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Primary Product of the Horseradish Peroxidase-Catalyzed Oxidation of Pentachlorophenol

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Peroxidases are a class of enzymes that catalyze the oxidation of various phenolic substrates by hydrogen peroxide. They are common enzymes in soil and are also available commercially, so that they have been proposed as agents of phenolic pollutant transformation both in the environment and in engineered systems. Previous research on the peroxidase-catalyzed oxidation of pentachlorophenol (PCP) has suggested that tetrachloro-*p*-benzoquinone (chloranil) is the principal product and that a considerable fraction of the PCP added to reaction mixtures appears to be resistant to oxidation. In experiments employing alternative methods of product separation and analysis, we found that both of these observations are artifacts of extraction and analytical methods used in previous studies. The major product of the horseradish peroxidase-catalyzed oxidation of pentachlorophenol from pH 4−7 was 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone (PPCHD), which is formed by the coupling of two pentachlorophenoxyl radicals. The yield of chloranil and other soluble products was negligible. PPCHD is insoluble and unreactive in aqueous media but is reactive when dissolved in various organic solvents. Substantial amounts of chloranil were formed when PPCHD was dissolved in benzene, ethyl acetate, or methanol; less was formed in hexane and acetonitrile; and negligible amounts were formed in dimethylformamide. High-pressure liquid chromatography (HPLC) analysis of PPCHD indicated that it is capable of undergoing dissociation and reduction to pentachlorophenol under typical reversed-phase HPLC conditions. Chlorinated oligomeric products are formed when PPCHD is stored in acetonitrile, either alone or with added pentachlorophenol. Our results demonstrate that the removal of PCP in peroxidase-catalyzed reactions can be much higher than indicated in previous work, as long as the initial product is separated by filtration or other physical means.

Introduction

Pentachlorophenol (PCP) has been used widely as a biocide in many agricultural and industrial applications (*1, 2*). It is a ubiquitous pollutant that has been placed on the U.S. Environmental Protection Agency priority pollutant list, and the use of PCP for wood preservation and other purposes has been restricted or banned in several countries (*3*). Although losses of PCP can occur in the environment (*3*), it is persistent at highly contaminated sites (*4*).

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PCP is known to be biodegradable by several bacterial and fungal species (*2, 4*−*6*). Full-scale bioremediation of PCP-contaminated soil has been achieved (*4*), but PCP can be difficult to biodegrade in contaminated soil and groundwater. PCP-degrading microbes are not always present at contaminated sites, requiring inoculation with axenic cultures in such situations (*2, 4*). High concentrations of PCP can be inhibitory to the degrading organisms (*2, 7*), and supplemental carbon sources are often required to sustain biodegradation over extended periods of time (*2, 8*).

The oxidation of phenolic compounds, including PCP, is readily catalyzed by a number of extracellular fungal and plant oxidative enzymes. Enzyme-catalyzed oxidation therefore has been proposed as an alternative method of removing PCP and other phenols from contaminated aqueous mixtures (*9*). The oxidation of PCP has been reported to be catalyzed by lactoperoxidase (*10*), horseradish peroxidase (HRP) (*11*−*13*), lignin peroxidase (LIP) (*13*−*18*), *Coprinus cinereus* peroxidase (CIP) (*19*), and a laccase from *Trametes* (*Coriolus*)-*versicolor* (*20*−*22*). These enzymes generally catalyze one-electron oxidations, which for phenolic substrates lead to phenoxyl radical intermediates that can undergo various postenzymatic reactions, including coupling reactions to form dimeric products (*23, 24*). Researchers have proposed that peroxidases might serve to detoxify PCP-contaminated media by catalyzing the conversion of PCP to less chlorinated products (*14, 15, 22*) or by facilitating the polymerization of PCP with natural organic matter precursors via the pentachlorophenoxyl radical intermediate (*18, 25*−*27*).

In several laboratory studies on the enzyme-catalyzed oxidation of PCP in aqueous media, the predominant reaction product was reported to be tetrachloro-*p*-benzoquinone (chloranil) (*11, 14, 15, 17, 20, 22*), and a substantial fraction of the added PCP was recovered after the reaction (*11, 14, 16, 19, 20, 24*). Other observations, however, conflict with these findings. For example, the HRP-catalyzed oxidation of PCP at pH 4 results in a decrease in sample toxicity that is far greater than predicted on the basis of recovered PCP (*13, 19*). In two other studies, significant concentrations of chloranil and residual PCP were measured by HPLC analysis of reaction mixture extracts, but the UV absorbance spectrum of the crude reaction mixture itself either failed to indicate the presence of these compounds (*11*) or indicated the presence of additional compounds (*14*). For reactions catalyzed by HRP over the pH range 4−7, we have found that direct filtration of the reaction mixture consistently yields a precipitate and that chloranil is not observed as a significant product. We report the characterization of the precipitate isolated from the HRP-catalyzed oxidation of PCP and explain why chloranil and PCP were the species determined following enzymatic oxidation in previous studies.

Methods

Enzyme and Chemicals. Purified HRP (type VIII acidic isoenzyme) was purchased from Sigma (St. Louis, MO). The enzyme was dissolved in 1 mL of reagent water and stored at −15 °C when not in use. Catalytic activity was assayed just prior to PCP oxidation by oxidation of 2,2′-azinodi(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (*28*). The assay reaction mixture contained 67 mM phosphate buffer (pH 6.0), 1.7 mM ABTS, 0.83 mM hydrogen peroxide (H_2O_2), and enzyme. One unit (U) of catalytic activity is defined as the amount of enzyme required to form 1 μmol of product/min.

PCP and ABTS were purchased from Aldrich (Milwaukee, WI). ^{14}C-Labeled PCP (specific activity 7.9 mCi/mmol; purity

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>98%), deuterated chloroform, trifluoroacetic acid, trifluoroacetic anhydride, and fuming nitric acid were purchased from Sigma. All solvents were HPLC or spectrophotometric grade, and all other chemicals were ACS reagent grade or equivalent.

2,3,4,5,6-Pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone (PPCHD) was synthesized by oxidizing 10.6 g of PCP with 4.2 mL of fuming nitric acid as a suspension in 30 mL of trifluoroacetic acid and 45 mL of trifluoroacetic anhydride, as described by Reed (29).

Enzymatic Oxidation of PCP. PCP oxidation was carried out at pH 4, 5, 6, and 7. Since the solubility of PCP increases with pH, we used higher initial PCP concentrations with increasing pH to facilitate product recovery. Reactions at pH 4 were conducted with 10 μM PCP, 6 μM H_2O_2, and 0.2 unit/mL of enzyme in a 10 mM sodium citrate/citric acid buffer. Reactions at pH 5 were conducted with 75 μM PCP, 45 μM H_2O_2, and 0.2 unit/mL of enzyme in a 10 mM sodium citrate/citric acid buffer. Reactions at pH 6 and 7 were conducted with 200 μM PCP, 120 μM H_2O_2, and 0.2 unit/mL of enzyme in a 10 mM potassium phosphate buffer. Controls were equivalent to sample vials except no H_2O_2 was added. For experiments with ^{14}C-labeled PCP, 20 000 disintegrations per min (dpm) were added to reaction vials and controls.

Reactions were initiated by adding H_2O_2, and the vials were shaken for 2 h on a rotary shaker at 250 rpm. The reaction mixtures were then vacuum filtered through a 0.2-μm polycarbonate filter (Poretics Corp.; Livermore, CA) or a 0.02-μm alumina membrane filter (Anodisc, Whatman Scientific; Maidstone, England). The precipitate collected on the filter was dissolved in 2 mL of acetonitrile for immediate analysis, and the unamended filtrate was analyzed separately. For the experiment in which a mass balance of radiolabel was evaluated, the filter and the filtrate were added to 9 mL of scintillation fluid (Scintisafe, Fisher Scientific; Pittsburgh, PA). Radioactivity (measured in dpm) was determined for 4 min on a scintillation counter (model 1900 TR, Packard Instruments; Meriden, CT).

Optical Spectra and HPLC Analysis. Ultraviolet/visible (UV/VIS) spectra were recorded on a U-3300 spectrophotometer (Hitachi Instruments, Danbury, CT) between 195 and 500 nm with a slit width of 0.5 nm. HPLC analysis was performed with a Waters (Milford, MA) system equipped with a model 616 pump, a 600S controller, a 717 plus autosampler, and a 996 photodiode array detector that allowed the spectra to be scanned between 200 and 500 nm. A C_8 column (25 cm × 4.6 mm, 5 μm particle size; Supelco, Inc., Bellefonte, PA) was used for product separation. The mobile phase was 55% acetonitrile: 45% H_2O containing 0.1% trifluoroacetic acid for 5 min, increasing linearly to 100% acetonitrile over 10 min, and holding for 10 min before returning to initial conditions.

^{13}C Nuclear Magnetic Resonance Spectroscopy (NMR). PPCHD (50 mg) was dissolved in 1 mL of deuterated chloroform ($CDCl_3$) and stored in an ice bath until analysis (~1 h). The ^{13}C NMR spectrum was recorded on a Bruker AM-500 spectrometer at 125 MHz, with shifts reported in ppm relative to the carbon resonance of tetramethylsilane (TMS). The centerline of the $CDCl_3$ triplet (77 ppm) served as a standard.

Mass Spectrometry. Mass spectrometric analysis was performed on a VG 70 250SEQ mass spectrometer. Electron impact (EI) spectra were obtained using a direct insertion probe at 60 and 15 eV. Synthetic PPCHD was added to the probe as a solid, while the enzymatic product was added to the probe tube dissolved in carbon tetrachloride, which was then allowed to evaporate.

Electron Paramagnetic Resonance (EPR) Spectroscopy. PPCHD (50 mg) was dissolved in 1 mL of deuterated chloroform ($CDCl_3$) immediately prior to analysis. X-Band EPR spectra were recorded on a JES-RE1X spectrometer (JEOL, USA Inc.) Typical acquisition parameters were microwave power of 1 mW, a sweep width of 50−100 G, a field modulation of 0.32−2 G, and a scan time of 2−4 min.

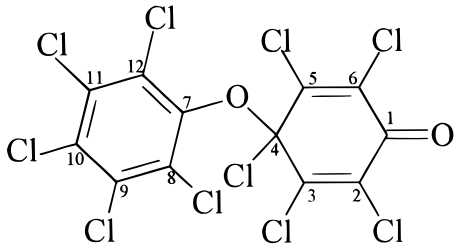

FIGURE 1. Structure of 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone. The assigned ^{13}C NMR shifts (in ppm) are C1 = 168.2; C2 (C6) = 132.4; C3 (C5) = 132.3; C4 = 95.1; C7 = 147.1; C8 (C12) = 132.3; C9 (C11) = 145.2; C10 = 128.9.

Results

NMR and EPR Spectra of PPCHD. The oxidation of pentachlorophenol by fuming nitric acid yielded an orange−yellow solid (mp 175−177 °C) whose color and melting point corresponded to that of PPCHD (Figure 1), as described by Reed (*29*). A well-resolved ^{13}C NMR spectrum of PPCHD was recorded in chloroform-*d* using a standard ^{13}C acquisition time (15 s). In contrast to other perchlorinated aromatic compounds, the addition of a spin relaxation reagent was not required. This finding is consistent with the observation by EPR spectroscopy (vide infra) indicating dissociation of PPCHD in chloroform to give a low concentration of pentachlorophenoxyl radical, which acts to enhance spin relaxation.

In accord with the σ-plane including C1, C4, C7, and C10, eight resonances were apparent as two sets of four, with an ~2:1 intensity ratio. The more intense set of resonances can be attributed to the carbons related by reflection in the σ-plane, and the less intense set can be atributed to the unique carbons. Assignment of the latter set is readily made by comparison with chemical shifts reported for chloranil and perchlorinated phenyl ethers: C1, 168.2; C7, 147.1; C10, 128.9; and C4, 95.1 ppm (*30*−*33*). The carbonyl carbon C1 and the sp^3-hybridized C4 are expected to appear at the lowest and highest fields, respectively. The relative shifts of C7 and C10 are established on the basis of an upfield position predicted for C10 para to the ether oxygen. The resonances of the symmetry-related carbons are assigned according to the following rationale. On the basis of relative intensities, resonances at 132.4 and 132.3 (partially resolved from a slightly less intense signal) have similar relaxation times and are assigned to vinylic carbons C2 (C6) and C3 (C5), respectively, a slight downfield shift assumed for the carbons adjacent to the carbonyl group. Since phenyl carbons ortho to the ether oxygen are expected to exhibit an upfield shift relative to the meta positions, the remaining signals at 132.3 and 145.2 ppm are assigned to C8 (C12) and C9 (C11), respectively.

PPCHD has been reported to be EPR active at ambient temperatures in carbon tetrachloride and benzene through dissociation of the dimer into pentachlorophenoxyl radicals (*31*). Chloroform solutions of PPCHD were also EPR active, giving a signal 37 G in width with no hyperfine structure, similar to the signals obtained in carbon tetrachloride and benzene. The *g*-value of 2.0076 in chloroform confirms that the signal originates from a pentachlorophenoxyl radical in this solvent as well. Dissociation to the radical species in chloroform is consistent with indications from ^{13}C NMR spectrometry of enhanced relaxation of the ^{13}C nuclear spins. A capillary sample of PPCHD in acetonitrile exhibited a similar

TABLE 1. Distribution of Radiolabel after HRP-Catalyzed Oxidation of ^{14}C-Labeled PCP at Different pH Values[a]

recovery of DPM (%)	pH 4	pH 5	pH 6	pH 7
filtrate				
sample	7.9	4.5	6.0	22.2
control	79.2	85.8	94.4	93.2
precipitate				
sample	87.4	91.7	87.1	71.0
control	9.1	7.4	4.3	3.7
total				
sample	95.3	96.2	93.1	93.2
control	88.3	93.2	98.7	96.9

[a] All samples and controls were filtered through a 0.02-μm filter.

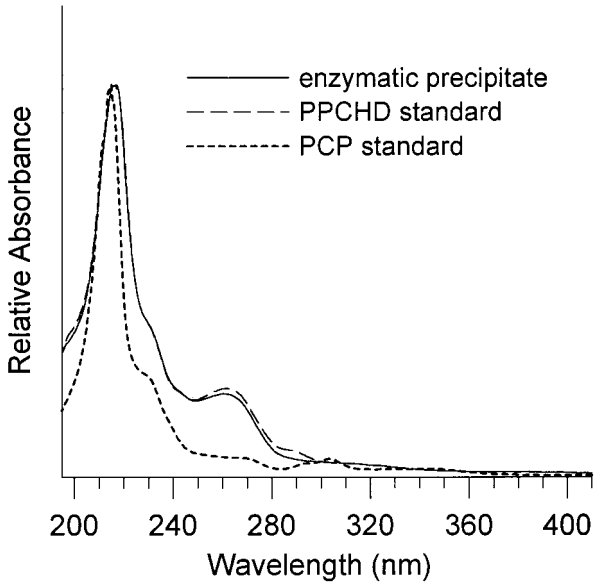

FIGURE 2. UV/VIS spectra of synthetic PPCHD and the precipitate obtained from the HRP-catalyzed oxidation of PCP at pH 5 as compared to the spectrum for PCP. Samples were dissolved in acetonitrile and analyzed immediately. Spectra were normalized with respect to the maximum absorbance of each species.

EPR spectrum, supporting the occurrence of monomer–dimer equilibration in polar media as well.

Enzymatic Oxidation of PCP. The HRP-catalyzed oxidation of PCP over the pH range 4–7 led to the formation of a yellow precipitate. The precipitate could be collected on a 0.2-μm filter except for the material obtained at pH 7, which required a 0.02-μm filter. Mass balance data for the HRP-catalyzed oxidation of ^{14}C-labeled PCP over this pH range revealed that most of the radioactivity added was recovered in the precipitate (Table 1). In contrast, most of the radiolabel in the control samples was recovered in the filtrate.

The UV/VIS spectrum of the precipitate from the enzymatic reaction (at all pH values) matched the spectrum of the synthetic PPCHD standard (Figure 2). The spectra, recorded in acetonitrile, were characterized by an absorbance maximum at 218 nm with a shoulder at 259 nm. This spectrum is distinct from that of chloranil in acetonitrile, which exhibits an absorbance maximum at 288 nm. The ratio of molar extinction coefficients $\epsilon_{288}/\epsilon_{218}$ for the precipitate (0.05) and chloranil (4.2) provides a strong indication that little or no chloranil is present in the HRP-derived product. The UV spectrum of PCP is characterized by the absence of significant absorbance at 259 nm.

The HPLC analysis of PPCHD proved to be difficult, initially yielding only a single peak that coeluted with a PCP

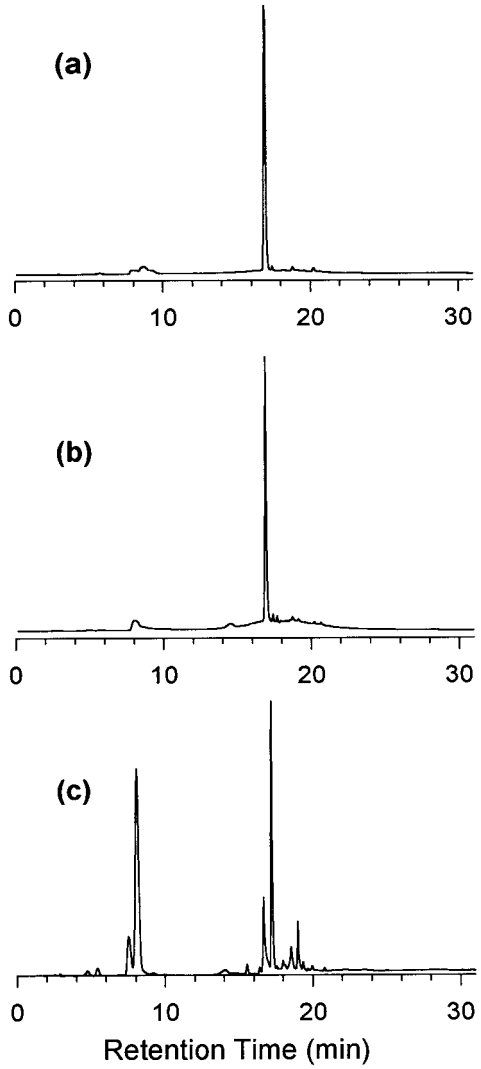

FIGURE 3. HPLC chromatograms (225 nm detection) of (a) an acetonitrile solution of the precipitate from the HRP-catalyzed oxidation of PCP at pH 6, dissolved and analyzed immediately after collection; (b) a solution of PPCHD in acetonitrile analyzed immediately; and (c) a solution of PPCHD in acetonitrile after incubation for 2 weeks. PCP standards eluted at 8 min and chloranil standards eluted at 5.8 min under the same HPLC conditions (not shown).

standard. This result may be explained by the dissociation of PPCHD and subsequent reduction, perhaps by trace metals in the column packing or other components of the HPLC system, since it has been shown that PPCHD is readily reduced to PCP by metals such as zinc in an alcohol or acid medium (*29*, *31*). The presence of trace reducing-contaminants associated with the column packing is supported by the observation that, after conditioning the column by repeated injections of PPCHD, it was possible to obtain consistent chromatograms having a predominant peak at a longer elution time (18 min) than that of PCP (8 min) (Figure 3, panels a and b). The precipitated enzymatic reaction product and a PPCHD standard produced identical chromatograms on the conditioned column, and the peak at 18 min had a UV spectrum identical to that of the PPCHD standard. No peak corresponding to chloranil was detected in either the standard or the enzymatic oxidation product.

The enzymatic reaction product and a PPCHD standard gave identical mass spectra (not shown), with a base cluster centered at m/z 266 corresponding to PCP. Weak ion clusters were centered at m/z 495 and 456, consistent with PPCHD$^{\bullet+}$–

TABLE 2. Solvent Effects on the Formation of Chloranil from the Decomposition of 200 μM PPCHD[a]

solvent	chloranil yield (μM)[b]
dimethylformamide	0.4 ± 0.4
hexane	12.8 ± 3.5
acetonitrile	17.3 ± 4.8
benzene[c]	64.9 ± 2.5
methanol	85.1 ± 3.9
ethyl acetate[c]	87.1 ± 0.9

[a] Samples were incubated for 24 h in 50 mL of solvent at 33 °C and then analyzed by HPLC. [b] Mean and standard deviation of three replicates. [c] Samples were blown to dryness, resuspended in an equivalent amount of acetonitrile, and then analyzed by HPLC.

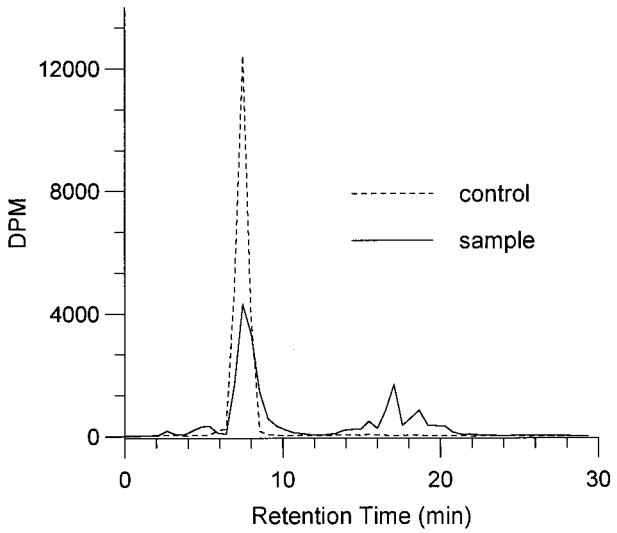

FIGURE 4. HPLC radiochromatogram of an acetonitrile solution initially containing 63 μM PPCHD and 12.5 μM ^{14}C-labeled PCP after incubation for 3 days. Chromatogram represents 56 samples collected at 32-s intervals. The radioactivity injected for both the sample and the control was 20 000 dpm.

Cl and $PPCHD^{\bullet+}-2Cl$, respectively. The base peak most likely arises from the protonation of the dissociation product—the pentachlorophenoxyl radical—in the ion source. Given the lability of PPCHD, the absence of the molecular ion is not surprising. No chloranil molecular ion (cluster with base peak at m/z 246) was observed in either spectrum.

Chloranil was reported to be a product of the peroxidase-catalyzed oxidation of PCP when organic solvents were used to extract products from the reaction mixtures or as cosolvents in enzymatic reactions (*11*, *14*, *15*, *17*, *20*, *22*). The recovery of chloranil from a 200 μM solution of PPCHD in various solvents varied from negligible in the case of dimethylformamide to 87 μM in the case of ethyl acetate (Table 2). Variable amounts of PCP were also found, but it is unclear how much of the PCP may have originated from dissociation and reduction of PPCHD on the HPLC column.

Incubation of PPCHD in acetonitrile for 2 weeks resulted in the formation of several other compounds, as indicated by HPLC analysis (Figure 3c). The reactivity of PPCHD in acetonitrile was evaluated further by incubating PPCHD with ^{14}C-labeled PCP in acetonitrile for 3 days. Some of the radiolabel initially associated with ^{14}C-labeled PCP was distributed among higher molecular weight compounds (Figure 4), suggesting that PPCHD reacts with PCP in acetonitrile to form oligomers.

Discussion

We infer from the following evidence that horseradish peroxidase catalyzes the extensive and essentially stoichiometric conversion of PCP to 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone (PPCHD): (i) the majority of the radioactivity in reactions carried out with ^{14}C-labeled PCP is associated with the precipitate (Table 1); (ii) analysis of the precipitate by UV/VIS spectroscopy (Figure 2), HPLC (Figure 3), and mass spectrometry indicated that the precipitate is virtually identical to the synthesized PPCHD; and (iii) we have seen no evidence for any other significant product of the reaction. PPCHD is formed by the coupling of two pentachlorophenoxyl radicals (*11*, *29*), the expected products of one-electron oxidation reactions catalyzed by HRP and other peroxidases. The formation of pentachlorophenoxyl radicals from the HRP-catalyzed oxidation of PCP has been observed (*11*), but the coupling product has not been identified previously as the major product of this reaction.

In earlier studies on the peroxidase- or laccase-catalyzed oxidation of PCP, chloranil and PCP were reported to be the predominant compounds remaining after the reactions were terminated (*11*, *14*, *15*, *17*, *20*, *21*, *25*). The apparent residual PCP was assumed to be resistant to oxidation based on its persistence after the addition of fresh enzyme (*17*, *19*). Chloranil has been proposed to form by an enzyme-mediated one-electron oxidation of the pentachlorophenoxyl radical to a carbocation, followed by the addition of water (*11*, *14*). However, in most of the previous studies on the enzymatic oxidation of PCP, the products were extracted from the reaction mixture with an organic solvent followed by chromatographic separation of the extract. In this study, we examined the behavior of PPCHD and demonstrated that it is unstable under these analytical conditions. PPCHD decomposes to chloranil and PCP on standing in several organic solvents, with a significant yield of chloranil within a 24-h period (Table 2). Particularly noteworthy is the formation of chloranil in ethyl acetate, a solvent commonly used for extraction, and in methanol, commonly used as a mobile phase in HPLC analysis. PPCHD is also easily reduced to PCP by sodium iodide and zinc in alcohol or acidic media (*29*, *34*) and is reduced by sodium borohydride and sodium iodide to chlorinated phenoxyphenols (*31*, *32*).

PPCHD is relatively insoluble in water and does not react nor decompose in water at temperatures up to 100 °C (*29*). Neither the precipitate from the enzymatic reaction nor the synthesized product decomposed in aqueous suspension at pH 4 over incubation periods as long as 12 days (not shown). We took advantage of these characteristics to isolate PPCHD directly from the enzymatic reaction mixture by filtration. Under acidic conditions, the collection by filtration is efficient, while at neutral pH the product is generated in fine suspension and must be collected on filters of pore size ≤0.02 μm.

The reaction of PPCHD with spin relaxation enhancers normally added to perchlorinated hydrocarbons for ^{13}C NMR spectrometry has prevented acquisition of NMR data (*31*). However, it proved possible to record the ^{13}C NMR spectrum of PPCHD in the absence of added relaxation enhancers. The explanation for this unexpected behavior lies in the dissociation of the dimer to a low concentration of pentachlorophenoxyl radical, which provided the necessary spin relaxation pathway. Such equilibration has been reported for oligomeric ethers of other perchlorinated compounds (*35*) and has been observed for PPCHD in carbon tetrachloride and benzene (*31*). We have demonstrated that dissociation also occurs in chloroform and acetonitrile. Consistent with reversible homolytic dissociation, on standing in acetonitrile, PPCHD forms other oligomeric products (Figure 3c) and incorporates ^{14}C-labeled PCP into oligomers (Figure 4).

It is clear that the removal of PCP by peroxidase-catalyzed oxidation can be much more extensive than has been indicated in other work and that potentially toxic products

such as chloranil are not likely to be found in the aqueous phase from such reactions. The present study also illustrates that sample preparation and analytical methods must be carefully evaluated in subsequent work on the enzyme-catalyzed oxidation of PCP and in other systems involving the one-electron oxidation of PCP.

Acknowledgments

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