Environmental Health Perspectives
Vol. 64, pp. 127-137, 1985

Free-Radical Metabolites of Acetaminophen and a Dimethylated Derivative

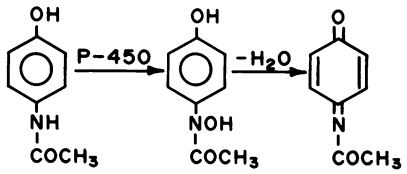
by Volker Fischer,* Paul R. West,* Laura S. Harman,* and Ronald P. Mason*†

The oxidation of acetaminophen (4′-hydroxyacetanilide) to the corresponding *N*-acetyl-*p*-benzoquinone imines by plant and mammalian peroxidases is discussed. The acetaminophen free radical (*N*-acetyl-4-aminophenoxyl) has been reported as an intermediate. It is very reactive and forms melanin-like polymeric products. Application of a fast-flow system makes it possible to detect the transient species and clearly distinguish it from persistent paramagnetic melanin polymers.

A model system, leading to more stable metabolites, can be obtained by introduction of methyl groups next to the oxygen, 3′,5′-dimethylacetaminophen (3′,5′-dimethyl-4′-hydroxyacetanilide). The ESR spectrum of the free radical formed could be completely analyzed and confirmed by deuterium substitution. The data are consistent with the assignment to a phenoxyl free radical (*N*-acetyl-2,6-dimethyl-4-aminophenoxyl). Its formation is discussed in terms of substrate, hydrogen peroxide and enzyme concentration dependence. It is believed to be formed via a direct one-electron oxidation of 3′,5′-dimethyl-4′-hydroxyacetanilide.

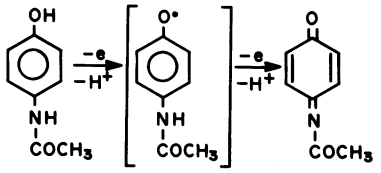
The radical does not form polymers or react with nucleophiles. Its redox behavior is discussed. The possible reaction of these phenoxyl free radicals with oxygen is thought to be negligible.

Acetaminophen (4′-hydroxyacetanilide) is a commonly used, mild analgesic drug which has gained acceptance as a salicylate (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*). This toxicity has been attributed to the formation of a highly reactive metabolic species, the *N*-acetyl-*p*-benzoquinone imine (*3,4*), which is thought to bind covalently to protein *in vivo*. Under therapeutic dose conditions, tissue glutathione appears to protect against hepatic damage by binding to the *N*-acetyl-*p*-benzoquinone imine, 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*-benzoquinone imine 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*-benzoquinone imine (*8*) [Eq. (1)].



(1)

However, more recent evidence indicates that *N*-hydroxyacetaminophen is not formed as an intermediate, and that acetaminophen undergoes overall two-electron oxidation to the quinone imine reactive species (*9,10*) [Eq. (2)].



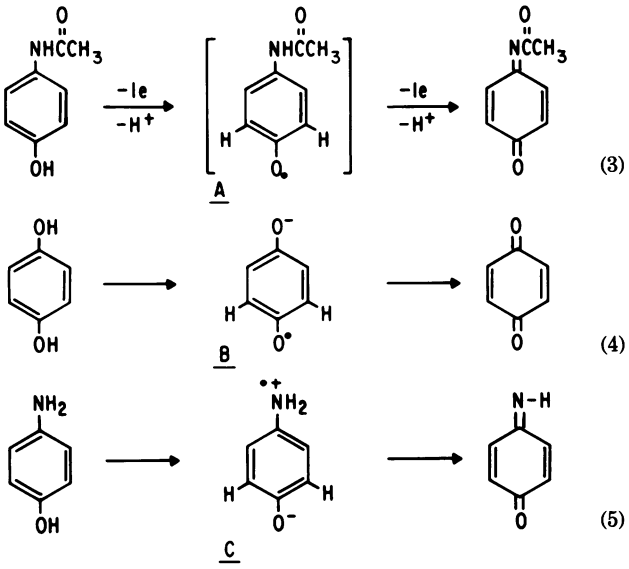
(2)

Hinson et al. suggested a two-electron oxidation of acetaminophen to the quinone imine by the cytochrome P-450 mixed-function oxidase (*11*). The one-electron oxidation of acetaminophen by cytochrome P-450 has also been proposed (*12,13*), but initially, only a single-line ESR signal characteristic of an acetaminophen-derived melanin-like polymer was detected (*14*). The formation of a free radical by prostaglandin hydroperoxidase has also been proposed, but not proven (*15–17*).

*Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P. O. Box 12233. Research Triangle Park, NC 27709.

†Author to whom correspondence should be sent.

As shown in Eqs. (3)–(5), this one-electron pathway is formally analogous to the familiar hydroquinone-quinone oxidation and could involve the generation of



(3)

(4)

(5)

transient free radical intermediates.

The zwitterionic and anionic radicals such as benzosemiquinone B and the aminophenol-derived C are relatively long-lived and can be observed in static systems. However, in acetanilide-derived radicals such as A, the electron-withdrawing acetyl group results in a higher pK_a, and one does not expect the electron-deficient radical to persist. To detect unstable radicals, flow systems with observation on a millisecond time scale are required. This technique consumes liters of solution, which limits the number of experiments that can be performed.

Introduction of methyl groups in the 3′ and 5′ positions of acetaminophen does not change the toxicity significantly. 3′,5′-Dimethylacetaminophen (3′,5′-dimethyl-4′-hydroxyacetanilide) is reported to be of comparable toxicity to acetaminophen itself (*18*). However, both the free radical and the benzoquinone imine metabolites of 3′,5′-dimethylacetaminophen are more stable and can be detected in static incubations. They do not undergo the very rapid dimerization and polymerization reactions which are characteristic of simple phenoxyl free radicals such as the acetaminophen free radical. [Eq. (6)].

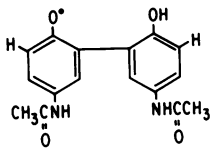
After the radical formation, a radical coupling reaction occurs, usually at the 3′ and 5′ position, followed by an enolization to regenerate the aromatic system. Analogous reaction of the 3′,5′-dimethylacetaminophen free radical should be much slower for two reasons. First, due to steric effects the 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 in the dimethyl analog without breaking carbon-carbon bonds.

Phenol derivatives can be oxidized by inorganic oxidizing agents such as PbO_2, $Pb(OAc)_4$ and $KMnO_4$. The horseradish peroxidase/H_2O_2 system, a generally accepted model for the peroxidase activity of prostaglandin H synthase, has an oxidizing potential similar to that of $KMnO_4$. Initial attempts to obtain spectra of the transient acetaminophen phenoxyl radical using horseradish peroxidase and H_2O_2 in a static system were unsuccessful. Only a stable, single broad line was observed which increased in intensity over the time course of the experiment (8 min) (*19*) (Fig. 1).

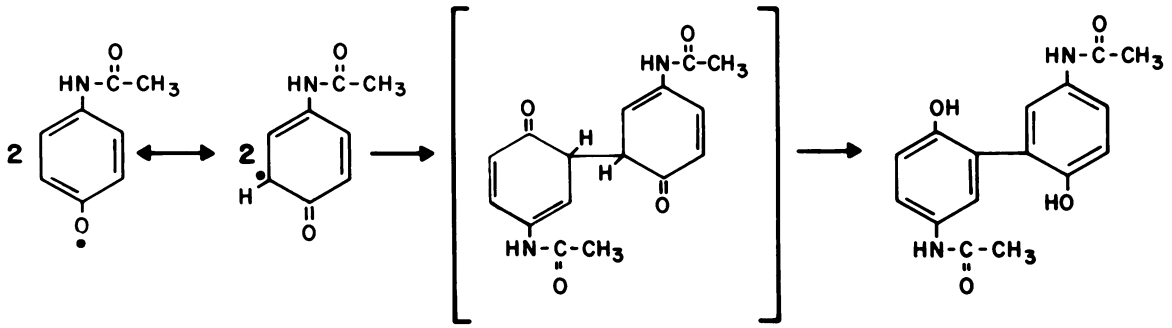
It appears that the broad signal is due to the subsequent formation of a melanin-like radical. Melanins are high-molecular weight polymers of quinone-, semiquinone (paramagnetic)-, and hydroquinone-type units in equilibrium. The observed ESR spectra of melanins (g = 2.004, line width of 4–10 G, and no detectable hyperfine structure) 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, is consistent with the anticipated formation of a melanin-related radical in the acetaminophen system. The slow rate of formation and the broad ESR signal are both inconsistent with the assignment of this species to the initial phenoxyl metabolite of acetaminophen as reported (*13*).

However, using the fast-flow technique, West et al. (*19*) found an intense, three-line ESR spectrum with amplitude ratios 1:2:1 between 20 and 100 msec after mixing (Fig. 2*A*). The signal has the characteristic g value of 2.0043 ± 0.0002 and can be assigned to an oxygen-centered phenoxyl free radical. It is similar to those obtained with other phenolic substrates in fast-flow horseradish peroxidase/H_2O_2 systems (*20,21*). The observed three-line pattern is characteristic of *para*-substituted phenoxyl radicals with a dominant large coupling to the two equivalent *ortho* hydrogens (*22*). For example, the acetaminophen coupling, 5.1 G, is comparable to phenoxyl itself, 6.6 G, but contrasts with the lower values of delocalized and stabilized species such as *p*-benzosemiquinone, a^{H}_{ortho} = 2.48 G (*22*). Hyperfine structure due to other magnetic nuclei present in the radical can be observed using a lower modulation amplitude (Fig. 2*B*) under the same flow conditions.

If the transient ESR signal arose from an acetaminophen dimer, one would anticipate entirely different



coupling constants for the *ortho* protons. For example, if the spin were localized on one ring, only a doublet pattern would be expected. Delocalization over both rings (i.e., four *ortho* positions) would give a triplet, but with one half the *ortho* splitting typical of substituted phenoxyl radicals.



(6)

West et al. (*19*) used a stopped-flow procedure to verify that this is the true transient phenoxyl radical and observed changes in the acetaminophen-derived free radical with time. The three-line spectrum 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. 3) (time 8 min). As expected, it is the same melanin signal as detected under static conditions (g value of 2.0048 ± 0.0003, peak-to-peak line width of 5 G).

Turning back to the question of metabolism, it is useful to consider the possible significance of this unstable transient radical which West et al. (*19*) have positively identified. The reactivity of the phenoxyl radical could render this species responsible for the toxicity usually attributed to *N*-acetyl-*p*-benzoquinone imine. Note that the relative instability of the acetaminophen radical compared to delocalized species, such as melanin radicals or zwitterions derived from *p*-aminophenol, is consistent with the enhanced electrophilic character of transient phenoxyl radicals (i.e., greater unpaired spin density at the oxygen and the *ortho* hydrogens).

The formation of this free radical by mammalian enzymes, cytochrome P-450 and prostaglandin H synthase has been proposed but not proven. We found that 3.0 units/mL of bovine lactoperoxidase could replace horseradish peroxidase in our experiments (*19*). The ESR signal was totally dependent on enzyme, H_2O_2 and acetaminophen, as is the case with the horseradish peroxidase. Prostaglandin H synthase gives the same result when H_2O_2 is used as the source of oxidative equivalents (unpublished data).

Recently Rosen et al. reported that the persistent melanin polymer signal is also observed in a static oxidation experiment employing phenobarbital-induced hamster hepatic microsomes (*14*). However, ESR detection of the more elusive short-lived acetaminophen phenoxyl radical was not reported, and observation of the transient species is a necessary condition for a free

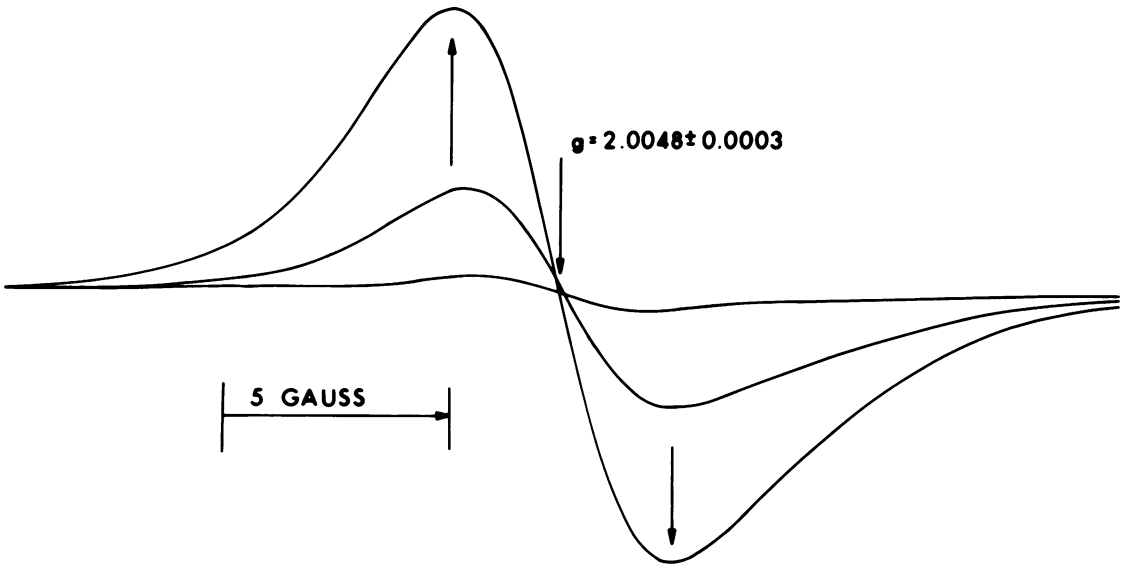


FIGURE 1. ESR spectrum of acetaminophen "melanin" formed by horseradish peroxidase and hydrogen peroxide showing growth of the melanin-type free radical signal. The concentrations of acetaminophen and H_2O_2 in the 3-mL incubation mixture of pH 7.4 phosphate buffer were 8.3 mM and 83 mM, respectively. The room temperature incubation mixture was deoxygenated with N_2 and the reaction initiated with 0.15 units/mL of horseradish peroxidase. From West et al. (*19*) with permission.

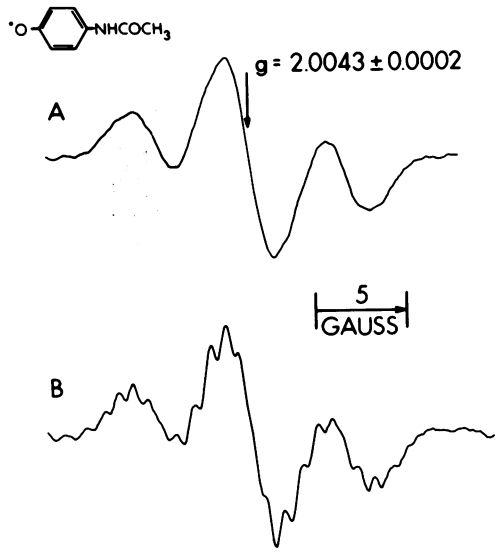


FIGURE 2. ESR fast-flow spectra of the acetaminophen free radical produced in the presence of horseradish peroxidase and hydrogen peroxide. The concentrations of acetaminophen, H_2O_2 and horseradish peroxidase in the flat cell were 5.0 mM, 12 mM, and 1.7 units/mL, respectively. Acetaminophen/H_2O_2 in 2 L of pH 7.5 phosphate buffer deoxygenated with N_2 was mixed with an equal volume of horseradish peroxidase in 2 L of deoxygenated, pH 7.5 phosphate buffer at a total flow rate of 100 mL/min. From West et al. (*19*) with permission.

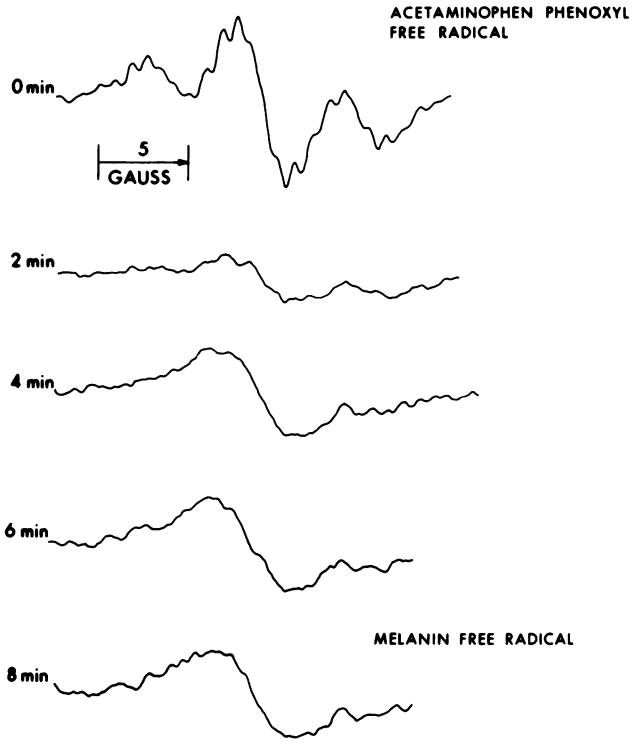


FIGURE 3. ESR stopped-flow spectra of acetaminophen-derived free radicals formed by horseradish peroxidase and hydrogen peroxide. The concentrations of acetaminophen, H_2O_2, and horseradish peroxidase in the flat cell were 5.0 mM, 12 mM, and 1.7 units/mL, respectively. Acetaminophen/H_2O_2 in 2 L of pH 7.5 phosphate buffer deoxygenated with N_2 was mixed with an equal volume of horseradish peroxidase in 2 L of deoxygenated, pH 7.5 phosphate buffer. The flow was terminated and the time-dependent signal recorded at room temperature. From West et al. (*19*) with permission.

radical mechanism of bioactivation. Nevertheless, 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, which are generated by prolonged incubation of acetaminophen in the presence of oxidizing enzymes.

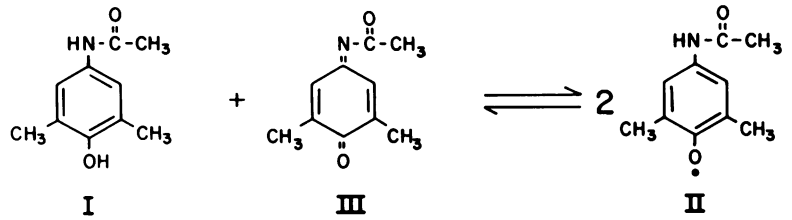
To obtain a more stable metabolite model, we synthesized 3′,5′-dimethylacetaminophen as reported by Fernando et al. (*18*). Nitrosation of 2,6-dimethylphenol with sodium nitrite led to 2,6-dimethyl-4-nitrosophenol, which was reduced with PtO_2/H_2 in a mixture of acetic acid and acetic anhydride to yield 3′,5′-dimethyl-4′-hydroxyacetanilide.

As expected, in contrast to acetaminophen, the enzymatic oxidation of 3′,5′-dimethylacetaminophen gave rise to a stable free radical as the primary metabolite (*23*). Its ESR signal is detectable in static systems. We never observed the single-line signal characteristic of acetaminophen-derived melanin formation.

The incubation of 3′,5′-dimethylacetaminophen, hydrogen peroxide and horseradish peroxidase in phosphate buffer, pH 7.4, resulted in an ESR spectrum showing 7 lines (Fig. 4*A*). The signal depends upon the presence of both horseradish peroxidase and hydrogen peroxide (Fig. 4B-C) and can be attributed to the 3′,5′-dimethylacetaminophen phenoxyl free radical formed by the one-electron oxidation of 3′,5′-dimethylacetaminophen. The nonenzymatic formation of the same radical is achieved by comproportionation of 3′,5′-dimethylacetaminophen and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine [Eq. (7)] (Fig. 4*E*).

Because the radical is in rapid equilibrium with 3′,5′-dimethylacetaminophen and N-acetyl-3,5-dimethyl-*p*-benzoquinone imine, it will be formed during the reaction even if the enzyme catalyzes a direct two-electron oxidation. The difficulty in distinguishing between one- and two-electron enzymatic oxidations of hydroquinones has been extensively discussed by Yamazaki (*24*), but horseradish peroxidase-catalyzed oxidations generally proceed by one-electron transfers in those cases where radical intermediates have been detected.

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. 4*F*). Apparently, decay of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine proceeds, at least in part, through reduction,



(7)

as does the decomposition of *N*-acetyl-*p*-benzoquinone imine itself (*4*). Solutions of 3′,5′-dimethylacetaminophen alone do not give an ESR signal, demonstrating that air oxidation will not form this free radical (Fig. 4*G*).

Comparison of the hyperfine constants from the phenoxyl radicals derived from acetaminophen (*19*) and *ortho*-substituted phenols, i.e., 2,4,6-trimethylphenoxyl, $a^{H}_{o\text{-}CH_3} = 6$ G (*24*), and 2,6-dimethyl-4-methoxyphenoxyl, $a^{H}_{o\text{-}CH_3} = 4.7$ G (*25*), made it possible to attribute the seven-line pattern to six 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. As can be seen (Fig. 5), these instrumental conditions also resulted in a poor signal/noise ratio. Only five out of seven groups representing the ring-methyl hydrogen splitting are visible, while the other two are now hidden in the noise. To achieve a definite assignment of splitting constants, the acetyl group of the 3′,5′-dimethylacetaminophen was deuterated. Part of the ESR spectrum resulting from this compound is shown in Figure 6. 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 (*23*).

In general, ESR spectroscopy shows that the phenoxyl radicals from these model compounds have similar

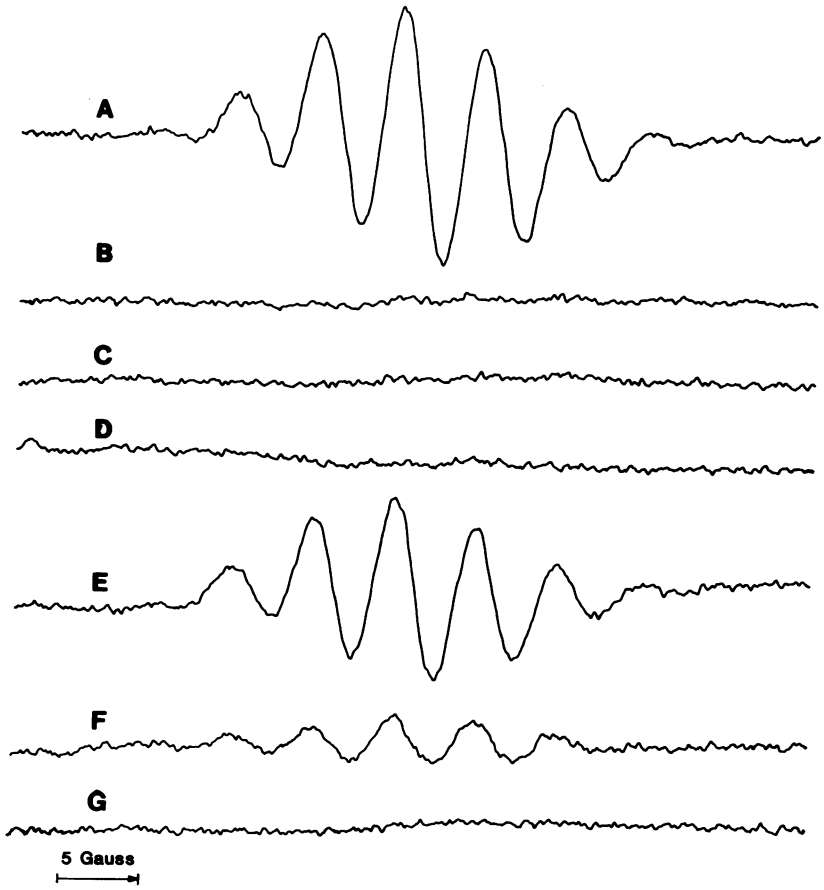


FIGURE 4. ESR spectra of the 3′,5′-dimethylacetaminophen phenoxyl free radical in phosphate buffer, pH 7.4. Enzymatic formation: (*A*) 5 mM 3′,5′-dimethylacetaminophen, 2.5 mM H_2O_2 and 0.2 μg/mL horseradish peroxidase; (*B*) H_2O_2 omitted; (*C*) horseradish peroxida omitted; (*D*) horseradish peroxidase heat-denatured. Comproportionation: (*E*) 2.5 mM 3′,5′-dimethylacetaminophen and 2.5 mM *N*-acet 3,5-dimethyl-*p*-benzoquinone imine; (*F*) 2.5 mM *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine; (*G*) 2.5 mM 3′,5′-dimethylacetaminophen. Fr Fischer and Mason (*23*) with permission.

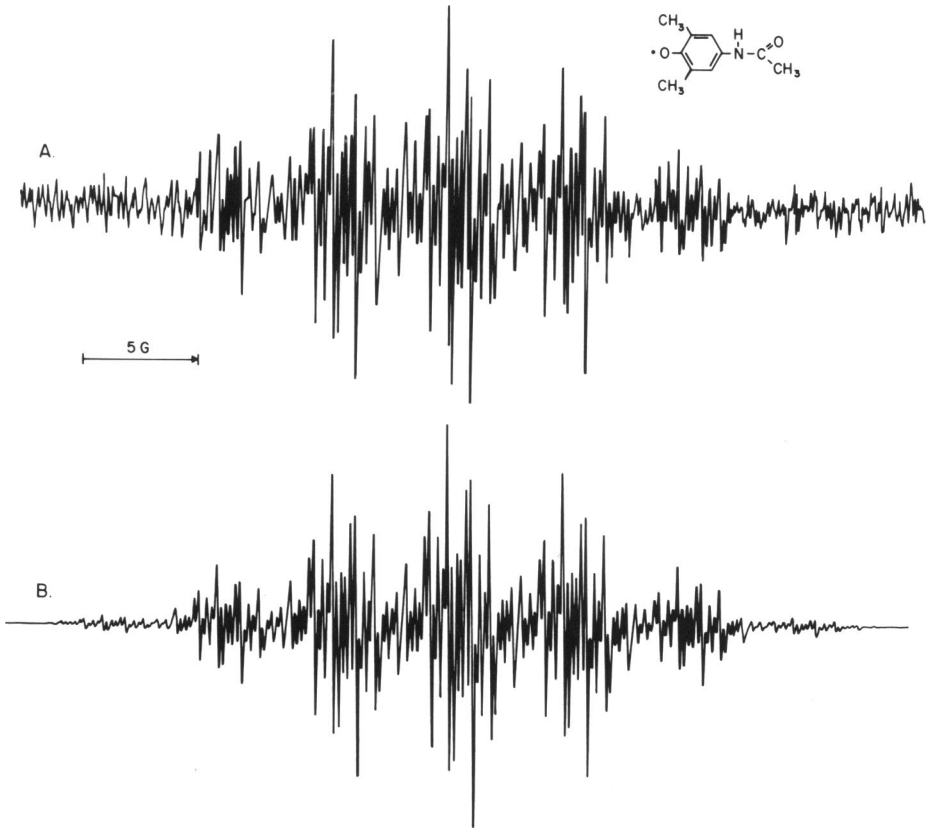


FIGURE 5. High resolution ESR spectrum of the 3′,5′-dimethylacetaminophen phenoxyl free radical obtained from an anaerobic incubation: (*A*) experimental spectrum obtained with 15 mM 3′,5′-dimethylacetaminophen, 10 mM H_2O_2 and 1.0 μg/mL horseradish peroxidase in phosphate buffer, pH 7.4, under a nitrogen atmosphere; (*B*) computer simulation. Hyperfine splitting constants were $a^{H}_{3',5'CH3}$ = 5.05 G, a^{N} = 0.404 G, $a^{H}_{2',6'}$ = a^{H}_{NH} = 0.82 G, and $a^{H}_{C(O)CH3}$ = 1.0 G. From Fischer and Mason (*23*) with permission.

splittings, electron distribution and, hence, reactivity, giving us confidence for further studies of 3′,5′-dimethyl acetaminophen as a model compound. With 3′,5′-dimethylacetaminophen it was easy to study the dependence of the maximum radical concentration on the H_2O_2, horseradish peroxidase and substrate concentrations. Increasing the hydrogen peroxide concentration resulted in an enhanced ESR signal intensity corresponding to the increased radical concentration (Fig. 7). When a concentration of 5.0 mM 3′,5′-dimethylacetaminophen was used, the maximum radical concentration was achieved at 2.5 mM hydrogen peroxide. Note that this value corresponds overall to one oxidizing equivalent per 3′,5′-dimethylacetaminophen. Higher hydrogen peroxide concentrations gave the same maximum radical concentration, but the signal was more transient.

The horseradish peroxidase concentration (Fig. 8) was varied over five orders of magnitude with enzyme concentrations as low as 1.0 ng/mL. The resulting radical concentration was enzyme-independent above ~0.1 μg/mL over more than three orders of magnitude, whereas it was proportional to the concentration of the horseradish peroxidase at concentrations less than 0.1 μg/mL, presumably due to inactivation of the enzyme. The maximum radical concentration was proportional to the substrate concentration (Fig. 9) over the range of 50 μM to the solubility limit, 10 mM. The absolute radical concentration was determined using one set of conditions: 2.0 mM 3′,5′-dimethylacetaminophen, 0.1 μg/mL horseradish peroxidase, and 2.0 mM H_2O_2. The time course of the radical formation in this experiment is shown (Fig. 10), demonstrating the approach to a limiting concentration of 2.5×10^{-7} M (*23*).

Through the use of ultraviolet-spectroscopy, the enzymatic formation of the two-electron oxidation product of 3′,5′-dimethylacetaminophen, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, was demonstrated (Fig. 11). Decay of the 3′,5′-dimethylacetaminophen 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. The resulting spectrum was identical to the one obtained from independently synthesized *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, which has nearly the same absorption maximum as found in *n*-hexane (*18*). Figure 12 shows the effect of the enzyme concentration on the reaction. As one can see, varying the concentration of horseradish peroxidase from 0.5 μg/mL to 4.0 μg/mL results in a linear increase in the rate of *N*-acetyl-3,5-dimethyl-*p*-benzo-

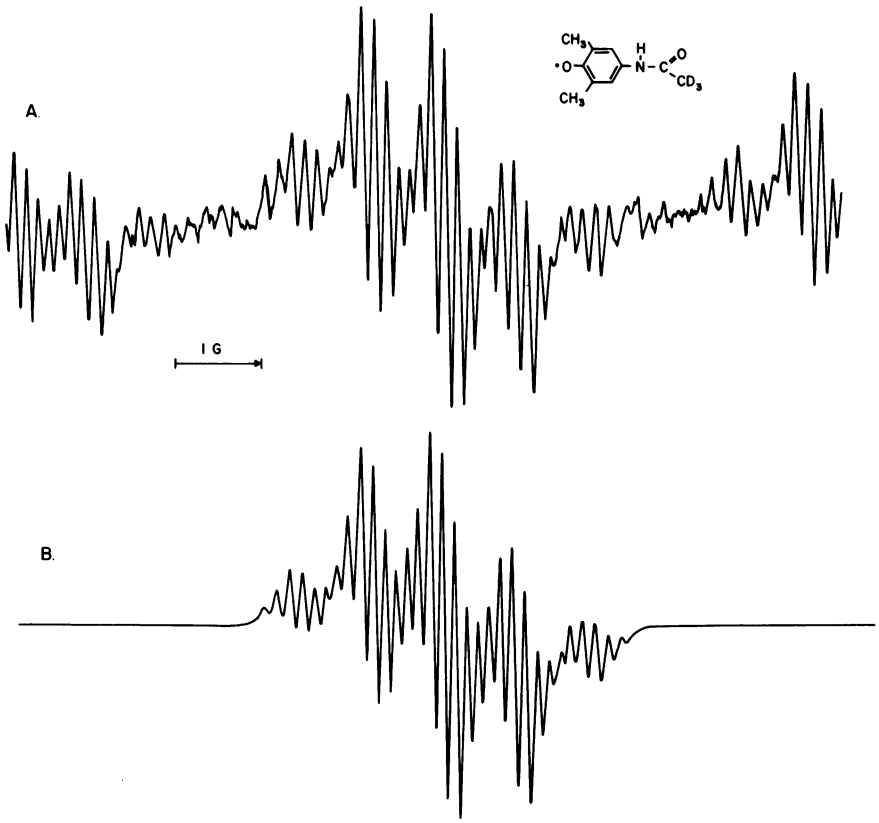
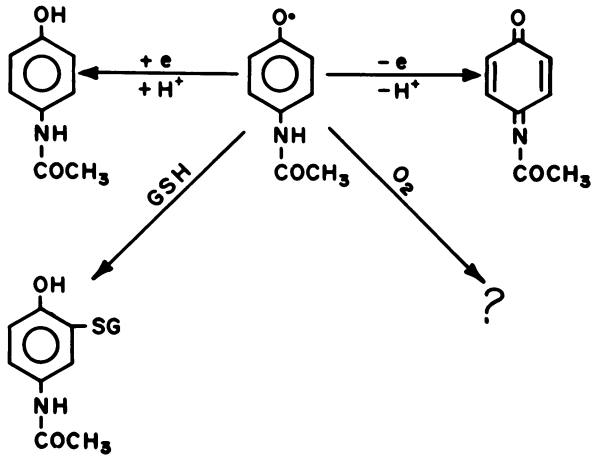


FIGURE 6. Third group of the ESR spectrum obtained from the $^2H_3$-3′,5′-dimethylacetaminophen phenoxyl free radical: (*A*) experimental conditions were: 10 mM $^2H_3$-3′,5′-dimethylacetaminophen, 10 mM H_2O_2, and 1.0 μg/mL horseradish peroxidase in phosphate buffer, pH 7.4, under a nitrogen atmosphere; (*B*) computer simulation. Hyperfine splitting constants were a^N = 0.404 G, $a^H_{2',6'}$ = a^H_{NH} = 0.82 G and $a^{2H}_{C(O)CH_3}$ = 0.154. From Fischer and Mason(*23*) with permission.

quinone imine formation, but does not affect the total amount of product formed when the 3′,5′-dimethylacetaminophen and H_2O_2 concentrations are held constant. The slow decay of the absorbance is probably due to hydrolysis of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (*18*). The stability of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine is in marked contrast to that of *N*-acetyl-*p*-benzoquinone imine (*26*).

We are still left with the question of why 3′,5′-dimethylacetaminophen and acetaminophen are both toxins, even though processes involving 3′,5′-attack, such as radical coupling and nucleophilic reaction with glutathione, are absent with 3′,5′-dimethylacetaminophen, though well documented for acetaminophen. It may be significant that electron transfer processes (i.e., oxidation/reduction) of both compounds are not affected by methyl substitution. Ultimately toxicity may involve an initial one-electron transfer to an electrophilic phenoxyl or quinone imine species. Some possible reactions of the acetaminophen radical are shown in Eq. (8). Except for the conjugation reaction with glutathione, these electrophilic transfer processes should be valid for both acetaminophen and its methylated analog.

The redox processes are demonstrated by the comproportionation reaction of 3′,5′-dimethylacetaminophen and its quinone imine as well as by the horseradish peroxidase/H_2O_2-dependent oxidation of 3′,5′-dimethylacetaminophen. Enzymatic reduction of the N-acetyl-*p*-benzoquinone imine to acetaminophen is shown by Powis et al. (*27*). However, they failed to show proof of a free-radical intermediate.



(8)

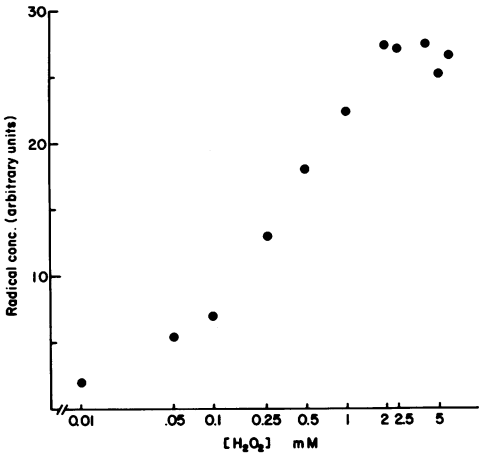


FIGURE 7. Hydrogen peroxide dependence of the maximum 3',5'-dimethylacetaminophen phenoxyl free radical concentration. All incubations contained 5.0 mM 3',5'-dimethylacetaminophen and 0.2 μg/mL horseradish peroxidase in phosphate buffer, pH 7.4. From Fischer and Mason (*23*) with permission.

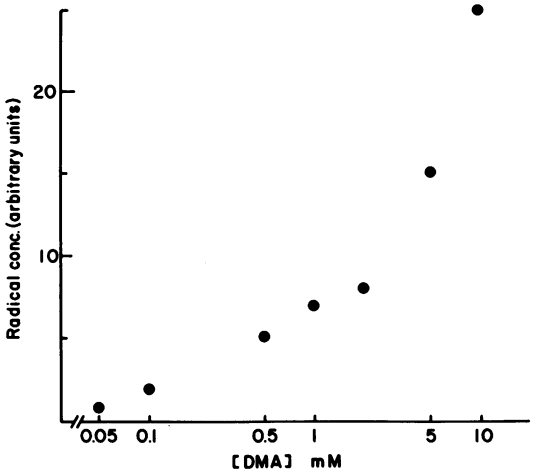


FIGURE 9. Substrate (DMA) dependence of the maximum 3',5'-dimethylacetaminophen phenoxyl free radical concentration. All incubations contained 0.2 mM hydrogen peroxide and 0.2 μg/mL horseradish peroxidase in phosphate buffer, pH 7.4.

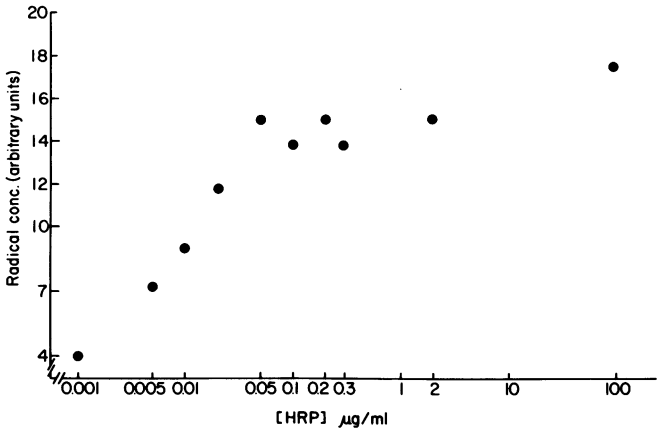


FIGURE 8. Horseradish peroxidase (HRP) dependence of the maximum 3',5'-dimethylacetaminophen phenoxyl free radical concentration. All incubations contained 5.0 mM 3',5'-dimethylacetaminophen, and 2.5 mM H_2O_2 in phosphate buffer, pH 7.4. From Fischer and Mason(*23*) with permission.

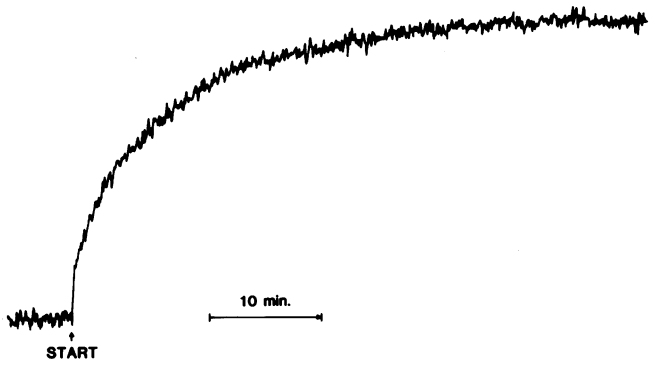


FIGURE 10. Time course of the amplitude of the ESR signal obtained from incubations containing 2.0 mM 3',5'-dimethylacetaminophen, 2.0 mM H_2O_2, and 0.1 μg/mL horseradish peroxidase in phosphate buffer, pH 7.4. From Fischer and Mason (*23*) with permission.

In Eq. (8), there is a question mark for the reaction with oxygen. Consider benzosemiquinone as a model, which is known to produce superoxide kinetically even though the thermodynamic data shows the equilibrium lays on the side of the semiquinone radical anion (*28,29*). It is important to note that oxidation of *p*-benzosemiquinone forms a stable neutral quinone, but still does not prefer to transfer an electron to oxygen. In the case of the acetaminophen radical, the initial product formed is the quinone imine cation with a charge right next to the acyl group. Clearly, net electron transfer to oxygen should be extremely difficult, and recent data indicates that this does not occur.

Initial experiments using the horseradish peroxidase/H_2O_2 oxidation of acetaminophen or 3',5'-dimethylacetaminophen, which attempted to spin trap the superoxide free radical with DMPO (5,5-dimethyl-1-pyrroline-1-oxide), have been unsuccessful in our laboratory. This is in agreement with the result of Powis et al. (*27*), who tried to measure superoxide formation during the reduction of *N*-acetyl-*p*-benzoquinone imine by reduction of acetylated-cytochrome c. *N*-acetyl-*p*-benzoquinone imine even inhibited superoxide formation during the reduction of 2,5-dimethyl-*p*-benzoquinone. In addition, no oxygen stress could be found, as determined by measurement of oxidized glutathione in bile (*30*), blood, or tissue (*31*). A recently published DMPO-superoxide adduct spectrum in an *N*-acetyl-*p*-benzoquinone imine/acetaminophen system (*32*) prompted us to carefully repeat our experiments. 3',5'-Dimethylacetaminophen free radical was also reported to react with oxygen and form superoxide (*32*); however, in contrast to our work, the authors could not find ESR-detectable concentrations of 3',5'-dimethylacetaminophen free

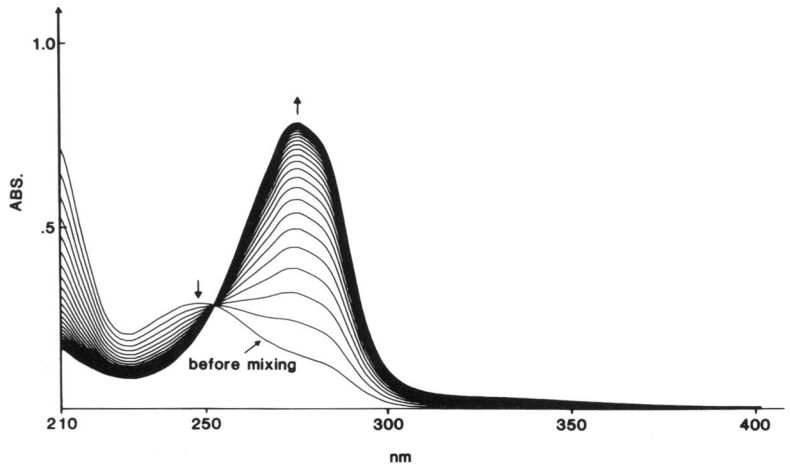


FIGURE 11. Oxidation of 3′,5′-dimethylacetaminophen by horseradish peroxidase/H_2O_2: optical spectra. The incubation mixture contained 25 μM 3′,5′-dimethylacetaminophen, 0.83 μg/mL horseradish peroxidase and 173 μM H_2O_2 in phosphate buffer, pH 7.4. At zero time, horseradish peroxidase was added and repetitive scanning started (10 nm/sec); a 15-sec interval was required to return the pen to the left edge at the end of each scan. From Fischer and Mason (*23*) with permission.

radical. Although we have been able to obtain the 3′,5′-dimethylacetaminophen radical in aerobic incubations, we could not detect a trace of superoxide. Therefore, several different approaches were employed. In addition to the spin trap experiments, oxidation of acetaminophen with horseradish peroxidase in the flow system was performed under oxygen as well as under nitrogen. Figure 13 shows the ESR signal obtained in both cases. All spectrometer conditions were the same. The oxygen-saturated system shows a lower signal amplitude or signal height, but a greater peak-to-peak linewidth, due to shorter relaxation times caused by the presence of oxygen in the system. The double integrations, which are proportional to the radical concentrations, are the same in both cases, implying the acetaminophen phenoxyl free radical is unreactive with molecular oxygen on the time scale of its rapid bimolecular self-reaction.

Oxygraph experiments confirmed this result. As seen in Figure 14, there is no detectable oxygen consumption in peroxidase systems, where the acetaminophen phenoxyl free radicals are readily detected. In case an unfavorable equilibrium had affected the results, the same experiment was performed in the presence of superoxide dismutase (40 μg/mL) to drive the reactions toward the *N*-acetyl-*p*-benzoquinone imines. Again no oxygen was consumed (data not shown). These results are consistent with our expectations for the rather electrophilic phenoxyl radical, whose immediate oxidation product is relatively unstable.

In summary, ESR spectroscopy employing a millisecond time scale fast-flow method has revealed the

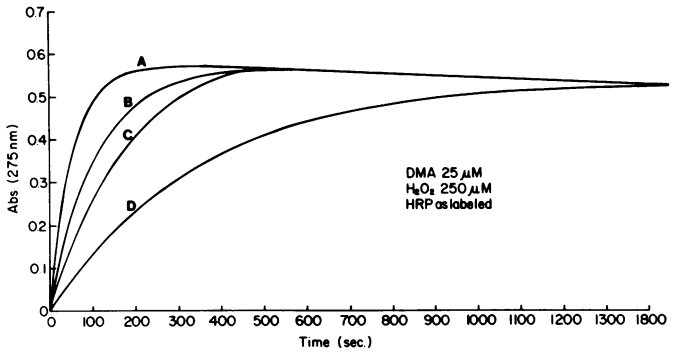


FIGURE 12. Effect of enzyme concentration on *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine formation. The time-dependent studies were performed applying the dual wavelength mode at 275 nm versus the isosbestic point at 252 nm. At zero time, hydrogen peroxide was added to a solution containing 3′,5′-dimethylacetaminophen and 4.0 μg/mL (*A*), 2.0 μg/mL (*B*), 1.0 μg/mL (*C*), and 0.5 μg/mL (*D*) of horseradish peroxidase in phosphate buffer, pH 7.4, respectively. From Fischer and Mason (*23*) with permission.

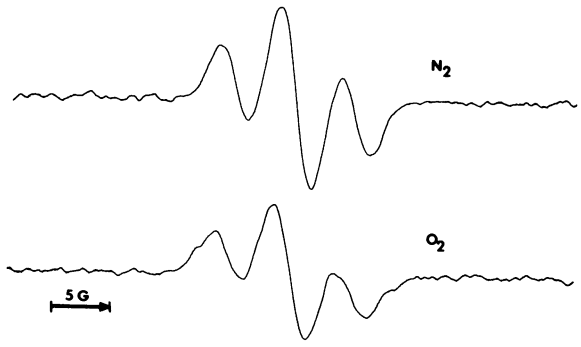


FIGURE 13. ESR spectra of the acetaminophen phenoxyl free radical obtained (*A*) under a nitrogen atmosphere and (*B*) under an oxygen atmosphere. The radical was produced in the presence of horseradish peroxidase and hydrogen peroxide in phosphate buffer, pH 7.4, and the spectra were recorded in a fast-flow system (*19*). The concentrations of acetaminophen, H_2O_2 and horseradish peroxidase in the flat cell were 5.0 mM, 12 mM, and 1.7 units/mL, respectively.

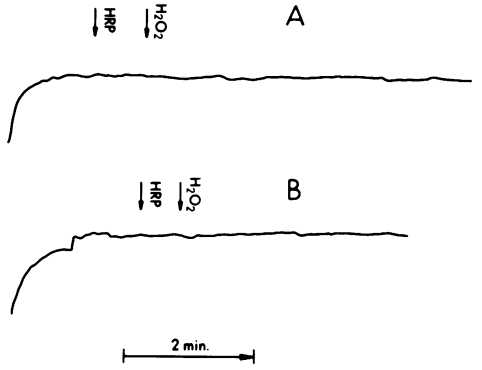


FIGURE 14. Oxygen uptake curves for the horseradish peroxidase/H_2O_2 and acetaminophen (*A*) or 3′,5′-dimethylacetaminophen (*B*) systems. Acetaminophen or 3′,5′-dimethylacetaminophen was placed in the chamber and other components added at the points indicated. Final concentrations were 4.0 mM acetaminophen (*A*); 5.0 mM 3′,5′-dimethylacetaminophen (*B*); 0.1 mM H_2O_2 and 1.0 mg/mL horseradish peroxidase.

formation of a transient phenoxyl radical in the reaction of acetaminophen with horseradish peroxidase/H_2O_2, bovine lactoperoxidase/H_2O_2 (*19*) and prostaglandin H synthase/H_2O_2 (unpublished data). 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. In contrast to acetaminophen, the *N*-acetyl-*p*-benzoquinone imine and radical metabolites of 3′,5′-dimethylacetaminophen are more stable and can be detected without resort to rapid-mixing techniques (*23*). The structure of the acetaminophen free radical and its *ortho*-substituted methylated analogs could be established by complete analysis of the ESR spectra and confirmed by deuterium isotope substitution. The acetaminophen derived free radical behaves like a typical phenoxyl radical, which has a very low reactivity towards oxygen (*33*). Specifically, electron donation by this radical to oxygen to form superoxide is negligible. The two-electron metabolite, the *N*-acetyl-*p*-benzoquinone imine, is also an electrophile that reacts with reducing agents. Our work with methylated-acetaminophen suggests that the mechanism of acetaminophen toxicity is via an electron transfer, the consequences of which are yet to be established.

REFERENCES

1. Prescott, L. F., Roscoe, P., Wright, N., and Brown, S. S. Plasma-paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. Lancet 1: 519–522 (1971).
2. Boyer, T. D. J., and Rouff, S. L. Acetaminophen-induced hepatic necrosis and renal failure. J. Am. Med. Assoc. 218: 440–441 (1971).
3. Miner, D. J., and Kissinger, P. T. Evidence for the involvement of *N*-acetyl-*p*-quinoneimine in acetaminophen metabolism. Biochem. Pharmacol. 28: 3285–3290 (1979).
4. Dahlin, D. C., and Nelson, S. D. Synthesis, decomposition kinetics, and preliminary toxicological studies of pure N-acetyl-*p*-benzoquinone imine, a proposed toxic metabolite of acetaminophen. J. Med. Chem. 25: 885–886 (1982).
5. Jollow, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillett, J. R., and Brodie, B. B. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. J. Pharmacol. Exptl. Therap. 187: 195–202 (1973).
6. Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gillett, J. R., and Brodie, B. B. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J. Pharmacol. Exptl. Therap. 187: 211–217 (1973).
7. Jollow, D. J., Thorgeirsson, S. S., Potter, W. Z., Hashimoto, M., and Mitchell, J. R. Acetaminophen-induced hepatic necrosis. VI. Metabolic disposition of toxic and nontoxic doses of acetaminophen. Pharmacology 12: 251–275 (1974).
8. Mitchell, J. R., Jollow, D. J., Gillette, J. R., and Brodie, B. B. Drug metabolism as a cause of drug toxicity. Drug Metab. Dispos. 1: 418–423 (1973).
9. Gemborys, M. W., and Mudge, G. H. Formation and disposition of the minor metabolites of acetaminophen in the hamster. Drug. Metab. Dispos. 9: 340–351 (1981).
10. Hinson, J. A., Pohl, L. R., and Gillette, J. R. *N*-Hydroxyacetaminophen: a microsomal metabolite of *N*-hydroxyphenacetin but apparently not of acetaminophen. Life Sci. 24: 2133–2138 (1979).
11. Hinson, J. A., Pohl, L. R., Monks, T. J., and Gillette, J. R. Minireview: acetaminophen-induced hepatotoxicity. Life Sci. 29: 107–116 (1981).
12. De Vries, J. Commentary: Hepatotoxic metabolic activation of paracetamol and its derivatives phenacetin and benorilate: oxygenation or electron transfer? Biochem. Pharmacol. 30: 399–402 (1981).
13. Nelson, S. D., Dahlin, D. C., Rauckman, E. J., and Rosen, G. M. Peroxidase-mediated formation of reactive metabolites of acetaminophen. Mol. Pharmacol. 20: 195–199 (1981).
14. Rosen, G. M., Singletary, W. V., Jr., Rauckman, E. J., and Killenberg, P. G. Acetaminophen hepatotoxicity, an alternative mechanism. Biochem. Pharmacol. 32: 2053–2059 (1983).
15. Moldéus, P., and Rahimtula, A. Metabolism of paracetamol to a glutathione conjugate catalyzed by prostaglandin synthetase. Biochem. Biophys. Res. Commun. 96: 469–475 (1980).
16. Boyd, J. A. and Eling, T. E. Prostaglandin endoperoxide synthetase-dependent cooxidation of acetaminophen to intermediates which covalently bind *in vitro* to rabbit renal medullary microsomes. J. Pharmacol. Exptl. Therap. 219: 659–664 (1981).
17. Moldèus, P., Andersson, B., Rahimtula, A., and Berggren, M. Prostaglandin synthetase catalyzed activation of paracetamol. Biochem. Pharmacol. 31: 1363–1368 (1982).
18. Fernando, C. R., Calder, I. C., and Ham, K. N. Studies on the mechanism of toxicity of acetaminophen. Synthesis and reactions of *N*-acetyl-2,6-dimethyl- and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imines. J. Med. Chem. 23: 1153–1158 (1980).
19. West, P. R., Harman, L. S., Josephy, P. D., and Mason, R. P. Acetaminophen: enzymatic formation of a transient phenoxyl free radical. Biochem. Pharmacol. 33: 2933–2936 (1984).
20. Shiga, T., and Imaizumi, K. Generation of phenoxy radicals by methemoglobin–hydrogen peroxide studies by electron paramagnetic resonance. Arch. Biochem. Biophys. 154: 540–547 (1973).
21. Shiga, T., and Imaizumi, K. Electron spin resonance study on peroxidase- and oxidase-reactions of horseradish peroxidase and methemoglobin. Arch. Biochem. Biophys. 167: 469–479 (1975).
22. Uber, W., and Stegmann, H. B. Aroxylradicals. In: Landolt-Börnstein: Numberical Data and Functional Relationships in Science and Technology, Vol. 9, Part C2, (H. Fischer and K.-H. Hellwege, Eds.), Springer Verlag, New York, 1979, p. 29.
23. Fischer, V., and Mason, R. P. Stable free radical and benzoquinone imine metabolites of an acetaminophen analogue. J. Biol. Chem. 259: 10284–10288 (1984).
24. Yamazaki, I. and Ohnishi, T. One-electron-transfer reactions in biochemical systems. I. Kinetic analysis of the oxidation-reduction equilibrium between quinol-quinone and ferro-ferricytochrome c. Biochim. Biophys. Acta 112: 469–481 (1966).
25. Stone, T. J. and Waters, W. A. Aryloxy-radicals. Part I. Electron spin resonance spectra of radicals from some substituted monohydric phenols. J. Chem. Soc. 112: 213–218 (1964).

26. Dahlin, D. C., Miwa, G. T., Lu, A. Y. H., and Nelson, S. D., N-Acetyl-*p*-benzoquinone imine: a cytochrome P-450 mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. U.S. 81: 1327–1331 (1984).
27. Powis, G., Svingen, B. A., Dahlin, D. C., and Nelson, S. D. Enzymatic and non-enzymatic reduction of *N*-acetyl-*p*-benzoquinone imine and some properties of the *N*-acetyl-*p*-benzosemiquinone imine radical. Biochem. Pharmacol. 33: 2367–2370 (1984).
28. Sawada, Y., Iyanagi, T., and Yamazaki, I. Relation between redox potentials and rate constants in reactions coupled with the system oxygen-superoxide. Biochemistry 14: 3761–3764 (1975).
29. Meisel, D., and Czapski, G. One-electron transfer equilibria and redox potentials of radicals studied by pulse radiolysis. J. Phys. Chem. 79: 1503–1509 (1975).
30. Lauterburg, B. H., Smith, C. V., Hughes, H., and Mitchell, J. R. Biliary excretion of glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. J. Clin. Invest. 73: 124–133 (1984).
31. Adams, J. D., Jr., Lauterburg, B. H., and Mitchell, J. R. Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. J. Pharmacol. Exptl. Therap. 227: 749–754 (1983).
32. Rosen, G. M., Raukman, E. J., Ellington, S. P., Dahlin, D. C., Christie, J. L., and Nelson, S. D. Reduction and glutathione conjugation reactions of *N*-acetyl-*p*-benzoquinone imine and two dimethylated analogues. Mol. Pharmacol. 25: 151–157 (1984).
33. Doba, T., Burton, G. W., Ingold, K. U., and Matsuo, M. α-Tocopheroxyl decay: lack of effect by oxygen. J. Chem. Soc., Chem. Commun. 1984: 461–462 (1984).