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The Metabolism of *N*-Acetyl-3,5-dimethyl-*p*-benzoquinone Imine in Isolated Hepatocytes Involves *N*-Deacetylation

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SUMMARY

3,5-Dimethyl-N-acetyl-p-benzoquinone imine (3,5-dimethyl-NAPQI) was cytotoxic to isolated hepatocytes from Sprague Dawley rats at levels between 200 and 300 μM. It rapidly oxidized intracellular glutathione within 10 sec, with the formation of oxidized glutathione. The cytotoxicity of 3,5-dimethyl-NAPQI could be prevented over a 3.5-hr period with the carboxylesterase inhibitor bis(*p*-nitrophenyl) phosphate, indicating that cytotoxicity involved *N*-deacetylation. The *N*-deacetylated product could be trapped with glutathione as 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol in 3,5-dimethyl-NAPQI-treated hepatocytes but not in hepatocytes pretreated with bis(*p*-nitrophenyl) phosphate, indicating that *N*-deacetylation activity had been inhibited. 3,5-Dimethyl-NAPQI was readily *N*-deacetylated by rat liver microsomes, in contrast to 3,5-dimethylacetaminophen. The latter was also not cytotoxic to hepatocytes at up to 2 mM. The *N*-deacetylated product 4-amino-2,6-dimethylphenol rapidly underwent autoxidation to form 2,6-dimethylbenzoquinone imine and was highly cytotoxic to hepatocytes at 200–300 μM. The latter reacted with glutathione to give the above conjugate and no glutathione oxidation occurred. Dithioerythritol (2 mM) added at 10, 20, and 30 min after 3,5-dimethyl-NAPQI delayed but did not prevent cytotoxicity. Dithioerythritol also resulted in the partial restoration of GSH, presumably as a result of reduction of protein mixed disulphides. The mechanism of cytotoxicity of 3,5-dimethyl-NAPQI therefore appears to be a result of a combination of oxidative stress and deacetylation resulting in arylation.

Acetaminophen (paracetamol), a widely used analgesic and antipyretic drug, can cause severe tissue necrosis at high doses in humans and experimental animals (1, 2). The hepato- and nephrotoxicity have been attributed to the formation of the electrophilic metabolite NAPQI, which is detoxified by reaction with GSH to form 3-(glutathion-*S*-yl)-acetaminophen (3). However, when tissue GSH is depleted, the quinone imine binds to proteins to initiate events leading to hepatic necrosis (4). The latter binding can involve protein thiol groups, to form a thioether linkage at the C-3 or C-5 positions of the acetaminophen (5).

In view of this, it would be expected that the C-3 and C-5 methyl groups of 3,5-dimethylacetaminophen would prevent protein binding and the subsequent cytotoxicity. Indeed, in a chemical system 3,5-dimethyl-NAPQI is fully reduced to 3,5-dimethylacetaminophen by GSH, and GSH is oxidised to GSSG (6). However, 3,5-dimethylacetaminophen has been shown to be as hepatotoxic as acetaminophen in rats and mice (7). This has been used as evidence against the theory that acetaminophen toxicity is initiated by the covalent binding of quinone imine to critical proteins. Furthermore, numerous antioxidants such as promethazine (8), α-tocopherol (8, 9), diphenyl-*p*-phenylenediamine (8, 9), and 3-*O*-methyl-(+)-catechin (10), as well as the metal chelator desferoxamine (9), protect hepatocytes against acetaminophen toxicity without inhibiting the degree of covalent binding. β-Mercaptopropionylglycine also prevents hepatotoxicity induced by acetaminophen *in vivo* without inhibiting covalent binding (11).

NAPQI cytotoxicity in isolated hepatocyte systems has been associated with GSH loss (12–14) and protein thiol loss (12, 14), both via oxidation and arylation and disturbances in Ca^{2+} homeostasis (14). The cytotoxicity of NAPQI has been suggested to be the result of its oxidative effects inasmuch as the restoration of protein thiol groups in NAPQI-treated rat hepatocytes with dithiothreitol prevented cytotoxicity (12). Acetaminophen cytotoxicity in hamster hepatocytes was also decreased when dithiothreitol was added after treatment with acetaminophen but preceding cell death (15). However, others have shown that acetaminophen hepatotoxicity *in vivo* is not accompanied by the release of oxidized glutathione into the plasma (16).

Another possible metabolic pathway that forms reactive in-

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ABBREVIATIONS: NAPQI, *N*-acetyl-p-benzoquinone imine; FAB, fast atom bombardment; HPLC, high pressure liquid chromatography; BNPP, bis(*p*-nitrophenyl) phosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Me_2SO, dimethylsulphoxide; DETAPAC, diethylenetriamine penta-acetic acid.

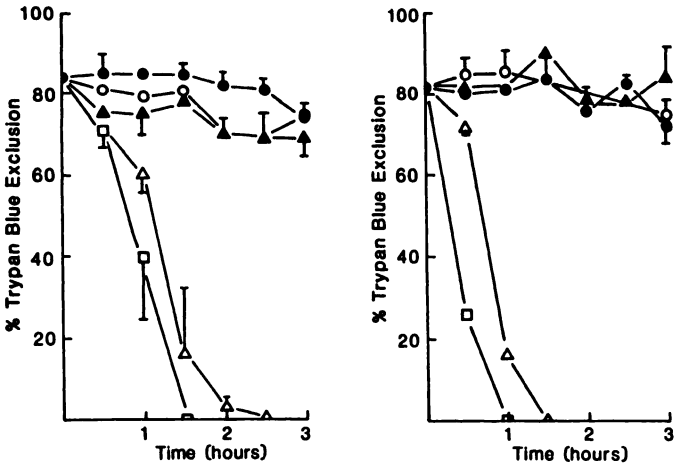

Fig. 1. Hepatocyte cytotoxicity induced by 3,5-dimethyl-NAPQI (A) and 4-amino-2,6-dimethylphenol (B). A, Hepatocytes (1×10^6 cell/ml) incubated with 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△), or 0.4 mM (□) 3,5-dimethyl-NAPQI. Control cells (●). B, Hepatocytes (1×10^6 cells/ml) incubated with 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△), or 0.4 mM (□) 4-amino-2,6-dimethylphenol hydrochloride. Control cells (●). Cell viability was determined by Trypan blue exclusion. Values represent averages of three experiments with error bars representing the standard deviations.

termediates involves an *N*-deacetylation reaction which has been suggested to explain the nephrotoxicity of acetaminophen observed in F344 rats (17). NAPQI and *N*-acetyl-3,5-dimethylbenzoquinone imine may be *N*-deacetylated by rat liver microsomes inasmuch as the 4-aminophenoxy radical and 2,6-dimethyl-4-aminophenoxy radical were detected by EPR when these quinone imines were incubated with microsomes (18).

In this paper, the cytotoxicity of 3,5-dimethyl-NAPQI in isolated hepatocytes was prevented by BNPP, a carboxylesterase inhibitor (19). Also, the *N*-deacetylated product 2,6-dimethylbenzoquinone imine was trapped with glutathione as 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol in 3,5-dimethyl-NAPQI-treated hepatocytes but not in hepatocytes preincubated with BNPP. 4-Amino-2,6-dimethylphenol was cytotoxic and reacted with glutathione (via autoxidation to 2,6-dimethylbenzoquinone imine) to form the above conjugate without causing glutathione oxidation. Thus, the toxicity of 3,5-dimethyl-NAPQI may not be entirely due to its oxidant properties.

Experimental Procedures

Materials. Glutathione, oxidized glutathione (disodium salt), DETAPAC, BNPP (sodium salt), fluoro-2,4-dinitrobenzene, iodoacetic acid, hydrogen peroxide (30%), 2,6-dimethylphenol, Trypan blue, and horseradish peroxidase (type VI) were obtained from Sigma Chemical Co. (St. Louis, MO). Dithioerythritol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Collagenase (from *Clostridium histoliticum*), HEPES, and NADPH were purchased from Boehringer-Mannheim (Montreal, Canada). HPLC solvents and Me_2SO were obtained from BDH Chemicals (Toronto, Canada).

Isolation and incubation of hepatocytes. Hepatocytes were prepared from the livers of male Sprague-Dawley rats (180–250 g) by collagenase perfusion (20). Animals were fed a standard chow diet *ad libitum*. Isolated cells were incubated in Krebs-Henseleit buffer, pH 7.4 (20×10^6 cells/20 ml), containing 12.5 mM HEPES, at 37° in rotating round bottom flasks under an atmosphere of 95% O_2/5% CO_2. Cell viability (normally 85–95%) was determined by Trypan blue exclusion (final concentration, 0.16%). Cells were preincubated for 15 min before the addition of the various chemicals in Me_2SO. In experiments with

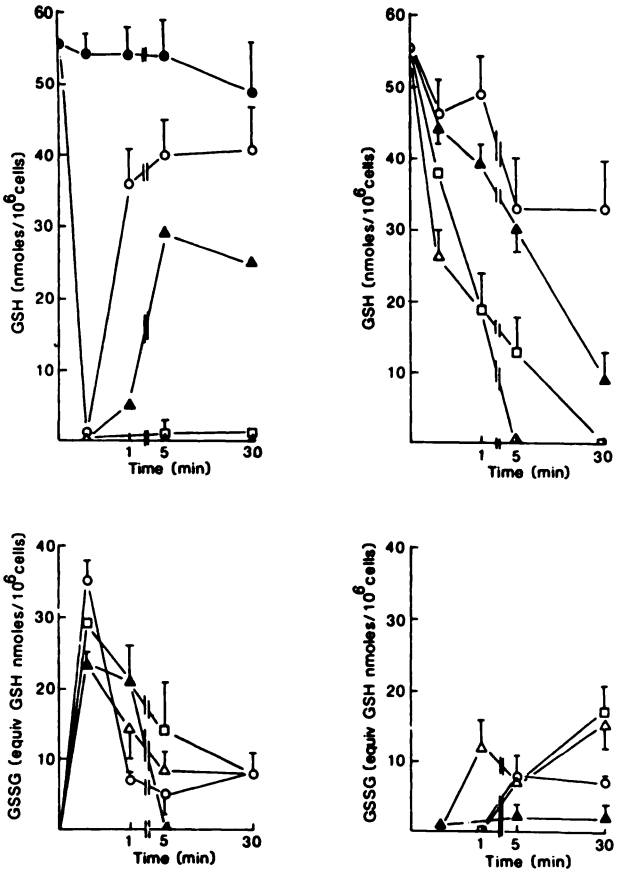

Fig. 2. Effect of 3,5-dimethyl-NAPQI and 4-amino-2,6-dimethylphenol on hepatocyte GSH and GSSG levels. A, Hepatocyte (1×10^6 cells/ml) GSH levels on treatment with 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△), or 0.4 mM (□) 3,5-dimethyl-NAPQI. Control cells (●). B, Hepatocyte (1×10^6 cells/ml) GSH levels on treatment with 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△), or 0.4 mM (□) 4-amino-2,6-dimethylphenol. C, Hepatocyte (1×10^6 cells/ml) GSSG levels on treatment with 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△), or 0.4 mM (□), 3,5-dimethyl-NAPQI. D, Hepatocyte (1×10^6 cells/ml) GSSG levels on treatment with 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△), or 0.4 mM (□) 4-amino-2,6-dimethylphenol. GSSG values were corrected for the small amount (5–10 nmol) of GSSG found in hepatocyte samples at $t = 0$, which was located in the media. Values represent averages of three experiments with error bars representing the standard deviations.

the carboxylesterase inhibitor BNPP, the cells were preincubated for an additional 5 min in the presence of inhibitor before the addition of 3,5-dimethyl-NAPQI or 4-amino-2,6-dimethylphenol.

Synthesis of 3,5-dimethylacetaminophen. 3,5-Dimethylacetaminophen was synthesized according to the procedure described by Fernando *et al.* (7). The white needles gave a UV λ_{max} (methanol) at 242 nm ($\epsilon = 3603$) and 282 nm ($\epsilon = 360$); ^1H NMR (80 MHz) ($CDCl_3$): 7.08 ppm (s, 2H), 4.3 ppm (broad s, 1H), 2.22 ppm (s, 6H), 2.13 ppm (s, 3H); mass spectrum, *m/z* 179 (M; relative intensity, 67%), 137 (M−$COCH_2$; relative intensity, 100%).

Synthesis of 4-amino-2,6-dimethylphenol hydrochloride. 4-Amino-2,6-dimethylphenol was synthesized from 2,6-dimethyl-4-nitrosophenol according to the general procedures described by Fernando *et al.* (7) but because it was extremely labile to oxidation under these conditions it was isolated as the hydrochloride salt. 2,6-Dimethyl-4-nitrosophenol (0.4 g, 2.48 mmol) in acetic acid (5 ml) was combined with platinum oxide (2.5 mg) and hydrogenated for 2 hr at room temperature under medium pressure (45 psi). The yellow solution turned greenish and after 2 hr became colorless. After the solution was removed from the hydrogenator it was rapidly filtered and 6 N HCl (2 ml) was added. The white crystals that separated on standing for 1 hr were isolated by filtration and dried. 4-Amino-2,6-dimethylphenol (hy-

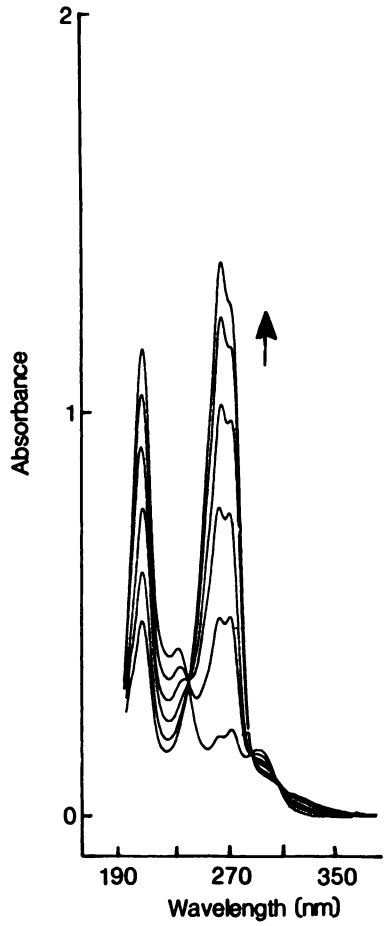

Fig. 3. Optical spectrum of the autoxidation of an aqueous solution of 4-amino-2,6-dimethylphenol with time. The reaction mixture contained 4-amino-2,6-dimethylphenol (100 μM) in 40 mM phosphate buffer, pH 7.4. The first scan represents t = 2 min and the last scan represents t = 15 min.

drochloride salt) gave a UV λ_{max} (methanol) at 214 nm (ϵ = 3900) and 272 nm (ϵ = 1480); ^{1}H NMR (80 MHz) (CD_3OD/D_2O): 7.00 ppm (s, 2H), 2.23 (s, 6H); FAB mass spectrum, m/z 138 (M + 1; relative intensity, 100%), 230 (M + 1 + glycerol; relative intensity, 14%).

Synthesis of 3,5-dimethyl-NAPQI. 3,5-Dimethyl-NAPQI was synthesized according to the general methods of Dahlin and Nelson (21) and Fernando *et al.* (7). After sublimation the yellow needles gave a UV λ_{max} (hexane) at 268 nm (s) (ϵ = 24,350) and 272 nm (ϵ = 24,900); ^{1}H NMR (80 MHz) ($CDCl_3$): 1.97 ppm (s, 6H), 2.25 ppm (s, 3H), 6.92 (s, 2H).

Synthesis of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol. 4-Amino-2,6-dimethylphenol hydrochloride (0.5 mM) was incubated with peroxidase (0.5 mg), H_2O_2 (0.5 mM), and GSH (1 mM) in 40 mM sodium acetate buffer, pH 5.5 (total volume, 500 ml). The 4-amino-2,6-dimethylphenol hydrochloride was dissolved in 50 ml of methanol before the addition of the other components; the reaction was started with the addition of H_2O_2. After 5 min, the light yellow reaction mixture was extracted with ethyl acetate (3 × equal volume); the remaining light yellow aqueous layer was concentrated *in vacuo* to a small volume. Aliquots of the aqueous layer were analyzed by HPLC using a C_{18} μBondapak column (3.9 mm i.d. × 30 cm; Waters, Milford, MA) eluted isocratically with water/2% acetic acid. Elution at 1.0 ml/min (254 nm) yielded one peak with a retention time of 5.5 min. Repetitive collections yielded material for analysis.

Isolation of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol from hepatocytes. Hepatocytes (2 × 10^7 cells/ml) were incubated with 4-amino-2,6-dimethylphenol hydrochloride (3 mM) or 3,5-dimethyl-NAPQI (3 mM) for 2 hr at 37°. Occasionally, the cells were exposed to 95% O_2/5% CO_2. Stock solutions (0.5 M) were made up in Me_2SO and an equivalent amount of Me_2SO was added to the control. Under these conditions the substrates were not toxic to the cells. In incubations containing the carboxylesterase inhibitor BNPP (10 mM), the cells were preincubated with the inhibitor for 5 min before addition of substrates. Incubations were terminated by the addition of metaphosphoric acid (final concentration, 5%); precipitated protein was removed by centrifugation and the pH of the supernatant was adjusted to pH 5.5. Samples were extracted with ethyl acetate (3 × equal volume) and stored at −20°. Aliquots were examined by HPLC (254 nm) using a C_{18} μBondapak column (3.9 mm i.d. × 30 cm; Waters) eluted (1.0 ml/min) with a linear gradient of H_2O/2% acetic acid to 7% methanol/H_2O/2% acetic acid.

TABLE 1

Summary of proton NMR assignments for GSH, 4-amino-2,6-dimethylphenol, and 3-(glutathion-S-yl)-4-amino-2,6-dimethylphenol

Chemical shifts are measured from trimethylsilane as external standard. Following the chemical shift, the splitting and the number of protons are in parentheses, 400 MHz.

Assignment	Chemical shifts		
	Glutathione[a]	4-Amino-2,6-dimethyl-phenol[b]	3-(Glutation-S-yl)-4-amino-2,6-dimethylphenol[c]
		ppm	
2 CH_3		2.23 (s,6)	2.24 (s,3)
6 CH_3			2.44 (s,3)
3,5 H		7.01 (s,2)	7.10 (s,1)
Gly_{α}	3.81 (m,2)		3.69 (d,1)
$Gly_{\alpha'}$			3.63 (d,1)
Cys_{α}	4.40 (t,1)		4.52 (t,1)
Cys_{β}	2.77 (m,1)		3.13 (dd,1)
$Cys_{\beta'}$	2.79 (m,1)		3.24 (dd,1)
Glu_{α}	3.66 (t,1)		3.76 (t,1)
$Glu_{\beta,\beta'}$	2.01 (m,2)		2.10 (m,2)
$Glu_{\gamma,\gamma'}$	2.40 (m,2)		2.43 (m,2)

[a] Glutathione spectra were recorded at room temperature in D_2O. Coupling constants are $J_{cys_{\alpha,\beta}}$ = 6.4 Hz, $J_{cys_{\beta,\beta}}$ = 1.8 Hz, $J_{glu_{\alpha,\beta}}$ = 6.4 Hz.

[b] 4-Amino-2,6-dimethylphenol spectra were recorded at room temperature in D_2O/CD_3OD.

[c] 3-(Glutathion-S-yl)-4-amino-2,6-dimethylphenol spectra were recorded at room temperature in D_2O. Coupling constants are $J_{gly_{\alpha,\alpha'}}$ = 18.6 Hz, $J_{cys_{\alpha,\beta}}$ = 4.9 Hz, $J_{cys_{\beta,\beta}}$ = 14.2 Hz, $J_{glu_{\alpha,\beta}}$ = 6.2 Hz.

Microsomal *N*-deacetylation of 3,5-dimethyl-NAPQI. Rat liver microsomes were prepared by the method of Ernster *et al.* (22). After being washed with 0.15 M Tris·HCl, pH 8.0, the microsomes were resuspended in the same buffer and kept in a −80° freezer. Protein determinations were made by the method of Bradford (23) using bovine serum albumin as standard. Incubations contained microsomes (0.74 mg), substrate (1 mM), and NADPH (0.5 mM) in 0.1 M phosphate buffer, pH 7.4, containing 1 mM DETAPAC (total volume, 1.0 ml) (18). In incubations containing the carboxylesterase inhibitor BNPP (1 mM), the mixtures were preincubated for 5 min before addition of substrate. After incubation at 37° for 60 min, the reaction was stopped by extracting the sample with ethyl acetate (1 ml). The amount of indophenol formed was determined by measurement of the absorbance at 500 nm (ϵ = 3162).

GSSG and GSH determinations. GSSG and GSH were determined by the HPLC method of Reed *et al.* (24) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene using a μBondapak NH_2 column (3.9 mm i.d. × 30 cm; Waters). Determination of GSH and GSSG in hepatocytes was carried out on the supernatant after precipitation of the protein with metaphosphoric acid (final concentration, 5%).

HPLC. GSH and GSSG determinations were carried out using a Waters 6000A solvent delivery system equipped with a WISP 710A automatic injector, a model 660 solvent programmer, and a Data Module. Analysis of metabolites from hepatocyte incubations were

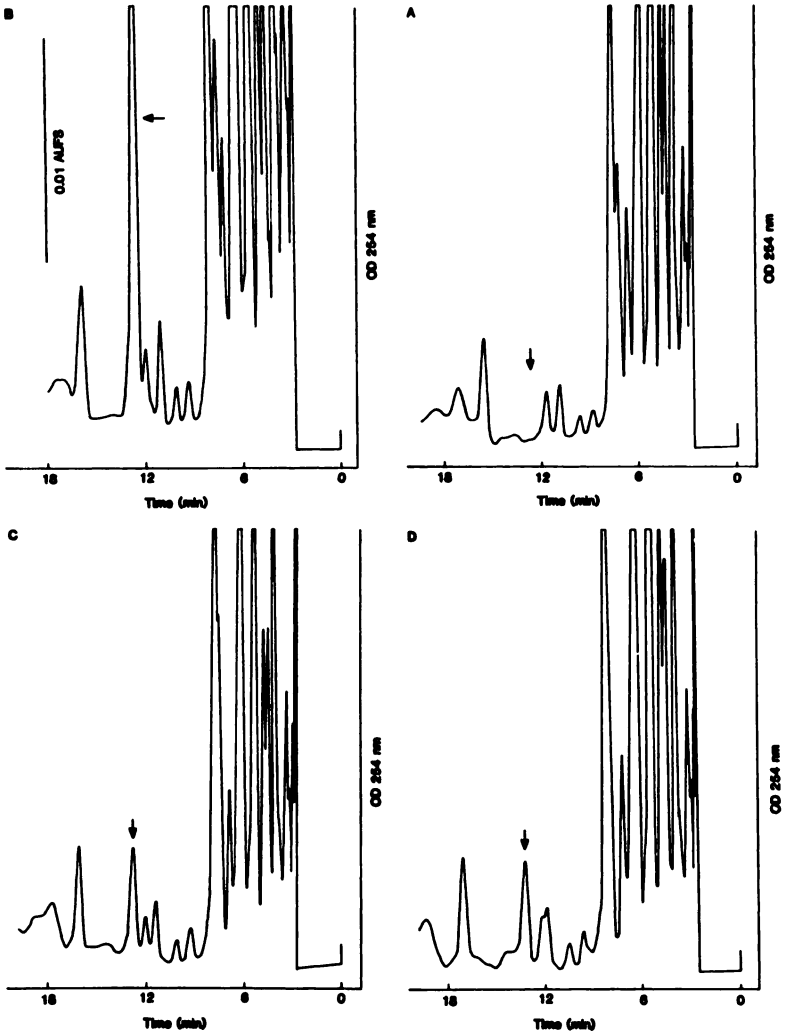

Fig. 4. HPLC tracings of hepatocyte extracts from cells treated with 3,5-dimethyl-NAPQI or 4-amino-2,6-dimethylphenol. A, Control cells. B, Control cell extract spiked with standard 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol. C, Cells treated with 3 mM 3,5-dimethyl-NAPQI. D, Cells treated with 3 mM 4-amino-2,6-dimethylphenol. In each case hepatocytes (2×10^7 cells/ml) were incubated at 37° for 2 hr with substrate and the reaction was terminated by the addition of metaphosphoric acid (final concentration, 5%). *AUFS*, absorbance units full scale.

carried out with a Beckmann 110A solvent delivery system, a 421 controller, a manual Altex 210A injector, a ERC-7210 UV detector, and a Linseis L 6512 recorder.

NMR spectrometry. A WH-400 MHz or WH-80 MHz Bruker instrument was used to record proton NMR spectra of samples.

Mass Spectrometry. A VG-Micromass 770E instrument or one with an Xe atom beam was used at ambient temperature to record spectra or FAB spectra of samples prepared in glycerol and deposited on the probe (25).

Results

Hepatocyte cytotoxicity. 3,5-Dimethyl-NAPQI was cytotoxic to hepatocytes (as determined by Trypan blue exclusion) in the concentration range above 200 μM (Fig. 1A). Within 30 min of addition to the cells, concentrations above 200 μM caused the cells to undergo surface "blebbing," a phenomenon described by other authors after exposure of isolated rat hepatocytes to NAPQI (12, 14). A similar result was obtained with 4-amino-2,6-dimethylphenol (Fig. 1B). 3,5-Dimethylacetaminophen up to 2 mM was not cytotoxic. 3,5-Dimethyl-NAPQI caused a rapid depletion in GSH levels (within 10 sec), which returned to control levels within 30 min with 0.1 mM 3,5-dimethyl-NAPQI but remained depressed with higher concentrations (Fig. 2A). Only approximately 50% of the depleted glutathione at 10 sec could be accounted for as GSSG but, in the case of 0.1 mM 3,5-dimethyl-NAPQI, 80% of the glutathione was eventually restored, indicating that some of the glutathione that was unaccounted for at the early time points was not irreversibly lost (Fig. 2C). With 4-amino-2,6-dimethylphenol, the glutathione gradually decreased with time at a rate dependent on 4-amino-2,6-dimethylphenol concentration (Fig. 2B) and only a small proportion of the missing glutathione could be accounted for as GSSG (Fig. 2D). 3,5-Dimethylacetaminophen (2 mM) did not cause any GSH disappearance.

Isolation and identification of the 4-amino-2,6-dimethylphenol glutathione conjugate. Autoxidation of 4-amino-2,6-dimethylphenol in phosphate buffer, pH 7.4, led to the rapid appearance of the 2,6-dimethylbenzoquinone imine, identified via its absorption spectrum at 266 nm and 272 nm (26) (Fig. 3). At pH 5.5, autoxidation did not occur. At higher concentrations (0.5 mM), the reaction mixture also gave a reddish colored pigment (λ_{max} 500 nm), which has previously been identified as the indophenol (18, 26). The 2,6-dimethylbenzoquinone imine could be immediately formed by oxidation with equimolar H_2O_2 and horseradish peroxidase at pH 5.5. The addition of 2 equivalents of glutathione to the 2,6-dimethyl-benzoquinone imine resulted in its immediate disappearance and the appearance of new water-soluble compound(s). The same compound(s) were formed upon autoxidation of the 4-amino-2,6-dimethylphenol in the presence of glutathione (2 equivalents) at pH 7.4.

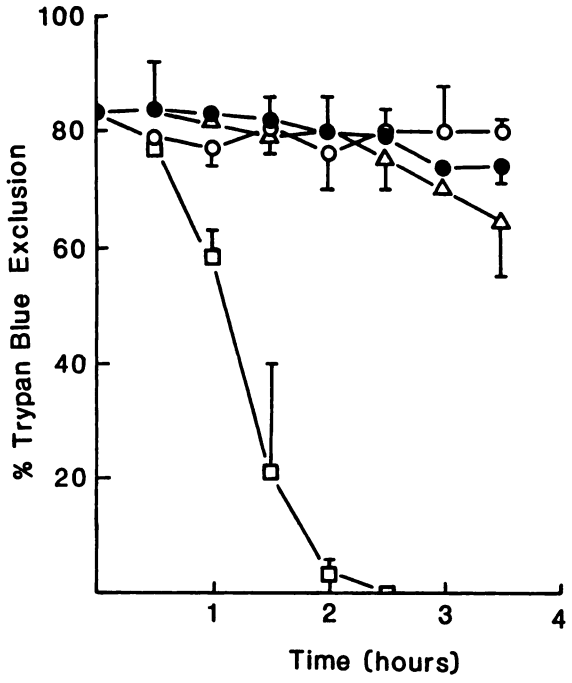

Fig. 5. Effect of BNPP on 3,5-dimethyl-NAPQI-induced cytotoxicity in hepatocytes. Hepatocytes (1×10^6 cells/ml) were treated with 0.5 mM BNPP, (○), 0.3 mM 3,5-dimethyl-NAPQI (□), or 0.5 mM BNPP and 0.3 mM 3,5-dimethyl-NAPQI (△). Control cells (●). Values represent averages of three experiments with error bars representing the standard deviations.

Repetitive HPLC gave enough material for NMR analysis (Table 1). In the conjugate, both the C-5 proton and 6-CH_3 protons were shifted downfield 0.1 and 0.2 ppm, respectively, consistent with the structure 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol. The FAB mass spectrum gave *m/z* 443 (M + 1; relative intensity, 3.5%), 320 (NSG; relative intensity, 52.5%), 322 (H_2NSG; relative intensity, 25.6%), 306 (SG; relative intensity, 25.0%), 137 (M + 1 − GSH; relative intensity, 5%), and 122 (M − NSG: relative intensity, 100%). This unusual mass spectrum deserves comment in that it gives a very weak molecular ion and the molecule appears to have undergone rearrangement in which the glutathione attaches to the amino group, giving rise to the fragments at 122 and 320. A similar rearrangement occurs with the monoglutathione conjugate of *p*-aminophenol.[2]

[2] L. G. McGirr and P. J. O'Brien, unpublished observations.

Formation of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol in hepatocytes. Incubation of hepatocytes (2×10^7 cells/ml) with nontoxic doses of 4-amino-2,6-dimethylphenol or with 3,5-dimethyl-NAPQI (3 mM) (equivalent to 150 μM substrate with 1×10^6 cells/ml) led to the formation of approximately equal amounts of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol as determined by HPLC (Fig. 4, C and D).

Effect of the carboxylesterase inhibitor BNPP on the toxicity of 3,5-dimethyl-NAPQI and the formation of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol in isolated hepatocytes. As shown in Fig. 5, the carboxylesterase inhibitor BNPP (0.5 mM) completely inhibited the toxicity of 3,5-dimethyl-NAPQI (300 μM) in isolated hepatocytes. It had no effect on the toxicity of 4-amino-2,6-dimethylphenol (results not shown). BNPP alone, at levels of 0.5 mM, did not cause any cytotoxicity or glutathione loss in the isolated hepatocytes. BNPP (10 mM BNPP with 2×10^7 cells/ml, equivalent to 0.5 mM BNPP with 1×10^6 cells/ml) also prevented the formation of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol in hepatocytes treated with 3,5-dimethyl-NAPQI (Fig. 6), although it did not affect its formation from 4-amino-2,6-dimethylphenol. BNPP did not affect the initial oxidation of GSH to GSSG catalyzed by 3,5-dimethyl-NAPQI. In hepatocytes treated with 3,5-dimethyl-NAPQI (300 μM), glutathione disappeared rapidly and did not return. In hepatocytes treated with 3,5-dimethyl-NAPQI in the presence of BNPP, some glutathione (15–40% of original) was restored between 5 and 20 min after its initial rapid disappearance. BNPP treatment of the cells resulted in the formation of substantial quantities of *p*-nitrophenol. *p*-Nitrophenol (100 μM) had no effect on the autoxidation of 4-amino-2,6-dimethylphenol (100 μM) and did not react with 3,5-dimethyl-NAPQI (100 μM).

Effect of dithioerythritol on the toxicity of 3,5-dimethyl-NAPQI in isolated hepatocytes. Dithioerythritol delayed the cytotoxicity of 3,5-dimethyl-NAPQI when it was added to the cells at 10, 20, and 30 min after the addition of 3,5-dimethyl-NAPQI; the addition at 10 min gave the most protection (Fig. 7). The cells rapidly removed the 3,5-dimethyl-NAPQI, $t_{1/2}$ at 100 μM, 12 sec; $t_{1/2}$ at 300 μM, 48 sec, presumably mainly as a result of reduction to 3,5-dimethylacetaminophen. Analysis of GSH 2 min after addition of dithioerythritol at 20 min indicated that GSH had returned to 45% (20–25 nmol/10^6 cells) that of the control values. Inasmuch as dithioerythritol can totally protect against oxidative stress situations by reduc-

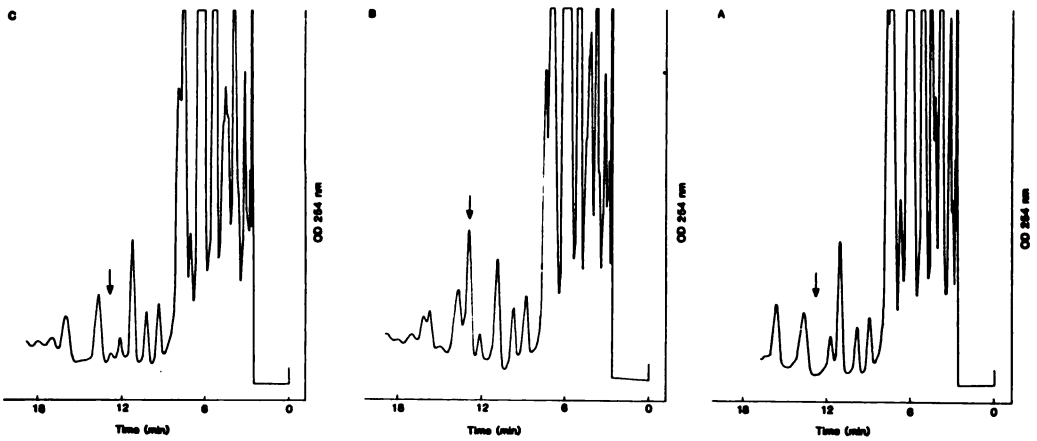

Fig. 6. HPLC tracings of hepatocyte extracts from hepatocytes treated with 3,5-dimethyl-NAPQI or 4-amino-2,6-dimethylphenol in the presence of the carboxylesterase inhibitor BNPP. A, Cells treated with 10 mM BNPP. B, Cells treated with 10 mM BNPP and 3 mM 4-amino-2,6-dimethylphenol. C, Cells treated with 10 mM BNPP and 3 mM 3,5-dimethyl-NAPQI. Hepatocytes (2×10^7 cells/ml) were preincubated at 37° for 5 min in the presence of BNPP before incubation with substrates.

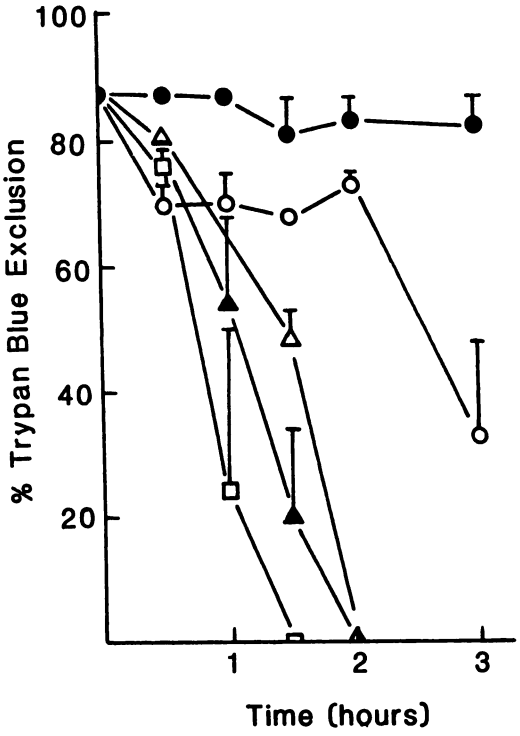

Fig. 7. Effect of dithioerythritol on 3,5-dimethyl-NAPQI-induced cytotoxicity in hepatocytes. Hepatocytes (1×10^6 cells/ml) were treated with 0.5 mM 3,5-dimethyl-NAPQI (□), 0.5 mM 3,5-dimethyl-NAPQI followed by 2 mM DTE at 10 min (○), 0.5 mM dimethyl-NAPQI followed by 2 mM DTE at 20 min (△), and 0.5 mM dimethyl-NAPQI followed by 2 mM DTE at 30 min (▲). Control cells (●). Values represent averages of three experiments with error bars representing the standard derivations.

ing GSSG to GSH or protein mixed disulfides, enough alkylation had already occurred at 10 min to eventually kill the cell.

Microsomal *N*-deacetylation of 3,5-Dimethyl-NAPQI. Rat liver microsomes catalyze the *N*-deacetylation of 3,5-dimethyl-NAPQI as determined by the formation of 2,2′,6,6′-tetramethylindophenol (Table 2). Although NADPH appears to stimulate *N*-deacetylation, it more likely reduces the 2,6-dimethylbenzoquinone imine to 4-amino-2,6-dimethylphenol, allowing the formation of the indophenol more readily. BNPP completely prevented the formation of indophenol in the absence or presence of NADPH. The mechanism of 2,2′,6,6′-tetramethylindophenol formation is extremely complex. Its formation from 4-amino-2,6-dimethylphenol is not linear with concentration but gives a hyperbolic curve. At levels of 4-amino-2,6-dimethylphenol below 150 μM, no indophenol is formed. Although 3,5-dimethylacetaminophen apparently was not deacetylated in this system, small amounts of deacetylation would not have been detected.

Discussion

3,5-Dimethyl-NAPQI was found to be cytotoxic to isolated hepatocytes in the same concentration range previously described for NAPQI (12–14). The initiation of NAPQI cytotoxicity has been largely attributed to oxidative stress caused by GSH oxidation and protein thiol oxidation, inasmuch as dithiothreitol can reverse processes leading to cytotoxicity. However, in this case, dithioerythritol only delayed the processes leading to cytotoxicity with 3,5-dimethyl-NAPQI. Furthermore, the cytotoxicity of 3,5-dimethyl-NAPQI in isolated hepatocytes was readily eliminated over a 3.5-hr time period with the carboxylesterase inhibitor BNPP, indicating that the cytotoxicity in hepatocytes involves *N*-deacetylation. The *N*-deacetylation of 3,5-dimethyl-NAPQI to 2,6-dimethylbenzoquinone imine was demonstrated in the hepatocyte by the isolation of 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol. Furthermore, this GSH conjugate was not formed in hepatocytes treated with BNPP. BNPP has previously been used *in vivo* to prevent methemoglobinemia caused by phenacetin deacetylation (27) and nephrotoxicity caused by acetaminophen deacetylation to *p*-aminophenol (17).

It was recently reported that 3,5-dimethyl-NAPQI was not cytotoxic to hepatocytes from male albino Wistar rats unless the GSH levels were lowered by prior treatment with diethylmaleate or *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea; GSH depletion upon 3,5-dimethyl-NAPQI treatment was also not found (28). In view of the toxicity and depletion of GSH, reported in this communication, via an *N*-deacetylation pathway, large strain and species differences in toxicity may occur as a result of differences in carboxylesterase activity. Large species differences in liver microsomal carboxylesterase activity have been reported with phenacetin and acetylaminofluorene and their *N*-hydroxy derivatives (29–31).

TABLE 2

Effect of bis(*p*-nitrophenyl) phosphate on the *N*-deacetylation of *N*-acetyl-3,5-dimethyl benzoquinone imine by rat liver microsomes

Incubations contained microsomes (0.74 mg of protein), substrate (1 mM), NADPH (0.5 mM), and BNPP (1 mM) in 0.1 M phosphate buffer, pH 7.4, containing 1 mM DETAPAC (total volume, 1.0 ml). After incubation at 37° for 1 hr, the 2,2′,6,6′-tetramethyl indophenol formed was extracted into ethyl acetate (1.0 ml) and the absorbance was determined at 500 nm. Results are expressed as averages ± standard deviation from three experiments.

Substrate	Incubation condition	A_{500}/mg of protein	Indophenol	Deacetylation
			nmol/mg	%
4-Amino-2,6-dimethylphenol	Buffer	1.20 ± 0.09	379.5 ± 28.5	
	Microsomes	0.93 ± 0.08	294.1 ± 25.3	
	Microsomes + NADPH	0.97 ± 0.03	306.8 ± 9.5	
	Microsomes + NADPH + BNPP	1.03 ± 0.08	325.7 ± 25.3	
3,5-Dimethyl-NAPQI	Buffer	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5
	Buffer + NADPH	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5
	Microsomes	0.06 ± 0.01	19.0 ± 3.2	22.0 ± 7.5
	Microsomes + NADPH	0.12 ± 0.01	38.0 ± 3.2	35.0 ± 7.5
	Microsomes + NADPH + BNPP	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5
	Microsomes + BNPP	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5
3,5-Dimethylacetaminophen	Buffer	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5
	Microsomes	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5
	Microsomes + NADPH	0.01 ± 0.01	3.2 ± 3.2	7.5 ± 7.5

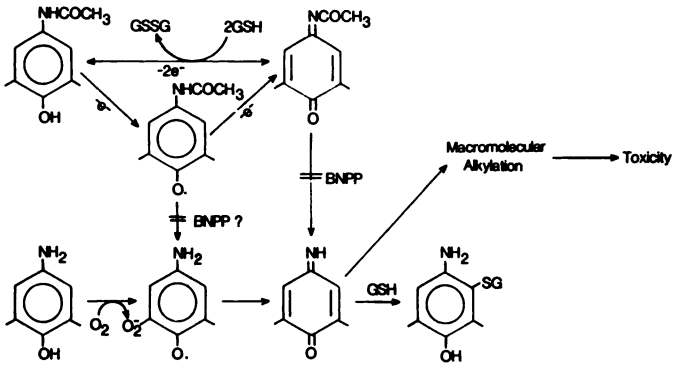

Fig. 8. Hypothetical scheme describing the route of metabolism and subsequent toxicity of 3,5-dimethyl-NAPQI.

It was found that 3,5-dimethyl-NAPQI rapidly and stoichiometrically oxidizes glutathione to GSSG and confirms the work of other investigators (6). In contrast, in hepatocytes not all the missing GSH could be accounted for as GSSG and yet, at low 3,5-dimethyl-NAPQI concentrations (100–200 μM) most of the missing GSH was restored within a short time. The missing glutathione at these early time points may be the result of protein mixed disulfide formation. Protein mixed disulfide formation occurs as a result of GSSG formation in menadione-treated hepatocytes (32). Interestingly, protein mixed disulfides can be reduced by dithiothreitol and, in our study, dithioerythritol restored glutathione levels to 45% of control values, which may explain the delay in cytotoxicity that occurs in the presence of dithioerythritol. NAPQI also oxidizes GSH to GSSG in a chemical system (6). In isolated hepatocytes, NAPQI oxidizes GSH to GSSG as well as causes protein thiol oxidation (12).

The rapid disappearance of GSSG and the simultaneous reappearance of GSH at low 3,5-dimethyl-NAPQI concentrations indicates that hepatocytes have a very high capacity to deal with oxidative stress situations. However, at higher 3,5-dimethyl-NAPQI concentrations GSSG levels remained higher for longer times and GSH was not restored. This may be attributed to an inactivation of glutathione reductase (results not shown) and/or enzymes involved in supplying NADPH. Alkylation may be involved in the enzyme inactivation, as GSH was restored if the hepatocytes were treated with BNPP before the addition of 3,5-dimethyl-NAPQI (300 μM). The consequence of this enzyme inactivation is an impairment of the ability of hepatocytes to detoxify 3,5-dimethyl-NAPQI. The accumulation of unmetabolized 3,5-dimethyl-NAPQI, GSSG, and mixed protein disulfides would be expected to cause an oxidative stress type of cytotoxicity. The slightly lower GSSG levels found with high 3,5-dimethyl-NAPQI concentrations than with low concentrations may reflect other metabolic pathways, such as *N*-deacetylation or reduction, which may compete at higher 3,5-dimethyl-NAPQI concentrations.

3,5-Dimethylacetaminophen was not toxic to hepatocytes in this study and other studies as well (33) but is toxic in hepatocytes from rats pretreated with phenobarbital (34). 3,5-Dimethylacetaminophen is apparently a poorer substrate for the carboxylesterases than is the quinone imine derivative because *N*-deacetylation of 3,5-dimethylacetaminophen should have given rise to the toxic 4-amino-2,6-dimethylphenol and caused GSH depletion. Experiments with isolated rat liver microsomes also suggested this because they were effective at *N*-deacetylating 3,5-dimethyl-NAPQI but were ineffective at *N*-deacetylating 3,5-dimethylacetaminophen. Phenobarbital has been found to induce specific isozymes of carboxylesterases (35) that may have contributed to the toxicity of 3,5-dimethylacetaminophen in phenobarbital treated rats.

4-Amino-2,6-dimethylphenol was cytotoxic in hepatocytes presumably because it readily autoxidises to 2,6-dimethylbenzoquinone imine, which readily reacts with GSH (or possibly protein thiols) to give 3-(glutathion-*S*-yl)-4-amino-2,6-dimethylphenol. Hydrogen peroxide formed during the autoxidation of 4-amino-2,6-dimethylphenol could cause the oxidation of GSH to GSSG and/or contribute to the cytotoxicity. Hydrogen peroxide is not toxic to isolated hepatocytes unless the enzymic defense system of the cell against oxidative stress is compromised in some manner, e.g., inhibition of catalase or glutathione reductase (36). However, inhibition of catalase in the hepatocyte with azide (5 mM) did not increase cytotoxicity to 4-amino-2,6-dimethylphenol, suggesting that cytotoxicity is the result of alkylation of protein thiols by 2,6-dimethylbenzoquinone imine rather than H_2O_2 formation. It is not likely that 4-amino-2,6-dimethylphenol is entirely responsible for the cytotoxicity of 3,5-dimethyl-NAPQI because 4-amino-2,6-dimethylphenol forms 10 times more quinone imine than 3,5-dimethyl-NAPQI in microsomes but produces about the same amount of cytotoxicity.

The cytotoxic mechanisms of 3,5-dimethyl-NAPQI therefore may involve a combination of oxidative stress and arylation of critical macromolecules after *N*-deacetylation. The initial oxidation of GSH to GSSG effectively removes the GSH so that the 2,6-dimethylbenzoquinone imine formed by *N*-deacetylation accumulates, causes protein arylation and enzyme inactivation, and prevents the restoration of GSH. Cytotoxicity likely than ensues as a result of 3,5-dimethyl-NAPQI, GSSG, and protein-glutathione mixed disulfides causing oxidative stress in the compromised hepatocyte.

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