

# Revisiting the Peroxidase Oxidation of 2,4,6-Trihalophenols: ESR Detection of Radical Intermediates

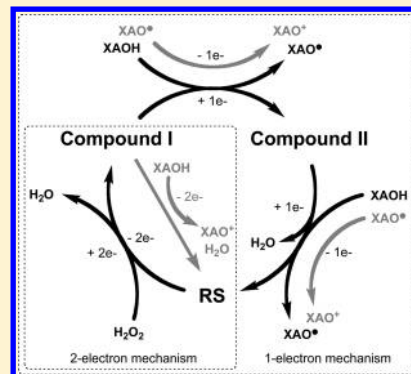
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 Supporting Information

**ABSTRACT:** The peroxidase oxidation of 2,4,6-trichlorophenol (TCP) has been clearly shown to result in 2,6-dichloro-1,4-benzoquinone (DCQ). DCQ is a 2-electron oxidation product of TCP that has undergone para dechlorination. Many peroxidases show similar oxidation of the substrate, TCP, to yield the quinone, DCQ. Depending on the substrate, peroxidases are thought to carry out both 1- and 2-electron oxidations; the mechanism can be confirmed by the detection of both enzyme and substrate intermediates. This article presents ESR evidence for the transient 2,4,6-trichlorophenoxy radical intermediate (TCP•), which exists free in solution, i.e., is not enzyme associated. These data are best explained as a 1-electron peroxidase oxidation of TCP to form TCP•, followed by enzyme-independent radical reactions leading to the 2-electron oxidized product. Also presented are data for the peroxidase oxidation of 2,4,6-trifluorophenol and 2,6-dichloro-4-fluorophenol.



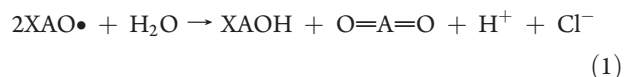
## INTRODUCTION

A peroxidase in the presence of  $\text{H}_2\text{O}_2$  will carry out the oxidation of phenolic compounds such as 2,4,6-trichlorophenol (TCP). In the case of ligin peroxidase,<sup>1</sup> horseradish peroxidase (HRP),<sup>2,3</sup> chloroperoxidase,<sup>4,5</sup> dehaloperoxidase-hemoglobin,<sup>6</sup> and myoglobin,<sup>7</sup> the main product is reported to be 2,6-dichloro-1,4-benzoquinone (DCQ). DCQ is the 2-electron oxidized form of TCP that has undergone para dechlorination (Scheme 1).

The typical peroxidase mechanism is outlined in Figure 1 and is initiated by the 2-electron oxidation of the enzyme resting state (RS) by an oxidizing substrate (eg.,  $\text{H}_2\text{O}_2$ ) to form compound I. Depending on the origin of the peroxidase, the structural form of compound I consists of  $\text{Fe(IV)=O}$  and either a heme radical cation or an amino acid radical.<sup>8</sup> Compound I then undergoes a 1-electron reduction by a reducing substrate (eg., a halophenol, XAOH) to form compound II and the corresponding radical, XAO•. Compound II then undergoes a second 1-electron reduction by a second molecule of the reducing substrate to regenerate RS and an additional radical.

This peroxidase mechanism is commonly referred to as an irreversible ping-pong mechanism.<sup>8</sup> The name makes reference to a very short-lived enzyme–substrate complex; the product departs as fast as the substrate contacts the enzyme, like a ping-pong ball when it strikes a paddle. To account for the formation of the 2-electron oxidized product, DCQ, three different pathways/reactions are considered. First, the radical, XAO•, can act as a reducing substrate for compound I and/or compound II forming a transient cyclohexadienone cation ( $\text{XAO}^+$ ). XAO• would rapidly react with water (or  $\text{OH}^-$ ), dehalogenate, and result in the quinone product ( $\text{O=A=O}$ )<sup>1–4,7</sup> (Figure 1, 1-electron mechanism). Second, the reducing substrate (eg., XAOH)

can act as a 2-electron reducing substrate for compound I, hence forgoing the formation of compound II, and resulting in  $\text{XAO}^+$ , then the quinone (Figure 1, 2-electron mechanism). Third, the radical generated via 1-electron oxidation of TCP can undergo an apparent radical–radical dismutation reaction resulting in the original reducing substrate and the quinone (reaction 1).



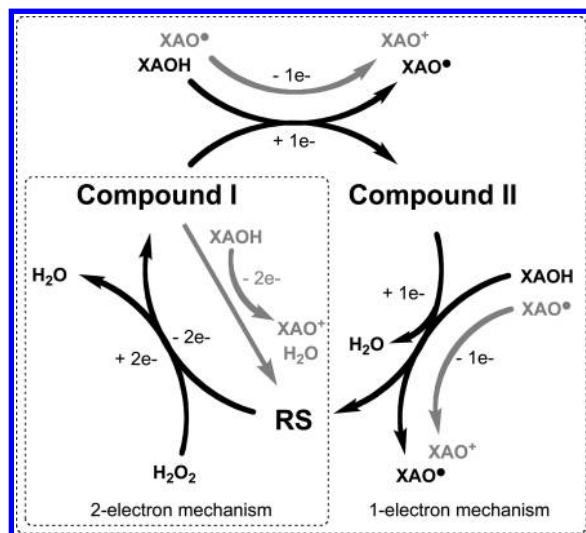
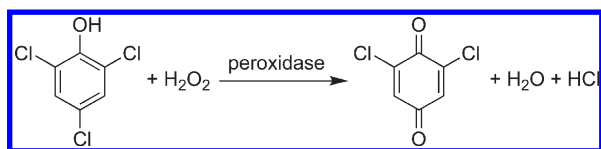
For a given enzyme/substrate system, it is important to know which mechanism is in operation. The difference between the 1-electron and 2-electron mechanism involves two key points: (1) compound II is formed during the 1-electron mechanism and not during the 2-electron mechanism, and (2) a radical (eg., XAO•) is formed during the 1-electron mechanism and not during the 2-electron mechanism. The significance of which mechanism is in operation can be briefly summarized by the following comments: (1) the formation of radical intermediates provides opportunities for other reactions/products to occur, and (2) the more involved enzyme kinetics of the 1-electron mechanism, the more intermediates that can be targeted to modulate enzyme function.

The 1-electron mechanism has been most frequently used to explain the formation of DCQ.<sup>1–4,7</sup> Compound II oxidation of the reducing substrate is usually the rate limiting step in the enzymatic cycle and hence can be detected under steady-state conditions. In cases where the form of compound II involves the

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**Scheme 1.** Peroxidase oxidation of 2,4,6-trichlorophenol to form the quinone product



**Figure 1.** Outline of peroxidase mechanism with  $\text{H}_2\text{O}_2$  as the oxidizing substrate and a halophenol (XAOH) as the reducing substrate. Enzyme intermediates are labeled as resting state (RS), compound I, and compound II. The dotted borders indicate the reactions specifically involved in the 1-electron and 2-electron mechanisms. Reactions presented in gray lead to the formation of a quinone (a two-electron oxidized product).

heme radical cation, UV-vis detection is fairly straightforward, although when the form of compound II involves a protein radical, UV-vis detection is more challenging.<sup>9</sup> This 1-electron mechanism is also supported by the observation of compound II under single-turnover conditions; if the 2-electron mechanism were involved, compound II would not be detected as is the case for the oxidation of thioanisole by CCPO,<sup>5</sup> iodide by HRP,<sup>10</sup> and sulfite by HRP.<sup>11</sup>

In this article, we present ESR data related to the reaction of HRP/ $\text{H}_2\text{O}_2$  with TCP and other TCP-analogues and present evidence to support the 1-electron oxidation mechanism mediated by both enzyme and radical chemistry.

## EXPERIMENTAL PROCEDURES

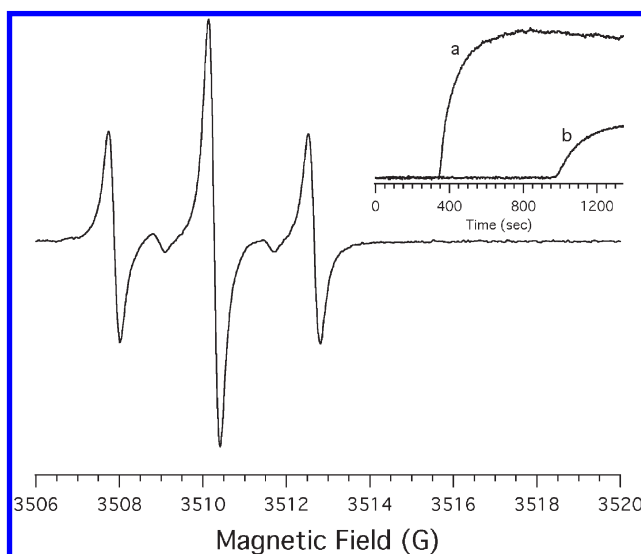
**Materials.** HRP (Type-VIa), TCP, TFP, DCFP, and horse heart myoglobin were obtained from Sigma Chemical Company (St. Louis, MO). Dehaloperoxidase was purified as described previously.<sup>12</sup> All other chemicals were of the highest purity available.

**UV-Visible Spectroscopy.** The oxidation of TCP by HRP/ $\text{H}_2\text{O}_2$  was followed using an HP8453 photodiode array UV-visible spectrometer. A cuvette containing TCP and  $\text{H}_2\text{O}_2$  was scanned (230–400 nm) prior to the addition of enzyme ( $t = 0$  s). The reaction was initiated by the addition of a negligible volume of HRP, mixed, and scanned at 30 s; additional scans were taken every 30 s. Since the absorption of TCP was well separated from DCQ, data were analyzed using the method presented by Harris<sup>13</sup> using the data in Table 1.

**Table 1.** Molar Absorptivity Values Used to Generate Concentration Data in Figure 3

pH	TCP		DCQ	
	$\epsilon_{274}^a$	$\epsilon_{312}^a$	$\epsilon_{274}^a$	$\epsilon_{312}^a$
6.2	1.18	3.39	12.64	0.43
7.4	0.96	4.17	12.17	0.41
9.0	1.03	4.58	8.37	1.89

<sup>a</sup> In units of  $\text{mM}^{-1} \text{cm}^{-1}$ .

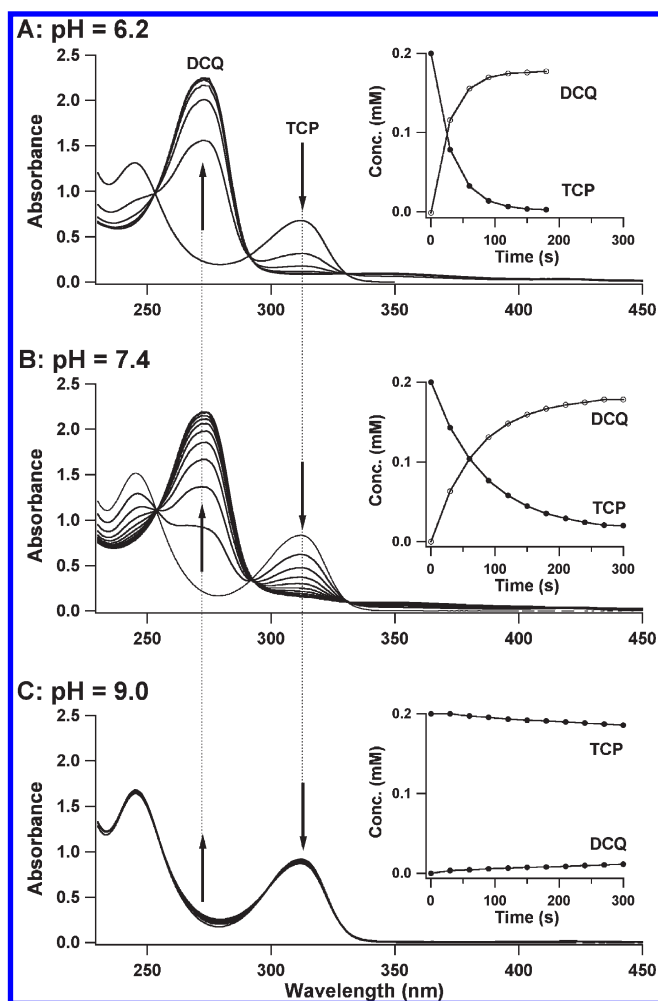


**Figure 2.** ESR spectrum observed 13 min after a solution of HRP ( $4.47 \mu\text{M}$ ),  $\text{H}_2\text{O}_2$  ( $0.5 \text{ mM}$ ), and TCP ( $0.5 \text{ mM}$ ) at pH 7.4 was mixed and transferred to the mounted Aqua-X ESR sample holder. The inset monitors the ESR intensity at 3510 G vs time for (a)  $4.47 \mu\text{M}$  HRP and (b)  $0.447 \mu\text{M}$  HRP. ESR parameters: 9.85 GHz microwave frequency, 1 mW microwave power, 100 kHz modulation frequency,  $0.2 G_{pp}$  modulation amplitude, and 82 s scan time with a 164 ms time constant.

**ESR Spectroscopy.** Detection of all radicals was done using a Bruker EMX spectrometer (Billerica, MA) equipped with a High Sensitivity cavity. X-band ( $\sim 9.8 \text{ GHz}$ ) ESR conditions are listed in the figure captions. When detecting radicals from a standard mixing experiment (Figure 2), the sample was introduced into the cavity using the Bruker Aqua-X sample holder. When conducting IE-ESR experiments, enzymes were loaded into a WG-812-Q flatcell from Wilmad Glass (Vineland, NJ) as described.<sup>14</sup> ESR simulations were performed using the WINSIM program;<sup>15</sup> all presented simulations use best-fit parameters as determined by the WINSIM program.

## RESULTS

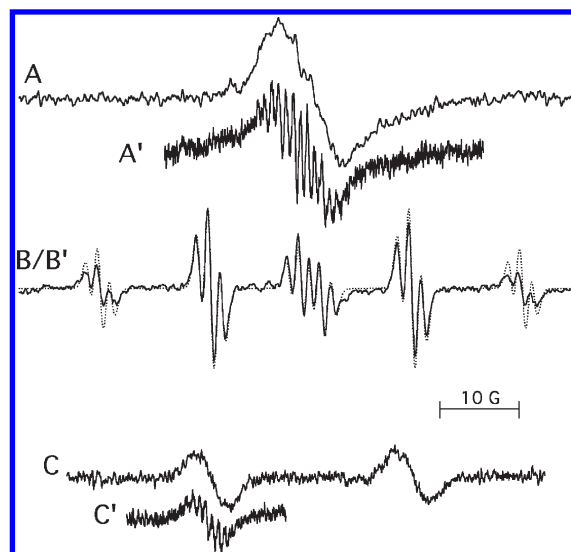
Shown in Figure 2 is the ESR spectrum detected when a solution of TCP/ $\text{H}_2\text{O}_2$  (pH 7.4) was mixed with HRP and quickly ( $\sim 20$  s) transferred into the ESR spectrometer. The resulting spectrum ( $a^H = 2.4 \text{ G}$ ) is equivalent to the ESR spectrum previously detected by Wiese et al. and is unlikely to be  $\text{TCP}\cdot$ .<sup>2</sup> The time course for the observed radical is shown in the inset of Figure 2. The radical signal was not detected immediately and was dependent on HRP concentration. At  $4.47 \mu\text{M}$  HRP (Figure 2, inset a), the radical signal appeared at  $\sim 350$  s after mixing, whereas at  $0.447 \mu\text{M}$  HRP (Figure 2, inset b), the radical signal appeared at  $\sim 970$  s after mixing. Previous work



**Figure 3.** UV–visible monitoring of TCP oxidation by HRP and H<sub>2</sub>O<sub>2</sub>. Solutions contained HRP (0.2  $\mu$ M), H<sub>2</sub>O<sub>2</sub> (0.2 mM), and TCP (0.2 mM) at A, pH 6.2, B, pH 7.4, and C, pH 9.0. Inset data are the calculated concentration of TCP and DCQ as described in the Experimental Procedures.

by Wiese et al.<sup>2</sup> used 23  $\mu$ M HRP, which apparently resulted in an almost immediate detection of this radical signal. The effect of TCP or H<sub>2</sub>O<sub>2</sub> concentration on the time course of the radical was not investigated. Similar attempts at ESR data collected with HRP (0.447  $\mu$ M), TCP, and H<sub>2</sub>O<sub>2</sub> at pH 6.2 and 9.0 observed no radical within a 40 min period. The time course of this reaction was followed by UV–visible spectroscopy at pH 6.2, 7.4, and 9.0 (Figure 3) indicating that oxidation of TCP was rapid at pH 6.2 and pH 7.4, although quite slow at pH 9.0.

Although the published TCP• ESR hyperfine couplings<sup>2</sup> are consistent with a diortho substituted phenoxyl radical, in the case of TCP• two questions arise: (1) the time course for the radical formation/persistence is unexpected for a phenoxyl radical, and (2) the spectrum is missing chlorine hyperfine couplings. Chlorine hyperfine couplings often go undetected since the gyromagnetic ratios for <sup>35</sup>Cl and <sup>37</sup>Cl are quite small (and different for each isotope), which typically result in inhomogeneous line broadening. As for the time course, a phenoxyl radical like TCP• is expected to form during the early events of the enzymatic cycle and is expected to be considerably less stable (more reactive) than the observed radical signal. The lack of chlorine hyperfine



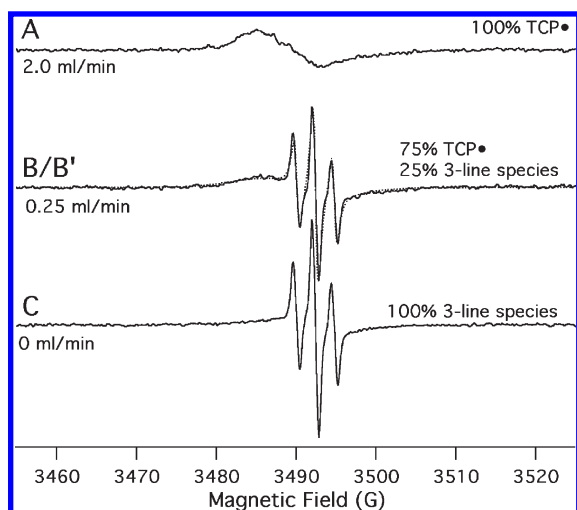
**Figure 4.** IE-ESR spectra collected using HRP<sub>i</sub>/H<sub>2</sub>O<sub>2</sub> and halophenols at pH 7.4. (A) ESR spectrum of TCP• ( $g = 2.008$ ) collected using HRP<sub>i</sub> (3 mg/mL), H<sub>2</sub>O<sub>2</sub> (1 mM), TCP (0.5 mM), and a 2.0 mL/min flow rate. ESR parameters: 9.798 GHz microwave frequency, 20 mW microwave power, 1  $G_{pp}$  modulation amplitude, and an average of 10–42 s scans with a time constant of 82 ms; (A') high-resolution TCP• collected using the same conditions as A but with 0.5  $G_{pp}$  modulation amplitude and an average of 20–167 s scans with a time constant of 167 ms; (B) TFP• ( $g = 2.006$ ) collected using HRP<sub>i</sub> (3.0 mg/mL), H<sub>2</sub>O<sub>2</sub> (1 mM), TFP (1.0 mM), and a 2.0 mL/min flow rate. ESR parameters: 9.802 GHz microwave frequency, 20 mW microwave power, 1  $G_{pp}$  modulation amplitude, and an average of 10–84 s scans with a time constant of 164 ms; (B') overlay of simulation (dotted) ESR spectrum, see text for simulation parameters; (C), DCFP• ( $g = 2.005$ ) collected using HRP<sub>i</sub> (0.7 mg/mL), H<sub>2</sub>O<sub>2</sub> (1 mM), DCFP (0.5 mM), and a 2.0 mL/min flow rate. ESR parameters: 9.775 GHz microwave frequency, 20 mW microwave power, 2  $G_{pp}$  modulation amplitude, and an average of 10–42 s scans with a time constant of 82 ms; and (C') high-resolution DCFP• collected using the same conditions as those in C, except for 0.5  $G_{pp}$  modulation amplitude and an average of 110–42 s scans with a time constant of 82 ms.

combined with the peculiar radical persistence lead us to investigate further the early events in the enzymatic cycle.

In order to observe highly reactive radicals formed during the early events of the enzymatic cycle, the ESR techniques of fast-flow ESR or immobilized enzyme ESR (IE-ESR) are required.<sup>14</sup> Shown in Figure 4A is the IE-ESR spectrum observed when a TCP/H<sub>2</sub>O<sub>2</sub> solution (pH 7.4) was flowed over immobilized HRP (HRP<sub>i</sub>). Under these experimental conditions, radicals with lifetimes of milliseconds have been detected.<sup>14,16,17</sup> The very broad, featureless spectrum (Figure 4A) provides no clear information with respect to the identification of TCP•, although when ESR conditions were set to be sensitive to spectral features and not signal-to-noise (i.e., modulation amplitude changed from 1.0  $G_{pp}$  to 0.5  $G_{pp}$  with signal averaging), the ESR spectrum shown in Figure 4A' was observed. This spectrum showed clear hyperfine interactions that can be simulated (Figure S1, Supporting Information) using two *ortho*-chlorine hyperfine coupling constants (hfcc) ( $a_o^{Cl} = 0.90$  G), a *para*-chlorine hfcc ( $a_p^{Cl} = 1.82$  G), and two *meta*-proton hfcc ( $a_m^H = 1.62$  G); this spectrum and simulation are consistent with the TCP• formed in organic solvents by photolysis.<sup>18</sup>

In order to overcome the complexity associated with chlorine nuclei, the same IE-ESR experiments were performed with the

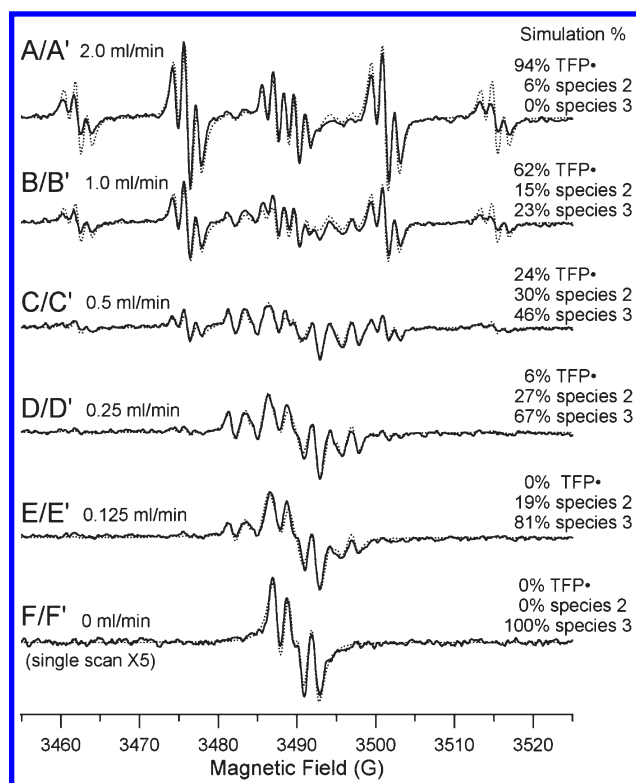




**Figure 5.** IE-ESR spectra collected at varying substrate flow rates for  $\text{HRP}_1/\text{H}_2\text{O}_2/\text{TCP}$  at pH 7.4. (A) ESR spectrum of  $\text{TCP}\bullet$  at 2.0 mL/min, same data as that presented in Figure 4A; (B) same as A, except for 0.25 mL/min; (B') simulation of B with relative contribution of  $\text{TCP}\bullet$  and the three-line spectrum indicated; (C) same as A, except for the flow is stopped, and only 5 scans are averaged.

fluoro-analogue of TCP, 2,4,6-trifluorophenol (TFP). Shown in Figure 4B is the IE-ESR spectrum observed when a  $\text{TFP}/\text{H}_2\text{O}_2$  solution (pH 7.4) was flowed over  $\text{HRP}_1$ . Similar spectra were observed when enzymatic oxidation was carried out by dehaloperoxidase or myoglobin (Figure S2, Supporting Information). Unlike TCP, this ESR spectrum was rich in hyperfine couplings. On the basis of previous ESR data on the tyrosyl radical<sup>19</sup> and the phenoxyl radical,<sup>20</sup> the observed ESR spectra can be simulated (Figure 4B') using two large *ortho*-fluorine hfcc ( $a_o^F = 13.5$  G), an even larger *para*-fluorine hfcc ( $a_p^F = 25.0$  G), and two *meta*-proton hfcc ( $a_m^H = 1.5$  G). Additionally, IE-ESR data were collected for the peroxidase oxidation of 2,6-dichloro-4-fluorophenol (DCFP). When a  $\text{DCFP}/\text{H}_2\text{O}_2$  solution (pH 7.4) was flowed over  $\text{HRP}_1$ , a doublet ESR spectrum was observed with a single hfcc ( $a_p^F = 25.2$  G) assigned to the *para*-fluorine nuclei (Figure 4C). When ESR conditions were set to be sensitive to spectral features and not signal-to-noise (ie., modulation amplitude changed from 2  $G_{pp}$  to 0.5  $G_{pp}$  with signal averaging), as was done with  $\text{TCP}\bullet$ , the ESR spectrum revealed clear hyperfine interactions ( $a_o^{\text{Cl}} = 0.84$  G and  $a_m^H = 1.44$  G) consistent with the values observed in  $\text{TCP}\bullet$  (Figure 4A'). The simulation (low field only) of the  $\text{DCFP}\bullet$  radical spectrum is presented in Figure S1 of the Supporting Information. IE-ESR experiments were performed under acidic conditions (pH 5) for TCP, TFP, and DCFP; the ESR spectra were nearly identical to the pH 7.4 data. No high resolution ESR data were collected at pH 5. No IE-ESR data were collected under basic (pH~9) conditions since UV-vis data (Figure 3) showed that the enzymatic activity was low.

A unique aspect of the IE-ESR experiment is that when the substrate ( $\text{XAOH}/\text{H}_2\text{O}_2$ ) flow rate is high (2 mL/min), early time (msec) events are observed. When flow rates are decreased, additional chemistry is allowed to occur in the active region of the ESR detection system. Shown in Figure 5 are the ESR spectra collected under a set of different flow conditions for  $\text{HRP}_1/\text{H}_2\text{O}_2/\text{TCP}$ . The most obvious result was that the ESR signal ( $a^H = 2.4$  G) seen under slow-flow (0.25 mL/min) (Figure 5B) and stopped-flow conditions (Figure 5C) is nearly identical to



**Figure 6.** IE-ESR data collected for  $\text{HRP}_1$  (8 mg/mL)/ $\text{H}_2\text{O}_2$  (1.0 mM)/TFP (1.0 mM) at varying substrate flow rates and the simulated ESR data. All data were collected using the same ESR data acquisition parameters. The hyperfine coupling constants used in the simulations are presented in the text; the % contribution to the overall spectrum is stated on the Figure. (A) 2.0 mL/min with ESR parameters similar to those in Figure 4B; (A') simulation of A; (B) 1.0 mL/min; (B') simulation of B; (C) 0.50 mL/min; (C') simulation of C; (D) 0.25 mL/min; (D') simulation of D; (E) 0.125 mL/min; (E') simulation of E; (F) 0 mL/min; and (F') simulation of F.

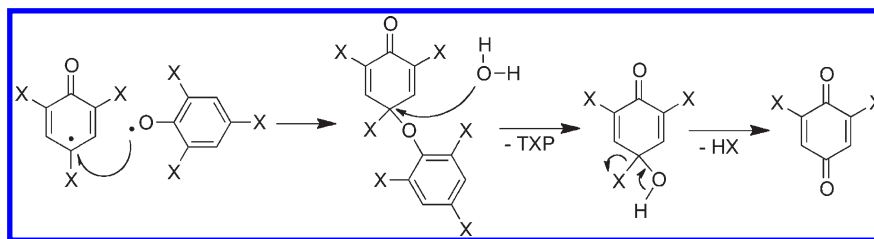
the ESR signal detected under standard mixing conditions (Figure 2). This result suggests that the radical seen under standard mixing conditions is not the primary phenoxyl radical but a secondary radical species. At an intermediate flow rate of 0.25 mL/min (Figure 5B), both the primary phenoxyl radical and the secondary radical are visible. Note the differences in radical  $g$ -values.

When variable flow rate IE-ESR data were collected for the  $\text{HRP}_1/\text{H}_2\text{O}_2/\text{TFP}$  system (Figure 6), the resolved fluorine hyperfine allowed for the observation of additional radical species. All of the data in Figure 6 can be simulated using three different radical species; species 1 ( $\text{TFP}\bullet$ ) [ $a_m^H(2) = 1.42$  G,  $a_o^F(2) = 13.90$  G, and  $a_p^F(1) = 25.08$  G], species 2 [ $a(1) = 1.99$  G,  $a(1) = 2.86$  G, and  $a(1) = 10.7$  G], and species 3 [ $a(1) = 1.86$  G and  $a(1) = 2.92$  G].

## DISCUSSION

The observation of the chlorine and fluorine hyperfine on the primary radicals generated from the  $\text{HRP}_1/\text{H}_2\text{O}_2/\text{TCP}$ ,  $\text{HRP}_1/\text{H}_2\text{O}_2/\text{TFP}$ , and  $\text{HRP}_1/\text{H}_2\text{O}_2/\text{DCFP}$  systems is clear evidence of a 1-electron peroxidase mechanism in  $\text{HRP}$ ; we believe this to be the case with dehaloperoxidase and myoglobin. This conclusion was correctly presented by Wiese et al.<sup>2</sup> but based on incorrectly assigned ESR data. It is important to stress that the detection of

Scheme 2. Enzyme Independent, Radical–Radical Coupling Reaction Leading to the Quinone Product



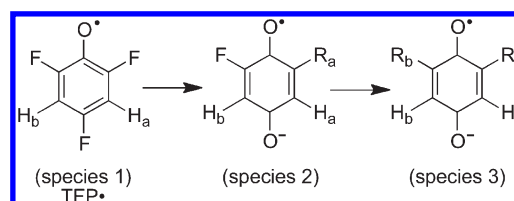
TCP•, TFP•, and DCFP• is support for the 1-electron peroxidase mechanism, although without correlating the radical concentration with  $\text{H}_2\text{O}_2$  consumed, which is difficult due to high radical reactivity, these results can only say that the 1-electron mechanism is clearly in operation; they do not rule out the 2-electron mechanism. In addition, the detection of such an isotropic (symmetric) ESR spectrum is clear evidence that the radical product is not associated with the reaction center, hence in solution. Our ESR data suggest that a most appropriate mechanism for the formation of DCQ is a combination of 1-electron peroxidase oxidation of the halophenol, followed by the radical–radical coupling chemistry (reaction 1) originally put forth, as one option, by Wiese et al.<sup>2</sup>

The presented ESR data show a large hfcc at the para position, indicating large unpaired spin density; hence, the ether intermediate would be a favorable radical–radical coupling product (Scheme 2). This enzyme/radical mechanism is much more likely than the halophenoxyl radical as a peroxidase substrate since the steady-state concentration of the halophenoxyl radical is quite low compared to the halophenol. This enzyme-independent radical chemistry of the peroxidase-generated radicals commonly dictates product formation as with the case of the tyrosyl radical forming dimers and trimers<sup>21</sup> and ascorbate radical dismutation.<sup>22</sup>

So, what is the origin of the radical shown in Figure 2 and presented by Wiese et al.<sup>2</sup> We believe that the radical shown in Figure 2 is 2,6-dichloro-1,4-benzosemiquinone for the following reasons: (1) the radical's persistence is consistent with those observed for chlorinated benzosemiquinones;<sup>23</sup> (2) the observed 1:2:1 structure of the ESR spectrum is consistent with the expected hfcc for the two equivalent ring protons; (3) the absence of the chlorine hyperfine is an indication of small couplings that will contribute to only line width;<sup>23,24</sup> and (4) the radical shown in Figure 2 was not observed when the reaction was carried out at  $\text{pH} < 6.2$ ; benzosemiquinones are known to not persist at or below pH values  $< \text{pK}_a$  of the benzosemiquinone.<sup>25</sup>

As presented in Figures 5 and 6, the IE-ESR method can vary the flow rate to detect secondary radicals. We were hopeful that the detection of secondary radicals for the fluoro-analogue of TCP (TFP•) would assist in the analysis of the TCP• secondary radical, although this was not the case. If the TFP• under went the same radical evolution as TCP•, then the final ESR spectrum seen under 0 mL/min flow conditions (Figure 6F) would have been from the unique 2,6-difluoro-1,4-benzosemiquinone. Apparently, the oxidation of TFP does not only result in para dehalogenation, as is the case with the oxidation of TCP,<sup>1–3</sup> but also in ortho dehalogenation as well. It is important to note that since the 0 mL/min flow ESR spectrum for TCP oxidation does not show any chlorine hyperfine splitting, these data cannot be used to prove that dechlorination does not occur at the ortho

Scheme 3. Proposed Radical Evolution Based on ESR Data in Figure 6



positions. Hammel and Tardone<sup>1</sup> have reported that only one inorganic chloride per TCP is detected during the peroxidase oxidation of TCP; hence, this result alone has led to the belief that there is no ortho dechlorination occurring in TCP. The ortho defluorination of TFP can be seen from the data presented in Figure 6. Under slower flow (1.0 mL/min  $>$  flow  $>$  0.125 mL/min; Figure 6B–E), additional radicals are observed. For the data presented in Figure 6, species 2 can be simulated as a mono-fluorinated radical ( $a_o^F = 10.7$  G) with two nonidentical protons ( $a_1^H = 1.99$  G and  $a_2^H = 2.96$  G), and species 3 can be simulated as an asymmetric radical (a proposed semiquinone) with two nonidentical protons ( $a_1^H = 1.86$ ,  $a_2^H = 2.92$ ) (Scheme 3, where  $R_a$  and  $R_b$  are ESR silent nuclei). Further investigation into the complete oxidation of TFP is required to fully understand the mechanism, although as a preliminary thought, it appears as though the radical–radical coupling reaction with TFP• involves the ether intermediate resulting from coupling at not only the para position but also the ortho position.

The ESR data requires additional discussion. Presented in Figure 4 are the experimental (4B) and simulated (4B') spectra of TFP•. These IE-ESR data are extremely reproducible; IE-ESR data have no scan time-dependent features.<sup>14</sup> The simulation of these data fit very well with the only exception being the intensity of the outer ESR transitions (3460–3470 G and 3515–3525 G); the experimental intensity of the outer transitions is lower in amplitude than that of the simulated. The broadening of the higher field outer transitions ( $>3500$  G) is a result of small hyperfine anisotropy commonly seen in ESR spectra with large hfcc, although the lower than expected intensity of the lowest and highest field lines (3460–3470 G and 3515–3525 G) remains unclear. Similar ESR observations have been seen by others for the fluoranil radical anion<sup>26</sup> and 9,10-perfluoroanthraquinone radical anion.<sup>27</sup>

In regard to the ESR simulation of the TCP•, the simulations did not require the use of both <sup>35</sup>Cl and <sup>37</sup>Cl isotopes. Reported simulations of TCP•<sup>18</sup> did use both isotopes, but this early publication did not have access to sophisticated computer methods. To further interpret the TCP• ESR data, the well-determined fluorine hfcc from TFP• ( $a_o^F = 13.5$  G,  $a_p^F = 25.0$  G)

were scaled using the gyromagnetic ratios to predict chlorine hfcc ( $a_o^{35\text{Cl}} = 1.4$  G and  $a_p^{35\text{Cl}} = 2.6$  G;  $a_o^{37\text{Cl}} = 1.2$  G and  $a_p^{37\text{Cl}} = 2.2$  G). These values, along with the *meta*-protons ( $a_m^{\text{H}} = 1.5$  G) and natural isotopic abundance, were used to generate a predicted TCP• ESR spectrum. Two observations were made: (1) the predicted  $a_o^{\text{Cl}}$  and  $a_p^{\text{Cl}}$  from the scaled TFP• hfcc were larger than the observed, indicating that the spin density on the fluorine nuclei is slightly larger than that for the chlorine nuclei, and (2) an adequate simulation could be achieved using only the  $^{35}\text{Cl}$  hfcc. When isotopic abundance of  $^{35}\text{Cl}$  and  $^{37}\text{Cl}$  was included in the simulation, the outermost lines were only slightly broadened. In our case, the low signal-to-noise in the high resolution ESR spectra (Figure 4A',C'; Figure S1, Supporting Information) does not allow for clear observation of these outermost lines.

## SUMMARY

We have detected the primary 2,4,6-trihalophenoxy radical intermediates resulting from the 1-electron peroxidase oxidation of TCP, TFP, and DCFP. Since the observed IE-ESR spectra are isotropic, we conclude that the primary radicals are not associated with the reaction center and are free to react further in solution. The ESR data provide support for the hypothesis that the formation of DCQ involves a 1-electron peroxidase oxidation of the TCP, followed by radical coupling chemistry resulting in TCP<sup>+</sup>, which is attacked by OH<sup>-</sup> in solution to form DCQ and Cl<sup>-</sup>. Secondary radicals observed under slower-flow IE-ESR experiments in both TCP (Figure 5) and TFP (Figure 6) are tentatively assigned as halogenated/dehalogenated 1,4-semiquinones; the mechanism of semiquinone formation will be presented in a subsequent paper.

## ASSOCIATED CONTENT

**S Supporting Information.** Details of the ESR simulation of TCP•/DCFP•; TFP• spectrum from dehaloperoxidase and myoglobin with kinetics. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ABBREVIATIONS

TCP, 2,4,6-trichlorophenol; TCP•, 2,4,6-trichlorophenoxy radical; TCP<sup>+</sup>, 2,4,6-trichlorocyclohexadienone cation; TFP, 2,4,

6-trifluorophenol; TFP•, 2,4,6-trifluorophenoxy radical; DCFP, 2,6-dichloro-4-fluorophenol; DCFP•, 2,6-dichloro-4-fluorophenoxy radical; XAOH, unspecified halophenol; XAO•, unspecified halophenoxy radical; XAO<sup>+</sup>, unspecified halocyclohexadienone cation; DCQ, 2,6-dichloro-1,4-benzoquinone; HRP, horseradish peroxidase; RS, enzyme resting state; HRP<sub>i</sub>, immobilized HRP; hfcc, hyperfine coupling constants; IE-ESR, immobilized enzyme ESR

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