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DIRECT ELECTRON SPIN RESONANCE DETECTION OF FREE RADICAL INTERMEDIATES DURING THE PEROXIDASE CATALYZED OXIDATION OF PHENACETIN METABOLITES

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SUMMARY

The oxidation of the phenacetin metabolites *p*-phenetidine and acetaminophen by peroxidases was investigated. Free radical intermediates from both metabolites were detected using fast-flow ESR spectroscopy. Oxidation of acetaminophen with either lactoperoxidase and hydrogen peroxide or horseradish peroxidase and hydrogen peroxide resulted in the formation of the *N*-acetyl-4-aminophenoxyl free radical. Totally resolved spectra were obtained and completely analyzed. The radical concentration was dependent on the square root of the enzyme concentration, indicating second-order decay of the radical, as is consistent with its dimerization or disproportionation. The horseradish peroxidase/hydrogen peroxide-catalyzed oxidation of *p*-phenetidine (4-ethoxyaniline) at pH 7.5—8.5 resulted in the one-electron oxidation products, the 4-ethoxyaniline cation free radical. The ESR spectra were well resolved and could be unambiguously assigned. Again, the enzyme dependence of the radical concentration indicated a second-order decay. The ESR spectrum of the conjugate base of the 4-ethoxyaniline cation radical, the neutral 4-ethoxyphenazyl free radical, was obtained at pH 11—12 by the oxidation of *p*-phenetidine with potassium permanganate.

Key words: Phenacetin — Acetaminophen — Free radicals — Peroxidase — Electron spin resonance

INTRODUCTION

Phenacetin (I), an analgesic and antipyretic drug, causes toxic effects to the kidney and the lower urinary tract, such as renal necrosis [1,2] and

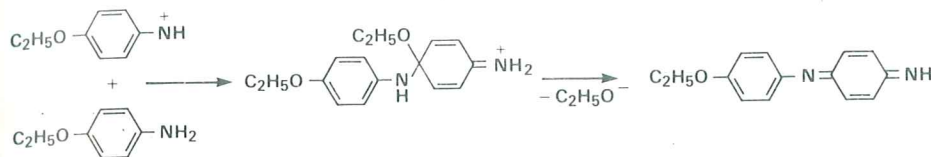Abbreviation: OXANOH, 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine.

tumors [3,4]. Phenacetin is metabolized extensively in vivo and forms acetaminophen (II) and p-phenetidine (III) as major metabolites [5]. Acetaminophen itself is a mild analgesic drug that has gained acceptance as an aspirin substitute. Ingestion of large quantities of acetaminophen can result in hepatic necrosis and acute renal failure in man [6,7].
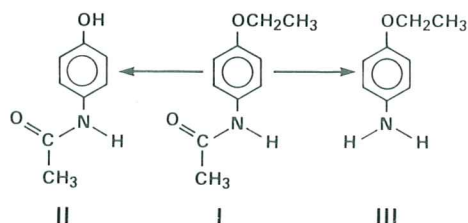
Both phenacetin and acetaminophen human nephrotoxicities are proposed to involve peroxidase-catalyzed activation to reactive species [8]. Although phenacetin is not a substrate for the peroxidase activity of prostaglandin H synthase [9], both p-phenetidine [8—10] and acetaminophen [11—13] are metabolized by the peroxidase activity of prostaglandin H synthase or horseradish peroxidase to form species which covalently bind to GSH or protein. In the case of p-phenetidine, these species also bind to DNA [14, 15] and induce strand breaks in cultured fibroblasts [9,15]. The metabolic activation of p-phenetidine and acetaminophen may proceed, in part, via a free radical mechanism catalyzed by peroxidases, but the free radicals have not been fully characterized. In an earlier communication, an unresolved three-line ESR spectrum was reported in acetaminophen/peroxidase systems [16]. On the basis of the five gauss hyperfine splitting, consistent with the presence of two *ortho* protons in phenoxyl radicals, and a g-value of 2.0043, this signal has been attributed to the N-acetyl-4-aminophenoxyl free radical. However, an unambiguous, complete assignment was not possible. A free radical metabolite has also been suggested as the initial oxidation product of p-phenetidine, yet only indirect evidence has been found [17]. This proposal is based on three observations: (1) The stoichiometric ratio of p-phenetidine consumed to hydrogen peroxide used is 2:1. (2) The addition of GSH to incubations thought to contain the p-phenetidine radical leads to gluthatione thiyl radical and GSSG formation [17—19] and this radical was proposed to be the oxidizing agent. (3) When a hydroxylamine (OXANOH) was added to incubations of p-phenetidine and horseradish peroxidase/ hydrogen peroxide, its one-electron oxidation product, a nitroxide radical, was observed [17].

The 2:1 stoichiometry of p-phenetidine and hydrogen peroxide removal during the horseradish peroxidase-catalyzed oxidation of p-phenetidine [17] is not definitive proof of a free radical intermediate. An ionic mechanism with an initial two-electron oxidation to an iminium cation followed

C_2H_5O—⟨benzene⟩—$\overset{+}{N}H$
+
C_2H_5O—⟨benzene⟩—NH_2
$\longrightarrow$
C_2H_5O—⟨benzene⟩—$\overset{H}{N}$—⟨cyclohexadiene⟩—OC_2H_5 $=\overset{+}{N}H_2$
$\xrightarrow{-C_2H_5O^-}$
C_2H_5O—⟨benzene⟩—N=⟨cyclohexadiene⟩=NH

by nucleophilic attack of the parent p-phenetidine could also account for this product. As previously pointed out, this non-free radical pathway would lead to identical products and stoichiometry [20]. Although indirect, the other evidence for a p-phenetidine-derived free radical is much more convincing [17—19]. Attempts to directly detect a p-phenetidine-derived free radical by ESR spectroscopy in static incubations were unsuccessful [17].

Using the fast-flow ESR technique, we attempted to characterize further the initial free radical metabolite of acetaminophen oxidation and to clarify whether a free radical intermediate is formed in the peroxidative oxidation of p-phenetidine, and whether these free radicals are responsible for the dimeric products. Because horseradish peroxidase is of plant origin and expensive, we have investigated using lactoperoxidase, a mammalian enzyme, and the inexpensive potassium permanganate as substitutes. It is important to demonstrate unambiguously a free radical oxidation pathway, especially since a p-phenetidine-derived radical has been proposed as the oxidation product responsible for p-phenetidine-dependent DNA single strand breaks and DNA binding [14,15].

MATERIALS AND METHODS

Deuterated acetic anhydride, deuterated acetic acid, acetaminophen, diethylenetriaminepentaacetic acid, lactoperoxidase (bovine milk), and horseradish peroxidase (type VI) were purchased from Sigma Chem. Co. (St. Louis, MO). Lactoperoxidase was denatured by heating in a closed vessel at 100°C for 30 min. p-Phenetidine and p-aminophenol were obtained from Aldrich Chem. Co. (Milwaukee, WI). p-Phenetidine was purified by distillation prior to the experiment. All other chemicals were used without further purification. $[^2H_3]$4-Hydroxyacetanilide was prepared via acetylation of p-aminophenol in a mixture of deuterated acetic acid and deuterated acetic anhydride.

ESR spectra were recorded at room temperature on a Varian E-109 ESR spectrometer equipped with a TM_{110} microwave cavity. Flow experiments were conducted as described previously [16] with a quartz fast-flow mixing chamber flat cell obtained from Wilmad Glass Co., Buena, NJ (Type WG-804, modified flat cell, 17 mm width). Reagents were prepared and bubbled with N_2 in two 4-l bottles. Outlets at the bases of the bottles were connected to the inlets of the flat cell with Tygon tubing. Gravity flow from a height of 6 ft. was regulated by Gilmont compact flow meters. Simulation of spectra were performed on an HP 9835B desktop computer or a Nicolet 1180 computer.
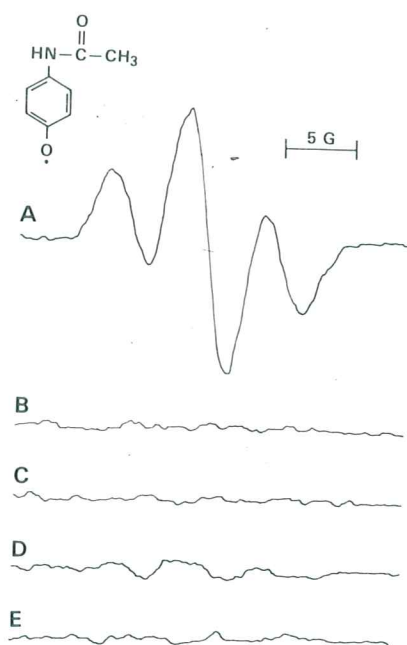
Fig. 1. The ESR fast-flow spectra of the N-acetyl-4-aminophenoxyl free radical produced in a system of acetaminophen, lactoperoxidase and hydrogen peroxide. The concentrations of acetaminophen, H_2O_2, and lactoperoxidase in the flat cell were 5.0 mM, 12.5 mM and 3.0 units/ml, respectively. Equal volumes of acetaminophen/H_2O_2 and lactoperoxidase in deoxygenated pH 7.5 phosphate buffer were mixed milliseconds prior to entering the flat cell at a total flow rate of 100 ml/min. A: complete system with acetaminophen, H_2O_2, and lactoperoxidase. B: same as in A, but no H_2O_2. C: same as in A, but no lactoperoxidase. D: same as in A, but with heat-denatured lactoperoxidase. E: same as in A, but no acetaminophen. Instrumental conditions: 20 mW microwave power, 1.33 G modulation amplitude, 1.0 s time constant and 12.5 G/min scan rate.

RESULTS

Using the fast-flow technique we were able to observe the previously reported ESR spectrum of the N-acetyl-4-aminophenoxyl free radical with an intensity ratio of $1:2:1$ in a system consisting of horseradish peroxidase, hydrogen peroxide and acetaminophen [16]. Figure 1 shows the same ESR signal observed when bovine lactoperoxidase was substituted for horseradish peroxidase [16]. No signal could be found if either hydrogen peroxide (Fig. 1B), lactoperoxidase (Fig. 1C), or acetaminophen (Fig. 1E) was omitted from the reaction mixture. The signal was also found to depend upon the presence of native enzyme (Fig. 1D). Lactoperoxidase has a soret optical spectrum which is similar to that of thyroid peroxidase, intestinal peroxidase, uterine peroxidase, eosinophil peroxidase, and prostaglandin H synthase [21,22]. As such, lactoperoxidase appears to be a useful prototype for most mammalian hemoprotein peroxidases.
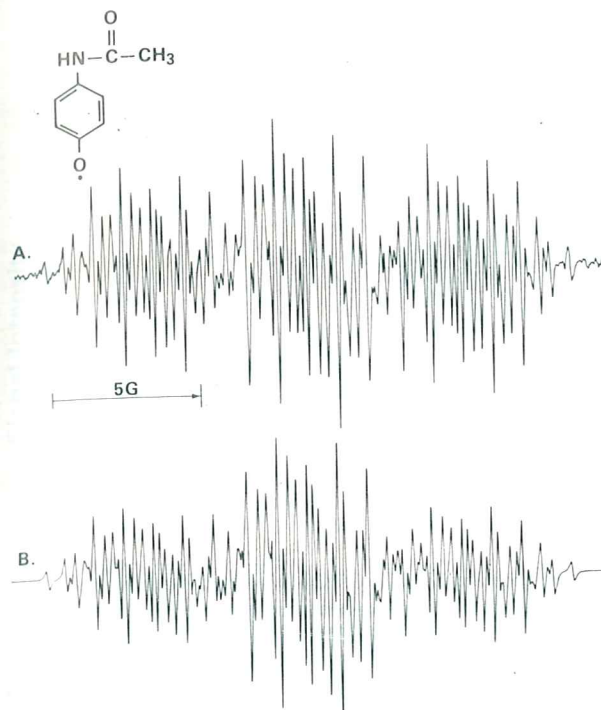
Fig. 2. The high-resolution ESR fast-flow spectrum of the N-acetyl-4-aminophenoxyl free radical produced by horseradish peroxidase and hydrogen peroxide. A: the experimental concentrations in the flat cell were 5.0 mM acetaminophen, 12.5 mM hydrogen peroxide and 1.7 units/ml horseradish peroxidase. Equal volumes of acetaminophen/H_2O_2 and horseradish peroxidase in deoxygenated pH 7.5 phosphate buffer were mixed milliseconds prior to entering the flat cell at a total flow rate of 100 ml/min. Instrumental conditions were 2.0 mW microwave power, 0.083 G modulation amplitude, 4 s time constant and 0.33 G/min scan rate. B: computer simulation. Hyperfine splitting constants were $a^N = 0.81_5$ G, $a_N^H = 1.35$ G, $a_{meta}^H = 0.64$ G, $a_{ortho}^H = 5.11$ G and $a_{C(O)CH_3}^H = 1.01$ G.

An improved, highly-resolved spectrum of the N-acetyl-4-aminophenoxyl free radical was obtained from the reaction of acetaminophen with horseradish peroxidase and hydrogen peroxide (Fig. 2A) using a drastically lower microwave power and modulation amplitude (Fig. 1A). The assignment of the hyperfine splitting constants was accomplished by computer simulation, Fig. 2B: $a_{NH}^N = 0.81_5$ G (triplet), $a_{NH}^H = 1.35$ G (doublet), $a_{meta}^H = 0.64$ G (triplet), $a_{ortho}^H = 5.11$ G (triplet), and $a_{C(O)CH_3}^H = 1.01$ G (quartet), with hyperfine interactions found for all nuclei with spin. The assignment was confirmed by employing acetaminophen deuterated at the acetyl group. Since the gyromagnetic ratio is known ($^\gamma H/^{\gamma^2}H = 6.514$), the deuterium splitting could be calculated a priori. The theoretical deuterium hyperfine of splitting constant agreed with that obtained by simulation of the experimental spectrum (Fig. 3).

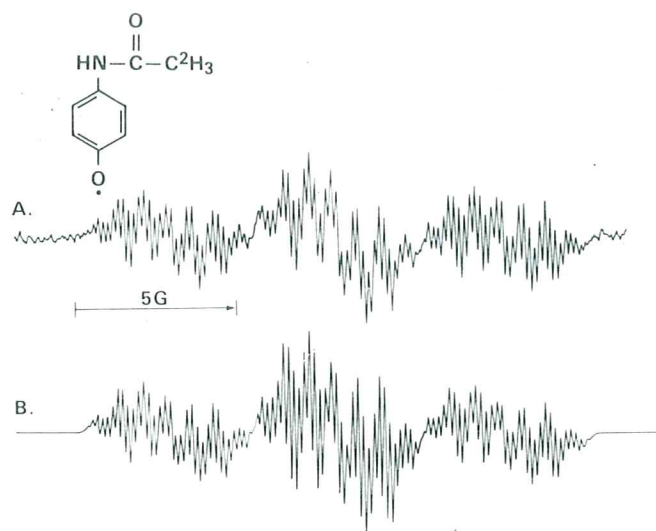The dependence of the N-acetyl-4-aminophenoxyl free radical concentra-

Fig. 3. The high resolution ESR fast-flow spectrum of the *N*-acetyl-4-aminophenoxyl free radical produced by the oxidation of deuterated acetaminophen by potassium permanganate. A: the experimental concentrations in the flat cell were 6.0 mM deuterated acetaminophen and 0.8 mM KMnO$_4$ in deoxygenated pH 7.5 phosphate buffer. Acetaminophen at 90 ml/min and KMnO$_4$ at 60 ml/min were mixed milliseconds prior to entering the flat cell. Instrumental conditions: 2.0 mW microwave power, 0.083 G modulation amplitude, 4 s time constant and 0.33 G/min scan rate. B: computer simulation. Hyperfine splitting constants were $a^N = 0.805$ G, $a^H_{NH} = 1.35$ G, $a^H_{meta} = 0.64$ G, $a^H_{ortho} = 5.11$ G and $a^{^2H}_{C(O)C^2H_3} = 0.155$ G.

tion on the enzyme concentration was determined a few milliseconds after mixing. To ensure that no underlying polymer signal interfered [16], the signal amplitude of both the first and the center line of Fig. 1A was plotted against the square root of the enzyme concentration. The results shown in Fig. 4 indicate that the radical concentration is linear with the square root of the enzyme concentration up to enzyme levels of 0.25 units/ml. At concentrations above 0.25 units/ml, the radical concentration begins to plateau.

The possible free radical formation from the other phenacetin metabolite, *p*-phenetidine, was investigated in a similar manner. As shown in Fig. 5A, rapidly mixing *p*-phenetidine and hydrogen peroxide with horseradish peroxidase at pH 7.5 resulted in a highly-resolved ESR spectrum. The formation of the free radical is dependent upon the presence of hydrogen peroxide (Fig. 5C), horseradish peroxidase (Fig. 5D) and *p*-phenetidine (Fig. 5E). The computer simulation (Fig. 5B) was obtained using the parameters: $a^N = 6.75$ G (triplet), $a^H_{NH_2} = 8.20$ G (triplet), $a^H_{ortho} = 4.25$ G (triplet) and $a^H_{OCH_2} = 2.37$ G (triplet). Although this is the first report of the 4-ethoxyaniline cation free radical as a metabolite, formation of this radical by oxidation with lead tetraacetate in trifluoroacetic acid has been
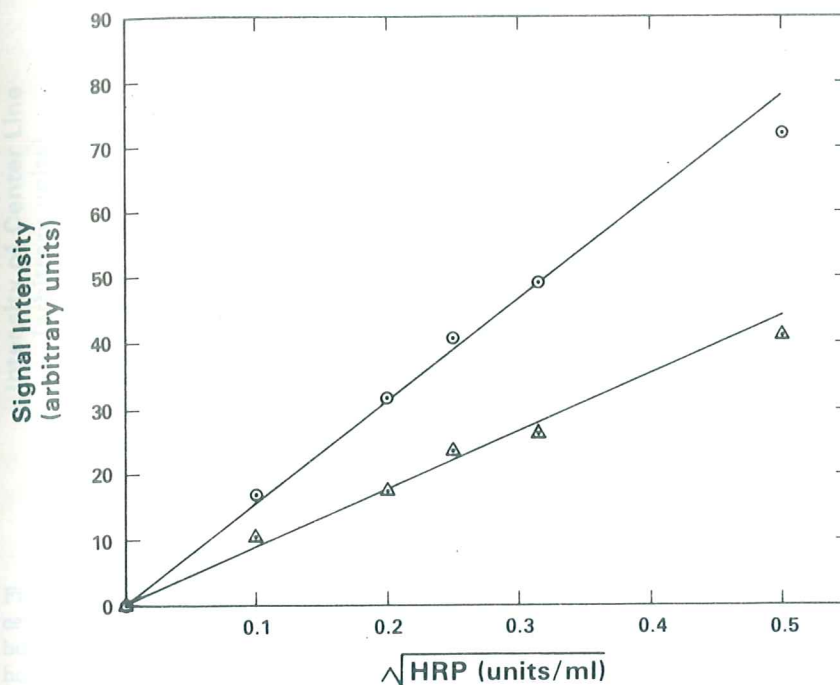
Fig. 4. Horseradish peroxidase-dependence of the N-acetyl-4-aminophenoxyl free radical concentration. 1.0 mM Acetaminophen with 2.5 mM H_2O_2 was flowed at 80 ml/min against variable concentrations of horseradish peroxidase at 80 ml/min. Final concentrations in the flat cell were 500 μM acetaminophen and 1.25 mM H_2O_2 in pH 7.5 phosphate buffer. Sample amplitudes were measured for the first ($\triangle$) and center ($\circ$) lines of the triplet in Fig. 1A.

reported previously [23]. As seen with the N-acetyl-4-aminophenoxyl free radical, the concentration of the 4-ethoxyaniline cation radical is linear with the square root of the horseradish peroxidase concentration up to 2.25 units/ml, but begins to plateau above this value.

The ESR spectrum of the 4-ethoxyaniline cation free radical can be observed up to pH 8.5 in the enzymatic system of p-phenetidine, hydrogen peroxide and horseradish peroxidase (Fig. 6). At pH 11—12, a different ESR spectrum is obtained by oxidation of p-phenetidine with potassium permanganate (Fig. 7A). The hyperfine parameters found by computer simulation, Fig. 7B: $a^N = 7.45$ G (triplet), $a_{NH}^H = 11.75$ G (doublet), $a_{ortho}^H = 5.60$ G (triplet), $a_{meta}^H = 1.30$ G (triplet) and $a_{OCH_2}^H = 1.30$ G (triplet), allow the assignment of this species to the neutral 4-ethoxyphenazyl free radical. The doublet hyperfine interaction of the single hydrogen clearly identifies this species as the conjugate base of the 4-ethoxyaniline cation.
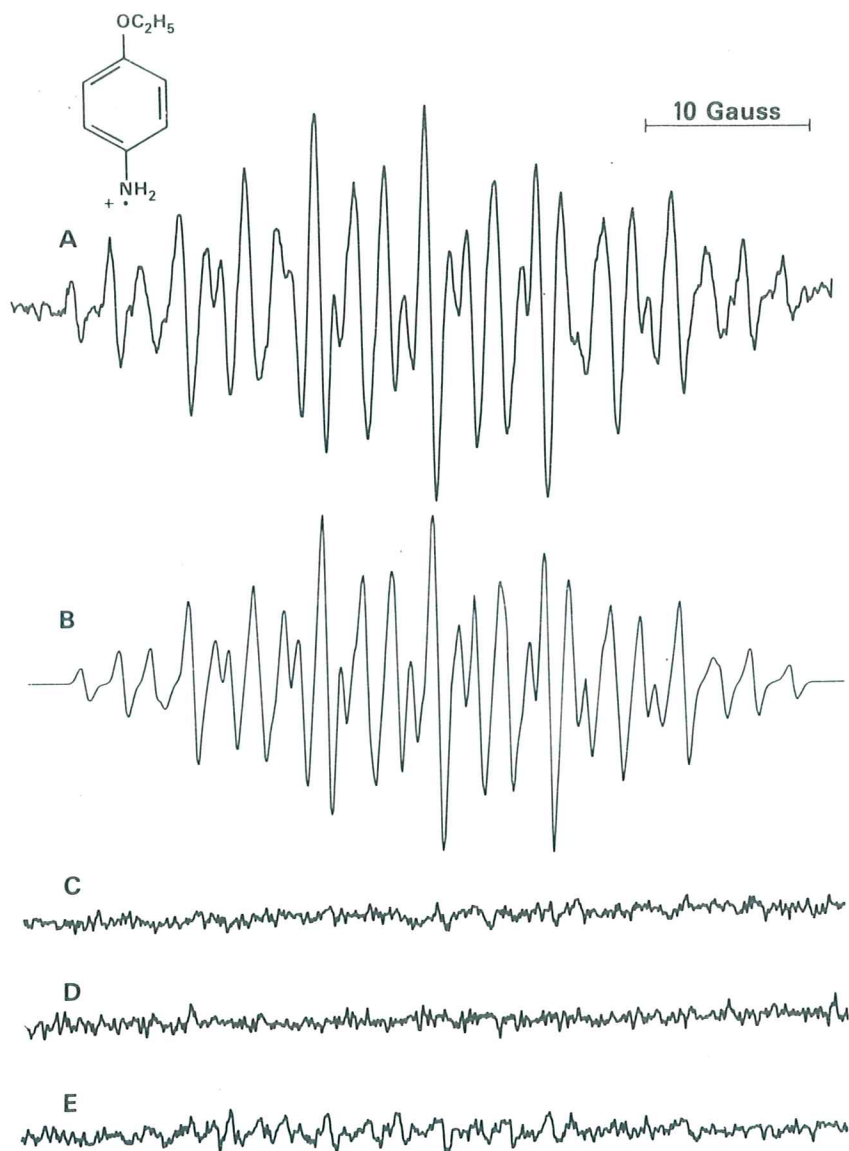
Fig. 5. The ESR fast-flow spectrum of the 4-ethoxyaniline cation free radical produced in a system of p-phenetidine, horseradish peroxidase and hydrogen peroxide. p-Phenetidine, 700 μM, with hydrogen peroxide, 350 μM, in pH 7.5 phosphate buffer with 1.0 mM diethylenetriaminepentaacetic acid was flowed against an equal volume of horseradish peroxidase, 3.0 units/ml, in phosphate buffer to give a total flow rate of 80 ml/min. A: complete system with p-phenetidine, hydrogen peroxide and horseradish peroxidase. B: computer simulation. Hyperfine splitting constants were $a^N = 6.75$ G, $a^H_{NH_2} = 8.20$ G, $a^H_{ortho} = 4.25$ G and $a^H_{OCH_2} = 2.37$ G. C: same as in A, but no H_2O_2. D: same as in A but no horseradish peroxidase. E: same as in A but no p-phenetidine. Instrumental conditions: 20 mW microwave power, 0.53 G modulation amplitude, 4 s time constant and 0.83 G/min scan rate.
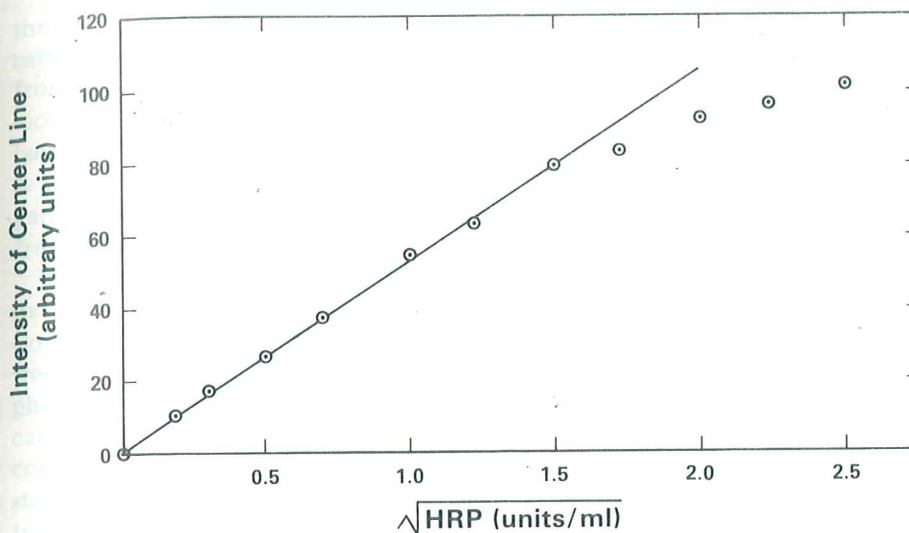
Fig. 6. Horseradish peroxidase-dependence of the 4-ethoxyaniline cation free radical concentration. *p*-Phenetidine, 350 μM, with 350 μM hydrogen peroxide in pH 7.5 phosphate buffer was flowed against an equal volume of buffer containing variable concentrations of horseradish peroxidase to give a total flow rate of 80 ml/min.

DISCUSSION

We were able to obtain a highly resolved ESR spectrum from the one-electron oxidation product of acetaminophen, the *N*-acetyl-4-aminophenoxyl free radical. The assignment of the hyperfine splitting constants is complete and was confirmed by deuterium substitution. In addition, the hyperfine splitting constants are similar to the ones determined for the 3,5-dimethyl-*N*-acetyl-4-aminophenoxyl free radical [24]. The *ortho* coupling of 5.11 G is dominant and characteristic of a phenoxyl free radical [16]. The second-order decay mechanism, as demonstrated by the square root-dependence of the radical concentration, can be explained in two ways. First, the radical may be, like its dimethyl analogue, in equilibrium with the parent acetaminophen and its two-electron oxidation product *N*-acetyl-*p*-benzoquinone imine [24]. By analogy, this equilibrium should favor the diamagnetic compounds and disproportionation should predominate. Secondly, the square root-
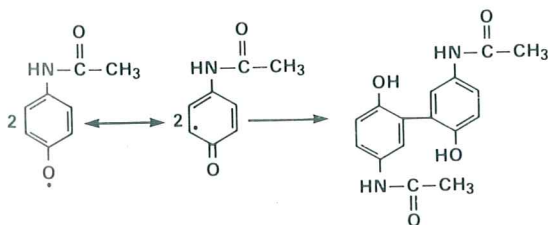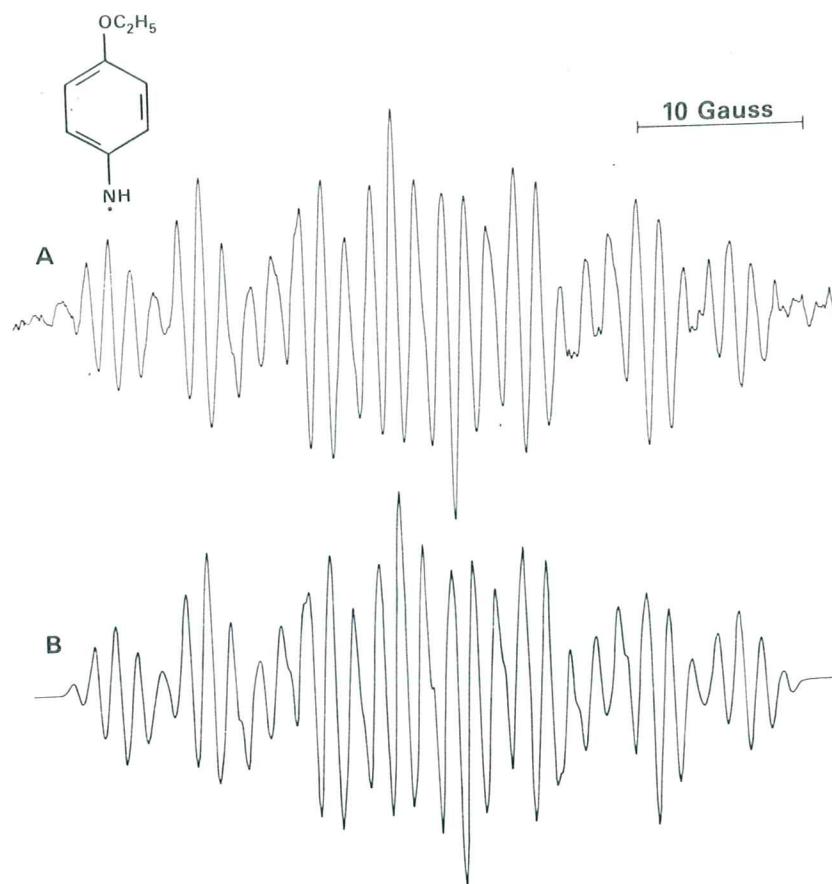
Fig. 7. The ESR fast-flow spectrum of the 4-ethoxyphenazyl free radical produced in a system of *p*-phenetidine and potassium permanganate at pH 12.0. A: *p*-phenetidine, 4.0 mM in phosphate/NaOH buffer, was rapidly mixed with an equal volume of 0.35 mM KMnO$_4$ in buffer to give a total flow rate of 160 ml/min. Instrumental conditions: 20 mW microwave power, 0.53 G modulation amplitude, 8 s time constant, and 0.83 G/min scan rate. B: computer simulation. Hyperfine splitting constants were: $a^N = 7.45$ G, $a^H_{NH} = 11.75$ G, $a^H_{ortho} = 5.60$ G, $a^H_{meta} = 1.30$ G and $a^H_{OCH_2} = 1.30$ G. The line shape was 50% Lorentzian and 50% Gaussian with a line width of 0.39 G.

dependence is also consistent with an initial dimer formation on a millisecond time scale. In fact, under our conditions of high acetaminophen concentration, the dimer resulting from covalent bond formation between the carbons *ortho* to the phenoxyl oxygen dominates product formation [25]. The covalent bond formation at the *ortho* positions is expected due to the high spin density at these carbon atoms, as reflected by the 5.11 G hydrogen hyperfine splitting. At enzyme concentrations above 0.25 units/ml, polymer formation was observed, as is typical for simple phenoxyl radicals [26]. Our results confirm the interpretation of West et al. [16], who attributed their

three-line signal to the short-lived free radical intermediate in the peroxidase-catalyzed acetaminophen oxidation, N-acetyl-4-aminophenoxyl, as distinct from the melanin-like polymer, a secondary product which had been previously identified as the primary radical intermediate [27,28]. Although lactoperoxidase could be substituted for horseradish peroxidase in this work, it was too expensive to use routinely. On the other hand, potassium permanganate gave comparable concentrations of the N-acetyl-4-aminophenoxyl free radical.

We were also able to confirm the proposal of Larsson et al. that the peroxidase-catalyzed oxidation of p-phenetidine forms a free radical [15,17,29]. Contrary to previous suggestions [15,17,29], the neutral 4-ethoxyphenazyl free radical is not present at physiological pH values. The neutral 4-ethoxyphenoxyl free radical is the dominant species only above pH 11. The radical cation should not dimerize as quickly as the neutral phenazyl radical due to coulombic repulsion. The observed square root-dependence of the steady-state radical concentration at pH 7.4 can be attributed to the coupling of two 4-ethoxyaniline cation radicals to ultimately form the dimeric product, N-(4-ethoxyphenyl)-p-benzoquinone imine [8,15]. It is also possible that coupling between the 4-ethoxyaniline cation radical and its conjugate base, the neutral 4-ethoxyphenazyl, is the fastest reaction at physiological pH values. In any case, the concentration of the neutral 4-ethoxyphenazyl is probably too low at pH 7.4 for its dimerization to account for product formation.
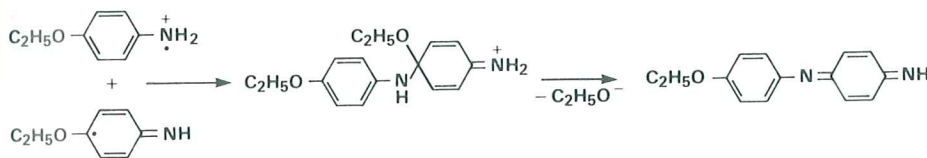
Although these investigations have clearly identified the free radical metabolites of acetaminophen and p-phenetidine, the stoichiometry of these free radicals to the diamagnetic product formation has not been determined. Nevertheless, the ESR detectable free radical metabolites formed by peroxidases have, in general, been found to be obligate intermediates of metabolism [30].

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