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Evidence That Acetaminophen and *N*-Hydroxyacetaminophen Form a Common Arylating Intermediate, *N*-Acetyl-*p*-Benzoquinoneimine

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SUMMARY

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Adding ascorbic acid to microsomal incubations containing acetaminophen inhibited covalent binding of the reactive metabolite. Adding ascorbic acid to incubations containing acetaminophen and cysteine markedly decreased acetaminophen–cysteine adduct formation. Ascorbic acid addition to aqueous incubations containing *N*-hydroxyacetaminophen and cysteine similarly inhibited the nonenzymatic formation of an acetaminophen–cysteine adduct. Therefore, the chemical reactions responsible for the nonenzymatic decomposition of *N*-hydroxyacetaminophen to yield acetaminophen–cysteine adducts were examined. In aqueous solutions above pH 7, *N*-hydroxyacetaminophen rapidly dehydrated to *N*-acetyl-*p*-benzoquinoneimine. In the absence of reducing compounds *N*-acetyl-*p*-benzoquinoneimine reacted with another molecule of *N*-hydroxyacetaminophen to give equal amounts of nitrosophenol and acetaminophen. The addition of cysteine or ascorbic acid slowed the decomposition of *N*-hydroxyacetaminophen and inhibited the formation of nitrosophenol. Cysteine effected these changes through decreasing the concentration of *N*-acetyl-*p*-benzoquinoneimine, primarily by reducing it to acetaminophen at low pH (5.5–7.0) or by conjugating with it to yield an acetaminophen–cysteine adduct at high pH (7.5–11.0). Ascorbic acid produced its effects only through reduction of the N-acetyl-*p*-benzoquinoneimine intermediate; thus acetaminophen was the only product. These observations provide strong evidence that the reactive intermediate formed in microsomes from acetaminophen and in solution from *N*-hydroxyacetaminophen is *N*-acetyl-*p*-benzoquinoneimine.

INTRODUCTION

The metabolic activation of acetaminophen to an arylating metabolite was originally postulated to occur via N oxidation to the hydroxamic acid (1, 2). Evidence in support of this hypothesis was provided by subsequent studies of species differences and of the effects of mixed-function oxygenase inducers and inhibitors on the N oxidation of 2-acetylaminofluorene (3, 4), 4-chloroacetanilide (3, 5), and phenacetin (6) to hydroxamic acids. Because of the formation of *meta*-substituted sulfhydryl adducts of acetaminophen, we proposed that *N*-hydroxyacetaminophen rapidly dehydrated to the reactive *N*-acetyl-*p*-benzoquinoneimine and this was the ultimate toxic species (7, 8).

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To investigate directly the possible role of *N*-hydroxyacetaminophen in acetaminophen-induced hepatic and renal necrosis, we prepared *N*-hydroxyacetaminophen by hydrogenolysis of the benzyl protecting group from 4-benzoxy-*N*-hydroxyacetanilide, as originally suggested by R. S. Andrews (Sterling Winthrop Laboratories, U.K.). We (9–11) further examined (i) the rate of decomposition of synthetic *N*-hydroxyacetaminophen, which was markedly decreased by acid conditions and the presence of sulfhydryl agents; (ii) the reaction of the compound with glutathione, *N*-acetylcysteine, and cysteine, which yielded adducts identical to those formed following metabolic activation of acetaminophen itself; and (iii) the toxicity of the compound, which produced centrilobular hepatic necrosis in mice at doses below the hepatotoxic dose of acetaminophen. *N*-Hydroxyacetaminophen and its reaction products with sulfhydryl compounds were assayed by gas chromatography, high-pressure liquid chromatography, and gas chromatography–mass spectrometry. In the presence of cysteine, *N*-hydroxyaceta-

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minophen decomposed to yield acetaminophen and the acetaminophen–cysteine adduct, which accounted totally for the disappearance of *N*-hydroxyacetaminophen. Conversely, in the absence of a sulfhydryl compound, the rate of decomposition of *N*-hydroxyacetaminophen was much faster and only 50% of the disappearance could be accounted for as acetaminophen (9, 10).

Two other groups concomitantly reported the successful synthesis of *N*-hydroxyacetaminophen by other approaches (12–14). They too found that the decomposition of *N*-hydroxyacetaminophen was pH and temperature dependent but did not examine the effect of sulfhydryl compounds on the rate of decomposition or on reaction products. Calder *et al.* (12, 13), however, did observe marked depletion of hepatic and renal glutathione and extensive hepatic and renal necrosis after *N*-hydroxyacetaminophen was administered to mice and rats.

Our preceding studies (9–11) of the reaction of *N*-hydroxyacetaminophen with sulfhydryl compounds have been consistent with the hypothesis that the hydroxamic acid undergoes a base-catalyzed dehydration to *N*-acetyl-*p*-benzoquinoneimine, which is the arylating species (Fig. 1A). The investigations of Calder *et al.* (12, 13) and Gemborys *et al.* (14) demonstrating a pH optimum of 9–10 for decomposition of *N*-hydroxyacetaminophen are also consistent with this hypothesis. We now report studies testing whether the decomposition products formed from *N*-hydroxyacetaminophen in the absence of sulfhydryl compounds also could arise from the *N*-acetyl-*p*-benzoquinoneimine intermediate (Fig. 1B). The investigations are extended to an analysis of the reactive intermediate formed from acetaminophen by microsomal mixed-function oxygenases to test whether the intermediate has reaction properties similar to those of *N*-acetyl-*p*-benzoquinoneimine (Fig. 1C).

MATERIALS AND METHODS

All reagents and materials were the highest grade

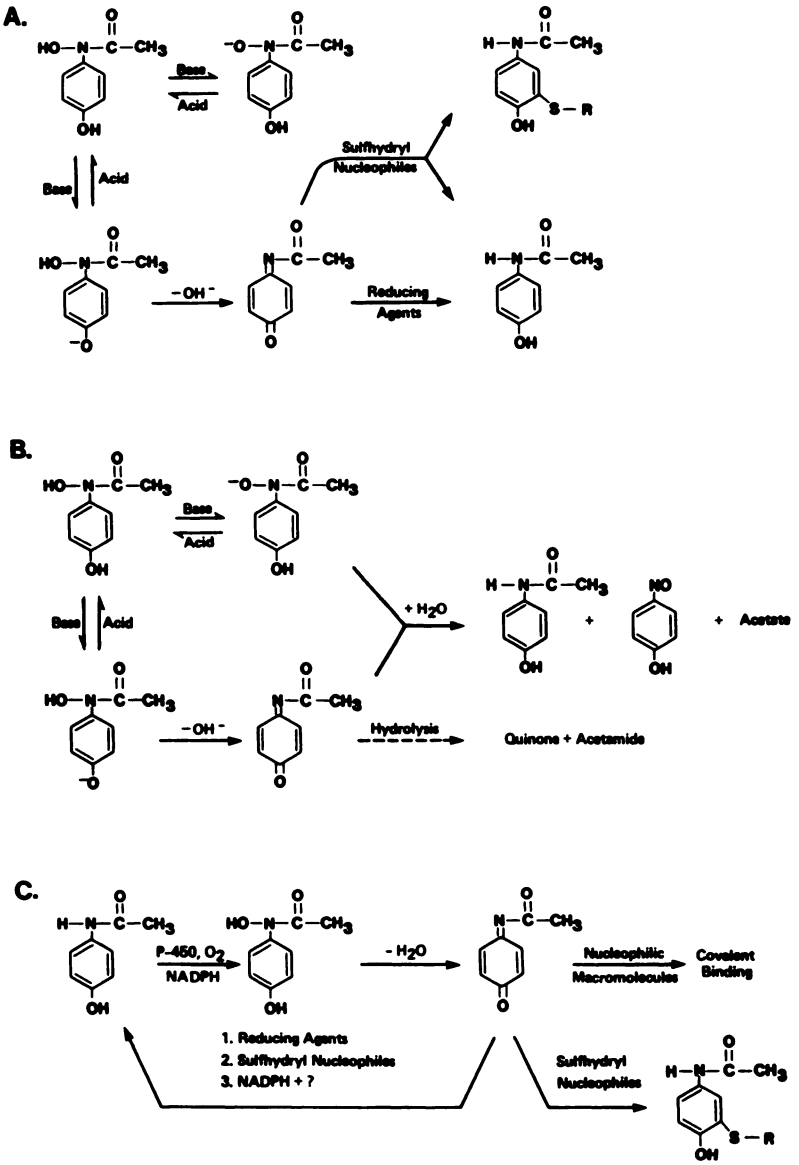

FIG. 1. *Postulated reactions of sulfhydryl compounds and reducing agents with the reactive intermediates formed from N-hydroxyacetaminophen and acetaminophen.*

commercially available. ^{14}C-(Carbonyl)- and ^{3}H-(general)-4-hydroxyacetanilides were purchased from New England Nuclear Corp., Boston, Mass., and purified as previously described (7).

N-Hydroxyacetaminophen (4.5 mM) was incubated in 0.1 M phosphate buffer at varying pH and at 37°C with shaking. Incubations (2 ml) were started by adding *N*-hydroxyacetaminophen at zero time as a distilled water solution, prepared immediately before use, and maintained at 0°C. All incubations contained 0.1 mg/ml *p*-chloroacetanilide and 0.4 mg/ml *p*-fluorophenol as internal standards. For sample analysis, see Table 1.

The acetaminophen–cysteine conjugate was measured as follows. Aliquots (0.1 ml) were withdrawn from incubations, added to 0.1 ml ice-cold methanol, and analyzed on the same day. Determinations were performed with a Waters Associates (Milford, Mass.) high-pressure liquid chromatographic (HPLC) system: Model U6K injector, Model 6000A solvent delivery system, Model 440 absorbance detector (254-nm filters), 0.4 × 30-cm μBondapak C_{18} column, and water:methanol:acetic acid (86.5:12.5:1.0) mobile phase at a flow rate of 2.0 ml/min. We observed the following retention times: acetaminophen, 4.9 min; acetaminophen–cysteine conjugate, 4.1 min; and *p*-fluorophenol, 10.8 min. Acetaminophen–cysteine conjugate concentrations were routinely determined by HPLC separation and by detection of absorbance at 254 nm. We established the relationship between concentration and the peak area ratio of the conjugate to internal standard (*p*-fluorophenol) by incubating ^{14}C-(carbonyl)-acetaminophen (sp act, 1720 dpm/nmol) with hepatic microsomes (3.22 mg protein/ml), NADPH, and 1 mM cysteine. Incubations (37°C for 30 min) were terminated by the addition of an equal volume of ice-cold methanol. After centrifugation, supernatant samples were filtered (0.5-μm filter) and injected directly onto the HPLC column. Column effluent was collected at 10-s intervals and counted in a Packard Model 3003 liquid scintillation spectrometer. After corrections for quench (channels ratio) and counting efficiency, dpm were converted to nanomoles of acetaminophen–cysteine conjugate. Conjugate absorptivity was 83.1% that of acetaminophen at 254 nm.

TABLE 1

Identification and quantitation of N-hydroxyacetaminophen and its decomposition products by mass spectrometry and gas chromatography

Acidified aliquots (0.2 ml) of incubation solutions were extracted with ethyl acetate. After extracts were evaporated, residues were treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide in CH_3CN and heated at 120°C for 10 min. Two-microliter samples were injected on a 6-ft, 1% OV-17 gas chromatographic column and the temperature was programmed from 100 to 200°C at 9°/min (flame ionization detection; carrier gas 70 cc N_2/min). Peak area ratios of *p*-nitrosophenol, *p*-nitrophenol, acetaminophen, and *N*-hydroxyacetaminophen to *p*-chloroacetanilide (retention time, 5.4 min) were proportional to their concentrations (r = 0.96, 0.99, 0.98, and 0.98, respectively) over the range of concentrations encountered experimentally (0.01 to 20 mM).

Silyl derivatives of	Retention	Mass spectral ions
	min	*m/z*
Quinone[a, b]	0.8	108 (M^+), 82, 80, 54, 53, 52
p-Nitrosophenol[b]	4.0	195 (M^+), 180, 166, 150, 91, 73
p-Nitrophenol[b]	6.6	211 (M^+), 196, 182, 150, 91, 73
Acetaminophen[b]	7.7	295 (M^+), 280, 254, 237, 206, 181, 180, 165, 116, 114, 73
N-Hydroxyacetaminophen[b]	10.7	311 (M^+), 296, 269, 268, 223, 206, 192, 179, 147, 73, 45, 43
N-Hydroxyacetaminophen[c]	—	352, 340, 312 (MH^+), 296, 222
Acetaminophen–cysteine, pentafluoropropionyl methyl ester derivative[d]	—	576 (M^+), 413, 398, 354, 342, 329, 312, 300, 287, 247, 234, 215, 183, 100, 45, 43

[a] Quinone does not form a silylated derivative.

[b] GC–electron impact mass spectrum obtained with an LKB Type 9000 gas chromatograph–mass spectrometer (conditions: ionizing voltage, 70 eV; ionizing current, 60 μA; accelerating voltage, 3.5 kV; source temperature, 270°C; separator temperature, 270°C).

[c] GC–chemical ionization mass spectrum obtained with a Varian Model 1700 gas chromatograph interfaced with a Finnigan Model 1015 mass spectrometer, on-line with a Systems Industries Model 150 data system (conditions: ionizing voltage, 140 eV; source temperature, ambient; methane served as the carrier and reagent gas; source pressure, 1 Torr).

[d] Electron impact mass spectrum obtained by direct probe insertion of the pentafluoropropionyl methyl ester, rather than the silyl derivative, into a Varian MAT CH7 mass spectrometer (conditions: ionizing voltage, 20 eV; ionizing current, 100 μA; accelerating voltage, 3kV; source temperature, 220°C; probe temperature, 100°C).

RESULTS

N-Hydroxyacetaminophen and its decomposition products were identified by analysis of full scan GC–electron impact mass spectra and by comparison with synthetic reference standards (Table 1). The identity of *N*-hydroxyacetaminophen was further verified by gas chromatography–chemical ionization mass spectrometry of the bis(trimethylsilyl)ether (Table 1). Thereafter, the rate of decomposition and the decomposition products of *N*-hydroxyacetaminophen were determined by gas chromatography (Tables 1 and 2).

Without the addition of other agents, *N*-hydroxyacetaminophen decomposed to give approximately equal amounts of acetaminophen and nitrosophenol and small amounts of benzoquinone and nitrophenol (Table 2B). The small concentration of benzoquinone (less than 1%) was not routinely quantitated, but the disappearance of *N*-hydroxyacetaminophen could always be accounted for by the formation of acetaminophen, nitrosophenol, and nitrophenol. Loss of *N*-hydroxyacetaminophen was maximal at pH 9–10 and minimal at pH 5 and 13 (Table 2), as previously reported (9–14).

The addition of sulfhydryl compounds such as cysteine markedly slowed the decomposition of *N*-hydroxyacetaminophen (9, 10) and altered product formation (Table 2). Again the rate of decomposition was fastest at pH 9.5. The distribution of products formed in the presence of cysteine was also influenced by pH. The amount of acetaminophen exceeded the amount of acetaminophen–cysteine adduct at pH 7.4 after 60 min, whereas the reverse relationship occurred at higher pH values.

Inclusion of other reducing agents such as ascorbic acid markedly slowed decomposition of *N*-hydroxyacetaminophen and altered product formation as well (Table 3). As seen with cysteine, 10 mM ascorbic acid completely inhibited formation of nitrosophenol and nitrophenol. However, ascorbate did not form a conjugate with the reactive intermediate of *N*-hydroxyacetaminophen that could be detected by HPLC analysis. The disappearance of *N*-hydroxyacetaminophen was accounted for by stoichiometric formation of acetaminophen (Table 3F).

TABLE 2

Effect of cysteine on decomposition of N-hydroxyacetaminophen and appearance of products

N-hydroxyacetaminophen was incubated for 15, 60, or 120 min at 37°C in 0.1 M phosphate buffers of differing pH, with equimolar cysteine (+), 10 mM cysteine (++), or no cysteine (0). Analyses are described in Table 1 and Methods. *N*-Hydroxyacetaminophen (NOH-PHAA); acetaminophen (PHAA); acetaminophen–cysteine (PHAA-CYS); *p*-nitrosophenol (p-NOP); *p*-nitrophenol (p-NO_2P); cysteine (Cys).

	Conditions			NOH-PHAA		PHAA[a]	PHAA-CYS	p-NOP	p-NO_2P	Total
	Cys	pH	min							
				mM	%	%	%	%	%	%
(A)	+	5.4	0	11.53	99.5	0.0	0.4			100.0
			15	11.27	97.3	0.0	0.5	ND[b]	ND	97.8
			60	10.20	88.1	0.0	0.6			88.7
	+	7.4	0	11.87	95.6	0.0	4.4			100.0
			15	10.67	86.0	3.5	6.5	ND	ND	96.0
			60	5.34	43.0	23.3	12.2			78.5
	++	7.7	0	7.18	99.3	0.0	0.7	0.0	0.0	100.0
			60	4.92	68.0	9.0	18.9	0.0	0.0	95.9
	+	8.2	0	10.19	97.4	0.0	2.6			100.0
			15	8.63	82.5	8.3	13.1	ND	ND	103.9
			60	4.08	39.0	26.0	43.8			108.8
	++	9.5	0	8.15	99.1	0.0	0.9	0.0	0.0	100.0
			60	0.24	2.9	27.7	67.0	0.0	0.0	97.7
	+	10.4	0	8.10	98.9	0.0	1.1			100.0
			15	4.70	53.4	5.0	30.2	ND	ND	92.6
			60	2.34	28.6	13.8	58.1			100.5
	++	11.4	0	5.86	99.5	0.0	0.5	0.0	0.0	100.0
			60	5.25	89.1	2.5	9.7	0.0	0.0	101.3
	+	13.0	0	8.14	98.1	0.0	1.9			100.0
			15	7.83	94.3	0.0	1.1	ND	ND	95.4
			120	7.87	94.8	0.0	4.3			99.1
(B)	0	7.7	0	4.51	100.0	0.0	0.0	0.0	0.0	100.0
			60	0.69	15.3	43.7	0.0	40.6	3.7	103.3
	0	9.0	0	4.85	100.0	0.0	0.0	0.0	0.0	100.0
			60	0.01	0.2	46.0	0.0	51.1	4.9	102.2
	0	11.7	0	5.35	100.0	0.0	0.0	0.0	0.0	100.0
				4.14	77.4	12.6	0.0	10.5	1.5	102.0

[a] Values are corrected to account for a small amount of acetaminophen (5–10%) present as an impurity in *N*-hydroxyacetaminophen.
[b] Not determined.

We compared the enzymatic formation of the reactive metabolite of acetaminophen by microsomal monooxygenases with the base-catalyzed (pH 7.4) formation of the reactive metabolite of *N*-hydroxyacetaminophen by investigating the effects of cysteine and ascorbate on these processes. The addition of cysteine alone trapped the reactive intermediate derived from the NADPH-dependent metabolism of acetaminophen (Table 4A) and from the chemical decomposition of *N*-hydroxyacetaminophen (Table 4B) as acetaminophen–cysteine adducts. Cysteine simultaneously inhibited the arylation of microsomal protein by acetaminophen (Table 4C) and the acetaminophen-stimulated oxidation of NADPH during microsomal metabolism (Table 4D).

When increasing amounts of ascorbic acid were introduced into microsomal incubations containing acetaminophen and cysteine or into aqueous solutions containing *N*-hydroxyacetaminophen and cysteine, the amount of acetaminophen–cysteine formed from acetaminophen or from *N*-hydroxyacetaminophen decreased in a concentration-dependent manner (Table 4A and B). The addition of ascorbic acid had a similar concentration-dependent effect on the arylation of microsomal protein by the reactive acetaminophen metabolite (Table 4C) but did not decrease acetaminophen stimulation of NADPH oxidation more than that caused by the addition of cysteine alone (Table 4D).

DISCUSSION

In aqueous solutions, *N*-hydroxyacetaminophen decomposes to acetaminophen and nitrosophenol and small amounts of nitrophenol and benzoquinone. Presumably, the nitrophenol arises from further oxidation of nitrosophenol, and benzoquinone arises from hydrolysis of *N*-acetyl-*p*-benzoquinoneimine (Fig. 1B). Decomposition of *N*-hydroxyacetaminophen in buffered solutions is maxi-

TABLE 3

Effects of ascorbic acid on decomposition of N-hydroxyacetaminophen and formation of products

Ascorbic acid and equimolar amounts of cysteine (+) were added to incubations of *N*-hydroxyacetaminophen in 0.1 M phosphate buffer. Analyses are described in Table 1 and Methods. Abbreviations are given in Table 2 except for ascorbic acid (ASC).

	Conditions				NOH-PHAA		PHAA[a]	PHAA-CYS	p-NOP	p-NO_2P	Total
	Cys	ASC	pH	min							
					mM	%	%	%	%	%	%
(A)	0	0	7.4	0	5.58	100.0	0.0	0.0			100.0
				10	2.73	48.9	15.6	0.0	ND[b]	ND	64.5
				30	1.48	26.5	22.6	0.0			49.3
(B)	0	1	7.4	0	5.58	100.0	0.0	0.0			100.0
				10	5.24	93.9	1.4	0.0	ND	ND	95.3
				30	4.38	78.5	10.8	0.0			89.3
(C)	+	0	7.4	0	5.71	99.7	0.0	0.3			100.0
				10	5.66	98.8	1.9	3.3	ND	ND	104.0
				30	5.10	89.0	5.4	7.3			101.7
(D)	+	1	7.4	0	6.20	99.7	0.0	0.3			100.0
				10	6.12	98.4	0.5	2.2	ND	ND	101.1
				30	5.30	85.2	4.2	6.1			95.5
(E)	0	0	9.4	0	4.24	100.0	0.0	0.0	0.0	0.0	100.0
				10	0.05	1.2	49.3	0.0	37.7	5.2	93.4
				30	0.00	0.0	50.0	0.0	44.3	3.1	97.4
(F)	0	10	9.4	0	4.43	100.0	0.0	0.0	0.0	0.0	100.0
				10	1.36	30.7	65.5	0.0	0.0	0.0	96.2
				30	0.41	9.2	97.3	0.0	0.0	0.0	106.5

[a] See Table 2, footnote *a*.

[b] Not determined.

mal around pH 9.5, which is consistent with *N*-acetyl-*p*-benzoquinoneimine formation being the rate-determining step (Fig. 1B). Nitrosophenol and acetaminophen are formed from *N*-hydroxyacetaminophen at an approximately 1:1 ratio. The appearance of equal amounts of both an oxidized and a reduced product suggests a two-electron redox couple in which the reactive *N*-acetyl-p-benzoquinoneimine formed by dehydration of *N*-hydroxyacetaminophen is reduced to acetaminophen by a second *N*-hydroxyacetaminophen molecule which becomes oxidized to nitrosophenol (Fig. 1B).

In reactions containing cysteine, the loss of *N*-hydroxyacetaminophen is markedly diminished and the formation of nitrosophenol but not of acetaminophen is abolished, because the reactive *N*-acetyl-*p*-benzoquinoneimine is either reduced or conjugated before it can react with *N*-hydroxyacetaminophen (Fig. 1A). This prevents paired nitrosophenol and acetaminophen formation by the bimolecular reaction pathway shown in Fig. 1B. In this system the formation of acetaminophen versus the acetaminophen–cysteine conjugate is pH dependent. Under acid conditions the reduction of *N*-acetyl-*p*-benzoquinoneimine to acetaminophen is predominant, whereas under basic conditions adduct formation is greater (Fig. 1A). Presumably, this occurs because cysteine is most nucleophilic as an anion and anion formation is suppressed by acidic conditions.

As with cysteine, the addition of ascorbic acid decreases the rate of decomposition of *N*-hydroxyacetaminophen and inhibits the formation of nitrosophenol. Ascorbic acid, which lacks the nucleophilic but not the reducing action of cysteine, converts *N*-hydroxyacetaminophen to acetaminophen stoichiometrically by reducing the *N*-acetyl-*p*-benzoquinoneimine intermediate (Fig. 1A). Agents such as cysteine and ascorbic acid do not reduce *N*-hydroxyacetaminophen directly to acetaminophen because they decrease the overall rate of decomposition of *N*-hydroxyacetaminophen. Moreover, they do not promote the formation of acetaminophen at acid and basic pHs which are not favorable for dehydration to the *N*-acetyl-*p*-benzoquinoneimine intermediate.

Both cysteine and ascorbic acid inhibit the arylation of microsomal protein by the toxic metabolite of acetaminophen formed oxidatively by cytochrome *P*-450 monooxygenases (Table 4C). The scheme in Fig. 1C predicts this behavior when the toxic metabolite of acetaminophen arises by initial *N*-hydroxylation as postulated. An important observation is the ability of increasing concentrations of ascorbic acid to decrease formation of the acetaminophen–cysteine adduct derived from the microsomal oxidation of acetaminophen and from the base-catalyzed decomposition of *N*-hydroxyacetaminophen (Table 4A and B). This effect of ascorbic acid on the acetaminophen adduct derived from oxidation of acetaminophen is explained by the reduction of the *N*-acetyl-p-benzoquinoneimine intermediate to acetaminophen before cysteine conjugation can occur (Fig. 1C). It apparently does not result from inhibition of microsomal

TABLE 4

Effect of ascorbic acid on acetaminophen–cysteine conjugate formation, covalent binding, and NADPH oxidation from microsomal metabolism of acetaminophen

Metabolic studies (A, C, and D) were conducted in 0.1 M phosphate at pH 7.4 and 37°C for 5 min with hepatic microsomes from male Swiss mice (1 mg protein/ml), 1 mM acetaminophen, 0.4 mM NADPH, and 1 mM cysteine. Values reported are means and standard deviations of 3 determinations. In B, *N*-hydroxyacetaminophen (4 mM) was reacted with cysteine (4 mM) at pH 7.4 and 37°C for 120 min. Products are expressed as % of initial *N*-hydroxyacetaminophen concentration. Covalent binding (^{3}H-acetaminophen; sp act, 1060 dpm/nmol) and NADPH oxidation were determined as described previously (17, 22). Other analyses are described in Table 1 and Methods.

Conditions		(A) Formation of acetaminophen–cysteine from microsomal metabolism of ^{3}H-acetaminophen	(B) Formation of acetaminophen–cysteine (%) and acetaminophen (%) from *N*-hydroxyacetaminophen (% remaining)		
Cysteine	Ascorbic acid		NOH-PHAA	PHAA	PHAA-CYS
	mM	*nmol/mg/5 min*			
+	0	2.39 ± 0.28 (100)	4.2	65.0	33.0
+	1	1.45 ± 0.21 (60)	7.1	71.4	28.0
+	5	0.84 ± 0.06 (35)	8.5	82.7	18.9
+	10	0.16 ± 0.03 (7)	9.6	89.0	5.8
+	30	0.02 ± 0.01 (1)	9.2	96.0	2.2
		(C) Covalent binding from microsomal metabolism of ^{3}H-acetaminophen	(D) NADPH oxidation from microsomal metabolism of ^{3}H-acetaminophen		
		nmol/mg/5 min	*nmol/mg/5 min*		
0	0	0.223 ± 0.025 (100)	16.4 ± 0.66 (100)		
+	0	0.009 ± 0.002 (4)	8.4 ± 0.90 (51)		
0	1	0.048 ± 0.006 (21)	11.4 ± 0.46 (70)		
0	5	0.014 ± 0.002 (6)	9.5 ± 1.50 (58)		
0	10	0.019 ± 0.002 (9)	9.0 ± 1.50 (55)		
0	30	0.003 ± 0.001 (1)	7.1 ± 1.20 (43)		

oxidation of acetaminophen because acetaminophen-stimulated NADPH oxidation in the presence of cysteine is not decreased further by the addition of ascorbic acid (Table 4D).

These results also explain why the amount of reactive metabolite formed from acetaminophen always appears to be greater when the metabolite is quantitated by measuring the amount of sulfhydryl adduct formed rather than by measuring the amount of covalent binding as an index of metabolite formation (Table 4A and C). In the absence of a high concentration of nucleophile, the strong reducing environment of the microsomal system probably favors the reduction of most of the *N*-acetyl-*p*-benzoquinoneimine back to acetaminophen before it has time to react with the nucleophilic sites present at low concentrations in protein macromolecules. Conversely, when present at high concentrations in microsomal incubations, sulfhydryl nucleophiles add efficiently to *N*-acetyl-*p*-benzoquinoneimine before reduction can occur, resulting in greater estimates of the rate at which the arylating intermediate is formed.

New studies applying electrochemical techniques have also provided evidence that *N*-acetyl-*p*-benzoquinoneimine is the arylating metabolite formed from acetaminophen. Kissinger recently reported (15) that *N*-acetyl-*p*-benzoquinoneimine can be generated electrochemically from acetaminophen and that the intermediate reacts with nucleophiles to produce sulfhydryl adducts that are indistinguishable from those formed during the microsomal metabolism of acetaminophen.

Thus, the collective data would appear to establish that the reactive intermediate resulting from *P*-450-mediated oxidation of acetaminophen is *N*-acetyl-*p*-benzoquinoneimine (7, 8). Furthermore, variations in the hepatotoxicity of acetaminophen with animal species and strains (16), enzyme induction and inhibition (1, 2, 17), and genetic responsiveness (18) correlate directly with differences in the ability to N hydroxylate other drugs (3–6).

One can speculate about alternative pathways. Isolation of 3-methoxyacetaminophen from urine (19) raised the possibility that the toxicity of acetaminophen could be due to a 3,4-epoxide metabolite or a 3,4-catechol derived from the epoxide. However, this pathway is not likely to be involved in toxicity. When the toxic arylating metabolite of acetaminophen is generated enzymatically under an $^{18}O_2$ atmosphere and trapped with glutathione, no ^{18}O is found in the adduct, indicating that it did not arise from 3,4 epoxidation (20). Moreover, the 3,4-catechol metabolite of acetaminophen contains ^{18}O and therefore arises from a pathway other than the toxic one (21). In addition, the trace amount of 3,4-catechol formed in microsomal incubations is not decreased by the addition of ascorbic acid or sulfhydryls (21), although ascorbic acid and sulfhydryls abolish covalent binding of acetaminophen (Table 4C).

Acetaminophen might also be oxidized to *N*-acetyl-*p*-benzoquinoneimine directly or through two one-electron steps. However, there is no precedent for reactions of this kind mediated by the cytochrome *P*-450 monooxygenases at the present time.

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