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ENFLURANE AND METHOXYFLURANE: THEIR INTERACTION WITH HEPATIC MICROSOMAL STEARATE DESATURASE*

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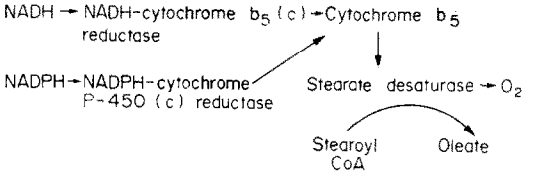
Abstract—The effects of the volatile anesthetic agents enflurane ($CClFHCF_2OCF_2H$) and methoxyflurane ($CCl_2HCF_2OCH_3$) on hepatic microsomal electron transfer components and stearate desaturase are reported. Both enflurane and methoxyflurane stimulated electron flow from NADH and NADPH through hepatic microsomal cytochrome b_5. The stimulation of electron flow from cytochrome b_5 by the anesthetic agents was not inhibited by metyrapone or CO, but was inhibited by 0.5 mM KCN. The effects of enflurane and methoxyflurane were influenced by the diet and pretreatment of the rat prior to death. A high-carbohydrate diet enhanced the effects, while fasting with or without phenobarbitone treatment diminished them. The anesthetic agents did not affect the rate constant for the autoxidation of purified trypsin-cleaved cytochrome b_5 or the activity of hepatic microsomal NADH- and NADPH-cytochrome *c* reductase, except that enflurane slightly increased the activity of NADH-cytochrome *c* reductase. The values of the equilibrium constants (K_{eq}) for the stimulation of the oxidation of hepatic microsomal cytochrome b_5 by enflurane and methoxyflurane were determined to be 1.2 and 0.5 mM, respectively. The K_{eq} for enflurane differed from the K_s and K_m values for the interaction of this anesthetic agent with cytochrome P-450, whereas the K_{eq} for methoxyflurane differed from the K_m for NADPH oxidation by cytochrome P-450, but not from the K_s for binding to cytochrome P-450 or the K_m for fluoride ion production from this anesthetic agent by cytochrome P-450. The K_i values of 0.08 and 0.11 mM obtained for cyanide inhibition of the enhancement of the oxidation of cytochrome b_5 by enflurane and methoxyflurane, respectively, are within experimental error of the K_i for cyanide inhibition of stearate desaturase. Enflurane and methoxyflurane, however, did not inhibit the conversion of stearoyl CoA to oleate by hepatic microsomal stearate desaturase. It is concluded that enflurane and methoxyflurane stimulate hepatic microsomal electron flow from NADH and NADPH through cytochrome b_5 *in vitro*, apparently by interacting with stearate desaturase.

The cytochrome P-450 mixed function oxidase of the hepatic endoplasmic reticulum catalyzes the primary step in the metabolism of many xenobiotics [1]. The first steps in the metabolism of the volatile anesthetic agents enflurane ($CClFHCF_2OCF_2H$) and methoxyflurane ($CCl_2HCF_2OCH_3$) involve dehalogenation and ether cleavage reactions mediated by hepatic microsomal cytochrome P-450 [2–4]. Detailed pathways for metabolism of these anesthetic agents *in vivo* have been proposed, but the pathways have not, as yet, been fully established, particularly for enflurane which is not metabolized extensively *in vivo* [2–6].

Stearate desaturase participates in another major electron transfer pathway of hepatic endoplasmic reticulum membranes. The physiological role of stearate desaturase appears to be to convert stearoyl CoA to oleate. This reaction can be supported by either NADH or NADPH and requires flavoprotein reductases and cytochrome b_5 as obligate intermediate electron carriers [7–11] as shown in Scheme 1.



Scheme 1. Electron transfer to stearate desaturase. Straight arrows indicate electron flow.

Stearate desaturase also exhibits mixed function oxidase activity in the oxidation of methyl sterols [12, 13]. Both the oxidase and desaturase activities are inhibited by cyanide ($K_i = 0.1$ mM) [10, 12]. Stearate desaturase has not been widely investigated with regard to its ability to interact with xenobiotics. It has, however, been reported to interact with phenolic xenobiotics [11] and with the volatile anesthetic agent halothane ($CF_3CHBrCl$) [14]. In an attempt to clarify further the metabolism and the physiological effects of the volatile anesthetic agents, enflurane and methoxyflurane, we have investigated their interaction with hepatic microsomal electron transfer proteins and with stearate desaturase. In the following investigation, in order to alter the levels of stearate desaturase, advantage was taken of the

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fact that the enzyme is under dietary control. The levels of stearate desaturase are negligible in fasted rats, measurable in fed rats, and elevated by feeding semi-purified high-carbonate diets [8, 15, 16].

The data indicate that enflurane and methoxyflurane enhance hepatic microsomal electron flow through cytochrome b_5 at clinically achievable anesthetic concentrations. The observed stimulation of electron transfer appears to arise from the interaction of enflurane and methoxyflurane with hepatic microsomal stearate desaturase.

EXPERIMENTAL

Materials. NADH and NADPH were obtained from Miles Laboratories, Cape Town, South Africa. Stearoyl CoA and [1-^{14}C]stearoyl CoA were obtained from Sigma Chemicals, Poole, England, and from New England Nuclear, Boston, MA, U.S.A., respectively. Dextrin was supplied by Merck Chemicals, Darmstadt, Germany, and by Sigma Chemicals.

The vitamin mixture used in the diet was constituted from vitamins received as a gift from Roche Pty. Ltd., Isando, Transvaal, South Africa. Choline chloride, sodium dithionite and cellulose were obtained from B. D. H. Chemicals Ltd., Poole, England. Casein was obtained from Merck Chemicals. Sodium phenobarbitone was supplied by Maybaker, Port Elizabeth, E. P., South Africa. Halothane (fluothane) was obtained from Halocarbon Laboratories Inc., Hackensack, NJ, U.S.A. Enflurane (ethrane) and methoxyflurane (penthrane) were obtained from Abbott Laboratories, Aeroton, Transvaal, South Africa. Trypsin-cleaved cytochrome b_5 was purified from rat liver microsomes by the method of Omura and Takesue [17].

Treatment of animals. In all experiments, male Long Evans rats weighing 250–300 g were used. Rats were fed on a normal laboratory diet of Epol Laboratory Chow, manufactured by Epol Ltd., Goodwood, Cape Town. This diet is referred to throughout as the normal diet, and is composed of protein (min. 20%), fat (2.5%), fibre (max. 6%), calcium (1.4%), and phosphorus (0.7%). Hepatic microsomal stearate desaturase was routinely induced by feeding rats a high-carbohydrate semi-purified diet of the following composition: dextrin, 126 g; sucrose, 30 g; cellulose, 4 g; casein, 30 g; NaCl, 4 g; KCl, 2 g; vitamin mixture, 6 g; and choline chloride, 0.2 g [8]. The vitamin mixture comprised the following: vitamin A, 2.5 g (325,000 I.U./g); vitamin D, 2.0 g (200,000 I.U./g); vitamin B_2 (Riboflavin), 500 mg; niacin, 7.5 g; and pantothenic acid, 1 g, made up to a total of 500 g with dextrin. This diet is referred to throughout as the high-carbohydrate diet. Rats were fed this diet for 2 days, fasted on day 3 and were refed the diet for 2 days [16]. The rats were killed and experiments were performed on day 6.

Isolation of hepatic microsomes. Rat hepatic microsomes were isolated by differential centrifugation, as described earlier [4]. The protein concentration of the microsomes was determined by the method of Lowry *et al.* [18], as modified by Chaykin [19]. Hepatic microsomes from rats fed a high-carbohydrate diet, suspended at a protein concentration of 1.5 mg/ml in 0.05 M Tris–HCl, pH 7.4, were used for all experiments unless indicated otherwise.

Cytochrome P-450 determination. Cytochrome P-450 concentrations were determined from measurements of the difference spectrum of CO-ferrocytochrome P-450 versus ferrocytochrome P-450, according to the method of Omura and Sato [20]. An extinction coefficient of 91 $cm^{-1}mM^{-1}$ for the difference in absorbance between 450 nm and 490 nm was utilized [20].

Re-oxidation of NADH-reduced cytochrome b_5. Hepatic microsomal cytochrome b_5 was reduced with a slight excess of NADH, and the first order re-oxidation of ferrocytochrome b_5, which occurs on exhaustion of the NADH, was monitored spectrally at 424 nm and 409 nm, using the method of Oshino *et al.* [8].

Redox steady-state of cytochrome b_5 *in the presence of NADPH.* The steady-state redox status of NADPH-reduced hepatic microsomal cytochrome b_5 was determined from the change in absorbance between 424 nm and 409 nm by the method of Oshino *et al.* [8]. The results are expressed as the percentage reduction of hepatic microsomal cytochrome b_5 by NADPH relative to dithionite. For both the reoxidation of NADH-reduced cytochrome b_5 and the NADPH steady state assays, the anesthetics, when present, were added to 3 ml of microsomal suspension and vortex mixed prior to the addition of NAD(P)H and cyanide.

NAD(P)H-cytochrome c reductase assays. The activities of NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase were determined by the method of Omura and Takesue [17]. The anesthetic agent was suspended in 2.10 ml of Tris–HCl, pH 7.4, by vortex mixing, prior to the addition of the NAD(P)H, cytochrome *c* and hepatic microsomes. The increase in the absorbance of ferrocytochrome *c* at 550 nm ($\varepsilon = 21.1\ cm^{-1}mM^{-1}$) was recorded.

Oxidation of purified trypsin-cleaved ferrocytochrome b_5. Purified trypsin-cleaved cytochrome b_5 was reduced by a modification of the method of Smith [21]. Purified ferricytochrome b_5 was bubbled with N_2 for 20 min, and 5% Palladium on asbestos (2% w/v, final concentration) was added to the cytochrome solution. The suspension was then bubbled with H_2 for 1–2 hr to convert the cytochrome b_5 to the ferrous form. Aliquots of ferrocytochrome b_5 were then removed from the reducing suspension, filtered through an 8 μ millipore filter, and 30 μl of the resultant solution (*ca.* 25 μM cytochrome b_5) were added to 1.25 ml of air equilibrated 0.1 M Tris–HCl, pH 7.4, in the presence or absence of the anesthetic agents. The oxidation of purified trypsin-cleaved ferrocytochrome b_5 was monitored spectrally at 424 nm.

Stearate desaturase assay. The activity of stearate desaturase was assayed via the conversion of [^{14}C]stearoyl CoA to [^{14}C]oleate, essentially by the method of Oshino *et al.* [10]. Incubation mixtures contained hepatic microsomes (1.0 mg protein), 1 mM NADH, and 40 mM [^{14}C]stearoyl CoA (12 μCi) in 0.5 ml of 0.1 M Tris–HCl, pH 7.25. Incubations were for 4 min with shaking at 37°. At the end of the incubation period, 2 mg each of carrier oleate and stearate were added to the reaction mixture just

prior to methylation. The fatty acids were methylated with $BF_3 \cdot CH_3OH$ by the method of McIntosh *et al.* [22], and the methyl esters were separated by argentation thin-layer chromatography on silica gel plates (25 cm × 25 cm × 0.25 mm), according to Berman *et al.* [14]. Scrapings from the thin-layer chromatograms were assayed for radioactivity in 7 ml of Instafluor scintillation mixture (Packard), using a Beckman model LS 8100 liquid scintillation counter. The results of the assay were expressed as the percentage of radioactivity in oleate/radioactivity in oleate + stearate [10].

Spectrophotometry. All spectral measurements were performed at 25° in a Unicam SP 1800 recording spectrophotometer using the thermostatically controlled compartment which is designed to accommodate turbid samples.

Calculations and statistical analyses. The observed first order rate constants (k_1) for the re-oxidation of NADH-reduced hepatic microsomal cytochrome b_5 in air saturated buffer were calculated from the slopes of plots of ln $(A_t - A_\infty)$ versus time, where A_t and A_∞ are the absorbance changes between 424 nm and 409 nm at time t and at infinity, respectively.

The observed first order rate constants (k_1) for the oxidation of purified trypsin-cleaved cytochrome b_5 were calculated from plots of ln $(A_{424_t} - A_{424_\infty})$ versus time. In all cases, the absorbance at infinite time was determined after approximately ten half-lives. Student's *t*-test for unpaired data was utilized to assess statistical significance, with $P < 0.05$ being probably significant, $P < 0.01$ being significant, and $P < 0.001$ being highly significant.

RESULTS

NADPH steady-state of hepatic microsomal cytochrome b_5. In the presence of enflurane, halothane and methoxyflurane, the redox status of NADPH-reduced cytochrome b_5 of hepatic microsomes from rats with elevated levels of stearate desaturase was shifted toward the ferric form of the hemoprotein (Table 1).

Table 1. Effects of anesthetic agents on the NADPH steady-state of hepatic microsomal cytochrome b_5*

Additions (mM)	NADPH steady-state (% reduction)
None	56.1 ± 13.5
Enflurane (14)	26.0 ± 4.5†
Halothane (18)	26.9 ± 5.5†
Methoxyflurane (1)	37.1 ± 1.6†

* Values reported are means ± S.D. for assays in duplicate for three to seven separate preparations of hepatic microsomes from rats fed a high-carbohydrate diet. The reaction mixture contained 3 ml of hepatic microsomes (1.5 mg protein/ml), 15 μM NADPH, and additions as indicated, at 25°.

† Differs significantly from no additions, $P < 0.01$.

Effects of anesthetic agents and inhibitors on the re-oxidation of hepatic microsomal ferrocytochrome b_5. For all studies of the re-oxidation of hepatic microsomal ferrocytochrome b_5, unless indicated otherwise, hepatic microsomes were from rats in which the levels of stearate desaturase were elevated by feeding a high-carbohydrate diet (see Experimental). That the levels of stearate desaturase in these microsomal preparations were elevated was demonstrated by the ability of stearoyl CoA to enhance the rate constant for the re-oxidation of cytochrome b_5 [8] (Tables 2–4).

The anesthetic agents, halothane, enflurane and methoxyflurane, stimulated the re-oxidation of microsomal cytochrome b_5 (Table 2), as was reported earlier for halothane [14]. The rate constants for the re-oxidation of cytochrome b_5 in the presence of stearoyl CoA or the anesthetic agents were decreased by 0.5 mM KCN ($P < 0.01$) (Table 2). The inhibitors of cytochrome P-450, namely metyrapone and carbon monoxide, had no effect on the re-oxidation of cytochrome b_5 in the presence or absence of stearoyl CoA or any of the anesthetic agents ($P > 0.1$) (Tables 3 and 4).

NAD(P)H-cytochrome c reductase activities. The effects of enflurane and methoxyflurane on the

Table 2. Effect of cyanide on the enhancement of the rate constants for the re-oxidation of cytochrome b_5 by stearoyl CoA and anesthetic agents*

Additions (mM)	First order rate constant for oxidation of ferrocytochrome b_5 $10^2 k_1$ (sec^{-1})
None	1.24 ± 0.35
KCN (0.5)	1.28 ± 0.20
Stearoyl CoA (0.012)	2.76 ± 0.73†
Stearoyl CoA (0.012) + KCN (0.5)	1.55 ± 0.37
Halothane (18)	1.73 ± 0.29†
Halothane (18) + KCN (0.5)	1.23 ± 0.30
Enflurane (14)	2.44 ± 0.63†
Enflurane (14) + KCN (0.5)	1.67 ± 0.54
Methoxyflurane (1)	1.98 ± 0.28†
Methoxyflurane (1) + KCN (0.5)	1.68 ± 0.08

* Values reported are means ± S.D. for assays in duplicate on each of three separate preparations of hepatic microsomes from rats fed a high-carbohydrate diet. Reaction mixtures contained 3 ml of hepatic microsomes (1.5 mg protein/ml), 1–5 μM NADH, and additions as indicated, at 25°.

† Differs significantly from no additions, $P < 0.01$.

Table 3. Effect of metyrapone on the enhancement of the rate constants for the re-oxidation of cytochrome b_5 by stearoyl CoA and anesthetic agents*

Additions (mM)	First order rate constant for the oxidation of ferrocytochrome b_5 $10^2 k_1$ (sec^{-1})
None	1.09 ± 0.22
Metyrapone (2.3)	1.17 ± 0.18
Stearoyl CoA (0.012)	4.19 ± 0.59†
Stearoyl CoA (0.012) + metyrapone (2.3)	4.15 ± 0.33†
Halothane (18)	1.68 ± 0.15†
Halothane (18) + metyrapone (2.3)	2.03 ± 0.48†
Enflurane (14)	2.23 ± 0.36†
Enflurane (14) + metyrapone (2.3)	2.13 ± 0.49†
Methoxyflurane (1)	1.78 ± 0.18†
Methoxyflurane (1) + metyrapone (2.3)	1.70 ± 0.12†

* Values reported are means ± S.D. for assays in duplicate on each of three separate preparations of hepatic microsomes from rats fed a high-carbohydrate diet. Reaction mixtures contained 3 ml of hepatic microsomes (1.5 mg protein/ml), 1–5 μM NADH, and additions as indicated, at 25°.

† Differs significantly from no additions, $P < 0.01$.

Table 4. Effect of carbon monoxide on the enhancement of the rate constants for the re-oxidation of cytochrome b_5 by stearoyl CoA and anesthetic agents*

Additions	First order rate constant for the oxidation of ferrocytochrome b_5 $10^2 k_1$ (sec^{-1})
None	0.97 ± 0.15
$CO–O_2$ (80:20, v/v)	1.04 ± 0.22
Stearoyl CoA (0.012 mM)	3.26 ± 1.05†
Stearoyl CoA (0.012 mM) + $CO–O_2$ (80:20, v/v)	3.31 ± 0.38†
Halothane (18 mM)	1.35 ± 0.28‡
Halothane (18 mM) + $CO–O_2$ (80:20, v/v)	1.10 ± 0.30
Enflurane (14 mM)	1.49 ± 0.08†
Enflurane (14 mM) + $CO–O_2$ (80:20, v/v)	1.62 ± 0.20†
Methoxyflurane (1 mM)	1.69 ± 0.31†
Methoxyflurane (1 mM) + $CO–O_2$ (80:20, v/v)	1.56 ± 0.42†

* Values reported are means ± S.D. for assays in duplicate on each of three separate preparations of hepatic microsomes from rats fed a high-carbohydrate diet. Reaction mixtures contained 3 ml of hepatic microsomes (1.5 mg protein/ml), 1–5 μM NADH, and additions as indicated, at 25°.

† Differs significantly from no additions, $P < 0.01$.

‡ Probably differs from no additions, $P < 0.05$.

Table 5. Effect of anesthetic agents on hepatic microsomal NADPH- and NADH-cytochrome *c* reductase and on the oxidation of purified trypsin-cleaved cytochrome b_5*

Additions (mM)	Autoxidation of purified ferrocytochrome b_5 $10^2 k_1$ (sec^{-1})	NADPH-cytochrome *c* reductase (units/mg protein)	NADH-cytochrome *c* reductase (units/mg protein)
None	0.85 ± 0.15	0.051 ± 0.005	0.95 ± 0.10
Enflurane (14)	0.89 ± 0.16	0.053 ± 0.005	1.34 ± 0.14†
Methoxyflurane (1)	0.97 ± 0.05	0.057 ± 0.001	1.09 ± 0.14

* For the NADPH- and NADH-cytochrome *c* reductase assays, 100 μl and 20 μl of hepatic microsomes (1.5 mg protein/ml) from rats fed a high-carbohydrate diet were added to 2.10 ml of 0.1 M Tris–HCl, pH 7.4, containing 20 μM cytochrome *c*, 0.1 mM of either NADPH or NADH, and additions as indicated. For the oxidation of purified, trypsin-cleaved ferrocytochrome b_5, the reaction mixtures contained 1.25 ml of 0.1 M Tris–HCl, pH 7.4, 30 μl of purified ferrocytochrome b_5, and additions as indicated. Reported values are means ± S.D.

† Differs significantly from no additions, $P < 0.01$.

Table 6. Effect of anesthetic agents and KCN on the rate constants for the re-oxidation of NADH-reduced hepatic microsomal cytochrome b_5 in differently pretreated rats*

Additions (mM)	10^2 k_1 (sec^{-1})		
	Fed a normal diet	Fasted	Fasted, phenobarbitone-induced
None	1.56 ± 0.17	1.63 ± 0.41	2.65 ± 0.07
KCN (0.5)	1.48 ± 0.20	1.34 ± 0.13	2.03 ± 0.43
Stearoyl CoA (0.012)	2.09 ± 0.12†	1.83 ± 0.27	2.78 ± 0.17
Stearoyl CoA (0.012) + KCN (0.5)	1.73 ± 0.30	1.55 ± 0.18	2.17 ± 0.25
Enflurane (14)	2.28 ± 0.26†	2.23 ± 0.38‡	3.61 ± 0.94‡
Enflurane (14) + KCN (0.5)	2.08 ± 0.15	2.02 ± 0.39	3.50 ± 0.78
Methoxyflurane (1)	1.90 ± 0.27‡	1.86 ± 0.09	3.00 ± 0.27‡
Methoxyflurane (1) + KCN (0.5)	1.73 ± 0.28	1.75 ± 0.18	2.82 ± 0.47
Cytochrome P-450 (nmoles/mg protein)	0.98 ± 0.05	1.24 ± 0.33	2.49 ± 0.12

* Values reported are means ± S.D. for assays in duplicate with each of three separate preparations of hepatic microsomes. Experimental details are as in Table 2.

† Differs significantly from no additions for similarly pretreated rats, $P < 0.001$.

‡ Probably differs from no additions for similarly pretreated rats, $P < 0.05$.

Table 7. Effect of anesthetic agents and KCN on the re-oxidation of NADH-reduced hepatic microsomal cytochrome b_5 from differently pretreated rats*

Additions	Per cent increase in first order rate constant (k_1)							
	High-carbohydrate diet		Normal diet		Fasted		Phenobarbitone-induced and fasted	
	−KCN	+KCN	−KCN	+KCN	−KCN	+KCN	−KCN	+KCN
Stearoyl CoA	100–800	50–200	34	16	12	17	5	7
Halothane	40	0	58†	10†	19†	22†	22†	30
Enflurane	97	30	46	40	37	24	36	32
Methoxyflurane	60	31	22	11	14	7	13	6

* Percentage increases were calculated from the values presented in Tables 2 and 4. Percentages are relative to the corresponding value for NADH alone

† Calculated from data in Ref. 14.

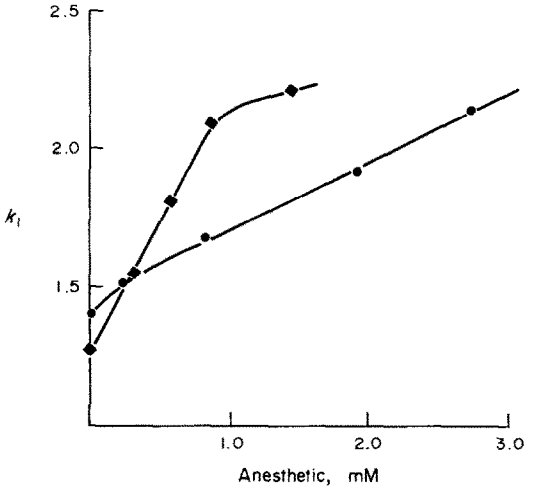

Fig. 1. Effects of enflurane (●) and methoxyflurane (◆) on the rate constants (k_1) for the re-oxidation of NADH-reduced hepatic microsomal cytochrome b_5. The first order rate constant k_1 is in units of 10^{-2} sec^{-1}. Experimental conditions are as given in Table 2.

activities of NADPH- and NADH-cytochrome *c* reductase are shown in Table 5. Neither enflurane nor methoxyflurane had a statistically significant effect on NADPH-cytochrome *c* reductase. Enflurane slightly enhanced the activity of NADH-cytochrome *c* reductase, whereas methoxyflurane did not.

Autoxidation of trypsin-cleaved ferrocytochrome b_5. The rate constants for the autoxidation of purified trypsin-cleaved ferrocytochrome b_5 in the presence of enflurane and methoxyflurane are given in Table 5. As can be seen, the rate constant for the autoxidation of purified ferrocytochrome b_5 is not altered in the presence of enflurane or methoxyflurane ($P > 0.1$).

Re-oxidation of hepatic microsomal ferrocytochrome b_5 *from fasted, fed or phenobarbitone-pretreated rats.* The rate constants for the re-oxidation of NADH-reduced cytochrome b_5 in microsomes from fasted, fed or phenobarbitone-induced rats are shown in Table 6, and the data are summarized in Table 7. Stearoyl CoA and enflurane increased the k_1 in fed rats, but not significantly in fasted rats

Table 8. Equilibrium constants for the interaction of anesthetic agents with hepatic microsomal enzymes

Compound	K_{eq} (mM) for cytochrome b_5 re-oxidation	K_s* (mM) for binding to cytochrome P-450	K_m* (mM) for oxidation of NADPH by cytochrome P-450	K_m* (mM) for fluoride ion production by cytochrome P-450
Enflurane	1.18 ± 0.16	0.46 ± 0.15	0.15 ± 0.10	0.36 ± 0.07
Methoxyflurane	0.48 ± 0.14	0.48 ± 0.13	0.10 ± 0.01	0.40 ± 0.12† 4.9 ± 0.9

* Data for hepatic microsomes from fasted, uninduced male rats from Ref. 4.
† Two K_m values were calculated from biphasic Eadie–Hofstee plots for this process.

pretreated or not with phenobarbitone. Cyanide inhibited the ability of stearoyl CoA to enhance the re-oxidation of cytochrome b_5 in microsomes from rats fed a normal diet ($P < 0.001$), but not in microsomes from fasted or phenobarbitone-treated rats ($P > 0.1$). Cyanide had no significant effect on the oxidation of cytochrome b_5 in the presence of enflurane or methoxyflurane in hepatic microsomes from fed, fasted or phenobarbitone-treated rats. The activity of stearate desaturase (as assessed by the stimulation of the re-oxidation of cytochrome b_5 by stearoyl CoA [8]) and the abilities of enflurane and methoxyflurane to stimulate the cyanide sensitive re-oxidation of NADH-reduced cytochrome b_5 decreased in the order of rats fed a high-carbohydrate diet > rats fed a normal diet > fasted rats ≅ fasted, phenobarbitone-treated rats (Table 7).

Determination of the equilibrium constants (K_{eq}) for the stimulation of hepatic microsomal electron transfer by enflurane and methoxyflurane. The effects of increasing concentrations of enflurane and methoxyflurane on the rate constants for the re-oxidation of NADH-reduced cytochrome b_5 were utilized to calculate the equilibrium constants (K_{eq}) for the stimulation of microsomal electron transfer by these anesthetic agents (Fig. 1, Table 8). The value of K_{eq} for enflurane differed from the K_s and K_m values for the binding and metabolism of enflurane by hepatic microsomal cytochrome P-450 (Table 8) ($P < 0.01$). The K_{eq} calculated for methoxyflurane differed ($P < 0.01$) from the K_m for NADPH oxidation and the high K_m for fluoride production by cytochrome P-450, but did not differ significantly from the K_s for binding to cytochrome P-450 or the low K_m for the production of fluoride ion from methoxyflurane by cytochrome P-450 ($P > 0.1$).

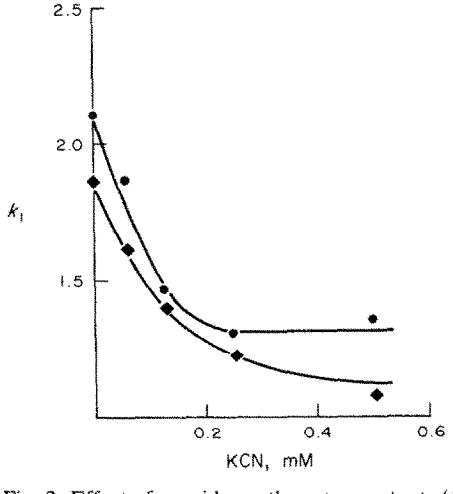

Fig. 2. Effect of cyanide on the rate constants (k_1) for the reoxidation of NADH-reduced hepatic microsomal cytochrome b_5 in the presence of (●) 1.4 mM enflurane and (◆) 0.6 mM methoxyflurane. Experimental conditions are as given in Table 2.

Inhibition of the re-oxidation of hepatic microsomal cytochrome b_5 *by KCN—Determination of* K_i. The effects of increasing concentrations of cyanide on the stimulation of the rate constants for the re-oxidation of NADH-reduced microsomal cytochrome b_5 by enflurane and methoxyflurane are shown in Fig. 2. The K_i values for cyanide were found to be 0.08 ± 0.01 mM for enflurane and 0.11 ± 0.02 mM for methoxyflurane. These constants compare well with the K_i of 0.14 mM determined for the cyanide inhibition of the stimulation of re-oxidation of hepatic microsomal cytochrome b_5 by halothane reported by Berman *et al.* [14] and with the value of K_i of 0.1 mM for cyanide inhibition of the conversion of stearoyl CoA to oleate by stearate desaturase reported by Oshino *et al.* [10].

Stearate desaturase assay. The effects of halothane, methoxyflurane and enflurane on the activity of stearate desaturase, as assayed via the conversion

Table 9. Effect of anesthetic agents on the stearate desaturase mediated conversion of stearate to oleate*

Additions (mM)	Oleate (oleate + stearate)
None	0.34 ± 0.16
Halothane (18)	0.38 ± 0.14
Enflurane (14)	0.36 ± 0.12
Methoxyflurane (1)	0.35 ± 0.16

* Values are means ± S.D. for three separate assays using microsomes from rats fed a high-carbohydrate diet. See Experimental Section for details.

of [^{14}C]stearoyl CoA to [^{14}C]oleate, are shown in Table 9. None of the anesthetic agents had any effect on this reaction ($P > 0.1$).

DISCUSSION

Enflurane and methoxyflurane appear to stimulate microsomal electron transfer from NADPH and NADH via cytochrome b_5, as shown by the ability of these anesthetic agents to shift the redox status of NADPH-reduced cytochrome b_5 toward the ferric form of the protein and to increase the rate constants for the reoxidation of NADH-reduced cytochrome b_5 (Tables 1 and 2). Since enflurane and methoxyflurane did not decrease the activity of microsomal NADH- and NADPH-cytochrome *c* reductase (Table 5), it would appear that these anesthetic agents probably do not decrease the rate of reduction of cytochrome b_5 but rather enhance the rate of oxidation of this heme protein.

The oxidation of ferrocytochrome b_5 can proceed via an autoxidation reaction or via the transfer of electrons to other microsomal proteins such as cytochrome P-450 or stearate desaturase. In the absence of added substrates for cytochrome P-450, stearate desaturase, or other microsomal enzymes which accept reducing equivalents from ferrocytochrome b_5, the oxidation of microsomal cytochrome b_5 *in vitro* is thought to arise in large part from the autoxidation of this hemoprotein [14, 23–26]. The autoxidation reaction involves the transfer of reducing equivalents from ferrocytochrome b_5 directly to oxygen to produce ferricytochrome b_5 and superoxide [23, 26].

Purified trypsin-cleaved cytochrome b_5, a heme peptide of approximately ninety residues which differs from the intact hemoprotein only in that the hydrophobic tail which attaches the protein to the membrane is lacking, was used as a model to assess the effects of enflurane and methoxyflurane on the autoxidation of ferrocytochrome b_5. Trypsin-cleaved cytochrome b_5 was chosen as a model system because it does not aggregate in water, as does cytochrome b_5 prepared by detergent solubilization, and the structure of the heme crevice and the rate of autoxidation of the hemoprotein are not altered following tryptic digestion of cytochrome b_5 [14, 27]. The first order rate constant for the autoxidation of purified trypsin-cleaved cytochrome b_5 reported herein (Table 5) is identical to values reported elsewhere [14] and is similar to the first order rate constant for the oxidation of membrane-bound hepatic microsomal ferrocytochrome b_5 in the absence of substrates for cytochrome P-450 and stearate desaturase [14, 23] (Tables 2 and 5). Since the rate constants for the autoxidation of trypsin-cleaved ferrocytochrome b_5 were not affected by enflurane and methoxyflurane, it would appear that these compounds probably do not affect the autoxidation of ferrocytochrome b_5. This conclusion is supported further by the observation that the autoxidation of cytochrome b_5 is not inhibited by KCN [14] (Table 2), while the effects of enflurane and methoxyflurane on hepatic microsomal electron transfer are. It would be anticipated, therefore, that enflurane and methoxyflurane may stimulate electron transfer from ferrocytochrome b_5 to another microsomal protein.

From several lines of evidence, it would appear that the microsomal electron acceptor in question is not cytochrome P-450. NADH, which does not effectively support cytochrome P-450-dependent reactions, supported the phenomenon as effectively as did NADPH (Tables 1 and 2). Neither prior induction of cytochrome P-450 with phenobarbital nor the presence of the inhibitors of cytochrome P-450—metyrapone and CO (see e.g. Refs. [28] and [29])—in the reaction mixture had any effect on the enhanced oxidation of cytochrome b_5 seen in the presence of enflurane and methoxyflurane (Tables 3, 4 and 6). In contrast, cyanide, which is not an effective inhibitor of cytochrome P-450, inhibited the process with a K_i which was well below the range of K_i values of 2.5 to 10 mM reported for the inhibition of cytochrome P-450 by cyanide [30, 31]. Finally, the K_{eq} for the stimulation of the re-oxidation of cytochrome b_5 by enflurane differed significantly from the K_s and K_m values for the interaction of enflurane with cytochrome P-450 [4] (Table 8).

It would appear that the microsomal 6-desaturase is not involved in the stimulation of microsomal electron transfer by enflurane and methoxyflurane, since its levels are not elevated by the feeding of a high-carbohydrate diet [15] which enhances the effects of the anesthetic agents (Tables 6 and 7). It is also possible to exclude catalase—a microsomal contaminant—as having an important role in mediating the effects of enflurane and methoxyflurane, because, although this enzyme is cyanide-sensitive, the K_i for cyanide inhibition of this enzyme (approximately 8 μM [32]) is 10-fold lower than the K_i calculated for cyanide inhibition of the re-oxidation of cytochrome b_5.

The results presented herein are consistent with the proposal that the transfer of electrons to stearate desaturase is responsible for the enflurane- and methoxyflurane-mediated enhancement of microsomal electron transfer through cytochrome b_5. The magnitude of the observed effect parallels the dietary induction of stearate desaturase; for example, the feeding of a high-carbohydrate diet, which induces stearate desaturase, results in maximal enhancement of electron transfer, while fasting, which reduces stearate desaturase to negligible levels [8, 15, 16], eliminates the effects of the anesthetic agents (Tables 6 and 7). In addition, the enhanced re-oxidation of cytochrome b_5 is inhibited by cyanide (Table 2), as is stearate desaturase. The K_i values calculated for cyanide inhibition of the stimulation of electron transfer by enflurane and methoxyflurane are within experimental error of the K_i of 0.1 mM reported for cyanide inhibition of the conversion of stearoyl CoA to oleate by stearate desaturase [10].

The lack of effect of enflurane and methoxyflurane on the conversion of stearoyl CoA to oleate is not inconsistent with the above proposal since the anesthetic agents may not bind to the substrate binding site of the enzyme. Other compounds which have been reported to interact with stearate desaturase, namely halothane and p-cresol, also do not inhibit the conversion of stearoyl CoA to oleate [11, 14].

In conclusion, it would appear that enflurane and methoxyflurane, at concentrations achieved in physiological fluids [33, 34], stimulate hepatic microsomal electron flow from NADH or NADPH by enhancing the oxidation of cytochrome b_5. The autoxidation of cytochrome b_5 and the transfer of electrons from cytochrome b_5 to catalase, to the 6-desaturase or to cytochrome P-450 appear not to be involved in the stimulation by these agents of microsomal electron transfer. That the enhancement of microsomal electron transfer by enflurane and methoxyflurane parallels the dietary induction of stearate desaturase and that the K_i for cyanide inhibition of the effect of enflurane and methoxyflurane equals the K_i for inhibition of stearate desaturation strongly suggest that the effects of enflurane and methoxyflurane are mediated via stearate desaturase. The nature of the interaction of enflurane and methoxflurane with stearate desaturase and the physiological and pathological effects thereof are under investigation.

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