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Oxidative Degradation of Phenanthrene by the Ligninolytic Fungus *Phanerochaete chrysosporium*

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The ligninolytic fungus *Phanerochaete chrysosporium* oxidized phenanthrene and phenanthrene-9,10-quinone (PQ) at their C-9 and C-10 positions to give a ring-fission product, 2,2′-diphenic acid (DPA), which was identified in chromatographic and isotope dilution experiments. DPA formation from phenanthrene was somewhat greater in low-nitrogen (ligninolytic) cultures than in high-nitrogen (nonligninolytic) cultures and did not occur in uninoculated cultures. The oxidation of PQ to DPA involved both fungal and abiotic mechanisms, was unaffected by the level of nitrogen added, and was significantly faster than the cleavage of phenanthrene to DPA. Phenanthrene-*trans*-9,10-dihydrodiol, which was previously shown to be the principal phenanthrene metabolite in nonligninolytic *P. chrysosporium* cultures, was not formed in the ligninolytic cultures employed here. These results suggest that phenanthrene degradation by ligninolytic *P. chrysosporium* proceeds in order from phenanthrene → PQ → DPA, involves both ligninolytic and nonligninolytic enzymes, and is not initiated by a classical microsomal cytochrome P-450. The extracellular lignin peroxidases of *P. chrysosporium* were not able to oxidize phenanthrene in vitro and therefore are also unlikely to catalyze the first step of phenanthrene degradation in vivo. Both phenanthrene and PQ were mineralized to similar extents by the fungus, which supports the intermediacy of PQ in phenanthrene degradation, but both compounds were mineralized significantly less than the structurally related lignin peroxidase substrate pyrene was.

Phanerochaete chrysosporium, a ligninolytic basidiomycete that causes white rot of wood, can also degrade a wide variety of compounds that are significant environmental pollutants, including certain polycyclic aromatic hydrocarbons (PAHs) (1–3, 11, 12, 20, 22). White-rot fungi are unique among eukaryotes in their ability to cleave carbon-carbon bonds in PAHs, the biological degradation of these compounds formerly having been considered an exclusively bacterial process (8).

The breakdown of most organopollutants by ligninolytic fungi is closely linked with ligninolytic metabolism, in that the process is stimulated by nutrient limitation, and it is generally thought that enzymes whose normal function is lignin degradation also catalyze the highly nonspecific xenobiotic oxidations that are characteristic of these organisms (1–3, 10–13, 22). Ligninolysis in white-rot fungi is catalyzed in part by extracellular lignin peroxidases (LiPs) (9, 14, 18, 24), and these enzymes have been shown also to oxidize PAHs that have ionization potentials less than about 7.6 eV (10, 13). LiPs are therefore prime candidates to be the catalysts of initial PAH oxidation in *P. chrysosporium*, and for anthracene, which is a LiP substrate, the role of these enzymes in biodegradation has been demonstrated (12).

However, many of the organopollutants degraded by *P. chrysosporium* are not LiP substrates, and it seems clear that other LiP-independent mechanisms must exist for their initial oxidation. Phenanthrene is one PAH which we previously found not to be a LiP substrate (13), but which was subsequently reported to undergo degradation in nutrient-limited *P. chrysosporium* cultures (1, 20). An understanding of the pathway for phenanthrene degradation in *P. chrysosporium* would be an important first step in the elucidation of LiP-independent xenobiotic mechanisms in this fungus. We now report that *P. chrysosporium* oxidizes phenanthrene at its C-9 and C-10 positions to give a ring-fission product, 2,2′-diphenic acid (DPA).

MATERIALS AND METHODS

Unlabeled reagents. Unlabeled phenanthrene, phenanthrene-9,10-quinone (PQ), and 2,2′-biphenyldimethanol were obtained from Aldrich. DPA, also from Aldrich, was dissolved as the neutral sodium salt in H_2O, and H_2O-insoluble impurities were removed from the H_2O phase by extraction with $CHCl_3$. DPA was then precipitated by adjusting the pH to 0, and the precipitate was recrystallized twice from H_2O.

Organic syntheses. Phenanthrene-*trans*-9,10-dihydrodiol was prepared by reducing PQ with $NaBH_4$ in 87% ethanol under an atmosphere of air (21) and was recrystallized from benzene-methanol (mp 183 to 186°C [literature mp 185 to 187°C]) (21); mass spectrum (diacetate) was *m/z* (relative intensity) 296 (M^+, 0.4); 236 ($-H_2CCO-H_2O$, 12.8); 194 ($-2H_2CCO-H_2O$, 100.0); 165 ($-2H_2CCO-H_2O-HCO$, 51.1). Phenanthrene-*cis*-9,10-dihydrodiol was obtained by oxidizing phenanthrene with OsO_4 in pyridine-benzene (5) and was recrystallized from $CHCl_3$ (mp 177 to 179°C [literature mp 178 to 179°C]) (6); mass spectrum (diacetate) was *m/z* (relative intensity) 296 (M^+, 4.5); 236 ($-H_2CCO-H_2O$, 8.7); 194 ($-2H_2CCO-H_2O$, 100); 165 ($-2H_2CCO-H_2O-HCO$, 72.8). All other reagents were of the highest quality commercially available.

^{14}C-labeled compounds. [9-^{14}C]phenanthrene (13.1 mCi mmol^{-1}), [1-^{14}C]naphthalene (8.0 mCi mmol^{-1}), and [7-^{14}C]benzo[*a*]pyrene (30.0 mCi mmol^{-1}), all from Sigma, were

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>98% radiochemically pure. [4-^{14}C]pyrene (10.1 mCi mmol^{-1}, >98% radiochemically pure) was obtained from Chemsyn Science Laboratories (Lenexa, Kans.).

[9-^{14}C]PQ was prepared from [9-^{14}C]phenanthrene by dichromate oxidation (26). Labeled phenanthrene (3.0 mg) was dissolved in 0.90 ml of hot (approximately 90°C) glacial acetic acid that was saturated in $K_2Cr_2O_7$. The solution was tightly capped, stirred at 110°C for 23 h, and then allowed to cool, after which about 2 ml (each) of ethyl acetate and H_2O was added and the mixture was shaken. The ethyl acetate fraction was collected, and the H_2O fraction was extracted five times more with ethyl acetate. The ethyl acetate fractions were then pooled and washed three times with saturated Na_2CO_3, dried over a short column of Na_2SO_4, and concentrated to a volume of about 1 ml under a stream of argon. The concentrated, crude [9-^{14}C]PQ, obtained in about 30% yield, was purified by preparative thin-layer chromatography (TLC) in CH_2Cl_2 (two cycles) on a 1-mm-thick plate of silica gel 60 (20 by 20 cm; Merck) to give a product with a radiochemical purity of about 75%. This partially purified [9-^{14}C]PQ was then subjected to preparative reversed-phase high-performance liquid chromatography (HPLC) on a 5-μm Hamilton PRP-1 (styrene divinylbenzene) column (4.1 by 150 mm) in H_2O-acetonitrile (55:45, vol/vol; flow rate, 1 ml min^{-1}; temperature, ambient). The pooled peak fractions, which eluted between 17 and 20 min, consisted of chromatographically homogeneous [9-^{14}C]PQ with a radiochemical purity of >98%. The final product was extracted into CH_2Cl_2, dried over Na_2SO_4, and concentrated to dryness by rotary vacuum evaporation. UV/visible absorption spectrum was as follows: λ (relative absorbance in tetrahydrofuran) 257 nm (1.00), 311 nm (0.14), 399 nm (0.06). Mass spectrum was *m/z* (relative intensity) 210 ([M^++2], 5.6); 208 (M^+, 22.6); 182 ([M^++2]−^{12}CO, 7.7); 180 ([M^++2]−^{14}CO, M^+−^{12}CO, 100.0); 152 ([M^++2]−^{14}CO−^{12}CO, M^+−2^{12}CO, 37.5).

[*carboxyl*-^{14}C]DPA was found as a by-product in the fractions that eluted with the void volume during HPLC purification of [9-^{14}C]PQ. A portion of this material (approximately 5 μCi) was repurified by reversed-phase HPLC on a Hamilton PRP-1 column in H_2O-acetonitrile-acetic acid (78:22:1, vol/vol; flow rate, 1 ml min^{-1}; temperature, ambient). The peak fraction, which eluted at 18 min, was chromatographically homogeneous and consisted of >98% radiochemically pure [*carboxyl*-^{14}C]DPA. Mass spectrum (dimethyl ester) was *m/z* (relative intensity) 272 (M^++2, 0.2); 270 (M^+, 1.5); 241 ([M^++2]−OCH_3, 0.3); 239 (M^+−OCH_3, 2.2); 213 ([M^++2]−^{12}CO−OCH_3, 7.7); 211 ([M^++2]−^{14}CO−OCH_3, M^+−^{12}CO−OCH_3, 100); 196 (15.9); 182 ([M^++2]−^{12}CO−$2OCH_3$, 1.3); 180 ([M^++2]−^{14}CO−$2OCH_3$, M^+−^{12}CO−$2OCH_3$, 14.7); 168 (8.8); 152 ([M^++2]−^{14}CO−^{12}CO−$2OCH_3$, M^+−2^{12}CO−$2OCH_3$, 19.5); 139 (14.8).

[*carboxyl*-^{14}C]DPA dimethyl ester was prepared by treating a methanol solution of [*carboxyl*-^{14}C]DPA with diazomethane in ether for 5 min at 0°C. The ether and remaining diazomethane were evaporated under a stream of argon, and the radiolabeled ester was used in mineralization studies without further purification.

Metabolic experiments. Cultures of *P. chrysosporium* (ATCC 24725) were set up for mineralization and metabolite analysis studies as described previously (12). The experiments were done with 25-ml rotary-shaken cultures, supplemented with 0.1% Tween 80, that contained a biomass of about 2 g of mycelia (dry weight) liter^{-1}. Low-N cultures contained 1.1 mM ammonium tartrate as the N source, whereas high-N cultures contained 30.0 mM ammonium tartrate. Stock solutions of ^{14}C-labeled substrates were added to the cultures as less than 0.1% of the culture volume. The PAHs and PQ were added in *N*,*N*-dimethylformamide, DPA was added in HPLC eluant (H_2O-acetonitrile-acetic acid, 78:22:1, vol/vol), and DPA dimethyl ester was added in methanol. In mineralization experiments, which were conducted as described previously (12), radiolabeled compounds were added at an initial concentration of 0.2 μM. When phenanthrene metabolites were to be extracted and analyzed by TLC, the labeled compounds were added at 2.0 μM initial concentration. In isotope dilution experiments, the initial concentration of [^{14}C]phenanthrene or [^{14}C]PQ was 4.0 μM. When direct analysis of the extracellular culture medium was to be performed by HPLC, the initial concentration of labeled substrate was 8.3 μM. The cultures were examined regularly by microscopy for bacterial contamination, with negative results.

Metabolite analysis. For the extraction of phenanthrene metabolites, four to six cultures were pooled and adjusted to pH 7. $CHCl_3$ (1 volume) was then added, and the mycelial pellets were homogenized by sonication, with the temperature of the mixture maintained at <20°C throughout the procedure. Cell debris was removed by centrifugation in stainless steel bottles, and the aqueous and $CHCl_3$ fractions were then collected separately. Subsequent steps were done essentially as described previously (12). The aqueous fraction was further extracted with $CHCl_3$-acetone (1:1, vol/vol) and then adjusted to pH 1.5 and reextracted with $CHCl_3$-acetone. The pH 7 organic extracts were pooled, dried, concentrated, and mixed with authentic standards of the following compounds: phenanthrene, PQ, phenanthrene-*trans*-9,10-dihydrodiol, phenanthrene-*cis*-9,10-dihydrodiol, and 2,2′-biphenyldimethanol. The samples were then analyzed for neutral phenanthrene metabolites by TLC on Merck silica gel 60 F_{254} plates (20 by 20 cm; 0.25 mm thick) in toluene-ethyl acetate-formic acid (100:100:2, vol/vol). ^{14}C-labeled phenanthrene metabolites were detected by autoradiography on Kodak X-OMAT AR film, and standards on the plates were viewed under UV illumination. The pH 1.5 organic extracts were methylated with diazomethane, mixed with an authentic standard of DPA dimethyl ester, and analyzed for acidic phenanthrene metabolites by TLC and autoradiography on Merck silica plates in *n*-hexane–ethyl acetate (3:1, vol/vol). The unmethylated pH 1.5 extract was also analyzed for acidic metabolites by ion-exclusion HPLC as described previously (12).

Direct analysis of extracellular phenanthrene metabolites in *P. chrysosporium* culture medium was done by reversed-phase HPLC on a Hamilton PRP-1 column under the eluant and flow conditions described above for the purification of radiolabeled DPA. Cultures (four replicates) were given [9-^{14}C]phenanthrene or [9-^{14}C]PQ on day 0, and at intervals thereafter, samples (0.20 ml) of culture medium were taken from each culture and pooled. A portion (0.60 ml) of the combined sample was centrifuged through a 0.45-μm-pore-size microcentrifuge filter, and the filter was rinsed twice by centrifugation with 0.08 ml of acetonitrile-acetic acid (100:1, vol/vol). Of the resulting combined filtrates, 0.50 ml was injected directly onto the HPLC column, and fractions (0.5 ml) were collected for scintillation counting. This procedure gave good estimations of DPA in the cultures, but it greatly underestimated the amount of residual phenanthrene, because most of this hydrophobic PAH was located in the mycelium rather than in the culture medium.

Peaks A and B (see Results) from the reversed-phase HPLC procedure described above were analyzed further by

normal-phase HPLC. The collected fractions from a series of metabolite analyses on the PRP-1 column were extracted into ethyl acetate, dried over Na_2SO_4, and concentrated under a stream of argon. Samples (0.27 ml), containing 2.6×10^5 dpm in the case of peak A and 7.3×10^5 dpm in the case of peak B, were then injected onto a 7-μm Dupont Zorbax silica column (4.6 by 250 mm), which was run in *n*-hexane–ethyl acetate (7:3, vol/vol; flow rate, 1 ml min^{-1}; temperature, ambient). Fractions (0.5 ml) were collected and assayed for ^{14}C by scintillation counting.

Determination of DPA by isotope dilution. For each experiment, four replicate 25-ml cultures that had been incubated for 4 days with [^{14}C]phenanthrene or [^{14}C]PQ were pooled, and a known amount of unlabeled crystalline DPA (approximately 1.9 g ± 1 mg) was added to the extracellular medium, which was then adjusted to pH 8 with NaOH and stirred until the sodium diphenate was completely dissolved. The mycelial pellets were then disrupted by sonication, the mixture was stirred for 1 h, and cell debris was removed by centrifugation (16,000 × *g*, 15 min). The supernatant fraction was extracted exhaustively with $CHCl_3$ to remove hydrophobic ^{14}C (mostly [^{14}C]phenanthrene) from which DPA could not be purified by recrystallization in H_2O. Crystals of DPA were then precipitated from the aqueous phase by adjusting the pH to 0 with HCl and were recrystallized repeatedly from H_2O. Samples (approximately 50 mg) from each crop of crystals were dried for 8 days over $CaSO_4$, weighed, and assayed by scintillation counting. Four to six recrystallizations were required to attain constant ^{14}C specific activity.

RESULTS

Fungal oxidation of phenanthrene to DPA. *P. chrysosporium* oxidized phenanthrene at positions 9 and 10 to give a ring-fission product, DPA. The major phenanthrene metabolite in N-limited fungal cultures chromatographed identically with authentic DPA by reversed-phase HPLC (Fig. 1, top) and ion-exclusion HPLC (data not shown), and after methylation with diazomethane it ran identically with DPA dimethyl ester by silica gel TLC (Fig. 2). HPLC analyses of pooled samples from four replicate cultures showed that DPA accumulated in the extracellular medium for about 10 days and finally accounted for 13% of initially added phenanthrene (Fig. 3). Other extracellular products were also observed and accounted for about 50% of the starting material (Fig. 1, top). TLC experiments showed that uninoculated cultures produced no acidic metabolites (Fig. 2).

The identification of DPA as a phenanthrene metabolite in *P. chrysosporium* was confirmed in an isotope dilution experiment. In ligninolytic cultures, approximately 9% of added phenanthrene was converted in 4 days to a product that recrystallized repeatedly with authentic DPA (Table 1). The experiment also showed that phenanthrene oxidation to DPA was partially suppressed under nonligninolytic (high-N) culture conditions, and it confirmed that no DPA was formed from phenanthrene in uninoculated cultures.

Neutral metabolites of phenanthrene. In an attempt to identify the immediate precursor to DPA, we analyzed the neutral $CHCl_3$-acetone extracts from fungal cultures for the presence of 9,10-oxidized phenanthrene metabolites. These fractions contained, in addition to unmetabolized phenanthrene, two other major components that were observed by HPLC and TLC analysis (Fig. 1, top; Fig. 4). The less-polar component (A) chromatographed indistinguishably from phenanthrene-*trans*-9,10-dihydrodiol, whereas the more-polar one (B) ran identically with standards of phenanthrene-*cis*-9,10-dihydrodiol and 2,2′-biphenyldimethanol, which were not resolved from each other in these chromatographic systems. However, when the collected peaks A and B from a series of reversed-phase HPLC runs were rechromatographed by normal-phase HPLC on silica, both components were shown to be mixtures, and none of the resolved peaks coeluted with either of the two dihydrodiol standards (Fig. 1,

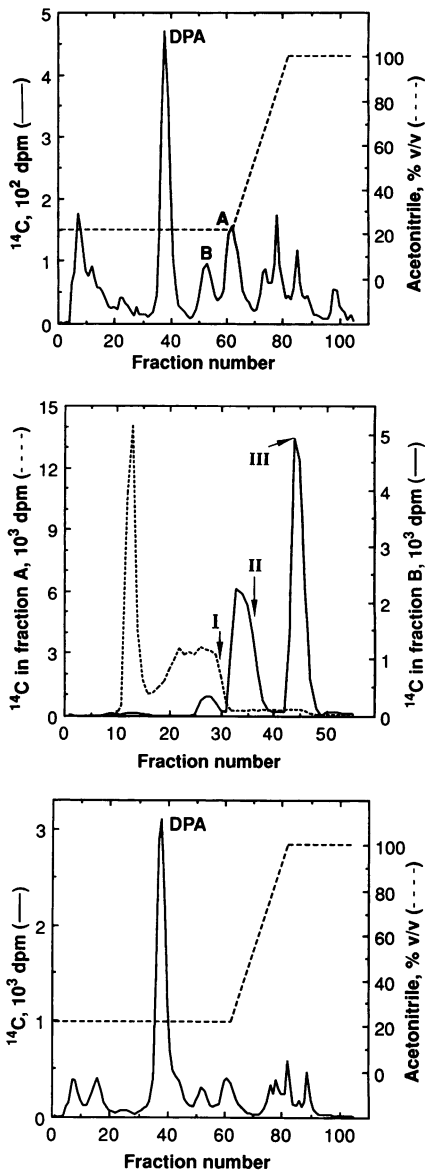


FIG. 1. HPLC analyses of extracellular metabolites formed in 2 days by N-limited *P. chrysosporium* from [9-^{14}C]phenanthrene and [9-^{14}C]PQ. Top: Reversed-phase HPLC of metabolites from [9-^{14}C] phenanthrene. Peaks A and B indicate fractions taken for further normal-phase HPLC analysis. The peak positions for authentic standards were as follows: PQ, fraction 83; phenanthrene, fraction 98. Middle: Normal-phase HPLC of the collected peaks A and B from a series of reversed-phase analyses of [9-^{14}C]phenanthrene metabolites. The peak positions for authentic standards are indicated as follows: I, phenanthrene-*trans*-9,10-dihydrodiol; II, phenanthrene-*cis*-9,10-dihydrodiol; III, 2,2′-biphenyldimethanol. Bottom: Reversed-phase HPLC of metabolites from [9-^{14}C]PQ. The peak position for an authentic PQ standard was fraction 83.

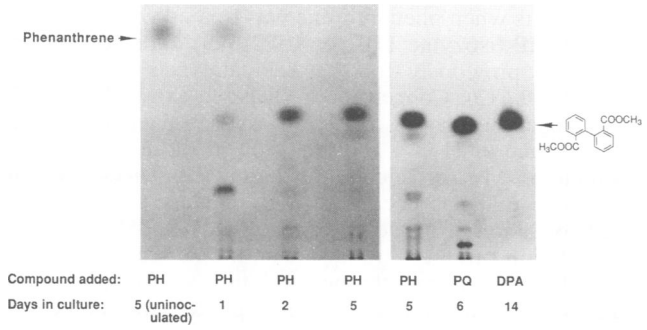


FIG. 2. Silica gel TLC and autoradiography of acidic metabolites formed from [9-^{14}C]phenanthrene (PH), [9-^{14}C]PQ, and [*carboxyl*-^{14}C]DPA by *P. chrysosporium* after the indicated times in N-limited culture. The metabolites were methylated with diazomethane before analysis. Some residual phenanthrene, not removed during the initial neutral extraction, is apparent in the uninoculated and day 1 samples.

middle). 2,2′-Biphenyldimethanol, however, was tentatively confirmed as a phenanthrene metabolite on the basis of its chromatographic properties. No neutral products derived from phenanthrene were found in uninoculated cultures (Fig. 4).

The absence of phenanthrene-*cis*-9,10-dihydrodiol in *P. chrysosporium* cultures was not surprising, since *cis*-dihydrodiols are characteristic of bacterial dioxygenase-mediated PAH metabolism (8, 17). However, the failure of phenanthrene-*trans*-9,10-dihydrodiol to accumulate was unexpected, because PAH *trans*-dihydrodiols are generally formed from PAHs by fungi (4, 7, 8), and the 9,10-isomer was previously reported to be the principal phenanthrene metabolite formed by nonligninolytic *P. chrysosporium* (23). To consider the possibility that phenanthrene-*trans*-9,10-dihydrodiol was in fact formed in these experiments but then rapidly cleaved to DPA by the fungus, we added the unlabeled dihydrodiol to ligninolytic cultures and then performed HPLC analyses to determine whether it was converted to other products. The results showed that phenanthrene-*trans*-9,10-dihydrodiol persisted in N-limited fungal cultures and that only trace quantities of DPA could be detected (data not shown).

Fungal oxidation of PQ. A search for other 9,10-oxidized phenanthrene metabolites that might be DPA precursors led us to examine the metabolism of PQ in *P. chrysosporium*. The chemical oxidation of this *o*-quinone is a facile reaction

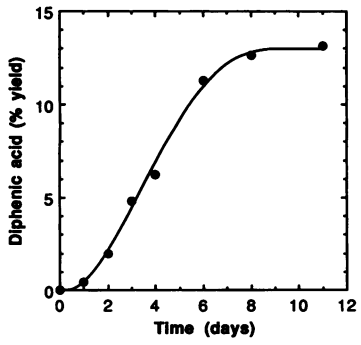


FIG. 3. Time course of extracellular DPA accumulation in N-limited *P. chrysosporium* cultures.

TABLE 1. Determination by isotope dilution of the [^{14}C]DPA produced by *P. chrysosporium* from [^{14}C]phenanthrene or [^{14}C]PQ

Culture conditions	^{14}C present as DPA (% of total ^{14}C added)
Plus [^{14}C]phenanthrene	
Low N	9.2
High N	5.8
Uninoculated (low N)	0.0
Plus [^{14}C]PQ	
Low N	41.5
High N	42.7
Uninoculated (low N)	15.5

(16), and DPA is formed as a side product when phenanthrene is chemically oxidized to produce PQ (see Materials and Methods). PQ was not observable as a metabolite of phenanthrene in *P. chrysosporium* (Fig. 4), but experiments with the unlabeled quinone showed that it was rapidly depleted from fungal cultures, none of the starting material being found after 24 h (data not shown). Phenanthrene, by contrast, was detectable in the cultures for at least 5 days (Fig. 4). HPLC and TLC analyses showed that the overall pattern of PQ oxidation in vivo resembled the pattern found for phenanthrene and that the major product formed from PQ was DPA (Fig. 1, bottom; Fig. 2). The isotope dilution experiment confirmed this identification and showed that the oxidation of PQ to DPA was considerably faster than the oxidation of phenanthrene to DPA (Table 1). DPA production from the quinone also occurred in uninoculated cultures to about one-third the extent found in vivo, which indicates that an appreciable component of PQ ring fission was abiotic. PQ cleavage to give DPA was unaffected by the level of N supplied to the cultures.

Mineralization of phenanthrene and PQ. *P. chrysosporium* oxidized [9-^{14}C]phenanthrene and [9-^{14}C]PQ (0.2 μM initial concentration) to $^{14}CO_2$ in similar yields (Table 2). The extent of phenanthrene mineralization tended to saturate as the initial PAH concentration was increased. For example, a typical set of four replicate low-N cultures, when given 0.2 μM phenanthrene, mineralized 5.8% ± 1.3% in 7 days, whereas four replicates given 2.0 μM phenanthrene mineralized only 2.8% ± 0.6% in this time. The mineralization of

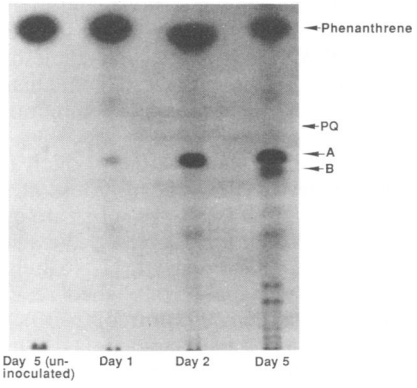
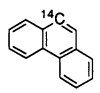
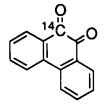
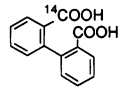
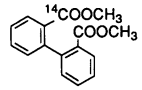
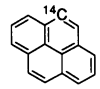


FIG. 4. Silica gel TLC and autoradiography of neutral metabolites formed from [9-^{14}C]phenanthrene by *P. chrysosporium* after the indicated times in N-limited culture.

TABLE 2. Mineralization of phenanthrene and structurally related compounds by *P. chrysosporium*[a]

Structure	% of total mineralized	
	Low-N cultures	High-N cultures
	6.2 ± 1.4 (1) 6.4 ± 1.3 (2) 6.4 ± 0.5 (3) 5.6 ± 1.1 (4)	0.11 ± 0.04 (2) 0.22 ± 0.07 (4)
	5.8 ± 2.2 (2) 5.4 ± 0.6 (4)	1.1 ± 0.2 (4)
	3.3 ± 0.9 (2) 2.2 ± 0.5 (4)	0.11 ± 0.03 (4)
	1.2 ± 0.5 (4)	0.04 ± 0.01 (4)
	16.1 ± 3.4 (1) 16.1 ± 2.9 (3)	ND[b]

[a] Experiments were conducted for 12 days, after which time mineralization had ceased. Values shown are means for four replicate cultures ± the standard deviation of the sample. Four independent experiments were conducted: numbers in parentheses indicate which one was the source of each data set. The chemical structures indicated are, from top to bottom: phenanthrene, PQ, DPA, DPA dimethyl ester, and pyrene.

[b] ND, not determined.

all compounds tested was suppressed under nonligninolytic (high-N) culture conditions (Table 2).

[*carboxyl*-^{14}C]DPA, the ring-fission product from [9-^{14}C] phenanthrene and [9-^{14}C]PQ, was also mineralized to a small extent, but these low CO_2 yields are not significant, considering the radiochemical purity of the [^{14}C]DPA that was supplied to the cultures. Moreover, no DPA intermediary metabolites that might indicate the pathway for this decarboxylation were detected in the chromatographic experiments (Fig. 2). One possible explanation for the low extent of DPA mineralization by *P. chrysosporium* is that uptake of the ionized acid by the fungus is slow, and in an attempt to investigate this possibility, we monitored the mineralization of a nonpolar DPA derivative, DPA dimethyl ester, in ligninolytic cultures. The results were inconclusive, in that a decrease rather than an increase in mineralization was observed.

It is noteworthy that phenanthrene, which is not a LiP substrate, was mineralized significantly less at its C-9 position than the structurally related LiP substrate pyrene was at its corresponding C-4 position (Table 2). We also found that mineralization of the LiP substrate [7-^{14}C]benzo[*a*]pyrene was consistent with previously published results (7.9% ± 1.9% in 14 days, four replicate cultures) (3, 10), whereas the mineralization of analogously labeled [1-^{14}C]naphthalene, which is not a LiP substrate, was negligible (0.5% ± 0.2% in 14 days, four replicate cultures).

Failure of LiP to oxidize phenanthrene. No UV/visible spectral changes were observable in spectrophotometric experiments when phenanthrene was treated with H_2O_2 and purified LiP (isozyme LiP1 or LiP2) (19) under conditions developed previously for the LiP-catalyzed oxidation of other PAHs (13). Concentrated, dialyzed extracellular fluid from fungal cultures also failed to oxidize phenanthrene in the presence of H_2O_2. The inclusion in these reactions of 3,4-dimethoxybenzyl alcohol, a natural *P. chrysosporium* metabolite that has been shown to enhance the LiP-catalyzed oxidation of borderline substrates (15, 25), also gave no oxidation products from phenanthrene. To address the possibility that phenanthrene oxidation was catalyzed by LiP, but at too low a rate for spectrophotometric detection, we performed overnight reactions with [9-^{14}C]phenanthrene, H_2O_2, 3,4-dimethoxybenzyl alcohol, and a crude LiP mixture by the procedure used previously to depolymerize lignin with LiPs in vitro (14). TLC analysis and autoradiography of these reactions showed again that there was no conversion of phenanthrene to other products.

DISCUSSION

These results show that 9,10-oxidation and ring cleavage to give DPA is a major fate of phenanthrene in ligninolytic *P. chrysosporium*. Phenanthrene metabolism in ligninolytic *P. chrysosporium* differs from the pathway employed by most bacteria, which cleave this PAH between positions 3 and 4 (8), and also differs from the process in nonligninolytic fungi and other eukaryotes, which are incapable of PAH ring fission (4, 8). Sutherland et al. (23) recently reported that nonligninolytic (i.e., high-N) cultures of *P. chrysosporium* ATCC 34541 fit the typical eukaryotic pattern, in that they metabolize phenanthrene to *trans*-dihydrodiols and phenanthrol conjugates. These investigators obtained the same result with *P. chrysosporium* ATCC 24725, the strain used in the work reported here, and in both strains phenanthrene-*trans*-9,10-dihydrodiol was found to be the major metabolite (22a, 23). Our results show that ligninolytic *P. chrysosporium*, by contrast, does not accumulate this metabolite. The explanation for this result cannot simply be that the dihydrodiol was both formed and rapidly degraded in our experiments, because we also found that an authentic standard of this compound, when added to low-N cultures, was readily detectable in the extracellular medium for several days. These results indicate that phenanthrene-*trans*-9,10-dihydrodiol is not an intermediate in DPA production from phenanthrene in ligninolytic cultures. We do not yet know why 9,10-dihydrodiol formation is suppressed under these conditions, but it may be that microsomal monooxygenases such as cytochrome P-450 are down-regulated during ligninolytic metabolism.

The data support a degradative pathway in which phenanthrene is cleaved to DPA via PQ. Both phenanthrene and PQ were degraded in ligninolytic cultures to give similar product profiles (Fig. 1). Although PQ was not detectable as a metabolite in cultures that had been given phenanthrene, the quinone was degraded to DPA about five times faster than the PAH was, and under these conditions, it is unlikely that PQ would accumulate to a detectable concentration. The proposed pathway thus consists of a slow initial oxidation to give PQ and then a rapid ring-fission reaction that yields DPA (Fig. 5). This oxidative 9,10-cleavage of PQ can occur spontaneously but is accelerated significantly by the fungus. 2,2′-Biphenyldimethanol also occurred as a minor phenanthrene metabolite in these experiments, but this dialcohol was probably a reduction product of DPA rather than a

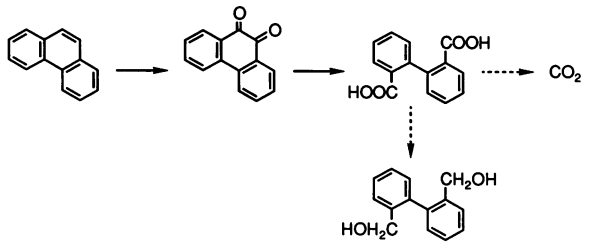


FIG. 5. Proposed pathway for phenanthrene degradation in ligninolytic *P. chrysosporium*. Major reactions (solid arrows) and minor ones (dashed arrows) are shown.

direct product of phenanthrene ring fission, because it was formed more slowly in culture than DPA was (Fig. 2 and 4).

The intermediacy of PQ in phenanthrene degradation is also supported by the mineralization studies, in that both the PAH and its quinone were oxidized to CO_2 in similar yields. However, rates of mineralization tended to saturate at higher initial phenanthrene concentrations, and CO_2 was a minor metabolite, compared with DPA, in cultures that were given micromolar concentrations of the PAH. It is likely that some of the DPA formed from phenanthrene and PQ in these experiments was further degraded, but it remains uncertain whether DPA is an obligatory intermediate in phenanthrene mineralization.

The mineralization data also suggest that PAHs which are LiP substrates are more susceptible to mineralization than PAHs which are not. The likely explanation for these findings is that the initial oxidation of nonsubstrate PAHs is a slow, and possibly rate-limiting, step in their biodegradation, as evidenced here by the persistence of phenanthrene in *P. chrysosporium* cultures (Fig. 4). The metabolism of PAHs that are LiP substrates is, by contrast, a rapid process: benzo[*a*]pyrene (22), pyrene (13), and anthracene (12) are all rapidly depleted from N-limited cultures. *P. chrysosporium* cultures grown under N-limited conditions are competent biodegraders for only a limited time, generally 10 to 30 days, with about 80% of mineralization occurring during the first half of the experiment (3, 12). Under these conditions, the presence of a slow initial step in PAH catabolism will limit the availability of oxidation products such as PQ and DPA during the brief period that systems are present to degrade them further, with the consequence that total degradation is decreased.

It is interesting that DPA production from phenanthrene was not strongly influenced by the level of N supplied to the cultures. This result suggests that phenanthrene ring fission is not a strict function of ligninolytic metabolism, although subsequent steps that lead to mineralization clearly are regulated by nutrient N levels. The agents responsible for phenanthrene ring fission in *P. chrysosporium* have yet to be identified. LiP is unlikely to be involved, not only because the production of this enzyme is strongly suppressed by high N levels in vivo (9, 18, 24), but also because attempts to oxidize phenanthrene with LiP preparations in vitro under various conditions have given uniformly negative results. The phenanthrene-oxidizing system of *P. chrysosporium* is evidently more oxidizing than LiP is and therefore might play a significant role in the xenobiotic metabolism of this white-rot fungus. Attempts to characterize the process are under way.

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