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Peroxidase-Catalyzed Oxidation of 2,4,6-Trichlorophenol

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Abstract. 2,4,6-Trichlorophenol (TCP) is an environmental contaminant that is toxic, mutagenic, and carcinogenic. We have investigated peroxidase-catalyzed oxidation of TCP as an alternative pathway of TCP bioactivation using horseradish peroxidase (HRP) as a model peroxidase. TCP was shown to function as a reducing substarte for HRP as evidenced by TCP-dependent, HRP-catalyzed reduction of 5-phenyl-4-penten-1-yl hydroperoxide (PPHP) to its corresponding alcohol. In addition, TCP was shown to undergo hydroperoxide (H_2O_2, ethyl hydroperoxide, or PPHP)-dependent metabolism as evidenced by electronic absorption spectroscopic analysis of reaction mixtures. A single major product was detected by reverse phase HPLC and was identified as 2,6-dichloro-1,4-benzoquinone (2,6-dichloro-2,5-cyclohexadiene-1,4-dione, CAS no. 697-91-6) on the basis of electronic absorption spectroscopy, mass spectrometry, and cochromatography with synthetic standard. In addition, HRP-catalyzed oxidation of TCP yielded EPR-detectable phenoxyl radical intermediates whose EPR spectrum consisted of a 1:2:1 triplet characterized by proton hyperfine coupling constants $a^{H(3,5)} = 2.35$ gauss. Mechanisms for the hydroperoxide-dependent, HRP-catalyzed oxidation of TCP are proposed that are consistent with these results.

Chlorophenols represent major and persistent worldwide environmental pollutants that have been used as biocides and/or have been generated during the manufacture of pentachlorophenol. 2,4,6-Trichlorophenol (TCP, CAS no. 88-06-2) has been used as an antimildew agent in the treatment of textiles and as a wood and glue preservative. TCP is also generated by treatment of phenol-containing waste waters with hypochlorite (NTP 1985) and represents a metabolite of hexachlorobenzene (Engst *et al* 1976) and lindane (Fitzloff *et al.* 1982), two widely used biocides. TCP is toxic and carcinogenic. TCP has been shown to be carcinogenic in rats and mice following chronic feeding bioassays (NCI 1979), is mutagenic in the L5178Y mouse lymphoma cell test (NTP 1987), and is positive in the lambda prophage induction assay when a liver S9 activation system is included (DeMarini *et al.* 1990). A major factor influencing TCP toxicity and carcinogenicity may involve metabolic activation by cytochrome P450. For example, TCP has been shown to undergo NADPH-dependent, cytochrome P450–catalyzed oxidation in Aroclor 1254–induced rat liver S9 fractions yielding 2,6-dichloro-1,4-benzene diol (Juhl *et al.* 1989). Incubation of the latter with PM2 DNA resulted in single strand breaks that appear to involve reactive oxygen species, generated during diol autoxidation.

Alternatively, TCP may undergo oxidative metabolic activation by mammalian peroxidases (*e.g.* prostaglandin H synthase, myeloperoxidase, thyroid peroxidase, salivary peroxidase, lactoperoxidase, or others) because various phenols have been shown to function as effective reducing cosubstrates for many peroxidases (reviewed in Everse *et al.* 1991). However, metabolic mechanisms associated with the oxidation of TCP or most other chlorophenols by mammalian peroxidases have not been investigated. Heme peroxidases reduce hydroperoxides to the corresponding alcohols resulting in the generation of peroxidase compound I, which is two oxidizing equivalents above the resting ferric state (Dunford 1991). Compound I consists of a ferryl intermediate (Fe(IV) = O) and, depending on the peroxidase, a porphyrin π-cation radical and/or protein radical. Peroxidase compound I undergoes one-electron reduction by peroxidase-reducing substrates, yielding the ferryl intermediate compound II, which undergoes a second one-electron reduction (by another equivalent of reducing substrate) to the resting ferric state. Both compounds I and II exhibit estimated redox potentials of $\sim$1 volt and oxidize numerous compounds by sequential electron transfer mechanisms including phenols, catechols, hydroquinones, aromatic amines, vinylic alcohols, various heterocyclic compounds, and others.

We have investigated the hydroperoxide-dependent oxidation of TCP by horseradish peroxidase (HRP) as a model peroxidase system and report that TCP functions as an excellent substrate for HRP and undergoes hydroperoxide-dependent, HRP-catalyzed oxidative dechlorination to 2,6-dichloro-1,4-benzoquinone. We also report EPR evidence that hydroperoxide-dependent, HRP-catalyzed oxidation of TCP results in the intermediacy of TCP-derived phenoxyl radicals. Mechanisms consistent with our results are proposed for the HRP-catalyzed oxidation of TCP.

Methods

Materials

HRP (EC 1.11.1.7, type VI, $A_{402}/A_{280} = 3.1$) was purchased from Sigma (St. Louis, MO), desalted by Sephadex G-25 (Sigma) chromatography, and quantitated by measurement of the Soret absorbance at 402

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nm ($\epsilon_{402} = 9.5 \times 10^4$ M^{-1} cm^{-1}) (Mauk and Girotti 1974). Heat-denatured HRP was prepared by subjecting HRP solutions to 100°C for 20 min in a sealed container. 2,4,6-Trichlorophenol (TCP) (CAS no. 88-06-2) and 2,6-dichloro-1,4-benzoquinone (2,,6-dichloro-2,5-cyclohexadiene-1,4-dione; CAS no. 697-91-6) were purchased from TCI American (Portland, OR) and the TCP was recrystallized from hexane/acetone mixtures. These compounds are toxic and/or carcinogenic; extreme care was taken, including the use of gloves and protective clothing. Cinnamyl alcohol (3-hydroxy-1-phenyl-1-propene) and hydrogen peroxide (30%) were purchased from Aldrich (Milwaukee, WI) and the latter was quantitated by iodometric titration using KIO_3-standardized $S_2O_3^{2-}$. Ethyl hydroperoxide was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). 5-Phenyl-4-penten-1-yl hydroperoxide was synthesized as described by Weller *et al.* (1985). Enzymatic reactions were carried out at 25°C (except EPR experiments, which were conducted at ambient temperature) in 0.1 M phosphate buffer (pH 7.0), which had been treated with iminodiacetic acid chelating resin (Sigma) to minimize any contaminating redox-active transition metals. All other reagents were obtained through commercial sources.

Analytical Methods

Electronic absorption spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer equipped with Pelltier temperature control. Analytical reverse phase HPLC analysis was carried out using a Waters µBondapak C18 125Å 10-µm 3.9 × 300 mm HPLC column and a Waters 600E HPLC solvent pumping system and 994M photodiode array detector run on Waters Millennium® software (version 2.1) using a Gateway 2000 P5-90 computer.

Mass Spectrometry: HPLC-purified samples were analyzed by electron ionization mass spectrometry at 70 volts electron energy from a direct exposure probe. The mass spectrometer was a model 4000 upgraded to 4500 capabilities (Finnigan MAT, San Jose, CA). The quadrupole analyzer was scanned from 45 to 645 daltons in 1 s, the ion source was set to 150°C (uncorrected), and the probe consisted of Finnigan direct exposure probe with a rhenium wire tip and a Finnigan MAT current controller. The sample was applied to the rhenium wire tip via a methanol solution and the solvent was allowed to partially evaporate in a hood before insertion into the probe vacuum lock for final solvent evaporation before mass spectrometric analysis. The probe current was ramped linearly from 0 to 650 mA at 5 mA/s as the data were collected. The acquired spectra were compared with the NIH/EPA/NBS mass spectral library (INCOS Library Search, version 8; 49,000 spectra).

EPR Spectroscopy: EPR spectra were obtained using a Varian E-104 spectrometer custom interfaced with an IBM-compatible computer for data acquisition and analysis. All spectra were stored on the computer for later analysis and signal intensities were measured from the stored spectra using locally written software. All reactions were carried out at room temperature. Immediately after preparation, solutions were aspirated into a 10.5-mm Wilmad flat cell centered in the TM_{110} microwave cavity. To avoid the possibility of transition metal contamination, polyethylene tubing, rather than stainless steel, was used for the aspiration. Because sample aspiration permits the flat cell to be cleaned and refilled without being distrubed, reproducibility between scans was greatly improved. (The composition of reaction mixtures and instrumental parameters employed in the ESR experiments are described in Figure legend 6.)

PPHP Reduction Assays

The PPHP reduction assays are a modification of the method reported by Marnett's group (Weller *et al.* 1985). Incubations were conducted in triplicate. PPHP (1.0×10^{-4}M) was incubated with HRP (1.0×10^{-7}M), TCP (2.0×10^{-4}M), or HRP + TCP in a total volume of 2.0 ml. Incubation of PPHP with HRP and ascorbic acid (2.0×10^{-4}M) served as a positive control. After 6 min, PPHP and PPA were isolated by solid phase extraction using 3 ml octadecylsilyl columns (J.T. Baker & Co., Phillipsburg, NJ) and eluted with HPCL-grade methanol (2×1.0 ml). An internal chromatographic standard (IS) of cinnamyl alcohol (3-hydroxy-1-phenyl-1-propene, 25 µl of 8 mM stock solution in methanol) was added to each sample and the samples were filtered and chromatographed by reverse-phase HPLC using isochratic 58% methanol as the mobile phase at a flow rate of 2.0 ml/min. Eluents were detected by absorbance at 257 nm. The concentrations of PPA and PPHP were calculated relative IS by peak integration and PPHP reduction is expressed as [PPA]/([PPA] + [PPHP]) (Figure 1).

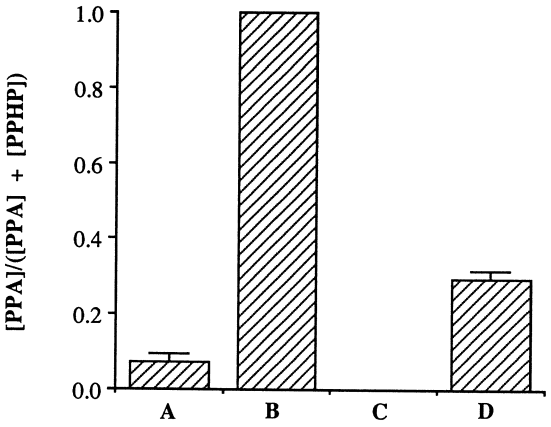

Fig. 1. TCP-dependent stimulation of HRP-catalyzed reduction of PPHP. PPHP (1.0×10^{-4}M) was incubated in 0.1 M phosphate buffer (pH 7.0) for 6 min at 25°C with A, HRP (1.0×10^{-7}M); B, HRP + TCP (2.0×10^{-4}M); C, TCP alone; or D, HRP + ascorbate (2.0×10^{-4}M). PPA and PPHP were extracted, chromatographed, and quantitated as described in the Methods section. The extent of PPHP reduction is expressed as $[PPA]([PPA] + [PPHP])^{-1}$ and the values represent the mean ± SD of triplicate measurements

Reverse-Phase HPLC of PCP Metabolites

TCP (2.0×10^{-4}M) was incubated with HRP (1.0×10^{-6}M) (or no addition) for 3 min followed by the addition of H_2O_2 (2.0×10^{-4}M) in a total volume of 1.0 ml. After 5 min, reaction mixtures were acidified with HCl to pH 2.0, saturated with NaCl, and extracted with ethyl acetate. The solvent was removed under a stream of argon or *in vacuo,* the residue was dissolved in HPLC-grade methanol (100 µl), and filtered. Reverse-phase HPLC was carried out using a mobile phase consisting of 50:50 solvent A in solvent B at $t = 0 - 2$ min followed by a linear gradient to 85:15 A/B over 32 min at a flow rate of 1.0 ml/min. Solvent A consisted of methanol and solvent B consisted of 0.02 M KH_2PO_4. Eluents were detected at 270 and 290 nm. Reaction mixtures for metabolite isolation for the acquisition of mass spectra were scaled-up 100-fold.

Results

TCP-Dependent, HRP-Catalyzed Reduction of PPHP

TCP was shown to function as a peroxidase reducing substrate for HRP as evidenced by its ability to stimulate the HRP-

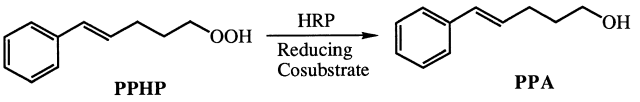

Fig. 2. Reaction equation

catalyzed reduction of 5-phenyl-4-penten-1-yl hydroperoxide (PPHP) to 5-phenyl-4-penten-1-ol (PPA) (Figure 2). Use of PPHP as a peroxidase probe was originally developed and utilized by Marnett and co-workers to evaluate the peroxidase reducing substrate efficiency of a variety of compounds for HRP (Weller *et al.* 1985) and prostaglandin H synthase (Markey *et al.* 1987). The method involves incubation of PPHP with peroxidase in the presence and absence of test compound(s), isolation of PPHP and PPA by solid phase extraction, and quantitation of PPHP reduction to PPA by reverse-phase HPLC analysis relative to an internal standard. Several modifications of the original method include the use of different reverse-phase HPLC stationary and mobile phases and utilization of cinnamyl alcohol as an internal standard (see Methods section). PCP stimulated HRP-catalyzed reduction of PPHP to PPA (Figure 1). The limited extent of hydroperoxide reduction in the absence of PCP may be due to some contaminating reductants in the enzyme preparation which were not completely removed by the desalting procedure. As shown in Figure 1, PCP-dependent stimulation of PPHP reduction (expressed as [PPA]/([PPA] + [PPHP])) was greater than that of ascorbic acid, which has previously been shown to function as an effective peroxidase reducing substrate for HRP (Weller *et al.* 1985). In contrast, PCP in the absence of HRP failed to promote any PPHP reduction (Figure 1).

Hydroperoxide-Dependent, HRP-Catalyzed Oxidation of TCP

HRP was shown to catalyze the hydroperoxide-dependent metabolism of TCP, using hydrogen peroxide, ethyl hydroperoxide, or PPHP as hydroperoxide substrates, as evidenced by time-dependent ultraviolet spectral changes of reaction mixtures (Figure 3). TCP exhibited an absorption maximum at 312 nm ($\epsilon_{312} = 4440 \pm 14\ M^{-1} \cdot cm^{-1}$) and a shoulder at 242 nm, both of which disappeared with the emergence of an absorption maximum at 274 nm upon addition of hydroperoxide. Identical spectral changes were observed using hydrogen peroxide, ethyl hydroperoxide, or PPHP, and all spectral changes were eliminated in the absence of HRP or when active enzyme was substituted with a heat-denatured preparation (not shown).

The products generated in reaction mixtures of TCP, HRP, and hydrogen peroxide were analyzed by reverse-phase HPLC as described in the Methods section. The product profile, obtained after reaction for 5 min, demonstrated the complete disappearance of TCP ($t_R = 23.8$ min) and the formation of a single major product ($t_R = 6.7$ min) (Figure 4). Product formation was not observed in reaction mixtures devoid of HRP or when active enzyme was replaced with a heat-denatured preparation. The major product cochromatographed with synthetic 2,6-dichloro-1,4-benzoquinone and exhibited an identical electronic absorption spectrum characterized by an absorption maximum at 274 nm. The mass spectrum of this metabolite (Figure 5) exhibited a molecular ion at *m/z* 176, which

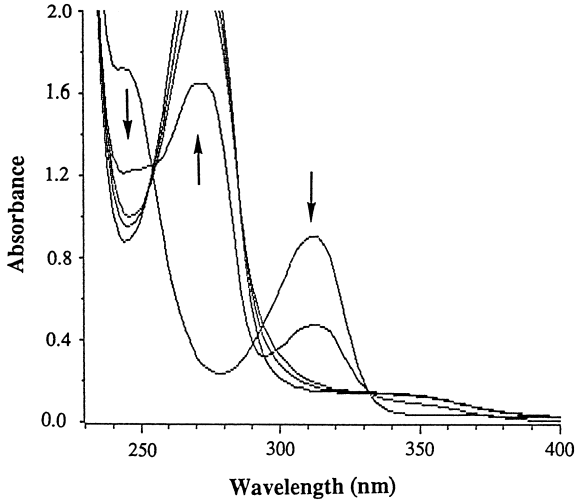

Fig. 3. Electronic absorption spectroscopic changes associated with HRP-catalyzed oxidation of TCP. TCP (1.75×10^{-4}M) was incubated at 25°C in 0.1 M phosphate (pH 7.0) with HRP (1.0×10^{-6}M) and H_2O_2 (3.50×10^{-4}M). Spectra were obtained at t = 0, 5 s, 30 s, 120 s, and 5 min

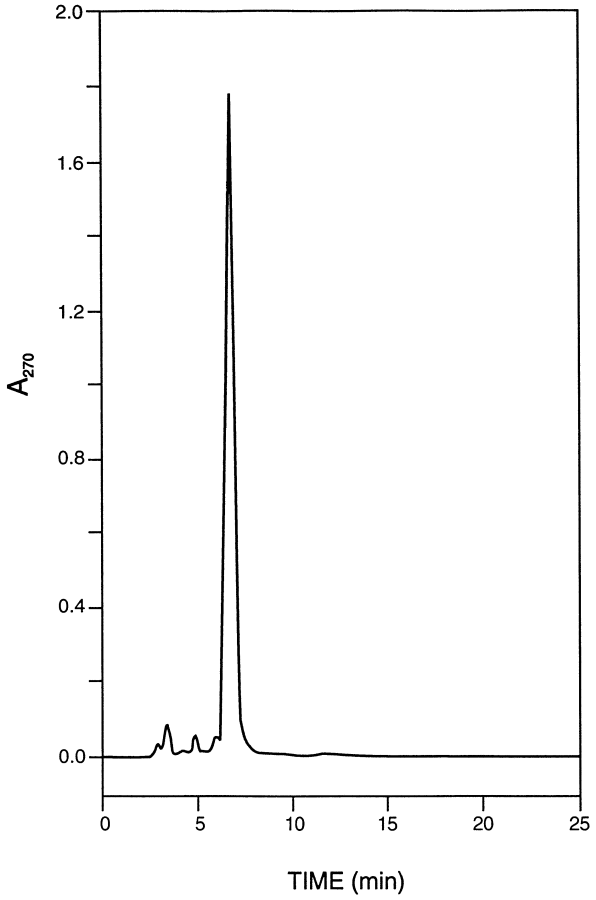

Fig. 4. Reverse-phase HPLC analysis of TCP oxidation products generated during hydroperoxide-dependent, HRP-catalyzed oxidation of TCP. Reaction conditions and analytical methods are described in the Methods section

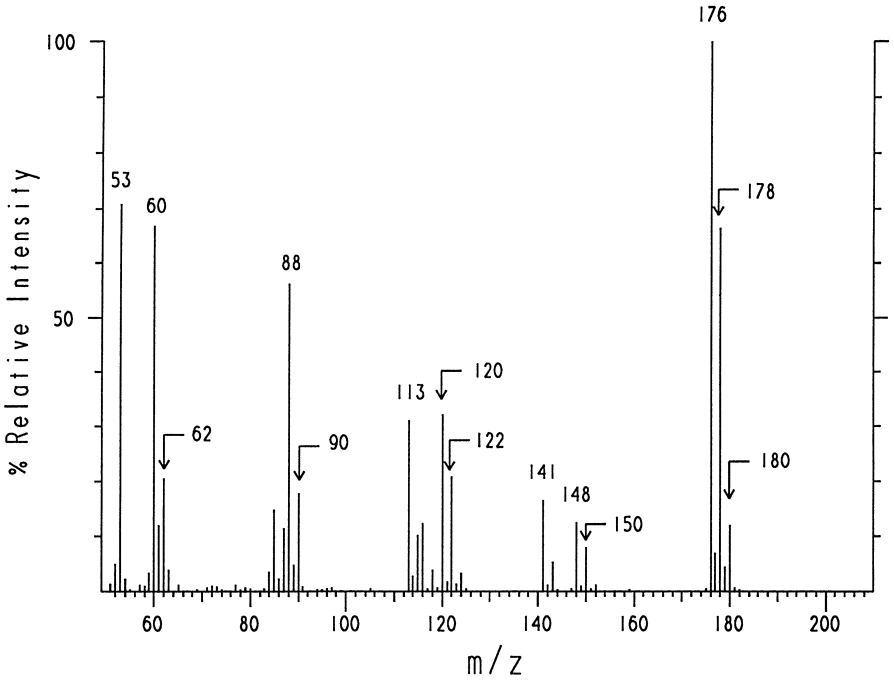

Fig. 5. Electron ionization mass spectrum of the major TCP oxidation product

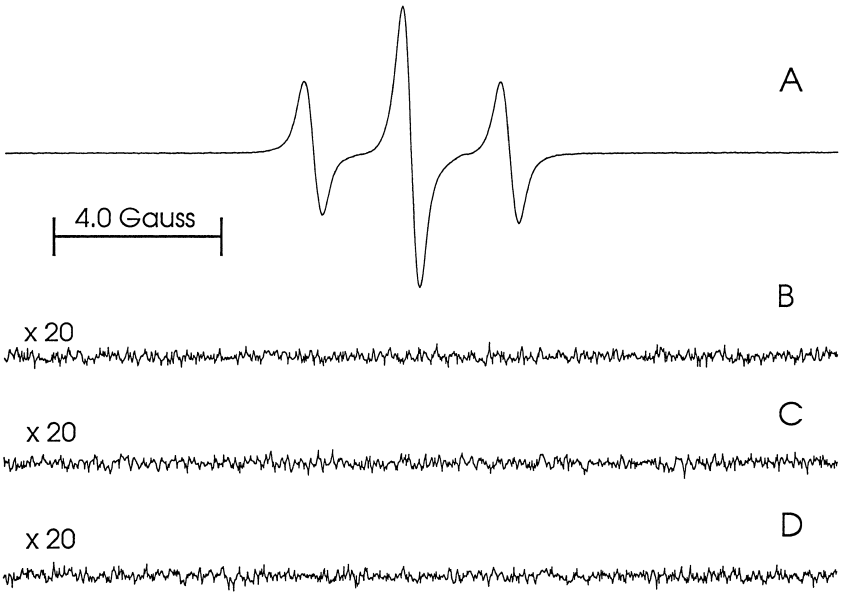

Fig. 6. EPR spectrum of 2,4,6-trichlorophenoxyl radicals generated during hydroperoxide-dependent, HRP-catalyzed oxidation of TCP. TCP (1.0×10^{-3}M) was incubated at ambient temperature in 0.1 M phosphate (pH 7.0) with HRP (1 mg/ml) and hydrogen peroxide (5.0×10^{-4}M). Spectra were obtained using a modulation amplitude of 0.5 G, time constant of 0.25 s, receiver gain of 4×10^3, microwave power of 20 mW, and a scan time of 4 min over 50 G

represents the base peak, and corresponds to the formula weight of the benzoquinone. Furthermore, the spectral fragmentation pattern matched the INCOS library spectrum for 2,6-dichloro-2,5-cyclohexadiene-1,4-dione with a 98.7% fit.

EPR Studies

We have also carried out ESR spectroscopic investigations. Incubations of TCP, HRP, and hydrogen peroxide yielded ESR-detectable formation of TCP-derived phenoxyl radicals (Figure 6A), which were not observed in reaction mixtures devoid of TCP (Figure 6B), hydroperoxide (Figure 6C), or HRP (Figure 6D). The ESR spectrum was characterized by a 1:2:1 triplet arising by coupling of the unpaired electron density with the *meta* protons ($a^{H3} = a^{H5} = 2.35$ G). These results indicate that HRP catalyzes the hydroperoxide-dependent oxidation of TCP to the corresponding trichlorophenoxyl radical (Figure 7).

Discussion

Our results demonstrate that TCP functions as a reducing cosubstrate for HRP as evidenced by its ability to stimulate HRP-catalyzed hydroperoxide reduction using PPHP as a molecular probe (Figure 1). We have also demonstrated that TCP undergoes hydroperoxide-dependent, HRP-catalyzed oxidation involving a dechlorination mechanism, yielding 2,6-dichloro-1,4-benzoquinone (2,6-dichloro-2,5-cyclohexadiene-1,4-dione) as a major oxidation product (Figures 3–5). HRP-

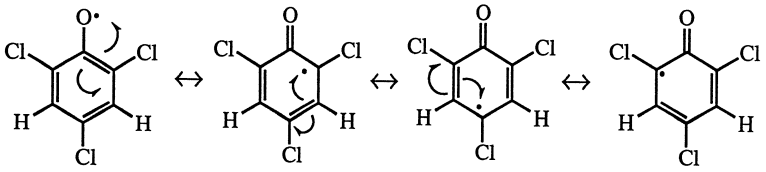

Fig. 7. Scheme 1

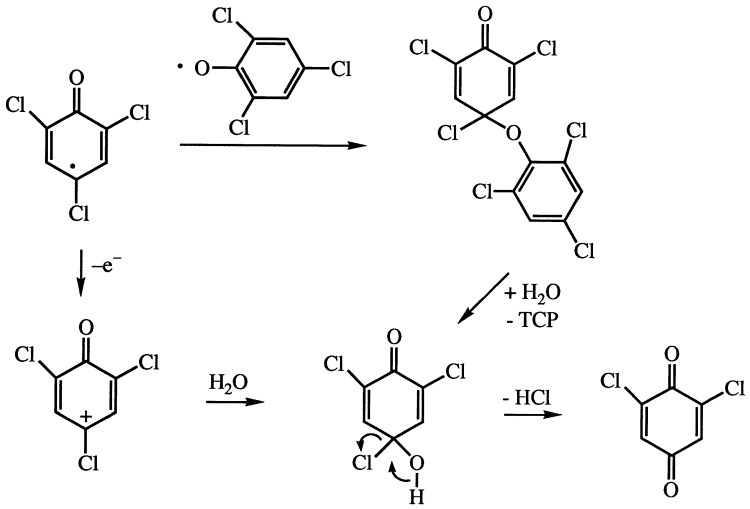

Fig. 8. Scheme 2

catalyzed oxidation of TCP was shown to proceed by one-electron oxidations, characteristic of peroxidase-catalyzed oxidation reactions including oxidation of phenolic compounds (Dunford 1991), as evidenced by ESR spectroscopy demonstrating the intermediacy of TCP-derived phenoxyl radicals (Figures 6, 7). Interestingly, TCP was shown to function as a more effective peroxidase substrate compared with pentachlorophenol (PCP) (Samokyszyn *et al.* 1995). This is evidenced by comparing the ratios of PPHP reduction (*i.e.* ([PPA]/([PPA] + [PPHP])) for TCP or PCP versus ascorbate. The chlorophenol/ascorbate PPHP reduction values for PCP and TCP are 0.88 ± 0.05 and 3.41 ± 0.30, respectively, under identical conditions. This difference may reflect differences of pKas which are 6.0 and 4.5 for TCP and PCP, respectively (Li *et al.* 1991). This is significant because our reactions with HRP were carried out at neutral pH and the reaction of phenolic compounds with the HRP higher oxidation states occurs with the intact phenolic function rather than the corresponding phenoxide (Dunford 1991). This is consistent with our observation that greater extents of hydrogen peroxide-dependent, HRP-catalyzed oxidation of PCP oxidation occur at pH 5.5 and 4.5 compared with pH 7.0 (data not shown), which are far below the pH optimum for HRP (Weller *et al.* 1985). However, at present we cannot rule out the involvement of other descriptors (electronic, hydrophobic, steric) in conferring the differences in chlorophenol substrate efficiencies.

Collectively, our results are consistent with a mechanism involving sequential one-electron oxidations of TCP, by compounds I and/or II, yielding TCP-derived phenoxyl radicals (Figure 7); these were observed and characterized by ESR spectroscopy (Figure 6). These radicals may undergo a second one-electron oxidation yielding carbocations which react with water giving *gem*-chlorohydrins (Figure 8). Alternatively, the TCP-derived phenoxyl radicals may undergo radical–radical coupling to yield an ether intermediate which subsequently hydrolyzes giving an equivalent of TCP and the *gem*-chlorohydrin (Figure 8). The latter mechanism is consistent with the demonstrated bimolecular radical–radical coupling reactions which characterize phenoxyl radicals (McDonald and Hamilton 1973). In addition, the phenoxide is expected to function as a good leaving group because of the relatively acidity of TCP ($pK_a = 5.99$)(Li *et al.* 1991). Furthermore, the latter mechanism, involving intermolecular dichlorophenoxyl radical coupling, is a much more likely mechanism kinetically because the concentration of TCP in the reaction mixtures is magnitudes higher compared with the steady state concentration of phenoxyl radicals. HRP catalysis involves sequential (and ordered) ping pong mechanisms involving reaction of one equivalent of reducing substrate with compound I (with diffusion of the resultant radical from the active site) followed by reaction of a second equivalent of the reducing substrate with compound II yielding a second equivalent of the corresponding free radical (Dunford 1991). Thus, the higher concentration of TCP compared with the concentration of phenoxyl radicals makes it unlikely that HRP catalyzes the second one-electron oxidation of the phenoxyl radical intermediates.

Intramolecular elimination of HCl from the resultant *gem*-chlorohydrin intermediate would give 2,6-dichloro-1,4-benzoquinone. This mechanism predicts that water would necessarily be the source of oxygen in the C4 carbonyl function. In fact, this has been demonstrated by Hammel and Tardone (1988), using ^{18}O-labeling/mass spectrometric analysis, for the TCP-derived benzoquinone generated during lignin peroxidase–catalyzed oxidation of TCP. Furthermore, this is consistent with our observation that hydroperoxide-dependent, HRP-catalyzed oxidation of TCP is not associated with any uptake of dioxygen (not shown). Thus, the phenoxyl radical does not appear to autoxidize. In addition, Ortiz de Montellano and colleagues have demonstrated that HRP and lignin peroxidase cannot catalyze oxidations via oxene transfer mechanisms because the

tertiary structures of these proteins do not allow substrate access to the ferryl intermediate of compound I (Ator and Ortiz de Montellano 1987; DePillis *et al.* 1990). If the latter occurred, this would result in the incorporation of hydroperoxide-derived oxygen. In fact the latter investigators have reported evidence suggesting that the peroxidase-catalyzed oxidations by both HRP and ligninase involve sequential one-electron transfer mechanisms through the methine carbon at the δ-edge of the heme prosthetic group.

Other than 2,6-dichloro-1,4-benzoquinone, our analysis of peroxidase-dependent, TCP-derived oxidation products failed to demonstrate any significant generation of other stable oxidation products including carbon–carbon or carbon–oxygen radical coupling products (*e.g.*, dimers). These represent major phenoxyl radical oxidation products involving radical–radical coupling reactions at *unsubstituted* carbon centers (McDonald and Hamilton 1973). Thus, chlorine substitution at the ortho and para positions in TCP, the carbons characterized by unpaired electron density in the phenoxyl radical intermediate (Figure 7), imposes steric restrictions that hinder intermolecular coupling reactions. This has previously been observed during peroxidase-catalyzed oxidation of PCP (Samokyszyn *et al.* 1995). Mechanistically, this also accounts for the absence of detectable formation of TCP-derived chlorinated dibenzodioxins and chlorinated dibenzofurans, which have been detected as major HRP- and lactoperoxidase-catalyzed oxidation products of 3,4,5- and 2,4,5-trichlorophenol (Oberg *et al.* 1990). An additional consequence of steric effects imposed by chlorine substitution is the relative stability of the TCP-derived phenoxyl radical intermediates, as evidenced by direct detection by EPR spectroscopy in reaction mixtures (Figure 6). This is consistent with our previous report demonstrating the generation of relatively stable phenoxyl radicals during peroxidase-catalyzed oxidation of PCP (Samokyszyn *et al.* 1995).

We predict that 2,6-dichloro-1,4-benzoquinone, the major peroxidase oxidation product of TCP, may function as an effective electrophile that reacts with nucleophiles by Michael addition reactions resulting in the formation of protein and DNA adducts as well as adducts derived from other cellular nucleophiles. This is consistent with the general electrophilic nature of quinones (Monks *et al.* 1992) as well as the demonstration by Rappaport and colleagues that the analogous PCP-derived tetrachlorobenzoquinones react with proteins *in vitro* and *in vivo* by reaction with cysteine sulfhydryls (Waidyanatha *et al.* 1994, 1996; Lin *et al.* 1996, 1997). In addition, we have recently demonstrated that synthetic 2,6-dichloro-1,4-benzoquinone reacts rapidly with low molecular weight primary amine- and thiol-containing molecular probes (data not shown) and we are in the process of elucidating the structures of these adducts. Thus, peroxidases may play a relevant role in the bioactivation of TCP (and perhaps other chlorophenols) *in vivo*, which may contribute to the observed toxicity and carcinogenicity of TCP.

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