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Investigation of Mechanisms of Acetaminophen Toxicity in Isolated Rat Hepatocytes with the Acetaminophen Analogues 3,5-Dimethylacetaminophen and 2,6-Dimethylacetaminophen

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SUMMARY

The toxicity of acetaminophen (4′-hydroxyacetanilide), 3,5-dimethylacetaminophen (3′,5′-dimethyl-4′-hydroxyacetanilide), and 2,6-dimethylacetaminophen (2′,6′-dimethyl-4′-hydroxyacetanilide) was investigated in hepatocytes isolated from phenobarbital-pretreated rats. At a concentration of 5 mM, acetaminophen was found to be the most cytotoxic of the three analogues. Inhibition of cellular glutathione reductase by pretreatment of hepatocytes with BCNU enhanced the toxicity of 3,5-dimethylacetaminophen without affecting the toxicity of either acetaminophen or 2,6-dimethylacetaminophen. In contrast, pretreatment with diethylmaleate preferentially enhanced the toxicity caused by 2,6-dimethylacetaminophen and, to a lesser extent, acetaminophen, without measurably affecting the toxicity of 3,5-dimethylacetaminophen. All three hydroxyacetanilides depleted cellular glutathione concentrations, but only the 3,5-dimethyl analogue caused measurable formation of glutathione disulfide. However, the cytotoxicity of all analogues could be decreased by the administration of the thiol agent, dithiothreitol. Moreover, all three analogues had antioxidant properties, and their ability to decrease cellular malondialdehyde formation correlated with their half-wave ($E_{1/2}$) oxidation potentials. The administration of the ferric ion chelator, desferrioxamine, which completely inhibited lipid peroxidation as measured by malondialdehyde formation, had no significant effects on cytotoxicity caused by acetaminophen or 3,5-dimethylacetaminophen, but partially protected against cytotoxicity caused by 2,6-dimethylacetaminophen, the poorest antioxidant of the three analogues. Covalent protein binding of all three analogues was measured. Whereas both acetaminophen and 2,6-dimethylacetaminophen bound to hepatocyte proteins under conditions where they were cytotoxic, 3,5-dimethylacetaminophen did not. Dithiothreitol was found to decrease the binding of radiolabel from both acetaminophen and its 2,6-dimethyl analogue, whereas desferrioxamine had no effect. These data indicate that the three analogues cause their cytotoxic effects by different mechanisms, although toxicity in all cases is probably mediated through their oxidation products, the quinone imines, which have as a common feature their ability to deplete cellular thiols.

The widely used analgesic acetaminophen (paracetamol, 4′-hydroxyacetanilide) is known to cause severe liver injury in both experimental animals and humans when high doses are administered (1, 2). The mechanism whereby acetaminophen causes liver injury involves metabolic conversion of the drug into a reactive intermediate by cytochrome(s) P-450 (3, 4). A cytotoxic metabolite of acetaminophen that is formed by hepatic cytochrome P-450 is the oxidation product, NAPQI (5–7). This electrophilic species is most likely responsible both for the depletion of hepatocellular GSH stores and the covalent binding to proteins that follows ingestion of acetaminophen. Recently, a thiol adduct of acetaminophen to mouse liver proteins has been characterized (8).

Whereas it is generally agreed that NAPQI is a reactive intermediate in the pathogenesis of liver injury caused by acetaminophen, there is much less agreement as to how NAPQI interacts with cellular constituents to cause cell death. From studies with isolated hepatocytes, the importance of covalent binding as a mechanism to account for acetaminophen-mediated cytotoxicity has been questioned as a result of studies which have shown that cell death does not necessarily occur, even when covalent binding is maximal in isolated cells (9). Therefore, investigators have suggested alternative explanations for mechanisms of cell death that involve oxidant stress mechanisms (10–12). However, more recent evidence from experiments in rats strongly indicates that acetaminophen hepatotoxicity is not accompanied by oxidant stress (13), and phy-

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ABBREVIATIONS: NAPQI, *N*-acetyl-*q*-benzoquinone imine; BCNU, 1,3-bis(2-chloroethyl)-*N*-nitrosourea; DEM, diethylmaleate; DTT, dithiothretiol; GSH, glutathione; GSSG, glutathione disulfide; MDA, malondialdehyde; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

siochemical evidence shows that neither the acetaminophen radical nor the radicals generated from two dimethylated analogues generate significant amounts of toxic oxygen species (14).

In order to further probe the mechanism of acetaminophen-mediated liver cell injury, we have compared the cytotoxic effects of acetaminophen and two of its dimethylated analogues in preparations of rat hepatocytes. Previous studies with these analogues indicated that, in rats, acetaminophen caused more extensive hepatic necrosis than 3,5-dimethylacetaminophen, which was in turn more hepatotoxic than 2,6-dimethylacetaminophen (15). Of further interest is the finding that the presumed reactive intermediates in hepatotoxicity, the respective quinone imines, react with GSH in different ways (16). Whereas NAPQI reacts to form both GSSG and a glutathione conjugate, only GSSG was detected from reaction of GSH and 3,5-dimethyl NAPQI, and only a GSH conjugate was characterized from reactions of GSH with 2,6-dimethyl NAPQI.

Because changes in the thiol status in hepatocytes appear to play a crucial role in pathogenic mechanisms of cytotoxicity caused by several agents, including acetaminophen and NAPQI (17), we investigated the cytotoxic effects of acetaminophen and its dimethylated analogues in hepatocytes with altered thiol status. The results of these studies show that alteration of cellular thiol status can markedly alter the time course of cytotoxicity caused by acetaminophen and its dimethylated analogues, and that the cytotoxicity of each compound is affected in a different way depending on how the cellular thiol status is manipulated.

Materials and Methods

Collagenase (grade II), DTT, and Hepes were obtained from Boehringer (Mannheim, FRC). Desferrioxamine (*N*-[5-(3-aminopentyl)-hydroxycarbonyl]-3-([5-(*N*-hydroxyacetamido))pentyl]-carbamoyl)-propionyl-hydroxamine-methansulfonic acid) was obtained from Ciba-Geigy LTD (Basel, Switzerland). Acetaminophen (4′-hydroxyacetanilide), DEM, *N*-acetylcysteine, GSH, and GSSG were purchased from Sigma Chemical Co. (St. Louis, MO), while 2,6-dimethylphenol, 3,5-dimethylphenol, and acetic anhydride were purchased from Aldrich (Milwaukee, WI). BCNU was a generous gift from Bristol-Meyers Laboratories (Stockholm, Sweden) and [1-^{14}C]acetic anhydride (10 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). All other reagents were obtained from local commercial sources and were of the highest available quality.

Synthesis of 2′,6′-dimethyl-4′-hydroxyacetanilide and 3′,5′-dimethyl-4′-hydroxyacetanilide was according to previously described procedures. Both compounds were recrystallized from chloroform to yield white crystalline solids that had melting points and spectroscopic properties virtually identical to those reported (15). Synthesis of [^{14}C-acetyl]acetaminophen (0.47 mCi/mmol), [^{14}C-acetyl]-2′,6′-dimethyl-4′-hydroxyacetanilide (0.33 mCi/mmol), and [^{14}C-acetyl]-3′,5′-dimethyl-4′-hydroxyacetanilide (0.18 mCi/mmol) were carried out using the same procedures with the exception that [^{14}C]acetic anhydride was employed for acetylation purposes. The radiochemical purity of each compound was determined by recrystallization to constant specific activity and high performance liquid chromatographic analysis on a Whatman Partisil 5 column (4.6 mm × 25 cm) using a mobile phase of ethyl acetate at a flow rate of 1.0 ml/min. Purity was determined to be 99.8% for radiolabeled acetaminophen, 99.7% for the 2,6-dimethyl analogue, and 99.5% for the 3,5-dimethyl analogue.

Male Sprague-Dawley rats (200–300 g) were used in all experiments; the rats received sodium phenobarbital (1 mg/ml) in the drinking water for at least 5 days prior to use.

Hepatocytes were isolated by collagenase perfusion of the liver as previously described (18), and cell incubations were performed at 37° in rotating, round-bottom flasks at a concentration of 10^6 cells/ml under an atmosphere of 95% O_2 and 5% CO_2. Acetone (100 mM) was included in incubations of hepatocytes to enhance activation of the compounds (19).

Inhibition of glutathione reductase in hepatocytes was performed as previously described (20, 21) in a modified Krebs-Henseleit buffer, supplemented with 25 mM Hepes and an amino acid mixture. After 25 min of treatment with BCNU, the cells were washed and resuspended in the same medium, without BCNU, and incubated for an additional 1 hr in order to restore the GSH level. The incubation medium was then changed to Krebs-Henseleit buffer without amino acids, pH 7.4, supplemented with 24 mM Hepes.

Depletion of GSH stores in hepatocytes was performed by the administration of DEM (0.05 ml/kg, intraperitoneally, in corn oil) 1 hr before isolation of hepatocytes. This treatment routinely depleted soluble thiols to 10–15% of control levels.

Assays were performed as follows. Concentrations of GSH and GSSG were determined by high performance liquid chromatography (22) with the following modification: HPO_3 was used in place of perchloric acid to precipitate proteins. Covalent binding of radiolabeled compounds was determined as previously described for acetaminophen (7, 23). Protein thiol determinations were performed by a modification (7) of the method of Sedlak and Lindsay (24), and protein measurements were made according to a modified method of Lowry (25). Lipid peroxidation was determined according to the method of Smith *et al.* (26). Hepatocyte integrity was monitored as previously described (27) by the exclusion of trypan blue.

Results

Incubations of acetaminophen, 3,5-dimethylacetaminophen, and 2,6-dimethylacetaminophen with isolated hepatocytes from phenobarbital-pretreated rats results in time-dependent cell death (Fig. 1A). The progression of cell death is different for each compound, with the onset occurring earliest with acetaminophen. The time course of cytotoxicity for 3,5-dimethylacetaminophen, but not that of acetaminophen or 2,6-dimethylacetaminophen, could be markedly accelerated by inactivation of the enzyme, glutathione reductase, with BCNU (Fig. 1B). A decrease of greater than 90% of glutathione reductase activity shifted the time course of cytotoxicity caused by 3,5-dimethylacetaminophen such that virtually all cells had lost viability by 3 hr as compared to 5 hr in cells not treated with BCNU.

A very different effect was observed when GSH levels were depleted by prior administration of DEM to rats (Fig. 1C). The time course of cytotoxicity caused by acetaminophen was shortened by approximately 1 hr. More striking was the enhanced toxicity of 2,6-dimethylacetaminophen which was only marginally cytotoxic in phenobarbital- or phenobarbital plus BCNU-treated cells. In contrast, DEM pretreatment had no significant effect on cytotoxicity caused by 3,5-dimethylacetaminophen.

Depletion of GSH also occurs in hepatocytes during incubation with all three analogues (Fig. 2). The extent of GSH depletion was greater, and the time course of depletion more rapid, for acetaminophen than for the other two analogues. The generation of oxidized glutathione (GSSG) could account for a major portion of the GSH loss only during incubation with the 3,5-dimethylated analogue.

The widely used thiol reducing agent, DTT, protected against the cytotoxicity caused by all three hydroxyacetanilides (Table 1). DTT was added after 1.5 hr of incubation with each substrate. This time was chosen because removal of substrates

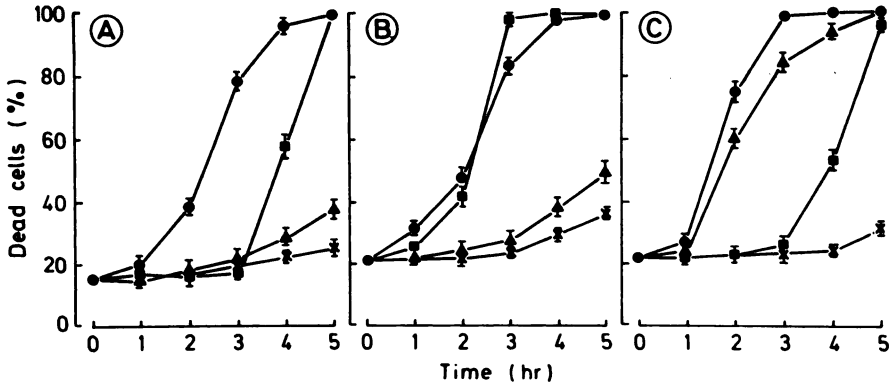

Fig. 1. Effects of acetaminophen and its dimethylated analogues on viability of isolated hepatocytes. The cytotoxic effects, as monitored by trypan blue uptake, of acetaminophen (●), 3,5-dimethylacetaminophen (■), and 2,6-dimethylacetaminophen (▲) were determined in hepatocytes isolated from phenobarbital-pretreated rats with no other treatment (A), with BCNU treatment (B), or with DEM treatment (C). All substrate concentrations were 5 mM. Control cells (×) were treated identically in each case except that 5 μl/ml of dimethyl sulfoxide only were added at the beginning of incubation periods rather than substrate in dimethyl sulfoxide. Values are means ± standard error from six different experiments.

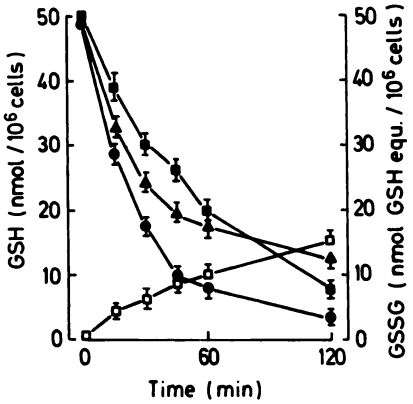

Fig. 2. Effects of acetaminophen and its dimethylated analogues on GSH and GSSG concentrations in hepatocytes. The effects of acetaminophen (●), 3,4-dimethylacetaminophen (■), and 2,6-dimethylacetaminophen (▲) on hepatocyte GSH concentration and on GSSG concentration after 3,5-dimethylacetaminophen (□). All substrate concentrations were 5 mM. Hepatocytes were isolated from phenobarbital-pretreated rats and then were treated with BCNU. GSSG is plotted as equivalents of GSH for comparative purposes. Values are corrected for a slow loss of GSH and formation of GSSG in cells without substrates (3–5 nmol GSH equivalents/10^8 cells over a 60-min period). GSSG could not be detected above control levels in cells treated with acetaminophen or 2,6-dimethylacetaminophen. Values are means ± standard errors from four different experiments.

after 1.5 hr did not change the progress of cell death, indicating that reactions leading to cell death which require the presence of substrate and/or unsequestered metabolites had already occurred.

Lipid peroxidation, as measured by MDA formation, was observed in incubations of isolated hepatocytes which had been pretreated with BCNU (Fig. 3) or DEM (Fig. 4). In the presence of 2,6-dimethylacetaminophen the amount of lipid peroxidation was only slightly less than that observed in cells without

TABLE 1

Effect of DTT on cytotoxicity of acetaminophen and its dimethylated analogues

The thiol agent DTT was added to incubations of acetaminophen, 3,5-dimethylacetaminophen, and 2,6-dimethylacetaminophen with hepatocytes isolated from phenobarbital-treated rats and other treatments as indicated.

Compound and cell treatments	Percentage of dead cells after 4-hr incubation period[a]	
	Control[b]	+DTT[c]
Acetaminophen		
BCNU-treated	98 ± 4	81 ± 6
DEM-treated	100 ± 0	85 ± 5
3,5-Dimethylacetaminophen		
BCNU-treated	97 ± 3	62 ± 7
2,6-Dimethylacetaminophen		
DEM-treated	94 ± 5	76 ± 6

[a] As determined by trypan blue uptake.
[b] Incubations contained substrates only (5 mM). Results are expressed as means ± SE of at least four separate experiments.
[c] DTT was added to cells after 1.5 hr of incubation with substrates. The final concentration of DTT was 10 mM. Results are expressed as means ± SE of at least four separate experiments.

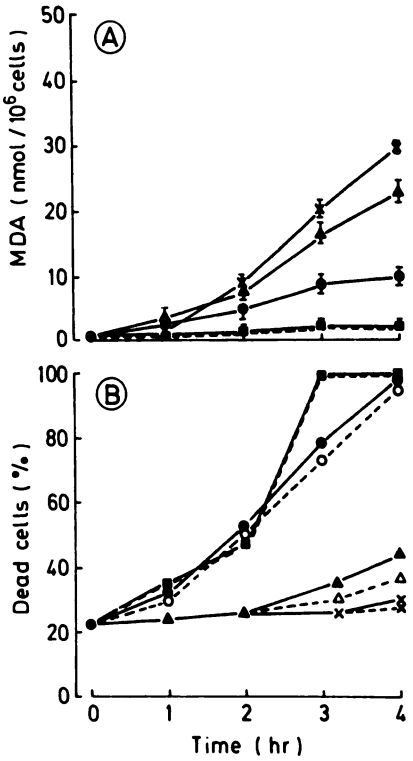

Fig. 3. Effects of acetaminophen and its dimethylated analogues on lipid peroxidation and toxicity in BCNU hepatocytes. The time course of MDA formation (A) and trypan blue uptake (B) was determined in hepatocytes isolated from phenobarbital-pretreated rats followed by treatment with BCNU. The cells were treated with vehicle only (×—×) and vehicle plus desferrioxamine (×– – –×); acetaminophen (●—●) and acetaminophen plus desferrioxamine (○– – –○); 3,5-dimethylacetaminophen (■—■) and 3,5-dimethylacetaminophen plus desferrioxamine (□– – –□); or 2,6-dimethylacetaminophen (▲—▲) and 2,6-dimethylacetaminophen plus desferrioxamine (△– – –△). Concentration of all substrates was 5 mM and of desferrioxamine, 250 μM. Values are means ± standard error from four different experiments. The standard errors for toxicity data are similar to those in Fig. 1. No significant difference in toxicity was observed in the presence of desferrioxamine.

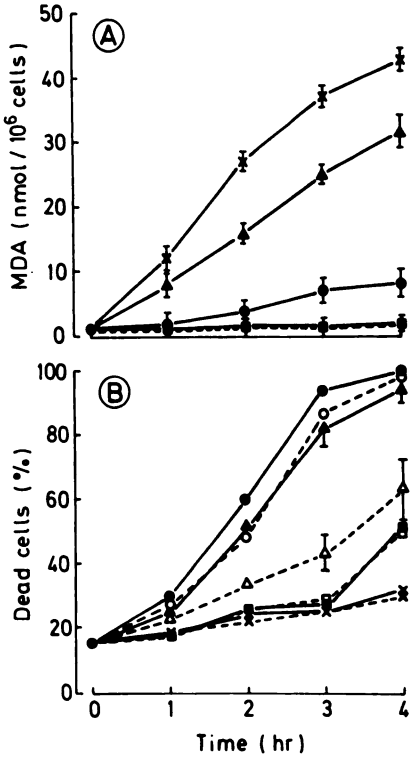

Fig. 4. Effects of acetaminophen and its dimethylated analogues on lipid peroxidation and toxicity in DEM hepatocytes. The time course of MDA formation (A) and trypan blue uptake (B) was determined in hepatocytes isolated from phenobarbital-pretreated and DEM-treated rats. *Symbols* and substrate concentrations are the same as those described in Fig. 4. Values are means ± standard error from three different experiments. The standard errors for toxicity data are similar to those in Fig. 1. The only significant difference in toxicity in the presence of desferrioxamine was observed for 2,6-dimethylacetaminophen.

TABLE 2

Half-wave oxidation potentials of acetaminophen and its dimethylated analogues

Half-wave oxidation potentials ($E_{\frac{1}{2}}$) were determined by sampled DC polarography in 0.1 M KNO_3. Analyte concentrations were approximately 10 mM. Scans were run in the positive direction at a scan rate of 2 mV/sec, with a working electrode of platinum wire and the reference electrode SCE.

Compound	$E_{\frac{1}{2}}$
	V
Acetaminophen	0.35
3,5-Dimethylacetaminophen	0.30
2,6-Dimethylacetaminophen	>0.65

substrate, but in the presence of acetaminophen and 3,5-dimethylacetaminophen the amount of lipid peroxidation was distinctly suppressed. The ability of the hydroxyacetanilides to suppress lipid peroxidation correlates with their chemical antioxidant capability (Table 2). It was possible to essentially eliminate MDA formation (<2 nmol/10^6 cells) in incubations containing the ferric ion chelator, desferrioxamine (Figs. 3 and 4). However, desferrioxamine had no significant effect on cytotoxicity caused by either acetaminophen or its 3,5-dimethylated analogue. In contrast, the cytotoxicity of 2,6-dimethylacetaminophen was decreased by nearly 50%.

Radiolabel from [^{14}C-acetyl]acetaminophen and its 2,6-dimethylated analogue bound to cellular proteins in a time-dependent manner when incubated under conditions where each substrate was cytotoxic (Fig. 5). Acetaminophen bound to

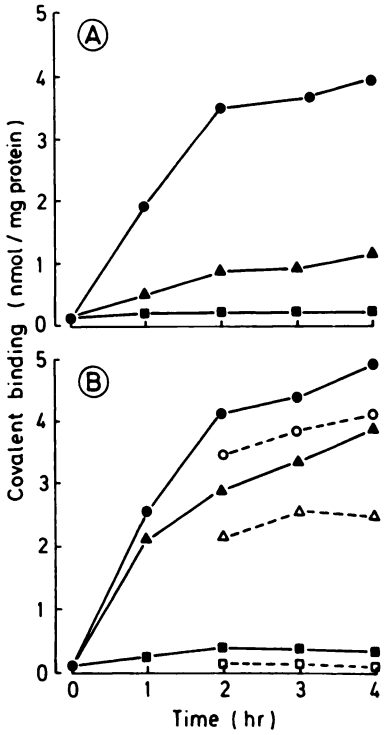

Fig. 5. Covalent binding of radiolabel from acetaminophen and its dimethylated analogues to hepatocyte proteins. Covalent binding of radiolabel from [^{14}C-acetyl]acetaminophen (●—●), [^{14}C-acetyl]-3,5-dimethylacetaminophen (■—■), and [^{14}C-acetyl]-2,6-dimethylacetaminophen (▲—▲) was determined in hepatocytes isolated from phenobarbital-pretreated rats and treated with either BCNU (A) or DEM (B). All substrates were incubated at a final concentration of 5 mM (specific activities were all approximately 0.1 mCi/mmol). DTT was added after 1.5 hr of incubating with substrates, and binding was determined where indicated in B for acetaminophen (○– – –○), 3,5-dimethylacetaminophen (□– – – –□), and 2,6-dimethylacetaminophen (△– – –△). Results are averages of two separate experiments with less than 9% difference between experiments.

a greater extent when cells were treated with DEM (Fig. 5B) than with BCNU (Fig. 5A), which parallels the increase observed in cytotoxicity (Fig. 1). Furthermore, treatment with DEM but not BCNU markedly enhanced both the binding and cytotoxicity of 2,6-dimethylacetaminophen (Figs. 1 and 5). In marked contrast, radiolabel from the 3,5-dimethylated analogue did not significantly bind to cellular proteins, even in BCNU-treated cells where it is as cytotoxic as acetaminophen. It is possible that the radiolabeled acetyl group is lost by hydrolysis from the 3,5-dimethylated analogue. However, paraoxon inhibition of esterase activity had no effects on the cytotoxicity of any of the analogues, and 94–97% of the radiolabel from 3,5-dimethylacetaminophen was recoverable as such after β-glucuronidase/sulfatase treatment of cell supernatants (data not shown).

Interestingly, DTT, which decreased the cytotoxicity caused by all analogues, also was found to decrease the binding of radiolabeled acetaminophen and its 2,6-dimethylated analogue to cellular proteins (Fig. 5B). However, desferrioxamine had no effect on covalent protein binding even though this agent partially protected against the cytotoxic effects of the 2,6-dimethylated analogue (the data are not shown in Fig. 5 for clarity because the time course of binding for each analogue in the presence of desferrioxamine was essentially identical to the time course of binding in its absence).

Discussion

In an attempt to better define the mechanism(s) by which acetaminophen causes hepatotoxicity, we have compared the cytotoxic effects of acetaminophen and two of its dimethylated analogues in hepatocytes isolated from phenobarbital-treated rats. The results of our experiments indicate that all three hydroxyacetanilides can deplete cellular thiols but that mechanisms of toxicity apparently differ in many respects for the three analogues.

In the experimental system that was employed for our studies, the cytotoxic effects of acetaminophen were not significantly enhanced by treatment of hepatocytes with BCNU (Fig. 1), and GSSG could not be detected in cells treated with acetaminophen (Fig. 2). This contrasts with results of experiments in which a major cytotoxic metabolite of acetaminophen, NAPQI, is added directly to hepatocytes, wherein BCNU treatment enhances cytotoxicity and GSSG formation is sustained (7). Under the conditions of the present experiments, relatively small amounts of NAPQI are probably formed continuously over the first 2 hr of incubation as indicated by declining GSH levels (Fig. 2), increasing levels of covalent binding (Fig. 5), and acetaminophen glutathione conjugate formation (data not shown). Thus, the rate of GSSG formation, if it is formed under these conditions, would be much slower than when NAPQI is added as a bolus dose, and even in the BCNU-treated cells there is probably sufficient residual GSH reductase activity to reduce the slowly generated GSSG back to GSH. It is noteworthy that other investigators have reported differences in effects of the antioxidant, ascorbic acid, on the reactive metabolite of acetaminophen generated under different conditions (28). Furthermore, BCNU treatment has been found to enhance hepatotoxicity in rats caused by the prooxidant, diquat, but not hepatotoxicity caused by acetaminophen (13).

Based on the cumulative results, we conclude that acetaminophen probably causes its cytotoxic effects without involvement of oxidative stress since BCNU had no effect on the cytotoxicity that was caused by acetaminophen, and lipid peroxidation was inhibited even in the presence of marked cytotoxicity (Fig. 4), an effect that has been observed by others (29). Covalent binding of the oxidative metabolite, NAPQI, to critical sulfhydryl groups provides the best explanation for the pathogenesis of cytotoxicity caused by acetaminophen. The sulfhydryl reagent, DTT, protected against acetaminophen toxicity (Table 1), and DTT, which is usually considered a thiol antioxidant, decreased covalent binding of acetaminophen to cell proteins (Fig. 5). However, we do not know how DTT is protecting the cell and we cannot rule out the possibility that it is acting through multiple mechanisms.

The results obtained in our model system differ from the results recently reported by Tee *et al.* (30), who removed acetaminophen from the incubations after 90 min. In their system, DTT markedly protected against the cytotoxic effects of acetaminophen without affecting the covalent binding of radiolabel from acetaminophen to hepatocyte proteins. Therefore, these investigators concluded that the probable reactive metabolite of acetaminophen, NAPQI, caused its cytotoxic effects by oxidizing sulfhydryl groups of critical proteins rather than by binding covalently to them. Why NAPQI appears to be reacting differently in the two hepatocyte systems is not presently known.

One of the analogues, 3,5-dimethylacetaminophen, does appear to cause its cytotoxic effects in our model system by oxidizing sulfhydryl groups. Although 3,5-dimethylacetaminophen is a good antioxidant and acts similarly to acetaminophen to inhibit MDA formation, its cytotoxic effect is markedly enhanced by BCNU treatment. It is the only analogue that leads to measurably increased concentrations of GSSG in the cell (Fig. 2). Under peroxidative or comproportionation conditions, 3,5-dimethylacetaminophen forms a relatively stable phenoxy radical (31). However, this radical stimulates neither oxygen consumption nor the oxidation of GSH (14).[2] Thus, the effects of the 3,5-dimethylated analogue are best explained by its two-electron oxidation to the quinone imine which is known to react chemically with GSH to form GSSG (16). No stable adducts of this quinone imine have been isolated and protein binding is not observed, even under markedly cytotoxic conditions. Thus, the pathogenesis of cytotoxicity of 3,5-dimethylacetaminophen probably involves direct oxidation of protein thiols by the quinone imine. In this regard it is interesting that DTT, which is known to reduce disulfides to thiols, is more effective in decreasing the cytotoxicity that is caused by 3,5-dimethylacetaminophen than in decreasing cytotoxicity caused by the other two analogues (Table 1). It is interesting that cytotoxicity caused by 3,5-dimethylacetaminophen is not accelerated significantly by DEM treatment which decreases the concentration of GSH to 10–15% of that in control cells. We can only speculate that this residual GSH is sufficient to maintain GSH reductase and other GSH-dependent repair processes near their normal activity during the time course of the experiments.

The only analogue that produces a cytotoxic reaction in which lipid peroxidative effects may play a role is 2,6-dimethy-

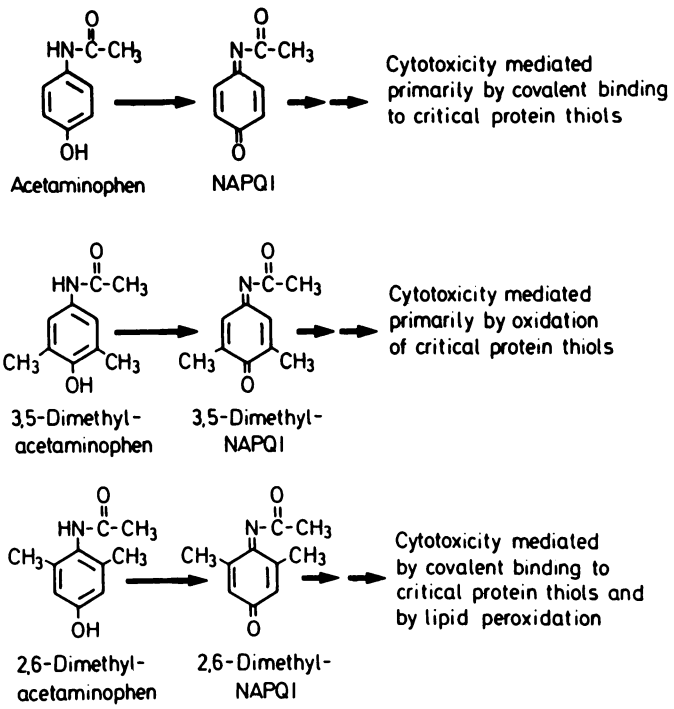

Fig. 6. Simplified scheme providing general hypotheses for mechanisms by which acetaminophen and its dimethylated analogues cause toxicity in hepatocytes.

[2] D. J. Porubek, M. Rundgren, P. J. Harvison, S. D. Nelson, and P. Moldéus, unpublished observations.

lacetaminophen. This analogue is a poor antioxidant and does not markedly inhibit MDA formation in the cell (Fig. 4). Of the three hydroxyacetanilides, it is probably oxidized the least rapidly to a quinone imine. BCNU treatment has no measurable effect on the cytotoxicity of 2,6-dimethylacetaminophen, whereas DEM pretreatment markedly accentuates its toxicity and binding to cellular proteins (Figs. 1 and 5). This indicates that GSH reacts efficiently to remove reactive metabolites of 2,6-dimethylacetaminophen. GSH is depleted by treatment of hepatocytes with 2,6-dimethylacetaminophen, but GSSG formation is not detectable (Fig. 2). At least two GSH conjugates of 2,6-dimethylacetaminophen are formed in hepatocytes (data not shown), but we have only been able to characterize one of these to date (16).

When GSH is depleted by DEM treatment, both binding to cellular proteins and lipid peroxidation contribute to cause cytotoxicity. Additional support for our interpretation of the results is the observation that desferrioxamine partially protects against cytotoxicity mediated by 2,6-dimethylacetaminophen without altering covalent protein binding. Conversely, DTT has a more marked effect on cytotoxicity caused by 2,6-dimethylacetaminophen than that caused by acetaminophen, probably because it decreases both the binding reaction and lipid peroxidative reactions.

In summary, acetaminophen and its dimethylated analogues, 3,5-dimethylacetaminophen and 2,6-dimethylacetaminophen, are all cytotoxic to hepatocytes isolated from phenobarbital-pretreated rats. As summarized diagrammatically (Fig. 6), we believe that the cytotoxic effects of the analogues are mediated by reactions of their oxidation products, the reactive quinone imines. Both the hydroxyacetanilides and the quinone imines have different physicochemical properties and, therefore, the cytotoxic mechanism of each hydroxyacetanilide depends on its redox potential, its rate of conversion to a quinone imine, and the nature of the interaction of the quinone imines with cellular constituents, particularly with sulfhydryl groups on proteins and peptides. We cannot rule out the possibility that some of the differences that we observe with BCNU and DEM treatments are caused by the effects of these agents on toxification or detoxification processes other than those we have described. However, the results clearly demonstrate that the three hydroxyacetanilides react in different ways to cause their cytotoxic effects. Reactions of the respective quinone imines in hepatocytes as they relate to cytotoxicity are being investigated presently.

Note added in proof. After submission of the manuscript, van de Straat *et al.* (32) reported that acetaminophen and 3,5-dimethylacetaminophen react similarly in hepatocytes from 3-methylcholanthrene-treated rats. That is, acetaminophen was toxic without evidence of GSSG formation, whereas 3,5-dimethylacetaminophen did not form a GSH conjugate but formed GSSG. Furthermore, these investigators provide evidence that cytochrome P-450 is required for the conversion of 3,5-dimethylacetaminophen to a reactive metabolite that oxidizes GSH to GSSG.

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