Enzymatic Reaction of Chlorotrifluoroethene with Glutathione: ^{19}F NMR Evidence for Stereochemical Control of the Reaction†

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ABSTRACT: Chlorotrifluoroethene, a potent nephrotoxin, is a substrate for the glutathione *S*-transferases present in the cytosolic and microsomal fractions of rat liver. The glutathione conjugate formed by both subcellular fractions has been identified as *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione by ^1H and ^{19}F NMR and by secondary ion mass spectrometry. The conjugate formed by the cytosolic fraction is an equimolar mixture of two diastereomers, whereas the conjugate formed by the microsomal fraction is predominantly one diastereomer, as judged by the ^{19}F NMR spectra. No evidence for the formation of *S*-(trihalovinyl)glutathione derivatives by an addition/elimination reaction was found. High-performance liquid chromatography was employed to measure the rates of glutathione conjugate formation in vitro. The rates of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione formation were 75–107 nmol min^{-1} (mg of protein)$^{-1}$ and 151–200 nmol min^{-1} (mg of protein)$^{-1}$ catalyzed by the cytosolic and microsomal fractions, respectively (measured at pH 7.4, 37 °C, with 5 mM glutathione). These results suggest that glutathione conjugation occurs at high rates in vivo to produce the highly nephrotoxic *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione.

Several halogenated alkanes and alkenes, including chlorotrifluoroethene (Clayton, 1977; Potter et al., 1981; Buckley et al., 1982), hexachloro-1,3-butadiene (Lock & Ishmael, 1979), 1,2-dichloroethane (Spencer et al., 1951), and tris-(2,3-dibromopropyl) phosphate (Elliott et al., 1982), have been identified as nephrotoxins. In addition, the cysteine conjugate of trichloroethene *S*-(1,2-dichlorovinyl)-L-cysteine is a potent nephrotoxin (Parker, 1965). These and subsequent observations [reviewed in Elfarra & Anders (1984)] have led to the hypothesis that glutathione conjugate formation followed by enzymatic processing by renal peptidases to yield the corresponding cysteine conjugates is involved in the production of nephrotoxicity by halogenated alkanes and alkenes. The cysteine conjugates may be direct-acting nephrotoxins or may be activated by cysteine conjugate β-lyase.

With CTFE,[1] the formation of a *S*-(haloethyl)glutathione derivative catalyzed by the hepatic glutathione *S*-transferases would be the first step in the reaction. The transport of this compound to the kidney followed by processing by γ-glutamyl transpeptidase and cysteinylglycine dipeptidase would yield the corresponding cysteine conjugate. Finally, metabolism of the cysteine conjugate by renal cysteine conjugate β-lyase would yield pyruvate, ammonia, and a highly reactive alkylating species, which is presumed to be the ultimate nephrotoxin (Figure 1). The organ specificity is explained by the low γ-glutamyl transpeptidase activity in the liver and the high levels of this enzyme along with high cysteinylglycine dipeptidase activity in the kidney (Hughey et al., 1978). Cysteine conjugate β-lyase is found in rat liver and kidney (Tateishi et al., 1978).

The validation of this hypothesis requires the demonstration of glutathione conjugate formation from CTFE and glutathione. Previous studies have reported high rates of CTFE-dependent glutathione depletion catalyzed by rat hepatic cytosolic and microsomal fractions and have identified *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione (CTFG) as the major product formed by cytosolic enzymes (Dohn & Anders, 1982a). We report here that this same product is formed by microsomal fractions. Moreover, conjugate formation by the hepatic microsomal glutathione *S*-transferases appears to operate under stereochemical control with CTFE as the substrate. These studies provide partial validation of the hypothesis that hepatic conjugate formation followed by renal processing of the conjugate is involved in the nephrotoxicity of CTFE. Studies on the nephrotoxicity of CTFG and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFC) will be described fully elsewhere.

Experimental Procedures

Synthesis of CTFG. A solution of 40 mmol of glutathione and 0.4 mmol of Na_2EDTA in 20 mL of water was adjusted to pH 9.6 with solid potassium hydroxide. This solution and 20 mL of ethanol containing 0.4 mmol of butylated hydroxytoluene were placed in a 3-L round-bottom flask, and 10 mL of water was added to give a clear solution. The reaction flask was evacuated and refilled 3 times with nitrogen and then evacuated and filled with CTFE (Matheson, East Rutherford, NJ). CTFE was added occasionally to maintain atmospheric pressure. After 5 h of stirring at room temperature, the contents of the flask were transferred to a beaker, the flask was washed twice with 20-mL portions of 50% ethanol, and the combined reaction solution and washings were diluted with

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[1] Abbreviations: CTFE, chlorotrifluoroethene; CTFG, *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione; CTFC, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; EDTA, ethylenediaminetetraacetic acid.

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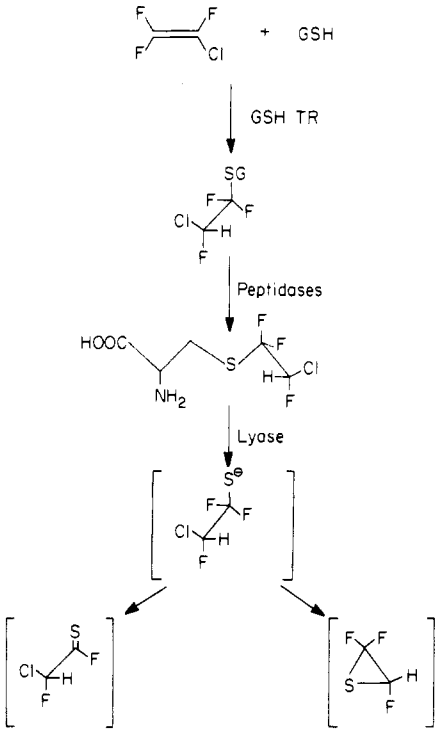


FIGURE 1: Proposed metabolic pathway for the bioactivation of CTFE. GSH = glutathione; GSH TR = cytosolic or microsomal glutathione *S*-transferases; peptidases = γ-glutamyl transpeptidase and cysteinylglycine dipeptidase; lyase = cysteine conjugate β-lyase. Proposed structures are shown in brackets.

80 mL of ethanol. The pH of this solution was adjusted to 4.6 with 70% perchloric acid. The solution was filtered, and a white precipitate (precipitate 1) and a cloudy filtrate were obtained. Analysis of precipitate 1 by thin-layer chromatography (Dohn & Anders, 1982a) indicated that it contained considerable CTFG in addition to potassium perchlorate. The filtrate was adjusted to pH 3.2 with trifluoroacetic acid and left uncovered overnight in a fume hood followed by storage for 24 h at 6 °C yielding a copious white precipitate (precipitate 2).

Precipitate 1 was suspended in 100 mL of boiling 85% ethanol and then filtered (the insoluble material contained negligible CTFG); the filtrate was diluted with 180 mL of boiling 85% ethanol, and precipitate 2 was added; a small amount of insoluble material was removed by filtration. After this was cooled slowly and left to stand for 24 h at room temperature and then 24 h at 6 °C, a white crystalline solid formed, which was collected and dried in vacuo. The yield was 8.54 g (50%). This material gave appropriate ^1H and ^{19}F NMR and secondary ion mass spectra (see below). Chromatography on an amino acid analyzer indicated 97.6% purity with a single impurity (not glutathione or glutathione disulfide). Repeated elemental analyses gave erratic results.

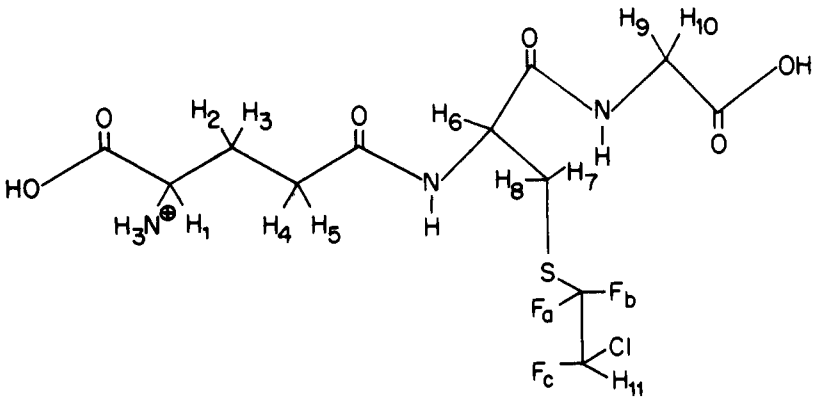
Preparation of Rat Hepatic Subcellular Fractions and Assay of Enzyme-Catalyzed CTFG Formation. Subcellular fractions were prepared from 270–370-g male Sprague-Dawley rats (Biolabs, White Bear Lake, MN), as described previously (Dohn & Anders, 1982b). Particulate fractions were resuspended in homogenizing media and resedimented once. The 100000*g* supernatant (cytosol) fraction was dialyzed overnight against 0.1 M potassium phosphate buffer, pH 7.4. The protein concentrations of the subcellular fractions were measured by the method of Lowry et al. (1951).

Assays of CTFG formation were conducted in 6-mL crimp-top vials (sealed with neoprene septa) containing enzyme, 5 mM glutathione, 0.2 mM EDTA, and 17 mM potassium phosphate buffer (pH 7.4) in a final volume of 1.5 mL. The vials were cooled on ice, evacuated, refilled with CTFE to 3 atm, transferred to a 37 °C water bath, and shaken vigorously for the duration of the assay. The reactions were stopped by immersing the vials in ice; the vials were opened in a fume hood, and 45 μL of trifluoroacetic acid followed by 130 μL of 2.0 M tripotassium phosphate was added. (The pH of the resulting solutions should be 2.2–2.3.) The samples were transferred to microfuge tubes and frozen to ensure complete precipitation of protein; after thawing and centrifuging, 1-mL portions of the supernatants were added to 0.25 mL of acetonitrile for analysis by high-performance liquid chromatography.

Twenty-microliter portions of the diluted samples were injected on a reverse-phase high-performance liquid chromatography column (Whatman 5-μm ODS-3 RAC II, 4.6 mm × 100 mm). The column was eluted at 1 mL/min with 13 mM trifluoroacetic acid and 0.2 mM EDTA (pH 2.2) in 20% aqueous acetonitrile with detection at 210 nm. Glutathione and glutathione disulfide eluted in the void volume; CTFG had a retention time of 3.8 min. This procedure is useful for measuring the production of as little as 25 nmol of CTFG per assay.

Purification of Biosynthetic CTFG. Protein from cytosol (94–144 mg), 100000*g* pellet (65–72 mg) or the 10000*g* pellet (63–88 mg), and 300 μmoles of glutathione, 6 μmol of EDTA, and 1.5 mmol of potassium phosphate buffer (pH 7.4) in a volume of 60 mL were placed in 125-mL crimp-top vials. The vials were sealed, evacuated, pressurized with CTFE to 3 atm, and incubated at 37 °C for 120 min with constant shaking. Reaction mixtures to be analyzed for fluorine-containing metabolites of CTFE were lyophilized and then reconstituted in 3 mL of deuterium oxide; Triton X-100 was added to give a clear solution. CTFG was purified from reaction mixtures after protein precipitation with an equal volume of cold ethanol for the cytosolic reactions and after ultracentrifugation for the reactions catalyzed by the 10000*g* and 100000*g* pellets. The preparations were lyophilized, reconstituted in a minimum volume of 13 mM trifluoroacetic acid, and applied to an air-driven 2 × 13 cm flash chromatography column filled with 20 g of 40-μm octadecyl (C_{18}) bonded phase (J. T. Baker, Phillipsburg, NJ). The column was eluted (5 mL/min) with 25 ml of 13 mM trifluoroacetic acid followed by 50 mL of 13 mM trifluoroacetic acid in 20% acetonitrile. Five-milliliter fractions were collected and analyzed for CTFG by thin-layer chromatography. Fractions (usually 9–13) containing CTFG were pooled, reduced to dryness, washed with chloroform/methanol (2/1) to remove residual trifluoroacetic acid, and dried in vacuo. This procedure gave pure CTFG from the cytosol and 100000*g* pellets, as judged by thin-layer chromatography and ^1H NMR. The material obtained from the 10000*g* pellet was impure and was not further purified. CTFG containing deuterium in the haloethyl group [*S*-(2-chloro-1,1,2-trifluoro[2-^2H]ethyl)glutathione] was prepared biosynthetically as described above with reactions conducted in deuterium oxide.

Instrumental Analyses. The ^{19}F NMR (339.7 MHz, 12-mm probe) and ^1H NMR (250 MHz, 5-mm probe) spectra of purified glutathione derivatives (6–9 mg/mL) were recorded in 50 mM deuterium chloride solution. The ^{19}F NMR spectra were recorded with a pulse length of 18 μs and a repetition rate of 1.6 s. Spectra were referenced with potassium fluoride in deuterium oxide (^{19}F NMR) in a coaxial reference tube or with internal 3-(trimethylsilyl)propanesulfonic acid (^1H NMR). Secondary ion mass spectra were obtained with a



Nucleus	Chemical Shift[a,b]	Multiplicity[c]	Coupling Constants (Hz)
H_1	4.12	t	$H_1 - H_{2,3} = 6.6$
$H_{2,3}$	2.26	m	
$H_{4,5}$	2.62	m	
H_6	4.75	d of d	$H_6 - H_7 = 8.3$
H_7	3.28	d of d	$H_6 - H_8 = 5.2$
H_8	3.50	d of d	$H_7 - H_8 = 14.5$
$H_{9,10}$	4.03	s	
H_{11}	6.66	d of d of d	$H_{11} - F_a = 5.0$; $H_{11} - F_b = 6.2$; $H_{11} - F_c = 47.8$
F_a	37.00	m	$F_a - F_b = 220$; $F_a - F_c = 18$; $F_a - H_{11} = 4.8$
F_b	35.03	m	$F_b - H_{11} = 6.3$
F_c	-26.23	m	$F_b - F_c = 18$; $F_c - H_{11} = 47$

FIGURE 2: Structure and NMR parameters of CTFG. Samples were dissolved in 50 mM deuterium chloride in deuterium oxide. [a]^{1}H chemical shifts are downfield from internal 3-(trimethylsilyl)propanesulfonic acid, which was assigned a chemical shift of 0.015 ppm. The spectral resolution was 0.427 Hz/point. [b]^{19}F positive shifts are downfield and negative shifts are upfield from potassium fluoride in neutral deuterium oxide contained in a coaxial reference tube. The spectral resolution was 0.782 Hz/point. [c]*s* = singlet, d = doublet, t = triplet, and m = multiplet.

Kratos MS-50S mass spectrometer fitted with a 6-keV cesium ion beam from samples dissolved in glycerol (Aberth et al., 1982).

Results

Enzyme-Catalyzed CTFG Formation. Rat hepatic subcellular fractions catalyzed CTFG formation from CTFE and glutathione (Table I). CTFG was formed at the rate of 2–3 nmol/min in the absence of liver subcellular fractions or in the presence of heat-denatured subcellular fractions. No CTFG was formed when either glutathione or CTFE was omitted from the reaction mixtures. The rate of CTFG formation was directly proportional to protein concentration in the ranges of 0.46–1.2 mg/mL for the 10000*g* pellet, 0.1–0.6 mg/mL for the 100000*g* pellet, and 0.25–0.75 mg/mL for the 100000*g* supernatant (measured after 10-min incubation time). The rate of CTFG formation in the presence of the 100000*g* supernatant (0.5 mg of protein/mL) was linear for 20 min, and the reaction catalyzed by the 100000*g* pellet (0.2 mg of protein/mL) was linear for 30 min. Kidney subcellular fractions, which were prepared as described above for liver subcellular fractions, catalyzed CTFG formation at low rates [$\leq$5 nmol min^{-1} (mg of protein)$^{-1}$]. The recovery of CTFG added to kidney subcellular fractions was low, which suggested that these fractions may metabolize CTFG.

NMR Studies on Synthetic and Biosynthetic CTFG. The ^{1}H NMR spectra of chemically synthesized CTFG and of CTFG isolated from cytosolic and microsomal incubations were identical. The assignments of ^{1}H chemical shifts and coupling constants are given in Figure 2. The assignments were based on ^{1}H–^{1}H decoupling experiments. The assignment of the nonexchangeable protons of the glutamyl residue was accomplished by irradiation of the multiplet at 2.26 ppm, which caused collapse of the triplet at 4.12 ppm to a singlet and collapse of the multiplet at 2.62 ppm to a closely spaced doublet of doublets. Irradiation of the multiplet at 2.62 ppm

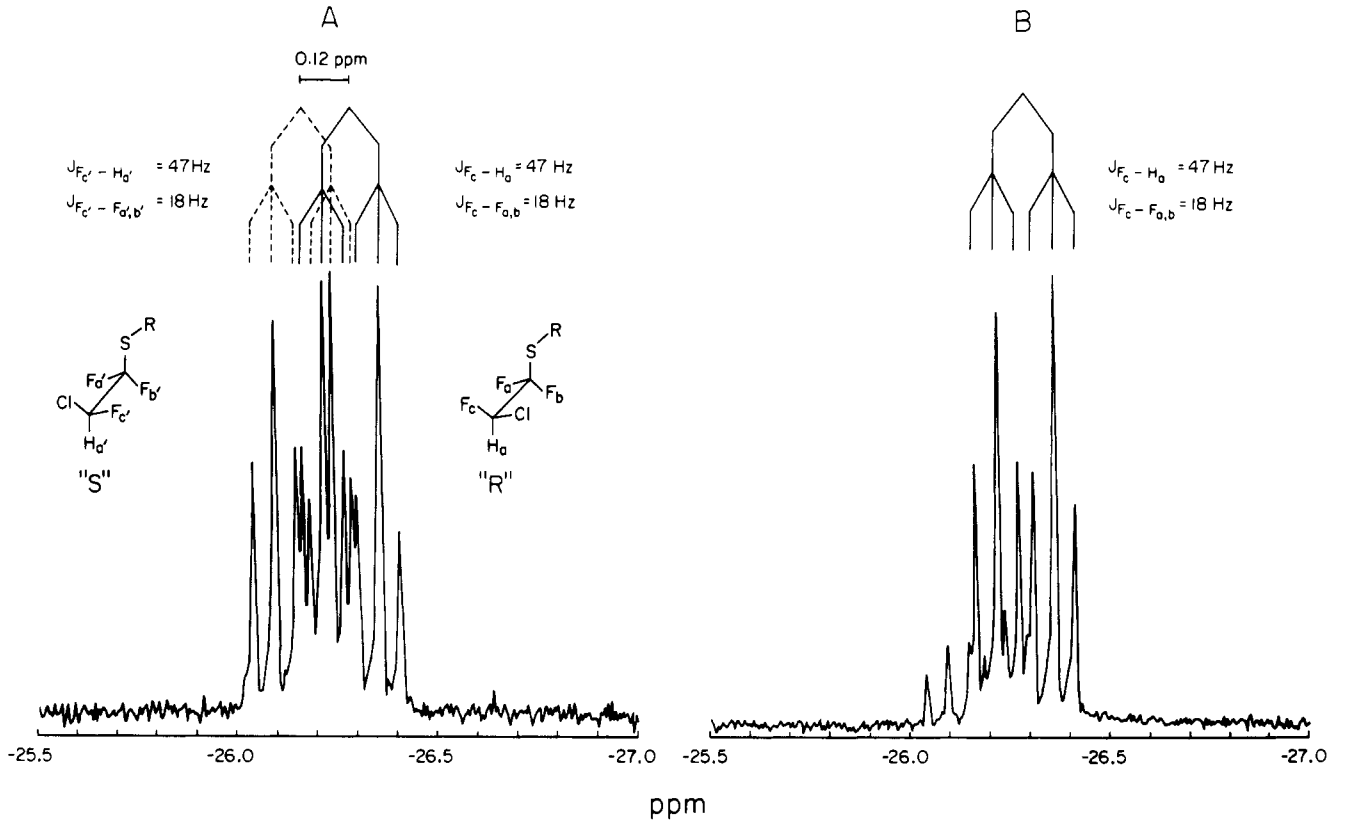


FIGURE 3: ^{19}F NMR of F_c in purified, biosynthetic CTFG. Chemical shifts are upfield from potassium fluoride. (A) CTFG produced by cytosolic transferase activity; (B) CTFG produced by microsomal transferase activity. The designation of stereoisomers as "*R*" and "*S*" is arbitrary.

Table I: CTFG Formation Catalyzed by Rat Hepatic Subcellular Fractions

subcellular fraction	sp act.[a] rat 1	rat 2	rat 3
10000*g* pellet	85 ± 5	116 ± 6	110 ± 10
100000*g* pellet	151 ± 4	200 ± 15	172 ± 7
100000*g* supernatant	75 ± 4	107 ± 2	89 ± 15

[a] Nanomoles of CTFG formed per minute per milligram of protein. Values are means ± SD from triplicate determinations for each rat and have been corrected for nonenzymatic CTFG formation. The reactions were conducted for 10 min, and the protein concentration was 0.5 mg/mL.

caused a simplification of the multiplet at 2.26 ppm. The nonexchangeable protons of the cysteinyl residue were assigned by irradiation of the proton at 4.75 ppm, which led to the collapse of the set of double doublets at 3.28 and 3.50 ppm to a set of doublets. Irradiation of the proton at 3.28 ppm caused the signals at 3.50 ppm and 4.75 ppm to collapse to doublets. The glycyl protons were identified as the only singlet in the ^1H NMR spectrum. The proton of the haloethyl group was identified by comparison of coupling constants in the ^1H and ^{19}F NMR spectra. The ^1H NMR spectra of biosynthetic conjugates prepared in deuterium oxide showed a marked reduction in the intensity of the multiplet centered at 6.66 ppm, which is assigned to the hydrogen in the haloethyl group. This demonstrates that this hydrogen arose from the solvent or from a pool of hydrogen atoms that readily exchanges with the solvent.

The ^{19}F NMR spectra of synthetic CTFG and CTFG formed by cytosolic fractions were identical, but the ^{19}F NMR spectrum of CTFG formed by microsomal fractions was different. The ^{19}F chemical shifts and coupling constants are given in Figure 2. The rationale behind these assignments has been presented (Dohn & Anders, 1982a). Figure 3 shows the upfield multiplets of CTFG formed by cytosolic and microsomal fractions, which were assigned to F_c (Figure 2) on the basis of F–F and H–F coupling constants. This multiplet in the spectrum of the cytosolic conjugate consists of two sets of lines, each set a doublet of triplets, which indicates that the methine fluorines in the two diastereomers differ in chemical shift by 0.12 ppm and that the two diastereomers are present in equal amounts. The spectrum of CTFG formed by microsomal fractions (Figure 3B) shows signals attributable to both diastereomers, but the isomer with the larger upfield shift predominates by a factor of 4.5–5.0 to 1.

The spectra of F_a and F_b (not shown) from synthetic CTFG and CTFG formed by cytosolic fractions also revealed differences in chemical shifts between the two diastereomers and indicated that the isomers were present in equal amounts. The spectra of F_a and F_b of CTFG formed by microsomal fractions again indicated the predominance of one diastereomer. Because of the smaller separation of the diastereomeric fluorines in this region of the spectra, it is easier to visualize the differences between the preparations by examination of the spectra of conjugates formed in the presence of deuterium oxide, which lack H–F couplings (Figure 4). The microsomal preparation was enriched in the diastereomer in which F_a is 0.04 ppm more upfield and F_b is 0.02 ppm more downfield. In the deuterated analogues, F_a and F_b resonate 0.16 ppm upfield and F_c resonates 0.72 ppm upfield from the corresponding resonances in the proton-containing analogues. This is consistent with the reported effects of deuterium substitution on ^{19}F chemical shifts (Hansen, 1983).

The production of unequal amounts of two diastereomers by the microsomal glutathione *S*-transferase system was observed in three experiments, including the reaction conducted in deuterium oxide. Similarly, production of equal amounts of the diastereomers was observed with three cytosolic prep-

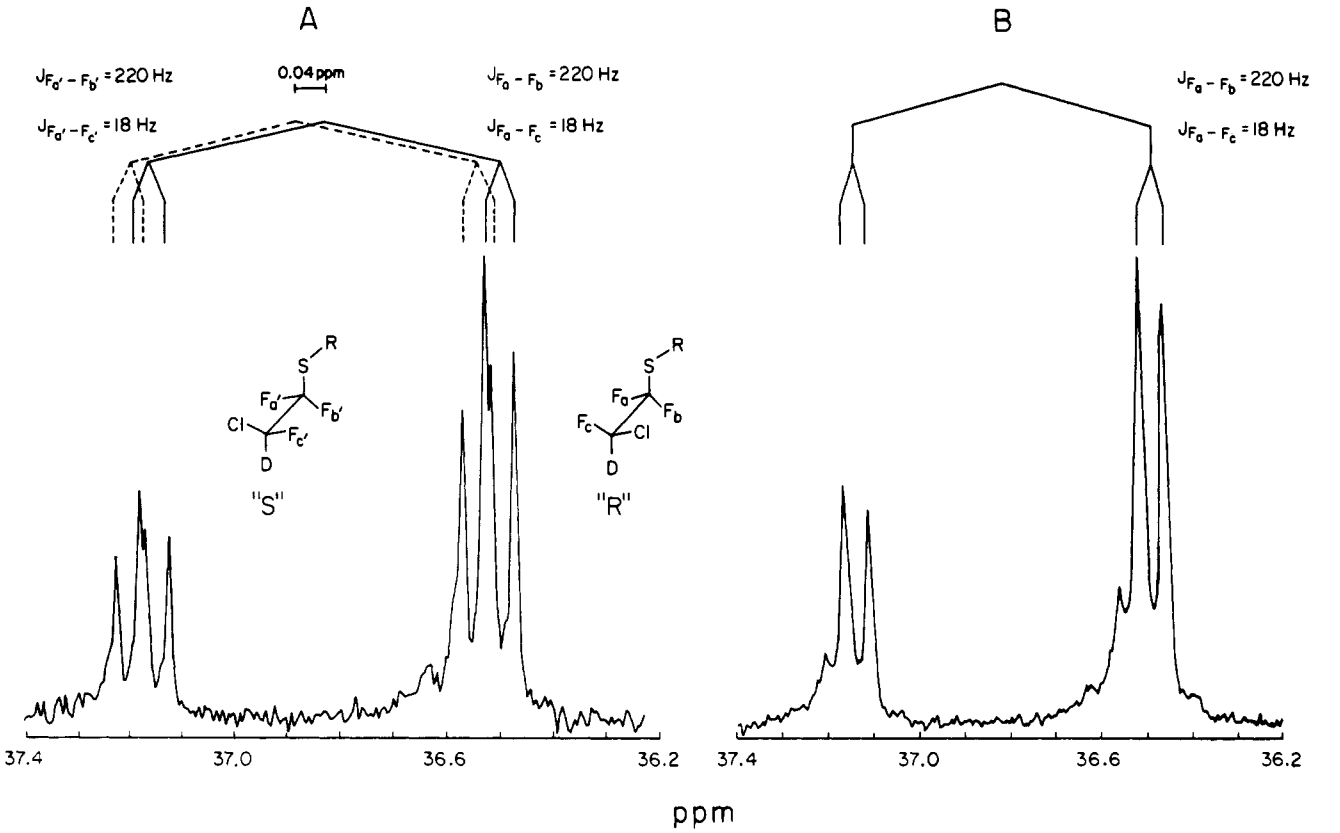


FIGURE 4: ^{19}F NMR of F_a in purified, biosynthetic CTFG from reactions conducted in deuterium oxide. Chemical shifts are downfield from potassium fluoride. (A) CTFG produced by cytosolic transferase activity; (B) CTFG produced by microsomal transferase activity. The designation of stereoisomers as "*R*" and "*S*" is arbitrary.

arations. A single preparation of partially purified CTFG from the 10000*g* pellet showed a diastereomeric enrichment similar to that seen in the microsomal product.

Bulk reactions of CTFE with glutathione catalyzed by the three subcellular fractions were lyophilized without prior removal of protein and then examined by ^{19}F NMR. There was no evidence of fluorine-containing metabolites other than CTFG, and the diastereomeric composition of the products was the same as described above for the purified material.

Secondary Ion Mass Spectrometry. The negative secondary ion mass spectra of the synthetic and the two biosynthetic preparations of CTFG were identical within the limiations of the technique. Exact comparisons of relative intensities of various ions in different mass spectra are not valid with this technique, because variation in the relative intensities of ions occurs in successive scans of the same sample. This variability applies to ions derived from the glycerol matrix as well as from the sample.

The major ions derived from CTFG and their postulated structures are shown in Figure 5. Ions containing the (2-chloro-1,1,2-trifluoroethyl)thio moiety were shifted to 1 mass unit higher in the spectra of the derivatives prepared in deuterium oxide. The ions at m/z 129, 131, 149, and 151 were also prominent in the mass spectrum of CTFC, which supports the postulated structures. The ion at m/z 128, which is assigned to the pyroglutamate anion, was almost absent in the mass spectrum of CTFC (≤1% of the base peak). All ions believed to contain chlorine were present in doublets separated by 2 mass units. The ratios of the doublets were not reproducible; the ^{37}Cl-containing ions were particularly overrepresented in the quasi-molecular ion cluster.

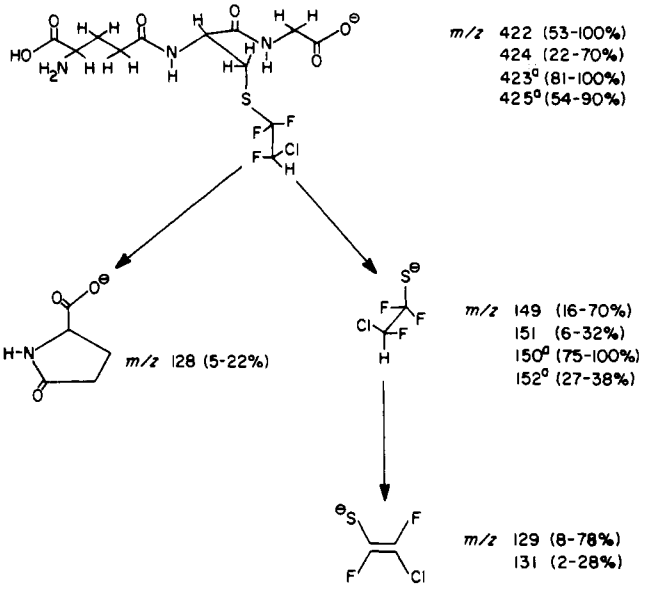


FIGURE 5: Negative ions generated from CTFG in glycerol by secondary ion bombardment with cesium ions. Values are percentages of the base peak, which was usually at m/z 183, corresponding to the glycerol dimer – H^+. In some spectra, however, the base peak was an ion derived from CTFG. Values are the ranges of two scans from synthetic, cytosolic, and microsomal CTFG and, in some cases, spectra from cytosolic and microsomal CTFG prepared in deuterium oxide. [a] Values from cytosolic and microsomal CTFG prepared in deuterium oxide.

Discussion

The identity of the ^1H NMR spectra, the mass spectra, and the HPLC retention times of the products isolated from cytosolic and microsomal incubations with synthetic CTFG support the conclusion that the three methods of preparation yield the same compound. Moreover, the magnitude of the H–F coupling constants indicates unequivocally that addition

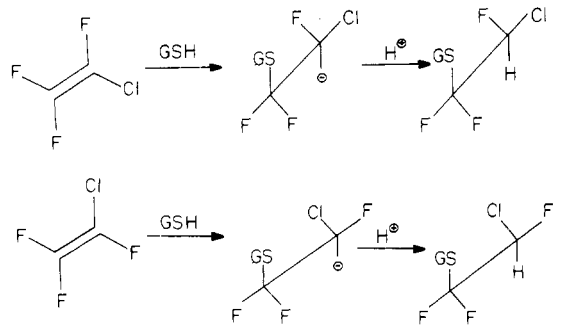


FIGURE 6: Possible orientations of CTFE relative to the thiolate portion of glutathione on the enzyme surface. GSH = glutathione.

of the thiol occurred at the carbon bearing two fluorines and not at the carbon bearing both chlorine and fluorine (Dohn & Anders, 1982a). This assertion is further substantiated by the effects of deuterium substitution on the ^{19}F NMR spectrum. Deuterium substitution caused a marked broadening of the F_c resonance, an effect consistent with geminal ^2H–F substitution; deuterium substitution also led to a simplification of the F_a and F_c resonances without line broadening, which occurred with vicinal ^2H–F substitutions. Finally, the relative magnitudes of the deuterium-induced chemical shifts are consistent with those expected for F_c being geminal to deuterium (Hansen, 1983). The differences seen in ^{19}F NMR spectra of the preparations are fully explicable by the formation of unequal amounts of two diastereomers by the microsomal enzyme system.

The formation of predominantly one diastereomer by the microsomal enzyme system requires that CTFE binds to the enzyme in only one of two possible orientations (Figure 6) and requires that protonation of the carbanionic intermediate occurs before either release from the enzyme or rotation about the carbon–carbon bond of the *S*-(haloethyl) group. It is possible that the microsomal enzyme contains a proton donor at the active site that rapidly protonates the newly formed carbanion. The lack of stereospecificity of the cytosolic enzyme might be explained by a number of factors. These include (i) binding of CTFE to the enzyme in either of the two possible orientations, (ii) release of the carbanionic intermediate before protonation, (iii) rotation about the carbon–carbon bond of the *S*-(haloethyl) group before protonation, and (iv) protonation of the carbanion from either side while on the enzyme surface, either by the solvent or by a nearby functional group on the enzyme.

CTFE is an excellent substrate for rat hepatic cytosolic and microsomal glutathione *S*-transferases (Table I). Because this substrate is a gas at room temperature, the kinetic constants cannot be easily determined. The high specific activities observed leave little doubt, however, that glutathione conjugation is an important route of CTFE metabolism in vivo. It is interesting that the specific activity of microsomal glutathione *S*-transferase activity is higher than the cytosolic transferase. Although the glutathione *S*-transferases were originally described and studied as soluble proteins in mammalian liver [reviewed in Chasseaud (1973)], recent reports have firmly established the existence of microsomal forms of these enzymes (Kraus & Gross, 1979; Friedberg et al., 1979; Morgenstern et al., 1980). These reports, however, dealt with substrates that were preferentially conjugated by the cytosolic transferases. The nephrotoxins hexachloro-1,3-butadiene and tetrafluoroethene are better substrates for microsomal glutathione *S*-transferases than for the cytosolic enzymes (Wolf et al., 1984; Odum and Green, 1984). It must be emphasized that these experiments, and the experiments reported here, dealt with crude subcellular fractions. No data are presently available on the reactivity of halogenated alkenes with purified transferase isozymes of either cytosolic or microsomal origin.

The results presented in this paper show that the conjugation of CTFE with glutathione proceeds as an addition reaction. Although the formation of products attributable to an addition/elimination reaction, namely, *S*-(trihalovinyl)glutathione conjugates, was sought in the present study, no such compounds were identified. Reactions of CTFE and other highly fluorinated analogues with sulfur nucleophiles proceed by displacement of fluoride to yield alkenes only in dry, aprotic solvents (Chambers & Mobbs, 1967). Reaction of CTFE with sulfur nucleophiles in protic solvents (e.g., ethanol) proceeds by addition yielding *S*-(2-chloro-1,1,2-trifluoroethyl) derivatives. Although the reaction of CTFE with sulfur nucleophiles in water has not been well studied, it is reasonable to assume that the reaction proceeds by addition and, therefore, CTFG is the expected product in the experiments described herein. Tetrafluoroethene also undergoes an addition reaction with glutathione under physiological conditions (Odum & Green, 1984), whereas the chlorinated alkene hexachloro-1,3-butadiene undergoes an addition/elimination reaction under similar conditions (Wolf et al., 1984).

When large-scale incubations of CTFE and glutathione with the various subcellular fractions were lyophilized without fractionation and examined by ^{19}F NMR, no products other than CTFG were observed. Specifically, no fluoride ion was observed. Although it is difficult to place a lower limit on the rate of fluoride ion formation in these experiments, this ion gives a sharp singlet in the ^{19}F NMR spectrum, whereas the individual fluorines of CTFG are split into complex multiplets. A conservative estimate of the lower limit of fluoride ion formation and, therefore, *S*-(trihalovinyl)glutathione formation is no more than 5% of the rate of CTFG formation.

Finally, the relevance of CTFG formation to CTFE nephrotoxicity is of importance. Both CTFG and CTFC are potent nephrotoxins, and the nephrotoxicity produced by the compounds is identical with that produced by CTFE (D. R. Dohn and M. W. Anders, unpublished observations). Recently, it has been reported that *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine is a substrate for rat kidney cysteine conjugate β-lyase (Odum & Green, 1984). CTFC is also a substrate for this enzyme (D. R. Dohn and M. W. Anders, unpublished observations), which is consistent with the known preference of cysteine conjugate β-lyase for a strongly electronegative group on the sulfur atom (Tateishi et al., 1978; Stevens & Jakoby, 1983). The action of cysteine conjugate β-lyase would produce 2-chloro-1,1,2-trifluoroethanethiol (Figure 1). This compound was synthesized by the X-ray-induced addition of hydrogen sulfide to CTFE (Harris & Stacey, 1963); in addition, passage of the gaseous thiol over potassium fluoride led to the formation of 2-chloro-2-fluorothionoacetyl fluoride (Figure 1). Elimination of fluoride ion from the thiolate may occur in aqueous solution and lead to the formation of the highly reactive thionoacyl fluoride, which may be the ultimate nephrotoxin produced from CTFE. This scheme accounts for the production of fluoride from CTFE and is consistent with the known increase in urinary and serum fluoride concentrations observed in rats after CTFE exposure (Potter et al., 1981; Buckley et al., 1982). Alternatively, 2-chloro-1,1,2-trifluoroethanethiol may cyclize to yield 2,2,3-trifluorothirane, but evidence for such a rearrangement is lacking. Identification of the reactive metabolite formed would advance the understanding of the mechanism by which CTFE causes kidney damage.

Finally, although the reactions of cytosolic glutathione *S*-transferases proceed with inversion of configuration (Mangold & Abdel-Monem, 1982) and with regioselectivity (Monks et al., 1982; Watabe et al., 1983), the present study is the first to demonstrate the induction of a chiral center by the microsomal glutathione *S*-transferases. Studies on the regio- and stereospecificities of the microsomal glutathione *S*-transferases are warranted and may reveal information on the mechanism of the reaction.

Registry No. Chlorotrifluoroethene, 79-38-9; glutathione *S*-transferase, 50812-37-8; glutathione, 70-18-8; (*R*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione, 97058-30-5; (*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione, 97058-31-6.

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Carbon Isotope Effect on Dehydration of Bicarbonate Ion Catalyzed by Carbonic Anhydrase†

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ABSTRACT: The carbon-13 kinetic isotope effect on the dehydration of HCO_3^- by bovine carbonic anhydrase has been measured. To accomplish this, bicarbonate was added to a buffer solution at pH 8 containing carbonic anhydrase under conditions where purging of the product CO_2 from the solution is rapid. Measurement of the isotopic composition of the purged CO_2 as a function of the concentration of carbonic anhydrase permits calculation of the isotope effect on the enzymic reaction. The isotope effect on the dehydration is $k^{12}/k^{13} = 1.0101 \pm 0.0004$. This effect is most consistent with a ping-pong mechanism for carbonic anhydrase action, in which proton transfer to or from the enzyme occurs in a step separate from the dehydration step. Substrate and product dissociation steps are at least 2–3-fold faster than the hydration/dehydration step.

Carbonic anhydrase catalyzes the hydration of carbon dioxide and its reverse, the dehydration of bicarbonate ion (Pocker & Sarkanen, 1978; Silverman & Vincent, 1984). The enzyme is nearly ubiquitous in living systems, playing a key role both in animal (Pocker & Sarkanen, 1978) and in plant (Reed & Graham, 1981) metabolism. Structurally, carbonic anhydrase is a simple enzyme, having a single subunit with a molecular weight near 30 000. All forms of the enzyme studied to date contain zinc, but there are no other cofactors (Pocker & Sarkanen, 1978). X-ray crystal structures have been reported for several forms of the enzyme (Liljas et al., 1972; Kannan et al., 1975).

The turnover number for carbonic anhydrase from mammalian erythrocytes is among the highest known for any enzyme. The overall reaction rate may be limited at least in part

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