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# Involvement of free radicals in peroxidatic reactions catalyzed by chloroperoxidase

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# INVOLVEMENT OF FREE RADICALS IN PEROXIDATIC REACTIONS CATALYZED BY CHLOROPEROXIDASE

by

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## Abstract

The mechanism of chloroperoxidase (CPO)-catalyzed peroxidatic reactions of several substituted hydroquinones was studied at various hydrogen peroxide concentrations. The pathway was studied using cytochrome c as the radical trapping agent. As the hydroquinones became more hindered there was a difference in the amount of radicals trapped. For hydroquinone, 59.3% radical pathway, and methylhydroquinone, 81.4% radical, the difference in radicals trapped is due to a difference in pathway. For 2,3-dimethylhydroquinone (75.4%), trimethylhydroquinone (44.5%), and *t*-butylhydroquinone (0%) other non-peroxidatic reactions are noticed. Thus, for the more substituted hydroquinones the difference in radicals trapped can not be assigned to a difference in radical pathway. Also, there were problems drawing conclusions for this system due to the catalatic reaction of hydrogen peroxide.

The radical trapping ability of 2,4,6-trimethylphenol was investigated for various other substrates. TMP reacted with the radicals generated in the enzymatic reactions of phenol, resorcinol, and *m*-methoxyphenol. Thus, this TMP system offers further potential as another radical trapping agent for use in these studies.

## Introduction

Chloroperoxidase (CPO) is a hemeprotein with an unusually broad range of catalytic properties. In addition to catalyzing reactions characteristic of peroxidases, peroxidatic reactions (Equation 2) of a variety of organic substrates, CPO also catalyzes the catalatic reaction (Equation 3) of peroxide. The initial step in the mechanistic sequence of CPO reactions involves the native enzyme and hydrogen peroxide forming an enzymatic intermediate known as compound I (Equation 1,$E_1$).

**Equations**: In these equations, E represents the native enzyme, $E_1$ is compound I, and $AH_2$ is a peroxidatic substrate.

$$E + H_2O_2 \rightleftharpoons E_1 + H_2O \qquad (1)$$

$$E_1 + AH_2 \rightleftharpoons E + A \qquad (2)$$

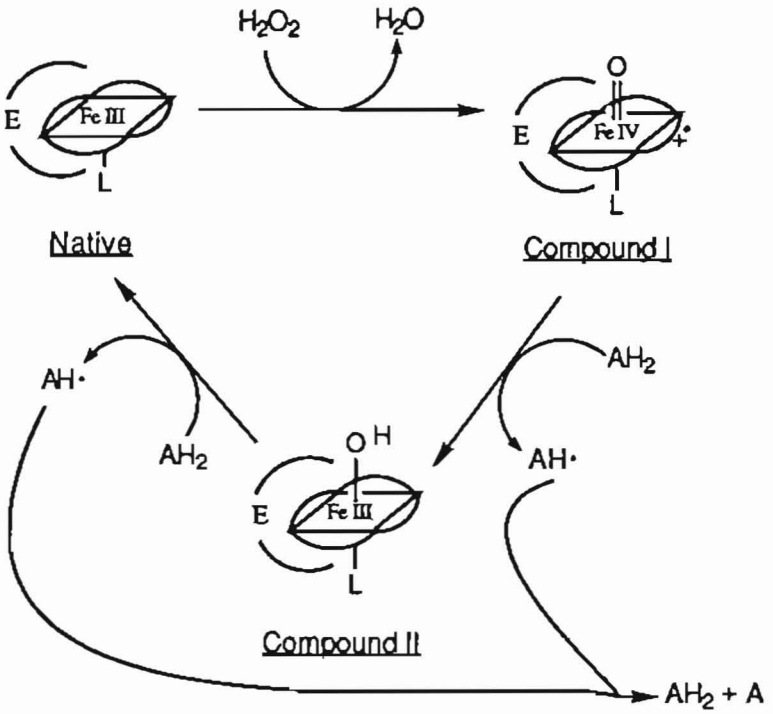
$$E_1 + H_2O_2 \rightleftharpoons E + O_2 \qquad (3)$$

Compound I then reacts with the organic substrate by either a one or two electron transfer mechanism (Schemes 1 and 2). Previous attempts to determine which pathway enzymatic reactions follow have been focused mostly on the enzyme horseradish peroxidase (HRP). Ortiz de Montellano suggested that HRP catalyzed reactions occur only through the one electron transfer mechanism[1-3]. He also proposed that peroxidatic reactions involving one electron transfer mechanisms occur at the heme edge, and reactions involving a two electron transfer mechanism occur at the metal center. This suggests that the protein structure of HRP completely blocks the metal center from organic substrates. If this hypothesis is correct, chloroperoxidase's ability to catalyze many types of reactions would

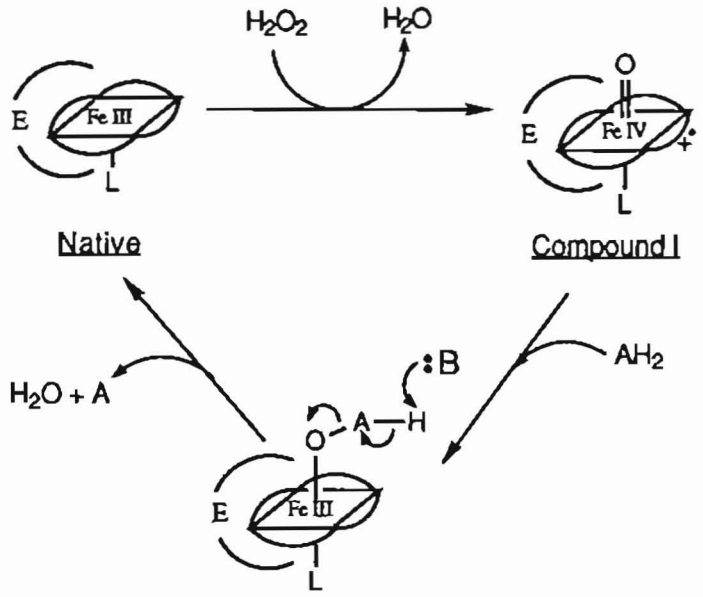
indicate that both the heme edge and the metal center are accessible to substrates, allowing for either the one or two electron mechanism of peroxidatic reactions.

Determining the factors that favor one electron reactions over two electron processes, and vice-versa, should provide information concerning the reaction sites for both types of reactions. The one electron mechanism (Scheme I) involves free radicals as intermediates, while there are no free radicals generated in the two electron mechanism (Scheme 2). Thus, it should be possible to determine which mechanism is operating in each CPO catalyzed reaction by measuring the extent of free radical formation in the reaction.

**SCHEME 1:** The one electron transfer mechanism for peroxidase reactions.



**SCHEME 2:** Possible two electron transfer mechanism for peroxidatic reactions.




In order to determine if radicals are present as intermediates in the reactions we developed an efficient system that traps any radicals produced during the course of the reaction. The percent free radical pathway for reactions catalyzed by chloroperoxidase can be determined by comparing the known 100% free radical HRP system to the data obtained from the CPO system.

## Experimental

*General-* All experiments were carried out in a Shimadzu UV-160 UV-VIS spectrophotometer at 25°C and pH 6.5 using 1-cm path length quartz cuvettes.

*Enzyme Preparations-* Horseradish peroxidase Type II (Lot# 66F-9690) was provided by Sigma Chemical Company and used as supplied. The enzyme had a specific activity of 220 units/mg.

Concentrations of HRP solutions were determined by the weight of the dried solid, assuming a molecular weight of 35,000 g/mol. Chloroperoxidase was produced and purified by Amy Shedd in our laboratories as previously discussed.[6] Concentrations of CPO solutions were determined by their absorbance at 398 nm using a molar absorption coefficient of 85,000 $M^{-1}cm^{-1}$.

*Hydrogen Peroxide Solutions*- All hydrogen peroxide solutions were prepared from reagent hydrogen peroxide (Lot# D17G) supplied by Morton Thiokol Inc. Concentrations of peroxide solutions were determined by the method of Cotton and Dunford.[7]

*Materials*- All other materials were of the highest quality available from commercial sources. Water was purified by reverse osmosis on a Barnstead system and then passed through one activated charcoal column, two deionizer columns, and a 0.2-μ filter.

*Determination of the Molar Absorption Coefficient of Benzoquinone*- Benzoquinone was purified by vacuum sublimation at room temperature. The absorbance at 247 nm was measured for the following concentrations of benzoquinone (μM): 1.3, 2.6, 3.8, 7.6, and 11.2. The molar absorption coefficient was determined from the slope of the plot of absorbance *versus* benzoquinone concentration.

*Determination of the Change in Molar Absorption Coefficient for reduction of Cytochrome C*- Cytochrome C Type III: from Horse Heart (Lot# 40H7260) was supplied by Sigma Chemical Company and

used as supplied. Ascorbic acid was supplied by Aldrich and also used as supplied. The change in absorbance of ferricytochrome C (70μM) at 550 nm was measured for the following concentrations of ascorbic acid (μM): 1.3, 2.6, 3.8, 7.6, and 11.2. The molar absorption coefficient was determined from a plot of absorbance *versus* twice the concentration of ascorbic acid.

*Determination of the Percent Free-Radical Pathway in reactions catalyzed by CPO*- For each substrate (45μM) the absorbance change at 550 nm was measured for the following peroxide concentrations (μM): 1.3, 2.6, 3.8, 7.6, and 11.2. The reactions were carried out in a phosphate buffer containing cytochrome C (70μM) and either HRP (either 25nM or 35nM) or CPO (either 50nM or 80nM). The concentration of radical trapped was determined from the absorbance change divided by our experimentally obtained epsilon value for cytochrome C. The moles of radicals trapped per mole of peroxide were determined from the slope of the plot of concentration of radicals trapped *versus* peroxide concentration. The % free-radical pathway in CPO catalyzed reactions was determined by the ratio of moles radicals trapped per mole of peroxide in CPO reactions to the moles of radical trapped per mole of peroxide in HRP reactions.

*Determination of the Fraction of Non-peroxidatic Reactions for Substituted Hydroquinones*- For each substrate (45μM) the absorbance change was measured at the appropriate wavelengths: 247nm for hydroquinone, 250nm for methylhydroquinone, 252nm for

2,3-dimethylhydroquinone, and 260nm for trimethylhydroquinone and *t*-butylhydroquinone; for the following peroxide concentrations (μM): 1.3, 2.6, 3.8, 7.6, and 11.2. The reactions were carried out in a phosphate buffer with HRP (25nM) and repeated using CPO (50nM). The molar absorption coefficient was determined from a simple Beer's Law plot of absorbance change *versus* peroxide concentration. If the molar absorption coefficients were found to be the same, regardless of the enzyme used, then there was no difference in the fraction of peroxidatic reaction. If a difference in the molar absorption coefficients was seen then the fraction of non-peroxidatic reactions could be determined by comparing the coefficients that were obtained.

*Determination of the Molar Absorption Coefficient for Trimethylhydroquinone*- The change in absorbance was measured at 260nm for the following concentrations of trimethylhydroquinone and ammonium persulfate (μM): 1.3, 2.6, 3.8, 7.6, and 11.2. The reactions were carried out in a pH 6.5 phosphate buffer at 80°C. The molar absorption coefficient was determined from a simple Beer's Law plot of absorbance change *versus* peroxide concentration.

*Investigation of 2,4,6-Trimethylphenol (TMP) as a potential radical trapping agent*- The reaction of phenol (45μM) and peroxide (15μM) with HRP (25nM) was repeatedly scanned as the reaction progressed. Also, the reaction was carried out in the presence of TMP (45μM and 70μM) using several different blank solutions. Among the blanks were phenol (45μM), phenol (45μM) and TMP (45μM

and 70μM, respectively), and a TMP blank (45μM and 70μM, respectively). The spectra of the reactions with the TMP were compared with those of reactions of phenol without TMP present. This was repeated for the following substrates: resorcinol, catechol, *p*-hydroxybenzaldehyde, *m*-methoxyphenol, and *p*-methoxyphenol.

## Results and Discussion

*Significance of catalatic pathway in CPO reactions for our system-* It is fairly well known that reactions catalyzed by horseradish peroxidase do not follow the catalatic pathway of peroxide.[1-3] However, reactions catalyzed by chloroperoxidase do have this pathway available. Therefore, it is necessary to determine, for each substrate, if a significant amount of peroxide is being consumed by this pathway. If peroxide reacts in this manner then the experimental molar absorption coefficient of CPO catalyzed reactions would appear to be smaller than the actual value and the percent radical pathway would also appear lower.

For hydroquinone the same amount of substrate reacted regardless of which enzyme catalyzed the reaction. We determined the molar absorption coefficient for the reaction of hydroquinone with HRP as the catalyst to be 22,200 $M^{-1}cm^{-1}$ and with CPO to be 20,500 $M^{-1}cm^{-1}$ (Figure 1). A result similar to that of hydroquinone was also found for methylhydroquinone. The molar absorption coefficient was found to be 23,700 $M^{-1}cm^{-1}$ for the HRP catalyzed reaction and 21,400 $M^{-1}cm^{-1}$ for the CPO reaction.

For both substrates, as the concentration of the peroxide increases the data points begin to curve downward slightly. This downward curve indicates that as the concentration of the peroxide is increased more peroxide goes through the catalatic pathway, as would be expected. However, over our range of concentrations the deviation is assumed to be insignificant and the difference between the two molar absorption coefficients is discounted as experimental error.

However, it is possible that the amount of peroxide that follows the catalatic pathway is significant and needs to be taken into account when calculating the percent radical pathway. When hydrogen peroxide reacts with chloroperoxidase compound I oxygen is produced, thus one way to more accurately determine the molar absorption coefficient for CPO reaction would be to use an oxygen electrode to measure the amount of oxygen produced from this pathway. The amount of peroxide that is consumed in the catalatic pathway could then be calculated into the molar absorption coefficient.

The molar absorption coefficient obtained for the hydroquinone reaction was verified by determining the molar absorption coefficient for benzoquinone, which is the product generated in the reaction of hydroquinone. The experimental molar absorption coefficient was found to be 21,900 $M^{-1}cm^{-1}$(Figure 2) which is identical to the values obtained in the enzymatic reactions, within experimental error.

**Figure 1.** Molar Absorption Coefficients for enzymatic reactions of Hydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 45μM hydroquinone; either 50nM CPO or 25nM HRP; 1.3, 2.6, 3.8, 7.6, and 11.2μM $H_2O_2$.

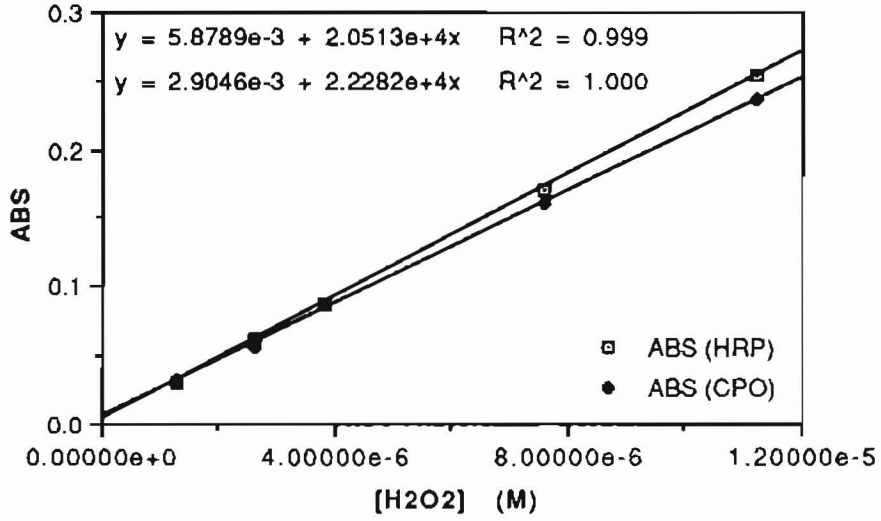
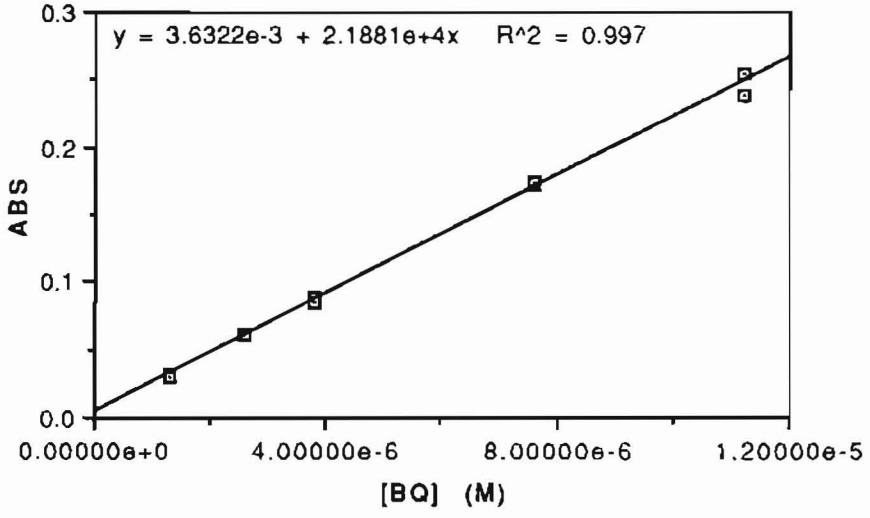



**Figure 2.** Molar Absorption Coefficients for Benzoquinone. Reaction conditions: 100mM phosphate, pH 6.5; 1.3, 2.6, 3.8, 7.6, and 11.2μM benzoquinone.




The results of these studies indicate that no significant amount of peroxide is consumed by the catalatic pathway for the chloroperoxidase catalyzed reaction of both hydroquinone and methylhydroquinone. This means that for these substrates the same

amount of substrate reacts with compound I, regardless of the enzyme used, and the differences in the amount of radical trapped can be credited to a difference in the pathway.

However, as more substituents were added to the hydroquinones larger molar absorptivities were seen with greater differences in the values for the HRP and CPO catalyzed reactions. The following table summarizes the values obtained (see also Figures 3-5):

**Table 1**: Summary of molar absorption coefficients for some substituted hydroquinones in ($M^{-1}cm^{-1}$).

| Substrate | HRP catalyzed | CPO catalyzed |
|---|---|---|
| 2,3-Dimethylhydroquinone | 73,200 | 61,700 |
| Trimethylhydroquinone | 149,000 | 56,700 |
| *t*-Butylhydroquinone | 54,100 | 15,600 |

**Figure 3.** Molar Absorption Coefficients for enzymatic reactions of 2,3-Dimethylydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 45μM hydroquinone; either 50nM CPO or 25nM HRP; 1.3, 2.6, 3.8, 7.6, and 11.2μM $H_2O_2$. The first four data points were fit with a linear curve fit to give the molar absorption coefficients.

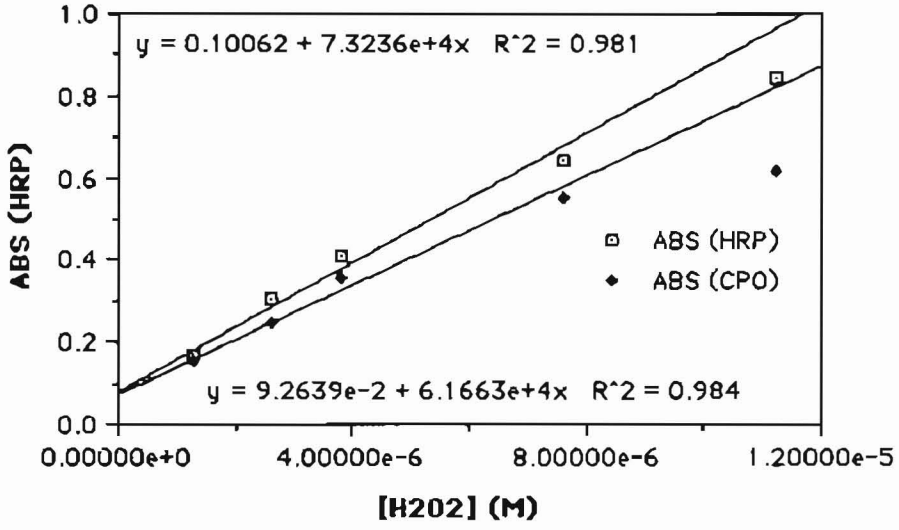


**Figure 4.** Molar Absorption Coefficients for enzymatic reactions of Trimethylydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 45μM hydroquinone; either 50nM CPO or 25nM HRP; 1.3, 2.6, 3.8, 7.6, and 11.2μM $H_2O_2$. The first three data points were fit with a linear curve fit to give the molar absorption coefficients.

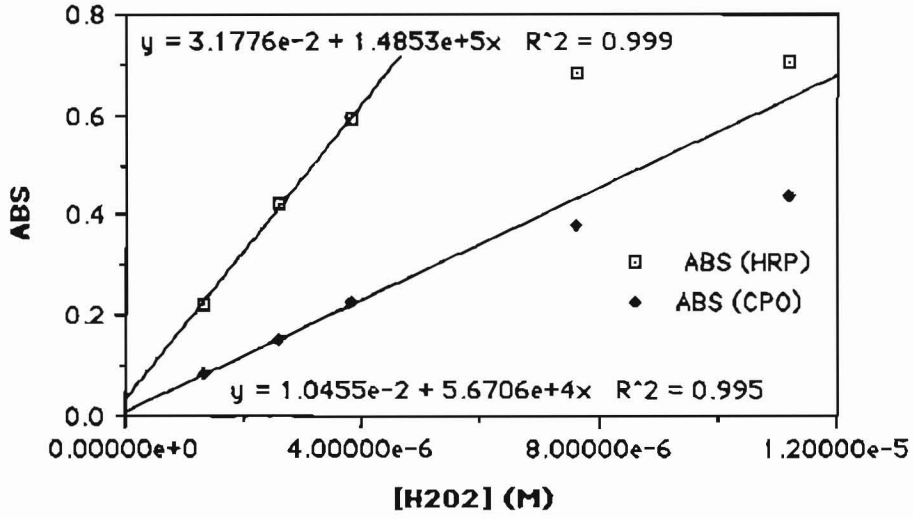
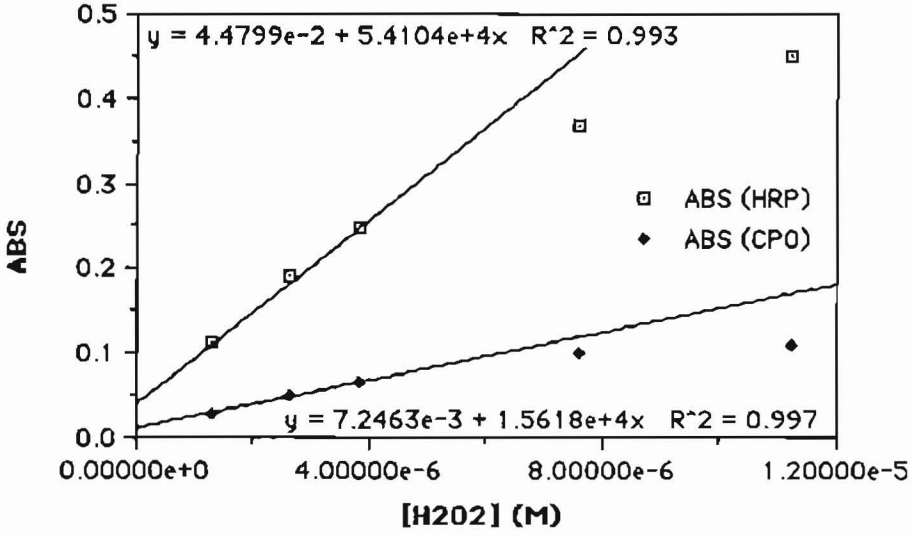



**Figure 5.** Molar Absorption Coefficients for enzymatic reactions of *t*-Butylhydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 45μM hydroquinone; either 50nM CPO or 25nM HRP; 1.3, 2.6, 3.8, 7.6, and 11.2μM $H_2O_2$. The first three data points were fit with a linear curve fit to give the molar absorption coefficients.



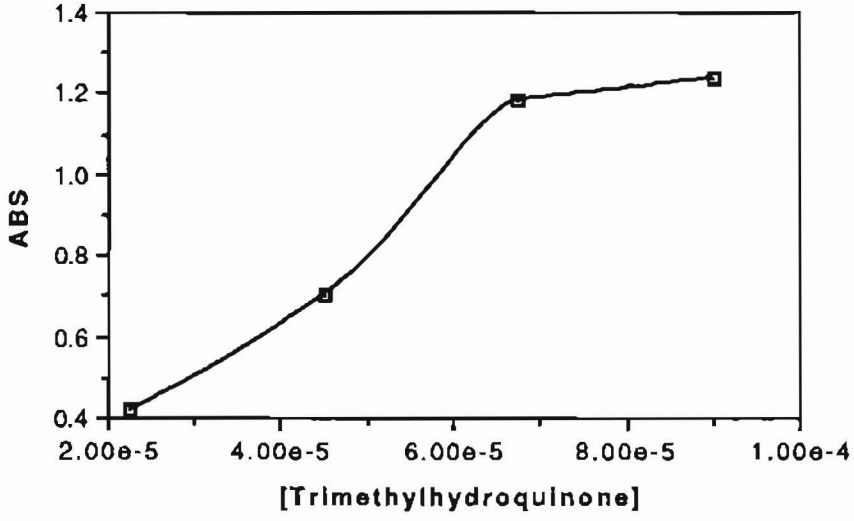

The large difference in the molar absorption coefficients for the two types of catalyzed reactions could be due to the binding of

the substrate to the enzyme. As the substrates become more substituted they should be less effective at binding to the active site of the enzyme, and for the CPO reactions as the substrate becomes less effective at binding to compound I, the binding of hydrogen peroxide to CPO compound I is likely to become more competitive. Less effective binding would not create as much of a change for the HRP reactions because hydrogen peroxide does not react with compound I so the organic substrate would have no competition. If the catalatic reaction is the only cause of the difference in consumption of the hydroquinones between HRP and CPO catalyzed reactions then it would again be possible to correct for the amount of catalatic reaction by using the oxygen electrode system as described above. Such a correction would increase in the observed molar absorption coefficient for the hydroquinone in the chloroperoxidase catalyzed reactions.

The Beer's Law plots for the more substituted hydroquinones (Figures 3-5) show a significant deviation from linearity at the higher peroxide concentrations for both the CPO and HRP reactions. The deviations in the CPO reaction could be due to hydrogen peroxide following the catalatic reaction. However, HRP does not catalyze the catalatic reaction so there must be some other effect. Possibly all of the substrate was being consumed at the higher peroxide concentrations. If the only reaction occurring is the peroxidatic reaction as there is an ample excess of hydroquinone over hydrogen peroxide so the peroxide should be the limiting reagent. A preliminary experiment was conducted with trimethylhydroquinone to determine if in fact all of the hydroquinone might react by some

paths other than the peroxidatic reaction. In this experiment the concentration of the substrate was increased from 45μM to 90μM, at a constant peroxide concentration of 11.2μM. The effect of this was to increase the overall absorbance change from 0.703 at the 45μM concentration to 1.234 for the 90μM case. Also, the quinone concentration was tested at 22.5μM and 67.5μM yielding absorbance changes of 0.419 and 1.183, respectively. As Figure 6 indicates the overall absorbance change seems to vary with hydroquinone concentration even though the hydrogen peroxide is constant.

**Figure 6**: Reaction conditions: 100mM phosphate, pH 6.5; 11.2μM $H_2O_2$; 25nM HRP; 22.5, 45, 67.5, and 90μM trimethylhydroquinone.




This certainly indicates that overall absorbance change is not dependent on the peroxide concentration, but instead on the concentration of trimethylhydroquinone. There is not enough peroxide present to react with all of the substrate, therefore there must be another reaction occurring in the mixture. Also, it was noted that when only the enzyme and quinone were in the mixture

there was a substantial amount of absorbance change, indicating that trimethylhydroquinone reacts directly with HRP without forming compound I first.

Based on these data we were unable to determine the nature of the reactions causing the observed changes in absorbance. It may involve semiquinone radicals since the problem is not observed when cytochrome C is present. The cytochrome could be reacting with radicals and preventing the undesired reaction from occurring. However, there are not enough data to confirm or refute this theory. To test whether the molecular oxygen might be involved in the non-peroxidatic reaction, reaction mixtures were purged with nitrogen for about 15 minutes. Such anaerobic reactions give no significant difference in absorbance change from those of aerobic reactions.

We were able to determine that for both the CPO and HRP catalyzed reactions the same product was being formed when trimethylhydroquinone was used as the substrate. The spectrum of the reaction shows a very clean reaction. For trimethylhydroquinone, the starting material shows a single peak around 285nm and as the reaction proceeds a single peak forms at around 260nm regardless of which enzyme is used to catalyze the reaction (Figures 7 and 8). The two spectra for the HRP and CPO catalyzed reactions are identical except for the overall absorbance change of the reaction. They both show the same isosbestic points at 230 and 279nm and this would only occur if the same product was being formed. However, the spectra were only taken for trimethylhydroquinone and the spectra of the other substituted

hydroquinones would have to be taken to see if the same products are being formed in those reactions.

**Figure 7.** Spectrum for the HRP catalyzed reaction of trimethylhydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 45μM trimethylhydroquinone; 7.6μM $H_2O_2$; 25nM HRP.

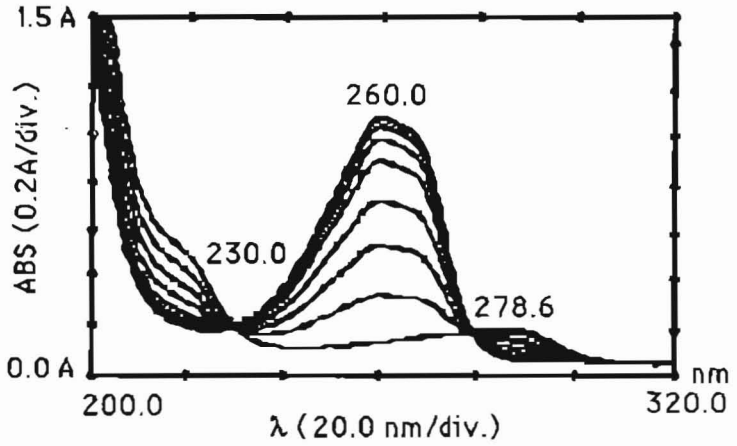
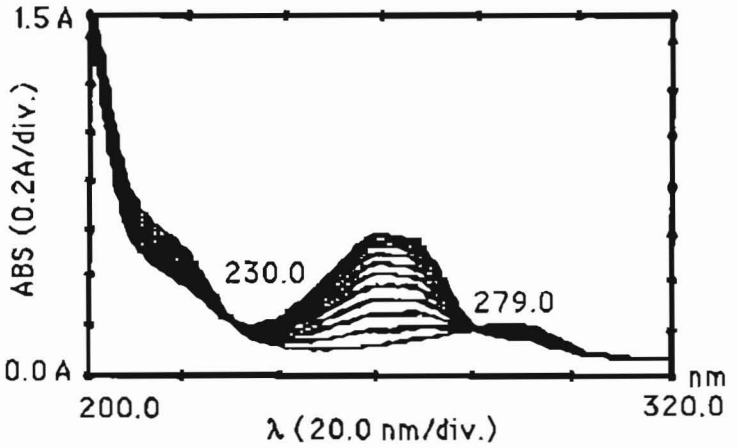



**Figure 8.** Spectrum for the CPO catalyzed reaction of trimethylhydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 45μM trimethylhydroquinone; 7.6μM $H_2O_2$; 50nM CPO.




An experiment was also carried out to determine the molar absorption coefficient of trimethylhydroquinone in a non-enzymatic reaction. Heating the trimethylhydroquinone reaction mixture, in the presence of persulfate, caused

trimethylhydroquinone to decompose into the same product that was formed by the enzymatic reactions (Figure 9). The value of the molar absorption coefficient for trimethylhydroquinone was determined to be 17,500 $M^{-1}cm^{-1}$ from this experiment (Figure 10). This value is considerably lower than that obtained for either enzyme. Thus, there must be a reaction other than the peroxidatic reaction occurring in the enzyme catalyzed reaction mixture.

**Figure 9.** Spectrum of reaction for the formation of the trimethylhydroquinone product in the presence of persulfate. Reaction conditions: 100mM phosphate, pH 6.5; 11.2μM trimethylhydroquinone; 11.2μM persulfate; 80°C.

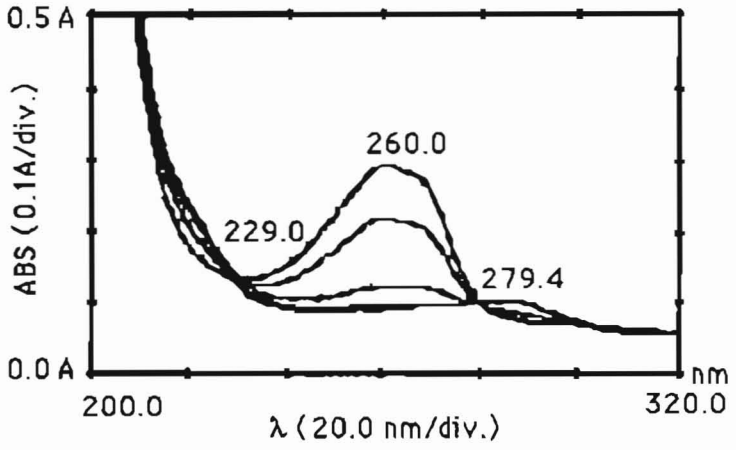
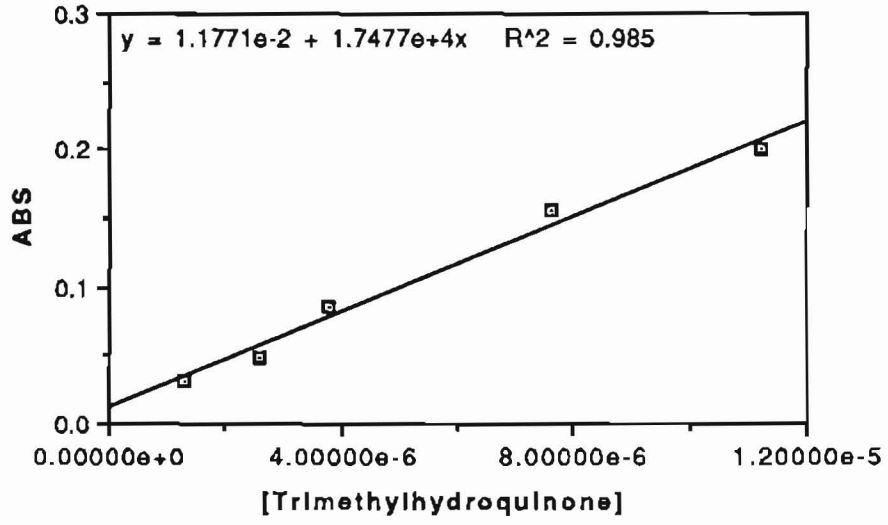
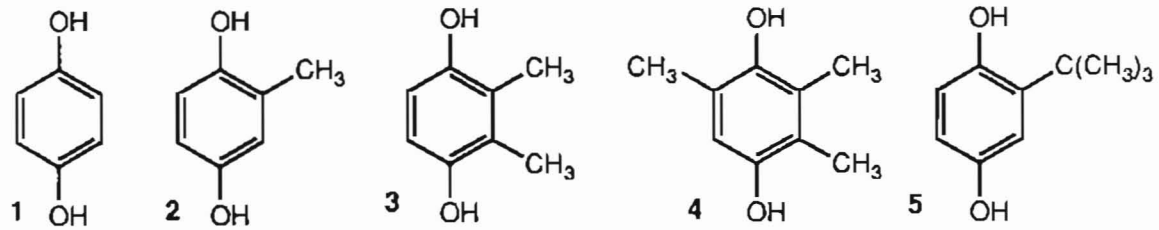


**Figure 10.** Molar Absorption Coefficient for non-enzymatic reaction of Trimethylhydroquinone. Reaction conditions: 100mM phosphate, pH 6.5; 1.3, 2.6, 3.8, 7.6, and 11.2μM trimethylhydroquinone; 1.3, 2.6, 3.8, 7.6, and 11.2μM persulfate, respectively.




*Percent Free Radical Pathway in Reactions Catalyzed by Chloroperoxidase-* In order to determine the percent free radical pathway in the CPO reactions we developed an efficient system that traps any radicals produced during the course of the reaction. Ferricytochrome C traps radicals efficiently[4] because it reacts with the radicals and at the same time is not a substrate for either HRP or CPO catalyzed reactions. When quinones react by a one electron mechanism the radicals that are produced are called semiquinones. Semiquinones of some substrates reduce ferricytochrome C producing ferrocytochrome C. We were able to isolate one class of quinones that produce semiquinones that reduce cytochrome C. These included hydroquinone (**1**, Figure 11) and various other substituted hydroquinones (**2-5**, Figure 11).

**Figure 11**: Summary of substrates that caused a reduction of ferricytochrome C.




After determining if the same amount of substrate reacts for each enzyme, it is possible to determine the percent free radical pathway of CPO reactions. However, the data presented here is uncorrected (Tables 2 and 3). Once the catalatic pathway and all other side reactions are taken into account the percent free radical pathway may change.

**Table 2**: Ratio of moles of radical trapped per mol of peroxide. Reaction conditions: 100mM phosphate, pH 6.5; 45µM hydroquinone; either 50nm CPO or 25nm HRP; 1.3, 2.6, 3.8, 7.6, and 11.2µM $H_2O_2$. Data generated from a linear curve fit of radical trapped versus peroxide concentration. The mole of radical trapped was determined by dividing the absorbance change by the experimental molar absorption coefficient for cytochrome C; 14,600$M^{-1}cm^{-1}$; which was determined by reduction with ascorbic acid.

| | Mole radical trapped per mole of peroxide | | | |
|---|---|---|---|---|
| Substrate | HRP (25nM) | CPO (50nM) | HRP (35nM) | CPO (80nM) |
| Hydroquinone | 2.00 | 1.18 | 1.84 | 1.06 |
| Methylhydroquinone | 2.12 | 1.73 | 2.08 | 1.65 |
| 2,3-Dimethylhydroquinone | 1.97 | 1.49 | 1.93 | 1.47 |
| Trimethylhydroquinone | 1.88 | 0.836 | 1.86 | 0.821 |
| t-Butylhydroquinone | 1.88 | 0.00 | ---- | ---- |

**Table 3**: Percent free radical pathway for CPO catalyzed reactions as compared to the 100% radical HRP reactions. Percent radical pathway determined by dividing the ratio of moles radical trapped per mole of peroxide for CPO reactions by the same for HRP reactions.

| Substrate | % Radical pathway | |
|---|---|---|
| | CPO(50nM) / HRP(25nM) | CPO(80nM) / HRP(35nM) |
| Hydroquinone | 59.3 | 57.7 |
| Methylhydroquinone | 81.4 | 79.4 |
| 2,3-Dimethylhydroquinone | 75.4 | 76.0 |
| Trimethylhydroquinone | 44.5 | 44.1 |
| *t*-Butylhydroquinone | 0.0 | ---- |

For HRP catalyzed reactions, since only the one electron transfer mechanism occurs[1-3], the expected ratio of moles of radical trapped per mole of peroxide is 2.00 since two moles of radical are produced for every mole of peroxide that reacts (Scheme 1). All of the data agree within experimental error with this theory, but the values obtained for trimethylhydroquinone and *t*-butylhydroquinone appear to be slightly lower than expected. If in fact there is some other type of radical reaction, such as dimerization, taking place for the more substituted hydroquinones, then some of the radicals could react with the substrate instead of with cytochrome C and the ratio would appear slightly lower than expected. The deviation is regarded as insignificant for our purposes.

The percent radical pathway for hydroquinone was found to be 59.3%, and for methylhydroquinone, the percent radical pathway was found to 81.4%. The difference between the percent radical pathway for these two substrates could be due to a couple of factors.

First of all, the increase in the radical pathway for methylhydroquinone could be due to a more stable radical being produced. Radicals are electron deficient species and the additional methyl group could donate a slight amount of electron density to the radical, making the one electron pathway slightly more favorable for methylhydroquinone that for hydroquinone.

Probably more important than the radical stability is the binding of the substrate to the enzyme. Since it has been suggested that two electron transfers occur at the heme iron and one electron transfers at the heme edge[1-3], it is possible that the additional methyl group of methylhydroquinone may make it more difficult for the substrate to bind to the heme iron active site of chloroperoxidase and follow the two electron mechanism (Scheme 2). As stated previously, when the binding of the substrate to compound I becomes worse, the binding of peroxide becomes more competitive and would need to be considered as the substrates become more hindered.

For 2,3-Dimethylhydroquinone we determined the percent radical pathway to be 75.4%. We expected a percentage higher than that for methylhydroquinone, 81.4%, based on both radical stability and binding, but the experimental value does not take into account the catalatic reaction. When considering the amount of peroxide consumed in the catalatic reaction instead of the peroxidatic reaction the percentage should increase. The amount of peroxide consumed should be larger than that of methylhydroquinone because 2,3-dimethylhydroquinone would have even more of a difficulty with binding and the catalatic reaction would be more competitive here.

Also using the argument of radical stability, 2,3-dimethylhydroquinone should be even more stable a radical than that for methylhydroquinone and should favor the one electron path more. It is also possible that radical stability has very little or nothing to do with the pathway that is followed and that binding need only be considered.

For trimethylhydroquinone the percent radical pathway was determined to be 44.5%. Again, this value is lower than what we expected to obtain based on the arguments of radical stability and binding. However, this data does not take into account any side reactions, such as the catalatic reaction. These would need to be considered here also in order to be sure of the actual percent radical pathway.

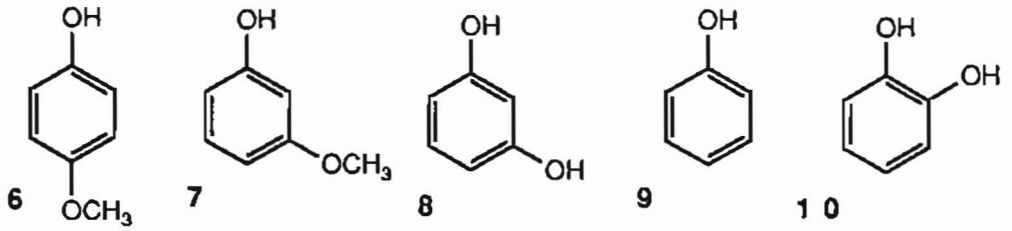
For the final substrate, *t*-butylhydroquinone, a completely unexpected result was obtained. The results of the HRP catalyzed reaction were consistent with the other substrates. However, there was no reaction of the cytochrome during the course of the CPO catalyzed reaction, indicating that no radicals were present. A reaction of *t*-butylhydroquinone was noticed for the CPO reaction in the absence of cytochrome C. The fact that a reaction occurs, but no radicals are present, indicates that the reaction is occurring by the two electron transfer mechanism. The theory of Ortiz de Montellano suggests that the two electron transfer takes place at the heme iron active site; they also suggest that for HRP the iron center is completely blocked from substrates by the protein structure.[1-3] If this heme iron versus heme edge theory is correct, *t*-butylhydroquinone, a very bulky substrate, should preferentially

react at the heme edge producing radicals. However, our results indicate no radical formation in the CPO catalyzed reactions of this substrate.

More research needs to be done to explain this fully, but it is possible that the iron site is less hindered than the heme edge site for CPO. Thus, two electron paths would predominate with more hindered substrates and one electron paths with less hindered ones. The difference between HRP and CPO would be that for HRP the iron site is more hindered and for CPO the heme edge site is more hindered. Such a structural difference could explain why it appears that fewer radicals are produced as the substrates become more hindered in CPO reactions.

*2,4,6-Trimethylphenol (TMP) as a Radical Trapping Agent-* For all of the substrates that we tested, any change of the hydroxyl functional groups, i.e. when a hydroxyl is replaced by another functional group or moved to the *ortho-* or *meta-* position (**6-10**, Figure 12), ferricytochrome C is not reduced. For some of these other substrates, like *p-* and *m*-methoxyphenol (**6,7**), resorcinol (**8**), and phenol (**9**), the production of semiquinones causes an oxidation of ferrocytochrome C and for others, catechol (**10**), the final peroxidatic product (A, Schemes I & II) reacts with ferrocytochrome C, making it impossible to detect intermediate semiquinone radicals.

**Figure 12:** Summary of substrates that did not cause a reduction of ferricytochrome C.




Since ferrocytochrome C was neither commercially available nor stable in our system, a different radical trapping agent, 2,4,6-trimethylphenol (TMP), was investigated for those substrates that caused an oxidation of ferrocytochrome C. TMP is not a substrate for the chloroperoxidase reactions in the absence of halide ions.[5] Also, TMP does not react with HRP at an appreciable rate in our system. Therefore, it may be possible to define a system in which TMP will be an effective radical trapping agent.

For those substrates that oxidized ferrocytochrome C to ferricytochrome C, instead of the reverse reduction, TMP was investigated as a possible radical trapping agent. As mentioned above TMP is not a substrate for the chloroperoxidase reactions in the absence of halides.[5] Also, it was determined through experimentation that TMP did not react with HRP under our reaction conditions. For our system the radical scavenging ability of TMP was investigated for three substrates: phenol, resorcinol, and *m*-methoxyphenol.

In the case of phenol, when the reaction mixture was scanned repeatedly as the reaction progressed a peak appeared at 400nm and there was a general increase in absorbance for the region around 250nm (Figure 13). When the reaction was carried out with TMP

(45μM) present the peak at 400nm formed only slightly and when the concentration of TMP was increased to 75μM the peak was not observed at all (Figures 14 and 15).

**Figure 13.** Spectral changes for the reaction of phenol with HRP. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$.

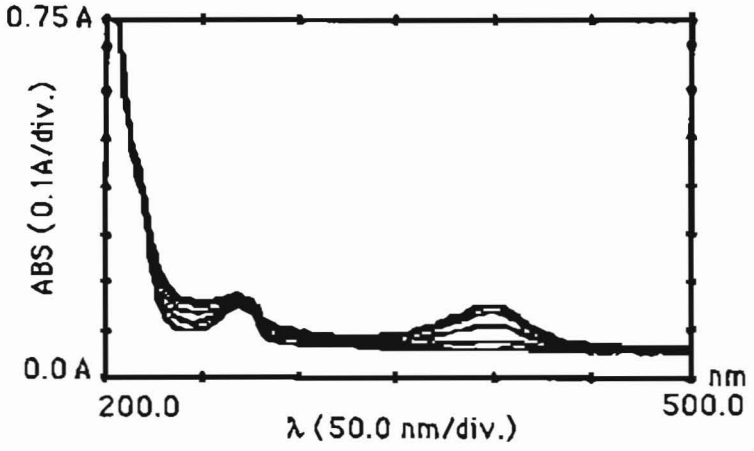



**Figure 14.** Spectral changes for the reaction of phenol with HRP in the presence of 45μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$; 45μM TMP.

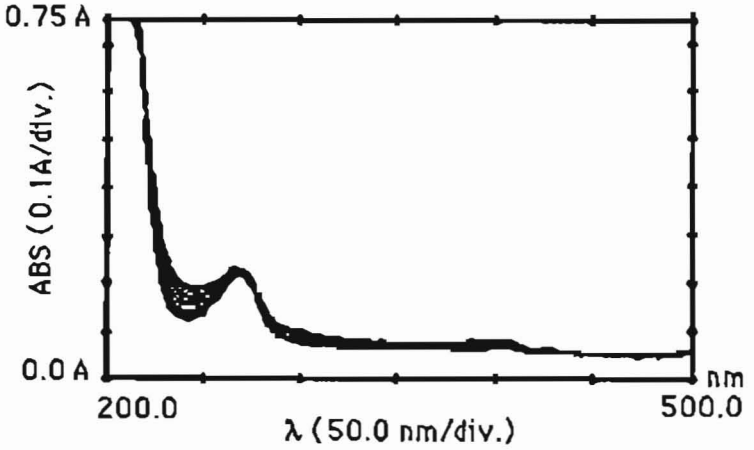
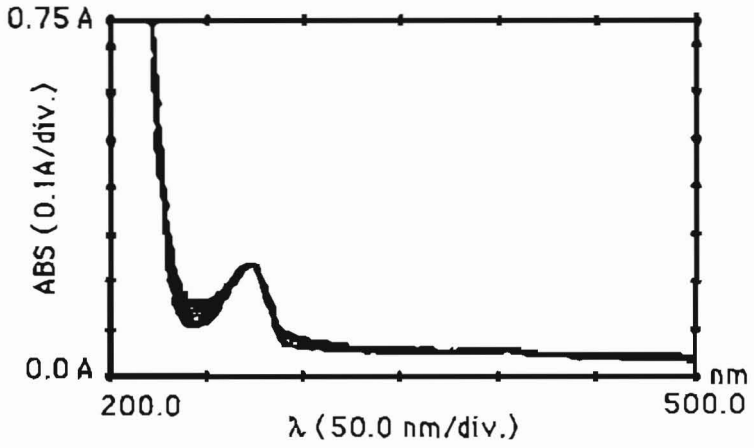


**Figure 15.** Spectral changes for the reaction of phenol with HRP in the presence of 75μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$; 75μM TMP.




A similar observation was made for both *m*-methoxyphenol and resorcinol. For *m*-methoxyphenol formation of a product peak was seen at 410nm in the absence of TMP (Figure 16), but was not observed at 45μM or 75μM TMP (Figures 17 and 18). The product peak for resorcinol appeared at about 405nM without TMP present (Figure 19). This peak decreased at 45μM TMP and decreased even further, but still observed, at 75μM (Figures 20 and 21).

**Figure 16.** Spectral changes for the reaction of *m*-methoxyphenol with HRP. Reaction conditions: 100mM phosphate, pH 6.5; 45μM *m*-methoxyphenol; 35nM HRP; 15μM $H_2O_2$.

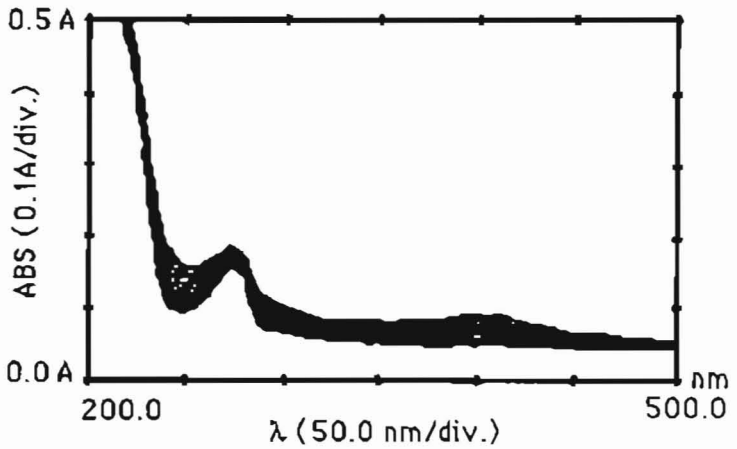


**Figure 17.** Spectral changes for the reaction of *m*-methoxyphenol with HRP in the presence of 45μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM *m*-methoxyphenol; 35nM HRP; 15μM $H_2O_2$; 45μM TMP.

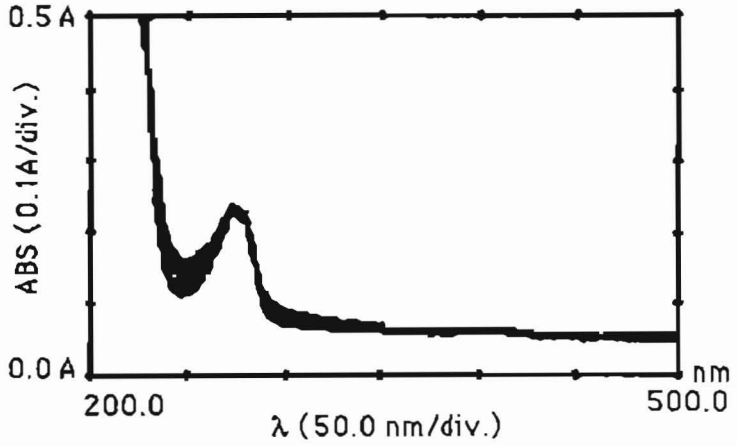



**Figure 18.** Spectral changes for the reaction of *m*-methoxyphenol with HRP in the presence of 75μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM *m*-methoxyphenol; 35nM HRP; 15μM $H_2O_2$; 75μM TMP.

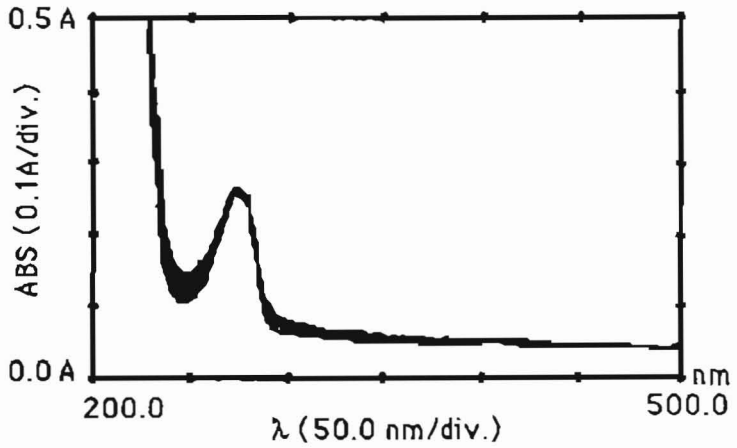


**Figure 19.** Spectral changes for the reaction of resorcinol with HRP. Reaction conditions: 100mM phosphate, pH 6.5; 45μM resorcinol; 35nM HRP; 15μM $H_2O_2$.

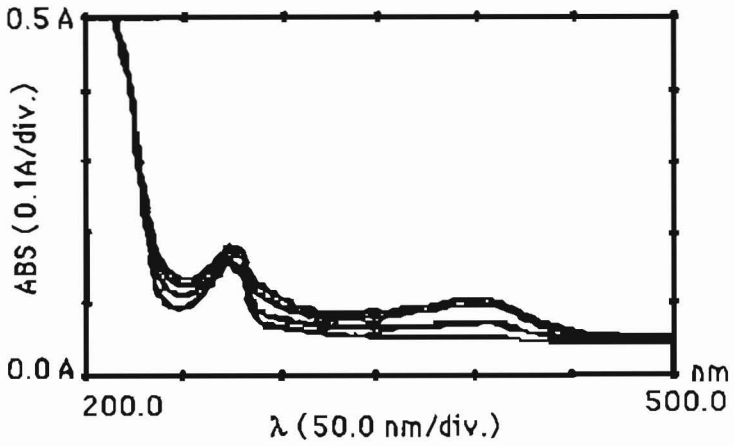



**Figure 20.** Spectral changes for the reaction of resorcinol with HRP in the presence of 45μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM resorcinol; 35nM HRP; 15μM $H_2O_2$; 45μM TMP.

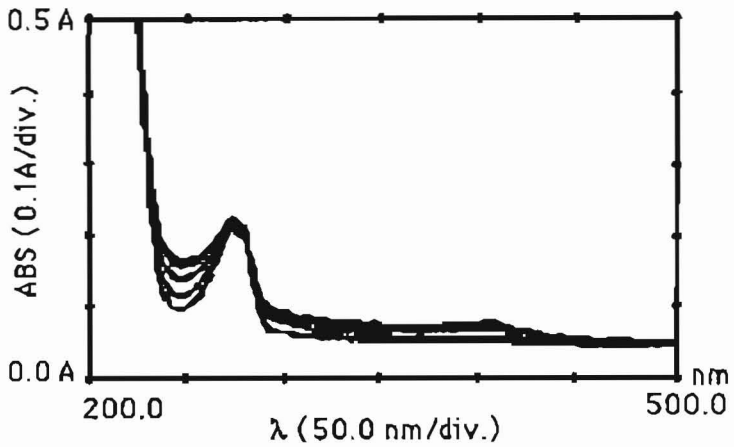
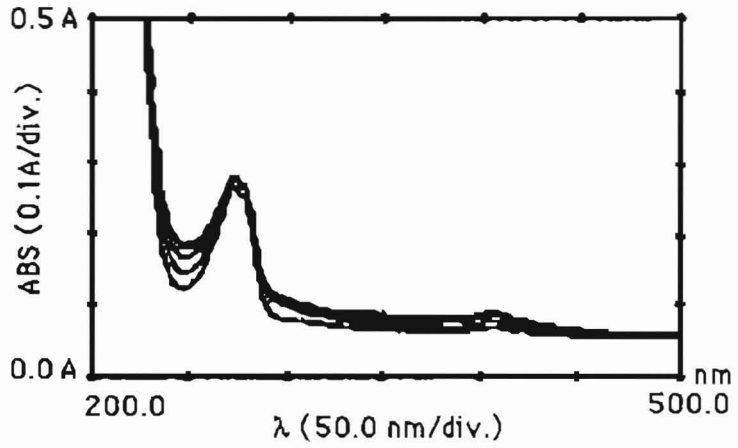


**Figure 21.** Spectral changes for the reaction of resorcinol with HRP in the presence of 75μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM resorcinol; 35nM HRP; 15μM $H_2O_2$; 75μM TMP.




Since TMP does not bind to CPO, its inhibition of the above peroxidatic reactions must occur off of the protein. A likely mechanism of inhibition could include reduction of intermediate radicals by TMP.

In an attempt to quantitate the amount of radical that reacted with the TMP, the spectrum of the system was scanned using various mixtures as blanks for each substrate. The blanks included TMP, substrate, and TMP with substrate (Figures 22-25). However, there is no peak that could be solely assigned to a TMP change. It appears from the spectra that the TMP may have an absorbance change in the 250nm region, but all of the substrates tested showed changes in this area also and it would not be possible to assign the absorbance change to a TMP reaction. Comparison of 250nm region of the spectrum for the reaction of phenol with no TMP present (Figure 22) to that of the reaction with 75μM TMP present (Figure 23) seems to indicate that a peak with a more definite shape is forming for the reaction with TMP present. It would be necessary to identify a

substrate that had no spectral change in the 250nm region in order to determine if TMP does in fact have an absorbance here.

**Figure 22.** Spectral changes for the reaction of phenol with HRP. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$. Blank: 100mM phosphate, pH 6.5; 45μM phenol.

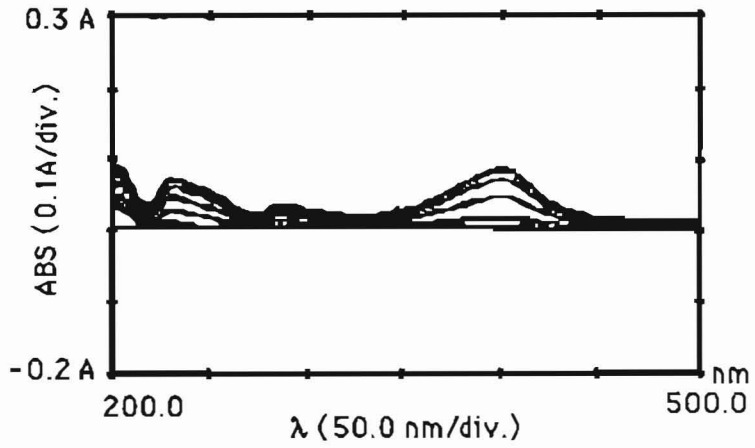



**Figure 23.** Spectral changes for the reaction of phenol with HRP in the presence of 75μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$; 75μM TMP. Blank: 100mM phosphate, pH 6.5; 45μM phenol; 75μM TMP.

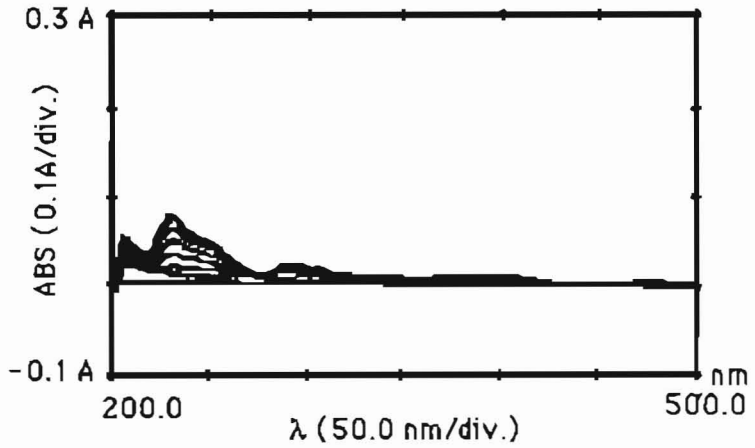


**Figure 24.** Spectral changes for the reaction of phenol with HRP in the presence of 75μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$; 75μM TMP. Blank: 100mM phosphate, pH 6.5; 45μM phenol.

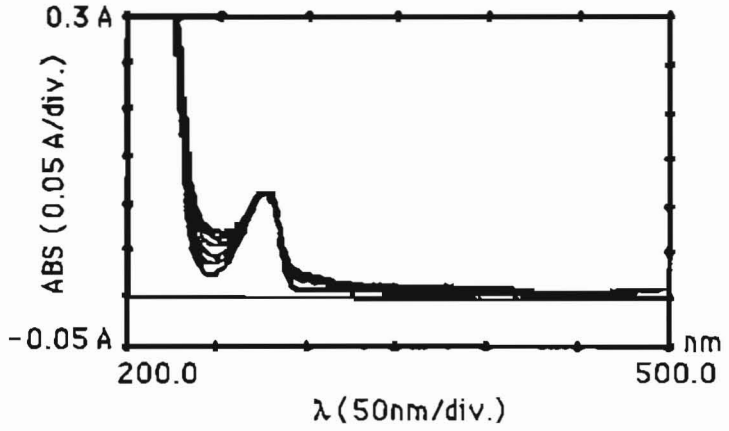
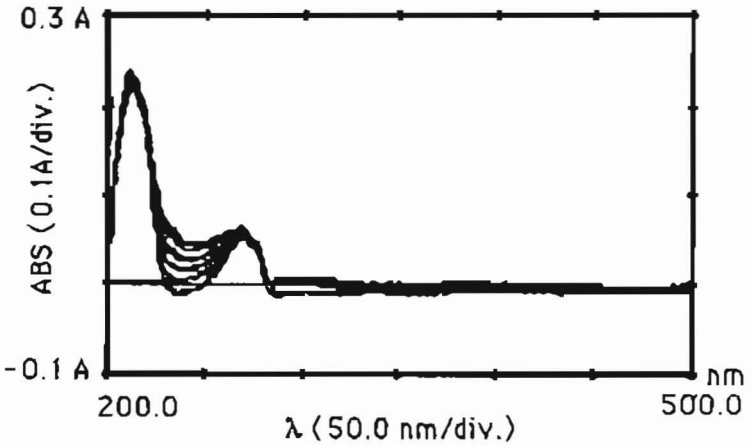



**Figure 25.** Spectral changes for the reaction of phenol with HRP in the presence of 75μM trimethylphenol. Reaction conditions: 100mM phosphate, pH 6.5; 45μM phenol; 35nM HRP; 15μM $H_2O_2$; 75μM TMP. Blank: 100mM phosphate, pH 6.5; 75μM TMP.




## Conclusions

On the basis of the research presented in this paper it is not possible to draw a final conclusion on how and why chloroperoxidase reactions proceed through both the one and two electron transfer mechanisms. However, it does give a definite direction to the project and an insight into what should be done to follow up on my

research. The catalatic reaction needs to be taken into account by the use of an oxygen electrode to detect any oxygen formed during the course of these reactions. Next, it must be determined why the molar absorption coefficients for the more substituted hydroquinones are so different for the type of enzyme used and why the values obtained for the enzymatic reaction are so different when persulfate is used to oxidize the substrate to the product. Perhaps most important, the reactivity of the binding sites must be developed further in order to determine which site is the most hindered for chloroperoxidase. Finally, the possibility of 2,4,6-trimethylphenol as a radical trapping agent must be developed further. When these problems have been taken into account it may be possible to determine the actual percent free radical pathway for any substrate.

## Acknowledgements

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