THE INTERACTION OF XENOBIOTICS AND ANAESTHETIC AGENTS WITH HEPATIC MICROSOMAL STEARATE DESATURASE.

BY

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"I am a part of all that I have met;
Yet all experience is an arch where thro'
Gleams that untravell'd world, whose margin fades
For ever and for ever when I move."

Lord Tennyson
"Ulysses"

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Organic halo compounds : their interaction with hepatic microsomal stearate desaturase. Manca, V., Harrison, G.G. and Ivanetich, K.M. Submitted for Publication. (1980).

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Halothane, ethrane and methoxyflurane - a novel hepatic interaction. K.M. Ivanetich, V. Manca and G.G. Harrison. S.A. Society of Anaesthetists Congress, Tygerberg, S.A., September 25 - 28, 1978.

Stearate desaturase and its interaction with halogenated anaesthetics. V. Manca, G.G. Harrison and K.M. Ivanetich. University of Cape Town 150 Academic Festival, December 7, 1979.

The interaction of halothane, methoxyflurane and enflurane with rat hepatic stearate desaturase. G.G. Harrison, V. Manca

and K.M. Ivanetich. Abstract accepted for 7th World Congress of Anaesthesiologists, Hamburg, Germany, September 14 - 21, 1980.

The interaction of xenobiotics and anaesthetic agents with hepatic microsomal stearate desaturase. V. Manca, G.G. Harrison and K.M. Ivanetich. Abstract accepted for 5th Congress of the South African Biochemical Society, Umhlanga Rocks, Natal, October 21 - 23, 1980.

ABSTRACT

This thesis comprises an investigation into the reaction of halogenated xenobiotics and anaesthetic agents, with hepatic microsomal stearate desaturase. The levels of stearate desaturase in the hepatic microsomes were routinely elevated by re-feeding the experimental animals a high carbohydrate diet. The interaction of the xenobiotics with stearate desaturase was assessed by monitoring their effects on the redox steady state of hepatic microsomal cytochrome $\underline{b}_5$, in the presence and absence of cyanide. Approximately half of the xenobiotics examined shifted the redox steady state of NADPH reduced ferrocytochrome $\underline{b}_5$ towards the ferric form of the protein. Those compounds which shifted the redox steady state of cytochrome $\underline{b}_5$ to a figure of 40% or below were examined further by assessing their effect on the re-oxidation of NADH reduced microsomal ferrocytochrome $\underline{b}_5$. The pseudo first order rate constant for the rate of re-oxidation of NADH reduced microsomal ferrocytochrome $\underline{b}_5$ was significantly elevated in the presence of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane, chloroacetaldehyde, halothane, enflurane and methoxyflurane. The enhanced re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ observed in the presence of the above compounds was significantly diminished by 0,5 mM cyanide. Furthermore, these compounds appeared to stimulate the oxidation of microsomal ferrocytochrome $\underline{b}_5$ without affecting its reduction.

The effects of these halogenated xenobiotics on the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ appeared to parallel the dietary induction of stearate desaturase. In those rats which had been re-fed a high carbohydrate diet to induce stearate desaturase, the effect of the compounds on microsomal cytochrome $\underline{b}_5$ was enhanced while fasting, which is known to drastically reduce stearate desaturase activity, eliminated these effects. Similarly, fasting together with phenobarbitone pretreatment, which is also known to reduce stearate desaturase activity, eliminated these effects. Furthermore, the effects of the halogenated hydrocarbons on microsomal cytochrome $\underline{b}_5$ paralleled the dietary induction of the Δ9-desaturase and not the Δ6-desaturase which is known to be differently regulated, thus excluding the Δ6-desaturase from involvement in the observed reactions.

The enhanced rate of re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ observed in the presence of bromotrichloromethane, halothane, enflurane and methoxyflurane was not affected by the presence of CO, an inhibitor of cytochrome P-450. The effect of chloroacetaldehyde and 1,2-dibromo-1,2-dichloroethane however, appeared to be decreased in the presence of CO. Metyrapone, another cytochrome P-450 inhibitor, was without effect on the enhanced rate of re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ observed in the presence of bromotrichloromethane, halothane, enflurane and methoxyflurane.

The equilibrium constant (K_{eq}) for the stimulation of the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ by bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane, enflurane and methoxyflurane has been determined. No K_{eq} value was calculable for chloroacetaldehyde inasmuch as the rate constant for the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ was decreased in the presence of relatively low concentrations (< 10 mM) of this compound. The first order rate constants (k_{obs}) for the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ in the presence of bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane were found to be similar to the K_s values determined for their binding to cytochrome P-450. The K_{eq} for enflurane differed from the K_s and K_m values for the interaction of this anaesthetic 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 anaesthetic agent by cytochrome P-450.

The K_i values obtained for cyanide inhibition of the enhancement of the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ by the three halo compounds and the anaesthetic agents were within experimental error of the K_i value of 0,1 mM determined by Oshino *et al*. (1966) for cyanide inhibition of stearate desaturase activity.

Neither the halo compounds nor the anaesthetic agents had any effect on the conversion of stearoyl CoA to oleate by

hepatic microsomal stearate desaturase.

The effect of the anaesthetic agents, enflurane and methoxyflurane on the rate constant for the oxidation of purified, trypsin-cleaved ferrocytochrome $\underline{b}_5$ was examined. Neither enflurane nor methoxyflurane had any significant effect on the oxidation of ferrocytochrome $\underline{b}_5$.

Neither bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane nor chloroacetaldehyde had any significant effect on the activities of either the NADH- or the NADPH-cytochrome $\underline{c}$ reductases. Enflurane and methoxyflurane also appeared to be without significant effect on either of these reductases although enflurane slightly increased the activity of the NADH-cytochrome $\underline{c}$ reductase.

From several lines of evidence, it is concluded that bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane, enflurane and methoxyflurane stimulate hepatic microsomal electron flow through cytochrome $\underline{b}_5$ by interacting with stearate desaturase : the effects of these xenobiotics on the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ paralleled the dietary induction of stearate desaturase, the equilibrium constants determined for the re-oxidation of ferrocytochrome $\underline{b}_5$ in the presence of these xenobiotics differed from the K_s and K_m values for their interaction with cytochrome P-450 and the K_i for cyanide inhibition of the enhancement of the re-oxidation of ferrocytochrome $\underline{b}_5$ agreed with that observed for the

inhibition of stearate desaturase activity.

In view of these results, the metabolism of these xenobiotics by stearate desaturase and their ability to uncouple the stearate desaturase enzyme system was assessed. A reconstituted system, as described by Shimakata *et al.* (1972) comprising hepatic microsomal stearate desaturase, cytochrome b_5 and NADH-cytochrome b_5 reductase was employed to study the *in vitro* metabolism of the anaesthetic agents halothane, enflurane and methoxyflurane. Hepatic microsomes treated with either iodomethane or potassium thiocyanate to deplete cytochrome P-450 levels while maintaining elevated levels of stearate desaturase were employed to study the *in vitro* metabolism of the anaesthetic agents.

Utilizing the reconstituted and microsomal systems, it appears that the stearate desaturase enzyme system does not metabolize halothane, enflurane or methoxyflurane. No fluoride ion or acid-labile fluorine compounds were released from the anaesthetic agents. No bromide ions were released from halothane. No volatile metabolites from any of the anaesthetic agents could be detected by gas liquid chromatography. No non-volatile metabolites of halothane were detected using $[1\text{-}^{14}C]$-halothane.

The uncoupling of the stearate desaturase enzyme system by xenobiotics was assessed by determining the levels of active oxygen species in hepatic microsomes. The enhanced electron

flow through cytochrome b_5 observed in the presence of the halo compounds and anaesthetic agents was correlated with the production of hydrogen peroxide. Chloroacetaldehyde and bromotrichloromethane produced a transient increase in hydrogen peroxide production, while a significant sustained increase was observed in the presence of halothane, enflurane and methoxyflurane. The anaesthetic agents did not increase the production of superoxide anion in hepatic microsomes. The increase in hydrogen peroxide in the presence of the anaesthetic agents provided evidence that they uncouple the stearate desaturase enzyme system as they bind to, but are not metabolized by stearate desaturase.

The effects *in vivo* of halothane, enflurane, ether and chloroform on the stearate desaturase enzyme system, cytochrome P-450, S.G.O.T. and histology were examined. A comparison was made between halothane and enflurane which have been shown to interact with the stearate desaturase enzyme system *in vitro*, and ether and chloroform which do not interact. The levels of stearate desaturase were unaffected by repeated exposures to any of these anaesthetic agents. Cytochrome P-450 levels were decreased by halothane anaesthesia while ether anaesthesia had an inductive effect on the cytochrome. S.G.O.T. levels were significantly increased after chloroform anaesthesia only. Hepatotoxicity, as assessed histologically and by raised S.G.O.T. levels was not evident following anaesthesia by halothane, enflurane or ether. Chloroform however, produced gross centrilobular

hepatic necrosis. It is concluded that halothane and enflurane are not potentially hepatotoxic.

A similar study of the effects on hepatotoxicity of these anaesthetic agents and methoxyflurane administered under hypoxic conditions was undertaken. Anaesthesia under hypoxic conditions is known to produce centrilobular hepatic necrosis. Anaesthesia with N_2O/O_2 under hypoxic conditions had no detrimental effect on stearate desaturase activity, mortality or hepatic histology. Methoxyflurane and enflurane, under hypoxic conditions produced negligible hepatotoxicity, while chloroform produced gross centrilobular necrosis of the hepatocytes, raised S.G.O.T. and high mortality, independantly of the levels of stearate desaturase in these animals. Anaesthesia with halothane under hypoxic conditions had no effect on stearate desaturase activity or cytochrome P-450 levels. Halothane had a deleterious effect on animals exposed under hypoxic conditions, as evidenced by raised S.G.O.T. levels, mortality and hepatic histology. This effect appeared to be decreased in those animals having elevated levels of stearate desaturase. It is therefore possible that stearate desaturase could play a protective role against halothane toxicity.

LIST OF ABBREVIATIONS

CoA	: Coenzyme A
C.S.F.	: Cyanide sensitive factor
Ci	: Curies
dpm	: Disintegrations per minute
EDTA	: Ethylenediaminetetra acetic acid
GSH	: Reduced glutathione
I.U.	: International Units
NADH	: Nicotinamide adenine dinucleotide (reduced)
NADP	: Nicotinamide adenine dinucleotide phosphate
NADPH	: Nicotinamide adenine dinucleotide phosphate (reduced)
S.G.O.T.	: Serum glutamic oxaloacetic transaminase
Tris	: Tris(hydroxymethyl)methylamine
hrs	: hours
k_{obs}	: first-order rate constant
K_i	: spectrally determined inhibition constant
K_s	: spectrally determined dissociation constant
K_m	: Michaelis-Menten constant
MAC	: minimum alveolar concentration
min	: minutes
sec	: seconds
k_{eq}	: spectrally determined equilibrium constant

Statistical analysis = Student's $\pm$ test

Symbol	Meaning
*	differs significantly from control value, $P < 0,001$
†	differs significantly from control value, $P < 0,01$
‡	probably differs from control value, $P < 0,05$
§	differs significantly from differently treated samples, $P < 0,001$
¶	differs significantly from differently treated samples, $P < 0,01$
‖	probably differs from differently treated samples, $P < 0,05$

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1. INTRODUCTION

DRUG METABOLISM

The last forty years has seen great advances in the field of drug metabolism. Drug metabolism is the process whereby compounds foreign to the body, known generally as "xenobiotics" undergo enzymatic biotransformation, leading to the production of metabolites. This observation is especially true in the case of the volatile anaesthetic agents which were initially assumed to be chemically inert substances, which exerted their anaesthetic properties without being metabolized. The classic work of Haggard (1924) on the absorption, distribution and elimination of diethyl ether provided a basis for this hypothesis. There was one exception to this hypothesis, namely trichloroethylene which as early as 1939, was shown to be metabolized *in vivo* (Barrett and Johnston, 1939). More recently, hepatic necrosis following anaesthesia provided a stimulus for a re-examination of the possible *in vivo* metabolism of anaesthetic agents. In the early 1960's, Stier (1964) and Van Dyke (Van Dyke *et al.*, 1964a, 1964b, Van Dyke and Chenoweth, 1965) demonstrated that the volatile anaesthetic agent halothane ($CF_3CHBrCl$) could be converted *in vivo* to urinary metabolites. It has subsequently been determined that virtually all xenobiotics undergo enzymatic biotransformation and this has led to a new field in biochemistry, namely the study of xenobiotic metabolism and its consequences.

The metabolism of xenobiotics has been established to occur primarily in the liver, while the enzymes of other organs and tissues such as the kidney, lungs and skin usually play minor roles. The main function of drug metabolizing enzymes is to protect the body against the deleterious effects of xenobiotics. These enzymes function via a 2-step process : the first step being the introduction of more polar side chains such as hydroxyl groups into the molecule. This process often involves hydroxylations, oxidations, reductions and hydrolyses. Once the xenobiotics have been hydroxylated, less toxic intermediates result which are more polar molecules and hence more water soluble than the parent compounds. Besides hydroxylation by cytochrome P-450, the first step in the metabolism of xenobiotics may be catalyzed by other enzymes situated in the endoplasmic reticulum, cytosol and mitochondria of liver cells. For example, the oxidation of amines is catalyzed by enzymes such as mitochondrial monoamine oxidase, while the oxidation and reduction of alcohols, aldehydes and ketones is performed by cytosolic alcohol and aldehyde dehydrogenases. The hydration of epoxides is catalyzed by epoxide hydrase, an enzyme of the endoplasmic reticulum (Handbook of Experimental Pharamcology, 1971).

Once a xenobiotic has been metabolized by one or more of these hepatic enzymes, the second step in its metabolism is often conjugation with a small polar molecule such as

glucuronic acid (Dutton, 1971) or reduced glutathione (Chasseaud, 1976). For glucuronidation to occur, the xenobiotic or its metabolite is conjugated with glucuronic acid by a heterogenous group of enzymes, the glucuronyl transferases (Mulder, 1974). Similarly, compounds are conjugated with glutathione by glutathione-S-transferases (Chasseaud, 1976). The metabolites resulting from these enzymatic reactions may then be excreted in the urine via the kidney.

The major hepatic enzymes involved in the first step of the metabolism of xenobiotics are known collectively as cytochrome P-450 although there is abundant evidence that they are a group of enzymes rather than a single enzyme (Conney, 1967; Gillette, 1971). Cytochrome P-450 is a group of b type cytochromes containing apoprotein moeities of molecular weight, 44 000 to 54 000 daltons. The term cytochrome P-450 is derived from the phenomenon that the CO-difference spectrum of the ferrocytochrome exhibits an absorption peak at 450 nm (Omura and Sato, 1962).

The presence of this CO-binding pigment in mammalian liver microsomes was first reported by Klingenberg (1958) and Garfinkel (1958) and evidence for its hemoprotein nature was provided by Omura and Sato (1964). Cytochrome P-450 has been shown to be a terminal oxygen-activating hemoprotein and is one of a group of enzymes known collectively as the mixed function oxidases (Gillette et al., 1972).

This term is used to describe any enzyme utilizing one oxygen molecule in its reactions, one oxygen atom being incorporated into the substrate while the other oxygen atom is reduced to water.

For the cytochrome P-450 mediated hydroxylation reactions, NADPH, oxygen and substrate are required, usually in a 1:1:1 molar stoichiometry. Electrons from NADPH are transferred to cytochrome P-450 via a flavoprotein called NADPH-cytochrome c (P-450) reductase (Lu et al., 1976). Cytochrome b_5 may also be involved in the transfer of electrons from NADH or NADPH to cytochrome P-450 (Hildebrandt and Estabrook, 1971; Mannering et al., 1974; Archakov et al., 1975), but it is not an obligate intermediate electron carrier. Cytochrome b_5, when reduced by NADH can usually only provide the second electron to cytochrome P-450 (Hildebrandt and Estabrook, 1971; Mannering et al., 1974). The pathways for electron transfer to cytochrome P-450 are shown in Figure I :-

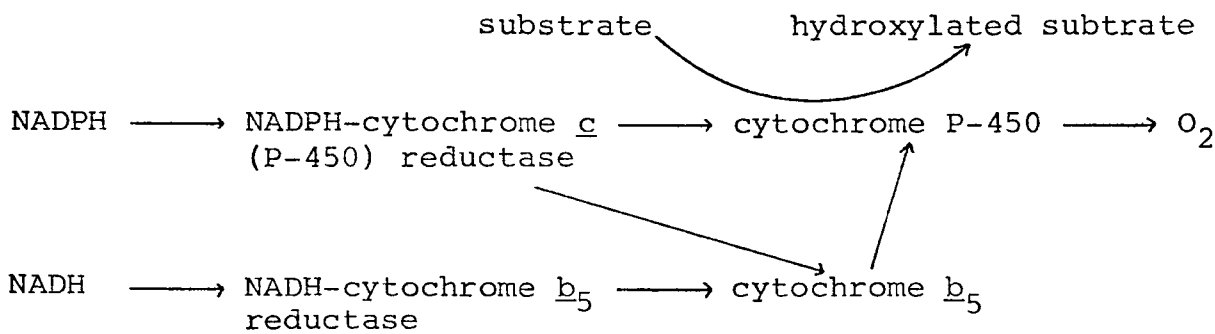


Figure 1 : Electron transport pathways in hepatic microsomes. (Straight arrows indicate electron flow).

The overall reaction catalysed by cytochrome P-450 is :-

$$NADPH + O_2 + H^+ + SH \longrightarrow NADP^+ + H_2O + SOH,$$

where SH indicates the substrate and SOH the hydroxylated substrate. The mechanism whereby cytochrome P-450 hydroxylates substrates is proposed to proceed as follows : (Estabrook et al., 1973; Coon et al.,1975; Dawson et al.,1976; Hrycay et al., 1976.)

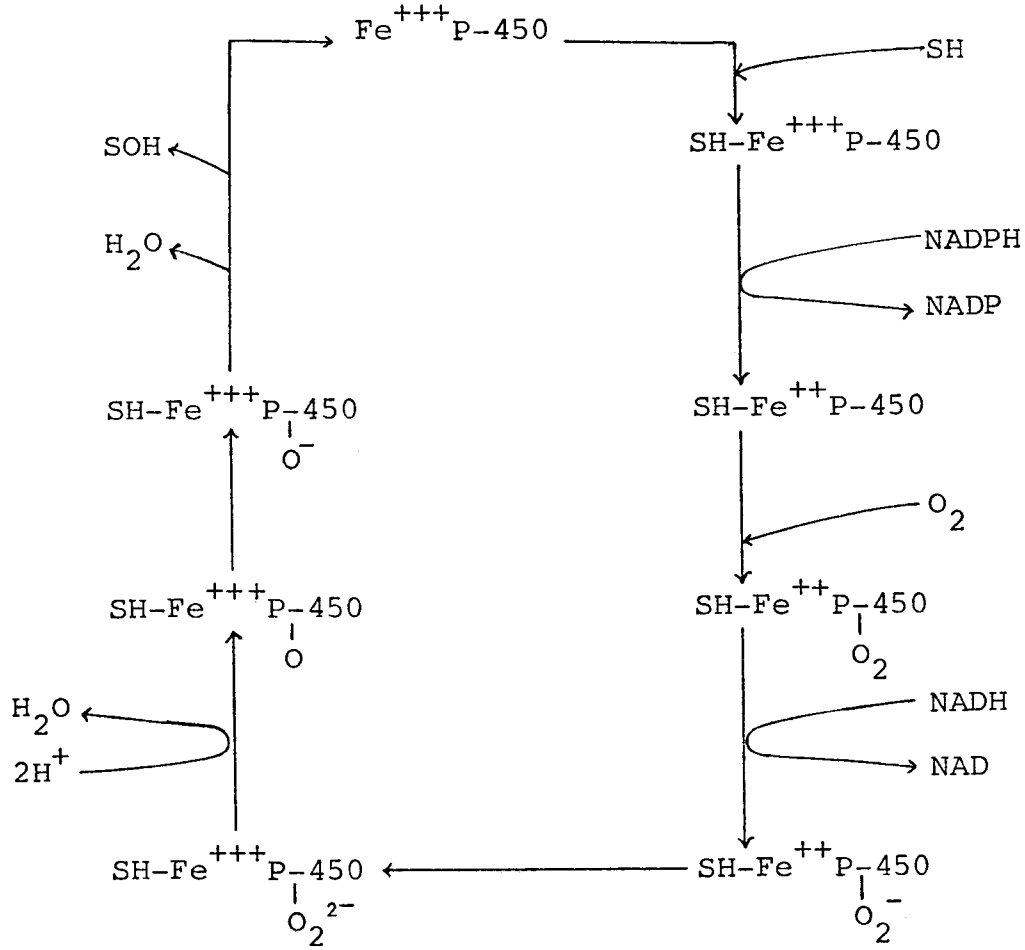


Figure 2 : Pathway of cytochrome P-450 catalyzed reactions.

SH indicates the substrate and SOH indicates the hydroxylated substrate.

Fe^{++} and Fe^{+++} indicate the oxidation state of the heme of cytochrome P-450.

While the metabolism of xenobiotics by these enzymes is generally advantageous in that it protects the body from the deleterious effects of some xenobiotics, it may also be detrimental to the body. Particularly, cytochrome P-450 mediated metabolites, which are usually hydroxylated compounds or derivatives thereof, are not always less toxic than the parent compounds. The metabolism of vinyl chloride by cytochrome P-450 gives rise to carcinogenic metabolites, probably chloroethylene oxide or chloroacetaldehyde (Kappus et al., 1975). The metabolism of the anaesthetic agent fluroxene by cytochrome P-450 not only gives rise to metabolites such as 2,2,2-trichloroethanol which are hepatotoxic, but also to reactive metabolites which are capable of chemically altering the heme of cytochrome P-450 apparently resulting in "suicide" inhibition of this important enzyme (Ivanetich et al., 1975; Ivanetich et al., 1976).

Cytochrome P-450 and several of the other drug metabolizing enzymes are situated in the endoplasmic reticulum. On homogenization of the liver, the endoplasmic reticulum is disrupted and segmented. The broken portions of the endoplasmic reticulum reseal to form vesicles which may be separated from the remaining cell debris by high speed centrifugation (Holtzman and Carr, 1972) or column chromatography (Tangen et al., 1973). The vesicles isolated from the endoplasmic reticulum are known as microsomes. They contain all of the enzymes originally present in the intact

endoplasmic reticulum and therefore provide a useful tool for investigations of drug metabolism.

The activities of microsomal drug metabolizing enzymes may be elevated in the livers of animals by pretreatment with various drugs, hormones or carcinogens such as the barbiturate phenobarbitone or the carcinogen 3-methylcholanthrene (Conney, 1967; Gillette, 1971). The increased enzymatic activity after treatment with these chemicals results from an increased level of the enzymes and is known as enzyme induction. Enzyme inducing agents provide an important tool for investigations of drug metabolism *in vitro* and *in vivo*.

Phenobarbitone is one of the most widely used inducing agents for cytochrome P-450 and other xenobiotic metabolizing enzymes. Phenobarbitone causes enhanced proliferation of the smooth endoplasmic reticulum of the hepatocytes which results in increased production of microsomal protein. In addition, phenobarbitone induces the levels of drug metabolizing enzymes such as cytochrome P-450, NADPH-cytochrome *c* reductase and cytochrome b_5 relative to the levels of total microsomal protein (Conney, 1967; Gillette, 1971).

The type of cytochrome P-450 induced by different chemicals differs. For example, the form of cytochrome P-450 elevated by phenobarbitone differs from that induced by 3-methylcholanthrene. The form of cytochrome P-450 induced by the

latter chemical is known as cytochrome P-448 because the CO-difference spectrum of the ferrocytochrome has a maximum absorption at 448 nm as opposed to the 450 nm absorption maximum found for the forms of cytochrome P-450 present in microsomes from phenobarbitone and uninduced rats (Mannering et al., 1969). The different forms of cytochrome P-450 differ in terms of their substrate specificity. Advantage has been taken of the different chemicals to induce different forms of cytochrome P-450 and this tool has been used extensively in studies on drug metabolism using hepatic microsomes.

METABOLISM OF ANAESTHETIC AGENTS

(1) Halothane.

Halothane, a volatile halogenated hydrocarbon of the structure $CF_3CHBrCl$, was introduced as an anaesthetic agent in 1956 (Bryce-Smith and O'Brien, 1956). It was the product of an extensive search for a non-explosive, non-flammable anaesthetic agent. Halothane has since become the most widely used anaesthetic agent in medical history due to the relative safety of its action, ease of administration, lack of emitic properties and rapid recovery characteristics.

The metabolism of halothane has been studied extensively. Up to 23% of the administered dose of halothane *in vivo* is excreted as non-volatile urinary metabolites

(Cascorbi et al., 1970) with the major urinary metabolite of halothane in man being trifluoroacetic acid (Stier, 1964; Van Dyke and Chenoweth, 1965, Rehder et al., 1967; Airaksinen et al., 1970; Cascorbi and Blake, 1971).

It has been proposed, but is disputed that trifluoroacetic acid is produced via trifluoroethanol and trifluoroacetaldehyde and it is known that trifluoroethanol is toxic (Blake et al., 1969; Airaksinen et al., 1970; Cohen, 1971). Bromide and chloride ions are other known halothane metabolites (Stier, 1964; Van Dyke et al., 1964b; Van Dyke and Chenoweth, 1965; Cohen, 1971).

Other urinary metabolites include trifluoroacetyl ethanolamine (Cohen, 1971), N-trifluoro-2-amino-ethanol and an N-acetyl-cysteine conjugate, identified as N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-cysteine (Cohen et al., 1975).

The initial step in the metabolism of halothane has been shown to occur in the microsomal fraction of the liver and to be mediated by cytochrome P-450 (Van Dyke, 1966; Karashima et al., 1977).

The metabolism of halothane by cytochrome P-450 was initially thought to be oxidative, but recent studies have shown that a reductive pathway involving cytochrome P-450 is also operative. Oxidative metabolism by cytochrome P-450 converts halothane to trifluoroacetic acid or to compounds which may react with phosphotidyl ethanolamine or glutathione, before being excreted in the urine (Figure 3).

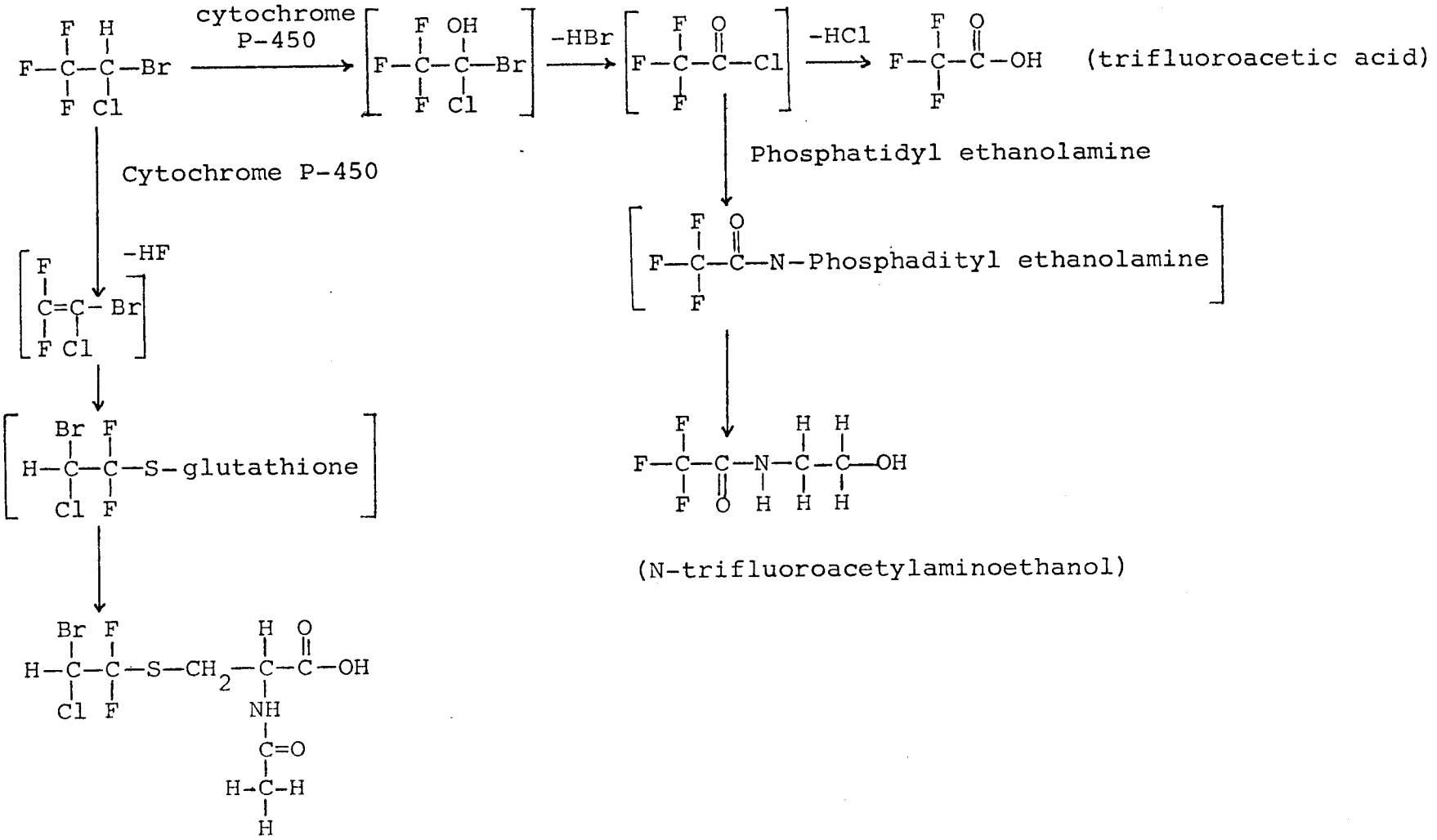
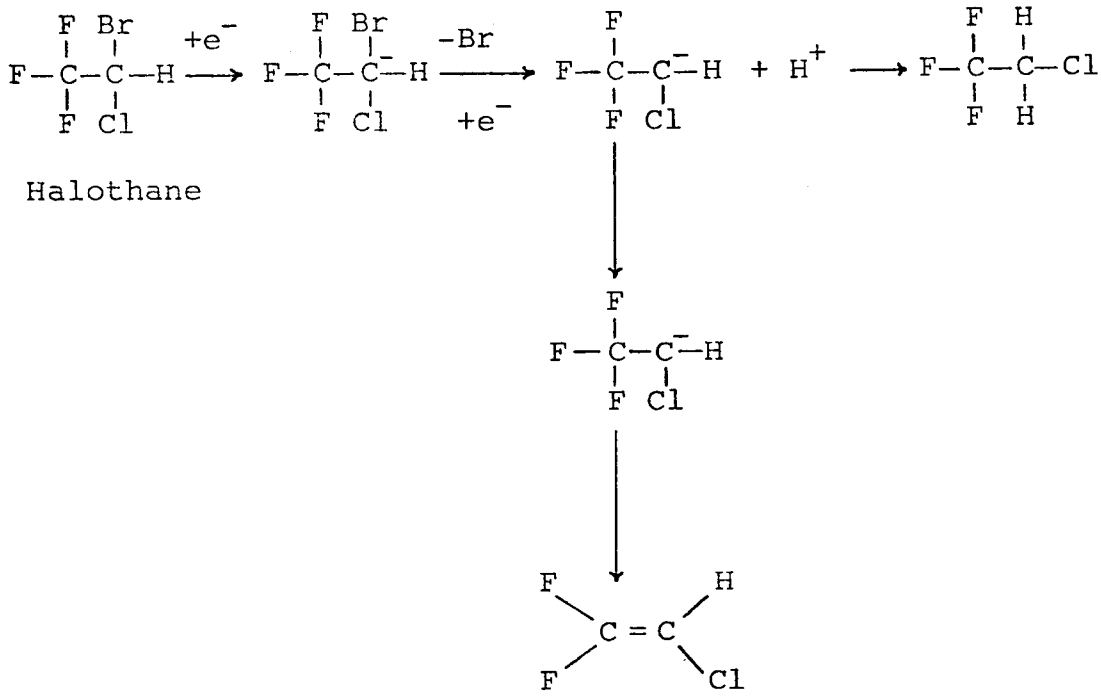


Figure 3 : Proposed pathway for the oxidative metabolism of halothane (Cohen et al., 1975).

The reductive metabolism of halothane, mediated by cytochrome P-450 occurs under hypoxic conditions. This pathway gives rise to volatile metabolites, two of which have recently been identified as CF_3CH_2Cl and CF_2=CHCl (Mukai et al., 1977). These volatile metabolites are thought to be produced via free radical and carbanion intermediates in the following manner (Sharp et al., 1979) :

Two electron reduction of halothane :



One electron reduction of halothane :

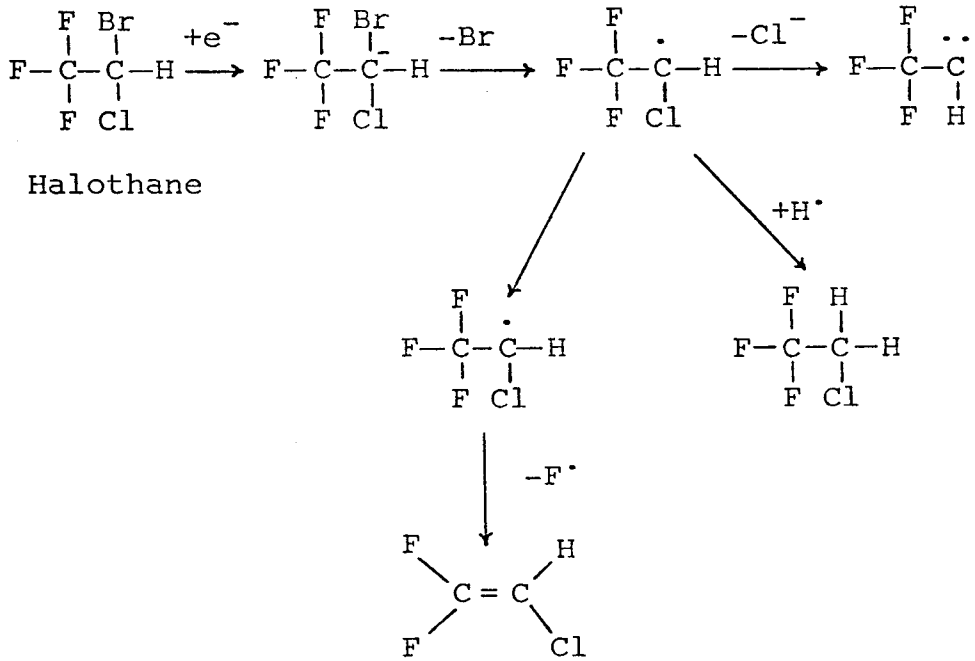


Figure 4 : Proposed pathways for the reductive metabolism of halothane by cytochrome P-450.

The observed hepatotoxicity following halothane anaesthesia in clinical practice, together with studies on its metabolism led to the realization that halothane was potentially hepatotoxic. Initially, halothane was not suspected to be the cause of the hepatotoxicity occasionally observed after halothane anaesthesia (Virtue and Payne, 1958). "Unexplained fever after halothane" or "halothane hepatitis" as the lesion has come to be called, is an extremely rare disorder and much controversy still surrounds it (The National Halothane Study, 1966). The lesion is characterized by fever occurring 2 to 3 days after exposure to halothane and is followed by jaundice (The National Halothane Study, 1966), chills and rashes (Klatskin and Kimberg, 1969; Klion et al., 1969) and anorexia leading to eventual coma (Trey et al., 1968; Peters et al., 1969). When examined microscopically, the livers of patients with halothane hepatitis reveal marked centrilobular necrosis, consistent with severe, chronic congestion (Burnap et al., 1958). Increases in serum enzymes also occur (Peters et al., 1969). Halothane hepatitis occurs more frequently in patients who undergo multiple exposures to halothane (The National Halothane Study, 1966; Klatskin, 1968; Trey et al., 1968), who are obese or aged (Peters et al., 1969) or have malignant disease (Simpson et al., 1971; Trowel et al., 1975). A similarity between halothane hepatitis and viral hepatitis has further complicated the understanding of halothane

hepatitis (The National Halothane Study, 1966; Klion et al., 1969). It has been suggested that a hypersensitive immune response to halothane or its metabolites may be the cause of the observed hepatitis (Belfrage, 1966; Klatskin and Kimberg, 1969).

Despite the controversy surrounding halothane, it is generally regarded as a safe anaesthetic agent; unexplained fever and hepatic necrosis have been observed to occur after exposure to other anaesthetic agents as well (Trey et al., 1968; Simpson et al., 1971). The unsatisfactory situation regarding halothane prevailing at present, coupled with the accumulating evidence that halothane is hepatotoxic, was a fundamental reason for the investigations of halothane reported in this thesis. To date, research into metabolism and hepatotoxicity of halothane has been confined mainly to the interaction of halothane with hepatic microsomal cytochrome P-450. Inasmuch as both the oxidative and reductive metabolic pathways of halothane already elucidated do not provide an explanation of the conditions of halothane hepatitis, the interaction of halothane with other microsomal enzymes becomes relevant. In this regard, the interaction of halothane with hepatic microsomal stearate desaturase was investigated.

(2) Enflurane.

Enflurane ($CClFHCF_2OCF_2H$) is a relatively new volatile

anaesthetic agent. It was first synthesised in 1963 and was introduced into clinical practice in 1966 (Dobkin et al., 1968). It is believed to be superior to many other currently used anaesthetic agents because of the virtual absence of toxic effects following enflurane anaesthesia ("Ethrane", 1972, Harrison et al., 1976).

In contrast to halothane, only about 2% of the administered dose of enflurane is detected in urine as fluoride ion and non-volatile organo-fluorine metabolites (Chase et al., 1971; Maduska, 1974; Cousins et al., 1976; Mazze et al., 1977; Corall et al., 1977), while 98% of the enflurane is excreted unchanged via the lungs. One mole of inorganic fluoride is proposed to be produced per mole of enflurane metabolised (Cousins et al., 1976).

The first step in the metabolism of enflurane appears to be mediated partly by cytochrome P-450. Enflurane is known to bind to the substrate binding site of cytochrome P-450 with the production of a type I difference spectrum (λ_{max} 386 nm; λ_{min} 419 nm). (Ivanetich et al., 1979). Evidence for the participation of cytochrome P-450 in the metabolism of enflurane is provided by the ability of enflurane to enhance the CO-inhibitable NADPH consumption by hepatic microsomes. The K_m for the oxidation of NADPH in the presence of enflurane has been determined to be approximately 2×10^{-4}M (Harrison et al., 1976; Ivanetich et al., 1979). It has been demonstrated that the metabolism of enflurane by

cytochrome P-450 gives rise to fluoride ion, but no acid-labile fluorine compounds could be detected (Ivanetich et al., 1979). Furthermore, enflurane is metabolized primarily by the form of cytochrome P-450 induced by phenobarbitone (Ivanetich et al., 1979).

The metabolism of enflurane has been proposed to occur via three pathways. These pathways are however, extremely tenuous and have been proposed in analogy to the pathways for the metabolism of methoxyflurane, inasmuch as the only metabolite of enflurane which has been identified is fluoride ion. The ratio of fluoride to inorganic fluoride detected in the urine of humans anaesthetised with enflurane suggested that dehalogenation of the beta carbon of the ethyl moeity of the enflurane molecule could occur (Chase et al., 1971; Cousins et al., 1976).

Cytochrome P-450 is proposed to catalyze the first step in each of the three pathways suggested for the metabolism of enflurane (Figure 5). The initial step in two of the pathways involves dehalogenation (Kuzava, 1973; Loew et al., 1974) while the third pathway involves O-dealkylation (Cousins and Mazze, 1974). It has been proposed that one pathway for the metabolism of enflurane proceeds via an initial dehalogenation and subsequent O-dealkylation to produce oxalic acid (Loew et al., 1974) (Figure 5, pathway 1). Enflurane has also been proposed to be metabolized by dehalogenation

to 1,1,2-trifluoro-2-hydroxyethyl difluoromethyl ether and subsequently by o-dealkylation to difluoromethoxy-difluoroacetic acid and finally to oxalic acid (Cousins and Mazze, 1974) (Figure 5, pathway 2). The third pathway involves o-dealkylation of enflurane to 2-chloro-1,1,2-trifluoroethanol and subsequently to chlorofluoroacetic acid and finally to oxalic acid (Cousins and Mazze, 1974) (Figure 5, pathway 3).

Although many substrates of cytochrome P-450 are inducing agents for this enzyme, enflurane does not appear to be one : exposure to enflurane at anaesthetic or subanaesthetic doses has been reported to be without effect on the levels or activities of several hepatic microsomal drug metabolizing enzymes (Marsh et al., 1979).

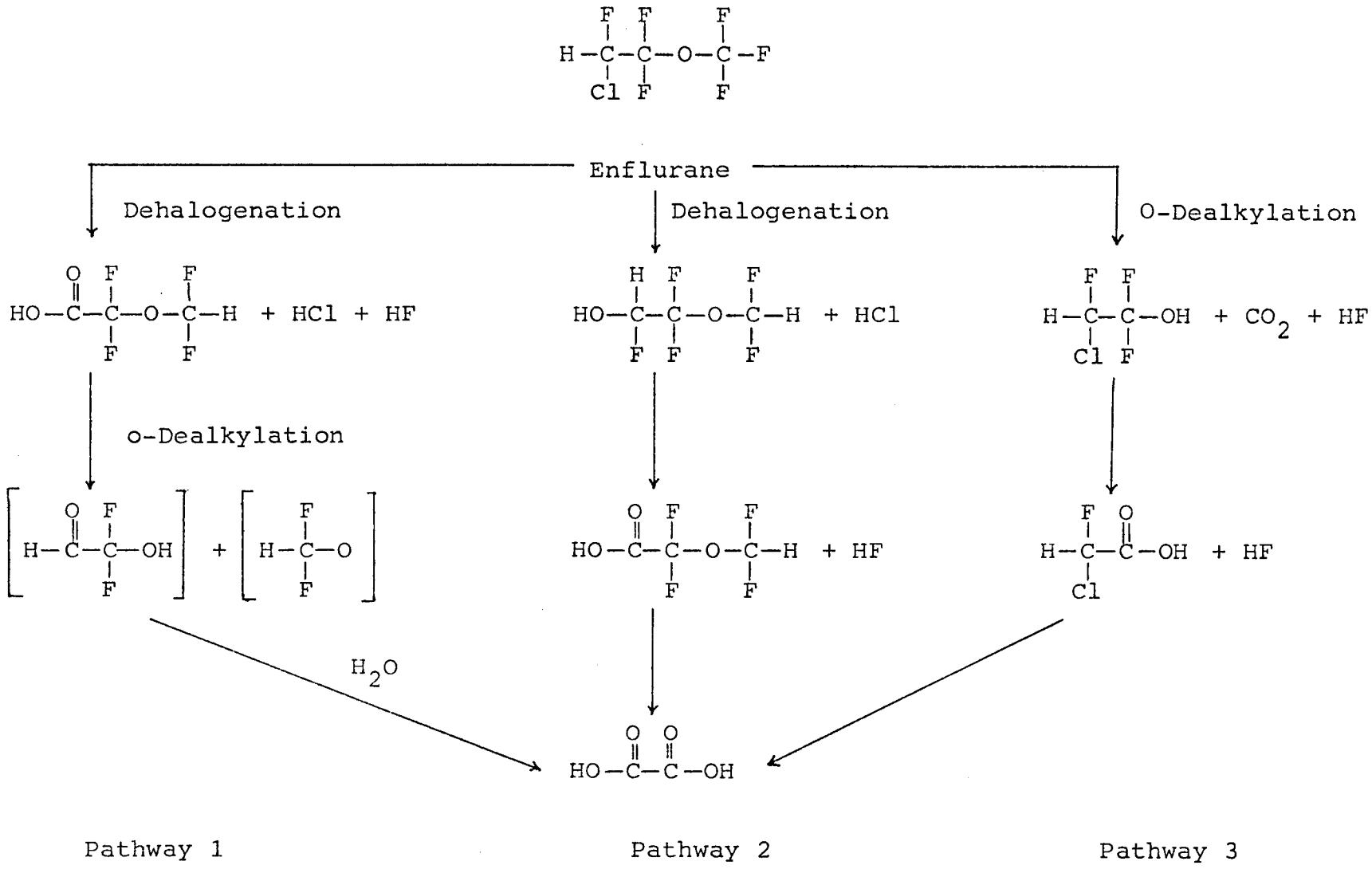


Figure 5 : Proposed pathways for the metabolism of enflurane.

The first step in each of the pathways is proposed to be catalyzed by cytochrome P-450.

Enflurane has not been implicated as an hepatotoxic or nephrotoxic drug ("Ethrane", 1972; Harrison et al., 1976), although it has been observed that exposure to enflurane can cause cell degeneration and necrosis (Van der Reis et al., 1974; Stevens et al., 1977). Renal dysfunction, similar to that produced by the anaesthetic agent methoxyflurane, has been rarely attributed to enflurane (Barr et al., 1974). Minimal, but reversible, changes in hepatic enzyme levels occur after exposure to enflurane (Thompson and Friday, 1978).

(3) Methoxyflurane.

Methoxyflurane ($CCl_2HCF_2OCH_3$) was introduced into clinical practice as a volatile anaesthetic agent in 1961 (Black and Clarke, 1971). It is non-flammable and is a good muscle relaxant, besides possessing excellent analgesic properties at low concentrations. About half of the methoxyflurane administered is exhaled unaltered in man and about 10% is converted to CO_2 which is also exhaled (Holaday et al., 1970). High levels of serum inorganic fluoride are also produced after exposure to methoxyflurane (Holaday et al., 1970; Taves et al., 1970; Fry et al., 1973; Brodeur et al., 1976). The urinary metabolites of methoxyflurane in man are inorganic fluoride, dichloroacetic acid and methoxydifluoroacetic acid which is the major urinary metabolite (Holaday et al., 1970; Yoshimura et al., 1976) (Figure 6). The ultimate metabolic product of methoxyflurane metabolism is proposed

to be oxalic acid (Lee Son et al., 1972) which may be produced via methoxydifluoroacetic acid and difluoro-hydroxyacetic acid (Mazze et al., 1971) (Figure 6).

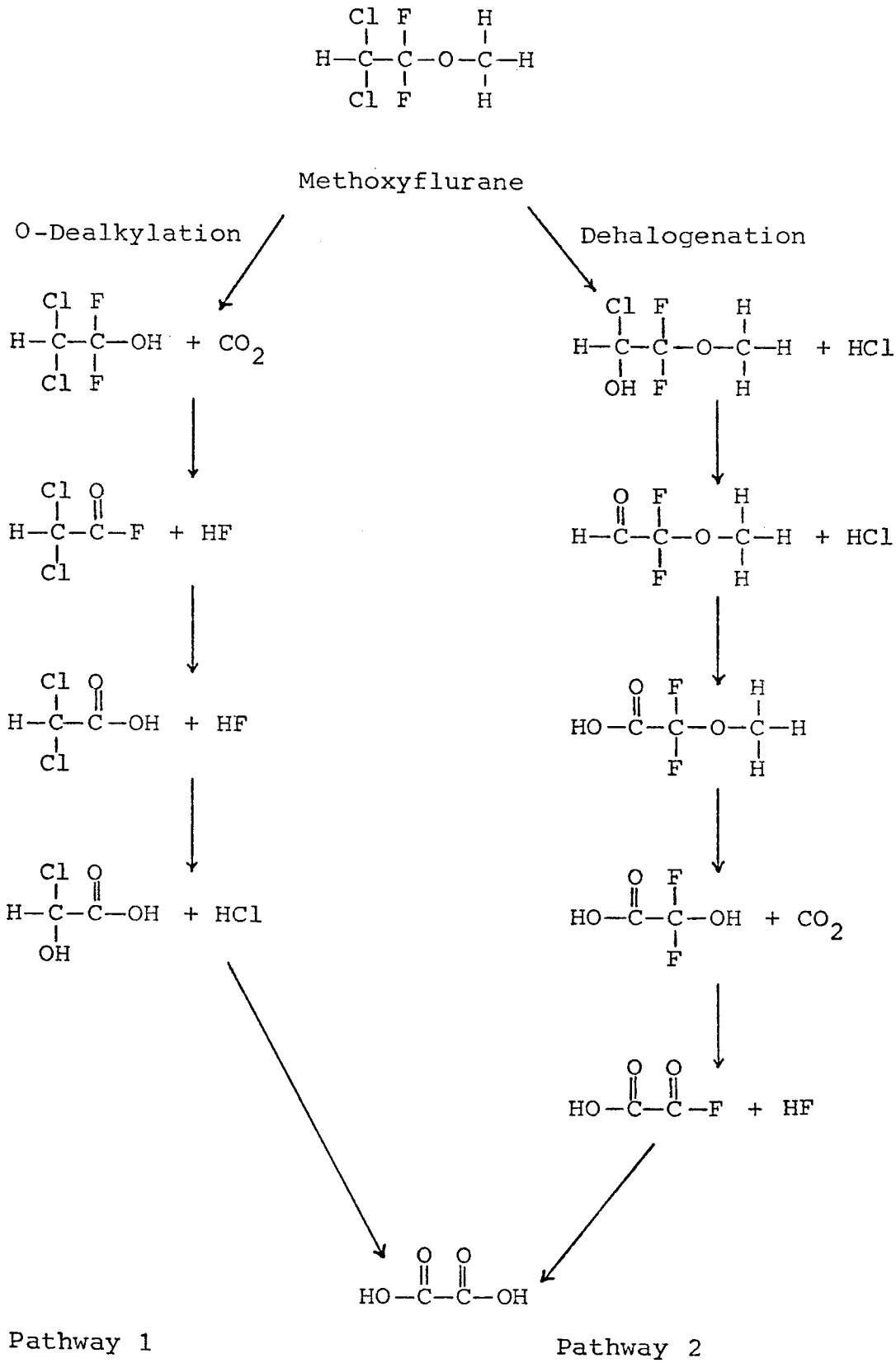


Figure 6 : Proposed pathways for the metabolism of methoxyflurane.

The first step in each of the pathways is proposed to be catalyzed by cytochrome P-450.

The metabolism of methoxyflurane is known to be mediated by cytochrome P-450. Methoxyflurane, like enflurane, binds to cytochrome P-450 with the production of a type I difference spectrum (Ivanetich et al., 1979). Methoxyflurane binds to the type of cytochrome P-450 induced by phenobarbitone and to at least one other form of this cytochrome, although not to the form of cytochrome P-450 induced by 3-methylcholanthrene (Ivanetich et al., 1979). Methoxyflurane has been found to stimulate the CO-inhibitable NADPH consumption by hepatic microsomes, and a K_m of $1 - 2 \times 10^{-4}$M has been determined for this process (Ivanetich et al., 1979). The metabolism of methoxyflurane by cytochrome P-450 gives rise to fluoride ion and to acid-labile fluorine compounds. The production of both fluoride ion and acid-labile fluorine compounds (i.e. methoxydifluoroacetic acid) from methoxyflurane in hepatic microsomes gave rise to biphasic kinetic data plots from which two sets of K_m and V_{max} values could be calculated (Ivanetich et al., 1979).

The metabolites of methoxyflurane are known to produce nephrotoxicity rather than hepatotoxicity and the degree of nephrotoxicity can be increased by phenobarbitone pretreatment (Lee Son et al., 1972; Brodeur et al., 1976). The urinary metabolite, oxalic acid, is also known to be a renal toxin (Lee Son et al., 1972; Hayler and Herman, 1973) while fluoride ions are also

responsible for nephrotoxicity (Mazze et al., 1971). The accumulated evidence against methoxyflurane has led to its being withdrawn from clinical use recently.

STEARATE DESATURASE ENZYME SYSTEM

Although hepatic microsomes contain some of the major enzymes involved in the metabolism of xenobiotics, they also contain a number of other enzymes which function primarily or exclusively in the metabolism of endogenous physiological compounds. An important member of this latter group of enzymes is stearate desaturase.

This enzyme system was identified by Bernhard et al. in 1959 and its components have since been isolated using detergent solubilization (Gurr and Robinson, 1970; Shimakata et al., 1972; Strittmatter et al., 1974; Safford et al., 1975). The isolated terminal oxidase consists of one polypeptide chain of 456 amino acid residues and has a molecular weight of 53 000 daltons. From amino acid analysis, it was determined that 62% of the amino acid residues are non-polar. Further, one equivalent of non-heme iron is associated with the desaturase enzyme (Strittmatter et al., 1974). Studies on the chemical modification of the desaturase enzyme indicate that several argenine and tyrosine residues may be directly involved in its action. The tyrosines may also participate in iron co-ordination, while the argenine residues are involved in ionic interactions (Enoch and

Strittmatter, 1978). Inasmuch as one of the major characteristics of stearate desaturase is its inhibition by cyanide, the terminal oxidase is also known as the "cyanide sensitive factor" or C.S.F. (Oshino et al., 1966). The main physiological function of stearate desaturase is the desaturation of CoA esters of fatty acids, converting them to Δ9-mono unsaturated fatty acids which are subsequently incorporated into biological membranes and are important in maintaining membrane fluidity. Stearate desaturase inserts a double bond between carbon atoms 9 and 10 of the long chain saturated fatty acid, stearic acid, converting it to the unsaturated fatty acid oleic acid (Holloway et al., 1963; Brett et al., 1971; De Gómez Dumm and Brenner, 1975). The enzyme shows a high degree of specificity for fatty acids with chain lengths of 16 - 18 carbon atoms (Paulsrud et al., 1970; Brett et al., 1971).

For enzymatic activity, stearate desaturase requires several components such as molecular oxygen and reduced pyridine nucleotides which serve as electron donors (Gellhorn and Benjamin, 1964; Oshino et al., 1966). NADH is the preferential electron donor (Jones et al., 1969; Joshi et al., 1977), but NADPH can also supply electrons. The purified enzyme has a lipid requirement (Holloway and Katz, 1972) in particular for phospholipids, triglycerides and fatty acids (Jones et al., 1969). Several proteins are known to be involved in the stearate desaturase enzyme system.

These are cytochrome b_5, NADH-cytochrome b_5 reductase and the cyanide sensitive factor itself.

It has recently been shown that several other protein factors enhance desaturase activity. A soluble protein with molecular weight of 24 000 has been shown to stimulate stearate desaturase activity *in vitro* (Jeffcoat *et al.*, 1976). A second factor required for desaturase activity has been isolated and found to be unstable to heat and tryptic digestion. This factor was concluded to be a specific protein and to be loosely bound to the microsomal membrane as it could be extracted with buffers of low ionic strength (Catalá *et al.*, 1975). Another heat sensitive protein factor which has a stimulatory effect on stearate desaturase has been identified as catalase (Baker *et al.*, 1976). Catalase appears to exert its stimulatory effect by preventing inactivation of stearate desaturase activity by hydrogen peroxide. A protein having a regulatory role in the desaturase reaction has recently been isolated from the cytosolic fraction of rat liver (Jones and Gaylor, 1979).

The overall reaction for the conversion of stearic acid to oleate is as follows : stearic acid is first converted to stearoyl CoA. Subsequently the stearoyl CoA is converted by the stearate desaturase enzyme system to oleate (Marsh and James, 1962; Masaro, 1968) :

Step 1 : stearic acid + CoA $\xrightarrow{ATP,\ Mg^{2+}}$ stearoyl CoA

Step 2 : (a) stearoyl CoA + enzyme $\xrightarrow{microsomes}$ stearoyl-enzyme + CoA

(b) stearoyl-enzyme $\xrightarrow{O_2,\ NAD(P)H}$ [hydroxystearoyl-enzyme]*

(c) [hydroxystearoyl-enzyme] $\xrightarrow{-H_2O}$ oleyl enzyme

(d) oleyl-enzyme + CoA $\longrightarrow$ oleyl-CoA + enzyme

* proposed intermediate, not proved.

The mechanism whereby a double bond is inserted between carbon atoms 9 and 10 of the 18 carbon chain by stearate desaturase is an intricate one : to insert the double bond, the two hydrogens must be in the cis configuration (Morris, 1970) and the substrate must be closely enfolded between C_5 - C_{15} and less closely enfolded at $C_{3,4,16}$ and $_{17}$. The substrate must then fit into a cleft in stearate desaturase which is 26 Å long by 4 Å wide, and to allow this, there must be a substantial conformational change in the enzyme substrate complex (Brett et al., 1971). It has been suggested that the methylene chain of stearoyl CoA assumes an "eclipsed" or gauche conformation at carbon atoms 9 and 10 of the enzyme substrate complex (Enoch et al., 1976). This could lead to rotation of the 9-10 carbon-carbon bond, bringing the 2 hydrogens together on the same

side of the chain in an eclipsed conformation. This allows a simultaneous, concerted removal of the 2 hydrogens by cis-elimination, this being the rate limiting step of the desaturase reaction (Morris, 1970; Brett et al., 1971; Enoch et al., 1976). There is no precedent for this reaction in model systems as the insertion of a double bond in a specific site along a carbon chain is unique.

A variety of compounds have been shown to enhance or decrease the activity of stearate desaturase. The enzyme is under dietary regulation : fasting alone diminishes desaturase activity (Inkpen et al., 1969; Lee and Sprecher, 1971; Oshino and Sato, 1972), while fasting followed by re-feeding a high carbohydrate diet induces stearate desaturase (Elovson, 1965; Inkpen et al., 1969; Oshino et al., 1971; Oshino and Sato, 1972) as does a fat-free diet (Holloway and Holloway, 1975; Holloway and Holloway, 1977; Jeffcoat and James, 1977). A variety of compounds stimulate desaturase activity by adaptive enzyme formation, including glucose (Donaldson, 1973), insulin (Gellhorn and Benjamin, 1964; Inkpen et al., 1969) and glycerol-3-phosphate (Raju and Reiser, 1972). Saturated fatty acids, fructose and glycerol also induce enzyme activity via increased protein synthesis (Mercuri et al., 1974; Jeffcoat and James, 1977). During induction of stearate desaturase increased enzymatic activity is due to an increase in the levels of the terminal oxidase of stearate desaturase, i.e. the cyanide sensitive factor (Oshino and Sato, 1972).

The best known inhibitor of stearate desaturase is cyanide (Oshino et al., 1966; Oshino et al., 1971) which, at concentrations of 0,5 mM, can cause full inhibition of the desaturase reaction in vitro (Joshi et al., 1977). Stearoyl CoA, at concentrations greater than 10 - 12 µM can cause substrate inhibition (Enoch et al., 1976; Jeffcoat et al., 1976; Joshi et al., 1977), while oleic acid can cause end-product inhibition. Linoleic acid, lysolecithin, deoxycholate and palmityl-DL-carnitine are also inhibitory (Uchiyama et al., 1967; Pande and Mead, 1968; Pande and Mead, 1970; Jeffcoat and James, 1978). Sterculate, a C19 fatty acid, has been found to inhibit stearate desaturase and equally effective inhibitors are cyclopropene fatty acids (Allen et al., 1967; Fogerty et al., 1972), which exert their inhibition by irreversible binding of the enzyme sulfhydryl group to the cyclopropene group (Raju and Reiser, 1967).

Stearate desaturase activity is decreased by compounds which are known to induce cytochrome P-450 in vivo such as the barbiturate phenobarbitone and the carcinogen 3-methylcholanthrene (Oshino and Sato, 1971; Montgomery and Holtzman, 1975). It is not known by what mechanism these drugs decrease the activity of stearate desaturase. Reduced enzymatic activity is observed following administration of ethionine (Lyman et al., 1970) cycloheximide and actinomycin (Oshino and Sato, 1972). Neoplastic tissue and tissue from copper deficient animals also have reduced

levels of stearate desaturase (Rao and Abrahams, 1975; Mercurio and De Tomás, 1978; Wahle and Davies, 1975).

Stearate desaturase appears to be identical to 4-methyl sterol oxidase (Gaylor and Mason, 1968; Siegfried and Gaylor, 1976). The 4-methyl sterol oxidase catalyzes the oxidation of the 4α-methyl group of sterols during the biosynthesis of cholesterol from lanosterol, resulting in the formation of a carboxylic acid (Gaylor et al., 1975). It is also suggested that the steroidal 4α-carboxylic acid is formed by methyl sterol oxidase from an hydroxymethyl intermediate, similar to the hydroxylated intermediate proposed for the action of stearate desaturase.

The properties of stearate desaturase and 4-methyl sterol oxidase are the same. Both enzymes require oxygen and reduced pyridine nucleotides (Gaylor and Mason, 1968; Siegfried and Gaylor, 1976). Both enzymes are inhibited by cyanide. Furthermore, fasting lowers the activity of both enzymes, while neither is inhibited by the inhibitors of cytochrome P-450, viz. CO and ethyl isocyanide (Oshino et al., 1966; Gaylor and Mason, 1968).

Microsomal electron transfer pathways.

Cytochrome b_5 plays a central role in hepatic microsomal electron transfer. It passes electrons from NADH and NADPH to stearate desaturase, cytochrome P-450 and oxygen (as shown in Figure 7) (Strittmatter et al., 1972; Oshino and Omura, 1973).

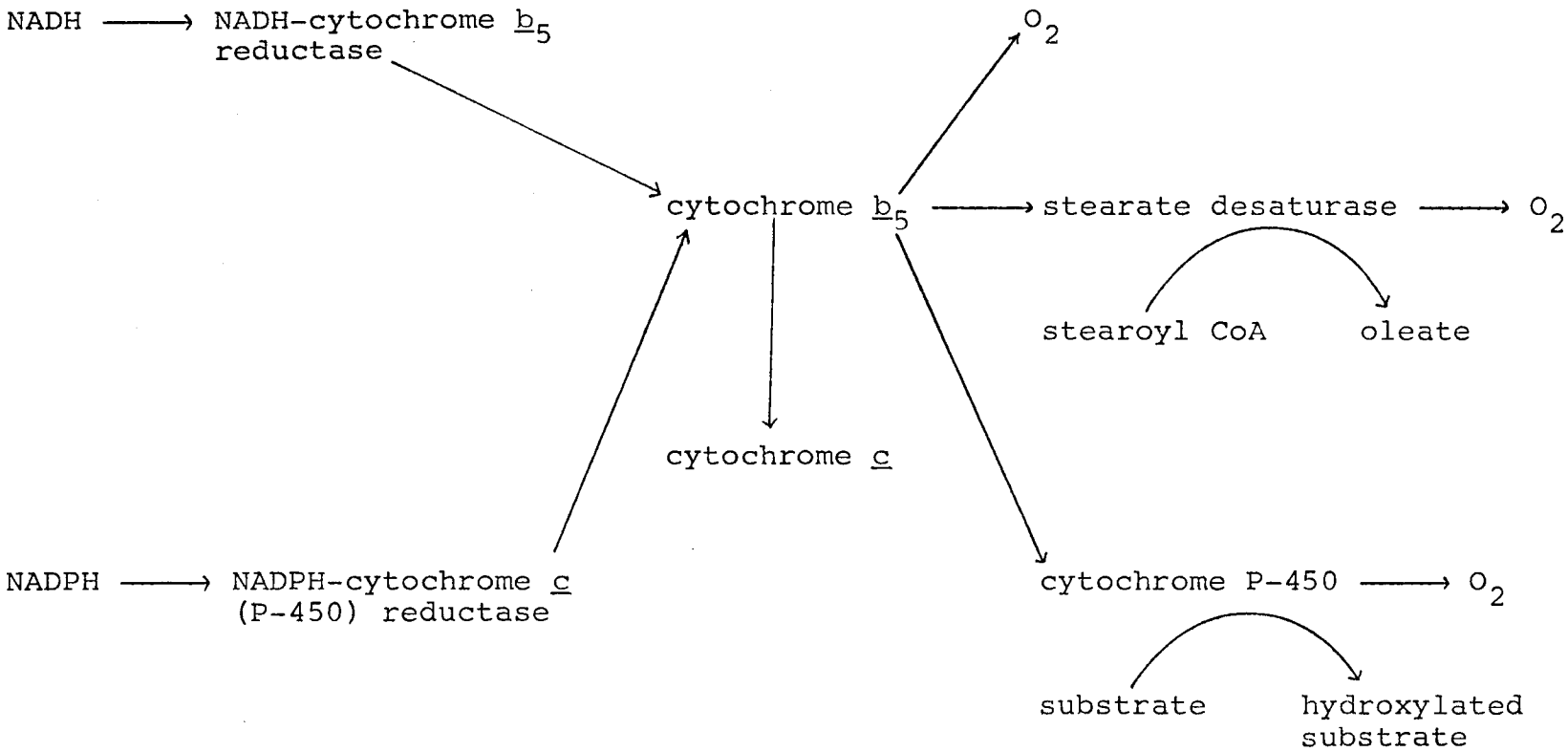


FIGURE 7 : Pathways for cytochrome b_5 electron transfer in hepatic microsomes.

(Cytochrome c is an artificial electron acceptor).

Straight lines indicate electron transfer.

Cytochrome $\underline{b}_5$ is an amphipathic molecule, whose hydrophobic portion attaches it to the microsomal membrane. This segment comprises amino acid residues near the amino terminal part of the molecule, while the hydrophilic end is exposed to the environment and is the enzymatically active part of the molecule (Spatz and Strittmatter, 1971; Rogers and Strittmatter, 1975). Cytochrome $\underline{b}_5$ has been sequenced (Ozols and Gerard, 1977) and contains a peptide chain of 141 amino acid residues per molecule of heme (Spatz and Strittmatter, 1971) with a molecular weight of 25 000 (Ito and Sato, 1968).

Proof that cytochrome $\underline{b}_5$ is involved in the transfer of electrons to stearate desaturase comes from several sources. Cytochrome $\underline{b}_5$ can accept electrons from NADH, which is the preferential electron donor for stearate desaturase, and from NADPH, which primarily provides electrons for cytochrome P-450 mediated reactions. Antisera prepared against the hydrophilic part of cytochrome $\underline{b}_5$ were found to inhibit the desaturase reaction, confirming the involvement of this section of cytochrome $\underline{b}_5$ in transferring electrons from NADH and NADPH to stearate desaturase (Oshino and Omura, 1973). Reconstitution experiments revealed that the absence of cytochrome $\underline{b}_5$ leads to complete loss of desaturase activity and restitution of activity was dependent on the amount of cytochrome $\underline{b}_5$ added, providing further evidence for its involvement (Shimakata *et al.*, 1972).

Electrons from NADPH are donated to cytochrome $\underline{b}_5$ via NADPH-cytochrome $\underline{c}$ (P-450) reductase in hepatic microsomes (Prough and Siler Masters, 1974; Enoch and Strittmatter, 1979). The NADPH-cytochrome $\underline{c}$ reductase is not as effective as the NADH-cytochrome $\underline{b}_5$ reductase in reducing cytochrome $\underline{b}_5$ and therefore in the presence of NADPH, cytochrome $\underline{b}_5$ is not fully reduced. NADPH-cytochrome $\underline{c}$ reductase is a membrane-bound protein and reduces cytochrome $\underline{b}_5$ directly. This reductase is capable of reducing cytochrome $\underline{c}$ indirectly, via cytochrome $\underline{b}_5$, with electron transfer between ferrocytochrome $\underline{b}_5$ and ferricytochrome $\underline{c}$ being rapid. The participation of NADPH-cytochrome $\underline{c}$ reductase in the desaturation of fatty acids has been demonstrated *in vitro* by Oshino (Oshino *et al.*, 1966; Oshino *et al.*, 1971.)

The reduction of cytochrome $\underline{b}_5$ by NADH is mediated via NADH-cytochrome $\underline{b}_5$ reductase (Okuda *et al.*, 1972; Rogers and Strittmatter, 1975). This reductase is an amphipathic molecule, whose ability to bind to the microsomal membrane is conferred by the hydrophobic segment of the molecule (Rogers and Strittmatter, 1975). The hydrophilic, catalytically active portion is therefore exposed to the cytoplasm (Spatz and Strittmatter, 1973). The reductase has a molecular weight of 200 000 and consists of six subunits each of molecular weight of 35 000 daltons. The hydrophobic portion of the peptide is responsible for the polymerization (Spatz and Strittmatter, 1972). The catalytically

active reductase is a more compact molecule than the inactive form and the flavin portion is in a more non-polar environment (Strittmatter, 1971).

It is believed that cytochrome $\underline{b}_5$ and NADH-cytochrome $\underline{b}_5$ reductase undergo translational diffusion in the plane of the microsomal membrane (Rogers and Strittmatter, 1974a; 1974b). Interactions between the lipid bilayer of the membrane and the hydrophobic portions of cytochrome $\underline{b}_5$ and NADH-cytochrome $\underline{b}_5$ reductase are essential for, and exert control over, the overall rate of reduction of cytochrome $\underline{b}_5$ by NADH. The amphipathic nature of these proteins is responsible for the lipid dependence of this interaction (Rogers and Strittmatter, 1973).

The mechanism of reduction of cytochrome $\underline{b}_5$ by NADH-cytochrome $\underline{b}_5$ reductase has been extensively investigated : it is proposed that cytochrome $\underline{b}_5$ interacts with NADH-cytochrome $\underline{b}_5$ reductase via complementary charge pair interactions involving the cytochrome $\underline{b}_5$ side chain carboxyls of glutamine 47, 48 and 52, the single exposed heme propionate and a 5th, as yet unidentified, side chain carboxyl group (Dailey and Strittmatter, 1979). Once cytochrome $\underline{b}_5$ has been reduced by NADH, and the NADH is exhausted, cytochrome $\underline{b}_5$ autoxidizes via first order kinetics. The autoxidation of cytochrome $\underline{b}_5$ involves the transfer of electrons from ferrocytochrome $\underline{b}_5$ directly to oxygen to produce water and superoxide and regenerates

ferricytochrome b_5 (Boveris et al., 1972; Berman et al., 1976). It has been shown that the re-oxidation of cytochrome b_5 is enhanced in the presence of a substrate for stearate desaturase. Furthermore, it has been demonstrated by Oshino et al., (1971) that this enhanced re-oxidation of cytochrome b_5 is directly proportional to the activity of stearate desaturase. This phenomenon is therefore the basis for a simple assay of stearate desaturase activity, as opposed to the more involved assay utilizing ^{14}C-stearoyl CoA.

Stearate desaturase and xenobiotics.

Stearate desaturase is generally not thought to interact with xenobiotics, and there have been very few reports of such interactions. It was first demonstrated by Oshino and Sato (1971) that p-cresol, together with several other phenolic compounds, enhanced the re-oxidation of NADH reduced cytochrome b_5 analagous to the enhancement seen in the presence of stearoyl CoA (Oshino et al., 1971). This stimulated rate of re-oxidation of cytochrome b_5 was inhibited by cyanide, suggesting involvement of stearate desaturase. Confirmation of the involvement of cytochrome b_5 in this reaction occurred when the rate of p-cresol stimulation of the re-oxidation of cytochrome b_5 was inhibited by antibodies to cytochrome b_5 (Oshino and Omura, 1973).

The first report on the interaction of an anaesthetic agent

with stearate desaturase was that of Berman et al., (1975). Halothane was found to accelerate microsomal electron transfer by interacting with the C.S.F. of stearate desaturase. Halothane accelerated stoichiometric consumption of NADPH and oxygen and increased the rate of re-oxidation of NADH reduced cytochrome b_5. It was confirmed that the observed effects of halothane on microsomal electron transfer were not due to interaction with cytochrome P-450 (Berman et al., 1975).

General aims.

This thesis covers a detailed investigation of the following :

(1) The interaction of xenobiotics, in particular the volatile anaesthetic agents halothane, enflurane and methoxyflurane and three selected halogenated xenobiotics, bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde with hepatic microsomal stearate desaturase was investigated. It was initially attempted to establish whether the xenobiotics might interact with stearate desaturase by monitoring their abilities to stimulate microsomal electron transfer via cytochrome b_5, namely their effects on the NADPH redox steady state of cytochrome b_5 and on the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome b_5, inasmuch as all xenobiotics known to interact with stearate desaturase enhance the re-oxidation of cytochrome b_5.

(2) It was attempted to establish whether the bound xeno-

biotics were metabolized by stearate desaturase or whether xenobiotics uncoupled the stearate desaturase enzyme system. A reconstituted system comprising NADH-cytochrome $\underline{b}_5$ reductase, cytochrome $\underline{b}_5$ and C.S.F. was employed, as well as hepatic microsomes having elevated levels of stearate desaturase activity, but artificially reduced levels of cytochrome P-450 to assess the metabolism of the anaesthetic agents.

(3) The ability of the levels of stearate desaturase to have an effect on the capacities of the anaesthetic agents to exert an hepatotoxic effect on the liver was examined <u>in vivo</u>.

2. EXPERIMENTAL

A. MATERIALS

(1) Pretreatment of animals.

Sodium phenobarbitone was supplied by Maybaker, Port Elizabeth, E.P., South Africa. The vitamin mixture used for the dietary induction of stearate desaturase was constituted from vitamins received as a gift from Roche (Pty) Ltd., Isando, Transvaal, South Africa. Dextrin was supplied by Merck Chemicals, Darmstadt, Germany and by Sigma Chemicals. Choline chloride and cellulose were obtained from B.D.H. Chemicals Ltd., Poole, England. Casein was purchased from Merck Chemicals, Darmstadt, Germany.

(2) Anaesthetic agents.

Halothane (fluothane) was obtained from Halocarbon Laboratories Inc., Hackensack, N.J., U.S.A. Enflurane (ethrane) and methoxyflurane (penthrane) were supplied by Abbott Laboratories, Aeroton, Transvaal, South Africa. Diethyl ether was obtained from the Natal Cane By-Products Ltd., Merebank, Natal, South Africa. Chloroform was purchased from Maybaker Ltd., Dagenham, England.

(3) Assays on hepatic microsomes.

NADH, NADPH, NADP and cytochrome c (horse heart) and

glucose-6-phosphate dehydrogenase were obtained from Miles Laboratories, Cape Town, South Africa. Stearoyl CoA and [1-^{14}C]-stearoyl CoA were supplied by Sigma Chemicals, Poole, England and New England Nuclear, Boston, Mass., U.S.A. Potassium cyanide and sodium dithionite were obtained from B.D.H. Chemicals Ltd., Poole, England. Glucose-6-phosphate was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

(4) Xenobiotics.

The xenobiotics used in the survey were obtained as follows : Allylbromide, 1-bromo-2-chloroethane, chloroacetaldehyde, 1,2-dibromo-1,1-dichloroethane, ethyl vinyl ether and 1,1,2-trichloroethane were from Fluka Chemicals, Buchs, Switzerland. Allyl chloride, bromoethane, carbon tetrachloride, diethyl ether, iodoform, chloromethyl methyl ether, 1,2-dichloroethane, bromoform, 2,2,2-trichloroethanol, trifluoroacetic acid and 2,2,2-trifluoroethanol were from Merck Chemicals, Darmstadt, Germany. Bromotrichloromethane and 1,1,1-trichloro-2,3-epoxypropane were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A. Chloroform, divinyl ether and ethyl iodide were purchased from Maybaker Ltd., Port Elizabeth, E.P., South Africa. 1-chloropropane and 1,2,3-trichloropropane were supplied by Eastman Organic Chemicals, Rochester, New York, U.S.A. Dibromomethane, 1,2-dibromopropane, dichloromethane, diiodomethane,

iodomethane, 1,1,2,2-tetrabromomethane, 1,1,1- and 1,1,2-trichloroethane and trichloroethylene were from B.D.H. Chemicals Ltd., Poole, England. 1,2-dibromo-1,2-dichloroethane, 1-fluorobutane, perfluorohexane, tetrabromomethane, tetraiodomethane and 2,2,2-trifluoroacetaldehyde hydrate were from ICN Pharmaceuticals Inc., Plainview, New York, U.S.A. Fluroxene was from Ohio Medical Products, Madison, Wisconsin, U.S.A. 1,2,2-trichloro-1,1,2-trifluoroethane (freon 113) was a gift from Anglo American Co., Cape Town, South Africa. Cylinders of vinyl chloride and other compressed gases were from Afrox Ltd., Cape Town, South Africa. 2,2,2-trifluoroethyl ethyl ether was synthesized as described earlier (Ivanetich et al., 1975).

Trypsin-cleaved cytochrome b_5 was purified from rat liver microsomes by the method of Omura and Takesue (1970).

(5) Inhibitors.

SKF 525A(2-diethylaminoethyl-2,2-diphenyl valerate) was a gift from Smith Kline and French Laboratories, Isando, Transvaal, South Africa. Metyrapone (2-methyl-1,2-bis-[3-pyridyl]-1-propanone) was a gift from Ciba-Geigy Ltd., Basle, Switzerland.

(6) Isolation of hepatic microsomal stearate desaturase.

Sodium deoxycholate and Triton X-100 were obtained from B.D.H. Chemicals Ltd., Poole, England. Reduced glutathione was obtained from Sigma Chemicals, Poole, England.

(7) Treatment of hepatic microsomes.

Dioxane, iodomethane, iso-butanol and sodium iodide were purchased from B.D.H. Chemicals, Ltd., Poole, England. Potassium thiocyanate was obtained from Merck Chemicals, Darmstadt, Germany.

(8) Gas liquid chromatography.

Di-iso-decylphthalate was supplied by Applied Science Laboratories Inc. Chromosorb P (acid-washed) and WHP Chromosorb/SE 30 were supplied by Johns-Manville, Denver, Colorado, U.S.A. Chromosorb 102 was purchased from Chemlab (Pty) Ltd., Cape Town, South Africa.

B. METHODS

(1) Treatment of animals.

Male Long-Evans rats weighing 250 - 300 g were used. Rats were fed routinely on a laboratory diet of Epol Laboratory Chow [protein (min. 20%); fat (2,5%); fibre (max. 6%); calcium (1,4%) and phosphorus (0,7%)] manufactured by Epol Ltd., Goodwood, Cape Town, South Africa. This diet is referred to throughout as the normal diet.

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. 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), 0,5 g; niacin, 7,5 g; 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 two days, fasted on the third day and re-fed the diet for two days (Oshino *et al*. 1971). The rats were sacrificed and experiments performed on the sixth day, unless otherwise indicated.

Where indicated, animals were treated with sodium pheno-

barbitone (80 mg/kg/day) by intraperitoneal injection for three days and were fasted overnight prior to sacrifice, in order to elevate the levels of hepatic microsomal cytochrome P-450.

(2) Isolation of hepatic microsomes.

Rats were sacrificed by cervical dislocation, the livers of three rats were excised and pooled and hepatic microsomes were isolated by differential ultracentrifugation as described by Holtzman and Carr (1972). The microsomal pellet was resuspended at a protein concentration of 1,5 mg protein/ml 0,02 M Tris-HCl, pH 7,4, unless indicated otherwise. The microsomes were in all cases used within four hours of preparation.

(3) Determination of protein.

The protein concentration of the hepatic microsomes was determined by the method of Lowry et al. (1951) as modified by Chaykin (1966), using bovine serum albumin as standard. A modified procedure described by Petersen (1977) was used to determine the protein concentration of purified stearate desaturase.

(4) Determination of hepatic microsomal cytochrome P-450.

Cytochrome P-450 concentrations were determined from measurements of the difference spectrum of CO-ferro-

cytochrome P-450 versus ferrocytochrome P-450, according to the method of Omura and Sato (1964). An extinction coefficient of 91 $mM^{-1}cm^{-1}$ for the difference in absorbance between 450 nm and 490 nm was utilized (Omura and Sato, 1964).

(5) Redox steady state of microsomal cytochrome b_5 in the presence of NADPH.

The redox steady state 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.* (1971). The results are expressed as the percentage reduction of hepatic microsomal cytochrome b_5 in the presence of NADPH relative to dithionite reduced cytochrome b_5. The xenobiotics, when present, were added to 3 ml of microsomal suspension (1,5 mg protein/ml) and vortex mixed for 30 seconds prior to the addition of NADPH (0,15 mM).

(6) Re-oxidation of NADH reduced microsomal cytochrome b_5.

The pseudo first order re-oxidation of microsomal ferrocytochrome b_5 was monitored spectrally at 409 nm and 424 nm by the method of Oshino *et al.* (1971). The xenobiotics, when present, were added to 3 ml of hepatic microsomes (1,5 mg protein/ml) and vortex mixed for 30 seconds. The reaction was initiated by the addition of NADH (1-5 µM).

For the determination of the effects of selected xenobiotics on k_{obs}, the rate constant for the re-oxidation of NADH reduced hepatic microsomal cytochrome $\underline{b}_5$, reaction mixtures were prepared as described above. For the determination of the K_i values for cyanide inhibition of the stimulation of the re-oxidation of microsomal cytochrome $\underline{b}_5$ by xenobiotics, reaction mixtures were prepared as described above, except that cyanide was added to the microsomal suspension before the xenobiotic.

(7) NADH- and NADPH-cytochrome c reductase assays.

The method of Omura and Takesue (1970) was used to determine the activities of hepatic microsomal NADH-cytochrome c reductase and NADPH-cytochrome c reductase. The xenobiotics, when present, were suspended in 2,10 ml 0,1 M Tris-HCl, pH 7,4, by vortex mixing for 30 seconds prior to the addition of ferricytochrome c, (0,45 mg/ml) NAD(P)H (0,084 mg/ml) and hepatic microsomes (1,5 mg protein/ml). The increase in absorbance of ferrocytochrome c at 550 nm was monitored (ε_{550nm} = 21,1 $mM^{-1}cm^{-1}$).

(8) Binding to cytochrome P-450.

Three ml of microsomal suspension (2 mg protein/ml) prepared from fasted or phenobarbital induced rats was added to each of two cuvettes. Spectra were

recorded before and after the addition of increasing amounts of xenobiotic to the sample cuvette. The xenobiotic was, where necessary, dispersed in the microsomal suspension by vortex mixing for 30 seconds. Absorbance difference spectra were recorded between 360 nm and 450 nm.

(9) Oxidation of purified trypsin-cleaved ferrocytochrome $\underline{b}_5$.

Purified trypsin-cleaved cytochrome $\underline{b}_5$ was reduced by a modification of the method of Smith (1955) : Purified ferricytochrome $\underline{b}_5$ 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 to 2 hours to convert ferricytochrome $\underline{b}_5$ to the ferrous form. Aliquots of ferrocytochrome $\underline{b}_5$ were then removed from the reducing suspension, filtered through an 8µ millipore filter, and 30 µl of the resultant solution (ca. 25 µM ferrocytochrome $\underline{b}_5$) was added to 1,25 ml of air equilibrated 0,1 M Tris-HCl, pH 7,4, in the presence or absence of the anaesthetic agents. The oxidation of purified trypsin-cleaved ferrocytochrome $\underline{b}_5$ was monitored spectrally at 424 nm.

(10) Determination of stearate desaturase activity.

The activity of stearate desaturase was assayed via the conversion of $[1\text{-}^{14}C]$-stearoyl CoA to $[1\text{-}^{14}C]$-oleate

essentially by the method of Oshino et al. (1966). Incubation mixtures contained hepatic microsomes (1,0 mg protein), NADH (1 mM) and [1-^{14}C]-stearoyl CoA (40 mM, 12 µCi) in 0,5 ml 0,1 M Tris-HCl, pH 7,25. Incubations were at 37°C for 4 minutes at 60 cycles per minute.

To determine the activity of purified stearate desaturase, one unit of purified NADH-cytochrome b_5 reductase, 0,5 nmoles cytochrome b_5 and 0,07 mg of purified C.S.F. protein were incubated with [1-^{14}C] -stearoyl CoA (40 mM, 12 µCi) in 1 ml 0,30 M Tris-HCl, pH 7,0 (Shimakata et al., 1972). The reaction was initiated by the addition of NADH (1 mM). Incubations were at 37°C for 20 minutes, with shaking at 60 cycles per minute. On completion of the incubation period, 2 mg each of carrier oleate and stearate were added to the reaction mixture. The fatty acids were methylated with $BF_3 \cdot CH_3OH$ by the method of McIntosh et al. (1977), and the methyl esters were separated by argentation thin layer chromatography on silica gel plates (25 cm × 25 cm × 0,25 mm) (Merck Chemicals) according to Berman et al. (1975). Scrapings from the thin layer chromatograms were assayed for radioactivity in 7 ml Instafluor scintillation cocktail (Packard) using a Beckman model LS 8100 liquid scintillation counter. The results of the assay were expressed as the percentage of

$$\frac{\text{radioactivity in oleate}}{\text{radioactivity in oleate + stearate}}$$

(11) Isolation of hepatic microsomal stearate desaturase, cytochrome b_5 and NADH-cytochrome b_5 reductase.

Hepatic microsomal stearate desaturase, cytochrome b_5 and NADH-cytochrome b_5 reductase were solubilized with the use of detergents essentially as described by Shimakata et al. (1972) :

Hepatic microsomes were isolated from a group of 15 rats which had been re-fed a high carbohydrate diet, by differential centrifugation (Holtzman and Carr, 1972). The freshly isolated hepatic microsomes were suspended at a protein concentration of 10 mg/ml 0,1 M Tris-HCl, pH 8,0 containing sodium deoxycholate (1%), Triton X-100 (1%), reduced glutathione (2 mM) and EDTA (5 mM). After stirring for 30 minutes, the suspension was centrifuged at 105 000 g, for 120 minutes. The supernatant was subjected to ammonium sulphate fractionation as described by Mihara and Sato (1972). The red pellicle collected between 25 - 45% saturation was then dissolved in a small volume of 20 mM Tris-HCl, pH 8,0, containing reduced glutathione (2 mM) and dialysed for two hours against two litres of the same buffer. The dialysed solution (5 ml) was diluted 5-fold with 20 mM Tris-HCl, pH 8,0, containing Triton X-100, (0,5%) reduced glutathione (2 mM), EDTA (2 mM) and KCl (5 mM). The solution was then chromatographed on a DEAE-Sephadex A-50 column (2 × 15 cm) which had been equilibrated with the same buffer. Elution was achieved

by the stepwise increases in the final KCl concentration of the buffer from 5 mM to 55, 105, 205 and 305 mM. Fractions of 10 ml were collected. The flow rate was 1 ml/minute. The fractions containing stearate desaturase, cytochrome $\underline{b}_5$ and NADH-cytochrome $\underline{b}_5$ reductase were concentrated to approximately 15 ml each using a Diaflo ultrafiltration apparatus fitted with a PM10 membrane. The resulting protein solutions were stored at -20°C until use, in all cases within 3 months of preparation.

(12) Determination of purified detergent solubilized cytochrome $\underline{b}_5$.

The concentration of purified detergent solubilized cytochrome $\underline{b}_5$ was determined by the method of Shimakata et al. (1972) from the intensity of the Soret band of ferrocytochrome $\underline{b}_5$ in the presence of sodium dithionite ($\varepsilon_{424nm} = 117\ mM^{-1}cm^{-1}$).

(13) Determination of the activity of detergent solubilized NADH-ferricyanide reductase.

The activity of detergent solubilized NADH-ferricyanide reductase was determined by the method of Mihara and Sato (1972). The reaction mixture contained 200 µl of enzyme and K_3FeCN_6 (1 mM) in a final volume of 1,5 ml, 0,1 M potassium phosphate, pH 7,5. The reaction was initiated by the addition of NADH (0,2 mM) and the decrease in

absorbance at 420 nm was monitored. (ε_{420nm} = 1,02 $mM^{-1}cm^{-1}$). One unit of activity corresponds to 1 µmole of ferricyanide reduced per minute.

(14) Xenobiotic concentration.

The concentration at which each xenobiotic was routinely utilized, was the highest concentration which would not disrupt the microsomal suspension (assessed visually) or convert hepatic microsomal cytochrome P-450 to cytochrome P-420 (assessed spectrally). Suspensions of hepatic microsomes (1,5 mg protein/ml) were divided equally between two 1-cm path length cuvettes. The xenobiotic was introduced below the surface of the microsomal suspension in the sample cuvette with a Hamilton µL syringe. The cuvette was then stoppered and vortex mixed for 30 seconds to disperse the added xenobiotic.

(15) Reconstitution of detergent solubilized stearate desaturase enzyme system.

The reconstitution of stearate desaturase activity was achieved essentially as described by Shimakata et al. (1972). The reconstituted system contained 0,06 - 0,07 mg purified stearate desaturase protein, one unit of purified detergent solubilized NADH-cytochrome b_5 reductase and 0,5 nmoles of purified detergent solubilized cytochrome b_5 in a total volume of 1 ml 0,30 M Tris-HCl, pH 7,0.

(16) Systems for assessing the possible metabolism of anaesthetic agents by stearate desaturase.

(a) The reconstituted system for the determination of metabolites of anaesthetic agents.

The reconstituted system was as described above (see section 15 of Methods). The anaesthetic agents, where necessary, were added to the incubation mixture and vortex mixed for 30 seconds prior to the addition of the NADH. The reaction was started by the addition of NADH (0,6 mM), the reaction was allowed to proceed at 37°C for 20 minutes. At that time, a further 0,6 mM NADH was added and the incubation was allowed to proceed at 37°C for a further 20 minutes. The reaction was terminated by plunging the tubes into ice. The incubates were immediately assayed for possible metabolites of the anaesthetic agents.

(b) Hepatic microsomal incubation system for the determination of metabolites of anaesthetic agents.

The anaesthetic agents were dispersed in 3 ml hepatic microsomes (1,5 mg protein/ml) by vortex mixing for 30 seconds. The reaction was initiated by the addition of NADH (0,3 mM) and the reaction mixture was incubated at 37°C with shaking at 60 cycles per minute. At that time, a further 0,3 mM NADH was added, and the reaction was allowed to proceed at 37°C for an additional 20 minutes. The reaction was terminated by plunging the

tubes into ice. The incubates were immediately assayed for possible metabolites of the anaesthetic agents.

(17) Assays for metabolites of anaesthetic agents from the stearate desaturase enzyme system.

(a) Determination of fluoride ion and acid-labile fluorine compounds.

Fluoride activities in millivolts were measured with an Orion solid state fluoride electrode (model 96-09) in combination with a single junction reference electrode (Orion 09-01-00) connected to a Radiometer model 22 pH meter. Reaction mixtures containing the reconstituted detergent solubilized stearate desaturase enzyme system or hepatic microsomes were terminated by the addition of 1 ml of 0,2 M sodium acetate, pH 4,9, to 1 ml of reaction mixtures containing the reconstituted enzyme system, or 0,1 ml of 3 M sodium acetate, pH 4,9 to 3 ml of the reaction mixtures containing hepatic microsomes. The samples were allowed to equilibrate to room temperature and were then analyzed for fluoride ion using the fluoride electrode. Known concentrations of sodium fluoride in 0,1 M sodium acetate, pH 4,9, were used to establish a standard curve each day. For the determination of acid-labile fluoride compounds, the incubation mixtures were brought to pH 1,5 by the addition of 10 µl of concentrated H_2SO_4. The fluoride standards were similarly treated and both samples and standards were incubated at room temperature for 90 - 95

hours. The pH was returned to pH 5,0 by the addition of 55 - 60 µl of NaOH (6,0 M) and total fluoride was measured using the fluoride electrode.

(b) Determination of bromide ion.

Bromide ion was determined essentially as described by Goodwin (1971) with minor modifications. The protein of the reconstituted stearate desaturase enzyme system and the hepatic microsomes was precipitated by the addition of 750 µl and 10 µl of sodium tungstate (10%) respectively, and 200 µl and 20 µl of H_2SO_4 (2 M) respectively. After centrifugation, the supernatant was used directly without filtering. Phosphate buffer (2,6 M) and sodium hypochlorite were added to the supernatant and the solution was heated at 100°C for 15 minutes. On cooling, 50% sodium formate was added to the samples, and the resulting solution was heated at 100°C for 10 minutes. After cooling, an equal volume of the rosaniline/molybdate mixture was added and the samples were mixed and allowed to stand for three minutes at room temperature after which tert-butanol and H_2SO_4 (7 M) were added with mixing. The absorbance of the samples at 570 nm was measured using a 4 cm path-length cuvette.

(c) Determination of volatile metabolites by gas liquid chromatography.

A Packard 428 gas liquid chromatograph equipped with a Ni^{63} electron capture detector (connected to a Pye-

Unicam DP 88 computing integrator) was used for the quantitative determination of volatile metabolites of the anaesthetic agents. The columns used were as follows :

(a) 1 m × 3 mm glass column of WHP Chromosorb/SE 30 (80 - 100 mesh). Column, detector and injector temperatures were 160, 170 and 190°C respectively. The gas flow was 40 ml N_2 per minute.

(b) 3 m × 3 mm glass column of 10% di-iso-decylphthalate on Chromosorb P (acid-washed) (60 - 80 mesh). Column, detector and injector temperatures were 120, 200 and 160°C respectively. The gas flow was 50 ml N_2 per minute.

(c) 2 m × 2,2 mm nickel column of Chromosorb 102 (80 - 100 mesh). The column, detector and injector temperatures were 150, 210 and 150°C respectively. The gas flow was 50 ml N_2 per minute.

Samples were extracted into 2 ml ether and a few crystals of anhydrous sodium sulphate were added. 1 - 5 µl of the dried ether extracts were injected onto the columns.

(d) Determination of non-volatile metabolites.
Halothane in petroleum ether (10 µl, 0,1 mCi [1-^{14}C]-halothane) was added to 3 ml hepatic microsomes (1,5 mg protein/ml) and vortex mixed for 30 seconds. The reaction was initiated with NADH (0,4 mM) and allowed to proceed at 37°C for 20 minutes with shaking at 60 cycles per minute. The reaction was terminated by plunging the

tubes into ice. Hydrophobic compounds were extracted with 4 × 3 ml volumes of ether, and the ether phases were combined. The combined ether phases and the residual microsomal suspension were assayed separately for radioactivity in Instagel scintillation cocktail (Packard) using a Beckman model LS 8100 liquid scintillation counter.

(18) Determination of heme.

The heme content of hepatic microsomes was determined by the alkali pyridine hemochrome method described by Omura and Takesue (1970). The absorbance difference between 557 nm and 575 nm was measured, and an extinction coefficient of 34,7 $mM^{-1}cm^{-1}$ was utilized for this difference.

(19) O-Demethylation of p-nitroanisole.

The O-demethylation of p-nitroanisole by hepatic microsomes was determined by a modification of the method of Netter and Seidel (1964). 1,5 ml of hepatic microsomes (1,5 mg protein/ml) and 1,5 ml p-nitroanisole (1 mM) were added to the reference and sample cuvettes. The cuvettes were equilibrated to 25°C, and the reaction was initiated by the addition of 30 µl of an NADPH generating system, [NADPH (0,4 mM), glucose-6-phosphate, (7,5 mM), glucose-6-phosphate dehydrogenase (0,5 U/ml), $MgCl_2$ (5 mM) nicotinamide (1 mM) and EDTA (0,2 mM)] to the sample cuvette. The absorbance change at 420 nm was monitored at 25°C

for 3 to 4 minutes, and initial reaction rates were calculated. The extinction coefficient for p-nitrophenol at 420 nm, pH 7,4, is 7,3 $mM^{-1}cm^{-1}$, (Netter and Seidel, 1964).

(20) 3,4-Benzpyrene hydroxylation.

The rate of hydroxylation of 3,4-benzpyrene to 3-hydroxybenzpyrene was determined spectrophotometrically by the method of Prough et al. (1976). 2,5 ml of hepatic microsomes (1,5 mg protein/ml) containing 3,4-benzpyrene (80 µM) in both reference and sample cuvettes were equilibrated to 25°C. NADH (200 µM) was added to both reference and sample cuvettes. The reaction was initiated by the addition of NADPH (100 µM) to the sample cuvette. The absorbance was monitored at 428 nm and 454 nm. An extinction coefficient of 13 200 $mM^{-1}cm^{-1}$ was utilized for this difference (Prough et al., 1976).

(21) Treatment of hepatic microsomes.

Chemicals used in the treatment of hepatic microsomes were chosen on the basis of earlier reports that they degrade cytochrome P-450 in vitro (Imai and Sato, 1967; Ichikawa et al., 1968).

(a) 1,4-Dioxane

To eliminate the presence of peroxides, 1,4-dioxane was refluxed with metallic sodium for 24 hours and subse-

quently redistilled. The fraction distilling between 101,5 and 102°C was utilized. Hepatic microsomes (1,5 mg protein/ml) from rats re-fed a high carbohydrate diet were incubated with 1,4-dioxane (1,0 M or 2,0 M) at 30°C for 0 to 90 minutes.

(b) Sodium iodide, iso-butanol and potassium thiocyanate.
After the 10 000 g centrifugation step in the isolation of hepatic microsomes from rats re-fed a high carbohydrate diet, the post-mitochondrial supernatant was treated with either sodium iodide (2 M), iso-butanol 10% (v/v) or 20% (v/v) or potassium thiocyanate (1 M). After standing at room temperature for 10 minutes, the procedure for the isolation of the hepatic microsomes was continued as described earlier.

(c) Iodomethane.
Iodomethane (0,27 M) was added to the post-mitochondrial supernatant of livers from rats re-fed a high carbohydrate diet, and the resulting suspension was vortex mixed for 1 minute. The solution was allowed to stand for 10 minutes on ice and was then bubbled with nitrogen for 10 minutes to drive off residual iodomethane. On completion of the microsomal isolation, the microsomes were bubbled with a mixture of $CO : O_2$ at a flow rate of 80 : 20 (v/v) for two minutes to inhibit any cytochrome P-450 activity remaining in the microsomes.

(22) Determination of hydrogen peroxide.

Hydrogen peroxide was determined according to the method of Hildebrandt et al. (1973). Aliquots (1,5 ml) of incubation mixtures were treated with 1,5 ml 5% trichloroacetic acid and the resulting suspension was centrifuged to precipitate protein. Ferroammonium sulphate (1,0 mM) and potassium thiocyanate (0,13 M) were added to the supernatant. The absorbance of the ferric thiocyanate complex was monitored at 480 nm in a Gilford single beam spectrophotometer.

(23) Determination of superoxide anion.

Superoxide anion was determined by a combination of the methods of Bartoli et al. (1977) and Misra & Fridorich (1972) by monitoring the formation of adrenochrome. The reaction mixture contained 3 ml of hepatic microsomes (0,5 or 1,5 mg protein/ml), EDTA (0,2 mM) and adrenaline (0,6 mM). The anaesthetic agents were dispersed in the microsomal suspension by vortex mixing for 30 seconds prior to the addition of adrenaline. The reaction was initiated by the addition of either NADH (1 mM) or NADPH (0,6 mM), and adrenochrome formation was monitored at 475 nm ($\varepsilon_{475nm} = 4020 M^{-1}cm^{-1}$) at 37°C.

(24) Administration of anaesthetic agents.

Groups of ten rats were fed on either a normal diet or

the high carbohydrate diet for five days and were then anaesthetised from standard vaporizers with 1 MAC anaesthetic agent (Eger, 1974), for three hours on days 6, 9 and 12 of their respective diets. The rats were sacrificed on day 13 of the programme, 24 hours after the last administration of the anaesthetic agent, or at an equivalent time for controls. Alternatively, groups of 5 or 10 rats were fed on either the normal diet or the high carbohydrate diet for five days and, in addition, were in some cases pretreated with phenobarbitone (80 mg/kg) for three days. The rats were anaesthetised from standard vaporizers with 1 MAC anaesthetic agent for two hours, under hypoxic conditions (14% O_2, 700 ml/min, using N_2O, 4,3 l/min as the carrier gas).

The anaesthetic chambers in which the rats were anaesthetised were two rectangular perspex boxes (60 cm × 30 cm × 30 cm) with a fenestrated floor 6 cm from the base. The area between the base and the floor was filled with anaesthetic soda lime. The anaesthetic gas was introduced into each chamber via a port below the floor in one corner and exhausted via a port on the top corner diagonally opposite from the inlet port. Where appropriate, a single gas mixture line with a T-junction splitting the gas flow to each chamber was used to ensure that the same anaesthetic gas mixture would be introduced into each anaesthetic chamber.

(25) Determination of serum glutamic oxaloacetic transaminase (S.G.O.T.).

Blood was obtained from the aorta of rats immediately following sacrifice. The blood was centrifuged at 3 000 rpm for five minutes to obtain the serum. The S.G.O.T. levels were determined spectrally at 340 nm by the method of Henry et al. (1960).

(26) Determination of hepatic reduced glutathione.

Liver glutathione levels were determined fluorimetrically by the method of Cohn and Lyle (1966). The liver was homogenized in EDTA (30 µM). Protein was removed by precipitation with metaphosphoric acid and subsequent centrifugation at 10 000 rpm for fifteen minutes. The supernatant was treated with o-phthalaldehyde. The fluorescence of the GSH-o-phthalaldehyde complex was monitored at 426 nm, using an excitation wavelength of 365 nm in a Perkin-Elmer model 203 fluorimeter.

(27) Hepatic histology.

Livers were excised from rats immediately after sacrifice and were placed in 10% formalin containing NaH_2PO_4 (0,26 M) and Na_2HPO_4 (0,46 M). The livers were then sectioned by dicing finely, stained with haematoxylin and eosin and examined by conventional light microscopy.

(28) Spectrophotometry.

Spectrophotometry was performed on a Unicam SP 1800 recording spectrophotometer unless otherwise indicated. For turbid suspensions, the cell holder adjacent to the photomultiplier was used, and the cuvettes were positioned so that the light path passed through their frosted faces in order to obtain more uniform light scattering. Spectral measurements were performed in thermostatically controlled compartments at 25°C, unless indicated otherwise.

(29) Calculations and statistical analyses.

The observed first order rate constants (k_{obs}) for the re-oxidation of NADH reduced hepatic microsomal ferro-cytochrome $\underline{b}_5$ were calculated from the slope of plots of ln ($A_t - A_\alpha$) versus time, where A_t and A_α are the absorbance changes between 409 nm and 424 nm at time t and at infinity, respectively. The first order rate constants (k_{obs}) for the oxidation of purified trypsin-cleaved cytochrome $\underline{b}_5$ were calculated from plots of ln ($A_{424\alpha} - A_{424t}$) versus time. In all cases, the absorbance at infinite time was determined after approximately ten half-lives.

Spectral binding constants (K_s) for the interaction of xenobiotics with hepatic microsomal cytochrome P-450 were calculated from Hanes and Eadie-Hofstee plots. The values of K_{eq}, the equilibrium constant for the

effect of xenobiotics on the re-oxidation of ferrocytochrome $\underline{b}_5$ and K_i, the equilibrium constant for cyanide inhibition of the effects of the xenobiotics on the re-oxidation of ferrocytochrome $\underline{b}_5$, were calculated from plots of k_{obs} versus the concentration of xenobiotic or cyanide, respectively. Transformations of this plot, such as those described by Lineweaver and Burke or Eadie and Hofstee, could not be employed, as the k_{obs} had a finite background value at substrate concentrations equal to zero. This background rate represents the autoxidation of ferrocytochrome $\underline{b}_5$ which is unaffected by KCN and xenobiotics.

All determinations, unless indicated otherwise, were in duplicate or triplicate on two or three separate preparations of hepatic microsomes or on two or three separate assay systems. Values reported are means $\pm$ standard deviations. Statistical analyses were performed on a Tektronix 31 progammable calculator. After testing for equal variances, Student's $\underline{t}$ test for unpaired data was utilized to assess statistical significance, unless indicated otherwise. Two-tailed P values are reported, with $P < 0,001$ representing a highly significant difference, $P < 0,01$ a significant difference and $P < 0,05$ a probably significant difference between means.

3. RESULTS

(1) The Interaction of Xenobiotics with Hepatic Microsomal Stearate Desaturase.

(a) A survey of the interaction of xenobiotics with hepatic microsomal stearate desaturase.

i) The effect of xenobiotics on the redox steady state of hepatic microsomal cytochrome b_5.

The effects of various xenobiotics, in particular halogenated hydrocarbons on the redox steady state of NADPH reduced hepatic microsomal cytochrome b_5 in microsomes from rats with elevated levels of stearate desaturase, are shown in Table 1. The effect of KCN (at a concentration equivalent to five times its K_i for stearate desaturase) on the redox steady state of cytochrome b_5 in the presence of xenobiotics is also shown in Table 1. That the hepatic microsomes utilized contained appreciable stearate desaturase activity is confirmed by the ability of stearoyl CoA to significantly decrease the redox steady state of cytochrome b_5 (Oshino et al., 1971) (Table 1).

Inasmuch as the purpose of this survey was to investigate whether xenobiotics could interact with stearate desaturase, each xenobiotic was used at the maximum concentra-

tion which would not disrupt the microsomal suspension or convert cytochrome P-450 to cytochrome P-420.

Virtually all of the halogenated alkanes investigated significantly shifted the redox status of cytochrome $\underline{b}_5$ towards ferricytochrome $\underline{b}_5$. The haloalkanes which were poly-halogenated and contained one or more types of halo atoms, e.g. F, Cl, Br or I appeared to be the most effective. In general, the haloethanes were more effective than, or at least as effective as the halomethanes, when compounds containing the same absolute number of halo atoms or of the same ratio of halo atoms per carbon atom were compared. The chlorinated ethanes, _viz_. 1,1,2-trichloroethane and 1,1,2,2-tetrachloroethane, were more effective than the chlorinated methane chloroform, at shifting the redox status of cytochrome $\underline{b}_5$ towards the ferric form while other halogenated ethanes such as 1,1,1-trichloroethane decreased the redox status of cytochrome $\underline{b}_5$ to the same extent as chloroform.

Compounds with multiple halo atoms proved to be more effective than singly substituted compounds in shifting the redox steady state of cytochrome $\underline{b}_5$ towards ferricytochrome $\underline{b}_5$. For instance, 1,2,3-trichloropropane, 1,1,1-trichloro-2,3-epoxypropane, bromotrichloromethane, and methoxyflurane shifted the redox status of cytochrome $\underline{b}_5$ towards the oxidized form to a greater extent than did chloropropane, dichloromethane and chloromethyl methyl

ether. However, no clear order of reactivity was evident for the different halogen substituents.

However, some comparisons do emerge :
Chloroacetaldehyde, fluroxene, ethyl vinyl ether, divinyl ether and 2,2,2-trifluoroethyl ether shifted the redox state of cytochrome $\underline{b}_5$ towards the oxidized form of the protein, while comparable concentrations of acetaldehyde, trifluoroeacetaldehyde hydrate, 2,2,2-trifluoroethanol, diethyl ether, trifluoroacetic acid and some vinyl and allyl halides did not affect the redox state of cytochrome $\underline{b}_5$.

The most striking effect on the NADPH steady state of cytochrome $\underline{b}_5$ occurred in the presence of bromotrichloromethane, 1,1,2,2-tetrachloroethane, 1,2,3-trichloro-1,1,2-trifluoroethane, 1,2-dibromo-1,1-dichloroethane, 1,2-dibromo-1,2-dichloroethane, 1,1,1-trichloro-2,3-epoxypropane and chloroacetaldehyde and the anaesthetic agents halothane, enflurane, methoxyflurane and ethyl vinyl ether.

As shown in Table I, the ability of the haloalkanes to shift the redox steady state of cytochrome $\underline{b}_5$ towards the oxidized form of the protein was diminished in the presence of cyanide. This effect was however, comparable to the magnitude of the effect of cyanide on the redox steady state of microsomal cytochrome $\underline{b}_5$ in

the absence of added xenobiotics.

ii) The effect of xenobiotics on the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome b_5.

The effects of those compounds which decreased the redox status of cytochrome b_5 to 40% or below, were further investigated for possible interaction with stearate desaturase by assessing their effects on the pseudo first order rate constants (k_{obs}) for the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome 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. For all studies of the re-oxidation of hepatic microsomal ferrocytochrome b_5, unless otherwise indicated, hepatic microsomes were from rats in which the levels of stearate desaturase were elevated by re-feeding the rats a high carbohydrate diet (Oshino et al., 1971). That the level of stearate desaturase in these microsomal preparations was elevated was demonstrated by the ability of stearoyl CoA to increase the rate constant for the re-oxidation of microsomal ferrocytochrome b_5 (Table 2) (Oshino et al., 1971).

Compounds which increased the rate constant for the re-oxidation of cytochrome b_5 in a cyanide inhibitable manner included diiodomethane, bromotrichloromethane,

1,1,2-trichloroethane, 1,2-dibromo-1,2-dichloroethane and probably chloroacetaldehyde (Table 2). The rate constant for the re-oxidation of cytochrome $\underline{b}_5$ was also enhanced by 1-bromo-2-chloroethane, 1,2-dibromo-1,1-dichloroethane, 1,2,3-trichloropropane, 1,1,1-trichloro-2,3-epoxypropane and ethyl vinyl ether, but the effects of these compounds on the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ were not significantly inhibited by cyanide.

The anaesthetic agents halothane, enflurane and methoxyflurane stimulated the re-oxidation of microsomal cytochrome $\underline{b}_5$ and their effects are inhibited by cyanide (Table 2). This confirms an earlier report by Berman *et al.*, (1975) that halothane stimulates the re-oxidation of hepatic microsomal ferrocytochrome $\underline{b}_5$ and that this effect is inhibited by cyanide.

TABLE 1. THE EFFECT OF XENOBIOTICS ON THE REDOX STEADY STATE OF HEPATIC MICROSOMAL CYTOCHROME b_5 IN THE PRESENCE OF NADPH.

Additions (mM)	% Reduction of cytochrome b_5	
	- KCN	+ 0,5 mM KCN
None	59,1 ± 5,0	70,2 ± 5,0[¶]
Stearoyl CoA (0,012)	44,6 ± 6,8*	-
Halogenated Methanes		
Dichloromethane (5,2)	58,2 ± 2,1	68,2 ± 1,3[¶]
Chloroform (8,2)	47,8 ± 4,0†	56,6 ± 0,1‖
Carbontetrachloride (3,4)	45,5 ± 3,7†	50,9 ± 3,0
Dibromomethane (48,0)	49,5 ± 4,8≠	50,4 ± 2,2
Tribromomethane (1,8)	50,2 ± 2,4≠	57,5 ± 3,8‖
Tetrabromomethane (0,01)	45,6 ± 10,6	51,5 ± 7,7
Diiodomethane (21,0)	40,5 ± 3,4*	-
Iodoform (0,42)	31,4 ± 4,5*	-
Tetraiodomethane (0,006)	48,7 ± 7,8	51,5 ± 7,7
Bromotrichloromethane (3,4)	31,2 ± 0,9*	-
Halogenated Ethanes		
1,2-Dichloroethane (42,0)	44,11 ± 4,0*	-
1,1,1-Trichloroethane (7,2)	45,7 ± 1,7*	49,3 ± 4,0
1,1,2-Trichloroethane (8,0)	37,6 ± 5,4*	49,9 ± 3,9[¶]
1,1,2,2-Tetrachloroethane (8,0)	34,4 ± 6,4*	44,7 ± 5,5[¶]

TABLE 1 (Cont.)

Additions (mM)	% Reduction of cytochrome b_5 -KCN	+ 0,5 mM KCN
Bromomethane (44,0)	48,3 ± 4,3†	49,7 ± 1,0
1,1,2,2-Tetrabromomethane (2,5)	41,9 ± 3,3*	51,5 ± 7,7‖
Ethyl iodide (8,4)	52,1 ± 1,5	54,9 ± 0,0
Halothane (18,0)	26,9 ± 5,5*	-
1,2,2-Trichloro-1,1,2-Trifluoroethane (14,1)	32,3 ± 4,0*	-
1-Bromo-2-chloroethane (12,1)	40,5 ± 8,1†	48,9 ± 4,3‖
1,2-Dibromo-1,1-dichloroethane (0,6)	28,7 ± 5,1*	32,0 ± 5,1¶
1,2-Dibromo-1,2-dichloroethane (0,6)	34,6 ± 9,0*	47,1 ± 3,3¶
<u>Halogenated Propanes</u>		
Chloropropane (3,8)	51,6 ± 2,0	64,6 ± 3,7¶
1,2,3-Trichloropropane (3,2)	39,9 ± 0,7*	48,2 ± 0,6§
1,3-Dibromopropane (1,6)	51,2 ± 3,5	61,1 ± 1,3‖
<u>Halogenated Butanes and Hexanes</u>		
1-Fluorobutane (17,0)	52,4 ± 1,1	62,8 ± 2,5¶
Perfluorohexane (8,4)	59,9 ± 5,5	76,1 ± 5,0‖
<u>Ether Compounds</u>		
Diethyl ether (32,4)	53,6 ± 2,9	-
2,2,2-Trifluoroethyl ether (28,4)	44,5 ± 2,3*	53,10 ± 2,5‖

TABLE 1 (Cont.)

Additions (mM)	% Reduction of cytochrome b_5 -KCN	% Reduction of cytochrome b_5 + 0,5 mM KCN
2,2,2-Trifluoroethyl vinyl ether (17,9)	45,7 ± 4,0†	57,5 ± 1,3‖
Enflurane (14,0)	27,39 ± 4,8†	-
Methoxyflurane (0,6)	39,1 ± 1,7†	-
Chloromethyl methyl ether (0,4)	64,7 ± 3,9	72,6 ± 5,4‖
Unsaturated Compounds		
Allyl chloride (40,5)	51,5 ± 7,0	49,4 ± 2,5
Allyl bromide (3,9)	49,5 ± 6,2	52,6 ± 1,8
Vinyl chloride (13,0)	68,4 ± 5,1	70,9 ± 0,6
Trichloroethylene (7,4)	51,1 ± 4,5	56,2 ± 0,6
Vinyl ether (22,0)	46,3 ± 2,9	46,4 ± 9,7
Ethyl vinyl ether (35,0)	35,7 ± 3,6*	35,0 ± 10,8
Miscellaneous Compounds		
Trifluoroacetic acid (200,0)	79,0 ± 2,0	84,1 ± 1,3‖
2,2,2-Trifluoroacetaldehyde hydrate (37,0)	54,4 ± 3,1	63,7 ± 2,5‖
2,2,2-Trifluoroethanol (69,0)	53,1 ± 5,3	66,4 ± 1,3‖
Chloroacetaldehyde (52,0)	25,8 ± 6,1*	33,3 ± 1,8
Chlorobenzene (3,3)	40,5 ± 4,4*	49,2 ± 6,2¶¶
2,2,2-Trichloroethanol (6,9)	46,5 ± 1,4*	61,1 ± 3,8¶¶
Acetaldehyde (50,0)	56,7 ± 4,4	60,9 ± 5,7
1,1,1-Trichloro-2,3-epoxy-propane	35,3 ± 2,8*	-

TABLE 1 (Cont.)

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* Differs significantly from "no additions", $P < 0,001$

† Differs significantly from "no additions", $P < 0,01$

≠ Probably differs significantly from "no additions", $P < 0,05$

§ Differs significantly from compound minus cyanide, $P < 0,001$

¶ Differs significantly from compound minus cyanide, $P < 0,01$

‖ Probably differs from compound minus cyanide, $P < 0,05$

TABLE 2. THE EFFECT OF XENOBIOTICS ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5.

Additions (mM)	Re-oxidation of ferrocytochrome b_5 $10^2 k_{obs}$ (sec^{-1})	
	-KCN	+ 0,5 mM KCN
None	1,35 ± 0,35	1,21 ± 0,28
Stearoyl CoA (0,012)	4,19 ± 1,49*	2,93 ± 0,76‖
Halogenated Methanes		
Diiodomethane (21,0)	1,60 ± 0,11≠	1,31 ± 0,09¶
Iodoform (0,4)	1,41 ± 0,15	1,40 ± 0,26
Bromotrichloromethane (3,4)	4,38 ± 0,85†	3,00 ± 0,19¶
Halogenated Ethanes and Propanes		
1,1,2-Trichloroethane (8,0)	1,75 ± 0,02†	1,44 ± 0,05¶
1,1,2,2-Tetrachloroethane (8,0)	1,10 ± 0,33	1,11 ± 0,14
1,2,2-Trichloro-1,1,2-trifluoroethane (14,1)	0,68 ± 0,09†	0,75 ± 0,05
1-Bromo-2-chloroethane (12,1)	1,79 ± 0,20†	1,83 ± 0,20
1,2-Dibromo-1,1-dichloroethane (0,6)	1,74 ± 0,38≠	1,48 ± 0,24
1,2-Dibromo-1,2-dichloroethane (0,6)	1,61 ± 0,15≠	1,10 ± 0,20¶
1,2,3-Trichloropropane (3,2)	1,71 ± 0,52≠	1,66 ± 0,38
Halothane (18,0)	2,62 ± 0,44†	1,87 ± 0,46¶

TABLE 2 (Cont.)

Additions (mM)	Re-oxidation of ferrocytochrome b_5 $10^2 k_{obs}$ (sec^{-1})	
	-KCN	+ 0,5 mM KCN
Miscellaneous Compounds		
1,1,1-Trichloro-2,3-epoxy-propane (3,0)	1,96 ± 0,30*	2,03 ± 0,42
Chloroacetaldehyde (52,0)	2,20 ± 0,20*	1,81 ± 0,20‖
Ethyl vinyl ether (35,0)	2,25 ± 0,06†	2,32 ± 0,87
Enflurane (14,0)	3,70 ± 0,96†	2,54 ± 0,82¶
Methoxyflurane (0,6)	3,00 ± 0,43†	2,55 ± 0,12¶

* Differs significantly from "no additions", $P < 0,001$

† Differs significantly from "no additions", $P < 0,01$

≠ Probably differs from "no additions", $P < 0,05$

¶ Differs significantly from compound minus cyanide, $P < 0,01$

‖ Probably differs from compound minus cyanide, $P < 0,05$

(b) The interaction of three xenobiotics with hepatic microsomal enzymes.

The ability of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde to increase the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ in a cyanide sensitive manner, suggests that these xenobiotics may stimulate microsomal electron transfer by interacting with hepatic microsomal stearate desaturase. Therefore, a detailed investigation on the interaction of these halogenated compounds with hepatic microsomal stearate desaturase and with other microsomal enzymes, such as cytochrome P-450 and several electron transfer proteins was undertaken. In these investigations, advantage was taken of the ability of fasting to reduce the levels and activity of stearate desaturase and of the induction of cytochrome P-450 by phenobarbitone as well as of the effects of known inhibitors of cytochrome P-450.

Hepatic microsomes from fasted rats were used to assess the ability of xenobiotics to affect the re-oxidation of cytochrome $\underline{b}_5$, in the absence of stearate desaturase activity. The inability of stearoyl CoA to significantly increase the rate constant for the re-oxidation of cytochrome $\underline{b}_5$ in these microsomes confirms that the stearate desaturase activities in these preparations is not measurable, although the levels of cytochrome P-450 were unaffected by fasting (Table 3).

The enhancement by bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane, but not by chloroacetaldehyde, of the rate constant for the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ was diminished by fasting (Tables 2 and 3). The cyanide sensitivity of the enhancement of the re-oxidation of microsomal cytochrome $\underline{b}_5$ by bromotrichloromethane and 1,2-dibromo-1,2-dichloromethane was eliminated by fasting (Tables 2 and 3).

CO and metyrapone, which are effective inhibitors of cytochrome P-450 (Roots and Hildebrandt, 1973; De Bruin, 1976) were utilized to assess the involvement of cytochrome P-450 in the abilities of xenobiotics to enhance the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$. As shown in Table 4, neither inhibitor significantly affected the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ in the absence of xenobiotics or stearoyl CoA, nor do they affect the enhancement of the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ in the presence of stearoyl CoA and bromotrichloromethane. CO and metyrapone did however significantly decrease the effects of 1,2-dibromo-1,2-dichloroethane and CO diminished the effect of chloroacetaldehyde, on the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$.

The effects of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde on the NADPH- and NADH-cytochrome $\underline{c}$ reductases and on stearate desaturase are shown in Table 5. None of these halo compounds

had a significant effect on either the NADPH- or the NADH-cytochrome c reductase, or the stearate desaturase mediated conversion of stearoyl CoA to oleate.

The effects of variable concentrations of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde on the rate constants for the re-oxidation of microsomal ferrocytochrome b_5 are shown in Table 6 and Figure 8. The equilibrium constants K_{eq}, for the effects of these xenobiotics on cytochrome b_5, were calculated from these data to be 2,2 $\pm$ 0,3 mM for bromotrichloromethane and 0,46 $\pm$ 0,1 mM for 1,2-dibromo-1,2-dichloroethane. It was not possible to calculate a K_{eq} value for chloroacetaldehyde because the rate constant for the re-oxidation of cytochrome b_5 was substantially decreased in the presence of ca. 10 mM chloroacetaldehyde. From Figure 8, however, the concentration of chloroacetaldehyde at which half maximal stimulation of the re-oxidation of cytochrome b_5 was obtained, was estimated to be greater than 35 mM.

Both bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane bound to cytochrome P-450 with the production of a type 1 difference spectrum (λ_{max} 387 nm, λ_{min} 421 nm). The K_s values for the binding of these compounds to cytochrome P-450 in hepatic microsomes from phenobarbitone pretreated rats were calculated from Figure 9 to be 1,7 $\pm$ 0,3 mM for bromotrichloromethane and 0,29 $\pm$

0,09 mM for 1,2-dibromo-1,2-dichloroethane. The K_s for the binding of chloroacetaldehyde to cytochrome P-450 in hepatic microsomes from phenobarbitone pre-treated rats was reported by Ivanetich et al., (1978) to be 30 mM. The K_s values for the binding of bromo-trichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde to cytochrome P-450 in hepatic microsomes from uninduced, fasted rats were calculated from Figure 10 to be 2,20 $\pm$ 0,30 mM, 0,15 $\pm$ 0,05 mM and 31,0 $\pm$ 8,0 mM, respectively.

The effects of variable amounts of KCN on the rate constants for the re-oxidation of microsomal ferro-cytochrome b_5 in the presence of fixed amounts of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde are shown in Table 7 and Figure 11. From these data, the K_i values for KCN were calculated to be 0,12 $\pm$ 0,03; 0,07 $\pm$ 0,02 and 0,10 $\pm$ 0,02 mM for the inhibition of the stimulation of microsomal electron transfer by KCN in the presence of bromotri-chloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde, respectively.

TABLE 3. THE EFFECT OF SELECTED XENOBIOTICS AND KCN ON THE RATE CONSTANTS FOR THE RE-OXIDATION OF NADH REDUCED FERROCYTOCHROME $\underline{b}_5$ IN HEPATIC MICROSOMES FROM FASTED RATS.

Additions (mM)	Re-oxidation of ferrocytochrome $\underline{b}_5$ $10^2 k_{obs}$ (sec^{-1})
None	1,31 ± 0,26
KCN	1,12 ± 0,46
Stearoyl CoA (0,012)	1,73 ± 0,21
Stearoyl CoA (0,012) + KCN (0,5)	1,46 ± 0,38
Bromotrichloromethane (3,4)	2,63 ± 0,34*
Bromotrichloromethane (3,4) + KCN (0,5)	2,36 ± 0,53*
1,2-Dibromo-1,2-dichloroethane (0,6)	1,27 ± 0,07
1,2-Dibromo-1,2-dichloroethane (0,6) + KCN (0,5)	0,95 ± 0,06≠
Chloroacetaldehyde (52,0)	2,52 ± 0,16*
Chloroacetaldehyde (52,0) + KCN (0,5)	2,67 ± 0,23*
Cytochrome P-450 (nmol/mg microsomal protein)	1,06 ± 0,14

* Differs significantly from "no additions", $P < 0,01$

≠ Probably differs from "no additions", $P < 0,05$

TABLE 4. THE EFFECTS OF CO AND METYRAPONE ON THE RATE CONSTANTS FOR THE RE-OXIDATION OF MICROSOMAL FERROCYTOCHROME b_5 IN THE PRESENCE AND ABSENCE OF SELECTED XENOBIOTICS.

Additions (mM)	Re-oxidation of ferrocytochrome b_5 $10^2 k_{obs}$ (sec^{-1})
None	1,31 ± 0,17
$CO:O_2$ (80:20 v/v)	1,20 ± 0,24
Metyrapone (2,3 mM)	1,16 ± 0,10
Stearoyl CoA (0,012 mM)	4,01 ± 0,37†
Stearoyl CoA (0,012 mM) + $CO:O_2$ (80:20 v/v)	4,07 ± 0,38†
Bromotrichloromethane (3,4 mM)	2,40 ± 0,22†
Bromotrichloromethane (3,4 mM) + $CO:O_2$ (80:20 v/v)	2,10 ± 0,42
Bromotrichloromethane (3,4 mM) + metyrapone (2,3 mM)	1,90 ± 0,37
1,2-Dibromo-1,2-dichloroethane (0,6 mM)	1,62 ± 0,14†
1,2-Dibromo-1,2-dichloroethane (0,6 mM) + $CO:O_2$ (80:20 v/v)	1,06 ± 0,17¶
1,2-Dibromo-1,2-dichloroethane (0,6 mM) + metyrapone (2,3 mM)	0,90 ± 0,12¶
Chloroacetaldehyde (52,0 mM)	2,50 ± 0,21†
Chloroacetaldehyde (52,0 mM) + $CO:O_2$ (80:20 v/v)	1,90 ± 0,14¶

† Differs significantly from "no additions", $P < 0,01$

¶ Differs significantly from same compound minus inhibitor, $P < 0,01$

TABLE 5. THE EFFECT OF SELECTED XENOBIOTICS ON HEPATIC MICROSOMAL REDUCTASES AND STEARATE DESATURASE.

Additions (mM)	NADPH-cytochrome c reductase (units/mg protein)	NADH-cytochrome c reductase (units/mg protein)	Stearate desaturase activity $\frac{\text{oleate}}{\text{(oleate + stearate)}}$
None	0,071 ± 0,01	1,12 ± 0,09	0,61 ± 0,01
Bromotrichloromethane (3,4)	0,061 ± 0,01≠	1,11 ± 0,08	0,65 ± 0,01
1,2-Dibromo-1,2-dichloroethane (0,6)	0,066 ± 0,004	1,21 ± 0,18	0,66 ± 0,06
Chloroacetaldehyde (52,0)	0,067 ± 0,01	1,05 ± 0,14	0,65 ± 0,04

≠ Probably differs from "no additions", $P < 0,05$

TABLE 6. DETERMINATION OF k_{obs} FOR THE STIMULATION OF THE RE-OXIDATION OF FERROCYTOCHROME b_5 IN THE PRESENCE OF SELECTED XENOBIOTICS.

Additions (mM)	Re-oxidation of ferrocytochrome b_5 $10^2 k_{obs}$ (sec^{-1})
None	1,32 ± 0,05
Bromotrichloromethane (0,68)	1,57 ± 0,29
Bromotrichloromethane (1,69)	1,90 ± 0,23
Bromotrichloromethane (3,38)	2,48 ± 0,42
Bromotrichloromethane (6,76)	2,79 ± 0,68
Bromotrichloromethane (16,9)	2,90 ± 0,82≠
1,2-Dibromo-1,2-dichloroethane (0,30)	1,49 ± 0,14
1,2-Dibromo-1,2-dichloroethane (0,59)	1,57 ± 0,05
1,2-Dibromo-1,2-dichloroethane (0,89)	1,63 ± 0,0
1,2-Dibromo-1,2-dichloroethane (2,95)	1,72 ± 0,01*
Chloroacetaldehyde (10,3)	0,83 ± 0,24≠
Chloroacetaldehyde (25,8)	1,42 ± 0,06
Chloroacetaldehyde (41,3)	2,58 ± 0,16
Chloroacetaldehyde (51,6)	2,88 ± 0,36
Chloroacetaldehyde (77,4)	3,78 ± 0,01*

* Differs significantly from "no additions", $P < 0,001$

≠ Probably differs from "no additions", $P < 0,05$

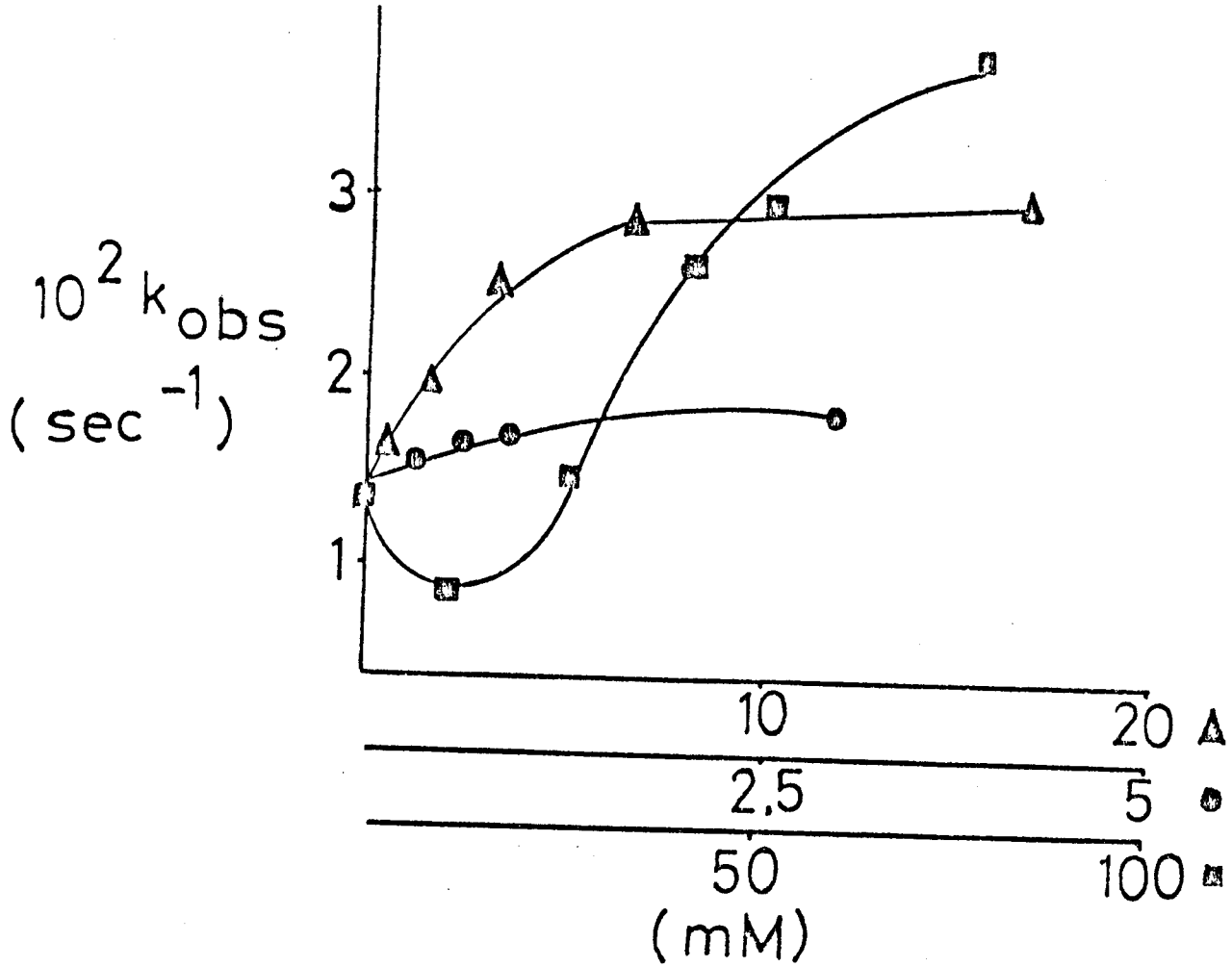

FIGURE 8 : The effect of variable concentrations of bromotrichloromethane (▲); 1,2-dibromo-1,2-dichloroethane (●) and chloroacetaldehyde (■) on k_{obs} for the stimulation of cytochrome b_5 re-oxidation.

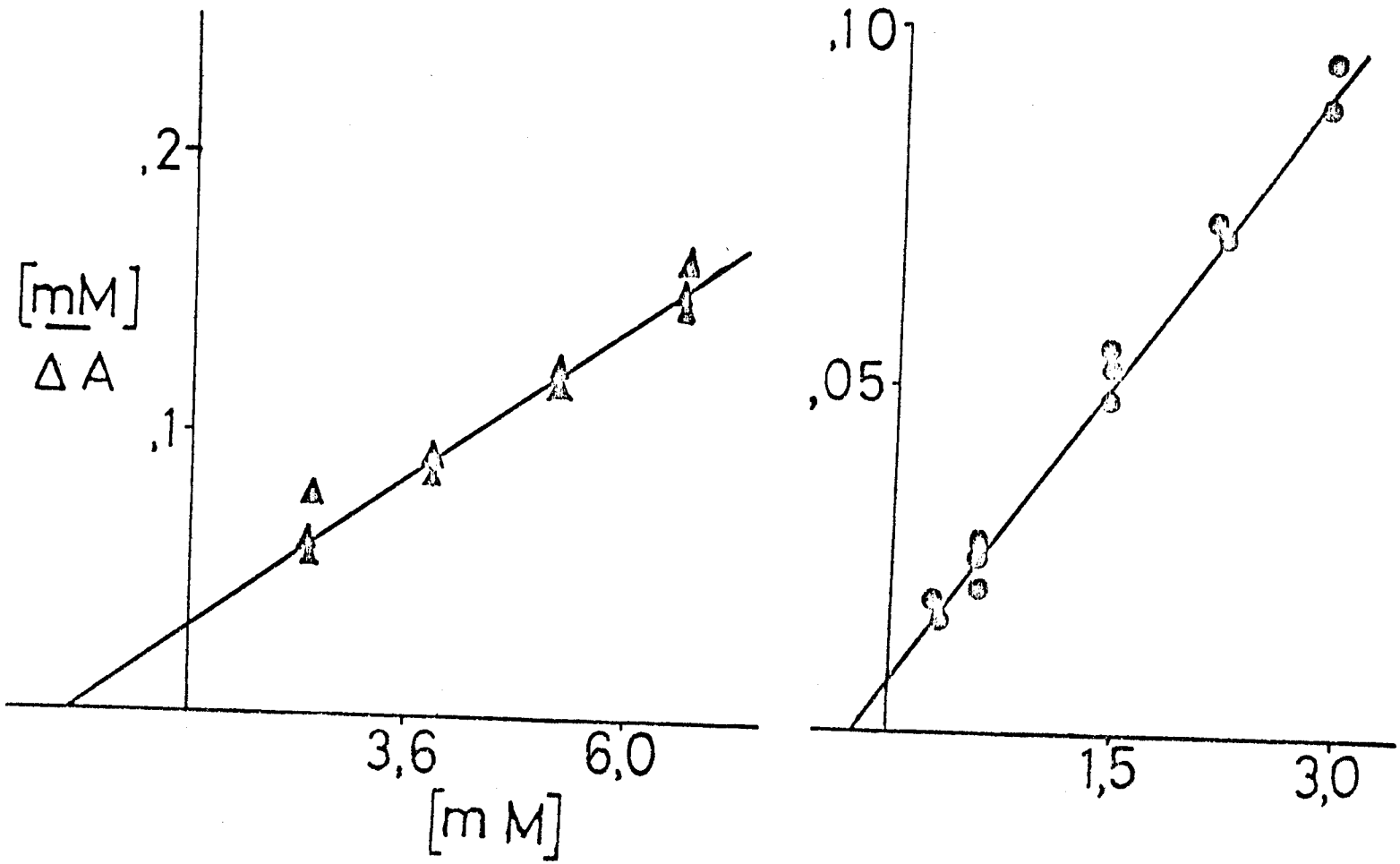

FIGURE 9 : Binding of bromotrichloromethane (▲) and 1,2-dibromo-1,2-dichloroethane (●) to cytochrome P-450 in microsomes from phenobarbitone treated rats $\Delta A = \Delta(A_{420nm} - A_{380nm})$.

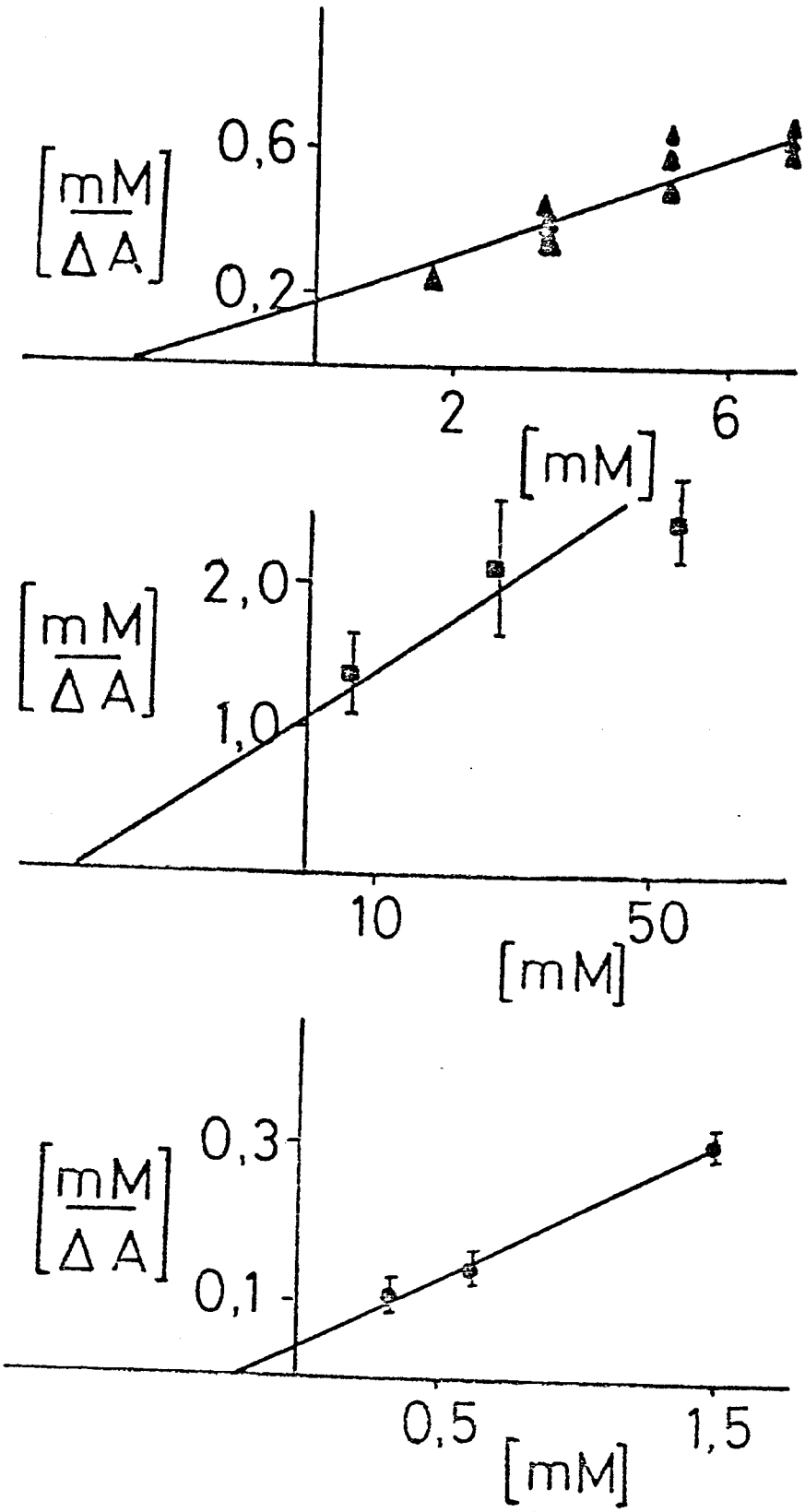

FIGURE 10 : Hanes plots of the binding of bromotrichloromethane (▲), chloroacetaldehyde (■) and 1,2-dibromo-1,2-dichloroethane (●) to cytochrome P-450 in microsomes from fasted rats.

TABLE 7. THE EFFECT OF INCREASING CONCENTRATIONS OF KCN ON THE RATE CONSTANTS FOR THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME $\underline{b}_5$ IN THE PRESENCE OF SELECTED XENOBIOTICS.

KCN (mM)	Other additions to hepatic microsomes (mM)	Re-oxidation of ferrocytochrome $\underline{b}_5$ $10^2 k_{obs}(sec^{-1})$
0,0	Bromotrichloromethane (3,4)	2,76 ± 0,06
0,06	Bromotrichloromethane (3,4)	-
0,12	Bromotrichloromethane (3,4)	2,29 ± 0,21
0,25	Bromotrichloromethane (3,4)	2,08 ± 0,37
0,38	Bromotrichloromethane (3,4)	1,94 ± 0,0
0,50	Bromotrichloromethane (3,4)	1,85 ± 0,02
0,0	1,2-Dibromo-1,2-dichloroethane (0,6)	1,75 ± 0,13
0,06	1,2-Dibromo-1,2-dichloroethane (0,6)	1,63 ± 0,21
0,12	1,2-Dibromo-1,2-dichloroethane (0,6)	1,49 ± 0,09
0,25	1,2-Dibromo-1,2-dichloroethane (0,6)	1,47 ± 0,15

TABLE 7 (Cont.)

KCN (mM)	Other additions to hepatic microsomes (mM)	Re-oxidation of ferrocytochrome $\underline{b}_5$ $10^2 k_{obs}$ (sec^{-1})
0,38	1,2-Dibromo-1,2-dichloroethane (0,6)	1,47 ± 0,15
0,50	1,2-Dibromo-1,2-dichloroethane (0,6)	-
0,0	Chloroacetaldehyde (51,6)	3,52 ± 0,17
0,06	Chloroacetaldehyde (51,6)	3,29 ± 0,0
0,12	Chloroacetaldehyde (51,6)	3,00 ± 0,0
0,25	Chloroacetaldehyde (51,6)	2,66 ± 0,23
0,38	Chloroacetaldehyde (51,6)	2,62 ± 0,05
0,50	Chloroacetaldehyde (51,6)	2,59 ± 0,28

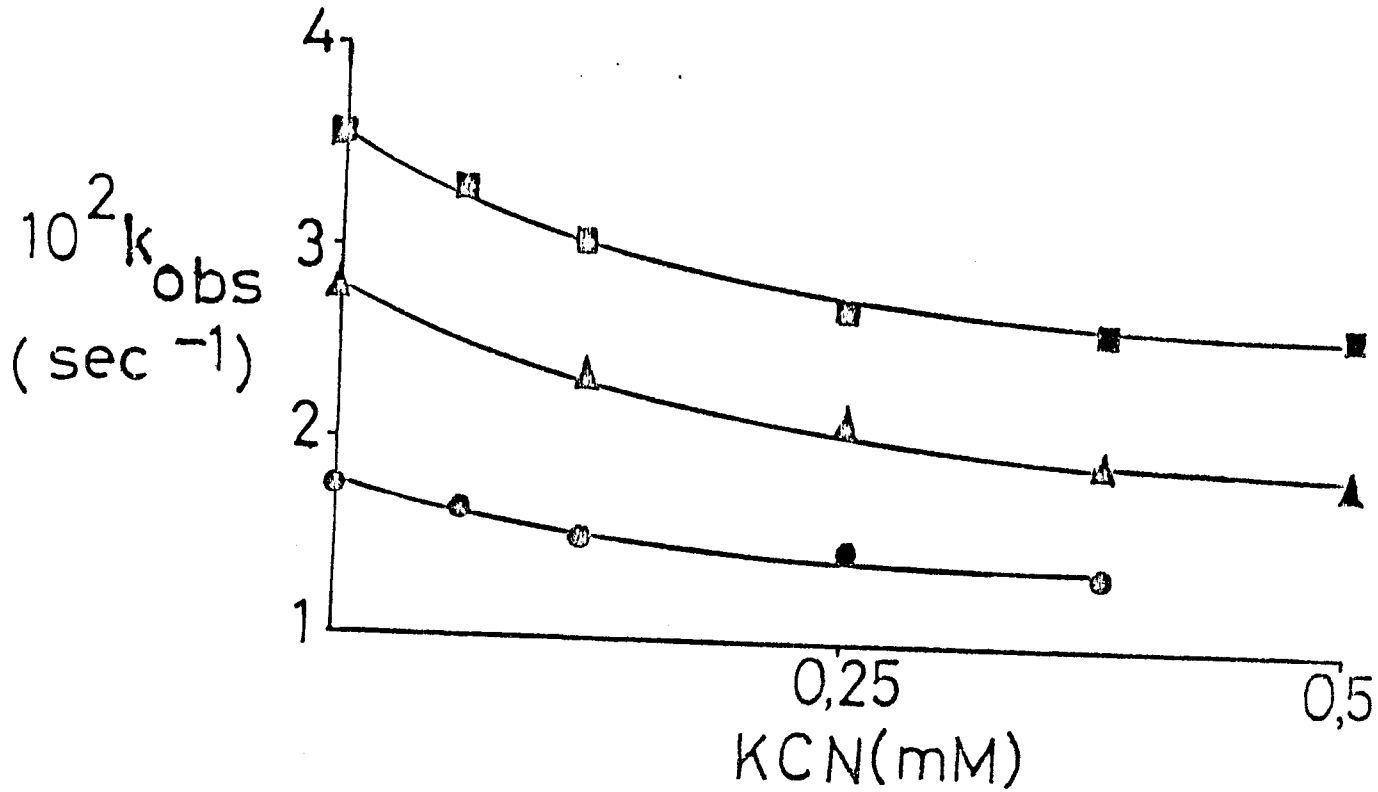

FIGURE 11 : The effect of KCN concentration on the rate constants (k_{obs}) for the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome b_5 in the presence of bromotrichloromethane (▲), 1,2-dibromo-1,2-dichloroethane (●) and chloroacetaldehyde (■).

(c) The interaction of three anaesthetic agents with hepatic microsomal enzymes.

The possibility that the anaesthetic agents halothane, enflurane and methoxyflurane enhanced the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ by interacting with stearate desaturase, was investigated. In addition, the ability of these anaesthetic agents to interact with other microsomal enzymes such as cytochrome P-450 and the electron transfer proteins, cytochrome $\underline{b}_5$ and the NADH- and NADPH-cytochrome $\underline{c}$ reductases, was assessed.

The effects of two inhibitors of cytochrome P-450, namely metyrapone and CO on the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ in the presence and absence of stearoyl CoA and the anaesthetic agents are presented in Tables 8 and 9. Neither of these inhibitors affected the rate of re-oxidation of cytochrome $\underline{b}_5$ observed in the presence of NADH alone, suggesting that they do not affect the background rate of re-oxidation of cytochrome $\underline{b}_5$. The inability of CO and metyrapone to affect the enhancement of the re-oxidation of ferrocytochrome $\underline{b}_5$ observed in the presence of stearoyl CoA or the anaesthetic agents (Tables 8 and 9), confirms that these inhibitors of cytochrome P-450 do not affect the activity of stearate desaturase.

The effects of enflurane and methoxyflurane on the NADPH-

and NADH-cytochrome c reductase activities are presented in Table 10. Neither enflurane nor methoxyflurane had a statistically significant effect on the NADPH-cytochrome c reductase. However, enflurane was found to significantly enhance the activity of NADH-cytochrome c reductase ($P < 0,01$) while methoxyflurane did not. Neither enflurane nor methoxyflurane had a significant effect on rate constants for the autoxidation of purified trypsin-cleaved ferrocytochrome b_5 (Table 10).

The rate constants for the re-oxidation of NADH-reduced ferrocytochrome b_5 in microsomes from fed, fasted or fasted, phenobarbitone treated rats are reported in Table 11 and these data are summarized in Table 12. Stearoyl CoA and enflurane increased k_{obs} in microsomes from fed rats, but not significantly in microsomes from fasted rats or fasted rats pretreated with phenobarbitone. Cyanide inhibited the ability of stearoyl CoA to enhance the re-oxidation of microsomal ferrocytochrome b_5 in microsomes from rats fed a normal diet, but not in microsomes from fasted or fasted, phenobarbitone treated rats. Cyanide had no significant effect on the re-oxidation of ferrocytochrome 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 enhancement of the re-oxidation of hepatic microsomal ferrocytochrome b_5 by stearoyl CoA (Oshino

et al., 1971)] and the ability of enflurane and methoxyflurane to stimulate the cyanide sensitive re-oxidation of NADH-reduced microsomal ferrocytochrome b_5 decreased in the following order : rats fed a high carbohydrate diet > fed a normal diet > fasted rats = fasted, phenobarbitone treated rats (Table 12).

The effects of increasing concentrations of enflurane and methoxyflurane on the rate constants for the re-oxidation of NADH-reduced microsomal ferrocytochrome b_5 are presented in Table 13 and Figure 12. These data were utilized to calculate K_{eq}, the equilibrium constants for the stimulation of microsomal electron transfer by these anaesthetic agents. The K_{eq} values are presented in Table 14 together with several equilibrium constants for the interaction of these anaesthetic agents with hepatic microsomal cytochrome P-450. Specifically, the K_s values for the binding of the anaesthetic agents to hepatic microsomal cytochrome P-450 and the K_m values for NADPH oxidation by hepatic microsomal cytochrome P-450 and the production of fluoride by cytochrome P-450 in the presence of enflurane and methoxyflurane, are presented in Table 14. The value of K_{eq} for the re-oxidation of cytochrome b_5 in the presence of enflurane differed from the K_s for the binding of enflurane to cytochrome P-450 and the K_m for NADPH oxidation by cytochrome P-450 in the presence of enflurane. The K_{eq}

for the re-oxidation of microsomal cytochrome $\underline{b}_5$ in the presence of methoxyflurane differed ($P < 0,01$) from the K_m for the stimulation of NADPH oxidation by methoxyflurane and from the high K_m for fluoride production from methoxyflurane by hepatic microsomal cytochrome P-450. The K_{eq} for methoxyflurane did not differ significantly from the K_s for binding of methoxyflurane to cytochrome P-450 or the low K_m for the production of fluoride ion from methoxyflurane by cytochrome P-450 ($P > 0,1$).

The effects of increasing concentrations of cyanide on the stimulation of the rate constants for the re-oxidation of NADH-reduced microsomal ferrocytochrome $\underline{b}_5$ by fixed concentrations of enflurane and methoxyflurane are shown in Table 15 and Figure 13. Increasing concentrations of cyanide resulted in greater extents of inhibition of the enhanced re-oxidation of cytochrome $\underline{b}_5$ observed in the presence of the anaesthetic agents. From these data, the K_i values for cyanide inhibition were determined to be $0,08 \pm 0,01$ mM for enflurane and $0,11 \pm 0,02$ 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 ferrocytochrome $\underline{b}_5$ by halothane, reported by Berman *et al.*, (1975), and with the 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.*, (1966).

Neither halothane, enflurane nor methoxyflurane had any effect on the stearate desaturase mediated conversion of stearoyl CoA to oleate ($P > 0,1$) (Table 16).

TABLE 8. THE EFFECT OF METYRAPONE ON THE ENHANCEMENT OF THE RATE CONSTANTS FOR THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME $\underline{b}_5$ BY STEAROYL CoA AND ANAESTHETIC AGENTS.

Additions (mM)	First order rate constant for the oxidation of ferrocytochrome $\underline{b}_5$ $10^2 k_{obs} (sec^{-1})$
None	$1,09 \pm 0,22$
Metyrapone (2,3)	$1,17 \pm 0,18$
Stearoyl CoA (0,012)	$4,19 \pm 0,59^{\dagger}$
Stearoyl CoA (0,012) + metyrapone (2,3)	$4,15 \pm 0,33^{\dagger}$
Halothane (18,0)	$1,68 \pm 0,15^{\dagger}$
Halothane (18,0) + metyrapone (2,3)	$2,03 \pm 0,48^{\dagger}$
Enflurane (14,0)	$2,23 \pm 0,36^{\dagger}$
Enflurane (14,0) + metyrapone (2,3)	$2,13 \pm 0,49^{\dagger}$
Methoxyflurane (1,0)	$1,78 \pm 0,18^{\dagger}$
Methoxyflurane (1,0) + metyrapone (2,3)	$1,70 \pm 0,12^{\dagger}$

† Differs significantly from "no additions" entry, $P < 0,01$

TABLE 9. THE EFFECT OF CO ON THE ENHANCEMENT OF THE RATE CONSTANTS FOR THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME $\underline{b}_5$ BY STEAROYL CoA AND ANAESTHETIC AGENTS.

Additions	First order rate constant for the oxidation of ferrocytochrome $\underline{b}_5$ $10^2 k_{obs}(sec^{-1})$
None	$0,97 \pm 0,15$
$CO:O_2$ (80:20; v/v)	$1,04 \pm 0,22$
Stearoyl CoA (0,012 mM)	$3,26 \pm 1,05^{\dagger}$
Stearoyl CoA (0,012 mM) + $CO:O_2$ (80:20; v/v)	$3,31 \pm 0,38^{\dagger}$
Halothane (18,0 mM)	$1,35 \pm 0,28^{\neq}$
Halothane (18,0 mM) + $CO:O_2$ (80:20; v/v)	$1,10 \pm 0,30$
Enflurane (14,0 mM)	$1,49 \pm 0,08^{\dagger}$
Enflurane (14,0 mM) + $CO:O_2$ (80:20; v/v)	$1,62 \pm 0,20^{\dagger}$
Methoxyflurane (1,0 mM)	$1,69 \pm 0,31^{\dagger}$
Methoxyflurane (1,0 mM) + $CO:O_2$ (80:20; v/v)	$1,56 \pm 0,42^{\dagger}$

† Differs significantly from "no additions" entry, $P < 0,01$

$^{\neq}$ Probably differs from "no additions" entry, $P < 0,05$

TABLE 10. THE EFFECT OF ANAESTHETIC AGENTS ON HEPATIC MICROSOMAL NADPH- AND NADH-CYTOCHROME *c* REDUCTASES AND ON THE OXIDATION OF PURIFIED TRYPSIN-CLEAVED FERROCYTOCHROME b_5.

Additions (mM)	Autoxidation of purified ferrocytochrome b_5 $10^2 k_{obs}$ (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)	0,89 ± 0,16	0,053 ± 0,005	1,34 ± 0,14†
Methoxyflurane (1,0)	0,97 ± 0,05	0,057 ± 0,001	1,09 ± 0,14

† Differs significantly from "no additions", $P < 0,01$

TABLE 11. THE EFFECT OF ANAESTHETIC AGENTS, STEAROYL CoA AND KCN ON THE RATE CONSTANTS FOR THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5 IN DIFFERENTLY PRETREATED RATS.

Additions (mM)	$10^2 k_{obs}$ (sec^{-1}) Pretreatment of rats: 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 (14,0)	2,28 ± 0,26*	2,23 ± 0,38≠	3,61 ± 0,94≠
Enflurane (14,0) + KCN (0,5)	2,08 ± 0,15	2,02 ± 0,39	3,50 ± 0,78
Methoxyflurane (1,0)	1,90 ± 0,27≠	1,86 ± 0,09	3,00 ± 0,27≠
Methoxyflurane (1,0) + 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

TABLE 11 (Cont.)

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* Differs significantly from "no additions" for similarly pretreated rats, $P < 0,001$

$\neq$ Probably differs significantly from "no additions", $P < 0,05$

TABLE 12. SUMMARY OF THE EFFECT OF ANAESTHETIC AGENTS, STEAROYL CoA AND KCN ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5 FROM DIFFERENTLY PRETREATED RATS.

Additions	% Increase in first order rate constant (k_{obs})							
	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 9 and 11. Percentages are relative to the corresponding value for the re-oxidation of cytochrome b_5 in the presence of NADH.

† Calculated from data of Berman et al., (1975).

TABLE 13. THE EFFECT OF VARIABLE CONCENTRATIONS OF ENFLURANE AND METHOXYFLURANE ON THE RATE CONSTANTS FOR THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5.

Additions (mM)	$10^2 k_{obs}$ (sec^{-1})
None	1,41 ± 0,13
Enflurane (0,28)	1,51 ± 0,09
Enflurane (0,83)	1,67 ± 0,17
Enflurane (1,93)	1,91 ± 0,10
Enflurane (2,74)	2,13 ± 0,14
None	1,27 ± 0,18
Methoxyflurane (0,29)	1,55 ± 0,15
Methoxyflurane (0,57)	1,81 ± 0,059
Methoxyflurane (0,86)	2,09 ± 0,00
Methoxyflurane (1,43)	2,20 ± 0,14

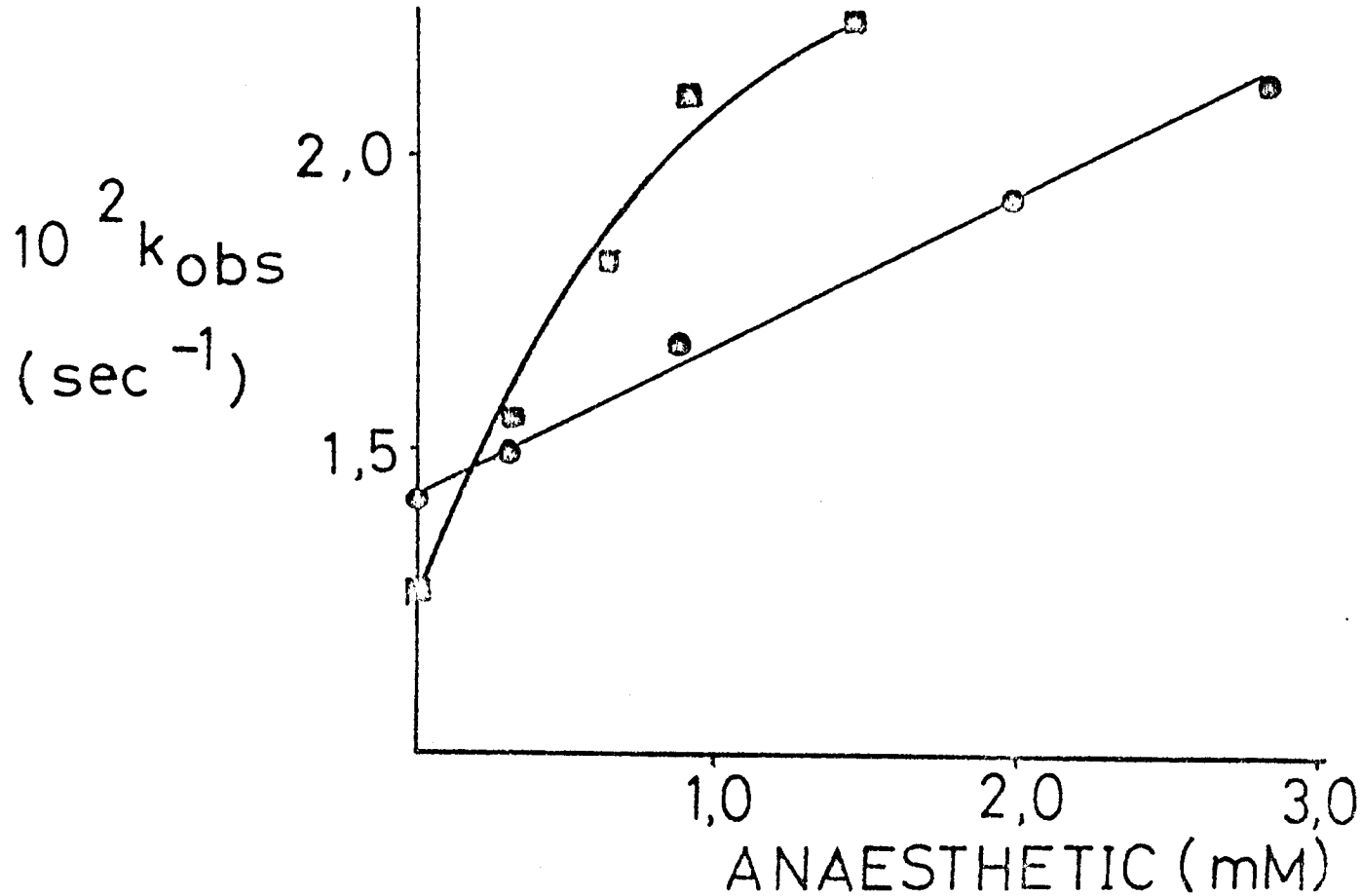


FIGURE 12. The effects of enflurane (●) and methoxyflurane (■) on the rate constants (k_{obs}) for the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome b_5.

TABLE 14. EQUILIBRIUM CONSTANTS FOR THE INTERACTION OF ANAESTHETIC 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 Ivanetich et al., (1979).

† Two K_m values were calculated from biphastic Eadie-Hofstee plots for this process, Ivanetich et al., (1979).

TABLE 15. THE EFFECT OF INCREASING CONCENTRATIONS OF KCN ON THE RATE CONSTANT FOR THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME $\underline{b}_5$ IN THE PRESENCE OF ENFLURANE AND METHOXYFLURANE.

KCN (mM)	Other additions to hepatic microsomes (mM)	Re-oxidation of ferrocytochrome $\underline{b}_5$ $10^2 k_{obs}(sec^{-1})$
0,0	Enflurane (1,4)	2,11 ± 0,0
0,06	Enflurane (1,4)	1,87 ± 0,15
0,12	Enflurane (1,4)	1,47 ± 0,25
0,25	Enflurane (1,4)	1,31 ± 0,05
0,50	Enflurane (1,4)	1,36 ± 0,06
0,0	Methoxyflurane (0,6)	1,86 ± 0,0
0,06	Methoxyflurane (0,6)	1,62 ± 0,0
0,12	Methoxyflurane (0,6)	1,40 ± 0,24
0,25	Methoxyflurane (0,6)	1,23 ± 0,04
0,50	Methoxyflurane (0,6)	1,07 ± 0,0

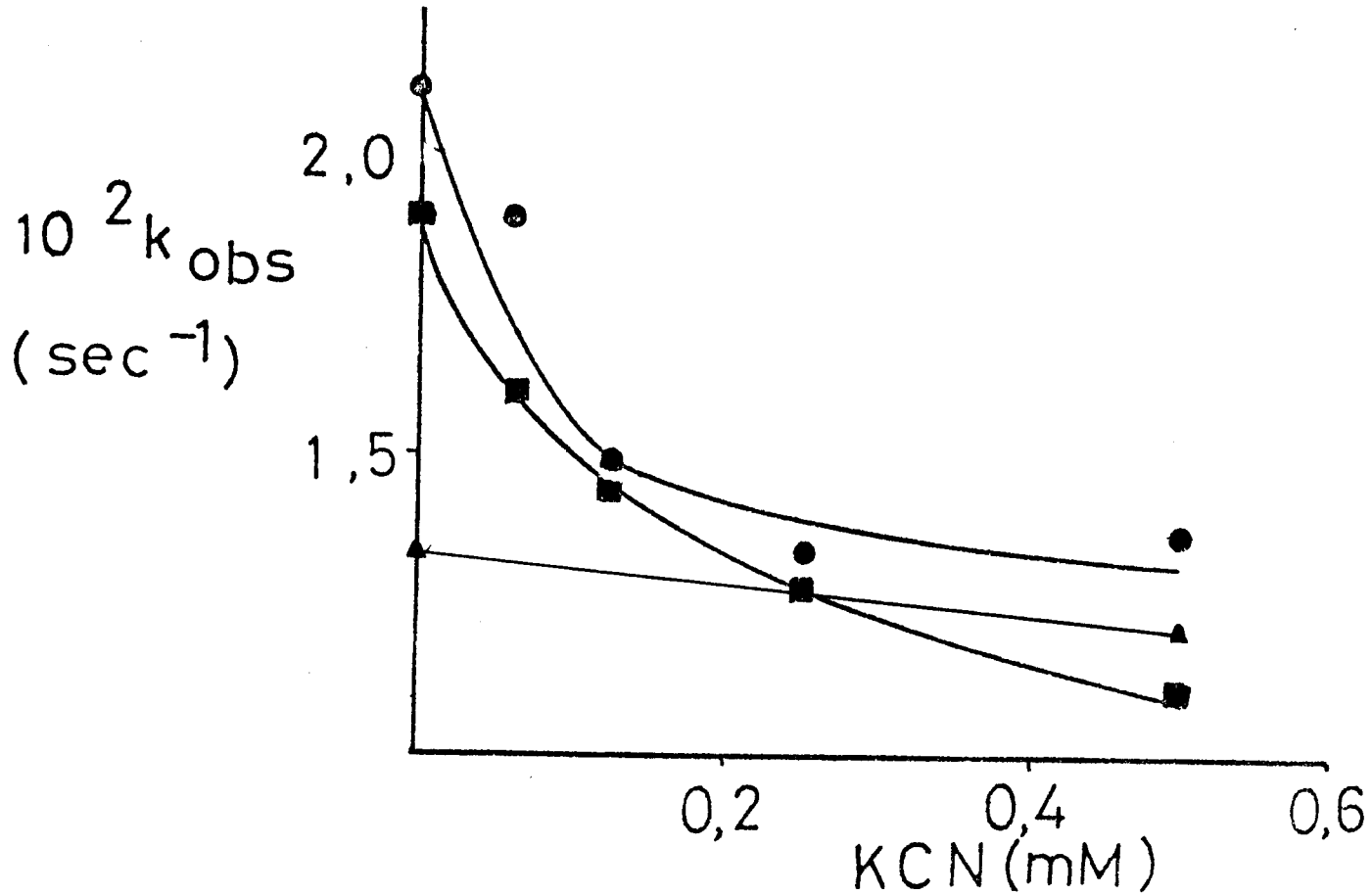


FIGURE 13 : The effect of KCN concentration on the rate constants (k_{obs}) for the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome b_5 in the presence of 1,4 mM enflurane (●) and 0,6 mM methoxyflurane (■) and in the absence of either anaesthetic agent (▲).

TABLE 16. THE EFFECT OF ANAESTHETIC AGENTS ON THE CONVERSION OF STEAROYL CoA TO OLEATE BY HEPATIC MICROSOMAL STEARATE DESATURASE.

Additions (mM)	$\frac{\text{oleate}}{\text{(oleate + stearate)}}$
None	0,34 ± 0,16
Halothane (18,0)	0,38 ± 0,14
Enflurane (14,0)	0,36 ± 0,12
Methoxyflurane (0,6)	0,35 ± 0,16

(2) Metabolism of anaesthetic agents by hepatic microsomal stearate desaturase and uncoupling of the stearate desaturase enzyme system by xenobiotics.

(a) Isolation of hepatic microsomal stearate desaturase, NADH-cytochrome b_5 reductase and cytochrome b_5 by detergent solubilization.

The three enzymes of the stearate desaturase enzyme system, viz. cytochrome b_5, NADH-cytochrome b_5 reductase and the stearate desaturase enzyme (C.S.F.) were isolated from the hepatic microsomes of rats re-fed a high carbohydrate diet, by the method of Shimakata et al., (1972). A typical elution profile of these proteins from the DEAE-Sephadex A-50 column used in the final purification step of this method is shown in Figure 14. Fractions 15 to 24 were pooled to obtain NADH-cytochrome b_5 reductase and fractions 26 to 35 were pooled to obtain cytochrome b_5. It was not possible to assay stearate desaturase activity in fractions eluted from the column because the activity of the enzyme in each of the 10 ml fractions was insufficient to be detected. On pooling fractions 1-12, where activity has been reported to occur by Shimakata et al., (1972) and concentrating by approximately 10 fold, stearate desaturase activity could be assayed in the presence of added purified detergent solubilized NADH-cytochrome b_5 reductase and cytochrome b_5.

The ability of the *in vitro* reconstituted stearate desaturase enzyme system and hepatic microsomes to convert stearoyl CoA to oleate is shown in Table 17. A comparison of the abilities of the reconstituted stearate desaturase enzyme system and hepatic microsomes to convert stearoyl CoA to oleate, indicates that the reconstituted system was functional, although less efficient than the microsomal system. The amount of C.S.F. protein employed in the reconstituted system was less than that used by Shimakata *et al.*, although the amounts of cytochrome b_5 and NADH-cytochrome b_5 reductase were in excess. Neither halothane, enflurane nor methoxyflurane appreciably affected the conversion of stearoyl CoA to oleate in the reconstituted stearate desaturase enzyme system (Table 17).

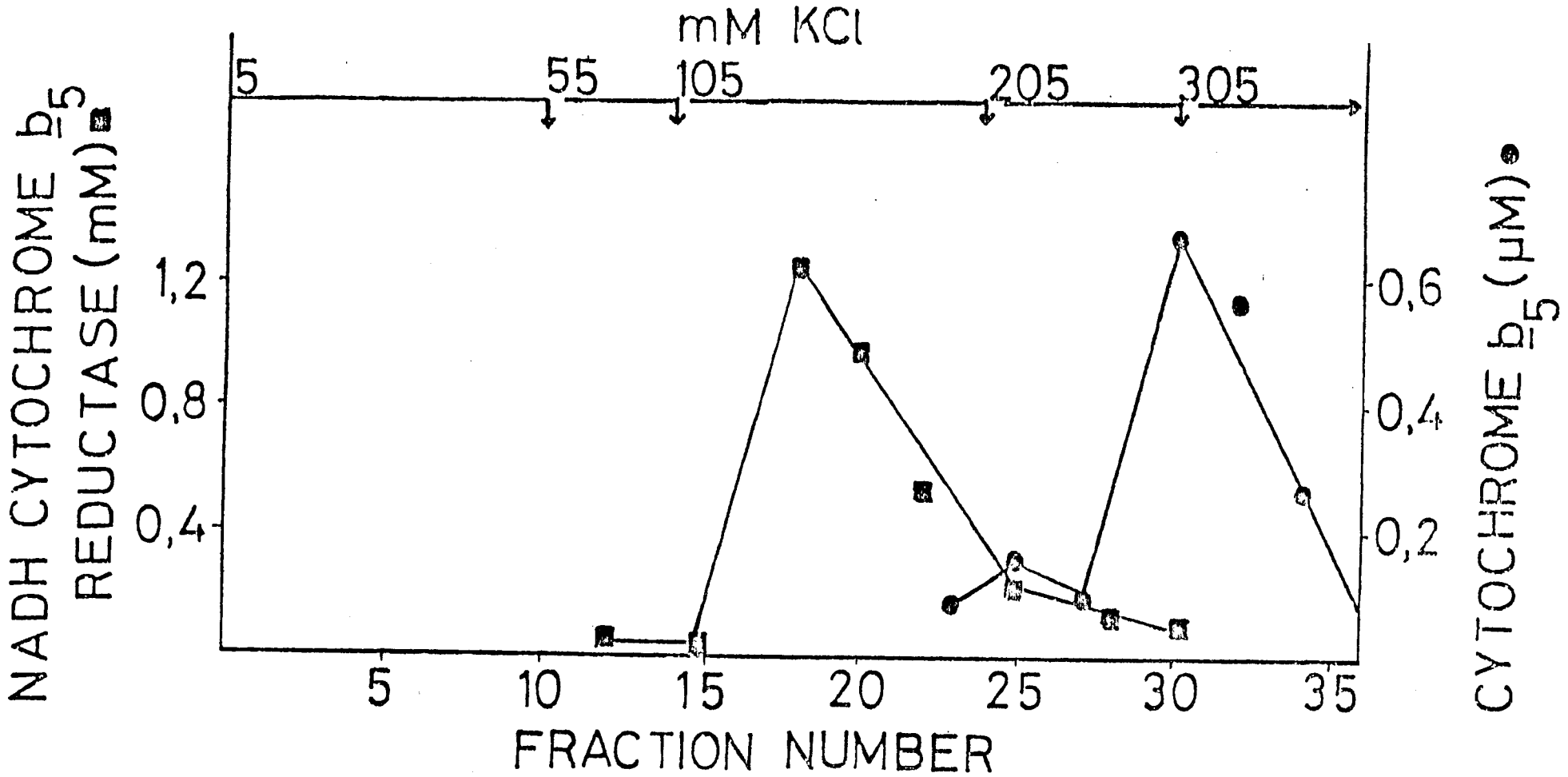


FIGURE 14 : Elution profile of hepatic microsomal enzymes from DEAE-Sephadex A-50 column, in the presence of increasing KCl concentrations. NADH-cytochrome b_5 reductase (■), cytochrome b_5 (●). Conditions of column elution described in Methods.

TABLE 17. STEAROYL CoA DESATURASE ACTIVITY OF THE RECONSTITUTED DETERGENT SOLUBILIZED STEARATE DESATURASE ENZYME SYSTEM AND OF HEPATIC MICROSOMES.

System	$\frac{\text{oleate}}{\text{(oleate + stearate)}}$	Stearate desaturase activity
Microsomes	0,46 ± 0,03	4,25 nmoles oleate formed/min/mg microsomal protein
Reconstituted (Preparation 1)	0,05 ± 0,03	1,50 nmoles oleate formed/min/mg C.S.F. protein
Reconstituted (Preparation 2)	0,10 ± 0,02	3,50 nmoles oleate formed/min/mg C.S.F. protein
Additions to reconstituted system (mM)		
None	0,05 ± 0,03	1,50 nmoles oleate formed/min/mg C.S.F. protein
Halothane (9,0)	0,03 ± 0,01	0,9 nmoles oleate formed/min/mg C.S.F. protein
Enflurane (7,0)	0,05 ± 0,01	1,5 nmoles oleate formed/min/mg C.S.F. protein
Methoxyflurane (3,0)	0,06 ± 0,04	1,8 nmoles oleate formed/min/mg C.S.F. protein

The reconstituted system contained 1 unit NADH-cytochrome $\underline{b}_5$ reductase, 0,5 nmoles cytochrome $\underline{b}_5$, 0,07 mg C.S.F. protein, 12 µCi (40 µM) [1-^{14}C]-stearoyl CoA in a total volume of 1 ml 0,30 M Tris-HCl, pH 7,0.

(b) Metabolism of three anaesthetic agents by the reconstituted stearate desaturase enzyme system.

The metabolism of the anaesthetic agents halothane, enflurane and methoxyflurane by stearate desaturase was assessed utilizing the reconstituted stearate desaturase enzyme system of Shimakata et al., (1972) which comprises the electron transfer proteins NADH-cytochrome b_5 reductase, cytochrome b_5 and the terminal oxidase stearate desaturase.* The possible metabolites of halothane, enflurane and methoxyflurane which were assayed include fluoride and bromide ions, acid-labile fluorine containing compounds and volatile halogenated derivatives.

Inasmuch as fluoride ions have been shown to be metabolites of enflurane, methoxyflurane and halothane, the production of fluoride ions and acid-labile fluorine compounds was assessed. A standard curve for the production of fluoride ion and acid-labile fluorine containing compounds, is shown in Figure 15. Acid-labile fluorine compounds were assayed by the difference between fluoride ion concentrations before and after treatment of the reaction mixtures with H_2SO_4 (see Methods). The amounts of fluoride ion and of acid-labile fluorine compounds produced from either 9 mM halothane, 7 mM enflurane or 3 mM methoxyflurane in the presence of the reconstituted stearate desaturase enzyme system

* The cytochrome P-450 content of the reconstituted system was less than 0,02 nmoles/mg protein.

were in all cases less than 1 μM.

Since bromide ions are known metabolites of halothane, the production of bromide ions was assessed. The standard curve for bromide ion is given in Figure 16. The release of bromide ions from halothane by the reconstituted stearate desaturase enzyme system was determined to be < 9 μM.

Inasmuch as halothane has been reported to give rise to volatile metabolites, and enflurane and methoxyflurane have been proposed to be converted to volatile metabolites, the production of volatile metabolites from these anaesthetic agents by the reconstituted stearate desaturase enzyme system, was assessed. No volatile intermediates have been detected for enflurane or methoxyflurane although several of the proposed intermediates of enflurane and methoxyflurane metabolism, such as the halo alcohols, could be volatile (see Figures 5 and 6).

Gas liquid chromatography was employed to assess the ability of the reconstituted stearate desaturase enzyme system to convert the anaesthetic agents to volatile metabolites. Two of the columns, viz. WHP Chromosorb/ SE 30 and Chromosorb P were used to detect compounds less volatile than the parent compounds, while the third column, Chromosorb 102 was used for the detection

of metabolites of halothane that are more volatile than the parent compound. Utilizing these three columns (see Methods for experimental details), the only compounds which were detected in incubation mixtures containing the anaesthetic agents, NADH and the reconstituted stearate desaturase enzyme system, were the anaesthetic agents themselves, as judged by their retention times. In the case of WHP Chromosorb/SE 30 column, the solvent ether, halothane, enflurane and methoxyflurane had retention times of ca. 13, 30, 25 and 20 seconds, respectively. In the case of enflurane, zero time incubations as well as incubations lacking NADH or containing KCN, were performed. Only enflurane could be detected. Using the Chromosorb P column, the solvent ether, halothane, enflurane and methoxyflurane had retention times of ca. 60, 120, 82 and 322 seconds, respectively. Incubation mixtures containing the anaesthetic agents as well as from zero time incubations and those containing KCN or lacking NADH, produced only an ether peak at 53 seconds and a peak having a comparable retention time to that of the anaesthetic agent in question. The Chromosorb 102 column was utilized to assess the metabolism of halothane only. Injections from incubation mixtures of the reconstituted system had only one peak at 505 seconds, which corresponds exactly to the retention time of halothane. Therefore, no volatile metabolites appeared to be produced from the interaction of the anaesthetic agents with the reconstituted stearate desaturase enzyme system.

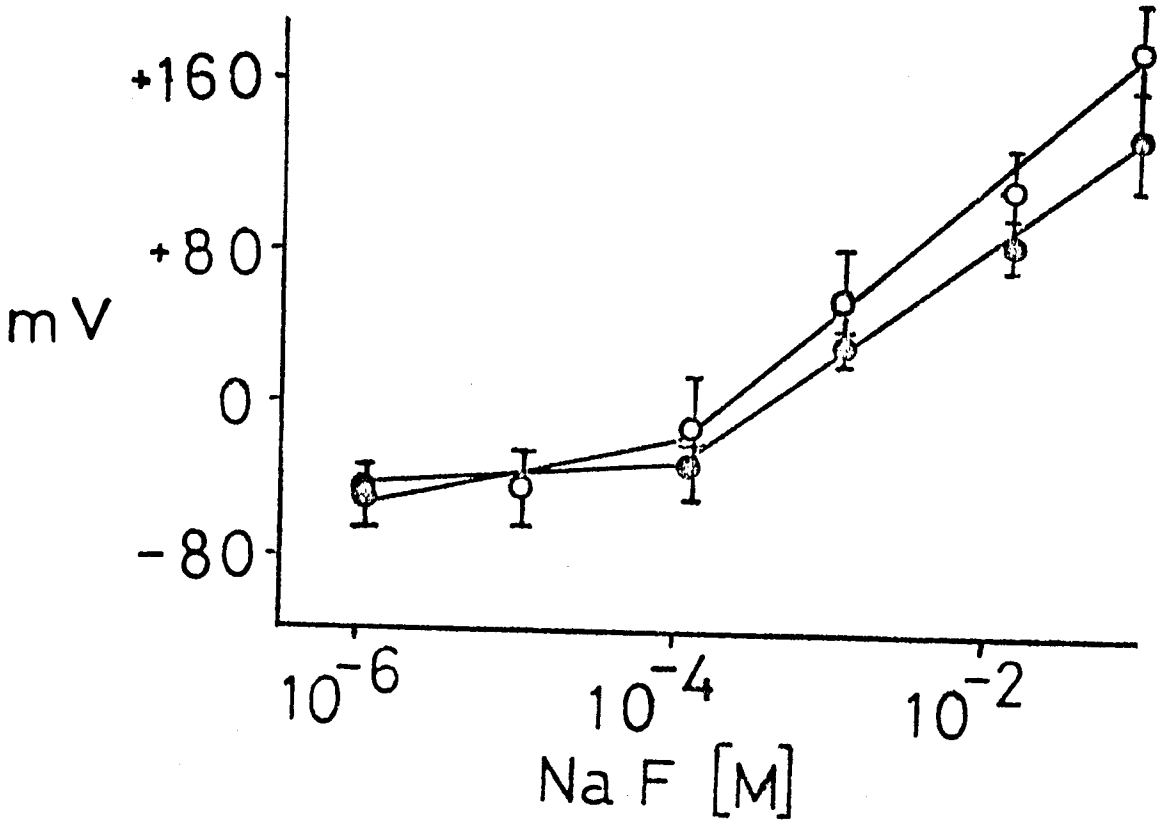


FIGURE 15. Standard curve for the production of fluoride ion (o) and acid-labile fluorine compounds (•).

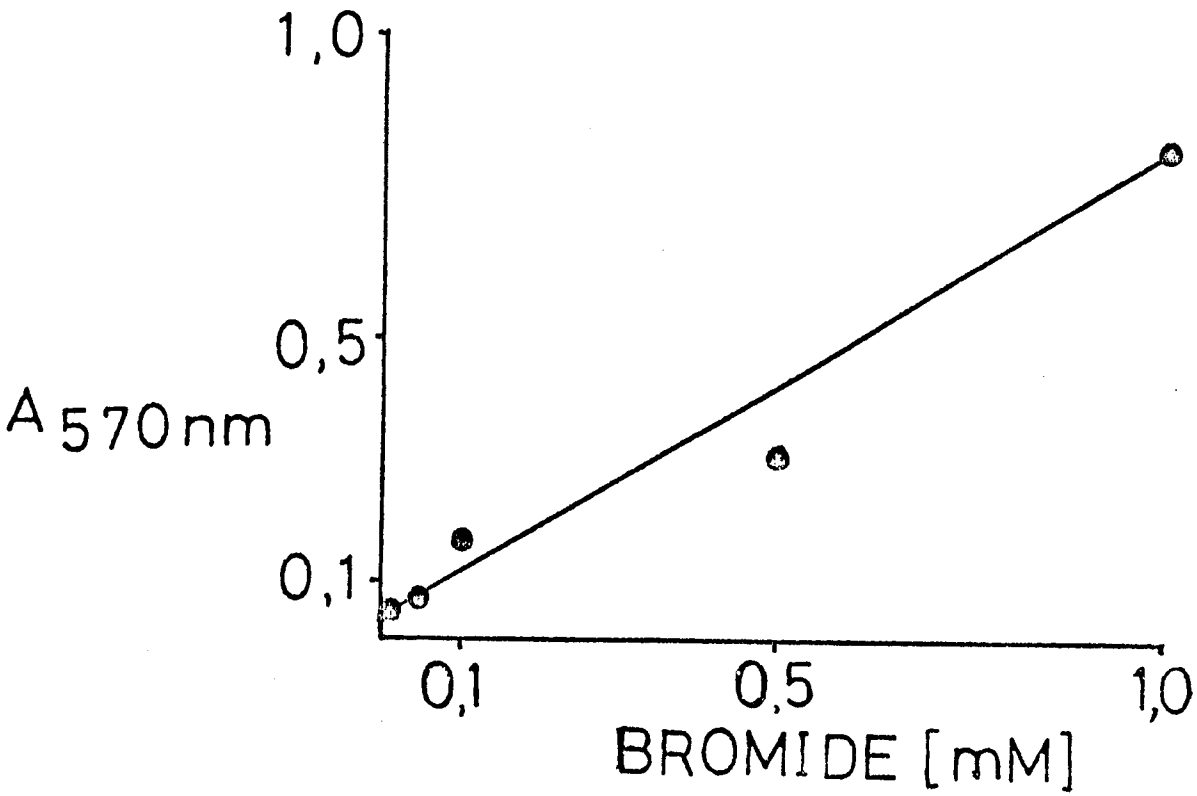


FIGURE 16. Standard curve for the production of bromide ion.

(c) The effect of chemical and physical treatment on the levels of hepatic microsomal enzymes and heme.

Hepatic microsomes isolated from rats with elevated levels of stearate desaturase were treated by either physical or chemical means in an attempt to selectively decrease the levels and activity of cytochrome P-450 in these microsomes, without affecting the activity of stearate desaturase. The activity of stearate desaturase was assessed via two methods viz. the re-oxidation of hepatic microsomal ferrocytochrome b_5 and by the conversion of [1-^{14}C]-stearoyl CoA to [1-^{14}C]-oleate. The increase in k_{obs} in the presence of stearoyl CoA versus its absence was taken as a measure of the stearate desaturase activity of the microsomes.

The effect of storage.

The effect of storing hepatic microsomes at 4^{o}C on cytochrome P-450 levels and stearate desaturase activity is reported in Table 18. After one week at 4^{o}C, the levels of cytochrome P-450 were significantly lowered compared to the levels in freshly prepared microsomes. The stearate desaturase activity was completely eliminated by storage. After two weeks storage at 4^{o}C, the cytochrome P-450 levels were further depressed and there was no stearate desaturase activity in these microsomes.

The choice of chemicals.

The following chemicals (viz. dioxane, sodium iodide, iso-butanol, potassium thiocyanate) were chosen on the basis of reports that they can decrease the content and activity of hepatic microsomal cytochrome P-450 in vitro, (Imai and Sato, 1967; Ichikawa et al., 1968). The chemicals were added to the purified hepatic microsomes at various stages during their preparation, in an attempt to selectively decrease cytochrome P-450 levels while not affecting the activity of stearate desaturase.

The effect of dioxane.

The effect of the addition of 1 M and 2 M dioxane to hepatic microsomes for up to 90 min at 30°C on the activity of microsomal stearate desaturase and the levels of cytochrome P-450 was assessed. The activity of stearate desaturase in the hepatic microsomes was found to decrease with time in the presence of dioxane. A greater decrease in stearate desaturase activity was observed in the presence of the higher concentration of dioxane (Table 19). The levels of cytochrome P-450 in the hepatic microsomes were also reduced significantly in the presence of dioxane, but cytochrome b_5 levels remained constant until 90 minutes of incubation when the cytochrome b_5 content appeared to be significantly lowered (Table 20). There is also a significant decrease

with time in the heme content of these microsomes. The observed decrease in cytochromes P-450 and b_5 appeared to be due to the degradation of the heme component of these proteins. Although the cytochrome P-450 levels were significantly lowered by dioxane treatment, stearate desaturase activity was also decreased. These microsomes could therefore not be used to assay the metabolism of anaesthetic agents by stearate desaturase as an essential criterion in the treatment of the microsomes was to maintain stearate desaturase activity.

The effect of sodium iodide and iso-butanol.

Sodium iodide and iso-butanol were added to the post mitochondrial supernatant during the isolation of hepatic microsomes from rats re-fed a high carbohydrate diet. Sodium iodide did not affect stearate desaturase activity, but did convert cytochrome P-450 to cytochrome P-420 (Tables 21 and 22). This observation was not pursued however, because sodium iodide was found to solubilize the microsomes.

Iso-butanol (10% v/v) had no significant effect on the activity of hepatic microsomal stearate desaturase or the content of cytochrome b_5 or heme. The cytochrome P-450 levels were significantly lowered by iso-butanol, although there was still 35% remaining. Iso-butanol

(20% v/v) did not affect the cytochrome b_5 or heme content of the microsomes, but did lower the cytochrome P-450 levels and correspondingly decreased the cytochrome P-450 mediated demethylation of p-nitroanisole (Table 22). Stearate desaturase activity in the presence of 20% v/v iso-butanol could not be assayed indirectly, namely via the re-oxidation of hepatic microsomal ferrocytochrome b_5 in the presence of stearoyl CoA (Table 21). However, when the activity of stearate desaturase was assessed directly, viz. via the conversion of stearoyl CoA to oleate, the activity of this enzyme system was decreased by only 50% (Table 21). This discrepancy in the effect of chemicals on the activity of the stearate desaturase enzyme system, when assayed by two methods, is not consistent with the results of Oshino (Oshino et al., 1971), who reported comparable activities when stearate desaturase was assayed via the re-oxidation of cytochrome b_5 and via the conversion of stearate to oleate. However, this discrepancy appears to be due to an effect of 20% iso-butanol on the ability of cytochrome b_5 to transfer electrons to microsomal proteins such as stearate desaturase, since there is no measurable microsomal electron transfer in the absence of stearoyl CoA (Table 21).

The effect of iodomethane.

Stearate desaturase activity, as determined by the re-oxidation of NADH reduced microsomal ferrocytochrome $\underline{b}_5$ in the presence of stearoyl CoA, appeared to be eliminated by iodomethane treatment, although, as shown in Table 23, the conversion of stearoyl CoA to oleate was only decreased by 50%. Iodomethane appeared to enhance electron transfer through cytochrome $\underline{b}_5$ in the absence of stearoyl CoA (Table 23). This enhanced background rate of electron flow through cytochrome $\underline{b}_5$ is comparable to that observed with microsomes not treated with iodomethane, in the presence of stearoyl CoA (Table 2). Under these circumstances, the re-oxidation of cytochrome $\underline{b}_5$ is not a reliable method of assessing stearate desaturase activity as no enhanced rate of re-oxidation of ferrocytochrome $\underline{b}_5$ can be observed if the background rate is already stimulated to levels comparable to that seen in the presence of stearoyl CoA. The conversion of stearoyl CoA to oleate indicated that although the activity of the stearate desaturase enzyme system is decreased, the enzyme system is still active. In fact, the conversion of stearoyl CoA to oleate in iodomethane treated microsomes, is two to four times greater than that obtained with the reconstituted detergent solubilized stearate desaturase enzyme system (Tables 17 and 23).

Iodomethane significantly lowered cytochrome P-450 levels and this appears to be due to degradation of the heme moeity of this enzyme as the loss of heme parallels the decrease in cytochrome P-450 in these microsomes (Table 24). The low cytochrome P-450 content is confirmed by the correspondingly decreased activity of p-nitroanisole demethylase and benzpyrene hydroxylase. Cytochrome b_5 levels were not decreased by iodomethane treatment, but appeared to be enhanced.

The effect of potassium thiocyanate.

Potassium thiocyanate does not significantly decrease stearate desaturase activity when assessed via the re-oxidation of cytochrome b_5. The activity is significantly lowered when assessed via the conversion of stearoyl CoA to oleate (Table 23). The activity of stearate desaturase in the potassium thiocyanate treated microsomes is 65 - 70% of that observed in untreated microsomes, regardless of which assay method is used.

Cytochrome P-450 levels were significantly reduced by potassium thiocyanate, due in part to the degradation of the heme moiety of the cytochrome (Table 25). The conversion of cytochrome P-450 to cytochrome P-420 is confirmed by the low rate observed for the demethylation of p-nitroanisole (Table 25). Cytochrome b_5 levels

appear to be slightly elevated, a phenomenon of no known explanation, which has been reported to occur in the presence of certain compounds (Wade et al., 1972; Ivanetich et al., 1978).

The treatment of microsomes with either 0,27 M iodomethane or 1 M potassium thiocyanate appeared to be most successful in selectively decreasing cytochrome P-450 levels while not significantly affecting stearate desaturase activity . These microsomes, treated with iodomethane or potassium thiocyanate were therefore used to assess the ability of stearate desaturase to metabolize the anaesthetic agents halothane, enflurane and methoxyflurane as their metabolism by cytochrome P-450 would be negligible in these microsomes.

TABLE 18. THE EFFECT OF STORING HEPATIC MICROSOMES AT 4°C ON CYTOCHROME P-450 LEVELS AND STEARATE DESATURASE ACTIVITY.

Time of storage at 4°C	Cytochrome P-450 (nmoles/mg microsomal protein)	(% decrease)	Re-oxidation of ferrocytochrome b_5 Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
Zero	1,00 ± 0,04	-	None	1,00 ± 0,01	97 ± 0,04
			Stearoyl CoA (0,012)	1,97 ± 0,05	
1 week	0,50 ± 0,01†	50	None	0,78 ± 0,24	0,08 ± 0,26≠
			Stearoyl CoA (0,012)	0,86 ± 0,02	
2 weeks	0,34 ± 0,03†	34	None	0,53 ± 0,07	-0,36 ± 0,16†
			Stearoyl CoA (0,012)	0,17 ± 0,23	

Assays were in duplicate on a single preparation of hepatic microsomes.

† Differs significantly from freshly prepared microsomes (zero time samples), $P < 0,01$

≠ Probably differs from freshly prepared microsomes (zero time samples), $P < 0,05$

TABLE 19. THE EFFECT OF DIOXANE ON THE ACTIVITY OF HEPATIC MICROSOMAL STEARATE DESATURASE.

Additions to microsomes	Time (min)	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA)
		Additions to assay medium	$10^2 k_{obs}$ (sec^{-1})	$10^2 k_{obs}$ (sec^{-1})
None	0	None	1,20 ± 0,27	3,35 ± 0,76
		Stearoyl CoA	4,55 ± 0,49	
1,0 M Dioxane	90	None	0,93 ± 0,0	2,49 ± 0,69
		Stearoyl CoA	3,42 ± 0,69	
None	0	None	1,33 ± 0,27	3,80 ± 0,80
		Stearoyl CoA	5,13 ± 0,76	
2,0 M Dioxane	30	None	1,41 ± 0,09	3,26 ± 0,18
		Stearoyl CoA	4,37 ± 0,52	
	60	None	1,60 ± 0,19	2,18 ± 0,61
		Stearoyl CoA	3,78 ± 0,42	

TABLE 19 (Cont.)

Additions to microsomes	Time (min)	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA)
		Additions to assay medium	10 k_{obs} (sec^{-1})	$10^2 k_{obs}$ (sec^{-1})
	90	None	1,34 ± 0,48	0,88 ± 0,27*
		Stearoyl CoA	2,22 ± 0,56	

Stearoyl CoA was used at a concentration of 0,012 mM

* Differs significantly from untreated microsomes (zero time samples), $P < 0,001$

TABLE 20. THE EFFECT OF DIOXANE ON HEPATIC MICROSOMAL CYTOCHROME P-450, CYTOCHROME b_5 AND HEME.

Additions to microsomes	Time incubated (min)	Cytochrome P-450 (nmoles/mg microsomal protein)	(% decrease)	Cytochrome b_5 (nmoles/mg microsomal protein)	(% decrease)	Heme (nmoles/mg microsomal protein)	(% decrease)	% loss heme / loss cytochrome P-450
None	0	1,09 ± 0,06	-	0,60 ± 0,01	-	1,85 ± 0,07	-	
1,0 M Dioxane	90	0,74 ± 0,10≠	32	0,57 ± 0,01	5	1,30 ± 0,14≠	30	30/32
None	0	1,03 ± 0,09	-	0,61 ± 0,02	-	1,70 ± 0,18	-	
2,0 M Dioxane	30	0,55 ± 0,02†	46	0,60 ± 0,01	2	0,65 ± 0,10†	62	62/46
	60	0,48 ± 0,08†	53	0,52 ± 0,01	15	0,63 ± 0,13†	63	63/53
	90	0,36 ± 0,09*	65	0,44 ± 0,02*	28	0,88 ± 0,05*	48	48/65

* Differs significantly from untreated microsomes, $P < 0,001$

† Differs significantly from untreated microsomes, $P < 0,01$

≠ Probably differs from untreated microsomes, $P < 0,05$

TABLE 21. THE EFFECT OF SODIUM IODIDE AND ISO-BUTANOL ON THE ACTIVITY OF HEPATIC MICROSOMAL STEARATE DESATURASE.

Additions to microsomes	Additions to assay medium	$10^2 k_{obs}$ (sec^{-1})	Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})	$\frac{\text{oleate}}{(\text{oleate + stearate})}$
None	None	$1,30 \pm 0,15$		-
	Stearoyl CoA	$4,82 \pm 0,77$	$3,57 \pm 0,85$	$0,55 \pm 0,02$
2 M Sodium Iodide	None	$1,54 \pm 0,13$		-
	Stearoyl CoA	$4,66 \pm 2,56$	$3,12 \pm 2,4$	
10% (v/v) Iso-butanol	None	$0,98 \pm 0,29$		-
	Stearoyl CoA	$4,10 \pm 0,53$	$3,12 \pm 0,59$	
20% (v/v) Iso-butanol	None	-*	-	
	Stearoyl CoA	-*		$0,25 \pm 0,06^{\neq}$

TABLE 21 (Cont.)

Stearoyl CoA used at a concentration of 0,012 mM

≠ Probably differs from untreated microsomes, $P < 0,05$

* Not measurable

TABLE 22. THE EFFECT OF SODIUM IODIDE AND ISO-BUTANOL ON THE LEVELS OF HEPATIC MICROSOMAL CYTOCHROMES AND HEME AND THE ACTIVITY OF CYTOCHROME P-450.

Additions to microsomes	Demethylation of p-nitro-anisole		Cytochrome P-450		Cytochrome b_5		Heme	
	(nmoles/min/mg microsomal protein)	% (decrease in activity)	(nmoles/mg microsomal protein)	% (decrease)	(nmoles/mg microsomal protein)	% (decrease)	(nmoles/mg microsomal protein)	% (decrease)
None	0,20 ± 0,09	-	0,98 ± 0,04	-	0,69 ± 0,04	-	1,59 ± 0,17	-
2 M Sodium iodide	-	-	0,02 ± 0,03*	98	-	-	-	-
10% (v/v) Iso-butanol	-	-	0,34 ± 0,17†	65	0,79 ± 0,0	0	2,10 ± 0,0	0
20% (v/v) Iso-butanol	0,04 ± 0,06≠	80	0,15 ± 0,10*	85	0,64 ± 0,29	7	1,71 ± 0,58	0

TABLE 22 (Cont.)

* Differs significantly from untreated microsomes, $P < 0,001$

† Differs signficantly from untreated microsomes, $P < 0,01$

≠ Probably differs from untreated microsomes, $P < 0,05$

TABLE 23. THE EFFECT OF IODOMETHANE AND POTASSIUM THIOCYANATE ON THE ACTIVITY OF HEPATIC MICROSOMAL STEARATE DESATURASE.

Additions to microsomes	Additions to assay medium	Re-oxidation of ferrocytochrome b_5 $10^2 k_{obs}$ (sec^{-1})	Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})	oleate / (oleate + stearate)
None	None	1,12 ± 0,32		0,49 ± 0,09
	Stearoyl CoA	3,67 ± 0,69	2,55 ± 0,43	
0,27 M	None	3,38 ± 0,49		0,23 ± 0,04*
Iodomethane	Stearoyl CoA	3,77 ± 1,09	0,39 ± 0,90*	
1 M Potassium	None	1,25 ± 0,37		0,38 ± 0,04*
Thiocyanate	Stearoyl CoA	2,91 ± 0,79	1,66 ± 0,78≠	

Stearoyl CoA was used at concentration of 0,012 mM

* Differs significantly from untreated microsomes, $P < 0,001$

≠ Probably differs from untreated microsomes, $P < 0,05$

TABLE 24. THE EFFECT OF IODOMETHANE ON HEPATIC MICROSOMAL ENZYMES AND HEME.

Assay	Additions to microsomes	
	None	0,27 M Iodomethane
Demthylation of p-nitroanisole		
(nmoles/mg microsomal protein) :	0,47 ± 0,07	0,095 ± 0,04*
% decrease in activity :	-	80
Hydroxylation of benzpyrene		
(nmoles/mg microsomal protein) :	0,067 ± 0,04	0,019 ± 0,02*
% decrease in activity	-	72
Cytochrome P-450		
(nmoles/mg microsomal protein) :	0,94 ± 0,08	0,037 ± 0,02*
% decrease :	-	96

TABLE 24 (Cont.)

Assay	Additions to microsomes	
	None	0,27 M Iodomethane
Cytochrome b_5		
(nmoles/mg microsomal protein) :	0,65 ± 0,03	1,04 ± 0,17
% decrease :	-	0
Heme		
(nmoles/mg microsomal protein) :	1,55 ± 0,10	0,83 ± 0,18*
% decrease :	-	46

* Differs significantly from untreated microsomes, $P < 0,001$

† Differs significantly from untreated microsomes, $P < 0,01$

TABLE 25. THE EFFECT OF POTASSIUM THIOCYANATE ON HEPATIC MICROSOMAL ENZYMES AND HEME.

Additions to microsomes	Demethylation of p-nitroanisole		Cytochrome P-450		Cytochrome b_5		Heme	
	(nmoles/min/mg microsomal protein)	% (decrease in activity)	(nmoles/mg microsomal protein)	% (decrease)	(nmoles/mg microsomal protein)	% (decrease)	(nmoles/mg microsomal protein)	% (decrease)
None	0,20 ± 0,09	-	0,98 ± 0,04	-	0,69 ± 0,02	-	1,59 ± 0,17	-
1 M Potassium thiocyanate	0,05 ± 0,05≠	75	0,06 ± 0,04*	94	0,85 ± 0,09†	0	1,03 ± 0,07*	35

* Differs significantly from untreated microsomes, $P < 0,001$

† Differs significantly from untreated microsomes, $P < 0,01$

≠ Probably differs from untreated microsomes, $P < 0,05$

(d) Metabolism of three anaesthetic agents by stearate desaturase in hepatic microsomes with negligible levels of cytochrome P-450.

The metabolism of the anaesthetic agents halothane, enflurane and methoxyflurane by stearate desaturase was assessed in hepatic microsomes previously treated with either iodomethane or potassium thiocyanate to reduce cytochrome P-450 levels. In these preparations, the metabolism of the anaesthetic agents by stearate desaturase could be monitored in the absence of cytochrome P-450 dependent metabolism of these anaesthetic agents.

A standard curve for the production of fluoride ion and acid-labile fluorine containing compounds is given in Figure 17. It can be seen in Table 26 that less than 1 μM fluoride ion and acid-labile fluorine containing compounds was released from halothane, enflurane and methoxyflurane by stearate desaturase in hepatic microsomes with elevated levels of this enzyme. Neither the iodomethane treated microsomes nor the potassium thiocyanate treated microsomes produced any detectable fluoride ion or acid-labile fluorine containing compounds (< 1 μM) on incubation with halothane, enflurane or methoxyflurane.

The standard curve for bromide production is given in

Figure 18. The release of bromide ions from 18 mM halothane by stearate desaturase was determined to be < 9 μM in hepatic microsomes from rats re-fed a high carbohydrate diet as well as in hepatic microsomes treated with iodomethane or potassium thiocyanate.

To detect volatile metabolites of the anaesthetic agents, a WHP Chromosorb/SE 30 column was utililized (see Methods). The retention times of halothane, enflurane and methoxyflurane were determined to be ca. 25, 19 and 25 seconds, respectively. Hepatic microsomes from rats re-fed a high carbohydrate diet and treated with either iodomethane or potassium thiocyanate were utilized. Injections from zero time or forty minute incubations containing either of the anaesthetic agents produced peaks having comparable retention times to the anaesthetic agents alone. It appeared therefore that no volatile metabolites are produced by stearate desaturase from the anaesthetic agents as no peaks corresponding to any metabolites were observed.

The results of the production of non-volatile metabolites of halothane by stearate desaturase are reported in Table 27. No non-volatile metabolites could be detected from the stearate desaturase mediated metabolism of halothane by assessing metabolism in hepatic microsomes from rats re-fed a high carbohydrate diet and treated with either iodomethane or potassium thiocyanate.

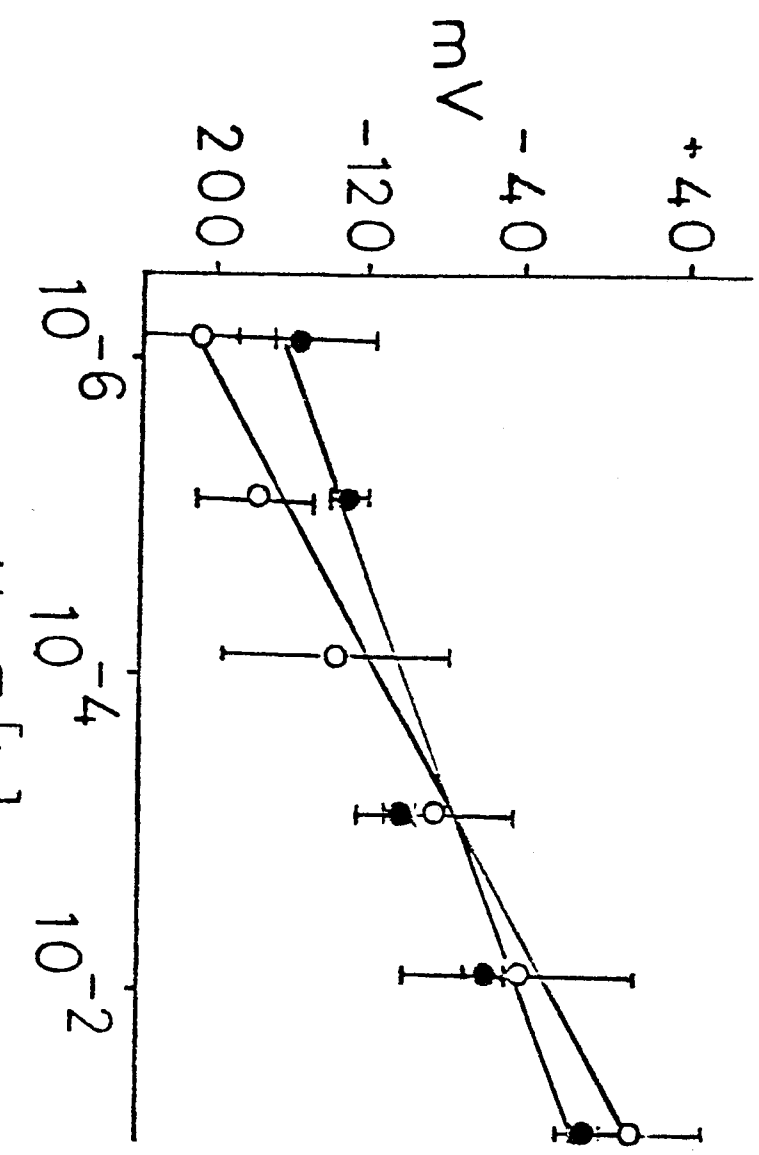


FIGURE 17 : Standard curve for the production of fluoride ion and acid-labile fluorine compounds. Fluoride ion (o), acid-labile fluorine compounds (●).

TABLE 26. RELEASE OF FLUORIDE ION AND ACID-LABILE FLUORINE COMPOUNDS FROM ANAESTHETIC AGENTS.

Pretreatment of microsomes	Additions to microsomes (mM)	mV	fluoride ion (μM)
None	Halothane (18,0)	-233 ± 16	<1
	Enflurane (14,0)	-233 ± 12	<1
	Methoxyflurane (0,6)	-230 ± 11	<1
Iodomethane (0,27 M)	Halothane (18,0)	-229 ± 19	<1
	Enflurane (14,0)	-235 ± 25	<1
	Methoxyflurane (0,6)	-240 ± 24	<1
Potassium thiocyanate (1 M)	Halothane (18,0)	-240 ± 26	<1
	Enflurane (14,0)	-244 ± 30	<1
	Methoxyflurane (0,6)	-246 ± 30	<1

			Acid-labile fluorine compounds (μM)
None	Halothane (18,0)	-210 ± 19	<1
	Enflurane (14,0)	-217 ± 15	<1
	Methoxyflurane (0,6)	-218 ± 15	<1
Iodomethane (0,27 M)	Halothane (18,0)	-234 ± 6	<1
	Enflurane (14,0)	-240 ± 4	<1
	Methoxyflurane (0,6)	-241 ± 3	<1

TABLE 26 (Cont.)

Pretreatment of microsomes	Additions to microsomes (mM)	mV	Acid-labile fluorine compounds (μM)
Potassium thiocyanate (1 M)	Halothane (18,0)	-247 ± 3	<1
	Enflurane (14,0)	-234 ± 17	<1
	Methoxyflurane (0,6)	-245 ± 8	<1

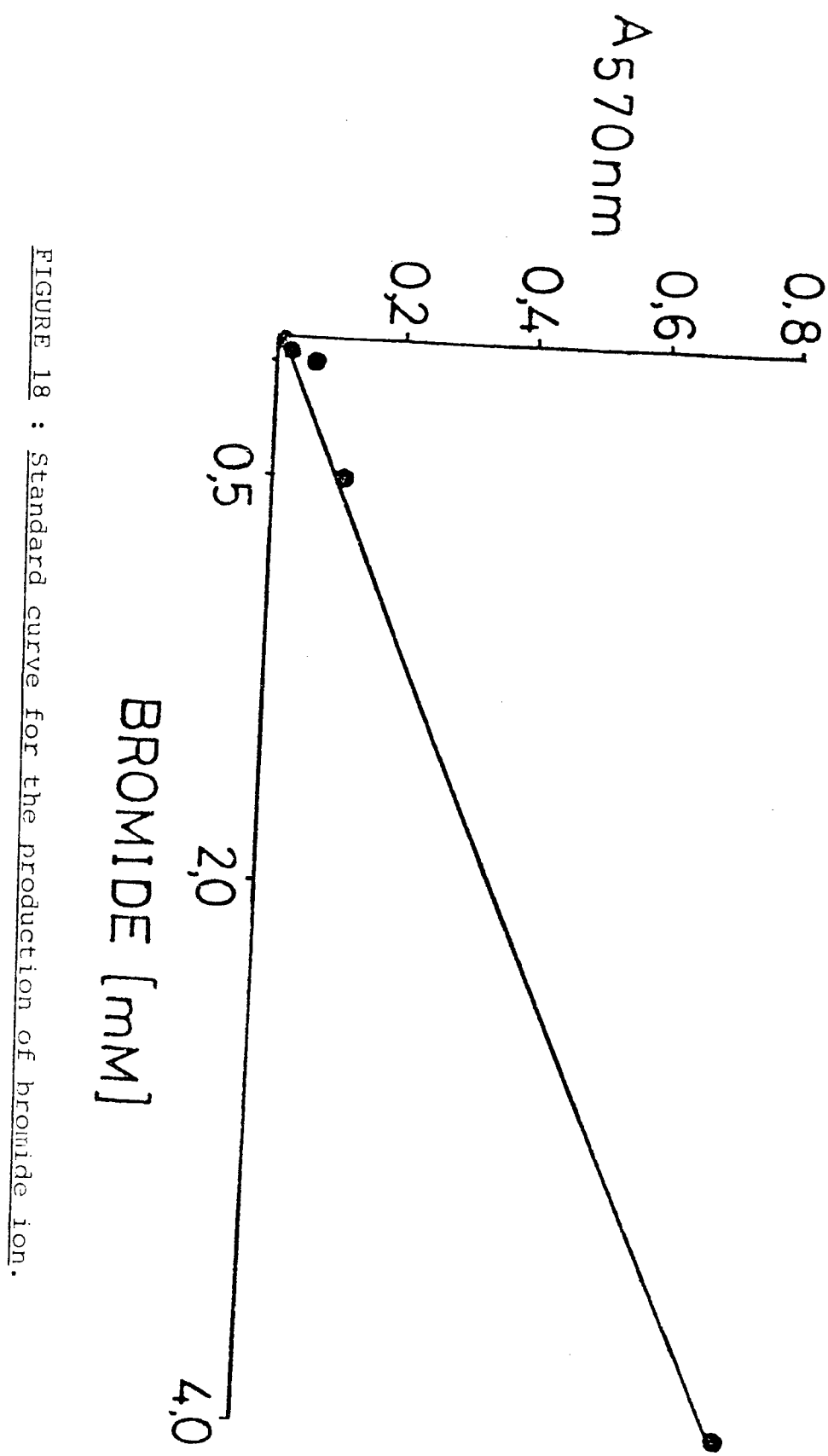


FIGURE 18 : Standard curve for the production of bromide ion.

TABLE 27. THE PRODUCTION OF NON-VOLATILE METABOLITES OF HALOTHANE BY THE STEARATE DESATURASE ENZYME SYSTEM IN HEPATIC MICROSOMES.

Treatment of microsomes	Additions*	dpm ^{14}C (ether)	dpm ^{14}C (microsomes)
	Halothane (2 µCi; 888 000 dpm)		503 542 ± 5 043
None	Halothane (2 µCi; 888 000 dpm)	332 395 ± 18 612	27 542 ± 6 851 Δ
	NADH (0,4 mM)	320 ± 112	579 ± 4
	Halothane (2 µCi; 888 000 dpm) + NADH (0,4 mM)	302 481 ± 93 730	22 158 ± 7 978 Δ
Iodomethane (0,27 M)	Halothane (2 µCi; 888 000 dpm)	248 876 ± 89 651	19 903 ± 2 881 Δ
	NADH (0,4 mM)	262 ± 88	83 ± 6
	Halothane (2 µCi; 888 000 dpm) + NADH (0,4 mM)	320 451 ± 59 253	23 076 ± 9 196 Δ

TABLE 27 (Cont.)

Treatment of microsomes	Additions	dpm ^{14}C (ether)	dpm ^{14}C (microsomes)
Potassium thiocyanate (1 M)	Halothane (2 µCi; 888 000 dpm)	303 790 ± 57 393	8 305 ± 1 495$^{\Delta}$
	NADH (0,4 mM)	282 ± 87	62 ± 31
	Halothane (2 µCi; 888 000 dpm) + NADH (0,4 mM)	282 582 ± 780	11 076 ± 1 310$^{\Delta}$

* To hepatic microsomes from rats fed a high carbohydrate diet (3 ml; 1,5 mg protein/ml)

$^{\Delta}$ The counts represent ^{14}C-CF_3COOH contamination of ^{14}C-halothane.

In an analagous experiment, 18 669 ± 4974 dpm of ^{14}C-CF_3COOH from ^{14}C-halothane were extracted from hepatic microsomes after acidification of the microsomes.

(e) The production of active oxygen species in hepatic microsomes.

The anaesthetic agents halothane, enflurane and methoxyflurane and the xenobiotics bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde stimulate microsomal electron transfer through cytochrome b_5 apparently due to their interaction with stearate desaturase (sections 1b and 1c). Neither halothane, enflurane nor methoxyflurane are metabolized by stearate desaturase (sections 2b and 2d). The enhanced microsomal electron flow observed and the lack of metabolism of the anaesthetic agents suggested that the anaesthetic agents might uncouple the stearate desaturase system. The electrons which are transferred by cytochrome b_5 would then be incorporated into various active oxygen species and an increased production of these species would be observed in a system uncoupled by these xenobiotics.

In iodomethane treated microsomes, the production of hydrogen peroxide is increased with time in the presence of NADH. The xenobiotics themselves have no effect on hydrogen peroxide production. Relative to the increase of hydrogen peroxide observed in the presence of NADH, the production of hydrogen peroxide was significantly increased after 1 minute in the presence of NADH plus chloroacetaldehyde or bromotrichloromethane. 1,2-

Dibromo-1,2-dichloroethane plus NADH did not produce elevated levels of hydrogen peroxide (Table 28; Figure 19).

The production of hydrogen peroxide on incubation of the anaesthetic agents with differently pretreated microsomes is reported in Tables 29, 30 and 31. In the absence of NADH, very low levels of hydrogen peroxide are produced in the presence of the anaesthetic agents. However, in the presence of both NADH and the anaesthetic agents, there is a statistically significant increase in the production of hydrogen peroxide with time (Tables 29, 30 and 31 and Figures 20, 21 and 22). This elevation of hydrogen peroxide levels was observed in microsomes from high carbohydrate fed rats pretreated or not with iodomethane or potassium thiocyanate, to decrease cytochrome P-450 levels.

The effect of the anaesthetic agents halothane, enflurane and methoxyflurane on the production of superoxide anion is reported in Table 32. Using NADPH as an electron donor, only halothane had a significant effect on the production of superoxide anion. At a higher protein concentration, none of the anaesthetic agents produced any superoxide anion. Using NADH as an electron donor, no superoxide anion could be detected in the presence of any of the anaesthetic agents with either the low or high concentrations of hepatic microsomes (Table 32). The oxidation of epinephrine to adrenochrome occurs in

the presence of superoxide anion as evidenced by the generation of superoxide anion and formation of adrenochrome by the xanthine/xanthine oxidase system. (The incubation system comprised adrenaline (0,6 mM), xanthine (300 µM) and xanthine oxidase (0,25 µg/ml) and generated 0,0012 $\pm$ 0,0002 moles adrenochrome per minute).

TABLE 28. THE EFFECT OF HALOGENATED COMPOUNDS ON HYDROGEN PEROXIDE PRODUCTION IN IODOMETHANE PRETREATED HEPATIC MICROSOMES FROM RATS FED A HIGH CARBOHYDRATE DIET.

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (µM)
NADH (1,0)	1	2,76 ± 0,90
	7	11,25 ± 4,50
	15	12,36 ± 2,76
Chloroacetaldehyde (51,6)	1	0,90 ± 0,93
	7	0,75 ± 0,72
	15	0,18 ± 0,24
Chloroacetaldehyde (51,6) + NADH (1,0)	1	8,67 ± 2,13†
	7	14,22 ± 5,19
	15	16,44 ± 4,86
1,2-Dibromo-1,2-dichloroethane (0,6)	1	0,33 ± 0,36
	7	0,21 ± 0,24
	15	0,42 ± 0,36
1,2-Dibromo-1,2-dichloroethane (0,6) + NADH (1,0)	1	4,74 ± 2,04
	7	6,81 ± 4,8
	15	11,79 ± 3,45

TABLE 28 (Cont.)

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (μM)
Bromotrichloro-methane (3,4)	1	0,51 ± 0,93
	7	0,36 ± 0,72
	15	0,48 ± 0,48
Bromotrichloro-methane (3,4) + NADH (1,0)	1	7,62 ± 2,94≠
	7	13,89 ± 4,92
	15	13,98 ± 3,42

† Differs significantly from NADH alone, $P < 0,01$

≠ Probably differs from NADH alone, $P < 0,05$

FIGURE 19 : The effect of xenobiotics on the production of hydrogen peroxide. The production of hydrogen peroxide by hepatic microsomes from rats fed a high carbohydrate diet, with the isolated hepatic microsomes being pretreated with iodomethane. Additions to incubations as follows : NADH (●), chloroacetaldehyde (▲) and chloroacetaldehyde + NADH (■) (Figure A); NADH (●), 1,2-dibromo-1,2-dichloroethane (▲) and 1,2-dibromo-1,2-dichloroethane + NADH (■) (Figure B); NADH (●), bromotrichloromethane (▲) and bromotrichloromethane + NADH (■) (Figure C).

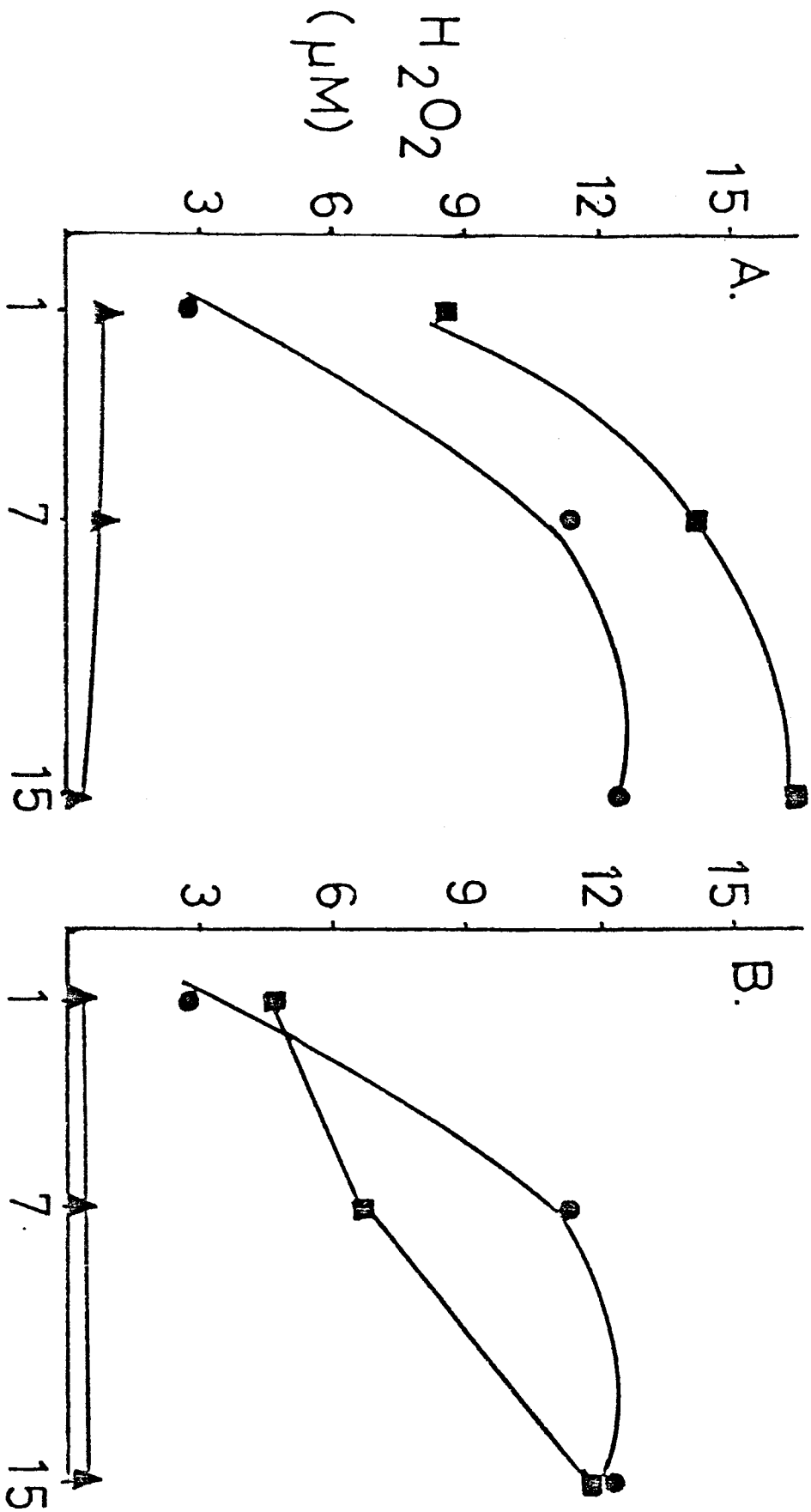


FIGURE 19A

FIGURE 19B

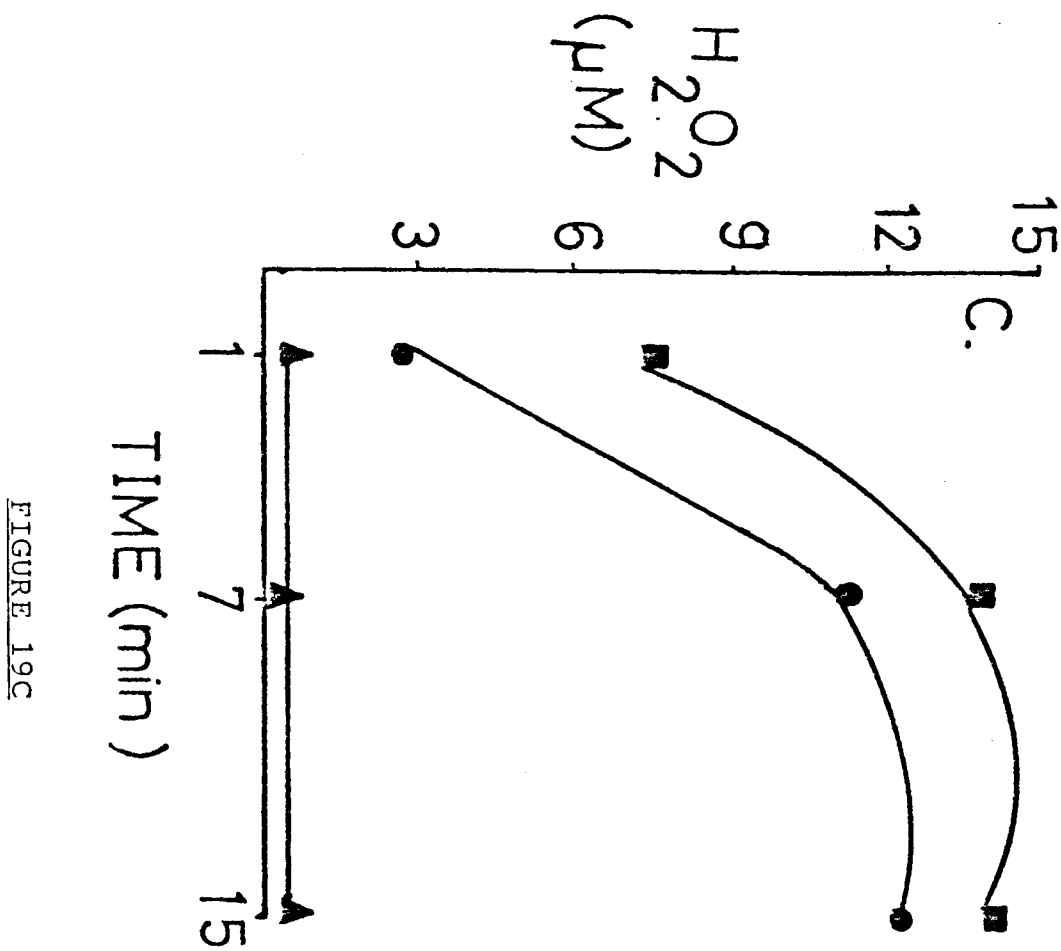


FIGURE 19C

TABLE 29. THE EFFECT OF ANAESTHETIC AGENTS ON HYDROGEN PEROXIDE PRODUCTION IN MICROSOMES FROM RATS FED A HIGH CARBOHYDRATE DIET.

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (µM)
NADH (1,0)	1	1,59 ± 0,90
	5	1,56 ± 1,44
	15	2,94 ± 1,68
	20	0,99 ± 0,75
Halothane (18,0)	1	0,0
	5	0,0
	15	0,0
	20	2,10 ± 2,94
Halothane (18,0) + NADH (1,0)	1	2,73 ± 1,56
	5	2,70 ± 1,92
	15	6,18 ± 2,10†
	20	6,78 ± 2,52†
Enflurane (14,0)	1	0,81 ± 1,14
	5	0,66 ± 1,02
	15	0,99 ± 1,14
	20	0,60 ± 0,78

TABLE 29 (Cont.)

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (µM)
Enflurane (14,0) + NADH (1,0)	1	2,94 ± 2,25
	5	4,80 ± 1,50*
	15	8,31 ± 3,99†
	20	7,29 ± 2,52*
Methoxyflurane (0,6)	1	3,72 ± 1,83
	5	3,00 ± 2,28
	15	1,38 ± 1,50
	20	0,81 ± 0,15
Methoxyflurane (0,6) + NADH (1,0)	1	2,34 ± 1,26
	5	5,43 ± 1,86*
	15	5,43 ± 1,26†
	20	3,18 ± 1,47†

* Differs significantly from NADH alone, $P < 0,001$

† Differs significantly from NADH alone, $P < 0,01$

FIGURE 20 : The effects of anaesthetic agents on the production of hydrogen peroxide. The production of hydrogen peroxide in microsomes from rats fed a high carbohydrate diet. Additions to incubations as follows : NADH (●), halothane (▲) and halothane + NADH (■) (Figure A); NADH (●), enflurane (▲) and enflurane + NADH (■) (Figure B); NADH (●), methoxyflurane (▲) and methoxyflurane + NADH (■) (Figure C).

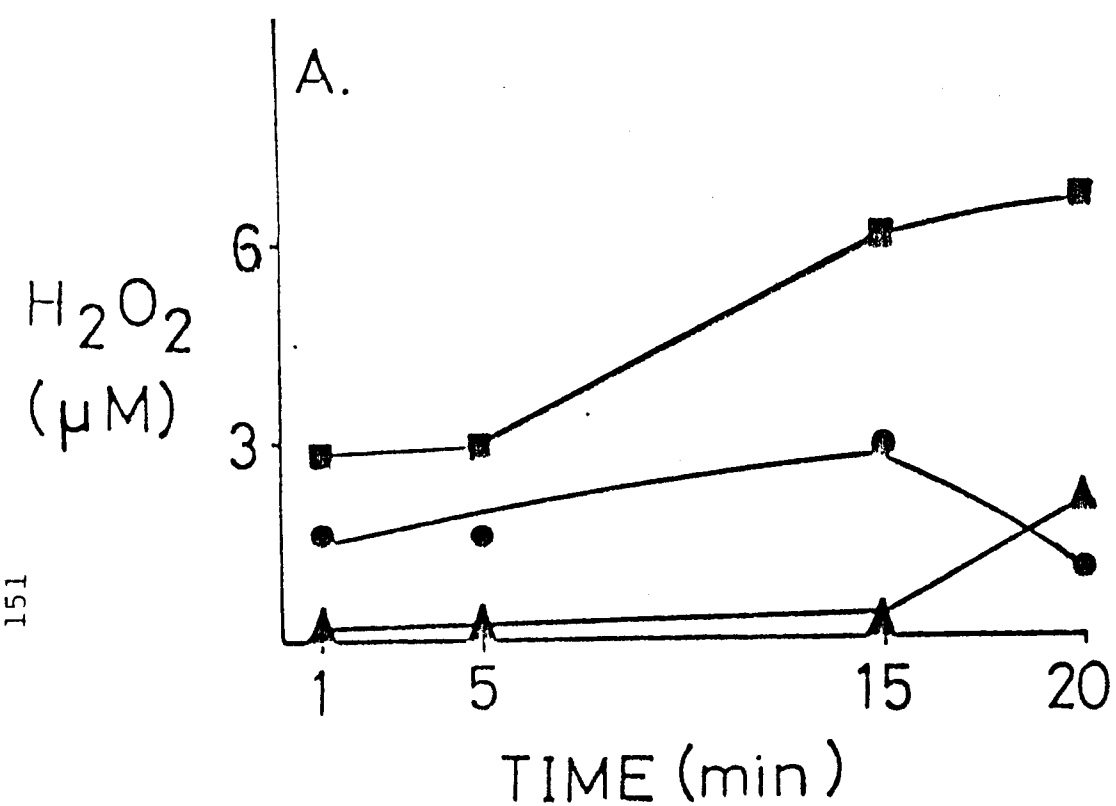


FIGURE 20A

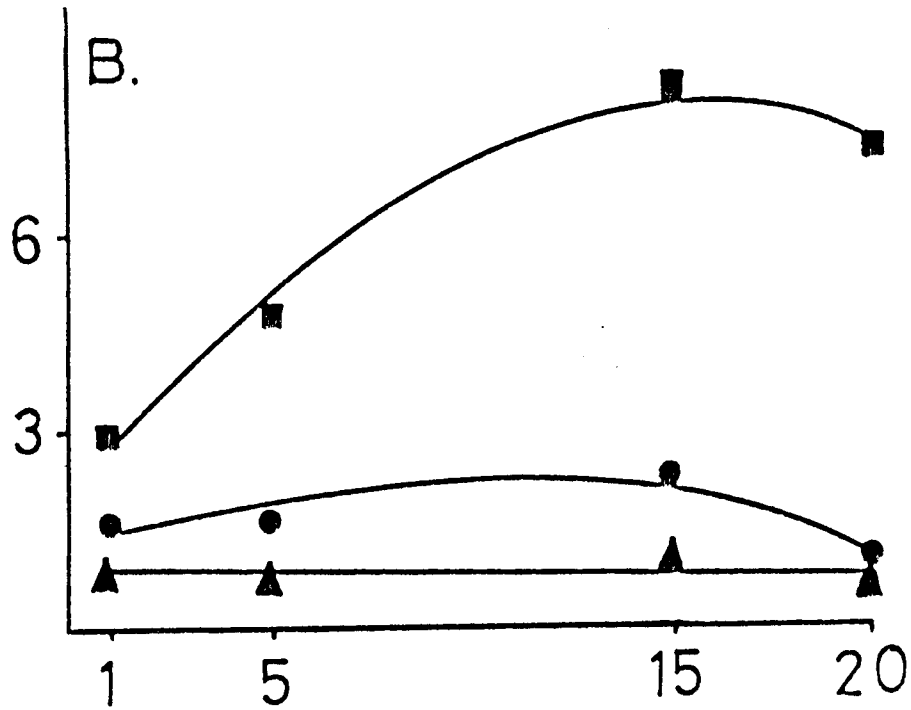


FIGURE 20B

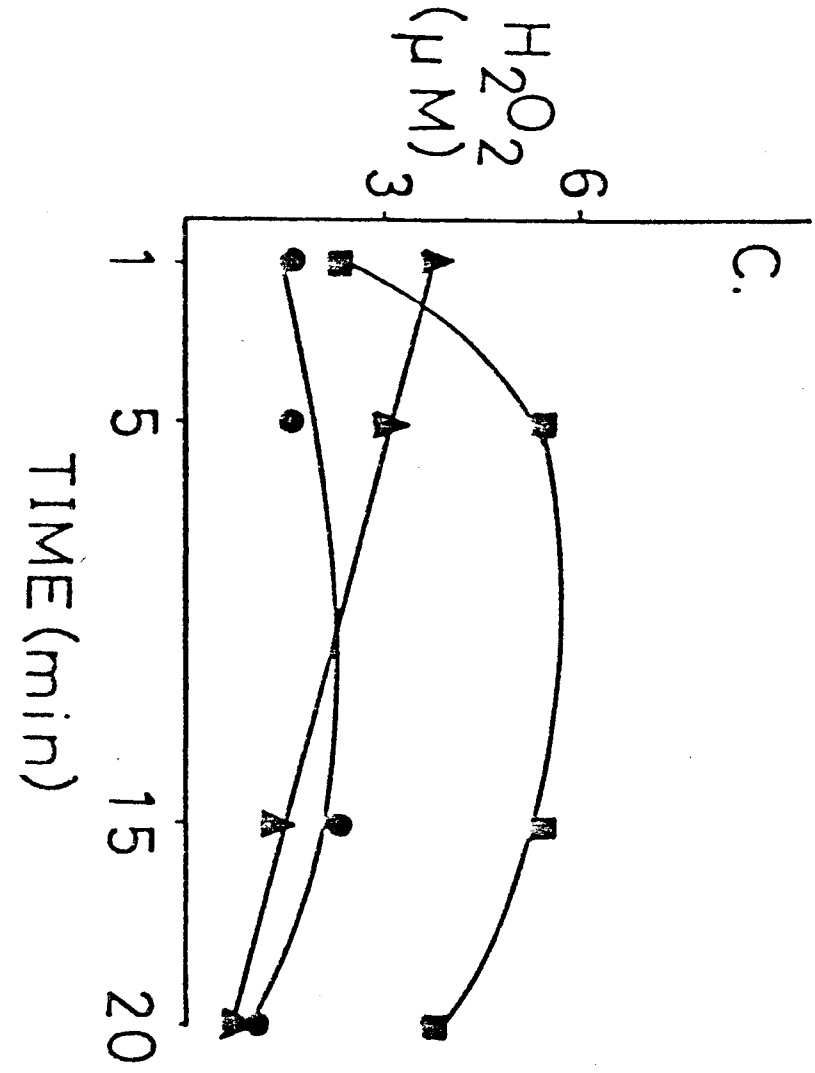


FIGURE 20C

TABLE 30. THE EFFECT OF ANAESTHETIC AGENTS ON HYDROGEN PEROXIDE PRODUCTION IN MICROSOMES FROM RATS FED A HIGH CARBOHYDRATE DIET, PRETREATED WITH IODOMETHANE.

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (μM)
NADH (1,0)	1	3,12 ± 1,14
	5	5,70 ± 1,98
	15	9,57 ± 0,66
	20	10,32 ± 2,34
Halothane (18,0)	1	2,19 ± 2,55
	5	1,23 ± 1,65
	15	1,95 ± 1,32
	20	2,52 ± 1,83
Halothane (18,0) + NADH (1,0)	1	4,80 ± 0,93≠
	5	7,68 ± 0,96
	15	10,41 ± 1,35
	20	9,99 ± 2,43
Enflurane (14,0)	1	3,09 ± 1,11
	5	4,80 ± 2,79
	15	6,30 ± 2,91
	20	7,47 ± 2,52

TABLE 30 (Cont.)

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (µM)
Enflurane (14,0) + NADH (1,0)	1	9,57 ± 1,86$^{\neq}$
	5	14,40 ± 2,94*
	15	17,01 ± 3,66†
	20	16,65 ± 2,94†
Methoxyflurane (0,6)	1	1,98 ± 1,59
	5	5,76 ± 2,55
	15	4,98 ± 1,71
	20	3,51 ± 1,08
Methoxyflurane (0,6) + NADH (1,0)	1	8,07 ± 2,34†
	5	13,32 ± 3,96†
	15	16,23 ± 4,86
	20	12,69 ± 4,20

* Differs significantly from NADH alone, $P < 0,001$

† Differs significantly from NADH alone, $P < 0,01$

$^{\neq}$ Probably differs from NADH alone, $P < 0,05$

FIGURE 21 : The effects of anaesthetic agents on the production of hydrogen peroxide. The production of hydrogen peroxide in microsomes from rats fed a high carbohydrate diet and with the isolated hepatic microsomes being pretreated with iodomethane. Additions to incubations as follows : NADH (●), halothane (▲), and halothane + NADH (■) (Figure A); NADH (●), enflurane (▲) and enflurane + NADH (■) (Figure B); NADH (●), methoxyflurane (▲) and methoxyflurane + NADH (■) (Figure C).

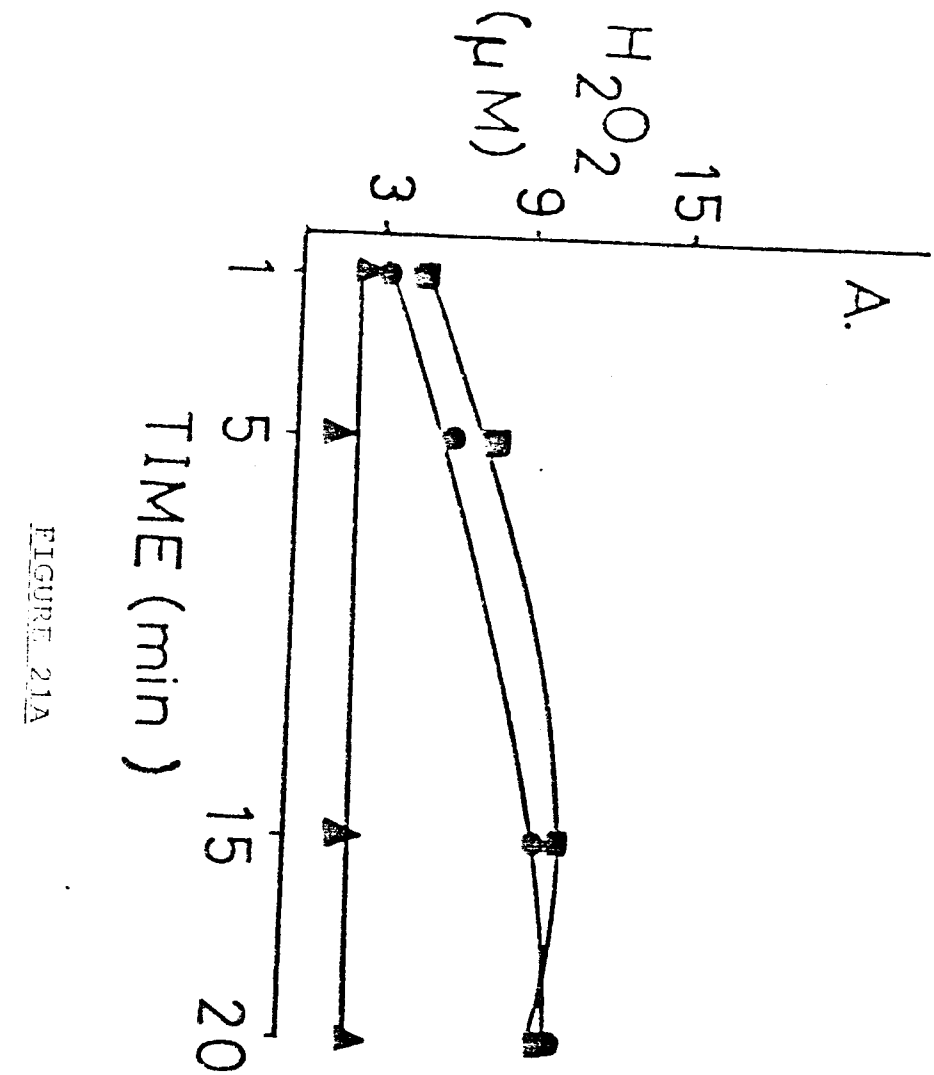


FIGURE 21A

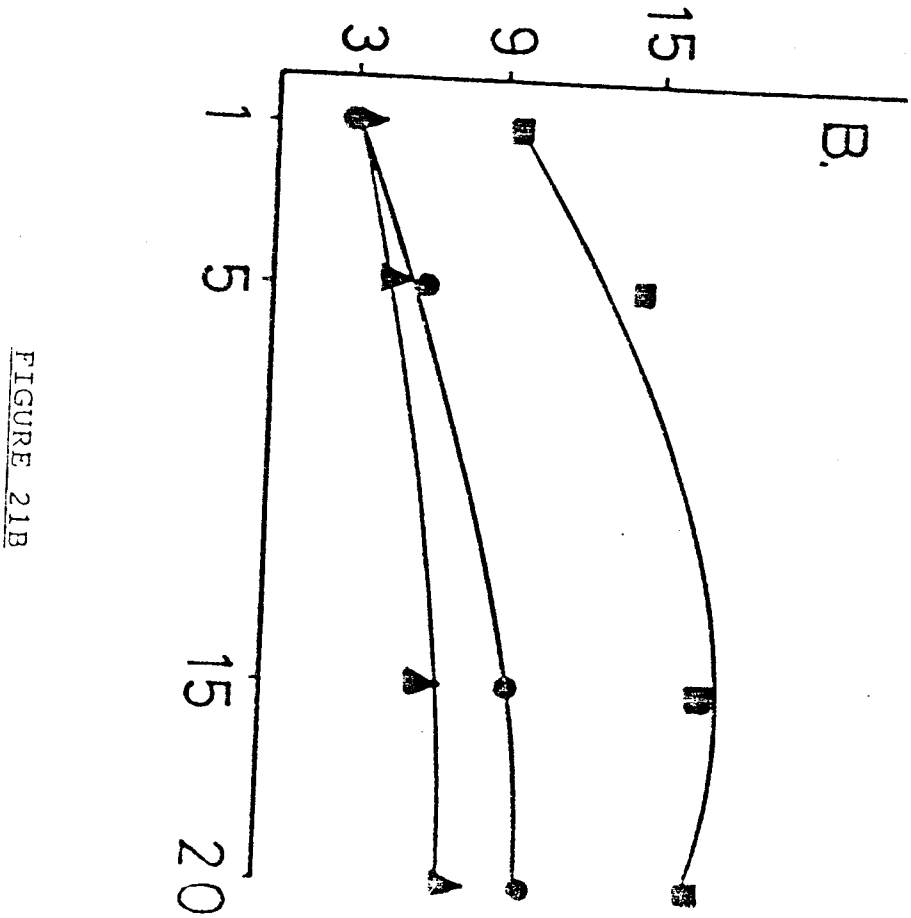


FIGURE 21B

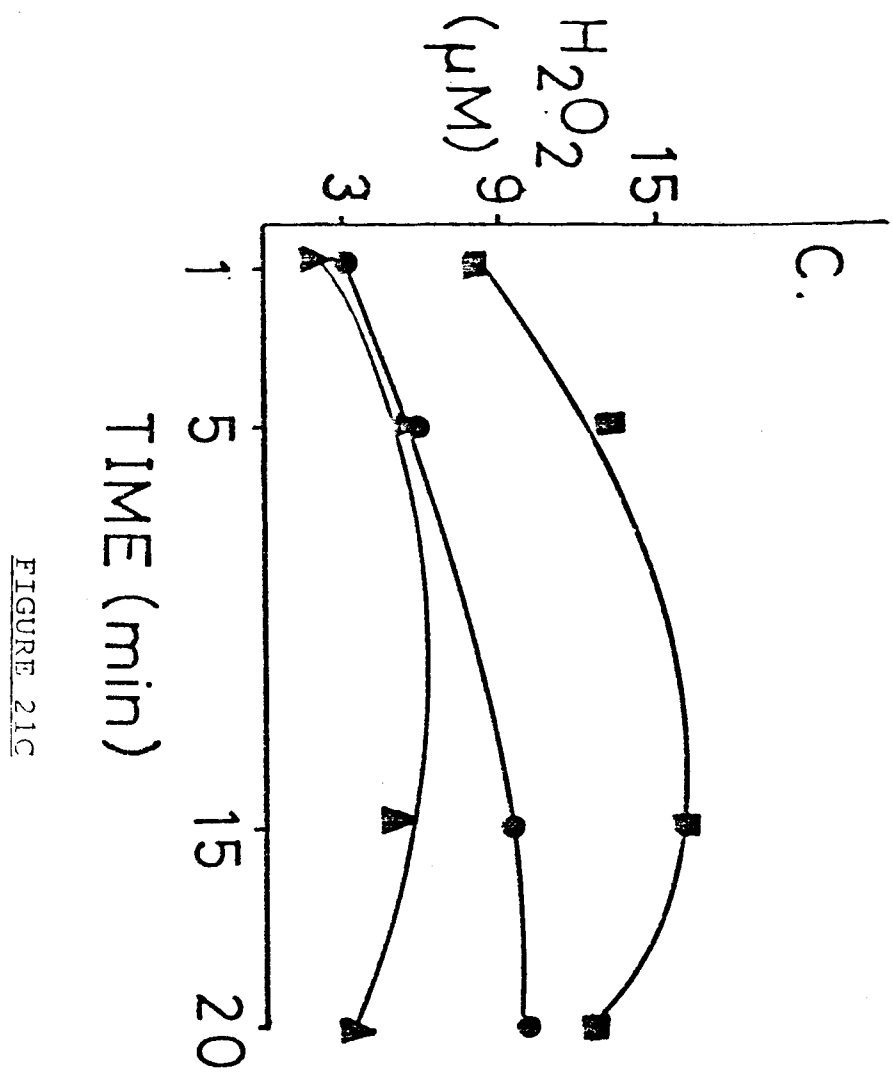


FIGURE 21C

TABLE 31. THE EFFECT OF ANAESTHETIC AGENTS ON HYDROGEN PEROXIDE PRODUCTION IN MICROSOMES FROM RATS FED A HIGH CARBOHYDRATE DIET, PRETREATED WITH POTASSIUM THIOCYANATE.

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (µM)
NADH (1,0)	1	2,52 ± 0,51
	5	3,30 ± 2,10
	15	5,82 ± 3,00
	20	9,00 ± 2,88
Halothane (18,0)	1	1,86 ± 2,22
	5	0,63 ± 0,75
	15	0,81 ± 0,75
	20	4,41 ± 6,15
Halothane (18,0) + NADH (1,0)	1	7,71 ± 3,63≠
	5	11,22 ± 0,24†
	15	7,59 ± 3,30
	20	12,42 ± 2,70
Enflurane (14,0)	1	1,32 ± 1,80
	5	0,39 ± 0,81
	15	1,23 ± 1,44
	20	2,61 ± 1,80

TABLE 31 (Cont.)

Additions to microsomes (mM)	Time (min)	Hydrogen peroxide produced (µM)
Enflurane (14,0) + NADH (1,0)	1	7,20 ± 2,46≠
	5	8,13 ± 3,18≠
	15	6,48 ± 3,30
	20	11,55 ± 4,11
Methoxyflurane (0,6)	1	0,39 ± 0,84
	5	0,57 ± 0,93
	15	0,12 ± 0,24
	20	0,24 ± 0,30
Methoxyflurane (0,6) + NADH (1,0)	1	8,46 ± 2,85†
	5	7,92 ± 4,35
	15	11,01 ± 3,12≠
	20	13,56 ± 3,36

† Differs significantly from NADH alone, $P < 0,01$

≠ Probably differs from NADH alone, $P < 0,05$

FIGURE 22 : The effects of anaesthetic agents on the production of hydrogen peroxide. The production of hydrogen peroxide in microsomes from rats fed a high carbohydrate diet and with the isolated hepatic microsomes being pretreated with potassium thiocyanate. Additions to incubations as follows : NADH (●), halothane (▲), and halothane + NADH (■) (Figure A); NADH (●), enflurane (▲) and enflurane + NADH (■) (Figure B); NADH (●), methoxyflurane (▲) and methoxyflurane + NADH (■) (Figure C).

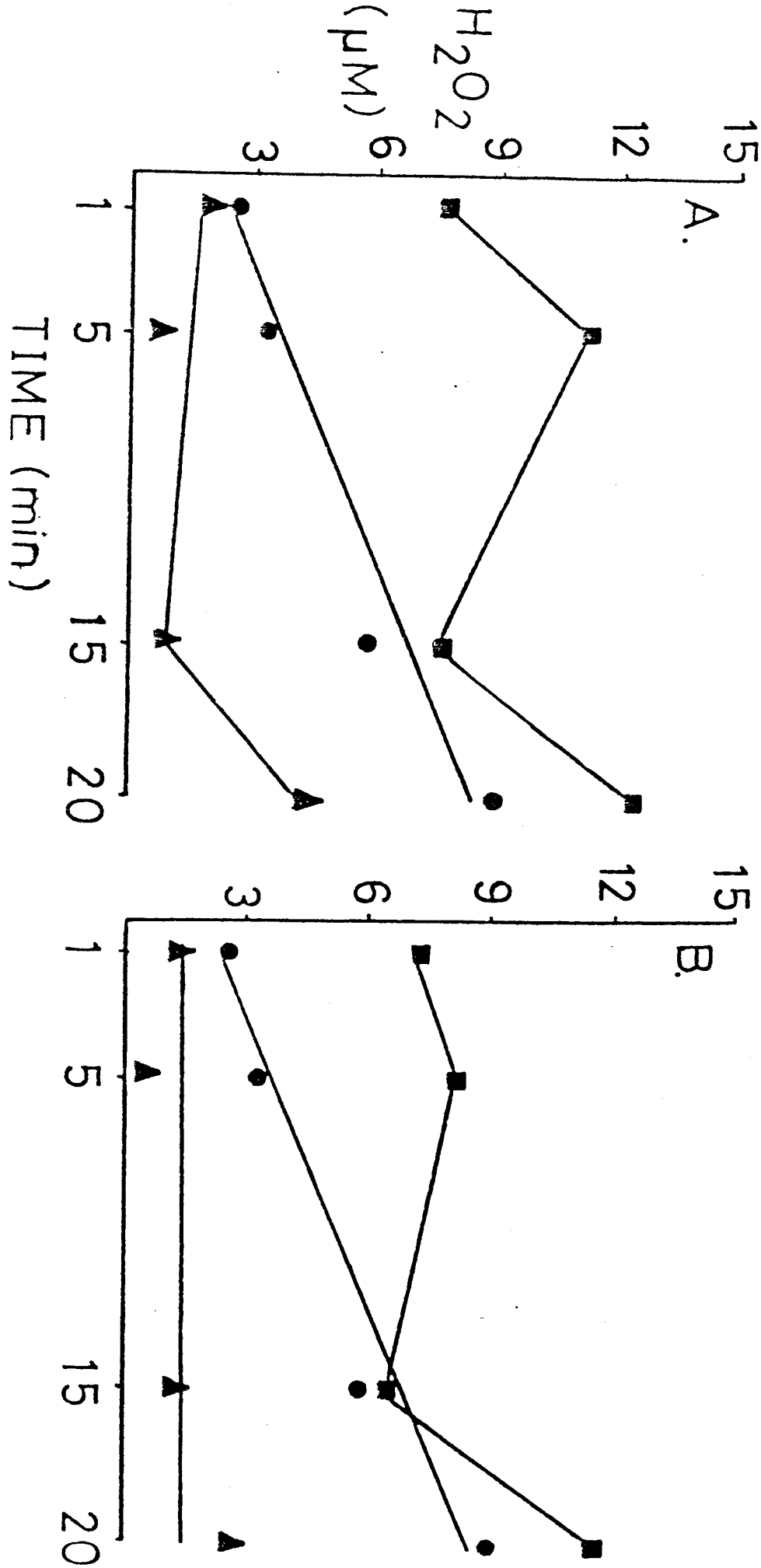


FIGURE 22A

FIGURE 22B

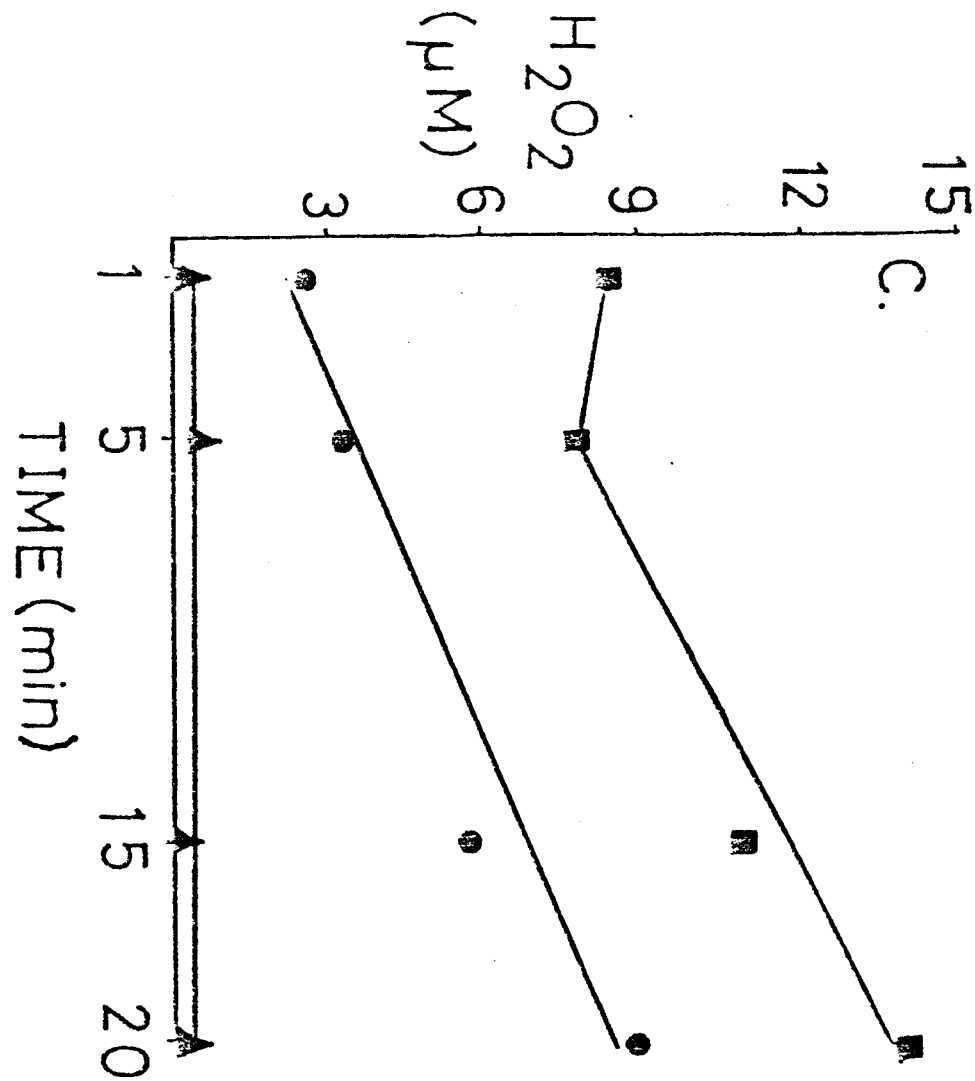


FIGURE 22C

TABLE 32. THE PRODUCTION OF SUPEROXIDE ANION IN HEPATIC MICROSOMES FROM RATS FED A HIGH CARBOHYDRATE DIET.

Components of reaction mixture			
mg microsomal protein/ml	Reductant (mM)	Anaesthetic agent (mM)	nmoles adrenochrome formed/min/mg microsomal protein
0,5	NADPH (0,6)	None	14,0 ± 0,71
0,5	NADPH (0,6)	Halothane (18,0)	16,0 ± 0,1†
0,5	NADPH (0,6)	Enflurane (14,0)	12,3 ± 2,1
0,5	NADPH (0,6)	Methoxyflurane (0,6)	14,0 ± 1,0
0,5	NADH (1,0)	None	0,0
0,5	NADH (1,0)	Halothane (18,0)	0,0
0,5	NADH (1,0)	Enflurane (14,0)	0,0
0,5	NADH (1,0)	Methoxyflurane (0,6)	0,0

TABLE 32 (Cont.)

Components of reaction mixture			
mg microsomal protein/ml	Reductant (mM)	Anaesthetic agent (mM)	nmoles adrenochrome formed/min/mg microsomal protein
1,5	NADPH (0,6)	None	5,0 ± 0,3
1,5	NADPH (0,6)	Halothane (18,0)	4,8 ± 0,3
1,5	NADPH (0,6)	Enflurane (14,0)	4,8 ± 1,0
1,5	NADPH (0,6)	Methoxyflurane (0,6)	4,3 ± 0,5
1,5	NADH (1,0)	None	0,0
1,5	NADH (1,0)	Halothane (18,0)	0,0
1,5	NADH (1,0)	Enflurane (14,0)	0,0
1,5	NADH (1,0)	Methoxyflurane (0,6)	0,0

Assays are in triplicate on a single preparation of hepatic microsomes.

† Differs significantly from no additions, $P < 0,01$

(3) Experiments in vivo.

(a) The effects of anaesthetic agents in vivo.

The effect of repeated anaesthesia of differently pre-treated rats on the stearate desaturase enzyme system and cytochrome P-450 levels was assessed in hepatic microsomes. The effects of this treatment on toxicity was assessed by monitoring S.G.O.T. levels in blood from animals exposed to repeated anaesthesia and by examining their livers by conventional light microscopy. Advantage was taken of dietary pretreatment, to alter the levels of hepatic microsomal stearate desaturase and phenobarbitone pretreatment, to increase the levels and activity of hepatic microsomal cytochrome P-450.

The effect of dietary pretreatment on the activity of the stearate desaturase enzyme system.

The activity of stearate desaturase was assessed via the re-oxidation of hepatic microsomal cytochrome b_5. The difference in k_{obs} in the presence minus absence of stearoyl CoA was taken as a measure of the activity of stearate desaturase. Stearate desaturase activity remained elevated 5, 8 and 11 days after the commencement of the high carbohydrate diet (Table 33), as evidenced by the increased rate of re-oxidation of ferrocytochrome b_5. Stearate desaturase activity was significantly lower in hepatic microsomes from rats fed

a normal diet when compared to the activity of the enzyme in hepatic microsomes from rats fed the high carbohydrate diet for 5 days (Table 34). The activity of stearate desaturase was negligible in hepatic microsomes from fasted or phenobarbitone treated rats (Table 34).

The effect of repeated anaesthesia on the hepatic microsomal stearate desaturase enzyme system.

Stearate desaturase activity, in hepatic microsomes from rats fed a high carbohydrate diet, was unaffected by repeated exposure to halothane, enflurane or chloroform when compared to stearate desaturase activity in unanaesthetized rats fed a high carbohydrate diet. The activity of stearate desaturase was decreased following exposure to ether however (Table 35). Similarly, stearate desaturase activity in hepatic microsomes from rats fed a normal diet, or fasted and pretreated with phenobarbitone, was unaffected by repeated exposure to halothane, ether or chloroform (Table 36 and 37) when compared to similarly pretreated unanaesthetized animals.

The effect of repeated anaesthesia on hepatic microsomal cytochrome P-450, S.G.O.T. and hepatic histology.

The levels of hepatic microsomal cytochrome P-450 were unaffected in hepatic microsomes from rats fed a high

carbohydrate diet in the absence of anaesthesia (Table 38). Repeated exposure to halothane resulted in decreased levels of cytochrome P-450 in hepatic microsomes from rats fed a high carbohydrate diet, while ether elevated the levels of cytochrome P-450 (Table 38). This is consistent with the known ability of diethyl ether to induce cytochrome P-450 (Brown and Sagalyn, 1974; Ross and Cardell, 1978). Repeated exposure of rats on the high carbohydrate diet to enflurane was without effect on cytochrome P-450 levels (Table 38).

The levels of cytochrome P-450 were slightly lowered in microsomes from rats fed a normal diet after repeated exposure to enflurane and decreased to a greater extent by chloroform, while the levels of cytochrome P-450 in these microsomes were unaffected by repeated exposure to halothane or ether (Table 39). Cytochrome P-450 levels in hepatic microsomes from fasted rats pretreated with phenobarbitone were significantly lowered following repeated exposure to halothane and ether and were slightly, but significantly elevated following exposure to enflurane (Table 40).

The levels of S.G.O.T. in hepatic microsomes from rats fed a high carbohydrate diet, in the absence of anaesthesia, were all within the normal range (less than 50 I.U.) determined from the mean $\pm$ three standard

deviations of values obtained for normal animals (Table 38). For all animals, S.G.O.T. levels greater than 50 I.U. were considered to be abnormal and indicative of hepatic damage. If the values of S.G.O.T. were less than 50 I.U., the mean $\pm$ standard deviation is given; if some values are greater than 50 I.U., the two categories have been split and the number greater or less than 50 I.U. given.

The levels of S.G.O.T. in hepatic microsomes from rats fed a high carbohydrate diet were within the normal range following repeated exposure to halothane, enflurane or ether. The S.G.O.T. levels were however, significantly raised following repeated chloroform anaesthesia of 2/7 animals indicating hepatic damage (Table 38). S.G.O.T. levels in hepatic microsomes from rats fed a normal diet or pretreated with phenobarbitone were within the normal range following repeated halothane, enflurane or ether anaesthesia (Tables 39 and 40) while exposure to chloroform in rats fed a normal diet resulted in raised S.G.O.T. levels in 3/8 rats (Table 39).

The livers of rats exposed to halothane, enflurane or ether appeared histologically normal regardless of dietary or phenobarbitone pretreatment (Tables 38, 39 and 40). Dietary pretreatment appeared not to affect the toxicity of chloroform anaesthesia, as animals on both high carbohydrate and normal diets anaesthetized

with chloroform, manifested gross centrilobular necrosis, a lesion characteristic of cytochrome P-450 mediated hepatic damage (Brown et al., 1974). It was not possible to assess the effects of chloroform on animals pretreated with phenobarbitone, due to the high mortality rate. However, gross hepatic necrosis was observed during chloroform anaesthesia of these animals.

TABLE 33. THE EFFECT OF A HIGH CARBOHYDRATE DIET ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5.

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}(sec^{-1})$
		Additions (mM)	$10^2 k_{obs}(sec^{-1})$	
No anaesthesia	High carbohydrate diet (5 days)	None	1,30 ± 0,31	
		KCN (0,5)	1,74 ± 0,16	
		Stearoyl CoA (0,012)	2,92 ± 0,51	1,62 ± 0,20
		Stearoyl CoA (0,012) + KCN (0,5)	2,62 ± 0,21	
No anaesthesia	High carbohydrate diet (8 days)	None	1,31 ± 0,04	
		KCN (0,5)	1,48 ± 0,04	
		Stearoyl CoA (0,012)	2,54 ± 0,01	1,23 ± 0,31
		Stearoyl CoA (0,012) + KCN (0,5)	1,92 ± 0,08	

TABLE 33 (Cont.)

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
		Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	High carbohydrate diet (11 days)	None	1,45 ± 0,04	
		KCN (0,5)	1,67 ± 0,01	
		Stearoyl CoA (0,012)	2,61 ± 0,16	1,16 ± 0,19
		Stearoyl CoA (0,012) + KCN (0,5)	2,41 ± 0,03	

TABLE 34. THE EFFECT OF DIETARY PRETREATMENT AND PHENOBARBITONE INDUCTION ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
			Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	High carbohydrate diet (5 days)	-	None	1,30 ± 0,31	
			KCN (0,5)	1,74 ± 0,16	
			Stearoyl CoA (0,012)	2,92 ± 0,51	1,62 ± 0,20
			Stearoyl CoA (0,012) + KCN (0,5)	2,62 ± 0,21	
No anaesthesia	Normal diet	-	None	1,56 ± 0,17	
			KCN (0,5)	1,48 ± 0,20	
			Stearoyl CoA (0,012)	2,09 ± 0,12	0,53 ± 0,21*
			Stearoyl CoA (0,012) + KCN (0,5)	1,73 ± 0,30	

TABLE 34 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
			Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	Fasted	-	None	1,63 ± 0,41	
			KCN (0,5)	1,34 ± 0,13	
			Stearoyl CoA (0,012)	1,83 ± 0,27	0,20 ± 0,03*
			Stearoyl CoA (0,012) + KCN (0,5)	1,55 ± 0,18	
No anaesthesia	Fasted	+	None	2,65 ± 0,07	
			KCN (0,5)	2,03 ± 0,43	
			Stearoyl CoA (0,012)	2,78 ± 0,17	0,13 ± 0,09*
			Stearoyl CoA (0,012) + KCN (0,5)	2,17 ± 0,25	

* Differs significantly from the levels in hepatic microsomes from rats fed a high carbohydrate diet, $P < 0,001$

TABLE 35. THE EFFECT OF REPEATED ANAESTHESIA ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5 IN MICROSOMES FROM RATS FED A HIGH CARBOHYDRATE DIET.

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
		Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	High carbohydrate diet (5 days)	None	1,30 ± 0,31	
		KCN (0,5)	1,74 ± 0,16	
		Stearoyl CoA (0,012)	2,92 ± 0,51	1,62 ± 0,20
		Stearoyl CoA (0,012) + KCN (0,5)	2,62 ± 0,21	
3 × 1 MAC halothane (3 hours)	High carbohydrate diet	None	1,41 ± 0,16	
		KCN (0,5)	1,60 ± 0,15	
		Stearoyl CoA (0,012)	3,08 ± 0,69	1,67 ± 0,62
		Stearoyl CoA (0,012) + KCN (0,5)	2,11 ± 0,35	

TABLE 35 (Cont.)

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
		Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
3 × 1 MAC enflurane (3 hours)	High carbohydrate diet	None	1,33 ± 0,25	
		KCN (0,5)	1,40 ± 0,24	
		Stearoyl CoA (0,012)	3,21 ± 0,23	1,88 ± 0,36
		Stearoyl CoA (0,012) + KCN (0,5)	2,38 ± 0,20	
3 × 1 MAC ether (3 hours)	High carbohydrate diet	None	1,23 ± 0,05	
		KCN (0,5)	0,97 ± 0,11	
		Stearoyl CoA (0,012)	2,19 ± 0,13	0,96 ± 0,13†
		Stearoyl CoA (0,012) + KCN (0,5)	1,77 ± 0,08	

TABLE 35 (Cont.)

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
		Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
3 × 1 MAC chloroform (3 hours)	High carbohydrate diet	None	1,07 ± 0,36	
		KCN (0,5)	1,05 ± 0,02	
		Stearoyl CoA (0,012)	2,31 ± 0,19	1,24 ± 0,31
		Stearoyl CoA (0,012) + KCN (0,5)	1,69 ± 0,11	

† Differs significantly from identically treated unanaesthetized controls, $P < 0,01$.

TABLE 36. THE EFFECT OF REPEATED ANAESTHESIA ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5 IN MICROSOMES FROM RATS FED A NORMAL DIET.

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
		Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	Normal diet	None	1,56 ± 0,17	
		KCN (0,5)	1,48 ± 0,20	
		Stearoyl CoA (0,012)	2,09 ± 0,12	0,53 ± 0,21
		Stearoyl CoA (0,012) + KCN (0,5)	1,73 ± 0,30	
3 × 1 MAC halothane (3 hours)	Normal diet	None	1,27 ± 0,18	
		KCN (0,5)	1,41 ± 0,38	
		Stearoyl CoA (0,012)	1,99 ± 0,13	0,72 ± 0,21
		Stearoyl CoA (0,012) + KCN (0,5)	1,69 ± 0,38	

TABLE 36 (Cont.)

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
		Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
3 × 1 MAC enflurane (3 hours)	Normal diet	None	1,29 ± 0,19	
		KCN (0,5)	1,39 ± 0,09	
		Stearoyl CoA (0,012)	1,68 ± 0,12	0,40 ± 0,13
		Stearoyl CoA (0,012) + KCN (0,5)	1,69 ± 0,11	
3 × 1 MAC ether (3 hours)	Normal diet	None	1,28 ± 0,13	
		KCN (0,5)	1,13 ± 0,18	
		Stearoyl CoA (0,012)	1,87 ± 0,06	0,59 ± 0,07
		Stearoyl CoA (0,012) + KCN (0,5)	1,61 ± 0,20	

TABLE 36 (Cont.)

Protocol	Diet	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA)
		Additions (mM)	$10^2 k_{obs}(sec^{-1})$	$10^2 k_{obs}(sec^{-1})$
3 × 1 MAC	Normal	None	0,78 ± 0,10	
chloroform	diet	KCN (0,5)	0,74 ± 0,17	
(3 hours)		Stearoyl CoA (0,012)	1,08 ± 0,20	0,31 ± 0,16
		Stearoyl CoA (0,012) + KCN (0,5)	1,09 ± 0,17	

TABLE 37. THE EFFECT OF REPEATED ANAESTHESIA ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME $\underline{b}_5$ IN MICROSOMES FROM FASTED RATS PRETREATED WITH PHENOBARBITONE.

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome $\underline{b}_5$		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
			Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	Fasted	+	None	2,65 ± 0,07	
			KCN (0,5)	2,03 ± 0,43	
			Stearoyl CoA (0,012)	2,78 ± 0,17	0,13 ± 0,09
			Stearoyl CoA (0,012) + KCN (0,5)	2,17 ± 0,25	
3 × 1 MAC halothane (3 hours)	Fasted	+	None	1,99 ± 0,57	
			KCN (0,5)	1,54 ± 0,37	
			Stearoyl CoA (0,012)	2,27 ± 0,61	0,29 ± 0,32
			Stearoyl CoA (0,012) + KCN (0,5)	1,75 ± 0,18	

TABLE 37 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA)
			Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	$10^2 k_{obs}$ (sec^{-1})
3 × 1 MAC enflurane (3 hours)	Fasted	+	None	0,77 ± 0,08	
			KCN (0,5)	0,58 ± 0,05	
			Stearoyl CoA (0,012)	0,65 ± 0,08	-0,12 ± 0,10†
			Stearoyl CoA (0,012) + KCN (0,5)	0,63 ± 0,18	
3 × 1 MAC ether (3 hours)	Fasted	+	None	2,14 ± 0,12	
			KCN (0,5)	1,57 ± 0,30	
			Stearoyl CoA (0,012)	2,27 ± 0,27	0,13 ± 0,20
			Stearoyl CoA (0,012) + KCN (0,5)	1,81 ± 0,30	

† Differs significantly from identically treated unanaesthetized controls, $P < 0,01$

TABLE 38. THE EFFECT OF A HIGH CARBOHYDRATE DIET AND REPEATED ANAESTHESIA ON HEPATIC MICROSOMAL CYTOCHROME P-450, S.G.O.T. AND HEPATIC HISTOLOGY.

Protocol	Diet	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic histology
No anaesthesia	5 days on high carbohydrate diet	0,78 ± 0,04	18,6 ± 3,4	-
No anaesthesia	8 days on high carbohydrate diet	0,52 ± 0,04	32,7 ± 14,1	-
No anaesthesia	11 days on high carbohydrate diet	0,74 ± 0,02	14,8 ± 2,2	-
3 × 1 MAC halothane (3 hours)	High carbohydrate diet	0,50 ± 0,12†	17,5 ± 4,6	Normal

TABLE 38 (Cont.)

Protocol	Diet	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic histology
3 × 1 MAC enflurane (3 hours)	High carbohydrate diet	0,66 ± 0,06	13,3 ± 2,2	Normal
3 × 1 MAC ether (3 hours)	High carbohydrate diet	1,22 ± 0,09*	33,5 ± 17,8	Normal
3 × 1 MAC chloroform (3 hours)	High carbohydrate diet	0,60 ± 0,01†	$\frac{5}{7}$ < 50 $\frac{2}{7}$ > 50	Centrilobular necrosis

* Differs significantly from identically treated unanaesthetized controls (5 days on high carbohydrate diet), P < 0,001

† Differs significantly from identically treated unanaesthetized controls (5 days on high carbohydrate diet), P < 0,01

TABLE 39. THE EFFECT OF A NORMAL DIET AND REPEATED ANAESTHESIA ON HEPATIC MICROSOMAL CYTOCHROME P-450, S.G.O.T. AND HEPATIC HISTOLOGY.

Protocol	Diet	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic histology
No anaesthesia	Normal diet	0,98 ± 0,05	37,8 ± 9,3	-
3 × 1 MAC halothane (3 hours)	Normal diet	0,85 ± 0,19	26,9 ± 5,5	Normal
3 × 1 MAC enflurane (3 hours)	Normal diet	0,82 ± 0,11$^{\neq}$	17,1 ± 1,7	Normal
3 × 1 MAC ether (3 hours)	Normal diet	1,12 ± 0,06	32,9 ± 3,2	Normal

TABLE 39 (Cont.)

Protocol	Diet	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic histology
3 × 1 MAC chloroform (3 hours)	Normal diet	0,66 ± 0,05*	$\frac{5}{8} < 50$ $\frac{3}{8} > 50$	Centrilobular necrosis

* Differs significantly from identically treated unanaesthetized controls, $P < 0,001$

≠ Probably differs from identically treated unanaesthetized controls, $P < 0,05$

TABLE 40. THE EFFECT OF FASTING, PHENOBARBITONE PRETREATMENT AND REPEATED ANAESTHESIA ON HEPATIC MICROSOMAL CYTOCHROME P-450, S.G.O.T. AND HEPATIC HISTOLOGY.

Protocol	Diet	Phenobarbitone pretreatment	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic histology
No anaesthesia	Fasted	-	1,24 ± 0,33	-	-
No anaesthesia	Fasted	+	2,49 ± 0,12	35,6 ± 7,5	-
3 × 1 MAC halothane	Fasted	+	1,49 ± 0,38≠	22,0 ± 8,3	Normal
3 × 1 MAC enflurane	Fasted	+	2,97 ± 0,30≠	39,0 ± 4,4	Normal

TABLE 40 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic histology
3 × 1 MAC ether	Fasted	+	2,19 ± 0,12†	40,4 ± 3,9	Normal

† Differs significantly from identically treated unanaesthetized controls, $P < 0,01$

≠ Probably differs from identically treated unanaesthetized controls, $P < 0,05$

(b) The effects of anaesthesia under hypoxic conditions in vivo.

It has been reported that exposure of phenobarbitone pretreated animals to halothane under conditions of hypoxia resulted in gross hepatic centrilobular necrosis (Cousins et al., 1979; McLain et al., 1979 and Ross et al, 1979). The effects of anaesthesia under hypoxic conditions on the stearate desaturase enzyme system, cytochrome P-450 levels and hepatic histology were assessed in the livers from rats fed a high carbohydrate or normal diet in the presence and absence of phenobarbitone pretreatment.

The effects of anaesthesia under hypoxic conditions on the hepatic microsomal stearate desaturase enzyme system.

Stearate desaturase activity in hepatic microsomes from rats fed a high carbohydrate diet was unaffected by N_2O or halothane administered under hypoxic conditions (O_2 700 ml/min), when compared to the activity in hepatic microsomes from rats fed a high carbohydrate diet, not exposed to anaesthesia (Table 41). Stearate desaturase activity was markedly decreased following chloroform anaesthesia under hypoxic conditions.

Stearate desaturase activity was decreased in hepatic microsomes from rats fed a high carbohydrate diet and

pretreated with phenobarbitone (Table 41) relative to those only fed a high carbohydrate diet. Halothane anaesthesia under hypoxic conditions did not further reduce the activity of stearate desaturase in hepatic microsomes from rats fed a high carbohydrate diet and pretreated with phenobarbitone.

Stearate desaturase activity in hepatic microsomes from rats fed a normal diet was measurable, although lower than that observed in microsomes from rats fed a high carbohydrate diet (Tables 41 and 42). Stearate desaturase activity in hepatic microsomes from rats fed a normal diet was unaffected by N_2O or halothane administration under hypoxic conditions, but was decreased by chloroform anaesthesia under hypoxic conditions (Table 42). Halothane anaesthesia under hypoxic conditions did not affect stearate desaturase activity in hepatic microsomes from rats fed a normal diet and pretreated with phenobarbitone (Table 42).

The effect of anaesthesia under hypoxic conditions on hepatic microsomal cytochrome P-450, S.G.O.T. and hepatic histology.

The levels of hepatic microsomal cytochrome P-450 in microsomes from rats fed a high carbohydrate diet were unaffected by N_2O, halothane or chloroform anaesthesia under hypoxic conditions (Table 43). The levels of

cytochrome P-450 were elevated by phenobarbitone pretreatment, but halothane anaesthesia under hypoxic conditions did not affect the raised levels of cytochrome P-450 present in hepatic microsomes from rats fed a high carbohydrate diet and pretreated with phenobarbitone (Table 43).

Similarly, the levels of hepatic microsomal cytochrome P-450 in microsomes from rats fed a normal diet, pretreated or not with phenobarbitone, were unaffected by N_2O, halothane or chloroform anaesthesia under hypoxic conditions (Table 44).

The levels of S.G.O.T. were raised, following exposure to halothane under hypoxic conditions, in animals fed either a normal or high carbohydrate diet, pretreated or not with phenobarbitone (Tables 43 and 44 and Figures 23 and 24). However, the levels of S.G.O.T. in animals on a normal diet, pretreated or not with phenobarbitone exposed to halothane and hypoxia, were significantly higher than in those animals on a high carbohydrate diet, pretreated or not with phenobarbitone exposed to halothane and hypoxia (Tables 43 and 44).

The levels of S.G.O.T. were raised following exposure to chloroform and hypoxia in animals fed either a normal diet or a high carbohydrate diet (Tables 43 and

44 and Figures 23 and 24). The levels of S.G.O.T. in animals fed a normal diet were raised following exposure to enflurane and methoxyflurane under hypoxic conditions (Table 44), but exposure to methoxyflurane appeared to be less deleterious than exposure to enflurane.

The hepatic histology of animals fed either the normal diet or the high carbohydrate diet and exposed to N_2O or halothane and hypoxia, was normal. When animals were fed either the normal diet or the high carbohydrate diet, pretreated with phenobarbitone and exposed to halothane and hypoxia, the hepatic histology was that of congestion and centrilobular necrosis (Tables 43 and 44). The hepatic histology of animals fed a normal diet, pretreated with phenobarbitone and exposed to enflurane and methoxyflurane, was normal. Exposure to chloroform and hypoxia resulted in congestion and centrilobular necrosis of hepatocytes from animals fed either a normal diet or a high carbohydrate diet (Tables 43 and 44).

The mortality of animals fed a normal diet and exposed to halothane and hypoxia, was significantly higher than that of animals fed a high carbohydrate diet and exposed to halothane and hypoxia (Tables 43 and 44 and Figures 23 and 24). The mortality of animals fed a normal diet and pretreated with phenobarbitone prior to exposure to halothane and hypoxia was not significantly

different from that of animals fed a high carbohydrate diet and pretreated with phenobarbitone prior to exposure to halothane and hypoxia (Tables 43 and 44).

The effect of exposure to halothane under hypoxic conditions on hepatic glutathione levels in rats fed a normal diet is reported in Table 45 and Figure 25. The levels of hepatic glutathione in animals exposed to N_2O/O_2 alone, were unaffected relative to the levels in unanaesthetized rats, while the levels of hepatic glutathione in animals exposed to halothane and hypoxia were significantly decreased after 30 and 45 minutes of exposure to halothane. This decrease however, was not maintained.

TABLE 41. THE EFFECT OF A HIGH CARBOHYDRATE DIET, PHENOBARBITONE INDUCTION AND A SINGLE ANAESTHESIA UNDER HYPOXIC CONDITIONS ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME b_5.

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
			Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
No anaesthesia	High carbohydrate diet	-	None	1,49 ± 0,17	
			KCN (0,5)	1,32 ± 0,42	
			Stearoyl CoA (0,012)	4,77 ± 1,70	3,28 ± 1,56
			Stearoyl CoA (0,012) + KCN (0,5)	4,14 ± 2,60	
N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	High carbohydrate diet	-	None (0,5)	1,13 ± 0,02	
			KCN (0,5)	1,29 ± 0,01	
			Stearoyl CoA (0,012)	3,68 ± 0,53	2,55 ± 0,51
			Stearoyl CoA (0,012) + KCN (0,5)	2,16 ± 0,37	

TABLE 41 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome $\underline{b}_5$		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
			Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	
1 MAC halothane N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	High carbohydrate diet	-	None	1,33 ± 0,24	
			KCN (0,5)	1,21 ± 0,20	
			Stearoyl CoA (0,012)	3,76 ± 1,22	2,43 ± 1,38
			Stearoyl CoA (0,012) + KCN (0,5)	2,21 ± 0,55	
1 MAC chloroform/ N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	High carbohydrate diet	-	None	1,21 ± 0,06	
			KCN (0,5)	1,01 ± 0,05	
			Stearoyl CoA (0,012)	1,89 ± 0,82	0,68 ± 0,75≠
			Stearoyl CoA (0,012) + KCN (0,5)	1,15 ± 0,05	

TABLE 41 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5 Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
No anaesthesia	High carbohydrate diet	+	None	2,07 ± 0,56	
			KCN (0,5)	1,89 ± 0,48	
			Stearoyl CoA (0,012)	2,89 ± 0,62	0,82 ± 0,35
			Stearoyl CoA (0,012) + KCN (0,5)	2,44 ± 0,45	
1 MAC halothane/ N_2O (4,3 l/min)/ O_2 (700 ml/min)	High carbohydrate diet	+	None	1,84 ± 0,13	
			KCN (0,5)	1,82 ± 0,29	
			Stearoyl CoA (0,012)	2,61 ± 0,28	0,77 ± 0,23
			Stearoyl CoA (0,012) + KCN (0,5)	2,53 ± 0,47	

≠ Probably differs from identically treated unanaesthetized controls, $P < 0,05$

TABLE 42. THE EFFECT OF A NORMAL DIET, PHENOBARBITONE INDUCTION AND A SINGLE ANAESTHESIA UNDER HYPOXIC CONDITIONS ON THE RE-OXIDATION OF NADH REDUCED HEPATIC MICROSOMAL FERROCYTOCHROME $\underline{b}_5$.

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome $\underline{b}_5$		Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}(sec^{-1})$
			Additions (mM)	$10^2 k_{obs}(sec^{-1})$	
No anaesthesia	Normal diet	-	None	1,36 ± 0,29	
			KCN (0,5)	1,25 ± 0,27	
			Stearoyl CoA (0,012)	2,10 ± 1,03	0,74 ± 0,69
			Stearoyl CoA (0,012) + KCN (0,5)	1,61 ± 0,25	
N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	Normal diet	-	None	0,67 ± 0,01	
			KCN (0,5)	0,79 ± 0,01	
			Stearoyl CoA (0,012)	1,46 ± 0,48	0,79 ± 0,48
			Stearoyl CoA (0,012) + KCN (0,5)	0,78 ± 0,0	

TABLE 42 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5 Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
1 MAC halothane/ N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	Normal diet	-	None	1,55 ± 0,42	
			KCN (0,5)	1,21 ± 0,19	
			Stearoyl CoA (0,012)	2,66 ± 0,72	1,11 ± 0,45
			Stearoyl CoA (0,012) + KCN (0,5)	2,11 ± 0,47	
1 MAC chloroform/ N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	Normal diet	-	None	1,09 ± 0,18	
			KCN (0,5)	0,99 ± 0,07	
			Stearoyl CoA (0,012)	1,17 ± 0,09	0,08 ± 0,21
			Stearoyl CoA (0,012) + KCN (0,5)	1,06 ± 0,07	

TABLE 42 (Cont.)

Protocol	Diet	Phenobarbitone pretreatment	Re-oxidation of ferrocytochrome b_5 Additions (mM)	$10^2 k_{obs}$ (sec^{-1})	Difference in k_{obs} (presence minus absence of stearoyl CoA) $10^2 k_{obs}$ (sec^{-1})
No anaesthesia	Normal diet	+	None	2,12 ± 0,63	
			KCN (0,5)	1,90 ± 0,54	
			Stearoyl CoA (0,012)	2,75 ± 0,95	0,63 ± 0,63
			Stearoyl CoA (0,012) + KCN (0,5)	2,64 ± 0,68	
1 MAC halothane/ N_2O (4,3 l/min)/ O_2 (700 ml/min)	Normal diet	+	None	1,65 ± 0,30	
			KCN (0,5)	1,71 ± 0,57	
			Stearoyl CoA (0,012)	2,11 ± 0,44	0,46 ± 0,24
			Stearoyl CoA (0,012) + KCN (0,5)	1,82 ± 0,31	

TABLE 43. THE EFFECT OF A HIGH CARBOHYDRATE DIET, PHENOBARBITONE INDUCTION AND A SINGLE ANAESTHESIA UNDER HYPOXIC CONDITIONS ON CYTOCHROME P-450, S.G.O.T., HEPATIC HISTOLOGY AND MORTALITY.

Protocol	Diet	Pheno-barbitone induction	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic Histology	Mortality
No anaesthesia	High carbo-hydrate diet	-	0,89 ± 0,10	25,0 ± 7,0	Normal	-
N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	High carbo-hydrate diet	-	1,15 ± 0,09	34,8 ± 9,2	Normal	$\frac{0}{10}$
1 MAC halothane/ N_2O (4,3 l/min)/ O_2 700 ml/mins) (2 hours)	High carbo-hydrate diet	-	1,27 ± 0,23	$\frac{30}{35} < 50$; $\frac{5}{35} > 50$ ¶	Normal	$\frac{2}{35}$ §

TABLE 43 (Cont.)

Protocol	Diet	Phenobarbitone induction	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic Histology	Mortality
1 MAC chloroform/ N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	High carbohydrate diet	-	1,09 $\pm$ 0,04	$\frac{7}{13} < 50$; $\frac{6}{13} > 50^{\neq}$	Some livers normal, majority displayed congestion and centrilobular necrosis	$\frac{16}{20}$ *
No anaesthesia	High carbohydrate diet	+	1,87 $\pm$ 0,52	33,9 $\pm$ 9,0	Normal	
1 MAC halothane/ N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hours)	High carbohydrate diet	+	1,14 $\pm$ 0,24	$\frac{5}{13} < 50$; $\frac{8}{13} > 50$	Some congestion and centrilobular necrosis	$\frac{4}{15}$

TABLE 43 (Cont.)

Statistical analyses were performed using a χ^2 distribution.

* Differs significantly from identically pretreated unanaesthetized rats, $P < 0,001$

≠ Probably differs from identically pretreated unanaesthetized rats, $P < 0,05$

§ Differs significantly from normal diet halothane anaesthetized rats, $P < 0,001$

¶ Differs significantly from normal diet halothane anaesthetized rats, $P < 0,01$

In certain cases, the number of S.G.O.T. assays performed was less than the number of rats anaesthetized. This discrepancy arose when the rats died during anaesthesia or overnight and it was impossible to obtain serum.

TABLE 44. THE EFFECT OF A NORMAL DIET, PHENOBARBITONE INDUCTION AND A SINGLE ANAESTHESIA UNDER HYPOXIC CONDITIONS ON CYTOCHROME P-450, S.G.O.T., HEPATIC HISTOLOGY AND MORTALITY.

Protocol	Diet	Induction	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic Histology	Mortality
No anaesthesia	Normal diet	-	0,97 ± 0,21	25,5 ± 7,1	Normal	-
N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hrs)	Normal diet	-	0,64 ± 0,06	$\frac{5}{10}$ < 50; $\frac{5}{10}$ > 50	Normal	$\frac{0}{10}$
1 MAC halothane N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hrs)	Normal diet	-	1,08 ± 0,37	$\frac{17}{33}$ < 50; $\frac{16}{33}$ > 50	Normal, some congestion	$\frac{12}{35}$ *
1 MAC chloroform N_2O (4,3 l/min)/ O_2 (700 ml/min) (2 hrs)	Normal diet	-	0,78 ± 0,16	$\frac{5}{8}$ < 50; $\frac{3}{8}$ > 50	Some livers normal, but majority displayed congestion and centrilobular necrosis	$\frac{17}{20}$ *

TABLE 44 (Cont.)

Protocol	Diet	Induction	Cytochrome P-450 (nmoles/mg microsomal protein)	S.G.O.T. (I.U.)	Hepatic Histology	Mortality
No anaesthesia	Normal diet	+	1,73 ± 0,55	32,0 ± 9,2	Normal	
N_2O (4,3 l/min)/ O_2 (700 ml/min)	Normal	+	-	$\frac{9}{14}$ < 50; $\frac{5}{14}$ > 50	Normal	$\frac{0}{14}$
1 MAC halothane/ N_2O (4,3 l/min)/ O_2 (700 ml/min)	Normal	+	1,23 ± 0,07	$\frac{5}{25}$ < 50; $\frac{20}{25}$ > 50 †	Congestion, centri-lobular necrosis	$\frac{7}{25}$ ≠
1 MAC enflurane/ N_2O (4,3 l/min)/ O_2 (700 ml/min)	Normal	+	-	$\frac{12}{30}$ < 50; $\frac{18}{30}$ > 50	Normal, occasional small areas of necrosis	$\frac{1}{30}$
1 MAC methoxy-flurane/N_2O (4,3 l/min)/ O_2 (700 ml/min)	Normal	+	-	$\frac{8}{10}$ < 50; $\frac{2}{10}$ > 50	Normal	$\frac{0}{10}$

TABLE 44 (Cont.)

Statistical analysis was performed using a χ^2 distribution.

* Differs significantly from identically pretreated N_2O anaesthetised rats, $P < 0,001$

† Differs significantly from identically pretreated N_2O anaesthetised rats, $P < 0,01$

≠ Probably differs from identically pretreated N_2O anaesthetised rats, $P < 0,05$

In certain cases, the number of S.G.O.T. assays performed was less than the number of rats anaesthetised. This discrepancy arose when the rats died during anaesthesia or overnight, and it was impossible to obtain serum.

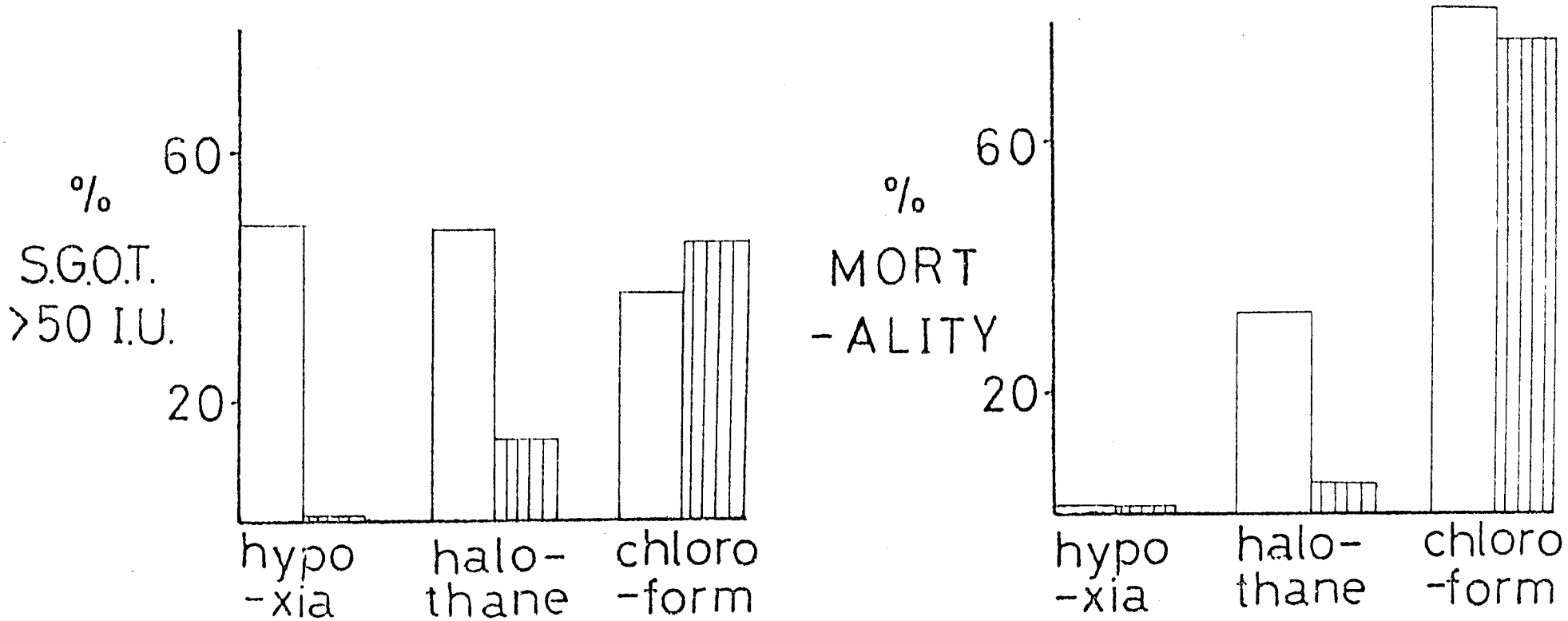


FIGURE 23 : The effect of dietary pretreatment and anaesthesia under hypoxic conditions on S.G.O.T. and mortality. Rats fed a normal diet (□); rats fed a high carbohydrate diet (▥).

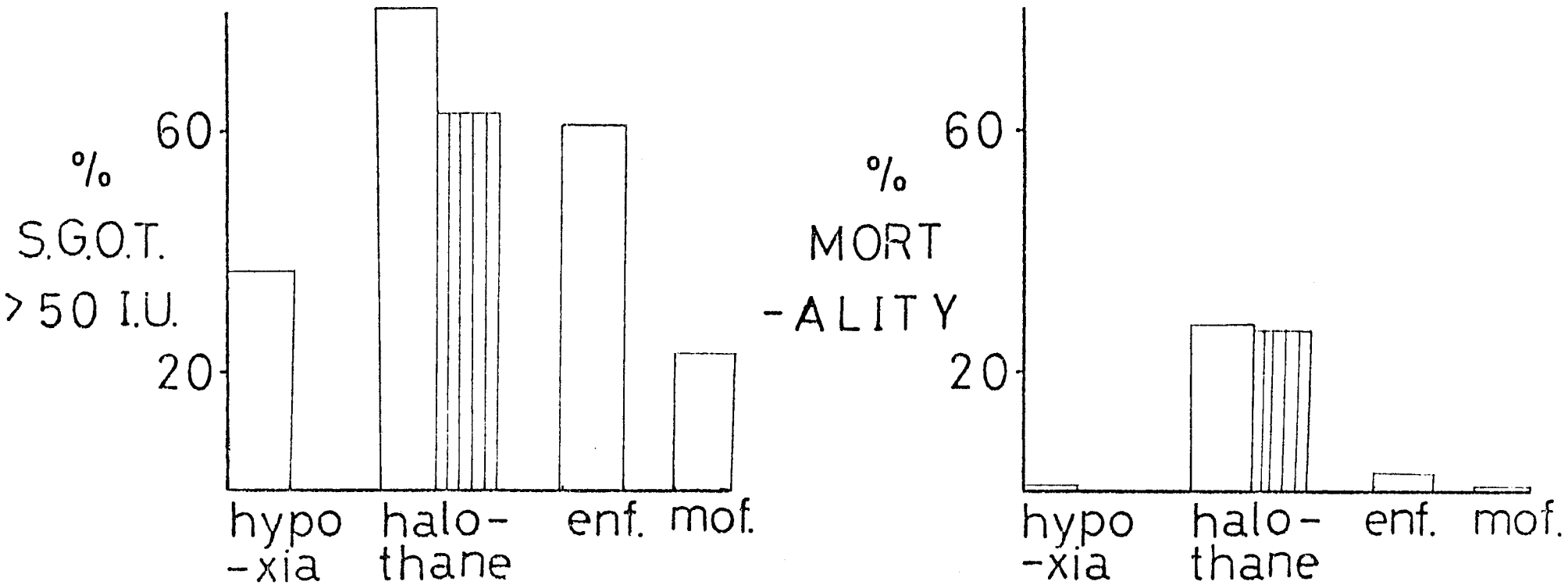


FIGURE 24 : The effect of dietary pretreatment, phenobarbitone induction and anaesthesia under hypoxic conditions on S.G.O.T. and mortality. Rats fed a normal diet (□); rats fed a high carbohydrate diet (▥). ENF = enflurane; MOF = methoxyflurane.

TABLE 45. THE EFFECT OF HALOTHANE ANAESTHESIA UNDER HYPOXIC CONDITIONS ON LIVER GLUTATHIONE.

Protocol	Time of exposure (min)	µg Glutathione/100 mg wet liver weight
No anaesthesia	-	133 ± 16
N_2O/O_2	30	138 ± 29
N_2O/O_2	90	123 ± 39
N_2O/O_2	180	156 ± 27
1 MAC halothane/N_2O/O_2	30	97 ± 18†
1 MAC halothane/N_2O/O_2	45	110 ± 9†
1 MAC halothane/N_2O/O_2	90	130 ± 24
1 MAC halothane/N_2O/O_2	120	110 ± 2
1 MAC halothane/N_2O/O_2	160	175 ± 0
1 MAC halothane/N_2O/O_2	180	160 ± 0

Assays are in duplicate for determinations on each of 3 to 5 livers of rats fed a normal diet, after a single anaesthetic exposure.

The rats were maintained on a normal diet and induced with phenobarbitone as described in Methods. N_2O was administered at a rate of 4,3 l/min. O_2 was administered at a rate of 700 ml/min.

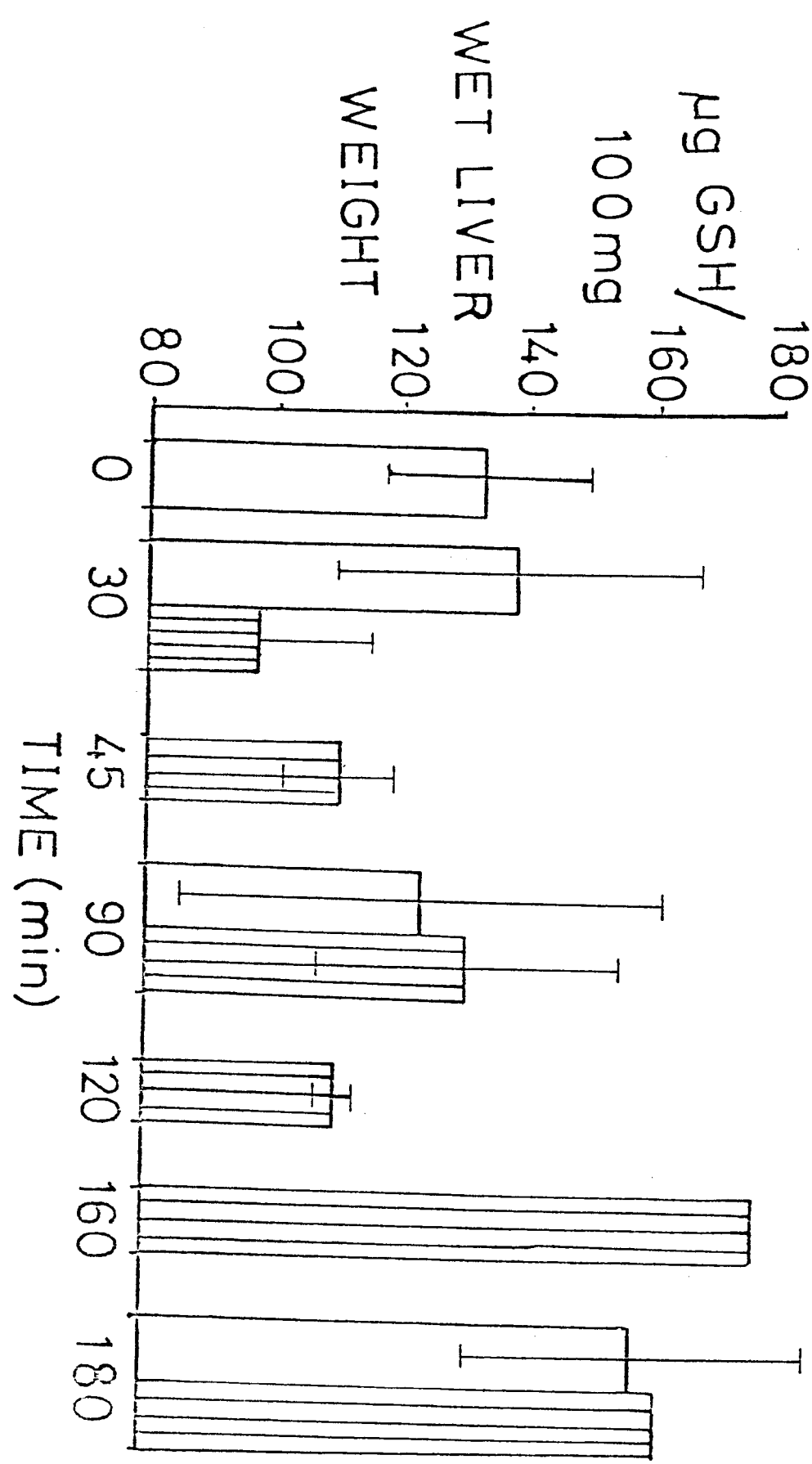


FIGURE 25 : The effect of halothane anaesthesia under hypoxic conditions on liver glutathione. N_2O/O_2 exposure (□); 1 MAC halothane/N_2O/O_2 (▥).

4. DISCUSSION

The interaction of xenobiotics with hepatic microsomal enzymes.

The interaction of xenobiotics with hepatic microsomal cytochrome P-450 has been the subject of extensive investigations over the past twenty years. The biochemical relevance of these investigations is of inestimable importance because the metabolism of xenobiotics, particularly by cytochrome P-450, is in general essential in maintaining the well-being of humans and animals exposed to xenobiotics such as medical drugs or environmental pollutants. However, it has recently been demonstrated that certain xenobiotics are capable of interacting with other hepatic microsomal enzymes, not generally thought to be involved in drug metabolism (Oshino and Sato, 1971). The relevance of such an interaction is of interest both biochemically and clinically as the interaction of xenobiotics with microsomal enzymes other than cytochrome P-450 could affect the extent of their metabolism or the metabolic status of the cell, with either beneficial or detrimental consequences to the body.

The observation that the hepatic microsomal enzyme stearate desaturase, which was previously known only to interact with its physiological substrate, interacts with p-cresol and halothane (Oshino and Sato, 1971; Berman et al., 1975)

suggested that other xenobiotics might also interact with hepatic microsomal stearate desaturase. This thesis reports on an investigation of the possible interaction of approximately fifty xenobiotics with hepatic microsomal stearate desaturase. This study represents the first extensive investigation of the interaction of xenobiotics with this enzyme. The majority of xenobiotics chosen for this study were halogenated hydrocarbons because one of the two xenobiotics previously shown to interact with stearate desaturase was the halogenated hydrocarbon halothane. However, halogenated ethers including the volatile anaesthetic agents enflurane ($CClFHCF_2OCF_2H$) and methoxyflurane ($CCl_2HCF_2OCH_3$), were also investigated. Non-halogenated structural analogues of some xenobiotics were included in the investigations in an attempt to draw some conclusions on the structural features of a xenobiotic required to interact with stearate desaturase.

It was not possible to assess the interaction of xenobiotics with stearate desaturase directly *viz*. via the conversion of stearoyl CoA to oleate, because none of the xenobiotics known to interact with stearate desaturase affect this process. Therefore, assays of the oxidation state of cytochrome b_5 were employed to indirectly assess the interaction of xenobiotics with stearate desaturase. This approach was utilized because compounds which interact with stearate desaturase are known to enhance the oxidation of hepatic microsomal ferrocytochrome b_5 (see Introduction). In these investigations, hepatic microsomes from rats fed a high carbohydrate diet to

enhance the activity of stearate desaturase were utilized and both NADH and NADPH were employed as electron donors to stearate desaturase. Furthermore, advantage was taken of the observation that cyanide effectively inhibits the stearate desaturase enzyme system by binding to the terminal oxidase, the C.S.F. (Oshino et al., 1966; Shimakata et al., 1971).

The first assay which was utilized to assess the interaction of xenobiotics with hepatic microsomal stearate desaturase was the redox steady state of microsomal cytochrome $\underline{b}_5$ in the presence of NADPH. This assay was chosen because it is the most rapid and simple assay which provides a measure of the oxidation state of cytochrome $\underline{b}_5$. This assay does not however directly measure the rate of oxidation of ferrocytochrome $\underline{b}_5$, but reflects a composite of the rate of reduction of ferricytochrome $\underline{b}_5$ by NADPH via NADPH-cytochrome $\underline{c}$ (P-450) reductase and the rate of oxidation of ferrocytochrome $\underline{b}_5$, a process which can occur via several pathways (see Figure 7).

In an attempt to assess the ability of the xenobiotics to interact with stearate desaturase, utilizing the NADPH redox steady state of microsomal cytochrome $\underline{b}_5$, it was observed that the majority of xenobiotics shifted the redox steady state of microsomal cytochrome $\underline{b}_5$ towards the ferric form of the protein (Table I). This assay provided only a gross indication of the ability of a xenobiotic to interact with stearate desaturase for several reasons. Firstly, NADPH is

not the preferential electron donor to stearate desaturase (but is for cytochrome P-450) and hence electron flow from NADPH through cytochrome $\underline{b}_5$ could be due to the interaction of xenobiotics with other microsomal proteins such as cytochrome P-450. Secondly, the attempt to assess the role of stearate desaturase on the effect of xenobiotics on the steady state of cytochrome $\underline{b}_5$ in the presence and absence of cyanide was unsuccessful because cyanide affected the redox steady state of NADPH reduced cytochrome $\underline{b}_5$ in the absence of xenobiotics.

In order to investigate with greater specificity the ability of selected xenobiotics to interact with stearate desaturase, the effect of those xenobiotics - particularly the haloalkanes - which shifted the redox steady state of cytochrome $\underline{b}_5$ to 40% or below, on the re-oxidation of NADH reduced hepatic microsomal cytochrome $\underline{b}_5$ was examined. This assay is more specific for the interaction of a xenobiotic with stearate desaturase than is the NADPH redox steady state assay because the assay of the re-oxidation of cytochrome $\underline{b}_5$ affords a direct measure of the rate of oxidation of cytochrome $\underline{b}_5$ and is not affected by the rate of reduction of cytochrome $\underline{b}_5$ by NADH. A further advantage of this assay is that the reductant NADH is the preferential electron donor for the stearate desaturase enzyme system (Jones *et al.*, 1969; Joshi *et al.*, 1977). Because the oxidation of cytochrome $\underline{b}_5$ can occur via several pathways (see Figure 7), the effects of cyanide were utilized to assess the role of stearate desatu-

rase in the enhanced oxidation of microsomal ferrocytochrome $\underline{b}_5$. A direct correlation between the rate of re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ and the rate of conversion of stearoyl CoA to oleate by stearate desaturase has been demonstrated by Oshino *et al.* (1971), confirming that the re-oxidation of cytochrome $\underline{b}_5$ provides a valid assay for the activity of stearate desaturase.

The rate of re-oxidation of NADH reduced microsomal ferrocytochrome $\underline{b}_5$ was significantly enhanced in the presence of the majority of the xenobiotics investigated (Table 2) with the exception only of iodoform, 1,1,2,2-tetrachloroethane, 1,2,2-trichloro-1,1,2-trifluoroethane, i.e. thirteen out of sixteen xenobiotics enhanced the rate of re-oxidation of microsomal ferrocytochrome $\underline{b}_5$.

In a comparison of the results obtained for the effect of xenobiotics on the NADPH steady state and on the re-oxidation of cytochrome $\underline{b}_5$, it was observed that of the xenobiotics which shifted the redox steady state of NADPH reduced cytochrome $\underline{b}_5$ towards the ferric form of the protein, some, for example, iodoform, 1,1,2,2-tetrachloroethane and 1,2,2-trichloro-1,1,2-trifluoroethane did not enhance the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$. Other xenobiotics, at comparable concentrations, such as iodomethane, 1,1,2-trichloroethane, 1-bromo-2-chloroethane and ethyl vinyl ether shifted the redox steady state of cytochrome $\underline{b}_5$ towards the ferric form and enhanced the re-oxidation of cytochrome $\underline{b}_5$.

Some xenobiotics such as 1,2-dibromo-1,1-dichloroethane, 1,2,3-trichloropropane, ethyl vinyl ether and 1-bromo-2-chloroethane did not exhibit a cyanide sensitive effect on the re-oxidation of cytochrome $\underline{b}_5$ while others such as diiodomethane and 1,1,2-trichloroethane did (Tables 1 and 2).

The ability of xenobiotics to enhance the rate of re-oxidation of cytochrome $\underline{b}_5$ in a cyanide insensitive manner suggested that these xenobiotics (e.g. 1,2-dibromo-1,1-dichloroethane, 1,2,3-trichloropropane, ethyl vinyl ether and 1-bromo-2-chloroethane) might enhance the electron flow through cytochrome $\underline{b}_5$ by interacting with microsomal proteins which are not inhibited by 0,5 mM cyanide, such as cytochrome P-450 (Jefcoate et al., 1969; Correia and Mannering, 1973), N-hydroxylamine oxidase and the phospholipid desaturase (Pugh and Kates, 1977) rather than microsomal stearate desaturase. It has been established that 1,2,3-trichloropropane and ethyl vinyl ether bind to the substrate binding site of cytochrome P-450 and stimulate microsomal NADPH oxidation (Ivanetich et al., 1978) suggesting that these xenobiotics are metabolized by the hepatic microsomal cytochrome P-450 enzyme system, which is consistent with the above proposal.

The ability of a xenobiotic to enhance the rate of re-oxidation of cytochrome $\underline{b}_5$ in a cyanide sensitive manner suggested that the xenobiotic might enhance electron flow through cytochrome $\underline{b}_5$ by interacting with one or more of the terminal oxidases which is inhibited by concentrations of cyanide of

0,5 mM such as the phospholipid desaturase and the acyl CoA Δ5-, Δ6- and Δ9-desaturases (see Oshino, 1978).

Those compounds which shifted the redox steady state of cytochrome $\underline{b}_5$ to 40% or below and which increased the rate of re-oxidation of cytochrome $\underline{b}_5$ in a cyanide sensitive manner were bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane, chloroacetaldehyde and the anaesthetic agents halothane, enflurane and methoxyflurane (Berman et al., 1975) (Tables 1 and 2). In order to establish whether the enhanced rate of re-oxidation observed in the presence of these xenobiotics was due to their interaction with hepatic microsomal stearate desaturase, the effect of these xenobiotics on cytochrome $\underline{b}_5$, the NADH- and NADPH-cytochrome $\underline{c}$ reductases, cytochrome P-450, N-hydroxylamine oxidase, the phospholipid desaturase and the acyl CoA Δ5- and Δ6-desaturases as well as the C.S.F. of the stearate desaturase enzyme system was examined. The possible interactions of the halogenated xenobiotics bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde with each of the above-mentioned microsomal proteins will be discussed first and those of the anaesthetic agents halothane, enflurane and methoxyflurane on these microsomal proteins will be discussed subsequently, since more extensive experimentation was undertaken on the interaction of the anaesthetic agents with hepatic microsomal proteins.

The interaction of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde with some hepatic microsomal proteins.

The ability of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde (as well as the anaesthetic agents halothane, enflurane and methoxyflurane) to shift the redox steady state of hepatic microsomal cytochrome b_5 towards the ferric form of the protein (Table 1) suggested that these xenobiotics are increasing the rate of oxidation and/or decreasing the rate of reduction of cytochrome b_5. The effect of these xenobiotics on the redox steady state of cytochrome b_5 is apparently due to an increased rate of oxidation of cytochrome b_5 rather than a decreased rate of reduction of the cytochrome since these xenobiotics enhance the rate constant for the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome b_5, which is a measure of the rate of oxidation of this hemoprotein (Berman et al., 1975; Oshino et al., 1971), but these xenobiotics do not increase the activity of the microsomal NADH- or NADPH-cytochrome c reductases* (Tables 2, 5 and 10).

The observed increase in the rate of oxidation of hepatic microsomal ferrocytochrome b_5 could therefore be due to an increased rate of autoxidation of this cytochrome, or due to

* Since electron transfer from ferrocytochrome b_5 to ferricytochrome c is rapid, the cytochrome c reductase activities provide a measure of the rate of reduction of ferricytochrome b_5 (Strittmatter and Velick, 1956).

an increased rate of electron transfer from ferrocytochrome $\underline{b}_5$ to one or more of the hepatic microsomal terminal oxidases (see above and Figure 7). The autoxidation of ferrocytochrome $\underline{b}_5$ refers to the direct transfer of electrons from ferrocytochrome $\underline{b}_5$ to molecular oxygen, by the following reaction :

$$Fe^{2+}\text{cytochrome } \underline{b}_5 + O_2 \rightleftharpoons Fe^{3+}\text{cytochrome } \underline{b}_5 + O_2^{-\cdot}$$

The enhancement of the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome $\underline{b}_5$ by bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde appeared not to be due to enhanced autoxidation of ferrocytochrome $\underline{b}_5$ because the stimulation of the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ by these xenobiotics is cyanide sensitive (Table 2) while the autoxidation of purified, trypsin-cleaved ferrocytochrome $\underline{b}_5$ is insensitive to cyanide (Berman et al., 1975).

Enhanced oxidation of hepatic microsomal ferrocytochrome $\underline{b}_5$ would therefore appear to be a consequence of the stimulation by these xenobiotics of electron transfer to one or more of the terminal microsomal oxidases (see above). The microsomal oxidase cytochrome P-450 is known to bind all three of these xenobiotics with the production of a type 1 difference spectrum (Ivanetich et al., 1978) (see Results) which is indicative of the binding of a substrate to the substrate-binding site of cytochrome P-450. In addition, the K_s values for the

binding of bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane to cytochrome P-450 were similar to their K_{eq} values for the stimulation of the re-oxidation of microsomal cytochrome $\underline{b}_5$ (Table 6 and Figures 8, 9 and 10).

It would appear however, that the terminal microsomal oxidase cytochrome P-450 is not responsible for the stimulation of the re-oxidation of microsomal cytochrome $\underline{b}_5$ by bromotrichloromethane, but may be at least in part responsible for that observed in the presence of 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde. The enhanced re-oxidation of cytochrome $\underline{b}_5$ observed in the presence of bromotrichloromethane was not decreased by CO and metyrapone (Table 4), two inhibitors of cytochrome P-450 which exert their effect by binding to the active site of this enzyme (De Bruin, 1976). The lack of effect of these inhibitors on the re-oxidation of cytochrome $\underline{b}_5$ in the presence of bromotrichloromethane suggested that cytochrome P-450 is not responsible for the enhanced re-oxidation observed in the presence of this xenobiotic. Furthermore, the re-oxidation of cytochrome $\underline{b}_5$ in the presence of bromotrichloroethane was inhibited by 0,5 mM cyanide at a concentration too low to inhibit cytochrome P-450 and decreased by fasting, which did not alter the levels of cytochrome P-450 significantly (Table 3). It appears therefore that cytochrome P-450 is not involved in the enhanced re-oxidation of ferrocytochrome $\underline{b}_5$ observed in the presence of bromotrichloromethane.

A role for cytochrome P-450 in the stimulation of the re-oxidation of cytochrome $\underline{b}_5$ by 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde is supported by the ability of CO to significantly inhibit the enhanced re-oxidation of cytochrome $\underline{b}_5$ in the presence of these two xenobiotics and of metyrapone to inhibit the enhanced re-oxidation of cytochrome $\underline{b}_5$ in the presence of 1,2-dibromo-1,2-dichloroethane (Table 4). Furthermore, fasting, which was without effect on the levels of cytochrome P-450, did not affect the enhanced re-oxidation of ferrocytochrome $\underline{b}_5$ observed in the presence of chloroacetaldehyde (Table 3).

The effects of the xenobiotics on another microsomal protein, N-hydroxylamine oxidase were considered. It appeared that N-hydroxylamine oxidase is not involved in the effects of bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde on the re-oxidation of cytochrome $\underline{b}_5$ because N-hydroxylamine oxidase is insensitive to cyanide and CO (Kadlubar et al., 1973) while the effects of these xenobiotics on the re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ are sensitive to cyanide and in the case of 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde, also to CO (Tables 2 and 4).

Furthermore, the microsomal phospholipid desaturase and the microsomal acyl CoA Δ5- and Δ6-desaturases would appear to play no role in the enhanced oxidation of microsomal ferrocytochrome $\underline{b}_5$ by the three halo xenobiotics. The acyl CoA

Δ5-desaturase and the phospholipid desaturase are less sensitive to cyanide than is the re-oxidation of cytochrome $\underline{b}_5$. The activities of the desaturases are inhibited by 1-5 mM cyanide while the effects of the xenobiotics on the re-oxidation of cytochrome $\underline{b}_5$ are inhibited by 0,5 mM cyanide. Furthermore, the activity of the Δ6-desaturase is not enhanced by refeeding a high carbohydrate diet (Inkpen et al., 1969) which enhances the effects of bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane on microsomal electron transfer (Tables 2 and 3).

In addition to the microsomal proteins discussed above, the microsomal contaminant catalase may also be excluded as a mediator of the enhanced oxidation of microsomal ferrocytochrome $\underline{b}_5$ in the presence of the three halo xenobiotics. The K_i for cyanide inhibition of catalase (approximately 8 μM) (Chance, 1952) is ten fold lower than the K_i for cyanide inhibition of the enhancement of the re-oxidation of cytochrome $\underline{b}_5$ by the three halo xenobiotics (Table 7 and Figure 11) which suggests that catalase is not responsible for the enhanced re-oxidation of cytochrome $\underline{b}_5$ observed in the presence of these xenobiotics.

In view of the above observations, it appeared that bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde at least in part, stimulate the oxidation of hepatic microsomal ferrocytochrome $\underline{b}_5$ by interacting with an hepatic microsomal enzyme other than those described above.

There are several lines of evidence to suggest that these xenobiotics enhance electron flow through cytochrome $\underline{b}_5$ by interacting with stearate desaturase:

The ability of bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane to shift the redox state of cytochrome $\underline{b}_5$ towards the ferric form of the protein and the ability of cyanide to inhibit this process, paralleled the dietary induction of stearate desaturase (Tables 2 and 3). In rats which had been re-fed a high carbohydrate diet to induce stearate desaturase, the effect of these xenobiotics on the re-oxidation of ferrocytochrome $\underline{b}_5$ was enhanced, whereas in fasted rats which have decreased levels and activity of stearate desaturase, the effect of the xenobiotics on the re-oxidation of ferrocytochrome $\underline{b}_5$ was not observable (Oshino and Sato, 1972) (Tables 2 and 3).

Further evidence that the xenobiotics are interacting with stearate desaturase is provided by the K_i values for cyanide inhibition of the enhancement of the re-oxidation of cytochrome $\underline{b}_5$ by bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane. The K_i values for this effect (Table 7 and Figure 11) are in good agreement with the K_i for cyanide inhibition of stearate desaturase (0,1 mM) reported by Oshino *et al.*,(1966), but differ from the K_i of 2,5 - 8 mM which has been reported for cyanide inhibition of cytochrome P-450 (Jefcoate *et al.*, 1969; Correia and Mannering, 1973). The observed agreement in K_i values provided further evidence that these two xeno-

biotics are indeed stimulating microsomal electron transfer by interacting with stearate desaturase.

Although bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and possibly chloroacetaldehyde appear to bind to stearate desaturase (see Results) none of these xenobiotics affect the stearate desaturase mediated conversion of stearoyl CoA to oleate (Table 5). This observation is consistent with reports that all other compounds such as phenols and halothane, which appear to stimulate microsomal electron transfer by interacting with stearate desaturase, also have no effect on the hepatic microsomal metabolism of stearoyl CoA (Oshino and Sato, 1970; Berman et al., 1975). The lack of effect of these xenobiotics on the conversion of stearoyl CoA to oleate could be because the xenobiotics do not bind to the active site of stearate desaturase, which is a cleft 26 Å long by 4 Å wide (Brett et al., 1971), but may bind at a distinct site which differs from the substrate binding site. Alternatively, since stearoyl CoA binds extremely tightly to the active site of stearate desaturase, the xenobiotics may bind at the same site as stearoyl CoA, but may not be able to displace stearoyl CoA. In either case, the binding of the xenobiotics to stearate desaturase might not affect the activity of the enzyme.

The two alternatives regarding the position on the C.S.F. at which these xenobiotics bind may be distinguished by the following means : if the two compounds bind at the same site,

at an equivalent value of $\frac{[\text{concentration}]}{K}$, each would be expected to interfere with the binding of the other. Thus, the xenobiotic under these circumstances would be expected to decrease the conversion of stearoyl CoA to oleate and conversely, stearoyl CoA would be expected to decrease the xenobiotic mediated stimulation of microsomal electron transfer.

Therefore, using the following values :

K_m for stearoyl CoA = 17 µM (Oshino et al., 1966)

[Stearoyl CoA] = 40 µM (Oshino et al., 1966)

K_{eq} for bromotrichloromethane = 2,2 mM (see Results)

[Bromotrichloromethane] = 3,4 mM (see Results)*

It can be calculated that :

$$\frac{[\text{Stearoyl CoA}]}{K_m} = \frac{40\ \mu M}{17\ \mu M} = 2,35 \quad \text{and}$$

$$\frac{[\text{Bromotrichloromethane}]}{K_{eq}} = \frac{3,4\ mM}{2,2\ mM} = 1,55$$

The ratio of these two values is 1,5 : 1. Therefore, under the experimental conditions used in this work, approximately a

* These concentrations of xenobiotics were the highest which would not disrupt the microsomal suspension. Therefore, it was not possible to conduct experiments using higher concentrations of the xenobiotics for less equivocal results.

40% decrease in the conversion of stearoyl CoA to oleate should be observed in the presence of bromotrichloromethane. However, no inhibition was observed (S.D. $\pm$ 2%) (Table 5), indicating that bromotrichloromethane binds to a site distinct from the binding site of stearoyl CoA.

Similar calculations for 1,2-dibromo-1,2-dichloroethane using

K_{eq} for 1,2-dibromo-1,2-dichloroethane = 0,46 mM (see Results)

and $\left[1,2\text{-dibromo-1,2-dichloroethane}\right]$ = 0,6 mM (see Results)* give a ratio of 1,8 : 1 which suggests that 35% inhibition of the conversion of stearoyl CoA to oleate should be observed in the presence of 1,2-dibromo-1,2-dichloroethane. However, no inhibition was observed (S.D. $\pm$ 9%) (Table 5), which suggests that 1,2-dibromo-1,2-dichloroethane also binds to a site on stearate desaturase distinct from that for stearoyl CoA.

Considering all of the above evidence, with regard to the interaction of the xenobiotics bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde with hepatic microsomal proteins, several conclusions regarding the ability of these xenobiotics to interact with stearate desaturase may be drawn : the abilities of 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde to stimulate the re-

* See footnote page 223

oxidation of NADH reduced microsomal ferrocytochrome $\underline{b}_5$ appeared to arise as a consequence of enhanced electron flow to both cytochrome P-450 and stearate desaturase. Bromotrichloromethane on the other hand, appeared to stimulate electron flow through cytochrome $\underline{b}_5$ by interacting only with stearate desaturase. Although all three of these xenobiotics appear to bind to stearate desaturase, none of them affect the stearate desaturase mediated conversion of stearoyl CoA to oleate, indicating that all three xenobiotics bind to a site on the enzyme distinct from the binding site of stearoyl CoA.

The interaction of halothane, enflurane and methoxyflurane with some hepatic microsomal proteins.

Similar experiments to those performed on the xenobiotics were conducted using the anaesthetic agents halothane, enflurane and methoxyflurane, in order to investigate in detail their interaction with some hepatic microsomal proteins. The consequences of the interaction of anaesthetic agents with microsomal proteins are of importance clinically, particularly in the case of halothane which is the most commonly used of these three anaesthetic agents and which has been implicated in the pathogenesis of halothane hepatitis (see Introduction).

The enhanced effects of the anaesthetic agents enflurane and methoxyflurane on the re-oxidation of NADH reduced microsomal ferrocytochrome $\underline{b}_5$ (Table 5) as well as their lack of effect

on the activity of either the NADH- or the NADPH-cytochrome *c* reductases (Table 10) suggested that these anaesthetic agents stimulate microsomal electron transfer by enhancing the oxidation of cytochrome b_5, while not affecting its reduction. Enflurane was found to slightly enhance the activity of NADH-cytochrome *c* reductase, which would however not affect the rate constant for the re-oxidation of microsomal ferrocytochrome b_5.

As stated above, the re-oxidation of hepatic microsomal ferrocytochrome b_5, which is enhanced in the presence of halothane, enflurane and methoxyflurane (Berman *et al.*, 1975) (Table 2) can proceed via the autoxidation of cytochrome b_5 or via the transfer of electrons from ferrocytochrome b_5 to other microsomal proteins such as cytochrome P-450 or stearate desaturase (see Figure 7). In order to assess the effects of these anaesthetic agents on the autoxidation of ferrocytochrome b_5, the purified, trypsin-cleaved cytochrome b_5 was used. This heme peptide differs from the intact microsomal hemoprotein as follows : the hydrophobic tail of this amphipathic molecule, which attaches cytochrome b_5 to the membrane, is lacking, but the hydrophilic region of the molecule, which is responsible for the electron transfer functions of cytochrome b_5 remains intact and active. Trypsin-cleaved cytochrome b_5 was chosen as a model system because it does not aggregate in water, as does intact cytochrome b_5 which has been prepared by detergent solubilization and because the structure of the heme crevice and the rate of autoxidation of

the hemoprotein are not altered by tryptic digestion of cytochrome b_5 (Strittmatter et al., 1972; Berman et al., 1975).

The pseudo first order rate constant for the autoxidation of purified trypsin-cleaved cytochrome b_5 (ca. $1,0 \times 10^2 sec^{-1}$) (Table 10) observed in the absence of enflurane and methoxyflurane was found to be similar to that reported by Berman et al., (1976) and by Strittmatter et al., (1972). This value is in good agreement with the reported first order rate constant for the re-oxidation of membrane bound hepatic microsomal ferrocytochrome b_5 in the absence of substrates for cytochrome P-450 and stearate desaturase (Boveris et al., 1972; Berman et al., 1975) (Tables 2 and 10). Furthermore, neither enflurane nor methoxyflurane had any significant effect on the rate constant for the autoxidation of purified ferrocytochrome b_5 (Table 10). In addition, the autoxidation of ferrocytochrome b_5 is not cyanide inhibitable (Berman et al., 1975), but the effects of enflurane and methoxyflurane on hepatic microsomal electron transfer are inhibited by 0,5 mM cyanide (Table 2). These observations therefore indicate that enflurane and methoxyflurane probably do not stimulate the autoxidation of ferrocytochrome b_5, but may stimulate electron transfer from ferrocytochrome b_5 to another microsomal protein (see pages 214 to 215 and Figure 7).

From several lines of evidence, it appeared that the terminal microsomal oxidase cytochrome P-450 is not involved in the

stimulation of the re-oxidation of cytochrome $\underline{b}_5$ observed in the presence of enflurane and methoxyflurane. Prior induction of cytochrome P-450 by phenobarbitone, which increases the levels of this enzyme in hepatic microsomes by 2 - 3 fold (Table 11), was without affect on the stimulation of the re-oxidation of hepatic microsomal ferrocytochrome $\underline{b}_5$ by enflurane and methoxyflurane (Tables 11 and 12). Furthermore, metyrapone and CO, which are inhibitors of cytochrome P-450, had no effect on the stimulation of the re-oxidation of NADH reduced hepatic microsomal ferrocytochrome $\underline{b}_5$ by halothane, enflurane or methoxyflurane (Berman et al., 1975) (Tables 8 and 9) which suggests that cytochrome P-450 is not responsible for the enhanced re-oxidation observed in the presence of these anaesthetic agents. This proposal is supported by the K_{eq} value for the stimulation of the re-oxidation of cytochrome $\underline{b}_5$ by enflurane which differed significantly from the K_s value for the binding of enflurane to cytochrome P-450 (Table 14) and further supported by the observation that the K_m for NADPH oxidation by cytochrome P-450 differed from the K_{eq} for the re-oxidation of ferrocytochrome $\underline{b}_5$ in the presence of both enflurane and methoxyflurane (Table 14). However, the observed K_{eq} for the re-oxidation of ferrocytochrome $\underline{b}_5$ in the presence of methoxyflurane was identical to the K_s for its binding to cytochrome P-450. In addition, the K_i of 0,1 mM which has been determined for the cyanide inhibition of the re-oxidation of ferrocytochrome $\underline{b}_5$ in the presence of the anaesthetic agents (see Results) differs from the K_i values of 2,5 - 10 mM which have been reported for the cyanide

inhibition of cytochrome P-450 (Jefcoate et al., 1969; Correia and Mannering, 1973), supporting the hypothesis that the observed effects of the anaesthetic agents on the re-oxidation of ferrocytochrome b_5 does not relate to cytochrome P-450.

Furthermore, it appears that the microsomal Δ6-desaturase is not involved in the stimulation of microsomal electron transfer by enflurane and methoxyflurane. The activity of the Δ6-desaturase is not increased by re-feeding a high carbohydrate diet (Inkpen et al., 1969) which enhances the effects of enflurane and methoxyflurane on the re-oxidation of ferrocytochrome b_5. Furthermore, the Δ6-desaturase is unaffected by fasting (Inkpen et al., 1969) which eliminates the effects of enflurane and methoxyflurane on the re-oxidation of ferrocytochrome b_5 (Tables 11 and 12). These results exclude the Δ6-desaturase from being involved in the observed enhanced re-oxidation of microsomal ferrocytochrome b_5. It is also possible to exclude catalase, a microsomal contaminant, as being responsible for mediating the effects of enflurane and methoxyflurane on the re-oxidation of NADH reduced microsomal ferrocytochrome b_5. As stated earlier (see page 220), although catalase is cyanide sensitive, the K_i for cyanide inhibition of this enzyme is vastly different from the K_i for cyanide inhibition of the enhanced re-oxidation of cytochrome b_5 by enflurane and methoxyflurane (ca. 0,1 mM).

It appears therefore that enflurane and methoxyflurane may

stimulate microsomal electron transfer through cytochrome $\underline{b}_5$ by accelerating the transfer of electrons to stearate desaturase. The magnitudes of the effects of these compounds on microsomal electron transfer parallel the dietary induction of stearate desaturase : the re-feeding of a high carbohydrate diet, which elevates the levels of stearate desaturase, results in the enhancement of electron transfer in the presence of these xenobiotics, relative to the rates observed in microsomes from rats fed a normal diet. In contrast, fasting, which decreases the levels and activity of stearate desaturase to negligible proportions, entirely eliminates the effects of enflurane and methoxyflurane on the re-oxidation of cytochrome $\underline{b}_5$ (Tables 11 and 12). Furthermore, the enhanced re-oxidation of cytochrome $\underline{b}_5$ observed in the presence of these anaesthetic agents, is inhibited by 0,5 mM cyanide (Table 2) as is stearate desaturase (Oshino et al., 1966; Oshino et al., 1971). The K_i values obtained 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 by Oshino et al., (1966) for the cyanide inhibition of the conversion of stearoyl CoA to oleate by stearate desaturase. Although it appears that enflurane and methoxyflurane affect microsomal electron transfer by interacting with stearate desaturase and therefore probably bind to stearate desaturase (Table 14), these compounds do not affect the stearate desaturase mediated conversion of stearoyl CoA to oleate (Table 16). The latter result is consistent with the ability of the

anaesthetic agents to bind to stearate desaturase if one proposes that the anaesthetic agents are not binding at the active site of the enzyme. Information on the location on the C.S.F. at which the anaesthetic agents bind may be obtained by means of the calculation shown above (see page 223) for the xenobiotics.

The K_m for stearoyl CoA = 17 µM (Oshino et al., 1966)

$[\text{Stearoyl CoA}]$ = 40 µM (Oshino et al., 1966)

K_{eq} for enflurane = 1,2 mM (see Results)

$[\text{Enflurane}]$ = 14 mM (see Results)*

It can be calculated that

$$\frac{[\text{stearoyl CoA}]}{K_m} = \frac{40\ \mu M}{17\ \mu M} = 2,35 \qquad \text{and}$$

$$\frac{[\text{enflurane}]}{K_{eq}} = \frac{14\ mM}{1,2\ mM} = 11,7$$

The ratio of these two values is 0,2 : 1 which suggests that approximately 80% inhibition of the conversion of stearoyl CoA should be observed. However, no inhibition (S.D. $\pm$ 30%) was observed (Table 16) which indicates that as in the case of the xenobiotics, enflurane does not bind to the same site on stearate desaturase as does stearoyl CoA†.

Similar calculations for methoxyflurane using K_{eq} for methoxyflurane = 0,48 mM (see Results) and $[\text{methoxyflurane}]$ =

* See footnote page 223

† This proposal is further supported by the observation that stearoyl CoA does not inhibit the production of hydrogen peroxide by the anaesthetic agents (Manca, V., Unpublished observations).

0,6 mM (see Results)*

give a ratio of 1,9 : 1 which suggests that approximately 30% inhibition of the conversion of stearoyl CoA to oleate should be observed. However, no inhibition was observed (Table 16), but the large standard deviations obtained make the case regarding methoxyflurane less clear than that of enflurane. However, in line with the results obtained for the other xenobiotics, it is possible that methoxyflurane also binds at a site on stearate desaturase distinct from that of stearoyl CoA.

The observation that enflurane and methoxyflurane appear to bind to stearate desaturase, but do not affect the enzyme's function is consistent with the observation that the halogenated alkanes bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde which also bind to stearate desaturase (Table 6 and Figures 9 and 10) do not affect the conversion of stearoyl CoA to oleate (Table 5). It has furthermore been shown by Berman *et al.*, (1975) and Oshino and Sato (1971) that halothane and *p*-cresol respectively, interact with stearate desaturase, but do not inhibit the activity of stearate desaturase.

Considering all of the above evidence with regard to the interaction of the anaesthetic agents halothane, enflurane and methoxyflurane with hepatic microsomal proteins, it appears that the ability of the anaesthetic agents to enhance the re-oxidation of microsomal ferrocytochrome b_5 results

*See footnote page 223

from enhanced electron flow through cytochrome $\underline{b}_5$ to stearate desaturase, by virtue of the ability of the anaesthetic agents to interact with the C.S.F. of stearate desaturase. It has been previously demonstrated by Berman *et al.*, (1975) that halothane enhanced electron flow through microsomal ferrocytochrome $\underline{b}_5$ by interacting with stearate desaturase. The results reported in this thesis confirm and extend this observation and demonstrate that enflurane and methoxyflurane also enhance electron flow through microsomal ferrocytochrome $\underline{b}_5$ apparently as a consequence of their interaction with stearate desaturase. Although all three of the anaesthetic agents appear to bind to stearate desaturase, none of them affect the conversion of stearoyl CoA to oleate suggesting that they do not bind to the same site on stearate desaturase as does stearoyl CoA.

The metabolism of anaesthetic agents by hepatic microsomal stearate desaturase and the uncoupling of the stearate desaturase enzyme system.

It was attempted to elucidate how the reducing equivalents from the enhanced electron flow arising from the interaction of the anaesthetic agents with hepatic microsomal stearate desaturase are utilized . The question was asked whether these reducing equivalents are utilized in the metabolism of these anaesthetic agents by stearate desaturase. A second question concerned whether or not the anaesthetic agents might stimulate the transfer of these reducing equivalents

to oxygen viz. essentially by uncoupling the stearate desaturase enzyme system.

A major problem in assessing these alternatives is the presence in hepatic microsomes of cytochrome P-450, a major drug metabolizing enzyme which has been shown to catalyze the first step in the metabolism of halothane (Van Dyke, 1966; Karashima et al., 1977), methoxyflurane and enflurane (Mazze et al., 1971; Harrison et al., 1976; Ivanetich et al., 1979) (see Figures 3, 4, 5 and 6) and which is also known to give rise to reduced oxygen species, such as hydrogen peroxide and superoxide anion (Hildebrandt et al., 1973; Bartoli et al., 1977). Investigation of the metabolism of xenobiotics by hepatic microsomal stearate desaturase or their stimulation of oxygen reduction by stearate desaturase would be hampered therefore by the presence of cytochrome P-450. Therefore to investigate the fate of the reducing equivalents from the proposed interaction of xenobiotics with hepatic microsomal stearate desaturase, systems deficient in cytochrome P-450 were employed.

A two-fold approach was adopted to achieve this goal. Firstly, the C.S.F. of stearate desaturase, cytochrome b_5 and NADH-cytochrome b_5 reductase were isolated from the hepatic endoplasmic reticulum by detergent solubilization, and the stearate desaturase enzyme system was reconstituted *in vitro* in the absence of cytochrome P-450 activity. The second approach was to employ hepatic microsomes obtained from animals with elevated levels of stearate desaturase and to chemically treat the microsomes in order to greatly reduce or entirely eliminate the levels and activity of cytochrome P-450.

The first system used was the reconstituted stearate desaturase enzyme system *in vitro*. This system was capable of supporting the stearate desaturase mediated conversion of stearoyl CoA to oleate. The activity of this reconstituted system (1,50 and 3,50 nmoles oleate formed/min/mg C.S.F. protein) exceeded that achieved by Shimakata *et al.*, (1972), when corrected for slightly different experimental conditions, by a factor of 3 - 6 fold. Although the stearate desaturase enzyme system requires phospholipid, no added exogenous phospholipid was required for an active reconstituted enzyme system (see Results). Presumably the C.S.F. and reductase fractions contain contaminating phospholipid, sufficient to satisfy the lipid requirement of the reconstituted desaturase system as was reported by Shimakata *et al.*, (1972).

The observation that the anaesthetic agents had no effect on

the conversion of stearoyl CoA to oleate in the reconstituted system (Table 17) is consistent with their lack of effect on this reaction in hepatic microsomes (Table 16). This observation, together with the functionality of the reconstituted enzyme system validated the use of the reconstituted system for the investigations of the fate of the reducing equivalents from the interaction of the anaesthetic agents with stearate desaturase.

The second experimental approach involved hepatic microsomes containing stearate desaturase activity, but devoid of cytochrome P-450 content. Such a system had not been previously described in the literature. Therefore, various physical and chemical methods were utilized in an attempt to achieve this end. It was anticipated that achieving this goal would be difficult due to the complex inter-relationships existing between proteins situated in the endoplasmic reticulum, and in fact, several initial attempts to achieve this goal were unsuccessful.

These difficulties are exemplified by the results of the investigations of the effect of storing hepatic microsomes on the levels of cytochrome P-450 and the activity of stearate desaturase. Storage of the hepatic microsomes at $4^{o}C$ (Table 18) completely eliminated stearate desaturase activity as judged indirectly by the re-oxidation of cytochrome $\underline{b}_5$ while cytochrome P-450 levels were reduced to one third of control values, indicating that both proteins have been deleteriously

affected by the storage treatment, although stearate desaturase to a greater extent than cytochrome P-450.

Chemical modification of cytochrome P-450 was attempted with various chemicals which were chosen on the basis of reports that they can decrease the activity of cytochrome P-450 in hepatic microsomes (Imai and Sato, 1967; Ichikawa et al., 1968) although their effects on hepatic microsomal stearate desaturase activity were not known.

Dioxane has been shown to be particularly effective at eliminating cytochrome P-450 activity in hepatic microsomes (Ichikawa et al., 1968). Incubation of the hepatic microsomes in the presence of dioxane reduced the cytochrome P-450 content of the hepatic microsomes apparently by degrading the heme moeity of the enzyme, as the heme loss to cytochrome P-450 loss was in the ratio of 1 : 1 (Table 20) while stearate desaturase activity was only slightly reduced (Table 19). In an attempt to decrease the cytochrome P-450 content to negligible levels, an increased concentration of dioxane was used. This however also failed to eliminate completely cytochrome P-450, as thirty per cent still remained, while stearate desaturase activity was drastically decreased.

The demonstration that dioxane affects the heme of cytochrome P-450 is contrary to the proposal of Ichikawa et al., (1968) that dioxane converts cytochrome P-450 to cytochrome P-420.

Iso-butanol (added during the isolation of the hepatic microsomes), had similar effects on the proteins of the endoplasmic reticulum to dioxane : namely at lower concentrations of the chemical, the activity of stearate desaturase was unaffected, while the levels of cytochrome P-450 were significantly lowered, but not completely eliminated (Tables 21 and 22). Increasing the concentration of iso-butanol lowered the cytochrome P-450 content further, but also did not completely eliminate it. In this case apparently by converting cytochrome P-450 to cytochrome P-420 as previously suggested by Ichikawa et al., (1968). The activity of the stearate desaturase enzyme system in the presence of 20% v/v iso-butanol was still 50% of that seen in untreated microsomes.

Sodium iodide (when added during the isolation of the hepatic microsomes) appeared to be a most successful chemical for retaining the stearate desaturase activity while reducing cytochrome P-450 levels (Tables 21 and 22). However, it was impractical to pursue this observation because sodium iodide solubilized the microsomes.

Of the chemical treatments employed to diminish cytochrome P-450 levels without greatly affecting the activity of stearate desaturase, iodomethane and potassium thiocyanate were the most effective. The levels of cytochrome P-450 were essentially reduced to zero by iodomethane treatment and this was accompanied by decreased activity of the cyto-

chrome P-450 mediated demethylation of p-nitroanisole and hydroxylation of benzpyrene (Table 24). The decrease in cytochrome P-450 levels by this chemical appeared to be due to degradation of the heme moeity of the enzyme because the level of the other microsomal hemoprotein - cytochrome b_5 - was not depleted by this chemical and the loss of cytochrome P-450 (0,90 nmoles/mg microsomal protein) was comparable to the loss of microsomal heme (0,72 nmoles/mg microsomal protein) (Ivanetich et al., 1978) (Table 24). Although stearate desaturase activity, as assessed by the re-oxidation of ferrocytochrome b_5, appeared to be entirely eliminated by iodomethane (Table 23), when stearate desaturase activity was assessed via the conversion of stearoyl CoA to oleate, 50% of the activity remained when compared to control values (Table 23). It is anticipated therefore that the re-oxidation of microsomal cytochrome b_5 in the presence of iodomethane may not accurately assess the activity of the stearate desaturase enzyme system, possibly because iodomethane enhances electron flow through cytochrome b_5 in the absence of added compounds (Table 23) (see page 117). In any case, the ratio of $\frac{\text{oleate}}{\text{oleate + stearate}}$ obtained in the iodomethane treated microsomes was far greater than that obtained with the reconstituted stearate desaturase system (compare with Table 17). Since the chemically treated microsomal system had greater stearate desaturase activity than the reconstituted system, it was anticipated that it might be preferable to assess the fate of the reducing equivalents from

the proposed interaction of the anaesthetic agents with stearate desaturase using the chemically treated microsomes than using the reconstituted enzyme system.

An identical pattern to that observed with iodomethane treated microsomes emerged for microsomes treated with potassium thiocyanate. There was a significant decrease in the content and activity of cytochrome P-450 (Table 25) which appeared to be due in part to the degradation of the heme moiety of the cytochrome : comparison of the loss of microsomal heme (0,56 nmoles/mg microsomal protein) to the loss of cytochrome P-450 (0,92 nmoles/mg microsomal protein) (Table 25) observed in the presence of potassium thiocyanate implied that the degradation of cytochrome P-450 in part reflects an alteration of the heme moeity of the enzyme. This is supported by the observation that as with iodomethane treated microsomes, no decrease in cytochrome $\underline{b}_5$ was detected following potassium thiocyanate treatment. In addition, potassium thiocyanate converted cytochrome P-450 to cytochrome P-420 as was previously reported by Imai and Sato (1967). Potassium thiocyanate also lowered stearate desaturase activity by approximately 30% (Table 23) as assessed by the re-oxidation of hepatic microsomal ferrocytochrome $\underline{b}_5$. When assessed via the conversion of stearoyl CoA to oleate, the activity of stearate desaturase was lowered by a comparable amount (approximately 20%). The activity of stearate desaturase in this microsomal preparation was more active than that in iodomethane treated micro-

somes and a further advantage is that microsomal electron transfer was not interrupted by potassium thiocyanate as is observed in microsomes treated with iodomethane.

On the basis of the above results of the investigations of various chemical treatments of hepatic microsomes, iodomethane and potassium thiocyanate treated microsomes were chosen to be utilized as a source of stearate desaturase activity, but negligible cytochrome P-450 activity. The chemically treated microsomes in fact, possessed far greater stearate desaturase activity than did the reconstituted stearate desaturase enzyme system (Tables 17 and 23). The interaction of xenobiotics with cytochrome P-450 in the chemically treated microsomes or the reconstituted stearate desaturase enzyme system , can be regarded as negligible as a consequence of the low cytochrome P-450 content and activity of both of these preparations.

Confirmation that cytochrome P-450 is not operative in the metabolism of the anaesthetic agents in the microsomal systems described above may be obtained as follows :-
It has been observed by Ivanetich et al., (1979) that 1,0 nmole cytochrome P-450/mg microsomal protein gave rise to 0,5 nmoles/fluoride ion produced from methoxyflurane/mg microsomal protein/min. The average concentration of cytochrome P-450 present in the hepatic microsomes treated with iodomethane or potassium thiocyanate was 0,05 nmoles cytochrome P-450/mg microsomal protein (Tables 24 and 25).

Therefore, by analogy :

$$\frac{\text{0,05 nmoles cytochrome P-450/mg microsomal protein}}{\text{1,0 nmole cytochrome P-450/mg microsomal protein}} = \frac{\text{x nmoles fluoride ion produced/mg protein/min}}{\text{0,5 nmoles fluoride ion produced/mg protein/min}}$$

x = 0,025 nmoles fluoride ion produced/mg microsomal protein/min. This value is far below the limit of detection for fluoride ion and therefore confirms that the cytochrome P-450 dependent metabolism of enflurane and methoxyflurane will not be measured in these hepatic microsomes.

These systems were therefore chosen as those best suited to assess the ability of the stearate desaturase enzyme system to metabolize the anaesthetic agents halothane, enflurane and methoxyflurane.

The metabolism of anaesthetic agents by stearate desaturase.

The enzymatic metabolism of the anaesthetic agents halothane, enflurane and methoxyflurane could result in the production of volatile metabolites such as haloalcohols or non-volatile metabolites such as halo ions (fluoride, chloride or bromide or halo carboxylic acids (see Figures 3, 4, 5 and 6). Using the experimental systems described above, the possible production of any of these metabolites from the anaesthetic agents by stearate desaturase was sought.

From the results of these investigations, it appeared that

the anaesthetic agents halothane, enflurane and methoxyflurane are not metabolized by hepatic microsomal stearate desaturase. In both experimental systems, i.e. the reconstituted and microsomal systems described above, it was not possible to detect measurable amounts of any volatile or non-volatile metabolites of halothane, enflurane or methoxyflurane.

No measurable levels of fluoride ion or acid-labile fluorine compounds were produced in either the reconstituted or microsomal systems from any of the anaesthetic agents by stearate desaturase (see Results). Although the carbon-fluoride bond has an energy of 107 Kcal/mole which is greater than the carbon-bromine, carbon-chlorine or carbon-hydrogen bonds, and energetically fluoride ion is the least likely of the halogens to be cleaved from any halogenated anaesthetic agents, there is virtually no way that enflurane or methoxyflurane could be metabolized without the release of fluoride ion (see Figures 5 and 6). In fact, halothane, enflurane and methoxyflurane are all known to be defluorinated *in vivo*, as measured by urinary fluoride ion excretion in humans exposed to these anaesthetic agents although the rate of defluorination of halothane and enflurane is far lower than that of methoxyflurane (Rehder *et al.*, 1967; Chase *et al.*, 1971; Cousins *et al.*, 1976; Holaday *et al.*, 1970; Fry *et al.*, 1973; Greenstein *et al.*, 1975).

An acid labile fluorinated metabolite is known to be produced

from methoxyflurane by cytochrome P-450 *in vitro* and *in vivo* (Holaday *et al.*, 1970; Ivanetich *et al.*, 1979). This compound is thought to be methoxydifluoroacetic acid (see Figure 6) (Mazze *et al.*, 1971). An analagous compound *viz.* chlorofluoroacetic acid may also be produced from enflurane (see Figure 5) (Cousins and Mazze, 1974). However, in general, no acid-labile fluorine compounds are produced from the interaction of halothane, enflurane or methoxyflurane with stearate desaturase and therefore specifically methoxydifluoroacetic acid and difluoromethoxy difluoroacetic acid are not produced by this interaction.

No measurable levels of bromide could be detected on incubating halothane with either the reconstituted or microsomal systems (see Results) which indicated that stearate desaturase does not remove bromide ions from halothane. Bromide ions are, however, known *in vivo* metabolites of halothane from the cytochrome P-450 enzyme system (Stier *et al.*, 1964). Van Dyke and Chenoweth (1965) have shown that the carbon-bromide bond of the halothane molecule is broken enzymatically and the debromination and dechlorination of halothane gives rise to the major oxidative metabolite, trifluoroacetic acid *in vivo* (Van Dyke and Chenoweth, 1965; Rehder *et al.*, 1967).

No volatile products of halothane were measurable following its interaction with stearate desaturase in either the reconstituted or microsomal systems. Among the volatile compounds assessed were several recently identified metabolites of

halothane from the action of cytochrome P-450 *in vivo*. These metabolites include 2-chloro-1,1-difluoroethylene and 2-chloro-1,1,1-trifluoroethane and 1,1-difluoro-2-bromo-2-chloroethylene and haloalcohols (such as the proposed metabolite 2,2,2-trifluoroethanol) (Maiorino *et al.*, 1979; Mukai *et al.*, 1977 and Sharp *et al.*, 1979) (Figure 4).

No volatile metabolites appeared to be produced from enflurane or methoxyflurane by the action of stearate desaturase, using either the reconstituted or microsomal enzymes. In contrast to halothane, there are no known cytochrome P-450 mediated volatile metabolites of either enflurane or methoxyflurane although several of the proposed intermediates in the metabolic pathways for enflurane and methoxyflurane are haloalcohols which would be volatile. Examples are 2-chloro-1,1,2-trifluoroethanol from enflurane and 2,2-dichloro-1,1-difluoroethanol from methoxyflurane (see Figures 5 and 6). Neither of these intermediates have been identified as yet, and no unknown peaks were seen on gas liquid chromatography columns specific for haloalcohols (see Results).

The production of non-volatile metabolites of halothane, such as trifluoroacetic acid which is known to be produced by the cytochrome P-450 mediated metabolism of halothane *in vivo* (Cohen *et al.*, 1975), was assessed. As may be seen in Table 27, no non-volatile metabolites of halothane could be detected using ^{14}C-halothane and the microsomal systems. There is therefore no support that stearate desaturase can chemically

transform halothane into non-volatile, polar metabolites such as the carboxylic acid, trifluoroacetic acid.

Further support for the proposal that the anaesthetic agents are not metabolized by stearate desaturase can be obtained by a comparison of the rate of re-oxidation of microsomal ferrocytochrome $\underline{b}_5$ and the calculated maximum rate of metabolite production from halothane, enflurane and methoxyflurane in both the reconstituted stearate desaturase enzyme system and the microsomal systems treated with iodomethane or potassium thiocyanate.

The rate of re-oxidation of cytochrome $\underline{b}_5$ may be represented as follows

$$\nu_{\substack{\text{cytochrome } \underline{b}_5 \\ \text{re-oxidation}}} = k_{obs}\left[Fe^{2+}\text{cytochrome } \underline{b}_5\right]$$

since the re-oxidation of cytochrome $\underline{b}_5$ is a pseudo first-order reaction. The rate constant for re-oxidation of cytochrome $\underline{b}_5$ (k_{obs}) in the absence of added substrates for cytochrome P-450 and stearate desaturase in hepatic microsomes or for autoxidation of purified cytochrome $\underline{b}_5$ is approximately $1 \times 10^{-2}sec^{-1}$ (Table 2) (Berman et al., 1975; 1976) and in the presence of halothane, enflurane or methoxyflurane, the rate constant for the re-oxidation of cytochrome $\underline{b}_5$ is approximately $3 \times 10^{-2}sec^{-1}$ (Table 2). The rate of re-oxidation of cytochrome $\underline{b}_5$ due to the transfer of electrons

from cytochrome $\underline{b}_5$ to stearate desaturase is therefore approximately $3 \times 10^{-2}sec^{-1}$ minus $1 \times 10^{-2}sec^{-1}$, or $2 \times 10^{-2}sec^{-1}$ in hepatic microsomes from rats fed a high carbohydrate diet. The concentration of cytochrome $\underline{b}_5$ in hepatic microsomes is approximately 0,4 nmoles/mg microsomal protein.

Therefore :

$$\nu_{\text{cytochrome } \underline{b}_5 \text{ re-oxidation}} = \frac{2 \times 10^{-2}}{\text{sec}} \left[\frac{0,4 \text{ nmoles}}{\text{mg protein}} \right]$$

$$= 8 \times 10^{-3} \text{ nmoles/mg microsomal protein/sec}$$

$$= 0,48 \text{ nmoles/mg microsomal protein/min.}$$

Based on the assumption that the rate of electron transfer from cytochrome $\underline{b}_5$ is approximately equal to the rate of electron transfer to stearate desaturase and further assuming that the rate of electron transfer to stearate desaturase is equivalent to the rate of metabolite production from the anaesthetic agents, the rate of re-oxidation of cytochrome $\underline{b}_5$ can be described as follows :

$\nu_{\text{cytochrome } \underline{b}_5 \text{ re-oxidation}} \simeq \nu_{\text{metabolite production}}$, if the electron transfer through cytochrome $\underline{b}_5$ involves a free radical. However, if the metabolism of the anaesthetic agents requires an electron pair, then

$2\nu_{\text{cytochrome } \underline{b}_5 \text{ re-oxidation}} \simeq \nu_{\text{metabolite production}}$.

The maximum rate of metabolite production from the rate of halo ion production may be assessed as follows :

$\nu_{\text{metabolite production}} = \nu_{F^-\text{production}} \leqslant 10\ \mu M/40\ \text{min}$, and

$\nu_{\text{metabolite production}} = \nu_{Br^-\ \text{production}} \leqslant 10\ \mu M/40\ \text{min}$

(the lower limit of detection of these assays for fluoride and bromide ion was 10 µM). $\nu_{\text{metabolite production}}$ is linear over 40 min.

Therefore, for fluoride ion production from halothane, enflurane and methoxyflurane, the maximum amount of metabolite expected to be produced is :

$\leqslant$ 10 nmoles/1,5 mg protein/40 min or

$\leqslant$ 0,17 nmoles/mg protein/min

For bromide ion production from halothane, the maximum rate of metabolism would be :

$\leqslant$ 10 nmoles/1,5 mg protein/40 min or

$\leqslant$ 0,17 nmoles/mg protein/min.

The maximum possible rates obtained for the metabolism of the anaesthetic agents (0,17 nmoles/mg/min) are approximately 3 fold lower than the rate for metabolite production calculated on the basis of the extent of electron transfer ,

(0,48 nmoles/mg/min) supporting the proposal described above that the stearate desaturase enzyme system does not appreciably metabolize the anaesthetic agents.

In conclusion therefore, there is no evidence for the release of halogen ions or for the production of volatile or non-volatile metabolites from the interaction of halothane, enflurane or methoxyflurane with stearate desaturase, in either the reconstituted stearate desaturase enzyme system or in hepatic microsomes treated with chemicals to decrease cytochrome P-450 content and activity *in vitro*. The lack of production of metabolites from these compounds together with the enhanced electron flow through cytochrome b_5 observed in the presence of these xenobiotics (Table 2) and their apparent ability to bind to stearate desaturase (Tables 1, 2, 13 and 14) suggests that the anaesthetic agents may stimulate the transfer of electrons from NADH via stearate desaturase to oxygen, i.e. these xenobiotics might uncouple the stearate desaturase enzyme system in hepatic microsomes. It is therefore anticipated that bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde, which enhance electron transfer via stearate desaturase, may also have similar effects.

The uncoupling of the stearate desaturase enzyme system by anaesthetic agents.

Uncoupling refers to the enhanced flow of electrons through an enzyme system to oxygen, without the metabolism of the

compound stimulating electron flow. An alternative definition of uncoupling is a situation in which mono-oxygenases display oxidase activity, rather than oxygenase activity (White, Stevens and Kamin, 1970; Ullrich and Diehl, 1971). Compounds which cause uncoupling appear to stimulate oxygen uptake due to the formation of an enzyme-substrate complex, but these compounds are unable to incorporate the active oxygen atom, as a consequence of their chemical structure (Staudt et al., 1974). Up to the present time, cytochrome P-450 has been the only microsomal enzyme shown to be uncoupled. The classic uncoupler of cytochrome P-450 is perfluorohexane which forms an enzyme-substrate complex with cytochrome P-450 : No metabolites are formed from perfluorohexane although two moles of NADPH are oxidized per mole of oxygen utilized (Ullrich and Diehl, 1971; Staudt et al., 1974). In general, in the presence of uncouplers, electrons from NADPH are utilized in the reduction of oxygen to hydrogen peroxide. Partial uncoupling of cytochrome P-450 occurs in the presence of n-hexane, aminopyrene and several other compounds (Hildebrandt et al., 1973; Staudt et al.,1974). It was anticipated that some xenobiotics might fully or partially uncouple the stearate desaturase enzyme system just as perfluorohexane and n-hexane uncouple the cytochrome P-450 enzyme system because some xenobiotics and anaesthetic agents stimulate electron flow, apparently without being metabolized.

Therefore, the ability of selected xenobiotics and anaesthetic agents to stimulate electron transfer through cytochrome b_5

and stearate desaturase (Table 2) to oxygen was assessed.

The reduction of oxygen can result in the production of "activated" oxygen species such as hydrogen peroxide, or superoxide anion. A further "activated" oxygen species, viz. singlet oxygen, can be formed non-enzymatically from superoxide.

There is precedent for the production of both hydrogen peroxide and superoxide in hepatic microsomes for example, the autoxidation of hepatic microsomal cytochrome b_5 generates superoxide anion (Berman et al., 1976) as does cytochrome P-450 hydroxylation reactions (Strobel and Coon, 1971) while the partial or full uncoupling of mixed function oxidation reactions in hepatic microsomes gives rise to hydrogen peroxide (Estabrook et al., 1968; Hildebrandt et al., 1973; Bartoli et al., 1977).

Singlet oxygen ($^1O_2$) differs from hydrogen peroxide and superoxide in that the oxygen molecule is in an excited electronic state rather than in a reduced state of molecular oxygen (Politzer et al., 1971). Singlet oxygen ($^1O_2$) differs from ground state molecular oxygen ($^3O_2$) in the distribution of the two highest-energy electrons in the pi molecular orbitals.

The "activated" oxygen species described above which may be produced in hepatic microsomes are interconvertible due to the action of enzymes such as superoxide dismutase and cata-

lase and via the non-enzymic production of $^1O_2$. For example, superoxide ($O_2^{-\cdot}$) can dismute enzymatically via superoxide dismutase as follows :

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide can also dismutate non-enzymically with the production of singlet oxygen ($^1O_2$) as follows :

$$2O_2^{-\cdot} + 2H^+ \longrightarrow {}^1O_2 + H_2O_2$$

H_2O_2 produced from these or other reactions may be degraded by the action of catalase to produce two molecules of water and a molecule of oxygen :

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Because of these interconversions, it was attempted to measure as many of the active oxygen species as possible. It was however not possible to assay the production of singlet oxygen in hepatic microsomes in the presence of the anaesthetic agents. Various chemical trapping agents such as 1,3-diphenylisobenzofuran and 2,5-diphenylfuran have been employed to monitor the formation of singlet oxygen (Piatt and O'Brien, 1979). These experiemnts have been conducted in closed, clearly defined systems. It is not possible to employ these trapping agents in microsomal systems as their sensitivity to autoxidation by ground state singlet oxygen in the presence of free radical

initiators renders them unsuitable in a system as complex as the microsomes (see Fee and Valentine, 1977).

Superoxide anion was not produced in hepatic microsomes from rats fed a high carbohydrate diet in the presence of the anaesthetic agents (Table 32).

Under certain experimental conditions, hydrogen peroxide was produced in hepatic microsomes in the absence and presence of the xenobiotics. The production of hydrogen peroxide in hepatic microsomes in the absence of xenobiotics, but in the presence of NADH is in the order of

microsomes treated with iodomethane >
microsomes treated with potassium thiocyanate >
untreated microsomes.

This same order of reactivity is observed in the re-oxidation of cytochrome $\underline{b}_5$ in the absence of the xenobiotics, suggesting that the enhanced electron flow seen in the microsomes treated with these chemicals in the absence of added xenobiotics results from the transfer of electrons from NADH via cytochrome $\underline{b}_5$ to oxygen to produce hydrogen peroxide. If electrons from NADH are passed through cytochrome $\underline{b}_5$ directly or indirectly to oxygen to form hydrogen peroxide, it would be expected that two molecules of cytochrome $\underline{b}_5$ would have to be re-oxidized to produce one molecule of hydrogen peroxide. As expected, twice the rate of cytochrome $\underline{b}_5$ re-oxidation was approximately equivalent to the rate of production of hydrogen peroxide for untreated, iodomethane or potassium thiocyanate treated microsomes (see Table 46).

TABLE 46. RE-OXIDATION OF FERROCYTOCHROME $\underline{b}_5$ AND THE PRODUCTION OF HYDROGEN PEROXIDE IN HEPATIC MICROSOMES.

Treatment of microsomes	Re-oxidation of cytochrome $\underline{b}_5$				Hydrogen peroxide production
	$10^2 k_{obs}$ (sec^{-1})	[cytochrome $\underline{b}_5$] (nmoles/mg microsomal protein)	2ν (nmoles/mg microsomal protein/min)	µM	ν (nmoles/mg microsomal protein/min)
None	1,1*	0,65†	0,88≠	1,59§	1,06
Iodomethane	3,4*	1,04†	4,20≠	3,12§	2,08
Potassium thiocyanate	1,3*	0,85†	1,28≠	2,52§	1,68

* Values obtained from Table 23

† Values obtained from Tables 23 and 24

≠ $\nu = k_{obs}$ [cytochrome $\underline{b}_5$]

§ Values obtained from Tables 29, 30 and 31 at one minute, because the reaction is not linear up to 5 minutes.

Therefore, reducing equivalents from cytochrome $\underline{b}_5$ appear to be transferred to oxygen to give rise to hydrogen peroxide in the absence of xenobiotics. This process may reflect a direct transfer of electrons from cytochrome $\underline{b}_5$ to oxygen via an autoxidation reaction, or may involve the transfer of electrons from cytochrome $\underline{b}_5$ to oxygen via a terminal oxidase.

Since the rate of re-oxidation of cytochrome $\underline{b}_5$ in untreated microsomes in the absence of added xenobiotics is approximately equal to the rate of autoxidation of purified, ferrocytochrome $\underline{b}_5$ (see Berman et al., 1975; 1976 and references therein), it appears that the re-oxidation of cytochrome $\underline{b}_5$ in these microsomes reflects an autoxidation reaction. Therefore, it appears that the autoxidation of cytochrome $\underline{b}_5$ in these microsomes gives rise to hydrogen peroxide. Since the autoxidation of purified cytochrome $\underline{b}_5$ has been reported to produce superoxide (Berman et al., 1976) it is possible that superoxide is an intermediate in the production of hydrogen peroxide, from the autoxidation of cytochrome $\underline{b}_5$ in these microsomes.

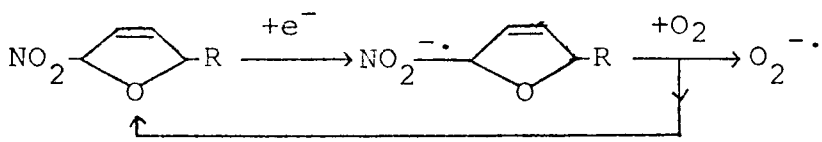
In the case of microsomes treated with iodomethane and potassium thiocyanate, the hydrogen peroxide produced could represent the enhanced autoxidation of cytochrome $\underline{b}_5$ or a stimulation of the transfer of electrons from cytochrome $\underline{b}_5$ to oxygen via one or more terminal oxidases. At present, these possiblities have not been distinguished.

In the presence of the halogenated xenobiotics bromotrichloromethane and chloroacetaldehyde, the production of hydrogen peroxide was increased while the production of hydrogen peroxide in the presence of 1,2-dibromo-1,2-dichloroethane was not elevated in hepatic microsomes from rats fed a high carbohydrate diet and treated with iodomethane (Table 28). The anaesthetic agents halothane, enflurane and methoxyflurane significantly increased the production of hydrogen peroxide in microsomes from rats fed a high carbohydrate diet, pretreated or not with iodomethane or potassium thiocyanate (Tables 29, 30 and 31). These increases in hydrogen peroxide production were significantly higher than those observed in the presence of NADH alone or the anaesthetic agents alone. The lack of metabolites of the anaesthetic agents produced, together with the elevated levels of hydrogen peroxide observed in the presence of the anaesthetic agents suggests that the anaesthetic agents stimulate electron transfer to oxygen. This may reflect a non-enzymatic process such as autoxidation of cytochrome $\underline{b}_5$ or the uncoupling of one or more microsomal terminal oxidases.

Autoxidation can be eliminated as being responsible for the production of hydrogen peroxide inasmuch as the enhanced electron flow through cytochrome $\underline{b}_5$ is cyanide inhibitable whereas the autoxidation of cytochrome $\underline{b}_5$ is not affected by cyanide (Berman *et al.*, 1975; 1976).

It would appear that a non-enzymic process such as that

proposed by Holtzman (1980) for nitrobenzofuran is not operative in the production of "activated" oxygen species in hepatic microsomes in the presence of the anaesthetic agents. Holtzman (1980) has suggested that certain compounds such as 5-nitrofuran may be reduced by NADPH-cytochrome P-450 reductase and then react non-enzymatically with oxygen to produce superoxide and regenerate substrate :



This produces a cyclic effect, with the concomitant production of superoxide and hydrogen peroxide. However, this mechanism is probably not operative in the case of the anaesthetic agents as the anaesthetic agents are not able to provide stable free radical intermediates in the same way that, for instance, 5-nitrofuran can.

A role for cytochrome P-450 in the production of hydrogen peroxide in the presence of the anaesthetic agents can be eliminated because the hydrogen peroxide production is not diminished in the hepatic microsomes which have no residual cytochrome P-450 levels or activity (Tables 30 and 31). That cytochrome P-450 is not involved in the hydrogen peroxide production by the anaesthetic agents is supported by the observation that CO : O_2 (80 : 20 v/v) does not diminish hydrogen peroxide production in hepatic microsomes from rats fed a high carbohydrate diet (Manca, V., Unpublished observations).

It appears therefore that the hydrogen peroxide produced in the presence of the anaesthetic agents could result from interaction of anaesthetic agents with stearate desaturase. This proposal arises from the observation that the enhanced electron flow through cytochrome $\underline{b}_5$ observed in the presence of the anaesthetic agents is cyanide sensitive and presumably these electrons give rise to the production of hydrogen peroxide. It was however not possible to monitor the effects of cyanide on the production of hydrogen peroxide by hepatic microsomes in the presence of the xenobiotics because cyanide interferes with the hydrogen peroxide assay that was used and with every other known assay for this active oxygen species (Orrenius, 1980). The definitive experiment in establishing this hypothesis would be to assess the production of hydrogen peroxide in the reconstituted stearate desaturase enzyme system.

Although hydrogen peroxide has been proposed to be produced during cytochrome P-450 catalyzed reactions (Hildebrandt *et al.*, 1973), hydrogen peroxide has not been proposed to be produced during stearate desaturase mediated reactions. The observation that the presence of stearoyl CoA does not lead to an increase in hydrogen peroxide production (Manca, V., Unpublished observations) supports this proposal and suggests that the hydrogen peroxide observed on interaction of the anaesthetic agents with stearate desaturase is produced during uncoupling of stearate desaturase by the anaesthetic agents.

The amounts of hydrogen peroxide produced in hepatic microsomes in the presence of the anaesthetic agents plus NADH is approximately 2,5 µM/per min (Tables 29, 30 and 31 and Figures 20, 21 and 22). These amounts correspond to approximately 1,7 nmoles hydrogen peroxide produced/mg microsomal protein. When corrected for hydrogen peroxide production in the presence of NADH alone, this figure becomes 0,6 nmoles hydrogen peroxide produced/mg protein/min. This latter figure is 3 - 18 fold lower than the range of 2 - 10 nmoles hydrogen peroxide/mg protein/min produced in phenobarbitone treated microsomes by partial uncouplers of cytochrome P-450 such as aminopyrene, aniline and hexobarbital (Hildebrandt et al., 1973). One would therefore expect much higher values of hydrogen peroxide production in the presence of a full uncoupler of cytochrome P-450 such as perfluorohexane.

The "activated" oxygen species, hydrogen peroxide, which is produced in hepatic microsomes as a consequence of the uncoupling of stearate desaturase by the anaesthetic agents (Tables 29, 30 and 31) may have a deleterious effect on the cell. However, enzymes such as superoxide dismutase and catalase are known to play a role in protecting the cell against the deleterious effects of active oxygen species, which include toxic effects on proteins, lipid peroxidation in membranes, peroxidation of unsaturated fatty acids and damage to DNA (Fong et al., 1973; Lavelle et al., 1973; Kellogg and Fridovich, 1975; King et al., 1975; Van Hemmen and Meuling, 1975).

The hydrogen peroxide produced during the uncoupling of stearate desaturase by the anaesthetic agents might play a role in the metabolism of xenobiotics by cytochrome P-450. It has been shown that cytochrome P-450 can utilize hydrogen peroxide as an "artificial electron donor" to support the metabolism of xenobiotics e.g. aminopyrene and trichloroethylene (Hildebrandt et al., 1973; Costa et al., 1979) and endogenous substrates (Hrycay et al., 1976).

In conclusion therefore, the anaesthetic agents halothane, enflurane and methoxyflurane appear to uncouple the stearate desaturase enzyme system. The results presented above are the first report in the literature of the possible uncoupling of stearate desaturase and provide the first suggestion of the uncoupling of any microsomal enzyme system other than cytochrome P-450.

Observations on the structural characteristics of xenobiotics interacting with hepatic microsomal stearate desaturase.

The observation that bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane, chloroacetaldehyde, halothane, enflurane and methoxyflurane enhance microsomal electron flow through cytochrome b_5 by interacting with stearate desaturase, suggested that other xenobiotics may also do so. In particular diiodomethane and 1,1,2-trichloroethane might be anticipated to do so as these xenobiotics stimulate the cyanide inhibitable re-oxidation of cytochrome b_5 (Table 2). It is anticipated that other xenobiotics, particularly halo-

alkanes or halogenated aliphatic ethers, having multiple halo substituents (e.g. carbontetrachloride, tetrabromomethane, 1,2-dichloroethane) which have been shown to shift the redox state of cytochrome $\underline{b}_5$ towards the ferric form (Table 1) might also stimulate microsomal electron transfer via cytochrome $\underline{b}_5$ by interacting with stearate desaturase.

The results of the investigations of the abilities of xenobiotics to enhance the re-oxidation of cytochrome $\underline{b}_5$ do not however allow a complete elucidation of the structural characteristics of a xenobiotic which may be required for its interaction with stearate desaturase. Some general conclusions, as regards these structural characteristics may however be drawn. All the xenobiotics which interact with stearate desaturase (bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane, chloroacetaldehyde, halothane, enflurane and methoxyflurane) are halogenated, but all halogenated compounds do not interact (see Tables 1 and 2). There appears to be no correlation between the number and type of the halogen substituents of a compound required for interaction with stearate desaturase inasmuch as xenobiotics containing multiple bromine or chlorine atoms such as bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane interact with stearate desaturase while 1,1,2,2-tetrachloroethane, 1-bromo-2-chloroethane do not (Table 2). In general however, compounds containing bromine or chlorine atoms such as those mentioned above, appear to be more reactive with stearate desaturase than compounds containing iodine atoms, such as diiodomethane and

iodoform. Xenobiotics containing fluorine atoms as the sole halogen substituent such as 1-fluorobutane and perfluorohexane do not appear to interact with stearate desaturase (Table 1) while compounds containing fluorine atoms plus other types of halogen atoms such as halothane and enflurane do (Tables 1 and 2). Of the six xenobiotics which interact with stearate desaturase, five contain multiple halogen substituents and only chloroacetaldehyde contains only one type of halogen. It appears therefore that multiple halo substituents may be favourable for the interaction of a xenobiotic with stearate desaturase.

The effects of anaesthetic agents in vivo

It was attempted to investigate whether the interaction of the anaesthetic agents, in particular, halothane, with stearate desaturase affected the activity of stearate desaturase in vivo. In addition, it was attempted to determine whether the potential hepatotoxicity of the anaesthetic agents could be connected with their ability to interact with stearate desaturase.

It general, it was observed that anaesthesia of rats with halothane, enflurane, ether or chloroform did not markedly affect the activity of stearate desaturase in vivo (Tables 35, 36 and 37) which was consistent with the lack of effect of halothane and enflurane on the conversion of stearoyl CoA to oleate in vitro (Table 16).

With regard to the hepatotoxic potential of the anaesthetic agents, halothane, enflurane and ether produced no evidence of hepatotoxicity in animals on a normal diet (Table 39). No significant changes in S.G.O.T. levels or in hepatic histology were observed following halothane, enflurane or ether anaesthesia of rats pretreated to enhance or reduce the activity of stearate desaturase (Tables 38, 39 and 40). These results suggest that these anaesthetic agents do not produce hepatic damage *in vivo* regardless of the activity of stearate desaturase. Only chloroform anaesthesia elevated S.G.O.T. levels and produced centrilobular necrosis (Tables 38 and 39). This is consistent with the known ability of chloroform to produce centrilobular necrosis of the liver, a cytochrome P-450 mediated effect (Brown *et al.*, 1974). The lack of effect of stearate desaturase activity on chloroform toxicity is consistent with the apparent inability of chloroform to interact with stearate desaturase (Table 1).

Although repeated halothane anaesthesia did not produce evidence of hepatic damage in the investigations described in this thesis (see Tables 38, 39 and 40), halothane has been reported to produce a dose-related reproducible hepatotoxicity (Hughes and Lang, 1972; Stevens *et al.*, 1975). The observation that repeated enflurane anaesthesia does not produce evidence of hepatic damage regardless of the activity of stearate desaturase in the animals thus exposed (Tables 38, 39 and 40) agrees with the implication that enflurane is not an hepatotoxin ("Ethrane", 1972; Harrison *et al.*, 1976). There

have however, been some reports that enflurane can cause cell degeneration and necrosis (Van der Reis et al., 1974; Stevens et al., 1977) although the extent of damage is always mild and far less than that produced by halothane (Stevens et al., 1977). Enflurane has been shown not to increase S.G.O.T. levels (Stacey et al., 1978; Thompson and Friday, 1978) which is in agreement with the normal range of S.G.O.T. levels observed after enflurane anaesthesia (Tables 38, 39 and 40). The observation that ether produced no hepatotoxicity (Tables 38, 39 and 40) is also in agreement with the lack of hepatotoxicity of this anaesthetic agent (Fairlie et al., 1951).

In order to investigate the possible protective effect of the interaction of halothane with stearate desaturase, it was essential to produce a reliable experimental model of cytochrome P-450 mediated hepatic necrosis, such as is produced by halothane in the rare disorder known as halothane hepatitis (see Introduction).

Recently, it has been established that halothane can be metabolized by cytochrome P-450 via a reductive pathway under hypoxic conditions, as opposed to the more general oxidative pathways (Sharp et al., 1979) (see Figures 3 and 4) and that such reductive biotransformation causes reproducible hepatic necrosis. The nature of the observed hepatotoxicity is that of centrilobular necrosis (Brown and Sipes, 1977; Nastaincyk et al., 1978; Cousins et al., 1979). Further evidence for

the hepatotoxicity of halothane under hypoxic conditions is provided by the observation that hepatic glutathione levels are decreased during such anaesthesia (Table 45 and Figure 25).

It was observed that anaesthesia with halothane under hypoxic conditions following phenobarbitone pretreatment as described by Brown and Sipes (1977), Nastaincyk (1978) and Cousins et al., (1979) caused congestion and centrilobular necrosis of the hepatocytes in animals fed a normal diet (Table 44). It was however, not possible to employ this model to study the effect of stearate desaturase activity on halothane induced hepatotoxicity because phenobarbitone decreased the activity of stearate desaturase to such an extent that no difference in this activity could be seen in rats fed a normal or a high carbohydrate diet (Tables 41 and 42).

Another model, consisting of halothane anaesthesia under hypoxic conditions in the absence of phenobarbitone pretreatment, was therefore employed to study the possible protective effect of the interaction of halothane with stearate desaturase. The S.G.O.T. levels were lower and the mortality significantly decreased in animals on the high carbohydrate diet anaesthetized with halothane under hypoxic conditions in the absence of phenobarbitone compared to these parameters in animals on the normal diet anaesthetized in the same way (Tables 43 and 44 and Figure 23). This result suggests that elevated levels of stearate desaturase may protect against the hepatotoxic potential of halothane.

In contrast to halothane, anaesthesia with enflurane and methoxyflurane under hypoxic conditions, does not give rise to hepatotoxicity (Table 44). This is consistent with the lack of evidence for the reductive metabolism of enflurane and methoxyflurane by hepatic cytochrome P-450 although there is much evidence for the oxidative metabolism of these anaesthetic agents.

As opposed to halothane, enflurane and methoxyflurane which interact with stearate desaturase (Tables 1, 2, 13 and 14), chloroform does not interact with this enzyme (Table 1). As expected, the induction of stearate desaturase was without effect on the chloroform mediated mortality and hepatic necrosis observed in animals exposed to chloroform under hypoxic conditions (Tables 43 and 44 and Figure 23). These observations further suggest that the ability of a xenobiotic to interact with stearate desaturase might have a protective effect in mitigating against the hepatotoxic potential of xenobiotics.

CONCLUSIONS.

The xenobiotics bromotrichloromethane, 1,2-dibromo-1,2-dichloroethane and chloroacetaldehyde and the anaesthetic agents halothane, enflurane and methoxyflurane enhance hepatic microsomal electron transfer through cytochrome b_5 in a cyanide sensitive manner, apparently as a consequence of their interaction with stearate desaturase. In addition to the xenobiotics mentioned above, it is anticipated that other halogenated compounds having multiple halo substituents (e.g. diiodomethane, 1,1,2-trichloroethane, carbontetrachloride, tetrabromomethane, 1,2-dichloroethane, 1,2-dibromo-1,1-dichloroethane) which shift the redox state of cytochrome b_5 towards the ferric form and / or stimulate the cyanide inhibitable re-oxidation of cytochrome b_5, might also interact with stearate desaturase. Although the anaesthetic agents halothane, enflurane and methoxyflurane and the xenobiotics bromotrichloromethane and 1,2-dibromo-1,2-dichloroethane appear to bind to stearate desaturase, it is anticipated that they do not bind to the same site on the enzyme as does stearoyl CoA, as the xenobiotics do not affect the stearate desaturase mediated conversion of stearoyl CoA to oleate. Although the anaesthetic agents appear to bind to stearate desaturase, they are apparently not metabolized by this enzyme system. This lack of metabolism of the anaesthetic agents, together with their ability to stimulate the production of hydrogen peroxide in hepatic microsomes free of cytochrome P-450, suggested that the anaesthetic agents may uncouple the stearate desaturase enzyme system. From *in vivo* studies, there

was no evidence that the enhanced production of hydrogen peroxide gave rise to hepatotoxic effects.

The hepatotoxicity of halothane under hypoxic conditions could be decreased by feeding rats a high carbohydrate diet, which elevates the levels and activity of hepatic microsomal stearate desaturase, suggesting that stearate desaturase might protect against the potential hepatotoxicity of halothane.

It has been demonstrated that xenobiotics and anaesthetic agents can interact with other hepatic microsomal enzymes not generally thought to be involved in xenobiotic metabolism. This interaction should be considered in work on drug metabolism employing hepatic microsomes. Microsomes should therefore not only be considered as a source of cytochrome P-450 but also as a medium where xenobiotics may interact with a variety of other microsomal proteins.

5. REFERENCES.

Airaksinen M.M., Rosenberg P.H., and Tammisto T. (1970). A possible mechanism of toxicity of trifluoroethanol and other halothane metabolites. Acta. Pharmacol. Toxicol. 28 299-304.

Allen E., Johnson A.R., Fogerty A.C., Pearson J.A. and Shenstone F.S. (1967). Inhibition by cyclopropene fatty acids of the desaturation of stearic acid in hen liver. Lipids 2 419-423.

Archakov A.I., Devichensky V.M. and Karjakin A.V. (1975). Electron transfer in the membranes of endoplasmic reticulum. Participation of cytochrome b_5 in the NADPH oxidation reaction. The evidence for two cytochromes b_5 in liver microsomes. Archs. Biochem. Biophys. 166 295-307.

Baker R.C., Wykle R.L., Schremmer Lockmiller J. and Snyder F. (1976). Identification of a soluble protein stimulator of plasmalogen biosynthesis and stearoyl-coenzyme A desaturase. Archs. Biochem. Biophys. 177 299-306.

Barr G.A., Cousins M.J., Mazze R.I., Hitt B.A. and Kosek J.C. (1974). A comparison of the renal effects and metabolism of enflurane and methoxyflurane on Fischer 344 rats. J. Pharmacol. Exp. Ther. 188 257-264.

Barrett H.M. and Jonston J.H. (1939). The fate of trichloroethylene in the organism. J. Biol. Chem. 127 765-770.

Bartoli G.M., Galeotti T., Palombini G., Parisi G. and Azzi A. (1977). Different contribution of rat liver microsomal pigments in the formation of superoxide anions and hydrogen peroxide during development. Archs. Biochem. Biophys. 184 276-281.

Belfrage S., Ahlgren I. and Axebon S. (1966). Halothane hepatitis in an anaesthetist. Lancet ii 1466-1467.

Berman M.C., Ivanetich K. and Kench J.E. (1975). The effects of halothane on hepatic microsomal electron transfer. Biochem. J. 148 179-186.

Berman M.C., Adnams C.M., Ivanetich K.M. and Kench J.E. (1976). Autoxidation of soluble trypsin-cleaved microsomal ferrocytochrome b_5 and formation of superoxide radicals. Biochem. J. 157 237-246.

Bernhard K., Von Bulow-Kogler J. and Wagner H. (1959). Enzymic dehydrogenation of stearic acid to oleic acid. Helv. Chim. Acta. 42 152-155.

Black G.W. and Clarke R.S.J. (1971). Recently introduced anaesthetic drugs. Int. Anesthesiology Clinics. 9 171-196.

Blake D.A., Cascorbi H.F., Rozman R.S. and Meyer F.J. (1969). Animal toxicity of 2,2,2-trifluoroethanol. Toxicol. and Applied Pharmacol. 15 83-91.

Boveris A., Oshino N. and Chance B. (1972). The cellular production of hydrogen peroxide. Biochem. J. 128 617-630.

Brett D., Howling D., Morris L.J. and James A.T. (1971). Specificity of the fatty acid desaturases. The conversion of saturated to monoenoic acids. Archs. Biochem. Biophys. 143 535-547.

Brodeur J., Paquin P., Authier L., Geadah D., Yamamauchi M. and Côté M.G. (1976). Influence of phenobarbital pretreatment in Fischer 344 rats. Toxicol. Appl. Pharmacol. 37 349-361.

Brown B.R. and Sagalyn A.M. (1974). Hepatic microsomal enzyme induction by inhalation anesthetics: mechanism in the rat. Anesthesiology 40 152-161.

Brown B.R., Sipes I.G. and Sagalyn A.M. (1974). Mechanisms of acute hepatic toxicity: chloroform, halothane and glutathione. Anesthesiology 41 554-561.

Brown B.R. and Sipes I.G. (1977). Biotransformation and hepatotoxicity of halothane. Biochem. Pharmacol. 26 2091-2094.

Bryce- Smith R. and O'Brien H.D. (1956). Fluothane: A non-explosive anaesthetic agent. Brit. Med. J. 2 969-972.

Burnap T.K., Galla S.J. and Vandam L.D. (1958). Anesthetic, circulatory and respiratory effects of fluothane. Anesthesiology 19 307-320.

Cascorbi H.F., Blake D.A. and Helrich M. (1970). Differences in the biotransformation of halothane in man. Anesthesiology 32 119-123.

Cascorbi H.F. and Blake D.A. (1971). Trifluoroethanol and halothane biotransformation in man. Anesthesiology 35 493-495.

Catalá A., Nervi A.M. and Brenner R.R. (1975). Separation of a protein factor necessary for the oxidative desaturation of fatty acids in the rat. J. Biol. Chem. 250 7481-7484.

Chase R.E., Holaday D.A., Fiserova-Bergerova V., Saidman L.J. and Mack F.E. (1971). The biotransformation of ethrane in man. Anesthesiology 35 262-267.

Chance B. (1952). The effect of pH upon the equilibria of catalase compounds. J. Biol. Chem. 194 483-496.

Chasseaud L. (1976). Conjugation with glutathione and mercapturic acid excretion. In "Glutathione: Metabolism and Function." pp. 77-114. Ed. I.M. Arias and W.B. Jacoby. Raven Press, New York.

Chaykin S. (1966). Biochemistry Laboratory Techniques. p. 20. Wiley, New York.

Cohen E.N. (1971). Metabolism of volatile anaesthetics. Anesthesiology 35 193-202.

Cohen E.N., Trudell J.R., Edmunds H.N. and Watson E. (1975). Urinary metabolites of halothane in man. Anesthesiology 43 392-401.

Cohn V.H. and Lyle J. (1966). A fluorometric assay for glutathione. Anal. Biochem. 14 434-440.

Conney A.H. (1967). Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19 317-366.

Coon M.J., Nordblom G.D., White R.E. and Hanger D.A. (1975). Purified liver microsomal cytochrome P-450: Catalytic mechanism and characterization of multiple forms. Biochem. Soc. Trans. 3 813-817.

Corall I.M., Knights K.M. and Strunin L. (1977). Enflurane (ethrane) anaesthesia in man. Br. J. Anaes. 49 881-885.

Correia M.A. and Mannering G.J. (1973). In "Microsomes and Drug Oxidations". p. 139. Ed. R.W. Estabrook, J.R. Gillette and K.C. Liebman. Williams and Wilkins, Baltimore.

Costa A.K., Katz I.D. and Ivanetich K.M. (1979). Trichloroethylene: Its interaction with hepatic microsomal cytochrome P-450 in vitro. Biochem. Pharmacol. 29 433-439.

Cousins M.J. and Mazze R.I. (1974). Biotransformation of enflurane (ethrane) and isoflurane (forane). Int. Anaes. Clinics. 12 111-119.

Cousins M.J., Greenstein L.R., Hitt B.A. and Mazze R.I. (1976). Metabolism and renal effects of enflurane in man. Anaesthesiology 44 44-53.

Cousins M.J., Sharp H.J., Gourlay G.K., Adams J.F. and Whitehead R. (1979). Hepatotoxicity and halothane metabolism in an animal model with application for human toxicity. Anaesthesia and Intensive Care 7 9-24.

Dailey H.A. and Strittmatter P. (1979). Modification and identification of cytochrome b_5 carboxyl groups involved in protein-protein interaction with cytochrome b_5 reductase. J. Biol. Chem. 254 5388-5396.

Dawson J.H., Holm R.H., Trudell J.K., Barth G., Linder R.E., Bunnenberg E., Djerassi C. and Tang S.C. (1976). Oxidized cytochrome P-450. Magnetic circular dichroism evidence for thiolate ligation in the substrate bound form. Implication for catalytic mechanism. J. Am. Chem. Soc. 98 3707-3709.

De Bruin A. (1976). In "Biochemical Toxicity of Environmental Agents". p. 368. Elsevier-North Holland, New York.

De Gómez Dumm I.N.T. and Brenner R.R. (1975). Oxidative desaturation of α-linolenic, linoleic and stearic acids by human liver microsomes. Lipids 10 315-317.

Dobkin A.B., Heinrich R.G., Israel J.S., Levy A.A., Nevile J.F. Jr. and Ounkasem K. (1968). Clinical and laboratory evaluation of a new inhalation agent: Compound 347 (CHF_2-O-CF_2-CHFCl). Anesthesiology 29 275-287.

Donaldson W.E. (1973). Glucose stimulation of fatty acid desaturation in liver of newly hatched chicks. Biochim. Biophys. Acta 316 8-12.

Dutton D.J. (1971). Glucuronide-forming enzymes in "Handbook of Experimental Pharmacology" 28 (Part 2). pp. 378-400. Ed. B.B. Brodie and J.R. Gillette. Springer-Verlag, Berlin, Heidelberg.

Eger E.I. (1974). MAC in "Anaesthetic Uptake and Action". pp. 1-25. Williams and Wilkins, Baltimore, Maryland.

Elovson J. (1965). Conversions of palmitic and stearic acid in the intact rat. Biochim. Biophys. Acta 106 291-303.

Enoch H.G., Catalá A. and Strittmatter P. (1976). Mechanism of rat liver microsomal stearoyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions and the function of lipid. J. Biol. Chem. 251 5095-5103.

Enoch H.G. and Strittmatter P. (1978). Role of tyrosyl and arginyl residues in rat liver microsomal stearyl coenzyme A desaturase. Biochemistry 17 4927-4932.

Enoch G. and Strittmatter P. (1979). Cytochrome b_5 reduction by NADPH-cytochrome P-450 reductase. J. Biol. Chem. 254 8976-8981.

Ernster L. and Orrenius S. (1965). Substrate-induced synthesis of the hydroxylating enzyme system of liver microsomes. Fed. Proc. 24 1190-1199.

Estabrook R.W., Hildebrandt A.G. and Ullrich V. (1968).Oxygen interaction with reduced cytochrome P-450. Z. Physiol. Chem. 349 1605-1608.

Estabrook R.W., Matsubara T., Mason J.I., Werringloer J. and Baron J. (1973). Studies on the molecular function of cytochrome P-450 during drug metabolism. Drug Metab. Disposit. 1 98-110.

—, Ethrane- A new dimension in inhalation anaesthetics in "Summary of technical data and clinical experience". Ohio Medical Products. 1972.

Fairlie C.W., Barss T.P., French A.B., Jones C.M. and Beecher H.K. (1951). Metabolic effects of anesthesia in man. A comparison of the effects of certain anesthetic agents on the normal liver. New Eng. J. Med. 244 615-622.

Fee J.A. and Valentine J.S. (1977). Chemistry of $O_2^{-\cdot}$ in "Superoxide and Superoxide Dismutases". p. 42. Ed. A.M. Michelson, J.M. McCord and I. Fridovich. Academic Press, London.

Fogerty A.C., Johnson A.R. and Pearson J.A. (1972). Ring position in cyclopropene fatty acids and stearic acid desaturation in hen liver. Lipids 7 335-338.

Fong K-L., McCay P.B., Poyer J.L., Keele B.B. and Misra H. (1973). Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl radicals produced during flavin enzyme activity. J. Biol. Chem. 248 7792-7797.

Fry B.W., Taves D.R. and Merin R.G. (1973). Fluorometabolites of methoxyflurane. Anesthesiology 38 38-44.

Garfinkel D. (1958). Studies on pig liver microsomes. I. Enzyme and pigment composition of different microsomal fractions. Archs. Biochem. Biophys. 77 493-509.

Gaylor J.H. and Mason H.S. (1968). Investigation of the component reactions of oxidative demethylation. Evidence against participation of cytochrome P-450. J. Biol. Chem. 243 4966-4972.

Gaylor J.L., Miyake Y. and Tamano T. (1975). Stoichiometry of 4-methyl sterol oxidase of rat liver microsomes. J. Biol. Chem. 250 7159-7167.

Gellhorn A. and Benjamin W. (1964). The intracellular localization of an enzymatic defect of lipid metabolism in diabetic rats. Biochem. Biophys. Acta 84 167-175.

Gillette J.R. (1971). Effect of various inducers on electron transport system associated with drug metabolism by liver microsomes. Metabolism 20 215-245.

Gillette J.R., Davis D.C. and Sasame H.A. (1972). Cytochrome P-450 and its role in drug metabolism. Ann. Rev. Pharmacol. 12 57-84.

Goodwin J.F. (1971). Colorimetric measurement of serum bromide with a bromate-rosaniline method. Clin. Chem. 17 544-547.

Greenstein L.R., Hitt B.A. and Mazze R.I. (1975). Metabolism in vitro of enflurane, isoflurane and methoxyflurane. Anesthesiology 42 420-424.

Gurr M.I. and Robinson M.P. (1970). Preliminary partial purification of hen liver microsomal stearoyl-CoA desaturase. Eur. J. Biochem. 15 335-341.

Haggard H.W. (1924). The absorption, distribution and elimination of ethyl ether. J. Biol. Chem. 59 737-802.

—, Handbook of Experimental Pharmacology. (1971). 28 (Part 2). Ed. B.B. Brodie and J.R. Gillette. Springer-Verlag, Berlin, Heidelberg.

Harrison G.G., Marsh J.A., Bradshaw J.J., Zeitsman I. and Ivanetich K.M. (1976). Some aspects of the hepatic metabolism of ethrane. S. Afr. Med. J. 50 2080-2082.

Hayler L. and Herman R.H. (1973). Oxalate metabolism. Am. J. Clin. Nutrit. 26 1073-1079.

Henry R.J., Chiamori N., Golub O.J. and Berkman S. (1960). Revised spectrophotometric methods for the determination of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and lactic acid dehydrogenase. Am. J. Clin. Path. 34 381-398.

Hildebrandt A. and Estabrook R.W. (1971). Evidence for the participation of cytochrome b_5 in hepatic microsomal mixed-function oxidation reactions. Archs. Biochem. Biophys. 143 66-79.

Hildebrandt A.G., Speck M. and Roots I. (1973). Possible control of hydrogen peroxide production and degradation in microsomes during mixed function oxidation reactions. Biochem. Biophys. Res. Commun. 54 968-975.

Holaday D.A., Rudofsky S. and Treuhaft P.S. (1970). The metabolic degradation of methoxyflurane in man. Anesthesiology 33 579-593.

Holloway P.W., Peluffo R. and Wakil S.J. (1963). On the biosynthesis of dienoic fatty acid by animal tissues. Biochem. Biophys. Res. Commun. 12 300-304.

Holloway P.W. and Katz J.T. (1972). A requirement for cytochrome b_5 in microsomal stearyl coenzyme A desaturation. Biochemistry 11 3689-3696.

Holloway C.T. and Holloway P.W. (1975). Stearyl coenzyme A desaturase activity in mouse liver microsomes of varying lipid composition. Archs. Biochem. Biophys. 167 496-504.

Holloway C.T. and Holloway P.W. (1977). The dietary regulation of stearyl coenzyme A desaturase activity and membrane fluidity in the rat aorta. Lipids 12 1025-1031.

Holtzman J.L. and Carr M.L. (1972). The temperature dependence of components of the hepatic microsomal mixed function oxidases. Archs. Biochem. Biophys. 150 227-234.

Holtzman J. (1980). Personal Communication.

Hrycay E.G., Gustafsson J., Ingelman-Sundberg M. and Ernster L. (1976). The involvement of cytochrome P-450 in hepatic microsomal steroid hydroxylation reactions supported by sodium periodate, sodium chlorite and organic hydroperoxides. Eur. J. Biochemistry 61 43-52.

Hughes H.C. and Lang C.M. (1972). Hepatic necrosis produced by repeated administration of halothane to guinea pigs. Anesthesiology 36 466-471.

Ichikawa Y., Uemura T. and Yamano T. (1968).The role of the hydrophobic bonding in hemoprotein P-450 and the effect of organic compounds on the conversion of hemoprotein P-450 to hemoprotein P-420 in "The Structure and Function of Cytochromes". pp. 534-644. Ed. K. Okunuki, M.D. Kamen and I. Sekuzu. University of Tokyo Press, Tokyo and University Park Press, Baltimore, Maryland.

Imai Y. and Sato R. (1967). Conversion of P-450 to P-420 by neutral salts and some other reagents. Eur. J. Biochem. 1 419-426.

Inkpen C.A., Harris R.A. and Quackenbush F.W. (1969). Differential responses to fasting and subsequent feeding by microsomal systems of rat liver 6- and 9-desaturation of fatty acids. J. Lipid Res. 10 277-282.

Ito A. and Sato R. (1968). Purification by means of detergents and properties of cytochrome b_5 from liver microsomes. J. Biol. Chem. 243 4922-4923.

Ivanetich K.M., Marsh J.A., Bradshaw J.J. and Kaminsky L.S. (1975). Fluroxene (2,2,2-trifluoroethyl vinyl ether) mediated destruction of cytochrome P-450 *in vitro*. Biochem. Pharmacol. 24 1933-1936.

Ivanetich K.M., Bradshaw J.J., Marsh J.A., Harrison G.G. and Kaminsky L.S. (1976). The role of cytochrome P-450 in the toxicity of fluroxene (2,2,2-trifluoroethyl vinyl ether) anaesthesia *in vivo*. Biochem. Pharmacol. 25 773-778.

Ivanetich K.M., Lucas S., Marsh J.A., Ziman M.R., Katz I.D. and Bradshaw J.J. (1978). Organic compounds. Their interactions with and degradation of hepatic microsomal drug-metabolising enzymes *in vitro*. Drug Met. and Dispos. 6 218-225.

Ivanetich K.M., Lucas S.A. and Marsh J.A. (1979). Enflurane and methoxyflurane. Their interaction with hepatic cytochrome P-450 *in vitro*. Biochem. Pharmacol. 28 785-792.

Jefcoate C.R.E., Gaylor J.L. and Calabrese R.L. (1966).Ligand interactions with cytochrome P-450. 1. Binding of primary amines. Biochemistry 8 3455-3463.

Jeffcoat R., Brawn P.R. and James A.T. (1976). The effect of soluble rat liver proteins on the activity of microsomal stearoyl-CoA and linoleoyl CoA desaturase. Biochim. Biophys. Acta 431 33-44.

Jeffcoat R. and James A.T. (1977). Interrelationship between the dietary regulation of fatty acid synthesis and the fatty acyl-CoA desaturases. Lipids 12 469-474.

Jeffcoat R. and James A.T. (1978). The control of stearoyl-CoA desaturase by dietary linoleic acid. Fed. Eur. Biochem. Soc. 85 114-118.

Jones P.D., Holloway P.W., Peluffo R.O. and Wakil S.J. (1969). A requirement for lipids by the microsomal stearyl coenzyme A desaturase. J. Biol. Chem. 244 744-745.

Jones D.P. and Gaylor J.L. (1979). Regulation of microsomal stearoyl-coenzyme A desaturase. Purification of a non-substrate-binding protein that stimulates activity. Biochem. J. 183 405-415.

Joshi V.C., Wilson A.C. and Wakil S.J. (1977). Assay for the terminal enzyme of the stearoyl coenzyme A desaturase enzyme system using chick embryo liver microsomes. J. Lipid Res. 18 32-36.

Kadlubar F.F., Mckee E.M. and Ziegler D.M. (1973). Reduced pyridine nucleotide-dependent N-hydroxy amine oxidase and reductase activities of hepatic microsomes. Archs. Biochem. Biophys. 156 46-52.

Kappus H., Bolt H.M., Buchter A. and Bolt W. (1975). Rat liver microsomes catalyse covalent binding of ^{14}C-vinyl chloride to macromolecules. Nature 257 (5522) 134-135.

Karashima D., Hirokata Y., Shigematsu A. and Furukawa T. (1977). The *in vitro* metabolism of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) by hepatic microsomal cytochrome P-450. J. Pharmacol. Exp. Ther. 203 409-416.

Kellogg E.W. III and Fridovich I. (1975). Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J. Biol. Chem. 250 8812-8817.

King M.M., Lai E.K. and McCay P.B. (1975). Singlet oxygen production associated with enzyme-catalyzed lipid peroxidation in liver microsomes. J. Biol. Chem. 250 6496-6502.

Klatskin G. (1968). Mechanisms of toxic and drug induced hepatic injury in "Toxicity of anaesthetics". Ed. B.R. Fink. Williams and Wilkins, Baltimore.

Klatskin G. and Kimberg D.V. (1969). Recurrent hepatitis attributable to halothane sensitization in an anaesthetist. New Eng. J. Med. 280 515-522.

Klingenberg M. (1958). Pigments of rat liver microsomes. Archs. Biochem. Biophys. 75 376-386.

Klion F.M., Schaffner F. and Popper H. (1969). Hepatitis after exposure to halothane. Ann. Inter. Med. 71 467-477.

Kuzava B.A. (1973). Ethrane: Is it a better anesthetic? J. Am. Assoc. of Nurse Anesthetists 41 515-526.

Lavelle F., Michelson A.M. and Dimitrijevic L. (1973). Biological protection by superoxide dismutase. Biochem. Biophys. Res. Commun. 55 350-357.

Lee C-J. and Sprecher H. (1971). An *in vitro* study of the effects of dietary alteration and fasting on the desaturation of palmitic, stearic eicosa-8,11-dienoic and eicosa-8,11,14-trienoic acids. Biochim. Biophys. Acta 248 180-185.

Lee Son S., Colella J.J. and Brown B.R. (1972). The effect of phenobarbitone on the metabolism of methoxyflurane to oxalic acid in the rat. Br. J. Anaesth. 44 1224-1228.

Loew G., Motulsky H., Trudell J., Cohen E. and Hjelmeland L. (1974). Quantum chemical studies of the metabolism of the inhalation anesthetics methoxyflurane, enflurane and isoflurane. Molec. Pharmacol. 10 406-418.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with folin phenol reagent. J. Biol. Chem. 193 265-275.

Lu A.Y.H., Junk K.W. and Coon M.J. (1969). Resolution of the cytochrome P-450-containing ω-hydroxylation system of liver microsomes into three components. J. Biol. Chem. 244 3714-3721.

Lu A.Y.H., Kuntzman R.and Conney A.H. (1976). The liver microsomal hydroxylation enzyme system. Front. Gastrointest. Res. 2 1-31.

Lyman R.L., Fosmire M.A., Giotas C. and Miljanich P. (1970). Inhibition of desaturation of stearic acid in livers of rats fed ethione. Lipids 5 583-589.

Maduska A.L. (1974). Serum inorganic fluoride levels in patients receiving enflurane anesthesia. Anesth. Analg. 53 351-353.

Maiorino R.M., Sipes I.G., Gandolfi A.J. and Brown B.R. (1979). Quantitative analysis of volatile halothane metabolites in biological tissues by gas chromatography. J. Chromatog. 164 63-72.

Mannering G.J., Sladek N.E., Parli C.J. and Shoeman D.W. (1969). Formation of a new P-450 hemoprotein after treatment of rats with polycyclic hydrocarbons in "Microsomes and Drug Oxidations". pp. 303-330. Ed. J.R. Gillette. Academic Press, New York.

Mannering G.J., Kuwahara S. and Omura T. (1974). Immunochemical evidence for the participation of cytochrome b_5 in the NADH synergism of the NADPH-dependent mono-oxidase system of hepatic microsomes. Biochem. Biophys. Res. Commun. 57 476-481.

Marsh J.B. and James A.T. (1962). The conversion of stearic to oleic acid by liver and yeast preparations. Biochim. Biophys. Acta 60 320-328.

Marsh J.A., Lucas S.A., Harrison G.G. and Ivanetich K.M. (1979). Do methoxyflurane and enflurane induce hepatic drug-metabolizing enzymes. S. Afr. Med. J. 55 871-877.

Masaro E.J. (1968). In "Physiological Chemistry of Lipids in Mammals". p.66. Philadelphia, Saunders.

Mazze R.I., Trudell J.R. and Cousins M.J. (1971). Methoxyflurane metabolism and renal dysfunction: clinical correlation in man. Anesthesiology 35 247-252.

Mazze R.I., Calverlay R.K. and Ty Smith N. (1977). Inorganic fluoride nephrotoxicity: prolonged enflurane and halothane anesthesia in volunteers. Anesthesiology 46 265-271.

McIntosh D.B., Berman M.C. and Kench J.E. (1977). Characteristics of sarcoplasmic reticulum from slowly glycolysing and from rapidly glycolysing pig skeletal muscle post mortum. Biochem J. 166 387-398.

McLain G.E., Sipes I.G. and Brown B.R. (1979). An animal model of halothane hepatotoxicity: Roles of enzyme induction and hypoxia. Anesthesiology 51 321-326.

Mercuri O., Peluffo R.O. and De Tomás M.E. (1974). Effect of different diets on the Δ9-desaturase activity of normal and diabetic rats. Biochim. Biophys. Acta 369 264-268.

Mercuri O. and De Tomás M.E. (1978). Δ9 Desaturase activity in normal mouse liver and hepatoma SSlK. Lipids 13 289-290.

Mihara K. and Sato R. (1972). Partial purification of NADH-cytochrome b_5 reductase from rabbit liver microsomes with detergents and its properties. J. Biochem. 71 725-735.

Misra H.P. and Fridovich I. (1972). The generation of superoxide radicals during autoxidation of hemoglobin. J. Biol. Chem. 247 6960-6962.

Montgomery M.R. and Holtzman J.L. (1975). Drug-induced alterations in hepatic fatty acid desaturase activity. Biochem. Pharmacol. 24 1343-1347.

Morris L.J. (1970). Mechanisms and stereochemistry in fatty acid metabolism. Biochem. J. 118 681-693.

Mukai S., Morio M., Fujii K. and Hanaki C. (1977). Volatile metabolites of halothane in the rabbit. Anesthesiology 47 248-251.

Mulder G.J. (1974). Heterogeneity of hepatic microsomal uridine diphosphate glucuronyltransferase: A critical evaluation. Biochem. Soc. trans. 2 1172-1174.

Nastaincyk W., Ullrich V. and Sies H. (1978). Effect of oxygen concentration on the reaction of halothane with cytochrome P-450 in liver microsomes and isolated perfused rat liver. Biochem. Pharmacol. 27 387-392.

Netter K.J. and Seidel G. (1964). An adaptively stimulated O-demethylating system in rat liver microsomes and its kinetic properties. J. Pharmacol. Exp. Ther. 146 61-65.

Okuda T., Mihara K. and Sato R. (1972). Interactions between NADH-cytochrome b_5 reductase and cytochrome b_5 preparations purified from liver microsomes. J. Biochem. 72 987-992.

Omura T. and Sato R. (1962). A new cytochrome in liver microsomes. J. Biol. Chem. 237 PC 1375-1376.

Omura T. and Sato R. (1964). The carbon monoxide-binding pigment of liver microsomes. 1. Evidence for its hemoprotein nature. J. Biol. Chem. 239 2370-2378.

Omura T. and Takesue S. (1970). A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome c reductase from rat liver microsomes. J. Biochem. 67 249-257.

Orrenius S. (1980). Personal Communication.

Oshino N., Imai Y. and Sato R. (1966). Electron-transfer mechanism associated with fatty acid desaturation catalyzed by liver microsomes. Biochim. Biophys Acta 128 13-28.

Oshino N. and Sato R. (1971). Stimulation by phenols of the re-oxidation microsomal bound cytochrome b_5 and its implication to fatty acid desaturation. J. Biochem. 69 169-180.

Oshino N., Imai Y. and Sato R. (1971). A function of cytochrome b_5 in fatty acid desaturation in rat liver microsomes. J. Biochem. 69 155-167.

Oshino N. and Sato R. (1972). The dietary control of the microsomal stearoyl-CoA desaturation enzyme system in rat liver. Archs. Biochem. Biophys. 149 369-377.

Oshino N. and Omura T. (1973). Immunochemical evidence for the participation of cytochrome b_5 in microsomal stearoyl-CoA desaturation reaction. Archs. Biochem. Biophys. 157 395-404.

Oshino N. (1978). Cytochrome b_5 and its physiological significance. Pharmac. Ther. 2 477-515.

Ozols J. and Gerard C. (1977). Covalent structure of the membranous segment of horse cytochrome b_5. Chemical cleavage of the native hemoprotein. J. Biol. Chem. 252 8549-8553.

Pande S.V. and Mead J.F. (1968). Inhibition of enzyme activities by free fatty acids. J. Biol Chem. 243 6180-6185.

Pande S.V. and Mead J.F. (1970). Inhibition of the stearyl coenzyme A desaturase system by sterculate. J. Biol. Chem. 245 1856-1861.

Paulsrud J.R., Stewart S.E., Gratt G. and Holman R.T. (1970). Desaturation of saturated fatty acids by rat liver microsomes. Lipids 5 611-616.

Peters R.L., Edmonson H.A., Reynolds T.B., Meister J.C. and Curphey T.J. (1969). Hepatic necrosis associated with halothane anesthesia. Am. J. Med. 47 748-764.

Peterson G.L. (1977). A simplication of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83 346-356.

Piatt J. and O'Brien P.J. (1979). Singlet oxygen formation by a peroxidase, H_2O_2 and halide system. Eur. J. Biochem. 93 323-332.

Politzer I.R., Griffin G.W. and Laseter J.L. (1971). Singlet oxygen and biological systems. Chem-Biol. Interactions 3 73-93.

Prough R.A. and Siler Masters B.S. (1974). The mechanism of cytochrome b_5 reduction by NADPH-cytochrome c reductase. Archs. Biochem. Biophys. 165 263-267.

Prough R.A., Patrizi V.W. and Estabrook R.W. (1976). The direct spectrophotometric observation of benzo(α)pyrene phenol formation by liver microsomes. Cancer Res. 36 4439-4443.

Pugh E.L. and Kates M. (1977). Direct desaturation of eicosatrienoyl lecithin to arachidonoyl lecithin by rat liver microsomes. J. Biol. Chem. 252 68-73.

Raju P.K. and Reiser R. (1967). Inhibition of fatty acyl desaturase by cyclopropene fatty acids. J. Biol. Chem. 242 379-384.

Raju P.K. and Reiser R. (1972). Stimulation of stearoyl-CoA desaturase activity by sn-glycero-3-phosphate in mouse liver microsomes. Biochim. Biophys. Acta 280 267-274.

Rao G.A. and Abrahams S. (1975). Stearoyl-CoA desaturase activity in mammary adenocarcinomas carried by C3H mice. Lipids 10 835-839.

Rehder K., Forbes J., Alter H., Hessler O. and Stier A. (1967). Halothane biotransformation in man: a quantitative study. Anesthesiology 28 711-715.

Rogers M.J. and Strittmatter P. (1973). Lipid-protein interactions in the reconstitution of the microsomal reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase system. J. Biol. Chem. 248 800-806.

Rogers M.J. and Strittmatter P. (1974a). Evidence for random distribution and translational movement of cytochrome b_5 in endoplasmic reticulum. J. Biol. Chem. 249 895-900.

Rogers M.J. and Strittmatter P. (1974b). The binding of reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase to hepatic microsomes. J. Biol. Chem. 249 5565-5569.

Rogers M.J. and Strittmatter P. (1975). The interaction of NADH-cytochrome b_5 reductase and cytochrome b_5 bound to egg lecithin lyposomes. J. Biol. Chem. 250 5713-5718.

Roots I. and Hildebrandt A.G. (1973). Non-competitive and competitive inhibition of mixed function oxidase in rat liver microsomes by metyrapone. Nauyn-Schmied. Arch. Pharmacol. 277 27-38.

Ross W.T. and Cardell R.R. Jr. (1978). Proliferation of smooth endoplasmic reticulum and induction of microsomal drug-metabolizing enzymes after ether or halothane. Anesthesiology 48 325-331.

Ross W.T., Daggy B.R. and Cardell R.R. (1979). Hepatic necrosis caused by halothane and hypoxia in phenobarbital-treated rats. Anesthesiology 51 327-333.

Safford R., Jeffcoat R. and James A.T. (1975). Factors effecting the solubilization of stearoyl-CoA desaturase of hen liver microsomes. Biochim. Biophys. Acta 409 86-96.

Sharp J.H., Trudell J.R. and Cohen E.N. (1979). Volatile metabolites and decomposition products of halothane in man. Anesthesiology 50 2-8.

Shimakata T., Mihara K. and Sato R. (1971). Lack of correlation between cyanide-binding spectrum and fatty acid desaturase activity in liver microsomes. Biochem. Biophys. Res. Commun. 44 533-538.

Shimakata T., Mihara K. and Sato R. (1972). Reconstitution of hepatic microsomal stearoyl-coenzyme A desaturase system from solubilized components. J. Biochem. 72 1163-1174.

Siegfried H.E. and Gaylor J.L. (1976). Investigation of microsomal oxygenases of biosynthetic processes. Stearoyl-CoA desaturase of adipose tissue and liver. J. Biol. Chem. 251 7468-7473.

Simpson B.R., Strunin L. and Walton B. (1971). The halothane dilemma: A case for the defence. Brit. Med. J. 4 96-100.

Smith L. (1955). Cytochromes a, a_1, a_2 and a_3. Meth. Enzymol. 2 732-740.

Spatz L. and Strittmatter P. (1971). A form of cytochrome b_5 that contains an additional hydrophobic sequence of 40 amino acid residues. Proc. Natl. Acad. Sci., U.S.A. 68 1042-1046.

Spatz L. and Strittmatter P. (1972). A polymerizing form of microsomal cytochrome b_5 reductase. Fed. Proc. 31 411 no. 1083.

Spatz L. and Strittmatter P. (1973). A form of reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. J. Biol. Chem. 248 793-799.

Stacey N.H., Priestly B.G. and Hall R.C. (1978). Toxicity of halogenated volatile anesthetics in isolated rat hepatocytes. Anesthesiology 48 17-22.

Staudt H., Lichtenberger F. and Ullrich V. (1974). The role of NADH in uncoupled microsomal monoxygenations. Eur. J. Biochem. 46 99-106.

Stevens W.C., Eger E.I., White A., Halsey M.J., Munger W, Gibbons R.D., Dolan W. and Shargel R. (1975). Comparative toxicities of halothane, isoflurane and diethyl ether at subanaesthetic concentrations in laboratory animals. Anesthesiology 42 408-419.

Stevens W.C., Eger E.I., White A., Biave C.G., Dibbons R.D. and Shargel R. (1977). Comparative toxicities of enflurane, fluroxene and nitrous oxide at subanaesthetic concentrations in laboratory animals. Canad. Anaes. Soc. J. 24 479-490.

Stier A. (1964). Trifluoroacetic acid as metabolite of halothane. Biochem. Pharmacol. 13 1544.

Stier A., Alter H., Hessler O. and Rehder K. (1964). Urinary excretion of bromide in halothane anesthesia. Anesth. Analg. 43 723-728.

Strittmatter P. and Velick S.F. (1956). A microsomal cytochrome reductase specific for diphosphopyridine nucleotide. J. Biol. Chem. 221 277-286.

Strittmatter P. (1971). The characterization and interconversions of two conformational states of cytochrome b_5 reductase. J. Biol. Chem. 246 1017-1024.

Strittmatter P., Rogers M.J. and Spatz L. (1972). The binding of cytochrome b_5 to liver microsomes. J. Biol. Chem. 247 7188-7194.

Strittmatter P., Spatz L., Corcoran D., Rogers M.J., Setlow B. and Redline R. (1974). Purification and properties of rat liver microsomal stearoyl CoA desaturase. Proc. Natl. Acad. Sci., U.S.A. 71 4565-4569.

Strobel H.W. and Coon M.J. (1971). Effect of superoxide generation and dismutation on hydroxylation reactions catalyzed by liver microsomal cytochrome P-450. J. Biol. Chem. 246 7826-7829.

Tangen O., Jonsson J. and Orrenius S. (1973). Isolation of rat liver microsomes by gel filtration. Anal. Biochem. 54 597-603.

Taves D.R., Fry B.W., Freeman R.B. and Gillies A.J. (1970). Toxicity following methoxyflurane anesthesia. Fluoride concentrations in nephrotoxicity. J. Amer. med. Assoc. 214 91-95.

—, The National Halothane Study. (1966). J. Amer. med. Assoc. 197 775-788.

Thompson D.S. and Friday C.D. (1978). Changes in liver enzyme values after halothane and enflurane for surgical anesthesia. Southern Med. J. 71 779-782.

Trey C., Lipworth L., Chalmers T.C., Davidson C.S., Gottlieb L.S., Popper H. and Saunders S.J. (1968). Fulminant hepatic failure. Presumable contribution of halothane. New Eng. J. Med. 279 798-801.

Trowel J., Peto R. and Crampton Smith A. (1975). Controlled trial of repeated halothane anaesthetics in patients with carcinoma of the uterine cervix treated with radium. Lancet i 821-823.

Uchiyama M., Nakegawa M. and Okin S. (1967). Effect of free unsaturated fatty acids on fatty acid desaturation by liver preparations. J. Biochem. 62 1-6.

Ullrich V. and Diehl H. (1971). Uncoupling of monoxygenation and electron transport by fluorocarbons in liver microsomes. Eur. J. Biochem. 20 509-512.

Van der Reis L., Askin S.J., Frecker G.N. and Fitzgerald W.J. (1974). Enflurane necrosis after enflurane anesthesia. J. Amer. mer Assoc. 227 76.

Van Dyke R.A., Chenoweth M.B. and Larsen E.R. (1964a). Synthesis and metabolism of halothane-1-^{14}C. Nature 204 471-472.

Van Dyke R.A., Chenoweth M.B. and Van Poznak A. (1964b). Metabolism of volatile anesthetics. Conversion in vivo of several anesthetics to $^{14}CO_2$ and chloride. Biochem. Pharmacol. 13 1239-1247.

Van Dyke R.A. and Chenoweth M.B. (1965). Metabolism of volatile anesthetics. Anesthesiology 26 348-357.

Van Dyke R.A. (1966). Metabolism of volatile anesthetics. Induction of microsomal dechlorinating and ether-cleaving enzymes. J. Pharmacol. Exp. Ther. 154 364-369.

Van Hemmen J.J. and Meuling W.J.A. (1975). Inactivation of biologically active DNA by γ-ray-induced superoxide radicals and their dismutation products singlet molecular oxygen and hydrogen peroxide. Biochim. Biophys. Acta 402 133-141.

Virtue R.W. and Payne K.W. (1958). Post operative death after fluothane. Anesthesiology 19 562-563.

Wade A.E., Wu B. and Greene F.E. (1972). Some in vitro assay conditions that affect quantitation and stability of cytochromes P-450 and b_5. Toxicol. Appl. Pharmacol. 22 503-512.

Wahle K.W.J. and Davies N.T. (1975). Effect of dietary copper deficiency in the rat on fatty acid composition of adipose tissue and desaturase activity of liver microsomes. Br. J. Nutrit. 34 105-112.

White-Stevens R.H. and Kamin H. (1970). Uncoupling of oxygen activation from hydroxylation in a bacterial salicylate hydroxylase. Biochem. Biophys. Res. Commun. 38 882-889.

Yoshimura N., Holaday D. and Fiserova-Bergerova V. (1976). Metabolism of methoxyflurane in man. Anesthesiology 44 372-379.