Biochemical Pharmacology, Vol. 28, pp. 785–792.

0006-2952/79/0315-0785 $02.00/0

ENFLURANE AND METHOXYFLURANE

THEIR INTERACTION WITH HEPATIC CYTOCHROME P-450 *IN VITRO**

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(*Received* 18 *February* 1978; *accepted* 26 *July* 1978)

Abstract—The binding and metabolism of enflurane ($CClFHCF_2OCF_2H$) and of methoxyflurane ($CCl_2HCF_2OCH_3$) were investigated *in vitro* with hepatic microsomes from male rats. The metabolism of these anesthetic agents was monitored by NADPH consumption and fluoride ion production. In addition, from methoxyflurane, the production of acid-labile fluoride was monitored. The effects of inducing agents for different forms of cytochrome P-450 on the binding (K_s, ΔA_{max}) and metabolism (K_M, V_{max}) of both anesthetic agents are reported. The effects of CO, SKF-525A and metyrapone on the production of fluoride from enflurane and on fluoride and acid-labile fluoride from methoxyflurane are compared. The results of these studies indicate that only a form of cytochrome P-450 induced by phenobarbital binds and metabolizes enflurane *in vitro*. In contrast, at least two forms of cytochrome P-450 are involved in the binding and metabolism of methoxyflurane *in vitro*. Methoxyflurane interacts with a form of cytochrome P-450 induced by phenobarbital and at least one other form of cytochrome P-450, but not with cytochrome P-448. The relative amounts of free and acid-labile fluoride produced from methoxyflurane *in vitro* are altered after induction with 3-methylcholanthrene but not with phenobarbital. Although K_s, ΔA_{max}, K_M and V_{max} (NADPH consumption) are similar in magnitude for enflurane and methoxyflurane, the rate of production of fluoride from enflurane is approximately 8-fold less than from methoxyflurane. The observed stoichiometry of 140:1 for NADPH consumption to fluoride production for enflurane suggests that enflurane is enhancing NADPH oxidation far in excess of its metabolism. The relevance of these results to the proposed pathways for the metabolism of enflurane and methoxyflurane is discussed. The results are compared with reports of the metabolism and toxicity of enflurane and methoxyflurane *in vivo*.

Enflurane ($CClFHCF_2OCF_2H$) and methoxyflurane ($CCl_2HCF_2OCH_3$) are halogenated methyl ethyl ethers in clinical use as inhalation anesthetics. Both agents are known to be metabolized by animals and man *in vivo* although enflurane is metabolized much less extensively than is methoxyflurane [1–4]. *In vivo* methoxyflurane is converted to fluoride and chloride ions, methoxydifluoroacetic acid, dichloroacetic acid, CO_2 and possibly oxalic acid, whereas the only known (urinary) metabolites of enflurane are fluoride ion and uncharacterized halogenated compounds [2–5]. Elevated levels of fluoride ion have been proposed to be responsible for the nephrotoxicity observed in animals and man after methoxyflurane anesthesia [6, 7].

The metabolism of enflurane and methoxyflurane *in vivo* occurs primarily in the liver. The initial step in the hepatic metabolism of enflurane and methoxyflurane *in vivo* is thought to involve cytochrome P-450 in an *O*-dealkylation and/or a dehalogenation reaction [8, 9]. The *O*-dealkylation reaction is thought to produce an unstable halogenated alcohol which decomposes in water with the production of dihaloacetic acid and two equivalents of fluoride ion, while the dehalogenation reaction reportedly gives rise initially to a halogenated ether acid.

In vitro enflurane and methoxyflurane produce type I difference spectra with hepatic microsomes from phenobarbital-induced rats [10]. In addition, the metabolism of enflurane and methoxyflurane by hepatic microsomes appears to be increased by phenobarbital [11–13]. Both observations suggest that enflurane and methoxyflurane are substrates for hepatic microsomal cytochrome P-450 *in vitro*. In the presence of hepatic microsomes plus NADPH, enflurane gives rise to fluoride ion while methoxyflurane is converted to fluoride ion and to acid-labile fluoride [11, 13]. The latter compound appears to be methoxydifluoroacetic acid, the major non-volatile urinary metabolite of methoxyflurane *in vivo* [5, 11].

Recent studies have shown that hepatic microsomal cytochrome P-450 is not a single entity but is actually a group of several related mixed-function oxidases of differing substrate specificities, sensitivities to inhibitors and spectral properties (e.g. Refs. 14–19). The levels of different forms of cytochrome P-450 can be elevated *in vivo* by inducing agents such as phenobarbital or 3-methylcholanthrene [20, 21]. A form of cytochrome P-450 induced by phenobarbital binds and metabolizes the volatile anesthetic agent fluroxene, whereas the form of cytochrome P-450 induced by 3-methylcholanthrene does not [22].

We report herein the results of an investigation of the interaction of enflurane and methoxyflurane with different forms of hepatic cytochrome P-450 *in vitro*. The purpose of this investigation was to achieve a greater

* This research was supported by grants from the Medical Research Council, the University of Cape Town Staff Research Fund and the Nellie Atkinson Bequest.

understanding of the roles of different forms of cytochrome P-450 in the binding and metabolism of enflurane and methoxyflurane and in the production of the toxic metabolite fluoride ion. While this paper was in preparation, an investigation of the metabolism of methoxyflurane *in vitro* was reported by Adler *et al.* [23].

EXPERIMENTAL

Materials. Enflurane ($CClFHCF_2OCF_2H$) and methoxyflurane ($CCl_2HCF_2OCH_3$) were supplied by Abbott Laboratories, S.A. SKF-525A (2-diethylaminoethyl-2,2-diphenyl valerate) and metyrapone (2-methyl-1,2-bis[3′-pyridyl]-1-propanone) were generous gifts from Smith, Kline & French Laboratories, Isando, Transvaal, S.A., and Ciba-Geigy Ltd, Basel, Switzerland respectively.

Treatment of animals. Male Wistar rats weighing between 180 and 220 g were used for all experiments. Treatment of animals and induction of drug-metabolizing enzymes were as described earlier [24].

Preparation of microsomes. Microsomes were prepared from fresh liver homogenates by differential centrifugation and were finally suspended at the required protein concentration in 0.02 M Tris–HCl, pH 7.4 [24].

Spectral assays. Difference spectra and initial rates of NADPH oxidation were determined at 30° as described earlier [22]. Initial rates of oxidation of NADPH in the presence of substrate in an atmosphere of $CO–O_2$ (80:20; v/v) were subtracted from observed rates of NADPH oxidation according to the method of Stripp *et al.* [25]. Spectral assays were performed using Unicam SP 1800 spectrophotometers. Turbid samples were positioned in the cell holder adjacent to the photomultiplier.

Metabolite production. Reaction mixtures for measurement of the production of metabolites from enflurane and methoxyflurane comprised a microsomal suspension (2.0 mg protein/ml of 0.02 M Tris–HCl, pH 7.4), NADPH generating system [26], 0.2 mM EDTA and varying amounts of anesthetic agent. The microsomal suspension was equilibrated to 30°, the anesthetic agent, EDTA, and the NADPH generating system were added, and the contents were vortex mixed for 20 sec to initiate the reaction. The vials were clamped in a horizontal position and incubated with shaking (100 cycles/min) at 30° in a Gallenkampstat water bath. The reaction was terminated by the addition of 0.1 ml of 3 M sodium acetate buffer (pH 4.9)/3.0 ml of reaction mixture, and the concentrations of free fluoride and acid-labile fluoride were then determined. The free fluoride ion concentration thereof was measured at room temperature with an Orion fluoride electrode using a Radiometer pH meter. Known concentrations of sodium fluoride in 0.1 M sodium acetate buffer, pH 4.9, were used to establish a standard curve daily; fluoride solutions in microsomal suspension (0.1 M sodium acetate buffer, pH 4.9) gave readings identical to fluoride standards in buffer alone.

For determination of acid-labile fluoride, 3.0 ml of the reaction mixture was brought to pH 1.5 with 10 μl of conc. H_2SO_4 [11] and incubated at room temperature for 85 hr or as specified. The mixture was returned to pH 5.0 with 55–60 μl of 6.0 M NaOH and the fluoride concentration was measured as described above. Solutions of fluoride in 0.1 M sodium acetate, pH 4.9, to which 10 μl H_2SO_4 and 55–60 μl of 6.0 M NaOH were added were used as standards for these determinations. Acid-labile fluoride refers to the difference between the fluoride ion concentrations of the reaction mixture before and after H_2SO_4 treatment.

Calculations and statistical analysis. Binding (K_s) and Michaelis (K_M) constants, maximal extents of binding (ΔA_{max}) and maximal rates of metabolism (V_{max}) were calculated from computerized Hanes and Eadie–Hofstee plots using linear regression analysis. Student's *t*-test was utilized to calculate significant differences between means. A significant difference was taken as $P < 0.01$, with $P < 0.05$ being probably significant. All reported values are means ± standard deviations.

RESULTS

Binding and NADPH oxidation. Enflurane and methoxyflurane bind to cytochrome P-450 in hepatic microsomes from uninduced and phenobarbital- or 3-methylcholanthrene-induced rats, resulting in the appearance of a type I difference spectrum (λ_{max} 386 nm, λ_{min} 419 nm). In addition, both anesthetic agents stimulate carbon monoxide inhibitable NADPH oxidation by hepatic microsomes. The enhanced NADPH oxidation observed in the presence of these anesthetic agents is not measurably inhibited by cyanide (0.5 mM).

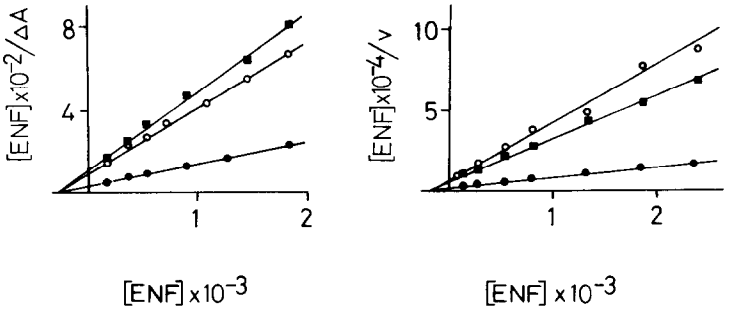

Fig. 1. Hanes plots of difference spectra and NADPH oxidation as a function of the concentration of enflurane (ENF) for hepatic microsomes from uninduced (○), 3-methylcholanthrene- (■) and phenobarbital- (●) induced rats. [ENF], molar; ΔA, $A_{386}–A_{419}$; and v, nmoles NADPH/mg of microsomal protein/min. Hepatic microsomes (2.0 mg protein/ml of 0.02 M Tris–HCl, pH 7.4), 30°.

Table 1. Interaction of enflurane with hepatic microsomal cytochrome P-450*

Pretreatment	Cyt P-450 (nmoles/mg microsomal protein)	Difference spectra: K_s (10^{-4} M)	Difference spectra: ΔA_{max} (A)	Difference spectra: ΔA_{max} (A/nmole cyt P-450)	NADPH oxidation: K_M (10^{-4} M)	NADPH oxidation: V_{max} (nmoles/mg microsomal protein/min)	NADPH oxidation: V_{max} (nmoles/nmole cyt P-450/min)
None	1.1 ± 0.1	4.6 ± 1.5	0.06 ± 0.01	0.053 ± 0.005	1.5 ± 1.0	3.4 ± 0.8	2.6 ± 0.2
MC	1.9 ± 0.2	5.0 ± 1.0	0.05 ± 0.01	0.029 ± 0.004†	3.5 ± 1.4	4.4 ± 0.6	2.4 ± 0.3
PB	2.4 ± 0.3	5.2 ± 0.9	0.16 ± 0.03†	0.070 ± 0.010†	2.3 ± 1.5	17.6 ± 2.1†	7.6 ± 1.0†

* Values reported are means ± S.D. for assays in triplicate for each of two to three separate preparations of hepatic microsomes. Abbreviations used are as follows: cyt, cytochrome; MC, 3-methylcholanthrene; and PB, phenobarbital.
† Differs from uninduced microsomes, $P < 0.001$.

Hanes plots of the extent of binding or the rate of NADPH oxidation with uninduced or induced microsomes are linear for both enflurane and methoxyflurane (see, e.g. Fig. 1). The parameters calculated from these plots are presented in Tables 1 and 2 for enflurane and methoxyflurane respectively. For enflurane, K_s and K_M (NADPH oxidation) are not significantly altered by either inducing agent. For methoxyflurane, K_s and K_M (NADPH oxidation) are generally altered by both phenobarbital induction and 3-methylcholanthrene induction. With uninduced and induced microsomes for both enflurane and methoxyflurane, K_M (NADPH oxidation) is generally smaller than K_s ($P \leqslant 0.05$ to < 0.001). The ΔA_{max} and V_{max} values for these anesthetic agents are not altered by 3-methylcholanthrene induction, but are elevated 3- to 5-fold by phenobarbital induction. Considering ΔA_{max} and V_{max}/nmole of cytochrome P-450, ΔA_{max} is decreased relative to controls after 3-methylcholanthrene induction while both ΔA_{max} and V_{max} are increased by phenobarbital induction (Tables 1 and 2).

Measurement of acid-labile fluoride. Treatment with H_2SO_4 for up to 70 hr of preincubated mixtures containing enflurane or methoxyflurane plus or minus hepatic microsomes but in the absence of NADPH does not result in measurable amounts of acid-labile fluoride. After the metabolism of enflurane by hepatic microsomes *in vitro*, incubation with H_2SO_4 for up to 90 hr does not produce measurable amounts of acid-labile fluoride. Subsequent to the metabolism of methoxyflurane, however, the concentration of fluoride ion increases with the length of time of H_2SO_4 treatment such that 85–90 hr are required for the hydrolysis of acid-labile fluorine compounds to approach completion (Fig. 2). The 18-hr acid incubation time utilized by

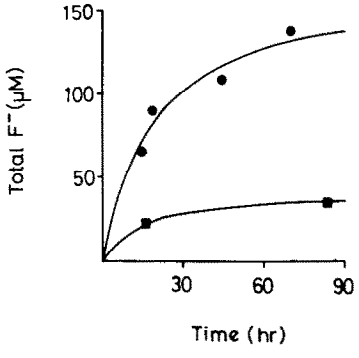

Fig. 2. Effect of time of incubation with H_2SO_4 on total fluoride ion concentration after incubation for 15 min of methoxyflurane, NADPH generating system and 3-methylcholanthrene- (■) or phenobarbital- (●) induced microsomes. Methoxyflurane (1.7 mM), microsomal suspension (2.0 mg protein/ml of 0.02 M Tris–HCl, pH 7.4), NADPH generating system and EDTA (0.2 mM) were incubated at 30° for 15 min prior to treatment with conc. H_2SO_4.

Table 2. Interaction of methoxyflurane with hepatic microsomal cytochrome P-450*

Pretreatment	Cyt P-450 (nmoles/mg microsomal protein)	Difference spectra: K_s (10^{-4} M)	Difference spectra: ΔA_{max} (A)	Difference spectra: ΔA_{max} (A/nmole cyt P-450)	NADPH oxidation: K_M (10^{-4} M)	NADPH oxidation: V_{max} (nmoles/mg microsomal protein/min)	NADPH oxidation: V_{max} (nmoles/nmole cyt P-450/min)
None	1.2 ± 0.1	4.8 ± 1.3	0.06 ± 0.01	0.054 ± 0.008	1.0 ± 0.1	3.3 ± 0.6	2.5 ± 0.2
MC	1.9 ± 0.2	6.1 ± 1.3	0.07 ± 0.01	0.038 ± 0.008†	2.4 ± 0.8†	4.3 ± 0.6	2.3 ± 0.4
PB	2.4 ± 0.3	2.9 ± 0.6‡	0.16 ± 0.03‡	0.066 ± 0.009†	0.66 ± 0.04‡	13.0 ± 2.4‡	6.5 ± 1.4†

* Values reported are means ± S.D. for assays in triplicate for each of two or three separate preparations of hepatic microsomes. Abbreviations used are as follows: cyt, cytochrome; MC, 3-methylcholanthrene; and PB, phenobarbital.
† Differs from uninduced microsomes, $P < 0.01$.
‡ Differs from uninduced microsomes, $P \leqslant 0.001$.

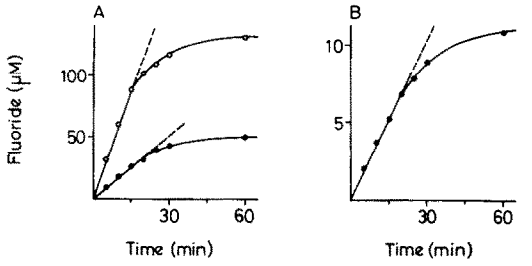

Fig. 3. Effect of time on the production of (A) free (●) and acid-labile (○) fluoride from methoxyflurane and of (B) free fluoride from enflurane by phenobarbital-induced microsomes. Methoxyflurane (2.9 mM) or enflurane (2.7 mM), microsomal suspension (2.0 mg protein/ml of 0.02 M Tris–HCl, pH 7.4), NADPH generating system and EDTA (0.2 mM) were incubated at 30°.

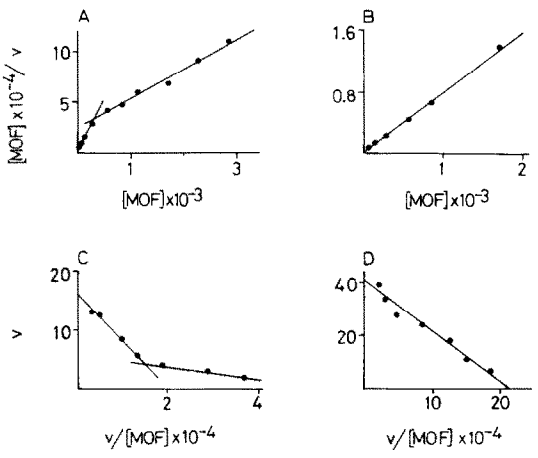

Fig. 4. Hanes and Eadie–Hofstee plots for the production of fluoride from methoxyflurane (MOF) by hepatic microsomes. Production of free fluoride by 3-methylcholanthrene-induced microsomes (A) and by phenobarbital-induced microsomes (B); production of acid-labile fluoride by 3-methylcholanthrene-induced microsomes (C) and by phenobarbital-induced microsomes (D). [MOF], molar; v, nmoles fluoride/mg of microsomal protein/time of incubation (20 and 15 min, respectively, for 3-methylcholanthrene- and phenobarbital-induced microsomes). Incubation conditions were as in Fig. 3.

other laboratories [23] would release less than 60 per cent of the total fluoride from the acid-labile fluorine compounds produced by the metabolism of methoxyflurane (Fig. 2).

Effect of time on the production of fluoride and acid-labile fluoride. The kinetics of the production of fluoride from enflurane and of fluoride and acid-labile fluoride from methoxyflurane by phenobarbital-induced microsomes are shown in Fig. 3. The rate of production of fluoride ion from enflurane is linear for approximately 20 min, whereas the rates of production of fluoride and acid-labile fluoride from methoxyflurane are linear for 15 min. Similar studies with microsomes from uninduced and 3-methylcholanthrene-induced animals reveal that the rates of production of fluoride from methoxyflurane are linear for 15 and 20 min, respectively, but that the levels of fluoride produced from enflurane with these types of microsomes are too low to be measured accurately by a fluoride electrode (< 1 μM). Incubation of either anesthetic agent with microsomes in the absence of the NADPH generating system results in fluoride concentrations of less than 1 μM. In all subsequent investigations of the production of fluoride and/or acid-labile fluoride from enflurane and methoxyflurane, the reaction times utilized correspond to the maximum time periods over which the metabolite production was linear.

Effect of induction of different forms of cytochrome P-450 on K_M and V_{max} for the production of fluoride and acid-labile fluoride. For the production of fluoride from enflurane with phenobarbital-induced microsomes, K_M and V_{max} were calculated as $3.55 \pm 0.67 \times 10^{-4}$ M and 0.13 ± 0.02 nmole F^-/mg of microsomal protein/min (0.055 ± 0.010 nmole F^-/nmole of cytochrome P-450/min).

The production of fluoride and acid-labile fluoride from methoxyflurane by uninduced or 3-methylcholanthrene-induced microsomes gives rise to biphasic Hanes and Eadie–Hofstee plots (e.g. Fig. 4). Two sets

Table 3. Production of free and acid-labile fluoride from methoxyflurane by rat hepatic microsomes*

Induction	Cyt P-450 (nmoles/mg microsomal protein)	Free fluoride K_M (10^{-4} M)	Free fluoride V_{max} (nmoles/mg microsomal protein/min)	Acid-labile fluoride K_M (10^{-4} M)	Acid-labile fluoride V_{max} (nmoles/mg microsomal protein/min)	Acid-labile F^- / Free F^-
None	1.0 ± 0.1	0.40 ± 0.12	0.14 ± 0.01	0.59 ± 0.17	0.34 ± 0.07	2.4 ± 0.2
		4.9 ± 0.9	0.37 ± 0.10	7.7 ± 5.0	0.66 ± 0.03	1.9 ± 0.6
MC	1.7 ± 0.1	0.37 ± 0.18	0.053 ± 0.023†	0.88 ± 0.47	0.28 ± 0.05	5.9 ± 2.2‡
		10.3 ± 2.2†	0.19 ± 0.04‡	10.9 ± 4.5	0.92‡ ± 0.10‡	5.0 ± 0.8‡
PB	2.4 ± 0.2	0.99 ± 0.08†	1.01 ± 0.15†	2.22 ± 0.09	2.93 ± 0.66†	3.0 ± 0.8

* Values reported are means ± S.D. for assays in triplicate for each of three to four separate preparations of microsomes. Abbreviations used are as follows: cyt, cytochrome; MC, 3-methylcholanthrene; and PB, phenobarbital.

† Differs from uninduced microsomes, $P < 0.01$.

‡ Probably differs from uninduced microsomes, $P < 0.05$.

of K_M and V_{max} values are, therefore, calculable in each case. In contrast, linear Hanes and Eadie–Hofstee plots are obtained for the production of fluoride and acid-labile fluoride from methoxyflurane by phenobarbital-induced microsomes (e.g. Fig. 4).

The effects of induction of different forms of cytochrome P-450 on K_M and V_{max} values for the production of fluoride and acid-labile fluoride from methoxyflurane are presented in Table 3. Induction by 3-methylcholanthrene does not alter the K_M values for production of these metabolites relative to uninduced microsomes except for the low affinity K_M for fluoride. Phenobarbital induction results in a single K_M each for the production of free fluoride and acid-labile fluoride. Relative to uninduced microsomes the former K_M differs significantly from both the high and low affinity K_M values for the production of fluoride, while the latter K_M differs significantly only from the high affinity K_M for the production of acid-labile fluoride.

With uninduced and 3-methylcholanthrene-induced microsomes the K_M values for production of free fluoride are within experimental error of the corresponding K_M values for production of acid-labile fluoride ($P > 0.05$). With phenobarbital-induced microsomes, however, the K_M for the production of free fluoride is significantly different from that for the production of acid-labile fluoride ($P < 0.001$).

Induction by 3-methylcholanthrene decreases V_{max} for the microsomal production of fluoride from methoxyflurane but does not affect or slightly increases V_{max} for the production of acid-labile fluoride. Induction by phenobarbital markedly increases V_{max} for the production of fluoride and acid-labile fluoride from methoxyflurane (Table 3). Considering V_{max} values/nmole of cytochrome P-450 does not alter the above, except that the low and high V_{max} values for the production of acid-labile fluoride with 3-methylcholanthrene-induced microsomes are decreased ($P < 0.01$) and unchanged ($P > 0.1$), respectively, relative to uninduced microsomes. In all cases V_{max} values for acid-labile fluoride production are greater than the corresponding V_{max} values for fluoride production ($P < 0.01$).

NADH (0.6 mM) supports the production of fluoride ions from enflurane in the presence of phenobarbital microsomes to less than 20 per cent of that observed in the presence of NADPH. With uninduced and 3-methylcholanthrene- or phenobarbital-induced microsomes, fluoride production from methoxyflurane in the presence of NADH was approximately 10 per cent of that supported by NADPH.

Effect of inhibitors on production of fluoride and acid-labile fluoride. The effects of inhibitors of cytochrome P-450 on the production of fluoride and acid-labile fluoride from methoxyflurane with microsomes from uninduced and induced animals are summarized in Table 4. The production of free fluoride is equally sensitive to inhibitors with control and 3-methylcholanthrene-induced microsomes but is relatively more sensitive to inhibition with phenobarbital-induced microsomes. Relative to uninduced microsomes, the production of acid-labile fluoride is generally less sensitive to inhibitors with 3-methylcholanthrene-induced microsomes and more sensitive with phenobarbital-induced microsomes. With uninduced microsomes or with phenobarbital-induced microsomes, the inhibitors affect the production of fluoride to the same extent as the production of acid-labile fluoride, except that in each case the production of acid-labile fluoride is inhibited to a greater extent than the production of fluoride by 200 μM metyrapone. In 3-methylcholanthrene-induced microsomes the production of acid-labile fluoride is much less sensitive to inhibition by metyrapone and CO than is the production of fluoride ion.

Production of fluoride ions from enflurane (2.4 mM) by phenobarbital-induced microsomes is inhibited by CO, 50 μM SKF-525A or 50 μM metyrapone to the same extent as is observed for the inhibition of free fluoride production from methoxyflurane by phenobarbital-induced microsomes.

Table 4. Effect of inhibitors of cytochrome P-450 on the production of free and acid-labile fluoride from methoxyflurane in rat hepatic microsomes*

Inhibitor (μM)	% Inhibition					
	Uninduced		3-Methylcholanthrene induced		Phenobarbital induced	
	Free F^-	Acid-labile F^-	Free F^-	Acid-labile F^-	Free F^-	Acid-labile F^-
SKF-525A:						
50	34 ± 8	37 ± 12	27 ± 8	22 ± 7†	41 ± 5	43 ± 8
200	50 ± 9	52 ± 9	43 ± 3‡	35 ± 6‡§	67 ± 1‡§	71 ± 2‡§
Metyrapone:						
50	29 ± 5	23 ± 8	34 ± 9	12 ± 8§‖	67 ± 2†	65 ± ‖†
200	43 ± 8	28 ± 6‖	51 ± 1‡	24 ± 13‡¶	79 ± 2†‡	68 ± 3†‡‖
CO	62 ± 10	62 ± 14	54 ± 16	23 ± 13†‖	84 ± 3†	87 ± 3†

* Means ± S.D. are reported for experiments performed in triplicate with two or three different preparations of hepatic microsomes. Methoxyflurane, 1.7 mM. For uninduced, 3-methylcholanthrene-induced and phenobarbital-induced microsomes, cytochrome P-450 was 1.0 ± 0.1, 1.7 ± 0.1 and 2.4 ± 0.2 nmoles/mg of microsomal protein and free and acid-labile fluoride production were 0.21 ± 0.01 and 0.77 ± 0.09, 0.11 ± 0.02 and 0.41 ± 0.04, and 0.65 ± 0.01 and 2.35 ± 0.3 nmoles/mg of microsomal protein/min respectively. CO was bubbled through microsomes at 10 ml/min for 2 min.

† Differs from uninduced microsomes, $P \leqslant 0.01$.

‡ Assays in triplicate with one preparation of hepatic microsomes.

§ Probably differs from uninduced microsomes, $P < 0.05$.

‖ Differs from free fluoride production with same type of microsomal preparation, $P \leqslant 0.01$.

¶ Probably differs from free fluoride production with same type of microsomal preparation, $P < 0.05$.

DISCUSSION

The demonstration that enflurane and methoxyflurane bind as type I compounds to cytochrome P-450 in microsomes from differently induced rats (Tables 1 and 2) confirms and extends the observation of Takahashi *et al.* [10] that both compounds produce type I difference spectra with phenobarbital-induced microsomes. The production of type I difference spectra by these anesthetic agents with rat hepatic microsomes *in vitro* indicates that these compounds bind to the substrate-binding site of cytochrome P-450 and suggests that they may be substrates for this enzyme [27].

The enhanced rate of NADPH oxidation by hepatic microsomes in the presence of enflurane and methoxyflurane appears to arise as a result of the interaction of these agents with cytochrome P-450 and may at least in part reflect the metabolism of these compounds by cytochrome P-450. This is suggested by the inhibition of NADPH oxidation by $CO–O_2$ (80:20). The lack of effect of potassium cyanide (0.5 mM) on the rate of NADPH oxidation indicates that hepatic microsomal stearate desaturase is not responsible for the stimulation of NADPH oxidation by enflurane or methoxyflurane although this enzyme appears to be involved in the stimulation of NADPH oxidation by the volatile anesthetic agent halothane [28].

The involvement of cytochrome P-450 in the production of fluoride from enflurane and methoxyflurane in hepatic microsomes from differently induced rats is indicated by the requirement of these reactions for NADPH, by the inability of NADH to support these reactions except to a slight extent, and by the inhibition of these reactions by compounds such as metyrapone, SKF-525A and CO, which are inhibitors of cytochrome P-450 (see e.g. Refs. 29–31) (Table 4).

For enflurane, since the K_s and the K_M for NADPH oxidation are not changed by induction (Table 1), it appears that one form of hepatic microsomal cytochrome P-450 binds enflurane and mediates CO-sensitive NADPH oxidation in the presence of this compound. Since the ΔA_{max} and the V_{max} for enflurane are increased by phenobarbital induction but unaffected or decreased by 3-methylcholanthrene induction, it appears that enflurane interacts with a form of cytochrome P-450 induced by phenobarbital but not with that induced by 3-methylcholanthrene. It is not clear why the K_M is generally somewhat smaller than the K_s for enflurane, although this discrepancy might reflect non-productive complex formation with one or more forms of cytochrome P-450.*

It appears that the form of cytochrome P-450 induced by phenobarbital also catalyzes the production of fluoride from enflurane. This proposal is supported by several observations: (1) the rate of fluoride production from enflurane is elevated by phenobarbital induction; (2) free fluoride production from enflurane is sensitive to metyrapone (which at *ca.* 50 μM is reportedly a specific inhibitor of phenobarbital-induced cytochrome P-450 [19]; and (3) for phenobarbital-induced microsomes, the K_M for fluoride production from enflurane is not statistically different from the K_M for NADPH oxidation ($P > 0.2$).

The demonstration that microsomal cytochrome P-450 metabolizes enflurane *in vitro* disproves the proposal of Barr *et al.* [32] to the contrary. The results of Barr *et al.* have also been questioned by Fiserova-Bergerova [33]. That the cytochrome P-450-dependent metabolism of enflurane does not give rise directly to acid-labile compounds is in accord with the proposed pathways for the metabolism of enflurane, inasmuch as difluoromethoxydifluoroacetate, the fluorinated ether acid that would be produced by 1,1-dehalogenation of enflurane, would not be expected to be nearly as acid-labile as methoxydifluoroacetate, the proposed acid-labile metabolite of the 1,1-dehalogenation of methoxyflurane [5, 9, 11]. The absence of measurable levels of acid-labile fluorine metabolites of enflurane provides no information, however, on whether the *O*-dealkylation or dehalogenation pathway would be favored in the metabolism of enflurane.

The observed stoichiometry of NADPH to fluoride ion of 140† is vastly different from the stoichiometry of less than 1 expected from the proposed pathways for the metabolism of enflurane. Since enflurane binds to the type I site of cytochrome P-450 but is relatively refractory to metabolism [2], one might anticipate that a large proportion of the CO-inhibitable NADPH oxidation observed in the presence of enflurane arises from reactions other than the cytochrome P-450-dependent metabolism of enflurane. There are several pathways for the hepatic microsomal oxidation of NADPH, such as those involving monoamine oxidase, stearate desaturase, and the autoxidation of cytochrome b_5, to mention a few. Enflurane may be stimulating one such pathway or alternatively may perhaps be acting as an uncoupler of microsomal electron transfer. An alternative explanation—that fluoride may not be a major metabolite of the cytochrome P-450-dependent metabolism of enflurane—is inconsistent with the proposed pathways for the metabolism of enflurane and with the observation that, although enflurane stimulates NADPH oxidation *in vitro* to a greater extent than methoxyflurane, methoxyflurane is metabolized much more extensively than enflurane *in vivo*.

The stoichiometry of NADPH to free fluoride of between 6 and 20 for methoxyflurane with differently induced microsomes is difficult to interpret in view of the complexity of the proposed pathways for the metabolism of methoxyflurane. Nevertheless, the observed stoichiometry does indicate that methoxyflurane stimulates NADPH oxidation in excess of substrate oxidation but not to the extent seen with enflurane.

For methoxyflurane, the variation in K_s and K_M (NADPH oxidation and metabolite production) with induction, and the lack of correlation between K_s, K_M (NADPH oxidation) and K_M (metabolite production) (Tables 2 and 3), strongly indicate that more than one form of cytochrome P-450 binds and metabolizes this anesthetic agent. Differences in the K_M for fluoride production and in the sensitivity of fluoride production to inhibitors with uninduced and phenobarbital-in-

* D. Piszkiewicz, University of California, Irvine, personal communication.

† Although the experiments leading to these values were not designed to demonstrate the stoichiometric relationship between NADPH oxidation and product formation with great accuracy, these experiments should provide a good approximation. Under similar experimental conditions the stoichiometry of 0.5 to 1.0 for NADPH:product was observed for fluroxene and 2,2,2-trifluoroethyl ethyl ether [22, 26, 34].

duced microsomes (Tables 3 and 4) suggest that the form of cytochrome P-450 induced by phenobarbital is not of major importance in the metabolism of methoxyflurane by uninduced microsomes. For uninduced microsomes, the lack of sensitivity of the production of fluoride ion and acid-labile fluoride to low concentrations of metyrapone supports this proposal. The same form of cytochrome P-450 involved in the production of fluoride and acid-labile fluoride in uninduced microsomes may catalyze the production of free fluoride in 3-methylcholanthrene-induced microsomes. The production of free fluoride and acid-labile fluoride by uninduced microsomes and the production of free fluoride by 3-methylcholanthrene-induced microsomes show similar sensitivities to inhibitors and are characterized by similar K_M values (Tables 3 and 4). Since the V_{max} for fluoride production/nmole of cytochrome P-450 is decreased by 3-methylcholanthrene induction, it appears that a form of cytochrome P-450 other than cytochrome P-448 would be involved.

Although the production of acid-labile fluoride from methoxyflurane by 3-methylcholanthrene-induced microsomes is characterized by K_M values similar to those for uninduced microsomes, the production of acid-labile fluoride with 3-methylcholanthrene-induced microsomes is relatively unaffected by metyrapone and CO and the ratio of acid-labile fluoride to free fluoride is increased by 3-methylcholanthrene induction (Tables 3 and 4). Interpretation of the role of different type P-450 cytochromes in the production of acid-labile fluoride from methoxyflurane with 3-methylcholanthrene-induced microsomes is, therefore, difficult.

The increases in the ΔA_{max} and in the V_{max} for NADPH oxidation and metabolite production for methoxyflurane after phenobarbital induction indicate that a cytochrome P-450 induced by phenobarbital is of predominant importance in the spectrally observable binding and in the metabolism of this volatile anesthetic agent with phenobarbital-induced microsomes (Tables 2 and 3). The observation that the production of free fluoride and acid-labile fluoride in phenobarbital-induced microsomes is more sensitive to low concentrations (50 μM) of metyrapone than of SKF-525A ($P < 0.01$) is consistent with this proposal [19] (Table 4). Although different forms of cytochrome P-450 appear to metabolize methoxyflurane in uninduced and phenobarbital-induced microsomes, there is no significant difference between these preparations in the relative rates of production of acid-labile fluoride to free fluoride (Table 3). The involvement of more than one form of hepatic microsomal cytochrome P-450 in the metabolism of methoxyflurane *in vitro* is in contrast to the observation that only the form of cytochrome P-450 induced by phenobarbital metabolizes the anesthetic agents enflurane and fluroxene [22].

A comparison of the relative rates of production of fluoride and acid-labile fluoride from methoxyflurane may provide insight into the relative rates of the two proposed pathways for the metabolism of methoxyflurane *in vivo*. Assuming that a major portion of the acid-labile fluoride produced from methoxyflurane *in vitro* represents methoxydifluoroacetic acid [5, 11], the dechlorination of methoxyflurane (which would directly produce methoxydifluoroacetic acid) occurs at least 2- to 6-fold more rapidly than the *O*-dealkylation pathway *in vitro*. This is consistent with the observation of Holaday *et al.* [5] that the dechlorination of methoxyflurane is more rapid than its *O*-dealkylation *in vivo* but is not in accord with the proposal—based on quantum mechanical considerations—by Loew *et al.* [9] that the *O*-dealkylation pathway should be favored over the dehalogenation pathway for methoxyflurane. In addition, it is apparent that 3-methylcholanthrene induction alters the relative rates of the *O*-dealkylation and dehalogenation pathways for the metabolism of methoxyflurane relative to uninduced microsomes whereas phenobarbital induction does not (Table 3).

The effects of inducing agents and inhibitors on the metabolism of enflurane and methoxyflurane *in vitro* reported here correlate well with the results of investigations of the metabolism and toxicity of these anesthetic agents *in vivo*. The toxic metabolite fluoride ion is produced in much lower levels from enflurane than from methoxyflurane *in vitro* (see Results) and *in vivo* [2–4], and enflurane is rarely nephrotoxic whereas methoxyflurane is known to produce nephrotoxicity with some regularity [3, 6, 7, 35]. Phenobarbital induction increases the production of fluoride from enflurane and methoxyflurane *in vitro* (see Results and Table 3) [11] and *in vivo* and enhances the nephrotoxicity of methoxyflurane *in vivo* [33, 36–40]. SKF-525A inhibits the conversion of these anesthetic agents to fluoride *in vitro* (see Results and Table 4) as well as *in vivo* [33, 36].

While the work presented here was in progress, the results of an investigation of the metabolism of methoxyflurane by rat hepatic microsomes *in vitro* were reported by Adler *et al.* [23]. In contrast to the results reported here, Adler *et al.* reported single K_M and V_{max} values for the production of free and total * fluoride from methoxyflurane by microsomes from uninduced rats and found that phenobarbital induction resulted in a greater increase in free fluoride than in total fluoride production. Adler *et al.*, however, ignored the nonlinearity of their Lineweaver–Burk plots for the production of free and total fluoride from uninduced rats (Fig. 4A,B [23]) from which were calculated their K_M and V_{max} values for microsomes from uninduced rats and on which their comparisons with microsomes from phenobarbital-induced rats were based. In addition, there was no indication by Adler *et al.* that the reported reaction rates were linear over the 30-min incubation time utilized in their studies. The aforementioned results and conclusions of Adler *et al.*, therefore, appear not to be valid.

In addition, the V_{max} for fluoride production by microsomes from phenobarbital-induced rats reported by Adler *et al.* does not agree with other reported values but is approximately 13-fold greater than the value reported here (Table 3), and approximately 20- to 100-fold greater than the reported rates of fluoride production from methoxyflurane by microsomes from phenobarbital-induced rats [11–13].

The rates of defluorination of methoxyflurane by hepatic microsomes reported by other laboratories range from 0.034 to 0.12 and from 0.14 to 0.80 nmole/min/mg of microsomal protein for uninduced and phenobarbital-induced microsomes respectively [11–13, 41]. The rate of defluorination of methoxyflurane

* Total fluoride equals the sum of free fluoride plus acid-labile fluoride.

was found not to vary with different strains of rats [11] and did not vary greatly between the different laboratories in spite of variations in experimental details. The above rates are similar to, but without exception lower than, the V_{max} values reported in this paper. That these rates are consistently lower than the corresponding V_{max} values reported herein is to be expected, inasmuch as the substrate concentrations utilized elsewhere were (when reported) in the region of 0.6 to 1.3 mM, which was not always greatly in excess of K_M (Table 3). In addition, in none of these reports was there any indication that the reaction rate was linear over the incubation time utilized which, if it were not, would also result in apparently lowered rates. It would be anticipated in any case that quantitative comparisons of the extent of metabolism of enflurane and methoxyflurane would not be apparent from such preliminary studies, but would have to await the characterization of the K_M and V_{max} values for these anesthetics as reported here.

Acknowledgement—We would like to acknowledge with thanks the advice and assistance of Professor James E. Kench in preparing this paper.

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