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Biodegradation of Crystal Violet by the White Rot Fungus *Phanerochaete chrysosporium*

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Biodegradation of crystal violet (*N,N,N',N',N'',N''*-hexamethylpararosaniline) in ligninolytic (nitrogen-limited) cultures of the white rot fungus *Phanerochaete chrysosporium* was demonstrated by the disappearance of crystal violet and by the identification of three metabolites (*N,N,N',N',N''*-pentamethylpararosaniline, *N,N,N',N''*-tetramethylpararosaniline, and *N,N',N''*-trimethylpararosaniline) formed by sequential N-demethylation of the parent compound. Metabolite formation also occurred when crystal violet was incubated with the extracellular fluid obtained from ligninolytic cultures of this fungus, provided that an H_2O_2-generating system was supplied. This, as well as the fact that a purified ligninase catalyzed N-demethylation of crystal violet, demonstrated that biodegradation of crystal violet by this fungus is dependent, at least in part, upon its lignin-degrading system. In addition to crystal violet, six other triphenylmethane dyes (pararosaniline, cresol red, bromphenol blue, ethyl violet, malachite green, and brilliant green) were shown to be degraded by the lignin-degrading system of this fungus. An unexpected result was the finding that substantial degradation of crystal violet also occurred in nonligninolytic (nitrogen-sufficient) cultures of *P. chrysosporium*, suggesting that in addition to the lignin-degrading system, another mechanism exists in this fungus which is also able to degrade crystal violet.

The triphenylmethane dye crystal violet (*N,N,N',N',N'',N''*-hexamethylpararosaniline) (Fig. 1) has seen extensive use in human and veterinary medicine, as a biological stain, and as a textile dye (4, 16, 19, 28, 32, 33). Unfortunately, wastewater treatment facilities are often unable to completely remove commercial dyestuffs, including triphenylmethane dyes such as crystal violet, from contaminated wastewater, thus contributing to the pollution of aqueous habitats (23, 24, 26, 34).

Also, triphenylmethane dyes have been found in soil and river sediment as a consequence of improper chemical waste disposal (25). For example, malachite green has been found in sediments from the Buffalo river, a tributary of Lake Erie (25). Significantly some triphenylmethane dyes have been shown to be carcinogenic (U.S. Environmental Protection Agency Genetox program, 1986) and crystal violet has been shown to be a potent clastogen (2, 3). Also, this class of chemicals has been suggested to be responsible for promotion of tumor growth in some species of fish (25).

The wood-rotting fungus *Phanerochaete chrysosporium* is able to degrade a broad spectrum of structurally diverse organopollutants (5–8, 11, 12, 30). Recent results in a number of laboratories have shown that the ability to degrade such an array of chemical compounds is due to the lignin-degrading system of this organism which occurs in response to nutrient (nutrient nitrogen, carbohydrate, or sulfur)-limiting conditions (5–8, 11, 12, 15, 27, 30).

In part, the lignin-degrading system consists of a number of peroxidases that are secreted by the fungus under these conditions of nutrient limitation. These peroxidases are commonly referred to as ligninases or lignin peroxidases and have the ability to catalyze the depolymerization of lignin as well as the initial oxidation of a wide variety of other compounds (11, 12, 27, 30). Included among the compounds shown to be degraded to CO_2 by nutrient nitrogen-deficient cultures of this fungus are DDT, 2,3,7,8-TCDD, polychlorinated biphenyls, benzo[*a*]pyrene, pentachlorophenol, chlordane, and phenanthrene (5–8, 30).

In this paper we report that triphenylmethane dyes also undergo extensive biodegradation in ligninolytic cultures of *P. chrysosporium*. Additionally, we report that the first reactions in the oxidative biodegradation of crystal violet are N-demethylation reactions catalyzed by a lignin peroxidase.

MATERIALS AND METHODS

Abbreviations. In this study, the trivial names of many common dyes are used as a matter of convenience. The following names of these dyes are those recognized by the Chemical Abstract Service: crystal violet, *N*-[4-[bis[4-(dimethylamino)phenyl]methylene] - 2,5 - cyclohexadien - 1 - ylidine] - *N* - methylmethanaminium chloride; cresol red, 4,4'-(3H-2,1 - benzoxathiol - 3 - ylidene)bis(2-methylphenol)-*S,S* - dioxide; bromphenol blue, 4,4' - (3H - 2,1 - benzoxathiol -3-ylidene)bis[2,6 - dibromophenol] - *S,S* - dioxide; brilliant green, *N* - [4 - [[4 - (diethylamino)phenyl]phenylmethylene] - 2,5 - cyclohexadien - 1 - ylidene] - *N* - ethylethanaminium sulfate (1:1); malachite green, *N* - [4 - [[4 - (dimethylamino)phenyl[phenyl]methylene] - 2,5 - cyclohexadien - 1 - ylidene] - *N* - methylmethanaminium chloride; ethyl violet, *N* - [4 - [bis[4 - (diethylamino)phenyl]methylene] - 2,5 - cyclohexadien -1 - ylidene] - *N* - ethylethanaminum chloride; pararosaniline, Benzenamine, 4-[(4-aminophenyl) (4-imino-2,5-cyclohexadien-1-ylidene)methyl]monohydrochloride.

Crystal violet is also referred to as *N,N,N',N',N'',N''*-hexamethylpararosaniline, and its mono-, di-, and tri-*N*-demethylation products are referred to as *N,N,N',N',N''*-penta-, *N,N,N', N''*-tetra-, and *N, N', N''*-trimethylpararosaniline, respectively.

Other abbreviations or trivial names used are as follows: DDT, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo[*p*]dioxin; chlordane, 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methano-1*H*-indene.

Microorganism. *P. chrysosporium* BKM-F-1767 was ob-

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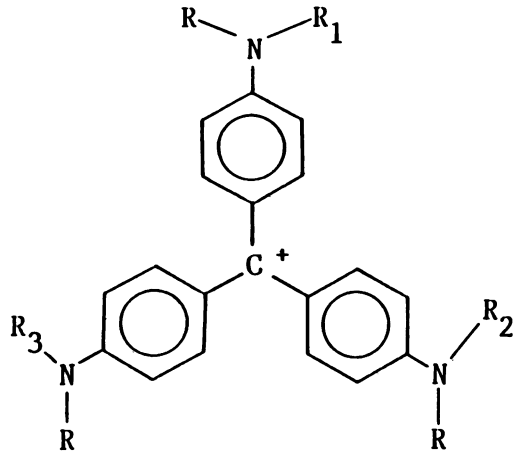

FIG. 1. Structure of crystal violet (*N,N,N',N',N'',N''*-hexamethylpararosaniline) (R, R_1, R_2, and R_3 = $-CH_3$), *N,N,N',N',N''*-pentamethylpararosaniline (R, R_1, R_2 = $-CH_3$; R_3 = $-H$), *N,N,N',N''*-tetramethylpararosaniline (R and R_1 = $-CH_3$; R_2 and R_3 = $-H$), and *N,N',N''*-trimethylpararosaniline (R = $-CH_3$; R_1, R_2, and R_3 = $-H$).

tained from the U.S. Department of Agriculture, Forest Products Laboratory, Madison, Wis., and maintained on malt agar slants at room temperature. Subcultures were routinely made every 30 to 60 days.

Culture conditions. *P. chrysosporium* was incubated at 37°C in the liquid culture medium (10 ml) previously described (9, 20) in 250-ml Wheaton bottles equipped with Teflon-sealed caps. This medium consists of 56 mM glucose, 1.2 mM ammonium tartrate, mineral salts, and thiamine (1 mg/liter) in 20 mM dimethylsuccinate (sodium) buffer (pH 4.5). Cultures were established by inoculating the medium with spores as described previously (20). During the first 3 days of incubation, cultures were allowed to grow in an atmosphere of air. After 3 days, the cultures were flushed with oxygen, resealed, and incubated for another 3 days.

Biodegradation. Cultures of *P. chrysosporium* were allowed to grow for 6 days as described above. At this time, 50 μl of crystal violet (1.0 mg/ml in H_2O) was added to cultures with gentle mixing to give a final concentration of 12.3 μM. Cultures were then flushed with oxygen and resealed. Crystal violet disappearance in the extracellular fluid was monitored by assaying the absorbance of the wavelength maximum of the dye at selected intervals during the incubation period. Biodegradation of other triphenylmethane dyes was assayed in a similar manner.

Crystal violet disappearance, as well as metabolite formation and disappearance, was also monitored by high-performance liquid chromatography (HPLC). Aliquots (0.5 ml) of the extracellular fluid were removed at selected intervals. Each aliquot was then extracted with 0.5 ml of methylene chloride, and 50-μl aliquots of the methylene chloride extracts were used for HPLC analysis.

Biodegradation experiments with only the extracellular fluid were performed in a similar manner. Fungal mycelium and extracellular fluid were separated by centrifugation (10,000 × *g* for 10 min).

Reverse-phase HPLC was performed by using a system equipped with an Altex model 110A pump (Anspec Co., Inc., Ann Arbor, Mich.), a Rheodyne injector (Rheodyne, Inc., Cotati, Calif.), an R-Sil C-18 reverse-phase column (4.6 by 250 mm) (Alltech Associates Inc., Deerfield, Ill.), and a Schoeffel 770 detector (ABI Analytical, Kratos Div., Ramsey, N.J.). For analytical procedures, isocratic elution was performed with acetonitrile-water (80:20, vol/vol) containing 0.01 M 2-naphthalenesulfonic acid and 0.01 M trifluoroacetic acid as described (1). In some experiments in which *N,N',N''*-trimethylpararosaniline was required for mass spectrometry, elution was performed with the same solvent system, except that 2-naphthalenesulfonic acid was omitted. Elution of crystal violet and crystal violet metabolites was monitored at 560 nm. Although neither crystal violet nor its N-demethylation products have absorption maxima at this wavelength, they all have substantial A_{560}, and it was for this reason that this wavelength was chosen to monitor the appearance and disappearance of these compounds.

In some experiments, crystal violet that had been adsorbed by fungal mycelium was resolubilized by adding 10 ml of methanol to the culture and then homogenizing it in a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged (10,000 × *g* for 1 min), and the A_{591} of the supernatant was determined. Also, the supernatant was analyzed by HPLC as described above.

It should be noted that in the culture medium and culture medium-methanol (1:1), the absorption maximum of crystal violet was found to occur at 591 nm. However, in 100% methanol, the absorption maximum occurred at 588 nm.

Lignin peroxidase purification. The lignin peroxidase used in this study was purified from the extracellular fluid of an agitated culture (1 liter) of *P. chrysosporium* BKM-F-1767 in a 2.8-liter Fernbach flask. The agitated culture was initiated from a 2-day-old mat culture as described (14), except that the culture medium of the mat culture consisted of 10 mM dimethyl succinate (sodium) (pH 4.5) containing 56 mM glucose, 1.2 mM ammonium tartrate, thiamine, and a mineral supplement (20) and the culture was grown under ambient atmosphere rather than 100% oxygen. The agitated culture medium consisted of 10 mM dimethylsuccinate (sodium) (pH 4.5) containing 56 mM glucose, 1.2 mM ammonium tartrate, 0.4 mM veratryl alcohol, 0.6 mM benzyl alcohol, 0.1% Tween 80, thiamine (1 mg/liter), and a mineral supplement that was sevenfold the concentration of that used in the mat culture (14). After 5 days of incubation (at 37°C and 200 rpm) under ambient atmosphere, the extracellular fluid was harvested and separated from the mycelium by centrifugation (10,000 × *g* for 15 min). The extracellular fluid was then concentrated to 37 ml by using a Minitan (Millipore Corp., Bedford, Mass.) concentrator equipped with a membrane having a molecular exclusion limit of 10,000.

The preparation was frozen, thawed, and centrifuged (15,000 × *g* for 20 min) twice to remove mucilaginous material which interfered with subsequent chromatography. The preparation was dialyzed overnight against 10 mM sodium acetate (pH 6.0), and the ligninases were purified by fast protein liquid chromatography with a Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden). The sample was applied in 10 mM sodium acetate (pH 6.0) and washed with 25 ml of the same buffer. The column was eluted with a linear gradient from 10 mM sodium acetate (pH 6.0) to 0.7 sodium acetate (pH 6.0) (2 ml/min for 70 min). The column was then further eluted with a linear gradient from 0.7 M sodium acetate (pH 6.0) to 1.0 M sodium acetate (pH 6.0) (2 ml/min for 20 min).

The peak which eluted at 0.18 M sodium acetate was the predominant lignin peroxidase in this preparation. Because another lignin peroxidase, which eluted at 0.21 M sodium

acetate, was incompletely resolved, only the material from the front base to the top of the predominant ligninase peak was collected and used in subsequent experiments. This material was assayed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and polyacrylamide gel isoelectric focusing. By these criteria, this protein was judged to be greater than 95% pure.

Enzyme assays. Lignin peroxidase activity was measured by the procedure described by Tien and Kirk (31). One unit of enzyme activity oxidized 1 μmol of veratryl alcohol in 1 min at room temperature. Oxidation of triphenylmethane dyes was monitored at the visible wavelength maximum of each dye. The reaction mixtures contained 50 mM sodium succinate buffer (pH 4.5) and 25 μM H_2O_2. The concentration of each dye was 12.3 μM.

Chemicals. Crystal violet, brilliant green, methyl violet, cresol red, pararosaniline, ethyl violet, and malachite green were purchased from Sigma Chemical Co., St. Louis, Mo. Bromphenol blue was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. *N,N,N',N',N''*-penta-, *N,N,N',N''*-tetra, and *N,N',N''*-trimethylpararosaniline are not commercially available. They are, however, present in preparations of methyl violet (1, 4). Therefore, analytical amounts of these compounds were obtained by thin-layer chromatography (TLC) of methyl violet with precoated Silica Gel 60 G F-254 plates (20 by 20 cm; thickness, 250 μm) (E. Merck AG, Darmstadt, Federal Republic of Germany). The solvent system used was propanol-H_2O-glacial acetic acid (90:9:1). Approximately 0.5 mg of methyl violet in 40 μl of methanol was applied in a line 2 cm from the bottom of the plate. The TLC plate was allowed to develop for 4 h, during which time four brightly colored bands were clearly separated. Band I (blue; R_f = 0.26), band II (bluish purple; R_f = 0.35), band III (purple; R_f = 0.43), and band IV (pink; R_f = 0.61) were designated as crystal violet, *N,N,N',N',N''*-pentamethylpararosaniline, *N,N,N',N''*-tetramethylpararosaniline, and *N,N',N''*-trimethylpararosaniline, respectively.

Although R_f values obtained in this study were in close agreement with those previously reported (Table 1) (4), the absorption maximum (in methanol) obtained for each compound was substantially different (Table 1). Therefore, it was necessary to confirm these structural assignments by mass spectrometry.

RESULTS

Mass spectrometry of crystal violet and its putative biodegradation products. The identity of reference standards, purified from methyl violet by TLC, was confirmed by mass spectrometry. The interpretation of mass spectra of triphenylmethane dyes is complicated by the fact that organic salts often undergo thermal decomposition or rearrangement during mass spectrometry. Therefore, in this study a number of other ions similar in magnitude to the ion characteristic of the cation molecular weight were found for each of the compounds identified. For example, for crystal violet, an intense ion characteristic of the cation molecular weight was found at *m/e* 372. However, an even larger peak was found at *m/e* 373, and a smaller peak was found at *m/e* 374. It has been shown that crystal violet is converted to leuco (reduced) crystal violet (molecular weight, 373) on being heated in the dark at 200°C for 3 h in a vacuum or in air (22). Therefore, leuco crystal violet may be formed by thermal reduction of crystal violet on the heated probe of the mass spectrometer, thus suggesting that the peak at *m/e* 373 is caused by ionization of leuco crystal violet. This interpretation is the same as that proposed by McEwen et al. (22). The less-intense peak of *m/e* 374 may be an ion formed by further rearrangement of neutral crystal violet in which a tertiary nitrogen is protonated to form the leuco crystal violet cation (molecular weight, 374). The interpretation of mass spectra of *N,N,N',N',N''*-penta and *N,N,N',N''*-tetramethylpararosaniline is even more complicated, because the removal of N-methyl groups allows formation of other ions which are consistent with a structural assignment as the imine of each dye and its corresponding reduced form. Thus, for *N,N,N',N',N''*-pentamethylpararosaniline, the expected ion (*m/e* 358) representative of the cation molecular weight was found, as were ions of *m/e* 359 and 360, characteristic of the leuco form and the cation formed by protonation of the leuco form, respectively. Also found was an ion of *m/e* 356, which we interpret as the imine formed from *N,N,N',N',N''*-pentamethylpararosaniline, and an ion of *m/e* 357, which we interpret as an ion formed from the reduced imine. For *N,N,N',N''*-tetramethylpararosaniline, the expected ion characteristic of the cation molecular weight was found (*m/e* 344), as were ions with *m/e* 345 and 346, which are representative of the leuco form and the protonated leuco form of the dye. Also found were ions of *m/e* 342 and 343, which are representative of the ionic and reduced imine, respectively. In all cases, fragment ions found in mass spectra of these compounds were consistent with structural assignments as crystal violet, *N,N,N',N',N''*-pentamethylpararosaniline and *N,N,N',N''*-tetramethylpararosaniline for TLC bands I, II, and III, respectively. Although band IV appeared as a single band on TLC, HPLC revealed that this material consisted of one predominant peak and a number of contaminants, which were removed by HPLC before mass-spectral analysis. The mass spectrum of this material was consistent with a structural assignment as *N,N',N''*-trimethylpararosaniline. The expected ion (*m/e* 330), which is characteristic of the cation molecular weight was found, as was an ion of *m/e* 331, which is characteristic of an ion formed from leuco *N,N',N''*-trimethylpararosaniline. Also found were ions of *m/e* 328 and 327, consistent with an ion formed from the

TABLE 1. R_f values in TLC and the wavelength of maximum absorption of crystal violet and its N-demethylation products[a]

Compound	Analytical standards		Biodegradation products		Analytical standard (4)		Photodegradation product (4)	
	Absorption max (nm)	R_f	Absorption max (nm)	R_f	Absorption max (nm)	R_f	Absorption max (nm)	R_f
Crystal violet	588	0.26	588	0.26	583	0.23	583	0.23
Pentamethylpararosaniline	579	0.35	580	0.35	573	0.32	574	0.33
Tetramethylpararosaniline	571	0.43	572	0.45	568.5	0.43	568.0	0.45
Trimethylpararosaniline	566	0.61	566	0.62		0.58	556.5	0.58

[a] The solvent system was propanol-water-glacial acetic acid (90:9:1). The wavelength of maximum absorption was determined in methanol.

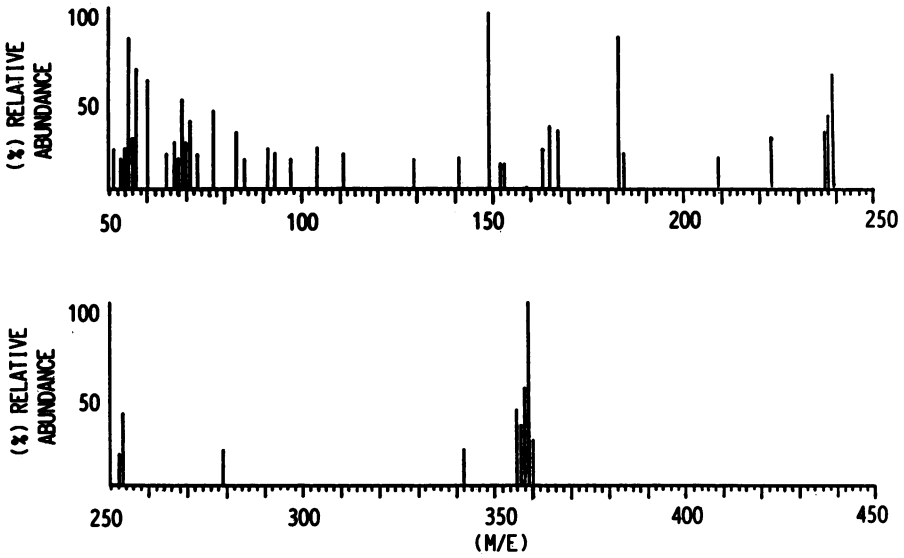

FIG. 2. Mass spectrum of *N,N,N',N',N''*-pentamethylpararosaniline.

imine and the reduced imine, respectively. Ions of *m/e* 327 and 326 were also present. The fact that a major fragment ion of *m/e* 104 (characteristic of an *N*-methylaniline fragment) was found, whereas no fragment of *m/e* 121 (characteristic of an *N,N*-dimethylaniline fragment) was found, confirmed a structural assignment of *N,N',N''*-trimethylpararosaniline.

The mass spectrum of *N,N,N',N',N''*-pentamethylpararosaniline is presented in Fig. 2 for illustrative purposes.

Crystal violet biodegradation products were identified by comigration in HPLC and TLC and by comparison of the absorption maximum of each metabolite with that of the appropriate analytical standard (Table 1; Fig. 3).

Crystal violet metabolism by cultures of *P. chrysosporium*. When crystal violet (12.3 μM) was added to nitrogen-limited ligninolytic cultures of *P. chrysosporium*, the dye underwent extensive degradation, as evidenced by a substantial decrease in the absorbance of the dye in the extracellular culture medium (Fig. 4A). After 6 h of incubation the absorbance was approximately 35% of that initially present, and, typically, upon continued incubation for 24 h the absorbance decreased to less than 1% of the initial level. In some experiments substantial absorbance (approximately 14% of that initially present) was still observed after 24 h of incubation. However, upon continued incubation for a total of 72 h, this absorbance decreased to undetectable levels. In addition to a decrease in absorbance, the wavelength of the absorbance maximum underwent a hypsochromic shift from 591 to 544 nm (Fig. 4A). Some of the dye appeared to be bound to the mycelium. However, even this material was decolorized at the end of the incubation period (72 h). Cultures were considered to be ligninolytic if veratryl alcohol oxidase activity was detectable. In nutrient nitrogen-deficient cultures, veratryl alcohol oxidase activity was shown to be 16.5 ± 1.8 U/liter. This activity was not detected in nutrient nitrogen-sufficient cultures.

In controls which contained no fungus, a 13% decrease in absorbance was noted after 72 h of incubation. However, most of this decrease in absorbance occurred within the first hour of incubation and was not associated with a spectral shift of the absorbance maximum of the dye. To ensure that the decrease in absorbance was due to biodegradation and not to a pH change or a nonbiological oxidation or reduction, we determined the effect of pH over the range pH 3.5 to pH 5.0 and the effect of 0.1% H_2O_2 and 0.1% ascorbate on crystal violet absorbance. The absorbance of crystal violet was not affected by pH over the range tested. Hydrogen

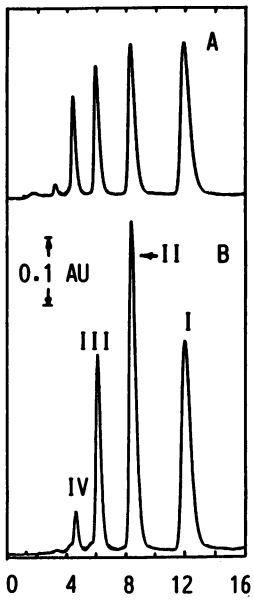

FIG. 3. HPLC elution profile of the metabolites formed upon incubation of crystal violet with nutrient nitrogen-deficient cultures of *P. chrysosporium*. (A) Two ligninolytic cultures (6 days old) of *P. chrysosporium* were incubated with crystal violet (12.3 μM). One culture was incubated for 2 h, and one was incubated for 4 h. The extracellular fluids of the two cultures were combined and extracted with an equal volume of methylene chloride, and 40 μl of the methylene chloride layer was used for HPLC analysis. (B) Methyl violet (40 μl, 52 μg/ml) served as a standard. This dye is a mixture of crystal violet (t_R = 12 min), *N,N,N',N',N''*-pentamethylpararosaniline (t_R = 8.4 min), *N,N,N',N''*-tetramethylpararosaniline (t_R = 6 min), and *N,N',N''*-trimethylpararosaniline (t_R = 4.6 min).

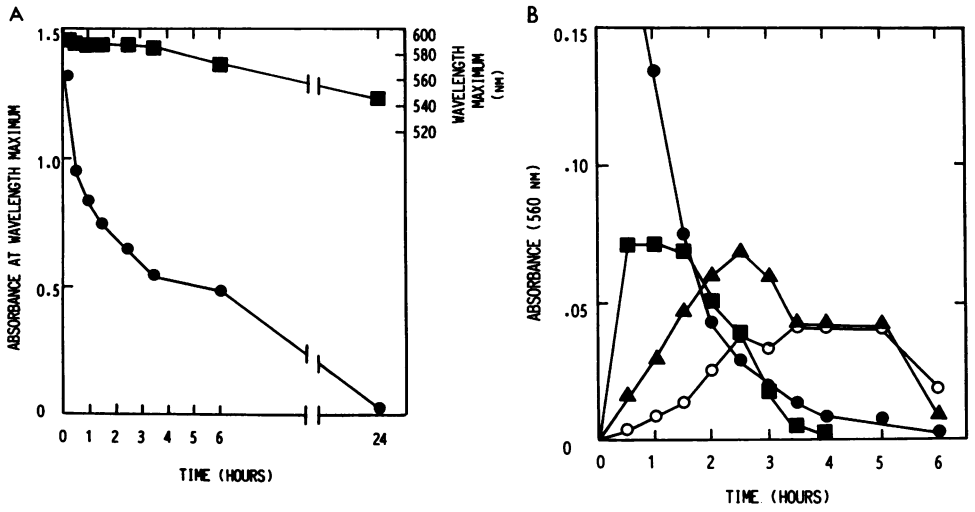

FIG. 4. Biodegradation of crystal violet in a ligninolytic culture (nitrogen limited) of *P. chrysosporium*. (A) Decolorization of crystal violet (●) and change in wavelength absorption maximum (■). (B) Disappearance of crystal violet (●) and formation of *N,N,N',N',N''*-pentamethylpararosaniline (■), *N,N,N',N''*-tetramethylpararosaniline (▲), and *N,N',N''*-trimethylpararosaniline (○).

peroxide (0.1%) caused a 6% decrease in A_{591} after 72 h of incubation, and ascorbate caused less than a 1% decrease in A_{591} after 72 h of incubation. A spectral shift of the absorbance maximum was not observed under any of these conditions.

The fact that the absorption maximum of the dye underwent a hypsochromic shift in nutrient nitrogen-limited cultures suggested that the dye might be undergoing N-demethylation, because the expected N-demethylation products (*N,N,N',N',N''*-penta-, *N,N,N',N''*-tetra-, and *N,N',N''*-trimethylpararosaniline) are known to have absorption maxima at wavelengths lower than that of crystal violet (4). Therefore, cultures of *P. chrysosporium* which had been incubated with crystal violet were extracted with methylene chloride and analyzed by TLC (Table 1) and by HPLC (Fig. 3). Three metabolites which comigrated with *N,N,N',N',N''*-penta-, *N,N,N',N''*-tetra-, and *N,N',N''*-trimethylpararosaniline were observed (Table 1; Fig. 3). The time course for crystal violet disappearance and for the appearance and disappearance of crystal violet metabolites was also studied (Fig. 4B). This study showed that *N,N,N',N',N''*-pentamethylpararosaniline appears to be the first metabolite formed, followed by *N,N,N',N''*-tetramethylpararosaniline and subsequently by *N,N',N''*-trimethylpararosaniline. Like crystal violet, these metabolites were also degraded.

The ability of nonligninolytic, nitrogen-sufficient (12 mM ammonium tartrate) 1-day-old cultures of *P. chrysosporium* to degrade crystal violet was also examined. After 72 h of incubation, the absorbance of crystal violet in the extracellular culture medium was 11% of that initially present and, unlike the situation with ligninolytic cultures, a substantial amount (22%) of the dye was found to be adsorbed to the fungal mycelium. No N-demethylation products of crystal violet were found in methanol extracts of these cultures. Also, the extracellular fluid from nonligninolytic cultures did not catalyze N-demethylation of crystal violet.

Degradation of crystal violet by extracellular fluid from ligninolytic cultures. Crystal violet was oxidized by extracellular fluid from ligninolytic nutrient cultures of *P. chrysosporium*, and the reaction products had shorter absorbance maxima than crystal violet (Fig. 5A). The time course for crystal violet disappearance and metabolite formation was similar to that observed in intact cultures (Fig. 5B). Thus, it was demonstrated that the first steps of crystal violet degradation by *P. chrysosporium* are extracellular. After 24 h of incubation, crystal violet, *N,N,N',N',N''*-penta-, and *N,N,N',N''*-tetramethylpararosaniline were no longer detected. Residual amounts of *N,N',N''*-trimethylpararosaniline were detected, as were two other metabolites, whose peak retention times during HPLC were 3.2 and 3.4 min. These putative crystal violet metabolites were not further characterized; however, their chromatographic characteristics and the fact that they are highly colored (pink) suggest that they may be dimethyl- or methylpararosaniline or pararosaniline.

Initial studies showed that the extracellular fluid had limited ability to degrade crystal violet unless H_2O_2 or an H_2O_2-generating system was added. We also found that supplementing the system with additional glucose (56 mM) promoted biodegradation.

Crystal violet N-demethylation by purified lignin peroxidase. Lignin peroxidase purified from nitrogen-limited cultures of *P. chrysosporium* was able to catalyze N-demethylation of crystal violet with results similar to those shown in Fig. 3A for the intact microorganism. These results confirm the hypothesis that the lignin-degrading system of this fungus catalyzes the initial steps in the oxidative biodegradation of crystal violet.

Biodegradation of other triphenylmethane dyes. Six other triphenylmethane dyes were degraded by ligninolytic cultures of *P. chrysosporium* as assayed by monitoring decolorization at their respective absorption maxima (Table 2). Involvement of the lignin-degrading system was confirmed by results in which the purified lignin peroxidase was shown to decolorize these dyes (Table 3).

Brilliant green, malachite green, and ethyl violet all contain N-alkyl groups. Therefore, the initial oxidation of these compounds may proceed via N-dealkylation in a manner similar to that shown for crystal violet. However, pararosaniline, cresol red, and bromphenol blue contain no N-alkyl groups. Thus, oxidation of these dyes occurs by a mecha-

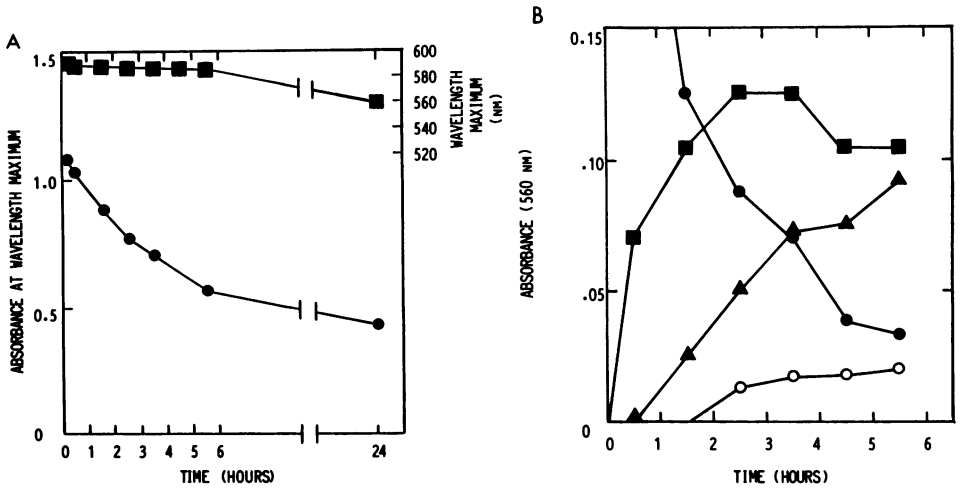

FIG. 5. Biodegradation of crystal violet in extracellular fluid obtained from a ligninolytic culture (nitrogen limited) of *P. chrysosporium*. (A) Decolorization of crystal violet (●) and change in wavelength absorption maximum (■). (B) Disappearance of crystal violet (●) and formation of *N,N,N',N',N''*-pentamethylpararosaniline (■), *N,N,N',N''*-tetramethylpararosaniline (▲), and *N,N',N''*-trimethylpararosaniline (○). The initial concentration of crystal violet was 12.3 μM. Glucose (56 mM) was added to initiate the reaction.

nism clearly different from that observed for crystal violet. These findings are consistent with the fact that the lignin-degrading system of *P. chrysosporium* is relatively nonspecific and that this nonspecific nature is due, at least in part, to the lignin peroxidases, which catalyze the initial oxidation of a wide variety of organic compounds (11, 12, 27).

DISCUSSION

Earlier studies of crystal violet biodegradation showed that this triphenylmethane dye is relatively resistant to biodegradation by microorganisms in the environment and in waste treatment facilities (23–26, 34). The inability of many bacteria to degrade crystal violet has been attributed to the fact that this dye is toxic to many microorganisms (23, 24, 32, 33). Some success in crystal violet decolorization in wastewater has been reported by using activated sludges that had been acclimated with crystal violet for 40 or 60 days (13). However, no dye degradation products were reported, and the possibility that decolorization was due to simple oxidation or reduction of the dye was not addressed. Thus, it is difficult to assess whether decolorization was due to adsorption by bacterial cells, simple oxidation or reduction of the dye to colorless forms, or true biodegradation. Michaels and Lewis (23) showed that among field-collected samples, between 6 and 28% of the bacterial colonies isolated from various environmental sources possessed the ability to decolorize crystal violet. However, these authors stated that transformation by these bacteria appeared to be slow when compared with the rate of transformation of other textile dyes. They also stated that none of the triphenylmethane dyes in their study were degraded to undetectable levels.

Biodegradation of triphenylmethane dyes by fungi has received less attention than that accorded to biodegradation by bacteria. However, Kwasniewska has shown that oxidative red yeasts (*Rhodotorula* sp. and *Rhodotorula rubra*) readily degrade crystal violet to undetectable levels (21). In these studies, neither H_2O_2 nor ascorbic acid was able to decolorize crystal violet. Results with these controls, combined with the fact that an intermediate degradation product formed by *R. rubra* appeared to undergo a hypsochromic shift of its absorbance maximum, relative to crystal violet, suggest that crystal violet in this system does undergo true biodegradation.

In the present study, we have shown that *P. chrysosporium* is also able to degrade crystal violet. Three degradation products were identified as *N,N,N',N',N''*-penta-, *N,N,N',N''*-tetra-, and *N,N',N''*-trimethylpararosaniline. These degradation products were formed by whole cultures of the

TABLE 2. Decolorization of triphenylmethane dyes by ligninolytic cultures of *P. chrysosporium*[a]

Dye	Absorption max (nm)	Decolorization (%) after:	
		24 h	48 h
Crystal violet	591	100	100
Pararosaniline	540	75.7	100
Cresol red	425	100	100
Bromphenol blue	591	97.7	100
Ethyl violet	577	94.7	100
Malachite green	617	76.8	100
Brilliant green	624	89.7	100

[a] Results are the average of two determinations.

TABLE 3. Decolorization of triphenylmethane dyes by a ligninase purified from *P. chrysosporium*

Dye	Rate of decolorization (% decolorization/min per μg)[a]
Crystal violet	14.2
Pararosaniline	33.0
Cresol red	2.8
Bromphenol blue	23.6
Ethyl violet	26.9
Malachite green	24.3
Brilliant green	45.0

[a] Results are the average of two determinations. The rate (% decolorization/min/μg protein) represents the initial rate of decolorization. It should be noted that the initial rate of decolorization was linear for less than 10 s. The initial concentration of each dye was 12.3 μM, and each assay mixture contained 1.75 μg of lignin peroxidase.

fungus, grown under nutrient nitrogen-limiting conditions, by extracellular fluid from these cultures, and by purified lignin peroxidase. Additionally, biodegradation appears to proceed beyond *N,N',N''*-trimethylpararosaniline, as evidenced by the fact that two additional, but as yet unidentified, colored crystal violet metabolites were found during HPLC. Also, these results showed that crystal violet is eventually degraded to a colorless product. Biodegradation of crystal violet in extracellular fluid from ligninolytic cultures required H_2O_2 or an H_2O_2-generating system. Supplemental glucose (56 mM) in extracellular fluid also promoted biodegradation of crystal violet, possibly by serving as a substrate for glucose oxidase. It should be noted, however, that glucose oxidase in *P. chrysosporium* is generally considered to be located in the periplasmic space (10). However, the fact that supplemental glucose supports crystal violet degradation suggests that small amounts of glucose oxidase might also be present in the extracellular fluid.

An unexpected result of this research was that substantial (89%) decolorization of crystal violet occurred in nonligninolytic cultures of *P. chrysosporium*. Although adsorption to fungal mycelium may account for some of the decolorization, only 22% of the decolorization observed could be attributed to this process. Therefore, it appears that this fungus may possess another mechanism for degrading crystal violet. This degradation process was not studied further. However, it should be noted that crystal violet was completely decolorized by ligninolytic cultures. While this did not occur under nonligninolytic conditions. Furthermore, N-demethylation products of crystal violet were not found under nonligninolytic conditions.

Workers in a number of laboratories have confirmed the fact that the nonspecific lignin-degrading system is able to degrade a wide variety of structurally diverse organic compounds, including some environmental pollutants that are among the most difficult to degrade (5–8, 11, 12, 27, 30). This biodegradative ability is due, at least in part, to the extracellular lignin peroxidases secreted by *P. chrysosporium*. These enzymes have been shown to be able to catalyze a wide variety of reactions including benzylic oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization, and O-demethylation (27, 31). The carbon skeletons of many of the compounds degraded by *P. chrysosporium* resemble substructures found within polymeric structures that have been proposed to be representative of lignin. However, lignin peroxidases from *P. chrysosporium* are also able to oxidize compounds such as benzo[*a*]pyrene, which, as noted by Sanglard et al. (30), bear little resemblance to lignin or lignin model substrates which have been used in lignin biodegradation studies. Like benzo[*a*]pyrene, crystal violet and the other dyes examined in this study bear little similarity to lignin or lignin model compounds. This fact is further emphasized by the seemingly anomalous fact that lignin does not contain nitrogen, yet attack at a nitrogen atom by a lignin peroxidase (i.e., N-demethylation) is the initial step in crystal violet degradation by *P. chrysosporium*.

Lignin physically protects cellulose from microbial attack. Therefore, the ability to degrade lignin presumably imparts a selective advantage to microorganisms which can degrade lignin and utilize cellulose, which is unavailable as a carbohydrate source to non-lignin degraders. However, biosynthesis and secretion of lignin peroxidases occur in response to nutrient nitrogen starvation, as well as to carbohydrate or sulfur starvation (11, 15). This, coupled with the fact that the lignin-degrading system degrades nitrogen-containing compounds as well as other compounds that bear little resemblance to lignin, leads one to speculate that the lignin-degrading system may, in fact, be a more generalized degradative system that, in addition to degrading lignin, nonspecifically attacks many organic compounds to secure a greater variety of nutrients for the cell.

Although a wide variety of substrates are oxidized by lignin peroxidases from *P. chrysosporium*, results of all or most studies to date are in accord with a free-radical mechanism for reactions catalyzed by this family of enzymes (30a). Furthermore, lignin peroxidases are similar to other peroxidases in that they also are activated by hydrogen peroxide to form compound I and compound II during their reaction cycle (29). Possibly the greatest difference between lignin peroxidases and other peroxidases is that lignin peroxidases are able to oxidize substrates with higher ionization potentials than those normally oxidized by conventional peroxidases (12).

Our results demonstrate that lignin peroxidases are also able to catalyze N-dealkylation reactions. Although this has not been previously shown, it was not totally unexpected, since it is well known that other peroxidases readily catalyze N-dealkylation of numerous substrates (17, 18). A more detailed biochemical characterization of N-demethylation of crystal violet by a purified lignin peroxidase is currently under study in this laboratory.

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