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Peroxidase-Mediated Formation of Reactive Metabolites of Acetaminophen

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SUMMARY

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Acetaminophen (4′-hydroxyacetanilide) is metabolized by horseradish peroxidase to a reactive metabolite or metabolites that become covalently (irreversibly) bound to either mouse liver microsomal protein or bovine serum albumin. The time-dependent reaction requires the presence of both the enzyme and hydrogen peroxide. Although the binding is almost completely inhibited by either catalase (0.2 mg/ml) or ascorbic acid (1.5 mM), it is unaffected by superoxide dismutase (20 μg/ml). Glutathione also inhibits the binding (~50% at a concentration of 0.1 mM) with formation of the same glutathione conjugate that is produced from acetaminophen and glutathione in the presence of mouse liver microsomal oxygenases. An acetaminophen radical is generated by horseradish peroxidase in the presence of hydrogen peroxide as determined by electron paramagnetic resonance spectroscopy. The radical rapidly disappears in the presence of microsomal protein, bovine serum albumin, or glutathione, and is quenched by ascorbic acid with the concomitant formation of the ascorbate radical. The acetaminophen radical can be photolytically generated and spin-trapped with 5,5-dimethyl-1-pyrroline-1-oxide. The hyperfine splitting constants A_H^β (18.7 G) and A_N (15.2 G) strongly suggest that the radical is primarily centered on a carbon atom. These results indicate that a reactive metabolite of acetaminophen is formed in a peroxidase-mediated reaction with properties similar to that which is produced in microsomal incubations, and that an acetaminophen radical is formed under the same peroxidative conditions.

INTRODUCTION

The widely used analgesic acetaminophen causes liver necrosis in man and experimental animals (1, 2). Metabolic activation of acetaminophen was originally postulated to occur via *N*-oxidation by cytochrome P-450 to *N*-hydroxyacetaminophen (*N*,4′-dihydroxyacetanilide) which dehydrated to the arylating agent, *N*-acetyl-*p*-benzoquinoneimine (3). Subsequently, *N*-hydroxyacetaminophen has been synthesized in crystalline form and has been found to have both chemical and toxicological characteristics consistent with its postulated role as an intermediate in the metabolism of acetaminophen to an arylating agent (4–7).

However, recent evidence has appeared (8, 9) which suggests that, if *N*-hydroxyacetaminophen is an intermediate in microsomal oxygenase-mediated covalent (irreversible) binding of acetaminophen to tissue protein, it must decompose at the enzymatic site of oxidation. Alternatively, *N*-hydroxyacetaminophen may not be formed as an intermediate, in which case other mechanisms for the formation of reactive metabolites should be considered. Because cytochrome P-450 can function as a peroxidase as well as an oxygenase (10, 11), we have investigated HRP[3]-mediated formation of reactive metabolites of acetaminophen. Experiments with generally labeled [^{3}H]acetaminophen revealed that, in the presence of H_2O_2, HRP can lead to irreversible binding of radiolabel to either microsomal protein or BSA, and this binding reaction is similar to that mediated by cytochrome P-450. In addition, an acetaminophen radical was detected in the HRP-mediated reaction. Although the radical was not detected in microsomal incubations, its formation in the HRP-mediated reaction may provide

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[3] The abbreviations used are: HRP, horseradish peroxidase; BSA, bovine serum albumin; SOD, superoxide dismutase; HPLC, high-pressure liquid chromatography; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide.

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further insight into mechanisms of reactive metabolite generation from acetaminophen.

MATERIALS AND METHODS

General comments. HRP Type II, SOD Type I from bovine blood, catalase from bovine liver, diethylenetriaminepentaacetic acid, BSA, and xanthine were purchased from Sigma Chemical Company (St. Louis, Mo.). Thin-layer chromatographic plates were obtained from Analtech, Inc. (Newark, Del.). Acetaminophen was purchased from Eastman Chemical Company (Rochester, N. Y.). Xanthine oxidase was obtained from Dr. Irwin Fridovich, Department of Biochemistry, Duke University. Chromatographic solvents were purchased from either Matheson, Coleman and Bell (Norwood, Ohio) or Waters Associates (Milford, Mass.) and were of nanograde purity. Radiolabeled [^{3}H]acetaminophen was obtained from New England Nuclear Corporation (Boston, Mass.) and was purified by a combination of chromatography on Analtech silica gel GF thin-layer plates (250 μm thickness), using ether as developing solvent, and by HPLC, using conditions previously described (12). ^{14}C-Acetyl-labeled *N*-methylacetaminophen and ^{14}C-ring-labeled phenacetin were prepared and purified by methods which will be described elsewhere. The spin trap, DMPO, was synthesized according to the method of Bonnett *et al.* (13).

Covalent binding experiments. Standard 3-ml reaction mixtures contained HRP (0.04 unit/ml), H_2O_2 (3 μmoles), purified radiolabeled substrate (0.3 μmole; ~1000 dpm/nmole), and either BSA or mouse liver microsomal protein (6 mg) in 0.05 M sodium phosphate buffer, pH 7.4. The order of addition was buffer (0.5 ml), BSA or microsomes (1 ml, 6 mg/ml in buffer), substrate (0.5 ml, 0.6 mM in buffer), H_2O_2 (20 μl, 150 mM in water), and HRP (1 ml, 0.12 unit/ml). Additions of other enzymes and inhibitors were made in the buffer. Reactions were run at 20° for varying times as described (see Results) and terminated by the addition of 2 ml of ice-cold 0.8 M trichloroacetic acid. The precipitated protein was washed successively with 80% methanol, 3:1 ethanol/ether, and 80% methanol as previously described for covalent binding determinations with [^{3}H]acetaminophen (14). In those incubations containing GSH, the glutathione conjugate of acetaminophen was analyzed by HPLC as previously described (12).

Acetaminophen free radical. The acetaminophen free radical was observed in the presence of HRP and hydrogen peroxide. The reaction medium contained 1 mM acetaminophen, 1 mM H_2O_2, 0.04 units/ml of HRP, and sufficient buffer (0.05 M sodium phosphate buffer with 1 mM diethylenetriaminepentaacetic acid, pH 7.4) to bring the final volume to 0.5 ml. There was no free radical generated if either HRP or hydrogen peroxide was omitted from the reaction mixture. Substituting a superoxide generating system, xanthine-xanthine oxidase at pH 7.8, in place of the HRP/H_2O_2 mixture, did not lead to the formation of the acetaminophen free radical.

Spin trapping studies on the acetaminophen free radical. The spin trapping of acetaminophen free radical was undertaken by using UV photolysis and the water-soluble spin trap, DMPO. The reaction contained 10 mM DMPO and 1 mM acetaminophen in a total volume of 0.5 ml of water. The reaction was commenced by exposing the above mixture to UV light [250-nm light source from Ultra-Violet Products, Inc. (San Gabriel, Calif.), SCT 1 Model] for 4 min. The light was removed and the EPR spectrum shown in Fig. 4 was recorded from the reaction mixture.

Radical formation from N-methylacetaminophen and phenacetin. Both *N*-methylacetaminophen and phenacetin appeared to form unstable radicals at rates considerably lower than the rate of radical formation from acetaminophen. Because of their apparent instability, no characterization of the radicals could be made.

RESULTS

Covalent binding studies. HRP in the presence of H_2O_2 leads to time-dependent covalent binding of radiolabel from [^{3}H]acetaminophen to BSA as an acceptor protein (Fig. 1). The absence of either HRP or H_2O_2 blocked this reaction. Results are not shown for the binding reaction to mouse liver microsomal protein, which when used as an acceptor protein provided curves almost identical with those generated in the presence of BSA as the acceptor. The binding reaction was almost totally inhibited (Fig. 2) in the presence of either catalase (0.2 mg/ml) or ascorbic acid (1.5 mM). In contrast, SOD (20 μg/ml) had no effect on the reaction. Glutathione (0.1 mM) inhibited the binding reaction approximately 50% with concomitant formation of a glutathione conjugate. This conjugate had the same retention time on HPLC as did that formed in microsomal oxygenase-mediated reactions of acetaminophen and glutathione (12). Hydrolysis of the conjugate with a glutathionase preparation from rat kidney homogenate (15) yielded 3-cysteinyl-acetaminophen which was differentiated from its 2-isomer by a method described elsewhere (16).

Radiolabeled analogues of *N*-methylacetaminophen

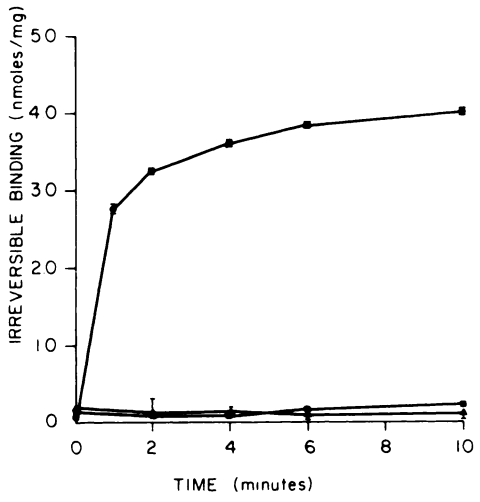

FIG. 1. *HRP/H_2O_2-mediated binding of [^{3}H]acetaminophen to BSA*

Results show the means ± standard deviation of at least four determinations for complete incubation (●——●), minus HRP (■——■), and minus H_2O_2 (▲——▲). Reaction conditions are described under Materials and Methods.

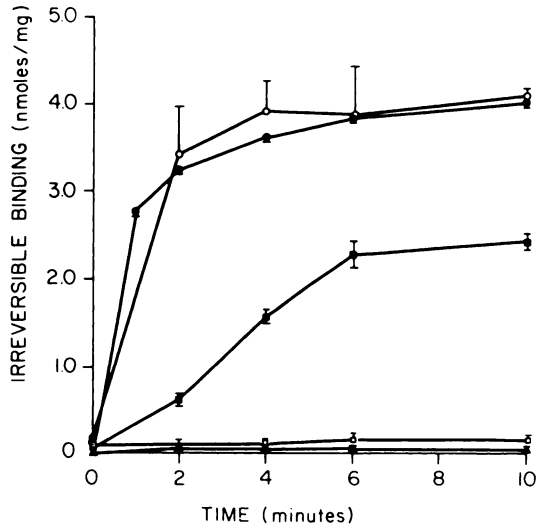

FIG. 2. *HRP/H_2O_2-mediated binding of [^{3}H]acetaminophen to BSA*

Results show the means ± standard deviation of at least four determinations for complete incubation (●——●) and the effects of the addition of glutathione, 0.1 mM (■——■); ascorbic acid, 1.5 mM (▲——▲); SOD, 20 μg/ml (○——○); and catalase, 0.2 mg/ml (□——□).

and phenacetin were not activated significantly by HRP to metabolites that covalently bound to BSA at concentrations (0.1 mM) equivalent to those at which acetaminophen binding reached levels of ~4 nmoles/mg.

Studies on acetaminophen free radical formation. Incubation of acetaminophen with the HRP/hydrogen peroxide system leads to the formation of a free radical which appears immediately upon addition of the enzyme to the reaction mixture and reaches its maximal concentration within 16 min, followed by a slow decay with a $t_{1/2}$ of 2 hr (Fig. 3). Since the free radical signal was not observed in the absence of acetaminophen, HRP, or

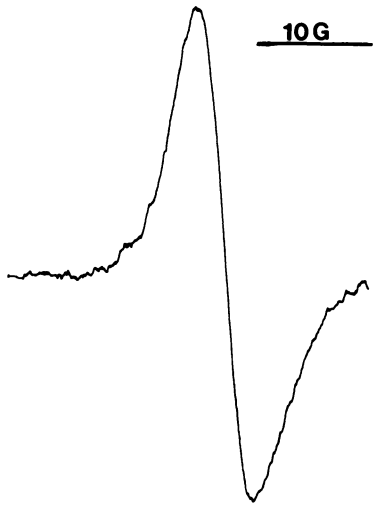

FIG. 3. *EPR spectrum of acetaminophen produced in the presence of HRP/H_2O_2 at pH 7.4*

The microwave power was 10 mW and the modulation frequency was 100 kHz with an amplitude of 1 G. The sweep time was 12.5 G/min at a response time of 3 sec.

H_2O_2, the formation of an acetaminophen radical is presumed. The acetaminophen free radical could not be produced by the superoxide-generating system of xanthine-xanthine oxidase at pH 7.8 and was unstable in the presence of reducing agents such as glutathione and ascorbic acid, producing the ascorbate radical in the presence of ascorbic acid. When mouse hepatic microsomes were added to the acetaminophen-HRP-H_2O_2 system, the acetaminophen free radical disappeared rapidly.

Spin-trapping studies were undertaken to determine whether an acetaminophen radical would be primarily centered on carbon, oxygen, or nitrogen, since this was not clear from the unresolved resonance absorption signal observed in the peroxidase reaction. The radical was produced via the UV photolysis of acetaminophen in the presence of the spin trap DMPO (Fig. 4). Janzen and Liu (17) have previously shown that spin trapping carbon-centered radicals results in the formation of adducts whose spectra show hyperfine splitting constants having $A_H^\beta > A_N$. On the other hand, oxygen-centered radicals result in spectra having $A_N > A_H^\beta$. Addition of a nitrogen free radical (spin of 1) to DMPO results in a spectrum with additional hyperfine splittings that were not observed. Thus, we conclude from the hyperfine splitting constants shown in Fig. 4 (A_H^β = 18.7 G and A_N = 15.2 G) that a carbon atom is bound to the spin trap, DMPO.

DISCUSSION

HRP/H_2O_2-mediated covalent binding of [^{3}H]acetaminophen to protein clearly is an enzymatic process, since binding levels were insignificant in the absence of either HRP or H_2O_2. Consistent with the requirement for H_2O_2 was the inhibition of binding with the addition of catalase to the complete incubation medium. In contrast, SOD had no effect on the binding reaction. SOD has also been observed to have no effect on the covalent binding of [^{3}H]acetaminophen mediated by microsomes prepared from livers of Swiss-Webster mice.[4] Two other results that paralleled those observed for microsomal oxygenase-effected covalent binding of acetaminophen to protein were the inhibition of peroxidase-mediated binding by ascorbic acid and by GSH. Ascorbic acid is a known inhibitor of the binding of acetaminophen to microsomal protein when the reaction is catalyzed by microsomal oxygenases (18), and inhibition of the peroxidase-catalyzed reaction by GSH yielded the same conjugate of acetaminophen that is formed from acetaminophen by microsomal oxygenases in the presence of GSH. These results imply that similar reactive intermediates of acetaminophen are formed by cytochrome P-450 and HRP.

One intermediate that was detected in the HRP-mediated reactions was an acetaminophen free radical. This radical is probably an aryl carbon radical according to the hyperfine splitting constants observed in the DMPO-spin adduct. Although the spin adduct was obtained only by UV photolysis, conditions clearly different from those in the HRP/H_2O_2 incubations, the radical did produce the same initial ESR signal that was observed in the HRP/H_2O_2 system.

Whether or not this radical is the species that binds is

[4] S. D. Nelson *et al.*, unpublished results.

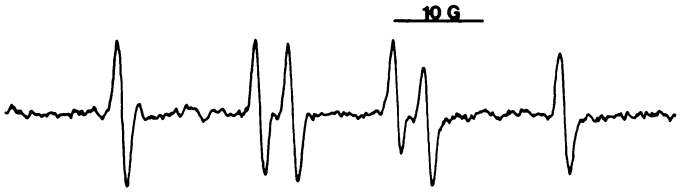

FIG. 4. *EPR spectrum of the DMPO-spin adduct of the acetaminophen free radical*

The adduct was obtained by UV photolysis of acetaminophen in the presence of an aqueous solution of DMPO. The microwave power was 10 mW and the modulation frequency was 100 kHz with an amplitude of 1 G. The sweep time was 25 G/min with a response time of 0.3 sec. $A_H{}^{\beta}$ was calculated to be 18.7 G, and A_N was 15.2 G.

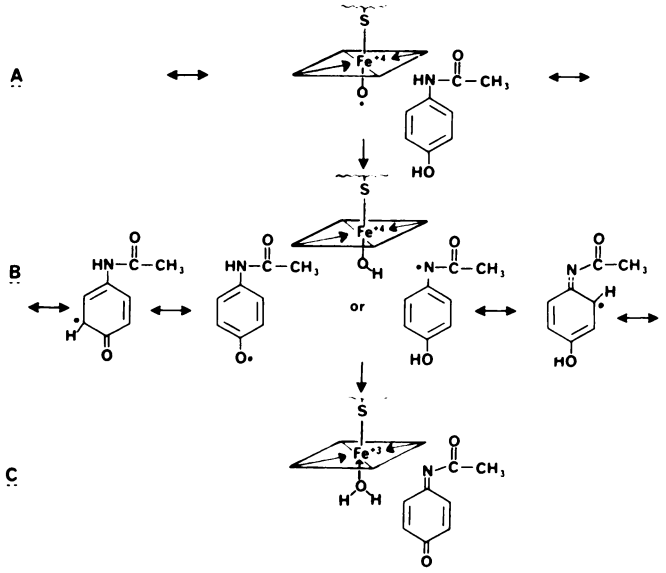

FIG. 5. *A hypothetical scheme for the oxidation of acetaminophen to an electrophilic metabolite via semiquinone radicals by a cytochrome P-450 microsomal oxygenase*

See Discussion for a description of the postulated states A, B, and C. ↔ indicates that additional resonance structures are possible.

open to question. Whereas protein binding of acetaminophen is maximal in 2–3 min, radical generation is maximal at 16 min. This may represent competition for some undefined intermediate in a possible quinone/semiquinone couple, or may simply reflect a different rate of radical formation when large amounts of exogenous protein are added. There is also the possibility that the binding reaction is limited because of destruction of the protein by the peroxidase system. In support of this, GSH prolonged the period of linear binding (Fig. 2).

However, the radical does meet many requirements for an intermediate in the HRP/H_2O_2-mediated covalent binding of acetaminophen, since all conditions which altered the binding reaction affected either the formation or degeneration of the radical in a parallel manner. The acetaminophen radical was not formed in the absence of either HRP or H_2O_2 and was rapidly quenched by the addition of microsomal protein, BSA, or GSH. The acetaminophen radical was apparently reduced by ascorbic acid, which thereby resulted in the formation of the ascorbate radical, as determined by EPR spectrometry.

Although the acetaminophen radical was not detected in microsomal oxygenase-mediated covalent binding reactions of acetaminophen, the results of these studies with a peroxidase enzyme suggest that acetaminophen may be oxidized to reactive intermediates without the intermediate formation of *N*-hydroxyacetaminophen (Fig. 5). According to several studies (for reviews see refs. 19 and 20), the most reactive oxygenating species generated by cytochrome P-450 can be visualized as a ferryl oxyradical complex (Fig. 5A). This structure is similar to that proposed for peroxidase Compound I, a postulated major difference being greater electrophilicity of the cytochrome P-450 complex which results from a probable sulfide ligand to the heme iron in the fifth liganding position (21). That the intermediates are not the same is recognized. Recently published studies provide strong evidence that some hydroperoxide-supported reactions of cytochrome P-450 proceed through mechanisms different from NADPH-supported reactions (22), and that hydroperoxide-generated intermediates do not correspond to intermediates in reactions catalyzed by peroxidases (23).

However, according to the elegant experiments of Groves *et al.* (24), whatever initial complex is formed apparently can carry out a radical abstraction reaction forming a possible radical cage complex of ferric cytochrome-hydroxyl radical and substrate radical (Fig. 5B). In the case of acetaminophen, this could produce either resonance-stabilized semiquinone or nitrenium radicals. If these radicals could escape the radical cage, theoretically they could bind to tissue macromolecules or cause other types of tissue damage. However, considering the nature of the radical cage complex, the acetaminophen radical could easily be oxidized by a rapid second electron transfer to produce *N*-acetyl-*p*-quinoneimine and a hydrated ferric P-450 complex (Fig. 5C). It is noteworthy that electrochemically generated *N*-acetyl-*p*-quinoneimine produces a pattern of metabolites virtually identical with that of acetaminophen in microsomal preparations (25).

Alternatively, the quinoneimine could arise in P-450-mediated reactions without the formation of semiquinone intermediates. This can best be visualized by considering the initial reaction of acetaminophen with the perferryl form of P-450 to give a ferric-oxyamide complex which could readily decompose by two-electron shifts and appropriate proton transfers to yield enzyme-product complex (Fig. 6).

Whether or not the quinoneimine is formed as an intermediate in the peroxidase-mediated binding reaction is unknown at the present time. The lack of binding of radiolabeled *N*-methylacetaminophen and phenacetin under conditions where acetaminophen binds extensively provides some very indirect evidence that both the amide nitrogen and phenolic oxygen cannot be substituted with alkyl groups. Microsome-mediated covalent binding of *N*-methylacetaminophen has also been found to be much less extensive than that of acetaminophen (26). Phenacetin, on the other hand, binds to about the same extent as acetaminophen in microsomal preparations; however, the mechanisms are quite different (27).

In conclusion, our results indicate that a reactive metabolite(s) of acetaminophen is formed in a peroxidase-

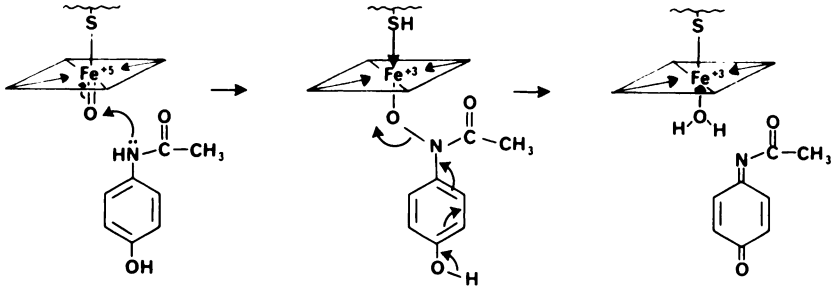

FIG. 6. *An alternative scheme for the generation of the same electrophilic intermediate without the intermediacy of semiquinone radicals* See Discussion for a description of this hypothetical scheme.

mediated reaction with properties similar to that which is produced in microsomal incubations, and that an acetaminophen radical is formed under the same conditions. Although this radical has properties which are consistent with its being an intermediate in both peroxidase and cytochrome P-450-mediated oxidation of acetaminophen to reactive metabolites, its actual role in these processes is yet to be determined. Recently, Moldéus and Rahimtula (28) have reported on the formation of a reactive metabolite of acetaminophen catalyzed by prostaglandin synthetase, an enzyme with peroxidase activity. Thus, more than one kind of oxygenase may be involved in the formation of toxic metabolites of acetaminophen, and we are now exploring the possibility of acetaminophen radical generation by prostaglandin synthetase.

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