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# $^{19}$F NUCLEAR MAGNETIC RESONANCE ANALYSIS OF TRIFLUOROETHANOL METABOLITES IN THE URINE OF THE SPRAGUE–DAWLEY RAT

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**Abstract**—2,2,2-Trifluoroethanol (TFE) is a common industrial solvent and a known metabolite of the inhalation anesthetics fluroxene (2,2,2-trifluoroethyl vinyl ether) and halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). The water-soluble metabolites of TFE were identified in the urine of Sprague–Dawley rats using $^{19}$F NMR spectroscopy. In rats dosed with 0.21 g TFE/kg body weight, approximately one-half of the administered TFE was excreted as the trifluoroethyl glucuronide. The remaining TFE was oxidized, primarily to trifluoroacetaldehyde hydrate, with a small percentage of the aldehyde oxidized further to trifluoroacetate. One additional fluorinated compound was found; after investigation, this was identified as a Schiff's base compound resulting from the addition of trifluoroacetaldehyde to urea. The time-dependent excretion of TFE metabolites was measured as a function of ethanol induction of hepatic enzymes. This study demonstrates the utility of $^{19}$F NMR for the analysis of drug metabolism in laboratory animals. In addition, the resistance of trifluoroacetaldehyde hydrate to further oxidation, coupled with its reactivity with common cellular amines, indicates the potential toxicity of this metabolite to mammalian tissues.

The anesthetic agents fluroxene (2,2,2-trifluoroethyl vinyl ether) and halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) are avoided in clinical anesthesiology due to potential toxic effects associated with their use. Toxicity has been ascribed to the formation of toxic metabolites from the relatively inert anesthetics [1, 2]. Both fluoroxene and halothane are metabolized by oxidation to either 2,2,2-trifluoroethanol (TFE§) or a trifluoroethanol-like intermediate [3, 4]. Hence, an improved understanding of TFE metabolism may elucidate the identity of the toxic intermediate(s) generated in anesthetic metabolism.

Previous studies of TFE metabolism have identified several metabolites generated either from conjugation or oxidation of the fluorinated alcohol [5–8]. Oxidation of TFE presumedly occurs through the microsomal ethanol oxidizing system, with possible contributions from peroxidase [7]. The stable end products of oxidation, trifluoroacetaldehyde hydrate (TFAld) and trifluoroacetic acid (TFAA), are not toxic to rats at concentrations similar to those generated from TFE metabolism [8]. Therefore, a reactive intermediate in TFE oxidation, possibly resulting from hydride ion removal from hydrated TFAld, has been proposed to explain TFE toxicity. This metabolite could covalently modify crucial cell components in lymphocytes, resulting in depression of immune system function and increased susceptibility to bacterial infection [8]. A similar intermediate has been proposed to explain covalent addition of a halothane metabolite to phospholipids [9].

Our laboratory is interested in studying the hepatic metabolism of fluorinated xenobiotics using $^{19}$F NMR. The utility of $^{19}$F NMR for the study of the metabolism of fluorinated anesthetics has been demonstrated previously [10–12]. Further, TFE may prove to be useful as a spectroscopic probe of anesthetic and intoxicant interactions with biological membranes.

In this study, $^{19}$F NMR is used to confirm the identity of the urinary metabolites of TFE in the Sprague–Dawley rat. The use of $^{19}$F NMR provides a simple method to detect and quantitate urinary TFE metabolites. Using this method, the effects of different ethanol treatments upon TFE metabolism have been measured. The goal of these studies is to improve our understanding of how TFE is metabolized *in vivo*, and also to observe the changes in TFE metabolism resulting from ethanol.

## METHODS

*Chemicals.* Trifluoroethanol and trifluoroacetic acid were obtained from Aldrich (Milwaukee, WI). Trifluoroacetaldehyde (as the hydrate) was obtained from Lancaster Synthesis (Windham, NH). β-Glucuronidase (rat liver Type I) was purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity commercially available and used without further purification.

*Synthesis of the trifluoroacetaldehyde-urea conjugate.* As described in Results, an unknown metabolite of TFE was found in urine, and presumed to be a

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§ Abbreviations: TFE, 2,2,2-trifluoroethanol; TFAld, hydrated trifluoroacetaldehyde; TFAA, trifluoroacetate; TFE-gluc, glucuronic acid conjugate of trifluoroethanol; TFE-urea, trifluoroacetaldehyde-urea covalent conjugate; and MEOS, microsomal ethanol oxidizing system.

covalent conjugate formed by the addition of TFAld to urea. To synthesize pure conjugate, 0.50 g (8.3 mmol) of urea was added to 3.9 mL (41.6 mmol) of hydrated TFAld in a ground glass Erlenmeyer flask. The flask was stoppered and incubated with occasional stirring at 37° for 24 hr. The white precipitate was collected by filtration and recrystallized in hot ethanol. The crystalline white solid was collected by filtration, dried in a 37° oven for 3 hr, and weighed (0.38 g; 32% yield). Analytical data: m.p. 151°; $^{19}$F NMR (DMSO-$d_6$, referenced to external TFAA in $D_2O$) 4.64 (d, $J$ = 5.7 Hz); $^{13}$C NMR (DMSO-$d_6$, referenced to TMS) 71.28 (o, CH, $J_H$ = 154.9 Hz; $J_F$ = 32.8 Hz), 124.05 (q, $CF_3$, $J_F$ = 283.2 Hz), 157.40 (s, CO); $^1$H NMR (DMSO-$d_6$, referenced to TMS) 4.58 (m, CH), 5.00 (s, $NH_2$), 6.09 (d, *trans* imide NH, $J$ = 10.0 Hz), 6.20 (*cis* imide NH, $J$ = 5.9 Hz). IR 3430 (imide NH), 3338 (amide $NH_2$), 3219 (amide $NH_2$), 1651 (CO), 1560 (amide $NH_2$).

The above analytical data indicated that the crystalline product was a mixture of *cis* and *trans* isomers of the protonated Schiff's base. To confirm, the sample was dissolved in basic $D_2O$ and reanalyzed. $^1$H NMR ($D_2O$/NaOD, referenced to DSS) 5.54 (q, CH, $J$ = 5.4 Hz). The presence of a single proton resonance for the compound when dissolved in aqueous solution confirms the identity of the crystalline solid as the protonated Schiff's base.

*Animals.* Male Sprague–Dawley rats (Ancare Corp., weights 250–450 g) were used throughout the study. Rats were given food (standard rat chow) and water *ad lib.* throughout all experiments. The animals used in these experiments were maintained in compliance with standards set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS, PHS, NIH Publication 86–23). Also, animal use in these studies was approved by the Animal Care Committee of Villanova University.

Rats were placed in metabolic cages (Nalgene) for at least 24 hr prior to TFE injection. Rats were injected with TFE (0.21 g/kg, i.p.) as a 20% solution in 0.9% NaCl. Urine was collected from rats at 8-hr intervals for 48 hr after TFE administration. Each animal received a single dose of TFE.

Animals were divided into four groups to describe different regiments of ethanol administration: Group I (control; N = 6): no ethanol treatment; Group II (pretreatment and cotreatment; N = 4): ethanol [10% (v/v) in drinking water] was begun 7 days before TFE injection and continued throughout urine collection; Group III (pretreatment; N = 4): ethanol [10% (v/v) in drinking water] was begun 7 days before TFE injection, and then replaced with tap water 12 hr before TFE injection; and Group IV (cotreatment; N = 6): ethanol [10% (v/v) in drinking water] was begun 12 hr before TFE injection and continued throughout urine collection.

*Statistical analysis of data.* Significant differences between treatment groups were identified by means of the one-factor analysis of variance (CLR ANOVA, Clear Lake Research, Houston, TX). The Newman–Keuls procedure was used for multiple comparisons. The unweighted means were used when treatment groups of different sizes were compared. A $P \leq 0.05$ was taken to reflect a statistically significant difference between treatment groups.

*Identification and measurement of trifluoroethanol metabolites.* Identification of TFE, TFAA and TFAld in rat urine was accomplished by direct addition of authentic compound to urine samples containing TFE metabolites. $^{19}$F NMR spectra were acquired before and after compound addition. Coresonance of the added compound with a fluorinated urinary component constituted a positive identification of that compound as a urinary metabolite.

The two covalent adducts resulting from TFE metabolism, the glucuronic acid conjugate of trifluoroethanol (TFE-gluc) and the trifluoroacetaldehyde-urea conjugate (TFAld-urea), were not commercially available. The TFAld-urea was therefore synthesized as described above. After synthesis, the presence of this compound in urine was confirmed by coresonance.

Evidence for the identity of the β-glucuronide conjugate was obtained by enzymatic analysis. Urine was treated with β-glucuronidase (1 mg/mL, corresponding to 624 units/mL) for 72 hr at 37°. $^{19}$F NMR analysis of enzyme-treated and control urine indicated that the enzyme specifically removed the $^{19}$F NMR resonance at 1.48 ppm with a concurrent increase in intensity at −0.94 ppm. This result is consistent with the assignment of the 1.48 $^{19}$F NMR resonance to TFE-gluc.

Concentrations of metabolites were determined by preparing several solutions of TFE with known concentrations. $^{19}$F NMR spectra were acquired from 0.4-mL samples of the standard solutions. The relative intensities of the $^{19}$F NMR resonances from the TFE standard solutions were determined by using the "cut and weigh" method. A plot of resonance intensity versus TFE concentration yields a straight line, which was used as a standard curve to determine the concentrations of urinary TFE metabolites. A new standard curve was generated daily to allow for variability in instrument performance.

*NMR methods.* $^{19}$F NMR spectra of urine samples were acquired using a Varian XL-200 spectrometer at 188.2 MHz for $^{19}$F. Into a 5-mm NMR tube was placed 0.4 mL of urine, followed by a capillary insert containing trifluoroacetic acid in $D_2O$ for use as an external standard and for field lock. A 90° (10 μsec) excitation pulse was used with a relaxation delay of 15.0 sec. As the longitudinal relaxation times for the fluorine nuclei ranged between 1.6 and 2.8 sec (determined by inversion recovery; data not shown), the chosen delay time allowed all nuclei to fully relax between acquisitions. Sixteen acquisitions were summed. Exponential line broadening of 1 Hz was applied before Fourier transformation. Urine samples were analyzed at ambient temperature.

*In vivo* $^{19}$F NMR spectra were acquired using a Vivospec imaging spectrometer (Otsuka Electronics) with a 4.7T horizontal bore magnet. Rats were dosed with TFE as described above, followed by sodium pentobarbital (50 mg/kg, i.p., in 0.9% NaCl) to induce anesthesia. A single turn surface coil with a diameter of 1.5 cm was placed over the lower abdomen of the rat for data acquisition. The animal was then placed in the magnet. Homogeneity was

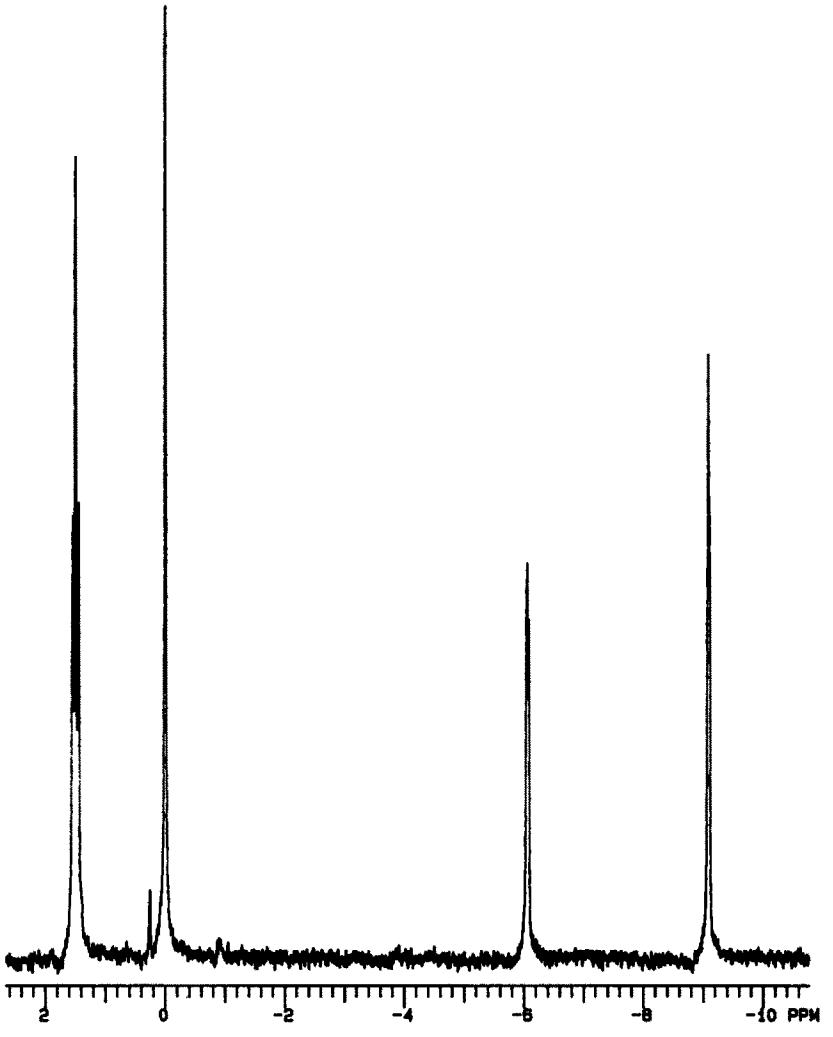


Fig. 1. $^{19}$F NMR spectrum of rat urine collected 16 hr after i.p. injection of 0.21 g/kg TFE. The spectrum was acquired with conditions described in Methods. External trifluoroacetic acid in deuterium oxide was used as a chemical shift reference. Resonance assignments are given in Table 1.

adjusted using the proton signal from water in the bladder of the animal. $^{19}$F NMR spectra were acquired using a 35-$\mu$sec acquisition pulse and a 2-sec relaxation delay. A spectral width of 10 kHz was described by 2048 data points. A total of 128 transients were summed per experiment, resulting in a total acquisition time of 4.3 min per spectrum. Exponential line broadening of 5 Hz was applied before Fourier transformation.

*Fluoride ion determination.* Fluoride ion concentrations were measured using an Orion Research model 901 microprocessor ionanalyzer and an Orion fluoride electrode. Samples were prepared by adding 0.5 mL of urine to 5.0 mL of distilled water, followed by 4.5 mL of ionic strength regulating buffer (0.1 M $Na_2HPO_4$, 0.04 M citric acid) [13]. Calibration of the ion electrodes was prepared by using 0.5 mL of untreated urine, 2.5 mL of distilled water, 4.5 mL of buffer, and 2.5 mL of known concentrations of KF (from 0.98 to 980 $\mu$M). A new standard curve was obtained daily.

## RESULTS

*Identification of urinary trifluoroethanol metabolites.* Rats were given trifluoroethanol at a dose of 0.21 g/kg, i.p.; this dose was chosen to allow for comparison with previous studies [7]. Immediately after injection, the animals were placed in metabolic cages and urine was collected at 8-hr intervals. Trifluoroethanol metabolites were measured using $^{19}$F NMR.

Figure 1 is a $^{19}$F NMR spectrum of rat urine collected 16 hr after TFE injection. Four sets of NMR resonances appeared in most $^{19}$F NMR spectra, with a fifth set occasionally observed. Since mammals contain no endogenous fluorinated compounds in sufficient concentration for NMR observation, all of the resonances shown in Fig. 1 must be derived metabolically from TFE.

Table 1 presents the final assignment of $^{19}$F NMR resonances observed in rat urine after TFE administration. Initial identification of the $^{19}$F NMR

Table 1. Assignment of $^{19}F$ NMR resonances of trifluoroethanol metabolites

| Chemical shift (ppm) | $J_{F-H}$ coupling (Hz) | Identity |
|---|---|---|
| 1.48 | 9.4 | β-Glucuronic acid conjugate of trifluoroethanol |
| 0.21 | — | Trifluoroacetate |
| 0.00 | — | Trifluoroacetic acid (chemical shift standard) |
| −0.94 | 8.3 | Trifluoroethanol |
| −6.08 | 5.3 | Trifluoroacetaldehyde-urea conjugate |
| −9.11 | 2.8 | Trifluoroacetaldehyde hydrate |

resonances in Fig. 1 was achieved by comparing the $^{19}F$ NMR spectrum of a urine sample before and after adding a known concentration of a suspected metabolite. Coresonance of the added metabolite with a peak from the untreated sample was considered a positive test. The previous identification of many urinary TFE metabolites [14], the knowledge of ethanol metabolism [15], and the large NMR chemical shift range of fluorine nuclei minimize the possibility for coresonance misassignment. The $^{19}F$ NMR resonances for TFE, TFAA, and TFAld were assigned by coresonance.

The set of resonances centered at 1.48 ppm had the correct splitting pattern for TFE-gluc. This metabolite had been determined previously to be the major urinary metabolite of TFE in the Sprague–Dawley rat [5]. Since the hepatic uridine 5'-diphosphoglucuronyltransferase forms only the β-glucuronide, sample urine was treated with β-glucuronidase for 72 hr, and the hydrolysis of the glucuronide followed using $^{19}F$ NMR. The urinary product was hydrolyzed to TFE, confirming the assignment of this resonance as the trifluoroethyl glucuronide.

Identification of the resonance at −6.08 ppm was aided by the observation that extended storage caused the intensity of the resonance at −6.08 ppm to increase with concurrent loss of intensity of the TFAld resonance. This fact, along with the observation that time-dependent excretion of the −6.08 resonance paralleled the time-dependent excretion of TFAld (data not shown), led to the conclusion that the −6.08 resonance was derived from the reaction of TFAld with some component in urine. The addition of pure TFAld to a 0.1 M urea solution resulted in the generation of an NMR resonance near −6.08 ppm, indicating that this resonance was derived from the reaction of TFAld with urea. The compound was then synthesized in sufficient quantity for chemical analysis (see Methods); $^{1}H$ NMR, $^{13}C$ NMR, and infrared spectroscopic analysis resulted in the compound described in Methods. Addition of the synthetic compound to a urine sample resulted in coresonance at −6.08 ppm, confirming the identify of this resonance.

There was no evidence of fluoride ion in any of the $^{19}F$ NMR spectra we obtained. We have demonstrated previously that the $^{19}F$ NMR resonance arising from fluoride ions may be broadened in biological samples, hindering the observation and quantitation of fluoride ions using NMR [11]. Therefore, some of the urine samples were checked for fluoride ions using an ion selective electrode. None of the samples measured showed observable concentrations of fluoride ions.

In vivo *confirmation of TFE metabolism.* The formation of a TFAld-urea conjugate could possibly be the result of sample heating or oxidation after urine exposure to air. To test this hypothesis, *in vivo* $^{19}F$ NMR spectra were acquired from the bladders of rats dosed with 0.21 g/kg TFE (Fig. 2). Spectra arising predominantly from urine in the rat bladder were recorded for 4 hr after TFE dosage. Figure 2 clearly shows the time-dependent accumulation of all TFE metabolites in the urine, including the TFAld-urea conjugate. The rapid formation of the TFAld-urea conjugate clearly demonstrates the high reactivity of TFAld in aqueous solution.

*Time-dependent elimination of trifluoroethanol metabolites.* Urine samples were collected at 8-hr intervals after TFE injection and analyzed for metabolites. Metabolite concentrations were determined using a standard curve of resonance intensity versus concentration. The concentrations of all metabolites generated from TFE were determined for 48 hr after TFE injection (Fig. 3). Since TFAld-urea is nonenzymatically derived from TFAld, and apparently only forms in the urine, throughout the remainder of this paper the concentrations of TFAld and TFAld-urea are combined and discussed together.

*Effect of ethanol on trifluoroethanol metabolism.* Since trifluoroethanol is metabolized using hepatic enzymes also used for ethanol metabolism [14], the effect of oral ethanol administration on TFE metabolism was investigated. Urinary metabolites were measured as a function of ethanol pretreatment (7 days oral administration of 10% ethanol, with a 12 hr rest before TFE administration), cotreatment (10% ethanol orally beginning 12 hr before TFE injection), or pre- and cotreatment (continuous ethanol administration beginning 7 days before trifluoroethanol injection). The time-dependent excretion of TFE metabolites as a function of ethanol treatment is demonstrated in Figs. 4–6.

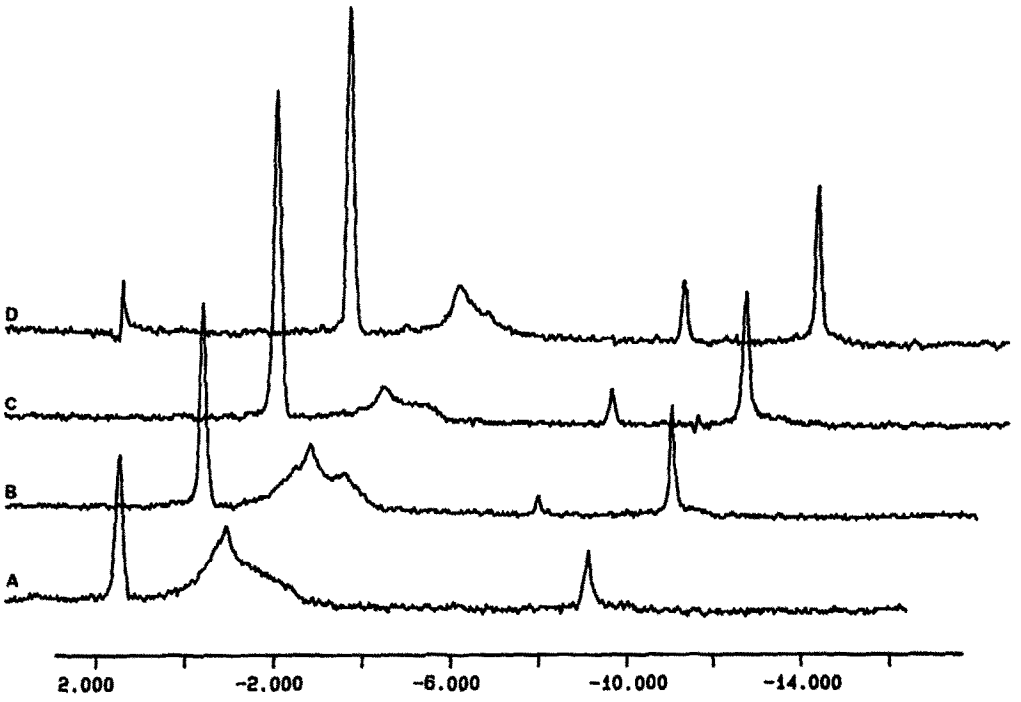


Fig. 2. *In vivo* $^{19}$F NMR spectra of urine within the bladder of the Sprague–Dawley rat. Rats were dosed with TFE (0.21 g/kg, i.p.), followed by anesthetization with sodium pentobarbital (50 mg/kg, i.p.). $^{19}$F NMR spectra were acquired using a surface coil beginning (A) 40 min, (B) 80 min, (C) 130 min, and (D) 240 min after injection of TFE. The spectra were acquired using conditions described in Methods; each spectrum took 4.3 min to acquire. The horizontal axis represents the chemical shift (in ppm units) using aqueous trifluoroacetate as a chemical shift standard. Note the time-dependent accumulation of the narrower resonances corresponding to urinary TFE metabolites, and in particular the resonance at approximately −6 ppm corresponding to TFAld-urea. The broad resonance in the center left of each spectrum arises from TFE in fatty tissue around the bladder of the rat. The apparent peak on the left side of the top spectrum is an instrumental artifact.

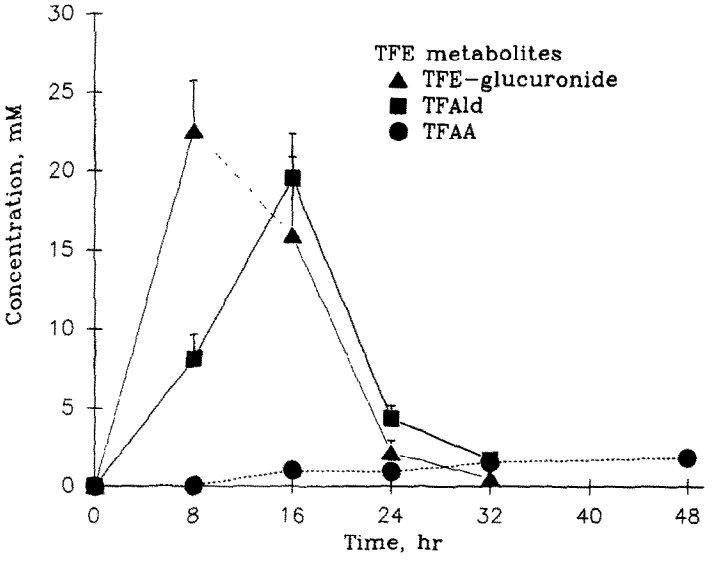


Fig. 3. Excretion of TFE metabolites in rat urine as a function of time after injection. Metabolite concentrations were determined using $^{19}$F NMR as described in Methods. Each point is the average of measurements made on 3–4 different animals, with the error bars representing one SEM. The line labeled TFAld represents the sum of the concentrations of the TFAld and TFAld-urea resonances, as the urea conjugate is derived directly from TFAld.

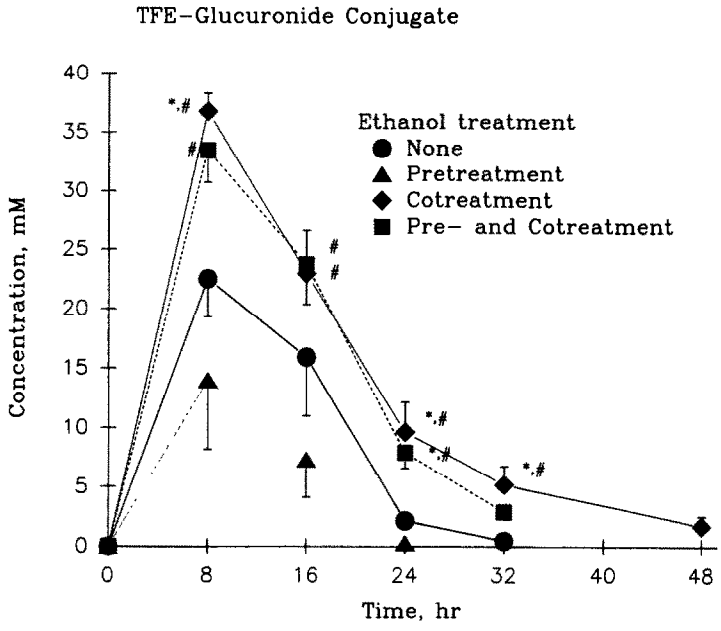


Fig. 4. Excretion of TFE-glucuronide conjugate as a function of alcohol treatment. Rats were given ethanol [10% (v/v) in drinking water] for 7 days before TFE injection (pretreatment), for 12 hr prior to TFE injection and continued throughout TFE metabolism (cotreatment), or beginning 7 days before TFE injection and continuing through the time of TFE metabolism (pre- and cotreatment). Each point is the average concentration of the metabolite in urine samples collected from 3–4 rats at the designated times, while the error bars represent one SEM. Key: (*) significantly different ($P \leq 0.05$) from the non-ethanol-treated control by the Newman–Keuls pairwise test after analysis of variance; and (#) significantly different ($P \leq 0.05$) from the pretreated values, by the Newman–Keuls pairwise test after analysis of variance.

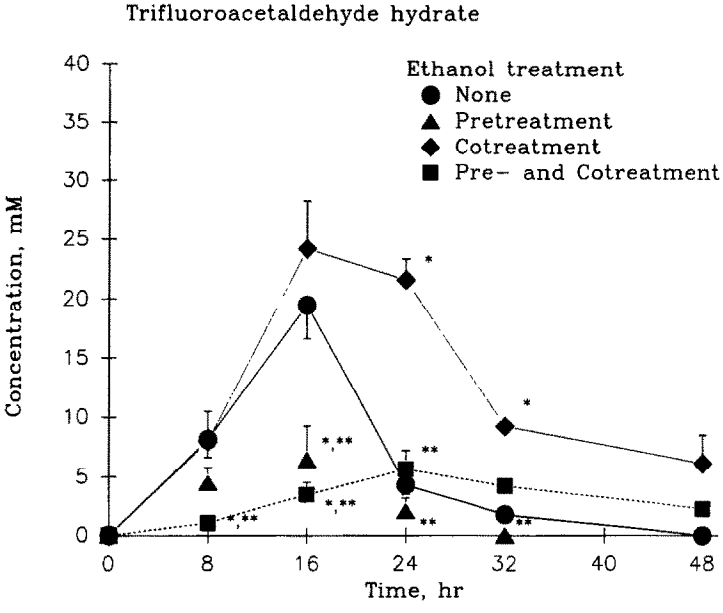


Fig. 5. Excretion of TFAld and the TFAld-urea conjugate as a function of alcohol treatment. See the legend to Fig. 4 and also Methods for an explanation of the different treatment conditions. Each point is the average concentration of the metabolite in urine samples collected from 3–4 rats at the designated times, while the error bars represent one SEM. Key: (*) significantly different ($P \leq 0.05$) from the non-ethanol-treated control by the Newman–Keuls pairwise test after analysis of variance; and (**) significantly different ($P \leq 0.05$) than the cotreated values, as measured by the Newman–Keuls pairwise test after analysis of variance.

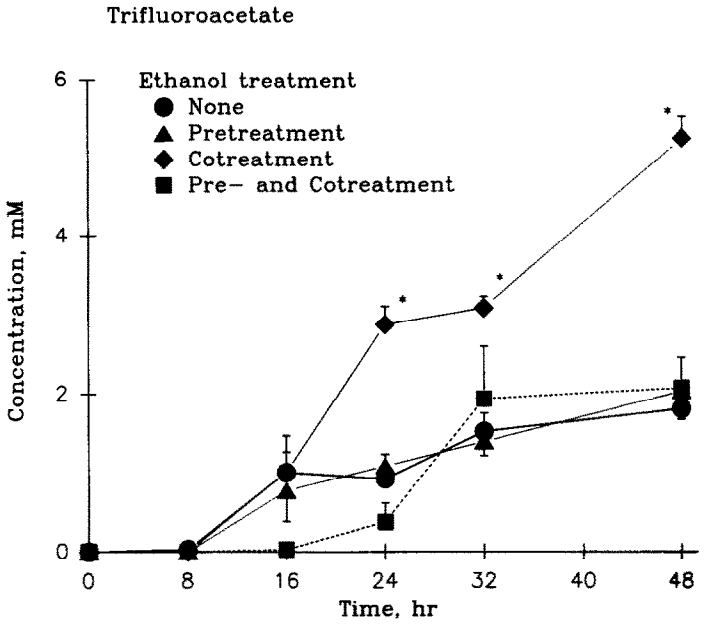


Fig. 6. Excretion of TFAA as a function of alcohol treatment. See the legend to Fig. 4 and also the Methods for an explanation of the different treatment conditions. Each point is the average concentration of the metabolite in urine samples collected from 3–4 rats at the designated times, while the error bars represent one SEM. Key: (*) significantly different ($P \leq 0.05$) from the non-ethanol-treated control by the Newman–Keuls pairwise test after analysis of variance.

## DISCUSSION

*Advantages of NMR analyses of fluorinated xenobiotic metabolism.* Several TFE metabolites have been identified previously in the serum [6, 7] and urine [5, 8, 16] of treated rats. Metabolites were identified using either radiochemical methods [17] or by gas chromatography after sample extraction and derivitization [6–8].

Nuclear magnetic resonance provides a simple method to assay water-soluble metabolites of many different chemicals. The advantages of both $^{1}$H and $^{19}$F NMR in the analysis of xenobiotic metabolites have been described previously [11, 12, 18]. As demonstrated in Fig. 1, all $^{19}$F NMR resonances resulting from TFE metabolites are clearly seen in a spectrum acquired from 0.4 mL of untreated urine. The spectrum shown in Fig. 1 was acquired in 4 min; however, all resonances were clearly visible after a single acquisition. Since no sample preparation is required, many hours of control experiments ensuring complete derivitization and reproducible extractions are unnecessary.

The discovery of the TFAld-urea conjugate illustrates the advantage of using $^{19}$F NMR to assay urinary TFE metabolites. Previous identification of TFAld as a TFE metabolite was performed by gas chromatographic analysis of trifluoromethane generated from the hydrolytic cleavage of TFAld by aqueous sodium hydroxide [8]. Basic hydrolysis of urine samples eliminated the $^{19}$F NMR resonance from both TFAld and the TFAld-urea conjugate (data not shown). Observation and quantitation of TFAld-urea, easily accomplished using $^{19}$F NMR, could not have been achieved using gas chromatographic analysis.

*Comparisons with previous studies.* The time-dependent excretion of TFE metabolites has been reported previously in both Sprague–Dawley [5, 6] and Wistar [7, 8] rats. The metabolite TFAld has been measured previously only in Wistar rats. We have confirmed that the major urinary metabolite of TFE in Sprague–Dawley rats is the glucuronide conjugate, as previously reported [6]. Comparing the relative concentrations of the oxidation and conjugation products (see Fig. 3), approximately half of the injected TFE is metabolized via each pathway. This is in contrast to the Wistar rat, where only 5% of TFE is excreted as the glucuronide conjugate [14].

In Wistar rats, TFAA production from TFE was maximal 24 hr after TFE injection, while TFAA production peaked only 6 hr after injection of TFAld. Therefore, the rate-limiting step in TFE oxidation to TFAA in the Wistar rat seemed to be the initial oxidation of TFE to TFAld [7]. In the Sprague–Dawley rat, this same conclusion cannot be made with confidence. Comparing the urinary output of TFAld and TFAA, most of the TFAld generated was excreted without further oxidation. Little if any TFE was excreted without metabolism. One possible explanation for the excretion of large amounts of TFAld is preferential tissue retention of TFE and TFAA relative to TFAld. We and others have shown previously that TFAA, when generated as a metabolite of the anesthetics halothane and methoxyflurane, is retained by tissues for extended

periods of time after anesthesia [11, 12, 19]. However, preliminary *in vivo* measurements of TFE metabolites in liver, brain, and muscle indicate that little TFAA is retained in tissue relative to TFAld and TFE*. TFE-gluc was observed only in liver and urine, consistent with the known distribution of UDP-glucuronyltransferase in mammals. While further experiments are required to fully understand the distribution of TFE and its metabolites in Sprague–Dawley rats, clearly the large excess of TFAld to TFAA observed in urine demonstrates that the rate-limiting step in the oxidation of TFE in this species is the conversion of TFAld to TFAA.

The microsomal ethanol oxidizing system (MEOS) is believed to be the major site of TFE oxidation in the liver [7]. Assuming this true, several hypotheses could be invoked to explain the rate difference between the two oxidation steps. One explanation would be competition at the oxidation site between TFE and TFAld. The binding affinity of TFE to the oxidation site would be much greater than the binding affinity of TFAld. Only when TFE concentrations had reduced to near zero would significant oxidation of TFAld to TFAA be observed. A second explanation would be inactivation of the oxidation site after oxidation of TFE to TFAld. Oxidation sites would need to regenerate before TFAld could be oxidized to TFAA, thereby explaining the difference in oxidation rates. The destruction of cytochromes that metabolize the anesthetic fluroxene has been observed previously [20–22].

*Interpretation of the effect of ethanol on TFE metabolism.* The effect of ethanol on TFE metabolism was of interest, since the same MEOS which metabolizes TFE also contributes to the metabolism of ethanol. To this end, ethanol was administered to rats before TFE injection (for enzyme induction), during TFE metabolism (to compete with TFE for common metabolic enzymes), and both before and during TFE metabolism.

Ethanol pretreatment, with removal before TFE injection, would be expected to induce cytochrome P450 (specifically P450IIE, IIB1, and IIB2 [23, 24]) and increase the rate of excretion of TFE oxidation products. In the results presented in Figs. 4–6, pretreatment with ethanol actually *decreased* the concentration of excreted TFAld relative to the nontreated control, with no significant change measured in the excretion of TFAA. Cotreatment with ethanol significantly *increased* the concentrations of all TFE metabolites in urine relative to controls given TFE without ethanol; if ethanol competes with TFE for metabolic enzymes, a significant *decrease* in metabolite production would be expected. Combined pre- and cotreatment with ethanol generated urinary TFE-gluc output similar to cotreatment, but depressed urinary TFAld concentrations similar to those observed for ethanol pretreatment alone.

To explain the effects of ethanol upon TFE metabolism, the effects of different ethanol treatments must be addressed separately. Pretreatment with ethanol without cotreatment depressed both the conjugation pathway (Fig. 4) and the oxidation pathway (as determined from the concentration of excreted TFAld; see Fig. 5). The simplest explanation for this result is that ethanol pretreatment, followed by a short period to allow for ethanol clearance, decreases the intracellular concentrations of both the MEOS enzymes utilized for TFE metabolism and the UDP-glucuronyltransferase at the time of TFE injection. To the best of our knowledge, the time dependence of the induction of the alcohol-metabolizing isoforms of cytochrome P450 has not been measured; we are presently pursuing these studies.

Ethanol, given 12 hr before TFE injection, resulted in increased urinary concentrations of both oxidative and conjugative metabolites. A short period of ethanol pretreatment may induce hepatic metabolic enzymes. At the time of TFE injection, these enzymes are at high levels within the liver, and therefore can metabolize TFE faster than in nontreated animals. Alternatively, the increase in conjugation pathway products could instead be the result of induction of UDP-glucuronic acid production in the liver resulting from ethanol treatment.

The results observed in animals that were both pre- and cotreated with ethanol do not support the above arguments. In animals that were both pre- and cotreated with ethanol the conjugation pathway was stimulated. The urinary concentrations of TFE-gluc in animals both pre- and cotreated were virtually identical to the output in animals that were simple cotreated with ethanol (Fig. 4). However, the oxidation of TFE was depressed in animals which both pre- and cotreated, resembling results obtained with animals pretreated with ethanol (Fig. 5).

One additional observation regarding the effects of ethanol on TFE oxidation is that the maximum urinary concentrations of TFAld were delayed when animals were cotreated with ethanol. This is clearly observed in Fig. 5, where TFAld output continued for extended times with ethanol cotreatment relative to nontreated or pretreated animals.

A possible explanation for the above observations is that pretreatment with ethanol somehow sensitizes a population of cytochrome P450 in rat liver to destruction upon treatment with TFE. The anesthetic fluoroxene has been demonstrated to inactivate cytochrome P450 in rat liver microsomes after phenobarbital induction [20–22]. Ethanol may induce several different types of cytochromes as a function of time. A short induction period would generate a type of cytochrome capable of TFE oxidation but not susceptible to TFE-mediated inactivation (hence explaining the increased rate of oxidation with ethanol cotreatment). However, extended pretreatment with ethanol would result in the inhibition of production of the aforementioned cytochrome, with preferential production of cytochrome P450(s) susceptible to destruction upon interaction with TFE (or TFAld). This interpretation is compatible with the earlier observation that when fluroxene is administered to phenobarbital-induced rats, about

* Selinsky BS, Pizzini M, Warsheski J and Joseph AP, manuscript in preparation

half of the hepatic cytochrome P450 is destroyed within 1 hr of fluoroxene administration, and cytochrome P450 levels are not fully recovered after 24 hr [3].

Alternatively, ethanol may affect separately the enzymes involved in oxidation and glucuronide conjugation. The urinary concentrations of TFE-gluc increased significantly following ethanol *cotreatment* (Fig. 4). Conversely, the urinary concentrations of TFAld significantly decreased following ethanol *pretreatment* (Fig. 5). Ethanol cotreatment may stimulate the conjugation pathway, while ethanol pretreatment may depress the oxidative pathway.

The oxidative and conjugative pathways for TFE metabolism use separate enzyme systems. Therefore, if both pathways are affected as described above, ethanol would have to work using two different and separate sites of action. The observed concentrations of TFE metabolites in urine may arise from the combination of two separate effects; however, an explanation invoking a single site of action is more palatable.

*Conclusions*. In this paper, the advantages of using $^{19}$F NMR for the study of the metabolism of fluorinated compounds have been reiterated. The effect of ethanol upon urinary excretion of TFE metabolites has been shown.

The extended lifetime of TFAld in tissues was an unexpected discovery. Extended incubation of TFAld with cellular components increases the likelihood of unfavorable reaction with cellular components. An enzymatically generated reactive TFE intermediate, suggested by Kaminsky and Fraser [14], may not be required for reaction with cellular species. TFAld may simply add to cellular amine due to the reactive nature of this fluorinated aldehyde. The reactivity of TFAld with biological amines under physiological conditions needs to be experimentally addressed.

Further studies are necessary to determine the molecular basis behind the effects of ethanol pretreatment and cotreatment on TFE metabolism. Ethanol is a known inducing agent of hepatic cytochromes, but the time-dependent induction of specific isozymes of cytochrome P450 is not known by these authors. The temporal induction of different cytochrome P450 species must be known before the above hypothesis can be fully tested.

To fully understand the results described in this report, better knowledge of which cytochrome P450 isoforms oxidize TFE and their susceptibility to inactivation by fluorinated substrates must be determined. Recently, ethanol has been determined to induce cytochrome P450 when added to cultured hepatocytes, with the identity of the induced isoforms and extent of induction comparable to that observed after *in vivo* induction [24]. Future studies in this laboratory will use isolated hepatocytes, in conjunction with enzymatic assay of cytochrome P450 isoforms, $^{19}$F NMR, and $^{31}$P NMR, to determine which P450 isoforms actively metabolize TFE, and also to assess how ethanol induction affects these isoforms.

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