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NEPHROTOXICITY AND HEPATOTOXICITY OF 1,1-DICHLORO-2,2-DIFLUOROETHYLENE IN THE RAT

INDICATIONS FOR DIFFERENTIAL MECHANISMS OF BIOACTIVATION

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Abstract—1,1-Dichloro-2,2-difluoroethylene (DCDFE) produced marked nephrotoxicity in rats upon an i.p. dose of 150 μmole/kg. At doses higher than 375 μmole/kg, DCDFE also produced hepatotoxicity. Aminooxyacetic acid, an inhibitor of cysteine conjugate β-lyase, appeared to be slightly nephrotoxic in Wistar rats. Nevertheless it exerted an inhibitory effect on the nephrotoxicity of DCDFE. The *N*-acetylcysteine conjugate of DCDFE was identified as a major urinary metabolite of DCDFE. When administered as such, this conjugate appeared to be a potent nephrotoxin, without any effect on the liver, indicating that glutathione conjugation of DCDFE is most likely a bioactivation step for nephrotoxicity. The appearance of traces of chlorodifluoroacetic acid in urine of rats treated with higher doses of DCDFE indicates the existence of an oxidative pathway of metabolism of DCDFE, probably involving epoxidation by hepatic mixed-function oxidases. It is speculated that the latter route might account for the hepatotoxicity at higher doses of DCDFE. The nephro- and hepatotoxicity of DCDFE, therefore, most likely are the result of two different mechanisms of bioactivation.

Halogenated ethylenes are subject to numerous toxicological studies since these industrially important compounds may cause nephro- and/or hepatotoxicity. The mechanisms of bioactivation for some of these compounds as yet are only partly elucidated. Tetrachloroethylene (TCE) which causes hepatotoxicity and hepatocellular carcinoma in rodents [1], has been shown to be metabolised by the hepatic microsomal mixed-function oxidase system to a reactive epoxide intermediate [2–4]. This epoxide or its rearrangement products (acyl halides and haloacetaldehydes) are believed to be responsible for the observed hepatotoxicity. 1,1-Dichloroethylene causes hepatotoxicity as well as nephrotoxicity [5, 6]. The nephrotoxicity of this compound is believed to be a result of oxidation by hepatic cytochrome P-450 to reactive intermediates which are then transported to the kidney. Only after induction of renal cytochrome P-450 has bioactivation *in situ* also been suggested to play a role [7].

Fluorinated ethylenes, like tetrafluoroethylene (TFE),† and chlorotrifluoroethylene (CTFE), however, all being important monomers in polymer industry, appeared to be specific nephrotoxins [8, 9]. Recent studies (reviewed by Elfarra and Anders [10]) indicated that the initial metabolic step in the bioactivation of this type of compound is conjugation to glutathione by hepatic glutathione-*S*-transferases (GSTs). The glutathione conjugates are subsequently degraded to the corresponding cysteine conjugates by biliary and renal hydrolases. Finally these cysteine conjugates are converted to pyruvate, ammonia and as yet unidentified reactive intermediates by a renal cysteine conjugate β-lyase [11]. A similar mechanism of bioactivation has recently been proposed for trichloroethylene, a compound which has been shown to cause nephrocarcinogenicity in rats [12, 13]. The occurrence of traces of *S*-1,2-dichlorovinyl-*N*-acetyl-cysteine in urine of rats treated with trichloroethylene indicated conjugation of trichloroethylene with glutathione [12]. In mice, however, trichloroethylene causes selective hepatocarcinogenicity due to more extensive metabolism by liver cytochrome P-450, involving the formation of short-lived reactive intermediates [14, 15].

In the present study we investigated in the rat nephro- and hepatotoxicity of 1,1-dichloro-2,2-difluoroethylene (DCDFE), a compound which is structurally related to the well-known hepatotoxin tetrachloroethylene as well as to the nephrotoxin tetrafluoroethylene. DCDFE has been shown to cause severe nephrodystrophy after chronic exposure to low levels. The threshold for toxicity was markedly lower than those of, the well-known nephrotoxins,

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† Abbreviations used: DCDFE, 1,1-dichloro-2,2-difluoroethylene: DCDFE-NAC, *N*-acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine; TFE, tetrafluoroethylene; CTFE, chlorotrifluoroethylene; CDFAA, chlorodifluoroacetic acid; AOA, aminooxyacetic acid; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NAG, *N*-acetyl-β-D-glucosaminidase; γ-GT, γ-glutamyl transpeptidase.

tetrafluoroethylene and chlorotrifluoroethylene [16]. Because of extensive industrial applications of DCDFE, characterisation of the toxicity caused by this compound is important. The biotransformation of DCDFE was also investigated, in order to find leads to explain the molecular mechanism of toxicity of this halogenated alkene.

MATERIALS AND METHODS

Chemicals

2,2-Difluorotetrachloroethane, *N*-acetylcysteine, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, aminooxyacetic acid (AOA, carboxymethoxylamine hemihydrochloride), and chlorodifluoroacetic acid (CDFAA) were purchased from Janssen Chimica (Beerse, Belgium).

1,1-Dichloro-2,2-difluoroethylene (DCDFE) was synthesised from 2,2-difluorotetrachloroethane according to the method of Sauer [17]. The product had a boiling point of 19° [18]. The purity and the identity was further assessed by gas chromatography/mass spectrometry (GC/MS), and ^{19}F-NMR: MS (only ions containing ^{35}Cl are given): *m/z* 132 (M^+, 100%), 113 (M^+-F,2.7%), 97 (M^+-Cl, 5%), 82 (M^+-CF_2, 25%); ^{19}F-NMR: δ-11.45 ppm (relative to the internal standard trifluoroacetic acid).

N-Acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine (DCDFE-NAC) was synthesised from DCDFE according to the procedure described for the synthesis of *N*-acetyl-*S*-(1,1,2,3,4-pentachloro-1,3-butadienyl)-L-cysteine [19], with slight modifications. Briefly, 50 mmole *N*-acetyl-L-cysteine was added to a solution of 100 mmole sodium methanolate in methanol. The solution was stirred for 30 min, and then cooled to 0°. DCDFE (75 mM) in cold methanol was added slowly under stirring. The reaction mixture was stirred for 12 hr at 4°. Then the solution was evaporated and the residue was dissolved in 25 ml of water. The solution was subsequently adjusted to pH 2 with HCl, and extracted three times with ethylacetate. The ethylacetate extracts were combined and evaporated. The residue was recrystallised from water after treatment with decolorising charcoal to yield ultimately 25% of product: melting point 137°. ^{1}H-NMR (90 MHz): δ (ppm, relative to 3-(trimethylsilyl)propane-sulfonic acid) 2.08 (s, 3H), 3.14–3.72 (m, 2H), 4.64–4.76 (m, 1H), 6.43 (t, J_{FH} = 7.0 Hz, 1H). ^{19}F-NMR (376 MHz): δ(ppm, relative to trifluoroacetic acid) −5.486 (AB-system with a fluorine-hydrogen coupling, JFH = 7.0 Hz; JFF = 207 Hz). After methylation of the product, the spectrum obtained with GC/MS was consistent with the expected structure (Fig. 1). MS (only ions containing ^{35}Cl are given): *m/z* 309 (M^+, 0.7%), 250 (M^+-CH_3CONH_2 and/or CH_3COO, 15%). The occurrence of ions at *m/z* 176 ($C_6H_{10}NO_3S^+$, 55%), 144 ($C_5H_6NO_2S^+$, 17%), and 88 ($C_3H_6NO_2^+$, 100%) indicate the characteristic fragmentation pattern of mercapturic acid methyl esters [20, 21]. Other *m/z* values of DCDFE-NAC: 208 (25%), 134 (29%), 117 (46%), 102 (14%), 59 (15%).

Phenyldiazomethane was synthesized as described by Karashima *et al.* [22]. Diazomethane was generated from *N*-methyl-*N*-nitroso-ureum [23].

Animals and treatment

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 different doses of DCDFE or DCDFE-NAC. DCDFE dissolved in arachidis oil was administered i.p. in a volume of 2.5 ml/kg. Doses of DCDFE were 75, 150, 375, 600 and 750 μmole/kg. DCDFE-NAC dissolved in a DMSO-saline mixture (with a minimum amount of DMSO) was administered i.p. in a volume of 2.5 ml/kg. Doses of DCDFE-NAC were 100, 210, 325 and 450 μmole/kg. Aminooxyacetic acid (AOA) dissolved in 0.2 M potassium phosphate buffer pH 6.5 was administered i.p. at a dose of 200 μmole/kg, in a volume of 2.5 ml/kg, 1 hr prior to the administration of 150 or 600 μmole/kg DCDFE. Control animals were given arachidis oil, a mixture of DMSO and saline, or 200 μmole/kg AOA followed by arachidis oil.

After treatment the rats were individually housed in all glass metabolism cages, designed for separate collection of urine and faeces. Urine was collected for 24 hr in cooled (0°) vessels.

During the experiment the rats were only provided with water *ad libitum*. Twenty-four hours after treatment, blood was collected by heart puncture. Blood was centrifuged subsequently (4000 *g*, 10 min) to obtain plasma. The animals were decapitated and the kidneys were removed for measurement of kidney to body weight ratio. For histopathological examination, kidneys and livers were fixed in 10% neutral buffered formalin. After fixation the kidneys and livers were embedded in paraffin, sectioned (5–7 μm) and stained with hemotoxylin and eosin.

Biochemical assessment of toxicity

Plasma urea, plasma and urinary glucose, plasma and urinary alkaline phosphatase (ALP), plasma alanine aminotransferase (ALT), urinary γ-glutamyl transferase (γ-GT), and plasma aspartate aminotransferase (AST) were determined using the respective J. T. Baker Kits. Plasma and urinary creatinine, and urinary protein were determined using Sigma Kits. Urinary *N*-acetyl-β-D-glucosaminidase (NAG) was determined according to the method of Von Knoll *et al.* [24]. Statistical significance of differences in plasma and urinary parameters was calculated using the two-tailed Student *t*-test.

Identification of urinary metabolites by GC/MS

DCDFE-NAC. Two-millilitre samples of urine of rats treated with DCDFE were acidified to pH 2 with HCl and subsequently extracted three times with 5 ml portions of ethylacetate. The combined ethylacetate fractions were dried over $MgSO_4$, and then evaporated under reduced pressure. The residue was dissolved in 0.5 ml methanol, and methylated by treating it with a freshly prepared diazomethane solution in diethylether. After evaporation of the diethylether and excess diazomethane by a mild air

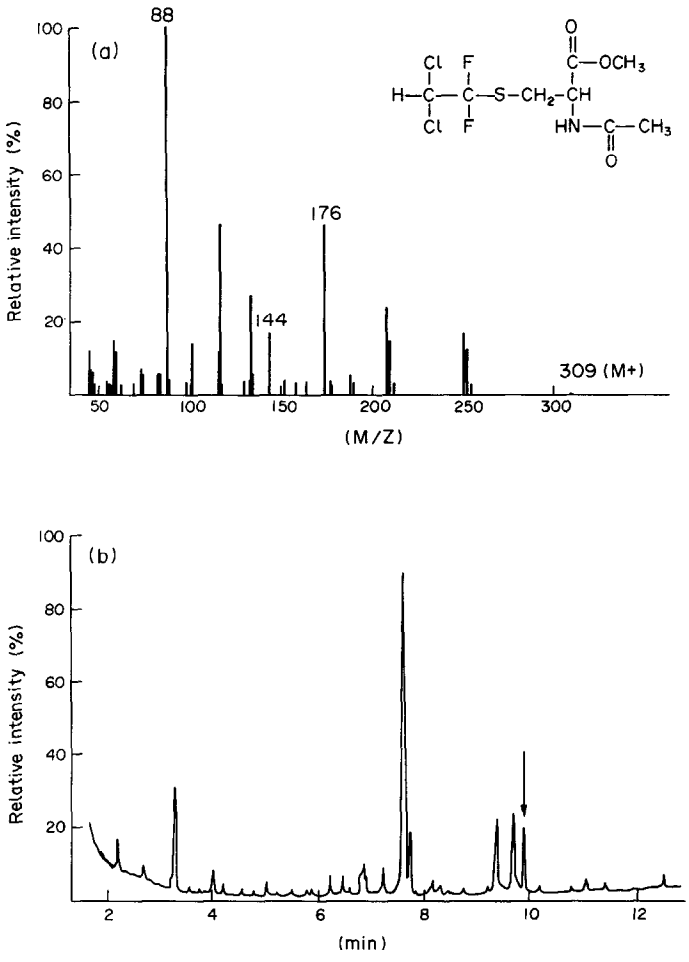

Fig. 1. Electron-impact mass spectrum of the methyl ester of authentic DCDFE–NAC (a). and total ion current chromatogram of a 24 hr urine extract of a rat treated i.p. with 600 μmole/kg of DCDFE, after methylation of the extract with diazomethane (b). The arrow indicates the peak corresponding to DCDFE–NAC.

stream, the remaining solution was examined with GC/MS.

Chlorodifluoroacetic acid. The presence of chlorodifluoroacetic acid (CDFAA) in urine was investigated by a procedure similar to a procedure for the detection of trifluoroacetic acid (TFA) in urine [22]. This procedure involves esterification of the acetic acid by phenyldiazomethane (PDM), providing the benzylester of CDFAA. For this purpose portions of 2 ml of urine of rats treated with 750 μmol/kg DCDFE were acidified to pH 0.5 with concentrated HCl, and subsequently extracted 3 times with 5 ml portions of diethylether. The diethylether phases were combined, treated with PDM at room temperature, concentrated by evaporation under reduced pressure, and examined with GC/MS.

Gas chromatography (GC) and mass spectrometry (MS)

GC analyses were made on a HP 5890 Chromatograph. The gas chromatograph was equipped with a splitless injector and nitrogen phosphorous detector. A SE-54 capillary column was prepared according to Xu and Vermeulen [29]. The operation temperatures of gas chromatography were 200° (injector) and 220° (detector). The oven temperature was programmed using a multiramp program. First ramp: 120° (2 min) to 160° at 60°/min. Second ramp: 160° (0.2 min) to 210° at 10°/min.

GC/MS analyses were made on a HP 5890/MSD system. A CP-Sil-19 capillary column obtained from Chrompack B.V. Middelburg was used. The operation temperatures were 280° (split injector), 280° (ion source, electron impact ionization, electron energy of 70 eV). In the case of the benzyl ester of CDFAA the oven temperature was programmed from 80° (2.5 min) to 280° at 20°/min. In case of methylated DCDFE-NAC, the oven temperature was programmed from 50° (2 min) to 280° at 20°/min.

Quantification of urinary DCDFE-NAC by GC

Urine samples (0.5 ml) of animals treated with different doses of DCDFE were adjusted to pH 1–2 with concentrated HCl. To quantitate the concentration of DCDFE-NAC, 0.1 mg of *N*-acetyl-*S*-

benzyl-L-cysteine was added as an internal standard. The acidified urine samples were extracted with 1 ml of methylethylketone by vortex-mixing during 20 sec. Then, 0.1 ml portions of the methylethylketone layer were separated and treated for 1 hr with an ethereal solution of diazomethane to methylate the mercapturic acids. After evaporation of the diethylether, 0.15 ml decane and 0.25 ml of methylethylketone were added to the solution. Finally, 1 μl of the ultimate solution was injected to the gas chromatograph.

Identification of urinary metabolites by ^{19}F-NMR

^{19}F-NMR spectra of untreated urine of rats dosed with 750 μmole/kg DCDFE and of control animals were recorded on a Bruker MSL 400 system (376.43 MHz, 10 mm probe). The spectra were referenced with trifluoroacetic acid.

RESULTS

Nephro- and hepatotoxicity in rats treated with DCDFE

The data on effects in the rat of i.p. administration of different doses of DCDFE on various plasma and urinary parameters are summarized in Table 1. The first responses indicative for nephrotoxicity due to DCDFE appeared already at the lowest dose of DCDFE (75 μmole/kg). At this dose an increase in kidney to body weight ratio, associated with an increased kidney to water content, of 13% was observed. The relatively small increase in urinary glucose level (570%) observed at this dose already indicates a slight damage to the proximal tubules of the kidney. Other biochemical parameters indicative for damage to the proximal tubules, i.e. urinary γ-GT, urinary ALP, and urinary NAG, were still unaltered. The elevation of the urinary protein, a less regioselective indicator for nephrotoxicity, was relatively small at the dose of 75 μmole/kg, namely 150%. No significant changes in plasma urea and plasma creatinine became apparent at this dose, suggesting that there was no significant damage of the glomerulus.

At a dose of 150 μmole/kg of DCDFE, the averaged urinary glucose and protein levels were much more elevated, namely 7100% and 1200% respectively. At this dose, however, due to high standard deviations these elevations were not statistically significant. The strongly elevated plasma urea and plasma creatinine levels indicate an impaired glomerular function at this dose. This might account for the reduced urine volume which was observed.

At the 375 μmole/kg dose, besides a very strong elevation of urinary protein and glucose contents, a statistically significant elevation of urinary enzyme activities was also observed: NAG, a 3-fold increase; ALP, a 29-fold increase; and γ-GT, a 19-fold increase. These observations clearly indicate severe damage to the proximal tubule region of the kidneys. At 600 and 750 μmole/kg the degree of nephrotoxicity, according to the changes in biochemical parameters, was even higher.

At the dose of 375 μmole/kg also a small, but statistically significant increase in plasma ALP and ALT was observed, namely of 49% and 110%

Table 1. Dose dependence of nephro- and hepatotoxicity of 1,1-dichloro-2,2-difluoroethylene (DCDFE) in the rat

Biochemical parameter	Dose of DCDFE (μmole/kg)					
	0	75	150	375	600	750
Plasma urea (mg%)	18 ± 6	24 ± 4	98 ± 10**	142 ± 25**	214 ± 15**	167 ± 44**
Plasma creatinine (mg%)	0.43 ± 0.15	0.44 ± 0.13	1.93 ± 1.26	2.21 ± 0.78**	3.91 ± 1.54*	4.43 ± 1.15**
Plasma ALP (U/l)	435 ± 47	452 ± 87	575 ± 51**	649 ± 95**	1268 ± 276**	1971 ± 334**
Plasma ALT (U/l)	12 ± 2	14 ± 3	12 ± 2	26 ± 6**	1311 ± 278**	2522 ± 1648*
Plasma AST (U/l)	23 ± 6	25 ± 4	18 ± 4	22 ± 3	624 ± 252**	1448 ± 1000*
Urine volume (ml/24 hr)	46 ± 15	25 ± 2	22 ± 16	30 ± 14	23 ± 4*	18 ± 4*
Urine glucose (mg/24 hr)	3.1 ± 0.4	21 ± 7**	223 ± 55**	233 ± 38**	179 ± 21**	135 ± 49**
Urine protein (mg/24 hr)	3.9 ± 1.8	9.6 ± 3.9*	51 ± 14**	62 ± 44*	55 ± 12**	43 ± 8**
Urine ALP (U/24 hr)	1.8 ± 1.4	1.0 ± 0.4	13 ± 19	53 ± 29*	49 ± 16**	43 ± 8**
Urine GGT (U/24 hr)	5.3 ± 3.4	4.1 ± 1.0	50 ± 44	99 ± 16**	91 ± 12**	76 ± 25**
Urine NAG (U/24 hr)	1.1 ± 0.3	1.1 ± 0.3	2.1 ± 0.9	3.6 ± 0.2**	7.0 ± 2.6**	8.3 ± 4.3*
Kidney: body wt (×100)	0.81 ± 0.06	0.92 ± 0.05*	0.99 ± 0.13*	0.93 ± 0.03*	1.00 ± 0.08*	1.02 ± 0.08**

Values are means ± SD of groups of four rats. Biochemical parameters were measured 24 hr after treatment with DCDFE.
*, ** Denotes statistically significant differences from control rats (* $P < 0.05$, ** $P < 0.01$).

respectively. At the higher doses, the elevation of these two enzymes was even much stronger. At these doses, a strong increase in plasma AST was also observed. Although elevation of plasma ALP, ALT and AST is not specifically indicative for liver damage, histopathological examination of the livers also showed only a more or less severe degree of liver damage at doses of DCDFE equal to or higher than 375 μmole/kg.

Urinary excretion of an N-acetylcysteine conjugate in rats treated with DCDFE

Ethylacetate extracts of acidified urine of rats treated with 600 μmole/kg of DCDFE were treated with diazomethane and examined with GC/MS. In the total ion current chromatogram, a small peak with a retention time and a mass spectrum identical to those of authentic methyl ester of DCDFE-NAC could be identified (Fig. 1). Quantitative determination of this N-acetylcysteine conjugate, performed with gas chromatography with nitrogen phosphorous detector, resulted in the 24 hr cumulative urinary excretion data as shown in Fig. 2. At the doses of 75 and 150 μmole/kg of DCDFE 45–60% of the administered compound was excreted as its mercapturic acid. However, at the higher doses the cumulative excretion of DCDFE-NAC was found to be only 10–30% of the administered dose.

Urinary excretion of chlorodifluoroacetic acid in rats treated with DCDFE

Diethylether extracts of acidified urine of rats treated with 750 μmole/kg of DCDFE were benzylated with phenyldiazomethane and examined by GC/MS. The mass spectrum of synthetic benzylester of chlorodifluoroacetic acid (CDFAA) is shown in Fig. 3. In the extracts of urine benzylated CDFAA was only measurable with single ion monitoring (SIM), because of its very low concentration. The SIM chromatograms of the masses at *m/z* 220 and 222, are shown in Fig. 3. The retention time on GC as well as the characteristic ratio of the masses at *m/z* 220 and 222 were identical to those of the synthetic benzylester of CDFAA.

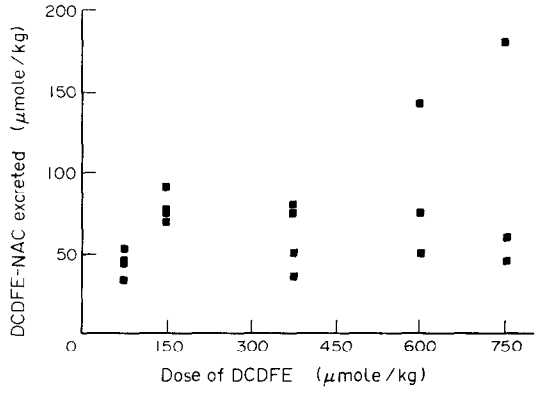

Fig. 2. Cumulative excretion of DCDFE–NAC in urine of rats treated i.p. with different doses of DCDFE. Urine was collected during the first 24 hr after DCDFE treatment. Points represent excretion data of the individual animals.

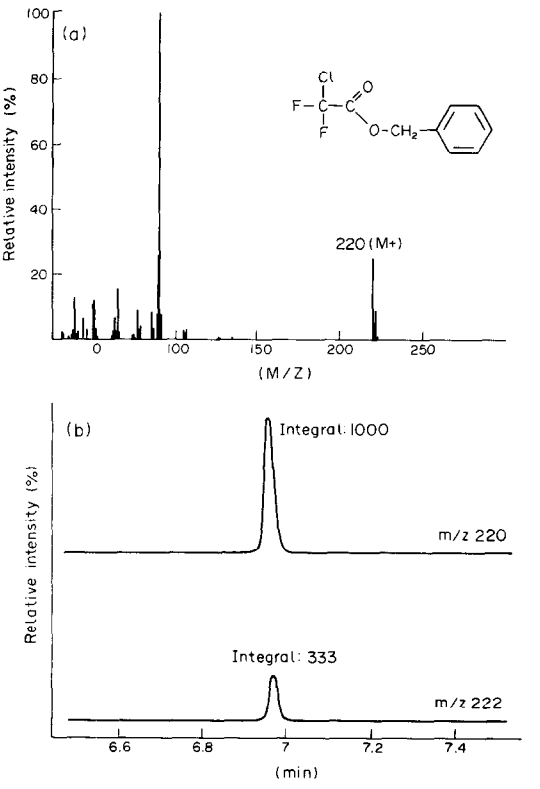

Fig. 3. Electron-impact mass spectrum of authentic chlorodifluoroacetic acid benzyl ester (a), and SIM-chromatograms of masses 220 and 222 of a 24 hr urine extract of a rat treated with 750 μmole/kg of DCDFE, after benzylation of the extract with phenyldiazomethane.

^{19}F-NMR of urine of rats treated with DCDFE

^{19}F-NMR-examination of untreated urine of rats dosed with 750 μmole/kg of DCDFE showed the presence of two types of signal, at $\delta -5.5$ ppm (AB-system) and $\delta -43.7$ ppm (singlet), respectively (Fig. 4a). The chemical shift of the first peak was identical to those of synthetical DCDFE-NAC (Fig. 4b). Because of the line broadening the small FH-coupling constant (7 Hz) is not visible in the urine sample. The chemical shift of the second signal was identical to that of the fluorine-anion [30]. Due to the extremely low concentration of CDFAA no signal of this compound ($\delta +12.5$ ppm, relative to the internal standard trifluoroacetic acid) could be detected. The ^{19}F-NMR spectrum of urine of untreated rats only indicated the presence of a small amount of F^-.

Nephro- and hepatotoxicity of the N-acetylcysteine conjugate of DCDFE

The effects of treatment of rats with the synthetical *N*-acetylcysteine conjugate of DCDFE (DCDFE–NAC) on the various biochemical parameters reflecting liver and kidney function are summarized in Table 2. DCDFE–NAC was a potent nephrotoxic agent at a dose of 100 μmole/kg, as indicated by the elevations of kidney to bodyweight ratio (19%), plasma urea (400%), plasma creatinine (245%), uri-

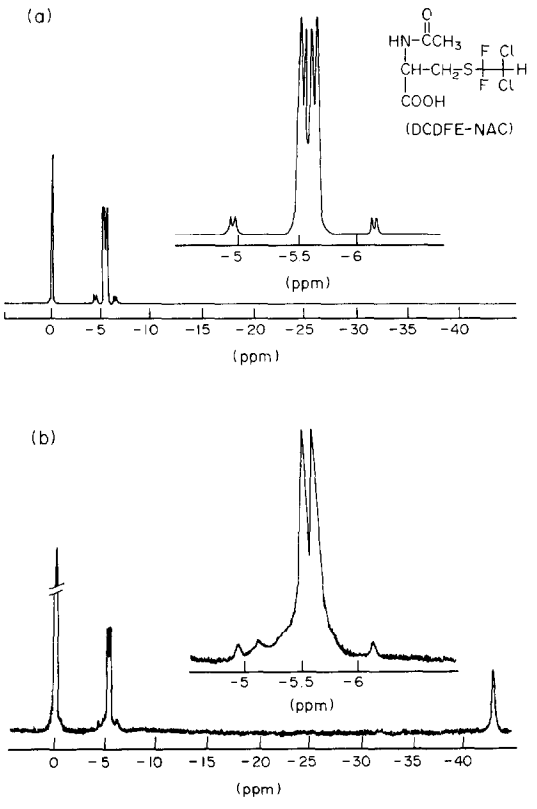

Fig. 4. ^{19}F-NMR spectra of authentic DCDFE–NAC (10 mM in potassium phosphate buffer, pH 7.0) (a), and of an untreated 24 hour urine sample of a rat dosed i.p. with 750 μmole/kg DCDFE (b).

nary protein (480%), urinary glucose (6900%) and urinary ALP (1670%). Upon higher doses of DCDFE-NAC the nephrotoxicity strongly increased. At the highest doses the urine production was almost blocked completely. Histopathological examination of the kidney showed an extensive necrotic band in the region of the inner cortex. At the doses of 325 and 450 μmole/kg white spots were observed on the outside of the kidneys. This indicates a greater extent of nephrotoxicity when compared to the highest dose of DCDFE. However, even at the highest doses of DCDFE–NAC no histopathological evidence for liver damage was observable. The relatively small increases in plasma ALT and plasma ALP, as compared with the increases seen with the highest dose of DCDFE, therefore, might result from the extremely extensive kidney damage produced by DCDFE–NAC.

Influence of aminooxyacetic acid on the nephro- and hepatotoxicity of DCDFE

The influence of pretreatment of rats with aminooxyacetic acid (AOA), as well known β-lyase inhibitor, 1 hr prior to administration of hepato- and/or nephrotoxic doses of DCDFE, is shown in Table 3. In animals only receiving a dose of 200 μmole/kg AOA, an increase in plasma urea (160%), in urinary glucose (3900%) and in urinary protein (165%) was observed when compared to untreated control animals only receiving arachidis oil. Despite the fact that this indicates a nephrotoxic potential of AOA itself, AOA-pretreatment nevertheless reduced the increase in plasma urea and urinary protein after a dose of 150 μmole/kg of DCDFE completely. Due to the high standard deviations, no statistically significant effect on urinary glucose could be seen. At 600 μmole/kg DCDFE, pretreatment with 200 μmole/kg of AOA did not reduce nephrotoxicity. AOA-Pretreatment did not protect at all against the hepatotoxicity of DCDFE at 600 μmole/kg, since the elevation of plasma ALP and ALT at this dose was not reduced.

DISCUSSION

The aim of the present study was to investigate the nephro- and hepatotoxic potential of 1,1-dichloro-2,2-difluoroethylene (DCDFE) in the rat, as well as part of its metabolism possibly relevant to the interpretation of the results. On the one hand, DCDFE has several industrial applications and has been shown to be more toxic than chlorotrifluoroethylene and tetrafluoroethylene [16]. On the other hand, DCDFE is structurally related to the specific nephrotoxin tetrafluoroethylene as well as to

Table 2. Dose dependence of nephro- and hepatotoxicity of the *N*-acetylcysteine conjugate of 1,1-dichloro-2,2-difluoroethylene (DCDFE–NAC) in the rat

Biochemical parameter	Dose of DCDFE–NAC (μmole/kg)				
	0	100	210	325	450
Plasma urea (mg%)	21 ± 7	105 ± 37**	139 ± 2**	157 ± 6**	196 ± 18**
Plasma creatinine (mg%)	0.8 ± 0.1	2.8 ± 0.7**	4.2 ± 2.5**	5.6 ± 2.0**	4.9 ± 0.3**
Plasma ALT (U/l)	14 ± 2	17 ± 3	8 ± 2	10 ± 3	320 ± 195**
Plasma ALP (U/l)	158 ± 21	177 ± 51	N.D.	N.D	208 ± 19**
Urine volume (ml/24 hr)	30 ± 5	17 ± 2**	4 ± 3**	1.1 ± 0.8**	5.0 ± 4.5**
Urine glucose (mg/24 hr)	3.6 ± 0.1	251 ± 41**	N.D.	N.D.	N.D.
Urine protein (mg/24 hr)	11 ± 3	62 ± 7	N.D.	N.D.	N.D.
Kidney:body wt (×100)	0.81 ± 0.06	0.96 ± 0.05**	1.10 ± 0.11**	1.17 ± 0.15**	1.08 ± 0.09**

Values are means ± SD of groups of four rats (N.D., not determined). Biochemical parameters were measured 24 hr after treatment with DCDFE–NAC. Animals were treated with DCDFE–NAC dissolved in DMSO/water (1/4); control animals received DMSO/water only.

*, ** Denotes statistically significant differences from control rats (*$P < 0.05$, ** $P < 0.01$).

Table 3. Influence of pretreatment of rats with aminooxyacetic acid (AOA) on nephro- and hepatotoxicity of 1,1-dichloro-2,2-difluoroethylene (DCDFE)

Biochemical parameter	Dose of DCDFE					
	0 μmole/kg		150 μmole/kg		600 μmole/kg	
	−AOA[a]	+AOA[b]	−AOA	+AOA[b]	−AOA	+AOA[b]
Plasma urea (mg%)	18 ± 6	46 ± 21†	98 ± 10**	49 ± 18††	214 ± 15**	232 ± 94%
Plasma ALT (U/l)	12 ± 2	18 ± 8	12 ± 2	12 ± 4	1311 ± 278**	546 ± 289*†
Plasma ALP (U/l)	435 ± 47	436 ± 153	575 ± 51**	506 ± 101	1268 ± 276**	1055 ± 109*
Urine glucose (mg/24hr)	3.1 ± 0.4	123 ± 101††	223 ± 55**	156 ± 162	179 ± 21**	121 ± 79
Urine protein (mg/24 hr)	3.9 ± 1.8	10.3 ± 5.6	51 ± 14**	14 ± 10††	55 ± 12**	35 ± 22
Kidney:body wt (×100)	0.81 ± 0.06	0.88 ± 0.02	0.99 ± 0.13*	0.92 ± 0.04*	1.00 ± 0.08*	1.06 ± 0.08

Values are means ± SD of groups of four rats. Biochemical parameters were measured 24 hr after treatment with DCDFE.

a. Control rats were treated i.p. with 2.5 ml/kg of arachidis oil.

b. Rats were pretreated i.p. with 200 μmole/kg of AOA dissolved in 0.2 M potassium phosphate buffer, one hour prior to the administration of the various doses of DCDFE.

*, ** Denotes significant differences from control animals with same pretreatment (* $P < 0.05$, ** $P < 0.01$).

†, †† Denotes significant differences from rats not pretreated with AOA († $P < 0.05$. †† $P < 0.01$).

the hepatotoxin tetrachloroethylene. The hepatotoxicity of tetrachloroethylene is believed to be the result of bioactivation to reactive intermediates by the hepatic microsomal mixed-function oxidase system [2–4]. The nephrotoxicity of tetrafluoroethylene, on the contrary, is believed to be the result of initial conjugation to glutathione (GSH) by hepatic, mainly microsomal, glutathione-*S*-transferases in the liver, followed by hydrolysis of the GSH-conjugate to the corresponding cysteine conjugate. The cysteine conjugate is then converted to an unknown sulfur-containing reactive intermediate in the kidney, presumably by cysteine conjugate β-lyase [11].

The present study was set up to elucidate the balance between nephrotoxicity and hepatotoxicity caused by DCDFE by investigating the effects of treatment of rats with different doses of DCDFE on various biochemical parameters reflecting liver and kidney function, as well as by histopathological examination.

The results, as summarized in Table 1, showed that DCDFE is already nephrotoxic at doses as low as 75 μmole/kg. The elevation of urinary glucose at the dose of 75 μmole/kg, without affecting plasma urea and creatinine levels, indicates that the primary site of nephrotoxicity is the proximal tubule area of the kidney [19]. At this site of the nephron, glucose is normally actively reabsorbed. The localisation of the DCDFE-induced nephrotoxicity was confirmed by histopathological examination of the kidneys at higher doses, showing an intense necrotic band in the inner cortex region. The elevation of plasma ALT, AST and ALP activities at doses equal or higher than 375 μmole/kg showed that DCDFE is also hepatotoxic at higher doses.

Metabolism of DCDFE in rats via glutathione conjugation was deduced from the presence of the *N*-acetylcysteine conjugate of DCDFE in urine of DCDFE-treated rats. This conjugate was identified with both GC/MS [20, 21] and ^{19}F-NMR. A molecular ion, as well as various peaks characteristic for mercapturic acid methyl esters [20] in the electron impact mass spectrum, a retention time in the gas chromatogram of authentic DCDFE–NAC methyl ester, as well as a ^{19}F-NMR spectrum identical to that of authentic DCDFE–NAC confirmed the identity of DCDFE-NAC present in rat urine. At doses of 75 and 150 μmole/kg of DCDFE 45–60% of the administered dose was excreted as *N*-acetylcysteine conjugate within 24 hr, as shown in Fig. 2. This indicates a relatively efficient conjugation to glutathione *in vivo*. Because of the high volatility of DCDFE (boiling point 19%) significant exhalation of the parent compound via the lungs has to be taken into account. At the higher doses of DCDFE the cumulative urinary excretion of the *N*-acetylcysteine conjugate within 24 hr was reduced markedly. A reduced glomerular filtration rate due to kidney damage might be an explanation of this phenomenon.

The presence of traces of chlorodifluoroacetic acid (CDFAA) in urine of rats treated with high doses of DCDFE suggests the existence of a minor oxidative pathway in the biotransformation of DCDFE (Fig. 5). The fact that at lower doses of DCDFE 60% of the administered dose is excreted as DCDFE–NAC suggests that conjugation of DCDFE to GSH in the

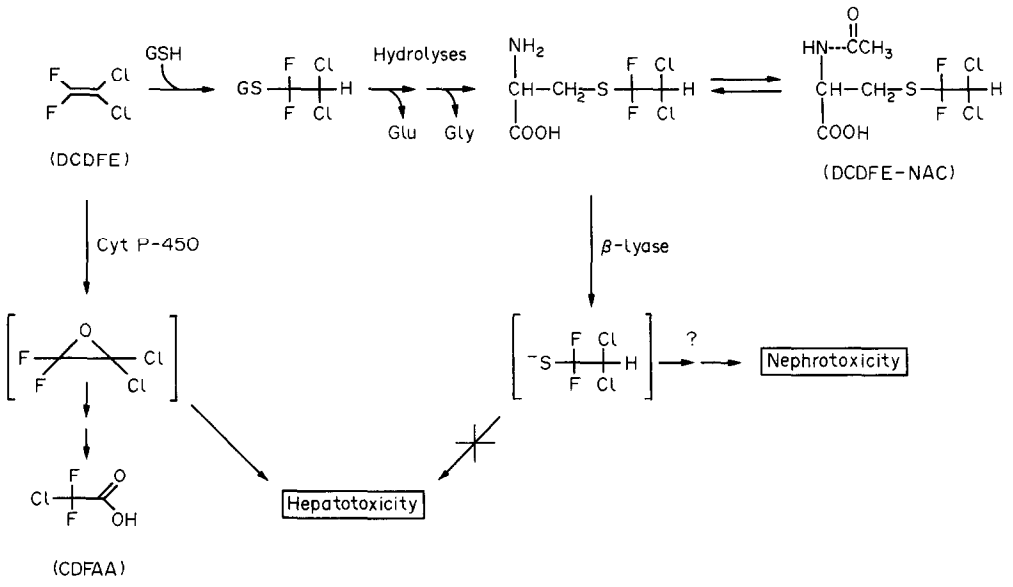

Fig. 5. Proposed routes of bioactivation of DCDFE leading to nephrotoxicity and hepatotoxicity.

liver is a much more important biotransformation route than epoxidation by mixed-function oxidases. In confirmity with this suggestion, ^{19}F-NMR in urine samples showed only signals derived from DCDFE–NAC and F^-. The presence of traces of chlorodifluoroacetic acid (CDFAA) were only detectable with single ion monitoring with GC/MS, a technique which for this compound is much more sensitive than ^{19}F-NMR. The ratio of DCDFE–NAC to CDFAA in urine, however, is not necessarily a proper reflection of the ratio of glutathione conjugation to oxygenation in the liver because further metabolism of the intermediary metabolic products involved in the respective metabolic pathways may occur before excretion in urine. At the highest doses of DCDFE, due to the glutathione conjugation, the hepatic glutathione content might be reduced markedly. As a result more DCDFE would become available for the oxidative pathway. In addition, because of the glutathione depletion, protection against the reactive epoxide would be diminished, thus resulting in more covalent binding to bio-macromolecules. In analogy with tetrachloroethylene [1], therefore, the hepatotoxicity of DCDFE might be the result of primary oxygenation to a reactive epoxide in the liver [2–4] (Fig. 5).

Recently, the *N*-acetylcysteine conjugate of hexachlorobutadiene (HCBD) was shown to cause lesions in the kidney identical to those described for HCBD itself [19]. This was explained by deacetylation of the *N*-acetylcysteine conjugate to the free cysteine conjugate, which then would become substrate for the renal β-lyase(s), thus leading to as yet unknown sulfur containing reactive intermediates. In the present study, DCDFE–NAC also caused kidney damage similar to that induced by the parent DCDFE. This, combined with the identification of DCDFE–NAC as a major urinary metabolite of DCDFE, strongly suggests that most probably conjugation of DCDFE to glutathione is indeed involved in the bioactivation of DCDFE to (a) nephrotoxic species. It is important to note that even at the highest dose of DCDFE–NAC, at which the extent of kidney damage was significantly greater than at the highest dose of DCDFE, still no signs of liver damage were observed. This leads to the suggestion that the hepatotoxicity of DCDFE is most probably the result of a mechanism different from that of the kidney damage.

To elucidate whether both the strong nephrotoxicity and the weak hepatotoxicity of DCDFE were the result of a bioactivation pathway involving β-lyase(s) [11, 25], rats were pretreated with 200 μmole/kg aminooxyacetic acid (AOA), one hour prior to administration of hepato- and/or nephrotoxic doses of DCDFE. The dose of AOA administered in this study was less than the 500 μmole/kg used by Elfarra *et al.* [26], because the latter dose was lethal to our Wistar rats. This might indicate a difference in susceptibility to the adverse effects of AOA between Wistar and Fischer 344 rats. The elevation of plasma urea and urinary glucose caused by AOA in our control animals (Table 3) demonstrates an intrinsic nephrotoxic potential of AOA. Nevertheless, AOA was able to reduce the increase in plasma urea and urinary protein caused by a dose of 150 μmole/kg of DCDFE (Table 3). This strongly suggests the involvement of β-lyase in the bioactivation of DCDFE to a nephrotoxin. This, as well as the strong and selective kidney toxicity of DCDFE–NAC observed, point at a similar bioactivation mechanism as that shown recently for tetrafluoroethylene and chlorotrifluoroethylene [11, 25]. However, in the present study AOA was not able to exert an inhibitory effect on the 600 μmole/kg DCDFE-induced nephrotoxicity. This might be due to inability of AOA to inhibit β-lyase-activity completely, or to the presence of β-lyase independent bioactivation mechanisms. Recently, a β-lyase independent conversion of *S*-(*p*-bromophenyl)-

L-cysteine to the corresponding thiol was proposed [27]. This pathway involves oxidative deamination of the cysteine conjugate, and subsequent cleavage of the thiopyruvic acid moiety.

In conclusion, it can be stated that 1,1-dichloro-2,2-difluoroethylene (DCDFE) causes nephrotoxicity at low doses, and, in addition, hepatotoxicity at much higher doses. The presence of up to 60% of the dose as *N*-acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine (DCDFE–NAC) in urine of rats dosed with DCDFE, and the severe kidney damage caused by synthetic DCDFE–NAC, without any observable effect on the liver, point to bioactivation of DCDFE to a nephrotoxin via primary glutathione conjugation. The presence of traces of chlorodifluoroacetic acid in urine of rats treated with the highest doses of DCDFE indicate that oxygenation of DCDFE by hepatic mixed-function oxidases, in principle, may also occur. This might explain the hepatotoxicity at higher doses. Therefore, the nephrotoxicity and hepatotoxicity caused by DCDFE are presumably the result of two different mechanisms of bioactivation (Fig. 5).

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