Biochemical Pharmacology, Vol. 43, No. 7, pp. 1493–1505, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

N-ACETYL-*p*-BENZOQUINONE IMINE-INDUCED PROTEIN THIOL MODIFICATION IN ISOLATED RAT HEPATOCYTES

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(*Received* 21 *August* 1991; *accepted* 26 *December* 1991)

Abstract—Incubation of isolated rat hepatocytes with *N*-acetyl-*p*-benzoquinone imine (NAPQI) or 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine (3,5-Me_2-NAPQI) resulted in a concentration-dependent decrease in the protein thiol content of the mitochondrial, cytosolic and microsomal fractions. On a concentration basis, 3,5-Me_2-NAPQI induced a more marked depletion of protein thiols than did NAPQI. Sodium dodecyl sulphate–polyacrylamide gel electrophoretic separation of the proteins of each fraction showed that different proteins had different susceptibilities to modification of their cysteine residues by the quinone imines. A few protein bands showed a decreased protein thiol content following incubation with non-toxic concentrations of quinone imines, whereas other proteins were affected by higher concentrations. Concentrations of quinone imines that were highly cytotoxic induced a general loss of protein thiols. NAPQI-induced protein thiol depletion occurred within 5 min and remained essentially unchanged for at least 30 min. In contrast, protein thiol depletion induced by 3,5-Me_2-NAPQI increased over the 30-min time course of the experiment. Toxic concentrations of 3,5-Me_2-NAPQI caused the formation of high molecular mass aggregates in all three subcellular fractions after 30 min of incubation. The observed crosslinking was not due to protein disulfide formation. However, no aggregate formation was observed after exposure of hepatocytes to NAPQI. One of the major target proteins of quinone imine-induced protein thiol depletion was a 17 kDa microsomal protein that was identified as the microsomal glutathione *S*-transferase. Exposure of hepatocytes and isolated liver microsomes to the quinone imines resulted in an up to four-fold increase in the specific activity of the microsomal glutathione *S*-transferase. In conclusion, our results are consistent with the suggestion of a critical role of protein thiol depletion in quinone imine-induced cytotoxicity.

Acetaminophen (*N*-acetyl-*p*-aminophenol, paracetamol, APAP‡) is a widely used analgesic and antipyretic drug. Taken at therapeutic doses, APAP is readily metabolized to non-toxic products by glucuronidation and sulphation. However, these conjugation pathways may become saturated, and APAP can cause severe hepatic necrosis in both man and experimental animals following an acute overdose [1]. It is generally believed that liver damage results from cytochrome P450-mediated oxidative bioactivation of APAP to a reactive intermediate [2] identified as *N*-acetyl-*p*-benzoquinone imine (NAPQI) [3]. Detoxification of NAPQI occurs mainly through conjugation with GSH; but GSH can also reductively react with NAPQI, regenerating the parent compound, APAP, with the concomitant formation of GSSG [4, 5].

‡ Abbreviations: APAP, *N*-acetyl-*p*-aminophenol (acetaminophen, paracetamol); CBB, Coomassie brilliant blue; mBBR, monobromobimane; NAPQI, *N*-acetyl-*p*-benzoquinone imine; 3,5-Me_2-NAPQI, 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonylfluoride; protSH, protein thiol; GSH, reduced glutathione; GSSG, oxidized glutathione; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Excessive production of NAPQI will result in the depletion of the cellular GSH levels and allow NAPQI to covalently bind to cellular macromolecules [6, 7]. Since a good correlation has been found between the level of covalent binding of the reactive metabolite to cellular proteins and the extent of liver necrosis, covalent binding to proteins has been implicated in the mechanism of APAP-induced liver damage [7]. Alternatively, NAPQI has been shown to produce oxidative changes in proteins [4] and a mechanism involving oxidative damage has also been proposed to explain liver injury caused by APAP.

It is well established that the amino acid residue cysteine is the main molecular target for the covalent and oxidative modifications of proteins by APAP and NAPQI [8–10]. Indeed, a number of investigators have observed a significant depletion of cellular protein thiol groups (protSH) in APAP- or NAPQI-treated hepatocytes [4, 11, 12]. However, measurements of the total cellular protSH content is a rather insensitive approach in view of the heterogeneity in the subcellular distribution of cysteine-containing proteins and the differences in their accessibility to thiol-modifying agents. Furthermore, chemical modification of cysteine residues is not necessarily accompanied by functional changes of the respective protein [13, 14], and

functional changes of some proteins may not lead to cytotoxity. It is therefore to be expected that a limited number of critical sites exist in the cell, whose inactness is essential for cell viability.

We have recently developed a new method for the rapid labelling of accessible and reactive protSH in intact hepatocytes with the thiol-specific fluorescent agent, monobromobimane (mBBr) [15]. Following subcellular fractionation of the hepatocytes, the individual labelled proteins can be visualized after separation by SDS–PAGE. We have used this method in the present study to investigate the effects of NAPQI on cytosolic, mitochondrial and microsomal protSH groups in hepatocytes. To distinguish between oxidative modification of protSH and adduct formation with NAPQI, we have compared the effects of NAPQI on protSH with those of its oxidizing analogue, 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine (3,5-Me_2-NAPQI) [5, 16]. The findings presented here show that both NAPQI and 3,5-Me_2-NAPQI produced a rapid and concentration-dependent decrease in hepatocyte protSH groups in all subcellular fractions. ProtSH loss was selectrive for some specific protein bands with low concentrations of the quinone imines, but appeared to be uniformly distributed with high concentrations of the quinone imines. The loss of protSH appeared to correlate with the degree of cytotoxicity, suggesting that protSH loss may be a critical factor in the mechanism of NAPQI-induced cytotoxicity.

MATERIALS AND METHODS

Chemicals. Collagenase (grade II), dithiothreitol, phenylmethylsulfonylfluoride (PMSF) and leupeptin were purchased from Boehringer–Mannheim (Mannheim, Germany). NAPQI and 3,5-Me_2-NAPQI were synthesized and purified as described previously [17, 18]. mBBr was purchased from Calbiochem (La Jolla, CA, U.S.A.). Acrylamide, *N,N'*-ethylene-bisacrylamide and sodium dodecylsulfate were obtained from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of the highest purity commercially available.

Hepatocyte isolation and incubation. Male Wistar rats (ALAB AB, Sollentuna, Sweden; 200–250 g, fed *ad lib.*) were used in all experiments. Hepatocytes were isolated as described previously [19] and incubated in round bottom flasks at a concentration of 10^6 cells/mL under an atmosphere of O_2/CO_2 (19:1) in modified Krebs–Henseleit buffer, pH 7.4, 37°. The quinone imines were added from freshly prepared stock solutions in dry Me_2SO; the final concentration of Me_2SO in the hepatocyte incubation did not exceed 0.3% (v/v). Cell viability was assayed by Trypan blue (0.16%) exclusion.

mBBr-derivatization and subcellular fractionation of hepatocytes. Aliquots (10 mL) of hepatocyte incubations were collected by centrifugation and resuspended in 1.6 mL of buffer A (250 mM sucrose, 1 mM EDTA, 5 mM Hepes, 25 mM *N*-ethylmorpholine, pH 7.4), supplemented with 4 mM mBBr. Following derivatization with mBBr for 5 min at room temperature, the cells were centrifuged, washed once in 5 mL of buffer A, and the final pellet resuspended in 1.6 mL of ice-cold buffer A, supplemented with 0.1 mM PMSF and 100 μg leupeptin/mL. PMSF was added from a 0.1 M stock in isopropanol immediately before use. The cell suspensions were then sonicated on ice using a Soniprep 150 probe sonicator (4 × 10 sec bursts, 5 μm amplitude). Mitochondrial, cytosolic, and microsomal fractions were obtained by differential centrifugation as described previously [15].

SDS–PAGE. Mitochondrial, microsomal and cytosolic proteins from control and quinone imine-treated hepatocytes were resolved electrophoretically on 1.5 mm thick, 10 or 11% SDS–polyacrylamide gels according to the method of Laemmli [20]. The mBBr-fluorescence of the proteins was visualized with a transilluminator equipped with a 365 nm light source and photographed with a Polaroid MP-4 camera equipped with a Wratten 460 nm long-pass emission filter. The gels were subsequently stained with Coomassie brilliant blue R-250 (CBB).

Activation of microsomal glutathione S-transferase in hepatocytes and in isolated microsomes. Following incubation with the agents described in Table 2, the hepatocytes were washed, sonicated as described above, and microsomal fractions were prepared by Ca^{2+} precipitation essentially as described [21]. Microsomes from whole liver were prepared as described [22], washed once with 0.15 M Tris–Cl, pH 8, to minimize cytosolic contamination [23], resuspended in 0.05 M Tris–Cl pH 7.5, 0.14 M NaCl and kept on ice until use. For the microsomal glutathione *S*-transferase activation experiments, microsomes (2 mg protein/mL in 150 mM KCl, 20 mM Hepes, pH 7.4) were incubated with quinone imines, *N*-ethylmaleimide (NEM) or mBBr. Aliquots were withdrawn at the times indicated and assayed for glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene as described [24]. The final concentration of the sulfhydryl modifying reagents added to the glutathione *S*-transferase assay medium never exceeded 0.5% of the GSH concentration.

Determination of protein content. Protein was determined using the method of Peterson (organelle fractions isolated from hepatocytes) [25] or the biuret method (whole liver microsomes) [26] using crystallized and lyophilized bovine serum albumin as standard.

Determination of the amino acid analysis of the 17 kDa protein band. The amino acid analysis was performed on SDS–polyacrylamide gel slices containing rat liver microsomal glutathione *S*-transferase, which had been purified as described [27] or on gel slices containing the 17 kDa protein of the microsomal fraction from control and quinone imine-treated hepatocytes. The gel slices were cut out while the gels were illuminated with a 365 nm light source before fixation of the gels. In the case of purified microsomal glutathione *S*-transferase, mBBr-derivatized fluorescent enzyme was prepared and used as a size marker in the lanes next to the non-derivatized enzyme. Gel slices were then hydrolysed for 24 hr in 6 M HCl in sealed and evacuated ampoules at 110°. The gel material was removed by centrifugation and the hydrolysed amino acids were applied to a Beckmann 121 M amino acid analyser.

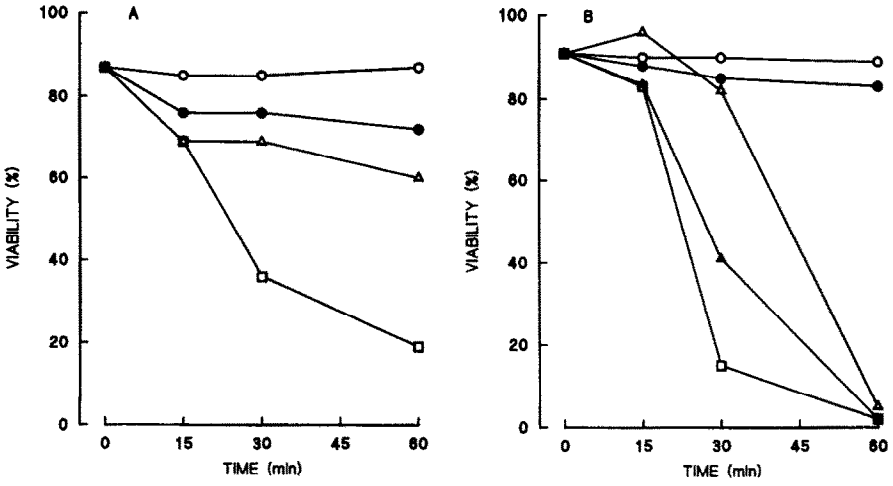


Fig. 1. Effect of NAPQI and 3,5-Me_2-NAPQI on the viability of isolated rat hepatocytes. Cytotoxicity of the quinone imines was monitored by Trypan blue uptake. The following concentrations of the quinone imines were used: Panel A: NAPQI, 100 μM (●), 250 μM (△), 450 μM (□); Panel B: 3,5-Me_2-NAPQI, 175 μM (●), 250 μM (△), 350 μM (▲), 450 μM (□); control incubations were in the presence of Me_2SO (○).

Statistical analysis. Duncan's multiple range test was performed to determine the significance of differences among individual group means. When appropriate, primary data were log-transformed prior to statistical evaluation in order to achieve homogeneity of variances. The significance level chosen for all statistical analyses was $P < 0.05$. The statistical analyses were carried out with the SPSS/PC$^+$ statistical package.

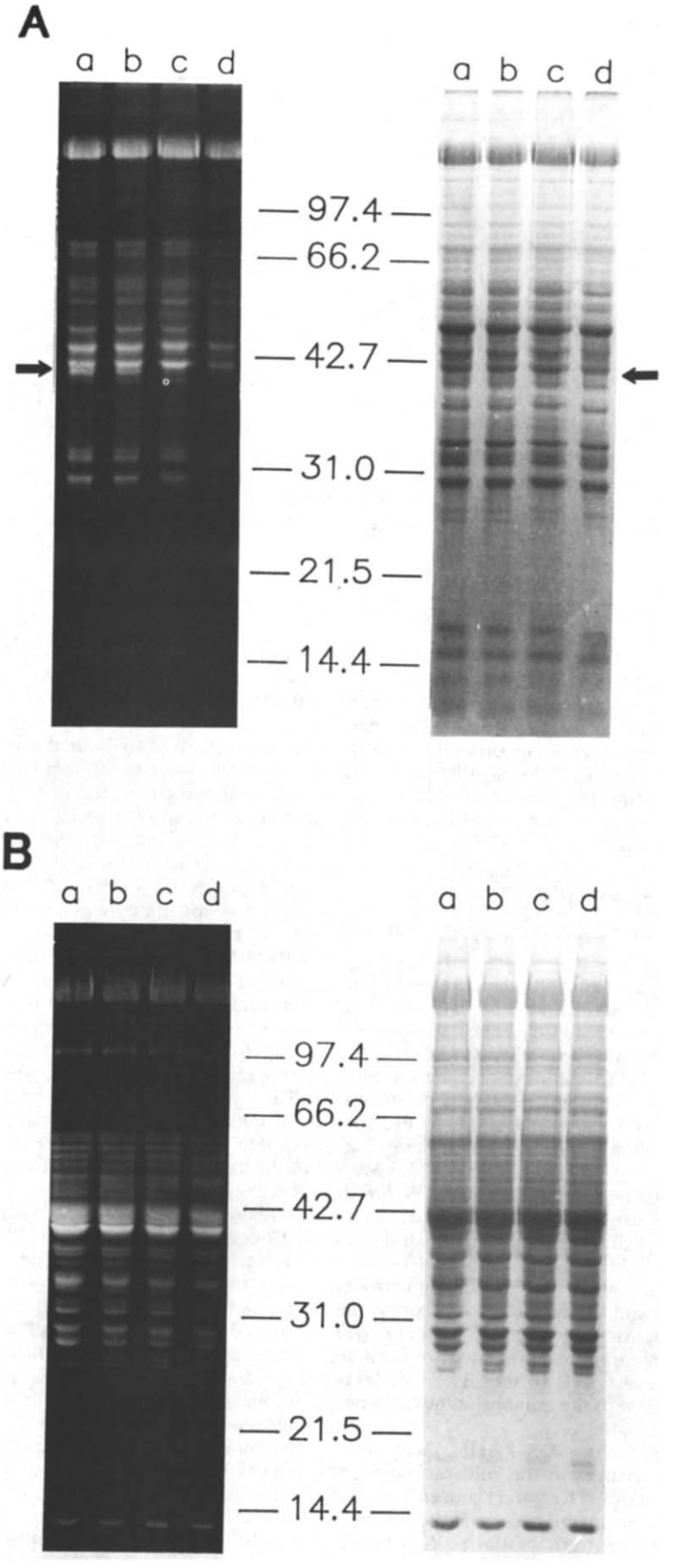
RESULTS

Cytotoxicity and protSH depletion by NAPQI and 3,5-Me_2-NAPQI

In agreement with previously reported results [16, 28], the addition of NAPQI or 3,5-Me_2-NAPQI to isolated rat hepatocytes caused a concentration- and time-dependent loss of cell viability. Concentrations of NAPQI of 100 and 250 μM produced only a small decrease in viability during the 60-min incubation period, whereas with 450 μM NAPQI, a 70% loss of viability was already observed at 30 min (Fig. 1A). As shown in Fig. 1B, 175 and 250 μM 3,5-Me_2-NAPQI (a dimethylated analogue of NAPQI with only oxidizing properties) was not markedly cytotoxic at 30 min, by which time 350 and 450 μM had decreased cell viability to 40 and 15% of control, respectively. To allow a better comparison between protSH depletion and the degree of cytotoxicity, the representative data shown in Figs 2–5 were obtained from the same preparations of hepatocytes as those used in Fig. 1 (see also Refs 16 and 28 for variability of cytotoxic response to the quinone imines between preparations of hepatocytes).

Figure 2 shows the SDS–PAGE analysis of NAPQI-induced changes in the sulfhydryl content of hepatocyte proteins. The lowest concentration of NAPQI (100 μM) did not induce any marked changes in the fluorescence pattern of the protein bands. Increasing the concentration of NAPQI to 250 μM caused a marked decrease in fluorescence of a number of protein bands, one of which having an apparent molecular mass of around 40 kDa. This band was present in both the mitochondrial (Fig. 2A) and the microsomal fractions (Fig. 2C). Several additional protein bands in the 28–32 kDa range, present in the mitochondrial and cytosolic fractions, and a 17 kDa band in the microsomal fraction showed a substantial loss of fluorescence after a 5-min exposure to NAPQI (250 μM). In addition, a slight decrease in fluorescence affecting many bands was observed at this concentration of NAPQI. With 450 μM NAPQI, the loss of fluorescence was more general; however, several bands did not show any apparent decrease in fluorescence, indicating that these sulfhydryl groups were not accessible to NAPQI even at highly cytotoxic concentrations. Comparison of the fluorescence pattern with the CBB-stain of the respective protein bands showed that the decreased fluorescence of most bands was not due to a decreased protein content. However, in the case of the 40 kDa microsomal protein (Fig. 2C), the decrease in fluorescence corresponded to a decreased CBB-stain of the band.

Compared to NAPQI, the decrease in protein fluorescence caused by 3,5-Me_2-NAPQI was more pronounced. Investigation of the samples taken 5 min after the addition of 3,5-Me_2-NAPQI (Fig. 3) revealed that in the case of this analogue, too, different proteins exhibited different susceptibilities towards thiol modification. Beside a slight general decrease in fluorescence in the cytosolic and mitochondrial fractions, several major bands (e.g. mitochondrial bands of 28–32 kDa) were affected by 175 μM 3,5-Me_2-NAPQI, a concentration that was essentially non-toxic to the isolated hepatocytes. With 250 μM 3,5-Me_2-NAPQI, there was a greater decrease in fluorescence in all three fractions,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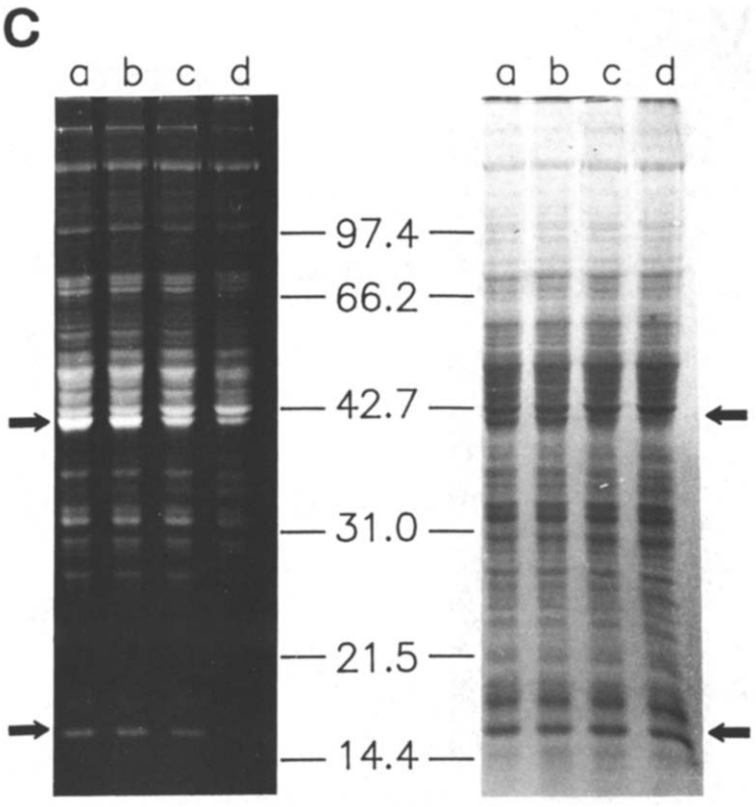


Fig. 2. Modification of protein thiols following treatment with NAPQI for 5 min. Hepatocytes were incubated with different concentrations of NAPQI for 5 min, before derivatization with mBBr and subfractionation into mitochondrial (A), cytosolic (B) and microsomal (C) fractions. The proteins were then separated by SDS–PAGE as described in Materials and Methods. Protein thiol-containing bands were identified by monitoring mBBr-fluorescence (left panel) and protein content was estimated following CBB staining (right panel). The following concentrations of NAPQI were used: 0 μM (lanes a), 100 μM (lanes b), 250 μM (lanes c), 450 μM (lanes d); control incubations were in the presence of Me_2SO. The electrophoretic mobility of the molecular mass standards (in kDa) is indicated in the middle.

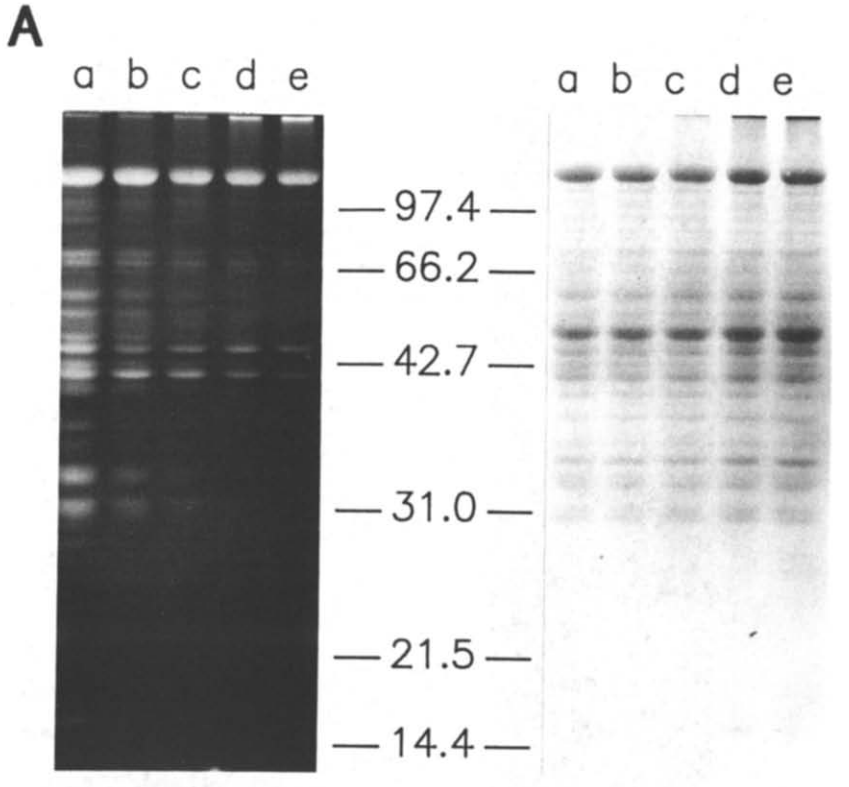
together with a substantial loss in three main microsomal protein bands of apparent molecular masses of 17, 40 and 47 kDa. As with NAPQI, a number of proteins, e.g. a 44 kDa mitochondrial protein band (Fig. 3A) and a 36 kDa microsomal protein band (Fig. 3C) showed little or no loss of mBBr-fluorescence at 5 min within the range of concentration of 3,5-Me_2-NAPQI used.

SDS–PAGE analysis revealed that incubating hepatocytes with NAPQI for 30 min did not alter the extent of fluorescence loss in the three subcellular fractions observed at 5 min (data not shown). In contrast, 3,5-Me_2-NAPQI-induced protein thiol depletion was markedly dependent on the duration of incubation (Fig. 4). After 30 min, mitochondrial and microsomal proteins showed an almost complete loss of fluorescence with 250 μM 3,5-Me_2-NAPQI. The loss of fluorescence was accompanied by a decreased CBB stain of the majority of the proteins and was associated with the formation of protein aggregates of high molecular mass in all three fractions (Fig. 4), that in part did not even enter the stacking gel (Fig. 5B; mitochondrial and microsomal fractions not shown). High molecular mass protein aggregate formation was not observed when hepatocytes were incubated under the same conditions with NAPQI (Fig. 5A).

Analysis of the 17 kDa microsomal protein

One of the major microsomal protein bands that was found to markedly lose its fluorescence upon incubation with a cytotoxic concentration of 3,5-Me_2-NAPQI, and to a lesser extent with NAPQI, was a protein of apparent molecular mass of 17 kDa. It has previously been shown that the most abundant microsomal protein migrating after the dye front on an 11% SDS–polyacrylamide gel with an apparent molecular mass of 17 kDa is the liver microsomal glutathione *S*-transferase (EC 2.5.1.18) [29]. This enzyme has a molecular mass of 17.3 kDa and contains one reactive cysteine residue at position 49 [27]. We therefore investigated the possibility that the 17 kDa microsomal protein identified here as a target for both NAPQI and 3,5-Me_2-NAPQI-induced protSH modification, was the microsomal glutathione *S*-transferase.

Purified microsomal glutathione *S*-transferase co-migrated on SDS–polyacrylamide gels with the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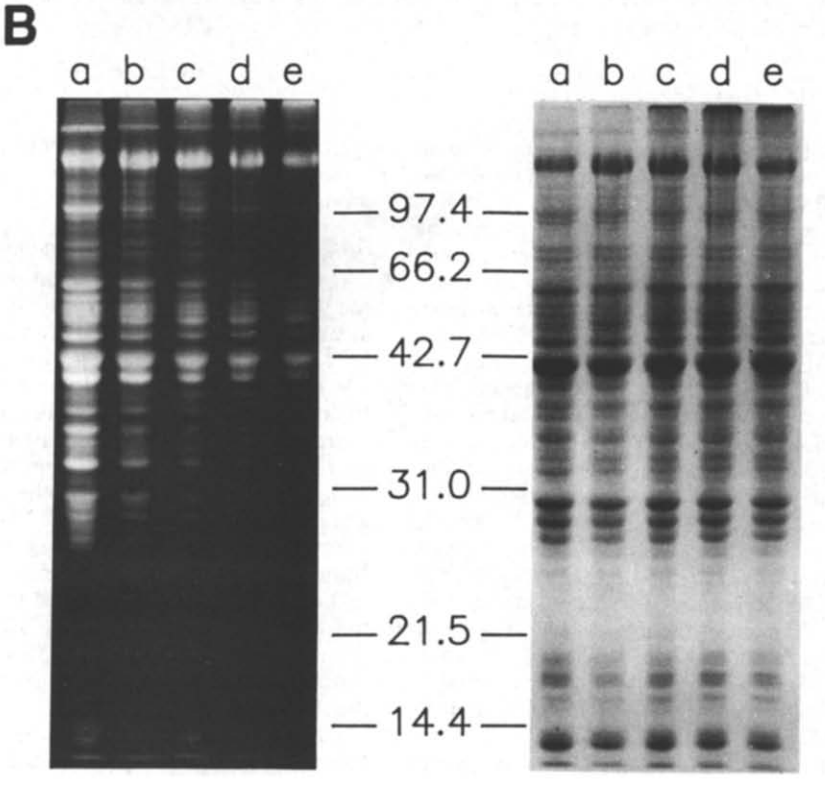
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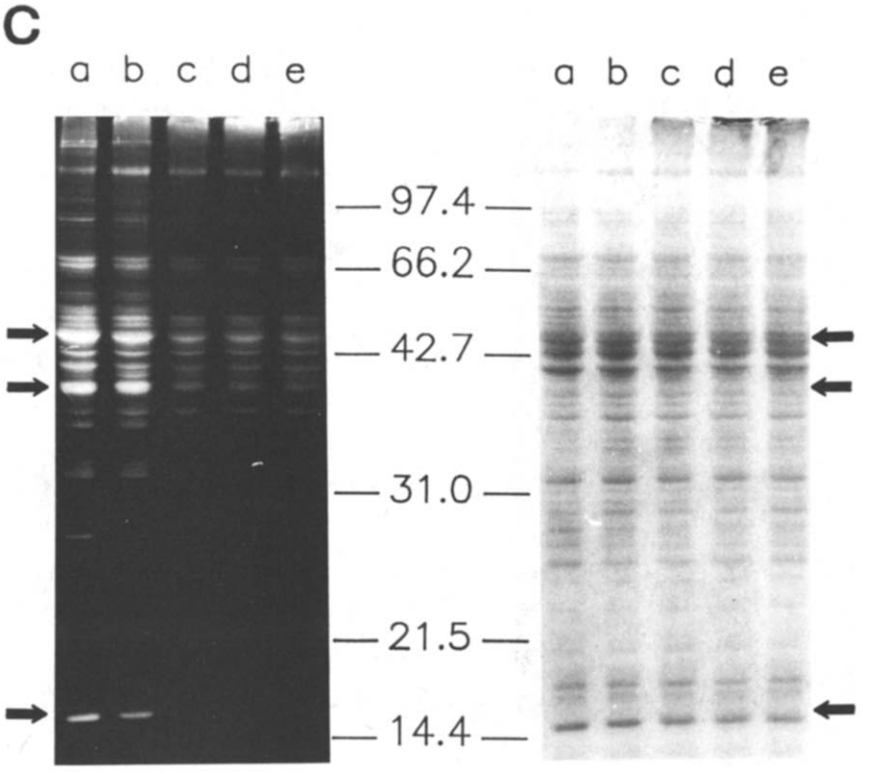


Fig. 3. Modification of protein thiols following treatment with 3,5-Me_2-NAPQI for 5 min. Hepatocytes were incubated with different concentrations of 3,5-Me_2-NAPQI for 5 min, before derivatization with mBBr and subfractionation into mitochondrial (A), cytosolic (B) and microsomal (C) fractions. The proteins were then separated by SDS–PAGE as described in Materials and Methods. Protein thiol-containing bands were identified by monitoring mBBr-fluorescence (left panel) and protein content is estimated following CBB staining (right panel). The following concentrations of 3,5-Me_2-NAPQI were used: 0 μM (lanes a), 175 μM (lanes b), 250 μM (lanes c), 350 μM (lanes d), 450 μM (lanes e); control incubations were in the presence of Me_2SO. The electrophoretic mobility of the molecular mass standards (in kDa) is indicated in the middle.

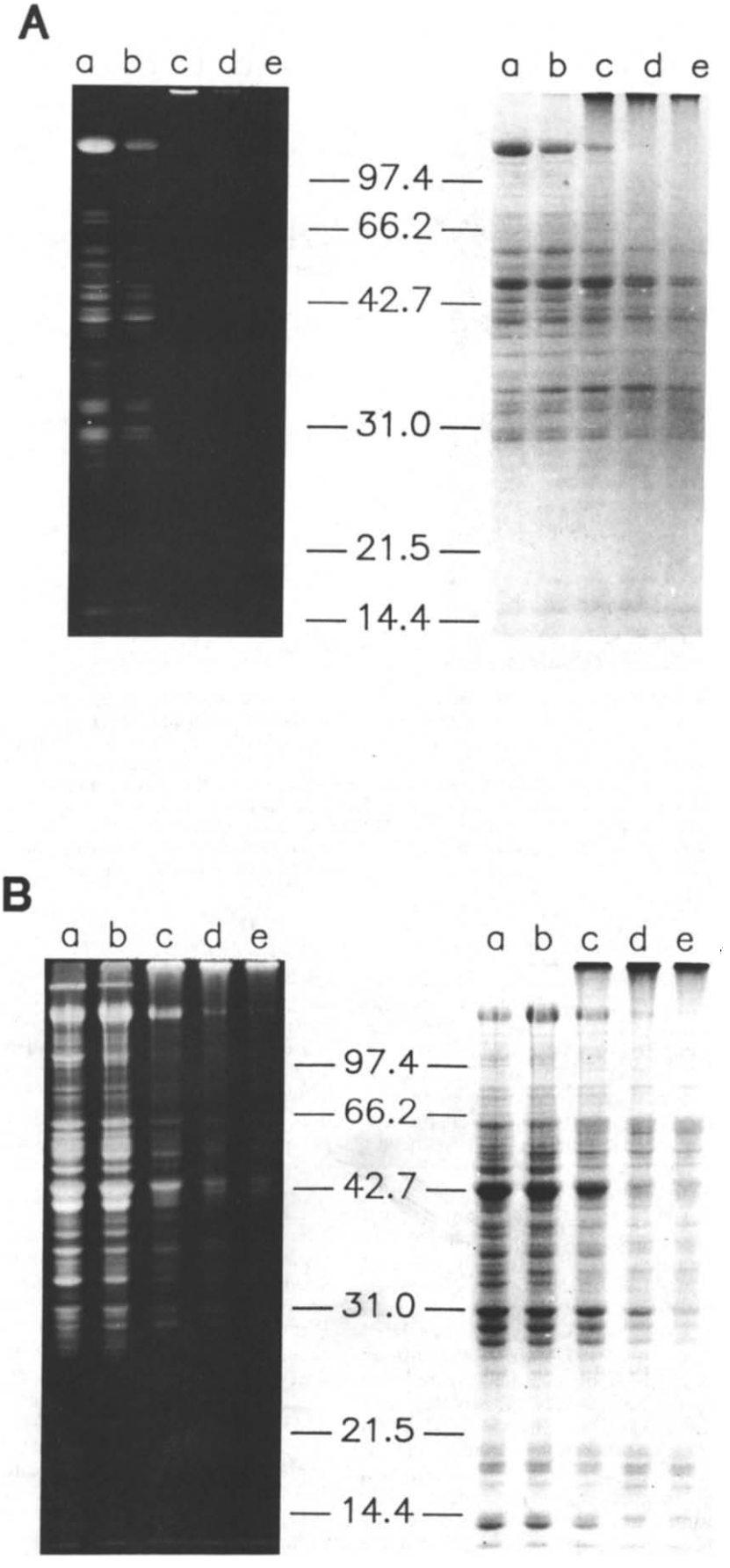
17 kDa microsomal protein of isolated rat hepatocytes (data not shown). In addition, the amino acid composition of the 17 kDa microsomal protein from either control or 3,5-Me_2-NAPQI-treated hepatocytes was essentially identical to that of purified glutathione *S*-transferase analysed from a gel slice (Table 1). The content of the amino acid arginine was decreased by incubation of hepatocytes with 3,5-Me_2-NAPQI, and the observed increase in valine content in the samples from 3,5-Me_2-NAPQI-treated hepatocytes was due to a broad peak underlying the valine peak, which made it impossible to accurately estimate the baseline for that particular amino acid.

Since the microsomal glutathione *S*-transferase can be activated following incubation of isolated microsomes with sulfhydryl modifying reagents, in particular with NEM [30], we investigated the effect of incubating hepatocytes and isolated liver microsomes with NAPQI and 3,5-Me_2-NAPQI on the activity of the microsomal glutathione *S*-transferase. NEM was included in this study as a positive control. Incubation of isolated hepatocytes with mBBr resulted in a marked activation of the enzyme (Table 2), showing that the enzyme's cysteine residue was derivatized by mBBr under the conditions used to monitor quinone imine-induced cellular protein thiol depletion. The two quinone imines caused a concentration dependent, up to 4-fold activation of the enzyme in hepatocytes (Table 2) and isolated microsomes (Table 3). Taken together, our data strongly suggest that the microsomal glutathione *S*-transferase was the main constituent of the 17 kDa microsomal protein.

DISCUSSION

A recently developed method which enables the *in situ* labelling and visualization of accessible protSH groups in subcellular fractions of isolated hepatocytes [15] was used to investigate whether the previously reported [4, 11] depletion of total cellular protSH by NAPQI was selective for subcellular fractions and individual proteins. We found that both NAPQI and its oxidizing analogue, 3,5-Me_2-NAPQI, caused a concentration-dependent decrease in protSH content of the mitochondrial, cytosolic and microsomal fractions.

Comparison of the degree of protSH depletion induced by similar cytotoxic concentrations of either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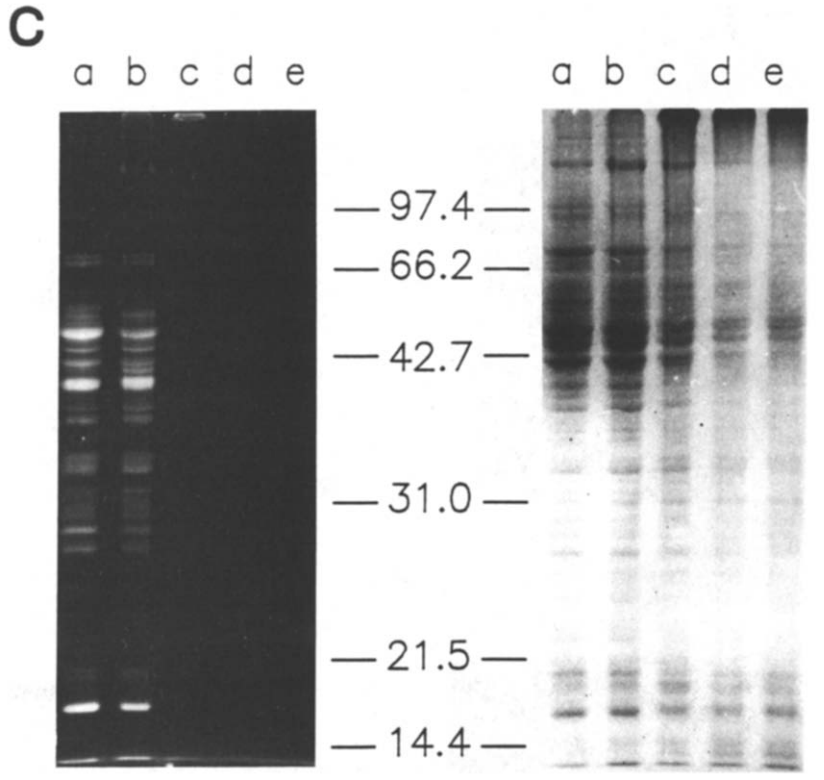

Fig. 4. Modification of protein thiols in the mitochondrial (A), cytosolic (B) and microsomal (C) fraction of isolated hepatocytes following treatment with 3,5-Me_2-NAPQI for 30 min. Protein thiol-containing bands were identified by monitoring mBBr-fluorescence (left panel) and protein content is estimated following CBB staining (right panel). For further details see legend to Fig. 2. The following concentrations of 3,5-Me_2-NAPQI were used: 0 μM (lanes a), 175 μM (lanes b), 250 μM (lanes c), 350 μM (lanes d), 450 μM (lanes e); control incubations were in the presence of Me_2SO. The electrophoretic mobility of the molecular mass standards (in kDa) is indicated in the middle.

quinone imine showed that protSH depletion induced by 3,5-Me_2-NAPQI was more extensive than after treating the cells with NAPQI. Furthermore, 3,5-Me_2-NAPQI-induced protSH depletion increased with the duration of the incubation, where NAPQI-induced protSH depletion was essentially complete within 5 min of its addition to the hepatocytes. In a recent study, Khairallah and co-worker [31] found that the parent phenolic compound of 3,5-Me_2-NAPQI, 3,5-Me_2-APAP, also produced a greater loss of protSH in cultured mouse hepatocytes than did equimolar concentrations of APAP. These authors suggested that, in contrast to APAP, 3,5-Me_2-APAP might undergo redox cycling, leading to a greater extent of protSH depletion [31]. However, unlike many semiquinones, the electrophilic phenoxyl radical enzymatically produced from 3,5-Me_2-APAP or spontaneously generated from 3,5-Me_2-NAPQI does not react with molecular oxygen [32, 33], and is much more stable than the radical formed from APAP, which is rapidly consumed through dimerization and polymerization reactions [32]. Also, Van de Straat *et al.* [33] have observed a slightly enhanced uncoupling of cytochrome P450 by 3,5-Me_2-NAPQI as compared to NAPQI. These properties may explain the greater and more continuous loss of protSH observed in the present study with 3,5-Me_2-NAPQI.

ProtSH depletion appeared, in part, to be a selective phenomenon, with different proteins showing different susceptibilities to modification of their cysteine residues by either of the quinone imines. The observed differences in susceptibility of the various proteins most likely reflect differences in accessibility and reactivity of the respective thiol groups, as determined by their local environment and individual pK_a values. In general, the phenomenon of selectivity was more pronounced with the oxidizing analogue 3,5-Me_2-NAPQI than with NAPQI. One-dimensional gels do, however, suffer from certain limitations of resolution so that a single band may contain several different but co-migrating proteins. Hence, a gradual loss of fluorescence of one protein band does not always allow a clear distinction to be made between a gradual thiol loss of a single protein or alterations of different proteins located in the same band on the gel. This, together with the loss of total protein through aggregate formation by 3,5-Me_2-NAPQI, makes it difficult to establish a causal relationship between the loss of certain protSH groups and the development of cytotoxicity with the quinone imines.

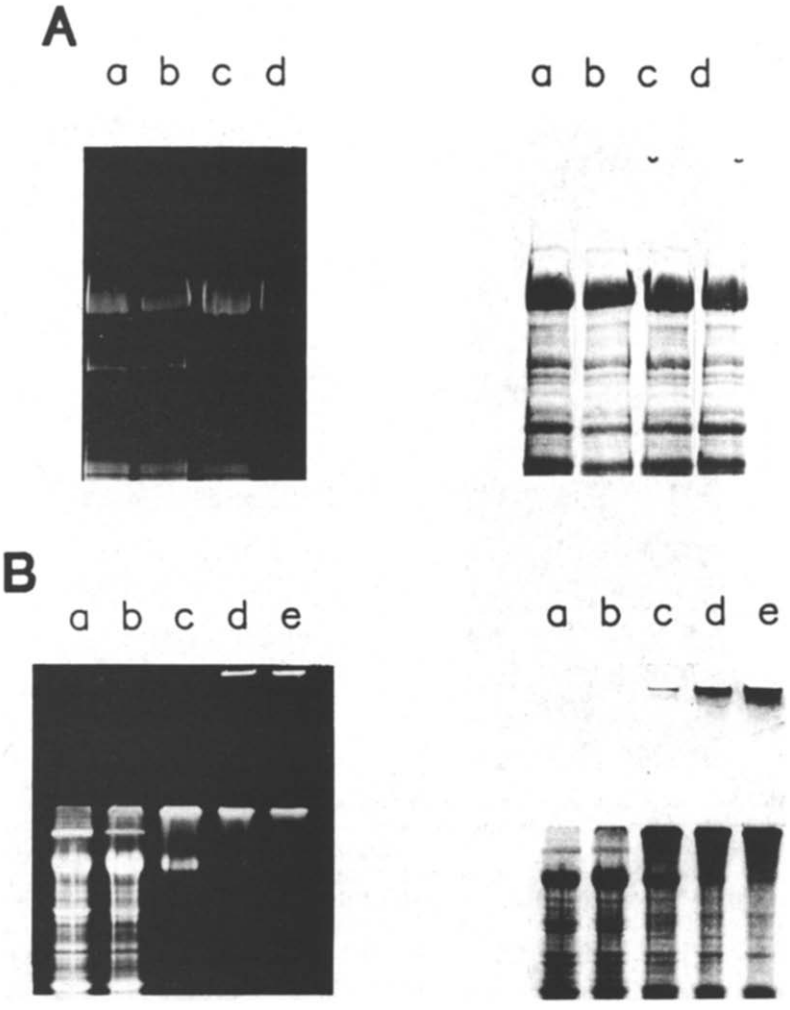

Fig. 5. Formation of high molecular mass aggregates after treatment of hepatocytes with 3,5-Me_2-NAPQI. Hepatocytes were exposed to NAPQI (Panel A) or 3,5-Me_2-NAPQI (Panel B) for 30 min, before derivatization with mBBr and preparation of the cytosolic fraction. For further details see legend to Fig. 2. Protein thiol-containing bands were identified by monitoring mBBr-fluorescence (left panel) and protein content is estimated following CBB staining (right panel). The following concentrations of quinone imines were used: NAPQI, 0 μM (lanes a), 100 μM (lanes b), 250 μM (lanes c), 450 μM (lanes d); 3,5-Me_2-NAPQI, 0 μM (lanes a), 175 μM (lanes b), 250 μM (lanes c), 350 μM (lanes d), 450 μM (lanes e); control incubations were in the presence of Me_2SO. The electrophoretic mobility of the molecular mass standards (in kDa) is indicated in the middle.

Concentrations of the quinone imines that were highly cytotoxic, additionally led to a general loss of protSH. However, the protSH content of certain bands remained unchanged even at these high quinone imine concentrations used. In the case of NAPQI and low concentrations of 3,5-Me_2-NAPQI, the loss of the mBBr-fluorescence of the bands was not due to a loss of the corresponding proteins (except for the 40 kDa microsomal protein, where with NAPQI the loss of fluorescence was paralleled with the loss of CBB staining). In contrast, high concentrations of 3,5-Me_2-NAPQI produced a general loss in mitochondrial, microsomal and cytosolic proteins. These proteins were in part recovered as high molecular mass protein aggregates that did not enter the stacking gels. The formation of such high molecular mass protein aggregates has also been observed by Khairallah and co-workers [31] using the parent phenolic compound. Although linkage of proteins via the quinone moiety is conceivable, in the case of 3,5-Me_2-NAPQI, blocking of the reactive 3 and 5 position by introduction of methyl groups has been shown to prevent covalent binding of 3,5-Me_2-NAPQI to proteins [16, 18]. Since in the present study, SDS–PAGE was performed under reducing conditions, disulfide bond formation can also be excluded as the cause of the observed protein crosslinking. Hence, the formation of these aggregates could have resulted from non-covalent protein aggregation or from a radical-mediated [32, 34] interaction with amino acid residues other than cysteine [35] or possibly with

Table 1. Amino acid composition of the 17 kDa microsomal protein

Amino acid	Amino acid content (%)			
	Purified mic GSH-T	Control hepatocytes	3,5-Me_2-NAPQI-treated hepatocytes (250 μM)	3,5-Me_2-NAPQI-treated hepatocytes (350 μM)
His	2.6	3.6	3.3	3.1
Arg	6.1	7.1	4.9	4.2
Asp	10.5	9.7	9.7	9.3
Thr	6.5	6.4	6.5	6.3
Ser	7.4	8.5	10.7	10.0
Glu	8.0	9.7	9.5	9.1
Pro	6.7	5.7	6.5	7.1
Ala	9.4	8.5	5.2	6.9
Val	7.0	5.8	9.9	10.8
Met	2.8	2.3	2.0	1.7
Ile	6.0	4.8	4.6	4.5
Leu	15.2	15.0	13.2	13.7
Tyr	4.9	5.2	5.2	4.7
Phe	7.2	7.5	8.5	7.4

The amino acid composition of the 17 kDa microsomal protein was determined from gel slices as described in Materials and Methods. Values are given as per cent of total amino acid content. The amino acids cysteine, tryptophane, lysine and glycine were not included for the calculation of the 100% values. Tryptophane and cysteine cannot be detected by the amino acid analysis method used here, and the lysine and glycine content was increased markedly in the samples of purified glutathione transferase from a gel slice compared to a gel-free sample, indicating interferences with components from gel or buffer.
mic GSH-T, microsomal glutathione *S*-transferase.

carbohydrate or lipid residues in the case of glycoproteins and lipoproteins, respectively.

Using an antibody specifically directed against protein-bound APAP, Khairallah and co-workers [31, 36] have investigated the covalent binding of the reactive metabolite of APAP to cellular proteins. In cultured mouse hepatocytes, covalent binding specifically occurred with a selected number of proteins, a 44 kDa protein mainly found in the microsomal and to a lesser degree in the cytosolic fraction, and protein of 56 and 58 kDA in the cytosolic fraction. Additionally, in *in vivo* conducted experiments, the same authors [36] observed covalent binding of APAP to a mouse liver 44 kDa protein in the 8000 *g* pellet. Incubation of isolated mouse hepatocytes with NAPQI also resulted in covalent binding to the 58 kDa protein but only minimal binding occurred to the 44 kDa protein [36]. APAP and 3,5-Me_2-APAP furthermore induced a marked protein thiol loss of a protein band of around 58 kDa in the microsomal fraction [31].

Proteins of the molecular masses reported above could only in part be identified as targets of quinone imine-induced protSH modification in isolated rat

Table 2. Effect of incubation of hepatocytes with NEM, mBBr or quinone imines on microsomal glutathione *S*-transferase activity

Treatment	Glutathione *S*-transferase activity (nmol/mg protein/min)
None (Me_2SO)	102 ± 20
NEM (200 μM	399 ± 63*
mBBr (4 mM)	510†
NAPQI (120 μM)	174 ± 36
NAPQI (160 μM)	244 ± 38*
3,5-Me_2-NAPQI (150 μM)	115 ± 22
3,5-Me_2-NAPQI (210 μM)	179 ± 36

Hepatocytes (10^6/mL) were incubated for 5 min in the absence or presence of NEM, mBBr or quinone imines before isolation of the microsomal fractions and measurements of glutathione *S*-transferase activity both as described in Materials and Methods.

Data are expressed as means ± SD of three to four separate hepatocyte preparations.

* $P < 0.05$.

† Mean of two separate preparations.

Table 3. Effect of treatment of rat liver microsomes with NEM or quinone imines on microsomal glutathione *S*-transferase activity

Treatment	Glutathione *S*-transferase activity (nmol/mg protein/min)
None (Me_2SO)	125 ± 18
NEM (200 μM)	1500 ± 266*
NAPQI (125 μM)	363 ± 35*
NAPQI (250 μM)	418 ± 54*
3,5-Me_2-NAPQI (125 μM)	344 ± 23*
3,5-Me_2-NAPQI (250 μM)	506 ± 12*

Rat liver microsomes were incubated (2 mg protein/mL) with sulfhydryl reagents at the indicated concentrations for 1 min and glutathione *S*-transferase activity measured on an aliquot as indicated in Materials and Methods.

Data are expressed as means ± SD of four separate microsome preparations.

* $P < 0.05$.

hepatocytes. The 44 kDa protein band has a low thiol content [36], and thus may not be detectable through labelling with mBBr. The 58 kDa protein band is the most intensely mBBr-fluorescent microsomal protein band in cultured mouse hepatocytes [31]; however, such a highly fluorescent band corresponding to 48 kDa was not detected in the microsomal fraction of freshly isolated rat hepatocytes, possibly indicating species differences in protSH distribution. The covalent binding of APAP to the 56 and 58 kDa protein bands of the cytosolic fraction described [31] may possibly be related to the loss of mBBr-fluorescence observed in the cytosolic fraction in this study.

In isolated rat hepatocytes an abundant microsomal protein band of an apparent molecular mass of 17 kDa is a major target with regard to protSH depletion by cytotoxic concentrations of the quinone imines. Both electrophoretic mobility and amino acid composition of the 17 kDa microsomal protein band strongly suggest that microsomal glutathione *S*-transferase (EC2.5.1.18) was the major constituent of this band. Microsomal glutathione *S*-transferase contains one reactive cysteine residue in position 49 and alkylation of this residue is accompanied by marked increase in the specific activity of the enzyme. With NAPQI, both covalent binding of the quinone imine to Cys 49 or protein mixed-disulfide formation of this residue with GSSG are conceivable; however, activation of the enzyme by GSSG has been found to require very high concentrations of the disulfide *in vitro* [37]. Since 3,5-Me_2-NAPQI does not covalently bind to thiol groups [16], this suggests that 3,5-Me_2-NAPQI-induced activation of the enzyme in isolated microsomes occurs via a yet different mechanism.

In conclusion, the results presented here show that in isolated rat hepatocytes, NAPQI and 3,5-Me_2-NAPQI produced a concentration-dependent decrease in protSH content, with protSH loss being selective for some specific protein bands with low concentrations of 3,5-Me_2-NAPQI and intermediate concentration of NAPQI. Since quinone imine-induced protSH loss appeared to correlate with cytotoxicity, our present results are consistent with a critical role of protSH loss in the mechanism of quinone-imine induced cytotoxicity. Microsomal glutathione *S*-transferase was identified as a major target protein for the quinone imines. The activation of the transferase may result in a more rapid conjugation of the NAPQI generated in the vicinity by cytochrome P450, and this could play a protective role against APAP-induced cytotoxicity.

Acknowledgements—This study was supported in parts by grants from the Swedish Medical Research Council, Swedish Cancer Society, by funds from Karolinska Institutet and NIH grant (GM 25418). We thank Prof. Hans Jörnvall and Carina Palmberg, Karolinska Institutet, Department of Chemistry I, for the analysis of the amino acid composition.

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