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Acetaminophen: enzymatic formation of a transient phenoxyl free radical

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Acetaminophen is a commonly used mild analgesic drug which has gained acceptance as an aspirin substitute. Although it is considered safe in normal dosage, ingestion of large quantities of acetaminophen can result in hepatic necrosis and acute renal failure in man [1, 2]. The reactive arylating species derived from acetaminophen is believed to be *N*-acetyl-*p*-benzoquinoneimine [3, 4]. Under therapeutic dose conditions, tissue glutathione appears to protect against hepatic damage by binding to the *N*-acetyl-*p*-benzoquinoneimine, as evidenced by acetaminophen dose-dependent depletion of liver glutathione [5, 6]. When acetaminophen is present in excess, however, the glutathione levels are depleted, leading to covalent binding of the arylating metabolite to tissue macromolecules and hepatic cell death [6, 7]. Several mechanisms for *N*-acetyl-*p*-benzoquinoneimine formation have been proposed. Metabolic activation might occur through *N*-oxidation of acetaminophen to *N*-hydroxyacetaminophen, followed by dehydration to the arylating *N*-acetyl-*p*-benzoquinoneimine [8]. However, more recent evidence indicates that *N*-hydroxyacetaminophen is not formed as an intermediate, and that acetaminophen undergoes 2-electron oxidation to the quinoneimine reactive species [9, 10].

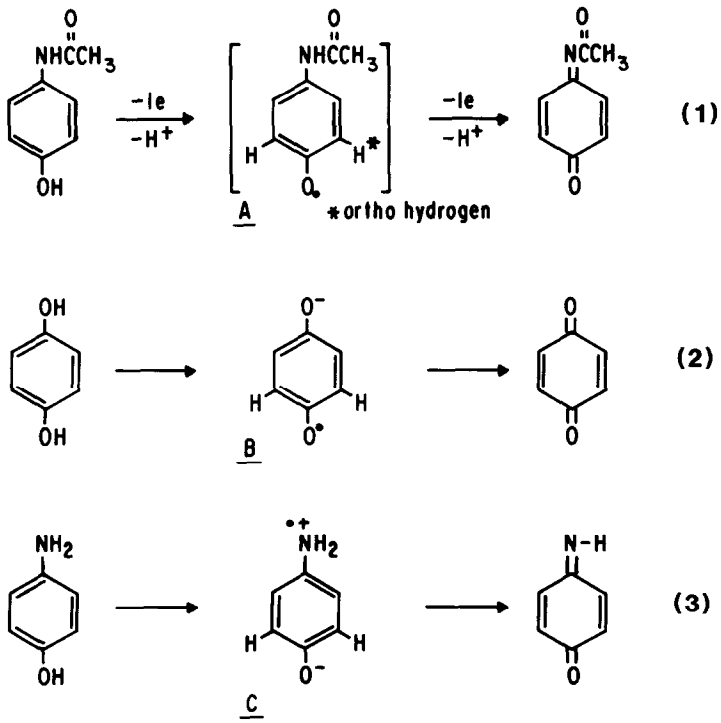
As shown in Scheme 1, this latter pathway (equation 1) is formally analogous to the familiar hydroquinone-quinone oxidation (equation 2), and could involve the generation of a transient phenoxyl free radical intermediate. The horseradish peroxidase/H_2O_2 system oxidizes phenol derivatives to free radicals that can be detected by direct ESR spectroscopy [11–13]. Stable radical ions, such as benzosemiquinone B, can be observed in static systems. In fact, the acetaminophen metabolite *p*-aminophenol is itself oxidized by horseradish peroxidase/H_2O_2 (equation 3), to produce the zwitterionic radical C with relatively long-term stability [14].

In sharp contrast, it is necessary to employ millisecond time scale flow methods to observe unstable phenoxyl radicals [12, 13].

Acetaminophen is a substrate for oxidation by horseradish peroxidase [15], and the stable radical observed after several minutes of incubation has been assigned to the primary radical metabolite. We now report characterization of the initial transient phenoxyl metabolite by ESR spectroscopy on the millisecond time scale, and reassign the long-term signal to a paramagnetic polymer similar to melanin.

Materials

Acetaminophen (*N*-acetyl-*p*-aminophenol), horseradish peroxidase (Type I), and bovine lactoperoxidase were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Hydrogen peroxide, 30%, was purchased from the Fisher Scientific Co. (Pittsburgh, PA, U.S.A.). All other chemicals were reagent grade. The conditions used for the fast-flow ESR experiments are described in the figure captions.



Scheme 1.

Results and discussion

Initial attempts to obtain spectra of the transient acetaminophen phenoxyl radical using horseradish peroxidase and H_2O_2 in a static system were unsuccessful. However, using the fast-flow technique, an intense, broad, three-line ESR spectrum with amplitude ratios 1:2:1 was observed 20–100 msec after mixing (Fig. 1A). The signal has the characteristic g value of 2.0043 ± 0.0002 attributable to an oxygen-centered phenoxyl free radical, and similar to those obtained with other phenolic substrates in fast-flow horseradish peroxidase/H_2O_2 systems [12, 13]. The observed three-line pattern is characteristic of *para*-substituted transient phenoxyl radicals with a dominant large coupling to the two equivalent *ortho*-hydrogens [16]. For example, the acetaminophen coupling, 5.2 G, is comparable to phenoxyl itself, 6.6 G, but contrasts with the lower values of delocalized and stabilized species such as benzosemiquinone B, a_H ortho = 2.48 G [16], and zwitterion C, a_H ortho = 2.77 G [14]. Hyperfine structure due to other magnetic nuclei present in the radical can be observed using a lower modulation amplitude (Fig. 1B) under the same flow conditions.

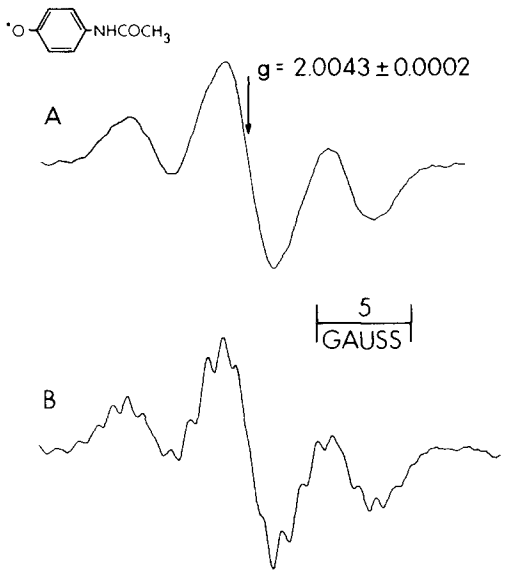
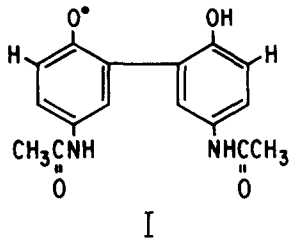


Fig. 1. ESR fast-flow spectra of the acetaminophen free radical produced in the presence of horseradish peroxidase and hydrogen peroxide. The concentrations of acetaminophen, H_2O_2 and horseradish peroxidase in the flat cell were 5 mM, 12 mM, and 1.7 units/ml respectively. Acetaminophen/H_2O_2 in 2 liters of pH 7.5 phosphate buffer deoxygenated with N_2 was mixed with an equal volume of horseradish peroxidase in 2 liters of deoxygenated pH 7.5 phosphate buffer at a total flow rate of 100 ml/min. Spectra were obtained on a Varian E-104 ESR spectrometer equipped with a TM_{110} microwave cavity at room temperature. A quartz fast-flow mixing chamber flat cell was obtained from the 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-liter bottles. Outlets at the bases of the bottles were connected to the inlets of the flat cell by Tygon tubing. Gravity flow from a height of 5 ft was regulated by two Gilmont compact flowmeters. (A) Microwave power was 5 mW; scan rate, 12.5 G/min; time constant, 1 sec; and modulation amplitude, 1.3 G. (B) Same as (A) but with modulation amplitude 0.65 G.

If the transient ESR signal arose from an acetaminophen dimer, I, we would anticipate entirely different coupling constants for the ortho protons. For example if the spin is localized on one ring, only a doublet pattern is expected. Delocalization over both rings (i.e. 4 ortho positions) would give a triplet, but with one-half the ortho splitting typical of substituted phenoxyl radicals.



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Using a stopped-flow procedure, we observed changes in the acetaminophen-derived free radical with time (Fig. 2). The three-line spectrum (Fig. 2; time 0) disappeared rapidly when the flow was terminated and was subsequently replaced by a single broad line (peak-to-peak line width 4.6 G), which increased in intensity over the time course of the experiment (Fig. 2; time 8 min). As would be expected, we obtained the same radical (Fig. 3) under static conditions (g value of 2.0048 ± 0.0003, peak-to-peak line width of 5 G). Other workers have also observed this single-line spectrum, which they attributed to the primary phenoxyl radical [15]. However, it is clear from the reaction time course that this spectrum belongs to a secondary species.

It appears that the broad signal reported here and by other workers is due to the subsequent formation of a melanin-like radical. Melanins are high molecular weight polymers of quinone-, semiquinone- (paramagnetic), and hydroquinone-type units in equilibrium [17]. Thus, the observed ESR spectra of melanins (g = 2.004, line width of 4–10 G, and no detectable hyperfine structure [17]) are close to those obtained for the acetaminophen polymer radical. This similarity and the knowledge that synthetic "melanins" arise from the prolonged enzymatic oxidation of a broad range of phenolic compounds, such as L-tyrosine, *p*-hydroquinone, and serotonin [17], are consistent with the anticipated formation of a melanin-related radical in the acetaminophen system.

The reactivity of the acetaminophen phenoxyl radical could render this species responsible for effects usually attributed to *N*-acetyl-*p*-benzoquinoneimine. Note that the relative instability of the acetaminophen radical compared to delocalized species such as melanin radicals [17] or zwitterion C is consistent with the enhanced electrophilic character of transient phenoxyls (i.e. greater unpaired spin density at oxygen and the *ortho*-hydrogens).

The formation of this free radical by mammalian enzymes, cytochrome P-450 [15, 18, 19] and prostaglandin synthase [20, 21] has been proposed but not proven. We found that 3 units/ml of bovine lactoperoxidase could replace horseradish peroxidase in the experiment described in Fig. 1. The ESR signal was totally dependent on enzyme, H_2O_2 and acetaminophen, as is the case with the horseradish peroxidase.

Recently Rosen *et al.* [22] reported that the persistent melanin polymer signal is also observed in a static oxidation experiment employing phenobarbital-induced hamster

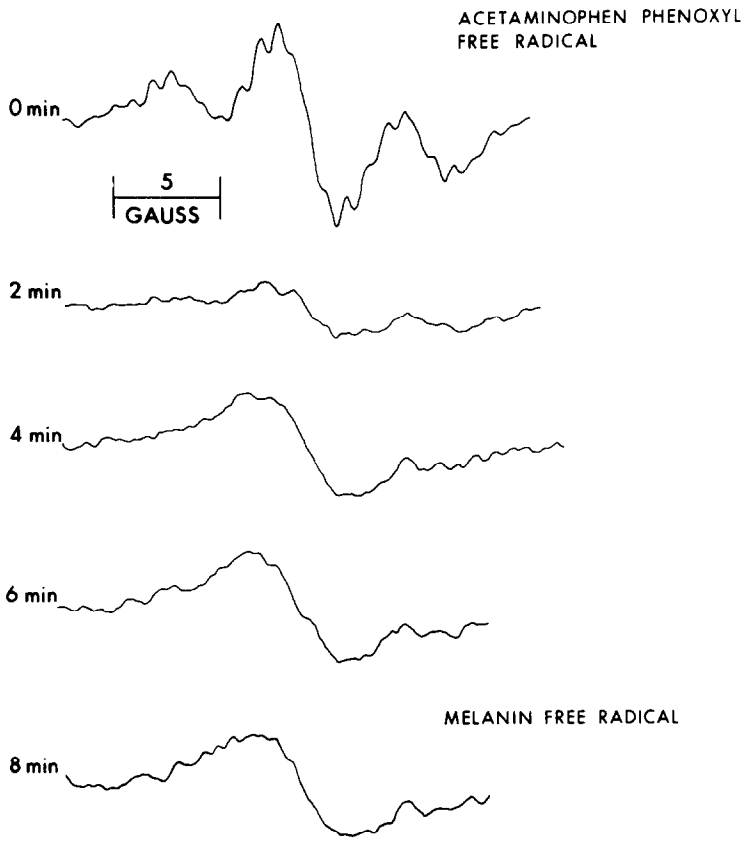

Fig. 2. ESR stopped-flow spectra of acetaminophen-derived free radicals formed by horseradish peroxidase and hydrogen peroxide. The concentrations of acetaminophen, H_2O_2 and horseradish peroxidase in the flat cell were 5 mM, 12 mM, and 1.7 units/ml respectively. Acetaminophen/H_2O_2 in 2 liters of pH 7.5 phosphate buffer deoxygenated with N_2 was mixed with an equal volume of horseradish peroxidase in 2 liters of deoxygenated pH 7.5 phosphate buffer. The flow was terminated and the time-dependent signal was recorded on a Varian E-104 ESR spectrometer equipped with a TM_{110} microwave cavity at room temperature. The microwave power was 5 mW; scan rate, 12.5 G/min; time constant, 1 sec; and modulation amplitude, 0.65 G.

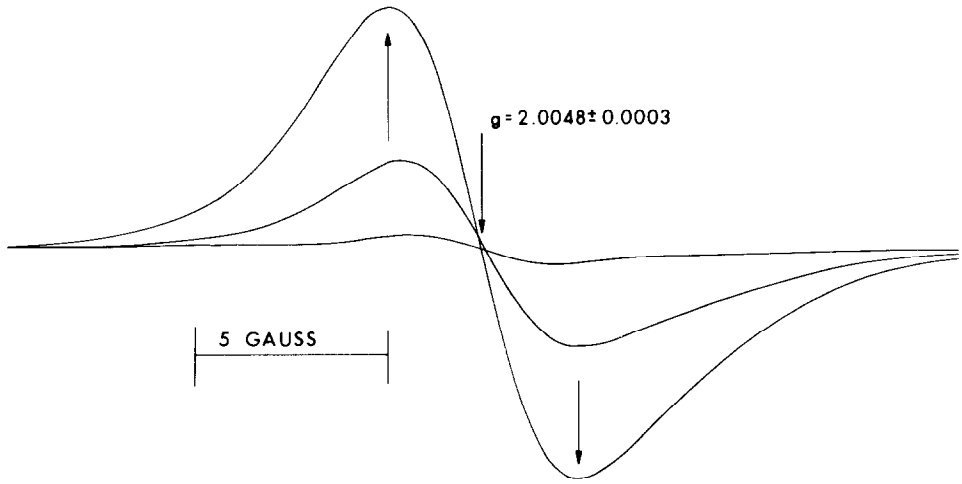

Fig. 3. ESR spectrum of acetaminophen "melanin" formed by horseradish peroxidase and hydrogen peroxide showing growth of the melanin-type free radical signal. The concentrations of acetaminophen and H_2O_2 in the 3-ml incubation mixture of pH 7.4 phosphate buffer were 8.3 and 83 mM respectively. The room temperature incubation mixture was deoxygenated with N_2, and the reaction was initiated with 0.15 units/ml of horseradish peroxidase. Magnetic field scans were begun at 0, 4, and 8 min after mixing. The microwave power was 20 mW; scan rate, 6.125 G/min; time constant, 1 sec; and modulation amplitude, 3.3 G.

hepatic microsomes. However, ESR detection of the more elusive short-lived acetaminophen phenoxyl radical A was not reported, and observation of the transient species is a necessary condition for a free radical mechanism for the bioactivation of acetaminophen. Notwithstanding, our flow experiments with peroxidase systems suggest that such radical pathways warrant consideration for mammalian enzymes, especially prostaglandin H synthase.

In summary, ESR spectroscopy employing a millisecond time scale fast-flow method has revealed the formation of a transient phenoxyl radical in the reaction of acetaminophen with horseradish peroxidase/H_2O_2 and bovine lactoperoxidase/H_2O_2. The short-lived radical is clearly distinguished from the persistent paramagnetic melanin polymers that are generated by prolonged incubation of acetaminophen in the presence of oxidizing enzymes.

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