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Stable Free Radical and Benzoquinone Imine Metabolites of an Acetaminophen Analogue*

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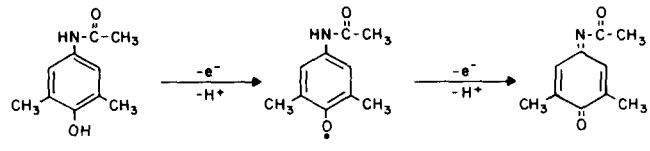
The enzymatic oxidation of the acetaminophen analogue 3',5'-dimethyl-4'-hydroxyacetanilide (3',5'-dimethylacetaminophen) with the horseradish peroxidase/hydrogen peroxide system forms a phenoxyl free radical metabolite. The structure of this free radical is established by a complete analysis of the ESR spectrum and confirmed by deuterium isotope substitution. Concomitant with phenoxyl radical formation, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine was detected by optical spectroscopy. The free radical is also formed by comproportionation in solutions of the quinone imine containing added 3',5'-dimethylacetaminophen. In contrast to acetaminophen, the imine and radical metabolites are stable and can be detected without resort to rapid-mixing techniques. Factors leading to the increased stability of these metabolites relative to those formed from acetaminophen are discussed in terms of the toxicity of acetaminophen.

Acetaminophen, a mild analgesic and antipyretic drug, was found to be hepatotoxic and nephrotoxic in man and experimental animals (1). This toxicity has been attributed to the formation of a highly reactive metabolic species, the *N*-acetyl-*p*-benzoquinone imine, which is thought to bind covalently to protein *in vivo*. Although the mechanism for the formation of this species is not clearly understood, it is almost certain that the activation does not occur via a pathway involving *N*-hydroxyacetaminophen (2, 3). Hinson *et al.* suggested a 2-electron oxidation of acetaminophen to the quinone imine by the cytochrome P-450 mixed-function oxidase (4). The 1-electron oxidation of acetaminophen by cytochrome P-450 has also been proposed (5, 6), but only a single-line ESR signal characteristic of acetaminophen-derived melanin-like polymer has been detected (7). The formation of a free radical by prostaglandin hydroperoxidase has also been proposed, but not proven (9–11). Using fast flow ESR, West *et al.* (8) detected a peroxidase-mediated acetaminophen phenoxyl free radical, which rapidly reacts further to give paramagnetic melanin-like polymeric products.

In order to elucidate this metabolic pathway (Scheme 1), the analogue DMA[1] was synthesized (12). DMA is reported to be of comparable toxicity to acetaminophen itself, whereas the 2',6'-dimethyl-4'-hydroxyacetanilide, like *N*-methylacetaminophen, shows only little toxicity in rats and mice (12). This suggests that the introduction of the methyl groups in the 3',5'-positions does not significantly change the metabolic pathway responsible for the toxic effects of acetaminophen. Both the radical formed from DMA and the corresponding quinone imine are more stable, so ESR and UV studies of this species were possible without the use of fast-flow mixing techniques, which require liters of solutions (8).



I II III

SCHEME 1

MATERIALS AND METHODS

Deuterated acetic anhydride, deuterated acetic acid, and horseradish peroxidase (type VI) were purchased from Sigma. 2,6-Dimethylphenol and galvinoxyl (2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxyl) were obtained from Aldrich.

3',5'-Dimethyl-4'-hydroxyacetanilide was prepared using the methods reported by Fernando *et al.* (12). Nitrosation of 2,6-dimethylphenol with sodium nitrite led to 2,6-dimethyl-4-nitrosophenol, which can be reduced with PtO_2/H_2 in a mixture of acetic acid and acetic anhydride to yield 3',5'-dimethyl-4'-hydroxyacetanilide. The acetyl group can be deuterated by performing the reduction in acetic acid d_4 and acetic anhydride d_6. A mixture of chloroform/ethanol was used for recrystallization.

N-Acetyl-3,5-dimethyl-*p*-benzoquinone imine was prepared by oxidizing DMA with AgO in chloroform in an analogous approach to methods previously described (12). The crude product was sublimed to yield *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine as yellow crystals, m.p. 114–115 °C (114–116 °C (12)). Horseradish peroxidase was denatured by heating the stock solution in a closed vessel at 100 °C for 60 min.

ESR spectra were recorded on Varian E-104 or E-109 spectrometers equipped with TM_{110} cavities. For the time-dependent intensity measurements and the high resolution spectra, a Varian field/frequency lock accessory was used. The spin concentration was determined by double integration of the ESR signal and comparison with the double integral of the signal from a galvinoxyl solution in ethanol. Simulations and calculations were performed on an HP 9835 B desktop computer or a Nicolet 1160 computer. Optical spectra were recorded on an Aminco model DW-2A spectrophotometer.

RESULTS

The peroxidase-mediated oxidation of DMA was studied using the horseradish peroxidase/hydrogen peroxide system. Through the use of UV spectroscopy, the enzymatic formation of the 2-electron oxidation product of DMA, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, was demonstrated (Fig. 1). Decay of the DMA absorption at 248 nm was accompanied by a simultaneous increase in the absorption at 275 nm. Characteristic of the reaction is an isosbestic point at 252 nm.

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[1] The abbreviation used is: DMA, 3',5'-dimethylacetaminophen with the IUPAC name 3',5'-dimethyl-4'-hydroxyacetanilide.

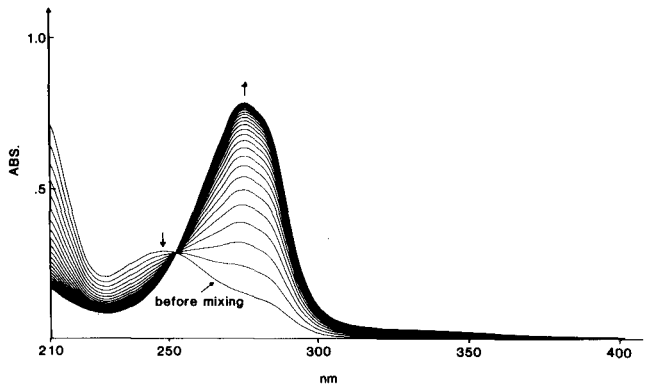


FIG. 1. **Oxidation of 3′,5′-dimethylacetaminophen by horseradish peroxidase/H_2O_2: optical spectra.** The incubation mixture contained 25 μM DMA, 0.83 μg/ml of horseradish peroxidase, and 172.5 μM H_2O_2 in phosphate buffer, pH 7.4. At zero time, horseradish peroxidase was added and repetitive scanning started (10 nm/s). 15 s were required to return the pen to the left edge at the end of each scan.

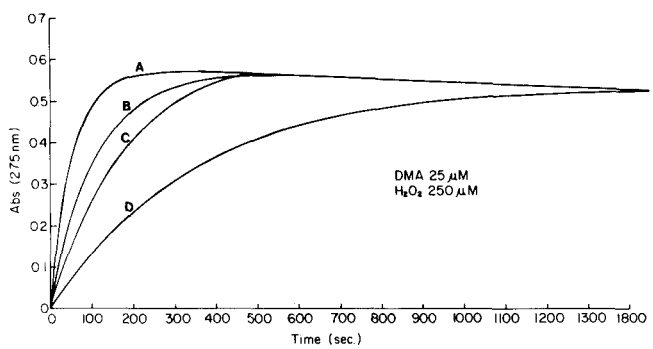


FIG. 2. **Effect of enzyme concentration on *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine formation.** The time-dependent studies were performed applying the dual wavelength mode at 275 nm *versus* the isosbestic point at 252 nm. At zero time, hydrogen peroxide was added to a solution containing DMA and 4 μg/ml (*A*), 2 μg/ml (*B*), 1 μg/ml (*C*), and 0.5 μg/ml (*D*) of horseradish peroxidase in phosphate buffer, pH 7.4, respectively.

The resulting spectrum was identical to the one obtained from independently synthesized *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (λ_{max} in phosphate buffer, pH 7.4, at 275 nm, 2.7×10^4 cm^{-1} M^{-1}), which has nearly the same absorption maximum as found in *n*-hexane (12). The reaction appears to be quantitative. Fig. 2 shows the effect of enzyme concentration on the reaction. As one can see, varying the concentration from 0.5 to 4 μg/ml results in a linear increase in the rate of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine formation, but does not affect the total amount of product formed. The slow decay of the absorbance is probably due to hydrolysis of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (12). The stability of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine is in marked contrast to that of *N*-acetyl-*p*-benzoquinone imine, which has recently been detected in an enzymatic system (13).

The incubation of DMA, H_2O_2, and horseradish peroxidase in phosphate buffer, pH 7.4, resulted in an ESR spectrum showing 7 lines (Fig. 3). The signal depends upon the presence of both horseradish peroxidase and H_2O_2 and can be attributed by computer simulation to the 3′,5′-dimethylacetaminophen phenoxyl free radical derived from 1-electron oxidation of DMA (Figs. 4 and 5). In Fig. 3*E* the nonenzymatic formation of the same radical is achieved by comproportionation of DMA and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (Scheme 2).

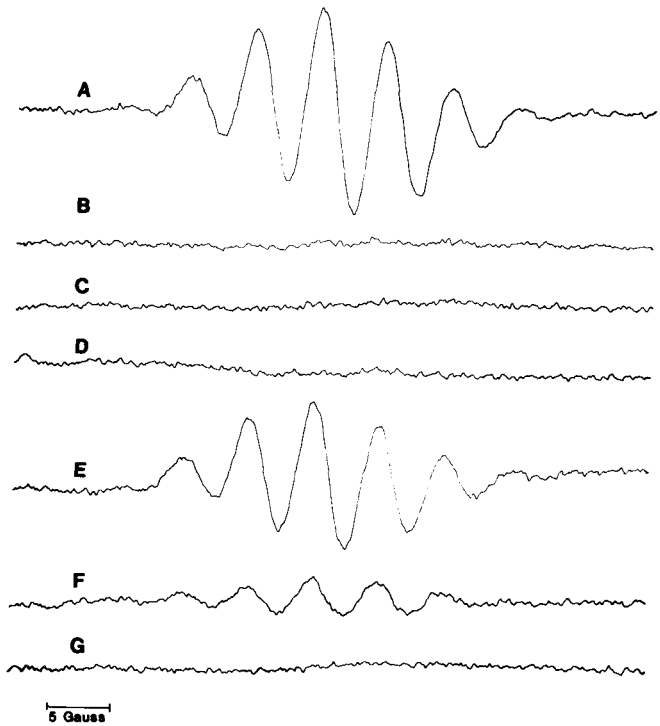
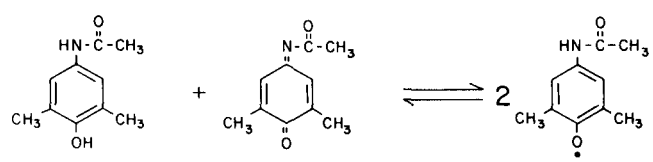


FIG. 3. **ESR spectra of the 3′,5′-dimethylacetaminophen phenoxyl free radical in phosphate buffer, pH 7.4.** Spectrometer settings: 20 milliwatts microwave power, 2.64 G modulation amplitude, 2 s time constant, 3.125 G/min scan rate. Enzymatic formation: *A*, 5 mM DMA, 2.5 mM H_2O_2, and 0.2 μg/ml of horseradish peroxidase; *B*, H_2O_2 omitted; *C*, horseradish peroxidase omitted; *D*, horseradish peroxidase heat denatured. Comproportionation: *E*, 2.5 mM DMA and 2.5 mM *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine; *F*, 2.5 mM *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine; *G*, 2.5 mM DMA.



SCHEME 2

A lower, but still significant, concentration of the free radical can be detected in an *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine solution in buffer (Fig. 3*F*). Apparently, decay of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine proceeds, at least in part, through reduction, as does the decomposition of *N*-acetyl-*p*-benzoquinone imine itself (14). Solutions of DMA alone do not give an ESR signal, demonstrating that air oxidation will not form this free radical at detectable levels (Fig. 3*G*).

Comparison of the hyperfine constants from the phenoxyl radicals derived from acetaminophen (8) and *ortho*-substituted dimethyl phenols, *i.e.* 2,4,6-trimethylphenoxyl $a^{H}_{ortho\text{-}CH_3}$ = 6 G (15) and 2,6-dimethyl-4-methoxyphenoxyl $a^{H}_{ortho\text{-}CH_3}$ = 4.7 G (15), made it possible to attribute the 7-line pattern to 6 equivalent methyl protons (hyperfine splitting constant: $a^{H}_{CH_3} \sim 5$ G). Improved resolution of the hyperfine structure could be obtained by using lower microwave power and modulation amplitude (Fig. 4*A*). As can be seen, these instrumental conditions also resulted in a poor signal/noise ratio. Only 5 out of 7 groups representing the *ortho*-methyl hydrogen splitting are visible, while the other two are now hidden in

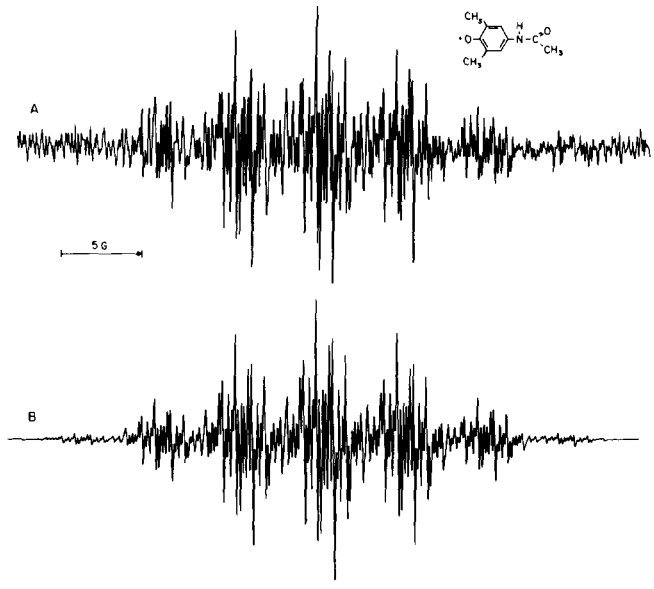


FIG. 4. **The high resolution ESR spectrum of the 3′,5′-dimethylacetaminophen phenoxyl free radical obtained from an anaerobic incubation.** *A*, the experimental spectrum was obtained with 15 mM DMA, 10 mM H_2O_2, and 1 μg/ml of horseradish peroxidase in phosphate buffer, pH 7.4, under a nitrogen atmosphere. Spectrometer settings: 1.5 milliwatts microwave power, 82.5 mG modulation amplitude, 1 s time constant, 1.33 G/min scan rate; *B*, computer simulation. Hyperfine splitting constants were $a^{H}_{ortho\text{-}CH_3} = 5.05$ G, $a^{N} = 0.404$ G, $a^{H}_{meta} = a^{H}_{NH} = 0.82$ G, and $a^{H}_{C(O)CH_3} = 1.0$ G.

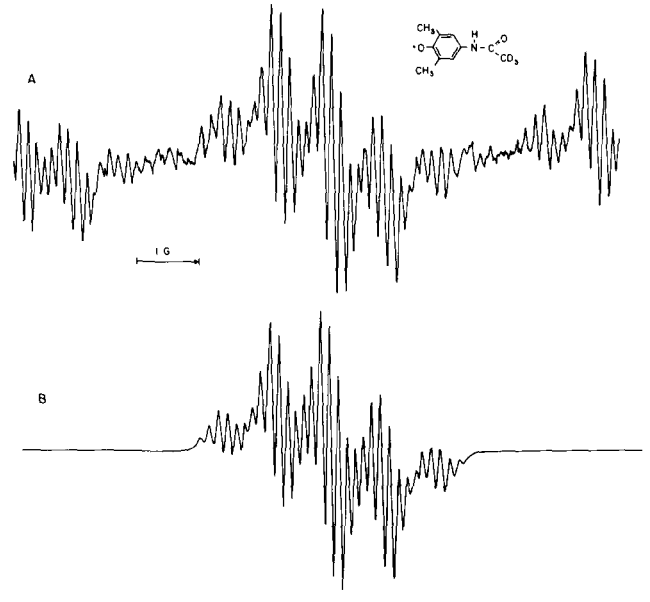


FIG. 5. **Third group of the ESR spectrum obtained from the d_3-3′,5′-dimethylacetaminophen phenoxyl free radical.** *A*, the experimental conditions were: 10 mM d_3-DMA, 10 mM H_2O_2, and 1 μg/ml of horseradish peroxidase in phosphate buffer, pH 7.4, under a nitrogen atmosphere. Spectrometer settings: 1.5 milliwatts microwave power, 66 mG modulation amplitude, 8 s time constant, 0.17 G/min scan rate; *B*, computer simulation. Hyperfine splitting constants were $a^{N} = 0.404$ G, $a^{H}_{meta} = a^{H}_{NH} = 0.82$ G, and $a^{2H}_{C(O)CH_3} = 0.154$ G.

the noise. To achieve a definite assignment of splitting constants, the acetyl group of the DMA was deuterated. Part of the expanded ESR spectrum resulting from this compound is shown in Fig. 5*A*. Due to the smaller gyromagnetic ratio of deuterium, the hyperfine splittings resulting from the acetyl deuterons are smaller than the corresponding hydrogen splittings. As a result, the overlap of the 7 main groups disappeared. This isotopic substitution and the known gyromagnetic ratios ($\gamma H/\gamma^2 H = 6.514$) make possible a complete assignment of the splitting constants to particular magnetic nuclei: $a^{H}_{ortho\text{-}CH_3} = 5.05$ G, $a^{N} = 0.404$ G, $a^{H}_{meta} = a^{H(^2H)}_{NH} = 0.82$ G (0.126 G), and $a^{H(^2H)}_{C(O)CH_3} = 1.0$ G (0.154 G). The computer simulations utilizing this data are shown in Figs. 4*B* and 5*B*. Ultimate proof of the assignment was obtained by exchange of the amide hydrogen of both acetyl-labeled and unlabeled compounds in buffer made with ^2H_2O.

In order to further characterize the system, investigations of the dependence of the maximum radical concentration on the H_2O_2 and horseradish peroxidase concentrations were performed with repetitive scans. Increasing the hydrogen peroxide concentration resulted in an enhanced ESR signal intensity corresponding to the increased radical concentration. At 2.5 mM hydrogen peroxide, the maximum radical concentration was achieved (Fig. 6). Higher hydrogen peroxide concentrations gave the same maximum radical concentration, but the duration of the signal was more transient, presumably due to the inactivation of the horseradish peroxidase (16) and overoxidation of the radical.

The horseradish peroxidase concentration was varied over 5 orders of magnitude with enzyme concentrations as low as 1 ng/ml. The resulting radical concentration was enzyme-independent above ~0.1 μg/ml over more than 3 orders of magnitude (Fig. 7), whereas it was proportional to the concentration of the horseradish peroxidase at concentrations less than 0.1 μg/ml. The maximum radical concentration was proportional to the substrate concentration over the range of 50 μM to 10 mM. The absolute radical concentration was determined using one set of conditions: 2 mM DMA, 0.1 μg/ml of horseradish peroxidase, and 2 mM H_2O_2. The time course of the radical formation in this experiment is shown in Fig. 8, demonstrating the approach to a limiting concentration of 2.5×10^{-7} M. This value and the observed decay of DMA (Fig. 1) and the formation of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (DMQI) allowed us to estimate the equilibrium

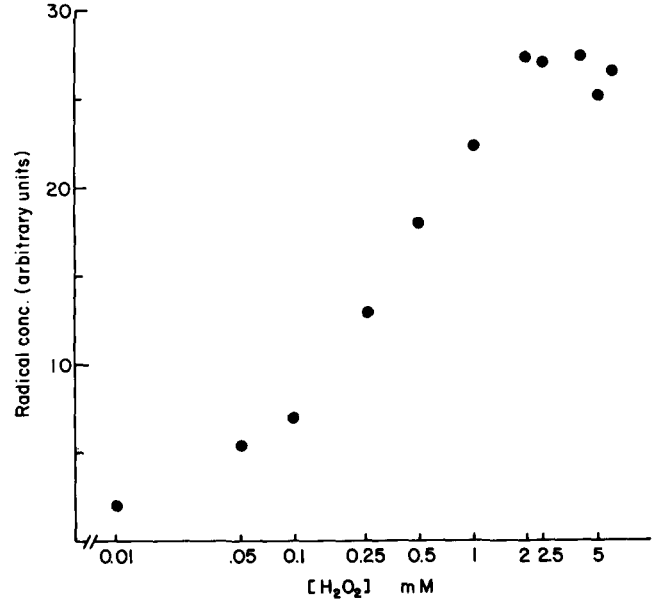


FIG. 6. **Hydrogen peroxide dependence of the maximum 3′,5′-dimethylacetaminophen phenoxyl free radical concentration.** All incubations contained 5 mM DMA and 0.2 μg/ml of horseradish peroxidase in phosphate buffer, pH 7.4.

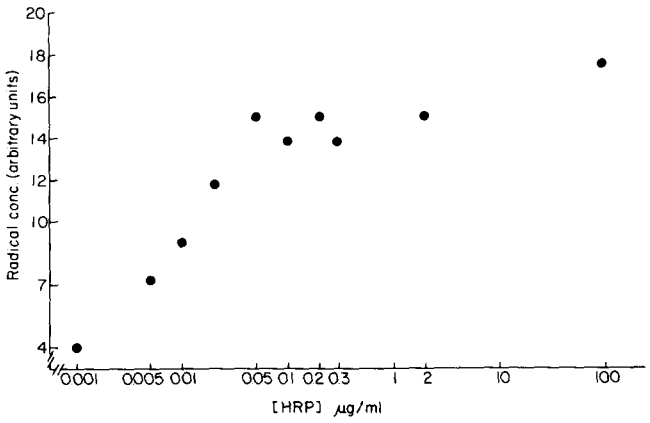


FIG. 7. **Horseradish peroxidase (*HRP*) dependence of the maximum 3′,5′-dimethylacetaminophen phenoxyl free radical concentration.** All incubations contain 5 mM DMA and 2.5 mM H_2O_2 in phosphate buffer, pH 7.4.

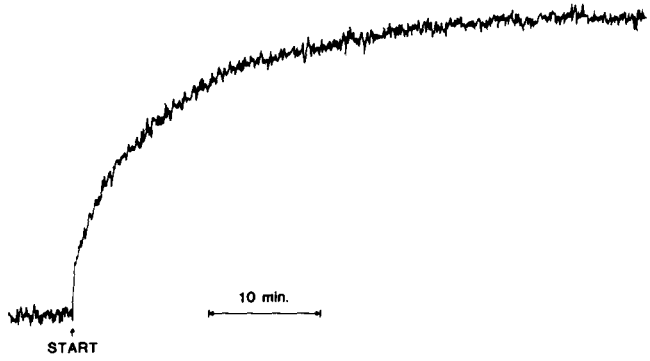


FIG. 8. **Time course of the amplitude of the ESR signal obtained from incubations containing 2 mM 3′,5′-dimethylacetaminophen, 2 mM H_2O_2, and 0.1 μg/ml of horseradish peroxidase in phosphate buffer, pH 7.4.**

constant

$$K_{eq} = \frac{[DMA\cdot]^2}{[DMA]\,[DMQI]}$$

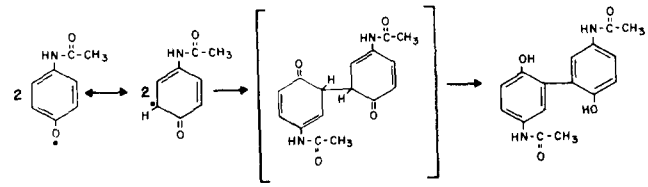
as 5×10^{-8}. The order of magnitude is comparable to the one reported by Yamazaki and Ohnishi (17) for the analogous hydroquinone/*p*-benzoquinone system.

DISCUSSION

ESR and UV spectroscopy have allowed us to detect the phenoxyl radical derived from DMA as well as its 2-electron oxidation product, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, during the peroxidase-mediated oxidation of DMA. Because the radical is in rapid equilibrium with DMA and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, it will be formed during the reaction even if the enzyme catalyzes a direct 2-electron oxidation. The difficulty in distinguishing between 1- and 2-electron enzymatic oxidations of hydroquinones has been extensively discussed by Yamazaki (18), but horseradish peroxidase-catalyzed oxidations generally proceed by 1-electron transfers in those cases where radical intermediates have been detected.

The reason that the 3′,5′-dimethylacetaminophen phenoxyl free radical is stable enough to detect in a static incubation, whereas the acetaminophen phenoxyl free radical can be observed only on the millisecond time scale (8), is due to the steric hindrance of the two methyl groups.

Rapid dimerization and polymerization reactions are characteristic of simple phenoxyl free radicals (19). In fact, in horseradish peroxidase incubations, dimerization of two acetaminophen phenoxyl free radicals occurs at the *ortho* positions (20), sites of high-electron spin density, as reflected by the large *ortho*-hydrogen hyperfine couplings (8) (Scheme 3). An analogous reaction of the 3′,5′-dimethylacetaminophen radical should be much slower for two reasons. First, due to steric effects the rate of dimer intermediate formation will be diminished. Second, the formation of the stable acetaminophen dimer from the intermediate requires rearomatization via enolization, which is impossible with the dimethyl analogue without the breaking of carbon-carbon bonds. The absence of this type of reaction is reflected by the fact that we never observed the single-line signal characteristic of acetaminophen oxidation to a melanin-like polymer (8). This steric effect also precludes the formation of the glutathione-acetaminophen conjugate (21) during the reaction of GSH with *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (22). If *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine or the corresponding phenoxyl free radical is formed *in vivo* by cytochrome P-450 or the peroxidase activity of prostaglandin H synthase, then the methyl groups blocking the reactive 3,5-positions would be expected to reduce the reactivity with cellular macromolecules such as protein (12). Conversely, reduction of both reactive species by NADPH-cytochrome P-450 reductase (22) or other quinone reductases is rapid and not expected to be greatly affected by dimethyl substitution. In conclusion, since electron transfer rates should not be greatly affected by this dimethyl substitution, redox chemistry may be directly involved in the toxicity of DMA and acetaminophen, because covalent bond formation (as reflected by both radical coupling and GSH conjugate formation) is diametrically different for DMA and acetaminophen, whereas the toxicity of these compounds is similar (12).



SCHEME 3

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