THE INTERACTION OF SOME HALOGENATED ANAESTHETIC AGENTS WITH HEPATIC DRUG METABOLIZING ENZYMES

by

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ABSTRACT

The interaction of some halogenated anaesthetic agents with hepatic drug metabolizing enzymes

This thesis comprises a report of investigations into the interaction of the volatile anaesthetic agents, fluroxene, 2,2,2-trifluoroethyl ethyl ether(TFEE), methoxyflurane and enflurane, with hepatic drug metabolizing enzymes *in vivo* and *in vitro*. Each of the anaesthetic agents interacts with the type P-450 cytochromes* of hepatic microsomes *in vitro* resulting in the appearance of a type I difference spectrum, enhancement of NADPH oxidation and production of potentially toxic metabolites, 2,2,2-trifluoroethanol (TFE) (from fluroxene and TFEE) and free fluoride ion (from methoxyflurane and enflurane). Values of K_s, ΔA_{max}, K_m and V_{max} have been determined for these interactions of the anaesthetic agents with the type P-450 cytochromes in microsomes from uninduced, polycyclic hydrocarbon induced and phenobarbital induced male Wistar rats.

Biphasic Hanes or Eadie-Hofstee plots were obtained with uninduced and polycyclic hydrocarbon induced microsomes for oxidation of NADPH and production of trifluoroethanol in the presence of TFEE, and for the production of free

*In this abstract, 'type P-450 cytochrome(s)' refers to any (or all) member(s) of the group of heterogeneous heme proteins of that name; 'cytochrome P-450' and 'cytochrome P-448' refer selectively to specific type P-450 cytochromes.

and acid-labile fluoride from methoxyflurane; two sets of K_m and V_{max} values were calculable in these cases. On the basis of linearity of Hanes plots and identity of K_s or K_m values in different types of microsomes, it appears that for fluroxene and enflurane, cytochrome P-450 is the predominant type P-450 cytochrome involved in the binding and metabolism of the agent in uninduced, polycyclic hydrocarbon induced and phenobarbital induced microsomes. Cytochrome P-448 appears to play no role in these interactions. Although activity of cytochrome P-450 also appears predominant in the spectrally observable binding of TFEE, involvement of at least one other type P-450 cytochrome in uninduced and polycyclic hydrocarbon induced microsomes is evident from the biphasic Hanes plots for NADPH oxidation and trifluoroethanol production. Differences in the type P-450 cytochromes involved in the binding and metabolism of methoxyflurane by microsomes from differently pretreated animals are also evident. In this case, cytochrome P-450 appears to play a predominant role only in phenobarbital induced microsomes; other type P-450 cytochromes are implicated in the binding and metabolism of methoxyflurane in uninduced and 3-methylcholanthrene induced microsomes.

For the interaction of fluroxene or TFEE with the type P-450 cytochromes of phenobarbital induced microsomes, the relationship $K_s = K_m$ (NADPH oxidation) $= K_m$ (TFE production) is observed. This same relationship is also evident for fluroxene with uninduced and 3-methylcholanthrene induced microsomes. For interactions of methoxyflurane and enflurane with the type P-450 cytochromes of hepatic microsomes, simple

correlations of this nature between values of K_s and K_m are not apparent and this situation probably reflects the greater complexity of the microsomal metabolism of these two agents relative to that of fluroxene or TFEE.

The stoichiometry of NADPH oxidation and metabolite (trifluoroethanol or fluoride) production was calculated from V_{max} values. For fluroxene and TFEE the observed stoichiometry of NADPH : TFE is 1 : 1-2; for methoxyflurane and enflurane the stoichiometry of NADPH : free F^- is 13 : 1 and 140 : 1, respectively. The observed stoichiometry for NADPH : F^- is not in agreement with that predicted by the postulated pathways of metabolism of methoxyflurane and enflurane. We suggest that spontaneous hydrolysis to fluoride of primary products of the microsomal metabolism of methoxyflurane does not occur, and propose that enflurane interacts with the type P-450 cytochromes of hepatic microsomes to a large extent as an uncoupler of substrate oxidation and NADPH and oxygen consumption.

Fluroxene in the presence of NADPH mediates the degradation of the type P-450 cytochromes *in vitro*. The process of degradation requires concomitant metabolism of fluroxene but is not related to the production of trifluoroethanol in different types of microsomes.

The interaction of trifluoroethanol, the toxic metabolite of fluroxene and TFEE, with hepatic enzymes postulated to be responsible for its metabolism was investigated. It appears that a pathway by which the alcohol may

be oxidatively metabolized involves the activity of the type P-450 cytochromes mixed function oxidation system. Trifluoroethanol exhibits a type I difference spectrum with hepatic microsomes and enhances microsomal NADPH oxidation with $K_s = K_m$ (NADPH oxidation) = 25 mM. Trifluoroethanol appears not to be a substrate for alcohol dehydrogenase *in vitro* although it competitively inhibits this enzyme with $K_i = 6.4 \times 10^{-6}$ M. In addition, trifluoroethanol is a non-competitive inhibitor of the activity of catalase *in vitro* with K_i = 28 mM. Metabolism of trifluoroethanol may also occur via a conjugation reaction *in vivo*, but glucuronidation of this alcohol *in vitro* was not observable in hepatic microsomes from the rats under investigation.

Conflicting reports have appeared in the literature on the ability of the anaesthetic agents to induce enzymes of the type P-450 cytochromes mixed function oxidation system. We have conducted an exhaustive investigation into the effects of methoxyflurane and enflurane on the hepatic drug metabolizing enzymes *in vivo*. Anaesthesia with either agent for four to sixteen days at sub-anaesthetic concentrations (0.1 MAC) results in few changes in the levels or activities of drug metabolizing enzymes relative to those in unanaesthetized control animals. Anaesthesia with methoxyflurane for one to four days at anaesthetic concentrations (1.0 MAC) results in a small increase in NADPH-cytochrome *c* reductase activity on the third day only.

Enflurane anaesthesia for one to four days at 1.0 MAC results in an increase in p-nitroanisole O-demethylation activity which is not paralleled by any increases in total type P-450 cytochromes, cytochrome b_5 or NADPH-cytochrome c reductase. We have concluded that fluroxene, methoxyflurane and enflurane may not be classified as inducers of hepatic drug metabolizing enzymes including enzymes responsible for conjugation with glucuronic acid of glutathione.

LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
I. CHEMICALS	
AIA	2-allyl-2-*iso*-propylacetamide
BP	3,4-benzpyrene
EDTA	ethylenediaminetetra-acetic acid
ENF	enflurane
MC	3-methylcholanthrene
MOF	methoxyflurane
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
PB	phenobarbital
SKF 525A	2-diethylaminoethyl-2,2-diphenyl valerate
TCA	trichloroacetic acid
TFE	2,2,2-trifluoroethanol
TFEE	2,2,2-trifluoroethyl ethyl ether
Tris	tris(hydroxymethyl)-aminomethane
UDP-	uridine 5'-diphospho-
II. MISCELLANEOUS	
ΔA	difference in absorbance
ΔA_{max}	maximum extent of difference spectrum
Cyt(s)	cytochrome(s)
hr	hour(s)
K_s	spectrally determined dissociation constant
K_m	Michaelis-Menten constant
LD_{50}	dose (of compound) lethal to 50% of animals in group tested
MAC	minimum alveolar concentration
MEOS	microsomal ethanol oxidizing system
min	minute(s)
N.D.	not done
ref.	reference
V_{max}	maximum reaction rate

III. STATISTICAL ANALYSIS : STUDENT'S *t*-TEST

SYMBOL (superscript)	MEANING
●	differs from values for uninduced or control animals, $p < 0.001$
■	differs from values for uninduced or control animals, $p < 0.01$
▼	probably differs from values for uninduced or control animals, $p < 0.05$

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I. INTRODUCTION

1. Review of hepatic metabolism of xenobiotics

Pathways of metabolism of nutrient carbohydrate, fat and protein are well documented and a large number of enzymes responsible for these metabolic transformations have been isolated and studied. These enzymes generally catalyse a single type of reaction and are specific for one particular type of substrate. In addition to ingesting compounds which have a role in life processes, living organisms are also subjected to a wide range of chemical compounds which are foreign to their normal biochemical functioning. Most of these drugs, carcinogens, environmental pollutants, and other xenobiotics are now known to be biochemically transformed by animals and man. The study of the processes involved has led to the growth over the past 20 to 30 years of such fields of research as biochemical pharmacology, molecular pharmacology, drug metabolism and disposition, and toxicology.

Some xenobiotics resemble substances normally synthesized and catabolized in the body and thus are metabolized by specific enzymes which synthesize and catabolize substances such as catacholamines, indoles, nucleic acids, amino acids, steroids, and fats (1). Most xenobiotics, however, have no endogenous analogue and are metabolized by non-specific enzymes.

In mammals, the liver is the major site of metabolism of

xenobiotics by non-specific enzymes although some activity is also to be found in extrahepatic organs and tissues such as the kidney, intestines, lungs, adrenals, blood and skin. The most common reactions of drug metabolism* involve oxidation, reduction, hydrolysis or conjugation. A drug may be subjected to two or more competing pathways simultaneously; alternatively its metabolic transformations may occur sequentially (2). A summary of the biochemical transformations of drugs and the hepatic enyzmes responsible is shown in Table 1, the information for which was drawn from the Handbook of Experimental Pharmacology (3).

Microsomal metabolism of drugs. Enzymes located in the endoplasmic reticulum of hepatic cells catalyse the majority of reactions of drug metabolism. On homogenization of the liver, the endoplasmic reticulum is disrupted giving rise to small vesicles which may be separated from the homogenate by high-speed ultracentrifugation (4), gel-filtration (5) or calcium precipitation (6); the resultant subcellular fraction is known as 'microsomes'. Many of the reactions undergone by drugs can be demonstrated in vitro with preparations of hepatic microsomes and suitable co-factors.

The microsomal enzymes protect the organism against an accumulation of lipid-soluble exogenous compounds by

* The term 'drug metabolism' is synonymous with 'metabolism of xenobiotics'; the former term is in more general usage than the latter.

Table 1. Hepatic Metabolism of Drugs

Reaction	Enzyme	Major subcellular location of enzyme
Oxidation of aliphatic and aromatic groups, N-oxidation, sulphoxidation, oxidative dealkylation, epoxidation	Cytochrome P-450	Endoplasmic reticulum
Reduction of azo-, nitro- compounds	Flavin enzymes or cytochrome P-450	Endoplasmic reticulum
Oxidation and reduction of alcohols, aldehydes and ketones	Alcohol dehydrogenase, aldehyde dehydrogenase	Soluble fraction
Oxidation of amines	e.g. Monoamine oxidases	Mitochondria
Hydrolysis of esters	Esterases	Soluble fraction and various organelles
Conjugation with glucuronic acid	UDP-glucuronyl transferases	Endoplasmic reticulum
Conjugation with glutathione	Glutathione-S-transferases	Soluble fraction
Conjugation with sulphate, glycine, other amino acids; acetylation and methylation	Various transferases	Soluble fraction and various organelles
Hydration of epoxides	Epoxide hydratase	Endoplasmic reticulum

converting them to more polar molecules which can be more readily excreted by the kidney. Some normal constituents of the body such as steroids, cholesterol, fatty acids, thyroxine and bilirubin are also substrates for these enzymes (7). The pathway by which most lipid-soluble compounds are rendered more water-soluble involves an oxidative reaction catalysed by the cytochrome P-450 drug metabolizing system. The oxidized compound may then be excreted without further change or may undergo a conjugation reaction, usually with glucuronic acid, to yield an even more polar metabolite. The enzymes of both the cytochrome P-450 system and those responsible for glucuronidation are tightly bound in the lipid portion of the endoplasmic reticulum (8,9).

Although oxidation mediated by the cytochrome P-450 enzyme system usually leads to detoxification or to inactive metabolites, there are a number of compounds which are converted to toxic or more active metabolites; for instance, vinyl chloride monomer becomes carcinogenic only after metabolic activation by microsomal enzymes (10); carbon tetrachloride is believed to be metabolized to highly reactive free radicals $CCl_3^{\cdot}$ and $Cl^{\cdot}$ (11); and the volatile anaesthetic agent, fluroxene is metabolized to the toxic compound, trifluoroethanol (12). In addition, during the metabolism of a compound by cytochrome P-450 a reactive metabolite may be formed such that destruction of cytochrome P-450 itself occurs (13,14,15).

In general, conjugation of drugs (or their oxidized products) with glucuronic acid or sulphate leads to pharmacologically inactive and highly polar compounds which are very readily excreted. There have been a few reports, however, of reactive metabolites formed during conjugation reactions; the glucuronide and sulphate conjugates of N-hydroxy-2-acetylaminofluorene (16) and N-hydroxyphenacetin (17,18) have been found to bind covalently to protein.

Although the deleterious effects of a number of xenobiotics are now known to occur as a result of metabolism of these compounds by microsomal enzymes, the role of other enzymes e.g. glutathione-S-transferases and epoxide hydratase, in protecting the cell against the effects of toxic or reactive metabolites is beginning to be understood (19,20).

The major enzyme systems of drug metabolism will be discussed at greater length in the following sections entitled:

a. The cytochrome P-450 drug metabolizing system
b. Conjugation with glucuronic acid
c. Conjugation with glutathione.

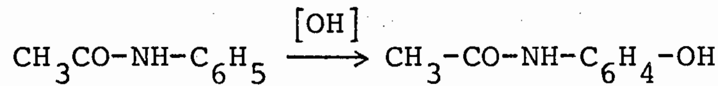
a. The Cytochrome P-450 Drug Metabolizing System

Since the first reports of reductive and oxidative metabolism of foreign compounds by hepatic microsomes (21,22), attention has been focused on the metabolism of drugs and other compounds by a group of non-specific enzymes in liver endoplasmic reticulum. The remarkable versatility of these enzymes with respect to choice of substrate is shown by their ability to catalyze such diverse reactions as those

illustrated in Figure 1 (2,23,24). Despite the diversity of these reactions they may all be regarded as proceeding via hydroxylation reactions.

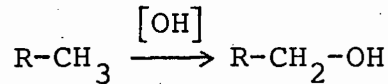
Figure 1. Reactions catalysed by the cytochrome P-450 drug metabolizing system.

Aromatic hydroxylation



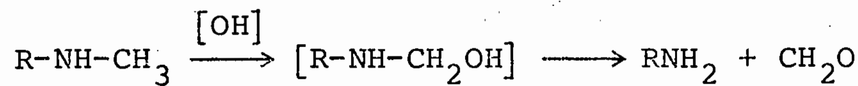
$$CH_3CO\text{-}NH\text{-}C_6H_5 \xrightarrow{[OH]} CH_3\text{-}CO\text{-}NH\text{-}C_6H_4\text{-}OH$$

Aliphatic hydroxylation



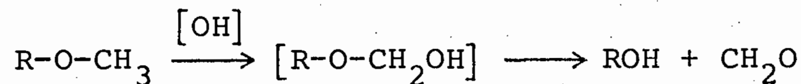
$$R\text{-}CH_3 \xrightarrow{[OH]} R\text{-}CH_2\text{-}OH$$

N-Dealkylation



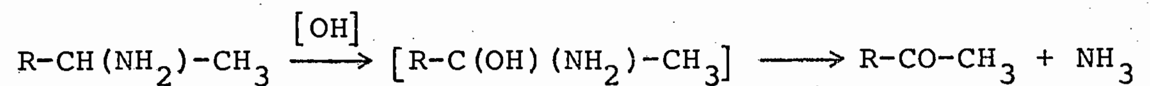
$$R\text{-}NH\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}NH\text{-}CH_2OH] \longrightarrow RNH_2 + CH_2O$$

O-Dealkylation



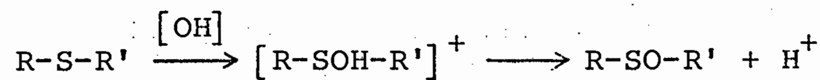
$$R\text{-}O\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}O\text{-}CH_2OH] \longrightarrow ROH + CH_2O$$

Deamination



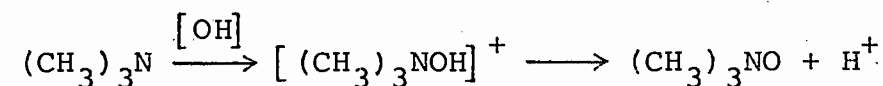
$$R\text{-}CH(NH_2)\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}C(OH)(NH_2)\text{-}CH_3] \longrightarrow R\text{-}CO\text{-}CH_3 + NH_3$$

Sulphoxidation



$$R\text{-}S\text{-}R' \xrightarrow{[OH]} [R\text{-}SOH\text{-}R']^+ \longrightarrow R\text{-}SO\text{-}R' + H^+$$

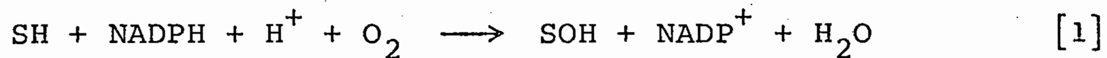
N-Oxidation



$$(CH_3)_3N \xrightarrow{[OH]} [(CH_3)_3NOH]^+ \longrightarrow (CH_3)_3NO + H^+$$

In addition, these enzymes may catalyse reductive reactions such as the reduction of azo- and nitro- compounds to primary aromatic amines and possibly the reductive cleavage of halogenated alkanes, such as carbon tetrachloride, to free radicals (25,26).

Since the oxidative reactions (Figure 1) in the hepatic endoplasmic reticulum require both NADPH and oxygen, the enzyme system responsible is frequently called a 'mixed function oxidase' according to the nomenclature of Mason (24). The enzyme system is also sometimes referred to as a 'monooxygenase' because the enzymes catalyse the consumption of one molecule of oxygen per molecule of substrate, one oxygen atom appearing in the product and the other being incorporated into a water molecule (24). The overall reaction is generally accepted to be of the following form:



$$SH + NADPH + H^+ + O_2 \longrightarrow SOH + NADP^+ + H_2O \qquad [1]$$

where SH represents the lipophilic substrate and SOH its hydroxylated product. NADH may replace NADPH as electron donor but the rate of reaction is reduced by about 85% (27). The metabolism of a substrate by the hepatic microsomal mixed function oxidase system may be monitored in an *in vitro* system comprising microsomes, substrate, NADPH and oxygen, by measuring NADPH oxidation, oxygen consumption, disappearance of substrate or formation of product (28). The theoretical stoichiometry for the microsomal mixed function oxidation reaction (equation 1) is a 1:1:1 consumption of substrate, NADPH and oxygen. In order to

demonstrate stoichiometry between disappearance of substrate (or appearance of product) and NADPH oxidation in hepatic microsomes, however, correction must be made for endogenous NADPH oxidation not related to substrate hydroxylation. Stripp et al. (29) have described one such correction based on the effects of carbon monoxide on substrate hydroxylation and NADPH oxidation.

Cytochrome P-450, a b type cytochrome, is now known to be the terminal oxidase for the electron transport system which is responsible for many of the diverse oxidative reactions by which xenobiotics may be metabolized (24). The cytochrome was so named because of the appearance of a difference spectral band at 450 nm when the reduced heme protein binds carbon monoxide (30). Cytochrome P-450 is no longer regarded as a single enzyme; evidence has accumulated which suggests the existence of multiple forms of the heme protein in liver microsomes. (See *Induction of enzymes of the cytochrome P-450 drug metabolizing system* below). 'Cytochrome P-450' is then a collective term used for the group of microsomal heme proteins which function as terminal oxidase in drug hydroxylations.

Present also in hepatic microsomes is a flavoprotein called NADPH-cytochrome c reductase (or NADPH-cytochrome P-450 reductase) which serves as an electron carrier between NADPH and cytochrome P-450 (31). Both names for this enzyme are used in the literature, the choice of name being dependent usually on the heme protein acceptor present in the assay

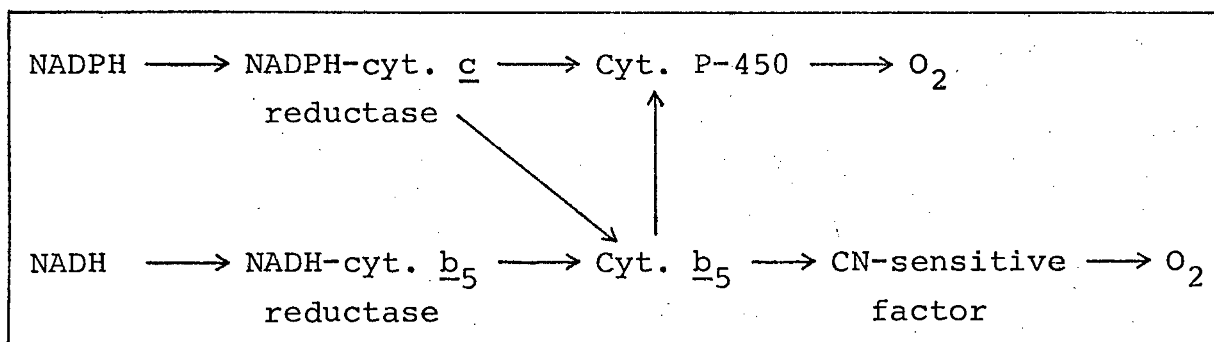
for the enzyme. Several lines of evidence suggested that this enzyme was involved in the hydroxylation of drugs by hepatic microsomes (31); studies with antibodies to NADPH cytochrome c reductase (32) and with purified components of the cytochrome P-450 drug metabolizing system (33,34) have established definitely the role of this enzyme in hepatic microsomal drug hydroxylation.

The isolation of components of the hepatic microsomal drug hydroxylating system in pure or partially pure form has been of great importance to the understanding of this enzyme system. Cytochrome P-450, NADPH-cytochrome c reductase, and a lipid fraction have been isolated from hepatic microsomes by detergent solubilization and DEAE-cellulose chromatography (35,36). All three components are required for the metabolism of fatty acids and drug substrates (33,34). The active constituent in the lipid fraction has been identified as phosphatidylcholine and has been shown to be essential for electron transfer from NADPH to cytochrome P-450 (37,38).

In addition to the NADPH-dependent cytochrome P-450 mixed function oxidase system, hepatic microsomes also contain (amongst many other enzymes) an NADH-dependent electron transport system which functions in desaturation of fatty acids (31,39). The components of this system consist of the flavoprotein NADH-cytochrome b_5 reductase, cytochrome b_5, and a non-heme iron protein known as the cyanide-sensitive factor (40). It appears that the components of the NADH-

and NADPH-dependent electron transport pathways interact with each other; both cytochrome P-450 and cytochrome $\underline{b}_5$ can be reduced by either NADPH or NADH (31) (Figure 2).

Figure 2. Cytochrome P-450 and $\underline{b}_5$ electron transport systems in hepatic microsomes (31).



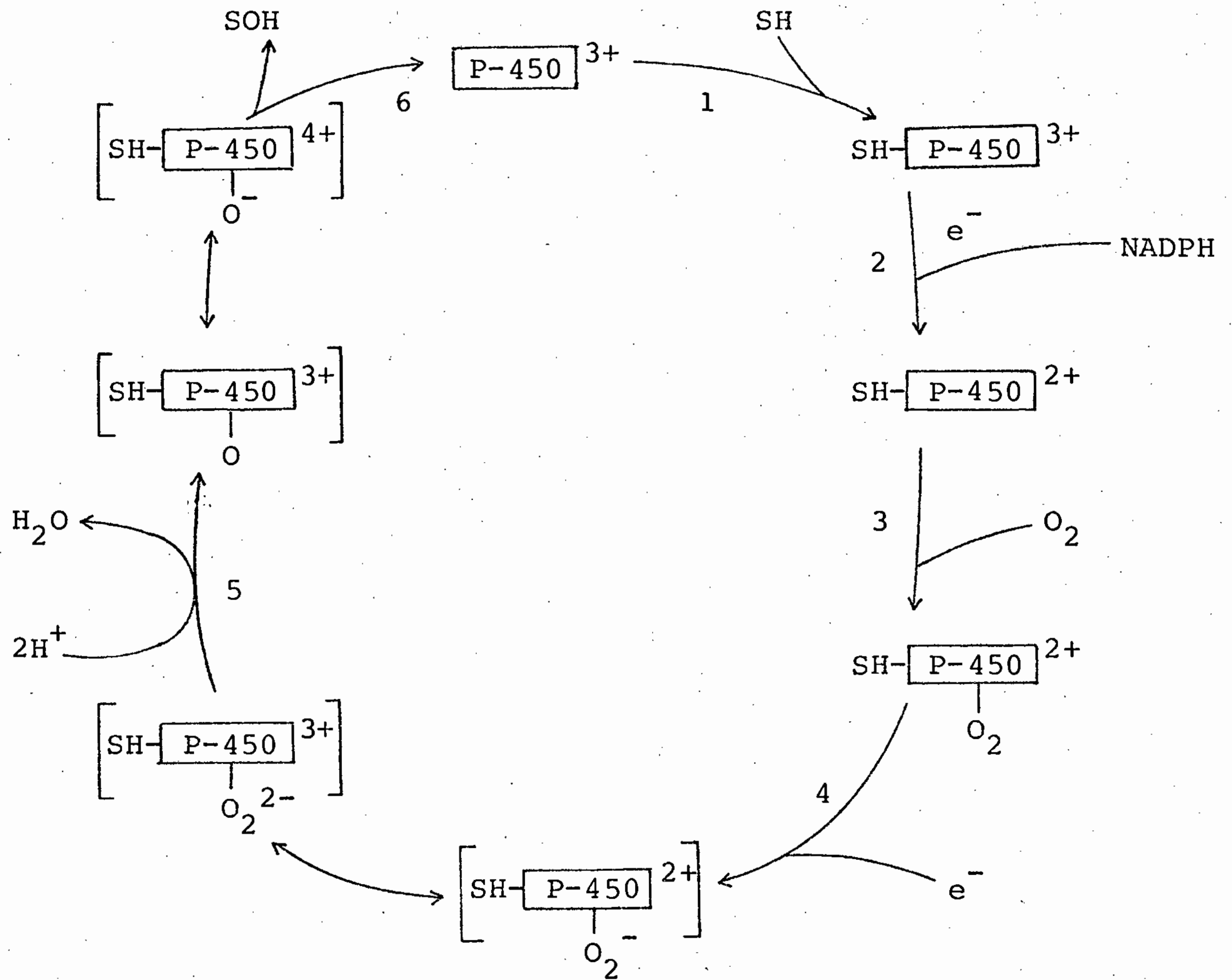
The role of NADH and cytochrome $\underline{b}_5$ in cytochrome P-450 dependent drug hydroxylation reactions is still a matter of uncertainty. Although NADPH is the preferred source of reducing equivalents for cytochrome P-450 linked hydroxylations, the use of both NADPH and NADH together as co-factors in studies _in vitro_ has long been known to produce greater than additive hydroxylation activity in many cases (27,39,41). It was originally suggested by Hildebrandt and Estabrook (42) that cytochrome $\underline{b}_5$ could provide the second electron for microsomal hydroxylation from either NADPH or NADH (see Figure 3). The involvement of cytochrome $\underline{b}_5$ in hydroxylation of substrates has been disputed in many cases however (39) and is clearly not required for hydroxylation reactions mediated by reconstituted systems comprising purified cytochrome P-450, NADPH-cytochrome $\underline{c}$ reductase and lipid (43). Nevertheless, in view of evidence

showing cytochrome $\underline{b}_5$ to be obligatory in certain reactions, it has been suggested that cytochrome $\underline{b}_5$ involvement in NADH or NADPH supported microsomal hydroxylation is dependent on the specific reaction studied and also on the particular species of cytochrome P-450 catalysing the reaction (39).

A generalized scheme describing the mechanism of cytochrome P-450 mediated hydroxylation of an organic compound has been developed by Estabrook and co-workers (44) and extended by others (45,46,47). The reaction sequence shown in Figure 3 depicts the individual reaction steps which may be described as follows:

1. A substrate (SH) for microsomal mixed function oxidation interacts directly with the low spin form of ferricytochrome P-450 to form a high-spin ferricytochrome P-450 enzyme-substrate complex (see *Binding of organic compounds to cytochrome P-450* below).
2. The enzyme-substrate complex was originally postulated to undergo a one electron reduction to a ferrocytochrome P-450 substrate complex (44,48). It has been suggested recently, however, that cytochrome P-450 is capable of accepting two electrons at a time, one by the heme iron atom and one by an acceptor which has not yet been identified (49,50).
3. The ferrous enzyme-substrate complex reacts with molecular oxygen to form an oxyferrous enzyme-substrate complex (51,52).

Figure 3. Proposed mechanism for mixed function oxidation reactions catalysed by cytochrome P-450



4. The oxyferrocytochrome P-450 - substrate complex undergoes a second stage of reduction, forming a superoxide ferrous enzyme-substrate intermediate which is a resonance form of the hydroperoxo ferric enzyme-substrate complex (45,46). Whether this second electron is transferred from a separate electron carrier (e.g. cytochrome b_5 or NADPH-cytochrome P-450 reductase) or from an unknown electron carrier already within the complex is uncertain (46).

5. It is proposed that the hydroperoxo ferric enzyme-substrate complex decomposes readily by heterolytic scission of the O-O bond to produce water and a ferric enzyme-monooxygen species which is a resonance form of the ferryl ion complex ($Fe^{4+}O^{-}$). This highly electrophilic species is presumed to be the actual hydroxylating agent (45,47).

6. The hydroxylated product is released from the site of binding on cytochrome P-450 and cytochrome P-450 in the low spin ferric state is regenerated.

Binding of organic compounds to cytochrome P-450. It was first reported in 1966 that drugs and other foreign compounds combine with oxidized hepatic microsomal cytochrome P-450 to produce characteristic difference spectra (53,54). These spectral changes have been interpreted as a reflection of the binding of substrates or inhibitors to the oxidized form of cytochrome P-450 and are classified into three categories termed type I, type II and modified type II,

based on the form of the spectral change resulting from interactions with different compounds (55). The modified type II change is also designated reverse type I (56). The three types of difference spectra are illustrated in Figure 4. The magnitude of the spectral change (the difference in absorbance between the peak and the trough) for all types of difference spectra is dependent on cytochrome P-450 content and substrate concentration, as well as the substrate employed (55).

The similarity observed for many substrates between the type I difference spectral dissociation constant (K_s) and the Michaelis constant (K_m), obtained by determination of rates of metabolism, gave rise to the conclusion that the type I change was a spectral manifestation of the enzyme-substrate complex. Because of the observation that compounds with widely differing chemical structures and modes of metabolic reaction give rise to the same spectral change (i.e. type I), it has been suggested that all substrates eliciting a type I spectrum interact at the same site on cytochrome P-450 (57). The type I difference spectrum has been shown to arise from substrate-induced conformational changes in the protein which alter the heme environment rather than from direct interaction of substrate with the heme (58). Nebert et al. have postulated that binding of type I substrates by cytochrome P-450 leads to loss of the sixth ligand (OH moiety from adjacent amino acid residue or group of similar ligand field strength) because of conformational changes in the protein (59).

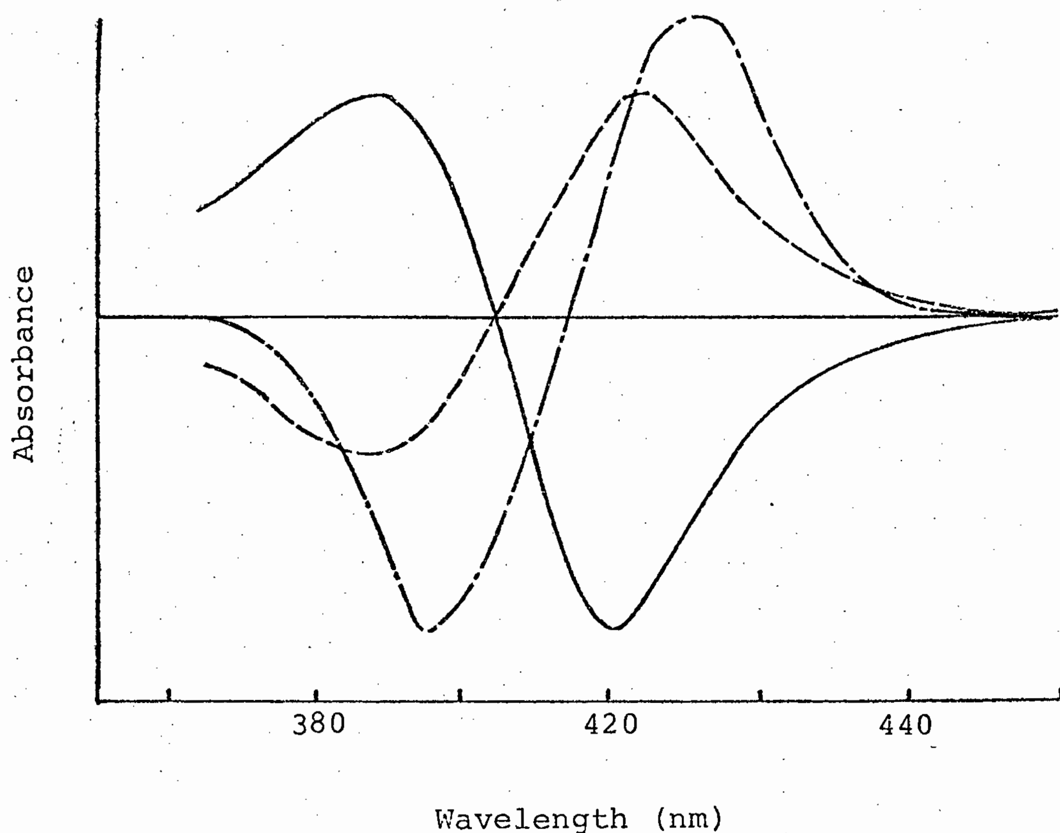


Figure 4. Difference spectral changes in hepatic microsomes. Sample cuvette contains microsomes and compound, and reference cuvette microsomes alone. Spectral changes represented: type I (———); type II (----); and modified type II (-·-·-·).

Type II spectral changes result from interaction of compounds with the cytochrome P-450 heme iron, resulting in formation of a modified ferri-hemochrome in the low-spin state with the type II compound as the sixth ligand (54,55,59,60). All type II compounds can provide a free electron pair, such as that from a heteroatom or a carbene, for liganding with the heme iron atom. These compounds can replace carbon monoxide as the ligand to iron in the ferrocytochrome P-450 - CO complex (61,62,63). Although the type II difference spectrum is a spectral manifestation of binding which does not lead to hydroxylation of the bound compound (51), a number of substrates are able to elicit a type II difference spectrum (59,61). In this case the resultant type II spectrum may be readily recognizable as such, e.g. the binding of aniline (60), or it may be obscured to a varied extent by the type I spectrum of the compound, e.g. the binding of amphetamine (64). Ullrich recommends that the terms 'type I' and 'type II' be replaced by the more descriptive terms 'substrate-binding' and 'ligand-binding' spectra respectively (62).

The modified type II spectrum, elicited by both substrates and non-substrates of cytochrome P-450 was initially postulated to reflect displacement by these compounds of some previously bound substrate at the type I site of the enzyme (57). Other workers have concluded more recently, however, that this is not the case and that modified type II compounds bind as sixth ligand to the cytochrome P-450 heme iron atom (59,61). As in the case of a type II

spectrum a modified type II spectrum can also occur simultaneously with a type I spectrum (57,61).

Induction of enzymes of the cytochrome P-450 drug metabolizing system. The concentrations of cytochrome P-450 and other components of the microsomal electron transport system in animals and man are affected by a number of factors. A wide variety of drugs, carcinogens and other xenobiotics (65,66) as well as environmental factors such as exposure to low temperatures (67) and gamma irradiation (68), are able to induce the synthesis of components of the microsomal mixed function oxidase system thereby increasing rates of drug metabolism.

The extent of the increase in the rate of drug metabolism after induction of cytochrome P-450 is dependent not only on the inducing agent but also on the substrate whose metabolism is monitored. Chemical inducing agents have therefore been grouped into two categories: (1) those that are able to stimulate the metabolism of a large variety of substrates e.g. phenobarbital, and (2) those that stimulate the metabolism of only a limited number of compounds e.g. the polycyclic hydrocarbons, such as 3-methylcholanthrene (31). Administration of phenobarbital or 3-methylcholanthrene to animals leads to elevated levels of hepatic microsomal cytochrome P-450; in addition phenobarbital but not 3-methylcholanthrene treatment results in proliferation of the endoplasmic reticulum and increased levels of NADPH-cytochrome c reductase (65,66).

There is considerable evidence demonstrating that the cytochrome P-450 induced by phenobarbital differs qualitatively from that induced by 3-methylcholanthrene, and that both differ from the predominant type P-450 cytochrome of uninduced animals (31). The cytochrome induced by 3-methylcholanthrene has been named cytochrome P-448 because its reduced CO-difference spectrum is located at 448 nm, in contrast to the peak at 450 nm found for the type P-450 cytochrome(s) in liver microsomes from untreated or phenobarbital treated animals (69). Schenkman *et al.* (70) have described a cytochrome P-450 species induced by pregnenolone 16α-carbonitrile which differs from the cytochromes P-450 and P-448 induced by phenobarbital and 3-methylcholanthrene respectively. Their tentative designation for this enzyme is cytochrome P-449*.

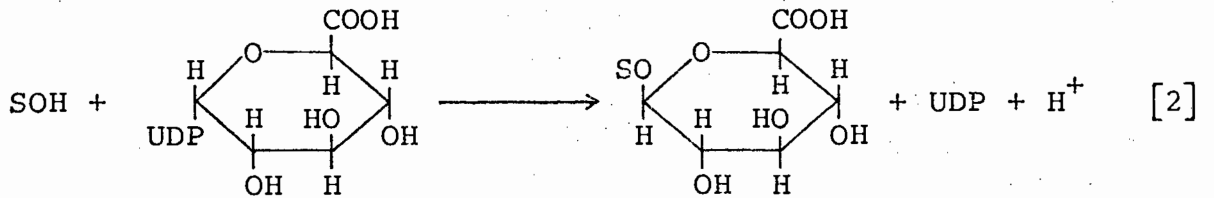
The technique of isolation and purification of components of the cytochromes P-450 drug metabolizing system has been used effectively as a probe into the multiplicity of cytochromes P-450 isolated from animals treated with different inducers (71,72). The spectral and catalytic properties of cytochromes P-450 purified from phenobarbital induced

* From this point, the terms 'cytochrome P-450' and 'cytochrome P-448' will be used to refer to the specific enzymes induced by phenobarbital and 3-methylcholanthrene respectively, and the terms 'cytochromes P-450' or 'type P-450 cytochrome(s)' will be used for the heterogeneous group of hemoproteins of that name.

rats and from 3-methylcholanthrene induced rats are quite different (71). From studies using antibodies raised against purified cytochrome P-448 (73), it is evident that cytochrome P-448 is immunochemically different from cytochrome P-450 from phenobarbital-treated rats. Moreover, from the available evidence it appears that multiple forms of the type P-450 cytochromes exist in hepatic microsomes from either untreated, phenobarbital induced or 3-methylcholanthrene induced animals and that each form possesses different or overlapping substrate specificities. Microsomes from untreated animals contain at least two types of cytochromes P-450 (74,75) and at least two also can be separated from hepatic microsomes of phenobarbital or 3-methylcholanthrene induced rats (76) and four from hepatic microsomes of phenobarbital or 3-methylcholanthrene induced mice (71).

b. <u>Conjugation with glucuronic acid</u>

Conjugation with glucuronic acid occurs in man and most mammalian species and is the most widespread and versatile of the conjugation reactions. Many compounds, with widely different molecular structures, can be metabolized by glucuronidation (77). In order for glucuronidation to occur, the glucuronic acid must be in the form of the high energy compound, uridine diphosphoglucuronic acid (UDP-glucuronic acid). The microsomal enzyme UDP-glucuronyl transferase catalyses the reaction which may be represented as follows (78):



where SOH represents a suitable substrate for the reaction.

The activity of UDP-glucuronyl transferase in hepatic microsomes may be measured by monitoring the disappearance of aglycone or the appearance of the glucuronide conjugate following incubation of microsomes, aglycone and UDP-glucuronic acid.

There is considerable evidence which indicates that rather than being a single enzyme UDP-glucuronyl transferase is heterogeneous (77,79). The existence of multiple forms of this enzyme was originally postulated to explain the finding that several animal species lack glucuronidating activity towards particular substrates (80). Further evidence for the multiplicity of the enzyme has accumulated mainly from studies of the kinetics and activation of UDP-glucuronyl transferase *in vitro* and from investigations into the development of UDP-glucuronyl transferase activities in the foetus and neonate (77). The isolation and purification of UDP-glucuronyl transferase(s) which could provide final evidence for the multiplicity of this enzyme has not yet been achieved.

UDP-glucuronyl transferase activities in hepatic microsomal preparations can be activated *in vitro* with agents which tend to disrupt the organization of the microsomal membrane.

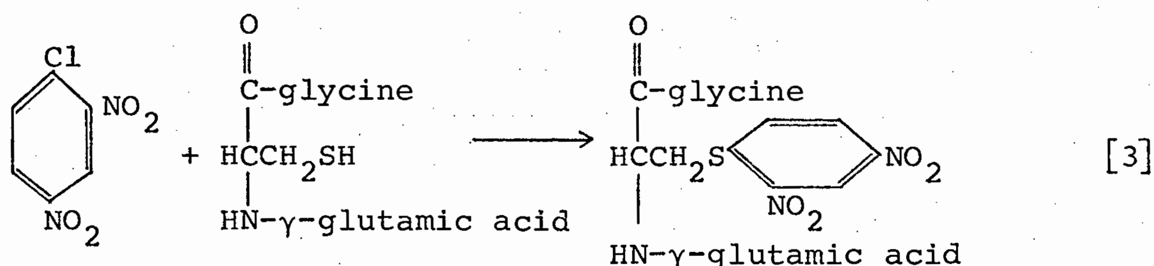
Treatment of microsomes with detergents (81,82), phospholipases, trypsin (83), organic solvents (84) and mild alkali (85) have been shown to enhance considerably the activity of UDP-glucuronyl transferase relative to that observed in untreated microsomes. Some form of activation, usually that by detergent, is common practice in assays of UDP-glucuronyl transferase activities *in vitro*.

UDP-glucuronyl transferase activity is enhanced by treatment of animals with a number of agents which are known to induce the cytochromes P-450 oxidation system. This enhancement of activity is ascribed to induction of UDP-glucuronyl transferase and is observed after treatment of animals with such compounds as phenobarbital, 3-methylcholanthrene (77), a polychlorinated biphenyl (86), or a chlorinated dibenzodioxin (87).

c. Conjugation with glutathione

Conjugation of compounds with glutathione by glutathione-S-transferases serves as a protective mechanism against the potential toxic effects of numerous reactive compounds (88). Glutathione-S-transferases located in the soluble fraction of liver and other organs appear to catalyse two main types of reaction with glutathione: (1) nucleophilic substitution, typified by the conjugation of glutathione with alkyl, aryl or aralkyl halides neutralizing their electrophilic sites, and (2) nucleophilic addition, typified by the conjugation of glutathione with epoxides or

αβ-unsaturated compounds (88). The conjugation of 1-chloro-2,4-dinitrobenzene with glutathione provides an example of the first type of reaction (89):



Assay of glutathione-S-transferase activity *in vitro* may be undertaken using a wide variety of substrates; the disappearance of substrate or formation of conjugate may be monitored in a system comprising soluble fraction isolated from liver, substrate and glutathione (90). The measurement of hepatic glutathione depletion *in vivo* can provide a useful indication of the protective role of glutathione against potentially toxic foreign compounds (91).

Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the conjugated cysteinyl residue to yield finally a mercapturic acid which is then excreted in the urine (92).

Six glutathione-S-transferases each with overlapping substrate specificities have been identified in the soluble fraction of rat liver (90,93). One of the five enzyme species that have been obtained in homogeneous form has been shown to be identical to ligandin (93,94) an organic anion binding protein which also exhibits glutathione-S-

transferase activity. Jakoby _et al_. (93) consider the glutathione-S-transferases to be a family of 'catalytic binding proteins' which also deactivate potentially toxic compounds by mechanisms other than conjugation with glutathione. Additional protection from toxic compounds arises (1) from the binding by these enzymes of compounds ('ligands') which do not contain a sufficiently electrophilic centre to undergo a conjugation reaction and (2) from the covalent linkage to these enzymes of compounds which are reactive (and which may also function as substrates if bound non-covalently).

Induction of hepatic glutathione-S-transferase activities by phenobarbital, 3-methylcholanthrene and 3,4-benzpyrene has been shown to occur in the rat (95,96,97).

2. Metabolism of volatile anaesthetic agents

Inhalation anaesthetics were considered as classic examples of pharmacologically active compounds that did not undergo metabolic transformation. Since 1964, however, investigations into the metabolism of anaesthetics in man and animals have been performed demonstrating that these compounds do in fact undergo biotransformations in the same manner as other xenobiotics. A number of reviews on the subject have been published (98-104).

An investigation into the interaction of four volatile anaesthetic agents with drug metabolizing enzymes of the

liver is reported in this thesis. The compounds studied are all halogenated ethyl ethers (Figure 5) and, except for 2,2,2-trifluoroethyl ethyl ether (TFEE), have been synthesized commercially and widely used as volatile anaesthetics. Fluroxene has recently been removed from the market but is still in clinical use to a limited extent.

Investigations of the metabolism of fluroxene, methoxyflurane and enflurane *in vivo* and *in vitro* have led to the identification of metabolites which indicate that these compounds generally undergo reactions involving ether cleavage (O-dealkylation) and dehalogenation (104).

Figure 5. Structures of anaesthetic agents

$CF_3CH_2OCH{=}CH_2$	Fluroxene
$CF_3CH_2OCH_2CH_3$	2,2,2-Trifluoroethyl ethyl ether (TFEE)
$CHCl_2CF_2OCH_3$	Methoxyflurane
$CHClFCF_2OCHF_2$	Enflurane

a. Metabolism of fluroxene and 2,2,2-trifluoroethyl ethyl ether

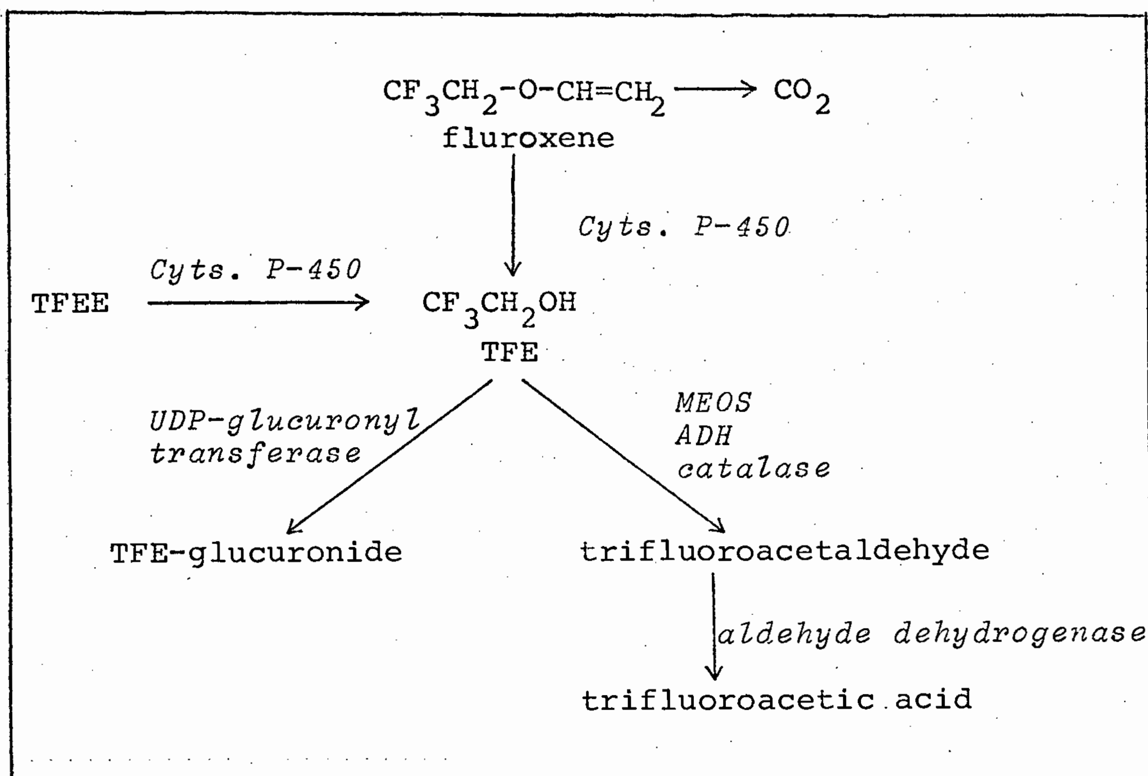
During its use from 1953 as an anaesthetic, fluroxene acquired a reputation for safe clinical usage in man with no evidence of organ toxicity (105,106). Recent laboratory investigations

have shown however, that fluroxene anaesthesia of animals may result in liver damage or death under certain circumstances (107,108,109); in addition, reports of fluroxene toxicity in man have also appeared (110-113).

The hepatotoxicity of fluroxene has been related to its metabolic transformation rather than to fluroxene itself. Factors known to affect drug metabolizing enzymes affect the toxicity of fluroxene, *e.g.* induction of drug metabolizing enzymes by phenobarbital potentiates the toxic effects of the anaesthetic while treatment of animals with agents that destroy drug metabolizing enzymes decrease the toxic effects (107,114,115,116). Blake *et al*. (12) identified trifluoroethanol glucuronide and trifluoroacetic acid as urinary metabolites of fluroxene in dogs and mice and subsequently postulated that the toxicity of fluroxene arises from its metabolic conversion to trifluoroethanol (117). Treatment of animals with trifluoroethanol itself results in toxic symptoms identical to those that develop after fluroxene anaesthesia (117). The possibility that the vinyl moiety of fluroxene is responsible for the toxic effects of the anaesthetic was dismissed as a result of investigations in our laboratory demonstrating that TFEE anaesthesia of rats is also lethal under certain conditions (116). The toxicity of both fluroxene and TFEE is therefore considered to arise from metabolism of the trifluoroethyl moiety common to both agents.

A scheme for the postulated pathways of biotransformation of fluroxene and TFEE is shown in Figure 6 (102).

Figure 6. Postulated pathways of metabolism of fluroxene and TFEE.



It has been postulated that cytochromes P-450 are responsible for the first step in the metabolism of the anaesthetic. The vinyl carbon atoms (C^{14}) of fluroxene are recovered only as $C^{14}O_2$ in mice and dogs after fluroxene anaesthesia (12). Defluorination of fluroxene does not appear to occur (118) and this observation is in keeping with the generally accepted concept of the stability of the carbon-fluorine bond in the trifluoromethyl group (119).

Possible pathways for metabolism of the primary metabolite trifluoroethanol include conjugation with glucuronic acid

catalysed by UDP-glucuronyl transferase and oxidation to trifluoroacetaldehyde (120).

Investigations into the mechanism of fluroxene toxicity have led to the proposal that further metabolism of trifluoroethanol is required before toxic symptoms develop (117,120). Various compounds known to inhibit the oxidation of alcohols have been shown to protect animals against the toxicity of fluroxene and trifluoroethanol (Table 2). Production of trifluoroacetaldehyde therefore has been suggested as the likely cause of the toxic effects arising after administration of fluroxene or trifluoroethanol to animals (125). As a result of studies with enzyme inhibitors _in vivo_, alcohol dehydrogenase and catalase have been implicated in the metabolism of trifluoroethanol; the most important pathway from trifluoroethanol to trifluoroacetaldehyde is postulated to be mediated by alcohol dehydrogenase (120). No conclusions regarding the activity of one particular enzyme in the metabolism of trifluoroethanol can validly be drawn from these studies because the agents used have been shown to inhibit microsomal drug metabolizing enzymes in addition to the specific enzymes usually held to be inhibited (Table 2).

The relative lack of toxicity of fluroxene in man compared to animal species has been ascribed to differences in the pathways of metabolism of the anaesthetic (125,131). The major metabolite in the urine of man after fluroxene anaesthesia is trifluoroacetate with free and conjugated

Table 2. Modification of the toxicity of fluroxene and 2,2,2-trifluoroethanol by compounds postulated to alter metabolism of these agents

Compound	Modification of toxicity[a]		Action of modifying compound
	Fluroxene	TFE	
Allopurinol		↓(117) ↑(121)	Xanthine oxidase inhibitor (121); also inhibits hepatic microsomal drug metabolism?
3-Amino-1,2,4-triazole	↓(120)	↓(117)	Catalase inhibitor; also inhibits hepatic microsomal drug metabolism(122)
Disulphiram	↓(120)	↓(117)	Acetaldehyde oxidase inhibitor; also inhibits hepatic microsomal drug metabolism (123,124)
Ethanol	↓(125)	↓(117, 121)	Competes with TFE for alcohol dehydrogenase, catalase or MEOS?
Isoniazid		↓(121)	Carbonyl reagent binding TFA[b] (121); also inhibits hepatic microsomal drug metabolism (126)
Phenobarbital		↓(127)	Increases hepatic drug metabolizing activity (65); also increases activities of aldehyde dehydrogenase (128,129)& UDP-glucuronyl transferase (77)
Pyrazole	↓(120)	↓(121)	Alcohol dehydrogenase inhibitor; also inhibits hepatic microsomal drug metabolism (130)

[a] Symbols: toxicity increased, ↑; toxicity decreased, ↓. References quoted in brackets.

[b] Abbreviation: trifluoroacetaldehyde, TFA.

trifluoroethanol occurring as minor metabolites (132); in animals, trifluoroethanol glucuronide is the major urinary metabolite (12). Results of investigations into the metabolism of fluroxene in man have been included in Table 3 for comparison with methoxyflurane and enflurane.

b. Metabolism of methoxyflurane and enflurane

Soon after the introduction of methoxyflurane as a volatile anaesthetic agent in 1960 (133), several reports linked the use of this drug to a renal syndrome known as 'high output renal failure' (134). It has now been established that this syndrome is due to high levels of inorganic fluoride that result from metabolism of methoxyflurane (135,136). Another renal toxin identified as a metabolite of methoxyflurane in the sera and urine of man and animals anaesthetized with this agent is oxalic acid (137,138). Methoxyflurane, still in clinical use, is expected to be withdrawn from the market shortly.

Utilizing the basic methyl ethyl ether structure of methoxyflurane, researchers investigated modifications of the molecule which would result in an effective anaesthetic agent but possessing fewer of the undesirable properties of methoxyflurane. Enflurane has recently been introduced as an alternative anaesthetic agent to methoxyflurane (134, 139). Defluorination of enflurane does occur in animals and man but peak serum inorganic fluoride levels in man are rarely above 50 μM/l (140,141,142) whilst those observed after methoxyflurane are usually greater than 100 μM/l (143).

A summary of results of investigations into the metabolism of methoxyflurane (144) and enflurane (145) in man is shown in Table 3 which includes for comparative purposes the results of similar studies with fluroxene (132). A considerable percentage of the anaesthetic agent remains unaccounted for particularly in the cases of methoxyflurane and fluroxene (Table 3). It has been postulated that a portion of the unrecovered anaesthetic becomes permanently bound to the tissues (132,144,146) but further investigations are required in order to confirm this.

Methoxyflurane is metabolized to a much greater extent than enflurane in man (Table 3) and in animals(147,148,149). Confirmation of the greater susceptibility of methoxyflurane than of enflurane to metabolic transformation is obtained from the quantum chemical studies of Loew *et al.* (150); in these studies the feasibility of ether cleavage and dechlorination reactions in methoxyflurane, enflurane, and in their metabolites was considered on the basis of ease of insertion of active oxygen into C-H bonds.

Pathways for the metabolism of methoxyflurane have been proposed (Figure 7) on the basis of metabolites identified *in vivo* and *in vitro* (143,150). Parallel pathways have been proposed (Figure 8) for the metabolism of the structurally similar enflurane although much less is known about metabolites of this agent(147,150); inorganic fluoride ion and uncharacterized organic fluoride compounds are the only metabolites of enflurane identified as yet (145).

Table 3. Recovery of anaesthetic agents and their metabolites in man.

	Methoxyflurane[a]	Enflurane[b]	Fluroxene[c]
	% dose absorbed		
Anaesthetic exhaled unaltered	19	83	58
Non-volatile urinary metabolites:			
Total	43.8	2.4	10.6[d]
Inorganic fluoride[e]	7.7	0.5	-
Organic fluoride	29.0	1.9	-
Oxalic acid	7.1	-	-
Unrecovered anaesthetic	37	15	32

[a] From reference 144; dose absorbed 18 g.

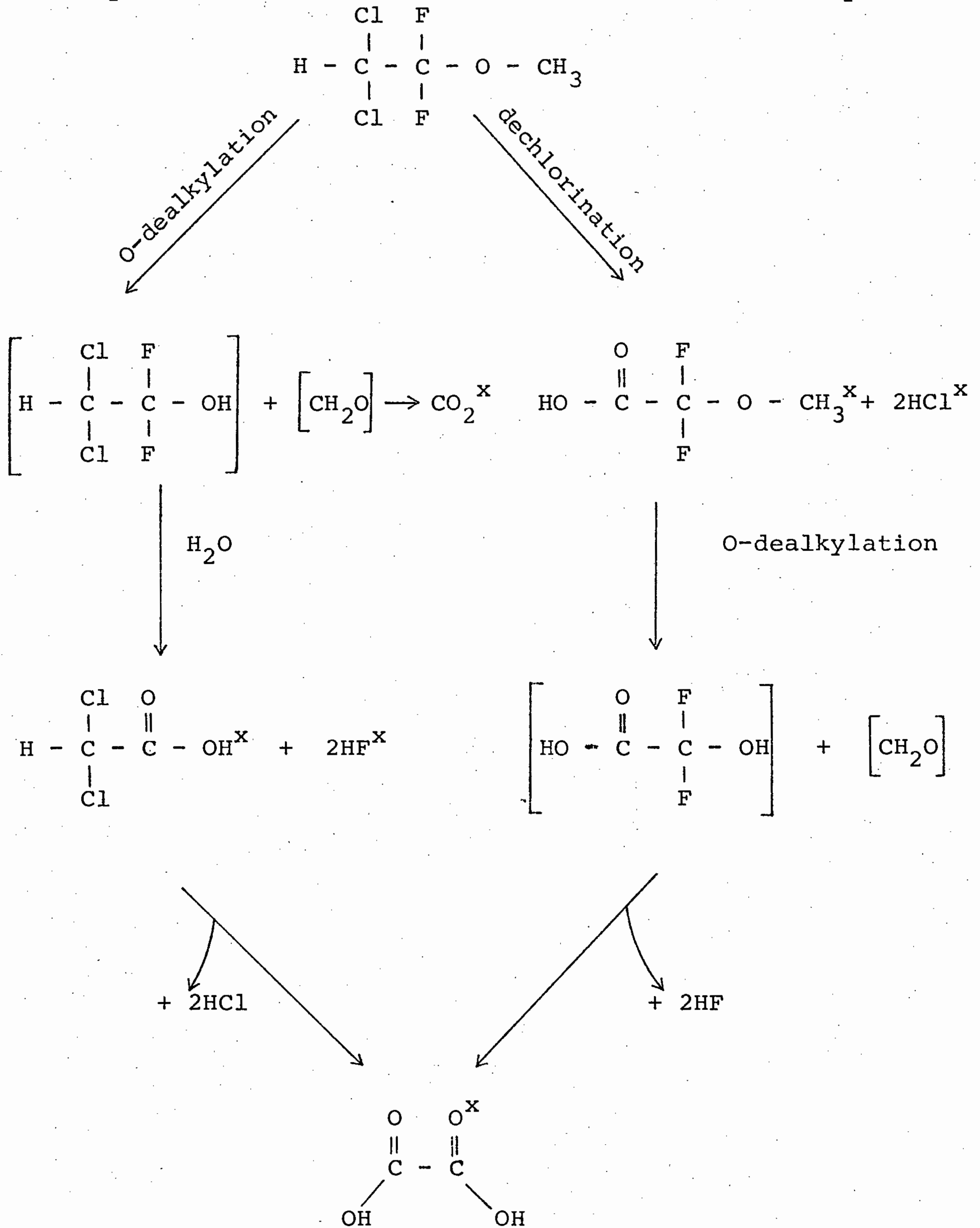
[b] From reference 145; dose absorbed 18 g.

[c] From reference 132; dose absorbed 32 g.

[d] Trifluoroacetic acid, 7.2%; free and conjugated trifluoroethanol, 0.6%; and unidentified organic compounds, 2.6%.

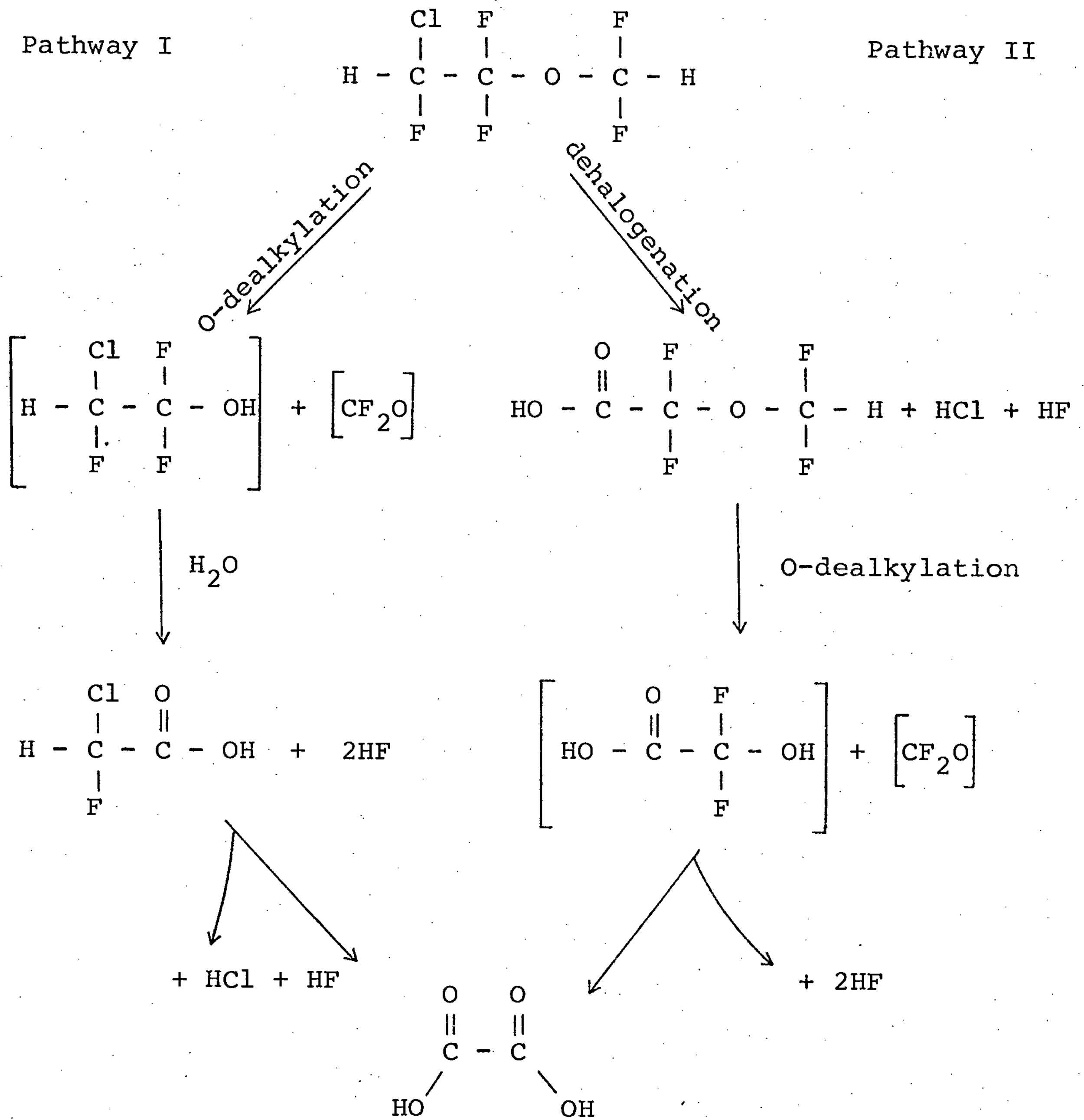
[e] Values should possibly be doubled in order to account for the free fluoride uptake from blood to bone (154).

Figure 7. Postulated pathways for metabolism of methoxyflurane (143, 150)



x Metabolites identified in refs. 143, 151 and 152.

Figure 8. Postulated pathways of metabolism of enflurane(150)



The metabolism of both methoxyflurane and enflurane is thought to be initiated by cytochromes P-450 with either an O-dealkylation or a dehalogenation reaction (Figures 7 and 8). Pathway I is believed to be favoured for methoxyflurane metabolism and pathway II for enflurane metabolism (150). The ether cleavage of either anaesthetic is postulated to result in an unstable 1,1-difluoroethyl alcohol which spontaneously decomposes to form a dihaloacetic acid with loss of 2 equivalents of inorganic fluoride. The compound dichloroacetic acid has in fact been identified as a metabolite of methoxyflurane (151). A dehalogenation reaction of the dihaloacetic acid possibly leads to the formation of oxalic acid.

In pathway II, the initial dehalogenation reaction is proposed to result in formation of a methoxyhaloacetic acid. After methoxyflurane anaesthesia, methoxydifluoroacetic acid has been detected (151) but the corresponding metabolite for enflurane has not. Ether cleavage of the methoxyhaloacetic acid intermediates leading to the formation of oxalic acid is then postulated to occur.

The finding of excessive amounts of urinary oxalic acid after methoxyflurane anaesthesia (143) is consistent with the proposed pathways for the metabolism of methoxyflurane. Oxalic acid has not been found in significant amounts, however, after enflurane anaesthesia (142) and Loew _et_ _al_. have concluded that it is unlikely that further metabolism of the products of the initial reactions postulated in the

pathways of Figure 8 occurs (150).

Induction of hepatic drug metabolizing enzymes in animals by phenobarbital pre-treatment leads to an increased rate of whole-body uptake of methoxyflurane, increases its metabolism to fluoride and oxalic acid and potentiates the nephrotoxic effects of the anaesthetic (138,148,153,154,155). Phenobarbital pretreatment, however, does not increase the defluorination of enflurane by rats *in vivo* (148) although it does increase fluoride deposition in bones of mice (but not of rats) after enflurane anaesthesia (154).

Metabolism of both methoxyflurane and enflurane has been demonstrated *in vitro*. The O-dealkylation, dechlorination and defluorination reactions of methoxyflurane have been shown to occur in rat hepatic microsomal preparations in the presence of NADPH (152,156,157); the defluorination of enflurane has been similarly demonstrated (157).

3. The interaction of volatile anaesthetic agents with hepatic drug metabolizing enzymes

The subject of this thesis was investigated in a three-fold manner:

a. The interaction of volatile anaesthetic agents with the cytochromes P-450 drug metabolizing system *in vitro*.

An investigation of the interactions of the volatile anaesthetic agents with the cytochromes P-450 drug metabolizing system *in vitro* was performed. The aim of this study was

to define such interactions and to discover the relationships between them. In addition, it was hoped that greater understanding of the role of different cytochromes P-450 in binding and metabolizing the anaesthetic agents would be reached, particularly with respect to those interactions which may result in effects deleterious to the animal.

b. The interaction of 2,2,2-trifluoroethanol with hepatic enzymes

The toxicity of fluroxene and TFEE is considered to arise as a consequence of the metabolism of these two agents to trifluoroethanol and further biotransformation of this metabolite appears to be a prerequisite for development of toxic symptoms. Investigation of the metabolism of trifluoroethanol itself is therefore relevant to a study of the metabolic fates of fluroxene and TFEE particularly with respect to the toxicity of these two agents. Accordingly, the interaction of trifluoroethanol with enzymes postulated to be responsible for its metabolism was investigated *in vitro*. The enzymes with which this study was undertaken include microsomal cytochromes P-450, alcohol dehydrogenase, catalase and microsomal UDP-glucuronyl transferase.

c. The effect of volatile anaesthetic agents on hepatic drug metabolizing enzymes *in vivo*

The inducibility by xenobiotics of hepatic drug metabolizing enzymes, in particular the cytochromes P-450 system, UDP-glucuronyl transferase and glutathione-S-transferases suggests the possibility that treatment of animals with

volatile anaesthetic agents may affect the concentrations and/or activities of these enzymes *in vivo*. Conflicting reports have appeared in the literature on the ability of volatile anaesthetic agents to induce the cytochromes P-450 system (See Tables 36 & 37, DISCUSSION). We have therefore conducted an exhaustive study into the effects of methoxyflurane and enflurane on the cytochromes P-450 system and also on other hepatic drug metabolizing enzymes. The aim of the study was not only to assess the inducing (or activating) properties of the anaesthetic agents but also to discover whether any anaesthetic-mediated destruction or inhibition of hepatic drug metabolizing enzymes occurs *in vivo*.

II. EXPERIMENTAL PROCEDURE

A. MATERIALS

1. Pretreatment of animals

Sodium phenobarbital (PB) and 3-methylcholanthrene (MC) were obtained from Maybaker, S.A. and Eastman-Kodak, respectively. 3,4-benzpyrene (BP) was from Sigma Chemical Co., and 2-allyl-2-*iso*propylacetamide (AIA) was a generous gift of Hoffman-La Roche, Nutley, New Jersey.

2. Anaesthetic agents

Fluroxene (2,2,2-trifluoroethyl vinyl ether) was supplied by Ohio Medical Products, Madison, Wisconsin. 2,2,2-Trifluoroethyl ethyl ether (TFEE) was prepared by hydrogenation of fluroxene at 30 lb/in^2 hydrogen using 5 g activated palladium on carbon (Merck) catalyst per 250 ml fluroxene. Hydrogenation was continued until samples of reaction mixture no longer exhibited the absorbance at 1600 cm^{-1} characteristic of carbon-carbon double bonds in the infrared spectrum. The TFEE was refluxed over sodium for at least 24 hr and was subsequently purified by fractional distillation. The fraction boiling between 50.0^o and 50.4^o was collected and stored in the dark under an atmosphere of nitrogen. The TFEE was found to be free of peroxides when tested with 5% aqueous KI (w/v) before use. Methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) and enflurane (2-chloro-1,1,2-trifluoroethyl difluoromethyl ether) were obtained from Abbott Laboratories S.A.

3. Assays on microsomal suspensions

NADH, NADPH, NADP , cytochrome *c* (horse heart, Type II), glucose-6-phosphate dehydrogenase, and enzymes for the coupled assay for glucuronidation were purchased from Miles Laboratories S.A.. Glucose-6-phosphate was obtained from Koch-Light Laboratories and uridine 5'-diphosphoglucuronic acid (ammonium salt) was from Sigma Chemical Co. Cylinders of carbon monoxide, nitrogen and oxygen were purchased from Afrox Ltd., S.A.

4. Inhibitors

SKF 525A (2-diethylaminoethyl-2,2-diphenyl valerate) was a generous gift from Smith Kline and French Laboratories, Isando, Transvaal, and metyrapone (2-methyl-1,2-bis[3'-pyridyl]1-propanone) was obtained from Ciba-Geigy Ltd.

5. Gas Liquid Chromatography

Di-*iso*-decylphthalate was supplied by Applied Science Laboratories Inc. and Chromosorb P (acid-washed) by Johns-Manville, Denver, Colorado.

6. Other assays

2,2,2-Trifluoroethanol (TFE) from Merck was of the highest purity available. NAD , alcohol dehydrogenase (horse liver), catalase (beef liver) and xanthine oxidase (buttermilk) were supplied by Miles Laboratories S.A. Xanthine and glutathione were from Sigma Chemical Co.

B. METHODS

1. Treatment of animals

Male Wistar rats weighing 160 - 220 g were used for all experiments. Animals were allowed free access to Epol Laboratory Chow (protein min. 20%, fat 2.5%, fibre max. 6%, calcium 1.4%, phosphorus 0.7%) and water unless otherwise specified, and were bedded on shredded paper. The temperature of the animal room was maintained at 22-24° and lighting was controlled to give 12 hr continuous light per day.

Induction of drug metabolizing enzymes was by intraperitoneal injection of sodium phenobarbital (80 mg/kg/day in 0.9% sterile saline), 3-methylcholanthrene (40 mg/kg/day in corn oil) or 3,4-benzpyrene (40 mg/kg/day in corn oil) for three consecutive days. Animals treated with AIA in addition to the three day regimen of induction were injected subcutaneously in the loose skin of the neck with 200 mg AIA/kg (20 mg/ml in 0.9% sterile saline) 1 hr prior to sacrifice. Animals were fasted overnight after the final injection as were also uninduced control rats and sacrificed by cervical fracture the following morning.

Animals were anaesthetized with methoxyflurane and enflurane for investigations of the effects of these agents on drug metabolizing enzymes *in vivo*. Anaesthesia was performed at sub-anaesthetic concentrations (0.1 MAC) for 6 hr on 4, 8, 12 or 16 days and at anaesthetic concentrations

(1.0 MAC) for 3 hr on 1, 2, 3 or 4 days.

The percentage concentrations (v/v) of methoxyflurane and enflurane used were as follows: methoxyflurane 0.02% (0.1 MAC) and 0.2% (1.0 MAC); enflurane 0.2% (0.1 MAC) and 2.0% (1.0 MAC). The animals were anaesthetized in groups of twelve or less in a perspex anaesthetic chamber (30 x 30 x 60 cm). Animals were placed on a fenestrated floor raised 8 cm from the base of the chamber, the space below the floor being filled with standard anaesthetic sodalime. The anaesthetic, vapourised by a 5 l/min stream of oxygen through a Cyprane temperature flow compensated Pentec or Enfluratek vapouriser, was passed as a continuous stream into the anaesthetic chamber through an inlet port sited at one corner beneath the fenestrated floor and exhausted through a port sited at the diagonally opposite upper corner. Concentrations of anaesthetic agents in gas samples taken from the exhaust port of the anaesthetic chamber were determined by gas-liquid chromatography on a Pye Unicam GCV chromatograph with a 10% di-*iso*-decylphthalate on chromosorb P column. The column temperature was 60° and 40° for methoxyflurane and enflurane determinations respectively. Unanaesthetized control animals were exposed to oxygen alone. Animals were fasted overnight after the final anaesthesia and sacrificed the following morning.

2. Preparation of microsomes

Livers were excised from animals immediately after death

and all subsequent procedures were performed at 4°. For investigations of effects of anaesthetic agents on drug metabolizing enzymes *in vivo* and production of fluoride from methoxyflurane and enflurane, microsomes were prepared by differential high speed centrifugation (4). Livers were homogenised in 3.0 vols 0.15 M KCl - 0.02 M Tris/HCl, pH 7.4. Cell debris, nuclei and mitochondria were spun down at 9000 g for 15 min in a Beckman J-21B centrifuge. The post-mitochondrial supernatant was spun at 105,000 g for 1 hr in a Beckman Model L ultracentrifuge. The resultant microsomal pellet was washed by resuspension in 0.15 M KCl - 0.02 M Tris-HCl, pH 7.4 and further centrifugation at 105,000 g for 45 min. The final microsomal pellet was resuspended to the required protein concentration in 0.02 M Tris-HCl, pH 7.4. In one experiment in which kidney microsomes were required their preparation was performed as described above.

Microsomes for use in all other studies were prepared by gel filtration according to the method of Tangen *et al.* (5). Livers were homogenized in 2.5 vols 0.25 M sucrose and the homogenate centrifuged at 9000 g for 15 min. The supernatant was layered on a Sepharose 2B column (20 cm x 7 cm) and microsomes eluted with 0.15 M KCl - 0.02 M Tris-HCl, pH 7.4, and diluted to the required protein concentration with 0.02 M Tris-HCl, pH 7.4. It should be noted that no significant differences in levels or activities of the drug metabolizing enzymes exist between microsomal suspensions

prepared by high speed centrifugation and those prepared by gel filtration (5).

3. Assays performed on microsomal preparations

All spectrophotometry was performed in Unicam SP1800 recording spectrophotometers unless otherwise stated. For recording spectra of turbid suspensions the cell holder adjacent to the photomultiplier was used with cuvettes positioned so that the light path was through their frosted faces. Unless otherwise specified the buffer used in the assays was 0.02 M Tris-HCl, pH 7.4.

a. Protein

Microsomal protein concentration was determined by the method of Lowry et al. (158) as modified by Chaykin (159) using bovine serum albumin as standard. Final absorbances were measured at 600 nm in a Gilford spectrophotometer.

b. Difference spectra

Suspensions of hepatic microsomes at 2.0 mg protein/ml were divided equally between two 1 cm path length cuvettes. The anaesthetic agent or trifluoroethanol was introduced below the surface of the microsomal suspension in the sample cuvette by means of a Hamilton syringe. The cuvette was then stoppered and vortex mixed for 5, 15 or 30 sec in order to disperse and solubilize the added trifluoroethanol, methoxyflurane and enflurane, or fluroxene and TFEE respectively. The magnitude of the resultant difference spectrum was measured as the difference in absorbance between the peak at about 390 nm and the trough at

about 420 nm and was corrected for the absorbance differences in the baseline at these wavelengths. In all cases maximum extents of difference spectra are reported as absorbance per 2.0 mg microsomal protein. Vortex mixing of the microsomal suspension without added anaesthetic agent did not produce a difference spectrum. In any one determination of the dissociation constant, K_s, sequential additions of anaesthetic agent or trifluoroethanol were made to the same sample cuvette. All difference spectra were recorded at room temperature.

c. NADPH oxidation

The rates of hepatic microsomal NADPH oxidation were determined in the presence of anaesthetic agent or trifluoroethanol. Equal quantities of microsomal suspension (2.0 mg protein/ml) were divided between two 1 cm path length cuvettes. Varying quantities of anaesthetic agent or trifluoroethanol were introduced into the sample cuvette as described for difference spectra determinations except when concentrations lower than 0.1 mM methoxyflurane or enflurane were required in which case the anaesthetic agent was added to 12 - 24 ml microsomal suspension and 3.0 ml of the mixture taken for assay. The reaction mixtures were equilibrated at 27° when monitoring fluroxene, TFEE and trifluoroethanol metabolism, and at 30° when monitoring methoxyflurane and enflurane metabolism. The reaction was initiated by addition of 50 - 100 µl NADPH solution to give a final NADPH concentration of 0.12 - 0.24 mM. NADPH oxidation was monitored spectrally at 340 nm using a

thermostatted cell compartment adjacent to the photomultiplier of the spectrophotometer. The extinction coefficient for NADPH at 340 nm is 6.2 $mM^{-1}cm^{-1}$. Correction was made for non-cytochrome P-450 NADPH oxidation by the method of Stripp _et al_. (29) in which the background rate of NADPH oxidation is determined in the presence of anaesthetic agent or trifluoroethanol in an atmosphere of carbon monoxide-oxygen (80:20; v/v). In these experiments, carbon monoxide and oxygen flow was controlled by Matheson Gas Products model 7600 flowmeters and the mixture of gases was allowed to bubble through the microsomal suspension for 30 sec at 20 ml/min prior to addition of substrate and cofactor.

d. _Incubation system for production of 2,2,2-trifluoroethanol or fluoride from anaesthetic agents_

The reaction mixture for the production of trifluoroethanol from fluroxene or TFEE and the production of fluoride from methoxyflurane or enflurane comprised microsomal suspension (2.0 mg protein/ml), NADPH generating system (0.4 mM NADP, 7.5 mM glucose-6-phosphate, 0.5 U/ml glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, 1 mM nicotinamide and 0.2 mM EDTA) and varying amounts of anaesthetic agent all in 0.02 M Tris-HCl, pH 7.4. The airspace above the incubation mixture constituted about 15% - 20% of the total volume (4.0 ml) of the glass reaction vial, which was stoppered with a serum cap. The reaction was initiated by addition of the anaesthetic agent and NADPH generating system, and mixed by vortex for 30 sec. The vials were clamped in a

horizontal position and incubated with shaking (100 cycles/min) at 30° in a Gallenkamp thermostatted water bath for a period of time (Table 4) during which the reaction was approximately linear. Two glass beads (diameter 5 mm) were included in the vial when production of trifluoroethanol was studied. In experiments under conditions of no shaking, vials were allowed to stand upright in the water bath. In one experiment, the microsomal incubation mixture was rendered anaerobic by inclusion of electron transport particles prepared from beef heart by Method 2 of Crane *et al*. (160), as described elsewhere (161).

Table 4. Duration of incubation of microsomal reaction mixtures in investigations of production of trifluoroethanol or fluoride from anaesthetic agents.

Anaesthetic agent	Pretreatment of animal source of microsomes	Duration of incubation (min)
Fluroxene	None All types induction	10
TFEE	None All types induction	10
Methoxyflurane	None PB induction	15
	MC induction	20
Enflurane	PB induction	20

e. Production of 2,2,2-trifluoroethanol

A Beckman GC-M chromatograph with a flame ionization detector connected to a Pye-Unicam DP 88 computing integrator was used for quantitative analysis of trifluoroethanol. The column used consisted of 3 m x 6 mm copper packed with 10% di-*iso*-decylphthalate on Chromosorb P (acid-washed) (132). Column temperature was 70° and injection port and detector compartment were at 200°. The carrier gas was nitrogen. Trifluoroethanol standards in water gave a linear response on the integrator over a range of concentration including and well above those produced in the microsomal system (Figure 9). A 5 µl sample of standard or of the microsomal mixture was injected without further treatment onto the column.

f. Production of fluoride

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 90-01-00) and connected to a Radiometer pH meter. After incubation of methoxyflurane and enflurane with microsomes and NADPH generating system as described in Section IIB.3.d., the reaction was terminated by addition of 0.1 ml 3 M sodium acetate buffer, pH 4.9, to 3.0 ml microsomal mixture. The mixture was allowed to equilibrate to room temperature and the free fluoride concentration determined. Known concentrations of sodium fluoride in 0.1 M sodium acetate buffer pH 4.9 were used to establish a standard curve each day; fluoride solutions in microsomes in 0.1 M sodium acetate, pH 4.9 gave identical mV readings

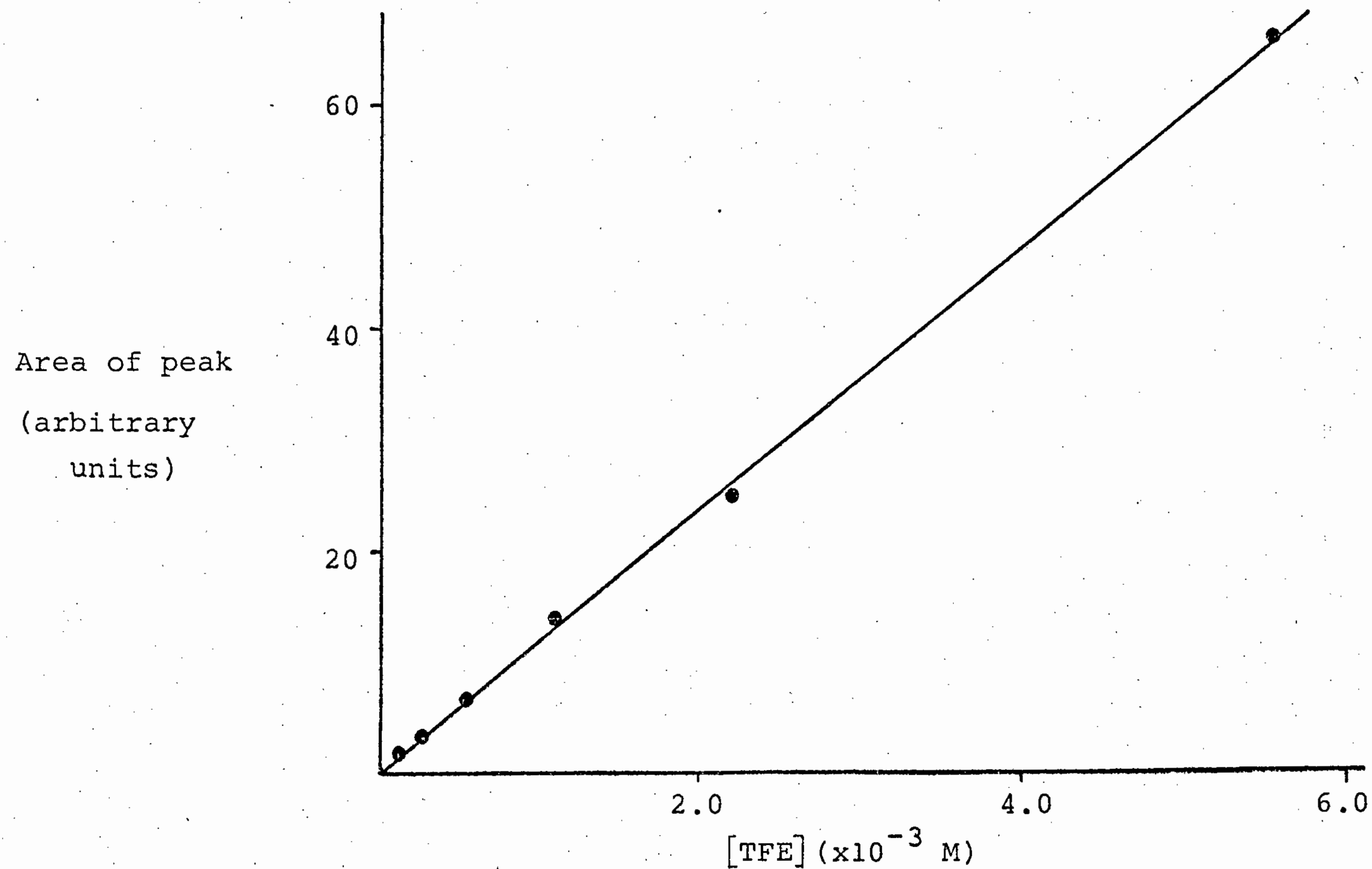


Figure 9. Effect of concentration of 2,2,2-trifluoroethanol on area of the peak obtained after gas-liquid chromatography of 2,2,2-trifluoroethanol solutions in water.

to those of fluoride in buffer alone. In order to determine acid-labile fluoride formed during incubation, the microsomal mixture was brought to pH 1.5 with 10 µl conc. H_2SO_4 after measurement of free fluoride activity and allowed to incubate at room temperature for 85 hours, or as indicated. The mixture was returned to pH 5.0 with 55 - 60 µl 6.0 M NaOH and the fluoride activity was measured. Solutions of fluoride in 0.1 M sodium acetate buffer, pH 4.9 to which 10 µl conc. H_2SO_4 and 55 - 60 µl 6.0 M NaOH were added were used as standards to determine fluoride concentrations after acid treatment.

In reports herein of production of fluoride from methoxyflurane and enflurane, 'free fluoride' refers to the free fluoride ion released during incubation of anaesthetic agents with microsomes and NADPH generating system; 'total fluoride' refers to the fluoride ion measureable after acid treatment of the reaction mixture, and 'acid-labile fluoride' refers to the difference between the total and free fluoride ion concentrations.

g. Cytochromes P-450

Microsomal cytochromes P-450 content was determined by the carbon monoxide difference spectral method of Omura and Sato (162). Microsomal protein was always 2.0 mg/ml. Carbon monoxide was bubbled at 10 ml/min for 30 sec. Fresh sodium dithionite was used to reduce the contents of reference and sample cuvettes and the extinction co-efficient used for the difference between the absorbances at 450 nm and 490 nm

($\Delta A_{450-490}$) was 91 $mM^{-1}cm^{-1}$. It should be noted that total type P-450 cytochromes are measured by this method, and that these values include the contributions of both cytochromes P-450 and P-448 to the total.

h. Cytochrome $\underline{b}_5$

Microsomal cytochrome $\underline{b}_5$ was determined by the NADH difference spectral method of Omura and Sato (162). Microsomal protein was always 2.0 mg/ml and the concentration of NADH was 0.2 mM. The extinction co-efficient used was 185 $mM^{-1}cm^{-1}$ for the difference in absorbance between 409 nm and 424 nm.

i. NADPH-cytochrome c reductase

Microsomal NADPH-cytochrome c reductase was determined with microsomal protein at 0.08 mg/ml as described by Omura and Takesue (163). 1 unit of enzyme is that which reduces 1 μmol cytochrome c /min. The extinction co-efficient difference between reduced and oxidized cytochrome c is 21.1 $mM^{-1}cm^{-1}$.

j. p-Nitroanisole O-demethylation

Microsomal O-demethylation of p-nitroanisole was determined by the method of Netter (164). Microsomal protein was 1.3 mg/ml, p-nitroanisole at 0.7 mM unless otherwise indicated, and the concentrations of components of NADPH generating system were as described in Section IIB.3.d. The reaction was monitored at 420 nm in cuvettes at 25 - 27° for 3 - 4 min and initial reaction rates calculated. The extinction co-efficient for p-nitrophenol at 420 nm, pH 7.4, is 7.3 $mM^{-1}cm^{-1}$ (165).

k. <u>3,4-Benzpyrene hydroxylation</u>

Hydroxylation of 3,4-benzpyrene to 3-hydroxybenzpyrene was determined spectrophotometrically by the method of Prough *et al*. (166). 3,4-Benzpyrene (80 μM) and NADH (200 μM) were present in both sample and reference cuvettes containing microsomal suspension at 2.0 mg protein/ml. The reaction was initiated by addition of NADPH (100 μM final concentration) and the increase in absorbance at 428 nm relative to 454 nm was determined at 25 - 27°. The extinction co-efficient of 3-hydroxybenzpyrene at 428 nm is 13.2 $mM^{-1}cm^{-1}$ (166).

l. <u>Glucose-6-phosphatase</u>

The activity of glucose-6-phosphatase was determined by measuring the μmol inorganic phosphate (P_i) released/min/mg microsomal protein during incubation of glucose-6-phosphate (20 mM) with microsomes (1.3 mg protein/ml)(167). The reaction was allowed to proceed for 20 min at 30° and was terminated by addition of 10% (w/v) trichloroacetic acid (TCA). Control samples containing microsomal suspension and glucose-6-phosphate as above were treated with TCA before the start of incubation. The mixture was then centrifuged at 1800 g in an MSE mistral 6L centrifuge for 5 min and the supernatant assayed for phosphorus by the method of King (168). Blanks and standards for the phosphorus assay were water and potassium dihydrogen phosphate respectively. The absorbance at 660 nm was read in a Gilford spectrophotometer.

m. <u>Glucuronidation of trifluoroethanol</u>

Monitoring concentration of free trifluoroethanol. Concentrations of free trifluoroethanol were monitored by gas-liquid chromatography (see Section IIB.3.e.) before and after incubation of microsomes with UDP-glucuronic acid and trifluoroethanol. The reaction mixture comprised microsomal suspension (0.8 - 0.9 mg protein/ml) 1.5 mM UDP-glucuronic acid, 1 mM saccharo-1,4-lactone (inhibitor of β-glucuronidase), and 5 mM $MgCl_2$, in a total volume of 1.0 ml 0.075 M Tris-HCl, pH 7.3. Reaction blanks contained all components except UDP-glucuronic acid. The reaction mixture, in glass vials stoppered with serum caps, was equilibrated at 37° and the reaction initiated by addition of microsomal suspension. The vials were incubated with shaking (80 - 100 cycles/min) in a Gallenkamp water bath at 37°. In some experiments inhibitors of pyrophosphatase, 0.4 mM sodium pyrophosphate or 1.5 mM ATP, were included in the reaction mixture to prevent decreased concentrations of UDP-glucuronic acid (169). Activation of UDP-glucuronyl transferase was attempted by inclusion in the incubation mixture of 0.04% Triton X-100 (81), by pre-incubation of microsomal suspensions at 37° for varied lengths of time (82), or by storage of microsomal pellets at -15° (81) for 12 days. In addition animals were pre-treated with phenobarbital or 3-methylcholanthrene, both compounds having been shown to increase the activities of UDP-glucuronyl transferase towards certain aglycones (77).

Coupled assay for UDP-glucuronyl transferase activity. Attempts to determine glucuronidation of trifluoroethanol were made by

means of the assay of Mulder and van Doorn (170) based on measurement of UDP production from UDP-glucuronic acid during the glucuronidation reaction; UDP production is coupled to the conversion of NADH into NAD through pyruvate kinase and lactate dehydrogenase. The reaction mixture contained 0.16 mg microsomal protein/ml and 0.04% Triton X-100 in addition to the other components described by Mulder and van Doorn (170). The reaction was allowed to proceed in 1 cm cuvettes at 30° and the absorbance at 340 nm monitored continuously for 5 min against a reference cuvette containing only microsomal suspension. Blanks in which either UDP-glucuronic acid or substrate was omitted resulted in a slow decrease in absorbance at 340 nm of about 0.002 and 0.005 O.D. units/min, respectively.

n. Glucuronidation of *p*-nitrophenol

The glucuronidation of *p*-nitrophenol was measured essentially as by Lueders and Kuff (81). The reaction mixture comprised microsomal suspension at 0.8 mg protein/ml, 0.4 mM *p*-nitrophenol, 1.5 mM UDP-glucuronic acid and 5 mM $MgCl_2$ in a total volume of 1.0 ml 0.075 M Tris-HCl, pH 7.3. Reaction blanks contained all components except UDP-glucuronic acid. Activation of UDP-glucuronyl transferase was achieved by inclusion of 0.04% Triton X-100 in the incubation mixture; this was considered necessary in view of the finding that induction of UDP-glucuronyl transferase can be better observed *in vitro* under conditions of activation of microsomes (171). The reaction was initiated by addition of microsomal suspension and reaction vials were shaken

(80 - 100 cycles/min) for 10 min at 30° in a Gallenkamp water bath. The reaction was terminated by addition of 2.0 ml 96% ethanol, and the mixture was then spun at 1800 *g* in an MSE Mistral 6L centrifuge. An aliquot of supernatant was added to 0.1 M NaOH and the absorbance at 400 nm read in a Gilford spectrophotometer. The extinction co-efficient for *p*-nitrophenol at alkaline pH is 20.8 $mM^{-1}cm^{-1}$ (81). The rate of disappearance of *p*-nitrophenol was calculated and taken as the rate of glucuronidation (172).

o. Destruction of cytochromes P-450

The incubation mixture for determination of the destruction of microsomal cytochromes P-450 by anaesthetic agents was the same as described in Section IIB.3.d. The concentration of cytochromes P-450 was determined as described in Section IIB.3.g. after incubation at 30° of microsomal suspension with anaesthetic agent and NADPH generating system against a reference cuvette containing microsomes similarly incubated but in the absence of anaesthetic agent and NADPH generating system. Cytochromes P-450 concentrations determined at zero incubation time in the presence of anaesthetic agent only were taken as control cytochromes P-450 levels. The addition of NADPH generating system to these control samples did not affect the $\Delta A_{450-490}$ at zero time.

4. Other assays

a. Alcohol dehydrogenase

Horse liver alcohol dehydrogenase oxidation of ethanol and trifluoroethanol was determined by monitoring the formation of NADH as described by Blake *et al*. (117). The soluble fraction from rat liver (post-microsomal supernatant) was assayed for the ability to oxidize ethanol and trifluoroethanol in a similar manner except that the concentrations of NAD and alcohol were 5 mM and about 0.5 M respectively.

b. Catalase

The decomposition of hydrogen peroxide by catalase was assayed spectrophotometrically at 240 nm by a modification of the method of Bergmeyer (173). The reaction mixture comprised 2.0 - 4.0 U catalase and 12 - 24 mM hydrogen peroxide in 0.05 M sodium phosphate buffer pH 8.0. The extinction co-efficient for hydrogen peroxide at 240 nm is 0.043 $mM^{-1}cm^{-1}$.

c. Xanthine oxidase

The oxidation of xanthine to uric acid by xanthine oxidase was assayed spectrophotometrically by a modification of the method of Kalckar (174). The reaction mixture comprised 0.05 mM xanthine and 0.005 U/ml xanthine oxidase in 0.05 M sodium phosphate buffer pH 8.0. The increase in absorbance at 290 nm was monitored, the extinction co-efficient for uric acid at this wavelength being 12.3 $mM^{-1}cm^{-1}$.

d. Peroxidation of alcohols by catalase

The incubation mixture for peroxidatic oxidation of trifluoroethanol and other alcohols comprised 0.005 U/ml xanthine oxidase, 0.05 mM xanthine, 100 U catalase and alcohol at

concentrations as indicated, in 0.05 M phosphate buffer, pH 8.0. In some experiments, catalase or catalase plus alcohol were omitted. The reaction mixture was allowed to equilibrate at 30° and the reaction was initiated by addition of xanthine oxidase. Rates of oxygen uptake were monitored by a Clark type oxygen electrode.

e. Glutathione-S-transferase

Glutathione-S-transferase activity in the soluble fraction (post-microsomal supernatant) of rat liver was assayed spectrophotometrically using 1-chloro-2,4-dinitrobenzene as substrate according to the method of Habig *et al*. (90). The reaction mixture comprised soluble fraction (0.06 - 0.09 mg protein/ml), 1.33 mM 1-chloro-2,4-dinitrobenzene, 2.5 mM glutathione in 0.1 M potassium phosphate buffer, pH 6.5. The reaction was allowed to proceed at 25° in cuvettes, and the increase in absorbance at 340 nm was monitored continuously for 3 - 4 min. The extinction coefficient for conjugated product at 340 nm is 9.6 $mM^{-1}cm^{-1}$ (90).

f. Glutathione

Glutathione levels in samples of rat liver were assayed fluorometrically by the method of Cohn and Lyle (175). Glutathione was extracted from the liver sample by homogenization of the tissue with 30 μM EDTA following which protein was removed by metaphosphoric acid and subsequent centrifugation. The resultant solution of glutathione was reacted with O-phthalaldehyde at pH 8.3 yielding a

highly fluorescent product. Standard solutions of glutathione and blanks of water were similarly treated with O-phthalaldehyde. The fluorescence at 420 nm resulting from activation at 365 nm was measured in a Perkin-Elmer Fluorescence Spectrophotometer 203.

5. Calculations

Spectral dissociation constants (K_s), maximum extents of binding (ΔA_{max}), Michaelis-Menten constants (K_m) and maximum reaction rates (V_{max}), were usually obtained from Hanes plots of paired [S] and ΔA, or [S] and v, values. [S] represents the concentration of anaesthetic agent or trifluoroethanol; ΔA represents the difference in absorbance between peak and trough observed in difference spectral studies; and v represents the initial rate of the reaction under investigation.

In all cases the data was also plotted by means of Eadie-Hofstee plots which are more sensitive to deviations from linearity in cases where simple Michaelis-Menten kinetics are not obeyed (176). In addition, use was made of Lineweaver-Burk or Dixon plots (177) to illustrate inhibition of certain reactions by trifluoroethanol.

A computer programme was utilized which allowed calculation, on the basis of linear regression analysis, of kinetic constants from the data by any of the linear transformations of the Michaelis-Menten equation.

Student's *t*-test was utilized to determine statistical significance (See ABBREVIATIONS, page viii).

6. Structure of experimental approach to investigations of the interactions of volatile anaesthetic agents with the cytochromes P-450 drug metabolizing system *in vitro*

For these studies, animals were induced for elevated levels of different cytochromes P-450 by pretreatment with 3,4-benzpyrene, 3-methylcholanthrene, 3-methylcholanthrene with 2-allyl-2-*iso*propylacetamide (MC/AIA), or phenobarbital. Investigations using hepatic microsomes isolated from uninduced and induced animals followed the scheme outlined in Table 5.

Table 5. Scheme for investigation of interactions of anaesthetic agents with the cytochromes P-450 drug metabolizing system *in vitro*

Investigation	Anaesthetic agent and induction															
	Fluroxene					TFEE					Methoxyflurane			Enflurane		
	None	BP	MC	MC/ AIA	PB	None	BP	MC	MC/ AIA	PB	None	MC	PB	None	MC	PB
Difference spectra	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
NADPH oxidation	✓	✓	✓	–	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓
TFE production	✓	✓	✓[a]	✓	✓[a]	✓	✓	✓	✓	✓[a]	–	–	–	–	–	–
F^- production	–	–	–	–	–	–	–	–	–	–	✓[a]	✓[a]	✓[a]	✓[a]	✓[a]	✓[a]
Cyts. P-450 destruction	✓	–	✓	–	✓	–	–	–	–	✓	–	–	✓	–	–	✓

[a] In addition, the effects of inhibitors of cytochromes P-450 were studied.

III. RESULTS

A. THE INTERACTION OF VOLATILE ANAESTHETIC AGENTS WITH THE CYTOCHROMES P-450 DRUG METABOLIZING SYSTEM *IN VITRO*

1. Binding of anaesthetic agents to cytochromes P-450

Fluroxene, TFEE, methoxyflurane and enflurane bind to cytochromes P-450 of hepatic microsomes isolated from uninduced and induced rats resulting in the appearance of a type I difference spectrum (e.g. Figure 10). The extent of binding of each of the anaesthetic agents increases with increasing anaesthetic concentration until saturating concentrations are reached (e.g. Figure 11). Hanes plots of the data from difference spectral studies are linear for each of the anaesthetic agents for all types of induction and allow calculation of spectral dissociation constants, K_s (55) and maximum extents of binding, ΔA_{max} (e.g. Figure 12).

The effects of inducers of cytochromes P-450 on the K_s and ΔA_{max} values for the four anaesthetic agents are presented in Tables 6, 7, 8 and 9. In these tables, averages and mean deviations for determinations of K_s and ΔA_{max} in three separate microsomal preparations are reported. Induction of either cytochrome P-448 with polycyclic hydrocarbons or of cytochrome P-450 with phenobarbital does not generally affect the K_s values for the binding of the anaesthetic agents to microsomal cytochromes P-450 relative to the respective K_s values obtained in uninduced microsomes (Tables 6-9). The value of K_s is changed only for methoxyflurane after phenobarbital induction.

The maximum extent of binding of each of the anaesthetic agents to hepatic microsomal cytochromes P-450 is identical in uninduced and polycyclic hydrocarbon induced microsomes. Induction of cytochrome P-450 by phenobarbital, however, increases the maximum extent of binding of each of the anaesthetic agents roughly in proportion to the increase in cytochromes P-450 levels.

Table 6. Binding of fluroxene to cytochromes P-450 of hepatic microsomes from variously induced animals

Induction	[Cyts.P-450] (nmol/mg microsomal protein)	K_s (mM)	ΔA_{max}
None	1.1 ± 0.1	0.93 ± 0.20	0.06 ± 0.01
BP	2.3 ± 0.1	0.90 ± 0.18	0.06 ± 0.02
MC	2.1 ± 0.2	0.94 ± 0.25	0.06 ± 0.02
PB	2.7 ± 0.2	0.95 ± 0.08	0.13 ± 0.03

Table 7. Binding of 2,2,2-trifluoroethyl ethyl ether to cytochromes P-450 of hepatic microsomes from variously induced animals

Induction	[Cyts.P-450] (nmol/mg microsomal protein)	K_s (mM)	ΔA_{max}
None	1.3 ± 0.1	1.5 ± 0.3	0.07 ± 0.01
BP	1.8 ± 0.1	1.7 ± 0.2	0.06 ± 0.01
MC	1.9 ± 0.2	2.5 ± 0.4	0.09 ± 0.02
PB	2.7 ± 0.2	1.8 ± 0.3	0.17 ± 0.02

Table 8. Binding of methoxyflurane to cytochromes P-450 of hepatic microsomes from variously induced animals

Induction	[Cyts.P-450] (nmol/mg microsomal protein)	K_s (mM)	ΔA_{max}
None	1.1 ± 0.1	0.48 ± 0.09	0.06 ± 0.01
MC	1.8 ± 0.1	0.62 ± 0.11	0.07 ± 0.01
PB	2.4 ± 0.3	0.29 ± 0.05●	0.16 ± 0.02

Table 9. Binding of enflurane to cytochromes P-450 of hepatic microsomes from variously induced animals

Induction	[Cyts.P-450] (nmol/mg microsomal protein)	K_s (mM)	ΔA_{max}
None	1.1 ± 0.1	0.49 ± 0.11	0.06 ± 0.01
MC	1.8 ± 0.1	0.50 ± 0.07	0.05 ± 0.01
PB	2.4 ± 0.3	0.52 ± 0.08	0.16 ± 0.03

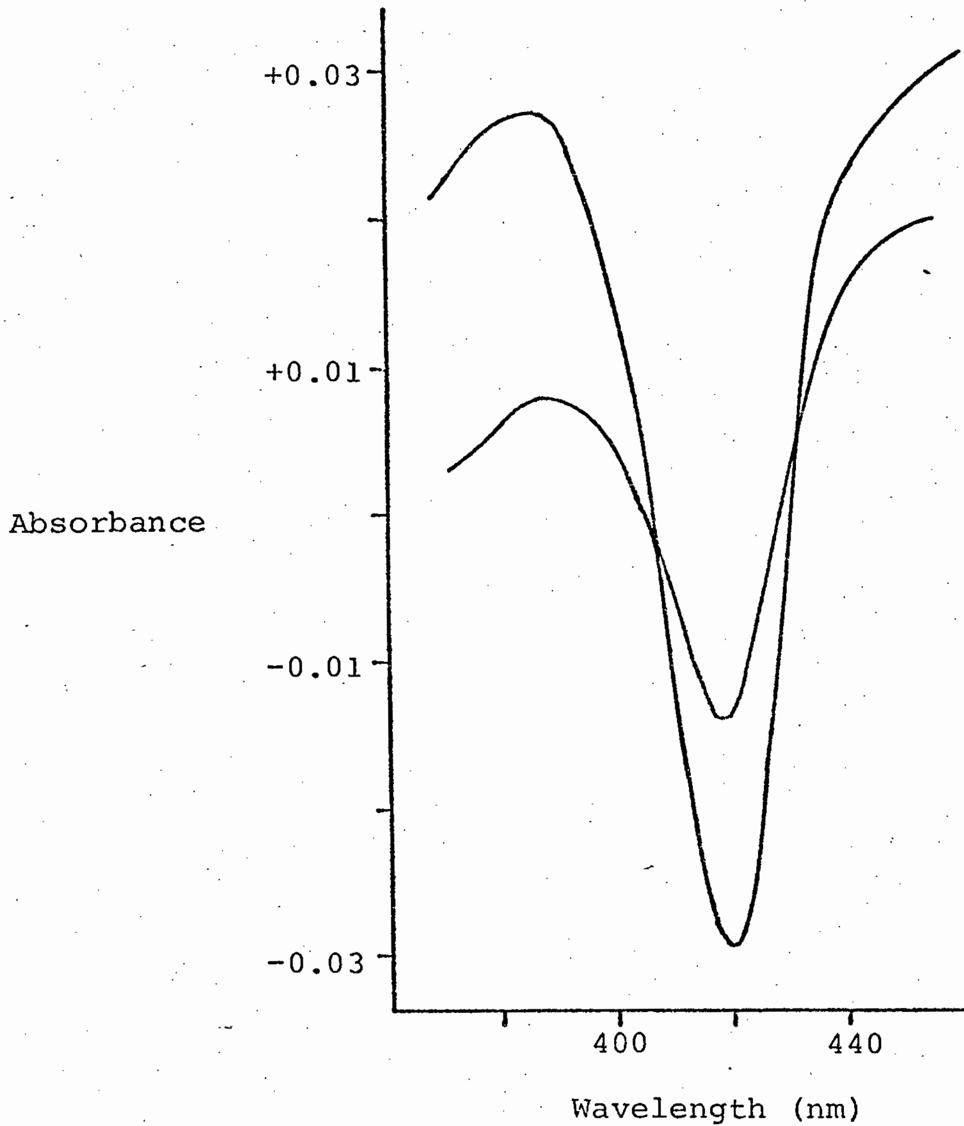


Figure 10. Type I difference spectral changes of enflurane with uninduced and phenobarbital induced hepatic microsomes. Enflurane, 0.27 mM; microsomal protein, 2.0 mg/ml in 0.02M Tris-HCl, pH 7.4; performed at 25°.

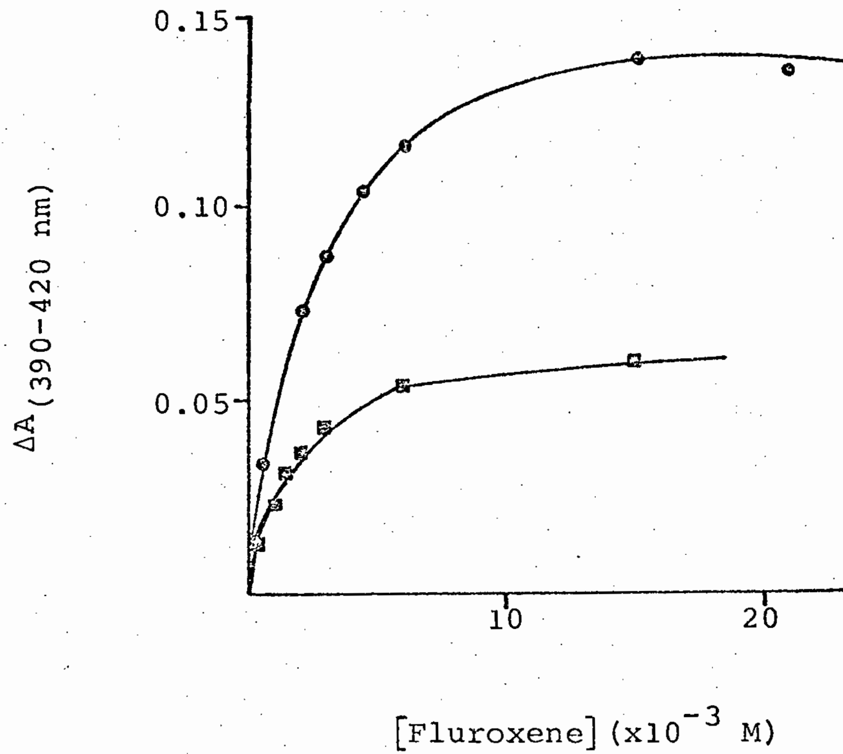


Figure 11. Effect of concentration of fluroxene on its difference spectra with hepatic microsomes from uninduced (■) and phenobarbital induced (●) animals. Microsomal protein, 2.0 mg/ml in 0.02 M Tris-HCl, pH 7.4; performed at 25°.

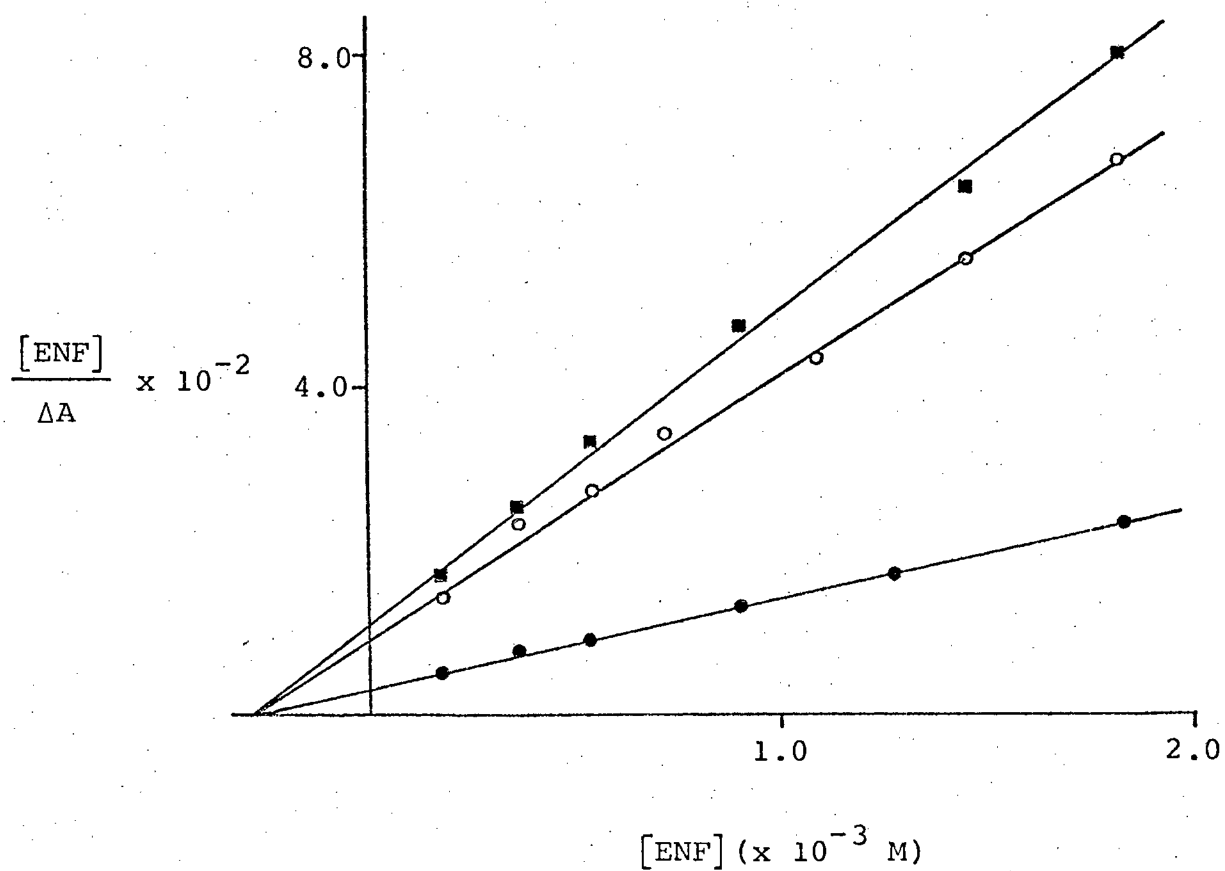


Figure 12. Hanes plots of difference spectra of enflurane with hepatic microsomes from uninduced (○), 3-methylcholanthrene induced (■) and phenobarbital induced (●) animals. Experimental conditions as for Figure 11.

2. Effects of anaesthetic agents on microsomal NADPH oxidation

Fluroxene, TFEE, methoxyflurane and enflurane stimulate carbon monoxide inhibitable NADPH oxidation by hepatic microsomes (*e.g.* Figure 13). The rate of NADPH oxidation by uninduced or induced microsomes in the presence of anaesthetic agent increases with increasing concentration of anaesthetic agent until saturating concentrations are reached. Hanes plots of the data from these studies permit calculation of Michaelis constants, K_m, and maximum rates of reaction, V_{max}, for the anaesthetic agents with microsomes from uninduced and induced animals.

Hanes plots are linear for NADPH oxidation by all types of microsomes in the presence of fluroxene, methoxyflurane and enflurane (*e.g.* Figure 14) but are linear only with phenobarbital induced microsomes in the presence of TFEE (Figure 15)*. The biphasic Hanes plots for NADPH oxidation by control or polycyclic hydrocarbon induced microsomes in the presence of TFEE allow calculation of two sets of K_m and V_{max} values.

The effects of induction of cytochromes P-450 on the K_m and V_{max} values for microsomal NADPH oxidation in the presence of the anaesthetic agents are shown in Tables 10, 11, 12 and 13 in which averages and mean deviations are reported for

* When linear Hanes plots were obtained for NADPH oxidation or metabolite production, concentrations of anaesthetic agents were investigated that were 10 to 20 times higher than the K_m values in each case in order to confirm the lack of biphasic character in the plots.

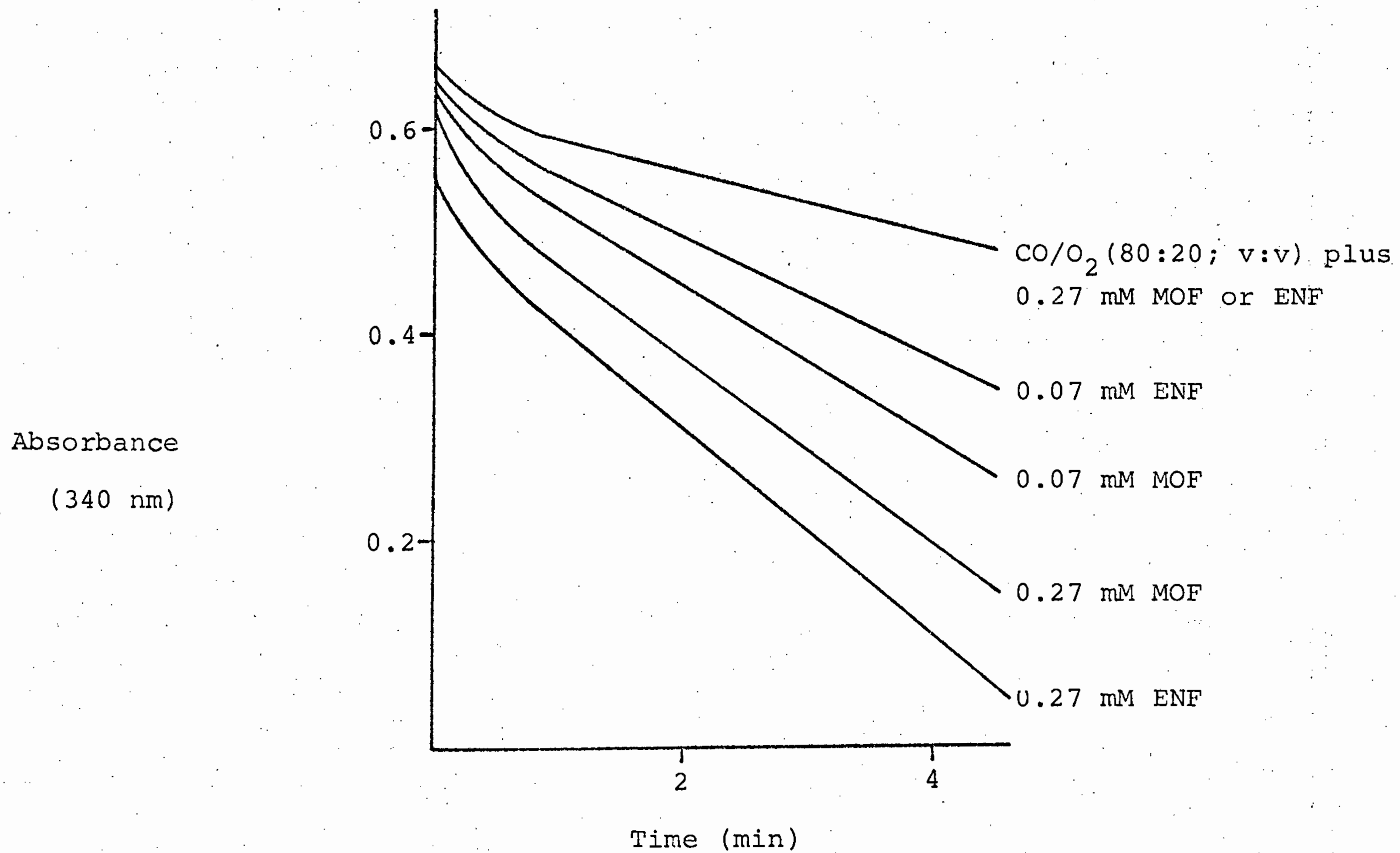


Figure 13. Oxidation of NADPH by phenobarbital induced hepatic microsomes in the presence of methoxyflurane and enflurane. NADPH, 0.24 mM; microsomal protein, 2.0 mg/ml in 0.02 M Tris-HCl, pH 7.4; reaction temperature, 30°C.

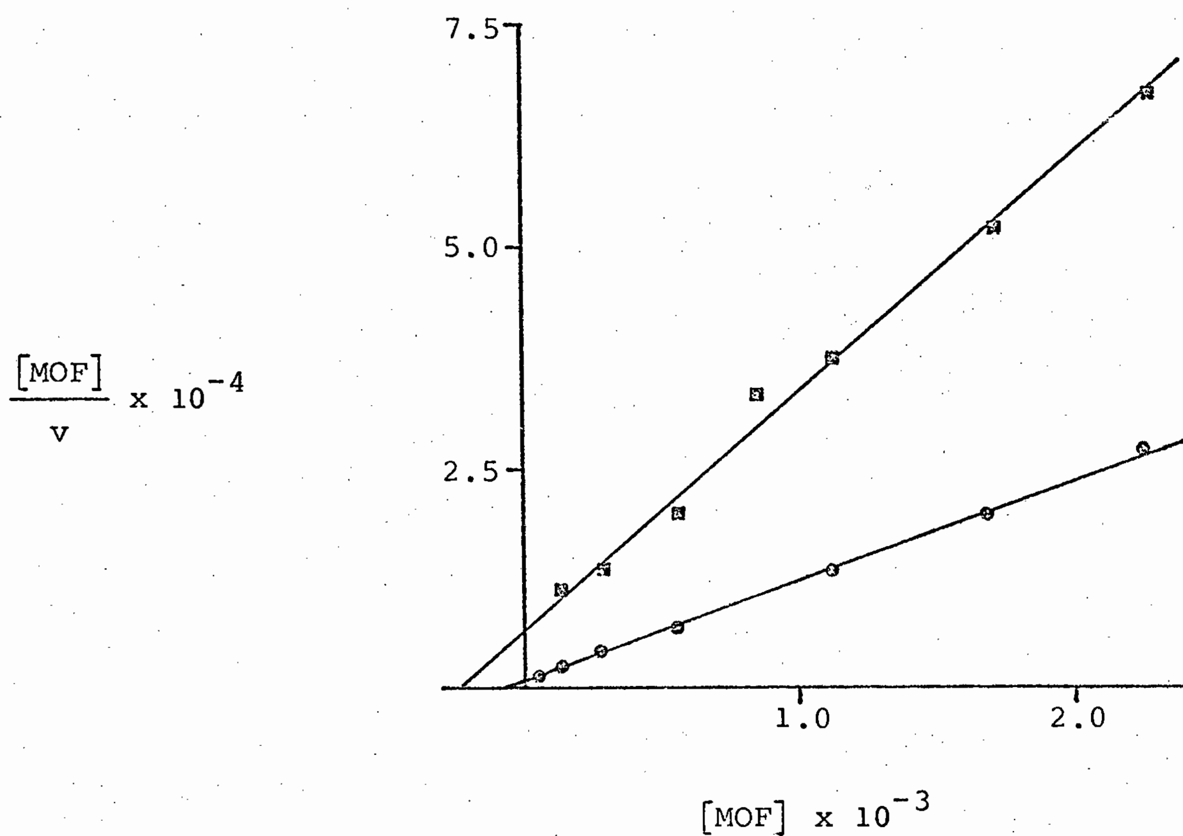


Figure 14. Hanes plots of NADPH oxidation by hepatic microsomes in the presence of methoxyflurane. 3-methylcholanthrene (■) and phenobarbital (●) induced microsomes; [MOF], molar; v, nmol NADPH/mg microsomal protein/min. Experimental conditions as for Figure 13.

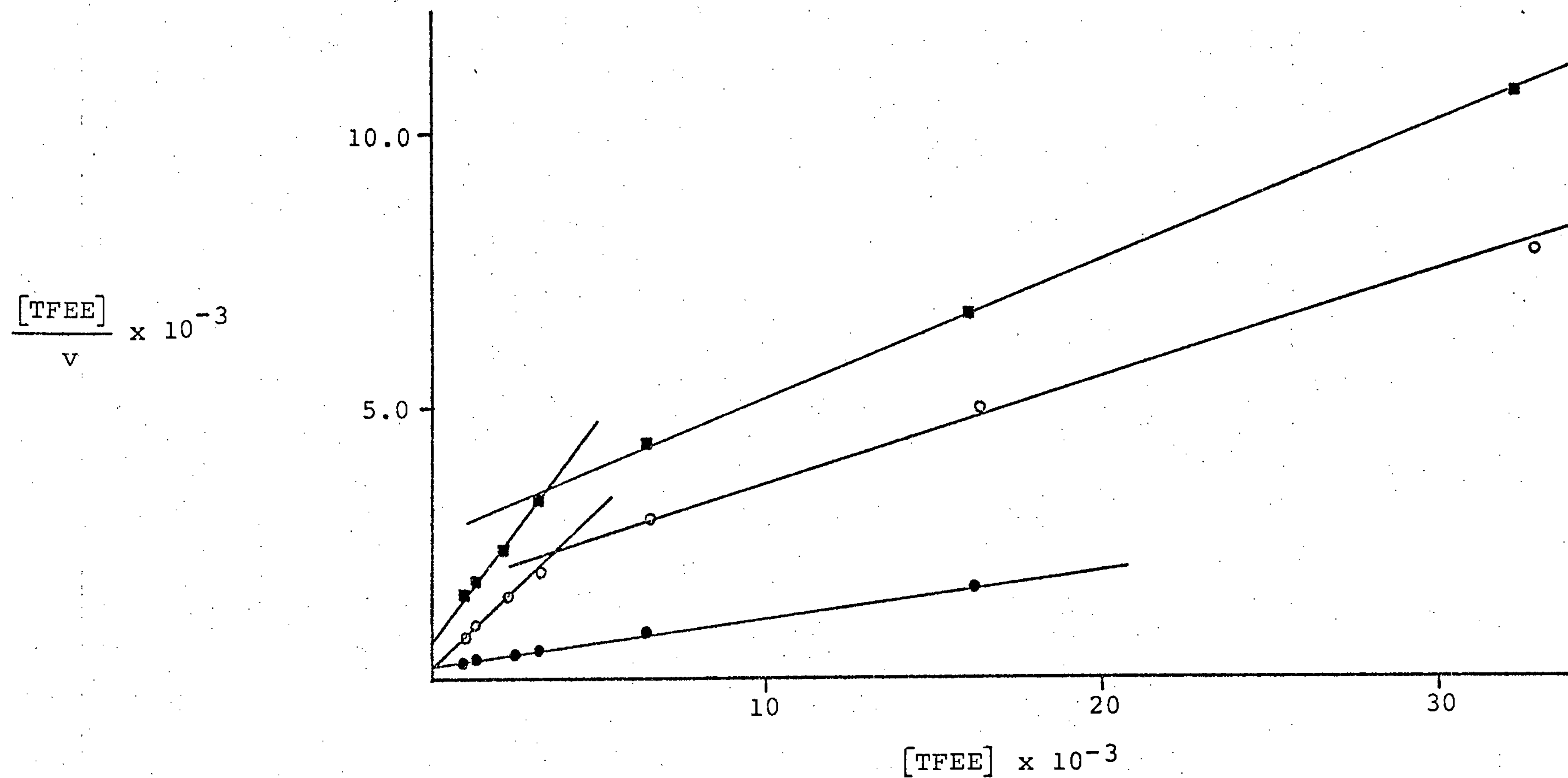


Figure 15. Hanes plots of NADPH oxidation by hepatic microsomes in the presence of 2,2,2-trifluoroethyl ethyl ether. Uninduced (o), 3-methylcholanthrene induced (■) and phenobarbital induced (•) microsomes; [TFEE], molar; v, nmol NADPH/mg microsomal protein/min. Experimental conditions as for Figure 13.

determinations of K_m and V_{max} with two or three separate preparations of hepatic microsomes.

Induction of cytochrome P-448 by polycyclic hydrocarbons or of cytochrome P-450 by phenobarbital does not significantly affect the K_m values for fluroxene or enflurane relative to the respective values in uninduced microsomes (Tables 10 and 13). In the case of TFEE the two K_m values observed each for uninduced, 3-methylcholanthrene induced and 3,4-benzpyrene induced microsomes are about 1.0 mM and 12.0 mM (Table 11). For methoxyflurane, phenobarbital but not 3-methylcholanthrene induction, affects the value of K_m (Table 12).

For each of the anaesthetic agents, the induction of cytochrome P-448 by polycyclic hydrocarbons does not significantly alter the V_{max} relative to that in uninduced microsomes. Induction of cytochrome P-450 by phenobarbital, however, elevates V_{max} three to five fold.

3. <u>The production of 2,2,2-trifluoroethanol from fluroxene or 2,2,2-trifluoroethyl ethyl ether by hepatic microsomes</u>

a. <u>Identification and quantitative assay of 2,2,2-trifluoroethanol</u>

2,2,2-trifluoroethanol was identified as a volatile product of the hepatic microsomal metabolism of fluroxene by gas-liquid chromatography in combination with mass spectrometry.*

* Identification of trifluoroethanol was performed by Dr L.S. Kaminsky at N.Y. State Department of Health, Division of Laboratories and Research, Albany, N.Y., U.S.A.

Table 10. Effect of induction of cytochromes P-450 on hepatic microsomal NADPH oxidation in the presence of fluroxene

Induction	Cyts. P-450 (nmol/mg microsomal protein)	K_m (mM)	V_{max} (nmol/mg microsomal protein/min)
None	1.1±0.1	0.76±0.40	4±1
BP	2.3±0.1	1.07±0.53	4±1
MC	2.1±0.2	0.95±0.15	3±1
PB	2.7±0.2	0.63±0.12	13±2

Table 11. Effect of induction of cytochromes P-450 on hepatic microsomal NADPH oxidation in the presence of 2,2,2-trifluoroethyl ethyl ether

Induction	Cyts. P-450 (nmol/mg microsomal protein)	K_m (mM)		V_{max} (nmol/mg microsomal protein/min)	
		K_m [a]	K_m [b]	V_{max} [a]	V_{max} [b]
None	1.3±0.1	0.6±0.2	11±1	2±0.5	6±1
BP	1.8±0.1	1.3±0.2	14±5	2±0.5	5±1
MC	1.9±0.1	0.9±0.3	11±2	1±0.5	4±1
PB	2.7±0.2	1.9±0.5	-	10±2	-

[a] Smaller K_m and corresponding V_{max}

[b] Larger K_m and corresponding V_{max}

Table 12. Effect of induction of cytochromes P-450 on hepatic microsomal NADPH oxidation in the presence of methoxyflurane

Induction	Cyts. P-450 (nmol/mg microsomal protein)	K_m (mM)	V_{max} (nmol/mg microsomal protein/min)
None	1.3±0.1	0.10±0.01	3±1
MC	1.9±0.2	0.25±0.06	4±1
PB	2.4±0.3	0.07±0.003	13±2

Table 13. Effect of induction of cytochromes P-450 on hepatic microsomal NADPH oxidation in the presence of enflurane

Induction	Cyts. P-450 (nmol/mg microsomal protein)	K_m (mM)	V_{max} (nmol/mg microsomal protein/min)
None	1.3±0.1	0.15±0.10	3±1
MC	1.9±0.2	0.35±0.10	4±1
PB	2.4±0.3	0.23±0.11	18±2

Gas-liquid chromatography (Section IIB.3.e.) of a reaction mixture after incubation of either fluroxene or TFEE with microsomal suspension and NADPH gives rise to a peak whose retention time is identical with that of a standard solution of trifluoroethanol.

Representative examples of gas-liquid chromatograms obtained during quantification of trifluoroethanol are shown in Figure 16. Fluroxene or TFEE is eluted with water within 3 to 4 min after injection of sample. An unidentified component is eluted at about 5 min. This peak is observed following incubation of microsomes and NADPH generating system in the presence or absence of fluroxene or TFEE.

b. Effect of experimental conditions on production of 2,2,2-trifluoroethanol from fluroxene

The effect of a variety of experimental conditions on the production of trifluoroethanol from fluroxene by phenobarbital induced hepatic microsomes was investigated and the results are presented in Table 14.

Increasing the microsomal protein concentration from 2.0 mg/ml to 10 mg/ml decreases by 75% the production of trifluoroethanol per mg microsomal protein; there is no difference in the K_m values determined at these two concentrations of microsomal protein. Under conditions of no shaking, production of trifluoroethanol decreases relative to shaken samples by 25% and 40% for 10 min and 30 min incubation periods, respectively. Prebubbling of the

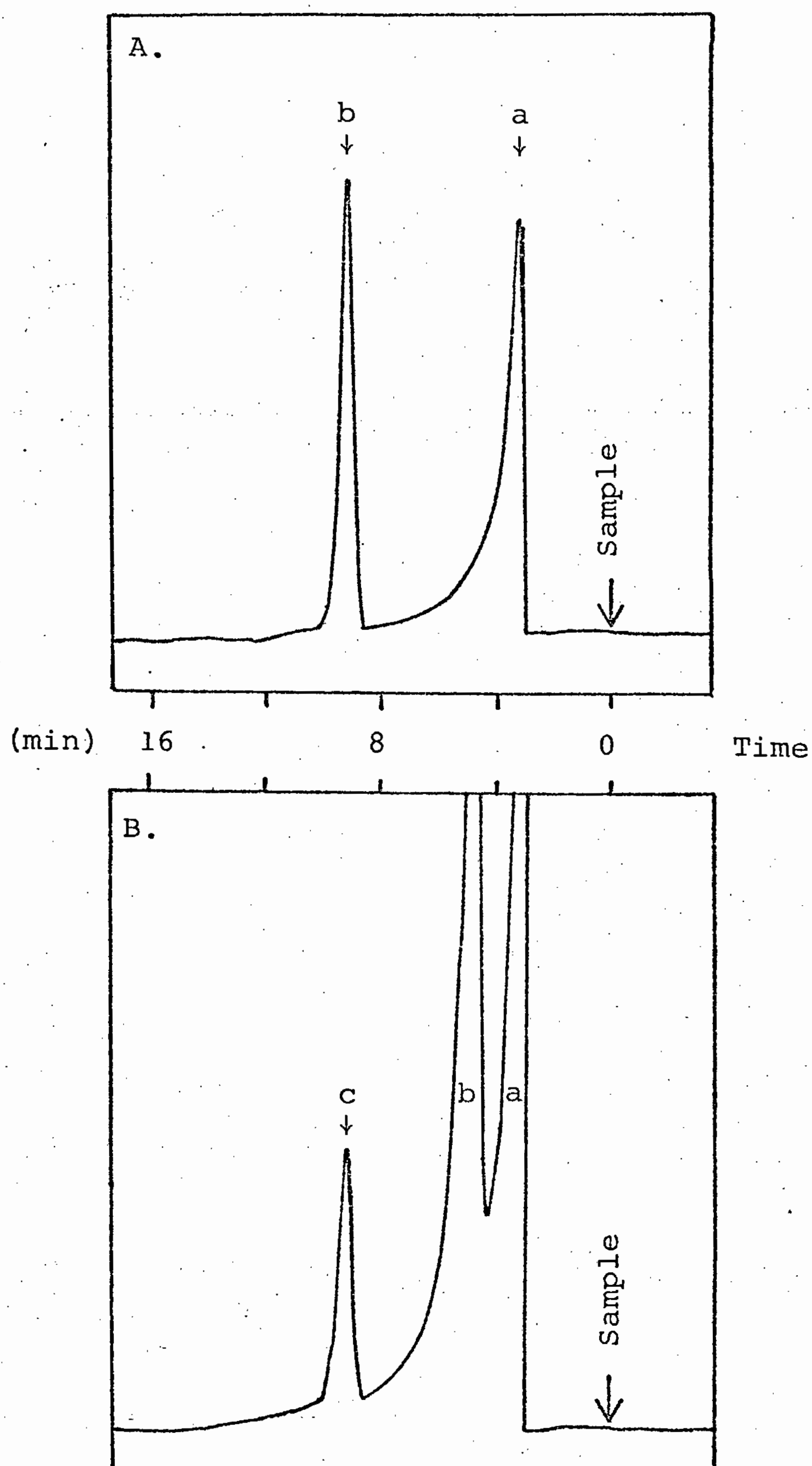


Figure 16. Gas-liquid chromatograms. A. Standard solution of 2,2,2-trifluoroethanol (0.7 mM) in water; a, water peak and b, trifluoroethanol peak. B. Sample of reaction mixture comprising phenobarbital induced microsomes (2.0 mg protein/ml), fluroxene (6.0 mM) and NADPH generating system after incubation at 30° for 10 min; a, water and fluroxene peak, b, unidentified peak and c, trifluoroethanol peak. Experimental details in METHODS.

Table 14. Effect of a variety of experimental conditions on the production of 2,2,2-trifluoroethanol from fluroxene by phenobarbital induced hepatic microsomes

Modification of experimental conditions[a]	Production of TFE[b] (nmol/mg microsomal protein)	
	10 min	30 min
None	112 ± 12	227 ± 17
Microsomal protein 10.0 mg/ml	27 ± 4	N.D.
No shaking	83 ± 9	123 ± 10
O_2 prebubbling[c]	101 ± 10	222 ± 20
Anaerobic; no shaking	<1.0	N.D.

a Unmodified experimental conditions were as described in Section IIB.3.d; fluroxene, 30 mM.

b Averages and mean deviations for determinations in triplicate with one microsomal preparation.

c Oxygen bubbled through microsomal suspension for 2 min at 10 ml/min.

microsomal suspension with oxygen has no effect on the rate of production of trifluoroethanol. Under completely anaerobic conditions, trifluoroethanol is not produced.

c. Effect of different electron and active oxygen donors on production of 2,2,2-trifluoroethanol from fluroxene

The effect of different electron and active oxygen donors on production of trifluoroethanol from fluroxene by phenobarbital induced hepatic microsomes is shown in Table 15. NADPH is the only effective electron donor for the microsomal production of trifluoroethanol; NADH supports the reaction but only to a limited extent. Periodate and hydrogen peroxide lead to non-enzymic degradation of fluroxene to trifluoroethanol.

d. Effect of time on the production of 2,2,2-trifluoroethanol

In order to determine a valid incubation period for kinetic studies of the process, production of trifluoroethanol from fluroxene and TFEE as a function of time was investigated in microsomes from phenobarbital induced animals. The results are illustrated in Figure 17. The production of trifluoroethanol from either fluroxene or TFEE is linear with time for 10 min after which the rate rapidly declines.

e. Effect of inhibitors on the production of 2,2,2-trifluoroethanol

The effect of inhibitors of cytochromes P-450 on production of trifluoroethanol from fluroxene and TFEE was investigated in microsomes from phenobarbital induced animals and the

Table 15. Effect of different electron and active oxygen donors on the production of 2,2,2-trifluoroethanol from fluroxene by phenobarbital induced hepatic microsomes[a]

Electron or active oxygen donor	Production of TFE (nmol/mg microsomal protein/10 min)
None	< 1.0
10 mM Sodium dithionite	< 1.0
10 mM Sodium ascorbate	< 1.0
NADPH[b]	109 ± 8
0.6 mM NADH	16 ± 2
7.5 mM Sodium Periodate	173
7.5 mM Sodium Periodate[c]	341
0.3% H_2O_2	69
0.3% H_2O_2[c]	921

[a] Incubation conditions as described in Section IIB.3.d.

[b] NADPH generating system.

[c] Buffer used in place of microsomes.

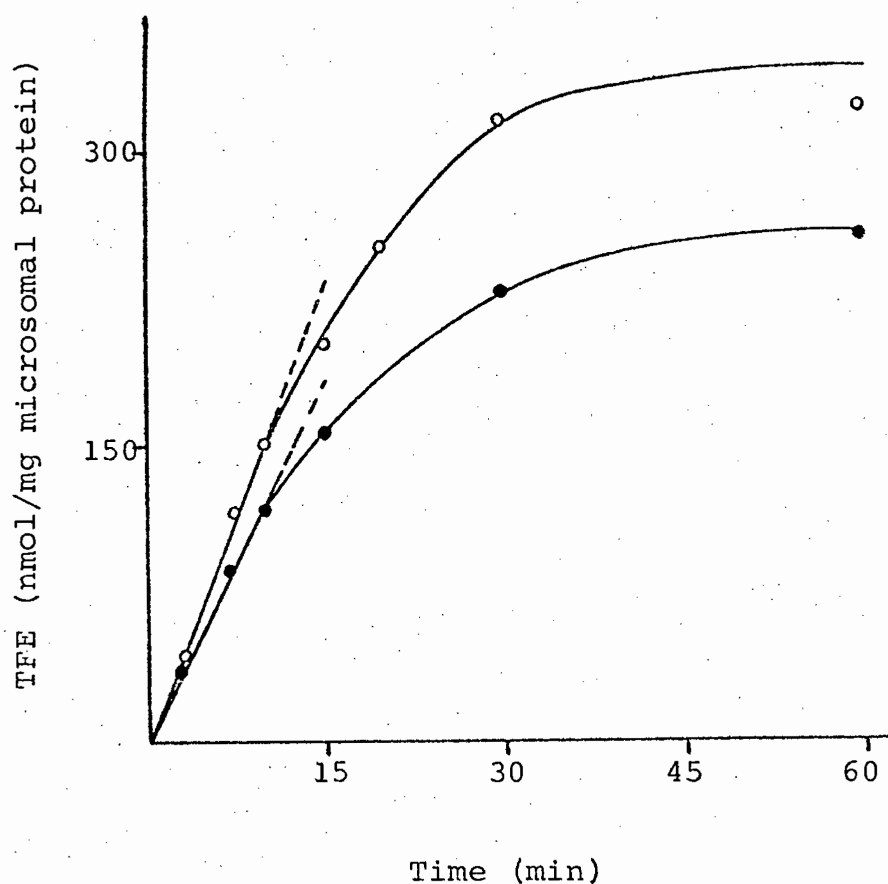


Figure 17. Effect of time on production of 2,2,2-trifluoroethanol from fluroxene (●) and 2,2,2-trifluoroethyl ethyl ether (O) by phenobarbital induced hepatic microsomes. Anaesthetic agent (5.0 mM), microsomal suspension (2.0 mg protein/ml) and NADPH generating system incubated together at 30° in 0.02 M Tris-HCl, pH 7.4.

results are presented in Table 16. At equimolar concentrations (50 μM) of the inhibitors, metyrapone is a more effective inhibitor of trifluoroethanol production than SKF 525A. Carbon monoxide inhibits the production of trifluoroethanol from fluroxene and TFEE by approximately 65%.

In 3-methylcholanthrene induced microsomes the extent of inhibition by metyrapone (50 μM) of trifluoroethanol production from fluroxene decreases by about 20% relative to that observed in phenobarbital induced microsomes although the extents of inhibition by SKF 525A (50 μM) are the same in both types of microsomes.

f. Effect of induction of cytochromes P-450 on production of 2,2,2-trifluoroethanol

Production of trifluoroethanol by microsomes as a function of fluroxene or TFEE concentration was investigated. The rate of microsomal production of trifluoroethanol increases with increasing concentration of anaesthetic agent until saturating concentrations are reached. Hanes plots of the data from these studies have been utilized to calculate K_m and V_{max} values. Hanes plots are linear for production of trifluoroethanol from fluroxene by all types of microsomes (Figures 18 and 19) and from TFEE by microsomes from 3-methylcholanthrene induced/AIA pretreated and phenobarbital induced animals only (Figures 20 and 21). Biphasic Hanes plots for production of trifluoroethanol from TFEE by microsomes from uninduced or polycyclic hydrocarbon induced

Table 16. Effect of inhibitors of cytochromes P-450 on the production of 2,2,2-trifluoroethanol from fluroxene and 2,2,2-trifluoroethyl ethyl ether by phenobarbital induced hepatic microsomes

Inhibitor	Production of TFE			
	from fluroxene[a]		from TFEE[a]	
	(nmol/mg microsomal protein/10 min)	(%)	(nmol/mg microsomal protein/10 min)	(%)
None	109 ± 8	100	145 ± 16	100
Metyrapone 50 μM	27 ± 4	25	18 ± 4	15
2.3 mM	18 ± 1	16	17 ± 4	12
SKF 525A 50 μM	73 ± 2	67	52 ± 6	36
330 μM	32 ± 1	29	26 ± 2	18
CO[b]	45 ± 1	41	46 ± 5	32

a Averages and mean deviations reported for determinations in triplicate on two separate microsomal preparations. Anaesthetic agents at 6.0 mM.

b Carbon monoxide bubbled through reaction mixture for 2 min at 10 ml/min prior to incubation.

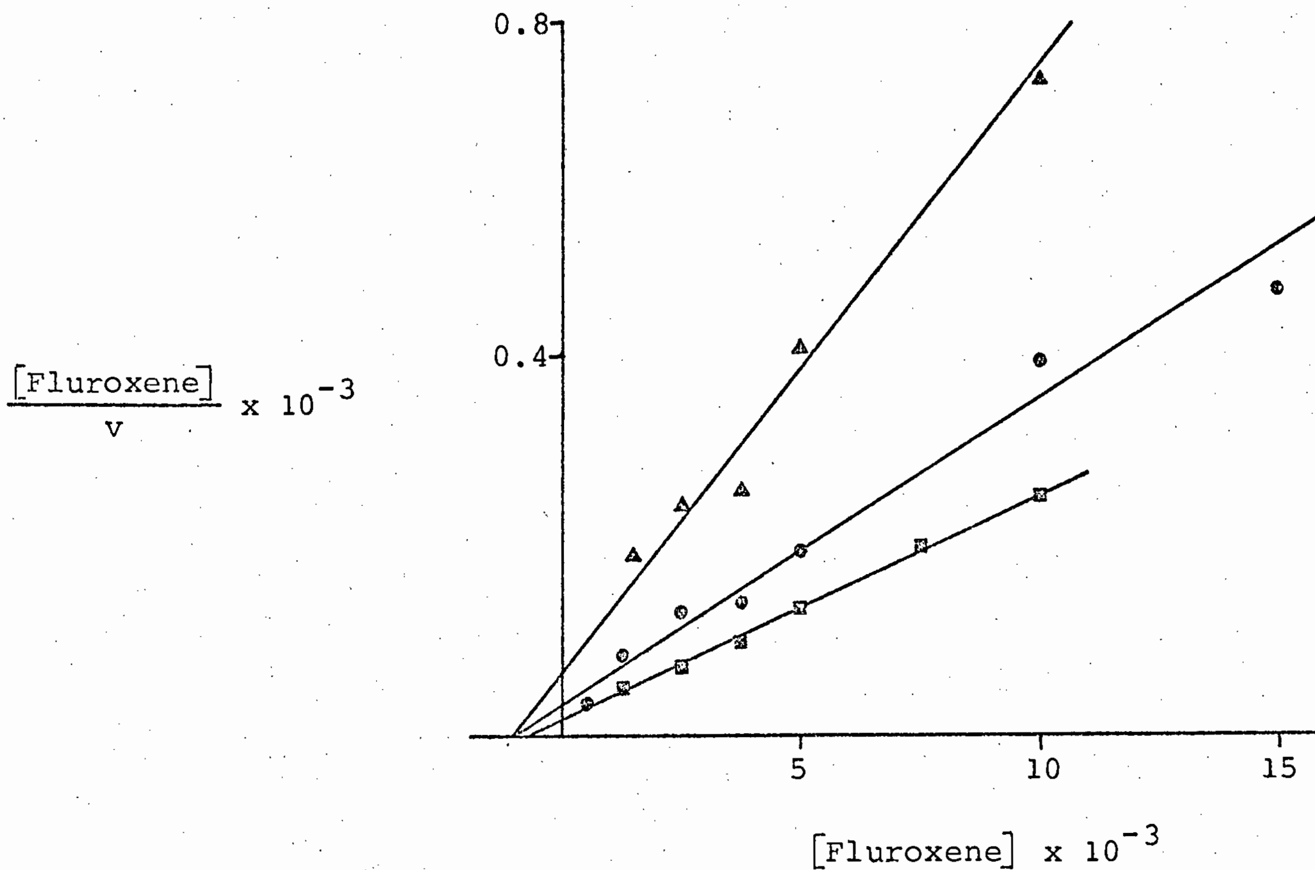


Figure 18. Hanes plots of production of 2,2,2-trifluoroethanol from fluroxene by hepatic microsomes from uninduced (●); 3-methylcholanthrene induced (■) and 3-methylcholanthrene induced/AIA treated (▲) animals; [Fluroxene], molar; v, nmol TFE/mg microsomal protein/10 min. Experimental conditions as for Figure 17.

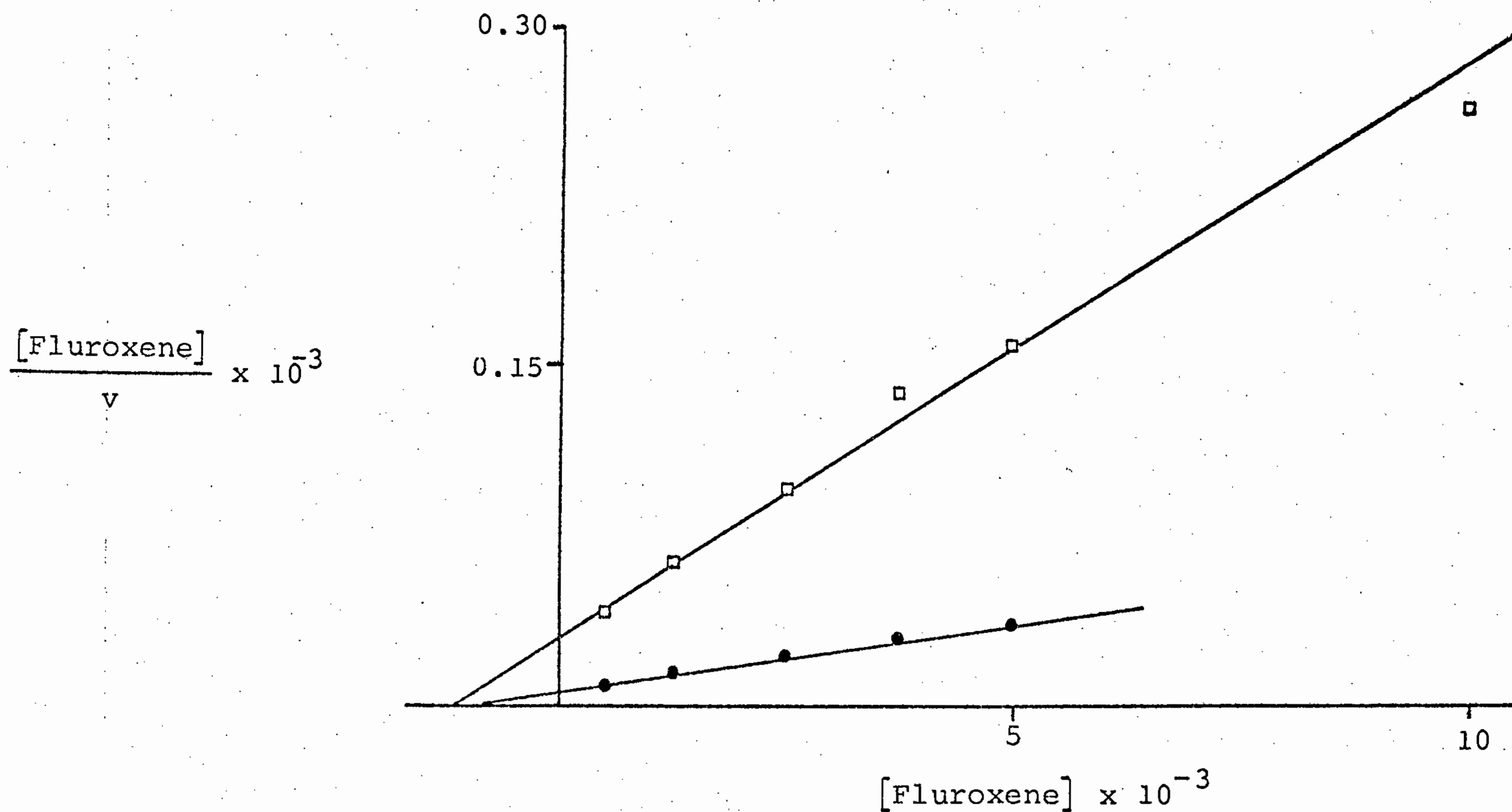


Figure 19. Hanes plots of production of 2,2,2-trifluoroethanol from fluroxene by hepatic microsomes from phenobarbital (●) and 3,4-benzpyrene (□) induced animals. [Fluroxene], molar; v, nmol TFE/mg microsomal protein/10 min. Experimental conditions as for Figure 17.

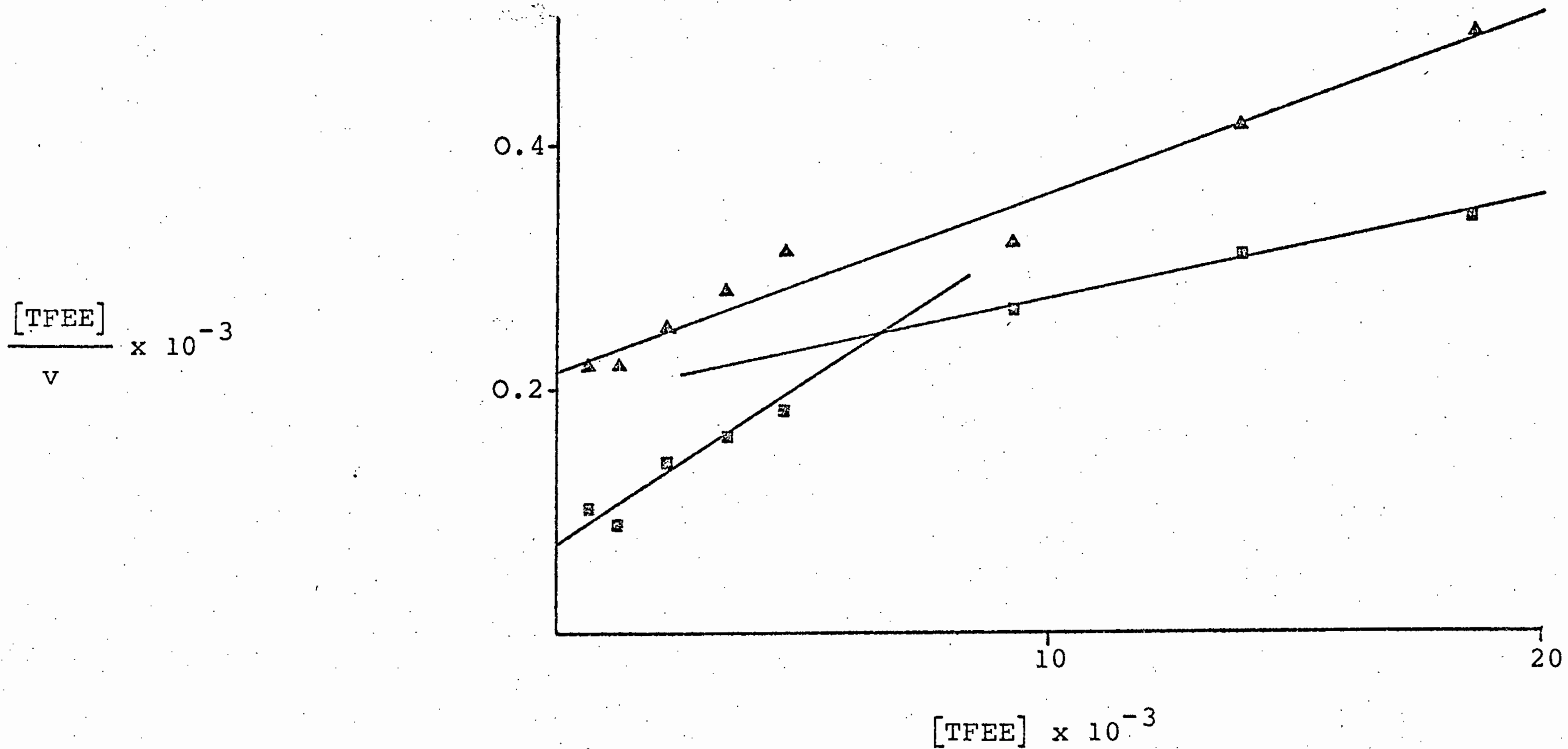


Figure 20. Hanes plots of production of 2,2,2-trifluoroethanol from 2,2,2-trifluoroethyl ethyl ether by hepatic microsomes from 3-methylcholanthrene induced (■) and 3-methylcholanthrene induced/AIA treated (▲) animals; [TFEE], molar; v, nmol TFE/mg microsomal protein/10 min. Experimental conditions as for Figure 17.

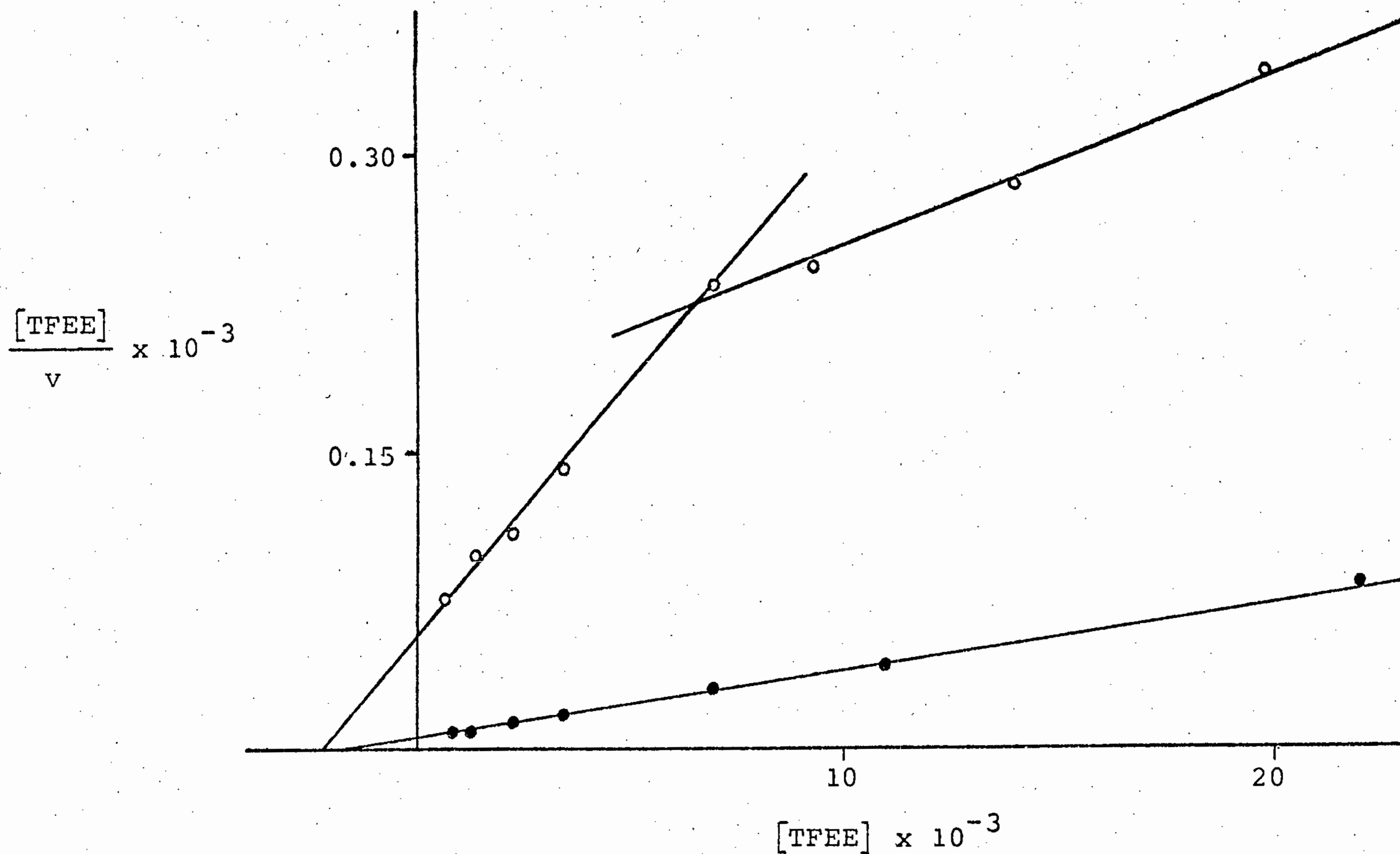


Figure 21. Hanes plots of production of 2,2,2-trifluoroethanol from 2,2,2-trifluoroethyl ethyl ether by hepatic microsomes from uninduced (O) and phenobarbital induced (●) animals; [TFEE], molar; v, nmol TFE/mg microsomal protein/10 min. Experimental conditions as for Figure 17.

animals allow calculation of two K_m and V_{max} values in each case.

The effects of induction of cytochromes P-450 on the values of K_m and V_{max} for production of trifluoroethanol from fluroxene and TFEE are shown in Tables 17 and 18 respectively. Averages and mean deviations for determinations of K_m and V_{max} with two or three separate preparations of hepatic microsomes are reported.

For production of trifluoroethanol from either fluroxene or TFEE, values of K_m obtained after polycyclic hydrocarbon induction are not significantly different from the K_m values observed with uninduced microsomes (Tables 17 and 18) except for the decrease in the larger K_m value observed with TFEE in 3,4-benzpyrene induced microsomes. AIA treatment of 3-methylcholanthrene induced animals leads to loss of the smaller K_m value for microsomal production of trifluoroethanol from TFEE (Table 18). Relative to values obtained with microsomes from uninduced animals, phenobarbital induction results in no significant change ($p > 0.05$) in the K_m value for microsomal production of trifluoroethanol from fluroxene (Table 17) and in the disappearance of the larger K_m for production of trifluoroethanol from TFEE (Table 18).

Values of V_{max} for production of trifluoroethanol from fluroxene (Table 17) or TFEE (Table 18) are unaffected by polycyclic hydrocarbon induction of cytochrome P-448 relative to values observed with uninduced microsomes.

Table 17. Effect of induction of cytochromes P-450 on hepatic microsomal production of 2,2,2-trifluoroethanol from fluroxene

Induction	Cyts. P-450 (nmol/mg microsomal protein)	K_m (mM)	V_{max} (nmol TFE/mg microsomal protein/min)
None	1.2 ± 0.1	1.4 ± 0.1	3.7 ± 0.5
BP	1.8 ± 0.1	1.8 ± 0.2	5.5 ± 1.2
MC	2.1 ± 0.2	1.3 ± 0.3	4.8 ± 0.4
MC/AIA	1.4 ± 0.1	1.2 ± 0.2	2.1 ± 0.4
PB	2.5 ± 0.3	0.7 ± 0.2	14.2 ± 0.8

Destruction of cytochrome P-450 by AIA results in decreases in the values of V_{max} for microsomal production of trifluoroethanol from fluroxene and TFEE; induction of cytochrome P-450 by phenobarbital results in substantial increases in the respective V_{max} values.

4. The production of fluoride from methoxyflurane and enflurane by hepatic microsomes

Free fluoride ion is produced from methoxyflurane and enflurane on incubation of either compound with microsomal suspension and NADPH generating system (157). During incubation, acid-labile fluoride is produced from methoxyflurane but not from enflurane. Control reaction mixtures in which the

Table 18. Effect of induction of cytochromes P-450 on hepatic microsomal production of 2,2,2-trifluoroethanol from 2,2,2-trifluoroethyl ethyl ether

Induction	Cyts. P-450 (nmol/mg microsomal protein)	K_m (mM)		V_{max} (nmol TFE/mg microsomal protein/min)	
		K_m[a]	K_m[b]	V_{max}[a]	V_{max}[b]
None	1.2±0.1	1.7±0.6	17.3±2.8	3.4±0.7	12.1±0.4
BP	1.8±0.1	1.0±0.1	7.0±0.8	4.4±1.0	8.3±0.2
MC	2.1±0.2	2.3±1.4	13.5±0.5	3.8±0.7	10.3±0.5
MC/AIA	1.4±0.1	-	15.5±1.7	-	5.3±1.3
PB	2.5±0.3	1.4±0.2	-	23.4±3.2	-

[a] Smaller K_m and corresponding V_{max}.

[b] Larger K_m and corresponding V_{max}.

anaesthetic agent is incubated with microsomes in the absence of NADPH generating system exhibit free fluoride concentrations of < 1.0 μM which is the lower limit of sensitivity of the fluoride electrode. Subsequent treatment of these reaction mixtures with conc. H_2SO_4 for 15 to 70 hr does not result in increased fluoride production, indicating that fluoride is not produced by acid hydrolysis of methoxyflurane or enflurane.

a. Determination of acid-labile fluoride

In order to ascertain the time required for complete hydrolysis of the acid-labile fluoride produced during microsomal metabolism of methoxyflurane, the effect of time of incubation with conc. H_2SO_4 on the concentration of total fluoride in the microsomal reaction mixtures was investigated. Concentrations of total fluoride increase with length of time of the acid treatment as illustrated in Figure 22. It is evident that incubation with concentrated H_2SO_4 for 85 to 90 hr is required in order that hydrolysis of acid-labile fluoride proceeds nearly to completion. An incubation time of 18 to 20 hr used in investigations of acid-labile fluoride production from methoxyflurane in other laboratories (156, 178) results in hydrolysis of only about 60% of the acid-labile fluoride.

b. Effect of time on production of fluoride

In order to determine a valid incubation period for kinetic studies of fluoride production, the production of fluoride from methoxyflurane and enflurane as a function of time was

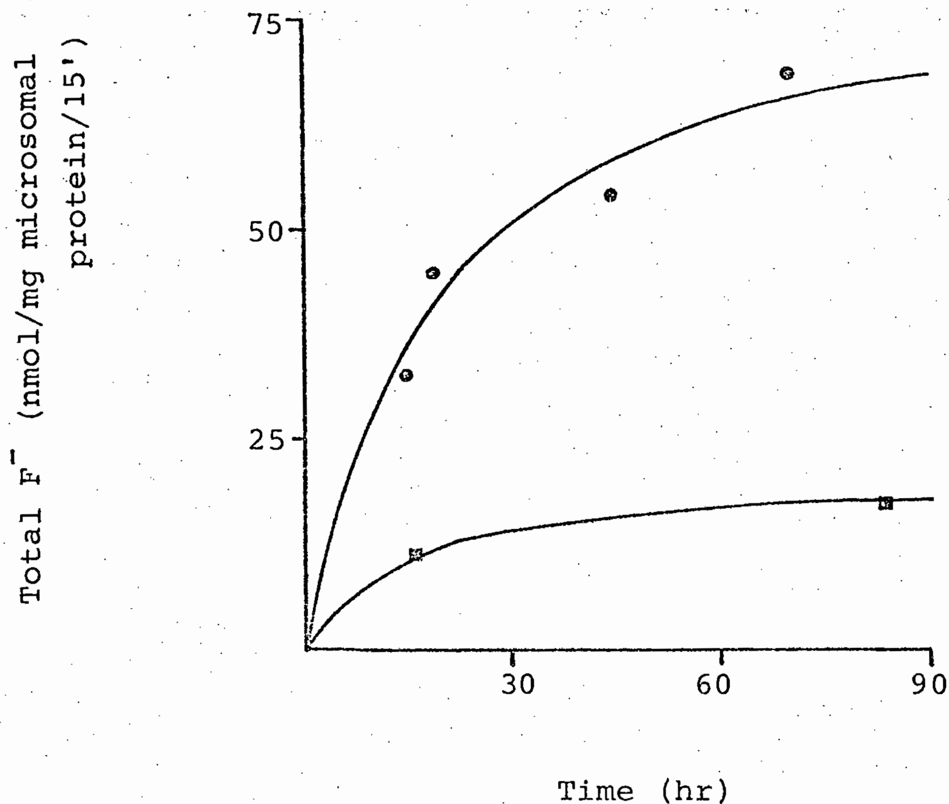


Figure 22. Effect of time of incubation with H_2SO_4 on total fluoride measureable after metabolism of methoxyflurane by 3-methylcholanthrene (■) and phenobarbital induced (●) microsomes. Methoxyflurane (1.7 mM), microsomal suspension (2.0 mg protein/ml) and NADPH generating system all in 0.02 M Tris-HCl, pH 7.4, incubated at 30° for 15' prior to treatment with conc. H_2SO_4.

investigated. Time courses for production of free and total fluoride from methoxyflurane by uninduced, 3-methylcholanthrene induced and phenobarbital induced microsomes are illustrated in Figures 23, 24 and 25; the rates of production of fluoride by these microsomes are linear for approximately 15, 20 and 15 min respectively. The production of free fluoride as a function of time from enflurane by phenobarbital induced microsomes is linear for approximately 20 min (Figure 26).

c. Effect of inhibitors on the production of fluoride

The effects of inhibitors of cytochromes P-450 on the microsomal production of fluoride from methoxyflurane and enflurane was investigated. With methoxyflurane, levels of free and total fluoride obtained in the absence and presence of inhibitors are indicated in Table 19 in which are reported averages and mean deviations for determinations performed in triplicate with one preparation of microsomes each from uninduced and induced animals.

Effects of the inhibitors on fluoride production from methoxyflurane are demonstrated more effectively by presentation of the results of such studies in the form of 'percentage inhibition' of free and acid-labile fluoride production. Accordingly, averages and mean deviations for percentage inhibition values determined with three different preparations of microsomes each from uninduced and induced animals are presented in Table 20.

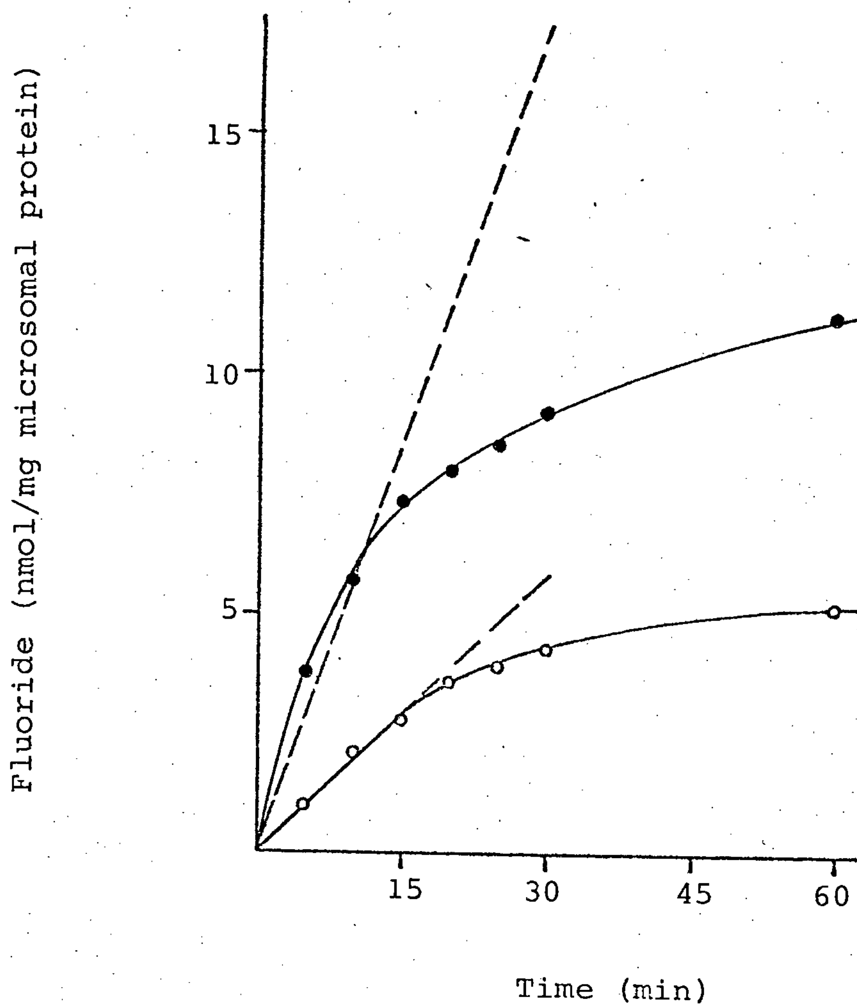


Figure 23. Effect of time on the production of free (○) and total (●) fluoride from methoxyflurane by uninduced microsomes. Methoxyflurane (2.9 mM), microsomal suspension (2.0 mg protein/ml) and NADPH generating system all in 0.02 M Tris-HCl, pH 7.4 incubated at 30°.

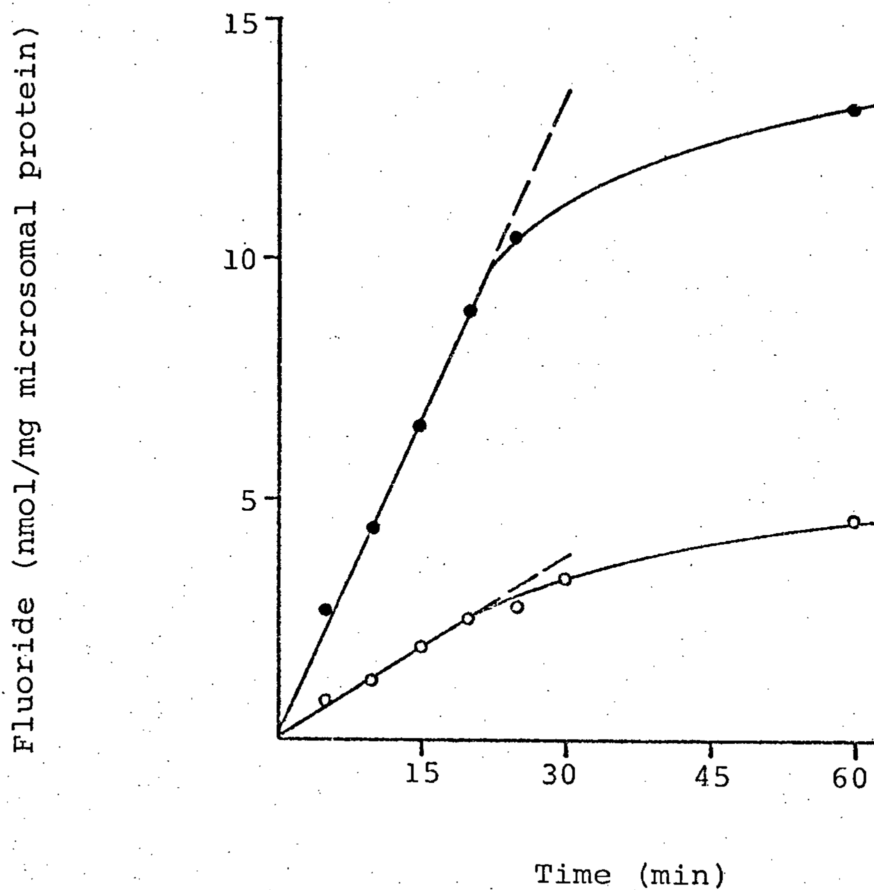


Figure 24. Effect of time on the production of free (O) and total (●) fluoride from methoxyflurane by 3-methylcholanthrene induced microsomes. Experimental conditions as for Figure 23.

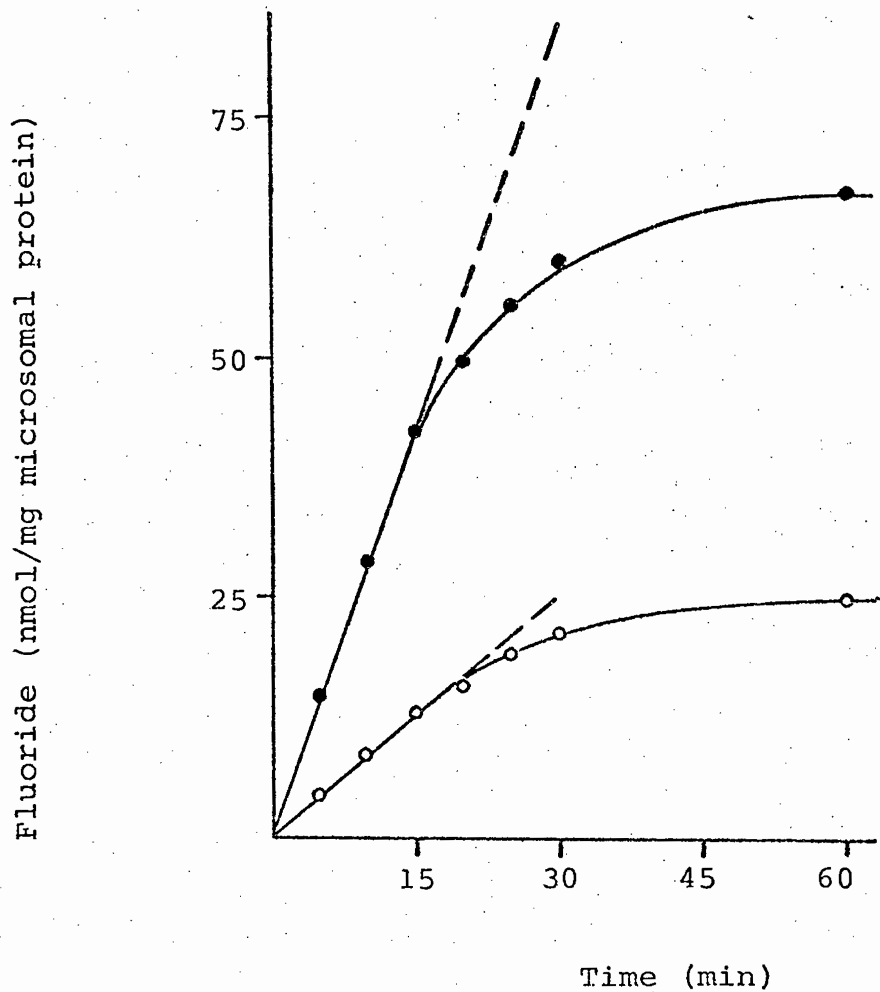


Figure 25. Effect of time on the production of free (O) and total (●) fluoride from methoxyflurane by phenobarbital induced microsomes. Experimental conditions as for Figure 23.

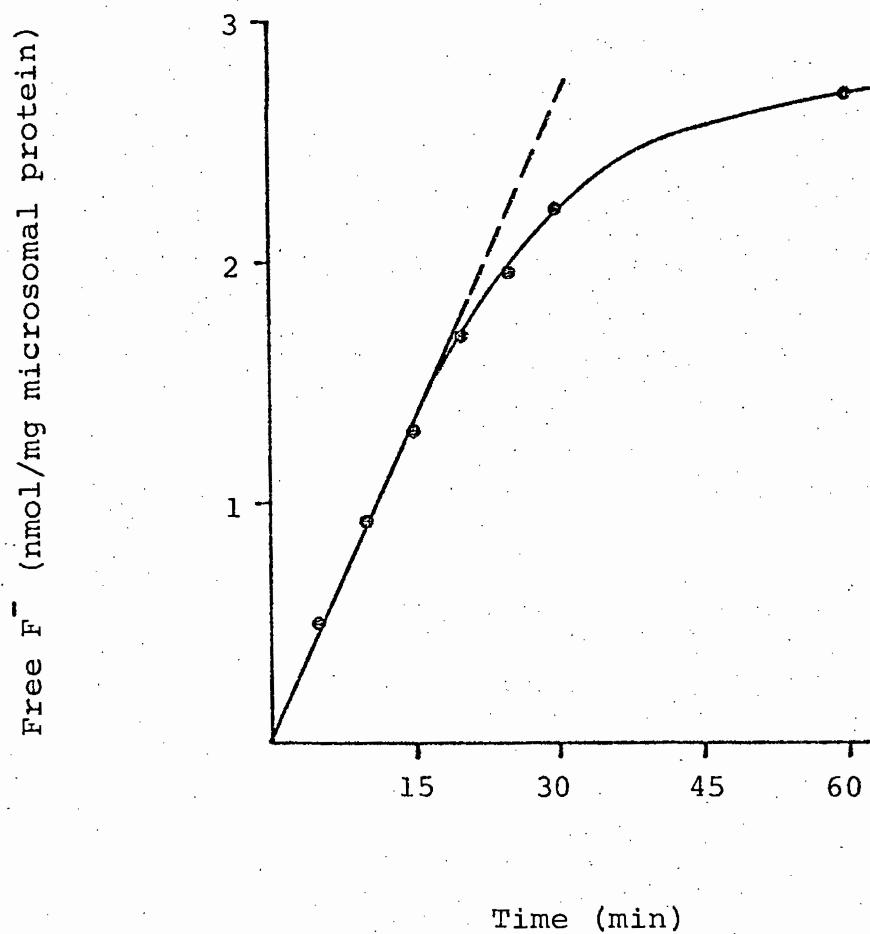


Figure 26. Effect of time on the production of free fluoride from enflurane by phenobarbital induced microsomes. Enflurane, 2.7 mM; other experimental conditions as for Figure 23.

Table 19. Effect of inhibitors of cytochromes P-450 on the production of fluoride from methoxyflurane in hepatic microsomes[a]

Inhibitor	Production of fluoride (nmol/mg microsomal protein/15 min)					
	Uninduced microsomes		MC induced microsomes		PB induced microsomes	
	Free F^-	Total F^-	Free F^-	Total F^-	Free F^-	Total F^-
None	3.2±0.1	14.7±1.3	1.7±0.3	7.8±0.6	9.6±0.2	45.0±4.5
Metyrapone:						
50 μM	2.2±0.0	11.0±0.6	1.2±0.1	7.0±0.4	3.2±0.1	16.6±0.4
200 μM	1.9±0.1	39.4±0.7	0.8±0.0	5.5±0.6	2.0±0.1	13.2±0.8
SKF 525A:						
50 μM	1.9±0.1	8.3±0.4	1.2±0.0	6.3±0.2	6.0±0.1	27.3±0.9
200 μM	1.3±0.0	6.1±0.5	1.0±0.0	4.9±0.3	3.1±0.1	13.2±0.6
CO[b]	1.0±0.2	5.5±0.6	0.9±0.0	6.5±0.1	1.5±0.3	6.7±0.2

[a] Methoxyflurane 1.7 mM.

[b] Carbon monoxide bubbled through reaction mixture for 2 min at 10 ml/min prior to incubation.

Table 20. Percentage inhibition of production of fluoride from methoxyflurane by hepatic microsomes

Inhibitor	% Inhibition of fluoride production					
	Uninduced microsomes		MC induced microsomes		PB induced microsomes	
	Free F^-	Acid-labile F^-	Free F^-	Acid-labile F^-	Free F^-	Acid-labile F^-
Metyrapone:						
50 µM	28±5	24±2	35±6	13±5 ▼ x	68±2 ■	65±2 ■
200 µM	37±3	31±3	52	24 y	79 ■	69 ■
SKF 525A:						
50 µM	33±7	37±7	27±7	24±3 ■	42±3	43±3
200 µM[a]	49±8	52±7	43	36 ▼	68 ▼	71 ▼
CO	62±5	62±2	57±14	24±11 ■ x	84±1 ■	86±1 ■

[a] % Inhibition determined with only one microsomal preparation except for investigations with control microsomes.

[x] Differs from free F^- production with same type of microsomal preparation, $p < 0.01$

[y] Differs from free F^- production with same type of microsomal preparation, $p < 0.05$

The production of free fluoride is generally inhibited to the same extent as the production of acid-labile fluoride by each inhibitor in uninduced microsomes and in phenobarbital induced microsomes (Table 20). With microsomes from 3-methylcholanthrene induced animals however, differences exist in the extents of inhibition of free fluoride production relative to acid-labile fluoride production with the inhibitors, metyrapone and carbon monoxide.

Metyrapone inhibits the production of fluoride to a lesser or to the same extent within experimental error as SKF 525A in microsomes from uninduced or 3-methylcholanthrene induced animals. In contrast, in phenobarbital induced microsomes metyrapone (50 μM) inhibits fluoride production to a greater extent than SKF 525A (50 μM) although this differential effect disappears at 200 μM concentrations of the inhibitors (Table 20).

Production of free and acid-labile fluoride by uninduced microsomes and production of free fluoride by 3-methylcholanthrene induced microsomes are inhibited to the same extents by each inhibitor. In contrast, production of acid-labile fluoride by 3-methylcholanthrene induced microsomes is generally less sensitive to inhibition by all inhibitors while production of both free and acid-labile fluoride by phenobarbital induced microsomes is more sensitive to inhibition (Table 20).

uninduced and 3-methylcholanthrene induced microsomes (Table 20).

The effect of inhibitors of cytochromes P-450 on the production of free fluoride from enflurane was investigated in microsomes from phenobarbital induced animals only (Table 21). Production of free fluoride is inhibited to a greater extent by metyrapone than by SKF 525A. The percentage inhibition observed in both cases is the same as that observed with free fluoride production from methoxyflurane by phenobarbital induced microsomes.

Table 21. Effect of inhibitors of cytochromes P-450 on the production of fluoride from enflurane by phenobarbital induced hepatic microsomes[a]

Inhibitor	Production of free fluoride (nmol/mg microsomal protein/20 min)[b]	(%)
None	2.0 $\pm$ 0.4	100
50 μM Metyrapone	0.8 $\pm$ 0.1	40
50 μM SKF 525A	1.2 $\pm$ 0.1	60
CO[c]	<0.5	<25

[a] Enflurane 2.4 mM.

[b] Averages and mean deviations of determinations with three separate microsomal preparations.

[c] Carbon monoxide bubbled through reaction mixture for 2 min at 10 ml/min prior to incubation.

d. <u>Effect of induction of cytochromes P-450 on production of fluoride</u>

The production of fluoride from methoxyflurane and enflurane was investigated in microsomes from uninduced and induced animals.

The production of free and total fluoride as a function of methoxyflurane concentration was studied in microsomes from uninduced, 3-methylcholanthrene induced and phenobarbital induced animals; production of acid-labile fluoride was calculated in each case. Microsomal production of fluoride increases with increasing concentration of anaesthetic agent until saturating concentrations are reached and Hanes or Eadie-Hofstee plots of the data have been used to calculate K_m and V_{max} values for free, acid-labile and total fluoride production by the different types of microsomes.

The production of free, acid-labile and total fluoride from methoxyflurane by uninduced or 3-methylcholanthrene induced microsomes gives rise to biphasic Eadie-Hofstee plots (Figures 27-32) which allow calculation of two K_m and V_{max} values in each case. The biphasic nature of the kinetics of fluoride production in these cases is not always so readily evident when the data is treated by means of the Hanes plot (e.g. Figure 33). In contrast, production of free, acid-labile and total fluoride from methoxyflurane by phenobarbital induced microsomes exhibits linear Hanes or Eadie-Hofstee plots (Figures 34 and 35).

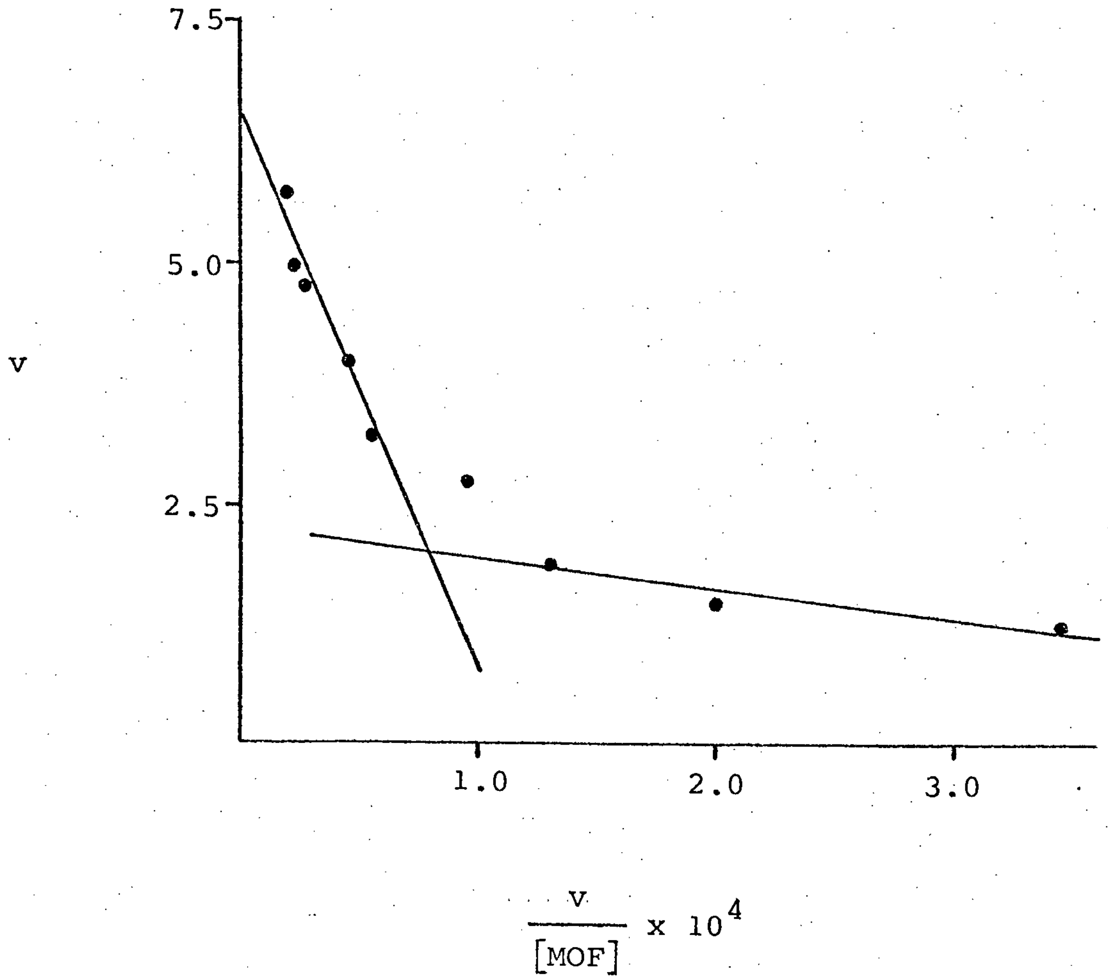


Figure 27. Eadie-Hofstee plot of production of free fluoride from methoxyflurane by uninduced microsomes. [MOF], molar; v, nmol free F^-/mg microsomal protein/15'. Experimental conditions as for Figure 23.

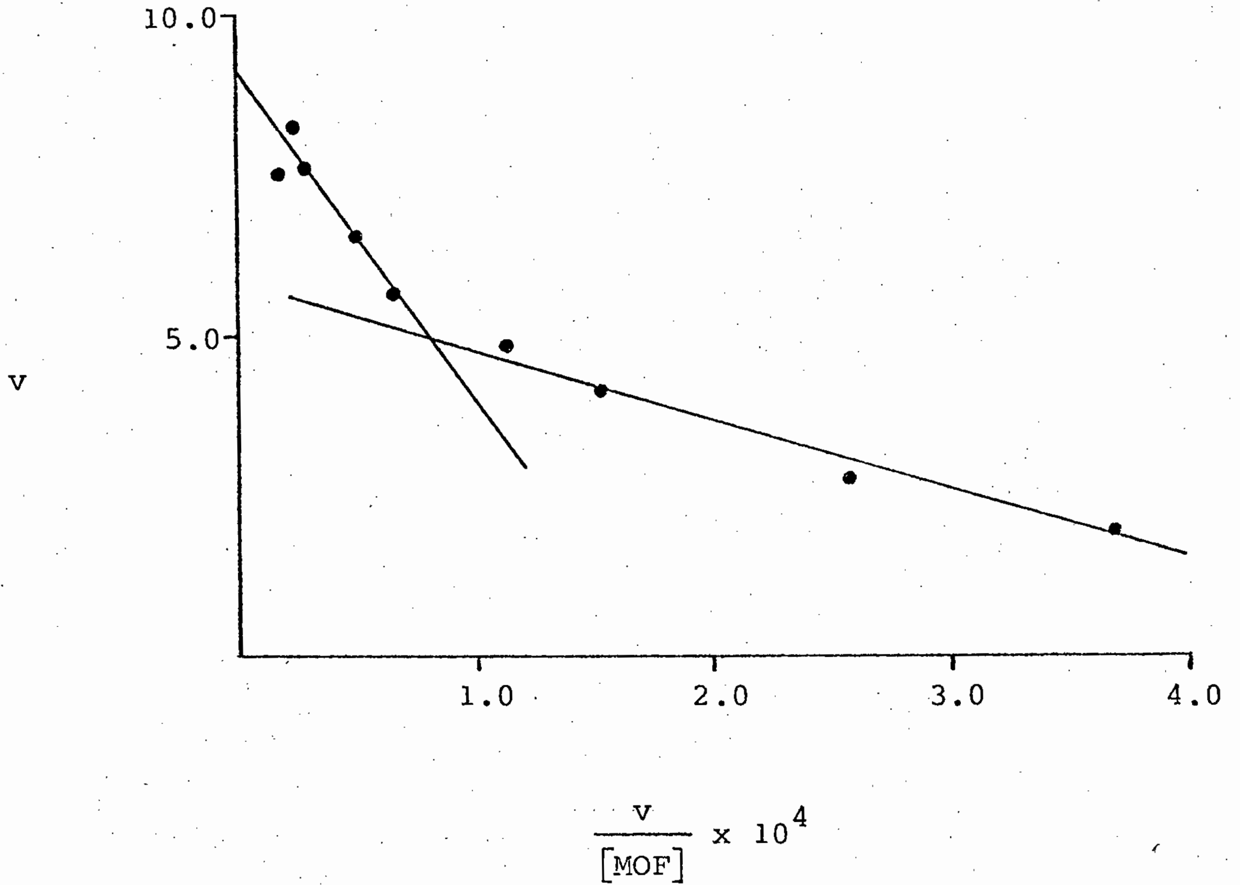


Figure 28. Eadie-Hofstee plot of production of acid-labile fluoride from methoxyflurane by uninduced microsomes. [MOF], molar; v, nmol acid-labile F^-/mg microsomal protein/15'. Experimental conditions as for Figure 23.

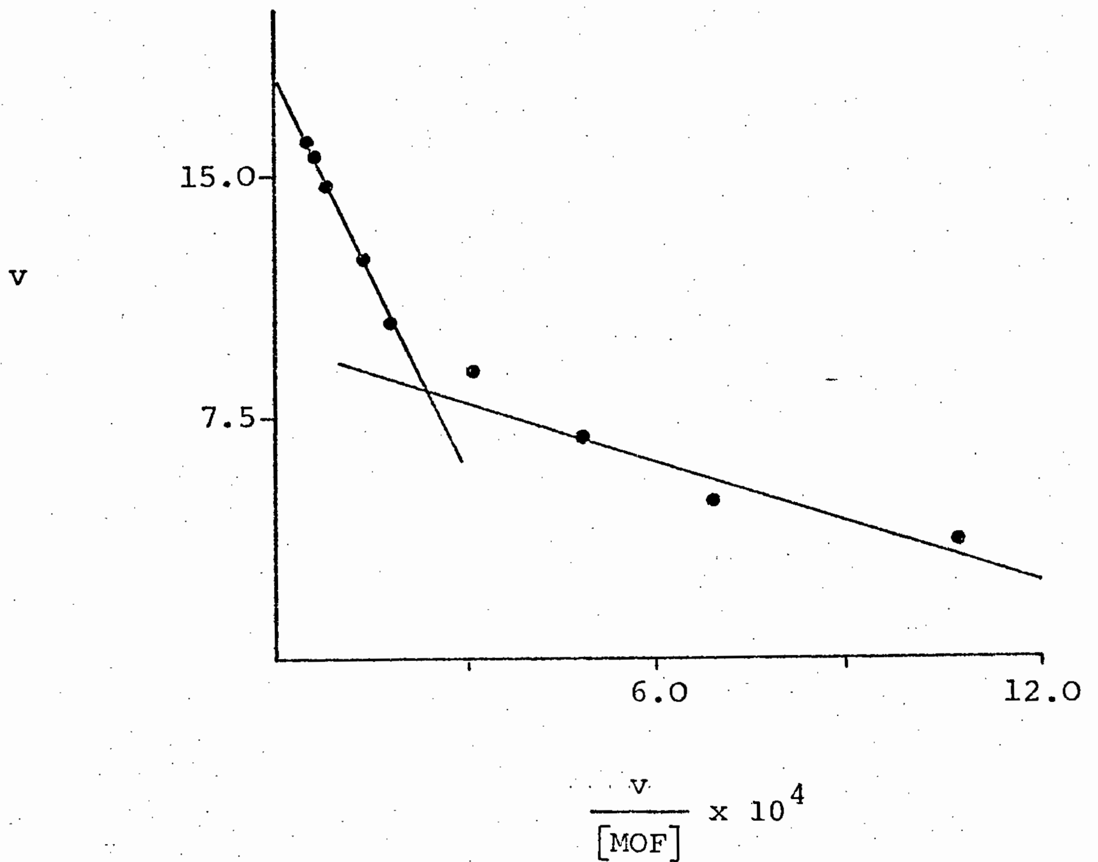


Figure 29. Eadie-Hofstee plot of production of total fluoride from methoxyflurane by uninduced microsomes. [MOF], molar; v, nmol total F^-/mg microsomal protein/15'. Experimental conditions as for Figure 23.

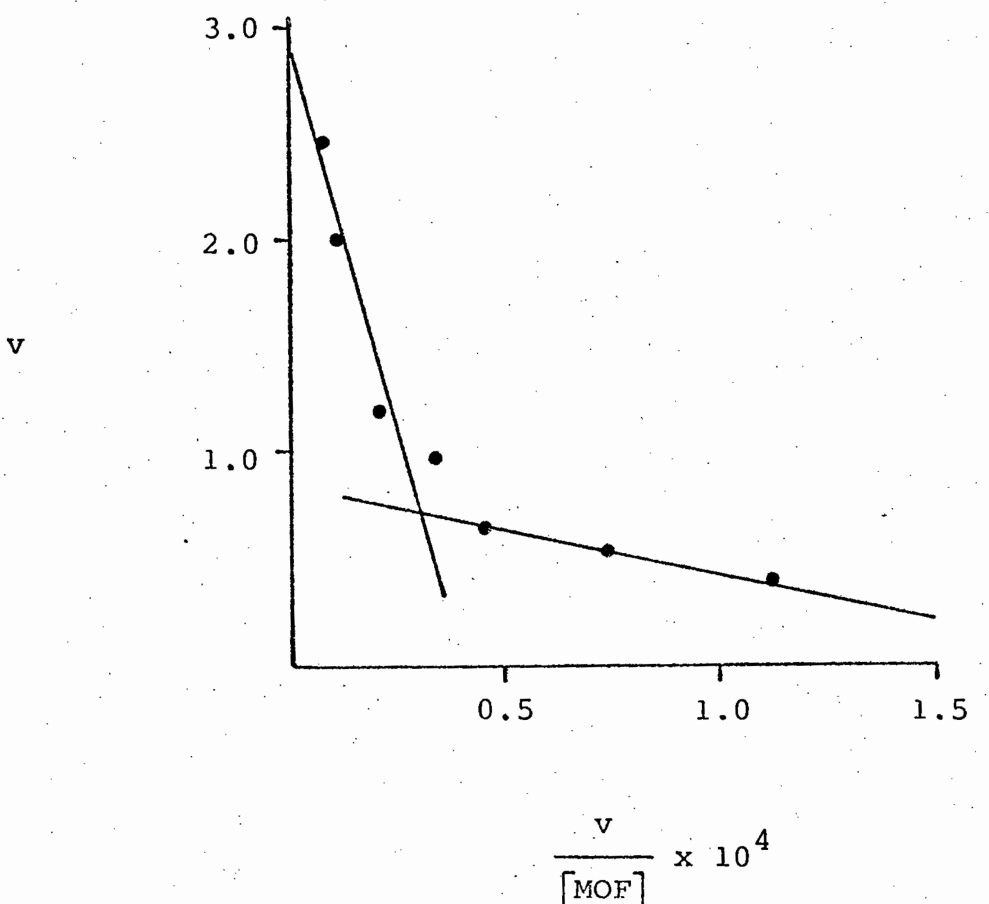


Figure 30. Eadie-Hofstee plot of production of free fluoride from methoxyflurane by 3-methylcholanthrene induced microsomes. [MOF], molar; v, nmol free F^-/mg microsomal protein/20'. Experimental conditions as for Figure 23.

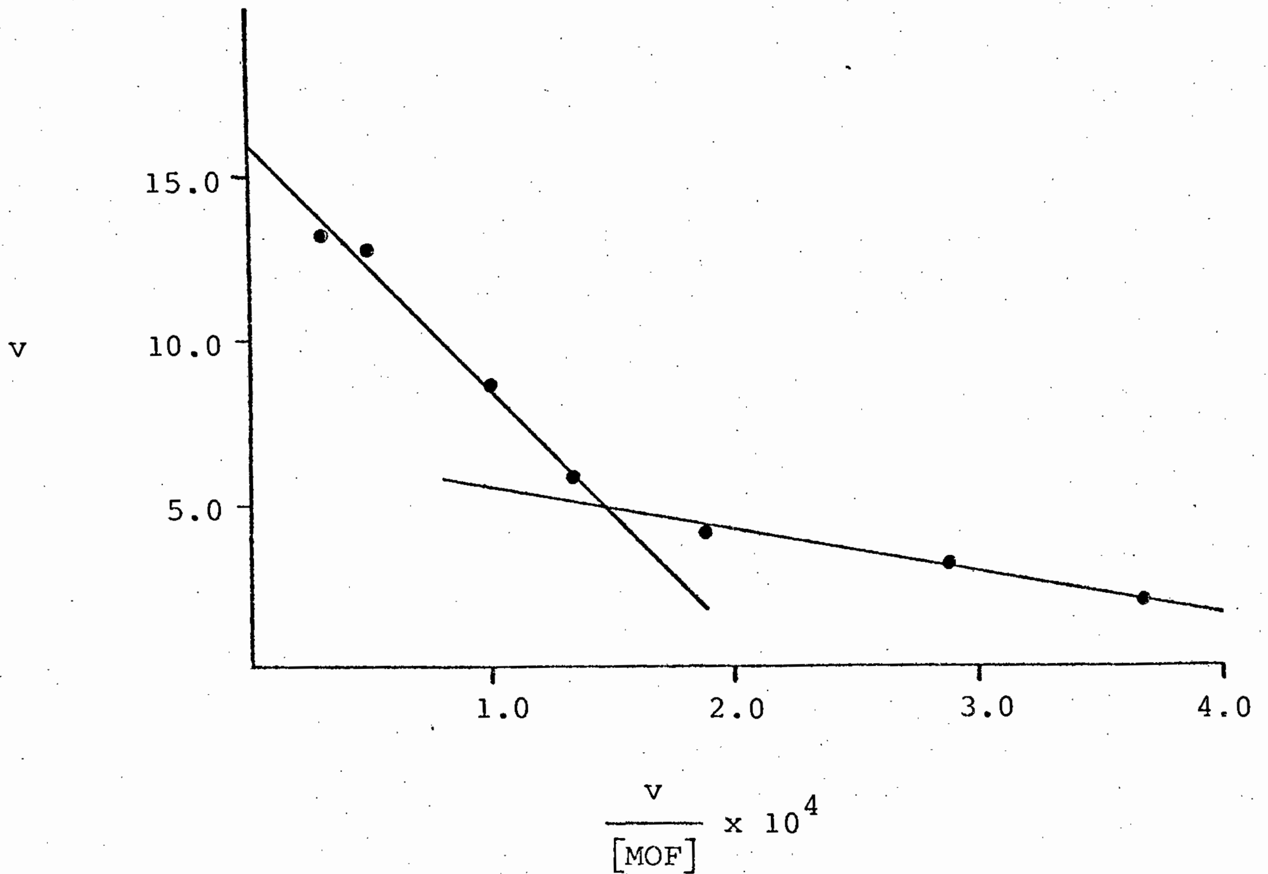


Figure 31. Eadie-Hofstee plot of production of acid-labile fluoride from methoxyflurane by 3-methylcholanthrene induced microsomes. [MOF], molar; v, nmol acid-labile F^-/mg microsomal protein/20'. Experimental conditions as for Figure 23.

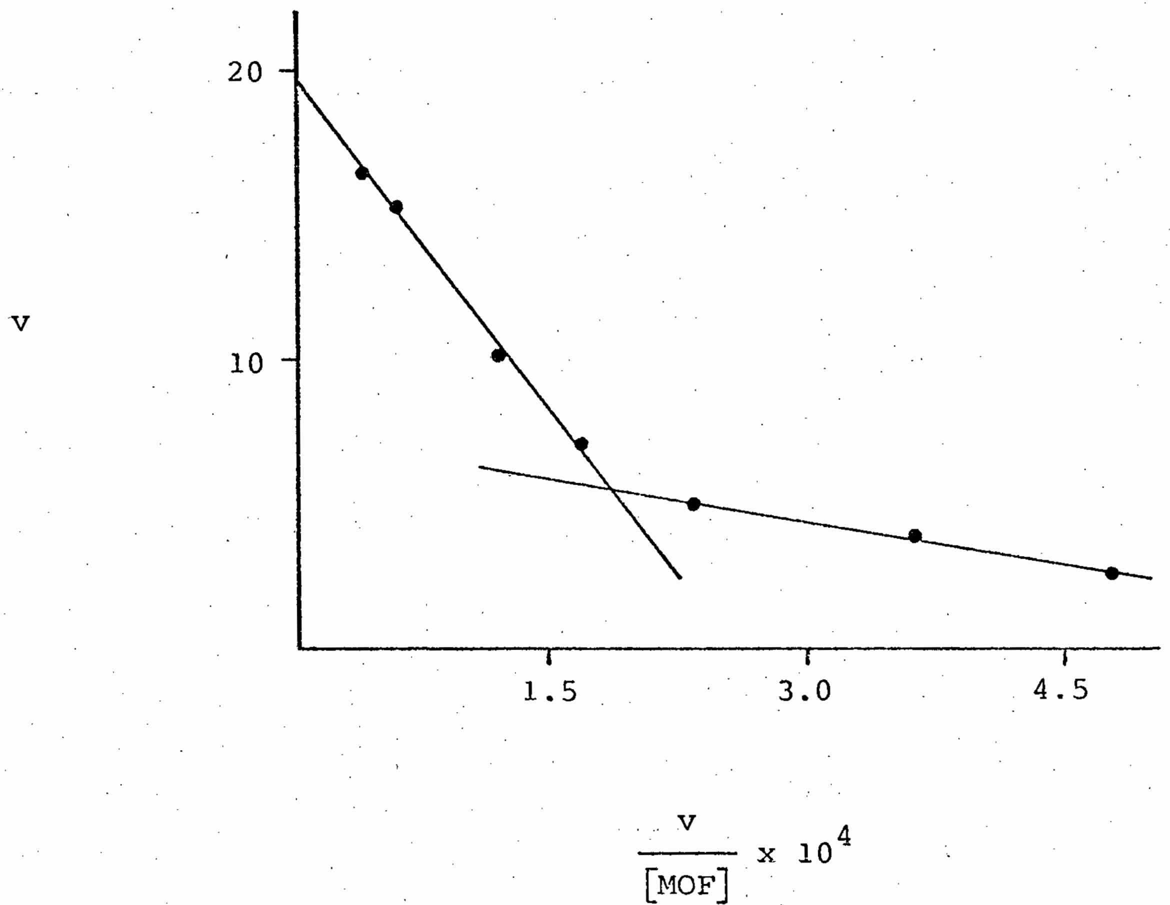


Figure 32. Eadie-Hofstee plot of production of total fluoride from methoxyflurane by 3-methylcholanthrene induced microsomes. [MOF], molar; v, nmol total F^-/mg microsomal protein/20'. Experimental conditions as for Figure 23.

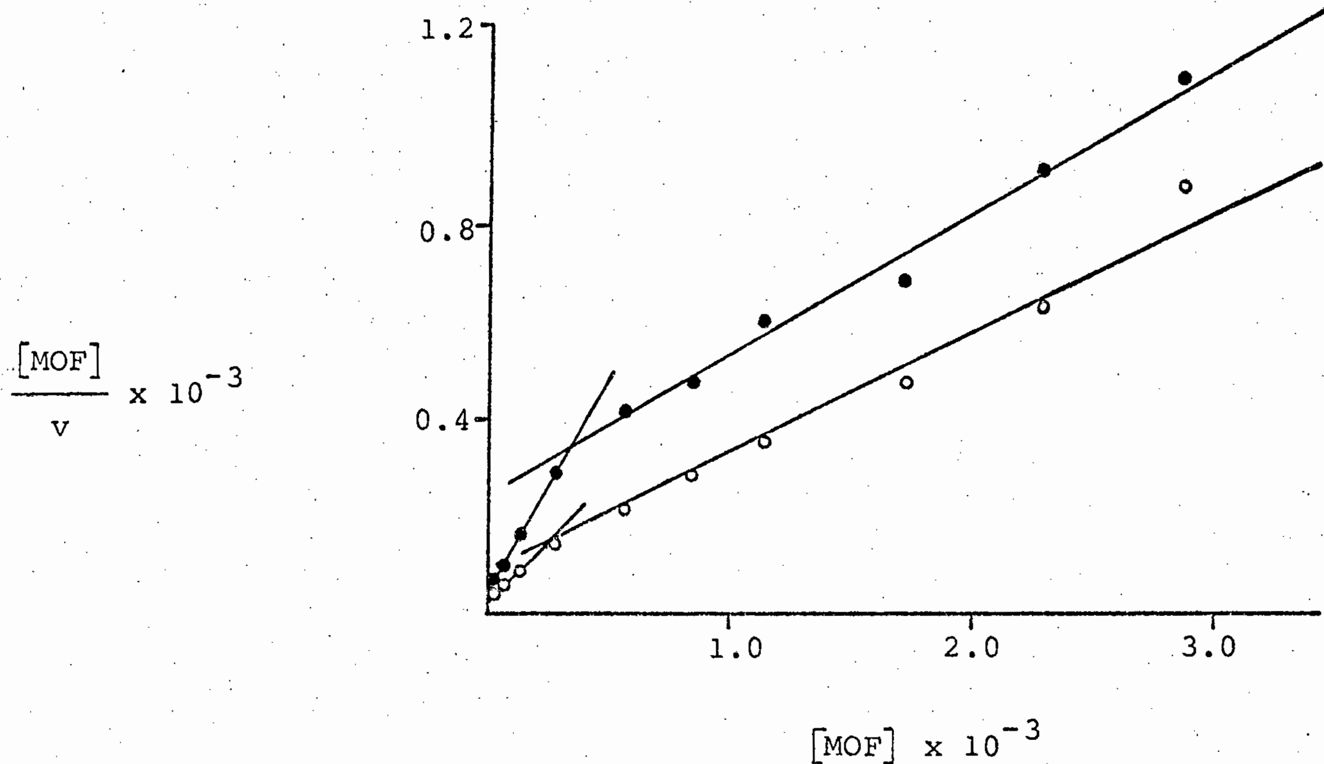


Figure 33. Hanes plots of production of free fluoride from methoxyflurane by hepatic microsomes from uninduced (○) and 3-methylcholanthrene induced (●) animals. [MOF], molar; v, nmol free F^-/mg microsomal protein/15 min for uninduced microsomes and /20 min for MC induced microsomes. Experimental conditions as for Figure 23.

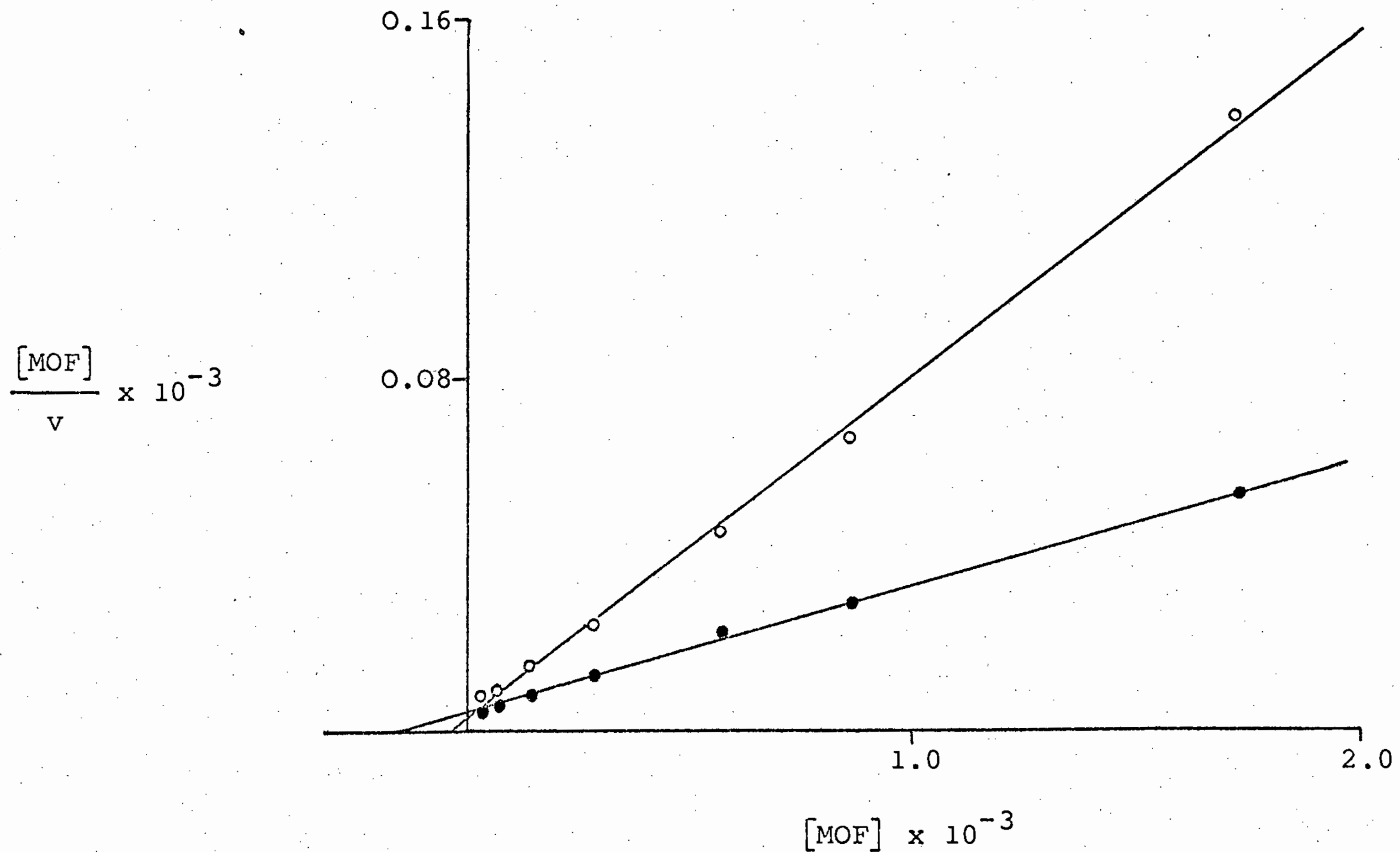


Figure 34. Hanes plots of production of free (O) and total (●) fluoride from methoxyflurane by phenobarbital induced microsomes. [MOF], molar; v, nmol F^-/mg microsomal protein/15'. Experimental conditions as for Figure 23.

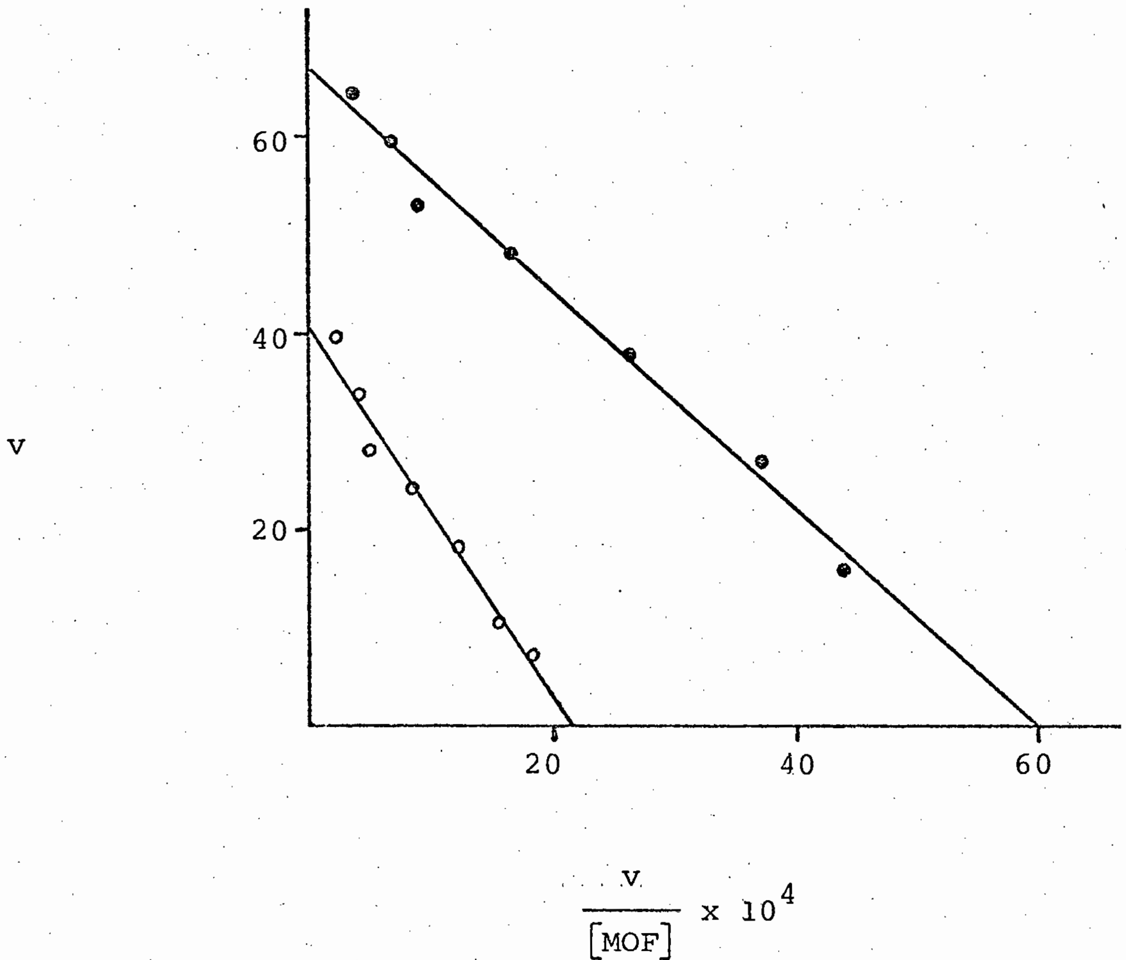


Figure 35. Eadie-Hofstee plots of acid-labile (O) and total (●) fluoride production from methoxyflurane by phenobarbital induced microsomes. [MOF], molar; v, nmol F^-/mg microsomal protein/15'. Experimental conditions as for Figure 23.

The effect of induction of cytochromes P-450 on the values of K_m and V_{max} for microsomal production of fluoride from methoxyflurane is presented in Table 22. Averages and mean deviations for determinations of K_m and V_{max} with three or four separate preparations of hepatic microsomes are reported. Production of free and acid-labile fluoride only will be considered in discussion of results; values of K_m and V_{max} for total fluoride production have been included in Table 22 for the sake of completeness.

The K_m values for production of free fluoride are the same within experimental error as those for production of acid-labile fluoride in control or 3-methylcholanthrene induced microsomes. Except for the larger K_m for free fluoride production, induction of cytochrome P-448 by 3-methylcholanthrene does not significantly affect the values of K_m for production of fluoride relative to those observed in uninduced microsomes. In microsomes from phenobarbital induced animals the K_m value for production of free fluoride is significantly different from the K_m value for production of acid-labile fluoride; these K_m values differ from both of the two K_m values obtained each for production of free or acid-labile fluoride in uninduced microsomes (Table 22).

Induction by 3-methylcholanthrene results in a decrease in V_{max} for microsomal production of free fluoride relative to that observed in uninduced microsomes whilst production of acid-labile fluoride is increased (Table 22).

Table 22. Effect of induction of cytochromes P-450 on hepatic microsomal production of fluoride from methoxyflurane

Induction	|Cyts. P-450| (nmol/mg microsomal protein)	K_m (mM)						V_{max} (nmol/mg microsomal protein/15 min)					
		Free F^-		Acid-labile F^-		Total F^-		Free F^-		Acid-labile F^-		Total F^-	
		K_m[a]	K_m[b]	K_m[a]	K_m[b]	K_m[a]	K_m[b]	V_{max}[a]	V_{max}[b]	V_{max}[a]	V_{max}[b]	V_{max}[a]	V_{max}[b]
None	1.0±0.1	0.04 ±0.007	0.4 ±0.1	0.06 ±0.01	0.7 ±0.1	0.06 ±0.003	0.4 ±0.1	2.3 ±0.4	5.4 ±0.9	4.6 ±1.2	9.6 ±0.4	8.8 ±1.0	16.3 ±1.2
MC	1.7±0.1	0.04 ±0.01	0.9■ ±0.2	0.08 ±0.03	0.9 ±0.3	0.06 ±0.02	0.8 ±0.1	0.8■ ±0.3	2.6▼ ±0.3	4.2 ±0.7	12.6▼ ±1.0	4.9 ±1.0	16.2 ±1.0
PB	2.4±0.2	0.10● ±0.01		0.22● ±0.01		0.14 ±0.02		15.1● ±1.6		43.9● ±7.3		66.6 ±6.0	

[a] Smaller K_m and V_{max} values.

[b] Larger K_m and V_{max} values.

Induction by phenobarbital substantially increases V_{max} values for production of free and acid-labile fluoride relative to uninduced microsomes.

The production of free fluoride from enflurane was investigated in microsomes from uninduced, 3-methylcholanthrene and phenobarbital induced animals at one concentration of enflurane (Table 23). Induction of cytochrome P-450 by phenobarbital elevates the rate of fluoride production approximately 2.8 times that observed in uninduced microsomes while induction of cytochrome P-448 by 3-methylcholanthrene does not affect the rate of fluoride production.

Table 23. Effect of induction of cytochromes P-450 on hepatic microsomal production of fluoride from enflurane[a]

Induction	Cyts. P-450 (nmol/mg microsomal protein)	Production of free fluoride[b] (nmol/mg microsomal protein/20 min)
None	1.0±0.1	0.9±0.2
MC	1.7±0.1	0.8±0.2
PB	2.4±0.2	2.5±0.2

[a] Enflurane 2.7 mM.

[b] Averages and mean deviations of determinations on two separate microsomal preparations.

Fluoride production as a function of enflurane concentration was studied with phenobarbital induced microsomes; levels of production of fluoride were too low to allow a similar investigation with uninduced and 3-methylcholanthrene induced microsomes. Hanes and Eadie-Hofstee plots for free fluoride production from enflurane by phenobarbital induced microsomes are linear (e.g. Figure 36) and allow calculation of K_m and V_{max} values of 0.36 ± 0.05 mM and 2.6 ± 0.7 nmol free fluoride/mg microsomal protein/20 min respectively.

5. Effect of anaesthetic agents on levels of hepatic drug metabolizing enzymes *in vitro*

The ability of the anaesthetic agents to degrade components of the hepatic drug metabolizing system *in vitro* was investigated with microsomes from phenobarbital induced animals, and the results are presented in Table 24.

After incubation of fluroxene with microsomes and NADPH generating system for 30 min the concentration of cytochromes P-450 is decreased by approximately 30% relative to zero time values. The concentration of total microsomal heme is decreased by an amount approximately equivalent to the decrease in concentration of cytochromes P-450 (179). A decrease in cytochromes P-450 concentration is not observed when NADPH is omitted from the incubation system. Incubation of TFEE, methoxyflurane or enflurane with microsomes and NADPH generating system does not affect the levels of cytochromes P-450. None of the anaesthetic agents inves-

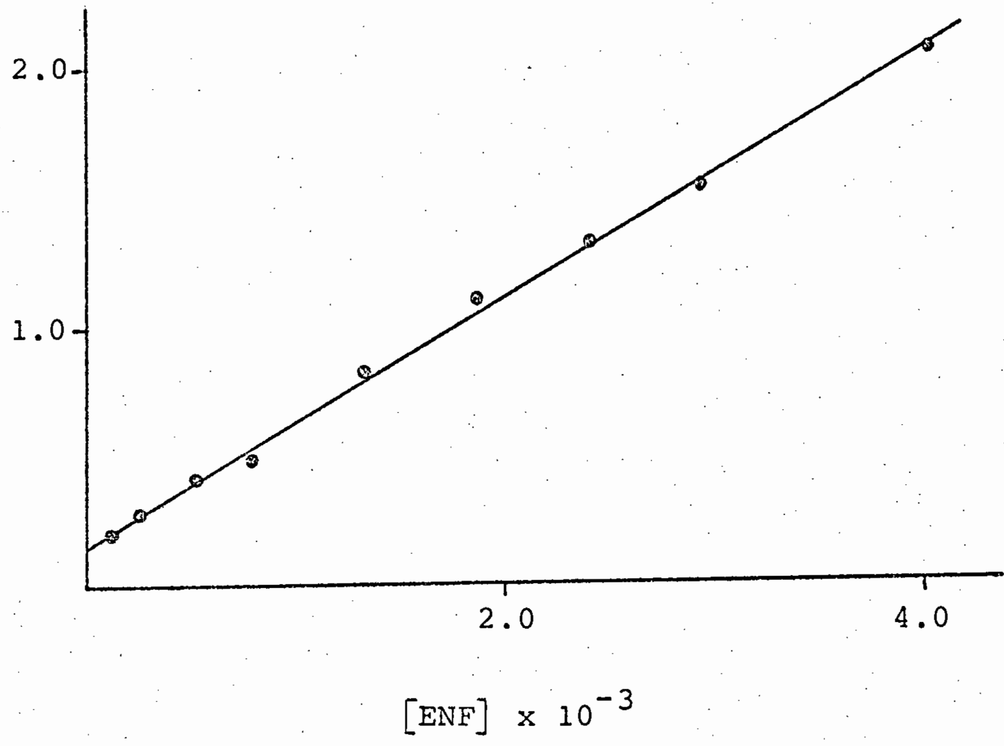


Figure 36. Hanes plot of production of free fluoride from enflurane by phenobarbital induced microsomes. [ENF], molar; v, nmol free F^-/mg microsomal protein/20'. Experimental conditions as for Figure 23.

Table 24. Effect of incubation of anaesthetic agents with phenobarbital induced microsomes and NADPH on the levels of microsomal drug metabolizing enzymes[a]

Anaesthetic agent	Cytochromes P-450 (nmol/mg microsomal protein)		Cytochrome b_5 (nmol/mg microsomal protein)		NADPH-cyt. c reductase (units/mg microsomal protein)	
	0'	30'	0'	30'	0'	30'
Fluroxene (30 mM)	2.4	1.7	0.54	0.58	0.11	0.12
TFEE (30 mM)	2.5	2.4	N.D		N.D	
Methoxyflurane (6 mM)	3.1	2.9	0.75	0.70	0.11	0.11
Enflurane (13 mM)	3.0	2.9	0.67	0.71	0.11	0.10

[a] Assay of drug metabolizing enzymes performed at zero time and after 30 min incubation at 30°; averages of triplicate determinations reported; mean deviations all <7%.

tigated affects the concentrations of cytochrome $\underline{b}_5$ or NADPH-cytochrome $\underline{c}$ reductase under the conditions of incubation (Table 24).

The relationship between the fluroxene mediated destruction of cytochromes P-450 and the metabolism of fluroxene to trifluoroethanol was investigated in microsomes from uninduced, 3-methylcholanthrene and phenobarbital induced animals. Destruction of cytochromes P-450 and production of trifluoroethanol were each studied as a function of time, the respective assays being performed on the same incubation mixtures usually at 5, 7, 10, 15, 20, 30 and 60 min. Plots of nmol cytochromes P-450 destroyed/mg microsomal protein versus nmol trifluoroethanol produced/mg microsomal protein are presented for uninduced, 3-methylcholanthrene and phenobarbital induced microsomes (Figure 37). For all types of microsomes there is a linear correlation between destruction of cytochromes P-450 and production of trifluoroethanol. The ratio of nmol trifluoroethanol produced to nmol cytochromes P-450 destroyed differs, however, for each of the three types of microsomes studied (Table 25).

6. Summary of the binding and metabolism of anaesthetic agents by microsomal cytochromes P-450 *in vitro*

A summary of the data from studies of the binding and metabolism of the anaesthetic agents by hepatic microsomal cytochromes P-450 *in vitro* is presented in Table 26. This provides a convenient means of referring to the results

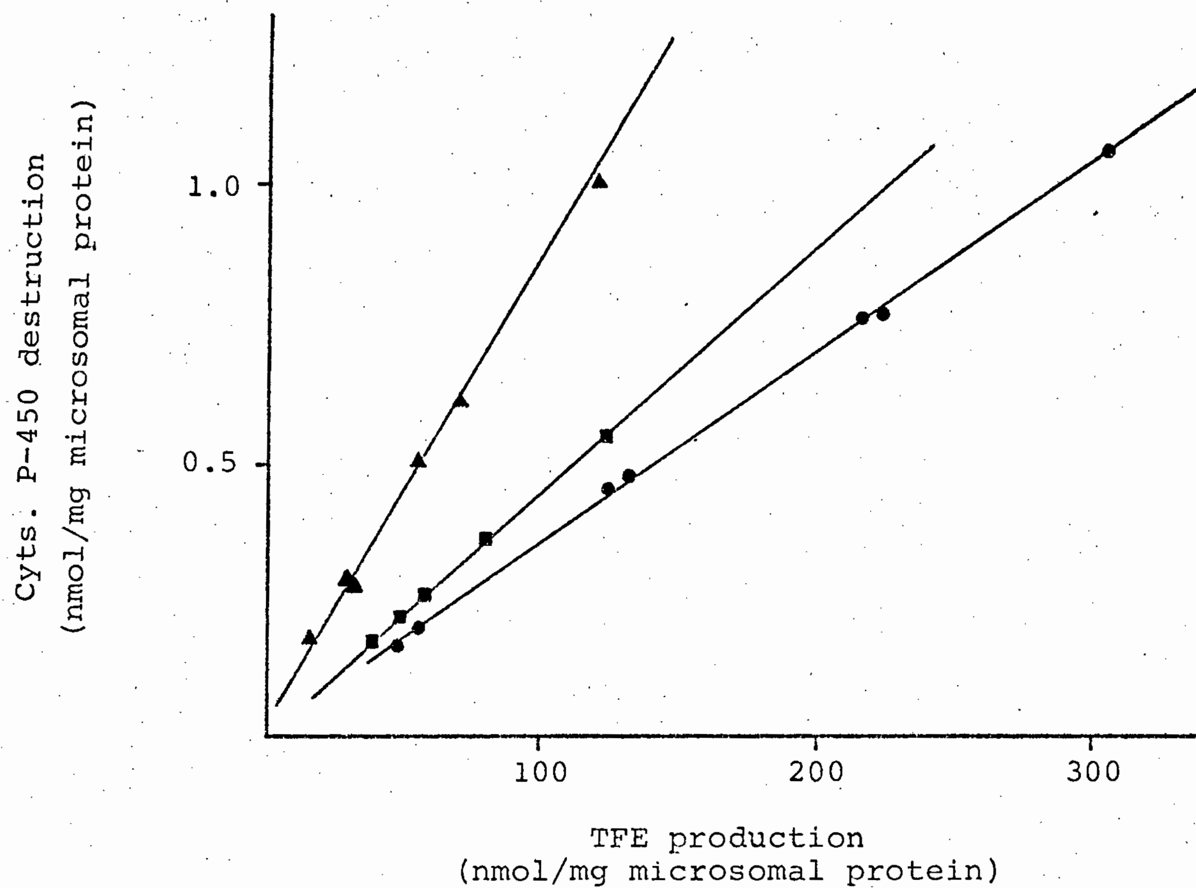


Figure 37. Relationship between 2,2,2-trifluoroethanol production and cytochromes P-450 destruction during metabolism of fluroxene by hepatic microsomes from uninduced (■), 3-methylcholanthrene induced (▲) and phenobarbital induced (●) animals. Experimental conditions as for footnote of Table 25 and microsomal protein 2.0 mg/ml in 0.02 M Tris-HCl, pH 7.4.

presented in the eleven tables from which the data for the summary are drawn; in addition, relationships between the different interactions of the anaesthetic agents with cytochromes P-450 may be observed more easily in Table 26.

For the interaction of fluroxene with cytochromes P-450, $K_s = K_m$ (NADPH oxidation) = K_m (TFE production). With microsomes from 3,4-benzpyrene induced animals these correlations are not so well observed (Table 26).

Table 25. Relationship between production of 2,2,2-trifluoroethanol from fluroxene and the fluroxene mediated destruction of cytochromes P-450 in microsomes from variously pretreated animals[a]

Induction	Initial Cyts. P-450 (nmol/mg microsomal protein)	nmol TFE produced / nmol Cyts.P-450 destroyed
None	1.4	226
MC	2.2	107
PB	2.6	296

[a] Fluroxene, 30 mM; booster NADPH generating system consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase added every 8 min at concentrations identical with generating system added at zero time.

Table 26. Summary of the binding and metabolism of anaesthetic agents by microsomal cytochromes P-450 *in vitro*

Anaesthetic agent	Induction	Binding K_s [a]	Binding ΔA_{max} [b]	NADPH oxidation K_m [a]	NADPH oxidation V_{max} [c]	Production of metabolites			
						Trifluoroethanol			
						K_m [a]	V_{max} [c]		
Fluroxene	None	0.9	0.06	0.8	4	1.4	4		
	BP	0.9	0.06	1.1	4	1.8	5		
	MC	0.9	0.06	1.0	3	1.3	5		
	PB	0.9	0.13	0.6	13	0.7	14		
TFEE	None	1.5	0.07	0.6 11	2 6	1.7 17	3 12		
	BP	1.7	0.06	1.3 14	2 5	1.0 7	4 8		
	MC	2.5	0.09	0.9 11	1 4	2.3 13	4 10		
	PB	1.8	0.17	1.9	10	1.4	23		
						Free F^-		Acid-labile F^-	
						K_m [a]	V_{max} [c]	K_m [a]	V_{max} [c]
Methoxy-flurane	None	0.5	0.06	0.10	3	0.04 0.4	0.15 0.36	0.06 0.7	0.31 0.64
	MC	0.6	0.07	0.25	4	0.04 0.9	0.05 0.17	0.08 0.9	0.28 0.84
	PB	0.3	0.16	0.07	13	0.1	1.01	0.2	2.93
Enflurane	None	0.5	0.06	0.15	3	N.D			
	MC	0.5	0.05	0.35	4	N.D			
	PB	0.5	0.16	0.23	18	0.4	0.13		

[a] mM Values quoted for K_s and K_m.

[b] Maximum extent of difference spectrum.

[c] Maximum rates of NADPH oxidation, TFE production or fluoride production in nmol/mg microsomal protein/min.

The K_s values obtained from the linear Hanes plots for the binding of TFEE to cytochromes P-450 of uninduced and polycyclic hydrocarbon induced microsomes are of the order of the smaller K_m values for NADPH oxidation or trifluoroethanol production. For the binding and metabolism of TFEE by phenobarbital induced microsomes, $K_s = K_m$ (NADPH oxidation) = K_m (TFE production)(Table 26).

Values of K_s for methoxyflurane binding to cytochromes P-450 in uninduced and 3-methylcholanthrene induced microsomes are approximately equal to the larger K_m values for free, and acid-labile fluoride production. The K_m values for NADPH oxidation by these microsomes in the presence of methoxyflurane do not correlate with K_s values for binding or with K_m values for production of fluoride from methoxyflurane. In addition, Hanes plots for NADPH oxidation do not reflect the biphasic nature of the plots for fluoride production. For the binding and metabolism of methoxyflurane by phenobarbital induced microsomes, the K_s and K_m values range between 0.07 mM and 0.3 mM.

The ratios of V_{max} values for acid-labile fluoride and free fluoride production from methoxyflurane by hepatic microsomes are presented in Table 27. It appears that 3-methylcholanthrene induction results in greater production of acid-labile fluoride relative to free fluoride whereas phenobarbital induction does not ($p > 0.05$).

For enflurane, correlation between the values of K_s

and K_m (NADPH oxidation) is not observed; with phenobarbital induced microsomes, $K_s = K_m$ (free F^- production)(Table 26).

Table 27. Effect of induction of cytochromes P-450 on relative proportions of acid-labile and free fluoride produced during hepatic microsomal metabolism of methoxyflurane

Induction	$\frac{\text{Acid-labile } F^-}{\text{Free } F^-}$	
None	2.1[a]	1.8[b]
MC	5.6[a] ▼	4.9[b] ▼
PB	2.9	

[a] Ratio of smaller V_{max} values.

[b] Ratio of larger V_{max} values.

Table 28. Relationship between oxidation of NADPH and production of metabolites during metabolism of anaesthetic agents by phenobarbital induced microsomes

Anaesthetic agent	$\frac{\text{NADPH oxidized}}{\text{Metabolite formed}}$	
Fluroxene	0.9	
TFEE	0.4	
Methoxyflurane	13.0[a]	3.0[b]
Enflurane	140	

[a] Ratio calculated for free F^- production.

[b] Ratio calculated for total F^- production.

The stoichiometry of NADPH oxidation and metabolite production for the anaesthetic agents is demonstrated in Table 28 by means of the ratios of V_{max} values for NADPH oxidation and trifluoroethanol or fluoride production in phenobarbital induced microsomes. Although the experiments leading to the values in Table 28 were not designed to demonstrate accurately the stoichiometry of NADPH consumed and product formed, they should nonetheless provide a good approximation of this stoichiometry; in all cases, the initial linear portion of the progress curve for the reaction was utilized to calculate rates of reaction.

Table 29. Relative effects of metyrapone and SKF 525A on the metabolism of anaesthetic agents by phenobarbital induced microsomes

Anaesthetic agent	Inhibition of production of metabolites Metyrapone[a] / SKF 525A
Fluroxene (6.0 mM)	2.3
TFEE (6.0 mM)	1.3
Methoxyflurane (1.7 mM) (free F^-)	1.6
(total F^-)	1.5
Enflurane (2.4 mM)	1.5

[a] Ratios of % inhibition of TFE or F^- production by metyrapone to that by SKF 525A at 50 μM concentrations of inhibitors (calculated from Tables 16, 20 and 21).

Inhibition of production of metabolites. The ratios of the extents of inhibition by metyrapone to that by SKF 525A for the production of trifluoroethanol or fluoride from the anaesthetic agents in phenobarbital induced microsomes are presented in Table 29. In all cases metyrapone inhibits production of metabolite to a greater extent than SKF 525A.

B. THE INTERACTION OF 2,2,2-TRIFLUOROETHANOL WITH HEPATIC ENZYMES

1. The interaction of 2,2,2-trifluoroethanol with cytochromes P-450

The interaction of trifluoroethanol with cytochromes P-450 was investigated with microsomes from phenobarbital induced animals.

Trifluoroethanol binds to hepatic microsomal cytochromes P-450 resulting in the appearance of a type I difference spectrum (Figure 38). The extent of the difference spectrum increases with increasing concentrations of trifluoroethanol until saturating concentrations are reached; Hanes plots of the data from such studies are linear (Figure 39) and allow calculation of K_s and ΔA_{max} values (Table 30).

Carbon monoxide inhibitable NADPH oxidation by phenobarbital induced microsomes increases slightly in the presence of trifluoroethanol; NADPH oxidation increases with increasing concentrations of this alcohol until saturating concentrations are reached. K_m and V_{max} values for microsomal NADPH

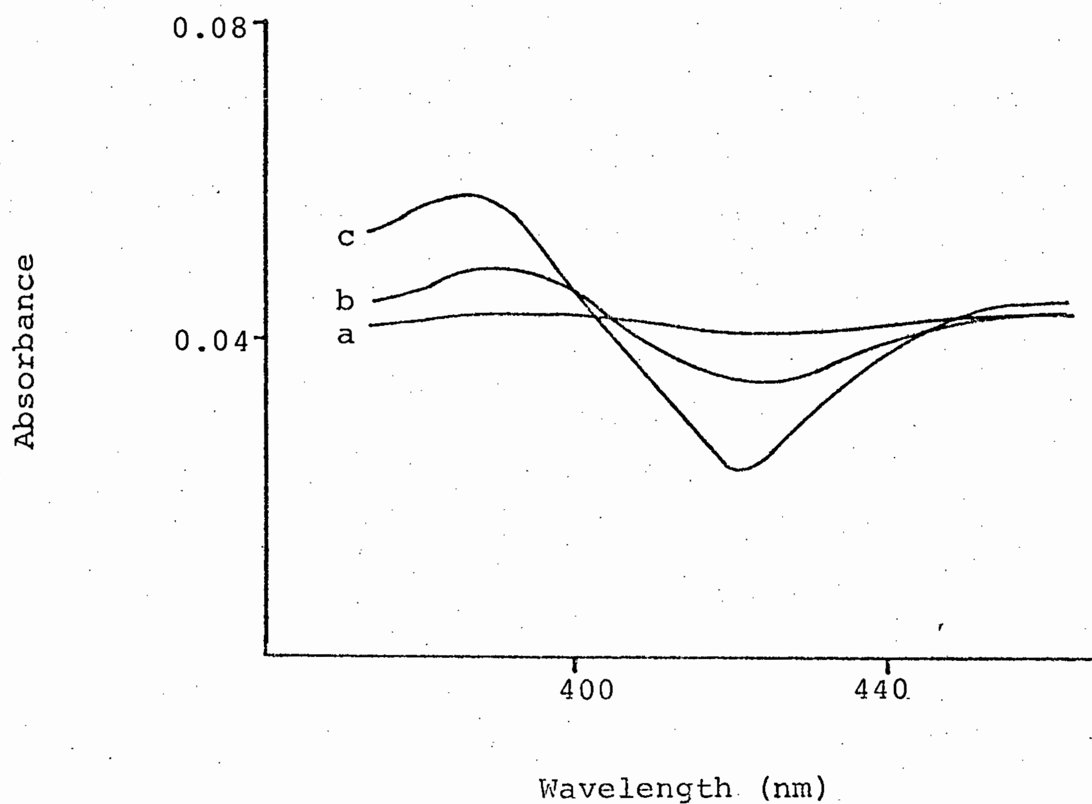


Figure 38. Type I difference spectral changes of 2,2,2-trifluoroethanol with phenobarbital induced microsomes. a, Baseline; b, 4.6 mM trifluoroethanol; c, 46 mM trifluoroethanol. Microsomal protein, 2.0 mg/ml in 0.02 M Tris-HCl, pH 7.4; performed at 25°.

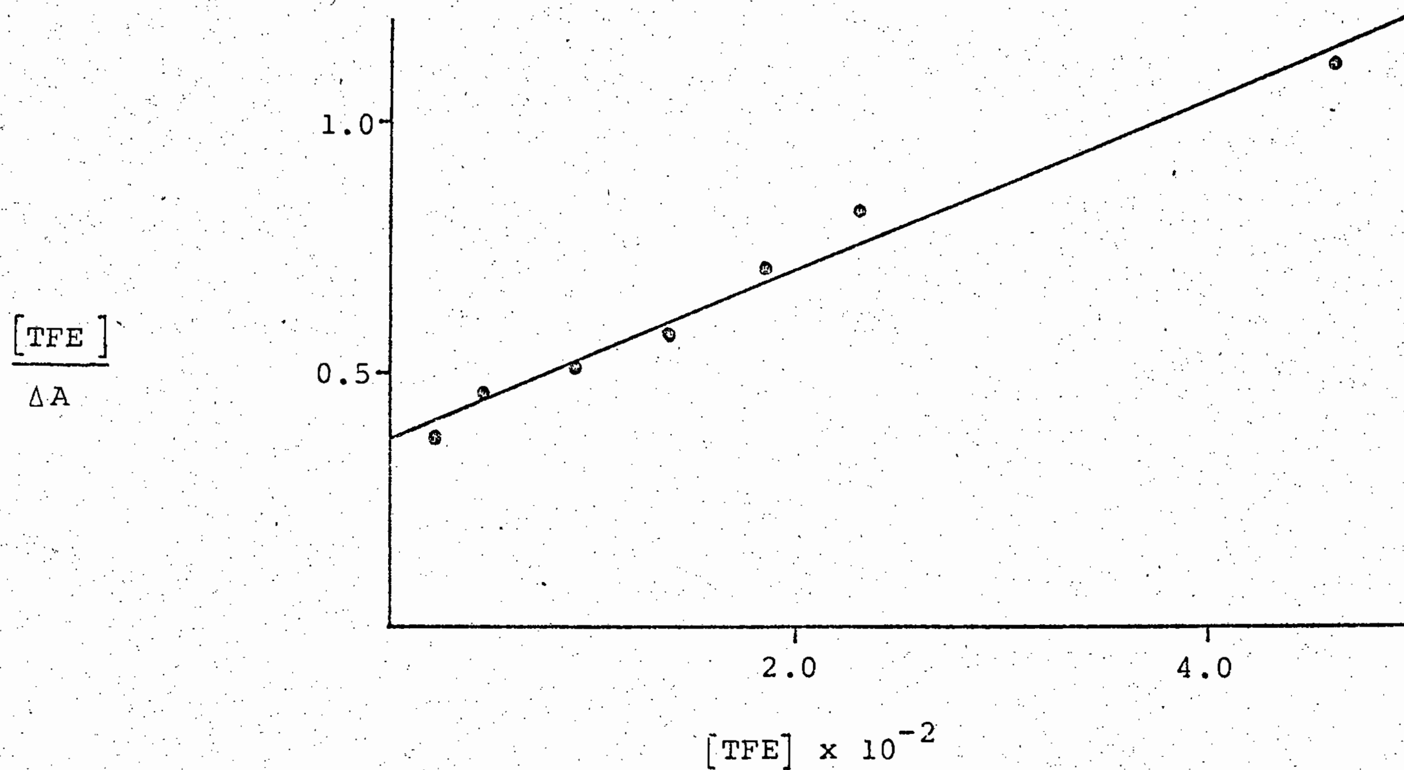


Figure 39. Hanes plot of difference spectra of 2,2,2-trifluoroethanol with phenobarbital induced microsomes. [TFE], molar. Experimental conditions as for Figure 38.

Table 30. The interaction of 2,2,2-trifluoroethanol with cytochromes P-450 in phenobarbital induced hepatic microsomes

Interaction	Affinity constant (mM)	Maximum extent of interaction
Type I difference spectrum	K_s: 23±4	ΔA_{max}: 0.06±0.01
NADPH oxidation	K_m: 25±11	V_{max}: 3.4±1.0 nmol NADPH/mg microsomal protein/min
Inhibition of fluroxene difference spectrum	K_i: 33±7	
Inhibition of *p*-nitroanisole O-demethylation	K_i: 35	

oxidation in the presence of trifluoroethanol (Table 30) were obtained from Hanes plots of the data (Figure 40).

In addition, trifluoroethanol inhibits the interactions of other compounds with microsomal cytochromes P-450 (Table 30). Trifluoroethanol inhibits the formation of a type I difference spectrum by fluroxene (Figure 41)* and inhibits the O-demethylation of *p*-nitroanisole (Figure 42). Inhibition is of a competitive type in both cases.

* Ethanol at concentrations between 2.6 - 100 mM has no effect on the fluroxene difference spectrum (*e.g.* Figure 41).

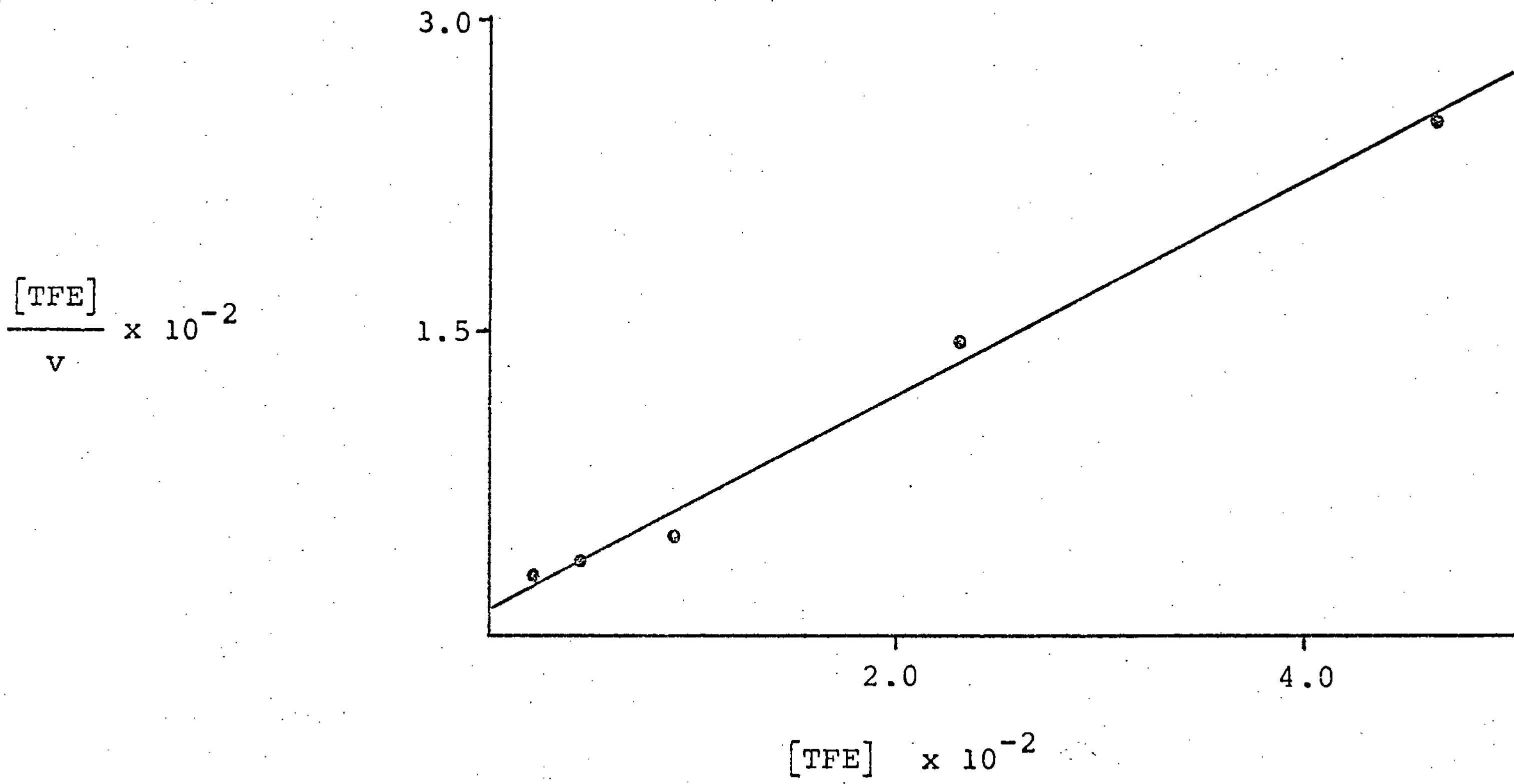


Figure 40. Hanes plot of NADPH oxidation by phenobarbital induced microsomes in the presence of 2,2,2-trifluoroethanol. [TFE], molar; v, nmol NADPH/mg microsomal protein/min. NADPH (0.12 mM), microsomal protein (2.0 mg/ml) in 0.02 M Tris-HCl, pH 7.4; reaction temperature, 27°.

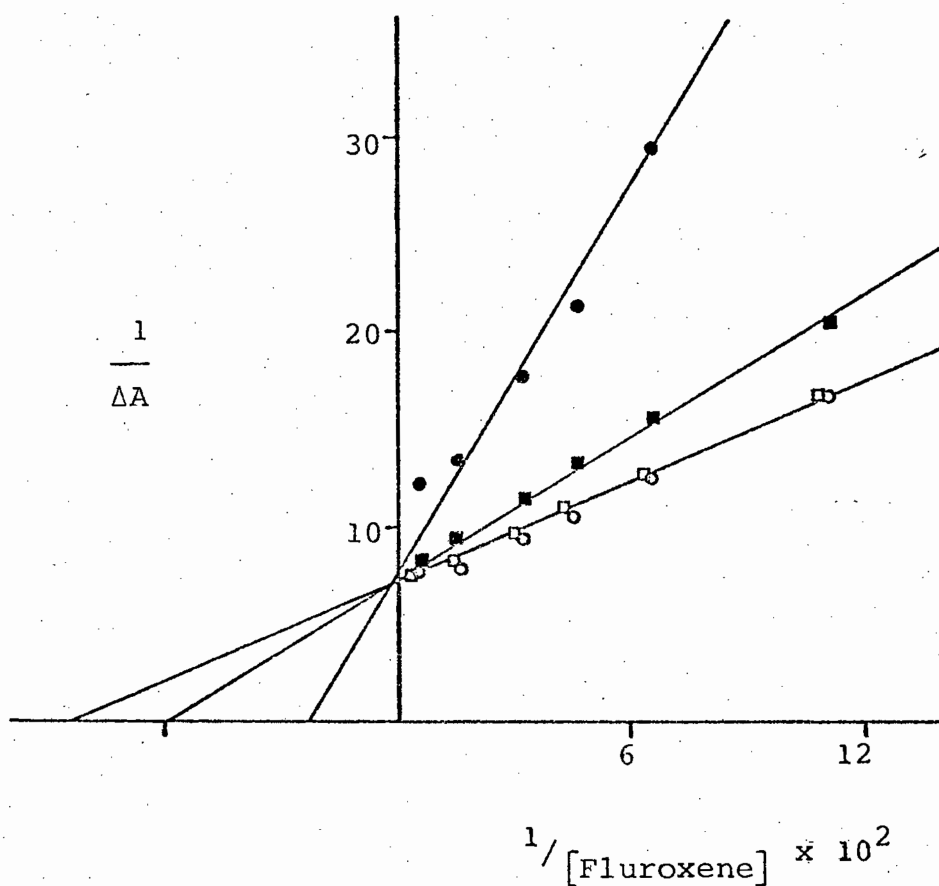


Figure 41. Lineweaver-Burk plots of inhibition by 2,2,2-trifluoroethanol of the type I difference spectral changes of fluroxene with phenobarbital induced microsomes. No trifluoroethanol (□), 6.6 mM trifluoroethanol (■), 40 mM trifluoroethanol (●), and 100 mM ethanol (○) present in both sample and reference cuvettes; [Fluroxene], molar; experimental conditions as for Figure 38.

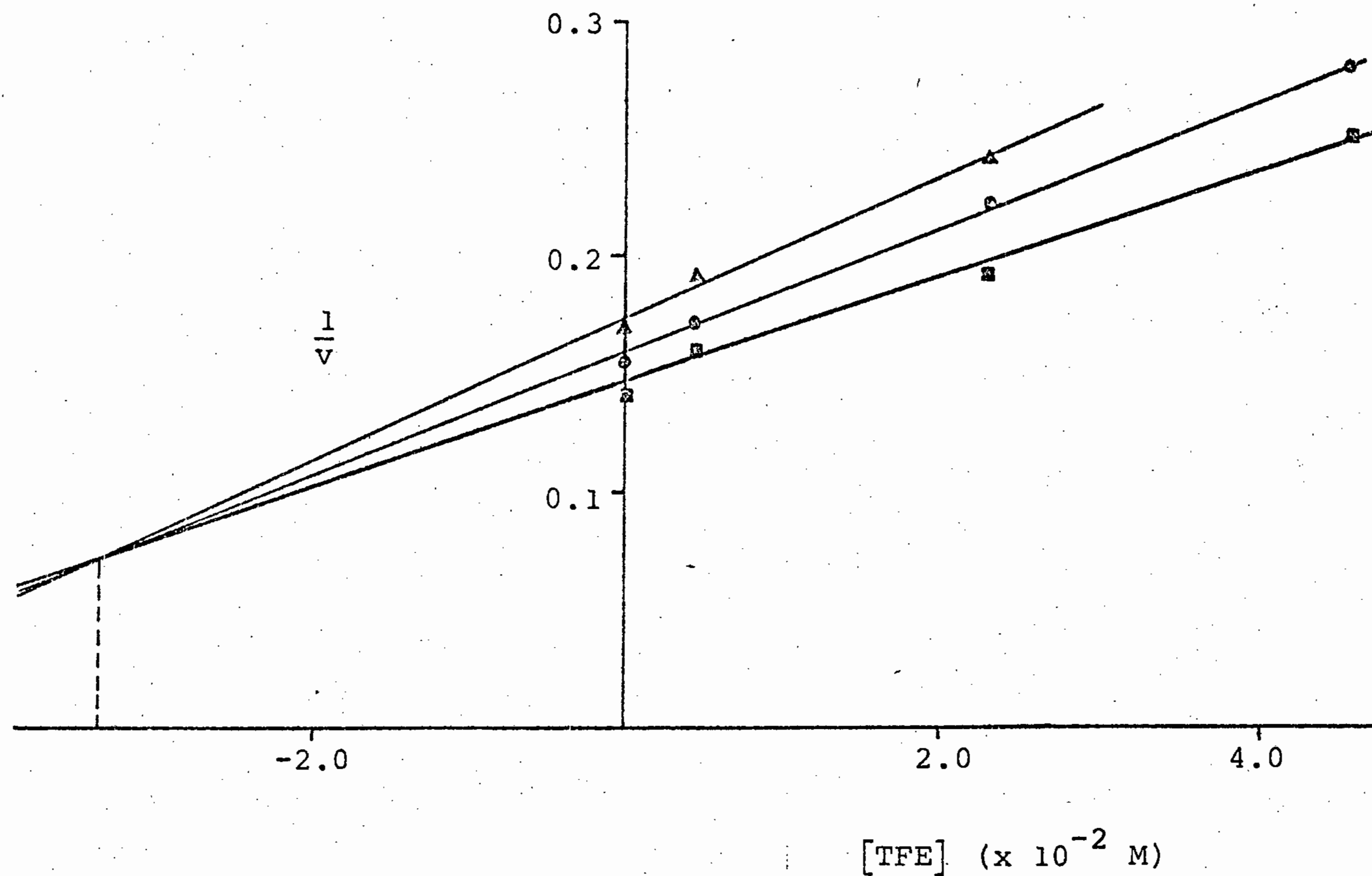


Figure 42. Dixon plots of inhibition by 2,2,2-trifluoroethanol of the O-demethylation of p-nitroanisole by phenobarbital induced microsomes. Concentration of p-nitroanisole 7×10^{-6} M (▲), 2×10^{-5} M (●) and 7×10^{-5} M (■); v, nmol p-nitrophenol/mg microsomal protein/min. Microsomal protein, 0.5 mg/ml in 0.02 M Tris-HCl, pH 7.4; reaction temperature, 25°.

2. The interaction of 2,2,2-trifluoroethanol with alcohol dehydrogenase

The NAD dependent oxidation of trifluoroethanol by alcohol dehydrogenase was investigated with horse liver alcohol dehydrogenase and the post-microsomal supernatant from rat liver. Trifluoroethanol is apparently not oxidized by horse liver alcohol dehydrogenase confirming the results of Blake *et al.* (117). In addition, oxidation of this alcohol by the soluble fraction of rat liver does not appear to occur although oxidation of ethanol under identical conditions is observed as judged spectrally by increase of NADH concentration in the reaction mixture.

Trifluoroethanol competitively inhibits the oxidation of ethanol by horse liver alcohol dehydrogenase with a K_i value of 6.4×10^{-6}M (Figures 43 and 44).

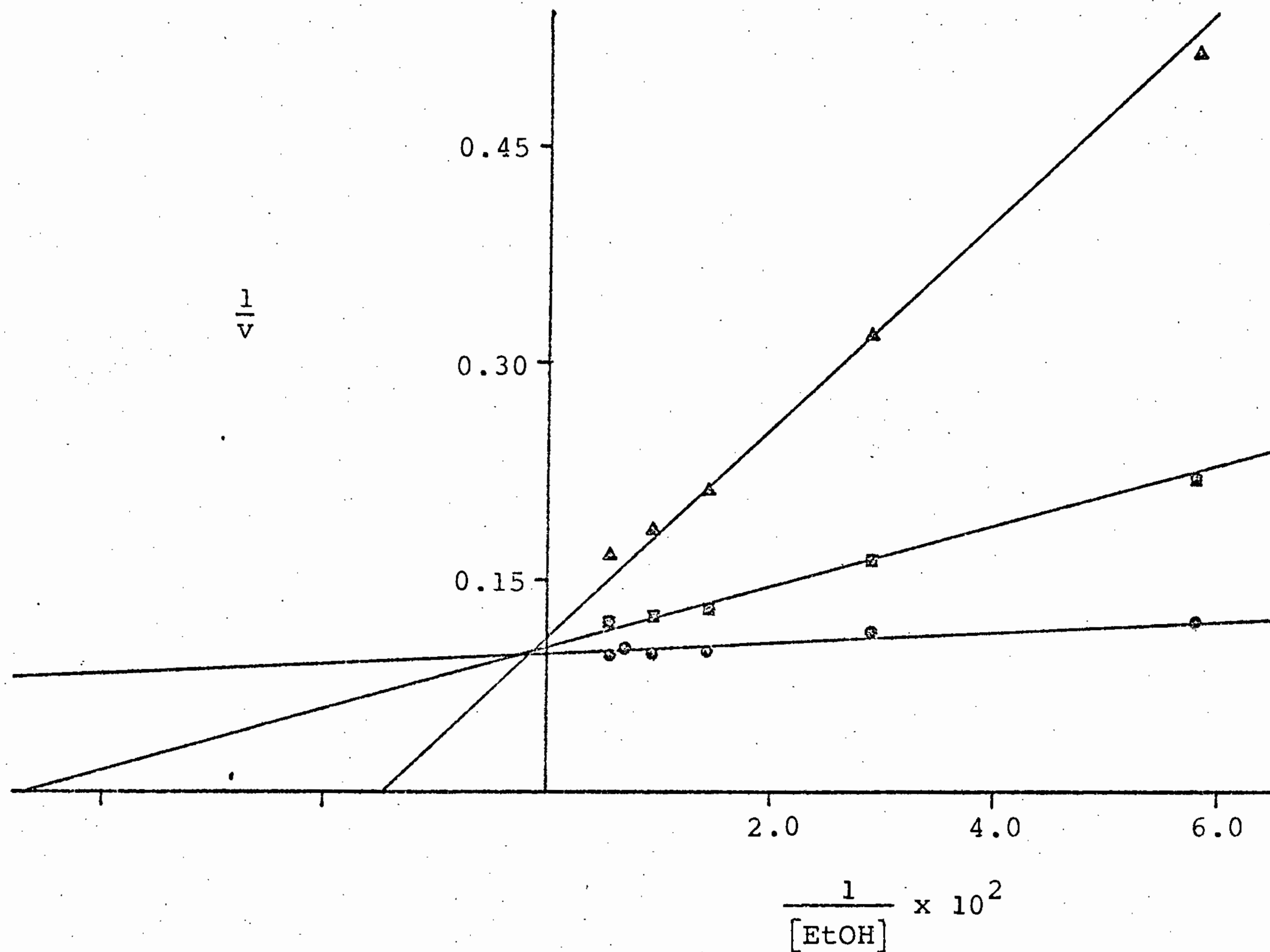


Figure 43. Lineweaver-Burk plots of inhibition by 2,2,2-trifluoroethanol of the reduction of NAD by alcohol dehydrogenase in the presence of ethanol. No trifluoroethanol (●), 2×10^{-5} M trifluoroethanol (■) and 9×10^{-5} M trifluoroethanol (▲) present in reaction mixture; [EtOH], molar; v, nmol NAD/ml/min. NAD, 0.07 mM; ADH, 0.03 U/ml; reaction at 25° in 0.05 M Tris-HCl, pH 8.2.

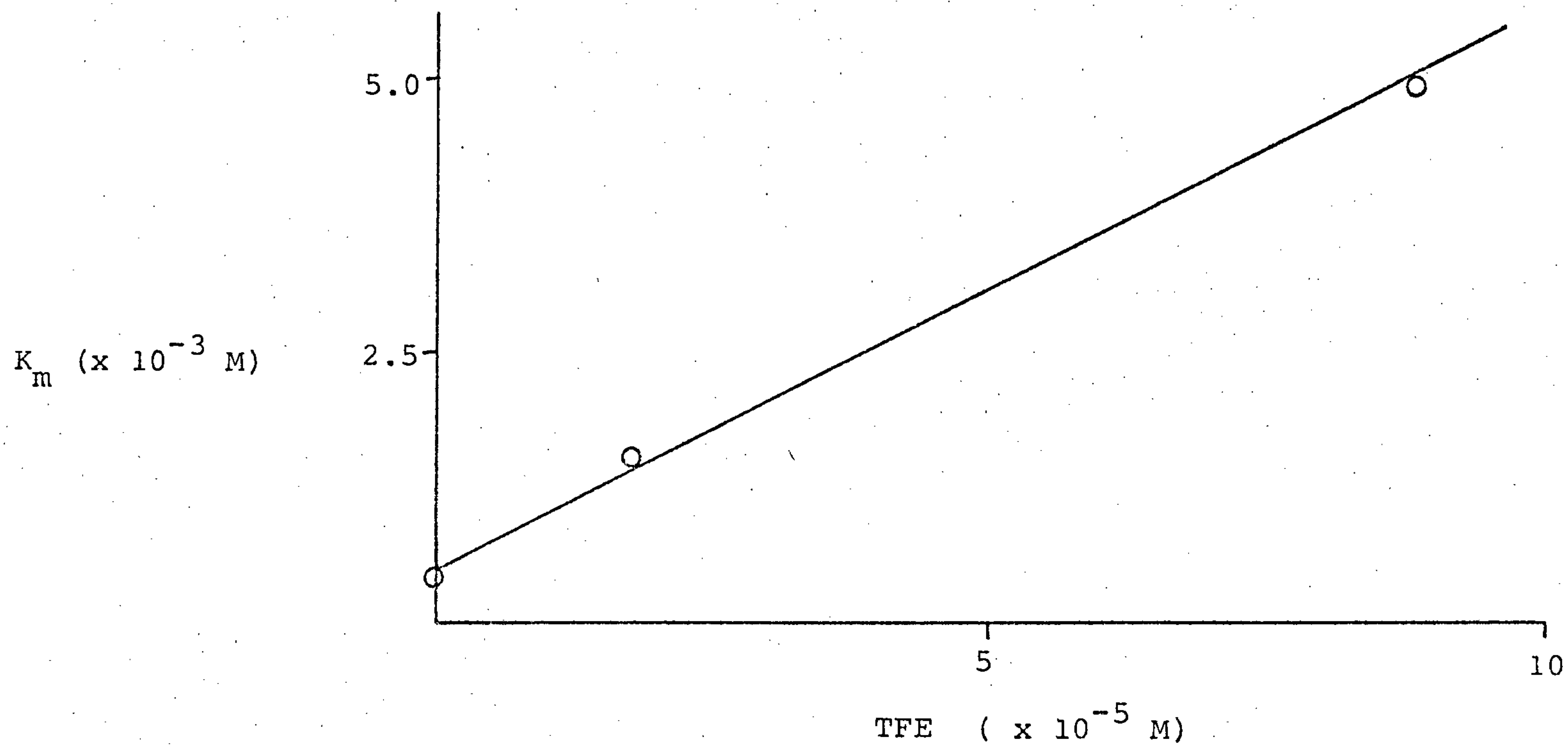


Figure 44. Effect of 2,2,2-trifluoroethanol on K_m values for ethanol with alcohol dehydrogenase. Alcohol dehydrogenase activity measured by monitoring reduction of NAD; experimental conditions as for Figure 43.

3. The interaction of 2,2,2-trifluoroethanol with catalase and xanthine oxidase

Peroxidatic oxidation of trifluoroethanol by catalase in combination with a hydrogen peroxide generating system was investigated by monitoring consumption of oxygen in a reaction mixture comprising catalase, xanthine oxidase, xanthine and trifluoroethanol. Time courses for oxygen uptake of various reaction mixtures are shown in Figure 45, and equations to illustrate the reactions occurring in the system in Figure 46. The maximum theoretical oxygen consumption for peroxidatic oxidation is exhibited by oxidation of xanthine by xanthine oxidase, and zero peroxidatic oxidation (or half the maximum theoretical consumption) is exhibited by oxidation of xanthine by xanthine oxidase in the presence of catalase (180, 181). Oxygen consumptions of reaction mixtures in the presence of trifluoroethanol and in the presence of alcohols known to be peroxidatically oxidized are illustrated (Figure 45).

The effect of trifluoroethanol on the catalytic activities of xanthine oxidase and of catalase was investigated in order to ascertain whether the oxygen uptake of the coupled peroxidatic oxidation system in the presence of trifluoroethanol could be attributed to a phenomenon other than peroxidatic oxidation of this alcohol. Oxidation of xanthine to uric acid by xanthine oxidase is not inhibited by trifluoroethanol or by ethanol at concentrations of about 50 mM (Figure 47). However, decomposition of hydrogen peroxide by catalase is inhibited by trifluoroethanol;

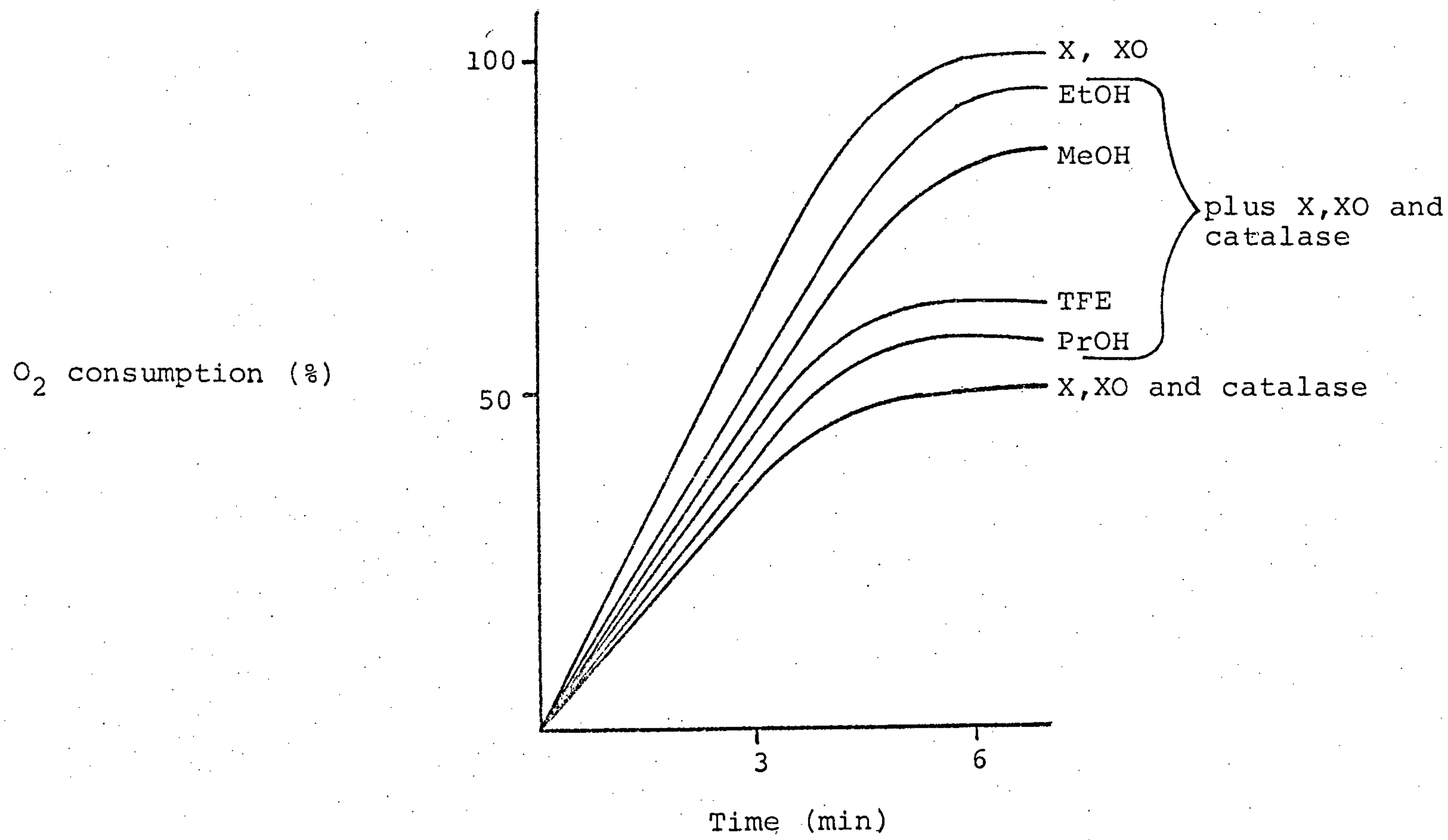
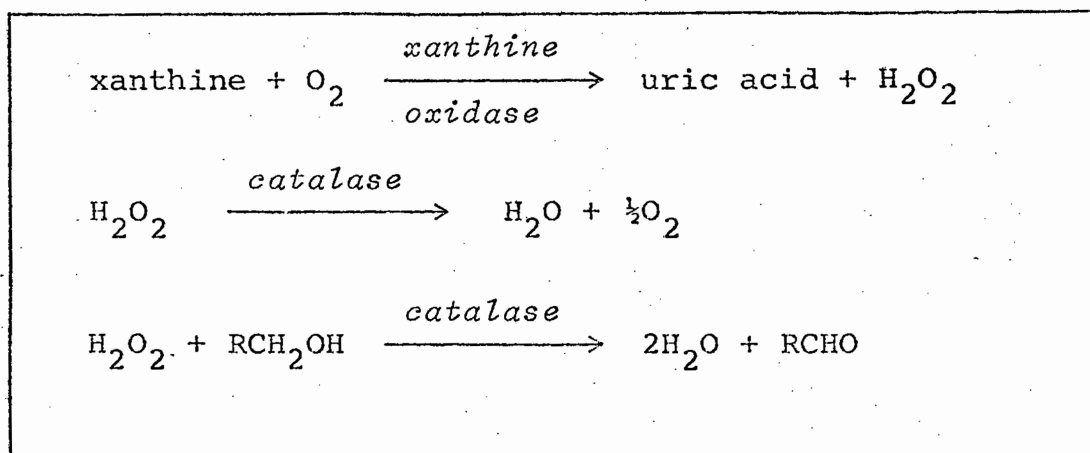


Figure 45. Time courses for consumption of oxygen by a peroxidatic system in the absence and presence of alcohols. Reaction mixtures comprising xanthine (X), xanthine oxidase (XO), catalase and ethanol (EtOH) (44 mM), methanol (MeOH)(44 mM), trifluoroethanol (TFE)(44 mM), or propanol (PrOH)(88 mM). Experimental details in METHODS.

Figure 46. Equations to illustrate reactions occurring in system for peroxidatic oxidation of alcohols



inhibition by ethanol at a similar concentration is not observed (Figure 47).

Inhibition of the catalase dependent decomposition of hydrogen peroxide by trifluoroethanol was studied at different concentrations of trifluoroethanol. A Dixon plot of the data from these experiments is presented in Figure 48. The inhibition of catalase by trifluoroethanol appears to be non-competitive and exhibits a K_i value of 28 mM.

4. The interaction of 2,2,2-trifluoroethanol with UDP-glucuronyl transferase

Trifluoroethanol does not appear to interact in any way with rat hepatic UDP-glucuronyl transferase *in vitro*. Glucuronidation of trifluoroethanol by hepatic microsomes is not observable by either of the two assay techniques under any of the experimental conditions described although glucuronidation of *p*-nitrophenol is observed under the same conditions. Furthermore, trifluoroethanol does not inhibit the glucuronidation of *p*-nitrophenol measured in the presence of 0.04% Triton X-100.

The possibility that glucuronidation of trifluoroethanol occurs in the kidneys was investigated with a preparation of kidney microsomes which had been activated by storage at -15° for 12 days prior to use. In this experiment, glucuronidation of trifluoroethanol (assayed by gas-liquid chromatography) was not observed although glucuronidation of *p*-nitrophenol by this microsomal preparation was measureable spectrally.

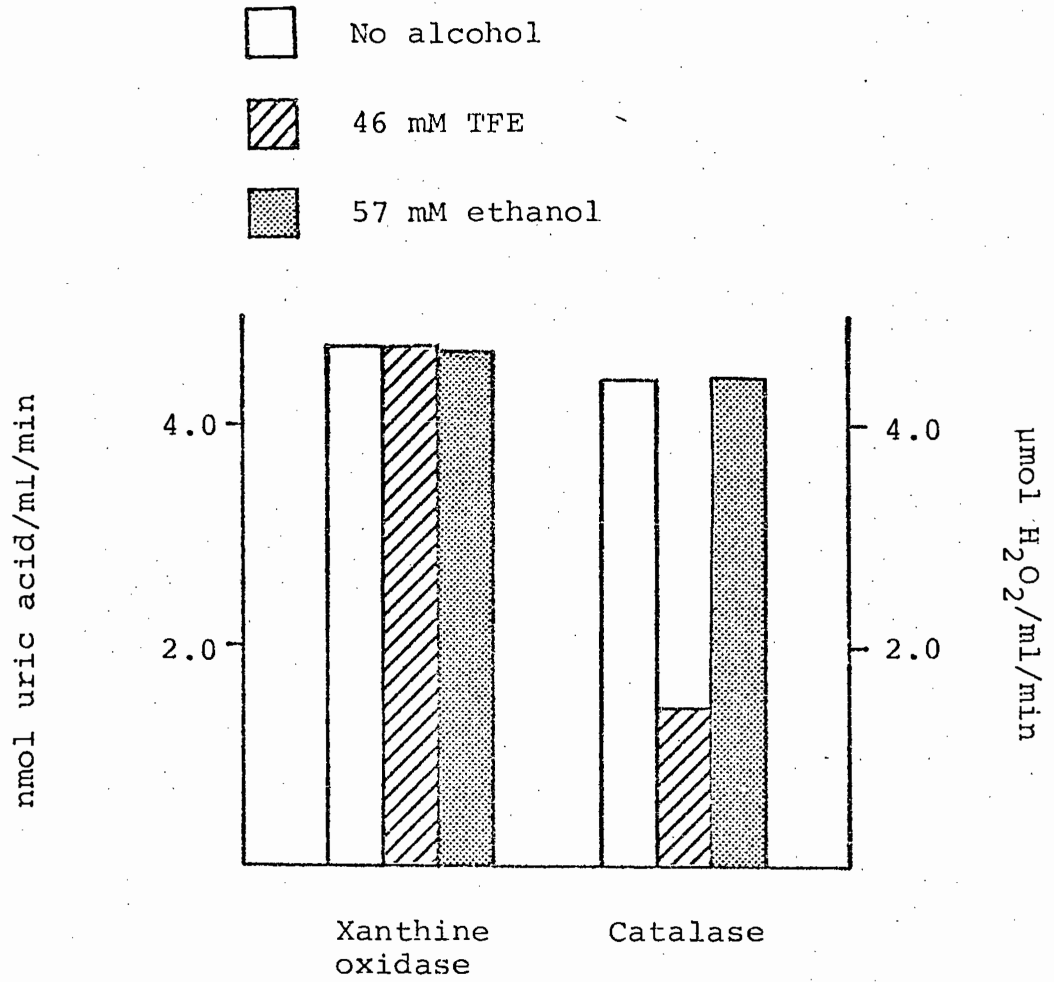


Figure 47. Effects of 2,2,2-trifluoroethanol and ethanol on the activities of xanthine oxidase and catalase. Experimental details in METHODS.

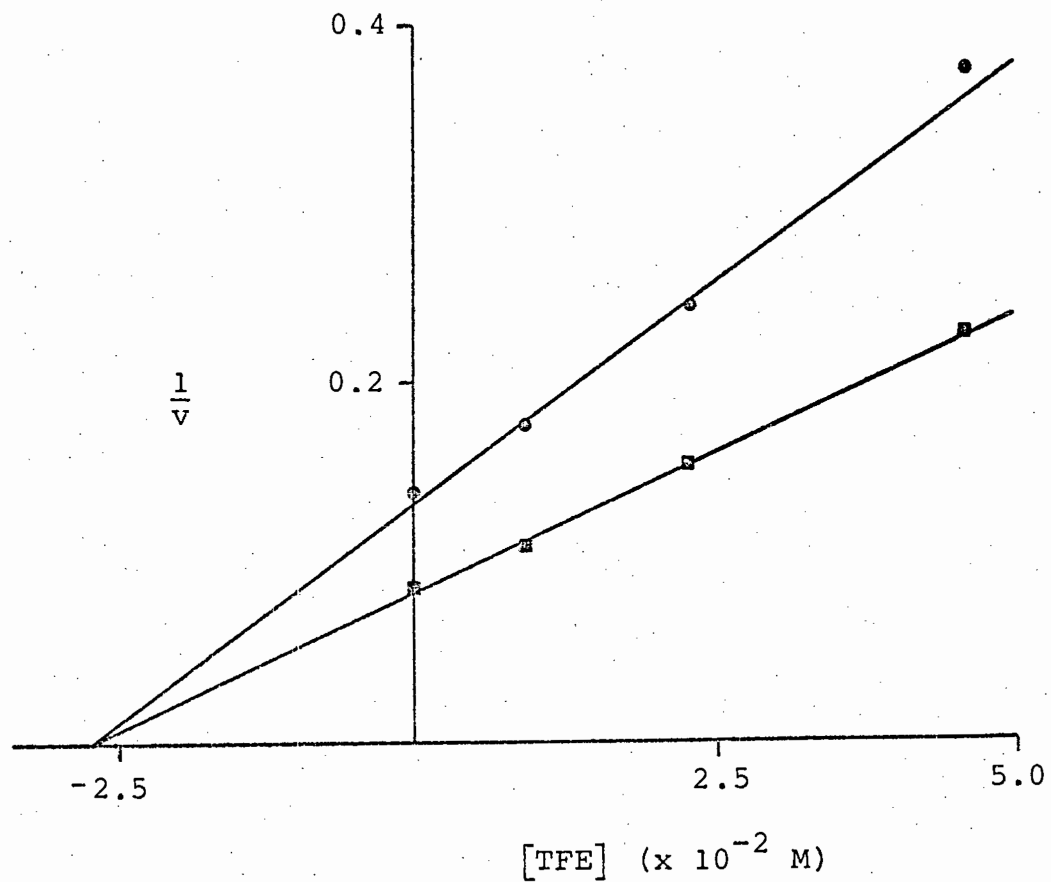


Figure 48. Dixon plots of inhibition by 2,2,2-trifluoroethanol of the decomposition of hydrogen peroxide by catalase. Concentration of hydrogen peroxide 1.2 x 10^{-2} M (●) and 2.4 x 10^{-2} M (■); v, μmol H_2O_2/ml/min. Experimental details in METHODS.

C. THE EFFECTS OF VOLATILE ANAESTHETIC AGENTS ON LEVELS OF HEPATIC DRUG METABOLIZING ENZYMES AND GLUTATHIONE *IN VIVO*

The effects of methoxyflurane and enflurane anaesthesia on levels of drug metabolizing enzymes in subcellular fractions isolated from livers of animals after anaesthesia were investigated. In considering results obtained from these experiments trends with respect to time in the effects of anaesthesia during the period of treatment and changes with respect to the unanaesthetized animals are sought. The results obtained after anaesthesia of animals with methoxyflurane at anaesthetic (1.0 MAC) and sub-anaesthetic (0.1 MAC) concentrations are shown respectively in Tables 31 and 32 in which averages and mean deviations are reported for determinations with liver fractions from three anaesthetized or two unanaesthetized animals in each case. Included in the tables is the activity of microsomal glucose-6-phosphatase, an enzyme which is not involved in drug metabolism and which is not usually inducible by compounds which induce drug metabolizing enzymes (181). Results of investigations with enflurane (Tables 33 and 34) are presented in the same manner as those with methoxyflurane.

During anaesthesia with methoxyflurane at 1.0 MAC for one to four days (3 hr/day) animals were observed to gain slightly less weight than unanaesthetized control animals. There appear to be no trends in levels or activities of any of the enzymes investigated during the four day period of treatment with methoxyflurane at 1.0 MAC (Table 31). Enzyme levels

and activities generally remain unchanged relative to those of unanaesthetized animals during the one to four days of treatment with methoxyflurane; exceptions to this occur only on isolated days. Hepatic glutathione levels increase in unanaesthetized control animals as well as during the treatment with anaesthetic; there are no significant differences between these two groups on any one day. NADPH-cytochrome c reductase activity is increased after the third day of anaesthesia only; whilst after the fourth day of anaesthesia there are apparent decreases in levels of hepatic microsomal cytochromes P-450, p-nitroanisole demethylation, 3,4-benzpyrene hydroxylation and glucose-6-phosphatase activity relative to unanaesthetized control values. These decreases, however, may be related to the fact that this group of anaesthetized and control rats were not starved before sacrifice. The apparent degradation of microsomal enzymes is being investigated further.

Anaesthesia of animals with methoxyflurane at 0.1 MAC for four to sixteen days (6 hr/day) does not affect the levels or activities of any of the hepatic enzymes investigated relative to unanaesthetized controls (Table 32). Hepatic glutathione levels are also not significantly changed ($p > 0.05$). There are generally no differences in gain of body weight between anaesthetized and unanaesthetized animals at 0.1 MAC methoxyflurane.

The effects of anaesthesia with enflurane at anaesthetic concentrations (1.0 MAC) on hepatic enzymes and glutathione are shown in Table 33. During the one to four days of anaesthesia (3 hr/day) no trends are noted in the results of any of the assays, and levels or activities of hepatic drug metabolizing enzymes and glutathione generally remain the same as those in the livers from unanaesthetized animals. The only change brought about by anaesthesia with enflurane at 1.0 MAC appears to be the increase in microsomal p-nitroanisole O-demethylation, a change which is not accompanied by any increase in cytochromes P-450 or NADPH-cytochrome c reductase. Differences in gain of body weight between anaesthetized and unanaesthetized animals become evident only after four days of anaesthesia with enflurane (1.0 MAC, 3 hr/day) when control animals exhibit a slightly greater weight gain than anaesthetized animals.

Anaesthesia with enflurane at 0.1 MAC for four to sixteen days (6 hr/day) does not affect the levels or activities of any of the hepatic enzymes investigated relative to the values obtained for unanaesthetized animals excepting for day twelve (Table 34). The apparent increases relative to unanaesthetized controls in enzyme levels and activities on day twelve cannot be ascribed to induction; the enzyme levels and activities of day twelve are the same ($p > 0.05$) as those for day eight which were not significantly different from control values for that day. Microsomal p-nitroanisole O-demethylation activity in livers from unanaesthetized animals decreased continuously from the fourth to

sixteenth day of anaesthesia; a similar decrease in this enzyme activity was observed in anaesthetized animals only after sixteen days of anaesthesia. Microsomal 3,4-benzpyrene hydroxylation activity decreased similarly in both anaesthetized and unanaesthetized animals from days four to sixteen. The decreases in cytochromes P-450 mediated reactions are not accompanied by decreases in cytochromes P-450 or NADPH-cytochrome c reductase. No differences may be observed in gain of body weight between anaesthetized and unanaesthetized animals at 0.1 MAC enflurane.

Table 31. Effect of anaesthesia with methoxyflurane at 1.0 MAC on concentrations and activities of hepatic enzymes and glutathione

Assay[a]	Methoxyflurane/O_2 (3 hr/day)				No anaesthetic agent/O_2 (3 hr/day)			
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 1	DAY 2	DAY 3	DAY 4
Cytochromes P-450 (nmol/mg)	0.81±.13	0.87±.16	0.91±.12	0.62±.02 ▼	0.65±.11	0.87±.05	0.94±.01	0.99±.04
Cytochrome $\underline{b}_5$ (nmol/mg)	0.41±.06	0.46±.10	0.47±.06	0.39±.01	0.32±.02	0.36±.01	0.42±.02	0.49±.03
NADPH-cytochrome $\underline{c}$ reductase (U/mg) x10^{-1}	0.68±.14	0.79±.14	0.87±.11 ■	0.73±.07	0.52±.04	0.78±.04	0.57±.03	0.63±.08
$\underline{p}$-Nitroanisole demethylation (nmol/mg/min)	1.13±.24	1.05±.31	1.00±.16	0.61±.23 ■	0.73±.21	0.98±.02	0.97±.09	1.14±.13
3,4-Benzpyrene hydroxylation (nmol/mg/min) x10^{-1}	0.49±.20	0.45±.10	0.34±.03	0.23±.02 ▼	0.23±.01	0.30±.03	0.27±.04	0.38±.02
UDP-glucuronyl transferase (nmol/mg/min)	19±3	19±1	18±2	21±2	17±1	16±2	19±1	17±2
Glucose-6-phosphatase (μg P_i/mg/min)	3.7±0.2	5.0±0.3	4.2±0.4	2.9±0.4 ▼	3.3±0.1	4.3±0.1	5.5±0.3	3.9±0.4
Glutathione-S-transferase (nmol/mg/min)	70±7	51±7	54±3	69±3	70±4	56±6	59±3	73±3
Glutathione (μg/100 mg wet liver)	97±6	120±14	121±11	167±9	119±7	151±11	-	153±5

[a] Levels of enzymes reported /mg microsomal protein or /mg cell supernatant. For details see METHODS.

Table 32. Effect of anaesthesia with methoxyflurane at 0.1 MAC on concentrations and activities of hepatic enzymes and glutathione

Assays[a]	Methoxyflurane/O_2 (6 hr/day)				No anaesthetic agent/O_2 (6 hr/day)			
	DAY 4	DAY 8	DAY 12	DAY 16	DAY 4	DAY 8	DAY 12	DAY 16
Cytochromes P-450 (nmol/mg)	1.03±.07	0.86±.18	0.89±.09	1.15±.08	0.96±.03	0.68±.04	0.91±.12	1.03±.08
Cytochrome $\underline{b}_5$ (nmol/mg)	0.44±.02	0.44±.05	0.41±.02	0.50±.04	0.35±.00	0.36±.03	0.39±.03	0.51±.04
NADPH-cytochrome $\underline{c}$ reductase(U/mg)x10^{-1}	0.60±.05	0.66±.05	0.64±.03	0.88±.01	0.62±.02	0.60±.11	0.56±.10	0.91±.01
$\underline{p}$-Nitroanisole demethylation (nmol/mg/min)	0.57±.05	0.59±.11	0.37±.06	0.51±.06	0.57±.10	-	0.39±.04	0.50±.02
3,4-Benzpyrene hydroxylation (nmol/mg/min)x10^{-1}	0.19±.01	0.23±.04	0.19±.02	0.30±.02	0.23±.02	0.23±.04	0.23±.04	0.32±.03
UDP-glucuronyl transferase (nmol/mg/min)	21±3	17±0	19±1	20±1	15±2	18±5	16±3	17±2
Glucose-6-phosphatase (μg P_i/mg/min)	8.7±0.9	7.5±0.4	7.5±0.2	6.9±0.5	7.4±0.1	8.3±0.2	7.6±0.8	6.6±0.3
Glutathione-S-transferase (nmol/mg/min)	71±6	69±4	50±6	70±3	65±3	74±6	63±3	74±11
Glutathione (μg/100 mg wet liver)	129±21	187±9	123±5	172±16	136±3	161±4	127±2	135±10

[a] Levels of enzymes reported/mg microsomal protein or /mg cell supernatant. For details see METHODS.

Table 33. Effect of anaesthesia with enflurane at 1.0 MAC on concentrations and activities of hepatic enzymes and glutathione

Assays[a]	Enflurane/O_2 (3 hr/day)				No anaesthetic agent/O_2 (3 hr/day)			
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 1*	DAY 2	DAY 3	DAY 4
Cytochromes P-450 (nmol/mg)	1.17±.10	1.00±.12	1.25±.15	1.13±.14	1.13	0.78±.09	0.96±.20	0.90±.07
Cytochrome b_5 (nmol/mg)	0.49±.03	0.41±.03	0.54±.04	0.47±.07	0.45	0.37±.02	0.38±.03	0.42±.05
NADPH-cytochrome c reductase (U/mg) x10^{-1}	0.71±.09	0.68±.06	0.65±.05	0.79±.05	0.67	0.54±.02	0.45±.03	0.83±.08
p-Nitroanisole demethylation (nmol/mg/min)	0.80±.24	0.69±.05	0.69±.08	1.24±.11 ▼	0.71	0.68±.30	0.50±.02	0.75±.09
3,4-Benzpyrene hydroxylation (nmol/mg/min) x10^{-1}	0.53±.04	0.23±.02	0.45±.04	0.53±.02	0.49	0.23±.04	0.23±.08	0.57±.02
UDP-glucuronyl transferase (nmol/mg/min)	16±1	11±2	21±4	15±2	12	15±6	24±1	18±0
Glucose-6-phosphatase (μg P_i/mg/min)	5.1±0.8	5.4±0.5	6.2±0.2	6.1±0.5	6.3	6.5±0.8	7.3±0.2	6.3±0.1
Glutathione-S-transferase (nmol/mg/min)	51±9	62±9	56±7	61±6	64	61±0	52±1	61±3
Glutathione (μg/100 mg wet liver)	128±21	115±1.4	123±11	99±12	120	109±8	109±3	93±1

[a] Levels of enzymes reported /mg microsomal protein or /mg cell supernatant. For details see METHODS.

* Values for one animal.

Table 34. Effect of anaesthesia with enflurane at 0.1 MAC on concentrations and activities of hepatic enzymes and glutathione

Assays[a]	Enflurane/O_2 (6 hr/day)				No anaesthetic agent/O_2 (6 hr/day)			
	DAY 4	DAY 8	DAY 12	DAY 16	DAY 4	DAY 8	DAY 12	DAY 16
Cytochromes P-450 (nmol/mg)	1.05±.06	1.19±.01	1.13±.04	0.99±.05	1.16±.05	1.24±.03	0.81±.09	0.99±.02
Cytochrome b5 (nmol/mg)	0.38±.01	0.53±.04	0.51±.00 ■	0.48±.04	0.46±.04	0.52±.06	0.39±.03	0.47±.00
NADPH-cytochrome c reductase (U/mg) x10^{-1}	0.62±.08	0.64±.02	0.65±.02	0.68±.06	0.62±.06	0.60±.11	0.56±.10	0.65±.03
p-Nitroanisole demethylation (nmol/mg/min)	1.19±.11	1.27±.18	1.28±.11 ▼	0.69±.07	1.26±.09	0.99±.04	0.64±.10	0.50±.02
3,4-Benzpyrene hydroxylation (nmol/mg/min) x10^{-1}	0.38±.03	-	0.30±.02	0.15±.02	0.38±.03	-	0.27±.02	0.15±.02
UDP-glucuronyl transferase (nmol/mg/min)	14±1	17±2	20±3	17±1	15±2	18±5	16±3	15±1
Glucose-6-phosphatase (µg P_i/mg/min)	7.5±0.4	8.1±0.1	9.1±0.4	7.3±0.5	8.9±0.7	9.6±0.1	6.5±0.8	6.7±0.2
Glutathione-S-transferase (nmol/mg/min)	51±5	71±6	74±2 ■	65±5	60±5	74±6	63±3	60±5
Glutathione (µg/100 mg wet liver)	80±2	127±3	133±6	112±11	97±3	123±13	123±17	133±25

[a] Levels of enzymes reported /mg microsomal protein or /mg cell supernatant. For details see METHODS.

IV. DISCUSSION

Elucidation of the pathways by which volatile anaesthetics are metabolized and identification of the enzymes involved in these pathways is a prerequisite to an understanding not only of the effectiveness of the compounds as anaesthetic agents but also of their potential as toxic compounds. Information on the metabolism of these compounds and their effect on drug metabolizing enzymes is necessary if safe clinical usage is to be assured. Investigations of the interactions of anaesthetic agents with drug metabolizing enzymes are relevant for both patients and operating theatre personnel, the former undergoing acute exposure at anaesthetic concentrations of agent and the latter undergoing chronic exposure at very low concentrations of agent.

Study of the metabolism of volatile anaesthetic agents or of any other xenobiotic is of importance not only for an understanding of the effects of that particular compound but also for an increased understanding of the mechanisms of drug metabolism in general. In this regard, the four anaesthetic agents under investigation may be useful as probes of the functioning of the cytochromes P-450 drug metabolizing system.

Binding of anaesthetic agents to hepatic microsomal cytochromes P-450. The four halogenated ethyl ethers studied all interact with cytochromes P-450 of rat hepatic microsomes to elicit a type I difference spectrum indicative of the binding of these compounds to the protein moiety

of the cytochrome. This confirms the observation of previous workers that methoxyflurane and enflurane give rise to a type I spectrum with phenobarbital induced microsomes (183, 184).

The similarity in binding constants for the four ethers (Table 26) is surprising in view of the importance of the hydrophobic nature of the compound in giving rise to a type I difference spectrum (59, 185). The lipid solubilities of the anaesthetic agents relative to each other are illustrated in Table 35 which demonstrates that methoxyflurane is very much more lipophilic than enflurane or fluroxene (TFEE is presumed to possess solubility coefficients of the order of those for fluroxene).

Table 35. Some physical constants for volatile anaesthetic agents

Anaesthetic agent	Boiling point[a] (^{o}C)	Oil/gas solubility[b] coefficient	Oil/water solubility[a] coefficient
Fluroxene	43.2	48	90
TFEE	50.3	-	-
Methoxyflurane	104.6	970	440
Enflurane	56.5	98	120

[a] From reference 119

[b] From reference 186

In studies of the binding of a series of haloalkanes to hepatic microsomal cytochromes P-450 (185, 187) it was demonstrated that the more lipophilic the haloalkane, the greater was its affinity of binding as measured by the K_s value. It is evident that in the case of the halogenated ethyl ethers factors other than the lipophilic characteristics of the compounds play a major role in determining the affinity of binding to cytochromes P-450.

The linearity of the Hanes plots for difference spectra and the fact that the K_s values remain unchanged by induction of either cytochrome P-450 or P-448 probably indicates that only one species of cytochromes P-450 is involved in the spectrally observable binding of each anaesthetic agent. In contrast, biphasic Hanes and Lineweaver-Burk plots for difference spectra with other halocarbon compounds have been noted as well as changes in K_s values after induction (185, 187).

Involvement of cytochromes P-450 in metabolism of anaesthetic agents. Inhibition of microsomal NADPH oxidation by carbon monoxide demonstrates that the enhanced rate of NADPH oxidation by hepatic microsomes in the presence of the anaesthetic agents arises as a result of interaction of these agents with cytochromes P-450. The results of inhibition studies with potassium cyanide in our laboratory (188) indicate that activity of the stearate desaturase system of hepatic microsomes is not responsible for the NADPH oxidation observed in the presence of any of the

agents under investigation although the desaturase system has been implicated in the enhancement of NADPH oxidation observed in the presence of another volatile anaesthetic agent, halothane ($CF_3CHBrCl$) (189).

The involvement of cytochromes P-450 in the production of trifluoroethanol or fluoride from the anaesthetic agents is indicated from the requirement of these processes for NADPH and from their inhibition by compounds which are accepted inhibitors of cytochromes P-450. In addition, in view of the method of preparation of microsomes used, it is highly unlikely that there is any contamination of microsomal suspensions by glutathione-S-transferase which has been implicated in defluorination of methoxyflurane *in vitro* (190, 191).

Production of metabolites from anaesthetic agents: some consideration of experimental techniques. The importance of performing assays of microsomal drug metabolism under optimal incubation conditions is stressed by Fouts (192) because of the significant effects of experimental factors (*e.g.* protein concentration, shaking) on rates of drug metabolism; under certain conditions the effects of inducing agents on microsomal drug metabolism may be masked (192). The incubation conditions for assay of production of metabolites of the anaesthetic agents were chosen after due consideration of the recommendations of Fouts and of the results presented in Table 14. In addition, the reaction was allowed to proceed for only that length of

time during which the reaction rate was linear with time.

Previous workers have noted that in rat hepatic microsomes the rates of cytochromes P-450 mediated reactions are linear with time for relatively short periods (*i.e.* 5 to 20 min) before tailing off is observed (193). This early deviation from linearity has been ascribed to the destruction of cytochromes P-450 which results from peroxidation of microsomal lipids in the presence of NADPH and oxygen (194). Inclusion of EDTA, a known inhibitor of lipid peroxidation, in the reaction mixture inhibits the destruction of cytochromes P-450 by lipid peroxides and extends the linearity of a number of cytochromes P-450 catalysed reactions (194, 195).

Although EDTA is included in the *in vitro* incubation system utilized to study trifluoroethanol or fluoride production from the anaesthetic agents, the progress curves for the respective reactions are linear with time for 10 to 20 min only. Inactivation of co-factors might explain in part this deviation from linearity; evidence for this suggestion arises from an observation in our laboratory that production of trifluoroethanol from TFEE is linear for 30 min if booster NADPH generating system is added at intervals during the incubation (188). In addition, in the case of metabolism of fluroxene to trifluoroethanol, the fluroxene mediated destruction of cytochromes P-450, which does not arise from or result in lipid peroxidation (161), is probably partially responsible for the early deviation from linearity.

Cytochromes P-450 mediated metabolism of fluroxene and 2,2,2-trifluoroethyl ethyl ether. It may be observed from Table 26 that per unit time, 1.6-fold more trifluoroethanol is produced from TFEE than from fluroxene although NADPH consumption in the presence of TFEE is in fact slightly less than that in the presence of fluroxene. In this regard, it should be noted that NADPH-independent production of trifluoroethanol from TFEE does not occur in vitro.

The discrepancy observed between rates of production of trifluoroethanol from fluroxene and TFEE probably arises as a result of the destruction of cytochromes P-450 which occurs during metabolism of fluroxene but not during metabolism of TFEE (Table 24). During the 10 min reaction time allowed for assay of trifluoroethanol production, 25 to 30% of the cytochromes P-450 initially present may be destroyed thereby resulting in decreased rates of trifluoroethanol production. Assay of rates of NADPH oxidation in the presence of fluroxene would not be expected to be affected by the degradation of cytochromes P-450 because less than 5% loss of the cytochromes occurs in the 2 to 3 min reaction time required for the assay. If correction to rates of trifluoroethanol production is made for fluroxene mediated destruction of cytochromes P-450, rates of NADPH oxidation for fluroxene become less than those of trifluoroethanol production (Table 26) and the stoichiometry of NADPH oxidation and trifluoroethanol production for fluroxene becomes similar to that for TFEE (Table 28).

That the observed stoichiometry for NADPH to trifluoroethanol is less than the expected ratio of 1:1 may be accounted for not only by differences in the methods of monitoring NADPH oxidation and trifluoroethanol production* but also by the finding that metabolism of both fluroxene and TFEE is not completely inhibited by carbon monoxide (Table 16). The correction for background NADPH consumption in the presence of substrate and carbon monoxide by the method of Stripp _et al_. (29) in these cases may be an overcorrection and may result in calculation of rates of NADPH oxidation which are accordingly too low. In experiments which are designed to demonstrate accurately stoichiometry between NADPH oxidation and product formation, investigations into the extent of inhibition of metabolism by carbon monoxide should be undertaken beforehand in order that a valid correction for background rates of NADPH consumption be made.

The major role played by cytochrome P-450, the predominant type P-450 cytochrome induced by phenobarbital, in the binding and metabolism of fluroxene and TFEE is demonstrated by the observation that only phenobarbital pretreatment of animals leads to increased extents of interaction of these

* Incubation temperatures for studies of metabolism of fluroxene and TFEE were 27° when NADPH oxidation was monitored, and 30° when trifluoroethanol production was followed; shaking of the reaction mixtures occurred only when metabolite production was monitored.

anaesthetic agents with cytochromes P-450 in vitro. Further evidence for the role of cytochrome P-450 in the metabolism of these compounds is obtained from the investigations of trifluoroethanol production by microsomes from 3-methylcholanthrene/AIA treated animals. As a result of specific destruction of cytochrome P-450 by AIA (13, 179), treatment of 3-methylcholanthrene induced animals with AIA gives rise to a preparation of microsomes whose content of cytochrome P-450 is considerably depleted relative to uninduced or 3-methylcholanthrene induced animals and which has a correspondingly decreased ability to metabolize fluroxene and TFEE relative to uninduced or 3-methylcholanthrene induced microsomes (Tables 17 and 18).

Cytochrome P-448, the predominant type P-450 cytochrome induced by polycyclic hydrocarbons generally appears to play no role (or only a very limited role) in the binding and metabolism of fluroxene and TFEE as demonstrated by the lack of effect of polycyclic hydrocarbon induction on the ΔA_{max} and V_{max} values for interactions of these anaesthetic agents with cytochromes P-450 (Table 26).

The K_s value for fluroxene binding to cytochromes P-450 appears to reflect formation of the enzyme-substrate complex which is prerequisite to both NADPH oxidation and trifluoroethanol production (Table 26). The observation that induction of cytochromes P-450 generally does not affect the values of K_s or K_m for fluroxene indicates that the same type P-450 cytochrome(s) is (are) responsible for interactions

of fluroxene in the different types of microsomes; the values of K_s and K_m for fluroxene probably reflect predominantly the activity of cytochrome P-450 (Table 26).

For metabolism of TFEE by uninduced and polycyclic hydrocarbon induced microsomes, the type P-450 cytochrome reflected by the larger K_m values for NADPH oxidation and trifluoroethanol production does not appear to bind TFEE in a manner which gives rise to a readily observable difference spectrum; the single K_s values correspond rather to the smaller K_m values for TFEE metabolism by these microsomes (Table 26). The K_s value for binding of TFEE in phenobarbital induced microsomes appears to reflect formation of the enzyme-substrate complex for both NADPH oxidation and trifluoroethanol production.

Differences in the cytochromes P-450 involved in metabolism of TFEE by uninduced and phenobarbital induced microsomes are immediately evident from the difference in the form of the respective Hanes plots (Figure 21). With uninduced (or polycyclic hydrocarbon induced) microsomes the involvement of at least two cytochromes P-450 or two binding sites on one type P-450 cytochrome is indicated by the biphasic nature of the Hanes plots for NADPH oxidation and trifluoroethanol production, in contrast to the apparent involvement of only one type P-450 cytochrome in phenobarbital induced microsomes. In addition, a qualitative difference in the polycyclic hydrocarbon induced cytochromes P-450 involved in metabolism of TFEE is suggested in microsomes from

3,4-benzpyrene induced animals where the smaller K_m for trifluoroethanol production is decreased in value relative to the corresponding values in uninduced and 3-methylcholanthrene induced animals. This difference, however, is not reflected in the corresponding K_m value for NADPH consumption (Table 26).

In view of the inducibility by phenobarbital of the component giving rise to the smaller K_m values for TFEE metabolism by uninduced microsomes and its destruction by AIA treatment (Table 18) it appears that the smaller K_m reflects the activity of cytochrome P-450 while the larger K_m reflects the activity of a type P-450 cytochrome which is not inducible by either phenobarbital or polycyclic hydrocarbons. Of the two (or more) type P-450 cytochromes apparently involved in the metabolism of TFEE in uninduced and polycyclic hydrocarbon induced microsomes, cytochrome P-450 would obviously play a major role in metabolism *in vivo* as it is unlikely that concentrations of TFEE obtained *in vivo* would be high enough that metabolism by the second type P-450 cytochrome were to become significant.

The production of trifluoroethanol from fluroxene and TFEE by hepatic microsomes *in vitro* correlates well with the observed effects of these two agents on uninduced and phenobarbital induced animals *in vivo* (116); for uninduced animals anaesthesia is non-toxic, while for phenobarbital induced animals it proves toxic. The death of phenobarbital induced animals after fluroxene or TFEE anaesthesia may be

explained by elevated rates of production of trifluoroethanol such that toxic concentrations of the compound are reached (117).

Cytochromes P-450 mediated metabolism of methoxyflurane and enflurane. Rates of production of total fluoride by hepatic microsomes represent the sum of the rates of free fluoride and acid-labile fluoride production during metabolism of methoxyflurane. The existence of an acid-labile metabolite which gives rise to inorganic fluoride has been reported both in vivo (196) and in vitro (156) for methoxyflurane. Methoxydifluoroacetic acid has been characterized as a urinary metabolite of methoxyflurane (151) and is the only metabolite identified which is acid-labile (156).

The possible significance of production of acid-labile and free fluoride with respect to the postulated pathways of metabolism of methoxyflurane should be mentioned. If methoxydifluoroacetic acid is considered to be the only acid-labile compound produced during the microsomal metabolism of methoxyflurane, the possibility then exists that production of acid-labile fluoride arises from the initial dechlorination reaction of pathway II (Figure 7) and is a measure of this reaction. Production of free fluoride could reflect either a direct defluorination reaction or the initial O-dealkylation of pathway I including spontaneous

hydrolysis of the products of this reaction (Figure 7). The inability to detect oxalic acid after metabolism of methoxyflurane in vitro (138) provides some basis for assuming that metabolism of methoxyflurane by hepatic microsomes in vitro proceeds only as far as the initial reactions of pathways I and II.

Interpretation of the stoichiometry of NADPH to fluoride for the metabolism of methoxyflurane and enflurane is not straightforward in that NADPH consumption in vitro reflects a number of reactions the immediate products of which are not known. According to the postulated pathways of metabolism of these anaesthetic agents, however (Figures 7 and 8), a ratio greater than 1.0 for NADPH oxidized to fluoride produced (either free or including acid-labile) would not be expected. The ratios of NADPH to fluoride observed for methoxyflurane and enflurane (Table 28) are far from fulfilling the stoichiometric requirements of Figures 7 and 8. In addition, an overcorrection is probably made in the measurement of rates of NADPH oxidation with methoxyflurane and enflurane in a similar manner to that described for fluroxene and TFEE (page 152); the observed ratios of NADPH to fluoride are therefore probably lower than they should actually be.

Possible explanations of the greater than expected consumption of NADPH relative to production of fluoride by hepatic microsomes in the presence of methoxyflurane and enflurane should be considered. Firstly, methoxyflurane and

enflurane may act to uncouple electron transport and oxygen activation from substrate oxidation. Uncouplers of microsomal oxidation e.g. perfluorohexane (197), are known to stimulate microsomal NADPH and oxygen consumption without themselves being oxidized; water and hydrogen peroxide have been postulated as products of uncoupling (197, 198, 199). The existence of partial uncouplers which function to dissociate to a greater or lesser extent NADPH and oxygen consumption from substrate oxidation has been postulated (199), and it is into this category that methoxyflurane and enflurane may fit. A second possible explanation for the observed stoichiometry of NADPH and fluoride is that the pathways of metabolism as represented in Figures 7 and 8 may be incorrect in a number of respects so that the theoretical stoichiometry is not actually that predicted by the pathways as postulated.

Methoxyflurane may function to uncouple to a limited extent the cytochromes P-450 mediated substrate oxidation from the oxidation of NADPH. However, the data for metabolism of anaesthetics shown in Table 3 (144) indicate that more organic than inorganic fluoride is formed from methoxyflurane in man (in contrast to the situation predicted by Figure 7). Therefore, in view of this observation and our demonstration of a stoichiometry of 13 for NADPH oxidized to free fluoride formed *in vitro*, it seems unlikely that spontaneous hydrolysis of products of the hepatic microsomal metabolism of methoxyflurane to free fluoride (as Figure 7) occurs to a significant extent.

The possibility of fluoride arising as a primary product of a cytochromes P-450 catalysed reaction should perhaps be considered. In addition, however, the possibility that NADPH consumption in the presence of methoxyflurane also reflects involvement of enzymes other than cytochromes P-450 should not be overlooked.

In contrast to the situation with methoxyflurane, a large proportion of the microsomal NADPH oxidation observed in the presence of enflurane appears likely to result from uncoupling by this agent. The ratio for NADPH oxidation to fluoride production (Table 28) is too high to be accounted for by postulating other pathways of metabolism for enflurane; the possibility that metabolism of the compound occurs without concomitant free fluoride production is discredited to a large extent by the results of <u>in vivo</u> studies (albeit in man) demonstrating a ratio of 4:1 for organic to inorganic fluoride metabolites after enflurane anaesthesia (Table 3) (145). The presence of C-F bonds in a compound is known to confer chemical stability on that compound (119); enflurane has five out of a possible eight C-X bonds as C-F, and this may render it refractory to oxidation by cytochromes P-450. Further studies are being performed to establish whether enflurane acts as an uncoupling agent for microsomal cytochromes P-450 electron transfer.

Although a compound such as enflurane may be relatively biologically inert, it may still participate in drug-drug

interactions: inhibition of the metabolism of other drugs may occur as a result of binding of the inert compound to cytochromes P-450 or by depletion of NADPH due to uncoupling of microsomal drug oxidation. In this respect, enflurane has been shown to inhibit metabolism of compounds in vivo (184) and in vitro (183) to the same extent as methoxyflurane.

The major role of phenobarbital induced cytochrome P-450 in the binding and metabolism of methoxyflurane and enflurane is demonstrated in the same manner as for fluroxene and TFEE by the results of the effects of induction on ΔA_{max} and V_{max} values for the interaction of these agents with cytochromes P-450 (Table 26); only induction with phenobarbital leads to increases in extents of the interaction of methoxyflurane or enflurane with cytochromes P-450. With regard to the extent of induction by phenobarbital pretreatment of animals, Greenstein et al. (157) concluded from in vitro studies at a single concentration of anaesthetic that the 'specific activity' of methoxyflurane defluorinase in microsomes from Fischer 344 rats was increased 7.3 times and that for enflurane defluorinase 1.6 times relative to uninduced controls. The results presented herein (either V_{max} values in Table 26, or values determined at a single concentration of anaesthetic in Tables 19 and 23) demonstrate that defluorination of both methoxyflurane and enflurane in vitro is increased by phenobarbital induction about 2.5 to 3.0 times the rate observed in uninduced controls. The difference in the results possibly arises from the species differences inherent in cytochromes P-450 catalysed reactions

or from failure by Greenstein _et al_. to utilize optimum conditions of assay _in vitro_, _i.e_. with respect to time, protein concentration and anaesthetic concentration.

Cytochrome P-448, the predominant cytochrome induced by 3-methylcholanthrene appears to play a limited role in the interactions of methoxyflurane and enflurane with cytochromes P-450 as demonstrated by the observation that induction of elevated levels of cytochrome P-448 generally has no effect on the extents of the interactions observed relative to those in uninduced microsomes. Induction by 3-methylcholanthrene does have an effect, however, on the relative proportions of acid-labile and free fluoride formed from methoxyflurane (Table 27) indicating that there are qualitative differences in the cytochromes P-450 responsible for methoxyflurane metabolism in uninduced and 3-methylcholanthrene induced microsomes. It could be speculated that the amount of anaesthetic metabolized via Pathway II (indicated by acid-labile fluoride production) relative to that metabolized via Pathway I (indicated by free fluoride production) (Figure 7) is increased by 3-methylcholanthrene pretreatment of animals. This interpretation of the results of Table 27 receives some corroboration from a report of the effect of induction with phenobarbital and 3-methylcholanthrene on O-dealkylation (_i.e_. Pathway I) and dechlorination (_i.e_. Pathway II) of methoxyflurane _in vitro_ (152). From data in this report we have calculated the ratios of dechlorination to O-dealkylation in microsomes from uninduced, 3-methylcholanthrene induced

and phenobarbital induced animals to be 10, 16 and 6 respectively. These ratios and the results presented in Table 27 appear to indicate that induction with 3-methylcholanthrene increases metabolism of methoxyflurane via Pathway II relative to Pathway I.

Differences in the cytochromes P-450 involved in the binding and metabolism of methoxyflurane in uninduced (or 3-methylcholanthrene induced) and phenobarbital induced microsomes are apparent from investigations of binding, NADPH oxidation and fluoride production. The biphasic nature of the Eadie-Hofstee and Hanes plots for fluoride production from methoxyflurane by uninduced and 3-methylcholanthrene induced microsomes (Figures 27-33) may result from involvement of at least two cytochromes P-450 catalysing one reaction, from the occurrence of two different reactions leading to free fluoride (and acid-labile fluoride) production, or from conformational changes of the enzyme at high substrate concentrations. In contrast, in phenobarbital induced microsomes the activity of one type P-450 cytochrome appears to predominate in the production of free or acid-labile fluoride from methoxyflurane (Figures 34 and 35). Phenobarbital treatment of animals does not appear to induce either of the two type P-450 cytochromes whose activity is reflected by the two K_m values obtained each for free and acid-labile fluoride production by uninduced microsomes; this is demonstrated by the lack of correspondence between these K_m values and the single

K_m values obtained each for free and acid-labile fluoride production by phenobarbital induced microsomes (Table 26). In addition, a difference between uninduced and 3-methylcholanthrene induced microsomes apparently exists for the type P-450 cytochrome represented by the larger K_m for free fluoride production (Table 26).

The binding and metabolism of methoxyflurane and enflurane by microsomal cytochromes P-450 is evidently much more complex than that of fluroxene or TFEE. In contrast to the situation observed with the latter agents, simple correlations between K_s and K_m values are not found for the interaction of methoxyflurane or enflurane with cytochromes P-450 (Table 26).

While the investigations reported in this thesis were in progress, results of a study of the metabolism of methoxyflurane _in vitro_ were published (178); K_m and V_{max} values are quoted for the production of free and total fluoride from methoxyflurane by uninduced and phenobarbital induced rat hepatic microsomes. These results are invalidated, however, by the fact that these workers determined free and total fluoride concentrations after a 30 min reaction time when rates of fluoride production are no longer linear (see Figures 23 and 25). In addition, the concentration of total fluoride was determined by Adler _et al_. (178) after treatment of reaction mixtures with H_2SO_4 for only 18 hr which we have shown

results in release of about 60% of the acid-labile fluoride present (Figure 22). Furthermore, the rates of production of free fluoride from methoxyflurane reported by these authors are at least ten times higher than the equivalent values reported herein and by other workers (156, 157, 200, 201).

Effect of inhibitors on production of metabolites from volatile anaesthetic agents. The compounds SKF 525A, metyrapone and carbon monoxide are accepted inhibitors of cytochromes P-450 (202, 203). The mechanism of the inhibitory action of SKF 525A is still not completely understood (203). SKF 525A itself is oxidatively metabolized by the cytochromes P-450 mixed function oxidase system and has been shown to inhibit competitively the metabolism of other drug substrates (204). In addition, however, mixed or noncompetitive inhibition kinetics for SKF 525A have been observed under certain conditions and other mechanisms of inhibition by SKF 525A have been proposed involving membrane disruption, uncoupling of electron transport or formation of a stable cytochromes P-450 - SKF 525A complex (202, 203, 205).

In contrast, metyrapone is not itself subject to oxidation but combines with both the oxidized and reduced forms of cytochromes P-450 to form a complex which absorbs at 446 nm (202). It has been suggested that metyrapone mimics the interaction of carbon monoxide with reduced cytochromes P-450 by liganding to the iron atom of cytochromes P-450

to form a complex nonfunctional in substrate oxidation (206). However, while carbon monoxide inhibits all cytochromes P-450 catalysed reactions to a greater or lesser extent, metyrapone diminishes the activity of only certain reactions (206) and actually stimulates the oxidation of type II substrates in vitro (203).

There have been reports which indicate that SKF 525A and metyrapone react selectively with different species of microsomal cytochromes P-450. Cytochrome P-450 induced by phenobarbital binds metyrapone tightly and SKF 525A weakly while the predominant type P-450 cytochrome(s) present in uninduced microsomes is (are) able to bind SKF 525A more tightly than is cytochrome P-450 (207). In addition, SKF 525A is considered to bind preferentially to the same species of cytochromes P-450 with which metabolites of amphetamines form inhibitory complexes absorbing at 455 nm (208). In this regard, 3,4-benzpyrene hydroxylase in phenobarbital induced microsomes was the most sensitive to inhibition by amphetamines and ethylmorphine N-demethylase the least sensitive of the mixed-function oxidase reactions investigated in vitro (209). Differences in the sensitivity of type P-450 cytochromes to inhibition by carbon monoxide have been suggested by a report on 3,4-benzpyrene hydroxylation in microsomes from phenobarbital and 3-methylcholanthrene treated animals (210).

The predominant role of the metyrapone sensitive cytochrome P-450 in the metabolism of the four anaesthetic agents is

indicated by the relative effects of metyrapone and SKF 525A on the production of trifluoroethanol and fluoride by phenobarbital induced microsomes (Table 29). In addition, it appears that there are differences between the cytochromes P-450 metabolizing fluroxene and the other anaesthetic agents: the metyrapone sensitive cytochrome P-450 plays a relatively greater role in the metabolism of fluroxene than in that of TFEE, methoxyflurane or enflurane.

Investigations into the inhibition of trifluoroethanol production from fluroxene in microsomes from differently induced animals provide the only evidence that the same type P-450 cytochromes may not be involved in the metabolism of fluroxene by different types of microsomes. The cytochromes P-450 present in 3-methylcholanthrene induced microsomes are less sensitive to metyrapone inhibition of trifluoroethanol production from fluroxene than are those in phenobarbital induced microsomes (RESULTS, page 79).

Inhibition of the metabolism of methoxyflurane was investigated more extensively than that of fluroxene with evidence presented for differences between the cytochromes P-450 responsible for fluoride production in uninduced, 3-methylcholanthrene induced and phenobarbital induced microsomes (Table 20). Support for the proposal (DISCUSSION, page 161) that 3-methylcholanthrene induces a type P-450 cytochrome that differs from those present in uninduced microsomes with respect to acid-labile fluoride production is provided by the demonstration that acid-labile fluoride production

in 3-methylcholanthrene induced microsomes is considerably less sensitive to metyrapone and carbon monoxide inhibition than that in uninduced microsomes (Table 20). The observation that fluoride production by uninduced or 3-methylcholanthrene induced microsomes is equally or less sensitive to metyrapone than to SKF 525A possibly indicates that cytochrome P-450 does not play a dominant role in fluoride production in these microsomes. This proposal is consistent with the observation that phenobarbital treatment of animals does not appear to induce the type P-450 cytochromes involved in fluoride production in uninduced or 3-methylcholanthrene induced microsomes (Table 26 and DISCUSSION, page 162).

The ability of metyrapone or SKF 525A to inhibit specific type P-450 cytochromes appears to decrease as the concentration of the inhibitor increases. At inhibitor concentrations of 50 μM, differences in the sensitivity to inhibition of cytochromes P-450 present in the different types of microsomes are usually readily evident, while at inhibitor concentrations greater or equal to 200 μM, inhibition of type P-450 cytochromes appears to become less specific (Tables 16 and 20).

Destruction of cytochromes P-450 by fluroxene in vitro. Destruction of microsomal cytochromes P-450 in vitro by carbon tetrachloride (211), halothane (212) and allyl-containing compounds such as allyl-iso-propylacetamide (213) and secobarbital (14) have been reported. Carbon tetra-

chloride results in a non-specific type of degradation which affects not only levels of cytochromes P-450 but also levels of other components of the endoplasmic reticulum, whilst halothane and the allyl-containing compounds destroy only cytochromes P-450.

Of the four anaesthetic agents investigated, only fluroxene is found to be capable of degrading hepatic microsomal cytochromes P-450 and this destruction is specific in that it is limited to cytochromes P-450 (Table 24). Anaesthesia of animals with fluroxene leads to destruction of cytochromes P-450 *in vivo* (116) which parallels that observed *in vitro*.

The fluroxene mediated destruction of cytochromes P-450 *in vitro* requires concomitant metabolism of fluroxene as demonstrated from studies with inhibitors (214) and the absence of destruction when NADPH or oxygen is omitted from the reaction mixture (161). In addition, the dependence of destruction of cytochromes P-450 on metabolism of fluroxene is indicated by the linear correlation between cytochromes P-450 destroyed and metabolite (trifluoroethanol) formed (Figure 37). It appears that both cytochromes P-450 and P-448 are destroyed by fluroxene (161) although the metabolism of fluroxene to trifluoroethanol is catalysed predominantly by cytochrome P-450; this situation is reflected in the lack of correlation between cytochromes P-450 destruction and trifluoroethanol production in microsomes from differently pretreated animals (Table 25). The observation that destruction of cytochromes P-450 in

microsomes from different sources does not depend on the absolute amounts of trifluoroethanol formed from fluroxene could indicate that:-

(1) the reactive metabolite of fluroxene which brings about the destruction of cytochromes P-450 is produced in different proportions to trifluoroethanol in the differently induced microsomes, or

(2) the cytochromes P-450 present in microsomes from 3-methylcholanthrene induced animals are more readily destroyed than those present in microsomes from phenobarbital induced microsomes.

These possibliities are being further investigated in our laboratory.

Metabolism of 2,2,2-trifluoroethanol. The appearance of a type I difference spectrum and enhanced NADPH oxidation with hepatic microsomes in the presence of trifluoroethanol (Table 30) is of interest with regard to its possible metabolism by the microsomal alcohol oxidizing system.

It has been established that a microsomal ethanol oxidizing system (MEOS) exists which is distinct from alcohol dehydrogenase and catalase (215). After purification of constituents of the microsomal membranes, the activity of MEOS has been reconstituted with the components, cytochromes P-450, NADPH-cytochrome c reductase and phospholipids in a way similar to the reconstitution of other drug metabolizing activities (215, 216, 217). The NADPH-dependent microsomal

alcohol oxidizing system exhibits a broad substrate specificity; in addition to ethanol, other primary alcohols such as methanol, propanol, butanol and pentanol have been shown to be metabolized to the corresponding aldehydes (215).

The interaction of trifluoroethanol with cytochromes P-450 differs, however, from that of ethanol. Trifluoroethanol exhibits a type I difference spectrum while ethanol exhibits a modified type II difference spectrum although longer chain alcohols are considered to exhibit dual interaction (type I and modified type II) with cytochromes P-450 (61). Furthermore, trifluoroethanol competitively inhibits the type I difference spectrum of fluroxene while ethanol at similar concentrations does not. The latter finding is surprising in view of the inhibition observed with 100 mM ethanol of the type I binding spectrum of hexobarbital (218). The similarity between the values of K_s and K_m and the values of K_i determined for trifluoroethanol interactions with cytochromes P-450 (Table 30) indicates that trifluoroethanol is possibly functioning as an alternative substrate to the type I compounds whose interactions are inhibited. The type I difference spectrum elicited by trifluoroethanol appears to reflect formation of the enzyme-substrate complex as judged by the identity of the K_s and K_m values (Table 30).

For metabolism of fluroxene *in vivo* and *in vitro*, the significance of inhibition by trifluoroethanol (product) of fluroxene (substrate) binding to cytochromes P-450 is probably negligible because of the much greater affinity of

cytochromes P-450 for fluroxene than for trifluoroethanol. The binding of hydroxylated product by cytochromes P-450 has been reported previously in the case of the metabolism of the substrate, desmethylimipramine (219); in addition, the hydroxylated product at high concentrations inhibited the metabolism of desmethylimipramine.

It appears from the results of our studies in vitro that of the enzymes postulated to be responsible for the oxidative metabolism of trifluoroethanol to trifluoroacetaldehyde only the cytochromes P-450 dependent microsomal alcohol oxidizing system is likely to be effective. As a result of investigations reported herein and from the evidence of other workers using purified preparations of alcohol dehydrogenase from yeast, animals (117) and man (220), it appears extremely unlikely that alcohol dehydrogenase is involved in the metabolism of trifluoroethanol. Alcohol dehydrogenase has in fact been shown to catalyse the reduction of trifluoroacetaldehyde to trifluoroethanol at an extremely slow rate (121, 188). We have demonstrated that alcohol dehydrogenase binds trifluoroethanol with a high affinity K_i resulting in inhibition of ethanol oxidation. There has been a report of the existence of a bacterial alcohol dehydrogenase which is able to oxidize trifluoroethanol at the same rate as ethanol (221) but it appears that this species of alcohol dehydrogenase has been lost in higher organisms.

Although catalase is extremely active in the oxidation of

ethanol *in vitro*, this enzyme is not considered to play a significant role in ethanol metabolism *in vivo* (222). The possibility that catalase is active in the oxidation of trifluoroethanol either *in vivo* or *in vitro* is eliminated by our finding that catalase may be inhibited by trifluoroethanol. Oxygen consumption in a peroxidatic system in the presence of trifluoroethanol (Figure 45) therefore arises as a result of inhibition of catalase rather than from peroxidatic oxidation of trifluoroethanol.

Our evidence suggesting that cytochromes P-450 may be involved in oxidation of trifluoroethanol rather than alcohol dehydrogenase or catalase is consistent with the observed effects of compounds which modify the toxicity of fluroxene and trifluoroethanol (Table 2); most of the agents listed are known to inhibit cytochromes P-450 reactions and could therefore also inhibit cytochromes P-450 mediated oxidation of trifluoroethanol. The proposal that metabolism of trifluoroethanol itself is required in order that toxic symptoms develop during or after treatment of animals with fluroxene or trifluoroethanol is therefore not invalidated by our findings. From our work and the results of other workers (Table 2), it appears that the toxicity of fluroxene and trifluoroethanol possibly arises as a result of cytochromes P-450 mediated oxidation of trifluoroethanol.

Metabolism of trifluoroethanol may also occur via a conjugation reaction. Conjugation with glucuronic acid provides

a mechanism by which the potentially toxic trifluoroethanol may be converted to a less toxic compound which can be rapidly excreted by the kidneys. In mice, glucuronidation appears to be the more important of the metabolic routes for trifluoroethanol in vivo as demonstrated by the recovery of 80% of a dose of C^{14}-trifluoroethanol as urinary glucuronide and 15% as urinary trifluoroacetate (117). In man the reverse situation occurs: 80% of injected C^{14}-trifluoroethanol is recovered as urinary trifluoroacetate and 15% as urinary trifluoroethanol-glucuronide (223).

Our failure to observe glucuronidation of trifluoroethanol in rat microsomal preparations may result from our inability to monitor rates less than 1-2 nmol/mg microsomal protein/min. The species of rats with which these experiments were performed may be relatively deficient in the ability to glucuronidate trifluoroethanol so that rates of reaction are too low to be monitored. Experiments in vivo would now be required in order to establish the existence of enzymes capable of glucuronidating trifluoroethanol in these rats.

The effects of volatile anaesthetic agents on the levels and activities of hepatic drug metabolizing enzymes and glutathione in vivo. As a result of studies demonstrating increased drug metabolizing activities after anaesthesia, the volatile anaesthetic agents fluroxene, methoxyflurane and enflurane have been proposed to induce hepatic drug metabolizing enzymes (103, 224, 225).

We have shown that anaesthesia with fluroxene (3% for 2 hr) leads in fact to decreased levels of hepatic microsomal cytochromes P-450 in phenobarbital and 3-methylcholanthrene induced animals; in addition, anaesthesia of uninduced animals with fluroxene does not affect levels of cytochromes P-450 relative to unanaesthetized animals at 2 or 24 hr after anaesthesia (116). The decrease in hexobarbital sleeping time observed 20 to 22 hr after fluroxene anaesthesia (1.5% for 7 hr) (224) cannot therefore be explained by postulating induction by fluroxene of enzymes of the cytochromes P-450 drug metabolizing system.

A number of investigations into the effects of anaesthesia with methoxyflurane and enflurane on drug metabolizing activities have been reported in the literature and the results of these studies, some of which conflict with each other, are summarized in Tables 36 and 37. Differences in the characteristics of drug metabolizing enzymes are known to exist between species and between different age-groups of one species (231) and these differences possibly play a major role in differences observed between the various reports of the effects of anaesthetic agents on the activities of drug metabolizing enzymes. It is doubtful whether any conclusions regarding the inducibility of drug metabolizing enzymes by methoxyflurane or enflurane may be drawn from the results presented in Tables 36 and 37. Hexobarbital sleeping time (224, 226, 229), the lethal dose of anaesthetic (226) and the ratio of urinary 6-β-hydroxy-cortisol to 17-hydroxycorticosteroids may be altered by

Table 36. Effects of methoxyflurane anaesthesia on drug metabolizing enzymes and activities

Dosage	Animal	Effect[a]	Ref.
0.03% 7hr/day (10 days)	Wistar (195-200 g)	↑ Dechlorination of methoxyflurane *in vitro*	152
0.13% 7hr/day (15 days)	Sprague-Dawley (40-50 g)	↓ Hexobarbital sleeping time ↑ LD_{50} methoxyflurane	226
(10 days)		↑ Aminopyrine demethylation *in vitro*	
1.0 g/kg/day i.p. (5 days)	Mice	No change in fraction of dose of methoxyflurane defluorinated	154
0.03 and 0.1% 7 hr/day (10 days)	Sprague-Dawley (170-180 g)	↓ Cyt. P-450, ↑ NADPH-cyt. *c* reductase, ↓ Hexobarbital oxidation, No change in aniline hydroxylation, *in vitro*	227
	(40-50 g)	No change in Cyt. P-450, ↑ NADPH-cyt. *c* reductase, No change in hexobarbital oxidation, ↑ Aminopyrine demethylation, No change in aniline hydroxylation, *in vitro*	
Unspecified	Sheep	↑ Cyt. P-450 in lung	228

[a] Increase or decrease in enzyme concentration or activity indicated by ↑ and ↓ respectively.

Table 37. Effects of enflurane anaesthesia on drug metabolizing enzymes and activities

Dosage	Animal	Effect[a]	Ref.
0.6% 7 hr/day (1-4 days)	Sprague-Dawley (40-60) g	↓Hexobarbital sleeping time	224
0.2% for 1, 2, 4, 8 days	Sprague-Dawley Fischer 344 unspecified weight	↑Cyt. P-450, ↑Serum inorganic F^- over 1-8 days	225
Repeated doses at anaesthetic & subanaesthetic concentrations	Wistar (200-250 g)	No change in Cyt. P-450, ↓Hexobarbital sleeping time	229
9.6 MAC-hours	Man	↑Ratio of urinary 6-β-hydroxycortisol to 17-hydroxycortico-steroids	230

[a] Increase or decrease in enzyme concentration or activity indicated by ↑ and ↓ respectively

effects of the anaesthetics on physiological processes unrelated to specific induction of drug metabolizing enzymes. Other factors which may invalidate conclusions that induction occurs are: dechlorination of methoxyflurane *in vitro* (152) was assayed using a mixture of microsomes and soluble fraction; in one case, cytochromes P-450 levels were assayed two weeks after exposure to methoxyflurane (228); Hitt *et al*. (225), who report that enflurane anaesthesia results in a two to three fold increase in cytochromes P-450 levels comparable to that after phenobarbital induction, have not reported values for unanaesthetized control animals and performed cytochromes P-450 assays after storage of livers in the frozen state; and, the increasing levels of serum inorganic fluoride observed during 1 to 8 days of enflurane anaesthesia (225) may be attributed to the additive effect on serum fluoride levels of repeated doses of enflurane (see Figure 3, in ref. 142).

The results reported in this thesis indicate that neither methoxyflurane nor enflurane is able to induce enzymes of the cytochromes P-450 mixed function oxidase system such that it may be classed in either of the two groups of accepted inducing agents (INTRODUCTION, page 17). Treatment of animals with either anaesthetic at 0.1 MAC for 4 to 16 days results in few changes in levels or activities of drug metabolizing enzymes (Tables 32 and 34). The only indication of induction of elevated levels of enzymes after anaesthesia by these agents is seen in the methoxyflurane

(1.0 MAC) induced slight elevation of NADPH-cytochrome *c* reductase after the third day of treatment (Table 31). This result is not in agreement with that of Brown and Sagalyn (227) who report activity of NADPH-cytochrome *c* reductase increased by methoxyflurane to the same extent as by phenobarbital induction. Cytochromes P-450 levels and drug metabolizing activities are in fact decreased after the fourth day of treatment with methoxyflurane (1.0 MAC) to levels below those observed in unanaesthetized animals (Table 31). Since the levels of the microsomal marker enzyme glucose-6-phosphatase are also decreased following methoxyflurane anaesthesia, it would appear that relatively non-specific damage to the proteins of the endoplasmic reticulum may follow exposure to methoxyflurane. Treatment of animals with enflurane (1.0 MAC for 3 hr/day) for four days appears to result in increased drug metabolizing activity, *viz.* *p*-nitroanisole-O-demethylation (Table 33), but again this increase in activity cannot be correlated with induction by enflurane of elevated levels of the drug metabolizing enzymes.

The apparent increases in drug metabolizing activities observed after anaesthesia with fluroxene, methoxyflurane or enflurane (224) (Tables 33, 36, 37) may be due to a mechanism unknown as yet and which is not related to induction *per se* of enzymes of the cytochromes P-450 drug metabolizing system. Alternatively, the relative amounts of different species of cytochromes P-450 may be altered by treatment with the anaesthetic agents such that levels

of total cytochromes P-450 remain unchanged but metabolism of particular compounds may be either increased or decreased. Further investigations are required in order to elucidate the mechanism of the apparent stimulation of activities of drug metabolizing enzymes by the volatile anaesthetic agents.

The variation in levels and activities of drug metabolizing enzymes observed in control unanaesthetized animals during the two month period of the investigations (Tables 31-34) indicates the importance of performing control experiments at the same time and with the same batch of animals as those used in experiments with anaesthetic agents or other xenobiotics.

Decreased levels of glutathione in the liver immediately after treatment of an animal by a particular compound is usually taken as an indication that that compound is toxic or potentially toxic and may result in damage to liver cells (91). Glutathione levels were measured 16 to 22 hr after the last anaesthetic treatment and may not therefore be a true reflection of the situation existing immediately after anaesthesia.

Summary of the interaction of volatile anaesthetic agents with hepatic drug metabolizing enzymes. Each of the anaesthetic agents under investigation interacts with cytochromes P-450 of hepatic microsomes resulting in the

appearance of a type I difference spectrum, enhancement of NADPH oxidation and production of potentially toxic metabolites, trifluoroethanol or fluoride. Enflurane is proposed to interact with cytochromes P-450 to a large extent as an uncoupler of substrate oxidation and NADPH and oxygen consumption. In addition, fluroxene in the presence of NADPH causes the degradation of cytochromes P-450; the process of degradation requires concomitant metabolism of fluroxene but is not related to the production of trifluoroethanol in different types of microsomes.

For fluroxene and enflurane, cytochrome P-450 appears to be the predominant type P-450 cytochrome involved in spectrally observable binding of the agent, enhanced microsomal NADPH oxidation and production of metabolites in different types of microsomes. Although activity of cytochrome P-450 also appears predominant in the spectrally observable binding of TFEE, involvement of at least one other type P-450 cytochrome is evident in the interaction leading to NADPH oxidation or production of trifluoroethanol in uninduced or polycyclic hydrocarbon induced microsomes. For methoxyflurane, differences in the cytochromes P-450 involved in its binding and metabolism by microsomes from differently pretreated animals are evident from studies of binding, NADPH oxidation and production of metabolites.

For the binding and metabolism of methoxyflurane, it appears that cytochrome P-450 plays a major role only in phenobarbital induced microsomes; in uninduced and 3-methylcholanthrene induced microsomes the activity of other type P-450 cytochromes is evident.

For the interaction of fluroxene or TFEE with cytochromes P-450 in phenobarbital induced microsomes, the enzyme-substrate complex, of which the difference spectrum is a spectral manifestation, appears to lead to both NADPH oxidation and trifluoroethanol production; this is demonstrated by the relationship, $K_s = K_m$ (NADPH oxidation) = K_m (TFE production). The same relationship is observable for fluroxene in uninduced and 3-methylcholanthrene induced microsomes. For TFEE, the type P-450 cytochrome represented by the larger K_m for NADPH oxidation and trifluoroethanol production in uninduced and polycyclic hydrocarbon induced microsomes appears not to bind TFEE in a manner which gives rise to a difference spectrum. For methoxyflurane and enflurane interactions with cytochromes P-450, simple correlations between K_s and K_m values are not observed and this situation probably reflects the greater complexity of the metabolism of these two agents relative to that of fluroxene or TFEE.

As a result of studies of the interactions of trifluoroethanol with hepatic enzymes *in vitro* we suggest that

the major pathway by which trifluoroethanol may be oxidatively metabolized involves the activity of the cytochromes P-450 mixed function oxidation system and that alcohol dehydrogenase and catalase are not active in this respect. Glucuronidation of trifluoroethanol is not observed in hepatic microsomes from the rats investigated; the reaction therefore occurs, if at all, at a very low rate in these animals.

Finally, it appears that fluroxene, methoxyflurane and enflurane may not be classified as inducers of hepatic drug metabolizing enzymes including enzymes responsible for conjugation with glucuronic acid or glutathione. The mechanism by which apparent increases in drug metabolizing activities are observed after anaesthesia with these agents does not appear to involve induction of enzymes of the cytochromes P-450 system.

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