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# Electron Paramagnetic Resonance Study of the Oxidation of *N*-Methyl-Substituted Aromatic Amines Catalyzed by Hemeproteins

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The oxidation of *N*-mono- and dimethyl-substituted toluidines and aniline by $H_2O_2$, catalyzed by horseradish peroxidase or metmyoglobin, produces organic free radicals, detectable by electron paramagnetic resonance spectroscopy at room temperature. The radical cation of *N,N*-dimethyl-*p*-toluidine was conclusively identified, but the other resolved EPR signals were assigned to radical cations of radical dimerization products, e.g., *N,N,N',N'*-tetramethylbenzidine formed from *N,N*-dimethylaniline. The *N*-demethylase activities of metmyoglobin were found to be uniformly smaller than those of horseradish peroxidase, consistent with the much faster reaction of the latter hemeprotein with $H_2O_2$. Detection of the monomeric radical cation of *N,N*-dimethyl-*p*-toluidine correlated with the largest rate of *N*-demethylation among this class of compounds. These findings emphasize the importance of radical stability (provided, for example, by the *para* methyl substituent) on subsequent competing reactions of the radical cation of the *N*-methyl substrate, i.e., one-electron oxidation leading to formaldehyde release or radical dimerization, which becomes more probable for the less stable radical intermediates. Attempts were made to correlate these results with data obtained for the $O_2$/NADPH-supported oxidation of these same substrates by liver microsomal cytochrome *P*-450. However, pronounced differences in physical state and kinetic properties of this heterogeneous, membrane-associated microsomal hemeprotein and the soluble "model" hemeprotein systems precluded firm conclusions concerning a radical mechanism of *N*-demethylation monooxygenase activities of microsomal fractions.

## INTRODUCTION

Studies carried out in this laboratory have established that several hemeproteins can catalyze the hydroperoxide-dependent *N*-demethylation of aminopyrine and *N*-methyl-substituted aromatic amines (*1, 2*), which are among the best electron donor substrates for both monooxygenation and peroxidatic reactions catalyzed by liver microsomal cytochrome *P*-450 (*3, 4*). Moreover, free radical species of several of these *N*-methyl compounds have been detected directly by EPR during their enzymatic oxidation (*1, 2*). These and other results

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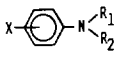
support the following radical mechanism for oxidative cleavage of the *N*-methyl group (1):

$$R_2\underset{(I)}{N{-}CH_3} \xrightarrow{-e^-} R_2\underset{(II)}{N{-}CH_3} \xrightarrow{-e^-,\,-H^+} R_2N^+{=}CH_2 \xrightarrow{H_2O} R_2NH_2^+ + H_2C{=}O. \qquad [1]$$

The substrate radical intermediate (II) undergoes further oxidation to an iminium cation, which, upon hydrolysis, yields the amine and formaldehyde. The oxidation of these electron donors by dehydrogenation, rather than oxygen insertion, is consistent with the well-studied chemical and electrochemical oxidation of this class of compounds (*5–7*), and also with the known catalytic function of hemeprotein peroxidases (*8*). Thus, several kinds of experimental evidence implicate $H_2O$, and not the hydroperoxide, as the immediate source of the oxygen atom of formaldehyde (Eq. [1]). Other workers, however, have suggested that cytochrome *P*-450 catalysis of the oxidation of this class of compounds by both hydroperoxides and $O_2$/NADPH involves a common "active oxidizing" species of the enzyme which transfers an oxygen atom directly from the oxidant (ROOH or $O_2$, respectively) to the substrate (*4, 9, 10*). Although this hypothetical enzyme intermediate has been presumed to be very similar to Compound I of horseradish peroxidase (HRP),[2] an oxygen-transfer function of peroxidase Compound I has never been demonstrated with any electron donor (*8*). Recently, we have demonstrated that the aminopyrine free radical is an intermediate in the oxidation of this drug by cumene hydroperoxide catalyzed by a purified preparation of liver microsomal cytochrome *P*-450 (*11*). This result has provided additional support for the generality of the proposed radical mechanism, Eq. [1].

The considerable evidence for oxidation of *N*-methyl compounds by one-electron abstraction, rather than direct oxygen insertion, in diverse chemical and enzymatic oxidizing systems (*1, 2, 5–7, 11*) has suggested that the cytochrome *P*-450-catalyzed *monooxygenation* of these compounds may also proceed by a similar mechanism. However, several properties of these microsomal oxidation reactions, such as extremely low rates and the concomitant formation of $H_2O_2$, have precluded a definitive experimental test of this hypothesis (*1, 11*). Therefore, as another experimental approach to this problem we have undertaken a comparative, systematic study of the oxidation of a class of closely related *N*-methyl compounds in several different hemeprotein–hydroperoxide systems and in liver microsomal fractions. The goals of this study were to determine if the relative rates of oxidation of these structural analogs showed parallel behavior with different hemeprotein catalysts and if the rate data could be correlated with EPR data, as additional evidence for the generality of this radical pathway. The compounds selected were *N*-mono- and dimethyl-substituted aniline and analogs (toluidine isomers) with a single methyl substituent at one of the three ring positions (Fig. 1). The experimental findings have clarified several features of

[2] Abbreviations used: HRP, horseradish peroxidase; DA, *N,N*-dimethylaniline; DPT, *N,N*-dimethyl-*p*-toluidine; DMT, *N,N*-dimethyl-*m*-toluidine; DOT, *N,N*-dimethyl-*o*-toluidine; MA, *N*-methylaniline; MPT, *N*-methyl-*p*-toluidine; MMT, *N*-methyl-*m*-toluidine; MOT, *N*-methyl-*o*-toluidine; TMB, *N,N,N',N'*-tetramethylbenzidine.



| AMINE | $R_1$ | $R_2$ | X |
|---|---|---|---|
| N,N-DIMETHYLANILINE ( DA ) | $-CH_3$ | $-CH_3$ | -H |
| N,N-DIMETHYL-p-TOLUIDINE ( DPT ) | $-CH_3$ | $-CH_3$ | PARA $-CH_3$ |
| N,N-DIMETHYL-m-TOLUIDINE ( DMT ) | $-CH_3$ | $-CH_3$ | META $-CH_3$ |
| N,N-DIMETHYL-o-TOLUIDINE ( DOT ) | $-CH_3$ | $-CH_3$ | ORTHO $-CH_3$ |
| N-METHYLANILINE ( MA ) | $-CH_3$ | -H | -H |
| N-METHYL-p-TOLUIDINE ( MPT ) | $-CH_3$ | -H | PARA $-CH_3$ |
| N-METHYL-m-TOLUIDINE ( MMT ) | $-CH_3$ | -H | META $-CH_3$ |
| N-METHYL-o-TOLUIDINE ( MOT ) | $-CH_3$ | -H | ORTHO $-CH_3$ |
| N,N,N',N'-TETRAMETHYLBENZIDINE ( TMB ) | $-CH_3$ | $-CH_3$ | PARA $-C_6H_4\ N(\ CH_3\ )_2$ |

FIG. 1. Chemical structures and abbreviations of the *N*-methyl-substituted aromatic amines of this study.

these complex enzyme-catalyzed radical reactions, in particular, the importance of chemical properties of the electron donor substrate and specific physical properties of the reaction system in determining which reaction of several possible competing reactions of a radical species will predominate.

## MATERIALS AND METHODS

The metmyoglobin used in this study was a highly purified preparation isolated from beef heart (*12*); HRP (type VI, salt-free powder) was purchased from Sigma and dialyzed extensively against distilled $H_2O$ prior to use. Microsomal fractions were prepared from the livers of phenobarbital-treated male rats, as previously described (*13*). The aromatic amine substrates were obtained from Aldrich (DPT, DMT, and DA) or Eastman (DOT, MA, MOT, MMT, and MPT), and were distilled before use. Hydrogen peroxide (Mallinckrodt, 30% AR grade, without stabilizers) was assayed spectrophotometrically at 240 nm (*14*). Cumene hydroperoxide was purified as the sodium salt (*15*) from a technical grade product of Matheson, Coleman and Bell; the purity of the salt was confirmed by iodometric titration (*16*) and hplc analysis. Formaldehyde was assayed by the standard Nash procedure (*17*) in aliquots of the reaction mixture which had been quenched with trichloroacetic acid and centrifuged to remove the protein precipitate. Incubations of microsomes with the amine substrates were performed at room temperature and contained the following components: 0.1 *M* Tris–HCl buffer at pH 7.5 or 8.0 (specified in Table 3), 0.15 *M* KCl, 10 m*M* $MgCl_2$, 2 m*M* 5'-AMP, an NADPH-regenerating system consisting of 5 m*M* isocitrate and 5 units of isocitrate dehydrogenase (Sigma), and 0.2 m*M* NADPH; the concentration of microsomal protein employed in the reaction was 1–2 mg/ml. Optimal concentrations of the amine substrates ranged from 2 m*M* for DPT to 20 m*M* for MA for the microsomal oxidations. A Beckman 25 spectrophotometer was employed for all absorbance measurements. The experimental techniques for the EPR experiments have been described in detail elsewhere (*1*); within 1 min after initiating the reaction, the

sample was transferred to a quartz capillary tube and the EPR signal recorded with a Varian E-4 spectrometer. Computer simulation of EPR signals was performed with a DECLAB 11/03 computer.

## RESULTS AND DISCUSSION

### *N-Demethylation Activities of HRP and Metmyoglobin*

The rates of $H_2O_2$-supported *N*-demethylation of each compound in this class, catalyzed by either horseradish peroxidase (HRP) or metmyoglobin, are given in Table 1. These values were measured under experimental conditions established to be optimal. On a heme basis, the catalytic activity of HRP was several hundredfold larger than that of metmyoglobin for each substrate, as has been reported for other electron donors (*1*). The optimal $H_2O_2$ concentration was generally larger for metmyoglobin than for HRP catalysis, whereas differences

TABLE 1

CATALYTIC ACTIVITIES OF HRP AND METMYOGLOBIN FOR OXIDATIVE *N*-DEMETHYLATION OF AROMATIC AMINES BY $H_2O_2$[a]

| Substrate | Rate ($\mu$mol $H_2C{=}O$/min/$\mu$mol hemeprotein) | |
|---|---|---|
| | HRP[b] | Metmyoglobin[c] |
| DA | $9.64 \times 10^3$ (pH 6.0) | 38 (pH 6.0) |
| DPT | $7.07 \times 10^4$ (pH 6.5) | 152 (pH 6.0) |
| DMT | $5.49 \times 10^3$ (pH 7.0) | 23 (pH 6.0) |
| DOT | $5.35 \times 10^3$ (pH 6.0) | 29 (pH 6.0) |
| MA | $1.10 \times 10^4$ (pH 6.0) | 42 (pH 6.0) |
| MPT | $4.68 \times 10^4$ (pH 5.0)[d] | 21 (pH 7.0) |
| MMT | $1.34 \times 10^4$ (pH 7.0) | 20 (pH 6.0) |
| MOT | $9.90 \times 10^3$ (pH 6.0) | 26. (pH 6.0) |

[a] Rates were measured at 37°C in 0.1 *M* potassium phosphate buffer at the indicated pH, with catalyst concentrations that ranged from 2–20 n*M* for HRP and 0.5–5.0 $\mu M$ for metmyoglobin.

[b] For the HRP-catalyzed reactions, the concentration of the amine substrate ranged from 1 m*M* for DMT to 10 m*M* for DOT, MOT, and MMT, while $H_2O_2$ concentrations ranged from 0.2 m*M* for DMT to 2 m*M* for MA and MOT.

[c] For the metmyoglobin-catalyzed reactions, the amine concentrations ranged from 3 m*M* for DA to 10 m*M* for DOT, MA, MPT, and MOT, while $H_2O_2$ concentrations ranged from 1.7 m*M* for DA to 10 m*M* for MA and MPT.

[d] No formaldehyde could be measured under experimental conditions comparable to those described in footnote *b*. The value given was determined in 0.1 *M* potassium acetate buffer, pH 5.0, at room temperature, with 5–12 n*M* HRP, 15 m*M* DPT, and 15 m*M* $H_2O_2$, under which conditions the EPR signal was readily detected (cf. Fig. 3 legend).

between the two catalysts with respect to optimal amine concentration were less consistent. The large differences in rates and in $H_2O_2$ requirements for the two hemeprotein catalysts reflect differences in their reactivity with $H_2O_2$; reported rate constants for this reaction are 1 to $12 \times 10^6 M^{-1}$ sec$^{-1}$ for HRP isozymes (*18*) and $1.4 \times 10^2 M^{-1}$ sec$^{-1}$ for metmyoglobin (*19*). Under certain experimental conditions, enzymatic oxidation of all substrates in Table 1 produced colored products, which were generally (but not always) purple.

*Detection and Identification of Enzymatically Produced Radicals*

With HRP as catalyst, the oxidation of each substrate in Table 1 by $H_2O_2$ yielded an EPR signal centered at $g = 2.003$, as expected for radicals derived from this class of compounds. In order to generate EPR signals sufficiently intense and stable for analysis, it was necessary to use higher concentrations of both the reactants and the enzymes and more acidic solutions than those described in Table 1. Under these conditions, the colors which formed were also more intense. While different *optimal* experimental conditions are specified for data in the tables and figures, respectively, for most substrates there was a common pH range where both the rate measurements and EPR experiments could be performed. Since our results are entirely consistent with many previous studies of radical formation during HRP catalysis, the use of a rapid-mixing EPR flow cell, which consumes large amounts of enzyme, was not considered necessary. With two substrates, DOT and MMT, the only EPR signals which could be detected under any experimental conditions, and only with HRP, were very weak and unresolved and could not be identified. With each of the remaining compounds, the same EPR signal was detected with the appropriate concentration of HRP or metmyoglobin. The EPR signals produced from DA and DPT in both enzymatic systems have been published in the preliminary communication of this work (*2*). The suggestion that the DPT-derived signal was the radical cation of this compound (*2*) has been confirmed by computer simulation. As shown in Table 2, the hyperfine splitting constants of this EPR signal (*2*) agree well with those previously reported for the DPT radical cation generated electrochemically (*7*), considering known effects of salt and solvent on $a_i$ values (*20*).

Of the two EPR signals previously reported (*2*) to arise from DA in the presence of HRP and $H_2O_2$, the one which developed first showed distinct hyperfine structure, while the later-appearing EPR signal was quite narrow, with no resolvable structure (*2*). However, the rate of oxidation of DA catalyzed by HRP is so fast that an accurate spectrum of the first-formed radical, free of the second species, could not be obtained in this system. Because the overall rate of oxidation of DA, and formation of the second, narrow EPR signal, is much smaller with metmyoglobin and either $H_2O_2$ or cumene hydroperoxide (*21*), a high-quality spectrum with well-resolved hyperfine splitting could be generated from DA with these systems. Moreover, a very similar EPR signal resulted from oxidation of *N,N,N',N'*-tetramethylbenzidine (TMB) in these several enzymatic systems. Computer simulation of these EPR spectra, generated experimentally from TMB or from DA (first signal), yielded hyperfine splitting constants (Table 2) in good

TABLE 2

SUMMARY OF HYPERFINE SPLITTING CONSTANTS DETERMINED BY COMPUTER SIMULATION OF EPR SIGNALS PRODUCED BY ENZYMATIC OXIDATION OF THE AMINE SUBSTRATES OF THIS STUDY

| Parent compound (Ref.) | $a_i$ (Gauss) | | | | |
|---|---|---|---|---|---|
| | $i$ = N | $i$ = H(N–$CH_3$) | $i$ = H(N–H) | $i$ = H(ring-$CH_3$) | $i$ = H(ring) |
| DPT (*2*)[a] | 10.55 | 11.35 (6H) | — | 9.70 (3H) | 5.20 (2H)<br>1.20 (2H) |
| DPT (*7*)[b] | 11.17 | 12.22 (6H) | — | 9.77 (3H) | 5.21 (2H)<br>1.36 (2H) |
| DA,I (*2*)[c] | 5.00 (2N) | 4.90 (12H) | — | — | 1.60 (4H)<br>0.80 (4H) |
| TMB (*22*)[c] | 4.86 (2N) | 4.74 (12H) | — | — | 1.63 (4H)<br>0.79 (4H) |
| DMT[d] | 5.00 (2N) | 5.00 (12H) | — | — | 1.80 (4H) |
| MA[e] | 6.4 (2N) | 6.4 (6H) | 6.4 (2H) | — | — |
| MPT[d] | 6.50 (2N) | 5.90 (6H) | 7.80 (2H) | 2.05 (6H) | 0.65 (2H) |
| MOT[e] | 6.4 (2N) | 6.4 (6H) | 8.0 (2H) | — | 6.4 (1H) |

[a] Estimated error ±0.1 G.
[b] Solvent, acetonitrile with 0.1 *M* $NaClO_4$.
[c] Solvent, acetonitrile with 0.1 *M* tetraethylammonium perchlorate.
[d] Estimated error ±0.1 G; $a_H$ for other ring protons <0.2 G.
[e] Estimated error ±1.0 G; $a_H$ for other ring protons <1.0 G.

agreement with those previously published for the radical cation of TMB (*22*). The first EPR signal detected during the HRP-catalyzed oxidation of DA (*2*) was shown by computer simulation to contain two major components, the TMB radical cation and a radical very similar to that produced from MA (Fig. 5). *N*-Methylaniline (MA) is the product of rapid *N*-demethylation of DA, Table 1 and Eq. [1], while TMB results from a competing dimerization of two radical cations of DA at their *para* positions (*23, 24*). The identity of the very narrow EPR signal produced from DA as the reaction progressed will be discussed later. However, we note that the radical cation of DA has thus far eluded detection by EPR in any chemical or electrochemical oxidizing system (*23*); this species would be expected to have hyperfine splitting constants comparable to those of the DPT radical (cf. Table 2), quite inconsistent with the features of the second EPR signal detected with DA.

The EPR signals produced from DMT, MPT, and MA with both HRP and metmyoglobin are shown in Figs. 2, 3, and 5. The hyperfine splitting constants determined by computer simulation of the DMT-derived EPR signal are very similar to those of the TMB radical cation, Table 2. Thus, the EPR signal of Fig. 2 is assigned to a radical species of a dimer, which arises from the coupling of two DMT radical monomers at their free *para* positions, analogous to the formation of TMB from DA (*24*). The well-resolved EPR signal produced enzymatically from MPT (Fig. 3) was readily simulated with the hyperfine splitting constants given in Table 2. Since the corresponding $a_i$ values are smaller than those of the DPT

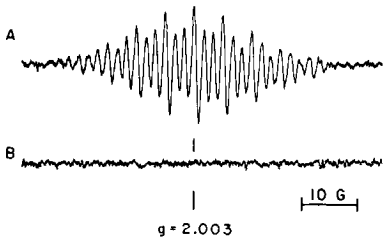



FIG. 2. EPR signal produced during the hemeprotein-catalyzed oxidation of DMT by $H_2O_2$. (A) The reaction was initiated by adding 43.9 $\mu M$ metmyoglobin to 10 m$M$ DMT and 10 m$M$ $H_2O_2$ in 0.1 $M$ potassium acetate buffer, pH 5.0. (B) Control experiments for A with the enzyme or one substrate omitted. Instrument settings for A and B were: power, 10 mW; modulation amplitude, 1.0 Gauss; gain, $4 \times 10^3$; microwave frequency, 9.159 GHz; magnetic field, approximately 3260 Gauss.

radical cation, this EPR signal is also assigned to a dimeric species. However, the *para* methyl substituent should prevent dimerization of MPT radicals at this most favorable, i.e., most electron-dense, ring position (*23*). Based on evidence from electrochemical studies for limited *ortho* coupling of DPT radical monomers (*25*), the MPT dimer is considered to have the structure shown in Fig. 4. The larger $a_N$ value for the MPT radical dimer compared to the TMB radical cation is attributed to the replacement of a methyl group by hydrogen on both nitrogen atoms of the former species.

The best resolution of the EPR signal produced by enzymatic oxidation of MA is shown in Fig. 5. This spectrum could be simulated with the $a_i$ values given in Table 2, but the estimated error is considerably larger than for the EPR signals

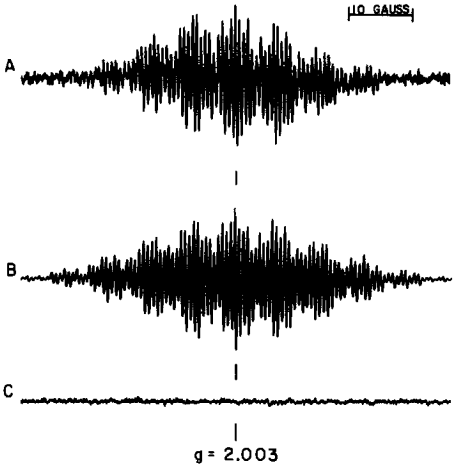



FIG. 3. Experimental EPR signal produced during the hemeprotein-catalyzed oxidation of MPT by $H_2O_2$ and the computer-simulated signal. (A) The reaction was initiated by adding 18 $\mu M$ metmyoglobin to 15 m$M$ MPT and 15 m$M$ $H_2O_2$ in 0.1 $M$ potassium acetate buffer, pH 5.0. (B) Computer simulation of A, with the $a_i$ values given in Table 2. (C) Control experiments for A with the enzyme or one substrate omitted. Instrument settings for A and C were identical to those described in the Fig. 2 legend, except for the modulation amplitude, 0.2 Gauss.

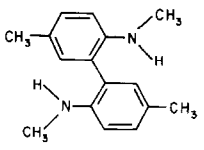


FIG. 4. Proposed structure for the dimeric oxidation product of MPT which gives rise to the EPR signal detected with this amine substrate.

which show good resolution of the ring proton splittings. This radical species (Fig. 5) cannot be conclusively identified from its EPR signal; however, the fact that the hyperfine splitting constants are in the same range as those determined for the MPT-derived radical dimer (Table 2) is indirect evidence that the two radicals are the same general type, i.e., benzidine species. Also shown in Table 2 are the hyperfine splitting constants determined for the radical derived from MOT (EPR signal not shown), which fit the pattern observed for other radicals produced from the *N*-monomethyl-substituted analogs. We note that there are few published data on the oxidation of *N*-monoalkyl-substituted anilines. The electrochemical oxidation of MA has been studied in aqueous solutions over a broad range of pH (*26*); the results of that study provided evidence for *para* coupling of MA radical monomers to form *N,N'*-dimethylbenzidine, quite analogous to DA oxidation, but EPR signal(s) arising from MA were not reported.

Although there is published evidence for the formation of nonbenzidine type dimers during the electrochemical oxidation of certain aromatic amines in nonaqueous solvents, these dimers were either not produced or not detected in the aqueous enzymatic systems characterized in this study. For example, the electrochemical oxidation of diphenylamines lacking *para* substituents yields isomeric benzidines (resulting from *para, ortho,* and mixed *para–ortho* coupling), as well as tetraphenylhydrazine, a product of *N–N* coupling (*27*). The EPR signals of radical cations of fully substituted hydrazines display $a_N$ values of approximately 7 G for tetraphenylhydrazine (*28*) or 13–16 G, for various types of tetraalkyl hydrazines (*29*). Thus, expected $a_i$ values for diphenyldimethylhydrazine radical cations would be intermediate between these limiting values, and would be larger than any observed for the dimeric radical species produced in this study

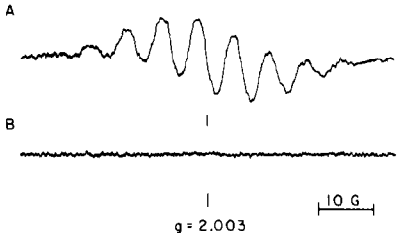



FIG. 5. EPR signal produced during the hemeprotein-catalyzed oxidation of MA by $H_2O_2$. (A) The reaction was initiated by adding 47.5 n*M* HRP to 15 m*M* MA and 15 m*M* $H_2O_2$ in 0.1 *M* potassium acetate buffer, pH 5.0. (B) Control experiments for A with the enzyme or one substrate omitted. Instrument settings for A and B were identical to those described in the Fig. 2 legend, except for the gain, $3.2 \times 10^3$.

(Table 2). A third type of radical dimerization has also been reported for aniline and its ring-substituted analogs: head-to-tail coupling involving the nitrogen of one radical and the *para* position of a second radical (*30*). The presence of the substituent on nitrogen, however, would make this type of coupling sterically unfavorable for the *N*-monomethyl-substituted compounds. Although *N*-demethylation of MA, to the extent that it can compete with radical dimerization under the conditions of the EPR experiments, would produce aniline (cf. Eq. [1]), which could then undergo the coupling reaction described, this reaction would require excess $H_2O_2$ relative to the amine and also active catalyst. Since HRP and most other hemeproteins are inactivated or destroyed by high peroxide concentrations, we employed stoichiometric $H_2O_2$ or less, with respect to the amine substrate, in all EPR experiments. Thus, it seems unlikely that head-to-tail dimers of the type described are responsible for any of the EPR signals detected in this study. Also, previous attempts to detect an EPR signal during the HRP-catalyzed oxidation of aniline were unsuccessful (*2*), suggesting that head-to-tail dimers, if actually formed, are unstable under these conditions. Finally, it should be mentioned that, although secondary and primary amines can be oxidized to nitroxides by $H_2O_2$ and other hydroperoxides, the experimental conditions required for these reactions (*20, 31*) are quite different from those employed for these enzymatic oxidations. Phenylmethyl nitroxides have $a_N$ values of 11–12 G (*32*), unlike the EPR signals produced from *N*-methyl aromatic amines in this study. Also, all radicals reported in this study had the same $g$ value of 2.003, compared to 2.006 which is characteristic of nitroxides (*20*). These arguments, and the evidence discussed earlier related to (i) the reported oxidation of MA to a benzidine species (*26*), (ii) the excellent resolution and computer simulation of the EPR signal produced from MPT, and (iii) the similarity of the $a_i$ values of all radicals derived from the *N*-monomethyl-substituted amines support the identification of these radicals as benzidine types.

There are published data which suggest that charge-transfer complex formation between DA and the radical (or fully oxidized) form of TMB may be responsible for the narrow EPR signal which developed subsequent to that of the TMB radical cation during the oxidation of DA. During cyclic voltammetry of DA, Galus and Adams (*33*) observed two redox couples (at ~0.6 and 0.45 V, respectively, vs SCE) in the pH range 2–4; these were clearly identified with oxidation of DA and TMB. However, at higher pH, a third redox couple with a lower potential, ~0.2 V vs SCE, became more prominent on successive cycles (*33*); this same species was also observed during the oxidation of mixtures of TMB and DA, but not solutions containing only TMB. Also, it was noted that a solution of the green TMB radical cation became blue when DA was added (*33*). Analogous studies of MA oxidation revealed very similar behavior, i.e., the appearance of a third redox couple with a low potential as the reaction progressed (*26*). In this case, it was possible to determine a stoichiometry of 2 MA molecules per benzidine molecule in the charge-transfer complex (*26*). Recent studies have shown that charge-transfer complex formation occurs between the fully oxidized form of *o*-tolidine (a *para* benzidine compound related to aniline) and its fully reduced form, resulting in a broad absorption near 630 nm (*34*); this complex, like those formed during the

oxidation of DA or MA, was dissociated in strongly acidic solutions (*34*). We also observed that the narrow EPR signal, and concomitant appearance of the blue color, did not develop during the chemical or enzymatic oxidation of TMB alone but absolutely required the presence of DA. A charge-transfer complex with an unpaired electron "jumping," or exchanging, rapidly among several aromatic rings would have an exchange-narrowed EPR signal with no resolved hyperfine splitting (*35*). Thus, based on several distinct kinds of experimental evidence, the identity of the radical with the very narrow EPR signal generated during DA oxidation is considered to be a charge-transfer complex between the TMB radical cation and one or more molecules of DA.

*Mechanism of Hemeprotein-Catalyzed Radical Formation*

The EPR signals detected in this study add considerable weight to other evidence suggesting that, with hydroperoxide oxidants, hemeproteins catalyze quite generally an initial one-electron abstraction of *N*-methyl-substituted aromatic amines (*1, 2, 11*). In the case of HRP and metmyoglobin, the one-electron oxidants have been shown to be the well-characterized higher oxidation states of these hemeproteins (*8*). The detection of only one radical monomer, the DPT radical cation, in this study is attributed to the inhibitory effect of the *para* methyl substituent on radical dimerization at the most reactive ring position. This does not, however, explain why a radical *dimer* was detected with MPT, which is identical to DPT except for one fewer methyl substituent on the nitrogen. A study of the electrochemical oxidation of a large series of *N,N*-dimethylaminoalkenes (or enamines), which give rise to stable purple "violene" radicals, demonstrated that each additional *N*-methyl group increases the stability of the radical cation (*36*). Our experimental results are consistent with this interpretation: dimerization is, in effect, the "default" reaction of unstable radical monomers which competes with their oxidation leading to formaldehyde (Eq. [1]). Thus, detection of the MPT radical dimer correlates with a lower rate of *N*-demethylation of MPT relative to DPT (Table 1). Indeed, MPT was the only compound which did not yield any measurable formaldehyde with HRP as catalyst when limiting $H_2O_2$ relative to the amine concentration was employed (Table 1). However, in those negative experiments with MPT, a noticeable color developed, indicative of the formation of radical coupling products. With higher concentrations of $H_2O_2$ relative to MPT, formaldehyde was readily measured (Table 1) and formation of the colored products was also observed. These findings indicate that, with limiting $H_2O_2$, the oxidant was rapidly depleted for the enzymatic oxidation of MPT to the radical cation. Since no $H_2O_2$ was available for subsequent oxidation of the MPT radicals, dimerization, presumably at the *ortho* position, was the only possible reaction of the species under these conditions. Moreover, the failure to detect the MPT radical monomer, under any conditions, i.e., with adequate oxidant, demonstrates the high probability of dimerization of this species. By comparison, the *ortho* coupling of DPT radicals (*25*) is probably retarded by the second $CH_3$ group on nitrogen.

The effect of the *para* methyl substituent on the relative rate of radical

dimerization vs radical oxidation appears to be quite substantial from the data in Table 1, since, for those analogs lacking a *para* substituent, the rate of *N*-demethylation of the *N*-monomethyl compound is greater than (HRP) or comparable to (metmyoglobin) that for the corresponding *N,N*-dimethyl-substituted compound. The most plausible explanation for this result is that those aromatic amines with a single *N*-methyl substituent encounter less steric hindrance in their approach to the oxidized heme group within the protein crevice, resulting in a faster rate of radical generation from these compounds (cf. Eq. [1]). It is clear that subtle alterations in the structure of the substrate may affect each reaction in the radical pathway of *N*-demethylation (Eq. [1]) differently. The complexity of these oxidations involving competing enzymatic and nonenzymatic reactions of free radical species does not permit a more detailed analysis of the effect of substrate structure on the overall rate of *N*-demethylation.

### *N-Demethylation Reactions Catalyzed by Liver Microsomal Fractions*

The next point to be considered is the relevance of these findings for $O_2$/NADPH-supported oxidation of this class of compounds by liver microsomal cytochrome *P*-450. In an extensive survey which included the *N,N*-dimethyl-substituted amines of this study, Schmid *et al.* (*37*) determined these activities for microsomal fractions from uninduced rats. Their data are shown in Table 3, along with similar data which we obtained for microsomal fractions from phenobarbital-induced rats. Table 3 also includes the rate data of Table 1, presented in the format

TABLE 3

RELATIVE RATES OF N-DEMETHYLATION OF *N,N*-DIMETHYL TOLUIDINE ISOMERS BY VARIOUS HEMEPROTEIN-CONTAINING SYSTEMS

| System | Rate (% of rate measured for DA for each system) | | |
|---|---|---|---|
| | DOT | DMT | DPT |
| Liver microsomes, $O_2$, NADPH[a] (uninduced rats) | 140 | 100 | 95 |
| Liver microsomes, $O_2$, NADPH[b] (phenobarbital-induced rats) | 147 | 108 | 57 |
| HRP, $H_2O_2$[c] | 55 | 57 | 733 |
| Metmyoglobin, $H_2O_2$[c] | 76 | 61 | 400 |
| Metmyoglobin, cumene hydroperoxide[d] | 31 | 94 | 242 |

[a] Data taken from Ref. (*38*).

[b] Experimental conditions described under Materials and Methods; the optimal pH was 7.5 except for DPT (pH 8.0). The rate of DA oxidation under optimal conditions was 32.2 nmol $H_2CO$/min/mg protein.

[c] Data taken from Table 1.

[d] Optimal experimental conditions: 0.1 *M* potassium phosphate buffer, pH 6.5 (DPT), 7.0 (DA and DMT) or 7.5 (DOT), 2–6 $\mu M$ metmyoglobin, 20–40 m*M* cumene hydroperoxide, 5–15 m*M* amine, and 37°C. The rate of DA oxidation was 22.7 $\mu$mol $H_2CO$/min/$\mu$mol metmyoglobin.

of relative rates employed by Schmidt *et al.* (*37*), and finally rates of oxidation of these compounds in the metmyoglobin-cumene hydroperoxide system (*38*). The latter system was investigated as a hemeprotein–hydroperoxide system for which the measured rates of *N*-demethylation are comparable to the very low rates characteristic of microsomal monooxygenation and peroxidatic reactions (*4, 37, 39*). The most noteworthy feature of Table 3 is the relatively larger rate of oxidation of DOT, and the relatively smaller rate for DPT, with both induced and uninduced microsomes compared to all of the hemeprotein–hydroperoxide systems. However, in view of the demonstrated complexity of radical *N*-demethylation reactions, quantitative comparisons of these data for systems as different as the heterogeneous, membrane-rich microsomes and the soluble hemeprotein solutions cannot be readily made. First, the very low rates of substrate oxidation in microsomes would result in very low concentrations of the postulated radical intermediates, which would most likely associate strongly with either protein (cytochrome *P*-450) or phospholipid. Under these conditions, dimerization of the radicals would be highly improbable. On the other hand, the HRP- and metmyoglobin-catalyzed reactions are sufficiently rapid to produce EPR signals of radical dimerization products with most substrates of this study. However, it is not possible to determine quantitatively the extent to which radical dimerization *decreases* the yield of formaldehyde from DA and DOT relative to DPT in these active, soluble oxidizing systems. Thus, radical dimerization is an unknown factor which affects differently the rate of *N*-demethylation by each of the oxidizing systems in Table 3.

Another important difference between the microsomal systems and the soluble systems relates to substrate "specificity" of the respective hemeproteins. HRP is known to exhibit low specificity for its electron donor substrate (*8, 18*). The remarkably broad range of substrate specificity of liver microsomal cytochrome *P*-450 can now be attributed, in part, to a very heterogeneous population of this hemeprotein in microsomes; many similar, but distinct, molecular species of liver microsomal cytochrome *P*-450 have been isolated and shown to have characteristic substrate specificities (*40*). Various inducers (drugs, etc.) increase the specific content of certain forms of liver microsomal cytochrome *P*-450, resulting in enhanced specific activity of the induced microsomes only for certain substrates (*4, 40, 41*). The data in Table 3 indicate that the activities of liver microsomes for *N*-demethylation of DA, DOT, and DMT increased similarly upon phenobarbital treatment of the animals, but that of DPT did not increase as much as expected. This result may be interpreted to mean that the cytochrome *P*-450 species most active in DPT oxidation was not induced, or was induced to a lesser extent than those form(s) responsible for oxidation of the other analogs. Since it is not clear at this time whether the same species of liver microsomal cytochrome *P*-450 catalyze the *N*-demethylation of all four analogs, further comparisons of these data for these different systems do not seem warranted.

Schmidt *et al.* (*37*) obtained a qualitative fit of their rate data for a large series of *N,N*-dialkyl-substituted aniline derivatives to an equation proposed by Hansch (*42*), relating the rate of oxidation to the lipid solubility and $pK_a$ of the substrate. For example, the rate of *N*-demethylation of *p*-nitro-*N,N*-dimethylaniline was

among the lowest of *para*-substituted DA analogs examined (*37*), a result predicted by the Hansch relationship based on the low $pK_a$ and poor lipid solubility of this compound (*37*). We note that the redox potential for removal of one electron from *p*-nitro-*N*,*N*-dimethylaniline is much larger ($E$ = 1.19 V vs SCE) than that of DA ($E$ = 0.71 V vs SCE) or DPT ($E$ = 0.65 V vs SCE) (*25*). Since the electronic properties of the substitutent would be expected to shift the lipid solubility, the $pK_a$, and the facility of electron abstraction in the same direction with respect to the parent compound (*43, 25*), the Hansch equation may actually provide a measure of the dependence of the overall rate of product formation on the one-electron redox potential of the substrate. The implications of such a correlation for the radical mechanism of *N*-demethylation (Eq. [1]) are that the first reaction (electron abstraction) is rate-limiting and that radical dimerization is negligible. The latter condition would certainly obtain in microsomes, as discussed earlier.

The results of this study have illustrated some of the many difficulties in testing the hypothesis that $O_2$/NADPH-supported *N*-demethylation reactions catalyzed by microsomal fractions involve a radical intermediate of the electron donor substrate. In particular, comparisons of rates for the same series of compounds which are oxidized by microsomes and by soluble "model" hemeprotein systems are inconclusive because of pronounced differences in the physical states, as well as the kinetic properties of radical generation and decay, characteristic of the two types of systems. In order to provide a valid test of the proposed radical mechanism of *N*-demethylation for liver microsomal cytochrome *P*-450, it will be necessary to use a reconstituted enzyme system containing purified NADPH–cytochrome *P*-450 reductase and a single species of the purified liver hemeprotein (*40*). Also, because the rates of substrate oxidation reactions catalyzed by cytochrome *P*-450 are so small (*39–41*), direct detection of radical intermediates (if possible) will require substrates which form radicals more stable than the compounds employed in this study. These approaches to the problem of mechanism of these microsomal oxidations are now under investigation.

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