Biochemical Pharmacology, Vol. 37, No. 23, pp. 4495–4504, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
© 1988. Pergamon Press plc

NEPHROTOXICITY OF MERCAPTURIC ACIDS OF THREE STRUCTURALLY RELATED 2,2-DIFLUOROETHYLENES IN THE RAT

INDICATIONS FOR DIFFERENT BIOACTIVATION MECHANISMS

J. N. M. COMMANDEUR, J. P. G. BRAKENHOFF, F. J. J. DE KANTER* and N. P. E. VERMEULEN†

Department of Pharmacochemistry (Molecular Toxicology), Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

(*Received* 7 *April* 1988; *accepted* 7 *June* 1988)

Abstract—The biotransformation and the hepato- and nephrotoxicity of the mercapturic acids (*N*-acetyl-1-cysteine *S*-conjugates) of three structurally related 2,2-difluoroethylenes were investigated *in vivo* in the rat. All mercapturic acids appeared to cause nephrotoxicity, without any measureable effect on the liver. The mercapturic acid of tetrafluoroethylene (TFE-NAC) appeared to be the most potent nephrotoxin, causing toxicity upon an i.p. dose of 50 μmol/kg. The mercapturic acids of 1,1-dichloro-2,2-difluoroethylene (DCDFE-NAC) and 1,1-dibromo-2,2-difluoroethylene (DBDFE-NAC) were nephrotoxic at slightly higher doses, i.e. at 75 and 100 μmol/kg, respectively. In the urine of TFE-NAC-treated rats significant amounts of difluoroacetic acid (DFAA) could be detected. With increasing doses, the relative amount of DFAA in urine increased progressively (5–18% of dose). In urine of rats treated with DCDFE-NAC and DBDFE-NAC, however, the corresponding dihaloacetic acids, dichloroacetic acid and dibromoacetic acid, could not be detected.

Formation of DFAA and pyruvate could also be observed during *in vitro* metabolism of the cysteine conjugate of tetrafluoroethylene (TFE-CYS) by rat renal cytosol. Inhibition by aminooxyacetic acid (AOA) pointed to a β-lyase dependency for the DFAA-formation. Next to DFAA and pyruvate, also formation of hydrogen sulfide and thiosulfate could be detected. These results suggest that TFE-CYS is bioactivated to a significant extent to difluorothionoacyl fluoride, which most likely is subsequently hydrolysed to difluorothio(no)acetic acid and difluoroacetic acid. According to formation of pyruvate, the cysteine conjugates derived from DCDFE-NAC and DBDFE-NAC also were efficiently metabolized by rat renal β-lyase. However, the formation of corresponding dihaloacetic acids, dichloroacetic acid and dibromoacetic acid, could not be detected *in vitro* at all. Only very small amounts of hydrogen sulfide and thiosulfate were detected. These results suggest that bioactivation of the latter two conjugates to a dichloro- or dibromothionoacyl fluoride represents only a minor route. Because of better leaving group abilities of chloride and bromide compared to fluoride, rearrangement of the initially formed ethanethiol to a thiirane might be favoured. Based on the present *in vivo* and *in vitro* data, it is concluded that the nephrotoxicity of the structurally related mercapturic acids of 2,2-difluoroethylenes is dependent on halogen substitution and presumably the result of at least two different mechanisms of bioactivation.

A very important pathway in the metabolism of electrophilic xenobiotics is conjugation to glutathione (GSH) by cytosolic, mitochondrial and microsomal GSH-*S*-transferases, which are present in various tissues [1, 2]. Usually, GSH conjugation is associated with detoxification of xenobiotics. However, it has been shown that conjugation to GSH may also play a role in several bioactivation reactions [3]. At present, different bioactivation mechanisms involving GSH conjugation have been recognized. Conjugation of GSH to 1,2-dihaloalkanes may lead to the formation of sulfur half-mustard compounds, which compounds may form electrophilic episulfonium ions by internal displacement of the second halogen atom by the thioether sulfur atom. Reaction of episulfonium ions with biological macromolecules, such as proteins, DNA or RNA, may result in toxicity [4, 5], and mutagenicity [6–8]. Another bioactivation mechanism has been identified for several nephrotoxic halogenated alkenes. In this mechanism, after conjugation of halogenated alkenes to GSH in the liver, the resulting GSH-conjugates are degraded to the corresponding cysteine conjugates by biliary and renal hydrolases. These cysteine conjugates ultimately are bioactivated by a renal cysteine conjugate β-lyase to presumably reactive thiol-containing compounds, ammonia, and pyruvate [9]. Apart from bioactivation to reactive metabolites, the cysteine conjugates of halogenated alkenes may be excreted unchanged in the urine, or may be converted by *N*-acetyltransferases to stable mercapturic acids, which are rapidly excreted in the urine [10, 11].

Recently, it has been shown that i.p. adminis-

* Department of Organic Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

† To whom correspondence should be addressed.

tration of the mercapturic acids of hexachlorobutadiene (HCBD) [12], and 1,1-dichloro-2,2-difluoroethylene (DCDFE) [13] to the rat at relatively low doses lead to severe nephrotoxicity, similar to what is observed upon administration of the parent compounds HCBD and DCDFE. This was explained by active deacetylation of the mercapturic acids to the corresponding cysteine conjugates *in vivo* and uptake in the proximal tubule cells in the kidney. The cysteine conjugates generated in turn can be bioactivated by cysteine conjugate β-lyase. More recently, it has been shown that mercapturic acids of halogenated alkenes are also transformed to reactive intermediates *in vitro* by rat renal cytosol [14]. Apparently, mercapturic acids are useful model compounds in the study of the reactive intermediates responsible for the nephrotoxicity of halogenated ethylenes.

The nature of the reactive intermediate(s) responsible for the nephrotoxicity of fluorinated ethylenes, like tetrafluoroethylene (TFE), chlorotrifluoroethylene (CTFE) and 1,1-dichloro-2,2-difluoroethylene (DCDFE), is not known. Green and Odum postulated an electrophilic thiol as being responsible for the nephrotoxicity of TFE [15]. Dohn *et al.* [16] however, suggested that the thiol compound formed from TFE rapidly rearranges to a thionoacyl fluoride and/or a thiirane, which would then react with biologically important macromolecules and cause toxicity. Very recently, in the course of this investigation, the formation of a chlorofluorothionoacyl fluoride from the cysteine conjugate of CTFE by a β-lyase mediated bioactivation mechanism indeed has been shown *in vitro* [17].

In the present study, the toxicity of the mercapturic acids of tetrafluoroethylene (TFE), 1,1-dichloro-2,2-difluoroethylene (DCDFE), and 1,1-dibromo-2,2-difluoroethylene (DBDFE) was compared in the rat. The dose dependency of the nephrotoxicity of these three mercapturic acids was studied and the influence of β-halogen substitution on nephrotoxicity of 2,2-difluoroethylenes. This was expected to give insight into the possible participation of thionoacyl fluorides and/or thiiranes in the pathogenesis of nephrotoxicity of 2,2-difluorinated ethylenes. Rearrangement of the initially formed ethanethiol compounds to a thiirane is expected to increase in importance when the β-carbon is substituted with: F < Cl < Br. In order to explain the chemical nature of reactive intermediate(s), we also investigated the appearance of hydrolysis products expected to arise from the elusive reactive intermediate(s) both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals. Difluoroacetic acid (DFAA), monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), and dibromoacetic acid (DBAA) were purchased from Janssen Chimica (Beerse, Belgium). 1,1-Dibromo-2,2-difluoroethylene (DBDFE) was purchased from Alfa Products. Tetrafluoroethylene (TFE) was puschased from Matheson Gas Products (Oevel, Belgium). Acylase I was purchased from Sigma Chemical Company (St. Louis, MO).

N-Acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-1-cysteine (DCDFE-NAC) was synthesized by reacting 1,1-dichloro-2,2-difluoroethylene (DCDFE) with *N*- acetyl-1-cysteine according to Commandeur *et al.* [13]. *N*-Acetyl-*S*-(1,1-dibromo-2,2-difluoro)-1-cysteine (DBDFE-NAC) was synthesized by a procedure analogous to the preparation of DCDFE-NAC. *N*-Acetyl-*S*-(1,1,2,2-tetrafluoro-ethyl)-1-cysteine (TFE-NAC) was prepared by stirring a methanolic solution of the disodium salt of *N*-acetyl-1-cysteine for 20 hr in an autoclave filled with a threefold excess of TFE. The reaction mixture was subsequently neutralized with diluted HCl, and the methanol was evaporated under reduced pressure. The residue was adjusted to pH 2, and finally extracted three times with ethylacetate. The ethylacetate phases were combined, treated with charcoal, dried with magnesium sulfate, and ultimately evaporated to dryness. All mercapturic acids were recrystallized twice from diethylether/petroleum ether. The analytical data (melting point, mass spectra, and NMR data) of the synthetic mercapturic acids are shown in Table 1. According to ^{1}H NMR, GLC and TLC the purity of the compounds was higher than 98%.

Cysteine conjugates of TFE, DCDFE and DBDFE were prepared both non-enzymatically and enzymatically. Non-enzymatical deacetylation involved heating a solution of mercapturic acid in 6 N HCl for 16 hr at 80°, analogous to Thomson *et al.* [18]. The solution was treated with charcoal, filtered and repeatedly evaporated to complete dryness under high vacuum to remove HCl. This procedure resulted in quantitative deacetylation, which was confirmed by ^{19}F NMR, TLC and GLC. Enzymic hydrolysis involved incubation of aqueous solutions of 10 mM of mercapturic acid at 37° for 1 hr with 2000 U/ml acylase I in a 50 mM potassium phosphate buffer, pH 7.4. According to their ^{19}F NMR spectra, and their behaviour on silica gel TLC, the products from acidic hydrolysis of the mercapturic acids of TFE, DCDFE and DBDFE were identical to those formed by enzymic hydrolysis using commercial acylase I from porcine kidney. The analytical data (mass spectra, and NMR data) of the synthetic cysteine conjugates were consistent with the expected structures (Table 1).

On silica gel TLC cysteine conjugates were detected on the plate by spraying with ninhydrin; while both cysteine and *N*-acetyl-1-cysteine conjugates were detected if sprayed with 0.1 M K_2CrO_7/acetic acid (1:1) followed by 0.1 M $AgNO_3$. [19]. The cysteine conjugates were free of mercapturic acids according to TLC, when developed with 70% *n*-propanol/water and according to GLC after methylation of the product with diazomethane.

The benzyl esters of DFAA, MCAA, DCAA and DBAA were synthesized by treating the corresponding acetic acids with phenyldiazomethane (PDM). PDM was synthesized as described by Karashima *et al.* [20]. Mass spectrum of benzyl ester of DFAA: *m/z* (intensity %): 51 (72), 65 (17, $C_5H_5^+$), 90 (25), 91 (100, $C_7H_7^+$), 186 (32, $M^{+\cdot}$). Mass spectrum of benzyl ester of MCAA: 51 (24), 65 (24, $C_5H_5^+$), 77 (24), 79 (16), 91 (100, $C_7H_7^+$),

BP 37/23-F

Table 1. Analytical data of the synthetic mercapturic acids (NAC) and cysteine conjugates (CYS) of tetrafluoroethylene (TFE), 1,1-dichloro-2,2-difluoroethylene (DCDFE) and 1,1-dibromo-2,2-difluoroethylene (DBDFE)

Compound	Mass spectrum m/z (intensity %)	^{1}H NMR* δppm (intensity)	^{19}F NMR† δppm (intensity, multiplicity)
DCDFE-NAC	*methylester:* 59 (16), 88 (100), 102 (14), 117 (44), 134 (25), 144 (15), 176 (44), 208 (32, 2Cl, $M^{+\cdot}$-$COOCH_3$), 250 (22, 2Cl), 309 (1, 2Cl, $M^{+\cdot}$)	2.01 (3H, s), 3.02–3.57 (2H, m‡), 4.32–4.51 (1H, m§), 6.36 (1H, t, $^3J_{FH}$: 9 Hz)	AB-system (2F, d): δ_A – 5.36, δ_B – 5.62, $^2J_{FF}$: 207 Hz; $^3J_{FH}$: 9 Hz
DBDFE-NAC	*methylester:* 59 (19), 88 (100), 102 (15), 117 (56), 134 (29), 144 (35), 176 (90), 296 (29, 2Br), 338 (25, 2Br, $M^{+\cdot}$-$COOCH_3$)	2.00 (3H, s), 3.01–3.60 (2H, m‡), 4.30–4.51 (1H, m§), 6.30 (1H, t, $^3J_{FH}$: 9 Hz)	AB-system (2F, d): δ_A – 0.88, δ_B – 1.02, $^2J_{FF}$: 203 Hz; $^3J_{FH}$: 9 Hz
TFE-NAC	*methylester:* 59 (19), 88 (68), 101 (9), 117 (19), 134 (10), 144 (8), 156 (12), 176 (100), 218 (28, $M^{+\cdot}$-$COOCH_3$ and $M^{+\cdot}$-NH_2COCH_3), 297 (1, $M^{+\cdot}$)	2.00 (3H), 3.02–3.58 (2H, m‡), 4.34–4.52 (1H, m§), 6.16 (1H, t of t, $^2J_{FH}$: 53 Hz, $^3J_{FH}$: 3 Hz)	AB-system (2F, d of t): δ_A – 15.47, δ_B – 16.09, $^2J_{FF}$: 230 Hz; $^3J_{FF}$: 9 Hz, $^3J_{FH}$: 3 Hz. −57.30 (2F, d of t): $^2J_{FF}$: 53 hZ, $^3J_{FF}$: 9 hZ
DCDFE-CYS	*N-trifluoroacetyl-methylester:* 59 (17), 69 (26), 117 (100), 236 (22, 2Cl), 250 (82, 2Cl, $M^{+\cdot}$-NH_2COCF_3), 304 (25, 2Cl, $M^{+\cdot}$-$COOCH_3$)	3.32–3.82 (2H, m‡), 4.22–4.38 (1H, m§), 6.46 (1H, t $^3J_{FH}$: 7 Hz)	AB-system (2F, d): δ_A – 4.78, δ_B – 5.38, $^2J_{FF}$: 206 Hz; $^3J_{FH}$: 7 Hz
DBDFE-CYS	*N-trifluoroacetyl, methylester:* 59 (13), 69 (20), 117 (100), 198 (14), 223 (12), 269 (11), 324 (150, 2Br), 338 (100, 2Br, $M^{+\cdot}$-NH_2COCF_3), 392 (12, 2Br, $M^{+\cdot}$-$COOCH_3$)	3.28–3.80 (2H, m‡), 4.20–4.39 (1H, m§), 6.36 (1H, t, $^3J_{FH}$: 9 Hz)	AB-system (2F, d): δ_A – 0.10, δ_B – 1.00, $^2J_{FF}$: 202 Hz; $^3J_{FH}$: 9 Hz
TFE-CYS	*N-trifluoroacetyl, methylester:* 51 (10), 59 (19), 117 (100), 147 (19), 184 (15), 198 (12), 218 (79, $M^{+\cdot}$-NH_2COCF_3) 252 (19, $M^{+\cdot}$-$COOCH_3$-HF), 272 (29, $M^{+\cdot}$-$COOCH_3$)	3.32–3.84 (2H, m‡), 4.28–4.51 (1H, m§), 6.24 (1H, t of t, $^2J_{FH}$: 53 Hz, $^3J_{FH}$: 3 Hz)	AB-system (2F, d of t): δ_A – 16.02, δ_B – 16.38, $^2J_{FF}$: 230 Hz; $^3J_{FF}$: 9 Hz, $^3J_{FH}$: 3 Hz. −57.31 (2F, d of t): $^2J_{FH}$: 53 Hz, $^3J_{FF}$: 9 Hz

* 90 MHz, solvent D_2O/Na_2CO_3; internal standard 3-(trimethylsilyl)propionic acid (assigned 0 ppm).
† 376 MHz, solvent potassium borate buffer pH 8.6; internal standard trifluoroacetic acid (assigned 0 ppm).
‡ AB part of ABX-system.
§ X part of ABX-system.

107 (19), 108 (49), 184 (38, 1Cl, $M^{+\cdot}$). Mass spectrum of benzyl ester of DCAA: 65 (21, $C_5H_5^+$), 91 (100, $C_7H_7^+$), 107 (7), 218-220-222 (10-7-1, 2Cl, $M^{+\cdot}$). Mass spectrum of benzyl ester of DBAA: 65 (19, $C_5H_5^+$), 91 (100, $C_7H_7^+$), 103 (26), 107 (23), 227–229 (23–23, 2Br, $M^{+\cdot}$).

Animals and treatments. Male Wistar rats (180–200 g), obtained from TNO (Zeist, The Netherlands), were housed in plastic cages in temperature (22°) and humidity (50%) controlled rooms equipped with a 12 hr lighting cycle. Food (Hope Farms) and water, were provided *ad libitum*. Groups of four rats were injected with equimolar doses of DCDFE-NAC, TFE-NAC, or DBDFE-NAC. The respective mercapturic acids, dissolved in a saline–DMSO mixture, were administered by i.p. injection in a volume of 2.5 ml/kg. Doses of mercapturic acids were 25, 50, 75 μmol/kg. DBDFE-NAC also was administered at a dose of 100 μmol/kg. Control animals received vehiculum only.

After treatment the rats were individually housed in all-glass metabolism cages, designed for separate collection of urine and faeces. During the experiment the rats were provided only with water *ad libitum*. Urine was collected for 48 hr in portions of 24 hr in cooled (0°) vessels. Blood was collected by heart puncture 48 hr after treatment. The blood was subsequently centrifuged (4000 *g*, 10 min) to obtain plasma. The animals were decapitated and their kidneys were removed for measurement of kidney-to-body weight ratios.

Biochemical assessment of toxicity. Plasma urea and plasma alanine transaminase (ALT) activity were determined 48 hr after treatment, using J. T. Baker kits. Urinary protein and glucose excretion were determined in the 48 hr period following treatment. Urinary protein was determined by Sigma kit No. 540. Urinary glucose was determined using the J. T. Baker kit. Statistical significance of differences in plasma and urinary parameters was calculated using the two-tailed Student's *t*-test.

In vitro *metabolism of cysteine conjugates and dihaloacetic acids.* Renal cytosol was prepared from male Wistar rats according to Wolf *et al.* [21]. Incubations of cysteine conjugates or dihaloacetic acids with renal cytosol were carried out at pH 8.6 in 0.1 M borate buffer according to Banki *et al.* [22]. Some incubations were performed in the presence of 0.2 mM aminooxyacetic acid, in order to inhibit β-lyase. All incubation mixtures contained 1 mg cytosolic protein/ml and 4 mM cysteine conjugate or 0.1 mM dihaloacetic acid in a total volume of 3 ml, and incubations were carried out at 37°. After 15 min the incubations were stopped by adding 0.5 ml concentrated HCl. The determination of halogenated acetic acids was carried out as described below. Pyruvic acid was determined spectrophotometrically by measuring the 2,4-dinitrophenylhydrazone derivative formed after derivization using 2,4-dinitrophenylhydrazine [23]. Formation of hydrogen sulfide was determined spectrophotometrically by the method of Siegel [24]. Formation of thiosulphate was determined spectrophotometrically by the method of Sörbo [25].

Identification of synthetic products and metabolites. The presence of halogenated acetic acids (HAAs) in urine and in *in vitro* incubation mixtures was investigated by a procedure similar to that described for the detection of trifluoroacetic acid (TFAA) in urine [20]. In short, to portions of 5 ml of rat urine, 0.5 μmol of monochloroacetic acid (MCAA) was added as an internal standard. These portions were acidified to pH 0.5 with concentrated HCl, and subsequently extracted three times with 5 ml portions of diethylether. The diethylether phases were combined, treated for 30 min with phenyldiazomethane (PDM) at room temperature, concentrated by evaporation and ultimately examined with GC/MS (described below). Standard curves were obtained by spiking blank urine with known amounts of the respective HAAs and the internal standard monochloroacetic acid.

For GC/MS analysis the cysteine conjugates were first treated with diazomethane and trifluoroacetic anhydride in order to synthesize the *N*-trifluoroacetyl methylester. Methylesters of the mercapturic acids were prepared by treatment of the mercapturic acids with diazomethane.

Gas chromatography and mass spectrometry. GC/MS analyses were carried out on a HP 5890/MSD system. A CP-Sil-19 capillary column obtained from Chrompack Ned. B.V. (Middelburg, The Netherlands) was used. The operating temperatures were 280° (split injector), 280° (ion source, electron impact ionization, electron energy of 70 eV). The column temperature was programmed from 80° (2.5 min) to 280° at 20°/min. The benzylesters of the HAAs were identified and quantified using selected ion monitoring of their respective molecular ions and the molecular ions of the internal standard MCAA.

Nuclear magnetic resonance. ^1H NMR spectra of mercapturic acids and cysteine conjugates were measured on a Bruker WH 90 spectrometer (90 MHz). The compounds were dissolved in deuterium oxide and chemical shifts were referenced with 3-(trimethylsilyl)-propionic acid as an internal standard. ^{19}F NMR spectra were measured on a Bruker MSL 400 System (376.43 MHz). For ^{19}F NMR the compounds were dissolved in potassium borate buffer pH 8.6 and chemical shifts were referenced with trifluoroacetic acid as an internal standard.

RESULTS

Toxicity of mercapturic acids in vivo

The effects of different i.p. doses (25, 50, 75 and 100 μmol/kg) of TFE-NAC, DCDFE-NAC and DBDFE-NAC on various functional blood and urinary parameters, indicative for nephrotoxicity, are shown in Fig. 1. All three mercapturic acids appeared to be nephrotoxins, as indicated by statistically significant increases of plasma urea, urinary protein, urinary glucose and relative kidney weights. TFE-NAC appeared to be the most potent nephrotoxin, causing nephrotoxicity at doses equal to and higher than 50 μmol/kg. The first indications of nephrotoxicity due to DCDFE-NAC appeared at a dose of 75 μmol/kg. Urinary data of doses of 100 μmol/kg of TFE-NAC and DCDFE-NAC were not determined due to some inhibition of urine production at these doses. DBDFE-NAC was the least potent nephro-

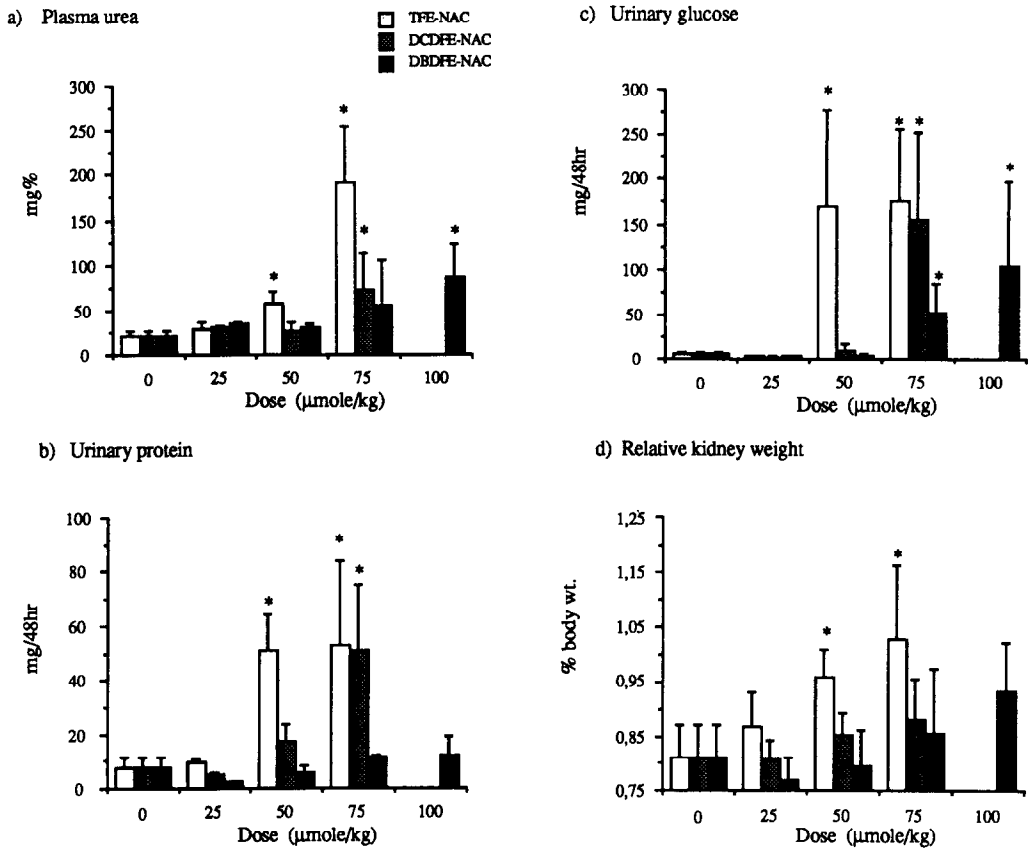

Fig. 1. Dose dependency of the parameters plasma urea, urinary protein, urinary glucose, and relative kidney weight, indicative for nephrotoxicity of mercapturic acids of 2,2-difluoroethylenes in the rat (N = 4). TFE-NAC: *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-1-cysteine; DCDFE-NAC: *N*-acetyl-*S*-(2,2-dichloro-1,1-difluoroethyl)-1-cysteine; DBDFE-NAC: *N*-acetyl-*S*-(2,2-dibromo-1,1-difluoroethyl)-1-cysteine. * Denotes statistical significance for differences from data from control rats ($P < 0.05$, Student's *t*-test).

toxin, causing significant nephrotoxicity only at a dose of 100 μmol/kg. Histological examination of the kidneys prepared 48 hr after treatment, showed that all three mercapturic acids caused necrosis in the region of the inner cortex. The appearance of this necrotic band paralleled the elevations of the biochemical parameters indicative for nephrotoxicity. Plasma ALT activities were not elevated even at the highest doses, indicating no toxic effect of the mercapturic acids on the liver [data not shown].

Urinary excretion of halogenated acetic acids and mercapturic acids

In urine of rats i.p. treated with different doses of TFE-NAC considerable amounts of difluoroacetic acid (DFAA) could be detected by GC/MS (Table 2). At a dose of TFE-NAC of 25 μmol/kg, approximately 5% of the dose of TFE-NAC administered was excreted as DFAA within 48 hr. At higher doses of TFE-NAC the percentage of excretion as DFAA was even higher: 10% at a 50 μmol/kg dose and 18% at a 75 μmol/kg dose. For all TFE-NAC doses, the main fraction (75–80%) of DFAA was excreted during the first 24 hr following treatment. DFAA could also be detected directly in untreated urine of TFE-NAC treated rats using ^{19}F NMR (Fig. 2).

In the urine of rats i.p. treated with DCDFE-NAC, and DBDFE-NAC, the corresponding HAAs, dichloroacetic acid (DCAA) and dibromoacetic acid (DBAA), could not be detected by GC/MS.

The unchanged mercapturic acids administered could also be identified in urine using ^{19}F NMR (Fig. 2) and GC/MS. The amount of excretion of the unchanged compounds was not quantified, however. Using ^{19}F NMR, next to DFAA, TFE-NAC and fluoride ion, an as yet unidentified fluorine-containing metabolite (41.5 ppm) could be detected in rat urine. Identification of this metabolite is still under progress, however.

In vitro *biotransformation of cysteine conjugates*

The results of the *in vitro* biotransformation of the cysteine conjugates of TFE, DCDFE and DBDFE by rat kidney cytosol are shown in Table 3. All three cysteine conjugates investigated were metabolized by cytosolic β-lyase as indicated by formation of pyruvic acid. TFE-CYS and DCDFE-CYS were

Table 2. Dose dependency of the excretion of difluoroacetic acid (DFAA) in urine of rats treated with *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-1-cysteine (TFE-NAC)

Dose of TFE-NAC (μmol/kg)	Excretion of DFAA					
	0–24 hr		24–48 hr		0–48 hr	
	μmol/kg	% of dose	μmol/kg	% of dose	μmol/kg	% of dose
25	1.0 ± 0.3	4.0 ± 1.1	0.2 ± 0.1	0.7 ± 0.1	1.2 ± 0.2	4.8 ± 1.0
50	4.3 ± 0.6	8.7 ± 1.3	0.7 ± 0.8	1.4 ± 0.8	5.0 ± 0.6	10.0 ± 1.2
75	9.9 ± 2.1	13.2 ± 2.8	3.3 ± 3.0	4.3 ± 4.0	13.2 ± 2.0	17.6 ± 2.7

Values are means ± SD of groups of four rats.

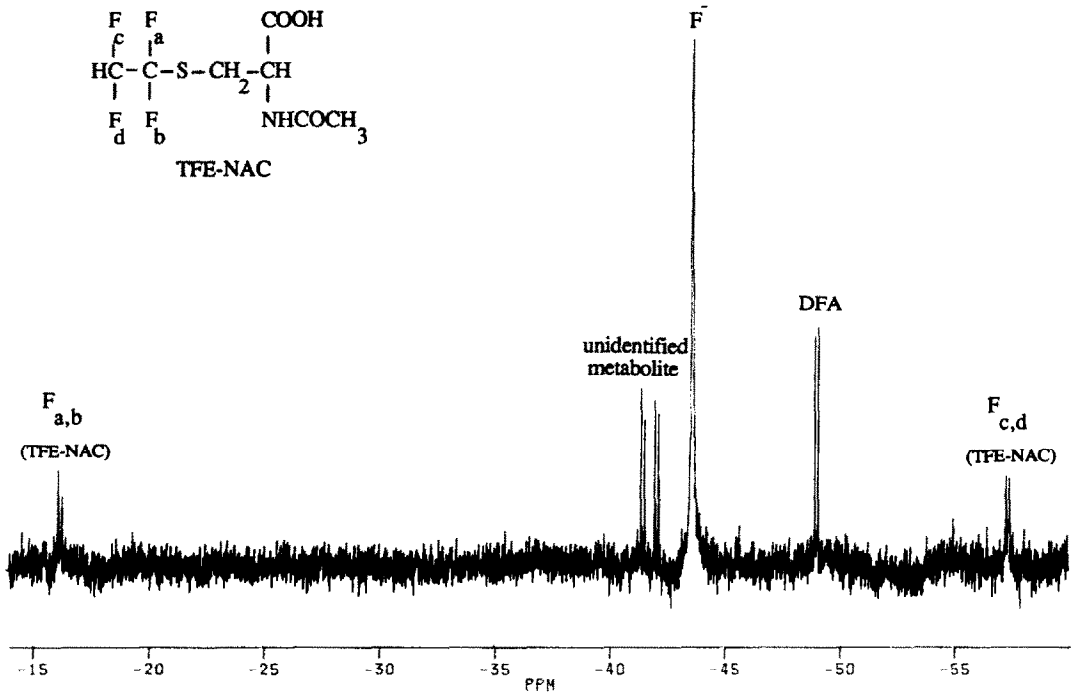

Fig. 2. ^{19}F NMR spectrum of 24-hr urine of rats treated with 75 μmol/kg *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-1-cysteine (TFE-NAC). DFA: difluoroacetic acid. Chemical shifts are referred to trifluoroacetic acid as an internal standard.

Table 3. *In vitro* metabolism of cysteine conjugates of 2,2-difluoroethylenes by rat renal cytosol. TFE-CYS: *S*-(1,1,2,2-tetrafluoroethyl)-1-cysteine; DCDFE-CYS: *S*-(2,2-dichloro-1,1-difluoroethyl)-1-cysteine; DBDFE-CYS: *S*-(2,2-dibromo-1,1-difluoroethyl)-1-cysteine

Substrate	Pyruvate (nmol/mg/min)	Hydrogen sulfide (nmol/mg/min)	Thiosulphate (nmol/mg/min)	Dihaloacetic acid (nmol/mg/min)
TFE-CYS	21.6 (0.2)	3.0 (0.1)	7.6 (1.5)	4.0 (0.5)
DCDFE-CYS	20.5 (0.4)	0.4 (0.1)	2.5 (0.5)	n.d.
DBDFE-CYS	11.9 (0.4)	n.d.	1.9 (0.6)	n.d.

Cysteine conjugates (4 mM) were incubated with rat kidney cytosol (1 mg protein/ml) for 15 min at 37° in borate buffer pH 8.6. The formation of pyruvate, hydrogen sulfide, thiosulphate, and dihaloacetic acid was quantified as described in Materials and Methods.

n.d. = not detectable.

degraded at approximately the same rate, whereas DBDFE-CYS was degraded at a slower rate. Addition of aminooxyacetic acid (AOA) to the incubation medium completely inhibited the formation of pyruvate from all three cysteine conjugates [data not shown], indicating that the reaction is indeed catalyzed by β-lyase. Next to pyruvic acid, in incubations of cytosol with TFE-CYS, significant formation of hydrogen sulfide, thiosulfate and DFAA could be detected (Table 3). In incubations of DCDFE-CYS, or DBDFE-CYS with cytosol, on the other hand, only traces of hydrogen sulfide and thiosulfate could be detected. In the latter two cases, the formation of dichloroacetic acid and dibromoacetic acid, respectively, could not be detected at all. After incubation of rat kidney cytosol with authentic difluoroacetic acid, dichloroacetic acid, or dibromoacetic acid no significant reduction of the concentration of these HAAs, as a result of secondary metabolism, could be observed upon an incubation period of 15 min [data not shown].

DISCUSSION

The nephrotoxicity and the biotransformation of fluorinated alkenes *in vivo* has already been subject to several studies. Kidney damage and fluoride ion excretion after exposure to fluoroalkenes was observed as early as 1974 by Dilley *et al.* [26] and more recently by Potter *et al.* [27]. Administration of fluoride to rats is known to cause nephrotoxicity. However, the amount of fluoride ions found in urine upon administration of tetrafluoroethylene (TFE) were significantly lower than that known to cause kidney damage [26, 28]. Therefore, another mechanism for the nephrotoxicity of fluoroethylenes was suggested. Recently Odum and Green [29] demonstrated that TFE was rapidly metabolized by glutathione conjugation both *in vivo* and *in vitro*, presumably by hepatic glutathione-*S*-transferases. The glutathione conjugate of TFE was shown to be excreted in bile, and subsequently to be rapidly degraded to the corresponding cysteine conjugate. Oral administration to rats of 100 mg/kg (450 μmol/kg) of the synthetic cysteine conjugate of TFE, TFE-CYS, was shown to cause severe nephrotoxicity. The similarity between the nephrotoxic effects of TFE and TFE-CYS *in vivo*, combined with the observed *in vitro* toxicity of TFE-CYS in renal kidney slices [15], led to the suggestion that TFE-CYS or a metabolite of it would be responsible for the TFE-induced nephrotoxicity. In early studies with *S*-(1,2-dichlorovinyl)-DL-cysteine (DCVC) it was already demonstrated that the formation of an unidentified reactive intermediate was the result of the action of renal cysteine conjugate β-lyase [30]. Other nephrotoxic compounds which appear to undergo a β-lyase dependent bioactivation are hexafluoropropene (HFP), chlorotrifluoroethylene (CTFE) [31], hexachlorobutadiene (HCBD) [12], bromobenzene [32] and 1,1-dichloro-2,2-difluoroethylene (DCDFE) [13]. At present, the precise chemical nature of the reactive intermediate(s) potentially responsible for the nephrotoxic effects still has to be elucidated. An electrophilic thiol, a thionoacyl fluoride and a thiirane meanwhile have been proposed as reactive intermediates which might be involved in the nephrotoxicity [15, 16].

Mercapturic acids are known to be absorbed and to be deacetylated rapidly by hepatic and renal acylases, both *in vivo* and *in vitro* [33, 34], and therefore they are useful precursors or model compounds in the study of the mechanism of cysteine conjugate-mediated nephrotoxicity of halogenated alkenes. The mercapturic acids of HCBD and DCDFE, for example, appeared to cause a nephrotoxicity similar to that caused by HCBD [12] and DCDFE itself [13].

In the present study, the nephrotoxicity and biotransformation of the mercapturic acids of three structurally related 2,2-difluorinated ethylenes were investigated *in vivo* in the male Wistar rat. As indicated by a significant elevation of urinary protein and glucose and plasma urea levels, the mercapturic acid of tetrafluoroethylene, TFE-NAC, appeared to be nephrotoxic in the rat upon i.p. doses as low as 50 μmol/kg (Fig. 1). Histopathological examination of the kidneys pointed to the proximal tubule region of the kidney as the primary site of toxicity. In urine of TFE-NAC-treated rats, apart from TFE-NAC itself, difluoroacetic acid (DFAA) could be detected as a metabolite. The presence of TFE-NAC in urine may be the result of direct excretion of unchanged TFE-NAC and/or of *N*-deacetylation to TFE-CYS followed by re-*N*-acetylation prior to excretion. DFAA, an ultimate product of a potentially reactive thiol-metabolite, was excreted in urine up to 18% of the dose of TFE-NAC. The relative amount of urinary DFAA in TFE-NAC-treated rats increased more than three-fold at higher doses. An explanation of the latter observation is difficult to give, because the amount of reactive thiol generated by β-lyase from a cysteine conjugate may depend upon a variety of factors [35]. A cysteine conjugate, once formed, (a) may be excreted unchanged, (b) may be *N*-acetylated to (re)generate the corresponding mercapturic acid, or (c) may be bioactivated by β-lyase to generate a reactive or a non-reactive thiol compound. Because of the inability to discriminate between unchanged mercapturic acid and mercapturic acid which is regenerated from the cysteine conjugate, the excretion of mercapturic acids in urine was not quantified in the present study. The formation of DFAA could also be observed *in vitro* upon degradation of synthetic TFE-CYS by rat kidney cytosol. Inhibition of the formation of DFAA by aminooxyacetic acid (AOA), a well-known inhibitor of pyridoxal phosphate-dependent enzymes, also indicated the involvement of renal cysteine conjugate β-lyase.

Figure 3 shows a likely bioactivation mechanism for TFE-NAC (X = F), which might be derived from the *in vivo* and *in vitro* data presented in this study and from previous *in vitro* studies [16, 17]. Possible rearrangement products derived from the initially formed ethanethiol compound are summarized in this figure. The fact that, both *in vivo* and *in vitro*, we could identify with GC/MS and ^{19}F NMR, DFAA as a metabolite of TFE-NAC suggests that the reactive ethanethiol compound (Fig. 3, I, X = F), resulting from a β-lyase-dependent degradation of TFE-CYS, is converted to difluorothionoacyl fluoride

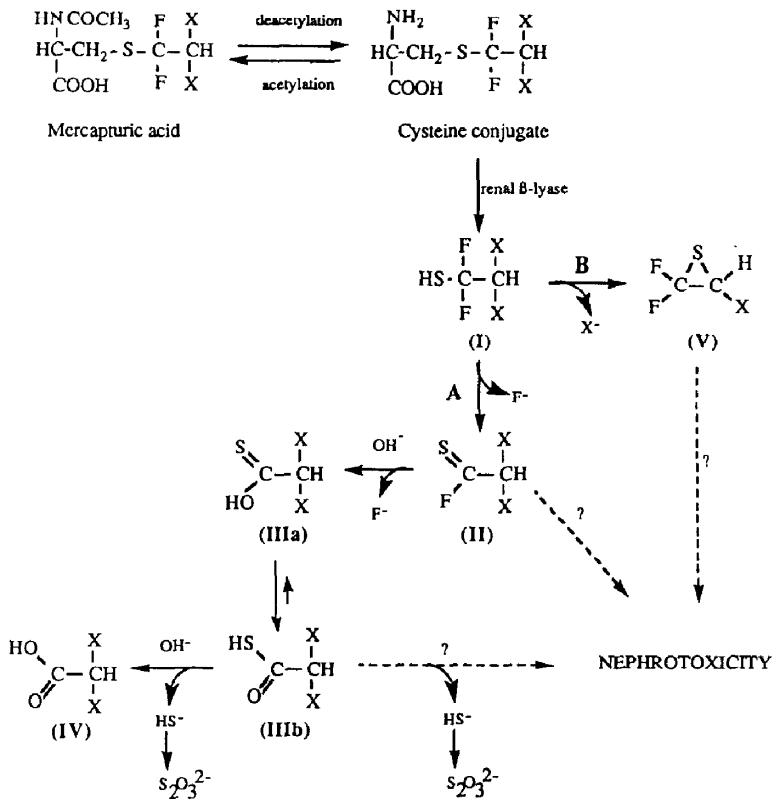

Fig. 3. Proposed routes of bioactivation of mercapturic acids and cysteine conjugates of 2,2-difluoroethylenes to reactive intermediates, and proposed mechanisms of degradation of reactive intermediates to dihaloacetic acids, hydrogen sulfide and thiosulphate. Route A: rearrangement to thionoacyl fluoride; route B: rearrangement to thiirane. I: 1,1-difluoro-2,2-dihaloethanethiol; II: dihalothionoacyl fluoride; IIIa: dihalothionoacetic acid; IIIb: dihalothioacetic acid; IV: dihaloacetic acid; V: 1,1-difluoro-2-halothiirane.

(Fig. 3, II, X = F, route A). This mechanism would be analogous to the *in vitro* conversion of chlorotrifluoroethanethiol to chlorofluorothionoacyl fluoride as proposed very recently by Dekant *et al.* [17]. Based on its chemical reactivity, we would expect difluorothionoacyl fluoride (Fig. 3, II, X = F) to bind covalently to nucleophilic centra in biomacromolecules, or to hydrolyse to difluorothionoacetic acid (Fig. 3, IIIa, X = F), which would subsequently rapidly tautomerise to difluorothioacetic acid (Fig. 3, IIIb, X = F). Thioacetic acids are expected to be the predominant forms in the tautomeric equilibria of this type of compounds and they are known to be potent acylating compounds [36]. Difluorothioacetic acid therefore might also bind covalently to nucleophilic centra or it might hydrolyse to DFAA (Fig. 3, IV, X = F); in both steps hydrogen sulfide would have to be released. Formation of hydrogen sulfide, which in the present study could indeed be observed during *in vitro* metabolism of TFE-CYS, supports this hypothesis.

The mercapturic acids of 1,1-dichloro-2,2-difluoroethylene (DCDFE-NAC) and 1,1-dibromo-2,2-difluoroethylene (DBDFE-NAC) also appeared to be potent nephrotoxins in the proximal tubular area, causing toxicity upon i.p. doses of 75 μmol/kg and 100 μmol/kg, respectively. However, in contrast to TFE-NAC, the corresponding dihaloacetic acids, dichloroacetic acid (DCAA) and dibromoacetic acid (DBAA), could not be detected in the urine of rats treated with DCDFE-NAC or DBDFE-NAC. After incubation of the cysteine conjugates of DCDFE and DBDFE with rat kidney cytosol, DCAA or DBAA could not be detected either. Degradation of authentic DCAA and DBAA by rat kidney cytosol appeared to be negligible too. Therefore, the lack of these metabolites after *in vitro* incubation of rat renal cytosol with DCDFE-CYS and DBDFE-CYS apparently cannot be explained by secondary metabolism of these dihaloacetic acids. It is not likely either that DCAA *in vivo* is completely biotransformed [37]. Upon renal cytosolic degradation of the cysteine conjugates DCDFE-CYS and DBDFE-CYS, the formation of hydrogen sulfide and thiosulfate was only found to be of minor importance (Table 3). These results suggest that conversion of DCDFE-CYS and DBDFE-CYS to the corresponding dichloro- and dibromothionoacyl fluorides (Fig. 3, route A, X = Cl, Br) most likely represents only a minor route in the rearrangement of the 2,2-dihalo-1,1-difluoroethanethiols initially formed from these cysteine conjugates. An alternative reactive intermediate

possibly derived from the ethanethiol might be a thiirane resulting from an intramolecular substitution reaction (Fig. 3; route B). In the case of DCDFE-NAC and DBDFE-NAC the formation of a thiirane (Fig. 3, V, X = Cl, Br; route B) might be favoured over the formation of the corresponding thionoacyl fluorides (Fig. 3, II, X = Cl, Br; route A) because chloride and bromide ions are better leaving groups than a fluoride ion. Previously, Dohn *et al.* already mentioned the formation of a thiirane as a potential reactive intermediate in the case of bioactivation of CTFE [16]. At present, however, the formation of a halogenated thiirane, its chemical reactivity and the nature of its secondary products still remains to be further proven and elucidated.

In conclusion, the three mercapturic acids investigated in this study (i.e. TFE-NAC, DCDFE-NAC and DBDFE-NAC) all appear to be potent and selective nephrotoxins. The order of toxicity, primarily located in the proximal tubular area, decreased slightly but consistently from TFE-NAC to DCDFE-NAC and to DBDFE-NAC. The identification of DFAA in urine of rats treated with TFE-NAC, combined with the formation of DFAA and, in addition to that, the formation of hydrogen sulfide, thiosulphate and pyruvate during renal cytosolic metabolism of TFE-CYS *in vitro*, point to the formation of difluorothionoacyl fluoride, or difluorothioacetic acid as reactive intermediates. The lack of excretion of the corresponding dihaloacetic acids in the urine of rats dosed with DCDFE-NAC and DBDFE-NAC, in addition to a minor hydrogen sulfide and thiosulphate formation observed during *in vitro* metabolism of DCDFE-CYS and DBDFE-CYS, however, strongly suggest that thionoacyl fluorides and/or thioacetic acids are not likely to be involved in the nephrotoxicity of these two mercapturic acids. Alternatively, the involvement of thiiranes in the nephrotoxicity is proposed in the case of 1,1-dichloro- and 1,1-dibromo-substituted 2,2-difluoroethylenes.

REFERENCES

1. Habig WH, Pabst MJ and Jacoby WB, Glutathione *S*-transferases. The first enzymic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
2. Jacoby WB and Habig WH, Glutathione transferases. In: *Enzymic Basis of Detoxification* (Ed. Jacoby WB), Vol. 2, pp. 63–94. Academic Press, Orlando, FL, 1980.
3. Igwe OJ, Biologically active intermediates generated by the reduced glutathione conjugation pathways. Toxicological implications. *Biochem Pharmacol* **35**: 2987–2994, 1986.
4. Elfarra AA, Baggs RB and Anders MW, Structure-nephrotoxicity relationships of *S*-(2-chloroethyl)-DL-cysteine and analogs: role for an episulfonium ion. *J Pharmacol Exp Ther* **233**: 512–516, 1985.
5. Webb WW, Elfarra AA, Webster KD, Thom R and Anders MW, Role for an episulfonium ion in *S*-(2-chloroethyl)-DL-cysteine induced cytotoxicity and its reaction with glutathione. *Biochemistry* **26**: 3017–3023, 1987.
6. Rannug U, Sundvall A and Ramel C, The mutagenic effect of 1,2-dichloroethane on Salmonella typhimurium. I. Activation through conjugation with glutathione *in vitro*. *Chem Biol Interact* **20**: 1–16, 1978.
7. Van Bladeren PJ, Breimer DD, Rotteveel-Smijs GMT, Van der Gen A and Mohn GR, The role of glutathione conjugation in the mutagenicity of 1,2-dibromoethane. *Biochem Pharmacol* **29**: 2975–2982, 1980.
8. Buijs W, Van Meeteren-Wälchi B, De Smidt PC, Vermeulen NPE, Booister-Schrijnemakers JGM, Van der Gen A and Mohn GR, The influence of metabolic activation on the mutagenic activity of a,ω-dihalogenoalkanes in Salmonella typhimurium TA 100. *Mutat Res*, in press.
9. Elfarra AA and Anders MW, Renal processing of glutathione conjugates. Role in nephrotoxicity. *Biochem Pharmacol* **33**: 3729–3732, 1984.
10. Jacoby WB, Stevens JL, Duffel MW and Weisiger RA, The terminal enzymes of mercapturate formation and the thiomethyl shunt. *Rev Biochem Toxicol* **5**: 97–115, 1984.
11. Chasseaud LF, Conjugation with glutathione and mercapturic acid excretion. In: *Glutathione: Metabolism and Function* (Eds. Jacoby WB and Arias IM), pp. 77–114. Raven Press, New York, 1976.
12. Nash JA, King LJ, Lock EA and Green T, The metabolism and disposition of hexachloro-1:3-butadiene in the rat and its relevance to nephrotoxicity. *Toxicol Appl Pharmacol* **73**: 124–137, 1984.
13. Commandeur JNM, Oostendorp RAJ, Schoofs PR, Xu B and Vermeulen NPE, Nephrotoxicity and hepatotoxicity of 1,1-dichloro-2,2-difluoroethylene in the rat. Indications for differential mechanisms of bioactivation. *Biochem Pharmacol* **36**: 4229–4237, 1987.
14. Vamvakas S, Dekant W, Berthold K, Schmidt S, Wild D and Henschler D, Enzymic transformation of mercapturic acids derived from halogenated alkenes to reactive and mutagenic intermediates. *Biochem Pharmacol* **36**: 2741–2748, 1987.
15. Green T and Odum J, Structure activity studies of the nephrotoxic and mutagenic action of chloro- and fluoroalkenes. *Chem Biol Interact* **54**: 15–31, 1985.
16. Dohn DR, Quebbemann AJ, Borch RF and Anders MW, Enzymic reaction of chlorotrifluoroethylene with glutathione: ^{19}F NMR evidence for stereochemical control of the reaction. *Biochemistry* **24**: 5137–5143, 1985.
17. Dekant W, Lash LH and Anders MW, Bioactivation mechanisms of the cytotoxic and nephrotoxic *S*-conjugate *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine. *Proc Natl Acad Sci USA* **84**, 7443–7447, 1987.
18. Thomson AER, Barnsley EA and Young L, Biochemical studies of toxic agents. 14. The biosynthesis of ethylmercapturic acid. *Biochem J* **86**: 145–152, 1963.
19. Knight RH and Young L, Biochemical studies of toxic agents. 11. The occurrence of premercapturic acids. *Biochem J* **70**: 111–119, 1958.
20. Karashima D, Shigematsu A, Furukawa T, Nagayoshi T and Matsumoto I, Esterification of trifluoroacetic acid with phenyldiazomethane for quantitative gas chromatographic analysis. Methods involving separation from biological materials. *J Chromat* **130**: 77–86, 1977.
21. Wolf CR, Berry PN, Nash JA, Green T and Lock EA, The role of microsomal and cytosolic glutathione-*S*-transferases in the conjugation of hexachloro-1:3-butadiene and its possible relevance to toxicity. *J Pharmacol Exp Ther* **228**: 202–208, 1984.
22. Banki K, Elfarra AA, Lash LH and Anders MW, Metabolism of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine to hydrogen sulfide and the role of hydrogen sulfide in *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine-induced mitochondrial toxicity. *Biochem Biophys Res Commun* **138**: 707–713, 1986.
23. Cooper AJL, Haber MT and Meister A, On the chemistry and biochemistry of 3-mercaptopyruvic acid, the a-keto acid analog of cysteine. *J Biol Chem* **257**: 816–826, 1982.

24. Siegel LM, A direct microdetermination for sulfide. *Anal Biochem* **11**: 126–132, 1965.
25. Sörbo B, A colorimetric method for the determination of thiosulphate. *Biochim Biophys Acta* **23**: 412–416, 1957.
26. Dilley JV, Carter VL and Harris ES, Fluoride excretion by male rats after inhalation of one of several fluoroethylenes or hexafluoropropene. *Toxicol Appl Pharmacol* **27**: 582–590, 1974.
27. Potter CL, Gandolfi AJ, Nagle R and Clayton JW, Effects of inhaled chlorotrifluoroethylene and hexafluoropropene on the rat kidney. *Toxicol Appl Pharmacol* **59**: 431–440, 1981.
28. Kessabi M, Braun JP, Benard V, Burgat-Sacaze V and Rico AG, Acute kidney toxicity of sodium fluoride in the rat. *Toxicol Lett* **5**: 169–174, 1980.
29. Odum J and Green T, The metabolism and nephrotoxicity of tetrafluoroethylene in the rat. *Toxicol Appl Pharmacol* **76**: 306–318, 1984.
30. Bhattacharya RK and Schultze MO, Properties of DNA treated with *S*-(1,2-dichlorovinyl)-L-cysteine and a lyase. *Arch Biochem Biophys* **153**: 105–115, 1972.
31. Dohn DR, Leininger JR, Lash LH, Quebbemann AJ and Anders MW, Nephrotoxicity of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-glutathione and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, the glutathione and cysteine conjugates of chlorotrifluoroethylene. *J Pharmacol Exp Ther* **235**: 851–857, 1985.
32. Monks TS, Lau SS, Highet RJ and Gillette JR, Glutathione conjugates of 2-bromohydroquinone are nephrotoxic. *Drug Metab Dispos* **13**: 553–559, 1985.
33. Tateishi M, Methylthiolated metabolites. *Drug Metab Rev* **14**: 1207–1234, 1983.
34. Suzuki S and Tateishi M, Purification and characterization of a rat liver enzyme catalyzing *N*-deacetylation of mercapturic acid conjugates. *Drug Metab Dispos* **9**: 573–577, 1981.
35. Monks TJ and Lau SL, Commentary: Renal transport processes and glutathione conjugate-mediated nephrotoxicity. *Drug Metab Dispos* **15**: 437–441, 1987.
36. Duus F, Thiocarbonyl compounds. In: *Comprehensive Organic Chemistry* (Ed. Jones DN), Vol. 3, pp. 373–487. Pergamon Press, Oxford, 1979.
37. Dekant W, Metzler M and Henschler D, Identification of *S*-(1,1,2-trichlorovinyl)-*N*-acetylcysteine as a urinary metabolite of trichloroethylene. Bioactivation through glutathione conjugation may explain its nephrocarcinogenity. *J Biochem Toxicol* **1**: 57–71, 1986.