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One-Electron Oxidation in the Degradation of Creosote Polycyclic Aromatic Hydrocarbons by *Phanerochaete chrysosporium*

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The abilities of whole cultures of *Phanerochaete chrysosporium* and *P. chrysosporium* manganese peroxidase-mediated lipid peroxidation reactions to degrade the polycyclic aromatic hydrocarbons (PAHs) found in creosote were studied. The disappearance of 12 three- to six-ring PAHs occurred in both systems. Both in vivo and in vitro, the disappearance of all PAHs was found to be very strongly correlated with ionization potential. This was true even for compounds beyond the ionization potential thresholds of lignin peroxidase and Mn^{3+}. Deviations from this correlation were seen in the cases of PAHs which are susceptible to radical addition reactions. These results thus begin to clarify the mechanisms of non-lignin peroxidase-labile PAH degradation in the manganese peroxidase-lipid peroxidation system and provide further evidence for the ability of this system to explain the in vivo oxidation of these compounds.

The ability of white rot basidiomycetes, particularly *Phanerochaete chrysosporium*, to degrade the polycyclic aromatic hydrocarbon (PAH) constituents of creosote and coal tar has been extensively studied. The first investigation in this area demonstrated $^{14}CO_2$ release from radiolabelled benzo[*a*]pyrene by *P. chrysosporium* liquid cultures (4). In the intervening years, extensive research to better define the range of PAH compounds subject to degradation by this fungus has been conducted. Liquid culture studies have documented the degradation and/or mineralization of a wide array of PAHs, with phenanthrene (3, 9, 12, 22, 31), fluorene and benzofluorenes (3), anthracene (3, 10, 13, 35), fluoranthene (3), pyrene (3, 14), benz[*a*]anthracene (3), and benzo[*a*]pyrene (4, 10, 27) among them.

Early research into xenobiotic degradation by *P. chrysosporium* implicated the highly nonspecific battery of enzymes used by this fungus to break down the lignin polymer as the primary agents in pollutant metabolism. The primary enzymatic constituents of the ligninolytic system of *P. chrysosporium* are thought to be lignin peroxidase (LiP) (32) and manganese peroxidase (MnP) (24).

LiP from *P. chrysosporium* directly catalyzes one-electron oxidations of aromatic substrates (15, 16). The resultant aryl cation radicals then undergo spontaneous rearrangements and degradation. The ability of LiP to oxidize PAHs was first demonstrated by Hammel et al. (14), who observed transformation of those PAHs with ionization potential (IP) values of $\leq$7.55 eV. LiP was unable to degrade compounds above this threshold value; the metabolism of these compounds in vivo therefore could not be explained by the direct action of this enzyme.

One-electron oxidations of several polycyclic aromatics by Mn^{3+} have been documented (5–7, 25), leading to the suggestion (11) that the MnPs of *P. chrysosporium* might play a key role in the degradation of PAHs in vivo. Manganic acetate, however, was found to be incapable of oxidizing PAHs with IPs equal to or greater than that of chrysene (approximately 7.8 eV) (5). Thus, it is not clear that Mn^{3+}-mediated oxidations can explain the degradation of these PAHs (i.e., fluoranthene and phenanthrene; see Fig. 2) in vivo.

Recently, an in vitro system mediated by the peroxidation of unsaturated lipids by MnP and Mn^{3+} was described by Moen and Hammel (21); this system catalyzed the oxidative cleavage of phenanthrene to diphenic acid, closely mimicking the action of intact fungal mycelium (12). These authors, therefore, invoked lipid peroxidation-based cooxidation to explain phenanthrene degradation by fungal cultures. The precise mechanism of the reaction, including the identity of the species which actually reacts with phenanthrene, has not yet been elucidated.

The objective of the present research was to characterize the behaviors of both the intact fungus and the MnP-based lipid peroxidation system with respect to a wider range of creosote PAHs. It was hoped that these studies would provide some insight into the mechanisms of degradation of these pollutants both in vitro and in vivo. This paper reports the disappearances of three- to six-ring creosote PAH constituents from intact fungal cultures and during lipid peroxidation in vitro. In each of these cases, the process is shown to be primarily dependent on IP, implying that the participation of one or more one-electron oxidants is involved across the full range of PAHs tested. The possible nature of this oxidant is discussed.

MATERIALS AND METHODS

Chemicals. An extract of weathered creosote was prepared from 30-year-old creosote-treated railroad ties. Ties were manually chipped and ground in a coffee grinder. Five 2-g samples of the resultant fines were then extracted by sonication in 1:1 hexane-methylene chloride (10 ml). Following vacuum filtration through glass fiber filters, the extracts were pooled, evaporated to dryness, and redissolved in 10 ml of *N*,*N*-dimethylformamide. Individual PAHs in the extract were first tentatively identified by matching high-pressure liquid chromatography (HPLC) retention times (Fig. 1) with those of authentic standards; these assignments were then confirmed by comparing UV absorption spectra with a 1090L series II HPLC apparatus equipped with a diode array detector (Hewlett-Packard, Little Falls, Del.). The concentrations of the 12 PAHs of interest in this final creosote extract were determined by comparison of peak areas with four-point standard curves ($r \geq 0.998$); concentrations of all PAHs except anthracene were between 10^{-3} and 10^{-4} M (Table 1).

^{14}C-labelled standards of benz[*a*]anthracene (5,6-^{14}C; 7.92 mCi/mmol; 97% pure) and benzo[*a*]pyrene (7,10-^{14}C; 9.65 mCi/mmol; 98% pure) were obtained from California Bionuclear Corporation (Los Angeles, Calif.). [^{14}C]phenanthrene (9-^{14}C; 10.9 mCi/mmol; 99% pure) was from Sigma (St. Louis, Mo.), as were unlabelled phenanthrene and benzo[*a*]pyrene and the lipids oleic acid and monolinoleoyl-rac-glycerol. Unlabelled benz[*a*]anthracene was purchased from

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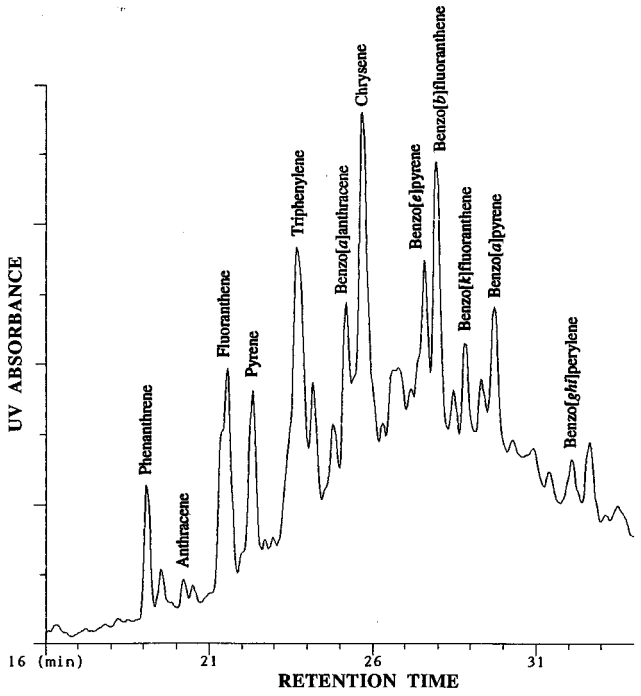

FIG. 1. Reverse-phase HPLC chromatogram of the creosote extract showing the retention times of the 12 PAHs examined in this study.

Supelco (Bellefonte, Pa.); all other PAH standards were from Aldrich (Milwaukee, Wis.).

Fungi. Stock cultures of *P. chrysosporium* Burdsall BKM-F-1767 (ATCC 24725) were obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. These were subcultured onto yeast extract-malt extract-peptone-glucose (YMPG) slants (18) and maintained at 4°C until use.

Liquid culture PAH degradation. *P. chrysosporium* spores were inoculated into 20 ml of BIII medium (17), which had 10 mM *trans*-aconitic acid substituted for dimethylsuccinic acid, in stoppered 125-ml Erlenmeyer flasks. The cultures were supplemented with Tween 20 (0.1%) and benzyl alcohol (825 μl/liter) and were maintained without agitation at 39°C. In order to avoid the potential inhibition of spore germination, creosote extract (20 μl) was added after 3 days of mycelial growth. Following 4-week incubations (with O_2 flushing every 3 to 5 days), the cultures ($n = 3$) were individually homogenized and extracted three times with equal volumes of ethyl acetate. The resultant extracts were dehydrated with anhydrous Na_2SO_4, evaporated to dryness with N_2, and redissolved in acetonitrile for HPLC analysis as described below. Percent disappearance values for each compound were determined by comparing the compounds' HPLC integrated peak areas with those for similarly extracted noninoculated controls ($n = 3$).

TABLE 1. Data on the 12 PAHs examined in this study, including concentration in creosote extract and recovery from *P. chrysosporium* liquid cultures

Compound	Mol wt	No. of rings	IP (eV)[a]	Concn (μM)	% Recovery (SD)
Phenanthrene	178	3	8.03	100	21 (3)
Anthracene	178	3	7.43	60	5 (3)
Fluoranthene	202	4	7.90	1,070	61 (5)
Pyrene	202	4	7.53	900	3 (0)
Triphenylene	228	4	8.10	1,210	78 (5)
Benz[*a*]anthracene	228	4	7.56	110	11 (4)
Chrysene	228	4	7.81	350	58 (5)
Benzo[*e*]pyrene	252	5	7.69	520	43 (5)
Benzo[*b*]fluoranthene	252	5	7.70	280	53 (2)
Benzo[*k*]fluoranthene	252	5	7.48	450	38 (5)
Benzo[*a*]pyrene	252	5	7.21	190	8 (3)
Benzo[*ghi*]perylene	276	6	7.31	220	46 (4)

[a] IPs for all the PAHs except benzo[*b*]fluoranthene and benzo[*k*]fluoranthene are from Pysh and Yang (26). The IPs were determined by the polarographic oxidation method. IPs for benzo[*b*]fluoranthene and benzo[*k*]fluoranthene are from the modified neglect of diatomic overlap calculations of Simonsick and Hites (30). These IPs were converted to experimental values with a graph based on Table I of reference 30.

Enzyme preparation. Fungal cultures for peroxidase production contained 750 ml of *trans*-aconitic acid-buffered BIII medium (17) with Tween 20 and benzyl alcohol added as described above. After 6 days (with agitation at 200 rpm and daily flushing with O_2), the resultant fungal pellets were removed by filtration. Extracellular fluid was then concentrated approximately 100-fold. High-molecular-weight polysaccharide was precipitated by freezing and thawing and was removed by centrifugation. After dialysis against 5 mM sodium acetate (pH 6.0), this crude peroxidase preparation was further concentrated (final volume ≈ 20 ml) and sterilized by filtration through low-protein-binding filters (Acrodisc 13; Gelman Sciences, Ann Arbor, Mich.). The enzyme activity in the final preparation was 2×10^4 nkat/ml for LiP by veratryl alcohol oxidation (33) and 3×10^4 nkat/ml for MnP on the basis of the vanillyl acetone assay (24).

In vitro reaction conditions. Lipid peroxidation-based reactions were based on those of Moen and Hammel (21). The reaction mixtures contained 10 μl of creosote extract as well as 20 mM sodium malonate buffer (pH 4.5), 200 μM Mn^{2+} (as $MnSO_4$), 0.5% *n*-dodecyl-β-D-maltoside, and 300 μM oleic acid (dispersed in the *n*-dodecyl-β-D-maltoside prior to addition). The reactions (1-ml amounts) were conducted in silanized, sterilized 7-ml borosilicate scintillation vials at room temperature as described previously (21). Crude enzyme was added daily in 5-μl aliquots. The reactions were terminated after 7 days by the addition of 1 ml of acetonitrile, and the mixtures were filtered through 0.2-μm-pore-size filters to remove particulates. Aliquots (20 μl) of these samples were injected for analysis by HPLC.

Chromatography analysis. All HPLC analysis described herein was performed with a Vydac 201TP54 C_{18} reverse-phase column (25 by 0.46 cm) (Nest Group, Southboro, Mass.). The HPLC gradient consisted of water-acetonitrile as follows: 0 to 5 min, 60:40; 5 to 30 min, ramp to 0:100; 30 to 35 min, hold at 0:100. The flow rate was 1 ml · min^{-1} throughout the gradient. Quantitation of ^{14}C was accomplished by passing column eluent through a Flo-One radiochromatography detector (Packard Instruments Co., Downers Grove, Ill.) operated in the TR-LSC mode with Flo-Scint V (5-ml/min flow rate) as the scintillation cocktail. Count-per-minute values thus obtained were converted to disintegrations per minute by applying a quench curve generated according to the manufacturer's specifications.

RESULTS

Degradation of PAHs by whole cultures of *P. chrysosporium*. Values for percentages of nontransformed PAHs recovered (calculated by comparison with noninoculated controls) for 12 three- to six-ring PAH creosote constituents from 4-week-old *P. chrysosporium* liquid cultures are listed in Table 1. For the compounds identified, their recoveries ranged from less than 10% (anthracene, pyrene, and benzo[*a*]pyrene) to approximately 80% (triphenylene). The values for the percent recoveries of most of the compounds within this group correlated very well with their IPs (Fig. 2). This was true across the full range of compounds tested (7.2 to 8.1 eV) and was particularly notable in the four- to six-ring PAHs ($r = 0.766$, $P = 0.0009$). Five compounds (phenanthrene, benz[*a*]anthracene, pyrene, anthracene, and benzo[*a*]pyrene) showed greater reductions in vivo than would be predicted from the near-linear relationship observed for the remaining seven. The likely reasons for this are discussed below.

Degradation of PAHs during in vitro lipid peroxidation. Experiments to determine the ability of lipid peroxidation-based cooxidative reactions to account for the observed in vivo disappearances of the PAHs in this study were conducted. Tracer experiments with radiolabelled three-, four-, and five-ring compounds (phenanthrene, benz[*a*]anthracene, and benzo[*a*]pyrene) were first conducted to guard against possible PAH losses from the reaction vessels via physical routes (i.e., volatilization, precipitation, or adherence to vessel walls). Essentially all input ^{14}C radioactivity (90% ± 1% for phenanthrene, 99% ± 1% for benz[*a*]anthracene, and 88% ± 2% for benzo[*a*]pyrene) was recovered from no-enzyme control reac-

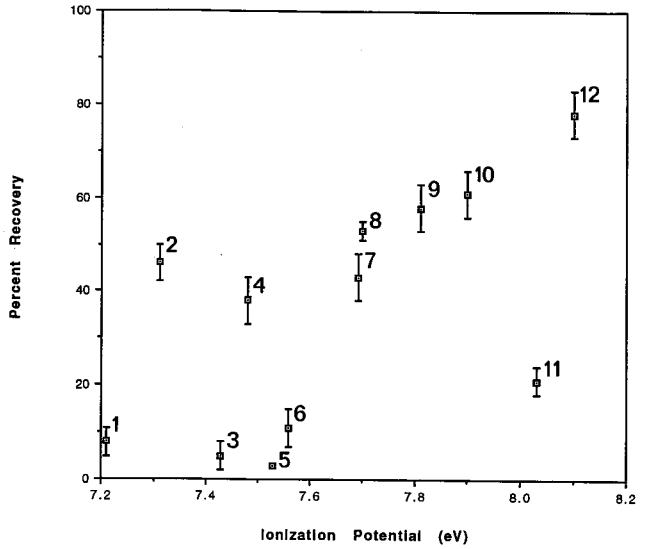

FIG. 2. Percent recoveries (calculated by comparison with noninoculated abiotic controls) of 12 three- to six-ring creosote extract PAHs from 4-week stationary liquid cultures of *P. chrysosporium* as a function of IP (26, 30). The PAHs are numbered as follows: 1, benzo[*a*]pyrene; 2, benzo[*ghi*]perylene; 3, anthracene; 4, benzo[*k*]fluoranthene; 5, pyrene; 6, benz[*a*]anthracene; 7, benzo[*e*]pyrene; 8, benzo[*b*]fluoranthene; 9, chrysene; 10, fluoranthene; 11, phenanthrene; and 12, triphenylene. Error bars indicate standard deviations.

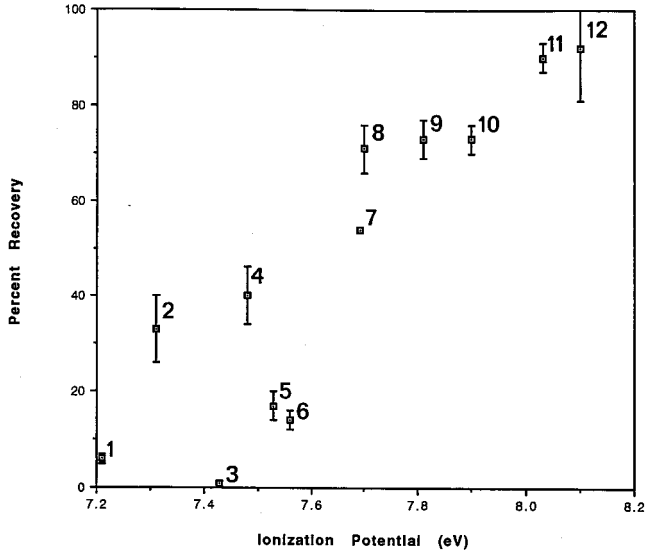

FIG. 3. Recovery values (calculated by comparison with no-enzyme controls) for the 12 PAHs from in vitro lipid peroxidation reaction mixtures. The compounds are numbered the same as in the legend to Fig. 2. Error bars indicate standard deviations.

tion mixtures at the conclusion of the 7-day incubation period. Additionally, all radioactivity recovered from these control reaction mixtures coeluted with the parent PAH when analyzed by HPLC (data not shown), implying that no nonenzymatic transformation had occurred. Thus, it can be stated with confidence that the disappearances observed in subsequent experiments were due exclusively to enzyme-dependent processes.

When whole-creosote extract was added to MnP-driven lipid peroxidation reaction mixtures, substantial losses of nearly all three- to six-ring PAHs were observed during the incubation period. Again, when the disappearance data for the 12 PAHs were plotted versus the IPs of the compounds, a very strong correlation ($r = 0.793$, $P = 0.0001$) was observed (Fig. 3); this was true whether an esterified fatty acid (monolinoleoyl-rac-glycerol) or free oleic acid was used as the lipid in the system (Fig. 4).

Clearly, those compounds with higher IPs were degraded to a much lesser extent than those with lower IPs in the above experiments, presumably because of, at least in part, the presence of much more readily oxidizable substrates. In order to better assess the apparent correlation between disappearance and IP among these compounds, six of the PAHs with high (>7.65 eV) IPs (triphenylene, phenanthrene, fluoranthene, chrysene, benzo[*b*]fluoranthene, and benzo[*e*]pyrene) were each added (final concentration, 20 μM) to individual reaction mixtures. Recovery values for these compounds ranged from 4% ± 0.5% for benzo[*e*]pyrene to 97% ± 5% for triphenylene, and again, these recovery values displayed an excellent correlation ($r = 0.683$, $P = 0.04$) with the IPs (Fig. 5).

DISCUSSION

Direct oxidation of PAHs by LiP is restricted to those compounds with IPs of ≤7.55 eV (14); Mn^{3+} in manganic acetate-acetic acid systems is incapable of oxidizing compounds with IPs of ≥7.8 eV (5). Nonetheless, degradation of three- to six-ring creosote PAHs with IPs between 7.2 and 8.1 eV is IP dependent, both in vivo and during lipid peroxidation in vitro. This implies that the participation of a one-electron oxidant stronger than LiP or Mn^{3+} is involved in both systems. The observed deviation of phenanthrene (a three-ring compound) from this pattern in vivo (Fig. 2) is most likely due to its higher solubility: phenanthrene is approximately an order of magnitude more soluble than the remainder of the PAHs examined in this work (reference 19 and references therein). The case of anthracene, a three-ring compound which exhibits lower-than-

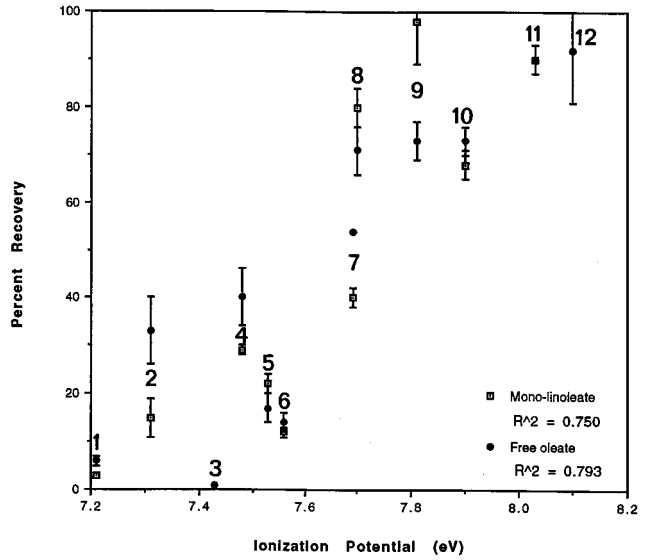

FIG. 4. Percent recoveries of PAHs from lipid peroxidation reaction mixtures with either the esterified fatty acid monolinoleoyl-rac-glycerol (monolinoleate) used by Moen and Hammel (21) or free oleic acid. The compounds are numbered the same as in the legend to Fig. 2. Error bars indicate standard deviations. R^2, correlation coefficient.

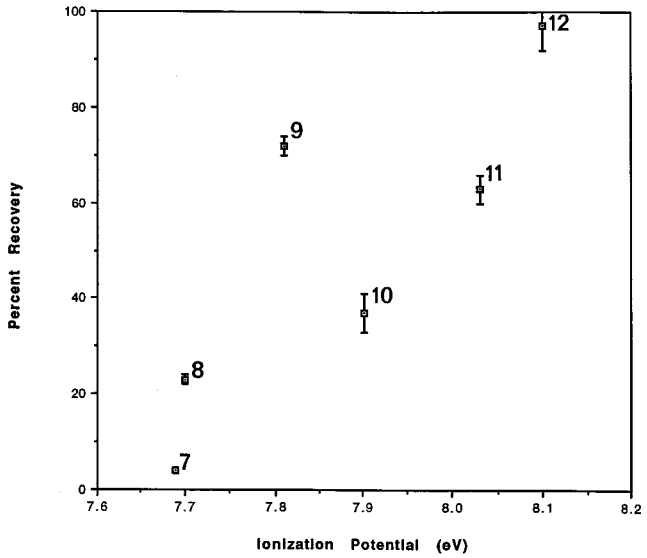

FIG. 5. Disappearance of six high-IP (non-LiP substrate) compounds when added individually (20 μM) to separate in vitro lipid peroxidation reactions. The compounds are numbered the same as in the legend to Fig. 2. Error bars indicate standard deviations.

expected recoveries both in vivo (Fig. 2) and in vitro (Fig. 3), is discussed below.

Lipid peroxidation takes place via a cascade of reactions which generate numerous radical species (2). Among these are carbon-centered lipid radicals (R·) and peroxyl radicals (ROO·), both of which are intermediates in the formation of lipid hydroperoxides (ROOH). Additionally, if homolytic cleavage of lipid hydroperoxides is catalyzed by LiP and/or MnP, as it is by cytochrome P-450 (28, 34) and bleomycin (23), alkoxyl radicals (RO·) will also be present. Of these three species, alkoxyl radicals are the strongest oxidants (2). Experiments to identify more precisely the radical species generated in this system as well as to elucidate their respective roles in PAH oxidation are currently in progress.

Although the primary reaction of peroxyl radicals with PAHs is hydrogen (electron) abstraction (19, 29), the peroxyl radicals also take part in addition reactions at olefinic bonds (19). This would likely lead to epoxidation, which would be followed by the formation of *trans*-dihydrodiols via epoxide cleavage. If peroxyl radicals are present, it may be assumed that radical addition reactions may also take place during PAH degradation both in vitro and in vivo. No data concerning the relative susceptibilities of PAHs to peroxyl or alkoxyl radical addition in liquid systems exist in the literature. Gas-phase reaction constants and half-lives for the reactions among PAHs and hydroxyl radicals, reactions which are dominated by radical addition pathways (1), have been determined (reference 20 and references therein). Four PAHs (benzo[*a*]pyrene, anthracene, pyrene, and benz[*a*]anthracene) which appear in Fig. 2 and 3 to lie below the lines defined by the remaining data points (higher disappearances than would be predicted by IP alone) also have very low half-lives in the gas-phase reaction with ·OH (Table 2). Thus, these compounds appear to be somewhat susceptible to oxy radical addition reactions. These observations imply that this mechanism may contribute to the degradation, both in vitro and in liquid culture, of at least these four PAHs.

Field evaluation (8) of soil remediation with white rot basidiomycetes has shown that PAHs with four or more fused rings were essentially not degraded; it was proposed that this was due to the extreme hydrophobicity, and resultant adsorption to soil organic matter, of these compounds. The data presented herein clearly show that compounds with up to six rings are degraded in vitro during MnP-dependent lipid peroxidation reactions and that these same compounds are depleted from liquid cultures of *P. chrysosporium*. Thus, there is the potential, via inoculation techniques which employ surfactants or similar approaches to enhance the solubility and availability of these compounds, to accelerate the pace of in situ remediation.

TABLE 2. PAH half-lives calculated for the gas-phase reaction with ·OH radicals[a]

Compound	Half-life (h)
Benzo[*ghi*]perylene	0.3–3
Benzo[*a*]pyrene	0.4–4
Anthracene	0.5–5
Pyrene	0.8–8
Benz[*a*]anthracene	0.8–8
Chrysene	0.8–8
Benzo[*k*]fluoranthene	1–11
Benzo[*b*]fluoranthene	1.4–14
Phenanthrene	2–20
Fluoranthene	2–20

[a] See reference 20 and references therein. Values for triphenylene and benzo[*e*]pyrene were not determined.

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