Effect of Glutathione Depletion on the Hepatotoxicity and Covalent Binding to Rat Liver Macromolecules of N-Hydroxy-2-acetylaminofluorene

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ABSTRACT

Glutathione plays an important role in the protection of the liver against several hepatotoxins. The hepatocarcinogen N-hydroxy-2-acetylaminofluorene is converted in the rat in vivo to reactive metabolites that bind covalently to cellular macromolecules. These metabolites may also react with glutathione, resulting in the formation of glutathione conjugates and in the detoxification of reactive metabolites. The role of glutathione in detoxification was investigated by depletion of glutathione in the rat in vivo with diethyl maleate.

When rats were pretreated with diethyl maleate, 45 min before the administration of N-hydroxy-2-acetylaminofluorene, excretion of 2-acetylaminofluorene:glutathione conjugates in bile was decreased by 60% as compared to controls. However, total covalent binding to rat liver protein was not increased, and total binding to DNA was even decreased (p < 0.1), apparently at the expense of the acetylated carcinogen-DNA adducts. Formation of deacetylated, 2-aminofluorene adducts to DNA was not affected by diethyl maleate. Pretreatment with diethyl maleate had no major effect on the acute hepatotoxic effects of N-hydroxy-2-acetylaminofluorene.

The results indicate that glutathione does not play a vital role in the detoxification of reactive metabolites generated from the carcinogen N-hydroxy-2-acetylaminofluorene, since glutathione is not very effective in competing with macromolecules for trapping of reactive metabolites of N-hydroxy-2-acetylaminofluorene. Thus, 1 mM glutathione did not decrease the covalent binding of 2-acetylaminofluorene:N-sulfate (one of the main reactive metabolites that is formed in vivo) to DNA in vitro, while 10 mM glutathione decreased the covalent binding to RNA by only 20% and to DNA by only 40%.

INTRODUCTION

A major part of the reactive metabolites that are generated from toxic compounds, such as benzo(a)pyrene (19), aflatoxin B1 (13, 15), acetaminophen (23, 31), and penicillin acid (11), can be detoxified by conjugation with glutathione before they can react with cellular macromolecules.

Until now, it is not clear whether glutathione plays a major role in trapping the reactive intermediates generated from aromatic amines and their hydroxamic acid metabolites like N-OH-AAF3 in vivo. Covalent binding of N-OH-AAF to macromolecules in rat liver (the main target organ for the carcinogenic action of N-OH-AAF in male rats) occurs while high concentrations of glutathione are still present (26, 30). This suggests that in vivo glutathione traps only very inefficiently the reactive intermediates of N-OH-AAF that react with cellular macromolecules. However, we have shown that the unstable and reactive N-acetoxy ester of the carcinogen N-OH-AAF (N-acetoxy-AAF) reacts in vitro with glutathione, yielding 4 conjugates which were identified as 1-, 3-, 4-, and 7-(glutathione-S-yl)-2-acetylaminofluorene (26). The synthetically prepared N-sulfate ester of N-OH-AAF (AAF-N-sulfate) also reacts in vitro with glutathione whereby the same 4 conjugates are formed (7). Two of these conjugates, 1- and 3-GS-AAF, are excreted in bile of rats that received an i.v. dose of N-OH-AAF (26). Kadlubar et al. (20) have obtained similar results for MAB N-sulfate, a presumed ultimate carcinogenic metabolite of the carcinogen N,N-dimethyl-4-aminobenzene. They found that, while MAB N-sulfate readily forms adducts with RNA and DNA, it also reacts with glutathione in vitro, whereby a conjugate is formed which is identical to a biliary in vivo N,N-dimethyl-4-aminobenzene metabolite (20).

To get insight into the role and efficiency of glutathione in trapping reactive intermediates generated from N-OH-AAF, we have depleted glutathione in the rat in vivo with DEM, and we have determined the effects of this depletion on the metabolism of N-OH-AAF and on the interaction of its reactive metabolites with cellular macromolecules in the liver and kidney. We also studied the effect of glutathione and l-cysteine on the covalent binding of AAF-N-sulfate to DNA and RNA in vitro in order to explain some of the in vivo data.

MATERIALS AND METHODS

Animals

Male Wistar rats, of the inbred strain of the Department of Pharmacology, University of Groningen, weighing 270 to 280 g, were used. A mixture of [ring-3H]- and [1'-acet[y-14C]-N-OH-AAF (specific activity 3H, 40 mCi/mmol; 14C, 5 mCi/mmol) was administered i.v. via a lateral tail vein at a dose of 120 μmol/kg under light diethyl ether anesthesia. The solution was prepared as described previously (28).

The abbreviations used are: N-OH-AAF, N-hydroxy-2-acetylaminofluorene; N-acetoxy-AAF, N-acetoxy-N-acetoxy-2-acetylaminofluorene; AAF-N-sulfate, N-sulfonoxyn-acetyl-2-acetylaminofluorene; MAB N-sulfate, N-methyl-4-aminobenzene-N-sulfate; DEM, diethyl maleate; PCP, pentachlorophenol; GPT, glutamate-pyruvate transaminase; GOT, glutamate-oxaloate transaminase; pNP, 4-nitrophenol; pNP-S, 4-nitrophenol sulfate; CDNB, 1-chloro-2,4-dinitrobenzene; 1-GS-AAF, 1-(glutathione-S-yl)-2-acetylaminofluorene; 2-GS-AAF, 2-(glutathione-S-yl)-2-acetylaminofluorene; dGuo-C8-AAF, 3-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dGuo-C8-2-AF, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dGuo-C8-2-AF, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene; HPLC, high-performance liquid chromatography.

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DEM was administered i.p. at a dose of 3.9 mmol/kg, 45 min before the administration of N-OH-AAF. It was mixed with an equal volume of propane-1,2-diol before injection. Control rats received a mixture of equal parts of 0.9% (w/v) NaCl solution and propane-1,2-diol. In experiments in which sulfation was inhibited, PCP (40 μmol/kg) was dissolved in the propane-1,2-diol.

Rats were anesthetized with diethyl ether 4 and 48 hr after the administration of N-OH-AAF. Blood was collected from the aorta at 48 hr for the determination of serum GPT and GOT activities. The liver and kidneys of a group of rats were removed at 4 hr, immediately frozen in liquid nitrogen, and stored at -20°C. The liver and kidneys were removed, immediately frozen in liquid nitrogen, and stored at -20°C for the determination of radioactivity covalently bound to macromolecules. Samples of the liver of rats killed at 48 hr were frozen in liquid Freon, and cryostal sections (10 μm thick) were made. These were stained with hematoxylin and eosin as described before (28).

Acid phosphatase, NADH reductase, and leucyl- and phenylalanine- dipeptidase activities were demonstrated as described by Hardonk et al. (17).

In some experiments, rats were anesthetized by i.p. injection of sodium pentobarbital, 15 min after the injection of DEM. The trachea and bile duct were cannulated and the urethra was ligated. Body temperature was kept at 38 ± 0.5°C. Bile was collected in tubes on ice for 4 hr after the administration of N-OH-AAF. Urine was collected from the bladder with a syringe at the end of the experiment. The liver and kidneys were removed, immediately frozen in liquid nitrogen, and stored at -20°C for the determination of radioactivity covalently bound to macromolecules.

In the experiments in which the influence of DEM on the metabolism of pNP was investigated, DEM was administered i.p. at a dose of 3.9 mmol/kg, 45 min before the administration of pNP (120 μmol/kg). This compound was dissolved in 0.9% (w/v) NaCl solution (40 μmol/ml) and administered i.v. under light diethyl ether anesthesia. The rats were placed in metabolism cages and urine was collected for 24 hr on ice.

Chemicals

[ring-3H]-N-OH-AAF (specific activity, 83 mCi/mmol) was synthesized according to the method of Westra (38), starting with [ring-3H]-2-nitrofluorene, which was obtained by acid-catalyzed exchange of unlabeled 2-nitrofluorene with tritiated water by the method of Breaman et al. (9). Purity was >98%.

[acetyl-1-14C]-OH-AAF (specific activity, 19 mCi/mmol) was obtained from the Midwest Research Institute, Kansas City, MO. [9-3H]-2-AAF-N-sulfate was synthesized as described in Ref. 7, using [9-3H]-2-OH-AAF (specific activity, 1.8 mCi/mmol; New England Nuclear, Boston, MA) as starting material.

HPLC. Glutathione was determined by a method based on that of Lowry et al. (24).

Determination of Radioactivity Covalent Bound to Protein and DNA in Livers and Kidneys of Rats

Livers and kidneys of rats were homogenized with 2 volumes of water in an X-1020 homogenizer and protein was isolated as described in Ref. 5. Protein was determined according to the method of Lowry et al. (24). After the extraction of proteins with phenol/chloroform, the aqueous layer was dialyzed twice against 20 volumes of water for 8 hr at 20°C. RNA and DNA were precipitated by the addition of 1 ml of 1 M aqueous NaCl solution and twice the volume of cold ethanol (−20°C). After centrifugation at 3,000 × g for 15 min, the pellet was dissolved in 50 ml of 0.05 M Tris-Cl containing 0.01 M EDTA and 0.1 M NaCl (pH 7.4). Five hundred units of α-amylase, 7,500 units of RNase A, and 10,000 units of RNase T1 were added, and the mixture was incubated at 37°C for 30 min at 50°C. The mixture was centrifuged at 3000 × g for 15 min, and 20 μl of the clear supernatant was analyzed by HPLC. A 0.39-× 15-cm 55 Spherisorb ODS II (Phase Separations, Quentinsey, Clwyd, United Kingdom) column was used, that was eluted with methanol:0.025 M aqueous ammonium formate buffer, pH 3.00 (38:62), at a flow rate of 1.5 ml/min. Detection was at 340 nm. The CDNB:glutathione conjugate eluted at 3.33 ± 0.08 min, while CDNB eluted at 10.12 ± 0.07 min. The glutathione conjugates of N-OH-AAF, 1-GS-AAF and 3-GS-AAF, and the N-Glucuronide conjugate of N-OH-AAF were determined on a 0.39 × 15-cm Nucleosil 5 C8 (Machery and Nagel, Düren, West Germany) column that was eluted with a methanol:0.025 M triethylamine acetate buffer (pH 3.85) gradient at a flow of 1.5 ml/min. The initial concentration of methanol in the gradient was 38%; it was changed at a linear rate to 43% in 15 min; detection was at 280 nm. The identification of peaks was by comparison of retention times with that of authentic glutathione conjugates, and by hydrolysis with β-glucuronidase as described in Ref. 29.

Deoxyguanosine adducts were separated on a 0.39-× 15-cm Nucleosil 5 C8 column which was eluted with a convex gradient [Program 3 of a Waters Model 660 programmer (Waters Associates, Milford, MA)] of 25 to 50% (w/v) methanol in H2O over a 20-min period at a flow rate of 1.5 ml/min. Thereafter, the solvent composition was changed at a linear rate to 100% methanol in 5 min. Detection was at 280 and 340 nm. The nonradioactive markers, dGuo-N2-AAF, dGuo-C8-AAF, and dGuo-C8-AF (synthesized as described below) eluted at 12.31 ± 0.07, 19.28 ± 0.09, and 22.47 ± 0.08 min, respectively. Fractions (0.8 min) were collected in scintillation vials, and radioactivity was determined by liquid scintillation counting after the addition of 5.0 ml of Hydro-Luma. Recovery of radioactivity from the column was 93.5 ± 2.5% (n = 6) for control rats and 92.0 ± 2.4 (n = 5) for DEM-pretreated rats.

pNP-S and pNP-G were determined in rat urine by HPLC as described in Ref. 14.
of 0.005 M 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3 diol buffer containing 0.001 M EDTA, pH 7.0, and radioactivity was determined by liquid scintillation counting after the addition of 10 ml of Plasmasol.

DNA Hydrolysis and Isolation of Carcinogen-DNA Adducts

DNA was hydrolyzed enzymatically as described by Howard et al. (18). The nonradioactive adduct markers dGuo-C8-AAF and dGuo-N2-AAF were synthesized as described in Ref. 6, while dGuo-C8-AF was obtained by deacetylation of dGuo-C8-AAF in aqueous 0.5 M EDTA (tetrasodium salt) solution for 1 hr at 95°C. The deacetylated adduct was isolated by adsorption onto a 2.6- x 20-cm Sephadex LH20 column (Pharmacia AB, Uppsala, Sweden) and eluted from the column with 70% (v/v) ethanol in water, after the EDTA had been washed out with water. It had an identical UV absorption spectrum and retention time on HPLC as described in Ref. 4. Nonradioactive adduct markers were added to the DNA hydrolysates and adducts were isolated by adsorption onto C18 Seppak-cartridges (Waters Associates). The cartridges were washed with 2.0 ml of 20% (v/v) methanol in water, and the adducts were recovered by elution with 5.0 ml of methanol. For control rats, 92 ± 3% (n = 8) of the total 3H radioactivity was recovered, while 93 ± 3% (n = 7) was recovered from DEM-pretreated rats. After evaporation of the methanol at 45°C with a stream of nitrogen, the residue was dissolved in 200 μl of 50% (v/v) methanol in water. A 100-μl aliquot was analyzed by HPLC.

Incubation of AAF-N-sulfate with RNA or DNA and Nucleophiles in Vitro

A 420-μl sample of a solution of AAF-N-sulfate in dimethylformamide (14 nm) was added to 6.0 ml of a solution of RNA or DNA (2.7 mg/ml) in 50 mM aqueous Tris-HCl buffer, pH 7.4, to yield to final (theoretical) concentration of AAF-N-sulfate to 1 μM. This mixture was incubated for 20 min at 37°C. In some experiments, the buffer also contained 1 or 10 mM glutathione or L-cysteine. A 5-ml aliquot of the incubations containing DNA were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), followed by extraction twice with an equal volume of diethyl ether. Incubations containing RNA and a 1-ml aliquot of the incubations with DNA were directly extracted twice with diethyl ether. The diethyl ether was evaporated from the combined fractions with a stream of nitrogen at 45°C, and the residue was dissolved in 1.0 ml of ethanol. An aliquot of the ethanol fraction was counted for radioactivity and 15 μl were analyzed by thin-layer chromatography. DNA and RNA were precipitated from the aqueous phase of the incubation by the addition of 1.0 ml of 2% (w/v) aqueous solution of cetyl trimethylammonium bromide, and the DNA and RNA were isolated and purified as described for DNA in Ref. 37. Recovery of RNA by this method was >95%. Recovery of DNA was >91%.

RESULTS

Depletion of Glutathione by DEM in Liver and Kidney of Rats Which Had Received N-OH-AAF. It is known that DEM treatment depletes hepatic and renal glutathione in the rat (33, 34); therefore, we used this compound to deplete glutathione in rats before the administration of N-OH-AAF.

N-OH-AAF (120 μmol/kg, i.v.) itself slightly decreased the hepatic glutathione concentration as observed 15 min after administration; however, at later times glutathione concentrations tended to be even above control levels (Chart 1). In rats that had been pretreated with DEM (3.9 mmol/kg, i.p.) the glutathione concentration decreased to 10% of controls; hepatic glutathione was still decreased, both at 15 min and 2 hr, after the administration of N-OH-AAF. Because metabolism of N-OH-AAF after i.v. administration occurs predominantly in the first 2 hr after administration, depletion of glutathione by DEM ensures a low glutathione concentration during the most relevant period after N-OH-AAF administration. After 24 hr, the hepatic glutathione concentration had returned to control values in these DEM-pretreated rats.

Depletion of renal glutathione also occurred in rats that had received DEM; the renal glutathione concentration, 30 min after injection of solvent in control rats was 0.67 ± 0.10 mm (n = 4), while this was 1.04 ± 0.10 mm (n = 4) in rats that had received N-OH-AAF, and 0.15 ± 0.02 mm (n = 4) in rats that had received DEM before the administration of N-OH-AAF. After 2 hr, renal glutathione had returned to control values in rats that had received DEM and N-OH-AAF.

Thus, our data show that DEM can be used to deplete hepatic and renal glutathione in rats which have received N-OH-AAF; further, that this depletion lasts for the period in which most of the N-OH-AAF is metabolized.

Influence of DEM Pretreatment of Rats on the Sulfation of pNP and N-OH-AAF in Vivo. N,O-Sulfation of N-OH-AAF is one of the main pathways of N-OH-AAF metabolism in the rat liver in vivo to form reactive metabolites that react with macromolecules in the cell (22, 26, 30). To find out whether DEM affects N,O-sulfation of N-OH-AAF, the influence of DEM on the sulfation of pNP was investigated, because the sulfotransferase that converts N-OH-AAF to its sulfate conjugate also accepts pNP as a substrate (41).

In control rats, 87.3 ± 7.5% (n = 4) of a dose of pNP (120 μmol/kg) was excreted in urine during 24 hr after administration, as the glucuronide and sulfate conjugates. In DEM-pretreated rats, this was 84.7 ± 9.6% (n = 3). The percentage of pNP that was excreted as sulfate conjugate in control rats was 25.9 ± 3.8%. These data indicate that DEM does not seem to affect the process of

*J. H. N. Meeran, unpublished results.
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sulfation.

It is not possible to measure the formation of AAF-N-sulfate itself, due to the high reactivity of this compound in aqueous media (25). Therefore, the amount of the N-O-glucuronide conjugate of N-OH-AAF that is excreted in bile and urine was determined in control rats and in rats that were pretreated with DEM, because inhibition of sulfation of N-OH-AAF can be detected by an increase in the amount of the N-O-glucuronide (26, 30).

Most of the N-OH-AAF metabolites in control rats are recovered in bile, while little is found in urine (Table 1). In rats pretreated with DEM, most of the metabolites were excreted in bile, but the amount excreted in urine had decreased as compared to controls (Table 1). This may be related to the decreased urine production in the DEM-pretreated rats (Table 1). In control rats, one-third of the dose of N-OH-AAF was excreted as the N,O-glucuronide conjugate, predominantly in bile (Table 2). The same amount of this metabolite was excreted in DEM-pretreated rats; however, less was excreted in urine, which was compensated for by an increased excretion in bile (Table 2).

Thus, these data show that it is very unlikely that DEM affects the N-O-sulfation of N-OH-AAF.

Influence of DEM Pretreatment on the Excretion of AAF: Glutathione Conjugates in Bile in Vivo. Depletion of glutathione by DEM decreased the excretion of the 1- and 3-glutathione:AAF conjugated in bile by 60%, as compared to controls (Table 2). No glutathione conjugates were found in urine, which is in agreement with our earlier data (26). At the end of the 4-hr period, the glutathione conjugates were no longer present in bile (results not shown).

Effects of DEM Pretreatment on the Covalent Binding of N-OH-AAF to Protein and DNA in the Liver and Kidney in Vivo. The covalent binding of N-OH-AAF to protein or DNA was determined under 2 conditions. First, the rats used in the experiments indicated in Table 1 were anesthesized during the 4-hr period after the administration of N-OH-AAF, while bile was collected via a bile duct cannula. In a second experiment, the animals were not anesthesized during this period and were allowed to move freely. Any metabolite that might be formed in the gut (e.g., by bacterial enzymes) can be reabsorbed and transported via the blood to the liver, and thus increase covalent binding as compared to the first group.

Part of the N-OH-AAF that was administered to control rats in both groups of animals became covalently bound to protein in the liver (Table 3); this covalent binding was not changed by DEM pretreatment. There was also considerable covalent binding of N-OH-AAF to protein in the kidneys; this, however, was significantly decreased in DEM-pretreated rats. The fluorene adducts that retain the N-acetyl group of N-OH-AAF were more decreased by DEM than were adducts generated from deacetylated metabolites, as indicated by the increase in the ratio of 3H over 14C (Table 3).

The formation of covalently bound adducts of N-OH-AAF to rat liver DNA in DEM-pretreated rats was decreased as compared to controls (Table 4). This was due to a decrease of AAF adducts, as indicated by a concomitant decrease in covalently bound 3H, as well as in 14C radioactivity. This was confirmed by analysis of the individual DNA adducts; the formation of the AAF adducts dGuo-N2-AAF and dGuo-C8-AAF (that still retain the N-acetyl group of N-OH-AAF) was decreased in DEM-pretreated rats (Table 4), while the formation of the aminofluorene adduct, dGuo-C8-AF had not changed.

Effect of Depletion of Glutathione by DEM on the Hepatotoxicity of N-OH-AAF. Glutathione plays a role in the protection of the liver against the toxic effects of several hepatotoxins (8, 11, 23, 31, 35). We therefore have studied the effect of depletion of hepatic glutathione by DEM on the hepatotoxicity of N-OH-AAF.

DEM itself was not toxic for the liver, as demonstrated by microscopic examination of liver slices (stained with hematoxylin and eosin) which showed no deviation from normal liver histology, while enzyme activities were normal. Moreover, GGT and GPT activities in serum taken 48 hr after the administration of DEM were not different from control values (Table 5).

N-OH-AAF induced a severe periportal necrosis, accompanied by infiltration of leukocytes as described before (28, 36). The

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<td>Effect of DEM pretreatment on the excretion of N-OH-AAF metabolites in bile and urine of the rat</td>
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<td>Effect of DEM pretreatment on the excretion of the N,O-glucuronide conjugate and the 1- and 3-GS-AAF conjugates of N-OH-AAF in bile and urine in vivo</td>
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<td>For experimental conditions refer to the legend of Table 1. The metabolites of N-OH-AAF in bile and urine were determined by HPLC, as described in &quot;Materials and Methods.&quot;</td>
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<td>1-GS-AAF</td>
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* No glutathione conjugates were detected in urine.

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not anesthetized and was allowed to move around freely. Livers and kidneys of all animals were removed 4 hr after administration of N-OH-AAF, and radioactivity
the experiments indicated in Table 1, and were cannulated while bile was collected during 4 hr after the administration of N-OH-AAF. The second group of animals
injection of N-OH-AAF. The first group of animals was anesthetized with pentobarbital 15 min after the administration of DEM or solvent. They had been used also for
covalently bound to protein was determined as described in *Materials and Methods.*

Anesthetized, bile duct-
cannulated rats

| No. of ani-
| H (pmol/mg) | H:14C |
| --- | --- | --- |
| Controls | 770 ± 90<sup>a</sup> | 1.15 ± 0.01 |
| DEM | 820 ± 70<sup>b</sup> | 1.21 ± 0.02<sup>c</sup> |

Intact, freely moving rats

| No. of ani-
| H (pmol/mg) | H:14C |
| --- | --- | --- |
| Controls | 740 ± 120 | 1.19 ± 0.02 |
| DEM | 770 ± 140<sup>d</sup> | 1.15 ± 0.02<sup>e</sup> |

<sup>a</sup> Mean ± S.E.  
<sup>b</sup> Not significantly different from controls by Wilcoxon's test (39).  
<sup>c</sup> Significantly different from controls at p = 0.1.  
<sup>d</sup> Significantly different from controls at p = 0.05.

Table 4

**Effect of DEM pretreatment on the covalent binding to liver DNA in vivo and on the formation of deoxyguanosine adducts of [ring-3H; acetyl-<sup>14</sup>C]-
N-OH-AAF**

For experimental conditions, refer to the legend of Table 3. The total amount of covalently bound radioactivity to DNA and the individual deoxyguanosine adducts were determined as described in *Materials and Methods.*

| No. of ani-
| H (pmol/mg) | H:14C |
| --- | --- | --- |
| Total binding to DNA | dGuo-N<sup>a</sup>-AAF | dGuo-C<sup>b</sup>-AAF | dGuo-C<sup>c</sup>-AF |
| Controls | 220 ± 43<sup>d</sup> | 165 ± 31<sup>e</sup> | 1.61 ± 0.06<sup>f</sup> | 8 ± 1<sup>g</sup> | 74 ± 6 | 58 ± 5 |
| DEM | 577 ± 100<sup>h</sup> | 250 ± 30<sup>i</sup> | 13 ± 1<sup>j</sup> | 5 ± 1<sup>k</sup> | 50 ± 10<sup>l</sup> | 54 ± 5<sup>m</sup> |

<sup>a</sup> Mean ± S.E.  
<sup>b</sup> Significantly different from controls by Wilcoxon's test (39) at p = 0.1.  
<sup>c</sup> Significantly different from controls at p < 0.05.  
<sup>d</sup> Not significantly different from controls.

Table 5

**Effects of DEM pretreatment of rats on the serum GOT and GPT activities in vivo, 48 hr after the administration of N-OH-AAF**

The rats were pretreated with an i.p. injection of DEM (3.9 mmol/kg), both compounds, or solvent, 45 min before the i.v. administration of N-OH-AAF (120 μmol/kg) or solvent. Blood was taken from the aorta under diethyl ether anesthesia, 48 hr after the administration of N-OH-AAF. Serum GOT and GPT activities were determined as described in *Materials and Methods.*

| Treatment | No. of ani-
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<td>Units/liter</td>
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<td>7</td>
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<td>8</td>
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<td>39 ± 3&lt;sup&gt;m&lt;/sup&gt;</td>
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<sup>a</sup> Mean ± S.E.  
<sup>b</sup> Six animals were treated; one of them died within 24 hr, another within 48 hr.  
<sup>c</sup> Significantly different from controls by Wilcoxon's test (39) at p = 0.1.  
<sup>d</sup> Five animals were treated; 2 of the animals died within 48 hr.

GPT activities were determined as described in "Materials and Methods."  
Liver damage resulted in increased serum GOT and GPT activities, 48 hr after the administration of N-OH-AAF. In rats pretreated with DEM before administration of N-OH-AAF a severe liver damage was still observed. The liver slices of the groups with and without DEM pretreatment were ranked by comparison of damage. Liver slices of rats (n = 5) that had received only N-OH-AAF were given higher rank order (corresponding to more damage) than were those of rats (n = 5) that had been pretreated with DEM (p < 0.1, Wilcoxon's rank sum test). However, GOT and GPT activities in the DEM-pretreated rats were higher than those in rats administered N-OH-AAF alone (Table 5).

To investigate whether N-O-sulfation is still responsible for the acute hepatotoxic effects of N-OH-AAF in DEM-pretreated rats, we have used the inhibitor of sulfation, PCP.  
PCP itself was not toxic for the liver, as demonstrated by normal liver histology and by GOT and GPT activities, which were not different from control values (Table 5).

Pretreatment with PCP prevented the liver damage by N-OH-AAF, as described before (28). The preventive effect of PCP on the occurrence of liver damage after N-OH-AAF administration was also observed in rats that had been pretreated with PCP and DEM simultaneously (Table 5); no histological signs of liver damage were observed.

**Effects of Glutathione and L-Cysteine on the Reaction of AAF-N-Sulfate with RNA and DNA in Vitro.** Because the foregoing experiments suggested that glutathione might not play an important role in the detoxification of a major part of the reactive metabolites that are formed from N-OH-AAF in the rat in vivo, we have determined the effects of glutathione and cysteine on the reaction of AAF-N-sulfate with RNA and DNA in vitro.

In control incubations, [9-<sup>14</sup>C]-AAF-N-sulfate reacted with RNA so that 40% of the radioactivity of AAF-N-sulfate became covalently bound to RNA. Only a little AAF-N-sulfate was converted to N-OH-AAF and AAF (Table 6). Because AAF-N-sulfate has a half-life in aqueous media of less than 1 min (25), it was assumed that all reactive ester had disappeared at the end of the incubations. When glutathione was present in the incubations, the covalent binding to RNA was decreased by 20%, while more was converted to AAF. Similar changes were observed with L-

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Table 3

**Effects of DEM pretreatment of rats on the covalent binding of [ring-3H; acetyl-<sup>14</sup>C]-N-OH-AAF to protein in the liver and kidney in vivo**

(ring=3H; acetyl=<sup>14</sup>C)-N-OH-AAF (120 μmol/kg) was administered i.v. The rats were pretreated with an i.p. injection of DEM (3.9 mmol/kg) or solvent, 45 min before the injection of N-OH-AAF. The first group of animals was anesthetized with pentobarbital 15 min after the administration of DEM or solvent. They had been used also for the experiments indicated in Table 1, and were cannulated while bile was collected during 4 hr after the administration of N-OH-AAF. The second group of animals was not anesthetized and was allowed to move around freely. Livers and kidneys of all animals were removed 4 hr after administration of N-OH-AAF, and radioactivity

| No. of ani-
| H (pmol/mg) | H:14C |
| --- | --- | --- |
| Liver | Controls | 770 ± 90<sup>a</sup> | 1.15 ± 0.01 |
| DEM | 820 ± 70<sup>b</sup> | 1.21 ± 0.02<sup>c</sup> |
| Kidney | Controls | 740 ± 120 | 1.19 ± 0.02 |
| DEM | 770 ± 140<sup>d</sup> | 1.15 ± 0.02<sup>e</sup> |

<sup>a</sup> Mean ± S.E.  
<sup>b</sup> Not significantly different from controls by Wilcoxon's test (39).  
<sup>c</sup> Significantly different from controls at p = 0.1.  
<sup>d</sup> Significantly different from controls at p = 0.05.

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cysteine; however, L-cysteine was more effective than glutathione. When DNA replaced RNA in the incubations, less of the AAF-N-sulfate became bound to DNA as compared to RNA. Glutathione (10 mM) decreased the covalent binding to DNA by 40%, while more was converted to AAF; 1 mM glutathione did not decrease covalent binding to DNA. Similar changes were observed with L-cysteine.

**DISCUSSION**

We have investigated in this study the role of glutathione in the detoxification of the reactive metabolites that are generated from N-OH-AAF in the rat in vivo by depletion of glutathione with DEM.

DEM does not seem to inhibit the formation of AAF-N-sulfate, because sulfation of pNP was not affected by pretreatment of rats with DEM. Furthermore, the amount of N-OH-AAF that was excreted as the N,O-glucuronide conjugate was not increased in the DEM-pretreated rats.

The glutathione conjugates of N-OH-AAF (1- and 3-GS-AAF) that are excreted in bile are formed mainly from the reactive AAF-N-sulfate (26). Therefore, the decreased formation of these conjugates after DEM pretreatment most likely results from a decreased reaction of AAF-N-sulfate with glutathione. This decreased reaction with glutathione, however, did not lead to an increased reaction of AAF-N-sulfate (or other reactive metabolites generated from N-OH-AAF) with protein or DNA in rat liver in vivo. In the liver, covalent binding to DNA was decreased; while in the kidneys, decreased covalent binding of N-OH-AAF to protein was observed. The latter might be due to decreased blood flow to kidneys in DEM-pretreated rats, which is suggested by the decreased urinary production and excretion of N-OH-AAF metabolites in urine (Table 1). However, the formation of AF adducts (that contain only 3H activity) to kidney protein is less decreased by DEM treatment than formation of AAF adducts (that contain both 3H and 14C activity). At present, we have no explanation for this.

The reason why the formation of AAF adducts to DNA is decreased in DEM-pretreated rats is not known. Our results suggest that it is not due to a decreased formation of the reactive AAF-N-sulfate. It might be due to some other effect of DEM on liver function related to the chance of the N-sulfate reaching the nucleus.

Administration of N-OH-AAF induces severe liver damage (12, 28, 36). This also occurs in DEM-pretreated rats (this study). It can be prevented in control and DEM-pretreated rats with PCP (Ref. 29; this study), which inhibits N-sulfation of N-OH-AAF.

Conflicting results were obtained on the effect of glutathione depletion on liver toxicity; histochemical stainings showed a reduced toxicity by DEM pretreatment, while the serum GOT and GPT activities indicated more damages. Because depletion of glutathione did not increase the covalent binding of N-OH-AAF to hepatic and renal macromolecules, this indicates that glutathione does not play a vital role in the detoxification of reactive metabolites generated from N-OH-AAF in the rat in vivo. This might imply that glutathione is not very effective in competing with macromolecules for trapping of reactive intermediates generated from N-OH-AAF. Therefore, we have investigated the reaction of AAF-N-sulfate with RNA and DNA in vitro, and determined the effects of glutathione and L-cysteine on these reactions. Glutathione (1 mM) was indeed of little effectiveness in reducing covalent binding to RNA and DNA; at 10 mM it was somewhat more effective. Similarly, glutathione only partially blocked covalent binding of MAB N-sulfate to nucleic acids in vitro (20). Moreover, glutathione was much less effective than cysteine. This is consistent with the results reported by Mulder et al. (32), who showed that cysteine was more effective than glutathione in reducing the amount of (enzymatically generated) AAF-N-sulfate binding to protein in vivo.

It is believed that AAF-N-sulfate breaks down to form a nitrenium ion, which is the ultimate reactive species that reacts with nucleophilic groups (16).

In general, "soft" nucleophiles like glutathione and cysteine do not readily react with "hard" electrophiles like the nitrenium ion. They rather engage in oxidation-reduction reactions (21, 26). This might explain why cysteine is more effective inasmuch as it...
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is a better reductor than glutathione. Yet, reduction cannot fully explain our results. Therefore, formation of glutathione conjugates (26), and possibly also cysteine conjugates, must contribute to the decreased covalent binding to RNA and DNA in vitro (even though reduction is much more effective).

We have shown previously that at least 20 to 30% of an i.v. dose of N-OH-AAF is converted to the reactive AAF-N-sulfate in the rat in vivo. Since inhibition of sulfation by the dose of PCP that was used in these studies, most likely is only 70% (29), the total amount of N-OH-AAF that is converted to AAF-N-sulfate therefore probably is 30 to 40%. The amount of N-OH-AAF that becomes covalently bound to macromolecules (mainly to proteins) in the liver of rats probably does not exceed 5%. This implies that most of the AAF-N-sulfate that is formed in the rat liver in vivo reacts with low-molecular-weight components (including glutathione), and most likely is detoxified thereby. As glutathione conjugates represent approximately 10 to 18% of the total amount of N-OH-AAF administered (Refs. 26 and 29; this study), a major part of the AAF-N-sulfate generated is detoxified by reaction with other low-molecular-weight compounds. Possible candidates are NADH and ascorbic acid. These compounds are present in liver in large amounts, and it has been shown that these compounds can reduce efficiently the nitrenium ion in vitro to AAF (1, 2, 32, 40). When glutathione has been depleted, these other detoxification pathways could easily take over, which may explain the small effect of depletion of glutathione on the covalent binding of N-OH-AAF to macromolecules in vivo.

However, other factors might also be involved. Compartmentalization of the cell might be one of them; AAF-N-sulfate might react with macromolecules in compartments that have a low glutathione concentration of their own. Depletion of glutathione (in other compartments) therefore probably does not affect covalent binding in the compartments that normally are devoid of glutathione.

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