Metabolic Pathways Utilized by *Phanerochaete chrysosporium* for Degradation of the Cyclodiene Pesticide Endosulfan

SETH W. KULLMAN AND FUMIO MATSUMURA*

Department of Environmental Toxicology, University of California, Davis, California 95616

Received 22 September 1995/Accepted 8 December 1995

Recent studies have shown that cultures of white rot fungi not favoring the production of lignin and manganese peroxidases are effective in degrading certain xenobiotics. In this study we have used endosulfan as a model xenobiotic to assess the enzymatic mechanisms of pesticide metabolism under ligninolytic (nutrient-deficient) and nonligninolytic (nutrient-rich) culture conditions. Rapid metabolism of this chlorinated pesticide occurred under each nutrient condition tested. However, the extent of degradation and the nature of the metabolic products differed for nutrient-deficient and nutrient-rich media. The pathways for endosulfan metabolism were characterized by analysis of the fungal metabolites produced. The major endosulfan metabolites were identified by gas chromatography-electron capture detection and gas chromatography-mass spectrometry as endosulfan sulfate, endosulfan diol, endosulfan hydroxyether, and a unknown metabolite tentatively identified as endosulfan dialdehyde. The nature of the metabolites formed indicates that this organism utilizes both oxidative and hydrolytic pathways for metabolism of this pesticide. Piperonyl butoxide, a known cytochrome P-450 inhibitor, significantly inhibited the oxidation of endosulfan to endosulfan sulfate and enhanced hydrolysis of endosulfan to endosulfan diol. We suggest that the metabolism of endosulfan is mediated by two divergent pathways, one hydrolytic and the other oxidative. Judging by the inactivity of extracellular fluid and partially purified lignin peroxidase in metabolizing endosulfan, we conclude that metabolism of this compound does not involve the action of extracellular peroxidases.

The commonly studied white rot fungus *Phanerochaete chrysosporium* has been shown to degrade and mineralize a wide variety of industrial and agricultural pollutants (4, 9). The ability to degrade such structurally diverse compounds has generally been attributed to the lignin-degrading enzyme system of this organism. The major components of the lignin-degrading enzyme system include lignin peroxidases (LiP), manganese-dependent peroxidases (MnP), and an H₂O₂-generating system, which are induced during secondary metabolism under nutrient-deficient culture conditions. This enzyme system has been shown to catalyze the initial oxidation of several persistent xenobiotics including polycyclic aromatic hydrocarbons, chloroaromatic compounds, and dioxins (6, 8, 19, 32). Recently, LiP and MnP have also been shown to facilitate both reductive and lipid peroxidation-mediated degradation of environmental pollutants (3, 21).

There are indications that enzyme systems other than LiP and MnP are also involved in pollutant degradation by these fungi. Yadav and Reddy (33–35) have reported that *P. chrysosporium* is capable of degrading several chlorinated xenobiotics under conditions which do not favor the production of LiP and MnP. Valli et al. (29, 30) have also suggested that for highly oxidized compounds such as nitroaromatics and chlorophenols, a series of reduction, methylation, and oxidation reactions are necessary for complete metabolism to occur. Most recently, Rieble et al. (24) have isolated from *P. chrysosporium* a 1,2-dioxygenase enzyme responsible for aromatic ring cleavage. However, despite considerable progress in our understanding of the metabolic capabilities of white rot fungi, many questions remain unanswered, particularly regarding the basic enzymatic mechanisms involved in the degradation of li-

pophilic organic pollutants. In order to effectively utilize these fungi for remediation of hazardous wastes in the future, it is essential to elucidate the metabolic pathways and major enzymes responsible for xenobiotic degradation.

Chlorinated organic pesticides are one of the major groups of chemicals responsible for environmental contamination. Many chlorinated pesticides are highly toxic and considered a potential risk to both human health and the environment. The cyclodiene pesticide endosulfan, 1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite, has been used extensively throughout the world as a broad-spectrum pesticide and is the only cyclodiene insecticide still registered for use in the United States. Because of such abundant usage, and the potential for environmental transport, endosulfan contamination is found throughout the environment. Endosulfan has been detected in the atmosphere, soils, sediments, surface waters, rain waters, and food stuffs (28).

As with most pesticides, the persistence and degradation of endosulfan are affected by the environmental conditions in which it is found. Endosulfan does not undergo direct photolysis but is transformed by chemical hydrolysis under alkaline conditions such as in seawater (1). In soil, endosulfan has been shown to be degraded by a wide variety of microorganisms (18). However, degradation rates are usually low and metabolism often results in the formation of endosulfan sulfate, an oxidative metabolite shown to be equally as toxic and persistent as the parent compound, endosulfan. Because of its persistence and toxicity, endosulfan contamination poses a significant environmental concern.

In this study we have used the cyclodiene pesticide endosulfan as a chemical probe to investigate the degradative capabilities of the white rot fungus *P. chrysosporium* BU-1 under both ligninolytic and nonligninolytic conditions. The results suggest that *P. chrysosporium* BU-1 employs two distinct metabolic pathways for the metabolism of this pesticide.

* Corresponding author. Mailing address: Department of Environmental Toxicology, University of California, Davis, CA 95616. Phone: (916) 752-4251. Fax: (916) 752-3394.

FIG. 1. Effect of culture condition on degradation of 1 ppm of endosulfan by *P. chrysosporium* BU-1 in stationary cultures containing 3 ml of either nitrogen-deficient (●), nitrogen-rich (▲), or carbon-deficient (■) medium and heat-killed controls (○). Values are the means of duplicate incubations. The percent error of the mean for endosulfan determinations were less than 6% for nitrogen-rich medium, 0.6% for carbon-deficient medium, 3.1% for nitrogen-deficient medium, and 9.5% for heat-killed mycelium.

MATERIALS AND METHODS

Microbial strain. The original strain of *P. chrysosporium* BKM-F-1767 was kindly provided by T. K. Kirk, Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wis. From this strain, *P. chrysosporium* BU-1 was selected for benomyl and UV tolerance (10). Cultures were maintained on agar medium containing 1% glucose, 1% malt, 0.2% Bacto-Peptone, 0.2% yeast extract, 0.1% asparagine, 0.2% KH_2PO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 mg of thiamine per liter and stored at 5°C (27).

Chemicals. All pesticide standards and metabolites were purchased from Chem Service Inc. (West Chester, Pa.). The pesticides and metabolites studied include endosulfan; endosulfan sulfate, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3,3-dioxide; endosulfan diol (alcohol), 1,4,5,6,7,7-hexachloro-2,3-bis(hydroxymethyl)bicyclo(2,2,1)-2-heptene; endosulfan ether, 1,4,5,6,7,7-hexachlorobicyclo(2,2,1)-5-heptene-2,3-bis(methylene)oxide; and endosulfan lactone, 5-hydroxymethyl-1,2,3,4,7,7-hexachloro-bicyclo(2,2,1)-2-heptene-6-carbonic acid. Piperonyl butoxide (PB), a-[2-(2-butoxyethoxy)-ethoxy]-4,5-methylenedioxy-2-propyltoluene; EDTA; and N,N,N',N' -tetramethylethylenediamine (TEMED) were purchased from Sigma Chemical Co. (St. Louis, Mo.). 3,4-Dimethoxybenzylalcohol (veratryl alcohol) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). All solvents used were of the highest analytical grade and were employed without further purification.

Culture conditions. *P. chrysosporium* BU-1 was grown from a conidial inoculum as stationary cultures in 35-mm-diameter polystyrene petri dishes and incubated in a moist atmosphere at 30°C. Each culture contained 3 ml of either nitrogen-deficient (2.4 mM N) or nitrogen-rich (24 mM N) medium. The contents of the medium were used as described by Kirk et al. (14), unless otherwise stated, with 1% glucose as the carbon source and asparagine and ammonium nitrate as nitrogen sources. Carbon-deficient medium contained 24 mM nitrogen and 0.1% glucose as a carbon source. The media were buffered with 10 mM sodium 2,2-dimethyl succinate (pH 4.5). Heat-killed controls were prepared by autoclaving cultures which had been pregrown in 25-ml Erlenmeyer flasks and incubated for the same period of time as indicated above.

Degradation studies. After 3 days of preliminary incubation, each culture received 15 μl of a solution of endosulfan in acetone-hexane (20:80 [vol/vol]) to give a final concentration of 1 $\mu\text{g}/\text{ml}$ (1 ppm). Individual cultures were incubated at various intervals up to 14 days after the addition of endosulfan. Following incubation, individual cultures were acidified with two drops of concentrated HCl and homogenized with a Teflon homogenizer (Wheaton). The homogenized material was then extracted with 3 ml of acetone-hexane (20:80 [vol/vol]) and 1 ml of 40% aqueous CaCl_2 , vortexed thoroughly, and centrifuged at $700 \times g$ for 1 min. Products were quantified by gas chromatography (GC) and identified by GC-mass spectrometry (GC-MS) either directly or after derivatization. Extracts

were derivatized by adding 5 μl of N,O -bis(trimethylsilyl)acetamide (BSA) to the organic extracts because trimethylsilylation improves the chromatographic behavior of endosulfan diol.

Instrumental analysis. Quantitative analysis of endosulfan and its metabolites was conducted by GC performed on a Varian model 3400 GC equipped with a ^{63}Ni electron capture detector. Separations were carried out by using a DB-1 column (0.53 mm [inside diameter] by 15 m; 0.25- μm film thickness; J&W Scientific). Nitrogen was used as both carrier and make-up gas. The oven temperature was programmed for an initial temperature of 110°C and raised to 245°C at 10°C/min. Injector and detector temperatures were 220 and 300°C respectively.

All GC-MS analyses were conducted with a Trio-2 MS (VG Masslab, Altrincham, United Kingdom) equipped for both electron ionization (EI) and negative ion electron capture ionization (EC). Electron ionization (70 eV) was performed with a trap current of 100 μA and a source temperature of 200°C. EC was performed in a manner similar to the procedures developed by Stemmler and Hites (25), with methane as the buffer gas at a pressure (measured at the source manifold) of 5×10^{-5} mbar (5 mPa) and holding the source at 150°C. Full-scan spectra were acquired over the ranges of m/z 40 to 500 (EI) and 30 to 500 (EC) at 1 s per scan. A DB-17 column (0.25 mm [inside diameter] by 30 m; 0.25- μm film thickness) was used for GC-MS and was coupled to the MS via direct interface. Helium was used as carrier gas with a linear velocity of 35 cm/s, and splitless injections of 2.0 μl were made with a purge delay of 45 s. The GC injector temperature was held at 270°C, and the column temperature was programmed from an initial temperature of 160°C (1 min) to 230°C at a rate of 6°C/min.

Inhibition studies. PB was added to cultures in 15 μl of methanol to make final concentrations of 1, 10, and 100 μM . Endosulfan was added to cultures with or without PB and incubated at 30°C for the indicated time period. Control and PB-containing cultures were maintained separately to avoid cross-contamination. EDTA (8 mM), TEMED (8 mM), and veratryl alcohol (0.4 and 1.5 mM) were added to fungal cultures containing 1 ppm of endosulfan and incubated as described above.

RESULTS

Degradation of endosulfan by whole cultures of *P. chrysosporium*. Endosulfan was extensively degraded in nitrogen-deficient, carbon-deficient, and nitrogen-rich cultures of *P. chrysosporium* BU-1 (Fig. 1). Degradation was determined by monitoring endosulfan disappearance by GC-EC detection.

TABLE 1. GC-MS ions and retention times of endosulfan and endosulfan metabolites

Substrate	Mol wt ^a	m/z [M-Cl+H] ^{-a}	RT (min) ^b
Endosulfan I	404	370	11.36
Endosulfan II	404	370	12.22
Sulfate	420	386	12.95
Diol ^c	358	324	11.58
Ether	340	306	8.65
Lactone	354	320	10.29
Hydroxyether	356	322	10.10
Putative dialdehyde	354	320	8.30

^a GC-MS conditions as described in Materials and Methods. Monoisotopic (³⁵Cl).

^b RT, retention time; GC conditions as described in Materials and Methods.

^c Derivatized with BSA.

Rapid degradation of endosulfan occurred in both nitrogen- and carbon-deficient media. The carbon-deficient medium was most effective at promoting degradation, with >95% disappearance of the substrate in a 50-h incubation period. Nitrogen-deficient cultures degraded >90% endosulfan in 50 h, and nitrogen-rich cultures degraded approximately 61% of the substrate in 95 h. Heat-killed controls retained approximately 95% of the substrate after 100 h, indicating that little degradation or volatilization of endosulfan had occurred.

Characterization of endosulfan metabolites. GC analysis of acetone-hexane extracts of fungal cultures indicated the formation of endosulfan sulfate, endosulfan hydroxyether, and endosulfan diol, which were identified on the basis of coelution with authentic standards and of EI and EC mass spectra which matched spectra obtained for authentic standards (Table 1). Endosulfan lactone and endosulfan ether were also identified as minor products. An additional major metabolite eluted with a retention time similar to that of endosulfan ether and has been tentatively identified as endosulfan dialdehyde on the basis of the retention time and mass spectra as described below. Authentic standards of the dialdehyde were not available for comparison.

Endosulfan and similar chlorinated cyclodienes yield complex EI spectra that often fail to give useful evidence of molecular weight or structure because of extensive fragmentation, and EC yields spectra that are more easily interpreted (24). In this study, EC was particularly useful for characterization of endosulfan metabolites. Endosulfan and its metabolites were identified on the basis of the presence of a molecular ion (M^-) or a specific fragment with a size of 34 Da less than the molecular mass corresponding to $[M+H-Cl]^-$. This fragment is presumed to be produced as a result of the presence of hydrogen atom donors formed in the MS ion source under typical conditions for EC.

The EC mass spectrum of the putative endosulfan dialdehyde (Fig. 2a) is similar to the spectra of endosulfan hydroxyether and endosulfan lactone. Unlike endosulfan, all three compounds yield a cluster of isotopomer peaks at m/z 282, 284, and 286 (because of the presence of ³⁵Cl and ³⁷Cl isotopes; elemental composition assigned as $C_6Cl_6^-$) and a weaker cluster at m/z 318, 320, and 322 $[M-HCl]^-$. The putative dialdehyde also yielded a set of peaks at m/z 254, 256, and 258 ($C_8H_2OCl_4^-$) that was not present in the spectra of endosulfan lactone, endosulfan diol, endosulfan hydroxyether, and endosulfan ether.

The EI mass spectrum of the putative dialdehyde (Fig. 2b) showed strong peaks at m/z 261, 263, and 265, which is a

prominent fragment ion observed in the EI spectrum of endosulfan dicarboxylic acid. This fragment was identified as $C_7H_2Cl_5^+$, which results from the loss of one chlorine atom and both aldehyde functional groups. Another cluster of peaks was assigned the composition $C_8H_2OCl_5^+$ and corresponds to $[M-HCl-CHO]^+$.

Formation of endosulfan metabolites by whole culture of *P. chrysosporium*. Endosulfan disappearance was determined concomitant with the formation of several endosulfan metabolites. The results presented in Fig. 3 show that the oxidation product, endosulfan sulfate, is the earliest appearing and most abundant metabolite formed under nitrogen-deficient culture conditions. Formation of the endosulfan sulfate occurred rapidly within the first 24 h and continued to accumulate throughout the duration of the incubation period, indicating the stability of this compound. Endosulfan diol and endosulfan hydroxyether both showed a characteristic rapid rise in formation followed by a steady decline. This type of rise and decline in product formation indicated that both metabolites were transient intermediates and precursors to subsequent metabolic products. Formation of the putative dialdehyde appeared subsequent to the formation of both endosulfan diol and endosulfan hydroxyether and accumulated slowly throughout the 95-h incubation.

Several of the identified endosulfan metabolites were added to cultures of *P. chrysosporium*, and their subsequent metabolisms were determined (Table 2). Endosulfan diol was metabolized to endosulfan hydroxyether and the putative dialdehyde. Endosulfan sulfate was not metabolized further to any product. Endosulfan lactone and endosulfan ether, metabolic products not readily formed by *P. chrysosporium*, were also added to fungal cultures to determine if they are substrates for metabolism. Endosulfan ether was metabolized to endosulfan hydroxyether and the putative dialdehyde. Endosulfan lactone proved to be resistant to further degradation, no metabolites were formed.

The proposed metabolic pathway for endosulfan metabolism is shown in Fig. 4. The parent endosulfan is either initially oxidized to endosulfan sulfate, which is a terminal end product, or hydrolyzed to the non-sulfur-containing endosulfan metabolites. An initial hydrolysis of endosulfan results in the formation of the intermediate metabolite endosulfan diol. Further oxidation of endosulfan diol results in the formation of endosulfan hydroxyether followed by formation of either endosulfan lactone or the putative endosulfan dialdehyde. It is presumed that the hydroxyether is produced through the formation of a transient aldehyde.

A comparison of the products and yields of endosulfan metabolites produced from nitrogen-deficient and nitrogen-rich cultures of *P. chrysosporium* are shown in Table 3. Nitrogen concentration had a significant influence on the types and yields of metabolic products produced. Nitrogen-deficient culture conditions produced abundant quantities of both the sulfur-containing and non-sulfur-containing metabolites but favored the formation of the oxidation product endosulfan sulfate. The hydrolysis products endosulfan hydroxyether and the putative endosulfan dialdehyde were produced in nearly equal yields. Nitrogen-rich conditions produced predominantly the non-sulfur-containing endosulfan metabolite endosulfan hydroxyether. Lower yields of endosulfan dialdehyde and endosulfan sulfate were produced under nitrogen-rich conditions.

Peroxidase-catalyzed oxidations. To determine if the extracellular peroxidases LiP and MnP were involved in the metabolism of endosulfan, whole-culture studies were performed with inhibitors and stabilizers of LiP. EDTA and TEMED were added as noncompetitive inhibitors to LiP (2). Both EDTA and TEMED failed to influence endosulfan metabo-

FIG. 2. Mass spectra of putative endosulfan dialdehyde. (A) EC spectrum; (B) EI spectrum at 70 eV. The conditions for MS are described in Materials and Methods.

lism, particularly the formation of the oxidation product endosulfan sulfate. The addition of veratryl alcohol (0.4 mM) to fungal cultures also failed to enhance endosulfan metabolism and at higher concentrations (1.5 mM) slightly inhibited endosulfan disappearance. Cell-free studies were performed with both partially purified LiP and concentrated extracellular fluid to test for their ability to oxidize endosulfan to endosulfan sulfate. Incubation of endosulfan with partially purified LiP or concentrated extracellular fluid and H_2O_2 failed to produce the oxidation product endosulfan sulfate under conditions previously developed for LiP-catalyzed oxidations (8). The addition of veratryl alcohol to these reaction mixtures as a stabilizer of LiP (31) also failed to enhance endosulfan sulfate formation.

Inhibition studies with PB. The addition of PB, a known cytochrome P-450 inhibitor (22), to cultures containing 1 ppm of endosulfan resulted in a concentration-dependent inhibition of endosulfan sulfate formation (Fig. 5). Those cultures to which PB was not added produced a rapid rise in the amount of endosulfan sulfate, which reached a maximum on day 7. Cultures to which 10 and 100 μ M PB were added showed a significant reduction in the quantity of endosulfan sulfate pro-

duced. Surprisingly, the additions of PB also resulted in an increase in endosulfan disappearance. The data shown in Fig. 6 illustrate that the addition of 100 μ M PB resulted in a significant increase in the rate of endosulfan disappearance from nitrogen-deficient cultures. To determine if the increase in endosulfan disappearance was due to an alteration in the metabolic pathway, the metabolites of PB-treated and untreated cultures were analyzed. A comparison of metabolic products formed indicated that the addition of PB indeed stimulated the hydrolysis pathway. Figure 7 represents the summation of total sulfur oxidation product and hydrolysis-derived metabolites formed at various concentrations of PB. After 48 h of incubation, cultures receiving 10 and 100 μ M PB produced higher percentages of hydrolytic metabolites than those containing 1 μ M or no PB.

DISCUSSION

P. chrysosporium has been shown to degrade and mineralize a large spectrum of environmentally significant organic pollutants including dioxins (32), polychlorinated biphenyls (9), chlo-

FIG. 3. Time course for disappearance of 1 ppm of endosulfan I (■) and formation of endosulfan metabolites: endosulfan sulfate (○), endosulfan hydroxyether (□), and putative endosulfan dialdehyde (△) by nitrogen-deficient cultures of *P. chrysosporium* BU-1. Values represent the means of duplicate incubations. The percent error of the mean for endosulfan disappearance was less than (12.0) for the formation of endosulfan metabolites.

rophenols (10, 30), and alkyl halides (13). Most studies suggest that the xenobiotic-degrading capabilities of this organism are closely associated with the production of the extracellular peroxidases, LiP and MnP (2). However, recent studies have shown that in some cases, cultures of *P. chrysosporium* not favoring the production of LiP and MnP were still effective in degrading certain xenobiotics such as DDT (dichlorodiphenyl-trichloroethane) (15); 2,4-dichlorophenoxyacetic acid (35); 2,4,5-trichlorophenoxyacetic acid (33, 35); benzene, toluene, ethylbenzene, and xylene (34), and phenanthrene (7, 26). While extensive characterization of the LiP has revealed their importance in pollutant degradation, subsequent studies have raised some question as to the involvement of additional enzyme systems that may contribute to the overall degradation of environmental chemicals. In this study we have used endosulfan as a model xenobiotic to assess the enzymatic mechanisms

of pesticide degradation by the white rot fungus *P. chrysosporium*.

Fungal metabolism of endosulfan was demonstrated by substrate disappearance and product formation. A sequential identification of the primary and secondary metabolic products enabled us to elucidate the metabolic pathways utilized for the degradation of this pesticide. These results suggest that *P. chrysosporium* possesses two major enzymatic activities, oxidation and hydrolysis, that participate in the metabolism of this compound.

In comparison to previous reports on the microbial metabolism of endosulfan, our results indicate that *P. chrysosporium* is unique in its capabilities to both oxidize and directly hydrolyze the parent form of this pesticide. While several soil fungi have been shown to metabolize endosulfan, it has been determined that most produce endosulfan sulfate as the major metabolic product and thus favor an oxidative rather than a hydrolytic mechanism for metabolism of this pesticide. Soil bacteria are capable of metabolizing endosulfan and produce predominantly endosulfan diol, favoring hydrolysis (18). While it is uncommon for most soil fungi to metabolize endosulfan to endosulfan diol, *P. chrysosporium* readily forms endosulfan diol under a variety of culture conditions. The fact that diol formation occurs under both nitrogen-rich and nitrogen-deficient conditions suggests that the enzyme system(s) responsible for endosulfan hydrolysis is not under the same regulatory mechanism as the LiP. Recently, Katayama and Matsumura (12) have shown that static cultures of *Trichoderma harzianum* were capable of producing endosulfan diol as a principal metabolite. They suggest that a hydrolytic enzyme, such as a sulfatase, is responsible for the indirect formation of endosulfan diol by hydrolysis of endosulfan sulfate. Our studies with *P. chrysosporium*, however, indicate that endosulfan sulfate is a terminal end product of oxidation and does not undergo further conversion under these conditions. These results suggest that endosulfan diol formation by *P. chrysosporium* must be due to a direct hydrolysis of the parent endosulfan itself and is not the result of a secondary metabolism of endosulfan sulfate.

Subsequent to hydrolysis of endosulfan, most soil bacteria are capable of further oxidizing endosulfan diol to several other non-sulfur-containing metabolites. Miles and Moy have proposed a succession of oxidation reactions converting endosulfan diol to endosulfan ether, endosulfan hydroxyether, and endosulfan lactone (19). While cultures of *P. chrysosporium* were able to oxidize endosulfan diol to endosulfan hydroxyether, they did not produce significant quantities of endosulfan ether or endosulfan lactone. When added directly to fungal cultures however, endosulfan ether was oxidized to endosulfan hydroxyether and other oxidation products whereas endosulfan lactone was not metabolized further. The oxidation state of endosulfan ether is equivalent to endosulfan diol; thus, the production of a similar product was expected. Why

TABLE 2. Metabolism of endosulfan and individual endosulfan metabolites^a

Original substrate added	Formation of metabolic product ^b :					
	Putative dialdehyde	Ether	Hydroxyether	Lactone	Sulfate	Other metabolites
Endosulfan	+++	+	+++	+	+++	—
Endosulfan Diol	+++	+	+++	+	—	—
Endosulfan Ether	+++	—	+++	—	—	—
Endosulfan Lactone	—	—	—	—	—	—
Endosulfan Sulfate	—	—	—	—	—	—

^a Each individual substrate was purchased from a commercial source and incubated in nitrogen-deficient cultures of *P. chrysosporium* BU-1 at 30°C for 7 days.

^b +++, formation of major product; +, formation of minor product; —, no product formation.

FIG. 4. Proposed metabolic pathway for metabolism of endosulfan by *P. chrysosporium* BU-1. Solid arrows indicate the major metabolic pathways; broken arrows indicate minor metabolic pathways.

P. chrysosporium fails to produce endosulfan ether and endosulfan lactone with such a highly oxidative capacity under nitrogen-deficient conditions remains to be determined. It is probable that oxidation of the diol to the hydroxyether is catalytically more favorable and competitive to the dehydration reaction forming the ether. The metabolite studies indicate that the disappearance of endosulfan hydroxyether is followed by the formation of a terminal metabolite not previously identified. Initial analysis of this compound by MS indicates that it is a hexachlorocyclopentadiene metabolite similar to both endosulfan hydroxyether and endosulfan dicarboxylic acid. However, distinctive fragmentation patterns in both the EI and EC mass spectra can be attributed to an open chain dialdehyde. The dialdehyde is an end product of metabolism and equivalent in redox potential to endosulfan lactone. The inability to metabolize either of these compounds further may

TABLE 3. Quantities of oxidative and hydrolytic metabolites formed in nitrogen-deficient and nitrogen-rich media by 7-day-old fungal cultures

Metabolite ^a	% Formation of metabolites in ^b :	
	Nitrogen-deficient medium	Nitrogen-rich medium
Endosulfan (parent)	0.39 ± 0.8	20.9 ± 10.7
Endosulfan sulfate (oxidation product)	65.7 ± 8.3	15.6 ± 5.2
Hydrolysis products		
Endosulfan dialdehyde	33.6 ± 13.0	12.3 ± 3.6
Endosulfan hydroxyether	27.3 ± 11.3	44.6 ± 15.8

^a Products were identified from the metabolism of endosulfan by *P. chrysosporium* BU-1. Cultures were incubated and analyzed as described in Materials and Methods.

^b The results are expressed as the percentage of endosulfan I. Values represent the means of four independent incubations of duplicate samples ± standard deviations.

FIG. 5. Inhibition of endosulfan sulfate formation by PB. Cultures were incubated with 1 ppm of endosulfan for the indicated period of time with either no PB addition (closed bars), 10 μM PB (hatched bars), or 100 μM PB (open bars). Values represent the means of duplicate incubations; Error bars indicate standard errors of the means.

FIG. 6. Effect of PB on metabolism of 1 ppm of endosulfan by *P. chrysosporium* BU-1 in stationary cultures containing 3 ml of nitrogen-deficient medium. PB (100 μ M) (●) was added with endosulfan after 3 days growth. Cultures not receiving PB (○) were incubated separately to avoid cross-contamination.

represent a limit in the oxidative capacity of this organism as seen with the LiP.

Medium composition was shown to have a significant influence on the metabolic outcome of endosulfan metabolism. Although *P. chrysosporium* extensively degrades endosulfan under a variety of nutrient conditions, nitrogen deficient, carbon deficient, and nitrogen rich, the relative ratios of the metabolic products formed under each condition were different. Similar changes in medium composition have also been shown to influence the metabolism of other xenobiotics. Phenanthrene metabolism by *P. chrysosporium* occurs by one of two distinct metabolic pathways dependent on the nitrogen concentration in the media (7, 26). In addition, buffer type, manganese concentration, and growth conditions have all been shown to have profound effects on LiP and MnP isozyme activities and profiles (17, 23). Such changes in medium composition may enable us to manipulate the metabolism of *P. chrysosporium* and facilitate the degradation of select xenobiotics. Barr and Aust have proposed such a mechanism for the degradation of trinitrotoluene (3). Altering the metabolic processes of microbial degrading systems may prove to be particularly important when controlling for the toxicity of specific degradation products produced. For example, while oxidation of endosulfan to endosulfan sulfate is efficient at eliminating the original pesticide, conversion to endosulfan sulfate is not regarded as detoxification or degradation.

Since the oxidation of endosulfan by *P. chrysosporium* was most abundant under nitrogen-deficient conditions, we sought to determine whether LiP or MnP is involved in endosulfan metabolism. While the onset of oxidative metabolism may occur under conditions favoring the production of LiP and MnP, it has been clearly established that LiP and MnP are not responsible for the oxidation of endosulfan to endosulfan sulfate. To test the hypothesis that endosulfan is oxidized by a monooxygenase, studies were performed with the addition of specific monooxygenase inhibitors. PB, a known cytochrome P-450 in-

FIG. 7. Summation of endosulfan metabolites produced by *P. chrysosporium* BU-1. Nitrogen-deficient cultures received 1, 10, or 100 μ M PB and no PB. Formation of endosulfan sulfate (solid bars) and total hydrolysis products (hatched bars) were determined after a 48-h incubation period.

hibitor (22), significantly inhibits formation of endosulfan sulfate when added to nitrogen- and carbon-deficient cultures. Often oxidative metabolism of sulfur-containing pesticides is attributed to either flavin-containing or cytochrome P-450 monooxygenases. Flavin-containing monooxygenases are known to catalyze a variety of S oxidations. However, cytochrome P-450 has been shown to specifically oxidize the cyclic sulfite ester of endosulfan to endosulfan sulfate in several organisms (16). Sutherland et al. (26) have also suggested the potential involvement of monooxygenase and epoxide hydrolase activity in the initial oxidation and hydration of phenanthrene in nitrogen-rich cultures of *P. chrysosporium*. Additionally, it is important to point out that while the oxidative action was significantly inhibited with the addition of PB, hydrolysis of endosulfan was enhanced. These results indicate that oxidation and hydrolysis of endosulfan occur by two separate processes and suggest that *P. chrysosporium* may contain several different mechanisms for the metabolism and degradation of xenobiotics. We are currently attempting to isolate and characterize the enzymes responsible for both oxidation and hydrolysis of this pesticide.

ACKNOWLEDGMENTS

This work was supported by NIEHS Superfund grant (5P42-ES-04699) and the U.S. EPA (CR81-9658) Center for Ecological and Health Research.

We thank A. D. Jones for his assistance with GC-MS and K. M. Scow for reviewing this paper.

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