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Electron Paramagnetic Resonance Probes of the Radical Decomposition of Cumene Hydroperoxide Initiated by Metmyoglobin

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Electron paramagnetic resonance studies have provided evidence for metmyoglobin initiation of the radical decomposition of cumene hydroperoxide, carried out in buffered aqueous solutions at ambient temperatures. The radicals formed oxidize aminopyrine to a free radical, readily detected at acidic pH, or react with the spin trap nitrosobenzene. The only species so trapped was the cumyl radical (optimal pH, 9.0), previously observed in a similar spin-trapping study of the chemical decomposition of cumene hydroperoxide in organic solvents. The earlier proposal that the cumyl radical arises from breakdown of an initially formed, unstable phenylcumyloxy nitroxide is consistent with the experimental findings of this study. Moreover, it was shown that the decomposition of cumene hydroperoxide initiated by ferrous ion or by other heme compounds occurs by the same mechanism. Thus, the very low peroxidatic activities of several hemeproteins with cumene hydroperoxide involve oxidizing free radicals, unlike H_2O_2-dependent oxidations catalyzed by true hemeprotein peroxidases, in which enzyme species are the functional oxidants.

INTRODUCTION

The horseradish peroxidase-catalyzed (HRP) oxidation of many compounds by H_2O_2 occurs via abstraction of single electrons by Compounds I and II, well-characterized forms of HRP which are two and one oxidizing equivalents, respectively, above the ferric hemeprotein (*1, 2*). However, it has been proposed that peroxidatic reactions of cytochrome *P*-450 with both H_2O_2 and lipid-soluble hydroperoxides proceed by a different mechanism, namely, direct transfer of an oxygen atom from the hydroperoxide to the electron donor substrate (*3, 4*). Although a Compound I-like species of cytochrome *P*-450 has been presumed to function as the oxygen transfer agent in these reactions, no definitive evidence for the existence of this species has been published (*3, 4*). With membrane-bound liver microsomal cytochrome *P*-450, cumene hydroperoxide has been shown to be the most active hydroperoxide in the oxidation of several classes of compounds (*5, 6*). However, it is well established that good chemical reductants readily induce a radical chain decomposition of cumene hydroperoxide (*7*). Thus,

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[3] Abbreviations used: HRP, horseradish peroxidase; MNP, 2-methyl-2-nitrosopropane.

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experimental evidence relating to both the chemistry of cumene hydroperoxide decomposition and the reaction mechanism of other hemeprotein peroxidases suggested that radical species derived from the hydroperoxide and/or the reducing substrate might be intermediates in these cytochrome *P*-450-catalyzed reactions.

In the present study, two different types of compounds have been employed as probes for reactive radicals arising during the interaction of cumene hydroperoxide with metmyoglobin. Metmyoglobin is a readily purified hemeprotein which catalyzes the *N*-demethylation of aminopyrine by cumene hydroperoxide at rates comparable to those reported for the cytochrome *P*-450-catalyzed reaction (*3*). Thus, metmyoglobin was considered to be an appropriate model hemeprotein for investigating this activity of cytochrome *P*-450. The probe molecules selected were: (1) aminopyrine, a drug substrate of cytochrome *P*-450, and a representative of the class of *N*, *N*-dimethyl-substituted amines which are oxidized to relatively stable radical cations (*8, 9*); and (2) nitrosobenzene, which can add to, i.e., "trap," free radicals to form stable nitroxide products (*10*). Thus, these probes can react with short-lived radicals in different ways, producing two distinct types of stable radical products detectable by electron paramagnetic resonance (epr) spectroscopy at ambient temperatures.

The choice of aminopyrine and nitrosobenzene for this study was suggested by earlier findings published from this laboratory. The aminopyrine *N*-demethylase activity of HRP, with H_2O_2 as oxidant, was found to be approximately 10^3 greater than the corresponding activity of cytochrome *P*-450 measured with any hydroperoxide (*11*). Moreover, a radical species of aminopyrine which attained significant concentrations during this reaction was shown to be an intermediate in the production of formaldehyde (*11, 12*). The one-electron oxidation of aminopyrine was quite consistent with the mechanism of HRP catalysis but incompatible with the direct oxygen-insertion scheme proposed for peroxidatic reactions catalyzed by cytochrome *P*-450 (*3, 4*). The remarkable stability of the aminopyrine radical in buffered aqueous solutions provided the opportunity to test whether the radical mechanism of *N*-demethylation of this compound occurs quite generally with hemeprotein–hydroperoxide systems.

More recently, we have published evidence, obtained by the use of the spin trap 2-methyl-2-nitrosopropane (MNP), that metmyoglobin stimulates the decomposition of cumene hydroperoxide to reactive radicals (*13*). The only hydroperoxide-derived radical trapped in those experiments was the methyl radical, previously shown to arise from decomposition of the cumyloxy radical (*7*). Although not trapped by MNP, the cumyloxy radical was considered to be the initial product of the reaction of cumene hydroperoxide with metmyoglobin. In an attempt to trap the cumyloxy radical and/or other possible radicals not trapped by MNP, we employed nitrosobenzene as a spin trap in the same hemeprotein–hydroperoxide system. The experimental results demonstrated the potential of this approach as a means of probing for reactive radicals produced in biological systems, and, in addition, suggested important mechanistic differences between hemeprotein catalysts which exhibit very low peroxidatic activities with cumene hydroperoxide and those which exhibit very large activities with H_2O_2.

MATERIALS AND METHODS

The metmyoglobin used in this study was a highly purified preparation isolated from beef heart (*14*); catalase (bovine liver, purified powder) and hemin (Equine, type III), purchased from Sigma, were used without further purification. Heme concentrations were assayed by the pyridine hemochromagen method (*15*). Aminopyrine and nitrosobenzene were obtained from Aldrich, and acetone-d_6 was a product of Merck Sharp and Dohme. The sodium salt of cumene hydroperoxide was prepared (*16*) from a technical grade product supplied by Matheson, Coleman, and Bell; the purity of the salt was confirmed by high-performance liquid chromatography and by iodometric titration (*17*). All other reagents were the highest quality commercially available. The experimental techniques employed for the epr experiments have been described in detail elsewhere (*12, 13*). Electron paramagnetic resonance spectra were recorded and integrated by use of a Digital Equipment Corporation PDP 11/05 computer interfaced to a Varian E-4 spectrometer.

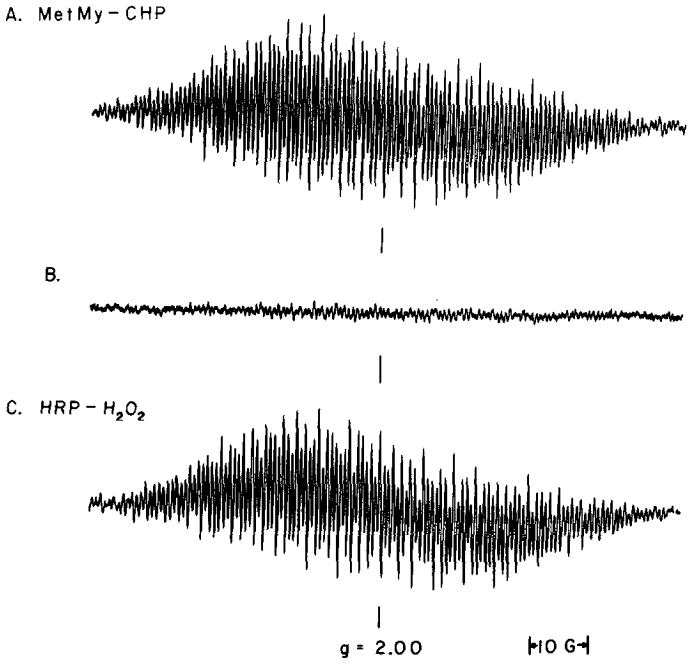


FIG. 1. Electron paramagnetic resonance signals resulting from oxidation of aminopyrine in the presence of cumene hydroperoxide and metmyoglobin or H_2O_2 and HRP. (A) The reaction mixture contained 20 m*M* aminopyrine, 30 m*M* cumene hydroperoxide (CHP), and 40 μM metmyoglobin (MetMy) in 0.1 *M* potassium acetate buffer, pH 5.0. (B) Control experiment: conditions were identical to (A) except metmyoglobin was omitted. (C) The reaction mixture contained 10 m*M* aminopyrine, 10 m*M* H_2O_2, and 1.9 μM HRP in 0.1 *M* potassium acetate buffer, pH 5.0, conditions which produced approximately the same epr signal intensity as (A). Instrument settings were identical for these epr spectra: power, 10 mW; modulation amplitude, 0.25 G; time constant, 0.3 sec; scan time, 4 min; magnetic field, 3260 G; microwave frequency, 9.16 GHz; instrument gain, 6.2×10^3. Temperature, 22°C.

RESULTS AND DISCUSSION

Aminopyrine Oxidation

Figure 1 shows that the epr signals of the radical species produced from aminopyrine in the presence of metmyoglobin and cumene hydroperoxide, or HRP and H_2O_2, are identical. All control experiments were negative, except that in which the hemeprotein was omitted, consistent with previous observations with H_2O_2 (*12*); the weak signal detected in this control (Fig. 1B) is a consequence of acid-promoted decomposition of hydroperoxides, together with the unusual stability of the aminopyrine free radical at acidic pH (*12*). Since the epr signal decayed slowly under these conditions, the effect of experimental variables on the maximal radical concentration attained in this system could be determined. The pH optimum occurred between pH 4.0 and 5.0. The maximal radical concentrations showed a linear dependence on the square root of the enzyme concentration, which is the expected dependence for steady-state levels resulting from rapid enzymatic production of the radical and a much slower second-order, nonenzymatic decay of the species (*18*). When the maximal radical concentration was measured as a function of the concentration of either substrate, saturation phenomena were observed; the substrate concentrations which produced 50% of the radical levels generated with saturating substrate were approximately 10 m*M* for cumene hydroperoxide and 2 m*M* for aminopyrine.

Oxidation of aminopyrine by chemical (*11*) and electrochemical (*19*) means has also been shown to generate this epr signal (Fig. 1), which has been conclusively identified as a radical cation of aminopyrine (*9*). Moreover, the H_2O_2-dependent oxidation of aminopyrine to formaldehyde catalyzed by both metmyoglobin and HRP has been clearly demonstrated to involve the radical species as a requisite intermediate, according to the following reaction sequence (*12*):

$$R_2N{-}CH_3 \xrightarrow{-e^-} R_2N{-}\overset{+}{C}H_3^{\cdot} \xrightarrow{-H\cdot} R_2N^+ = CH_2 \xrightarrow{H_2O} R_2NH_2^+ + H_2C = 0. \qquad [1]$$

The aminopyrine *N*-demethylase activity of metmyoglobin is much smaller with cumene hydroperoxide than with H_2O_2 (*12*), i.e., 8.1 mol/min/mol heme compared with 44 mol/min/mol heme, and both activities are considerably smaller than that reported for the HRP–H_2O_2 system, i.e., 2.5×10^4 mol/min/mol heme (*11*). Under optimal experimental conditions, the relative steady-state radical levels measured in each of these systems, normalized by the hemeprotein concentration, were qualitatively consistent with these values: 1.0 for metmyoglobin–cumene hydroperoxide; 5.7 for metmyoglobin–H_2O_2; and 4.6×10^2 for HRP–H_2O_2. These comparisons are not strictly quantitative because epr detection of this radical requires considerably larger reactant and enzyme concentrations than are necessary for assay of enzymatic activity. The rate of formaldehyde production depends not only on the steady-state radical concentration, but also on the rate of subsequent oxidation of the radical (cf. Eq. [1]); this radical decay process may be enzymatic (*12*), nonenzymatic, i.e., by dismutation (*9*) or other means, or may be a combination of these various reactions in each hemeprotein–hydroperoxide system. The proposed mechanism of enzymatic *N*-demethylation (Eq.

[1]) is essentially identical to proposed mechanisms of chemical *N*-demethylation effected with various oxidizing agents (*20, 21*); moreover, it is consistent with the formation of epr-detectable radical species from a variety of unsaturated *N,N*-dimethyl-substituted amines by electrochemical and chemical means (*8, 9*). Thus, the radical mechanism of *N*-demethylation of aminopyrine, rather than a direct oxygen-insertion pathway, appears to be rather general for chemical oxidizing systems, as well as several hemeprotein–hydroperoxide systems with a broad range of activities.

Spin-Trapping with Nitrosobenzene

The epr signal produced when metmyoglobin was added to a buffered solution of nitrosobenzene and cumene hydroperoxide is shown in Fig. 2. Low concentrations of acetone were employed, as in the previous spin-trapping study, to increase the solubility, i.e., the trapping efficiency, of the nitroso compound; fully deuterated solvent eliminated possible interference from trapped radicals derived from the solvent. There are two obvious advantages of nitrosobenzene over MNP in this system: (1) more sensitive detection of the nitroxide (Fig. 2), thus, permitting the use of lower concentrations of both the spin trap and the solvent; and (2) greater stability of this nitroxide, by some hours, than the MNP-trapped methyl radical under similar conditions. The relative amplitude of the epr signal of Fig. 2 increased in a near-linear fashion with increasing pH, to pH 9.0; a similar

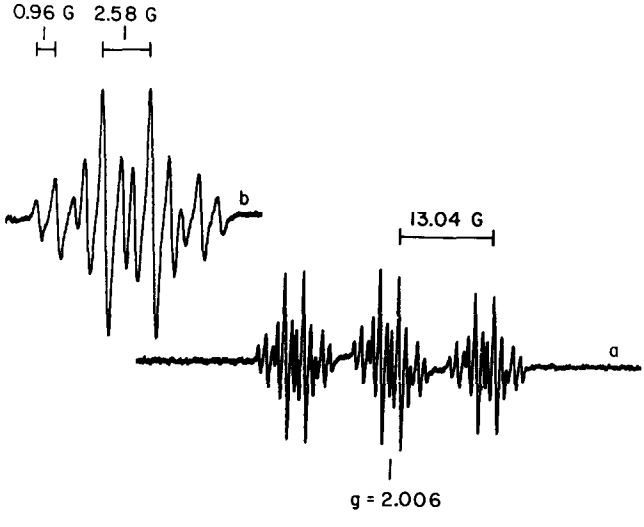


FIG. 2. Electron paramagnetic resonance signal resulting from trapping by nitrosobenzene of a radical produced in the metmyoglobin–cumene hydroperoxide system. The reaction mixture contained 30 m*M* cumene hydroperoxide, 25 m*M* nitrosobenzene, 27.5 μM metmyoglobin, and 5% acetone-d_6 in 0.1 *M* Tris–HCl buffer, pH 9.0. (a) Complete signal. (b) Low-field portion of the signal on an expanded magnetic field scale. Instrument settings: power, 20 mW; modulation amplitude, 0.4 G; time constant, 0.1 sec; scan time, 4 min; magnetic field, 3257 G; microwave frequency, 9.153 GHz; gain, 8×10^2 for (a) and 10×10^2 for (b). Temperature, 22°C.

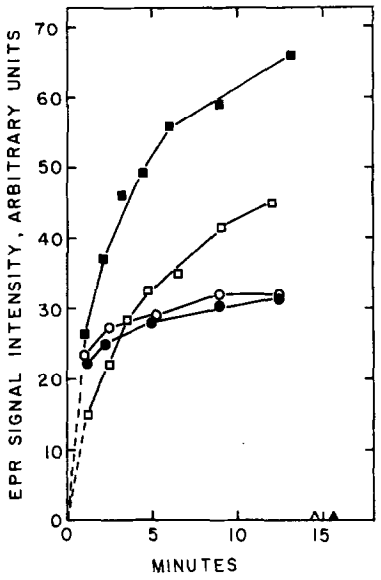


FIG. 3. Effect of aminopyrine on the epr signal intensity of the nitrosobenzene-trapped radical produced in the metmyoglobin–cumene hydroperoxide system. The reaction mixtures contained 27.3 μM metmyoglobin, either no aminopyrine (open symbols) or 40 mM aminopyrine (closed symbols), and additional components, as indicated, in 0.1 M Tris–HCl buffer, pH 9.0: □, ■, 7.5 mM nitrosobenzene and 10 mM cumene hydroperoxide; ○, ●, 25 mM nitrosobenzene and 30 mM cumene hydroperoxide. Metmyoglobin was omitted in the control experiments, △, ▲. Instrument settings were similar to those given in the legend of Fig. 2 except that the gain was 1.5×10^3 (○, ●) or 2.5×10^3 (□, ■). Temperature, 22°C.

pH dependence was previously observed for generation of the methyl radical in this same system (*13*).

Evidence for the identity of this nitroxide radical will be presented later, but it clearly is *not* the nitrosobenzene-trapped methyl radical, which has been previously reported (*19*). In order to determine why the epr signal of the methyl radical trapped by nitrosobenzene was not also detected, an experiment was performed in which both MNP and nitrosobenzene were added to the metmyoglobin–cumene hydroperoxide system. Since the outermost epr lines of the MNP-trapped methyl radical do not overlap the narrower epr signal of Fig. 2, the former nitroxide should be detected, if actually produced in this system. However, with MNP present the epr signal was completely unchanged, and no component of the MNP-trapped methyl radical was detected. This result suggested that the methyl radical, a product of decomposition of the cumyloxy radical, is not formed in this system in the presence of these concentrations of nitrosobenzene (Fig. 2). In the spin-trapping study with MNP, it was shown that aminopyrine very effectively inhibited trapping of the methyl radical by MNP (*13*). With nitrosobenzene as the spin-trap probe, an effect of aminopyrine was observed only when the aminopyrine concentration was much larger than the nitrosobenzene concentration, as shown in Fig. 3. Under these conditions, an increase, rather than a decrease, of the signal amplitude of the nitroxide product was observed. A control experiment

in which aminopyrine was added after the epr signal had attained its maximal intensity demonstrated that aminopyrine did not react directly with this nitroxide. The optimal pH for detecting radicals trapped by nitroso compounds in this system is alkaline, while the aminopyrine radical is most stable at acidic pH (cf. Fig. 1) (*12*); consequently, the aminopyrine radical was not detected under the conditions of the spin-trapping experiments, even when large aminopyrine concentrations were added. The effect of aminopyrine and the failure to detect a trapped methyl radical in this system are consistent with the following interpretation.

The unimolecular decomposition of the cumyloxy radical yields acetophenone, in addition to the methyl radical (Fig. 4) (*7*). Thus, by measuring the amounts of acetophenone and cumenol produced in this system in the absence and presence of aminopyrine, we previously established why aminopyrine inhibits so effectively MNP trapping of the methyl radical (*13*). The results indicated that aminopyrine reacts much more readily with the methyl radical than with the cumyloxy radical, presumably yielding the aminopyrine radical and methane as products. The results of the present study indicate that nitrosobenzene traps the cumyloxy radical very efficiently, thereby preventing formation of the methyl radical. An effect of aminopyrine was observed only at low concentrations of the spin trap (Fig. 3), conditions which would permit some cumyloxy radicals to escape this trapping reaction and to react with aminopyrine, if present. However, reduction of the cumyloxy radical required high concentrations of aminopyrine, consistent with previous conclusions about the relative reactivities of the methyl and cumyloxy radicals with aminopyrine (*13*). The data in Fig. 3 indicate that aminopyrine actually stimulates decomposition of cumene hydroperoxide, as was clearly demonstrated in the previous spin-trapping study by quantitative product assays in the absence and presence of aminopyrine (*13*). The mechanism of aminopyrine stimulation is probably very similar to that proposed for hydroquinone stimulation of the Fe^{2+}-induced decomposition of cumene hydroperoxide (*7*): the radical species of each compound is oxidized more readily than the fully reduced compound and can sustain the chain reaction by reducing another

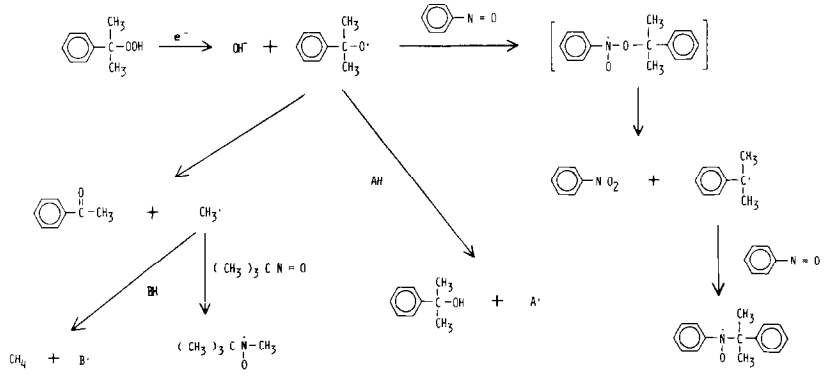


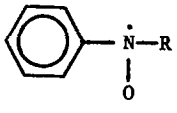
FIG. 4. Mechanism of the radical decomposition of cumene hydroperoxide, including the trapping reactions of MNP and nitrosobenzene. AH and BH are electron donors.

molecule of cumene hydroperoxide. It should be noted that oxidation of the aminopyrine radical to the iminium cation is a necessary reaction in the proposed mechanism of formaldehyde production (Eq. [1]). Thus, the aminopyrine radical, by stimulating the radical chain decomposition of cumene hydroperoxide, effectively "catalyzes" oxidation of more aminopyrine. The essential role of the radical species of aminopyrine in this process was demonstrated by a control experiment, shown in Fig. 3: in the absence of metmyoglobin, high concentrations of aminopyrine could not initiate formation of the radical trapped by nitrosobenzene. As shown in Fig. 3, other control experiments were also negative under the conditions of these experiments. However, over long periods of time, the control containing only nitrosobenzene and cumene hydroperoxide developed the same epr signal which formed in a matter of minutes in the presence of metmyoglobin (Fig. 3).

The next question to be considered is whether the identity of the nitroxide radical detected in this system (Fig. 2) supports this mechanism. The hyperfine splitting pattern is consistent with that expected for a trapped cumyloxy radical, but does not provide an unambiguous identification. Since the observed splittings arise solely from magnetic nuclei of the nitrosobenzene moiety, the same general splitting pattern would be observed for any tertiary alkyl or tertiary alkoxy radical trapped by nitrosobenzene. The values of the hyperfine splitting constants of this nitroxide, in aqueous solution, are compared in Table 1 with values reported for related nitroxides produced by various means in organic solvents. Since hyperfine splitting constants are known to depend on the solvent, no conclusions can be drawn from these data concerning the identity of the trapped radical. Moreover, we have found no literature reference to the epr signal of the nitrosobenzene-trapped cumyloxy radical.

TABLE 1

HYPERFINE SPLITTING CONSTANTS REPORTED FOR TERTIARY ALKYL AND TERTIARY ALKOXY PHENYL NITROXIDES



R	i =	A_i (G) N	$H_{o,p}$	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	(*22*)
		11.21	2.46	0.88	Cumene	(*23*)
$-OC(CH_3)_3$		14.9	3.1	1.0	$CFCl_3$	(*28*)
		14.7	3.03	0.94	Benzene	(*29*)
$-C(CH_3)_3$		12.4	2.0	0.9	*o*-Xylene	(*30*)
		13.35	1.7	0.85	CH_2Cl_2	(*31*)

The best evidence for the origin and identity of this radical was published by Terabe and Konaka, who studied the radical-trapping reactions of aromatic nitroso compounds in organic solvents (*22*). They produced an epr signal similar to that in Fig. 2 by heating a benzene solution of cumene hydroperoxide and nitrosobenzene, and we have confirmed their result. Moreover, when the stable nitroxide produced in the aqueous metmyoglobin–cumene hydroperoxide system was extracted into benzene, the hyperfine splitting constants became identical to those of the radical produced under Terabe and Konaka's conditions. They identified this radical as a nitrosobenzene-trapped cumyl radical; this structure was also rather conclusively established by Malatesta and Ingold, who generated the same nitroxide by several distinct photochemical reactions (*23*). Based on a series of control experiments with cumene, Terabe and Konaka proposed (*22*) that the nitroxide formed by addition of the cumyloxy radical to nitrosobenzene was very unstable and decomposed to nitrobenzene and the cumyl radical, which was subsequently trapped by nitrosobenzene. This explanation is entirely consistent with the results of our study. Thus, the failure to detect methyl radicals, trapped by nitrosobenzene or by MNP added with nitrosobenzene, indicates that the precursor of the methyl radical was very efficiently trapped by nitrosobenzene. Efficient trapping of the cumyloxy radical by the spin trap can also explain the experimental conditions required for observing any effect of aminopyrine.

It is clear that the formation of nitrobenzene and the cumyl radical may lead to other possible reactions in an already complex radical chain reaction. For example, the relatively large aminopyrine–nitrosobenzene concentration ratio required for aminopyrine stimulation of formation of the epr signal could conceivably result in reaction of the cumyl radical with aminopyrine instead of nitrosobenzene. The effective cumyl radical concentration would be immediately decreased by this reaction, but the aminopyrine radical so formed could sustain the decomposition of cumene hydroperoxide. However, each cumyl radical removed in this way could produce, at most, a single replacement cumyl radical. Thus, assuming 100% trapping efficiency for both the cumyloxy and cumyl radicals, no net increase of the nitrosobenzene-trapped cumyl radical would occur, and with lower trapping efficiency, a decrease would be expected. However, the observation that aminopyrine increased the concentration of this species (Fig. 3) indicates that the reaction of the cumyl radical with aminopyrine does not occur.

At low nitrosobenzene concentrations, in the absence of aminopyrine, the cumyloxy radicals not trapped by nitrosobenzene would be expected to decompose to methyl radicals. Why, then, was the latter species not trapped in the metmyoglobin–cumene hydroperoxide system under these conditions? There are two possible explanations. First, since the methyl radical is a highly reactive species, only a fraction of these radicals may be trapped, even with large concentrations of a spin trap. For example, the concentration of the nitrosobenzene-trapped cumyl radical has been estimated to be at least 20–30 times larger than that of the methyl radical trapped by MNP under similar conditions in the metmyoglobin–cumene hydroperoxide system. Other possible reactions of the methyl radical yield methanol, methane, or ethane (*7*), but it is unlikely that the latter product is formed in these aqueous solutions. Thus, the conditions required

for formation of the methyl radical, namely, low concentrations of nitrosobenzene, are also those which decrease the probability of trapping this very reactive species. A second factor related to detection of methyl radicals trapped quite generally by nitroso spin traps is the facile dismutation reaction of nitroxide radicals with protons on the alpha carbons (*24*). These nitroxides are considerably less stable than nitroxides having no alpha carbon protons, i.e., the nitrosobenzene-trapped cumyl radical. Thus, instability of the nitrosobenzene-trapped methyl radical most likely prevented its detection under conditions where very low concentrations of this species might have been formed.

Since the cumyl radical trapped by MNP is expected to be a very stable species, the failure to detect this nitroxide in the previous study with MNP supports the proposed mechanism for formation of the cumyl radical, i.e., by decomposition of the unstable phenylcumyloxy nitroxide. An alternate route for formation of the cumyl radical is by one-electron oxidation of cumene present as an impurity in the cumene hydroperoxide. Although the hydroperoxide was highly purified as the sodium salt and contained no measurable cumene, this possibility was tested by some control experiments. When cumene, dissolved in acetone, was added to a reaction mixture containing cumene hydroperoxide, nitrosobenzene, and metmyoglobin, there was no stimulation of the epr signal, as would be expected if the cumyl radical arose solely from oxidation of cumene. The significance of the slight inhibition actually observed is not clear, since very small concentrations of cumene produced a noticeable turbidity due to the low solubility of this compound in water.

In order to test the generality of the proposed radical pathway of decomposition of cumene hydroperoxide initiated by metmyoglobin, we have performed some preliminary spin-trapping experiments with other heme compounds. Catalase has been shown to catalyze aminopyrine *N*-demethylation by cumene hydroperoxide (*25*), and hemin functions similarly, with an activity of 17 mol/min/mol heme (B. W. Griffin, unpublished results). Both heme compounds stimulated the decomposition of cumene hydroperoxide to the same radical species which were trapped by MNP and nitrosobenzene, respectively, in the metmyoglobin-containing system; the pH dependence for this reaction in all three systems was very similar. However, HRP, a hemeprotein which cannot utilize cumene hydroperoxide for peroxidatic reactions (*3*), was completely ineffective at initiating formation of these radicals. Moreover, ferrous ion functioned similarly to these heme compounds, with some minor differences: (1) the reaction occurred most readily in acidic solution (pH $\sim$ 4.0), a consequence of the redox properties and solubility of unchelated ferrous ion (*26*); and (2) the nitrosobenzene-trapped methyl radical was clearly detected, along with the trapped cumyl radical, immediately after initiating the reaction. However, the latter nitroxide radical persisted, while the trapped methyl radical decayed in a matter of minutes. These observations support the conclusions discussed earlier concerning the relative stabilities of these two nitroxides and demonstrate that nitrosobenzene can trap the methyl radical in aqueous solutions. That the methyl radical trapped by nitrosobenzene was detected only in the ferrous ion–cumene hydroperoxide system may relate to the acidity of these solutions.

These experimental results provide strong evidence for a common radical mechanism of cumene hydroperoxide decomposition initiated by ferrous ion or by several ferric heme compounds known to "catalyze" peroxidatic reactions with this hydroperoxide. Presumably, the electron-rich heme group supplies an electron for the reduction of cumene hydroperoxide to the cumyloxy radical and OH^-; it is expected that an oxidized heme species analogous to that formed from metmyoglobin with H_2O_2 (*27*) would be produced concomitantly. However, in numerous attempts we have been unable to detect such a species during the oxidation of metmyoglobin by cumene hydroperoxide. The hydroperoxide concentrations required to produce any measurable change in absorbance of the hemeprotein, as well as measurable oxidation of electron donors, also caused irreversible loss of the heme, as established by the pyridine hemochromagen assay. The rapid destruction of cytochrome *P*-450 heme by cumene hydroperoxide, and the inhibition of this reaction by added *N*-methyl substrates, has been reported (*3*). We observed that both aminopyrine and nitrosobenzene were effective inhibitors of metmyoglobin destruction by cumene hydroperoxide (B. W. Griffin, unpublished results). These findings indicate that the observed heme destruction results from attack of the heme group by the reactive radicals derived from cumene hydroperoxide. Thus, this study has established some important distinctions between the very low peroxidatic activities typical of those hemeproteins which react with cumene hydroperoxide and the remarkably efficient catalysis characteristic of the true hemeprotein peroxidases which utilize H_2O_2. In the former case, the functional oxidants are organic free radicals arising from heme-initiated decomposition of the hydroperoxide, whereas well-characterized heme species are known to be the catalytically functional oxidants in the latter reactions (*1*, *2*).

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