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MODEL COMPOUND STUDIES RELATED TO PEROXIDASES—II

THE CHEMICAL REACTIVITY OF A HIGH VALENT PROTOHEMIN COMPOUND

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Abstract—The chemical reactivity of the model analog to compound I of the peroxidases resulting from the reaction of "chelated protohemin" and *m*-chloroperbenzoic acid is examined. The model intermediate shows no H-atom abstraction or O insertion activity and substrate reactivity depends only on the $E_{1/2}$ value of the substrate. A Marcus theory treatment of the available kinetic data for HRP suggests that the oxidative pathway for substrate oxidations is an outer-sphere electron transfer. From the results of the model catalyzed oxidation of 1,4-cyclohexadiene to benzene, an alternate mechanism for cytochrome P-450 catalyzed hydroxylations is suggested.

Among the biological catalysts for the reduction of dioxygen or one of its partially reduced forms (H_2O_2, O_2^-, ROOH), hemoproteins constitute a large and important class.[1,2] These catalysts all employ similar iron-porphyrin complexes when bound to a fifth ligand, usually an imidazole.[3] By variations in the protein environment, this seemingly common catalytic center carries out a wide variety of chemical processes. For example, hemoglobin, myoglobin and several other heme proteins exist in the Fe(II) state and reversibly bind dioxygen without oxidation to Fe(III).[4] Oxidases and peroxidases, on the other hand, exist as Fe(III) complexes, have Fe(II) forms which are unstable toward oxidation by dioxygen, and generally perform the function of reducing dioxygen (or H_2O_2, ROOH, etc.) to water or an equivalent organic compound such as an alcohol or an epoxide.[4-7] Catalase, horseradish peroxidase (HRP), cytochrome c peroxidase, cytochrome oxidase and cytochrome P-450 are examples of this class. In the latter case, cytochrome P-450, the rather unusual insertion of an oxygen atom into a C–H bond is easily accomplished.[6]

$$R\text{–}H + O_2 \xrightarrow[2e^-,\,2H^+]{\text{P-450}} R\text{–}OH + H_2O. \qquad (1)$$

Because all of these oxidation catalysts involve a product-forming step which returns the enzyme to the Fe(III) state, it has been concluded that the oxidation states of the intermediates exceed that of the Fe(III) porhyrin. In addition, both the peroxidases and cytochrome P-450 can utilize hydroperoxides plus the Fe(III) form of the enzyme to carry out their rather different reactions.[8,9]

$$ROOH + \text{phenol} \xrightarrow{\text{HRP(FeIII)}} \text{"oxidized phenol"} + H_2O \qquad (2)$$

$$ROOH + RH \xrightarrow{\text{P-450(FeIII)}} ROH + H_2O. \qquad (3)$$

This has led to the postulate that the intermediate formed in both enzymes is very similar.[6,10,11]

There are two important differences between cytochrome P-450 and HRP. First, the fifth ligand on HRP is an imidazole[12] and on P-450 it is a thiolate.[13] Secondly, HRP has been shown to accomplish hydrogen peroxide reduction through two distinctly separate one-electron substrate oxidations,[14] whereas P-450 inserts an O atom into a substrate in what has been assumed by most researchers to be a single two-electron reduction or a slow formation of a caged radical pair which collapses without diffusion.[15]

In the case of cytochrome P-450, the intermediate high oxidation states of the catalyst have not been isolated. Their structure has been inferred from studies on HRP. Horseradish peroxidase reacts with hydrogen peroxide to produce a green compound,[16] known as compound I, whose one-electron reduction produces a red species, compound II, which is then reduced by one more electron to the resting Fe(III) state.[17]

$$HRP + H_2O_2 \rightarrow \underset{\text{(green)}}{HRP(I)} \xrightarrow{e^-} \underset{\text{(red)}}{HRP(II)} \xrightarrow{e^-} HRP. \qquad (4)$$

Both compounds I and II react rapidly with phenols to produce short-lived phenoxyl radicals.[18]

Compound I is two electron equivalents above the resting Fe(III) state. Most physical measurements are consistent with the cation radical formation of Dolphin *et al.* in which the iron is Fe(IV) and the second electron has been removed from an orbital which is principally located on the porphyrin.[19] Compound II has been assigned an Fe(IV) structure based on Mossbauer,[20,21] magnetic susceptibility[22] and electron paramagnetic resonance studies.[23]

Because these oxidized iron porphyrins seem to be similar in many heme protein oxidation catalysts and because the intermediates carry out interesting and quite varied oxidation processes, a better understanding of the structure and function of such intermediates has become a widely sought objective.[24-26]

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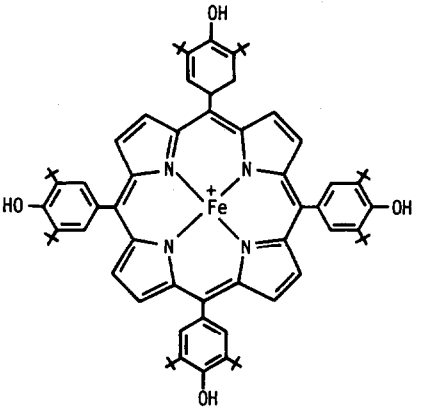
One approach to this problem, which has enjoyed some success in understanding the behavior of dioxygen carriers, is the preparation of biomimetic heme or hemin compounds followed by structure reactivity studies. This approach suffers a serious, but not insurmountable, problem. In the case of dioxygen carriers, it was necessary to prepare model dioxygen complexes of sufficient lifetime to be studied.[27,28] In oxidase investigations, complexes equivalent to Fe(V) porphyrins or Fe(IV) porphyrins must be maintained for sufficient time to allow their study.

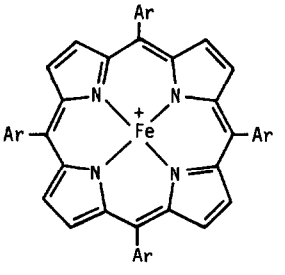
Several model systems have been devised for HRP and cytochrome P-450 catalyzed reactions. In most cases, simple hemins have been reacted with oxidants such as dioxygen, hydroperoxides, peracids and iodosobezene. Intermediates resembling both compound I and compound II in some of their spectrosocpic properties and in their ability to carry out the appropriate oxidations have been observed.[29,30] However, these intermediates are seen only at low temperature, and efficient catalytic oxidation of, for example, olefins has not been accomplished. Destruction of the catalyst usually competes with product oxidation and low turnover ratios are observed.[31] In one recent example, tetramesityl hemin chloride was treated with iodosylbenzene to produce an intermediate at $-30°$, which oxidized norbornene.[32]

Just as in the case of the studies of dioxygen binding to model compounds, the present study of the mechanism of formation and subsequent reduction of "oxidized hemins" (Compounds I and II of model systems) has begun with two approaches. These consist of either synthesizing model compounds which form stable oxidized intermediates or the use of fast kinetic methods to accomplish measurements before the intermediate is destroyed. As with oxygen carriers, the synthetic approach invariably involves the preparation of model compounds with structures which differ substantially from that of the enzyme active site,[31-38] while the kinetic method attempts to use models which mimic the active site as closely as possible, *vis-a-vis* protohemin imidazole complex. In the present case, it appears that either extremely electron-withdrawing[37] or electron-donating groups[38] on tetraphenylhemins accomplish the stabilization of the doubly oxidized species corresponding to compound I as shown in Fig. 1.

Because we wished to determine reaction mechanisms with compounds which resemble the HRP site as much as possible, we have taken the kinetic approach. First, we used chelated protohemin chloride, 1^+Cl^- (Fig. 2), which contains a proximal imidazole similar to the protein. We have generated a compound I analog using peracids and hydroperoxides and studied thoroughly the catalyzed one-electron oxidation of 2,4,6-tri-t-butylphenol (TBPH) to the stable blue phenoxyl radical.[39] This phenol, at high concentrations (10^{-3}–10^{-1} M), effectively traps



Electron Donation by Proton Loss[38]



Ar = Mesityl,[32] C_6F_5[37]

Steric or Electronegative Effects

Fig. 1. Hemins which form stable "Fe^{IV}" complexes.

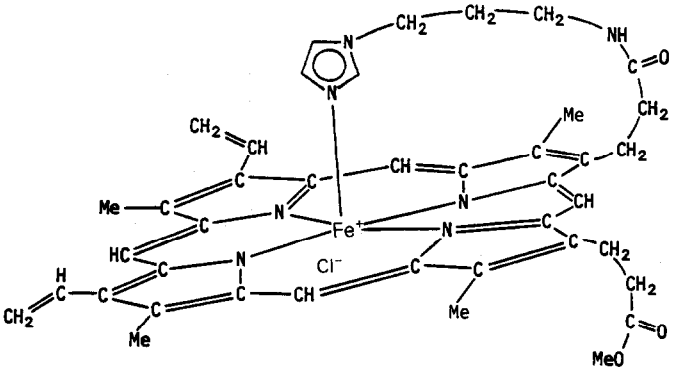
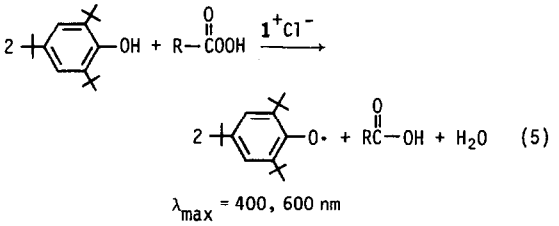


Fig. 2. Protohemin monomethyl ester mono-3-[1-imidazolyl]propylamide, 1^+Cl^-.

the first formed intermediate before it can react with another hemin and therefore prevents hemin destruction. Reaction 5 does not proceed without the catalyst and can be made to turn over up to 50,000 times without appreciable loss of catalyst. It is, in fact, an effective synthetic oxidizing agent and a true catalytic system.

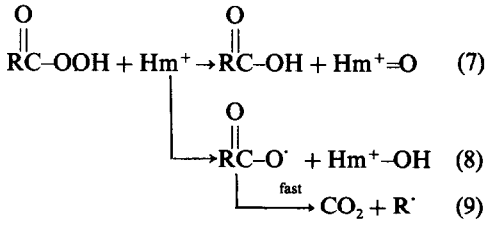


λ_{max} = 400, 600 nm

The stoichiometry of the reaction was found to be two moles of phenoxyl radical per mole of peracid as indicated by eqn (5) and the rate was first order in peracid, first order in chelated protohemin chloride, and zero order in phenol. Since this means the rate of intermediate formation is rate-limiting, we studied the structural and solvent effects on the formation reaction (eqn 6), in which $Hm^+{=}O$ represents a species which is

$$ROOH + Hm^+ \rightarrow ROH + Hm^+{=}O \quad (6)$$

two electron equivalents more oxidized than the hemin (Hm^+). The reaction was accelerated by increasing solvent polarity, by increasing the leaving group ability of RO^- (as judged by the acidity of ROH), and by the presence of a proximal imidazole. Furthermore, the reaction was general-base catalyzed, responding to the concentration of collidine buffer. Heterolytic cleavage of the O–O bond was confirmed by the absence of CO_2 in the catalytic oxidation of TBPH by phenyl peracetic acid and perlauric acid, both of which give CO_2 upon formation of their acyloxy radicals.



These observations are consistent with and strongly favor the following process for the formation of "oxohemin", the model compound for HRP compound I.

The general features of our model system confirm many of the postulates of Schonbaum,[40] and Poulos and Kraut[41] that proximal imidazole as well as distal imidazole catalysis are important in the reduction of hydrogen peroxide.

Having provided some evidence for the catalyzed heterolytic cleavage of the peroxide bond to form the two-electron oxidized intermediate, we now address the mechanism of reaction of such an intermediate with various substrates. This presents additional problems since hemin destruction prevents the build-up of this intermediate. Consequently we have been forced to use competitive methods to elucidate the reaction mechanism.

EXPERIMENTAL

Materials

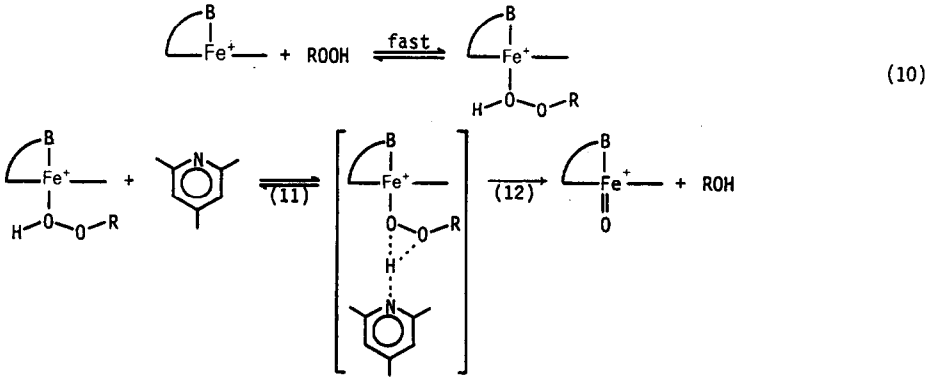
Protohemin mono - 3 - (1 - imidazoyl)propylamide, monomethyl ester (1^+Cl^-) was prepared from hemin chloride according to published procedures.[42] *m*-Chloroperbenzoic acid (MCPBA) was obtained from the Aldrich Chemical Co. and determined to be 81% active by iodometric analysis.[43] Identical results were obtained at the same calculated concentrations using purified MCPBA (98%).[44] Perlauric acid was prepared as described in the literature[45] and shown to be 93% active by iodometric titration. Zn and Cu were inserted into tetraphenylporphyrin (Aldrich) by lit. methods.[46] 2,4,6-Tri-t-butylphenol (TBPH) was recrystallized twice from 95% EtOH. Hydroquinone was recrystallized two times from abs. EtOH. Anhyd. MeOH ($H_2O < 0.2\%$) was Mallinckrodt Spectra AR grade and not purified further. CH_2Cl_2 was distilled under argon from CaH_2 and stored over 4Å molecular sieves.

Deuterio-2,4,6-tri-t-butylphenol was synthesized by dissolving TBPH (5.0 g, 2.1 mmole) in 25 ml C_6H_6 and shaking twice with 10 ml soln of 10^{-3} M NaOD in D_2O. The benzene layer was separated and the solvent removed on the roto-evaporator to yield deuterio-tri-t-butylphenol. The isotopic purity was checked by NMR (88%).

Diphenylpicrylhydrazine (DPPH) was prepared by dissolving diphenylpicrylhydrazyl radical (1.0 g, 2.5 mmol) in 10 ml of CH_3OH. To this soln, 4 equiv of aqueous sodium dithionite (1.74 g, 10 mmole) were added. The color of the soln immediately changed from purple to a brown-red. The soln was then diluted with 200 ml of a sat. NaCl aq and then extracted four times with 50 ml $CHCl_3$. The $CHCl_3$ soln was dried over $MgSO_4$ and evaporated to yield a dark red oil. The oil was crystallized by dissolving it in $CHCl_3$/EtOH (2 : 3) and cooling to 0°. The crystals were filtered and dried under vacuum, yield: 0.2 g (20%), m.p. 162–166° (lit. 171–172°).[47]

All other chemicals were used as received from the manufacturers.

Methods. UV-visible spectra and kinetic data were recorded on three spectro-photometers: Cary 15,



Kontron/Uvikon 810, and a Hewlett Packard 8450. Absorbance vs time data recorded on the Cary 15 and the Kontron/Uvikon 810 were manually digitized and used for kinetic analysis. The temp. in the cell holders of the Cary 15 and the Uvikon 810 was maintained by a thermostatted circulating water bath. NMR spectra were recorded on a Varian 390 spectrometer.

Extinction coefficients in MeOH for 1^+Cl^- were calculated by weighing out pure compound and dilution to the appropriate volume. Since the nature of the ligand and pH affect the ϵ value for 1^+Cl^-, stock soln were converted entirely to the μ-oxo species by the addition of tetabutylammonium hydroxide ($\sim 100\,\mu l$–0.1 M, to 5 ml of hemin soln) and a standard value calculated for this species. For each kinetic series, the concentration of 1^+Cl^- was calculated by comparison of the soret absorbance to that of the μ-oxo species generated under identical experimental conditions.

Kinetic data for the oxidation of TBPH, DPPH and hydroquinone were all obtained using a similar procedure. Typically, a 5 ml soln of substrate (10^{-2} M–10^{-3} M) was added to an 8 in. 1 cm path length silica curvette. A septum was placed over the cuvette and a calibrated amount of hemin soln was injected (5–10 μl). O_2 was removed by bubbling solvent-saturated argon through the cuvette for 10 min. To this soln, 1–10 μl quantities of 0.1 M MCPBA were added with a microliter syringe. The soln was shaken vigorously and placed in the spectrophotometer where absorbance vs time data were recorded. The maximum lag time was 6 sec. Depending on the extinction coefficients of the products, up to four MCPBA additions could be made to each sample. The oxidations were run under pseudo-first-order conditions.[48]

The DPPH oxidation was followed by the build-up of diphenylpicrylhydrazyl radical at 530 nm.[49] The oxidation of TBPH was monitored by the build-up of phenoxyl radical at 400 and 630 nm.[50] In the case of hydroquinone, the disappearance of hydroquinone was monitored at 294 nm. The stoichiometry of the reaction was calculated using a $\Delta\epsilon = 1700$. Both *m*-chlorobenzoic acid and benzoquinone absorb at 294. Benzoquinone was identified as the product of hydroquinone oxidation by its UV spectrum. Identical rates were obtained with DPPH and hydroquinone in deoxygenated or air-saturated solns.

Oxidation of zinc tetraphenylporphyrin. The 1^+Cl^- catalyzed oxidation of ZnTPP by MCPBA in MeOH was monitored with time by the decrease in the absorbance at 555 nm[51] on the Kontron/Uvikon 810 spectrophotometer. The data was analyzed using the method of initial rates.

Three separate experiments were carried out to determine the order in 1^+Cl^-, MCPBA and ZnTPP. The $\Delta\epsilon$ was calculated from the average of three ΔODs obtained upon addition of MCPBA (9.3×10^{-7} mole, 1.86×10^{-4} M) to a 5 ml soln of ZnTPP (1.03×10^{-4} M) and 1^+Cl^- (4.32×10^{-7} M). The spectrum was recorded within 10 sec of the MCPBA addition ($\Delta\epsilon = 9.0 \pm 0.8 \times 10^3$). The concentration of hemin was calibrated by preparing a stock soln of concentrated hemin ($\sim 10^{-4}$ M) in CH_3OH and adding 100 μl to 5 ml of spectral grade MeOH. From the absorbance at 399 (ϵ = 103,000), the concentration was calculated (4.32×10^{-8} mole/100 μl stock hemin).

The initial rate as a function of 1^+Cl^- concentration was determined by adding 5 μl of 0.093 M MCPBA soln to 5 ml solns of ZnTPP (1.03×10^{-4} M) containing 8.64×10^{-8} M, 1.73×10^{-7} M, 2.59×10^{-7} M, 3.46×10^{-7} M, and 4.32×10^{-7} M 1^+Cl^-. Upon addition of the MCPBA through a rubber septum into the 8 in. glass cuvette, the soln was shaken vigorously and placed in the spectrophotometer. The maximum lag time between addition of MCPBA and observation was 6 sec. In identical procedures, the rate as a function of [MCPBA] and [ZnTPP] was determined. The MCPBA concentration was varied by adding 1, 2, 3, 4 and 5 μl quantities of a stock 0.093 M MCPBA soln to separate solns containing 1.03×10^{-4} M ZnTPP and 1.73×10^{-7} M 1^+Cl^-. The ZnTPP concentration was varied by dilution of the stock 1.03×10^{-4} M ZnTPP soln.

Competition by 1,4 - cyclohexadiene and 2,4,6 - tri - t - butylphenol for intermediate. In MeOH, 5 ml solns containing 5.5×10^{-3} M TBPH and 0, 10^{-2} M, 5×10^{-3} M, 7.5×10^{-2} M, 10^{-1} and 2×10^{-1} M 1,4-cyclohexadiene were prepared in 8 in. septum-capped, 1 cm Pyrex cuvettes. Each cuvette was deoxygenated with solvent–saturated argon for 10 min. To each cuvette, 30 μl of a stock 1^+Cl^- soln was added giving a resultant hemin concentration of 1.44×10^{-5} M. A separate 1.4×10^{-1} M perlauric acid soln was prepared in methanol. Reactions were begun by adding 5 μl of the perlauric acid soln to the cuvette containing the hemin/phenol-cyclohexadiene soln, shaking vigorously and then monitoring the change in absorbance vs time at 630 nm. The $[phenoxyl]_\infty$ with no 1,4-cyclohexadiene present was 1.8×10^{-4} M. From the ΔOD time plots, the slower decrease in phenoxyl radical due to abstraction of an H- atom from the cyclohexadiene could also be analyzed by pseudo-first-order methods. At 0.1 M cyclohexadiene, 1.2×10^{-5} M 1^+Cl^- and 5.5×10^{-3} M TBPH, the addition of perlauric acid (1.4×10^{-4} M) resulted in the fast formation of phenoxyl radical and a slower phenoxyl decay ($k = 3.1 \times 10^{-2}\,sec^{-1}$).

The quantitative analysis for benzene from the oxidation of 1,4-cyclohexadiene by tri-t-butylphenoxyl radical was carried out as follows. Four 0.5 ml solns containing 0.1 M TBPH, 0.53 M 1,4-cyclohexadiene, and 4×10^{-5} M 1^+Cl^- (2 μl stock) were prepared. Each soln was placed in a 2 ml culture tube, covered a septum and bubbled slowly with solvent–saturated argon for 2 min. A 100 μl addition of a stock perlauric acid soln (0.59 M) in MeOH was added slowly to two of the solns. At the end of the perlauric acid addition, 2 μl more of stock 1^+Cl^- soln was added to each soln. The solns were all diluted to 2 ml with MeOH and analyzed on a Varian 3700 gas chromatograph at 50° using a 3% SE 30 column.[52] Integration of peak areas was done by cutting out and weighing the peaks. (Benzene/cyclohexadiene: control: Calc. 0.03; exp. 0.06, 0.05. Reaction: Calc. 0.35; exp. 0.37, 0.35.)

The rate of tri-t-butylphenoxyl radical oxidation of 1,4-cyclohexadiene was determined as follows. To a deoxygenated MeOH soln of TBPH (10^{-2} M) and 1^+Cl^- (8.2×10^{-6} M), 15 μl of a 0.1 M MCPBA soln (3.0×10^{-4} m) was added. The resultant absorbance due to phenoxyl radical was 1.05 at 400 nm (5.8×10^{-4} M). 1,4-Cyclohexadiene (47 μl, 5.0×10^{-4} mole) was then injected into the soln and the absorbance vs time monitored at 400 nm. The data was analyzed using pseudo-first-order kinetics where the slope of $\ln[A_t - A_\infty]$ vs time divided by the concentration of cyclohexadiene is equal to the second-order rate constant for H-atom abstraction from cyclohexadiene by phenoxyl radical ($k = 2.85 \times 10^{-2}\,M^{-1}\,sec^{-1}$).

Substrate reactivity with intermediate. Substrates were evaluated for reactivity with the intermediate based on whether the substrate was able to prevent the destruction of the hemin. Typically 1×10^{-5} M solns of 1^+Cl^- were prepared in an 8 in. silica cuvette containing 5 ml MeOH and $\geq 10^{-2}$ M substrate. *m*-Chloroperbenzoic acid, 10 μl of a 0.1 M soln, was then syringed into the cuvette resulting in a 20-fold excess of MCPBA (2×10^{-4} M) to 1^+Cl^-. The spectral change in the soret region was monitored within 20 sec after addition of MCPBA. In the case of cyclohexene, phenanthrene and thianthrene the test was also carried out in CH_2Cl_2 at substrate concentrations ≥ 1.0 M.

Hemin as a competitive substrate. Solns of 0.5, 0.25, 0.125, 0.1, 0.05, 0.03, 0.015, 0.01 and 0.005 M 2,4,6 - tri - t - butylphenol in 5 ml CH_2Cl_2 were prepared in 8 in., 1 cm silica cuvettes. The solns were deoxygenated by blowing through solvent–saturated argon for 10 min each. To each cuvette was added 6 μl of a concentrated stock 1^+Cl^- solution in CH_2Cl_2 resulting in an average absorsorbance of

0.60. A stock MCPBA soln (0.28 M) was prepared in 25 ml of CH_2Cl_2. Experiments were run by making three injections of 10 μl of stock MCBPA soln. The spectrum at 630 nm was monitored after each injection of peracid. The total ΔOD after three additions of MCPBA was plotted as a function of the concentation of 2,4,6-tri-t-butylphenol. The identical procedure was carried out using deuterated 2,4,6-tri-t-butylphenyl (88%) at 0.5, 0.25, 0.125, 0.01, 0.05, 0.025 0.0125, 0.01 and 0.005 M concentrations. After the three peracid additions, the phenoxyl radical was reduced by adding 500 μl of a 0.1 M soln of *p*-methoxyphenol. The concentration of hemin could then be calculated from the absorbance at 400 nm. With both the proto- and deuteriophenols, reduced concentrations of hemin were observed at 0.1 M phenol ($A_{400} \approx 0.36$) and no absorbance due to hemin remained at 0.05 M phenol and lower concentrations after the addition of peracid.

RESULTS

The hemin model used exclusively in the present study is protohemin monomethyl ester mono-3-[1-imidazoly]propylamide chloride (1^+Cl^-), shown in Fig. 2. The kinetics of the reaction between this compound and MCPBA, reviewed above, were thoroughly studied in our earlier work.[39] *m*-Chloroperbenzoic acid was employed as the primary oxidant due to its relative stability and rate of reaction with 1^+Cl^-.

Oxidation of 2,4,6-*tri-t-butylphenol.* The reaction of MCPBA (10^{-4} M) with catalytic amounts of 1^+Cl^- (10^{-6}–10^{-5} M) in the presence of excess TBPH results in the formation of a stable blue phenoxyl radical characterized by its visible spectrum.[50] The radical is stable for hours in thoroughly deoxygenated solvents. Figure 3 shows the catalyzed production of phenoxyl radical at specific time intervals. The kinetics of phenoxyl radical formation were monitored at 400 nm and 630 nm. In pure MeOH, only a slight molar excess of TBPH over MCPBA was required to obtain reliable first-order kinetics (3 ×). In CH_2Cl_2 or benzene, it was necessary to maintain the TBPH concentration at 10^{-1} M ([MCPBA] $\approx 10^{-4}$ M) in order to avoid destruction of the catalyst. In all solvents the rate law was shown to be first order in MCPBA and first order in 1^+Cl^-.

$$\frac{d[\text{phenoxyl}]}{dt} = k[1^+Cl^-][\text{MCPBA}]. \qquad (13)$$

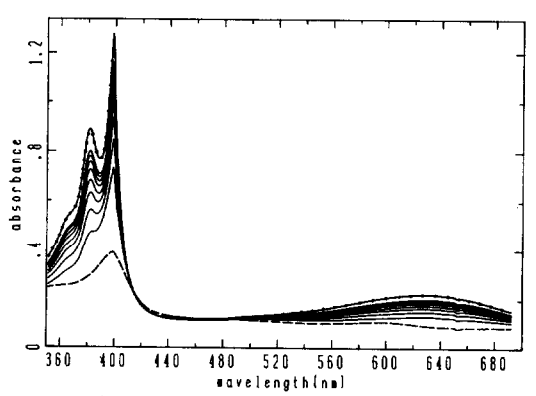


Fig. 3. The 1^+Cl^- catalyzed oxidation of TBPH by MCPBA in CH_3OH. ——, 4.0×10^{-6} M 1^+Cl^- and 10^{-3} M TBPH; —, after the addition of 2.5×10^{-4} M MCPBA at 5, 10, 15, 20, 25, 30, 35 and 40 sec; ⊖⊖⊖, after 170 sec.

Oxidation of zinc tetraphenylporphyrin. ZnTPP does not react at an appreciable rate with MCPBA in MeOH under the conditions of the experiment. In the presence of 1^+Cl^-, ZnTPP is readily oxidized to a species identified from its visible spectrum as the cation radical.[51] The radical is not stable and slowly decomposes with a decrease in the characteristic 504 nm peak and a subsequent increase in the band at 640 and 490 nm due to a product of methanol attack at the meso positions of $ZnTPP^{+\cdot}$.[53] These changes are shown in Fig. 4. The radical is sufficiently stable that reasonable rate data can be obtained within the first 30 sec. The kinetics of the ZnTPP oxidation were followed using the method of initial rates. The change in absorbance of ZnTPP over this time is essentially linear, consistent with a zeroth-order reaction when MCPBA is in (pseudo-first-order) excess. The decrease in the 555 nm band of ZnTPP due to the formation of $ZnTPP^{+\cdot}$ was monitored. Rates of oxidation as functions of 1^+Cl^-, MCPBA, and ZnTPP concentrations are plotted in Figs. 5(a–c). The initial rate of absorbance change is seen to obey the rate law

$$\frac{-d\text{Abs}}{dt} = k_1[1^+Cl^-][\text{MCPBA}] \qquad (14)$$

being independent of ZnTPP and first order in 1^+Cl^- and MCPBA. The rate constant, k_1, calculated using ($\Delta\epsilon_{555\,nm} = 9 \times 10^3$)

$$k_1 = \frac{\text{rate(abs/sec)}}{2\Delta\epsilon[1^+Cl^-][\text{MCPBA}]} \qquad (15)$$

is seen to be constant with the experimental uncertainty in $\Delta\epsilon$ and the MCPBA concentration (Table 1). A factor of 2 is present in the denominator to account for the stoichiometry of the reaction, 2 mole of ZnTPP oxidized per mole of MCPBA. The oxidation of copper tetraphenylporphyrin (CuTPP) to a species identifiable as $CuTPP^+$[51] was carried out in the same manner and gave similar results.

Oxidation of 2,2 - *diphenyl* - 1 - *picrylhydrazine*

2,2-Diphenyl-1-picrylhydrazine (DPPH) was also used as a substrate and its oxidation in CH_3OH by

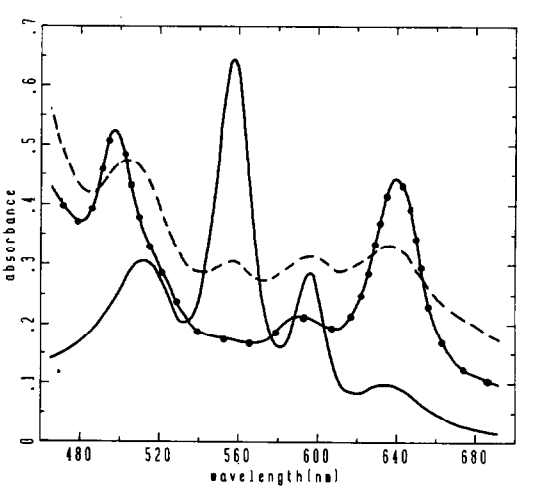


Fig. 4. The oxidation of ZnTPP by MCPBA catalyzed by 1^+Cl^- in CH_3OH. —, 3.6×10^{-5} M ZnTPP and 2×10^{-6} M 1^+Cl^- in CH_3OH; ——, 20 sec after addition of 10^{-3} M MCPBA; ⊖⊖⊖, after 5 min.

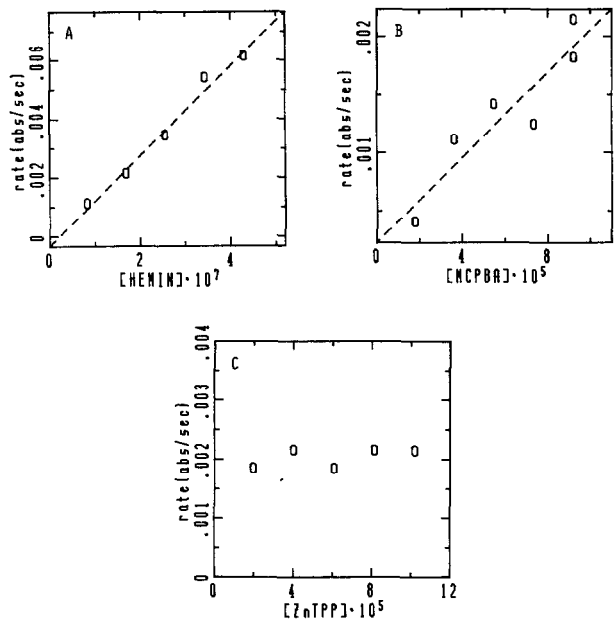


Fig. 5. A, The initial rate of $ZnTPP^+$ formation as a function of $[1^+Cl^-]$ upon addition of 9.3×10^{-5} M MCPBA to a solution of 1.03×10^{-4} M ZnTPP and 1^+Cl^- in CH_3OH. B, The initial rate as a function of [MCPBA] upon addition to a solution of 1.03×10^{-4} M ZnTPP and 1.73×10^{-7} M 1^+Cl^-. C, The initial rate as a function of [ZnTPP] upon addition of 9.3×10^{-5} M MCPBA to a solution of 1.73×10^{-7} M 1^+Cl^- and ZnTPP. T = 25°.

Table 1. The oxidation of ZnTPP by MCPBA catalyzed by 1^+Cl^- in CH_3OH. T = 29°

A. The Initial Rate Dependence of ZnTPP (1.03×10^{-4} M) Oxidation by MCPBA (9.30×10^{-5} M) on $[1^+Cl^-]$ in CH_3OH at 25°.

$[1^+Cl^-] \times 10^7$	Rate (abs/sec) $\times 10^3$	$k_1 \times 10^{-3}$ (M^{-1} sec^{-1})
0.864	1.13	7.81
1.73	2.15	7.44
2.59	3.47	8.00
3.46	5.42	9.36
4.32	6.15	8.51

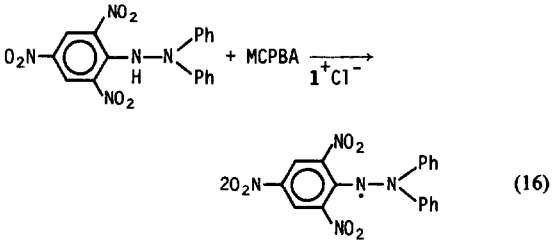
B. The Initial Rate Dependence of ZnTPP (1.03×10^{-4} M) Oxidation as a Function of [MCPBA] in CH_3OH at 25°. $[1^+Cl^-] = 1.73 \times 10^{-7}$ M.

[MCPBA] $\times 10^5$	Rate (abs/sec) $\times 10^3$	$k_1 \times 10^{-3}$ (M^{-1} sec^{-1})
1.86	0.41	7.04
3.72	1.12	9.67
5.58	1.42	8.17
7.44	1.25	5.39
9.30	1.82	6.28
9.30	2.15	7.44

C. The Initial Rate Dependence of ZnTPP Oxidation by MCPBA (9.30×10^{-5} M) as a Function of [ZnTPP] in CH_3OH at 25°. $[1^+Cl^-] = 1.73 \times 10^{-7}$ M.

[ZnTPP] $\times 10^5$	Rate (abs/sec) $\times 10^3$	$k_1 \times 10^{-3}$ (M^{-1} sec^{-1})
10.3	2.15	7.44
8.24	2.16	7.46
6.18	1.85	6.39
4.12	2.16	7.46
2.06	1.85	6.39

MCPBA and 1^+Cl^- could be followed spectrophotometrically by the formation of the hydrazyl radical (DPP˙) at 530 nm[49] analogous to the TBPH oxidation studies. A complete rate law study was not carried out, but the data is consistent with a rate-limiting formation of an oxidizing intermediate, followed by a rapid DPPH oxidation. The oxidation of DPPH is a one-electron oxidation and the stoichiometry of the reaction was 2DPP˙/MCPBA, also consistent with the TBPH oxidations.



(16)

Oxidation of hydroquinone. The oxidation in MeOH by MCPBA and 1^+Cl^- was followed spectrophotometrically by the decrease in absorbance at 294 nm. The data is shown in Table 3. The conditions were identical to those for the oxidations of DPPH and TBPH except that deoxygenation of the reaction solution was unnecessary. The spectral changes occurring during the 1^+Cl^- catalyzed oxidation of hydroquinone by perlauric acid are shown in Fig. 6. The oxidation product of hydroquinone was identified from its UV-visible spectrum as benzoquinone. The stoichiometry of the reaction was determined to be 1 : 1 from the decrease in absorbance at 294 nm ($\Delta\epsilon = 1700$).

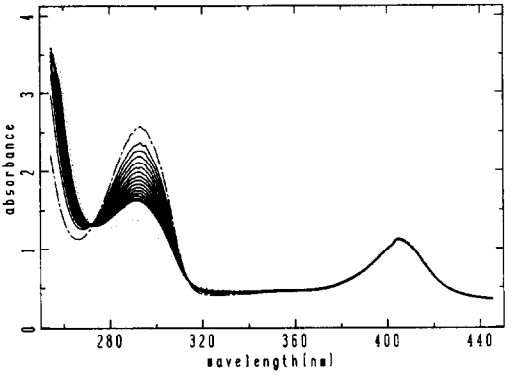


Fig. 6. Spectral changes occurring during 1^+Cl^- catalyzed oxidation of hydroquinone by MCPBA. ---, a solution of 1×10^{-3} M hydroquinone and 8×10^{-6} M 1^+Cl^- in CH_3OH; —, spectral changes from 3 to 45 sec at 3-sec intervals upon addition of 8.8×10^{-4} perlauric acid; ···, final spectrum after 120 sec.

Table 2. The oxidation of DPPH by MCPBA catalyzed by 1^+Cl^- in CH_3OH [DPPH] = 1.1×10^{-3} M; T = 29°

$[1^+Cl^-]$ $\times 10^6$ M	[MCPBA] $\times 10^4$ M	k_{obs}[a] (sec^{-1})	$k_{obs}1/k_{obs}2$[b]	k_1 (M^{-1} sec^{-1})[c] $\times 10^{-4}$
2.2	1.04	0.0215	1.03	0.98 ± 0.04
2.2	0.52	0.0307	1.14	1.39 ± 0.06
2.2	1.04	0.0225	0.965	1.02 ± 0.05
2.9	1.04	0.0263	1.01	.91 ± 0.03
3.6	1.04	0.0367	1.02	1.01 ± 0.03

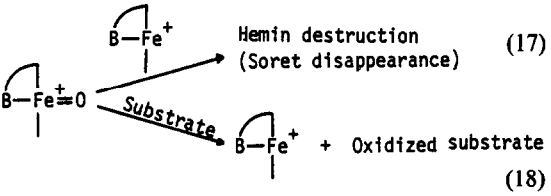
[a]The k_{obs} is the average k_{obs} for the first two half-lives. [b]The ratio of the k_{obs} for the first half-life over the k_{obs} for the second half-life. [c]The second order rate constant $k_1 = k_{obs}/[1^+Cl^-]$.

Table 3. The oxidation of hydroquinone by MCPBA catalyzed by 1^+Cl^- in CH_3OH. [Hydroquinone] = 10^{-3} M; T = 25°

$[1^+Cl^-]$ $\times 10^6$ M	[MCPBA] $\times 10^4$ M	k_{obs} (sec^{-1}) $\times 10^2$	k_1 (M^{-1} sec^{-1}) $\times 10^{-3}$
2.44	2.45	2.12	8.7 ± .1
2.44	4.9	1.53	6.3 ± .1
2.64	2.45	1.69	6.4 ± .1
2.64	4.9	1.95	7.4 ± .1
4.25	4.9	2.76	6.5 ± .1
4.25	4.9	2.67	6.28 ± .02
4.68	4.9	3.13	6.69 ± .04
4.68	4.9	2.29	6.40 ± .05
5.16	2.45	3.29	6.4 ± .1
5.16	2.45	3.46	6.7 ± .1
5.16	2.45	3.11	6.0 ± .1
5.16	2.45	2.94	5.7 ± .1
7.55	4.9	4.72	6.2 ± .1
7.55	2.45	4.50	6.0 ± .1

The second-order rate constants, k_1, for TBPH, ZnTPP, DPPH and hydroquinone oxidations are summarized in Table 4. Within experimental error they are all identical and are consistent with a rate-limiting formation of the oxidizing intermediate.

Substrate activity. Since the reaction of substrates with the intermediate occurs after the rate-limiting step, kinetic data concerning the reactivity can only be obtained via competitive methods. Competition between hemin destruction and substrate oxidation is a useful measure of substrate reactivity.

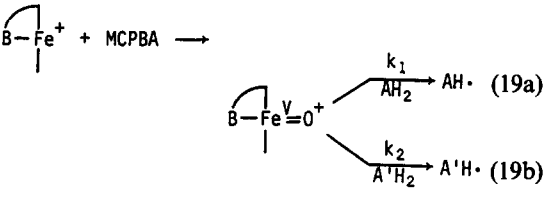


With rapidly reacting substrates such as phenols and aromatic amines, there is no heme destruction and oxidized product can be detected. Slowly reacting substrates do not compete with hemin destruction and the Soret band of the hemin disappears as soon as the peracid is added (i.e. within seconds). When this occurs, no oxidized product is found.

m-Chloroperbenzoic acid ($\sim 2.5 \times 10^{-4}$ M) was added to solutions containing the potential substrate ($\geq 10^{-2}$ M) and 10^{-5} M 1^+Cl^-. Those substrates which protect the hemin from destruction are considered oxidizable by the intermediate complex. Table 5 lists the 28 compounds tested, along with their $E_{1/2}$ values and selected bond dissociation energies.

Isotope effects

Isotope effects and relative rates may also be investigated using the following scheme.



In most cases exchange between the products made this method impractical. For example, oxidation of a mixture of zinc tetraphenylporphyrin and TBPH resulted in a mixture of the two radicals. But the zinc tetraphenylporphyrin cation radical oxidized TBPH about as rapidly as did the intermediate itself and an equilibrium mixture of the four components was obtained.

The one competing process which did not interfere with the formed phenoxyl radicals or produce them in a subsequent step seemed to be hemin destruction. At low phenol concentrations hemin destruction is observed upon addition of MCPBA and this destruction resulted in iron products which are incapable of catalyzing TBPH oxidation. Protection of the hemin against destruction is provided by TBPH in the reaction which produced the phenoxyl radical. The extent of this protection can then be used to assess the relative rates of phenoxyl radical production and heme destruction. Because the rate of phenoxyl radical production is independent of TBPH concentration, but first order in 1^+Cl^- concentration, we use the total integrated amount of phenoxyl radical in a process in which 1^+Cl^- is completely destroyed and phenoxyl radical production stops. At higher phenol concentrations, the lifetime of 1^+Cl^- is extended and more phenoxyl radical is produced.

The amounts of phenoxyl radical produced at 1.7×10^{-3} M MCPBA, 1×10^{-5} M 1^+Cl^- as a function of TBPH concentration in methylene chloride are shown in Fig. 7 as ΔOD at 630 nm, a maximum for the phenoxyl radical. Above 10^{-1} M TBPH all of the peracid is used to produce the phenoxyl radical and the hemin is not destroyed. Below 0.1 M TBPH the heme is destroyed while producing amounts of phenoxyl radical which depends upon TBPH concentration. The observation that the O-deuterated TBPH gave essentially identical results at all concentrations of phenol indicates an absence of an isotope effect. A primary effect of 5 would have the same effect as changing the concentration by a factor of 5 and would be easily observable.

Competition between 1,4-cyclohexadiene and TBPH. We have attempted to observe the competition of 1,4-cyclohexadiene and TBPH for our intermediate by using perlauric acid as the oxidant. In CH_3OH, perlauric acid was found to be unreactive towards 1,4-cyclohexadiene under the conditions of our kinetic experiments. This was determined by preparaing a solution of perlauric acid and cyclohexadiene in CD_3OD and monitoring the NMR spectrum. The solution was stable for at least 35 min at room temperature. The rate constant for the direct reaction between phenoxyl radical and cyclohexadiene was found to be $2.8 \times 10^{-2}\,M^{-1}\,sec^{-1}$, therefore making it possible to carry out competitive experiments at fixed TBPH and varying concentrations of cyclohexadiene and observing the yield of phenoxyl radical. Figure 8 shows the ΔOD for phenoxyl radical production at different cyclo-

Table 4. Rate constants (k_1) obtained from the oxidation of selected substrates by MCPBA catalyzed by 1^+Cl^- in CH_3OH

Substrate	k_1 (M^{-1} sec^{-1}) × 10^{-3}
2,4,6-tri-t-butylphenol[a]	7.6 ± .2
Zn tetraphenylporphyrin[b]	8.2 ± .7
Diphenylpicrylhydrazine[c]	11 ± 2
Hydroquinone[a]	6.5 ± .7
Hydroquinone[c]	9.9 ± .1

[a] T = 25°. [b] T = 28°. [c] T = 29°.

Table 5. Substrate reactivity towards the intermediate formed from 1^+Cl^- and MCPBA

Substrate[a]	$E_{\frac{1}{2}}$ (V)[b] vs. SCE	BDE[c] (kcal/mole)	Activity[d]
Mesidine			+
Aniline	0.625[e]		+
p-Methoxyphenol	0.75[e]	84.0[f]	+
Guaiacol	0.8[e]		+
Hydroquinone	0.537[g]	84.6[f]	+
4-t-Butylphenol	0.578[e]	86.5[f]	+
Phenol	0.633	88.3[f]	
Dimethylaniline	0.680		+
Tri-t-butylphenol	.984[h]	81.6[i]	+
$DPPH_2$	0.77		+
ZnTPP[j]	0.71		+
CuTPP[j]	0.90		+
Pyrene	1.16		+
m-Cyanophenol			partial
p-Nitrophenol	.924		-
Thianthrene	1.05		-
Phenanthrene	1.50		-
trans-Stilbene	1.51		-
Dimethylsulfoxide	>2.1		-
Anisole	1.76[e]		-
Cumene	2.18[e]		-
Toluene	1.98	85	-
1,3-Cyclohexadiene		70.1	-
1,4-Cyclohexadiene		69.8	-
Cyclooctene			-
Cyclohexene	1.94[e]		-
Triphenylmethane			-
Cyclohexane	>2.4[e]		-

[a]Substrate concentrations are $\geq 10^{-2}$ M. [b]Values are taken from from C. K. Mann and K. K. Barnes, "Electrochemical Reactions in Nonaqueous Systems," Marcel Dekker, New York, 1970, unless otherwise noted. [c]BDE for the appropriate CH bonds were taken from Ref. 61. [d]+ Designates substrate protects 1^+Cl^- at ~10^5 M with addition of 2×10^{-4} M MCPBA. - Designates hemin is destroyed. [e]L. Meites and P. Zuman, "Electrochemical Data," part I, John Wiley and Sons, New York, 1974. [f]L. R. Mahoney and M. A. DaRooge, J. Am. Chem. Soc. 97, 4722 (1975). [g]S. D. Ross, M. Finkelstein and E. Rudd, "Anodic Oxidation," Academic Press, New York, 1975. [h]I. M. Sosonkin, V. A. Subbotin and G. N. Strogor, Zh. Obshch. Khim. 44, 1215 (1973). [i]Ref. 62. [j]R. H. Felton, in "The Porphyrins," ed. D. Dolphin, Vol. V, Academic Press, New York, 1978.

hexadiene/TBPH ratios. At high ratios, the direct reaction between phenoxyl radicals and cyclohexadiene decreased the yield of phenoxyl radical. Figure 9 shows a plot of the ΔOD for phenoxyl radical formation vs time. The phenoxyl radical absorption at 400 nm is seen to increase rapidly and then decay more slowly, both first-order processes. The rate of formation of the phenoxyl radical is equal to the rate in the absence of cyclohexadiene and the decay rate constant is equal to that separately determined for the reaction of cyclohexadiene with the phenoxyl radical.

By increasing the 1,4-cyclohexadiene concentration to 1.9 M at a 1^+Cl^- concentration of $\sim 5 \times 10^{-6}$ M and a tri-t-butylphenol concentration of 10^{-2} M, we have observed the catalytic oxidation of 1,4-cyclohexadiene with no visible build-up of phenoxyl radical. In separate experiments, analysis of the reac-

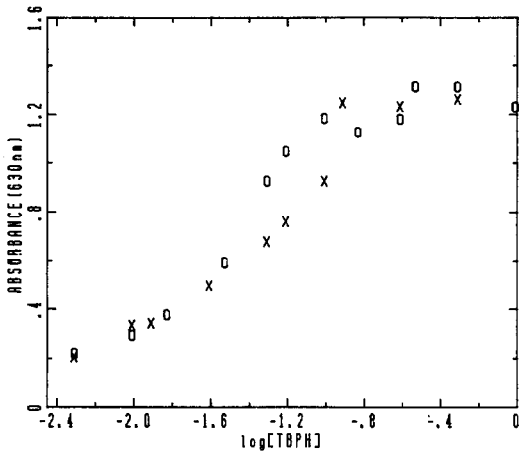


Fig. 7. The absorbance change at 630 nm due to the production of phenoxyl radical as a function of [TBPH] in CH_2Cl_2. [MCPBA] = 1.7×10^{-3} M; [1^+Cl^-] = 1×10^{-5} M; T = 25°. O, Proto; ×, deuterio.

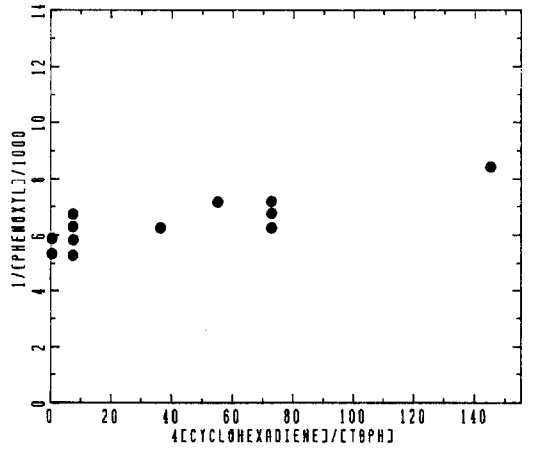


Fig. 8. The production of phenoxyl radical as a function of [cyclohexadiene] in CH_3OH. [TBPH] = 5.5×10^{-3} M; [perlauric acid] = 1.4×10^{-4} M; [1^+Cl^-] = 1.28×10^{-5} M; T = 25°.

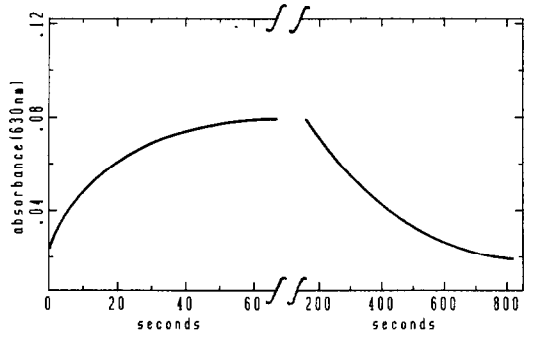
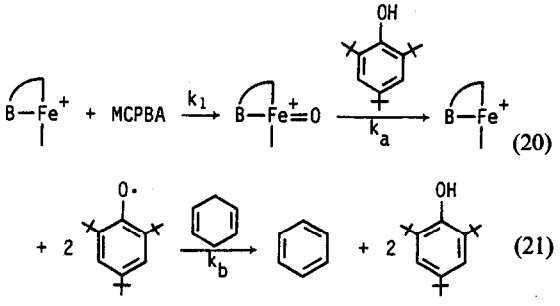


Fig. 9. The absorbance change at 630 nm vs time due to tri-t-butyl-phenoxyl radical in CH_3OH upon addition of perlauric acid to a solution of TBPH, 1^+Cl^- and 1,4-cyclohexadiene. The phenoxyl radical is slowly quenched by the cyclohexadiene. [1^+Cl^-] = 1.2×10^{-5} M; [TBPH] = 5.5×10^{-3} M; [perlauric acid] = 1.4×10^{-4} M; [cyclohexadiene] = 10^{-1} M; T = 25°.

tion mixture by GC showed quantitative formation of benzene. The reaction scheme is outlined below.



DISCUSSION

Direct kinetic studies of the reactions of our intermediaties by analogy with HRP methods[5] is not currently possible with simple hemins, owing to the instability of the higher oxidation state intermediaties. This instability may include a coproportionation reaction to give an Fe(IV) dimer, via an inner-sphere process, or a coproportionation to give two Fe(IV) species via an outer-sphere process. Either process is expected to be rapid ($ZnTPP^{2+}$ + $ZnTPP \xrightarrow{k} 2ZnTPP^+$, $k = 10^{10}\ M^{-1}\ sec^{-1}$).[54] Jones has suggested that his intermediate formed from deuterohemin and hydrogen peroxide is the dimer species.[55]

$$Hm^+{=}O + Hm^+ \rightarrow Hm^+OHm^+. \quad (22)$$

Subsequently or in a separate step, rapid destruction of hemin also occurs.

$$Hm^+{=}O + Hm \rightarrow \text{loss of } Hm^+ \text{ spectrum.} \quad (23)$$

By using reactive substrates, such as phenols, we have avoided both of these problems. At phenol concentrations of 10^{-3} M to 10^{-1} M, depending upon solvent, hemin destruction is eliminated. Furthermore, assuming a rate constant of $> 10^6\ M^{-1}\ sec^{-1}$ for phenol oxidation,[56] the rate of dimerization at 5 μM hemin concentration would have to greater than the diffusion-controlled rate to compete with the reaction of $Hm^+{=}O$ with 10^{-3}–10^{-1} M phenol. At such concentrations the formation of $Hm^+{=}O$ is rate-limiting (eqns 24–26).

$$RCO_3H + Hm^+ \xrightarrow{\text{slow}} RCO_2H + Hm^+{=}O \quad (24)$$

$$Hm^+{=}O + PhOH \xrightarrow{\text{fast}} Hm{=}O + PhO^{\cdot} + H^+ \quad (25)$$

$$Hm{=}O + PhOH \xrightarrow{\text{fast}} HmOH + PhO \quad (26)$$

Therefore, in order to compare reactivities of substrates toward $Hm^+{=}O$, competitive processes were employed.

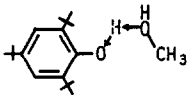
Types of reactive substrates. In order for a substrate to be studied in our system, it must compete with reaction 23, and for O transfer, probably with reaction 22 as well. We thought it instructive to determine the nature of substrates which are

sufficiently reactive to protect the hemin by competing with reaction 23. With reactive substrates present, the hemin is left unchanged through many turnovers (up to 50,000)—thus the hemin has been protected. As a general test for an upper limit of reactivity of a substrate, at least 0.01 M substrate was exposed to an MCPBA concentration of 5×10^{-4} M and 1^+Cl^- concentration of about 10^{-5} M. If the hemin was immediately destroyed the substrate was considered unreactive. The test has a number of limitations: (1) the rate of reaction of the intermediate with a substrate must be faster than reaction with 10^{-5} M hemin; (2) partial protection of the hemin is difficult to interpret; (3) direct reactions between MCPBA and high concentrations of substrate occur. Table 5 lists our results along with the $E_{1/2}$ and the bond dissociation energies for some of the reagents. These results reveal the qualitative reactivity of our intermediate. Our intermediate will oxidize substrates whose $E_{1/2}$ value is ~ 1 V or less. Substrate activity appears to be entirely independent of the bond dissociation energy. Rapidly epoxidized substrates (i.e. phenanthrene) are unreactive. These results all point to electron transfer as the mechanism for oxidation in our system and are in agreement with the available data for HRP. A limiting value of 1 V for the $E_{1/2}$ of a reagent is in accord with the published data for the $E_{1/2}$ of compound I and compound II of HRP (I, 0.92 V; II, 0.94 V).[57] The results of Job and Dunford[56] and Shiga and Imaizumi,[18] in which the substituents on phenol were varied, led both groups to conclude that the rate of oxidation is dependent only on the $E_{1/2}$ of the substrate. Like our model system, *p*-nitrophenol will not reduce compound I of HRP.[18] The model shows a pattern of reactivity that closely matches HRP; the main difference between HRP and the model is the greater stability of the oxidizing intermediaties of HRP. This is most certainly due to the encapsulation of the hemin by the protein.

Isotope effects. Although the survey of substrates suggests electron transfer as the reaction pathway under our conditions, abstraction of H-atoms from phenols was not excluded. Two pathways, an electron transfer from the phenol (27) or hydrogen abstraction (28) could be envisioned. Reaction 27 should have no primary isotope effect whereas reaction 28 should show the usual k_H/k_D for hydrogen-atom abstraction from hindered phenols of 10–15.[58]

Fixed concentations of 1^+Cl^- were added to solutions of a fixed concentration of peracid and varying concentrations of either the phenol TBPH or deuterated phenol. A plot of the concentration of phenoxyl radical which developed against the concentration of phenol is shown in Fig. 9. The two phenols gave similar results, indicating that the deuterated phenol competes with hemin destruction just as well as does the phenol; thus $k_H/k_D \cong 1$. This can be taken as strong evidence for electron transfer before hydrogen removal.

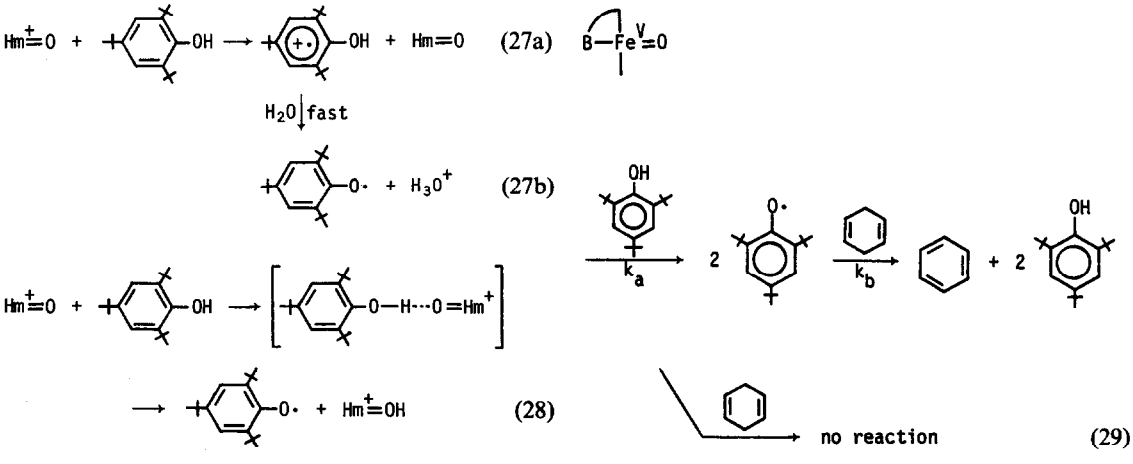
The lower concentrations of phenol required to protect the heme in methanol than in methylene chloride suggests that H-bonding to the phenol serves to lower its oxidation potential.



A comparison of the gas–phase ionization potentials of anisole and phenol (8.22 eV, 8.50 eV)[59] vs their respective $E_{1/2}$ values in solution (1.76 V, 0.62 V)[60] reveals a similar trend. H-atom bonding to the phenol and solvation of the proton lower its $E_{1/2}$ value relative to anisole. The $E_{1/2}$ value is not for the ionized phenol (~ 0.2 V).

Competition between 1,4-cyclohexadiene and TBPH. As another test for H-atom abstraction, we competed cyclohexadiene against TBPH for our intermediate. The bond dissociation energy for the C–H bond is significantly lower than that for the OH bond, 69 kcal/mole[61] and 81.6 kcal/mole,[62] respectively.

The results show that the yield of phenoxyl radical is unaffected by cyclohexadiene except at high concentrations where the direct reaction between phenoxyl radical and cyclohexadiene becomes significant. This is further evidence against a hydrogen–atom abstraction mechanism. The direct reaction of phenoxyl radical with cyclohexadiene produced quantitative yields of benzene by GC analysis. Interestingly, by increasing the cyclohexadiene concentration to about 1 M and decreasing the concentration of 1^+Cl^- we were able to catalyze the oxidation of 1,4-cyclohexadiene to benzene without ever building up detectable amounts of phenoxyl radical.



Model reactivity vs peroxidase reactivity. In our earlier work we were able to evaluate parameters which have been proposed to control the rate of compound I formation in the peroxidases.[39] If we were to likewise interpret the reactivity of the peroxidase intermediates based on a model study, it was necessary that our models have similar reactivity. Our model, in fact, has almost identical reactivity towards substrates as peroxidase: (1) oxidation by sequential one-electron steps, (2) correct stoichiometry, (3) approximately the same substrate oxidizing potential requirement, (4) identical substrate specificity, (5) no evidence for hydrogen-atom abstraction or O-atom transfer. The major difference between our model system and the proteins is that the protein intermediates are stable in the absence of substrates whereas in the model, destruction of hemin occurs, although in the absence of a substrate, compound I will decay to compound II, presumably by oxidation of the protein.[63]

From our competitive experiments with substrates vs hemin destruction and the lack of an isotope effect, we suggest the mechanism of oxidation by our intermediate is consistent with an outer-sphere electron-transfer pathway. We also believe this to be the case with the peroxidases. In the oxidation of substrate by HRP, it does not seem necessary, or even likely in some cases, that the substrate enters into the enzyme pocket. In the formation of compound I the reaction of t-butylhydroperoxide with HRP is 8 orders of magnitude slower than methyl hydroperoxide primarily due to steric problems.[64] A survey of the substrates oxidized by compounds I and II reveals many of these to be substantially larger than t-butylhydroperoxide and their rates of oxidation are not correlated to size (i.e. $K_4Fe(CN)_6$, $k = 7.5 \times 10^6\ M^{-1}\ sec^{-1}$; phenol, $k = 2.8 \times 10^6\ M^{-1}\ sec^{-1}$).[56,65] It has also been shown that HRP can be oxidized to compound I by K_2IrCl_6.[66,67] Clearly this species would have difficulty entering the enzymatic pocket and therefore it may oxidize the hemin via the porphyrin edge, near the surface of the protein. The known porphyrin π cation radical structure of compound I would be well suited for an electron–transfer process at the protein surface.

Marcus theory treatment of HRP. We have proposed that the principle mode of reactivity of the intermediate in our studies and HRP is outer-sphere electron transfer. If this is true for HRP, the available kinetic data for substrate oxidations by compounds I and II should be interpretable in terms of Marcus theory.

A large body of data concerning electron–transfer reactivity of metalloproteins has been reported in recent years.[68] In particular, the redox chemistry of the Fe(II) and Fe(III) states of cytochrome c have been extensively investigated with a variety of small redox agents.[69] The predominant mode of electron transfer in cytochrome c is believed to be an outer-sphere process via the exposed porphyrin periphery. The kinetic data for such reactions are interpreted in terms of Marcus theory.[69,70] The analysis is based on the principle that calculated self-exchange rates for the protein should be invariant to the nature of the substrate if electron transfer proceeds by the same mechanism in cross reactions as it does in its self-exchange reaction. Deviations from constancy are then interpreted in terms of (1) protein-substrate interactions that are not canceled by interaction in the self exchange of substrate, or (2) differences in the activation processes for the cross reactions compared to the respective self-exchange processes.[71] Corrections for electrostatic work terms have been applied for cytochrome c.[72] Inaccessibility of the active site has been deduced for other metalloproteins.[73,74] The advantage of applying Marcus theory to the two successive one-electron processes of HRP is that many of the complicating factors drop out by virtue of the fact that the same protein, assuming only minor structural changes, is involved in both steps.

For HRP the rate of the cross reactions, eqn (30) and (31), are related to the self-exchange reactions for compounds I (eqn 32) and II (eqn 33), and the substrate (eqn 34) by eqns (35) and (36), respectively (S = substrate).

$$HRP(I) + S_{red} \underset{}{\overset{k_{I,S}}{\rightleftharpoons}} HRP(II) + S_{ox} \quad (30)$$

$$HRP(II) + S_{red} \overset{k_{II,S}}{\rightleftharpoons} HRP + S_{ox} \quad (31)$$

$$HRP(II) + HRP(I) \xrightarrow{k_{I,I}} HRP(I) + HRP(II) \quad (32)$$

$$HRP + HRP(II) \xrightarrow{k_{II,II}} HRP(II) + HRP \quad (33)$$

$$S_{ox} + S_{red} \overset{k_{SS}}{\rightleftharpoons} S_{red} + S_{ox} \quad (34)$$

$$2 \log k_{I,S} = \log k_{I,I} + \log k_{SS} + 16.95(E_I^\circ - E_S^\circ) \quad (35)$$

$$2 \log k_{II,S} = \log k_{II,II} + \log k_{SS} + 16.95(E_{II}^\circ - E_S^\circ) \quad (36)$$

$$\log k_{I,S}/k_{II,S} = \frac{1}{2}(\log k_{I,I}/k_{II,II}) + 8.47(E_I^\circ - E_{II}^\circ). \quad (37)$$

The ratio of the rates of reaction of substrate with compounds I and II (eqn 37), then, depends only on the difference in self-exchange rates of HRP(I) and HRP(II) and $E_I^\circ - E_{II}^\circ$. If all substrates react at the same site via outer-sphere electron transfer, $\log k_{I,S}/k_{II,S}$ should be a constant. In fact, for the substrates listed in Table 6, this is generally the case. The average ratio ≈ 40 is consistent with a potential difference of 0.2 V if $k_{I,I} = k_{II,II}$. Based on the fact that a spin change occurs in going from compound II to HRP resting state, one might expect $k_{II,II}$ to be smaller. If this is the case E° would have to be smaller than 0.2 V based on the above analysis.

The relative magnitude of the self-exchange terms $k_{I,I}$ and $k_{II,II}$ will depend on possible differences in protein structure in solution and structural differences in the porphyrin and iron–ligand bonds in the different oxidation states. Differences in the protein structure are impossible to assess. However, there exists a considerable amount of data on structural differences in metalloporphyrins as a result of oxidation and spin-state differences.[75,76] In simple metal complexes spin-state changes have much larger structural effects than oxidation state differences involving only t_{2g} electrons.[77] In hemes, these effects are typically manifested in much longer iron–axial ligand bond lengths in high-spin complexes compared to low-spin systems. The effects of oxidation in the

Table 6. Rates of reaction of HRP(I) and HRP(II) with substrates

Substrate	pH	$k_{I \to II}$	$k_{II \to HRP}$	$\frac{k_{I \to II}}{k_{II \to HRP}}$
p-Aminobenzoic acid[a]	5.4	5×10^4	1.3×10^3	38
Luminol[b]	8	2.3×10^6	7.2×10^4	32
Nitrite[a]	5.4	2×10^5	2.4×10^3	83
Ferrocyanide[c]	5.0	7.5×10^6	2.1×10^5	36
	5.9	1.9×10^6	4.4×10^4	43
	6.9	9.1×10^5	1.8×10^4	51
p-Cresol[d,e]	6.83	-	1.07×10^6	
	7.0	4.2×10^7	-	39
Resorcinol[e,f]	7.0	8.0×10^6	3×10^5	27
Guaiacol[g]	7	9×10^6	3×10^5	30
Ascorbic Acid[d]	4.26	2.3×10^6	2.6×10^5	9
I^- [h,i]	5.4	1.8×10^5	4.5×10^2	400
	6.0	5.6×10^4	1.2×10^2	466

[a]B. Chance, Arch. Biochem. Biophys. 41, 416 (1952). [b]M. J. Cormier and J. Prichard, J. Biol. Chem. 243, 4706 (1968). [c]M. L. Cotton and H. B. Dunford, Can. J. Chem. 51, 582 (1973). [d]J. E. Critchlow and H. B. Dunford, J. Biol. Chem. 247, 3714 (1972). [e]Ref. 56. [f]B. Chance, Adv. Enzymol. 12, 153 (1951). [g]I. Yamazaki and K. Yokota, Mol. Cell. Biochem. 2, 39 (1973). [h]P. Roman, H. B. Dunford and M. Evett, Can. J. Chem. 49, 3059 (1971). [i]P. Roman and H. B. Dunford, Biochemistry 11, 2076 (1972).

porphyrin ring, while not known for hemes, may be reasonably estimated based on known structural data for the zinc tetraphenylporphyrin cation radical.[78] This radical, containing a coordinated perchlorate in one axial site, shows little if any structural differences from a normal five-coordinated zinc porphyrin. Thus, we would not expect a metalloporphyrin cation radical to show significantly different electron–transfer rates than those of a higher oxidation level metalloporphyrin involving a t_{2g} transfer without a spin charge. In the case of HRP where we assume HRP(I) is a low-spin Fe(IV) porphyrin cation radical, HRP(II) is a low-spin Fe(IV) porphyrin and HRP in its resting state is a high-spin Fe(III), $k_{I,I}$ is expected to be faster than $k_{II,II}$ owing to the Franck–Condon barrier associated with the spin change in going to the resting state of the protein. This feature takes on added significance in view of the recent report that the oxidation level of compound I of HRP is actually about 50 mV *lower* than that of compound II at acidic or neutral pH.[57] The slower rate of oxidation of substrates by compound II may, in part, be due to the larger Franck–Condon barrier associated with the spin change. A 0.1 Å change in the iron histidine bond length in going from high-spin HRP to low-spin compound II is expected. This, coupled with a 0.5 Å movement of the iron into the plane and a slight shortening of Fe–N in-plane distances, could easily offset the 50 mV greater oxidation potential of compound II and explain the slower rates of substrate oxidation by compound II.

While the Marcus treatment does involve a number of assumptions, the relative constancy of $k_{I,S}/k_{II,S}$ with a variety of different substrates provides support for our suggested outer-sphere transfer via the heme periphery in HRP. This reactivity pattern is consistent with an Fe(IV) porphyrin radical cation formulation reacting nonspecifically via electron transfer through the heme edge.

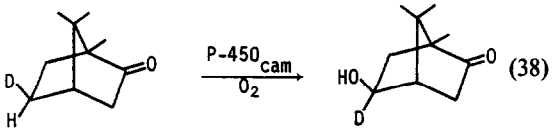
Relevance to cytochrome P-450. In examining the various reactions which cytochrome P-450 is able to carry out and the mechanistic studies concerning these reactions, it becomes apparent that this enzyme can react by H-atom abstraction,[79] O-atom transfer,[80] or electron transfer.[81] There are stereochemical and isotope effect studies strongly supporting H-atom abstraction by P-450. Stereochemical evidence favors direct O-atom transfer,[80] although evidence has been presented indicating a radical epoxidation as well.[82] Both isotope effects[79] and electron-spin resonance studies favor electron transfer from thioethers and arylamines to P-450.[81,83]

Cytochrome P-450 seems to be very capable of acting as a peroxidase in certain low potential oxidations, but HRP has never been shown to function as an oxygenase. We believe that the major difference between these two proteins is access to the active site by the substrate. We have suggested that all HRP oxidations occur via an outer-sphere electron–transfer pathway and that substrates do not enter into the active site. With P-450, substrate binding inside the active site is an integral part of the catalytic cycle.[84,85] Because of facile electron–transfer reactions of high oxidation state hemes, the binding of a substrate in the vicinity of the active site is a reasonable mechanism to make use of the high oxidation state intermediate before it is short-circutited via an outer-sphere process. The reactivity of the per-

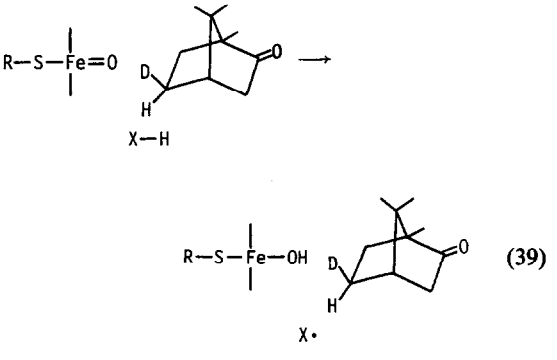
oxidases and P-450 may be readily understood in terms of this concept.

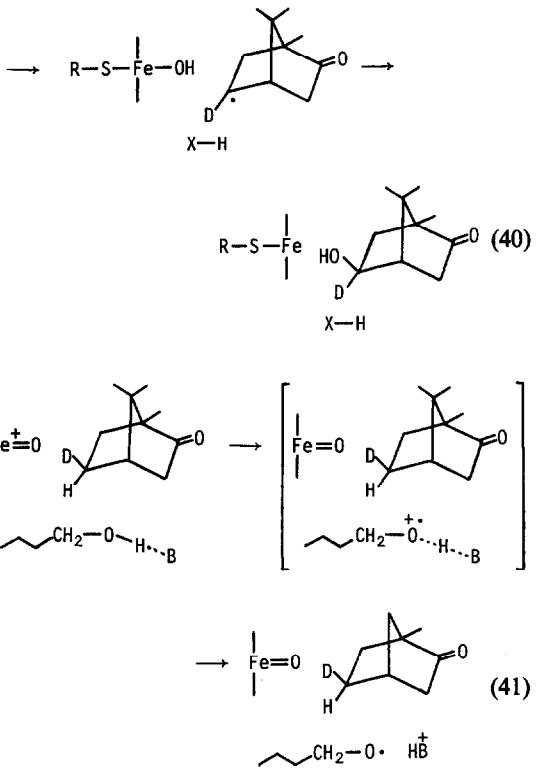
It should not be concluded that the intermediate in P-450 reactions and compound I of the peroxidases are identical. Recent experiments with electron-deficient, tetrapentafluorophenyl-,[37] and electron-rich, tetra-*p*-methoxylphenylhemin[38] have revealed different ratios and yields of hydrogen abstraction. It has been suggested that presence of an axial thiolate ligand in P-450 should serve to reduce a porphyrin cation radical structure, thereby differentiating it from compound I of the peroxidases and that this somehow makes it a better O-transfer reagent.[86] On the other hand, the high mobility of the oxidizing equivalent in a porphyrin cation radical may be essential to HRPs function as an outer-sphere electron-transfer reagent. Further model studies should help clarify what differentiates a high oxidation state heme into being an electron-transfer agent or an oxygen-atom transfer reagent.

The rather nonstereospecific hydroxylation of norbornane by liver microsome P-450 provided strong evidence for a two-step oxidation process.[79] More recently, the stereospecific oxidation of camphor to exo-5-hydroxy camphor by bacterial P-450 was shown to involve, at least in part, hydrogen-atom abstraction from the endo site.[87]



Since free radicals at the 5 position collapse to give both *exo* and *endo* products,[88] this was offered as evidence for a remote free radical. This poses the question of the mechanism for formation of X·, which must be capable of abstracting H from a ~95 kcal/mole C–H bond. Our electron-transfer, proton-loss, abstraction mechanism for 1,4-cyclohexadiene oxidation presents a possible pathway. However, a phenoxyl radical such as we have prepared will not carry out the desired abstraction. As a tentative scheme, we suggest that the hydrogen bonding which plays an important role in phenoxyl radical production might be effective in catalyzing the formation of an alkoxyl·or other reactive O or N radical in the process (41) (B could be imidazole).[89] Thus an electron-transfer process could lead to hydrogen abstraction in an electron-transfer, deprotonation, abstraction (ETDA) process.





CONCLUSION

Our studies of the oxidation of various substrates, using the compound I analog of HRP derived from chelated protoheim chloride, indicate that it acts in a similar fashion to HRP, as an electron-transfer reagent. The mechanism of phenol oxidation is concluded to be an outer-sphere electron-transfer process. Likewise, from a Marcus theory treatment of available data for HRP oxidations, we suggest that compounds I and II of HRP oxidize substrates by an outer-sphere electron-transfer pathway. An indirect peracid oxidation of cyclohexadiene to benzene through an intermediate phenoxyl radical is described and its similarity to proposed indirect oxidation of substrates in P-450 noted. Possible biological implications of this ETDA sequence have been suggested.

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REFERENCES

[1]T. E. King, S. Mason and M. Morrison, *Oxidases and Related Redox Systems*. Vol. 1, University Park Press, Baltimore, Maryland (1973).
[2]Osamu Hayaishi, *Molecular Mechanisms of Oxygen Activation*. Academic Press, New York (1974).
[3]R. J. Sundberg and R. B. Martin, *Chem. Rev.* **74**, 471 (1974).
[4]E. Antonini and M. Brunori, *Hemoglobin and Myoglobin in Their Reactions with Ligands*. North Holland, Amsterdam (1971).
[5]H. B. Dunford and J. S. Stillman, *Coord. Chem. Rev.* **19**, 187 (1976).
[6]R. E. White and M. J. Coon, *Ann. Rev. Biochem.* **49**, 315 (1980).

[7]D. F. Wilson and M. Erecinska, *The Porphyrins* (Edited by D. Dolphin), Vol. VII, pp. 2–70. Academic Press, New York (1978).
[8]B. Chance, *Arch. Biochem. Biophys.* **37**, 235 (1952).
[9]G. O. Nordblom, R. E. White and M. J. Coon, *Ibid.* **175**, 524 (1976).
[10]B. W. Griffin, J. A. Peterson and R. W. Eastabrook, *The Porphyrins* (Edited by D. Dolphin), Vol. VII pp. 333–377. Academic Press, New York (1978).
[11]G. Hamilton, *Molecular Mechanisms of Oxygen Activation* (Edited by O. Hayaishi), pp. 405–451. Academic Press, New York (1974).
[12]A. Brill and H. Sanberg, *Biochemistry* **7**, 4254 (1968).
[13]J. O. Stern and J. Peisach, *J. Biol. Chem.* **249**, 7495 (1974).
[14]P. George, *Adv. Catal.* **4**, 367 (1952).
[15]J. T. Groves and G. A. McClusky, *J. Am. Chem. Soc.* **98**, 859 (1976).
[16]B. Chance, *Arch. Biochem. Biophys.* **21**, 416 (1949).
[17]B. Chance, *Science* **109**, 204 (1949).
[18]T. Shiga and K. Imaizumi, *Arch. Biochem. Biophys.* **167**, 469 (1975).
[19]D. Dolphin, *Israel J. Chem.* **21**, 67 (1981).
[20]Y. Maeda and Y. Morita, *Biochim. Biophys. Res. Commun.* **29**, 680 (1967).
[21]T. H. Moss, A. Ehrenberg ad A. J. Bearden, *Biochemistry* **8**, 4159 (1969).
[22]A. Ehrenberg, *Sr. Kem. Tidskr.* **74**, 103 (1962).
[23]W. E. Blumberg, J. Peisach, B. A. Wittenberg and J. B. Wittenberg, *J. Biol. Chem.* **243**, 1854 (1968).
[24]J. T. Groves, W. J. Kruper, T. E. Nemo and R. S. Myers, *J. Mol. Catal.* **7**, 169 (1980).
[25]I. Tabushi and N. Koga, *Adv. Chem. Ser.* **191**, 291 (1980).
[26]C. K. Chang and D. Dolphin, *Bioorganic Chemistry* (Edited by E. E. van Tamelen), Vol. IV, pp. 37–80. Academic Press, New York (1978).
[27]T. G. Traylor, M. J. Mitchell, S. Tsuchiya, D. H. Campbell, D. V. Stynes and N. Koga, *J. Am. Chem. Soc.* **103**, 5234 (1981).
[28]J. P. Collman, R. R. Gagne, C. A. Reed, T. R. Halbert, G. Lang and W. T. Robison, *Ibid* **97**, 1427 (1975).
[29]C. K. Chang and M.-S. Kuo, *Ibid.* **101**, 3413 (1979).
[30]D. Chin, A. Balch and G. LaMar, *Ibid.* **102**, 1446 (1980).
[31]P. Shannon and T. C. Bruice, *Ibid.* **103**, 4580 (1981).
[32]J. T. Groves, R. C. Haushalter, M. Nakamura, T. E. Nemo and B. J. Evans, *Ibid.* **103**, 2884 (1981).
[33]C. L. Hill and B. C. Schardt, *Ibid.* **102**, 6374 (1980).
[34]J. T. Groves, T. E. Nemo and R. S. Myers, *Ibid.* **101**, 1032 (1979).
[35]A. Gold, W. Ivey and M. Brown, *J. Chem. Soc.* Chem. Commun. **293**, (1981).
[36]C. K. Chang and F. Ebina, *Ibid.* 778 (1981).
[37]T. G. Traylor, K. Nolan and R. Hildreth, *J. Am. Chem. Soc.* in press.
[38]I. Tabushi and A. Yazaki, *Ibid.* **103**, 7371 (1981).
[39]T. G. Traylor, W. A. Lee and D. V. Stynes, *Ibid*, in press.
[40]M. Morrison and G. R. Schonbaum, *Ann. Rev. Biochem.* **45**, 861 (1976).
[41]T. L. Poulos and J. Kraut, *J. Biol. Chem.* **225**, 8199 (1980).
[42]T. G. Traylor, C. K. Chang, J. Geibel, A. Berzinis, T. Mincey and J. Cannon, *J. Am. Chem. Soc.* **101**, 6716 (1979).
[43]R. D. Mair and A. Grawpner, *J. Anal. Chem.* **36**, 194 (1964).
[44]N. N. Schwartz and J. H. Blumbergs, *J. Org. Chem.* **29**, 1976 (1964).
[45]W. E. Parker, C. Ricciuti, C. L. Ogg and D. Swern, *J. Am. Chem. Soc.* **77**, 4037 (1955).
[46]J. H. Fuhrhop and K. Smith, *Porphyrins and Metalloporphyrins* (Edited by K. Smith), pp. 757–869. Elsevier, Amsterdam (1975).
[47]P. B. Ayscargh and K. E. Russell, *Can. J. Chem.* **43**, 3039 (1965).
[48]T. G. Traylor, D. Campbell, V. Sharma and J. Geibel, *J. Am. Chem. Soc.* **101**, 5376 (1979).
[49]R. H. Poirier, E. J. Kahler and F. Benington, *J. Org. Chem.* **17**, 1437 (1952).
[50]E. J. Land, G. Porter and E. Strachen, *Faraday Trans.* 1885 (1961).
[51]A. Wolberg and J. Manassen, *J. Am. Chem. Soc.* **92**, 2982 (1970).
[52]G. D. Mendenhall and K. U. Ingold, *Ibid.* **95**, 2963 (1973).
[53]D. Dolphin, R. H. Felton, D. C. Borg and J. Fajer, *Ibid.* **92**, 743 (1970).
[54]W. Potter, R. N. Young and G. Levine, *Ibid.* **102**, 2471 (1980).
[55]P. Jones, D. Mantle, D. M. Davies and H. C. Kelly, *Biochemistry* **16**, 3974 (1977).
[56]D. Job and H. B. Dunford, *Eur. J. Biochem.* **66**, 607 (1976).
[57]Y. Hayashi and I. Yamazaki, *J. Biol. Chem.* **254**, 9101 (1979).
[58]M. Simonyi and F. Tudos, *Adv. Phys. Org. Chem.* **9**, 127 (1971).
[59]A. Streitwieser, *Prog. Phys. Org. Chem.* **1**, 1 (1963).
[60]L. Meites and P. Zuman, *Electrochemical Data*, Part I. Wiley, New York (1974).
[61]K. W. Egger and A. T. Cocks, *Helv. Chim. Acta* **56**, 1516 (1973).
[62]L. R. Mahoney and M. A. DaRooge, *J. Am. Chem. Soc.* **97**, 4722 (1975).
[63]M. Santimone, *Biochimie* **57**, 265 (1975). Authors report decay of HRP(I→II) occurs by two pathways: (1) reduction of HRP(I) by endogenous donor; (2) coproportionation, HRP(I) + HRP→2HRP(II).
[64]K. G. Paul, P. I. Ohlsson and S. Wold, *Acta Chem. Scand.* Ser. B **33**, 747 (1979).
[65]B. B. Hasinoff and H. B. Dunford, *Biochemistry* **9**, 4930 (1970).
[66]P. George, *Science* **117**, 220 (1953).
[67]P. George, *Arch. Biochem. Biophys.* **45**, 21 (1953).
[68]C. E. Castro, *The Porphyrins* (Edited by D. Dolphin), Vol. V, pp. 1–80. Academic Press (1978).
[69]S. Ferguson-Miller, D. Braughtigan and E. Margoliash, *The Porphyrins* (Edited by D. Dolphin), Vol. VII, pp. 149–240. Academic Press, New York (1978).
[70]N. Sutin, *Bioinorganic Chemistry II* (Edited by K. Raymond), *Adv. Chem. Ser.* 162, pp. 156–172. Am. Chem. Soc., New York (1977).
[71]R. A. Holwerda, R. A. Read, R. A. Scott, S. Wherland, H. Gray and F. Millett, *J. Am. Chem. Soc.* **100**, 5028 (1978).
[72]S. Wherland and H. B. Gray, *Proc. Natl Acal. Sci. U.S.A.* **73**, 2950 (1976).
[73]J. V. McArdle, C. L. Coyle, H. B. Gray, G. S. Yoneda and R. A. Holwerda, *J. Am. Chem. Soc.* **99**, 2483 (1977).
[74]J. G. Reynolds, C. L. Coyle and R. H. Holm, *Ibid.* **102**, 4350 (1980).
[75]W. R. Scheidt, *The Porphyrins* (Edited by D. Dolphin), Vol. III, pp. 463–512. Academic Press, New York (1978).
[76]J. L. Hoard, *Porphyrins and Metalloporphyrins* (Edited by K. Smith), pp. 317–380. Elsevier, Amsterdam (1975).
[77]H. C. Stynes and J. A. Ibers, *Inorg. Chem.* **10**, 2304 (1971).
[78]L. D. Spaulding, P. G. Eller, J. A. Bertrand and R. H. Felton, *J. Am. Chem. Soc.* **96**, 982 (1974).
[79]J. T. Groves and G. A. McClosky, *Biochem. Biophys. Res. Commun.* **81**, 154 (1978).
[80]F. P. Guengerich, W. M. Crawford and P. G. Watanabe, *Biochemistry* **18**, 5177 (1979).
[81]Y. Watanabe, S. Oae and T. Iyanagi, *Bull. Chem. Soc. Japan* **55**, 188 (1982).
[82]P. R. Ortiz de Montellano, K. L. Kunze, H. S. Beilan and C. Wheeler, *Biochemistry* **21**, 1331 (1982).
[83]B. W. Griffin and P. L. Ting, *Ibid.* **17**, 2206 (1978).
[84]B. W. Griffin and J. A. Peterson, *Ibid.* **11**, 4740 (1972).
[85]R. W. Eastabrook, G. Martinez-Zedillo, S. Young, J. A.

Peterson and J. McCarthy, *J. Steroid Biochem.* **6**, 419 (1975).
[86]V. Ullrich, *J. Mol. Catal.* **7**, 159 (1980).
[87]P. V. Gould, M. H. Gelb and S. G. Sligar, *J. Biol. Chem.* **256**, 6686 (1981).
[88]E. C. Kooyman and G. C. Vegter, *Tetrahedron* **4**, 382 (1958).
[89]J. Peisach, W. B. Mims and J. L. Davis, *J. Biol. Chem.* **254**, 12379 (1979).