APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1998, p. 399–404 Vol. 64, No. 2
0099-2240/98/$04.00+0
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# Enzymatic Combustion of Aromatic and Aliphatic Compounds by Manganese Peroxidase from *Nematoloma frowardii*

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Received 3 September 1997/Accepted 31 October 1997

**The direct involvement of manganese peroxidase (MnP) in the mineralization of natural and xenobiotic compounds was evaluated. A broad spectrum of aromatic substances were partially mineralized by the MnP system of the white rot fungus *Nematoloma frowardii*. The cell-free MnP system partially converted several aromatic compounds, including [U-$^{14}$C]pentachlorophenol ([U-$^{14}$C]PCP), [U-$^{14}$C]catechol, [U-$^{14}$C]tyrosine, [U-$^{14}$C]tryptophan, [4,5,9,10-$^{14}$C]pyrene, and [ring U-$^{14}$C]2-amino-4,6-dinitrotoluene ([$^{14}$C]2-AmDNT), to $^{14}CO_2$. Mineralization was dependent on the ratio of MnP activity to concentration of reduced glutathione (thiol-mediated oxidation), a finding which was demonstrated by using [$^{14}$C]2-AmDNT as an example. At [$^{14}$C]2-AmDNT concentrations ranging from 2 to 120 μM, the amount of released $^{14}CO_2$ was directly proportional to the concentration of [$^{14}$C]2-AmDNT. The formation of highly polar products was also observed with [$^{14}$C]2-AmDNT and [U-$^{14}$C]PCP; these products were probably low-molecular-weight carboxylic acids. Among the aliphatic compounds tested, glyoxalate was mineralized to the greatest extent. Eighty-six percent of the $^{14}$COOH-glyoxalate and 9% of the $^{14}$CHO-glyoxalate were converted to $^{14}CO_2$, indicating that decarboxylation reactions may be the final step in MnP-catalyzed mineralization. The extracellular enzymatic combustion catalyzed by MnP could represent an important pathway for the formation of carbon dioxide from recalcitrant xenobiotic compounds and may also have general significance in the overall biodegradation of resistant natural macromolecules, such as lignins and humic substances.**

Manganese peroxidase (MnP) (EC 1.11.1.13) is a heme-containing glycoprotein that requires hydrogen peroxide ($H_2O_2$) as an oxidant (7, 17). This enzyme is produced only by ligninolytic basidiomycetes (white rot fungi and litter-decaying fungi) (3, 11). MnP oxidizes Mn(II) to Mn(III), which then oxidizes phenolic rings to phenoxy radicals, leading finally to the decomposition of compounds (8, 32). Due to its high reactivity, Mn(III) has to be stabilized via chelation by dicarboxylic acids, such as malonate or lactate (37). In addition to phenolic structures, the MnP system has been reported to catalyze cleavage of nonphenolic lignin model compounds (5, 9, 14, 20). Evidence that MnP plays a crucial role in biodegradation of macromolecular substances is accumulating; e.g., this enzyme plays a role in the depolymerization of lignin (14, 36), in the bleaching of pulp (10, 27), and in the decomposition of humic substances (13).

Due to the nonspecificity of Mn(III), the MnP system is also able to oxidize a variety of organic pollutants and xenobiotic compounds. Thus, the conversion of 4-amino-2-nitrotoluene (33), polycyclic aromatic hydrocarbons, including creosote (2, 4, 26), and chlorolignin-containing wastes (18) has been described previously. We have recently described partial mineralization of [ring U-$^{14}$C]2-amino-4,6-dinitrotoluene ([$^{14}$C]2-AmDNT), a main metabolite of the explosive 2,4,6-trinitrotoluene, by a crude preparation of MnP from the South American white rot fungus *Nematoloma frowardii* (29). The present paper describes the enzymatic combustion of a broad spectrum of aromatic and aliphatic substances by the MnP system of *N. frowardii* and demonstrates the universal validity of the degradation principle. Furthermore, dependence of the mineralization process on the concentration of the peptide glutathione (GSH) is demonstrated. The concept of enzymatic combustion was adopted from wood microbiology; this concept has been used to describe the depolymerization of high-molecular-weight lignin by nonspecific extracellular peroxidases from ligninolytic fungi (15).

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## MATERIALS AND METHODS

**Organism and chemicals.** The South American white rot fungus *N. frowardii* (Horak) b19 (= DSM 11239) was isolated and characterized as described previously (12, 13).

The following radioactively labeled chemicals were used. [ring U-$^{14}$C]2,4,6-trinitrotoluene (2.2 mCi mmol$^{-1}$) and [$^{14}$C]2-AmDNT (2.2 mCi mmol$^{-1}$) were obtained from W. Fels (Department of Organic Chemistry, University of Paderborn, Paderborn, Germany); [U-$^{14}$C]pentachlorophenol ([U-$^{14}$C]PCP) (10.4 mCi mmol$^{-1}$), [U-$^{14}$C]2,4-dichlorophenol (9.3 mCi mmol$^{-1}$), [U-$^{14}$C]phenol (8.7 mCi mmol$^{-1}$), and [U-$^{14}$C]catechol (2 mCi mmol$^{-1}$) were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany); and [4,5,9,10-$^{14}$C]pyrene (56 mCi mmol$^{-1}$) was purchased from Amersham Buchler (Braunschweig, Germany). We also used [U-$^{14}$C]tyrosine (457 mCi mmol$^{-1}$), [U-$^{14}$C]tryptophan (599 mCi mmol$^{-1}$), [U-$^{14}$C]phenylalanine (580 mCi mmol$^{-1}$), [U-$^{14}$C]glutamic acid (293 mCi mmol$^{-1}$), [U-$^{14}$C]aspartic acid (219 mCi mmol$^{-1}$), [U-$^{14}$C]leucine (182 mCi mmol$^{-1}$), [U-$^{14}$C]glycine (212 mCi mmol$^{-1}$), $^{14}$COOH-glyoxalate (4.8 mCi mmol$^{-1}$), $^{14}$CHO-glyoxalate (7.0 mCi mmol), [$^{14}$C]urea (53.5 mCi mmol$^{-1}$), [U-$^{14}$C]glucose (298 mCi mmol$^{-1}$), and [U-$^{14}$C]fructose (275 mCi mmol$^{-1}$). All of these radiochemicals were obtained from New England Nuclear Co. (Boston, Mass.).

All other chemicals, reagents, and solvents were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich Chemie GmbH.

**Culture conditions and enzyme preparation.** The basal medium used for the production of MnP was an N-sufficient medium described previously (12), except that the Mn(II) concentration was 300 instead of 168 μM. Inoculation material was prepared from malt agar plates, which were precultivated for 7 days. Cultivation was carried out in 11 conical flasks containing 300 ml of the medium; each flask was inoculated with 10 agar plugs (1 cm in diameter) of active mycelium and incubated for 25 days. The culture broth was harvested and filtered through glass wool. The filtrate was concentrated by two steps of ultrafiltration, one performed with an Ultralab 2L Minisette filter with a 10-kDa molecular mass cutoff (Pall Filtron GmbH, Karlstein, Germany) and the other performed with an Amicon Chamber polysulfone filter with a 10-kDa cutoff. Low-molecular-weight substances in the crude extract were removed by diafiltration (MicroProDiCon; 25-kDa cutoff; Spectrum, Houston, Texas) performed with sodium malonate buffer (10 mM, pH 5.0). The final preparation had an MnP activity of 10.2 U

$ml^{-1}$; activities of lignin peroxidase (LiP) or other peroxidases were not found, and only traces of laccase (0.1 U $ml^{-1}$) were detectable. This MnP preparation was used for most experiments. In addition, a partially purified MnP preparation (isoenzymes MnP1 and MnP2 combined) (30) obtained from anion-exchange chromatography (fast protein liquid chromatography) (Mono Q column; Pharmacia, Uppsala, Sweden) and highly purified isoenzyme MnP2 were used in certain experiments. The latter was prepared by anion-exchange chromatography and subsequent preparative isoelectric focusing as reported recently (30); it has a molecular mass of 44 kDa and a pI of 3.2.

**Mineralization experiments.** Mineralization experiments in which MnP preparations and $^{14}$C-labeled compounds were used were carried out in sterile 10-ml reaction tubes tightly closed with rubber septa and sealed with plastic screw caps. Each reaction tube contained in a total volume of 1 ml the following filter-sterilized components: 30 mM sodium malonate buffer (pH 4.5), 1 mM $MnCl_2$, 15 mM glucose, glucose oxidase (0.04 U; from *Aspergillus niger*; low in catalase activity; Sigma-Aldrich GmbH), 10 mM reduced GSH, and 2 U of the diafiltered MnP preparation or 2 U of the partially purified MnP preparation or 0.1 U of the highly purified isoenzyme MnP2 preparation. To ensure simple handling of $^{14}$C-labeled substances, they were added in all cases to a final concentration of 0.1 μCi $ml^{-1}$ ($2.2 \times 10^5$ dpm) and were dissolved in *N*,*N*-dimethylformamide or water. The samples were incubated at 37°C on a rotary shaker (160 rpm) for 24 or 72 h. Released $^{14}CO_2$ was trapped and measured as described previously (24, 28, 29). Control experiments were performed with boiled MnP.

To determine the dependence of [$^{14}$C]2-AmDNT mineralization on the GSH concentration, the reaction mixture described above was used, but the concentration of GSH was varied from 0 to 20 mM. Furthermore, the concentration of [$^{14}$C]2-AmDNT and the MnP activity were modified (0.005 to 0.4 μCi $ml^{-1}$ [$1.1 \times 10^4$ to $8.8 \times 10^5$ dpm], corresponding to 2.25 to 180 μM [$^{14}$C]2-AmDNT and 0.2 to 5 U of MnP $ml^{-1}$) to determine the influence of these parameters on the extent of mineralization.

In the case of [$^{14}$C]2-AmDNT and [$^{14}$C]PCP, the distribution of residual radioactivity in the reaction solution was analyzed by high-performance liquid chromatography (HPLC) (Merck Hitachi, Darmstadt, Germany) after treatment with MnP. The HPLC system was equipped with a model L4500 diode array detector operating at a wavelength range of 210 to 500 nm. An UltraSep ES FS column (250 by 3 mm; Knauer, Groß-Umstadt, Germany), which was developed for separation of low-molecular-weight organic acids in food chemistry, was employed to separate the complex reaction mixture by using 10 mM phosphoric acid as the solvent (flow rate, 0.55 ml $min^{-1}$; detection wavelength, 210 nm; injection volume, 100 μl) under isocratic conditions. Every 2 min, fractions (1.1 ml) were collected in scintillation vials, and radioactivity was determined by liquid scintillation counting.

**Enzyme assay.** MnP activity was directly measured by measuring the formation of Mn(III)-malonate complexes ($\varepsilon_{270}$ = 11.59 $mM^{-1}$ $cm^{-1}$) as described by Wariishi et al. (37). Each modified assay mixture (1 ml) contained 5 to 50 μl of an MnP preparation, 0.5 mM $MnCl_2$, and 0.1 mM $H_2O_2$ in 50 mM sodium malonate buffer (pH 4.5). LiP was assayed by the veratryl oxidation method (16). Laccase activity was estimated by following the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) at 420 nm (12, 38). Each assay solution (1 ml) contained 5 to 50 μl of enzyme solution and 0.3 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) in 100 mM citrate buffer (pH 5.0).

**Statistical procedures.** In all experiments, measurements were obtained from triplicate parallel reaction mixtures. The values reported are means, and in all cases the standard deviations were less than 3%.

## RESULTS

**Influence of GSH concentration on the extent of [$^{14}$C]2-AmDNT mineralization.** The extent of [$^{14}$C]2-AmDNT mineralization was strongly dependent on the concentration of the thiol GSH (Fig. 1). In the absence of GSH, the MnP system mineralized only 2% of the initial [$^{14}$C]2-AmDNT within 24 h (1 μM $CO_2$ was released), but the addition of GSH at a concentration as low as 0.1 mM led to twice as much mineralization and up to 10 mM, additional increases in GSH concentration resulted in nonlinear increases in $^{14}CO_2$ evolution. Twice-logarithmic scaling of GSH concentration versus released $^{14}CO_2$ led to linearity between the two parameters (Fig. 1). At GSH concentrations between 10 and 15 mM the amount of released $^{14}CO_2$ remained nearly constant (12 to 13 μM $^{14}CO_2$, corresponding to 27 to 29% of the initial radioactivity). Concentrations of GSH higher than 15 mM led to nearly complete inhibition of the mineralization process. The drastic differences in $^{14}CO_2$ release observed with 10 mM GSH and different MnP activities are clearly visible in Fig. 2. Between 1 and 2 U of MnP per ml there was a considerable increase in the extent of mineralization, and additional increases in MnP activity did not lead to further increases in $^{14}CO_2$ release ($^{14}CO_2$ remained nearly constant). Additional experiments demonstrated that the absolute concentration of GSH was not the decisive factor for extent of mineralization; rather, the ratio of GSH concentration to MnP activity was the most critical factor. Thus, when we used 0.5 U of MnP $ml^{-1}$ instead of 2 U $ml^{-1}$, the optimum GSH concentration was found to be 2.5 mM, and inhibition of [$^{14}$C]2-AmDNT mineralization occurred with concentrations higher than 5 mM. The optimum GSH concentration per unit of MnP activity for mineralization of [$^{14}$C]2-AmDNT was approximately 5 mM.

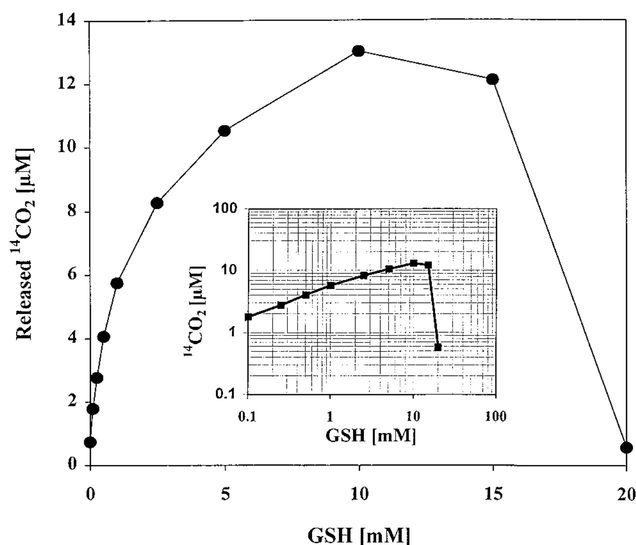


FIG. 1. Influence of GSH concentration on the extent of MnP-catalyzed [$^{14}$C]2-AmDNT mineralization. Samples were incubated for 24 h. Twice-logarithmic scaling resulted in a linear relationship between GSH concentration and $^{14}CO_2$ release. In control experiments performed with boiled MnP less than 0.2 μM $^{14}CO_2$ (<0.5% of the initial radioactivity) was released.

**Dependence of the extent of mineralization of the [$^{14}$C]2-AmDNT concentration.** At [$^{14}$C]2-AmDNT concentrations between 2.25 and 113.5 μM, the extent of mineralization was directly proportional to the concentration of the substrate (Fig. 3). Consequently, the percentage of mineralization (percentage of released $^{14}CO_2$ relative to the initial radioactivity) was

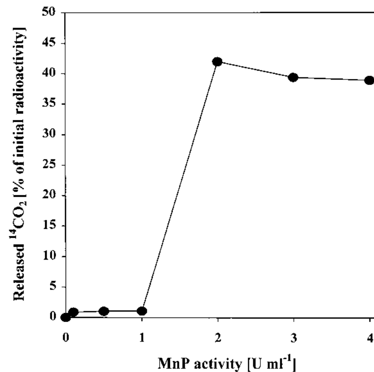


FIG. 2. Levels of mineralization of [$^{14}$C]2-AmDNT (0.1 μCi per ml) when 10 mM GSH and different MnP activities were used. Samples were incubated for 72 h. The graph shows the drastic increase in the extent of mineralization at MnP activities between 1 and 2 U $ml^{-1}$ (this only applies to a GSH concentration of 10 mM).

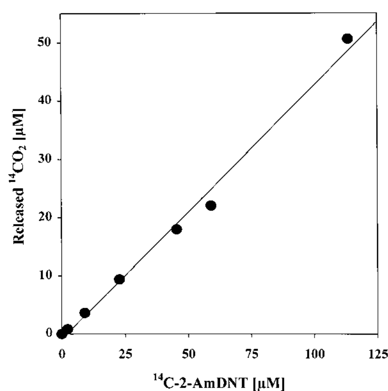


FIG. 3. Dependence of $^{14}CO_2$ release from [$^{14}$C]2-AmDNT on substrate concentration. The concentration of [$^{14}$C]2-AmDNT was varied between 2.25 and 113.5 μM (0.005 and 0.25 μCi per ml); in this concentration range, the amounts of $^{14}CO_2$ released were directly proportional to the concentration of [$^{14}$C]2-AmDNT. Samples were incubated for 72 h.

nearly independent of the concentration of [$^{14}$C]2-AmDNT in that range and was nearly the same (about 40%) in all experiments. Using a [$^{14}$C]2-AmDNT concentration as high as 180 μM only resulted in a further nonlinear increase in $^{14}CO_2$ release (data not shown).

**Mineralization of [$^{14}$C]2-AmDNT by purified MnP.** Because only small amounts of highly purified MnP2 were available, only low enzyme activities could be employed in mineralization studies. Even so, an MnP activity as low as 0.1 U $ml^{-1}$ (which corresponds to the activity found in liquid cultures of *N. frowardii* [12]) was able to mineralize about 3% of the [$^{14}$C]2-AmDNT within 72 h (in this experiment we used the same [$^{14}$C]2-AmDNT concentration as in the experiments described above, 45.4 μM, corresponding to 0.1 μCi $ml^{-1}$). Because of the low enzyme activity, the GSH concentration had to be reduced to 0.5 mM (the presence of 10 mM GSH led to a $^{14}CO_2$ release of only 0.6%). In control experiments performed with boiled MnP2 no $^{14}CO_2$ was released. This result demonstrates that MnP is responsible for the direct mineralization of [$^{14}$C]2-AmDNT. Additional experiments, performed with partially purified MnP (2 U $ml^{-1}$), led, within 72 h, to a level of [$^{14}$C]2-AmDNT mineralization of 37%, which was similar to the value obtained with diafiltered MnP preparations (42%) (Table 1).

**Mineralization of additional $^{14}$C-labeled aromatic compounds.** All $^{14}$C-labeled aromatic compounds tested were partially mineralized by the MnP system (Table 1). On the basis of our finding that the level of mineralization of [$^{14}$C]2-AmDNT was independent of its concentration over a wide range, it was reasonable to compare the levels of mineralization of $^{14}$C-labeled substances with different specific activities.

Less polar aromatic compounds, such as pyrene and phenylalanine, as well as aromatic ring compounds with high electron deficiencies, such as 2,4,6-trinitrotoluene (electron deficient due to the three symmetric electron-withdrawing nitro groups), were slightly mineralized (4 to 8%). Among the phenols tested, 2,4-dichlorophenol was mineralized to the lowest extent (9%). In spite of its five chlorine substituents, PCP was rapidly degraded by the MnP system (level of mineralization, 36%), and the degradation of PCP was even greater than the degradation of phenol (18%). Catechol was the phenolic compound which was mineralized to the greatest extent (49%). The amino acid tyrosine containing a phenolic ring was also extensively mineralized (42%). Tryptophan, which has a heterocyclic ring system (indole), also served as a substrate for the MnP system and was converted at the same rate as phenol (18%).

TABLE 1. MnP-catalyzed enzymatic combustion of several $^{14}$C-labeled substances[a]

| Compound | Concn (μM) | Mineralization: % $^{14}CO_2$ released by MnP | Mineralization: % $^{14}CO_2$ released by controls |
|---|---|---|---|
| Aromatic compounds | | | |
| [ring U-$^{14}$C]2-AmDNT | 45.4 | 42 | 0.04 |
| [ring U-$^{14}$C]2,4,6-trinitrotoluene | 45.4 | 4.8 | 0 |
| [U-$^{14}$C]catechol | 50.0 | 49 | 3.8 |
| [U-$^{14}$C]PCP | 9.6 | 36 | 0.07 |
| [U-$^{14}$C]phenol | 11.5 | 18.3 | 0.05 |
| [U-$^{14}$C]2,4-dichlorophenol | 10.3 | 9.4 | 0.1 |
| [U-$^{14}$C]tyrosine | 0.22 | 42.8 | 2.6 |
| [U-$^{14}$C]phenylalanine | 0.17 | 5.9 | 1.5 |
| [U-$^{14}$C]tryptophan | 0.17 | 19.1 | 1.1 |
| [4,5,9,10-$^{14}$C]pyrene | 1.8 | 7 | 0 |
| Aliphatic compounds | | | |
| $^{14}$COOH-glyoxalate | 20.8 | 86 | 19 |
| $^{14}$CHO-glyoxalate | 14.3 | 9 | 1.7 |
| [U-$^{14}$C]glycine | 0.47 | 3.9 | 1.2 |
| [U-$^{14}$C]aspartic acid | 0.46 | 6.4 | 0.7 |
| [U-$^{14}$C]glutamate | 0.34 | 3.6 | 1 |
| [U-$^{14}$C]leucine | 0.55 | 0.8 | 0 |
| [U-$^{14}$C]urea | 1.9 | 0.4 | 0 |
| [U-$^{14}$C]glucose | 0.34 | 1.7 | 0.2 |
| [U-$^{14}$C]fructose | 0.36 | 1.9 | 0.7 |

[a] Each reaction mixture (1 ml) contained 2 U of diafiltered MnP, 30 mM sodium malonate (pH 4.5), 1 mM $MnCl_2$, 10 mM GSH, 15 mM glucose, 0.04 U of glucose oxidase, and 0.1 μCi of a labeled aromatic or aliphatic substrate. The mixtures were incubated on a rotary shaker at 37°C for 72 h. Control experiments were performed with the complete MnP system and boiled enzyme. All values are the means of three replicates; the standard deviation was in all cases less than 3%.

As an example of mineralization of an aromatic compound by MnP from *N. frowardii*, the time course of $^{14}CO_2$ release from [$^{14}$C]PCP is shown in Fig. 4 for different GSH concentrations and an incubation period of 8 days. The greatest amounts of $^{14}CO_2$ were released within the first hours of incubation, and then the mineralization process slowed down; in the end, up to 44% of the initial radioactivity was released as

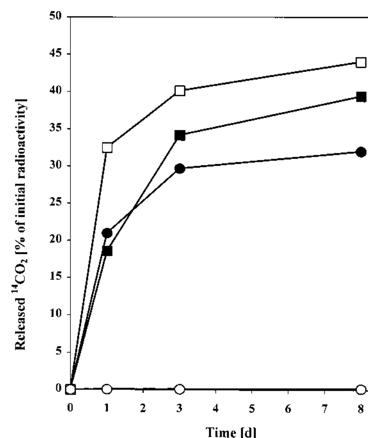


FIG. 4. Time courses of [$^{14}$C]PCP mineralization by MnP obtained with different GSH concentrations. Symbols: ●, no GSH; □, 1 mM GSH; ■, 10 mM GSH. d, day.

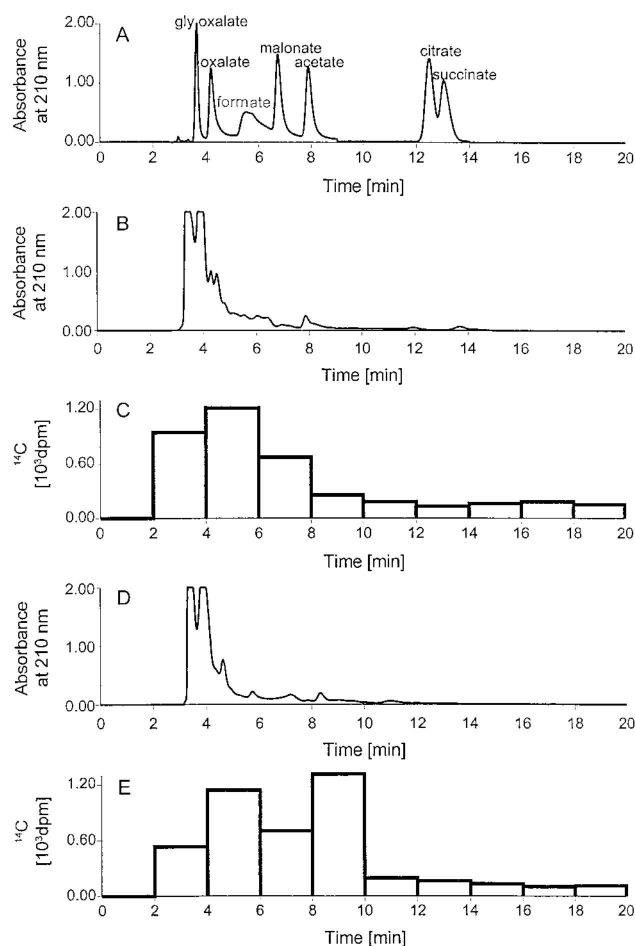


FIG. 5. Analysis of residual radioactivity in samples of [$^{14}$C]PCP and [$^{14}$C]2-AmDNT, which were treated with MnP for 72 h. An UltraSep ES FS column (Knauer) was used for separation. (A) Low-molecular-weight organic acid standards (concentration range, 3 to 15 mM). (B) Chromatogram of the reaction mixture containing [$^{14}$C]PCP. (C) Distribution of residual radioactivity in the reaction mixture containing [$^{14}$C]PCP. (D) Chromatogram of the reaction mixture containing [$^{14}$C]2-AmDNT. (E) Distribution of residual radioactivity in the reaction mixture containing [$^{14}$C]2-AmDNT. In both cases, the most radioactivity was eluted from the column at the same time as glyoxalate, oxalate, formate, malonate, and acetate; however, unambiguous identification of individual acids was not possible due to the broad distribution of radioactivity.

$^{14}CO_2$ within 8 days. Interestingly, the greatest extent of PCP mineralization was achieved with 1 mM GSH, whereas 10 mM GSH led to a lower release rate (38%). Even in the absence of GSH more than 30% of the [$^{14}$C]PCP was converted to $^{14}CO_2$.

**Analysis of residual radioactivity in the reaction mixture.** When a special column for the separation of organic acids was used, in the case of [$^{14}$C]PCP about 32% of the residual radioactivity was found in early fractions (2 to 8 min) (Fig. 5B and C) and broad nonspecific distribution of radioactivity was observed. Organic acid standards (glyoxalate, oxalate, formate, malonate, and acetate) were eluted from this column at approximately the same time, indicating that the fission products formed by MnP had similar structures (Fig. 5A), whereas aromatic substances (e.g., benzoic acid) or unsaturated carboxylic acids (e.g., fumarate) were not eluted from the column. Due to the broad distribution of radioactivity, unambiguous identification of individual substances was not possible, but the maximum radioactivity between 4 and 6 min and the HPLC chromatogram (Fig. 5B) of the reaction solution suggest that formate, glyoxalate, and/or oxalate might have been the main products formed. A similar distribution of residual radioactivity was found for samples of [$^{14}$C]2-AmDNT treated with MnP; 62% of the residual radioactivity was even associated with highly polar substances (Fig. 5D and E). Interestingly, the maximum radioactivity was found in fraction 4 (8 to 10 min), indicating that acetate was formed. No radioactivity was eluted from the column when samples which had been incubated with boiled MnP preparations were separated. These results demonstrate that [$^{14}$C]PCP and [$^{14}$C]2-AmDNT were degraded by the MnP system to $^{14}CO_2$ and polar $^{14}$C-labeled fragments (ring fission products), which were probably low-molecular-weight organic acids.

**Mineralization of $^{14}$C-labeled aliphatic compounds.** Most of the $^{14}$C-labeled aliphatic compounds tested were mineralized to lesser extents than aromatic compounds (Table 1). Thus, sugars, such as glucose or fructose, the $C_1$ compound urea, and the hydrophobic amino acid leucine were only slightly mineralized by the MnP system (<2% $^{14}CO_2$). Levels of $^{14}CO_2$ of 4 to 6% were detected for aspartic acid, glutamic acid, and glycine. In contrast, the levels of mineralization of the carboxylic group of $^{14}$COOH-glyoxalate and the aldehyde group of $^{14}$CHO-glyoxalate were 86 and 9%, respectively. Thus, approximately 48% of glyoxalate was converted to $^{14}CO_2$ by the MnP system. With $^{14}$COOH-glyoxalate as the substrate, controls containing boiled MnP released 19% of the $^{14}$C as $^{14}CO_2$, indicating that even the $H_2O_2$ generated by glucose oxidase in combination with GSH and Mn(II) can attack glyoxalate. In additional control experiments in which glucose oxidase, GSH, and Mn(II) were omitted there were no noticeable releases of $^{14}CO_2$ (<3%).

## DISCUSSION

Diafiltered and purified preparations of MnP from the white rot fungus *N. frowardii* were capable of degrading a broad spectrum of aromatic and aliphatic substances directly to carbon dioxide and polar fission products. On the basis of this finding, the use of the term enzymatic combustion, used for the depolymerization of lignin, has to be evaluated again. Not only are nonspecific oxidations leading to a potpourri of diverging reactions and a high diversity of intermediate products affected by ligninolytic enzymes (15), but in the case of MnP from *N. frowardii* partial direct mineralization of aromatic and aliphatic substrates also occurs. In the end this means that MnPs from certain basidiomycetous fungi may represent an important system for the formation of carbon dioxide, especially from recalcitrant aromatic rings. This enzyme works extracellularly, and thus uptake of substrates into cells to mineralize the compounds is not necessarily required.

The effect of the MnP system on aromatic substrates resembles the effect described for abiotic Fenton's reagent, which chemically mineralizes aromatic substrates via formation of aggressive hydroxyl radicals (34). Hydroxyl radicals have been presumed to be the ultimate oxidants in the mineralization of the fluoroquinolone antibiotic enrofloxacin by the brown rot fungus *Gloeophyllum* sp., but the enzyme system responsible for generating hydroxyl radicals could not be identified (21). It has been shown that the cellobiose dehydrogenase of brown rot fungi and the LiP of white rot fungi (40) can provide a direct enzyme source for Fenton's reagent, but nothing about the ability of these systems to mineralize aromatic substrates has been reported. Whether hydroxyl radicals and/or other radical species are somehow involved in the mineralization process catalyzed by *N. frowardii* MnP is currently under investigation.

The extent of mineralization was considerably enhanced in the presence of the thiol GSH, a natural peptide produced by eucaryotic cells which protects cells against reactive oxygen species and free radicals (23), and it was found to be dependent on the ratio of GSH concentration to MnP activity. GSH amplified the oxidative strength of the primary mediator, Mn(III), probably by acting as a "secondary mediator," but it is still unclear whether GSH is a real redox mediator (which undergoes the whole reaction cycle again) or a cosubstrate. It is known that GSH enables MnP to convert veratryl alcohol to veratryl aldehyde (5, 6) and to cleave lignin model compounds via thiol-mediated oxidation (35). It has been postulated that these oxidations occur via benzylic radicals (side chain radicals) generated from thiyl radicals and/or hypothetic GSH-Mn(III) complexes (22), but in the presence of GSH the aromatic ring was not cleaved (5). Our findings, however, demonstrate that the MnP-GSH system is able to cleave different aromatic structures, since only the breakdown of aromatic rings allows the formation of $CO_2$. The formation of carboxylic acids after ring fission is probably the basis for the subsequent release of $CO_2$. Our finding that the level of residual radioactivity associated with highly polar substances eluted from the specific column for organic acids was in the same range as the levels of formate, glyoxalate, oxalate, malonate, and acetate supports this assumption and indicates that decarboxylation reactions may be the final step in the mineralization process. Direct mineralization of oxalate by MnP has been reported by Shimada et al. (31). In the present study, we found that glyoxalate, the direct precursor of oxalate, was also mineralized by MnP and that other aliphatic carboxylic acids can also be attacked. Sugars, the hydrophobic amino acid leucine, and the $C_1$ compound urea were unsuitable substrates for the MnP system.

The amount of $CO_2$ released from [$^{14}$C]2-AmDNT increased with the concentration of this aromatic substrate. Such a behavior is characteristic of pseudo-first-order kinetics that are observed for free radical reactions (1). Within an approximate concentration range of 2 to 120 $\mu$M, the percentages of mineralized [$^{14}$C]2-AmDNT were almost identical and the extent of relative mineralization was independent of the substrate concentration. For this reason, it is possible to compare the levels of mineralization of labeled substances with far different specific radioactivities by using the same initial radioactivity. Similar data were obtained for the mineralization of different concentrations of $^{14}$C-labeled polycyclic aromatic hydrocarbons in straw cultures of the white rot fungus *Pleurotus* sp. strain Florida (39). Moreover, recent evidence has shown that *Pleurotus* MnP is directly involved in the mineralization of pyrene in solid substrates (19).

The MnP system also mineralizes other aromatic compounds to a greater or lesser extent. Thus, all of the phenols tested were partially mineralized, and the highest level of release of $^{14}CO_2$ was observed for [$^{14}$C]PCP, whereas the level of degradation of [$^{14}$C]2,4-dichlorophenol was threefold less. We concluded that a high number of chlorine substituents makes attack by the MnP system easier. Furthermore, mineralization of [$^{14}$C]PCP required less GSH than mineralization of [$^{14}$C]2-AmDNT required, probably because the relative reactive hydroxyl group of PCP makes primary attack by MnP and the subsequent mineralization of the molecule easier. In vivo mineralization of PCP has been described for *Phanerochaete chrysosporium* (25), but as in the case of other pollutants, the actual role of ligninolytic enzymes in the mineralization process has remained unclear. In addition to a high number of chlorine substituents, the amino acid side chain in tyrosine and the second hydroxyl group in catechol also supported attack by MnP.

The present paper makes a contribution to the discussion about how ligninolytic fungi mineralize organic pollutants and xenobiotic compounds. The extracellular enzymatic combustion catalyzed by the MnP system provides an explanation for the way in which these fungi mineralize aromatic substances without ruling out the possibility that intracellular reactions are also involved in the mineralization process. More work is needed to characterize the MnP-mediator system of *N. frowardii* and compare it with the systems of other basidiomycetes. Furthermore, whether MnP is able to mineralize macromolecular substrates should also be examined. Our first experiments in which labeled straw lignin and synthetic humic substances were used indicated that the MnP-catalyzed depolymerization of these substrates is accompanied by release of carbon dioxide.

## ACKNOWLEDGMENTS

This work was supported by grants 0327051D and 145082A2 from the German Ministry for Education and Research (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie), as well as by the Fonds der Chemischen Industrie.

We thank I. Schwabe for excellent technical assistance and D. Ziegenhagen for assistance with the computer graphics.

## REFERENCES

1. **Barr, D. P., and S. D. Aust.** 1994. Mechanisms white rot fungi use to degrade pollutants. Environ. Sci. Technol. **28:**78A–87A.
2. **Bogan, B. W., R. T. Lamar, and K. Hammel.** 1996. Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. Appl. Environ. Microbiol. **62:**1788–1792.
3. **Bonnen, A., L. H. Anton, and A. B. Orth.** 1994. Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus*. Appl. Environ. Microbiol. **60:**960–965.
4. **Collins, P. J., and A. D. W. Dobson.** 1996. Oxidation of fluorene and phenanthrene by Mn(II) dependent peroxidase activity in whole cultures of *Trametes* (*Coriolus*) *versicolor*. Biotechnol. Lett. **18:**801–804.
5. **D'Annibale, A., C. Crestini, E. Di Mattia, and G. G. Sermanni.** 1996. Veratryl alcohol oxidation by manganese-dependent peroxidase from *Lentinus edodes*. J. Biotechnol. **48:**231–239.
6. **Forrester, I. T., A. C. Grabski, R. R. Burgess, and G. F. Leatham.** 1988. Manganese, Mn-dependent peroxidases and the biodegradation of lignin. Biochem. Biophys. Res. Commun. **157:**992–999.
7. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242:**329–341.
8. **Glenn, J. K., L. Akileswaran, and M. H. Gold.** 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251:**688–696.
9. **Hammel, K. E., P. J. Tardone, M. A. Moen, and L. A. Paice.** 1989. Biomimetic oxidation of nonphenolic lignin models by Mn(III): new observations on the oxidizability of guaiacyl and syringyl substructures. Arch. Biochem. Biophys. **270:**404–409.
10. **Harazono, K., R. Kondo, and K. Sakai.** 1996. Bleaching of hardwood Kraft pulp with manganese peroxidase from *Phanerochaete sordida* YK-624 without addition of $MnSO_4$. Appl. Environ. Microbiol. **62:**913–917.
11. **Hatakka, A.** 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13:**125–135.
12. **Hofrichter, M., and W. Fritsche.** 1997. Depolymerization of low-rank coal by extracellular fungal enzyme systems. II. The ligninolytic enzymes of the coal-humic-acid-depolymerizing fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol. **47:**419–424.
13. **Hofrichter, M., and W. Fritsche.** 1997. Depolymerization of low-rank coal by extracellular fungal enzyme systems. III. In vitro depolymerization of coal humic acids by a crude preparation of manganese peroxidase of the white-rot fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol. **47:**566–571.
14. **Jensen, K. A., Jr., W. B. Bao, S. Kawai, E. Srebotnik, and K. E. Hammel.** 1996. Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. Appl. Environ. Microbiol. **62:**3679–3686.
15. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
16. **Kirk, T. K., S. Croan, M. Tien, E. Murtagh, and R. L. Farell.** 1986. Produc-

tion of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8:**27–32.

17. **Kuwahara, M., J. K. Glenn, M. A. Morgan, and M. H. Gold.** 1984. Separation and characterization of two extracellular $H_2O_2$-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169:** 247–250.
18. **Lackner, R., E. Srebotnik, and K. Messner.** 1991. Oxidative degradation of high molecular weight chlorolignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **178:**1092–1098.
19. **Lang, E., F. Nerud, E. Novotna, F. Zadrazil, and R. Martens.** 1996. Production of ligninolytic exoenzymes and pyrene mineralization by *Pleurotus* sp. in lignocellulose substrate. Folia Microbiol. **41:**489–493.
20. **Maltseva, O. V., M.-L. Niku-Paavola, A. A. Leontievsky, N. M. Myasoedova, and L. A. Golovleva.** 1991. Ligninolytic enzymes of the white rot fungus *Panus tigrinus*. Biotechnol. Appl. Biochem. **13:**291–302.
21. **Martens, R., H.-G. Wetzstein, F. Zadrazil, M. Capelari, P. Hoffmann, and N. Schmeer.** 1996. Degradation of the fluoroquinolone enrofloxacin by wood-rotting fungi. Appl. Environ. Microbiol. **62:**4206–4209.
22. **McEldoon, J. P., and J. S. Dordick.** 1991. Thiol and $Mn^{2+}$-mediated oxidation of veratryl alcohol by horseradish peroxidase. J. Biol. Chem. **266:**14288–14293.
23. **Meister, A., and M. E. Anderson.** 1983. Glutathione. Annu. Rev. Biochem. **52:**711–760.
24. **Michels, J., and G. Gottschalk.** 1994. Inhibition of lignin peroxidase of *Phanerochaete chrysosporium* by hydroxylaminodinitrotoluene, an early intermediate in the degradation of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. **60:**187–194.
25. **Mileski, G. J., J. A. Bumpus, M. A. Jurek, and S. D. Aust.** 1988. Biodegradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **54:**2885–2889.
26. **Moen, M. A., and M. H. Hammel.** 1994. Lipid peroxidation by manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. **60:**1956–1961.
27. **Paice, M. G., I. D. Reid, R. Bourbonnais, F. S. Archibald, and L. Jurasek.** 1993. Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies Kraft pulp. Appl. Environ. Microbiol. **59:**260–265.
28. **Scheibner, K., M. Hofrichter, A. Herre, J. Michels, and W. Fritsche.** 1997. Screening of fungi intensively mineralizing 2,4,6-trinitrotoluene. Appl. Microbiol. Biotechnol. **47:**452–457.
29. **Scheibner, K., M. Hofrichter, and W. Fritsche.** 1997. Mineralization of 2-amino-4,6-dinitrotoluene by manganese peroxidase of the white-rot fungus *Nematoloma frowardii*. Biotechnol. Lett. **19:**835–839.
30. **Schneegaß, I., M. Hofrichter, K. Scheibner, and W. Fritsche.** 1997. Purification of the main manganese peroxidase isoenzyme MnP2 from the white-rot fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol. **48:**602–605.
31. **Shimada, M., D.-B. Ma, Y. Akamatsu, and T. Hattori.** 1994. A proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. FEMS Microbiol. Rev. **13:**285–296.
32. **Tuor, U., H. Wariishi, H. E. Schoemaker, and M. H. Gold.** 1992. Oxidation of phenolic arylglycerol β-aryl ether lignin model compounds by manganese peroxidase from *Phanerochaete chrysosporium*: oxidative cleavage of an α-carbonyl model compound. Biochemistry **31:**4986–4995.
33. **Valli, K., H. B. J. Brock, D. K. Joshi, and M. H. Gold.** 1992. Degradation of 2,4-dinitrotoluene by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**221–228.
34. **Venkatardi, R., and R. W. Peters.** 1993. Chemical oxidation technologies: ultraviolet light/hydrogen peroxide, Fenton's reagent, and titanium dioxide-assisted photocatalysis. Hazard. Waste Hazard. Mater. **10:**107–149.
35. **Wariishi, H., K. Valli, V. Renganathan, and M. H. Gold.** 1989. Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. J. Biol. Chem. **264:**14185–14191.
36. **Wariishi, H., K. Valli, and M. H. Gold.** 1991. In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **176:**269–275.
37. **Wariishi, H., K. Valli, and M. H. Gold.** 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelator. J. Biol. Chem. **267:**23688–23695.
38. **Wolfenden, B. S., and R. L. Willson.** 1982. Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions. J. Chem. Soc. Perkin. Trans. II **2:**805–812.
39. **Wolter, M., F. Zadrazil, R. Martens, and M. Bahadir.** Degradation of eight high condensed EPA-PAHs by *Pleurotus* sp. Florida in solid substrate. Appl. Microbiol. Biotechnol., in press.
40. **Wood, P. M.** 1994. Pathways for production of Fenton's reagent by wood-rotting fungi. FEMS Microbiol. Rev. **13:**313–320.