Biochemical Pharmacology, Vol. 38, No. 21, pp. 3731–3741, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

TOXICITY OF THE CYSTEINE-*S*-CONJUGATES AND MERCAPTURIC ACIDS OF FOUR STRUCTURALLY RELATED DIFLUOROETHYLENES IN ISOLATED PROXIMAL TUBULAR CELLS FROM RAT KIDNEY

UPTAKE OF THE CONJUGATES AND ACTIVATION TO TOXIC METABOLITES

PIETER J. BOOGAARD,*† JAN N. M. COMMANDEUR,‡ GERARD J. MULDER,* NICO P. E. VERMEULEN‡ and J. FRED NAGELKERKE*

*Division of Toxicology, Center for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9503, 2300 RA Leiden, The Netherlands; and ‡Department of Pharmacochemistry (Molecular Toxicology), Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

(*Received* 15 *February* 1989; *accepted* 16 *May* 1989)

Abstract—Isolated proximal tubular cells from rat kidney were incubated with the cysteine-*S*-conjugates and corresponding mercapturates of the potent nephrotoxicants tetrafluoroethylene (TFE), chlorotrifluoroethylene (CTFE), 1,1-dichloro-2,2-difluoroethylene (DCDFE) and 1,1-dibromo-2,2-difluoroethylene (DBDFE). Toxicity of these *S*-conjugates was determined by their ability to inhibit α-methylglucose uptake by the cells. The cytotoxicity of the cysteine-*S*-conjugates and mercapturates of TFE and CTFE was similar, but the cysteine-*S*-conjugates of DCDFE and DBDFE were more toxic than their mercapturates. The cytotoxicity of the conjugates decreased in the following order TFE ≈ CTFE > DCDFE > DBDFE, which is the same as observed *in vivo*. Inhibition of renal cysteine-*S*-conjugate β-lyase by aminooxyacetic acid alleviated the cytotoxicity of both the cysteine-*S*-conjugates and the mercapturic acids of the four haloethylenes. The cytotoxicity of the mercapturates, but not of the cysteine-*S*-conjugates, could be reduced by probenecid, suggesting that the cysteine-*S*-conjugates are transported by a different carrier system than the mercapturates. The deacetylation of the mercapturates of TFE and CTFE in the cells was much higher than that of the mercapturates of DCDFE and DBDFE. The cysteine-*S*-conjugates of DCDFE and DBDFE were N-acetylated by the cells whereas the other cysteine-*S*-conjugates were not (TFE) or only marginally (CTFE) N-acetylated. The observed differences in cytotoxicity may be explained by differences in (1) the balance between acetylation/deacetylation by the cells, (2) the conversion rate of the *S*-conjugates to toxic metabolites by renal β-lyase and (3) the transport into the proximal tubular cells.

An important pathway in the elimination of electrophilic xenobiotics from the body is their excretion as mercapturic acids by the kidney. The first step in the mercapturic acid biosynthesis is conjugation with the nucleophilic sulfhydryl-group of glutathione (GSH§), which in general leads to detoxification. In the past few years, however, it has been found that GSH conjugation may convert some compounds into selective nephrotoxins [1, 2].

The GSH-conjugates are synthesized intracellularly, for instance in the liver, and are subsequently excreted into blood and/or bile. Conversion of the GSH-conjugates to cysteine-*S*-conjugates is catalysed by γ-glutamyltranspeptidase and dipeptidases present in the biliary tree, the gut or the kidney. The cysteine-*S*-conjugates can subsequently be converted to the corresponding mercapturates by N-acetylation in the liver and the kidney. Intact GSH-conjugates released into the blood can be converted very efficiently by peptidases in the membranes of the proximal tubular cells in the kidney, to the cysteine-*S*-conjugates, which are subsequently taken up by these cells [3, 4]. Thus, compounds which are initially conjugated with GSH can enter the proximal tubular cells either as GSH-conjugates, cysteine-*S*-conjugates or mercapturic acids.

† To whom correspondence should be addressed.

§ Abbreviations used: AOA, aminooxyacetic acid; BSA, bovine serum albumin; DBDFE, 1,1-dibromo-2,2-difluoroethylene; DBDFE-Cys, *S*-(1,1-difluoro-2,2-dibromoethyl)-L-cysteine; DBDFE-NAc, *N*-acetyl-*S*-(1,1-difluoro-2,2-dibromoethyl)-L-cysteine; DCDFE, 1,1-dichloro-2,2-difluoroethylene; DCDFE-Cys, *S*-(1,1-difluoro2,2-dichloroethyl)-L-cysteine; DCDFE-NAc, *N*-acetyl-*S*-(1,1-difluoro-2,2-dichloroethyl)-L-cysteine; CTFE, chlorotrifluoroethylene; CTFE-Cys, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; CTFE-NAc, *N*-acetyl-*S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; GSH, glutathione; HCBD, hexachloro-1,3-butadiene; HCBD-GSH, *S*-(1,2,3,4,4 - pentachloro - 1,3 - butadienyl) - glutathione; HCBD-Cys, *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine; HCBD-NAc, *N*-acetyl-*S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine; HEPES, 4-(2-hydroxyethyl)-piperazinethanesulfonic acid; α-MG, α-methylglucose; pAH, *p*-aminohippuric acid; TFE, tetrafluoroethylene; TFE-Cys, *S*-tetrafluoroethyl-L-cysteine; TFE-NAc, *N*-acetyl-*S*-tetrafluoroethyl-L-cysteine; TFE-PMS, *N*-difluorothionoacetyl-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

Uptake of certain cysteine-*S*-conjugates and mercapturates, followed by activation by renal β-lyase, induces severe nephrotoxicity; β-lyase converts cysteine-*S*-conjugates with unsaturated or electronegative substituents into ammonia, pyruvate and reactive sulfur compounds [1–3, 5]. Thus, several metabolites of hexachloro-1,3-butadiene (HCBD), i.e. HCBD-GSH, HCBD-Cys and HCBD-NAc, induce the same type of acute necrosis of the proximal tubules in the rat *in vivo* as the parent compound, HCBD [6]. The same holds true for the mercapturic acid of 1,1-dichloro-2,2-difluoroethylene (DCDFE-NAc), and its precursor DCDFE [7, 8]. Recently, it was shown that the mercapturic acids of TFE, DCDFE and DBDFE were all nephrotoxic in the rat, presumably due to a rapid deacetylation *in vivo*, and subsequent activation by β-lyase. Most probably, different kinds of reaction intermediates are involved [8].

In isolated proximal tubular cells both DCDFE-NAc and DCDFE-Cys caused loss of cellular function, although it took a longer incubation period for the mercapturate to exert the same effect as the cysteine-*S*-conjugate [9]. This might indicate that the transport rate of the mercapturate into the cell is lower than that of the cysteine-*S*-conjugate. Alternatively, deacetylation of the mercapturic acid, which has to occur before it is a substrate for β-lyase, may be rate limiting in the process leading to toxicity.

The kidney also has a capacity to acetylate cysteine-*S*-conjugates. The balance between acetylation and deacetylation might determine the nephrotoxicity of the fluoroethylenes. Other organs than the kidney, in particular the liver, have acetyltransferase activity too, which makes the interpretation of results of *in vivo* experiments more complex: does the liver play an important role in the nephrotoxicity of that compound? Since acetyltransferases in sub-cellular systems were reported to be labile [10, 11], isolated renal proximal tubular cells provide a more suitable *in vitro* system to study these conversions at the kidney level exclusively. Moreover, they enable studies both of the transport processes and the mechanism of toxicity.

In the present work, we have used suspensions of isolated rat proximal tubular cells to study the uptake and toxicity of both the cysteine-*S*-conjugates and mercapturic acids of TFE, CTFE, DCDFE and DBDFE, four structurally related geminal difluoroethylenes. The involvement of the renal β-lyase and influence of the acetylation/deacetylation balance is investigated in relation to the toxicity of the compounds.

MATERIALS AND METHODS

Chemicals

Products were purchased as follows: collagenase (from Clostridium histolyticum) from Boehringer Mannheim (Mannheim, F.R.G.); [U-^{14}C]-α-methyl-glucose (150 mCi/mmol) and *p*-[glycyl-1-^{14}C-]-amino-hippuric acid (50 mCi/mmol) from Amersham (Amersham, U.K.); probenecid, α-methyl-glucose (α-MG, 1-*O*-methyl-α-D-glucopyranoside), Bovine Serum Albumin (fraction V) and Acylase I from Sigma (St Louis, MO); aminooxyacetic acid (AOA, carboxymethoxylamine hemihydrochloride) from Aldrich (Brussels, Belgium); and Nycodenz (iohexol) from Nycomed AS (Oslo, Norway).

S-(1,1-Difluoro-2,2-dibromoethyl)-L-cysteine (DBDFE-Cys), *N*-acetyl-*S*-(1,1-difluoro-2,2-dibromoethyl)-L-cysteine (DBDFE-NAc), *S*-(1,1-difluoro-2,2-dichloroethyl)-L-cysteine (DCDFE-Cys), *N*-acetyl-*S*-(1,1-difluoro-2,2-dichloroethyl)-L-cysteine (DCDFE-NAc), *S*-tetrafluoroethyl-L-cysteine (TFE-Cys) and *N*-acetyl-*S*-tetrafluoroethyl-L-cysteine (TFE-NAc) were synthesized as described previously [7, 8].

N-Acetyl-*S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-NAc) was prepared by stirring a methanolic solution of the disodium salt of *N*-acetyl-L-cysteine for 20 hr in an autoclave filled with a threefold excess of chlorotrifluoroethylene (CTFE). The reaction mixture was subsequently neutralized with diluted hydrochloric acid, and the methanol was evaporated under reduced pressure. The residue was adjusted to pH 2, and finally extracted three times with ethylacetate. The ethylacetate fractions were combined, treated with charcoal, dried with anhydrous magnesium sulfate, and evaporated to dryness. The mercapturic acid was recrystallized twice from diethylether/petroleum ether. Due to the introduction of a chiral carbon, this procedure resulted in a racemic mixture of 50:50% (according to ^{19}F-NMR-data). For correct assignment of the ^{19}F-NMR chemical shifts of the two diastereomers the glutathione conjugate of CTFE was prepared according to Dohn *et al.* [12]. This biosynthetical procedure results in enrichment of one of the two diastereomers. By heating the glutathione conjugate at 80° in 6 N HCl for 16 hr, the conjugate was degraded to the corresponding cysteine-*S*-conjugate. This cysteine-*S*-conjugate was subsequently acetylated using acetic anhydride, thus yielding the mercapturic acid. The GC/MS spectrum of the methyl ester of this semi-biosynthetically synthesized mercapturic acid was identical to that of the chemically prepared compound. However, according to the ^{19}F-NMR-spectra the ratio of the two diastereomers was 2:1.

Chemical characterization. ^{1}H-NMR: (D_2O/Na_2CO_3) δ (ppm, multiplicity) = 2.00 (3H, singlet), 3.03–3.58 (2H, AB-part of ABM-system), 4.35–4.52 (2H, M-part of ABM-system), 6.58 (1H, doublet ($^{2}J_{FH}$ 48 Hz) of triplet ($^{3}J_{FH}$ 5 Hz)). ^{19}F-NMR: (H_2O, relative to trifluoroacetic acid): *diastereomer 1* AB-system (2F, doublet of triplet): δ_A −9.73 ppm, δ_B 12.18 ppm, $^{2}J_{FF}$ 219 Hz; $^{3}J_{FF}$ 17 Hz, $^{3}J_{FH}$ 5 Hz. −72.74 ppm (1F, doublet of triplet) $^{2}J_{FH}$ 48 Hz. $^{3}J_{FF}$ 17 Hz. *diastereomer 2* AB-system (2F, doublet of triplet): δ_A −9.64 ppm, δ_B −11.18 ppm, $^{2}J_{FF}$ 219 Hz; $^{3}J_{FF}$ 17 Hz, $^{3}J_{FH}$ 5 Hz. −72.93 ppm (1F, doublet of triplet) $^{2}J_{FH}$ 48 Hz. $^{3}J_{FF}$ 17 Hz. Mass-spectrum (methyl ester): *m/z* (intensity %) 234 (3%, 1Cl, $M^{+\cdot}$ − $COOCH_3$ and NH_2COCH_3), 192 (8%, 1Cl), 176 (15%), 144 (6%), 134 (11%), 117 (27%), 88 (100%), 67 (14%), 63 (15%).

The cysteine-*S*-conjugate of CTFE (CTFE-Cys) was prepared by heating a solution of CTFE-NAc in 6 N HCl for 16 hr at 80° [8]. The solution was treated with charcoal, filtered and repeatedly evaporated to complete dryness under high vacuum to remove hydrochloric acid. This procedure resulted in quanti-

tative deacetylation of the CTFE-NAc. The ^{1}H-NMR and ^{19}F-NMR spectra of the product were identical to that of CTFE-Cys as described by Dohn *et al.* [12]. Mass spectrum (*N*-trifluoroacetyl, methyl ester): *m/z* (intensity %) 288 (21%, 1Cl, $M^{+\cdot} - COOCH_3$), 268 (10%, 1Cl), 234 (50%, 1Cl, $M^{+\cdot} - NH_2COCF_3$), 184 (11%), 163 (11%), 138 (10%), 117 (100%), 69 (33%), 59 (17%).

All other chemicals were of the highest purity available.

Cell isolation

For all experiments male Wistar rats (180 to 220 g body weight), from the Sylvius Laboratories (University of Leiden) were used; the animals had free access to a commercial diet (SRM-A, Hope Farms, Woerden, The Netherlands) and tap water, and were kept on a 12 hr day/night cycle.

Proximal tubular cells were isolated by collagenase perfusion and purified by isopycnic centrifugation as described previously [13, 14]. Briefly, rats were anesthetized and the abdomen opened. The coeliac and upper mesenteric arteries and the lower vena cava were ligated, the aorta was subsequently cannulated with a dripping cannula and the renal veins opened. While being perfused with 150 ml Ca^{2+}-free Hanks'-HEPES buffer containing 0.5 mM EGTA the kidneys were removed and transferred to a circulating perfusion system. The kidneys were subsequently perfused with Hanks'-HEPES buffer containing 4 mM Ca^{2+} and 0.12% (w/v) collagenase. After 18 min the cortical tissue was gently dispersed in chilled Hanks'-HEPES buffer supplemented with 2.5% (w/v) BSA and filtered through nylon gauze (pore size 80 μm). The cell suspension was washed and viable proximal tubular cells were separated from dead cells, cell debris and tubular fragments by isopycnic centrifugation on a discontinuous gradient, using Nycodenz as density medium.

Preparation of subcellular fractions from rat kidney

To prepare subcellular fractions, rats were killed by decapitation, kidneys were removed and a 30% (w/v) homogenate was made on ice-cold 50 mM potassium phosphate buffer (pH = 7.40) using a Potter homogenizer. The homogenates were then centrifugated at 11,000 *g* for 20 min at 4° and the supernatants (containing cytosol and microsomes [15]) were collected. Part of these supernatants were centrifugated at 100,000 *g* for 1 hr to prepare cytosolic fractions (100,000 *g* supernatant). Subcellular fractions were stored at −20° until use.

Incubations

The cells were incubated in Hanks'-buffer (pH = 7.40) supplemented with 25 mM HEPES and 2.5% (w/v) bovine serum albumin (fraction V), at 37° under 95% O_2/5% CO_2, on a rotatory shaker (160 cycles/min).

For the studies on metabolism and relative toxicity with renal proximal tubular cells, the cells were preincubated for 15 min, before the conjugates under study, dissolved in the Hanks'-HEPES-BSA-buffer and neutralized with sodium bicarbonate (pH = 7.40), were added. For the studies with inhibitors, AOA or probenecid, the cells were first preincubated for 15 min, and subsequently incubated with the inhibitor for another 15 min before the conjugates under study were added. In toxicity studies the cells were incubated at a concentration of about 3.5×10^6 cells/ml, with various concentrations of the conjugates. The uptake of α-methylglucose (α-MG), recently shown to be a very sensitive parameter to assess toxicity in isolated renal proximal tubular cells [9, 13, 14], was used in all experiments. In the metabolism studies cells (at a concentration of about 10^7 cells/ml) were incubated for 15 min with the cysteine-*S*-conjugates and for 30 min with the mercapturates before the reaction was stopped by adding 250 μl/ml 38% (w/v) hydrochloric acid. The concentration of the conjugates of TFE and CTFE was 500 μM and that of the conjugates of DCDFE and DBDFE 2 mM.

Incubations of renal cytosol on 11,000 *g* fractions of rat kidney with the *S*-conjugates were carried out at 37° in potassium phosphate buffer (50 mM, pH = 7.40) in a total volume of 3.0 ml. The concentration of subcellular fractions during the incubations was 4 mg protein/ml.

Studies on transport by isolated proximal tubular cells

To determine the uptake of α-methylglucose (α-MG) and *p*-amino-hippuric acid (pAH), 800-μl samples from the cell suspension (about 3 million cells) were taken from the incubation flasks. The samples were mixed with 100 μl Hanks'-HEPES-buffer (carbogen saturated, 37°) containing either 5 mM [^{14}C]-α-MG (25 mCi/mmol) or 100 μM [^{14}C]-pAH (1230 mCi/mmol). After 2 min of incubation at 37°, aliquots were pipetted into 10 vol. of ice-cold Krebs–Henseleit–bicarbonate buffer and cells were separated from the medium by rapid centrifugation. They were washed three times with 10 vol. ice-cold buffer. The cells were lysed in 500 μl distilled water and the samples deproteinated by centrifugation after addition of 500 μl aqueous trichloroacetic acid (10% w/v final concentration). The supernatant was mixed with Emulsifier Safe (Packard) and the radioactivity determined by liquid scintillation counting. α-MG and pAH uptake determinations were always performed in triplicate.

Identification of metabolites by gas chromatography/mass spectrometry (GC/MS) and HPLC

For GC/MS analysis of reaction products incubations were stopped at several time points by addition of 0.5 ml cold 38% (w/v) hydrochloric acid. Subsequently, 0.2 mg *N*-acetyl-*S*-benzyl-1-cysteine was added as an internal standard and the incubation mixtures were extracted twice by 5 ml ethyl acetate. The combined ethyl acetate fractions were reduced to 0.5 ml by evaporation and treated with an excess of etherial diazomethane. GC/MS analyses were carried out on a HP 5890/MSD system. A CP-Sil SE-30 capillary column (25 m, 0.22 mm i.d.) obtained from Chrompack Nl. B.V. (Middelburg, The Netherlands) was used. The operation 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. Products were analysed by selected ion monitoring (SIM) of charac-

teristic ions. Ions at w/z 117, 144 and 176 are characteristic fragments for mercapturic acids. The formation of the "pseudo-mercapturic acid", *N*-difluorothionoacetyl - *S* - (1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-PMS), a covalent adduct formed during β-lyase mediated activation of TFE-Cys, was investigated by SIM of ions at m/z 196, 219, 284 and 296 as described previously [16].

Formation of cysteine-*S*-conjugates was determined by reversed phase HPLC after derivation of the cysteine-*S*-conjugates with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol according to Lindroth and Mopper [17]. The OPA-reagent was prepared by mixing 270 mg OPA in 5 ml ethanol, 200 μl 2-mercaptoethanol and 45 ml 0.4 potassium borate buffer (pH = 9.50). Samples (20 μl) of the incubation mixtures were taken at different time points, reacted for 2 min with 100 μl OPA-reagent and investigated with HPLC. A Lichrosorb 5RP18 column (150 × 4.6 mm) was used and OPA-adducts of TFE-Cys, CTFE-Cys and DCDFE-Cys were separated from cytosolic amines by isocratic elution with 60% methanol/40% 12.5 mM potassium phosphate buffer, pH = 7.20 at a flow rate of 0.7 ml/min. The OPA-adduct of DBDFE-Cys was determined by using a gradient starting from 30% acetonitrile/70% 12.5 mM potassium phosphate buffer (pH = 7.20) and moving at a rate of 1.2%/min to 50% acetonitrile/50% 12.5 mM potassium phosphate buffer (pH = 7.20). Fluorimetric detection of all OPA-adducts took place at 330 nm excitation and 426 nm emission wave lengths.

Pyruvic acid was determined as its 2,4-dinitrophenylhydrazone by reversed phase HPLC using a Lichrosorb 5RP18 column (150 × 4.6 mm). Samples of incubation mixtures were treated by an equal volume of 0.2% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid, incubated for 20 min at 37°, centrifugated and investigated by HPLC. The eluent used was 55% methanol/44% 50 mM tetraethylammonium iodide/1% acetic acid at a flow rate of 1.0 ml/min. Detection took place at 365 nm.

RESULTS

Toxicity of cysteine-S-conjugates and mercapturic acids in isolated proximal tubular cells

The proximal tubular cell were incubated with the cysteine-*S*-conjugates and the mercapturates of the four difluoroethylenes at a concentration of 100 μM for 2 hr. The toxicity of the conjugates as determined by their effect on α-MG uptake decreased in the order TFE ≈ CTFE > DCDFE > DBDFE (Fig. 1). The mercapturic acids and the corresponding cysteine-*S*-conjugates of TFE and CTFE were equally toxic; these four conjugates caused significant inhibition of α-MG uptake at concentrations as low as 10 μM and inhibited α-MG transport completely after 1 hr at a concentration of 250 μM. For the less toxic conjugates, those of DCDFE and DBDFE, the mercapturates could exert the same degree of toxicity as the corresponding cysteine-*S*-conjugates, but it took more time to do so (Fig. 2C and D). DBDFE-Cys and DBDFE-NAc had no effect on α-MG uptake at concentrations below 25 μM (data not shown). At 250 μM DCDFE-Cys and DBDFE-Cys completely inhibited α-MG uptake, but this occurred only after 3 hr of incubation.

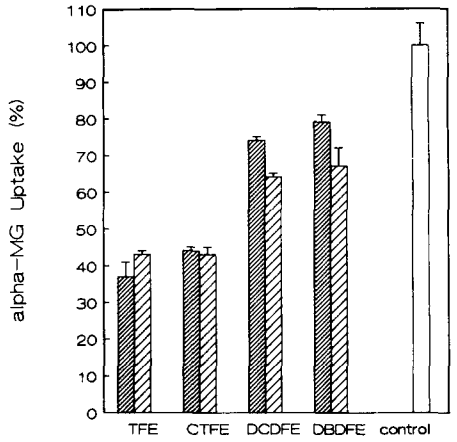


Fig. 1. Relative toxicity of cysteine-*S*-conjugates and the corresponding mercapturic acids, as determined by α-MG uptake. Rat renal proximal tubular cells (3.5 × 10^6 cells/ml) were incubated with 100 μM of the conjugates for 2 hr. ■ Mercapturates, ▨ cysteine-*S*-conjugates, □ control. Values are means ± SE of triplicates.

Toxicity of the cysteine-*S*-conjugates and mercapturic acids of TFE, CTFE (100 μM), DCDFE and DBDFE (250 μM) could be inhibited by AOA, an effective inhibitor of the cysteine-*S*-conjugate β-lyase (Fig. 2).

Organic anion transport

The cysteine-*S*-conjugates as well as the mercapturates possess a carboxylate-group. Therefore, they might be concentrated in the renal proximal tubular cell by organic anion carriers, which can competitively be blocked by probenecid. The active uptake of pAH by proximal tubular cells has been reported elsewhere [13]. The uptake was time dependent and linear for at least 2 min, and showed apparent K_m and V_{max} of 0.08 mM and 6.7 nmol/mg protein/min respectively. The pAH uptake could be reduced by a 10-min preincubation with the competitive inhibitor of organic anion transport, probenecid. Probenecid at a concentration of 1.0 mM inhibited pAH uptake by 76 ± 2%. Almost complete inhibition (97 ± 2%) could be obtained by preincubation with 5 mM probenecid (Fig. 3). At concentrations higher than 5 mM, probenecid decreased α-MG uptake (data not shown); therefore all experiments with probenecid were performed at a concentration of 1 mM although only 76% inhibition of pAH-uptake was observed at that concentration.

Effects of probenecid on toxicity of the conjugates

In order to determine whether the probenecid-sensitive organic anion carrier mediated the uptake of the cysteine-*S*-conjugates and the mercapturic acids, the effects of probenecid preincubation on toxicity were studied. The toxicity of the mercapturic acids was reduced by a 10-min preincubation with 1.0 mM probenecid (Fig. 4A). The toxicity of the cysteine-*S*-conjugates, on the contrary, was not

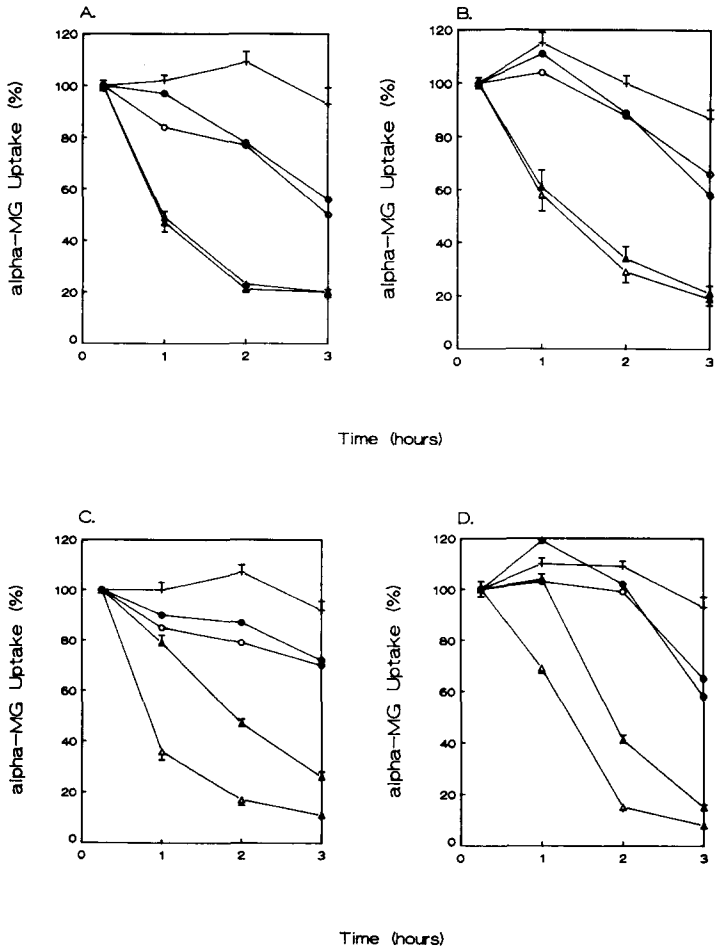


Fig. 2. Effect of AOA on the toxicity of cysteine-*S*-conjugates and the corresponding mercapturic acids in isolated rat renal proximal tubular cells. Cells (3.5×10^6 per ml) were incubated with 1.0 mM AOA for 15 min before conjugates were added. All values are means ± SE, figures are representative of three separated experiments. (A) 100 μM TFE-*S*-conjugates, (B) 100 μM CTFE-*S*-conjugates, (C) 250 μM DCDFE-*S*-conjugates, and (D) 250 μM DBDFE-*S*-conjugates. +, control; ○, cysteine-*S*-conjugate + AOA: ●, mercapturate + AOA; △, cysteine-*S*-conjugate; ▲, mercapturate.

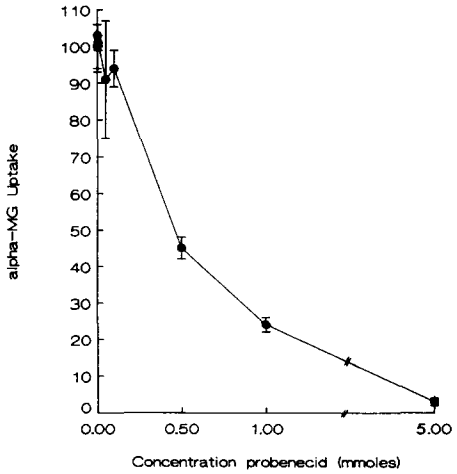


Fig. 3. Inhibition by probenecid of [^{14}C]-pAH uptake by proximal tubular cells. Cells (3.5×10^6/ml) were pre-incubated with probenecid for 15 min before [^{14}C]-*p*-amino-hippuric acid (100 μM final concentration) was added.

altered at all (Fig. 4B). The protective effect of 1.0 mM probenecid against the toxicity of TFE-NAc and CTFE-NAc was more prominent at lower concentrations (25 μM) of these mercapturates (data not shown); again, no protection against the corresponding cysteine-*S*-conjugates was observed.

Metabolism of S-*conjugates of* 2,2-*difluoroethylenes by proximal tubular cells*

The metabolism of the *S*-conjugates of DCDFE and DBDFE by rat renal proximal tubular cells was studied at a concentration of 2.0 mM. To avoid extracellular metabolism due to enzyme leakage as a result of toxicity, the conjugates were incubated for only 15 min (cysteine-*S*-conjugates) or 30 min (mercapturic acids). Due to the much higher toxicity of the *S*-conjugates of TFE and CTFE (Fig. 4) the metabolism of these conjugates was studied at the lower concentration of 0.5 mM.

After 15 min of incubation with the cysteine-*S*-conjugates no or only very low levels of the corresponding mercapturic acids were detected (Fig. 5).

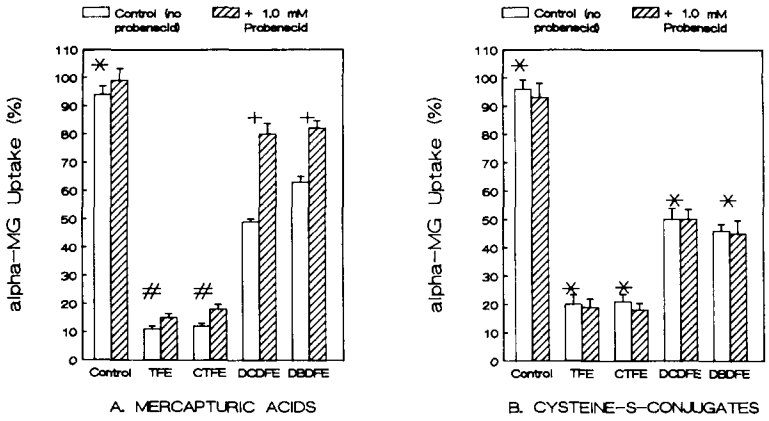


Fig. 4. Effect of probenecid on the cytotoxicity of mercapturates (A) and corresponding cysteine-*S*-conjugates (B). Isolated rat renal proximal tubular cells (3.5×10^6 cells/ml) were preincubated with probenecid for 15 min before the conjugates were added in a final concentration of 100 μM. α-MG uptake was determined 2 hr after addition of the conjugates. Results were statistically analysed using an unpaired, one-tailed student *t*-test (*t*-ease). * Not significantly different, $P = 0.50$. # Significantly different, $P < 0.05$. + Significantly different, $P < 0.02$.

In incubations with TFE-Cys the formation of TFE-NAc could not be detected at all, both in absence or presence of AOA. This observation cannot be attributed to a lack of uptake of TFE-Cys by the cells, because TFE-Cys was toxic to the cells (Fig. 2A), and formation of TFE-PMS after intracellular β-lyase dependent activation was observed (Table 1). Similarly CTFE-Cys yielded only traces of the mercapturic acid. However, DCDFE-Cys and DBDFE-Cys yielded significantly higher concentrations, 30–50 μM, of the corresponding mercapturates after 15 min, amounting to 1.5–2.5% of the substrate.

When the cells were incubated with the mercapturic acids, significant reduction of their concentration could be observed (Fig. 6). The mercapturates of TFE and CTFE were metabolized for over 90% after 30 min of incubation. These results indicate that uptake as well as the subsequent deacetylation of the TFE-NAc and CTFE-NAc is very efficient, and therefore probably not rate limiting for the onset of toxicity. However, only 5–20% of DCDFE-NAc and DBDFE-NAc had been converted after 30 min (Fig. 6). This might be due to a rate limiting cellular uptake and/or a slow rate of intracellular deacetylation. Due to the four-fold higher initial concentration of DCDFE-NAc and

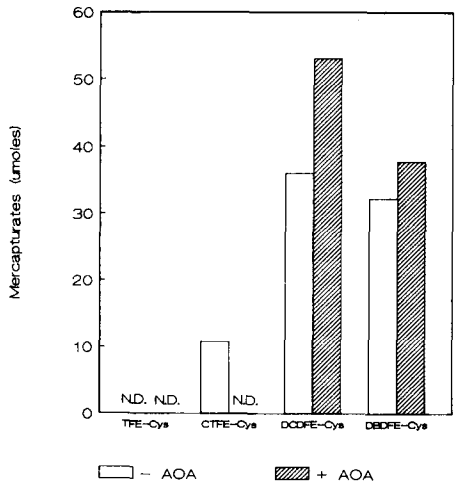


Fig. 5. Acetylation by renal proximal tubular cells of the cysteine-*S*-conjugates of TFE, CTFE, DCDFE and DBDFE. Cells (10^7/ml) were preincubated with or without 1.0 mM AOA for 15 min and subsequently incubated with the cysteine-*S*-conjugates for another 15 min. Initial concentration of TFE- and CTFE-Cys was 0.5 mM, and of DCDFE-Cys and DBDFE-Cys 2.0 mM. Values have an analytical variance of 5%.

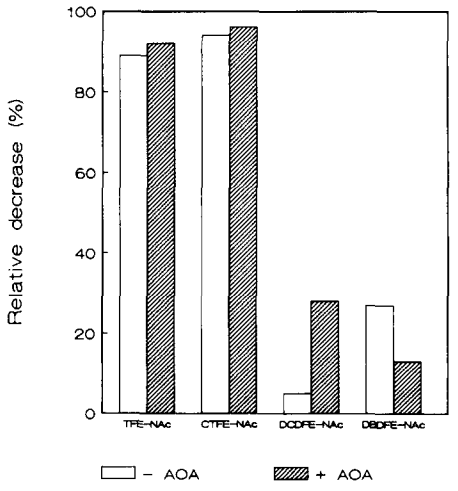


Fig. 6. Relative deacetylation of mercapturates by renal proximal tubular cells. Cells (10^7/ml) are incubated, after preincubation with or without 1.0 mM AOA, for 30 min with TFE-NAc (0.5 mM), CTFE-NAc (0.5 mM), DCDFE-NAc (2.0 mM) or DBDFE-NAc (2.0 mM). Values have an analytical variance of 5%.

DBDFE-NAc the absolute decreases (μmole converted) were comparable to those of TFE-NAc and CTFE-NAc.

Metabolism of S-conjugates of 2,2-difluoroethylenes by subcellular fractions of rat kidney

To investigate whether the difference in toxicity of the *S*-conjugates of fluoroethylenes might be attributed to differences in rates of activation (either deacetylation or β-lyase-dependent degradation) the conjugates were also incubated with subcellular fractions of rat kidney.

When the mercapturic acids of the halogenated ethylenes were incubated with rat renal 11,000 *g* supernatants, containing the acylases, a time-dependent decrease of their concentration could be observed (Fig. 7). The formation of the corresponding cysteine-*S*-conjugates in these incubations indicated that the mercapturic acids were metabolized by acylase(s). Aminooxyacetic acid (AOA) did not influence the rate of deacetylation. The rate of deacetylation of TFE-NAc and CTFE-NAc was much higher than that of DCDFE-NAc and DBDFE-NAc (Fig. 7).

To determine the activity of renal cysteine-*S*-conjugate β-lyase, the cysteine-*S*-conjugates were incubated with renal cytosol. Activity of β-lyase (measured by formation of pyruvate) decreased significantly in the order TFE-Cys > CTFE-Cys > DCDFE-Cys > DBDFE-Cys (Table 2). In the presence of 0.5 mM AOA, pyruvate production was inhibited completely.

DISCUSSION

The toxicity of the cysteine-*S*-conjugates and the mercapturates of four structurally related difluoroethylenes was studied in isolated proximal tubular cells from rat kidney. To explain the observed differences in toxicity, the transport of these conjugates into the cells and their metabolism by activating and deactivating enzymes was also investigated.

All *S*-conjugates were toxic to the proximal tubular cells as determined by inhibition of α-MG uptake.

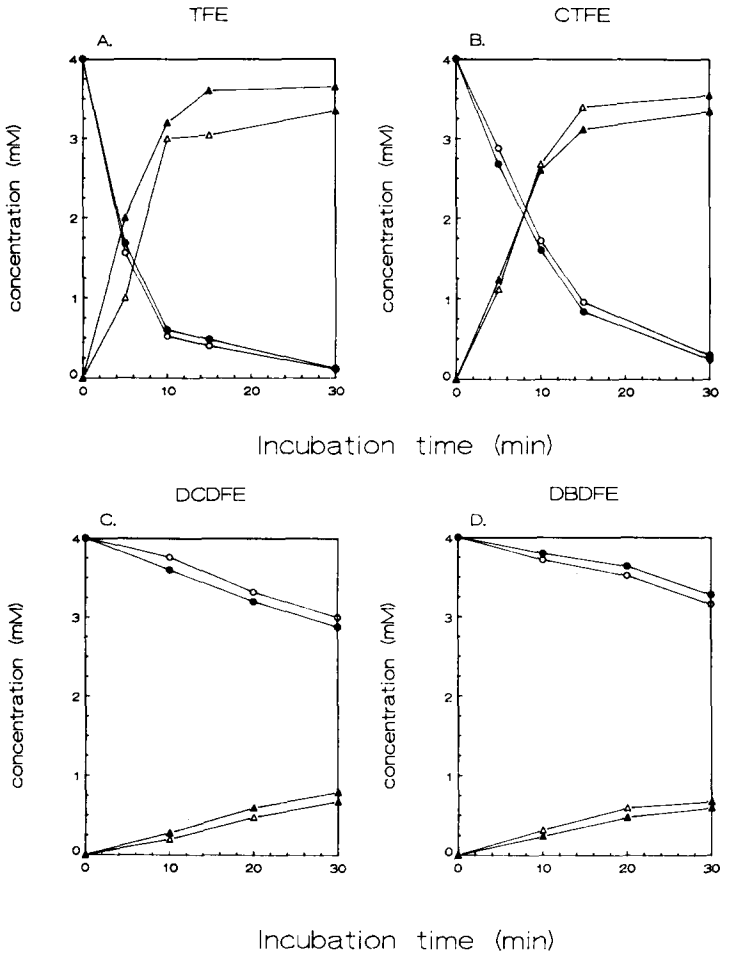


Fig. 7. Deacetylation of mercapturic acids and concomitant formation of corresponding cysteine-*S*-conjugates by rat renal 11,000 *g* supernatants. Incubations were performed in potassium phosphate buffer (50 mM, pH = 7.40, 37°) with (closed symbols) or without (open symbols) 0.2 mM AOA. ○,●, mercapturates; △,▲, cysteine-*S*-conjugates formed.

Table 1. Formation of the pseudomercapturate, TFE-PMS (*N*-difluorothionoacetyl-*S*-(tetrafluoroethyl)-L-cysteine) upon incubation of rat renal proximal tubular cells with *S*-conjugates of TFE

Conjugate	Incubation time (min)	Concentration TFE-PMS in μM
TFE-Cys (0.5 mM)	15	8.8
TFE-Cys (0.5 mM) + AOA (1.0 mM)	15	N.D.
TFE-NAc (0.5 mM)	30	20.8
TFE-NAc (0.5 mM) + AOA (1.0 mM)	30	2.5

N.D.: not detectable.

The inhibition of their toxicity by preincubation of the cells with aminooxyacetic acid (AOA) indicates a β-lyase-dependent mechanism of bioactivation. The *S*-conjugates of TFE and CTFE appeared to be more toxic than those of DCDFE and DBDFE. The cysteine-*S*-conjugates and the mercapturic acids of TFE and CTFE were equally toxic, which can be explained by a very active deacetylation of TFE-NAc and CTFE-NAc to their cysteine-*S*-conjugates, as was confirmed in both cellular and subcellular incubations. The toxicity of DCDFE-NAc and DBDFE-NAc was delayed as compared with the toxicity exerted by the corresponding cysteine-*S*-conjugates. This may be due to either differences in rate of cellular uptake and/or by a rate limiting deacetylation step for these mercapturic acids. The slow rate of deacetylation of both DCDFE-NAc and DBDFE-NAc by 11,000 *g* supernatants, when compared with that of TFE-NAc and CTFE-NAc tends to support the second explanation.

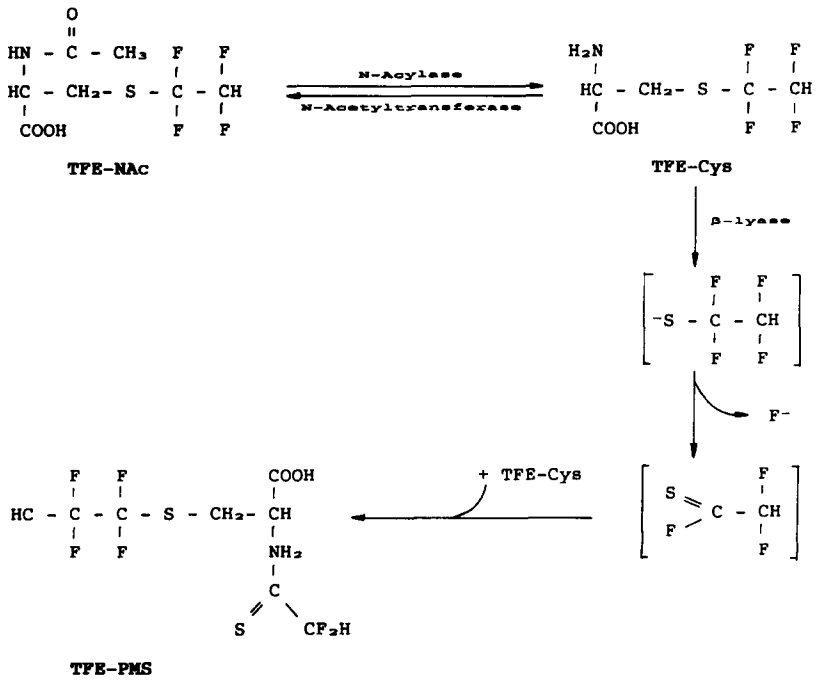
Little formation of mercapturic acids was observed in incubations of the proximal tubular cells with the cysteine-*S*-conjugates. However, *in vivo* a high percentage (>60%) of the dose of DCDFE is excreted as DCDFE-NAc in the urine of rats treated with 150 μmol DCDFE/kg [7]. One possibility seems that the proximal tubular cells have lost their ability to acetylate cysteine-*S*-conjugates after isolation for instance by deactivation of *N*-acetyltransferase or depletion of acetyl-coenzyme A. However, the alternative explanation is that *in vivo* DCDFE-Cys is acetylated in the liver rather than in the kidney. Others have shown, indeed, that a S-substituted GSH conjugate can be hydrolysed to the cysteine-*S*-conjugate in the kidney and subsequently is transferred to the liver for N-acetylation of the conjugate. The mercapturate was finally excreted in the urine [18, 19].

As depicted in Scheme 1, *N*-acetyltransferases (both renal and hepatic) can convert cysteine-*S*-conjugates to mercapturic acids. As mercapturic acids are readily excreted by the kidney, this is a detoxification route. However, renal acylases can convert the mercapturates into the corresponding cysteine-*S*-conjugates which are substrate for the renal β-lyase. As the *N*-acetyltransferase activity of the proximal tubular cells appeared to be rather low, the toxicity of the *S*-conjugates will primarily be determined by the uptake rate, and by the activity of the renal acylase and β-lyase. In 11,000 *g* supernatants the activity of acylase as well as β-lyase for their TFE- and CTFE-conjugate substrates was much higher than that for the *S*-conjugates of DCDFE and DBDFE. The latter conjugates exert much less toxicity both *in vivo* and *in vitro*, which suggests an important role of the acylase as well as the β-lyase activities in determining the relative nephrotoxicity of the fluoroethylenes.

No data on the renal transport of fluorinated ethylene-*S*-conjugates are available, but some related substances—among which chlorinated alkene-*S*-conjugates—have been studied [6, 20–24]. In our *in vitro* system we observed that the toxicity of the mercapturates of the four 2,2-difluoroethylenes was reduced by preincubation of the cells with probenecid. Probenecid is an inhibitor of the basolateral organic anion and Na^+-coupled GSH transport systems [20, 25]. Probenecid, however, did not provide any protection towards the toxicity caused by the cysteine-*S*-conjugates of the same 2,2-difluoroethylenes. This observation shows that the cysteine-*S*-conjugates are not transported via a probenecid-sensitive basolateral carrier (which transports mercapturates [20]), but by another transporter, probably an L-amino acid transporter [3, 21, 22], as depicted in Scheme 2. *In vivo* studies seem to contradict this since administration of probenecid inhibited the nephrotoxicity caused by *S*-(1,2-dichlorovinyl)-L-cysteine [23] and HCBD-Cys [6] *in vivo*. This apparent contradiction can be explained

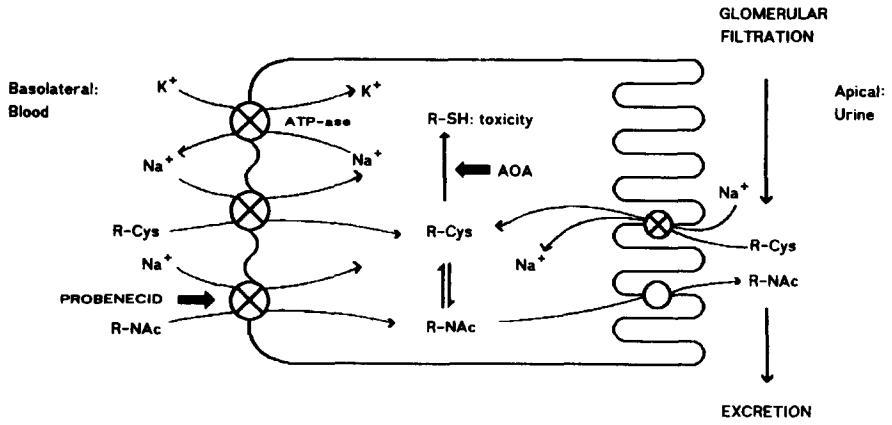
Table 2. Activity of β-lyase of rat renal cytosol towards cysteine-*S*-conjugates of difluorinated ethylenes

	Activity (nmol pyruvic acid/min/mg protein)			
Conjugate	0.5 mM	1.0 mM	2.0 mM	4.0 mM
TFE-Cys	2.8 ± 0.2	5.9 ± 0.3	9.7 ± 0.3	13.7 ± 0.3
CTFE-Cys	2.3 ± 0.2	4.2 ± 0.2	7.2 ± 0.3	9.5 ± 0.2
DCDFE-Cys	2.1 ± 0.1	3.4 ± 0.3	4.7 ± 0.2	7.6 ± 0.3
DBDFE-Cys	1.9 ± 0.2	2.2 ± 0.2	2.7 ± 0.2	3.0 ± 0.1



Scheme 1. Bioactivation mechanisms of TFE-*S*-conjugates. The upper part, including the conversion to a reactive intermediate by β-lyase cleavage of the cysteine-*S*-conjugate, applies to all 2,2-difluoroethylenes. The formation of the pseudomercapturate (TFE-PMS) was only observed in the case of TFE-*S*-conjugates.

by efficient hepatic acetylation of the cysteine-*S*-conjugates to the mercapturates, which are then transported to the kidney and taken up by a probenecid-inhibitable process. The fact that probenecid provided partial protection against nephrotoxicity may indicate that the accumulation of the cysteine-*S*-conjugate in the kidney is determined by uptake and deacetylation of hepatic mercapturic acids, as well as uptake of circulating unchanged cysteine-*S*-conjugate. Probenecid also inhibits nephrotoxicity of HCBD in rats and mice *in vivo* [5, 22], which suggests that delivery of the GSH-conjugate or mercapturic acid from the blood, followed by intracellular degradation, is more important for providing the cysteine-*S*-conjugate to the kidney than direct delivery of the latter from the blood. The fact that probenecid also inhibits the nephrotoxicity of HCBD-GSH *in vivo*, but only very



Scheme 2. Renal proximal tubular handling of *S*-conjugates derived from 2,2-difluoroethylenes. Different uptake mechanisms for cysteine-*S*-conjugates (R-Cys) and the corresponding mercapturic acids (R-NAc) by the renal proximal tubular cell exist. Both types of conjugates accumulate intracellularly via carrier-mediated transport systems (⊗), which depend on the Na^+-gradient provided by the basolateral Na^+/K^+-ATP-ase. Only the mercapturates are taken up by a probenecid inhibitable process, and can leave the cell by facilitated diffusion (○). Black arrows indicate inhibition of the process.

slightly in isolated rat renal epithelial cells [6, 26] suggests that the mercapturic is responsible for accumulation in the kidney and subsequent bioactivation. This implies that next to β-lyase, acylase activity is a very important factor in determining the relative toxicity of this type of nephrotoxicants.

In contrast to the present result it has been reported that probenecid protected renal cells *in vitro* against the cytotoxicity of *S*-(1,2-dichlorovinyl)-L-cysteine and afforded a slight protection against HCBD-Cys as well [27, 28]. As Lock stated in a recent review this is somewhat surprising; he suggested that metabolism yielding the mercapturates may have occurred in these cell suspensions [28]. Our results support this suggestion.

In incubations of proximal tubular cells with TFE-Cys the formation of another interesting metabolite was observed: *N*-difluorothionacetyl-*S*-(tetrafluoroethyl)-L-cysteine (Table 1). This so-called pseudomercapturic acid (TFE-PMS) was also detected in cellular incubations in the presence of TFE-NAc. Pre-incubation of the cells with AOA inhibited formation of this metabolite, which has been shown to result from attack of the free amino group of TFE-Cys by the reactive intermediate, difluorothionoacetyl fluoride [16]. Thus, TFE-Cys accumulates in the proximal tubular cells on exposure to either TFE-Cys or TFE-NAc, and can act as scavenger of its own reactive intermediates formed upon cleavage by β-lyase as illustrated in Scheme 1. The corresponding pseudomercapturic acids derived from CTFE-Cys, DCDFE-Cys and DBDFE-Cys, however, could not be detected in the incubations with proximal tubular cells. Only traces of these compounds have been detected in incubations with subcellular fractions (Commandeur *et al.*, manuscript in preparation). Therefore, although chlorothionoacylfluoride already has been identified as a reactive intermediate derived from CTFE-Cys [29], our results point to another reactive intermediate, presumably a thiirane, as the main reactive intermediate derived from CTFE-Cys, DCDFE-Cys and DBDFE-Cys (Commandeur *et al.*, manuscript in preparation).

In conclusion, these investigations show (1) that both the cysteine-*S*-conjugates and the mercapturic acids of the four geminal difluoroethylenes TFE, CTFE, DCDFE and DBDFE are cytotoxic in renal proximal tubular cells *via* the β-lyase pathway; (2) that the differences in toxicity of the cysteine-*S*-conjugates and the corresponding mercapturic acids (TFE ≈ CTFE > DCDFE > DBDFE) agree with the observed nephrotoxicity *in vivo*, and can be explained by differences in deacetylation as well as β-lyase activities; and (3) that both cysteine-*S*-conjugates and mercapturic acids are readily taken up by the proximal tubular cells, but only the mercapturates are transported by the organic anion carrier (Scheme 2).

REFERENCES

1. Elfarra AA and Anders MW, Renal processing of glutathione conjugates. Role in nephrotoxicity. *Biochem Pharmacol* **33**: 3279–3732, 1984.
2. Anders MW, Elfarra AA and Lash LH, Cellular effects of reactive intermediates: nephrotoxicity of *S*-conjugates of amino acids. *Arch Toxicol* **60**: 103–108, 1987.
3. Monks TJ and Lau SS, Renal transport processes and glutathione conjugate-mediated nephrotoxicity. *Drug Metab Disp* **15**: 437–441, 1987.
4. Ross BD and Guder WG, Heterogeneity and compartmentation in the kidney. In: *Metabolic Compartmentation* (Ed. Sies H) Academic Press, London, 1982.
5. Lash LH, Elfarra AA and Anders MW, Renal cysteine conjugate β-lyase. Bioactivation of nephrotoxic cysteine-*S*-conjugates in mitochondrial outer membrane. *J Biol Chem* **261**: 5930–5935, 1986.
6. Lock EA and Ishmael J, Effect of the organic transport inhibitor probenecid on renal cortical uptake and proximal tubular toxicity of hexachloro-1,3-butadiene and its conjugates. *Toxicol Appl Pharmacol* **81**: 32–42, 1985.
7. 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.
8. Commandeur JNM, Brakenhoff JPG, de Kanter FJJ and Vermeulen NPE, Nephrotoxicity of mercapturic acids of three structurally related 2,2-difluoroethylenes in the rat. *Biochem Pharmacol* **37**: 4495–4504, 1988.
9. Boogaard PJ, Mulder GJ and Nagelkerke JF, Isolated proximal tubular cells from rat kidney as an *in vitro* model for studies on nephrotoxicity. II. α-Methylglucose uptake as a sensitive parameter for mechanistic studies of acute toxicity by xenobiotics. *Toxicol Appl Pharmacol*, in press.
10. Duffel MW and Jakoby WB, Cysteine *S*-conjugate *N*-acetyltransferase from rat kidney microsomes. *Molec Pharmacol* **21**: 444–448, 1982.
11. Vamvakas S, Dekant W, Berthold K, Schmidt S, Wild D and Henschler D, Enzymatic transformation of mercapturic acids derived from halogenated alkenes to reactive and mutagenic intermediates. *Biochem Pharmacol* **36**: 2741–2748, 1987.
12. Dohn DR, Leininger JR, Lash HL, Quebbemann AJ and Anders MW, Nephrotoxicity of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, the glutathione and cysteine conjugates of chlorotrifluoroethene. *J Pharmacol Exp Ther* **235**: 851–857, 1985.
13. Boogaard PJ, Mulder GJ and Nagelkerke JF, Isolated proximal tubular cells from rat kidney as an *in vitro* model for studies on nephrotoxicity. I. An improved method for preparation of a homogeneous suspension of proximal tubular cells and characterization of α-methylglucose uptake. *Toxicol Appl Pharmacol*, in press.
14. Boogaard PJ, Mulder GJ and Nagelkerkef JF, Alpha-methylglucose uptake by isolated rat kidney proximal tubular cells as a parameter for cell integrity *in vitro*. In: *Nephrotoxicity: Extrapolation from* in Vitro *to* in Vivo *and Animals to Man* (Eds. Bach PH and Lock EA), pp. 337–342. Plenum Press, London, 1989.
15. 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.
16. Commandeur JNM, de Kanter FJJ and Vermeulen NPE, Bioactivation of the cysteine *S*-conjugate and mercapturic acid of tetrafluoroethylene to acylating reactive intermediates in the rat. Dependence of activation and deactivation activities on acetyl coenzyme A availability, submitted for publication.
17. Lindroth P and Mopper K, High performance liquid chromatographic determination of subpicomole

amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. *Analyt Chem* **51**: 1667–1674, 1979.
18. Inouhe M, Okajima K and Morino Y, Metabolic coordination of liver and kidney in mercapturic acid biosynthesis *in vivo*. *Hepatology* **2**: 311–316, 1982.
19. Bakke JE, Rafter J, Larsen GL, Gustafsson JÅ and Gustafsson BE, Enterohepatic circulation of the mercapturic acid and cysteine conjugates of propachlor. *Drug Metab Disp* **9**: 525–528, 1981.
20. Lock EA, Odum J and Ormond P, Transport of *N*-acetyl-*S*-pentachloro-1,3-butadienylcysteine by rat renal cortex. *Arch Toxicol* **59**: 12–15, 1986.
21. Schaeffer VH and Stevens JL, Mechanism of transport for toxic cysteine conjugates in rat kidney cortex membrane vesicles. *Molec Pharmacol* **32**: 293–298, 1987.
22. Schaeffer VH and Stevens JL, The transport of *S*-cysteine conjugates in LLC-PK_1 cells and its role in toxicity. *Molec Pharmacol* **31**: 506–512, 1987.
23. Elfarra AA, Jakobson I and Anders MW, Mechanisms of *S*-(1,2-dichlorovinyl)glutathione-induced nephrotoxicity. *Biochem Pharmacol* **35**: 283–288, 1986.
24. Ban M and de Céaurriz J, Probenecid-induced protection against acute hexachloro-1,3-butadiene and methylmercury toxicity to the mouse kidney. *Toxicol Lett* **40**: 71–76, 1988.
25. Lash LH and Jones DP, Renal glutathione transport. Characteristics of the sodium dependent system in the basal-lateral membrane. *J Biol Chem* **259**: 14508–14515, 1984.
26. Jones TW, Wallin A, Thor H, Gerdes RG, Ormstad K and Orrenius S, The mechanisms of pentachlorobutadienyl-glutathione nephrotoxicity studied with isolated rat renal epithelial cells. *Arch Biochem Biophys* **251**: 504–513, 1986.
27. Lash LH and Anders MW, Cytotoxicity of *S*-(1,2-dichlorovinyl)-glutathione and *S*-(1,2-dichlorovinyl)-L-cysteine in isolated rat kidney cells. *J Biol Chem* **261**: 13076–13081, 1986.
28. Lock EA, Studies on the mechanism of nephrotoxicity and nephrocarcinogenicity of halogenated alkenes. *CRC Crit Rev Toxicol* **19**: 23–42, 1988.
29. Dekant W, Lash LH and Anders MW, Bioactivation mechanism 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.