Use of spin traps to elucidate radical mechanisms of oxidations by hydroperoxides catalyzed by hemeproteins

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The use of the spin traps nitrosobenzene and 2-methyl-2-nitrosopropane has established that metmyoglobin and liver microsomal cytochrome P-450 initiate a radical decomposition of cumene hydroperoxide. With metmyoglobin and the alkyl nitroso compound, the only radical product of cumene hydroperoxide trapped was the methyl radical formed by β scission of the cumyloxy radical. With both hemeprotein initiators, nitrosobenzene trapped only the cumyl radical, considered to be a decomposition product of the unstable spin adduct phenylcumyloxynitroxide. Support for this proposal includes: (1) previous spin trapping studies of the chemical decomposition of cumene hydroperoxide; and (2) significant inhibition by nitrosobenzene of the one-electron oxidation of aminopyrine and the autoxidation of unsaturated membrane lipids resulting from addition of the hydroperoxide to liver microsomes. Aminopyrine altered the epr signal amplitudes of the spin adducts produced with both nitroso compounds, indicative of oxidation of aminopyrine by the methyl radical and reduction of cumene hydroperoxide by the aminopyrine radical. The participation of hydroperoxide-derived radicals in the low peroxidatic activities of certain hemeproteins is quite distinct from the catalytic function of the true hemeprotein peroxidases, which bring about an efficient two-electron reduction of specific hydroperoxides.

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L'utilisation du nitrosobenzène et du méthyl-2 nitroso-2 propane comme capteurs de spin a permis de démontrer que la métmyoglobine et le cytochrome microsomique P-450 provoquent la décomposition radicalaire de l'hydroperoxyde de cumène. Avec la métmyoglobine et le composé nitrosoalkyle, le radical méthyle, formé par la scission β du radical cumylpéroxy, est le seul produit radicalaire piégé de l'hydroperoxyde de cumène. Avec les deux initiateurs du type hèmeprotéine, le nitrosobenzène piège seulement le radical cumyle qui serait un produit de décomposition du phenylcumyloxynitroxyde, un adduit de spin instable. Cette proposition est basée sur: (1) les études antérieures de piégeage de spin lors de la décomposition chimique de l'hydroperoxyde de cumène; (2) le fait que le nitrosobenzène inhibe de façon significative l'oxydation, par un électron, de l'aminopyrine et l'autoxydation des lipides de la membrane insaturées résultant de l'addition de l'hydroperoxyde aux microsomes du foie. L'aminopyrine modifie les amplitudes du signal de rpe des adduits de spin provenant des deux composés nitrosés indiquant ainsi une oxydation de l'aminopyrine par le radical méthyle et une réduction de l'hydroperoxyde de cumène par le radical aminopyrine. La participation des radicaux provenant de l'hydroperoxyde dans des activités peroxydantes faibles de certaines hèmeprotéines est tout à fait distincte de la fonction catalytique des vraies peroxydases de l'hèmeprotéines qui provoquent une réduction efficace par deux électrons d'hydroperoxydes spécifiques.

[Traduit par le journal]

Introduction

Hemeprotein peroxidases are among the most efficient and best studied protein catalysts. These enzymes utilize H_2O_2, and in certain cases, a limited number of organic hydroperoxides and peroxy acids, to oxidize various electron donors (1). A class of peroxidase isozymes isolated from horseradish root exhibits rather low specificity for the reducing substrate, which includes aromatic amines, phenols, and many other compounds (1). However, catalases from mammalian and other sources exhibit their largest activities with H_2O_2 as the reducing substrate, thereby effecting a rapid decomposition of H_2O_2 to O_2 and H_2O (2). It has been clearly established that the mechanism of catalysis by horseradish peroxidase (HRP) involves the following sequence of reactions (1):

$$[1a]\quad \text{HRP } (Fe^{3+}) + H_2O_2 \rightarrow \text{Compound I}$$

$$[1b]\quad \text{Compound I} + AH \rightarrow \text{Compound II} + A\cdot$$

$$[1c]\quad \text{Compound II} + AH \rightarrow \text{HRP } (Fe^{3+}) + A\cdot$$

$$[1d]\quad A\cdot + A\cdot \rightarrow A\text{–}A$$

Reaction of the ferric hemeprotein with H_2O_2 produces the spectroscopically distinct enzyme species designated as Compound I (1); this species retains both oxidizing equivalents of the peroxide, as evidenced by regeneration of the ferric enzyme (via another hemeprotein species compound II), upon the sequential transfer of two electrons from two molecules of the reducing substrate AH (1). Some of the more stable radical species (A·) formed in this catalytic cycle have been detected directly by epr (1); the final oxidation products of AH result from non-enzymatic reactions of the A· radicals; dimerization, disproportionation, or other reactions are possible, depending on the chemical reactivity of the particular radical and the experimental conditions (1).

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The catalysis of H_2O_2 decomposition by catalase also involves the formation of a species very similar to Compound I of horseradish peroxidase (2); however, reduction of this enzyme species by H_2O_2 appears to be a concerted two-electron transfer reaction, without the involvement of a Compound II-like enzyme intermediate (2). The electronic structure of Compound I is considered to be that of a porphyrin π cation radical associated with Fe(IV) (3); the single oxidizing equivalent of Compound II of horseradish peroxidase remains as Fe(IV) (3). It is very significant that, although the catalysis of H_2O_2-supported oxidations by free and complexed metal ions often produces radical species from the peroxide ($HO_2\cdot$ and $OH\cdot$) (4), these species play no role in the formal catalytic cycle of hemeprotein peroxidases given by reaction [1]. However, low levels of peroxy radicals and superoxide radical anion may be produced during the peroxidase-catalyzed oxidation of certain electron donors; these species arise from non-enzymatic reactions of the substrate radicals $A\cdot$ with molecular O_2 present in the reaction system (5).

The studies described herein were initiated after several reports of the "peroxidatic" activities of cytochrome P-450 appeared (6). This hemeprotein is found in many mammalian and non-mammalian organisms, where it catalyzes the oxidation by molecular O_2 of compounds, such as hydrocarbons and steroids, having relatively unactivated C—H bonds (7). These reactions are designated as monooxygenations because one atom of the O_2 molecule is incorporated into the organic substrate and the other is reduced to H_2O by two electrons originating in reduced pyridine nucleotides (NADH or NADPH), the electron-transfer agents in biological systems (7):

$$[2] \quad NADPH + H^+ + O_2 + BH \rightarrow NADP^+ + H_2O + BOH$$

The mechanism by which cytochrome P-450 "activates" molecular O_2 for specific insertion into an organic compound is of considerable interest. The reports that organic hydroperoxides could function as the oxidant in certain cytochrome P-450-catalyzed oxidations led to the proposal that these reactions were altogether similar to those of other hemeprotein peroxidases, involving the formation of a Compound I-like species of cytochrome P-450 (8). This species was presumed to function as the oxygen-transfer agent in both the peroxidatic and monooxygenase reactions catalyzed by this hemeprotein (8). However, several features of the cytochrome P-450-catalyzed reactions with hydroperoxides were inconsistent with the well-known chemistry of peroxidase catalytic function: the catalytic activities were extremely low, yet substantial destruction of the heme group occurred (8); also, H_2O_2 was much less effective as the oxidant than were organic hydroperoxides, such as cumene hydroperoxide (6, 8) which is a very poor oxidizing substrate for horseradish peroxidase (1, 8). On the other hand, these properties and others were compatible with cytochrome P-450 initiation of a radical decomposition of cumene hydroperoxide, as occurs with various metal ions (9). We have tested this hypothesis by using epr and other techniques to characterize the decomposition of cumene hydroperoxide by well-defined model systems and by cytochrome P-450.

Experimental

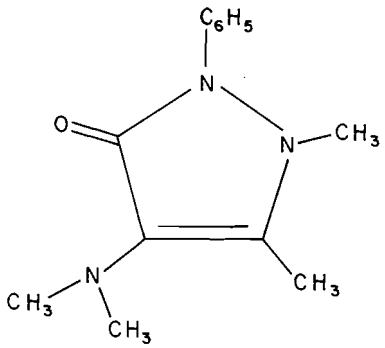
Metmyoglobin was a highly purified preparation isolated from beef heart (10); other purified hemeproteins and hemin were purchased from commercial sources. Microsomal fractions were prepared from the livers of phenobarbital-treated male rats, as described in (11). Aminopyrine, 4-aminoantipyrine, nitrosobenzene, 2-methyl-2-nitrosopropane (MNP), and metyrapone were purchased from Aldrich; acetone-d_6 was a product of Merck, Sharp and Dohme.[2] The sodium salt of cumene hydroperoxide was prepared (12) from a technical grade product supplied by Matheson, Coleman, and Bell; the purity of the salt was confirmed by high-performance liquid chromatography and iodometric titration. All other reagents were the highest quality commercially available. The experimental techniques employed for the epr experiments, which made use of a Varian E-4 spectrometer interfaced to a Digital Equipment Corporation PDP 11/05 computer, have been described in detail elsewhere (13, 14). Details of the high-performance liquid chromatographic analysis of the products of decomposition of cumene hydroperoxide have been published (14).

Results and discussion

Model system studies

Aminopyrine (Fig. 1) has served in this study as a convenient spectroscopic probe for monitoring the formation of one-electron oxidants in enzymatic reactions. The *N*-demethylation of this drug has been commonly used to assay the monooxygenase activity of liver microsomal cytochrome P-450, a membrane-associated hemeprotein (7). The electrochemical oxidation of aminopyrine was reported by Sayo and Masui (15) to produce a relatively stable, violet radical species of this compound. In

[2]The common chemical names and abbreviations used are: aminopyrine, 4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; 4-aminoantipyrine, 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; MNP, 2-methyl-2-nitrosopropane; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; cumene hydroperoxide, α,α-dimethylbenzyl hydroperoxide; Tris, tris-(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NAD, NADP, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide.



Aminopyrine

FIG. 1. Structure of aminopyrine.

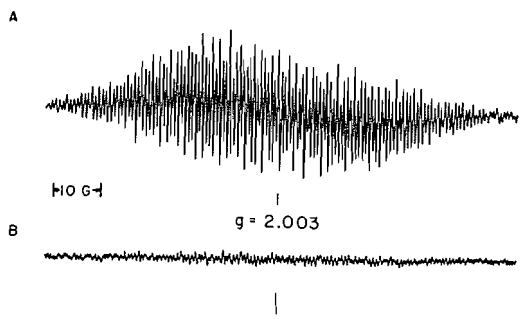


FIG. 2. Electron paramagnetic resonance spectrum of the aminopyrine free radical produced with cumene hydroperoxide and metmyoglobin. (*A*) The reaction mixture contained 20 m*M* aminopyrine, 30 m*M* cumene hydroperoxide, and 40 μ*M* metmyoglobin in 0.1 *M* potassium phosphate buffer, pH 5.0 (*B*) Control experiment: identical to *A*, except for omission of metmyoglobin. Microwave power, 10 mW; modulation amplitude, 0.25 G; temperature, 22°C.

our laboratory, it was shown that the horseradish peroxidase-catalyzed oxidation of aminopyrine with H_2O_2 produced the same radical species, which was proposed to be an intermediate in the *N*-demethylation of aminopyrine by this enzymatic system (13):

$$[3]\quad R_2N{-}CH_3 \xrightarrow{-e^-} R_2N{-}CH_3{\cdot}^+ \xrightarrow{-H\cdot} R_2N^+{=}CH_2 \xrightarrow{H_2O} R_2NH_2^+ + H_2C{=}O$$

This reaction sequence is consistent with: (*1*) the mechanism of peroxidase-catalyzed generation of radical species from many reducing substrates (1); (*2*) non-enzymatic *N*-demethylation reactions effected by various chemical oxidants (16); and (*3*) the chemical structure of aminopyrine, which is closely related to the well-studied violenes, such as *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (17). The unusual stability of the aminopyrine radical, coupled with its rather intense absorbance in the visible region (with a maximum near 565 nm in aqueous solution (18)), is an obvious advantage for studying oxidizing systems with low catalytic activities, a characteristic property of cytochromes P-450 (6–8).

In model-system studies of the oxidation of aminopyrine by cumene hydroperoxide catalyzed by metmyoglobin, the epr signal of the aminopyrine radical was readily detected in aqueous buffered solutions at room temperature, as shown in Fig. 2. Metmyoglobin was selected for these studies because it not only exhibits peroxidatic activities with cumene hydroperoxide comparable to those reported for cytochrome P-450 (6, 8, 19), but it also has somewhat larger peroxidatic activities with H_2O_2 as oxidant (13, 20). Indeed, the reaction of metmyoglobin with a two-fold excess of H_2O_2 has been shown to produce an enzyme species with the spectral properties of Compound II of horseradish peroxidase, which also retains a single oxidizing equivalent of the oxidant (20). Both the rate of formation of this so-called higher oxidation state of the heme iron and its subsequent reduction by electron donors are considerably slower than for horseradish peroxidase (13, 20), consistent with large differences in their catalytic activities toward common electron donors. Our efforts to demonstrate that a discrete higher oxidation state of metmyoglobin comparable to Fe(IV) is formed by reaction of the hemeprotein with cumene hydroperoxide were unsuccessful; a large excess of the hydroperoxide was required to perturb appreciably the absorbance spectrum of the hemeprotein, and the observed changes could be correlated with irreversible destruction of the heme group (18).

The chemistry of the induced radical decomposition of cumene hydroperoxide has been shown to be complex (9), and the radical species produced in this reaction (alkoxy, peroxy, and alkyl) are not particularly persistent (21). As expected, no epr signals were detected at room temperature when metmyoglobin was mixed with cumene hydroperoxide in aqueous buffered solutions. However, when 2-methyl-2-nitrosopropane (MNP) was present, the reaction produced an epr signal, Fig. 3, which contained two nitroxide radicals, assigned to spin adducts of MNP with a methyl radical and a butyl radical (14). The latter nitroxide has been shown to arise from light-induced decomposition of the spin trap (22), and was, accordingly, observed in all control experiments. However, the epr signal of the spin adduct of MNP and the methyl radical was detected only when both metmyoglobin and cumene hydroperoxide were present with the spin trap. Among the large number of control experi-

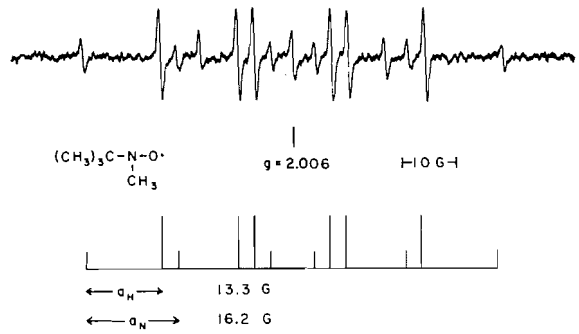


FIG. 3. Electron paramagnetic resonance signals detected in a solution of metmyoglobin, cumene hydroperoxide and MNP. The reaction mixture contained 24 μM metmyoglobin, 30 mM cumene hydroperoxide, 50 mM MNP, and 10% acetone-d_6 in 0.1 M Tris–HCl buffer, pH 9.0. Microwave power, 20 mW; modulation amplitude, 0.82 G; temperature, 22°C. (Reprinted from ref. 14 with permission.)

ments performed were variation of the pH and the solvent, which was required at low levels to increase the solubility of the spin trap in these aqueous solutions; because alcohols were oxidized to radicals which were trapped by MNP, deuterated acetone proved to be the most suitable solvent. At the level (10%) employed in these epr experiments, acetone did not have any significant effect on the measured aminopyrine N-demethylase activity of metmyoglobin with cumene hydroperoxide.

Addition of aminopyrine to the reaction mixture containing metmyoglobin, cumene hydroperoxide, and MNP caused the amplitude of the epr signal of the MNP-trapped methyl radical to decrease dramatically, as shown in Fig. 4. One possible explanation of this effect, namely a direct reaction of aminopyrine with the spin adduct, was eliminated by appropriate control experiments, also shown in Fig. 4. Product assays showed that the metmyoglobin-initiated decomposition of cumene hydroperoxide produced significant amounts of acetophenone, in addition to cumenol (14). Acetophenone formation was clearly incompatible with the usual mechanism of peroxidase catalysis, which would be expected to yield cumenol as the sole product of reduction of cumene hydroperoxide. In the presence of aminopyrine, under experimental conditions comparable to those of the epr experiments, the total yield of both acetophenone and cumenol was increased, but their ratio was not significantly affected, Table 1. These experimental results are consistent with the known chemistry of the radical decomposition of cumene hydroperoxide: formation of the cumyloxy radical, which undergoes a one-electron reduction to cumenol or

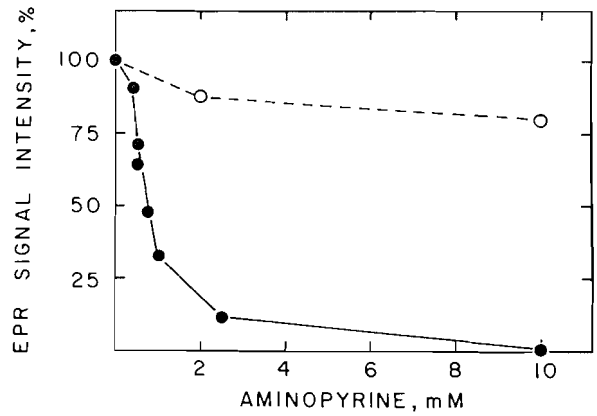
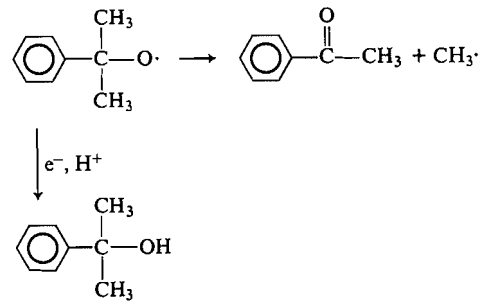


FIG. 4. Effect of aminopyrine on the relative EPR signal amplitude of the MNP-trapped methyl radical produced by the reaction of cumene hydroperoxide with metmyoglobin. The reaction mixture (●) was identical to that described in Fig. 3, except that the cumene hydroperoxide concentration was 20 mM and aminopyrine was present at the indicated concentration. For the control experiment (○), the epr signal amplitude of the spin adduct of MNP and the t-butyl radical (decomposition product of MNP) was measured in the presence of cumene hydroperoxide alone, or metmyoglobin and H_2O_2, in 0.1 M Tris–HCl buffer, pH 9.0. (Reprinted from ref. 14 with permission.)

β-scission to yield acetophenone and the methyl radical (9).

[4] 

The finding that aminopyrine stimulated acetophenone formation, but inhibited trapping of the methyl radical, formed in equimolar amounts with

TABLE 1. Effect of aminopyrine on the products of cumene hydroperoxide decomposition stimulated by metmyoglobin[a]

pH	Aminopyrine added, mM	Cumenol formed, mM	Acetophenone formed, mM
7.0	0	0.71	0.38
	0.44	1.42	0.82
9.0	0	0.68	0.44
	0.44	1.04	0.58

[a]The reaction mixtures contained 22 μM metmyoglobin, 20 mM cumene hydroperoxide, and stated concentration of aminopyrine in 0.1 M buffer (Tris–HCl, pH 9.0, or potassium phosphate, pH 7.0) at 22°C. Reaction products were assayed as described in the Experimental section 5 min after the reaction was initiated.

acetophenone, suggested that aminopyrine reacted predominantly with the more reactive radical species (CH_3·) arising from cumene hydroperoxide. According to the simple scheme above, one-electron reduction of the cumyloxy radical by aminopyrine would be expected to decrease significantly the ratio of acetophenone to cumenol produced. Later experiments suggested that the aminopyrine radical can function as an electron donor to cumene hydroperoxide, thereby promoting the radical chain decomposition of the oxidant.

We next investigated nitrosobenzene as a potential trap of the cumyloxy radical produced in the metmyoglobin – cumene hydroperoxide system (23). In these experiments an unusually stable nitroxide was detected (Fig. 5), which exhibited no hyperfine splitting from nuclei of the trapped moiety. This splitting pattern is that expected for a trapped *tertiary* alkyl or *tertiary* alkoxy radical, and is quite distinct from that previously reported for the methyl radical trapped by nitrosobenzene (24). Indeed, when both nitrosobenzene and MNP were present, the wings of the broader epr signal of the MNP-trapped methyl radical were not detected, and the epr signal of the nitrosobenzene spin adduct was unchanged. These results suggested that CH_3· was not formed in the presence of this concentration of nitrosobenzene, consistent with efficient trapping of the cumyloxy radical by the spin trap. The effect of aminopyrine on the epr signal amplitude of the spin adduct was stimulation, as shown in Fig. 6, which was observed only

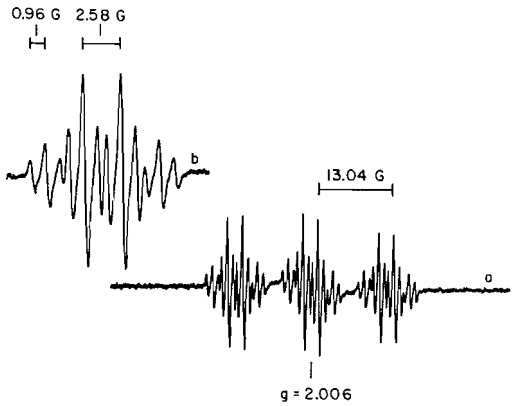


FIG. 5. Electron paramagnetic resonance signal produced in a solution of metmyoglobin, cumene hydroperoxide, and nitrosobenzene. The reaction mixture contained 27.5 μ*M* metmyoglobin, 30 m*M* cumene hydroperoxide, 25 m*M* nitrosobenzene, and 5% acetone-d_6 in 0.1 *M* Tris–HCl buffer, pH 9.0. (*a*) Complete signal; (*b*) low-field portion of the signal on an expanded magnetic field scale. Microwave power, 20 mW; modulation amplitude, 0.4 G; temperature, 22°C. (Reprinted from ref. 23 with permission.)

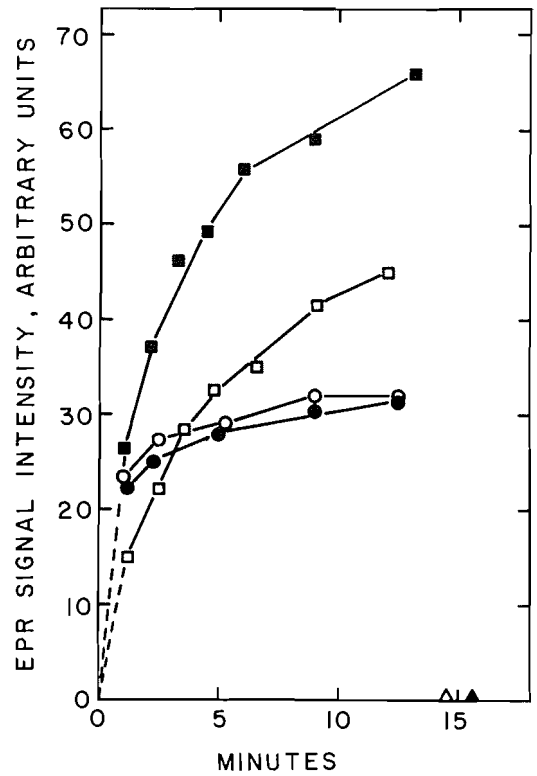


FIG. 6. Effect of aminopyrine on the epr signal amplitude of the nitrosobenzene spin adduct produced by the reaction of cumene hydroperoxide with metmyoglobin. The reaction mixtures contained 27.3 μ*M* metmyoglobin, either no aminopyrine (open symbols) or 40 m*M* aminopyrine (closed symbols), and additional components, as described, in 0.1 *M* Tris–HCl buffer, pH 9.0 with 5% acetone-d_6: (□, ■), 7.5 m*M* nitrosobenzene and 10 m*M* cumene hydroperoxide; (○, ●), 25 m*M* nitrosobenzene and 30 m*M* cumene hydroperoxide. Control experiments (△, ▲): metmyoglobin was omitted. Other conditions as given in Fig. 5 legend. For clarity of presentation, the instrument gain was 1.67 times larger for the open and filled square symbols. (Reprinted from ref. 23 with permission.)

when the concentration of aminopyrine was much larger than that of nitrosobenzene. This experiment was possible because the aminopyrine radical (Fig. 2) was not detected at the alkaline pH which was optimal for formation of the spin adduct of nitrosobenzene (23). Also, addition of aminopyrine to the reaction after the epr signal had attained its maximal intensity produced no effect, indicating that aminopyrine does not react directly with the nitroxide.

The reaction scheme shown in Fig. 7 provides a consistent explanation of the experimental results obtained by the use of two nitroso spin traps as probes of the reaction of metmyoglobin with cumene hydroperoxide. With nitrosobenzene, the initially-formed spin adduct is considered to be the unstable

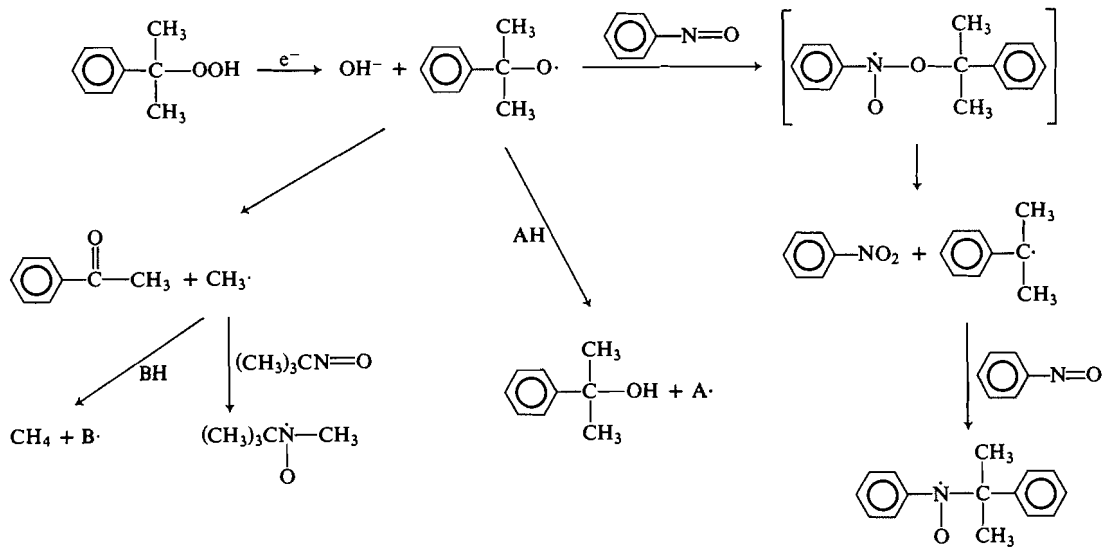


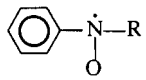
FIG. 7. Proposed mechanism for the metmyoglobin-initiated radical decomposition of cumene hydroperoxide, including the trapping reactions of MNP and nitrosobenzene. AH and BH are electron donors.

phenylcumyloxynitroxide, which decomposes to nitrobenzene and the cumyl radical; subsequent trapping of the cumyl radical by nitrosobenzene produces the stable nitroxide detected by epr. Evidence for the identity of this trapped species as the cumyl radical was previously published by Terabe and Konaka (25), who studied the trapping reactions of a series of aromatic nitroso compounds in purely chemical systems. They reported the hyperfine splitting constants of an epr signal which developed when a solution of cumene hydroperoxide and nitrosobenzene in benzene was heated (25). We observed that benzene extraction of the nitroxide produced in aqueous solutions of metmyoglobin, cumene hydroperoxide, and nitrosobenzene decreased the hyperfine splitting constants to those observed under Terabe and Konaka's experimental conditions (23, 25), Table 2. Those workers based their identification upon a number of control experiments with cumene, which gave identically the same epr signal (25). The same nitroxide has been generated by photochemical means, which established the structure rather conclusively as phenylcumylnitroxide (26). A comparison of the hyperfine splitting constants of this nitroxide with those reported for phenylbutylnitroxide (27) and phenylbutoxynitroxide (28), shown in Table 2, supports the assignment of a trapped cumyl radical. Finally, the failure to detect the methyl radical trapped by nitrosobenzene or by MNP in the presence of nitrosobenzene can be attributed to very efficient trapping of the cumyloxy radical by nitrosobenzene, Fig. 7.

The stimulatory effect of aminopyrine on the nitroxide radical produced in the presence of nitrosobenzene can also be explained by this scheme, Fig. 7: the requirement for a large excess of aminopyrine relative to nitrosobenzene is consistent with efficient trapping of the cumyloxy radical by the nitroso compound. Those cumyloxy radicals which escape trapping at low concentrations of the trap will lead to one-electron oxidation of aminopyrine, either directly or by the methyl radical product of β scission of the cumyloxy radical (9). The additional phenylcumylnitroxide formed in the presence of aminopyrine is attributed to one-electron reduction of cumene hydroperoxide by the aminopyrine radical (cf. reaction [3]), which sustains the radical chain reaction. The stimulation of the Fe^{2+}-induced decomposition of cumene hydroperoxide by hydroquinone (9) has been proposed to involve the semiquinone radical in a similar role. Control experiments showed that aminopyrine, even at high concentrations, could not initiate formation of phenylcumylnitroxide in the absence of metmyoglobin. However, aqueous solutions of cumene hydroperoxide and nitrosobenzene, in the absence of the hemeprotein, slowly developed the same epr signal which formed rapidly in the presence of the enzyme (Fig. 6).

Although the experimental observations support the proposed origin of the cumyl radical, i.e., as a decomposition product of phenylcumyloxynitroxide, other possible sources of the cumyl radical were considered and eliminated. Cumene present as a contaminant in the hydroperoxide could be

TABLE 2. Hyperfine splitting constants reported for *t*-alkyl-and *t*-alkoxyphenyl-nitroxides:



R	A_i, G i = N	i = $H_{o,p}$	i = H_m	Solvent	Reference
—	13.04	2.58	0.96	H_2O	This work
$-C(C_6H_5)(CH_3)_2$	11.53	2.45	0.9	Benzene	25
	11.21	2.46	0.88	Cumene	26
$-OC(CH_3)_3$	14.9	3.1	1.0	$CFCl_3$	27
$-C(CH_3)_3$	13.35	1.7	0.85	CH_2Cl_2	28

oxidized to the cumyl radical. However, the hydroperoxide was highly purified, with no detectable cumene. Moreover, addition of low levels of cumene to the metmyoglobin – cumene hydroperoxide system containing nitrosobenzene produced no significant effect on the amplitude of the epr signal. Another potential source of the cumyl radical is reversible dissociation of O_2 from the cumylperoxy radical (29). While we obtained no direct evidence for formation of the cumylperoxy radical in these reactions, it should arise by transfer of a hydrogen atom from the hydroperoxide to the cumyloxy and methyl radicals in the absence of any other electron donors (28). However, in the presence of a good electron donor like aminopyrine, the cumylperoxy radical is not expected to be formed; even if it were formed, it should play no significant role in the oxidation of aminopyrine because of the characteristically low reactivity of peroxy radicals in hydrogen atom abstraction (29). This has been clearly demonstrated by the recent publication (30) of absolute rate constants for hydrogen atom abstraction from a carbon α to a nitrogen (quite analogous to the oxidation of aminopyrine, reaction [3]) effected by *t*-butoxy and *t*-butylperoxy radicals; the alkoxy radical was more reactive by a factor of 10^5 to 10^6 (30).

Rosen and Rauckman detected in a system containing hematin, cumene hydroperoxide, and DMPO a nitroxide assigned to the cumylperoxy adduct of the nitrone (31). However, this species was detected only weakly at pH 3.0, where nitrones readily undergo acid-promoted hydrolysis. There is other experimental evidence (40) that alkylperoxy spin adducts of nitrones are highly unstable, particularly at temperatures greater than 0°C. We must conclude that the identity of the nitroxide reported by Rosen and Rauckman (31) is uncertain, and that the proposed role of the cumylperoxy radical is not supported by their data. Available data indicate that in aqueous solutions the common nitroso and nitrone spin traps are not suitable for positive identification of oxygen-centered organic radicals. However, the use of nitrones appears to be subject to more oxidation artefacts, i.e., 5,5-dimethylpyrrolidone-(2)-oxyl-(1) (31, 40), than is the use of nitroso spin traps.

Studies with liver microsomes

For spin trapping experiments with cumene hydroperoxide and liver microsomal fractions containing the membrane-associated hemeprotein cytochrome P-450, nitrosobenzene was chosen because of the stability of the spin adduct and the greater detection sensitivity for this radical. As shown in Fig. 8, the epr signal detected in this system clearly contained the sharp resonances of phenylcumylnitroxide and another broad component, considered to be the membrane-associated fraction of the same nitroxide (32); the broadening is attributed to slower rotational motion of the nitroxide in the membrane and measurable paramagnetic exchange interaction with O_2, present at a higher concentration in the lipid phase of this system. It was observed that: (*1*) this epr signal was considerably less stable in microsomes than in the model system, and (*2*) all control experiments except one were negative: microsomes containing only nitrosobenzene gave a very weak epr signal quite distinct from that detected with both cumene hydroperoxide and nitrosobenzene present. This epr signal was consistent with that reported for the phenylhydronitroxide radical (33), suggesting that it arises from reduction of nitrosobenzene by substances in the microsomes. This epr signal was destroyed by oxidants such as *t*-butyl hydroperoxide, and also was not detected in the presence of cumene hydroperoxide. Significantly, metyrapone, a bipyridyl compound which is a specific inhibitor of cytochrome P-450, produced measurable inhibition (46%) of the epr signal detected with liver microsomes, cumene hydroperoxide, and nitro-

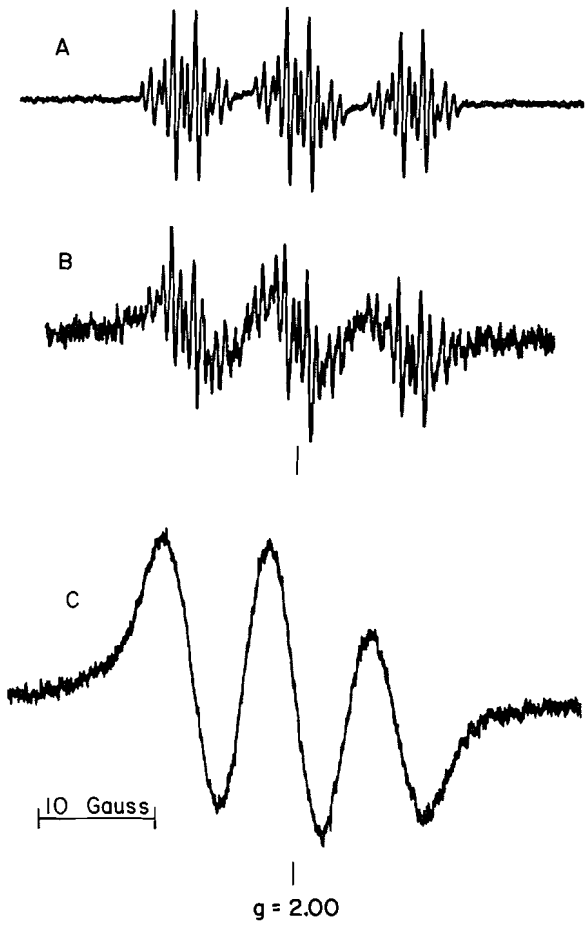


FIG. 8. Comparison of epr signals resulting from addition of metmyoglobin or rat liver microsomes to cumene hydroperoxide in the presence of nitrosobenzene. The reaction with metmyoglobin (*A*) was that described in the Fig. 5 legend. The reaction mixtures for *B* and *C* contained 18.8 μ*M* liver microsomal cytochrome P-450, 30 m*M* cumene hydroperoxide, 12.5 m*M* nitrosobenzene, and 2.5% acetone-d_6 in 0.1 *M* Hepes–Tris buffer, pH 6.5. Microwave power, 20 mW; modulation amplitude, 0.4 G (*A*, *B*) or 5.0 G (*C*); gain, 8×10^2 (*A*); 5×10^3 (*B*); 1.5×10^3 (*C*).

sobenzene, but had no effect on metmyoglobin-dependent generation of this nitroxide (32). Also, the metmyoglobin system was much more sensitive to inhibition by cyanide than was the microsomal system (32), consistent with the relative affinities of the two hemeproteins for this ligand. These results strongly implicated cytochrome P-450 as the initiator of cumene hydroperoxide decomposition in these liver microsomal fractions.

The possibility that inorganic iron, a likely contaminant of microsomes, could be responsible for the results obtained with microsomes was eliminated by control experiments. The addition of ferrous (but not ferric) ion to aqueous solutions of cumene hydroperoxide and nitrosobenzene (pH ~ 4.0) produced the phenylcumylnitroxide and also detectable levels of the less stable spin adduct of nitrosobenzene and the methyl radical. The Fe^{2+}-dependent reaction was also distinguished from that initiated by the hemeproteins by: (*1*) the requirement for Fe^{2+} concentrations approximately 20-fold larger than the hemeprotein concentrations employed; and (*2*) complete inhibition of the epr signal by 10 m*M* EDTA. Neither hemeprotein–cumene hydroperoxide system displayed any sensitivity to EDTA. Moreover, the addition of 200 μ*M* Fe^{2+} to microsomes had no effect on the epr signal detected in the absence of this metal ion.

The epr experiments indicated that nitrosobenzene trapped efficiently the initial radical arising from cumene hydroperoxide in the presence of hemeproteins or Fe^{2+}; but the failure to detect the spin adduct of the cumyloxy radical made independent confirmation of these results important, especially for the microsomal system. Spectral changes observed during the microsomal oxidation of aminopyrine by cumene hydroperoxide demonstrated the rapid formation of the violet aminopyrine radical, Fig. 9; however, the absorbance con-

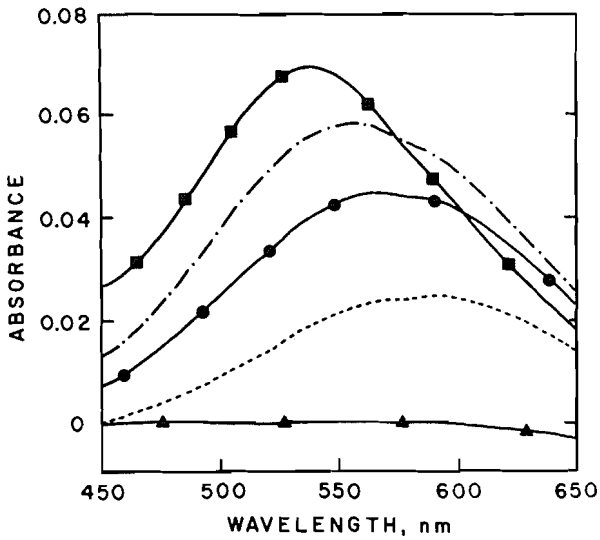


FIG. 9. Spectral changes observed during the microsomal oxidation of aminopyrine by cumene hydroperoxide. A solution containing 1 mg total microsomal protein/mL in 50 m*M* Hepes–Tris buffer, pH 6.5, with 0.15 *M* KCl, 10 m*M* $MgCl_2$, and 30 m*M* aminopyrine was added to both sample and reference cuvets, and the baseline was recorded (▲). The spectrum was scanned at the indicated times after addition of 0.25 m*M* cumene hydroperoxide to the sample cuvet: 15 s (---), 1 min (●), 3 min (—·—·), and 5 min (■), which corresponded to the maximal absorbance increase. The spectrum recorded at 1 min is nearly identical to that of the aminopyrine radical produced in other enzymatic oxidizing systems (18). (Reprinted from ref. 34 with permission.)

tinued to increase with a concomitant shift in the absorbance maximum from 560 nm to 535 nm, followed by a slower decay and a further shift of the absorbance maximum to 527 nm (34). It was shown that oxidation of 4-aminoantipyrine by microsomes and cumene hydroperoxide, or other oxidizing systems, produced a 525 nm-absorbing chromophore; this species had been identified from earlier studies of the chemistry of 4-aminoantipyrine as antipyrine red, a coupling product of two molecules of the parent compound (35). We note that 4-aminoantipyrine is the product of oxidative cleavage of two *N*-methyl groups from the amino group of aminopyrine (cf. Fig. 1) and is oxidized much more easily than aminopyrine (13). Thus, the spectral changes of Fig. 9 are considered to represent initial formation of the aminopyrine radical, followed by rapid loss of two *N*-methyl groups from the radical, and subsequent oxidation of the product, 4-aminoantipyrine, to antipyrine red. This reaction sequence apparently occurs even though the aminopyrine concentration is considerably larger than the concentration of both cumene hydroperoxide and 4-aminoantipyrine which is formed; the localization of these reactions in the microsomal membrane, which has a limited capacity for the very water-soluble aminopyrine, is probably responsible for this phenomenon. The important point for this study, however, is that nitrosobenzene very effectively inhibited the absorbance changes which occurred during the microsomal oxidation of aminopyrine by cumene hydroperoxide (Fig. 10), or the oxidation of 4-aminoantipyrine by this system (34). The sensitivity of spectrophotometric detection of the aminopyrine radical (15, 18) and the adequate solubility of nitrosobenzene in H_2O made the use of organic solvents unnecessary in these experiments.

Nitrosobenzene also inhibited another reaction – the consumption of molecular O_2 — which occurs when cumene hydroperoxide is added to liver microsomes (34); the concentrations of nitrosobenzene which produced significant inhibition of this reaction (Fig. 11) and the microsomal oxidation of aminopyrine by cumene hydroperoxide (Fig. 10) were very similar. Both phenomena dependent on cumene hydroperoxide — aminopyrine oxidation and O_2 consumption — were similarly inhibited by metyrapone and known substrates of liver microsomal cytochrome P-450. The O_2 consumption was attributed to autoxidation (peroxidation) of unsaturated membrane lipids initiated by one or more of the radical species derived from cumene hydroperoxide. This proposal was tested by control experiments with a model system in which metmyoglobin

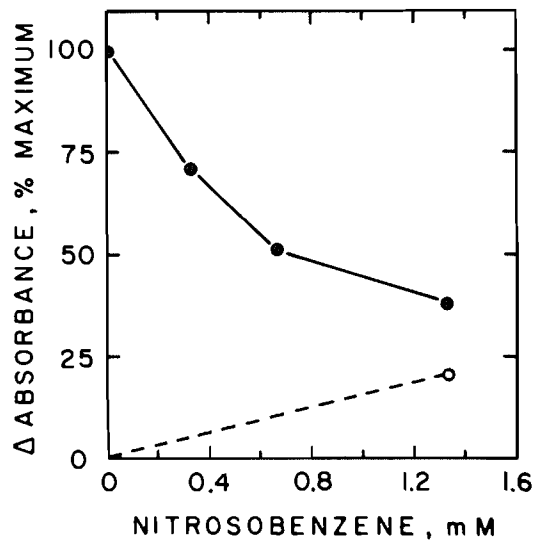


FIG. 10. Effect of nitrosobenzene on the maximal absorbance measured during the microsomal oxidation of aminopyrine by cumene hydroperoxide. Experimental conditions were identical to those described in the Fig. 9 legend, with nitrosobenzene added at the indicated concentrations (●). The maximal absorbance increase which developed near 535 nm was measured. The control experiment (○), in which aminopyrine was omitted, developed a measurable, but featureless, absorbance in the visible region. (Reprinted from ref. 34 with permission).

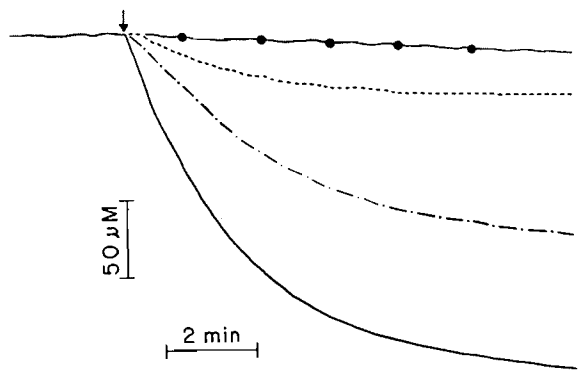


FIG. 11. Effect of nitrosobenzene on O_2 consumption resulting from addition of cumene hydroperoxide to rat liver microsomes. Experimental conditions were identical to those described in the Fig. 9 legend, except that aminopyrine was omitted and nitrosobenzene was added at the stated concentrations: —, 0; ·---·, 0.33 m*M*; ---, 0.67 m*M*; ●, 1.33 m*M*. The reaction was initiated by adding 0.25 m*M* cumene hydroperoxide at the point indicated by the arrow. The O_2 concentration was monitored continuously by a Clark-type electrode in stirred solutions within a cell which was closed to the atmosphere and maintained at 25°C. (Reprinted from ref. 34 with permission.)

and arachidonic acid were substituted for cytochrome P-450 and for the unsaturated membrane lipids of the microsomes. In the absence of arachidonic acid, no measurable O_2 uptake occurred in aqueous solutions of metmyoglobin and cumene

hydroperoxide under the experimental conditions of Fig. 11. With 0.3 m*M* arachidonic acid also present, the rate of O_2 consumption dependent on cumene hydroperoxide was somewhat lower than the control rate observed for the microsomal system (Fig. 11). At relatively higher concentrations of arachidonic acid, low levels of contaminating hydroperoxides formed by air oxidation of this compound were sufficient to sustain O_2 consumption with metmyoglobin without any requirement for cumene hydroperoxide. Significantly, both the cumene hydroperoxide-dependent and -independent O_2 uptake in the metmyoglobin/arachidonic acid system were inhibited by levels of nitrosobenzene which were effective in the analogous microsomal reaction (Fig. 11). Finally, it was shown that cyanide, but not metyrapone, could inhibit metmyoglobin-initiated autoxidation of arachidonic acid. The inhibitory effect of nitrosobenzene on hemeprotein-initiated autoxidation of unsaturated lipids is also consistent with the trapping of a carbon-centered radical produced by hydrogen atom abstraction from the lipid molecule. Enzymatic oxygenation reactions of polyunsaturated fatty acids have been probed by 2-methyl-2-nitrosopropanol (36), MNP (37), and nitrosobenzene (38); in each case, a secondary carbon-centered radical adduct of the nitroso compound was identified by its epr spectrum (36–38). However, epr data obtained with the lipid-free model system in our study have provided the best evidence for the point of nitrosobenzene inhibition of the radical decomposition of cumene hydroperoxide initiated by hemeproteins. Based on various kinds of experimental data obtained with two spin traps, as well as numerous similarities between the model system containing metmyoglobin and the microsomal system containing cytochrome P-450, we conclude that the reaction sequence of Fig. 7 accounts for the major reactions that occur in the hemeprotein-initiated decomposition of cumene hydroperoxide, in the absence or presence of nitroso spin traps.

These experimental results indicate that reactive one-electron oxidants produced by the addition of cumene hydroperoxide to liver microsomes are responsible for both aminopyrine oxidation and initiation of autoxidation of unsaturated lipids. The findings that rather low levels of nitrosobenzene significantly inhibited both reactions, taken with the epr spin trapping data, strongly implicate the cumyloxy and/or methyl radicals as the oxidizing species. The reactions initiated by adding cumene hydroperoxide to liver microsomes clearly required cytochrome P-450, since metyrapone, a specific and high-affinity ligand of this hemeprotein, was an excellent inhibitor of these reactions. The results obtained with metmyoglobin and cumene hydroperoxide were analogous, with this system, however, showing sensitivity to inhibition by cyanide, a known ligand of metmyoglobin. The ability of H_2O_2 (and certain organic hydroperoxides and peroxy acids) to oxidize ferric hemeprotein peroxidases to species containing Fe(IV) (1–3) suggests that those hemeproteins which react with cumene hydroperoxide may transfer a single electron to this oxidant. The products of this reaction would presumably be the cumyloxy radical and an Fe(IV) heme species of unknown structure. It also seems likely that the heme group in this or other oxidation state of the enzyme is attacked by one or both of the hydroperoxide-derived radicals which are responsible for oxidation of other electron donors. Indeed, we have observed that destruction of the heme group of metmyoglobin by cumene hydroperoxide can be inhibited by aminopyrine or nitrosobenzene. Also, recent evidence was published for the reaction of methyl radicals with ferric, ferrous, and iron-free deuteroporphyrin (39). The low selectivity of the organic free radical oxidants derived from cumene hydroperoxide in the presence of hemeproteins can, thus, account for all of the phenomena which have been observed in these systems.

These experiments are now being extended to other hemeproteins, as well as other organic hydroperoxides. With hemin and catalase, the same cumene hydroperoxide-derived radicals trapped by MNP and nitrosobenzene have been detected; both heme compounds also exhibit low peroxidatic activities with this hydroperoxide. However, horseradish peroxidase shows no reactivity with cumene hydroperoxide by any of the techniques employed in this study. These results suggest that, while the high catalytic activities of hemeprotein peroxidases with H_2O_2 and a limited number of other peroxidic agents derive from the characteristic reactivity of iron and its chelated forms with peroxides, evolutionary development of the protein structures of peroxidases has modified this reactivity to prevent the formation of reactive oxidizing radicals from the oxidant. The advantage to these enzymes of such altered reactivity would appear to be self-preservation, so that "the show (efficient catalysis) may go on". Those hemeproteins with biological functions not directly related to efficient catalytic reduction of hydroperoxides have retained to varying degrees the ability to effect one-electron reduction of hydroperoxides, which accounts for their low peroxidatic activities. Finally, it seems appropriate to emphasize what

others (40) have cautioned about spin trapping applications, especially in biological systems: a critical approach is essential. In addition to using several spin traps to investigate a given system, and other experimental techniques in conjunction with epr, it is necessary to perform all possible control experiments. Only then can one draw definitive conclusions about the formation and possible significance of free radicals in the system under study.

Acknowledgment

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1. M. MORRISON and G. SCHONBAUM. Ann. Rev. Biochem. **45**, 861 (1976).
2. G. SCHONBAUM and B. CHANCE. Enzymes. **13C**, 363 (1976).
3. J. E. ROBERTS, B. M. HOFFMAN, R. RUTTER, and L. P. HAGER. J. Biol. Chem. **256**, 2118 (1981).
4. C. WALLING. Acc. Chem. Res. **8**, 125 (1975).
5. I. YAMAZAKI. *In* Molecular mechanisms of oxygen activation. *Edited by* O. Hayaishi. Academic Press, New York. 1974. pp. 535–558.
6. A. D. RAHIMTULA and P. J. O'BRIEN. Biochem. Biophys. Res. Commun. **60**, 440 (1974).
7. B. W. GRIFFIN, J. A. PETERSON, and R. W. ESTABROOK. *In* The porphyrins. Vol. VII. *Edited by* D. Dolphin. Academic Press, New York. 1969. pp. 333–375.
8. G. D. NORDBLOM, R. E. WHITE, and M. J. COON. Arch. Biochem. Biophys. **175**, 524 (1976).
9. M. S. KHARASCH, A. FONO, and W. NUDENBERG. J. Org. Chem. **15**, 763 (1950).
10. T. GOTOH and K. SHIKAMA. Arch. Biochem. Biophys. **163**, 476 (1974).
11. J. WERRINGLOER and R. W. ESTABROOK. Arch. Biochem. Biophys. **167**, 270 (1975).
12. R. D. MAIR and R. T. HALL. *In* Organic peroxides. Vol. 2. *Edited by* D. Swern. Wiley-Interscience, New York. 1971. pp. 535–635.
13. B. W. GRIFFIN and P. L. TING. Biochemistry, **17**, 2206 (1978).
14. B. W. GRIFFIN and P. L. TING. FEBS Lett. **89**, 196 (1978).
15. H. SAYO and M. MASUI. J. Chem. Soc. Perkin Trans. II, 1640 (1973).
16. D. H. ROSENBLATT, L. A. HULL, D. C. DELUCA, G. T. DAVIS, R. C. WEGLEIN, and K. K. R. WILLIAMS. J. Am. Chem. Soc. **89**, 1158 (1967).
17. J. M. FRITSCH, H. WEINGARTEN, and J. D. WILSON. J. Am. Chem. Soc. **92**, 4038 (1970).
18. B. W. GRIFFIN, C. MARTH, Y. YASUKOCHI, and B. S. S. MASTERS. Arch. Biochem. Biophys. **205**, 543 (1980).
19. B. W. GRIFFIN. *In* Fundamental research in homogeneous catalysis. Vol. 3. *Edited by* M. Tsutsui. Plenum Press, New York. 1979. pp. 773–791.
20. T. YONETANI and H. SCHLEYER. J. Biol. Chem. **242**, 1974 (1967).
21. K. U. INGOLD. *In* Free radicals. Vol. 1. *Edited by* J. K. Kochi. Wiley-Interscience, New York. 1973. pp. 37–112.
22. E. G. JANZEN. Acc. Chem. Res. **4**, 31 (1971).
23. B. W. GRIFFIN and D. RAMIREZ. Bioorg. Chem. **10**, 177 (1981).
24. M. J. PERKINS and P. WARD. J. Chem. Soc. Sect. B, 395 (1970).
25. S. TERABE and R. KONAKA. J. Chem. Soc. Perkin Trans. II, 2163 (1972).
26. V. MALATESTA and K. U. INGOLD. Tetrahedron Lett. 3307 (1973).
27. G. CHAPELET-LETOURNEUX, H. LEMAIRE, and A. RASSAT. Bull. Soc. Chim. Fr. 3283 (1965).
28. A. MACKOR, TH. A. J. W. WAJER, TH. J. DE BAER, and J. D. W. VAN VOORST. Tetrahedron Lett. 385 (1967).
29. B. C. GILBERT and A. J. DOBBS. *In* Organic peroxides. Vol. 3. *Edited by* D. Swern. Wiley-Interscience, New York. 1972. p. 284.
30. D. GRILLER, J. A. HOWARD, P. R. MARRIOTT, and J. C. SCAIANO. J. Am. Chem. Soc. **103**, 619 (1981).
31. G. M. ROSEN and E. J. RAUCKMAN. Mol. Pharmacol. **17**, 233 (1980).
32. B. W. GRIFFIN. *In* Microsomes, drug oxidations and chemical carcinogenesis. Vol. 1. *Edited by* M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'BRIEN. Academic Press, New York. 1980. pp. 319–322.
33. H. LEMAIRE, A. RASSAT, and J-P. RAVET. Tetrahedron Lett. **47**, 3507 (1964).
34. P. L. ASHLEY and B. W. GRIFFIN. Mol. Pharmacol. **19**, 146 (1981).
35. E. EISENSTAEDT. J. Org. Chem. **3**, 153 (1939).
36. J. J. M. C. DEGROOT, G. J. GARSSEN, J. F. G. VLIEGENTHART, and J. BOLDINGH. Biochim. Biophys. Acta, **326**, 279 (1973).
37. R. P. MASON, B. KALYANARAMAN, B. E. TAINER, and T. E. ELING. J. Biol. Chem. **255**, 5019 (1980).
38. H. AOSHIMA, T. KAJIWARA, A. HATANAKA, and H. HATANO. J. Biochem. Tokyo, **82**, 1559 (1977).
39. D. BRAULT and P. NETA. J. Am. Chem. Soc. **103**, 2705 (1981).
40. E. G. JANZEN. *In* Free radicals in Biology. Vol. 4. *Edited by* W. A. Pryor. Academic Press, New York. 1980. pp. 115–154.