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In Vivo Nuclear Magnetic Resonance Studies of Hepatic Methoxyflurane Metabolism. I. Verification and Quantitation of Methoxydifluoroacetate

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SUMMARY

The elimination and metabolism of the fluorinated inhalation anesthetic methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) in rats has been monitored using *in vivo* ^{19}F nuclear magnetic resonance at 8.45 T. The elimination of methoxyflurane from rat liver as measured using a surface coil is a first order process when measured beginning 2–3 hr after the end of methoxyflurane anesthesia over a period of 12 hr. The rate constant for hepatic methoxyflurane elimination is dependent upon the duration of anesthesia, varying from 0.24 hr^{-1} for 15 min of anesthesia to 0.07 hr^{-1} for 1 hr of anesthesia. Methoxyflurane was shown to be metabolized in the liver to methoxydifluoroacetate using the surface coil method. No resonance for hepatic fluoride ion could be observed *in vivo*. Pure sodium methoxydifluoroacetate was synthesized in order to confirm the identity of the resonances in liver and urine. ^{19}F NMR spectra of urine collected from anesthetized rats contain resonances for two methoxyflurane metabolites, methoxydifluoroacetate and inorganic fluoride. Studies with liver homogenates imply that fluoride is quickly cleared from the liver and eliminated from the body through the urine, explaining the inability to observe hepatic fluoride using a surface coil. The ^{19}F NMR resonance for inorganic fluoride in urine was found to be broadened by interaction with metal ions, since the broadening could be eliminated by treatment with chelating resin.

Various fluorinated inhalation anesthetics have been widely used clinically during the past 20 years. However, the gradual recognition of associated toxicity has resulted in the limited use or abandonment of several of these anesthetics. In most cases, evidence suggests that this toxicity is associated with metabolites of the anesthetics rather than with the anesthetics themselves. One of the first fluorinated anesthetics used clinically was methoxyflurane ($CHCl_2CF_2OCH_3$). As the use of methoxyflurane increased, reports of renal damage following methoxyflurane anesthesia surfaced, which led to extensive investigations of possible toxicity in humans and in model animal systems. The results of these investigations implicated fluoride ion, a methoxyflurane metabolite, as the primary cause of renal failure (1, 2). Methoxyflurane was eventually discontinued as an operating room anesthetic.

Anesthetic clearance has been a subject of major interest. The ideal surgical anesthetic should be rapidly eliminated from the body without any biotransformation. In this regard, methoxyflurane is less than ideal, since 75% of the inhaled anesthetic is reported as being metabolically transformed (3). Comparing methoxyflurane with other fluorinated anesthetics, isoflurane is reported to be eliminated without significant biotransformation, while only 8.5% of inspired enflurane is metabolized (3). As mentioned earlier, strong evidence exists that the renal damage observed following methoxyflurane administration is caused by high concentrations of serum fluoride which poison the kidney via an unknown mechanism (4). According to existing models for methoxyflurane metabolism (5, 6), fluoride can be generated from methoxyflurane in one of two ways, as described in Eqs. 1 and 2. Methoxyflurane may either be dechlorinated to form the intermediate methoxydifluoroacetate, which may decompose to fluoride ion plus oxalate, or demethylated and lose fluoride ions to yield dichloroacetate. However, in the initial report which proposed methoxydifluoroacetate as a methoxyflurane metabolite, insufficient analytical data were reported to positively identify this metabolite (7).

$$CHCl_2CF_2OCH_3 \xrightarrow{2\ Cl^-} {}^-OOCCF_2OCH_3 \xrightarrow{HCHO} 2\ F^- + {}^-OOCCOO^- \quad (1)$$

$$CHCl_2CF_2OCH_3 \xrightarrow{HCHO} 2\ F^- + CHCl_2COO^- \quad (2)$$

In vivo NMR spectroscopy provides a nondestructive means of monitoring the elimination and metabolism of xenobiotics in living tissue (8). Fluorinated compounds are particularly amenable to this type of analysis as a consequence of the inherent sensitivity of the fluorine nucleus for NMR detection and the absence of background resonances arising from endogenous fluorinated compounds. Recently, several laboratories,

ABBREVIATION: EDTA, ethylenediaminetetraacetic acid.

including our own, have used ^{19}F NMR to examine the metabolism and elimination of fluorinated xenobiotics in humans and in laboratory animals. Studies on the metabolism of 5-fluorouracil in rat liver (9), the elimination of halothane from rabbit brain (10), and the elimination and metabolism of halothane from rat liver (11) have been published.

In this paper, we describe studies of the hepatic metabolism and elimination of methoxyflurane as measured by ^{19}F NMR spectroscopy. The time-dependent appearance of fluorinated methoxyflurane metabolites in the urine is also reported. In the following paper (12), we will describe a more detailed analysis of methoxyflurane metabolism in the rat. These studies provide a more complete understanding of methoxyflurane elimination in an animal model.

Materials and Methods

Chemicals. Methoxyflurane [with 0.01% (w/w) butylated hydroxytoluene] was obtained from Pitman-Moore, Washington Crossing, NJ. Chelating resin (sodium form) was obtained from Sigma Chemical Company, St. Louis, MO. Other chemicals were the highest purity commercially available and used without further purification.

Synthesis of sodium 2,2-difluoro-2-methoxyacetate (sodium methoxydifluoroacetate). Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Proton and ^{13}C NMR spectra were obtained on a GN-500 NMR spectrometer (General Electric Company Medical Systems, Fremont, CA), the latter with proton noise decoupling; chemical shifts are reported in ppm. Fluorine NMR spectra were recorded as described later for urine analysis. Fast atom bombardment was utilized for the mass spectra, using a dithiothreitol/dithioerythritol matrix. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

A solution of 0.72 mol of sodium methoxide in 130 ml of anhydrous methanol (prepared with 16.56 g of sodium) was evaporated *in vacuo* to yield a moist paste. Dry dimethylformamide (100 ml) was added and most of the solid dissolved by stirring at 100° under an argon atmosphere. After cooling the suspension in an ice bath, chlorodifluoroacetic acid (39.14 g, 0.3 mol) was added dropwise over 25 min with stirring. The reaction was then heated in a 130° oil bath and the residual methanol distilled off over the course of 1 hr, by which time the rate of distillation had greatly diminished (a total of 100 ml of methanol was collected here and during the evaporation of the sodium methoxide solution conducted earlier), and a fine precipitate was present. Fluorine NMR indicated that the reaction had reached completion. It was thus cooled under argon and stored at −20° for a few days.

The mixture was allowed to reach room temperature, and the supernatant was decanted from precipitate and treated with ether to yield further precipitate. The combined solids were added in small portions with stirring to 2 N aqueous HCl (250 ml), with the pH being adjusted to less than 1.0 after each addition with 5 N HCl. The resulting solution was extracted with ether (in two portions, with two 300-ml volumes each), and the organic layers were dried over anhydrous sodium sulfate and evaporated *in vacuo* at 15° to an oil (30.7 g).

Since purification of the acid could not be achieved by vacuum distillation due to the co-distillation of volatile decomposition products, the sodium salt was prepared directly from a portion of the product. Thus, the oil (14.4 g) was added to 0.01 M sodium bicarbonate (40 ml) and the biphasic solution was treated slowly with vigorous stirring, first with 0.1 M bicarbonate (200 ml) to yield a pH of 1.5, and then 0.5 M bicarbonate until the pH was 2.0. This was then extracted with ether (12 volumes of 100 ml), readjusting the pH after each addition to 2.0 with 1 N HCl. After analyzing the extracts by fluorine NMR, the first one was discarded and the rest were combined, dried over anhydrous sodium sulfate, and evaporated to a pale yellow oil. This was dissolved in anhydrous methanol (150 ml) and stirred with Bio-Rex 70 (Na+) resin until the pH was 6.6. The resin was washed with methanol and the combined solutions were filtered through Celite and evaporated to dryness. Trituration with ether provided white, crystalline solid (3.54 g, 17%). Recrystallization from methanol yielded an analytical sample: m.p. 155–156° (softens), 177° (decomposes; ^1H NMR (DMSO-d_6, referenced to residual solvent resonance) 3.41 (s, MeO); ^{19}F NMR (0.5 M sodium phosphate buffer, pH 7.5, internal trifluoroacetate reference standard) 7.42; ^{13}C NMR (DMSO-d_6, referenced to solvent resonance) 50.19 (t, MeO, J = 5.78 Hz), 117.64 (t, CF_2, J = 275.8 Hz), 162.08 (t, COONa, J = 32.6 Hz); MS, *m/z* (relative intensity) 125 ($(M-H)^-$, 100), 81 ($(M-H-CO_2)^-$, 11). Analysis Calculated for $C_3H_3F_2O_3Na$: C, 24.34; H, 2.04. Found: C, 24.40; H,2.12.

Animals. Sprague-Dawley rats (Charles River Breeding Laboratories, 300–400 g body weight) were used throughout this study. Rats were fed standard rat chow and water *ad libitum* at all times. Each animal received a single exposure to methoxyflurane; each experiment was performed with a new animal. Animal use in these studies was approved by the Animal Care Committee at the National Institute of Environmental Health Sciences.

Urine was collected for analysis using a Nalgene metabolic cage. Urine samples were collected twice a day, frozen immediately after collection, and stored at −20°. All samples collected from the same animal were thawed at the same time for analysis of methoxyflurane metabolites.

Measurements of hepatic metabolism. Measurements of hepatic methoxyflurane metabolism were performed using the *in vivo* NMR technique of London *et al.* (13). In brief, the abdominal wall directly over the liver of the rat was surgically removed to allow better observational selectivity for liver tissue using an externally placed surface coil. The rat surgery was performed under anesthesia using a ketamine/xylazine mixture (100 mg of ketamine + 32 mg of xylazine/kg rat). The animals were allowed to recover for 48–72 hr after surgery before methoxyflurane dosage.

Methoxyflurane treatment (3% for 5 min, 2% for remainder) was carried out in a closed anesthesia machine (Summit Hill Laboratories, Navesink, NJ). Three different dosage times of 15 min, 30 min, and 60 min were utilized. The animals were allowed to revive (i.e., regain righting reflex) following methoxyflurane exposure; the revival time ranged from 15 min to 2 hr depending upon the duration of methoxyflurane exposure. After the rat had revived, Inactin [5-*sec*-butyl-5-ethyl-2-thiobarbituric acid (Lockwood Associates Imports, East Lansing, MI) 100 mg/kg intraperitoneally] was administered to anesthetize the animal during the NMR observation. Inactin is a long-acting anesthetic which allows continual observation for up to 12 hr without removing the animal from the bore of the NMR magnet (14).

Identification of methoxyflurane metabolites. Methoxyflurane metabolites were identified by adding measured amounts of authentic compound to rat liver homogenates. Co-resonance was used as the criterion for positive identification. Homogenates were prepared from rats treated with methoxyflurane for 15 min. After allowing 4 hr for generation of methoxyflurane metabolites, as observed in the *in vivo* experiments, the rats were sacrificed by decapitation and their livers were excised. Minced liver was homogenized using a DenBroeck homogenizer in 2 volumes of buffer (0.1 M potassium phosphate buffer, 5 mM $MgCl_2$, pH 7.4) and centrifuged for 10 min at low speed to remove nonhomogenized material. The homogenate was quick-frozen in liquid nitrogen and stored at −20° for subsequent analysis by ^{19}F NMR.

***In vivo* NMR methods.** A Nicolet NT-360 NMR spectrometer, with an 8.9-cm vertical bore, 8.45-T superconducting magnet, was used in all studies. *In vivo* hepatic ^{19}F and ^{31}P NMR spectra were obtained using a "home-built" NMR probe consisting of two concentric transmit/receive coils. The outer coil is 1.7 cm in diameter and tuned to 146 MHz to receive ^{31}P, while the inner coil is 1.0 cm in diameter and tuned to 339 MHz to receive ^{19}F. In this probe, the anesthetized rat is positioned upright, and its movement is restricted by the tight quarters of the NMR probe and by restraint with surgical tape across the neck and lower abdomen. The optimum pulse length to excite liver tissue in the surgically prepared rat was determined by using appropriate phan-

toms placed at a distance from the coil to approximate the coil-liver distance in the *in vivo* experiment. The ability to observe signals from the liver in rats was then evaluated using ^{31}P NMR. The criterion for liver specificity was the lack of a significant phosphocreatine resonance in the ^{31}P NMR spectrum. Fig. 1 displays two ^{31}P NMR spectra, one collected from rat hindleg (Fig. 1A) and the other from the liver of one of our surgically prepared rats (Fig. 1B). Whereas the major resonance in the muscle spectrum corresponds to phosphocreatine, the liver spectrum shown here has only a small phosphocreatine resonance. The liver spectrum in Fig. 1B can be compared to published ^{31}P NMR spectra of exposed or perfused liver (13, 15). This method previously has been used both in ^{31}P NMR studies of ethionine toxicity (16) and in ^{2}H studies of methionine metabolism in rat liver (17).

For some experiments, ^{19}F and ^{31}P NMR spectra were collected sequentially to monitor the energy state and positioning of the rat during the NMR experiment. The intensity of the ^{31}P NMR resonances of hepatic ATP remained within 10% of initial spectra throughout the experiment. No other attempts were made to monitor the physiological status of the rat while in the NMR magnet.

The spin lattice relaxation time T_1 of methoxyflurane in excised rat liver was determined using the inversion recovery sequence in conventional NMR probes. The ^{19}F T_1 of methoxyflurane in excised liver tissue collected from rats after a 20-min exposure to methoxyflurane was determined to be 0.5 ± 0.1 sec. The T_2 of methoxyflurane was estimated to be 1.7 msec (±20%) from the linewidth of the ^{19}F methoxyflurane resonance. Relaxation parameters were determined from excised liver rather than *in vivo* due to difficulties in determining T_1 *in vivo* due to radiofrequency field inhomogeneities (18), and also due to intensity changes arising from methoxyflurane flux.

In acquiring *in vivo* ^{31}P NMR spectra, a 60-μsec pulse length, 1-sec relaxation delay, ±5,000 Hz spectral width, and 4,000 data points were used. *In vivo* ^{19}F NMR spectra were acquired using a 60-μsec pulse length, 2-sec ($4 \times T_1$) relaxation delay, ±15,000 Hz spectral width, and 4K data points. As evidenced later, the large sweep width was necessary to observe all of the fluorinated methoxyflurane metabolites. The number of acquisitions averaged per spectrum and the elapsed time per spectrum varied between experiments; further details are provided in the legend to Fig. 2 (under Results). For fluorine, 20-Hz exponential line broadening was applied to the free induction decays before Fourier transform; for phosphorus, 30-Hz exponential line broadening was applied.

***In Vitro* NMR methods.** For liver homogenates, a specially built 12-mm ^{19}F NMR probe (Doty Scientific) was used. The volume of liver homogenate placed in the NMR tube was 2 ml. A 30° (20-μsec) pulse was employed with a 0.3-sec interpulse delay. Normally, 2,000 or 5,000 acquisitions were averaged per spectrum. A spectral width of ±15,000 Hz was described by 4,096 data points. An exponential line broadening of 20 Hz was applied to the summed free induction decays before Fourier transform. Chemical shifts are reported relative to 50 mM trifluoroacetate in water contained in a sealed capillary.

For urine analysis of methoxydifluoroacetate and fluoride by ^{19}F NMR, a high resolution Nicolet ^{1}H probe was used. ^{19}F NMR spectra were obtained by retuning the proton coil to 339.7 MHz. The sample volume used was 0.5 ml. Other conditions were identical to those used for measuring homogenates, except that with the high resolution probe the 30° pulse length was 6 μsec. Methoxydifluoroacetate concentrations in urine samples were determined using sodium methoxydifluoroacetate solutions in urine prepared at known concentrations as standards. New standard curves were generated on each day of analysis.

Fluoride ion determination. Fluoride ion concentrations in urine were determined using an ion-specific electrode (Corning) with a Ag/AgCl reference electrode. Samples were prepared from 3 ml of urine diluted with 7 ml of distilled water and 10 ml of ionic strength regulating buffer (0.1 M Na_2HPO_4, 0.04 M citric acid) (19). Electrode calibration standards were prepared by adding known amounts of 0.1 M KF standard solution to 3 ml of control (untreated) urine and diluting as above.

Results

Hepatic Metabolism of Methoxyflurane. To monitor *in vivo* hepatic metabolism by NMR, selectivity for the liver over surrounding tissue needed to be established. The method chosen to study selectively liver metabolism by NMR is to use a surgically prepared rat, with ^{31}P NMR monitoring to ensure

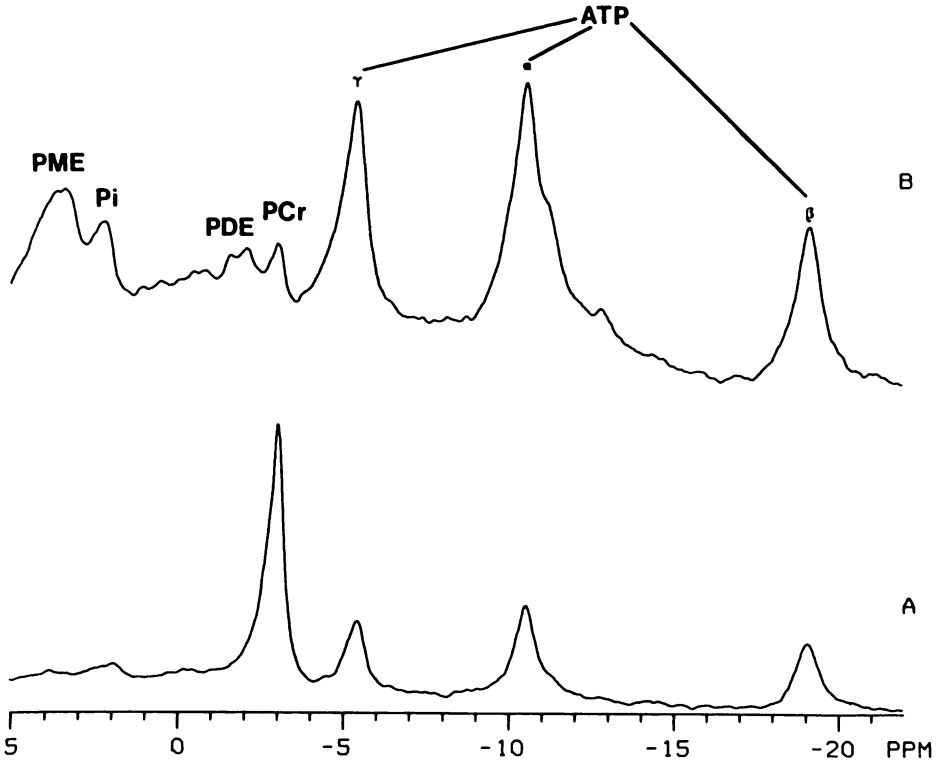

Fig. 1. ^{31}P NMR spectra of rat flank muscle (A) and rat liver (B) measured on the surgically prepared rat described under Materials and Methods. Both spectra were obtained using a 1.7-cm surface coil at 8.45 T. Other details can be found under Materials and Methods. PME, phosphomonoesters; PCr, phosphocreatine; PDE, phosphodiesters.

liver selectivity (13). Details of this method can be found under Materials and Methods.

After liver specificity was determined, the hepatic metabolism of methoxyflurane could be studied. A series of ^{19}F NMR spectra of the liver of methoxyflurane-dosed rats is given in Fig. 2. The spectra shown in the stack plot in Fig. 2 contain two resonances, a large resonance at −11.0 ppm (relative to external trifluoroacetate) which declines with time, and a second resonance at −7.1 ppm which increases with time. The resonance at −11.0 ppm can be immediately identified as methoxyflurane, based on: 1) the high initial intensity of this resonance which declines with time, 2) the appearance of this resonance immediately after methoxyflurane administration without any other observable ^{19}F NMR resonances, and 3) the co-resonance of the −11.0 ppm peak observed in liver homogenates prepared from a rat immediately after a 15-min methoxyflurane dose and when authentic methoxyflurane is added to the same homogenate.

The second intrahepatic ^{19}F resonance observed exhibits a chemical shift which differs significantly from that observed for solutions of potassium fluoride, and hence was tentatively assigned to methoxydifluoroacetate based on the previously proposed metabolic fate of methoxyflurane. To positively assign this resonance, a direct synthesis of methoxydifluoroacetate was performed by reaction of chlorodifluoroacetic acid with sodium methoxide in dimethylformamide. Purification by a series of extractions and subsequent treatment with cation exchange resin yielded pure, crystalline sodium methoxydifluoroacetate. The structure of the synthesized species was confirmed by ^{1}H, ^{13}C, and ^{19}F NMR, mass spectroscopy, and elemental analysis. The ^{31}C NMR spectrum of synthetic methoxydifluoroacetate shows fluorine coupling with all three carbons and indicates the high degree of purity of the product (Fig. 3). Solution spectra of the synthesized methoxydifluoroacetate exhibit a ^{19}F chemical shift (−7.4 ppm) very similar to that of the unassigned resonance in liver. To provide further evidence for assigning the −7.1 ppm resonance to methoxydifluoroacetate, the synthesized compound was added to a liver homogenate prepared from a rat 4 hr after a 15-min methoxyflurane exposure. The resulting spectrum shows that the synthesized methoxydifluoroacetate co-resonates with the methoxyflurane metabolite. The chemical shift values for methoxyflurane and its metabolites in liver and liver homogenates are given in Table 1.

Curiously, no resonance corresponding to fluoride could be detected *in vivo*. To determine whether the resonance for fluoride ion in the liver is broadened by binding to some cellular component, liver homogenates were prepared from rats 4 hr after a 15-min methoxyflurane exposure. The homogenate was separated into aliquots. For the first aliquot, a control ^{19}F NMR spectrum was recorded; no fluoride resonance was visible in the homogenates. To the same aliquot 100 mM EDTA was added and a second ^{19}F NMR spectrum was recorded. Again, no fluoride ion resonance is observed. A second aliquot was heated to 100° for 1 min, cooled to room temperature, and centrifuged to remove solid debris. The supernatant was then checked for fluoride ion by ^{19}F NMR. This sample has no observable resonance for fluoride ion. The third aliquot was treated with an equal volume of chelating resin for 30 min at room temperature. The sample was then centrifuged to remove resin and solid material, and the supernatant was measured for fluoride ion using ^{19}F NMR. Again, no resonance for fluoride ion could be detected (see Fig. 4). ^{19}F NMR spectra obtained using homogenates prepared from rats 30 hr after a 15-min methoxyflurane exposure also did not exhibit any resonance corresponding to fluoride ion (data not shown). Therefore, no measurable concentrations of inorganic fluoride are detected in liver by NMR following methoxyflurane treatment.

Hepatic elimination of methoxyflurane. From integration of the methoxyflurane resonance in a series of ^{19}F NMR spectra taken over time, the rate of methoxyflurane elimination from the liver can be determined. Elimination rates of methoxy-

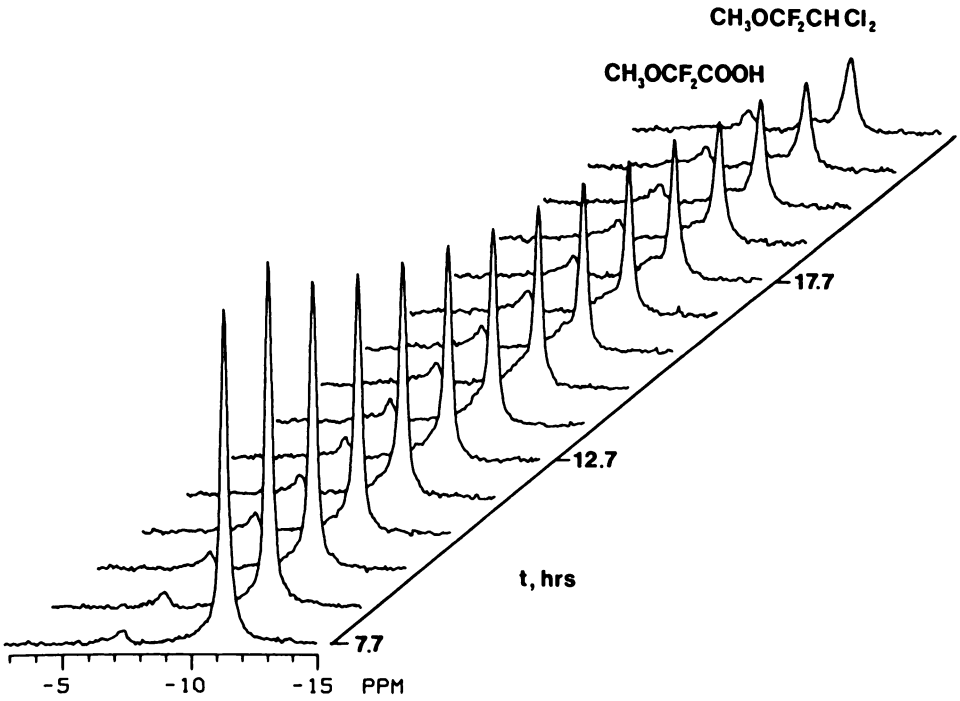

Fig. 2. Stacked plot of consecutive ^{19}F NMR spectra of the liver of a rat dosed with methoxyflurane. Each spectrum in the stacked plot is the time average of 3400 transients, and took 1 hr to accumulate. The first spectrum was begun 6.67 hr after the end of a 1-hr administration of methoxyflurane, as described under Materials and Methods.

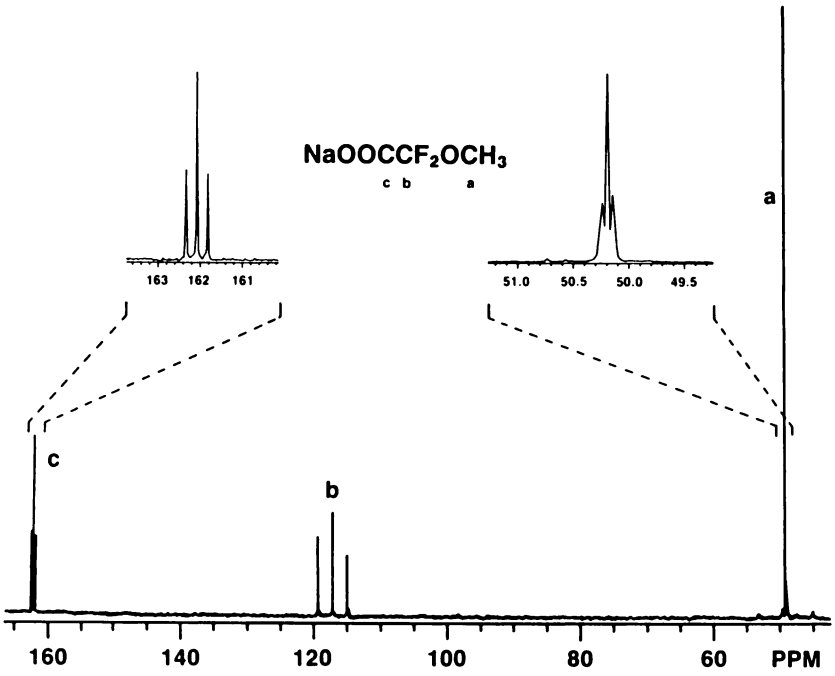

Fig. 3. ^{13}C NMR spectrum of the putative methoxyflurane metabolite methoxydifluoroacetate.

TABLE 1

^{19}F NMR chemical shifts (in ppm relative to external trifluoroacetate) of methoxyflurane and its metabolites in liver homogenates and in urine

	Chemical shift		
	Methoxyflurane	Methoxydifluoroacetate	F^-
Urine		−7.3	−43.5
Liver homogenate	−10.7	−7.6	
In vivo	−11.0	−7.1	

flurane from the liver have been calculated at three different methoxyflurane doses and are reported in Table 2. Two points are to be noted from this table. First, there is a significant difference in hepatic elimination rates of methoxyflurane calculated in these studies, depending upon the extent of methoxyflurane dose. Second, the rate of elimination of methoxyflurane from the liver is much less than the rate for elimination of halothane, determined to have a first order decay rate constant of 0.21 hr^{-1} for a 1-hr halothane exposure carried out under similar conditions (11).

Urine studies of methoxyflurane elimination. To determine the whole body elimination of methoxyflurane, ^{19}F NMR analysis of rat urine collected from animals following methoxyflurane exposure was carried out. Previous studies of urinary metabolites of methoxyflurane lacked quantitative data on the amount of methoxydifluoroacetate in the urine, and therefore, the relative concentrations of fluoride and methoxydifluoroacetate could not be determined. The ^{19}F NMR analysis can measure the concentration of the two metabolites mentioned above in urine samples, and also may discover other fluorine-containing methoxyflurane metabolites which exist in concentrations too low to be identified previously.

The ^{19}F NMR spectrum of rat urine collected in the first 7 hr following a 1-hr exposure to methoxyflurane is shown in Fig. 5A. Two resonances are visible which correspond to methoxydifluoroacetate and to inorganic fluoride. In the ^{19}F NMR spectra of urine collected from methoxyflurane-treated rats, we note that the resonance corresponding to fluoride ion is broadened to a variable extent in different samples. The ^{19}F NMR resonance for fluoride is known to be broadened if paramagnetic ions are in urine (20); additionally, diamagnetic ions such as Ca^{2+} and Mg^{2+} will broaden the fluoride resonance due to chemical exchange.

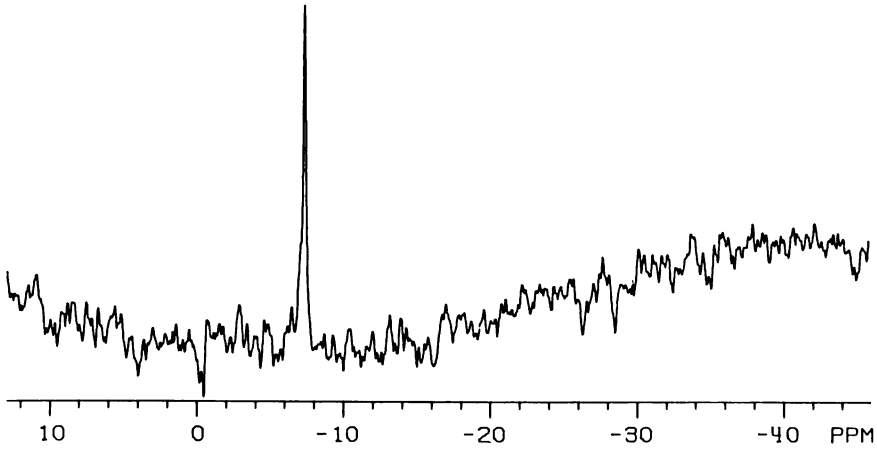

Fig. 4. ^{19}F NMR spectrum of a liver homogenate prepared from a methoxyflurane-treated rat after treatment with chelating resin, as described under Results. The number of transients was 80,000.

TABLE 2

Hepatic elimination rates of methoxyflurane from rats as a function of dose

Dose	k_1[a]	n[b]	r^2[c]
	hr^{-1}		
15 min	0.24 ± 0.06	3	0.97–0.99
30 min	0.14 ± 0.05	3	0.89–0.96
60 min	0.07 ± 0.04[d]	3	0.75–0.99

[a] k_1 represents the first order decay constant of the methoxyflurane resonance measured *in vivo*. Values are ± standard deviation.

[b] n represents the number of experiments. Each first order decay constant was determined from one rat; nine rats were used to generate the data in this table.

[c] The range of r^2 curve-fitting values for modeling methoxyflurane signal loss as a first order decay.

[d] Significantly different from 15 mM dose ($p < 0.05$) by the two-tailed Student's t test.

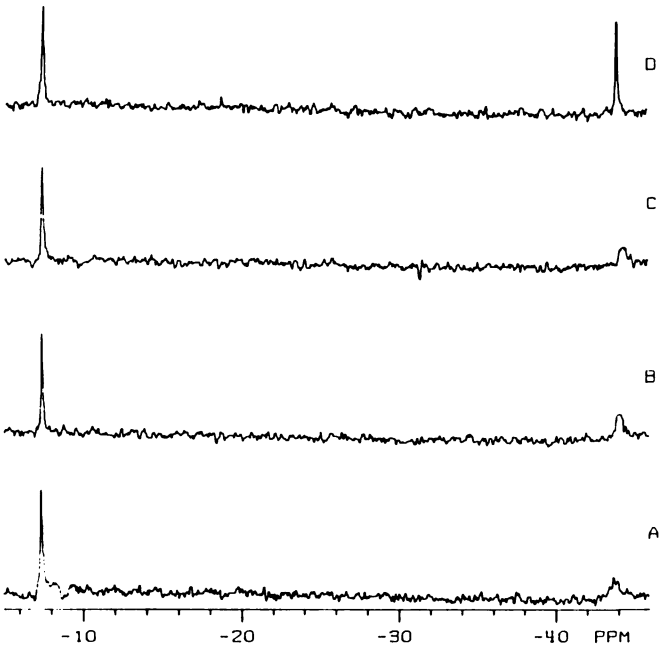

Fig. 5. ^{19}F NMR spectrum of urine collected for 7 hr after the administration of a 1-hr dose of methoxyflurane. The spectra shown were accumulated as described under Materials and Methods. A. Untreated urine. B. Urine after the addition of 10 mM EDTA. C. Urine after the addition of 100 mM EDTA. D. Urine after treatment with chelating resin, as described under Results. In all spectra, 2000 transients were accumulated.

To determine the cause of broadening of the fluoride resonance, a procedure similar to that used for the liver microsomes was employed. When 10 mM EDTA was added to urine and the ^{19}F NMR spectrum obtained, the resonances were only slightly sharpened (Fig. 5B). Increasing the EDTA concentration to 100 mM shows no added reduction in the linewidth of the fluoride ion resonance (Fig. 5C). However, when urine was allowed to stand at room temperature for 30 min with an equal volume of chelating resin, the fluoride ion resonance was narrowed to give a linewidth similar to that observed for methoxydifluoroacetate (Fig. 5D). These experiments indicate that some multivalent cation is interacting with fluoride ion, leading to a broadened NMR resonance, and that EDTA does not significantly reduce the observed broadening.

To determine the urinary concentrations of fluorinated methoxyflurane metabolites, a standard curve of intensity versus concentration of fluoride and methoxydifluoroacetate was generated by adding known amounts of these two compounds to urine collected from untreated rats. Using standard curves, the concentrations of the two methoxyflurane metabolites could be determined. However, the variable linewidths of the fluoride ion resonances make quantitation difficult. Therefore, fluoride ion concentrations in urine were determined using a fluoride ion-specific electrode. Fluoride ion concentrations determined by the two methods generally agreed within 20%. The fluoride ion concentrations reported here were those obtained using the ion-specific electrode.

To measure the whole body elimination of methoxyflurane in the rat, urine samples were collected for 1 week following methoxyflurane dosage. The concentrations of the methoxyflurane metabolites were determined as a function of time. The results of this experiment are plotted in Fig. 6. From this figure, it is seen that the excretion of both methoxydifluoroacetate and fluoride is maximal approximately 24–30 hr after a 1-hr methoxyflurane dose, and also that measureable amounts of methoxyflurane metabolites are still excreted up to 7 days after methoxyflurane exposure.

Discussion

Lack of NMR-observable hepatic fluoride. In the ^{19}F NMR studies of hepatic methoxyflurane metabolism, it was noted that no inorganic fluoride could be observed either in rat liver measured *in vivo* or in homogenates of rat livers extracted immediately following methoxyflurane dosage. From the urine studies described above, fluoride is certainly being generated from methoxyflurane in the rat. The lack of inorganic fluoride in the liver could be attributed to: (a) the quick excretion of

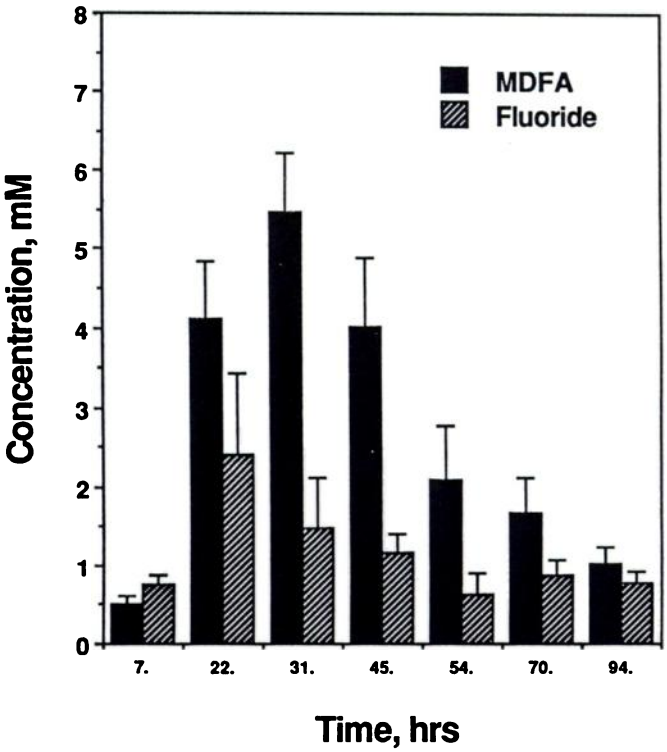

Fig. 6. Time course of excretion of methoxydifluoroacetate (*MDFA*) and inorganic fluoride, as measured by analysis of rat urine. Each time point represents the averaged concentrations of metabolites from six rats. Methoxydifluoroacetate production was monitored by ^{19}F NMR as described under Materials and Methods, whereas fluoride production was monitored by a fluoride ion-specific electrode. The error bars represent 1 SE.

fluoride from the liver after its formation from methoxyflurane, (b) broadening of the liver fluoride resonance due to binding to metal ions or immobile cellular components, or (c) the formation of fluoride being extrahepatic. The third possibility is unlikely, since in hepatic microsome experiments metabolism of methoxyflurane to inorganic fluoride has been observed (21). We do note that the enzymes necessary to metabolize methoxyflurane to fluoride ion do exist in tissues other than liver (22). To distinguish between fast excretion of liver-generated fluoride and broadening of the ^{19}F NMR resonance for fluoride, liver homogenates were prepared from livers excised from rats 4 and 30 hr after a 15-min methoxyflurane exposure. Since NMR resonances, and fluoride resonances in particular, are often broadened by interaction with paramagnetic metal ions (20), the liver homogenates were treated in several ways which would remove the agent causing the broading of the NMR resonance. The lack of observable fluoride in liver homogenates under all conditions indicates that fluoride generated in the liver is quickly excreted into the bloodstream. This is a reasonable hypothesis, given how rapidly fluoride appears in the urine after methoxyflurane exposure (Fig. 6).

Broadening of the urinary fluoride resonance. The broadening of the urinary fluoride resonance and the inability of additions of mM levels of EDTA to significantly reduce this broadening illustrate the difficulties of using NMR methodology to assay for fluoride in biological systems. Since treatment with chelating resin is able to eliminate this broadening, the effect can be ascribed to some chelatable metal ions. Although paramagnetic ions are known to broaden the NMR resonances of halide ions via dipolar relaxation, urine is known to contain mM levels of calcium and magnesium as well. This effect may reflect chemical exchange of the fluoride between uncomplexed and complexed environments, as well as the limited solubility of calcium and magnesium fluorides. The limited effectiveness of EDTA in reducing this broadening may reflect the strength of the metal-fluoride bond, as well as the possibility of additional complexation of the chelated ions with fluoride. Crystal structures of magnesium-EDTA indicate that the magnesium is seven coordinate, with a water molecule representing the seventh ligand (23). Clearly, the absence of a significant fluoride resonance in the liver and the broadening observed in the urine limit the use of ^{19}F NMR for this type of application.

Hepatic elimination of methoxyflurane. As seen in Table 2, there is a significant difference in elimination rates of methoxyflurane from rat liver dependent upon anesthetic dose time. This difference is not likely to be due to different rates of metabolism in rat liver. As determined from microsome studies, the K_m for methoxyflurane is approximately 0.2 mM in noninduced rats (21). Since the mean alveolar concentration for methoxyflurane is 0.9 mM (24), and noting that the liver-blood solubility partition coefficient for fluorinated anesthetics is greater than 1 (25, 26), the concentration of methoxyflurane in the liver of a fully anesthetized rat is near saturating for the enzymes which metabolize it (21). In fact, at our longest exposure times, the rats remain asleep for up to 1 hr after ceasing anesthetic exposure and are visibly slowed due to the anesthesia for up to 2 hr longer. Methoxyflurane is readily deposited into fat and other fatty tissues (25). Therefore, differences noted in hepatic methoxyflurane elimination are probably due to ongoing recruitment of methoxyflurane into liver from storage in fat and fatty tissue concurrent with metabolism of methoxyflurane by the liver.

In this regard, we note that the methoxyflurane elimination rates from liver were calculated using a model for first order decay. Although there was some variability in the calculated decay rates between animals (as noted by the relatively large standard deviations), most of the data fit well to a first order decay model. We do not expect the actual elimination of methoxyflurane from the liver to be a monophasic first order process. Since it was necessary to wait for the animal to revive from methoxyflurane anesthesia before beginning the measurement of anesthetic elimination, other, faster decay processes may have occurred before methoxyflurane clearance was monitored.

All elimination rates in this report were determined from inactin-anesthetized rats. Inactin might well inhibit the cytochrome P-450-mediated metabolism of methoxyflurane. Although the effects of inactin on drug metabolism are not well known, the metabolic interactions of volatile anesthetics with other barbituates have been examined (27–30). Therefore, methoxyflurane elimination rates actually may be faster than reported here. One study has demonstrated that inactin does not affect either hepatic ATP levels or hepatic response to acute ethionine poisoning (16).

Urinary clearance of methoxyflurane metabolites. Urinary analysis of methoxyflurane metabolism demonstrates that methoxyflurane is metabolized *in vivo* to methoxydifluoroacetate and fluoride ion. The urine concentration of both metabolites reaches a maximum at between 24 and 30 hr after a 1-hr exposure to methoxyflurane, which is consistent with previous studies which measured only inorganic fluoride as a function of time (31, 32). The concentrations of both fluoride ion and methoxydifluoroacetate decrease to less than 20 μM (which is our limit of detection using our standard assay conditions) after approximately 7 days. Homogenates of livers extracted from rats 7 days post-anesthesia also show no measurable fluorine resonances.

In conclusion, the ^{19}F NMR analysis of methoxyflurane metabolism reported here illustrates the broad range of information related to absorption, transformation, and elimination which can be derived from such studies. Additionally, the NMR technique is of value for making a more definitive identification of fluorinated organic species. Although the metabolic conclusions summarized in Eqs. 1 and 2 are based largely on the observation of the organic metabolite, methoxydifluoroacetate, in methoxyflurane-treated animals, the synthesis of this compound and study of its NMR properties were needed to establish its identity and to provide quantitation. The present studies provide clear confirmation of the presence of this intermediate. Additional aspects of the metabolism of methoxyflurane are treated in the accompanying paper (12).

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