PYRIDINE NUCLEOTIDE INVOLVEMENT IN RAT HEPATIC MICROSOMAL DRUG METABOLISM

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by

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CONTENTS

SUMMARY

The apparent kinetic constants (K_m, V) of NADPH for the hepatic microsomal mixed function oxidase enzyme complex have been determined using the Type I substrates, aminopyrine, ethylmorphine or benzphetamine and the Type II substrate, aniline.

The presence of other enyzmes in the heterogeneous microsomal fraction used, notably nucleotide pyrophosphatase, have been shown to exert a marked influence on the resultant kinetic constants. The addition of inorganic pyrophosphate, which inhibits nucleotide pyrophosphatase activity has been shown to:-

(1) protect NADPH from destruction by nucleotide pyrophosphatase particularly at low NADPH concentrations, and

(2) markedly reduce the apparent K_m (NADPH) value for aminopyrine-N-demethylase.

The kinetic constants of NADPH-cytochrome c reductase and NADPH-cytochrome P_{450} reductase (in the presence of aminopyrine, ethylmorphine or aniline) have also been determined.

The apparent K_m (NADPH) value for demethylation reactions were reduced by approximately 50% when determined in the presence of NADH. It is proposed that the NADH mediated alteration in apparent K_m (NADPH) values represent a change in the rate limiting step from NADPH-cytochrome P_{450} reductase.

The following experimental evidence also supports this proposal:-

(1) NADH, when added with NADPH was shown to increase the apparent V of NADPH-cytochrome P_{450} reductase in the presence of either aminopyrine or ethylmorphine (by 280% and 180% respectively) when compared to the corresponding rates obtained with NADPH alone. There was no change in the apparent K_m (NADPH) values in the

presence of NADH for both substrates, and

(2) the addition of NADH with NADPH significantly increased ($P < 0.001$) the initial rapid phase of the biphasic reduction kinetics of cytochrome P_{450} in the presence of aminopyrine.

These observations suggest that NADH exerts a heterotropic co-operative effect with respect to NADPH on cytochrome P_{450} reduction. Similar but less pronounced effects were obtained when the 1,4,5,6 tetrahydronicotinamide analogue of NADH was added in place of NADH. However, the analogue was unable to reduce the cytochrome P_{450}- aminopyrine or ethylmorphine complexes or support the demethylation of these two substrates.

These findings suggested that the structure of NADH as well as its capacity to donate an electron are jointly responsible for the observed NADH synergism of hepatic drug metabolism.

The present results are not completely compatible with any mechanism that has been proposed to explain microsomal electron transport. It is, however, possible to explain the present results by making some modifications to the model proposed by Estabrook & Associates.

Finally, as the major component of this thesis deals with the determination of enzyme kinetic constants, several digital computer programmes that statistically analyse enzyme kinetic data have been compared. It was shown that different weight factors relating to the variability of replicate velocity determinations at each substrate concentration can have a marked influence on the resultant numerical values of the kinetic constants.

Three criteria have been established that must be satisfied before any set of kinetic constants are acceptable for inclusion in the results section.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge and belief, contains no material previously published by any other person, except where due reference is made in the text.

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ABBREVIATIONS

DiHAP	2,6-dihydroxyacetophenone
EDTA	Ethylene diamine tetra acetic acid
FAD	Flavine adenine dinucleotide
FMN	Flavine mono nucleotide
HCHO	Formaldehyde
IDH	Isocitrate dehydrogenase
K_m	Michaelis-Menten constant
K_{ii}	Enzyme-inhibitor-substrate complex dissociation constant
K_{is}	Enzyme-inhibitor complex dissociation constant
K_s	Spectral dissociation constant
MFO	Hepatic microsomal mixed function enzyme complex
N	Number of determinations
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
p-AP	para-aminophenol
S.D.	Standard deviation of the mean
V	Maximum velocity
ΔA_{max}	Maximum change in absorbance between a spectral peak and trough
i.p.	intraperitoneal
x g	Times gravity; relative centrifugal force

C H A P T E R 1.

GENERAL INTRODUCTION

1.1 HISTORICAL

One of the earliest reports suggesting that man was capable of transforming foreign molecules was the identification of hippuric acid as a urinary product of ingested benzoic acid (Keller, 1842). This was followed by evidence for the conversion of benzene to phenol (Schultzen & Naunyn, 1867), ethereal sulphate (Baumann,1876) and glucuronide conjugations (Schmiedeberg & Meyer, 1879) and mercapturic acid synthesis (Jaffe, 1879; Baumann & Preusse, 1879).

The initial report that suggested these reactions were mechanisms to reduce the toxicity of ingested compounds appears to have been made by Neumaster (1895) when discussing glucuronide conjugation.

Since that time, other transformations have been described, but perhaps the next most notable advance was the observation, by a number of workers, that the enzymes effecting these transformations were located in the endoplasmic reticulum of the intact liver cell. Mueller & Miller (1949) showed that in rat liver homogenates, the reductive cleavage of the azo link of the carcinogenic dye 4-dimethylamino-azobenzene was dependent on two enzyme systems:- one system present in the 120,000 x g supernatant (soluble fraction) reduced the added NADP to NADPH, while the other, which was localised in the microsomal fraction utilised the NADPH to cleave the dye. They further showed significant destruction of the dye occurred when the microsomes were incubated with NADPH, thus confirming the role of the soluble fraction in generating this co-factor.

It was also found at this time that NADH could not effectively substitute for NADPH in this reaction as the amount of dye cleaved in the presence of NADH alone was about 10% of

that obtained with NADPH.

Subsequently, Mueller & Miller (1953) showed that oxygen and NADPH were required for the oxidative demethylation of 3-methyl-4-monomethylazobenzene by rat liver homogenates. Similar requirements were also found for the hydroxylation of aromatic compounds (Mitoma *et al.*, 1956) and alkyl hydrocarbon chains (Cooper & Brodie, 1955 a,b,), the O-dealkylation of codeine to morphine (Axelrod, 1955b) the N-demethylation of aminopyrine (LaDu *et al.*,1955) and N-methylaniline (Gaudette and Brodie,1959) N-oxide formation (Baker & Chaykin, 1962), and the sulphoxidation of chlorpromazine (Gillette & Kamm, 1960). These studies also showed that the liver had a greater capacity for oxidative biotransformations than other organs such as the brain, spleen, kidney and muscle.

The great diversity of these enzymic transformations of foreign molecules raised two possibilities of whether these reactions were either catalysed by a few relatively nonspecific enzymes or by numerous, highly specific enzymes within the microsomal fraction of liver homogenates. (Brodie & Axelrod, 1950). It was thought initially that the latter proposition was correct and that drugs underwent chemical modification because their structures were similar to substrates of intermediatry metabolism, (Gaudette & Brodie, 1959).

However, while a number of xenobiotics were demethylated (e.g. aminopyrine, N-methylaniline, monomethyl-4-aminoantipyrine) it was found that naturally occurring molecules with N-methyl groups (e.g. adrenalin, sarcosine, choline and creatine) were not demethylated by the rat hepatic microsomal systems . (Guadette & Brodie, 1959).

This observation favoured the former of the two proposals

and it was further suggested (Brodie *et al.*, 1955) that the microsomal system was responsible for detoxifying foreign compounds that gain access to the body via the alimentary tract.

While Mitomi *et al.*, (1956) and Axelrod (1955a) both suggested that the main function of the system was to reduce the toxicity and/or therapeutic activity of non polar aromatic compounds, the observation that codeine was converted to morphine (Axelrod, 1955b) indicated that some microsomal oxidation products still retained pharmacological activity.

1.2. DISTRIBUTION OF DRUG METABOLISING ACTIVITY WITHIN THE MICROSOMAL FRACTION.

The endoplasmic reticulum of the hepatocyte exists in two forms based upon ribosomal attachment:

(1) a rough surfaced form which consists of lipid tubules with numerous small dense particles (ribosomes) attached, and

(2) a smooth surfaced form which is the lipid tubules without the ribosomes. (Palade & Siekevitz, 1956).

On mechanical disruption, the endoplasmic reticulum forms small vesicles which can be isolated as rough and smooth surfaced microsomes by differential centrifugation, (Gillette, 1963). It has been shown that the ribosomes, which are obligatory for protein synthesis, are not involved in drug metabolism (Gillette, 1963).

Fouts (1961) has shown that there was a greater rate of metabolism of a range of drug substrates and NADPH oxidation in smooth surfaced microsomes when compared to that obtained for rough surfaced microsomes. The actual ratio of the activities in the microsomal fractions was dependent on:-

(1) the method used to fractionate the microsomal fraction

into rough and smooth microsomes, and

(2) the animal species used (Fouts *et al.*,1966; Gram *et al.*, 1967 a,b; Gram & Fouts, 1967).

Similar results were obtained by Remmer & Merker (1963, 1965) although the distribution of activity between the two fractions was generally not as marked as that obtained by Fouts (1961). The study by Remmer & Merker also indicated that microsomal enzymes not involved in drug metabolism (e.g. glucose-6-phosphatase, NAD glycohydrolase) are equally distributed between rough and smooth surfaced microsomes.

1.3. EARLY PROPOSALS OF THE MECHANISM OF DRUG METABOLISM.

A large number of structurally different drugs and xenobiotic compounds undergo oxidative transformations catalysed by the liver microsomal enzyme system. The common requirements of all these reactions for NADPH and molecular oxygen, led Brodie *et al.*, (1958) to suggest that all oxidative pathways in microsomes can be envisaged to occur via an initial hydroxylation, followed in some cases by rearrangement as shown in Table 1.1.

TABLE 1.1.

Hydroxylation of aromatic rings.

$$CH_3 - CO - NH - C_6H_5 \xrightarrow{(OH)} CH_3 - CO - NH - C_6H_4 - OH$$

Oxidation of sidechains

$$R - CH_2CH_2CH_2 - CH_3 \xrightarrow{(OH)} R - CH_2CH_2 - CH_2OH - CH_3$$

N-Dealkylation

$$R - NH - CH_3 \xrightarrow{(OH)} (R - NH - CH_2OH) \longrightarrow R - NH_2 + HCHO$$

Unstable

Table 1.1. continued

O-Dealkylation

$$R - O - CH_3 \xrightarrow{(OH)} (R - O - CH_2 - OH) \longrightarrow R - OH + HCHO$$

Unstable

Deamination

$$R - CH_2 - CH(NH_2) - CH_3 \xrightarrow{(OH)} (R - CH_2 - COH\ (-NH_2) - CH_3)$$

Unstable

$$\longrightarrow R - CH_2 - CO - CH_3 + NH_3$$

Sulphoxide formation

$$R - S - CH_3 \longrightarrow (R - S - CH_2 - OH) \longrightarrow R - SO - CH_3$$

Unstable

Table 1.1. The mechanism of oxidative pathways in hepatic microsomes (from Brodie *et al.*, 1958).

Further it was suggested that a possible common step in microsomal oxidations may be the formation of an intermediate hydroxyl donor (OH), which, in conjunction with a number of non specific enzymes, can transfer a hydroxyl radical to an appropriate acceptor substrate.

Gillette (1963) postulated the following sequence for microsomal hydroxylations:-

(1) $NADPH + A + H^+ \longrightarrow AH_2 + NADP^+$

(2) $AH_2 + O_2 \longrightarrow$ "active oxygen"

(3) "active oxygen" + Drug $\longrightarrow$ oxidised drug + $A + H_2O$

Overall, $NADPH + O_2$ + Drug $\longrightarrow$ oxidised drug + $NADP^+ + H_2O$

It was suggested that NADPH reduces a component present in

the microsomes that reacts with molecular oxygen to produce an active oxygen intermediate. The active oxygen then oxidises drug substrates with the aid of a group of non specific enzymes localised in the microsomes. Alternatively, NADPH reduces the microsomal enzymes which then react with oxygen to produce a group of active oxygen enzyme complexes.

A number of suggestions as to the nature and chemical composition of the intermediate "active oxygen" have been proposed:-

(1) Brodie *et al.*, (1954) suggested that the active hydroxyl group is electrophilic (OH+) and that it hydroxylates aromatic or heterocyclic rings at electronegative sites.

(2) Breslow & Lukens (1960) suggested a hydroxyl free radical (OH) rather than a positive ion.

(3) Staudinger *et al.*, (1965) agreed in part with the suggestion of Breslow & Lukens (1960) and suggested in addition a hydroxonium free radical ($OH_2^{\cdot}$).

(4) Gillette *et al.*, (1957) has shown that hydrogen peroxide is formed during the oxidation of NADPH by liver microsomes in the absence of any drug substrates. Although hydrogen peroxide has been excluded as an "active oxygen", it has been suggested (Gillette, 1963) that an intermediate which leads to the formation of hydrogen peroxide could be the hydroxylating species. More recent suggestion as to the nature of"active oxygen" are given later.

1.4. THE DISCOVERY OF CYTOCHROME P_{450}

Chance & Williams (1954) while examining the reactions of cytochrome b_5 in liver microsomes noted that more NADH was required than was theoretically necessary to reduce this cytochrome,

and an additional electron acceptor was proposed to exist in liver microsomes. Although not reported in the published article, Williams recognised the presence of a carbon monoxide binding pigment in liver microsomes (Estabrook, 1971a).

Klingenberg (1958) first recorded the spectral details of the microsomal haemoprotein which, when reduced in the presence of carbon monoxide forms a broad peak with a maximum absorption at 450 nm, thus it was called cytochrome P_{450}. Omura & Sato (1962, 1964a,b,) agreed with Klingenberg that the pigment was a haemoprotein although Garfinkel (1958) had earlier proposed that it was not an iron haemoprotein.

A pigment of a similar nature was also shown to be present in adrenal microsomes and mitochondria (Estabrook *et al.*,1963; Cooper *et al.*,1965; Omura *et al.*,1965) and microsomes from kidney and intestinal mucosa (Sato *et al.*,1965).

Under anaerobic conditions, low concentration of carbon monoxide can produce maximal formation of the 450 nm peak whereas much higher concentrations are required under aerobic conditions to obtain an equivalent absorbance change. This suggested that in the absence of carbon monoxide but in the presence of air, cytochrome P_{450} would exist in the oxidised form possibly complexed with oxygen, (Gillette, 1967).

Estabrook *et al.*, (1963) showed that the inhibitory effects of carbon monoxide on the C-21 hydroxylation of 17-hydroxy progesterone reported by Ryan & Engel (1957) could be reversed by monochromatic light with a wavelength of 450 nm suggesting that cytochrome P_{450} might be a component of the adrenal microsome C-21 hydroxylase system. Later, Omura *et al.*,(1965, 1966) showed that cytochrome P_{450} was also a component of the 11β-hydroxylase system of adrenal mitochondria.

It was suggested that cytochrome P_{450} functions in these systems by reacting with oxygen to produce "active oxygen" which is introduced into the steroid substrate.

The following indirect evidence suggested that cytochrome P_{450} present in liver microsomes could be involved in the transfer of "active oxygen" into drug substrates:-

(1) Deoxycholate and snake venom inhibited NADPH oxidase (Gillette *et al.*,1957; Gillette, 1963) and converted cytochrome P_{450} to an inactive form, cytochrome P_{420}, (Omura & Sato, 1964a,b).

(2) Carbon monoxide which binds to the reduced form of the haemoprotein was shown to inhibit a range of transformations (Conney *et al.*1957a; Orrenius *et al.*,1964; Gillette, 1967).

(3) Monochromatic light with a wavelength of 450 nm reversed the inhibitory effects of carbon monoxide on demethylation and hydroxylation reactions (Cooper *et al.*, 1965).

(4) Treatment of animals with phenobarbital (see next section) caused approximately parallel increases in cytochrome P_{450} content and barbiturate hydroxylation (Remmer & Merker, 1965).

1.5. THE EFFECT OF PRETREATING ANIMALS WITH XENOBIOTICS.

Conney *et al.*,(1956) reported that one consequence of treating rats with small doses of polycyclic hydrocarbons (e.g. 3,4-benz(a)pyrene or 3-methyl cholanthrene) was to increase the rate at which the isolated liver microsomal fraction N-demethylated and reduced the azo linkage of aminoazo dyes.

This observation was subsequently extended to show similar increases in the hydroxylation of 3,4 benz(a)pyrene (Conney

et al. 1957b) and the ring hydroxylation of 2-acetylaminofluorence (Cramer *et al.*, 1960).

It was suggested (Conney *et al.*, 1956, 1957b) and subsequently confirmed using the protein synthesis inhibitors (puromycin and actinomycin D) that pretreatment of animals with polycyclic hydrocarbons increased enzymic activity by inducing the synthesis of new enzyme protein. (Conney & Gilman, 1963; Gelboin & Blackburn, 1964; Orrenius *et al.*, 1965).

The stimulatory effects of barbiturate pretreatment on the rate of metabolism of drugs was first shown by Remmer (1958, 1959 a,b) and subsequently by Conney & Associates (Conney *et al.*,1960, 1961a,b).

Since the initial discovery, numerous drugs, insecticides and carcinogens have been shown to induce the hepatic microsomal drug metabolising enzymes (Conney, 1967, Remmer, 1972). The inductive effects of chlorinated insecticides were found by accident when it was noticed that spraying animal rooms with halogenated hydrocarbon insecticides resulted in increased levels of hepatic microsomal drug metabolism (Hart & Fouts, 1963; Hart *et al.*,1963). The actual amounts of different inducers required to produce maximal elevation of enzyme levels varies considerably (Conney, 1967).

It has been shown (Fouts & Rogers, 1965; Remmer & Merker, 1965) that pretreatment of rats with either phenobarbital, chlordane or tolbutamide produced marked proliferation of the smooth endoplasmic reticulum with little or no change in the rough surfaced endoplasmic reticulum. In contrast, treatment with 3, 4-benz(a)pyrene or 3-methylcholanthrene, while stimulating the metabolism of certain drugs (Conney *et al.*, 1956,1957b ; Crammer *et al.*, 1960) produced little change in the smooth endoplasmic reticulum (Fouts & Rogers, 1965).

After multiple injections of phenobarbital the activity of aminopyrine-N-demethylase and cytochrome P_{450} content were higher in smooth surfaced microsomes when compared to rough surfaced microsomes (Orrenius & Ernster, 1964). Gram *et al.*,(1967b) also reported greater mixed function oxidase activity was present in the smooth microsomes after either phenobarbital or 3-methylcholanthrene pretreatment.

The increased rates of metabolism of two barbiturates, hexobarbital and 5-(2-bromallyl)-5isopropyl-1-methyl barbituric acid, closely parallels the amount of cytochrome P_{450} present in the microsomal fraction, (Remmer & Merker, 1965). Such pretreatment of animals also induces NADPH oxidase (Conney & Burns, 1963; Remmer & Merker, 1963; Conney *et al.*,1961,b) and NADPH cytochrome c reductase (Ernster & Orrenius, 1965; Kato, 1966; Orrenius & Ernster, 1964) but does not significantly elevate cytochrome b_5 content (Remmer & Merker, 1965; Remmer, 1972) or other enzymes which are localised in the endoplasmic reticulum but are not associated with the metabolism of drugs. (Remmer & Merker, 1963).

Studies of the half lives of the microsomal haemoproteins (Remmer, 1972) have also suggested that increased levels of the mixed function oxidase system are due to an enhanced rate of synthesis rather than a diminished rate of catabolism. While the majority, if not all of the oxidative reactions catalysed by cytochrome P_{450} are induced to some extent by phenobarbital, DDT and related drugs, this is not the case with polycyclic hydrocarbon induction. For example, the hydroxylation of 3,4-benz(a)pyrene is increased 10-30 times by 3,4-benz(a)pyrene induction, (Conney *et al.*1957b) whereas the hydroxylation of barbiturates (Conney *et al.*,1960) and the ethylmorphine demethylation (Mannering, 1968) show little increase, (Remmer, 1972).

This observation suggested that the nature of phenobarbital and polycyclic hydrocarbon induction of cytochrome P_{450} are different.

It was subsequently shown (Alvares *et al.*,1967; Hildebrandt *et al.*,1968) that the λ_{max} of cytochrome P_{450} from rats previously treated with polycyclic hydrocarbon was not identical to that from control and phenobarbital pretreated rats. Phenobarbital treatment produces a haemoprotein that still has its λ_{max} at 450 nm although its intensity/mg protein is greatly increased, (Remmer & Merker, 1965).

In contrast, the λ_{max} of P_{450} from rats previously treated with polycyclic hydrocarbon has been reported at 448 nm (Alvares *et al.*, 1967) and 446 nm (Hildebrandt *et al.*, 1968). However, some doubts have been raised as to the method used by Hildebrandt *et al.*, to determine the λ_{max} in polycyclic hydrocarbon induced rats, (Mannering, 1971).

It has been suggested (Mannering, 1971) that the treatment of rats with polycyclic hydrocarbons prior to sacrifice induces the biosynthesis of a molecular species of cytochrome P_{450} that is present in small amounts in untreated animals. It was further suggested that both cytochrome P_{450} and P_{448} exist in inter-convertible forms and the latter haemoprotein is not a combination of cytochrome P_{450} with polycyclic hydrocarbons or their metabolites.

However, Hashimoto & Imai (1976) have suggested that cytochrome P_{450} purified from rats that had been previously treated with 3-methylcholanthrene is in the form of a cytochrome P_{450}-3-methylcholanthrene complex.

Comai & Gaylor (1973) have proposed that there are possibly three different forms of cytochrome P_{450} in control microsomes.

Welton & Aust (1974) confirmed this observation and suggested molecular weights of 44,000, 50,000 and 53,000.

The component with a molecular weight of 50,000 predominates in microsomes from untreated rats while pretreatment with phenobarbital or 3-methylcholanthrene selectively induces the compounds with molecular weights of 44,000 and 53,000 respectively.

In contrast, NADPH-cytochrome c reductase solubilised from microsomes isolated from untreated rats and rats previously treated with either phenobarbital or 3-methylcholanthrene are immunological similar with identical molecular weights (Welton & Aust, 1975).

Various other factors, such as animal strain, animal species, age, sex and nutritional status have been shown to exert a profound influence on drug mediated enzyme induction (Conney, 1967; Remmer, 1972; Campbell & Hayes, 1974).

1.6. POSSIBLE COMPONENTS AND MECHANISMS OF THE HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE COMPLEX.

It has been stated the hepatic enzyme complex that is responsible for the biotransformation of xenobiotics is dependent on NADPH and molecular oxygen. This finding places this enzyme system in the "Mixed Function Oxidase " (MFO) category of Mason (1957,) or the "Monooxygenase" classification of Hayaishi (1962). Once characteristic of the mixed function oxidase classification is that the enzyme system should catalyse the formation of one molecule of product with the consumption of one molecule of oxygen; one oxygen atom appears in the product while the other undergoes a two equivalent reduction to form water.

Experimental evidence in support of this classification was the demonstration, using ^{18}O, that atmospheric oxygen and not the oxygen of water was involved in the hydroxylation of acetanilide (Posner *et al.*, 1961), triethylamine oxide formation (Baker & Chaykin, 1962) and the hydroxylation of 11-deoxycorticosterone

to form corticosterone, (Hayano *et al.*, 1955,a,b). The demonstration of ^{18}O incorporation into formaldehyde arising from the N-demethylation of various substrates has been hampered by the rapid exchange of the oxygen of formaldehyde with aqueous oxygen (Gillette, 1967; Mannering, 1968).

1.7. THE INVOLVEMENT OF NADPH-CYTOCHROME C REDUCTASE.

While cytochrome P_{450} was generally recognised as the terminal oxidase in the mixed function oxidase enzyme complex, the question arose as to whether NADPH reduced the haemoprotein directly or indirectly through an intermediate electron carrier.

The following indirect evidence suggested that the flavoprotein NADPH-cytochrome c reductase mediated the reduction of cytochrome P_{450}:-

(1) Cytochrome c had been shown to be a potent inhibitor of demethylation (Mueller & Miller, 1953; La Du *et al.*, 1955) and hydroxylation (Cooper & Brodie, 1955,a) reactions, and

(2) there was no known function for NADPH-cytochrome c reductase in liver microsomes (Gillette, 1967) and

(3) the flavoprotein was induced by phenobarbital treatment (Remmer & Merker, 1963, 1965; Orrenius & Ernster, 1964) and

(4) the percentage of NADPH-cytochrome c reductase activity retained in microsomes treated with steapsin was similar to the percentage of NADPH-cytochrome P_{450} reductase and aniline hydroxylase retained. (Sato *et al.*, 1965).

However, variations in the kinetic constants of NADPH for demethylation reactions in intact microsomes (Orrenius, 1965; Schenkman *et al.*, 1967) and for purified NADPH-cytochrome c

reductase have cast some doubts as to the role of this enzyme in the mixed function oxidase system. Sasame & Gillette (1970) have suggested that this discrepancy might be due to action of nucleotide pyrophosphatase in the heterogeneous microsomal fraction.

Since then, experimental evidence has accumulated to provide strong support for the involvement of NADPH-cytochrome c reductase in the mixed function oxidase system. For example, antibodies prepared against the purified flavoprotein have inhibited aniline hydroxylase (Omura, 1969) and ethylmorphine demethylase (Sasame *et al.*, 1973).

The flavoprotein reductase has been shown to be distributed homogenously on the outer surfaces of microsomes but heterogenously within microsomes in groups of three to five enzyme molecules (Morimoto *et al.*, 1976).

NADPH-cytochrome c reductase has been purified and its properties characterised since the initial report of Horecker, (1950). Masters *et al.*, (1965) have shown that:-

(1) the enzyme catalyses a one electron transfer from NADPH to cytochrome c, and

(2) it's prosthetic group, flavine adenine dinucleotide (FAD) undergoes cyclic oxidation-reductions between the half reduced (FADH) and fully reduced states ($FADH_2$).

More recently Iyanagi & Mason (1973) reported that the purified enzyme has a molecular weight between 68,000 - 79,000 with two flavines per molecule. The flavines were FAD and flavine mononucleotide (FMN) which were present in equimolar amounts. This latter observation has been confirmed by Masters (1974).

1.8. THE RESOLUTION OF THE MIXED FUNCTION OXIDASE COMPLEX INTO VARIOUS COMPONENTS AND RE-CONSTITUTED HYDROXYLASE ACTIVITY.

Although the steroid 11β hydroxylase system of beef adrenal cortex mitochondria had been separated into three components, similar studies using liver microsomes were unsuccessful (Omura *et al.*, 1965, 1966).

The steroid hydroxylase system was resolved into three fractions that were essential for hydroxylase activity:-

(1) a particular fraction containing cytochrome P_{450},

(2) a flavoprotein, NADPH-cytochrome c reductase,

(3) a non-haem iron protein called "adrenodoxin" by Suzuki & Kimura (1965).

Omura *et al.*, (1966) proposed the following scheme for the steroid 11β hydroxylase system.

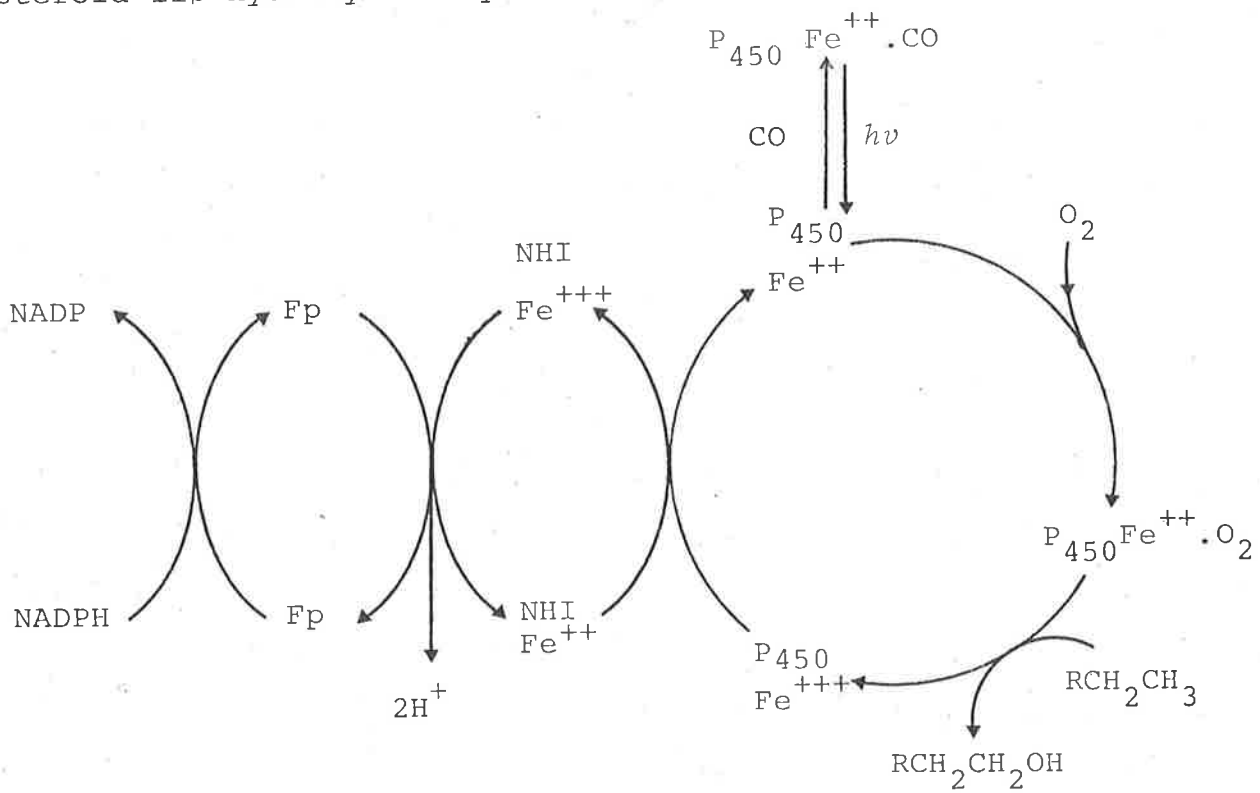


FIGURE 1.1. Proposed Scheme for 11β hydroxylase in mitochondria (redrawn from Omura *et al.*, 1966).

Fp represents NADPH-cytochrome c reductase

NHI represents adrenodoxin.

It was postulated that reduced cytochrome P_{450} combines with molecular oxygen to form "active oxygen", one oxygen atom re-oxidises the haemoprotein while the other reacts with the substrate molecule and results in the introduction of a hydroxyl group into the steroid molecule. In such a scheme, a 1:1:1 stoichiometry for NADPH oxidised: oxygen consumed: hydroxylated product formed would be expected. Such a stoichiometry has been shown for the C-21 hydroxylase system of adrenal cortex microsomes (Cooper *et al.*, 1962), the C-11 hydroxylation of deoxycorticosterone by adrenal cortex mitochondria (Cammer & Estabrook, 1966) and the demethylation of aminopyrine by rat liver microsomes from phenobarbital treated rats (Orrenius, 1965).

In view of the similarities between the adrenal cortex mitochondrial and microsomal systems and the liver microsomal MFO system, it was proposed that a similar electron transport system could operate in hepatic microsomes.

The first successful report that the MFO system could be resolved into it's different components and hydroxylase activity reconstituted was made by Lu & Coon (1968). Three components again appeared obligatory for hydroxylase activity:- (Lu & Coon, 1968; Lu *et al.*, 1969,b, 1970),

(1) cytochrome P_{450},

(2) NADPH-cytochrome c reductase,

(3) a microsomal lipid component which could be replaced by phosphatidyl choline.

Earlier, Remmer *et al.*, (1966) reported that two types of spectral interaction occurred when drug substrates were added to intact hepatic microsomes. The drug substrates could be divided into one of two main groups based on the difference spectrum produced when they were added to microsomes; either a Type I

substrate(λ_{max} in the range 385 - 390 nm,λ_{min} 418 - 427 nm) or a Type II substrate (λ_{max} 425 - 435 nm,λ_{min} 390 - 405 nm), (Remmer *et al.*,1966; Schenkman *et al.*,1967,a). It was further suggested that these interactions represent the first step, the formation of a complex between drug and oxidised cytochrome P_{450}, in microsomal drug hydroxylations. These studies were based upon a report that a small but reproducible spectral change occured when 17-hydroxylprogesterone was added to adrenal cortex microsomes (Narasimhula *et al.*,1965).

For both drugs and steroids, the magnitude of the spectral change is dependent on the concentration of steroid or drug added and the amount of cytochrome P_{450} present.

1.9. THE INFLUENCE OF VARIOUS PYRIDINE NUCLEOTIDE CONCENTRATIONS ON HEPATIC DRUG METABOLISM.

It had been known for some time that NADH could not effectively substitute for NADPH in MFO reactions (Mueller & Miller, 1949; Axelrod, 1956; Fouts & Brodie, 1957). However, Conney *etal.*, (1957a), reported a synergistic increase in product formation in the presence of both reduced pyridine nucleotides when compared with the individual rates. This observation has been confirmed for a range of substrates by various workers.

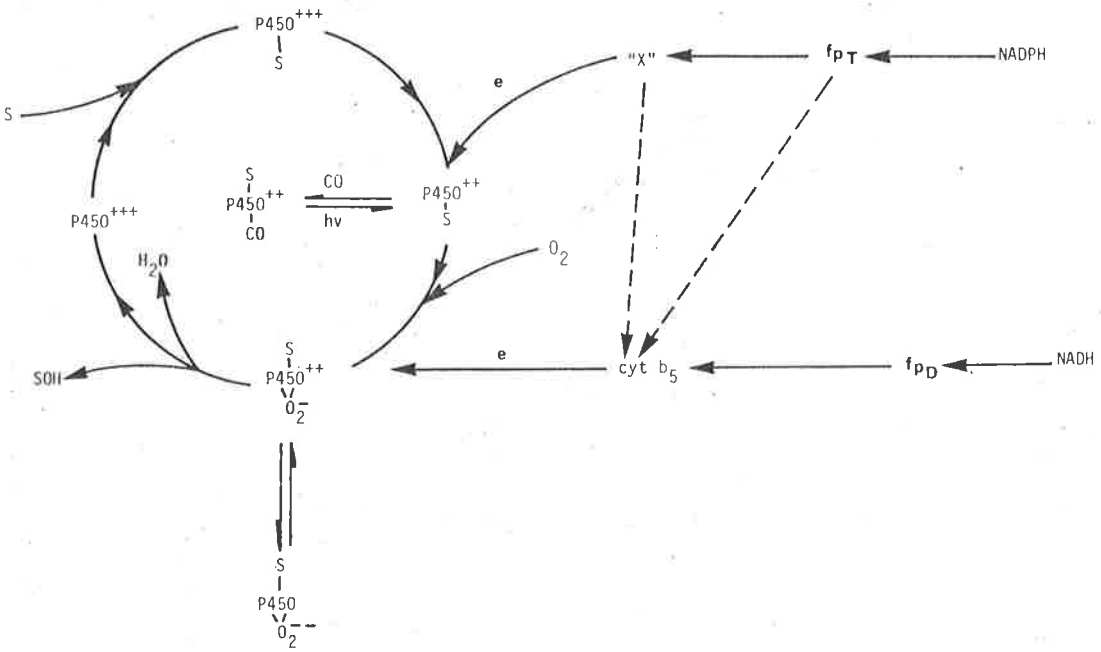
However, no real attempt was made to explain the mechanism of this effect until Estabrook & Associates (Cohen & Estabrook, 1971,a,b,c; Hildebrandt & Estabrook, 1971) re-examined the pyridine nucleotide involvement in hepatic drug metabolism. It was apparent that sound experimental evidence favoured the proposal that the oxidised cytochrome P_{450} substrate-complex was reduced by an electron from NADPH which was transferred via NADPH-cytochrome c reductase. It was proposed that the other microsomal haemoprotein cytochrome b_5 was involved in the donation of the

second electron from either NADPH or NADH. As NADH was more efficient than NADPH in reducing cytochrome b_5 a synergistic increase in product formation was evident in the presence of both pyridine nucleotides.

Figure 1. 2. represents a proposed mechanism of microsomal electron transport during drug hydroxylation (Cohen & Estabrook, 1971,c; Hildebrandt & Estabrook, 1971).

FIGURE 1.2.

SCHEMATIC REPRESENTATION OF MICROSOMAL DRUG OXIDATION



The following sequence of events were suggested during hepatic drug hydroxylation reactions :-

(1) drug substrates interact reversibly with the low spin form of ferric cytochrome P_{450} to produce a high spin form of the ferric-cytochrome P_{450}- substrate complex,

(2) the complex undergoes a reduction by an electron,which originates from NADPH, via NADPH-cytochrome c reductase to form a ferrous cytochrome P_{450}- substrate complex,

(3) oxygen reversibly interacts with the ferrous cytochrome

P_{450}- substrate complex,

(4) a second one electron reduction generates an intermediate which was previously called "active oxygen". Active oxygen may be a hydroperoxide derivitive of ferric cytochrome P_{450}- substrate complex, or a super oxide anion of the ferrous cytochrome P_{450}- substrate complex.

(5) re-arrangement occurs with the introduction of one oxygen as a hydroxyl group into the substrate and the reduction of the other oxygen to water,

(6) the hydroxylated product dissociates from the ferric cytochrome P_{450} with the regeneration of the low spin form of ferric cytochrome P_{450}.

Sasame *et al.*, (1973, 1974a) using an antibody to cytochrome b_5 has confirmed that the second electron from NADH but not NADPH is transferred by this haemoprotein.

Experimental evidence in support of the proposed oxygenated ferrous cytochrome P_{450}- substrate complex was obtained by Estabrook *et al.*, (1971,b,) when a new spectral species with a λ_{max} of 440 nm was observed during hexobarbital metabolism. The appearance of the 440 nm absorbing species was dependent on oxygen, NADPH and a Type I substrate. The addition of NADH with NADPH reduces the magnitude of the spectral species which further supports the concept that cytochrome b_5 is the donor of the second electron from NADH to the oxygenated ferrous cytochrome P_{450}- substrate complex (Estabrook *et al.*, 1971,c).

Shleyer *et al.*, (1972) have observed the presence of an oxygenated form of cytochrome P_{450} in a partially purified preparation from adrenal cortex mitochondria and demonstrated it's function in steroid hydroxylation. More recently, Baron *et al.*, (1973) reported that the magnitude of the spectral change

(440 - 500 nm - the latter wavelength being the isobestic point) in liver microsomes was dependent on the temperature and the nature of the added substrate. However, in similar experiments, Coon (1974) was unable to reproduce the formation of the 440 nm spectral species.

RECENT ATTEMPTS TO ELUCIDATE THE MECHANISMS OF HEPATIC DRUG METABOLISM.

More recently, there have been four main areas of research to further elucidate the mechanism and characterise intermediates of the hepatic MFO enzyme system:-

(1) The use of the detergent solubilised and reconstituted hydroxylating system to identify obligatory and facilitatory components of the system.

Cytochrome P_{450} has been solubilised from intact microsomes by deoxycholate in the presence of glycerol , (Lu & Coon, 1968) and in addition, NADPH-cytochrome c reductase as well as a lipid factor have been identified as obligatory components (Coon & Lu, 1969) for the hydroxylation of fatty acids and hydrocarbons (Lu & Coon, 1968; Lu *et al.*,1969,a) and a range of drug substrates (Lu *et al.*,1969,a,b; 1970; 1971; 1972; Fugita & Mannering, 1973).

The active component of the heat stable lipid factor was shown to be phosphatidylcholine (Strobel *et al.*, 1970). Extraction of lypholised microsomes with organic solvents has shown that while microsomal lipid is required for enzymic activity, it is not necessary for the binding of substrates to cytochrome P_{450} (Vore *et al.*,1974).

It has been shown (Lu *et al.*,1973) using cytochrome

P_{450} solubilised from contron and phenobarbital treated rats, cytochrome P_{448} from 3-methylcholanthrene treated rats together with the other obligatory components that the substrate specificity and catalytic activity reside in the cytochrome P_{450} or P_{448} fractions.

The addition of purified cytochrome b_5 to cytochrome P_{450}, NADPH-cytochrome c reductase and phosphatidyl choline inhibited benzphetamine demethylation in the presence of NADPH alone (Lu *et al.*, 1974; West *et al.*, 1974). The results of these studies have suggested that cytochrome b_5 is an obligatory component of the NADH electron transport pathway while being facilitatory in the transfer of the electron from NADPH for some drug substrates.

Turnover numbers (i.e. nmoles product formed/nmole cytochrome P_{450}) for the resolved and reconstituted system have been shown to be equal to or greater than the corresponding numbers for intact microsomes for all the drug substrates studied to date (Lu, 1974). This observation argues against the involvement of the hypothetical intermediate "X"(proposed by Dallner *et al.*, 1966; Estabrook *et al.*, 1969, 1971,a, 1972; Dallman *et al.*, 1969; Kupfer & Orrenius, 1970,; Estabrook & Cohen, 1969; Ichihara *et al.*,1973).

(2) The demonstration that flavoprotein reductases and cytochrome b_5 could be isolated and purified in a form that still retained catalytic activity enabled rabbit and goat antibodies to be prepared against these components. These studies have provided evidence for the involvement of NADPH-cytochrome c reductase in

transferring the first electron to the ferric cytochrome P_{450}- substrate complex (Masters *et al.*, 1973). The results from antibody studies have also suggested that cytochrome b_5 mediates the introduction of the second electron from NADH but not NADPH (Sasame *et al.*, 1973, 1974 a).

(3) Currently there is divided opinion as to the nature and chemical composition of the actual hydroxylating species ("active oxygen"). Gillette *et al.*, (1957) has shown the formation of hydrogen peroxide with the methanol/ catalase system (Keilin & Hartree, 1945), when NADPH is oxidised in hepatic microsomes. However, hydrogen peroxide has been excluded as an intermediate in hepatic mixed function oxidase reactions (Gillette, 1963, 1967; Ullrich & Staudinger, 1968). Nevertheless, the stoichiometry of MFO enzymes suggest that active oxygen exists at the oxidation level of hydrogen peroxide or atomic oxygen (Ullrich & Staudinger, 1968).
More recently, methods with greater sensitivity have confirmed the observation of Gillette *et al.*,(1957) relating to hydrogen peroxide formation during NADPH oxidation (Thurman *et al.*,1972; Hildebrandt *et al.*, 1973). This finding is significant in view of the recent suggestion that cytochrome P_{450} can function as a peroxidase, (Hrycay & O'Brien, 1972; Hrycay & Prough, 1974; Hrycay *et al.*,1975). It has also been shown that certain organic hydroperoxides can substitute for NADPH and oxygen and support the hydroxylation of various drugs and fatty acids (Rahimtula & O;Brien, 1974, 1975; Kadluban *et al.*, 1973; Ellin & Orrenius, 1975). Hrycay *et al.*, (1975a,b,1976) have shown that in

addition to NADPH, either $NaIO_4$, $NaClO_2$, hydrogen peroxide or organic hydroperoxides supported androstenedione hydroxylation in the presence of paritally purified rat liver microsomal cytochrome P_{450} preparations.

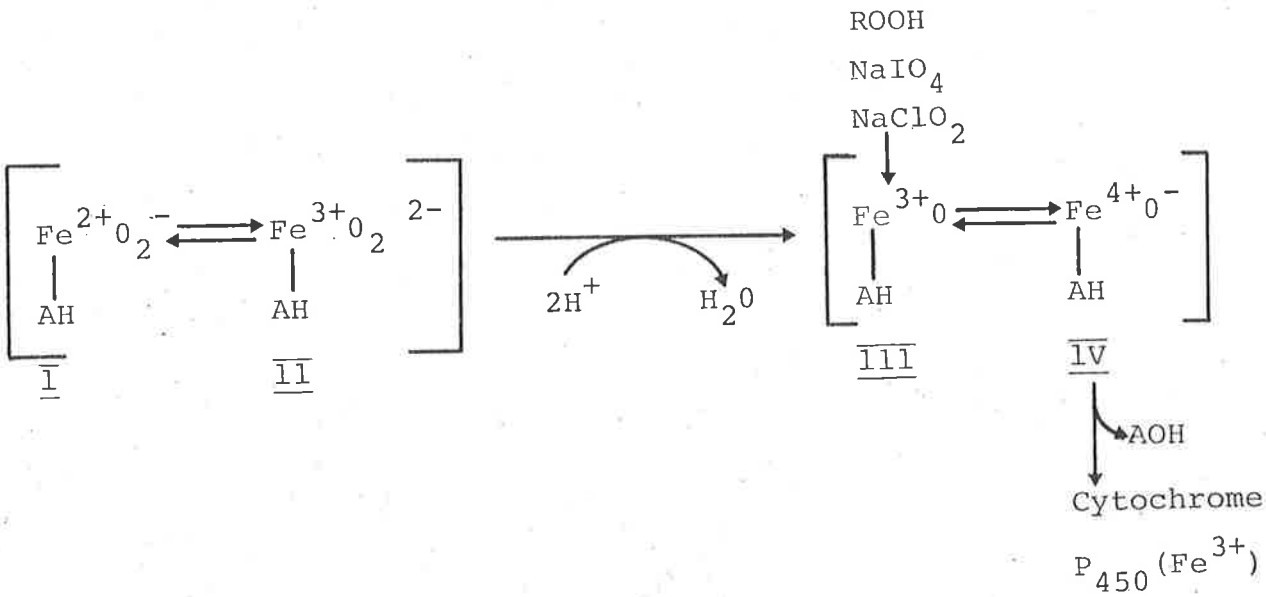


FIGURE 1.3. Active oxygen intermediates proposed by Hrycay *et al.*, 1975.

These workers suggested that the ferrous cytochrome P_{450}- substrate complex binds molecular oxygen and accepts the second electron to form the ferrous cytochrome P_{450}- superoxide intermediate (I)which is in resonance with the ferric cytochrome P_{450}- hydroperoxide complex (II). This species is unstable and decomposes with the liberation of water to give a ferric cytochrome P_{450}- mono-oxygen species (III) which is in resonance with the ferryl ion complex (IV) and which decomposes to produce the hydroxylated substrate. Organic hydroperoxides, $NaIO_4$, $NaClO_2$ bypass the reduction steps and enter where shown on the scheme.

In contrast Debey & Balney (1973) have suggested that when rat liver microsomes are incubated with NADPH,

superoxide ions (O_2^-) can be detected by their reaction with various peroxidases. It therefore appears that the exact nature of "active oxygen" is still not completely resolved.

(4) It is now well established that catalytic activity resides in the haemoprotein cytochrome P_{450}. Studies designed to reveal the electronic structure of the haem in mammalian cytochrome P_{450} have been hampered because the partially purified cytochrome P_{450} samples used are contaminated by other haemoproteins. Lipscomb & Gunsalus (1973) proposed that cytochrome P_{450} consisits of a single molecule of ferriprotoporphyrin IX with two amino acids ligands, probably cysteine and histidine, and four pyrrole nitrogens bound to the iron. Studies using partially purified preparations have shown that cytochrome P_{450} has an anomalous absorption spectrum for a protohaem and this has been attributed to thiolate anion of a cysteinyl residue binding to one of the axial ligands. (Watanabe & Horie, 1976; Stern & Peisach, 1976). Recently, however, cytochrome P_{450} has been obtained in homogeneous form (Imai & Sato, 1974; Van der Hoever *et al.*, 1974). Magnetic circular dichroism studies on such samples (Shimizu *et al.*, 1975) have suggested that oxidised cytochrome P_{450} exists in a state between the ferric low spin and ferrous low spin states. However, direct magnetic measurements are necessary to further characterise the environment of the haem of cytochrome P_{450}.

These approaches have yielded valuable information regarding the intermediates and mechanisms of microsomal hydroxylation reactions. However, one approach that has not been utilized to it's fullest extent is the estimation of the kinetic parameters of

both reduced pyridine nucleotides in hepatic drug metabolism.

The mechanism of microsomal electron transport proposed from the most recent detailed examination of the pyridine nucleotide involvement (Cohen & Estabrook, 1971,a,b,c,; Hildebrandt & Estabrook, 1971) has become an accepted working hypothesis. Apart from this study however, relatively little attention has been given to the estimation of accurate kinetic constants of the pyridine nucleotides during drug hydroxylation. The only available values using microsomes isolated from rat liver are from Orrenius (1965) and Schenkman *et al.*, (1967) who obtained apparent K_m (NADPH) values of 25μM and 28μM respectively for the N-demethylation of aminopyrine, while Oshino *et al.*, (1966) calculated a value of 15μM for aniline p-hydroxylase and Ullrich (1969a), a value of 10μM for the hydroxylation of cyclohexane.

The presence in the heterogeneous microsomal fraction of other enzymes that can both degrade and consume electrons from the pyridine nucleotides during drug hydroxylation is a significant problem which can be responsible for erroneous estimates of the kinetic parameters. The major examples of the former group of enzymes are nucleotide pyrophosphatase and NAD glycohydrolase, while the latter group is represented by fatty acid desaturase and lipid peroxidation. Most of the above workers have failed to consider the influence of these competing reactions on the numerical estimates of the kinetic constants.

This thesis reports the determination of the kinetic constants of NADPH for the metabolism of a range of drug substrates once the effects of these competing reactions have been minimised. Further, the influence of NADH and a structural analogue of NADH ($NADH_3$) on the kinetic parameters of NADPH have also been evaluated.

The results from these studies are not entirely consistent with any mechanism of microsomal electron transport that has been proposed to date. A modified mechanism based on the scheme of Estabrook & Associates is proposed, that is compatible with the results obtained.

C H A P T E R 2.

MATERIALS & METHODS

2.1. MATERIALS

Pyridine nucleotides, (both oxidised and reduced), cytochrome c, isocitrate (monopotassium salt), isocitrate dehydrogenase (E.C. 1.1.1.42, threo-Ds-Isocitrate:NADP oxidoreductase-decarboxylating; in 50% glycerin, approx. 40 IU/ml.), glutamic dehydrogenase (E.C. 1.4.1.3, L-glutamate:NADP oxidoreductase-deaminating, in 50% glycerin) and crystalline bovine serum albumin were obtained from Calbiochem. (Aust.) Sydney. All products were A grade.

Tetrasodium pyrophosphate (LR), perchloric acid (70%,AR), sucrose (AR) and acetylacetone (LR) were obtained from Ajax chemicals, Sydney.

Aminopyrine was the product of K & K Laboratories (Lot No. 8949 - A).

Ethylmorphine HCl was the product of T & H. Smith, England (Lot EY 3392).

Aniline HCl was prepared according to the method in Vogel (1962a, p 627) and recrystallised from water.

2,6 dihydroxyacetophenone (Mp = 151-153^{o}C) was kindly donated by Dr. A.J. Ryan, University of Sydney.

Tris ((Hydroxymethyl) aminomethane, 99.9%) was obtained from Sigma Chemical Co., U.S.A.

Tris buffers (1.0 M or 0.5 M) were adjusted to pH 7.25 with 10N HCl at 37^{o}C. All other Tris buffers were dilutions of the above stock solutions. α-ketoglutaric acid was also obtained from Sigma.

Carbon monoxide (Matheson Gas products, U.S.A.) gas was bubbled through a solution containing 16G sodium dithionite, 6.6G sodium hydroxyide, 2.0G sodium anthroquinone - β-sulphonate in 100 mls of water (Vogel 1962b, p1081) to remove trace amounts of oxygen.

Ammonium Chloride (AR) was obtained from Fluka (AG - Buchs).

Formaldehyde solution (37% w/v, AR) was the product of British Drug Houses (BDH), p-aminophenol (LR,BDH) was recrystallised from water before use.

Glass distilled water, which was subsequently passed through an ion exchange resin, was used for all solutions.

Diethyl ether (LR) was the product of May and Baker. The ether (approx. 2 litres) was washed with 200 mls of ferrous sulphate solution (5%), 200 mls of sodium hydroxide (1N) and distilled water (3 x 200mls) to remove any peroxides which might be present. Potassium chloride (LR), trisodium phosphate (LR) and ammonium acetate (LR) were obtained from May and Baker.

All other inorganic chemicals used were the highest grade commercially available.

2.2. METHODS

2.2.1. ANIMALS

Male hooded Wistar rats weighing 250-300 Gm. were housed under controlled conditions of light and temperature in suspended cages. Animals were allowed free access to both food(Charlick's rat and mouse cubes) and water to the time of killing, which was standardised (between 7.30am and 8.00am) to minimise diurnal variation in enzyme activities. (Radzialowski and Bousquet, 1968)

2.2.2. PRETREATMENT OF ANIMALS

Phenobarbital induced rats were administered an aqueous solution of 75mg phenobarbital/kg body weight, intraperitoneally in water for 3 days. The last dose was administered 20h before killing.

3,4-Benz(a)pyrene induced rats received 40mg/kg body weight in peanut oil, intraperitoneally, as a single dose 20h before

killing. Controls for the latter group received an equivalent intraperitoneal dose of oil. All microsomes other than those indicated were isolated from untreated male rats.

2.2.3. ISOLATION OF MICROSOMAL ENZYMES

Animals were killed by cervical dislocation, their livers quickly excised and chilled in 0.25M sucrose buffered with 2mM Tris-Cl, pH 7.5 (Buffered sucrose) at 4°C. All subsequent procedures were performed at 4°C.

The livers were weighed and then perfused with buffered sucrose to remove haemoglobin which might interfere with some spectral assays. Following the perfusion, the liver was minced and then homogenised (two strokes) with 3mls of buffered sucrose per g of liver (wet weight) using a motor driven Potter homogeniser. Identical perfusion and homogenisation technique was used for all liver samples.

Cell debris, nuclei, mitochondria and other heavy organelles were removed from the homogenate by successive centrifugations at 600 x g for 10 min and then 10,000 x g for 10 min. This procedure increased the yield of microsomal protein by minimising the trapping of microsomes in the pellet if the homogenate was centrifuged at 10,000 x g for 20 min (Cinti *et al.*, 1972a). The post mitochondrial supernatant was obtained by careful aspiration. Care was taken to avoid the fluffy layer at the top.

Microsomal preparations were then obtained from the 10,000 x g supernatant by either (a) centrifuging at 105,000 x g for 60 min (in a Beckman L2-65B ultracentrifuge). The microsomes were washed with buffered sucrose and sedimented again at the same speed for 45 min, or by (b) calcium aggregation by the method of Cinti *et al.*, (1972a). Calcium chloride was added to the post mitochondrial supernatant to give a final calcium concentration

of 8 mM.

The solution was allowed to stand at 4°C for 20 min and the microsomal fraction isolated by centrifuging at 27,000 g for 15 min. The original method of Cinti *et al.*, (1972a) was modified to the extent that the microsomes were washed with buffered sucrose, sedimented and finally suspended in 1.15% w/v KCl buffered with 10mM phosphate buffer, pH 7.5. The protein content of the microsomal suspension was determined by the method of Lowry *et al.*, (1951) using crystalline bovine serum albumin as standard.

2.2.4. ASSAYS CONDITIONS FOR THE METABOLISM OF DRUG SUBSTRATES AMINOPYRINE OR ETHYLMORPHINE-N-DEMETHYLASE

The *in vitro* incubation system consisted of 100mM Tris-Cl buffer, (pH 7.25 at 37°C) 20mM tetrasodium pyrophosphate (pH 7.5) 10 µmoles of isocitrate, 20 µmoles magnesium chloride, 0.6 IU isocitrate dehydrogenase, 10 µmoles semicarbazide HCl, NADP and/or NADH at the concentrations indicated in the text and 2.5mg of microsomal protein in a final volume of 2.5mls. Either aminopyrine or ethylmorphine was included at final concentrations of 4mM or 6mM respectively. Marbles were added to incubation beakers to improve mixing (Fouts, 1970). The microsomal enzyme suspension and the reaction mixture (see above) were separately incubated at 37°C for 5 min to reduce all the NADP to NADPH and equilibrate temperatures prior to initiating the reaction by the addition of the microsomal suspension at 15 s intervals. The reaction was stopped after a 5 min incubation period by the addition of 5 mls of 0.6N perchloric acid. The contents of the incubation beakers were transferred to glass tubes and centrifuged at 2,000 x g for 30 min The formaldehyde in the supernatant (3mls) was measured by the method of Nash (1953) as modified by Cochin & Axelrod (1959). The results have been expressed as

nmoles of formaldehyde formed/5min/mg microsomal protein.

ANILINE PARA-HYDROXYLASE

The incubation system was identical to that described above except that semicarbazide was omitted and aniline (4mM final concentration) replaced the other substrates. Further, the NADPH regenerating system in this assay consisted of 20 µmoles of glucose-6-phosphate and 0.25mls of soluble fraction (i.e. 105,000 x g supernatant from untreated rats) as the source of glucose-6-phosphate dehydrogenase instead of isocitrate and isocitrate dehydrogenase. The reaction was stopped after a 15 min incubation by the addition of 1ml of saturated NaCl solution. The p-aminophenol was estimated by the ether extraction method of Kato & Gillette (1965) as modified by Gram *et al.*, (1967a). This method was found to be more reproducible than the more rapid method of Imai *et al.*, (1966). The results have been expressed as nmoles p-aminophenol formed/15min/mg microsomal protein.

PARA-NITROANISOLE-O-DEMETHYLASE

The composition of the incubation media was identical to that used for aniline para-hydroxylase except p-nitroanisole replaced aniline as the substrate at a final concentration of 2mM. The reaction was stopped after a 30 min incubation by the addition of 2.5mls of a 10% trichloracetic acid solution. The amount of p-nitrophenol in the supernatant obtained by centrifuging of the reaction media at 2,000 x g for 15 min was estimated by the method of Netter & Seidel, (1964). The results have been expressed as nmoles p-nitrophenol formed/30 min/mg microsomal protein.

PARA-NITROBENZOIC ACID NITROREDUCTASE

The composition of the incubation media was identical to

that used for the aniline para-hydroxylase assay except that p-nitrobenzoic acid (6 µmoles/2.5mls of reaction medium) replaced aniline as the substrate and the incubation was performed under oxygen free nitrogen gas. The reaction was stopped after a 30 min incubation period by the addition of 5 mls of 10% trichloracetic acid solution. The amount of p-aminobenzoic acid formed was measured by the method of Fouts & Brodie (1957). The results have been expressed as nmoles p-aminobenzoic acid formed/30 min/mg microsomal protein.

LIPID PEROXIDATION

Endogenous lipid peroxidation was measured by malondialdehyde formation with the thiobarbituric acid reagent (Bernheim *et al.*, 1948) as described by Ernster & Nordenbrand (1967). The microsomes were isolated and washed as described above. The surface of the final particulate fraction was rinsed with buffered KCl prior to resuspension to reduce the amount of residual sucrose which interferes with the colour reaction (Ernster & Nordenbrand, 1967; Wills & Wilkinson, 1967).

For the estimation of endogenous malondialdehyde the composition of the reaction media was identical to that used for the demethylation of either aminopyrine or ethylmorphine except that semicarbazide was omitted because it has been shown to interfere with the development of the coloured malondialdehyde-thiobarbituric acid complex (Wilbur *et al.*,1949; Kohn & Liversedge, 1944). The colour of the complex was developed at 80°C instead of 100°C as this minimised the interference caused by residual sucrose. (Fortney & Lynn, 1964; Wills & Wilkinson, 1967). A molar extinction coefficient of 156,000 cm^{-1} at 535 nm was used to calculate the concentration of the malondialdehyde

(Sinnhuber *et al.*, 1958). It should be noted that this method does not measure lipid peroxidation directly, but rather malondialdehyde and other breakdown products of oxidised unsaturated fatty acids. This factor has raised some doubts as to the validity of this widely used method to measure endogenous lipid peroxidation (Philpot, 1963). An alternative method has been suggested (Wills, 1966) in which the absorbance at 535 nm of samples under standard assay conditions is measured and expressed as the thiobarbituric acid value.

Nevertheless, Wills & Rotblat (1964) and Wills (1966) have shown that measurements of lipid peroxidation by the thiobarbituric acid method correlated well with other methods (e.g. the ferric thiocyanate method of Wagner *et al.*, 1947, and measuring $A_{232\ nm}$ (Holman, 1954) for peroxide determination), and that the thiobarbituric acid method was the most convenient for studying peroxide formation in tissues.

2.2.5. SPECTRAL DETERMINATIONS

(a) CYTOCHROMES

Cytochrome P_{450}

Hepatic cytochrome P_{450} was estimated by the method of Omura & Sato (1964a,b). Microsomal suspensions (5mg/ml) were diluted to (3mg/ml) with phosphate buffer (pH 7.5.) to give a final buffer concentration of 0.1M. Sodium dithionite (5mg approximately) was then added to the suspension which was dispensed equally into two silica cuvettes. After establishing a baseline between 400 nm and 500 nm in a Pye Unicam SP 8,000 recording spectrophotometer, the test cuvette was bubbled with carbon monoxide for one min and the spectrum re-recorded. The amount of cytochrome P_{450} was estimated by the difference in absorbance between 450 nm and 490 nm using a millimolar extinction coefficient of $91cm^{-1}$ (Omura & Sato,

1964a,b). The results have been expressed as nmoles cytochrome P_{450}/mg microsomal protein.

Cytochrome b_5

The amount of cytochrome b_5 was estimated according to the method of Omura & Sato (1964a). Microsomes were diluted to a protein concentration of 2mg/ml in a media containing 100mM Tris-Cl buffer (pH 7.5), 20 µmoles magnesium chloride and 20mM sodium pyrophosphate (pH 7.5).

When an NADPH regenerating system was included, isocitrate (10 µmoles) and 0.6 IU of isocitrate dehydrogenase were added in each 2.5ml of reaction medium. The concentration of either aminopyrine or ethylmorphine when included in this assay were identical to those used in metabolic studies (i.e., 4mM and 6mM respectively). The reaction media was dispensed into two silica cuvettes and a baseline between 350 nm and 450 nm was measured in the SP 8,000. NADH (to give a final concentration of 100 µM) was added to one cuvette and an equal amount of buffer to the reference cuvette and the difference spectrum recorded. The difference in absorbance between 424 nm and 409 nm was taken as the amount of cytochrome b_5 present using a milli-molar extinction coefficient of 185 (Omura & Sato, 1964a).

(b) ENZYME ASSAYS

NADPH Oxidase

NADPH oxidase activity was determined by the method of Gillette *et al.*, (1957) at a protein concentration of 1.6mg/ml. The reduction in absorbance at 340 nm after the addition of NADPH (20 µl of a 12.6mM solution in 10mM Tris-Cl buffer pH 7.5) was monitored for at least one min. The results have been expressed as nmoles NADPH oxidised/min/mg microsomal protein.

NADPH-Cytochrome c Reductase

NADPH-cytochrome c reductase activity was measured in 10 x 10mm silica cuvettes containing 2.5mls of reaction medium as described by Phillips & Langdon (1962).

The assay medium contained 0.1mg of microsomal protein/ml, 0.3mM potassium cyanide, 0.05mM cytochrome c, 10 µmoles magnesium chloride and 0.1M Tris-Cl buffer (pH 7.5). The reduction of cytochrome c at 25°C was followed at 550 nm, using the initial linear phase to calculate the rate of the reaction and a millimolar extinction coefficient of 18.5.

The results have been expressed as nmoles cytochrome c reduced/min/mg microsomal protein.

NADPH-Cytochrome P_{450} Reductase

NADPH-cytochrome P_{450} reductase was measured in 10mm x 10mm silica cuvettes containing 2.5mls of reaction medium as described by Holtzman *et al.*, (1968) with the following modifications:-

The bulk buffer medium which contained 125mM Tris-Cl buffer (pH 7.5), 25mM pyrophosphate (pH 7.5), and 25 µmoles of magnesium chloride at 37°C, was bubbled with dithionite scrubbed nitrogen (Vogel,1962a, P1081) for 60 min. The microsomal suspension was resuspended in 1.15% KCl buffered with 10mM sodium phosphate (pH 7.5) to a protein concentration of 15mg/ml and then diluted to 3mg/ml with the nitrogen bubbled bulk buffer medium.

The concentrations of Tris buffer, magnesium chloride, and pyrophosphate in the final assay medium were therefore identical to that used for metabolic experiments.

The diluted microsomal suspension was first bubbled with dithionite scrubbed nitrogen for 10 min. An aliquot (2.5mls) was then transferred to an Aminco anaerobic cuvette and 50 µl of an

appropriate dilution of NADPH standard solution placed in a plunging device which was fitted to the top of the cuvette.

Dithionite scrubbed carbon monoxide was passed through the cuvette for a further 5 min while the cuvette contents were adjusted to 26°C. The cuvette was sealed, placed in the spectrophotometer and the reaction initiated by depressing the plunger once. When substrates were incorporated into this assay, the final concentrations were identical to those used in metabolic experiments.

However, it was observed that when aniline HCl was the added substrate, considerable clumping of the microsomal suspension occurred while the temperature of reaction components was being adjusted to 26°C. This effect was overcome by the addition of a small magnetic bar to the cuvette which allowed the cuvette contents to be completely mixed without contamination of the two solutions, just prior to the initiation of the reaction.

The assay was found to be more reproducible when the assay temperature was 26°C instead of the usual 37°C, confirming the observation of Fouts & Pohl (1971).

Both standard NADH and NADPH solutions were thoroughly mixed and an aliquot added to the plunging device in experiments employing both reduced pyridine nucleotides. A milli-molar extinction coefficient of 91 (Omura & Sato, 1964a) was used to calculate the number of nmoles of cytochrome P_{450} reduced/min/mg microsomal protein.

In experiments concerned with the biphasic reduction kinetics of cytochrome P_{450}, (Chapter 5), the absorbance at 450 nm was monitored until asyptotic (4 min). The amount of cytochrome P_{450}-CO complex at time, t, seconds was calculated thus:-

$$A_{450,\infty} - A_{450,t}$$

where $A_{450,\infty}$ represents the absorbance at 450 nm at infinite time (4 min) and $A_{450,t}$ represents the absorbance at time, t, sec. This reading at time, t, was divided by $A_{450,\infty}$ to give % unreduced cytochrome P_{450}-CO complex, which was plotted logarithmically as a function of time.

NADPH oxidase, NADPH-cytochrome c reductase and NADPH-cytochrome P_{450} reductase assays were all performed using a Gilford 2,400S recording spectrophotometer.

2.2.6. STANDARDISATION OF THE PYRIDINE NUCLEOTIDE CONCENTRATION FOR KINETIC EXPERIMENTS.

Reduced pyridine nucleotides were standardised spectrophotometrically at 340 nm using a milli-molar extinction coefficient of 6.22 (Horecker & Kornberg, 1948), and were cell corrected. Cell corrections were generally in the range of 0.01 to 0.02 for the matched cells used throughout the study. Oxidised pyridine nucleotides were first reduced (isocitrate/isocitrate dehydrogenase for NADP) and then standardised as above.

2.2.7. DETERMINATION OF NADPH CONCENTRATION DURING HYDROXYLATION REACTIONS.

The concentration of NADPH remaining during aminopyrine demethylation and the para hydroxylation of aniline was determined essentially by the method of Klingerberg (1963). One millilitre samples were removed from the incubation medium at selected time intervals and mixed with 0.5mls of 2.8% KOH in 50% aqueous ethanol (Vortex mixer). The pH was reduced to 7.5 - 8.0 by the addition of 2mls of 2.0M Tris buffer (pH 7.5) mixed as before and clarified by centrifuging at 2,000 x g for 30 min. A 2ml aliquot was added to a silica cuvette together with 0.5mls of 25mM

ammonium chloride and 0.5mls of 25mM α keto glutaric acid and the absorbance at 340 nm determined (Gilford 2,400S spectrophotometer). The reduction in absorbance at 340 nm after the addition of 5 µl glutamic dehydrogenase (in 50% aqueous glycerin) is taken as the amount of NADPH present (EmM=6.22).

The initial NADPH concentration in the incubation medium was determined as described previously (2.2.6.). The concentration of NADPH in zero time samples that were determined by this method were within 4% of stated concentration.

2.2.8. DETERMINATION OF KINETIC PARAMETERS.

Kinetic parameters were evaluated from experiments which employed eight duplicate substrate concentrations which were arranged in geometric progression ranging from approximately $0.2K_m$ to 20 - $50K_m$. Preliminary experiments were carried out to approximate the K_m value. At least two but usually three substrate concentrations were below the K_m, one was at the K_m value, while the rest were above the K_m value. Each value for a kinetic constant quoted was obtained from at least three animals or three pools of animals. The actual number are given in the tables or the legends to the figures.

There was no statistically significant difference in the results obtained when many of the experiments were repeated on different days with fresh liver samples. The time lag between these duplicate determinations was, in some cases, approximately 12 months, indicating the reproducability of assay techniques.

All K_m and V values reported in this thesis are apparent values determined under the conditions described in the Methods section.

2.2.9. COMPUTATION OF KINETIC CONSTANTS.

The computer programme HYPER, written by Cleland (1967) was translated into BASIC (Appendix 1) and run on either a PDP 11/20 or a PDP 11/40 computer. This programme is based on an earlier programme of Wilkinson (1961) and calculates K_m, V, 1/V, V/K_m and K_m/V values and their standard deviations. The programme was modified to calculate a correlation coefficient,r, once convergence had occurred which has been defined by Wagner & Sedman (1973) as,

$$r^2 = \frac{\Sigma obs^2 - \Sigma(obs-calc)^2}{\Sigma obs^2}, \qquad r = \sqrt{r^2}$$

where "obs" represents the experimental readings and "calc" represents the calculated velocity (at that substrate concentration) using the computed K_m and V values. For the hypothetical case where every point lies on the calculated line, $\Sigma(obs - calc)^2 = 0$, therefore r = 1, i.e., the closer the r value is to unity, then the velocity readings must lie in close proximity to the calculated curve. These modifications to the programme are lines 430 to 455 and 980 to 985 (Appendix 1).

This programme has the facility for the data to be weighted during the computations. A mean, standard deviation and the coefficient of variation (standard deviation/mean x 100) was calculated for the velocity readings at each substrate concentration.

The velocity readings at each substrate concentration were weighted with the reciprocal of the variance (1/VAR) and the reciprocal of the coefficient of variation (1/CV) for that substrate concentration. Further, a weight factor of unity which corresponds to an unweighted regression was also used.

The criteria on which selection of kinetic data to be used was based on the following:-

(1) that the CV of any parameter be not more than 20%,

(2) that the data points be equally distributed about the computer generated line of best fit, especially in the area where the rate of change in slope of the tangents to the curve is greatest,

(3) that the correlation coefficient r, be greater than 0.98. The correlation coefficient gives an indication of the fit of the data to the model, (i.e., equation describing Michaelis-Menten kinetics). If any one of the above criteria were not satisfied the experiment was repeated.

A more detailed discussion of why HYPER was the programme selected and the influence of the different weight factors on the kinetic constants is given in Chapter 3. This selection procedure was performed on every kinetic constant quoted in this thesis.

2.2.10. PREPARATION OF 1,4,5,6 TETRAHYDRONICOTINAMIDE ANALOGUE OF NADH ($NADH_3$).

NAD (150 - 200mg) was hydrogenated using 1% Palladium suspended on barium carbonate at $4^{\circ}C$ and atmospheric pressure according to Dave *et al.*, (1968). The hydrogenation was performed in a 100ml Quickfit flask with a side arm sealed with an air tight rubber closure. This apparatus allowed the removal of samples to monitor the extent of the reduction during hydrogenation. The hydrogenation was stopped before all the NAD was reduced at a point when the 289/265 nm absorbance ratio reached 0.75.

The catalyst was removed by centrifuging at 2,000 x g at 4°C for 20 min and the solution further clarified by passage through a 0.2 μ membrane filter[1] in a Swinney adaptor.[2]

$NADH_3$ was purified by the method described by Stock (1969). The solution was applied to an 8mm x 20cm DEAE cellulose column (bicarbonate form) at 4°C.

Samples of 6mls were collected when the column was eluted with a linear gradient of 0 - 0.2M ammonium bicarbonate, (150mls in resevoir and mixing chambers). Samples with a 289/265 nm ratio of greater than 0.85 were bulked and lypholised.

The residue was reconstituted with 10mM Tris buffer (pH 7.5) and clarified by filtering using a 0.2 μ membrane filter in a Swinney adaptor and stored at 4°C until required. A milli-molar extinction coefficient of 17.7 at 289 nm (Stock, 1969) was used to calculate the concentration of $NADH_3$ in solution. Although previous work (Stock, 1969) has shown that $NADH_3$ lost only 7% of the 289 nm chromophore over 34 days at 4°C, all samples were used within one week of preparation.

2.2.11. CLEANING OF SPECTROPHOTOMETRIC CELLS AND GLASSWARE.

All cells (both silica and glass) used in this study were stored in a solution of 3% sodium nitrate, 3% sodium perchlorate in concentrated sulphuric acid in a closed glass container. The cells were rinsed thoroughly with distilled water and then with 10mM Tris-Cl buffer, (pH 7.5) and allowed to drain before use.

All other glassware was washed in detergent, (Pyroneg, Diversey Products, Australia) rinsed with distilled water (5 times) and dried in an oven.

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1 Cellulose Acetate, OXOID, England.

2 25mm Sartorius Membrane Filter holder, Germany.

2.2.12. STATISTICS

The unpaired Student t test (two tailed) was used to compare the results of different experiments with a level of significance of at least $p < 0.05$ to reject the null hypothesis.

C H A P T E R 3

FACTORS THAT INFLUENCE NADPH KINETIC ESTIMATIONS DURING MIXED FUNCTION OXIDASE REACTIONS

3.1. A COMPARISON OF THE ENZYME ACTIVITIES IN MICROSOMES ISOLATED BY ULTRACENTRIFUGING AND CALCIUM AGGREGATION.

3.1.1. INTRODUCTION

The majority of drug hydroxylation reactions are catalysed by the enzyme system which is localised within the endoplasmic reticulum of the intact liver cell. After mechanical disruption of the liver, this enzyme complex can be isolated from some other cellular components by centrifuging. Initial centrifugation at 10,000 x g removes the cell debris and heavy organelles such as nuclei and mitochondria,etc. Then, the microsomal fraction can be isolated from the post mitochondrial supernatant by one of the following methods:- ultracentrifugation, (Schneider & Hogeboom, 1950), calcium aggregation (Cinti *et al.*, 1972a), iso electric point precipitation (Fry & Bridges, 1975), and a gel filtration procedure (Tangen *et al.*, 1973).

The ultracentrifuging procedure has been most widely used, but suffers from the disadvantage that it is a lengthy one. On the other hand, the calcium aggregation method, originally reported by Kamath & Narayan (1972) is rapid and requires a maximum force of only 27,000 x g. Therefore the activities of microsomes isolated by calcium aggregation and ultracentrifugation are compared in the following section.

3.1.2. RESULTS

The content of cytochrome P_{450} and b_5, and the activities of aniline hydroxylase, aminopyrine demethylase, p-nitroanisole demethylase and p-nitrobenzoic acid reductase for microsomes isolated by calcium aggregation and ultracentrifugation from untreated rats are compared in Table 3.1. Further, the interaction of a Type I substrate (Hexobarbital) and a Type II substrate

(Aniline) with cytochrome P_{450} estimated from binding studies, (K_s, ΔA_{max} values) are also given in this Table.

TABLE 3.1.

A comparison of the activities of microsomes from control rats isolated by Calcium Aggregation and Ultracentrifugation.

PARAMETER	Ca AGGREGATION	ULTRA-CENTRIFUGATION
Cytochrome P_{450} [a]	0.77 ± 0.036	0.79 ± 0.051
Cytochrome b_5 [a]	0.42 ± 0.022	0.37 ± 0.04
Aniline p-Hydroxylase [b]	22.6 ± 1.6	20.8 ± 0.86
Aminopyrine Demethylase [b]	144.9 ± 6.7	152.6 ± 6.6
p-Nitroanisole Demethylase [b]	28.2 ± 1.26	27.6 ± 2.1
p-Nitrobenzoic Acid Reductase [b]	45.7 ± 4.33	46.9 ± 5.93
BINDING STUDIES		
Hexobarbital K_s [c]	0.134± 0.039	0.111± 0.042
ΔA_{max} [d]	0.046± 0.004	0.045± 0.005
Aniline K_s	0.58 ± 0.13	0.62 ± 0.12
ΔA_{max}	0.057± 0.003	0.057± 0.003

a. Expressed as nmoles/mg microsomal protein.

b. Expressed as nmoles product formed/mg microsomal protein/30min.

c. Spectral dissociation constant.

d. Maximum spectral change between the wave length pairs of 419 nm and 385 nm for hexobarbital and 430 nm and 392 nm for aniline.

Livers from 12 rats were divided into four pools. The homogenate (in buffered sucrose) was centrifuged at 10,000 x g for 20 min. The post mitochondrial supernatant was divided into 2 equal portions and the microsomal fraction isolated by ultra-

Table 3.1. continued

centrifugation (from one portion) and calcium aggregation (from other portion). The microsomes were washed with buffered sucrose and resedimented. Assays were performed as described in Chapter 2.

There is no significant difference in any parameter between the two different methods used to prepare the microsomal fraction.

Tables 3.2. and 3.3. show the results obtained from similar experiments when the rats were treated prior to sacrifice with sodium phenobarbital and 3,4 benz(a)pyrene respectively. The two methods isolate functionally similar microsomal fractions from induced rats except for aniline para-hydroxylase activity in 3,4 benz(a)pyrene treated rats.

3.1.3. DISCUSSION

Various reports (Kupfer & Levin, 1972; Kamath *et al.*,1971; Kamath & Narayan, 1972; Schenkman & Cinti, 1972) have indicated that the calcium method yields a microsomal fraction which is functionally similar to that obtained by the conventional ultra-centrifugation procedure. Further, Cinti *et al.*,(1972a) have shown that the kinetic parameters (K_m, V) of ethylmorphine and aminopyrine demethylases, aniline hydroxylase, hexobarbital oxidase and p-nitroanisole demethylase are also identical in both methods. The results obtained in the present study reinforce this conclusion.

While the calcium method has been used to isolate the microsomal fraction from rats treated with phenobarbital, 3-methylcholanthrene and acetone, (Rao *et al.*, 1975) the justification for doing so has not been established by comparative studies. However, the present work suggests that the microsomal fraction

isolated by the calcium method from phenobarbital and 3,4 benz(a)-pyrene pretreated rats is identical in activity to conventionally isolated microsomes.

Microsomes were isolated by the calcium aggregation method for all subsequent experiments reported in this thesis.

TABLE 3.2.

A comparison of the activities of microsomes isolated from rats previously treated with phenobarbital by Calcium Aggregation and Ultracentrifugation.

PARAMETER	Ca AGGREGATION	ULTRA-CENTRIFUGATION
Cytochrome P_{450}	1.79 ± 0.1	1.83 ± 0.17
Cytochrome b_5	0.46 ± 0.024	0.47 ± 0.04
Aniline p-Hydroxylase	51.83 ± 3.5	55.54 ± 2.6
Aminopyrine Demethylase	378.8 ±10.2	370.0 ± 5.5
p-Nitroanisole Demethylase	68.7 ± 2.55	70.8 ± 1.42
p-Nitrobenzoic Acid Reductase	143.6 ± 8.04	154.9 ± 9.22
BINDING STUDIES		
Hexobarbital K_s	0.157 ± 0.084	0.145 ± 0.06
ΔA_{max}	0.094 ± 0.019	0.12 ± 0.017
Aniline K_s	0.935 ± 0.25	0.778 ± 0.15
ΔA_{max}	0.103 ± 0.009	0.117 ± 0.009

The activities (i.e. nmoles/mg microsomal protein/time) are identical to those given in the legend of Table 3.1.

Rats were treated with sodium phenobarbital (75mg/kg body weight) daily for three consecutive days prior to killing. Microsomes were isolated as described in the legend to Table 3.1.

TABLE 3.3.

A comparison of the activities of microsomes isolated from rats previously treated with 3,4 Benz(a)pyrene, Calcium Aggregation and Ultracentrifugation.

PARAMETER	Ca AGGREGATION	ULTRA-CENTRIFUGATION
Cytochrome P_{450}	1.02 ± 0.18	1.35 ± 0.21
Cytochrome b_5	0.565 ± 0.035	0.568 ± 0.01
Aniline p-Hydroxylase[a]	22.86 ± 1.52	28.27 ± 1.52
Aminopyrine Demethylase	142.1 ± 5.27	147.7 ± 4.7
p-Nitroanisole Demethylase	79.9 ± 1.96	79.2 ± 1.55
p-Nitrobenzoic Acid Reductase	60.3 ± 6.37	57.77 ± 0.94
BINDING STUDIES		
Hexobarbital K_s	0.046 ± 0.049	0.089 ± 0.079
ΔA_{max}	0.014 ± 0.002	0.018 ± 0.004
Aniline K_s	0.339 ± 0.102	0.318 ± 0.065
ΔA_{max}	0.085 ± 0.007	0.097 ± 0.008

a. significantly different at $P < 0.005$.

The activities (nmoles/mg microsomal protein/time) are identical to those given in legend of Table 3.1. Rats were treated with 3,4 benz(a)pyrene in oil (40mg/kg body weight), as a single intraperitoneal dose 20 hr before killing. Microsomes were isolated as described in the legend to Table 3.1.

3.2. THE INFLUENCE AND CONTROL OF ENZYMES COMPETING WITH THE MIXED FUNCTION OXIDASE ENZYME COMPLEX FOR PYRIDINE NUCLEOTIDES.

3.2.1. INTRODUCTION

The microsomal fraction, (isolated by differential centrifugation) is a heterogenous fraction which contains, in addition to the mixed function oxidase system, enzymes that can either utilise the reduced forms of the pyridine nucleotides or degrade them. Certain intermediates of the multi-component cytochrome P_{450} system have been implicated in lipid peroxidation (Orrenius *et al.*, 1964; Wills, 1969a; Kamataki & Kitagawa, 1973; and Kamataki *et al.*, 1974), fatty acid desaturation (Oshino *et al.*, 1971), w-hydroxylation of long chained fatty acids (Preiss & Block, 1964), and steroid hydroxylations (Kuntzman *et al.*, 1964). Such potentially competing reactions could influence the concentration of NADPH available for drug hydroxylations.

In addition to these NADPH requiring reactions, there is also a high activity of NAD glycohydrolase and a nucleotide pyrophosphatase enzyme localised in the microsomal fraction. Both of these enzymes degrade the pyridine nucleotides.

This section describes the influence and control of these reactions. It also considers the effect of various NADPH regeneration systems on NADPH concentrations remaining at different times during the demethylation of aminopyrine and hydroxylation of aniline. The effects of protein concentration and linearity of reaction velocity with respect to time are also examined.

3.2.2. RESULTS

(a) Effect of various NADPH regenerating systems

Figure 3.1. shows the amount of p-aminophenol formed from aniline and the concentrations of NADPH remaining at selected time

FIGURE 3.1.

The effect of various NADPH regenerating systems on the amount of p-aminophenol formed and the concentration of NADPH remaining at selected time intervals.

The regenerating systems used were:-

■——■ soluble fraction/glucose-6-phosphate (0.25mls of soluble fraction (from control rat liver) and 20 µmoles per 2.5mls of complete reaction mixture respectively),

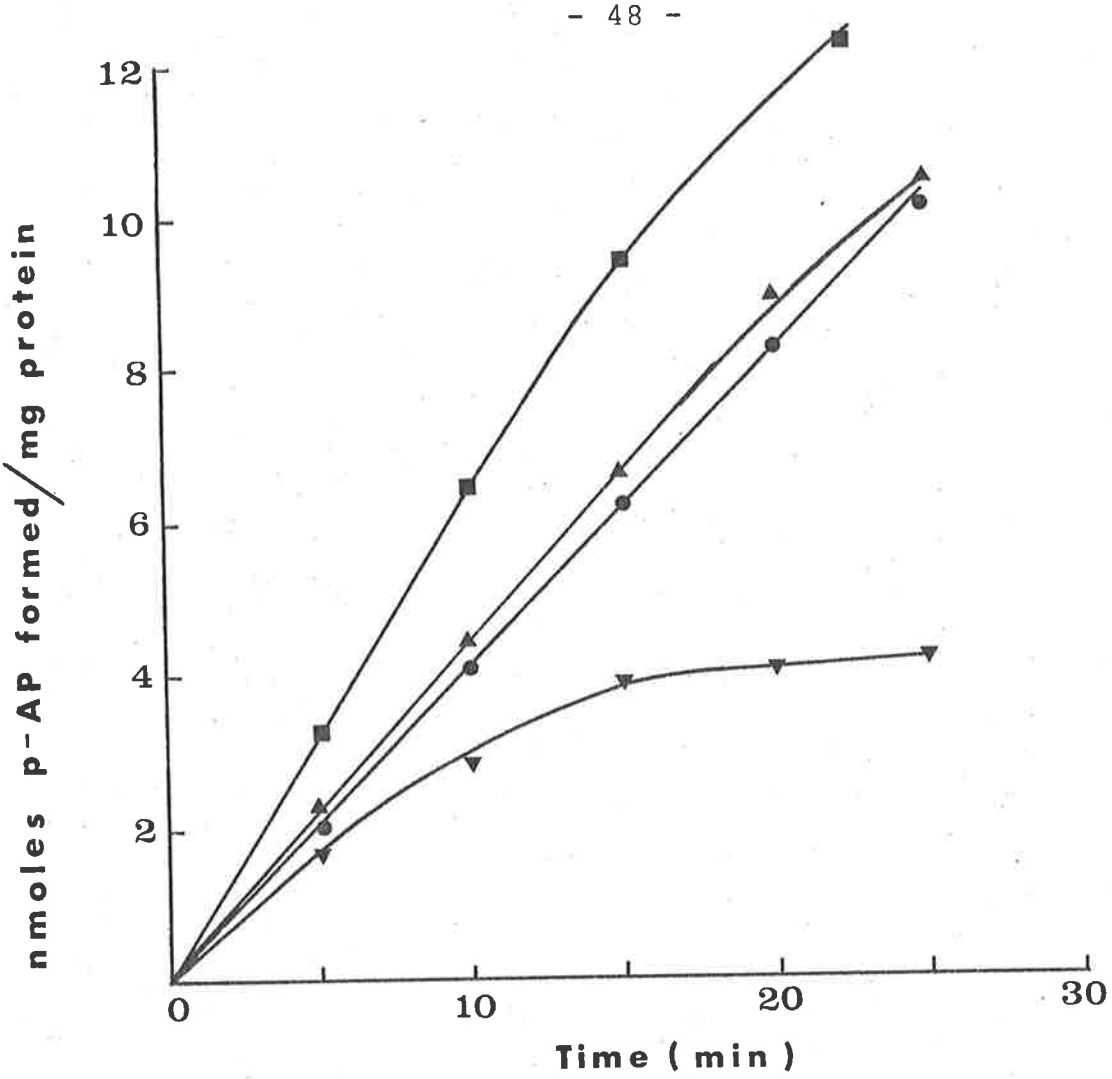
●——● isocitrate/IDH (10 µmoles and 0.6 IU per 2.5mls of complete reaction mixture respectively),

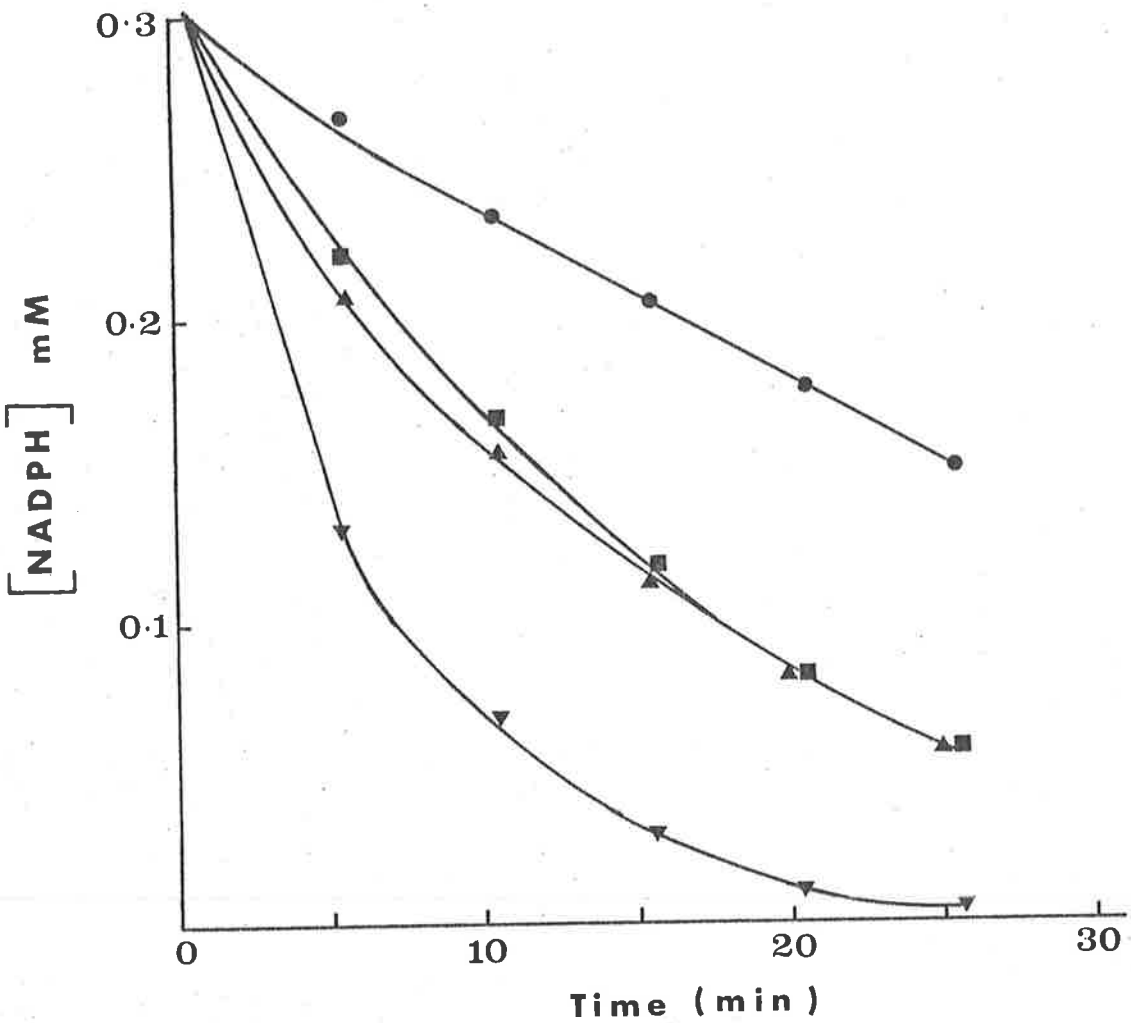
▲——▲ ammonium sulphate fractionated soluble fraction/ glucose-6-phosphate (0.25mls of 0-60% cut of the soluble fraction from control rat liver (resuspended to the original volume) and 20 µmoles per 2.5mls of complete reaction mixture respectively),

▼——▼ NADPH in the absence of a regenerating system.

The reaction medium (25mls) contained 100mM Tris-Cl buffer (pH 7.5), 100 µmoles magnesium chloride, 4mM aniline, 300 µM NADP and one of the NADPH regenerating systems above. For the NADPH alone experiment (▼——▼), 300 µM NADPH replaced NADP.

The microsomal enzyme suspension and the reaction mixture (minus microsomal protein) were incubated separately at 37°C for 5 min to equilibrate termperature and reduce NADP to NADPH where appropriate. The reaction was initiated by the addition of microsomal protein to give a final concentration of 1mg/ml, Aliquots (2.5mls) were removed at 5 min intervals for the estimation of p-aminophenol. The NADPH concentration remaining was estimated in 1ml aliquots which were removed 30 sec after the sample for product formation (Methods 2.2.7). This experiment was repeated twice and similar results were obtained.


12
10
8
6
4
2
nmoles p-AP formed/mg protein
0
10
20
30
Time (min)


0·3
0·2
0·1
[NADPH] mM
0
10
20
30
Time (min)

intervals in the presence of various NADPH regenerating systems. Included in Figure 3.1. are the results obtained when NADPH was added in the absence of a regenerating system. The composition of the remainder of the incubation medium is given in the Methods. The hydroxylation of aniline was greatest with the 100,000 x g supernatant/glucose-6-phosphate system and least when NADPH was added in the absence of any regenerating system. The isocitrate/IDH and the ammonium sulphate fractionated soluble fraction systems produced nearly identical rates of formation of p-aminophenol. However, the concentration of NADPH remaining at the different time intervals did not follow the above trend. For example, destruction of NADPH was minimal with the isocitrate/IDH system, greater with both soluble fraction systems and the greatest decay was observed with NADPH alone in the absence of a regenerating system.

However, the latter readings represent NADPH oxidation as well as NADPH destruction. Because it gave consistently elevated levels of NADPH, the isocitrate/IDH regenerating system was used in all subsequent experiments reported in this thesis.

(b) The Influence and Control of Nucleotide Pyrophosphatase and NAD Glycohydrolase Activities.

Enzymes which can destroy NADPH are NAD glycohydrolase and nucleotide pyrophosphatase (Figure 3.2.) both of which occur in the microsomal fraction. Of these, nucleotide pyrophosphatase has been inhibited by pyrophosphate, AMP, sodium fluoride and EDTA in detergent solubilised rat liver microsomes to various degrees (Bachorik & Dietrich, 1972).

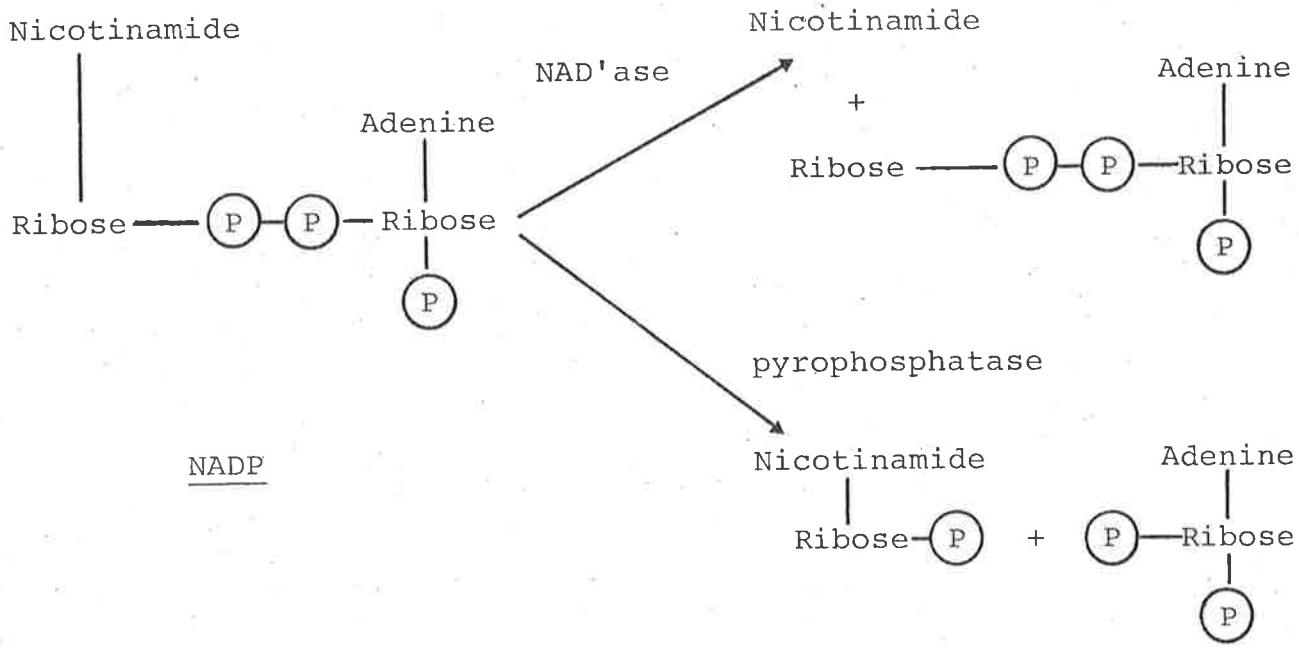


FIGURE 3.2. The products of the reactions catalysed by NAD glycohydrolase (NAD'ase) and nucleotide pyrophosphatase with NADP as substrate.

Figure 3.3. shows the rate of product formation (from both aminopyrine (figure 3.3.A) and aniline (figure 3.3.B))and the levels of NADPH remaining as a function of time in the presence and absence of 20mM pyrophosphate (adjusted to pH 7.5). With both substrates, the inclusion of pyrophosphate was responsible for an elevated concentration of NADPH during the mixed function oxidase reactions.

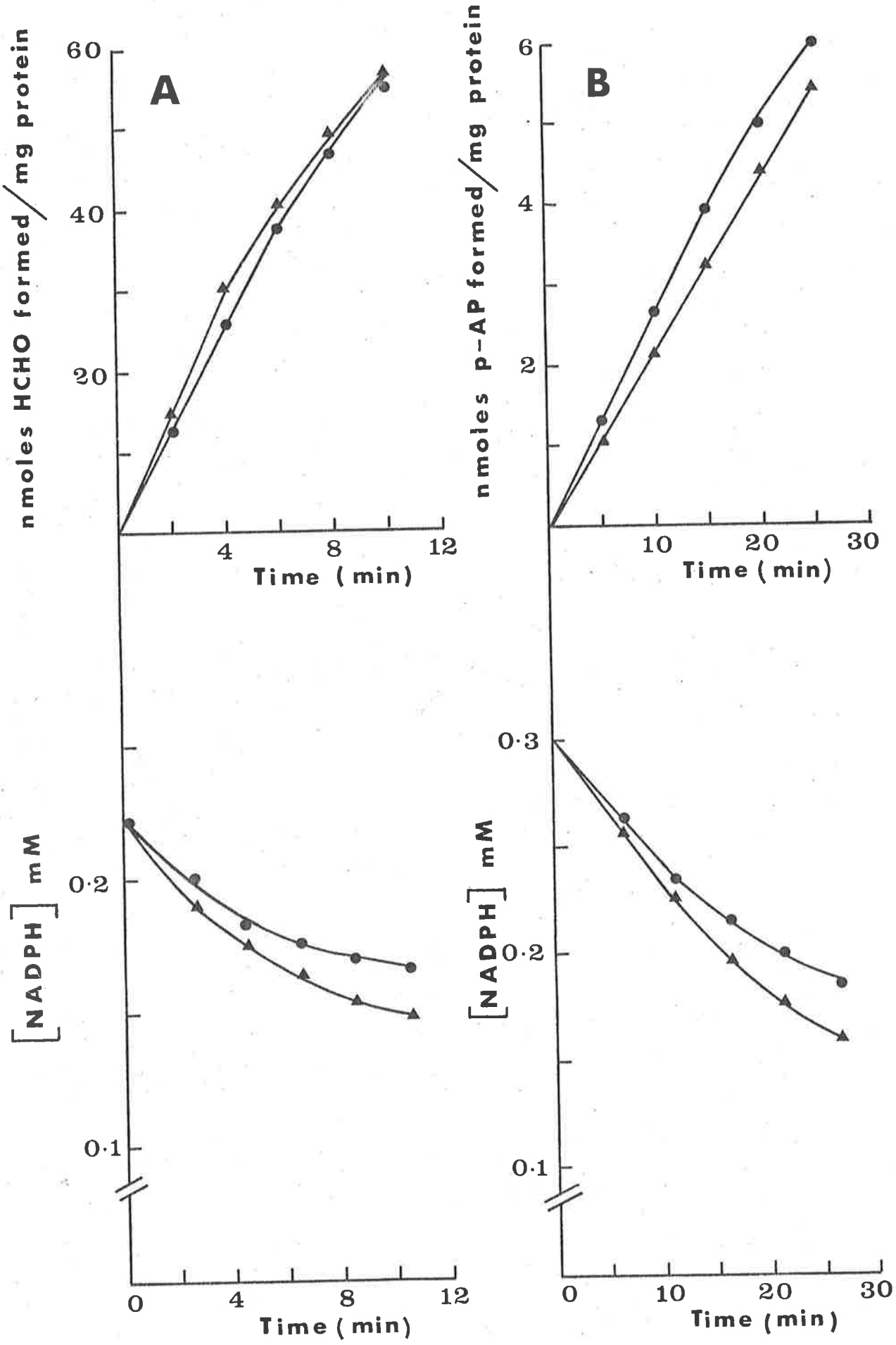
However, the production of formaldehyde from aminopyrine was slightly inhibited when pyrophosphate was included in the incubation medium where as p-aminophenol formation appears to be slightly stimulated. Higher pyrophosphate concentration (30mM) increased the inhibition of aminopyrine demethylation and also inhibited aniline hydroxylation without showing any greater stabilisation of NADPH levels. Therefore, sodium pyrophosphate was routinely incorporated in kinetic experiments at a final concentration of

FIGURE 3.3.

The influence of pyrophosphate on the rate of product formation and NADPH levels remaining during aminopyrine demethylation and aniline hydroxylation.

The reaction medium (25mls) contained 100mM Tris-Cl buffer (pH 7.5), 100 µmoles of isocitrate, 200 µmoles of magnesium chloride, 100 µmoles of semicarbazide (for aminopyrine demethylase assay only), 6.0 IU isocitrate dehydrogenase, NADPH at the concentrations indicated below and 1mg/ml of microsomal protein. The substrates included in the assay were aminopyrine (Figure 3.3.A, final concentrations of 4mM) and aniline (Figure 3.3.B, final concentration of 4mM). The initial concentrations of NADPH determined as described in the Methods (2.2.6) were 230 µM (aminopyrine) and 310 µM (aniline). The NADPH concentration in the zero time samples were within 4% of the stated concentration.

The assays were performed in the presence (●——●) and absence (▲——▲) of 20mM pyrophosphate. The microsomal enzyme suspension and the reaction mixture (minus microsomal protein) were incubated separately at 37^{o}C for 5 min to reduce all the NADP to NADPH and equilibrate temperatures. At selected time intervals after the initiation of the reaction, the amount of formaldehyde (Fig. A) and p-aminophenol (Fig. B) were estimated in 2.5ml aliquots of the reaction medium as described in the Methods. The concentration of NADPH remaining was estimated using 1ml aliquots which were removed 30 sec after the sample for product formation, as described in the Methods (2.2.7). The experimental readings are the means of three determinations.


A
B
nmoles HCHO formed/mg protein
nmoles p-AP formed/mg protein
60
40
20
6
4
2
4
8
12
10
20
30
Time (min)
Time (min)
[NADPH] mM
0·3
0·2
0·1
0
4
8
12
10
20
30
Time (min)
Time (min)

20mM. NADPH levels could not be detected after two min incubation in the absence of pyrophosphate during aminopyrine demethylation with an NADPH concentration of 52 µM. The NADPH levels at the same time in the presence of 20mM pyrophosphate was 80% of the initial concentration.

This experiment was repeated with initial NADPH concentrations of 20 µM and 10 µM, but the results fluctuated because these NADPH concentrations were at the limit of the assay sensitivity. Similar pyrophosphate stabilisation of NADPH levels was observed with the soluble fraction/glucose-6-phosphate regenerating system.

NAD glycohydrolase, has been reported to utilise the oxidised (Zatman *et al.*, 1953) but not the reduced form (Hofman & Rapoport, 1955) of the pyridine nucleotides, and is competitively inhibited by nicotinamide (Mann & Quastel, 1941).

When nicotinamide (10mM) was included in the reaction medium in place of pyrophosphate, the profile of NADPH degraduation was identical to that obtained in the presence of isocitrate/IDH alone, (Figure 3.1.). Further, the results obtained in the presence of both pyrophosphate (20mM) and nicotinamide (10mM) were identical to those obtained in the presence of pyrophosphate alone, (Figure 3.3.B).

Rates of formaldehyde production from aminopyrine were not increased in the presence of 10mM nicotinamide with NADPH concentrations ranging from 110 µM to 5 µM.

(c) Influence of Lipid Peroxidation

Aminopyrine (Wills, 1969a), ethylmorphine (Jeffrey & Mannering, 1974) high concentrations of pyrophosphate (Ernster & Nordenbrand, 1967) and commercial preparations of isocitrate dehydrogenase (Kotake *et al.*, 1975) have all been shown, individually to inhibit endogenous NADPH supported lipid

peroxidation. The extent of endogenous lipid peroxidation was measured as described in the Methods. Malondialdehyde, the end product of lipid peroxidation, could not be detected in the complete assay medium used for drug metabolism studies in the presence or absence of either aminopyrine or ethylmorphine. However, a rate of 1.6 nmoles malondialdehyde formed/mg microsomal protein/5min was observed in the presence of 0.1M Tris buffer and NADPH (no regenerating system) which compares favourably with previously reported values (Orrenius *et al.*,1964).

(d) Influence of Different Isocitrate/IDH Concentrations

The effect of various isocitrate/IDH concentrations on p-aminophenol formation at selected intervals is given in Figure 3.4. Identical rates of p-aminophenol formation were obtained with isocitrate/IDH concentrations ranging from 15 µmoles/0.6 IU to 20 µmoles/1.0 IU respectively. Therefore, isocitrate (20 µmoles) and IDH (0.6 IU) were used routinely in subsequent experiments. Similar results were obtained with aminopyrine as the substrate. Doubling the IDH concentration at low NADPH concentrations (5 µM and 10 µM) did not increase the rate or the amount of formaldehyde formation from aminopyrine.

The rate of reduction of NADP to NADPH by IDH has been shown to involve an initial lag phase, the length of which is dependent on the composition of the assay medium. (Sanner & Ingebretsen, 1976; Carlier & Pantaloni, 1976). However, all of the NADP was in the form of NADPH (concentrations up to 200 µM were tested) at the end of the 5 min "preincubation" used in the present study (Results not shown).

FIGURE 3.4.

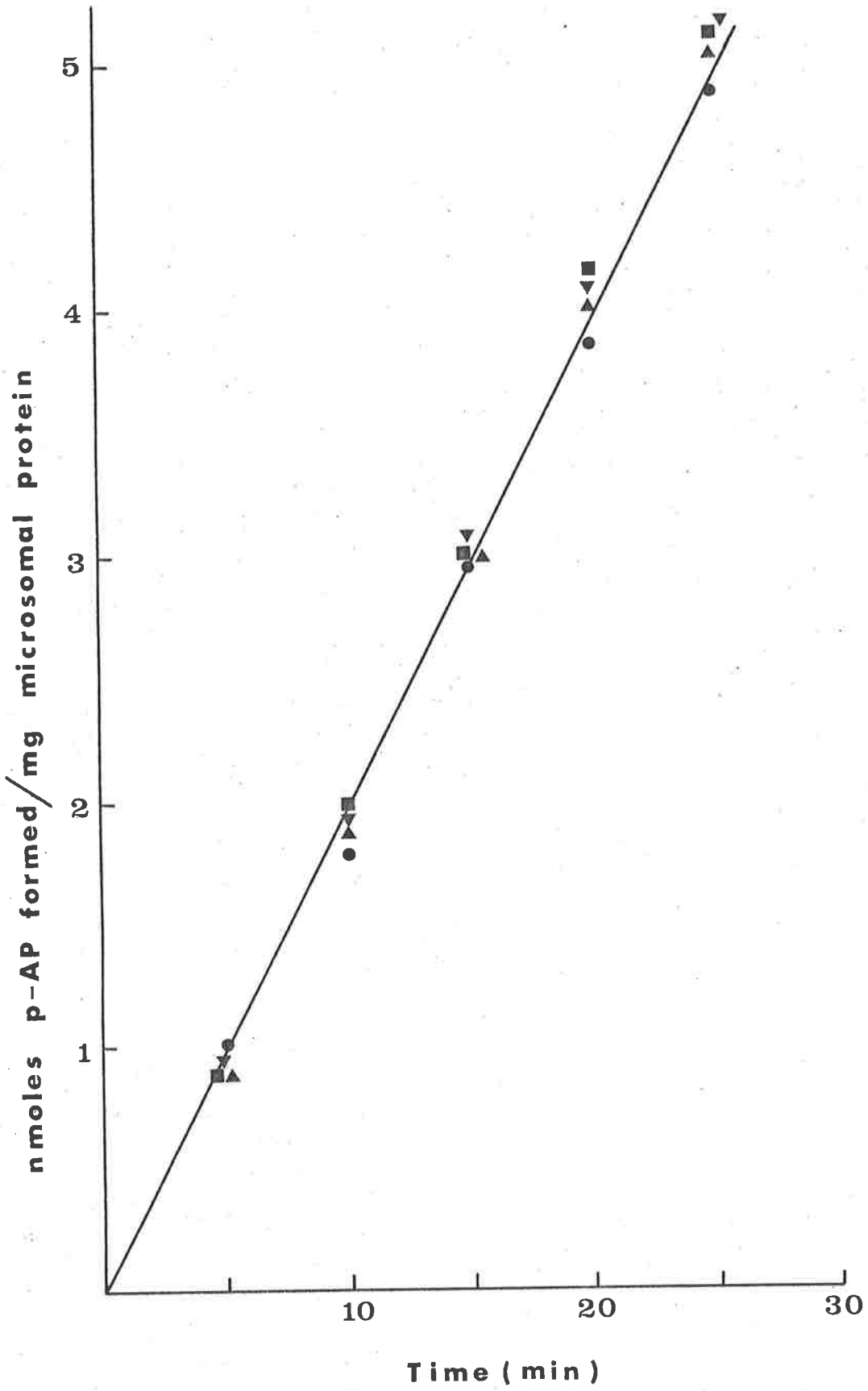
The influence of various concentrations of isocitrate and IDH on p-aminophenol productions.

The experiment was similar to that described in Figure 3.1. except that various concentrations of isocitrate and IDH were used.

SYMBOL	ISOCITRATE[a] (µmoles)	IDH[a] (IU)
●——●	20	1.0
■——■	15	0.8
▲——▲	15	0.6
▼——▼	10	0.8

a : concentration of either isocitrate or IDH per 2.5mls of reaction medium.

Identical results were obtained when the experiment was repeated on two separate occasions.


nmoles p-AP formed/mg microsomal protein
1
2
3
4
5
10
20
30
Time (min)

(e) Influence of Protein Concentration

Figure 3.5. shows a linear relationship between p-aminophenol formation and microsomal protein concentration (mg/ml) in the incubation medium determined in the presence of 312 µM NADPH. A similar linear relationship was observed for the N-demethylation of aminopyrine. The microsomal protein concentration of 1mg/ml was used in subsequent experiments.

(f) Linearity of Product Formation with respect to Time

Figure 3.3. also shows that a linear relationship exists between the amount of p-aminophenol formed and time for at least 15 min. Similar experiments using aminopyrine as substrate were linear for only 6 min (Figure 3.6.) with NADPH concentration of 100 µM. Further, linearity existed for 5 min when the initial NADPH concentrations were 5 µM and 10 µM.

3.2.3. DISCUSSION

Meaningful kinetic estimations are extremely difficult in systems where the variable substrate is utilised in more than one reaction. Therefore, before any reliance can be placed on the kinetic values obtained by a system such as the hepatic microsomal mixed function oxidase (MFO), competing reactions must be eliminated as far as practical.

The MFO enzyme complex is localised in the endoplasmic reticulum together with a large number of other enzymes. Two of these enzymes, NAD glycohydrolase and nucleotide pyrophosphatase can degrade NADPH, the principal source of electrons for the MFO complex.

The cleavage of NADPH by nucleotide pyrophosphatase, however, can be inhibited by a range of substances including inorganic pyrophosphate, AMP, sodium fluoride and EDTA (Bachorik & Dietrich, 1972). Tetra sodium pyrophosphate (adjusted to pH 7.5) was found

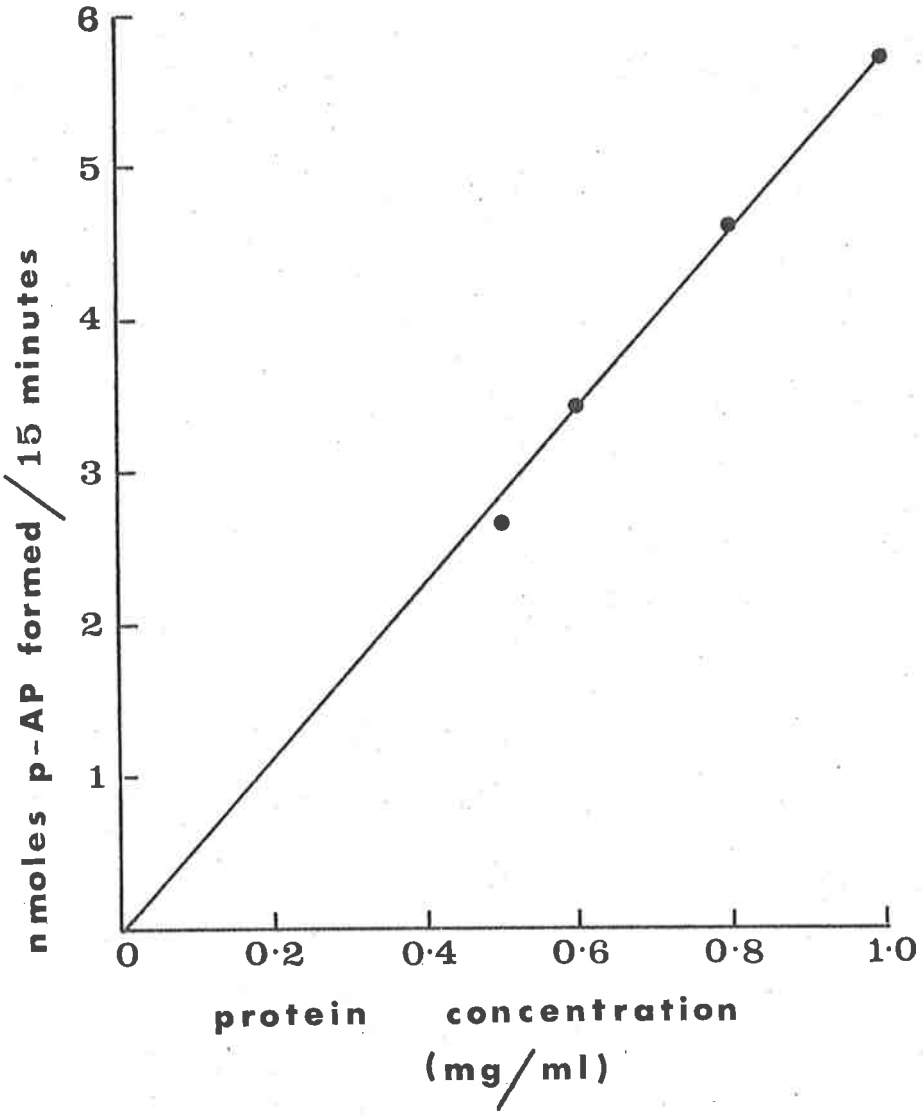


FIGURE 3.5. The influence of microsomal protein concentration on p-aminophenol formation.

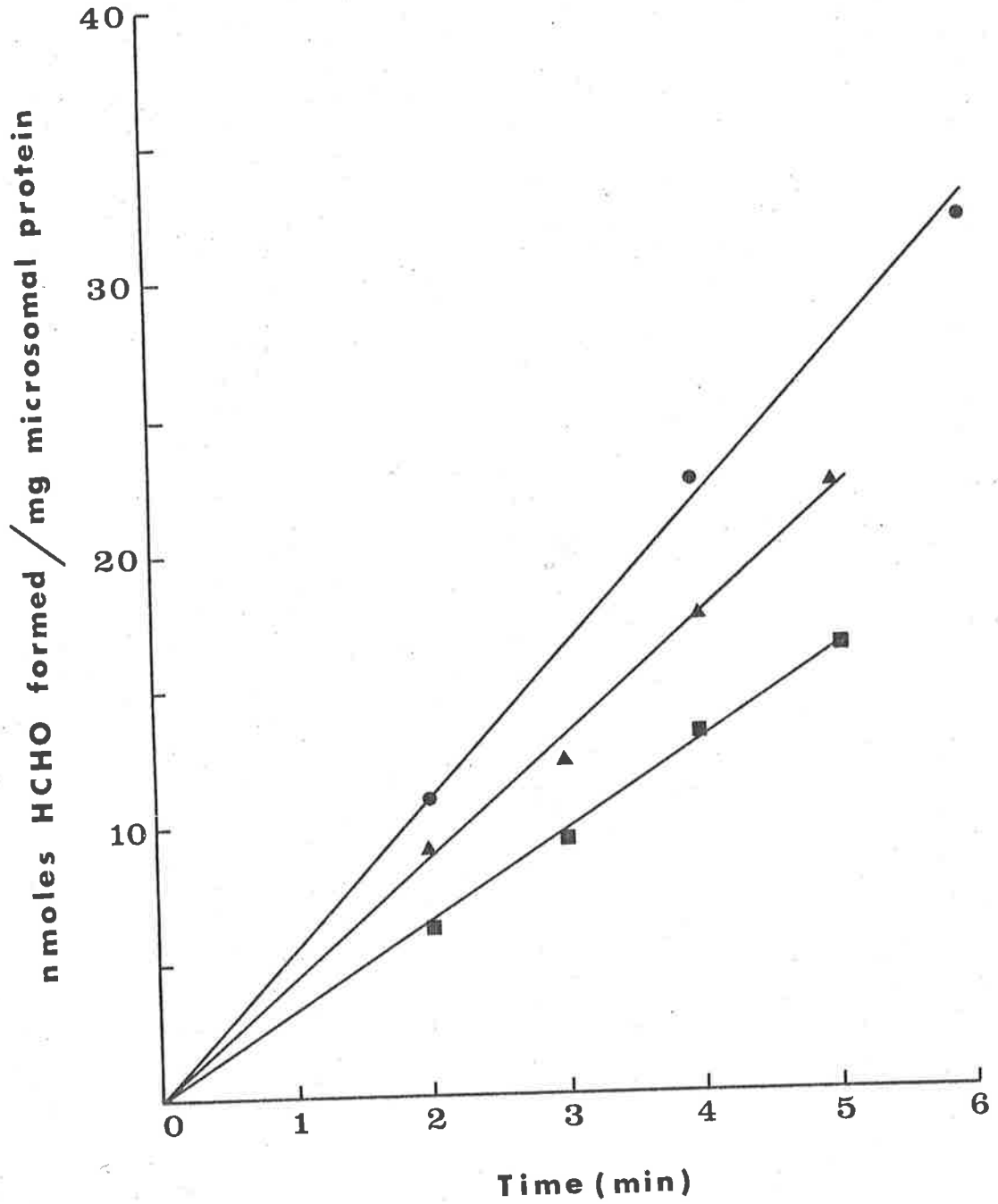
Protein concentration (mg of microsomal protein/ml of reaction medium) was varified from 0.5 to 1mg/ml. The composition of the reaction medium and the estimation of p-aminophenol has been described in the Methods.

FIGURE 3.6.

Linearity between formaldehyde formation from aminopyrine and time with different NADPH concentrations.

The composition of the reaction mixture was similar to that described for Figure 3.3. The NADPH concentrations used were 100 µM (●——●), 10 µM (▲——▲) and 5 µM (■——■). The microsomal enzyme suspension and the reaction mixture were separately incubated at 37°C for 5 min to reduce all the NADP to NADPH and equilibrate temperatures. The reaction was initiated by the addition of microsomal protein to give a final concentration of 1mg/ml.

The amount of formaldehyde present in 2.5ml aliquots removed at appropriate times was estimated as described in the Methods.


40
30
20
10
nmoles HCHO formed / mg microsomal protein
0
1
2
3
4
5
6
Time (min)

in the present work to inhibit nucleotide pyrophosphatase activity in the microsomal preparations used. This compound was shown to stabilise NADPH levels during drug hydroxylations, particularly at low initial NADPH concentrations. The inclusion of 20 mM pyrophosphate in the incubation medium resulted in a reduced rate of formaldehyde production from aminopyrine but had no significant effect on p-aminophenol formation from aniline. However, higher concentrations did show inhibition of both aniline hydroxylase and aminopyrine demethylase without further stabilising NADPH levels. Further support for the inclusion of pyrophosphate is given by the K_m(NADPH) for aminopyrine-N-demethylase determined in the presence and absence of 20mM pyrophosphate, (Chapter 4).

Some workers (Estabrook *et al.*, 1973) consider pyrophosphatase activity to have only a minor effect when assay times are short, however, the results obtained in the present study do not support this conclusion.

The comprehensive study of the pyridine nucleotide involvement in drug hydroxylation by Cohen & Estabrook (1971a,b,c,) revealed that NAD glycohydrolase activity had a significant effect on the NADPH concentration available for hydroxylation reactions. This study utilised male rabbits which had been previously treated with phenobarbital. While NAD glycohydrolase activity has been shown to occur in rat liver microsomes when NAD was used as the substrate (Sung & Williams, 1952; Bojarski & Wynne, 1957; Jacobson & Kaplan, 1957; Bock *et al.*, 1971; and Green & Dobrajansky, 1971), little activity of the enzyme was observed in erythrocytes when either NADH or NADPH replaced NAD as substrate (Hofman & Rapoport, 1955). The present results suggest that NADPH is a poor substrate for NAD glycohydrolase in rat liver microsomal preparations.

The NADPH regenerating system appears to effectively compete with NAD glycohydrolase for the $NADP^+$ released during drug hydroxylation. If this was not the case, nicotinamide would be expected to exert some stabilising influence on NADPH concentration during hydroxylation reactions and increase product formation above that observed in the absence of nicotinamide, particularly at low NADPH concentration.

However, the results of the present study showed that nicotinamide, when added to the assay media instead of pyrophosphate, gave an identical degradation profile for NADPH to that obtained with the isocitrate/IDH regenerating system. Moreover, the combination of nicotinamide and pyrophosphate gave the same results as pyrophosphate above. It was found that there was only 2-3% of the 340 nm chromophore of NADPH was lost at 37^oC over 5 min during the demethylation of aminopyrine , while the addition of nicotinamide did not increase HCHO production with low initial NADPH concentrations.

These results are supported by Buening & Franklin, (1974), and Correia & Mannering (1974a) who also suggest that NAD glycohydrolase activity is minimal in rat liver microsomes.

In addition to the oxidation of drugs, the mixed function oxidase enzyme system has been implicated in lipid peroxidation, steroid hydroxylation and in part in fatty acid desaturation. All of the above reactions compete with drug substrated for NADPH electrons. Orrenius *et al.*, (1964), has shown that 5mM aminopyrine inhibits endogenous NADPH lipid peroxidation by approx. 95%. This finding was confirmed by Wills, (1969a) who extended the study to show that codeine and phenobarbital inhibited lipid peroxidation to a lesser extent while hexobarbital did not inhibit at all. Ethylmorphine (Jeffrey & Mannering, 1974), high

concentrations of pyrophosphate (Ernster & Nordenbrand, 1967) and commercial preparations of isocitrate dehydrogenase (Kotake *et al.*, 1975) have also been shown individually to inhibit NADPH supported endogenous lipid peroxidation.

The component present in commercial preparations of Sigma Type IV IDH has been identified as EDTA at a concentration between 10 to 40mM (Warner & Neims, 1976). Although the present work uses a different commercial brand of IDH (Calbiochem) it's specification is similar to the Sigma product. A final EDTA concentration of 100-400 µM would be expected at the dilution used in demethylation reactions (approximately 1/100) which has been shown to almost completely inhibit lipid peroxidation. (Kamataki & Kitagawa, 1973).

The effect of these compounds together in the complete assay medium used for drug metabolism studies was to reduce malondialdehyde production to a level that could not be detected by the thiobarbituric acid reagent. However, a rate of 1.6 nmoles of malondialdehyde formed/5min/mg microsomal protein was observed in the presence of 0.1M Tris buffer, microsomal protein and NADPH (no generating system). This rate compares favourably with base rates reported by Orrenius *et al.*, (1964), and also by Wills, (1969b) when corrected for variations in protein concentration and the nature and strength of the buffer.

Cinti (1975) has also shown that lipid peroxidation is undetectable with aminopyrine as substrate and negligable with ethylmorphine as substrate (<0.1 nmoles malondialdehyde formed/5min/mg microsomal protein) using calcium aggregated microsomes and an incubation medium similar in composition to that employed in the present study.

Wills & Wilkinson (1967) have also shown that lipid peroxidation increases with storage of microsomes even at the low temperature of 4°C. The present study used fresh microsomes with the enzyme incubations completed within 5hr of removing the liver from the rats.

The magnesium chloride present in the assay medium would stimulate lipid peroxidation to some extent as Wills (1969b) found that 10mM $MgCl_2$ stimulated peroxidation by 40%, however, calcium at the same concentration inhibited peroxidation by 97%. Kamath *et al.*, (1971) has estimated a calcium concentration of approximately 1mM in microsomes isolated by the calcium aggregation method. Therefore the opposing effects of calcium and magnesium would probably be self-eliminating. It is concluded from this study that lipid peroxidation does not occur to any significant extent in the systems used for the following studies.

Microsomal cytochrome b_5 has been implicated in fatty acid desaturation, (Oshino *et al.*, 1971; Oshino & Omura, 1973), while inhibition experiments with ethylisocyanide have suggested the non involvement of cytochrome P_{450}, (Oshino *et al.*,1966). Desaturase activity is dependent upon electrons donated to cytochrome b_5 by NADH and to a lesser extent NADPH and ascorbic acid in *in vitro* systems, (Oshino *et al.*, 1966). There appears to be no additive or synergistic increase in activity in the presence of both reduced pyridine nucleotides compared to the rates observed in the presence of NADPH or NADH alone, (Oshino *et al.*, 1966). In this respect, fatty acid desaturation differs from the metabolism of Type I substrates by the mixed function oxidase complex (Cohen & Estabrook, 1971a,b,c; Correia & Mannering, 1973a,b,c).

However, unlike endogenous lipid peroxidation, desaturase activity can only be demonstrated in a substrate fortified

(usually stearyl CoA) incubation medium. Further, assay conditions are critical to the demonstration of significant activity. Oshino *et al.*, (1966) has shown that desaturase activity was linear for only 6-7min and refortification of the incubation medium with more microsomal protein did not restore linearity. These workers also demonstrated that desaturase activity was markedly reduced if stearyl CoA and the microsomes were mixed prior to initiating the reaction with NADPH.

The longer the "pre-incubation", the greater the reduction in desaturase activity. It was concluded that substantial hydrolysis or transacylase activity could occur when stearyl CoA was left in contact with the microsomal suspension.

The endogenous levels of fatty acyl CoA compounds in the microsomal suspension is not known, but any natural CoA derivitives present were in contact with the hydrolase and transacylase enzymes for at least 4 hr during the isolation and washing of the microsomes prior to the initiation of drug metabolism studies.

A rabbit antibody to rat liver cytochrome b_5 which is specific for NADH was shown (Sasame *et al.*, 1973) to have no effect on endogenous rates of NADPH and NADH oxidation. This study suggests, but does not prove, that the activity of fatty acid desaturation and any other reactions which occur via cytochrome b_5 are very low in microsomal suspensions in the absence of specific added substrates. Therefore, as we do not add a specific substrate for the desaturating reaction, it is considered that this pathway does not utilise electrons from either NADPH or NADH to any marked extent.

The w-hydroxylation medium and long chained fatty acids is another NADPH and molecular oxygen-dependent reaction catalysed

by liver microsomal preparations (Priess & Block, 1964). Priess & Block (1964) have shown that w-hydroxylation activity is low in fresh microsomal suspensions, but the freezing and thawing of microsomes increases w-hydroxylation at the expense of fatty acid desaturation.

There were a number of similarities between drug and steroid hydroxylase activities of liver microsomes that led Kuntzman *et al.*, (1964) to conclude that both drugs and steroids are substrates for the one hydroxylating enzyme complex. Both steroid hydroxylase and w-hydroxylase activities, like fatty acid desaturase, can only be demonstrated in a substrate fortified medium.

Perhaps the most efficient method for estimating the effects of lipid peroxidation and hydroxylation, fatty acid desaturation, steroid hydroxylation, NAD glycohydrolase and nucleotide pyrophosphatase activities on NADPH utilisation is to examine the stoichiometry between NADPH oxidised (2 electron donor): oxygen consumed: product formed by drug hydroxylation reactions. The desired stoichiometry of 1:1:1 has been shown for the C-21 hydroxylation of 17-hydroxyprogesterone by bovine adrenal microsomes (Cooper *et al.*, 1962), and also for aminopyrine-N-demethylation by liver microsomes isolated from phenobarbital treated rats (Orrenius, 1965).

However, Bock *et al.*, (1971); Buening & Franklin, (1974); Jeffrey & Mannering, (1974), have shown that the specific activities of both NAD glycohydrolase and nucleotide pyrophosphatase in microsomes from phenobarbital treated rats are approximately half of the activities in microsomes from control rats.

The stoichiometry has also been shown for the N-demethylation of benzphetamine by a solubilised and reconstituted microsomal

enzyme system (Lu *et al.*, 1970). This reconstituted system is free of nucleotide pyrophosphatase and NAD glycohydrolase activities.

However, a stoichiometry of 2 NADPH oxidised: 1 formaldehyde formed was obtained by Cohen & Estabrook, (1971a) for Type I substrates using microsomes isolated from phenobarbital treated rabbits. This finding has led Stripp *et al.*, (1972), and Sasame *et al.*, (1973) to propose various methods of estimating and correcting for endogenous NADPH oxidations in an attempt to re-establish stoichiometry, none of which is completely satisfactory.

Recently reports have indicated that a 1:1 stoichiometry exists between NADPH oxidised:oxygen consumed (Buening & Franklin, 1974) and also between NADPH oxidised and product formed from ethylmorphine (Jeffrey & Mannering, 1974) when endogenous nucleotide pyrophosphatase activity is controlled.

Jeffrey & Mannering, (1974) have shown that 5-AMP (0.5mM), ATP, sodium fluoride and EDTA (0.2mM) markedly inhibited nucleotide pyrophosphatase. Sodium pyrophosphate, the inhibitor used in the present study has been shown to completely inhibit rat liver nucleotide pyrophosphatase at a concentration of 25mM (Sasame & Gillette, 1970).

In view of the demonstrated stoichiometry, it is probable that the other reactions that can oxidise NADPH (i.e. lipid peroxidation, fatty acid desaturation, etc.,) collectively use only a small amount of the NADPH available during drug hydroxylation. Nevertheless, these other reactions must exert some effect and it is probable that the true K_m values will be slightly lower than those obtained in the present work. Obviously, in view of the complex inter-relationships that exist between the various reactions which compete with the mixed function oxidase for reduced pyridine nucleotides, any K_m (NADPH) value determined

for drug hydroxylation must still be an approximation.

The use of the detergent solubilised and reconstituted microsomal hydroxylating system originally reported by Lu & Coon, (1968), may justify further study for this purpose. However, while this system is free of contaminating enzymes and gives turnover numbers (nmoles product formed/nmole cytochrome P_{450}) equal to or above that obtained with the crude microsomal fraction for most of the drug substrates studied, recent evidence suggests that the reconstituted and the crude system are not identical in every respect (Mannering, 1974).

One of the most significant findings is the loss of the NADH synergistic increase in formaldehyde production from benzphetamine in the reconstituted system (Lu *et al.*, 1974). A NADH synergistic increase has been shown for this substrate in intact microsomes (Correia & Mannering, 1973a,c).

Therefore, until these discrepancies are resolved, the most accurate and meaningful kinetic estimates available are those obtained using intact microsomes, with due precautions taken to eliminate competing reactions without adversely affecting mixed function oxidase activity.

With the limitations of the system in mind, the present work evaluates apparent K_m and V values under the assay conditions described. The changes in the kinetic constants for both NADPH and NADH under various, but carefully controlled conditions are used in an attempt to further characterise the pyridine nucleotide involvement in hepatic microsomal drug metabolism.

The reaction rates were linear for 15 min for aniline hydroxylase and at least 5 min for aminopyrine with NADPH concentration as low as 5 μM in the latter case. Recently it has been suggested (Cinti, 1975) that many studies have employed suboptimal amounts

of IDH in demethylation reactions. It was shown that the addition of the 105,000 x g supernatant fraction (soluble fraction) stimulated both aminopyrine and ethylmorphine demethylases by effectively increasing the amount of IDH present in the incubation medium. The stimulatory effects of the soluble fraction were not apparent if the concentration of IDH was increased from 0.2 to 1.0 IU/3mls of incubation medium (i.e. 0.83 IU/2.5mls of incubation medium). The concentration of IDH employed in the present study (0.6 IU/2.5mls) was almost at the level where the addition of the soluble fraction was without effect on the demethylation reactions. Further, the present work has shown that the concentrations of isocitrate and IDH were not limiting as doubling the IDH concentration at low NADPH concentrations (5 μM and 10 μM) did not increase formaldehyde production from aminopyrine.

A microsomal protein concentration of 1mg/ml was used throughout the present study because lower concentrations would have resulted in unacceptably low levels of formaldehyde production at low NADPH concentrations.

The majority of the experimental work for this thesis was completed before a more sensitive method of formaldehyde estimation was available (Chrastil & Wilson, 1975).

A recent review (Lenk, 1976) has considered many of the problems involved in the acquisition and interpretation of kinetic data for the mixed function oxidase enzyme complex in the heterogenous microsomal fraction. Many of the procedures adopted in the present work are in general agreement with the suggestions contained in the above review.

Lenk, (1976) also raised the question of the applicability of using Michaelis-Menten kinetics (which were derived for purified soluble enzymes) to describe a membrane bound, multicomponent

enzyme system. It was suggested that the thickness of microsomal membranes was far below the critical value of 1mm which eliminated interphase diffusion and the transport of reactants and metabolites as possible rate limiting steps.

While it was concluded that microsomal membranes constitute a system applicable for the determination of rate constants, changes in apparent K_m values can only be taken as an indication of a change in the rate limiting step.

The isocitrate/IDH regenerating system was chosen because NADPH decomposition was less with this system compared to the soluble fraction/glucose-6-phosphate system. There appears to be some component present in the soluble fraction which is not nucleotide pyrophosphatase (Sasame & Gillette, 1970), that potentiates NADPH degradation. The unknown factor was not removed when the 105,000 x g supernatant was fractionated with solid ammonium sulphate. The 0-60% fraction, when reconstituted to the same volume as the soluble fraction gave the same profile of NADPH degradation as the unfractionated 105,000 x g supernatant.

However, product formation was greater in the soluble fraction system than in the isocitrate/IDH system.

Nelson *et al.*, (1973) and Kamataki *et al.*, (1975) have suggested that there is a component present in the soluble fraction which "reverses the inhibitory effect of NADPH supported lipid peroxidation". There is also a similar component present in commercial preparations of IDH (Kotake *et al.*,1975), which has been identified as EDTA (Warner & Neims, 1976). However, the results of the present study and those of Cinti (1975) indicate that lipid peroxidase activity is undetectable(using the thiobarbituric acid reagent) under the assay conditions used.

Therefore, the discrepancy in aniline metabolism between the two systems is not completely resolved.

The rapid decline in NADPH concentration with respect to time when NADPH was added without a regenerating system reflects NADPH oxidation as well as NADPH degradation. The low levels of p-aminophenol formed are due to the rapid decline in NADPH concentrations and emphasise the need for a NADPH regenerating system.

3.3. COMPUTATIONAL METHODS USED TO CALCULATE KINETIC CONSTANTS.

3.3.1. INTRODUCTION

Numerous graphical and computer methods are available to estimate the values of the kinetic constants (K_m, V) of the Michaelis-Menten equation. As this equation describes a hyperbolic function, various linear transformations are used to estimate K_m and V values.

If both the substrate concentration and velocity determinations were error free, it would be of little consequence which graphical method was chosen to evaluate the constants. Indeed, there would probably be no great advantage in using digital computer methods in preference to graphical procedures.

However, while it is generally accepted that the substrate concentrations are accurately known and error free, this is not the case with velocity determinations which are subject to some experimental error. Under these conditions, the linear transformations no longer provide equally accurate estimates of the parameters.

Appreciation of this fact has had the effect of creating a greater awareness of iterative digital fitting techniques for obtaining estimates of the K_m and V. Therefore, in this section,

various digital computer techniques are compared using the same set of experimentally obtained data. Further, the influence of various weight factors on the resultant numerical values assigned to the parameters are also evaluated. Finally, three criteria are established which must be met before the results of any kinetic experiment can be accepted.

3.3.2. EXPERIMENTAL

Three different computer techniques were compared in the present study. The programme HYPER, which was written by Cleland (1967) is based on the residual least squares method of Wilkinson (1961). This programme was translated from FORTRAN to BASIC language run on a PDP 11/20 or a PDP 11/40 computer (Appendix 1 is a source listing of this programme).

The HYPERB programme which was written by Hanson *et al.*, (1967), is based upon the maximum likelihood procedure of Bliss & James (1966) and was also run in BASIC.

NONLIN is a general non linear least squares curve fitting programme (Metzler, 1969) which uses the procedure of Hartley & Booker (1965). This programme was run in FORTRAN on an ICL 1903A computer. When using NONLIN, the equations, either algebraic or differential to which the data is being fitted are supplied in a subroutine, D FUNC.

HYPER and HYPERB were modified to calculate a correlation coefficient, r, once convergence had occurred which has already been defined in the Methods section (2.2.8.). NONLIN calculates r as part of it's normal operation.

3.3.3. RESULTS

The numerical values assigned to the constants of the Michaelis-Menten equation by the three computer programmes in question are given in Table. 3.4.

TABLE 3.4.

K_m and V values computed by HYPER, HYPERB and NONLIN using the same data set.

	HYPER	HYPERB	NONLIN
K_m [a]	9.26	9.26	9.26
	(1.14)	(1.14)	(1.12)
V [b]	52.1	52.1	52.1
	(1.96)	(1.96)	(1.94)

Values in parenthesis are standard error of the determination.
Weight factors for HYPER and NONLIN were unity.

a K_m expressed as μM.

b V expressed as nmoles of HCHO formed/5min/mg of microsomal protein.

N = 3.

The data used in the above comparison was the titration of the oxidative demethylation of aminopyrine as a function of NADPH concentration in the presence of 20mM sodium pyrophosphate.

The three programmes do not differ in their capability to converge on the same apparent K_m and V given the same data set. While both HYPER and NONLIN enable the experimental velocity determinations to be weighted, HYPERB does not provide this facility because of the assumption that there is a constant local variance in velocity readings at different substrate concentrations.

The effect of weighting the velocity readings at each substrate concentration with the reciprocal of the coefficient of variation (1/CV) and the reciprocal of the variance (1/VAR) of

those velocity readings, which were obtained from replicate determinations, is shown in Figure 3.7.

The values of the kinetic constants obtained from these weight factors are compared to those obtained when the weight factor was unity, i.e. the data is not weighted at all in the latter case.

The data was obtained from the titration of aniline para hydroxylation as a function of NADPH concentration. There was no significant difference between the K_m values of 5.86 µM and 6.29 µM or the V values of the 7.16 and 7.26 nmoles of p-aminophenol formed/15min/mg of microsomal protein when the velocity readings at each substrate concentration are weighted with 1/CV or 1/VAR of those velocity readings at that substrate concentration, respectively. However, when the weight factor was unity, the K_m and V were 23.1 µM and 10.33 nmoles p-aminophenol formed/15min/mg microsomal protein respectively.

3.3.4. DISCUSSION

The numerical values of the constants of the Michaelis-Menten equation are usually obtained by using one or more transformations which convert the equation from a hyperbolic function to a linear relationship. The three most common graphical procedures are 1/velocity(v)versus, 1/substrate concentration (S), (Lineweaver & Burk, 1934), v versus v/S, (Eadie, 1942; Hofstee, 1952), and S/v versus S (Hanes, 1932; Lineweaver & Burk, 1934).

Since the experimental velocity readings obtained are subject to experimental error to a greater or lesser extent, the three linear transformations no longer provide identical estimates of K_m and V. Furthermore, unweighted linear regression of any of these transformations does little to improve the accuracy of the values of the kinetic constants.

FIGURE 3.7.

The effect of weighting kinetic data with three weight factors using the programme HYPER.

The data was obtained from the titration of aniline parahydroxylation as a function of NADPH concentration (Table 4.1.). The weight factors used were unity (curve A), 1/CV and 1/VAR (both shown as curve B because the resultant kinetic parameters were essentially equivalent). The points are the mean ± SD of three duplicate determinations while the lines are the computer generated lines of best fit using the kinetic constants below.

The kinetic constants computed were:-

unity K_m = 23.1 ± 4.23 μM.

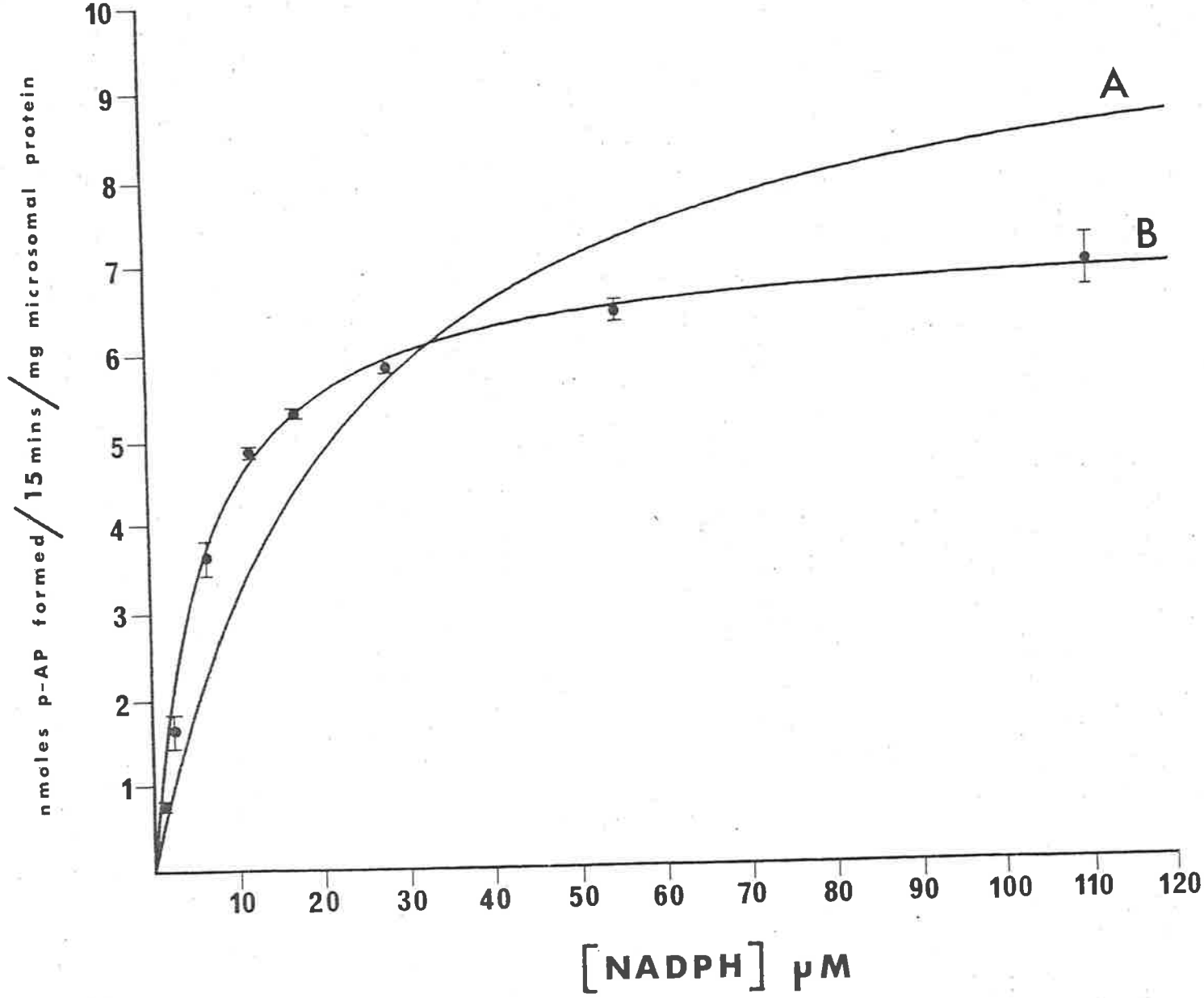
V = 10.33 ± 1.08 nmoles p-aminophenol formed/15min/mg microsomal protein. (r = .97).

1/CV K_m = 5.86 ± 0.4 μM.

V = 7.16 ± 0.11 nmoles p-aminophenol formed/15min/mg microsomal protein. (r = .997)

1/VAR K_m = 6.29 ± 0.47 μM.

V = 7.26 ± 0.15 nmoles p-aminophenol formed/15min/mg microsomal protein. (r = .997)


nmoles p-AP formed / 15 mins / mg microsomal protein
10
9
8
7
6
5
4
3
2
1
A
B
10
20
30
40
50
60
70
80
90
100
110
120
[NADPH] μM

With these limitations of the graphical procedures in mind, several authors have written digital computer programmes for the statistical analysis of enzyme kinetic data. These procedures aim to eliminate the bias inherent in the various graphical procedures, (Markus *et al.*, 1976).

The three programmes tested converge on the same apparent K_m and V values, when all data points were weighted equally (Table 3.4.). Equal weighting assumes constant variance in velocity readings at different substrate concentrations. However, the results of Figure 3.7. illustrate the importance of correct data weighting to compute kinetic parameters. When the data at each substrate concentration were weighted either with 1/CV or 1/VAR of velocity readings at that substrate concentration, essentailly equivalent results were obtained. However, when the data were weighted equally, HYPER converged on a false value of apparent K_m and V.

Dowd & Riggs (1965) support the use of correct weight factors to compute more realistic apparent K_m and V values from kinetic data. They demonstrate, convincingly, the inherent dangers in using graphical methods with linear transformations of the Michaelis-Menten equation or unweighted linear regression to obtain the best fit of the data. Atkins (1974), Ottaway (1973) and Bartfai & Mannervik (1973) have, more recently, considered other weight factors which can be used to obtain better estimates of kinetic constants from kinetic data.

Generally, there was no statistical difference in K_m and V values when the same data was weighted with either 1/CV or 1/VAR. There was, however, often a significant difference when equal weight was applied to kinetic data compared to the other two weight factors.

Three criteria were used to evaluate the results of different weight factors in any particular kinetic experiment.

(1) that the CV of any parameter be not more than 20%;

(2) that the data points be equally distributed about the computer generated line of best fit, especially in the area where the rate of change in slope of the tangents to the curve is greatest;

(3) that the correlation coefficient r, be greater than 0.98.

The correlation coefficient gives an indication of the fit of the data to the model. (i.e. Equation describing Michaelis-Menten kinetics). If any of the above criteria were not satisfied the experiment was repeated.

The results obtained in the present work and those of Storer *et al.*, (1975) suggest that the common assumption that there is a constant variance in velocity readings at different substrate concentrations can result in erroneous values for K_m and V. If digital computer techniques are to be used to statistically analyse kinetic data, a programme which provides the facility to weight kinetic data should be selected. Also the same data set should be weighted with various factors to obtain the best possible estimates of the kinetic constants.

C H A P T E R 4.

NADPH KINETICS DURING HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE REACTIONS

4.1. INTRODUCTION

The hepatic microsomal mixed function oxidase enzyme system is dependent on both molecular oxgen and NADPH (Gillette, 1963, 1967) and while NADPH is not absolutely specific it has been shown that rates of product formation are reduced by approximately 90% when NADH replaces NADPH as the electron donor (Axelrod, 1955b; Orrenius, 1965).

Although there has been considerable interest in the kinetic behaviour of the mixed function oxidase system for various substrates in the presence of saturating concentrations of NADPH, relatively little attention has been devoted to the kinetics when the concentration of NADPH is varied for this system. Orrenius, (1965) and Schenkman *et al.*, (1967) calculated K_m (NADPH) values of 25 µM and 28 µM respectively for the demethylation of aminopyrine. Further, Oshino *et al.*, (1966) computed a K_m (NADPH) of 15 µM during the parahydroxylation of aniline, while Ullrich, (1969a) obtained a value of 10µM for the hydroxylation of cyclohexane.

However, all of these reports have failed to acknowledge the influence of the reactions that can degrade and possibly consume electrons from NADPH during drug hydroxylations. In the previous Chapter, it was shown that the inclusion of 20mM pyrophosphate inhibited nucleotide pyrophosphatase activity while microsomal NAD glycohydrolase did not appear to significantly degrade the NADPH added to the total incubation mixture.

This Chapter therefore reports K_m values for NADPH in the presence of pyrophosphate using various drug substrates. The kinetics of NADPH are evaluated for NADPH-cytochrome P_{450} reductase which has been proposed as the rate limiting step in

drug hydroxylations (Gigon *et al.*, 1969) and also for NADPH-cytochrome c reductase.

4.2. RESULTS

4.2.1. K_m (NADPH) FOR AMINOPYRINE DEMETHYLASE IN THE ABSENCE AND PRESENCE OF PYROPHOSPHATE

Figure 4.1. shows the titration of aminopyrine demethylation as a function of NADPH concentration in the presence and absence of 20mM pyrophosphate. The hyperbolic nature of both curves indicates the NADPH involvement in drug hydroxylations obeys classical Michaelis-Menten kinetics. The inclusion of pyrophosphate in the assay reduced the K_m (NADPH) from 25 µM to 7.92 µM.

In the previous Chapter, the inclusion of pyrophosphate was shown to inhibit p-aminophenol production from aniline. Figure 4.1. also shows that for aminopyrine demethylase, the V values are reduced from 59 nmoles to 50 nmoles HCHO formed/5min/mg microsomal protein when 20mM pyrophosphate was present in the assay medium.

4.2.2. INFLUENCE OF DIFFERENT DRUG SUBSTRATES

Table 4.1. shows the K_m and V values of NADPH using either aminopyrine, ethylmorphine or benzphetamine as Type I substrates or aniline as a Type II substrate.

There was no significant difference in K_m (NADPH) values when any of the three Type I substrates were included in the assay, but there was a significant difference between aniline and aminopyrine. There was, however, a highly significant variation in V values between the four drug substrates tested. This variation in V values reflects the intrinsic rate of metabolism of structurally different substrates.

FIGURE 4.1.

Titration of aminopyrine demethylation as a function of NADPH concentration.

The kinetic constants were determined in the presence (●——●) and absence (O——O) of 20mM pyrophosphate using microsomes isolated from untreated rats. The points are mean ± S.D. of six (●——●) and three (O——O) determinations performed in duplicate, and the lines are the computer generated lines of best fit, using all the points. The velocity readings at each substrate concentration were weighted with 1/CV arising from those readings for that substrate concentration.

The kinetic constants computed were:-

K_m = 7.92 ± 0.82 μM.

V = 50.3 ± 1.5 nmoles HCHO formed/5min/mg microsomal protein in the presence of 20mM pyrophosphate

K_m = 27.9 ± 1.8 μM.

V = 59.95 ± 1.23 nmoles HCHO formed/5min/mg microsomal protein in the absence of 20mM pyrophosphate.

The K_m and V values are significantly different at $P < 0.005$. The data obtained in the presence of 20mM pyrophosphate represents six determinations which were performed on two separate occasions. The substrate concentrations were within 1% of each other and the true values were used in HYPER, although only one set is displayed in the graph for the sake of clarity. The spread in the velocity readings at each substrate concentration were comparable.

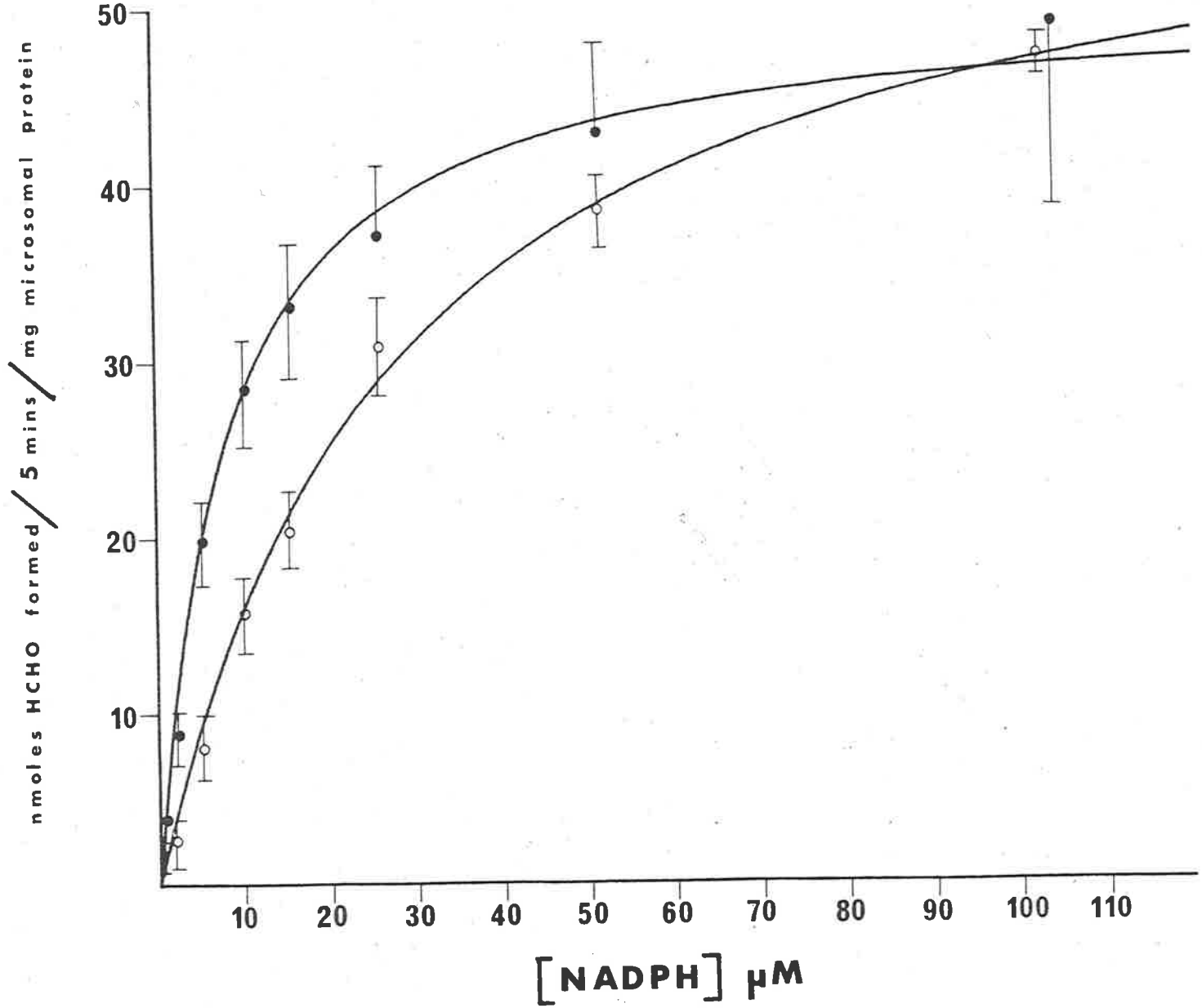

nmoles HCHO formed / 5 mins / mg microsomal protein
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
110
[NADPH] μM

TABLE 4.1.

K_m and V values for NADPH using aminopyrine, ethylmorphine, benzphetamine or aniline as substrates.

	Aminopyrine	Ethylmorphine	Benzphetamine	Aniline
K_m [a]	7.92 ± 0.82 [e]	8.49 ± 1.48	8.78 ± 1.75	5.86 ± 0.4 [e]
V [b]	50.3 ± 1.48	33.8 ± 2.25	24.9 ± 1.5	7.16 ± 0.11
N [c]	6	3	3	3
r [d]	0.985	0.989	0.98	0.997

a K_m expressed as μmolar : mean ± S.D.

b V expressed as nmoles HCHO formed/5min/mg microsomal protein for aminopyrine, ethylmorphine, benzphetamine and nmoles p-aminophenol formed/15min/mg microsomal protein for aniline: mean ± S.D.

c Number of experiments.

d Correlation coefficient as defined in computational Methods (2.2.9)

e Significantly different at $P < 0.01$.

Weight factor used in computations was 1/CV

These results do not agree with those of Schenkman *et al.*, (1967) who found no significant difference in apparent K_m (NADPH) values with aniline or aminopyrine, but he used microsomes isolated from phenobarbital treated rats. The inclusion of 20mM pyrophosphate in the medium for aniline hydroxylase reduced the K_m (NADPH) value by approximately 60% from 15 μM (Oshino *et al.*, 1966) to 5.86 μM (Table 4.1.). The reduction in K_m (NADPH) values

effected by the addition of pyrophosphate is of the same order as that obtained for aminopyrine demethylase (68% reduction).

4.2.3. KINETICS OF THE PYRIDINE NUCLEOTIDES FOR NADPH AND NADH CYTOCHROME C REDUCTASE.

The kinetic constants of NADPH (in the presence and absence of 20mM pyrophosphate) and NADH (in the presence of 20mM pyrophosphate) for these enzymes are shown in Table 4.2.

TABLE 4.2.

K_m and V values of the pyridine nucleotides for NADPH- and NADH-cytochrome c reductase under various conditions.

	NADPH		NADH
	Without pyrophosphate	With pyrophosphate	With pyrophosphate
K_m [a]	3.09 ± 0.51	4.76 ± 0.55	6.5 ± 0.39
V [b]	134.5 ± 5.2	128.5 ± 3.87	760 ± 11.8
r [c]	0.99	0.992	0.997

a K_m is expressed as μmolar : mean ± S.D. : significantly different from each other at $P < 0.025$

b V is expressed as nmoles cytochrome c reduced/min/mg microsomal protein/ml of cuvette contents : mean ± S.D.

c Correlation coefficient as defined in computational Methods (2.2.9.).

N = 3 for all determinations; weight factor = 1/CV.

Microsomes used were isolated from untreated rats.

Pyrophosphate was without significant effect on V values when NADPH was the electron donor. The K_m (NADPH) was approximately 70% of K_m (NADH) although the V (NADH) was almost six times the value of V (NADPH). The reduction of cytochrome c has been shown

to be mediated via cytochrome b_5 reductase. (Okuda *et al.*, 1972; Rogers & Strittmatter, 1973). The K_m (NADPH) obtained in the presence of pyrophosphate was signigicantly different from the values obtained for the Type I substrates and the para hydroxylation of aniline.

4.2.4. KINETICS OF NADPH FOR NAPDH-CYTOCHROME P_{450} REDUCTASE.

Figure 4.2. shows the rate of reduction of the cytochrome P_{450}-aminopyrine complex at various NADPH concentrations. The hyperbolic nature of this curve would suggest that the classical Michaelis-Menten equation can be used to describe this reaction. The kinetic constants of NADPH for NADPH-cytochrome P_{450} reductase determined in the presence of various drug substrates is shown in Table 4.3.

TABLE 4.3.

K_m and V values of NADPH for NADPH-cytochrome P_{450} reductase in the presence and absence of drug substrates.

	No Substrate	Aminopyrine	Ethylmorphine	Aniline
K_m [ad]	10.93 ± 2.15	1.56 ± 0.28[e]	2.5 ± 0.5[e]	3.7 ± 0.71
V [bd]	3.7 ± 0.23	5.28 ± 0.13[f]	8.92 ± 0.38[f]	2.2 ± 0.1
r [c]	0.980	0.991	0.98	0.982

a K_m expressed as μmolar : mean ± S.D.

b V expressed as nmoles cytochrome P_{450} reduced/minute/mg microsomal protein : mean ± S.D.

c correlation coefficient as defined in computational Methods.

d values obtained in the presence of a substrate are significantly different from "No Substrate" values at $P<0.005$.

e significantly different at $P < 0.05$.

f significantly different at $P < 0.05$.

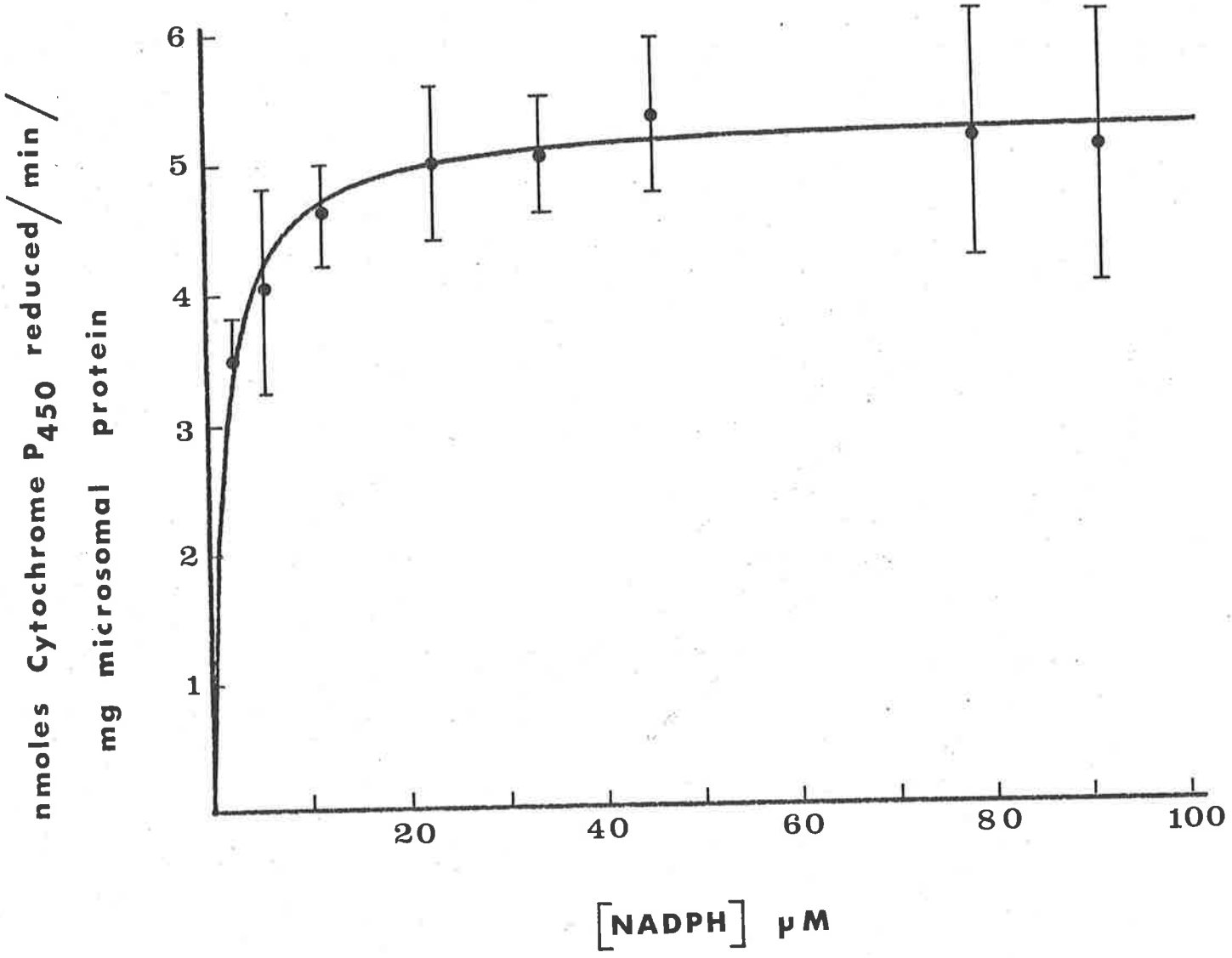


FIGURE 4.2. Titration of NADPH-cytochrome P_{450} reductase as a function of NADPH concentration.

The points are the mean ± S.D. of three duplicate determinations. While the line is the computer generated line of best fit. The microsomal suspension used for each determination was obtained by pooling the livers from three control rats.

NADPH-cytochrome P_{450} reductase was assayed as described in the Methods in the presence of 4mM aminopyrine.

Table 4.3. continued

The values assigned to the kinetic parameters were obtained from three duplicate independent determinations. The final concentrations of aminopyrine, ethylmorphine and aniline in the reaction were 4mM, 6mM and 4mM respectively.

The numerical values for the kinetic parameters were dependent on the substrate. However, all three substrates caused a lowering of the apparent K_m from that obtained in the absence of any substrate. There was a significant difference in the K_m values between the two Type I substrates, aminopyrine and ethylmorphine. Further, these two substrates caused a significant increase in apparent V, while the Type II substrate aniline was responsible for a lower than control value.

4.2.5. EFFECT OF TREATING RATS WITH PHENOBARBITAL.

The values of the kinetic parameters for NADPH using either aminopyrine or ethylmorphine as substrate and microsomes isolated from rats that had been treated with sodium phenobarbital (75mg/kg, i.p.) daily for three days prior to sacrifice are given in Table 4.4.

TABLE 4.4.

	Aminopyrine	Ethylmorphine
K_m [a,d]	11.04 ± 0.95 [c]	14.78 ± 1.31 [c]
V [b]	104.2 ± 4.0	100.2 ± 3.2
r	0.993	0.994

---

a K_m expressed as μM : mean ± S.D.

b V expressed as nmoles HCHO formed/5min/mg microsomal protein : mean ± S.D.

Table 4.4. continued

c significantly different at $P < 0.025$.

d both K_m and V values above are significantly different from the values obtained using microsomes isolated from control rats for both substrates (Table 4.1.), at $P < 0.01$.

N = 3 for both substrates.

Microsomes were isolated from rats treated with Phenobarbital (75mg/kg, i.p. daily) for three days prior to sacrifice.

The K_m (NADPH) values, but not the V values are significantly different when either aminopyrine or ethylmorphine was the substrate with microsomes isolated from rats treated with phenobarbital. Further, the kinetic constants for NADPH when either aminopyrine or ethylmorphine was the added substrate (Table 4.4.) are significantly different from the values obtained in similar experiments using microsomes isolated from control rats (Table 4.1.). The increase in V values was expected as it is now well established that such treatment stimulates the metabolism of a wide range of xenobiotics. Comai & Gaylor, (1973) have shown that there are possibly three different forms of cytochrome P_{450} in control microsomes with molecular weights of 44,000, 50,000 and 53,000 (Welton & Aust, 1974). The haemoprotein with a molecular weight of 50,000 predominates in control microsomes while pretreatment of the rats with phenobarbital increases the 44,000 component at the expense of the other two.

It is possible that the higher K_m (NADPH) values are a consequence of the predominance of the cytochrome P_{450} species with a molecular weight of 44,000.

4.3. DISCUSSION

The inclusion of 20mM pyrophosphate in the *in vitro* incubation medium has been shown to protect NADPH from the destructive activity of nucleotide pyrophosphatase during the demethylation of aminopyrine and the para hydroxylation of aniline (Chapter 3). The results of the present Chapter support this finding.

The K_m (NADPH) of 27.9 µM obtained during the demethylation of aminopyrine in the absence of pyrophosphate is in agreement with the results in the literature for N-demethylation reactions (Orrenius, 1965; Schenkman *et al.*, 1967). However, when the experiment was repeated in the presence of 20mM pyrophosphate, the K_m value was reduced to 7.92 µM, indicating that pyrophosphate afforded significant protection particularly at low NADPH levels. While pyrophosphate stabilised NADPH concentration during metabolism, formaldehyde formation from aminopyrine was inhibited (Figure 4.1.) as was para aminophenol production from aniline, (Chapter 3).

This inhibitory effect has been attributed by Sasame & Gillette, (1970) to an inhibition of NADPH cytochrome c reductase in studies using microsomes isolated from mice. The results of Table 4.2. show that pyrophosphate slightly increases the apparent K_m (NADPH) with little change to the V value, suggesting that pyrophosphate may be a weak competitive inhibitor (with respect to NADPH) of NADPH cytochrome c reductase.

While the apparent V of NADH was almost six times that of NADPH in the crude microsomal preparation, the activity of purified NADPH cytochrome c reductase (obtained by trypsin digestion of microsomes) when NADH was the electron donor was 1% of that obtained with NADPH as the sole source of electrons,

(Phillips & Langdon, 1962). The NADH mediated reduction of cytochrome c in the crude microsomal preparation has been shown to proceed via cytochrome b_5 and cytochrome b_5 reductase (Okuda *et al.*, 1972; Rogers & Strittmatter, 1973).

The apparent K_m (NADPH) of 2 μM, for purified NADPH cytochrome c reductase (Phillips & Langdon, 1962) is an order of magnitude smaller than the apparent K_m (NADPH) derived during the metabolism of Type I substrate by Orrenius (1965) and Schenkman *et al.*, (1967). This difference has cast some doubts on the role of NADPH cytochrome c reductase in the mixed function oxidase system. (Cohen & Estabrook, 1971a; Sasame & Gillette, 1970). The apparent K_m values of approximately 8 μM for different substrates obtained in the presence of pyrophosphate indicates, as suggested by Sasame & Gillette (1970) that the discrepancy can most probably be attributed to pyrophosphatase activity.

The K_m (NADPH) value of 7.92 μM obtained in the presence of pyrophosphate is in good agreement with the value of 7.04 μM reported by Lu & West (1972) using a resolved and reconstituted microsomal hydroxylating system that would be free of nucleotide pyrophosphatase activity (3,4-benz(a)pyrene was the substrate). A 1:1:1 stoichiometry for NADPH oxidised : O_2 consumed:formaldehyde produced with benzphetamine as the substrate has been shown with this reconstituted system (Lu *et al.*, 1970). The K_m (NADPH) value obtained in the present work for benzphetamine was not significantly different from the values for the other Type I substrates. The close agreement between the K_m (NADPH) values and the demonstrated stoichiometry for benzphetamine, supports the proposal that pyrophosphate effectively controls nucleotide pyrophosphatase activity to an extent that it's influence on K_m values is minimal.

Although it might appear that the comparison of K_m (NADPH) values for aminopyrine or ethylmorphine demethylation and 3,4-benz(a)pyrene hydroxylation are not strictly valid, the finding of Holtzman & Carr (1970, 1972a) that ethylmorphine demethylase and 3,4-benz(a)pyrene hydroxylase are inhibited to the same extent when D_2O is substituted for H_2O would suggest a common rate limiting step for these two substrates.

There was no significant difference between K_m (NADPH) values when either aminopyrine or ethylmorphine was used as drug substrate (Table 4.1.). However, in spite of the demonstrated stoichiometry of benzphetamine in the resolved and reconstituted hydroxylating system, this substrate undergoes debenzylation as well as N-demethylation in intact microsomes, (Gorrod, 1973), apparently due to non-cytochrome P_{450} dependent enzymes which would not be present in the resolved and reconstituted system, (Coon, 1973). It was therefore decided that benzphetamine would not be used in subsequent experiments.

The metabolism of aminopyrine to 4-aminoantipyrine involves two consecutive demethylation reactions and proceeds via the intermediate formation of monomethyl-4-aminoantipyrine. The accumulation of relatively large amounts of monomethyl-4-aminoantipyrine has been demonstrated with a fortified 9,000 x g supernatant from rat liver during a 30 min incubation period. (Gram *et al.*, 1968). Further, it was shown that aminopyrine, at concentrations greater than 2mM inhibited the demethylation of monomethyl-4-aminoantipyrine to 4-aminoantipyrine. (Gram *et al.*, 1968). It was proposed that the conversion of aminopyrine to monomethyl-4-aminoantipyrine was a fast reaction, while the second demethylation of monomethyl-4-aminoantipyrine to 4-aminoantipyrine was a slow reaction . Therefore with the short

incubation time of 5 min, the accumulation of a sufficient concentration and subsequent demethylation of this intermediate would be minimal, (Gram *et al.*, 1968; Cohen & Estabrook, 1971a). Further, there has been an isolated report (Ullrich, 1969b) in the discussion of the paper presented by Estabrook & Cohen to the symposium on "Microsomes & Drug Oxidations", that demethylation only accounts for approximately 50% of aminopyrine metabolism while hydroxylation accounts for the rest. However, as far as we are aware, the experimental results leading to this conclusion have not been published.

The possibility that more than one enzyme system is involved in the metabolism of aminopyrine has been proposed on the basis of non linear, Lineweaver & Burk plots (aminopyrine was the varied substrate - Pederson & Aust, 1970) and the inhibition caused by dieldrin and DDT (Aust & Stevens, 1971). Subsequently it was proposed that multiplicity of aminopyrine demethylase was apparent because microsomes isolated from untreated rats contained three different forms of cytochrome P_{450}, (Welton & Aust, 1974).

While the inhibitory effects of dieldrin and DDT have not been examined in the present work, the Lineweaver & Burk plots of all kinetic experiments reported (NADPH being the varied substrate) were strictly linear,suggesting that only one enzyme system was active under the experimental conditions employed. However, the possibility still exists that more than one system was active, but the linearity of such plots indicates that the total contribution of any "other" systems is minimal.

The value reported by Lu & West (1972) agrees with the value obtained at a microsomal protein concentration of 0.1 mg/ml reported by Sasame & Gillitte (1970) using 3,4-benz(a)-pyrene as substrate. However, by reducing the incubation time

and doubling the amount of IDH, the K_m (NADPH) value was reduced to 0.26 μM. The present work (Chapter 3) has shown that doubling the IDH concentration at low NADPH concentrations (5 μM and 10 μM) did not increase formaldehyde production from aminopyrine. This finding coupled with the effective elimination of pyrophosphatase activity (by pyrophosphate) indicates that higher IDH concentrations would not reduce apparent K_m (NADPH) values to any significant extent. Lower protein concentrations could not be used in *in vitro* incubations as this would result in almost undetectable levels of formaldehyde at low NADPH concentrations used in kinetic experiments.

There is a large variation in the K_m (NADPH) value of 7.04 μM reported by Lu & West (1972), and the smallest value of 0.26 μM reported by Sasame & Gillette (1970). Both studies used 3,4-benz(a)pyrene as substrates. Sasame & Gillette (1970) concluded that variations in K_m (NADPH) values could most likely be attributed to nucleotide pyrophosphatase activity. The assay conditions used also influenced the resultant values although there was essentially little variation in the assay for 3,4-benz(a)pyrene between the two reports. The resolved and reconstituted system would be free of nucleotide pyrophosphatase activity.

The most likely explanation for the discrepancy would be the loss of some component during solubilisation of the microsomal components which is present in the crude microsomal suspension. This unknown factor which is not obligatory, would facilitate the transfer of electrons from NADPH to the cytochrome P_{450} substrate complex. Such a factor has been already proposed to exist between NADPH cytochrome c reductase and cytochorme P_{450}, (Dallner *et al.*, 1966; Estabrook, 1969, 1971a; Dallman

et al., 1969; Kupfer & Orrenius, 1970; Estabrook & Cohen, 1969; Ichihara *et al.*, 1973). More recently, Mull *et al.*, (1975) has proposed that Factor "X" appears to be a non-haem iron protein with a molecular weight of 48,000.

Turnover studies (Lu *et al.*, 1974) with the resolved and reconstituted system using 3,4-benz(a)pyrene and benzphetamine as substrate have suggested that this unkown factor if it does exist would have little influence on the overall rates of product formation.

The K_m (NADPH) value of 10 μM reported by Ullrich, (1969a) with the Type I substrate, cyclohexane, is much closer to the values obtained in the present study. No attempt was made to control or assess the influence of nucleotide pyrophosphatase or NAD glycohydrolase activities in the microsomal fraction used, which was isolated from male Wistar rats. It might be possible that the lower *in vitro* incubation temperature of 25°C could reduce to a greater extent the activity of the enzymes that degrade the pyridine nucleotides. Further, the phosphate buffer used by Ullrich (1969a) in the *in vitro* incubation medium has been shown to exert only an indirect inhibitory effect on nucleotide pyrophosphatase activity (Gang *et al.*, 1972). However, it is unlikely that this latter observation can be used to explain why the value reported by Ullrich is lower than those reported by Schenkman *et al.*, (1967), or Orrenius (1965) when they all used Type I substrates.

Remmer (1966) has shown that drug substrates combine with oxidised cytochrome P_{450} to produce difference spectra which were classified as either a Type I (peak and trough at approximately 385 nm, 420 nm, respectively) or a Type II (peak ca. 430 nm, trough ca. 390 nm) spectral change. This observation of complex

formation between drug substrates and oxidised cytochrome P_{450} raised the possibility that the reduction of the cytochrome P_{450}- substrate complex might be the rate limiting step.

There was a greater correlation between the rate of ethylmorphine demethylation and NADPH-cytochrome P_{450} reductase activity than between ethylmorphine demethylase and either cytochrome P_{450} content, NADPH cytochrome c reductase or the magnitude of the Type I spectral change in various species (Davies *et al.*, 1969). Gigon *et al.*, (1968, 1969), has shown that Type I substrates stimulate the NADPH mediated reduction of cytochrome P_{450} while Type II substrates retard cytochrome P_{450} reduction. It was proposed that Type I substrates induce a perturbation in cytochrome P_{450} such that the electron from NADPH is transferred more readily to the cytochrome-P_{450}-substrate complex than to cytochrome P_{450} alone. Moreover, it was demonstrated (Gigon *et al.*, 1968) that a 1:1 stoichiometry existed between the extent of ethylmorphine demethylation and the difference between the unstimulated and ethylmorphine stimulated rate of cytochrome P_{450} reduction. Holtzman *et al.*, (1968) supported the proposition that cytochrome P_{450} reduction might be the rate limiting step in rabbit microsomes, but the actual amount of this haemoprotein was more likely to be rate limiting in rat microsomes.

The failure to observe a marked isotope effect with 4,4-dideuterioNADPH on (1) either ethylmorphine demethylase or aniline hydroxylase activity, (2) the oxidation of NADPH and (3) the uptake of oxygen suggests that the fission of the C-H bond of NADPH is not the rate limiting step for microsomal mixed function oxidase hydroxylation reactions (Holtzman, 1970).

In contrast to his earlier statement, Holtzman has repeatedly demonstrated (Holtzman & Rumack, 1971, 1973; Holtzman & Carr, 1972a; Holtzman, 1974), a 1:1 stoichiometry in rat liver microsomes between ethylmorphine demethylation and the difference between NADPH-cytochrome P_{450} reductase activity in the presence and absence of ethylmorphine (Δ P_{450} reductase). The conclusion from these studies was that cytochrome P_{450} reduction governs the overall rate of mixed funcion oxidase reactions. (Holtzman & Carr, 1970, 1972a; Holtzman & Rumack, 1971, 1973; Holtzman, 1974).

Schenkman & Cinti (1970) have calculated activation energies for aminopyrine demethylase and NADPH cytochrome P_{450} reductase, and shown these to be identical. The activation energy for NADPH-cytochrome P_{450} reductase did not change when either aminopyrine or hexobarbital was added to the reaction. Also the Q10 value for aminopyrine demethylase of 2.0 was in close agreement with the value of 1.9 obtained for NADPH-cytochrome P_{450} reductase. The similarity in activation energies was confirmed in a later publication (Schenkman, 1972) and stoichiometry between aminopyrine demethylation and Δ cytochrome P_{450} reductase in the absence and presence of aminopyrine demonstrated. These studies also eliminated oxygen activation, the interaction of activated oxygen with the donor substrate and the release of products as possible rate limiting steps.

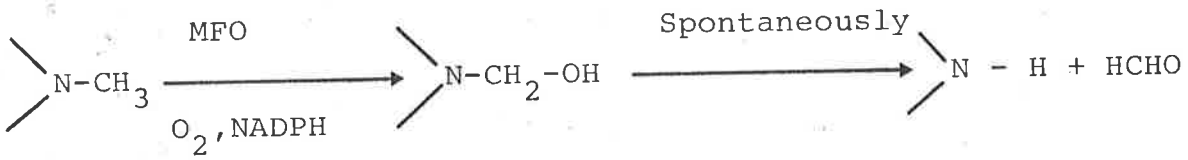
The conclusion from these studies was that the rate of reduction of the cytochrome P_{450}-substrate complex was rate limiting when aminopyrine was the substrate. This might not necessarily be the case when either ethylmorphine or aniline was the substrate because of dissimilar activation energies of aniline hydroxylase and ethylmorphine demethylase to that of NADPH-cytochrome P_{450} reductase.

However, similar experiments by Holtzman & Carr, (1972b), have suggested that when either aniline or ethylmorphine was the added substrate, cytochrome P_{450} reduction might be rate limiting. Moreover, it was also suggested that these correlations might be fortuitous and the possibility that the introduction of the second electron was rate limiting proposed. Cytochrome b_5 or a second cytochrome P_{450} molecule were suggested as possible donors of this second electron.

Estabrook *et al.*, (1973) suggested that one consequence of phenobarbital induction of the hepatic mixed function oxidase system is to change the rate limiting step from the transfer of an electron from NADPH to the ferric cytochrome P_{450}- substrate complex (in untreated animals) to another reaction step in the overall cyclic function of cytochrome P_{450}. It was suggested that the introduction of the second electron to the oxygenated ferrous cytochrome P_{450}- substrate complex assumes this role.

More recently, Matsubara *et al.*, (1976) have proposed that the rate limiting step for ethylmorphine demethylase in microsomes isolated from phenobarbital induced rats occurs after the oxygen binding reaction and before the binding of another substrate molecule.

The actual mechanism of N-demethylation has been suggested (Brodie *et al.*, 1958; Holtzman, 1970) to proceed via an intermediate hydroxylation reaction according to Scheme 4.1.



Scheme 4.1. Mechanism of microsomal N-demethylation.

Recently, Thompson & Holtzman (1974) have suggested that the breaking of one of the C-H bonds in the N-CH_3 group when the

"active oxygen (OH))" is substituted for one of the N-methyl hydrogen atoms is a rate limiting step for ethylmorphine demethylase. Further, the previous suggestion that the introduction of the second electron is also rate limiting was reaffirmed.

It is evident that various investigators consider different sites in the overall hydroxylation sequence to be rate limiting. At the present time, however, the bulk of the experimental evidence would suggest that the rate of reduction of the cytochrome P_{450}-substrate complex is the rate limiting step.

The present study shows that the rate of ethylmorphine demethylation is approximately 65% of the aminopyrine demethylation rate which agrees with the results of Schenkman. However, Holtzman using the same strain of rats that was used by Schenkman has found the converse to be true, i.e. ethylmorphine is more rapidly demethylayted than aminopyrine (Schenkman, 1973). Such variations in basic demethylation rates emphasises the difficulties in comparing and extrapolating results from different laboratories.

NADPH-cytochrome P_{450} reductase activity was assayed at $26^{o}C$ instead of the usual $37^{o}C$ because the reduction rate was found to be more reproducible at various NADPH concentrations at the lower temperature. This finding confirms the observation of Fouts & Pohl (1971). Therefore, stoichiometry calculations between the demethylation of either aminopyrine or ethylmorphine and the Δ cytochrome P_{450} reductase activity plus or minus the respective substrate, were not attempted because of the different assay temperatures.

The percentage increase above control values at $26^{o}C$ of NADPH-cytochrome P_{450} reductase activity was 143% and 240% for aminopyrine and ethylmorphine respectively.

Increases of 142% for aminopyrine and 180% for ethylmorphine have been reported for NADPH-cytochrome P_{450} reductase at 37°C. (Gigon *et al.*, 1968; Gillette, 1969). The reason why ethylmorphine stimulates cytochrome P_{450} reduction to a greater extent is, at the moment, unclear, but it could possibly be due to the different rat strain used in the present study.

The results of Table 4.3. suggest that the nature of the substrate might influence the cytochrome P_{450} binding site for the introduction of the first electron, which originated in NADPH. A possible alternative explanation of these results would be the influence of various substrates on NADPH-cytochrome P_{450} reductase activity.

This study shows that the inclusion of Type I substrates in the assay medium for NADPH-cytochrome P_{450} reductase caused a significant reduction in the K_m value and an increase in V.

Further, the magnitude of these changes were dependent on the nature of the added substrate. These results are compatible with the suggestion of Gigon *et al.*, (1969) that Type I substrate induces a structural change such that the oxidised cytochrome P_{450} substrate complex is more readily reduced than cytochrome P_{450} alone, while Type II substrates retard the reduction of cytochrome P_{450}- substrate complex. The K_m and V values reported for NADPH-cytochrome P_{450} reductase (Table 4.1) do not correlate with those obtained in metabolism experiments. Such variation may be due to the anaerobic environment necessary to measure NADPH-cytochrome P_{450} reductase activity.

C H A P T E R 5

THE INFLUENCE OF NADH ON NADPH KINETICS DURING HEPATIC MIXED FUNCTION OXIDASE REACTIONS.

5.1. INTRODUCTION

While it is well established that NADPH is a more efficient electron donor than NADH during drug hydroxylations, it has also been known for some years that there is a synergistic increase in product formation in the presence of both of these reduced pyridine nucleotides. (Conney *et al.*, 1957a; Nilsson & Johnson, 1963).

In 1971, Cohen & Estabrook (1971a,b,c) and Hildebrandt & Estabrook (1971) proposed a model to explain the observed increases in enzymic activity in the presence of both NADPH and NADH. Most of the evidence obtained subsequently has with minor modifications supported this proposal. Nevertheless, alternative explanations of the NADH stimulation of NADPH linked drug hydroxylation have been suggested by Correia & Mannering (1973a, b,c) and Staudt *et al.*, (1974).

In the previous Chapter, K_m (NADPH) values were evaluated for NADPH-cytochrome P_{450} reductase (in the presence of various substrates) and for the demethylation of aminopyrine and ethylmorphine. This Chapter records the influence of NADH on NADPH kinetics for these reactions in an attempt to further characterise the pyridine nucleotide involvement in hepatic mixed function oxidase hydroxylations. The results obtained by varying NADH concentrations while holding the NADPH concentration constant are also reported.

5.2. RESULTS

5.2.1. EFFECT OF NADH ON NADPH KINETICS DURING DRUG METABOLISM

The K_m and V values of NADPH for the mixed function oxidase system using either aminopyrine or ethylmorphine as substrate in the presence of three fixed NADH concentrations are shown in Table 5.1.

TABLE 5.1.

K_m and V values for NADPH in the presence of substrates and NADH.

	Aminopyrine			Ethylmorphine		
NADH CONCENTRATION	96.1 (μM)	19.2 (μM)	12.0 (μM)	96.1 (μM)	19.2 (μM)	12.0 (μM)
K_m [a]	3.57 ± 0.12[e]	3.31 ± 0.31[e]	5.0 ± 0.86[e]	5.77 ± 0.82[f]	6.5 ± 0.92	5.7 ± 0.92[f]
V [b]	52.7 ± 0.31	41.1 ± 0.63[e]	36.0 ± 1.9[e]	47.6 ± 1.7[e]	36.7 ± 1.3	33.8 ± 1.3
r [c]	.999	.991	.982	.986	0.99	.986
N [d]	3	6	3	3	3	3

a K_m expressed as μmolar : mean ± S.D.

b V expressed as nmoles HCHO formed/5min/mg microsomal protein : mean ± S.D.

c correlation coefficient, as defined in the computational Methods (2.2.9)

d number of determinations.

e significantly different from appropriate control at $P < 0.005$.

f significantly different from appropriate control at $P < 0.05$.

The kinetic parameters obtained in the absence of NADH (Table 4.1.) are as follows:-

Aminopyrine

K_m = 7.92 ± 0.82 μM.

V = 50.3 ± 1.48 nmoles HCHO formed/5min/mg microsomal protein.

Ethylmorphine

K_m = 8.49 ± 1.48 μM.

V = 33.8 ± 2.25 nmoles formed/5min/mg microsomal protein.

The K_m (NADPH) value of 7.92 µM obtained in the absence of NADH (Table 4.1.) was reduced to 3.57 µM in the presence of 96.1 µM NADH with aminopyrine as substrate. A similar reduction from the control K_m (NADPH) value of 8.49 µM to 5.77 µM was obtained when ethylmorphine replaced aminopyrine as substrate.

The reduction in K_m was also significant when the NADH concentration was reduced to either 19.2 or 12.0 µM. In the presence of 96.1 µM NADH, there was a 5% and a 41% increase in apparent V values with aminopyrine and ethylmorphine respectively. It can be seen (Table 5.1.) that V, in the presence of either 19.2 or 12.0 µM NADH and with aminopyrine as substrate, was significantly lower than the V recorded in the absence of NADH. This contrasts with the lack of significant difference from control V found with these concentrations of NADH when ethylmorphine was the substrate.

5.2.2. EFFECT OF NADH ON NADPH CYTOCHROME P_{450} REDUCTASE KINETICS.

Figure 5.1. shows the titration of cytochrome P_{450} reductase as a function of NADPH concentration in the presence and absence of 119 µM NADH. The curves are the computer generated lines of best fit. Both reduced pyridine nucleotides were introduced simultaneously to initiate the reaction when the K_m for NADPH was measured in the presence of NADH and aminopyrine.

The V value increased from a control value of 5.28 to 14.6 nmoles cytochrome P_{450} reduced/min/mg microsomal protein in the presence of 119 µM NADH. There was, however, no significant change in K_m (NADPH) values.

NADPH-cytochrome P_{450} reductase activity with NADH and either aminopyrine or ethylmorphine was approximately 10% of that obtained with NADPH. Both graphs are hyperbolic, suggesting that NADH exerts a heterotropic co-operative effect with respect to NADPH.

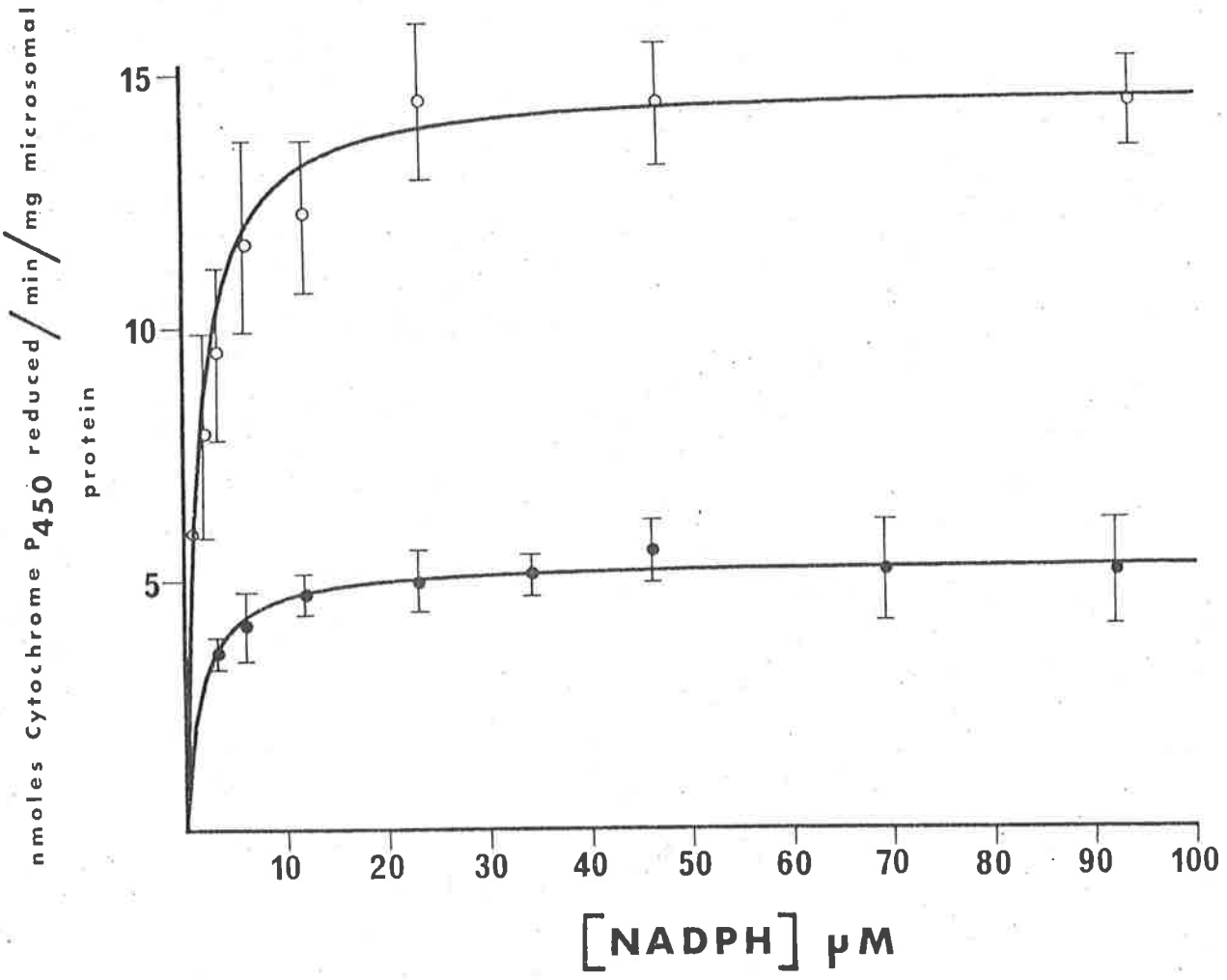


FIGURE 5.1. Titration of NADPH-cytochrome P_{450} reductase activity as a function of NADPH concentration in the presence and absence of NADH.

The points are the mean ± S.D. of three duplicate determinations, while the lines are the computer generated lines of best fit in the absence (●——●) and presence (○——○) of 119 μM NADH. The microsomal suspension used for each determination was obtained by pooling the livers from three control rats.

NADPH-cytochrome P_{450} reductase was assayed as described in the Methods in the presence of 4mM aminopyrine.

The data for NADPH-cytochrome P_{450} reductase in the absence of NADH has been redrawn from Figure 4.2. The velocity readings at each substrate concentration were weighted with 1/CV arising from these readings for that substrate concentration.

Similar results were obtained when the NADH concentration was reduced to 20.2 μM (Table 5.2.). While there was no significant difference in V values obtained in the presence of the higher NADH concentration with either aminopyrine or ethylmorphine, there was a difference in the V values of NADPH-cytochrome P_{450} reductase for these two substrates in the absence of NADH (Table 4.3).

The marked increase in V value was still apparent when the NADH concentration was lowered to 20.4 μM in the presence of ethylmorphine. However, when aniline was the added substrate, the K_m (NADPH) value dropped from 3.7 μM (in the absence of NADH) to 1.46 μM. Further, the apparent V value was increased by 94% above the value obtained in the absence of NADH to 4.26 nmoles cytochrome P_{450} reduced/min/mg microsomal protein.

An increase in NADPH-cytochrome P_{450} reductase activity from 3.6 $\pm$ 0.2 in the absence of NADH to 6.6 $\pm$ 0.7 nmoles of cytochrome P_{450} reduced/min/mg microsomal protein in the presence of 123 μM NADH was observed in the absence of added substrates. (N = 3).

5.2.3. ANILINE HYDROXYLASE ACTIVITY IN THE PRESENCE OF SATURATING PYRIDINE NUCLEOTIDE CONCENTRATIONS.

The activity of aniline hydroxylase in the presence of NADPH, NADH or both pyridine nucleotides together is given in Table 5.3. The activity with NADH as the sole electron donor is only 11% of that obtained in the presence of NADPH. However, a synergistic increase in p-aminophenol production was observed when both pyridine nucleotides were present when compared to the rates obtained with either NADPH or NADH alone.

TABLE 5.2.

K_m and V values of NADPH or NADPH-cytochrome P_{450} reductase in the presence of NADH.

	Aminopyrine		Ethylmorphine		Aniline
NADH CONCENTRATION [f]	119	20.2	98	20.4	99.1
	(μM)	(μM)	(μM)	(μM)	(μM)
K_m [a]	1.43 ± 0.23	1.9 ± 0.19	2.01 ± 0.22	1.54 ± 0.21[d]	1.46 ± 0.17[d]
V [be]	14.61 ± 0.32	14.53 ± 0.24	14.75 ± 0.49	12.43 ± 0.31	4.26 ± 0.14
r [c]	0.990	0.994	0.986	0.992	0.988

a K_m expressed as μmolar : mean ± S.D.

b V expressed as nmoles cytochrome P_{450} reduced/min/mg microsomal protein : mean ± S.D.

c correlation coefficient as defined in the computational Methods (2.2.9).

d significantly different from the value obtained in the absence of NADH at $P < 0.05$.

e significantly different from the value obtained in the absence of NADH at $P < 0.001$.

f mean concentration : the coefficient at variation of NADH concentration was always < 4%.

The values assigned to the kinetic parameters were obtained from three duplicate independent determinations. The final concentrations of aminopyrine and ethylmorphine and aniline in the reaction were 4mM, 6mM and 4mM respectively.

TABLE 5.3.

Aniline Hydroxylase activity in the presence of saturating pyridine nucleotide concentrations.

PYRIDINE NUCLEOTIDE CONCENTRATION	ANILINE HYDROXYLASE [a]	% OF CONTROL ACTIVITY
NADPH (191 μM)	3.76 ± 0.11 [b]	100
NADH (202 μM)	0.43 ± 0.2	11.4
NADPH (191 μM) + NADH (202 μM)	5.52 ± 0.09	147

a : nmoles p-aminophenol formed/10min/mg microsomal protein.

b values are the average of two determinations : mean ± difference from the mean.

5.2.4. EFFECT OF DIFFERENT NADH CONCENTRATIONS IN THE PRESENCE OF A FIXED NADPH CONCENTRATION.

Figure 5.2. shows the titration of aminopyrine and ethylmorphine demethylase as a function of NADH concentration in the presence of 104.7 μM NADPH. Each point represents the total value obtained in the presence of both the fixed concentration of NADPH and the various concentrations of NADH less the value obtained for NADPH alone. These control values were 40 nmoles and 43.3 nmoles HCHO formed/5min/mg microsomal protein for aminopyrine and ethylmorphine respectively. The data could not be successfully fitted to the Michaelis-Menten equation using HYPER, based upon criteria established in the Methods. The same data were fitted to the Hill equation (Equation 5.1.) using the

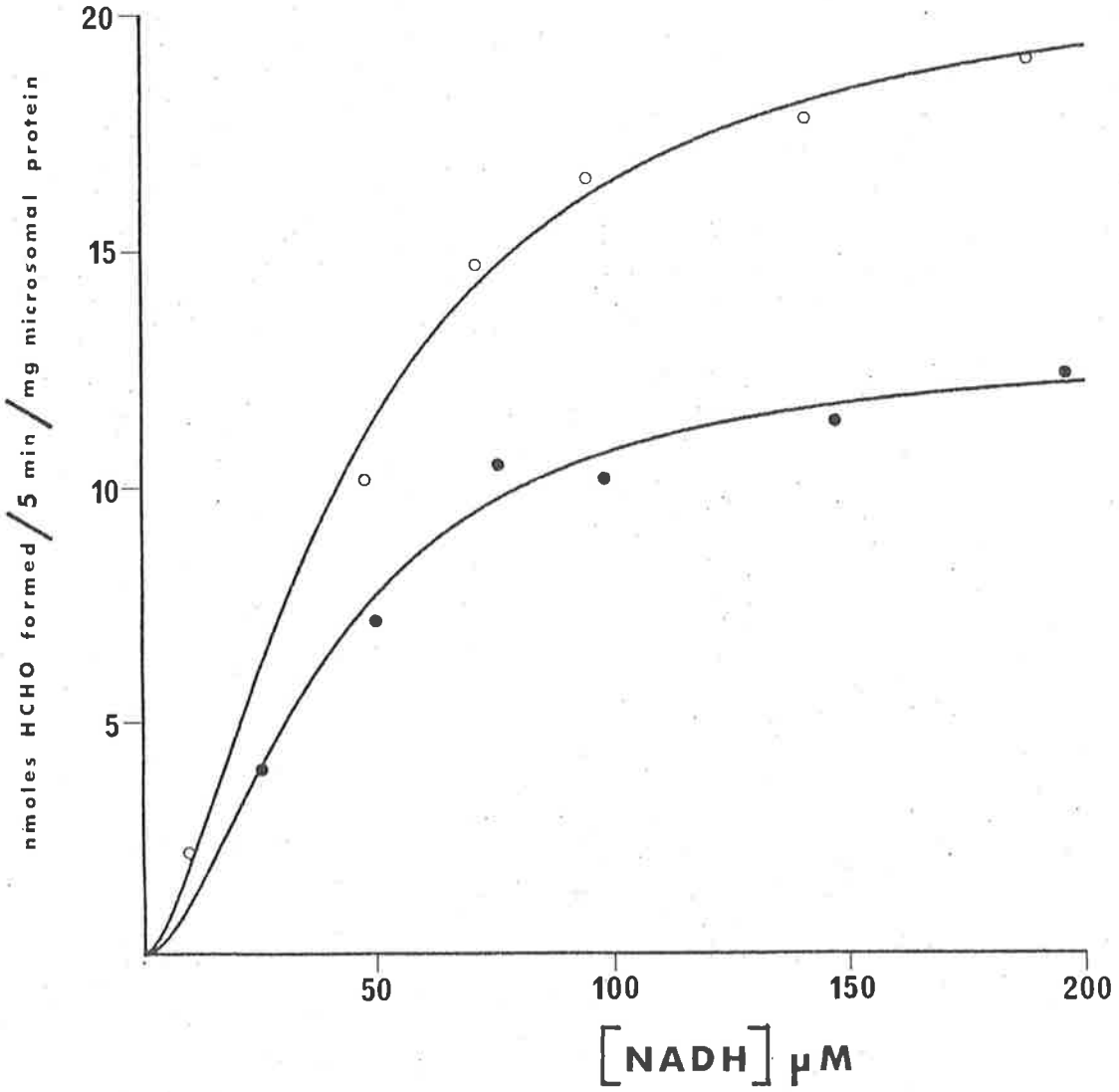


Figure 5.2. Titration of aminopyrine and ethylmorphine demethylase activity as a function of NADH concentration in the presence of 104.7 µM NADPH.

The substrates, aminopyrine (●——●) and ethylmorphine (○——○) were included at a final concentration of 4mM and 6mM respectively. The activity obtained in the presence of NADPH alone was subtracted from all readings obtained in the presence of both reduced pyridine nucleotides. These control values were 40 nmoles and 43.4 nmoles HCHO formed/5min/mg microsomal protein. The points are the mean values of three determinations while the lines are the computer generated lines of best fit by NONLIN using all the individual data points.

digital computer programme NONLIN (Metzler, 1969).

$$v = \frac{V.S^n}{K + S^n} \quad \ldots . \quad (5.1.)$$

where v is the initial velocity, V is the maxiumum velocity, S is the substrate concentration and n and K are constants. This equation, which is applied to the kinetics of allosteric enzymes, reverts to the normal Michaelis-Menten equation, when n = 1.

In Figure 5.2. the curves are the computer generated lines of best fit. The sigmoidal nature of these curves with either aminopyrine or ethylmorphine are indicative of a homotropic co-operative NADH effect. The values of the constants of the Hill equation using the above data are given in Table 5.4.

With both substrates, the n values approach a value of 2 with an NADPH concentration of 104.7 μM.

However, when the experiments were repeated in the presence of approximately 350 μM NADPH, the n value was reduced from 1.74 to 1.38 for aminopyrine and from 1.53 to 1.0 for ethylmorphine. Similar numerical values of K, V and n were obtained when the experiments were repeated, but the correlation coefficient, r, was not significantly improved.

5.2.5. REDUCTION KINETICS OF THE CYTOCHROME P_{450}-AMINOPYRINE COMPLEX BY NADPH.

A semi-logarithmic plot of the percent unreduced cytochrome P_{450}-aminopyrine complex as a function of time is shown in Figure 5.3. The reaction was initiated by the introduction of NADPH to give a final concentration of 107.4 μM. The graph consists of two phases and therefore can be expressed in mathematical form thus:-

$$P = Ae^{-r_1 t} + Be^{-r_2 t} \quad \ldots . \quad (5.2.)$$

TABLE 5.4.

Hill Equation Parameters of NADH determined in the presence of NADPH.

	AMINOPYRINE		ETHYLMORPHINE	
NADPH CONCENTRATION	104.7 (μM)	379 (μM)	104.7 (μM)	347.6 (μM)
K	453 ± 116.6	100 ± 23.4	338.6 ± 21.3	80 ± 12.0
V [a]	10.2 ± 0.5	10.2 ± 0.8	21.3 ± 2.6	20 ± 3.9
n	1.74 ± 0.1	1.38 ± 0.12	1.53 ± 0.1	1.0 ± 0.11
r	0.971	0.96	0.94	0.95

a V expressed as nmoles HCHO formed/5min/mg microsomal protein.

The data (obtained as previously described in the legend of Figure 5.2.) was fitted to the Hill equation using the digital computer programme NONLIN, (Metzler, 1969).

Initial estimates required by NONLIN were obtained by first fitting the data to the Hill equation using Atkins (1973) programme. The initial estimates were:-

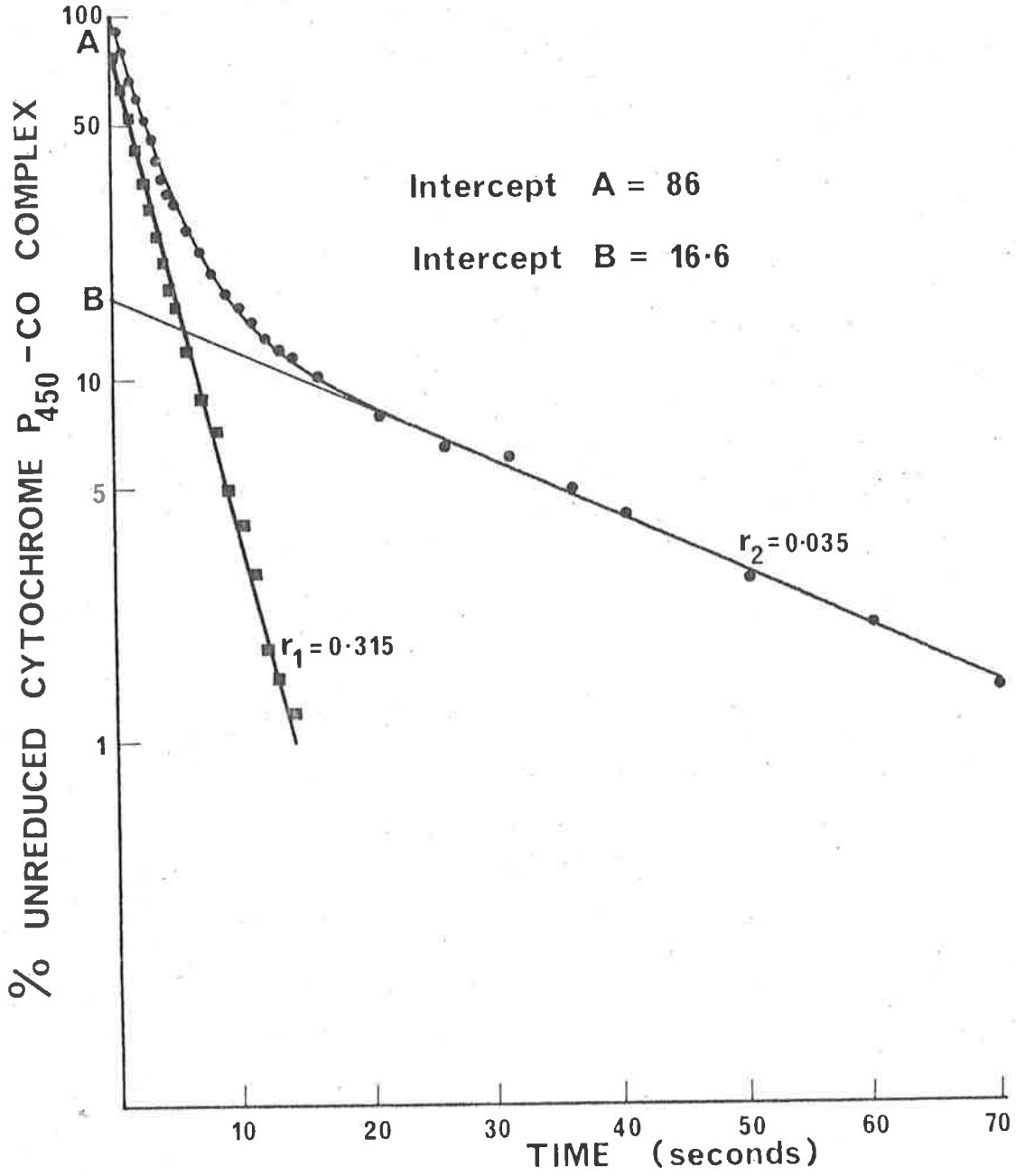
	AMINOPYRINE		ETHYLMORPHINE	
NADPH CONCENTRATION	104.7 (μM)	379 (μM)	104.7 (μM)	347.6 (μM)
K	457	131.6	338.7	105.1
V	10.1	9.6	21.33	15.95
n	1.74	1.49	1.53	1.2

FIGURE 5.3. The reduction of the cytochrome P_{450}-aminopyrine complex by NADPH as a function of time.

The reduction of the cytochrome P_{450}-aminopyrine complex by NADPH (107.4 μM) was measured as described in the Methods.

The points (●) represent the amount of unreduced cytochrome P_{450}-aminopyrine complex at a particular time, t. The straight line (■——————■) was obtained by "peeling" the curve, i.e., at each time interval t, the difference between the curve and the value at the same time of the straight line obtained by back projection of the terminal linear section of the curve is plotted as a function of time - r_1 and r_2 are the slopes of the straight lines.

The graphical estimates of A,B,r_1 and r_2 were used as initial estimates in NONLIN for the data in Table 5.5.


100
A
50
B
10
5
1
% UNREDUCED CYTOCHROME P450-CO COMPLEX
Intercept A = 86
Intercept B = 16·6
r2 = 0·035
r1 = 0·315
10
20
30
40
50
60
70
TIME (seconds)

where P represents the amount of unreduced cytochrome P_{450}-aminopyrine complex at time t, and A, r_1, B and r_2 are constants. The data obtained from three separate duplicate experiments was fitted to the above equation using NONLIN.

Equation 5.2., which describes the reduction kinetics was supplied in the sub-routine D FUNC. The data from each individual experiment was fitted to the above equation using a weight factor of 1. Then, at each time interval, t, the data from the three duplicate experiments were bulked and a mean and standard deviation calculated. Weight factors of unity, reciprocal of the variance and reciprocal of the coefficient of variation at each time interval were used to weight the bulked data for that time interval. The numerical values assigned to A, r_1, B, and r_2 by NONLIN are shown in Table 5.5.

No significant differences were found in the values of the constants using the various weight factors. The excellent fit of the data to equation 5.2. is shown by the small standard deviations of all constants and the high value of the correlation coefficient, cor.

5.2.6. REDUCTION OF THE CYTOCHROME P450-AMINOPYRINE COMPLEX BY NADPH IN THE PRESENCE OF NADH.

Figure 5.4. shows the reduction of the cytochrome P_{450}-aminopyrine complex as a function of time when the reaction was initiated by the addition of NADPH and NADH together. The final concentrations of NADPH and NADH were 107.4 µM and 101.1 µM respectively.

The graph of the results obtained is biphasic and data was therefore fitted to equation 5.2. (Table 5.6.).

TABLE 5.5.

Numerical values of the constants of Equation 5.2. when the reaction was initiated by NADPH alone.

EXPERIMENT	PARAMETERS [a]				
	A	r_1	B	r_2	cor [b]
1.	87.8 ± 1.2	0.39 ± 0.01	21.3 ± 1.05	0.043 ± 0.003	0.999
	80.1 ± 1.4	0.42 ± 0.01	22.7 ± 1.0	0.036 ± 0.002	1.000
2.	80.1 ± 1.26	0.47 ± 0.013	24.3 ± 0.6	0.023 ± 0.001	0.999
	73.0 ± 1.54	0.41 ± 0.016	25.0 ± 1.1	0.033 ± 0.002	0.999
3.	77.6 ± 1.05	0.36 ± 0.01	23.4 ± 0.8	0.031 ± 0.002	1.000
	79.8 ± 0.74	0.41 ± 0.01	22.1 ± 0.6	0.039 ± 0.001	1.000
Bulked	80.2 ± 1.17	0.41 ± 0.01	23.0 ± 0.8	0.033 ± 0.002	0.996

a mean ± S.D.

b correlation coefficient as defined in the computational Methods (2.2.9.).

The data was obtained from the reduction of cytochrome P_{450}-aminopyrine -CO complex as a function of time when the reaction was initiated by NADPH (107.3 μM).

Aminopyrine was included in the reaction at a final concentration of 4mM.

The weight factor used for the computations was the reciprocal of the variance.

FIGURE 5.4. The reduction of the cytochrome P_{450}- aminopyrine complex by the simultaneous addition of NADPH and NADH as a function of time.

The reduction of the cytochrome P_{450}- aminopyrine complex by the simultaneous addition of NADPH (107.4 μM) and NADH (100.1 μM) was measured as described in the Methods.

The points (●) represent the amount of unreduced cytochrome P_{450}- aminopyrine complex at a particular time, t. The straight line (■——————■) was obtained by "peeling" the curve, i.e., at each time interval t, the difference between the curve and the value at the same time of the straight line obtained by back projection of the terminal linear section of the curve is plotted as a function of time - r_1 and r_2 are the slopes of the straight lines.

The graphical estimates of A,B,r_1 and r_2 were used as initial estimates in NONLIN for the data in Table 5.6.

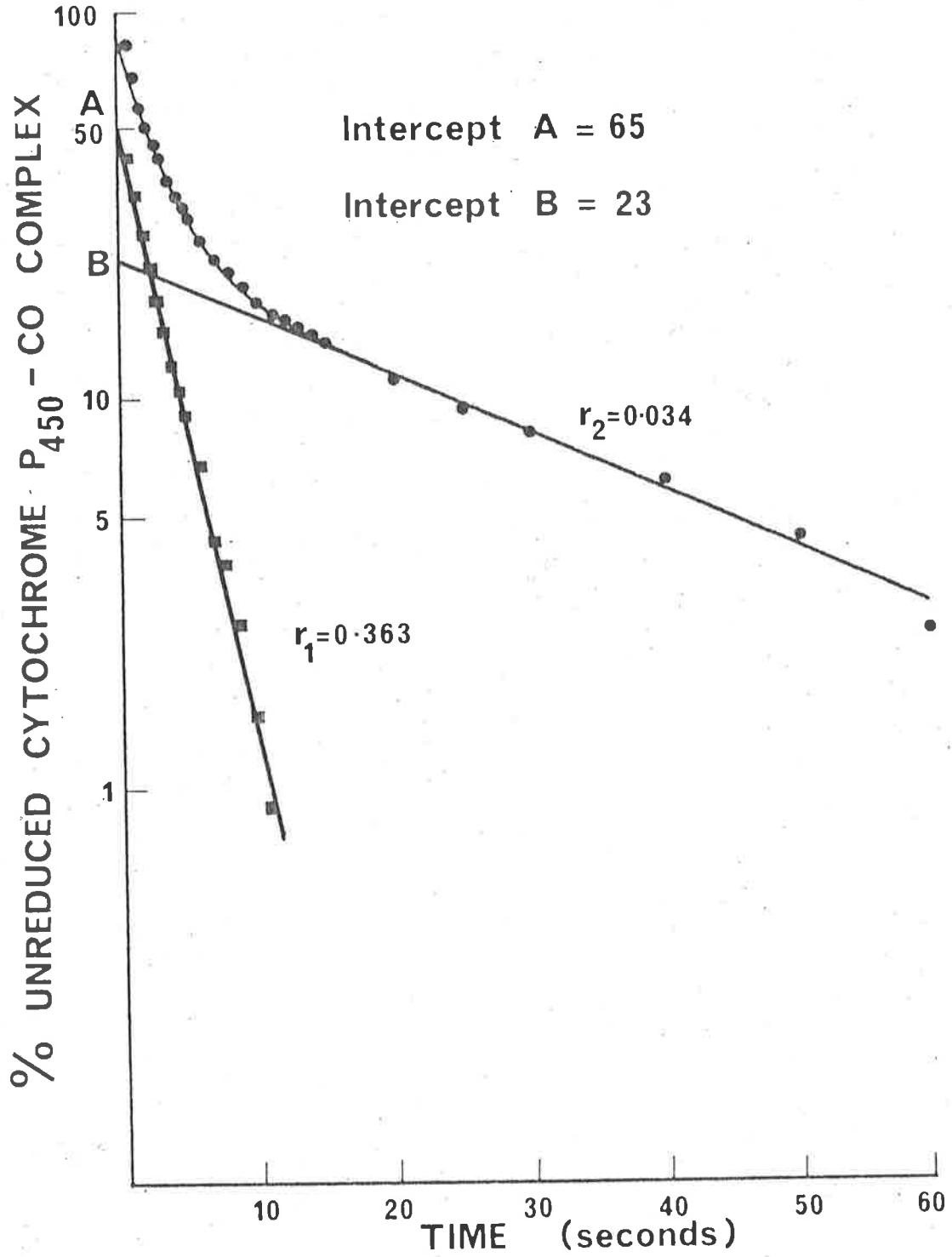

100
A
50
B
10
5
1
% UNREDUCED CYTOCHROME P_{450} - CO COMPLEX
Intercept A = 65
Intercept B = 23
r_2=0·034
r_1=0·363
10
20
30
40
50
60
TIME (seconds)

TABLE 5.6.

Numerical values of the constants of Equation 5.2. when the reaction was initiated by the simultaneous addition of NADPH and NADH.

EXPERIMENT	PARAMETERS [a]				
	A	r_1	B	r_2	cor [b]
1.	70.2 ± 0.47	0.47 ± 0.004	26.7 ± 0.16	0.039 ± 0.001	1.000
	71.7 ± 1.07	0.489 ± 0.01	25.3 ± 0.36	0.039 ± 0.001	1.000
2.	62.8 ± 0.66	0.50 ± 0.006	27.5 ± 0.2	0.039 ± 0.001	0.998
	61.7 ± 0.66	0.49 ± 0.006	29.3 ± 0.27	0.037 ± 0.001	0.999
3.	71.2 ± 0.64	0.5 ± 0.006	24.2 ± 0.21	0.04 ± 0.001	0.999
	66.8 ± 0.74	0.47 ± 0.007	28.6 ± 0.25	0.037 ± 0.001	0.999
Bulked	67.5 ± 0.7	0.49 ± 0.006[c]	26.9 ± 0.22	0.039 ± 0.001[d]	0.998

a mean ± S.D.

b correlation coefficient.

c significantly different compared to r_1 (Table 5.5.) at $P < 0.001$.

d significantly different compared to r_2 (Table 5.5.) at $P < 0.01$.

The data was obtained from the reduction of the cytochrome P_{450}-aminopyrine-CO complex as a function of time when the reaction was initiated by the simultaneous addition of NADPH (107.3μM) and NADH (100.1 μM). Aminopyrine was included in the reaction at a final concentration of 4mM. The data was weighted with the reciprocal of the coefficient variation.

The half life ($0.691/r_1$) of the initial rapid phase (r_1 of equation 5.2.) determined in the presence of both reduced pyridine nucleotides (1.4. sec) was significantly lower than that obtained in the absence of NADH (1.7 sec $P < 0.001$).

5.2.7. STEADY STATE CYTOCHROME b_5 LEVELS DURING THE METABOLISM OF AMINOPYRINE AND ETHYLMORPHINE.

Table 5.7. shows the steady state levels of reduced cytochrome b_5 during the metabolism of aminopyrine or ethylmorphine. The incubation medium for these experiments was identical to that used for *in vitro* N-demethylation studies except that the final protein concentration was adjusted to 3mg/ml. The microsomal suspension used for each determination was obtained by combining the microsomal fraction from three rats.

After adjusting the cuvette contents to 37°C and establishing a baseline, 25 µl of an NADPH solution (final concentration of 93.1 µM) was added to the test cuvette and an equivalent amount of buffer to the reference cuvette and the spectrum determined from 400 nm to 500 nm. Immediately following this, the same procedure was used to add an appropriate amount of NADH. The cuvettes were incubated at 37°C for 5 min and the spectrum determined again.

It is evident that NADPH reduces approximately 0.25 nmoles cytochrome b_5/mg microsomal protein in the presence of either aminopyrine or ethylmorphine. The further addition of 10.6 µM NADH increases the steady state level of reduced cytochrome b_5 to 0.46 nmoles/mg microsomal protein. However, after incubation at 37°C for 5 min, the level of reduced cytochrome b_5 had almost returned to that obtained for NADPH. When 106.1 µM NADH is added to 93.1 µM NADPH, almost complete reduction of cytochrome b_5 is maintained during the 5 min incubation at 37°C in the presence of

TABLE 5.7.

Steady state b_5 levels during the metabolism of aminopyrine and ethylmorphine.

PYRIDINE NUCLEOTIDE (A)	CONCENTRATION OF A (μM)	REDUCED CYTOCHROME b_5 LEVELS DURING METABOLISM (nmoles/mg microsomal protein	
		AMINOPYRINE	ETHYLMORPHINE
NADPH	93.1	0.261	0.234
NADH	10.6	0.468	0.459
	21.2	0.477	0.459
	106.1	0.459	0.45
LEVELS OF CYTOCHROME b_5 REMAINING AFTER ADDITION OF NADH (5MIN AT 37°C)	10.6	0.324	0.252
	21.2	0.342	0.27
	106.1	0.414	0.432

The incubation medium was identical to that used in metabolism studies except that the protein concentration was 3mg/ml. The cuvette contents were adjusted to 37°C and a baseline established. The spectrum was re-recorded after NADPH (25 μl, final concentration 93.1 μM) was added to one cuvette and an equivalent volume of buffer to the reference cuvette. 25 μl of approriate NADH solution was added to the test cuvette and the spectrum recorded immediately and after 5 min at 37°C.

both drug substrates. When 21.2 μM NADH was added after the NADPH solution, the level of reduced cytochrome b_5 (after the 5 min incubation) was intermediate between these two extremes.

There appears to be a constant ratio between the maximum velocity for N-demethylation (obtained from Table 5.1.) and the steady state concentration of reduced cytochrome b_5 after the 5 min incubation at 37°C (from Table 5.7.) at various NADH concentrations (Table 5.8.).

TABLE 5.8.

Ratio of V values for the demethylation of aminopyrine and ethylmorphine to the steady state concentration of reduced cytochrome b_5 in the presence of various NADH concentrations.

APPROXIMATE NADH CONCENTRATION (μM)	V VALUE (TABLE 5.1.) (A)	STEADY STATE CYTOCHROME b5 LEVELS DURING METABOLISM (TABLE 5.7.) (B)	A/B RATIO
	AMINOPYRINE		
100	52.7	0.414	127
20	41.1	0.342	120
10	36.0	0.32	113
	ETHYLMORPHINE		
100	47.6	0.43	111
20	36.7	0.27	136
10	33.8	0.25	135

NADH concentrations have been approximated to 100, 20 and 10 μM. The actual concentrations are 96.1 μM (metabolism) and 106.1 μM (b_5 levels) for the highest NADH concentration (i.e. 100 μM). The actual concentrations are given in Table 5.1. and 5.7.

5.2.8. INFLUENCE OF NADH ON THE KINETIC CONSTANTS OF NADPH USING MICROSOMES ISOLATED FROM PHENOBARBITAL TREATED RATS.

Table 5.9. shows the apparent K_m and V values of NADPH during the demethylation of aminopyrine or ethylmorphine using microsomes isolated from phenobarbital treated rats. For both substrates, the inclusion of NADH in the incubation medium resulted in a lowering of apparent K_m value compared with values obtained in the absence of NADH using microsomes isolated from phenobarbital treated rats. There was also significant increases in apparent V values in the presence of NADH, except with aminopyrine in the presence of 21.2 µM NADH. The influence of NADH on the kinetic constants of NADPH in the presence of either aminopyrine or ethylmorphine is similar in microsomes from both control and phenobarbital induced rats.

TABLE 5.9.

K_m and V values of NADPH in the presence of NADH during the demethylation of aminopyrine and ethylmorphine by microsomes isolated from phenobarbital treated rats.

NADH CONCENTRATION	AMINOPYRINE 107.7 (µM)	AMINOPYRINE 21.1 (µM)	ETHYLMORPHINE 107.7 (µM)	ETHYLMORPHINE 21.1 (µM)
K_m [a]	5.09 ± 0.63[e]	8.5 ± 0.8[f]	6.37 ± 0.48[e]	11.4 ± 1.07[f]
V [b]	134.4 ± 6.1[g]	99.5 ± 3.2	153.7 ± 3.0[f]	129.2 ± 3.8[f]
r [c]	0.982	0.994	0.997	0.994
N [d]	3	3	3	3

a K_m expressed as µM : mean ± S.D.

b V expressed as nmoles formaldehyde formed/5min/mg microsomal protein.

c correlation coefficient

d Number of determinations

Table 5.9. continued

e significantly different from value obtained in the absence of NADH (Table 4.4.) at $P < 0.001$.

f significantly different from value obtained in the absence of NADH (Table 4.4.) at $P < 0.05$.

g significantly different from the value obtained in the absence of NADH (Table 4.4.) at $P < 0.005$.

5.3. DISCUSSION

The relatively low activity of the mixed function oxidase complex with NADH as the sole electron donor has been well established (Gillette, 1967; Conney, 1967; Mannering, 1968). However, early reports (Conney *et al.*, 1957a; Nilsson & Johnson, 1963) have indicated that, in the presence of both pyridine nucleotides, there is a synergistic increase in reaction rates of approximately 50-70% above the rate obtained when NADPH was the only electron donor. This finding has been subsequently confirmed by Cohen & Estabrook (1971a,b,c); Gillette (1971b); Cinti *et al.*, (1972); Correia & Mannering (1973a,b,c); Bjorkheim & Danielsson (1973); Sitar & Mannering (1973) and Netter & Illing (1974).

The reduction in apparent K_m (NADPH) for both aminopyrine and ethylmorphine-N-demethylase in the presence of NADH could indicate one of two things. Either greater affinity of the mixed function oxidase for NADPH in the presence of NADH, or a change in the rate limiting step. The latter is generally accepted to be the reduction of the oxidised cytochrome P_{450}-substrate complex by an electron from NADPH (see Chapter 4) via NADPH-cytochrome c reductase. (Masters *et al.*, 1971; Lu *et al.*, 1969a).

Because of the multicomponent nature of this system, it has been suggested (Estabrook & Cohen, 1969) that change in K_m values is more likely to represent a change in the rate limiting step than a variation in affinity of the enzyme system for that particular substrate. Therefore based on this proposition the reduction in the apparent K_m (NADPH) value in the presence of three fixed NADH concentrations for the demethylation of aminopyrine and ethylmorphine (Table 5.1.) suggest that in the presence of both pyridine nucleotides, NADPH-cytochrome P_{450} reductase activity is no longer rate limiting. While it is generally accepted that the NADPH-cytochrome P_{450} reductase step is the rate limiting one, little attention has been given to the kinetics of NADPH in this reaction. The hyperbolic nature of both curves (Figure 5.1.) obtained for the reduction of the cytochrome P_{450}- aminopyrine complex with varying NADPH concentrations suggests that the Michaelis-Menten equation can be used to describe these reactions.

A hypothesis that NADH in the presence of NADPH alters the rate limiting step, would require that NADH stimulates NADPH-cytochrome P_{450} reductase. The results of Figure 5.1. and Table 5.2. show that in the presence of NADH, there is no alteration in the K_m (NADPH) value from that obtained in the absence of NADH, but there is a 280% increase in V value for the reduction of the cytochrome P_{450}- aminopyrine complex. This finding would suggest that NADH may exert a heterotropic co-operative effect with respect to NADPH. The possibility also exists that NADPH may stimulate the NADH mediated reduction of cytochrome P_{450}- substrate complex although this appears to be remote since the activity with NADH alone is only 10% of the rate obtained with NADPH.

These above results do not agree with those of Hildebrandt & Estabrook (1971), who reported there was no variation in the rate of reduction of the cytochrome P_{450}- ethylmorphine complex when the reaction was initiated with either NADPH or NADPH plus NADH. These workers performed the assay in the presence of 150mM KCl in an attempt to stimulate NADPH-cytochrome P_{450} reductase activity to a level that it would no longer be rate limiting (Hildebrandt & Estabrook, 1970). However, the synergistic effect of NADH on the reduction of NADPH-cytochrome P_{450} reductase was not observed when the experiment was repeated in this laboratory in the presence of 150mM KCl. This would indicate that KCl in this concentration does mask the changes in the rate of reduction of the cytochrome P_{450}- aminopyrine complex. Further, the published work of Hildebrandt & Estabrook (1971) used microsomes isolated from phenobarbital treated rats. It has also been shown (Penglis *et al.*,(1)) that there was no stimulation of cytochrome P_{450} reduction by NADH with microsomes isolated from rats treated with phenobarbital.

The same extent of stimulation of NADPH-cytochrome P_{450} reductase occurred when the NADH concentration was reduced to 20 μM, and similar results were also obtained with ethylmorphine as the substrate.

The inclusion of aniline in the medium for NADPH-cytochrome P_{450} reductase reduced the V value when compared to that obtained in the absence of any drug substrate, a finding which agrees with the results of Gigon *et al.*,(1968, 1969), (Chapter 4). However, there was an increase in V value of 94% above that obtained with NADPH alone (Table 4.3.) when the reduction of the cytochrome P_{450}- aniline complex was initiated by the simultaneous addition of both pyridine nucleotides.

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(1) S. Penglis, G.K. Gourlay & B.H. Stock - manuscript in preparation.

While NADH, as the sole electron donor gave only 11% of the p-aminophenol formation obtained with NADPH , there was a synergistic increase of 47% in the presence of both pyridine nucleotides. This latter finding is in direct conflict with the results of Correia & Mannering (1973a,c), who showed that there was no NADH synergistic increase in product formation with the Type II substrates, aniline and N-methylaniline. They also correlated the magnitude of the NADH synergistic increase in ethylmorphine demethylation with the magnitude of the Type I binding using microsomes which had been treated by various methods to modify this binding site. The reason for the discrepancy between the results of Correia & Mannering (1973a,c) and present work is not immediately clear. However, a consistent increase, although of different magnitude, was observed in the present work for cytochrome P_{450} reduction in the presence of aniline and for aniline hydroyxlase.

There are conflicting reports in the literature regarding the rate limiting step for aniline hydroxylase. The studies of Holtzman & Carr (1970, 1972b) relating to;

(1) the D_2O inhibition of both aniline hydroxylase and NADPH-cytochrome P_{450} reductase in the presence of aniline, and

(2) the activation energies of aniline hydroxylase and NADPH-cytochrome P_{450} reductase in presence of aniline suggests that the reduction of the cytochrome P_{450}-aniline complex is rate limiting. However, similar activation energy studies of Schenkman (1972) do not support this proposal.

Therefore, it is possible that the NADH synergistic increase in aniline hydroxylase is not related to the NADH stimulated reduction of the cytochrome P_{450}- aniline complex.

Recently, a number of propositions have been advanced to explain the synergistic effect of NADH in the presence of NADPH on *in vitro* reaction rates. Based on studies using phenobarbital-induced rabbits, Cohen & Estabrook (1971a,b,c) have proposed a model of electron transport during drug hydroxylation. It was suggested that the oxidised cytochrome P_{450}- substrate complex is reduced by an electron from NADPH via NADPH-cytochrome c reductase. The reduced complex so formed binds molecular oxygen and is further reduced by another electron which is supplied either by NADPH or NADH via NADH-cytochrome b_5 reductase. Further, they showed that NADH does not appear to specifically inhibit endogenous NADPH oxidase activity. This mechanism was reaffirmed in a subsequent communication (Hildebrandt & Estabrook, 1971), which implicated cytochrome b_5 as the donor of the second electron from either NADPH or NADH.

Studies by Sasame *et al.*, (1973, 1974a) using a rabbit antibody to trypsin solubilised rat liver cytochrome b_5 have indicated that cytochrome b_5 is involved in transferring an electron from NADH but not NADPH to the partially reduced oxygenated cytochrome P_{450}- substrate complex.

Similar studies by Mannering *et al.*, (1974) confirmed that cytochrome b_5 mediates the introduction of second electron from NADH, but did not conclusively show the involvement of cytochrome b_5 in transferring the second electron from NADPH.

However, Correia & Mannering (1973a,b,c) have proposed an alternative mechanism based on an examination of the role of cytochrome b_5 in drug oxidations and NADH synergism. These investigators activated the fatty acid desaturase pathway by the addition of stearyl CoA to examine the possibility that electrons from NADH could be diverted from the mixed function oxidase complex via cytochrome b_5 and thus reduce or abolish NADH synergism.

It was proposed that when NADPH is the only source of electrons, the rate limiting step is the introduction of the second electron and not the rate of reduction of the oxidised cytochrome P_{450}- substrate complex. However, this latter step is rate limiting in the presence of both pyridine nucleotides. The addition of Type I substrates causes an increased demand for both first and second electrons because of the stimulatory effect on cytochrome P_{450} reductase. (Gigon *et al.*, 1968, 1969) NADPH, it was proposed could satisfy the demand for first electrons but not for second electrons. Therefore, the introduction of the second electron becomes the rate limiting step. However, in the presence of both pyridine nucleotides, NADH is more efficient than NADPH in donating the second electron and therefore the introduction of the first electron again becomes rate limiting.

It was shown that cyanide, at a final concentration between 0.1-0.5mM, reversed the inhibitory effect of stearyl CoA on ethylmorphine demethylation. However, cyanide, in the absence of exogenous stearyl CoA enhanced NADH synergism. The proposed explanation of this finding was that desaturation of membrane fatty acids occurs when microsomes are incubated with NADPH or NADH and that this endogenous reaction normally inhibits ethylmorphine demethylation by diverting electrons from the mixed function oxidase system via cytochrome b_5. The added potassium cyanide, it was claimed inhibits the diversion of electrons, and hence an increased amount of formaldehyde is produced because of the greater availablity of electrons for the mixed function oxidase system.

Cyanide at concentrations which enhanced NADH synergism, was without appreciable effect on ethylmorphine demethylation when NADPH was the sole source of electrons. This was interpreted to

mean that in the absence of stearyl CoA, the drain of electrons from NADPH to the desaturase system via cytochrome b_5 was not great enough to limit the rate of turnover of the monoxygenase system (Mannering, 1974).

These two statements appear to be contradictory. It was first proposed that in the presence of both pyridine nucleotides, cyanide is able to inhibit endogenous fatty acid desaturase activity. However, when NADPH is present alone, cyanide has no effect because there are apparently low levels of endogenous fatty acid desaturase activity. Both NADPH and NADH alone have been shown to support approximately the same extent of fatty acid desaturase activity (Oshino *et al.*, 1966; Schenkman & Jansson, 1974; Jansson & Schenkman, 1975). It is likely that the latter statement is correct because Sasame *et al.*, (1973) have shown little variation in endogenous NADH oxidation in the presence or absence of an antibody to cytochrome b_5.

However, similar experiments by Schenkman & Jansson (1974) failed to reproduce the counteractivity effects of cyanide on the stearyl CoA inhibition of ethylmorphine demethylation. It was concluded that little of the inhibition of stearyl CoA is due to electron drain by the operation of the desaturase system because the microsomes used were isolated from desaturase induced animals (Oshino & Sato, 1972). Similar results were obtained with control rats. Further, Mannering, when working with Sato in Japan was unable to reproduce these experiments and concluded that, "obviously Japanese rats and Minnesota rats are different", (Mannering,1974).

Sasame & Gillete (1976) have recently suggested that in the presence of both reduced pyridine nucleotides, approximately 80% of the second electrons required for ethylmorphine demethylation and peroxide formation originate from NADH. This finding argues

against the drain of electrons from NADH to the fatty acid desaturase pathway to any significant extent during drug hydroxylation reactions.

While the proposition advanced by Correia & Mannering may appear plausible with the saturating NADH concentrations used (1mM), it would be difficult to accept that this mechanism would operate with NADH concentrations of 10 μM. This concentration of NADH has been shown, in present study to reduce the apparent K_m value for aminopyrine or ethylmorphine-N-demethylase. Further, the stimulatory effects of NADH on the V value and the rate constant for the initial rapid phase of the biphasic reduction kinetics for NADPH-cytochrome P_{450} reductase are inconsistent with their proposal.

Staudt *et al.*, (1974) have proposed a role for NADH in uncoupling hepatic mixed function oxidase reactions. The stoichiometry of hydroxylation of n-hexane, cyclohexane and per flouro-n-hexane was studied using microsomes isolated from phenobarbital treated rats. With cyclohexane as the substrate, the stoichiometry of cyclohexane hydroxylated:NADPH oxidised: oxygen consumed approached 1:1:1. However, with the uncoupler, per fluoro-n-hexane, no product was formed and NADPH:O_2 was 2:1. The stoichiometry for n-hexane suggested that partial uncoupling had occurred. NADH did synergise n-hexane hydroxylation but not cyclohexane hydroxylation.

An increase in the oxidation of NADH via cytochrome b_5 was shown to occur during hydroxylation. It was proposed that the active oxygen cytochrome P_{450} complex not used for product formation is reduced to water by the NADH-cytochrome b_5 system rather than the NADPH-cytochrome c system. This sparing effect accounted, it was suggested, for the synergistic effect of NADH

during mixed function oxidase reactions. However, as the authors concede, the question as to the importance of uncoupling during the hydroxylation of typical substrates remains unanswered. Recent studies (Buening & Franklin, 1974; Jeffrey & Mannering, 1974) have shown a 1:1:1 stoichiometry for formaldehyde formation:NADPH oxidised:oxygen consumed when nucleotide pyrophosphatase activity is effectively controlled.

The results of Mannering *et al.*, (1974) and Sasame *et al.*, (1973, 1974a) provide experimental evidence to suggest that NADH provides the second of two electrons required for the oxidation of drug substrates.

However, the present results indicate that, in addition to this role, NADH might also induce a perturbation in the cytochrome P_{450}- substrate complex, allowing the electron from NADPH to be supplied at a much greater rate. This concept is supported by the following:-

(a) the K_m value of both aminopyrine and ethylmorphine-N-demethylases, determined in the presence of three NADH concentrations, is almost half the value determined in the absence of NADH. This suggests that the reduction of the cytochrome P_{450}- substrate complex is no longer rate limiting;

(b) the three-fold increase in V over control values for NADPH-cytochrome P_{450} reductase when the reaction is initiated by NADPH plus NADH. The half life of the NADH effect must be very short because both pyridine nucleotides are introduced simultaneously. Further, NADPH-cytochrome P_{450} reductase activity in the presence of NADH alone was only 10% of that obtained with NADPH.

Substrate-induced difference spectra designed to show the NADH induced perturbation of the cytochrome P_{450}- substrate complex were unsuccessful because of the different steady state levels of reduced cytochrome b_5 obtained with NADPH or NADH (Estabrook *et al.*, 1972).

A similar proposal has been advanced for horse liver alcohol dehydrogenase where NADH, in addition to donating an electron, also induces a conformational change in the enzyme (Wong & Williams, 1968; Biellmann & Jung, 1971).

Lenk (1976), has proposed that the integration of the mixed function oxidase complex into a protein membrane does not appear to hinder any possible conformational change in the enzyme complex.

Original reports (Gigon *et al.*, 1969; Degwitz *et al.*, 1969 and Kupfer & Orrenius, 1970) on the *in vitro* reduction of the cytochrome P_{450}- substrate complex from hepatic microsomes showed the system to exhibit biphasic kinetics in both intact, and subsequently in the resolved and reconstituted system (Coon *et al.*, 1971).

Gillette (1971a,b) has proposed that the reduction of the cytochrome P_{450}- substate complex can give polyphasic kinetics under certain conditions. Nevertheless, individual determinations (Tables 5.5. & 5.6.) in the present work show that under the experimental conditions used, the reduction of the cytochrome P_{450}- aminopyrine complex fits a biphasic model. The present work shows that when the reduction of the cytochrome P_{450}- aminopyrine complex is initiated by the simultaneous addition of NADPH and NADH, the half lives of both phases are significantly lower than the values obtained with NADPH alone.

Recently, Matsubara *et al.*, (1976) and Peterson *et al.*, (1976) have reported that NADPH-cytochrome P_{450} reductase activity in microsomes isolated from rats that had previously been treated with phenobarbital also exhibited biphasic reduction kinetics which, it was suggested was most probably composed of two concurrent first order reactions. This particular study utilised a dual wavelength stopped flow spectrophotometer to assay NADPH-cytochrome P_{450} reductase activity.

It may well be true that the absolute values of the rate constants determined with the more sophisticated stopped flow spectrophotometer are different from those determined using the more conventional spectrophotometric technique used in this study. Nevertheless, meaningful comparisons can still be made using the spectrophotometric technique between the results obtained when NADPH-cytochrome P_{450} reductase activity was initiated by the introduction of NADPH alone or NADPH and NADH together. The present study places emphasis on the changes in apparent V values or the change in slope of the initial rapid phase of the biphasic reduction kinetics rather than the absolute values of these parameters.

The rates of change in absorbance at 450 nm obtained when NADPH cytochrome P_{450} reductase activity was initiated by the simultaneous addition of both NADPH and NADH (Table 5.2.) are lower than the maximum rates obtained with microsomes isolated from rats previously treated with phenobarbital at the same protein concentration (i.e. 33-36 nmoles cytochrome P_{450} reduced/min/mg microsomal protein), i.e. these absorbance changes are well within the capabilities of the instrument used to measure them (a Gilford 2400S recording spectrophotometer).

One other potential problem with the technique used in the

present study to measure NADPH-cytochrome P_{450} reductase activity is that trace amounts of oxygen can still be present in the "anaerobic" reaction medium. The trace amounts of oxygen have been shown to exert a significant effect on the slow phase but not the rapid phase of the biphasic cytochrome P_{450} reduction kinetics (Gillette, 1971a,b; Gillette *et al.*, 1973). Gillette (1971a,b; Gillette *et al.*, 1973) has also shown that the rapid phase of cytochrome P_{450} reduction accounts for virtually all of the N-demethylation of ethylmorphine. This being the case, the trace amounts of oxygen, if present, will not alter the significance of the results presented in this thesis.

The presence of such trace amounts of oxygen in the "anaerobic" incubation medium has been one of the proposals advanced to explain the biphasic cytochrome P_{450} reduction kinetics , (Gillette, 1971a,b). Other propositions include:-

(a) a difference in the reduction rate of the cytochrome P_{450}- substrate complex when compared to free cytochrome P_{450} (Gigon *et al.*, 1969; Sasame & Gillette, 1969) and

(b) in the microsomal membrane, one NADPH-cytochrome P_{450} reductase molecule has been proposed to reduce approximately twenty molecules of cytochrome P_{450}. (Estabrook *et al.*, 1971a; Estabrook, 1971d). Further, a certain percentage of the haemoprotein molecules exist in clusters around the flavoprotein reductase (Estabrook *et al.*, 1971d; Franklin & Estabrook, 1971). It has been suggested (Peterson *et al.*, 1976) that the rapid phase of the biphasic reduction kinetics represents the random reduction of the cytochrome P_{450} molecules clustered around the flavoprotein reductase,while the slow phase involves the translational movement of NADPH-cytochrome

P_{450} reductase to the cytochrome P_{450} molecules not directly associated with the clusters.

The question arises as to the number of NADH molecules that are involved with the mixed function oxidase system to produce the results obtained. Hill (1910) has proposed a kinetic equation (equation 5.1.) to describe sigmoidal curves which was based on the binding of oxygen to haemoglobin. Until recently, the numerical value of the Hill coefficient, n, was estimated from a plot of log v/(V - v) versus log (S). However, Atkins (1973) has written a digital computer programme based on a modified Fabonacci search to estimate these parameters. (Appendix 2 is a source listing of the BASIC-PLUS version of this programme).

The graphs and results (Figure 5.2. and Table 5.4.) obtained for the demethylation of either aminopyrine or ethylmorphine in the presence of approximately 100 μM NADPH and varying NADH concentrations would suggest a homotropic NADH co-operative effect. Sigmoidal binding kinetics have been reported for the binding of a drug substrate (14-deoxy-14-[(2-diethylaminoethyl)-mercaptoacetoxy]-di-hydromutilin hydrogen fumarate) to rat liver cytochrome P_{450}, (Schuster *et al.*, 1975). This co-operative behaviour was interpreted to indicate a possible conformational change in cytochrome P_{450} when it complexes with the drug. Further, sigmoidal kinetics have also been shown for the binding of oxygen for the N-oxygenation of N,N dimethylaniline by rabbit liver microsomes (Hlavica, 1972).

NONLIN was used to obtain numerical estimates for the parameters of the Hill equation in Table 5.4., because this programme gives an estimate of the variation in each parameter and also a correlation between the data and the model. While Atkins' programme does compute reliable estimates of the parameters, it fails to provide this type of statistical analysis.

It would appear therefore, that the more general programme written by the same author (Atkins, 1971a;b) NONLIN or a similar programme would be more suitable. The n values in presence of 104.7 μM NADPH of 1.71 (with aminopyrine) and 1.53 (with ethylmorphine) suggest that there are at least two NADH binding sites.

However, when this experiment was repeated in the presence of approximately 350 μM NADPH, the n value was reduced from 1.74 to 1.38 (aminopyrine) and from 1.53 to 1.0 (ethylmorphine). Variations in n values with different effector concentrations have been reported for sheep liver pyruvate carboxylase.[1]

It was postulated that sigmoidal curves in the absence of supporting evidence obtained from binding studies are not necessarily proof of homotropic co-operative kinetics, i.e., only one NADH molecule would be responsible for the sigmoidal kinetics obtained in Figure 5.2. and Table 5.4. It should be noted, however, that this interpretation does not alter the NADH heterotropic effect with respect to NADPH for cytochrome P_{450} reductase.

The kinetic analyses involving the Hill equation have been performed using microsomal membranes rather than purified enzymes. In such a heterogenous system, it is not possible to examine the exclusive binding of the pyridine nucleotides to the mixed function oxidase system. Therefore, at the present time, the number of NADH molecules involved in the NADH synergistic effect is not completely resolved. The variation in n value with different NADPH concentrations would probably suggest that a single NADH molecule is responsible for the observed effects.

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1. Keech D.B. - personal communication - submitted for publication, Arch Biochem Biophys.

The data used to calculate the apparent K_m and V values for NADPH in the presence of three fixed NADH concentrations using either aminopyrine or ethylmorphine as substrate (Table 5.1.) was fitted to the Hill equation (Table 5.10.) using Atkins' programme.

TABLE 5.10.

Kinetic parameters for NADPH obtained by refitting the data to Table 5.1. to the Hill equation.

SUBSTRATE	NADH CONCENTRATION (μM)	K	V [a]	n
AMINOPYRINE	96.1	4.68	51.35	1.19
	19.2	3.97	43.35	1.06
	12.0	4.39	38.83	0.80
ETHYLMORPHINE	96.1	7.23	45.48	1.17
	19.2	7.51	35.79	1.1
	12.0	6.4	33.0	1.1

a V expressed as nmoles HCHO formed/5min/mg microsomal protein.

The data was fitted to the Hill equation using the digital computer programme of Atkins (1973), run in BASIC on a PDP 11/40.

The value of n is a function of two factors:-

(a) the number of binding sites, and

(b) the strength of interaction between the sites.

The n value of approximately 1.1 for both aminopyrine and ethylmorphine in presence of three NADH concentrations probably suggests that there is only one NADPH binding site in the presence of both reduced pyridine nucleotides. As the number of binding

sites can never be less than n (Wyman, 1964), the possibility of two NADPH binding sites must be considered. However, with a n value of 1-1.2, there would be little interaction between the sites. Further, the data obtained for NADPH-cytochrome P_{450} reductase in the presence of both pyridine nucleotides was also fitted to the Hill equation. These results (Table 5.11.) indicate that there is only a single NADPH binding site on this enzyme. Therefore, these results cannot be explained by the co-operative binding of NADPH molecules.

TABLE 5.11.

Kinetic parameters of NADPH for NADPH cytochrome P_{450} reductase obtained by refitting the data of Table 5.2. to the Hill equation.

SUBSTRATE ADDED	NADH CONCENTRATION (μM)	K	V [a]	n
AMINOPYRINE	119	1.27	15.29	.74
	20.2	1.91	15.76	.72
ETHYLMORPHINE	98	2.31	15.62	.86
	20.4	1.6	14.7	.56

a V expressed as nmoles cytochrome P_{450} reduced/min/mg microsomal protein.

The data used in Table 5.2. was refitted to the Hill equation, using the digital computer programme of Atkins (1973), run in BASIC on a PDP 11/40.

If the addition of both pyridine nucleotides in *in vitro* demethylation studies stimulates NADPH-cytochrome P_{450} reductase to such an extent that it is no longer the rate limiting step, the question

arises as to which subsequent step assumes this role.

NADPH-cytochrome P_{450} reductase activity can also be stimulated by the prior treatment of rats with phenobarbital, (Remmer, 1972). Estabrook *et al.*, (1973) has suggested from stopped flow experiments using microsomes isolated from phenobarbital treated rats, that the introduction of the second electron becomes rate limiting. It would therefore appear that stimulation of NADPH-cytochrome P_{450} reductase activity by either the *in vitro* addition of NADH, or by treatment of rats with phenobarbital, changes the rate limiting step possibly to the introduction of the second electron.

Gunsalus & Lipscomb (1973) have also reported that the introduction of the second electron is rate limiting in the bacterial cytochrome P_{450} cam system.

The following results from the present work also suggest, but do not prove that the introduction of the second electron from NADH via cytochrome b_5 is the rate limiting step:-

(a) the similar numerical V values for aminopyrine and ethylmorphine at each NADH concentration (Table 5.1.) and

(b) the constant ratio between V values for the demethylation of aminopyrine and ethylmorphine and the steady state concentration of reduced cytochrome b_5 after the 5 min incubation at $37^{o}C$ (Table 5.8.).

The influence of treating rats with phenobarbital on the kinetic constants of NADPH in the presence of NADH for either aminopyrine or ethylmorphine demethylation are similar to the results obtained from control rats, i.e. the addition of NADH generally lowers the apparent K_m (NADPH) value and increases the apparent V value.

However, in contrast to the results obtained in control animals, Penglis *et al.*,(1) has shown that NADH does not exert a heterotropic co-operative effect with respect to NADPH for NADPH-cytochrome P_{450} reductase in phenobarbital induced animals. The addition of NADH lowers the apparent K_m value but does not alter the apparent V value. Studies are currently in progress to further characterise the pyridine nucleotide involvement in phenobarbital treated rats. However, these findings together with those of Estabrook *et al.*, (1973) suggest that the rate limiting step in the mixed function oxidase complex is different in the microsomal fractions from control and phenobarbital induced rats.

The following experimental evidence supports this suggestion:-

(1) significant amounts of phenobarbital given to induce hepatic microsomal mixed function oxidase reactions prior to killing have been shown to remain bound to microsomes even after a number of washings. (Holtzman *et al.*, 1976). The bound drug is at least 70% unmetabolised and interferes with the *in vitro* binding of either ethylmorphine or phenobarbital to the Type I binding site, (although the bound drug appears not to bind to the Type I site) and,

(2) phenobarbital pretreatment has been shown to selectively induce a form of cytochrome P_{450} with a molecular weight of 44,000 that is present only in small amounts in microsomes isolated from untreated rats, (Welton & Aust, 1974).

Further, these results strongly suggest that mechanisms derived using microsomes isolated from phenobarbital treated rats may not necessarily be operative in microsomes from control rats.

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(1) Penglis S., G.K.Gourlay & B.H.Stock, manuscript in preparation.

C H A P T E R 6.

THE INFLUENCE OF THE 1,4,5,6 - TETRAHYDRONICOTINAMIDE ANALOGUE OF NADH ON NADPH KINETICS DURING HEPATIC MIXED FUNCTION OXIDASE REACTIONS.

6.1. INTRODUCTION

One characteristic of most enzyme catalysed reactions is the high degree of specificity of the enzyme for its substrate(s). Alterations to the structure of the substrate frequently renders the substrate inactive but in some instances, minor alterations can produce compounds that are still capable of binding to the enzyme but without undergoing the chemical modification of the natural substrate.

If a structural analogue of the natural substrate is able to bind to the catalytic site of the enzyme,in the presence of both substrate and analogue, there would be a competition for this binding site and the degree to which this competition reduces the rate of the reaction will be a function of :-

(1) the effectiveness of the analogues fit to the active centre, and

(2) the concentration of both the analogue and natural substrate.

However, the analogue can also influence the enzyme reaction without binding to the active site. It could bind at some other site leading either to non-competitive inhibition or in enzymes that are under allosteric control, associate with an effector site, thereby modifying enzymic activity.

Stock (1969) has examined the influence of tetrahydronicotinamide and the corresponding analogue of nicotinamide mononucleotide, (NMN) designated tetrahydro NMN, both as substrates for and competitors with the natural substrates for the enzymes involved in NAD biosynthesis.

This worker showed that while the corresponding analogue of NAD ($NADH_3$) by itself, was incapable of substituting for NADH as a co-factor for lactic, malic and alcohol dehydrogenases, it was

an effective competitor with respect to NADH for these enzymes. It was, however, somewhat less effective as an inhibitor when oxidised co-factor, NAD, was involved with these enzymes.

These findings suggested that the $NADH_3$ analogue (and also the corresponding hydrogenated analogue of NADP) could provide a useful tool to examine and further characterise pyridine nucleotide involvement in hepatic microsomal drug metabolism.

The present Chapter, therefore, describes a study of the influence of $NADH_3$ on the kinetic constants of NADPH for the demethylation of aminopyrine and ethylmorphine and for NADPH-cytochrome P_{450} reductase in the presence of these two Type I substrates.

6.2. RESULTS

6.2.1. NADPH CYTOCHROME P_{450} REDUCTASE ACTIVITY IN THE PRESENCE OF $NADH_3$ ANALOGUE.

Figure 6.1. shows the reduction of the cytochrome P_{450}-aminopyrine complex by the NADPH, $NADH_3$, and NADPH in the presence of $NADH_3$. $NADH_3$ by itself was incapable of reducing the cytochrome P_{450}- aminopyrine complex, however, the simultaneous addition of $NADH_3$ and NADPH increased the reduction rate compared to the rate for NADPH alone.

It has been shown that the reduction of the cytochrome P_{450}-aminopyrine complex by either NADPH alone or both NADPH and NADH together, could successfully be fitted to a two exponential equation, (Equation 5.2.). The results of similar studies where $NADH_3$ was added with NADPH were also fitted to equation 5.2. and are shown in Table 6.1. The concentrations of reduced pyridine nucleotides and $NADH_3$ were determined spectrophotometrically at 340 nm and 289 nm respectively as described in the Methods section (Chapter 2).

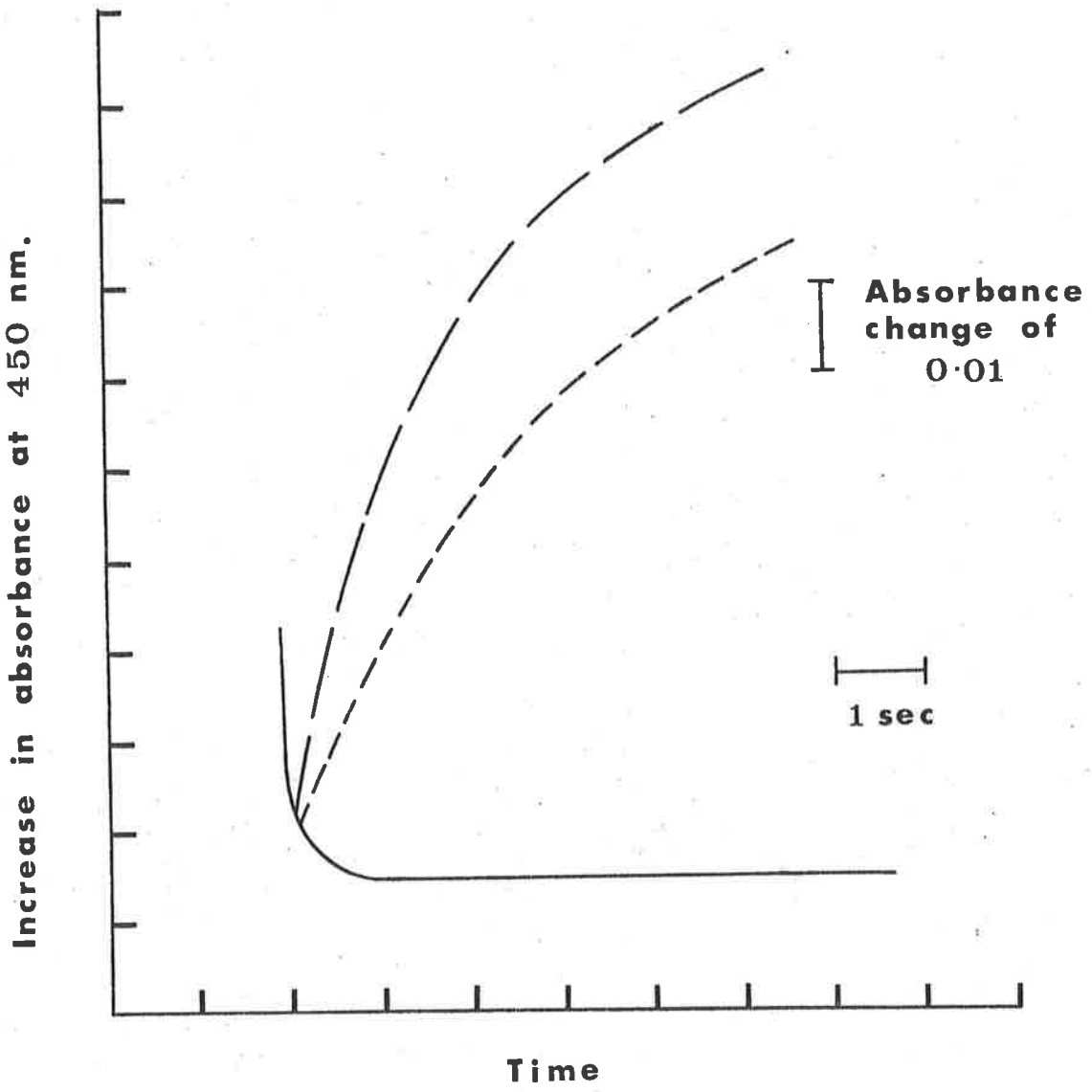


FIGURE 6.1. The reduction of the Cytochrome P_{450}- Aminopyrine Complex by NADPH alone, $NADH_3$ alone and NADPH and $NADH_3$ together.

The reduction of the cytochrome P_{450}- aminopyrine complex was initiated by the introduction of $NADH_3$ to give a final concentration of 100 µM (————), NADPH, 93.6 µM (– – – – –) or both $NADH_3$ and NADPH together (—— —— —).

TABLE 6.1.

Numerical values of the constants of Equation 5.2. determined in the presence of NADPH and $NADH_3$.

EXPERIMENT	PARAMETERS[a]					
	A	r_1	B	r_2	cor[b]
1.	74.7 ± 0.53	0.42 ± 0.005	23.2 ± 0.005	0.035 ± 0.002	1.000
	67.4 ± 0.4	0.47 ± 0.006	29.2 ± 0.26	0.035 ± 0.001	0.999
2.	70.5 ± 0.39	0.431 ± 0.005	26.7 ± 0.27	0.045 ± 0.001	1.000
	67.6 ± 0.27	0.42 ± 0.004	29.4 ± 0.2	0.042 ± 0.001	1.000
3.	72.1 ± 0.36	0.486 ± 0.006	25.0 ± 0.25	0.039 ± 0.001	1.000
	72.9 ± 0.23	0.456 ± 0.004	24.2 ± 0.17	0.045 ± 0.001	1.000
Bulked	70.2 ± 0.64	0.433 ± 0.007[c]	26.9 ± 0.32	0.044 ± 0.001[d]	0.997

a mean ± S.D.

b correlation coefficient as defined in the Methods.

c significantly different from r_1 (Table 5.5.) at $P < 0.05$

d significantly different from r_2 (Table 5.5.) at $P < 0.005$

$NADH_3$ (64 μM) was pre-incubated in the reaction medium for 5 min prior to initiating the reaction with NADPH weight factor used in computations was reciprocal of the coefficient variation.

An r_1 value of 0.426 ± 0.016 was obtained for the reduction of the cytochrome P_{450}- aminopyrine complex when the reaction was initiated by the simultaneous addition of NADPH and $NADH_3$. This value was not statistically different from the value of 0.41 ± 0.01 obtained in the presence of NADPH alone (Table 5.5.). As $NADH_3$ has been shown to be unable to reduce the cytochrome P_{450}- aminopyrine complex, the experiment was repeated and the $NADH_3$ was added 5 min before initiating the reaction by NADPH. Under these conditions, there was a statistically significant increase in the value of r_1 to 0.433 ± 0.007 (Table 6.1.).

Table 6.2. shows the kinectic constants of NADPH for NADPH-cytochrome P_{450} reductase determined in the presence and absence of $NADH_3$.

TABLE 6.2.

K_m and V values of NADPH for NADPH-cytochrome P_{450} reductase determined in the absence and presence of 100 µM $NADH_3$.

	$NADH_3$ ABSENT	$NADH_3$ PRESENT
K_m [a]	1.56 ± 0.28	2.0 ± 0.4
V [b]	5.28 ± 0.13[d]	9.05 ± 0.4[d]
r [c]	0.991	0.998

a K_m expressed as µM : mean ± S.D.

b V expressed as nmoles cytochrome P_{450} reduced/min/mg microsomal protein : mean ± S.D.

c correlation coefficient as defined in the Methods.

d significantly different at $P < 0.001$.

N = 3 for both determinations.

NADPH-cytochrome P_{450} reductase activity was measured as described in the Methods. Aminopyrine was included in the assay at a final concentration of 4mM.

The values obtained in the absence of $NADH_3$ have been taken from Table 4.3.

The inclusion of $NADH_3$ did not change the apparent K_m (NADPH) value, but increased the apparent V by approximately 80% to 9.05 nmoles cytochrome P_{450} reduced/min/mg microsomal protein.

The rate of reduction of the cytochrome P_{450}- aminopyrine complex by the simultaneous addition of NADPH (94 μM), NADH (121.2 μM) and $NADH_3$ (139.4 μM) was 13.05 ± 0.87 nmoles reduced/min/mg microsomal protein (N = 3). This value is significantly lower ($P < 0.05$) compared to the V value of 14.61 ± 0.32 nmoles cytochrome P_{450} reduced/min/mg microsomal protein obtained in the presence of 119 μM NADH (Table 5.2.).

The rate of reduction of the cytochrome-P_{450}-ethylmorphine complex by saturating concentrations of NADPH (94.3 μM) and $NADH_3$ (139.4 μM) was 11.15 ± 1.03 nmoles reduced/min/mg microsmal protein (N = 3) which is significantly higher from the V value for NADPH-cytochrome P_{450} reductase determined in the presence of ethylmorphine (Table 4.3.). The control rate for the same microsomal suspension when the reduction was initiated by the simultaneous addition of NADPH (94.3 μM) and NADH (121.2 μM) was 13.68 ± 1.4 nmoles cytochrome P_{450}-ethylmorphine reduced/min/mg microsomal protein which is not significantly different from the V value of NADPH-cytochrome P_{450} reductase determined in the presence of ethylmorphine and 98 μM NADH (Table 5.2., 14.75 ± 0.49 nmoles cytochrome P_{450} reduced/min/mg microsomal protein). The rate of 13.64 ± 1.03 nmoles cytochrome P_{450}- ethylmorphine complex reduced/min/mg microsomal protein obtained when the reduction was initiated with NADPH, NADH and $NADH_3$ together (same concentrations as for aminopyrine), was lower but not significantly different from the rate determined with NADPH and NADH (Table 5.2.).

These results suggest that NADH and $NADH_3$ may be competing for the same site on the cytochrome-P_{450}- substrate complex.

The results of Table 6.3. show the rate of reduction of the cytochrome P_{450}- substrate complex upon the simultaneous addition of both NADPH and NADH. The reduction was initiated 5 min after the addition of $NADH_3$ to the assay medium which was contained in a silica cuvette. For both aminopyrine and ethylmorphine, the rate of reduction of the cytochrome P_{450}- substrate complex was not significantly different from the rate determined in the presence of NADPH and $NADH_3$ together. However, both rates were significantly lower than the corresponding V values for NADPH cytochrome P_{450} reductase obtained in the presence of approximately 100 µM NADH (Table 5.2.).

TABLE 6.3.

Activity of NADPH-cytochrome P_{450} reductase in the presence of substrates by the simultaneous addition of NADPH (92.4 µM) and NADH (98 µM).

SUBSTRATE	NADPH-CYTOCHROME P_{450} REDUCTASE [a]
Aminopyrine (4mM)	10.9 ± 0.97 [b]
Ethylmorphine (6mM)	10.74 ± 1.78

a expressed as nmoles cytochrome P_{450}- substrate complex reduced/min/mg microsomal protein.

b value is significantly different ($P < 0.05$) from the value obtained by the simultaneous addition of NADPH, NADH and $NADH_3$ (13.04 ± 0.87 nmoles cytochrome P_{450} reduced/min/mg microsomal protein).

N = 3 for both substrates.

$NADH_3$ (89 µM) was added to the assay media 5 min prior to initiating the reaction by the simultaneous addition of NADPH (92.4 µM) and NADH (98 µM).

6.2.2. KINETIC CONSTANTS OF NADPH IN THE PRESENCE OF $NADH_3$ DURING DRUG HYDROXYLATIONS.

The K_m and V values of NADPH determined in the presence of $NADH_3$ for aminopyrine or ethylmorphine-N-demethylase are given in Table 6.4. The K_m (NADPH) value determined with ethylmorphine as the substrate was significantly greater than the value obtained in the presence of NADPH alone (Table 4.1.). This contrasts with the lack of significant change in K_m (NADPH) values when aminopyrine was the substrate. However, the V values for both substrates determined in the presence of $NADH_3$ are greater than the corresponding values determined in the presence of NADPH alone (Table 4.1.).

TABLE 6.4.

K_m and V values for NADPH determined in the presence of 68 µM $NADH_3$.

	AMINOPYRINE	ETHYLMORPHINE
K_m [a]	7.1 ± 0.53	13.6 ± 0.95 [d]
V [b e]	78.6 ± 1.3	59.9 ± 1.2
r [c]	0.996	0.997

a K_m expressed as µM : mean ± S.D.

b V expressed as nmoles HCHO formed/5min/mg microsomal protein : mean ± S.D.

c correlation coefficient as defined in the Methods.

d significantly different from the value obtained in the absence of $NADH_3$ with ethylmorphine as substrate at $P < 0.01$ (Table 4.1.).

e both V values significantly different from the value obtained in the absence of $NADH_3$ at $P < 0.001$ (Table 4.1.).

N = 3 for both determinations.

$NADH_3$, by itself was unable to support the demethylation of either aminopyrine or ethylmorphine (results not shown) or the para hydroxylation of aniline (Table 6.5.).

Table 6.5. also shows the influence of $NADH_3$ on aniline hydroxylase activity determined in the presence of optimal concentrations of either NADPH alone or both NADPH or NADH together.

$NADH_3$, when added with NADPH did not alter the amount of p-aminophenol formed from that obtained with NADPH alone. NADH was however, able to synergise the production of p-aminophenol in the presence of NADPH and $NADH_3$, (33% increase) but not to the extent obtained in the presence of NADPH and NADH (47% increase, Table 5.3.).

TABLE 6.5.

Aniline hydroxylase activity in the presence of the $NADH_3$ analogue.

PYRIDINE NUCLEOTIDE	ANILINE HYDROXYLASE [a]	% ACTIVITY
NADPH (191 µM)	3.76 ± 0.11[b]	100
$NADH_3$ (170 µM)	0	0
NADPH (191 µM) + $NADH_3$ (170 µM)	3.72 ± 0.02	99
NADPH (191 µM) + NADH (202 µM) + $NADH_3$ (170 µM)	5.0 ± 0.16	133

a expressed as nmoles p-aminophenol formed/10min/mg microsomal protein.

b values are mean ± difference from the mean for two determinations.

6.2.3. K_m AND V VALUES FOR NADPH DETERMINED IN THE PRESENCE OF NADH AND $NADH_3$.

The K_m and V values for NADPH determined in the presence of NADH and $NADH_3$ are given in Table 6.6. With either aminopyrine or ethylmorphine as substrate, the K_m (NADPH) values are significantly lower than the values determined in the presence of $NADH_3$ (Table 6.4.). The values are, however, not as low as those determined in the presence of NADH (Table 5.1.). The V value determined with ethylmorphine, but not aminopyrine, as substrate was significantly higher than the value determined in the presence of $NADH_3$ (Table 6.4.). The V values determined in the presence of both NADH and $NADH_3$ are similar when either aminopyrine or ethylmorphine was the added substrate.

There was, however, a significant difference in these V values when the kinetic constants for NADPH were determined in the presence of $NADH_3$ alone (Table 6.4.).

TABLE 6.6.

K_m and V values of NADPH for demethylation reactions determined in the presence of $NADH_3$ and NADH.

	AMINOPYRINE	ETHYLMORPHINE
K_m [a] [d]	4.66 ± 0.52 [e]	6.88 ± 0.7
V [b] [f]	79.7 ± 1.9	80.1 ± 1.6 [g]
r [c]	0.994	0.994

a K_m expressed as μM : mean ± S.D.

b V expressed as nmoles HCHO formed/5min/mg microsomal protein : mean ± S.D.

c correlation coefficient as defined in the Methods.

d significantly different from values obtained in the presence of 68 μM $NADH_3$ (Table 6.4.) for respective substrate at $P < 0.005$.

Table 6.6. continued

e significantly different from the value obtained in the presence of NADPH alone (Table 4.1.) at $P < 0.005$.

f significantly different from the values obtained in the presence of 96.1 μM NADH (Table 5.1.) at $P < 0.001$.

g significantly different from the value obtained in the presence of 68 μM $NADH_3$ (ethylmorphine Table 6.4.) at $P < 0.001$.

N = 3 for both determinations.

The concentrations of NADH and $NADH_3$ were 108.4 μM and 100 μM (aminopyrine) and 92.6 μM and 106.2 μM (ethylmorphine) respectively.

6.2.4. THE INFLUENCE OF $NADH_3$ ON THE STEADY-STATE LEVELS OF REDUCED CYTOCHROME b_5.

In the previous chapter, the addition of NADH after NADPH approximately doubled the levels of reduced cytochrome b_5 obtained with NADPH. When $NADH_3$ was added after NADPH, there was no increase in the steady-state levels of reduced cytochrome b_5. Further, the addition of $NADH_3$ after NADPH and NADH failed to increase reduced cytochrome b_5 levels above that obtained in the presence of NADH or NADPH and NADH.

Identical results were obtained when either aminopyrine or ethylmorphine were the added drug substrates.

6.3. DISCUSSION

The tetrahydronicotinamide analogue of NAD, $NADH_3$ does not function as an electron source in the demethylation of either aminopyrine or ethylmorphine nor in the reduction of the cytochrome P_{450}- substrate complex. However, while $NADH_3$ was incapable of reducing the cytochrome P_{450}- aminopyrine complex, a synergistic increase in reduction rate was observed in the presence of NADPH and $NADH_3$ (Figure 6.1.).

There was also a significant increase in the value of the slope of the initial rapid phase (r_1 of equation 5.2.) of the cytochrome P_{450}- aminopyrine complex reduction when $NADH_3$ was previously incubated with the microsomal suspension (Table 6.2.). Although not statistically significant, there was an increase in r_1 of cytochrome P_{450} reduction (in the presence of aminopyrine) when both NADPH and $NADH_3$ were added simultaneously.

The K_m (NADPH) values for NADPH-cytochrome P_{450} reductase determined in the presence or absence of $NADH_3$ were not significantly different, but the V value in the presence of $NADH_3$ was increased approximately 80%. An increase in the maximal rate of reduction of the cytochrome P_{450}- ethylmorphine complex was also apparent with both NADPH and $NADH_3$ when compared to the rate in the presence of NADPH alone.

It would therefore appear that while $NADH_3$ was incapable of reducing the complex it was capable of inducing a conformational change in the cytochrome P_{450}- substrate complex, similar to that produced by NADH. However, $NADH_3$ appears to be less efficient than NADH in inducing this conformational change. These results with $NADH_3$ also suggest that the structure of NADH, rather than it's capacity to donate an electron is responsible for the conformational change.

An identical proposal that $NADH_3$ induces the same conformational change as NADH has been advanced for octopine dehydrogenase, the terminal enzyme for the glycolytic pathway in some invertebrates. This proposal was based upon both crystallographic (Biellmann & Jung, 1971; Olomucki *et al.*, 1975) and spectroscopic evidence (Olomucki *et al.*, 1975). It was suggested that $NADH_3$ (referred to as h_2 [5,6] NADH in these references) while being unable to form ternary complexes could form good binary complexes. Similar

studies could not be attempted in the present study because of the heterogenous nature of the microsomal fraction.

A conformational change suggests that there is an alteration of the tertiary structure of the cytochrome P_{450}- substrate complex upon the binding of NADH or $NADH_3$.

It should be noted, however, that this may not necessarily be the case. The binding of NADH or $NADH_3$ may modify the site for introduction of the electron from NADPH without any change to the tertiary structure of the complex. It would be a difficult proposition to show which of the two possibilities actually occurs in the heterogenous microsomal suspension used for these studies. Although the following discussion suggests a conformational change, it is equally possible that the latter of the above two proposals could operate.

The rate of reduction of the cytochrome P_{450}- aminopyrine complex by a combination of NADPH, NADH and $NADH_3$ was significantly lower than the rate obtained with optimal concentrations of NADPH and NADH. A similar reduction although not significant was apparent when ethylmorphine was the added substrate. These results raised the possibility that NADH and $NADH_3$ were competing for the same site on the cytochrome P_{450}- substrate complex.

Evidence in support of this proposal was obtained when the reduction of the cytochrome P_{450}- substrate complex was initiated by the addition of NADPH and NADH 5min after the addition of $NADH_3$ (Table 6.3.). The lack of stimulation by NADH under such circumstances suggests that the NADH binding site has been either partially or completely blocked, by $NADH_3$. Further, it also suggests that in the time taken for the cytochrome P_{450}- substrate to be reduced by an electron from NADPH, NADH cannot effectively remove $NADH_3$ from this site after it has been bound.

$NADH_3$, when included in the assay medium for either aminopyrine or ethylmorphine-N-demethylase resulted in a significant increase in the V values for both substrates when compared to the values obtained in it's absence (Table 4.1.).

The increases observed in the presence of $NADH_3$ were of a greater magnitude compared to the values obtained in the presence of NADH (Table 5.1.). However, unlike NADH, $NADH_3$ did not reduce the apparent K_m (NADPH) value obtained during the demethylation of either aminopyrine or ethylmorphine.

This latter finding would suggest that while $NADH_3$ is able to significantly stimulate NADPH-cytochrome P_{450} reductase activity, the stimulation is not sufficient to change the rate limiting step from the reduction of the cytochrome P_{450}- substrate complex.

The observation that there are approximately parallel increases in V values for aminopyrine-N-demethylase (56%) and NADPH-cytochrome P_{450} reductase (71%) in the presence of aminopyrine, upon the addition of both NADPH and $NADH_3$ supports this proposal, i.e., the reduction of the cytochrome P_{450}- aminopyrine complex is still the rate limiting step in the presence of $NADH_3$.

However, when ethylmorphine was the substrate, parallel increases in the V values did not occur. Thompson & Holtzman (1974) have suggested that the rate limiting step for the demethylation of ethylmorphine is shared between; (a) introduction of the second electron and (b) the exchange between the active oxygen (OH) and one of the three hydrogen atoms of the N-methyl group (see scheme 4.1.). If this proposal is correct, the extent of $NADH_3$ stimulation of the demethylation of ethylmorphine would be unrelated to the stimulation of NADPH-cytochrome P_{450} reductase in the presence of ethylmorphine.

However, a possible alternative explanation of the apparently anomolous results with ethylmorphine could be the influence of this substrate on cytochrome P_{450}. The addition of $NADH_3$ with NADPH stimulates ethylmorphine demethylase by 77% and NADPH-cytochrome P_{450} reductase in the presence of ethylmorphine by only 25%. It is significant that, while there are differences in V values for NADPH-cytochrome P_{450} reductase in the presence of either aminopyrine or ethylmorphine (Table 4.3.), such differences are not apparent when the reduction is initiated by NADPH and NADH (Table 5.2.) and NADPH and $NADH_3$ (Table 6.3.). It would therefore appear that the perturbation induced by either NADH or $NADH_3$ is complimentary to the conformational change induced by the substrate (i.e., either aminopyrine or ethylmorphine).

The control V values obtained for cytochrome P_{450} reduction in the presence of either aminopyrine or ethylmorphine, NADPH and either NADH or $NADH_3$ would suggest that, under these conditions, the reduction of the ferric cytochrome P_{450}-substrate complex to the ferrous cytochrome P_{450}- substrate complex is occurring at the maximum possible rate. Alternatively, some step preceeding this step is rate limiting. This step may not necessarily be rate limiting for the overall hydroxylation sequence.

Two possibilities would be (1) the reduction of the flavine nucleotides of the enzyme NADPH cytochrome c reductase by NADPH, or (2) transfer of the electrons from NADPH cytochrome c reductase to the as yet unidentified intermediate, "X" proposed by Dallner *et al.*, (1966) and Estabrook *et al.*, (1969).

In support of this latter possibility, Matsubara & Estabrook, (1973), Estabrook *et al.*, (1973a) have proposed the presence of a new, unidentified electron acceptor unique to the NADPH-cytochrome P_{450} reductase sequence. Further, the rate of transfer of electrons

from NADPH to cytochrome c is approximately 10 times faster than the rate of transfer to the cytochrome P_{450}- substrate complex in the presence of NADH, which would tend to discount the first possibility. Therefore, even though parallel increases in the V values are not apparent for ethylmorphine, the possibility exists that the reduction of the cytochrome P_{450}- ethylmorphine complex is still rate limiting in the presence of $NADH_3$.

As previously discussed (Chapter 5, Discussion), the results obtained for NADPH cytochrome P_{450} reductase when the reaction was initiated by either NADPH and NADH or NADPH and $NADH_3$ are within the capabilities of the spectrophotometer used to measure the absorbance change.

The mechanism by which the $NADH_3$ analogue influences aniline hydroxylase appears to be different to that proposed for either aminopyrine or ethylmorphine. Aniline hydroxylase activity in the presence of both NADPH and $NADH_3$ was similar to that obtained with NADPH alone. This observation is contrary to that obtained with the Type $\underline{I}$ substrates where $NADH_3$ increased the extent of demethylation. Further, the combination of NADPH, NADH and $NADH_3$ reduced the amount of p-aminophenol formed compared to that observed with NADPH and NADH (Table 5.3.).

In the previous Chapter, it was suggested that the reduction of the cytochrome P_{450}- aniline complex may not necessarily be the rate limiting step. The results obtained in the present Chapter would tend to support this proposition.

It appears that NADH and $NADH_3$ still compete for the same site on the cytochrome P_{450}-aniline complex, but the capacity to donate an electron is more important than the ability to induce a perturbation in this complex. Therefore, it is suggested that when aniline is the substrate, $NADH_3$ acts as a competitive inhibitor with NADH,

thus reducing the extent of p-aminophenol formation. There was a significant difference between the V values for aminopyrine-N-demethylase and ethylmorphine-N-demethylase when the kinetic constants of NADPH were determined in the presence of $NADH_3$, (Table 6.4.). In contrast, there was no difference in the V values for aminopyrine and ethylmorphine demethylation when the K_m and V values for NADPH were determined in the presence of NADH and $NADH_3$.

A similar trend was apparent when examining the influence of either $NADH_3$ alone or NADH and $NADH_3$ together, on the V values of NADPH cytochrome P_{450} reductase in the presence of either aminopyrine or ethylmorphine.

It is proposed that during the demethylation of either aminopyrine or ethylmorphine in the presence of NADPH, NADH and $NADH_3$, there would initially be a competition between NADH and $NADH_3$ for the same site on the cytochrome-P_{450}- substrate complex. Irrespective of whether either NADH or $NADH_3$ was bound to this site, the first electron for the reduction of the ferric cytochrome P_{450}-substrate complex would originate in NADPH. When $NADH_3$ is bound to the site, the second electron which is required for the complete reduction of the oxy-ferrous cytochrome P_{450}- substrate complex must necessarily come from a second NADPH molecule.

However, in the presence of an NADH molecule, the second electron could come from either another molecule of NADPH or the NADH molecule which was associated with cytochrome P_{450} during the transfer of the first electron. If this applies, the NADH would be oxidised to NAD with it's associated change in charge.

Such an alteration in charge is associated with a change from the boat conformation of the reduced dihydronicotinamide ring (Oppenheimer *et al.*, 1971) to the planar aromatic ring of the

oxidised ring. It is proposed that this conformational change is sufficient to dissociate the NAD from the cytochrome P_{450}- substrate complex.

When $NADH_3$ is present in lieu of NADH, it is not oxidizable by the cytochrome P_{450} system and therefore will retain it's original non planar conformation. It is proposed that in this situation, the $NADH_3$ stays associated with cytochrome P_{450} during the transfer of the second electron from NADPH.

Further, it appears (Table 6.3.) that NADH is not capable of displacing $NADH_3$ from this site once it is bound. As the demethylation reactions proceed, the number of sites occupied by $NADH_3$ would quickly increase at the expense of NADH. Similar values for the demethylation of aminopyrine and ethylmorphine are observed because there is no significant difference in the V values of NADPH cytochrome P_{450} reductase (in presence of aminopyrine or ethylmorphine) when the reduction was initiated after $NADH_3$ had been bound to the cytochrome P_{450}- substrate complex (Table 6.3.). However, this hypothesis is inconsistent with the finding that the V for ethylmorphine demethylation in the presence of $NADH_3$ is 59.9 $\pm$ 1.2 nmoles HCHO formed/5min/mg microsomal protein (Table 6.4.), whereas in the presence of both NADH and $NADH_3$, a value of 80.1 $\pm$ 1.6 nmoles HCHO formed/5min/mg microsomal protein (Table 6.6) is obtained.

Necessarily, all of the effector sites must be occupied by $NADH_3$ when the kinetic constants for NADPH are determined in the presence of $NADH_3$.

This finding suggests that the proposal of Thompson & Holtzman (1974) relating to the rate limiting step for ethylmorphine demethylation may well be correct.

Thus it appears that the introduction of the second electron

from cytochrome b_5 and possibly the exchange of active oxygen (OH) for one of the N-CH_3 hydrogens may be rate limiting. As NADH maintains a higher steady state level of reduced cytochrome b_5 than NADPH, a greater ethylmorphine demethylation rate is apparent in the presence of NADPH, NADH and $NADH_3$ than NADPH and $NADH_3$.

However, the demethylation rate with ethylmorphine as substrate in the presence of both reduced pyridine nucleotides and $NADH_3$ is substantially greater than the corresponding rate in the presence of NADPH and NADH (Table 5.1.). It therefore appears that in the presence of NADPH, NADH and $NADH_3$, $NADH_3$ can still bind to the cytochrome P_{450}- ethylmorphine complex thereby inducing a conformational change which facilitates the input of the second electron and greatly increases the demethylation rate above that obtained in the presence of NADPH and NADH. The apparently anomolous results in the proposition are the kinetic constants of NADPH for ethylmorphine-N-demethylase determined in the presence of $NADH_3$. When this experiment was repeated, the values assigned to the kinetic constants were not statistically different from the values in Table 6.4. (for ethylmorphine).

Table 6.6. also shows that there is a reduction in K_m (NADPH) values for both substrates in the presence of $NADH_3$ and NADH when compared to similar values determined in the presence of $NADH_3$ alone (Table 6.4.). NADH, in the absence of $NADH_3$, has been shown to significantly reduce the K_m (NADPH) value (Table 5.1.).

During the initial stages of the demethylation, in the presence of NADPH, NADH and $NADH_3$, NADH would occupy a certain percentage of the available sites on the cytochrome P_{450}- substrate complex, but as previously suggested, this percentage would rapidly decrease as the demethylation proceeded.

Consistent with this hypothesis is the observation that the reduction in K_m (NADPH) value during aminopyrine demethylation in the presence of NADH and $NADH_3$ (Table 6.6.) is not as marked as the reduction observed with 96.1 μM NADH alone (Table 5.1., $P < 0.05$). Further,the same trend is apparent although not statistically significant during ethylmorphine demethylation.

Overall, the results of this Chapter suggest that two factors, the structure of NADH as well as it's capability to donate an electron are responsible for the NADH syngergistic effects observed during the demethylation of aminopyrine and ethylmorphine.

C H A P T E R 7.

THE INHIBITORY EFFECTS OF 2,6-DIHYDROXYACETOPHENONE ON THE HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE ENZYME REACTIONS.

7.1. INTRODUCTION

2,6-dihydroxyacetophenone and some of it's derivatives have been shown to inhibit aniline hydroxylase and aminopyrine demethylase in hepatic microsomal preparations (Bobik *et al.*, 1975). The parent compound 2,6-dihydroxyacetophenone but not the 2-methoxy-6-hydroxy nor the 2,6-dimethoxy derivatives was shown to inhibit NADPH-cytochrome P_{450} reductase activity.

Bobik (1974) suggested that the inhibition of aniline hydroxylase by 2,6-dihydroxyacetophenone occurred at or prior to the reduction of the cytochrome P_{450}- substrate complex. However, it was also proposed that aminopyrine demethylase was subject to inhibition at some point after cytochrome P_{450} reduction.

Spectral evidence was presented (Bobik, 1974) which suggested that 2,6-dihydroxyacetophenone inhibited the transfer of the second electron from reduced cytochrome b_5 to the oxygenated ferrous cytochrome P_{450}- aminopyrine complex.

In view of these results, 2,6-dihydroxyacetophenone has been considered as a possible tool in further characterising the mechanism of electron transport during drug hydroxylation reactions.

This Chapter therefore reports a more detailed examination of the inhibitory effects of 2,6-dihydroxyacetophenone on the kinetics of NADPH for aminopyrine and ethylmorphine demethylases both with NADPH alone and in the presence of NADH.

7.2. EXPERIMENTAL

2,6-dihydroxyacetophenone was added to the incubation medium by an appropriate dilution of a 2.5mM concentrated solution in 10mM Tris-Cl buffer, pH 7.5. Complete solution of the 2,6-dihydroxyacetophenone concentrate could only be effected by warming to 50°C. The exact concentration of 2,6-dihydroxyacetophenone

was verified using an E^{1cm}_{mM} coefficient of 10.965 at 268 nm (Dell & Kamp, 1967).

7.3. RESULTS

7.3.1. THE INFLUENCE OF 2,6-DIHYDROXYACETOPHENONE ON AMINOPYRINE AND ETHYLMORPHINE DEMETHYLATION.

The effect of varying the concentration of 2,6-dihydroxyacetophenone on the velocity of aminopyrine demethylase with NADPH as the variable substrate (Figure 7.1.) suggests that the nature of the inhibition is non-competitive.

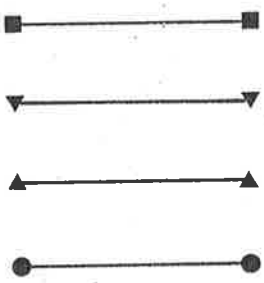
The kinetic data obtained at each inhibitor concentration were fitted initially to the Michaelis-Menten equation (Cleland, 1963) using the procedure detailed in the Methods section. In Figure 7.1. the lines are the computer generated lines of best fit at each concentration of inhibitor, while the points represent the means of three duplicate determinations at each NADPH concentration.

Following the primary fitting procedure (above), secondary plots of the slopes (obtained from HYPER output) versus inhibitor concentration, and the ordinate intercept (HYPER output) versus inhibitor concentration were constructed. The secondary plots for aminopryine demethylase (Figure 7.2.) were both linear indicating that 2,6-dihydroxyacetophenone may be classes as a linear non-competitive inhibitor. (i.e., S-linear, I-linear, non-competitive inhibitor).

FIGURE 7.1.

Lineweaver & Burk plot for aminopyrine-N-demethylase in the presence of various 2,6-dihydroxyacetophenone (DiHAP) concentrations.

The different 2,6-dihydroxyacetophenone concentrations used were:-



357.5 µM.

194.8 µM.

89.6 µM.

no DiHAP (data taken from Table 4.1.).

Velocity is expressed as nmoles HCHO formed/5min/mg microsomal protein, and NADPH concentration as µM.

The points are the means of three duplicate determinations while the lines are the computer generated lines of best fit.

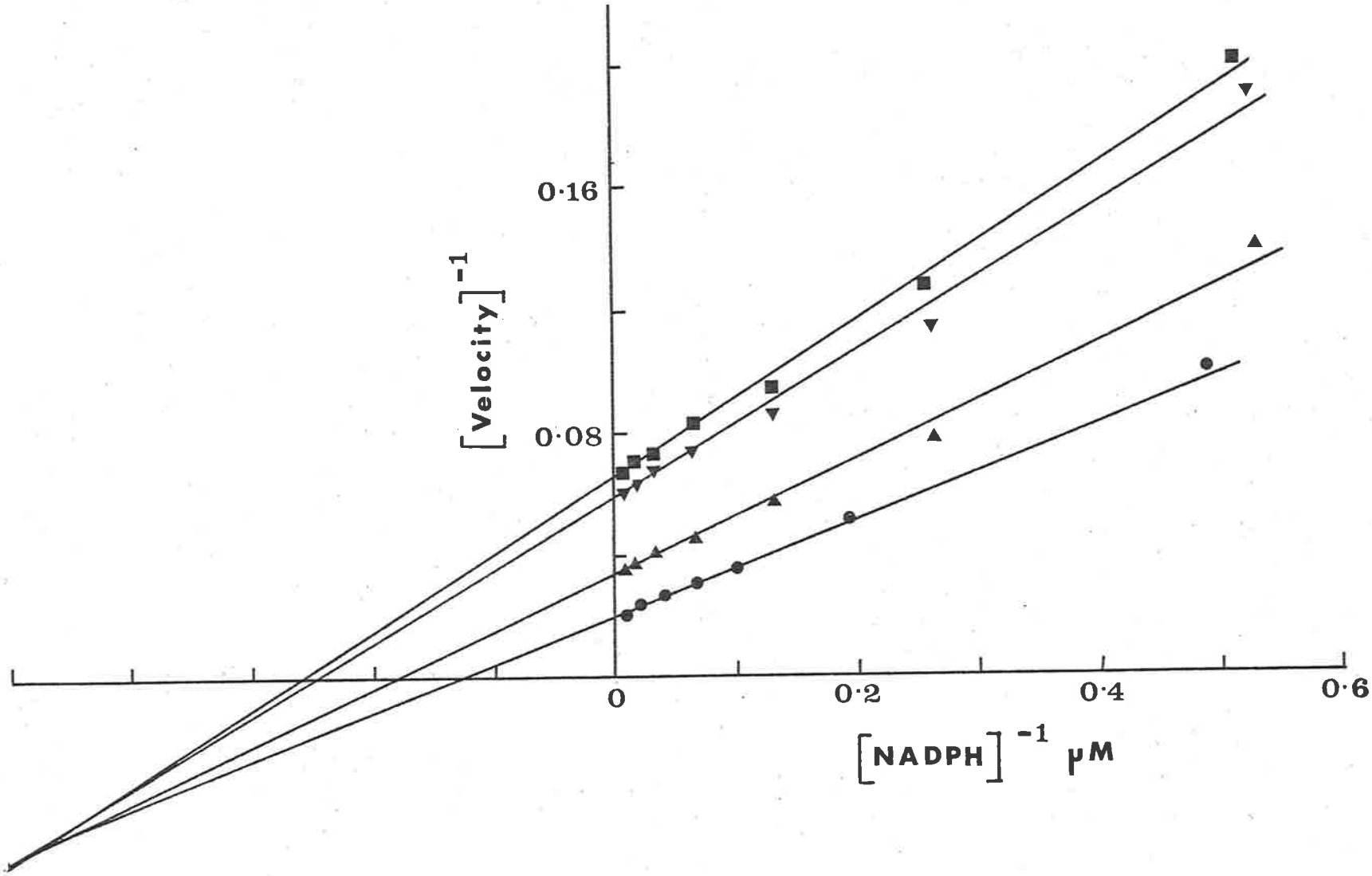
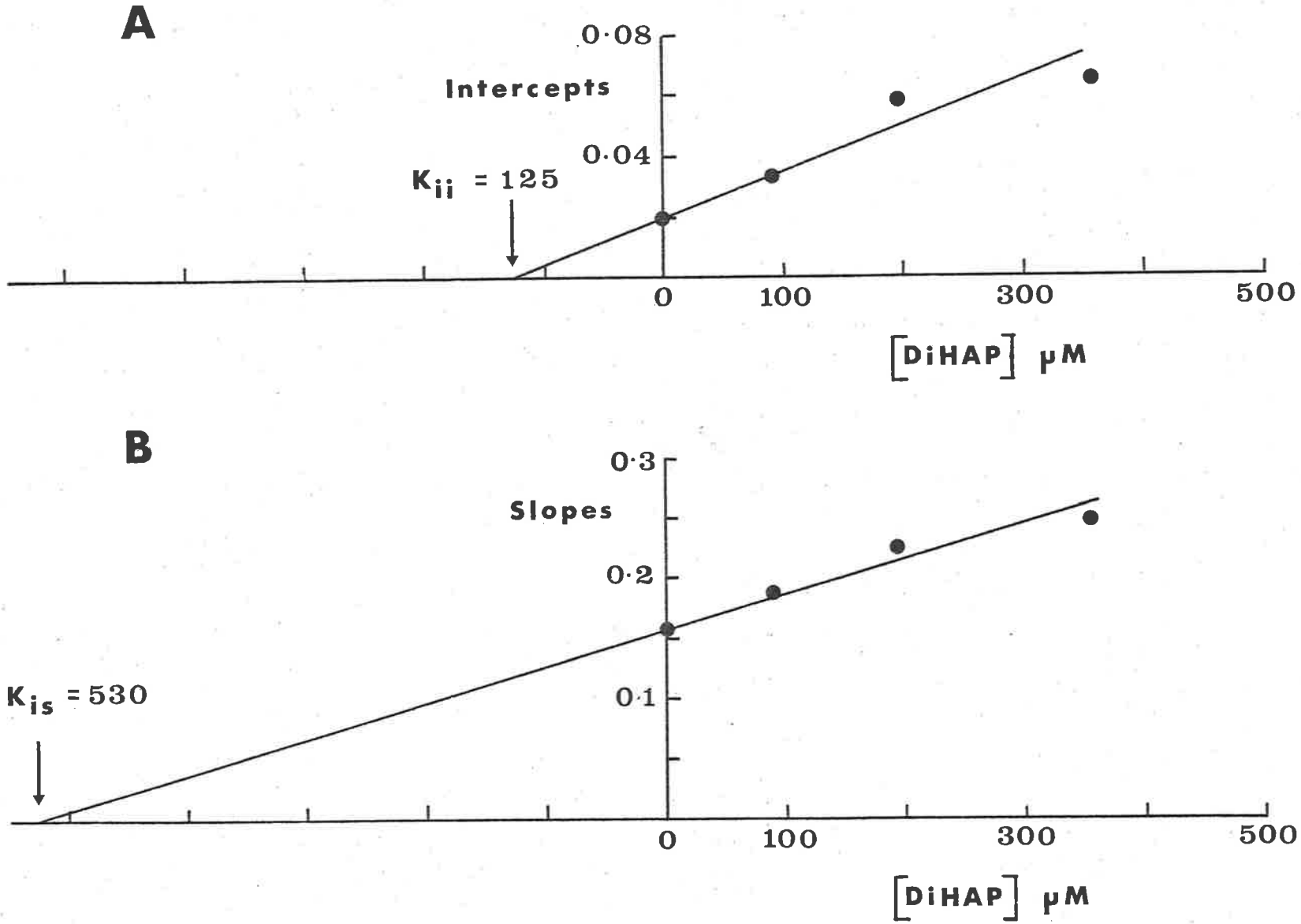

0·16
0·08
[Velocity]$^{-1}$
0
0·2
0·4
0·6
[NADPH]$^{-1}$ μM

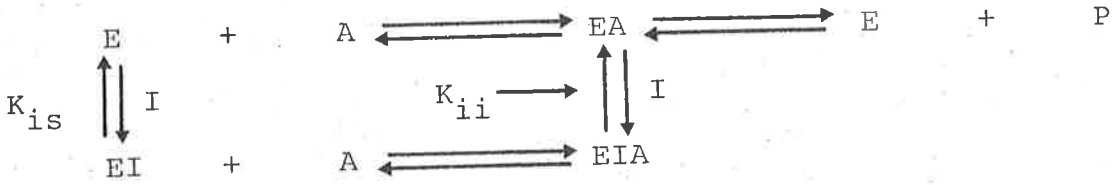
FIGURE 7.2.

Secondary plots of 1/V intercepts versus DiHAP concentration (Figure 7.2.A) and slope versus DiHAP concentration (Figure 7.2.B) for aminopyrine-N-demethylase.

The slopes and 1/V intercept values at each DiHAP concentration were obtained from the HYPER output for that concentration.


A
0·08
Intercepts
0·04
K_{ii} = 125
0
100
300
500
[DiHAP] μM
B
0·3
Slopes
0·2
0·1
K_{is} = 530
0
100
300
500
[DiHAP] μM

The following scheme and Equation 7.1. describe this type of inhibition.



and

$$v = \frac{V\,A}{K_m\,(1 + I/K_{is}) + A\,(1 + I/K_{ii})} \quad \ldots\ldots 7.1.$$

where I represents the inhibitor concentration, K_{is}, K_{ii}, v, V, K_m and A represent the enzyme-inhibitor complex dissociation constant, the enzyme-inhibitor-substrate complex dissociation constant, velocity, maximum velocity, Michaelis-Menten constant and substrate (NADPH) concentration respectively.

The double reciprocal form of equation 7.1. takes the form of an equation of a straight line at constant inhibitor concentrations:-

$$\frac{1}{v} = \frac{K_m}{V}.(1 + I/K_{is}).\frac{1}{A} + \frac{1}{V}.(1 + I/K_{ii}) \quad \ldots\ldots 7.2.$$

with a slope of $\frac{K_m}{V}.(1 + I/K_{is})$ and an ordinate intercept of $\frac{1}{V}.(1 + I/K_{ii})$.

However, at different inhibitor concentrations, both the slopes and ordinate intercepts will vary as a linear function of the inhibitor concentration, thus:-

$$\text{Slope} = \frac{K_m}{V}.(1 + I/K_{is}) = \frac{K_m}{V.K_{is}}.\,I + \frac{K_m}{V} \quad \ldots\ldots 7.3.$$

and

$$\text{Intercept} = \frac{1}{V}.(1 + I/K_{ii}) = \frac{1}{V.K_{ii}}.\,I + \frac{1}{V} \quad \ldots\ldots 7.4.$$

It is therefore apparent that the X-axis intercepts of the slopes and 1/V intercept versus inhibitor concentration secondary plots provides numerical estimates of K_{is} and K_{ii} respectively.

The significance of slope-linear-intercept-linear non-competitive inhibition is that only one inhibitor molecule combines with either the free enzyme (E) or the enzyme-substrate complex (EA).

The secondary plots from similar experiments when ethylmorphine was the substrate are shown in Figure 7.3.

Table 7.1. gives the numerical estimates of the constants of equation 7.1. for both aminopyrine and ethylmorphine demethylases in the presence of 2,6-dihydroxyacetophenone using NONCOM.

When the data for aminopyrine demethylase was fitted to equation 7.1. using the NONCOM programme, the value of K_{is} became negative after the first iteration and increasingly more negative with each subsequent iteration. Possible reasons for this apparently anomolous behaviour are given in the discussion. Therefore the value of K_{is} in Table 7.1. with aminopyrine as the substrate was obtained by a linear regression of the points in Figure 7.2.B.

TABLE 7.1.

The numerical estimates of the constants of Equation 7.1. for aminopyrine and ethylmorphine demethylases in the presence of 2,6-dihydroxyacetophenone.

PARAMETER [a]	AMINOPYRINE[e]	ETHYLMORPHINE[f]
K_m [b]	7.93 ± 0.56	8.35 ± 0.9
V [c]	50.28 ± 1.01	33.5 ± 1.36
K_{is}	650.0[d]	172.4 ± 84.7
K_{ii}	128.1 ± 8.5	336.0 ± 72.6
r	0.985	0.972

a each parameter mean ± S.D.

b expressed as μM.

c nmoles HCHO formed/5min/mg microsomal protein.

Table 7.1. continued

d from linear regression of the points in Figure 7.2.B.

e values derived from 225 data points.

f values derived from 176 data points.

The numerical values of K_m(NADPH) and V obtained in the presence of either aminopyrine or ethylmorphine and 2,6-dihydroxyacetophenone (Table 7.1.) are not significantly different from values obtained in the absence of the inhibitor, (Table 4.1.).

7.3.2. THE EFFECTS OF 2,6-DIHYDROXYACETOPHENONE ON AMINOPYRINE OR ETHYLMORPHINE DEMETHYLASE IN THE PRESENCE OF NADH.

The secondary plots of the data obtained to study the influence of 2,6-dihydroxyacetophenone on NADPH kinetics for aminopyrine demethylase in the presence of 120 μM NADH are shown in Figure 7.4. The nature of the inhibition still appears to be linear non competitive. The numerical values of the constants of equation 7.1. for aminopyrine demethylase in the presence of 120 μM NADH and various inhibitor concentrations are given in Table 7.2.

TABLE 7.2.

The numerical estimates of the constants of Equation 7.1.for aminopyrine in the presence of NADH and 2,6-dihydroxyacetophenone.

PARAMETER	NUMERICAL ESTIMATES [a]
K_m [b]	3.54 ± 0.14
V [c]	52.52 ± 0.37
K_{is}	234.2 ± 50.0
K_{ii}	185.7 ± 7.0
r	0.994

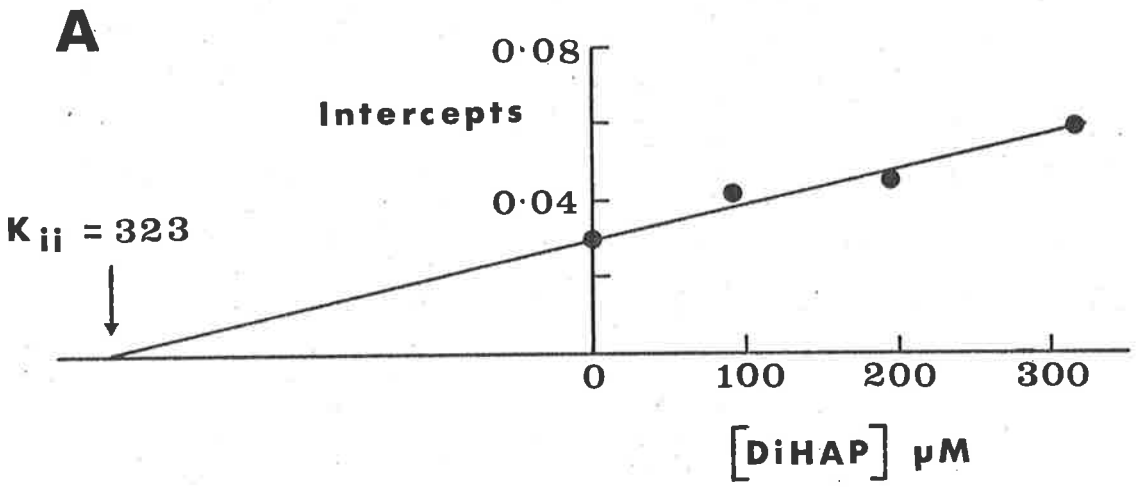
---

a mean ± S.D. : values derived from 187 data points using NONCOM.

FIGURE 7.3.

Secondary plots of 1/V intercepts versus DiHAP concentration (Figure 7.3.A.) and slopes versus DiHAP concentration (Figure 7.3.B.) for ethylmorphine-N-demethylase.

The slopes and 1/V intercept values at each DiHAP concentration were obtained from the HYPER output for that concentration.


A
0·08
Intercepts
0·04
K_{ii} = 323
0
100
200
300
[DiHAP] μM

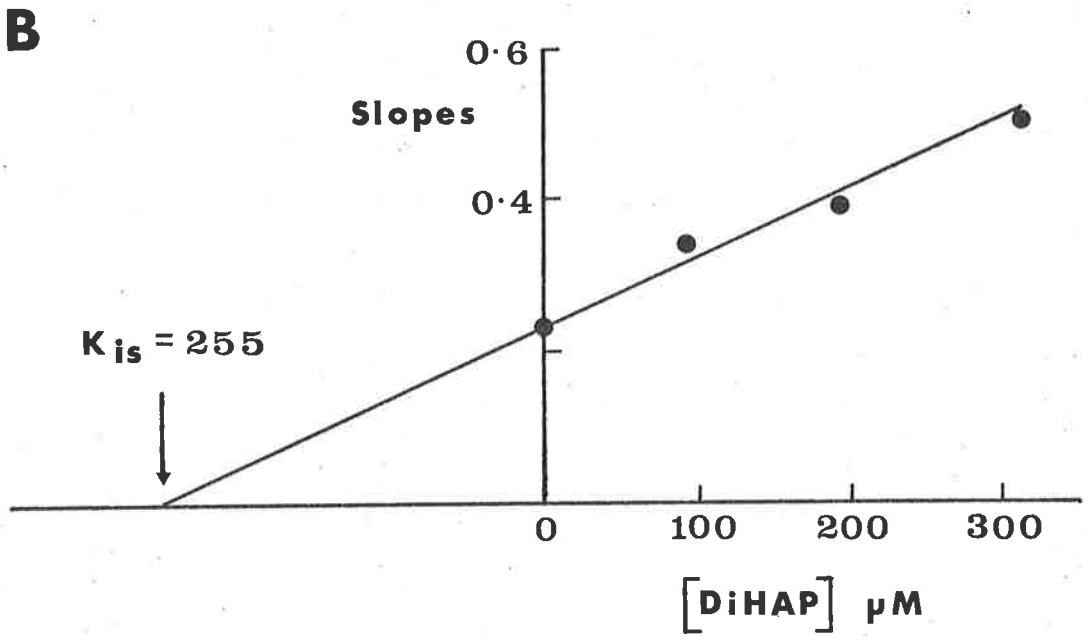
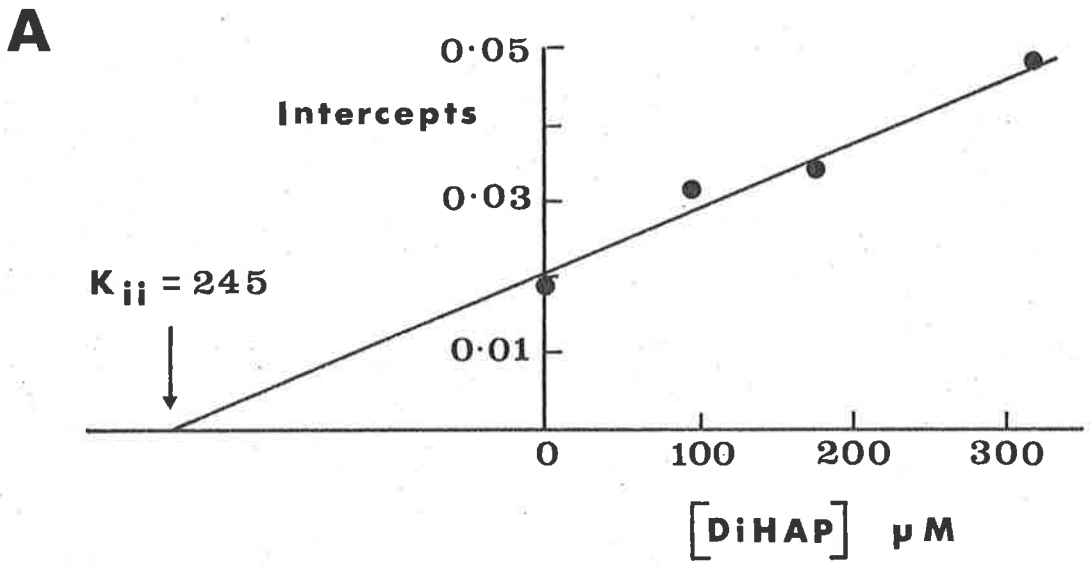

B
0·6
Slopes
0·4
K_{is} = 255
0
100
200
300
[DiHAP] μM

FIGURE 7.4.

Secondary plots of 1/V intercepts versus DiHAP concentration (Figure 7.4.A) and slopes versus DiHAP concentration (Figure 7.4.B) for aminopyrine-N-demethylase in the presence of 120 μM. NADH.

The slope and 1/V intercept values at each DiHAP concentration were obtained from the HYPER output for that concentration.


A
0·05
Intercepts
0·03
K_{ii} = 245
0·01
0
100
200
300
[DiHAP] μM

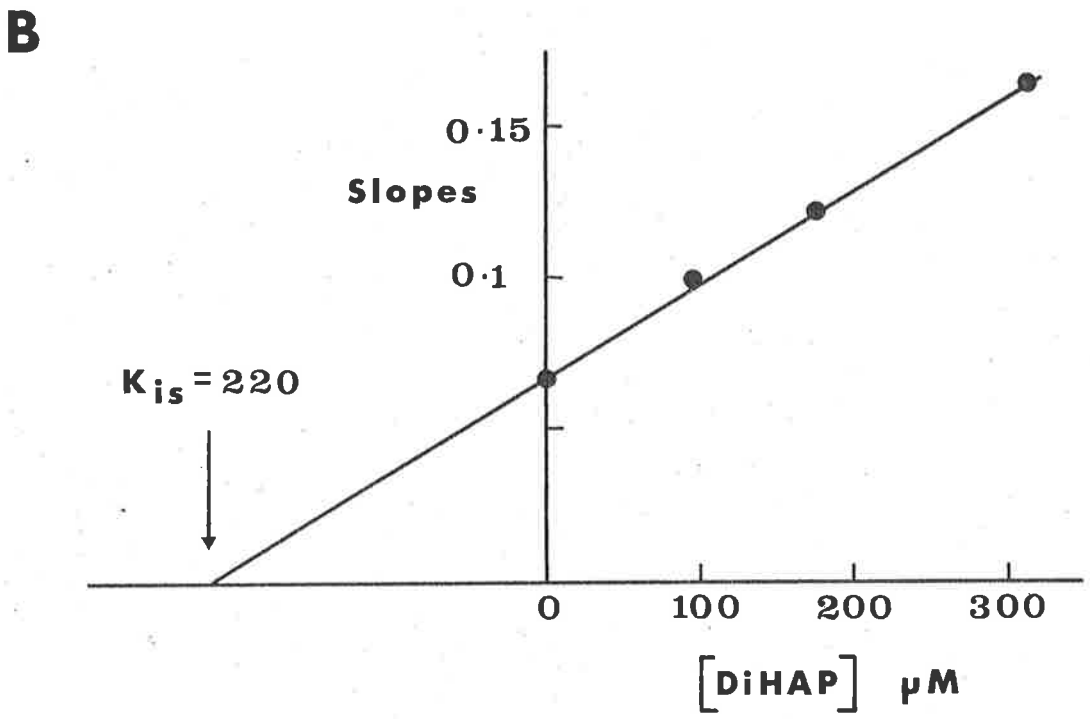

B
0·15
Slopes
0·1
K_{is} = 220
0
100
200
300
[DiHAP] μM

Table 7.2. continued

b expressed as μM.

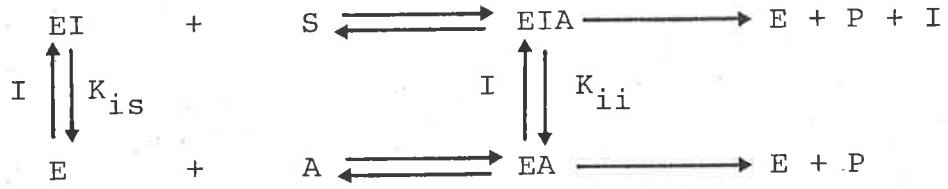
c expressed as nmoles HCHO formed/5min/mg microsomal protein.

In contrast, however, the secondary plots for ethylmorphine obtained in the presence of 96.1 μM NADH and the inhibitor suggest that the inhibition has changed in nature to intercept-hyperbolic, slope-hyperbolic non-competitive inhibition, (Figure 7.5.).

Equation 7.5 describes this type of inhibition (from Cleland, 1963).

$$v = \frac{V.A}{K_m\left(\frac{1 + I/K_{is}}{1 + I/K_{ii}}\right) + A\left(\frac{1 + I/K_{is}}{1 + I/K_{ii}}\right)} \quad \ldots 7.5.$$

This equation is similar to the equation which describes "hyperbolic competitive inhibition" which occurs when both substrate and inhibitor are present on the enzyme at the same time (Equation 7.6.). This is a special case for competitive inhibition for it is generally accepted that both inhibitor and substrate cannot be present on the enzyme at the same time.



$$v = \frac{V.A}{K_m\left(\frac{1 + I/K_{is}}{1 + I/K_{ii}}\right) + A} \quad \ldots 7.6.$$

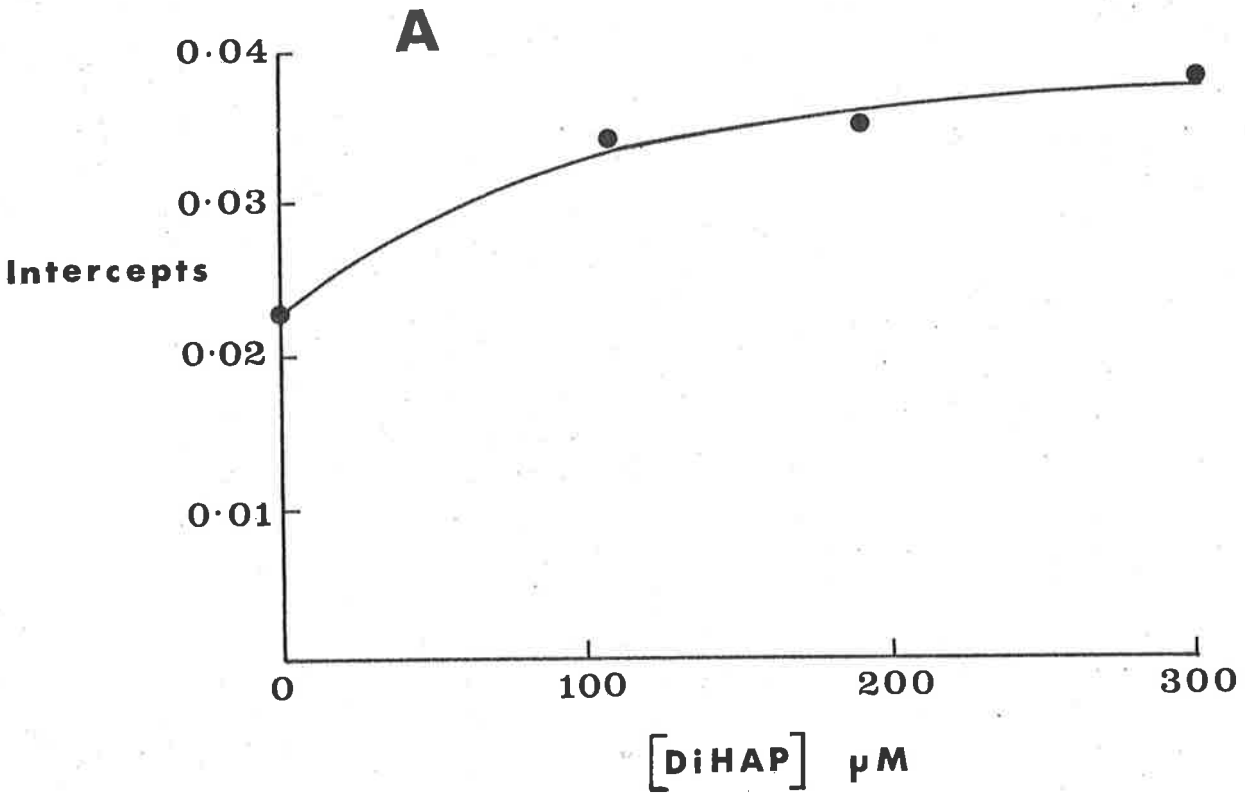
A secondary plot of slope versus inhibitor concentration is in this case hyperbolic.

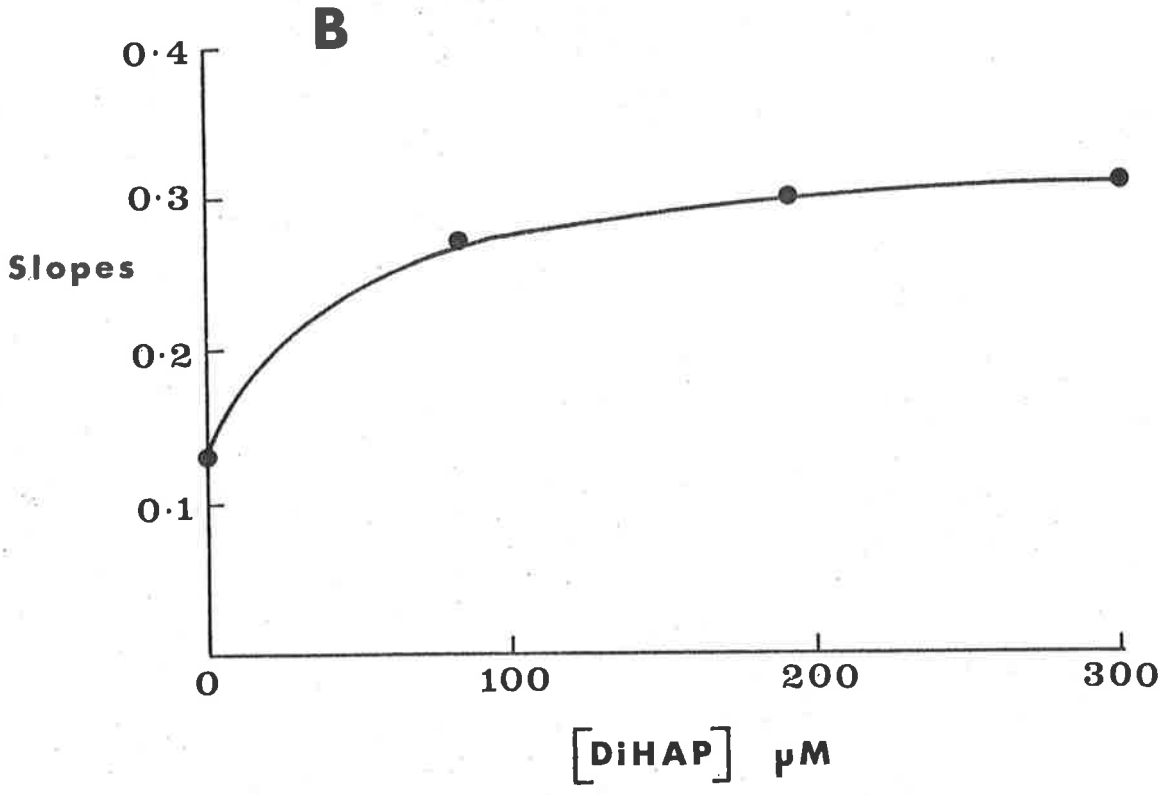
It has been postulated that the effect of I is to make it

FIGURE 7.5.

Secondary plots of 1/V intercepts versus DiHAP concentration (Figure 7.5.A.) and slopes versus DiHAP concentration (Figure 7.5.B.) for ethylmorphine-N-demethylase in the presence of 96.1 μM NADH.

The slope and 1/V intercept values at each DiHAP concentration were obtained from the HYPER output for that concentration.


A
0·04
0·03
Intercepts
0·02
0·01
0
100
200
300
[DiHAP] μM


B
0·4
0·3
Slopes
0·2
0·1
0
100
200
300
[DiHAP] μM

more difficult for A to react with the enzyme, but the EIA complex once formed breaks down at the same rate as the EA complex. At saturating concentrations of A, the enzyme will be in the form of both EA and EIA, but since both complexes decompose at identical rates, apparent v is the same in the presence and absence of I. (Cleland, 1963).

If it is assumed that the EIA complex decomposes at a rate that is either different from, or identical to that of EA, then the scheme representing S -linear, I-linear non-competitive inhibition (Scheme 7.1.) resembles that describing hyperbolic competitive inhibition (above, equation 7.6.). In all cases of non-competitive inhibition, both substrate and inhibitor are considered to be present together on the enzyme surface at the same time. It is therefore possible that the EIA complex could decompose to produce product, inhibitor and free enzyme. If this occurs, secondary plots of slopes versus inhibitor concentration and 1/V intercept versus inhibitor concentration could be expected to be hyperbolic in nature.

Alternatively the hyperbolic nature of the secondary plots (Figure 7.5.) for ethylmorphine-N-demethylase in the presence of NADH could indicate that nature of 2,6-dihydroxyacetophenone inhibition has changed to mixed inhibition, i.e., the inhibition observed is composed of both competitive and non-competitive components.

7.3.3. THE INFLUENCE OF 2,6-DIHYDROXYACETOPHENONE ON NADPH-CYTOCHROME P_{450} REDUCTASE IN THE PRESENCE OF EITHER AMINOPRYINE OR ETHYLMORPHINE.

The addition of 2,6-dihydroxyacetophenone (210 μM) caused significant inhibition of NADPH-cytochrome P_{450} reductase activity in the presence of either aminopyrine or ethylmorphine. Marked

aggregation of the microsomal suspension was observed in the presence of the inhibitor while the incubation medium was being adjusted to 26°C.

A small magnetic bar was added to the cuvette which allowed aggitation of the contents prior to initiating the reaction. The reduction rates in the presence of either substrate under these conditions were not readily reproduced.

A reduction from 5.28 ± 0.13 (Table 4.3.) to 1.4 ± 0.7 nmoles cytochrome P_{450} reduced/min/mg microsomal protein (N = 3) was observed with aminopyrine as the substrate when the reduction was initiated by the addition of 125 µM NADPH. When ethylmorphine replaced aminopyrine as the substrate, cytochrome P_{450} reductase activity was reduced from 8.92 ± 0.38 (Table 4.3.) to 4.58 ± 1.02 nmoles cytochrome P_{450} reduced/min/mg microsomal protein (N = 3).

The addition of NADPH (125 µM) and NADH (130 µM) together to initiate the reaction resulted in an increase from 1.4 ± 0.7 to 2.5 ± 0.9 (aminopyrine) and from 4.58 ± 1.02 to 7.16 ± 1.68 (ethylmorphine) nmoles cytochrome P_{450} reduced/min/mg microsomal protein. (N = 3 for both substrates).

The reduction in the rates observed in the presence of the inhibitor when the reaction was initiated by the simultaneous addition of NADPH and NADH are significantly lower than the corresponding rates observed in the absence of the inhibitor, (Table 5.2.).

7.3.4. THE INFLUENCE OF 2,6-DIHYDROXYACETOPHENONE ON THE STEADY-STATE LEVELS OF REDUCED CYTOCHROME b5.

The addition of 2,6-dihydroxyacetophenone (305 µM final concentration) to the incubation medium did not alter the steady-state level of reduced cytochrome b_5 upon the addition of NADPH (210 µM) or NADPH (210 µM) and NADH (244 µM) together compared to values

obtained in the absence of the inhibitor (Table 5.7.), (with either aminopyrine or ethylmorphine as the drug substrate). There was no alteration in the steady-state levels of reduced cytochrome b_5 when the spectra were recorded after a 5 min incubation at $37^{o}C$.

Further, there was no evidence of an absorbance peak at 440 nm. This absorbance peak has been attributed to the presence of the oxygenated form of the ferrous cytochrome P_{450}-substrate complex.

7.4. DISCUSSION

The linearity of the secondary plots of 1/V intercept versus inhibitor concentration and slopes versus inhibitor concentration for both aminopyrine and ethylmorphine demethylase suggests that 2,6-dihydroxyacetophenone is a slope-linear, intercept-linear non-competitive inhibitor.

All the data obtained at the different inhibitor concentration for each drug substrate were fitted to the equation describing this type of inhibition (Equation 7.1.) using the iterative digital computer programme NONCOM. This programme (written by Cleland[1]) was translated into BASIC and run on a PDP 11/40 digital computer, (Appendix 3 is a listing of this programme). The programme was modified to:-

(1) calculate a correlation coefficient, r, as defined in the computational Methods, once convergence has occurred (Lines 450-460 and 630) and

(2) allow the experimental data to be weighted (Lines 455, 1100).

The BASIC programme was tested for any errors which might occur during translation by the use of simulated data. The simulated data was obtained using the following numerical values

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1. The programme was obtained from Prof. W.W.Cleland, University of Wisconsin.

for the constants in Equation 7.1.:-

$V = 100,000$; $K_m = 0.36$; $K_{is} = 0.59$; $K_{ii} = 0.51$.

The selected substrate concentrations were 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5., 5 and 10 at inhibitor concentrations of 0.01 and 0.05. (All arbitary units).

The output from the programme, which is also shown in Appendix 3 indicates that the programme converged on values for the constants of Equation 7.1., that were identical to those used to simulate the data, i.e., the BASIC version is an accurate translation of the original programme which will converge on the correct value of K_m, V, K_{is} and K_{ii}.

However, when the data obtained with aminopyrine as the substrate was fitted to the linear non-competitive equation using NONCOM, the value of K_{is} became negative after the first iteration and increasingly more negative with each subsequent iteration.

Inspection of the graphical estimates for aminopyrine demethylase (Figure 7.2.) revealed that the numerical value of K_{is} was more than four times the value of K_{ii}. Equation 7.1. contains the factors $[I]/K_{is}$ and $[I]/K_{ii}$ and therefore, the relative importance of $[I]/K_{ii}$ will necessarily be greater than $[I]/K_{is}$. Because of this factor, the programme (which is a residual least squares programme) attempts to obtain accurate estimates of K_{ii}, K_m and V at the expense of K_{is}.

The computer programme was not considered to be in error because:-

(1) it converged on identical numerical estimates of the constants of Equation 7.1. that were used to simulate the data (described above) and

(2) it converged quickly on positive values of K_{is} and K_{ii} which were in good agreement with graphic estimates when

the values of these two constants were close together, i.e., the anomolous behaviour was only apparent when the numerical value of one constant was much greater than the other, and

(3) the K_m and V values were not significantly different from the values obtained in the absence of the inhibitor.

The numerical values of K_{ii} and K_{is} obtained with aminopyrine as the substrate suggest that 2,6-dihydroxyacetophenone combines more readily with the ferric cytochrome P_{450}- aminopyrine complex (E in the scheme) than with the ferrous cytochrome P_{450}- aminopyrine compex (EA in the scheme, A represents NADPH, the varied substrate).

However, the converse appears to be true when ethylmorphine was the drug substrate, i.e., the inhibitor appears to combine more readily with the ferrous cytochrome P_{450}- ethylmorphine complex than with the oxidised cytochrome P_{450}- ethylmorphine complex.

Gigon *et al.*, (1968, 1969) has suggested that Type I substrates combine with and induce a conformational change in cytochrome P_{450} such that the cytochrome P_{450}- substrate complex is reduced at a greater rate than cytochrome P_{450} alone. It has been shown previously (Table 4.3.) that the reduction rates of the cytochrome P_{450}- aminopyrine complex, cytochrome P_{450}- ethylmorphine complex and cytochrome P_{450} alone were 5.28, 8.92 and 3.7 nmoles cytochrome P_{450} reduced/min/mg microsomal protein, respectively.

It is therefore apparent that different Type I substrates induce the proposed conformational change to varying degrees, which could explain why 2,6-dihydroxyacetophenone combines more readily with the ferrous cytochrome P_{450} - ethylmorphine complex and the ferric cytochrome P_{450}- aminopyrine complexes.

It should be noted that the proposed conformational change relating to the interaction of Type I substrates with oxidised cytochrome P_{450} implies an alteration to the tertiary structure of cytochrome P_{450}. The observed increase in reduction rate upon the binding of substrates, similar to the proposed binding of NADH, may just as readily be explained by the interaction of the substrate without an obligatory alteration in the tertiary structure of the complex.

If the proposal that NADH supplies the second electron via cytochrome b_5 after the first has been supplied by NADPH (Cohen & Estabrook, 1971c; Hildebrandt & Estabrook, 1971) is correct, similar numerical values of K_{ii} and K_{is} would be expected when these inhibition experiments were repeated in the presence of a constant NADH concentration.

The nature of the inhibition by 2,6-dihydroxyacetophenone still appeared to be linear non-competitive (Figure 7.4.) when the inhibition experiments for aminopyrine demethylase were repeated in the presence of 120 μM NADH.

However, the numerical value of K_{is} was reduced from 650 (no NADH) to 234 $\pm$ 50 (120 μM NADH) and K_{is} was increased from 128.1 $\pm$ 8.5 (no NADH) to 185.7 $\pm$ 7.0 (120 μM NADH), (Tables 7.1. and 7.2.).

When the inhibition experiments with ethylmorphine as substrate were repeated in the presence of 96.1 μM NADH, the nature of the inhibition changed from linear non-competitive to slopes-hyperbolic, intercept-hyperbolic non-competitive inhibition. (Figure 7.5.).

Although other explanation may be possible, the alteration in the values of K_{ii} and K_{is} (with aminopyrine as substrate) and the change in the nature of 2,6-dihydroxyacetophenone inhibition (with ethylmorphine as substrate) suggests that NADH is interacting with

the oxidised cytochrome P_{450}- substrate complex before the electron is transferred from NADPH (the varied substrate (A) in these inhibition experiments).

These results provide further experimental evidence in support of the proposed interaction of NADH with the oxidised cytochrome P_{450}- substrate complex prior to the reduction of this complex by an electron originating in NADPH.

C H A P T E R 8.

GENERAL DISCUSSION

The following mechanisms have been proposed to account for the observed NADH synergistic increase in product formation during the metabolism of certain drugs by the hepatic microsomal enzyme system. A feature common to all proposals is the role of NADPH-cytochrome c reductase in transferring the first electron from NADPH to the ferric cytochrome P_{450}- substrate complex:-

(1) the mechanism proposed by Estabrook & Associates (Cohen & Estabrook, 1971a,b,c; Hildebrandt & Estabrook, 1971) suggested that cytochrome b_5 mediates the introduction of the second electron from either NADPH or NADH. Because NADH is more efficient than NADPH in donating the second electron, a synergistic increase in product formation is therefore apparent in the presence of both pyridine nucleotides.

(2) Correia & Mannering (1973a,b,c) have supported the role of cytochrome b_5 in donating the second electron. In their scheme, it was proposed that the extent of NADH synergism was related to the levels of endogenous fatty acid desaturase activity.

(3) Staudt *et al.*, (1974) have proposed an uncoupling mechanism to explain NADH synergism. The active oxygen that is not incorporated into the substrate is reduced to water by the NADH cytochrome b_5 system and this sparing effect is the main mechanism of NADH synergism.

The relative strengths and deficiencies of these three proposals have been discussed in detail in Chapter 5.

In contrast to these mechanisms which were proposed from experiments with intact microsomes, Lu *et al.*, (1974), (using the resolved and reconstituted system) reported the addition of cytochrome b_5 to the three obligatory components inhibited benz-

phetamine demethylation. The addition of NADH and cytochrome b_5 reductase reversed the inhibitory effect.

It was postulated that in the presence of NADPH alone, there is a competition between cytochromes P_{450} and b_5 for the electron from NADPH-cytochrome c reductase. When both reduced pyridine nucleotides are added together, NADH reduces cytochrome b_5 thus eliminating the competition between the two cytochromes for NADPH-cytochrome c reductase which results in NADH synergism.

It is apparent that of all the models, the mechanism proposed by Estabrook & Associates has been the one most widely accepted. Further, the data obtained in present work is more compatible with this proposal than any of the other three mechanisms. Therefore, this scheme of microsomal electron transport during drug hydroxylation has been selected as the working hypothesis. After the proposal of this hypothesis, Jansson & Schenkman (1973) reported an inhibition of aminopyrine-N-demethylase when detergent solubilised cytochrome b_5 was added and bound to intact microsomes. The addition of NADH with NADPH did not prevent the inhibition which, it was suggested, argued against the involvement of cytochrome b_5 in hepatic MFO reactions.

Earlier, Ichikawa & Loehr (1972) had proposed that electrons from NADH could reach cytochrome P_{450} in the absence of cytochrome b_5. In contrast, however, Hrycay & Estabrook (1974) have suggested that the addition of detergent solubilised cytochrome b_5 to intact microsomes markedly stimulates the NADH mediated reduction of cytochrome P_{450}. Mannering (1974) has raised some doubts regarding the validity of such experiments as a means of establishing a role for cytochrome b_5 in the microsomal MFO system.

Studies using antibodies to cytochrome b_5 (Sasame *et al.*, 1973; Mannering *et al.*, 1974) have suggested that cytochrome b_5 mediates the introduction of the second electron from NADH but not from NADPH. Later, Sasame *et al.*,(1974b) proposed that cytochrome b_5 could be involved in certain NADPH dependent cytochrome P_{450} reactions. Correia & Mannering (1973b) have questioned the interpretation of the antibody studies of Sasame *et al.*,(1973) although subsequently Mannering *et al.*, (1974) supports their proposal. Thus, it is apparent that the role of cytochrome b_5 in transferring electrons from NADPH and NADH is not fully resolved although, at the present time, the bulk of the experimental evidence supports the involvement of this cytochrome in transferring electrons from NADH but not NADPH.

The present work assumes that cytochrome b_5 mediates the introduction of the second electron from NADH but not NADPH. It further assumes that in the presence of both reduced pyridine nucleotides, the second electron is derived preferentially from NADH via cytochrome b_5 rather than NADPH via it's, as yet, unidentified carrier.

These assumptions are perhaps not unreasonable in view of the demonstrated NADH synergistic increase in the metabolism of Type I substrates (Cohen & Estabrook, 1971a,b,c; Hildebrandt & Estabrook, 1971; Correia & Mannering, 1973a,b,c). Under such circumstances, the second electron could be derived from NADH or NADPH via their respective routes, but appears to come from NADH. If this were not the case, NADH could not exert it's proposed synergistic effect on product formation, assuming the working hypothesis is correct.

This assumption was supported by the observation (Cohen & Estabrook, 1971c) that NADH oxidation was greatly enhanced in the

presence of low concentrations of NADPH. The mechanism which has been proposed on the basis of studies with microsomes isolated from phenobarbital pretreated rats (Hildebrandt & Estabrook, 1971) assumed that the reduction of the cytochrome P_{450}- substrate complex by the flavoprotein reductase is rate limiting.

However, in the present study, the effect of NADH in significantly reducing the K_m (NADPH) value during both aminopyrine and ethylmorphine-N-demethylase suggests that there has been a change in the rate limiting step (Estabrook & Cohen, 1969). If the working hypothesis is correct, NADH would not be expected to exert any marked effect on apparent K_m (NADPH) values but would be expected to increase V values.

The present results suggest that NADH was interacting with the cytochrome P_{450}- substrate complex or some other component at or prior to the introduction of the first electron which originated in NADPH. Any significant effects gained by the interaction of NADH with NADPH-cytochrome c reductase can be excluded because the rate at which electrons can be transported through the flavoprotein when cytochrome c is the electron acceptor, is at least an order of magnitude above that obtained with cytochrome P_{450}.

The interaction of NADH with the cytochrome P_{450}- substrate complex was further supported by the following experimental evidence:-

(1) NADH significantly increased the V values of NADPH-cytochrome P_{450} reductase in the presence of either aminopyrine or ethylmorphine. There was no change in the apparent K_m (NADPH) value. These results suggest that NADH exerts a heterotropic co-operative effect with respect to NADPH, and

(2) NADH significantly stimulates the initial rapid phase

of the biphasic reduction kinetics of cytochrome P_{450} in the presence of aminopyrine, and

(3) the change in the nature of 2,6-dihydroxyacetophenone inhibition (with ethylmorphine as substrate) and the numerical values of the inhibition constants (with aminopyrine as substrate) when the inhibitor experiments were repeated in the presence of NADH (Chapter 7).

It is therefore proposed that in the presence of both reduced pyridine nucleotides with microsomes isolated from control rats the reduction of the cytochrome P_{450}- substrate complex is no longer rate limiting.

Preliminary experiments have correlated the V values for the demethylation of aminopyrine and ethylmorphine in the presence of both NADPH and NADH to the steady state level of reduced cytochrome b_5 in the presence of both pyridine nucleotides, (Table 5.8.). Further, the actual magnitude of the V values were similar at each NADH concentration when the kinetic constants of NADPH were determined for both aminopyrine and ethylmorphine, (Table 5.1.).

Although these results suggest that in the presence of both pyridine nucleotides, the introduction of the second electron is rate limiting, other possibilities must be considered. For example, the exchange of the active oxygen (depicted as OH) with one of the $N\text{-}CH_3$ hydrogens has been suggested to be partially rate limiting for ethylmorphine-N-demethylase (Thompson & Holtzman, 1974). The binding of molecular oxygen to the ferrous cytochrome P_{450}- substrate complex and any steps that occur after the introduction of active oxygen would be expected to be rapid and not rate limiting. The concept that the introduction of the second electron is rate limiting has been advanced by Estabrook *et al.*, (1973) from

the results of stopped flow experiments using microsomes isolated from phenobarbital pretreated rats and by Gunsalus & Lipscomb, (1973) for the bacterial cytochrome $P_{450\ cam}$ system.

On the basis of the experimental evidence reported in this thesis, the following sequence of events are proposed to occur during either aminopyrine or ethylmorphine demethylation in the presence of both reduced pyridine nucleotides:-

(1) the substrate combines with ferric cytochrome P_{450} to form a ferric cytochrome P_{450}- substrate complex.

(2) the NADH molecule binds to and induces a conformational change in the ferric cytochrome P_{450}- substrate complex thereby allowing the electron from NADPH to be transferred at a greater rate. As discussed in Chapter 6, the proposed conformational change implies that there is an alteration to the tertiary structure of the cytochrome P_{450}- substrate complex. It is equally possible that the binding of NADH somehow modifies the input of the first electron without changing the tertiary structure of the complex. NADPH-cytochrome P_{450} reductase is stimulated by the binding of NADH to the extent that it is no longer rate limiting.

(3) the NADH that binds to cytochrome P_{450} also donates one electron via NADH-cytochrome b_5 reductase to reduce cytochrome b_5. Both cytochrome b_5 (Strittmatter *et al.*, 1972; Rogers & Strittmatter, 1974a) and cytochrome b_5 reductase (Rogers & Strittmatter, 1974b) have been postulated to undergo translational diffusion within microsomal membranes. Further, the reduction of cytochrome b_5 by cytochrome b_5 reductase is much more rapid than the reoxidation of the cytochrome by the donation

of an electron (Strittmatter *et al.*, 1972; Hildebrandt & Estabrook, 1971; Cohen & Estabrook, 1971c). It is therefore possible that the cytochrome b_5 and the flavoprotein reductase could be in the vicinity of the NADH binding site on the cytochrome P_{450}- substrate complex. It is postulated that the reduction of cytochrome b_5 by an electron from the cytochrome P_{450} bound NADH molecule occurs after the reduction of the cytochrome P_{450}- substrate complex by an electron from NADPH,

(4) molecular oxygen binds to the ferrous cytochrome P_{450}- substrate complex either before or after cytochrome b_5 reduction,

(5) the second electron required for the activation of the bound oxygen is transferred from reduced cytochrome b_5. This step could involve, if necessary, the translational diffusion of reduced cytochrome b_5,

(6) the active hydroxylating species is introduced into the substrate,

(7) the complex dissociates liberating hydroxylated substrate, water and regenerates ferric cytochrome P_{450}.

Although the results of Chapter 5 suggest that only one NADH molecule is involved in the NADH synergistic effect during both aminopyrine and ethylmorphine demethylation, they also raised the possibility that two NADH molecules may be involved and that there was a homotropic co-operative interaction between them. A minor modification to the proposed mechanism can accomodate these results if they prove to be correct.

In such a modified mechanism, it is suggested that the first NADH molecule binds to and induces the same conformational change in the cytochrome P_{450}- substrate complex as previously proposed.

It does not, however, donate any electrons to cytochrome b_5, but rather influences the site for the introduction of the second electron via cytochrome b_5. The second NADH molecule binds in a co-operative manner with the first NADH molecule and donates the electron to reduce cytochrome b_5. A NADH homotropic co-operative interaction between the two NADH binding sites is observed because the NADH reduction of cytochrome b_5 is very rapid compared to the rate at which it is reoxidised by donating it's electron to the oxygenated-ferrous cytochrome P_{450}- substrate complex.

If the mechanism proposed in this thesis is correct, the actual structure of NADH would be expected to be a significant factor responsible for the proposed NADH induced conformational change.

Other investigators have advanced a similar proposal that NADH, in addition to donating an electron, also induces a conformational change in both horse liver alcohol dehydrogenase (Wong & Williams, 1968; Biellmann & Jung, 1971) and octopine dehydrogenase (Olomucki *et al.*, 1975).

The structural analogue of NADH, $NADH_3$, which was shown to be totally ineffective in supporting the reduction of the cytochrome P_{450}- aminopyrine or ethylmorphine complexes or the demethylation of these two substrates was used to examine whether or not the structure of NADH was responsible for the conformational change.

While the analogue could not reduce the cytochrome P_{450}- substrate complex, it was responsible for a synergistic increase in the NADPH dependent reduction rate of this cytochrome (Figure 6.1.). Further, in the presence of $NADH_3$ there was a significant increase in the V values of NADPH-cytochrome P_{450} reductase (in the presence of either aminopyrine or ethylmorphine) and an increase in the initial rapid phase of the biphasic reduction kinetics of

the cytochrome P_{450}- aminopyrine complex.

The $NADH_3$ analogue has been shown by both crystallographic (Biellmann & Jung, 1971) and spectroscopic evidence (Olomucki *et al.*, 1975) to induce the same conformational change as NADH in horse liver alcohol dehydrogenase. Similarly, spectral evidence suggests that $NADH_3$ induces the same conformational change as NADH in octopine dehydrogenase (Olomucki *et al.*, 1975)

Although the $NADH_3$ analogue had much the same influence as NADH on NADPH-cytochrome P_{450} reductase, the magnitude of the $NADH_3$ effects were somewhat lower than the corresponding NADH effects. It is suggested that while $NADH_3$ is capable of inducing a conformational change similar to that produced by NADH, it is however, less efficient than NADH in inducing the change.

One possible explanation of the reduced efficiency of $NADH_3$ relative to NADH is the conformation adopted by the nicotinamide ring in both nucleotides. The pyridine nucleotides have been suggested (Oppenheimer *et al.*, 1971) to exist in solution, in a folded conformation where the adenine and nicotinamide rings are parallel to one another. It was further suggested that NADH and other reduced analogues exist predominately with the B side of the dihydropyridine ring folded against the adenine ring. (See Figure 8.1.). Such a folded interaction, it was proposed, puckers the dihydropyridine ring such that the $C\text{-}4_b$ proton moves equitorial and the $C\text{-}4_a$ proton moves axial.

However, the nicotinamide ring of the more fully hydrogenated analogue, although able to adopt the boat conformation of the NADH nicotinamide ring, has lost the C5-C6 double bond (of NADH) and therefore would also exist in other possible conformations. The fact that the nicotinamide ring of $NADH_3$ may not exist predmoninately in the proposed boat conformation could be responsible for

the reduced efficiency of $NADH_3$ relative to NADH in inducing the conformational change in the cytochrome P_{450}- substrate complex.

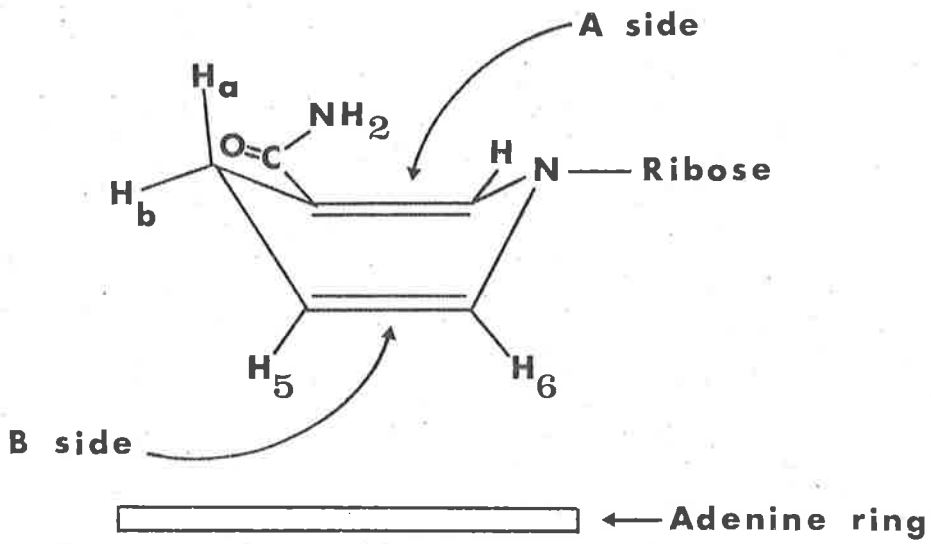

FIGURE 8.1. Proposed conformation of reduced pyridine nucleotides in solution (from Oppenheimer *et al.*, 1971).

Olomucki *et al.*, (1975) have also postulated that the conformation adopted by the nicotinamide ring of $NADH_3$ is different from that adopted by the same ring in NADH.

Overall, these results support the suggestion that the structure of NADH rather than it's electron donating capacity is the significant factor in the proposed conformational change which occurs when NADH interacts with the ferric-cytochrome P_{450}-substrate complex.

The results obtained when the kinetic constants for NADPH for the MFO system were determined in the presence of $NADH_3$ are compatible with the scheme proposed in this thesis, with minor modifications.

It is suggested that in the presence of NADPH and $NADH_3$:-

(1) the substrate combines with ferric cytochrome P_{450} to form a ferric cytochrome P_{450}- substrate complex.

(2) $NADH_3$ combines with the NADH binding site on the complex inducing the conformational change that results in an increased rate of reduction of the cytochrome P_{450}-substrate-$NADH_3$ complex. However, as previously suggested, $NADH_3$ is not as efficient as NADH in inducing the conformational change and therefore NADPH-cytochrome P_{450} reductase although stimulated is still rate limiting for aminopyrine-N-demethylase. When the kinetic constants of NADPH for aminopyrine-N-demethylase were determined in the presence of $NADH_3$ there was a significant increase in the V value but no change in the apparent K_m value (Table 6.4.). Further, in the presence of $NADH_3$, there were parallel increases in V values for aminopyrine demethylase and NADPH-cytochrome P_{450} reductase (plus aminopyrine). These results are consistent with the mechanism proposed. The lack of reduction in the apparent K_m (NADPH) value in the presence of $NADH_3$ compared to the value obtained in the absence of $NADH_3$ supports the concept that NADPH-cytochrome P_{450} reductase is still rate limiting.

Table 6.4. also shows that there is a significant increase in K_m (NADPH) value in the presence of $NADH_3$ when ethylmorphine was the substrate compared to the corresponding value obtained in the absence of $NADH_3$ (Table 4.1.). This suggests that when ethymorphine was the substrate, the reduction of the cytochrome P_{450}- ethylmorphine complex by the electron from NADPH in the presence of $NADH_3$ is no

longer rate limiting. Further experimental evidence in support of this concept was the finding that parallel increases in V values were not observed for ethylmorphine-N-demethylase and NADPH-cytochrome P_{450} reductase (plus ethylmorphine) when both activities were determined in the presence of $NADH_3$. It is evident however, that $NADH_3$ has a pronounced effect because the V value for ethylmorphine demethylase in the presence of $NADH_3$(Table 6.4.) is significantly greater than the corresponding V values determined in the presence of NADH (Table 5.1.). The rate limiting step under such circumstances remains to be elucidated. $NADH_3$ did not increase the steady state level of reduced cytochrome b_5 above that obtained with NADPH in the presence of ethylmorphine or aminopyrine. Nevertheless, the introduction of the second electron and/or the exchange of active oxygen with the $N-CH_3$ hydrogens are two possibilities.

(3) molecular oxygen binds to the ferrous cytochrome P_{450}-substrate complex and is then activated by the introduction of the second electron from a second NADPH molecule via cytochrome b_5 or some other, as yet unidentified intermediate. The remainder of the mechanism is identical to that proposed earlier.

During the demethylation of either substrate in the presence of $NADH_3$ it is proposed that $NADH_3$ remains firmly bound to the cytochrome P_{450}- substrate complex. Theoretically, it is possible to test this assumption by examining the binding of radioactive $NADH_3$ to the hepatic MFO system. However, in the heterogenous microsomal fraction used in the present study, such experiments are not really feasible. When NADH is present with NADPH, the

mechanism proposed in this thesis suggests that the second electron required for the production of active oxygen is ultimately derived from the NADH molecule that binds to and induces the conformational change in the cytochrome P_{450}- substrate complex. In donating this electron, the oxidised pyridine nucleotide NAD would be formed with an obligatory change in the conformation of the nicotinamide ring of the nucleotide from the boat dihydro form (Oppenheimer *et al.*, 1971) to the planar conformation of the oxidised pyridine nucleotide.

It is proposed that such a conformational change is sufficient to dissociate NAD from the cytochrome P_{450}- substrate complex.

In contrast, $NADH_3$ when present does not donate any electrons and therefore would remain associated with the cytochrome P_{450}- substrate complex because there is no conformational change.

The kinetic constants of NADPH obtained for aminopyrine or ethylmorphine-N-demethylase in the presence of both NADH and $NADH_3$ are consistent with the proposed mechanism. During the demethylation of either substrate, there would initially be a competition between NADH and $NADH_3$ for the binding site on the cytochrome P_{450}- substrate complex. As previously proposed, $NADH_3$ remains firmly associated with the complex once bound, whereas NADH dissociates from the complex once it has donated the electron required to activate molecular oxygen. Therefore, as the demethylation proceeds, there will be a rapid increase in the amount of $NADH_3$ bound at the expense of NADH.

When the kinetic constants for NADPH were determined in the presence of NADH and $NADH_3$ for aminopyrine or ethylmorphine-N-demethylase, the prediction based upon the proposed mechanism would be a reduction of apparent K_m (because of initial binding of NADH) but not to the extent obtained when similar kinetic constants for

NADPH were determined in the presence of NADH (Table 5.1.). Further, the V values in the presence of the three nucleotides for demethylation of both substrates should be similar to the corresponding values obtained when the kinetic constants of NADPH were determined in the presence of $NADH_3$ because of the predicted relatively high occupancy of the binding site by $NADH_3$ and the apparent inability of NADH to displace $NADH_3$ from the site once bound (Chapter 6.). The results obtained with aminopyrine, and in part with ethylmorphine are consistant with the predictions made.

As discussed in Chapter 6, the reason for the apparently anomolous high V with ethylmorphine is not immediately clear, but similar values were obtained when the experiment was repeated in another set of animals (N = 3).

Stier (1976) suggests that intact microsomes are heterogenous with respect to their lipid composition and that there are areas of immobilised lipid that surround the cytochrome P_{450} system. It was further proposed that a metastable lipid halo rigidly couples the enzymes of the multicomponent enzyme system. The binding of substrates or the reduction of cytochrome P_{450} may alter the lipid stability which in turn may couple or uncouple the mult-enzyme system.

Although unlikely, the possibility that NADH may exert it's synergistic effect by binding to some lipid component which induces a coupling phenomenon in the multienzyme complex cannot be excluded.

The influence of NADH on NADPH kinetics in microsomes isolated from phenobarbital pretreated rats suggest the mechanism of electron transport during drug hydroxylation in induced rats may well be different from that which operates in untreated rats. This could be a significant finding because mechanisms proposed in the past (Cohen & Estabrook, 1971c; Hildebrandt & Estabrook, 1971) and

thought to be generally applicable as an explanation of microsomal electron transport,have been based on studies using microsomes from phenobarbital pretreated rats and rabbits.

APPENDIX 1.

SOURCE LISTING AND TEST DATA FOR HYPER.

The following listing is a BASIC-PLUS version of HYPER, an iterative least-squares digital computer programme that fits data to the Michaelis-Menten equation. The programme written in FORTRAN by Cleland (1967), was modified to calculate a correlation co-efficient once conversion had occurred as previously described.

The data input to the programme was also modified because the original method used by Cleland (1967) was very repetitious when there were multiple velocity determinations at each substrate concentration.

Lines 1500-1999 are for data input which takes the following format, (Lines 130-218):-

All the replicate velocity determinations at each substrate concentration, starting from the highest substrate concentration and working to the lowest concentration

Then for the highest substrate concentration, the following data is supplied:-

(a) the number of velocity determinations at that substrate concentration, and

(b) the actual substrate concentration, and

(c) the weight factor to be applied to the velocity determinations.

This procedure is repeated at each substrate concentration.

The accuracy of the BASIC-PLUS version was tested using simulated data by assigning a K_m value = 10, and V = 50. Velocity readings were calculated by substituting these values into the Michaelis-Menten equation at the following substrate concentrations, 1, 2, 5, 10, 20, 30, 50, 100 and 120.

The programme converged on the following kinetic constants when supplied with the test data:-

$$K_m = 9.99 \pm 0.01$$

and

$$v = 49.97 \pm 0.01,$$

indicating that the BASIC-Plus version is an accurate translation of the original programme.

```
10 REM - PROGRAM NAME- HYPER, TRANSLATED INTO BASIC-PLUS BY
11 REM G.K. GOURLAY, SCHOOL OF PHARMACY, S.A.I.T
12 REM PROGRAM WRITTEB BY W.W.CLELEAD
20 DIM V(100),A(100),W(100),S(3,4),Q(3), S9(3),Y8(3)
30 J9=0
40 INPUT" NUMBER OF ITERATIONS REQUIRED "$B
50 INPUT" NUMBER OF DATA POINTS "$N9
55 INPUT "NUMBER OF DATA SETS"$G5
60 IF N9 < =0 GO TO 1460
70 M=1
80 N=2
90 P=N9-N
100 N1=N+1
110 N2=N+2
130 FOR I=1 TO N9
135 READ V(I)
140 NEXT I
150F2=1$F3=0
160 FOR F=1 TO G5
170 READ G1,G2,G3
175 F3=F3+G1
180 GO SUB 190
184 NEXT F
188 GO TO 215
190 FOR I=F2 TO F3
200 A(I)=G2
205 W(I)=G3
208 NEXT I
210 F2=F2+G1
212 RETURN
215 FOR I=1 TO N9
216 PRINT V(I),A(I),W(I)
218NEXT I
219 GO TO 1000
220 IF W(I)>0 GO TO 240
230 W(I)=1
240 Q(1)=V(I)^2/A(I)
250 Q(2)=V(I)^2
```

```
260 Q(3)=V(I)
270 GO TO 1080
280 C9=S(1,1)/S(2,1)
282 PRINT"THE PRELIMINARY ESTIMATES OF THE PARAMETERS ARE"
283 PRINT "K= "$C9
285 PRINT " V MAX= "$S(1,1)
290 J9=J9+1
300 PRINT " NUMBER OF          K          V MAX"
301 PRINT " ITERATIONS"
302 PRINT " PERFORMED"
310 N8=0
320 M=2
330 GO TO 1000
340 D=C9+A(I)
350 Q(1)=A(I)/D
360 Q(2)=Q(1)/D
370 Q(3)=V(I)
380 GO TO 1080
390 C9=C9-S(2,1)/S(1,1)
400 N8=N8+1
405 PRINT N8,C9,S(1,1)
410 IF (N8-B)<0 GO TO 1000
420 S2=0
421 R8 =0
425 R9=0
430 FOR I = 1 TO N9
440 S2=S2+(V(I)-S(1,1)*A(I)/(C9+A(I)))^2*W(I)
442 R8=R8+(V(I)-S(1,1)*A(I)/(C9+A(I)))^2
445 R9=R9+(V(I)*V(I))
450 NEXT I
455 R2=(R9-R8)/R9
460 S2=S2/P
470 S1=SQR(S2)
480 Y9=C9/S(1,1)
490 V9=1/S(1,1)
500 V8=1/Y9
```

```
510 FOR J = 2 TO N1
520 FOR K = 1 TO N
530 S(K,J)=S(K,J)*S9(K)*S9(J-1)
540 NEXT K
550 NEXT J
560 S8=S1*SQR(S(1,2))
570 S7=S1*SQR(S(2,3))/S(1,1)
580 S6=S8/S(1,1)^2
590 S(1,3)=S1*SQR(C9^2*S(1,2)+S(2,3)+2*C9*S(1,3))
600 S5=S(1,3)/S(1,1)^2
610 S4=S(1,3)/C9^2
620 Z9=1/S7^2
630 Z8=1/S8^2
640 Z7=1/S5^2
650 Z6=1/S6^2
660 Z5=1/S4^2
670 PRINT
680 PRINT " MICHAELIS CONSTANT,K = "$C9
690 PRINT " STANDARD ERROR OF K = "$S7
700 PRINT " WEIGHT FACTOR FOR K FOR FUTURE ANALYSIS= "$Z9
710 PRINT
720 PRINT
730 PRINT " MAXIMUN VELOCITY, V MAX = "$S(1,1)
740 PRINT " S.E. OF V MAX = "$ S8
750 PRINT " WEIGHT FACTOR FOR V MAX = "$Z8
760 PRINT
770 PRINT
780 PRINT "K /V MAX (SLOPE OF PLOT OF 1/V V'S 1/S) = "$Y9
790 PRINT "S.E. OF K /V MAX = "$S5
800 PRINT " WEIGHT FACTOR OF K /V MAX = "$Z7
810 PRINT
820 PRINT
830 PRINT
840 PRINT " 1/ V MAX = "$V9
850 PRINT " S.E. OF 1/ V MAX = "$S6
860 PRINT " WEIGHT FACTOR OF 1/ V MAX = "$Z6
870 PRINT
880 PRINT
890 PRINT
900 PRINT " V MAX / K = "$V8
910 PRINT " S.E. OF V MAX / K = "$S4
920 PRINT " WEIGHT FACTOR OF V MAX /K = "$Z5
930 PRINT
940 PRINT
950 PRINT "EXPERIMENTAL VARIANCE(RESIDUAL LEAST SQUARES)="$S2
960 PRINT "SQUARE ROOT OF EXPERIMENTAL VARIANCE(SIGMA)="$S1
970 PRINT
980 PRINT "R2 = "$R2
985 PRINT "R  = "$ SQR(R2)
990 GO TO 2000
```

```
1000 FOR J = 1 TO N2
1010 FOR K = 1 TO N1
1020 S(K,J)=0
1030 NEXT K
1040 NEXT J
1050 FOR I = 1 TO N9
1060 ON M GO TO 240,340
1080 FOR J = 1 TO N1
1090 FOR K = 1 TO N
1100 S(K,J)= S(K,J)+Q(K)*Q(J)*W(I)
1110 NEXT K
1120 NEXT J
1130 NEXT I
1140 FOR K = 1 TO N
1150 S9(K)=1/SQR(S(K,K))
1160 NEXT K
1170 S9(N1)=1
1180 FOR J = 1 TO N1
1190 FOR K = 1 TO N
1200 S(K,J)=S(K,J)*S9(K)*S9(J)
1210 NEXT K
1220 NEXT J
1230 Y8(N1)=-1
1240 S(1,N2)=1
1250 FOR L = 1 TO N
1260 FOR K = 1 TO N
1270 Y8(K)= S(K,1)
1280 NEXT K
1290 FOR J = 1 TO N1
1300 FOR K = 1 TO N
1310 S(K,J)=S(K+1,J+1)-Y8(K+1)*S(1,J+1)/Y8(1)
1320 NEXT K
1330 NEXT J
1340 NEXT L
1350 FOR K = 1 TO N
1360 S(K,1)= S(K,1)*S9(K)
1370 NEXT K
1380 ON M GO TO 280,390
1460 PRINT J9
1500 DATA 4.54,8.33,16.67,25,33.33,37.5,41.67,45.45,46.1
1501 DATA 1,1,1,1,2,1,1,5,1,1,10,1,1,20,1,1,30,1,1,50,1
1502 DATA 1,100,1,1,120,1
 2000 END
```

```
 NUMBER OF ITERATIONS REQUIRED ? 10
 NUMBER OF DATA POINTS ? 9
NUMBER OF DATA SETS? 9
 4.54            1               1
 8.33            2               1
 16.67           5               1
 25              10              1
 33.33           20              1
 37.5            30              1
 41.67           50              1
 45.45           100             1
 46.1            120             1
THE PRELIMINARY ESTIMATES OF THE PARAMETERS ARE
K=  9.98678
 V MAX=  .199846
 NUMBER OF          K            V MAX
 ITERATIONS
 PERFORMED
 1              9.98677         49.9723
 2              9.98677         49.9723
 3              9.98677         49.9723
 4              9.98677         49.9723
 5              9.98677         49.9723
 6              9.98677         49.9723
 7              9.98677         49.9723
 8              9.98677         49.9723
 9              9.98677         49.9723
 10             9.98677         49.9723

 MICHAELIS CONSTANT,K =  9.98677
 STANDARD ERROR OF K =  .104963E-1
 WEIGHT FACTOR FOR K FOR FUTURE ANALYSIS=  9076.77


 MAXIMUN VELOCITY, V MAX =  49.9723
 S.E. OF V MAX =  .136069E-1
 WEIGHT FACTOR FOR V MAX =  5401.12


K /V MAX (SLOPE OF PLOT OF 1/V V'S 1/S) =  .199846
S.E. OF K /V MAX =  .170252E-3
 WEIGHT FACTOR OF K /V MAX =  .344996E 8



 1/ V MAX =  .200111E-1
 S.E. OF 1/ V MAX =  .544877E-5
 WEIGHT FACTOR OF 1/ V MAX =  .336824E 11



 V MAX / K =  5.00385
 S.E. OF V MAX / K =  .426287E-2
 WEIGHT FACTOR OF V MAX /K =  55029.5


EXPERIMENTAL VARIANCE(RESIDUAL LEAST SQUARES)= .261375E-3
SQUARE ROOT OF EXPERIMENTAL VARIANCE(SIGMA)= .161671E-1

R2 =  1
R  =  1

Ready
```

APPENDIX 2.

SOURCE LISTING AND TEST DATA FOR ATKINS.

Atkins is a BASIC-PLUS version of the programme of Atkins (1973) that fits data to the Hill equation using a modified Fabonacci search.

The substrate and corresponding velocity determinations are read into the programme as data pairs (line 140),

i.e., S1, V1, S2, V2 . . . . S_nV_n.

lines 10 to 19 or 1500 to 1999 are available for data input.

The programme was tested using simulated data. The velocity determinations were calculated at the following substrate concentrations, 1, 3, 5, 7, 9, 15, 21, 25, 31, 35, 39 and 45 by assigning the following values to the constants:-

V = 52, K = 7.9, n = 1.81.

The final values computed by the programme when supplied to the test data were:-

V = 52.00, K = 7.95, n = 1.81.

I acknowledge the assistance of Mr. A.J. Ross, applications programmer, Computer Centre, South Australian Institute of Technology in the translation of this programme.

```
ATKINS  04:08 PM          28-Oct-76
1!      BASIC-PLUS VERSION OF THE SUBJECT OF THE PAPER
        "A SIMPLE DIGITAL-COMPUTER PROGRAM FOR ESTIMATING THE
         PARAMETERS OF THE HILL EQUATION",   BY GORDON L. ATKINS,
        EUR. J. BIOCHEM. 33,175-180 (1973)
2!
        VARIABLES HAVE BEEN RENAMED AS FOLLOWS:

        ORIGINAL          NEW
        NMIN              N5
        NMAX              N9
        NH0               N0
        NH1               N1
        NH2               N2
        NH3               N3
        SSQ0              S0
        SSQ1              S1
        SSQ2              S2
        SSQ3              S3
        SSQ4              S4
3!      SV2               S9
        DA                D8
        NH                N4
        VM                V9
        SSQ               S8
4 REM TRANSLATED INTO BASIC-PLUS BY G.K. GOURLAY
5 REM SCHOOL OF PHARMACY
10 DATA 1,5,84,3,24,9,5,36,38,7,42,16,9,45,29,15,49,11
11 DATA 21,50,39,25,50,82,31,51,19,35,51,35,39,51,46
12 DATA 45,51,59
20 N% = 0%
30 PRINT : PRINT
60 PRINT "HILL EQUATION  :  USING MODIFIED FIBONACCI SEARCH"
70 PRINT : PRINT : PRINT
80 INPUT "NUMBER OF POINTS"; N%
90 !
100 DIM S(100),V(100)
140 FORI%=1%TO N%:READ S(I%),V(I%): NEXT I%
150 N5 = .5 : N9 = 5
160 N1 = N5 + .382*(N9 - N5) : N2 = N9 - .382*(N9 - N5)
170 N4 = N1
175 GOSUB 1100 : S1 = S8 : K1 = K : V1 = V9
176 N4 = N2
178 GOSUB 1100 : S2 = S8 : K2 = K : V2 = V9
180 IF S1<1E-30 THEN GO TO 650
190 IF S2<1E-30 THEN GO TO 650
200 K% = 0
210 K% = K% + 1% : P% = 0%
220 IF ABS((K2-K1)/(K2+K1))>1E-4 THEN P% = 1%
230 IF ABS((V2-V1)/(V2+V1))>1E-4 THEN P% = 1%
240 IF P%=0% THEN GO TO 360
250 IF S2>S1 THEN GO TO 290
```

```
ATKINS  04:09 PM          28-Oct-76
260 N5 = N1 : V1 = V2 : K1 = K2 : N1 = N2 : S1 = S2
270 N2 = N9 - .382*(N9 - N5)
280 N4 = N2
285 GOSUB 1100
286 K2 = K : S2 = S8 : V2 = V9
287 GO TO 340
290 N9 = N2 : V2 = V1 : K2 = K1 : N2 = N1 : S2 = S1
300 N1 = N5 + .382*(N9 - N5)
310 N4 = N1
315 GOSUB 1100
316 K1 = K : S1 = S8 : V1 = V9
320 IF S1<1E-30 THEN GO TO 650
330 IF S2<1E-30 THEN GO TO 650
340 IF K%<40% THEN GO TO 210 ELSE PRINT : PRINT : PRINT
350 PRINT "MAXIMUM NUMBER OF CYCLES" : GO TO 1340
360 IF S2>S1 THEN GO TO 390
370 N3 = N2 : S3 = S2 : N2 = N9
380 N4 = N2
385 GOSUB 1100
386 K2 = K : S2 = S8 : V2 = V9
390 N3 = N1 : S3 = S1 : N1 = N5
400 N4 = N1
405 GOSUB 1100
406 K1 = K : S1 = S8 : V1 = V9
410 IF S1<1E-30 THEN GO TO 650
420 IF S2<1E-30 THEN GO TO 650
430 B1 = (S3-S1)/(N3-N1)
440 B2 = ((S2-S1)/(N2-N1) - (S3-S1)/(N3-N1))/(N2-N3)
450 N0 = .5*(N1+N3-B1/B2)
460 IF N1<N0 AND N0<N2 THEN GO TO 480 ELSE PRINT : PRINT : PRINT
470 PRINT "RERUN WITH A NEW RANGE FOR N" : GO TO 1340
480 N4 = N0
485 GOSUB 1100
486 K0 = K : S0 = S8 : V0 = V9
490 IF S0<1E-30 THEN GO TO 650
500 PRINT : PRINT : PRINT : PRINT : PRINT
510 PRINT "FINAL VALUES"
520 PRINT : PRINT
530 PRINT "        V ="V0"              ";
540 PRINT "K = "K0"          ";
550 PRINT "N ="N0 : PRINT
560 PRINT "        SD OF RESIDUALS ="SQR(S0) : PRINT : PRINT
580 PRINT "VALUES FOR GRAPH" : PRINT
590 PRINT "     S                    V:OBSERVED          ";
600 PRINT "V:CALCULATED              RESIDUAL"
```

```
ATKINS  04:11 PM        28-Oct-76
610 FOR I% = 1% TO N%
612 PRINT USING"####.##"S(I%);
613 PRINT TAB(20);
614 PRINT USING "#######.###" V(I%);
620 B1 = EXP(NO*LOG(S(I%))) : B2 = (VO*B1)/(KO+B1)
630 PRINT USING "             #######.###" B2;
631 PRINT USING "             #######.###" V(I%)-B2
632 NEXT I%
640 GO TO 1340
650 PRINT "DETERMINANT IS TOO SMALL" : PRINT : PRINT
660 GO TO 1340
1100 !SUBROUTINE WILKINSON(V(1,,100),S(1,,100),NH,K,SSQ,VM,N%)
1120 DIM W(100)
1140 A,B,C,D,E = 0
1150 FOR I% = 1% TO N% : W(I%) = EXP(N4*LOG(S(I%)))
1160 X = V(I%)*V(I%) : Y = X/W(I%) : A = A + X*V(I%) : B = B + X*X
1170 C = C + Y*V(I%) : D = D + X*Y : E = E + Y*Y : NEXT I%
1180 D8 = A*E - C*D
1190 IF D8<1E-30 THEN GO TO 1330 ELSE
       K = (B*C - A*D)/D8 : V9 = (B*E - D*D)/D8
1220 A,B,C,D,E,S9 = 0
1230 FOR I% = 1% TO N%
1240 Z = W(I%)+K : X = V9*W(I%)/Z : Y = -X/Z
1250 A = A + X*X : B = B + Y*Y : C = C + X*Y
1260 D = D + X*V(I%) : E = E + Y*V(I%) : S9 = S9 + V(I%)*V(I%) :
       NEXT I%
1270 D8 = A*B - C*C
1280 IF D8 < 1E-30 THEN GO TO 1330 ELSE
       B1 = (B*D - C*E)/D8 : B2 = (A*E - C*D)/D8
1290 V9 = B1*V9 : K = K + B2/B1
1300 IF ABS(1-B1)<1E-6 AND B2<1E-6 THEN GO TO 1310 ELSE GO TO 1220
1310 S8 = (S9-B1*D-B2*E)/(N%-3) : RETURN
1330 S8 = 0 : RETURN
1335 RETURN
1340 PRINT
2000 END
```

ATKINS 04:03 PM 28-Oct-76

HILL EQUATION : USING MODIFIED FIBONACCI SEARCH

NUMBER OF POINTS? 12

FINAL VALUES

V = 52.0005 K = 7.9488 N = 1.81227

SD OF RESIDUALS = .017064

VALUES FOR GRAPH

S	V:OBSERVED	V:CALCULATED	RESIDUAL
1.00	5.840	5.811	0.029
3.00	24.900	24.934	-0.034
5.00	36.380	36.361	0.019
7.00	42.160	42.148	0.012
9.00	45.290	45.287	0.003
15.00	49.110	49.116	-0.006
21.00	50.390	50.392	-0.002
25.00	50.820	50.818	0.002
31.00	51.190	51.194	-0.004
35.00	51.350	51.351	-0.001
39.00	51.460	51.465	-0.005
45.00	51.590	51.587	0.003

Ready

APPENDIX 3.

SOURCE LISTING AND TEST DATA FOR NONCOM.

NONCOM is a BASIC-PLUS version of an iterative least-squares programme that fits data to the equation for slope-linear, intercept linear non-competitive inhibition. (Equation 7.1.).

The original programme was written by W.W. Cleland (University of Wisconsin, Madison, Wisconsin) and is available on request from the author.

The data input to NONCOM is similar to that of HYPER. All the replicate velocity determinations (at each substrate concentration, highest concentration to lowest), then, for each substrate concentration the following data is given:-

(a) number of velocity determinations at that substrate concentration,

(b) the actual substrate concentration,

(c) the inhibitor concentration,

(d) the weight factor to be applied to the velocity determinations.

The data at different inhibitor concentrations for the same drug substrate (i.e. either aminopyrine or ethylmorphine) was fitted simultaneously using this programme in Tables 7.1. and 7.2.

The test data was calculated by assigning the following values to the constants:-

K_m = 0.35, V = 100,000, K_{is} = 0.59, K_{ii} = 0.51

The velocity determinations were calculated at the following substrate concentrations, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2,5, 5 and 10 at two inhibitor concentrations, 0.01 and 0.05

NONCOM converged on the following values for the constants when given the test data:-

K_m = 0.36, V = 100,001, K_{is} = 0.591, K_{ii} = 0.51

```
LIST 1-450
NONCOM  02:43 PM          10-Jun-76
1 DATA 2657.5,6384.2,11988,21364,40257,57083,72165,85759,91506,94677.4
2 DATA 2490.7,5981,11225.6,19987.5,37593,53218,67179,79729,85023,87943
3 DATA 1,.01,.01,1,1,.025,.01,1,1,.05,.01,1,1,.1,.01,1,1,.25,.01,1
4 DATA 1,.5,.01,1,1,1,.01,1,1,2.5,.01,1,1,5,.01,1,1,10,.01,1
5 DATA 1,.01,.05,1,1,.025,.05,1,1,.05,.05,1,1,.1,.05,1,1,.25,.05,1
6 DATA 1,.5,.05,1,1,1,.05,1,1,2.5,.05,1,1,5,.05,1,1,10,.05,1
80!FITS DATA TO THE EQUATION FOR NON-COMPETITIVE INHIBITION
81! PROGRAM WRITTEN BY W.W.CLELAND, TRANSLATED INTO BASIC-PLUS
82! BY G.K.GOURLAY, SCHOOL OF PHARMACY
90 DIM V(200),A(200),B(200),W(200),S(5,6),Q(5),S9(5),Y8(5)
95 INPUT"NUMBER OF DATA SETS";G5
98 INPUT"NUMBER OF DATA POINTS";N9
100INPUT"NUMBER OF ITERATIONS REQUIRED";E
102 READ V(I) FOR I=1 TO N9
110 F2=1:F3=0
120 FOR F=1 TO G5
123 READ G1,G2,G3,G4:F3=F3+G1: GO SUB 150
140 NEXT F
145 GO TO 215
150 FOR I=F2 TO F3:A(I)=G2:B(I)=G3:W(I)=G4:NEXT I
200 F2=F2+G1
210 RETURN
215 PRINT V(I),A(I),B(I),W(I) FOR I=1 TO N9
220 M=1:N=4:P=N9-N:N1=N+1:N2=N+2
230 GO TO 1000
240 Q(1)=V(I)^2/A(I):Q(2)=V(I)^2*B(I)/A(I)
250 Q(3)=V(I)^2: Q(4)=V(I)^2*B(I): Q(5)=V(I)
270 GO TO 1080
275 C8=1/S(3,1):C9=S(1,1)/S(3,1):C7=S(1,1)/S(2,1)
280 C6=S(3,1)/S(4,1)
290 PRINT"THE PRELIMINARY ESTIMATES OF THE PARAMETERS ARE"
295 PRINT"K=";C9:PRINT"V MAX=";C8:PRINT"K IS=";C7
300 PRINT"K II=";C6:PRINT:PRINT:PRINT
310 PRINT"NUMBER OF          K              V MAX          K IS          K II"
315 PRINT"ITERATIONS":PRINT"PERFORMED"
320 N8=0:M=2
330 GO TO 1000
335 D=(1+B(I)/C7)*C9/A(I)+1+B(I)/C6:Q(1)=1/D
340 Q(2)=(1+B(I)/C7)/A(I)/D^2
341 Q(3)=B(I)/A(I)/D^2
345 Q(4)=B(I)/D^2:Q(5)=V(I): GO TO 1080
390 C8=S(1,1):C9=C9-S(2,1)/S(1,1):C7=C7*(1+S(3,1)*C7/S(1,1)/C9)
400 C6=C6*(1+S(4,1)*C6/S(1,1))
410N8=N8+1
420 PRINT N8,C9,C8,C7,C6
430 IF(N8-E)<0 GO TO 1000
440 S2=0:R9=0:R8=0
450 FOR I=1 TO N9
```

```
450 FOR I=1 TO N9
452 P3=(V(I)-C8/((1+B(I)/C7)*C9/A(I)+1+B(I)/C6))^2
455 S2=S2+P3*W(I):R8=R8+P3:R9=R9+V(I)^2: NEXT I
460 R2=(R9-R8)/R9:S2=S2/P:S1=SQR(S2)
465 S(K,J)=S(K,J)*S9(K)*S9(J-1) FOR K=1 TON FOR J=2 TO N1
468 S8=S1*SQR(S(1,2)): S7= S1*SQR(S(2,3))/S(1,1)
470 S3=S1*C7^2*SQR(S(3,4))/S(1,1)/C9
480 S5=S1*C6^2*SQR(S(4,5))/S(1,1)
490 Z8=1/S8^2:Z9=1/S7^2:Z3=1/S3^2:Z5=1/S5^2
500 PRINT:PRINT:PRINT
510 PRINT "K=";C9
515 PRINT" S.E. OF K=";S7
520 PRINT"W.F. FOR K=";Z9:PRINT:PRINT:PRINT
530 PRINT"V MAX=";C8
540 PRINT "S.E. OF V MAX=";S8
550 PRINT "W.F. FOR V MAX=";Z8:PRINT:PRINT:PRINT
560 PRINT"K IS=";C7
570 PRINT " S.R. OF K IS=";S3
580 PRINT " W.F. OF K IS=";Z3:PRINT:PRINT:PRINT
590 PRINT" K II=";C6
600 PRINT "S.E. OF K II=";S5
610 PRINT " W.F. OF K II=";Z5
620 PRINT:PRINT:PRINT
630 PRINT "R2="; R2: PRINT "R1="; SQR(R2)
999 GO TO 2000
1000 FOR J = 1 TO N2
1010 FOR K = 1 TO N1
1020 S(K,J)=0
1030 NEXT K
1040 NEXT J
1050 FOR I = 1 TO N9
1060 ON M GO TO 240,335
1080 FOR J = 1 TO N1
1090 FOR K = 1 TO N
1100 S(K,J)= S(K,J)+Q(K)*Q(J)*W(I)
1110 NEXT K
1120 NEXT J
1130 NEXT I
1140 FOR K = 1 TO N
1150 S9(K)=1/SQR(S(K,K))
1160 NEXT K
1170 S9(N1)=1
1180 FOR J = 1 TO N1
1190 FOR K = 1 TO N
1200 S(K,J)=S(K,J)*S9(K)*S9(J)
1210 NEXT K
1220 NEXT J
1230 Y8(N1)=-1
1240 S(1,N2)=1
1250 FOR L = 1 TO N
1260 FOR K = 1 TO N
1270 Y8(K)= S(K,1)
1280 NEXT K
1290 FOR J = 1 TO N1
1300 FOR K = 1 TO N
```

```
LIST 1310-2000
NONCOM  02:49 PM          10-Jun-76
1310 S(K,J)=S(K+1,J+1)-Y8(K+1)*S(1,J+1)/Y8(1)
1320 NEXT K
1330 NEXT J
1340 NEXT L
1350 FOR K = 1 TO N
1360 S(K,1)= S(K,1)*S9(K)
1370 NEXT K
1380 ON M GO TO 275,390
1460 PRINT J9
2000 END

Ready
```

```
RUN
NONCOM  02:29 PM          10-Jun-76
NUMBER OF DATA SETS? 20
NUMBER OF DATA POINTS? 20
NUMBER OF ITERATIONS REQUIRED? 10
 2657.1          .01             .01             1
 6383.4          .025            .01             1
 11986.7         .05             .01             1
 21362.6         .1              .01             1
 40254.7         .25             .01             1
 57081.3         .5              .01             1
 72163.8         1               .01             1
 85759.7         2.5             .01             1
 91505.7         5               .01             1
 94677           10              .01             1
 2490.7          .01             .05             1
 5981            .025            .05             1
 11225           .05             .05             1
 19987.5         .1              .05             1
 37593           .25             .05             1
 53218           .5              .05             1
 67179           1               .05             1
 79729           2.5             .05             1
 85025           5               .05             1
 87943           10              .05             1
 THE PRELIMINARY ESTIMATES OF THE PARAMETERS ARE
K= .360053
V MAX= 100001
K IS= .590957
K II= .509949


NUMBER OF          K              V MAX          K IS           K II
ITERATIONS
PERFORMED
 1                 .360053        100001         .590957        .509949
 2                 .360053        100001         .590957        .509949
 3                 .360053        100001         .590957        .509949
 4                 .360053        100001         .590957        .509949
 5                 .360053        100001         .590957        .509949
 6                 .360053        100001         .590957        .509949
 7                 .360053        100001         .590957        .509949
 8                 .360053        100001         .590957        .509949
 9                 .360053        100001         .590957        .509949
 10                .360053        100001         .590957        .509949


K= .360053
 S.E. OF K= .718264E-5
W.F. FOR K= .193835E 11

•

V MAX= 100001
S.E. OF V MAX= .486076
W.F. FOR V MAX= 4.23244
```

```
K IS= .590957
 S.R. OF K IS= .183454E-3
 W.F. OF K IS= .297131E 8


 K II= .509949
S.E. OF K II= .396874E-4
 W.F. OF K II= .634884E 9


R2= 1
R1= 1

Ready
```

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