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Mechanisms of Acetaminophen Oxidation to *N*-Acetyl-*P*-benzoquinone Imine by Horseradish Peroxidase and Cytochrome P-450*

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Horseradish peroxidase rapidly catalyzed the H_2O_2-dependent polymerization of acetaminophen. Acetaminophen polymerization was decreased and formation of GSSG and minor amounts of GSH-acetaminophen conjugates were detected in reaction mixtures containing GSH. These data suggest that horseradish peroxidase catalyzed the 1-electron oxidation of acetaminophen and that GSH decreased polymerization by reducing the product, *N*-acetyl-*p*-benzosemiquinone imine, back to acetaminophen. Analyses of reaction mixtures that did not contain GSH showed *N*-acetyl-*p*-benzoquinone imine formation shortly after initiation of reactions. When GSH was added to similar reaction mixtures at various times, 3-(glutathion-*S*-yl)-acetaminophen was formed. The formation and disappearance of this product were very similar to *N*-acetyl-*p*-benzoquinone imine formation and were consistent with the disproportionation of 2 mol of *N*-acetyl-*p*-benzosemiquinone imine to 1 mol of *N*-acetyl-*p*-benzoquinone imine and 1 mol of acetaminophen followed by the rapid reaction of *N*-acetyl-*p*-benzoquinone imine with GSH to form 3-(glutathion-*S*-yl)acetaminophen. When acetaminophen was incubated with NADPH, oxygen and hepatic microsomes from phenobarbital-pretreated rats, 1.2 nmol 3-(glutathion-*S*-yl)acetaminophen/nmol cytochrome P-450/10 min was formed. Formation of polymers was not observed indicating that *N*-acetyl-*p*-benzoquinone imine was formed via an overall 2-electron oxidation rather than a disproportionation reaction. However, when cumene hydroperoxide was replaced by NADPH in microsomal incubations, polymerization was observed suggesting that cytochrome P-450 might also catalyze the 1-electron oxidation of acetaminophen.

High doses of acetaminophen[1] cause liver and kidney damage in humans and in laboratory animals (1–6). Acetaminophen is thought to be metabolized by cytochrome P-450 and possibly other oxidative enzymes to reactive intermediate(s) that lead to the toxic response. Current evidence indicates that acetaminophen may be converted to reactive intermediates via 1-electron oxidation to give *N*-acetyl-*p*-benzosemiquinone imine or 2-electron oxidation to give *N*-acetyl-*p*-benzoquinone imine.

The metabolism of acetaminophen by cytochrome P-450 has been suggested to occur via a 2-electron oxidation pathway since *N*-acetyl-*p*-benzoquinone imine reacts with GSH to form GS-A (7, 8), and this conjugate is also formed *in vivo* (9) and by purified cytochrome P-450 isozymes (10). *N*-Acetyl-*p*-benzoquinone imine has also been isolated from incubations of acetaminophen with purified cytochrome P-450 and cumene hydroperoxide as an oxidizing agent (11). Other mechanisms have also been proposed that would account for these results. De Vries (12) has suggested that cytochrome P-450 may catalyze the 1-electron oxidation of acetaminophen to give *N*-acetyl-*p*-benzosemiquinone imine which may then react with glutathionyl radical to form GS-A. Rosen *et al.* (13) also proposed a mechanism where cytochrome P-450 oxidizes acetaminophen to *N*-acetyl-*p*-benzosemiquinone imine which is then further oxidized by molecular oxygen to give superoxide anion and *N*-acetyl-*p*-benzoquinone imine. However, this latter explanation is unlikely since it appears that *N*-acetyl-*p*-benzosemiquinone imine does not react with oxygen to give superoxide anion (14). Alternatively, if *N*-acetyl-*p*-benzosemiquinone imine were a cytochrome P-450-mediated intermediate it might disproportionate in a manner similar to benzosemiquinone (15) and 3′,5′-dimethyl-*N*-acetyl-*p*-benzosemiquinone imine (16) to give the small amount of *N*-acetyl-*p*-benzoquinone imine previously observed with cytochrome P-450 (11).

We have been involved in developing methods to distinguish between the overall 1- and the overall 2-electron oxidation products of acetaminophen and using these methods to study enzyme reactions. Recently, we showed that horseradish peroxidase-catalyzed polymerization of acetaminophen was consistent with a free radical-coupling mechanism (17). We also demonstrated that in incubation mixtures which contained GSH, horseradish peroxidase and H_2O_2, GSH-acetaminophen conjugates were formed; however, the mechanism of GSH-acetaminophen conjugation was not established (18). Further studies demonstrated that *N*-acetyl-*p*-benzoquinone imine reacted with acetaminophen to give *N*-acetyl-*p*-benzosemiquinone imine which reacted to give acetaminophen polymers (7).

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[1] The abbreviations and trivial names used are: A or acetaminophen, 4′-hydroxyacetanilide; A_2 or acetaminophen dimer, 4′,4‴-dihydroxy-3′,3‴-biacetanilide; A_3 or acetaminophen trimer, 4′,4‴,4⁗-trihydroxy-3′,3‴:5‴,3⁗-teracetanilide; A_4 or acetaminophen tetramer, 4′,4‴,4⁗,4⁗′-tetrahydroxy-3′,3‴:5‴,3⁗:5⁗,3⁗′-quarteracetanilide; *N*-A_2 or *N*-acetaminophen dimer, 4′,4‴-dihydroxy-*N*,3‴-biacetanilide; *N*″-A_3 or *N*″-acetaminophen trimer; 4′,4‴,4⁗-trihydroxy-3′,*N*″:3‴,3⁗-teracetanilide; *N*-A_3 or *N*-acetaminophen trimer, 4′,4‴,4⁗-trihydroxy-*N*,3‴:5‴,3⁗-teracetanilide; GS-A or 3-(glutathion-*S*-yl)acetaminophen, *N*-[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-[(5-acetamido-2-hydroxyphenyl)thio]-ethyl]-L-glutamine; GS-A_2 or 3-(glutathion-*S*-yl)diacetaminophen, *N*-[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-[(5,5′-diacetamido-2,2′-dihydroxy-3-biphenylyl)thio]ethyl]-L-glutamine; DPEA, 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine; HPLC, high-performance liquid chromatography.

In this report, we have presented evidence that horseradish peroxidase catalyzes the 1-electron oxidation of acetaminophen to *N*-acetyl-*p*-benzosemiquinone imine which leads primarily to polymerization or to a lesser extent disproportionation to give *N*-acetyl-*p*-benzoquinone imine. Evidence is also presented that the *N*-acetyl-*p*-benzoquinone imine, rather than the *N*-acetyl-*p*-benzosemiquinone imine, reacts with GSH to give GS-A. Comparative studies using microsomes suggest that cytochrome P-450 catalyzes the overall 2-electron oxidation of acetaminophen to *N*-acetyl-*p*-benzoquinone imine and may also oxidize acetaminophen by a 1-electron oxidation mechanism.

MATERIALS AND METHODS[2]

RESULTS

Formation of GSH-acetaminophen Conjugates and Acetaminophen Polymers with Horseradish Peroxidase—Horseradish peroxidase and H_2O_2 have been shown previously to catalyze the polymerization of acetaminophen and in the presence of GSH to catalyze the formation of GSH-acetaminophen conjugates (17, 18). These products have been identified (17, 18) and their structures are shown in Fig. 1.

In reaction mixtures containing 100 μM GSH, the horseradish peroxidase-mediated polymerization of acetaminophen was observed and low levels of GS-A and GS-A_2 were formed (Fig. 2). A_2 was the major product and its formation was increased with increasing concentrations of acetaminophen. Similarly, *N*-A_2 formation was increased with higher concentrations of acetaminophen; however, formation of the *N*-A_2 was about 10-fold less than that of the A_2. The other acetaminophen polymers decreased with increasing concentrations of acetaminophen. Although six acetaminophen polymers were detected at low concentrations of acetaminophen, only four acetaminophen polymers were detected at 10 mM acetaminophen.

Above 100 μM acetaminophen, two GSH-acetaminophen conjugates were detected. With acetaminophen concentrations below 4 mM, GS-A_2 was the major conjugate. Above 4 mM acetaminophen, the formation of GS-A increased and its formation with 10 mM acetaminophen was approximately 2.5 times greater than GS-A_2.

In reaction mixtures containing 1.0 mM acetaminophen, the effect of different concentrations of GSH on polymerization and conjugation was examined. As shown in Fig. 3, GSH decreased acetaminophen polymer formation in a concentration-dependent manner. The formation of the GSH-acetaminophen conjugates was optimal at about 60 μM GSH. As with the acetaminophen polymerization, the formation of the GSH-acetaminophen conjugates decreased with increasing concentrations of GSH above 60 μM. Table I shows the ratio of GS-A to A_2 formation. This ratio was highest at 60 μM GSH. Above this concentration, the ratio was approximately 0.08–0.09 and remained nearly constant up to 6.0 mM GSH while total product formation decreased by 72%. These data suggest that both A_2 and the GS-A may have been formed via a common acetaminophen intermediate. Similarly, the ratio of total GSH-acetaminophen conjugates to A_2 formation, although higher, was nearly constant with increasing concentrations of GSH (Table I).

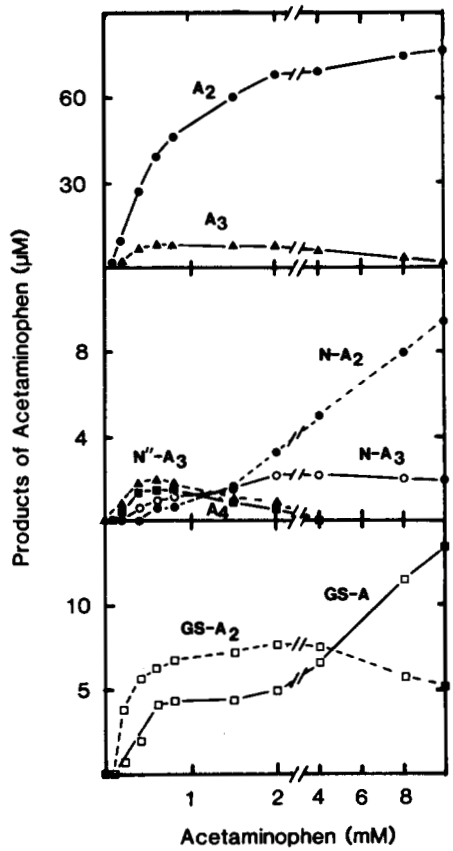

FIG. 2. **Effect of acetaminophen concentration on horseradish peroxidase-mediated formation of the acetaminophen products in the presence of GSH.** Reaction mixtures (1 ml) contained 0–10 mM acetaminophen, 80 nM of horseradish peroxidase, 200 μM H_2O_2, 100 μM GSH, and 100 mM potassium phosphate, pH 7.4. Mixtures were equilibrated at 25 °C, and reactions were initiated by the addition of H_2O_2. After 5 min a 1-ml solution of ice-cold methanol/water (90:10, v/v) containing 2 mM ascorbic acid was added to mixtures. Reaction products were analyzed by HPLC as described under "Materials and Methods."

To determine if GSH decreased polymerization by competing with acetaminophen to reduce enzymatically H_2O_2 to H_2O or if GSH reduced the free radical of acetaminophen, the oxidation of GSH to GSSG was examined in reaction mixtures containing horseradish peroxidase and H_2O_2 with or without acetaminophen. Fig. 4 shows that GSH was oxidized to GSSG by horseradish peroxidase alone; however, GSSG formation was substantially higher when acetaminophen was included in reaction mixtures. The amount of GSSG formation without acetaminophen was between 25–30% of the amount of GSSG formation with acetaminophen. The difference in GSH oxidation, with and without acetaminophen, suggests that GSH could decrease acetaminophen polymerization by reducing the acetaminophen free radical after it was formed by horseradish peroxidase.

It has previously been shown that ascorbic acid decreased acetaminophen polymerization (17). Fig. 5 shows that ascorbic acid similarly decreased GSH-acetaminophen conjugation as well as polymer formation. The decrease in GSH-acetaminophen conjugation and acetaminophen polymerization was nearly proportional, further suggesting that the glutathione conjugates and polymers may have formed via a common intermediate.

Since the data with GSH and ascorbic acid (Table I and in

[2] Portions of this paper (including "Materials and Methods," Tables I and II, and Figs. 1, 4, and 8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86 M-1381, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

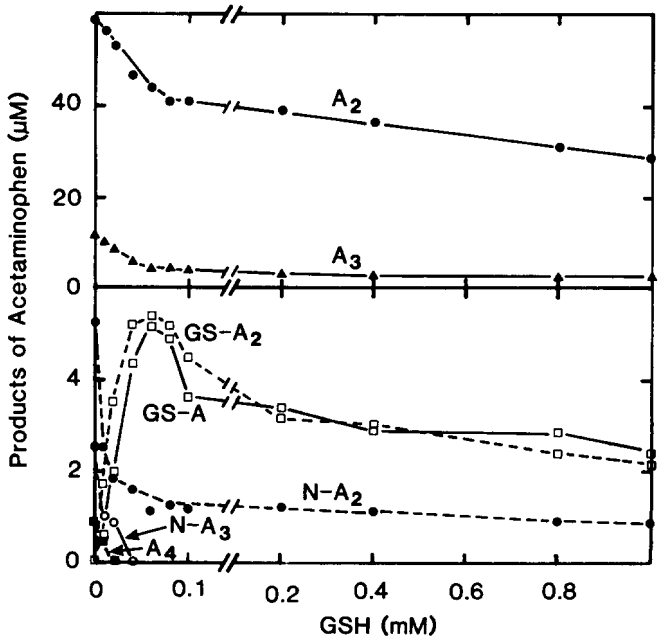

FIG. 3. **Effect of GSH concentration on horseradish peroxidase-mediated catalysis of acetaminophen.** Reaction conditions were the same as those described in the legend to Fig. 2 except all samples contained 1.0 mM acetaminophen and 100 μM H_2O_2 with 0–1.0 mM GSH.

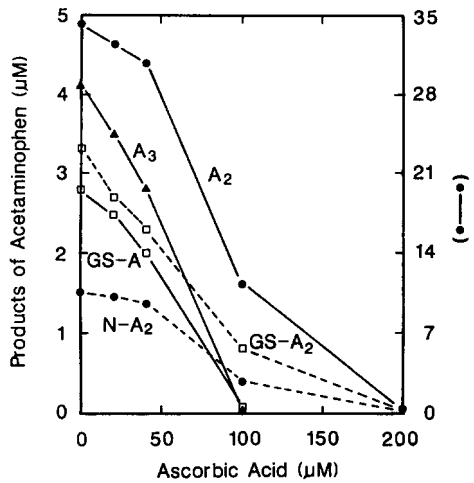

FIG. 5. **Effect of ascorbic acid concentration on horseradish peroxidase-mediated formation of the acetaminophen products in the presence of GSH.** Reaction conditions were the same as those described in the legend to Fig. 3 except all samples contained 100 μM GSH with 0–200 μM ascorbic acid. Since the amount of A_2 was greater than the other products, its quantitation is shown on the right *y* axis. The quantitation of the other products is shown on the left *y* axis.

Fig. 5), indicated that GS-A and A_2 may have been formed via a common intermediate, experiments were performed to examine the formation of acetaminophen polymers and conjugates when GSH was added at different times after initiation of the horseradish peroxidase-catalyzed polymerization reaction. As shown in Fig. 6, low amounts of GS-A were formed when reaction mixtures were incubated for 15 s with GSH before addition of the ascorbic acid solution (designated as time 0). Acetaminophen polymerization was nearly maximal when GSH was added at 1 min. In contrast, GS-A and GS-A_2 formation were maximal at about 15 s after addition of GSH, and the amount of each conjugate decreased with longer

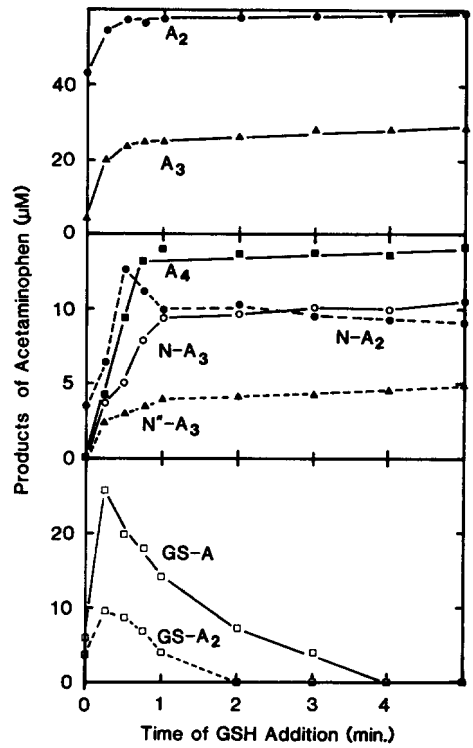

FIG. 6. **Horseradish peroxidase-catalyzed conversion of acetaminophen to products with the addition of GSH at different time points.** Reaction conditions were the same as those described in the Fig. 3 legend except that 200 μM H_2O_2 was included in all experiments. GSH (2 mM) was added at the indicated time points (0–5 min) after incubation was initiated and a methanol/water solution containing 2 mM ascorbic acid was added 15 s after GSH addition to ensure that reactions were completely quenched. Zero time means that reactions were incubated for 15 s with 1 mM GSH before addition of ascorbic acid solution.

incubation times. GS-A_2 was only detected within the first 2 min of the reaction, whereas GS-A was detected for up to 4 min after initiation of reaction.

These data indicated that the intermediate which led to polymer formation and the intermediate which conjugated with GSH might be different. Thus, the possibility that *N*-acetyl-*p*-benzoquinone imine may be formed was examined by direct HPLC analyses of incubation mixtures which did not contain GSH (Fig. 7). In these incubation mixtures, a product was detected that coeluted with *N*-acetyl-*p*-benzoquinone imine but not with any of the other products. This product was collected in vials containing either ascorbic acid or GSH and reanalyzed by the HPLC system described for the separation of acetaminophen polymers and conjugates. The isolated product reacted with ascorbic acid to give acetaminophen and reacted with GSH to give acetaminophen and GS-A. These results were uniquely consistent with the isolated product being *N*-acetyl-*p*-benzoquinone imine.

Under similar reaction conditions, the amount of *N*-acetyl-*p*-benzoquinone imine and GS-A formed at the various time points was nearly the same (Fig. 7). As with GS-A formation, the concentration of *N*-acetyl-*p*-benzoquinone imine was maximal at 15 s and slowly decreased with longer incubation times. Neither *N*-acetyl-*p*-benzoquinone imine nor GS-A were detected after a 5-min incubation. The decrease in GS-A and *N*-acetyl-*p*-benzoquinone imine was presumably due to the slow comproportionation of *N*-acetyl-*p*-benzoquinone imine with acetaminophen. The second order rate constant for the comproportionation reaction was previously shown to be 33 M^{-1} s^{-1} (7).

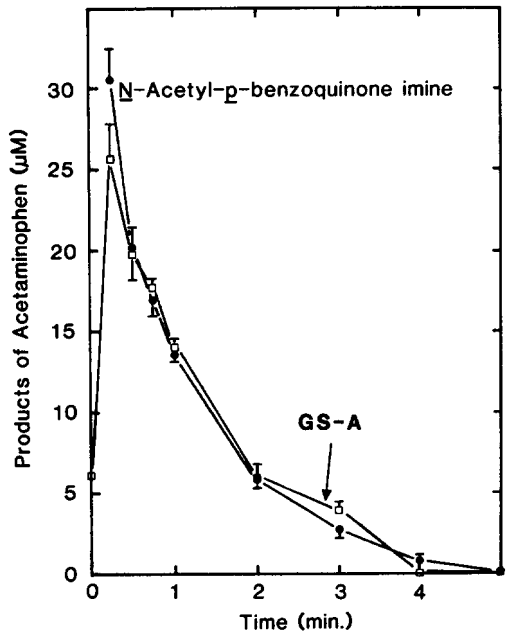

FIG. 7. **Time course of horseradish peroxidase-catalyzed conversion of acetaminophen to *N*-acetyl-*p*-benzoquinone imine or GS-A.** Reaction conditions were the same as those described in the legend to Fig. 6. Reaction mixtures were incubated for various lengths of time and *N*-acetyl-*p*-benzoquinone imine was determined in incubations which did not contain GSH by HPLC as described under "Materials and Methods." Quantitation of GS-A is taken from Fig. 6 and included here for comparison.

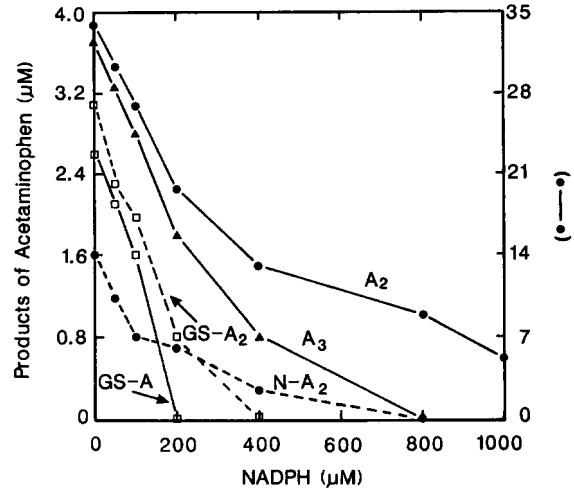

FIG. 9. **Effect of NADPH concentration on horseradish peroxidase-mediated formation of the acetaminophen products in the presence of GSH.** Reaction conditions were the same as those described in the legend to Fig. 3 except all samples contained 100 μM GSH with 0–1.0 mM NADPH. Since the amount of A_2 was greater than the other products, its quantitation is shown on the right *y* axis. The quantitation of the other products is shown on the left *y* axis.

Formation of Acetaminophen Products with Hepatic Microsomes from Rats Pretreated with Phenobarbital—When [^{14}C] acetaminophen (1.0 mM) was incubated with hepatic microsomes, NADPH, and oxygen, the formation of GS-A was linear with time for at least 20 min. The turnover number for GS-A formation was 1.2 nmol/nmol P-450/10 min. In incubations containing 100 μM DPEA, a cytochrome P-450 inhibitor, the formation of GS-A was 0.2 nmol/nmol P-450/10 min.

Further analyses showed that horseradish peroxidase catalyzed the H_2O_2-dependent conversion of acetaminophen to polymers in the presence of microsomal enzymes as well as it did in the absence of microsomes. However, in the presence of 1.0 mM NADPH, polymerization was greatly inhibited. Likewise, horseradish peroxidase-dependent polymerization of acetaminophen in the absence of microsomal preparation was equally inhibited by NADPH.

Fig. 8 shows the effect of increasing NADPH concentration on the horseradish peroxidase-catalyzed conversion of acetaminophen to total polymers and Fig. 9 shows the effect of NADPH on product formation in the presence of 100 μM GSH. Polymerization was decreased with increasing concentrations of NADPH, greater than 92% by 1 mM NADPH (Fig. 8). As with acetaminophen polymerization, the formation of the GSH-acetaminophen conjugates was also decreased by NADPH, and the decrease in polymer formation was nearly proportional to the decrease in conjugate formation. In order to determine whether the decrease in acetaminophen polymerization was due to the reduction of *N*-acetyl-*p*-benzosemiquinone imine or the reduction of H_2O_2 by NADPH, spectrophotometric analyses were performed. These analyses showed that 100 μM NADPH was slowly oxidized by horseradish peroxidase and 100 μM H_2O_2 (less than 1% was oxidized min^{-1}). Upon addition of 300 μM acetaminophen, nearly 50% of the NADPH was oxidized within 10 s after addition. These experiments indicated that the *N*-acetyl-*p*-benzosemiquinone imine caused NADPH oxidation.

Since the direct detection of acetaminophen polymerization products, as a means of showing free radical formation, was not possible, additional experiments were performed to further examine whether or not cytochrome P-450 might catalyze acetaminophen by a 1-electron oxidation pathway. The rate of NADPH oxidation to the rate of product formation was examined. In the absence of acetaminophen, the hepatic microsomes from rats pretreated with phenobarbital caused the oxidation of NADPH at a rate of 144.2 nmol/nmol P-450/10 min when incubation mixtures contained 1 mM GSH and 122.3 nmol/nmol P-450/10 min without GSH. Acetaminophen stimulated NADPH oxidation to 148.5 nmol/nmol P-450/10 min with GSH and 135.2 ± 1.0 nmol/nmol P-450/10 min without GSH. Thus, acetaminophen stimulated NADPH oxidation by 4.3 and 12.9 nmol/nmol P-450/10 min in the presence and absence of GSH, respectively. Under similar conditions 1.2 nmol of GS-A/nmol P-450/10 min was formed, and the other previously identified cytochrome P-450-catalyzed metabolite of acetaminophen, 3′-hydroxyacetaminophen (26, 27), was not detected. Since, acetaminophen-dependent stimulation of NADPH oxidation could not be accounted for by product formation, these results suggest that cytochrome P-450 may also catalyze the 1-electron oxidation of acetaminophen.

Formation of Acetaminophen Polymers from Reaction Mixtures Containing Cumene Hydroperoxide and Hepatic Microsomes from Phenobarbital-Pretreated Rats—In contrast to microsomal incubation mixtures which contained NADPH, acetaminophen polymerization was observed in incubations where 2 mM cumene hydroperoxide replaced NADPH. Polymers were detected in incubations where microsomes were used that had previously been immersed in boiling water for 5 min; yet, polymerization was about 3-fold greater with microsomes not boiled (Table II). DPEA and SKF-525A decreased polymerization by 2.0- to 2.6-fold, respectively. KCN had no appreciable effect on polymerization. Similarly, GS-A was formed in reaction mixtures which contained 1.0 mM GSH. Utilization of boiled microsomes also decreased GS-A formation by about 3-fold.

DISCUSSION

Horseradish peroxidase is generally thought to be divalently oxidized by H_2O_2 and then reduced by various electron-donating substrates in two separate 1-electron transfer steps (16, 28–31). Consistent with this mechanism horseradish peroxidase has been shown to catalyze the 1-electron oxidation of acetaminophen as demonstrated by electron spin resonance observations (33) and by the identification of acetaminophen polymers that are indicative of a free radical-coupling reaction (17). Even though the phenoxyl radical is the primary radical found in solution (32), the radical-coupling data suggested that both the nitrogen-center radical and the phenyl carbon radical *ortho* to the hydroxyl group form the acetaminophen polymers since these were the only identified sites of polymerization (17). When GSH was added to the horseradish peroxidase system, two GSH-acetaminophen conjugates, GS-A and GS-A_2, were formed (18).

In this work, we have examined the mechanism by which GSH reacts with the oxidized acetaminophen intermediates formed during the catalysis by horseradish peroxidase. GSH decreased acetaminophen polymerization in this system (Fig. 3), apparently by reacting with *N*-acetyl-*p*-benzosemiquinone imine to form GSSG and acetaminophen (Fig. 4). Both ascorbic acid and NADPH also decreased polymerization as well as conjugate formation (Figs. 5 and 9). Collectively these data indicate that the various reducing agents decrease polymerization by reducing *N*-acetyl-*p*-benzosemiquinone imine back to acetaminophen; however, since GSH (Fig. 4) as well as ascorbic acid (15) were also substrates for horseradish peroxidase, some enzymatic inhibition may have occurred. Other experiments indicated that the formation of GS-A occurred via conjugation of GSH with the *N*-acetyl-*p*-benzoquinone imine rather than with the *N*-acetyl-*p*-benzosemiquinone imine intermediate. These data showed that the initial rate of formation, subsequent disappearance, and the amount of *N*-acetyl-*p*-benzoquinone imine paralleled the amount of GS-A formed in the absence of GSH or when GSH was added at various times after initiation of reactions, respectively (Figs. 6 and 7). The data further suggested that *N*-acetyl-*p*-benzoquinone imine was not formed via an overall horseradish peroxidase-catalyzed 2-electron oxidation of acetaminophen but rather that acetaminophen was oxidized by a 1-electron transfer and that the resulting *N*-acetyl-*p*-benzosemiquinone imine disproportionated to give acetaminophen and *N*-acetyl-*p*-benzoquinone imine. If *N*-acetyl-*p*-benzoquinone imine was formed via an overall 2-electron oxidation, the formation of GS-A should either remain the same or increase as the concentrations of GSH, ascorbic acid, or NADPH were increased; however, the decrease in GSH-acetaminophen conjugation was proportional to the decrease in acetaminophen polymerization (Table I and Figs. 3, 5, and 9).

Although the data suggested that GS-A primarily was formed via GSH conjugation with *N*-acetyl-*p*-benzoquinone imine, the mechanism of GS-A_2 formation appears to be more complex. We have previously presented evidence that shows one mechanism of GS-A_2 formation is apparently via GS-A oxidation by *N*-acetyl-*p*-benzosemiquinone imine to give acetaminophen and 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine and that this semiquinone reacts with the acetaminophen semiquinone via a radical termination mechanism (18). Although this may be a pathway for GS-A_2 formation, it does not adequately explain all of the GS-A_2 formation observed in Fig. 6. Since the concentration of *N*-acetyl-*p*-benzosemiquinone imine after 1 min was apparently quite low as demonstrated by the decreased rate of polymerization (Fig. 6), it seems plausible that the quinone imine of A_2 may have been formed and subsequently reacted with GSH to give GS-A_2.

A summary of the horseradish peroxidase reactions of acetaminophen is shown in Fig. 10. Horseradish peroxidase catalyzed the 1-electron oxidation of acetaminophen. GSH can then reduce the *N*-acetyl-*p*-benzosemiquinone imine intermediate back to acetaminophen with concomitant formation of GSSG. The phenyl- or the nitrogen-centered radicals are the likely intermediates that form polymers primarily through a covalent bond between carbons *ortho* to the hydroxyl groups and, to a lesser extent, between the carbon *ortho* to the hydroxyl group and the amino group of another acetaminophen molecule. Alternatively, two molecules of *N*-acetyl-*p*-benzosemiquinone imine may disproportionate to *N*-acetyl-*p*-benzoquinone imine and acetaminophen. The evidence suggests that the disproportionation is the major pathway leading to GSH-acetaminophen conjugate formation. If GSH-acetaminophen conjugates are formed via a radical mechanism, the data suggest that it is a minor reaction.

In contrast to the metabolites formed during the catalytic oxidation of acetaminophen by horseradish peroxidase, the metabolites formed by microsomal cytochrome P-450 in the presence of NADPH are quite different. Although GS-A was formed, the formation of acetaminophen polymers was not observed in the NADPH-dependent microsome-mediated reactions. If GS-A was formed via *N*-acetyl-*p*-benzosemiquinone imine disproportionation, as was observed with the horseradish peroxidase system, then polymer formation should have been at least five times greater than GS-A formation assuming the two system would give comparable results. Since the horseradish peroxidase studies suggest that GSH-acetaminophen conjugation is via the *N*-acetyl-*p*-benzoquinone imine intermediate, the microsomal studies indicate that cytochrome P-450 catalyzes the overall 2-electron oxidation of acetaminophen.

In addition to NADPH and microsomes catalyzing the overall 2-electron oxidation of acetaminophen, the micro-

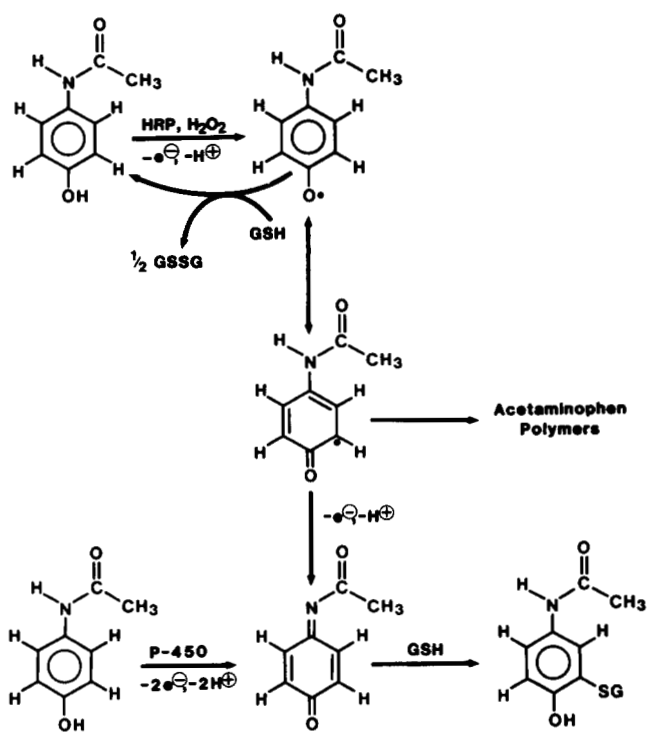

FIG. 10. **Schematic representation of the proposed mechanisms of acetaminophen polymer and GS-A formation.**

somes may have catalyzed the 1-electron oxidation which went undetected, since incubations were performed in the presence of high concentrations of NADPH. It was shown that NADPH was very effective at reducing *N*-acetyl-*p*-benzosemiquinone imine back to acetaminophen (Fig. 8). Furthermore, NADPH (Figs. 8 and 9) decreased acetaminophen polymerization more effectively than did GSH (Fig. 3), and ascorbic acid (Fig. 5) was more effective than either NADPH or GSH at decreasing acetaminophen polymerization.

Although no direct evidence demonstrating the 1-electron oxidation of acetaminophen by cytochrome P-450 and NADPH has been obtained, indirect evidence suggests that cytochrome P-450 may have catalyzed the 1-electron oxidation of acetaminophen. Rate studies have shown that acetaminophen stimulated NADPH oxidation with microsomes, which was substantially greater than what could be accounted for by product formation. Thus, formation of *N*-acetyl-*p*-benzosemiquinone imine followed by its reduction by NADPH would be consistent with these results. Additionally, formation of acetaminophen polymers was observed in microsomal incubations where cumene hydroperoxide replaced NADPH (Table II). It is well-established that microsomal (33, 34) and purified preparation (35) of cytochrome P-450 can utilize hydroperoxides as oxidizing agents in place of NADPH and oxygen; however, the metabolism of certain compounds may not always be identical when NADPH is replaced by cumene hydroperoxide (36). Our experiments have shown that cumene hydroperoxide and hepatic microsomes caused a nonenzymatic oxidation of acetaminophen to polymers in boiled preparation. Although the formation of acetaminophen polymerization products in incubation mixtures with boiled microsomal preparations is not understood, the data indicate that cytochrome P-450 probably does play a role in the cumene hydroperoxide-dependent catalysis of acetaminophen since polymerization was increased by 2- to 3-fold over the amount of polymer observed with boiled microsomes. These experiments do indicate a strong likelihood that cytochrome P-450 may catalyze the 1-electron oxidation of acetaminophen; however, additional studies are needed for a better understanding of the cytochrome P-450-dependent oxidation of acetaminophen.

In conclusion, these data suggest that horseradish peroxidase catalyzed the 1-electron oxidation of acetaminophen to *N*-acetyl-*p*-benzosemiquinone imine, which then reacts primarily to form acetaminophen polymers, and to a lesser degree disproportionates to form *N*-acetyl-*p*-benzoquinone imine and acetaminophen. GSH reduces *N*-acetyl-*p*-benzosemiquinone imine to acetaminophen, whereas it conjugates with *N*-acetyl-*p*-benzoquinone imine to form GS-A. In comparative studies using microsomes and NADPH, acetaminophen polymerization products were not observed, while GS-A was a major product. However, when cumene hydroperoxide replaced NADPH, both GS-A and polymerization products were observed. These results suggest that cytochrome P-450 may catalyze acetaminophen by both overall 1- and 2-electron oxidation mechanisms.

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Supplementary Material to

MECHANISMS OF ACETAMINOPHEN OXIDATION TO N-ACETYL-P-BENZOQUINONE IMINE BY HORSERADISH PEROXIDASE AND CYTOCHROME P-450

David W. Potter and Jack A. Hinson

MATERIALS AND METHODS

[phenyl-U-^{14}C]Acetaminophen (1.65 mCi/mmol; >99% pure) was obtained from Dr. Robert W. Roth of Midwest Research Institute, Kansas City, MO. Horseradish peroxidase, EC number 1.11.1.7 (type VI), acetaminophen, 30% H_2O_2, GSH, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and ascorbic acid were purchased from Sigma Chemical Co., St. Louis, MO. N-Acetyl-p-benzoquinone imine was synthesized as previously described (7). All other reagents were of the highest grade available.

Reaction Procedures - Reaction mixtures (1 ml) contained 100 mM potassium phosphate, pH 7.4, and various amounts of H_2O_2, GSH and acetaminophen. Mixtures were equilibrated at 25° and reactions were initiated with H_2O_2. Reactions usually were terminated by the addition of 1 ml ice-cold methanol:water (90:10, v/v) containing 2 mM ascorbic acid. Specific details of the reaction mixtures are described in the legends to figures and tables. Concentrations of GSSG were determined spectrophotometrically by the method of Sies and Summer (19). For the determination of GSSG, reaction mixtures contained 0 or 1 mM acetaminophen, 0-2 mM GSH, 80 nM horseradish peroxidase, 100 μM H_2O_2 and 100 mM potassium phosphate, pH 7.4. NADPH followed by glutathione reductase were added to incubation mixtures 1 min after reactions were initiated with H_2O_2. Oxidation of NADPH was not observed without glutathione reductase.

Hepatic Microsomes - Hepatic microsomes from Sprague-Dawley rats (NCTR breeding colony) pretreated with phenobarbital were prepared as previously described (20). Protein concentrations were estimated by the biuret method (21) and cytochrome P-450 concentrations were estimated by the method of Omura and Sato (22). Microsomal preparations contained 2.5 nmol cytochrome P-450/mg protein. Incubations (1 ml) contained 1 mM [^{14}C]acetaminophen, 1.0 mg microsomal protein/ml, 1 mM NADPH or 2 mM cumene hydroperoxide, 0 or 1 mM GSH, and 100 mM potassium phosphate, pH 7.4. Reactions were initiated by the addition of NADPH or cumene hydroperoxide. Mixtures were incubated at 37° for 10 min, and reactions were terminated by the addition of 1 ml ice-cold methanol:water (90:10, v/v) containing 2 mM ascorbic acid.

A Cary 219 spectrophotometer from Varian Associates, Inc., Palo Alto, CA, was utilized to determine concentrations of NADPH using the extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm. Using a modification of the method of Sasame and Boyd (23) the oxidation of NADPH was determined in reaction mixtures (1 ml) containing 1.0 mg phenobarbital-pretreated rat liver microsomes, 1.0 mM NADPH, 0 or 1.0 mM acetaminophen, 0 or 1.0 mM GSH and 100 mM potassium phosphate, pH 7.4. Reactions were initiated with NADPH, incubated at 37° for 10 min and terminated by the addition of 333 μl 10% perchloric acid. Samples were placed on ice for 1 hr, centrifuged for 20 min, 1.0-ml of the supernatant was removed and added to 333 μl of 2 M dibasic potassium phosphate. Samples were again centrifuged for 10 min, 1.0-ml of the supernatant was removed and added to 133 μl 2N sodium hydroxide and 33 μl glucose-6-phosphate (1.7 mg). The concentration of NADPH was determined by optical absorbance spectroscopy after the addition of 1 unit glucose-6-phosphate dehydrogenase.

Liquid Chromatography - Acetaminophen polymers and GSH-acetaminophen conjugates were analyzed by reversed-phase HPLC (17,18). The analytical HPLC system consisted of two model 6000 HPLC pumps, a Waters Associates, Inc. model 440 UV detector (254 nm) and a model 660 microprocessor (Milford, MA), a Micromeritics (Norcross, GA) model 725 automatic injector, a Hewlett Packard model 3390A Reporting Integrator (Palo Alto, CA), and a 5-μm C_{18} Altex Ultrasphere ODS reversed-phase column (4.6 X 250 mm) (Berkeley, CA). A binary solvent system with a flow rate of 1.0 ml/min was used for acetaminophen metabolite separation. One solvent was methanol and the other solvent, solvent A, consisted of 87.9% water, 10% methanol, 2% acetic acid and 0.1% ethyl acetate. Solvent A was maintained at 100% for 10 min, followed by a 15-min linear gradient to give 81% A and 19% methanol.

N-Acetyl-p-benzoquinone imine formation was determined by HPLC (11) using an isocratic solvent system which consisted of 20% methanol and 10 mM potassium phosphate, pH 7.4.

H_2O_2 and Horseradish Peroxidase Quantitation - Deionized glass-distilled water was used to prepare stock solutions of H_2O_2 and horseradish peroxidase. Concentrations were estimated by optical absorbance spectroscopy using the extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm for H_2O_2 (24) and 89.5 X 10^3 M^{-1} cm^{-1} at 403 nm for horseradish peroxidase (25).

TABLE I

Ratio of GSH-acetaminophen conjugates to acetaminophen dimer formation

GSH (mM)	GS-A/A_2[a]	GS-A + GS-A_2/A_2
0.02	.037	.106
0.04	.091	.204
0.06	.119	.221
0.08	.087	.204
0.1	.087	.194
0.2	.080	.168
0.8	.081	.170
2.0	.075	.142
6.0	.081	.146

[a]The data for GS-A, GS-A_2 and A_2 formation with 0.02-0.8 mM GSH are taken from Fig. 3. The amounts of GS-A, GS-A_2 and A_2 formed with 2 mM GSH were 2.0 μM, 1.8 μM and 26.7 μM, respectively, and with 6 mM GSH were 1.7 μM, 1.4 μM and 21.0 μM, respectively.

TABLE II

Products of acetaminophen formed during incubations containing cumene hydroperoxide and hepatic microsomes from phenobarbital-pretreated rats

Treatment	Reaction Products[a] (nmol/nmol P-450/10 min)			
	- GSH		+ GSH[b]	
	A_2	N-A_2	GS-A	A_2
None	4.6	0.5	1.5	0.3
Boiled[c]	1.5	0.3	0.5	0.2
DPEA (100 M)	2.2	0.2	0.4	0.2
SKF-525A (2 mM)	1.8	0.1	0.7	0.3
KCN (5 mM)	4.5	0.3	1.1	0.2

[a]Reaction mixtures (1 ml) contained 1 mg microsomal protein, 1.0 mM [^{14}C]acetaminophen, 2.0 mM cumene hydroperoxide and 100 mM potassium phosphate, pH 7.4. Reactions were initiated with cumene hydroperoxide, incubated at 37° for 10 min and reactions terminated as previously described in the legend to Fig. 2.

[b]GSH content was 1.0 mM.

[c]Microsomes were immersed in boiling water for 5 min.

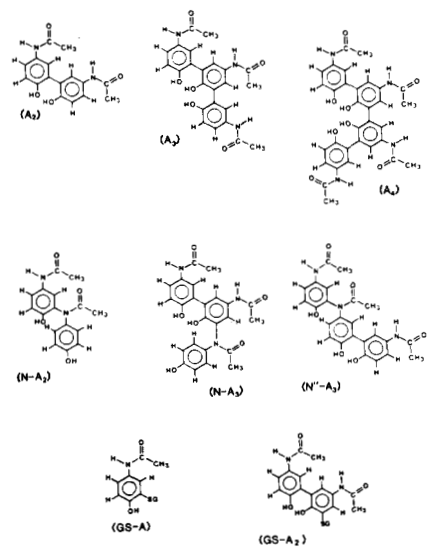

Fig. 1. Structures of acetaminophen polymers and GSH-acetaminophen conjugates. The acetaminophen polymers and GSH-acetaminophen conjugates have been previously identified (17,18) and shown to be acetaminophen dimer (A_2), acetaminophen trimer (A_3), acetaminophen tetramer (A_4), N-acetaminophen dimer (N-A_2), N''-acetaminophen trimer (N''-A_3), N-acetaminophen trimer (N-A_3), 3-(glutathion-S-yl)acetaminophen (GS-A) and 3-(glutathion-S-yl)diacetaminophen (GS-A_2).

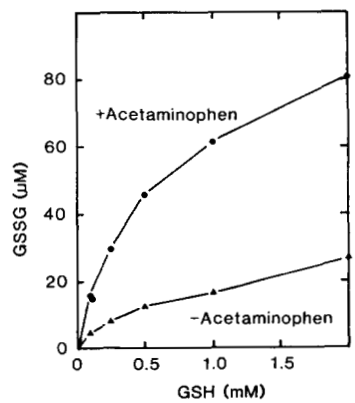

Fig. 4. Effect of acetaminophen on the oxidation of GSH to GSSG by horseradish peroxidase. Reaction conditions were the same as those described in the legend to Fig. 3 except GSSG concentrations were determined in the presence or absence of 1.0 mM acetaminophen. The GSSG concentrations were determined spectrophotometrically as described in "Materials and Methods."

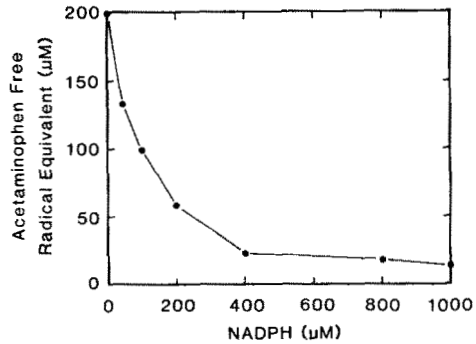

Fig. 8. Effect of NADPH concentration on the horseradish peroxidase-catalyzed conversion of acetaminophen to polymers. Reaction conditions were the same as those described in Fig. 3 except samples contained 0-1.0 mM NADPH. The acetaminophen free radical equivalent was the sum of the total free radical equivalents which was determined for each polymer by multiplying polymer concentration by 2 (n-1) where n is the number of acetaminophen molecules/polymer.