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METABOLIC ACTIVATION OF THE NEPHROTOXIC HALOALKENE 1,1,2-TRICHLORO-3,3,3-TRIFLUORO-1-PROPENE BY GLUTATHIONE CONJUGATION*

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Abstract—1,1,2-Trichloro-3,3,3-trifluoro-1-propene (TCTFP) is structurally closely related to the stable and non-toxic tetrachloroethylene. However, in TCTFP, the trifluoromethyl group enhances chemical reactivity with nucleophiles. This fact suggested that TCTFP may be metabolized intensively by glutathione (GSH) conjugation and therefore, like hexachlorobutadiene, would be expected to be nephrotoxic. We have investigated the nephrotoxicity and metabolism of TCTFP. Administration of 20 and 40 mg/kg to male rats resulted in a large, dose-dependent increase in urinary excretion of γ-glutamyl transpeptidase (GGT) indicative of proximal tubular damage. No increase in plasma transaminase concentrations indicative of liver damage was found. In rats, *N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine was a major urinary metabolite of TCTFP. TCTFP was transformed by microsomal and cytosolic GSH *S*-transferases from rat liver to *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)glutathione (DCTFPG) (identified by NMR and mass spectrometry). DCTFPG was toxic to rat renal cortex cells. Inhibition of GGT and cysteine conjugate β-lyase blocked DCTFPG cytotoxicity. These results suggest the following TCTFP bioactivation: conjugation with GSH in the liver, catabolism of the GSH *S*-conjugate to the cysteine *S*-conjugate and cleavage of the cysteine *S*-conjugate by β-lyase with formation of reactive intermediates in the kidney.

Halogenated alkenes may be bioactivated by two different pathways in rats: oxidation by cytochrome P-450 and glutathione (GSH) *S*-transferase catalysed conjugation with glutathione. Oxidative metabolism has been demonstrated for vinyl chloride, 1,1-dichloroethene, tri- and tetrachloroethene (for review, see Ref. 1). Cytochrome P-450 dependent monooxygenases transform these haloalkenes to chlorooxiranes and chlorinated aldehydes which may react with macromolecules, thus initiating haloalkene-induced hepatotoxicity and carcinogenicity [1]. Conjugation with GSH to form haloalkyl- and haloalkenyl-GSH *S*-conjugates has been demonstrated for hexachlorobutadiene, tetrachloroethene and several fluoroalkenes [2]. These GSH *S*-conjugates or catabolites formed by processing of the glutathionyl-residue by GGT‡ and dipeptidases are concentrated in the kidney by renal transport systems; cysteine *S*-conjugates present in the kidney are ultimately metabolized by cysteine conjugate β-lyase. The β-lyase dependent metabolism of haloalkyl- and haloalkenyl-cysteine *S*-conjugates results in formation of cytotoxic and genotoxic intermediates [2]. This reaction sequence presumably accounts for the nephrotoxicity and, perhaps, nephrocarcinogenicity of several haloalkenes [2].

1,1,2-Trichloro-3,3,3-trifluoro-1-propene(TCTFP) is structurally closely related to the very stable and nontoxic tetrachloroethene. With respect to steric and electron withdrawing effects, the trifluoromethyl group resembles chlorine [3]. However, the trifluoromethyl substituent does not participate in mesomeric stabilization of the double bond; moreover, trifluoromethyl groups strongly stabilize carbanions [3]. Therefore, TCTFP is more reactive towards nucleophiles. These chemical considerations suggest that TCTFP is less likely to be metabolized by cytochrome P-450 and much more likely to be metabolized by GSH-conjugation; TCTFP should thus be nephrotoxic like hexachlorobutadiene [4] and fluoroalkenes [5].

To verify these predictions for metabolic pathways based on known chemical reactivity, we have investigated the nephrotoxicity of TCTFP and its metabolism in rats. The expectation proved to be correct: TCTFP is nephrotoxic in rats, metabolized to toxic GSH *S*-conjugates in rat liver homogenates and to mercapturic acids *in vivo*.

MATERIALS AND METHODS

Biochemical assays. Plasma urea, plasma-ASAT, plasma-ALAT, urinary GGT and urinary glucose were determined by commercially available methods (Boehringer Mannheim, F.R.G. and Sigma Chemical Co., F.R.G.). Urinary protein concentrations

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‡ Abbreviations used: TCTFP, 1,1,2-trichloro-3,3,3-trifluoro-1-propene; DCTFPG, *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-glutathione; DCTFPC, *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine; N-Ac-DCTFPG, *N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine; GGT, γ-glutamyltranspeptidase; AOAA, aminooxyacetic acid; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

were determined with the BioRad Protein assay kit.

Animals and treatment. Female Wistar rats (weighing from 220–260 g) from the Institute für Versuchstierkunde (Hannover, F.R.G.) were used for the studies. Single doses of TCTFP (40 mg/kg) in corn oil (0.5 ml) were administered by stomach tube between 9 and 10 a.m. and the animals were transferred by an all-glass metabolic cage immediately after application of TCTFP. Urine was collected for 24 hr in a cooled (4°) flask. Standard diet (Altromin®) and tap water were supplied *ad libitum*.

Preparation of subcellular fractions from rat liver and kidney. Rat liver microsomes and cytosol were prepared as described previously [6].

Enzymatic assays. All incubations with TCTFP (4 mM) were carried out at 37° in 0.1 M potassium phosphate buffer, pH 7.4, containing microsomal (0.1–0.5 mg/ml) or cytosolic protein (0.25–1 mg/ml), 0.1 mM tetrasodium EDTA and glutathione (10 mM) in a final volume of 3 ml. Closed flasks were used for all incubations. Samples (0.5 ml) were removed with a syringe through an airtight septum. In some experiments, a NADPH-generating system [7] or 1-chloro-2,4-dinitrobenzene (4 mM) were included.

Separation and quantification of S-*conjugates.* The reactions were stopped by addition of 0.1 ml 30% trichloroacetic acid. After storage at 4° for 24 hr, the precipitated protein was removed by centrifugation. Samples of the supernatant (0.01–0.05 ml) were fractionated by HPLC (linear gradient over 40 min from 0 to 100% methanol in water containing 0.1% trifluoroacetic acid; flow rate, 1.0 ml/min; column: Supelco LC-18S, 250 × 4 mm). The absorption of the eluate was monitored at 268 nm (Hewlett–Packard 1040 diode array detector).

Urine samples were filtered through HV-filters (pore size 0.45 μm; Millipore, Eschborn, F.R.G.) and aliquots (50 μl) fractionated by HPLC as described above. Peak areas were integrated with the software provided with the 1040 detection system, and the concentrations of the conjugates were determined by computerized comparison with standard curves. UV-spectra of the eluate were recorded at 1.28 sec intervals with a threshold setting of 5 mAU.

Instrumental analyses. ^{13}C NMR spectra were recorded in 5 mm tubes with a Bruker WH 400 spectrometer. Chemical shifts are reported in parts per million downfield from tetramethylsilane (set at δ = 0.00 ppm) as internal reference. Four thousand individual spectra were collected for Fourier transformation. Chemical derivatization reactions and preparative HPLC were performed as described [6]. GC/MS was performed with a Hewlett–Packard 5790 MSD with splitless injection (12 m × 0.2 mm column, 0.33 μm HP-1) with a linear temperature gradient of 10°/min from 40° to 250°. Injector and transfer line temperatures were 250° and 260°, respectively.

Isolation and incubation of renal epithelial cells. Isolated rat renal cortex cells were prepared by a modification of the collagenase perfusion method of Ormstad *et al.* [8] from male Wistar rats (Institut für Versuchstierkunde, Hannover, F.R.G.) weighing 250–300 g.

Differences from the surgical procedure described in [8] consisted in making the incision for the insertion of the cannula above the lower ligature (and not below the upper ligature) which was placed at the end of the abdominal aorta close to the bifurcation into the common iliac arteries. Also, at the end of the preparation the dispersed cells were harvested by 60 sec centrifugation at 80 *g*. This additional step increased the yield of cells by approximately 10% without increasing the proportion of smaller cells of endothelial and reticuloendothelial origin or resulting in cell aggregates or impairing the viability of the cells. The cell pellet was gently resuspended in Krebs–Henseleit buffer, pH 7.4, supplemented with 25 mM HEPES, 2.5 mM $CaCl_2$, 15 mM $NaHCO_3$, 5 mM glucose and 2% (w/v) bovine serum albumin at a density of approximately 1.2×10^6 cells/ml. All buffers were equilibrated with 95% O_2, 5% CO_2.

Cells were incubated at 37° in a shaker-waterbath with the test compounds dissolved in DMSO (final DMSO concentration 0.4%) and samples were taken at the beginning of the incubation and after 90 and 180 min for viability determination.

In experiments with aminooxyacetic acid (AOAA) [9] or Acivicin (AT-125) [10], the cell suspension was preincubated for 10 min with the inhibitors dissolved in 0.9% NaCl (final concentration of AOAA, 0.5 mM and AT-125, 0.25 mM) before addition of the test compounds.

Determination of cytotoxicity of S-*conjugates.* Viability was estimated by Trypan Blue exclusion. For determination of Trypan Blue exclusion 200 μl of the cell suspension were mixed with an equal volume of sterile filtered Trypan Blue solution (0.4%) and the number out of 500 cells that stained was counted immediately.

Identification of biosynthetic S-(1,2-*dichloro*-3,3,3-*trifluoro*-1-*propenyl*)*glutathione.* Microsomal protein from female rats (200 mg) was incubated with TCTFP (0.2 mmol) and glutathione (0.5 mmol) in 100 ml of potassium phosphate buffer, pH = 7.4, containing 0.1% Triton X-100 and 1% ethanol. After 1 hr, DCTFPG was isolated and purified by HPLC as described previously for *S*-(1,2,2-trichlorovinyl)glutathione [6]. Total yield of DCTFPG was 24 mg. This sample was subjected to ^{13}C NMR and a small aliquot hydrolysed to the corresponding cysteine *S*-conjugate followed by chemical derivatization [6].

Chemicals. 1,1,2-Trichloro-3,3,3-trifluoro-1-propene was obtained from Pfalz and Bauer Chemicals (Waterbury, CT) and redistilled before use. Purity was 99.5% as determined by GC/MS.

S-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)glutathione and *N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine were prepared by methods described previously for the synthesis of haloalkene derived GSH *S*-conjugates and mercapturic acids [11, 12]. Purity of synthetic *S*-conjugates was greater than 99% as checked by HPLC and TLC [6].

For the new compounds: *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)glutathione: ^{13}C NMR (CD_3OD, D_2O; ^1H-decoupled): δ (ppm) = 26.8, 32.3, 35.1, 42.2, 53.9. 54.3, 117.3 (*q*, J_{C-F} = 292 Hz), 138.3, (*q*,

Table 1. Toxicity of 1,1,2-trichloro-3,3,3-trifluoro-1-propene (TCTFP) in rats

Parameter	Control	TCTFP (25 mg/kg)	TCTFP (50 mg/kg)
Plasma urea (mg/%)	38 ± 4	85 ± 22**	143 ± 32**
Plasma ASAT (U/l)	68 ± 16	54 ± 17	70 ± 8
Plasma ALAT (U/l)	16 ± 3	11 ± 4	10 ± 3
Urine glucose (mg/24 hr)	2 ± 0.6	85 ± 31**	125 ± 27**
Urine protein	5 ± 1	30 ± 13**	65 ± 14**
Urine GGT (U/24 hr)	3540 ± 783	22490 ± 1370**	39470 ± 2170**

Female rats dosed p.o. with TCTFP dissolved in corn oil (1 ml) between 9 and 10 a.m. Twenty-four hr after dosing, the rats were killed with diethylether, blood and the 24-hr urine sample were taken for biochemical analysis. Results are mean ± SD (N = 4).

ASAT, aspartate amino transferase; ALAT, alanine amino transferase; GGT, γ-glutamyltranspeptidase.

** Significantly different from control ($P < 0.001$).

J_{C-F} = 7 Hz), 162.3 (q, J_{C-F} = 34 Hz), 172.5, 173.0, 173.7, 175.3.

The mass spectrum was recorded by GC/MS after hydrolysis to the cysteine *S*-conjugate and derivatization by *N*-bis(trifluoroacetyl)methylamine and BCl_3/methanol to give *N*-trifluoroacetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine methyl ester: Mass spectrum (electron impact, 70 eV): m/z (^{35}Cl) = 393 (5.3%, 2Cl, M^+); 334 (6.8%, 2Cl, M^+-CO_2CH_3); 280 (49.2%, 2Cl, M^+-CF_3CONH_3); 245 (100%, 1Cl, M^+-CF_2CONH_2, -Cl); 209 (17%, 2Cl); 198 (13%); 197 (9.4%, 2Cl); 184 (6%, 1Cl); 166 (4.7%); 160 (10.3%, 2Cl); 138 (16%); 129 (7%); 117 (21%); 113 (10%); 110 (5%); 96 (9.9%); 79 (16.6%); 70 (11.1%); 69 (41.9%); 59 (26.5%).

N-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine: ^{13}C NMR (CD_3OD; ^1H-decoupled: δ (ppm) = 23.2, 33.5, 53.2, 117.4 (q, J_{C-F} = 293 Hz), 138.4 (q, J_{C-F} = 7 Hz), 162.2 (q, J_{C-F} = 33 Hz), 172.4, 176.3.

Mass spectrum (electron impact, 70 eV, methyl ester): m/z (^{35}Cl) = 339 (3.2%, 2Cl, M^+); 280 (45.9%, 2Cl, M^+-CH_3CONH_2); 245 (100%, 1Cl, M^+-CH_3CONH_2,-Cl); 238 (28.3%, 2Cl); 182 (9.3%, 1Cl); 144 (15%); 130 (9.3%); 117 (15.1%); 88 (63.3%); 59 (12.3%).

RESULTS

Nephrotoxicity of TCTFP

In rats, oral administration of TCTFP produced marked, dose-dependent changes in all the parameters used to assess kidney damage after 24 hr. Increases in plasma urea, urinary glucose and protein indicated a TCTFP-induced reduction of glomerular filtration and/or tubular reabsorption (Table 1). Damage to the proximal tubule is also indicated by the marked increase in the level of the enzyme γ-glutamyltranspeptidase (GGT). Biochemical parameters indicative of hepatocellular damage were not influenced by TCTFP administration suggesting the absence of hepatotoxicity.

Metabolism of TCTFP in vitro

When TCTFP was incubated with hepatic microsomes or cytosol in the presence of 10 mM glutathione, the time and protein concentration-dependent formation of two polar metabolites in a ratio of 2:1 was observed. Their retention times and electronic spectra were identical to those of synthetic DCTFPG. Both metabolites exhibited identical UV-spectra (λ_{max} = 268 nm) and were isolated for mass spectrometry and NMR-analysis. Since it was presumed that the metabolites represent glutathione *S*-conjugates, they were hydrolysed under conditions known to hydrolyse GSH *S*-conjugates to the corresponding cysteine *S*-conjugates to permit analysis by GC/MS after chemical derivatisation with *N*-*bis*(trifluoroacetyl)methylamine and BCl_3/methanol [6]. Using this procedure, two volatile derivatives having identical mass spectra were obtained in a ratio of 2:1 (Fig. 1). The mass spectra showed several fragments with the characteristic isotopic clusters of chlorine atoms (m/z (^{35}Cl) = 393, M^+; m/z = 334, M^+-$COOCH_3$; m/z = 280, M^+-CF_3CONH_2; m/z = 245, M^+-CF_3CONH_2, -Cl) in addition to fragment indicative of *N*-trifluoroacetylated cysteine *S*-conjugate methyl esters (m/z = 198, M^+-$CF_3C_2Cl_2S$; m/z = 69, CF_3^+). By comparison with the mass spectra obtained when synthetic TCTFP was subjected to identical procedures, the substances were identified as *N*-(trifluoroacetyl)-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine methyl ester. The identification of this cysteine *S*-conjugate after hydrolysis indicates the structure of the metabolite as *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)glutathione. For further confirmation of the proposed structure, the TCTFP-metabolites were analysed by ^{13}C NMR spectrometry. The ^{13}C NMR spectrum (Fig. 2) revealed several resonances assigned to four carbonyl carbon atoms (δ 172.5–176.4) and six aliphatic carbon atoms (δ 26.8–55.1) which

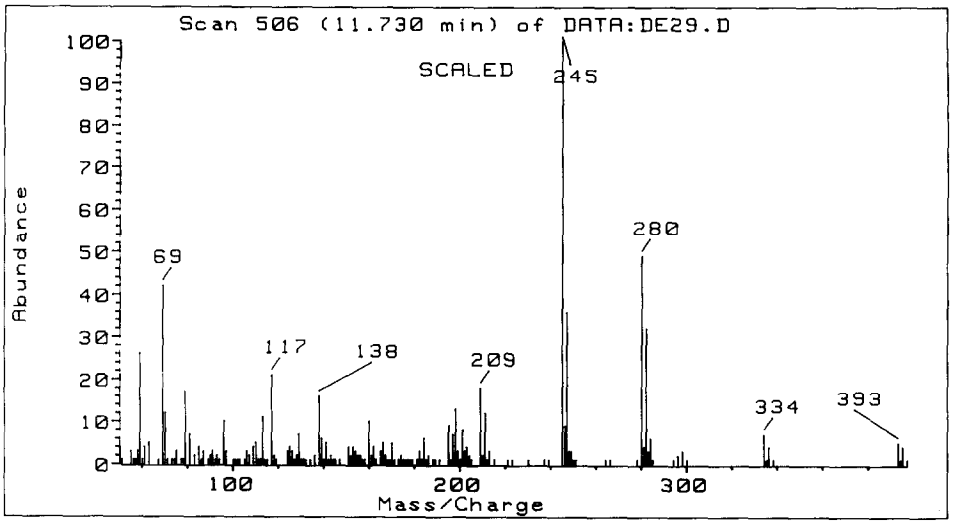

Fig. 1. Mass spectrum (GC/MS, electron impact, 70 eV) of metabolite formed by hepatic microsomes from 1,1,2-trichloro-3,3,3-trifluoro-1-propene (2 mM) in the presence of glutathione (5 mmol). Mass spectrum was recorded after hydrolysis, *N*-trifluoroacetylation and esterification. For method, see [6] and [12].

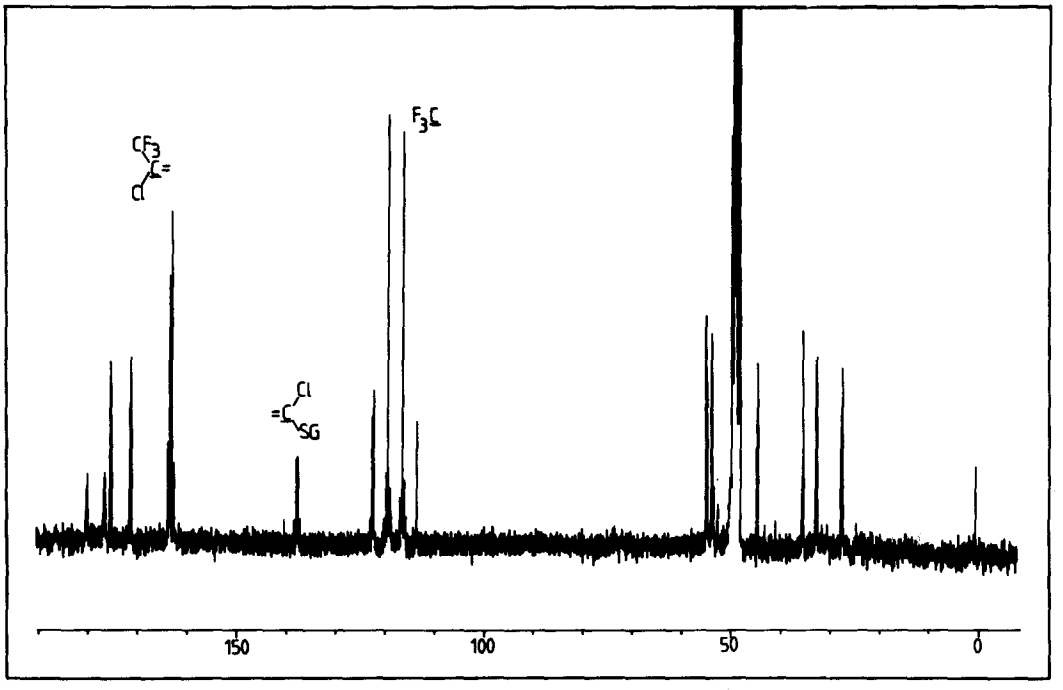

Fig. 2. ^{13}C-NMR spectrum of metabolite formed from glutathione and 1,2,2-trichloro-3,3,3-trifluoro-1-propene by rat liver microsomes. For assignment of ^{13}C-resonances, see [12–14].

correspond closely to the reported chemical shifts observed in the ^{13}C NMR spectra of glutathione *S*-conjugates [12, 13, 14]. Besides these resonances three quartets (δ 117.3, 162.3 and 138.3) were observed. The C–F coupling constant (292 Hz) and chemical shift of the quartet at 117.3 are consistent with the values reported for the carbon atoms of trifluoromethyl groups [14]; it is thus assigned to the trifluoromethyl group (carbon atom *a*, Fig. 3) in DCTFPG. The quartet at 162.3 is coupled with fluorine with a coupling constant of 34 Hz, which is in the range of coupling constants reported for carbon atoms in β-position to fluorine groups and is thus assigned to the carbon atom *b* (Fig. 2). The resonance at δ 138.3 ($J_{C-F} = 7$ Hz) is assigned to the carbon atom *c* (Fig. 2). Coupling constants

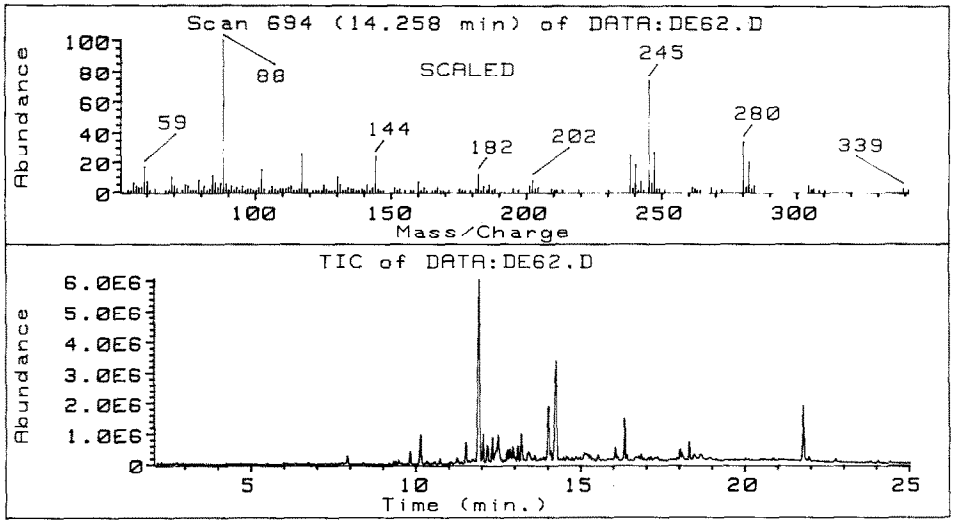

Fig. 3. Gas chromatographic separation of urine fraction after esterification and mass spectrum (electron impact, 70 eV) of *N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine methyl ester present in rat urine after administration of 1,2,2-trichloro-3,3,3-trifluoro-1-propene.

Table 2. Rates of *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)glutathione (DCTFPG) formation from 1,1,2-trichloro-3,3,3-trifluoro-1-propene (4 mM) and glutathione (10 mM) catalysed by hepatic cytosolic (protein concentrations 0.25–1 mg/ml) and microsomal (protein concentrations 0.1–0.5 mg/ml) from male and female Wistar rats and the effect of the competitive GSH *S*-transferase inhibitor 1-chloro-2,4-dinitrobenzene (CDNB: 4 mM)

Fraction	Conjugate formation (nmol/min/mg protein)
Male rat	
liver microsomes	530 ± 58
liver microsomes + NADPH	496 ± 62
liver microsomes + CDNB	113 ± 48
liver cytosol	120 ± 12
Female rat	
liver microsomes	513 ± 38
liver microsomes + NADPH	521 ± 46
liver microsomes + CDNB	96 ± 13
liver cytosol	161 ± 29
Non-enzymatic formation of DCTFPG in boiled microsomes	12 ± 7

DCTFPG formation was quantified by HPLC. Results are mean ± SD from 4 independent incubations, samples taken at 15 and 30 min after the start of the incubation.

Peak areas for *E*- and *Z*-DCTFPG are summed.

between fluorine and carbon atoms in γ-position are reported between 3 and 6 Hz [14]. Combined, the electron impact mass spectrum and the ^{13}C NMR spectrum unequivocally identify the TCTFP-metabolites as *E*- and *Z*-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)glutathione.

Formation of DCTFPG in subcellular fractions was quantified by HPLC (Table 2). DCTFPG formation was dependent on the presence of GSH, on protein concentration up to 0.5 mg/ml of protein and on time up to 30 min. Microsomal GSH *S*-transferases exhibited a much higher activity with DCTFPG than did cytosolic transferases (Table 2). No significant differences in DCTFPG formation were observed in subcellular fractions obtained from male and from female rats. The presence of the competitive GSH *S*-transferase substrate 1-chlor-2,4-dinitrobenzene inhibited DCTFPG formation from TCTFP. Addition of a NADPH-generating system did not significantly decrease DCTFPG formation. In rat kidney cytosol and microsomes incubated with TCTFP and GSH, only a very low rate of DCTFPG production was observed. The reaction rates observed were not significantly different from the

rate of the non-enzymatic reaction of TCTFP with glutathione and were, therefore, not further quantified.

Identification of N-*acetyl*-S-(1,2-*dichloro*-3,3,3-*trifluoro*-1-*propenyl*)-L-*cysteine as a urinary metabolite of DCTFPC*

Urine of rats given 40 mg/kg TCTFP orally (total dose for two rats was 0.1 mmol) was collected during 6 hr intervals over a period of 24 hr after administration. HPLC-analysis of an aliquot (100 μl) of the urine sample collected 6 hr after administration contained two peaks whose retention times (32.8 min and 33.4 min) and electronic spectra (not shown) were identical to those of the two isomers of synthetic *N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine. For further analysis, these metabolites were isolated by prep. HPLC and subjected to GC/MS after esterification with boron trichloride/methanol. The mass spectrum obtained for the peak at 14.258 min (Fig. 3) contained several fragments suggesting the presence of chlorine atoms in the molecule (m/z (^{35}Cl) = 339 (2Cl, M^+); 280 (2Cl, M^+-CH_3CONH_3); 245 (1Cl, M^+-CH_3CONH_2, -Cl); 238 (2Cl, M^+-$COOCH_3$, $-COCH_2$)) and two fragments characteristic of the methyl esters of mercapturic acids (m/z = 144 ($C_5H_6NO_2S$); 88 ($C_3H_6NO_3$)). The peak with a retention time of 14.013 min (Fig. 3) showed an identical mass spectrum in respect of fragmentation and intensity of fragments. Moreover, the obtained spectra were identical to those of the two isomer obtained by chemical synthesis of *N*-acetyl-DCTFPC and the retention times of the synthetic reference compounds were identical with those of the metabolites in HPLC and GC under various separation conditions. These observations identify *E*- and *Z*-*N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine (*N*-Ac-DCTFPC) as urinary metabolites of TCTFP. Urinary excretion of *N*-Ac-DCTFPC amounted to 3.8 μmol in the first 6 hr after administration and gradually decreased (urine collected from 6–12 hr after administration 4.7 μmol *N*-Ac-DCTFPC; 12–18 hr, 2.9 μmol and 18–24 hr, 1.4 μmol). Total urinary excretion of *N*-Ac-DCTFPC within 24 hr after administration amounted to 9 μmol.

Cytotoxicity of DCTFPG

Freshly isolated rat renal cortex cells have been used to determine *S*-conjugate-induced cytotoxicity [15, 16, 17]. Isolated kidney cells were incubated with DCTFPG and cell viability, determined by Trypan Blue exclusion, was assessed at 0, 90 and 180 min after the start of the incubation.

DCTFPG resulted in a dose- and time-dependent decrease in cell viability. At a concentration of 1 mM, DCTFPG reduced cell viability from approximately 90% at the start of the incubation to less than 30% at 3 hr. A decrease in cell viability was observed at DCTFPG-concentrations down to 0.01 mM. Both the GGT-inhibitor Acivicin and the β-lyase inhibitor aminooxyacetic acid (AOAA) protected cells from DCTFPG-toxicity (Fig. 4).

DISCUSSION

The chemical structure and reactivity of TCTFP and the known pathways of haloalkene biotransformation indicated that TCTFP should be biotransformed to a glutathione conjugate and should thus be nephrotoxic. This expectation was confirmed.

TCTFP was found to be a much more potent nephrotoxin than the structurally related tetrachloroethene [1]. TCTFP is selectively nephrotoxic at doses causing no detectable hepatic damage. The biochemical parameters determined after treatment of rats suggest that TCTFP, like hexachlorobutadiene [18] and nephrotoxic fluoroalkenes, induces severe proximal tubular damage.

The *in vitro* metabolism studies demonstrate that TCTFP is conjugated with glutathione to yield two isomers of DCTFPG giving identical mass spectra and ^{13}C NMR spectra. Considerations of the probable chemical mechanisms of DCTFPG formation support the assigned structure of the major isomer as *E*-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-glutathione. The substitution of a chlorine atom of halogenated olefins by thiolates proceeds by an addition/elimination reaction [19]: first, the thiolate adds to the double bond to form an intermediate carbanion. Since trifluoromethyl substitition strongly carbanions [3], glutathione adds to TCTFP at the C-1 atom to form a carbanion with the negative charge at the C-2 atom. Because *trans*-eliminations are thermodynamically favored [19], chloride elimination from this carbanion is expected to result in the favored formation of the isomer with glutathione bound in the *trans*-position to the CF_3-residue.

Microsomal glutathione *S*-transferases from rat liver exhibited a much higher activity with TCTFP than did cytosolic *S*-transferases. As discussed previously [6, 12], this difference in activity between soluble and membrane-bound glutathione transferases, which has been observed with several haloalkenes, is very likely due to a preferential distribution of the lipophilic alkenes into lipid membranes resulting in higher local concentrations of the substrate for conjugation by the membrane-bound glutathione *S*-transferases.

The absence of hepatic damage induced by TCTFP *in vivo* suggests that metabolic oxidation reactions do not take part in the bioactivation of TCTFP. Also, since addition of a NADPH-generating system did not influence *S*-conjugate formation rates from TCTFP in rat liver microsomes, it is concluded that little oxidative metabolism of TCTFP occurs *in vitro*.

In rats, *N*-Ac-DCTFPC, which is formed by renal processing of DCTFPG by the enzymes of mercapturic acid formation (Fig. 5), was definitively identified as a urinary metabolite of TCTFP. The amount of *N*-Ac-DCTFPC found in rat urine within 24 hr after administration of TCTFP indicates that about 10% of the TCTFP-dose is converted to the mercapturic acid. The data presented suggest that GSH-conjugation reactions are also operative in TCTFP-metabolism *in vivo* and that GSH conjugation reactions represent a major pathway of biotransformation for this compound. GSH *S*-conjugates formed may also be excreted with feces as demonstrated for hexachlorobutadiene-derived *S*-conjugates [18]. Moreover, intensive metabolism of DCTFPC by renal β-lyase likely occurs.

The studies on the metabolism of TCTFP *in vivo*

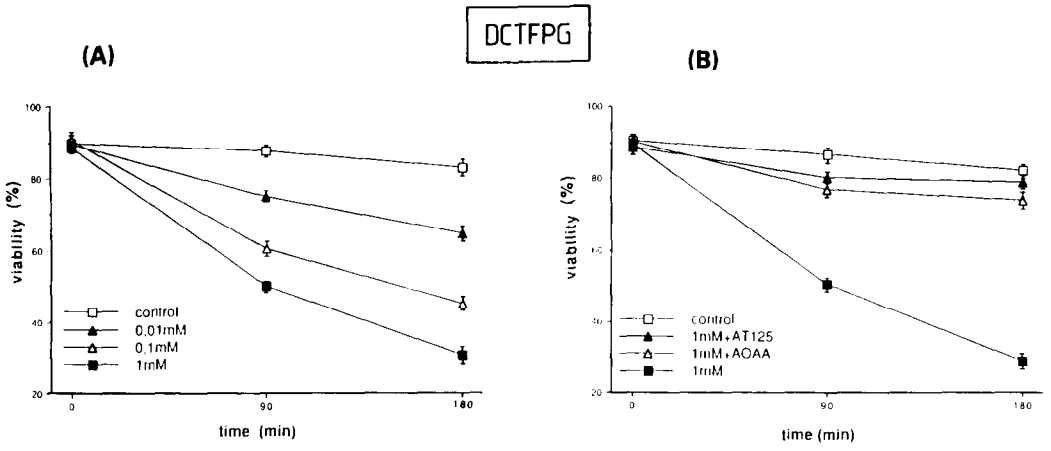

Fig. 4. Cytotoxicity of *S*-(1,2-dichloro-3,3-trifluoro-1-propenyl)glutathione (DCTFPG) in freshly isolated rat renal cortex cell preparations (A) containing 0 (□), 0.01 (▲), 0.1 (△) and 1 mM (■) DCTFPG. (B) Cells were preincubated for 10 min with AT-125 (0.25 mM) (▲) and aminooxyacetic acid (0.5 mM) (△) before addition of DCTFPG. Cell viability was determined by Trypan Blue exclusion. Values represent mean ± SD of four experiments.

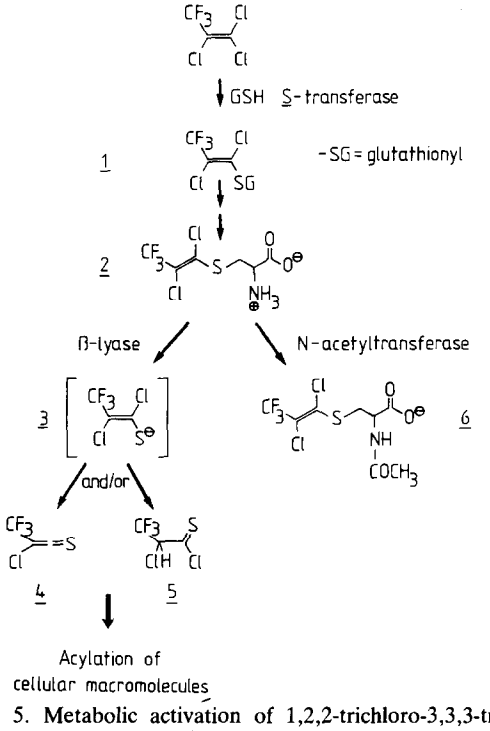

Fig. 5. Metabolic activation of 1,2,2-trichloro-3,3,3-trifluoro-1-propene. *1* *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-glutathione, *2* *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine, *3* 1,2-dichloro-3,3,3-trifluoro-1-mercapto-1-propene, *4* chlorotrifluoromethylthioketene, *5* 2-chloro-3,3,3-trifluorothiopropionic acid chloride, *6* *N*-acetyl-*S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine.

and *in vitro* and on the toxicity of the metabolites formed suggest that the activation scheme as depicted in Fig. 5 is responsible for the organ specific toxicity. The further mechanism of DCTFPG processing proceeds along pathways already established for other halovinyl glutathione *S*-conjugates [15, 16]. Like DCTFPG, the *S*-conjugate *S*-(1,2-dichlorovinyl)glutathione (DCVG) is cytotoxic in rat renal cortex cells. The major steps in DCVG-bioactivation have been elucidated. In analogy, further processing of DCTFPG results in toxic intermediates. DCTFPG (*1*, Fig. 5) is metabolized to *S*-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine (*2*) (DCTFPC) by GGT and dipeptidases. As indicated by the effect of the β-lyase inhibitor AOAA on DCTFPC cytotoxicity, DCTFPC is a substrate for renal β-lyases [16, 20, 21], which cleave DCTFPC to pyruvate, ammonia and the enethiol *3*. This enethiol is instable and rapidly converts to a thioacylating intermediate which could be either the thioketene *4* or the thioacyl choride *5* [22, 23]. Interaction of these highly reactive intermediates with lipids and proteins may cause the toxicity observed with DCTFPC. Alternatively, DCTFPC may be acetylated by renal acetyl-transferases to yield *N*-Ac-DCTFPC *6*, which is excreted with urine and is a urinary metabolite of TCTFP in rats. The intensive metabolism of TCTFP by glutathione conjugation may well explain the high nephrotoxicity. Conjugation rates for TCTFP determined *in vitro* are 500–1000-fold those observed for tetrachloroethene.

These results show that considerations of the chemical structure and reactivity of xenobiotics combined with a knowledge of the biotransformation of structurally similar compounds may predict the biotransformation pathway and, thus, the toxicity.

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REFERENCES

1. Henschler D, Halogenated alkenes and alkines. In: *Bioactivation of Foreign Compounds* (Ed. Anders MW). Academic Press, Orlando, 1985.

2. Anders MW, Lash LH, Dekant W, Elfarra AA and Dohn DR. Biosynthesis and metabolism of glutathione *S*-conjugates to toxic forms. *CRC Crit Rev Toxicol* **18**: 311–341, 1988.
3. Chambers RD, *Fluorine in Organic Chemistry*. Wiley-Interscience, New York, 1973.
4. Lock EA and Ishmael J, The acute toxic effects of hexachlorobutadiene on the rat kidney. *Arch Toxicol* **43**: 47–57, 1979.
5. Clayton JW, Toxicology of the fluoroalkenes: Review and research needs. *Environ Health Perspect* **21**: 255–267, 1977.
6. Dekant W, Martens G, Vamvakas S, Metzler M and Henschler D, Bioactivation of tetrachloroethylene: Role of glutathione *S*-transferase catalyzed conjugation versus cytochrome P-450 dependent phospholipidalkylation. *Drug Metab Dispos* **15**: 702–709, 1987.
7. Wolf RC, Berry PB, Nash JA, Green T and Lock EA, 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.
8. Ormstad K, Orrenius S and Jones DP, Preparation and characteristics of isolated kidney cells. *Methods Enzymol* **77**: 137–146, 1981.
9. Wallach DP, The inhibition of gamma-aminobutyric-alpha-keto-glutaric acid transaminase *in vitro* and *in vivo* by aminooxyacetic acid. *Biochem Pharmacol* **5**: 166, 1960.
10. Reed DJ, Ellis WW and Meck RA, The inhibition of γ-glutamyl-transpeptidase and glutathione metabolism of isolated rat kidney cells by L-(S,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125; NSC-163501). *Biochem Biophys Res Commun* **94**: 1273–1277, 1980.
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. Dekant W, Vamvakas S, Henschler D and Anders MW, Enzymatic conjugation of hexachloro-1,3-butadiene with glutathione: Formation of 1-(glutathion-*S*-yl)-1,2,3,4,4-pentachlorobuta-1,3-diene and 1,4-*bis*(glutathion-*S*-yl)-1,2,3,4-tetrachlorobuta-1,3-diene. *Drug Metab Dispos* **16**: 701–706, 1988.
13. Hinson JA, Monks TJ, Honig MH, Highet RJ and Pohl LR, *S*-(Glutathion-*S*-yl)acetaminophen. A biliary metabolite of acetaminophen. *Drug Metab Dispos* **10**: 47–50, 1982.
14. Hesse M, Meier H and Zeeh B, *Spektroskopische Methoden in der organischen Chemie*. Thieme, Stuttgart, 1987.
15. 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.
16. 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.
17. Lash LH and Jones DP, Uptake of the glutathione conjugate *S*-(1,2-dichlorovinyl)glutathione by renal basal-lateral membrane vesicles and isolated kidney cells. *Mol Pharmacol* **28**: 278–282, 1985.
18. 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.
19. Rappoport Z, The rich mechanistic world of nucleophilic vinylic substitution. *Rec Trav Chim Pays-Bas* **104**: 309–349, 1985.
20. Stevens JL, Robbins JD and Byrd RA, A purified cysteine conjugate β-lyase from rat kidney cytosol. *J Biol Chem* **261**: 15529–15537, 1986.
21. Stevens JL, Ayoubi N and Robbins JD, The role of mitochondrial matrix enzymes in the metabolism and toxicity of cysteine *S*-conjugates. *J Biol Chem* **263**: 3395–3400, 1988.
22. Dekant W, Berthold K, Vamvakas S, Henschler D and Anders MW, Thioacylating intermediates as metabolites of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2,2-trichlorovinyl)-L-cysteine formed by cysteine conjugate β-lyase. *Chem Res Toxicol* **1**: 175–178, 1988.
23. Dekant W, Berthold K, Vamvakas S and Henschler D, Thioacylating agents as ultimate intermediates in the β-lyase catalysed metabolism of *S*-(pentachlorobutadienyl)-L-cysteine. *Chem Biol Interact* **67**: 139–148, 1988.