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Reduction and Glutathione Conjugation Reactions of *N*-Acetyl-*p*-Benzoquinone Imine and Two Dimethylated Analogues

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SUMMARY

N-Acetyl-3,5-dimethyl-*p*-benzoquinone imine, *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, and *N*-acetyl-*p*-benzoquinone imine were synthesized via the oxidation of 3,5-dimethylacetaminophen, 2,6-dimethylacetaminophen, and acetaminophen, respectively. All three quinone imines were rapidly reduced to their corresponding semiquinone imines by NADPH-cytochrome P-450 reductase. All three benzoquinone imines underwent comproportionation with their respective phenols to yield the corresponding semiquinone imines, which in the presence of oxygen gave superoxide. Identification of this latter free radical was based on spin-trapping techniques. Reduced GSH was found to be an excellent nucleophile toward *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, whereas this thiol behaved as a one-electron reductant toward *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine. Finally, GSH was determined to act as both a nucleophile and a reductant toward *N*-acetyl-*p*-benzoquinone imine.

INTRODUCTION

The widely used analgesic acetaminophen is known to cause hepatic necrosis in humans and experimental animals after high doses have been administered (1, 2). In 1973, Mitchell and co-workers proposed that acetaminophen is oxidized by cytochrome P-450 to *N*-hydroxyacetaminophen, which dehydrates to give water and the hypothetical toxic intermediate *N*-acetyl-*p*-benzoquinone imine (3–6). Once this intermediate is generated, it can be detoxified by covalent reaction with reduced GSH; however, when GSH is depleted, *N*-acetyl-*p*-benzoquinone imine binds primarily to cellular macromolecules (7). This process is believed to be the event which leads to hepatic necrosis. In recent years, aspects of this mechanism have come into question. For example, Hinson *et al.* (8) and Nelson *et al.* (9) have determined that if *N*-acetyl-*p*-benzoquinone imine is the toxic species, it is formed by a pathway that does not include *N*-hydroxyacetaminophen.

Validating the existence of *N*-acetyl-*p*-benzoquinone imine in biological milieu and defining the role this quinone imine might play in acetaminophen-mediated hepatotoxicity have been surprisingly difficult. For example, in 1979 Miner and Kissinger (10) prepared *N*-acetyl-*p*-benzoquinone imine by coulometric oxidation of acetaminophen. Although they could identify this compound by liquid chromatography using electrochemical detection, these investigators were unable to detect *N*-acetyl-*p*-benzoquinone imine in microsomal mixtures containing acetaminophen and NADPH. Part of the problem with the identification of *N*-acetyl-*p*-benzoquinone imine is its short half-life, which has been reported to be 11 min in sodium phosphate buffer (11) and no greater than 7 sec in microsomal preparations (10). More encouraging is the recent study by Hinson *et al.* (12) in which 3-(glutathion-*S*-yl)acetaminophen was isolated as a biliary metabolite of acetaminophen. This finding further supports the hypothesis that GSH detoxifies acetaminophen by acting as a nucleophile in which GSH covalently binds to *N*-acetyl-*p*-benzoquinone imine. Recently, Dahlin and Nelson (11) prepared *N*-acetyl-*p*-benzoquinone imine in stable crystalline form and found that this compound is considerably more toxic both *in vitro* and *in vivo* than acetaminophen. Of interest is the observation that 50% of mice receiving *N*-acetyl-*p*-benzoquinone imine (20 mg/kg, i.p.) die not from hepatic failure but from blood coagulation.

In recent years, the importance of covalent binding as a mechanism to account for acetaminophen-mediated hepatotoxicity has been questioned. For example, Labadarios *et al.* (13) and Devalia *et al.* (14) have found that, in the presence of certain thiols and flavones, covalent binding of acetaminophen to cellular macromolecules is

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only minimally decreased while these compounds prevent hepatotoxicity. This result demonstrates that covalent binding alone is not sufficient to cause cellular necrosis. Likewise, Gerber and co-workers (15) have reported that *N*-acetylcysteine protects against hepatotoxicity without altering covalent binding of an acetaminophen metabolite to hepatic protein, even if administered after covalent binding was maximal. Recent studies by Fernando *et al.* (16) also provide evidence that covalent binding is not sufficient to mediate hepatic necrosis. These investigators synthesized two analogues of acetaminophen, namely 3,5-dimethylacetaminophen and 2,6-dimethylacetaminophen. When these compounds were tested for hepatotoxicity in mice, it was found that 3,5-dimethylacetaminophen was considerably more toxic than 2,6-dimethylacetaminophen. Assuming that these analogues of acetaminophen are equivalently oxidized by cytochrome P-450 to their corresponding quinone imines and that the mechanism of hepatotoxicity is covalent binding at position 3 or 5 of the quinone imine to cellular molecules, then only 2,6-dimethylacetaminophen should exhibit hepatotoxicity. Since the contrary was demonstrated, the covalent binding hypothesis to account for hepatotoxicity of these acetaminophen analogues must be seriously questioned. The uncertainty as to the role of *N*-acetyl-*p*-benzoquinone imine in acetaminophen-initiated hepatotoxicity prompted us to synthesize *N*-acetyl-*p*-benzoquinone imine, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, and to study their reactivity in model systems as a means to better understand their role in initiating hepatotoxicity.

MATERIALS AND METHODS

General comments. DETAPAC,[3] bovine erythrocyte SOD, reduced GSH, and GSH reductase were purchased from Sigma Chemical Company (St. Louis, Mo.). Chelex-100 ion exchange resin was obtained from Bio-Rad (Richmond, Calif.). All buffers were passed through a Chelex-100 ion exchange column according to the method of Poyer and McCay (17) to remove trace metal ion impurities.

Synthesis and identification of acetaminophen analogues. The structures of acetaminophen, 3,5-dimethylacetaminophen, 2,6-dimethylacetaminophen, *N*-acetyl-*p*-benzoquinone imine, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine are shown in Fig. 1. The synthesis of 3,5-dimethylacetaminophen and 2,6-dimethylacetaminophen and their corresponding quinone imines were undertaken using the methods outlined by Fernando *et al.* (16). Briefly stated, the synthesis of 3,5-dimethylacetaminophen requires the reaction of 2,6-dimethylphenol with sodium nitrite to yield 2,6-dimethyl-4-nitrosophenol. Catalytic reduction of this compound with platinum and hydrogen in a mixture of acetic acid and acetic anhydride gave, after recrystallization from water, 3′,5′-dimethyl-4′-hydroxyacetanilide (3,5-dimethylacetaminophen), m.p. 160–162° [reported, m.p. 161–162° (16)].

In the case of 2,6-dimethylacetaminophen, sulfanilic acid was diazotized and then allowed to react with 3,5-dimethylphenol to yield 4-amino-3,5-dimethylphenol. Acetylation followed by recrystallization from chloroform/light petroleum gave 2′,6′-dimethyl-4′-hydroxyacetanilide (2,6-dimethylacetaminophen), m.p. 180–182° [reported, m.p. 182.5–183.5° (16)].

N-Acetyl-*p*-benzoquinone imine [both unlabeled and (*ring*-^{14}C)-labeled] was prepared by a modification of the procedure already reported (11) in which 2 ml of HPLC-grade chloroform (previously washed successively with sulfuric acid and water, and then dried over calcium carbonate and distilled) were added to a mixture of 20 mg of pure acetaminophen, 20 mg of anhydrous sodium sulfate, and 40 mg of silver oxide (Aldrich Chemical Company (Milwaukee, Wisc.). The suspension was stirred for 1 hr at room temperature. To the mixture was added approximately 1 mg of butylated hydroxytoluene and 20 mg of activated charcoal. The mixture was then adsorbed onto a Sil Sep Pak cartridge (Waters Associates, Milford, Mass.) and washed with 2–3 ml of purified chloroform. *N*-Acetyl-*p*-benzoquinone imine was eluted with 2 ml of analytical-grade ether. The ether solution was added to a sublimation apparatus, and the ether was removed in a gentle stream of nitrogen. Pure *N*-acetyl-*p*-benzoquinone imine sublimated at 45°, 0.07 mm Hg (yield 20%).

N-Acetyl-3,5-dimethyl-*p*-benzoquinone imine and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine were prepared by the lead tetraacetate oxidation of 3,5-dimethylacetaminophen and 2,6-dimethylacetaminophen as described by Fernando *et al.* (16). In the case of crude *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, this compound was recrystallized from ether/light petroleum giving yellow crystals; m.p. 112–114° [reported, m.p. 114–116° (16)]. For crude *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, this compound was recrystallized from light petroleum giving yellow crystals; m.p. 70–72° [reported, m.p. 70–72° (16)].

Identification of end products resulting from the reaction of *N*-acetyl-*p*-benzoquinone imine with a number of different reactants was conducted using HPLC methodologies as follows. A Waters Associates HPLC apparatus consisting of a Model 6000 A solvent delivery system with Model 660 solvent programmer, Model 440 absorbance detector with a 254-nm filter, and a U6 K injector with a 2-ml sample loop were used throughout. Separations were carried out on an Altex 5-μm Ultraspere ODS column starting with a mobile phase of 5% methanol and 95% 0.01 M monobasic potassium phosphate in 1% acetic acid. After 8 min, solvent program 10 was initiated to bring the methanol concentration to 30% by 12 min. Under these conditions, at a flow rate of 2 ml/min, hydroquinone eluted at 4.0 min, benzoquinone imine at 8.6 min, acetaminophen at 10.2 min, and 3-(glutathion-*S*-yl)acetaminophen at 15.5 min as determined using synthetic standards. In the case of radiolabeled *N*-acetyl-*p*-benzoquinone imine, collections were made every 0.5 min in 10 ml of ACS scintillation cocktail (Amersham/Searle, Arlington Heights, Ill.), and radioactivity was quantified by scintillation spectroscopy on a Beckman LS-7500 counter.

Spin-trapping studies. Spin-trapping and spin-exchange techniques were used to identify a number of free radical species generated during the oxidation of acetaminophen and its analogues to their corresponding quinone imines. Classical spin-trapping studies involve the reaction of a reactive free radical with a nitrone or nitroso compound to give a more stable nitroxyl free radical. For these types of experiments, DMPO was used. This nitrone was synthesized according to the methods outlined by Bonnett *et al.* (18). An equal molar concentration of acetaminophen and *N*-acetyl-*p*-benzoquinone imine (1 mM) was incubated with DMPO (0.1 M), and superoxide was identified by the characteristic EPR spectrum of its spin-trapped adduct, DMPO-OOH. In a similar manner, the two other analogues of acetaminophen along with their corresponding benzoquinone imines were allowed to react together, and the production of superoxide was monitored by spin-trapping techniques.

To verify the production of acetaminophen free radical by NADPH-cytochrome P-450 reductase, the oxidation of the cyclic hydroxylamine, OXANOH, to its corresponding nitroxide, OXANO, was employed. OXANO was synthesized as previously described (19). OXANOH was prepared by bubbling a 10 mM water solution of OXANO containing 1 mM DETAPAC with hydrogen in the presence of platinum oxide for 45 min (20). The hydroxylamine should be prepared fresh just prior to its use, since OXANOH is unstable when stored under reducing con-

[3] The abbreviations used are: DETAPAC, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase; HPLC, high-pressure liquid chromatography; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-OOH, 5,5-dimethyl-2-hydroperoxylpyrrolidinoxyl; OXANOH, 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine; OXANO, 2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl; DSS, 2,2-dimethyl-2-silopentane-5-sulfonate.

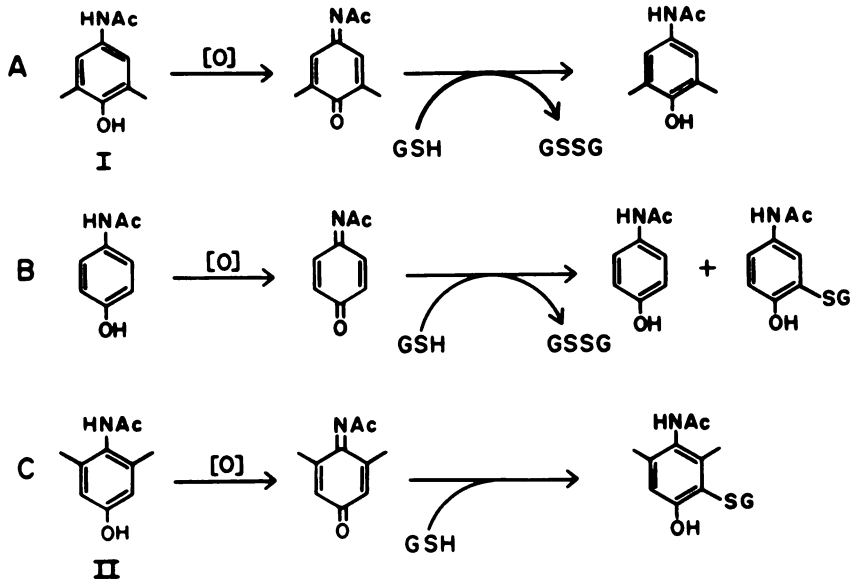

FIG. 1. *Reaction of reduced GSH with benzoquinone imines*

A. 3,5-Dimethylacetaminophen (*I*) is oxidized to *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine. Reduction with GSH gives 3,5-dimethylacetaminophen. B. Acetaminophen is oxidized to *N*-acetyl-*p*-benzoquinone imine. Reaction with GSH gives acetaminophen and 3-(glutathion-*S*-yl)acetaminophen. C. 2,6-Dimethylacetaminophen (*II*) is oxidized to *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine. Reaction with GSH gives 3-(glutathion-*S*-yl)-2,6-dimethylacetaminophen.

ditions. However, the corresponding nitroxide is very stable, lasting for weeks in a water solution and for years in a desiccator. The rate of OXANOH oxidation (to produce the corresponding nitroxide) was determined by measuring the appearance of the EPR signal as a function of time using a Varian Associates Model E-9 spectrometer. The reaction mixture consisted of *N*-acetyl-*p*-benzoquinone imine, or *N*-acetyl-3,5-dimethyl-*p*-benzoquinone (1 mM of each quinone imine), NADPH-cytochrome P-450 reductase (44 nm), NADPH (250 μM), SOD (5 μg/ml), and OXANOH (1 mM). In the absence of the quinone imines, oxidation of OXANOH was not observed.

NADPH-cytochrome P-450 reductase was purified from rat hepatic microsomes according to the procedure of Yasukochi and Masters (21), with minor modifications. Emulgen 913 (KAO-Atlas, Tokyo, Japan) at 30% higher concentration was used instead of Renex 690, and the reductase was purified through the affinity column step and no further. Microsomes were prepared according to published procedures (22).

Identification of the products from the reaction of reduced GSH with *N*-acetyl-*p*-benzoquinone imine and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine was carried out by the use of HPLC, UV spectrophotometric analysis, and NMR. HPLC analyses were performed as already described. The product from the reaction of GSH with *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine had a retention time of 31 min. The NMR spectrum of a sample of the product that was obtained after lyophilization was recorded on a Brucker WM-500 superconducting spectrometer operating at 500 MHz. The spectrum was obtained in D_2O using DSS as an internal standard. Routine parameters were as follows: pulse width, 15 μsec; sweep width, 6600 Hz; data points 16K.

The appearance of GSSG was monitored by measuring GSH reductase (0.1 unit/ml)-stimulated NADPH (0.13 mM) oxidation in 50 mM chelexed sodium phosphate buffer (pH 7.4) containing 1 mM DETAPAC. Similar studies were conducted for *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine.

RESULTS AND DISCUSSION

The role that reduced GSH plays in the protection against *N*-acetyl-*p*-benzoquinone imine-mediated hepatotoxicity theoretically may be 2-fold. First, GSH may act as a nucleophile, as in the case of its reaction with *N*-acetyl-*p*-benzoquinone imine to give 3-(glutathion-*S*-yl)acetaminophen (12). Second, GSH is a one-electron reductant in which two molecules of GSH would reduce *N*-acetyl-*p*-benzoquinone imine to give acetaminophen and GSSG (23) (Fig. 2).

N-Acetyl-2,6-dimethyl-*p*-benzoquinone imine (0.5 mM) (λ_{max} 255 nm in deionized water) was found to react slowly with GSH (1 mM) to produce a new species (λ_{max} 296 nm in water). When the above reaction had gone to completion (as determined by monitoring the shift in absorption from 255 to 296 nm). NADPH and GSH reductase were added and the rate of NADPH oxidation was measured. Under these conditions, we found that NADPH was not oxidized, indicating that during the reaction of GSH with *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, GSSG was not produced. The product from the reaction of GSH with *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine eluted as a single peak from HPLC. Reduction of this compound with activated Raney nickel (K & K Laboratories) in refluxing ethanol produced 2,6-dimethylacetaminophen as determined by comparison with synthetic standard material. Retention times (18.6 min) were the same on HPLC using the system described herein, and EI mass spectra were identical at a nominal resolution of 100 (10% valley) on a VH Micromass 7070 H double-focusing instrument with major ions recorded at m/z 179 (M^+), 137 ($M\text{-}COCH_2$), and 122.

High-resolution ^{1}H-NMR analysis of the GSH conjugate gave the spectrum shown in Fig. 3. This spectrum is similar to that reported for 3-(glutathion-*S*-yl)acetaminophen (12) with expected changes in the aromatic and aliphatic regions. The singlet at 6.63 ppm integrates for one proton and is assigned to C-5 of the aromatic ring. Resonance absorption occurs at 2.08 ppm for the aryl methyl proton groups. Some differences in chemical shifts for protons on the glutathionyl residue are apparent when comparing the spectrum of this GSH

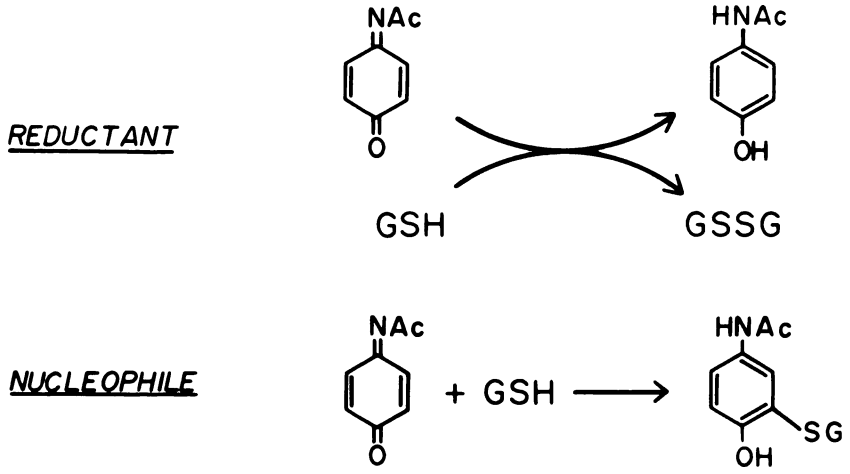

FIG. 2. *Role that reduced GSH plays in preventing acetaminophen-mediated hepatotoxicity*

GSH can behave as a reductant and reduce *N*-acetyl-*p*-benzoquinone to acetaminophen. GSH can also act as a nucleophile to give 3-(glutathion-*S*-yl)acetaminophen.

conjugate with that of acetaminophen, in particular the more highly deshielded α-cysteine resonance and α-glycine resonance. These changes may be caused partially by the proximity of an aryl methyl group and differences in pH, inasmuch as our spectrum was recorded at pH 3.0 versus pH 7.4 as reported for the GSH conjugate of acetaminophen (12). However, decoupling experiments with irradiation of the α-cysteinyl proton resonance at 4.75 ppm caused the β-cysteinyl quartet at 2.95 ppm and the β'-cysteinyl quartet at 3.24 ppm to collapse to doublets (J gem = 14.5 Hz). Similarly, irradiation of the β-glutamyl resonance at 2.12 ppm caused the α-glutamyl triplet at 3.79 ppm and the γ-glutamyl quartet at 2.51 ppm to collapse to singlets. Thus, the assignment of these resonances is further confirmed. Therefore, in the case of *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, it appears that GSH behaves, not as a reductant, but only as a nucleophile (Fig. 1).

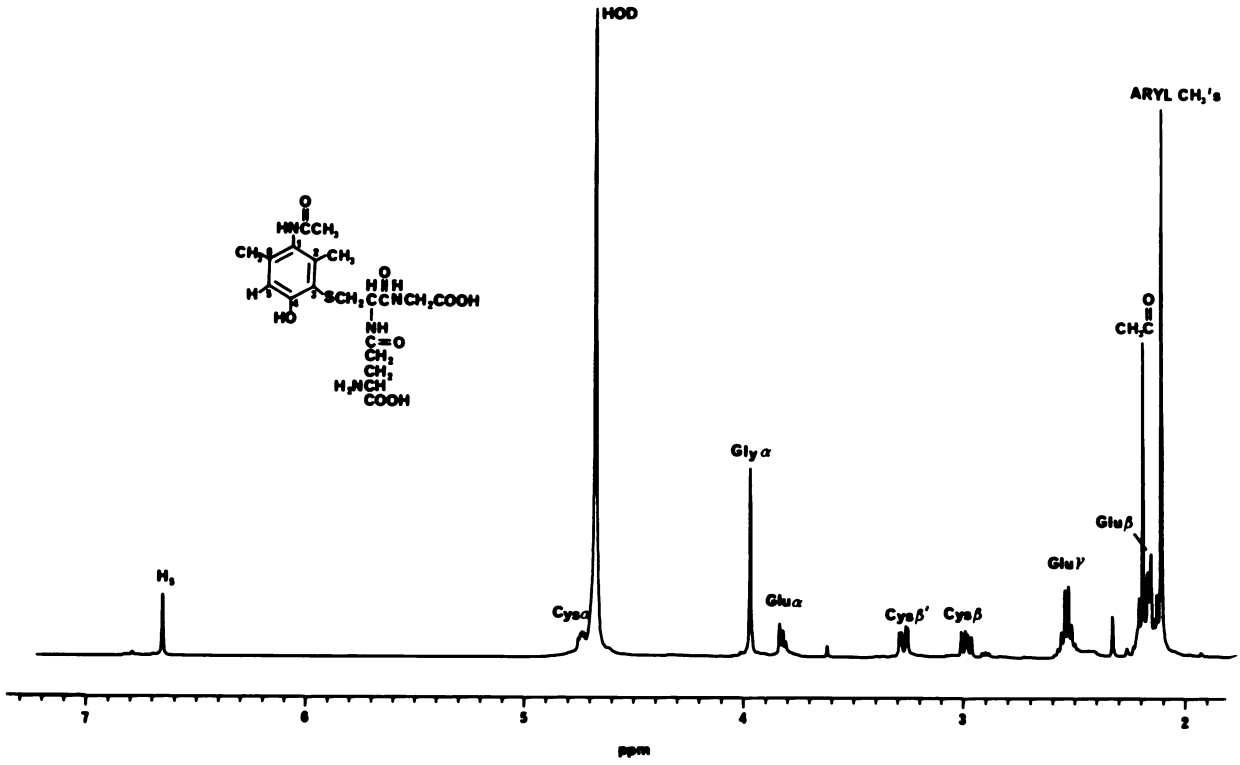

FIG. 3. *^{1}H-NMR spectrum (500) of the GSH conjugate of 2,6-dimethylacetaminophen*

Chemical shifts are in parts per million downfield from DSS, and coupling constants are in Hertz units as follows: Aryl CH_3 (2.08), Glu-β (2.12, J = 7), CH_3CO (2.16), Glu-γ (2.51, 7 Hz), Cys-β (2.95, J = 8.8, 14.5), Cys-β' (3.24, J = 4.8, 14.5), Glu-α (3.79, J = 7), Gly-α (3.9), Cys-α (4.75, J = 4.8, 8.8), and H_5 (6.63). Small resonance signals at 2.31, 2.89, and 3.61 ppm are residual proton signals from DSS (the internal standard).

In the case of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (0.5 mM), reaction with GSH (1 mM) led to a rapid shift in the λ_{max} from 272 nm to 240 nm along with the concomitant production of GSSG, which was determined by adding NADPH and GSH reductase (to the completed reaction), and monitoring the rate of NADPH oxidation. Assignment of the product from the above reaction was based on the UV absorption spectrum, thin-layer chromatography, and NMR, which indicated that the reaction of GSH with *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine gave only 3,5-dimethylacetaminophen. Thus, in the case of *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, GSH acts exclusively as a one-electron reductant (Fig. 1), and not as a nucleophile.

Reaction of *N*-acetyl-*p*-benzoquinone imine (0.5 mM) with GSH (1 mM) gave 3-(glutathion-*S*-yl)acetaminophen, and acetaminophen, which was based on HPLC analysis of the reaction mixture. Under these conditions, 36% of the reaction product was acetaminophen, 50% was found to be 3-(glutathion-*S*-yl)acetaminophen, and 14% other products, including the hydrolysis product acetamide and what is tentatively identified as a GSH conjugate or hydroquinone with a retention time of 12.4 min, the same retention time as the major, as of yet uncharacterized, reaction product of *p*-benzoquinone and glutathione. Thus, we conclude that GSH acts both as a reductant and as a nucleophile toward *N*-acetyl-*p*-benzoquinone imine.

It is known that benzoquinone and 1,4-dihydroxybenzene react to give the corresponding semiquinone free radicals (24). Thus, by analogy, we hypothesize that *N*-acetyl-*p*-benzoquinone imine might react with acetaminophen to give acetaminophen free radical. This theory was verified using several different experiments. Since acetaminophen free radical in the presence of oxygen is not stable long enough to be observed using conventional EPR techniques, we measured the formation of this free radical by following the one-electron oxidation of OXANOH by acetaminophen free radical to give acetaminophen and OXANO, which is a stable nitroxyl free radical (Fig. 4). Since we have previously demonstrated (25) that superoxide can also oxidize OXANOH to OXANO, a 5 μg/ml concentration of SOD was included in the reaction mixture (Fig. 5). When *N*-acetyl-*p*-benzoquinone imine, acetaminophen, and OXANOH were incubated together

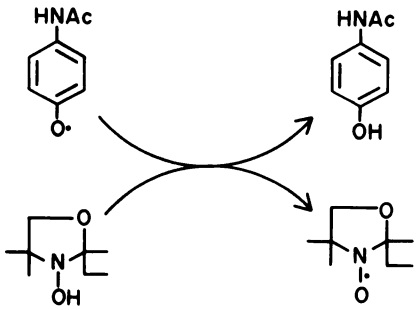

FIG. 4. *Oxidation of OXANOH by acetaminophen free radical*
The cyclic hydroxylamine, OXANOH, can be oxidized by free radicals such as acetaminophen free radical to give OXANO, a nitroxyl free radical. OXANO is sufficiently stable to be observed by conventional EPR spectrometric techniques.

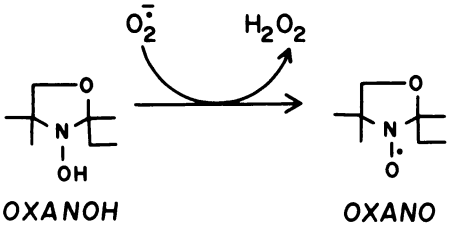

FIG. 5. *Oxidation of OXANOH by superoxide*
The cyclic hydroxylamine, OXANOH, can be oxidized by superoxide to give the corresponding stable nitroxide, OXANO.

in the presence of SOD, we found a rapid production of OXANO, indicating that acetaminophen and *N*-acetyl-*p*-benzoquinone imine comproportionated to acetaminophen free radical. For a control, *N*-acetyl-*p*-benzoquinone imine was mixed with OXANOH and SOD. Under these conditions, OXANO was produced, but at a concentration of only 10% of that observed when acetaminophen was included in the reaction mixture. In a similar manner, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine and 3,5-dimethylacetaminophen as well as *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine and 2,6-dimethylacetaminophen were found to undergo comproportionation to give the corresponding free radicals, which were observed indirectly via oxidation of OXANOH to OXANO.

The formation of acetaminophen free radical should generate superoxide by reaction with oxygen. Under these conditions, one of the best methods for determining the presence of superoxide is spin trapping. Reaction of *N*-acetyl-*p*-benzoquinone imine with acetaminophen in the presence of the spin-trap DMPO (0.1 M) gave DMPO-OOH (Fig. 6). Structural assignment was based on the EPR spectrum obtained by the reaction of tetra-

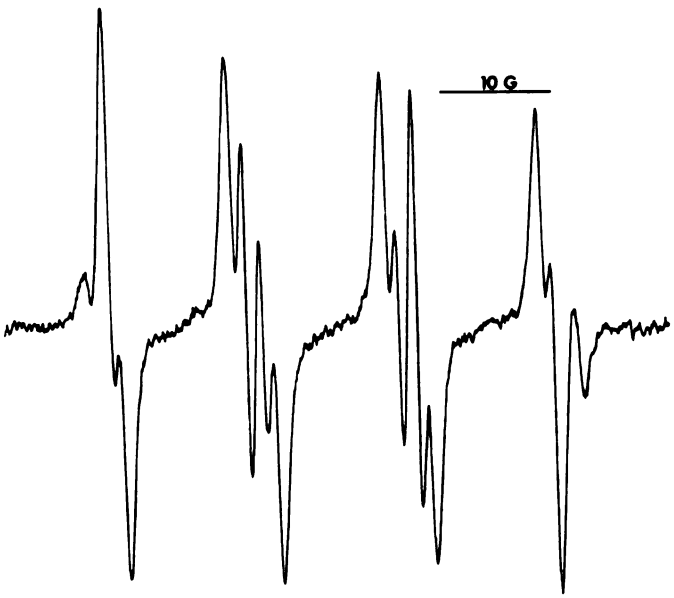

FIG. 6. *EPR Spectrum of the spin-trapped adduct of superoxide, DMPO-OOH*
This spectrum was obtained by the reaction of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in the presence of the spin trap DMPO. The spectrum is identical with that observed when DMPO is incubated with tetramethylammonium superoxide: $A_N = 14.3$ G; $A_H^{\beta} = 11.7$ G; and $A_H^{\gamma} = 1.25$ G.

methylammonium superoxide with DMPO (26). The formation of DMPO-OOH is verification for the production of superoxide when acetaminophen and *N*-acetyl-*p*-benzoquinone imine are allowed to react together in the presence of oxygen. Similarly, superoxide was detected from the reaction of 3,5-dimethylacetaminophen free radical and 2,6-dimethylacetaminophen free radical with oxygen.

Since quinones are known to be excellent substrates for NADPH-cytochrome P-450 reductase (24), we investigated whether or not *N*-acetyl-*p*-benzoquinone imine could be reduced by this enzyme. When *N*-acetyl-*p*-benzoquinone imine was incubated with purified NADPH-cytochrome P-450 reductase and NADPH in 0.1 M chelexed sodium phosphate buffer (pH 7.4) containing 1 mM DETAPAC, we could not observe acetaminophen free radical by direct EPR spectrometry. There are several explanations as to why this free radical was not noted. First, once acetaminophen free radical is generated, its reaction with oxygen is so fast that a high enough steady-state concentration of the free radical is not present to be observed by conventional EPR techniques. However, this is probably not the limiting factor, since the acetaminophen free radical was not observed even when the reaction was conducted under anaerobic conditions. Second, the reduction of acetaminophen free radical, perhaps by NADPH-cytochrome P-450 reductase, could be so fast that EPR-detectable concentrations of acetaminophen free radical are not achieved. The oxidation of OXANOH to OXANO by acetaminophen free radical (Fig. 4) could be used to determine whether acetaminophen free radical is produced under these experimental conditions. Incubation of OXANOH with *N*-acetyl-*p*-benzoquinone imine, NADPH-cytochrome P-450 reductase, and NADPH in the presence of SOD (5 μg/ml) led to the rapid formation of OXANO as monitored by EPR spectrometry. This result suggests that acetaminophen free radical was generated. The two necessary control experiments, incubation of OXANOH with NADPH-cytochrome P-450 reductase and NADPH in the presence of SOD and the reaction of OXANOH with NADPH-cytochrome P-450 reductase and *N*-acetyl-*p*-benzoquinone imine in the presence of SOD, were conducted. Neither of these experiments gave substantial quantities of OXANO (no greater than 10% of that observed when the reaction mixture included all of the reactants). Finally, using HPLC techniques, it was determined that acetaminophen was the predominate product (96%) when *N*-acetyl-*p*-benzoquinone imine was incubated with purified NADPH-cytochrome P-450 reductase and NADPH.

In a similar manner, *N*-acetyl-*p*-benzoquinone imine and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine were incubated with purified NADPH-cytochrome P-450 reductase and NADPH. As in the case of *N*-acetyl-*p*-benzoquinone imine, when *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, purified NADPH-cytochrome P-450 reductase, and NADPH were mixed together, no EPR spectrum was observed in either the presence or absence of oxygen. Verification for the formation of 3,5-dimethylacetaminophen free radical was obtained by monitoring the oxidation of OXANOH to OXANO by EPR in the presence of SOD (5 μg/ml). Like acetaminophen free radical produced during the reduction of *N*-acetyl-*p*-benzoquinone imine by the reductase, electron transfer is so rapid that sufficiently high concentrations of 3,5-dimethylacetaminophen free radical required for EPR spectrometric studies were never achieved. HPLC analysis of the above reaction mixture determined that 3,5-dimethylacetaminophen was the only product isolated.

In the case of *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, reaction with purified NADPH-cytochrome P-450 reductase and NADPH led to formation of 2,6-dimethylacetaminophen free radical, which was directly observed by EPR spectrometric techniques (Fig. 7). In the absence of either the enzyme of NADPH, no EPR spectrum was noted. 2,6-Dimethylacetaminophen was the only product isolated from the above reaction mixture. Why, in the case of *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine, one can observe the free radical during its enzymatic reduction, whereas for both *N*-acetyl-*p*-benzoquinone imine and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine the corresponding free radical is so reactive that it never achieves concentrations high enough to be measured by conventional EPR techniques, remains unclear. One possible explanation is that the electrochemical potential of 2,6-dimethylacetaminophen free radical is sufficiently low that its reactivity is reduced, leading to sufficiently high steady-state concentrations of the free radical and thus allowing us to observe its EPR spectrum. It seems improbable that geometric restrictions (due to the methyl groups on the aromatic ring) would present a problem in light of the rapid reactivity of 3,5-dimethylacetaminophen free radical and acetaminophen free radical.

We conclude that purified NADPH-cytochrome P-450 reductase in the presence of NADPH reduces *N*-acetyl-*p*-benzoquinone imine, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine to their corresponding phenols by means of two one-electron reductions in which free radicals are intermediates. In addition, acetaminophen and *N*-acetyl-*p*-benzoquinone imine, 3,5-dimethylacetaminophen and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, and 2,6-dimethylacetaminophen and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine comproportionate to give the cor-

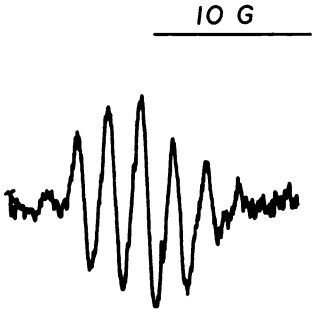

FIG. 7. *EPR spectrum of 2,6-dimethylacetaminophen free radical*
This spectrum was obtained by the one-electron reduction of *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine by purified NADPH-cytochrome P-450 reductase in the presence of NADPH. No spectrum was observed in the absence of either purified enzyme or NADPH. The microwave power was 20 mW, and the modulation amplitude was 0.5 G at 100 KHz. The sweep time was 12.5 G/min, and the response time was 0.3 sec.

responding free radicals which, in the presence of oxygen, generate superoxide. Finally, we have demonstrated that GSH can react with *N*-acetyl-*p*-benzoquinone imine by two distinct pathways, acting as both a reductant and a nucleophile, whereas GSH acts only as a reductant toward *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine, and only as a nucleophile toward *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine.

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