THE INTERACTION OF THREE LOCAL ANAESTHETIC AGENTS WITH HEPATIC MICROSOMAL CYTOCHROME P-450

by

Leonard Howard van den Honert, B.Sc.(Med.) Hons.

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2 ABSTRACT

The effect of inducing agents of cytochrome P-450 on the binding and metabolism of three local anaesthetic agents: lidocaine, mepivacaine and bupivacaine has been investigated. All three local anaesthetic agents bound to the type I binding site of cytochrome P-450, which is characteristic of substrate binding to cytochrome P-450, and stimulated the CO-inhibitable oxidation of NADPH. Lidocaine is shown to be metabolized by cytochrome P-450 to the products MEGX and acetaldehyde. The forms of cytochrome P-450 elevated with phenobarbital and/or pregnenolone-16α-carbonitrile were shown to play an important role in the binding of lidocaine to cytochrome P-450. Cytochrome P-448 did not appear to be involved in the binding of lidocaine to cytochrome P-450. These findings are supported by the ability of the inhibitors of cytochrome P-450 viz. metyrapone, SKF 525-A and $CO:O_2$ to inhibit binding of lidocaine to cytochrome P-450.

No single form of cytochrome P-450 appears to preferentially metabolize lidocaine, but rather multiple forms of the enzyme appear to be involved in the metabolism of lidocaine.

The phenobarbital inducible form of cytochrome P-450 appears to play a major role in the binding of mepivacaine to cytochrome P-450. Cytochrome P-450 in microsomes from rats pretreated with β-naphthoflavone and pregnenolone-16α-carbonitrile does

not appear to have a significant role in the binding of mepivacaine to cytochrome P-450.

All forms of cytochrome P-450 are involved in the metabolism of mepivacaine to metabolic products as assessed by the oxidation of NADPH. However, the form of cytochrome P-450 induced by pretreatment of rats with phenobarbital may play a predominant role in the total metabolism of mepivacaine.

Multiple forms of cytochrome P-450 appear to be involved in the binding and total metabolism of bupivacaine. As in the case of mepivacaine, the total metabolism of bupivacaine, as assessed by the oxidation of NADPH, may be predominantly catalyzed by the form of cytochrome P-450 found in microsomes from rats pretreated with phenobarbital. Partially purified cytochrome P-450 was found to bind lidocaine in a type I manner and, in the presence of the artificial electron donor H_2O_2, produce MEGX. This further supports the role of cytochrome P-450 in the in vitro metabolism of lidocaine.

Hepatocytes were found to metabolize lidocaine to MEGX, indicating that lidocaine metabolism in vivo might well be mediated by cytochrome P-450

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7 ABBREVIATIONS

A	absorbance
cyt	cytochrome
GX	glycinexylidide
MEGX	monoethylglycinexylidide
MET	metyrapone-[2-methyl-1,2 bis (3-pyridyl)-1-propane]
mic	microsomal
PB	phenobarbital
PCN	pregnenolone-16α-carbonitrile
SKF 525-A	β-diethylaminoethyl-2,2-diphenyl-valerate
UDP	uridine 5'-diphosphate

I INTRODUCTION

In general, the metabolism of xenobiotics, which are compounds foreign to the body, occurs primarily in the liver. The enzymes involved in the metabolism of xenobiotics are known as drug metabolizing enzymes. They occur in other tissues in the body i.e. the brain, kidney, lung and skin, but these tissues usually contribute less than 5% to the total drug metabolizing activity of the body. It is thought that the drug metabolizing enzymes of extrahepatic tissues, particularly those such as the adrenal and testes, function primarily in the metabolism of endogenous substrates (see e.g. 1).

Drug metabolism is considered to occur in two distinct stages:

I) The oxidative conversion of the hydrophobic substrate into more hydrophilic product by dealkylation, deamination and hydroxylation reactions amongst others.

II) The conjugation of the oxidized intermediate or of the parent compound with a small organic molecule such as glucuronic acid, glutathione or sulfate.

The overall process of drug metabolism converts a hydrophic xenobiotic which would be stored in the fat depots of the body into a hydrophilic conjugate which is readily excreted into the urine, via the kidney. This overall process generally results in detoxification since it converts a hydrophobic compound, the storage of which in the body could be associated with toxic effects, into a conjugate which almost without

exception is devoid of physiological effects. There are exceptions to the above statement, viz. a growing number of compounds have been shown to be converted to reactive metabolites which are far more toxic than the parent compound and may, in fact, be ultimate carcinogens or toxins. This activation usually involves the enzymes of the first stage (phase I) of the pathway but can also involve enzymes of phase II. Examples of compounds which are activated include a) vinyl chloride which is converted by phase I enzymes to chloroethylene oxide and/or chloroacetaldehyde, one or both of which is/are proposed to mediate the carcinogenicity of the parent compound (2) and b) benzpyrene, which is not in itself carcinogenic, is metabolized by both phase I and phase II enzymes to the ultimate carcinogen benzpyrene-7,8-diol-9,10-epoxide (3, 4).

The first stage of drug metabolism is catalyzed predominantly by the cytochrome P-450 enzyme system of the hepatic endoplasmic reticulum. This enzyme system is frequently studied <u>in vitro</u> using the subcellular particles known as hepatic microsomes. Microsomes are vesicles which form spontaneously from segments of endoplasmic reticulum following the disruption of the endoplasmic reticulum by homogenization of the liver tissue. The advantage of microsomes for studies of drug metabolism, in comparison to the use of purified cytochrome P-450, is that NADPH-cytochrome P-450 reductase, the obligate electron transport enzyme for passing electrons from NADPH to cytochrome P-450 is present in its physiological relationship to cytochrome P-450. Microsomes are thus a useful tool to study phase I of

drug metabolism. Although many enzymes involved in phase II of drug metabolism, i.e. the glucuronyl transferases and epoxide hydrase, are also present in microsomes, microsomes are a poor model system for assessing the activation and detoxification of xenobiotics in vivo because they lack the soluble phase II enzymes, for example glutathione transferases and the sulfotransferases.

Cytochrome P-450 is a group of haem containing isoenzymes so named because of a striking spectral feature of the CO-ferrocytochrome P-450 complex i.e. its absorbance at 450 nm. This group of enzymes is of great importance in phase I of drug metabolism because of their extremely broad substrate specificity. For example, substrates for one or more forms of cytochrome P-450 include: aryl hydrocarbons, polycyclic hydrocarbons (benzpyrene, benzanthrene, naphthalene, 3-methyl-cholanthrene, phenanthrene); barbiturates (pento-, hepto-. phenobarbital); halogenated hydrocarbons (vinyl chloride, trichloroethylene, halothane, trichloroethane); amphetamines and polychlorinated biphenyls. In addition to the broad substrate specificity of cytochrome P-450, this group of enzymes also catalyzes a wide variety of oxidative reactions such as C-oxidation, O-dealkylation, N-dealkylation, de-amination and sulfoxidation[1].

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[1]Cytochrome P-450 has also been shown to catalyze reductive reactions recently, such as the reductive dehalogenation of halothane, which is thought to be associated with the production of halothane hepatitis (6).

The overall reaction catalyzed by the microsomal cytochrome P-450 enzyme system is accepted to be of the following form, even though in many cases, the final product may not be hydroxylated.

$$SH + NADPH + H^+ + O_2 \rightarrow SOH + NADP^+ + H_2O \qquad (1)$$

where SH represents the hydrophobic substrate and SOH its hydroxylated product.

As shown in equation (1), the rate of metabolism of a compound by cytochrome P-450 can be assessed by the measuring of the rate of oxidation of NADPH. According to this equation one mole of NADPH is oxidized per mole of substrate hydroxylated. Thus, monitoring the oxidation of NADPH provides an easy spectral assay for assessing the metabolism of compounds by cytochrome P-450. In practice, in experiments conducted with hepatic microsomes, the theoretical stoichiometry of 1:1 is not observed due to oxidation of NADPH by side reactions. Following inhibition of cytochrome P-450 mediated reactions by CO, corrections for background rates of NADPH oxidation in the presence of substrate and $CO:O_2$ (80:20; v/v) can be made, resulting in a closer approximation to the theoretical stoichiometry of one mole of NADPH oxidized per mole substrate hydroxylated (5).

At least six distinct forms of cytochrome P-450 have been identified in rat liver endoplasmic reticulum on the basis of electrophoretic and immunochemical evidence (7, 8). The different forms of cytochrome P-450 have different amino acid compositions, molecular weights, spectral characteristics and

sensitivities to inhibitors. In addition, the different forms of cytochrome P-450 differ in their substrate specificities (see below). For example, different forms of cytochrome P-450 may catalyze one type of reaction (e.g. biphenyl hydroxylation) at two or more different sites on one substrate (9) or they may catalyze different reactions (e.g. N-demethylation and C-hydroxylation) on one substrate (see below for details). However, there are several activities that are common to several of the multiple forms of cytochrome P-450 i.e. p-nitroanisole demethylase, benzpyrene hydroxylase and aniline hydroxylase. Assessing the role of different forms of cytochrome P-450 in the metabolism of a xenobiotic, using hepatic microsomes is complicated because a mixture of the different forms of cytochrome P-450 is present in hepatic microsomes regardless of the pretreatment of the animal from which the microsomes were isolated.

The levels of cytochrome P-450 can be increased by a wide variety of inducing agents (10). The inducing agents often selectively increase the levels of one particular form of cytochrome P-450 in the mixture of different forms of cytochrome P-450 present in the hepatic microsomes. The ability of an inducing agent to increase a particular form of cytochrome P-450 provides a very useful tool for the study of the role of a particular form of cytochrome P-450 in the metabolism and toxicity of xenobiotics.

The most widely studied inducing agent for cytochrome P-450 is phenobarbital. This agent causes an increase in one

specific form of cytochrome P-450 known as cytochrome P-450 LM_1 (rat liver) and having a molecular weight of 51 000 daltons (11, 12). This form of cytochrome P-450 catalyzes the biotransformation of a wide variety of compounds, including amongst others the halogenated hydrocarbons fluorexene and trichloroethylene. The form of cytochrome P-450 predominating in microsomes from rats pretreated with phenobarbital also displays activities such as benzamphetamine demethylase, aminopyrine demethylase and p-nitrobenzoate reductase (16). Phenobarbital drastically increases the ability of liver to metabolize certain xenobiotics because it causes proliferation of the smooth endoplasmic reticulum in the hepatocyte and in addition elevates the levels of NADPH cytochrome c reductase activity, cytochrome b_5 and cytochrome P-450 relative to the level of microsomal protein (13-15).

Pregnenolone-16α-carbonitrile, another inducing agent for cytochrome P-450, increases a specific form of cytochrome P-450 distinct from the form elevated by phenobarbital. The form of cytochrome P-450 induced by pregnenolone-16α-carbonitrile has a molecular weight of 54 000 daltons (11, 12) and is involved in the metabolism of hexobarbital, ethylmorphine, benzo(a)pyrene and benzphetamine (16). Pregnenolone-16α-carbonitrile induction results in an increase in the levels of both cytochrome P-450 and NADPH-cytochrome c reductase relative to the levels of microsomal protein, and in proliferation of the smooth-surface endoplasmic reticulum in hepatocytes. Pregnenolone-16α-carbonitrile does not elevate the levels of conjugating enzymes *in vivo* (14).

The polycyclic hydrocarbons, β-naphthoflavone and 3-methylcholanthrene are responsible for the elevation of yet another form of cytochrome P-450, which is distinct from the forms of cytochrome P-450 elevated by pretreatment of rats with phenobarbital or pregnenolone-16α-carbonitrile. The specific form of cytochrome P-450 induced by the polycylic hydrocarbons is termed cytochrome P-448 due to the absorbance maximum at 448 nm for the difference spectrum of CO-ferrocytochrome P-450 versus ferrocytochrome P-450. β-naphthoflavone and 3-methylcholanthrene induction causes an increase in cytochrome P-448. Cytochrome P-448 predominantly metabolizes polyaromatic aryl hydrocarbons such as: benzo(a)pyrene, benzanthrene, naphthalene and phenanthrene and is therefore also known as aryl hydrocarbon hydroxylase (15). The molecular weight of cytochrome P-448 is 55 000 daltons and cytochrome P-448 has been termed LM_2 (rat liver) due to its electrophoretic mobility (12). Polycyclic hydrocarbons do not alter the levels of the microsomal components cytochrome $\underline{b}_5$ and NADPH-cytochrome $\underline{c}$ reductase (phase I components) or cause proliferation of the smooth-surface endoplasmic reticulum. The polycyclic hydrocarbons do, however, increase the levels of some phase II enzymes, viz. epoxide hydrase and UDP-glucuronyl transferase.

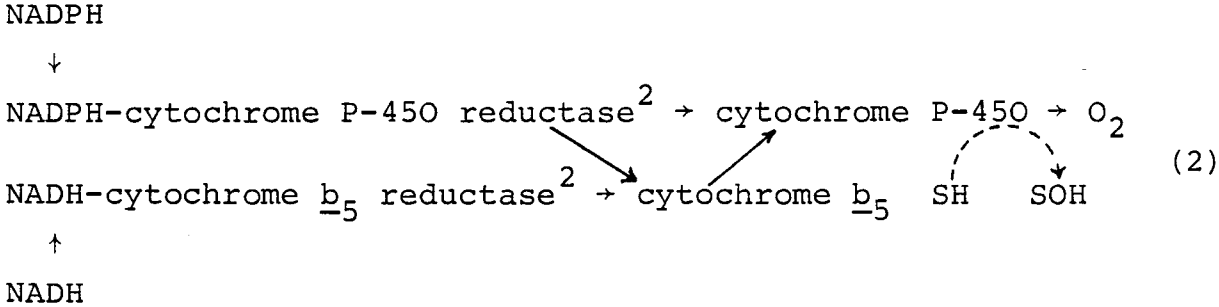
Other compounds are known to induce forms of cytochrome P-450 which are distinct from the forms of cytochrome P-450 described above. For example, ethanol induces a form of cytochrome P-450 which catalyzes the hydroxylation of benzpyrene and aniline. The form of cytochrome P-450 induced by ethanol is proposed to be involved in ethanol metabolism <u>in vivo</u> in the system is known

as MEOS (microsomal ethanol oxidizing system)(17).

The existence of other forms of cytochrome P-450 have been proposed based on the use of novel inducing agents (18) for example, iso-safrole, but these forms require further characterization before they can be unequivocally identified as novel forms of cytochrome P-450.

Cytochrome P-450 haemoproteins are not in themselves capable of metabolizing xenobiotics, but require one or more electron carriers to transport electrons one at a time from the preferred two electron donor NADPH or secondary, less effective donor NADH, to the cytochrome P-450-substrate complex.

The overall scheme for electron transport for cytochrome P-450 mediated reactions is as outlined below:



(2)

The flow of electrons is indicated by straight arrows.

For NADPH dependent drug metabolism NADPH-cytochrome P-450 reductase is an obligate electron carrier in the microsomal cytochrome P-450 enzyme system. Cytochrome $\underline{b}_5$ can also function as an intermediate electron carrier in the passage of electrons from NADH via its corresponding reductase to cytochrome P-450. Cytochrome $\underline{b}_5$ is not an

obligatory intermediate electron carrier for the NADPH dependent metabolism of most xenobiotics by cytochrome P-450, although it has recently been shown to be required for the NADH dependent metabolism by cytochrome P-450 of a very few xenobiotics such as hydroxyamine (19).

As mentioned above, NADPH is usually the most effective electron donor for cytochrome P-450 mediated reactions. Other compounds such as NADH and ascorbate may also act as electron donors, but are usually only about 10% as effective as NADPH in supporting cytochrome P-450 dependent reactions.

The accepted mechanism for the metabolism of compounds by cytochrome P-450 is depicted diagrammatically in Figure 1 and may be summarised as follows:

a) The substrate binds to active site of cytochrome P-450 resulting in the formation of a high spin ferricytochrome P-450-substrate complex.

b) The ferricytochrome P-450-substrate complex is reduced to the ferrous form by one electron from NADPH via NADPH-cytochrome P-450 reductase.

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[2] Both NADH-cytochrome $\underline{b}_5$ reductase and NADPH-cytochrome P-450 reductase are also known as cytochrome $\underline{c}$ reductases because they can transfer electrons to the non-physiological acceptor cytochrome $\underline{c}$ via cytochrome $\underline{b}_5$. The activity of these reductases is often measured as cytochrome $\underline{c}$ reductase activity because of ease of assay. This is thought to be a valid assay for reductase activity because electron transfer to cytochrome $\underline{c}$ is a fast step in the overall reaction (20).

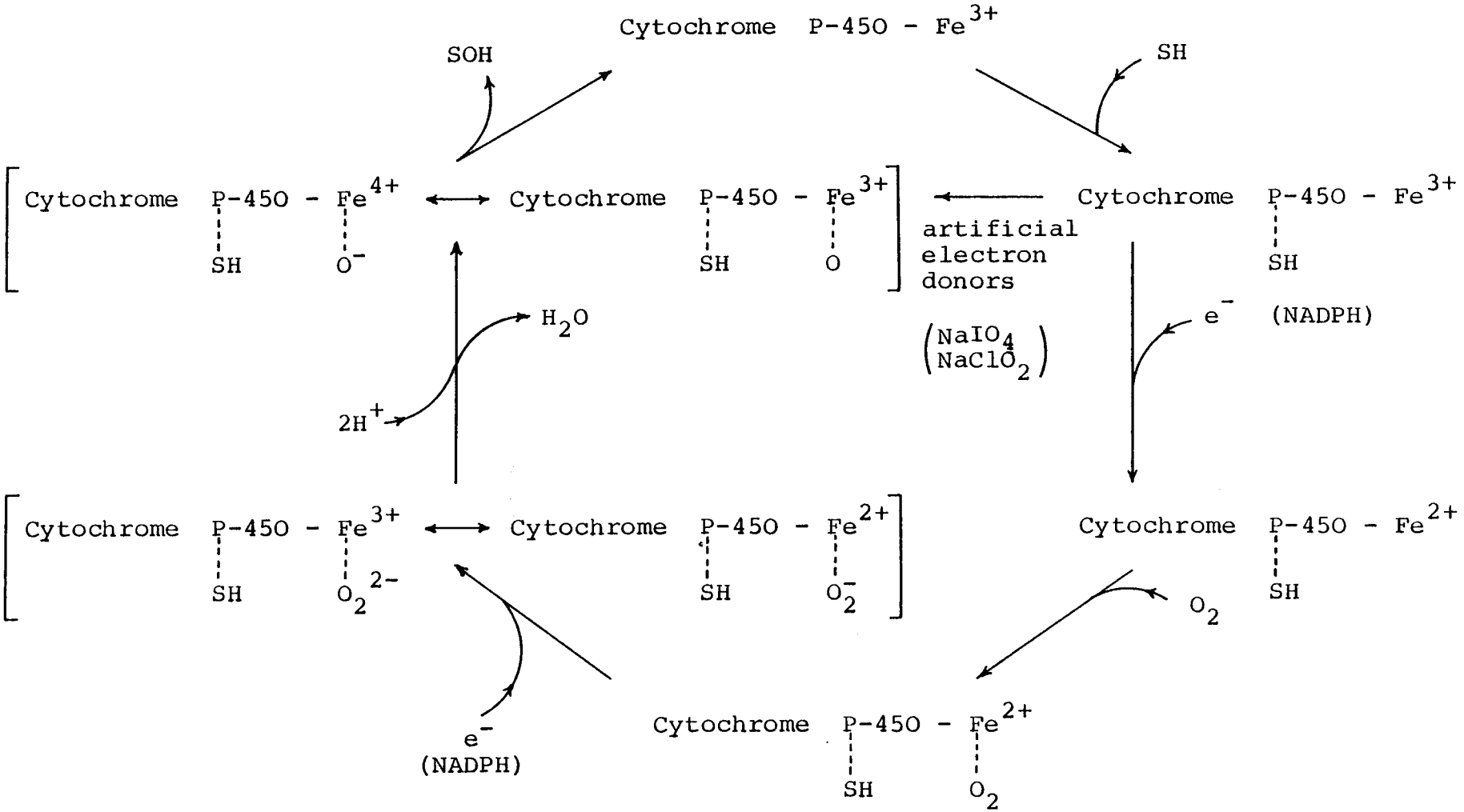

FIGURE 1. Mechanism of cytochrome P-450 dependent reactions

SH, substrate; SOH, hydroxylated product

c) Molecular oxygen binds to the ferrous ion of the reduced complex and this complex is reduced by a second electron from NADPH via the appropriate reductase. The superoxide ferrous enzyme-substrate intermediate which is formed is in resonance with the hydroperoxo ferric enzyme-substrate complex (21, 22).

d) The O-O bond of the hydroperoxo ferric enzyme-substrate complex is heterolytically cleaved to yield water and a ferric enzyme-mono-oxygen species which is in resonance with the ferryl ion-oxene complex.

e) The hydroxylated product is released from cytochrome P-450, thereby regenerating the cytochrome P-450 in the ferric state.

Artificial electron donors such as $NaIO_4$, $NaClO_2$, H_2O_2 and organic hydroperoxides can support the hydroxylation of a variety of substrates by purified cytochrome P-450 in the absence of NADPH, NADPH-cytochrome P-450 reductase and molecular oxygen (23-25). These artificial electron donors are thought to function by directly providing cytochrome P-450 with the active oxygen species necessary for hydroxylation. In addition, various model systems have been shown to catalyze reactions typical of cytochrome P-450. These systems usually comprise haem, a ligand or not, and an electron donor such as a thiol or NADPH, and have been shown to exhibit aniline hydroxylase and other activities typical of cytochrome P-450 (see e.g. 23, 24).

As shown in Figure 1, the first step in the metabolism of a compound by cytochrome P-450 is the binding of the substrate

to ferricytochrome P-450. Cytochrome P-450 perhaps is unique in that one can easily ascertain whether or not a compound is a substrate by the type of visible spectrum that it produces with cytochrome P-450, and one can readily obtain spectral binding constants in order to characterize substrate binding. This favourable situation occurs because the spin state and coordination chemistry of the ferric ion of the haem group of cytochrome P-450 are extremely sensitive to ligands and to conformational changes in the enzyme caused by binding to the active site or to other sites on the enzyme. The binding of compounds to at least three sites on cytochrome P-450 is distinguishable spectrally in the visible region of the spectrum (Figure 2). The three spectrally detectable ways in which compounds have been shown to bind to ferricytochrome P-450 in hepatic microsomes, are known as the type I, IR and II difference spectra.

Type I binding is typical of the binding of a substrate to the active site of ferricytochrome P-450. Type IR and II, which will be considered below, reflect binding to sites on the cytochrome P-450 molecule which do not allow metabolism of the compound to occur.

The type I difference spectrum is characterized by a peak at ca. 385 nm and a trough at ca. 420 nm and arises from a change in spin state of the haem iron ion of ferricytochrome P-450 from a low spin state to a high spin state. This alteration in spin state of ferricytochrome P-450 results from alterations in the conformation of the enzyme, and <u>not</u> from the direct

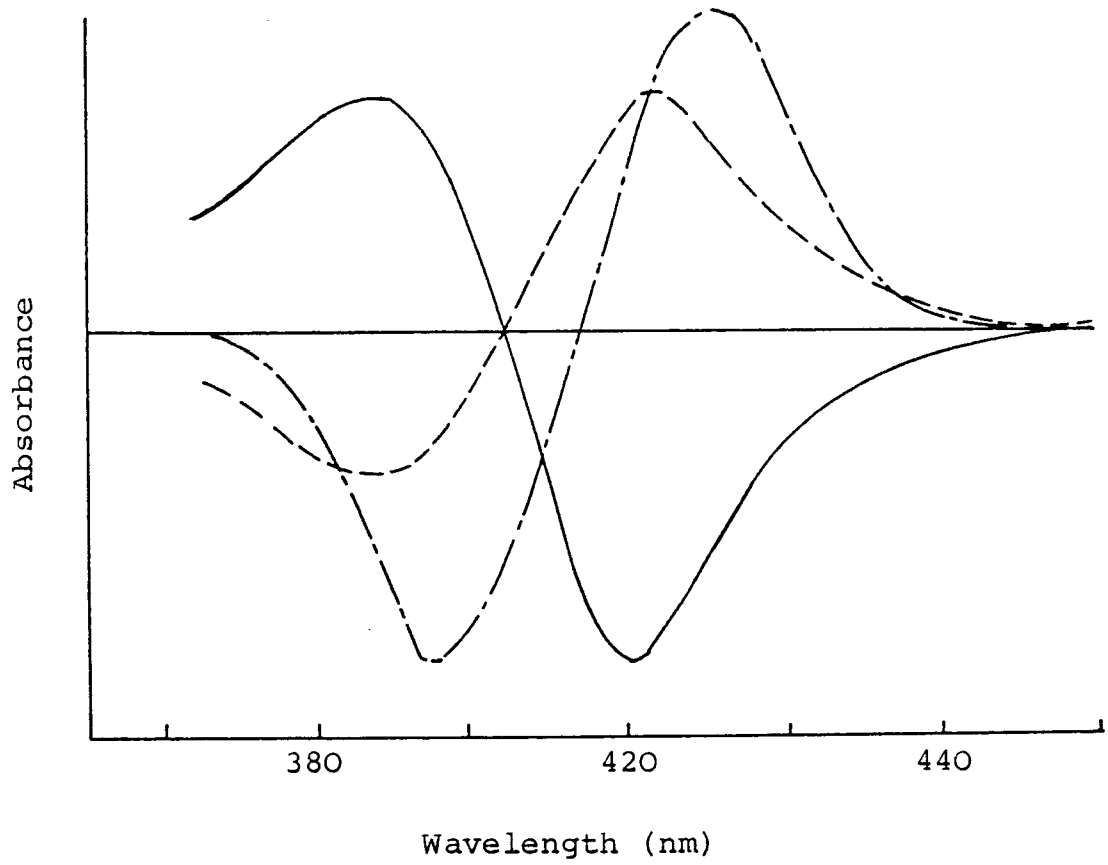

FIGURE 2. Difference spectral changes in hepatic microsomes. Sample cuvette contains microsomes and compound, and reference cuvette microsomes alone. Spectral changes represented:
type I (———); type II (-----); and type IR (-·-·-)

interaction of the substrate with the haem iron (26-28). It must be borne in mind that in hepatic microsomes there is a mixture of forms of cytochrome P-450 and, consequently, the spin state changes reflected by the observed difference spectra may represent alterations in one or more of the multiple forms of cytochrome P-450 present in the hepatic microsomes. As mentioned above, most compounds producing type I difference spectra, for example, hexobarbital, phenacetin, vinyl chloride and trichloroethylene - are metabolized by cytochrome P-450 (26). A notable exception to this statement is n-perfluorohexane, which binds to hepatic microsomal cytochrome P-450 with a type I difference spectrum and stimulates CO-inhibitable NADPH oxidation, and is yet not metabolized by cytochrome P-450 (30). n-Perfluorohexane is thus termed an uncoupler of cytochrome P-450 because it stimulates the flow of electrons from NADPH to oxygen by binding to cytochrome P-450 and stimulating its reduction, without in itself being metabolized.

The type IR difference spectrum reflects the opposite change in spin state to that seen with type I compounds, viz. the type IR difference spectrum arises from the conversion of the haem ion of ferricytochrome P-450 from a high spin to a low spin state (28). This change results in a difference spectrum which is the exact reverse of the type I difference spectrum, and is characterized by a trough at ca. 385 nm and a peak at ca. 420 nm (26, 31). Compounds displaying type IR difference spectra are not bound in the region of the haem moiety and consequently are not metabolized by cytochrome P-450.

Alcohols and carboxylic acids such as ethanol, phenol, tryptophan and agroclavine give type IR difference spectra.

The remaining type of spectral change, the type II, is quite different to the preceding two types. This spectral change results from the binding of a strong field ligand to the 6th coordination position of the haem iron of ferricytochrome P-450 (32). The type II difference spectrum is characterized by a trough at ca. 390 nm and a peak at ca. 430 nm (26-28) (Figure 2). All compounds which exhibit type II difference spectra in the presence of microsomes are, without exception, strong field ligands which are able to donate an electron pair to the haem iron of ferricytochrome P-450. Typical ligands include amines, thiols and carbenes, and the binding of these ligands to ferricytochrome P-450 results in an increase in the amount of ferricytochrome P-450 in the low spin state. Type II compounds compete with oxygen as a ligand for the haem iron ion of ferrocytochrome P-450, and thus inhibit cytochrome P-450 non-competitively as seen for CO and metyrapone (33).

Certain compounds are known to inhibit the metabolism of xenobiotics by cytochrome P-450 (9). Included here are compounds such as metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propane], SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate), SKF 8742-A(2-ethylaminoethyl-2,2-diphenylvalerate), Lilly 18947 (2,4-dichloro-6-phenylphenoxyethyl-diethylamine), and CO. Most of these compounds specifically inhibit cytochrome P-450 without affecting the activities of other drug metabolizing enzymes. SKF 525-A, however, has been shown to inhibit the activity of

the microsomal UDP-glucuronyl transferases (34).

The inhibitors of cytochrome P-450 fall into two categories:

(a) those that bind to the substrate binding site of cytochrome P-450 giving rise to competitive inhibition of cytochrome P-450 mediated reactions, as exemplified by most substrate for cytochrome P-450, such as hexobarbital.

(b) those that bind to the central iron atom of the haem of ferrocytochrome P-450 and thus interfere with the binding of oxygen to this enzyme, giving rise to non-competitive inhibition of cytochrome P-450 mediated reactions. An example of a non-competitive inhibitor is CO.

Some inhibitors viz. those which can bind to both substrate and oxygen binding sites can exhibit competitive, non-competitive or mixed inhibition kinetics, depending on the reaction investigated. Examples of inhibitors in this class are metyrapone and SKF525-A (35-37).

Some of the above compounds selectively inhibit one or more of the multiple forms of cytochrome P-450 without affecting the activity of the remainder of forms. Metyrapone is thought to inhibit the form of cytochrome P-450 induced by phenobarbital but not affect that form induced by 3-methylcholanthrene (38). The effect of metyrapone on the remaining forms of cytochrome P-450 is not known. In contrast, SKF 525-A has been proposed to inhibit reactions catalyzed by cytochrome P-448 and to affect reactions catalyzed by other forms of cytochrome P-450 to lesser extents (37). CO is generally

accepted to inhibit multiple forms of cytochrome P-450, but not all activities of the multiple forms of cytochrome P-450 are equally inhibited (37, 39), which somewhat clouds its use as a non-specific inhibitor of cytochrome P-450 dependent reactions.

Recently, a large number of studies of drug metabolism have utilized isolated liver cells, known as hepatocytes (40, 41). Hepatocytes have been proposed to provide a better model system for drug metabolism *in vivo* than do microsomes, because the intact cells contain in addition to the drug metabolizing enzymes in the endoplasmic reticulum, the co-factors and enzymes of the cytosol which are required for phase II of drug metabolism. Hepatocytes thus contain all of the hepatic detoxifying systems and toxifying systems found *in vivo* while the microsomes contain the primary drug metabolizing enzymes of phase I, viz. the cytochrome P-450 enzyme system, as well as the glucuronyl transferases and epoxide hydrase of phase II, but lack the soluble enzymes catalyzing phase II reactions. Thus hepatocytes are thought to give closer approximation to the intricacies of drug metabolism *in vivo* than do studies of drug metabolism using isolated microsomes. I have therefore performed preliminary investigations with hepatocytes to compare results to those obtained with microsomal studies.

Lidocaine, mepivacaine and bupivacaine (structures shown in Figures 3-5) are local anaesthetics of the amide linked class. Lidocaine which was synthesized by Löfgren in 1943 (42), represented a major breakthrough in the chemistry of the local anaesthetics, in that it did not produce allergic or

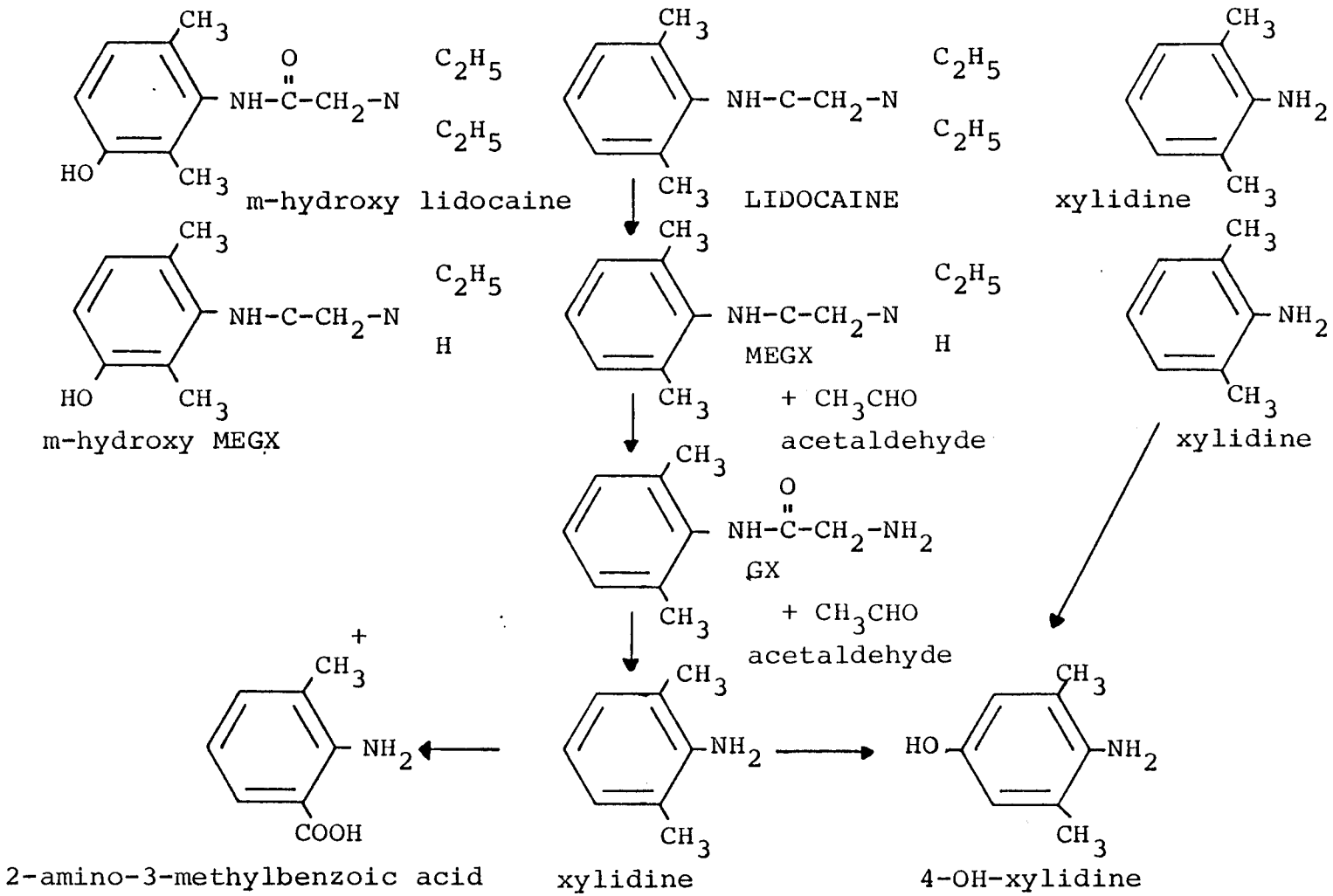

Figure 3. Summary of the proposed pathways for the biotransformation of lidocaine in humans and rats in vivo (57, 59). (+ postulated)

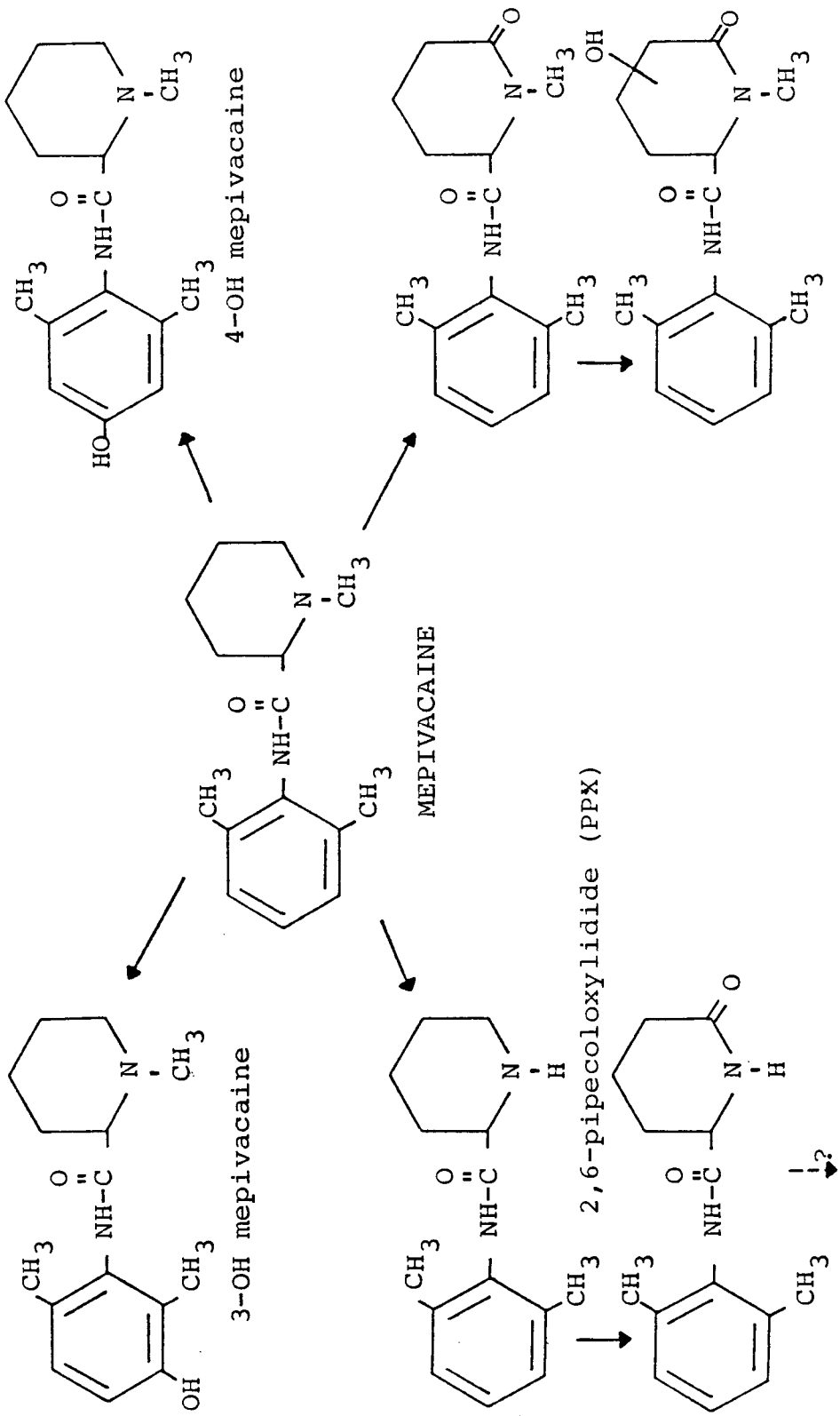

Figure 4. Summary of the proposed pathway for the biotransformation of mepivacaine in humans in vivo (59).

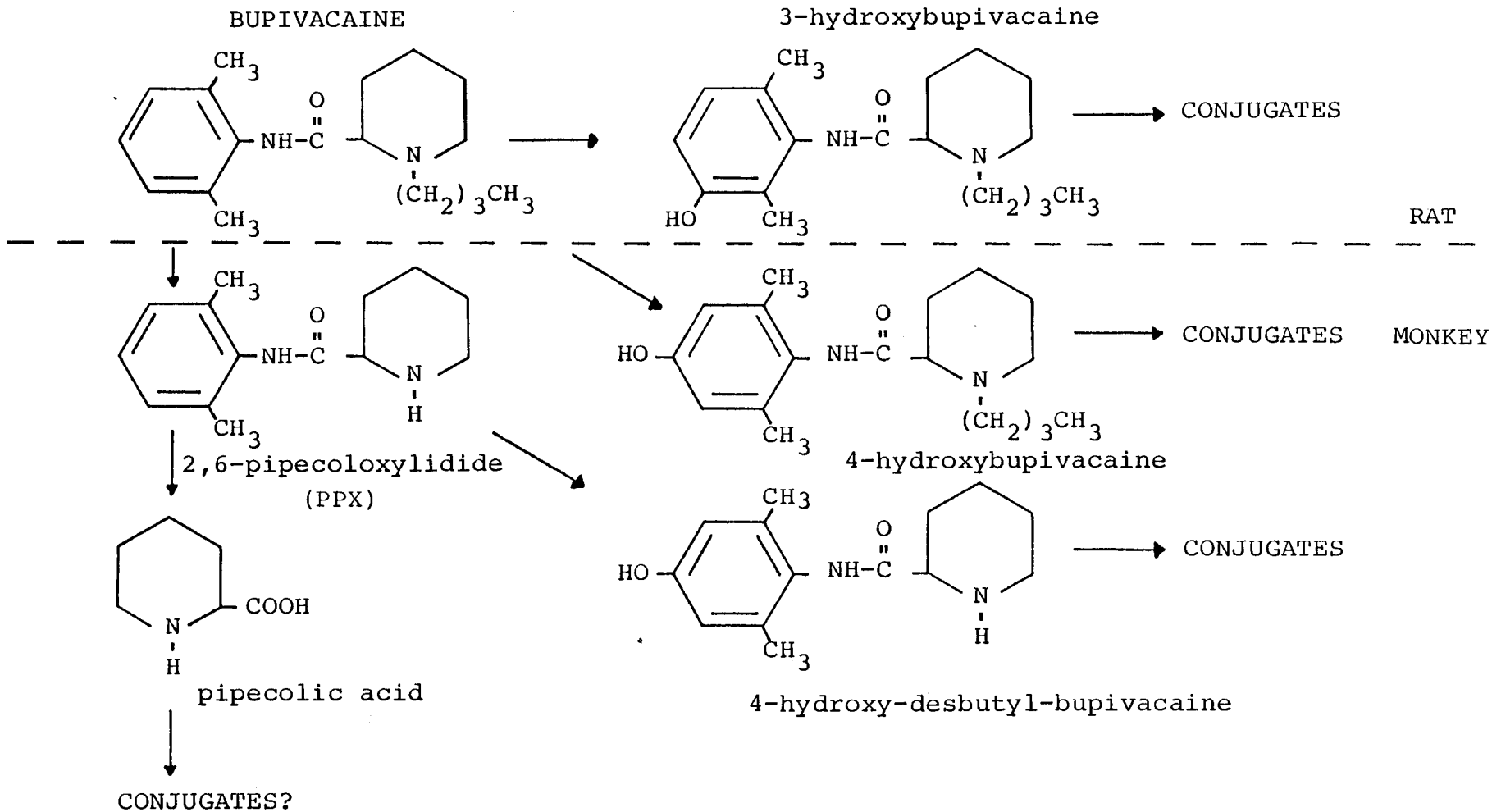

Figure 5. Summary of the proposed pathway for the biotransformation of bupivacaine in the rat and monkey *in vivo* (52).

sensitizing-type reactions. Mepivacaine and bupivacaine which were synthesized in 1957 and 1963, respectively, shared the advantages of lidocaine, but were of greater anaesthetic potency (43, 44).

Lidocaine is recognized as a local anaesthetic displaying low toxicity (45). The LD_{50} for lidocaine (88 mg/kg) is far higher than the dose required for maximum anaesthetic potency (1.5 - 15 mg/kg). At concentrations below the LD_{50}, the only toxic effects of lidocaine reported include CNS depression and occasionally convulsions (44).

Interestingly, lidocaine has the potential to inhibit the replication of tumour cells much more effectively than of normal cells; 1.2 mM lidocaine reduçes the growth of rat hepatoma cells by 50% whereas the replication of non-malignant cells is virtually unaffected by this concentration of lidocaine (46).

Lidocaine has been shown neither to be mutagenic in cultures of Salmonella strain TA-1538 bacteria nor carcinogenic in laboratory animals (mice) (45). The lack of carcinogenicity of lidocaine is somewhat surprising in as much as N-hydroxy metabolites of lidocaine and of its major metabolite monoethyl glycinexylidide (MEGX) have been reported, and have been proposed to be carcinogenic (47). In addition, aromatic ring hydroxylation which is known to occur during the metabolism of lidocaine, has been proposed to occur via the formation of labile arene oxide intermediates which are capable of reacting

with cellular macromolecules such as RNA, DNA and protein and are proposed to mediate the carcinogenicity of many non-reactive parent compounds (48).

Lidocaine, mepivacaine and bupivacaine have been shown to undergo metabolism in animals and man *in vivo* (49-58) (Figures 3-5). The proposed metabolic pathways for lidocaine *in vivo* are shown in Figure 3 (52, 57-59). Lidocaine is metabolized *in vivo* to MEGX, 3-hydroxylidocaine, 3-hydroxy MEGX, glycine xylidide (GX), xylidine and 4-hydroxyxylidine (60-62). In the urine of humans and dogs, the major metabolite of lidocaine was 4-hydroxyxylidine, whereas in the urine of rats, the major metabolites were 3-hydroxylidocaine and 3-hydroxy MEGX. In the urine of guinea pigs MEGX was the major metabolite of lidocaine (60).

In mammals, the metabolism of lidocaine appears to occur primarily in the liver: viz. liver tissue was able to appreciably metabolize lidocaine (63, 64, 65) while other tissues i.e. the kidney, brain and lung displayed a far lower capacity for this process. The metabolism of lidocaine by liver slices *in vitro* is dependent on the presence of oxygen and NADPH, both of which are required for the hepatic drug metabolizing enzymes situated in the endoplasmic reticulum.

The proposed metabolic pathways for the metabolism of mepivacaine and bupivacaine are shown in Figure 4 and 5. Both mepivacaine and bupivacaine are metabolized *in vivo* to 2,6-pipecoloxylidide (PPX) by N-dealkylation of the parent

compounds (49, 56, 59). The 3-hydroxy and 4-hydroxy derivatives of mepivacaine and bupivacaine have also been identified as urinary metabolites in humans, rats and monkeys (50, 52).

At least in the case of mepivacaine, its metabolism appears to occur in the liver. Mepivacaine has been shown to be converted to PPX in liver slices (66). In the case of bupivacaine there are no reports concerning its site of metabolism in vivo, but it may be anticipated by analogy to lidocaine and mepivacaine, that this compound would also be metabolized by the liver.

Hepatic cytochrome P-450 appears to catalyze the first step or steps in the metabolism of lidocaine. This proposal is supported by the observations that the metabolism of lidocaine occurred predominantly in the endoplasmic reticulum of the liver and exhibited an absolute requirement for NADPH and oxygen (64). The inhibitor of microsomal cytochrome P-450, SKF 525-A was shown to inhibit the metabolism of lidocaine in rat liver homogenates (66), and phenobarbital pretreatment, which increases cytochrome P-450 amongst other effects, resulted in an increased rate of metabolism of lidocaine by rat liver homogenates (67).

Lidocaine has been shown to bind to, and to be metabolized by cytochrome P-450 in hepatic microsomes. Lidocaine binds to the type I binding site of cytochrome P-450 in vitro which is typical of substrates for cytochrome P-450 (66, 68, 69, 70). The binding of lidocaine to cytochrome P-450 was characterized by two values of K_s of ca. 2 μM and ca. 160 μM (69). The high

affinity binding site for lidocaine was proposed to catalyze the aromatic hydroxylation of lidocaine to 3-hydroxylidocaine, while the lower affinity binding site was proposed to catalyze the deethylation of lidocaine to MEGX (69).

The production of MEGX from lidocaine by hepatic microsomal cytochrome P-450 according to Nyberg et al. was characterized by a K_m of 250 μM and a V_{max} of 15 nmol/mg protein × min (70). In contrast, von Bahr et al. found that the metabolism of lidocaine to MEGX by hepatic microsomal cytochrome P-450 was catalyzed at two sites on cytochrome P-450. He found values of K_m of 0.04 μM (high affinity) and 40 μM (low affinity) and values of V_{max} of 0.05 nmol/mg protein × min (high affinity) and 3.64 nmol/mg protein × min (low affinity) (69). The lack of agreement of the results of Nyberg et al. and von Bahr et al. is surprising since they both work in the same laboratory on the same strain of rats.

It appears that cytochrome P-450 plays a role in the metabolism of lidocaine in humans. The metabolism of lidocaine by human liver microsomes in vitro has been shown to require NADPH generating system and to produce MEGX and 3-hydroxylidocaine (68). These results are consistent with known metabolites of lidocaine in humans in vivo (57-60).

A role for cytochrome P-450 in the metabolism of mepivacaine and bupivacaine has not been established. However, the ability of an inhibitor of cytochrome P-450, SKF 525-A, to inhibit the metabolism of mepivacaine in rat liver homogenates, and of

pretreatment with the inducing agent phenobarbital, to increase mepivacaine metabolism in rat liver homogenates (67) are consistent with a role for cytochrome P-450. Investigation of the binding or metabolism of mepivacaine and bupivacaine by cytochrome P-450 in isolated hepatic microsomes has not been reported.

The interaction of the three related local anaesthetic agents - lidocaine, mepivacaine and bupivacaine - with hepatic microsomal cytochrome P-450, especially with respect to the following points, is reported in this thesis:

a) To identify the form(s) of cytochrome P-450 involved in the binding of lidocaine, mepivacaine and bupivacaine.
b) To identify the form(s) of cytochrome P-450 involved in the metabolism of the three local anaesthetics by cytochrome P-450 as assessed by the stimulation of CO-inhibitable oxidation of NADPH.
c) To identify the form(s) of cytochrome P-450 involved in the metabolism of lidocaine by assessing its conversion to acetaldehyde, MEGX and other metabolites.
d) To investigate the effect of artificial electron donors on the conversion by partially purified cytochrome P-450 of lidocaine to acetaldehyde and MEGX.
e) To investigate the effect of lidocaine on the levels and activities of hepatic microsomal components <u>in vitro</u>.

II EXPERIMENTAL

1. Materials

Sodium phenobarbital and β-naphthoflavone were obtained from Maybaker, R.S.A. and Aldrich Chemical Company, Milwaukee, Wisconsin, USA, respectively. Pregnenolone-16α-carbonitrile was a gift from G.D. Searle and Co., Chicago, Illinois, USA. Lidocaine was supplied by Petersons Ltd., Cape Town, R.S.A. The metabolites of lidocaine, viz. 3-hydrolidocaine, 3-hydroxyMEGX, GX and 4-hydroxyxylidine were obtained from Astra A.B., Södertälje, Sweden. Bupivacaine (marcaine) and mepivacaine (carbocaine) were supplied by Winthrop, Claremont, C.P., R.S.A. NADP, NADH, NADPH, glucose-6-phosphate disodium salt, hyaluronidase and cytochrome c were obtained from Miles Laboratories, Cape Town, R.S.A. Glucose-6-phosphate dehydrogenase (type VII) and collagenase (type IV) were supplied by Sigma Chemicals, St Louis, Missouri, USA. Cylinders of pure gases were obtained from Afrox Ltd. and Air Products S.A. (Pty) Ltd., Cape Town, R.S.A. Metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propane] and SKF525-A (β-diethylaminoethyl-2,2-diphenyl valerate) were gifts from Ciba-Geigy Limited, Basle, Switzerland and Smith, Kline and French Ltd., Isando, Transvaal, R.S.A., respectively. Stationary phases and packing for gas liquid were supplied by Applied Science Laboratories Inc., State College, Penn., USA. Chemicals for the isolation of hepatocytes were primarily obtained from Merck Chemicals, Darmstadt, Germany. p-Phenyl-

phenol was supplied by Eastman Kodak Company, Rochester, N.Y., USA. Sodium dithionite was obtained from Merck Chemicals, Darmstadt, Germany. All other reagents were the best grade available. Water was glass distilled and deionized.

2. Treatment of animals

Male Long-Evans rats weighing between 180 g and 220 g were used for all experiments. The animals were allowed free access to Epol Laboratory Chow (protein min. 20%, fat 2.5%, fibre max. 6%, calcium 1.4%, phosphorous 0.7%) and water unless otherwise indicated. Different forms of cytochrome P-450 were induced by interperitoneal injection of sodium phenobarbital (80 mg/kg/day) or pregnenolone-16α-carbonitrile (50 mg/kg/day) each for three consecutive days, the last injection 16 hrs prior to sacrifice or by one injection of β-naphthoflavone (80 mg/kg/day) 40 hrs prior to sacrifice. All animals were starved overnight following the last injection or at a comparable time for untreated rats, and were sacrificed by cervical fracture the following morning.

3. Preparation of hepatic microsomes

Microsomal suspensions were prepared at 4° from fresh rat liver homogenates by differential ultracentrifugation by a modification of the method of Holtzman and Carr (71): The livers from three rats were homogenized in 0.15 M KCl - 0.02 M Tris-HCl, pH 7.4 (3 ml per gram wet liver). The cell debris,

nuclei and mitochondria were removed by centrifugation at 10 000 g for 25 min in a Beckman J-21B centrifuge. The microsomes were sedimented from the supernatant by centrifugation at 105 000 g for 55 min in a Beckman model L ultracentrifuge. The microsomal pellet was resuspended in 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 at a concentration of 2 mg microsomal protein/ml unless otherwise stated. Hepatic microsomes were used within 4 hrs of preparation. The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (72) as modified by Chaykin (73) using bovine serum albumin as a standard.

4. Preparation of Standard Solutions

4.1 Ethanolic solutions of lidocaine, mepivacaine and bupivacaine

Lidocaine - 305 mg lidocaine hydrochloride was dissolved in 0.5 ml distilled water. The pH was adjusted to 7.3 - 7.5 with ca.0.08 ml 4 M NaOH, and 0.2 ml 96% ethanol was added to the lidocaine solution. The final concentration of lidocaine in this solution was 1.4 M. A 1:10 (v/v) dilution of the stock lidocaine solution with distilled water was used in addition to the concentrated stock solution for studies of the binding and metabolism of lidocaine. The dilute lidocaine solution had a concentration of 0.15 M.

Mepivacaine - 302 mg mepivacaine hydrochloride was dissolved in 0.7 ml distilled water. The pH was adjusted to ca pH 7.2 - 7.4 using ca 0.1 ml 4 M NaOH and 0.2 ml 96% ethanol was added to the mepivacaine solution. The final concentration of mepivacaine in this solution was 1.07 M.

Bupivacaine - 309 mg bupivacaine hydrochloride was added to 2.0 ml distilled water. 0.8 ml ethanol was added to the solution of bupivacaine. The final concentration of bupivacaine in this solution was 0.34 M.

Routinely, 1-20 µl of ethanolic solution of anaesthetic agent was added per 3 ml of microsomal suspension. No correction was made for the dilution of microsomal suspension. Ethanol (1-20 µl) was without effect in all experiments.

4.2 Solutions of metabolites of lidocaine

Dilute solutions of MEGX, GX, 4-hydroxyxylidine, 3-hydroxy MEGX and 3-hydroxylidocaine were prepared in water for standards for gas liquid chromatography.

a) MEGX: A stock solution of 2 mg/ml was prepared using distilled water. This stock solution was further diluted 1:50 (v/v) and 1:100 (v/v) with distilled water and routinely 1 µl of the diluted stocks were injected onto the GLC to construct a standard curve for quantification of MEGX produced in incubation mixtures.

b) GX: A stock solution of 2 mg/ml was prepared using distilled water. Routinely 1 µl of solution of GX was

injected onto the GLC to identify possible peaks from incubation mixtures.

c) 4-hydroxyxylidine: A stock solution of 3.2 mg/ml was prepared using distilled water. This stock solution was diluted 1:10 (v/v) with distilled water and routinely 1 µl was injected onto the GLC.

d) 3-hydroxy MEGX: A stock solution of 4 mg/ml was prepared using distilled water. This stock solution was diluted 1:10 (v/v) with distilled water and routinely 2 µl was injected onto the GLC.

e) 3-hydroxylidocaine: A stock solution of 2 mg/ml was prepared using distilled water. 2 µl of this stock solution was injected onto the GLC.

5. Spectral assays on microsomes

5.1 Difference spectra

Two teflon stoppered 4 ml cuvettes each containing 3.0 ml of microsomal suspension were equilibrated to 25°. A small volume (0.5 - 20 µl) of a solution of the compound under investigation was added to the sample cuvette below the surface of the microsomal suspension using a Hamilton µl syringe. The cuvette was then stoppered and the suspension shaken by hand. The magnitude of the difference spectrum was measured as the difference in absorbance between the peak at ca. 385 nm and the trough at ca. 420 nm. Correction was made for the intrinsic differences in absorbance at these wavelengths for

cuvettes containing microsomal suspension only.

5.2 NADPH oxidation

The metabolism of lidocaine, mepivacaine and bupivacaine was measured by monitoring the rate of hepatic microsomal NADPH consumption in the presence of each of the anaesthetic agents as follows: Two teflon stoppered 4 ml cuvettes each containing 2.5 ml of microsomal suspension were equilibrated to 25°. Varying amounts of a solution of the anaesthetic agent under investigation were added to the sample cuvette as described for the difference spectra (see Section 5.1). The reaction was initiated with the addition of 50 µl of NADPH (0.12 mM, final concentration) to the sample cuvette. The decrease in absorbance at 340 nm due to the oxidation of NADPH was recorded as a function of time. In all cases, reported rates of the oxidation of NADPH were corrected for background rates of hepatic microsomal NADPH oxidation in the presence of anaesthetic agent under an atomsphere of $CO:O_2$ (80:20; v/v) (5). The $CO:O_2$ was introduced as described in section 7.

5.3 Cytochrome P-450 assay

The concentration of cytochrome P-450 was determined from measurements of the difference spectrum of CO-ferrocytochrome P-450 versus ferrocytochrome P-450, according to the method of Omura and Sato (74). Two teflon stoppered 4 ml cuvettes each containing 3 ml of microsomal suspension were equilibrated to 25°. The sample cuvette was bubbled with CO at 30 ml/min

for 30 sec. A few grains of sodium dithionite were added to each cuvette, and the absorbance difference spectrum was measured from 410 nm to 500 nm. The extinction coefficient used for the difference between the absorbance at 450 nm and 490 nm was 91 $mM^{-1}cm^{-1}$ (74).

5.4 Cytochrome b_5 assay

The concentration of cytochrome b_5 was determined from the difference spectrum of ferrocytochrome b_5 versus ferricytochrome b_5 as described by Omura and Sato (74). Two teflon stoppered 4 ml cuvettes each containing 3 ml of microsomal suspension were equilibrated to 25°. 100 µl NADH (0.2 mM, final concentration) was added to the sample cuvette and the absorbance difference spectrum was measured over the range of 390 nm to 430 nm. An extinction coefficient of 185 $mM^{-1}cm^{-1}$ was used for the difference in absorbance between 424 nm and 409 nm (74).

5.5 NADPH-cytochrome c reductase assay

The activity of the NADPH-cytochrome c reductase was determined from the rate of reduction of cytochrome c at 550 nm as described by Omura and Takesue (75). Two one ml cuvettes each containing 1.05 ml 0.02 M Tris-HCl, pH 7.4 buffer, 100 µl cytochrome c (35 µM, final concentration) and 50 µl NADPH-CN (0.11 mM, and 10 mM final concentration, respectively) were equilibrated to 25°. The reaction was initiated by adding 50 µl microsomal suspension (0.08 mg microsomal protein/ml,

final concentration) to the sample cuvette. An extinction coefficient of 21.1 $mM^{-1}cm^{-1}$ was used for the difference in absorbance of reduced minus oxidized cytochrome c at 550 nm (75).

5.6 Microsomal haem assay

The concentration of microsomal haem was determined as the reduced pyridine haemochromogen as described by Omura and Sato (74). The sample cuvette contained one ml microsomal suspension (0.8 mg microsomal protein/ml, final concentration), 0.5 ml pyridine, one ml NaOH (0.1 M, final concentration) and a few grains of sodium dithionite. No reference was utilized. The sample was scanned from 540 nm to 580 nm. An extinction coefficient of 32.4 $mM^{-1}cm^{-1}$ was used for the difference between the absorbance between 557 nm and 575 nm (74).

5.7 Spectrophotometry

Unless otherwise stated, all spectral measurements were performed using a Beckman 5230 recording spectrophotometer. The thermostatted cell position adjacent to the photomultiplier was used for turbid samples. In all cases cuvettes with a pathlength of 1 cm were used.

6. Metabolism of lidocaine

6.1 Incubation mixtures

Possible metabolites of lidocaine were assayed in incubation mixtures containing lidocaine (1 mM), NADPH generating system [NADP (0.4 mM, final concentration), glucose-6-phosphate (7.5 mM, final concentration), glucose-6-phosphate dehydrogenase (0.5 units per ml), $MgCl_2$ (5 mM, final concentration), nicotinamide (1 mM, final concentration), EDTA (0.2 mM, final concentration)] and hepatic microsomes (2 mg microsomal protein/ml) in 0.02 M Tris-HCl, pH 7.4, unless otherwise indicated. Where inhibitors of cytochrome P-450 were present, they were added to the microsomal suspension prior to the introduction of the lidocaine. Incubations were carried out at 37° in a Gallenkamp shaking water bath with shaking at 100 oscillations per min.

6.2 Acetaldehyde production

Acetaldehyde formation was measured spectrally at 560 nm according to the method of Stotz (76) in incubation mixtures containing lidocaine, NADPH generating system and microsomal suspension (preparation - incubation of reaction mixtures as described above, see section 6.1). At the end of the incubation period, microsomal protein was precipitated using H_2SO_4 (0.02 M, final concentration) plus $NaWO_4$ (5 mM, final concentration) and the precipitate was removed by centrifugation at 1 000 g for 10 min. To one ml of the supernatant, 50 µl of 5% aqueous $CuSO_4$, 8 ml of concentrated H_2SO_4 and 200 µl p-hydroxybiphenyl reagent [p-phenylphenol (0.06 M, final concentration) NaOH (0.5 M, final concentration)] were added. The above sample was vortex mixed, incubated for

30 mins at 30° without shaking and the absorbance was read at 560 nm on a Gilford single beam spectrophotometer.

Incubation mixtures containing hepatic microsomal suspension and NADPH generating system or hepatic microsome and lidocaine, treated exactly as described above, were utilized as reaction blanks.

6.3 Assay of volatile metabolites of lidocaine by gas-liquid chromatography

MEGX and GX were identified in incubation mixtures (see section 6.1) containing hepatic microsomes, NADPH generating system and lidocaine by gas-liquid chromatography according to DiFazio and Brown (77), viz. using a 2 ft × ¼ inch glass column packed with Chromosorb W (AW) coated with 2% Carbowax 20 M + 3% KOH. Operating conditions were: injection port temperature, 220°; column temperature, 180°; detector temperature, 250°; carrier gas (nitrogen) 35 ml/min. Retention times were as follows: Lidocaine, 240 sec; MEGX, 387 sec; GX, 940 sec. Identification of 3-hydroxy MEGX and 4-hydroxy-2,6-xylidine was with a 6 ft ×¼ inch glass column packed with Chromasorb W (AW) 80/100 mesh with 3% GEXE 60[1]. Operating conditions were: injection port and detector temperature, 280°; column temperature (temperature programmed), 170°-210°; initial time 13 min at 170°, rise time 10°/min and final time 14 min at 210°; carrier gas (nitrogen), 35 ml/min. Retention times were: 3-hydroxy MEGX, 1170 sec;

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[1] Keenaghan, J.B. personal communication, December 1979.

4-hydroxyxylidine, 193 sec.

All gas-liquid chromatography experiments were performed on a Packard 428 gas chromatograph equipped with a Pye-Unicam DP-88 computing integrator.

7. Inhibitors

Inhibitors were in all cases added to microsomal suspensions prior to the addition of lidocaine and NADPH generating system. SKF525-A and metyrapone were added to microsomal suspension at final concentrations of 200 mM and 2.33 mM, respectively. After addition of SKF525-A or metyrapone, the reaction mixtures were shaken before any further additions were made. $CO:O_2$ (80:20; v/v) was bubbled through the incubation mixture at 25 ml/min for 30 sec using an 18 gauge syringe needle.

8. Interaction of lidocaine with partially purified cytochrome P-450

Partially purified cytochrome P-450 was isolated from phenobarbital induced Long-Evans rats (weighing 250-300 g) according to the method of v.d. Hoeven and Coon (78). This preparation consists of a mixture of forms of cytochrome P-450 free from other microsomal components, such as NADPH cytochrome c reductase and cytochrome b_5.

Difference spectra for the binding of lidocaine to partially purified cytochrome P-450 from phenobarbital induced rats

(2 μM) in 0.02 M Tris-HCl, pH 7.4 were obtained as described for obtaining difference spectra with hepatic microsomes (see Section 5.1).

The ability of artificial electron donors to support the metabolism of lidocaine by partially purified cytochrome P-450 was assessed in incubation mixtures (3.0 ml) containing lidocaine (1 mM), partially purified hepatic microsomal cytochrome P-450 from phenobarbital induced rats (ca. 2 μM) (25) and an artificial electron donor such as $NaClO_2$ (5 mM), H_2O_2 (10 mM), $NaIO_4$ (7.5 mM) or m-chloroperbenzoic acid (3.3 mM) in 0.02 M Tris-HCl, pH 7.4. Incubations were for 10 min at 37° with shaking at 100 oscillations per min.

9. Isolation of hepatocytes

Hepatocytes were isolated from rat liver according to the method of Fry et al. (79). The freshly removed liver was sliced thinly (ca. 0.5 mm) and the slices were incubated for 10 min at 37° with shaking (50 oscillations per min). This incubation procedure was repeated three times, each with a fresh change of buffer [phosphate buffered saline, pH 7.4; NaCl (0.14 M), Na_2HPO_4 (0.02 M)]. Thereafter followed two incubations for 10 min at 37° with fresh phosphate buffered saline plus EDTA (0.5 mM, final concentration) with shaking. The slices were then incubated for 60 min at 37° with shaking with collagenase (5 mg) and hyaluronidase (10 mg) per 10 ml of Hanks buffered saline [NaCl (0.14 M), Na_2HPO_4 (0.3 mM), KH_2PO_4 (0.4 mM), glucose (0.01 M)], pH 7.4. The cells were then

washed with phosphate buffered saline and collected by centrifugation at 50 g for one min three times and were finally suspended in Leibowitz L-15 medium supplemented with 10% fetal calf serum and penicillin/streptomycin (100 IU and 100 µg/ml, respectively). The cells were counted on a haemocytometer using the Trypan Blue exclusion test to ascertain viability. Cells were diluted to 10^6 cells/ml and used within one hr of preparation. In all cases cell viability at the time of preparation was greater than 95%. Cells were stored on ice until used.

10. Calculations

Reported values are means $\pm$ standard deviation. Determinations were in triplicate with each of three or more separate preparations of hepatic microsomes or hepatocytes and in triplicate with one preparation of partially purified cytochrome P-450. Binding constants (K_s), maximum extent of binding ($^{\Delta}A$ max), K_m and V_{max} values were determined from Hanes plots. Statistical analyses were performed using the Student's t-test for unpaired data, with a significant difference being taken as $P < 0.01$. A probably significant difference was taken as $P < 0.05$.

III RESULTS

1. The binding of lidocaine to hepatic microsomal cytochrome P-450

Hanes plots for the binding of lidocaine to cytochrome P-450 in hepatic microsomes from uninduced, phenobarbital and pregnenolone-16α-carbonitrile induced rats were biphasic, with K_s values for a low affinity and a high affinity binding site being calculable (Figures 6, 8 and 9). Pretreatment of rats with β-naphthoflavone led to a monophasic binding plot (Figure 7) with the single K_s value calculated being comparable to that for the high affinity binding site for microsomes from uninduced rats. The effect of induction of different forms of cytochrome P-450 on the binding constant (K_s) and the maximum extent of binding ($^{\Delta}A$ max) for the type I binding of lidocaine to hepatic microsomal cytochrome P-450 is presented in Table 1.

Pretreatment of rats with phenobarbital, or pregnenolone-16α-carbonitrile did not result in significant alterations in the value of K_s for either binding site relative to microsomes from uninduced rats. Pretreatment of rats with β-naphthoflavone did not result in a significant alteration in the value of K_s for the high affinity binding site while the low affinity binding site disappeared following pretreatment of rats with β-naphthoflavone. The values of $^{\Delta}A$ max and $^{\Delta}A$ max/nmol microsomal cytochrome P-450 for the low affinity site were significantly increased following pregnenolone-16α-

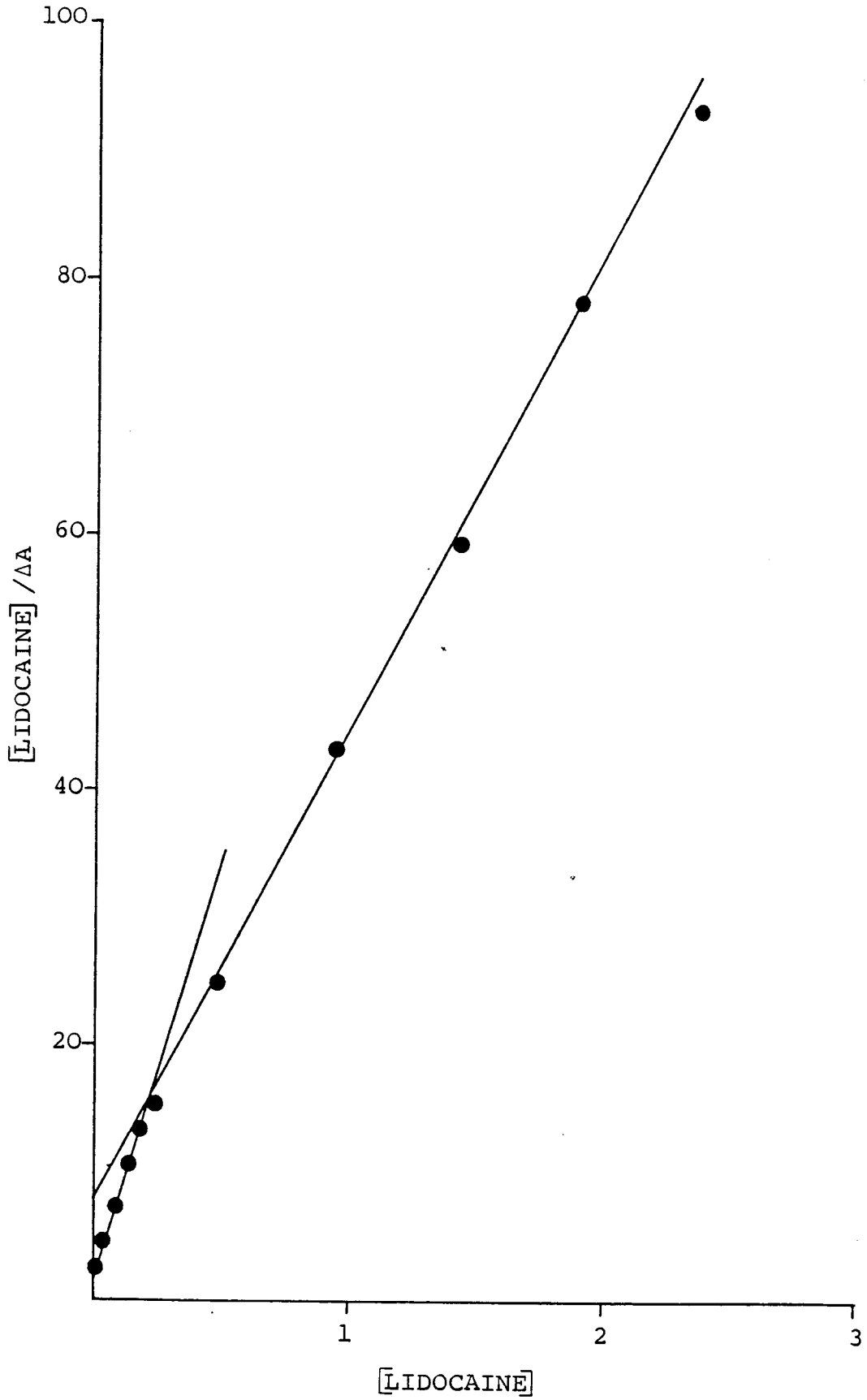

Figure 6. Hanes plot for the binding of lidocaine to cytochrome P-450 in hepatic microsomes from uninduced rats.

[LIDOCAINE], mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

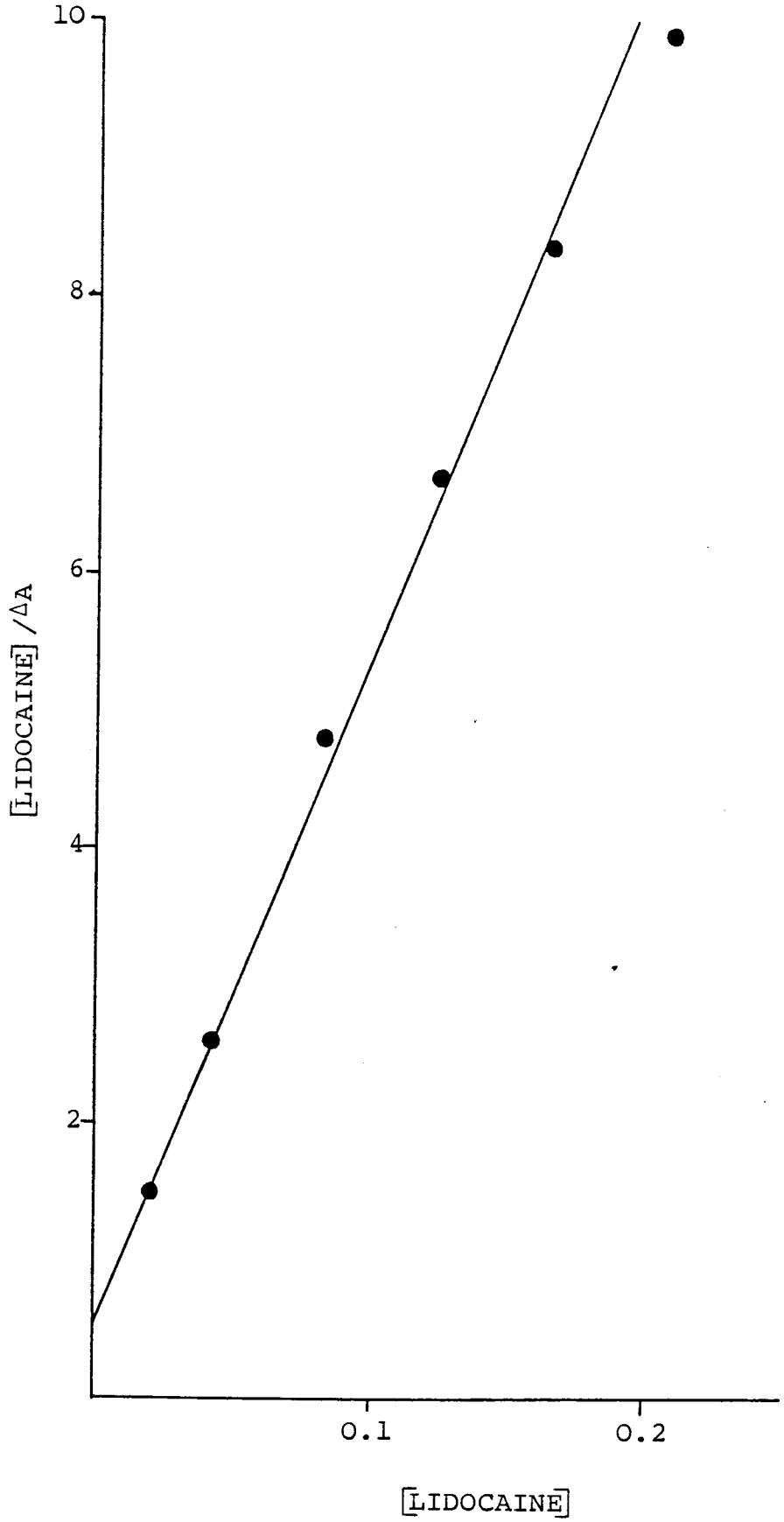

Figure 7. Hanes plot for the binding of lidocaine to cytochrome P-450 in hepatic microsomes from β-naphthoflavone pretreated rats.

[LIDOCAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

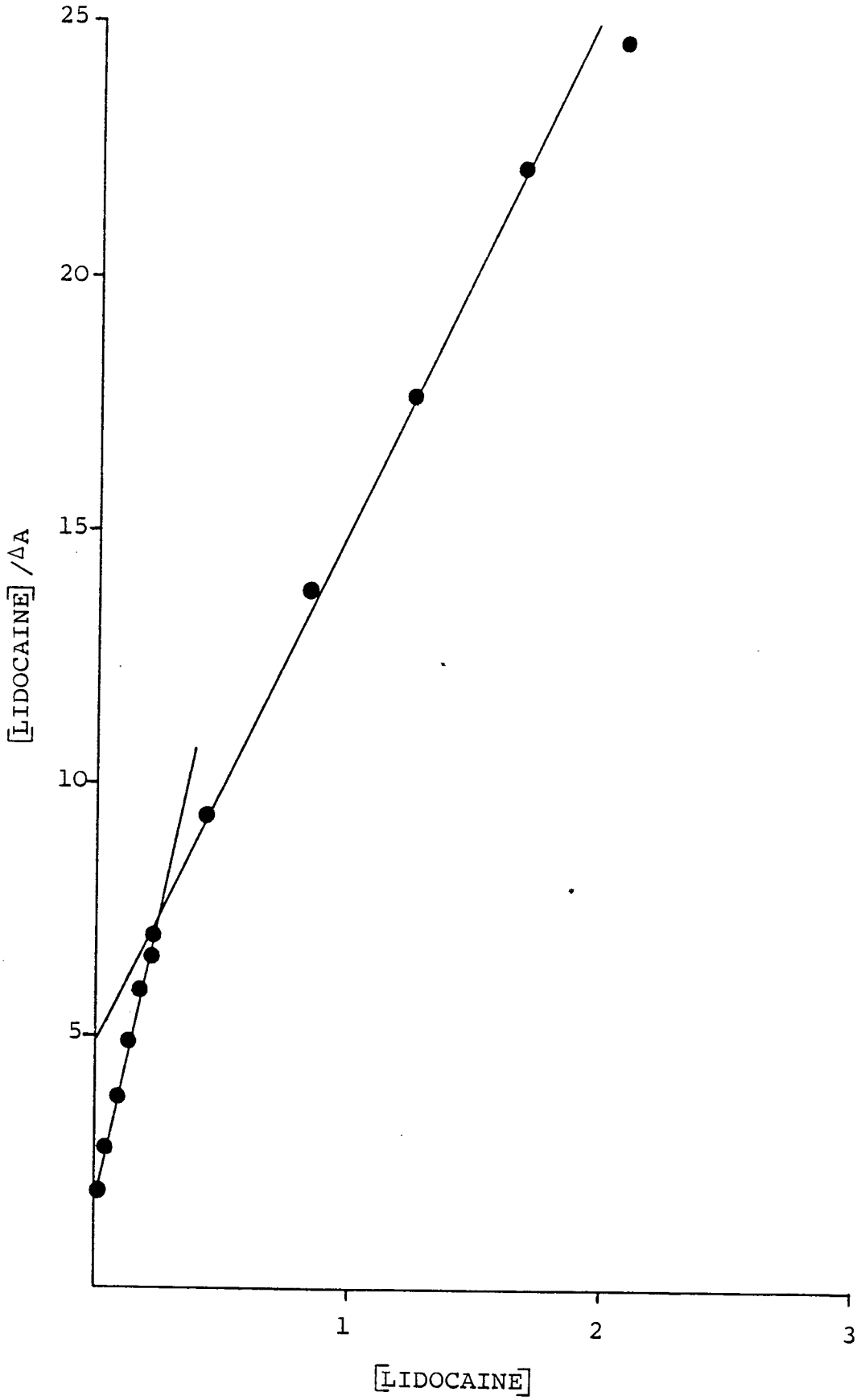

Figure 8. Hanes plot for the binding of lidocaine to cytochrome P-450 in hepatic microsomes from pregnenolone-16α-carbonitrile pretreated rats. [LIDOCAINE], mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

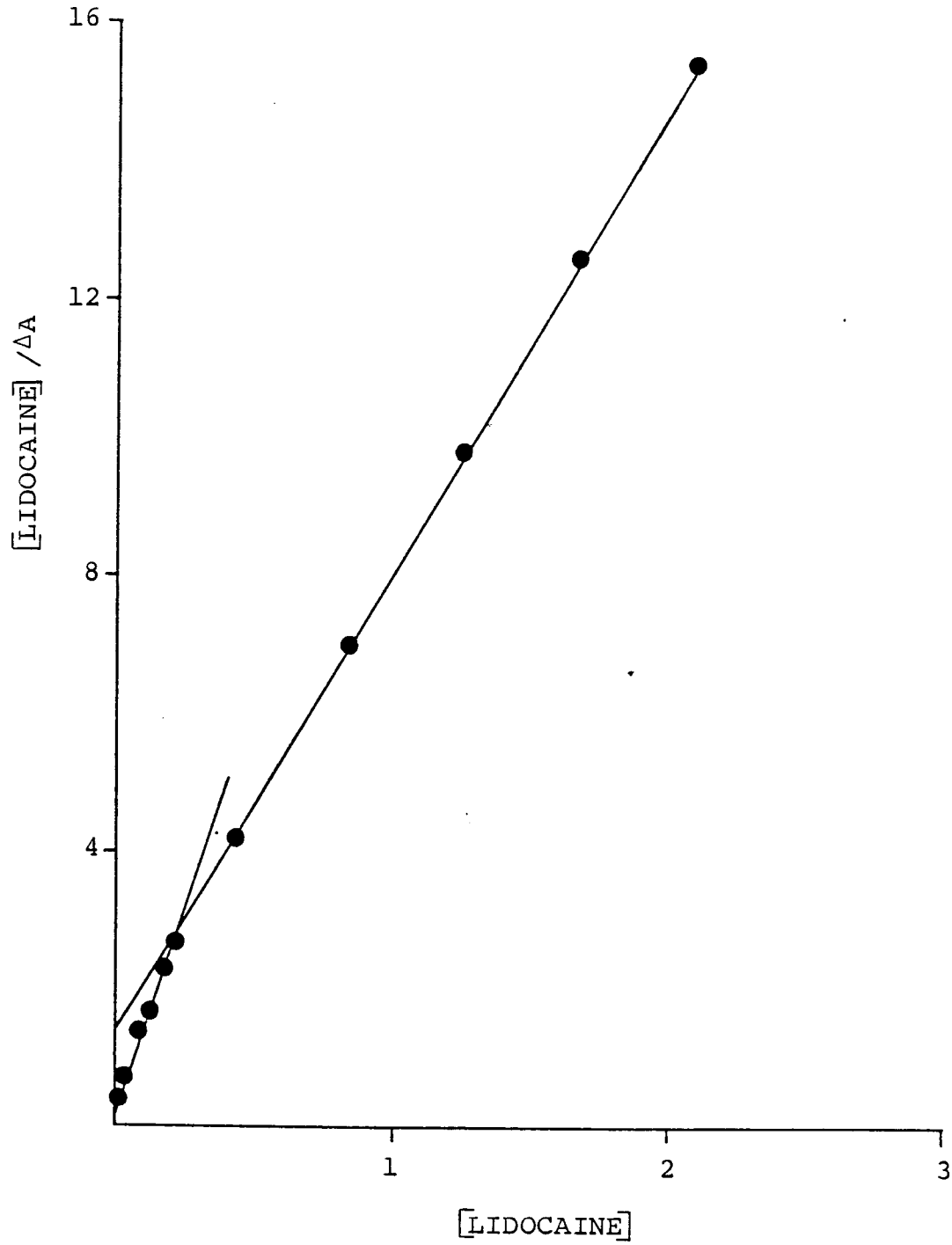

Figure 9. Hanes plot for the binding of lidocaine to cytochrome P-450 in hepatic microsomes from phenobarbital pretreated rats.

[LIDOCAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

TABLE 1. Effect of induction of different forms of cytochrome P-450 on the binding of lidocaine to hepatic microsomal cytochrome P-450.

	K_s (mM)		ΔA max		ΔA max/nmol cyt. P-450	
Induction	low affinity site	high affinity site	low affinity site	high affinity site	low affinity site	high affinity site
NONE	0.40 ± 0.03	0.030 ± 0.020	0.025 ± 0.004	0.017 ± 0.001	0.034 ± 0.006	0.023 ± 0.003
BNF	-	0.009 ± 0.004	-	0.017 ± 0.006	-	0.013 ± 0.004*
PCN	0.55 ± 0.14	0.037 ± 0.007	0.095 ± 0.031*	0.031 ± 0.010†	0.058 ± 0.011*	0.019 ± 0.005
PB	0.27 ± 0.18	0.032 ± 0.008	0.197 ± 0.032*	0.108 ± 0.023*	0.054 ± 0.006*	0.030 ± 0.002*

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

carbonitrile pretreatment, and the values of $^{\Delta}A$ max and $^{\Delta}$max/nmol cytochrome P-450 for both the high and the low affinity sites were significantly increased following phenobarbital pretreatment. When compared to microsomes from uninduced rats, the value of $^{\Delta}A$ max/nmol cytochrome P-450 for microsomes from β-naphthoflavone pretreated rats were decreased significantly relative to the microsomes from uninduced rats.

2. The binding of mepivacaine to hepatic microsomal cytochrome P-450

Mepivacaine is shown to display a Type I binding spectrum in preparations of hepatic microsomes (Figure 10). Hanes plots for the binding of mepivacaine to cytochrome P-450 in hepatic microsomes from uninduced, β-naphthoflavone and pregnenolone-16α-carbonitrile pretreated rats were monophasic (Figures 11-13), while those from rats pretreated with phenobarbital were biphasic (Figure 14).

The effect of induction of different forms of cytochrome P-450 on the values of K_s and $^{\Delta}A$ max for the binding of mepivacaine to hepatic microsomal cytochrome P-450 is presented in Table 2.

On one day out of five days that the binding of mepivacaine to hepatic microsomal cytochrome P-450 from pregnenolone-16α-carbonitrile pretreated rats was investigated, a biphasic Hanes plot was calculable. The low affinity value of K_s was 0.05 mM.

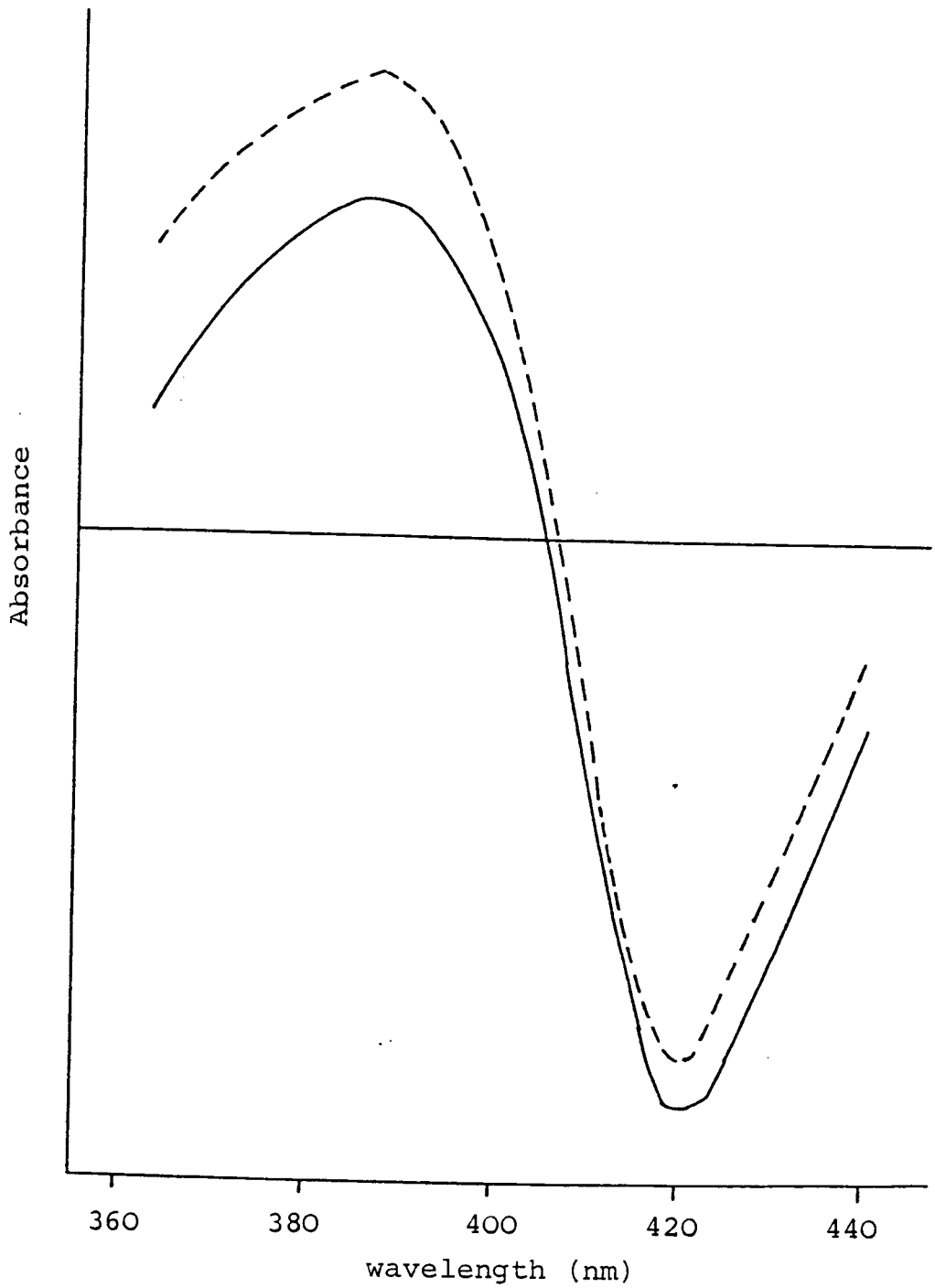

Figure 10. Type 1 difference spectra for the binding of mepivacaine (1.8 mM, final concentration) (—) and bupivacaine (11 mM, final concentration) (---) to cytochrome P-450 in microsomes from phenobarbital pretreated rats.

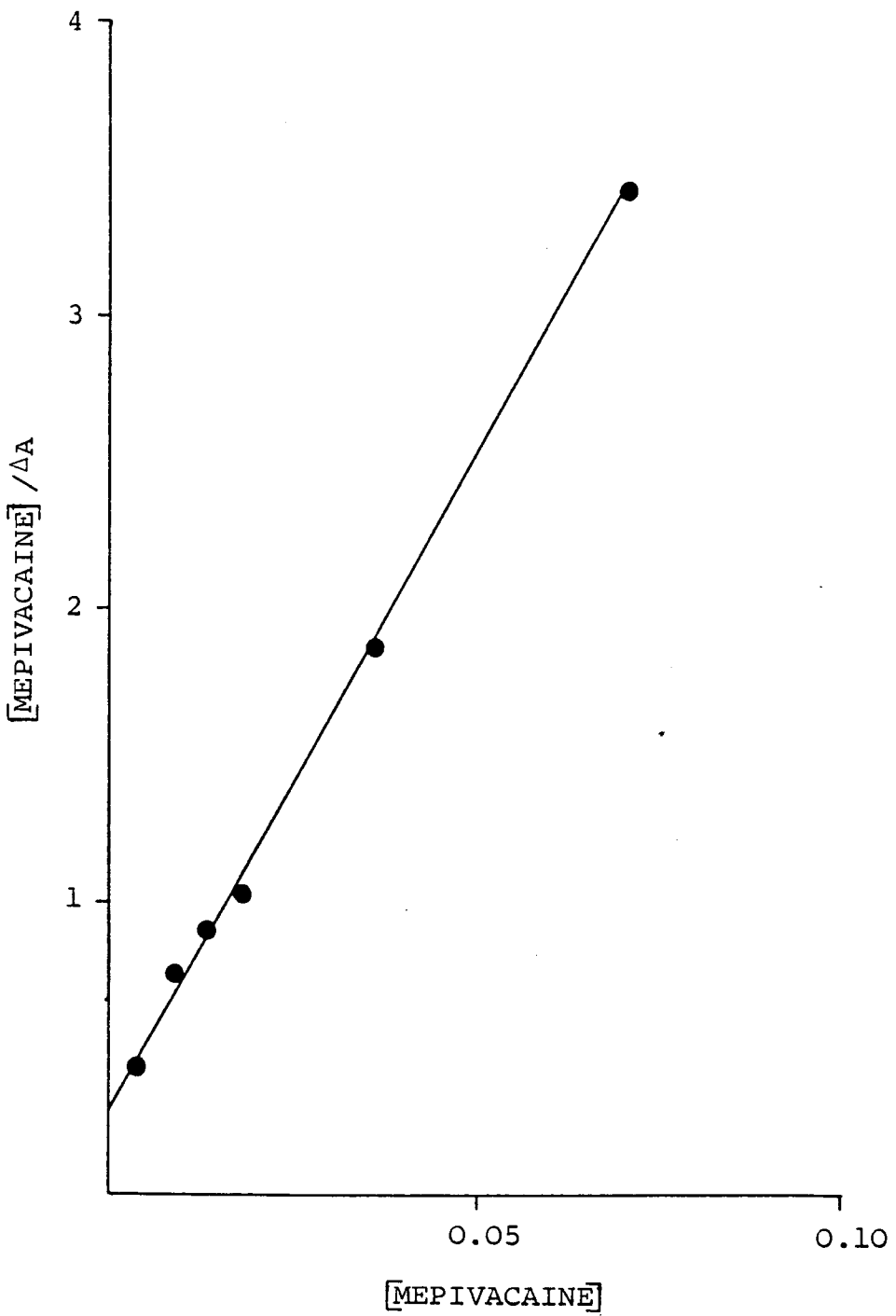

Figure 11. Hanes plot for the binding of mepivacaine to cytochrome P-450 in hepatic microsomes from uninduced rats.

[MEPIVACAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

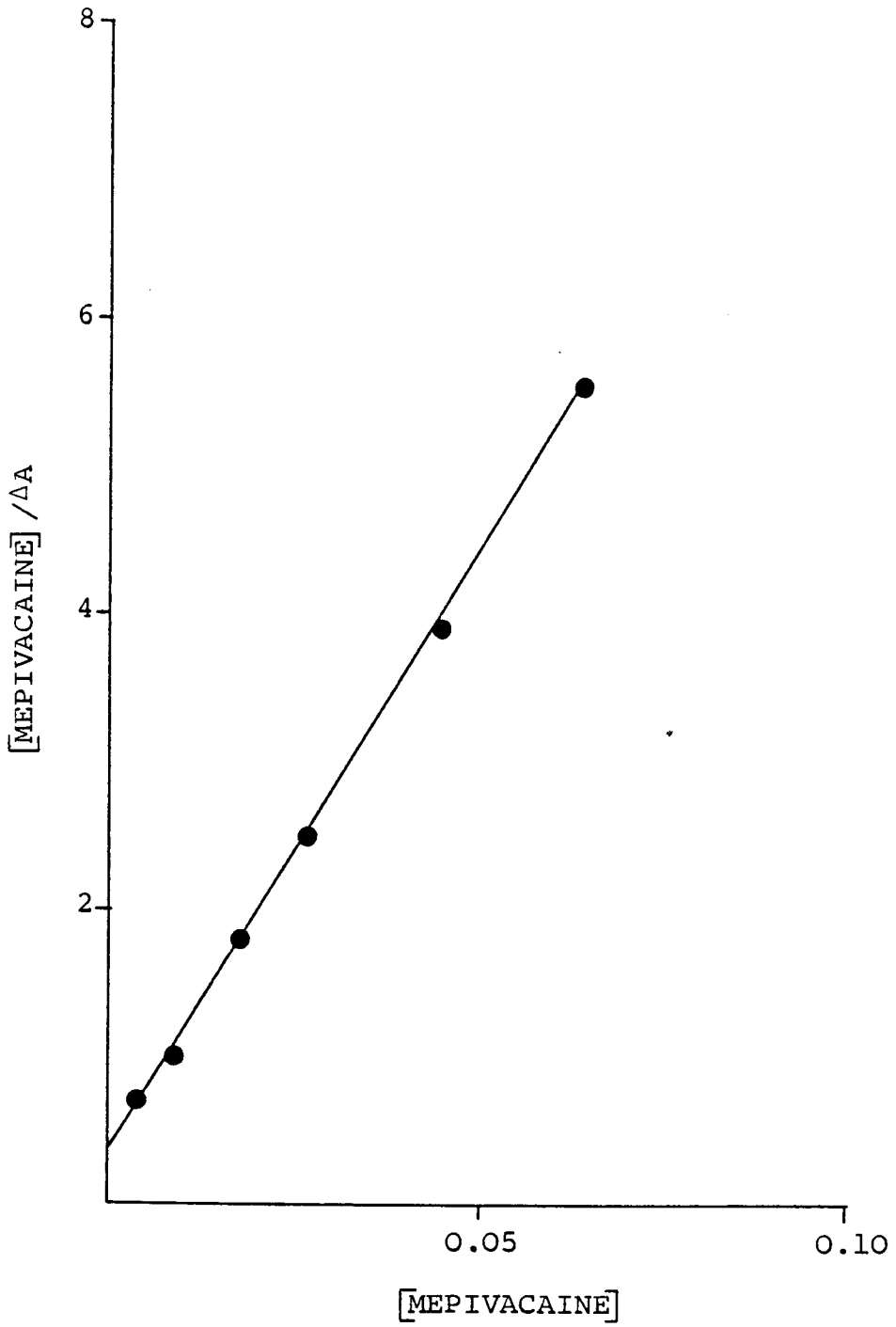

Figure 12. Hanes plot for the binding of mepivacaine to cytochrome P-450 in hepatic microsomes from β-naphthoflavone pretreated rats. [MEPIVACAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

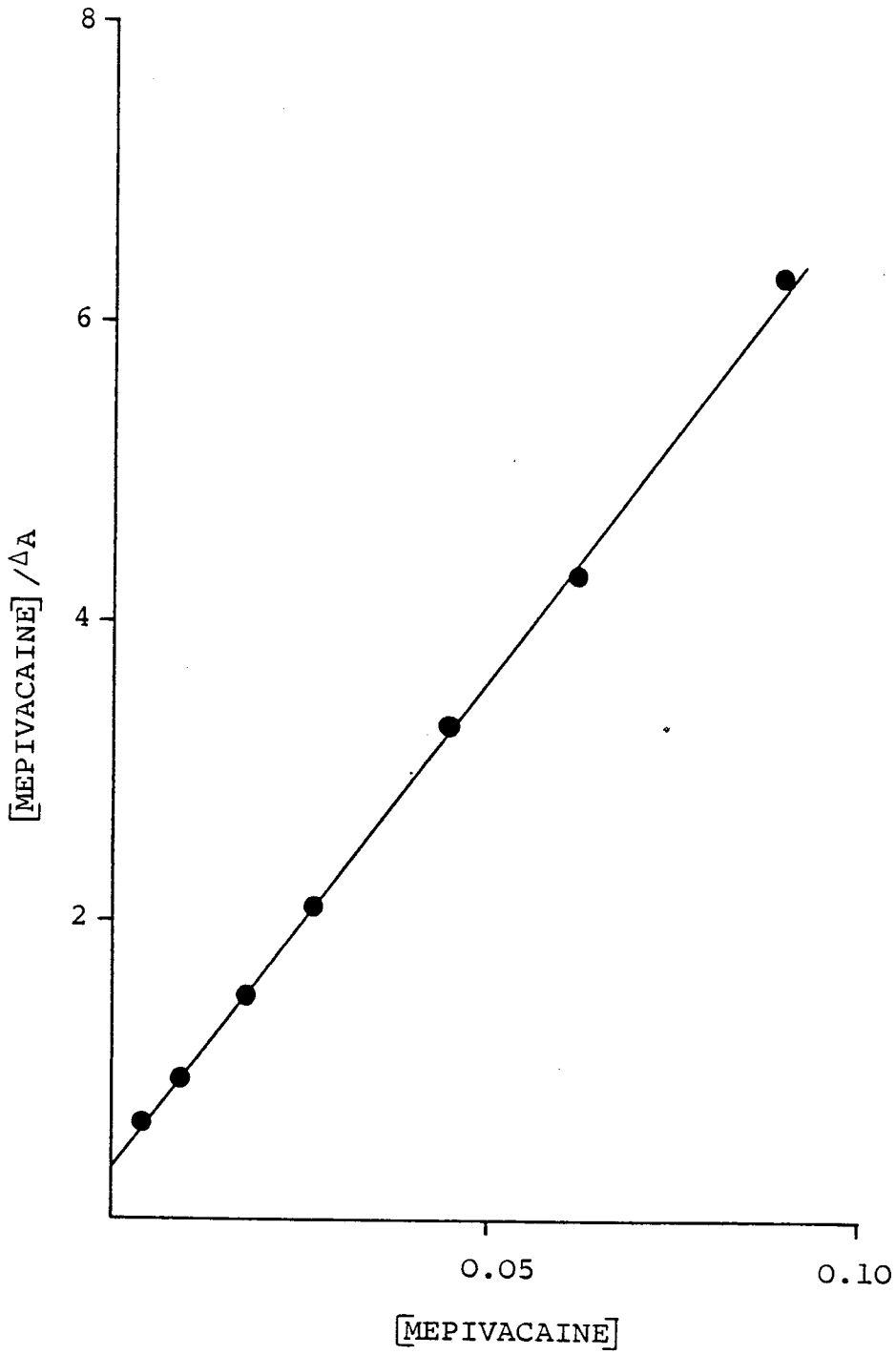

Figure 13. Hanes plot for the binding of mepivacaine to cytochrome P-450 in hepatic microsomes from pregnenolone-16α-carbonitrile pretreated rats. [MEPIVACAINE], mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

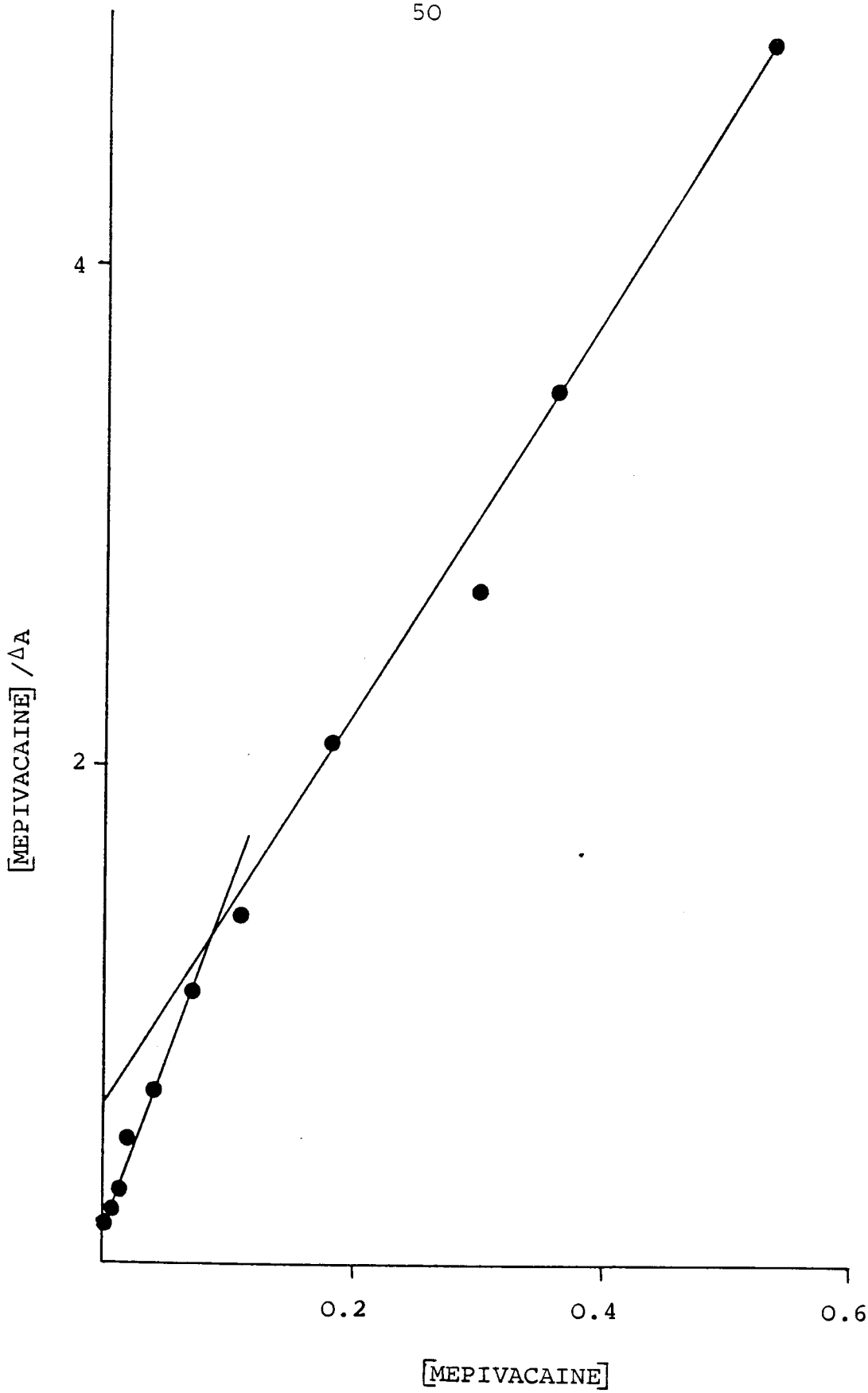

Figure 14. Hanes plot for the binding of mepivacaine to cytochrome P-450 in hepatic microsomes from phenobarbital pretreated rats.

[MEPIVACAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

TABLE 2. Effect of induction of different forms of cytochrome P-450 on the binding of mepivacaine to hepatic microsomal cytochrome P-450.

	K_s (mM)		ΔA max		ΔA max/nmol cyt. P-450	
Induction	low affinity site	high affinity site	low affinity site	high affinity site	low affinity site	high affinity site
NONE	-	0.008 ± 0.005	-	0.015 ± 0.006	-	0.017 ± 0.005
BNF	-	0.008 + 0.004	-	0.014 ± 0.003	-	0.012 ± 0.003
PCN	-	0.014 ± 0.005	-	0.014 ± 0.008	-	0.009 ± 0.005†
PB	0.045 ± 0.014	0.009 ± 0.005	0.096 ± 0.020	0.052 ± 0.012*	0.039 ± 0.005	0.022 ± 0.005

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

When compared to the binding of mepivacaine to hepatic cytochrome P-450 in microsomes from uninduced rats, no significant change in the values of K_S or ΔA max/nmol microsomal cytochrome P-450 for binding to the high affinity site was observed for any type of induction. Pretreatment of rats with phenobarbital led to a significant increase in the value of ΔA max for the high affinity site over values obtained using uninduced rats and resulted in the appearance of a low affinity site.

3. The binding of bupivacaine to hepatic microsomal cytochrome P-450

Bupivacaine is shown to display a Type I difference spectrum in the presence of hepatic microsomes (Figure 10). Microsomes from uninduced and β-naphthoflavone pretreated rats yielded biphasic Hanes plots for the binding of bupivacaine to hepatic cytochrome P-450 (Figures 15 and 16) with a high affinity K_S value of ca.0.01 mM and a low affinity value of K_S of ca. 0.05 mM Monophasic Hanes plots were obtained for the binding of bupivacaine to hepatic cytochrome P-450 in microsomes from pregnenolone-16α-carbonitrile and phenobarbital pretreated rats (Figures 17 and 18). The effect of induction of different forms of cytochrome P-450 on the values of K_S and ΔA max for the binding of bupivacaine to hepatic microsomal cytochrome P-450 is shown in Table 3. The value of K_S following induction of rats by pregnenolone-16α-carbonitrile and phenobarbital (ca. 0.07 mM) corresponded to the low affinity value of K_S obtained in the case of bupivacaine binding to hepatic

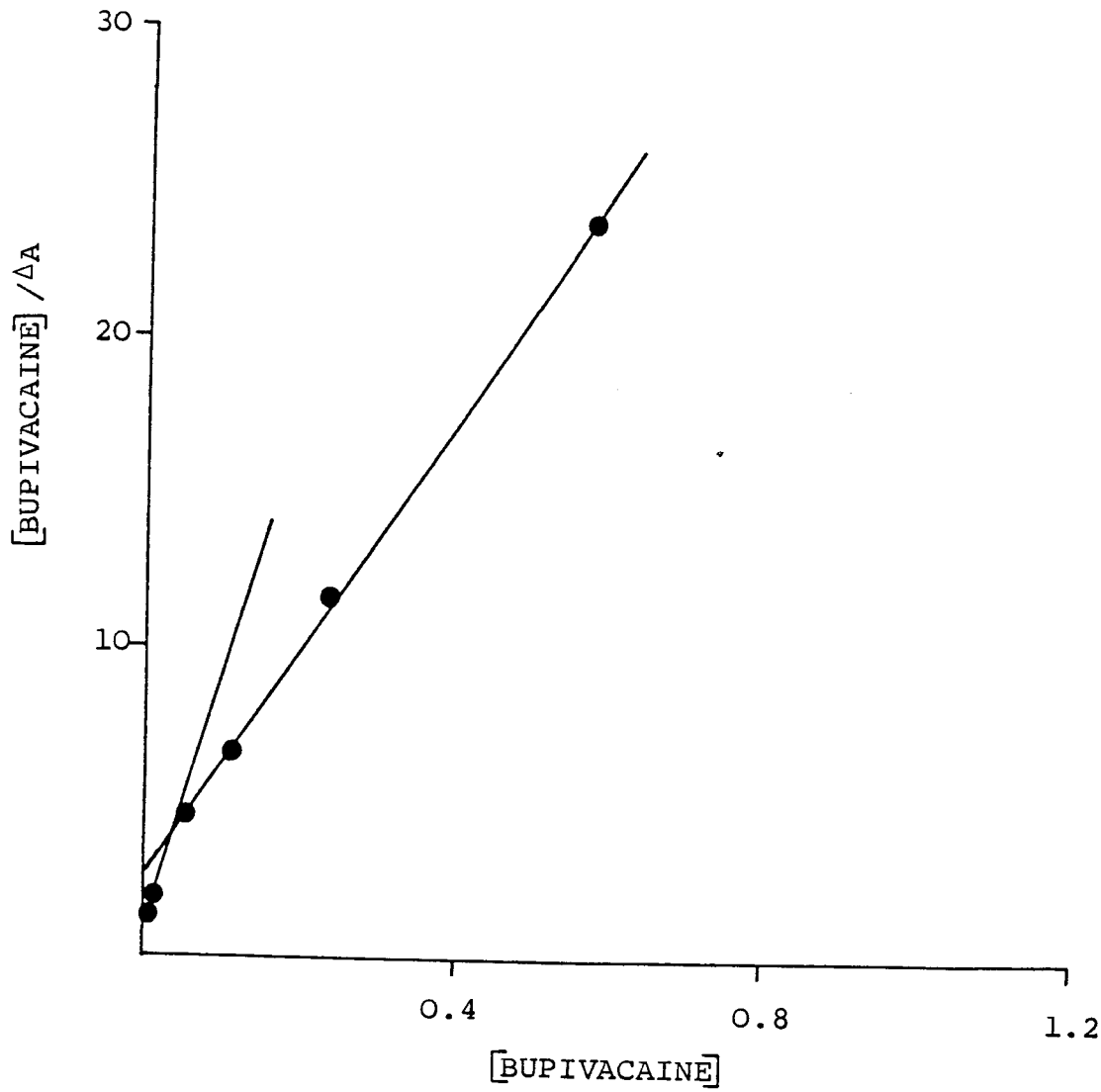

Figure 15. Hanes plot for the binding of bupivacaine to cytochrome P-450 in hepatic microsomes from uninduced rats.

[BUPIVACAINE], mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

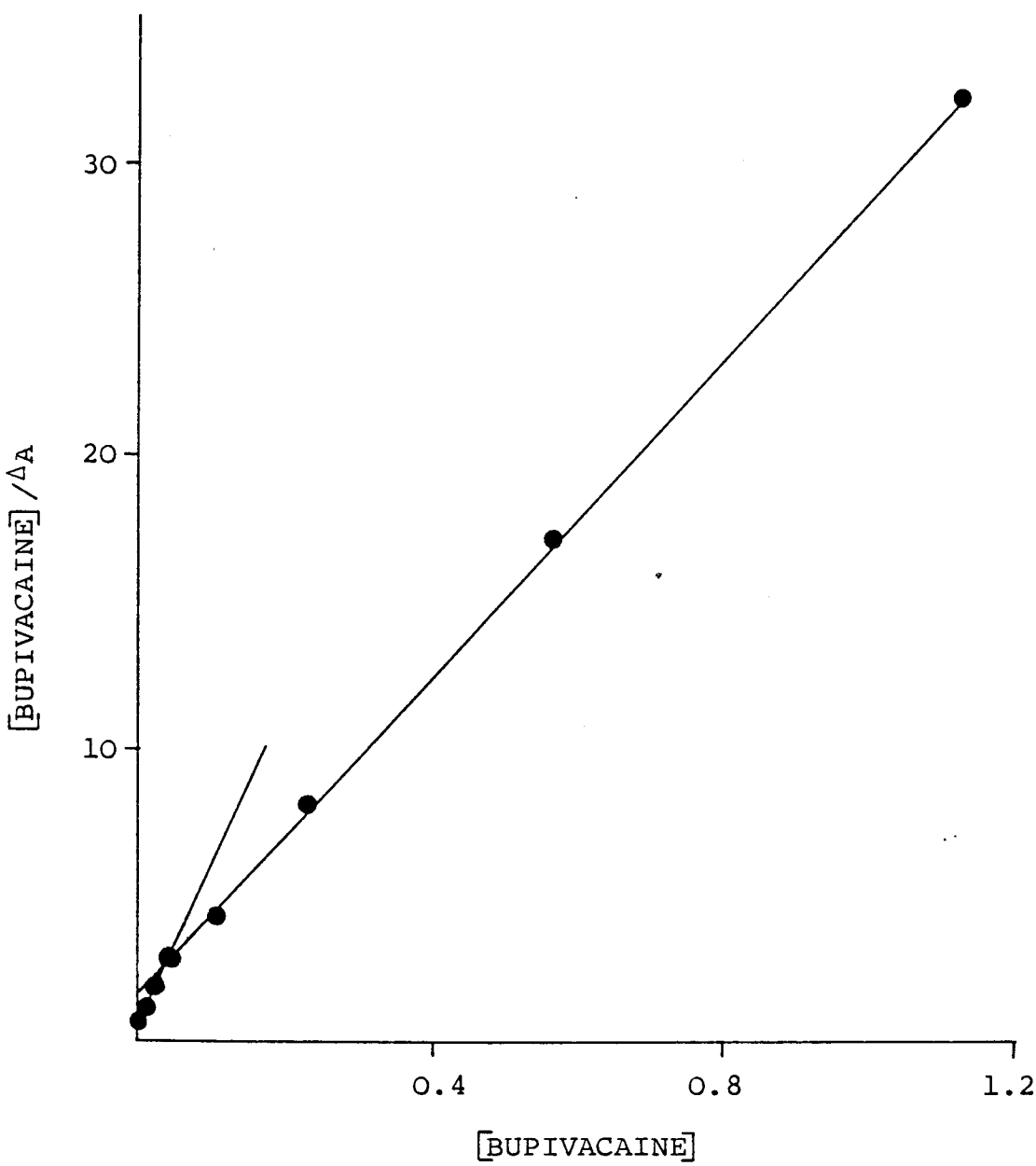

Figure 16. Hanes plot for the binding of bupivacaine to cytochrome P-450 in hepatic microsomes from β-naphthoflavone pretreated rats.

[BUPIVACAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

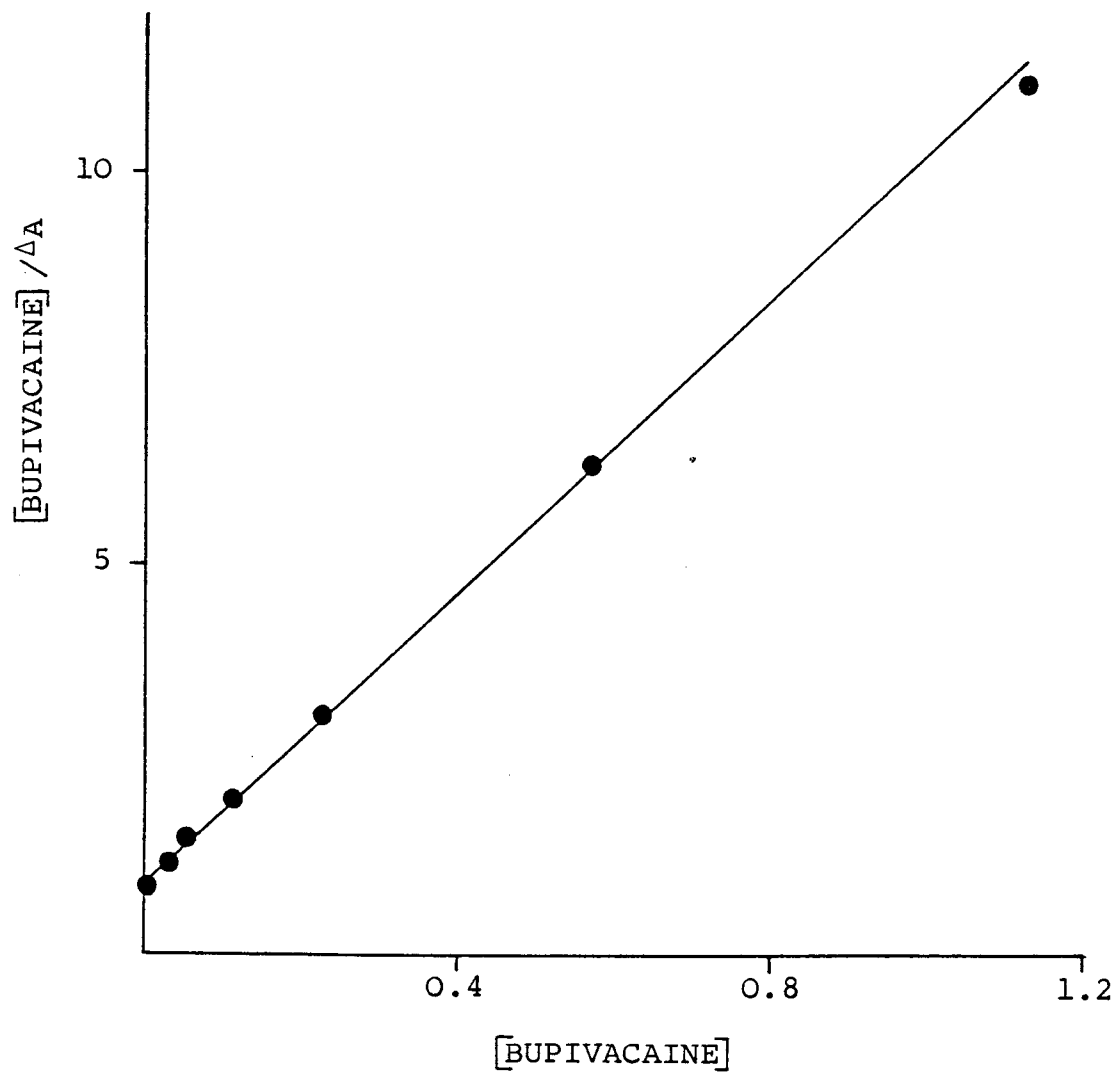

Figure 17. Hanes plot for the binding of bupivacaine to cytochrome P-450 in hepatic microsomes from pregnenolone-16α-carbonitrile pretreated rats. [BUPIVACAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

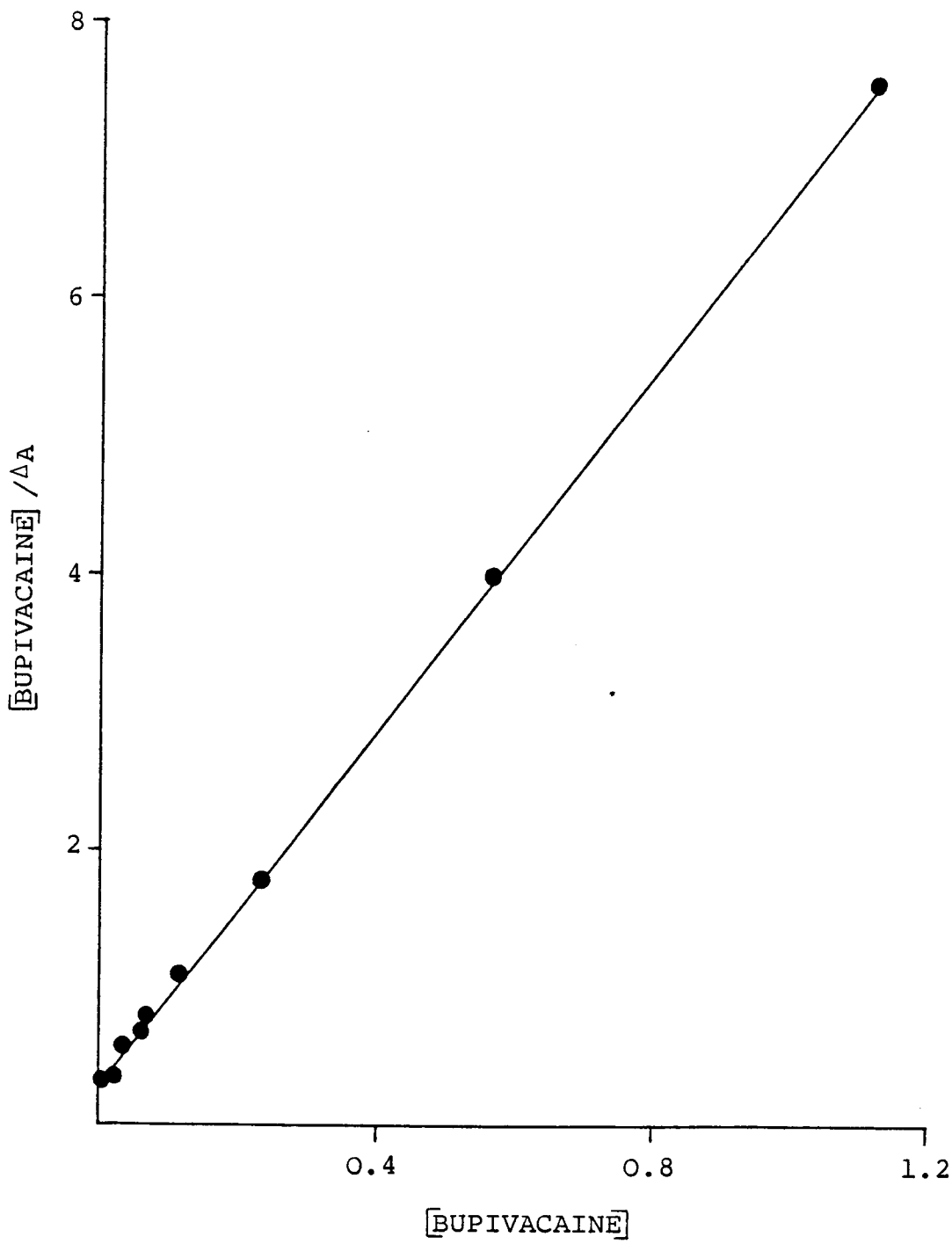

Figure 18. Hanes plot for the binding of bupivacaine to cytochrome P-450 in hepatic microsomes from phenobarbital pretreated rats.

[BUPIVACAINE] , mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

cytochrome P-450 in microsomes from uninduced and β-naphthoflavone pretreated rats.

The values of ΔA max and ΔA max/nmol microsomal cytochrome P-450 for the binding of bupivacaine to the low affinity site of hepatic microsomal cytochrome P-450 were significantly increased following pretreatment of the rats with pregnenolone-16α-carbonitrile and phenobarbital relative to the values for the binding of bupivacaine to hepatic cytochrome P-450 in microsomes from uninduced rats. Pretreatment of rats with pregnenolone-16α-carbonitrile also significantly increased the value of K_s relative to the low affinity value of K_s obtained for the binding of bupivacaine to cytochrome P-450 in hepatic microsomes from uninduced rats. Pretreatment of rats with β-naphthoflavone did not appear to significantly alter the values of K_s, ΔA max or ΔA max/nmol microsomal cytochrome P-450 for either the high or low affinity sites relative to the values calculated for the binding of bupivacaine to hepatic cytochrome P-450 in microsomes from uninduced rats (Table 3).

On one day out of three days that the binding of bupivacaine to cytochrome P-450 in hepatic microsomes from rats pretreated with pregnenolone-16α-carbonitrile was investigated, a biphasic Hanes plot was calculable, with a corresponding high affinity value of K_s of 23 μM. The value of ΔA max was 0.058 and the value of ΔA max/nmol microsomal cytochrome P-450, 0.035.

TABLE 3. Effect of induction of different forms of cytochrome P-450 on the binding of bupivacaine to hepatic microsomal cytochrome P-450.

	K_s (mM)		ΔA max		ΔA max/nmol cyt. P-450	
Induction	low affinity site	high affinity site	low affinity site	high affinity site	low affinity site	high affinity site
NONE	0.057 ± 0.013	0.007 ± 0.004	0.035 ± 0.016	0.012 ± 0.006	0.035 ± 0.009	0.014 ± 0.006
BNF	0.045 ± 0.021	0.010 ± 0.001	0.034 ± 0.005	0.024 ± 0.001†	0.024 ± 0.002	0.017 ± 0.001
PCN	0.095 ± 0.002*	-	0.086 ± 0.020*	-	0.053 ± 0.003*	-
PB	0.060 ± 0.001	-	0.156 ± 0.021*	-	0.067 ± 0.002*	-

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

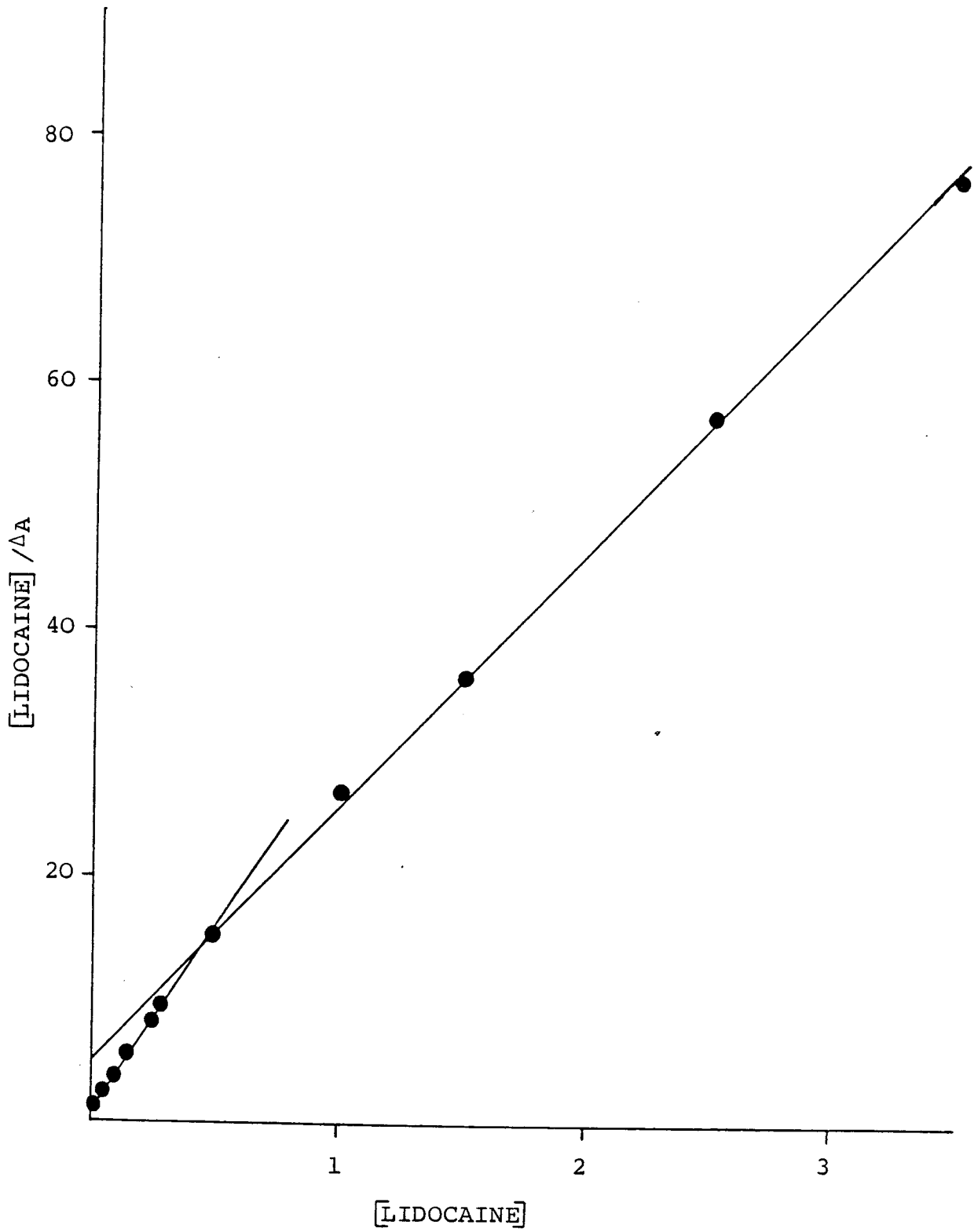

Figure 19. Hanes plot for the binding of lidocaine to partially purified cytochrome P-450 isolated from hepatic microsomes from phenobarbital pre-treated rats.

[LIDOCAINE], mM; $\Delta A = A_{385\ nm} - A_{420\ nm}$

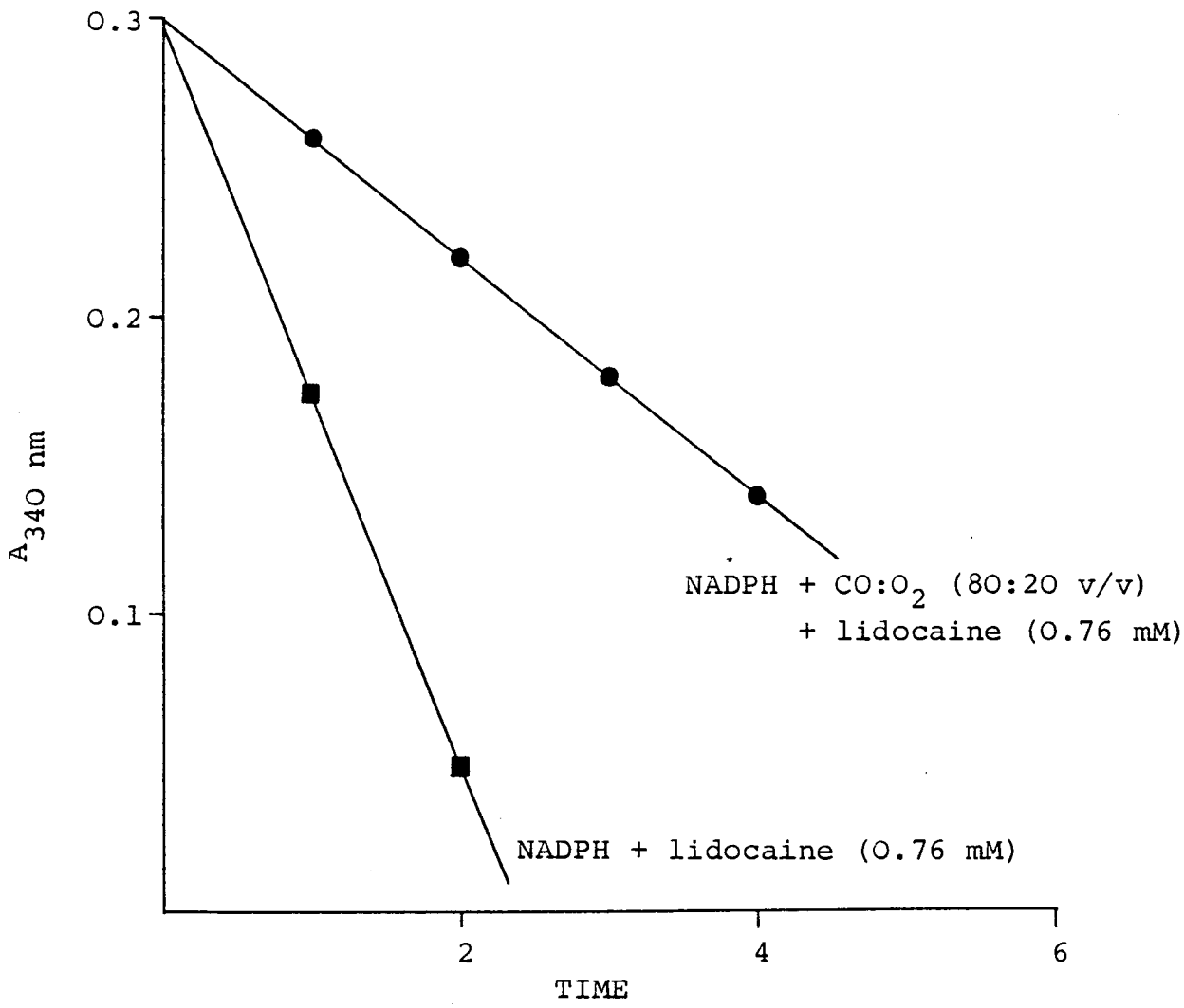

Figure 20. Stimulation of CO-inhibitable NADPH oxidation by cytochrome P-450 in hepatic microsomes from phenobarbital pretreated rats in the presence of 0.76 mM lidocaine.
Time, min; $A_{340\ nm}$, absorbance units.

TABLE 4. Effect of induction of different forms of cytochrome P-450 on the hepatic microsomal NADPH oxidation of Lidocaine.

Induction	N A D P H OXIDATION			
	nmol/min/mg microsomal protein		nmol/min/nmol cytochrome P-450	
	Lidocaine		concentration	
	0.76 mM	0.06 mM	0.76 mM	0.06 mM
NONE	1.43 ± 0.29	0.81 ± 0.24	1.33 ± 0.04	0.91 ± 0.16
BNF	1.55 ± 0.26	0.61 ± 0.14	0.59 ± 0.14*	0.44 ± 0.13*
PCN	2.77 ± 1.31*	1.09 ± 0.14	0.71 ± 0.17*	0.68 ± 0.01*
PB	7.71 ± 0.66*	8.13 ± 1.64*	3.15 ± 0.50*	2.64 ± 0.27*

* Differs from corresponding value for microsomes from uninduced rats $P < 0.001$

4. Binding of lidocaine to partially purified cytochrome P-450 from phenobarbital pretreated rats

Lidocaine produces a Type I difference spectrum with partially purified cytochrome P-450 (2 μM) isolated from hepatic microsomes from phenobarbital pretreated rats (data not shown). The Hanes plot for the binding of lidocaine to partially purified cytochrome P-450 was biphasic (Figure 19). The values of K_s for the binding of lidocaine to partially purified cytochrome P-450 were 20 ± 1 μM and 300 ± 2 μM with the corresponding values of $^{\Delta}A$ max/nmol cytochrome P-450 being 0.020 ± 0.003 and 0.031 + 0.006, respectively.

5. Lidocaine stimulated hepatic microsomal NADPH oxidation

CO-inhibitable NADPH oxidation by hepatic microsomes from uninduced and pretreated rats was stimulated by lidocaine. A typical plot of the oxidation of NADPH by hepatic microsomes in the presence of lidocaine is shown in Figure 20. The rate of lidocaine stimulated oxidation of NADPH was significantly greater with microsomes from rats pretreated with phenobarbital than with microsomes from uninduced rats (Table 4). Pretreatment of rats with β-naphthoflavone and pregnenolone-16α-carbonitrile did not significantly alter the rate of oxidation of NADPH per mg microsomal protein relative to the rate observed in microsomes from uninduced rats (Table 4).

6. Mepivacaine stimulated hepatic microsomal NADPH oxidation

Mepivacaine in the presence of microsomes from untreated and pretreated rats stimulated CO-inhibitable NADPH oxidation in vitro (Table 5). The rate of mepivacaine stimulated oxidation of NADPH per mg microsomal protein and per nmol cytochrome P-450 was significantly greater with microsomes from rats pretreated with phenobarbital than with microsomes from uninduced rats. Pretreatment of rats with β-naphthoflavone or pregnenolone-16α-carbonitrile significantly increased the rate of hepatic microsomal oxidation of NADPH per mg of microsomal protein, but did not significantly increase the rate per nmol of cytochrome P-450 in the presence of mepivacaine relative to the rates observed with microsomes from untreated rats (Table 5).

7. Bupivacaine stimulated hepatic microsomal NADPH oxidation

CO-inhibitable NADPH oxidation was stimulated by bupivacaine (0.7 mM) using hepatic microsomes from untreated and pretreated rats in vitro (Table 5). The rate of bupivacaine stimulated oxidation of NADPH was significantly greater per mg microsomal protein and per nmol cytochrome P-450 with microsomes from rats pretreated with phenobarbital than with microsomes from uninduced rats. The rate of bupivacaine stimulated oxidation of NADPH was significantly greater per mg microsomal protein, but not per nmol cytochrome P-450 with microsomes from rats pretreated with β-naphthoflavone or pregnenolone-16α-carbonitrile than with microsomes from uninduced rats (Table 5).

TABLE 5. Effect of induction of different forms of cytochrome P-450 on the stimulation of hepatic microsomal NADPH oxidation by mepivacaine and bupivacaine.

Induction	N A D P H OXIDATION			
	nmol/min/mg protein	nmol/min/nmol cytochrome P-450	nmol/min/mg protein	nmol/min/nmol cytochrome P-450
	MEPIVACAINE (0.7 mM)		BUPIVACAINE (0.7 mM)	
NONE	1.02 ± 0.06	1.38 ± 0.29	1.49 ± 0.08	1.99 ± 0.18
BNF	2.27 ± 0.27*	1.43 ± 0.10	2.10 ± 0.23*	1.32 ± 0.09*
PCN	3.00 ± 0.06*	1.68 ± 0.04	4.16 ± 0.30*	2.34 ± 0.17
PB	8.52 ± 0.01*	2.42 ± 0.03*	9.55 ± 0.17*	2.71 ± 0.01*

* Differs from microsomes from uninduced rats $P < 0.01$

8. Hepatic microsomal metabolism of lidocaine

Typical gas liquid chromatograms of the lidocaine metabolites MEGX, GX, 3-hydroxy MEGX and 4-hydroxyxylidine are shown in Figures 21 and 22. Retention times are slower (± 200 sec) than the literature values, which is probably due to a slower gas flow through the column.

The production of MEGX from lidocaine by cytochrome P-450 in hepatic microsomes from uninduced and pregnenolone-16α-carbonitrile pretreated rats was linear for 5 min (Figure 23) from β-naphthoflavone pretreated rats for 3 min and from phenobarbital pretreated rats, for 1 min (Figure 23). These incubation times were then used for all further investigations of the production of MEGX.

Acetaldehyde production from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with phenobarbital and pregnenolone-16α-carbonitrile was linear for 5 min (Figure 24) and from uninduced and β-naphthoflavone pretreated rats, the production of acetaldehyde was linear for 10 min. The above incubation times were then used for all further investigations of the production of acetaldehyde.

8.1 Production of MEGX

Hanes plots for the production of MEGX from lidocaine in the presence of microsomal suspension were biphasic for microsomes from rats pretreated with pregnenolone-16α-carbonitrile and

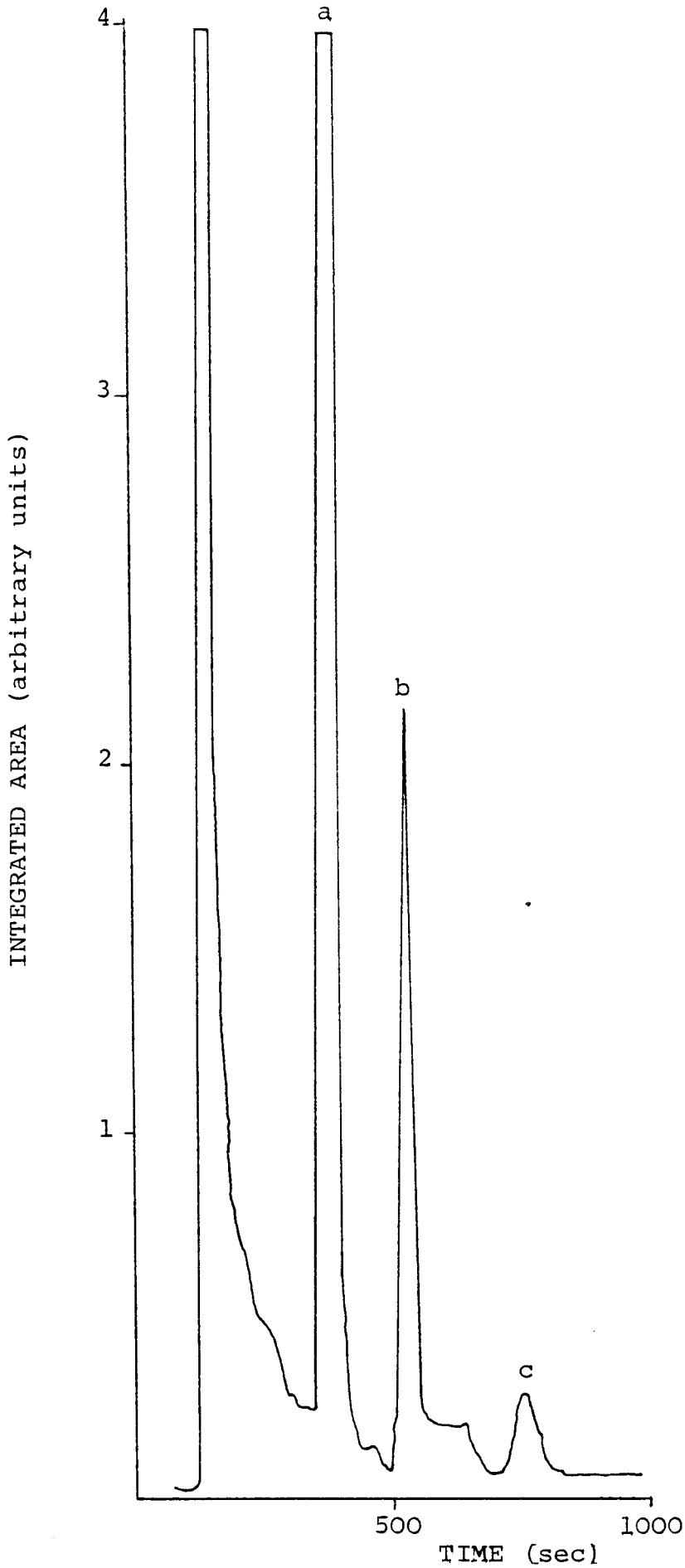

Figure 21. Typical chromatogram of mixture of lidocaine (a), MEGX (b) and GX (c). 5 µl of mixture containing lidocaine (8.7 mM), MEGX (3 mM) and GX (4 mM) was injected onto the column. Retention times for incubation mixtures injected onto the column were identical.

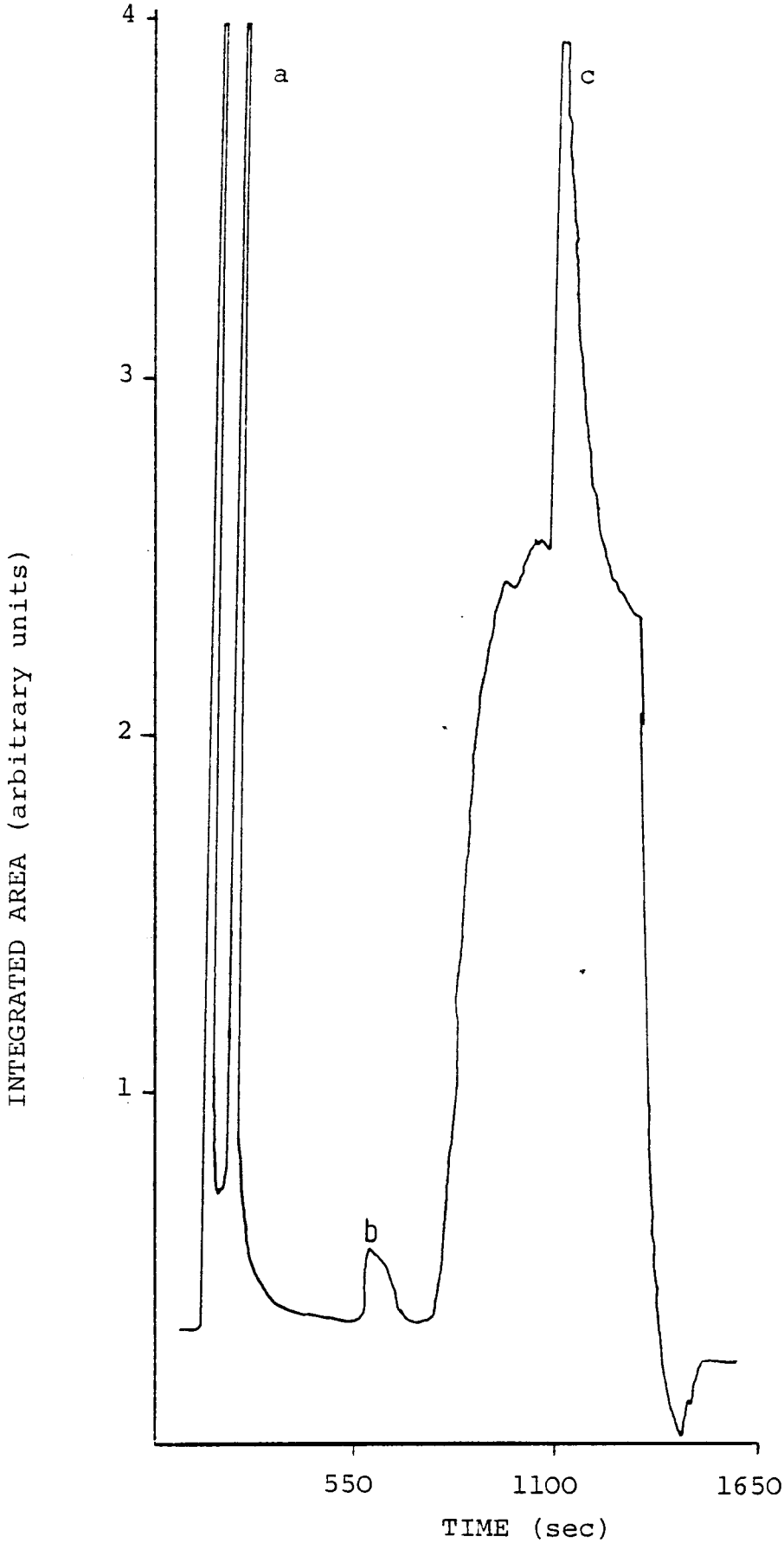

Figure 22. Chromatogram of 4-hydroxyxylidine (a) (23 mM, final concentration), GX (b) (4 mM, final concentration) and 3-hydroxy MEGX (18 mM, final concentration). 5 µl of the mixture was injected onto the column. Retention times for metabolites in incubation mixtures were identical.

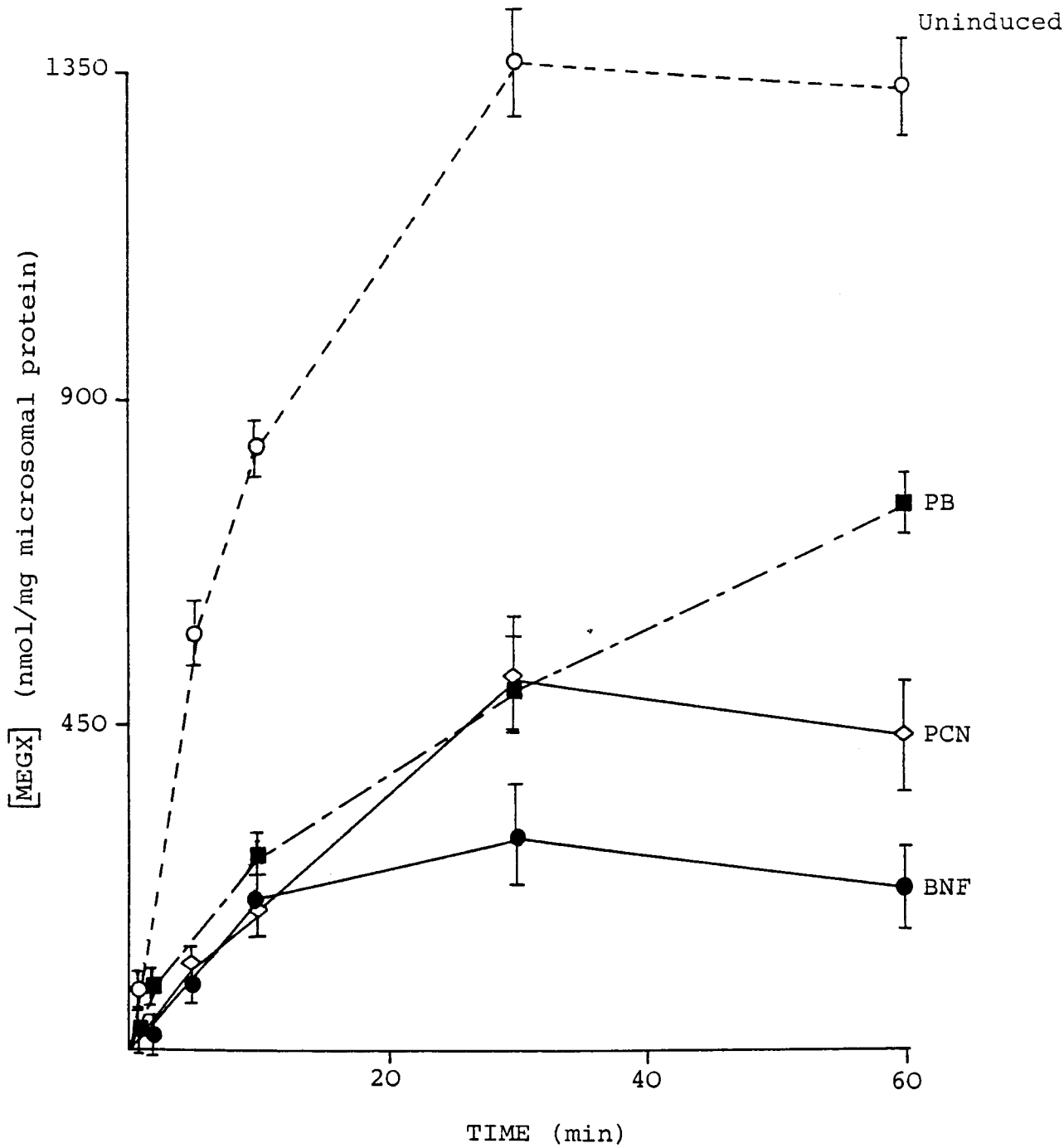

Figure 23. The production of MEGX from lidocaine by hepatic microsomes from untreated and pretreated rats as a function of time.

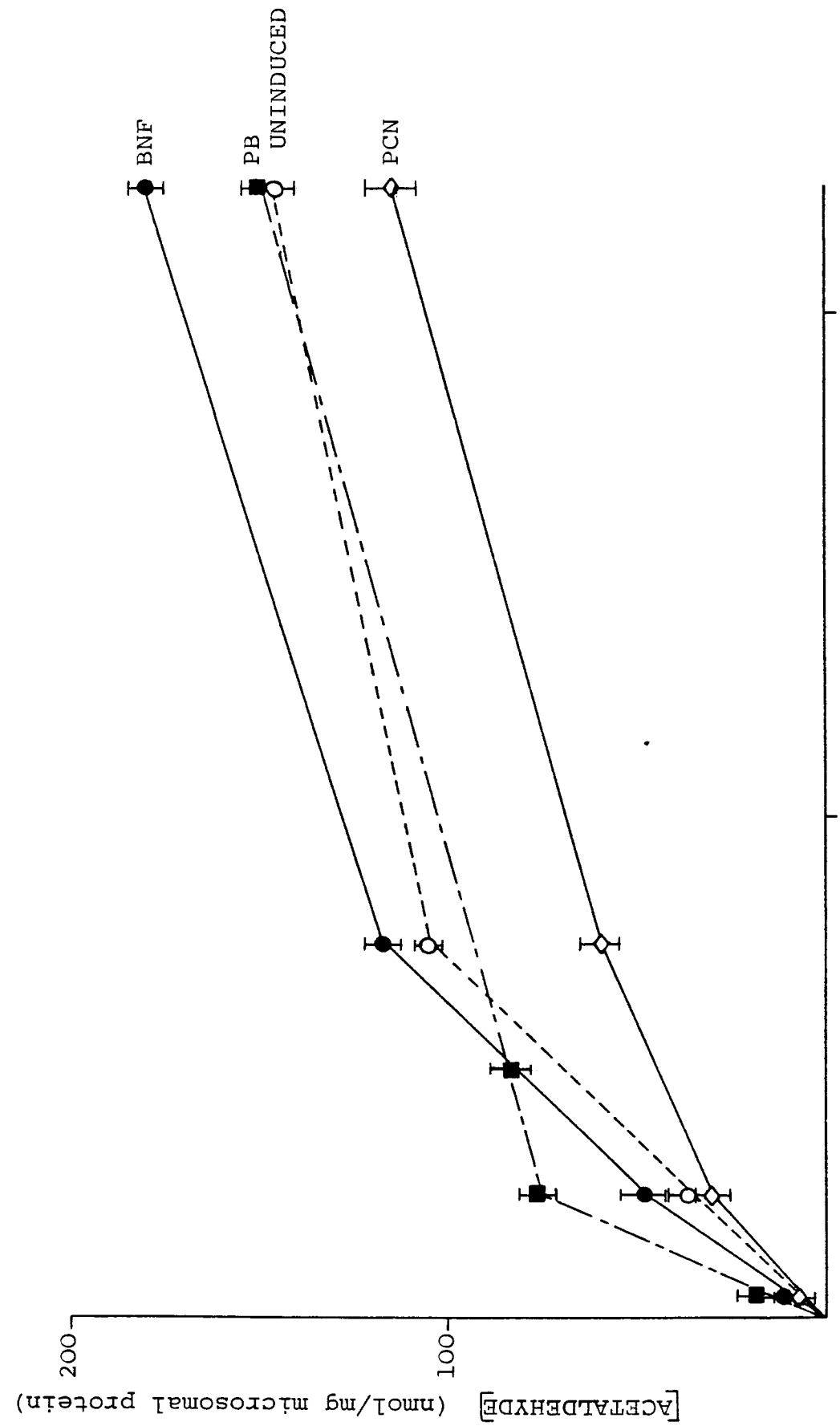

Figure 24. The production of acetaldehyde from lidocaine by hepatic microsomes from untreated and pretreated rats as a function of time.

phenobarbital and from uninduced rats (Figures 25, 27 and 28). Two distinct values of K_m, representing a high and a low affinity site were calculated and did not differ significantly following pretreatment with phenobarbital or pregnenolone-16α-carbonitrile relative to the values calculated for microsomes from uninduced rats (Table 6). Pretreatment of rats with β-naphthoflavone led to a monophasic Hanes plot for hepatic microsomal metabolism of lidocaine to MEGX (Figure 26) with the value of K_m slightly, but significantly higher than the high affinity value observed with other types of inducing agent (Table 6).

Pregnenolone-16α-carbonitrile pretreatment of rats caused a significant decrease in the value of V_{max}/nmol cytochrome P-450 for the low affinity site relative to microsomes from uninduced rats. Pretreatment of rats with β-naphthoflavone or phenobarbital did not appear to significantly alter the values of V_{max} or V_{max}/nmol cytochrome P-450 relative to microsomes from uninduced rats (Table 6).

8.2 Production of acetaldehyde

Biphasic Hanes plots for the production of acetaldehyde from lidocaine by hepatic microsomal suspensions, were obtained for all types of induction (Figures 29-32). Pretreatment of rats with β-naphthoflavone and phenobarbital led to a significant decrease in the value of K_m for the low affinity site when compared to microsomes from uninduced rats (Table 7). In addition pretreatment of rats with β-naphthoflavone led to a significant decrease in the value of K_m for the high affinity site relative

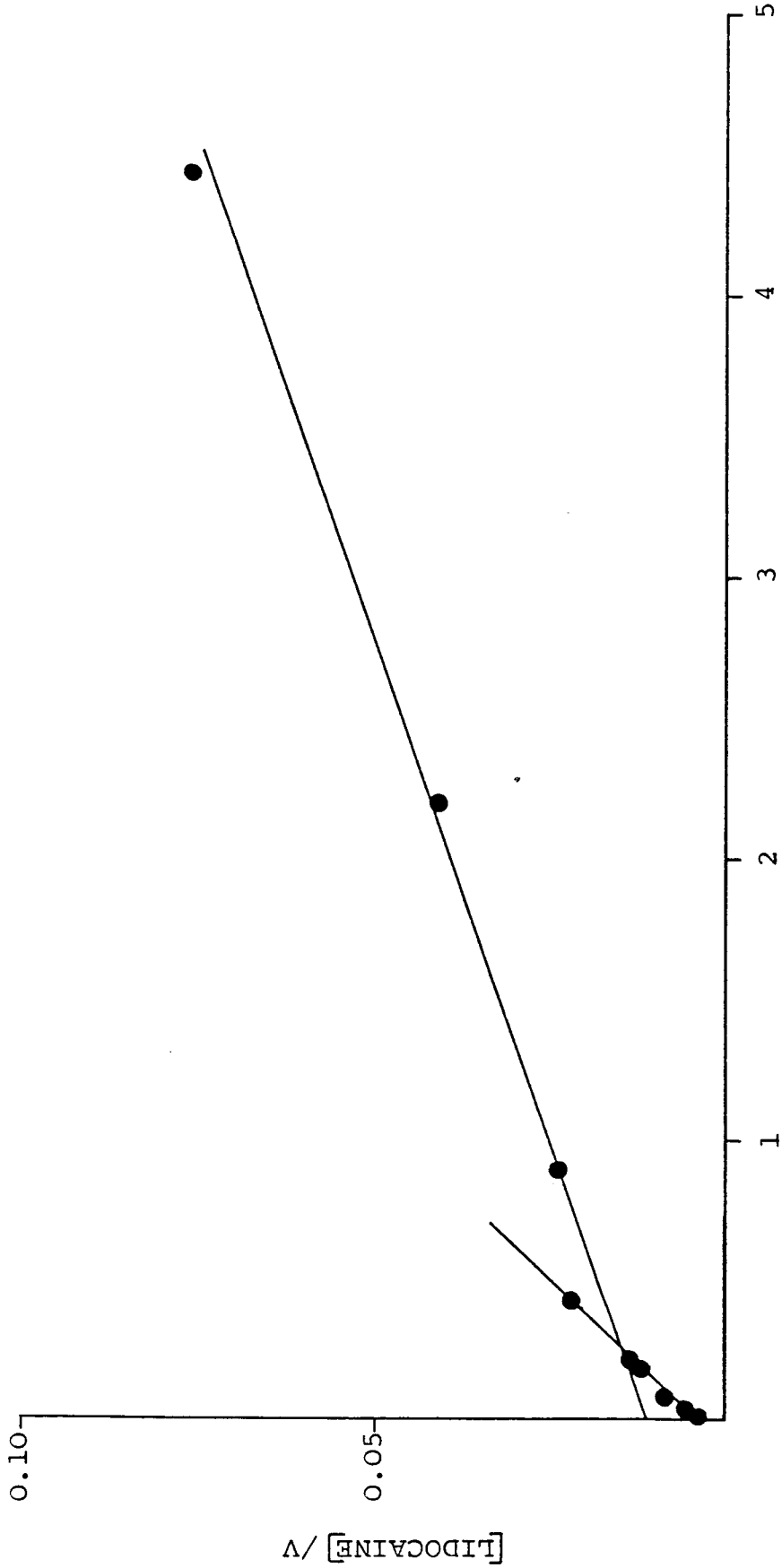

Figure 25. Hanes plot for the production of MEGX from lidocaine by cytochrome P-450 in hepatic microsomes from uninduced rats.
[LIDOCAINE], mM; V, nmol MEGX produced/min/nmol cytochrome P-450

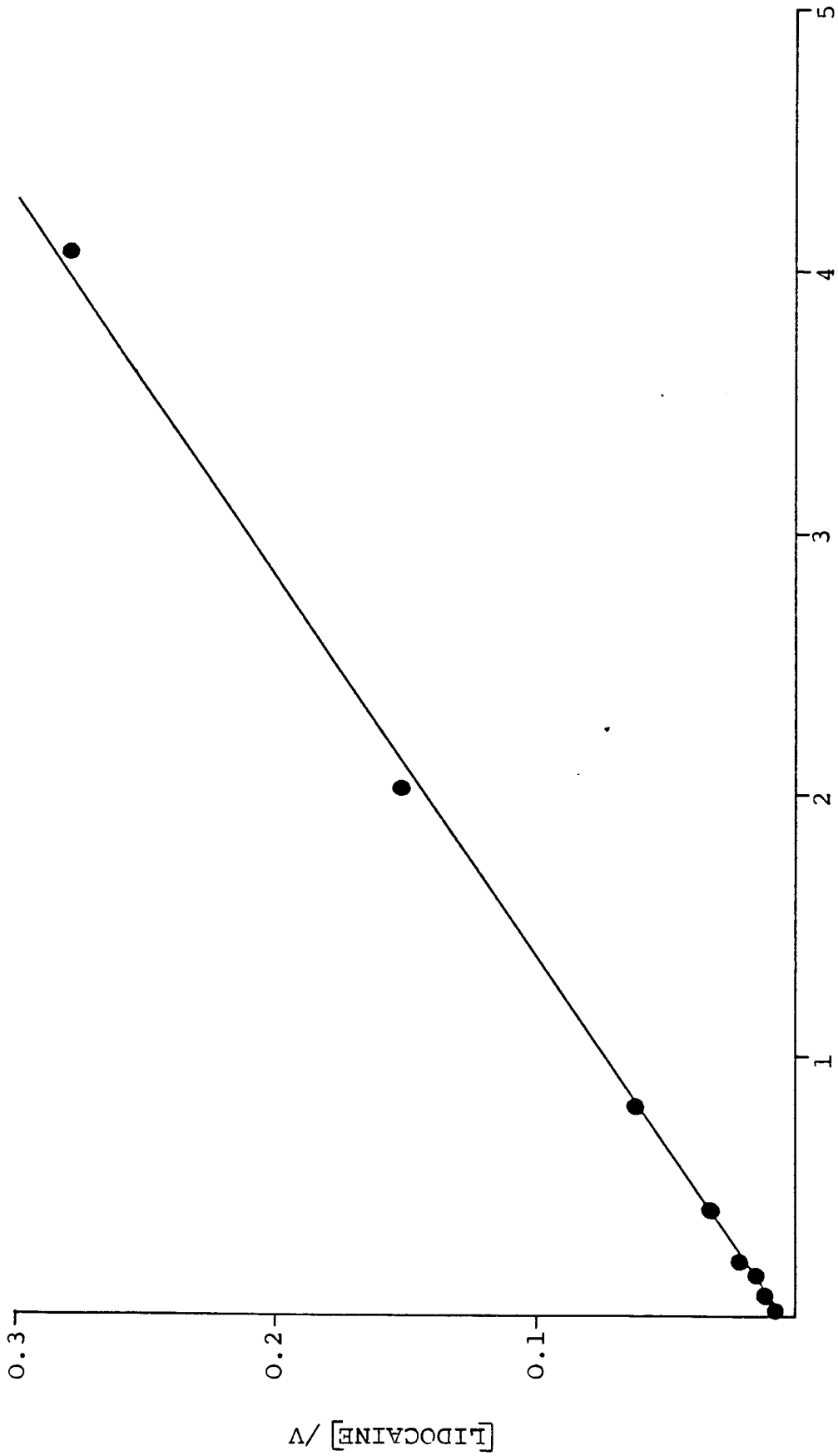

Figure 26. Hanes plot for the production of MEGX from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with β-naphthoflavone.
[LIDOCAINE], mM; V, nmol MEGX produced/min/nmol cytochrome P-450

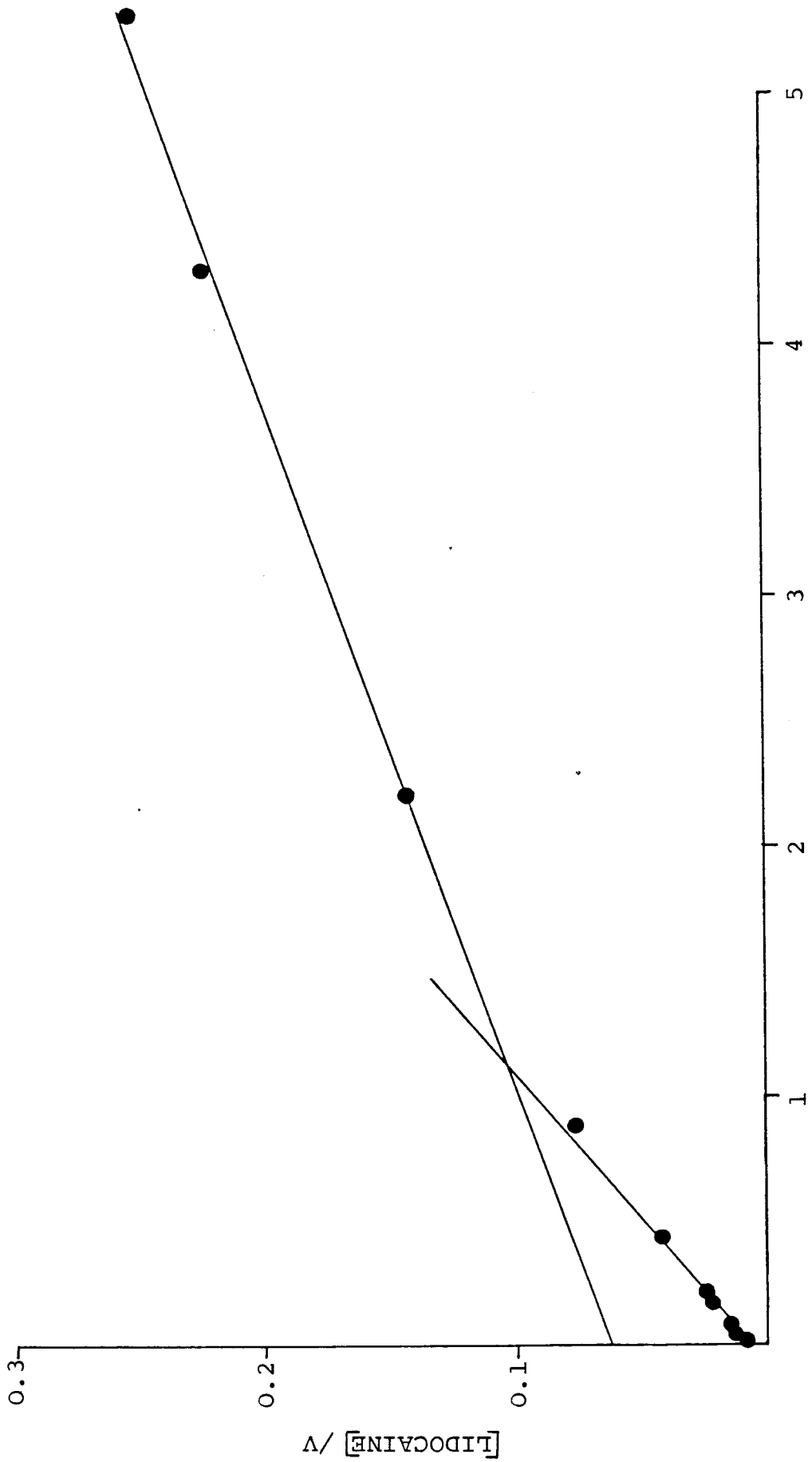

Figure 27. Hanes plot for the production of MEGX from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with pregnenolone-16α-carbonitrile. [LIDOCAINE], mM; V, nmol MEGX produced/min/nmol cytochrome P-450

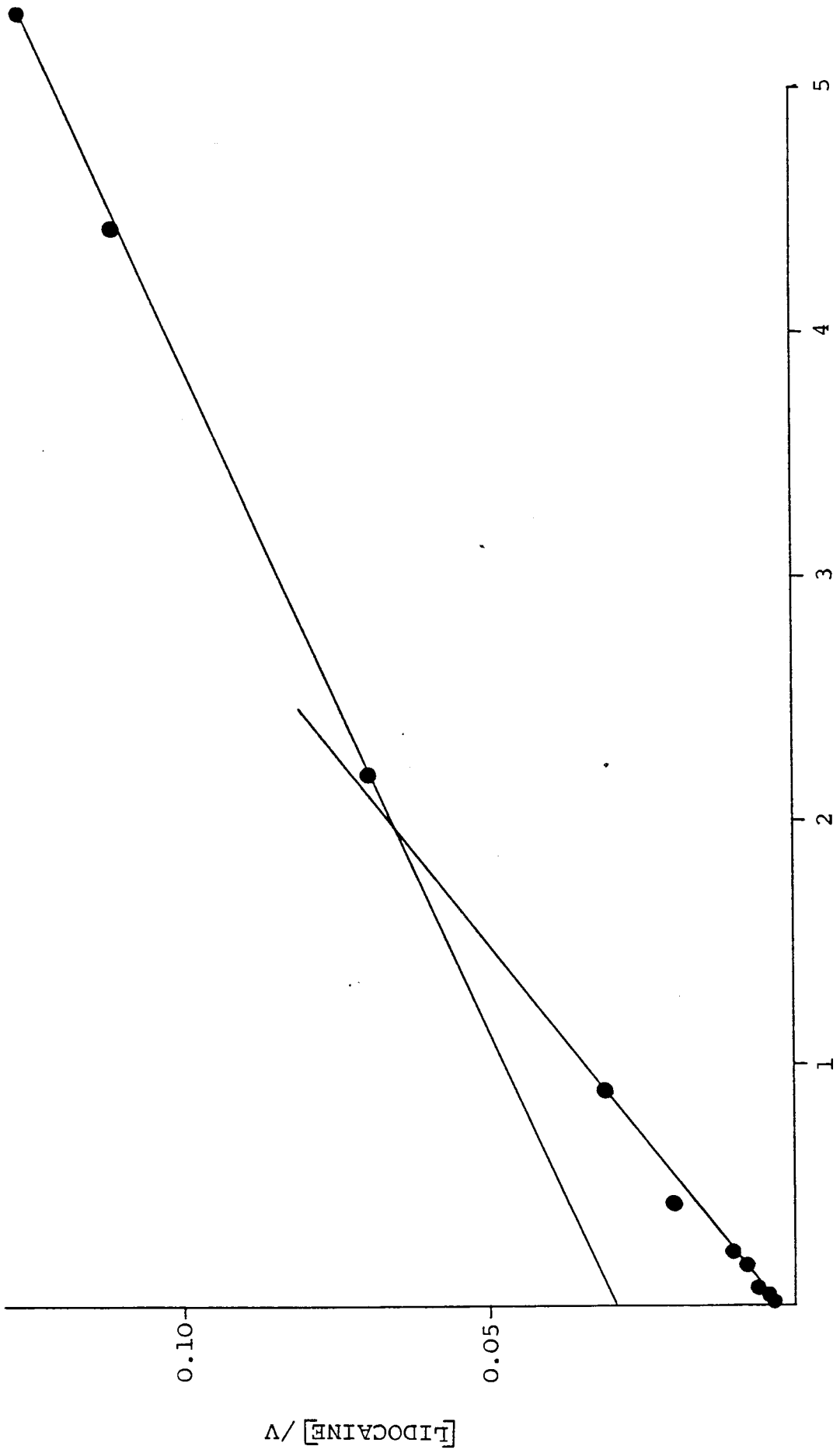

Figure 28. Hanes plot for the production of MEGX from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with phenobarbital.
[LIDOCAINE], mM; V, nmol MEGX produced/min/nmol cytochrome P-450

TABLE 6. Effect of induction of different forms of cytochrome P-450 on the metabolism of lidocaine by microsomal cytochrome P-450. A] MEGX production.

INDUCTION	K_m (mM)		V_{max}			
			nmol/min/mg microsomal protein		nmol/min/nmol cytochrome P-450	
	low affinity site	high affinity site	low affinity site	high affinity site	low affinity site	high affinity site
NONE	1.32 ± 0.76	0.090 ± 0.001	71.1 ± 13.5	23.5 ± 4.9	68.6 ± 11.9	23.1 ± 5.4
BNF	-	0.120 ± 0.001*	-	21.3 ± 3.1	-	17.5 ± 1.5
PCN	1.18 ± 0.60	0.070 ± 0.026	36.8 ± 3.5*	19.0 ± 8.1	25.5 ± 0.2*	13.1 ± 4.2†
PB	1.45 ± 0.21	0.057 ± 0.015	79.1 ± 46.1	37.1 ± 21.5	36.7 ± 26.4	20.3 ± 11.5

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

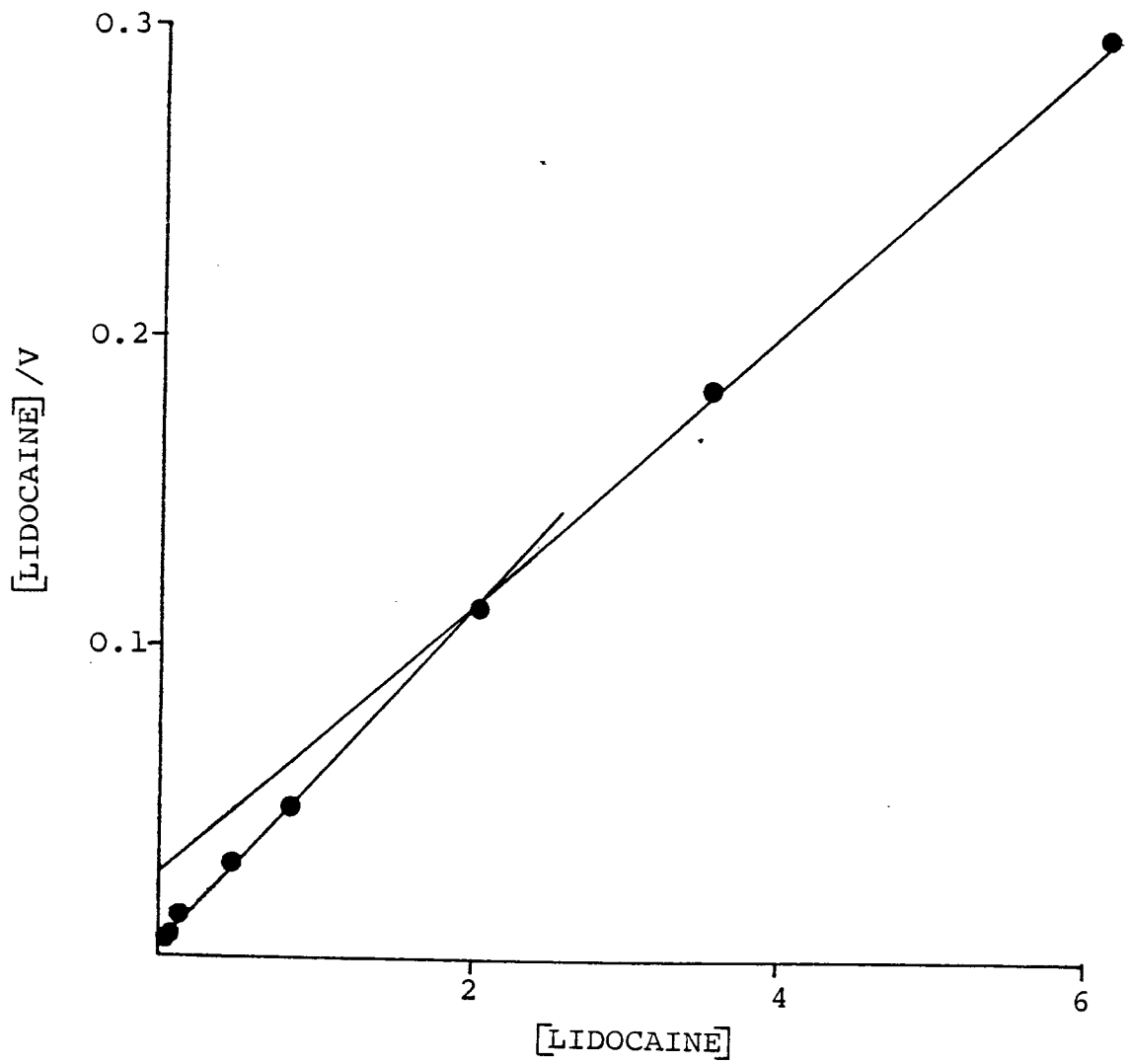

Figure 29. Hanes plot for the production of acetaldehyde from lidocaine by cytochrome P-450 in hepatic microsomes from untreated rats.

[LIDOCAINE], mM; V, nmol acetaldehyde produced/min/nmol cytochrome P-450

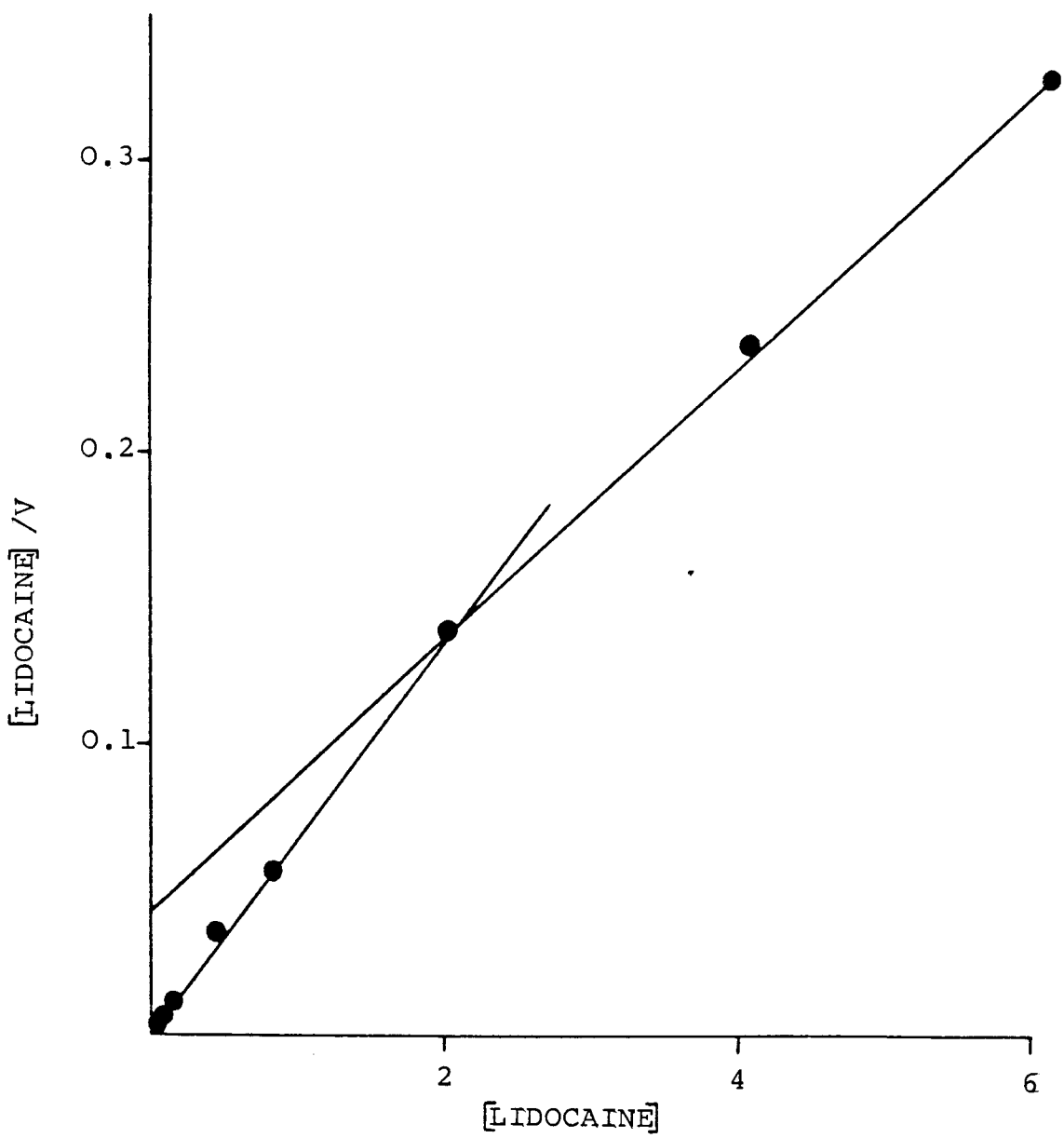

Figure 30. Hanes plot for the production of acetaldehyde from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with β-naphthoflavone.
[LIDOCAINE], mM; V, nmol acetaldehyde produced/min/nmol cytochrome P-450

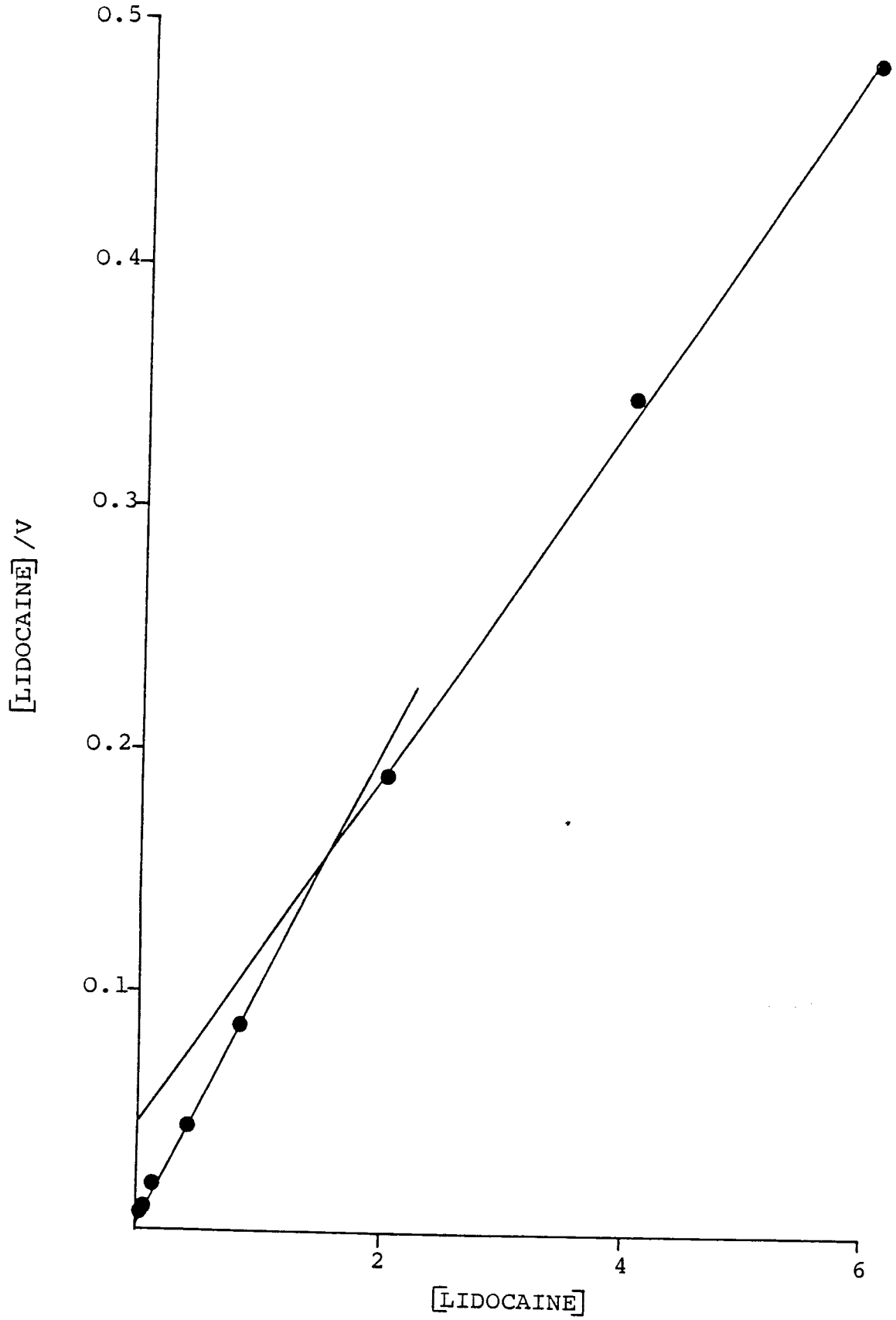

Figure 31. Hanes plot for the production of acetaldehyde from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with pregnenolone-16α-carbonitrile.
[LIDOCAINE], mM; V, nmol acetaldehyde produced/min/nmol cytochrome P-450

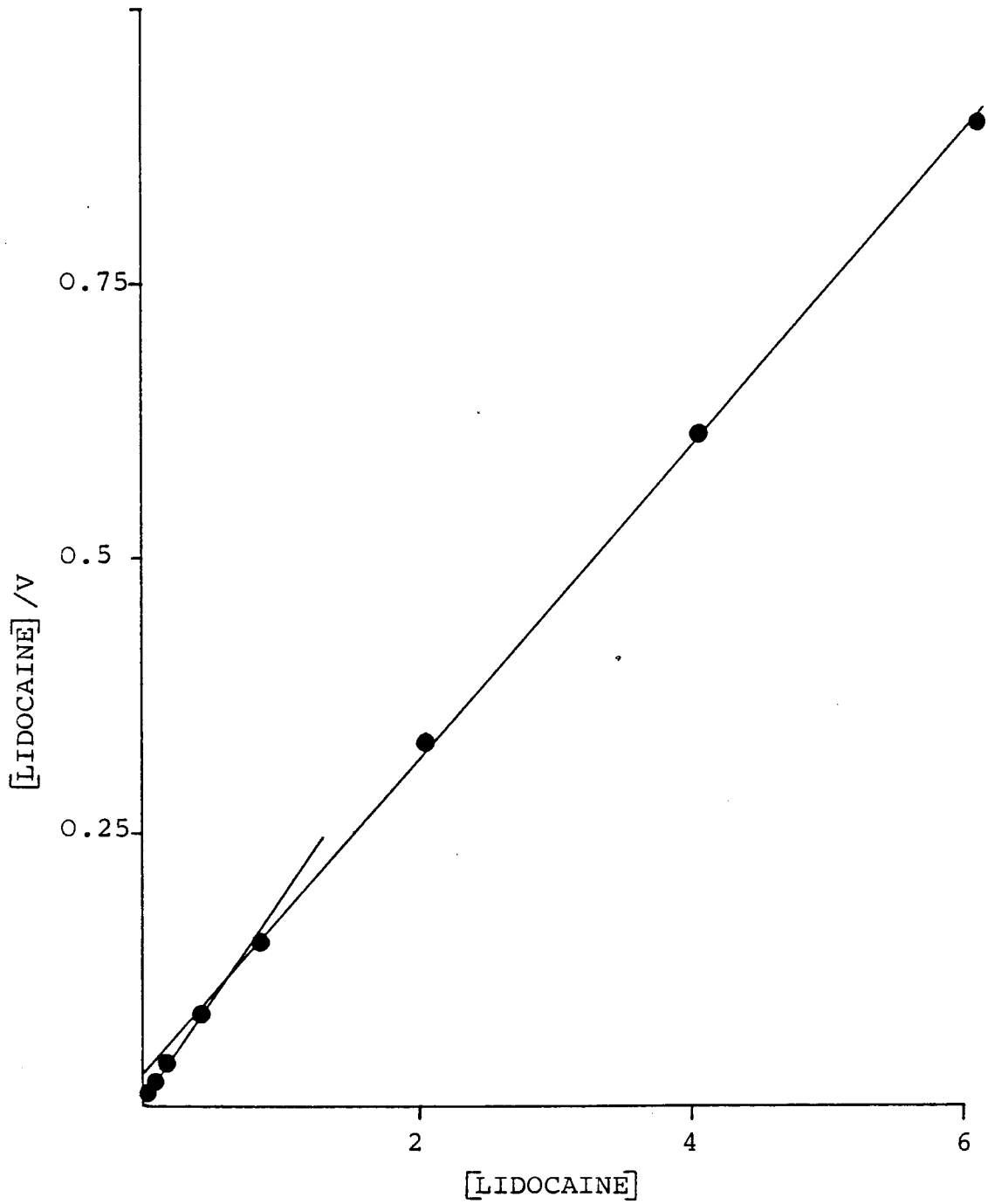

Figure 32. Hanes plot for the production of acetaldehyde from lidocaine by cytochrome P-450 in hepatic microsomes from rats pretreated with phenobarbital.
[LIDOCAINE], mM; V, nmol acetaldehyde produced/min/nmol cytochrome P-450

to microsomes from uninduced rats (Table 7).

The values of V_{max}/mg microsomal protein and V_{max}/nmol cytochrome P-450 remained unchanged following induction, except in the case of the high affinity site following pretreatment of rats with phenobarbital, where the value of V_{max}/nmol cytochrome P-450 was significantly decreased (Table 7).

8.3 Production of other lidocaine metabolites

Attempts to identify other metabolites of lidocaine in incubation mixtures containing microsomal suspension (3.0 ml), NADPH-generating system and EDTA and lidocaine (1.0 mM) proved unsuccessful except in the case of 3-hydroxy MEGX (limit of detection, 37 nmol/ml) which was produced in trace amounts (<38 nmol/ml). GX (limit of detection, 0.2 μmol/ml) and 4-hydroxyxylidine (limit of detection, 23 nmol/ml) were not found in measurable amounts (see Experimental Section 6.3).

9 Metabolism of lidocaine by partially purified cytochrome P-450

Incubation of lidocaine (1 mM), partially purified cytochrome P-450 from phenobarbital pretreated rats (2 μM) and H_2O_2 (10 mM), resulted in appreciable production of MEGX, relative to zero time samples (16.4 nmol/nmol cytochrome P-450). The conversion of lidocaine to MEGX by partially purified cytochrome P-450 was not supported by $NaClO_2$, $NaIO_4$, or $C_7H_5ClO_3$ (limit of detection, 7.2 nmol MEGX/ml).

TABLE 7. Effect of induction of different forms of cytochrome P-450 on the metabolism of lidocaine by hepatic microsomal cytochrome P-450. B] Acetaldehyde production.

INDUCTION	K_m (mM)		V_{max}			
			nmol/min/mg microsomal protein		nmol/min/nmol cytochrome P-450	
	low affinity site	high affinity site	low affinity site	high affinity site	low affinity site	high affinity site
NONE	0.64 ± 0.16	0.039 ± 0.002	21.3 ± 4.7	13.8 ± 3.8	28.2 ± 11.8	16.1 ± 3.9
BNF	0.33 ± 0.01*	0.025 ± 0.007*	20.0 ± 3.6	10.5 ± 0.1	20.8 ± 2.3	10.9 ± 0.6
PCN	0.61 ± 0.16	0.047 ± 0.006	23.4 ± 1.7	16.5 ± 3.4	15.8 ± 0.7	10.5 ± 2.8
PB	0.29 ± 0.12*	0.053 ± 0.015	31.4 ± 4.3†	15.8 ± 2.8	15.4 ± 1.3	7.7 ± 1.6*

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

Incubation of lidocaine (1 mM), partially purified cytochrome P-450 (2 μM) and the artificial electron donors; H_2O_2, $NaIO_4$, $NaClO_2$ or $C_7H_5ClO_3$ (concentrations given in Experimental Section 8) did not result in the formation of acetaldehyde (limit of detection, 5.4 nmol acetaldehyde/ml) following 10 min incubation at 37°. Incubation for 10 min of the artificial electron donors, mentioned above, with acetaldehyde (0.45 mM) did not result in significant decreases in the levels of acetaldehyde.

Zero time samples comprised as above did not produce measurable amounts of MEGX or acetaldehyde for any type of artificial electron donor used.

10 Inhibition of the interaction of lidocaine with hepatic microsomal cytochrome P-450

The effect of inhibitors of cytochrome P-450 on the binding and metabolism of lidocaine in hepatic microsomes from rats pretreated or not to increase levels of cytochrome P-450 is given in Tables 8 and 9. Metyrapone was an effective inhibitor of the binding of lidocaine to hepatic microsomal cytochrome P-450 and of the ability of lidocaine to stimulate CO-inhibitable hepatic microsomal NADPH oxidation (Table 8) ($P < 0.01$).

Metyrapone (2.33 mM) inhibited the binding of lidocaine to cytochrome P-450 and the stimulation by lidocaine of CO-inhibitable NADPH oxidation more effectively in microsomes from rats pretreated with pregnenolone-16α-carbonitrile and phenobarbital than in microsomes

from uninduced rats and rats pretreated with β-naphthoflavone (Table 8).

$CO:O_2$ (80:20 v/v), metyrapone (2.33 mM) and SKF 525-A (200 mM) significantly inhibited the conversion of lidocaine to MEGX and acetaldehyde by hepatic microsomes from uninduced and pretreated rats ($P < 0.01$) (Table 9). For the production of MEGX and acetaldehyde, the effectiveness of the inhibitors was in the order of metyrapone > $CO:O_2 \geq$ SKF 525-A. In general, the production of MEGX from lidocaine by hepatic microsomal cytochrome P-450 was more sensitive to inhibitors than was the production of acetaldehyde.

Metyrapone (2.33 mM) and $CO:O_2$ (80:20 v/v) were significantly ($P < 0.01$) more effective inhibitors of MEGX production in microsomes from rats pretreated with β-naphthoflavone, pregnenolone-16α-carbonitrile or phenobarbital than with microsomes from uninduced rats. The extent of inhibition by SKF 525-A of the production of MEGX was equivalent in microsomes from untreated and phenobarbital, β-naphthoflavone and pregnenolone-16α-carbonitrile pretreated rats ($P > 0.05$) (Table 9).

Pretreatment of rats with β-naphthoflavone and pregnenolone-16α-carbonitrile did not alter the effectiveness of $CO:O_2$ (80:20 v/v) or metyrapone as inhibitors of the production of acetaldehyde. In contrast, phenobarbital pretreatment resulted in significantly increased ($P < 0.01$) effectiveness of $CO:O_2$

TABLE 8. Effect of inhibitors of cytochrome P-450 on the binding and NADPH oxidation of lidocaine.

INDUCTION	% INHIBITION OF BINDING	% INHIBITION OF CO-INHIBITABLE NADPH OXIDATION
INHIBITOR →	MET (2.33 mM)	MET (2.33 mM)
NONE	76.0 ± 6.5	64.5 ± 1.6
BNF	63.0 ± 20.8	58.7 ± 11.5
PCN	95.3 ± 1.3*	70.8 ± 6.0†
PB	94.5 ± 0.6*	76.3 ± 2.2*

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

TABLE 9. Effect of inhibitors of cytochrome P-450 on the metabolism of lidocaine.

INDUCTION	% INHIBITION OF					
	MEGX PRODUCTION			ACETALDEHYDE PRODUCTION		
	$CO:O_2$ (80:20 v/v)	MET (2.33 mM)	SKF 525-A (200 mM)	$CO:O_2$ (80:20 v/v)	MET (2.33 mM)	SKF 525-A (200 mM)
NONE	49 ± 2	88 ± 1	48 ± 15	38 ± 12	62 ± 5	37 ± 8
BNF	61 ± 4*	97 ± 2*	66 ± 9†	50 ± 9	67 ± 4	46 ± 3†
PCN	80 ± 7*	94 ± 1*	64 ± 17	47 ± 11	68 ± 6	21 ± 10†
PB	76 ± 8*	96 ± 1*	61 ± 6	67 ± 4*	84 ± 1*	31 ± 14

* Differs from corresponding value for microsomes from uninduced rats $P < 0.01$

† Probably differs from corresponding value for microsomes from uninduced rats $P < 0.05$

(80:20 v/v) and metyrapone (2.33 mM) as inhibitors of acetaldehyde production. The extent of inhibition of acetaldehyde production by SKF 525-A was not affected by any type of pretreatment of the rats ($P > 0.05$).

11 The effects of lidocaine treatment on hepatic microsomal components in vitro

Lidocaine (0.76 mM or 0.06 mM, final concentration) does not affect the levels of cytochrome P-450, cytochrome *b*5 or microsomal haem, or the activity of NADPH-cytochrome *c* reductase *in vitro* (Tables 10 and 11).

The levels of cytochrome P-450, cytochrome *b*5, microsomal haem and the activity of NADPH-cytochrome *c* reductase in microsomes from uninduced rats and rats pretreated with β-naphthoflavone, pregnenolone-16α-carbonitrile or phenobarbital were not affected by either concentration of lidocaine (Tables 10 and 11).

12 Studies using isolated rat hepatocytes

Hepatocytes (10^6 cells /ml) were incubated at 37° without shaking with varying concentrations of lidocaine (0.1 mM, 1.0 mM and 10 mM, final concentration) and cell viability was assessed as a function of time (Figure 33). The cell viability in the absence of lidocaine remained constant for ca. 20 min, thereafter a striking decrease in viability was observed. Over the time period investigated, lidocaine (0.1 mM, final concentration) did not significantly affect cell viability

TABLE 10. The effect of 0.06 mM lidocaine on hepatic microsomal enzymes and haem.

Induction	Lidocaine	[cytochrome P-450] (nmol/mg microsomal protein)	[cytochrome b_5] (nmol/mg microsomal protein)	NADPH-cytochrome c reductase (U/mg microsomal protein)	[Haem] (nmol/mg microsomal protein)
NONE	NONE	0.82 ± 0.04	0.20 ± 0.02	0.054 ± 0.012	1.51 ± 0.03
NONE	0.06 mM	0.87 ± 0.05	0.26 ± 0.03	0.047 ± 0.003	1.44 ± 0.07
BNF	NONE	1.48 ± 0.36	0.30 ± 0.08	0.034 ± 0.003	2.50 ± 0.71
BNF	0.06 mM	1.39 ± 0.40	0.40 ± 0.12	0.043 ± 0.006	2.49 ± 0.70
PCN	NONE	1.62 ± 0.19	0.19 ± 0.01	0.049 ± 0.001	2.41 ± 0.24
PCN	0.06 mM	1.58 ± 0.21	0.25 ± 0.01	0.053 ± 0.002	2.29 ± 0.33
PB	NONE	2.87 ± 0.15	0.22 ± 0.03	0.110 ± 0.001	3.94 ± 0.15
PB	0.06 mM	2.84 ± 0.18	0.33 ± 0.05	0.110 ± 0.002	3.70 ± 0.28

TABLE 11. The effect of 0.76 mM lidocaine on hepatic microsomal enzymes and haem.

Induction	Lidocaine	[cytochrome P-450] (nmol/mg microsomal protein)	[cytochrome b_5] (nmol/mg microsomal protein)	NADPH-cytochrome c reductase (U/mg microsomal protein)	[Haem] (nmol/mg microsomal protein)
NONE	NONE	0.82 ± 0.04	0.20 ± 0.02	0.054 ± 0.012	1.51 ± 0.03
NONE	0.76 mM	0.91 ± 0.05	0.25 ± 0.02	0.049 ± 0.004	1.48 ± 0.10
BNF	NONE	1.48 ± 0.36	0.30 ± 0.08	0.034 ± 0.003	2.50 ± 0.71
BNF	0.76 mM	1.49 ± 0.35	0.42 ± 0.12	0.039 ± 0.002	2.40 ± 0.61
PCN	NONE	1.62 ± 0.19	0.19 ± 0.01	0.049 ± 0.001	2.41 ± 0.24
PCN	0.76 mM	1.57 ± 0.13	0.21 ± 0.01	0.052 ± 0.001	2.26 ± 0.29
PB	NONE	2.87 ± 0.15	0.22 ± 0.03	0.110 ± 0.001	3.94 ± 0.15
PB	0.76 mM	2.95 ± 0.09	0.31 ± 0.06	0.114 ± 0.002	3.40 ± 0.32

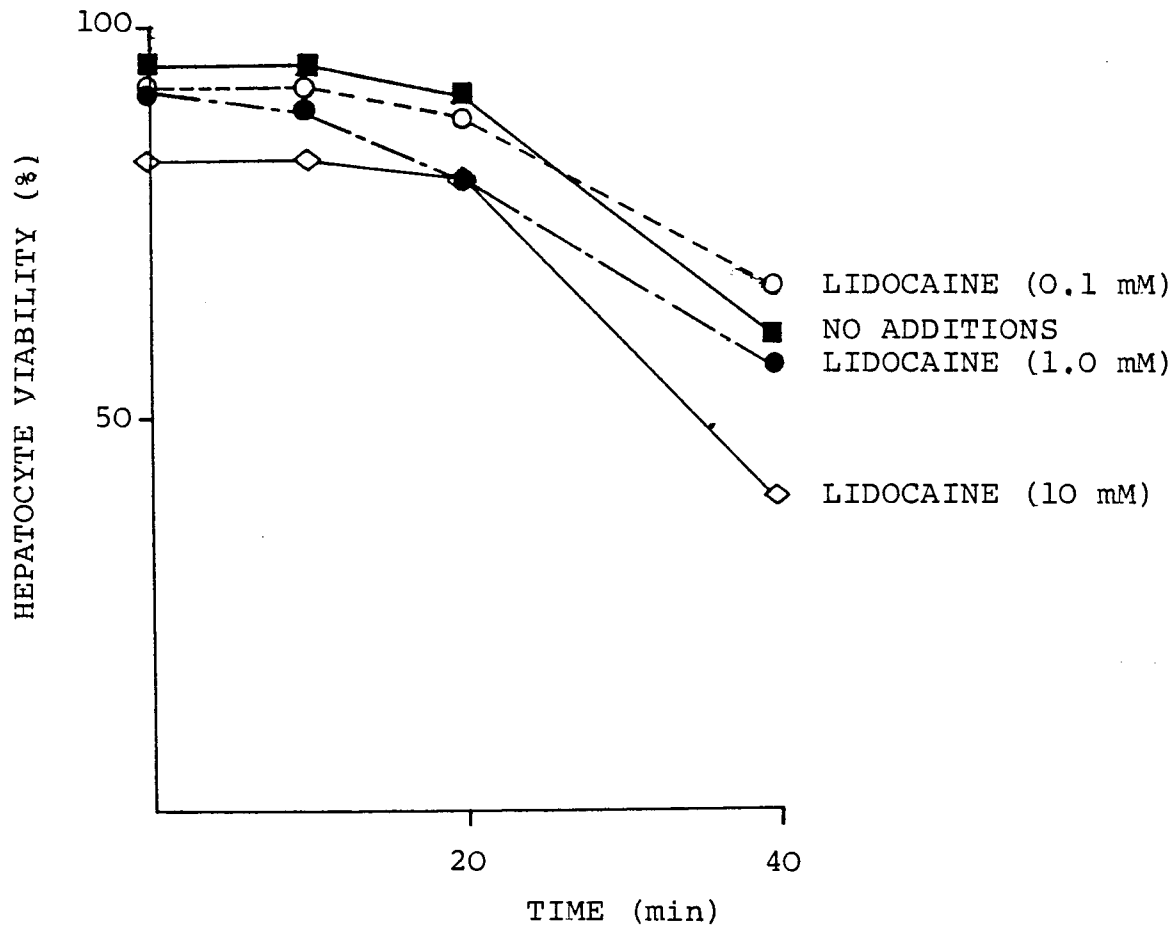

Figure 33. Hepatocyte viability in the presence of varying concentrations of lidocaine as a function of time.

relative to that seen in the absence of lidocaine. Lidocaine (1 mM, final concentration) slightly decreased cell viability between 10 min and 40 min. In the presence of lidocaine (10 mM, final concentration) an initial drop in viability was observed and was maintained throughout the experiment.

The production of MEGX from lidocaine in the presence of hepatocytes was investigated as a function of time (Figure 34). Hepatocytes isolated from uninduced rats converted lidocaine (0.1 mM, final concentration or 1.0 mM, final concentration) to MEGX: the reaction was linear for up to 30 min (Figure 34). Measurable levels of MEGX were not produced in the absence of lidocaine.

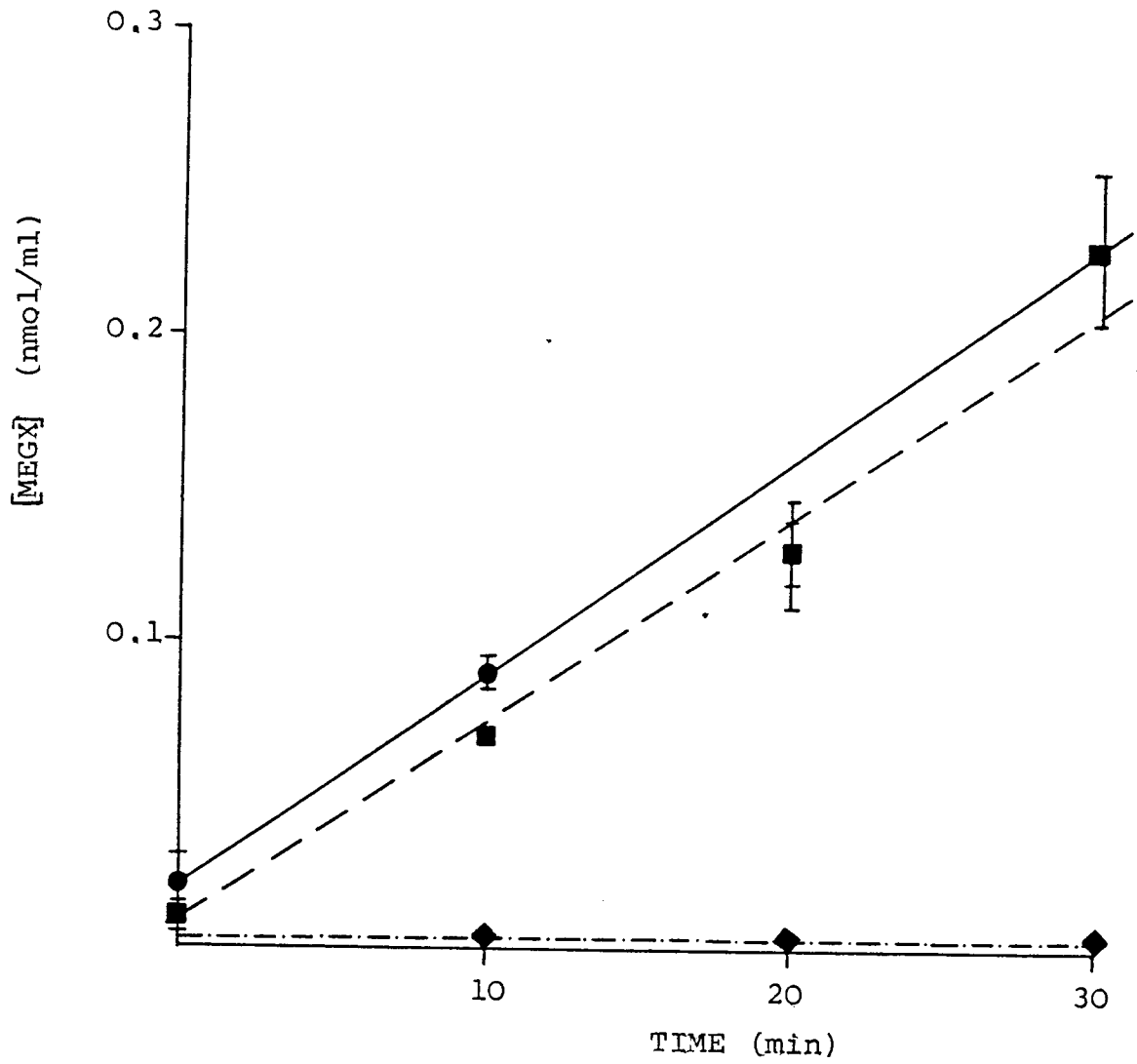

Figure 34. MEGX production by hepatocytes from uninduced rats in the presence of lidocaine (1.0 mM -●-) and (0.1 mM -■-) and its absence (—·◆·—) as a function of time.

IV DISCUSSION

The results reported herein confirm that lidocaine binds to hepatic microsomal cytochrome P-450 and is metabolized by the hepatic microsomal cytochrome P-450 enzyme system. As reported earlier by Von Bahr et al (69) (A) lidocaine is shown to bind to rat hepatic microsomal cytochrome P-450 with the production of a type I spectral change, which is characteristic of substrates for this enzyme (29) and (B) the binding of lidocaine to cytochrome P-450 is characterized by two distinct binding constants (Table 1) (Figure 6).

That lidocaine is a substrate for cytochrome P-450 is further supported by the observation that lidocaine stimulates the CO-inhibitable oxidation of NADPH by hepatic microsomes (Table 4), which is a measure of cytochrome P-450 dependent metabolism (see Introduction). This proposal is further supported by the observation that metyrapone, which is an effective inhibitor of cytochrome P-450 mediated reactions (10), decreases the binding of lidocaine to hepatic microsomal cytochrome P-450 (Table 8) and that several inhibitors of cytochrome P-450, viz. metyrapone, SKF 525-A and $CO:O_2$ (80:20 v/v) (10), (see Results - Section 10) all decreased the hepatic microsomal metabolism of lidocaine (Table 9).

The products of the cytochrome P-450 catalyzed metabolism of lidocaine, using hepatic microsomes, are confirmed to be MEGX and acetaldehyde, as reported earlier by Hollunger (64) and

by Heinonen (67) (Tables 6 and 7).

That lidocaine is metabolized by cytochrome P-450 is unequivocally demonstrated by the results of the studies of the interaction of lidocaine with partially purified cytochrome P-450. Partially purified cytochrome P-450 isolated from hepatic microsomes from phenobarbital pretreated rats is shown to bind lidocaine with a type I difference spectrum (see Results - Section 4) which, as mentioned above is characteristic of substrates for this enzyme. Furthermore, in the presence of the artificial electron donor H_2O_2, partially purified cytochrome P-450 was capable of metabolizing lidocaine to MEGX (see Results - Section 9).

The role of different forms of hepatic microsomal cytochrome P-450 in the binding and metabolism of lidocaine has been investigated using inducing agents such as β-naphthoflavone, phenobarbital and pregnenolone-16α-carbonitrile to elevate the levels of specific forms of cytochrome P-450.

The forms of cytochrome P-450 elevated with phenobarbital and pregnenolone-16α-carbonitrile were found to play an important role in the binding of lidocaine by cytochrome P-450. Pregnenolone-16α-carbonitrile treatment elevated the $^{\Delta}A$ max/nmol cytochrome P-450 for the low affinity binding site of cytochrome P-450 (Table 1) indicating that the form of cytochrome P-450 induced by pregnenolone-16α-carbonitrile has a definite role in the low affinity binding site for lidocaine. In contrast, phenobarbital treatment enhanced the extent of binding ($^{\Delta}A$ max/nmol

cytochrome P-450) of lidocaine to both the low and the high affinity binding sites on cytochrome P-450 indicating that the phenobarbital inducible form of cytochrome P-450 is important in the binding of lidocaine to both of these sites (Table 1).

Further evidence for the binding of lidocaine to the phenobarbital inducible form of cytochrome P-450 is provided by the observation that metyrapone, which is reported to be a preferential inhibitor of the phenobarbital inducible form of cytochrome P-450 but not to affect cytochrome P-448 (38) inhibited the binding of lidocaine to hepatic microsomal cytochrome P-450 to a significantly greater extent in microsomes from phenobarbital pretreated rats than in microsomes from untreated rats.

From the results of our studies of the inhibition of the binding of lidocaine to cytochrome P-450 (Table 8), it would appear that metyrapone is not a specific inhibitor of the phenobarbital form of cytochrome P-450: Since metyrapone inhibits the binding of lidocaine as effectively as in microsomes from pregnenolone-16α-carbonitrile treated rats as in microsomes from phenobarbital treated rats.

The form of cytochrome P-450 induced by β-naphthoflavone does not appear to be involved in the binding of lidocaine to cytochrome P-450: This proposal is supported by (A) the decrease in the value of $^{\Delta}$A max/nmol cytochrome P-450 for the high affinity binding site for lidocaine following β-naphtho-

flavone induction, (B) the elimination of the low affinity binding site for lidocaine following β-naphthoflavone induction and (C) the sensitivity of the binding of lidocaine in microsomes from β-naphthoflavone treated rats to metyrapone inhibition relative to microsomes from uninduced rats (Tables 1 and 8).

The low and high affinity binding sites for lidocaine on cytochrome P-450 could represent either two distinct binding sites on one form of cytochrome P-450 or one binding site on each of two forms of cytochrome P-450. The latter possibility would appear more likely, since a differential effect on the values of $^{\Delta}A$ max and $^{\Delta}A$ max/nmol cytochrome P-450 for the low and the high affinity binding sites is observed for the binding of lidocaine to cytochrome P-450 in microsomes from rats treated with pregnenolone-16α-carbonitrile: the values of $^{\Delta}A$ max and $^{\Delta}A$ max/nmol cytochrome P-450 for the low affinity binding site are significantly increased ($P < 0.01$) for the binding of lidocaine to cytochrome P-450 in microsomes from pregnenolone-16α-carbonitrile treated rats (Table 1), whereas the values of $^{\Delta}A$ max and $^{\Delta}A$ max/nmol cytochrome P-450 for the high affinity binding site are not significantly increased following pregnenolone-16α-carbonitrile pretreatment of rats relative to uninduced rats.

Different conclusions regarding the role of different forms of cytochrome P-450 in the metabolism of lidocaine would appear to be drawn depending on the method which was used to assess the metabolism of lidocaine. For example, effects of

inducing agents for different forms of cytochrome P-450 on the ability of lidocaine to stimulate CO-inhibitable hepatic microsomal NADPH oxidation - which is a facile way of measuring the total metabolism of a substrate by cytochrome P-450 (see Introduction) - suggest that the form of cytochrome P-450 induced by phenobarbital, but not the forms elevated by β-naphthoflavone or pregnenolone-16α-carbonitrile, appears to play a predominant role in the metabolism of lidocaine: viz. the induction of the phenobarbital inducible form of cytochrome P-450 resulted in a significant stimulation of CO-inhibitable NADPH oxidation by lidocaine, while elevation of the β-naphthoflavone or pregnenolone-16α-carbonitrile inducible forms of cytochrome P-450 resulted in decreased rates of lidocaine metabolism as assessed by CO-inhibitable oxidation of NADPH (Table 4). In addition, metyrapone which is reported to inhibit the form of cytochrome P-450 elevated by phenobarbital, but not cytochrome P-448 (38), is a more effective inhibitor of lidocaine stimulated NADPH oxidation in microsomes from phenobarbital treated rats than from uninduced rats ($P < 0.01$) (Table 8).

In contrast, direct measurement of the effects of the inducing agents for different forms of cytochrome P-450 on the conversion of lidocaine to MEGX and acetaldehyde, suggest that no single form of cytochrome P-450 preferentially metabolizes lidocaine. Firstly, none of the inducing agents used in this investigation, namely β-naphthoflavone, pregnenolone-16α-carbonitrile or phenobarbital significantly increased the values of V_{max}/nmol cytochrome P-450 relative to the values observed in microsomes from uninduced rats (Tables 6 and 7). The role of multiple

forms of cytochrome P-450 in the conversion of lidocaine to acetaldehyde and MEGX is supported by the ability of the inhibitors metyrapone and SKF 525-A which are reported to preferentially inhibit the form of cytochrome P-450 elevated by phenobarbital and the form of cytochrome P-450 elevated by β-naphthoflavone (cytochrome P-448), respectively, (38) - to inhibit the production of these two metabolites significantly in microsomes from untreated, β-naphthoflavone, pregnenolone-16α-carbonitrile or phenobarbital pretreated rats (Table 9). The far greater effectiveness of metyrapone than SKF 525-A for each type of induction suggests that the form of cytochrome P-450 elevated by phenobarbital plays a more important role than cytochrome P-448 (the form of cytochrome P-450 elevated by β-naphthoflavone) ($P < 0.01$) in the metabolism of lidocaine.

Although the interpretation of the results of MEGX and acetaldehyde production presented above are identical, it is thought that the data for MEGX production may provide a better index of the extent of metabolism of lidocaine than does acetaldehyde production. Although the K_m values for acetaldehyde production are generally similar to the K_m values for MEGX production for each type of induction (Tables 6 and 7) ($P > 0.05$), the V_{max}/nmol cytochrome P-450 values for acetaldehyde production were, for most types of induction, lower than the corresponding V_{max}/nmol cytochrome P-450 values for MEGX production (Tables 6 and 7) ($P < 0.01$ to $P < 0.05$). We interpret these results to mean that, although acetaldehyde and MEGX are produced in 1:1 stoichiometry in agreement with proposed metabolic pathway for lidocaine (Figure 3), that acetaldehyde, once formed may react

rapidly with one or more components of the microsomes or with cytochrome P-450 itself: this proposal is supported by (A) the shorter time that acetaldehyde production remains linear for each type of induction relative to the time for which MEGX production remained linear (Figures 23 and 24) and (B) the observation that partially purified cytochrome P-450 can convert lidocaine to MEGX in the presence of the artificial electron donor H_2O_2, whereas this system does not produce measurable amounts of acetaldehyde from lidocaine[1].

A discrepancy in the results of our investigation of lidocaine metabolism was that the rate of total metabolism of lidocaine as assessed by the oxidation of NADPH was a factor of 7-100 fold lower than the rate of conversion of lidocaine to MEGX and acetaldehyde. At least in part, the lower rate of NADPH oxidation reflects a relatively low concentration of lidocaine viz. a concentration of lidocaine below or equivalent to the values of K_m for the low affinity site on cytochrome P-450 (see Tables 6 and 7). However, the observed rates of oxidation of NADPH are still strikingly below the values of V_{max} for acetaldehyde or MEGX production for only the high affinity site. This discrepancy does not appear to reflect incomplete inhibition by CO of the stimulation of the oxidation of NADPH by lidocaine - since, even when reaction rates are not corrected for background rates of NADPH oxidation, the

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[1] The latter result does not reflect the reaction of acetaldehyde with artificial electron donors (see Results - Section 9).

observed rates of lidocaine stimulated NADPH oxidation i.e. those for lidocaine + NADPH + microsomal suspension are still a factor of 10-20 times lower than the rate of conversion of lidocaine to MEGX and acetaldehyde. The discrepancy is not due to too low a concentration of NADPH being present in reaction mixtures - the initial concentration of NADPH is 144 μM and during 3 min over which the reaction was followed and for which the rate was linear, 59 μM was oxidized thereby leaving over 50% of the NADPH (85 μM) remaining in the microsomal suspension at the end of the reaction. It appears that possibly some component of the NADPH generating system perhaps such as $MgCl_2$ or EDTA, may be enhancing the metabolism of lidocaine in as much as the replacement of NADPH by the NADPH generating system is the only difference in reaction mixtures for the measurement of the oxidation of NADPH versus the production of the metabolites MEGX and acetaldehyde from lidocaine. This phenomenon of the rate of NADPH oxidation being far lower than the rate of production of metabolites has not been observed in our laboratories during studies of the cytochrome P-450 dependent metabolism of numerous compounds, such as trichloroethylene (80), 1,1,1-trichloroethane (81), perchloroethylene (82) and fluorexene (83).

The results reported herein on the binding and metabolism of lidocaine, viz. the values of K_s, ΔA max, K_m and V_{max}, were generally in agreement with the published results where such were available in the literature. No data has been published reporting the effects of phenobarbital, pregnenolone-16α-carbonitrile or β-naphthoflavone induction on the binding and

metabolism of lidocaine by cytochrome P-450. The effect of sex, species and strain differences of uninduced laboratory animals on the hepatic microsomal binding and metabolism of lidocaine by cytochrome P-450 has been reported (69, 70).

The results for the binding of lidocaine to cytochrome P-450 (values of K_s and $^{\Delta}A$ max) for microsomes from uninduced rats reported herein (Table 1) were generally comparable, i.e. within a factor of 2 to 6, of reported results, except the high affinity binding constant of 2 μM reported by von Bahr et al. was 15 fold lower than our results. The value of the binding constant for the low affinity binding site reported by Nyberg et al. (7 μM) is comparable to that for our high affinity binding site (Table 1). The value of K_s for the high affinity site for lidocaine determined by Nyberg et al. (0.2 μM) was however a factor of 160 lower than the high affinity K_s value determined by our laboratory (30 μM). With the concentrations of lidocaine that were used in our laboratory, one would not expect to see the high affinity binding constant reported by Nyberg et al., and Nyberg et al. would also not expect to see our low affinity binding constant.

The differences seen between results published by von Bahr et al. and Nyberg et al. and those determined in our laboratory can to a certain extent be explained by strain differences of the experimental animals, von Bahr and Nyberg used Sprague-Dawley rats while Long-Evans rats were used in our studies. However, there is no apparent explanation for the discrepancy observed between the results of von Bahr et al. and Nyberg

et al. - who were using the identical methods and were working in the same laboratory with the same strain of rats.

The values of K_m and V_{max} for the production of MEGX from lidocaine in the presence of hepatic microsomal cytochrome P-450 reported by von Bahr et al. (69) and Nyberg et al. (70) were in some cases comparable to results obtained in this laboratory. The value of K_m for the low affinity site (0.04 mM) as calculated from the results of von Bahr et al. (see Figure 35) corresponded to the value of K_m for the high affinity site determined in this laboratory (0.09 mM), with the corresponding value of V_{max} calculated from the data reported by von Bahr et al. being 7 fold lower than reported herein (Table 6). The value of K_m (0.6 mM) reported by Nyberg et al. corresponded to our value of K_m for the low affinity site (1.3 mM) with the value of V_{max} reported by Nyberg et al. being 3 fold lower than the V_{max}/mg microsomal protein × min for microsomes from uninduced rats reported herein (Table 6).

The values of K_m and V_{max} for the high affinity site for the production of MEGX (ca. 0.6 μM and 0.05 nmol/mg protein × min) which were calculated from results of von Bahr et al. (see Figure 36) were not reported by either Nyberg et al. or found in this study. The inability to observe this high affinity site in the experiments reported herein is that the minimum concentration of lidocaine which was added to incubation mixtures (1000 μM) was far higher than the K_m (0.4 - 1.0 μM) calculated from the data reported by von Bahr et al. (69).

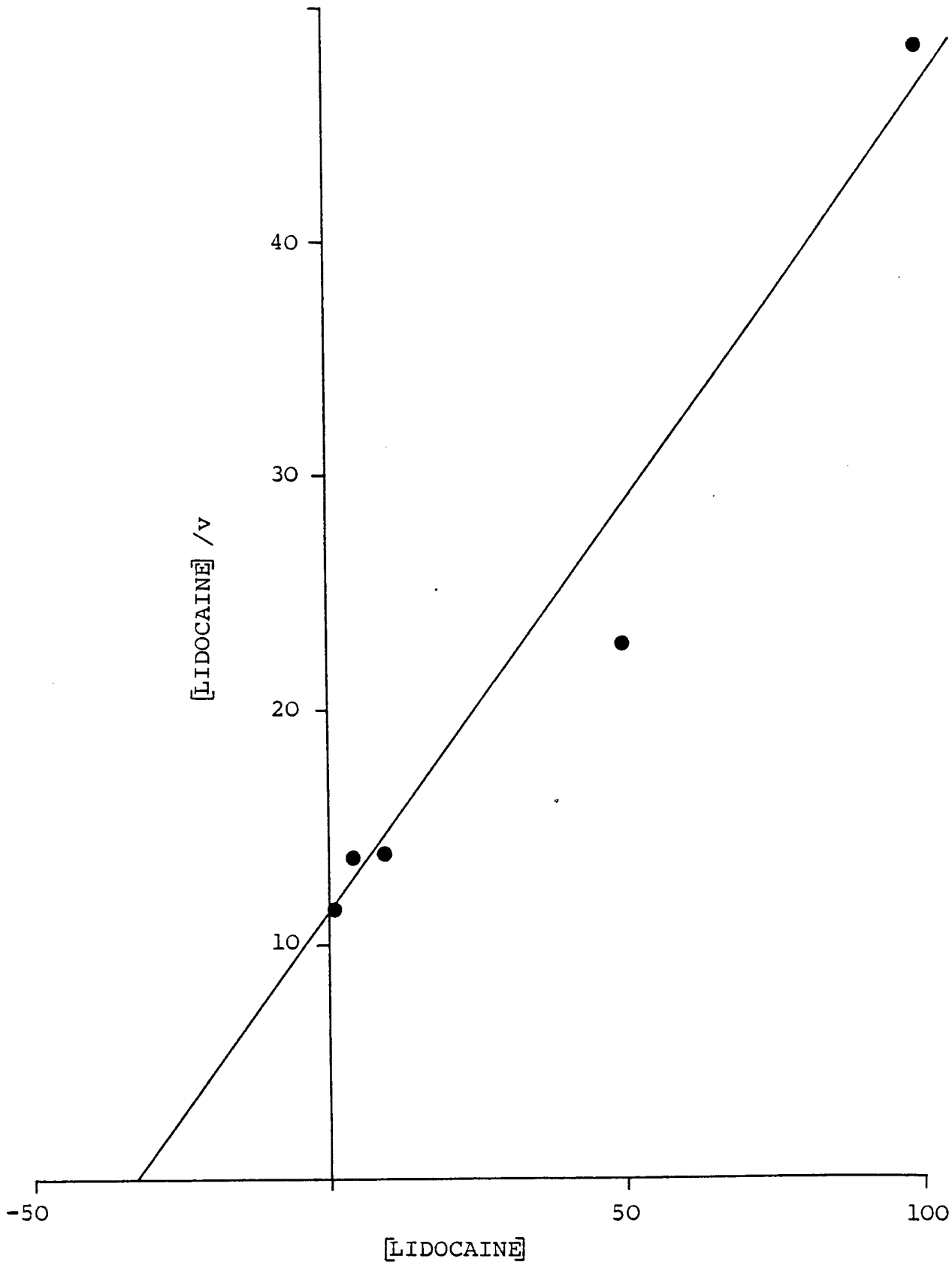

Figure 35. Hanes plot of the data of von Bahr et al. for the low affinity site for the production of MEGX from lidocaine by hepatic microsomes from untreated Sprague-Dawley rats (69).

[LIDOCAINE] , μM; V, nmol MEGX/mg protein × min.

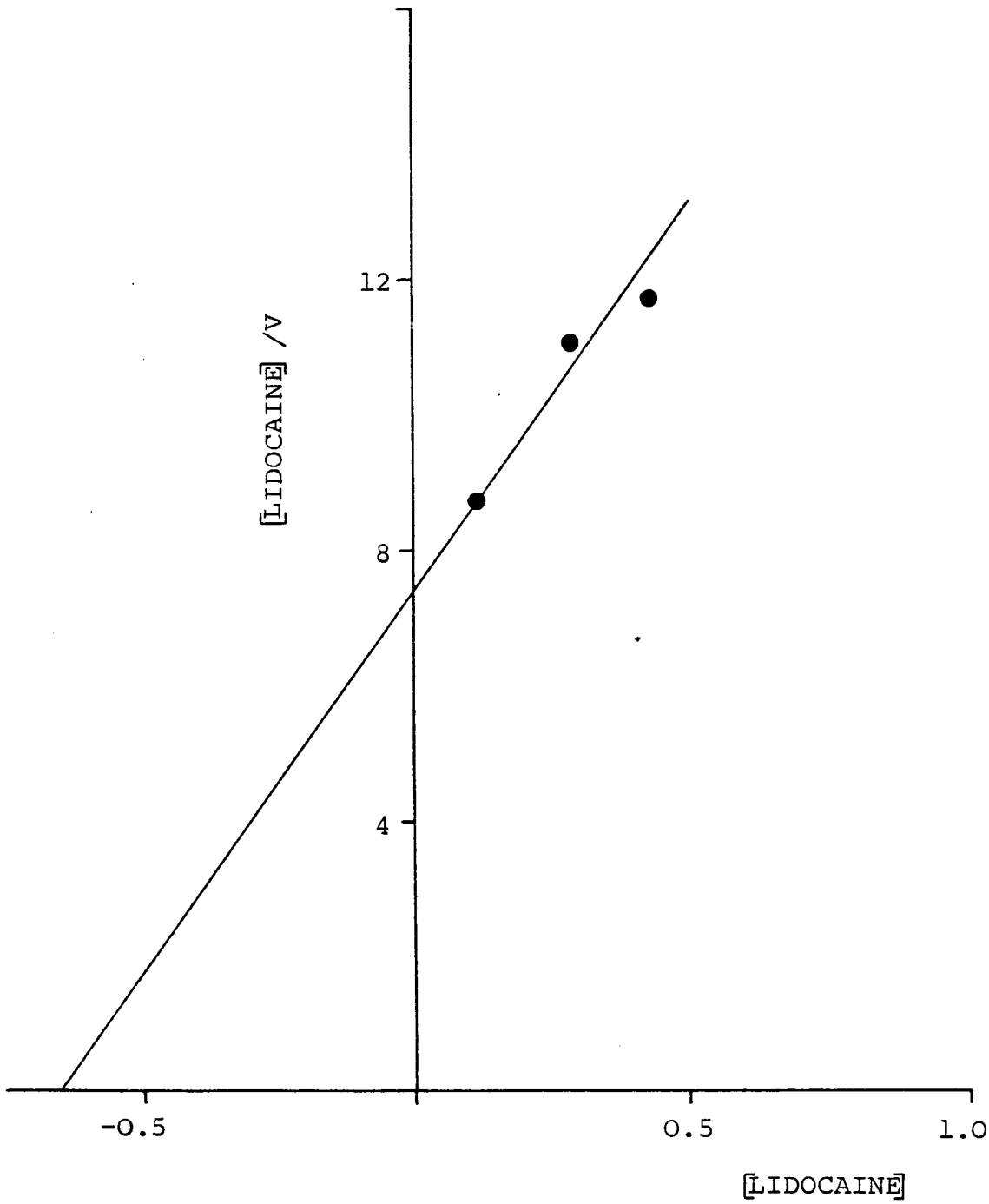

Figure 36. Hanes plot of the data of von Bahr et al. for the high affinity site for the production of MEGX from lidocaine by hepatic microsomes from untreated Sprague-Dawley rats (69).

[LIDOCAINE] , μM; V, nmol MEGX/mg protein × min.

However, Nyberg *et al.* using concentrations of lidocaine similar to those used by von Bahr *et al.* did not observe the high affinity site reported by von Bahr *et al.*

From results reported herein, the metabolism of lidocaine by cytochrome P-450 does not appear to convert lidocaine to reactive species. Lidocaine showed no effect on various microsomal components-levels of microsomal cytochrome P-450, haem and cytochrome b_5 and the activity of NADPH-cytochrome P-450 reductase (Tables 10 and 11). These results are consistent with the observations that (A) lidocaine is not toxic to cultured cells from normal tissues (84) and that (B) lidocaine is generally of low toxicity *in vivo* (47). The apparent lack of production of reactive species from the metabolism of lidocaine by hepatic microsomal cytochrome P-450 (see Results - Section 11) is consistent with the observation that lidocaine and its metabolites are not mutagenic or carcinogenic.

That cytochrome P-450 is involved in the metabolism of lidocaine *in vivo* is supported by the observation that MEGX - a product of the metabolism of lidocaine by partially purified cytochrome P-450 in the presence of electron donors is also produced from lidocaine by hepatic microsomal cytochrome P-450 (see Results - Section 9), by isolated hepatocytes (see Results - Section 12) and *in vivo* as a urinary metabolite of lidocaine in humans and rats (56, 60). The reported reduction in the rate of hepatic clearance of lidocaine in the cat in the presence of SKF 525-A an inhibitor of cytochrome P-450 (85), and the reported increase

in the rate of *in vivo* metabolism of lidocaine to MEGX in dogs following phenobarbital pretreatment (67), support a role for cytochrome P-450 in the metabolism of lidocaine *in vivo* and suggest that cytochrome P-450 may catalyze the rate limiting step in the metabolism of lidocaine.

There appear to be multiple products from the interaction of lidocaine and its metabolites with hepatic cytochrome P-450. Using hepatic microsomes, cytochrome P-450 metabolizes lidocaine to MEGX *in vitro* (69, 70). In addition, in incubation mixtures of microsomal suspension, lidocaine and NADPH generating system, lidocaine has been reported to be converted to 3-hydroxylidocaine (66) and has been shown to be converted to trace amounts of 3-hydroxy MEGX in this laboratory (see Results - Section 8.3). The low amounts of 3-hydroxy MEGX produced from lidocaine by hepatic microsomal cytochrome P-450 reported herein are to be expected since only low concentrations of the precursor MEGX (under 1 μM) are produced in reaction mixtures. In addition to the above hydroxylation and deethylation reactions, cytochrome P-450 could mediate the deethylation of MEGX to glycine xylidide (GX), and the hydroxylation of xylidine to 4-hydroxyxylidine. It would be anticipated that the conversion from MEGX to GX by cytochrome P-450 would not be seen *in vitro* because of very low levels of precursor MEGX and the relative lack of sensitivity of the assay for GX. (This assay is approximately 5 fold less sensitive than the assay for 3-hydroxy MEGX (see Results - Section 8.3).

In the proposed metabolic pathways of lidocaine (Figure 3), there are several metabolites of lidocaine that do not appear to be produced by cytochrome P-450, viz. xylidine and 2-amino-3-methyl benzoic acid. These urinary products would appear to be further metabolic products from metabolites of the cytochrome P-450 mediated metabolism of lidocaine, produced by non-microsomal enzyme systems. One enzyme that could catalyze some reactions in the proposed pathway is amidase, which is present in the liver cell and could catalyze the conversion of lidocaine to xylidine, of MEGX to xylidine and of GX to xylidine.

Evidence for the interaction of mepivacaine and bupivacaine with hepatic microsomal cytochrome P-450 has not been previously reported in the literature. The results presented herein, however, clearly demonstrate that mepivacaine and bupivacaine are substrates for hepatic microsomal cytochrome P-450: both compounds bind to hepatic microsomal cytochrome P-450 in a type I manner (Figure 14) which is characteristic of substrates of this enzyme, and stimulate the CO-inhibitable oxidation of NADPH by hepatic microsomes (Table 5), which is a measure of cytochrome P-450 dependent metabolism.

The role of different forms of hepatic microsomal cytochrome P-450 in the binding and metabolism of mepivacaine and bupivacaine was investigated using inducing agents to elevate the levels of specific forms of cytochrome P-450: from the results of these studies, it would appear that the phenobarbital inducible form of cytochrome P-450 plays an important

role in the binding of mepivacaine to cytochrome P-450 and that multiple forms of cytochrome P-450 are involved in the metabolism of mepivacaine. Firstly, phenobarbital pretreatment resulted in an increased maximum extent of binding ($^{\Delta}$A max/mg microsomal protein) for the high affinity binding site for the binding of mepivacaine to cytochrome P-450 relative to microsomes from untreated rats and secondly, phenobarbital pretreatment resulted in the appearance of a low affinity binding site for mepivacaine which is not observable following β-naphthoflavone or pregnenolone-16α-carbonitrile pretreatment of rats, or for the binding of mepivacaine to cytochrome P-450 in microsomes from untreated rats (Table 2). It would appear that the forms of cytochrome P-450 induced by β-naphthoflavone and pregnenolone-16α-carbonitrile do not play a major role in the binding of mepivacaine, in as much as induction of multiple-forms of cytochrome P-450 with these inducing agents did not result in an increase in values of $^{\Delta}$A max or $^{\Delta}$A max/nmol cytochrome P-450 relative to the values observed for the binding of mepivacaine to cytochrome P-450 in microsomes from untreated rats (Table 2).

In contrast to the results from the binding of mepivacaine to cytochrome P-450, it appears that multiple forms of cytochrome P-450 are involved in the metabolism of mepivacaine. Elevation of the levels of cytochrome P-450 with phenobarbital, β-naphthoflavone and pregnenolone-16α-carbonitrile resulted in enhanced stimulation by mepivacaine of the oxidation of NADPH (nmol NADPH oxidized/mg protein), relative to hepatic microsomes from untreated rats, while phenobarbital induction

slightly increased the rate of NADPH oxidation/nmol cytochrome P-450 (Table 5). These results indicate that although several forms of cytochrome P-450 play a role in the stimulation of CO-inhibitable oxidation of NADPH by mepivacaine, that the form of cytochrome P-450 elevated by phenobarbital induction may play a predominant role in microsomes from phenobarbital treated rats.

Multiple forms of cytochrome P-450 appear to be involved in the binding and metabolism of bupivacaine. The forms of cytochrome P-450 elevated following phenobarbital and pregnenolone-16α-carbonitrile induction play a role in the low affinity binding site of cytochrome P-450 for bupivacaine since pregnenolone-16α-carbonitrile and phenobarbital pretreatment of rats elevated the values of $^{\Delta}A$ max and $^{\Delta}A$ max/nmol cytochrome P-450 for the low affinity binding site for bupivacaine (Table 3). In contrast, cytochrome P-448 may have no role in the low affinity site but a significant role in the high affinity binding site for bupivacaine since β-naphthoflavone pretreatment of rats had no effect on the low affinity binding site but did elevate the $^{\Delta}A$ max value for the high affinity site (Table 3). That cytochrome P-448 plays a major role for the high affinity site is supported by the apparent absence of the high affinity site in microsomes from pregnenolone-16α-carbonitrile and phenobarbital pretreated rats.

There appear to be multiple forms of cytochrome P-450 involved in the metabolism of bupivacaine as assessed by the stimulation of CO-inhibitable NADPH oxidation. Elevation of cytochrome

P-450 in hepatic microsomes by pretreatment of rats with phenobarbital, pregnenolone-16α-carbonitrile and β-naphthoflavone resulted in stimulation of the rate of NADPH oxidation in the presence of bupivacaine (nmol NADPH oxidized/mg protein), whereas only phenobarbital pretreatment increased the rate of NADPH oxidation/nmol cytochrome P-450 (Table 5), relative to microsomes from untreated rats. These results indicate that multiple forms of cytochrome P-450 are involved in the stimulation of hepatic microsomal oxidation of NADPH by bupivacaine, with possibly the phenobarbital inducible form of cytochrome P-450 playing an important role in the total metabolism of bupivacaine.

In conclusion, it has been demonstrated that the three local anaesthetic agents bind to and are metabolized by cytochrome P-450, but it appears that different forms of cytochrome P-450 are of differing importance in the binding and metabolism of lidocaine, mepivacaine and bupivacaine.

It appears that the phenobarbital form of hepatic microsomal cytochrome P-450 plays a major role in the binding of lidocaine and mepivacaine to both the high and the low affinity binding sites and in the binding of bupivacaine to the low affinity binding site (Tables 1, 2 and 3), the pregnenolone-16α-carbonitrile inducible form of cytochrome P-450 appears to be important in the binding of lidocaine and bupivacaine to the low affinity binding site of cytochrome P-450 (Tables 1 and 3). Multiple forms of cytochrome P-450, with the β-naphthoflavone inducible form being important, are involved in the high affinity binding

site for bupivacaine (Table 3). (See Results - Sections 1, 2 and 3).

The overall rates for the metabolism of lidocaine, mepivacaine and bupivacaine by cytochrome P-450 appear to be similar, since the stimulation of the rate of oxidation of NADPH (nmol NADPH oxidized/nmol cytochrome P-450 × min) by hepatic microsomes was similar for lidocaine, mepivacaine and bupivacaine (Tables 4 and 5). Multiple forms of cytochrome P-450 appear to be involved in the oxidation of NADPH in the presence of mepivacaine and bupivacaine, with the phenobarbital inducible form of cytochrome P-450 playing a slight, but significant role, whereas the phenobarbital inducible form of cytochrome P-450 appears to play a major role in the stimulation of NADPH oxidation by lidocaine (see Results - Sections 5, 6 and 7).

It was not possible to establish a correlation between the extent of *in vitro* metabolism and the *in vivo* metabolism of the local anaesthetics, lidocaine, mepivacaine and bupivacaine, because not all the metabolites detected *in vivo* were able to be detected *in vitro*.

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