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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

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SUMMARY

The β-lyase-dependent bioactivation of *S*-conjugates of tetrafluoroethylene by subcellular fractions from rat liver and rat kidney was studied. Incubation of both hepatic and renal cytosol with *S*-(1,1,2,2-tetrafluoroethyl)-*l*-cysteine (TFE-Cys) resulted in the formation of previously unidentified difluorothionamides, indicating difluorothionoacyl fluoride as the main reactive intermediate derived from the β-lyase-dependent bioactivation of TFE-Cys. The presence of *N*-difluorothionoacetyl-*S*-(1,1,2,2-tetrafluoroethyl)-*l*-cysteine (TFE-PMS) and difluoroacetic acid in urine of rats treated with *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-*l*-cysteine (TFE-NAC) points to a similar mechanism of bioactivation *in vivo*. When TFE-NAC was incubated with 11,000 × *g* supernatants of rat kidney and liver in the absence of exogenous acetyl coenzyme A (acetyl-CoA), *N*-deacetylation and subsequent β-lyase-dependent activation to difluorothionoacyl fluoride could be observed. Both the *N*-deacetylation of TFE-NAC and the β-lyase-dependent activation of TFE-Cys were much faster in rat kidney then in rat liver. When TFE-Cys was incubated with 11,000 × *g* supernatants of rat kidney and rat liver, formation of TFE-NAC could only be observed in the presence of 2 mM exogenous acetyl-CoA; the initial rate of *N*-acetylation was 5-fold higher in renal then in hepatic fractions. Under these conditions, formation of TFE-PMS was very low. The low urinary excretion of unchanged TFE-NAC (3–5% of dose) upon administration of TFE-NAC points to a high *N*-deacetylation/*N*-acetylation ratio *in vivo*. Due to a very high turn-over of TFE-NAC/TFE-Cys, the availability of the cofactor for *N*-acetylation, acetyl-CoA, might be rate limiting in the kidney, resulting in accumulation of TFE-Cys followed by increasing β-lyase-dependent bioactivation of TFE-Cys to reactive intermediates.

GSH is an endogenous tripeptide that has several important physiological and toxicological roles. A toxicologically important role of GSH includes the bioinactivation of electrophilic xenobiotics or chemically reactive metabolites. However, as reviewed by Elfarra and Anders (1) and Igwe (2), some bioactivation mechanisms also involve glutathione-*S*-conjugate formation. Firstly, GSH conjugates of 1,2-dihaloalkanes have been shown to form episulfonium ions by a nonenzymatic intramolecular rearrangement, thus causing mutagenicity, carcinogenicity, and nephrotoxicity (3–5). A second GSH conjugation-dependent bioactivation mechanism involves degradative processing of GSH conjugates derived from halogenated ethylenes to the corresponding cysteine conjugates and, finally, cleavage of the cysteine conjugate by a renal β-lyase with the formation of chemically reactive metabolites in the proximal tubular area (1).

Cysteine conjugate β-lyases are pyridoxal phosphate-dependent enzymes that are present in mammals in the liver, kidney, and intestinal microflora. In liver and kidney, β-lyase activity is located in cytosol (6) and in the outer membrane and/or matrix of the mitochondria (7, 8). Both cytosolic and mitochondrial β-lyase cleave cysteine conjugates with certain chemical characteristics to ammonia, pyruvate, and corresponding thiol compounds. There are some indications that renal mitochondria might be the primary targets of toxic cysteine conjugates (9, 10). However, at present, the relative contribution of the cytosolic and mitochondrial enzymes to the nephrotoxicity is not known.

In the case of nephrotoxic cysteine conjugates, the thiol compound resulting from β-lyase-catalyzed cleavage or, more likely, chemically reactive electrophilic rearrangement products derived from it are believed to bind covalently to biomacro-

ABBREVIATIONS: β-lyase, cysteine conjugate β-lyase; TFE-Cys, *S*(1,1,2,2-tetrafluoroethyl)-*l*-cysteine; TFE, tetrafluoroethylene; TFE-NAC, *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-*l*-cysteine; DFA, difluoroacetic acid; DFTA, difluorothio(no)acetic acid: DFTAF, difluorothionoacyl fluoride; TFE-PMS, *N*-difluorothionoacetyl-*S*-(1,1,2,2-difluoroethyl)-*l*-cysteine; TFE-PMO, *N*-difluoroacetyl-*S*-(1,1,2,2-difluoroethyl)-*l*-cysteine; CTFE-Cys, *S*-(2-chloro-1,1,2-trifluoroethyl)-*l*-cysteine; acetyl coenzyme A; GC, gas chromatography; Ms, mass spectrometry; HPLC, high pressure liquid chromatography.

molecules and, as a result of that process, to cause renal toxicity. According to Green and Odum (11), it was first postulated that the reactive intermediate derived from TFE-Cys would be tetrafluoroethylmercaptane. Because of four electron-withdrawing fluorine subtitutents, this thiol was suggested to possess an electrophilic nature and, thus, it would be able to bind covalently to nucleophilic thiols and disulfides of proteins. Dohn *et al.* (12), however, also without experimental evidence, postulated that the thiol resulting from the β-lyase-mediated cleavage of CTFE-Cys would rearrange to a thionoacyl fluoride and/or to a thiirane. More recently, the formation of a thionoacyl fluoride from CTFE-Cys was demonstrated indirectly by Dekant *et al.* (13), by trapping this electrophile with diethylamine in an *in vitro* system. Covalent binding of 2-chloro-1,1,2-trifluoroethanethiol to benzyl bromide also pointed to a nucleophilic rather than an electrophilic character of the mercaptane. By demonstrating the excretion of significant amounts of DFA in urine of rats treated with TFE-NAC, we have shown that rearrangements of the thiol compound to a thionoacyl fluoride might also occur in the bioactivation of TFE-Cys *in vivo* (14).

Mercapturic acids of TFE, 1,1-dichloro-2,2-difluoroethylene, 1,1-dibromo-2,2-difluoroethylene, and hexachloro-1,3-butadiene have been shown to be specific nephrotoxins in the rat (14–16). As shown by Lock and Ishmael (17), the mercapturic acid derived from the nephrotoxin hexachloro-1,3-butadiene is accumulated in the renal cortex by a probenecid-inhibitable transport system. It was shown by Pratt *et al.* (18) that renal cytosol of the rat was also able to *N*-deacetylate this mercapturic acid and to activate the resulting cysteine conjugate to reactive intermediates that were not further identified. Vamvakas *et al.* (19) recently showed that the mercapturic acids of hexachloro-1,3-butadiene, trichloroethylene, and tetrachloroethylene could also be bioactivated to mutagenic species by rat renal and hepatic cytosol. At present, at least two cytosolic *N*-deacetylases are known to be involved in *N*-deacetylation of mercapturic acids (6, 20). Therefore, and because mercapturic acids show comparable toxicities (14, 19), mercapturic acids are suitable model compounds to investigate the mechanism of β-lyase-mediated nephrotoxicity of the chlorinated and fluorinated ethylenes both *in vivo* and *in vitro*. However, the cysteine conjugates once formed also can be *N*-acetylated again by microsomal *N*-acetyltransferases (21).

In the present study, we investigated the bioactivation mechanism of the *N*-acetyl-*l*-cysteine and cysteine *S*-conjugates of TFE by rat hepatic and renal subcellular fractions. In order to identify the reactive intermediate presumably responsible for the renal toxicity, we identified the nature of covalent adducts to various nucleophiles. The enzymes involved in metabolism of *N*-acetyl-*l*-cysteine and cysteine *S*-conjugates, notably cysteine conjugate β-lyase, cysteine conjugate *N*-acetyltransferase, and *N*-deacetylase, are present in both rat liver and rat kidney. We also investigated the organ dependency of the balance of bioactivating and bioinactivating enzymes by incubating the *S*-conjugates of TFE with 11,000 × *g* supernatants in the absence and presence of the various cofactors and inhibitors of the competing enzymes involved in biotransformation. These fractions contain both cytosolic (*N*-deacetylase and β-lyase) and microsomal (*N*-acetyltransferase) enzymes. Finally, to evaluate the relevance of the *in vitro* results for the *in vivo* situation, we also investigated the metabolism of TFE-NAC *in vivo*.

Experimental Procedures

Materials and synthesis. DFA, aniline, benzylamine, diethylamine, α-keto-γ-methiolbutyric acid, phosphorous pentasulfide, and pyridoxal hydrochloride were obtained from Aldrich (Brussels, Belgium). Acetyl coenzyme A was purchased from Sigma Chemical Company (St. Louis, MO). Difluoroacetic anhydride was prepared as described by Sawicki (22). TFE-NAC, TFE-Cys and *N*-acetyl-*S*-benzyl-*l*-cysteine were prepared as described previously (14).

N-Difluoroacetylaniline and *N*-difluoroacetylbenzylamine were prepared by derivization of aniline and benzylamine with difluoroacetic anhydride in toluene. *N*-Difluorothionoacetylaniline and *N*-difluorothionoacetylbenzylamine were prepared by treatment of *N*-difluoroacetylaniline and *N*-difluoroacetylbenzylamine with phosphorous pentasulfide in the presence of sodium bicarbonate in acetonitrile (23). *S*-Methyl esters of *N*-difluorothionoacetylaniline and *N*-difluorothionoacetylbenzylamine were prepared by treating the parent compound with diazomethane. The methyl ester of TFE-PMO was prepared by derivization of TFE-Cys with diazomethane and difluoroacetic anhydride, respectively.

^{19}F NMR and GC-MS data of the newly synthesized reference compounds are shown in Table 1.

Animals and treatment. Male Wistar rats (180–200 g of body weight) from Harlan S. D. (Zeist, The Netherlands) were used in the present tudy. They were housed in humidity (50%)- and temperature (22°)-controlled rooms with a 12-hr lighting cycle. Food and water were provided *ad libitum*. For *in vivo* metabolism studies, rats were injected intraperitoneally with 75 μmol/kg of body weight. TFE-NAC dissolved in arachidis oil. After treatment, rats were placed in all-glass metabolism cages designed for separate collection of urine and feces. To quantitate urinary metabolites, 0.2 mg of *N*-acetyl-*S*-benzyl-*l*-cysteine was added as an internal standard to urine collected for 24 hr. A 10-ml fraction of urine was acidified to pH 2 with concentrated hydrochloric acid and extracted three times with 2 volumes of ethyl acetate. The combined ethyl acetate fractions were evaporated *in vacuo* to 1ml and treated then with an excess of ethereal diazomethane.

Preparation of subcellular fractions from rat kidney and liver. Male Wistar rats were killed by decapitation, kidneys and livers were removed, and a 30%, w/v, homogenate was made in ice-cold 50 mM potassium phosphate buffer, pH 7.4, using a Potter homogenizer. The homogenates were then centrifuged at 11,000 × *g* for 20 min, at 4° and the supernatants [containing cytosol and microsomes (24)] were collected. Part of these supernatants were centrifuged at 100,000 × *g* for 1 hr to prepare cytosolic fractions (100,000 × *g* supernatant). Subcellular fractions were stored at −20° until use.

Incubation of TFE-Cys and TFE-NAC with subcellular fractions of rat liver and kidney. The overall metabolism by the crude enzyme fractions used (cytosol and 11,000 × *g* supernatant) depends on several factors: temperature, pH, enzyme concentrations, K_m and V_{max} values, substrate concentrations, availability of cofactors, and presence or absence of inhibitors. We limited the number of variables as much as possible. Therefore, temperature, buffer, enzyme concentration, and substrate concentration were kept constant.

The *S*-conjugates of TFE (4 mM) were incubated with cytosol or 11,000 × *g* fractions of both rat kidney and rat liver at 37°, in potassium phosphate buffer (50 mM, pH 7.4), in a total volume of 3 ml. The concentrations of enzymic fractions in incubations were 30 mg organ/ml, which resulted in the following protein concentrations: kidney cytosol, 3 mg/ml; kidney 11,000 × *g* fraction, 4 mg/ml; liver cytosol, 4 mg/ml; and liver 11,000 × *g* fraction, 5 mg/ml. In order to trap reactive intermediates with nucleophilic amines, cytosolic incubations were performed in the presence of 4 mM aniline, benzylamine, or diethylamine. The contribution of nonenzymic degradation of *S*-conjugates was investigated by incubation with heat-inactivated (4 min at 95°) subcellular fractions. To investigate the involvement of β-lyase, incubations were performed in the presence of 1 mM aminooxyacetic acid, a potent inhibitor of the pyridoxal phosphate-dependent enzyme β-lyase (1).

N-Acetylation of TFE-Cys (initial concentration, 4 mM) in incuba-

TABLE 1
Electron impact mass spectra and ^{19}F NMR data of the synthetic *N*-difluoroacetyl and *N*-difluorothioacetyl compounds

Compound	Electron impact mass spectrum	^{19}F NMR[a]
	m/z (intensity %)	*δ ppm (intensity, multiplicity)*
N-Difluoroacetylaniline	171 (61, $M^{+\cdot}$), 120 (60, $M^{+\cdot} - CF_2H$), 92 [60, $(M - COCF_2H)^+$], 77 (100), 65 (44), 51 (71)	−51.64 (2F, d), $^2J_{FH}$ = 54 Hz
N-Difluorothionoacetylaniline	187 (35, $M^{+\cdot}$), 186 [39, $(M - H)^+$], 136 [50, $(M - CF_2H)^+$] *S*-Methyl ester: 201 (40, $M^{+\cdot}$), 154 [54, $(M - SCH_3)^+$], 150 [21, $(M - CF_2H)^+$], 135 (15), 104 (31), 77 (100), 51 (62)	−39.69 (2F, d), $^2J_{FH}$ = 57 Hz
N-Difluoroacetylbenzylamine	185 (25, $M^{+\cdot}$), 134 [6, $(M - CF_2H)^+$], 106 (7), 91 (100), 77 (14), 65 (19), 51 (50)	−51.90 (2F, d), $^2J_{FH}$ = 54 Hz
N-Difluorothionoacetylbenzylamine	201 (20, $M^{+\cdot}$), 168 [6, $(M - HS)^+$], 123 (6), 117 (8), 104 (5), 91 (100), 92 (30), 77 (6), 65 (20), 51 (17) *S*-Methyl ester: 215 (2, $M^{+\cdot}$), 167 [15, $(M - SCH_3)^+$], 91 (100), 65 (20), 51 (13)	−41.62 (2F, d), $^2J_{FH}$ = 56 Hz
TFE-PMO	*O*-Methyl ester: 313 (1, $M^{+\cdot}$), 254 [15, $(M - COOCH_3)^+$), 234 [6, $(M - COCF_2H)^+$], 218 [34, $(M - H_2NCOCF_2H)^+$], 166 (21), 156 (10), 147 (13), 136 (23), 117 (100), 101 (15), 88 (48), 59 (45), 51 (77)	AB system (2F, d of t): δ_A, −15.78, δ_B, −16.28, $^2J_{FF}$ = 235 Hz $^3J_{FF}$ = 9 Hz, $^3J_{FH}$ = 3 Hz; −52.01 (2F, d), $^2J_{FH}$ = 54 Hz; −57.33 (2F, d of t), $^2J_{FH}$ = 53 Hz, $^3J_{FF}$ = 9 Hz

[a] 376 MHz; solvent, potassium phosphate buffer, pH 7.4; internal standard, trifluoroacetic acid (assigned 0 ppm).

tions with 11,000 × *g* supernatants was determined in the absence as well as in the presence of exogenous acetyl-CoA (2 mM).

To investigate the biotransformation of the fluorine-containing *S*-conjugates in a noninvasive way, incubations were performed in 10-mm NMR tubes at 37° and, 30 min after the start of incubation were measured by ^{19}F NMR, using a Bruker MSL 400 system operating at 376.43 MHz. Chemical shifts were referenced with trifluoroacetic acid as an internal standard.

For GC and GC-MS analysis of reaction products, at several time points reactions were stopped by addition of 0.5 ml of cold 2 N hydrochloric acid to 0.4-ml fractions of incubation mixtures. *N*-Acetyl-*S*-benzyl-*l*-cysteine (0.2 mg) was added as an internal standard and, subsequently, the incubation mixtures were extracted twice by 5 ml ethylacetate. The combined ethylacetate fractions were reduced to 0.5 ml by evaporation and treated with an excess of etherial diazomethane. For quantification of TFE-Cys, 1 ml of cold 2 N sodium hydroxide and 50 μl of acetic anhydride were added to 0.5 ml of the extracted water phase. The mixture was vortexed for 2 min. After 5 min at 0°, this procedure was repeated. After 5 min at 25°, the mixture was acidified to pH 1 with concentrated hydrochloric acid and 0.2 mg of *N*-acetyl-*S*-benzyl-*l*-cysteine was added as an internal standard. The mixture was then extracted twice with 5 ml of ethyl acetate, evaporated, and treated with ethereal diazomethane. The amount of TFE-NAC formed was also quantified with GC. Using this procedure, more than 95% of TFE-Cys could be measured as its mercapturic acid.

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 with an equal volume of 0.2% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid, incubated for 20 min at 37°, and then centrifuged. The supernatant was diluted with an equal volume of methanol and investigated by HPLC with UV detection at 365 nm. The eluent employed was 55% methanol/44% tetraethylammonium iodide (50 mM)/1% acetic acid, at a rate of 1 ml/min.

GC and MS. 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 Ned. B.V. (Middleburg) was used. The operation temperatures were 280° (split injector) and 280° (ion source). Electron impact ionization (electron energy of 70 eV) was used. The carrier gas was helium, at a flow of about 3 ml/min, and the column headpressure was 80 kPa. The column temperature was programmed from 80° (2.5 min) to 280° at 20°/min. Retention times of the analyzed compounds under these GC conditions were: *N*-difluoroacetylaniline, 6.63 min; *N*-difluorothionoacetylaniline, 7.50 min; *N*-difluorothionoacetylaniline *S*-methyl ester, 7.52 min; *N*-difluoroacetylbenzylamine, 7.38 min; *N*-difluorothionoacetylbenzylamine, 8.50 min; *N*-difluorothionoacetylbenzylamine *S*-methyl ester, 8.54 min; TFE-NAC methyl ester, 8.28 min; TFE-PMO methyl ester, 7.52 min; TFE-PMS *O,S*-dimethyl ether, 8.75 min; and *N*-acetyl-*S*-benzyl-*l*-cysteine methyl ester, 12.40 min.

Results

***In vitro* metabolism of TFE-Cys by cytosol of rat kidney and rat liver.** Incubation of TFE-Cys with cytosol of both rat kidney and rat liver resulted in the formation of several fluorine-containing metabolites. In the ^{19}F NMR spectra of both renal and hepatic incubation mixtures, after 30 min of incubation, new signals of an AB system centered at −16.12 ppm, of multiplets in the region −40.30 to −42.50 ppm, and of a singlet at −44.35 ppm could be observed, in addition to those of unchanged TFE-Cys (Fig. 1 and, in more detail, Fig. 2). Minor changes were observed in the coupling pattern of the signal at −57.30 ppm (not shown in detail). The singlet at −44.35 ppm was attributed to the fluoride anion. The AB system centered at −16.12 ppm was slightly but significantly different from that of TFE-Cys; metabolite: δ_A, −15.72 ppm, δ_B, −16,52 ppm, $^2J_{FF}$, 235; TFE-Cys: δ_A, −16.02 ppm, δ_B, −16.38 ppm; $^2J_{FF}$, 230 Hz (14). This strongly suggested that this product was a derivative of TFE-Cys. The AB system at −41.30 ppm (δ_A, −40.90 ppm, δ_B, −41.70 ppm, $^2J_{FF}$, 290 Hz, $^2J_{FH}$, 54 Hz) appeared to belong to a fluorine-containing electrophile covalently bound to the nucleophilic amino group of a free cysteine conjugate. DFTAF and DFTA[1] are two TFE-Cys derived reactive intermediates that were recently proposed to be capable of acetylating nucleophilic amino groups (14). Covalent binding of these reactive intermediates to the free amino group of TFE-Cys would result in the formation of mercapturic acid-like compounds, which we will here refer to as 'pseudomercap-

[1] Thio(no)acetic acid refers to the tautomeric equilibrium between a thionoacetic acid and its thioacetic acid. The latter form is the predominant form and has acetylating properties (25).

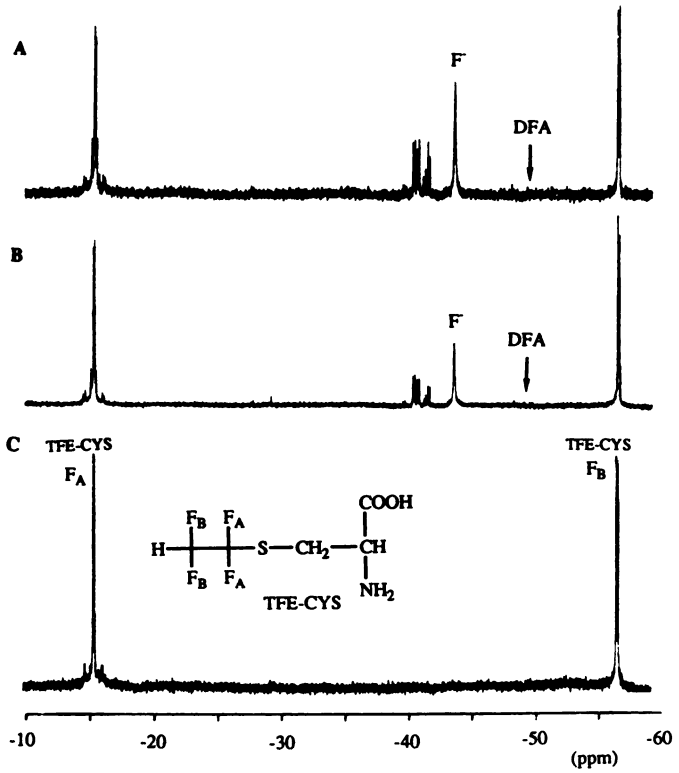

Fig. 1. ^{19}F NMR spectra of incubation mixtures containing TFE-Cys (initial concentration, 4 mM) and rat kidney cytosol (A), rat liver cytosol (B), or rat kidney cytosol in the presence of 1 mM aminooxyacetic acid (C), in potassium phosphate buffer, pH 7.4. Incubations were performed in a 10-mm NMR tube at a temperature of 37°. Concentrations of enzymic fractions were: kidney cytosol, 3 mg of protein/ml, and liver cytosol, 4 mg/ml. Thirty minutes after the start of the incubations, ^{19}F NMR spectra were recorded at 376.43 MHz, and chemical shifts are relative to trifluoroacetic acid as the internal standard. The chemical shift of authentic DFA is indicated by an *arrow*.

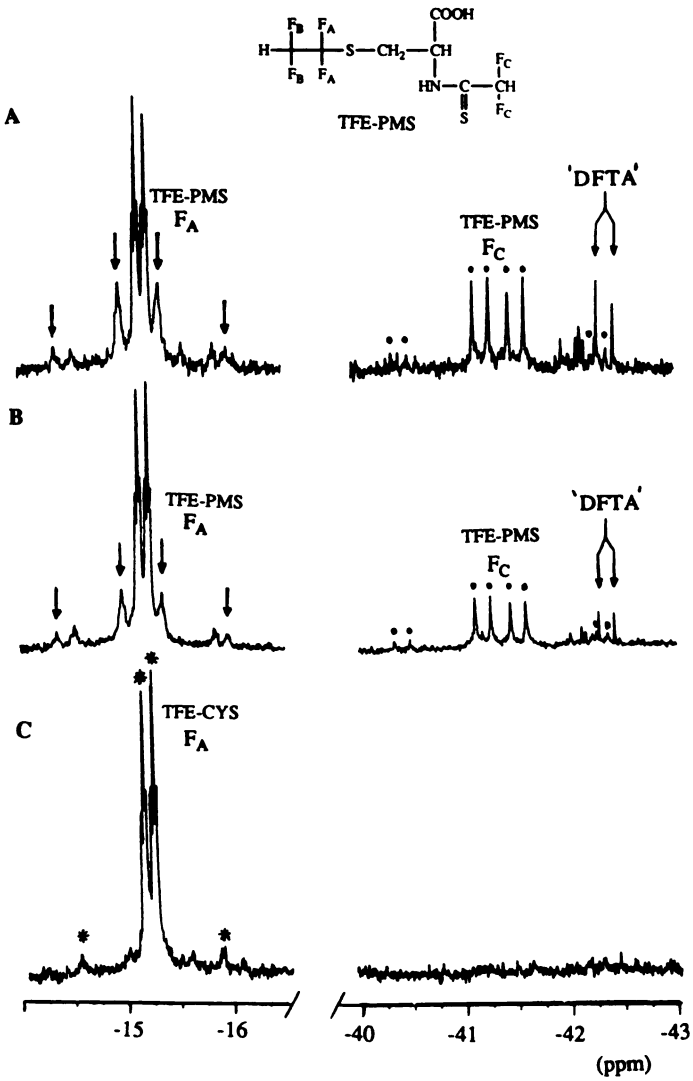

Fig. 2. Details of the ^{19}F NMR spectra of incubation mixtures containing TFE-Cys (initial concentration, 4 mM) and rat kidney cytosol (A), rat liver cytosol (B), or rat kidney cytosol in the presence of 1 mM aminooxyacetic acid (C), in potassium phosphate buffer, pH 7.4. Conditions are as described in the legend to Fig. 1. The AB systems attributed to the F_A fluorine atoms of TFE-PMS and TFE-Cys are indicated by the *arrows* and *asterices*, respectively. The AB system attributed to F_C of TFE-PMS is indicated by *points*. *'DFTA'*, presumably DFTA.

turic acids.' Conjugation of DFTAF to TFE-Cys would lead to the formation of a pseudomercapturic acid in which the sulfur atom of the reactive intermediate is retained, TFE-PMS.[2] Conjugation of DFTA to TFE-Cys, however, would result in the loss of hydrogen sulfide and formation of TFE-PMO.[2] These so-called pseudomercapturic acids were analyzed with GC/MS in a way similar to the analysis of mercapturic acids. After acidification, extraction with ethyl acetate, and methylation with diazomethane, GC-MS analysis of the extract showed the appearance of a major product with a retention time at 8.75 min (Fig. 3A) and with the mass spectrum shown in Fig. 4A. This mass spectrum was assigned to the *O,S*-dimethyl ester of TFE-PMS, *S*-methylation being the result of tautomerism of the difluorothionamide group to a difluorothiolimide group (26). Fragmentation of TFE-PMS: 343 (1%, M^+), 296 [11%, $(M\text{-}SCH_3)^+$], 284 [30%, $(M\text{-}COOCH_3)^+$], 242 [5%, $M-CF_2CF_2H)^+$], 219 [60%, $(M-N{=}C(SCH_3)(CF_2H))^+$], 196 [50%, $(M\text{-}CH_2SCF_2CF_2H)^+$]. To further characterize this novel metabolite, TFE-PMS present in the ethyl acetate extract of a large scale incubation was purified by preparative thin layer chromatography (silica gel; eluent, 80% *n*-propanol). TFE-PMS and its *O,S*-dimethyl ester were thus further characterized by ^{19}F NMR,^{1}H NMR, and exact mass measurements (Table 2). Dimethylation of TFE-PMS by diazomethane was confirmed by ^{1}H NMR. Due to the sensitivity to solvent changes, the ^{19}F NMR chemical shifts of TFE-PMS in chloroform are different from those in the incubation mixtures.

Upon quantification using ^{19}F NMR and GC-MS, the amount of TFE-PMS formed after 30 min in renal and hepatic incubations was determined to be 60–70% of the amount of pyruvic acid formed. Pyruvic acid represents the β-lyase-dependent metabolism of TFE-Cys. In addition to the pseudomercapturic acid TFE-PMS, in both renal and hepatic incubations small amounts of TFE-PMO could be identified, as its *O*-methyl ester, by GC-MS (Figs. 3A and 4B). Both TFE-PMS and its *O,S*-dimethyl ester appeared to be oxidized slowly to TFE-PMO and its methyl ester, respectively, in the presence of an oxygen-containing atmosphere. When incubations were per-

[2] The rationale for the abbreviations used here is: PMS, *p*seudo*m*ercapturic acid *s*ulfur-containing derivative; PMO: *p*seudo*m*ercapturic acid *o*xygen-containing derivative.

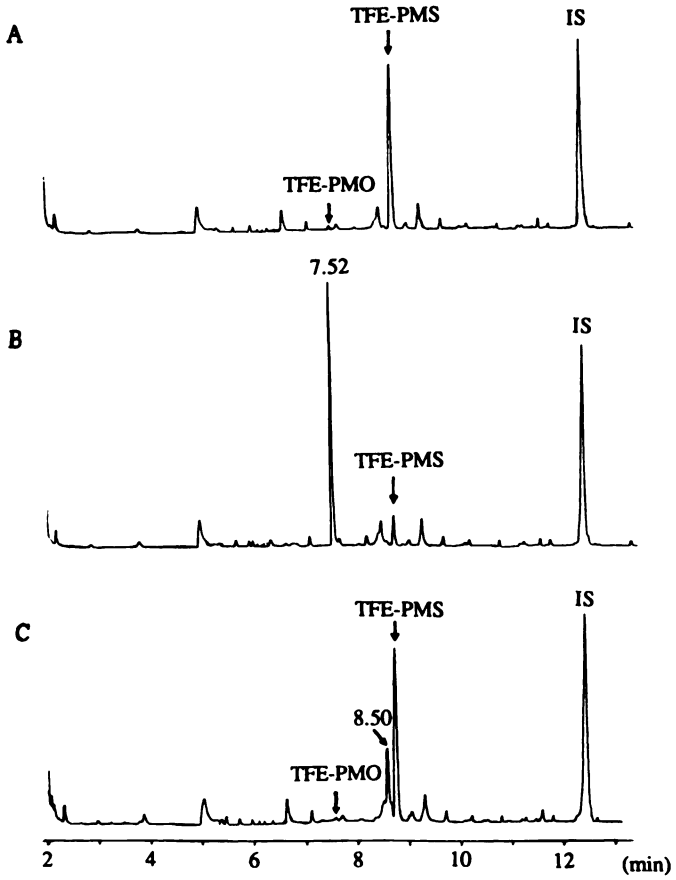

Fig. 3. Total ion current of GC-MS analyses of methylated ethyl acetate extracts after 60 min of incubation of kidney cytosol (3 mg/ml) with TFE-Cys (4 mM initial concentration) in the absence and presence of exogeneous nucleophilic amines. A, Incubation in the absence of nucleophilic amines; B, incubation in the presence of 4 mM aniline; C, incubation in the presence of 4 mM benzylamine. Products formed during incubation are indicated by their retention times. *IS*, internal standard.

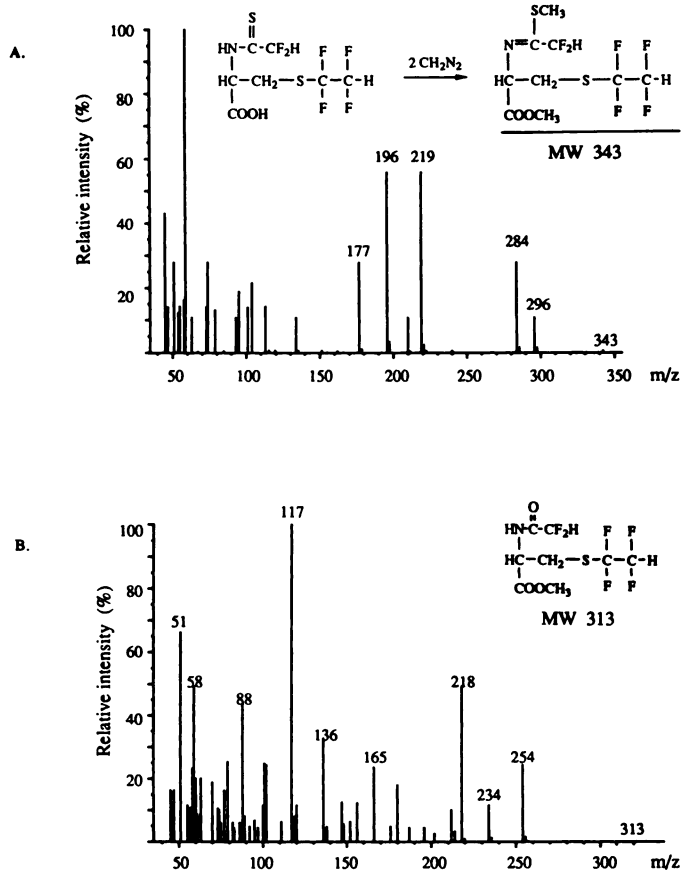

Fig. 4. Electron impact mass spectra of two metabolites formed during incubation of TFE-Cys with cytosol of rat kidney and rat liver. Incubation mixtures contained TFE-Cys (4 mM initial concentration) and cytosol (kidney, 3 mg/ml; liver, 4 mg of protein/ml), dissolved in 50 mM potassium phosphate buffer, pH 7.4. After 2 hr at 37°, the incubations were stopped by the addition of concentrated hydrochloric acid. Metabolites were extracted with ethyl acetate and subsequently methylated by diazomethane. GC-MS analyses of the extract were carried out on a HP 5890/MSD using electron impact ionization. The presumed structures of the metabolites are: TFE-PMS (A) and TFE-PMO (B).

formed under anaerobic conditions, only traces of TFE-PMO could be detected.

DFA has been shown to be an important urinary metabolite after administration of TFE-NAC to rats (14). With ^{19}F NMR, in cytosolic incubation mixtures with TFE-Cys, however, no DFA (chemical shift, −49.00 ppm; $^2J_{FH}$, 54 Hz) could be detected (Fig. 1). The metabolite with the ^{19}F NMR signal at −42.34 ppm and a coupling constant of 55 Hz, however, was converted very rapidly and completely into DFA upon treatment with acid or base. Because thio(no)acetic acids are known to be converted to the corresponding acetic acids upon acid and base treatment, the metabolite at −42.34 ppm most likely is DFTA. Because of its acid lability, this metabolite could not have been identified by the GC-MS procedure previously used successfully for detection for halogenated acetic acids (14, 15). Attempts to synthesize DFTA chemically by treating difluoroacetic anhydride with hydrogen sulfide in pyridine resulted in the formation of several products. The most abundant product, apart from DFA, had a chemical shift of −42.34 ppm and a coupling constant of 55 Hz (indicative of a $^2J_{FH}$ coupling of a CF_2H group), identical to those of the metabolite presumed to be DFTA. This compound, indeed, was converted to DFA upon acid or base treatment. We did not succeed, however, in isolating this product.

The remaining signals in the −42 ppm region (Fig. 2, A and B) are most probably the result of covalent binding of DFTAF to cytosolic amine-containing molecules, because the chemical shift is similar to that of the difluorothioamide group of TFE-PMS (Fig. 2). The identity of these adducts at present is still unknown, however.

When incubations were performed in the presence of 1 mM aminooxyacetic acid, the metabolism of TFE-Cys was inhibited completely (Fig. 1C), indicating the involvement of β-lyase in bioactivation of TFE-Cys. Heat treatment of renal and hepatic cytosol before incubation also resulted in a complete abolishment of metabolism of TFE-Cys. Nonenzymic degradation of TFE-Cys, therefore, does not play a role in these *in vitro* incubations.

Trapping of reactive intermediates generated by cytosolic bioactivation of TFE-Cys by nucleophilic amines. To further identify the nature of the reactive intermediate(s) formed from TFE-Cys, different nucleophilic amines were added to the incubation mixtures and the formation of covalent adducts was investigated by using ^{19}F NMR and GC-MS. When incubations of renal cytosol with TFE-Cys were performed in the presence of 4 mM aniline, a strong signal at −39.68 ppm (doublet, $^2J_{HF}$ = 56 ppm) could be observed with ^{19}F NMR,

TABLE 2

Characterization of TFE-PMS and its *O,S*-dimethyl ester by ^1H NMR, ^{19}F NMR, and exact mass analysis

Compound	^1H NMR[a]	^{19}F NMR[b]
	δ ppm (intensity, multiplicity)	
TFE-PMS Exact mass: Observed, 314.9825 Calculated, 314.9823	AB system (2H, d): δ_A, 3.54 ppm, δ_B, 3.84 ppm, $^2J_{HH}$ = 15 Hz, $^3J_{HH}$ = 4 Hz; 5.44 ppm (1H, d of t): $^3J_{HH}$ = 6 Hz, $^3J_{HH}$ = 4 Hz; 5.80 ppm (1H, t of t): $^2J_{FH}$ = 54 Hz, $^3J_{FH}$ = 3 Hz; 6.18 ppm (1H, t): $^2J_{FH}$ = 56 Hz; 7.90 ppm (1H, s); 8.56 ppm (1H, d): $^3J_{HH}$ = 6 Hz	AB system (2F, d of t): δ_A, −12.77 ppm, δ_B, −14.40 ppm, $^2J_{FF}$ = 231 Hz, $^3J_{FF}$ = 8 Hz, $^3J_{FH}$ = 3 Hz; AB system (2F, d): δ_A, −40.89 ppm, δ_B, −42.86 ppm, $^2J_{FF}$ = 288 Hz, $^2J_{FH}$ = 54 Hz; −55.70 ppm (2F, d of t): $^2J_{FH}$ = 54 Hz, $^3J_{FF}$ = 8 Hz
TFE-PMS *O,S*-dimethyl ester Exact mass: Observed, 343.0133 Calculated, 343.0135	2.67 ppm (3H, s); 3.14–3.58 ppm (2H, m[c]); 3.77 ppm (3H, s); 4.23–4.90 ppm (1H, m[d]); 5.80 ppm (1H, t of t): $^2J_{FH}$ = 54 Hz, $^3J_{FH}$ = 3 Hz; 6.13 ppm (1H, t): $^2J_{FH}$ = 55 Hz	AB system (2F, d of t): δ_A, −15.26 ppm, δ_B, −16.16 ppm, $^2J_{FF}$ = 231 Hz, $^3J_{FF}$ = 8 Hz, $^3J_{FH}$ = 3 Hz; AB system (2F, d): δ_A, −37.07 ppm, δ_B, −38.84 ppm, $^2J_{FF}$ = 318 Hz, $^2J_{FH}$ = 55 Hz; −56.02 ppm (2F, d of t): $^2J_{FH}$ = 54 Hz, $^3J_{FH}$ = 9 Hz

[a] Samples are dissolved in $CDCl_3$. ^1H NMR chemical shifts are referenced to internal 3-trimethylsilane (assigned 0 ppm).
[b] Samples are dissolved in $CDCl_3$. ^{19}F = NMR chemical shifts are referenced to internal trifluoroacetic acid (assigned 0 ppm).
[c] AB part of ABM spectrum.
[d] M part of ABM spectrum.

pointing to the formation of a fluorine-containing aniline adduct. This adduct was extracted with ethyl acetate and methylated. When examined with GC-MS, the metabolite at 7.52 min (Fig. 3B) had a retention time and mass spectrum identical to that of chemically synthesized *N*-difluorothionoacetylaniline S-methyl ester. As with TFE-PMS, the difluorothionamide group of *N*-difluorothionoacetylaniline could also be *S*-methylated by diazomethane, due to tautomerism. TFE-PMS could not be detected in the aniline-containing incubation mixtures using ^{19}F NMR; however, small amounts of TFE-PMS were detected in ethyl acetate extracts using the more sensitive GC-MS (retention time, 8.75 min (Fig. 3B). The presence of aniline in incubation mixtures resulted in some inhibition of β-lyase, when measured as the formation of pyruvic acid, in both renal (30% inhibition) and hepatic (5% inhibition) incubations.

When incubations were performed in the presence of 4 mM benzylamine, TFE-PMS formation was still important (Fig. 3C). After extraction, methylation, and examination with GC-MS, the product at 8.54 min could be identified as *N*-difluorothionoacetylbenzylamine *S*-methyl ester. Examination of the incubation mixture directly with ^{19}F NMR, revealed only a very weak doublet at −41.61 ppm ($^2J_{FH}$ = 55 Hz), which coincided with the signals of the difluorothionamide group of TFE-PMS (data not shown). Benzylamine did not affect β-lyase activity in renal or hepatic incubations. In incubations with diethylamine as a scavenger of reactive intermediates, no additional fluorine-containing metabolites could be detected with ^{19}F NMR, when compared with incubations without diethylamine. Also, the GC-MS chromatogram of methylated extracts of these incubation mixtures was identical to that shown in Fig. 3A.

Metabolism of TFE-Cys and TFE-NAC by 11,000 × supernatants of rat kidney and liver. The rate of formation of the pseudomercapturic acid TFE-PMS in incubations of TFE-Cys (initial concentration, 4 mM) with 11,000 × *g* supernatants of rat kidney and rat liver (containing both cytosolic and microsomal enzymes) was determined by GC-MS. The initial rate of formation of TFE-PMS in incubations with rat renal supernatant was about 3 times higher than that with hepatic supernatant (Fig. 5). In addition to this, the initial rate of formation of pyruvic acid also appeared to be approximately 3-fold higher in renal incubations (12 nmol of pyruvic acid/min/mg of protein) then in hepatic incubations (3.8 nmol of pyruvic acid/min/mg of protein). Formation of pyruvic acid was linear for at least 10 min. In renal as well as in hepatic incubations, approximately 60–70% of the amount of DFTAF formed was bound covalently to the amino group of the parent cysteine conjugate. To investigate whether the observed organ dependency of β-lyase activity was regulated by the availability of α-keto acids (27), incubations also were carried out in the presence of 0.5 mM α-keto-γ-methiolbutyric acid. The formation of pyruvic acid indeed was slightly stimulated by this α-keto acid, 10% with renal and 25% with hepatic incubations. With both renal and hepatic fractions, the formation of TFE-PMS and pyruvic acid from TFE-Cys could be completely inhibited by 1 mM aminooxyacetic acid or by heat inactivation before incubation with TFE-Cys. Although the initial rate of formation of TFE-PMS was higher with renal fractions, after

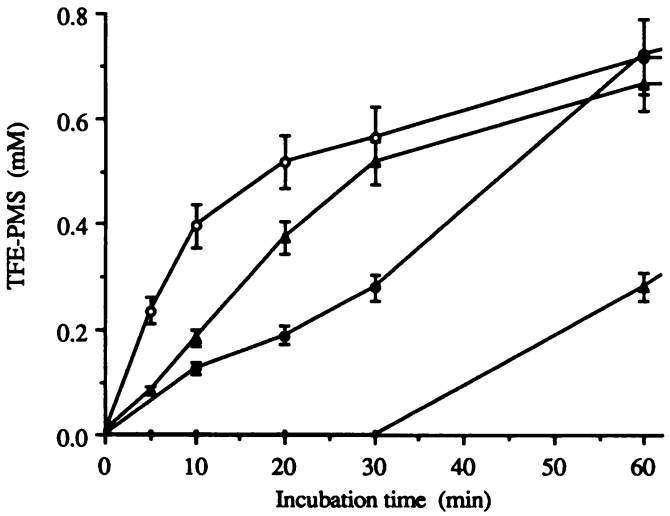

Fig. 5. Time dependence of the formation of TFE-PMS in incubations of 11,000 × *g* supernatants of rat kidney (*open symbols*) and rat liver (*closed symbols*) in the case of TFE-Cys (*circles*) or TFE-NAC (*triangles*). Incubations were carried out in 50 mM potassium phosphate buffer, pH 7.4, at 37° with *S*-conjugates of TFE at an initial concentration of 4 mM. The concentration of renal and hepatic 11,000 × *g* supernatants corresponded to 4 mg of renal protein/ml or 5 mg of hepatic protein/ml. The concentration of TFE-PMS in the incubation mixtures was determined as described in Experimental Procedures.

60 min of incubation the final concentration of TFE-PMS in the hepatic incubations was equal to that observed in the renal incubations. Renal 11,000 × *g* supernatant appeared to be able to degrade TFE-PMS; after 60 min, 15% of TFE-PMS (initial concentration, 2.5 mM) was degraded. Degradation of TFE-PMS was neglegible in hepatic 11,000 × *g* supernatant.

A more striking organ dependency was seen when renal and hepatic 11,000 × *g* supernatants were incubated with the mercapturic acid of TFE, TFE-NAC. The initial rate of *N*-deacetylation of TFE-NAC in renal 11,000 × *g* supernatant was approximately 8 times higher (75 versus 9 nmol/mg of protein/min) then in hepatic 11,000 × *g* supernatant (Fig. 6). The initial rate of disappearance of TFE-NAC corresponded to the initial rate of formation of TFE-Cys (Fig. 6). Aminooxyacetic acid did not influence the rate of deacetylation of TFE-NAC in either renal or hepatic incubations. Due to inhibition of secondary metabolism of β-lyase after 30 and 60 min, the final concentration of TFE-Cys in these incubations was significantly higher, when compared with those in the absence of aminooxyacetic acid (Fig. 6). In incubations of TFE-NAC with both renal and hepatic 11,000 × *g* supernatants, the formation of TFE-PMS could also be observed (Fig. 5). In incubations of TFE-NAC with hepatic 11,000 × *g* supernatant, however, TFE-PMS could only be detected after 60 min (Fig. 5).

In incubations of TFE-Cys with 11,000 × *g* supernatants of rat liver or rat kidney, no formation of TFE-NAC was observed. However, when exogenous acetyl-CoA (end concentration, 2 mM) was included in the incubation mixtures with both renal and hepatic 11,000 × *g* supernatants, the formation of TFE-NAC was significant (Fig. 7). The initial rate of formation of TFE-NAC in renal incubations was approximately 5-fold higher then in hepatic incubations, in spite of the much higher *N*-deacetylation activity of the renal fraction. After 30 min of incubation, however, the amount of TFE-NAC in the renal incubations was strongly reduced again, while in the hepatic incubations it still increased. When, after 30 min of incubation, an additional amount of acetyl-CoA was added to the renal incubation, the acetylation further increased, (Fig. 7). In the presence of exogenous acetyl-CoA, only very small amounts of TFE-PMS could be detected in renal as well as in hepatic incubations with TFE-Cys.

***In vivo* metabolism of TFE-NAC.** To investigate the formation of the pseudomercapturic acids (TFE-PMS and TFE-PMO) as potential indicators of the reactive intermediates DFTAF and DFTA formed from TFE-Cys *in vivo*, urine extracts of rats treated with TFE-NAC (50 μmol/kg of body weight) were examined by GC-MS and selected ion monitoring of characteristic ions derived from both TFE-PMS and TFE-PMO (Fig. 4). Ions at *m/z* 177, 196, 219, 284, and 296 were indeed found in urine extracts of TFE-NAC-treated animals at a retention time and with relative peak areas identical to those obtained from the *O,S*-dimethyl ester of TFE-PMS generated *in vitro*. The amount of TFE-PMS in urine was estimated to be 30 nmol, corresponding to 0.2% of the dose of TFE-NAC administered. Ions at *m/z* 117, 176, 218, and 254, which are characteristic for TFE-PMO, however, could not be detected in urinary extracts at the retention time of chemically synthesized TFE-PMO. The amount of unchanged TFE-NAC excreted in urine within 48 hr was found to be only 3–5% of the administered dose.

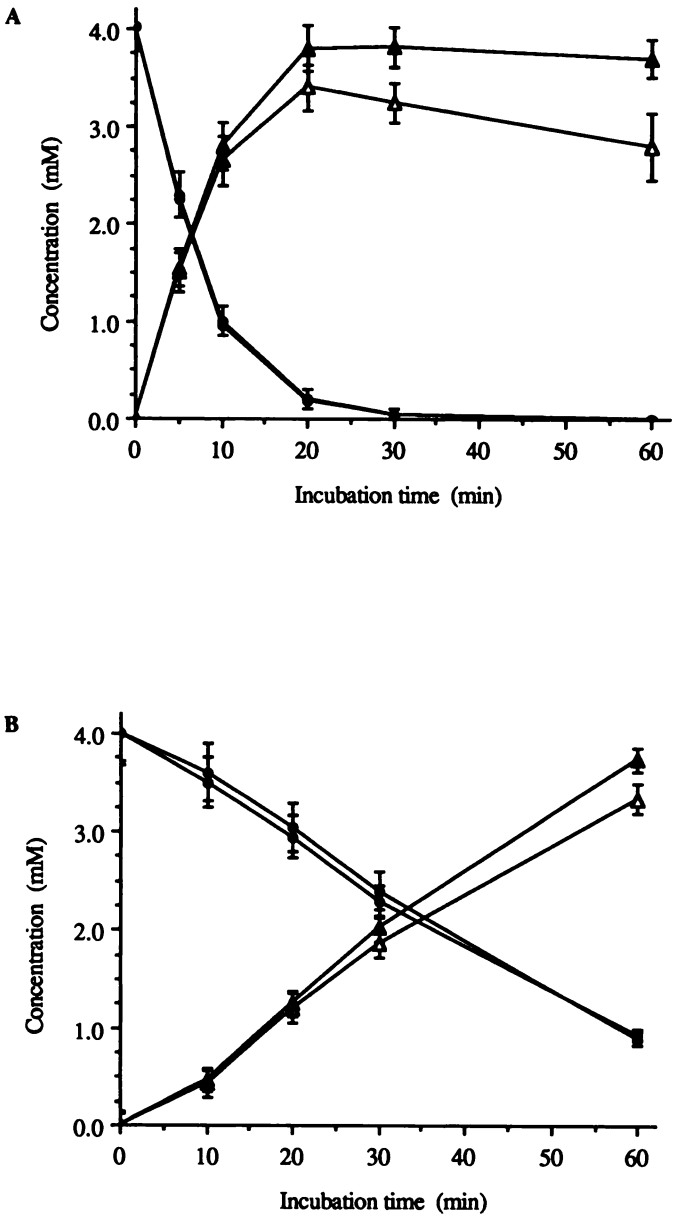

Fig. 6. Metabolism of TFE-NAC by 11,000 × *g*- supernatants of rat kidney (A) and rat liver (B). Incubations were carried out in 50 mM potassium phosphate buffer, pH 7.4, at 37°, with 11,000 × *g* supernatant at a concentration corresponding to 4 mg of renal protein/ml or 5 mg of hepatic protein/ml. The initial concentration or TFE-NAC was 4 mM. Total metabolism was determined by measuring TFE-NAC disappearance (*circles*). *N*-Deacetylation was determined by measuring TFE-Cys (*triangles*). To study the influence of secondary metabolism by β-lyase, incubations were carried out in the absence (*open symbols*) and presence (*closed symbols*) of aminooxyacetic acid. Concentrations of TFE-NAC and TFE-Cys in the incubation mixtures were determined as described in Experimental Procedures.

Discussion

The aim of the present study was to identify reactive intermediates derived from the β-lyase-dependent bioactivation of TFE-Cys and TFE-NAC and to investigate whether the organ-selective nephrotoxicity of these compounds (14, 28) could be explained by relative differences in activities of the enzymes involved in the bioactivation of these conjugates.

The results presented in this tudy are supportive of a bioactivation mechanism of TFE as illustrated in Fig. 8. The previous

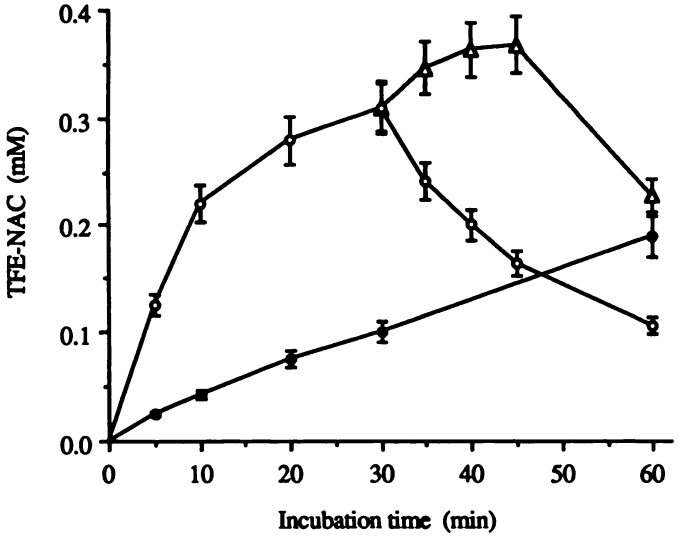

Fig. 7. Time dependence of *N*-acetylation of TFE-Cys by 11,000 × *g* supernatants of rat kidney (○) and rat liver (●) in the presence of exogenous acetyl-CoA. Incubations (total volume, 1.5 ml) were carried out in 50 mM potassium phosphate buffer, pH 7.4, at 37°, with 11,000 × *g* supernatants at concentrations of 30 mg of organ/ml, which correspond to 4 mg of renal protein/ml or 5 mg of hepatic protein/ml. The initial concentration of TFE-Cys and acetyl-CoA were 4 and 2 mM, respectively. In a parallel renal incubation, after 30 min an additional dose (3 μmol) of acetyl-CoA (△) was added to the medium. Concentrations of TFE-NAC in the incubation mixtures were determined as described in Experimental Procedures.

identification of DFA (Fig. 8, **IV**) as a metabolite formed from TFE-Cys in rat renal cytosol was the first indirect indication of the formation of DFTAF (Fig. 8, **II**) and/or DFTA (Fig. 8, **IIIa** and **b**), both potentially acylating agents (14). The identification of TFE-PMS in the present study points to DFTAF as the main reactive intermediate derived from bioactivation of TFE-Cys by both rat renal and rat hepatic cytosol. Aminooxyacetic acid, an inhibitor of pyridoxal phosphate-dependent enzymes (1), inhibited the degradation of TFE-Cys and the formation of TFE-PMS completely, indicating a crucial involvement of β-lyase. When nucleophilic amines such as aniline or benzylamine were added to the incubation mixtures in order to trap reactive intermediates, the corresponding difluorothionamides of aniline and benzylamine could be identified as products measurable with GC-MS (Table 1, Fig. 3, B and C). Aniline appeared to be the most effective nucleophile in trapping the reactive intermediate; aniline nearly completely inhibited the formation of TFE-PMS from TFE-Cys, as a result of competition with the amino group of free TFE-Cys. Diethylamine, which was recently used by Dekant *et al.* (13) to trap reactive intermediates, did not inhibit the formation of TFE-PMS at all, nor did it result in detectable fluorine-containing metabolites measurable with ^{19}F NMR. This points to a very poor trapping ability of this nucleophile. The very low level of *N*-difluoroacetylation of the amines investigated points to a poor electrophilic reactivity of DFTA. This is probably due to an inactivating substitutent effect of the fluorine atoms.

β-Lyase-dependent degradation of TFE-Cys and CTFE-Cys *in vitro* has been reported to yield DFA and chlorofluoroacetic acid, (13, 14). In the present study, however, using ^{19}F NMR, the formation of DFA from TFE-Cys in renal or hepatic cytosol could not be shown (Fig. 1). The apparent discrepancy between the present and previous results (14) is attributed to the presence of an unstable metabolite in the incubation mixtures, which can only be observed directly with the noninvasive ^{19}F NMR and which is converted rapidly to DFA upon acidification. The method previously used to detect DFA involved acidification before extraction and derivization (14). During this procedure, the unstable metabolite is converted to DFA. Treatment of our incubation mixtures with strongly acidic reagents used for detection of hydrogen sulfide, according to the method of Siegel (29), also converted the unstable metabolite to DFA. Treatment of difluoroacetic anhydride with hydrogen sulfide resulted in formation of a product with identical ^{19}F NMR chemical shift, which suggests that this unstable metabolite might be DFTA. In that case, in addition to DFA, hydrogen sulfide will be released upon acidification. This would imply that the formation of hydrogen sulfide *in vitro*, as de-

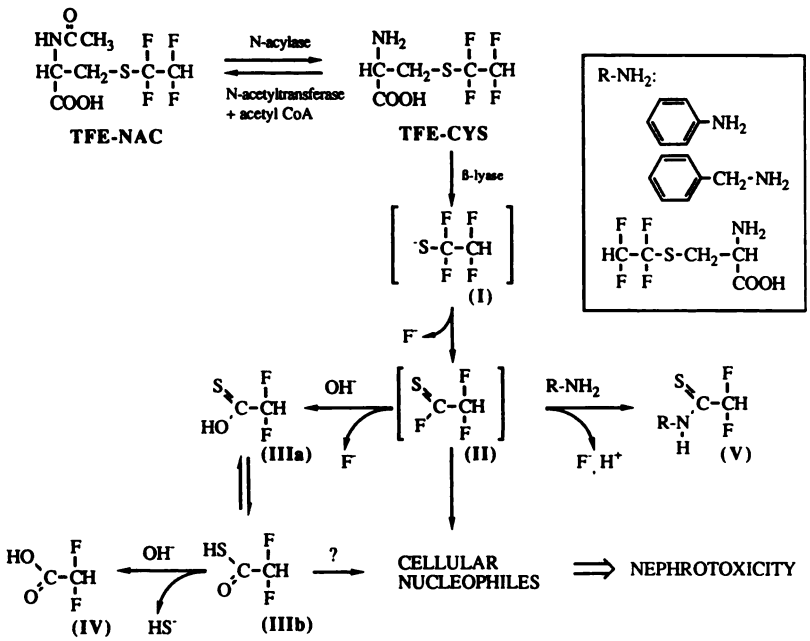

Fig. 8. Metabolism of TFE-NAC and TFE-Cys by *N*-deacetylase(s), *N*-acetyltransferase(s), and β-lyase(s), formation of acylating reactive intermediates, and covalent binding to amine-containing nucleophiles. **I**, 1,1,2,2-tetrafluoroethanethiol; **II**, DFTAF; **IIIa**, difluorothionoacetic acid; **IIIb**, difluorothioacetic acid; **IV**, DFA; **V**, difluorothionamides of aniline, benzylamine, and TFE-Cys.

scribed earlier (14, 30), might have been overestimated due to the derivization method used. However, to fully support this hypothesis, the identity of the acid-labile product should be proved unequivocally. The formation of chlorofluoroacetic acid, as described by Dekant *et al.* (13), might also have been the result of stopping the enzymic reactions by acidifying the incubation mixtures with 30% trichloroacetic acid before analyzing with ^{19}F NMR.

In the present study, we also investigated whether the organ-selective nephrotoxicity of TFE-NAC previously reported (14) could be explained by relative differences in activity of the enzymes involved in the biotransformation of these compounds, notably the cytosolic enzymes *N*-deacetylase (6, 20) and cysteine conjugate β-lyase and the microsomal enzyme cysteine conjugate *N*-acetyltransferase (21). *N*-Deacetylation of TFE-NAC occurred both in hepatic and renal 11,000 × *g* supernatants. However, the renal supernatants exhibited an 8-fold higher initial *N*-deacylating activity than the hepatic supernatants (Fig. 6). In incubations of TFE-NAC with 11,000 × *g* supernatants of both rat liver and rat kidney, at this concentration of TFE-NAC, *N*-deacetylating activity was apparently very high compared with the subsequent β-lyase activity, because TFE-Cys accumulated in these incubations (Fig. 6). This rapid accumulation of TFE-Cys combined with a relatively high β-lyase-dependent bioactivation of TFE-Cys to DFTAF results in the formation of TFE-PMS, which could be detected already within 5 min (Fig. 5). Due to much lower *N*-deacetylase activities and a lower β-lyase activity, in hepatic 11,000 × *g* supernatant the formation of TFE-PMS from TFE-NAC was delayed when compared with incubations in renal 11,000 × *g* supernatants.

When the 11,000 × *g* supernatants were incubated with TFE-Cys, a rapid formation of TFE-PMS was also observed with both renal and hepatic fractions (Fig. 4). The initial formation of both TFE-PMS and pyruvic acid, a product of β-lyase-dependent degradation of cysteine conjugates, was 3-fold faster in renal then in hepatic incubations. The small effect of α-keto-γ-methiolbutyric acid on β-lyase activities (10–25% stimulation) indicated that the inavailability of α-keto acids cannot explain the much lower β-lyase activity in the hepatic incubation. In both renal and hepatic incubations, the ratio of TFE-PMS/pyruvic acid was 60–70%. Therefore, the 3-fold difference between the rates of formation of TFE-PMS from TFE-Cys in renal and hepatic incubations cannot be explained by a higher partitioning of reactive intermediates to endogenous amino acids in the hepatic incubation mixtures, despite the fact that the protein concentration was slightly higher in the latter.

N-Acetylation of TFE-Cys can be seen as a bioinactivation step that would compete with the β-lyase-mediated bioactivating step (Fig 8). Formation of TFE-NAC, however, could not be demonstrated in incubations of TFE-Cys with hepatic or with renal 11,000 × *g* supernatants. However, when 2 mM exogenous acetyl-CoA, the cofactor for *N*-acetyltransferases, was added to the incubation mixtures, the formation of significant amounts of TFE-NAC could be observed with both renal and hepatic fractions; the initial rate of formation of TFE-NAC in the renal incubations was 5 times higher then that in the hepatic incubations (Fig. 7). Despite a very high *N*-deacetylase activity observed in renal fractions at 4 mM TFE-NAC (Fig. 6), TFE-NAC formation could be measured in incubations with 4 mM TFE-Cys (Fig. 5). In the latter incubations, the initial concentration of TFE-NAC is probably low compared with K_m of *N*-deacetylation of TFE-NAC. In this case, the rate of deacetylation might still be low, compared with the rate of acetylation. However, due to the presence of competing enzymes in the 11,000 × *g* fractions, we were not able to determine K_m values of the individual enzymes. In the renal incubations with TFE-Cys in the presence of acetyl-CoA, after 30 min of incubation a rapid decrease of the TFE-NAC formed was observed (Fig. 7). Addition of a second dose of acetyl-CoA, however, resulted in restoration of the *N*-acetylation of TFE-Cys. We, therefore, suggest that depletion of acetyl-CoA as a result of a high turn-over of TFE-Cys/TFE-NAC, rather then instability of *N*-acetyltransferases in these crude enzyme fractions (21), limited the *in vitro* *N*-acetylation of TFE-Cys after 30 min of incubation.

As previously reported, in urine of rats treated with a nephrotoxic dose of TFE-NAC, DFA, fluoride anion, and an as yet unidentified fluorine-containing compound could be identified as metabolites, using ^{19}F NMR (14). Using GC-MS analysis, in the present study small amounts (0.2% of the dose) of TFE-PMS could be identified in urine of rats treated with a nephrotoxic dose of TFE-NAC. This proves that DFTAF, identified here for the first time *in vitro*, is also formed *in vivo*. The as yet unidentified urinary metabolite (14) has a chemical shift in ^{19}F NMR similar to that of chemically synthesized difluorothionamides (Table 1). The identification of DFA in urine with the noninvasive ^{19}F NMR technique might indicate that the initially formed acid-labile metabolite (most likely DFTA; **IIIa** and **b**, Fig. 8) is hydrolysed to DFA *in vivo*.

The excretion of unchanged TFE-NAC in 24-hr urine of rats treated with 50 μmol/kg TFE-NAC, to an extend of only 3–5% of the dose, also points to a high *N*-deacetylation/*N*-acetylation ratio *in vivo*. As demonstrated by the present *in vitro* studies, the balance of *N*-deacetylation/*N*-acetylation might be largely determined by the availability of acetyl-CoA, a necessary cofactor for *N*-acetyltransferase. In principle, the selective nephrotoxicity of TFE-NAC might even be the result of depletion of acetyl-CoA in the kidney due to the very high turn-over of TFE-Cys/TFE-NAC in that organ. This would result in accumulation of TFE-Cys in the kidney, followed by β-lyase-dependent bioactivation to DFTAF. The identification of TFE-PMS in rat urine supports this hypothesis. A much lower turnover of TFE-Cys/TFE-NAC in the liver in addition to a lower β-lyase activity in that organ might explain the relative insensitivity of that organ to TFE-Cys-mediated toxicity.

Although our present results tend to explain the organ-selective toxicity of TFE-NAC on enzymic bases, inter-organ differences in transport and accumulation mechanisms also might be of importance in determining the organ selectivity of nephrotoxic cysteine conjugates. Studies with systems more related to the *in vivo* situation are required to solve these questions.

In conclusion, the identification of various *N*-difluorothionamides (**V**, Fig. 8) both *in vitro* and *in vivo* has demonstrated that DFTAF (**II**, Fig. 8) is the main reactive intermediate formed by β-lyase from TFE-Cys. DFTAF is presumably also responsible for the selective nephrotoxicity of TFE-NAC *in vivo*. Both renal and hepatic subcellular fractions were shown to be able to *N*-deacetylate TFE-NAC to TFE-Cys, which then can be *N*-acetylated again to TFE-NAC or can be bioactivated by cysteine conjugate β-lyase to DFTAF. The availability of

acetyl-CoA was shown to be a important factor determining the balance of *N*-acetylation versus *N*-deacetylation, as well of *N*-acetylation versus β-lyase-dependent activation. The very low percentage of excretion of TFE-NAC in urine of TFE-NAC-treated rats might be the reflection of a depletion of acetyl-CoA in the kidney due to the very high turn-over of TFE-NAC/TFE-Cys, as was demonstrated *in vitro*. This would result in accumulation of TFE-Cys in this organ and subsequent β-lyase-catalyzed activation to DFTAF (II, Fig. 8), which causes nephrotoxicity and the formation of TFE-PMS *in vivo*.

References

1. Elfarra, A. A., and M. W. Anders. Renal processing of glutathione conjugates: role in nephrotoxicity. *Biochem. Pharmacol.* **33**:3729–3732 (1984).
2. Igwe, O. J. Biologically active intermediates generated by the reduced glutathione conjugation pathways: toxicological implications. *Biochem. Pharmacol.* **35**:2987–2994 (1986).
3. Rannug, U., A. Sundvall, and C. Ramel. The mutagenic effect of 1,2-dichloroethane on *Salmonella typhimurium*. I. Activation through conjugation with glutathione *in vitro*. *Chem-Biol. Interact.* **20**:1–16 (1978).
4. Van Bladeren, P. J., D. D. Breimer, G. M. T. Rotteveel-Smijs, A. van der Gen, and G. R. Mohn. The role of glutathione conjugation in the mutagenicity of 1,2-dibromoethane. *Biochem. Pharmacol.* **29**:2975–2982 (1980).
5. Elfarra, A. A., R. B. Baggs, and M. W. Anders. Structure-nephrotoxicity relationships of *S*-(2-chloroethyl)-*dl*-cysteine and analogs: role for an episulfonium ion. *J. Pharmacol. Exp. Ther.* **233**:512–516 (1985).
6. Tateishi, M. Methylthiolated metabolites. *Drug Metab. Rev.* **14**:1207–1234 (1983).
7. Lash, L. H., A. A. Elfarra, and M. W. Anders. Renal cysteine conjugate β-lyase: bioactivation of nephrotoxic cysteine *S*-conjugates in mitochondrial outer membrane. *J. Biol. Chem.* **261**:5930–5935 (1986).
8. Stevens, J. L., N. Ayoubi, and J. D. Robbins. The role of mitochondrial matrix enzymes in the metabolism and toxicity of cysteine conjugates. *J. Biol. Chem.* **263**:3395–3401 (1988).
9. Stonard, M. D., and V. H. Parker. 2-Oxoacid dehydrogenases of rat liver mitochondria as the site of action of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-3-mercaptoproprionic acid. *Biochem. Pharmacol.* **20**:2417–2427 (1971).
10. Wallin, A., T. W. Jones, A. E. Vercesi, I. Cotgreave, K. Ormstad, and S. Orrenius. Toxicity of *S*-pentachlorobutadienyl-*l*-cysteine studied with isolated rat renal cortical mitochondria. *Arch. Biochem. Biophys.* **258**:365–372 (1987).
11. Green, T., and J. Odum. Structure activity studies of the nephrotoxic and mutagenic action of chloro- and fluoroalkenes. *Chem. Biol. Interact.* **54**:15–31 (1985).
12. Dohn, D. R., A. J. Quebbemann, R. F. Borch, and M. W. Anders. Enzymic reaction of chlorotrifluoroethylene with glutathione: ^{19}F-NMR evidence for stereochemical control of the reaction. *Biochemistry* **24**:5137–5143 (1985).
13. Dekant, W., L. H. Lash, and M. W. Anders. Bioactivation mechanisms of the cytotoxic and nephrotoxic *S*-conjugate *S*-(2-chloro-1,1,2-trifluoroethyl)-*l*-cysteine. *Proc. Natl. Acad. Sci. USA* **84**:7443–7447 (1987).
14. Commandeur, J. N. M., J. Brakenhof, F. J. J. de Kanter, and N. P. E. Vermeulen. Nephrotoxicity of mercapturic acids of three structurally related 2,2-difluoroethylenes in the rat: indications for different bioactivation mechanisms. *Biochem. Pharmacol.* **37**:4495–4505 (1988).
15. Commandeur, J. N. M., R. A. J. Oostendorp, P. R. Schoofs, B. Xu, and N. P. E. Vermeulen. 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).
16. Nash, J. A., L. J. King, E. A. Lock, and T. Green. The metabolism and disposition of hexachloro-1,3-butadiene in the rat and its relevance to nephrotoxicity. *Toxicol. Appl. Pharmacol.* **73**:124–137 (1984).
17. Lock, E. A., and J. Ishmael. Effect of the organic acid transport inhibitor probenecid on renal cortical uptake and proximal tubular toxicity of hexachlorobutadiene and its conjugates. *Toxicol. Appl. Pharmacol.* **81**:32–42 (1985).
18. Pratt, I. S., T. Ormond, and E. A. Lock. Metabolism of the mercapturic acid of hexachloro-1,3-butadiene by rat kidney cytosol *in vitro*. Proceedings of the 27th Congress of the European Society of Toxicology. Harrogate, England, 39 (1986).
19. Vamvakas, S., W. Dekant, K. Berthold, S. Schmidt, D. Wild, and D. Henschler. Enzymic transformation of mercapturic acids derived from halogenated alkenes to reactive and mutagenic intermediates. *Biochem. Pharmacol.* **36**:2741–2748 (1987).
20. Suzuki, S., and M. Tateishi. Purification and characterization of a rat liver enzyme catalyzing *N*-deacetylation of mercapturic acid conjugates. *Drug Metab. Dispos.* **9**:573–577 (1981).
21. Duffel, M. W., and W. B. Jacoby. Cysteine *S*-conjugate *N*-acetyltransferase from rat kidney microsomes *Mol. Pharmacol.* **21**:444–448 (1982).
22. Sawicki, E. Polyfluoroacyl derivates of carcinogenic and allied Amines. *J. Org. Chem.* **21**:376 (1956).
23. Scheeren, J. W., P. H. J. Ooms, and R. J. F. Nivard. A general procedure for the conversion of a carbonyl group into a thione group with tetraphosphorous decasulphide. *Synthesis* 149–151 (1973).
24. Wolf, C. R., P. N. Berry, J. A. Nash, T. Green, and E. A. Lock. 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).
25. Duus, F. Thiocarbonyl compounds, in *Comprehensive Organic Chemistry* (D. N. Jones, ed.), Vol. 3. Pergamon Press, Oxford, 373–487 (1979).
26. Walter, W., and J. Voss. The chemistry of thioamides, in *The Chemistry of Amides*. (J. Zabicky, ed). Interscience Publishers, London, 385–475 (1970).
27. Stevens, J. L., J. D. Robbins, and R. A. Byrd. A purified cysteine conjugate β-lyase from rat kidney cytosol: requirement for an α-keto acid or an amino acid oxidase for activity and identity with soluble glutamine transaminase K. *J. Biol. Chem.* **261**:15529–15537 (1986).
28. Odum, J., and T. Green. The metabolism and nephrotoxicity of tetrafluoroethylene in the rat. *Toxicol. Appl. Pharmacol.* **76**:306–318 (1984).
29. Siegel, L. M. A direct microdetermination for sulfide. *Anal. Biochem.* **11**:126–132 (1965).
30. Banki, K., A. A. Elfarra, L. H. Lash, and M. W. Anders. Metabolism of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine to hydrogen sulfide and the role of hydrogen sulfide in *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine-induced mitochondrial toxicity. *Biochem. Biophys. Res. Commun.* **138**:707–713 (1986).

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