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RAT LIVER METABOLISM AND TOXICITY OF 2,2,2-TRIFLUOROETHANOL

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Abstract—2,2,2-Trifluoroethanol (TFE) is a metabolite of anesthetic agents and chlorofluorocarbon alternatives. Its toxicity in rats is a consequence of its metabolism to 2,2,2-trifluoroacetaldehyde (TFAld) and then to trifluoroacetic acid (TFAA). The enzymes involved in the toxic metabolic pathway have been investigated in this study. For the reaction of TFE to TFAld, the major hepatic metabolism associated with toxicity (as assessed by pyrazole-inhibitability) was NADPH dependent and occurred in the microsomes, whereas for TFAld conversion to TFAA, NADPH-dependent microsomal metabolism was significant, but mitochondrial and cytosolic metabolism in the presence of NADPH were also major contributors. NADPH-dependent hepatic microsomal metabolism of TFE to TFAld and TFAld to TFAA was inhibited by carbon monoxide, 2-allyl-2-isopropylacetamide, SKF-525A, metyrapone, imidazole, and pyrazole, and both reactions were oxygen dependent. The metabolism of TFE to TFAld was inhibited by diethyldithiocarbamate, a specific inhibitor of cytochrome P4502E1, and by a monoclonal antibody to P4502E1, whereas the metabolism of TFAld was inhibited by neither. Ethanol pretreatment of rats enhanced the V_{max} for hepatic microsomal metabolism of TFE to TFAld from 5.3 to 9.7 nmol/mg protein/min, while for TFAld to TFAA the V_{max} was increased from 4.3 to 6.5 and the K_m was unaffected for both reactions. Phenobarbital pretreatment of the rats did not affect any of these kinetic parameters. Coadministration of ethanol and a lethal dose of TFE very markedly decreased the lethality. Both the lethality (LD_{50} 0.21 to 0.44 g/kg) and the metabolic kinetic parameters $[(V_{max}/K_m)_H(V_{max}/K_m)_D = 4.2]$ were affected markedly when deuterated TFE replaced TFE. In contrast, deuteration of TFAld did not affect its lethality or rates of metabolism, but did affect its K_m. Taken together these results indicate that P4502E1 catalyzed toxicity-associated hepatic metabolism of TFE to TFAld, while TFAld metabolism was catalyzed by a P450 which was not P4502E1. The hepatic metabolism of TFAld was not associated with its toxicity, which has been determined previously to be associated with its intestinal metabolism.

Metabolism of the volatile anesthetic agent fluroxene to 2,2,2-trifluoroethanol (TFE)§ in rats is catalyzed by a variety of cytochromes P450 [1, 2], with toxic consequences. The product, TFE, is further metabolized to 2,2,2-trifluoroacetaldehyde (TFAld), and subsequently to trifluoroacetic acid (TFAA), with the toxicity of the anesthetic agent being ultimately associated with an intermediate in the pathway from TFAld to TFAA [3, 4]. Furthermore, TFAA is a metabolite of the clinically important anesthetic agents isoflurane and halothane, and TFAld is also a metabolite of isoflurane [5–7]. TFAA is a putative environmental product [8] of the hydrochlorofluorocarbons proposed as alternatives to the currently heavily used chlorofluorocarbons [9], and consequently it has the potential to be formed in the environment in enormous quantities. One of these hydrofluorocarbon alternatives, 1,1,1,2-tetrafluoroethane, is also presumed to be metabolized to TFE [10]. Taken together these results highlight the importance of developing mechanisms for the metabolism and toxicity of TFE and its metabolites.

Although TFE is a competitive inhibitor of ethanol oxidation by horse, human, and rat liver alcohol dehydrogenase [3, 11–13], it is not a substrate for this enzyme [3, 11]. Studies using inhibitors have also excluded possible roles for the enzymes aldehyde oxidase and aldehyde dehydrogenase in the *in vivo* metabolism of TFAld in rodents [3]. Indirect evidence however, has implicated cytochrome P4502E1 in the *in vivo* metabolism and toxicity of TFE [3, 14]. In particular, disulfiram and diethyldithiocarbamate, which specifically inhibit P4502E1 [15], inhibit the metabolism and toxicity of TFE in rats [3]. However, at the initiation of this study no evidence of the potential of P450 to catalyze the oxidation of an aldehyde to an acid had been reported. Subsequently, it was demonstrated in a mouse liver microsomal system [16], and in a reconstituted mouse liver P4502C system [17], that P450 can catalyze the conversion of aldehydes to carboxylic acids. In both studies confirmatory evidence for the involvement of P450 was the incorporation of oxygen-18 from molecular oxygen into the carboxylic acid formed. It has also been reported

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§ Abbreviations: TFE, 2,2,2-trifluoroethanol; TFAA, trifluoroacetic acid; TFAld, 2,2,2-trifluoroacetaldehyde; and P450, cytochrome P450.

recently that reconstituted rat P4502E1 and hepatic microsomes from acetone-treated rats catalyze the conversion of acetaldehyde to acetate [18]. Overall there is a dearth of information on which enzymes catalyze the reactions interconverting TFE, TFAld, and TFAA, and most importantly on which enzymes are involved in the toxicity of these compounds.

In this paper we report on our studies to elucidate the enzymes involved in the rat liver biotransformations of TFE and TFAld to TFAA, and relate these biotransformations to the toxicity of these compounds.

MATERIALS AND METHODS

Reagents. TFE and TFAA were obtained from the Aldrich Chemical Co. (Milwaukee, WI) and TFAld hydrate was from K & K Rare and Fine Chemicals (Plainview, NY). TFAld hydrate was incubated at 90°, in an open container, for several hours to remove contaminating TFE. The cofactors NAD, NADP, NADH, and NADPH, and the inhibitors diethyldithiocarbamate, pyrazole, imidazole, and metyrapone, were purchased from the Sigma Chemical Co. (St. Louis, MO). SKF- 525-A and 2-allyl-2-isopropylacetamide were gifts from Smith, Kline & French Laboratories (Philadelphia, PA) and Hoffmann-LaRoche (Nutley, NJ), respectively.

Deuterated TFE was prepared by a general method proposed for the preparation of low-boiling alcohols [19]. TFAA (13.6 g, 0.12 mol) was added dropwise over 1 hr to a stirred mixture of $LiALD_4$ (5.0 g, 0.12 mol) in diglyme (60 mL). Next the reaction mixture was heated at 100° for 1 hr, and then cooled to 0–10°, and *n*-butoxyethanyl alcohol (42 g, 0.36 mol) was added. The product was then distilled off in a fraction which boiled at 140–160°, and which, on redistillation, yielded a fraction boiling at 75–80°, 3.41 g (29% of theoretical). The preparation yielded a single peak on gas chromatography. The proton NMR of an authentic sample of TFE exhibited resonances at δ 3.2 (1H, triplet) and δ 2.9 (2H, quintet), which represent the hydroxyl and methylene protons, respectively. The corresponding proton NMR of deuterated TFE exhibited a resonance signal only at δ 3.4 (1H, singlet) representing the hydroxyl proton, and indicating that the methylene carbon was fully deuterated. This was confirmed with deuterium NMR of the deuterated TFE, which exhibited a resonance signal at δ 3.8 (2D, quartet). The intensity of this signal, relative to that of an internal standard of deuterated chloroform, indicated complete deuteration of d_2-TFE.

Deutero TFAld hydrate was prepared by methods based on published syntheses of TFAld hydrate [20, 21]. To TFAA (28.5 g, 0.25 mol) in dry either (250 mL) at 0–5°, under dry nitrogen, was added $LiALD_4$ (5.0 g, 0.13 mol), slurried in dry ether (200 mL). $LiALD_4$ was added over a period of 1 hr. The reaction mixture was stirred for a further 1 hr and H_2O (10 mL) and then H_2SO_4 [20 mL in H_2O (50 mL)] were added, and the ether layer was separated off. The aqueous layer was extracted with ether, which was added to the original ether solution. The ether was removed *in vacuo* and the residue was taken up in dilute NaOH solution, which was extracted twice with ether. The ether solution was dried with Na_2SO_4 and the ether removed *in vacuo*. The residue was distilled to yield deuterated TFAld hydrate, b.p. 107°, 20.6 g (71% yield), which yielded a single peak on gas chromatography. The MS exhibited a molecular ion at 99, while TFAld exhibited an ion at 98.

Animals and treatment. Male Wistar rats (200–250 g) were obtained from a colony maintained by this Center. Animals were acclimated for at least 5 days at 22° with a 12-hr on/12-hr off light cycle, and had free access to feed (Purina Laboratory Rodent Chow) and water, except in the case of the feed for the 24 hr before death by carbon dioxide overdose. All animal procedures were approved by the Institutional Animal Care and Use Committee of this institution.

Subcellular fractions were prepared from rat liver homogenates by differential ultracentrifugation: mitochondria by the method of Fowler *et al.* [22], microsomes as previously described [23], and cytosol was the supernatant from the 100,000 *g* spin. The preparations were immediately frozen and used after being thawed only once. The purity of the subcellular fractions was not determined.

For studies of the lethality of TFE and TFAld and their deuterated analogs, three doses of each test compound diluted 1 to 5 in saline were injected i.p. into groups of five rats per dose; the rats were fasted for 12 hr prior to drug administration. Rats were observed for 6 days and all moribund animals were killed. In one study, rats were injected i.p. with TFE (0.25 g/kg) and then immediately with ethanol (12.5%, w/v) in saline (1.2 g ethanol/kg), followed by the same doses of ethanol at 4 and 8 hr after TFE administration. Controls received TFE and saline only.

For induction studies rats were treated as follows: phenobarbital (100 mg/kg/day) was administered i.p. for 2 days and animals were killed on day 4; or ethanol was administered in the drinking water at 5% on days 1 and 2, 10% on days 3 and 4, and 20% on days 5–15 and animals were killed on day 15.

Metabolism of TFE and TFAld. TFAld and TFAA in microsomal reaction mixtures were analyzed by gas chromatography as previously described by Fraser and Kaminsky [4]. The rates of TFE metabolism were determined from the rates of TFAld formation, and the rates of TFAld metabolism were determined from the rates of TFAA formation.

For investigations of the kinetics of the metabolism of TFE or TFAld or their deuterated analogs by rat hepatic subcellular fractions, TFE or TFAld (0–200 mM) was incubated with 2 mg subcellular fraction protein at 37° in Tris–HCl buffer (50 mM, pH 7.4) containing $MgCl_2$ (10 mM) in a final volume of 1.0 mL. After preincubation at 37° for 1 min the reaction was initiated by the addition of NAD, NADH, NADP, or NADPH (1.5 mg/mL) and terminated after 5 min (TFE) or 20 min (TFAld) by cooling in an ice bath. In all other metabolic studies TFE and TFAld concentrations were 100 mM. In some reactions pyrazole (10 mM) was added prior to initiation of the reaction. Inhibitors of microsomal

metabolism were preincubated for 5 min with the reaction mixture prior to initiation of the reaction. Other details are included in the tables and figures.

The role of ethanol-inducible cytochrome P4502E1 in rat liver microsomal metabolism was probed using a monoclonal antibody to cytochrome P4502E1, designated 1-91-3 [24], which was provided by Dr. C. S. Yang, Rutgers University. Antibody was incubated with microsomes (20 mg/mL) at room temperature for 30 min. The microsomes were then diluted to a final concentration of 2 mg/mL with Tris–HCl buffer, TFE or TFAld (300 mM) was added, and the mixture was incubated at 37° for 1 min. The reaction was initiated with NADPH (2.0 mg/mL) and continued for 5 min with TFE or for 15 min with TFAld before being terminated by cooling in an ice-bath. The entire reaction volume of 0.2 mL was assayed for TFAld or TFAA. Ascites fluid at equivalent quantities to antibody was used in controls.

RESULTS

Time courses of the NADPH-dependent hepatic microsomal metabolism of TFE to TFAld (Fig. 1) and TFAld to TFAA (Fig. 2) were essentially linear for 5 and 20 min, respectively.

The rates of metabolism of TFE to TFAld were determined in subcellular rat liver preparations in the presence of various cofactors, with and without the inhibitor pyrazole (Table 1). The microsomal fraction produced the highest rates of metabolism/mg protein with NADPH as cofactor. NADH also supported microsomal metabolism of TFE but at 49% of the rate with NADPH, while NAD and NADP were no more effective than endogenous cofactors. Pyrazole (10 mM) only inhibited NADPH-supported microsomal metabolism.

Cytosolic enzymes catalyzed the metabolism of TFE, but at low rates relative to microsomal metabolism (24% at best). Metabolism was not stimulated by added cofactor and was not susceptible to pyrazole (10 mM) inhibition.

Both NADH and NADPH supported mitochondrial metabolism of TFE to TFAld, but at 37 and 56%, respectively, of the corresponding rates

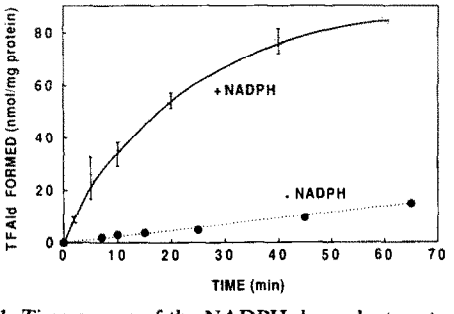

Fig. 1. Time course of the NADPH-dependent, untreated rat liver microsomal metabolism of 2,2,2-trifluoroethanol to 2,2,2-trifluoroacetaldehyde (TFAld). Reactions were conducted at 37° and with 2 mg of microsomal protein/mL. Other details are as described in Materials and Methods. Values are means ± SD, N = 3.

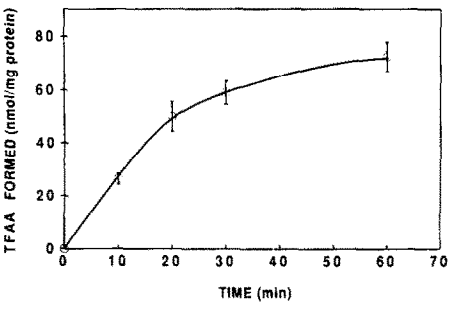

Fig. 2. Time course of the NADPH-dependent, untreated rat liver microsomal metabolism of 2,2,2-trifluoroacetaldehyde to trifluoroacetic acid (TFAA). Reactions were conducted at 37° and with 2 mg of microsomal protein/mL. Other details are as described in Materials and Methods. Values are means ± SD, N = 3.

with microsomes. Only the NADPH-dependent metabolism was inhibited by the 10 mM pyrazole.

The rates of metabolism of TFAld to TFAA were determined in subcellular rat liver preparations in the presence of various cofactors, with and without the inhibitor pyrazole (Table 2). With microsomes TFAld oxidation was supported most effectively by NADPH, with NADH being 27% as effective. Only the NADPH-dependent metabolism was inhibited by 10 mM pyrazole.

Cytosolic enzymes catalyzed the metabolism of TFAld, but at 64% of the rate of NADPH-dependent microsomal metabolism. Metabolism was apparently independent of added cofactor and, at 10 mM pyrazole, only the metabolism in the presence of NADPH was inhibited.

Only NADPH of the cofactors tested produced a significant increase in TFAld metabolism by mitochondria. The rate was equivalent to the NADPH-dependent, microsome-catalyzed rate, and metabolism was inhibited by pyrazole.

The NADPH-dependent microsomal metabolism of TFE to TFAld was statistically significantly inhibitable by all of the reagents tested, particularly by diethyldithiocarbamate (Table 3). Metabolism of TFAld to TFAA under the same conditions pertaining to TFE metabolism was more susceptible to inhibition by SKF 525-A, imidazole, and pyrazole, was not inhibited significantly by diethyldithiocarbamate, and was similarly inhibited by carbon monoxide (Table 4). Both reactions were inhibited when the oxygen in these solutions was replaced by nitrogen, indicating an oxygen requirement.

Ethanol, but not phenobarbital, induction of the rats caused a significant increase relative to uninduced controls in the V_{max} value for NADPH-dependent hepatic microsomal metabolism of TFE to TFAld and TFAld to TFAA (Table 5) when expressed per mg protein, but not when expressed per nmol P450. The corresponding K_m values were not affected by phenobarbital or ethanol treatment of the rats. Ethanol induction, however, did not increase rates of NADH-dependent hepatic microsomal metabolism of TFE to TFAld (data not shown).

TFE competitively inhibited the NADPH-dependent hepatic microsomal metabolism of TFAld to

Table 1. 2,2,2-Trifluoroethanol (TFE) metabolism to 2,2,2-trifluoroacetaldehyde (TFAld) by rat liver subcellular fractions; the influence of cofactors and pyrazole*

Subcellular fraction and cofactor	TFAld (nmol/min/mg protein)	% Inhibition by pyrazole† (10 mM)
Microsomes		
None	0.29 ± 0.02‡	0.0
NAD	0.46 ± 0.04	0.0
NADP	0.36 ± 0.03	0.0
NADH	2.30 ± 0.15	0.0
NADPH	4.73 ± 0.10	33.0
Boiled	0.07 ± 0.01	—
Cytosol		
None	0.85 ± 0.05	0.0
NAD	1.14 ± 0.25	0.0
NADP	0.37 ± 0.04	0.0
NADH	0.38 ± 0.04	0.0
NADPH	0.33 ± 0.02	0.0
Mitochondria		
None	0.37 ± 0.03	0.0
NAD	0.33 ± 0.04	0.0
NADP	0.30 ± 0.05	0.0
NADH	0.86 ± 0.02	0.0
NADPH	2.63 ± 0.12	33.2
Boiled	0.21 ± 0.06	—

* Rates of metabolism were determined as described in Materials and Methods.
† Pyrazole inhibition is designated as 0 when pyrazole produced no significant ($P < 0.01$) decrease in rate. A "—" indicates that the experiment was not conducted.
‡ Values are means ± SD with N = 3 different microsomal preparations.

Table 2. 2,2,2-Trifluoroacetaldehyde (TFAld) metabolism to trifluoroacetic acid (TFAA) by rat liver subcellular fractions; the influence of cofactors and pyrazole*

Subcellular fraction and cofactor	TFAA (nmol/min/mg protein)	% Inhibition by pyrazole† (10 mM)
Microsomes		
None	0.19 ± 0.05‡	46.1
NAD	0.34 ± 0.10	0.0
NADP	0.14 ± 0.02	0.0
NADH	0.44 ± 0.11	0.0
NADPH	1.63 ± 0.15	57.3
Boiled	0.18 ± 0.08	—
Cytosol		
None	0.93 ± 0.18	0.0
NAD	0.97 ± 0.12	0.0
NADP	0.84 ± 0.14	0.0
NADH	1.05 ± 0.05	0.0
NADPH	0.89 ± 0.14	30.1
Boiled	0.19 ± 0.12	—
Mitochondria		
None	0.87 ± 0.20	0.0
NAD	0.93 ± 0.20	0.0
NADP	0.65 ± 0.04	0.0
NADH	0.93 ± 0.12	0.0
NADPH	1.62 ± 0.18	32.6
Boiled	0.22 ± 0.09	—

* Rates of metabolism were determined as described in Materials and Methods.
† Pyrasole inhibition is designated as 0 when pyrazole produced no significant ($P < 0.01$) decrease in rate. A "—" indicates that the experiment was not conducted.
‡ Values are means ± SD with N = 3 different microsomal preparations.

Table 3. Inhibitors of NADPH-dependent hepatic microsomal metabolism of 2,2,2-trifluoroethanol (TFE) to 2,2,2-trifluoroacetaldehyde (TFAld)*

Inhibitor	Concentration	Rate of TFAld formation† (nmol/min/mg protein)	% Inhibition
None		4.01 ± 0.11	
Metyrapone	1 mM	3.42 ± 0.10‡	14.7
	10 mM	3.25 ± 0.08‡	18.9
SKF 525-A	1 mM	3.82 ± 0.20	4.8
	10 mM	2.97 ± 0.05‡	26.0
AIA§	10 mM	3.05 ± 0.12‡	24.0
Imidazole	1 mM	2.32 ± 0.17‡	42.3
Pyrazole	1 mM	2.99 ± 0.09‡	25.6
	10 mM	2.34 ± 0.07‡	41.6
	100 mM	1.81 ± 0.12‡	55.0
CO‖		2.70 ± 0.11‡	32.6
N_2¶		0.80 ± 0.11‡	80.2
Diethyldithiocarbamate**	100 μM	1.12 ± 0.12‡	72.0

* Rates of metabolism were determined as described in Materials and Methods. Reactions were run for 10 min.

† Values are reported as means ± SD with N = 3–4.

‡ Significantly different from control (none), $P < 0.005$ (Student's t-test).

§ 2-Allyl-2-isopropylacetamide (AIA) was incubated with microsomes and NADPH for 10 min at 37°; then TFE and more NADPH were added and the reaction mixture was incubated for a further 10 min. The control consisted of the same treatment but without AIA.

‖ CO mixed 9:1 with O_2 was bubbled through the microsomal suspension for 1 min prior to the addition of TFE and NADPH. The control was bubbled with N_2 mixed 9:1 with O_2.

¶ The microsomal suspension was bubbled with N_2 for 1 min prior to the addition of TFE and NADPH.

** Diethyldithiocarbamate was incubated with microsomes and NADPH for 10 min prior to the addition of TFE.

TFAA (Fig. 3 and Table 6). TFE at 75 and 150 mM did not affect significantly the V_{max} for TFAld oxidation, but at 150 mM TFE the K_m for this reaction was increased significantly.

The NADPH-dependent, ethanol-induced microsomal metabolism of TFE to TFAld was dose-dependently inhibited by a monoclonal antibody to rat hepatic cytochrome P4502E1 (Fig. 4). At an antibody ratio of 2 mg/mg microsomal protein the reaction was inhibited by 81%. The control ascites fluid produced no comparable inhibition under the same conditions. The NADPH-dependent microsomal metabolism of TFAld to TFAA was not inhibited by the anti P4502E1.

The lethalities of both TFE and TFAld are very sensitive to dose, and thus LD_{50} studies utilized narrow dose ranges of these compounds and their deuterated analogs. For TFE, doses ranged from 0.15 to 0.25 g/kg, for d_2-TFE from 0.30 through 0.50 g/kg, and for TFAld and D_1-TFAld from 0.25

Table 4. Inhibition of NADPH-dependent hepatic microsomal metabolism of 2,2,2-trifluoroacetaldehyde (TFAld) to trifluoroacetic acid (TFAA)

Inhibitor	Concentration	Rate of TFAA formation* (nmol/min/mg protein)	% Inhibition
None		1.33 ± 0.06	
SKF 525-A	10 mM	0.57 ± 0.04†	57.1
Imidazole	1 mM	0.40 ± 0.05†	70.2
Pyrazole	10 mM	0.51 ± 0.06†	61.4
CO‡		0.81 ± 0.10†	38.8
N_2§		0.47 ± 0.06†	81.4
Diethyldithiocarbamate‖	300 μM	1.32 ± 0.07	0

* Rates of metabolism were determined as described in Materials and Methods. Reactions were run for 20 min. Values are means ± SD with N = 3–4.

† Significantly different from control (none), $P < 0.005$ (Student's t-test).

‡ CO mixed 9:1 with O_2 was bubbled through the microsomal suspension for 1 min prior to the addition of TFAld and NADPH. The control was bubbled with N_2 mixed 9:1 with O_2.

§ Microsomal suspension was bubbled with N_2 for 1 min prior to the addition of TFAld and NADPH.

‖ Diethyldithiocarbamate was incubated with microsomes and NADPH for 10 min prior to the addition of TFAld.

Table 5. Effects of ethanol or phenobarbital pretreatment of rats on the rates of NADPH-dependent, hepatic microsomal metabolism of 2,2,2-trifluoroethanol (TFE) and 2-trifluoroacetaldehyde (TFAld)*

Reaction	Inducer†	V_{max} (nmol/min/mg protein)	K_m (mM)
TFE → TFAld	None	5.3 ± 0.4 (4.7)‡	49.3 ± 6.7
	EtOH	9.7 ± 1.1§ (5.0)	60.2 ± 3.0
	PB	6.1 ± 0.1 (4.4)	45.5 ± 5.0
TFAld → TFAA	None	4.3 ± 0.1 (3.8)	110.6 ± 23.5
	EtOH	6.5 ± 0.8‖ (3.4)	138.5 ± 9.9
	PB	4.2 ± 0.7 (3.0)	86.4 ± 14.9

* Rats were induced and rates of metabolism determined as described in Materials and Methods. Results were obtained from Lineweaver–Burk plots of at least three experiments and are reported as means ± SD.

† Cytochrome P450 concentrations in hepatic microsomes from untreated, phenobarbital (PB)-induced or ethanol (EtOH)-induced rats were 1.13 ± 0.07, 1.93 ± 0.02, and 1.38 ± 0.03 nmol/mg protein, respectively.

‡ V_{max} values in parentheses are expressed in nmol/min/nmol P450.

§ Significantly different from untreated rat, $P < 0.01$ (Student's t-test).

‖ Significantly different from untreated rat, $P < 0.05$ (Student's t-test).

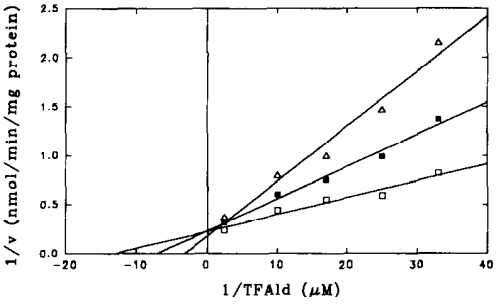

Fig. 3. Lineweaver–Burk plots of the rates of NADPH-dependent, untreated rat liver microsomal metabolism of (□) 2,2,2-trifluoroacetaldehyde to trifluoroacetic acid in the presence of 2,2,2-trifluoroethanol [(■) 75 mM, or (△) 150 mM]. Reactions were conducted at 37° with 2 mg of microsomal protein/mL. Lines are best fits by linear regression ($r > 0.98$). Other conditions are as described in Materials and Methods.

Table 6. Effect of 2,2,2-trifluoroethanol (TFE) on 2,2,2-trifluoroacetaldehyde (TFAld) metabolism to trifluoroacetic acid (TFAA) by hepatic microsomes*

Reaction conditions	V_{max}† (nmol/min/mg protein)	K_m† (mM)
Control	5.4 ± 1.2	101.8 ± 27.1
+ 75 mM TFE	4.1 ± 0.4	141.8 ± 19.3
+ 150 mM TFE	4.5 ± 0.8	293.8 ± 53.1‡

* Hepatic microsomes (2 mg/mL) were incubated with TFAld (30, 40, 60, 100, or 400 mM) in Tris–HCl buffer (50 mM, pH 7.4) containing $MgCl_2$ (10 mM) and TFE (0, 75, or 150 mM) for 5 min at 37° in a final volume of 1 mL. The reaction was initiated with NADPH (1.5 mg/mL), incubated for 20 min, and terminated by cooling in ice water. The reaction mixture was washed three times with diethyl ether (5 mL), evaporated almost to dryness, and resuspended in water (0.5 mL).

† V_{max} and K_m were calculated from Lineweaver–Burk plots. Values are means ± SD (N = 3).

‡ Significantly different from controls, $P < 0.025$ (Student's t-test).

to 0.30 g/kg. The LD_{50} of TFE administered i.p. to fasted rats (0.21 g/kg [2]) was increased to 0.44 g/kg with d_2-TFE, a 2.1-fold decrease in lethality. With TFAld hydrate the LD_{50} of approximately 0.26 g/kg was not altered when d_1-TFAld hydrate was administered in place of TFAld hydrate.

Co-administration of ethanol with TFE to rats overcame the lethal effects of the TFE. At 0.25 g TFE/kg four of five rats died, whereas at the same dose of TFE administered with ethanol zero of five died.

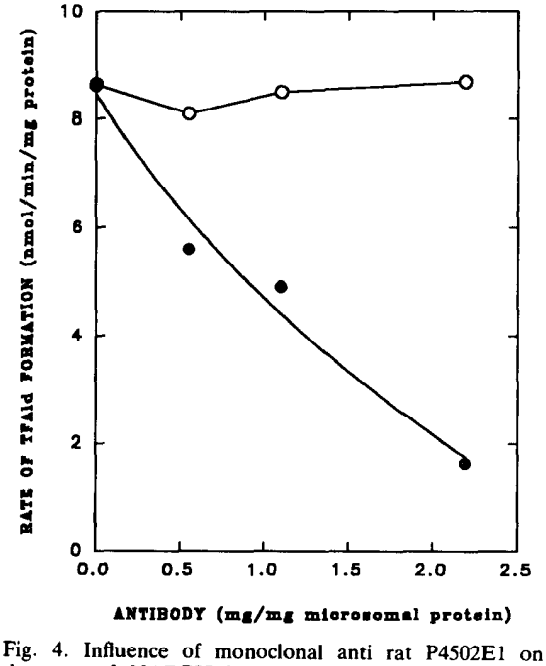

Fig. 4. Influence of monoclonal anti rat P4502E1 on the rate of NADPH-dependent, rat liver microsomal metabolism of 2,2,2-trifluoroethanol to 2,2,2-trifluoroacetaldehyde (TFAld). Key: (●) anti-P4502E1; and (○) control ascites fluid. Other conditions are as described in Materials and Methods.

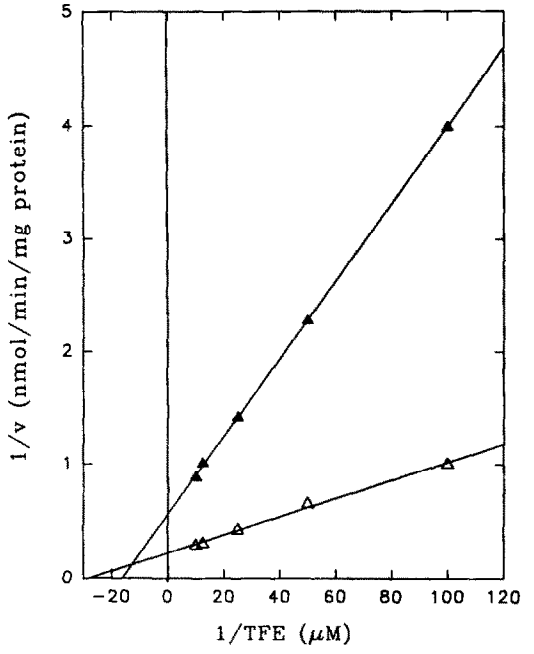

Fig. 5. Lineweaver–Burk plots of the rates of NADPH-dependent, uninduced rat liver microsomal metabolism of (△) 2,2,2-trifluoroethanol (TFE) and (▲) deuterated TFE to 2,2,2-trifluoroacetaldehyde. Reactions were conducted at 37° with 2 mg of microsomal protein/mL. Lines are best fits by linear regression ($r > 0.98$). Other conditions are as described in Materials and Methods.

The effect of replacement of C-1 protons by deuterium in TFE and TFAld hydrate on the kinetics of untreated rat hepatic microsomal metabolism to TFAld and TFAA, respectively, is shown in Figs. 5 and 6. In the case of TFE metabolism, V_{max} was decreased by deuteration from 4.4 to 1.7 nmol TFAld/min/mg protein, and K_m increased from 37.5 to 59.7 mM. For TFAld hydrate, V_{max} was unaffected by substitution with the deuterated substrate, 3.5 vs 3.6 nmol TFAA/min/mg, while K_m was increased from 71 to 213 mM. Thus, for TFE metabolism the observed deuterium isotope effect $(V_{max}/K_m)_H/(V_{max}/K_m)_D = 4.2$, while $V_H/V_D = 2.6$. For TFAld metabolism, the observed $(V_{max}/K_m)_H/(V_{max}/K_m)_D = 2.9$, with $V_H/V_D = 1$.

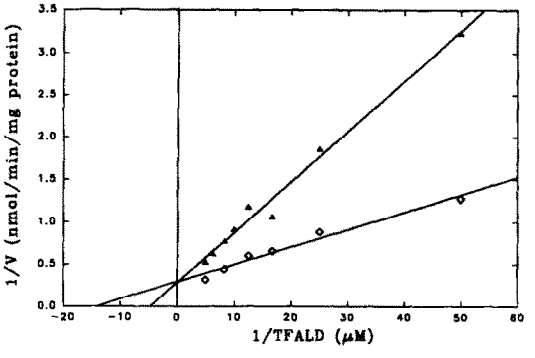

Fig. 6. Lineweaver–Burk plots of the rates of NADPH-dependent, untreated rat liver microsomal metabolism of (◇) 2,2,2-trifluoroacetaldehyde (TFAld), and (▲) deuterated TFAld to trifluoroacetic acid. Reactions were conducted at 37° with 2 mg of microsomal protein/mL. Lines are best fits by linear regression ($r > 0.98$). Other conditions are as described in Materials and Methods.

DISCUSSION

It has been clearly established previously that the expression of TFE toxicity in rats is dependent on its metabolism to TFAld and TFAA [2, 3]. While alcohol dehydrogenase [3, 11], aldehyde oxidase, and aldehyde dehydrogenase [3] have all been excluded as possible participants in TFE toxicity-related metabolism, the actual enzymes involved were unknown.

The objective of this study was to elucidate the TFE metabolism associated with its toxicity. Initial insights into the specific enzymes involved in TFE metabolism associated with its toxicity were gained from the elucidation of which hepatic subcellular metabolism of TFE and TFAld was pyrazole-inhibitable. Pyrazole has been demonstrated to readily inhibit TFE toxicity in rats [3]. The most prominent pyrazole-inhibitable TFE metabolism by the liver was NADPH dependent and occurred in the microsomal fraction, but the mitochondrial fraction also produced some pyrazole-inhibitable metabolism (Table 1). Thus, while all three subcellular fractions provide systems capable of metabolizing TFE to TFAld, microsomal and mitochondrial NADPH-dependent metabolism are most prominently associated with the toxicity of TFE. While the extent of microsomal contamination of the mitochondrial preparations was not evaluated, it would require in excess of 50% contamination to account for the observed mitochondrial activity—where actual contamination is likely to be only a few percent [25]. Because the mitochondrial pyrazole-inhibitable metabolism played a minor role relative to microsomal metabolism, only the latter was investigated further. The enzymes involved in the various other activities are not investigated in this paper.

In the case of TFAld, microsomal NADPH-dependent metabolism was also one of the most prominent of those that were pyrazole-inhibitable. Mitochondrial NADPH-dependent metabolism was also pyrazole-inhibitable, while the cytosolic metabolism, in the presence of only NADPH of the cofactors tested, was also pyrazole-inhibitable (Table 2). This latter result is difficult to explain since cytosolic metabolism of TFAld was apparently exogenous nicotinamide coenzyme independent, although the apparent independence could be a result of the presence of endogenous cofactors. Thus, all three subcellular fractions appear to support metabolism of TFAld associated with its toxicity. In this study emphasis is placed on NADPH-dependent microsomal enzymes.

The NADPH-dependent microsomal metabolism of both TFE and TFAld was oxygen dependent, carbon monoxide inhibitable, and was also inhibitable by a number of P450 inhibitors including pyrazole (Tables 3 and 4). Taken together these results

implicate one or more forms of P450 in the metabolism of TFE and TFAld which are associated with their toxicity. The observation that the hepatic microsomal metabolism of TFAld to TFAA was competitively inhibited by TFE (Table 6 and Fig. 3) suggests that TFE binds competitively to the enzyme which catalyzes the metabolism of TFAld to TFAA, and is not metabolized since other data (see later) indicated that NADPH-dependent microsomal metabolism of TFE and TFAld is not catalyzed by the same enzyme.

A possible enzyme candidate for the role of metabolizing TFE is P4502E1, which has been demonstrated to be capable of catalyzing the metabolism of a growing list of low molecular weight substrates including ethanol, benzene, styrene, CCl_4, $CHCl_3$, CH_2Cl_2, CH_3Cl, CH_3CCl_3, 1,2-dichloropropane, ethylene dichloride, ethylene dibromide, vinyl chloride, vinyl bromide, acrylonitrile, vinyl carbamate, ethyl carbamate, and trichloroethylene [15]. Several factors were presented in support of a role for P4502E1 in metabolizing these substrates, including use of the selective inhibitors diethyldithiocarbamate and its oxidized derivative disulfiram [15].

The involvement of P4502E1 as a major contributor to the hepatic microsomal metabolism of TFE to TFAld is strongly supported by the observations that the reaction was inhibited by diethyldithiocarbamate, a specific inhibitor of P4502E1 at the concentrations used [15, 26] (Table 3), and by anti P4502E1 (Fig. 4). The inhibition by anti P4502E1 of 81% of the microsomal activity indicates that P4502E1 is the major enzyme involved in NADPH-dependent hepatic microsomal metabolism of TFE.

There is substantial support in the literature for P4502E1-catalyzed metabolism of a primary alcohol to an aldehyde—most notably with ethanol as a substrate [27, 28]. Catalysis of the metabolism of an aldehyde to a carboxylic acid has also been reported previously for P4502E1 and for a P4502C form [16–18]. Even aldehyde hydrates were reported to be substrates for the P450 although their rates of oxygenation were significantly lower than those for the corresponding aldehydes [16, 17]. In the case of the hepatic metabolism of TFAld to TFAA, the failure of either diethyldithiocarbamate or anti P4502E1 to inhibit suggests that P4502E1 is not involved, although the other evidence presented supports a role for a P450. Apparently the fluoro substituents on TFAld prevent its metabolism by P4502E1, since acetaldehyde is a substrate for this enzyme [18].

Further insights into the relationship of P4502E1 metabolism of TFE to its toxicity were gained from studies where ethanol was co-administered to rats and where deuterated TFE and TFAld metabolism and toxicity were correlated. In the former case co-administration of ethanol, which competes for P4502E1, essentially prevented the lethality to rats of an otherwise lethal dose of TFE—further support for P4502E1 involvement in the toxifying process of TFE. The marked effects following replacement of the methylene protons of TFE with deuterium in decreasing the lethality of TFE, and on both the V_{max} and K_m for the metabolism of TFE to TFAld (Fig. 5), strongly support a role for P4502E1 in TFE metabolism and toxicity. The observed deuterium isotope effect of 4.2 for the metabolism of TFE to TFAld is consistent in magnitude with values observed for other P4502E1 methylene proton abstraction reactions [29, 30]. The kinetic isotope effect for ethanol oxidation to acetaldehyde by P4502E1 using a noncompetitive comparison of unlabeled and [1,1-$^2H_2$]ethanol with rabbit liver microsomes is reported to be 3.7 [29]. The value for the same reaction, but using a comparison of [$^2H_6$]-ethanol and [1-^{13}C]ethanol, and an untreated rat liver microsomal preparation, is 3.0 [29]. At least in the case of the rabbit liver preparation, substitution of ethanol with the deuterated analog increased the K_m and decreased the V_{max} for the oxidation reaction [29], consistent with our observations with TFE and deuterated TFE.

In contrast to the effects of deuteration of TFE on its hepatic metabolism and toxicity, deuterated TFAld exhibited the same toxicity as TFAld, and although the rate of hepatic microsomal metabolism was also not affected, the K_m for the oxidation of TFAld to TFAA was. This apparent contradiction is consistent with our previous studies, which implicated intestinal and not hepatic metabolism of TFAld in the toxicity of TFE and TFAld [4]. The complex relationship between the various organ-specific toxicities of TFE and its hepatic metabolism has not been resolved completely. The current results are consistent with our previous suggestions that the metabolism of TFE to TFAld occurs primarily in the liver and that subsequently the TFAld is metabolized to TFAA in other organs, with toxic consequences.

In summary, hepatic P4502E1 is the primary source for metabolism of TFE on a pathway leading to toxicity. TFAld is also metabolized by P450 but not by P4502E1. The hepatic metabolism of TFAld was not associated with its toxic effects, which have been demonstrated previously to arise from intestinal metabolism.

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REFERENCES

1. Murphy MJ, Dunbar DA, Guengerich FP and Kaminsky LS, Destruction of highly purified cytochromes P-450 associated with metabolism of fluorinated ether anesthetics. *Arch Biochem Biophys* **212**: 360–369, 1981.
2. Murphy MJ, Dunbar DA and Kaminsky LS, Acute toxicity of fluorinated ether anesthetics: Role of 2,2,2-trifluoroethanol and other metabolites. *Toxicol Appl Pharmacol* **71**: 84–92, 1983.
3. Fraser JM and Kaminsky LS, Metabolism of 2,2,2-trifluoroethanol and its relationship to toxicity. *Toxicol Appl Pharmacol* **89**: 202–210, 1987.
4. Fraser JM and Kaminsky LS, 2,2,2-Trifluoroethanol intestinal and bone marrow toxicity: The role of its metabolism to 2,2,2-trifluoroacetaldehyde and trifluoroacetic acid. *Toxicol Appl Pharmacol* **94**: 84–92, 1988.

5. Hitt BA, Mazze RI, Cousins MJ, Edmunds HN, Barr GA and Trudell JR, Metabolism of isoflurane in Fisher 344 rats and man. *Anesthesiology* **40**: 62–67, 1974.
6. Maiorino RM, Gandolfi AJ and Sipes IG, Gas-chromatographic method for the halothane metabolites, trifluoroacetic acid and bromide, in biological fluids. *J Anal Toxicol* **4**: 250–254, 1980.
7. Bradshaw JJ and Ivanetich KM, Isoflurane: A comparison of its metabolism by human and rat hepatic cytochrome P-450. *Anesth Analg* **63**: 805–813, 1984.
8. Niki H, As assessment of potential degradation products in the gas-phase reactions of alternative fluorocarbons in the troposphere. In: *Scientific Assessment of Stratospheric Ozone*, Vol. II, pp. 235–245. NASA, Washington, DC, 1989.
9. Manzer LE, The CFC-ozone issue: Progress on the development of alternatives to CFCs. *Science* **249**: 31–35, 1990.
10. Olson MJ, Reidy CA, Johnson JT and Pederson TC, Oxidative defluorination of 1,1,1,2-tetrafluoroethane by rat liver microsomes. *Drug Metab Dispos* **18**: 992–998, 1990.
11. Blake DA, Cascorbi HF, Rozman RS and Meyer FJ, Animal toxicity of 2,2,2-trifluoroethanol. *Toxicol Appl Pharmacol* **15**: 83–91, 1969.
12. Kvassman J and Pettersson G, Effect of pH on the binding of decanoate and trifluoroethanol to liver alcohol dehydrogenase. *Eur J Biochem* **103**: 557–564, 1980.
13. Blair AH and Vallee BL, Some catalytic properties of human liver alcohol dehydrogenase. *Biochemistry* **5**: 2026–2034, 1966.
14. Feierman DE and Cederbaum AI, Inhibition of microsomal oxidation of ethanol by pyrazole and 4-methylpyrazole *in vitro*. *Biochem J* **239**: 671–677, 1986.
15. Guengerich FP, Kim D-H and Iwasaki M, Role of human cytochrome P-450IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4**: 168–179, 1991.
16. Watanabe K, Narimatsu S, Yamamoto I and Yoshimura H, Hepatic microsomal oxygenation of aldehydes to carboxylic acids. *Biochem Biophys Res Commun* **166**: 1308–1312, 1990.
17. Watanabe K, Narimatsu S, Yamamoto I and Yoshimura H, Oxygenation mechanism in conversion of aldehyde to carboxylic acid catalyzed by a cytochrome P-450 isozyme. *J Biol Chem* **266**: 2709–2711, 1991.
18. Terelius Y, Norsten-Höög C, Cronholm T and Ingelman-Sundburg M, Acetaldehyde as a substrate for ethanol-inducible cytochrome P450 (CYP2E1). *Biochem Biophys Res Commun* **179**: 689–694, 1991.
19. Friedman L and Jurewicz A, A convenient procedure for the preparation of low-boiling alcohols and amines via lithium aluminum hydride and deuteride reduction. *J Org Chem* **33**: 1254–1255, 1968.
20. Pierce OR and Kane TG, A new synthesis of perfluoroaldehydes. *J Am Chem Soc* **74**: 300–301, 1954.
21. Hustad D and Ahlbrecht AH, The chemistry of the perfluoro acids and their derivatives. III. The perfluoro aldehydes. *J Am Chem Soc* **74**: 5422–5426, 1952.
22. Fowler BA, Lucier GW and Hayes AW, Organelles as tools in toxicology. In: *Principles and Methods of Toxicology* (Ed. Hayes W), pp. 635–658. Raven Press, New York, 1982.
23. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P-450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
24. Ko I-Y, Park SS, Song BY, Patten C, Tan Y, Hah YC, Tang CS and Gelboin HV, Monoclonal antibodies to ethanol-induced rat liver cytochrome P-450 that metabolizes aniline and nitrosamines. *Cancer Res* **47**: 3101–3109, 1987.
25. Niranjan BG, Wilson NM, Jefcoate CR and Avadhani NG, Hepatic mitochondrial cytochrome P-450 system. *J Biol Chem* **259**: 12495–12501, 1984.
26. Brady JF, Xiao F, Wang M-H, Li Y, Ning SM, Gapac JM and Yang CS, Effects of disulfiram on hepatic P450IIE1, other microsomal enzymes, and hepatotoxicity in rats. *Toxicol Appl Pharmacol* **108**: 366–373, 1991.
27. Puntarulo S and Cederbaum AI, Temperature dependence of the microsomal oxidation of ethanol by cytochrome P450 and hydroxyl radical-dependent reactions. *Arch Biochem Biophys* **269**: 569–575, 1989.
28. Lieber C, The microsomal ethanol oxidizing system: Its role in ethanol and xenobiotic metabolism. *Biochem Soc Trans* **16**: 232–239, 1988.
29. Miwa GT, Walsh JS and Lu AYH, Kinetic isotope effects on cytochrome P-450 catalyzed oxidation reactions. *J Biol Chem* **259**: 3000–3004, 1984.
30. Ekström G, Norsten C, Cronholm T and Ingelman-Sundberg M, Cytochrome P-450 dependent ethanol oxidation. Kinetic isotope effects and absence of stereoselectivity. *Biochemistry* **26**: 7348–7354, 1987.