation of Chlorophenols Incubated with Peroxidase, Laccase, Tyrosinase, and Birnessite in the Presence of Humic Constituents

	transformation of chlorophenols (%)					
	2-CP	3-CP	4-CP	2,4-DCP	2,5-DCP	2,4,5-TCP
Peroxidase						
substrate alone	22.9 ± 1.7	11.6 ± 0.1	32.3 ± 2.5	75.1 ± 1.2	25.1 ± 1.9	42.2 ± 1.8
substrate + syringaldehyde	62.5 ± 0.6	9.1 ± 0.5	82.0 ± 3.1	86.2 ± 0.7	31.7 ± 3.6	68.3 ± 0.5
+ 4-HBA	34.7 ± 2.8	13.2 ± 0.7	36.5 ± 2.2	75.2 ± 1.4	37.5 ± 1.1	39.7 ± 1.2
+ catechol	18.5 ± 2.2	7.9 ± 3.0	21.4 ± 1.0	27.2 ± 3.3	7.7 ± 0.8	21.1 ± 3.4
Laccase						
substrate alone	43.1 ± 1.6	8.1 ± 1.1	34.0 ± 0.2	95.9 ± 0.9	21.0 ± 0.5	27.1 ± 1.1
substrate + syringaldehyde	95.2 ± 0.2	32.5 ± 2.0	100.0 ± 0.1	96.4 ± 0.5	68.3 ± 1.1	96.0 ± 0.7
+ 4-HBA	79.7 ± 0.3	7.0 ± 3.9	49.2 ± 0.7	96.1 ± 0.4	22.4 ± 0.2	29.2 ± 0.6
+ catechol	66.0 ± 0.8	7.4 ± 1.5	16.6 ± 0.5	93.5 ± 0.3	10.9 ± 3.6	8.9 ± 0.3
Tyrosinase						
substrate alone	11.4 ± 2.2	32.2 ± 0.4	100.0 ± 0.5	20.4 ± 1.5	1.6 ± 1.5	1.2 ± 0.1
substrate + syringaldehyde	6.2 ± 0.2	32.6 ± 0.5	100.0 ± 0.1	6.5 ± 1.8	2.5 ± 3.0	2.0 ± 0.5
+ 4-HBA	13.3 ± 2.1	29.1 ± 5.4	100.0 ± 1.0	21.6 ± 1.7	3.6 ± 3.9	3.8 ± 0.9
+ catechol	35.4 ± 1.5	47.9 ± 1.5	100.0 ± 0.1	52.6 ± 2.4	10.5 ± 1.5	9.6 ± 1.4
Birnessite						
substrate alone	62.0 ± 0.6	32.8 ± 5.6	45.4 ± 3.8	77.6 ± 6.4	28.4 ± 3.1	37.6 ± 7.9
substrate + syringaldehyde	75.1 ± 3.3	57.0 ± 9.7	100.0 ± 0.1	94.4 ± 1.0	62.1 ± 7.6	77.5 ± 2.0
+ 4-HBA	58.6 ± 2.1	21.8 ± 5.9	45.4 ± 3.3	74.6 ± 0.8	29.5 ± 4.1	72.9 ± 7.6
+ catechol	20.8 ± 1.4	16.0 ± 2.7	21.6 ± 0.4	16.7 ± 0.8	13.5 ± 1.0	9.9 ± 0.9

TABLE 3. Transformation of Chloroanilines Incubated with Peroxidase, Laccase, Tyrosinase, and Birnessite in the Presence of Humic Constituents

	transformation of chloroanilines (%)				
	2-chloroaniline	3-chloroaniline	4-chloroaniline	2,4-dichloroaniline	2,4,5-trichloroaniline
Peroxidase					
substrate alone	4.4 ± 4.7	28.8 ± 1.5	78.3 ± 0.7	4.1 ± 3.9	5.5 ± 0.7
substrate + syringaldehyde	6.0 ± 4.1	30.4 ± 1.2	76.7 ± 0.4	0.7 ± 0.8	14.2 ± 2.6
+ 4-HBA	12.3 ± 0.1	55.9 ± 0.1	77.5 ± 0.4	8.0 ± 1.0	6.9 ± 1.4
+ catechol	17.8 ± 1.9	76.9 ± 0.6	83.1 ± 0.2	25.0 ± 0.7	35.8 ± 3.1
Laccase					
substrate alone	9.4 ± 0.1	0.8 ± 0.3	31.0 ± 3.6	3.2 ± 0.2	3.6 ± 1.8
substrate + syringaldehyde	23.0 ± 3.6	59.6 ± 1.5	83.6 ± 0.5	59.7 ± 0.9	67.6 ± 0.7
+ 4-HBA	10.0 ± 0.2	4.2 ± 0.6	35.0 ± 0.1	5.0 ± 0.3	6.2 ± 0.6
+ catechol	63.0 ± 0.7	65.8 ± 0.8	98.6 ± 0.6	70.1 ± 2.7	78.5 ± 0.3
Tyrosinase					
substrate alone	10.1 ± 0.9	4.0 ± 0.1	39.1 ± 0.7	5.1 ± 0.4	3.9 ± 2.2
substrate + syringaldehyde	12.5 ± 0.3	8.3 ± 0.6	5.4 ± 0.5	7.0 ± 0.2	6.9 ± 2.4
+ 4-HBA	12.6 ± 0.4	11.6 ± 0.6	77.6 ± 0.6	14.5 ± 0.6	6.9 ± 2.5
+ catechol	53.9 ± 0.2	86.6 ± 0.3	82.2 ± 0.9	68.1 ± 0.2	73.1 ± 6.1
Birnassite					
substrate alone	99.6 ± 0.8	63.7 ± 0.5	85.7 ± 4.2	22.0 ± 3.4	56.1 ± 6.0
substrate + syringaldehyde	53.4 ± 1.8	70.9 ± 1.4	81.8 ± 3.0	31.0 ± 5.3	71.4 ± 0.6
+ 4-HBA	97.4 ± 2.4	71.6 ± 2.7	86.3 ± 2.0	26.3 ± 5.6	41.3 ± 0.3
+ catechol	73.2 ± 2.9	95.8 ± 1.9	95.7 ± 1.1	85.3 ± 3.5	95.4 ± 0.7

Transformation reactions mediated by oxidoreductases or birnessite involve two stages: (a) enzyme- or birnessite-mediated oxidation of the substrates (xenobiotic chemicals or humic constituents) and (b) chemical coupling of the oxidation products. The transformation mechanisms may differ depending on molecular structure of the substrates and the type of the oxidizing agent (Figure 4). With peroxidase, laccase, or birnessite, most substrates (including xenobiotic phenols, phenolic humic constituents, and anilines) are oxidized in the first stage to free radicals (*4, 16, 18, 19*). Hydroxylated phenols, such as catechol, are oxidized by the same agents to *o*-quinones. Catechol molecules may occur as phenoxide anions that readily couple to *o*-quinones through nucleophilic substitution (*20*). *o*-Quinones are also generated (via ortho-hydroxylation of phenols and anilines) in the presence of tyrosinase (*16, 21*).

In the second stage of one-substrate reactions, the oxidized compound undergoes coupling to itself. When cosubstrates are present, however, cross-coupling may occur between different oxidation products. It can be expected that in order for cross-coupling to prevail, considerable amounts of the cosubstrates must undergo the same transformation mechanism. This requirement, however, was not always met. For instance, when 4-CP and catechol were incubated together with laccase, the former was oxidized with the formation of free radicals, whereas the latter formed phenoxide anions and *o*-quinones (Figure 4) (*20*). In addition, catechol was transformed at a much greater rate than 4-CP (Figures 1 and 2), causing competitive inhibition of the transformation of the latter. Because the oxidation products of 4-CP and catechol differed in their oxidized forms and the rates of formation, they were not capable of efficient cross-

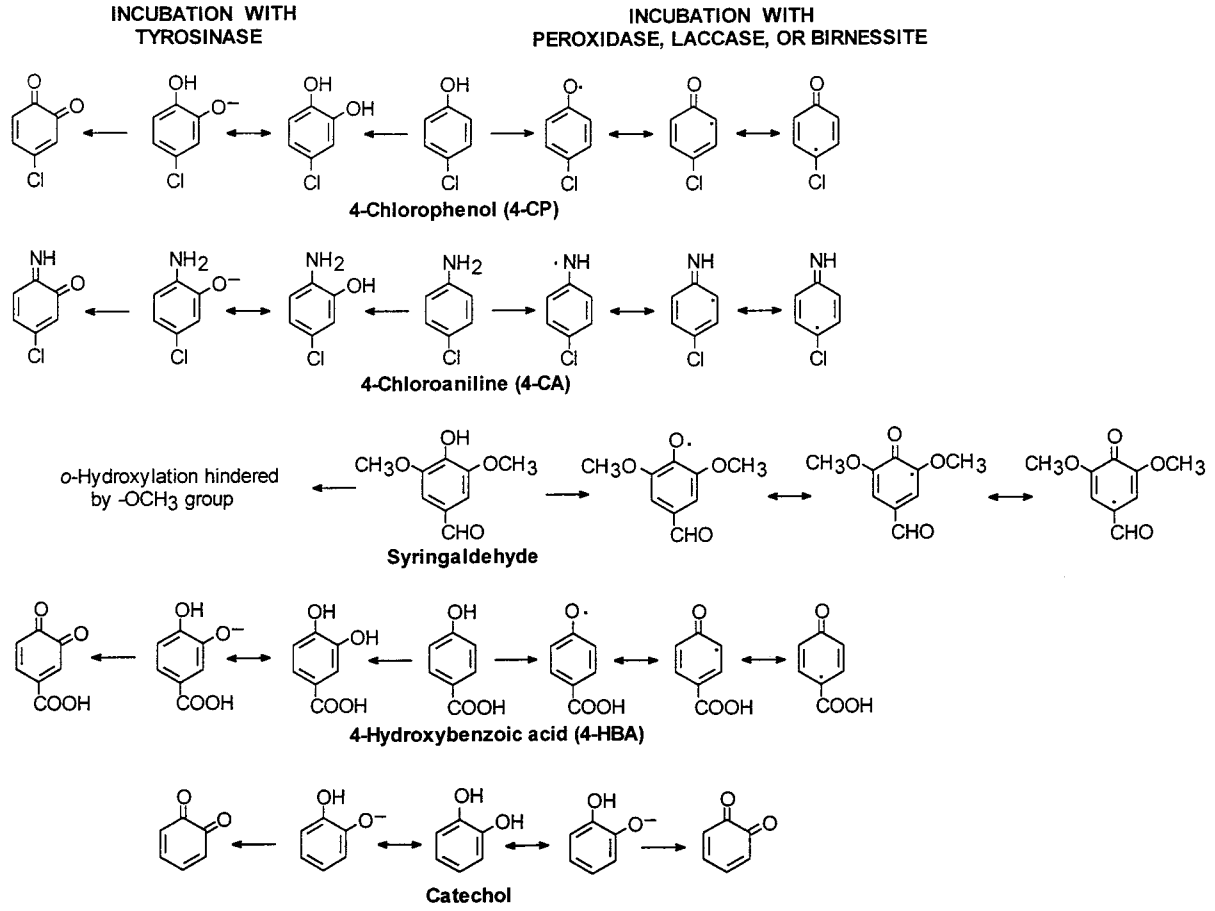

FIGURE 4. Oxidation pathways of chlorinated substrates and humic constituents in the presence of various enzymes and birnessite.

coupling, especially at high concentrations of catechol (Figure 3). Apparently, phenoxide anions from catechol were predominately involved in nucleophilic coupling to *o*-quinones, whereas free radicals originating from 4-CP coupled mostly to each other. The overall result of this incompatibility was the reduced transformation of 4-CP relative to the control (Figures 1 and 3, Table 2). For the same reason, reduced transformation was observed for other chlorophenols incubated with catechol in the presence of laccase, peroxidase, or birnessite. Additionally, minimal effect or reduced transformation of 4-CP was determined for hydroquinone, phloroglucinol, gallic acid, protocatechuic acid, and caffeic acid that, like catechol, have two or more hydroxyl groups and are rapidly transformed (Table 1).

In contrast, during the laccase-mediated reaction of 4-CP with syringaldehyde as a cosubstrate, both compounds were oxidized to free radicals (Figure 4). According to Dordick et al. (*24*) and Fossey et al. (*25*), free radicals can propagate through radical transfer. Apparently, the relatively slow formation of 4-CP free radicals was enhanced by radical transfer from the rapidly formed free radicals of syringaldehyde. Since the respective oxidation products represented the same oxidized form, they could readily couple to each other. As a result of this cross-coupling, the rate of 4-CP transformation increased from 34% (incubation of 4-CP without syringaldehyde) to 100% (incubation of 4-CP with syringaldehyde) (Figures 1 and 3). Similarly, enhanced transformation was observed for other chlorophenols incubated with syringaldehyde in combination with laccase, peroxidase, or birnessite (Table 2).

With tyrosinase, catechol proved to be more effective than syringaldehyde in enhancing the transformation of chlorophenols (Table 2). This outcome can again be attributed to differences in the reaction mechanisms. Laccase, peroxidase, and birnessite all oxidize chlorophenols to free radicals (*10*, *16*). With tyrosinase, chlorophenols first undergo ortho-hydroxylation and then are further oxidized to the respective *o*-quinones (Figure 4) (*16*, *21*). Catechol in the presence of tyrosinase is directly oxidized to *o*-quinone. Since, in the present study, both components of the reaction mixture assumed the same oxidized form, they were capable of efficient cross-coupling (through nucleophilic substitution), thereby enhancing the transformation rate of the chlorophenols. In the case of syringaldehyde, the tyrosinase-mediated ortho-hydroxylation and *o*-quinone formation were apparently hindered by the presence of the two methoxy groups at the *o*-positions, and consequently, the transformation of most chlorophenols was either reduced or unaffected (Table 2).

Contrary to the observations made for chlorophenols, transformation of chloroanilines was greatly enhanced by catechol in the presence of peroxidase, laccase, or birnessite (Figure 1, Table 3). Previous research has demonstrated that chlorinated anilines are nucleophiles and can undergo nucleophilic addition to quinone components and carbonyl groups of humic substances even in the absence of enzymes or birnessite (*5*−*8*). In experiments with laccase, peroxidase, or birnessite, chloroanilines apparently underwent nucleophilic addition to *o*-quinone molecules and quinone oligomers resulting from the oxidation and polymerization of catechol. As indicated in Figure 4, catechol was oxidized to *o*-quinone and formed quinone oligomers regardless of the oxidizing agent used; therefore, enhanced transformation was observed in the presence of both tyrosinase and other mediating agents. On the other hand, a reduction was found in the laccase-mediated transformation of catechol in the presence of 4-CA (Figure 2), because the phenoxide anion generated from catechol had to compete with 4-CA for

o-quinone. This observation supports the notion that 4-CA undergoes cross-coupling to *o*-quinone generated from catechol.

With some exceptions, either insignificant or minor enhancements of chloroaniline transformation were observed in reactions involving syringaldehyde and 4-HBA (Table 3). In general, anilines are less reactive than phenols when incubated alone with oxidoreductases. Berry and Boyd (*22*) determined that the rate constants for the peroxidase-mediated transformation of chloroanilines were lower at least by 1 order of magnitude than those determined for the respective chlorophenols. Apparently fewer free radicals per enzyme unit were generated during incubation with chloroanilines than with chlorophenols. In mixed reactions, humic constituents, which were oxidized to free radicals much faster than chlorinated anilines, seemed primarily to undergo coupling to themselves with limited cross-coupling to the sparse chloroaniline free radicals (*9*, *23*). On the other hand, unlike chlorophenols, the unoxidized chloroanilines are known to undergo nucleophilic addition to quinone oligomers originating from the free radical coupling of humic cosubstrates (*9*). For that reason, in some cases the transformation of chloroanilines in the presence of syringaldehyde (e.g., 4-CA incubated with laccase) and 4-HBA (e.g., 3-CA incubated with horseradish peroxidase) was considerably enhanced despite the slow production of chloroaniline free radicals (Figures 1 and 3, Table 2).

4-HBA incubated with laccase was transformed to a limited extent (Figure 2); therefore, only negligible amounts of the unoxidized 4-CA could undergo nucleophilic addition to quinone oligomers originating from this humic constituent. Little enhancement was also observed for the transformation of 4-CP. As in the case of chlorophenols, the enhancement of the tyrosinase-mediated transformation of chloroanilines in the presence of syringaldehyde was negligible (reduced transformation was even observed for 4-CA) due to the presence of the two methoxy groups at the *o*-position of the cosubstrate molecule, which interfered with ortho-hydroxylation. Negligible enhancement of chloroaniline transformation (and a reduction in the case of 2,4-DCA) was also observed for peroxidase combined with syringaldehyde, apparently due to the competitive transformation of the latter. Slightly higher enhancement was observed with the less reactive and, therefore, not competitive 4-HBA (Table 3).

To summarize, the outcome of the two-substrate reaction can be predicted in many cases on the basis of knowledge regarding the mechanism of transformation. The enhancement of chloroaniline and chlorophenol transformation by humic constituents is only possible if the mechanism is common to both cosubstrates, whether it involves nucleophilic addition or free radical coupling. Frequent instances of no enhancement or even reduced transformation despite the same mechanism of coupling appear to indicate the involvement of additional controlling factors (e.g., steric hindrance or competitive transformation) rather than a contradiction of the general principle.

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