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Lipid Peroxidation by the Manganese Peroxidase of *Phanerochaete chrysosporium* Is the Basis for Phenanthrene Oxidation by the Intact Fungus

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The manganese peroxidase (MnP) of *Phanerochaete chrysosporium* supported Mn(II)-dependent, H_2O_2-independent lipid peroxidation, as shown by two findings: linolenic acid was peroxidized to give products that reacted with thiobarbituric acid, and linoleic acid was peroxidized to give hexanal. MnP also supported the slow oxidation of phenanthrene to 2,2′-diphenic acid in a reaction that required Mn(II), oxygen, and unsaturated lipids. Phenanthrene oxidation to diphenic acid by intact cultures of *P. chrysosporium* occurred to the same extent that oxidation in vitro did and was stimulated by Mn. These results support a role for MnP-mediated lipid peroxidation in phenanthrene oxidation by *P. chrysosporium*.

The ligninolytic fungi that cause white rot of wood are unique among eukaryotes in their ability to degrade polycyclic aromatic hydrocarbons (PAHs) (8, 20, 22). PAHs are ubiquitous, toxic organopollutants of both natural and anthropogenic origin. Their toxicity underlies the use of creosote, a PAH mixture, as a fungicidal wood preservative. However, many fungi, including certain white rot basidiomycetes, tolerate this treatment and grow on creosote-treated wood (40). White rot fungi have also been shown to deplete and detoxify PAHs in contaminated soils (1, 2, 10).

The ability of white rot fungi to degrade PAHs stems in part from the action of ligninolytic enzymes on these hydrocarbons. For example, the first step in anthracene oxidation by *Phanerochaete chrysosporium* is catalyzed by extracellular lignin peroxidases (22). However, some PAHs that are not lignin peroxidase substrates are nevertheless degraded by white rot fungi (7). We recently reported (21) that *P. chrysosporium* cleaves one such PAH, phenanthrene, to 2,2′-diphenic acid (DPA) by an unknown mechanism (Fig. 1). We now show that this reaction is a cooxidative consequence of lipid peroxidation by another ligninolytic fungal enzyme, manganese peroxidase (MnP).

MATERIALS AND METHODS

Reagents. Crude fungal peroxidases were isolated from the extracellular medium of 5-day-old, N-limited, rotary-shaken cultures of *P. chrysosporium* by ultrafiltration and dialysis. MnP isozyme H4 was purified from this preparation by quaternary aminoethyl ion-exchange chromatography and Cibacron blue agarose chromatography as described previously (18, 27). For one experiment, we used MnP H4 that had been purified from an overproducing strain of *P. chrysosporium* (34) by chromatography and preparative isoelectric focusing (23). This preparation, a generous contribution from M. Tien (Pennsylvania State University), was homogeneous by analytical isoelectric focusing.

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[9-^{14}C]phenanthrene (13.1 mCi mmol^{-1}; >98% radiochemically pure) was obtained from Sigma. Monolinolein (1-monolinoleoyl-rac-glycerol), monoolein (1-monooleoyl-rac-glycerol), monostearin (1-monostearoyl-rac-glycerol), linoleic acid, and linolenic acid were obtained from Sigma, by dry ice shipment in the case of unsaturated lipids, and stored under argon at −20°C. Surfactants were the low peroxide-low carbonyl grade (Surfact-Amps) provided by Pierce Chemical Co. All other chemicals were reagent grade or better.

Oxidation of phenanthrene in vitro. Reagents were sterilized before use by filtration through 0.45-μm-pore-size microcentrifuge filters, and reactions were conducted in silanized sterile containers: loosely capped 7-ml vials for aerobic reactions or 10-ml serum vials sealed under argon for anaerobic reactions. Complete reactions (2.0 ml) contained sodium glycolate (20 mM; pH 4.5), Tween 20 (0.1%, wt/vol), a glyceryl fatty acid ester as indicated (1.0 mM), $MnSO_4$ (0.20 mM), [^{14}C]phenanthrene (10.4 μM; 6.00×10^5 dpm), and MnP as indicated below. Ingredients were added in the following order. The glyceryl fatty acid ester was added at a 100 mM concentration to the Tween 20 stock solution (10%, wt/vol), and 20 μl of the resulting solution was added to the reaction vial with the required amounts of H_2O and buffer. Vortex mixing of these ingredients yielded a clear emulsion, to which $MnSO_4$ and [^{14}C]phenanthrene (as a 5.8 mM solution in *N,N*-dimethylformamide) were added.

Reactions were then initiated by adding 0.5 nmol of chromatographically purified MnP, and an additional 0.1 nmol of enzyme was added daily thereafter for a total reaction time of 8 days. One experiment was done with isoelectric focusing-purified MnP, using 0.25 nmol of enzyme on the first day and an additional 0.05 nmol of enzyme daily thereafter. The reaction vials were rotary shaken at room temperature throughout the experiment, and samples (250 μl) were periodically removed and filtered through 0.45-μm-pore-size microcentrifuge filters. The filters were rinsed twice by centrifugation with 35 μl of acetonitrile-acetic acid (19:1), and a portion (250 μl) of the combined filtrates was analyzed by high-performance liquid chromatography (HPLC) as described below. All manipulations involving reagent addition or sample removal were conducted aseptically in a microbiological transfer hood.

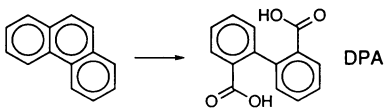

FIG. 1. Cleavage of phenanthrene to DPA.

Assays for lipid peroxidation. Assays by the thiobarbituric acid (TBA) method were done as described by Buege and Aust (6), using TBA reagent that was supplemented with 0.01% (wt/vol) butylated hydroxytoluene. Complete reaction mixtures (4.0 ml) contained sodium glycolate (20 mM; pH 4.5), Tween 20 (0.1%, wt/vol), linolenic acid (0.33 mM), and $MnSO_4$ (0.20 mM). H_2O_2 was also included in some assays at a concentration of 0.40 mM. Reactions were initiated with MnP H4 (0.1 nmol), after which samples (0.5 ml) were removed at intervals, combined with 1.0 ml of TBA reagent, capped to prevent evaporation, and incubated at 100°C for 15 min. The cooled samples were clarified by centrifugation, and their absorbances were measured at 535 nm.

For the determination of hexanal production during lipid peroxidation, the complete assay (25.0 ml) contained sodium glycolate (20 mM; pH 4.5), Tween 20 (0.1%, wt/vol), linoleic acid (0.33 mM), and $MnSO_4$ (0.20 mM). Reactions were initiated with concentrated, dialyzed *P. chrysosporium* extracellular fluid (approximately 35 μg of total hemoprotein) and allowed to proceed for 18 h. They were then terminated by adding 25 ml of a saturated 2,4-dinitrophenylhydrazine solution in 1 N HCl, stirred for 1 h, and extracted four times with 50 ml of CCl_4. The combined CCl_4 extracts were washed twice with 100 ml of 1 N HCl, dried over Na_2SO_4, and concentrated to dryness by rotary vacuum evaporation. Samples were redissolved in 0.2 to 0.5 ml of CH_3OH and filtered, and portions (2 to 20 μl) were analyzed by reversed-phase HPLC as described below.

Oxidation of phenanthrene in vivo. *P. chrysosporium* (ATCC 24725) was grown from conidial inocula in low-N (1.1 mM ammonium tartrate) growth medium that contained the basal concentration of trace elements and 0.1% (wt/vol) Tween 20 (25, 26). The same medium was used in experiments with low-Mn fungal cultures, except that $MnSO_4$ was omitted from the trace element solution. The medium without added Mn contained approximately 5 μM Mn that was adventitiously present in the other ingredients (4). The Mn concentration of the basal medium was approximately 35 μM. Experiments on the Mn dependence of phenanthrene oxidation were done in 25-ml rotary-shaken cultures that were set up with fourfold-concentrated biomass as described previously (22). Additional experiments on phenanthrene biodegradation were done in 10-ml stationary cultures. All cultures were grown under O_2 at 37°C.

For rotary-shaken cultures, [^{14}C]phenanthrene (6.9 μM; 5.0 × 10^6 dpm) was added when the biomass was concentrated, after 48 h of growth (22). For stationary cultures, [^{14}C]phenanthrene (12.6 μM; 4.0 × 10^6 dpm) was added after 72 h of growth, by which time the fungus had formed firm mycelial mats. A 140 μM emulsion of the radiolabeled PAH was prepared in 0.1% (wt/vol) Tween 20, and 1.0 ml was added to the surface of each mat. Samples of the extracellular medium (250 μl) were periodically removed from both stationary and agitated cultures and prepared for HPLC as described above for the in vitro studies.

Analytical techniques. Reversed-phase HPLC of ^{14}C-labeled phenanthrene metabolites was done on a styrene-divinylbenzene column (PRP-1; Hamilton) in acetonitrile-H_2O-acetic acid as described previously (21). Fractions (1.0 ml) were collected, and radiolabeled products were quantitated by liquid scintillation counting.

Reversed-phase HPLC of hexanal 2,4-dinitrophenylhydrazone was done on a C_{18} column (Vydac 201TP; 4.6 by 250 mm; 10-μm particle size). The column was eluted with CH_3OH-H_2O, 3:1, at 1.0 ml min^{-1} and ambient temperature, and the eluate was monitored spectrophotometrically at 360 nm. The reaction product that ran identically with an external standard of hexanal 2,4-dinitrophenylhydrazone was collected for solid-probe mass spectral analysis.

Solid-probe mass spectrometry and gas chromatography-mass spectrometry were performed on a Finnigan MAT 4510 gas chromatograph-mass spectrometer. Gas chromatography was done on a 15-m DB-5 (nonpolar silicone polymer) fused silica capillary column (J & W Scientific) operated at various temperatures. Electron impact mass spectra were obtained at 70 eV.

RESULTS

Characteristics of cell-free phenanthrene oxidation. Preliminary work showed that the oxidation of phenanthrene to DPA could be reconstituted in a *P. chrysosporium* cell-free system that contained dialyzed extracellular concentrate from N-limited fungal cultures, Mn(II), and Tween 80. HPLC analysis of such reactions showed that they gave product profiles similar to those obtained from intact fungal cultures. To confirm the identification of DPA in cell-free experiments, we collected and extracted the major HPLC peak from a large-scale reaction, methylated it with diazomethane, and subjected the product to gas chromatography-mass spectrometry analysis. The methylated oxidation product ran identically with authentic DPA dimethyl ester by gas chromatography, and both compounds exhibited the same mass spectrum: *m/z* (relative intensity) 270 (M^+, 2); 239 ($M^+ - \cdot OCH_3$, 1); 211 ($M^+ - \cdot OCH_3$, − CO, 100); 196 (10); 180 ($M^+ - 2 \cdot OCH_3$, − CO, 9); 168 (4); 152 ($M^+ - 2 \cdot OCH_3$, − 2CO, 11); 139 (10).

Purified MnP was as effective as crude enzyme in the cell-free system. However, it was clear that phenanthrene oxidation was not a result of straightforward MnP catalysis, because the reaction required unsaturated lipids. The initial evidence for this requirement was that the system failed to oxidize phenanthrene when Tween 80 was replaced by other surfactants such as Tween 20, *n*-dodecyl-β-D-maltoside, or 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, despite the findings that all of these detergents were able to emulsify phenanthrene and none of them inhibited MnP at the concentrations used. Tween 80 contains unsaturated fatty acid esters, whereas the other surfactants tested do not.

Further investigation showed that Tween 80 could be replaced by unsaturated fatty acid esters in the cell-free system. Thus, monoolein (18:1) or monolinolein (18:2) supported phenanthrene oxidation in the presence of Tween 20, whereas monostearin (18:0) did not. Free unsaturated fatty acids could also be used, but they gave lower DPA yields. Table 1 shows the requirements of the final, reconstituted cell-free system. Oxygen and an unsaturated lipid were essential for DPA production, whereas the free radical scavenger butylated hydroxytoluene inhibited the reaction completely. These results show that phenanthrene oxidation by the cell-free system involves oxyradical reactions of unsaturated fatty acids, i.e., lipid peroxidation (19).

Active MnP and Mn(II) were required for phenanthrene oxidation (Table 1). However, no reaction was obtained when MnP was replaced by Mn(III)-α-hydroxy acid chelates, which

TABLE 1. DPA production by the cell-free system under various conditions

Lipid	Reaction conditions[a]	DPA yield (%)
Monostearin	Complete	1.4
Monoolein	Complete	7.4
Monolinolein	Complete	15.6, 13.6, 13.9, 14.2[b]
	Under argon	0.3
	Plus 200 μM BHT	0.2
	With boiled MnP	0.2
	Minus MnP	0.1
	Minus Mn(II)	1.0
	Complete with IEF-purified MnP	11.6

[a] BHT, butylated hydroxytoluene; IEF, isoelectric focusing.
[b] Results of multiple experiments.

are products of MnP turnover that act as intermediary oxidants in previously described MnP catalyses (17, 38). These findings indicate that the MnP heme plays some essential role besides Mn(III) generation in the cell-free system.

H_2O_2 was not required in the cell-free system, and catalase had no inhibitory effect (data not shown). However, other experiments indicated that the system was capable of supporting peroxidase turnover. When phenanthrene was replaced with the MnP substrate 2,6-dimethoxyphenol, the system without H_2O_2 generated a product that exhibited a UV absorption maximum at 270 nm, indicative of 2,2′,6,6′-tetramethoxydiphenoquinone (38). When MnP was replaced with lignin peroxidase and phenanthrene was replaced with veratryl alcohol, again in the absence of H_2O_2, the system generated veratraldehyde (data not shown).

Lipid peroxidation by MnP. MnP with linolenic acid gave a positive result in the TBA assay, the standard test for polyunsaturated lipid peroxidation (19). The reaction required both active enzyme and Mn(II). Short-term enzymatic reactions (Fig. 2A) required H_2O_2, but extended assays (Fig. 2B) showed that MnP-dependent lipid peroxidation occurred, after a lag, in its absence. H_2O_2-independent lipid peroxidation by MnP was unaffected by the addition of catalase (data not shown).

The TBA assay is designed to detect malondialdehyde, a product diagnostic for the peroxidative scission of fatty acids with two or more double bonds. However, malondialdehyde is

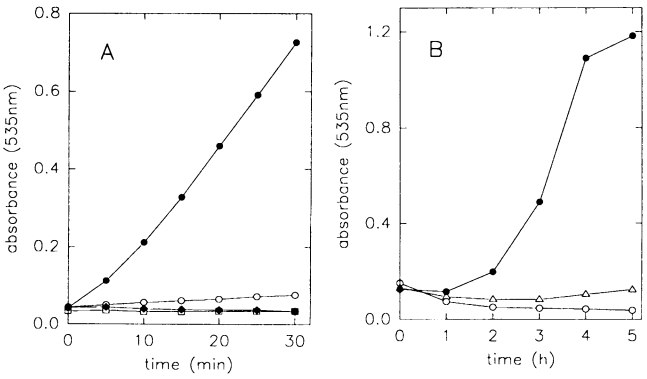

FIG. 2. Peroxidation of linolenic acid by MnP and Mn(II) as shown in the TBA assay. (A) Short-term, H_2O_2-dependent peroxidation in the complete reaction (●) and in reactions lacking Mn(II) (○), H_2O_2 (◆), or MnP (□). (B) Long-term, H_2O_2-independent peroxidation in the complete reaction (●), in a reaction without Mn(II) (△), and in a reaction with boiled MnP (○).

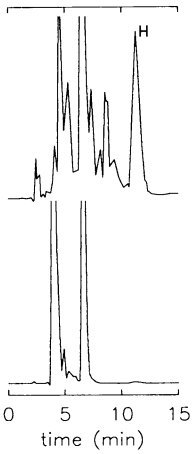

FIG. 3. Peroxidation of linoleic acid by MnP and Mn(II) as shown by HPLC of 2,4-dinitrophenylhydrazine-derivatized reaction extracts. (Top) Chromatogram of products from the complete reaction. H indicates hexanal 2,4-dinitrophenylhydrazone. (Bottom) Chromatogram of products from the reaction without Mn(II). The reaction without enzyme (not shown) also failed to produce hexanal.

formed in small quantities during lipid peroxidation, and other compounds also give positive colorimetric reactions with this test (19). We therefore considered it important to obtain evidence for MnP-mediated lipid peroxidation by some other method. The detection of fatty acid-derived alkanals that are late-stage scission products of lipid peroxidation forms the basis for such a method. For example, linoleic acid is cleaved during peroxidation to give an aldehyde mixture rich in hexanal (13).

Analysis of reactions with crude MnP and linoleate confirmed that the enzyme cleaved this fatty acid in an H_2O_2-independent reaction to give hexanal. When the reaction mixtures were extracted, derivatized with 2,4-dinitrophenylhydrazine, and subjected to reversed-phase HPLC, a major product that chromatographed identically with hexanal 2,4-dinitrophenylhydrazone was found (Fig. 3). Solid-probe mass spectrometry of the collected HPLC peak confirmed the structure: *m/z* (relative intensity) 280 (M^+, 78); 224 ($M^+ - H_2C{=}CHCH_2CH_3$, 5); 223 ($M^+ - \cdot CH_2CH_2CH_2CH_3$, 9); 206 ($M^+ - \cdot CH_2CH_2CH_2CH_3 - \cdot OH$, 54); 83 (100). The MnP-dependent system produced 0.03 μmol of hexanal per μmol of linoleate in 18 h, a rate similar to that observed previously during linoleate autoxidation (13). No hexanal was produced in the absence of Mn(II) or enzyme (Fig. 3).

Relevance of the system to phenanthrene oxidation in vivo. The cell-free system in its final form (Table 1; Fig. 4A; Fig. 5A) oxidized 12 to 16% of initially added phenanthrene to DPA in 6 to 8 days and also produced other, unidentified products that accounted for an additional 40 to 50% of the starting material. Similar results are obtained when intact *P. chrysosporium* oxidizes phenanthrene (21) (Fig. 4B; Fig. 5B).

Additional, albeit indirect, support for MnP involvement was obtained from the observation that DPA production in whole fungal cultures occurred faster in medium that contained the basal Mn concentration (35 μM) than in cultures that contained low Mn (5 μM) (Fig. 4B). Mn(II) concentration affects MnP activity not only because Mn(II) is an obligatory substrate for the enzyme (37, 38) but also because this transition metal ion stimulates the transcription of MnP genes (5). Of course, this experiment does not exclude the possibility

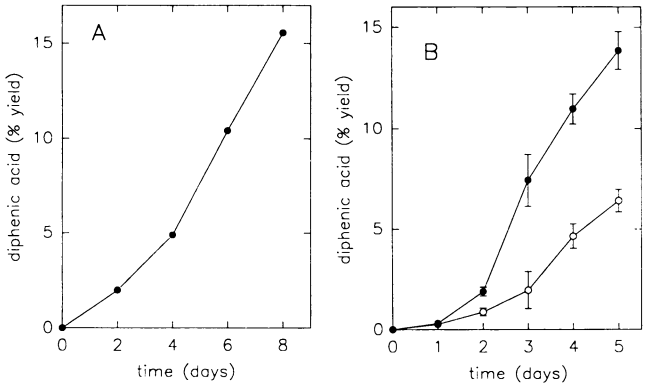

FIG. 4. Time course of DPA production from [^{14}C]phenanthrene. (A) Results obtained with MnP in the presence of Mn(II) and monolinolein under air. (B) Results obtained with rotary-shaken cultures of intact *P. chrysosporium* in basal medium (●) and low-Mn medium (○) with Tween 20. The datum points show the mean ± the standard deviation of the sample for four replicate cultures. After 8 days, the DPA yields were 13.9% ± 2.4% for basal medium cultures and 8.2% ± 0.8% for low-Mn cultures.

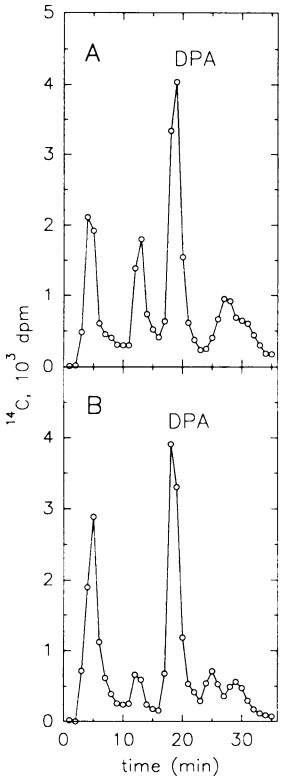

FIG. 5. HPLC of polar products obtained from the oxidation of [^{14}C]phenanthrene. Unidentified nonpolar products and residual phenanthrene eluted later and are not shown. (A) Products obtained with MnP after 8 days in the presence of Mn(II) and monolinolein under air. The DPA yield in this experiment was 13.6%. (B) Products obtained after 6 days with stationary cultures of intact *P. chrysosporium* in basal medium with Tween 20. The DPA yield in this experiment was 10.2%.

that some other effect of Mn, unrelated to MnP, might have influenced phenanthrene oxidation in vivo. However, it is clear that the result is not merely due to a general inhibition of cell growth under Mn limitation, because past work has shown that *P. chrysosporium* biomass levels are unaffected within the Mn concentration range employed here (4).

Our previous study on phenanthrene oxidation by *P. chrysosporium* employed agitated cultures in a growth medium that included exogenous unsaturated fatty acids in the form of Tween 80 (21). The data reported here raised the possibility that phenanthrene oxidation by the intact fungus might be a culture artifact reproducible only under these conditions, i.e., that the requisite lipid peroxidation might occur in vivo only with agitation and unsaturated lipid supplements. However, an investigation of this problem showed that stationary cultures grown in media containing Tween 20 (Fig. 5B) or no surfactant (data not shown) oxidized phenanthrene to DPA as rapidly as Tween 80-supplemented cultures do (21).

DISCUSSION

MnP supported the Mn-dependent peroxidation of unsaturated fatty acids. It also oxidized phenanthrene to DPA in a reaction that required unsaturated lipids, oxygen, and Mn(II) and that was inhibited by the free radical scavenger butylated hydroxytoluene. Intact *P. chrysosporium* oxidized phenanthrene to DPA to the same extent that the MnP-lipid system did, using a pathway that was stimulated by Mn(II). These results support the involvement of extracellular MnP-mediated lipid peroxidation in phenanthrene oxidation by *P. chrysosporium*.

The operation of this mechanism in vivo would require that white rot fungi produce extracellular lipids that are accessible to MnP. Electron microscopic and immunolabeling data support this scenario. These studies show that fungal peroxidases are found to be associated with the plasma membrane, where they might initiate the peroxidation of membrane lipids (9, 36). The data also show that extracellular membranes are frequently attached to the hyphae of white rot fungi (9, 15) and that peroxidases are found on these structures (9). The chemical composition of these extracellular membranes cannot be stated with certainty, but one attempt to isolate and characterize them indicated that they contain lipids (14). A previous analysis of the extractable lipids in *P. chrysosporium* mycelium showed, not surprisingly, that they contain unsaturated fatty acids (28). Our results demonstrate that, whatever the identity of the extracellular peroxidizable species in *P. chrysosporium*, the fungus is able to produce it without the addition of exogenous unsaturated lipids.

Lipid peroxidation is frequently initiated by transition metal ions that react with endogenous lipid hydroperoxides, either oxidizing them to peroxy radicals or reducing them to alkoxy radicals. In the case of the *P. chrysosporium* cell-free system, possible initiators include Mn(II), Mn(III), and the peroxidase heme (16, 19, 33, 39). It is not yet clear what role Mn(III) plays in the system, but if it is a direct participant, it is presumably generated via H_2O_2-independent peroxidase turnover, a process that does appear to occur in the cell-free system. The agents that support enzyme turnover remain to be identified, but possibilities include lipid hydroperoxides and lipid-derived aldehydes (24, 29).

The oxygen-centered radicals produced during lipid peroxidation are known to trigger xenobiotic cooxidations. For example, aromatic compounds that contain olefinic double bonds, such as benzo[*a*]pyrene-7,8-dihydrodiol (11, 12), cyclopenteno[*c*,*d*]pyrene (35), and styrene (3), are epoxidized by peroxy radicals. Fully aromatic molecules such as benzo[*a*]pyrene are also cooxidized during lipid peroxidation but

evidently via direct hydrogen or electron abstraction by peroxidases or lipid oxyradicals (31, 32). Phenanthrene is most likely to be oxidized by the latter route, which would yield phenanthrene-9,10-quinone as an intermediate that subsequently undergoes facile 9,10-cleavage to give DPA (21).

Past work on PAH cooxidations during lipid peroxidation has shown the importance of this process in mammalian xenobiotic metabolism (30), but it does not appear that lipid peroxidation has been considered a mechanism for the microbial cooxidation of aromatics. The MnP-lipid peroxidation system may have general significance in white rot fungal metabolism and merits further study.

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