furacarb in these plants is summarized by the scheme given in Figure 2. The results clearly indicated N–S bond cleavage as the initial step in the metabolic pathway, giving rise to carbofuran as the first major metabolite. Carbofuran then was further metabolized to 3-OH-CF which, in turn, was conjugated. Overall, carbofuran and 3-OH-CF were the major metabolites, with carbofuran being more significant in the early period after treatment and 3-OH-CF becoming more important in the later periods. These results are in good agreement with those obtained in the metabolism study of [*carbonyl*-^{14}C]benfuracarb in the cotton plant (Umetsu et al., 1985). Except for the identification of 3-C=O-P as one of the major metabolites in the corn plant after stem injection, all other metabolites were of minor significance. Therefore, N–S bond cleavage, oxidation, hydrolysis, and conjugation remain the principal reactions in the metabolism of benfuracarb in the bush bean, cotton, and corn plants.

In the previous study on the metabolism of benfuracarb in the cotton plant following foliar application of [*carbonyl*-^{14}C]benfuracarb (Umetsu et al., 1985), the metabolic fate of the ring moiety following ester hydrolysis of carbamate linkage remained unknown. The present study clearly reveals CF-P, 3-OH-P, and 3-C=O-P, all in the form of conjugates, as the metabolites resulting from hydrolysis of the carbamate moiety.

It is noteworthy that 3-OH-benfuracarb and 3-C=O-benfuracarb, metabolites in which the N-substituted β-alanine ethyl ester moiety remained attached to the carbamate moiety through the sulfur bridge, were detected in significant amounts. *N*-(2-Toluenesulfenyl)-3-ketocarbofuran was previously reported to be a metabolite of *N*-(2-toluenesulfenyl)carbofuran (Black et al., 1973).

Registry No. CF-S_2-N(ip)(EP), 98859-42-8; CF-SO_2-N(ip)(EP), 98874-78-3; CF-S_2-CF, 39995-74-9; 3-OH-benfuracarb, 98859-43-9; 3-C=O-benfuracarb, 98859-44-0; CF, 1563-66-2; 3-OH-CF, 16655-82-6; 3-C=O-CF, 16709-30-1; *N*-CH_2OH-CF, 18999-70-7; 3-OH-*N*-CH_2OH-CF, 17781-14-5; 3-C=O-*N*-CH_2OH-CF, 19019-30-8; NH_2-CF, 4790-87-8; 3-OH-NH_2-CF, 98874-79-4; 3-C=O-NH_2-CF, 98859-45-1; CF-P, 1563-38-8; 3-OH-P, 17781-15-6; 3-C=O-P, 17781-16-7; EtOC(O)$(CH_2)_2$N(*i*-Pr)SCl, 83129-89-9; (EtOC(O)$(CH_2)_2$N(*i*-Pr)S)$_2$, 95255-55-3; benfuracarb, 82560-54-1.

LITERATURE CITED

Black A. L.; Chiu, Y. C.; Fukuto, T. R.; Miller, T. A. *Pestic. Biochem. Physiol.* **1973**, *3*, 435.
Umetsu, N.; Fahmy, M. A. H.; Fukuto, T. R. *Pestic. Biochem. Physiol.* **1979**, *10*, 104.
Umetsu, N.; Kuwano, E.; Fukuto, T. R. *J. Environ. Sci. Health, Part B* **1980**, *B15* (1), 1.
Umetsu, N.; Nishioka, T.; Fukuto, T. R. *J. Agric. Food Chem.* **1981a**, *29*, 711.
Umetsu, N.; Nishioka, T.; Fukuto, T. R. *J. Agric. Food Chem.* **1981b**, *29*, 1280.
Umetsu, N.; Tanaka, A. K.; Fukuto, T. R. *J. Pestic. Sci.*, in press.

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Mineralization of Chloroaniline/Lignin Conjugates and of Free Chloroanilines by the White Rot Fungus *Phanerochaete chrysosporium*

Masood Arjmand and Heinrich Sandermann, Jr.*

Lignin conjugates of [ring-U-^{14}C]-4-chloroaniline and [ring-U-^{14}C]-3,4-dichloroaniline were prepared enzymatically and characterized by gel permeation chromatography and chemical degradation. These samples, as well as the free [ring-U-^{14}C]chloroanilines and a control lignin made from [ring-U-^{14}C]coniferyl alcohol, were fermented with the white rot fungus *Phanerochaete chrysosporium*. The chloroaniline/lignin conjugates were mineralized to carbon dioxide to the same extent as the control lignin, about 60% of the initial radioactivity being trapped as [^{14}C]CO_2 after 30 days. The free [ring-U-^{14}C]-4-chloroaniline and -3,4-dichloroaniline were mineralized to 35% and 50%, respectively. Several fungal chloroaniline-derived metabolites were formed, but the free chloroanilines or their azo and azoxy derivatives could not be detected. It is concluded that lignin incorporation, followed by fungal oxidation, represents a pathway for the complete removal of chloroanilines from the environment.

INTRODUCTION

Chlorinated anilines are widely applied in agriculture as components of acylanilide, *N*-phenylcarbamate, and *N*-phenylurea herbicides. Plant metabolic studies have shown that usually the free chloroanilines are released as primary herbicide metabolites, followed predominantly by incorporation into the plant "insoluble" residue fraction. Lignin has been proposed as a primary binding site in the plant "insoluble" residue on the basis of solubilization and degradation experiments (Balba et al., 1979; Still, 1968; Still et al., 1976; Still et al., 1981; Sutherland, 1976; Yih et al., 1968). Chloroaniline/lignin conjugates have also been prepared enzymatically (Balba et al., 1979; Still et al., 1981; von der Trenck et al., 1981), and the nucleophilic addition of the chloroanilines to the side chain α-carbon atom of lignol quinone methide intermediates has been identified as a major chemical mechanism of copolymerization (Still et al., 1981; von der Trenck et al., 1981). NMR spectral evidence indicated that incorporation via aromatic linkages also occurred (von der Trenck et al., 1981).

In spite of the high amounts of "insoluble" chloroaniline plant residues usually formed, knowledge of their bioavailability is extremely limited. The bioavailability of the total "insoluble" residue formed in rice from propanil labeled in the 3,4-dichloroaniline moiety has been studied in rats and dogs (Sutherland, 1976). Most (76%) of the radioactivity appeared in feces. A small amount (2.4%) of the initial "insoluble" residue appeared in urine and had apparently become bioavailable. The chemical nature of the excreted radioactivities and the fate of the nonrecovered radioactivity (21.5%) were not reported. Lignin itself

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is thought to pass undigested through the intestinal tract of animals, although steer, millipede, and termites can apparently modify lignin structure (Crawford, 1981; Zeikus, 1981). In contrast, certain microorganisms, in particular white rot fungi, are known to completely mineralize lignin. This process plays an important role in the biological carbon cycle (Crawford, 1981; Zeikus, 1981). The fate of lignin-incorporated xenobiotics during fungal lignin turnover has apparently so far not been studied although it seems important to clarify whether lignin can act as an accumulation site or a true metabolic sink for environmental chemicals (Sandermann et al., 1983).

The free chloroanilines are known to be but slowly mineralized by soil fungi and bacteria, toxic azo and azoxy derivatives and other condensation products as well as polymers being preferentially formed (Bartha et al., 1983; Cripps and Roberts, 1978; Fishbein, 1984).

The purpose of the present study was to analyze the fate of lignin-incorporated chloroanilines during lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. The free chloroanilines and unmodified lignin were studied for comparison.

EXPERIMENTAL SECTION

Materials and General Methods. [Ring-U-^{14}C]coniferyl alcohol was synthesized from [ring-U-^{14}C]vanilline (Amersham-Buchler, Braunschweig) by a published procedure (Freudenberg and Hübner, 1952) with only one modification. Instead of distilling the feruloyl ethyl ester, it was purified on a silica gel 60 (Merck, Darmstadt) column, using petroleum ether (40–60 °C)/diethyl ether (3:1, v/v) as eluting solvent (Kirk et al., 1975). The yield of [ring-U-^{14}C]coniferyl alcohol was 32%, and the product was ≥98% pure, as shown by TLC, HPLC, and GLC. The electron-impact mass spectrum of the acetyl derivative showed the following major fragments: m/z 264, 222, 179, 163, 159, 147, 137, 124, 119, 103, 91. The spectrum was identical with that of the authentic compound, the fragment at m/z 264 corresponding to the molecular ion.

[Ring-U-^{14}C]-4-chloroaniline and [ring-U-^{14}C]-3,4-dichloroaniline were purchased from Pathfinder Laboratories Inc., St. Louis, MO. These compounds were ≥98% pure by TLC.

TLC was carried out on precoated silica gel G plates (Merck, Darmstadt, No. 5554) in the following solvent systems: A, benzene/dioxane/acetic acid (90:25:4, v/v/v); B, *n*-hexane/benzene/acetone (7:3:1, v/v/v). HPLC was carried out on a 4.6 × 250 mm ODS column (5 μm) with isocratic elution by acetonitrile/water (2:3, v/v) (1.5 mL/min). GC was carried out as previously described (Arjmand and Sandermann, 1985) with temperature program (iii) starting at 100 °C. Radioactivity was determined by the previous methods (Arjmand and Sandermann, 1985), lignin samples being counted in a dioxane-based scintillation fluid (Bray, 1960).

Preparation of Lignin Samples. Reference [Ring-U-^{14}C]-Labeled Lignin. [Ring-U-^{14}C]coniferyl alcohol was polymerized with a final yield of 78% by a published procedure (Kirk et al., 1975). The specific radioactivity was 4.8 × 10^4 dpm/mg of polymer.

[Ring-U-^{14}C]-4-chloroaniline/Lignin Conjugate. Nonradioactive coniferyl alcohol was polymerized in the presence of [ring-U-^{14}C]-4-chloroaniline as described (von der Trenck et al., 1981). The incorporation of the aniline was 10% (w/w), and the specific radioactivity was 1.4 × 10^5 dpm/mg of polymer.

[Ring-U-^{14}C]-3,4-dichloroaniline/Lignin Conjugate. Nonradioactive coniferyl alcohol was polymerized in the presence of [ring-U-^{14}C]-3,4-dichloroaniline as described (von der Trenck et al., 1981). The incorporation of the aniline was 41% (w/w), and the specific radioactivity was 1.5 × 10^5 dpm/mg of polymer.

Growth of Fungus. The white rot fungus *P. chrysosporium* Burds. (ATCC 24725) was obtained from Professor A. Fiechter, ETH Zürich, Zürich, Switzerland. The growth conditions used were adopted from the literature (Kirk et al., 1975, 1978; Ulmer et al., 1983). The fungus was maintained at room temperature on 2% (w/v) malt agar slants. Aqueous suspensions of fungal conidia were used for inoculation of the liquid growth medium. The basal growth medium described (Kirk et al., 1978) (100 mL) was used in 1-L Fernbach flasks. The basal medium was supplemented with 56 mM D-glucose and 0.6 mM each of NH_4NO_3 and L-asparagine as nitrogen sources, as well as 30 mM 2,2-dimethylsuccinate, with pH adjusted to 4.5 by addition of aqueous NaOH. The buffered glucose solution was autoclaved. Basal medium and nitrogen source were filter sterilized (Millex-GS, 0.22-μm filter). The sample to be tested and 1 mL of conidial suspension was added. Aliquots (1 mL) from the complete media were used for determination of radioactivity, and these values served as the basis to calculate percent conversion rates. The Fernbach flasks were fitted with silicon stoppers through which two 6-mm glass tubes were inserted. One of these tubes extended into the flask to about 1 cm above the surface of the culture fluid. On the other side, the tube was connected via silicon tubing to a sterilizing filter that consisted of a Pasteur pipet filled with glass wool. The second glass tube extended about 4 cm into the Fernbach flask and ended in the shape of a funnel. The latter was filled with a polyurethane foam stopper (2-cm diameter, 3-cm height) for trapping of volatile organic compounds. On the other side, the tube was connected via silicon tubing to another glass tube that was inserted into a 20-mL glass vial containing 10 mL of a solution of 20% (v/v) 2-aminoethanol in methanol. Screw clamps on both silicon tubings allowed the flasks to be closed. The flasks were flushed every 3–5 days by opening the clamps and forcing pure oxygen gas through the glass wool filter at a rate of about 100 mL/min for 1 h. The oxygen displaced most of the evolved [^{14}C]CO_2 that was absorbed in the 2-aminoethanol solution. Trapped radioactivity was determined after addition of 10 mL of a Triton X-100 based scintillation fluid (Rotiszint 22, Roth, Karlsruhe) to the 2-aminoethanol solution. The efficiency of scintillation counting was 57% as shown by internal standardization with [^{14}C]toluene. Control experiments indicated that the trapping efficiency of the 2-aminoethanol solution was >98%.

The fungal cultures were incubated without shaking at the optimal growth temperature of 39 °C. The evolution of [^{14}C]CO_2 was followed for about 1 month at which time the fungal growth rate was very low and CO_2 evolution was minimal.

Study of 4-Chloroaniline. [Ring-U-^{14}C]-4-chloroaniline (100 μg in 50 μL of methanol; 4.2 × 10^5 dpm), reference [ring-U-^{14}C]-labeled lignin (3 mg in 30 μL of dimethylformamide), and [ring-U-^{14}C]-4-chloroaniline/lignin conjugate (3 mg in 30 μL of dimethylformamide) were separately added to each of five culture flasks. One flask in each series containing all components except for fungal inoculum was used as a control. All flasks were for 27 or 33 days incubated under the usual conditions of growth.

Study of 3,4-Dichloroaniline. [Ring-U-^{14}C]-3,4-dichloroaniline (100 μg in 50 μL of methanol; 5.4 × 10^5 dpm), reference [ring-U-^{14}C]-labeled lignin (3 mg in 30 μL of

dimethylformamide), and [ring-U-^{14}C]-3,4-dichloroaniline/lignin conjugate (3 mg in 30 μL of dimethylformamide) were studied as described above for 4-chloroaniline.

Workup Procedure. At the end of each experiment, the fungal mycelia were isolated by vacuum filtration. Aliquots (1 mL) of the culture filtrates were used for determination of radioactivity. The mycelia from each flask were homogenized in 50 mL of 80% (v/v) aqueous acetone by means of a high-speed homogenizer (Ultraturrax Model TP 18/2N, Janke-Kunkel, Staufen). The mycelial residues were removed by filtration. Aliquots (1 mL) of the filtrates were used for determination of radioactivity. Radioactivity of the mycelial residues was determined after lyophilization and combustion. Acetone was largely removed from the filtrates by use of a rotary evaporator at <40 °C. The resulting solution with always less than 6% of the initial radioactivity was combined with the initial culture filtrate. This was followed by extraction with two equal volumes of ethyl acetate. Radioactivity of the aqueous and organic phases was determined, followed by TLC analysis and further metabolite purification by HPLC and GLC.

The polyurethane foam plugs were extracted with 20 mL of neat acetone, and 1-mL aliquots were used for determination of radioactivity. Control experiments indicated that acetone completely eluted adsorbed radioactivity.

Chemical Degradation Reactions. Acid Hydrolysis [after You et al., 1982]. Sulfuric acid [70% (w/v), 250 μL] was added to 1 mg of the lignin sample in a glass vial that was then sealed and kept for 1 h at 100 °C. The sample was neutralized with 30% (w/v) NaOH, and water was added to give a volume of 3 mL. The solution was extracted with a 3-mL portion of ethyl acetate, and the organic extract was analyzed by TLC in solvent systems A and B.

Alkaline Hydrolysis [after You et al., 1982]. Sodium hydroxide [50% (w/v), 250 μL] was added to 1 mg of the lignin sample in a glass vial that was then sealed and kept for 1 h at 100 °C. Water was added to give a volume of 3 mL. The solution was extracted with a 3-mL portion of ethyl acetate, and the organic extract was analyzed by TLC in solvent systems A and B.

2-Aminoethanol Treatment [after Noodén, 1970]. Neat 2-aminoethanol (250 μL) was added to 1 mg of the lignin sample in a glass vial that was then sealed and kept at 190 °C for 2 h. Water was added to give a volume of 3 mL. The solution was extracted with a 3-mL portion of ethyl acetate, and the organic extract was analyzed by TLC in solvent systems A and B.

RESULTS

Characterization of Chloroaniline/Lignin Conjugates. The synthetic [ring-U-^{14}C]chloroaniline/lignin conjugates were chromatographed on a calibrated column of Sephadex LH-60 in dimethylformamide (Figure 1). Most of the applied material appeared at a molecular weight of about 1100, but there were also molecular weight species of up to 20000. Residual free [ring-U-^{14}C]chloroanilines or low molecular weight transformation products were not detected. As previously reported (von der Trenck et al., 1981), rechromatography of individual eluted fractions led to defined peaks at the expected elution positions. The ultraviolet absorption of the lignin species and the incorporated radioactivity showed parallel elution curves over the entire molecular weight range of Figure 1. The results therefore ruled out the occurrence of inclusion compounds and rather indicated that true covalent binding had occurred by some random mechanism [cf. Sandermann et al., 1983].

Horseradish peroxidase is known to transform 4-chloroaniline (Daniels and Saunders, 1953). It appeared therefore important to examine the content of unchanged chloroanilines in the lignin conjugates. For this purpose, three drastic hydrolysis procedures were adopted from the literature (Noodén, 1970; You et al., 1982). The results obtained are summarized in Table I. As was to be expected from the published control experiments (Noodén, 1970; You et al., 1982), the free chloroanilines could be completely recovered in unchanged form after each of the treatments. Up to 74% of the radioactivity of the [ring-U-^{14}C]-4-chloroaniline/lignin conjugate and up to 92% of the radioactivity of the [ring-U-^{14}C]-3,4-dichloroaniline/lignin conjugate were released in the form of the unchanged free chloroanilines.

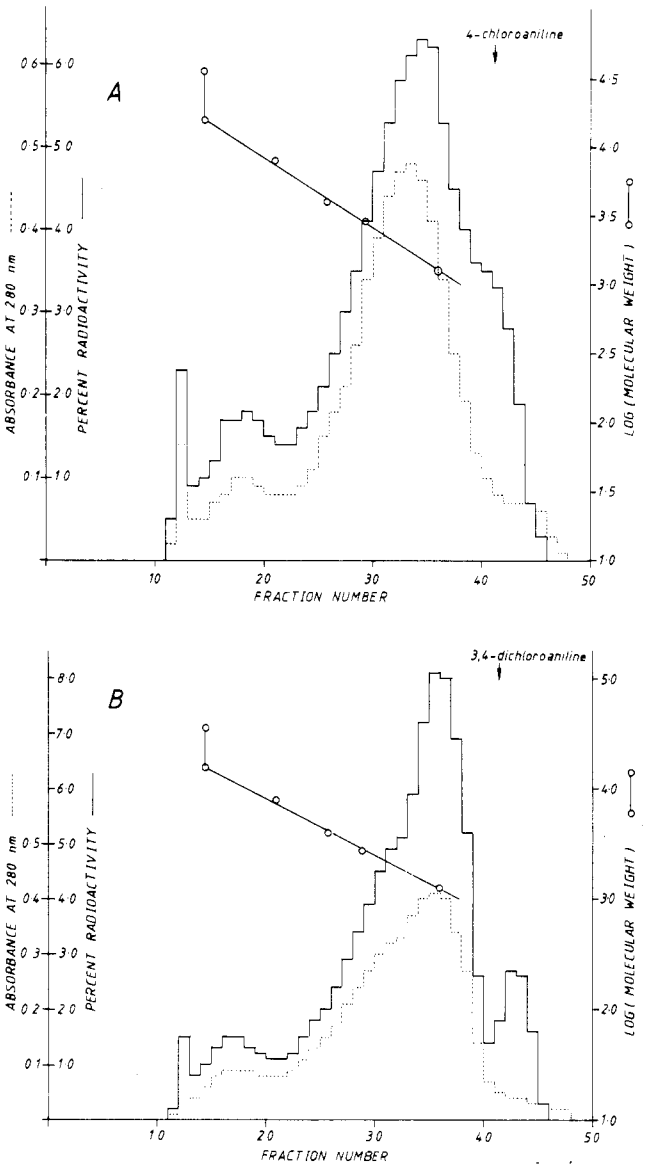

Figure 1. Gel permeation chromatography of chloroaniline/lignin conjugates on a column (2.1 × 100 cm) of Sephadex LH-60: (A) 4-chloroaniline/lignin conjugate; (B) 3,4-dichloroaniline/lignin conjugate. The solvent used was dimethylformamide, and fractions of 7 mL were collected. Radioactivity (—, percent of applied) and UV absorption at 280 nm (- - -) are plotted against fraction number. The elution positions of the free chloroanilines are indicated by arrows. In addition, a calibration curve was determined in independent runs using polystyrene molecular weight markers (○).

Biodegradation of Chloroaniline/Lignin Conjugates. 4-Chloroaniline. Monitoring of [^{14}C]CO_2 showed that there was a rapid mineralization during the first 10 days of incubation, followed by a much slower phase

Table I. Chemical Degradation of the Chloroaniline/Lignin Conjugates (All Samples [Ring-U-^{14}C] Labeled)

sample treatment	radioactivity, % ethyl acetate extracted[a]	nonextracted[b]	recovery, %
acid hydrolysis			
free 4-chloroaniline	107		107
free 3,4-dichloroaniline	109		109
4-chloroaniline/lignin conj	75	17	92
3,4-dichloroaniline/lignin conj	92	10	102
alkaline hydrolysis			
free 4-chloroaniline	112		112
free 3,4-dichloroaniline	98		98
4-chloroaniline/lignin conj	26	72	98
3,4-dichloroaniline/lignin conj	32	62	94
2-aminoethanol treatment			
free 4-chloroaniline	105		105
free 3,4-dichloroaniline	105		105
4-chloroaniline/lignin conj	46	47	93
3,4-dichloroaniline/lignin conj	84	24	108

[a] Upon TLC in solvent systems A and B ≥90% of the material migrated as the free chloroaniline, the remaining material being immobile. [b] Upon TLC in solvent systems A and B all of the material was immobile.

(Figure 2A). About 60% of the applied radioactivity was recovered as [^{14}C]CO_2 during 27 days in the case of the [ring-U-^{14}C]-4-chloroaniline/lignin conjugate as well as of the control lignin made with [ring-U-^{14}C]coniferyl alcohol. Free [ring-U-^{14}C]-4-chloroaniline was during 27 days mineralized to about 35%. No [^{14}C]CO_2 was developed in any of the control incubations where fungal inoculum was omitted.

The total amount of 4-chloroaniline present in the lignin conjugate of Table IIA was 300 μg, which was 3 times as much as used in the form of the free 4-chloroaniline.

After 27 days of incubation, the fungal mycelia were isolated by filtration. Dry-weight determination (Table IIA) showed no reduction caused by 4-chloroaniline. The mycelia were extracted with aqueous acetone as described under the Experimental Section. The "insoluble residues" contained less than 2% of the initial radioactivity (Table IIA). The volatile radioactivity also was only around 2% of the initial radioactivity and was not further studied. The volatile radioactivity of the control incubations was significantly higher (12.5 and 19%, respectively).

The "soluble" fraction of the mycelia and the culture fluid was partitioned between ethyl acetate and water. The

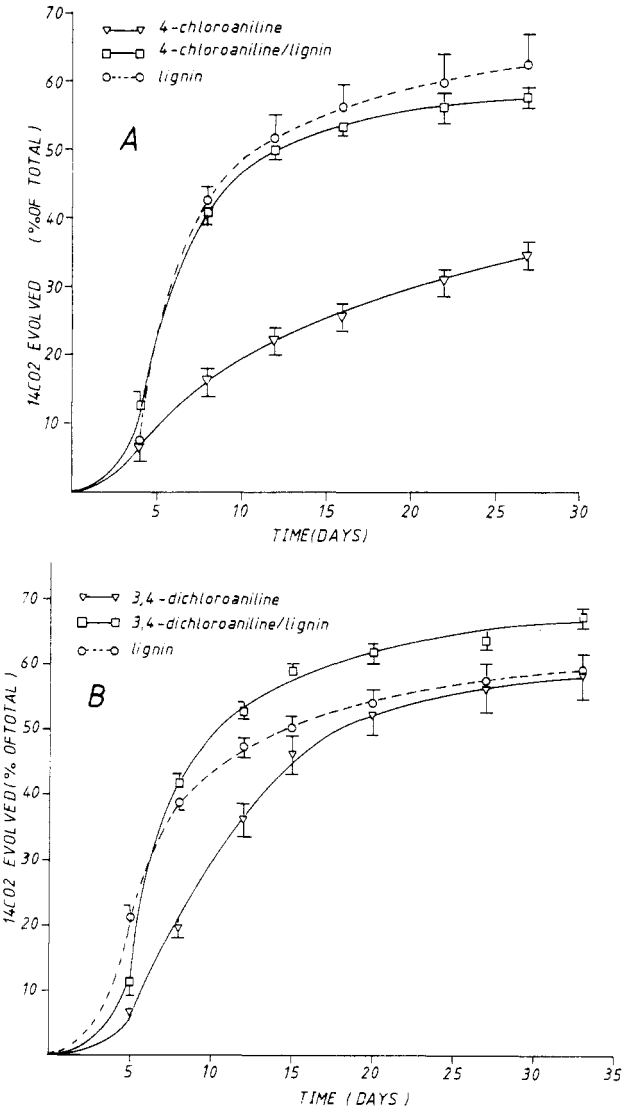

Figure 2. Biodegradation of [ring-U-^{14}C]chloroaniline/lignin conjugates (□), of reference [ring-U-^{14}C] lignin without xenobiotic (○) and of free [ring-U-^{14}C]chloroanilines (▽) by the white rot fungus *Phanerochaete chrysosporium*: (A) experiments with 4-chloroaniline; (B) experiments with 3,4-dichloroaniline. The trapped [^{14}C]CO_2 (cumulative percent of applied radioactivity) is plotted against fermentation time (days). No [^{14}C]CO_2 was developed in control fermentations without fungal inoculum. The standard deviations shown were derived from n = 4 parallel experiments.

Table II. Distributions of Radioactivity in the Fermentations of Chloroaniline/Lignin Conjugates (Percent of Applied Radioactivity ± Standard Deviation, n = 4)

sample	free aniline	control	aniline/lignin conj	control	lignin	control
A. Distributions in Experiments with 4-Chloroaniline						
CO_2 (cf. Figure 2A)	34.5 ± 2.0		57.8 ± 1.2		62.2 ± 4.6	
volatiles	1.6 ± 0.3	19.0	2.4 ± 0.9	12.5		
mycelial res	0.9 ± 0.1		1.8 ± 0.2		1.8 ± 0.3	
ethyl acetate phase	61.9 ± 2.4	72.3	19.7 ± 0.6	92.6	6.8 ± 1.4	75.2
aq phase	6.9 ± 0.2	6.5	18.8 ± 1.9		34.0 ± 4.2	21.4
total [^{14}C] recovered	105.4 ± 4.3	97.8	100.4 ± 2.6	105.1	104.8 ± 5.2	96.6
mycelial dry wt, mg	76.3 ± 9.6		92.8 ± 8.2		83.8 ± 8.7	
B. Distributions in Experiments with 3,4-Dichloroaniline						
CO_2 (cf. Figure 2B)	57.8 ± 3.4		64.1 ± 1.3		61.0 ± 2.2	
volatiles	3.6 ± 0.5	47.4	1.4 ± 0.1	13.6		
mycelial res	0.6 ± 0.1		0.4 ± 0.1		3.4 ± 0.8	
ethyl acetate phase	27.7 ± 1.9	46.6	17.9 ± 0.2	87.9	9.2 ± 1.4	72.4
aq phase	9.2 ± 0.5	4.3	11.8 ± 0.4		27.3 ± 2.8	24.6
total [^{14}C] recovered	98.9 ± 4.1	98.3	95.6 ± 0.9	101.5	100.9 ± 4.3	97.0
mycelial dry wt, mg	46.0 ± 2.6		48.5 ± 9.8		89.3 ± 7.1	

amounts of radioactivity in both phases are listed in Table IIA. The water-soluble material was immobile upon TLC in solvent systems A and B and was not further studied. The TLC profiles of the ethyl acetate soluble fractions are shown in Figure 3A. Free 4-chloroaniline could not even in the control incubations be recovered. Transformation products migrating in the area of potentially toxic azo derivatives (Poland and Knutson, 1982) occurred only in the control incubations where other transformation products were also detected (Figure 3A). A single major transformation product (R_f 0.60) was formed from both free [ring-U-^{14}C]-4-chloroaniline and its lignin conjugate. The R_f value of free 4-chloroaniline in solvent system A was 0.76. In solvent system B, the R_f values of the metabolite and of free 4-chloroaniline were 0.10 and 0.24, respectively. Upon HPLC, the metabolite had a retention time of 5.7 min, compared to 6 min for free 4-chloroaniline. The GLC retention times were 10.4 min for the metabolite and 6.9 min for 4-chloroacetanilide.

The chemical structure of the novel metabolite remains to be elucidated.

3,4-Dichloroaniline. Monitoring of [^{14}C]CO_2 showed again that there was a rapid mineralization during the first 10 days of incubation, followed by a much slower release (Figure 2B). Between 55 and 65% of the applied radioactivity was recovered as [^{14}C]CO_2 after 33 days of incubation of the three samples studied. The total amount of 3,4-dichloroaniline contained in the lignin conjugate sample was 1.2 mg, which was 12 times more than used in the experiments with the free 3,4-dichloroaniline. In comparison with Figure 2A, free 3,4-dichloroaniline was a better substrate for mineralization than free 4-chloroaniline. No [^{14}C]CO_2 was developed in any of the control incubations where fungal inoculum was omitted. The dry-weight results of Table IIB indicated that 3,4-dichloroaniline was somewhat growth inhibitory.

Volatile products amounted to 3.6% for free 3,4-dichloroaniline and 1.4% for its lignin conjugate. The volatilization rates of the control incubations were again significantly higher and amounted to 47.4 and 13.6%, respectively. The "insoluble residue" of the mycelial extractions contained less than 1% of the initial radioactivity.

Partitioning between ethyl acetate and water led to metabolite fractions whose amounts are listed in Table IIB. The water-soluble material was immobile upon TLC in solvent systems A and B and was not further characterized. The TLC profiles of the ethyl acetate soluble fractions are shown in Figure 3B. Free 3,4-dichloroaniline could not even in the control incubations be detected. Transformation products migrating in the area of azo derivatives were only detected in the control fermentations. A major transformation product at R_f 0.65 appeared to correspond to the R_f 0.60 product formed from 4-chloroaniline (see above). Free 3,4-dichloroaniline had an R_f value of 0.76 in solvent system A. In solvent system B, the metabolite had R_f 0.11, and free 3,4-dichloroaniline had R_f 0.25. There were additional metabolite peaks, but these products have so far not been further studied.

As in the case of the 4-chloroaniline experiments, the metabolite peaks observed were the same in incubations employing the free chloroanilines or the corresponding lignin conjugates. The multiple transformation products formed in the parallel control incubations were clearly different on the basis of the TLC profiles of Figure 3.

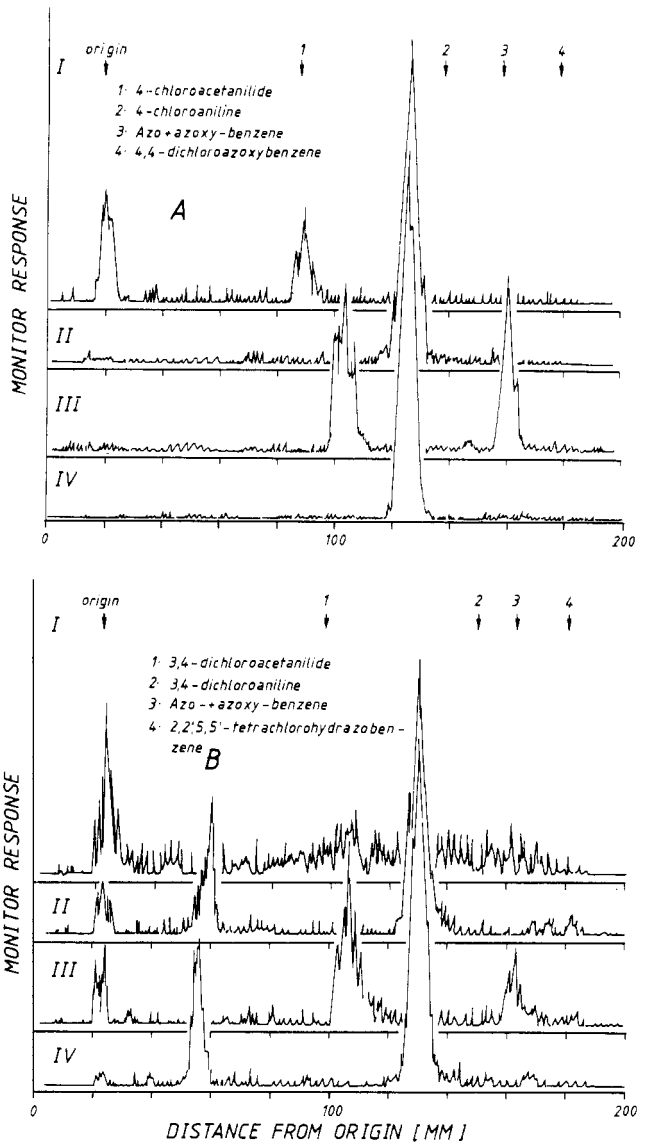

Figure 3. Thin-layer chromatography of the ethyl acetate extracts of Table II in solvent system A. The distributions of radioactivity shown were determined with a Berthold Model LB 2842 automatic TLC linear analyzer system. (A) Ethyl acetate extracts from the experiments with 4-chloroaniline: (I) 4-chloroaniline/lignin conjugate, control without fungal inoculum; (II) 4-chloroaniline/lignin conjugate; (III) free 4-chloraniline, control without fungal inoculum; (IV) free 4-chloroaniline. The positions of the following reference compounds are indicated by arrows: (1) 4-chloroacetanilide; (2) 4-chloroaniline; (3) azo- and azoxybenzene; (4) 4,4'-dichloroazoxybenzene. (B) Ethyl acetate extracts from the experiments with 3,4-dichloroaniline: (I) 3,4-dichloroaniline/lignin conjugate, control without fungal inoculum; (II) 3,4-dichloroaniline/lignin conjugate; (III) free 3,4-dichloroaniline, control without fungal inoculum; (IV) free 3,4-dichloroaniline. The positions of the following reference compounds are indicated by arrows: (1) 3,4-dichloroacetanilide; (2) 3,4-dichloroaniline; (3) azo- and azoxybenzene; (4) 2,2',5,5'-tetrachlorohydrazobenzene.

DISCUSSION

Characterization of Chloroaniline/Lignin Conjugates. The gel permeation profiles of the present chloroaniline/lignin conjugates (Figure 1) were in agreement with the previously reported molecular weight distributions (von der Trenck et al., 1981). Chloroaniline/lignin conjugates isolated from plant sources (Balba et al., 1979; Still et al., 1976; 1981; Yih et al., 1968) have not been characterized by gel permeation chromatography, but the plant lignin conjugates formed from 2,4-D (Scheel and Sandermann, 1981) and pentachlorophenol (Scheel et al., 1984) had elution profiles resembling those of Figure 1. An acetylated model chloroaniline/lignin conjugate has previously been fractionated on a Micro-Styragel gel permeation column, but no calibration of the column or mo-

lecular weight values of the lignin species were reported. It was even left open whether noncovalent chloroaniline/lignin inclusion compounds were present (Still et al., 1981). The present and previous (von der Trenck et al., 1981) gel permeation results establish that the chloroanilines were covalently incorporated by some random mechanism.

Three drastic hydrolysis procedures were applied in order to examine how much unchanged chloroaniline could be liberated from the lignin conjugates. 2-Aminoethanol is a known lignin solubilizing agent (Allan, 1971) and has been shown not to modify maleic hydrazide under drastic hydrolysis conditions (Noodén, 1970). The strong acid and alkali hydrolysis procedures used have previously been shown not to modify the parent chloroanilines (You et al., 1982). These procedures led to up to 92% release of unchanged chloroaniline from the lignin conjugates. High yields of unchanged free chloroanilines have previously been obtained by pyrolysis of chloroaniline/lignin conjugates (Balba et al., 1979; Still et al., 1981), but the hydrolysis procedures are much easier to carry out.

Biodegradation of Chloroaniline/Lignin Conjugates. The chloroaniline/lignin conjugates appeared to be fully bioavailable to the white rot fungus since the incorporated chloroanilines were as readily mineralized as lignin itself. Furthermore, the mineralization rates of the free chloroanilines were much higher than the rates previously reported for other microorganisms (Bartha et al., 1983; Cripps and Roberts, 1978; Fishbein, 1984). *P. chrysosporium* may therefore possess an unusually high and perhaps rather general capacity for the mineralization of recalcitrant xenobiotics. Recent preliminary results indicate that [^{14}C]chloroaniline/lignin conjugates isolated from wheat straw are extensively mineralized by *P. chrysosporium*. The organism has furthermore been found to mineralize lignin sulfonic acids (Haider and Trojanowski, 1981; Kern, 1983; Lundquist et al., 1977), a polyguaiacol polymer (Crawford et al., 1981) and polymeric dyes (Glenn and Gold, 1983; Haars et al., 1982).

There was a possibility that under the oxidative conditions of lignin degradation by *P. chrysosporium* the oxidative conversion of the chloroanilines to the potentially toxic azo and azoxy derivatives (Poland and Knutson, 1982) could also occur. However, no evidence for the generation of such compounds was detected in the fungal cultures although the control incubations contained some unidentified transformation products with the TLC mobility of azo derivatives.

At this point one may conclude that lignin incorporation of chlorinated anilines followed by fungal lignin degradation represents a pathway for the final removal of the anilines from the environment. It remains to be studied whether this also applies to other xenobiotics.

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Registry No. *p*-$ClC_6H_4NH_2$, 106-47-8; CO_2, 124-38-9; 3,4-dichloroaniline, 95-76-1; lignin, 9005-53-2; 4-chloroaniline–lignin conjugate, 98541-31-2; 3,4-dichloroaniline–lignin conjugate, 77716-53-1.

LITERATURE CITED

Allan, G. G. In "Lignins. Occurrence, Formation, Structure and Reactions"; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley: New York, 1971; pp 511–573.

Arjmand, M.; Sandermann, H. *Pesticide Biochem. Physiol.* **1985**, *23*, 389–397.

Balba, H. M.; Still, G. G.; Mansager, E. R. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 237–240.

Bartha, R.; You, I.-S.; Saxena, A. In "Pesticide Chemistry. Human Welfare and the Environment"; Miyamoto, J., Kearney, P. C., Eds.; Pergamon Press: Oxford, New York, 1983; Vol. 3, pp 345–350.

Bray, G. A. *Anal. Biochem.* **1960**, *1*, 279–285.

Crawford, R. L. "Lignin Biodegradation and Transformation"; Wiley: New York, 1981.

Crawford, R. L.; Robinson, L. E.; Foster, R. D. *Appl. Environ. Microbiol.* **1981**, *41*, 1112–1116.

Cripps, R. E.; Roberts, T. R. In "Pesticide Microbiology"; Hill, I. R., Wright, S. J. L., Eds.; Academic Press: New York, 1978; pp 669–730.

Daniels, D. G. H.; Saunders, B. C. *J. Chem. Soc.* **1953**, 822–826.

Fishbein, L. In "The Handbook of Environmental Chemistry"; Hutzinger, O. Ed.; Springer-Verlag: Berlin, 1984; Vol. 3, pp 1–40.

Freudenberg, K.; Hübner, H. H. *Chem. Ber.* **1952**, *85*, 1181–1191.

Glenn, J. K.; Gold, M. H. *Appl Environ. Microbiol.* **1983**, *45*, 1741–1747.

Haars, A.; Milstein, O.; Lohner, S.; Hüttermann *Holzforsch.* **1982**, *36*, 85–91.

Haider, K.; Trojanowski, J. *Holzforsch.* **1981**, *35*, 33–38.

Kern, H. W. *Holzforsch.* **1983**, *37*, 287–292.

Kirk, T. K.; Connors, W. J.; Bleam, R. D.; Hackett, W. F.; Zeikus, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2515–2519.

Kirk, T. K.; Schultz, E.; Connors, W. J.; Lorenz, L. F.; Zeikus, J. G. *Arch. Mikrobiol.* **1978**, *117*, 277–285.

Lundquist, K.; Kirk, T. K.; Connors, W. J. *Arch. Mikrobiol.* **1977**, *112*, 291–296.

Noodén, L. D. *Plant Physiol.* **1970**, *45*, 46–52.

Poland, A.; Knutson, J. C. *Ann. Rev. Pharmacol. Toxicol.* **1982**, *22*, 517–554.

Sandermann, H.; Scheel, D.; von der Trenck, T. *J. App. Polym. Sci., Appl. Polym. Symp.* **1983**, *37*, 407–420.

Scheel, D.; Sandermann, H. *Planta* **1981**, *152*, 253–258.

Scheel, D.; Schäfer, W.; Sandermann, H. *J. Agric. Food Chem.* **1984**, *32*, 1237–1241.

Still, G. G. *Science (Washington, D.C.)* **1968**, *159*, 992–993.

Still, G. G.; Balba, H. M.; Mansager, E. R. *J. Agric. Food Chem.* **1981**, *29*, 739–746.

Still, G. G.; Norris, F. A.; Iwan, J. In "Bound and Conjugated Pesticide Residues"; Kaufman, D. D., Still, G. G., Paulson, G. D., Bandal, S. K., Eds.; American Chemical Society: Washington, DC, 1976; ACS Symp. Ser. No. 29, pp 156–165.

Sutherland, M. L. In "Bound and Conjugated Pesticide Residues"; Kaufman, D. D., Still, G. G., Paulson, G. D., Bandal, S. K., Eds.; American Chemical Society: Washington, DC, 1976; ACS Symp. Ser. No. 29, pp 153–155.

von der Trenck, K. T.; Hunkler, D.; Sandermann, H. *Z. Naturforsch. C, Biosci.* **1981**, *36C*, 714–720.

Ulmer, D. C.; Leisola, M. S. A.; Schmidt, B. H.; Fiechter, A. *Appl. Environ. Microbiol.* **1983**, *45*, 1795–1801.

Yih, R. Y.; McRae, D. H.; Wilson, H. F. *Science (Washington, D.C.)* **1968**, *161*, 376–377.

You, I.-S.; Jones, R. A.; Bartha, R. *Bull. Environm. Contam. Toxicol.* **1982**, *29*, 476–482.

Zeikus, J. G. In "Advances in Microbial Ecology"; Alexander, M., Ed.; Plenum Press: New York, 1981; Vol. 5, pp 211–243.

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