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Horseradish Peroxidase-Catalyzed Oxidation of Acetaminophen to Intermediates that Form Polymers or Conjugate with Glutathione

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SUMMARY

Horseradish peroxidase catalyzed the polymerization of acetaminophen. Addition of reduced glutathione (GSH) to reaction mixtures resulted in decreased polymerization and formation of minor amounts of GSH-acetaminophen conjugates. The conjugates were identified as 3-(glutathion-*S*-yl)acetaminophen and 3-(glutathion-*S*-yl)diacetaminophen. Horseradish peroxidase also catalyzed polymerization of synthetic 3-(glutathion-*S*-yl)acetaminophen to a dimer conjugate. In contrast to acetaminophen, 3-(glutathion-*S*-yl)acetaminophen oxidation was slowly catalyzed by horseradish peroxidase. However, in reaction mixtures containing equimolar concentrations of acetaminophen and synthetic 3-(glutathion-*S*-yl)acetaminophen, the formation of 3-(glutathion-*S*-yl)diacetaminophen and 3-(diglutathion-*S*-yl)diacetaminophen was rapid and accounted for approximately 95% of the products, whereas acetaminophen polymers accounted for only 5% of the products. These findings suggest that horseradish peroxidase catalyzed the one-electron oxidation of acetaminophen to *N*-acetyl-*p*-benzosemiquinone imine which preferentially polymerized rather than reacted with GSH. *N*-Acetyl-*p*-benzosemiquinone imine may also oxidize 3-(glutathion-*S*-yl)acetaminophen to form acetaminophen and 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine. The data indicate that once this conjugate radical is formed it reacts with either *N*-acetyl-*p*-benzosemiquinone imine or 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine via a radical termination mechanism.

Acetaminophen (4′-hydroxyacetanilide) is a commonly used analgesic and antipyretic drug. Although acetaminophen is reportedly safe in low doses, it causes hepatic necrosis and renal damage in both man and laboratory animals when administered in large doses (1–6). Both toxicities are believed to be a result of reactive metabolites. Cytochrome P-450 and prostaglandin H synthase metabolize acetaminophen to intermediate(s) that bind to protein and conjugate with GSH (7–16). Current evidence suggests that cytochrome P-450 catalyzes the two-electron oxidation of acetaminophen to the reactive intermediate, NAPQI (7–13). This conclusion is supported by two observations: NAPQI is a metabolite of cytochrome P-450 using cumene hydroperoxide, although it is not detected when using NADPH (10), and synthetic NAPQI reacts with GSH to form the same GSH-acetaminophen conjugate formed in cytochrome P-450 incubation mixtures (13, 17–19). Although these data suggest that NAPQI may be a cytochrome P-450-derived metabolite of acetaminophen, an alternative mechanism of forming the GSH conjugate of acetaminophen has been proposed in which the one-electron oxidation intermediate of acetaminophen, NAPSQI, reacts with a glutathionyl radical (20). If NAPSQI were a cytochrome P-450-mediated intermediate it might disproportionate in a manner similar to benzosemiquinone (21) and 3′,5′-dimethyl-*N*-acetyl-*p*-benzosemiquinone imine (22) to give the small amount of NAPQI previously observed with cytochrome P-450 (10). Although unproven, the one-electron oxidation of acetaminophen by prostaglandin H synthase has been proposed (14–16). The prostaglandin H synthase-mediated intermediate(s) appears to react with protein and GSH in a manner similar to the cytochrome P-450-mediated intermediates.

We have been interested in the chemistry of NAPSQI as an oxidative intermediate of acetaminophen. Recently, we reported that acetaminophen was polymerized by horseradish peroxidase (23) to yield two acetaminophen dimers, three

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ABBREVIATIONS: GSH, reduced glutathione; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NAPSQI, *N*-acetyl-*p*-benzosemiquinone imine; ESR, electron spin resonance; GS-A or 3-(glutathion-*S*-yl)acetaminophen, *N*-[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-[(5-acetamido-2-hydroxyphenylyl)thio]ethyl]-L-glutamine; HPLC, high pressure liquid chromatography; GS-A_2 or 3-(glutathion-*S*-yl)diacetaminophen, *N*-[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-[(5,5′-diacetamido-2,2′-hydroxy-3-biphenylyl)thio]ethyl]-L-glutamine; GS_2-A_2 or 3-(diglutathion-*S*-yl)diacetaminophen, *N,N′*-[(5,5′-diacetamido-2,2′-dihydroxy-3,3′-biphenylene)bis[thio[(1*R*)-1-[(carboxymethyl)carbamoyl]ethylene]]]di-L-glutamine; Me_2SO, dimethyl sulfoxide.

acetaminophen trimers, and one acetaminophen tetramer (see Fig. 1). Formation of polymers is indicative of a free radical termination mechanism (23) which is consistent with ESR studies of West *et al.* (24) who showed that NAPSQI is formed by peroxidation of acetaminophen.

In the present studies, we have examined the effect of GSH on acetaminophen polymerization reactions. The data indicate that NAPSQI formed polymers more readily than reacting with GSH to form conjugates. However, formation of NAPSQI in the presence of the GSH-acetaminophen conjugate, GS-A, resulted in preferential formation of polymers of GS-A which is consistent with NAPSQI oxidizing GS-A to give the 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinoneimine intermediate.

Materials and Methods

[phenyl-UL-^{14}C]Acetaminophen (1.65 mCi/mmol) was obtained from Dr. Robert W. Roth of Midwest Research Institute (Kansas City, MO) and was purified by thin layer chromatography and HPLC to >99% (23). [glycine-2-^{3}H]GSH (50 μCi/mmol) was purchased from New England Nuclear (Boston, MA). Horseradish peroxidase (EC 1.11.1.7) (type VI), acetaminophen, 30% H_2O_2, GSH, and ascorbic acid were from Sigma Chemical Co. (St. Louis, MO). Silver oxide (I) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Deuterated water was purchased from Merck, Isotopes, Inc. (Montreal, Quebec, Canada).

Synthesis of 3-(glutathion-*S*-yl)acetaminophen. NAPQI was synthesized by a modification of the method of Dahlin and Nelson (18). Acetaminophen (100 mg) was oxidized with 100 mg of silver oxide (I) in 5 ml of freshly redistilled chloroform containing 10 mg of anhydrous sodium sulfate for 2 hr at room temperature. Activated charcoal (10 mg) was added to chloroform and the suspension was filtered. The chloroform layer was applied to a Waters silica Sep-Pak and the NAPQI was eluted with anhydrous diethyl ether. Dry Me_2SO (200 μl) was added to the ether and the ether was evaporated under reduced pressure which left the NAPQI in Me_2SO. This solution was used in the preparation of GS-A. A 1-liter volume of deionized glass-distilled water containing 2.0 mM GSH and 100 mM potassium phosphate, pH 7.4, was placed on ice and the Me_2SO containing NAPQI was slowly added with continuous mixing. This reaction resulted in the formation of acetaminophen and GS-A. Acetaminophen was removed from the aqueous phase by continuous liquid:liquid extraction with ethyl acetate for 72 hr. The aqueous phase was then evaporated under reduced pressure. The residue was redissolved in 10 ml of H_2O and the GS-A was purified by HPLC using a 10-μm Waters μBondapak-C_{18} column (0.78 × 30 cm).

Five hundred-MHz ^{1}H NMR spectroscopy of the synthetic GS-A revealed three aromatic ^{1}H resonances (H_2, H_5, and H_6) and one acetyl resonance of acetaminophen as well as the typical seven resonances of glutathione (see Table 2). Integration of the resonances showed that acetaminophen and glutathione were present in equal amounts. By comparison of the ^{1}H NMR spectrum of this product with the ^{1}H NMR spectrum of GS-A isolated from the bile of rats (12), it was concluded that the compound was GS-A.

Metabolism of acetaminophen. Analytical incubations (1 ml) contained 100 mM potassium phosphate (pH 7.4), 80 nM horseradish peroxidase, and various amounts of acetaminophen, GSH, GS-A, and H_2O_2. Specific details of the contents of the reaction mixtures are given in the legends to figures and tables. Samples were equilibrated at 25° for 2 min and then initiated by adding H_2O_2. Reactions were terminated as previously described by adding 1 ml of ice-cold methanol:H_2O (90:10) containing 2.0 mM ascorbate and placing the incubation mixtures on ice (23).

Preparative incubations (25 ml) contained 100 mM potassium phosphate (pH 7.4), 80 nM horseradish peroxidase, 2.0 mM acetaminophen, 2.0 mM GS-A, and 500 μM H_2O_2. Reactions were initiated with H_2O_2 and were allowed to go to completion at 25°.

Liquid chromatography. Acetaminophen metabolites were analyzed by reversed-phase HPLC (23). The analytical HPLC system consisted of two model 6000 HPLC pumps, a model 440 UV detector (254 nm), and a model 660 microprocessor from Waters Associates, Inc. (Milford, MA), a Micromeritics (Norcross, GA) model 725 automatic injector, a model 3390A Reporting Integrator from Hewlett-Packard (Palo Alto, CA), and a 5-μm C_{18} Ultrasphere ODS reversed-phase column (4.6 × 250 mm) from Altex (Berkeley, CA). A binary solvent system with a flow rate of 1.0 ml/min was used for acetaminophen metabolite separation. Solvent A, which consisted of 87.9% water, 10% methanol, 2% glacial acetic acid, and 0.1% ethyl acetate (v/v) was maintained at 100% for 10 min, followed by a linear gradient to give 81% solvent A and 19% methanol in 15 min.

For UV analyses of compounds, the analytical HPLC system was equipped with a Hewlett-Packard model 1040A spectrophotometric detector. The HPLC column was equilibrated with 90% 10 mM acetic acid and 10% methanol. After the sample was injected, separation was performed with a 15-min linear gradient to a mixture of 71% 10 mM glacial acetic acid and 29% methanol which was maintained until separation was completed. Retention times of compounds with this solvent system were: acetaminophen (13 min), GS-A (18 min), GS-A_2 (31 min), and GS_2-A_2 (43 min).

The three glutathione conjugates of acetaminophen were isolated by semipreparative HPLC using a 10-μm Waters μBondapak-C_{18} column (0.78 × 30 cm). Synthetic GS-A was purified using isocratic conditions of 100% solvent A pumped at 3 ml/min. The retention time of GS-A was approximately 12 min. The two other GS-A conjugates of acetaminophen were isolated from preparative incubation mixtures using the same gradient system used for analytical analysis except that the flow rate was 3 ml/min. The other conjugates had retention times of approximately 30 and 38 min.

NMR spectroscopy. Proton NMR spectra were obtained using a Bruker ^{1}H WM-500 NMR spectrometer. Spectra were obtained at room temperature from samples in deuterated water using tetramethylsilane as an external standard.

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 (25) and 89.5 × 10^3 M^{-1} cm^{-1} at 403 nm for horseradish peroxidase (26).

Results

Horseradish peroxidase and H_2O_2 have been previously shown to catalyze the polymerization of acetaminophen. These products, shown in Fig. 1, were isolated by HPLC and identified by a combination of 500-MHz ^{1}H NMR spectroscopy and mass spectrometry (23).

Using the same analytical HPLC system previously developed to separate and quantitate the various polymers, we have examined acetaminophen oxidation by horseradish peroxidase when GSH was included in the reaction mixtures. An HPLC chromatogram of the horseradish peroxidase-mediated products is shown in Fig. 2 (*upper pannel*). Acetaminophen is shown as compound *A*. The acetaminophen polymerization products (23) are labeled according to the type of polymer formed. Compound *B* had a retention time which was identical to that of synthetic GS-A and compound *C* was an unknown product which was formed only in the presence of glutathione. The effect of glutathione concentration on the formation of glutathione conjugates and acetaminophen polymers is shown in Table 1. Formation of GS-A (compound B) and compound C was relatively low in comparison to acetaminophen polymerization.

Identification of GSH-dependent metabolites. When

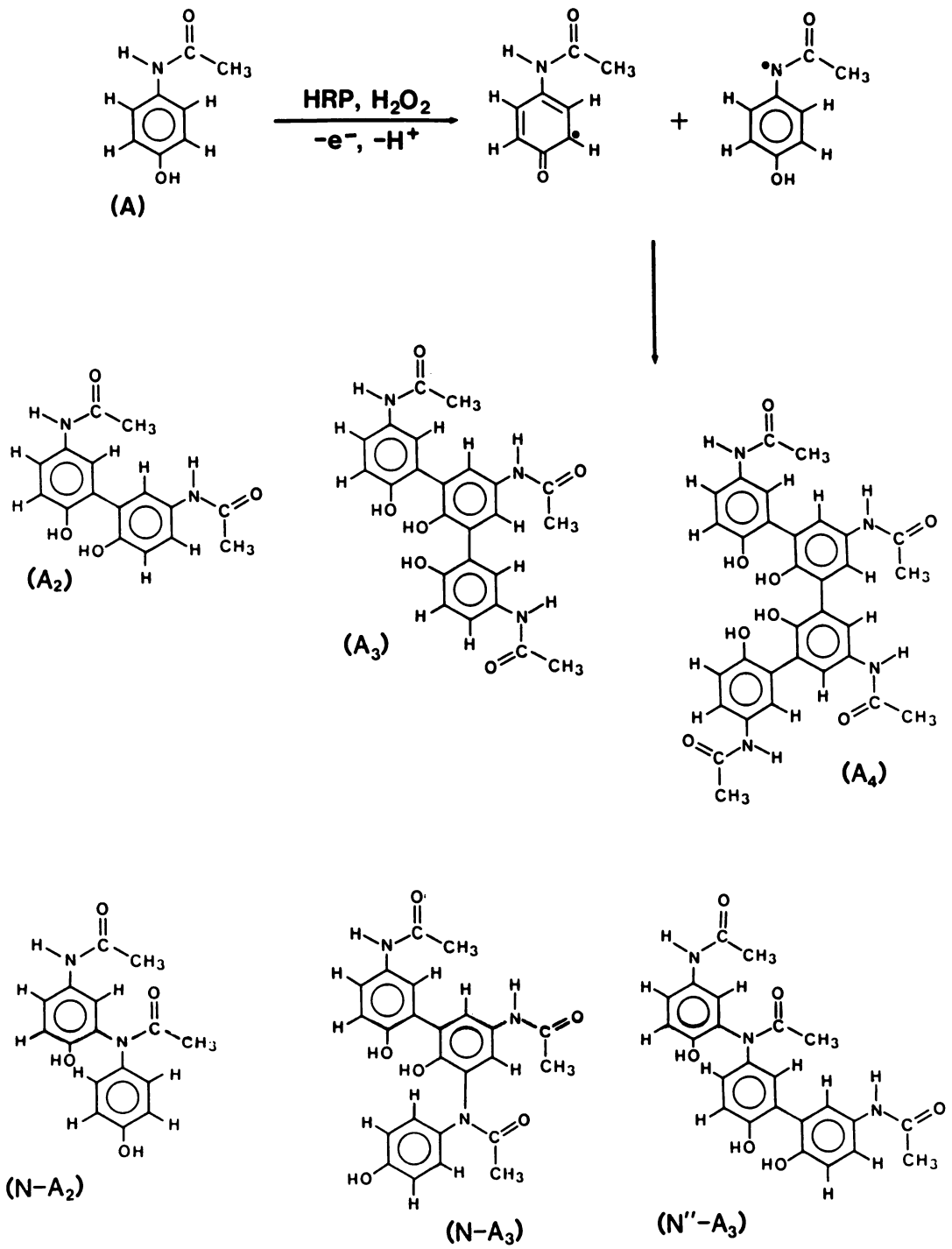

Fig. 1. Schematic representation of acetaminophen (*A*) polymerization catalyzed by horseradish peroxidase (*HRP*) and H_2O_2. The polymers were previously identified (23) as acetaminophen dimer (A_2), acetaminophen trimer (A_3), *N*-acetaminophen dimer (*N*-A_2), *N''*-acetaminophen trimer (*N''*-A_3), acetaminophen tetramer (A_4), and *N*-acetaminophen trimer (*N*-A_3).

horseradish peroxidase was incubated with [^{3}H]GSH and [^{14}C] acetaminophen both compounds B and C contained the two radiolabels, thus indicating that both compounds B and C were GSH-acetaminophen conjugates.

Since horseradish peroxidase catalyzed the conversion of acetaminophen to numerous acetaminophen polymers, it was considered that once GS-A was formed, it too could be oxidized and the oxidation product could polymerize. In reaction mixtures which contained [^{14}C]acetaminophen, GS-A, horseradish peroxidase, and H_2O_2, without GSH, compound C was formed (Fig. 2, *lower panel*). In addition, another peak was observed by HPLC with a retention time of 36.7 min (compound D). Radioanalysis demonstrated that [^{14}C]acetaminophen was incorporated into compound C, but not into compound D (data not shown). Under these conditions, acetaminophen polymerization was minor.

Large-scale incubations were performed similar to those described in Fig. 2 (*lower panel*), and compounds C and D were

isolated by semipreparative HPLC. The 500-MHz ^{1}H NMR spectral data of compounds C and D are shown in Table 2 along with spectral data of synthetic GS-A (compound B). Aromatic structures for the three GSH-acetaminophen conjugates are shown in Fig. 3. The ^{1}H NMR spectra of the glutathionyl moiety of compounds C and D were nearly identical to the glutathionyl moiety of GS-A with the respective proton resonances being within 0.1 ppm of one another. Integration of proton resonances revealed that equimolar amounts of acetaminophen and glutathione were present in compound D and 2 mol of acetaminophen for each mole of glutathione were present in compound C. The aromatic resonances of the three compounds were, however, different. In contrast to GS-A, which had three aromatic resonances, compound C had five and compound D had two aromatic resonances.

Homonuclear decoupling experiments showed that the aromatic protons of compound C with resonances at 7.42 ppm (H_2) and 7.32 ppm (H_6) were interrelated, as were protons with resonances at 7.37 ppm ($H_{2'}$), 7.29 ppm ($H_{6'}$), and 6.96 ppm ($H_{5'}$). H_2 and H_6 were *meta* ($J_{2,6}$ = 1.7 Hz), $H_{2'}$ and $H_{6'}$ were *meta* ($J_{2',6'}$ = 2.2 Hz), and $H_{5'}$ and $H_{6'}$ were *ortho* to one another ($J_{5',6'}$ = 8.2 Hz). The doublet resonance at 6.96 ppm was assigned as $H_{5'}$ since protons *ortho* to a hydroxyl group are shifted upfield by approximately 0.5 ppm (27). ^{1}H NMR analysis of compound C was consistent with acetaminophen coupled

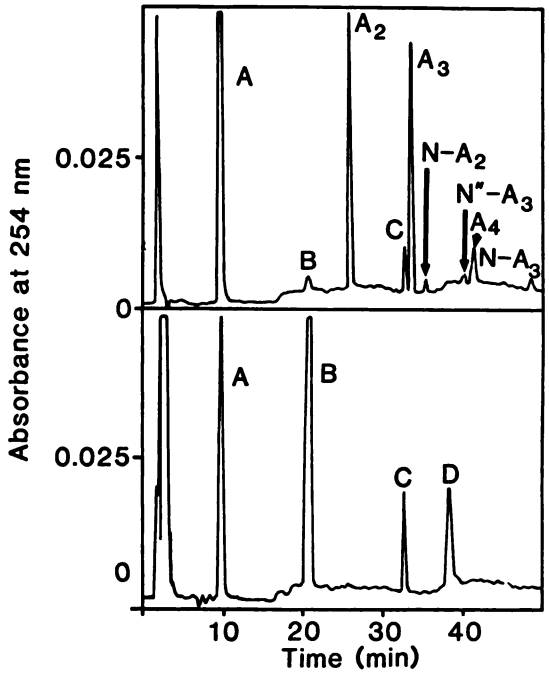

Fig. 2. Analytical HPLC chromatograms of acetaminophen metabolites catalyzed by horseradish peroxidase and H_2O_2. Chromatograms are the HPLC separations of products as detected by UV absorbance at 254 nm. The *upper* chromatogram is the HPLC separation of products from a 50-μl aliquot of a 1-ml reaction mixture containing 1.0 mM acetaminophen, 20 μM GSH, 200 μM H_2O_2, 80 nM horseradish peroxidase; and 100 mM potassium phosphate (pH 7.4). The reaction mixture was incubated for 5 min at 25° and the reaction terminated with 1 ml of ice-cold methanol:H_2O (90:10, v/v) containing 2 μmol of ascorbate. The products were previously identified (23) as acetaminophen (*A*), acetaminophen dimer (A_2), acetaminophen trimer (A_2), *N*-acetaminophen dimer (*N*-A_2), *N"*-acetaminophen trimer (*N"*-A_3), acetaminophen tetramer (A_4), and *N*-acetaminophen trimer (*N*-A_3). Compounds *B* and *C* were only formed in the presence of GSH. The *lower* chromatogram is the HPLC separation of products from a 50-μl aliquot of a 1-ml reaction mixture containing 200 μM acetaminophen, 200 μM 3-(glutathion-*S*-yl)acetaminophen (compound *B*), 70 μM H_2O_2, 80 nM horseradish peroxidase, and 100 mM potassium phosphate (pH 7.4).

TABLE 2
The 500-MHz ^{1}H NMR spectral parameters of GS-A, GS-A_2, and GS$_2$-A_2

Assignments	Chemical shift in ppm from TMS as external standard[a]		
	GS-A[b]	GS-A_2[c]	GS$_2$-A_2[d]
H_2	7.47 (d,1)	7.42 (d,1)	7.43 (d,1)
H_5	6.96 (d,1)		
H_6	7.24 (dd,1)	7.32 (d,1)	7.33 (d,1)
$H_{2'}$		7.37 (d,1)	
$H_{5'}$		6.96 (d,1)	
$H_{6'}$		7.29 (dd,1)	
N-Acetyl	2.16 (s,3)	2.17 (s,3), 2.15 (s,3)	2.18 (s,3)
Cys-α	4.48 (dd,1)	4.54 (dd,1)	4.58 (dd,1)
Glu-α	3.70 (t,1)	3.70 (t,1)	3.65-3.7 (m,3)
Gly-α,α′	3.68 (m,2)	3.68 (m,2)	3.65-3.7 (m,3)
Cys-β′	3.41 (dd,1)	3.41 (dd,1)	3.45 (dd,1)
Cys-β	3.26 (dd,1)	3.26 (dd,1)	3.29 (dd,1)
Glu-γ	2.45 (m,2)	2.45 (m,2)	2.44 (m,2)
Glu-β	2.09 (m,2)	2.09 (m,2)	2.08 (m,2)

[a] Spectra were obtained at room temperature from samples in deuterated water.
[b] 3-(Glutathion-*S*-yl)acetaminophen (GS-A, compound B) coupling constants are: $J_{A\text{-}2,6}$ = 2.6 Hz, $J_{A\text{-}5,6}$ = 8.6 Hz, $J_{cys\text{-}\alpha,\beta'}$ = 4.7 Hz, $J_{cys\text{-}\alpha,\beta}$ = 8.6 Hz, $J_{cys\text{-}\beta,\beta'}$ = 14.6 Hz, $J_{gly\text{-}\alpha,\alpha'}$ = 17.2 Hz, $J_{glu\text{-}\alpha,\beta'}$ = 6.5 Hz, $J_{glu\text{-}\beta,\gamma}$ = 7.3 Hz, $J_{glu\text{-}\beta,\alpha}$ = 3.4 Hz.
[c] 3-(Glutathion-*S*-yl)diacetaminophen (GS-A_2, compound C) coupling constants are: $J_{A\text{-}2,6}$ = 1.7 Hz, $J_{A\text{-}2',6'}$ = 2.2 Hz, $J_{A\text{-}5',6'}$ = 8.2 Hz, $J_{cys\text{-}\alpha,\beta'}$ = 4.3 Hz, $J_{cys\text{-}\alpha,\beta}$ = 8.2 Hz, $J_{cys\text{-}\alpha,\beta}$ = 8.2 Hz, $J_{cys\text{-}\beta,\beta'}$ = 14.6 Hz, $J_{gly\text{-}\alpha,\alpha'}$ = 17.2 Hz, $J_{glu\text{-}\alpha,\beta}$ = 6.5 Hz.
[d] 3-(Diglutathion-*S*-yl)diacetaminophen (GS$_2$-A_2, compound D) coupling constants are: $J_{A\text{-}2,6}$ = 2.2 Hz, $J_{cys\text{-}\alpha,\beta'}$ = 4.7 Hz, $J_{cys\text{-}\alpha,\beta}$ = 8.2 Hz, $J_{cys\text{-}\beta,\beta}$ = 14.6 Hz, $J_{gly\text{-}\alpha,\alpha}$ = 17.2 Hz.

TABLE 1
Effect of GSH concentration on acetaminophen polymerization
Reaction mixtures (1 ml) contained 1.0 mM acetaminophen, 80 nM horseradish peroxidase, 100 μM H_2O_2, 0–6.0 mM GSH, and 100 mM potassium phosphate, pH 7.4. The reaction mixtures were incubated for 5 min at 25° and reactions were terminated with 1 ml of ice-cold methanol:water (90:10, v/v) containing 2 mM ascorbate.

GSH	Reaction products							
	GSH conjugates[a]		Acetaminophen polymers					
	GS-A	GS-A_2	Dimer	Trimer	*N*-Dimer	*N"*-Trimer	Tetramer	*N*-Trimer
mM	μM		μM					
0			60.9 ± 1.3	11.8 ± 0.2	3.2 ± 0.1	0.3 ± 0.1	0.9 ± 0.2	1.0 ± 0.2
0.4	2.9 ± 0.3	3.2 ± 0.2	36.6 ± 1.2	3.3 ± 0.2	1.3 ± 0.1	ND[b]	ND	ND
1.0	2.4 ± 0.0	2.4 ± 0.1	29.6 ± 0.1	2.6 ± 0.1	1.3 ± 0.1	ND	ND	ND
2.0	1.9 ± 0.6	2.0 ± 0.1	27.4 ± 1.0	2.3 ± 0.4	1.0 ± 0.1	ND	ND	ND
4.0	1.7 ± 0.0	1.3 ± 0.2	22.9 ± 0.4	1.4 ± 0.0	0.9 ± 0.1	ND	ND	ND
6.0	1.6 ± 0.1	1.2 ± 0.1	19.2 ± 0.5	1.0 ± 0.4	0.5 ± 0.2	ND	ND	ND

[a] Identification of 3-(glutathion-*S*-yl)acetaminophen (GS-A) and 3-(glutathion-*S*-yl)diacetaminophen is shown in Table 2.
[b] ND, not detected. The limit of detection was judged to be 0.1 μM.

ortho to the hydroxyl group of GS-A. In accordance with IUPAC nomenclature, compound C was named *N*-[(1R)-1-[(carboxymethyl)carbamoyl]-2-[(5,5′-diacetamido-2,2′-hydroxy-3-biphenylyl)thio]ethyl]-L-glutamine which for convenience was called 3-(glutathion-*S*-yl)diacetaminophen (GS-A_2).

Homonuclear decoupling experiments showed that the aromatic protons of compound D with resonances at 7.43 ppm (H_2) and 7.33 ppm (H_6) were interrelated and *meta* to one another ($J_{2,6}$ = 2.2 Hz). Since only downfield resonances were observed, compound D was consistent with two molecules of GS-A coupled *ortho* to the hydroxyl groups. Compound D was named *N,N*′-[(5,5′-diacetamido-2,2′-dihydroxy-3,3′-biphenylylene)bis[thio[(1*R*)-1-[(carboxymethyl)carbamoyl]ethylene]]]di-L-glutamine and was given the trivial name 3-(diglutathion-*S*-yl)diacetaminophen (GS_2-A_2).

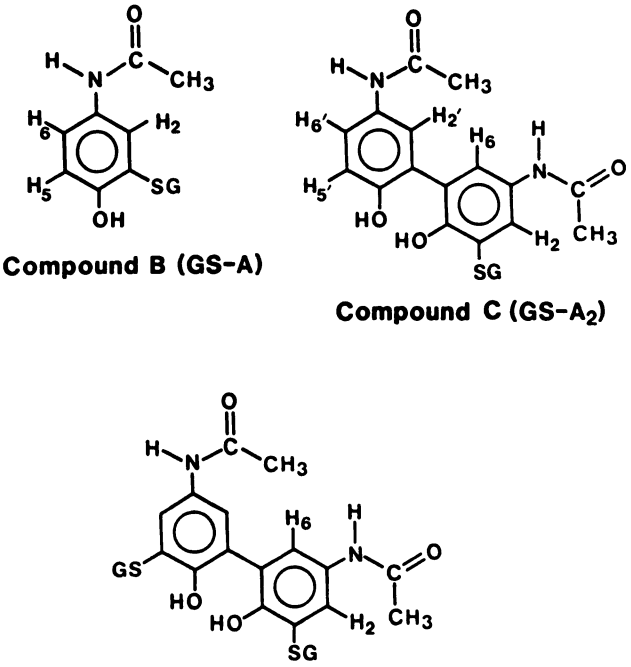

Fig. 3. Structures of 3-(glutathion-*S*-yl)acetaminophen (GS-A, compound B), 3-(glutathion-*S*-yl)diacetaminophen (GS-A_2, compound C), and 3-(diglutathion-*S*-yl)diacetaminophen (GS_2-A_2, compound D). The GS is the glutathionyl moiety of the conjugate. The numbering of aromatic protons corresponds to the numbering system used in Table 2.

UV spectra of acetaminophen and the three GSH-acetaminophen conjugates are shown in Fig. 4. The spectra were obtained with a Hewlett-Packard 1040A spectrophotometer. Acetaminophen has a relative absorbance maximum at 244 nm. The glutathione conjugates also absorb light in this region; however, all three conjugates have relative absorbance maxima around 210 nm, which is presumably absorbance due to the peptide bond.

Conditions for 3-(glutathion-*S*-yl)acetaminophen polymerization. When horseradish peroxidase was incubated with GS-A, minor amounts of GS_2-A_2 were detected (Fig. 5). These data indicated that GS-A was a poor substrate for horseradish peroxidase. However, addition of acetaminophen to the incubation mixture resulted primarily in the formation GS-A_2 and GS_2-A_2. Below 300 μM acetaminophen, GS_2-A_2 was the major product; above 300 μM acetaminophen, the formation of GS-A_2 was the major product. Acetaminophen polymerization was a minor reaction. At equimolar concentrations of acetaminophen and GS-A less than 5% of the reaction products was the acetaminophen dimer, whereas greater than 95% of the reaction products were GSH-acetaminophen polymers. Even at 10 mM acetaminophen, approximately 66% of the reaction products were either GS-A_2 and GS_2-A_2, whereas 34% were identified as acetaminophen polymers. In contrast, six acetaminophen polymerization products were observed when GS-A was not included in reaction mixtures. However, acetaminophen polymerization was decreased with increasing amounts of GS-A (Fig. 6). GS-A_2 was the major product at concentrations of less than 100 μM GS-A and GS_2-A_2 was the major polymerization product at higher concentrations of GS-A. As the concentration of GS-A increased, the formation of acetaminophen polymers decreased (Fig. 6).

Table 3 shows the stoichiometry of H_2O_2 consumption to product formation in reaction mixtures containing horseradish peroxidase and equimolar amounts of acetaminophen and GS-A. Mixtures were reacted for 30 min and product analyses demonstrated that approximately 1 mol of H_2O_2 was consumed per mole of polymer formed.

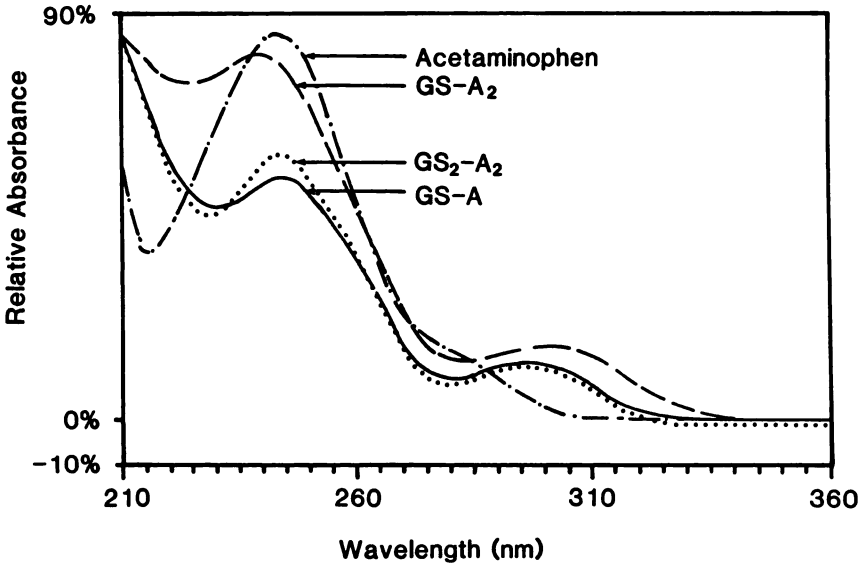

Fig. 4. UV spectra of acetaminophen and GSH-acetaminophen conjugates. The compounds were separated by analytical HPLC and spectra were obtained with a Hewlett-Packard model 1040A spectrophotometer as described in Materials and Methods.

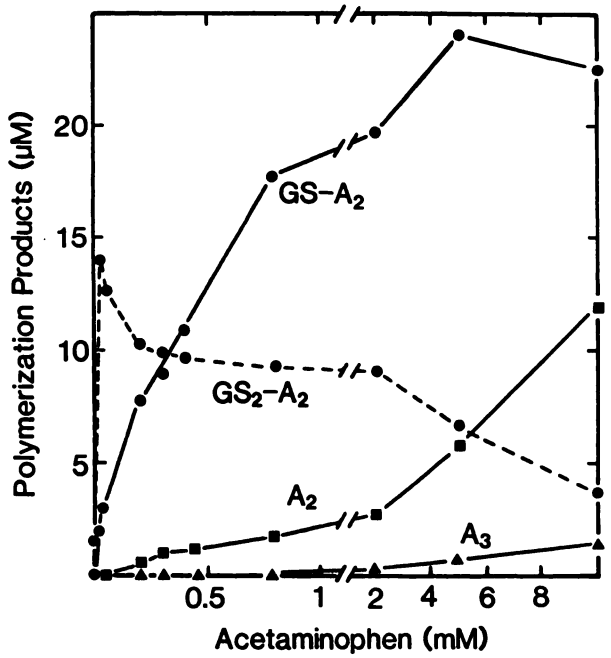

Fig. 5. Effect of acetaminophen concentration on horseradish peroxidase-catalyzed formation of acetaminophen metabolites in the presence of GS-A. Reactions were the same as those described in the legend to Fig. 2 except that all samples contained 200 μM GS-A and 0–10 mM acetaminophen. The detected products were GS-A_2, GS_2-A_2, acetaminophen dimer, and acetaminophen trimer.

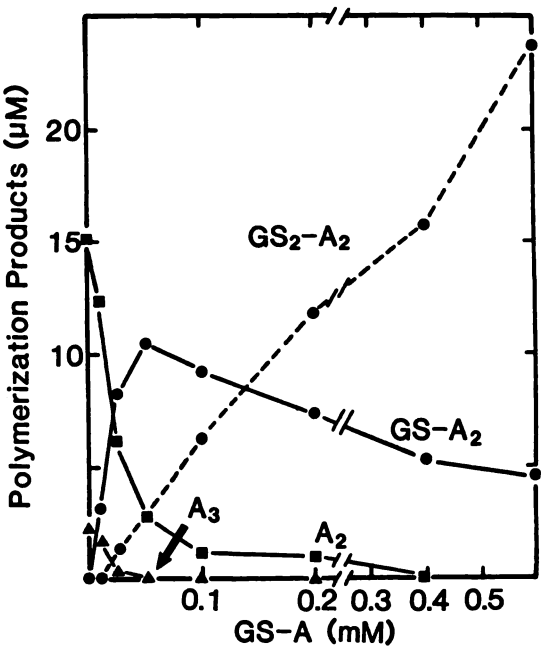

Fig. 6. Effect of GS-A concentration on horseradish peroxidase-catalyzed formation of acetaminophen metabolites in the presence of acetaminophen. Reaction mixtures of 1 ml contained 0–600 μM GS-A, 200 μM acetaminophen, 70 μM H_2O_2, 80 nM horseradish peroxidase, and 100 mM potassium phosphate (pH 7.4). Samples were incubated for 5 min at 25° and analyzed by analytical HPLC as previously described in the legend to Fig. 2. The detected products were GS-A_2, GS_2-A_2, acetaminophen dimer, and acetaminophen trimer. Four other acetaminophen polymers were detected when GS-A was not included in reaction mixtures (data not shown).

Discussion

Glutathione, a tripeptide normally maintained at intracellular concentrations of 1–12 mM (28), has numerous roles in the biological system, including protection against cellular damage caused by free radical and electrophilic intermediates. For example, *in vivo* acetaminophen is metabolized to an intermediate that reacts with GSH to yield GS-A (12). This reaction is generally considered to be a detoxification event and the conjugate to be chemically inert.

In this study, we have examined the reactions of GSH with the acetaminophen oxidative intermediates generated by horseradish peroxidase and H_2O_2. GSH had two effects: a concentration-dependent decrease in acetaminophen polymerization and the formation of small amounts of GSH-acetaminophen conjugates. GSH did not decrease polymerization as effectively as ascorbate (23). We previously found that 400 μM ascorbate decreased polymerization greater than 98% (23). In contrast, 400 μM GSH decreased polymerization only by about 50% and 6 mM GSH decreased polymerization by 74%. Preliminary data indicate that GSH decreased polymerization by reacting with NAPSQI to form oxidized glutathione and acetaminophen.

The mechanism of GSH-acetaminophen conjugation with the horseradish peroxidase system is still unknown; however, three mechanisms seem possible: NAPSQI could react with a glutathionyl radical to form a conjugate, NAPSQI might disproportionate to form NAPQI and in turn form GS-A, or horseradish peroxidase could directly catalyze the formation of a small amount of NAPQI. A disproportionation mechanism may be the more plausible route for GSH-acetaminophen conjugation since similar compounds like *p*-benzosemiquinone (21) and 3′,4′-dimethyl-*N*-acetyl-*p*-benzosemiquinone imine (22) disproportionate. However, the importance of each mechanism in conjugate formation will require further investigation.

TABLE 3
Stoichiometry of H_2O_2 consumption to product formation
Reaction mixtures contained equimolar concentrations of acetaminophen and GS-A. Assay conditions were the same as those described in the legend to Fig. 4 with 80 nM horseradish peroxidase, 200 μM acetaminophen, and 200 μM GS-A. Samples were incubated for 30 min to ensure completion of reactions.

H_2O_2	Reaction products			Total	Products/H_2O_2
	A_2	GS-A_2	GS_2-A_2		
μM		μM		μM	
10	ND*	6.0	4.0	10.0	1.0
20	ND	11.3	13.3	24.6	1.2
40	ND	22.9	21.0	43.9	1.1
60	1.4	28.4	26.9	56.7	0.9
80	3.0	34.9	36.1	74.0	0.9
					$\bar{x} = 1.0 \pm 0.1$

* ND, not detected. The limit of detection was judged to be 0.1 μM.

Our present studies indicate that GS-A may not be inert as commonly assumed but may be easily oxidized to a free radical intermediate. In reaction mixtures containing GSH, horseradish peroxidase catalyzed acetaminophen polymerization and the formation of minor amounts of GS-A and GS-A_2 (Table 1). Formation of the latter conjugate suggested the involvement of the GS-A free radical intermediate, 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine. Subsequent experiments demonstrated that GS-A was slowly converted to GS_2-A_2 (Fig. 5). When reaction mixtures contained equimolar amounts of acetaminophen and GS-A, 95% of the reaction products were GS-A_2 and GS_2-A_2. Thus, the polymerization products involving GS-A were predominant.

The GSH-acetaminophen conjugates (GS-A, GS-A_2, GS_2-A_2)

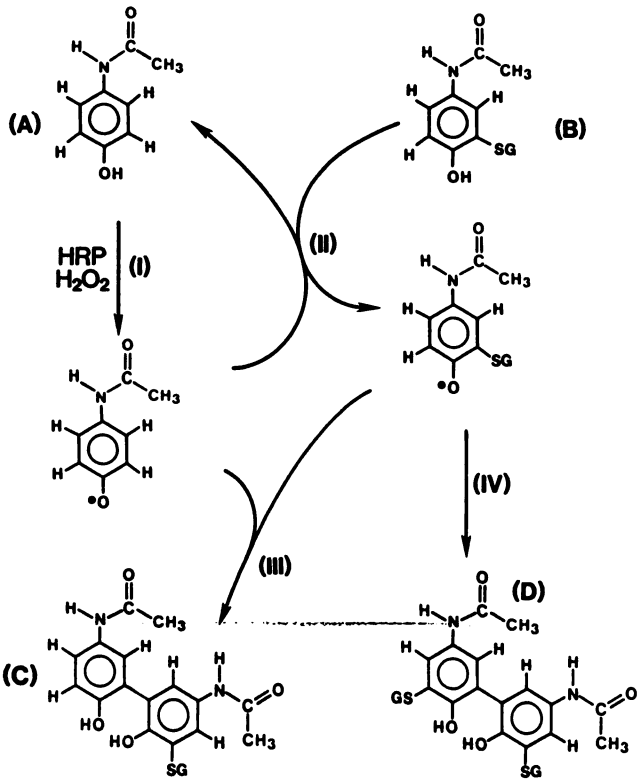

Fig. 7. Schematic representation of the proposed mechanism for GS-A polymerization. Reaction *I* represents the one-electron oxidation of acetaminophen (*A*) to NAPSQI catalyzed by horseradish peroxidase (*HRP*) and H_2O_2. Reaction *II* represents nonenzymatic reaction of NAPSQI and GS-A (*B*) to yield acetaminophen and 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine. Reaction *III* is the free radical termination reaction between NAPSQI and 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine to yield GS-A_2 (*C*). Reaction *IV* represents the free radical termination reaction between two 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine to yield GS_2-A_2 (*D*).

were identified by 500-MHz ^{1}H NMR. Unpublished results[2] have confirmed these identifications. Fast atom bombardment mass spectrometry results have demonstrated that GS-A has a molecular ion + 1 of 457 *m/z*, GS-A_2 has a molecular ion + 1 of 606 *m/z*, and GS-A_2 has a molecular ion + 1 of 911 *m/z*.

The schematic representation of GS-A polymerization, shown in Fig. 7, has been constructed from the data presented under Results. Since acetaminophen was rapidly and GS-A was slowly oxidized by horseradish peroxidase, the free radical initiation step apparently is the formation of NAPSQI. The horseradish peroxidase-mediated formation of the phenoxyl free radical of acetaminophen has been observed by ESR spectroscopy (24). In reaction mixtures without GS-A, the free radical of acetaminophen undergoes a coupling reaction (radical termination) to produce acetaminophen polymers as shown in Fig. 1. In reaction mixtures containing GS-A, NAPSQI nonenzymatically oxidizes GS-A to give acetaminophen and 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine (step II). Although the free radical of GS-A has not been examined by ESR spectroscopy, if the phenoxyl free radical of GS-A is formed it should be in resonance with the carbon *ortho* to the oxygen substituent since this is the site of polymerization. Once the GS-A free radical is formed, it can then couple with either NAPSQI (step III), to yield GS-A_2 (product C), or GS-A free radical (step IV), to yield GS_2-A_2. These products may result from the direct coupling of carbon-centered radicals *ortho* to oxygen substitution, followed by rearomatization.

The relevance of NAPSQI as a biological reactive intermediate remains unknown. There are mammalian enzymes, such as prostaglandin H synthase, that might catalyze the *in vivo* oxidation of acetaminophen to NAPSQI. However, to our knowledge, neither acetaminophen polymers nor GSH-acetaminophen polymers have been detected as *in vivo* metabolites. Further analyses are needed to determine the significance of these metabolites *in vivo*.

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