Plant Biochemistry of Xenobiotics.
Mineralization of Chloroaniline/Lignin Metabolites from Wheat by the White-Rot Fungus, *Phanerochaete chrysosporium*

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The lignin metabolites formed in wheat plants from [ring-u-^{14}C]-4-chloroaniline and -3,4-dichloroaniline were isolated by a Björkman-type procedure and characterized by gel permeation chromatography and chemical degradation. The isolated metabolite fractions were then incubated with the white-rot fungus, *Phanerochaete chrysosporium*.

Mineralization to [^{14}C]carbon dioxide occurred in high yields, about 65% of the initial radioactivity of the lignin-bound chloroanilines being trapped as CO_2 after an incubation period of 32—33 days. The lignin metabolites with the ^{14}C-label in the aromatic ring of the chloroanilines were mineralized as readily as a non-xenobiotic control lignin with a ^{14}C-label in the aromatic ring of coniferyl alcohol.

It is concluded that the white-rot fungus studied has an unusually high capacity for the removal of chloroanilines and possibly other xenobiotic contaminants from the environment.

Introduction

Chlorinated anilines and their derivatives are frequent contaminants of agricultural plants and soils, being introduced mainly as components of acylanilide, N-phenylcarbamate and N-phenylurea herbicides [1—5]. The yearly production figure for one of the component chloroanilines, 4-chloroaniline, is > 500 tons for West Germany alone [6]. The plant metabolism of the herbicide classes mentioned proceeds by enzymatic liberation of the free chloroanilines, followed by oxidation, conjugation or polymerization reactions. The latter reactions usually predominate and lead to high "insoluble" residue fractions [1, 7—15]. Lignin appeared to be a major binding site in these "insoluble" residues [7—14]. However, as previously discussed in detail [14] the chemical characterization of the presumed chloroaniline/lignin metabolites was inadequate. For example, the occurrence of non-covalent lignin inclusion compounds rather than covalent lignin conjugates could not be excluded.

Enzymatically prepared artifical chloroaniline/lignin conjugates have previously been characterized as truly covalent adducts which were formed by copolymerization via radical as well as quinone-methide intermediates [12, 13, 16, 17].

The "insoluble" chloroaniline residues persist over rather long time periods in plants [1, 7—15], lignin itself being a highly persistent natural polymer [18, 19]. However, white-rot fungi are known to catalyze the rapid mineralization of plant lignin, and this degradative reaction is of great importance for the global carbon cycle [18, 19]. We have recently determined that chloroanilines copolymerized into artificial lignin are extensively mineralized by the white-rot fungus, *Phanerochaete chrysosporium* [17]. The free chloroanilines were also well mineralized, so that *Phanerochaete chrysosporium* appeared to have the capacity for the complete removal of chloroaniline contaminants from its environment. In order to further test this conclusion with substrates more similar to real agricultural residues, we have now studied lignin metabolites formed from [ring-u-^{14}C]-chloroanilines in wheat plants. The isolation, characterization and fungal mineralization of these lignin metabolites will be reported.

Materials and Methods

Materials

[Ring-u-^{14}C]-4-chloroaniline (10.24 µCi/µmol) and [ring-u-^{14}C]-3,4-dichloroaniline (6.12 µCi/µmol) were purchased from Pathfinder Laboratories, St. Louis, USA. These compounds were $\geq 98\%$ pure as determined by thin layer chromatography. The preparation of artificial control lignin (specific radioactivity, 4.8×10^4 dpm/mg) from [ring-u-^{14}C]coniferyl alcohol and other materials used here have been previously described [17].

General methods

Thinlayer chromatography was carried out on pre-coated silica gel G plates (Merck No. 5554) using the following solvent systems: A, benzene/dioxane/acetic acid, 90:25:4 (v/v/v); and B, n-hexane/benzene/acetone, 7:3:1 (v/v/v). Radioactivity was determined by the previous methods [17], lignin samples being counted in a dioxane based scintillation fluid [20]. Gel permeation chromatography on Sephadex LH-60 in dimethylformamide was performed as described [17, 21]. Samples of 3 mg were applied in 30 µl dimethylformamide.

Hydrolytic methods

Acid hydrolysis [22], alkaline hydrolysis [22] and treatment with 2-aminoethanol [23] were carried out as described [17].

Isolation of 4-chloroaniline/lignin metabolites

Seeds of wheat (*Triticum aestivum* L., var. Kolibri) were surface-sterilized with 6.5% (w/v) aqueous sodium hypochlorite, and then washed with distilled water. The seeds were germinated in the dark over a period of 48 h. The wheat plants were grown hydroponically at 25 °C under constant white light (9500 lux, Osram L 40 W/25), as previously described [24]. After the formation of mature spikes (about 6 weeks), the plants were removed from the growth medium, the roots were thoroughly washed with destilled water, and 28 plants were placed separately in glass vials (9×1.4 cm), each of which contained 5.12 µCi (1 µmol) 4-chloroaniline in 5 ml hydroponic medium. The plants were placed under the constant light source, and fresh hydroponic medium was added as needed during the incubation. After 8 days, the plants were removed from the vials. The roots were washed with destilled water, and roots plus about 5 cm stalk section (together 6.2 g dry weight) were submitted to a Björkman-type lignin extraction procedure [25, 26]. The cut and dried plant material was ground in liquid nitrogen by means of a mortar and pestle. This was followed by lyophilization and Soxhlet extractions with benzene/ethanol, 2:1 (v/v; 300 ml, 24 h) and ethanol (250 ml, 24 h). The residue was rinsed with 500 ml water that had been pre-warmed to 60 °C, lyophilized and ground under toluene for 5 days at 25 °C in a Fritsch model 601 mill with 1 cm steel balls. After centrifugation ($10,000 \times g$, 10 min), the residue was dried under a stream of nitrogen at 25 °C and was then extracted with three 100-ml portions of dioxane/water, 9:1 (v/v). Sonication with the microtip attachment of a Branson model B 12 sonifier (6 min, 25 °C) was employed to facilitate the extraction. After centrifugation ($10,000 \times g$, 10 min), the dioxane/water extracts were combined and concentrated at < 40 °C to about 5 ml by use of a rotary evaporator. The lignin fraction was precipitated by gradual addition of 10 ml water with constant rapid stirring. The suspension was purged with nitrogen and centrifuged ($20,000 \times g$, 20 min). The final lignin fraction was lyophilized, a dry weight of 76 mg was determined and the material was dissolved in 760 µl dimethylformamide. The specific radioactivity amounted to 1.3×10^5 dpm/mg dry weight.

Isolation of 3,4-dichloroaniline/lignin metabolites

The procedure used was essentially identical to that described above for the 4-chloroaniline/lignin metabolites. Each wheat plant (total, 40 plants) was incubated with 6.12 µCi (1 µmol) [ring-u-^{14}C]-3,4-dichloroaniline. In this case, the washed roots and the combined stalks and leaves were extracted separately. The dry weights after homogenization in liquid nitrogen were 1.3 g (roots) and 6.3 g (stalks and leaves), respectively. The final root-lignin fraction (21 mg dry weight) had a specific radioactivity of 1.3×10^5 dpm/mg dry weight. The final lignin fraction prepared from stalks and leaves had a dry weight of 250 mg and a specific radioactivity of 6.3×10^4 dpm/mg dry weight. Solutions of 1 mg lignin per 10 µl dimethylformamide were used for further study.

Fungal culture

The white-rot fungus, *Phanerochaete chrysosporium* Burds. (ATCC 24725), was obtained from

Professor A. Fiechter, ETH Zürich, Switzerland. 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. All experimental details of fungal culture, of carbon dioxide trapping and of radioactivity determination were as previously reported [17].

Incubation of [ring-u-^{14}C]-4-chloroaniline/lignin metabolites

Aliquots of the lignin metabolite fraction (3 mg in 30 µl dimethylformamide, 3.9×10^5 dpm) were added to each of 4 culture flasks. One additional flask which contained all components except for the fungal inoculum was used as a control. Another additional flask with fungal inoculum received the previous amount [17] of a control lignin made enzymatically from [ring-u-^{14}C]coniferyl alcohol. All flasks were incubated for 32 days.

Incubation of [ring-u-^{14}C]-3,4-dichloroaniline/lignin metabolites

Aliquots of the lignin metabolite fraction isolated from roots (3 mg in 30 µl dimethylformamide, 3.9×10^5 dpm) were added to each of 4 culture flasks. These flasks, as well as one control flask lacking fungal inoculum and two flasks with control lignin (see above) were incubated for 33 days.

Work-up procedure

The previous procedure [17] was followed. In brief, the fungal mycelia were isolated by centrifugation ($10,000 \times g$, 30 min) at the end of each experiment. Aliquots (1 ml) of the supernatants were used for determination of radioactivity. The mycelia from each flask were homogenized in 50 ml 80% (v/v) aqueous acetone. The mycelial residues were isolated by filtration, and their radioactivity was determined after lyophilization and combustion. Aliquots (1 ml) of the filtrates were used for determination of radioactivity. Acetone was largely removed from the filtrates by use of a rotary evaporator at < 40 °C, and the resulting solution was added to the initial culture filtrate. The combined solution was extracted twice with equal volumes of ethylacetate. Radioactivity of the aqueous and organic phases was determined. The ethylacetate phase was concentrated and adsorbed to a silica gel Sep-Pak cartridge (Waters Associates), followed by elution with a stepwise petrolether/diethylether gradient. Fractions containing radioactive material were combined and further analyzed by thinlayer chromatography in solvent systems A and B.

Radioactive material associated with the polyurethane foam plugs of the culture flasks was studied after extraction with 20 ml acetone.

Results

Incorporation of chloroanilines into "insoluble" plant residues

Initial metabolic experiments were carried out with soybean and wheat cell suspension cultures under the published standardized conditions [26, 27]. With 1 ppm of the [ring-u-^{14}C]chloroanilines, the soybean cells incorporated 2.1 and 2.5%, and the wheat cells 65.9 and 30.3% of 4-chloroaniline and 3,4-dichloroaniline, respectively, into the "insoluble" residue fraction (R. Winkler and H. Sandermann, unpublished results). Although the wheat cells formed high amounts of "insoluble" residues, further studies were carried out with intact wheat plants in order to better simulate agricultural conditions. The incorporation rate of [ring-u-^{14}C]-3,4-dichloroaniline into the total "insoluble" residue of roots, stalks and leaves (7.6 g dry weight) of the experiment described under Methods was determined to be 24%. In autoradiographs of the treated wheat plants, most of the radioactivity appeared to be associated with the roots, although radioactivity was present throughout the plant including the spikes (data not shown). As described in detail under Methods, "insoluble" wheat plant residues labeled with [ring-u-^{14}C]-4-chloroaniline or [ring-u-^{14}C]-3,4-dichloroaniline were sequentially extracted by a Björkman-type procedure. This led to solubilized lignin metabolite fractions, in analogy to previous studies carried out with 2,4-dichlorophenoxyacetic acid [28] and with pentachlorophenol [26].

Characterization of chloroaniline/lignin metabolites

In order to differentiate between non-covalent and covalent lignin association of the chloroanilines, gel permeation chromatography on Sephadex LH-60 in dimethylformamide was employed. The elution patterns of the 4-chloroaniline- and 3,4-dichloroaniline/lignin metabolite fractions used subsequently for fungal incubation are shown in Fig. 1A and 1B, respec-

tively. Calibration of the column with polystyrenes of known molecular weights indicated that the lignin metabolites were polydisperse and were present in the molecular weight range between 600 and 35,000. There were also metabolite species of molecular weight in excess of 35,000, and of molecular weight below 600. Thinlayer chromatographic examination of the lignin metabolite fractions indicated that the free chloroanilines and their known dimeric transformation products were absent. The ultraviolet absorption reflecting mainly lignin content and the radioactivity of the incorporated chloroaniline moieties showed roughly parallel elution over the entire molecular weight range.
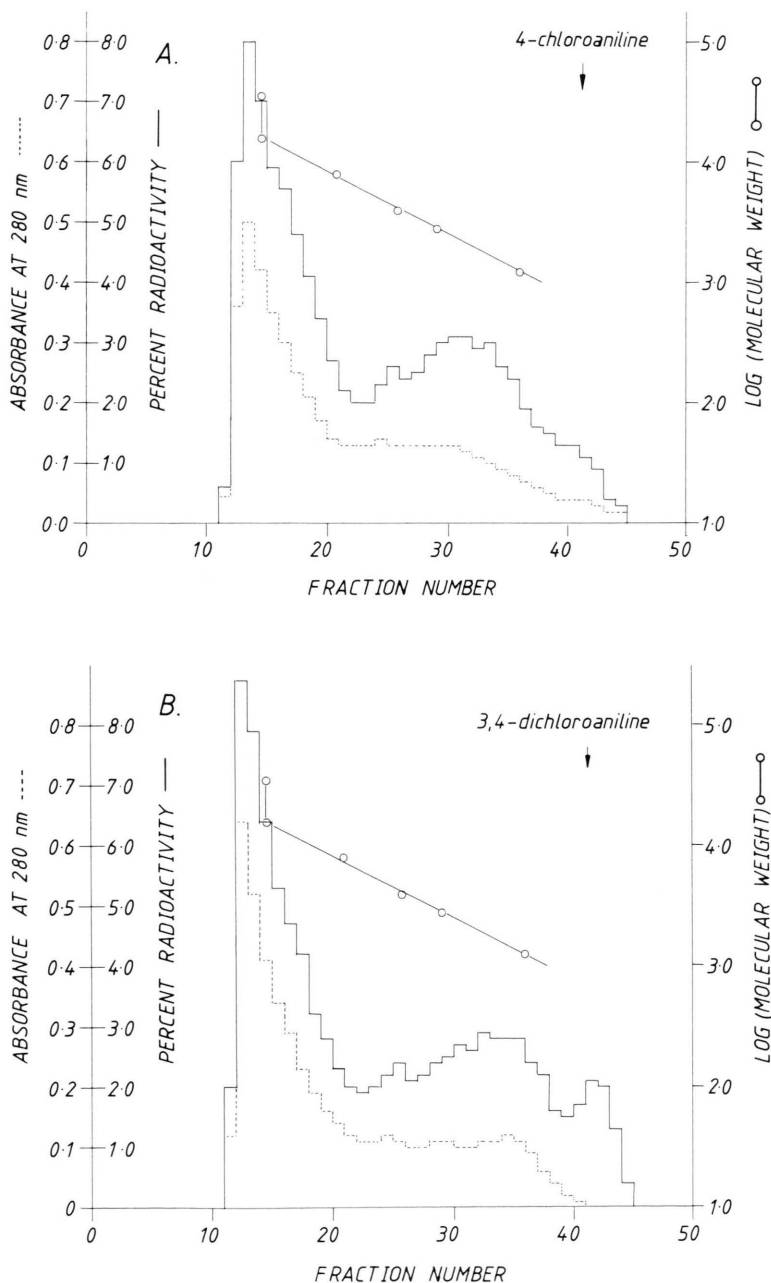

Fig. 1. A, B. Gel permeation chromatography of wheat chloroaniline/lignin metabolites on Sephadex LH-60. A. [ring-u-^{14}C]-4-chloroaniline/lignin metabolite, B. [ring-u-^{14}C]-3,4-dichloroaniline/lignin metabolite. A column of 2.1×100 cm was used with dimethylformamide as eluting solvent. Fractions of 7 ml were collected. The column was calibrated in independent runs using known polystyrene molecular weight markers (O———O). The elution positions of the free chloroanilines are indicated by arrows. The eluted metabolite fractions were studied for UV-absorption at 280 nm (– – – – –) and for radioactivity (———; % of total radioactivity applied to column).
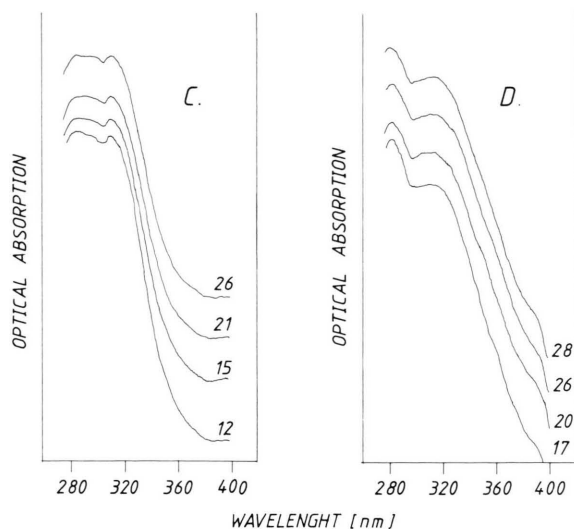
Fig. 1. C, D. UV-spectra of selected metabolite fractions. The indicated fractions were studied in a Perkin Elmer model 554 spectrophotometer using a reference cuvette containing dimethylformamide.

The ultraviolet spectra of selected fractions of Fig. 1A and 1B are shown in Fig. 1C and 1D, respectively.

In order to determine a minimum percentage of chloroaniline incorporated in unchanged form, the chloroaniline/lignin metabolites were subjected to the drastic degradative reactions previously applied to artificial chloroaniline/lignin conjugates [17]. After treatment with strong acid, base or 2-amino-ethanol, the amount of released chloroaniline was determined by thinlayer chromatography in solvent systems A and B. Without these hydrolytic treatments, all of the radioactivity remained at the origin of the chromatograms. Radioactive material released by the hydrolytic treatments migrated exclusively in the position of the free chloroanilines. The quantitative results of hydrolytic cleavage are summarized in Table I. Strong acid hydrolysis was the most effective method for cleavage, 79% and 73% of the incorporated radioactivity being released in the cases of the 4-chloroaniline/lignin metabolite and the 3,4-dichloroaniline/lignin metabolite, respectively.

Table I. Chemical degradation of [ring-u-^{14}C]chloroaniline/lignin metabolites isolated from wheat plants. All data are given as % of applied radioactivity. Quantitation was done by thinlayer chromatography in solvent systems A and B, followed by scanning. The lignin metabolites appeared as a single peak at the origin of the chromatograms ('start material'). Degradation led in all cases to the free chloroanilines as the only mobile split product.

Treatment	Free chloroaniline	Start material	Recovery
Acid hydrolysis of			
4-chloroaniline/lignin	79.0	14.0	93.0
3,4-dichloroaniline/lignin	73.8	33.2	107.0
Alkaline hydrolysis of			
4-chloroaniline/lignin	0.0	93.6	93.6
3,4-dichloroaniline/lignin	20.0	85.8	105.8
2-Aminoethanol treatment of			
4-chloroaniline/lignin	64.7	39.7	104.4
3,4-dichloroaniline/lignin	68.4	32.8	101.2

Fungal degradation

4-Chloroaniline/lignin metabolite

The formation of $[^{14}C]CO_2$ during incubation of the [ring-u-^{14}C]-4-chloroaniline/lignin metabolite fraction with the white-rot fungus, *Phanerochaete*

chrysosporium, is depicted in Fig. 2 A. The corresponding data for a pure lignin sample prepared with [ring-u-^{14}C]coniferyl alcohol are also shown. After the incubation period of 32 days, 59.2% and 67% of the initial radioactivity were trapped as $[^{14}C]CO_2$ in the case of the 4-chloroaniline/lignin metabolite and the control lignin, respectively. No volatile incubation products associated with the polyurethane stopper were detected, although the control incubation without fungal inoculum developed 5.5% volatile
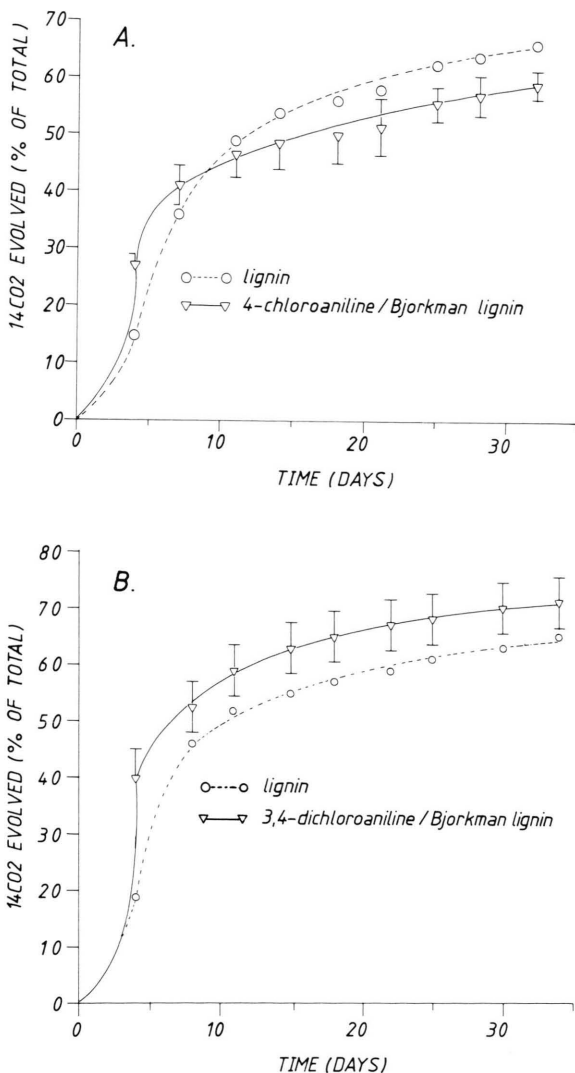

Fig. 2. Mineralization of ^{14}C-labeled lignin samples by *Phanerochaete chrysosporium*. A non-xenobiotic control lignin [ring-u-^{14}C]-labeled in coniferyl alcohol [○−−−○] was studied in comparison with A. the [ring-u-^{14}C]-4-chloroaniline/Björkman lignin fraction and B. the [ring-u-^{14}C]-3,4-dichloroaniline/Björkman lignin fraction (▽——▽). Radioactivity trapped as $[^{14}C]CO_2$ (cumulative % of applied radioactivity) is plotted against incubation time (days). The standard deviations shown were derived from n = 4 parallel incubation experiments.
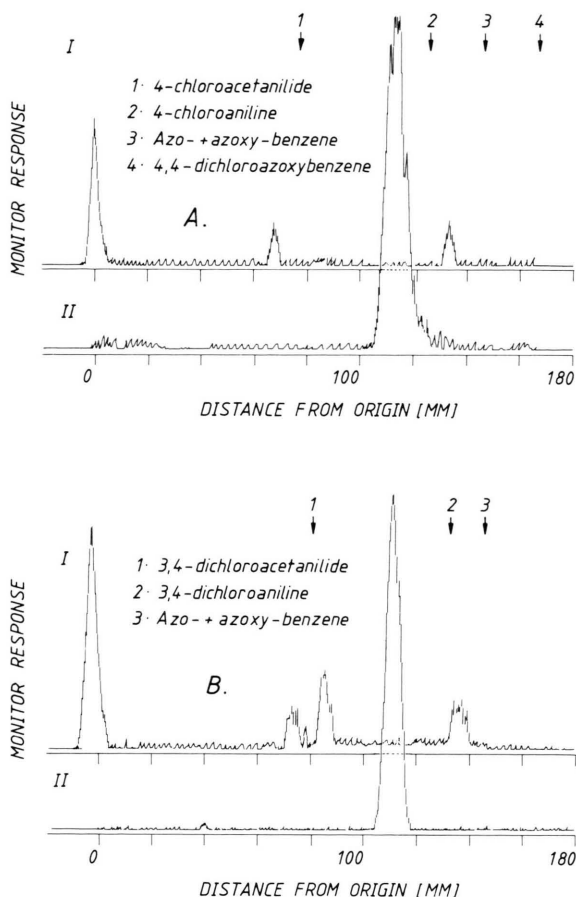

Fig. 3. Thinlayer chromatography of the ethylacetate extracts of Table II in solvent system A. The distributions of radioactivity shown were determined with a Berthold model LB 2842 Automatic TLC-Linear Analyzer System.
A. Ethylacetate extract from the experiment with 4-chloroaniline. I. Scan from control incubation without fungal inoculum. II. Scan from incubation with fungal inoculum.
B. Ethylacetate extract from experiment with 3,4-dichloroaniline. I. Scan from incubation without fungal inoculum. II. Scan from incubation with fungal inoculum. The chromatographic positions of the listed well-known metabolites are indicated by arrows.

radioactivity, presumably the free 4-chloroaniline (see below).

Significant amounts of radioactivity were associated with the ethylacetate and aqueous extracts of the work-up procedure. The radioactivity of the aqueous phase has not been studied in detail. The radioactivity of the ethylacetate extract was mainly associated with a single peak of R_f 0.76 in solvent system A (Fig. 3A, scan II) and of R_f 0.24 in solvent B (not shown). The ethylacetate phase of the control incubation without fungal inoculum contained three different minor radioactive components that were not identified (Fig. 3A, scan I). Recent mass-spectroscopic studies have shown that the R_f-0.76 material represents a previously unreported metabolite, N-(4-chlorophenyl)-succinimide (manuscript in preparation).

3,4-Dichloroaniline/lignin metabolite

The formation of $[^{14}C]CO_2$ during fungal degradation is depicted in Fig. 2B along with the mineralization curve for the control lignin prepared with [ring-u-^{14}C]coniferyl alcohol. After 33 days of incubation, 71.3% and 65.4% of the initial radioactivity was trapped as $[^{14}C]CO_2$ in the case of the 3,4-dichloroaniline/lignin metabolite and the control lignin, respectively. Volatile radioactivities amounted to 1.3% and 11.4% for the 3,4-dichloroaniline/lignin metabolite and the control lignin, respectively. In both cases, the radioactivity co-migrated with free 3,4-

dichloroaniline upon thinlayer chromatography in solvent systems A and B.

The total distributions of radioactivity are summarized in Table II. Radioactive material associated with the mycelial residue and the aqueous extract phase was not studied in detail. The radioactivity of the ethylacetate extract was mainly associated with a single peak at R_f 0.76 in solvent system A (Fig. 3B, scan II) and R_f 0.25 in solvent system B (not shown). The ethylacetate phase from the incubation with control lignin showed four other minor radioactive components that have not been identified (Fig. 3B, scan I). Mass-spectroscopic studies have shown the R_f-0.76 component to be N-(3,4-dichlorophenyl)-succinimide (manuscript in preparation).

Discussion

Incorporation of chloroanilines into "insoluble" plant residues

The high incorporation rates of 4-chloroaniline and 3,4-dichloroaniline into the "insoluble" residue of wheat plants are in agreement with previous studies where high incorporation rates of chlorinated anilines into the "insoluble" residue of monocot crop plants, such as rice, barley and wheat, have been observed [1, 7−15]. Cultured wheat cells also gave high incorporation rates of the chloroanilines into the "insoluble" residue, as previously also observed for 2,4-dichlorophenoxyacetic acid [28] and penta-

Table II. Distributions of radioactivity after incubation of the [ring-u-^{14}-C]-chloroaniline/lignin metabolites from wheat with *Phanerochaete chrysosporium*. The data are given as % of applied radioactivity ± standard deviation ($n = 4$). The work-up procedure leading to the fractions listed is described under Methods. The mycelial dry weights were 63.8 ± 3.9 mg in the experiments with the 4-chloroaniline/lignin metabolite and 64.5 ± 11.8 mg in the experiments with the 3,4-dichloroaniline/lignin metabolite. The incubations without fungal inoculum were performed in order to examine for non-biological transformation reactions.

Fraction	4-chloroaniline/lignin metabolite		3,4-dichloroaniline/lignin metabolite	
	with	without	with	without
	fungal inoculum		fungal inoculum	
CO$_2$	59.2 ± 2.2	−	71.3 ± 4.3	−
Volatiles	−	5.5	1.3 ± 0.1	11.4
Mycelial residue	1.6 ± 0.3	−	1.3 ± 0.2	−
Ethylacetate phase	28.4 ± 2.6	59.8	13.3 ± 1.1	65.8
Aqueous phase	11.9 ± 1.1	26.5	9.3 ± 1.4	19.7
Total^{14}C recovered	101.1 ± 4.1	91.8	96.4 ± 3.4	96.9

chlorophenol [26]. However, cultured soybean cells incorporated chloroanilines and pentachlorophenol [26] to a much lower extent into the "insoluble" residue. The low incorporation rates seem to be due to high levels of competing enzyme reactions in the soybean cells. The formation of N-malonyl conjugates of chloroanilines was found to be much higher in soybean than in wheat cells (R. Winkler, R. Schmitt and H. Sandermann, unpublished results). In the case of pentachlorophenol, the soybean cells formed high amounts of β-D-glucosyl and (O-malonyl)-β-D-glucosyl conjugates rather than high "insoluble" residues [29]. The "insoluble" residue formed in wheat plants from 4-chloroaniline and 3,4-dichloroaniline was sequentially extracted by a Björkman-type procedure. This method which is rather specific for lignin has previously been successfully applied to the "insoluble" residues formed from 3-chloroaniline and 3,4-dichloroaniline in rice [12, 13] as well as artificial chloroaniline/lignin conjugates [12, 13, 16, 17].

Characterization of chloroaniline/lignin metabolites

Gel permeation chromatography on Sephadex LH-60 in dimethylformamide has been developed as a method to distinguish non-covalent lignin inclusion compounds from truly covalent lignin conjugates [14, 21]. Previous studies of chloroaniline/lignin complexes did not rule out that the test chloroanilines were incorporated into lignin merely as inclusion products [12, 13].

The co-chromatography of UV-absorbing material (reflecting mainly lignin) and of incorporated radioactivity in Fig. 1 A, B provided strong evidence for a covalent linkage between lignin and the incorporated chloroanilines. Since the specific radioactivity of the lignin species was rather constant over the entire molecular weight range, some random mechanism seemed to be responsible for incorporation.

The lignin nature of the eluted fractions was further shown by the UV-spectra of Fig. 1 C, D. These spectra, in particular the characteristic double peak, were similar to those reported previously for wheat lignin fractions [26, 28]. The degradation reactions

indicated that the chloroanilines were to 70−80% incorporated in chemically unchanged form. The latter finding, as well as the present molecular weight distributions, are similar to the results obtained recently with artificial chloroaniline/lignin conjugates [17], thus providing additional justification for the use of artificial xenobiotic/lignin conjugates as models for plant lignin metabolites (*cf.*, [14]).

Fungal degradation

The fungal conversion of about 65% of the copolymerized [ring-u-^{14}C]chloroanilines to [^{14}C]CO_2 was as efficient as the mineralization of the non-xenobiotic control lignin with a [ring-u-^{14}C]-label in the coniferyl alcohol moiety. Similarly high mineralization rates by *Phanerochaete chrysosporium* have recently been obtained with artificial chloroaniline/lignin conjugates as well as the free chloroanilines [17]. These results suggest that the white-rot fungus can catalyze the final removal of plant chloroaniline residues even though such contaminants are rather persistent [1−5, 7−15]. When considered in terms of the global carbon cycle [18, 19], plant lignin metabolites of xenobiotics are therefore not necessarily 'terminal' residues. *Phanerochaete chrysosporium* is known to also mineralize a multitude of structurally diverse phenolic compounds [30], as well as persistent environmental chemicals such as DDT, chlorinated biphenyls, 2,3,7,8-tetrachlorodioxine, lindane and benzo[α]pyrene [31] and lignin-sulfonates [32−35]. It remains to be studied whether the unusual degradative capacity of *Phanerochaete chrysosporium*, or perhaps related organisms, can be exploited for biological decontamination.

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