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In Vivo Nuclear Magnetic Resonance Studies of Hepatic Methoxyflurane Metabolism. II. A Reevaluation of Hepatic Metabolic Pathways

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SUMMARY

Methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) is believed to be metabolized via two convergent metabolic pathways. The relative flux through these two metabolic pathways has been investigated using a combination of *in vivo* surface coil NMR techniques and *in vitro* analyses of urinary metabolites. Analysis of the measured concentrations of inorganic fluoride, oxalate, and methoxydifluoroacetate in the urine of methoxyflurane-treated rats for 4 days after anesthesia indicates that the anesthetic is metabolized primarily via dechlorination to yield methoxydifluoroacetate. The methoxydifluoroacetate is largely excreted without further metabolism, although a small percentage of this metabolite is broken down to yield fluoride and oxalate, as determined by urine analysis of rats dosed with synthetic methoxydifluoroacetate. At early times after methoxyflurane exposure, the relative concentrations of methoxyflurane metabolites indicate that a significant fraction of the metabolic flux occurs via a different pathway, presumably demethylation, to yield dichloroacetate as an intermediate. Direct analysis of dichloroacetate in the urine using water-suppressed proton NMR indicates that the level of this metabolite is below the detection threshold of the method. Measurements made on the urine of rats dosed directly with dichloroacetate indicate that this compound is quickly metabolized, and dichloroacetate levels in urine are again found to be below the detection threshold. These results demonstrate the quantitative importance of the dechlorination pathway in the metabolism of methoxyflurane in rats.

The development of *in vivo* NMR spectroscopy has led to increased insight into the biochemistry and physiology of organs and intact tissue (1). The applications of this technique to the study of normal and diseased tissue have increased steadily over the past several years (2). In contrast, applications of NMR to *in vivo* toxicology are still in their infancy. The most promising aspect of *in vivo* toxicology is the use of ^{19}F NMR to measure the metabolism and elimination of fluorinated xenobiotics. Using this method, the hepatic metabolism of 5-fluorouracil (3, 4) and halothane (5) have been studied in humans and in laboratory animals. In the previous paper (6), ^{19}F NMR has been used to measure the hepatic metabolism and elimination of the fluorinated inhalation anesthetic methoxyflurane in rats. The methoxyflurane metabolite methoxydifluoroacetate (MDFA) has been unequivocally identified, and its presence as the major fluorinated methoxyflurane metabolite in the liver and in urine has been demonstrated.

The presently accepted model for methoxyflurane metabolism (Fig. 1) indicates that the anesthetic is metabolized to the end products fluoride and oxalate via two different metabolic pathways. One pathway begins with dechlorination and oxidation of the $—CHCl_2$ moiety of the methoxyflurane molecule, resulting in MDFA (hereafter referred to as the "dechlorination pathway"). MDFA may be capable of decomposing, either chemically or enzymatically, to oxalate and fluoride (7). The second pathway begins with oxidation of the methoxy group of methoxyflurane, with further decomposition to dichloroacetate and fluoride (the "demethylation pathway"). Dichloroacetate may either be excreted without further metabolism (8) or broken down to chloride and either oxalate, glycine, or carbon dioxide (9).

However, the data used to develop this model are incomplete. The original reported analytical data which supported the identification of MDFA were not conclusive since the metabolite was not isolated in pure form (8). In addition, the identification of dichloroacetate as a methoxyflurane metabolite (8) was refuted in a later report by the same research group (10). Although the capability of hepatic enzymes to metabolize methoxyflurane to dichloroacetate in vitro has been demonstrated (11), the flux through this pathway in the *in vivo* metabolism of methoxyflurane is still in question. Furthermore, since fluoride and oxalate are the only methoxyflurane metabolites which have been previously quantitated, no data exist on the relative flux of methoxyflurane metabolism through the dechlorination and demethylation pathways.

In the present study, these aspects of methoxyflurane metabolism have been evaluated using *in vitro* and *in vivo* NMR techniques. To aid in the evaluation, a new assay for urinary

ABBREVIATION: MDFA, methoxydifluoroacetate.

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dichloroacetate using water-suppressed proton NMR of urine has been developed. The ability to quantitate the metabolites MDFA, dichloroacetate, fluoride, and oxalate has allowed for a more complete understanding of methoxyflurane metabolism. To summarize the results of these studies, we have not been able to identify dichloroacetate in rat urine following methoxyflurane anesthesia. Since no dichloroacetate is observed, only the dechlorination pathway can be corroborated for the *in vivo* metabolism of methoxyflurane. However, measurements of *in vivo* dichloroacetate metabolism by urine analysis indicate that dichloroacetate is quickly and completely metabolized after administration. A comparison of the excreted concentrations of methoxyflurane metabolites as a function of time after methoxyflurane anesthesia indicates that, whereas most of the anesthetic is metabolized via the dechlorination pathway, a small percentage is metabolized via a different pathway, presumably the demethylation pathway.

Materials and Methods

Chemicals. Methoxyflurane [with 0.01% (w/w) butylated hydroxytoluene] was obtained from Pitman-Moore, Washington Crossing, NJ. Sodium methoxydifluoroacetate was synthesized as previously described (6). Dichloroacetic acid was purchased from Aldrich Chemical Co. All other chemicals were the highest purity commercially available and used without further purification.

Animals. Sprague-Dawley rats (Charles River Breeding Laboratories; 300–400 g body weight) were used throughout this study. Rats were fed with standard rat chow and water *ad libitum*.

***In vivo* metabolism of MDFA.** Measurements of *in vivo* metabolism of MDFA were performed on rats whose abdominal wall had been surgically excised (6). Forty-eight to 72 hr after surgery, rats were anesthetized with Inactin (5-*sec*-butyl-5-ethyl-2-thiobarbituric acid, Lockwood Associated Imports, East Lansing, MI; 100 mg/kg, intraperitoneally), after which MDFA was injected intraperitoneally at a dose of 50 mg/kg dissolved in 1 ml of isotonic saline. The animal was then placed in the magnet and hepatic metabolism was measured with a surface coil as previously described (6).

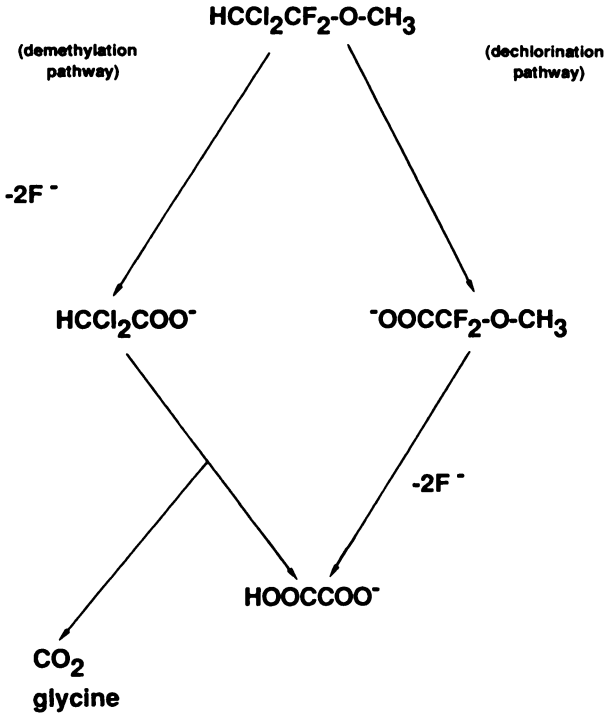

Fig. 1. Abbreviated model of methoxyflurane metabolism. This model is a modified version of the model proposed originally by Mazze *et al.* (7), with added details of dichloroacetate metabolism derived from Ref. 9.

MDFA metabolism was also measured by NMR and chemical analysis of urine samples from MDFA-treated rats. Rats were dosed with 50 mg/kg MDFA as above. Urine samples were collected for 4 days after MDFA administration and analyzed for MDFA, fluoride, and oxalate as described below. Seven rats were dosed to study urinary metabolites of MDFA.

***In vivo* metabolism of dichloroacetate.** Dichloroacetate was administered to rats as a 50 mg/ml solution of dichloroacetic acid in isotonic saline, neutralized to pH 7 with 5 N NaOH. Rats were injected intraperitoneally at a dose of 50 mg/kg. After administration, urine samples were collected for 4 days and analyzed for dichloroacetate and oxalate. Five rats were treated with dichloroacetate for measurement of metabolism by urine analysis.

Urine analysis of methoxyflurane metabolites. For analysis of urinary methoxyflurane metabolites, two rats were anesthetized for 1 hr (3% for 5 min, 2% for 55 min) using a veterinary anesthesia chamber (Summit Hill Laboratories, Navesink, NJ). After anesthesia, the rats were immediately placed in plastic metabolic cages (Nalgene). 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 and analyzed for methoxyflurane metabolites on the same day. These experiments were repeated three times, using a total of six rats.

Assays. Concentrations of MDFA were determined as previously described (6). Concentrations of inorganic fluoride were determined using a fluoride-specific ion electrode (6). Fluoride standards were prepared by adding 0.1 M NaF solution to urine collected from non-treated, nonoperated rats.

Urinary oxalate concentrations were determined using an oxalate oxidase enzymatic assay kit purchased from Sigma Chemical Company (St. Louis, MO). This assay is identical to that developed by Laker *et al.* (12), except that oxalate oxidase isolated from barley rather than moss was used. The sensitivity limit for this assay is estimated to be 10 μM in urine. The reported oxalate concentrations were corrected for normal oxalate concentrations found in urine of rats before exposure to the chemical of interest.

Urine analysis of dichloroacetate. Samples were prepared for assay of dichloroacetate by adding deuterium oxide to urine samples to a final concentration of 20%. ^{1}H NMR was used to identify and quantitate dichloroacetate, using the resonance from the single proton on the chlorine-containing carbon observed at a chemical shift of 6.08 ppm relative to tetramethylsilane. In the assay, proton chemical shifts were referenced to a strong singlet resonance at 5.397 ppm corresponding to allantoin (13). Water-suppressed ^{1}H NMR spectra were obtained using a GN-500 spectrometer (General Electric Company Medical Systems, Fremont, CA) at 500.1 MHz. Water suppression was achieved using presaturation at the water resonance frequency. Typically, a 6-sec presaturation pulse was used, followed by a 90° (18-μsec) pulse and data acquisition. A relaxation delay of 20 sec was allowed between pulses to allow for the complete relaxation of the dichloroacetate proton. One-Hz exponential line broadening was applied to the free induction decay before Fourier transformation.

Solution stability of MDFA. The stability of MDFA in solution as a function of pH was determined by making 5 mM solutions of MDFA in 50 mM Tris/50 mM citric acid buffer, with the pH adjusted to values between 4.0 and 9.0. The solutions were stored in 5-mm NMR tubes and incubated at 37°. Tubes were removed from incubation at fixed times following the beginning of the incubation, and ^{19}F NMR spectra were recorded to measure the relative concentrations of MDFA present. The percentage of decomposition was calculated from the ratio of fluoride to methoxydifluoroacetate present in the NMR spectrum. Conditions used to accumulate ^{19}F NMR spectra were similar to those used for the accumulation of urine spectra, except that a 30° (5-μsec)

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excitation pulse was used with a 5-sec relaxation delay, to allow for full relaxation of the fluorine nuclei in both compounds of interest. These spectra were accumulated at 37°.

Results

As summarized in Fig. 1 and in the introduction, two metabolic pathways have been proposed to contribute significantly to the hepatic metabolism of methoxyflurane (6). As discussed in the preceding study (6), the observation of a ^{19}F resonance in both the urine and the liver of methoxyflurane-treated rats with a chemical shift identical to that of synthesized MDFA provides direct evidence for the significance of the dechlorination pathway. Two further questions of interest are: 1) to what extent does the alternate demethylation pathway contribute to the total metabolism of methoxyflurane, and 2) to what extent is MDFA metabolized to yield inorganic fluoride and oxalate? The studies described below were performed in order to obtain further insight into these questions.

Urine analysis of methoxyflurane-treated rats. Analysis of the levels of inorganic fluoride, MDFA, and oxalate in urine as a function of time were carried out using the assays described under Materials and Methods. Dichloroacetate concentrations in urine were determined using water-suppressed ^1H NMR. The feasibility of this approach results from the chemical shift of the dichloroacetate proton at 6.08 ppm, a relatively uncrowded region of the ^1H NMR spectrum of urine (Fig. 2a). One possible limitation of this approach arises as a consequence of the absence of good relaxation mechanisms for the observable methine proton of dichloroacetate. We have measured the spin lattice relaxation time T_1 of the methine proton of dichloroacetate to be 6.7 sec in a 10% solution of dichloroacetate in water. Experimentally, a 20-sec relaxation delay was used which, when coupled with a 6-sec saturation pulse at the water resonance frequency, allows for full relaxation of the dichloroacetate proton and maximum signal intensity (Fig. 2, b and c). A second limitation of the assay is evident in Fig. 2c. There is a resonance at 6.06 ppm which overlaps the resonance for the methine proton of dichloroacetate at 6.08 ppm. The assay can still be carried out, but the endogenous resonance at 6.06 ppm limits the practical sensitivity of the assay to 0.5 mM in urine. In the concentration range 0.5–10.0 mM, a linear standard curve of resonance intensity versus dichloroacetate concentration can be generated.

The proton resonance for dichloroacetate in urine shown in Fig. 2c appears to be split into two resonances of unequal intensity. Although the cause for this apparent splitting is not known, the relative intensity of the resultant resonances may indicate an isotope effect on the methine proton resonance. The observation of chlorine isotope effects in chloromethanes has been reported recently (14, 15).

Urine samples were collected from rats for a period of 1 week following a 1-hr exposure to methoxyflurane. Concentrations of inorganic fluoride, MDFA, and oxalate were determined as a function of time following exposure and are reported in Fig. 3. Dichloroacetate was also assayed in these urine samples; there was no evidence for excreted dichloroacetate following methoxyflurane anesthesia.

An examination of Fig. 3 shows that the concentrations of excreted MDFA and fluoride reach a maximum at approximately 24–30 hr post-methoxyflurane exposure. This is consistent with tissue retention of methoxyflurane for extended times after anesthesia, followed by release back into the blood and metabolism by the liver. As determined previously (6), the metabolism and elimination of both methoxyflurane and MDFA from the liver take place over a period of many hours. At the 7- and 22-hr time points after exposure, the ratio of fluoride to oxalate excreted is much greater than at times later than 30 hr after exposure (see *inset* to Fig. 3). The concentrations of fluoride and oxalate remain relatively constant from 30 hr until 98 hr after exposure, whereas the concentration of excreted MDFA decreases. Measurable amounts of methoxyflurane metabolites are still excreted for 4 days after methoxyflurane exposure.

***In vivo* metabolism of dichloroacetate.** Using the assay reported above, there is no evidence for significant concentrations of dichloroacetate in the urine of methoxyflurane-anesthetized rats. If dichloroacetate, formed by the demethylation pathway of methoxyflurane catabolism, is further metabolized to yield only oxalate, as indicated by Plummer *et al.* (16), then the final ratio of urinary fluoride/oxalate should be 2:1. However, studies with isolated hepatocytes (9) indicate that dichloroacetate may be metabolized to other species as well as

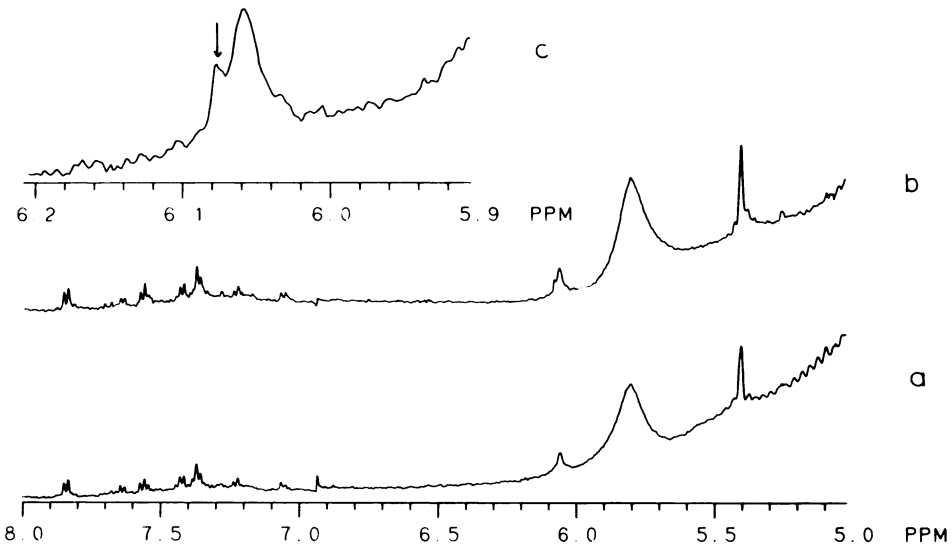

Fig. 2. Illustration of the dichloroacetate assay using water-suppressed ^1H NMR. a. A ^1H NMR spectrum of urine collected from an untreated rat. The procedure used to accumulate this spectrum is described under Materials and Methods. b. A spectrum of urine collected from an untreated rat to which was added dichloroacetic acid to a final concentration of 1.0 mM. c. An expansion of spectrum b from 5.9 to 6.2 ppm showing the region of interest. The resonance for dichloroacetate is marked with an *arrow*. The broad resonance overlapping the dichloroacetate resonance is found in all spectra and has not been identified.

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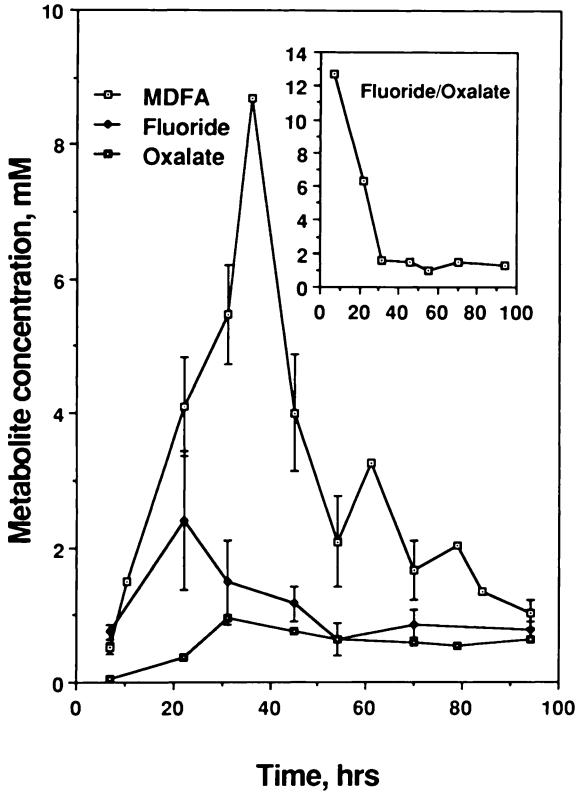

Fig. 3. Time course of excretion of the metabolites MDFA, fluoride, and oxalate in rat urine following a 1-hr exposure to methoxyflurane. Rats were exposed to methoxyflurane as described under Materials and Methods. The *symbols* which correspond to the three metabolites are described in the figure. The points shown for MDFA and fluoride are the average values for six rats, with the *error bars* representing 1 SE. The points for oxalate represent the average values measured for two rats. *Inset:* Ratio of fluoride/oxalate excreted in rat urine as a function of time after methoxyflurane anesthesia.

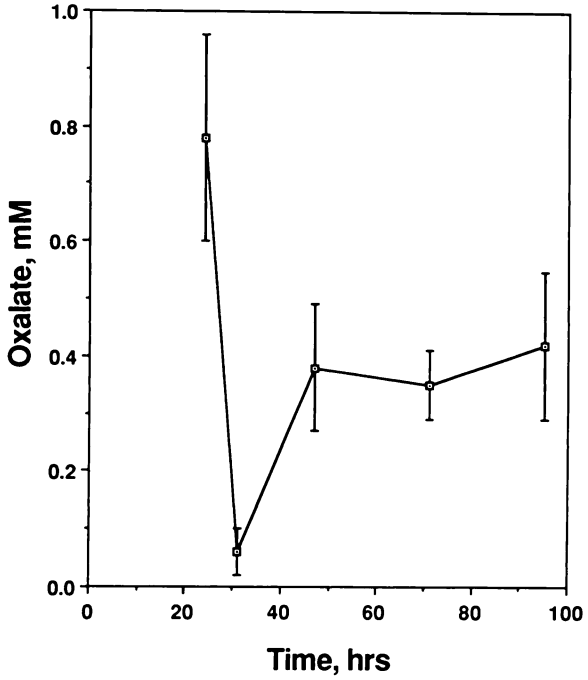

Fig. 4. Time course of oxalate excretion in the urine of rats dosed with 50 mg/kg dichloroacetate. The points represent the average of five rats, with the *error bars* representing 1 SE.

TABLE 1

Mass balance of metabolism of methoxydifluoroacetate and dichloroacetate

Compound	Percent recovered[a]	*n*
Methoxydifluoroacetate	67 ± 6	7
Dichloroacetate[b]	17 ± 5	3

[a] Values are ± standard error.
[b] Recovered as oxalate.

oxalate, which would decrease the concentration of excreted oxalate relative to fluoride. In order to determine whether dichloroacetate is metabolized *in vivo* or excreted without biotransformation, dichloroacetate was administered to rats intraperitoneally at a dose of 50 mg/kg. Urine samples were collected for 4 days following dichloroacetate administration and assayed for oxalate and dichloroacetate. Subject to the detection threshold noted under Materials and Methods, no dichloroacetate was observed in the urine of rats injected with dichloroacetate. There was an increase in the concentration of excreted oxalate as compared to control urine, with the greatest increase noted at 24 hr after dichloroacetate administration (Fig. 4). The inability to measure unmetabolized dichloroacetate and the rapid appearance of oxalate in urine support the idea that dichloroacetate is quickly metabolized *in vivo*.

The percentage of dichloroacetate metabolized to oxalate was determined by summing the μmol of excreted oxalate for 4 days after dichloroacetate injection. The recovery of metabolized dichloroacetate as oxalate was determined to be 17% (Table 1). This percentage is less than that predicted from studies with isolated hepatocytes, where 40% of administered dichloroacetate is recovered as oxalate (9).

Hepatic metabolism of MDFA. To determine whether MDFA could be metabolized to fluoride plus oxalic acid *in vivo*, MDFA was given to rats by intraperitoneal injection, and its hepatic metabolism was followed using the surface coil ^{19}F NMR method, described under Materials and Methods, and by urine analysis by ^{19}F NMR. Fig. 5 shows a stack plot of ^{19}F NMR spectra of rat liver accumulated at 1-hr intervals beginning 30 min after the administration of 50 mg/kg MDFA intraperitoneally into the rat. A single resonance is observed which corresponds to MDFA. This figure indicates that MDFA can be absorbed into the liver after an intraperitoneal dose but does not appear to be metabolized. No resonance corresponding to fluoride ion is observed; however, as shown previously, fluoride ions generated in the liver may be quickly removed from this organ (6).

To better determine the *in vivo* metabolism of MDFA, urine samples were collected from rats for 4 days after a 50-mg/kg intraperitoneal dose. The concentrations of MDFA, fluoride, and oxalate were determined as described under Materials and Methods as a function of time after MDFA administration. The results of these determinations are described in Fig. 6. It is clear from this figure that measurable amounts of fluoride and oxalate are generated from MDFA *in vivo*. Note also that the ratio of excreted fluoride to oxalate never exceeds 2:1. The recovery of administered MDFA in urine was also determined, and is reported in Table 1.

Previous studies have suggested the possibility that MDFA is unstable in solution and that this compound is not enzymatically degraded but instead is excreted from the liver intact,

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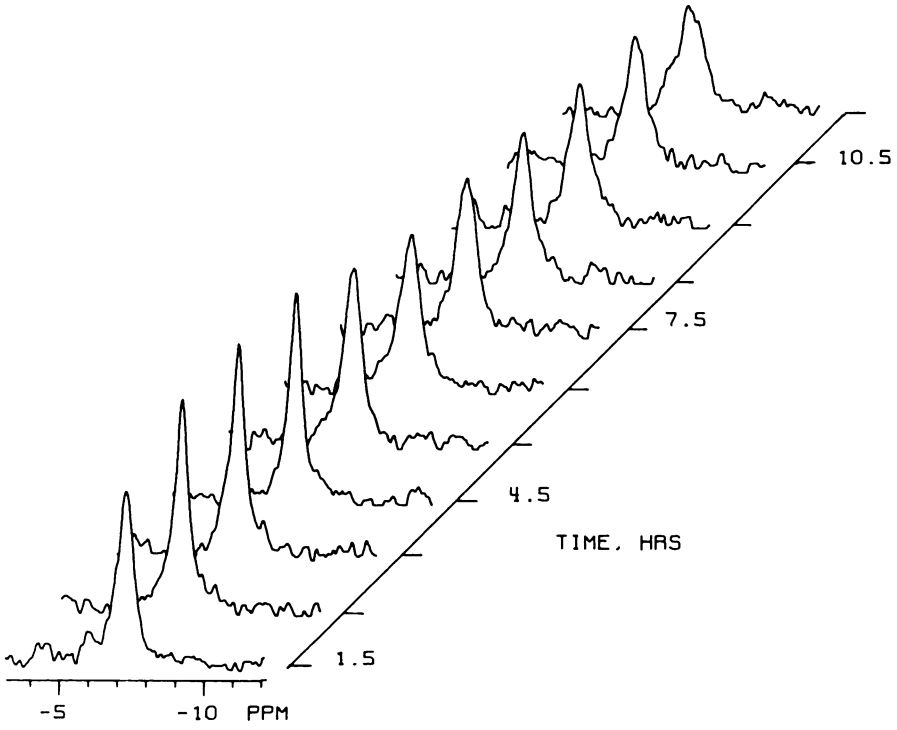

Fig. 5. Stack plot of hepatic ^{19}F NMR spectra obtained from a rat after a 50 mg/kg dose of MDFA. The first spectrum was accumulated beginning 0.5 hr after the administration of MDFA, and each spectrum took 1 hr to accumulate. Other details of the experiment can be found in Materials and Methods.

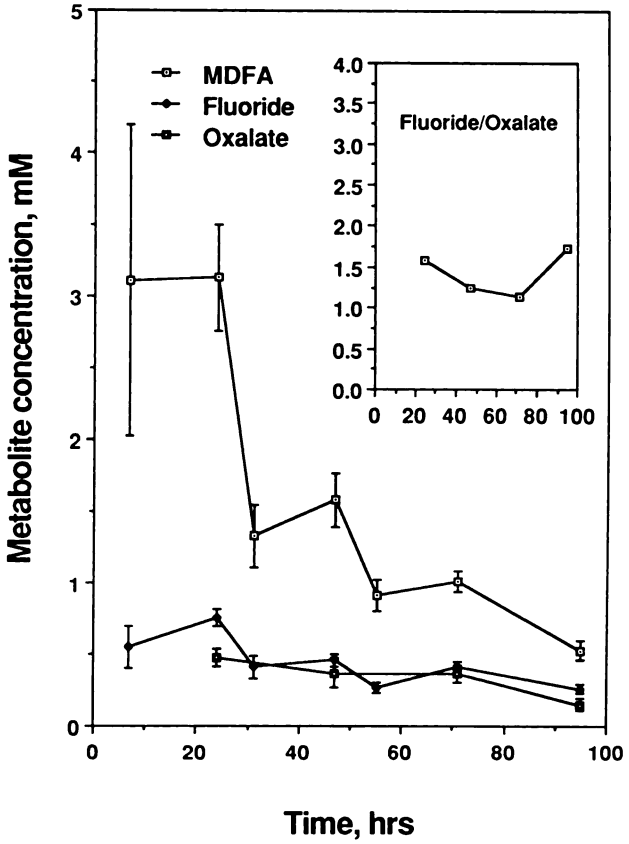

Fig. 6. Time course of excretion of MDFA, fluoride, and oxalate following administration of MDFA to the rat as described in Fig. 5. Metabolite concentrations were determined as described under Materials and Methods. The data points represent the average concentration of the metabolite in urine collected from seven rats, with the *error bars* representing 1 SE. *Inset:* ratio of excreted fluoride/oxalate in rat urine as a function of time after MDFA administration.

collected in the urine, and then chemically degrades at the lower pH of the urine to fluoride ion and oxalate (17, 18). To address this hypothesis, solutions of MDFA, synthesized as described in the previous paper (6), in Tris/citrate buffer at various pH values were incubated at 37°, and the stability of MDFA was determined by ^{19}F NMR. The results of this study are displayed in Fig. 7. In the pH range of 4.0–9.0 that was studied, it is clear that MDFA is most stable between pH 4.0 and 5.0, and that between pH 5.0 and 9.0 the stability of this compound in solution does not vary significantly.

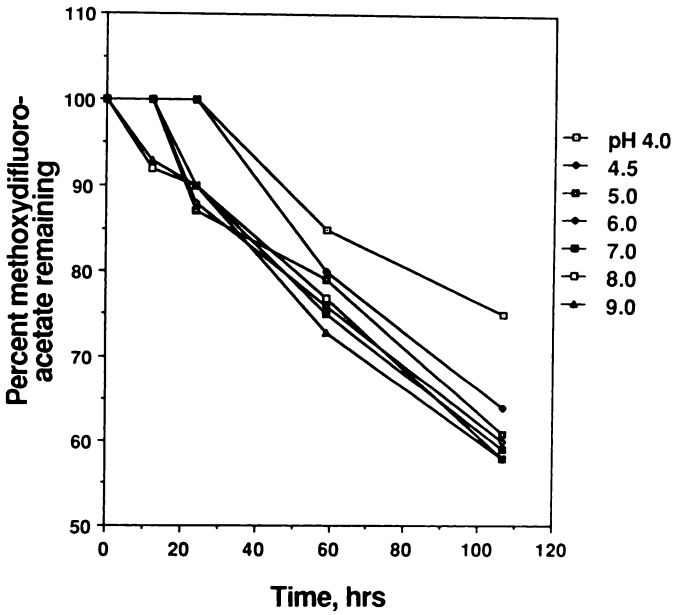

Fig. 7. Solution stability of MDFA as a function of pH. Experimental details can be found under Materials and Methods. Each *symbol* is identified in the figure.

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Discussion

Do two metabolic pathways contribute to methoxyflurane metabolism *in vivo*? Interest in understanding the metabolism of methoxyflurane is based on the hypothesis that metabolic transformation may play a significant role in mediating the toxic side effects associated with the use of this anesthetic. Thus, it has been proposed that the metabolic degradation of methoxyflurane to inorganic fluoride may constitute the primary basis for the observed renal toxicity (7). However, as discussed by Plummer *et al.* (16), fluoride can be produced via at least two independent metabolic pathways. A detailed evaluation of the relative flux through the two proposed pathways is limited by several factors. First, the different metabolites are excreted at varying rates and may be absorbed into other body tissues prior to excretion. For example, inorganic fluoride in urine may be reduced due to bone deposition. It has been reported that up to 50% of a single intraperitoneal injection of sodium fluoride is deposited in bone (19). Bell *et al.* (20) attempted to simulate fluoride ion generation from methoxyflurane anesthesia by subcutaneous injections of 120 mmol/kg sodium fluoride in rats. Urine was collected during the injections and for 2 days following. Quantitation of urinary fluoride showed that only 50% of injected fluoride was excreted. An examination of Fig. 6 demonstrates that while MDFA is metabolized to inorganic fluoride and oxalate, the measured ratio of the latter two metabolites is less than 2:1 throughout the period of measurement.

Second, both fluoride and oxalate can form calcium and magnesium salts with relatively low solubility products (21). This low solubility is the basis for the well established tendency of calcium oxalate to form kidney stones and may also contribute to the renal toxicity of fluoride ions. Precipitated metabolites will not be observable by NMR techniques or fluoride ion electrodes. Third, it has been shown that liver cells can dechlorinate dichloroacetate to yield glyoxalate, which is subsequently converted to glycine and carbon dioxide, as well as to oxalate (9). In one study utilizing [2-^{14}C]dichloroacetate, the relative rates of formation of oxalate/glycine/CO_2 were determined to be 1:1:0.5 (9). In our *in vivo* studies of dichloroacetate metabolism described in Fig. 4, the conversion of dichloroacetate to oxalate is evident, especially at early times after administration. However, only 17% of the administered dichloroacetate is recovered as oxalate (Table 1). Hence, the simple metabolic picture described by Plummer *et al.* (16) becomes more complex.

Despite these limitations on the analysis of urinary metabolites of methoxyflurane, two conclusions can be drawn from the studies described here. First, the dechlorination is the primary catabolic pathway for the degradation of methoxyflurane. This conclusion is supported by the following observations.

1. The total amount of MDFA excreted is much greater than the total amount of fluoride excreted (Fig. 3). This is true even if urinary fluoride concentrations are doubled to approximate the potential fluoride loss via bone deposition.

2. The observed urinary fluoride/oxalate ratio, although initially elevated, approaches a limiting value of slightly less than 2:1 (Fig. 3). Similarly, studies in which the metabolic intermediate of the dechlorination pathway, MDFA, was administered directly yielded a urinary fluoride/oxalate ratio slightly under 2:1 (Fig. 6).

3. According to the scheme of Plummer *et al.* (16), the demethylation pathway would also result in the production of a fluoride/oxalate ratio of 2:1. However, studies on the catabolism of directly administered dichloroacetate suggest that much of this material is metabolized to products other than oxalate (see Fig. 4 and Ref. 9).

4. Methoxyflurane metabolites continue to be excreted for 95 hr after anesthesia.

5. The time courses of metabolism and elimination of methoxyflurane and MDFA, as described by the data in Figs. 3 and 6, are qualitatively similar.

The second conclusion of the study is that the relative concentrations of methoxyflurane metabolites observed at relatively short times post-anesthesia suggest that a second metabolic pathway for methoxyflurane is recruited at high methoxyflurane concentrations. The large excess of excreted fluoride to oxalate is consistent with a significant flux through the demethylation pathway described previously. The absence of an observable dichloroacetate resonance in the urine presumedly reflects its rapid catabolism to other products.

Fig. 6 demonstrates that MDFA is broken down *in vivo* to form fluoride and oxalate. Enzymatic metabolism of MDFA may occur either via a cytosolic glutathione-dependent enzyme which defluorinates both methoxyflurane and fluoroacetate (22, 23), or by a microsomal cytochrome P-450/cytochrome b_5 complex (24). These enzymes have both been demonstrated to catalyze the demethylation of methoxyflurane *in vitro*. Conversely, since MDFA is somewhat unstable in solution near neutrality (Fig. 7), the observed metabolism of the compound (Fig. 6) may simply correspond to chemical decomposition. We are unable to distinguish between the two alternatives in these studies. A controlled study using purified hepatic enzymes would distinguish between the two alternatives.

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