THE FLUROXENE MEDIATED DEGRADATION OF CYTOCHROMES P-450

by

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2. ABSTRACT

The degradation of cytochromes P-450 by fluroxene (2,2,2-trifluoroethyl vinyl ether) has been investigated. Fluroxene is shown to specifically degrade cytochromes P-450 *in vivo* and *in vitro* without affecting the levels of the other microsomal enzymes, cytochrome $\underline{b}_5$ and NADPH-cytochrome *c* reductase. Fluroxene appears to degrade the haem moiety of cytochromes P-450 but does not affect the level of the apoprotein. The degradation of cytochromes P-450 by fluroxene is accompanied by a loss of *p*-nitroanisole O-demethylase and biphenyl 4-hydroxylase activities and a decrease in the extent of aniline binding is observed.

By using cytochromes P-450 dependent reactions which are catalysed by specific type P-450 cytochromes, *e.g.* the hydroxylation of benzpyrene, the N-demethylation of ethylmorphine and the binding of ethyl isocyanide, it is established that only cytochrome P-450 is degraded by fluroxene *in vivo* following phenobarbital induction of animals, and both cytochrome P-450 and cytochrome P-448 following methylcholanthrene induction. The same type P-450 cytochromes are shown to be degraded by fluroxene *in vitro* in phenobarbital and methylcholanthrene induced microsomes. This was established from studies of the kinetics of the fluroxene mediated degradation of cytochromes P-450. In addition, the K_m values for the fluroxene mediated degradation of cytochromes P-450 differ with

the different inducing agents and indicate the involvement of two different type P-450 cytochromes in the degradation reaction in methylcholanthrene induced microsomes.

Metabolic activation of cytochromes P-450 by the cytochromes P-450 drug metabolising pathway appears to be essential for the fluroxene mediated degradation of cytochromes P-450. Since none of the known or proposed metabolites of fluroxene can mimic the degradation of cytochromes P-450 by fluroxene, a reactive species is proposed to be involved. By varying the experimental conditions, and with the use of inhibitors of cytochromes P-450, the likely sequence of events in the fluroxene mediated degradation of cytochromes P-450 is shown to be as follows: fluroxene is metabolised by cytochrome P-450 to a transient reactive intermediate which has the ability to degrade the haem moiety of cytochrome P-450 and cytochrome P-448.

By comparing the ability of various analogues of fluroxene to degrade cytochromes P-450, it is established that the formation of the proposed reactive intermediate is dependent on the presence of the vinyl moiety of the molecule. Initial studies indicate that the reactive species may take the form of an epoxide.

3. ACKNOWLEDGEMENTS

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7. ABBREVIATIONS

K_s	spectral binding constant which is equal to the concentration of the substrate that gives half the numerical maximal binding, ΔA_{max}, of the substrate to the enzyme
K_m	Michaelis-Menten constant which is equal to the concentration of the substrate that gives half the numerical maximal velocity, V_{max}, of the reaction
ΔA_{max}	the numerical maximal binding of the substrate to the enzyme
V_{max}	the numerical maximal velocity of the reaction
S	substrate
V	velocity of reaction
A	absorbance
cyt(s)	cytochrome(s)
mics	microsomal

AIA	2-allyl-2-iso-propylacetamide
benzpyrene	3,4-benzpyrene
DVE	divinyl ether
EVE	ethyl vinyl ether
GSH	reduced glutathione
methylcholanthrene	3-methylcholanthrene
MC	3-methylcholanthrene
MP	metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propane]
PB	phenobarbital
SKF 525-A	β-diethylaminoethyl-2,2-diphenyl valerate

ABBREVIATIONS (cont.)

SDS	sodium dodecyl sulfate
trifluoroacetic acid	2,2,2-trifluoroacetic acid
trifluoroacetaldehyde	2,2,2-trifluoroacetaldehyde
trifluoroethanol	2,2,2-trifluoroethanol
trifluoroethyl ethyl ether	2,2,2-trifluoroethyl ethyl ether
TFEE	2,2,2-trifluoroethyl ethyl ether

I INTRODUCTION

Fluroxene (2,2,2-trifluoroethyl vinyl ether) is a volatile anaesthetic agent that was introduced into clinical practice in 1953 (1) and was the first volatile fluorinated compound utilized to anaesthetise man. Although fluroxene accumulated a considerable record of clinical safety (2,3), it has recently been shown to be toxic to many animal species (4-7) and, under certain conditions, to man (8-11). As a result of these toxicity studies, fluroxene was withdrawn from the market by the manufacturers in 1977, but is still in limited use as an anaesthetic agent.

Fluroxene has been shown to undergo metabolism in animals and man *in vivo* (12-15). The structures of fluroxene and possible metabolites thereof are shown in figure 1.

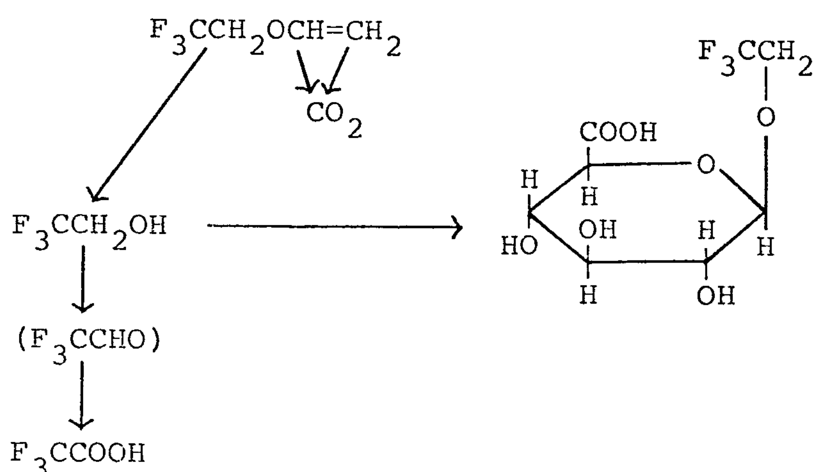


FIGURE 1 Structures of fluroxene and possible metabolites

The only known metabolite of the vinyl portion of the molecule is carbon dioxide, but this compound only accounts for 10% of the fluroxene metabolised (12). In animal species the main metabolite arising from the trifluoroethyl portion of fluroxene is trifluoroethanol, which appears in the urine as the glucuronide, whereas only traces of trifluoroacetic acid are produced (12). In contrast, trifluoroacetic acid has been found to be the main metabolite of the trifluoroethyl portion of fluroxene in man, with trifluoroethanol-glucuronide appearing as a minor metabolite (13). Trifluoroacetaldehyde has been implicated, but never identified, as an intermediate in the formation of trifluoroacetic acid from fluroxene (7,14). Metabolites of fluroxene, and not the anaesthetic agent itself, are thought to result in the toxic effects of fluroxene administration. Trifluoroethanol, the major metabolite of fluroxene in many animal species, is itself toxic to laboratory animals (7,16).

The endoplasmic reticulum has been shown to be the site of the metabolism of fluroxene and other organic compounds _in vivo_ (12). This subcellular structure cannot be isolated from the cell intact. On homogenization of the cell, the endoplasmic reticulum is disrupted and segments of the membrane seal to form tiny vesicles, known as microsomes (17). Microsomes form a convenient system for _in vitro_ studies of drug metabolism by the enzymes of the endoplasmic reticulum.

Microsomal enzymes are responsible for the detoxification

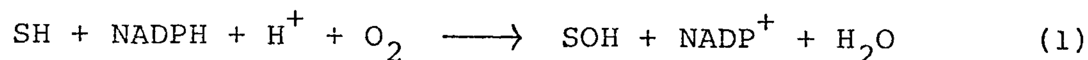
of potentially harmful substances such as drugs, environmental pollutants and carcinogens. In general, the microsomal drug metabolising system renders lipophilic compounds more hydrophilic so that they can be readily excreted by the kidneys. This process diminishes the harmful effects of the accumulation of hydrophobic substances in the body. Although the metabolism of compounds by hepatic microsomal drug metabolising enzymes is generally a detoxification process, there are cases where the biotransformation of a compound by this pathway results in the formation of metabolite(s) of increased toxicity or carcinogenicity relative to the parent compound. For example, the toxicity of vinyl chloride appears to result from the conversion of this compound to reactive species such as chloroethylene oxide or chloroacetaldehyde by the microsomal mixed function oxidases (18). In addition, the metabolism of benzpyrene gives rise to a number of hydroxylated products, one or more of which are carcinogenic, whereas the parent compound is not (19,20).

The metabolism of drugs and xenobiotics is considered to occur in two phases: the first phase involves the oxidation of lipophilic compounds, and the second phase involves conjugation of the oxidized compound with a small polar molecule. The first phase of the microsomal electron transfer pathway is catalysed predominantly by the cytochromes P-450 dependent drug metabolising pathway*. The

* In this thesis, the terms "cytochromes" P-450, "type P-450 cytochromes" and "cytochrome P-450 haemoproteins" refer to the heterogeneous mixture. The terms "cytochrome P-450" and "cytochrome P-448" refer to the specific enzymes.

second phase gives rise to a relatively hydrophilic compound for excretion by the kidneys (21,22). The components of the microsomal drug metabolising pathway are found in highest concentrations in the liver, with the next highest amount in the kidneys. Low, but measurable, levels are found in most organs and tissues (23).

The cytochromes P-450 drug metabolising pathway consists of a group of enzymes of broad substrate specificity. These enzymes are responsible for the metabolism of a wide variety of xenobiotics, such as drugs, insecticides, herbicides, food preservatives and carcinogens. The basic reaction catalysed by the cytochromes P-450 drug metabolism pathway is as follows:



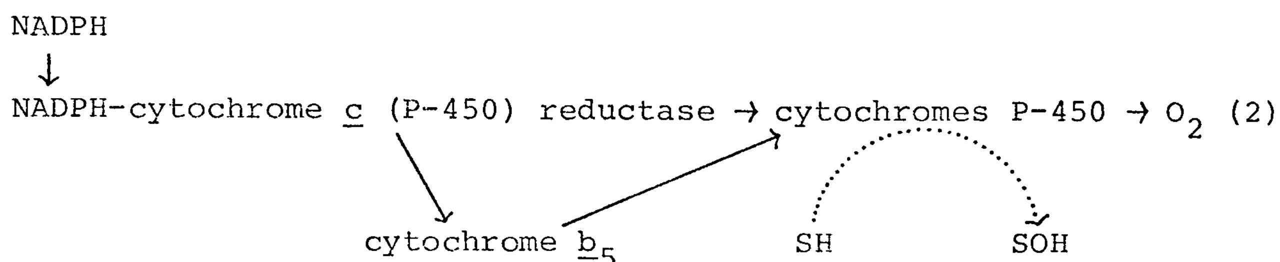
$$SH + NADPH + H^{+} + O_2 \longrightarrow SOH + NADP^{+} + H_2O \quad (1)$$

where SH and SOH represent substrate and hydroxylated product respectively. The types of oxidative reactions catalysed by the cytochromes P-450 include deamination, O- and N-dealkylation, N-oxidation, sulfoxidation, and hydroxylation of aryl and alkyl amines.

Cytochromes P-450 are a heterogeneous group of enzymes of diverse substrate specificities. These enzymes were named for their most striking spectral feature, i.e. the strong absorbance at 450 nm of the carbon monoxide-ferrocytochromes P-450 complex. Within the heterogeneous cytochromes P-450 group of enzymes, there are two spectrally distinct forms which have been well characterized. For

the one form, the absorbance maximum of the carbon monoxide-ferrocytochromes P-450 is at 450 nm, while for the other, the absorbance maximum is at 448 nm. At first, these were thought to represent different conformational forms of a single enzyme. However, recently, several different type P-450 cytochromes have been separated and purified and these different absorbance maxima are therefore attributable to distinct enyzmes. Cytochrome P-450 and cytochrome P-448 vary in physical properties, such as molecular weight, substrate specificity, antibody specificity and spectral properties (24-29). It is not known how many different type P-450 cytochromes exist, but six distinct cytochromes P-450 have been identified in rat liver on the basis of immunochemical evidence (30). Cytochromes P-450 are known as the terminal oxidases in the microsomal mixed function oxidase system.

The microsomal electron transport pathway for the metabolism of drugs by cytochromes P-450 is as follows:



The arrows indicate the direction of flow of the electrons. Cytochromes P-450 bind substrate and oxygen and catalyse the reaction shown in equation 1. NADPH-cytochrome c

reductase fulfils a role as electron carrier* (31,32). Recent studies on the reconstitution of purified and solubilized components of the cytochromes P-450 drug metabolising pathway indicate that, in addition to cytochromes P-450 and NADPH-cytochrome c reductase, a phospholipid fraction is essential for drug metabolism in vitro (33-35). The active component of the phospholipid fraction, phosphatidylcholine, has been shown to be essential for the transfer of electrons from NADPH to cytochromes P-450, although its mode of action is unknown (36).

It is uncertain as to whether cytochrome b_5, the only other microsomal haem protein, plays a role in the cytochromes P-450 drug metabolising pathway. Cytochrome b_5 is not essential for drug metabolism although it is capable of transferring electrons to cytochromes P-450 (32). It seems likely that cytochrome b_5 plays a role in the synergistic effects observed when both NADH and NADPH are used to support hepatic microsomal drug metabolism (37).

The levels of the cytochrome P-450 haemoproteins have been shown to be increased by a large variety of compounds, including environmental agents and drugs (38). These compounds are known as enzyme inducers. The inducing agents for cytochromes P-450 fall into two categories. Phenobarbital is a typical inducer of the first category

* NADPH cytochrome c reductase can reduce artificial electron acceptors such as ferricyanide and cytochrome c. This enzyme is commonly named NADPH-cytochrome c reductase because its activity is usually measured by the reduction of cytochrome c. It is also known as NADPH-cytochrome P-450 reductase (33).

(32,38). Phenobarbital causes proliferation of the endoplasmic reticulum and elevation of NADPH-cytochrome c reductase, cytochrome b_5 and cytochrome P-450 (38,39) and consequently stimulates the metabolism of a large variety of substrates (32). The second category of inducing agents is typified by the polycyclic hydrocarbons, benzpyrene and methylcholanthrene. The polycyclic hydrocarbons elevate the levels of cytochromes P-450 without affecting the levels of the other microsomal enzymes or causing proliferation of the endoplasmic reticulum. The type P-450 cytochrome preferentially induced by the polycyclic hydrocarbons is known as cytochrome P-448. Induction of cytochrome P-448 by the polycyclic hydrocarbons results in a stimulation of the metabolism of only a limited number of substrates, predominantly the hydroxylation of polycyclic aromatic hydrocarbons. For this reason, cytochrome P-448 is also known as aryl hydrocarbon hydroxylase.

A wide variety of organic compounds bind to cytochromes P-450. There are several different binding sites on the cytochromes P-450, some of which are spectrally observable. The binding of compounds to these latter sites alters the environment of the haem moiety of cytochromes P-450 and gives rise to absorbance changes in the visible region of the spectrum. These structural changes have been most extensively characterised in terms of the formation of difference spectra by organic compounds in the presence of microsomes. More recently, these structural changes have been characterised in terms of the spectral changes

observed with purified solubilized cytochromes P-450 (24,25,29).

There are three commonly observed types of difference spectra, known as type I, II and IR. The type I difference spectrum is characterised by a peak at about 385 nm and a trough near 420 nm (40-42) and arises from a change in the environment of the haem moiety. The spin state of the haem iron ion is changed from a relatively low spin form to a relatively high spin form (43). Most compounds producing type I difference spectra undergo cytochromes P-450 dependent metabolism. Consequently, a type I change is often associated with the formation of an enzyme-substrate complex. A wide variety of compounds give rise to type I difference spectra including insecticides, herbicides, carcinogens and many drugs, of which the barbiturates are examples.

The type II spectral change results from the liganding of a compound to the haem iron ion of ferricytochromes P-450. The type II difference spectrum is characterised by a peak near 430 nm and a trough at about 390 nm (40-42). Type II compounds are good ligands, such as primary and secondary amines, sulfides, carbenes and alkoxides. Aniline is the most common example of the type II compounds and is one of the few type II compounds which are substrates for cytochromes P-450 (40,44). Type II compounds compete with carbon monoxide and oxygen as ligands for the ferrous ion of reduced cytochromes P-450.

In addition to compounds which bind to the type I and type II binding sites, there are compounds which give rise to a type IR difference spectrum (42,45). This type of difference spectrum is the reverse of a type I difference spectrum. The type IR difference spectrum is characterised by a peak at about 420 nm and a trough at about 385 nm. The type IR difference spectrum arises from a change in the spin state of the haem iron towards a relatively low spin form (43). Type IR compounds are not usually substrates for cytochromes P-450. Examples of type IR compounds are alcohols and acids. Both type I and type IR compounds are unable to displace carbon monoxide from ferrocytochromes P-450.

The binding of the substrate to ferricytochromes P-450 is the first step in the cytochromes P-450 dependent metabolic pathway. The detailed mechanism for cytochromes P-450 catalysed reactions is shown diagramatically in figure 2. As shown in the scheme, the ferric form of cytochromes P-450 first binds the substrate (SH) and then accepts one electron from NADPH to form the ferrous cytochromes P-450-substrate complex. This complex then combines with molecular oxygen and accepts a second electron from NADPH. At this stage events become uncertain, but it has been proposed that a ferrous enzyme-superoxide intermediate is formed which is in resonance with the ferric-hydroperoxo complex (46). This complex is unstable yielding a ferric enzyme-monooxygen species which is in resonance with an unstable ferryl ion complex. The latter

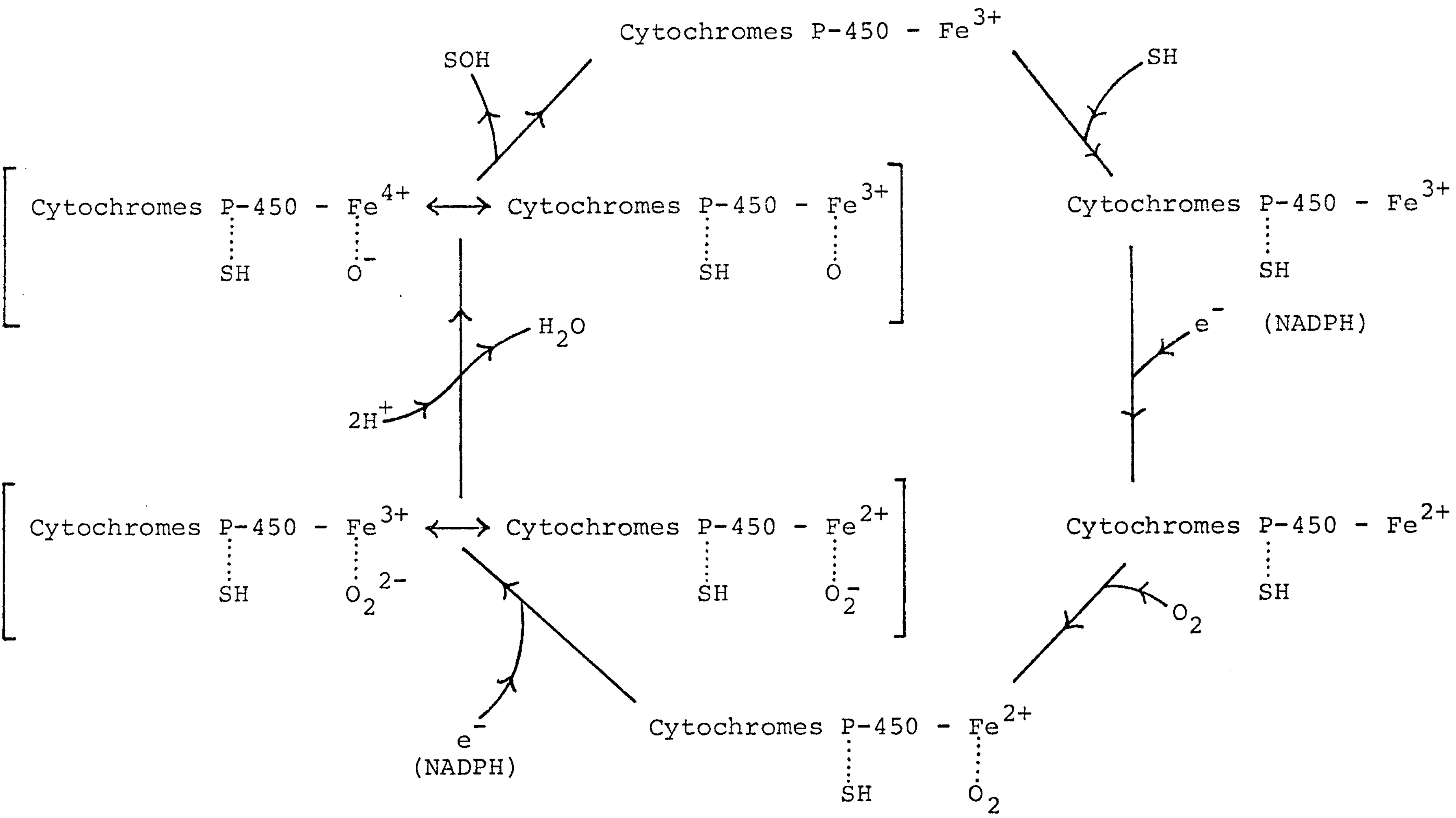


FIGURE 2 Mechanism of cytochromes P-450 dependent reactions

species decomposes giving rise to the hydroxylated product (SOH) and regenerating the ferric form of the enzyme.

The hydroxylated product usually undergoes further reaction via other enzymes such as conjugation with a small physiological polar molecule. Conjugation with glucuronic acid (glucuronidation) or sulphate (sulphation) are common examples of such reactions (47). The conjugated product is usually devoid of biological activity and is readily excreted in the urine or bile.

The interaction of fluroxene with the cytochromes P-450 dependent drug metabolising pathway has been studied in detail in our laboratories (48). Fluroxene has been shown to bind to the type I binding site of cytochromes P-450 *in vitro*. The K_s values for the binding of fluroxene to cytochromes P-450 (approximately 0,9 mM) are identical for differently induced microsomes. The metabolism of fluroxene by the cytochromes P-450 dependent pathway has been investigated by measuring the rates of NADPH consumption (48) and of 2,2,2-trifluoroethanol production (96). A stoichiometric relationship between the rates of trifluoroethanol production and the rates of NADPH oxidation was observed for the metabolism of fluroxene indicating that the metabolism of fluroxene follows the equation for a typical cytochromes P-450 reaction (equation 1). The K_m values for the metabolism of fluroxene as measured by NADPH consumption were found to be approximately 0,8 mM and did not vary with differently induced microsomes. The close

agreement between the K_s and K_m values for the binding and metabolism of fluroxene suggests that the spectrally observable binding constant (K_s) for fluroxene reflects the formation of the substrate-cytochromes P-450 complex. Since the K_s and K_m values are similar, the rate limiting step for the metabolism of fluroxene is probably subsequent to the binding of fluroxene to ferricytochromes P-450.

In contrast to the similarity between the K_s and K_m values for the metabolism of fluroxene by differently induced microsomes, the ΔA_{max} and V_{max} values differ with different inducing agents. The ΔA_{max} value is approximately two-fold greater in phenobarbital induced microsomes than in uninduced, benzpyrene and methylcholanthrene induced microsomes. Similarly, V_{max} is shown to be elevated approximately three-fold by phenobarbital induction compared to control or other inductions. From the binding and metabolism data, it was concluded that cytochrome P-450, the type P-450 cytochrome induced by phenobarbital, is primarily responsible for the binding and metabolism of fluroxene, although cytochrome P-448 may be involved to a small extent.

One unexpected aspect of the interaction of fluroxene with the cytochromes P-450 drug metabolising pathway, was the ability of fluroxene to degrade cytochromes P-450 *in vivo* and *in vitro*. This phenomenon has been demonstrated for a number of compounds, including the allyl containing barbiturates, AIA, and carbon tetrachloride (49-54).

AIA has been shown to degrade the haem moiety of cytochromes P-450 *in vivo* and *in vitro* without affecting the levels of other microsomal enzymes. In addition to the ability of AIA to destroy cytochromes P-450, AIA stimulates the activity of 5-aminolevulate synthetase, which causes an over production of porphyrins, leading to experimental porphyria (55-58). In contrast, carbon tetrachloride is non-specific in its degradation of cytochromes P-450 and results in the loss of other microsomal proteins, and the mechanism of degradation of cytochromes P-450 by carbon tetrachloride appears to differ from that of AIA. Lipid peroxidation has been proposed to be involved in the degradation of microsomal enzymes by carbon tetrachloride (50, 59-61).

This thesis investigates the degradation of hepatic cytochromes P-450 by fluroxene, especially with respect to the following:

1. Which of the type P-450 cytochromes are degraded by fluroxene.
2. The stage during the metabolism of fluroxene at which the destruction of hepatic cytochromes P-450 occurs.
3. The role played by different cytochromes P-450 in the fluroxene mediated destruction of hepatic cytochromes P-450.
4. The portion of the fluroxene molecule that is responsible for the destruction of cytochromes P-450.

II MATERIALS

Sodium phenobarbital and methylcholanthrene were obtained from Maybaker, S.A. and Eastman Kodak, respectively. Fluroxene (2,2,2-trifluoroethyl vinyl ether) was supplied by Ohio Medical Products, Madison, Wisconsin. Ethyl vinyl ether and divinyl ether were ob ained from Fluka, Buchs, Switzerland and Maybaker, S.A. respectively. Trifluoroethyl ethyl ether was prepared by hydrogenation of fluroxene as previously described (62). Trifluoroacetaldehyde was purchased from ICN Pharmaceuticals, Plainview, New York, and trifluoroethanol and trifluoroacetic acid were from Merck. NADP, NADH and glucose-6-phosphate dehydrogenase were obtained from Miles Laboratories, Cape Town. NADPH, cytochrome c (Type III), benzpyrene and reduced glutathione were obtained from Sigma Chemicals. p-Nitroanisole was obtained from Eastman Kodak and was recrystallised from petroleum ether (b.p. 100° - 120°). Glucose-6-phosphate was obtained from Koch-Light Laboratories. Biphenyl and 4-hydroxybiphenyl were purchased from Merck-Schuchard and recrystallised from ethanol and petroleum ether (b.p. 100° - 120°) respectively. 2-Hydroxybiphenyl was obtained from BDH Chemicals Limited and was recrystallised from petroleum ether (b.p. 100° - 120°). Succinic acid was obtained from Merck and recrystallised from distilled water. Cylinders of pure gases were obtained from Afrox Limited. Metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propane] and

SKF 525-A (β-diethylaminoethyl-2,2-diphenyl valerate) were generous gifts from Ciba-Geigy Limited, Basle, Switzerland, and Smith, Kline and French, Ltd. respectively. AIA (2-allyl-2-*iso*-propylacetamide) was a generous gift of Hoffman-La Roche, Nutley, New Jersey. Mr. G.H. Blekkenhorst (Department of Medicine, University of Cape Town) generously supplied the ethyl isocyanide. All other reagents were the best grade available. Water was glass distilled.

III METHODS

1. Induction of animals

Male Wistar rats weighing between 180 g and 250 g were used in all experiments. The 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 indicated. Cytochromes P-450 were induced by intraperitoneal injections of sodium phenobarbital (80 mg/kg/day) or methylcholanthrene (40 mg/kg/day) for 3 consecutive days. All animals were starved overnight after the last injection and sacrificed by cervical fracture the following morning.

2. Preparation of microsomes

Microsomes were prepared at 4° from fresh rat liver homogenates by gel filtration on Sepharose 2B equilibrated in 0,15 M KCl - 0,02 M Tris-HCl pH 7,4 according to the method of Tangen *et al*. (63). Microsomes were also prepared by differential ultracentrifugation according to the method of Holtzman and Carr (64) with the following modifications: the liver was homogenized in 3 ml 0,15 M KCl - 0,02 M Tris-HCl pH 7,4, for each gram wet liver weight. The debris and mitochondria were removed by centrifugation at 10 000 g for 15 min in a Beckman J-21B centrifuge. The microsomes were sedimented from the supernatant by centrifugation at 105 000 g for 1 hr in a Beckman model L ultracentrifuge. The microsomal pellet

washed with 0,15 M KCl - 0,02 M Tris-HCl and resedimented by centrifugation at 105 000 g for 45 min. The microsomes were finally suspended in 0,02 M Tris-HCl pH 7,4. This preparation of microsomes was used following *in vivo* destruction of cytochromes P-450 by fluroxene. Immediately after isolation of the microsomes, the protein concentration of the microsomal suspension was determined by the method of Lowry *et al*. (65) as modified by Chaykin (66), using bovine serum albumin as a standard. The microsomes were diluted to a concentration of 2 mg microsomal protein/ml with 0,02 M Tris-HCl and used at this concentration in this buffer in subsequent experiments unless otherwise stated.

3. Preparation of electron transport particles

Mitochondria were prepared from fresh beef heart by differential ultracentrifugation (67) using 0,9% KCl in the final wash. Electron transport particles were prepared from the mitochondria by the method II of Crane *et al*. (68) and were finally suspended in 0,25 M sucrose. The activity of the electron transport particles under the conditions of the anaerobic experiments was determined in the presence of succinate using a Clark type oxygen electrode. The recorder was calibrated with air saturated solutions (278 μM oxygen) and zeroed on solutions containing an excess of the oxygen scavenger sodium dithionite (0 μM oxygen). By comparison, the activity of the electron transport particles was found to be 0,05 - 0,20 μmol oxygen consumed/min.

4. <u>Destruction of cytochromes P-450 *in vitro*</u>

Destruction studies were carried out in the presence of a NADPH generating system containing 0,4 mM NADP, 7,4 mM glucose-6-phosphate, 0,5 U/ml glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, 1 mM nicotinamide and 0,2 mM EDTA (final concentrations). 3,0 ml of microsomal suspension was pipetted into vials of appropriate size so that the air space in the vial was approximately 10 - 15% of the total volume once the vial was stoppered with a serum cap. Samples and reaction blanks, the latter comprised only of microsomal suspension, were preincubated at 30° for 2 min. The NADPH generating system - EDTA mixture and then the compound to be tested were introduced below the surface of the microsomes in the sample. The vial was sealed immediately with a serum cap and the reaction was initiated by mixing. In the case of lipophilic reagents, vortex mixing was performed for 30 sec to initiate the reaction. Samples and blanks were incubated with shaking in a Galenkampstat shaking water bath at 30°. At the end of the incubation period, samples and blanks were removed and assayed immediately for the levels of the different microsomal enzymes.

5. <u>Determination of the rate constants for the destruction of cytochromes P-450 by fluroxene *in vitro*</u>

Determination of the rate constants for the destruction of cytochromes P-450 was performed as described earlier, except that a booster consisting of 0,4 mM NADP, 7,4 mM glucose-6-

phosphate, and 0,5 U/ml glucose-6-phosphate dehydrogenase (final concentrations) was added at every eighth min during the course of the reaction. Cytochromes P-450 values were corrected for the dilution caused by addition of the booster and also for the small, but significant, loss of cytochromes P-450 observed only in the presence of NADPH generating system plus phenobarbital induced microsomes.

6. <u>Experiments under total anaërobic conditions</u>

A mixture of microsomal suspension (2 mg protein/ml, final concentration) and electron transport particles (0,4 mg protein/ml, final concentration) in 0,02 M Tris-HCl, pH 7,4, was deoxygenated for 15 min by repeated flushing with deoxygenated nitrogen and evacuation by aspiration. This treatment resulted in a decrease in the oxygen content to approximately 15% that of air saturated microsomes, as measured by a Clark type oxygen electrode. All subsequent procedures were carried out in a nitrogen bag which was continually flushed with argon or oxygen free nitrogen. All solutions added to the microsomes in subsequent steps had been previously deoxygenated by bubbling with oxygen free nitrogen for 15 min. To further decrease the oxygen content of the incubation mixture, succinate (10 mM, final concentration) was added to the suspension mixture and 3,0 ml aliquots were pipetted into 4 ml cuvettes with gas tight teflon stoppers. NADPH generating system and fluroxene were then added to the microsomes as previously

described, and the solution was vortex mixed. The cuvettes were removed from the nitrogen bag and incubated at room temperature without shaking. Assay by oxygen electrode of these samples indicated that the levels of oxygen were effectively zero and could not be decreased following addition of the oxygen scavenger sodium dithionite. In addition, the O-demethylation of p-nitroanisole, which proceeds aerobically with a first order rate constant of 0,2 min^{-1}, did not proceed measurably over 10 min under the anaerobic experimental conditions.

7. Destruction of cytochromes P-450 *in vivo*

Two groups of 3 to 4 rats each of almost identical weights (190 ± 5 g) were induced with sodium phenobarbital or methylcholanthrene as described. The rats were starved overnight after the last injection and one group received fluroxene by intraperitoneal injection or AIA by subcutaneous injection the next morning. In the case of phenobarbital induction, the rats received 2 ml/kg fluroxene or 200 mg/kg AIA and were killed 55 ± 5 min thereafter. In the case of methylcholanthrene induction, the rats received 5 ml/kg fluroxene and were killed 85 ± 5 min after fluroxene treatment.

8. Spectrophotometry

Unless otherwise stated, all spectral measurements were performed using a Unicam SP 1800 recording spectrophotometer with a Unicam AR 25 recorder. The thermostatted

cell position adjacent to the photomultiplier was used throughout and cuvettes were placed so that the frosted cell surface was in the light path.

9. Difference spectra

9.a Determination of binding constants

The binding constants (K_s) of analogues and metabolites of fluroxene were determined spectrally. 3,0 ml of microsomal suspension in 4 ml cuvettes were equilibrated to 30°. The compound under investigation was added to the sample cuvette below the surface of the microsomal suspension. The cuvette was then stoppered and, in the case of lipophilic compounds, vortex mixed. The magnitude of the resultant difference spectrum was measured as the difference in absorbance between the peak centred near 385 nm and the trough at approximately 420 nm. The spectrum was corrected for baseline irregularities recorded before the addition of the compound. K_s and ΔA_{max} values were calculated from computer generated Hanes plots of $[S]$ versus $\Delta A/[S]$ and were checked for linearity using the more sensitive Eadie Hofstee plots of ΔA versus $\Delta A/[S]$.

9.b Ethyl isocyanide binding

The crossover pH of ethyl isocyanide binding was determined by a modification of the methods of Imai and Sato (69) and Sladek and Mannering (70) as follows:
3 volumes of microsomal suspension (2 mg protein/ml) were added to 1 volume of 0,4 M potassium phosphate buffer of

various pH. The microsomal suspension was divided equally between 2 cuvettes and 0,6 µl (final concentration, 2,8 mM) of ethyl isocyanide was added to the sample cuvette and the suspension was then vortex mixed for 10 sec. The contents of both cuvettes were reduced with sodium dithionite and the resulting difference spectrum and pH were recorded at room temperature. The magnitudes of the absorbance peaks at 430 nm and 454 nm were measured relative to 500 nm.

9.c <u>Aniline binding</u>

The binding of aniline to cytochromes P-450 was measured at 87 mM aniline (final concentration). The resultant type II difference spectrum was measured as the difference in absorbance between 430 nm and 385 nm. This concentration of aniline was shown to give a difference spectrum equivalent to ΔA_{max}.

10. <u>NADPH oxidation</u>

The metabolism of analogues and metabolites of fluroxene were measured by monitoring the rate of NADPH consumption in the presence of these compounds. 3,0 ml of microsomal suspension in 4 ml cuvettes was equilibrated to 30°. Varying amounts of the compound under investigation were added as described for the difference spectra. Sample cuvettes were vortex mixed and the reaction was initiated with the addition of 50 µl (0,12 mM, final concentration) of NADPH. The decrease in absorbance at 340 nm due to

NADPH oxidation was recorded spectrally against a reaction blank containing only microsomes. All values were corrected for any non-cytochromes P-450 dependent NADPH oxidation according to the method of Stripp *et al.* (71). K_m and V_{max} values were calculated as were the K_s and ΔA_{max} values.

11. Enzyme assays

11.a Cytochromes P-450

The concentration of cytochromes P-450 was determined by measuring the difference spectrum between carbon monoxide plus sodium dithionite reduced microsomes versus sodium dithionite reduced microsomes as described by Omura and Sato (72).

11.b Cytochrome b_5

The concentration of cytochrome b_5 was determined by the difference spectrum between NADH reduced microsomes and microsomes as described by Omura and Sato (72).

11.c NADPH-cytochrome *c* reductase

The activity of NADPH-cytochrome *c* reductase was determined spectrally by the rate of reduction of cytochrome *c* as described by Omura and Takesue (73).

11.d Microsomal haem

The concentration of microsomal haem was determined as the reduced pyridine haemochromogen as described by Omura and Sato (72).

11.e Glucose-6-phosphatase

The activity of glucose-6-phosphatase was determined using a modified method of Nordlie and Arion (74). Microsomal suspension (1,3 mg protein/ml, final concentration) was incubated with glucose-6-phosphate (0,02 M, final concentration) in a volume of 1,5 ml in 0,02 M Tris-HCl, pH 7,4, for 20 min in a shaking water bath at 30°. The reaction was quenched by the addition of 1,0 ml 10% trichloroacetic acid and the precipitated protein was spun down in a MSE 6L centrifuge at 2 000 rpm for 10 min. Reaction blanks were prepared by the addition of glucose-6-phosphate to the incubation medium after precipitation of the protein. 1 ml of the supernatant was used to determine the inorganic phosphorus content as described by King (75). The absorbance was read at 660 nm on a Gilford spectrophotometer, and the inorganic phosphorus released/mg microsomal protein was determined by comparison with potassium dihydrogen phosphate standards.

11.f O-Demethylation of *p*-nitroanisole

The O-demethylation of *p*-nitroanisole was measured by a modification of the method of Netter *et al*. (76). A 2 mM solution of *p*-nitroanisole in 0,02 M Tris HCl, pH 7,4, was prepared by heating to 45°. The stock solution of *p*-nitroanisole was kept at 37° for the duration of the assay. Microsomal suspension (0,6 mg protein/ml, final concentration) and *p*-nitroanisole (0,6 mM, final concentration) in 0,02 M Tris-HCl, pH 7,4 in a total

volume of 3,0 ml, were equilibrated to 37°. The reaction was initiated by the addition of NADPH generating system and initial reaction rates were recorded for about 5 min at 420 nm against a reference without NADPH generating system.

11.g N-Demethylation of ethylmorphine

The N-demethylation of ethylmorphine was measured according to the method of Stripp *et al*. (77). 1,5 ml microsomal suspension (0,6 and 2,0 mg protein/ml, final concentration, for phenobarbital and methylcholanthrene induced microsomes respectively) was equilibrated to 37° with NADPH generating system. The reaction was initiated by addition of 0,1 ml ethylmorphine (10 mM, final concentration) and incubated for 10 min with shaking. The reaction was quenched by the addition of 0,5 ml 15% zinc sulfate and 5 min later, 0,5 ml saturated barium hydroxide was added. The precipitated protein was removed by centrifugation at 2 000 rpm, and 1,25 ml of the supernatant was used for the determination of formaldehyde by the Hantzsch reaction of Nash (78). 0,5 ml Nash reagent was added to the supernatant and the colour allowed to develop at 60° for 30 min. The absorbance was read at 415 nm. A standard curve was constructed from solutions containing 0,5 - 4,0 μg formaldehyde per ml.

11.h Hydroxylation of benzpyrene

The hydroxylation of benzpyrene was measured spectrophotometrically by the method of Prough *et al*. (79) and

fluorimetrically by the method of Nebert and Gelboin (80) as modified by Pelkonen _et al_. (81). In the spectrophotometric method, 2,5 ml microsomal suspension containing 80 μM benzpyrene was equilibrated to 30° in cuvettes. 100 μl NADH (200 μM final concentration) was then added to both cuvettes and the reaction was immediately initiated with 50 μl NADPH (100 μM final concentration). The absorbance changes at 454 nm, 401 nm and 428 nm were recorded with time, and initial rates were calculated for the change in absorbance at 401 nm and 428 nm relative to 454 nm. In the fluorimetric method, 3,0 ml microsomal suspension was equilibrated with benzpyrene in acetone (80 μM - 320 μM, final concentrations) to 37°. The reaction was initiated with NADPH generating system and incubated for 15 min with shaking. The reaction was terminated with the addition of 0,1 ml cold acetone. The products were extracted from the incubation mixture by shaking with 3,25 ml _n_-heptane for 10 min. After centrifugation at 2 000 rpm on a MSE 6L centrifuge for 10 min, 1 ml of the organic layer was added to 5 ml 1M NaOH, and extracted and centrifuged as before. Finally, the organic phase was removed, and the fluorescence of the aqueous phase was read on a Perkin Elmer 203 fluorimeter with excitation wavelength of 396 nm and emmission wavelength of 522 nm.

11.i Hydroxylation of biphenyl

The 2- and 4-hydroxylation of biphenyl was measured fluorimetrically by the method of Creaven _et al_. (82) as

modified by Burke et al. (83). The incubation mixture (total volume 2,0 ml) contained microsomal suspension, NADPH generating system and biphenyl (2 μmol, final concentration), in 0,02 M Tris-HCl, pH 7,6. The microsomes and generating system were preincubated for 90 sec in teflon stoppered tubes in a shaking water bath. The reaction was initiated with 0,5 ml biphenyl and oxygen was blown over the surface of the incubation mixture for 20 sec. The incubation proceeded under oxygen for 5 min and the reaction was terminated with 0,5 ml 5N HCl. The 2- and 4-hydroxybiphenyl products were extracted from the reaction mixture by shaking with 7 ml n-heptane (containing 1,5% iso-amyl alcohol to prevent emulsification) for 5 min at room temperature. After centrifugation for 10 min, 2 ml of the heptane layer was added to 5 ml 0,1 M NaOH in a clean teflon stoppered tube and extracted and centrifuged as before. The heptane phase was removed and 2 ml of the aqueous phase pipetted into quartz fluorimeter cuvettes. 0,5 ml 0,25 M succinic acid was added, and after mixing, the fluorescence was read with excitation and emission wavelengths of 275 nm and 330 nm respectively for the 4-hydroxylated product, and 290 nm and 415 nm respectively for the sum of the 2- and 4-hydroxylated products. Reaction blanks containing biphenyl added to the inactivated microsomes and standards of 2- and 4-hydroxybiphenyl (in 96% ethanol) which were also added to the inactivated microsomes, were extracted in the same way. Standard curves of 4-hydroxybiphenyl over the range of 2,5 to

25 nmol/mg microsomal protein were prepared and the absorbance read at both sets of wavelengths. 2-hydroxybiphenyl (2,5 nmol/mg microsomal protein) and 4-hydroxybiphenyl (12,4 nmol/mg microsomal protein) standards were run daily. The concentration of the 2-hydroxybiphenyl products was determined as described by Creaven *et al.* (82).

12. Lipid peroxidation

Malonaldehyde formation, an index of lipid peroxidation, was measured as described by Ernster and Nordenbrand (84). The protein in the incubation sample was precipitated with trichloroacetic acid and the malonaldehyde production was measured colorimetrically with the thiobarbituric acid reaction.

13. Reduced glutathione determination

The reduced glutathione content of the sample used was estimated by the *p*-chloromercuribenzoate method of Stadtman (85). The increase in optical density due to 0,1 μmol reduced glutathione after 5 min incubation was 0,595 $\pm$ 0,005 at 255 nm, indicating that the sample was 89-96% reduced.

14. Sodium dodecyl sulfate gel electrophoresis

Sodium dodecyl sulfate gel electrophoresis was run on 5,6% polyacrylamide gels containing 0,1% SDS using a modification of the method of Fairbanks *et al.* (86) and Welton and Aust (87). 10 cm glass electrophoresis tubes were thoroughly cleaned by soaking in warm SDS solution. The gels were

prepared by mixing the solutions in Table 1 in the following proportions and order: 7 ml acrylamide, 5 ml 10x buffer, 2,5 ml SDS, 28 ml distilled water, 5 ml ammonium persulfate and 2,5 ml N,N,N',N'-tetramethylethylenediamine. The solution was mixed thoroughly and pipetted into the clean dry electrophoresis tubes. Each tube was carefully overlaid with a solution of 0,1% SDS, 0,15% ammonium persulfate and 0,05% N,N,N',N'-tetramethylethylenediamine using a pasteur pipette, to insure a uniform gel surface. The gels took about ½ hr to polymerize. When polymerization was complete, the surface of the gels was washed with electrophoresis buffer (Table 1) and the gel tubes were transferred to a Pleuger Acrylophor electrophoresis chamber containing the electrophoresis buffer. The gels were left to stand overnight at room temperature, and were pre-electrophoresed for about 2 hr at 5 V per cm using a LKB 3371E DC power supply before application of the sample.

Microsomal samples for electrophoresis were prepared as follows: a mixture of 10 ml microsomal suspension (approximately 3,5 mg protein/ml) containing 10% sucrose, approximately 0,01% bromophenol blue (tracking dye), 1 mM EDTA and 1% SDS was prepared. 5 or 10 μl of the microsomal suspension were then applied to the gel. Molecular weight markers bovine serum albumin, catalase, alcohol dehydrogenase and pepsin were prepared in the same way at a protein concentration of 0,5 mg/ml. Electrophoresis was performed in the dark at 4° with a voltage gradient

Table 1. Solutions for SDS gel electrophoresis

Solution	Ingredients
Acrylamide	40 g acrylamide, 1,5 bisacrylamide/ 100 ml water
SDS	2% w/v /H_2O
Ammonium persulfate	1,5% w/v /H_2O
N,N,N',N'-tetramethyl-ethylenediamine	0,5% v/v /H_2O
10x buffer pH 7,4	0,4 M Tris, 0,2 M sodium acetate, 0,02 M EDTA, pH 7,4
Electrophoresis buffer	100 ml 10x buffer, 50 ml 2% SDS, 850 ml water

of 5 V/cm which resulted in a current of 2 to 3 ma per tube, for approximately 8 hr.

The gels were either stained for protein using Coomasie blue without shaking as described by Fairbanks et al. (86) or for cytochromes P-420 peroxidase activity using the benzidine-hydrogen peroxide stain of Clausen (88). The solutions used for the Coomasie blue staining procedure are given in Table 2. They were used in descending order for the times indicated. The benzidine-hydrogen peroxide stain was utilized after soaking the gels in 0,02 M Tris-HCl containing 50% methanol to lower the SDS concentration. The gels stained with Coomasie blue were scanned at 550 nm using a Varian Techtron model 635 spectrophotometer with gel-scanning attachment and the cytochromes P-450 peaks quantitated with a Unicam SP 88 Computing Integrator.

Table 2. Coomasie blue staining procedure for proteins after SDS gel electrophoresis

Coomasie blue % w/v	Solvent	Time for staining
0,025	25% *iso*-propyl alcohol 10% acetic acid	overnight
0,0025	10% *iso*-propyl alcohol 10% acetic acid	ca 8 hr
0,001	10% acetic acid	overnight
	10% acetic acid	24 hr and for storage

IV RESULTS

1. The effects of fluroxene treatment on the levels and activities of hepatic microsomal enzymes in induced animals

Fluroxene treatment of phenobarbital and methylcholanthrene induced animals results in the degradation of cytochromes P-450, and the loss of cytochromes P-450 is accounted for by an equivalent loss of microsomal haem (Table 3). There appears to be no measurable loss of microsomal protein as measured by the microsomal marker enzyme, glucose-6-phosphatase (Table 3). The activities of this enzyme are identical in microsomes from induced rats with and without fluroxene treatment. There is also no decrease in the levels of microsomal NADPH-cytochrome c reductase or cytochrome b_5 (Table 3).

p-Nitroanisole O-demethylase and biphenyl 4-hydroxylase activities and aniline binding are reactions which are catalysed non-specifically by type P-450 cytochromes (76, 89-91) and are decreased in proportion to the decrease in the levels of cytochromes P-450 following fluroxene injection (Tables 4, 5 and 6).

It is confirmed that the activity of ethylmorphine N-demethylase is catalysed by cytochrome P-450 (26,32,92) because it is approximately three times greater in phenobarbital induced microsomes, where cytochrome P-450 is induced, than in methylcholanthrene induced microsomes (Table 7). Ethylmorphine N-demethylase activity is decreased following fluroxene injection of phenobarbital

Table 3. The effects of fluroxene treatment on concentrations and activities of microsomal enzymes in phenobarbital and methylcholanthrene induced animals

Induction	Fluroxene treatment	[Cyts P-450] (nmol/mg mics protein)	[Cyt b_5] (nmol/mg mics protein)	NADPH-cyt *c* reductase (U/mg mics protein)	[Haem] (nmol/mg mics protein)	Glucose-6-phosphatase (mg phosphorus released/mg mics protein/ 20 min)
PB	+	1,79±,39	0,63±,06	0,12±,02	2,20±,42	0,21±,03
PB	None	3,01±,04	0,66±,04	0,14±,02	3,27±,49	0,22±,03
MC	+	1,06±,03	0,64±,08	0,08±,02	2,10±,05	0,21±,06
MC	None	2,27±,07	0,66±,04	0,09±,02	3,19±,01	0,25±,07

Table 4. The effect of fluroxene treatment on the activity of p-nitroanisole O-demethylase in phenobarbital and methylcholanthrene induced animals

Induction	Fluroxene treatment	Cytochromes P-450		p-Nitroanisole O-demethylase	
		(nmol/mg mics protein)	% Relative to controls	(nmol p-nitroanisole/ mg mics protein/min)	% Relative to controls
PB	+	1,70 ± ,02	68	5,30 ± ,11	68
PB	None	2,49 ± ,27	-	7,84 ± ,06	
MC	+	0,74 ± ,07	45	2,52 ± ,65	47
MC	None	1,66 ± ,01	-	5,34 ± ,16	

Table 5. The effect of fluroxene treatment on the activity of biphenyl 4-hydroxylase in phenobarbital induced animals

Fluroxene treatment	Cytochromes P-450		Biphenyl 4-hydroxylase	
	(nmol/mg mics protein)	% Relative to controls	(nmol/mg mics protein/5 min)	% Relative to controls
+	1,66 ± ,15	66	15,6 ± 1,2	71
None	2,53 ± ,12	-	21,9 ± 2,0	-

Table 6. The effect of fluroxene treatment on aniline binding in phenobarbital and methylcholanthrene induced animals

Induction	Fluroxene treatment	Cytochromes P-450		Aniline binding	
		(nmol/mg mics protein)	% Relative to controls	(A_{430nm} - A_{395nm})	% Relative to controls
PB	+	1,70 ± ,02	68	0,132 ± ,009	64
PB	None	2,49 ± ,27	-	0,207 ± ,019	-
MC	+	0,74 ± ,07	45	0,073 ± ,008	39
MC	None	1,66 ± ,01	-	0,188 ± ,015	-

Table 7. The effect of fluroxene treatment on the activity of ethylmorphine N-demethylase in phenobarbital and methylcholanthrene induced animals

Induction	Fluroxene treatment	Cytochromes P-450		Ethylmorphine N-demethylase	
		(nmol/mg mics protein)	% Relative to controls	(nmol formaldehyde/mg mics protein/10 min)	% Relative to controls
PB	+	1,39 ± ,32	65	8,9 ± 0,8	72
PB	None	2,14 ± ,08	-	12,3 ± 1,3	-
MC	+	0,74 ± ,07	45	3,0 ± 0,6	73
MC	None	1,66 ± ,01	-	4,1 ± 0,3	-

and methylcholanthrene induced animals (Table 7).

The level of benzpyrene 3-hydroxylase activity is increased following induction of cytochrome P-448 by methylcholanthrene compared to phenobarbital induction of cytochrome P-450 (Table 8), confirming that benzpyrene 3-hydroxylation is catalysed by cytochrome P-448 (26,27,93,94,98). In phenobarbital induced rats, spectrophotometric measurement of the formation of the 3-hydroxylated product showed that there was no decrease in the level of benzpyrene 3-hydroxylase activity following fluroxene treatment (Table 8). In methylcholanthrene induced rats, similar measurements indicated that a large proportion of the benzpyrene 3-hydroxylase activity was lost after fluroxene treatment (Table 8). The results of the spectrophotometric measurement of the formation of the 9-hydroxylated product ($A_{401nm} - A_{454nm}$) of benzpyrene showed that fluroxene treatment resulted in a decrease in activity in methylcholanthrene induced microsomes but no decrease in activity in phenobarbital induced microsomes. The decrease in the formation of 9-hydroxybenzpyrene (Table 9) was comparable to the decrease of the 3-hydroxylated product (Table 8).

The results of fluorimetric measurements of benzpyrene hydroxylase activity are shown in Table 9. In phenobarbital induced microsomes, the results are identical using the spectrophotometric and fluorimetric methods. In methylcholanthrene induced microsomes, no decrease in activity is seen following fluroxene treatment with the

Table 8. The effect of fluroxene treatment on the activity of benzpyrene 3-hydroxylase in phenobarbital and methylcholanthrene induced animals

Induction	Fluroxene treatment	Cytochromes P-450		Benzpyrene 3-hydroxylase	
		(nmol/mg mics protein)	% Relative to controls	(nmol 3-hydroxybenzpyrene/ mg mics protein/min)	% Relative to controls
PB	+	1,70 ± ,04	69	0,21 ± ,02	105
PB	None	2,48 ± ,27	—	0,20 ± ,02	—
MC	+	0,83 ± ,03	53	0,18 ± ,05	35
MC	None	1,58 ± ,07	—	0,51 ± ,04	—

Table 9. The effect of fluroxene treatment on benzpyrene hydroxylase activity - a comparison between the spectrophotometric and fluorimetric assay methods used

Assay Method	Induction	[Benzpyrene] (μM)	% Relative to controls
Spectrophotometric ($A_{401nm} - A_{454nm}$)	PB	80	103, 164[a]
	MC	80	34 ± 4[b]
Fluorometric	PB	80	95 (72-125)[c]
	PB	160	159 (108-190)[c]
	PB	320	105 (90-128)[c]
	MC	80	99 ± 4[b]
	MC	160	61 ± 1[b]
	MC	320	67 (68-66)[c]

[a] Values for experiments done on two different preparations of microsomes.

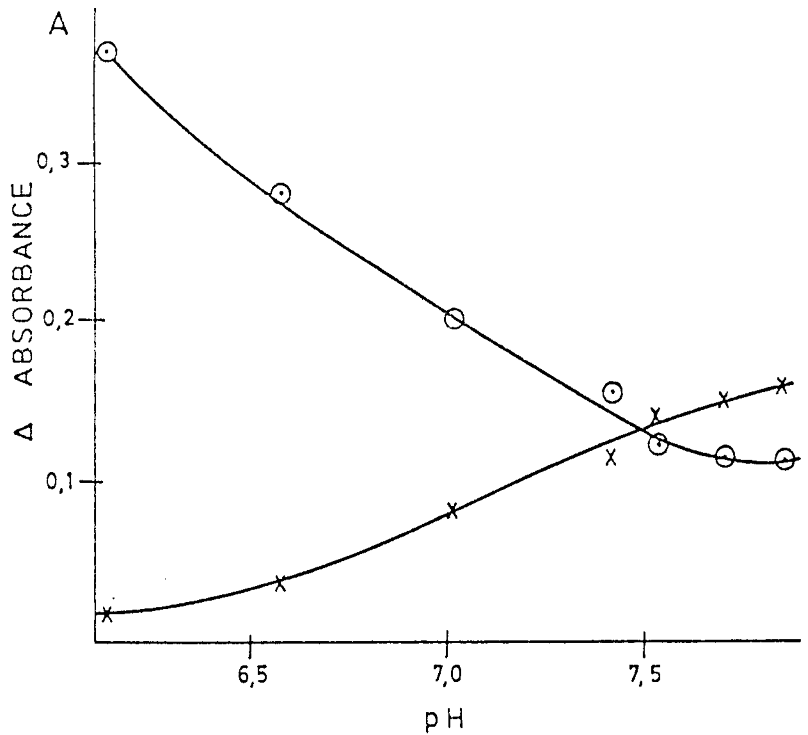
[b] Standard deviation for experiments on different preparations of microsomes.

[c] Value, with range given in parenthesis, for experiments on one preparation of microsomes.

fluorimetric method using the same concentration of benzpyrene as in the spectrophotometric method (80 μM). However, as the concentration of benzpyrene is increased, a loss of activity is seen after fluroxene treatment, although not as great as the loss seen using the spectrophotometric method. The results obtained using the spectrophotometric method appear to be more reliable than those obtained using the fluorimetric method: in the fluorimetric method, with the long incubation time (15 min), initial reaction rates are not being measured; in addition, the products are extracted from the reaction medium in two stages and each step in the extraction procedure leads to a source of error. In contrast, in the spectrophotometric method, the formation of the products is determined by directly measuring initial reaction rates.

The determination of biphenyl 2-hydroxylase activity, which is proposed to be specific for cytochrome P-448 (89,93), was found to be too inaccurate, and hence the results are not reported.

The binding of ethyl isocyanide as a function of pH in phenobarbital and methylcholanthrene induced microsomes with and without fluroxene treatment is shown in Figures 3 and 4 respectively. Following phenobarbital induction, the crossover pH is the same in animals treated or not with fluroxene (Table 10, figure 3). In microsomes from methylcholanthrene induced, fluroxene treated animals, the crossover pH is considerably higher than that in the absence



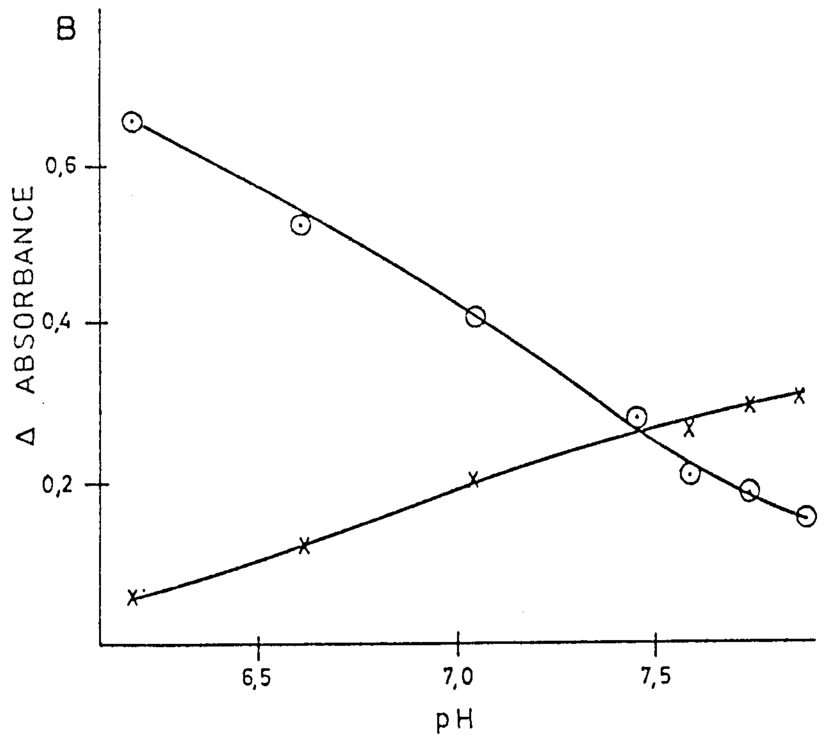


FIGURE 3 The effect of pH on the 430 nm (⊙) and 454 nm (x) absorbtion maxima of ethyl isocyanide binding to cytochromes P-450 in phenobarbital plus fluroxene (A) and phenobarbital microsomes (B)

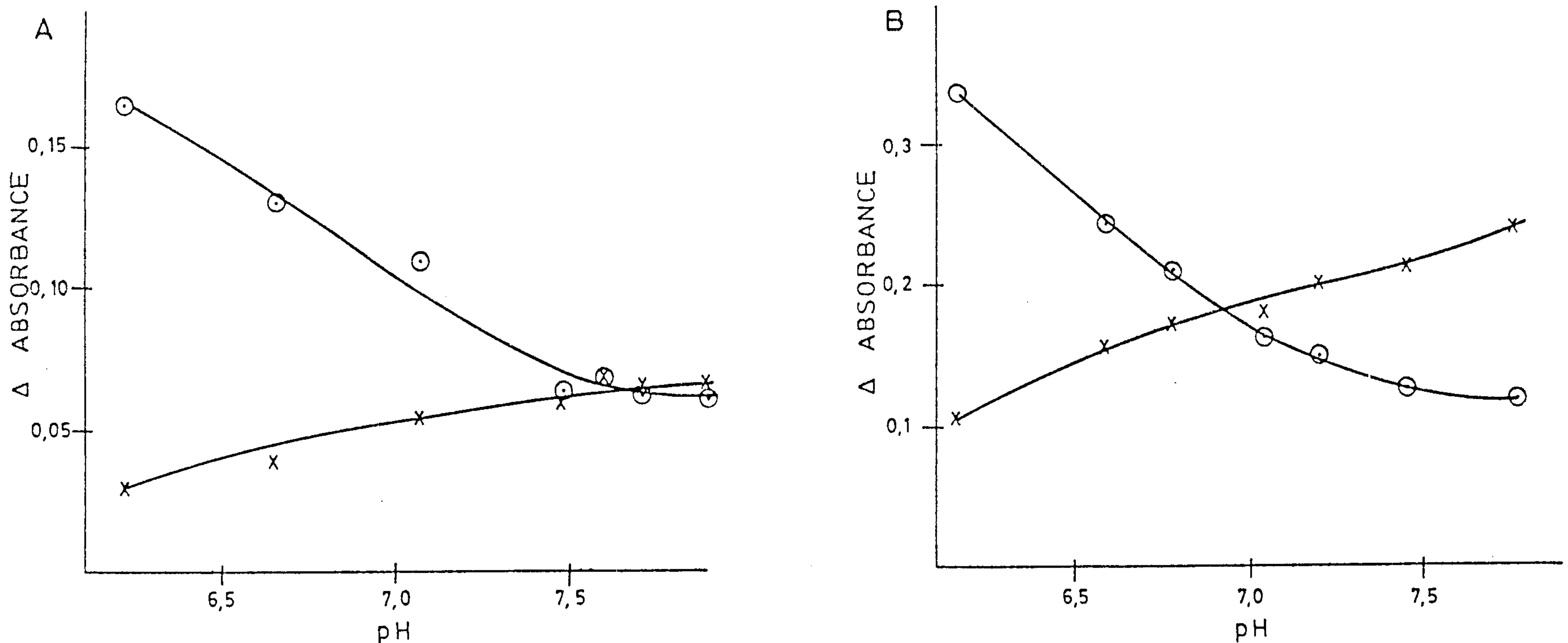


FIGURE 4 The effect of pH on the 430 nm (⊙) and 454 nm (x) absorbtion maxima of ethyl isocyanide binding to cytochromes P-450 in methylcholanthrene plus fluroxene (A) and methylcholanthrene microsomes (B)

Table 10. The effect of fluroxene treatment on the ethyl isocyanide binding crossover pH in phenobarbital and methylcholanthrene induced animals

Induction	Fluroxene treatment	Cytochromes P-450		Ethyl isocyanide crossover pH
		(nmol/mg mics protein)	% Relative to controls	
PB	+	1,79 ± ,39	59	7,56 ± ,06
PB	None	3,01 ± ,40	—	7,57 ± ,07
MC	+	1,06 ± ,03	47	7,56 ± ,13
MC	None	2,27 ± ,07	—	6,98 ± ,02

of fluroxene treatment (Table 10, figure 4).

Microsomes from rats induced with phenobarbital or methylcholanthrene and treated with fluroxene were green in colour in contrast to microsomes from animals not treated with fluroxene. The greenish colouration was similar to that found in microsomes from animals treated with AIA, which mediates, as does fluroxene, the destruction of cytochromes P-450. In the case of AIA the colour has been attributed to green pigments which have been identified as oxyphlorins and porphyrins and are proposed to be degradation products of haem (95). The green colour resulting from fluroxene treatment was more apparent in microsomes from methylcholanthrene induced rats than phenobarbital induced rats and may also reflect the degradation of the haem moiety of cytochromes P-450.

2. The effects of fluroxene on the levels of hepatic microsomal enzymes *in vitro*

The carbon monoxide difference spectrum of reduced cytochromes P-450 utilized in the measurement of cytochromes P-450 is shown in figure 5. The levels of cytochromes P-450 before and after incubation of the microsomal suspension with fluroxene and NADPH generating system for 30 min are shown for phenobarbital induced microsomes. The extent of degradation of cytochromes P-450 and haem in phenobarbital and methylcholanthrene induced microsomes *in vitro* is demonstrated in Table 11. Incubation of induced microsomes with fluroxene does not affect the levels

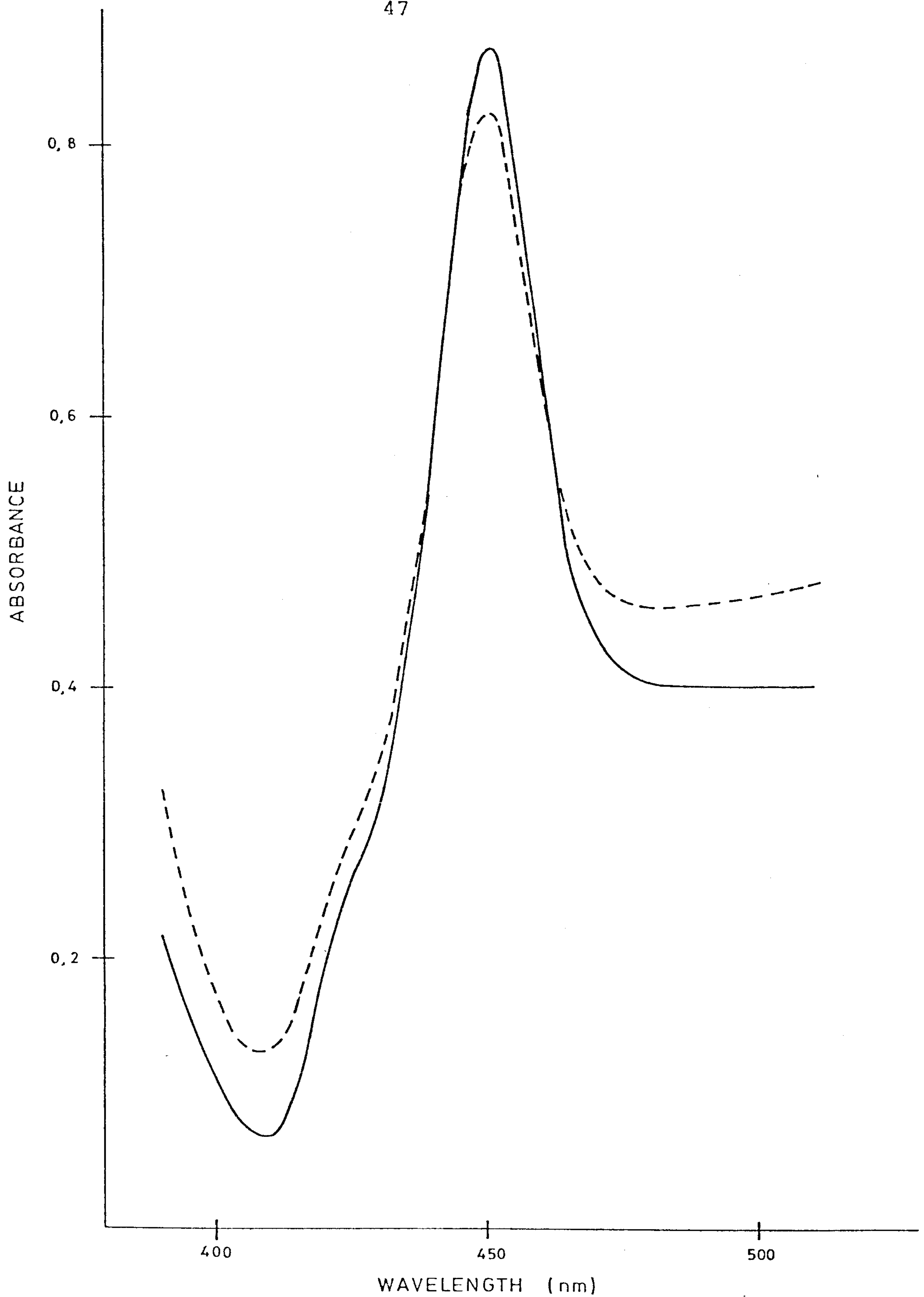


FIGURE 5 The carbon monoxide difference spectrum of reduced cytochromes P-450 before (—) and after (---) incubation of phenobarbital induced microsomes with fluroxene and NADPH generating system for 30 min

Table 11. The destruction of cytochromes P-450 and haem by fluroxene in phenobarbital and methylcholanthrene induced microsomes *in vitro*

Induction	Fluroxene (30 mM)	[Cyts P-450] (nmol/mg mics protein)		% Cyts P-450 lost	[Haem] (nmol/mg mics protein)		Loss of cyts P-450 (nmol/mg mics protein)	Loss of haem (nmol/mg mics protein)	% Loss haem/loss cyts P-450
		0 min	30 min		0 min	30 min			
PB	-	3,35±,01	3,22±,01	4	3,92±,03	3,62±,02	0,13	0,30	
PB	+	2,88±,04	2,32±,03	19	3,96±,01	3,36±,09	0,56	0,60	107
PB	+[a]	2,22±,04	2,20±,05	1					
MC	-	1,97±,03	1,78±,07	10	2,43±,04	2,36±,06	0,19	0,07	
MC	+	1,90±,01	0,94±,05	51	2,50±,01	1,96±,01	0,96	0,54	56

[a] Without added NADPH generating system.

of cytochrome $\underline{b}_5$ or NADPH-cytochrome $\underline{c}$ reductase (Table 12).

3a. Kinetics of the destruction of hepatic cytochromes P-450 by fluroxene *in vitro*

The destruction of cytochromes P-450 by fluroxene *in vitro* followed first order kinetics. The decrease in the levels of cytochromes P-450 as a function of time in phenobarbital and methylcholanthrene induced microsomes is shown in figure 6. The first order rate plots for the destruction of cytochromes P-450 in phenobarbital and methylcholanthrene induced microsomes are linear as shown in figures 7 and 8. The first order rate constants for the degradation of cytochromes P-450 by fluroxene were calculated to be $4{,}4 \times 10^{-2}$ min^{-1} for phenobarbital and $4{,}9 \times 10^{-2}$ min^{-1} for methylcholanthrene induced microsomes. The total loss of cytochromes P-450 was 58% over 65 min in phenobarbital and 51% over 60 min in methylcholanthrene induced microsomes.

3b. K_m and V_{max} for the destruction of hepatic cytochromes P-450 by fluroxene *in vitro*

The K_m and V_{max} for the fluroxene mediated destruction of cytochromes P-450 *in vitro* were determined from Hanes plots (figures 9, 10 and 11) for various types of induction. The K_m values were found to vary with the different types of induction, and V_{max} values were elevated in phenobarbital and methylcholanthrene induced microsomes compared to uninduced microsomes as shown in Table 13.

Table 12. The effects of fluroxene on the levels of cytochrome $\underline{b}_5$ and NADPH-cytochrome $\underline{c}$ reductase in phenobarbital and methylcholanthrene induced microsomes *in vitro*

Induction	Fluroxene (30 mM)	[Cyt $\underline{b}_5$] (nmol/mg mics protein) 0 min	30 min	% Cyt $\underline{b}_5$ lost	NADPH-cyt $\underline{c}$ reductase (U/mg mics protein) 0 min	30 min	% NADPH-cyt $\underline{c}$ reductase lost
PB	-	0,65$\pm$,02	0,70$\pm$,02	0	0,12$\pm$,01	0,12$\pm$,01	0
PB	+	0,54$\pm$,01	0,58$\pm$,01	0	0,11$\pm$,01	0,11$\pm$,01	0
MC	-	0,79$\pm$,04	0,87$\pm$,02	0	0,07$\pm$,01	0,08$\pm$,01	0
MC	+	0,70$\pm$,02	0,80$\pm$,01	0	0,09$\pm$,01	0,11$\pm$,01	0

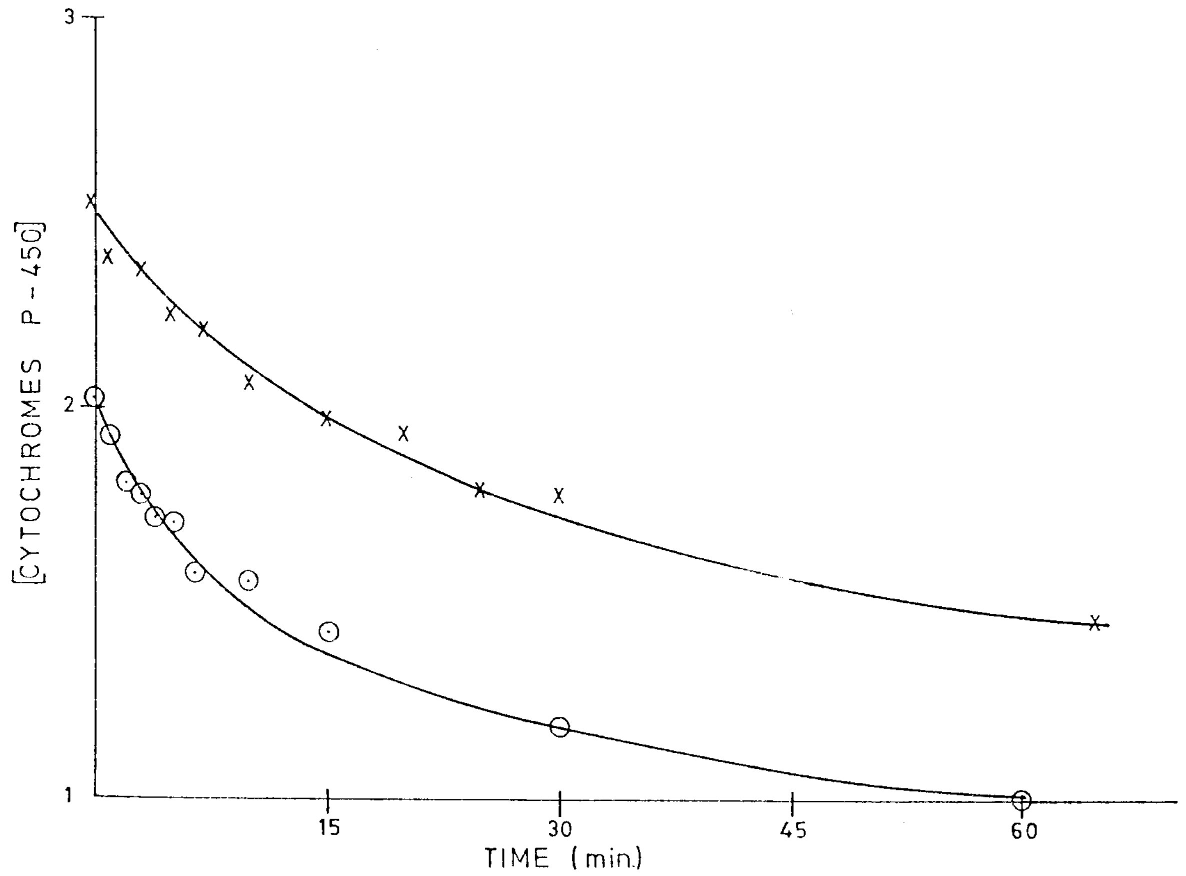


FIGURE 6 The destruction of cytochromes P-450 by fluroxene as a function of time in phenobarbital (x) and methylcholanthrene ⊙ microsomes _in vitro_. Cytochromes P-450 concentration, nmol / mg microsomal protein

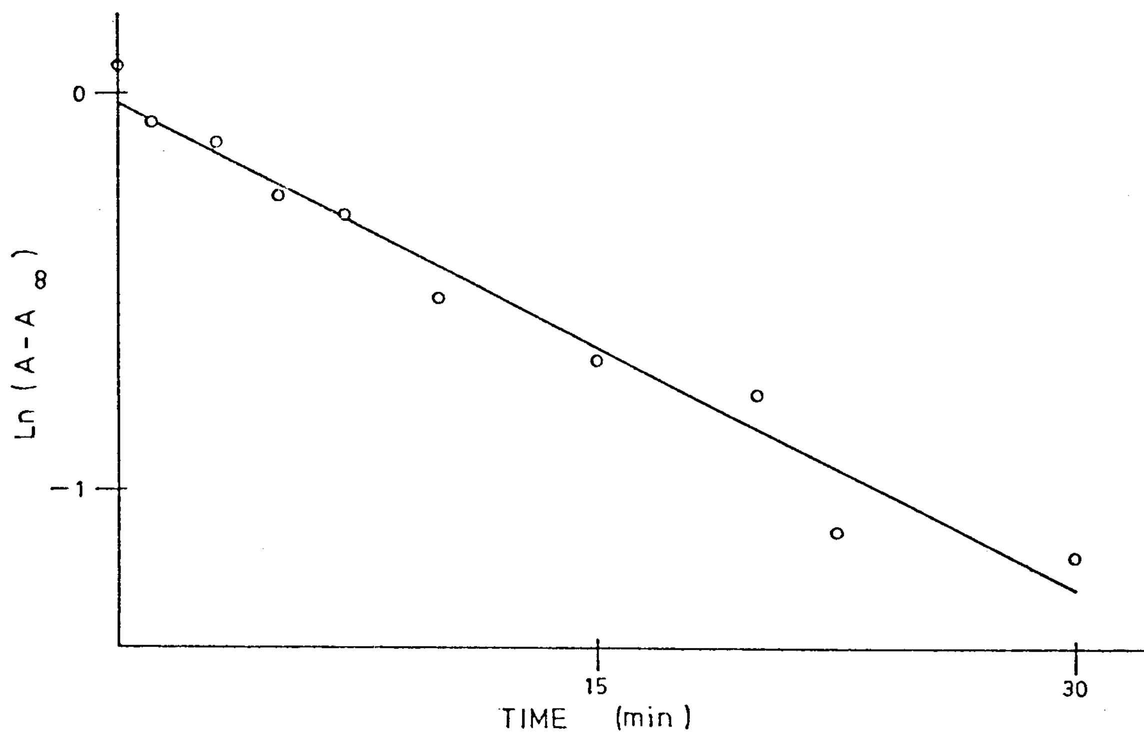


FIGURE 7 The first order rate plot for the destruction of cytochromes P-450 by fluroxene in phenobarbital induced microsomes *in vitro*

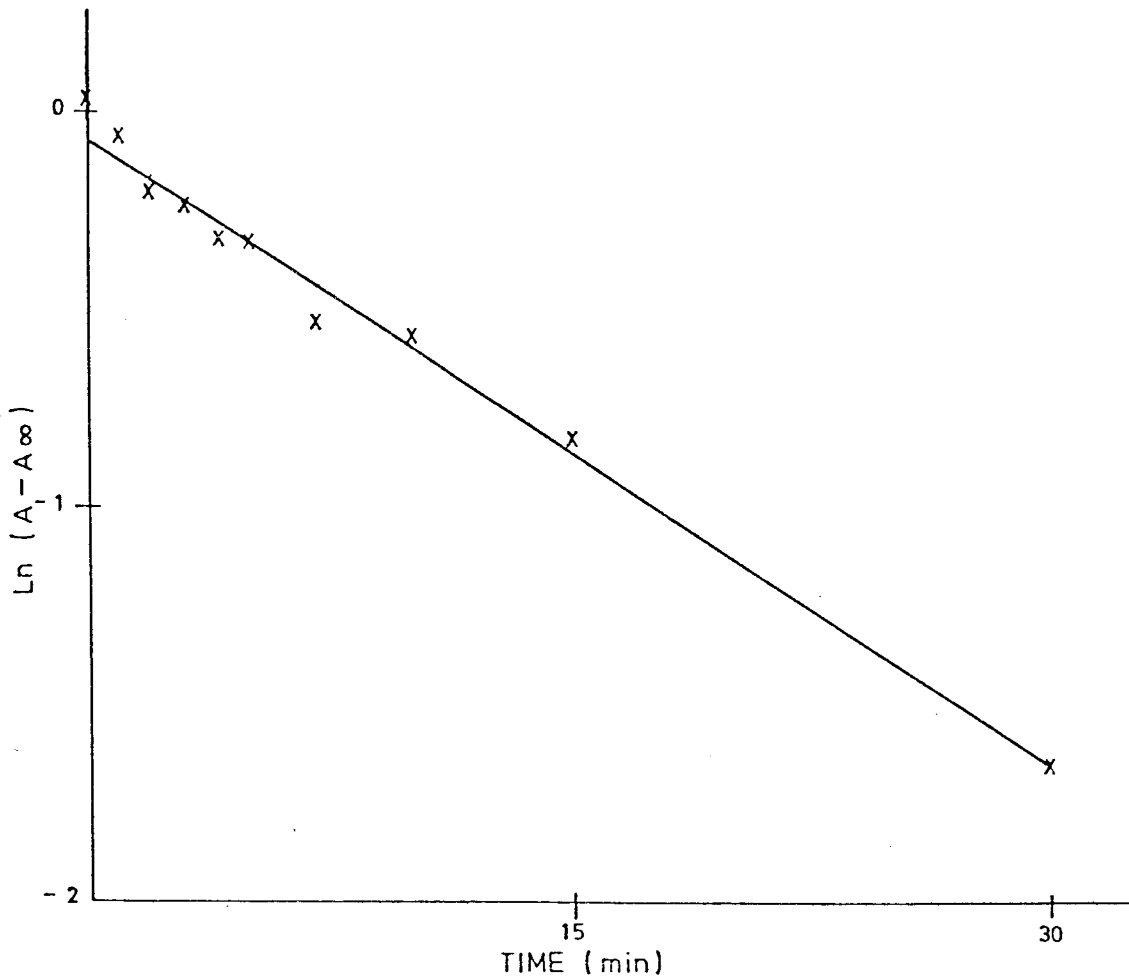


FIGURE 8 The first order rate plot for the destruction of cytochromes P-450 by fluroxene in methylcholanthrene induced microsomes *in vitro*

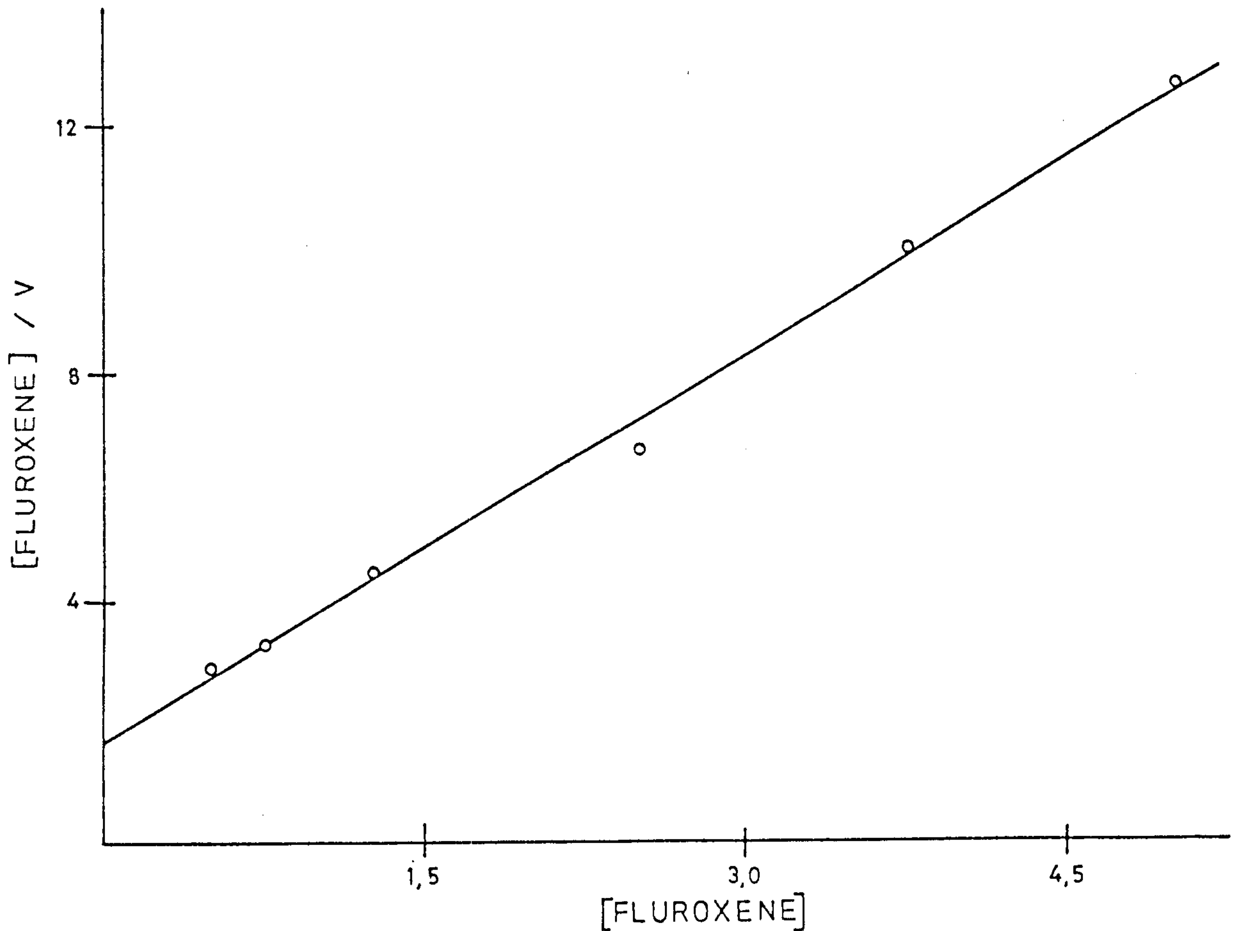


FIGURE 9 Hanes plot for the destruction of cytochromes P-450 by fluroxene in phenobarbital induced microsomes. Fluroxene concentration, mM; V, nmol cytochromes P-450 destroyed / mg microsomal protein / 7 min

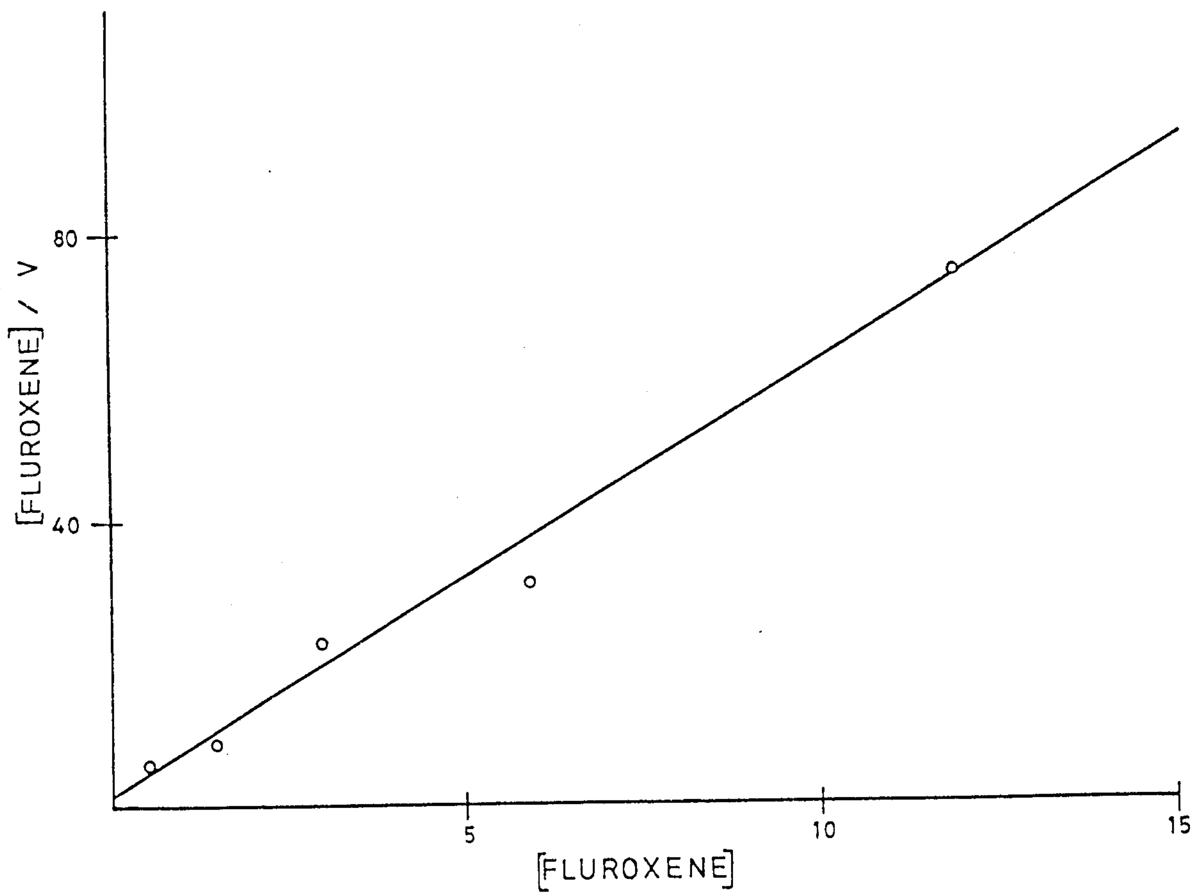


FIGURE 10 Hanes plot for the destruction of cytochromes P-450 by fluroxene in methylcholanthrene induced microsomes. Fluroxene concentration, mM; V, nmol cytochromes P-450 destroyed / mg microsomal protein / 7 min

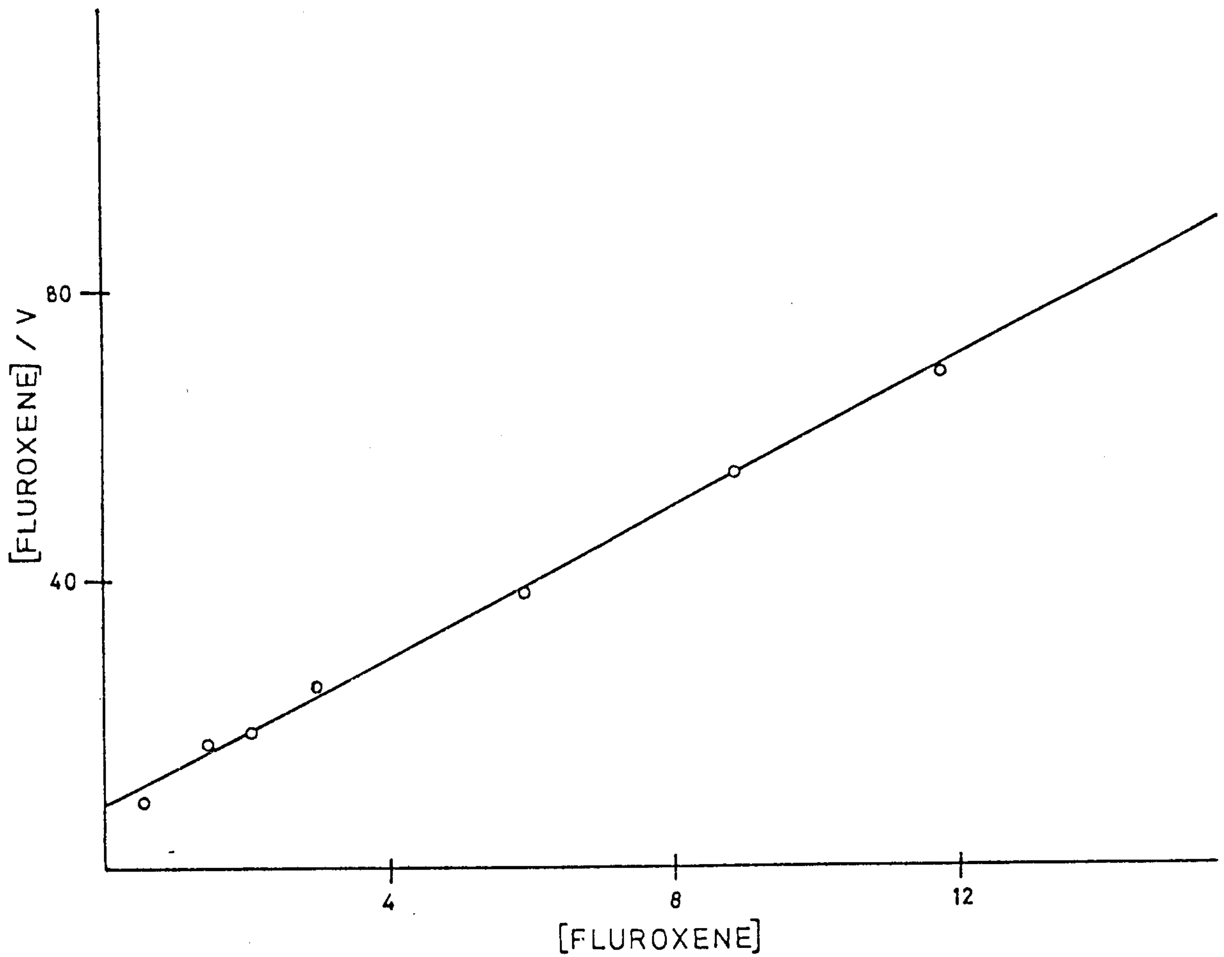


FIGURE 11 Hanes plot for the destruction of cytochromes P-450 by fluroxene in uninduced microsomes. Fluroxene concentration, mM; V, nmol cytochromes P-450 destroyed / mg microsomal protein / 10 min

Table 13. The effect of induction on the K_m and V_{max} values for the destruction of cytochromes P-450 by fluroxene *in vitro*

Induction	K_m (mM)	V_{max} (nmol cyts P-450/ mg mics protein/ 7 min)
None	1,76 ± ,10	0,19 ± ,02[a]
PB	0,87 ± ,35	0,45 ± ,21
MC	3,32 ± ,85	0,48 ± ,16

[a] nmol cyts P-450/mg mics protein/10 min

4a. Interaction of metabolites of fluroxene with hepatic cytochromes P-450 in phenobarbital induced microsomes *in vitro*

Four metabolites of fluroxene are trifluoroethanol, trifluoroacetic acid, trifluoroacetaldehyde and bicarbonate (figure 1). Of these, only trifluoroethanol and trifluoroacetaldehyde bind to cytochromes P-450 giving a type I difference spectrum, whereas trifluoroacetic acid and bicarbonate do not interact with cytochromes P-450 in a spectrally observable manner in phenobarbital induced microsomes. The K_s value for trifluoroacetaldehyde binding to cytochromes P-450 was determined as 3,3 ± 0,9 mM (figure 12).

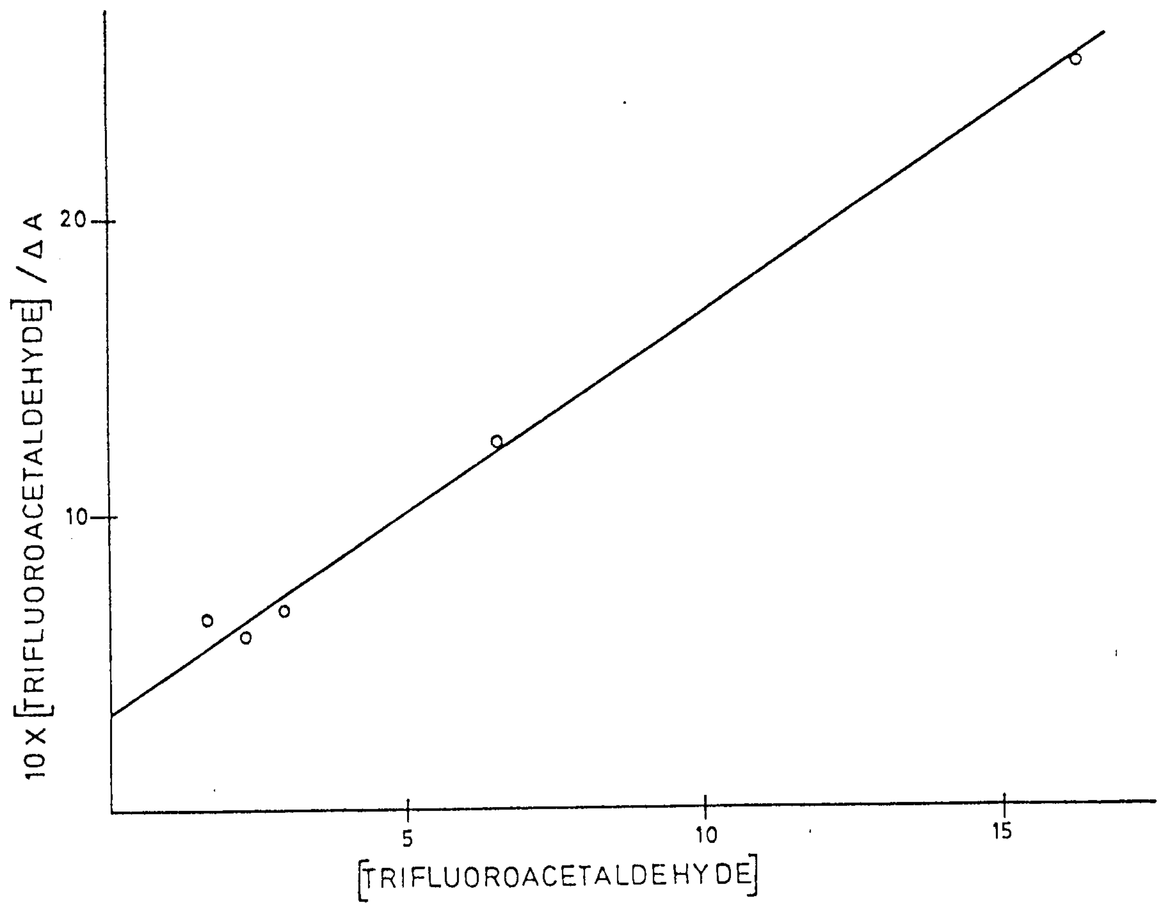


FIGURE 12 Hanes plot for the binding of trifluoroacetaldehyde to cytochromes P-450. Trifluoroacetaldehyde concentration, mM; $\Delta A = A_{380nm} - A_{420nm}$

Trifluoroacetaldehyde appears to be negligibly metabolised, with an apparent V_{max} value of 0,9 $\pm$ 0,4 nmol NADPH consumed/mg microsomal protein/min being calculated at a concentration of 38 mM. Neither trifluoroacetic acid nor bicarbonate enhance carbon monoxide sensitive NADPH consumption and, therefore, apparently do not undergo cytochromes P-450 dependent metabolism. The K_s and K_m values for trifluoroethanol binding and metabolism are presented elsewhere (96).

4b. <u>The effects of metabolites of fluroxene on the levels of hepatic microsomal enzymes *in vitro*</u>

The lack of destruction of cytochromes P-450 in phenobarbital and methylcholanthrene induced microsomes by metabolites of fluroxene is shown in Table 14. None of the metabolites appreciably degrade cytochromes P-450, and this is supported by the lack of destruction of microsomal haem by trifluoroacetaldehyde and bicarbonate in phenobarbital induced microsomes. None of the metabolites of fluroxene affect the levels of cytochrome $\underline{b}_5$ and NADPH-cytochrome $\underline{c}$ reductase in phenobarbital induced microsomes (Table 15).

5. <u>The effects of various compounds and experimental conditions on the fluroxene mediated degradation of hepatic cytochromes P-450 *in vitro*</u>

The effects of various compounds and different experimental conditions on the fluroxene mediated destruction of cytochromes P-450 are shown in Table 16. In phenobarbital

Table 14. The effects of metabolites of fluroxene on the levels of cytochromes P-450 and haem in phenobarbital and methylcholanthrene induced microsomes *in vitro*

Metabolite (mM)	Induction	[Cyts P-450] (nmol/mg mics protein)		% Cyts P-450 lost	[Haem] (nmol/mg mics protein)	
		0 min	30 min		0 min	30 min
-	PB	2,42±,03	2,20±,15	9	2,88±,03	2,72±,04
CF_3CO_2H (200)	PB	2,02±,03	2,07±,04	0		
CF_3CH_2OH (69)	PB	2,99±,02	2,89±,03	3		
CF_3CHO (37)	PB	2,34±,04	2,26±,08	3	2,84±,01	2,77±,08
HCO_3^- (32)	PB	2,39±,06	2,24±,01	6	2,85±,04	2,65±,03
-	MC	1,67±,06	1,70±,07	0		
CF_3CO_2H (190)	MC	1,87±,02	1,77±,09	5		
CF_3CH_2OH (69)	MC	1,79±,03	1,73±,12	3		
CF_3CHO (38)	MC	1,61±,05	1,65±,02	0		
HCO_3^- (32)	MC	1,84±,10	1,88±,06	0		

Table 15. The effects of metabolites of fluroxene on the levels of cytochrome b_5 and NADPH-cytochrome *c* reductase in phenobarbital induced microsomes *in vitro*

Metabolite (mM)	[Cyt b_5] (nmol/mg mics protein)		% Cyt b_5 lost	NADPH-cyt *c* reductase (U/mg mics protein)		% NADPH-cyt *c* reductase lost
	0 min	30 min		0 min	30 min	
-	0,60±,01	0,65±,01	0	0,11±,01	0,11±,01	0
CF_3CO_2H (210)	0,59±,01	0,63±,01	0	0,11±,01	0,11±,01	0
CF_3CH_2OH (69)	0,53±,01	0,57±,01	0	0,11±,01	0,10±,01	9
CF_3CHO (37)	0,61±,01	0,70±,01	0	0,08±,01	0,09±,01	0
HCO_3^- (32)	0,60±,01	0,66±,01	0	0,28±,01	0,26±,01	7

Table 16. The effects of various compounds and experimental conditions on the destruction of cytochromes P-450 by fluroxene (6 mM) *in vitro*

Addition (mM)	Induction	[Cyts P-450] (nmol/mg mics protein)			% Cyts P-450 lost	TFE formed[+] (nmol/mg mics protein/min)
		0 min	10 min	30 min		
-	PB	2,42±,12		1,75±,01	28	
-	PB	2,02±,03	1,77±,01		12	9,7±1,0
$CO:O_2$	PB	2,08±,23	2,21±,04		0	3,5±0,1
O_2	PB	1,71±,01	1,48±,03		13	8,9±1,1
Anaerobic	PB	2,01±,03	2,09±,06		0	< 0,01
GSH (5,0)	PB	2,83±,01		2,09±,02	26	
KCN (0,1)	PB	2,79±,01		1,98±,01	29	
-	MC	2,17±,08		1,44±,02	34	
GSH (5,0)	MC	2,29±,09		1,46±,04	36	

[+] Data from Marsh *et al.* (97).

induced microsomes, $CO:O_2$ (80:20; v/v) completely inhibited the fluroxene mediated destruction of cytochromes P-450 as did totally anaerobic conditions. The extent of the destruction of cytochromes P-450 was not enhanced by saturating the microsomes with oxygen. Reduced glutathione did not inhibit the fluroxene mediated destruction of cytochromes P-450 in phenobarbital or methylcholanthrene induced microsomes. Cyanide did not inhibit the degradation of cytochromes P-450 by fluroxene in phenobarbital induced microsomes.

6. The effects of inhibitors on the fluroxene mediated degradation of hepatic cytochromes P-450 *in vitro*

Total inhibition of the fluroxene mediated degradation of cytochromes P-450 was seen in the presence of 0,05 and 2,3 mM metyrapone in phenobarbital and methylcholanthrene induced microsomes (Table 17). The destruction of cytochromes P-450 by fluroxene was totally inhibited by high concentrations of SKF 525-A (330 μM) but low concentrations of this inhibitor (50 μM), did not diminish the fluroxene mediated degradation of cytochromes P-450 in phenobarbital or methylcholanthrene induced microsomes.

7. Lipid Peroxidation

Malonaldehyde production, under conditions of fluroxene mediated destruction of cytochromes P-450 *in vitro* in phenobarbital induced microsomes, is shown in Table 18.

Table 17. The effects of inhibitors on the destruction of cytochromes P-450 by fluroxene (6 mM) *in vitro*

Inhibitor (mM)	Induction	[Cytochromes P-450] (nmol/mg mics protein)			% Cyts P-450 lost	TFE formed[+] (nmol/mg mics protein/min)
		0 min	10 min	30 min		
-	PB	2,42±,12		1,75±,01	28	
-	PB	2,02±,03	1,77±,01		12	9,7±1,0
MP (2,3)	PB	2,74±,01		2,80±,03	0	
MP (0,05)	PB	1,88±,04	1,95±,07		0	2,4±0,4
SKF 525-A (0,05)	PB	2,30±,03	1,96±,03		15	6,8±1,7
SKF 525-A (0,33)	PB	2,37±,03	2,34±,06		1	4,1±0,4
-	MC	1,84±,01	1,53±,02		17	
MP (0,05)	MC	1,42±,08	1,40±,02		1	
SKF 525-A (0,05)	MC	1,83±,01	1,59±,04		13	

[+] Data from Marsh *et al*. (97).

As shown therein, the increase in malonaldehyde production caused by the addition of generating system was the same in the absence and presence of fluroxene.

Table 18. Malonaldehyde production in phenobarbital induced microsomes *in vitro*

Additions (mM)	[Malonaldehyde] (nmol/mg mics protein) 0 min	[Malonaldehyde] (nmol/mg mics protein) 30 min	[Malonaldehyde] (nmol/mg mics protein/30 min)
-	0,14±0,10	0,40±0,07	0,26
NADPH generating system	0,02±0,01	0,64±0,10	0,62
Fluroxene (30 mM)	0,83±0,18	0,93±0,32	0,10
NADPH generating system + fluroxene (30 mM)	1,56±0,70	2,13±0,40	0,57

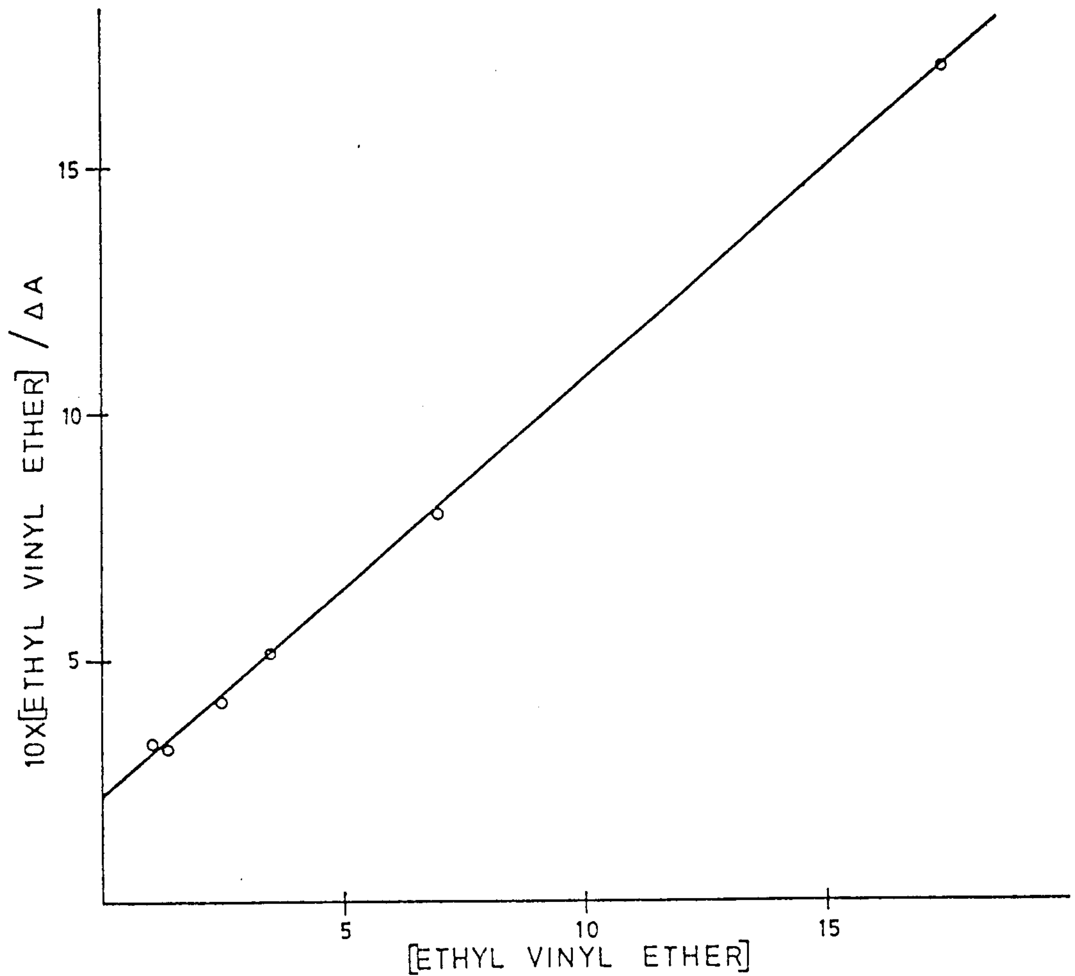
8a. The interaction of structural analogues of fluroxene with hepatic cytochromes P-450 in phenobarbital induced microsomes *in vitro*

The structural analogues of fluroxene, *e.g.* ethyl vinyl ether, divinyl ether and 2,2,2-trifluoroethyl ethyl ether, all bind to cytochromes P-450 in a type I manner. The binding constants for these interactions were calculated to be 2,65 ± 0,09 mM and 2,70 ± 0,85 mM for ethyl vinyl

ether and divinyl ether, respectively. Both of the vinyl ethers are metabolised by cytochromes P-450 as measured by NADPH consumption. K_m values of 3,33 ± 0,04 mM and 1,25 ± 0,21 mM and V_{max} values of 7,65 ± 0,67 and 7,74 ± 0,54 nmoles NADPH consumed/mg microsomal protein/min were calculated for ethyl vinyl ether and divinyl ether, respectively. The Hanes plots were similar for both ethyl vinyl ether and divinyl ether and those for the binding and metabolism of ethyl vinyl ether are illustrated in Figures 13 and 14. Trifluoroethyl ethyl ether also binds to cytochromes P-450 in a type I manner and is metabolised by cytochromes P-450. Details of the interaction of trifluoroethyl ethyl ether with cytochromes P-450 are reported elsewhere (48).

8b. Effects of analogues of fluroxene on hepatic microsomal enzymes *in vitro*

In phenobarbital induced microsomes, neither trifluoroethyl ethyl ether, ethyl vinyl ether nor divinyl ether destroy cytochromes P-450 (Table 19). However, in methylcholanthrene induced microsomes, ethyl vinyl ether and divinyl ether degrade cytochromes P-450, whereas trifluoroethyl ethyl ether does not (Table 20). Microsomal haem is also destroyed by ethyl vinyl ether and divinyl ether in methylcholanthrene induced microsomes and the loss of microsomal haem roughly parallels the loss of cytochromes P-450 (Table 20). In both cases, the % loss of haem/cytochromes P-450 is greater than that observed with



<u>FIGURE 13</u> Hanes plot for the binding of ethyl vinyl ether to cytochromes P-450
Ethyl vinyl ether concentration, mM; $\Delta A = A_{385nm} - A_{420nm}$

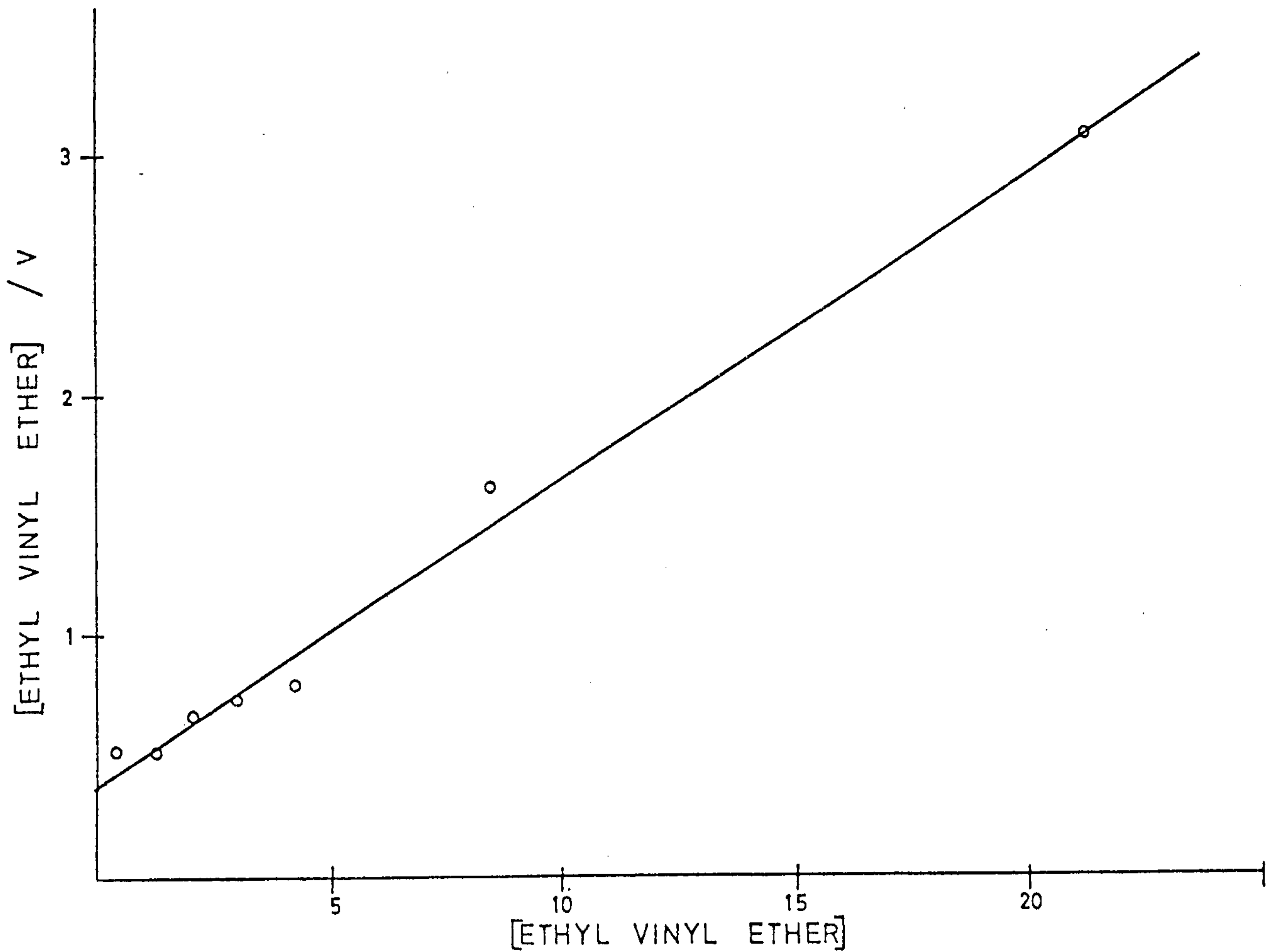


FIGURE 14 Hanes plot for the metabolism of ethyl vinyl ether by cytochromes P-450. Ethyl vinyl ether concentration, mM; V, nmol NADPH consumed / mg microsomal protein / min.

Table 19. The effects of analogues of fluroxene on the levels of cytochromes P-450 in phenobarbital induced microsomes *in vitro*

Compound (mM)	[Cyts P-450] (nmol/mg mics protein)		% Cyts P-450 lost
	0 min	30 min	
-	2,85 ± ,04	2,72 ± ,08	5
EVE (35)	2,63 ± ,02	2,71 ± ,02	0
DVE (22)	3,02 ± ,03	3,19 ± ,04	0
TFEE (28)	2,28 ± ,05	2,16 ± ,05	5

Table 20. The destruction of cytochromes P-450 and haem by analogues of fluroxene in methylcholanthrene induced microsomes *in vitro*

Compound (mM)	[Cyts P-450] (nmol/mg mics protein) 0 min	[Cyts P-450] (nmol/mg mics protein) 30 min	% Cyts P-450 lost	[Haem] (nmol/mg mics protein) 0 min	[Haem] (nmol/mg mics protein) 30 min	Loss cyts P-450 (nmol/mg mics protein)	Loss Haem (nmol/mg mics protein)	% Loss haem/ Loss cyts P-450
-	1,67±,04	1,70±,07	0	2,43±,04	2,36±,06	0	0,07	
EVE (35)	1,81±,02	1,59±,01	12	2,42±,12	2,21±,01	0,22	0,21	95
EVE (35)[a]	1,73±,05	1,71±,05	1					
DVE (22)	1,93±,02	1,38±,02	28	2,51±,01	2,13±,01	0,55	0,38	69
DVE (22)[a]	1,81±,06	1,70±,04	6					
TFEE (28)	1,73±,02	1,72±,10	1					

[a] Without NADPH generating system.

fluroxene (Table 11). Reduced glutathione does not inhibit the destruction of cytochromes P-450 by ethyl vinyl ether or divinyl ether in methylcholanthrene induced microsomes (Table 21).

Table 21. The effect of reduced glutathione on the destruction of cytochromes P-450 by ethyl vinyl ether and divinyl ether in methylcholanthrene induced microsomes *in vitro*

Analogue (mM)	GSH (5mM)	[Cyts P-450] (nmol/mg mics protein) 0 min	30 min	% Cyts P-450 lost
-		2,08±,04	2,15±,05	0
EVE (35)	+	2,21±,08	1,85±,04	16
	-	2,13±,03	1,94±,09	9
DVE (22)	+	2,27±,01	1,55±,04	32
	-	2,21±,06	1,65±,05	25

Neither ethyl vinyl ether nor divinyl ether affect the levels of cytochrome $\underline{b}_5$ and NADPH-cytochrome $\underline{c}$ reductase in methylcholanthrene induced microsomes (Table 22).

9. SDS gel electrophoresis of hepatic cytochrome P-450 haemoproteins

The SDS gel electrophoresis of cytochromes P-450 was performed

Table 22. The effects of ethyl vinyl ether and divinyl ether on the levels of cytochrome b_5 and NADPH-cytochrome *c* reductase in methylcholanthrene induced microsomes *in vitro*

Analogue (mM)	[Cyt b_5] (nmol/mg mics protein)		% Cyt b_5 lost	NADPH-cyt *c* reductase (U/mg mics protein)		% NADPH-cyt *c* reductase lost
	0 min	30 min		0 min	30 min	
-	0,79±,04	0,87±,02	0	0,07±,01	0,08±,01	0
EVE (35)	0,75±,01	0,85±,01	0	0,11±,01	0,12±,01	0
DVE (22)	0,73±,02	0,79±,01	0	0,14±,01	0,13±,01	7

in order to establish which type P-450 cytochromes are degraded by fluroxene *in vivo*.

Figures 15 and 16 show the scans of the cytochromes P-450 band patterns after SDS gel electrophoresis of phenobarbital and methylcholanthrene induced microsomes. Normally only four protein bands corresponding to the haem proteins located by the peroxidase stain could be detected, but when good separation was achieved, a fifth band was detected between bands 3 and 4. This band appeared to merge with band 3 when separation was not optimal. Phenobarbital and methylcholanthrene induction gave rise to different cytochromes P-450 protein band patterns. The band patterns for phenobarbital induced microsomes were similar to those reported by Welton and Aust (87). In methylcholanthrene induced microsomes, the protein bands differed considerably from those reported by Welton and Aust (87) but were similar to those reported by Welton *et al*. (28). Fluroxene treatment of induced animals did not alter the protein band patterns relative to animals receiving the corresponding inducing agent only. This observation was confirmed by integration of the cytochromes P-450 peaks: the loss of cytochromes P-450 as measured by integration is negligible and within experimental error (Table 23).

To test the validity of the results obtained using SDS electrophoresis, this technique was utilized to quantitate the cytochromes P-450 bands from phenobarbital induced animals treated with AIA and were compared to those from

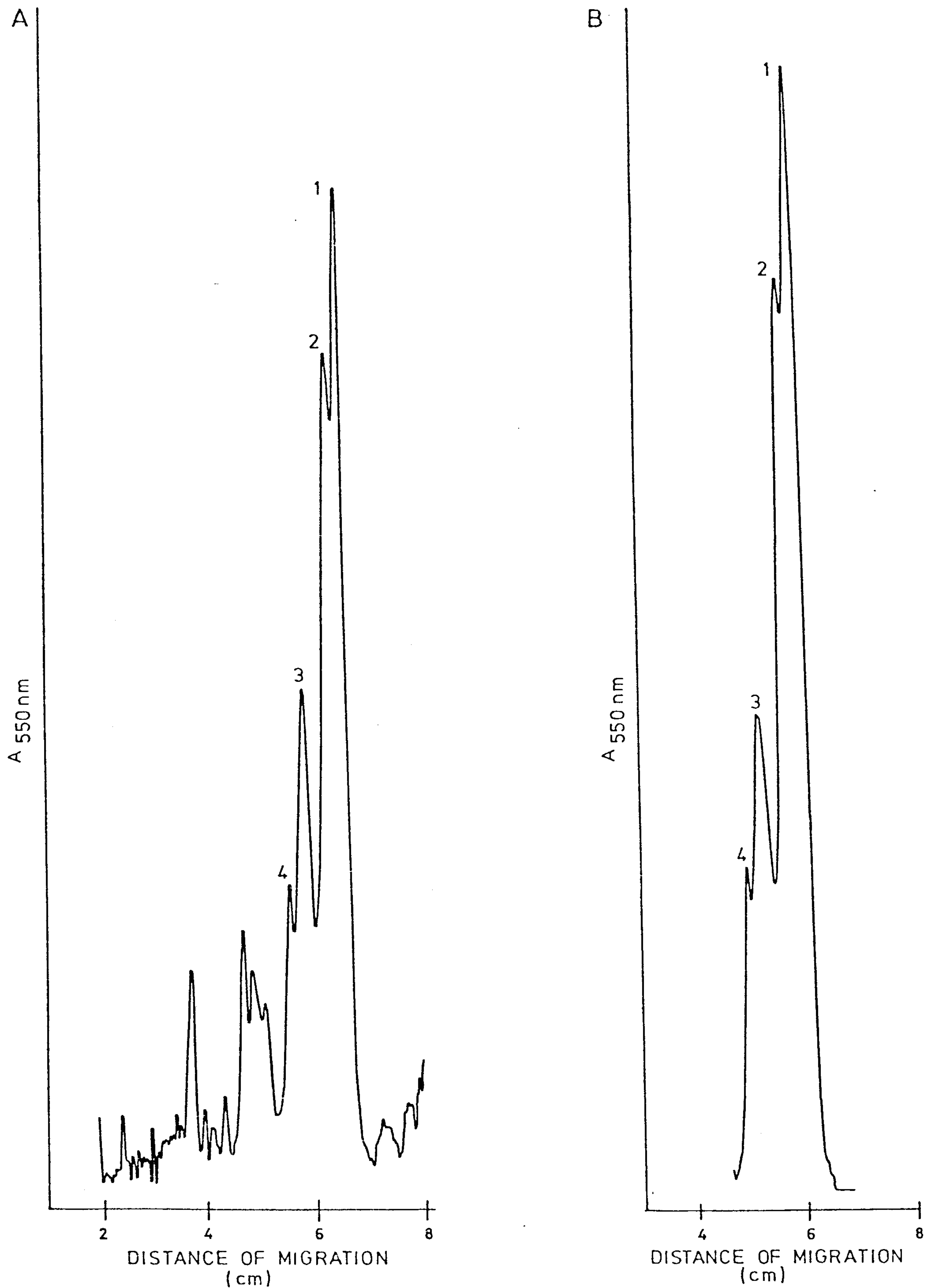


FIGURE 15 Scans of the cytochromes P-450 protein bands after separation by SDS gel electrophoresis of phenobarbital plus fluroxene (A) and phenobarbital (B) microsomes

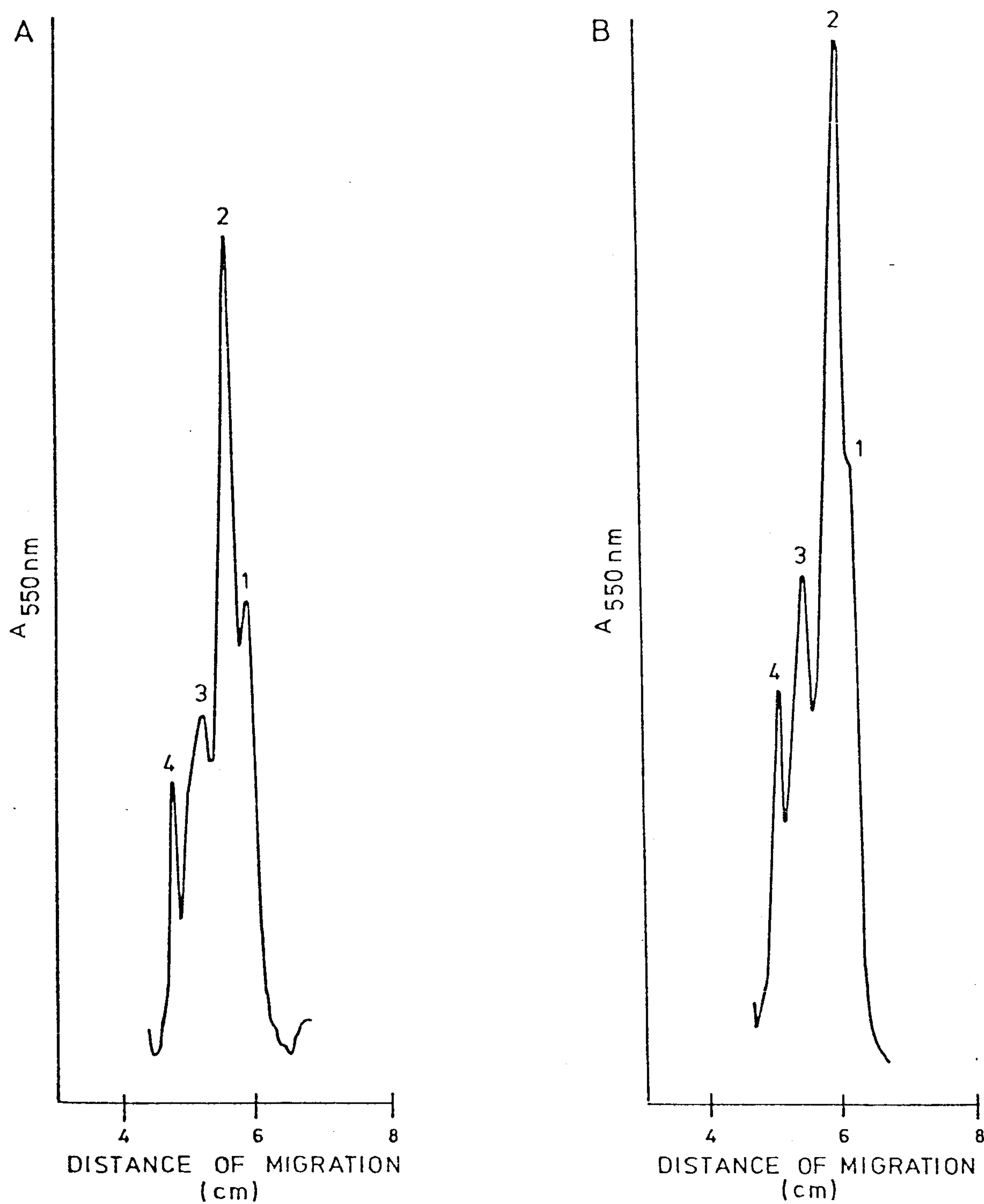


FIGURE 16 Scans of the cytochromes P-450 protein bands after separation by SDS gel electrophoresis of methylcholanthrene plus fluroxene (A) and methylcholanthrene (B) microsomes

Table 23. The effects of fluroxene or AIA treatment on cytochrome P-450 haemoproteins as measured by SDS gel electrophoresis

Induction	Further treatment	[Cytochromes P-450][a] (nmol/mg mics protein)	Cytochromes P-450 haemoprotein bands (% of total area)				Total area (arbitrary units)
			Peak 1	Peak 2	Peak 3	Peak 4	
PB	None	3,01±,04	41±2	29±1	23±1	8±1	38±5
PB	Fluroxene	1,79±,39	40±3	25±3	27±4	8±1	32±3
MC	None	2,27±,07	22[b]	45[b]	24±1	10±1	29±5
MC	Fluroxene	1,06±,03	22±2	46±3	23±1	11±1	26±4
PB	None	2,06±,03	45±2	30±2	17±4	8±1	16±3
PB	AIA	0,63±,05	45±1	27±2	21±2	8±1	19±3

[a] Assayed by the method of Omura and Sato (72).

[b] The peaks were only integrated separately on one occasion. The value obtained for the combined peaks was 66±1%.

phenobarbital induced animals. AIA has been proposed to specifically destroy the haem moiety of cytochrome P-450 (51). The scans of the cytochromes P-450 protein bands of hepatic microsomes isolated from animals treated or not treated with AIA are identical although considerable degradation of cytochromes P-450 is observed by spectral assay (Table 23). In addition, no loss of cytochromes P-450 is measurable by integration (Table 23).

These results are inconsistent with the proposed specificity of AIA to degrade cytochrome P-450 in vivo and with the degradation of cytochromes P-450 by fluroxene demonstrated in this thesis (see DISCUSSION). The discrepancy in the results possibly arises because the SDS gel electrophoresis is performed at high detergent concentration which results in the disruption of the environment of the haem causing the denaturation of cytochromes P-450. During SDS gel electrophoresis of isolated, purified cytochromes P-450, haem is shown to be liberated from the cytochrome P-450 haemoproteins, and appears as a forerunning band of free haem. In addition, a single haem plus protein band is detected (99). It appears, therefore, that the areas corresponding to the different type P-450 cytochromes reflect the sum of the concentrations of the haemoprotein plus apoprotein. Since SDS gel electrophoresis of microsomes from animals treated with fluroxene or AIA show no changes in the relative amounts of the cytochromes P-450 present compared to animals not treated (Table 23), it appears that fluroxene and AIA degrade

cytochromes P-450 by destroying the haem moiety of the protein, without affecting the apoprotein. Furthermore, it appears that this technique cannot be utilized to quantitate cytochromes P-450 where the haem but not the protein moiety has been degraded, as was attempted by Baird _et al_. (100).

V DISCUSSION

Fluroxene has been shown to be toxic to many animal species and to man (4-11). Since the toxicity of fluroxene appears to arise from the metabolic conversion of fluroxene by the mixed function oxidases to toxic intermediates or metabolites, we have investigated the metabolism of fluroxene by hepatic microsomal mixed function oxidases *in vivo* and *in vitro*. Aspects of the metabolism of fluroxene and the fluroxene mediated degradation of cytochromes P-450, will be discussed herein.

The effects of fluroxene on the levels and activities of cytochromes P-450 and proteins associated with the cytochromes P-450 drug metabolising pathway have been investigated (INTRODUCTION, equation 2). Of these enzymes, fluroxene appears to degrade only cytochromes P-450 and does not affect the levels of cytochrome $\underline{b}_5$ or NADPH-cytochrome *c* reductase *in vivo* or *in vitro* (Tables 3, 11 and 12). In addition, there appears to be no loss of total microsomal protein as measured by the microsomal marker enzyme, glucose-6-phosphatase (Table 3)(101).

Fluroxene degrades cytochromes P-450 by chemically modifying only the haem moiety of the haemoprotein *in vivo*. This is evident because the loss of microsomal haem is equivalent to the loss of cytochromes P-450 *in vivo* (Table 3), whereas the apoprotein is not degraded, as measured by SDS gel electrophoresis (Table 23). Fluroxene also appears to only degrade the haem moiety of cytochromes

P-450 in vitro; the loss of haem is equivalent to the loss of cytochromes P-450 in phenobarbital induced microsomes, although not in methylcholanthrene induced microsomes (Table 11). In addition, there is no appearance of cytochromes P-420 (figure 5), the denatured form of cytochromes P-450 which results from the binding to microsomal proteins of the free haem moiety released following denaturation of cytochromes P-450 (102). The presence of cytochromes P-420 would have been expected if the conformation of the enzyme was altered but the haem moiety not destroyed.

In order to establish whether the degradation of cytochromes P-450 is accompanied by a loss of activity of these haemoproteins in vivo and to establish which type P-450 cytochromes are degraded by fluroxene, a series of cytochromes P-450 dependent reactions were measured after fluroxene treatment of animals. Some of the cytochromes P-450 dependent reactions utilized are known to be catalysed non-specifically by type P-450 cytochromes, e.g. p-nitroanisole O-demethylation and biphenyl 4-hydroxylation (76, 89). In addition, the binding of aniline to cytochromes P-450 appears to be non-specific (90,91). In contrast, other cytochromes P-450 dependent reactions utilized have been shown to be catalysed by particular type P-450 cytochromes. For example the hydroxylation of benzpyrene is catalysed specifically by cytochrome P-448 (26,27,93,94,98), whereas the N-demethylation of ethylmorphine appears to be catalysed by cytochrome P-450 but definitely not by cytochrome P-448* (26,32,92). Furthermore, the binding of ethyl

* The specificity of ethylmorphine N-demethylase activity in this thesis refers to cytochromes P-450 in male rats.

isocyanide to ferrocytochromes P-450 differs with cytochrome P-450 and cytochrome P-448 and can be utilized to distinguish these forms of the haemoproteins (25,26).

Since, following fluroxene treatment, the losses of p-nitroanisole O-demethylase and biphenyl 4-hydroxylase activities and aniline binding are equivalent to the loss of cytochromes P-450 (Tables 4, 5 and 6), it would appear that the decrease in concentration of cytochromes P-450, as measured by spectral assay, reflects a decrease in enzymatic activity of this group of enzymes. The reactions which are specifically catalysed by different type P-450 cytochromes were used to distinguish which type P-450 cytochromes are preferentially degraded by fluroxene in phenobarbital and methylcholanthrene induced microsomes. Since, following fluroxene treatment of phenobarbital induced animals, ethylmorphine N-demethylase activity is decreased (Table 7), but benzpyrene hydroxylase activity is not (Tables 8 and 9), it would appear that cytochrome P-450 is degraded by fluroxene, and cytochrome P-448 is not. In methylcholanthrene induced animals the loss of both ethylmorphine N-demethylase and benzpyrene hydroxylase activities (Tables 7 and 8) indicate that both cytochrome P-450 and cytochrome P-448 are degraded by fluroxene *in vivo* following this type of induction. In order to try and establish which of these type P-450 cytochromes is preferentially degraded by fluroxene in methylcholanthrene induced microsomes, values have been assigned for the levels of cytochrome P-450 and cytochrome P-448 in this type of

preparation of microsomes determined on the basis of a large body of scientific literature (26,28,89,93,103).

In methylcholanthrene induced microsomes, where the total cytochromes P-450 content is 1,6 nmol/mg microsomal protein, approximately 1 nmol/mg microsomal protein appears to be cytochrome P-448 which catalyses the hydroxylation of benzpyrene, and the remaining 0,6 nmol/mg microsomal protein constitutes the other type P-450 cytochromes. There is a 65% loss of benzpyrene hydroxylase activity following the degradation of cytochromes P-450 by fluroxene <u>in vivo</u> in methylcholanthrene induced microsomes (Table 8) and thus, of the 1 nmol/mg microsomal protein cytochrome P-448, 0,65 nmol/mg microsomal protein is degraded. Of the 0,6 nmol/mg microsomal protein type P-450 cytochromes remaining, 0.2 nmol/mg microsomal protein is degraded by fluroxene (Table 7). Thus, of the 0,8 nmol/mg microsomal protein cytochromes P-450 degraded by fluroxene in methylcholanthrene induced animals, 0,65 nmol/mg microsomal protein is cytochrome P-448 and 0,2 nmol/mg microsomal protein the other type P-450 cytochromes. Although the exact amounts of the different type P-450 cytochromes in microsomes following methylcholanthrene induction are unknown, it appears that cytochrome P-448 is preferentially degraded by fluroxene <u>in vivo</u> following methylcholanthrene induction. Further support for these proposals was obtained from the changes in the crossover pH following the degradation of cytochromes P-450 <u>in vivo</u>.

The ethyl isocyanide crossover pH of purified preparations of cytochrome P-450 and cytochrome P-448 are 7,85 and 6,85

respectively (25,26). In methylcholanthrene induced animals, the change in crossover pH from 6,9 to 7,5 following fluroxene treatment (figure 4, Table 10) indicates that cytochrome P-448 is preferentially degraded by fluroxene *in vivo*. Following fluroxene treatment of phenobarbital induced animals, the crossover pH remains the same as that found for microsomes from phenobarbital induced (figure 3, Table 10) and uninduced animals (69), indicating that either cytochrome P-450, which is induced by phenobarbital, or the type P-450 cytochrome predominating in uninduced microsomes, is degraded by fluroxene. However, the lack of degradation of cytochromes P-450 following fluroxene anaesthesia of uninduced rats (104) suggests that fluroxene degrades cytochrome P-450 in preference to the type P-450 cytochrome predominating in uninduced microsomes.

The degradation of cytochrome P-450 in phenobarbital induced microsomes and cytochrome P-448 in methylcholanthrene induced microsomes has also been demonstrated *in vitro*. From studies of the kinetics of the degradation of cytochromes P-450 by fluroxene, it was found that the total losses of cytochromes P-450 were equivalent for phenobarbital and methylcholanthrene induced microsomes indicating that both cytochrome P-450 and cytochrome P-448 are degraded by fluroxene. In contrast, AIA has been proposed to specifically degrade cytochrome P-450 (51). Support for this proposal arises from studies of the kinetics of the degradation of cytochromes P-450 by AIA *in vitro* (105). The extent of loss of cytochromes P-450

following degradation by AIA differs considerably for phenobarbital and methylcholanthrene induced microsomes: in phenobarbital induced microsomes, 50% of the cytochromes P-450 was lost over 65 min, whereas in methylcholanthrene induced microsomes, only 16% was lost over the same period of time indicating that only cytochrome P-450 is degraded by AIA in both phenobarbital and methylcholanthrene induced microsomes.

Although fluroxene degrades both cytochrome P-450 and cytochrome P-448, this anaesthetic agent does not appear to affect the levels of any other type P-450 cytochromes in hepatic microsomes _in vitro_. In uninduced microsomes, where neither cytochrome P-450 nor cytochrome P-448 are thought to predominate (25,87,106), attempts to determine the first order rate constant for the degradation of cytochromes P-450 by fluroxene _in vitro_ were not successful because the decrease in the levels of cytochromes P-450 was very slight. In addition, the V_{max} value for the degradation of cytochromes P-450 by fluroxene in uninduced microsomes was considerably lower than that observed for phenobarbital and methylcholanthrene induced microsomes (Table 13). The decreased ability of fluroxene to degrade cytochromes P-450 in uninduced microsomes may result from the initial low levels of cytochromes P-450, but more likely reflects the ability of fluroxene to degrade cytochrome P-450 and cytochrome P-448, but not the other type P-450 cytochromes.

Since fluroxene appears to degrade cytochrome P-450 in

phenobarbital induced microsomes and both cytochrome P-450 and cytochrome P-448 in methylcholanthrene induced microsomes, K_m values for the degradation of cytochromes P-450 by fluroxene were measured in order to establish whether different mechanisms exist for the fluroxene mediated degradation of cytochromes P-450 in the differently induced microsomes. The K_m values for the degradation of cytochromes P-450 by fluroxene are shown to differ for the variously induced microsomes (Table 13). The K_m value for the degradation of cytochromes P-450 by fluroxene in phenobarbital induced microsomes is identical to K_s for the binding of fluroxene to cytochrome P-450 and the K_m for the cytochrome P-450 dependent conversion of fluroxene to trifluoroethanol (48,96). Therefore, in phenobarbital induced microsomes, it appears that the binding of fluroxene to cytochrome P-450 is rapid and prior to the rate limiting step in the degradation of cytochromes P-450 by fluroxene. In phenobarbital induced microsomes, only cytochrome P-450 is involved in the metabolism of fluroxene, and only cytochrome P-450 appears to be degraded by fluroxene.

The K_m value for the degradation of cytochromes P-450 by fluroxene in methylcholanthrene induced microsomes is higher than that in phenobarbital induced microsomes and differs from the K_m for the conversion of fluroxene to trifluoroethanol in methylcholanthrene induced microsomes (96). The difference between the K_m values for the metabolism of fluroxene by cytochrome P-450 and the fluroxene mediated degradation of cytochromes P-450 in methylcholanthrene induced microsomes probably reflects the involvement of different type P-450 cytochromes in the metabolism of

fluroxene and in the fluroxene mediated degradation of cytochromes P-450 with this type of microsomes. It has, in fact, been demonstrated in methylcholanthrene induced microsomes that cytochrome P-450 is the main type P-450 cytochrome catalysing the conversion of fluroxene to trifluoroethanol (48), whereas cytochrome P-450 and cytochrome P-448 are both degraded by fluroxene.

Metabolic activation of fluroxene appears to be a prerequisite for the degradation of cytochromes P-450 by fluroxene. The degradation of cytochromes P-450 requires NADPH and oxygen (Tables 11 and 16), both of which are cofactors for cytochromes P-450 dependent reactions as well as for other microsomal reactions such as haem oxygenase and stearate desaturase (107-109). Carbon monoxide, an inhibitor of cytochromes P-450 dependent reactions (110), fully inhibits the degradation of cytochromes P-450 by fluroxene (Table 16), whereas cyanide (0,1 mM), an inhibitor of the other microsomal reactions mentioned (111,112), has no effect on the fluroxene mediated degradation of cytochromes P-450 (Table 16). Other inhibitors of the cytochromes P-450 dependent drug metabolising pathway, metyrapone and SKF 525-A (113-116), also inhibit the fluroxene mediated degradation of cytochromes P-450 (Table 17), although inhibition by these compounds is more complex and will be discussed later (DISCUSSION, pages 89,92). Thus the cytochromes P-450 dependent drug metabolising pathway is definitely involved in the degradation of cytochromes P-450 by fluroxene as well as the metabolism of fluroxene (48,96).

Since metabolic activation of fluroxene appears to be essential for the fluroxene mediated degradation of cytochromes P-450, the possible sequence of events during the metabolism of fluroxene which results in the degradation of cytochromes P-450 is as follows: fluroxene binds to ferricytochrome P-450 to form the fluroxene-ferricytochrome P-450 complex which accepts one electron from NADPH to form the fluroxene-ferrocytochrome P-450 complex. The rate limiting step in the cytochromes P-450 drug metabolising pathway has been shown to be subsequent to the binding of molecular oxygen to the ferrocytochromes P-450-substrate complex (117). Therefore, since oxygen does not enhance the fluroxene mediated destruction of cytochromes P-450 (Table 16), the rate limiting step in the degradation of cytochromes P-450 by fluroxene occurs after the fluroxene-ferrocytochrome P-450 oxygenated complex has accepted the second electron from NADPH. The degradation of cytochromes P-450 takes place at either of the subsequent two steps in the metabolism of fluroxene as illustrated in figure 17.

Since metabolic activation of fluroxene is essential for the fluroxene mediated degradation of cytochromes P-450, it is possible that the metabolism of fluroxene results in a reactive metabolite which catalyses the degradation of cytochromes P-450. However, none of the known or proposed stable metabolites of fluroxene can mimic the degradation of cytochromes P-450 by fluroxene (Table 14), although some bind to and are metabolised by cytochromes P-450. Of all the known or proposed metabolites of fluroxene, only the

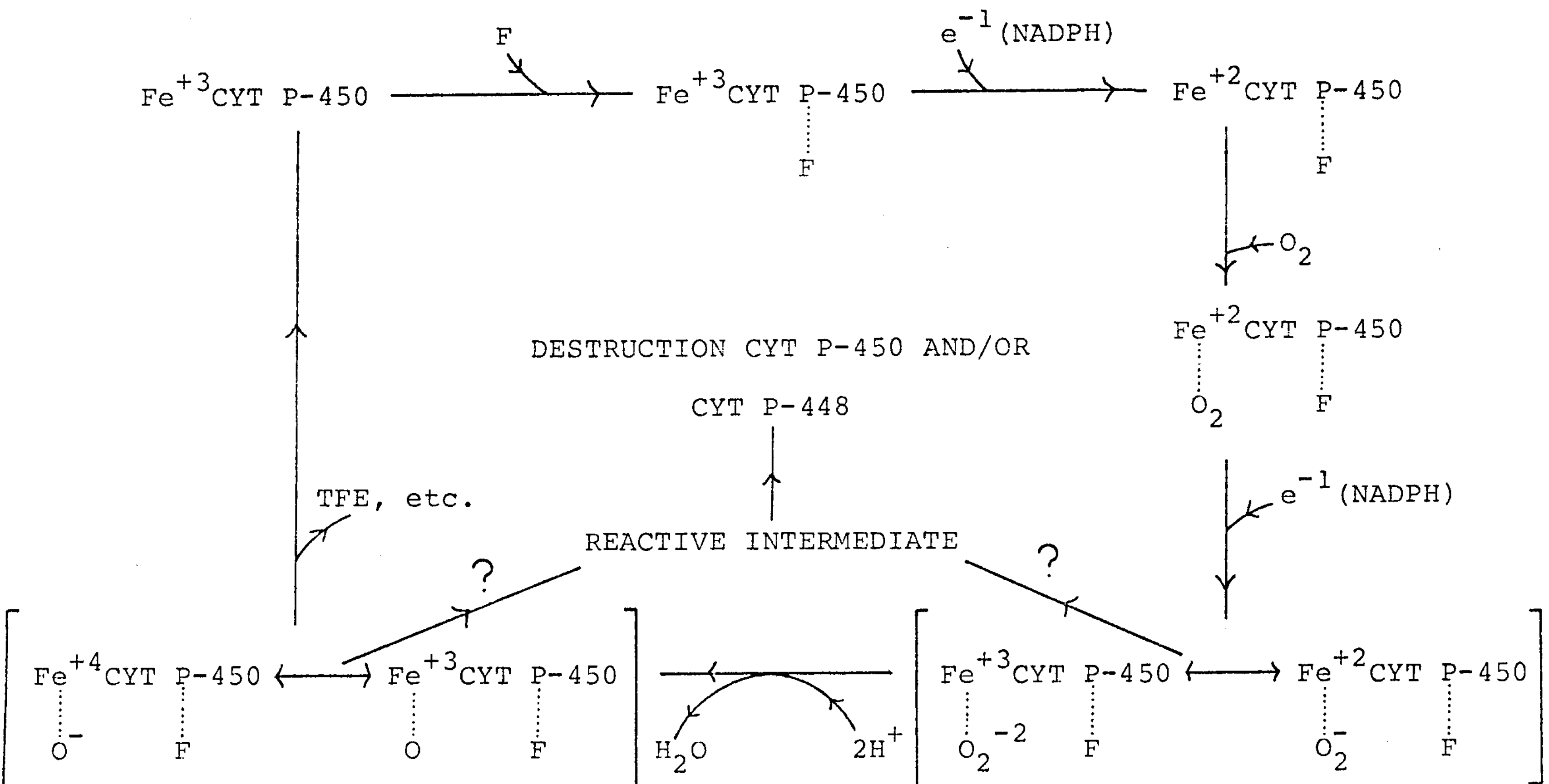


FIGURE 17. A possible mechanism for the destruction of cytochromes P-450 by fluroxene

glucuronide of trifluoroethanol is biologically inactive and as a result was not studied. Hence, it is possible that metabolic activation of fluroxene by cytochromes P-450 may give rise to a transient reactive intermediate which is responsible for the degradation of cytochromes P-450. Since cytochrome P-450 is primarily responsible for the metabolism of fluroxene to trifluoroethanol, it is possible that this enzyme also catalyses the formation of the proposed reactive intermediate which then can degrade cytochrome P-450 and cytochrome P-448. Alternatively, cytochrome P-450 may catalyse the metabolism of fluroxene to trifluoroethanol, but cytochrome P-450 and/or cytochrome P-448 may catalyse the formation of the reactive intermediate from fluroxene which can then react with cytochrome P-450 or cytochrome P-448. By using inhibitors of specific cytochromes P-450 dependent reactions, it was possible to distinguish between these alternatives.

The inhibitors of cytochromes P-450 dependent reactions were selected because of their proposed specificity for different type P-450 cytochromes. Metyrapone has been proposed to bind specifically to cytochrome P-450 (118) and to specifically inhibit cytochrome P-450 dependent reactions (119). There was total inhibition of the fluroxene mediated degradation of cytochromes P-450 in phenobarbital induced microsomes in the presence of low (50 μM) and high (2,3 mM) concentrations of metyrapone (Table 17); since cytochrome P-450 is the main type P-450 cytochrome degraded by fluroxene in phenobarbital induced microsomes

and fluroxene is primarily metabolised by cytochrome P-450, inhibition of the fluroxene mediated degradation of cytochromes P-450 by metyrapone in phenobarbital induced microsomes was anticipated.

In methylcholanthrene induced microsomes, where fluroxene degrades both cytochrome P-450 and cytochrome P-448, there is also total inhibition of the fluroxene mediated degradation of cytochromes P-450 by metyrapone (Table 17). Since metyrapone reportedly inhibits cytochrome P-450 dependent reactions specifically and since fluroxene is metabolised primarily by cytochrome P-450, the total inhibition of the fluroxene mediated degradation of cytochromes P-450 by metyrapone probably indicates that metyrapone inhibits the fluroxene mediated degradation of cytochromes P-450 by inhibiting the formation of the reactive intermediate by cytochrome P-450. Therefore, it appears that two type P-450 cytochrome molecules are involved in the degradation of cytochromes P-450 by fluroxene: cytochrome P-450 alone is involved in the formation of the reactive metabolite which can then degrade cytochrome P-450 or cytochrome P-448. Inhibition of the formation of the reactive metabolite by cytochrome P-450 results in total inhibition of the fluroxene mediated degradation of cytochromes P-450 in phenobarbital and methylcholanthrene induced microsomes. However, the partial inhibition of the conversion of fluroxene to trifluoroethanol in phenobarbital induced microsomes remains unexplained. Perhaps, for some reason, the metabolism of fluroxene to trifluoroethanol is not as susceptible to

inhibition as the fluroxene mediated degradation of cytochromes P-450. It is interesting to note that carbon monoxide also totally inhibits the fluroxene mediated degradation of cytochromes P-450 but not the metabolism of fluroxene to trifluoroethanol (Table 16) and the same explanation may hold for both of these inhibitors of cytochromes P-450 dependent reactions.

In methylcholanthrene induced microsomes, where two type P-450 cytochrome molecules are involved in the degradation of cytochromes P-450 by fluroxene, the kinetics of degradation are pseudo first order with respect to cytochromes P-450. Oxygen, fluroxene and NADPH are the other components of the degradation reaction on whose concentrations the first order kinetics could depend, but these compounds are all present in excess. Therefore, because two different type P-450 cytochromes appear to be involved in the degradation of cytochromes P-450 by fluroxene in methylcholanthrene induced microsomes, the pseudo first order kinetics observed suggest that only one type P-450 cytochrome is involved in the rate limiting step of the reaction. From the similarity between the rate of degradation of cytochromes P-450 by fluroxene in phenobarbital induced microsomes where only cytochrome P-450 is degraded, and methylcholanthrene induced microsomes, where cytochrome P-450 and cytochrome P-448 are degraded, it appears that the type P-450 cytochrome involved in the rate limiting step is the same for both types of induction. Since only cytochrome P-450 appears to be involved in the

formation of the reactive metabolite in both types of induction, this could be the rate limiting step of the reaction.

The inhibition of the fluroxene mediated degradation of cytochromes P-450 by SKF 525-A was also studied. SKF 525-A has been proposed to specifically bind to a type P-450 cytochrome other than that induced by phenobarbital. This type P-450 cytochrome may be cytochrome P-448 or any of the other type P-450 cytochromes (118). Since fluroxene appears to bind to cytochrome P-450 and SKF 525-A is proposed to bind to any type P-450 cytochrome, except cytochrome P-450, no inhibition of the binding of fluroxene to cytochrome P-450 was anticipated. However, although SKF 525-A does not inhibit the binding of fluroxene to cytochrome P-450 at concentrations below 100 μM, it inhibits the binding of fluroxene to cytochromes P-450 at concentrations of 100 μM to 330 μM (97). The type of inhibition observed was of a non-competitive or mixed type in contrast to the competitive inhibition usually observed with SKF 525-A where it competes for binding to the type I binding site of cytochromes P-450 (113,120). Therefore, the specificity of SKF 525-A for binding to a type P-450 cytochrome other than cytochrome P-450 appears to occur only at concentrations below 100 μM.

Since low concentrations of SKF 525-A (50 μM) do not inhibit the fluroxene mediated degradation of cytochromes P-450 in phenobarbital or methylcholanthrene induced microsomes (Table 17), SKF 525-A is probably specific for a type

P-450 cytochrome other than cytochrome P-450 or cytochrome P-448. The role played by the type P-450 cytochrome for which SKF 525-A is specific in the fluroxene mediated degradation of cytochromes P-450, appears to be minor since inhibition of this type P-450 cytochrome does not measurably affect the degradation of cytochromes P-450 by fluroxene; this result confirms the proposal that fluroxene does not affect the levels of type P-450 cytochromes other than cytochrome P-450 and cytochrome P-448*. The inhibition of the fluroxene mediated degradation of cytochromes P-450 seen at higher concentrations of SKF 525-A (330 μM) in phenobarbital induced microsomes, supports the proposal that SKF 525-A non-specifically inhibits type P-450 cytochromes at this concentration (Table 17). Therefore, the inhibition studies done at 330 μM SKF 525-A confirm that cytochromes P-450 are involved in the degradation of cytochromes P-450 by fluroxene, but do not assist in the determination of the role played by the different type P-450 cytochromes.

In an attempt to establish the nature of the reactive intermediate in the fluroxene mediated degradation of cytochromes P-450, the ability of various analogues of fluroxene to degrade cytochromes P-450 was investigated. As a result of these studies, it is established that the reactive intermediate arises from the vinyl moiety of the

* Alternatively, perhaps the site of inhibition of cytochromes P-450 by SKF 525-A is not the same as the site of degradation of this enzyme by fluroxene, but this seems unlikely as SKF 525-A binds to the haem moiety of cytochromes P-450 (113,115,118,120) which is also the site of degradation of the haemoprotein by fluroxene.

molecule. The saturated analogue of fluroxene, trifluoroethyl ethyl ether, does not contain a vinyl moiety and is unable to degrade cytochromes P-450 in phenobarbital or methylcholanthrene induced microsomes *in vitro* (Tables 19 and 20) and *in vivo* (104). However, the analogues of fluroxene which contain the vinyl moiety, ethyl vinyl ether and divinyl ether, both degrade cytochromes P-450 in methylcholanthrene induced microsomes although they do not degrade cytochromes P-450 from phenobarbital induced microsomes (Tables 20 and 19). As with fluroxene, metabolic activation of ethyl vinyl ether and divinyl ether appears to be a prerequisite for the degradation of cytochromes P-450 since no destruction of cytochromes P-450 is observed in the absence of the electron donor NADPH (Table 20). The extent of degradation of cytochromes P-450 is greater with divinyl ether which contains the two vinyl moieties than with ethyl vinyl ether which contains a single vinyl moiety.

Should ethyl vinyl ether and divinyl ether degrade cytochromes P-450 by the same mechanism as fluroxene, it would appear that the difference between the types of cytochromes P-450 degraded by these ethers lies in the difference between the reactive intermediates formed from fluroxene and from ethyl vinyl ether or divinyl ether. With ethyl vinyl ether and divinyl ether, the lack of degradation of cytochromes P-450 in phenobarbital induced microsomes could be explained if cytochrome P-448 only is responsible for catalysing the formation of the reactive intermediates from these ethers. Alternatively, cytochrome P-450 could catalyse the formation of the reactive intermediate which

then specifically degrades cytochrome P-448 but not cytochrome P-450.

Lipid peroxidation is one mechanism whereby the degradation of cytochromes P-450 is known to occur (50,121,122). Lipid peroxidation can occur in the presence of microsomes and NADPH _in vitro_ (123). For this reason, the fluroxene mediated degradation of cytochromes P-450 _in vitro_ was measured in the presence of EDTA, a potent inhibitor of lipid peroxidation (123,124). Furthermore, the production of malonaldehyde, a measure of the extent of lipid peroxidation, was not enhanced in incubation mixtures in which degradation of cytochromes P-450 had occurred to a considerable extent (Table 18). These results confirm that lipid peroxidation is not involved in the degradation of cytochromes P-450 and the reactive intermediate which appears to be involved in the fluroxene mediated degradation of cytochromes P-450 does not arise from or result in the peroxidation of lipids.

Since the degradation of cytochromes P-450 involves the vinyl moiety of the ethers, a likely form of the reactive intermediate would be that of an epoxide. Epoxides are highly strained molecules and as a result are extremely reactive. Epoxides appear to be formed during the metabolism of allyl containing barbiturates, pesticides and polycyclic hydrocarbons by cytochromes P-450 (19,20,125). The degradation of cytochromes P-450 by AIA has been proposed to involve the formation of an epoxide across the double bond of the allyl moiety of the molecule (49).

Fluroxene, ethyl vinyl ether and divinyl ether each contain a vinyl moiety which could be oxidised to an epoxide.

Although reduced glutathione which is a strong nucleophile capable of deactivating epoxides (125) or glutathione S-transferase, a cytoplasmic enzyme, alone do not inhibit the fluroxene mediated degradation of cytochromes P-450, the combination of glutathione S-transferase and reduced glutathione partially inhibit the fluroxene mediated degradation of cytochromes P-450 (126). This combination has been shown to react with strong electrophiles, such as epoxides (127-129). Therefore, it appears that the reactive intermediate produced from fluroxene may take the form of an epoxide. Studies in progress with the microsomal enzyme epoxide hydrase are expected to confirm whether an epoxide is involved in the fluroxene mediated degradation of cytochromes P-450.

The implications of the results of this investigation into the degradation of cytochromes P-450 by fluroxene appear to be two-fold. One important aspect of this investigation is the elucidation of the degradation of cytochromes P-450 as a possible mechanism for drug-drug interactions. Fluroxene is amongst the first medically used compounds shown to degrade cytochromes P-450 _in vivo_ and _in vitro_ and the degradation of cytochromes P-450 should be considered, along with induction, inhibition of and competition with the components of the drug metabolising pathway in explaining the complexities of drug-drug interactions in drug therapy when more than one drug is prescribed. Studies have been

recently completed in our laboratory to establish in which functional groups the ability to specifically degrade cytochromes P-450 resides; these include the allyl, alkyne, nitro, nitrile, and some halogenated moieties (130). Compounds such as AIA and the allyl containing barbiturates contain the allyl functional group and have been shown to degrade cytochromes P-450. It is hoped that the knowledge obtained from establishing which functional groups have the ability to degrade cytochromes P-450 can be extrapolated to medically used compounds and thus the use of drugs which degrade cytochromes P-450 can be avoided or at least cognizance taken of their ability to alter the metabolism of themselves and other xenobiotics. This should especially be noted in the case of anaesthetic agents where not only the patient is affected but also medical personnel involved in administration of the anaesthetic.

Secondly, the possibility was revealed that fluroxene, in its ability to degrade the haem moiety of cytochromes P-450, may produce experimental porphyria. AIA, a compound known to produce porphyria, degrades the haem moiety of cytochromes P-450 _in vivo_ to green pigments, which have been proposed to be oxyphlorins and unidentified porphyrins (95). Furthermore, it has been postulated that the ability of AIA to induce experimental porphyria may be related to its ability to degrade cytochrome P-450 _in vivo_ (56,58,131). Fluroxene is also capable of degrading the haem moiety of cytochromes P-450 and a similar green colouration was observed after the fluroxene mediated destruction of cytochromes

P-450 *in vivo*. Although the mechanism of fluroxene destruction of cytochromes P-450 may be different to the destruction of cytochrome P-450 by AIA, the similarities in the ability of both agents to specifically destroy cytochromes P-450 by haem degradation to green products, has led us to believe that fluroxene may be a chemical inducer of porphyria. This is currently under investigation in our laboratory and preliminary studies have shown that fluroxene treatment of animals elevates the levels of porphyrins and precursors relative to controls and therefore probably does induce experimental porphyria.

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