The Effects of Halothane on Hepatic Microsomal Electron Transfer

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1. The effects of halothane (CF_3CHBrCl), a volatile anaesthetic agent, on electron transfer in isolated rat liver microsomal preparations were examined. 2. At halothane concentrations achieved in tissues during clinical anaesthesia (1–2 mm), halothane shifts the redox equilibrium of microsomal cytochrome b_5 in the presence of NADPH towards the oxidized form. Halothane accelerates stoichiometric consumption of NADPH and O_2 , increases the rate of reoxidation of NADH-reduced microsomal ferrocycytochrome b_5 , but does not affect NADPH- or NADH-cytochrome c reductase activity. The enhanced microsomal electron flow seen in the presence of halothane is not diminished by CO nor is it increased by pretreatment of the animals with phenobarbital. 3. The effects of halothane are maximum in microsomal preparations isolated from animals fed on a high-carbohydrate diet to induce stearate desaturase activity. Changes in microsomal electron transfer caused by halothane are in all cases abolished by low concentrations (1–2 mm) of cyanide. Microsomal stearate desaturase activity is unaffected by halothane. 4. The first-order rate constant for oxidation of membrane-bound ferrocycytochrome b_5 in the absence of added substrate ($k_1 = 1.5 \times 10^{-2} \text{ s}^{-1}$) is similar to that for autoxidation of purified ferrocycytochrome b_5 ($k_1 = 7 \times 10^{-3} \text{ s}^{-1}$). The rate of autoxidation of soluble ferrocycytochrome b_5 is unaffected by halothane. 5. It is concluded that the effects of halothane on microsomal electron transfer are not related to cytochrome P -450-linked metabolism but rather arise from the interaction of halothane with the cyanide-sensitive factor of the stearate desaturase pathway.

Halothane is a widely used general anaesthetic agent and although relatively safe there are many reports that it may capriciously give rise to jaundice which has an unusually high mortality (Miller & Hunter, 1970). This halogenated hydrocarbon is metabolized only very slowly *in vivo* by the hepatic endoplasmic reticulum, and its anaesthetic action is thought to be elicited by the intact molecule, not by a metabolite (Greene, 1968). Although the mechanism of anaesthesia is thought not to involve alterations in cellular metabolism, significant alterations of metabolic function can be demonstrated in normal tissues at concentrations of halothane achieved during clinical anaesthesia. For instance, halothane has been shown to affect mitochondrial electron transfer (Harris *et al.*, 1971) and carbohydrate metabolism (Biebuyck *et al.*, 1972).

Although the metabolism of halothane *in vitro* has been demonstrated to be localized in hepatic microsomal fractions, the effects of the intact molecule on hepatic microsomal electron transfer have not been elucidated. We have therefore investigated the effects of halothane on hepatic microsomal electron transfer with particular reference to the stearate desaturase and the cytochrome P -450 drug-metabolizing pathways.

Experimental

Materials

NADH and NADPH were obtained from Miles Laboratories Ltd., Cape Town, South Africa. Unlabelled and [$1\text{-}^{14}\text{C}$]stearoyl-CoA (57.6 mCi/mmol) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and The Radiochemical Centre, Amersham, Bucks., U.K. respectively. Halothane (CF_3CHBrCl), stabilized with 0.01% thymol, was purchased from I.C.I. South Africa (Pharmaceuticals) Ltd., Johannesburg, South Africa. Ethylmorphine was a gift from Fine Chemical Corp., Cape Town, South Africa. Trypsin-cleaved cytochrome b_5 was isolated from calf liver and purified by the method of Omura & Takesue (1970), except that the purified protein was desalted by ultrafiltration through an Amicon UM-2 membrane.

Methods

Treatment of animals. Male albino rats of the Wistar strain, weighing between 180 and 350 g, were used. The animals were housed in experimental cages providing free access to water and food, which was normally Epol Laboratory Chow (Epol Animal

Feeds, Cape Town, South Africa). Most animals were fed before death. Where indicated, animals were starved overnight before death or were fed on a semi-purified high-carbohydrate diet to increase hepatic microsomal stearate desaturase activity (Oshino *et al.*, 1971). In the latter instance, the animals were fed with the high-carbohydrate diet for 1–4 days, starved for 24h, re-fed on the same diet and killed 16–24h after the beginning of the second feeding period (Shimakata *et al.*, 1971). Phenobarbital-treated animals were injected intraperitoneally with 50mg of sodium phenobarbital/kg body wt. per day for 5 days. After the last dose, the animals were starved overnight and killed the next morning.

Preparation of microsomal fraction. Microsomal fractions were prepared from rat liver homogenates by differential ultracentrifugation in 0.15M-KCl (Ernster & Nordenbrand, 1967). Microsomal protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Mitochondrial contamination was estimated by succinate oxidase activity (King, 1967) to be 3.8%. The mitochondrial contamination was effectively decreased to 1.5% by the presence of 0.2mM- Na_2S (Wilson & Gilmour, 1967), which was added to reaction mixtures where indicated.

Assays. The oxidation of NADH and NADPH was monitored spectrophotometrically at 340nm ($\epsilon = 6.2 \times 10^3 \text{M}^{-1} \cdot \text{cm}^{-1}$) (Bergmeyer, 1963). NADH- and NADPH-cytochrome *c* reductase activities were measured as described by Siler-Masters *et al.* (1967). Cytochrome *P*-450 was determined by the method of Omura & Sato (1964). The *N*-demethylation of ethylmorphine was monitored by formaldehyde production as described by Stripp *et al.* (1971).

The stearate desaturase assay was performed essentially as described by Oshino *et al.* (1966), with 40nCi of [$1\text{-}^{14}\text{C}$]stearoyl-CoA substrate per experiment. The extracted free fatty acids were methylated with anhydrous BF_3 -methanol complex (14% BF_3) (BDH Chemicals Ltd., Poole, Dorset, U.K.) and separated on silica-gel G thin-layer plates (5cm $\times$ 20cm $\times$ 0.25mm) (E. Merck AG, Darmstadt, Germany) which had been lightly sprayed with saturated aq. AgNO_3 and activated at 110°C for 30min immediately before use. Chromatography was performed in a solvent system of diethyl ether–light petroleum (b.p. 60–80°C; 1:19, v/v) at 18–20°C. The fatty acid esters were detected on the developed chromatograms by spraying with water or aq. 0.05% Rhodamine 6G. The areas corresponding to the methyl esters were dried and scraped into counting vials containing 10ml of Bray's solution (Stern *et al.*, 1969). The samples were then counted for radioactivity in a Beckman liquid-scintillation system LS 233. Results were expressed as the percentage of oleate/(oleate+stearate), a parameter that is independent of the recovery of the radioactive sample.

Spectral determinations were performed on a Unicam SP.1800 spectrophotometer with a SP.1805 program controller. O_2 uptake was measured with a Clark-type oxygen electrode. Reactions were performed at 30°C unless otherwise stated. Reaction mixtures were equilibrated with air unless otherwise indicated and stoppered where necessary to prevent escape of volatile components. Gas mixtures of CO and O_2 were prepared with the aid of a Fluotec mark 2 mixing chamber. Concentrations of halothane greater than 18mm were achieved by adding halothane in dimethylformamide unless otherwise stated. The amounts of dimethylformamide added (less than 50 μl per 3ml of reaction mixture) were without effect on the reactions investigated.

Kinetics. NADH was utilized as reductant for studies of the reoxidation of microsomal cytochrome *b*₅ (Oshino *et al.*, 1971). The reoxidation reaction or steady-state redox status of microsomal cytochrome *b*₅ was determined spectrally by the difference in optical absorbance between 424 and 409nm (Omura & Sato, 1964). For these measurements, microsomal fractions were suspended at a concentration of 2.0mg of protein/ml in 0.1M-Tris-HCl buffer, pH 7.2, containing 0.2mM- Na_2S . Appropriate additions were made to thermally equilibrated samples. Spectral determinations on these samples were performed in a thermostatically controlled compartment of the spectrophotometer designed for turbid samples.

Solutions of purified trypsin-cleaved cytochrome *b*₅ were purged with O_2 -free N_2 for 20min to minimize H_2O_2 formation during the subsequent reduction by H_2 in the presence of palladium catalyst (Smith, 1955). The trypsin-cleaved ferrocytochrome *b*₅ was recovered by filtration and used immediately in the autoxidation experiments. The autoxidation of trypsin-cleaved ferrocytochrome *b*₅ was monitored spectrally at 424nm.

The observed first-order rate constants (k_1) for the oxidation of microsomal and purified cytochrome *b*₅ were calculated from plots of $\ln[(E_{424} - E_{409})_t - (E_{424} - E_{409})_\infty]$ or $\ln(E_t - E_\infty)$ versus time respectively, where 'infinite time' is after at least five half-lives of the reaction.

Results

Effect of halothane on microsomal consumption of NADPH and O_2

The results of studies of the effect of halothane on the consumption of NADPH and O_2 by hepatic microsomal preparations are shown in Fig. 1. Halothane enhances utilization of NADPH and O_2 by approximately 100%. The effect of halothane is reversed by 2.5mM-cyanide, the presence of which returns consumption of NADPH and O_2 to the lower values seen in the presence of NADPH and cyanide.

Fig. 1. Effect of halothane and cyanide on NADPH and O₂ consumption by rat liver microsomal fractions

Suspensions of microsomal fractions (1.5–2.0 mg of protein/ml) prepared from liver of rats fed on a high-carbohydrate diet were incubated at 30°C in 3.0 ml of 0.1 M-Tris-HCl buffer, pH 7.2, containing 0.2 mM-Na₂S. Halothane (15 mM) and/or NaCN (2.5 mM) were added as indicated. The reaction was initiated by the addition of 0.45 μmol of NADPH. Initial rates of NADPH oxidation (□) and O₂ consumption (■) were monitored as described under 'Methods'.

NADPH oxidation and O₂ uptake remain stoichiometric in all experiments (Fig. 1). Na₂S, added as an inhibitor of mitochondrial electron transfer (Wilson & Gilmour, 1967), decreases the consumption of NADPH and O₂ in our microsomal preparations by less than 5% in either the presence or the absence of halothane.

Effect of halothane on the redox status of microsomal cytochrome b₅

In the presence of NADPH and O₂, microsomal cytochrome b₅ exists in a redox steady state (Oshino *et al.*, 1971). Under these conditions, in the absence of added oxidizable substrate for the cytochrome P-450 or stearate desaturase pathways, approx. 65% of the cytochrome b₅ is reduced at equilibrium (Fig. 2). The redox status of the equilibrium mixture is shifted increasingly towards the oxidized form in the presence of increasing quantities of halothane. The use of redistilled halothane, which was free from thymol, did not alter the effect of halothane on the redox status of cytochrome b₅. Halothane, at concentrations up to 40 mM, has no effect on NADPH- or NADH-cytochrome c reductase activity (Fig. 2).

Reoxidation of microsomal cytochrome b₅

Microsomal suspensions were reduced with limiting amounts of NADH, and the reoxidation of the endo-

Fig. 2. Effect of halothane on the redox equilibrium of microsomal cytochrome b₅ and on NADH- and NADPH-cytochrome c reductase activities

The redox equilibrium of cytochrome b₅ in microsomal suspensions (2 mg of protein/ml) prepared from fed rats was determined in 0.15 M-Tris-HCl, pH 7.2, at 30°C in the presence of various concentrations of halothane as described under 'Methods'. The mol fraction of reduced cytochrome b₅ (●) was calculated after reduction with Na₂S₂O₄. The activities of NADH-cytochrome c reductase (▲) and of NADPH-cytochrome c reductase (○) in the presence of various concentrations of halothane as a percentage of controls was determined at 25°C as described under 'Methods'.

Fig. 3. Reoxidation of microsomal ferrocytochrome b₅

Microsomal suspensions (1.5 mg of protein/ml) in a total volume of 3.0 ml of 0.1 M-Tris-HCl buffer, pH 7.2, containing 0.2 mM-Na₂S, were incubated at 30°C. After addition of 15 nmol of NADH to the sample cuvette, the absorbance was monitored alternately at 424 and 409 nm. The first-order constant ($k_1 = 2.0 \times 10^{-2} \text{ s}^{-1}$) for reoxidation of ferrocytochrome b₅ (see insert) was calculated as described under 'Methods'.

genous ferrocytochrome b_5 was monitored. Since NADH rapidly reduces ferricytochrome b_5 , once the supply of NADH is exhausted the oxidation of microsomal ferrocytochrome b_5 promptly follows first-order kinetics (Fig. 3), and the first-order rate constant (k_1) for oxidation of ferrocytochrome b_5 is a valid index of microsomal electron flow from cytochrome b_5 (Oshino *et al.*, 1971). The rate constants for the oxidation of ferrocytochrome b_5 in the absence of added substrate for terminal microsomal oxidases were found to be ($\pm$ S.D., $n = 3-4$) $1.5 \times 10^{-2} \pm 0.3 \times 10^{-2} \text{ s}^{-1}$ in microsomal fractions from control animals. Microsomal fractions isolated from rats pretreated with a high-carbohydrate diet to induce the stearate desaturase pathway exhibited slightly increased rate constants of $1.8 \times 10^{-2} \pm 0.3 \times 10^{-2} \text{ s}^{-1}$. Cyanide at concentrations of up to 2 mM had no appreciable effect on the rate constants for starved uninduced or starved phenobarbital-induced preparations (Table 1). As reported by Oshino *et al.* (1971), in carbohydrate-induced microsomal suspensions stearoyl-CoA increases the rate of oxidation of ferrocytochrome b_5 severalfold and the effect of stearoyl-CoA is completely blocked by concentrations of cyanide greater than 0.5 mM (Table 2). Cyanide also decreased oxidation of ferrocytochrome b_5 in the absence of substrate in these preparations.

Halothane (8.0 mM) increases the rate constant for cytochrome b_5 reoxidation in microsomal preparations from uninduced, phenobarbital-induced and carbohydrate-treated animals (Table 3). The enhancement is more marked in the animals fed on a normal or a high-carbohydrate diet before death than in the other cases. The effect of increasing concentrations of cyanide on the halothane-enhanced reoxidation of ferrocytochrome b_5 in microsomal fractions from carbohydrate-induced animals is shown in Fig. 4.

Table 1. *Effect of cyanide and CO on rate constants for reoxidation of NADH-reduced microsomal cytochrome b_5*

For experimental details see the text. Values are means $\pm$ S.D. of three or four experiments on each of two microsomal preparations. * $P < 0.05$ compared with the relevant control (no additions) preparations.

Addition	$10^2 \times k_1 \text{ (s}^{-1}\text{)}$	
	Uninduced	Phenobarbital-induced
—	1.20 ± 0.03	1.49 ± 0.23
NaCN (0.5 mM)	1.42 ± 0.08	1.76 ± 0.21
NaCN (1.0 mM)	1.40 ± 0.10	1.71 ± 0.36
NaCN (2.0 mM)	1.08 ± 0.17	1.44 ± 0.2
NaCN (10.0 mM)	—	$0.78 \pm 0.04^*$
—	—	1.87 ± 0.16
CO + O ₂ (50:50)	—	2.07 ± 0.22
CO + O ₂ (80:20)	—	$1.48 \pm 0.10^*$

Cyanide, at concentrations greater than 0.6 mM, completely inhibits the halothane enhancement of reoxidation of cytochrome b_5 . The K_i value for cyanide inhibition of this effect was calculated from Fig. 4 to be 0.14 mM.

Role of cytochrome P-450

The rate constants for the reoxidation of NADH-reduced microsomal cytochrome b_5 are similar for

Table 2. *Effect of stearoyl-CoA and cyanide on rate constants for reoxidation of NADH-reduced cytochrome b_5 in carbohydrate-induced microsomal fractions*

For experimental details see the text. The desaturase activity of microsomal suspensions used in Expt. 1 was such that oleate/(oleate + stearate) = 10% per min measured over 4 min. Values are means $\pm$ S.D. with number of determinations in parentheses. * $P < 0.02$ versus controls; † $P < 0.01$ versus controls.

Expt. no.	Stearoyl-CoA (12 μ M)	NaCN (0.5 mM)	$10^2 \times k_1 \text{ (s}^{-1}\text{)}$
1	—	—	$2.11 \pm 0.25 \text{ (4)}$
	—	+	$1.25 \pm 0.17 \text{ (3)}^*$
	+	—	$4.65 \pm 1.48 \text{ (3)}^*$
	+	+	$0.81 \pm 0.01 \text{ (2)}$
2	—	—	$1.77 \pm 0.21 \text{ (2)}$
	+	—	$4.47 \pm 1.45 \text{ (3)}$
3	—	—	$1.44 \pm 0.20 \text{ (5)}$
	+	—	$4.92 \pm 1.09 \text{ (3)}^\dagger$

Table 3. *Effects of halothane on the rate constants for reoxidation of NADH-reduced cytochrome b_5 in hepatic microsomal fractions*

For experimental details see the text. Microsomal fractions from starved uninduced and starved phenobarbital-induced rats contained approx. 1.2 and 3.2 nmol of cytochrome P-450/mg of microsomal protein respectively. Values are means $\pm$ S.D. of three or more determinations. * $P < 0.01$ versus controls; † $P < 0.05$ versus controls; ‡ $P < 0.02$ versus halothane alone.

Pretreatment	Halothane (mM)	NaCN (mM)	$10^2 \times k_1 \text{ (s}^{-1}\text{)}$
Uninduced, fed	—	—	1.31 ± 0.12
	8.0	—	$2.07 \pm 0.07^*$
	—	—	1.99 ± 0.10
	—	0.6	$1.18 \pm 0.09^*$
Carbohydrate-induced	8.0	—	$2.72 \pm 0.18^\dagger$
	8.0	0.6	$1.29 \pm 0.16^\ddagger$
	8.0	—	1.18 ± 0.02
	8.0	—	$1.40 \pm 0.11^\dagger$
Uninduced, starved	8.0	0.6	1.44 ± 0.12
	—	—	1.63 ± 0.05
	8.0	—	$2.00 \pm 0.02^*$
	8.0	0.6	$1.19 \pm 0.09^\ddagger$

uninduced and phenobarbital-induced microsomal preparations (Table 3). In microsomal fractions obtained from animals fed on a high-carbohydrate diet, saturation of the reaction suspension with $\text{CO} + \text{O}_2$ (80:20) was of slight effect on the reoxidation reaction (Table 1). Under the same conditions 10mM-

Fig. 4. Effect of cyanide on the first-order rate constants (k_1) for reoxidation of microsomal ferrocytochrome b_5 in the presence of halothane

First-order rate constants for reoxidation of ferrocytochrome b_5 in suspensions of hepatic microsomal fractions prepared from rats fed on a high-carbohydrate diet were determined as described in Fig. 3. Halothane (10mM) and various amounts of cyanide were added to sample cuvettes before the addition of NADH.

halothane increased the rate constant, k_1 , for reoxidation of microsomal cytochrome b_5 from $1.1 \times 10^{-2} \pm 0.2 \times 10^{-2} \text{ s}^{-1}$ to $2.4 \times 10^{-2} \pm 0.1 \times 10^{-2} \text{ s}^{-1}$ ($\pm \text{s.d.}$; $n = 2-5$). Further addition of CN^- (1.6mM) decreased k_1 to $0.9 \times 10^{-2} \pm 0.1 \times 10^{-2} \text{ s}^{-1}$. Thus CO did not prevent halothane from enhancing reoxidation of microsomal cytochrome b_5 , nor the inhibition of this enhancement by cyanide.

The NADPH-dependent demethylation of ethylmorphine in microsomal preparations from phenobarbital-induced rats was 5.6 ± 0.4 and 4.8 ± 0.1 nmol of formaldehyde/min per mg of microsomal protein, in the absence and the presence of halothane (10mM) respectively.

Microsomal stearyl-CoA desaturase activity

The results of the stearate desaturase assays are presented in Table 4. In accord with the finding of Shimakata *et al.* (1971), microsomal preparations from the livers of starved rats have low stearate desaturase activity and pretreatment of the animals with a high-carbohydrate diet increases desaturase activity markedly. Low concentrations of cyanide (2.7mM) effectively inhibit the reaction. Halothane, at concentrations of up to 40mM, has no effect on the desaturation of stearyl-CoA when either NADH or NADPH is used as the electron donor (Table 4).

Autoxidation of trypsin-cleaved ferrocytochrome b_5

The autoxidation of purified trypsin-cleaved cytochrome b_5 followed a first-order rate law. The first-order rate constant for autoxidation of trypsin-cleaved cytochrome b_5 at pH 7.5 and 30°C was $7.0 \times 10^{-3} \pm 0.6 \times 10^{-3} \text{ s}^{-1}$ in 0.1M-Tris-HCl buffer. The presence of cyanide (2mM) or halothane (15mM)

Table 4. Effects of halothane and cyanide on stearate desaturase activity of rat liver microsomal preparations

For experimental details see the text. Microsomal fractions were prepared from livers of starved rats and from rats in which stearate desaturase activity was induced by feeding with a high-carbohydrate diet. Where applicable pure halothane was added to microsomal suspensions in capped tubes and vigorously agitated to give the final concentrations shown below. Values are means of two determinations $\pm \text{s.d.}$, for a reaction time of 4 min. * $P < 0.01$ versus control; † $P < 0.01$ versus starved control.

Pretreatment	Electron donor	Addition	Oleate/(oleate+stearate) (%)
Starved	NADH	—	3.8 ± 0.4
Induced	NADH	—	$22.6 \pm 0.3^\dagger$
		2.7mM-NaCN	$3.1 \pm 0.5^*$
		17mM-Halothane	17.3 ± 0.8
		41mM-Halothane	17.1 ± 0.8
Induced	NADH	—	12.0 ± 0.6
		43mM-Halothane	12.9 ± 0.4
Induced	NADH	—	18.8 ± 0.4
		39mM-Halothane	20.7 ± 2.6
Induced	NADPH	—	13.4 ± 2.1
		43mM-Halothane	12.8 ± 1.3

in the reaction solution did not affect the rate of autoxidation.

Discussion

Halothane enhances the stoichiometric utilization of NADPH and O_2 by isolated rat liver microsomal fractions (Fig. 1), indicating that this halogenated hydrocarbon stimulates electron transfer through one or more components of the microsomal electron-transfer chain between NADPH and the terminal electron acceptor, O_2 . Since halothane does not affect the activity of microsomal NADH- or NADPH-cytochrome *c* reductase but enhances the oxidation of NADH-reduced microsomal-bound cytochrome *b*₅ (Table 3) and shifts the redox equilibrium of NADPH-reduced cytochrome *b*₅ towards the oxidized form (Fig. 2), the locus of action of halothane is presumably at or after the oxidation of ferrocytochrome *b*₅.

The oxidation of microsomal-bound cytochrome *b*₅ can proceed via an autoxidation reaction or via the direct transfer of electrons to other components of the microsomal electron-transfer chain. The components of microsomal fractions capable of accepting electrons from ferrocytochrome *b*₅ are the two terminal mixed-function oxidases: (1) the cytochrome *P*-450 components of the microsomal drug-metabolizing system and (2) the cyanide-sensitive factor of the fatty acid desaturase pathway. Reducing equivalents from NADPH and, possibly in small measure, from NADH can be supplied to cytochrome *P*-450 via cytochrome *b*₅ (Baron *et al.*, 1973; Correia & Mannering, 1973). Although cytochrome *b*₅ is not an essential intermediate in the transfer of electrons to this system (Ichikawa & Loehr, 1972; Sasame *et al.*, 1973), it is an obligate intermediate in the passage of electrons from either NADH or NADPH to the cyanide-sensitive factor of the stearate desaturase pathway (Jones *et al.*, 1969; Oshino *et al.*, 1971; Shimakata *et al.*, 1972).

Ferrocytochrome *b*₅ is slowly autoxidizable when bound to microsomal membranes (Krisch & Staudinger, 1959) or when isolated and purified (Strittmatter & Velick, 1956). Autoxidation is taken to indicate direct interaction of the haemoprotein with some oxidant present in aqueous solution such as molecular O_2 , but probably not with peroxide (Boveris *et al.*, 1972).

Trypsin-cleaved cytochrome *b*₅, a haem peptide containing approximately 90 residues of the intact protein, was used for studies of the autoxidation of purified ferrocytochrome *b*₅. Tryptic cleavage of cytochrome *b*₅ removes the hydrophobic tail of the protein that is responsible for binding to hydrophobic portions of the membrane. The cleavage does not, however, appreciably affect the structure of the haem crevice (Strittmatter *et al.*, 1972). The trypsin-cleaved

cytochrome *b*₅ was thought to be a better model than detergent-solubilized cytochrome *b*₅, since the latter preparation readily aggregates in water in the absence of detergent (Spatz & Strittmatter, 1971).

The first-order rate constant for the oxidation of NADH-reduced microsomal-bound cytochrome *b*₅ in the absence of added substrate, reported here, of $1.5 \times 10^{-2} s^{-1}$ is similar to the value of $2.2 \times 10^{-2} s^{-1}$, calculated from the results of Oshino & Sato (1971), and that of approx. $1.2 \times 10^{-2} s^{-1}$ reported by Boveris *et al.* (1972) for similar conditions. These rate constants are also similar to the first-order constants for the autoxidation of purified trypsin-cleaved ferrocytochrome *b*₅, of approx. $7 \times 10^{-3} s^{-1}$ (the present work) and $1.6 \times 10^{-2} s^{-1}$ (pH 8.1, 25°C) (P. Strittmatter, personal communication), suggesting that in the absence of added substrates for the desaturase or cytochrome *P*-450 pathways, oxidation of microsomal-bound cytochrome *b*₅ is independent of reductant and in large part arises from autoxidation of this haemoprotein rather than from the transfer of electrons to endogenous physiological substrates.

Concentrations of halothane which caused enhanced oxidation of microsomal ferrocytochrome *b*₅ did not alter the rate of autoxidation of trypsin-cleaved ferrocytochrome *b*₅. This suggests that halothane is not affecting the autoxidation of microsomal-bound ferrocytochrome *b*₅ but is acting on one or more components of the electron-transfer chain, e.g. cytochrome *P*-450 or the cyanide-sensitive factor, which couple the oxidation of ferrocytochrome *b*₅ to O_2 .

Halothane binds to microsomal cytochrome *P*-450 with the production of a 'type 1' difference absorption spectrum under aerobic conditions (Uehleke *et al.*, 1973). Its metabolism, shown to be localized in the microsomal fraction of rat liver (Cohen, 1971; Van Dyke & Wood, 1973) and to be increased in phenobarbital-treated rats (Scholler, 1968; Clauberg, 1970), might account for the enhanced consumption of NADPH and O_2 by halothane observed in our studies. However, the effect is reversed by concentrations of cyanide too low to inhibit cytochrome *P*-450-mediated reactions (Jefcoate *et al.*, 1969; Gaylor *et al.*, 1970). Several observations also indicate that the halothane-enhanced reoxidation of cytochrome *b*₅ is not mediated by cytochrome *P*-450. Halothane only slightly enhances the reoxidation of ferrocytochrome *b*₅ in microsomal preparations from starved control or starved phenobarbital-induced animals (Table 3); the threefold increase in hepatic microsomal cytochrome *P*-450 content in the latter animals does not result in a significant alteration in the magnitude of the halothane effect. An atmosphere of $CO+O_2$ (80:20), which inhibits reactions involving cytochrome *P*-450 (Omura *et al.*, 1965), does not diminish the halothane

enhancement of cytochrome b_5 reoxidation. The observation that halothane enhances microsomal oxidation of NADH (results not shown), which in the absence of NADH is not an effective electron donor to cytochrome $P-450$ (Mannering, 1971), provides additional evidence against the participation of cytochrome $P-450$. These observations further indicate that it is unlikely that a cytochrome $P-450$ metabolite of halothane is responsible for enhanced electron flow.

Since cyanide abolishes the halothane enhancement of cytochrome b_5 reoxidation at concentrations well below the reported values of K_i for cyanide inhibition of cytochrome $P-450$ (2.5–8.0 mM) (Jefcoate *et al.*, 1969; Gaylor *et al.*, 1970; Correia & Mannering, 1973), a cyanide-sensitive factor appears to be involved.

A variety of observations indicates that the cyanide-sensitive factor is identical with that required for stearate desaturase activity. Halothane enhancement of cytochrome b_5 reoxidation was of the greatest magnitude in microsomal fractions isolated from rats fed on a high-carbohydrate diet before death (Table 3). These feeding conditions are known to induce the cyanide-sensitive factor of the stearate desaturase pathway of hepatic endoplasmic reticulum (Inkpen *et al.*, 1969; Oshino *et al.*, 1971; Shimakata *et al.*, 1971). The halothane effect is seen with NADH or NADPH as electron donor (Fig. 2 and Table 3), either of which can provide electrons to the stearate desaturase pathway (Jones *et al.*, 1969; Holloway, 1971; Oshino *et al.*, 1971). Comparison of the K_i values for cyanide inhibition of the halothane effect, found here to be 0.14 mM (Fig. 4), with the K_i of 0.10 mM (Oshino *et al.*, 1966) for cyanide inhibition of desaturation of stearoyl-CoA, provides strong evidence that enhanced electron flow to (or through) the cyanide-sensitive factor is responsible for the halothane-enhanced reoxidation of cytochrome b_5 .

It is possible that another cyanide-sensitive factor is mediating the effect of halothane. A component of the microsomal fraction possibly responsible for this effect could be the other known desaturase. A 6-desaturase has been shown to occur in hepatic microsomal preparations and to be responsible for the desaturation of oleate, linoleate and linolenate (Brenner & Peluffo, 1966). The 6- and 9-desaturases respond differently to dietary factors; for instance, starvation does not decrease 6-desaturase activity, nor does feeding with a high-carbohydrate diet after starvation enhance 6-desaturase activity (Inkpen *et al.*, 1969). In contrast, starvation lowers 9-desaturase activity and the magnitude of the halothane effect, whereas feeding with a high-carbohydrate diet after starvation enhances 9-desaturase activity and the magnitude of the halothane effect. These findings appear to exclude 6-desaturase involvement in the action of halothane.

Catalase, a contaminant of isolated microsomal preparations (Tangen *et al.*, 1973), is several orders of magnitude more sensitive to cyanide inhibition ($K_i = 8 \mu\text{M}$) (Chance, 1952) than is the process which is stimulated by halothane, suggesting that this enzyme is also not responsible for any of the observed effects of halothane.

The above evidence is consistent with the involvement of the cyanide-sensitive factor of stearate desaturase in mediating the effect of halothane. If the cyanide-sensitive factor of the stearate desaturase pathway is involved, halothane must bind weakly to this factor relative to its natural substrate stearoyl-CoA, since halothane does not inhibit desaturation of stearoyl-CoA. Our data indicate that halothane does bind weakly to its site of action, since its effects are elicited only in the millimolar concentration range. Oshino & Sato (1971) have demonstrated that stearoyl-CoA binds extremely tightly to the cyanide-sensitive factor of sterol demethylase, which has been demonstrated to be identical with the cyanide-sensitive factor of stearate desaturase (Gaylor & Mason, 1968; Gaylor *et al.*, 1970; Oshino & Sato, 1971). Phenols have been shown to stimulate the oxidation of microsomal ferrocyclochrome b_5 but not to decrease the desaturation of stearoyl-CoA (Oshino & Sato, 1971), analogous to the effects seen here with halothane.

Our results indicate that halothane stimulates microsomal electron transfer through interaction with the cyanide-sensitive factor of stearate desaturase. This factor is known to be a non-haem redox enzyme (Oshino *et al.*, 1966; Shimakata *et al.*, 1972). It is possible that halothane is enhancing the oxidation of the cyanide-sensitive factor, by binding to the enzyme and altering its conformation. Alternatively, halothane may be metabolized by this pathway, as suggested by the results of studies of the interaction of catechol with the cyanide-sensitive factor, where oxidation of this phenol was observed (Oshino & Sato, 1971).

Halothane and various phenols are the only xenobiotics that have been shown to interact with the cyanide-sensitive factor of the hepatic microsomal fraction, and halothane does so at concentrations (1–2 mM) encountered during general anaesthesia (Saidman, 1968). It appears likely that other foreign compounds, in particular other halogenated hydrocarbons, may also interact with this enzyme and enhance microsomal electron flow. It is possible that the known hepatotoxic effects of halothane and other halogenated compounds are in part mediated by interaction with the cyanide-sensitive factor or by the depletion of reduced nicotinamide nucleotide cofactors as a consequence of enhanced microsomal electron transfer through this pathway.

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