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DISSOCIATION OF COVALENT BINDING FROM THE OXIDATIVE EFFECTS OF ACETAMINOPHEN

STUDIES USING DIMETHYLATED ACETAMINOPHEN DERIVATIVES*

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Abstract—The cytotoxic effects of 10 mM acetaminophen (APAP) in primary cultures of non-induced mouse hepatocytes are accompanied by depletion of intracellular glutathione (GSH), arylation of protein, and loss of protein sulfhydryl (PSH) groups. Investigation of the stoichiometry of the covalent binding and PSH loss after APAP exposure demonstrated a greater loss in PSH than could be accounted for by covalent binding to proteins and suggests that APAP exhibits both oxidative and arylative actions in cell culture. Subcellular fractionation revealed that the PSH oxidation induced by APAP was greatest in the microsomal fraction. Exposure of the hepatocytes to 10 mM 3,5-dimethyl-acetaminophen (3,5-DMA) or 2,6-dimethyl-acetaminophen (2,6-DMA) permitted dissociation of the oxidative and arylative properties of APAP. Even though treatment of cultured hepatocytes with 3,5-DMA did not result in covalent binding, there was a more rapid depletion of intracellular GSH, oxidation of PSH, and cytotoxicity compared to APAP. This investigation also provides the first evidence that the cytotoxic effects of both APAP and 3,5-DMA are accompanied by the formation of protein aggregates of high molecular weight that are not disulfide linked. The aggregates probably reflect the oxidative properties of these drugs and may be a mediator of their toxic effects. By contrast, 2,6-DMA, which did bind to cellular proteins and deplete GSH, did not lead to PSH loss, protein aggregation, or cytotoxicity. Since PSH oxidation and protein aggregation correlated well with cytotoxicity, these data suggest that the oxidative component of APAP and 3,5-DMA can play a significant role in eliciting cellular damage in cultured hepatocytes.

Acetaminophen (APAP) is a widely used, safe, antipyretic, analgesic which when taken in excessive doses causes severe centrilobular hepatic necrosis [3–5]. Hepatotoxicity is thought to result from the cytochrome P-450 mediated oxidation of APAP to *N*-acetyl-*p*-benzoquinone imine (NAPQI) [6–8]. Upon exposure to toxic doses of APAP, intracellular levels of GSH become exhausted, allowing NAPQI to react freely with cellular macromolecules [3, 9]. Although arylation of proteins through covalent binding is often implicated in hepatotoxicity [5, 10], several studies have suggested that oxidative or free radical reactions initiated by APAP may also be of importance [11–14]. Free radical reactions could result from a one-electron oxidation of acetaminophen generating a semiquinone radical [15] or from the enzymatic redox cycling of the quinone imine [16]. The involvement of an oxidative mechanism is supported by the fact that antioxidants [11] and thiol reducing agents [14] protect against APAP-induced toxicity, whereas agents which augment oxidative stress, such as 1-3-(2-chloroethyl-1-nitrosourea) (BCNU) or $FeCl_3$, hasten the onset and enhance the extent of hepatocyte injury [12, 17]. Therefore, NAPQI may play a bifunctional role in APAP hepatotoxicity [8, 18, 19]. It can act as an electrophile through the 3′ or 5′ carbon positions of the aromatic ring to mediate covalent binding or, alternatively, the quinone moiety can be converted to a semiquinone-imine radical that may initiate oxidative stress.

Recent attempts to evaluate the mechanistic importance of covalent binding and oxidative stress in APAP hepatotoxicity have utilized the 2,6-dimethyl (2,6-DMA) and 3,5-dimethyl (3,5-DMA) derivatives of APAP [20, 21]. When tested in mice, 3,5-DMA and APAP were shown to be equally toxic but 2,6-DMA failed to elicit detectable liver injury [20]. Analysis of the cytochrome P-450 mediated oxidation of 3,5-DMA indicates that it is activated to its quinone imine [22]. In addition, both APAP and 3,5-DMA can be oxidized to phenoxy free radicals by peroxidase mediated catalysis [23], and it has been suggested that the dimethylated derivatives retain the capacity for electron transfer reactions [24, 25].

In vitro studies have shown that *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine oxidizes GSH to GSSG, whereas *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine becomes conjugated with GSH to form 3-(glutathion-*S*-yl),2-6-dimethyl APAP [24]. In contrast, NAPQI reacts with GSH to generate both GSSG and the 3-(glutathion-*S*-yl) APAP conjugate [24]. In addition, NAPQI added exogenously to sus-

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pensions of rat hepatocytes results in the depletion of protein sulfhydryls (PSH) [13, 18]. To evaluate the oxidative role of NAPQI generated intracellularly, the present study investigates both PSH oxidation and covalent binding upon the direct addition of APAP to primary cultures of hepatocytes from non-induced mice, and attempts to resolve whether the two processes can be dissociated by comparing the effects of APAP and its dimethylated derivatives.

MATERIALS AND METHODS

Source of materials. [^{3}H(G)], [^{3}H]acetic anhydride, *N*-ethyl-2-[^{3}H]maleimide, and ^{125}I-goat anti-rabbit IgG were purchased from New England Nuclear-Dupont, Inc. (Boston, MA). Ring-labeled [^{14}C]APAP was obtained from Amersham-Searle (Arlington Heights, IL). Collagenase CLS-II and peroxidase-linked goat anti-rabbit IgG were obtained from Organon Teknika (BCA Cappel, Westchester, PA). Monobromobimane (Thiolyte) was purchased from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO) and were of the highest grade available.

Synthesis of dimethylated acetaminophen derivatives. The 2,6- and 3,5-dimethyl derivatives were synthesized as described [20] except that the 4-amino-3,5-dimethyl phenol was recrystallized in hot toluene rather than benzene. The synthesis of [^{3}H]2,6-DMA was achieved by adding 5 mCi of [^{3}H]acetic anhydride (0.5 mCi/mmol) to 1.0 g of the purified 4-amino-3,5-dimethyl phenol in 6% sodium acetate. Purity (99.9%) was verified by melting point determination, reverse phase HPLC analysis [26] and NMR spectroscopy. Reverse phase HPLC analysis using a Waters C_{18} μBondapak Z-module column run isocratically with 20% methanol/water at 2 ml/min resulted in retention times of 5.1 min for 2,6-DMA and 12.5 min for 3,5-DMA.

Preparation of hepatocytes and cell culture. Hepatocytes were isolated from 10- to 14-week-old male (20–25 g) C57-BL/6 mice (Jackson Laboratories, Bar Harbor, ME) by a two-step collagenase perfusion method as described [27, 28]. Briefly, livers were initially perfused with a Ca^{2+}-free buffer consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 0.5 mM EDTA, 7 units/ml heparin, 140 mM NaCl, and 6.7 mM KCl (pH 7.6) for 10 min, and then perfused for another 10 min with collagenase (37 units/ml) buffer containing 67 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2$, 100 mM HEPES, 50 μg/ml gentamicin, 1 mg/ml glucose, and 0.3% bovine serum albumin (BSA), pH 7.6. After washing, the isolated hepatocytes were plated on 35 mm Falcon tissue culture dishes (0.75×10^6 cells/plate) in Eagle's Minimum Essential Medium (MEM) supplemented with nonessential amino acids, 10% fetal bovine serum, 10 μM dexamethasone, 2 μM insulin, 15 mM nicotinamide, 50 μg/ml gentamicin, 200 μg/ml streptomycin, and 200 units/ml penicillin. Cells were allowed to adhere for at least 15 hr prior to drug exposure in fresh medium lacking nicotinamide.

Isotopic measurements of APAP covalent binding and protein sulfhydryls. For APAP and 2,6-DMA covalent binding studies, a 10 mM concentration of either [^{3}H]APAP (0.1 mCi/mmol) or [^{3}H]2,6-DMA (0.45 mCi/mmol) was added to the cultures. At the indicated exposure times, the cells were harvested and homogenized in a hypotonic buffer (10 mM Tris–HCl, 15 mM KCl, and 5 mM EDTA, pH 7.4). Aliquots were removed in duplicate and the proteins were precipitated with perchloric acid to a final concentration of 1.0 N and washed twice with ice-cold 80% methanol [5] containing excess unlabeled APAP or 2,6-DMA to minimize nonspecific adsorption of the isotopes. The proteins were subsequently solubilized in NCS (Amersham Searle) and counted in 0.6% 2,5-diphenyloxazole (PPO)/toluene scintillation fluid in a Beckman LS 3801 liquid scintillation spectrometer. No significant differences in binding were noted when ring-labeled [^{14}C]APAP was used in place of [^{3}H(G)] (data not shown).

PSH levels were determined isotopically by monitoring the binding of [^{3}H]*N*-ethylmaleimide (NEM) to sulfhydryl groups on proteins under non-denaturing conditions. This sulfhydryl reagent was chosen because it titrates only the most reactive protein thiol groups [29]. In these studies, cultured hepatocytes were treated with 10 mM APAP, 2,6-DMA, or 3,5-DMA for up to 24 hr and homogenized in hypotonic buffer containing 5 mM [^{3}H]NEM (0.1 mCi/mmol). Aliquots of cultured cells were prepared for isotopic analysis as described above.

For subcellular fractionation studies, cells dosed with 10 mM [^{14}C]APAP (0.1 mCi/mmol) were homogenized in an isotonic buffer (the hypotonic buffer described above supplemented with 1/10 volume of 200 mM HEPES and 1.2 M KCl, pH 7.4) containing [^{3}H]NEM and partitioned by differential centrifugation into an 8,500 *g* nuclear-mitochondrial fraction, a 105,000 *g* microsomal fraction, and a supernatant fraction [30]. Subcellular fractions were prepared for isotopic analysis as described above. This double label technique permitted simultaneous and rapid measurement of both PSH and APAP covalent binding on identical protein extracts from APAP-treated cells.

Detection of PSH groups by fluorescence. Proteins from control and treated cells were resolved electrophoretically on 10% polyacrylamide gels in sodium dodecyl sulfate (SDS) according to the method of Laemmli [31]. The PSH containing bands were identified using the fluorescent sulfhydryl probe, monobromobimane (mBBr) [32]. Hepatocytes were incubated with APAP, 2,6-DMA, or 3,5-DMA for selected times and then homogenized in hypotonic buffer containing 0.3 mM mBBr. This concentration of mBBr provided at least a 2-fold molar excess over the total thiol content of control cells as determined by titration with Ellman's reagent [33]. Homogenates were separated by centrifugation at 105,000 *g* into particulate and supernatant fractions. Samples were dialyzed (1000:1) twice against ice-cold phosphate-buffered saline (PBS) to remove excess monobromobimane prior to electrophoresis. This minimized the possibility of artifactual binding to any protein thiols which may become exposed during protein disulfide reduction with mercaptoethanol in the electrophoretic sample

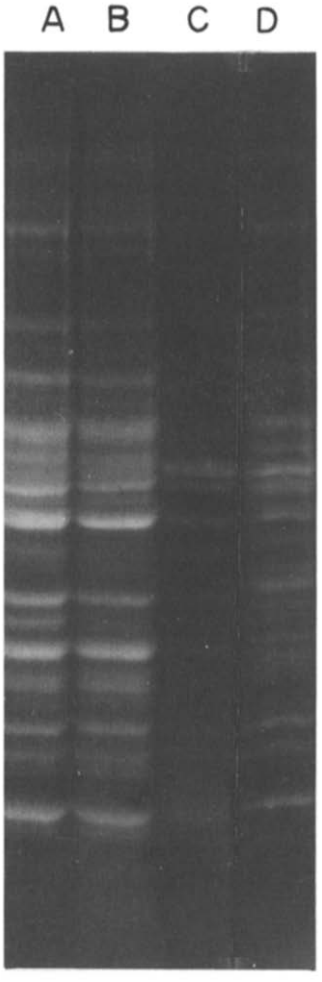

Fig. 1. Effect of NEM pretreatment on mBBr derivatization under native and denatured conditions. C57 mouse liver was homogenized 1:40 (w/v) in STM buffer (pH 7.5) and reacted either immediately (lane A) or 30 min post-homogenization (lane B) with 1.9 mM mBBr. PSH derivatization was allowed to proceed for 30 min at room temperature, and the homogenates were centrifuged at 105,000 *g* for 60 min to yield a cytosolic fraction. For NEM-pretreated samples, a portion of the liver was homogenized in STM buffer containing 5 mM NEM, and cytosolic proteins were isolated after 30 min at 4°. After passing the sample through a G-25 Sephadex column to remove unreactive NEM, proteins collected in the void volume were either directly reacted with mBBr (lane C) or denatured by the addition of 4 M urea prior to reaction with mBBr (lane D). All samples were dialyzed against several changes of PBS to remove residual mBBr. Samples were diluted 1:1 in electrophoresis buffer containing 2% SDS, 10% mercaptoethanol, 20% glycerol, and 0.025% bromophenol blue. Proteins (30 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis [26] under reducing conditions. Subsequently, the gels were fixed in a 50% methanol, 7% glacial acetic acid solution. Monobromobimane fluorescence was visualized and photographed as described under Materials and Methods.

buffer. After electrophoresis, gels were visualized with a transilluminator (Polaroid Model No. 3-3034) equipped with a 366 nm UV light source and photographed with a Polaroid MP-4 Camera equipped with a Wratten 460 nm longpass emission filter. Subsequently, the gels were stained with Coomassie Brilliant Blue R-250 for detection of protein bands.

Evaluation of the use of NEM to quantitate reactive protein thiols. To examine the effectiveness of NEM in detecting the most exposed PSH groups, a comparison between the NEM method [34] and the mBBr method [35] for monitoring exposed thiols was conducted. Livers obtained from untreated C57-BL/6 mice were homogenized 1:40 (w/v) in 0.25 M sucrose, 10 mM Tris–HCl, 1 mM $MgCl_2$ buffer (STM) containing 5 mM NEM, pH 7.5. After 30 min at 4°, cytosolic proteins were obtained by differential centrifugation at 105,000 *g* for 60 min. Unbound NEM was removed from the proteins by passing the sample through a G-25 Sephadex column (1.5 × 25 cm), and the proteins collected in the void volume were reacted immediately with mBBr as a 2-fold molar excess over tissue thiols. In some experiments, proteins were denatured by urea (4 M final concentration) prior to reaction with mBBr. All samples were dialyzed against PBS prior to polyacrylamide gel electrophoresis. As shown in Fig. 1, little or no PSH loss was detected after 30 min standing at 4° prior to mBBr derivatization (compare lane A vs lane B), indicating that the loss of PSH observed after APAP is not a mere consequence of non-specific oxidation of thiols during the 30 min utilized for NEM titration. Although NEM pretreatment drastically reduced mBBr derivatization, it is clear that NEM did not mask all the mBBr reactive sites (lane A vs lane C). Moreover, if proteins are unfolded by the addition of urea after NEM pretreatment, several additional protein thiol sites become assessible to mBBr (lane C vs lane D). These data indicate that the proteins modified by NEM are indeed representative of the most exposed thiols in the native structure. The differential reactivity of these two reagents towards native thiols could partially explain the lower PSH values reported here compared to those reported by others [35].

Immunochemical detection of APAP- and 3,5-DMA-bound proteins. An affinity-purified antibody (Ab) was utilized to detect protein-bound APAP [30]. Characterization of the substrate specificity of the anti-APAP antibody towards 2,6-DMA and 3,5-DMA was achieved by competitive ELISA. For these studies microtiter plates were coated with 50 μl/well of NAPQI which had been covalently linked to BSA [23], and 20 μl of 0.12 to 500 μg/ml of APAP, 3,5-DMA, or 2,6-DMA in PBS containing 0.05% Tween-20 was added to separate wells. Following incubation for 60 min at 37° in the presence of affinity-purified antibody (20 μl of a 1:75 Ab dilution in PBS–Tween), peroxidase-linked goat anti-rabbit IgG was added for an additional hour to permit reaction with the Ab–antigen complex. Peroxidase activity was monitored at 405 nm on a computer-interfaced Artek plate reader. Figure 2 demonstrates a similar affinity of the antibody for both 3,5-DMA and APAP and a much lower affinity for 2,6-DMA.

The specificity of the antibody towards free 3,5-DMA made it possible to immunochemically evaluate the potential binding of 3,5-DMA to cellular proteins. Proteins from particulate and cytosolic fractions of 3,5-DMA- or APAP-treated cells were separated by electrophoresis and the resolved proteins

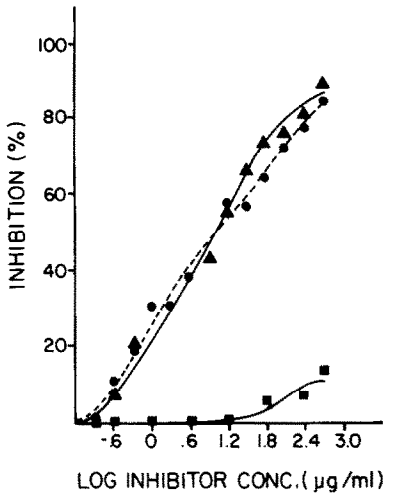

Fig. 2. Characterization of the substrate specificity of the affinity-purified anti-APAP antibody. The cross-reactivity of the antibody with free APAP (●——●), 3,5-DMA (▲——▲), or 2,6-DMA (■——■) was determined by competitive ELISA. All data are expressed as the average ±SE of at least three different experiments.

were electroblotted to nitrocellulose membranes for 6 hr at 75 V in buffer containing 25 mM Tris–HCl, 192 mM glycine, and 20% methanol. Subsequently, the membranes were washed for several minutes in 25 mM Tris–HCl buffer, pH 7.6, containing 0.8% NaCl and 0.05% Tween-20 to remove residual SDS, and then placed for 12 hr at 4° in Tris–HCl-buffered saline containing 3.0% BSA to block any remaining protein reactive positions on the membranes [36]. The transblotted proteins were incubated with affinity-purified anti-APAP antibodies and washed several times prior to the addition of ^{125}I-conjugated goat anti-rabbit IgG. Immediately following, the blotted nitrocellulose membranes were exposed at −70° to Kodak XAR-5 film for 24–48 hr.

Analytical procedures. Total intracellular glutathione was assayed by the procedure of Griffith [37] except that perchloric acid replaced picric acid as a protein precipitant. Cell toxicity was assessed from the percentage of total intracellular glutamate oxaloacetate transaminase (GOT) activity which was released into the extracellular medium [38]. Protein was determined by the method of Lowry *et al.* [39] using BSA as a protein standard. Statistical differences between the APAP-treated samples and controls were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

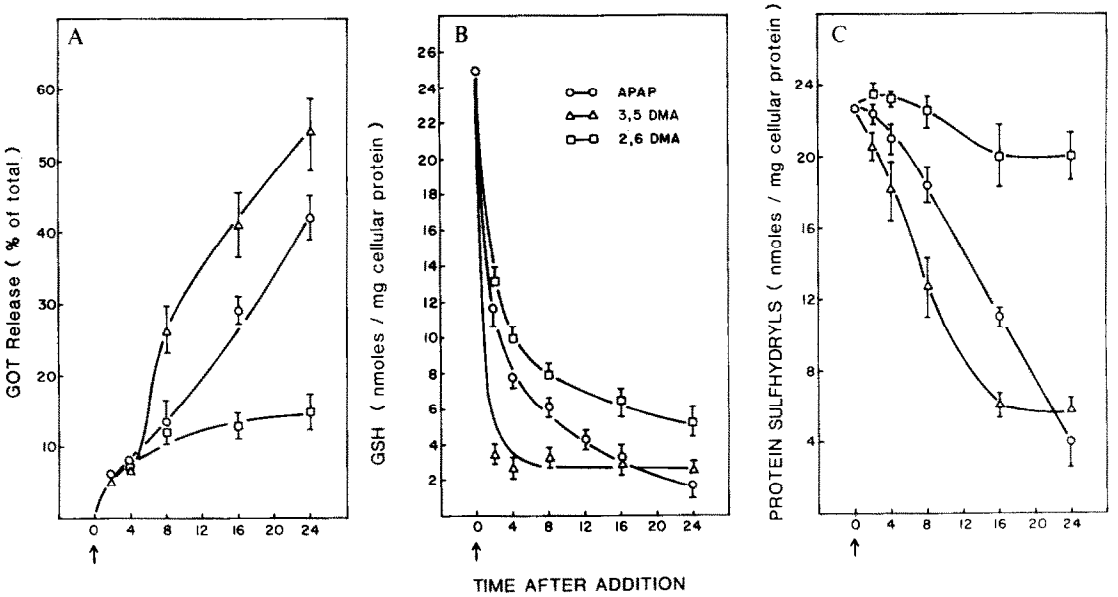

Fig. 3. Effects of APAP and its dimethylated derivatives on cytotoxicity, GSH, and PSH. Cultured hepatocytes were treated with 10 mM APAP (○——○), 3,5-DMA (△——△), or 2,6-DMA (□——□) for up to 24 hr (A) The cytotoxicity was monitored as the percent of total GOT activity that is released into the extracellular medium. Enzyme leakage from control cells did not exceed more than 15% of the total GOT activity per plate for the duration of the experiment. All data are expressed as the average ±SE of at least three different experiments and are corrected for the GOT content initially found in the fetal bovine sera added to the culture medium. (B) The effects of APAP and its dimethylated derivatives on GSH content were determined in hepatocytes homogenized in 1.0 N perchloric acid. The acid-soluble extracts were neutralized with 3 N $KHCO_3$ to pH 7.4 and assayed enzymatically for intracellular GSH [28]. No significant changes in GSH levels were noted in control cells through 24 hr. (C) The effects of APAP and its dimethylated derivatives on PSH were determined in hepatocytes homogenized in hypotonic buffer containing 5.0 mM [^{3}H]NEM (0.1 mCi/mmol) to titrate available PSH groups. After 30 min at 4°, the proteins were precipitated by the addition of an equal volume of 2 N perchloric acid, and the samples were washed and counted as described under Materials and Methods. No significant loss of PSH in control cells was observed through 24 hr in culture.

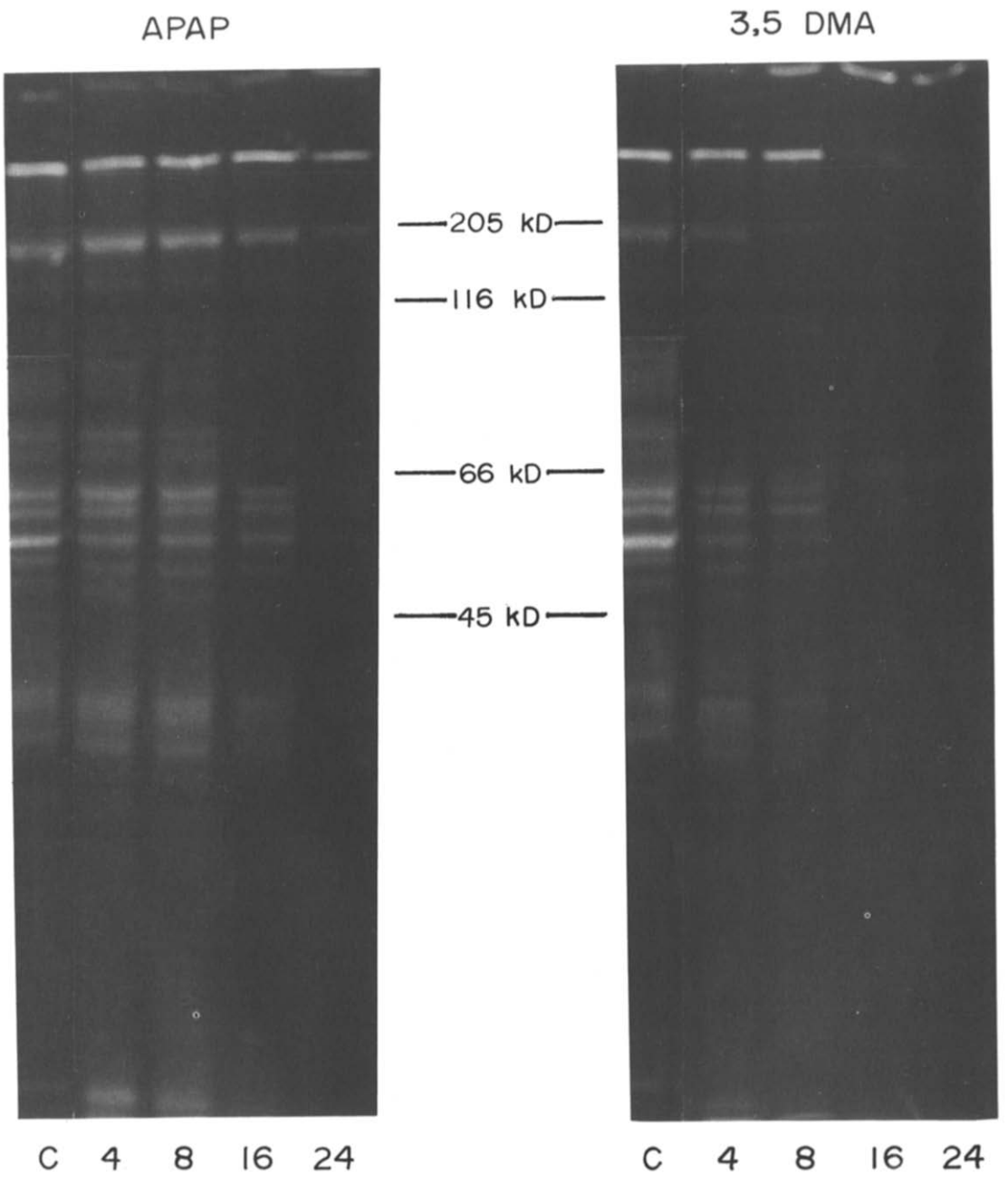

Fig. 4. Electrophoretic assessment of PSH loss induced by APAP and 3,5-DMA. Hepatocytes were treated as described in the legend of Fig. 3 and homogenized in hypotonic buffer containing 0.3 mM monobromobimane. PSH derivatization was allowed to proceed for 30 min at room temperature, the homogenates were centrifuged at 105,000 *g* for 60 min, the particulate fractions were resuspended in 1.0 ml of PBS (pH 7.4) and dialyzed against several changes of PBS to remove any residual monobromobimane. Samples were prepared for electrophoresis as described in the legend of Fig. 1. The electrophoretic mobility of each molecular weight standard is noted.

RESULTS

Cytotoxicity of APAP, 2,6-DMA, and 3,5-DMA and effects on hepatocyte GSH and PSH. The toxicity of 10 mM APAP and its dimethylated derivatives was monitored by the extent of GOT release into the culture medium (Fig. 3A). The cytotoxicity increased as a function of the duration of exposure to either APAP or 3,5-DMA, but the rate of enzyme release during the first 8 hr was more rapid after 3,5-DMA than after APAP. In contrast, GOT leakage in 2,6-DMA-treated hepatocytes was not significantly different from that observed in controls.

Although only APAP and 3,5-DMA resulted in cytotoxicity, all three compounds depleted intracellular GSH. However, the kinetics of GSH depletion differed among them (Fig. 3B). By 2 hr after 3,5-DMA addition, the loss of GSH exceeded

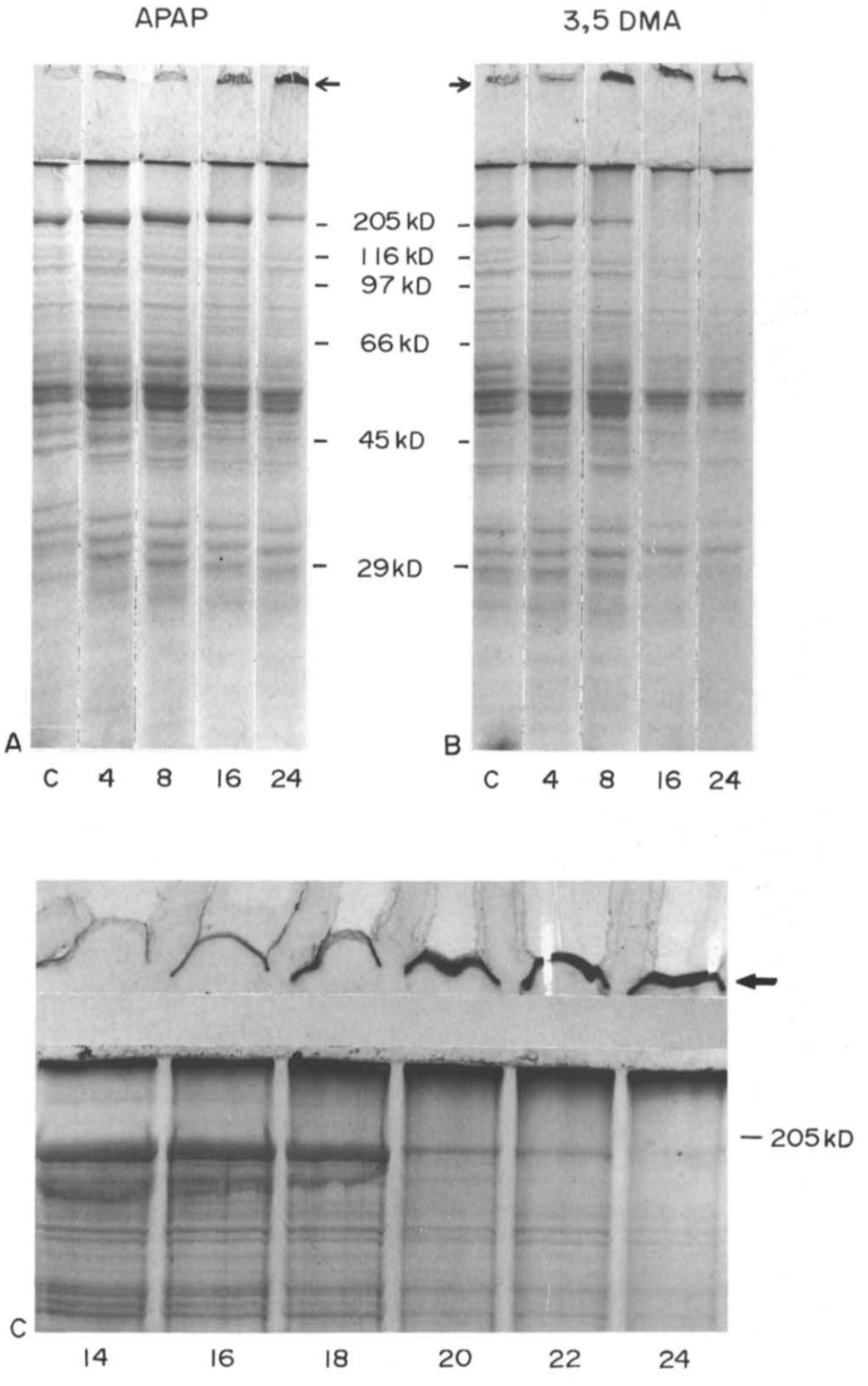

Fig. 5. Electrophoretic detection of high molecular weight protein aggregates induced by APAP and 3,5-DMA. Hepatocytes were treated for up to 24 hr with 10 mM APAP (A and C) or 3,5-DMA (B). Reaction conditions are similar to those described in the legend of Fig. 1 except that electrophoretic gels were stained with a 3% Coomassie Brillant Blue solution to enable direct comparison of the protein content of the gels with the fluorescent profile shown in Fig. 4. Figure 5C illustrates the high molecular weight region of the electrophoretic gels for APAP exposures from 14 to 24 hr. An arrow indicates the localization of crosslinked proteins that do not enter the 3% polyacrylamide stacking gel. The electrophoretic mobility of each molecular weight standard is noted.

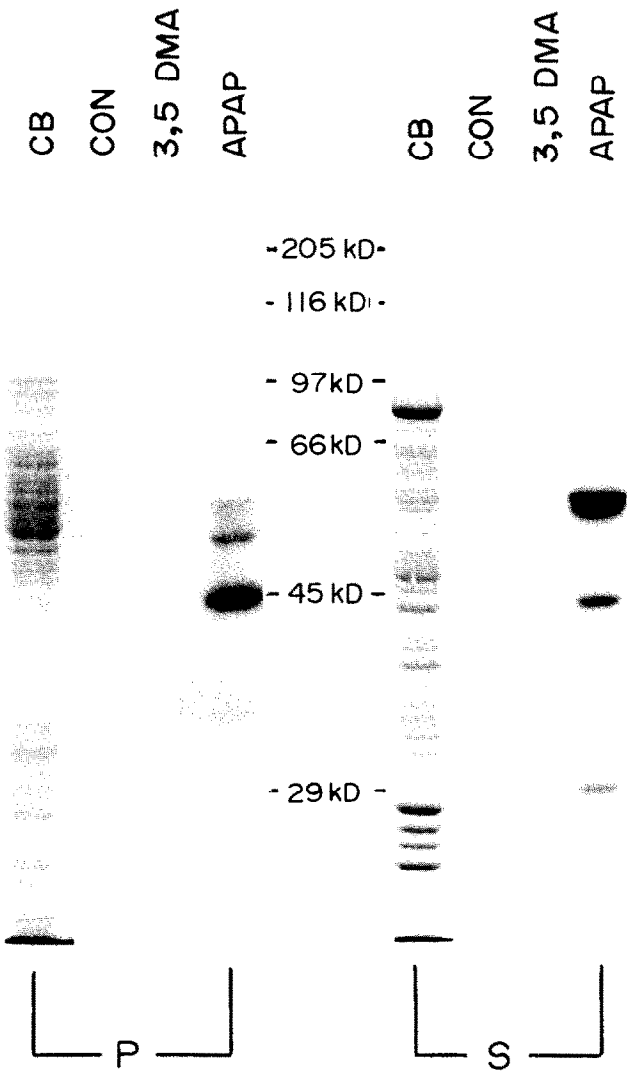

Fig. 6. Immunochemical detection of APAP-bound proteins. Hepatocytes were treated with 10 mM APAP or 3,5-DMA for 4 hr, and subcellular fractions were isolated and designated (P) for the 105,000 *g* pellet and (S) for the 105,000 *g* supernatant. Electrophoretic conditions are the same as those described in the legend of Fig. 1. For Western blotting studies the protein bands were transferred to nitrocellulose membranes as described under Materials and Methods. Lanes are labeled for Coomassie Brillant Blue protein staining (CB), and for the Western blots of untreated cells (CON), or those treated with APAP or 3,5-DMA. The electrophoretic mobility of each molecular weight standard is noted.

85%, whereas neither APAP nor 2,6-DMA resulted in GSH depletion greater than 50%. However, by 16 hr both 3,5-DMA and APAP had depleted GSH to about 10% of control as compared to 25% of control for 2,6-DMA.

Furthermore, both APAP and 3,5-DMA induced extensive PSH depletion (Fig. 3C). The delayed effects of APAP in eliciting GSH depletion (Fig. 3B) paralleled a slower onset of PSH loss compared to that observed following 3,5-DMA administration. Within 8 hr, PSH decreased by 43% after 3,5-DMA, but only by 18% after APAP. Similarly, maximal PSH depletion (to approximately 25% of control) was achieved after 16 hr with 3,5-DMA, but not until 24 hr with APAP. By contrast, an equimolar concentration of 2,6-DMA, which also readily depleted GSH, did not alter PSH levels significantly even by 24 hr.

To examine the specificity of the losses in PSH, proteins from the particulate fractions from APAP- and 3,5-DMA-treated hepatocytes were resolved electrophoretically and visualized with monobromobimane (Fig. 4). Consistent with the results obtained by [^{3}H]NEM titration (Fig. 3C), fluorescence diminished in individual protein bands as a function of the duration of exposure. The PSH depletion induced by 3,5-DMA appeared more rapid and more extensive than that observed with APAP. For both compounds, the decrease in fluorescence with increased time of drug exposure appeared uni-

Table 1. Assessment of covalent binding to proteins by APAP and its dimethylated derivatives in cultured mouse hepatocytes

Hr	Drug bound (nmol/mg cellular protein)		
	2,6-DMA	APAP	3,5-DMA
2	0.41 ± 0.02	0.94 ± 0.3	ND*
4	0.49 ± 0.04	1.45 ± 0.4	ND
8	0.62 ± 0.04	2.75 ± 0.3	ND
16	1.03 ± 0.21	4.01 ± 1.1	—
24	1.14 ± 0.07	13.5 ± 1.0	—

Covalent binding was determined isotopically after treating hepatocytes with 10 mM [^{3}H]APAP (0.1 mCi/mmol) or [^{3}H]2,6-DMA (0.45 mCi/mmol). The values represent the average ±SE of at least four different experiments.

* ND indicates that no binding of 3,5-DMA was detected immunochemically after Western blotting (see also Fig. 6).

formly distributed among the separated proteins, indicating that the loss in PSH may be a non-selective event.

In addition to decreasing PSH, both 3,5-DMA and APAP exposure also resulted in decreased staining with Coomassie Brillant Blue (Fig 5, A and B). Visual inspection of the stained gels revealed that by 16–24 hr after 3,5-DMA, the intensity of several proteins bands (i.e. 200, 118, 90, 66, 55, 42, and 20 kD) appeared selectively decreased, whereas only the 200 and 42 kD bands were decreased after APAP. This apparent loss of selected proteins was associated with the formation protein aggregates of high molecular weight which did not penetrate the 3.0% polyacrylamide electrophoretic stacking gel (represented by the stained bands at the top of the lanes in Fig. 5). These aggregates are indicative of protein crosslinking and become particularly prominent after 16 hr of APAP exposure (Fig. 5C). By contrast, Coomassie staining of electrophoretically resolved proteins from 2,6-DMA-treated hepatocytes did not reveal any protein loss or crosslinking (data not shown).

Assessment of covalent binding of APAP, 3,5-DMA, and 2,6-DMA to hepatocyte protein. Hepatocytes exposed to 10 mM APAP or 3,5-DMA for 2, 4, or 8 hr were harvested and fractionated into particulate (105,000 *g* pellet) and supernatant fractions. Western blot profiles of the electrophoretically resolved proteins revealed selective binding to several bands from the APAP-treated cells, but none from cells exposed to 3,5-DMA. Figure 6 shows the data obtained after a 4-hr exposure and reveals that the most intensively stained band after APAP exposure was approximately 44 kD in the particulate fraction, whereas in the supernatant fraction the most intense immunochemical staining was associated with bands of 56 and 58 kD. Even though these findings suggest that 3,5-DMA does not result in covalent binding and is consistent with the suggestion of others [21, 24], the possibility remains that an electrophilic metabolite of 3,5-DMA did bind either before or after deacetylation [23] and the resulting conjugate may not have been detected by the anti-APAP antibody.

Since the anti-APAP antibody exhibited much lower affinity to 2,6-DMA (Fig. 2), the covalent binding of this compound to electrophoretically resolved proteins was not studied. However, to quantitate covalent binding of APAP and 2,6-DMA, additional studies were conducted with ^{3}H-labeled compounds, and binding to homogenate proteins was determined. As shown in Table 1, there was a significant time-dependent increase in covalent binding of both APAP and 2,6-DMA to hepatic proteins.

Table 2. Contribution of covalent binding to the loss of PSH in cultured hepatocytes exposed for 8 hr with APAP

Cell fraction	A Control PSH	B APAP-treated PSH	C Loss in PSH	D APAP covalent binding	E Estimated PSH oxidation
Total 8500 *g*	22.4 ± 1.6	18.3 ± 1.2*	4.2 ± 1.1	1.3 ± 0.1	2.8
Pellet 105,000 *g*	17.8 ± 1.4	14.9 ± 0.9*	2.8 ± 0.5	1.6 ± 0.1	1.2
Pellet	45.1 ± 2.7	33.3 ± 1.4†	11.8 ± 0.4	1.0 ± 0.2	10.8
Soluble	25.4 ± 2.4	22.3 ± 2.2	3.1 ± 0.9	0.7 ± 0.2	2.4

Cultured hepatocytes were treated with 10 mM [^{14}C]APAP (0.1 mCi/mmol) for 8 hr. Control and treated hepatocytes were monitored for PSH and covalent binding as described under Materials and Methods. Approximately 5×10^6 cells were homogenized in buffer containing 5 mM [^{3}H]NEM (0.1 mCi/mmol), and subcellular fractions were obtained by differential centrifugation into a 8500 *g* pellet, a 105,000 *g* pellet, and a soluble fraction. Fractions were resuspended in perchloric acid to a final concentration of 1.0 N. The loss in PSH was calculated as the difference between control PSH (column A) and those obtained after an 8-hr APAP exposure (column B). Similarly, (column E) was calculated as the difference between the loss in PSH (column C) and the extent of APAP covalent binding (column D) and represents an estimate of PSH oxidation. All data are expressed as nmol/mg cellular protein and are the average ± SE of five different experiments. Statistical significance of the differences was evaluated by Student's *t*-test.

*† The differences of PSH values between APAP-treated and control cells (columns A and B) are denoted as significant at $^*P < 0.05$ or at $^\dagger P < 0.01$.

However, there was much less binding with 2,6-DMA than with APAP, and the difference became more exaggerated with increasing time of exposure. Thus, APAP binding was 2.3-fold higher than 2,6-DMA after 2 hr and increased to 3.9-fold by 16 hr. Furthermore, the large increase in APAP binding observed between 16 and 24 hr was not observed after 2,6-DMA administration.

Contribution of covalent binding to the loss of PSH by APAP. A comparison of Fig. 3 and Table 1 revealed that the extent of covalent binding exceeded PSH loss only after 16 hr of exposure; the loss in PSH prior to 16 hr could not be accounted for solely on the basis of APAP arylation of protein thiols. Between 8 and 16 hr PSH decreased by 7.3 nmol/mg protein (from 18.4 to 11.1), whereas covalent binding only increased by 1.2 nmol/mg protein (from 2.8 to 4.0). To more carefully define the contribution of the early covalent binding to the loss in PSH, cells were exposed to APAP for 8 hr, and both PSH and covalent binding were measured in subcellular fractions from the same cultures using the double isotope technique. This procedure involved the use of [^{14}C]APAP to monitor covalent binding, and [^{3}H]NEM to provide an index of available PSH. PSH oxidation was assessed as the difference between PSH loss and the extent of APAP covalent binding. Results in Table 2 indicate that the decrease in total PSH by 8 hr was 18%. However, of the 4.2 nmol of PSH that were depleted, only 1.3 nmol (32%) could be accounted for by covalent binding. When individual fractions were examined, a similar dissociation was noted for the 8500 *g* pellet and the cytosol where only 30 and 50%, respectively, of the PSH depletion could be accounted for by covalent binding. Moreover, in the microsomal fraction, which contained the highest PSH content, over 90% of the PSH loss could not be accounted for by APAP binding. In addition, on a per milligram protein basis, the greatest loss in PSH (11.8 nmol) was detected in this fraction. Since it contained only about 10% of total cellular protein, the large oxidative effect observed in this fraction was masked when expressed in terms of total cellular PSH.

DISCUSSION

Several reports have demonstrated that the putative reactive metabolite of APAP, NAPQI, contains both electrophilic and oxidizing properties [8, 18, 19]. Rosen *et al.* [24] showed *in vitro* that NAPQI can react with GSH to form both a GSH-NAPQI adduct as well as oxidize GSH to the disulfide. More recently, Albano *et al.* [18] demonstrated that in isolated hepatocytes from induced mice the extracellular addition of NAPQI similarly resulted in the formation of both GSH conjugates and glutathione disulfide. The present findings demonstrate that the protein thiol oxidation that has been observed in hepatocyte suspensions with exogenously added NAPQI [13, 18] also occurs, albeit more slowly, after exposure of primary cultures of mouse hepatocytes to APAP directly. The slower kinetics observed in the present study may be explained by the slow rate of P-450 conversion of APAP to NAPQI in cultures from non-induced mice.

To adequately assess the APAP-induced thiol perturbations, it was necessary to utilize a sulfhydryl reagent that would monitor only the most reactive thiols while maintaining proteins in their native configuration. The use of NEM to monitor PSH involves the attack of the NEM on a thiol anion species [40]. Since the pK_a of a free thiol in solution is 9.0 [29], only the most reactive thiols with low effective pK_a values would be titrated by NEM at pH 7.5 [29]. The enhanced nucleophilicity that would render such proteins NEM-reactive would also enhance the likelihood that such proteins would be involved in thiol disulfide exchange reactions [29] as well as in NAPQI-mediated covalent binding. In support of this latter notion, pretreatment of PSH with NEM has been shown to block completely NAPQI binding to proteins assayed immunochemically with an anti-APAP antibody [41]. These data suggest that the pools of PSH which binds NAPQI are similar to those which are alkylated by NEM. Therefore, the double label approach utilized to monitor the contribution of [^{14}C]APAP covalent binding to the loss in [^{3}H]NEM reactive protein thiols under non-denaturing conditions provides a reliable estimate of those thiols which would be readily accessible to the actions of NAPQI in the intracellular environment.

The administration of cytotoxic concentrations of APAP resulted in a loss of protein sulfhydryls that could not be accounted for by the extent of APAP covalent binding to proteins. During the first 8 hr after APAP exposure, the loss of PSH was less than 20% of the total PSH content of control cells. However, the covalent binding of APAP in culture could account for no more than one-third of that PSH loss. This suggests that two-thirds of the PSH loss during this period must have resulted from PSH oxidation. The loss of PSH at this time was not the result of APAP-induced cell death since we have observed that, under similar conditions, the removal of APAP within 8 hr results in the total repletion of glutathione as well as the recovery of the rates of both protein synthesis and degradation [42]. Moreover, covalent binding accounted for less than 20% of the PSH loss observed in hepatocytes between 8 and 16 hr after APAP administration (Fig. 3C and Table 1). Since covalent binding cannot account for the amount of PSH lost, these observations provide evidence for an oxidative component of APAP and support the relevance of the bifunctional properties of NAPQI in cultured hepatocytes.

Subcellular fractionation revealed that the PSH loss occurring during the first 8 hr of APAP exposure was greatest in the microsomal fraction. This fraction, which is the primary site of APAP activation [3], is also abundantly rich in PSH (Table 2). It is not certain whether the increased sensitivity of the microsomal fraction to PSH loss is the result of the physical proximity to the site of NAPQI formation or of the high thiol content of this fraction. However, it should be emphasized that the PSH loss does not appear to be a selective phenomenon. All electrophoretically resolved protein bands that exhibit thiol content detectable by the fluorescent thiol marker, monobromobimane, tended to lose PSH upon prolonged APAP exposure. It is interesting

that the 42–44 kD protein band, which is the earliest [43] and most extensively targeted protein for APAP covalent binding ([30] and Fig. 6) in the microsomal fraction, does not appear to be enriched in PSH. This observation suggests that the selectivity observed in covalent binding may not be merely a function of the free sulfhydryl content of the affected proteins.

The present study has shown that, although 3,5-DMA does not covalently bind proteins, it can mimic several properties of APAP including GSH depletion, PSH depletion, formation of protein aggregates of high molecular weight, and cytotoxicity. These results are in contrast to recent reports that 3,5-DMA does not induce toxicity in isolated hepatocytes from induced rats [22]. The reason for this discrepancy is not clear. In the present study, the effects of 3,5-DMA are more pronounced than those of equimolar concentrations of APAP. The more rapid oxidative effects of 3,5-DMA are consistent with the demonstration of a direct relationship between the extent of alkyl substitution of benzoquinones and the degree of redox cycling events leading to thiol oxidation [44]. In addition, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine as well as the phenoxy free radical of 3,5-DMA are more stable than the corresponding APAP intermediates [23]. It is possible that the increased stability of the 3,5-dimethyl quinone imine would enable it to react more readily with protein thiols and/or quinone reductases to initiate a more potent oxidative stress to the hepatocyte. However, the lack of binding of 3,5-DMA observed here may not only explain the more rapid oxidative action of 3,5-DMA, but also suggests that the binding of NAPQI to thiols (GSH as well as PSH) might, in part, play a protective role by effectively reducing the intracellular concentration of NAPQI.

The notion that covalent binding to protein may, in part, represent a protective mechanism is supported by the observation that structural isomers of APAP (3-hydroxy-APAP and 2-hydroxy-APAP) result in covalent binding to hepatic protein to a similar extent as native APAP, but do not induce hepatotoxicity [45, 46]. In the present study, 2,6-DMA resulted in covalent binding which was about 30% of that noted after equimolar concentrations of APAP, yet 2,6-DMA caused no detectable hepatocyte damage. Recent studies with suspensions of hepatocytes from phenobarbital-induced rats confirm the relative differences in covalent binding and demonstrate only minimal toxicity after 2,6-DMA [21]. Although the present data cannot rule out the possibility that the extent or specificity of the covalent binding may be critical for the progression of the hepatotoxicity, they do argue that covalent binding may not, *per se*, mediate toxicity. It is possible that the proteins arylated after 2,6-DMA exposure may differ from those targeted by APAP. Recently, we have shown that the covalent binding of APAP to hepatic proteins, both *in vivo* [30] and in cultured hepatocytes ([43] and Fig. 6), is highly selective with respect to proteins arylated. Hence, some covalent binding may represent a detoxification mechanism which functions to sequester the reactive electrophile/oxidizing metabolite as an inert adduct. We are currently examining by immunochemical analysis the specificity of covalent binding after 2,6-DMA to permit comparisons to the proteins targeted by APAP.

To our knowledge, this report is the first to document the formation of protein aggregates of high molecular weight in hepatocytes exposed to a toxic concentration of APAP or 3,5-DMA. These aggregates are impenetrable to a 3.0% electrophoretic stacking gel run under reducing conditions and, therefore, are not crosslinked through disulfide bonds. It is likely that they may exceed one million daltons in molecular weight [47]. Because radical scavengers can prevent protein aggregation, it has been suggested that such crosslinking (assayed by the inability of proteins to enter electrophoretic gels) may be mediated by a free radical mechanism [48, 49]. Although we have no direct evidence to implicate free radical events, this interpretation is consistent with the observed protein crosslinking and decrease in PSH in cultured hepatocytes exposed to toxic concentrations of 3,5-DMA and APAP.

Under oxidative conditions *in vitro*, APAP has been reported to undergo free radical-induced polymerization reactions generating trimers and tetramers [15, 50]. If an APAP polymer were bound to proteins in hepatocytes exposed to APAP, one would expect that an isotopic analysis of covalent binding would overestimate the actual number of covalent adducts formed. Binding of polymerized APAP could explain the large increase in covalent binding noted between 16 and 24 hr (Table 1). In support of this, covalent binding exceeded PSH loss between 16 and 24 hr. This later binding was associated with a dark brown coloration of the precipitated proteins (data not shown). A melanin-like chromophore has also been associated with free radical-mediated APAP polymerization, *in vitro* [23]. Thus, it is likely that the large increase of APAP covalent binding is mediated by an oxidative mechanism. Consistent with this is our observation that 2,6-DMA, which did not exhibit any oxidative action (Fig. 3C), also did not exhibit any accelerated binding between 16 and 24 hr. It should be emphasized, however, that although both protein crosslinking and APAP polymerization can be explained by free radical mechanisms, these events are most likely independent, since 3,5-DMA, which we have observed to induce protein crosslinking, has been shown to be incapable of undergoing free-radical-mediated polymerization [23].

This study has demonstrated that APAP toxicity to cultured hepatocytes may involve both arylative and oxidative mechanisms. The poor stoichiometric relationship between the selective APAP-induced covalent binding and the more general PSH depletion suggests that these processes may occur independently. Use of both 2,6-DMA (arylation) and 3,5-DMA (oxidation) permitted dissociation of these processes for comparison with the effects of APAP. Data obtained with 3,5-DMA indicate that oxidation in the absence of covalent binding may mediate toxicity. The covalent binding and lack of toxicity of 2,6-DMA indicate that some covalent binding may be innocuous or, possibly, even protective. Although we cannot discount the possibility that, for APAP, some selective covalent bind-

ing may be mechanistically important, the present results suggest that in cultured hepatocytes the oxidative actions of endogenously generated NAPQI also play an important role in the cytotoxic process.

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