Mechanism of N-Demethylation of Aminopyrine by Hydrogen Peroxide Catalyzed by Horseradish Peroxidase, Metmyoglobin, and Protohemin†

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ABSTRACT: Hemeproteins and protohemin have been investigated as models for oxidative N-demethylation reactions catalyzed by liver microsomal cytochrome P-450. At 37 °C, catalytic activities for the H_2O_2-supported N-demethylation of aminopyrine were (mol of formaldehyde per min per mol of heme): 7.8 for protohemin and 44 for metmyoglobin, compared with the value of 2.5×10^4 previously reported for horseradish peroxidase (Griffin, B. W. (1977) *FEBS Lett. 74,* 139–143). Reduction of horseradish peroxidase compound I to compound II by aminopyrine was rapid and stoichiometric (0.53 mol of aminopyrine/mol of compound I), while reduction of the higher oxidation state of metmyoglobin required a large excess of aminopyrine. Aminopyrine analogues containing fewer *N*-methyl groups functioned similarly as electron donors. In all three heme catalyst–H_2O_2 systems, the same transient free radical of aminopyrine was detected by electron paramagnetic resonance at room temperature. With horseradish peroxidase as catalyst, several correlations were established between free radical concentrations and rates of formaldehyde production measured under identical conditions; the most important of these was the close correspondence of the kinetics of free radical generation and product formation. Based on these findings, as well as the structural similarity of aminopyrine to violenes (dialkylaminoalkenes), a reaction mechanism is proposed in which aminopyrine undergoes two successive one-electron oxidations to an iminium cation, followed by hydrolysis to the formaldehyde and secondary amine products. Thus, the oxygen atom of formaldehyde comes from H_2O and not directly from the oxidant, as has been proposed for cytochrome P-450 catalysis of this reaction.

Oxidative N-demethylation of substrates catalyzed by liver microsomal cytochrome P-450, requiring O_2 and NADPH,[1] is considered a characteristic monooxygenation reaction of this enzyme (Conney, 1967; McMahon, 1966). The reaction product is presumed to be a carbinolamine intermediate (Parke, 1968), analogous to the hydroxylated products which are formed in many cytochrome P-450 dependent reactions (Griffin et al., 1978). Subsequent nonenzymatic breakdown of this unstable product is considered to give rise to an amine and formaldehyde, the product typically assayed. Reports that several cytochrome P-450 activities (Rahimtula & O'Brien, 1974, 1975; Ellin & Orrenius, 1975), including aminopyrine N-demethylation, are supported by cumene hydroperoxide in the absence of both O_2 and NADPH have implicated a "peroxidase" activity of the liver enzyme under these conditions. It has been suggested that the "peroxidase" and monooxygenase reactions catalyzed by cytochrome P-450 proceed through a common enzyme oxidant (Rahimtula & O'Brien, 1975; Rahimtula et al., 1974; Nordbloom et al., 1976), analogous to compound I of horseradish peroxidase (HRP) and catalase. However, unlike the higher oxidation states of HRP and catalase, which oxidize substrates by electron abstraction (Schonbaum & Chance, 1976; Morrison & Schonbaum, 1976), this species of cytochrome P-450 is presumed to oxidize substrates by direct oxygen insertion (Rahimtula et al., 1974; Nordbloom et al., 1976).

Other hemeproteins reported to catalyze aminopyrine N-demethylation include catalase, in the presence of several organic hydroperoxides (Kadlubar et al., 1973), and HRP in the presence of hydrogen peroxide (Gillette et al., 1958); however, these activities are not well characterized. Since simple systems consisting of a purified hemeprotein and a hydroperoxide may serve as useful models for the reaction mechanism of the complex, membrane-bround cytochrome P-450 enzyme system, we have undertaken detailed studies of the oxidation of typical cytochrome P-450 substrates catalyzed by various hemeproteins.

A preliminary account of the HRP-catalyzed N-demethylation of aminopyrine by hydrogen peroxide was recently reported by this laboratory (Griffin, 1977). The significant findings of that report were: (1) a catalytic activity of 2.5×10^4 mol of formaldehyde per min per mol of heme, which is about 1000-fold larger than any reported values of the aminopyrine *N*-demethylase activity of cytochrome P-450 (Griffin, 1977); and (2) detection of millimolar concentrations of a transient aminopyrine free radical, implicated by several kinds of evidence as an intermediate in the reaction. The present study, which characterizes further hydrogen peroxide supported N-demethylation of aminopyrine catalyzed by hemeproteins and protohemin, indicates that oxidative N-demethylation may be a rather general catalytic activity of heme compounds. Based on the experimental findings of this study, a reaction mechanism involving the aminopyrine free radical is proposed for these systems which is quite different from the presently accepted mechanism of cytochrome P-450 catalyzed N-demethylation reactions.

Materials and Methods

Oxymyoglobin purified from fresh beef heart (Gotoh &

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[1] Abbreviations used: NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); HRP, horseradish peroxidase; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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Shikama, 1974) was oxidized with a slight excess of $K_3Fe(CN)_6$ which was removed by chromatography on a Sephadex G-25 column (Antonini & Brunori, 1971). HRP (type VI, salt-free powder) with a RZ value of approximately 3.0 and protohemin (equine, type III) were used as supplied from Sigma. Aminopyrine and antipyrine were purchased from Aldrich; 4-aminoantipyrine and *N*-methyl-4-aminoantipyrine were generously provided by Winthrop Laboratories. The *N*-methyl-4-aminoantipyrine was purified by recrystallization from ether–petroleum ether to remove colored contaminants resulting from air oxidation of this compound. All other chemicals used were reagent grade.

Heme concentrations were determined by the pyridine hemochromagen method (Falk, 1964). Hydrogen peroxide concentration was assayed spectrophotometrically at 240 nm (Nelson & Kiesow, 1972). Solutions of ascorbate were standardized by titration with $K_3Fe(CN)_6$. Formaldehyde was assayed by the Nash procedure (Nash, 1953), after the reaction had been quenched with 15% trichloroacetic acid and centrifuged to remove precipitated protein.

A Waters Associates high-performance liquid chromatograph with μ Bondapak C_{18} column was employed to monitor the time dependence of substrate and product concentrations in the HRP-catalyzed reactions. The flow rate was maintained at 2 mL/min and detection was at 254 nm. The solvent system employed was methanol/water (50:50), the composition of which was increased linearly to 100% methanol. An aliquot (150 μL) of the reaction mixture was withdrawn at the indicated time points and quenched by mixing with an equal volume of methanol. Each point is the average of two determinations on 10-μL injections of the quenched sample. Concentrations were evaluated by measurement of peak areas and comparison with a standard curve.

All spectrophotometric measurements were made with a Beckman Model 25 UV-visible spectrophotometer. EPR spectra were recorded at room temperature with a Varian E-4 spectrometer. After initiating the reaction by addition of catalyst, the mixture was transferred to a calibrated capillary tube and the EPR signal scanned immediately, about 1 min after mixing. For comparison of EPR signals generated in different systems or determination of radical concentrations, experimental conditions were chosen to optimize the stability of the transient radical species. Aqueous solutions of $CuSO_4$ and Fremy's salt (peroxylaminedisulfonate) were employed as $S = \frac{1}{2}$ intensity standards, with excellent agreement between the two. Radical concentrations were quantitated by collecting and doubly integrating EPR spectra with a dedicated Digital Equipment Corp. PDP11/05 computer interfaced to the EPR spectrometer. It was established that the computed area of the Fremy's salt signal is independent of modulation amplitude. Because limitations on the number of data points available in computer memory prevented integration of the resolved aminopyrine radical signal, the effect of modulation amplitude could be examined only under conditions of overmodulation; however, no effect on the computed area was observed.

Under many experimental conditions of this study, rapid decay of the aminopyrine free radical prevented quantitative double integration of the EPR signal. For this reason, all reported radical concentrations were determined from the maximal EPR signal amplitude recorded at a fixed magnetic field as a function of time. For these experiments, the signal was overmodulated, resulting in a typical first derivative spectrum with a peak-to-peak width of about 35 G. Under these conditions, the value of the low-field signal maximum was rather insensitive to low-frequency, low-amplitude fluctuations in magnetic field. The kinetic EPR scan was started within 50–60 s after initiating the reaction by manual mixing. Where the radical concentration could be determined by double integration of a stable EPR signal, a comparison was made between the computed area of the overmodulated signal and the maximal signal amplitude determined in a kinetic experiment under identical experimental conditions. The relationship so established was employed to determine radical concentrations in all kinetic EPR experiments.

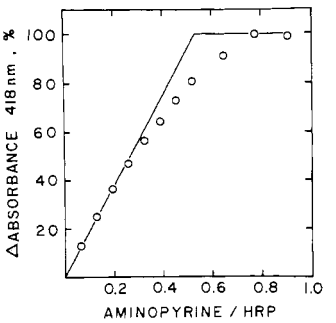
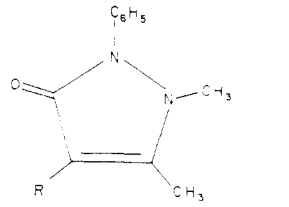


FIGURE 1: Stoichiometry of reduction of horseradish peroxidase compound I to compound II by aminopyrine. The absorbance spectrum of compound I, formed by adding a slight excess (1.5- to 4.0-fold) H_2O_2 to 4.6 nmol of HRP in 2.0 mL of 0.1 M potassium phosphate buffer (pH 7.0), was recorded. Then the increase in absorbance at the absorbance maximum (418 nm) of compound II, produced by adding increasing amounts of aminopyrine to the solution, was measured. With aminopyrine (and all electron donors examined), the titration was taken to complete formation of compound II, corresponding to $\Delta A = 100\%$. The same stoichiometry was determined when the titration was monitored at 428 nm, isosbestic for ferric peroxidase and compound I.

Results

As reported previously (Griffin, 1977), the maximal rate of the HRP-catalyzed reaction occurs near pH 7.0. In potassium phosphate buffer, pH 7.0, 37 °C, the reaction rate was maximal near 0.4 mM H_2O_2 and was inhibited by larger peroxide concentrations; under optimal conditions, the apparent K_m value for aminopyrine was determined from a Lineweaver–Burk plot to be 8.5 mM. The metmyoglobin-catalyzed N-demethylation of aminopyrine by H_2O_2 exhibited maximal rates near pH 5.0 to 5.5; for the protohemin-catalyzed reaction, a broad pH optimum, at pH 7.0 to 8.0, was observed. For protohemin and metmyoglobin, apparent K_m values for H_2O_2 were determined from Lineweaver–Burk plots. At pH 7.7, 37 °C, K_m and V_{max} were determined for protohemin to be 1.45 mM H_2O_2 and 7.8 mol of formaldehyde per min per mol of heme, respectively; for metmyoglobin (pH 6.2, 37 °C), these values were 1.30 mM H_2O_2 and 44 mol of formaldehyde per min per mol of heme, respectively. However, at pH 7.2, these parameters for metmyoglobin catalysis decreased to 0.13 mM H_2O_2 and 6.9 mol of formaldehyde per min per mol of heme. The rate of the protohemin-catalyzed reaction as a function of aminopyrine concentration was maximal near 3 mM aminopyrine, with inhibition at higher substrate concentrations. For metmyoglobin catalysis, maximal rates were attained at larger aminopyrine concentrations (6–12 mM), and no aminopyrine inhibition was observed.

Since we previously reported that aminopyrine can function as an electron donor to both compounds I and II of HRP (Griffin, 1977), the stoichiometry of the reduction of compound I to compound II was determined. The titration data shown in Figure 1 indicate that this reaction occurs very rapidly and that the molar ratio of aminopyrine to HRP compound I extrapolated to the end point is 0.53. Several analogues of aminopyrine containing fewer *N*-methyl groups or lacking the amino group (Figure 2) were examined for their ability to



	R
ANTIPYRINE	-H
4-AMINOANTIPYRINE	$-NH_2$
N-METHYL-4-AMINOANTIPYRINE	$-NHCH_3$
AMINOPYRINE	$-N(CH_3)_2$

FIGURE 2: Structure of aminopyrine and various analogues.

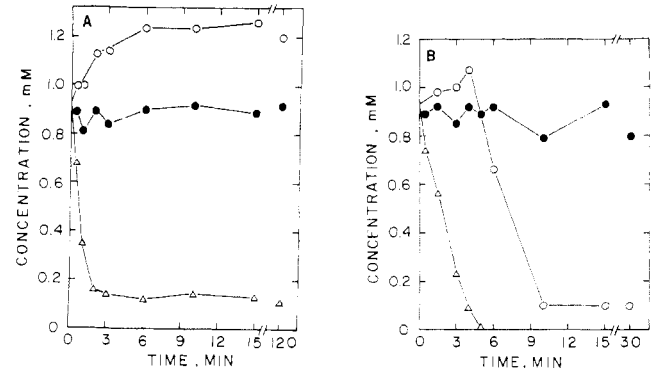


FIGURE 3: Time course of oxidation of aminopyrine, *N*-methyl-4-aminoantipyrine, and 4-aminoantipyrine by hydrogen peroxide catalyzed by horseradish peroxidase. The reaction mixture contained approximately 1 mM each of aminopyrine and the two analogues, variable hydrogen peroxide as indicated, and 78 nM horseradish peroxidase in 0.1 M potassium phosphate buffer (pH 7.5), 37 °C. After the reaction was initiated by adding the enzyme, the concentrations of the three substrates were monitored as a function of time by the HPLC assay described. (A) $[H_2O_2]$ = 1.0 mM; (B) $[H_2O_2]$ = 3.0 mM. In identical control experiments with the enzyme omitted, no significant decrease of any substrate was observed after 35 min. (●) Aminopyrine; (○) 4-aminoantipyrine; (△) *N*-methyl-4-aminoantipyrine.

donate electrons to compound I. Of these analogues, only antipyrine was ineffective; reduction of 1 mol of HRP compound I to compound II required 0.45 mol of 4-aminoantipyrine and 0.27 mol of *N*-methyl-4-aminoantipyrine. Thus, under the same experimental conditions, aminopyrine and 4-aminoantipyrine function as two-electron donors, and *N*-methyl-4-aminoantipyrine functions as a four-electron donor, in the one-electron reduction of compound I. An identical control experiment confirmed that ascorbate donates two electrons for the conversion of compound I to compound II.

The ability of aminopyrine and its analogues to reduce the higher oxidation state of metmyoglobin (George & Irvine, 1952; Yonetani & Schleyer, 1967) was also established. At pH 8.0, approximately 90% of the Soret absorbance of metmyoglobin could be recovered by adding a large excess of aminopyrine to the higher oxidation state of metmyoglobin, formed by adding a twofold excess of H_2O_2 to the ferric hemeprotein. The reaction exhibited a distinct isosbestic point but occurred too slowly for determination of stoichiometry. Under more acidic conditions, where this oxidized form of metmyoglobin is known to decay in a complex manner to other species (Fox et al., 1974), aminopyrine functioned similarly, but with smaller recovery of metmyoglobin. As observed with HRP, both 4-aminoantipyrine and *N*-methyl-4-aminoantipyrine reduced the higher oxidation state of metmyoglobin, but antipyrine did not. However, none of these electron donors reduced ferric HRP or metmyoglobin, even under a carbon monoxide atmosphere.

Because millimolar concentrations of 4-aminoantipyrine and *N*-methyl-4-aminoantipyrine interfere with the Nash assay for formaldehyde, we have employed high performance liquid chromatography (HPLC) to study the HRP-catalyzed oxidation of these compounds. The time course of disappearance of aminopyrine and these two analogues in a reaction mixture containing equimolar amounts of the three substrates was monitored at two different H_2O_2 concentrations. When the H_2O_2 concentration was one-third the total molar substrate concentration, Figure 3A, *N*-methyl-4-aminoantipyrine rapidly decreased concomitantly with the appearance of 4-aminoantipyrine. This indicates that the former compound was converted, by N-demethylation, to the latter, but the final substrate levels indicate significant oxidation of 4-aminoantipyrine. When the peroxide concentration was equal to the total substrate concentration, Figure 3B, 4-aminoantipyrine completely disappeared after the rapid and complete loss of *N*-methyl-4-aminoantipyrine. Since aminopyrine was not oxidized to any significant extent in either experiment, clearly the two analogues are oxidized more readily than the parent compound in the HRP-H_2O_2 system. Although 4-aminoantipyrine may undergo N-demethylation in the HRP-catalyzed reaction, other routes of oxidation of this compound are suggested by the HPLC profile of the reaction products, which we are attempting to identify.

The aminopyrine free radical EPR signals produced by protohemin, metmyoglobin and HRP in the presence of H_2O_2 are compared in Figure 4a; all have a *g* value of 2.003 and appear to be identical.[2] For each system of Figure 4a, experimental conditions were chosen for both maximal signal intensity and stability of the radical. The radical exhibits greatest stability below pH 6.0. Under these conditions only one set of control experiments, those with the catalyst omitted (Figure 4b), showed a very weak signal identical with those of Figure 4a. The hydroxyl radical, formed by acid-promoted decomposition of H_2O_2, is probably the one-electron oxidant in these control experiments. The aminopyrine free radical has been previously detected with Fenton's reagent (Griffin, 1977), known to generate hydroxyl radicals (Walling, 1975).

The HRP-catalyzed reaction has been examined in detail to provide additional support for the involvement of the radical in the N-demethylation reaction. In Figures 5 and 6, the initial rate of formaldehyde production and the maximal radical concentration (determined in a kinetic EPR experiment, as described) were measured under identical conditions. The H_2O_2 dependence of these two parameters is shown in Figure 5. The rate of N-demethylation is considerably inhibited at these large peroxide concentrations required for the EPR experiments; the radical concentration shows a similar, but less pronounced, inhibition at large peroxide concentration. Because of limitations on the EPR experiments (i.e., mixing time and spectrometer sensitivity), peroxide concentrations less than 2 mM could not be used: although this concentration is inhibitory, the reaction was over before the signal could be scanned. The effects of varying aminopyrine concentration on both the maximal radical concentration and the initial rate of formaldehyde production are shown in Figure 6. Of necessity, these experiments were also carried out at an inhibitory concentration of peroxide; however, the two parameters show a similar dependence on concentration of the *N*-methyl substrate.

[2] In the preliminary communication of a portion of this work (Griffin, 1977), the magnetic field scale of the aminopyrine free radical EPR signal was incorrectly labeled; the scale in Figure 4 is correct.

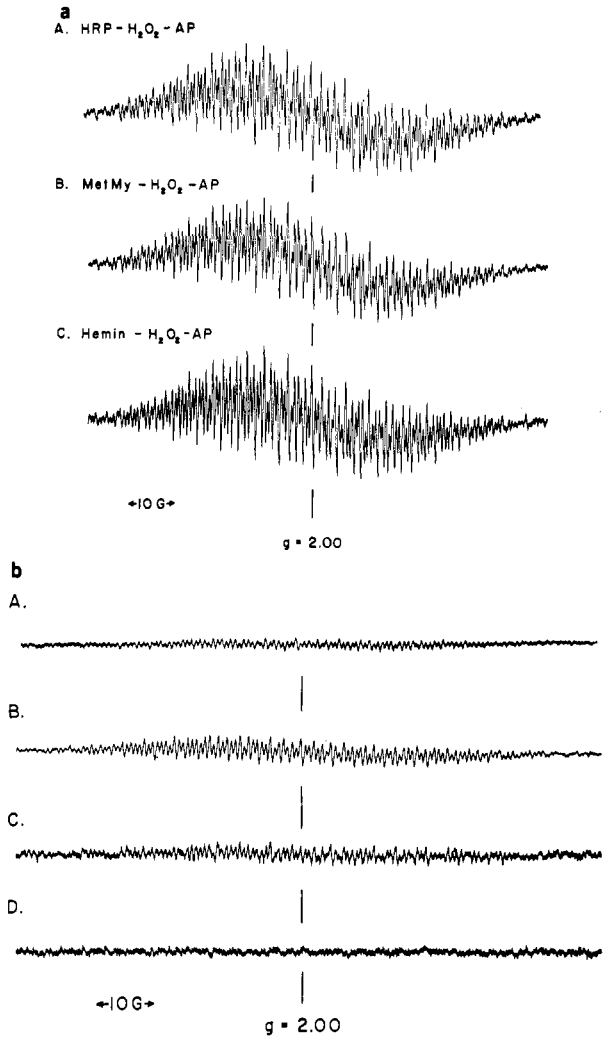


FIGURE 4: Aminopyrine free radical EPR signal generated by horseradish peroxidase, metmyoglobin, and protohemin in the presence of hydrogen peroxide. All EPR spectra were recorded at room temperature (22 °C) with the following instrument settings: power, 10 mW; modulation amplitude, 0.82 G; scan time, 4 min; time constant, 0.3 s; instrument gain, variable as indicated. (Panel a) These experiments contained the indicated concentrations of hydrogen peroxide, aminopyrine, and the catalyst in 0.1 M buffer at the stated pH and instrument gain. (A) Horseradish peroxidase: 10 mM hydrogen peroxide, 10 mM aminopyrine, 2.0 μM horseradish peroxidase; potassium acetate (pH 5.8); gain, 2.5×10^3. (B) Metmyoglobin: 20 mM hydrogen peroxide, 20 mM aminopyrine, 10 μM metmyoglobin; potassium acetate (pH 5.0); gain, 3.2×10^3. (C) Protohemin: 20 mM hydrogen peroxide, 20 mM aminopyrine, 31 μM protohemin; potassium phosphate (pH 6.0); gain, 6.2×10^3. (Panel b) For these control experiments, all experimental conditions were identical with those of the appropriate experiment described in panel a. (A) Horseradish peroxidase: enzyme omitted. (B) Metmyoglobin: enzyme omitted. (C) Protohemin: catalyst omitted. (D) Protohemin: aminopyrine or hydrogen peroxide omitted (similar controls for horseradish peroxidase and metmyoglobin were also negative).

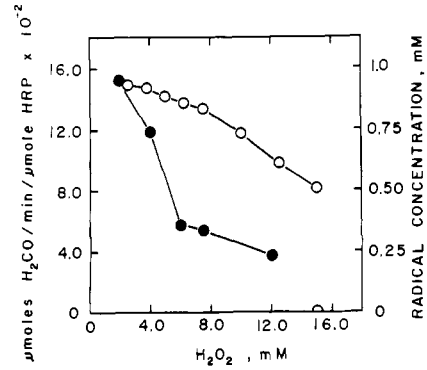


FIGURE 5: Dependence of aminopyrine free radical concentration and initial rate of formaldehyde production on hydrogen peroxide concentration. Reaction mixtures contained 10 mM aminopyrine, 0.5 μM horseradish peroxidase, and variable hydrogen peroxide as indicated in 0.1 M potassium phosphate buffer (pH 7.0), 22 °C. (○) Radical concentration; (●) rate of formaldehyde production. In the absence of hydrogen peroxide, both parameters have zero values. The single point shown on the abscissa is a control for the EPR experiments with the enzyme omitted.

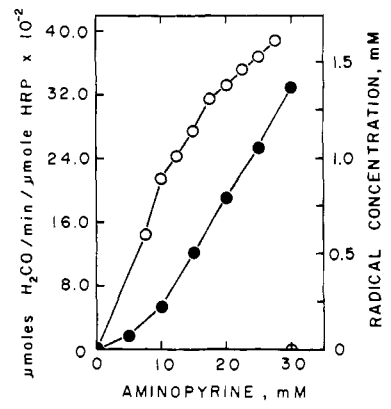


FIGURE 6: Dependence of aminopyrine free radical concentration and initial rate of formaldehyde production on aminopyrine concentration. Experimental conditions were 7.5 mM hydrogen peroxide, 0.5 μM horseradish peroxidase, and variable aminopyrine as indicated in 0.1 M potassium phosphate buffer (pH 7.0), 22 °C. (○) Radical concentration; (●) rate of formaldehyde production. Also shown (on the abscissa) is a control for the EPR experiments with the enzyme omitted.

Kinetic experiments were undertaken to determine the time dependence of free radical generation and formaldehyde production in separate, but otherwise identical, experiments. As can be seen from Figure 7, the concentrations of both the radical and formaldehyde attain their maximal values at very nearly the same time, after which the formaldehyde level remains constant as the radical EPR signal decays rapidly. Moreover, the largest free radical concentration measured in this experiment, 1.5 mM, represents a significant fraction of the final formaldehyde concentration. These kinetic experiments provide strong evidence for the direct involvement of the free radical in aminopyrine N-demethylation. It can be seen in Figure 7 that the radical concentration maintains an apparent steady-state value for a short period of time, after which it increases to the maximal value. This behavior is attributed to relief of peroxide inhibition by conversion of inactive HRP compound III to an active form as the peroxide is depleted. Moreover, the observed acceleration of the rate of formaldehyde production in this experiment supports this interpretation. The maximal radical concentrations measured in kinetic EPR scans showed a linear dependence on the square root of the HRP concentration. This correlation has been predicted and experimentally confirmed for steady-state radical concentrations arising during HRP catalysis (Yamazaki et al., 1960). Thus, the maximal aminopyrine radical concentrations determined in this study reflect approximate steady-state values.

Discussion

The present study has established several correlations, in addition to those previously published (Griffin, 1977), between concentrations of the aminopyrine free radical and rates of N-demethylation of this substrate catalyzed by HRP in the presence of H_2O_2. The strongest evidence for the involvement

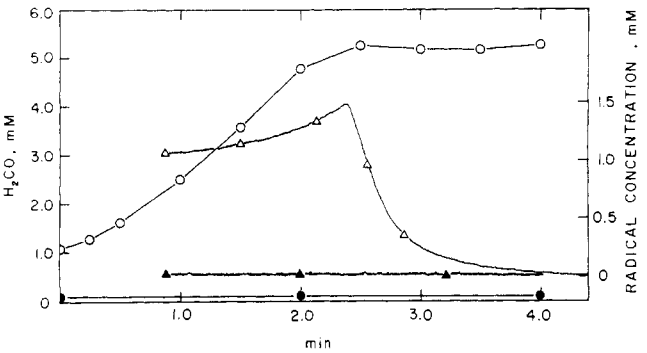


FIGURE 7: Time dependence of generation of the aminopyrine free radical and production of formaldehyde. The reaction mixtures contained 10 mM hydrogen peroxide, 10 mM aminopyrine, and 1.9 μM horseradish peroxidase in 0.1 M potassium phosphate buffer (pH 7.0), 22 °C. (○) Formaldehyde concentration, measured at discrete time points; (△) kinetic EPR scan of the free radical concentration. (●, ▲) Controls with the enzyme omitted, but otherwise identical with the above experiments. Instrument settings for the EPR time scans were: power, 10 mW; modulation amplitude, 4 G; scan time, 4 min; time constant, 0.3 s; magnetic field, 3246 G; microwave frequency, 9.160 GHz; gain, 1.5×10^3.

of this free radical species in the reaction pathway was the parallel kinetic behavior of the free radical and product concentrations. Moreover, two other heme compounds which catalyze the N-demethylation of aminopyrine by H_2O_2 generate the same aminopyrine free radical. There is considerable evidence that this species is a one-electron oxidized free radical cation of aminopyrine, which can be generated by enzymatic (Griffin, 1977), chemical (Griffin, 1977), and electrochemical (Sayo & Masui, 1973) oxidation. A reaction mechanism consistent with all these results can be proposed:

$$R_2N{-}CH_3 \xrightarrow{-e^-} R_2N{-}CH_3^+\cdot \xrightarrow{-H} R_2N^+{=}CH_2 \xrightarrow{H_2O} R_2NH_2^+ + H_2C{=}O \quad (1)$$

As indicated, aminopyrine undergoes a one-electron oxidation to the free radical species, which is further oxidized to an iminium cation. Hydrolysis of the iminium cation yields the amine and formaldehyde.

Although aminopyrine has three *N*-methyl groups, the experiments carried out with the analogues suggested that those on the 4-dimethylamino are cleaved first. The 4-dimethylamino group is one of several characteristic structural features which aminopyrine shares with dialkylamino-substituted alkenes (violenes). In organic solvents these compounds (Gilbert et al., 1970; Fritsch et al., 1970) undergo facile one-electron oxidation to a violet-colored free radical cation, which can be further oxidized to an iminium cation (Figure 8). The classification of aminopyrine as a violene is supported by the report of Sayo & Masui (1973), and our own observation, of an intense violet color associated with large concentrations of the free radical of aminopyrine. The *g* value of this species ($g = 2.003$) is nearly identical with that of violene radical cations (Wertz & Bolton, 1972), but measurably different from that of nitroxides, which exhibit *g* values near 2.006 (Freed, 1976). Also, the relative stability of the aminopyrine free radical and its complex hyperfine splitting pattern are indicative of the resonance stabilization of violene radical cations (Fritsch et al., 1970).

Several examples of purely chemical oxidative N-dealkylations support the mechanism proposed for the enzymatic reaction. Oxidation of benzyldimethylamines by ClO_2 in aqueous solution produces formaldehyde and benzaldehyde, both of which arise from hydrolysis of an intermediate iminium cation with several resonance structures (Rosenblatt et al., 1967). An

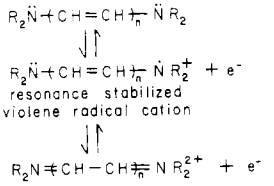


FIGURE 8: Structures of violenes and their one- and two-electron oxidized products.

iminium cation is also considered to be an intermediate in the dealkylation of tertiary amines by peroxidic reagents (*tert*-butyl peroxide, benzyol peroxide, etc.) in organic solvents (Walling, 1957; de la Mare, 1960). Finally, we note the oxidation of *N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD) to a stable violene-type radical known as Wurster's blue cation (Forrester et al., 1968; Hammond et al., 1955; Bolton et al., 1962), and its further oxidation by peroxides to formaldehyde and the corresponding amine. The mechanism of formaldehyde formation from TMPD is entirely analogous to that proposed for aminopyrine N-demethylation.

In stoichiometric experiments, aminopyrine can supply two electrons for the one-equivalent reduction of HRP compound I, indicating that the aminopyrine free radical is readily oxidized to the iminium cationic species by compound I. However, the product of hydrolysis of this iminium cation, *N*-methyl-4-aminoantipyrine, functions as a four-electron donor in the conversion of compound I to compound II. Based on the latter result and the proposed mechanism, aminopyrine is expected to be a six-electron donor. This apparent discrepancy indicates that some reaction beyond the electron transfer steps of eq 1 is rate limiting; that step is probably hydrolysis of the iminium cation. The second-order rate constant for hydrolysis of these compounds by OH^- is considerably larger than the first-order rate constant for hydrolysis by H_2O (Bruylants & Feytmants-de Medicis, 1970); however, at the pH of the titrations, both hydrolysis reactions are expected to be relatively slow. Also, it is known that the structure of the iminium cation greatly influences the rate of hydrolysis (Smith, 1965). With a fully substituted nitrogen atom, the iminium cation derived from aminopyrine should be more resistant to attack by H_2O because it can exist in several resonance forms and is also sterically hindered by the pyrazolone ring. It is also possible that the carbinolamine, considered to be the immediate, short-lived product of hydrolysis (Bruylants & Feytmants-de Medicis, 1970), has a measurable lifetime in the case of aminopyrine. As used here, the term stability implies only that the iminium cation of aminopyrine is hydrolyzed much more slowly than the rapid electron transfer steps by which it is formed; it is unlikely that this species could be isolated.[3] The detection of large steady-state concentrations of the aminopyrine radical under catalytic conditions also confirms that a reaction beyond radical generation is rate limiting. That step may be the hydrolysis reaction or possibly enzymatic oxidation of the free radical, which will occur slowly in the presence of a large excess of aminopyrine. As expected from the results in Figure 3, both *N*-methyl-4-aminoantipyrine and 4-aminoantipyrine inhibit very effectively generation of the aminopyrine free radical in the HRP-H_2O_2 system. However, conditions for detecting free radical species of these compounds have not

[3] A referee has suggested that a dication, rather than the proposed iminium cation, may be formed by the two-electron equivalent oxidation of aminopyrine. The dication, if formed, would be unstable because constraints imposed by the pyrazolone ring would prevent maximal separation of the two positive charges (localized on nitrogen atoms). Thus, it is expected that a dication formed from aminopyrine would rapidly lose a proton to form the more stable iminium cation.

been found. Thus, these radicals, which are presumably formed during the oxidation of both substrates by HRP, must be rather unstable; this may explain why they are oxidized more rapidly than aminopyrine (Figure 3).

Although it seems likely that the cytochrome P-450 catalyzed N-demethylation of aminopyrine supported by hydroperoxides proceeds by the proposed free radical mechanism, direct EPR detection of the free radical species in this system may be difficult because of the inherent low activities of the microsomal enzyme. With the O_2/NADPH-supported reaction, there is the more serious problem of H_2O_2 production concomitant with formaldehyde formation, reported for microsomes (Hildebrandt & Roots, 1975; Estabrook & Werringloer, 1977) as well as a purified, reconstituted system (Nordbloom & Coon, 1976). This H_2O_2 formation makes the experimental problem of distinguishing the proposed oxygen insertion and peroxidatic mechanisms of N-demethylation very difficult. For example, one study of ^{18}O incorporation from $^{18}O_2$ into the aldehyde product of *N*-benzyl-4-phenyl-4-carbethoxypiperidine (McMahon et al., 1969) has been taken as evidence for the oxygen insertion mechanism. However, since catalase inhibitors were not employed in that study, it is possible that rapid labeling of H_2O (by $H_2{}^{18}O_2$ decomposition) introduced ^{18}O into the aldehyde, according to eq 1. Because the H_2O_2 produced could support the peroxidatic pathway, detection of the aminopyrine free radical during the O_2/NADPH reaction would not eliminate the oxygen insertion mechanism. Although there is little conclusive evidence for the direct oxygen insertion mechanism of N-demethylation, it is conceivable that both mechanisms may operate simultaneously in different pools of cytochrome P-450 (Welton et al., 1974).

In attempts to answer these questions, we are extending these studies to both membrane-bound and purified forms of liver microsomal cytochrome P-450. Also the N-demethylation of other typical cytochrome P-450 substrates catalyzed by various hemeproteins is under investigation in order to determine if the proposed mechanism of aminopyrine oxidation is completely general.

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