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Reactions of *N*-Acetyl-*p*-benzoquinone Imine with Reduced Glutathione, Acetaminophen, and NADPH

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SUMMARY

Synthetic *N*-acetyl-*p*-benzoquinone imine reacted with reduced glutathione (GSH), [^{14}C]acetaminophen, and NADPH. It reacted rapidly with GSH to yield acetaminophen (33%) and 3-(glutathion-*S*-yl)acetaminophen (67%), and with acetaminophen or NADPH to yield acetaminophen polymers. The data suggested that *N*-acetyl-*p*-benzoquinone imine was reduced by GSH to form acetaminophen but primarily reacted with GSH to form 3-(glutathion-*S*-yl)acetaminophen. The evidence further suggested that *N*-acetyl-*p*-benzoquinone imine comproportionated with [^{14}C]acetaminophen to yield a mixture of radioactive and nonradioactive *N*-acetyl-*p*-benzosemiquinone imine which subsequently formed acetaminophen polymers by a radical coupling reaction. [^{14}C]Acetaminophen was incorporated into the acetaminophen polymers. The amount of ^{14}C incorporation was dependent on the initial concentration of [^{14}C]acetaminophen and *N*-acetyl-*p*-benzoquinone imine. An increase in the ratio of [^{14}C]acetaminophen to *N*-acetyl-*p*-benzoquinone imine resulted in an increase in [^{14}C]acetaminophen incorporation into the acetaminophen polymers. NADPH reduced *N*-acetyl-*p*-benzoquinone imine to acetaminophen and acetaminophen polymers were formed. When [^{14}C]*N*-acetyl-*p*-benzoquinone imine was incubated without acetaminophen, only minor amounts of acetaminophen polymerization were observed.

The two-electron oxidation product of acetaminophen (4′-hydroxy-acetanilide) is NAPQI. This product may be a reactive intermediate responsible for the hepatic necrosis caused by acetaminophen (1, 2). NAPQI has been formed by electrochemical techniques (3), by dehydration of *N*-hydroxyacetaminophen (4–6), or by oxidation with silver oxide (7, 8). Synthetic NAPQI reacts with GSH to form 3-(glutathion-*S*-yl)acetaminophen and acetaminophen, covalently binds to protein, and is reportedly toxic to hepatocytes and mice (8).

The two-electron oxidation of acetaminophen mediated by NADPH and the cytochrome P-450 mixed-function oxidase system has been suggested (1, 2) but remains unproven. The principal reason for this proposal is that 3-(glutathion-*S*-yl)acetaminophen is a product when acetaminophen, GSH, and NADPH are incubated with hepatic microsomes (9, 10). However, Miner and Kissinger (3) were unable to detect microsome-dependent formation of NAPQI using electrochemical detection. Similarly, Dahlin *et al.* (11) were unable to show the formation of NAPQI with a purified cytochrome P-450 system using NADPH as an electron donor. Dahlin *et al.* (11) suggest that this may be due to the rapid reduction of NAPQI by NADPH, since they were able to detect NAPQI formation with purified cytochrome P-450 using cumene hydroperoxide as an oxidizing agent.

Other investigators suggest that the one-electron oxidation product of acetaminophen, NAPSQI, is a reactive intermediate that binds to GSH and protein (12). It has been suggested that NAPSQI could be a cytochrome P-450-dependent (12) as well as a prostaglandin H synthase-dependent intermediate (13–15).

In the *in vitro* enzyme reaction systems described above, nonenzymatic reactions of NAPQI with other components of the reaction mixture could complicate the interpretation of data relating to the enzymatic reaction mechanism. For example, it is still unclear whether the acetaminophen free radical is formed during the metabolism of acetaminophen by either cytochrome P-450 or prostaglandin H synthase. Nor is it clear

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ABBREVIATIONS: NAPQI, *N*-acetyl-*p*-benzoquinone imine; NAPSQI, *N*-acetyl-*p*-benzosemiquinone imine; A or acetaminophen, 4′-hydroxyacetanilide; acetaminophen-d_3, *N*[$^2H_3$]acetyl-4′-hydroxyaniline; 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⁗′-quteracetanilide; 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]-L-glutamine; Me_2SO, dimethyl sulfoxide; HPLC, high performance liquid chromatography.

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whether the radical of acetaminophen reacts with GSH to give the GSH-acetaminophen conjugates. We have previously examined the horseradish peroxidase-dependent metabolism of acetaminophen to NAPSQI which subsequently reacted to form acetaminophen polymers. Identification of these polymers from reaction mixtures may be an important method for determining formation of the free radical intermediates. Furthermore, we hope to use these studies as a basis to examine further the mechanism of other enzymes that oxidize acetaminophen. However, to understand the enzymatic reactions, it seems that an understanding of the nonenzymatic reaction mechanisms of the various acetaminophen oxidation products is needed. Therefore, we have examined the different types of reaction products formed when NAPQI reacts with GSH, acetaminophen, and NADPH. The evidence presented in this paper suggests that NAPQI reacted with GSH to form 3-(glutathion-*S*-yl)acetaminophen (GS-A) and acetaminophen, reacted with acetaminophen by comproportionation to form NAPSQI which subsequently formed acetaminophen polymers, and reacted with NADPH to form acetaminophen and acetaminophen polymers.

Materials and Methods

[phenyl-U-^{14}C]Acetaminophen (1.65 mCi/mmol) was synthesized by Dr. Robert W. Roth of Midwest Research Institute, Kansas City, MO. [^{14}C]Acetaminophen was purified by thin layer chromatography and HPLC to >99% (16). *N*-[$^{2}H_3$]Acetyl-4′-hydroxyaniline was a gift from Dr. Lance R. Pohl and its synthesis has been described previously (17). Horseradish peroxidase (EC 1·11·1·7), type VI, acetaminophen, 30% H_2O_2, GSH, and ascorbic acid were purchased from Sigma Chemical Co., St. Louis, MO. Silver oxide (I) was purchased from Aldrich Chemical Co., Milwaukee, WI.

NAPQI was synthesized by a modification of the method of Dahlin and Nelson (8). Acetaminophen was oxidized by silver oxide (I) in redistilled chloroform. The solution containing NAPQI was filtered and applied to a Waters Associates, Inc. (Milford, MA) silica Sep-Pak. NAPQI was eluted with chloroform, collected, and applied to another Waters silica Sep-Pak. NAPQI was subsequently eluted with anhydrous diethyl ether. Dry Me_2SO (400 μl) was added to the ether and the ether was evaporated under pressure leaving the NAPQI in Me_2SO. HPLC analyses indicated the purity of the various preparations to be between 94 and 97%. Acetaminophen was the only impurity detected. NAPQI in Me_2SO was stored in liquid nitrogen and was stable for at least 1 month.

Extinction coefficients. [^{14}C]Acetaminophen polymers were purified from a 50-ml incubation mixture containing 100 mM potassium phosphate (pH 7.4), 100 nM horseradish peroxidase, 2.0 mM [^{14}C] acetaminophen, and 200 μM H_2O_2. Reactions were initiated with H_2O_2 and incubated at 25° for 30 min. The mixture was evaporated under reduced pressure and the [^{14}C]acetaminophen polymers were extracted with 10 ml of methanol. Individual polymers were purified by semipreparative reverse phase HPLC as previously described (16). The HPLC system consisted of two model 6000 HPLC pumps from Waters Associates, a Tracor variable wavelength detector (Tracor, Inc., Austin, TX) set at 254 nm, and a 10-μm Waters μBondapak-C_{18} column (0.78 × 30 cm). The solvent system used for separation of polymers consisted of 67.9% water, 30% methanol, 2% glacial acetic acid, and 0.1% ethyl acetate (v/v). The flow rate was 3 ml/min. The ^{14}C-polymers were isolated and dried under reduced pressure. Purity of the ^{14}C-polymers was estimated to be >98%.

Extinction coefficients of ^{14}C-polymers were determined in both methanol and 100 mM potassium phosphate buffer (pH 7.4) with a Cary 219 spectrophotometer from Varian Associates, Inc. (Palo Alto, CA). An absorbance spectrum for each compound was obtained and the concentration of ^{14}C-polymer was determined by removing aliquots from the cuvette and determining the radioactivity in Scintisol (Isolabs, Inc., Akron, OH) using a Searle Analytic (Des Plaines, IL) Mark III scintillation counter.

Reaction procedure. Reaction mixtures of 1 ml contained 100 mM potassium phosphate (pH 7.4) and various amounts of GSH, acetaminophen, or NAPQI. Specific details of reaction mixtures' contents are given in the legends to figures and tables. In general, samples were preincubated for 2 min at 25° in a Dubnoff metabolic shaking incubator. In mixtures containing NAPQI, reactions were initiated by adding 3–10 μl of Me_2SO containing NAPQI. All experiments were performed on at least two separate occasions.

Liquid chromatography. Acetaminophen polymers resulting from the reactions of [^{14}C]acetaminophen and NAPQI were routinely analyzed by an HPLC system equipped to monitor UV absorbance and radioactive isotopes. 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, a model 727 automatic injector from Micromeritics Instrument Corp. (Norcross, GA), a model 3390A reporting integrator from Hewlett-Packard (Palo Alto, CA), a FLO-ONE HS radioactive flow detector from Radiomatic Instruments and Chemical Co., Inc. (Tampa, FL), and a linear recorder from Linear Inc. (Irvin, CA). The acetaminophen polymers were separated on a 5-μm C_{18} Ultrasphere ODS reverse phase liquid chromatography column (4.6 × 250 mm) from Altex (Berkeley, CA) using a binary gradient system with a flow rate of 1.0 ml/min. Solvent A consisted of 87.9% water, 10% methanol, 2% glacial acetic acid, and 0.1% ethyl acetate. After the HPLC column was equilibrated with 100% solvent A, samples were automatically injected. Solvent A was maintained at 100% for 10 min, followed by a linear gradient to 81% A and 19% methanol in 15 min, and isocratic conditions were maintained for an additional 20 min. Finally, 100% methanol was obtained by a linear gradient for 10 min. The detection limits of the HPLC system were judged to be about 0.1 μM based on results observed with acetaminophen standards. Isotope specific activity was determined with the FLO-ONE radioactive flow detector using FLO-Scint III pumped at 5.0 ml/min. [^{14}C]NAPQI formation was determined by HPLC (11) using an isocratic solvent system which consisted of 20% methanol and 80% 10 mM potassium phosphate (pH 7.4).

Kinetic determinations. The oxidation of NADPH by NAPQI was quantitated using a Cary 219 spectrophotometer at 340 nm using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Second order rate constants were determined from the least squares slope of the ln NADPH/NAPQI or the ln [^{14}C]acetaminophen/NAPQI versus time.

Identification of acetaminophen polymers. Standards of acetaminophen polymers were obtained by incubating acetaminophen, horseradish peroxidase, and H_2O_2 as previously described (16). These standards have been identified by 500–MHz ^{1}H NMR spectroscopy and mass spectrometry (16).

The analytical HPLC system, described above, was equipped with a Hewlett-Packard (Palo Alto, CA) model 1040A spectrophotometric detector for UV analyses of acetaminophen polymers. The HPLC solvent system was modified. The HPLC column was equilibrated with 90% 10 mM acetic acid and 10% methanol. After the sample was injected, 90% 10 mM acetic acid and 10% methanol was maintained for 10 min, followed by a linear gradient to give 71% 10 mM acetic acid and 29% methanol in 15 min. With this HPLC system acetaminophen had a retention time of 8.7 in, and the retention times of the standard acetaminophen polymers (Fig. 1) were: acetaminophen dimer (A_2) (25.4 min), acetaminophen trimer (A_3) (31.9 min), *N*-acetaminophen dimer (N-A_2) (32.7 min), *N″*-acetaminophen trimer (N″-A_3) (37.7 min), acetaminophen tetramer (A_4) (39.1 min), and *N*-acetaminophen trimer (N-A_3) (44.4 min). UV spectra were obtained by analyzing the polymers as they eluted from the column. These spectra were compared with spectra of products generated from reactions of acetaminophen and NAPQI.

Mass spectrometry. Electron impact mass spectrometry was used to determine the amount of deuterium incorporated into the acetamino-

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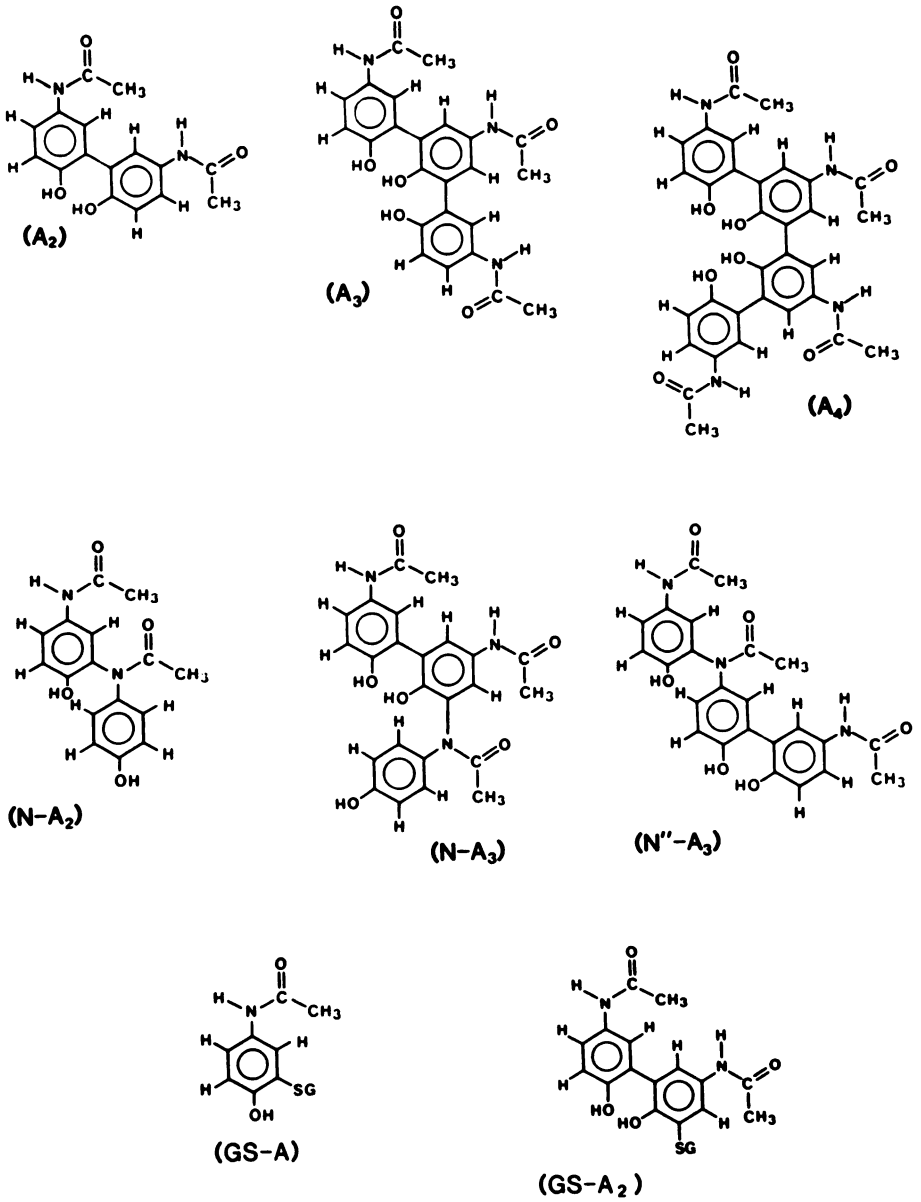

Fig. 1. Structures of acetaminophen polymers and GSH-acetaminophen conjugates. The compounds have been previously identified (16, 17) as acetaminophen dimer (A_2), acetaminophen trimer (A_3), acetaminophen tetramer (A_4), *N*-acetaminophen dimer (N-A_2), *N''*-acetaminophen trimer (N-A_3), *N*-acetaminophen trimer (N-A_3), 3-(glutathion-*S*-yl)acetaminophen (*GS-A*) and 3-(glutathion-*S*-yl)diacetaminophen (*GS-A*$_2$).

phen dimer during the reaction of NAPQI with acetaminophen-d_3. The acetaminophen dimer was purified by semipreparative HPLC, dried under reduced pressure, and redissolved in methanol (1 μg/μl). Approximately 1 μg of the acetaminophen dimer was introduced into the source of a Kratos MS-50 mass spectrometer by thermal vaporization from a glass tip probe. The ion source was 200°. Mass spectra were obtained at an electron beam energy of 70 eV. Multiple scans over about 8 atomic mass units (in 3 sec) were obtained in the selected ion monitoring mode with an oscillographic recorder. Mass resolution was 10,000.

Results

Identification of reaction products. The reaction of NAPQI with GSH to give acetaminophen and GS-A [3-(glutathion-*S*-yl)acetaminophen] has been reported previously (7, 11, 18). Analyses presented here show that formation of these products was dependent on the initial concentrations of GSH and NAPQI (Fig. 2). NAPQI rapidly reacted with excess GSH to form acetaminophen as the minor product (33%) and 3-(glutathion-*S*-yl)acetaminophen as the major product (67%). When concentrations of GSH were lower than that of NAPQI, proportionally less 3-(glutathion-*S*-yl)acetaminophen was formed. Acetaminophen was the major product at low concentrations of GSH. This result was due to the addition of ascorbic acid to reaction mixtures 30 sec after the reaction was initiated. Albano *et al.* (19) have recently published similar results showing that NAPQI in the presence of excess GSH formed 3-(glutathion-*S*-yl)acetaminophen and acetaminophen in a ratio of about 3:2. At 40 μM GSH, 0.45 μM acetaminophen dimer, and 5.2 μM 3-(glutathion-*S*-yl)diacetaminophen (GS-A_2) were also detected.

HPLC analyses demonstrated that numerous products were formed when NAPQI reacted with acetaminophen. Fig. 3 (*bot-*

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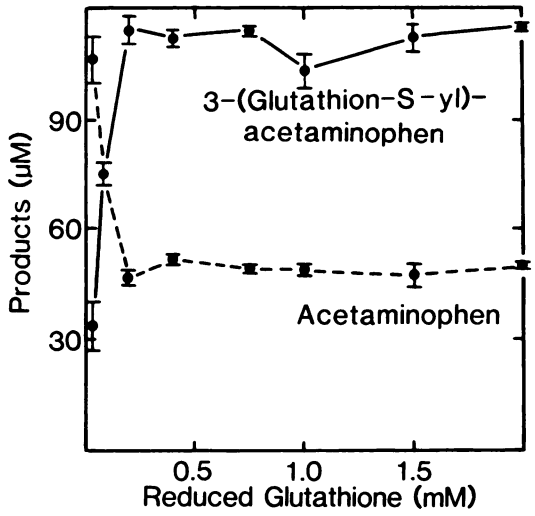

Fig. 2. Reactions of NAPQI with various concentrations of GSH to form acetaminophen and GS-A. Reaction mixtures (1 ml) contained 100 mM potassium phosphate buffer (pH 7.4), 0.04–2.0 mM GSH, and 190 μM NAPQI. Reactions were initiated by adding NAPQI in 5 μl of Me_2SO. Reaction mixtures were incubated for 30 sec and then reactions were terminated with a 1-ml methanol:water solution containing 2 mM ascorbate. Formation of acetaminophen and GS-A was analyzed by HPLC as described in Materials and Methods. Similar results were also observed when NAPQI dissolved in diethyl ether or chloroform instead of Me_2SO was added to buffer containing GSH.

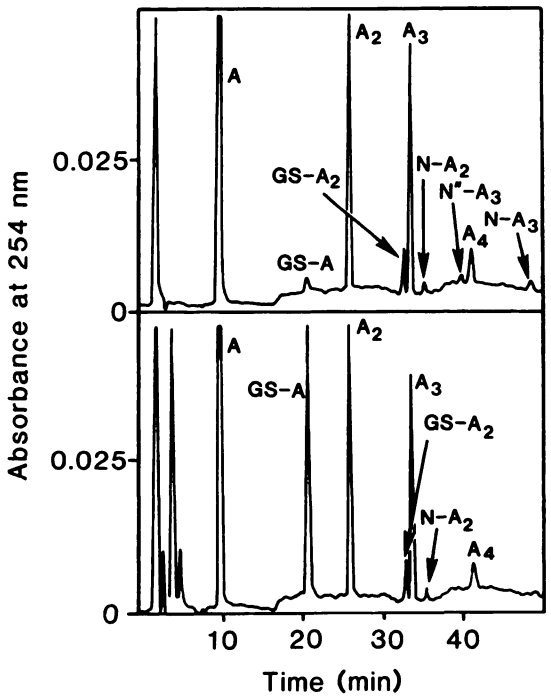

Fig. 3. HPLC profile of acetaminophen reaction products. The *upper panel* shows acetaminophen polymers from a 1-ml incubation mixture containing 100 mM potassium phosphate (pH 7.4), 1.0 mM acetaminophen, 80 nM horseradish peroxidase, 20 μM GSH, and 200 μM H_2O_2. The *lower panel* shows reaction products from a 1-ml incubation mixture containing 200 μM acetaminophen, 200 μM NAPQI, and 100 μM potassium phosphate buffer. Reactions were initiated with NAPQI in 7 μl of Me_2SO and incubated at 25° for 5 min before terminating the reaction by adding GSH (2.0 mM). Chromatography conditions are described in Materials and Methods.

tom) shows an HPLC chromatogram of these products after equimolar concentrations of acetaminophen and NAPQI were reacted. The reaction was terminated after 5 min by addition of 2 mM GSH. These products had retention times similar to those of the horseradish peroxidase-mediated acetaminophen polymerization products that have been identified previously [Fig. 3, *top*, (16)].

Comparison of HPLC profiles strongly suggested that the products resulting from reactions of acetaminophen with NAPQI were the same acetaminophen polymers that formed during horseradish peroxidase-mediated reactions. UV spectroscopy was employed for further confirmation. The UV spectra (obtained with a Hewlett-Packard model 1040A spectrophotometric detector) of compounds with the same retention times were compared (data not shown). Compounds with similar retention times had identical UV spectra. Collectively, the HPLC retention times and the UV spectral data demonstrated that acetaminophen reacted with NAPQI to form the same acetaminophen polymers that were formed when acetaminophen was catalyzed by horseradish peroxidase.

Acetaminophen polymer extinction coefficients are listed in Table 1. UV absorbance maxima and extinction coefficients were determined on a Cary 219 spectrophotometer at room temperature. The K-band maxima were greater in methanol than in 100 mM potassium phosphate buffer, pH 7.4 (Table 1). The acetaminophen polymers which were coupled via the nitrogen to carbon bonds had K-band maxima at slightly longer wavelength than did the polymers which were coupled via the carbon to carbon bonds.

NAPQI reactions in buffer and with acetaminophen. The stability of NAPQI in buffer was examined by incubating [^{14}C]NAPQI in potassium phosphate buffer for 0–60 min (Table 2). At various time points, 2.0 mM GSH was added to the mixtures to terminate the reactions and to derivatize the remaining NAPQI to form the stable GSH conjugate. Products

TABLE 1
Extinction coefficients of [^{14}C]acetaminophen polymers

Polymer	Wavelength	Extinction coefficient[a]	
		Methanol	0.1 M KPO_4, pH 7.4
	nm	*mM^{-1} cm^{-1}*	
A_2	240	35.4 ± 1.2	
	300	6.7 ± 0.4	
	239		32.5 ± 0.4
	319		5.7 ± 0.2
A_3	242	61.3 ± 2.1	
	308	10.4 ± 0.14	
	240		58.5 ± 2.1
	328		10.7 ± 0.2
N-A_2	250	28.0 ± 2.5	
	285	5.7 ± 0.3	
	246		24.0 ± 2.0
	277		8.0 ± 0.6
A_4	242	86.6 ± 4.1	
	308	17.6 ± 0.6	
	238		78.0 ± 3.1
	327		12.5 ± 0.4
N-A_3	247	54.3 ± 1.1	
	295	8.9 ± 0.3	
	244		51.9 ± 0.4
	319		9.3 ± 0.1

[a] Extinction coefficients were determined with a Cary 219 spectrophotometer from Varian Associates, Inc. Concentrations of ^{14}C-polymers were determined by liquid scintillation counting. Structures of acetaminophen polymers are shown in Fig. 1.

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TABLE 2

Time course of product formation from [^{14}C]NAPQI in potassium phosphate buffer

Time	Reaction products[a]						Total ^{14}C equivalent
	A_2	A_3	A_4	N-A_2	A	GS-A	
min				*μM*			
0	ND[b]	ND	ND	ND	31.2	51.0	82.2
0.5	ND	ND	ND	ND	29.6	48.8	78.4
1	ND	ND	ND	ND	30.1	52.3	82.4
2	ND	ND	ND	ND	33.7	54.0	87.7
3	ND	ND	ND	ND	32.7	48.3	81.0
4	ND	ND	ND	ND	28.5	56.7	85.2
5	0.3	ND	ND	ND	35.6	46.2	82.4
10	0.3	ND	ND	ND	30.3	49.7	80.5
20	0.6	0.3	ND	ND	30.9	48.1	81.1
30	2.8	1.4	ND	0.2	31.1	46.5	87.7
60	6.3	2.4	1.4	0.2	33.5	23.7	78.7

[a] Reaction mixtures containing 82 μM [^{14}C]NAPQI and 100 mM potassium phosphate (pH 7.4) were incubated at 25°. GSH (2 mM) was added at different time points (0–60 min) to terminate the reaction and a 1-ml methanol:water solution containing 2 mM ascorbate was added 15 sec after GSH addition to ensure that reactions were completely quenched. Assay conditions are described in the legend to Fig. 3. Structures of products are shown in Fig. 1.

[b] ND, not detected. Limit of detection was judged to be 0.1 μM.

were then quantitated by HPLC (Fig. 3). As shown in Table 2, the [^{14}C]NAPQI was relatively stable in potassium phosphate buffer; [^{14}C]3-(glutathion-*S*-yl)acetaminophen was the major product when incubated for less than 30 min. Although [^{14}C]acetaminophen was also formed, its formation could be mainly accounted for as a GSH reduction product of [^{14}C]NAPQI similar to what is shown in Fig. 2. After NAPQI was incubated for 60 min, the amount of acetaminophen formed was about 50% higher than what could be accounted for by a simple reduction of NAPQI by GSH. Thus, the additional formation of this acetaminophen must have been formed by a mechanism that is not yet understood. The amount of [^{14}C]3-(glutathion-*S*-yl)acetaminophen detected slowly decreased with time, indicating a decrease in the amount of [^{14}C]NAPQI. Formation of [^{14}C]acetaminophen polymers was also observed as the incubation time was increased. After 5 min, a small amount of the [^{14}C]acetaminophen dimer was observed, followed by the [^{14}C]acetaminophen trimer at 20 min. The [^{14}C]acetaminophen N-dimer and the [^{14}C]acetaminophen tetramer were detected at 30 and 60 min, respectively. The total amount of radioactivity recovered as identified products was greater than 96%. A small amount of radioactivity also eluted before [^{14}C]acetaminophen. This may have been hydroquinone or benzoquinone, which have been reported as minor hydrolysis products of NAPQI and previously estimated to be about 1–2% of the total reaction products (11, 19).

In contrast to the stability of NAPQI in buffer, NAPQI was less stable when [^{14}C]acetaminophen was included in reaction mixtures. The stability of NAPQI was dependent on the initial concentration of [^{14}C]acetaminophen (Table 3). High concentrations of [^{14}C]acetaminophen resulted in lower amounts of NAPQI [quantitated as 3-(glutathion-*S*-yl)acetaminophen] and greater amounts of acetaminophen polymers 5 min after incubations were initiated. NAPQI completely reacted with 1.7 mM [^{14}C]acetaminophen within 5 min, and the only products detected were the six acetaminophen polymers (Table 3). Both NAPQI (quantitated as the GSH-acetaminophen conjugate) and acetaminophen polymers were observed at lower [^{14}C]acetaminophen concentrations. An acetaminophen dimer-GSH conjugate was also observed. The ratio of ^{14}C incorporation into the acetaminophen dimer was increased with increasing amounts of [^{14}C]acetaminophen.

Table 4 shows the time-dependent formation of acetaminophen polymers when NAPQI was reacted with [^{14}C]acetaminophen. The formation of acetaminophen polymers increased, whereas NAPQI (quantitated as 3-(glutathion-*S*-yl)acetaminophen) decreased with time. Radioactivity was incorporated into all polymers, and about 47% of the total amounts of acetaminophen dimer contained ^{14}C. The amount of [^{14}C]acetaminophen incorporated into the acetaminophen trimer (A_3) and the acetaminophen tetramer (A_4) was slightly less than that incorporated into the acetaminophen dimer (A_2). A small amount of ^{14}C was also incorporated into 3-(glutathion-*S*-yl)acetaminophen and a larger amount of ^{14}C was incorporated into 3-(glutathion-*S*-yl)diacetaminophen which was about the same as the amount incorporated into the acetaminophen dimer.

A second order rate constant for [^{14}C]acetaminophen reacting with NAPQI was determined. NAPQI concentrations were estimated by dividing 3-(glutathion-*S*-yl)acetaminophen by the factor 0.67. A plot of the ln [^{14}C]acetaminophen/NAPQI versus time was linear, and a rate constant of 33 $M^{-1}sec^{-1}$ was determined.

NAPQI reacted with equimolar, 4-fold or 8-fold concentrations of acetaminophen-d_3 to give the previously observed acetaminophen polymers. The acetaminophen dimer (A_2) was isolated from incubation mixtures by HPLC, and the relative abundance of acetaminophen-d_3 incorporated into the acetaminophen dimer was determined by electron impact mass spectrometry as shown in Table 5. A molecular ion of *m/z* 300 should result from the dimerization of 2 mol of NAPQI, *m/z* 303 from 1 mol of NAPQI and 1 mol of acetaminophen-d_3, and *m/z* 306 from 2 mol of acetaminophen-d_3. The molecular ion of *m/z* 303 gave the greatest relative abundance at all concentrations of acetaminophen-d_3. The acetaminophen-d_3 dimer/total acetaminophen dimer ratio was roughly the same as the ratio of [^{14}C]acetaminophen dimer to total acetaminophen dimer shown in Table 3. The relative abundance of the molecular ion at *m/z* 300 was greatest when NAPQI was reacted with an equimolar concentration of acetaminophen-d_3; however, when reacted with 4–8-fold excess acetaminophen d_3, the relative abundance of *m/z* 300 decreased and the relative abundance of *m/z* 306 increased (Table 5).

In additional studies, the mechanism of [^{14}C]3-(glutathion-

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TABLE 3

Effect of [^{14}C]acetaminophen concentration in reactions with NAPQI

[^{14}C]Acetaminophen	Reaction products[a]								
	Glutathione conjugates		Acetaminophen polymers						[^{14}C]A_2/total A_2
	GS-A	GS-A_2	A_2	A_3	A_4	N-A_2	N″-A_3	N-A_3	
mM					*μM*				
0	41.6	ND[b]	ND	ND	ND	ND	ND	ND	
0.03	34.0	0.9	4.1	1.1	0.3	0.3	ND	ND	0.44
0.05	28.9	1.1	8.1	2.0	0.6	0.3	ND	ND	0.47
0.11	25.4	1.1	14.3	3.0	0.7	0.3	ND	ND	0.49
0.21	15.5	1.0	20.7	4.2	0.9	0.6	ND	ND	0.51
0.43	8.5	0.8	31.0	5.9	1.0	1.5	0.1	0.6	0.56
0.85	2.4	0.4	47.4	5.6	1.0	2.3	0.1	0.7	0.59
1.70	ND	ND	54.1	4.2	0.4	3.3	0.1	1.1	0.61

[a] Reaction mixtures contained 64 μM NAPQI, various amounts of [^{14}C]acetaminophen (0–1.7 mM), and 100 μM potassium phosphate (pH 7.4). Reaction mixtures were incubated at 25° and GSH (2 mM) was added after 5 min to terminate the reaction. A 1-ml methanol:water solution containing 2 mM ascorbate was added 15 sec after GSH addition to ensure that reactions were completely quenched. Assay conditions as described in the legend to Fig. 3. Structures of products are shown in Fig. 1.

[b] ND, not detected. The limit of detection was judged to be 0.1 μM.

TABLE 4

Time course of product formation in reaction mixtures containing NAPQI and [^{14}C]acetaminophen

Time	Reaction products[a]							
	Glutathione conjugates		Acetaminophen polymers					
	GS-A	GS-A_2	A_2	A_3	A_4	N-A_2	N″-A_3	N-A_3
min				*μM*				
0	79.2 (0.01)[b]	ND[c]	ND	ND	ND	ND	ND	ND
0.5	77.3 (0.01)	0.5	8.0 (0.52)	0.4	ND	0.6	ND	ND
1.0	72.3 (0.01)	1.1 (0.40)	13.0 (0.48)	0.9	ND	0.9	ND	ND
2.0	60.0 (0.06)	2.3 (0.42)	23.3 (0.42)	3.4 (0.38)	0.4	0.7	ND	0.1
4.0	38.7 (0.10)	3.2 (0.50)	30.1 (0.49)	8.4 (0.43)	1.7 (0.52)	0.6	0.1	0.2
6.0	23.8 (0.19)	3.2 (0.42)	34.3 (0.43)	11.9 (0.40)	2.3 (0.40)	0.6	0.1	0.6
8.0	14.7 (0.32)	2.7 (0.44)	34.4 (0.47)	14.5 (0.40)	3.2 (0.39)	0.5	0.2	1.4
10.0	8.4 (0.40)	2.4 (0.41)	37.9 (0.44)	16.6 (0.40)	3.3 (0.39)	0.4	0.2	1.5

[a] Reaction mixtures contained 120 μM NAPQI, 200 μM [^{14}C]acetaminophen, and 100 mM potassium phosphate (pH 7.4). Reaction mixtures were incubated at 25° and GSH (2 mM) was added at various time points (0–10 min) to terminate the reaction. A 1-ml methanol:water solution containing 2 mM ascorbate was added 15 sec after GSH addition to ensure that reactions were completely quenched. Assay conditions are described in the legend to Fig. 3.

[b] The ratio of ^{14}C-product to total product.

[c] ND, not detected. Limit of detection was judged to be 0.1 μM.

TABLE 5

Incorporation of acetaminophen-d_3 into the acetaminophen dimer (A_2) in reactions of NAPQI with various amounts of acetaminophen-d_3

NAPQI/ acetaminophen-d_3[a]	Relative Abundance[b]			[^{3}H]A_2/ total A_2[c]
	300	303	306	
1	22.7	66.3	11.1	0.44
4	6.6	75.6	17.9	0.56
8	4.9	78.1	17.0	0.56

[a] Reaction mixtures (3 ml) contained 200 μM NAPQI, 200–1600 μM acetaminophen-d_3, and 100 mM potassium phosphate. Reaction mixtures were incubated for 15 min at 25° and reactions were terminated with a 3-ml methanol:water solution containing 2 mM ascorbic acid. The acetaminophen dimer (A_2) was isolated by HPLC and *m/z* determined by electron impact mass spectrometry as described in Materials and Methods.

[b] Relative abundance expressed as intensity/sum of intensities for *m/z* 300, 303, and 306.

[c] The ratio of acetaminophen-d_3 dimer to total dimer was calculated by the percentage of relative abundance: $\frac{303 + 2(306)}{2(300 + 303 + 306)}$.

TABLE 6

Time course of [^{14}C]NAPQI and [^{14}C]GS-A formation in reaction mixtures containing NAPQI and [^{14}C]acetaminophen

Time	Reaction products[a]	
	[^{14}C]NAPQI[b]	[^{14}C]GS-A[c]
min	*μM*	
0	1.8	0.2
2.5	6.3	6.8
5.0	7.7	6.3
7.5	5.1	4.7
10.0	3.5	2.9
15.0	0.8	0.6

[a] Reaction mixtures contained 200 μM NAPQI, 200 μM [^{14}C]acetaminophen, and 100 mM potassium phosphate (pH 7.4).

[b] Reaction mixtures were incubated without GSH for various lengths of time and [^{14}C]NAPQI formation was determined directly by HPLC (11).

[c] GS-A formation was examined by methods described in the legend to Table 4.

S-yl)acetaminophen formation was examined. It was considered that ^{14}C might be incorporated into 3-(glutathion-*S*-yl)acetaminophen by the radical of [^{14}C]acetaminophen conjugating with the radical of GSH, or by NAPSQI disproportionating to [^{14}C]NAPQI. The data shown in Table 6 are from experiments similar to those described in Table 4 except that the formation of [^{14}C]NAPQI as well as the formation of [^{14}C]3-(glutathion-*S*-yl)acetaminophen was evaluated in parallel experiments. Table 6 shows that, when equimolar amounts of NAPQI and [^{14}C]acetaminophen were incubated for various lengths of time, ^{14}C was incorporated into 3-(glutathion-*S*-yl)acetaminophen and NAPQI to nearly the same extent. Maximal amounts of [^{14}C]acetaminophen were incorporated into both compounds at 5 min, and at 15 min less than 1 μM was incorporated into either 3-(glutathion-*S*-yl)acetaminophen or NAPQI.

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Reduction of NAPQI by NADPH. The results of incubating NAPQI with various amounts of NADPH are shown in Table 7. Without NADPH, NAPQI was stable for at least 5 min (addition of GSH at 5 min resulted in only GS-A and acetaminophen formation). In reaction mixtures containing NADPH, greater amounts of acetaminophen and acetaminophen polymers were formed and less NAPQI was detected when quantitated as 3-(glutathion-*S*-yl)acetaminophen. As the concentration of NADPH was increased, the amount of acetaminophen formation increased. The acetaminophen dimer (A_2) and the acetaminophen trimer (A_3) were maximal when NADPH concentration was 200 μM. Above 200 μM NADPH the formation of both polymers decreased. In reaction mixtures containing greater than 400 μM NADPH, NAPQI was not detected after 5 min (quantitated as 3-(glutathion-*S*-yl)acetaminophen).

A second order rate constant for the oxidation of NADPH by 70 μM NAPQI was determined over a range of three concentrations of NADPH, 100, 200 and 300 μM. The second order rate constant was 80 ± 6 $M^{-1}sec^{-1}$.

Discussion

Identification of acetaminophen polymers (16) and GSH-acetaminophen conjugates (18) has allowed us to analyze the differences in NAPQI and NAPSQI reactions. Synthetic NAPQI rapidly reacted with GSH to give 3-(glutathion-*S*-yl)acetaminophen (GS-A). Acetaminophen polymers were not detected when NAPQI was reacted with excess GSH; only acetaminophen and 3-(glutathion-*S*-yl)acetaminophen were formed (Fig. 2). Thus, NAPQI acted as an electrophile as well as an oxidizing agent of GSH, as has been previously reported (7). We previously found that NAPSQI was an intermediate generated by the horseradish peroxidase system that formed acetaminophen polymers and minor amounts of GSH-acetaminophen conjugates (18). Since polymers were the major products, it was suggested that they were formed via a free radical coupling mechanism (16). In agreement, electron spin resonance studies also suggested that horseradish peroxidase catalyzed acetaminophen to NAPSQI (20).

Currently, we are investigating the mechanism of 3-(glutathion-*S*-yl)diacetaminophen formation ($GS\text{-}A_2$). One mechanism has been proposed (18) where 3-(glutathion-*S*-yl)acetaminophen was oxidized to 3-(glutathion-*S*-yl)-*N*-acetyl-*p*-benzosemiquinone imine and then reacted with NAPSQI to give 3-(glutathion-*S*-yl)diacetaminophen. An alternative to this mechanism might be that the quinone of the acetaminophen dimer was formed and then reacted with GSH. The data, shown in Table 3, suggest that only minor amounts of 3-(glutathion-*S*-yl)diacetaminophen may have been formed via the oxidation of 3-(glutathion-*S*-yl)acetaminophen. This conjugate was probably formed from an acetaminophen oxidation intermediate since the ratio of ^{14}C incorporation in 3-(glutathion-*S*-yl)diacetaminophen was about the same as the ratio of ^{14}C incorporated into the acetaminophen dimer. If 3-(glutathion-*S*-yl)diacetaminophen had been formed via 3-(glutathion-*S*-yl)acetaminophen, the ratio of ^{14}C incorporation should have been much lower. Under these conditions, GSH may have formed a GSH-acetaminophen conjugate with either NAPQI or the quinone imine of the acetaminophen dimer.

Reactions between NAPQI and acetaminophen resulted in the formation of acetaminophen polymers. The concentration of acetaminophen was important. Without acetaminophen, [^{14}C]NAPQI was relatively stable. Only small amounts of [^{14}C]acetaminophen polymers were detected 30 min after incubation in buffer alone. Addition of GSH after 30 min resulted in the formation of [^{14}C]3-(glutathion-*S*-yl)acetaminophen, which was about 91% of the amount observed when GSH was added immediately after [^{14}C]NAPQI. Analyses of total ^{14}C equivalents demonstrated that 96% of the radioactivity after a 60-min incubation was associated with [^{14}C]acetaminophen, [^{14}C]3-(glutathion-*S*-yl)acetaminophen, or [^{14}C]acetaminophen polymers. In contrast, when NAPQI was reacted with acetaminophen, polymers were readily formed with a concomitant decrease in NAPQI [quantitated as 3-(glutathion-*S*-yl)acetaminophen]. This suggests that NAPQI reacted with acetaminophen by an initial comproportionation reaction to give two equivalents of NAPSQI. Once NAPSQI was formed, the data suggest that it then reacted by a number of different mechanisms. NAPQI primarily reacted to give acetaminophen polymers, but it appears that NAPSQI also disproportionated to form acetaminophen and NAPQI. This is suggested by [^{14}C]acetaminophen incorporation into [^{14}C]3-(glutathion-*S*-yl)acetaminophen and NAPQI as shown in Table 5. The reactions were further complicated since NAPSQI also transproportionated with acetaminophen. Thus, when NAPQI was reacted with high concentrations of [^{14}C]acetaminophen, a high

TABLE 7

Effect of NADPH concentration in reactions with NAPQI

NADPH	Reaction products[a]							
	GS-A	$GS\text{-}A_2$	A	A_2	A_3	A_4	$N\text{-}A_2$	$N\text{-}A_3$
μM				μM				
0	179.2	ND[b]	110.9	ND	ND	ND	ND	ND
25	144.4	2.1	109.2	4.4	2.6	1.4	ND	2.0
50	120.2	2.9	107.9	7.2	4.3	1.6	0.6	2.2
100	80.7	3.1	112.0	12.1	6.7	2.1	0.7	2.3
150	55.3	2.6	148.3	15.9	8.0	2.3	0.9	2.0
200	29.8	1.9	165.0	18.8	8.2	2.0	0.9	1.1
300	3.4	0.8	207.8	18.1	5.1	1.1	1.3	ND
400	ND	ND	250.0	14.7	3.2	ND	1.4	ND
500	ND	ND	251.0	10.7	1.3	ND	1.2	ND
600	ND	ND	265.0	9.3	1.0	ND	1.1	ND

[a] Reaction mixtures (1 ml) contained 300 μM NAPQI, 0–600 μM NADPH, 100 mM potassium phosphate (pH 7.4). GSH (2 mM) was added at 5 min to terminate the reaction and 1 ml of methanol:water solution containing 2 mM ascorbate was added 15 sec after GSH addition to ensure that reactions were completely quenched. Structures of products are shown in Fig. 1.

[b] ND, not detected. The limit of detection was judged to be 0.1 μM.

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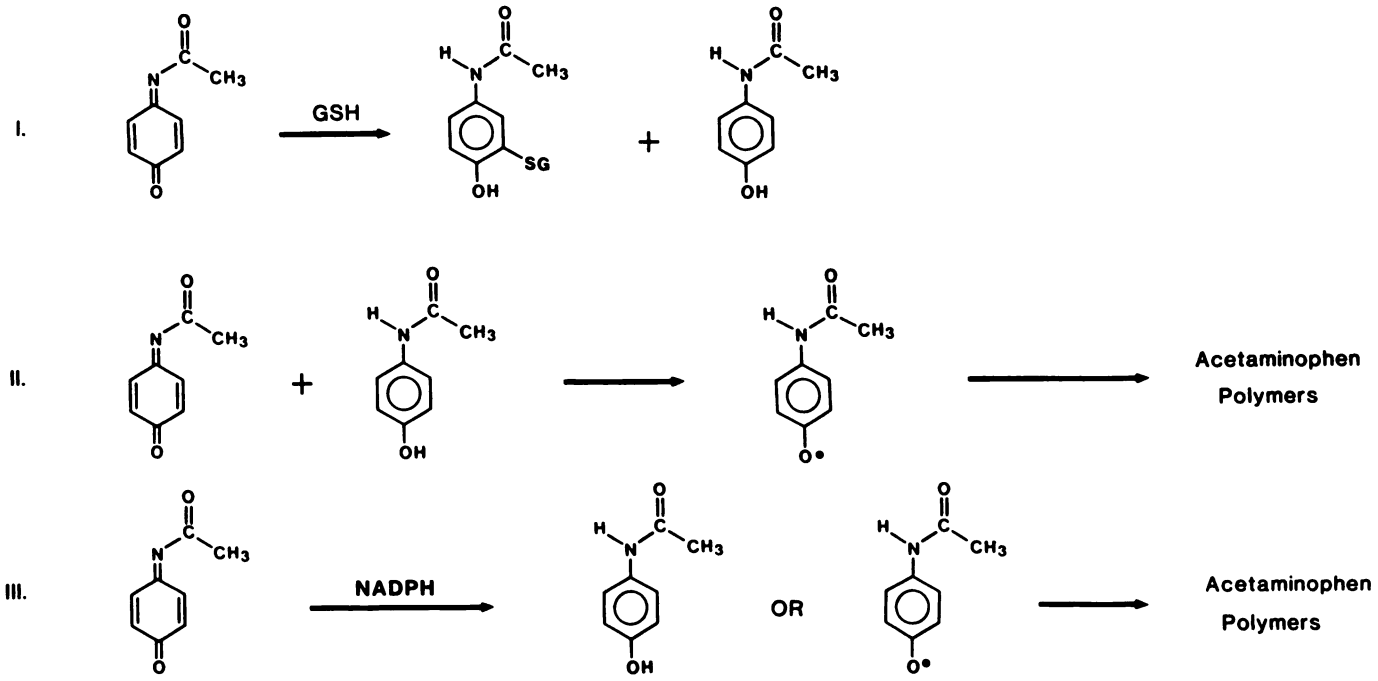

Fig. 4. Reaction of NAPQI with GSH, acetaminophen, and NADPH. Although the radical is shown as a phenoxy radical, the phenyl- and the nitrogen-centered radicals are the likely intermediates that form the acetaminophen polymers (see Fig. 1 for structures).

proportion of ^{14}C was incorporated into the acetaminophen polymers.

The results indicate that [^{14}C]acetaminophen and NAPQI comproportionated to NAPSQI which then reacted primarily via a coupling reaction to give slightly less than a 50:50 ratio of labeled to unlabeled compound. The data do not suggest that an appreciable amount of NAPSQI reacts with NAPQI to form the semiquinone imine of the acetaminophen dimer. If this were a major reaction pathway, then the expected incorporation of [^{14}C]acetaminophen into the acetaminophen trimer would be considerably lower than the values shown in Table 4. Instead, the [^{14}C]acetaminophen trimer/total acetaminophen trimer ratio is only slightly less than the ratio observed for the acetaminophen dimer (Fig. 4). The [^{14}C]acetaminophen tetramer/total acetaminophen tetramer ratio was about the same as that of the acetaminophen trimer and only slightly less than that of the acetaminophen dimer, suggesting that the acetaminophen tetramer may have formed either by the coupling of two acetaminophen dimer radicals or by the coupling of an acetaminophen trimer radical with NAPSQI.

The data presented in Table 5 show that acetaminophen-d_3 reacted with NAPQI to give the acetaminophen dimer with molecular ions of m/z 300, 303, and 306, indicating dimerization between deuterium-labeled and unlabeled acetaminophen radicals. These data further demonstrate the comproportionation of acetaminophen with NAPQI to give radicals that, at least in part, randomly polymerize with other free radicals. However, this may be an overly simplified explanation since a totally random polymerization process would yield an expected relative abundance of 25% m/z 300 to 50% m/z 303 to 25% m/z 306. The relative abundance of m/z 303 was considerably greater than would be expected for a random process. One explanation for this might be that, once 2 mol of NAPSQI are formed from the NAPQI and acetaminophen comproportionation reaction, they polymerize before they diffuse. Moreover, the formation of greater amounts of m/z 306 than 300 in reaction mixtures with high concentrations of acetaminophen-d_3 is consistent with the transproportionation reaction described in mixtures containing high concentrations of [^{14}C]acetaminophen (Table 3).

In other experiments we have observed that NADPH reacts with NAPQI to give acetaminophen and acetaminophen polymers. Powis *et al.* (21) have postulated that NADPH reduced NAPQI via a one-electron transfer mechanism, yet they were unable to detect NAPSQI by electron spin resonance spectroscopy (21). Our results indicate that NAPSQI was formed in the reactions of NADPH with NAPQI since acetaminophen polymers were formed. Our experiments further indicate that, at low concentrations of NADPH, the reductions of NAPQI would be almost exclusively via a one-electron reduction since the decrease in NAPQI as calculated from the amount of GS-A formed was about twice that of the NADPH concentration. It is unlikely that these results occurred via an acetaminophen NAPQI comproportionation reaction since the rate of NAPQI reacting with NADPH is greater than 2-fold the rate at which it reacts with acetaminophen. Unlike reactions of NAPQI with acetaminophen to give polymers, NADPH primarily reduces NAPQI to acetaminophen.

These studies indicate that both NADPH and acetaminophen react with NAPQI, and, in microsomal or *in vivo* reactions where NAPQI might be expected to be formed, it would appear that these components might react with NAPQI. However, the second order rate constants are rather low. The second order rate constant for the reaction of NADPH with NAPQI was 80 $M^{-1}sec^{-1}$ and the second order rate constant of acetaminophen reaction with NAPQI was 33 $M^{-1}sec^{-1}$. In contrast, GSH rapidly reacts with NAPQI. Preliminary data[1] have demonstrated that

[1] I. Wilson, J. A. Hinson, B. Coles, S. D. Nelson, P. Wardman, and B. Ketterer, unpublished observations.

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GSH rapidly reacts with NAPQI at pH 6.5 with a second order rate constant of about 1×10^4 $M^{-1}sec^{-1}$. Given that the concentrations of acetaminophen, NADPH, and GSH were similar, then GSH would react with NAPQI about 300 times faster than acetaminophen and over 100 times faster than NADPH. Thus, when considering these competing reactions, the formation of NAPSQI via the reduction of NAPQI by acetaminophen or NADPH in the presence of GSH should be minor.

Our results indicate that determination of the enzymatic mechanism by which acetaminophen is oxidized may be complicated by non-enzymatic reactions. For example, if acetaminophen is oxidized to NAPQI by prostaglandin H synthase or cytochrome P-450, then NAPQI may comproportionate with acetaminophen to produce NAPSQI. In this case the formation of acetaminophen polymers would be relatively low as compared to the formation of 3-(glutathion-*S*-yl)acetaminophen in the presence of GSH. Careful analysis of the various reaction products is needed for a more complete understanding of the enzymatic mechanisms. Our data (Fig. 4) suggest, however, that NAPQI reacts with GSH to form primarily 3-(glutathion-*S*-yl)acetaminophen, and that NAPQI reacts with acetaminophen to give NAPSQI, which primarily reacts to give acetaminophen polymers. NADPH appears to reduce primarily NAPQI to acetaminophen.

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