FACTORS AFFECTING ANTIPYRINE METABOLISM

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by

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C O N T E N T S

D E C L A R A T I O N

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 contains no material previously published by any other person, except where due reference is made in the text. Material from this thesis has formed part of the following publications:

Harman, A.W., Penhall, R.K., Priestly, B.G., Frewin, D.B., Phillips, P.J. and Clarkson, A.R.: Aust. N.Z. J. Med. 7, 385-390 (1977).

Harman, A.W., Russell, W.J., Frewin, D.B., and Priestly, B.G.: Anaesth. Intens. Care. 6, 210-214 (1978).

Harman, A.W., Frewin, D.B., Priestly, B.G., and Alexander, C.B.J.: Br. J. Clin. Pharmac. 7, 45-48 (1979).

Results of this thesis have also been presented to meetings of the Australasian Society of Clinical and Experimental Pharmacologists in Adelaide (November, 1976), Sydney (November, 1977) and Sydney (August, 1979).

ANDREW HARMAN

A C K N O W L E D G E M E N T S

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ABSTRACT OF THESIS

The aims of this thesis were to assess the value of salivary antipyrine elimination kinetics in the study of factors that influence the activity of hepatic mixed function oxidase enzymes.

Antipyrine metabolism in patients with chronic liver disease was found to be impaired when compared with a control population of unmedicated volunteers. Chronic renal dysfunction did not appear to alter antipyrine elimination.

The half-life of antipyrine was significantly reduced in a group of epileptics on long-term phenytoin therapy. Used as a measure of enzyme induction, half-life was unable to discriminate those patients at risk from osteomalacia or to detect any additional enzyme inducing effect on the addition of a barbiturate to the therapeutic regimen. Antipyrine half-life did not correlate with either phenytoin dose or plasma level.

Patients receiving chlorpromazine therapy for psychotic illness metabolized antipyrine faster than control subjects; however, in patients receiving fluphenazine decanoate there was no change. The difference may be related to plasma levels attained by each agent after dosing, fluphenazine being given intramuscularly and achieving only very low plasma concentrations.

The effects of chronic ethanol intake on the elimination kinetics were determined in a group of male alcoholics. Antipyrine metabolism was impaired in this group when compared to controls.

These results demonstrated that a significant proportion of the alcoholics studied had impaired hepatic drug metabolizing capacity and that the activity of the hepatic microsomal enzymes may be related to the extent of ethanol induced liver damage in these subjects.

Antipyrine elimination kinetics were measured in anaesthetists during a period when they were giving general anaesthetics and a period when they were working in intensive care where they were not using volatile anaesthetic agents. During the anaesthetic work period there was a reduction in antipyrine half-life and the clearance of antipyrine was increased. Analysis of the data was performed using each anaesthetist as his/her own control. When the data was analysed on a group basis, no change in elimination kinetics was detected because of the wide variation in metabolism between subjects. Exposure to anaesthetic agents under operating theatre conditions appeared to enhance hepatic metabolism.

Many chemicals in the environment are known to alter the disposition of drugs. The effect of petrol on the activity of mixed function oxidase activity was investigated in man and the rat. Antipyrine half-lives in a group of male petrol station workers were shorter than in controls. The rates of oxidative metabolism of antipyrine, aminopyrine, ethylmorphine, aniline and benzo(a)pyrene were all increased by more than 45% in 10,000 x g hepatic microsomal supernatant preparations from rats exposed to petrol vapour over a period of three weeks. These results indicated that petrol vapour was a moderately potent inducer of microsomal mixed function oxidase

activity in rats, and that occupational exposure to petroleum may result in enhanced microsomal drug metabolism.

Isolated rat hepatocytes offer a useful *in vitro* model for the study of drug metabolism. They retain activity of Phase I and II reactions without the necessity for added cofactors and hence are more indicative of the metabolic activity of intact tissue than are microsomal preparations. Metabolic drug interactions between antipyrine and inhibitor concentrations of SKF525-A, phenobarbitone and chlormethiazole were investigated in rat hepatocytes isolated by a collagenase perfusion technique. Chlormethiazole and SKF525-A competitively inhibited antipyrine metabolism. Phenobarbitone produced a mixed-type inhibition. Chlormethiazole and phenobarbitone were found to be weak inhibitors and acute inhibitory drug interactions between these agents and antipyrine are unlikely to be observed at normal therapeutic blood concentrations. Pretreatment of rats with phenobarbitone prior to hepatocyte isolation resulted in induction of antipyrine metabolism. The results showed that classical microsomal induction and inhibition can be demonstrated in isolated hepatocytes.

The overall results of this thesis indicate that (i) the salivary elimination kinetics of antipyrine in man is a sensitive qualitative test for assessing those factors that can alter the activity of the hepatic mixed function oxidase enzymes; (ii) isolated hepatocyte preparations may be a useful *in vitro* model in which to study potential drug interactions.

GENERAL INTRODUCTION

The oxidative metabolism of a wide variety of lipid soluble compounds is mediated by an enzyme system located in the smooth endoplasmic reticulum, predominantly in liver cells. This enzyme system is often referred to as the mixed function oxidase enzymes. It has the capacity to oxidize both endogenous compounds, such as steroid hormones, lipids and bile acids, as well as exogenous compounds such as drugs, environmental pollutants and dietary constituents which enter the body. Since many of these compounds are highly lipid soluble, they would be very slowly excreted if not converted by oxidation to more polar water soluble compounds.

The enzyme system referred to in the foregoing, consists of a haemoprotein called cytochrome P450, NADPH-cytochrome c reductase and a lipid (phosphatidylcholine) embedded in the membrane of the smooth endoplasmic reticulum. It is essentially a multi-component electron transport system catalysing oxidation reactions with cytochrome P450 acting as the terminal oxygenase. A compound binds to cytochrome P450 and in the presence of molecular oxygen and NADPH is oxidized by the incorporation of one oxygen atom. The rate limiting step appears to be the transfer of electrons to the cytochrome P450 moiety, however the precise nature of this step has not been elucidated (Bjorkhem, 1977).

Some compounds, if presented to this enzyme system in sufficiently high concentrations for a sufficient length of time, cause an increase in enzyme activity. This increase has been termed "induction". Induction can be promoted by a variety of

chemicals including barbiturates, anaesthetic gases, CNS stimulants, antiinflammatory agents, steroids and environmental pollutants. Induction is usually associated with an increase in liver weight, proliferation of the smooth endoplasmic reticulum and an increase in cytochrome P450 and NADPH cytochrome c reductase concentrations. It is believed that these inducers can be divided into two classes: the barbiturate type which are characterized by producing an increase in cytochrome P450 content and the polycyclic hydrocarbon type which induce the activity of a much smaller number of enzymic reactions catalysed by a modified cytochrome P450, called cytochrome P448.

Other compounds have the ability to inhibit the activity of the mixed function oxidase enzymes. Among these are metyrapone and α-naphthoflavone which have differential specificity for cytochrome P450 and cytochrome P448 respectively (Kahl et al., 1978), SKF525-A (Anders and Mannering, 1966) thio-sulphur containing compounds such as disulfiram (Freundt, 1978) and lead (Meredith et al., 1977). Hence the activity of the mixed function oxidase enzyme system can be increased or decreased by various chemicals.

A knowledge of the distribution of compounds absorbed by the body is important both from a clinical and toxicological viewpoint. It is useful to know how the concentration of an agent will change with time so as to make predictions as to its pharmacological or toxicological effect. Since the mixed function oxidase enzymes play an important role in determining the rate of elimination of a compound, knowledge of their activity is of value as an aid to making these predictions. However, the activity of the mixed function

oxidase enzymes is not the only factor that determines the elimination rate of a compound. The rate of liver blood flow, binding to proteins, excretion of the compound in the unchanged form and metabolism by other enzyme systems may also play a role. Nevertheless, changes in mixed function oxidase activity can have profound effects on the pharmacological action of a compound. An example of this is the potentially lethal interaction of oral anticoagulants and hypnotics (McDonald et al., 1968). Many hypnotic drugs produce marked induction of mixed function oxidase activity. If a patient is being treated with a combination of anticoagulant and hypnotic and the hypnotic therapy is discontinued, a rise in plasma anticoagulant concentration may result due to the withdrawal of the enzyme inducing effect of the hypnotic. If the dose of the anticoagulant is not adjusted, fatal haemorrhage may result.

In animal studies, the activity of these enzymes is commonly measured using hepatic microsomal preparations. Subcellular fractions of the liver are prepared by centrifugation or sedimentation techniques, and the microsomes, a membrane fraction which contain the mixed function oxidase enzymes, can be incubated with various substrates to obtain an in vitro measure of their activity. A method that is now becoming popular is the use of isolated hepatocytes. The latter are prepared by enzymic digestion of the extracellular matrix of the liver by a perfusion technique. The hepatocytes retain many cellular functions that are lost in microsomal preparations, such as NADPH generating system and type II metabolism (conjugation reactions). Another experimental technique for the study of metabolic activity is the use of the isolated perfused liver. In this system the

metabolic organization of the liver as a whole is retained which allows insight into the interrelationships between pathways in the cell and the organ. Since it is perfused, processes such as uptake of substrate and release of product into the perfusate can be measured.

While the above systems are useful for studying drug metabolism in animals, they are obviously of little use when one wants to determine the activity of drug metabolizing enzymes in man. The availability of liver biopsy material in man is limited and biopsy sampling is rarely justified as a test for enzyme activity. Methods have been developed that use in vivo tests of liver function as indices of enzyme activity. The rationale is to measure products of the enzyme step(s) as an index of the activity of the enzyme system.

1. Urinary D-glucaric acid (DGA) excretion

DGA is a product of the glucuronic acid pathway in man, which, among other things, is involved in type II metabolism (glucuronide formation). Aarts (1965) reported increased excretion of DGA in the urine of subjects treated with phenobarbitone and Hunter et al. (1971) found increased DGA excretion in a population of epileptics on a variety of anticonvulsant drugs. These workers suggested that the excretion of DGA could be used as an index of enzyme induction in man. It has also been suggested that reduced DGA excretion can be used as a test of inhibition of enzyme activity. Decreased urinary excretion of DGA has been found in patients with

congestive heart failure (Tokola et al., 1975), starvation (Sotaniemi et al., 1969) and in severly burned patients (Ciaccio & Fruncillo, 1979) conditions known to decrease the rate of drug elimination in man. Hence there seems to be a correlation between drug metabolizing enzyme activity and DGA excretion.

However, recent evidence suggests that DGA excretion may not always reflect mixed function oxidase activity. Sorrell et al. (1976) showed that increased excretion of DGA occurs in the acute phase of viral hepatitis while the clearance of antipyrine, a drug metabolized by the mixed function oxidase enzymes, was depressed. Carrella et al. (1978) had similar findings and they suggested that this effect could be due to leakage of DGA from damaged liver cells or possibly increased input of DGA precursors due to the presence of jaundice. The excretion of DGA has been found to be increased by oral contraceptives (Mowat, 1968) whereas many other workers have found that these agents decrease the rate of drug metabolism (Carter et al., 1975; O'Malley et al., 1972; Homeida et al., 1978). Thus, the excretion of DGA may not always reflect mixed function oxidase activity. The glucuronic acid pathway is complex. Some of the enzymes involved are found in the cytosol while others are membrane bound and found in the microsomes. Furthermore, the rate limiting step in the production of D-glucaric acid has yet to be determined (Hunter et al., 1973). Hence, while DGA excretion seems to reflect hepatic microsomal enzyme activity in some situations, further evaluation of the relationship between the enzymes that metabolize drugs and the enzymes of the glucuronic acid pathway is required before the utility of DGA excretion as a test of mixed function

oxidase enzyme activity can be fully appreciated.

2. Urinary 6-beta-hydroxycortisol excretion

Cortisol is metabolized by two main pathways: oxidation to 6-beta-hydroxycortisol and reduction, resulting in A-ring derivatives, including 17-hydroxycorticosteroids. Induction of mixed function oxidase enzymes should theoretically increase the ratio of 6-beta-hydroxycorticosteroids/17-hydroxycorticosteroids excreted in the urine. This method has been used to demonstrate enzyme induction in patients receiving anticonvulsants (Conney et al. 1965) and in workers exposed to the pesticide Endrin (Jaeger, 1970).

It has recently been shown that the ratio increases in liver disease (Eade et al., 1977), yet this is a condition where it is well recognised that drug metabolizing enzyme activity is decreased (Branch et al., 1973; Andreasen et al., 1974). One assumption in using this method is that the ability of the cell to reduce the A-ring of cortisol is not impaired. Structural damage to the cell may affect both oxidative and reductive pathways and this assumption may not always hold. Another limitation to this method is that the assay for urinary corticosteroids is time consuming. It seems that further evaluation of this technique is required before it can be used as a general index of mixed function oxidases.

3. Gamma-glutamyl transpeptidase (GGTP) activity, (EC 2.3.2.2.)

Increased activity of the mixed function oxidase system has been associated with increased GGTP activity in plasma. This activity is increased on chronic ethanol ingestion in man (Teschke,

1977) and on administration of phenobarbitone (Rosalki et al., 1971; Hildebrandt et al., 1975). In epileptics there is a good positive correlation between DGA excretion and GGPT activity (Davidson et al., 1974). However its use as a general index of the activity of microsomal drug metabolizing enzymes may be more limited. Extra-hepatic cholestasis causes an increase in GGPT activity in plasma (Kryszewski, 1973; Adjarov et al., 1976), whereas other evidence suggests that drug metabolism is normal in this condition (Elfstrom and Lindgren, 1974). In pregnancy, GGPT activity in plasma was found to be normal, whereas DGA excretion was markedly increased suggesting enzyme induction (Herzberg et al., 1977). Smith and Rawlins (1974) noted a lack of correlation between GGPT and the excretion of DGA and 6-beta-hydroxycortisol in the urine of healthy subjects. Hildebrandt et al. (1975) also failed to find a correlation between GGPT activity and DGA excretion.

Another shortcoming of using GGPT activity as a test of microsomal enzyme activity is that although it can be used to test enzyme induction it does not appear to be of use in testing inhibition of enzyme activity. Very little is known of the mechanisms governing the release of GGPT into the plasma. It has been suggested that this enzyme is located in the endoplasmic reticulum (Pastel and O'Gorman, 1975; Teschke, 1977); however, recent evidence suggests that it is predominantly concentrated in the plasma membrane (Ratanasavanh et al., 1979). Hence there seems to be a tenuous relationship between the release of GGTP from the liver cell and the activity of mixed function oxidase enzymes.

4. Cultured Lymphocytes

Kellermann et al. (1976) found a strong correlation between antipyrine half-life, a drug extensively metabolized by the mixed function oxidase enzymes, and the percent induction of aryl hydrocarbon hydroxylase by 3-methylcholanthrene in mitogen stimulated lymphocytes. They suggested that induction of aryl hydrocarbon hydroxylase activity in cultured human lymphocytes by 3-methylcholanthrene may be a useful index of the genetic contribution to rates of drug metabolism. However it appears that the extent of inducibility varies with the length of culture and is dependant on the blastogenic state of the cells at the time of harvest. It has been suggested that there is a need to standardize time of culture and extent of blast transformation if this test is to be useful (Hart et al., 1977).

Since this test only measures the genetic component of drug metabolism it is of no use in studying the effects of environmental factors, drug therapy or disease states on drug metabolizing ability.

5. Liver Biopsy

Mixed function oxidase activity in animals is commonly measured in hepatic microsomal preparations. This technique has been applied to samples of human liver obtained by biopsy sampling or post-mortem specimens (Alvares et al., 1969; Schoene et al., 1972; Sotaniemi et al., 1977). This method has the capability of providing a direct measure of various enzyme activities and cytochrome P450 content and allows multiple comparisons to be

made within the same piece of tissue (Schoene et al., 1972). However, predicting *in vivo* metabolism from *in vitro* studies is often hampered by the difficulty in optimizing cofactor and substrate concentrations *in vitro*. Also, *in vitro* studies are unable to predict the influence transport processes, hormones and NADPH supply have on drug metabolizing ability in the *in vivo* situation. Naturally, the major drawback with this technique is that liver biopsy sampling is seldom warranted ethically unless liver damage is suspected. Thus this method is of little use in obtaining an index of enzyme activity in the general population.

6. Metabolic Probes

By far the most commonly used methods for examining the activity of hepatic microsomal drug metabolizing enzymes are the use of probe drugs. The basis of these methods is that the elimination rate of a drug is used as an index of the activity of the enzymes that metabolize it. To be useful, a probe drug should have the following properties:

- be safe to use with no toxic side effects;
- be able to be administered orally with complete bioavailability as this simplifies dosing procedures;
- have low clearance rate so that elimination is not influenced by changes in hepatic blood flow;
- be extensively metabolized by hepatic mixed function oxidase enzymes.

Amobarbitone (Inaba et al., 1976), phenylbutazone (Poland et al., 1970; Smith and Rawlins, 1974), warfarin (Orme et al., 1972), phenacetin (Baty and Robinson, 1977; Pantuck et al., 1979) pentobarbitone (Reidenberg et al., 1976; Benowitz and Jones, 1977) and theophylline (Kappas et al., 1976) fulfil most of the above criteria and have been used as probe drugs to study the effects of a variety of factors on microsomal drug metabolizing enzymes. Their rate of elimination is estimated from the decline in concentration of the parent compound in plasma with time.

A recent development in the use of the so called aminopyrine breath test (ABT) where the percentage of radioactivity administered as (^{14}C-N-methyl)-aminopyrine appearing as $^{14}CO_2$ in exhaled breath at time intervals after dosing is used as an index of aminopyrine concentration. Carbon dioxide is a product of N-demethylation of aminopyrine. The ABT has been found to correlate well with the clearance rate of aminopyrine (Hepner and Vesell, 1974; Bircher et al., 1976) and this test has been used to examine the changes induced in drug metabolism due to hepatocellular disease, cholestasis (Hepner and Vesell, 1977) and cirrhosis in man (Bircher et al., 1976). However, the major problem with the ABT would be to persuade subjects to ingest radioactive drug. As so much coverage is given in the media to the potential dangers of radioactivity there is a stigma attached to ingesting this form of compound even though the levels used (about 2 uCi/subject) may be innocuous on a theoretical basis.

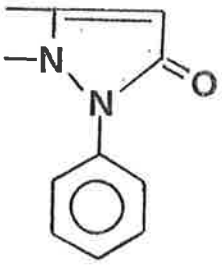
The most commonly used probe drug is antipyrine (phenazone).

This drug has properties that not only satisfy the criteria given above for a compound to be a useful metabolic probe, but it also has certain other advantages as well.

Work by Brodie and Axelrod (1950) demonstrated that antipyrine was completely absorbed when given as an oral dose, had negligible protein binding and was extensively metabolized in the body with very little appearing in urine as the parent compound. There are several metabolites of antipyrine in man (Fig. 1). The two main metabolites are 4-hydroxyantipyrine and 3-hydroxymethylantipyrine which each account for about 30% of the dose. A minor metabolite is N-demethylantipyrine which accounts for about 9% (Kellermann and Luyten-Kellermann, 1978). There are also trace amounts of 3-carboxyantipyrine (Yoshimura et al., 1971) and a dihydrodiol (Stafford et al., 1974).

Antipyrine has both high lipid and water solubility. It distributes freely throughout all body compartments and has been used to measure total body water. Soberman et al. (1949) found that antipyrine had similar distribution in the body to tritiated water. Its protein binding has been estimated to be less than 10% (Brodie and Axelrod, 1950). Antipyrine elimination has been shown to follow first-order kinetics at doses of the order of 10-20 mg/kg bodyweight described by a one-compartment model (Greisen & Andreasen, 1976). It has no significant first-pass effect through the liver (Gugler et al., 1975) and plasma levels after oral and intravenous administration are very similar (Andreasen and Vesell, 1974). Since it has a low extraction ratio

Figure 1. Structures of antipyrine (I) and its metabolites: 3- hydroxymethylantipyrine (II), 4-hydroxyantipyrine (III), N-demethylantipyrine (IV), 3-carboxy-antipyrine (V), and the dihydrodiol (VI).



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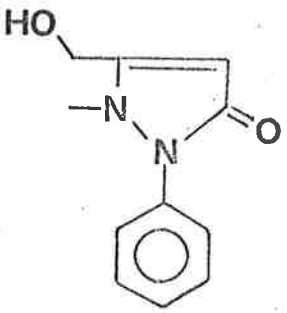

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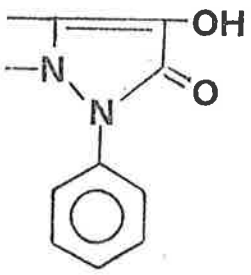

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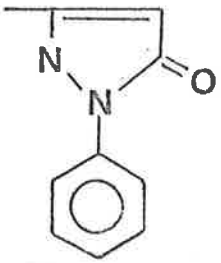

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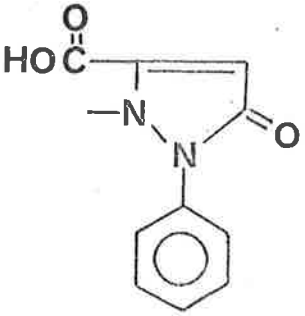

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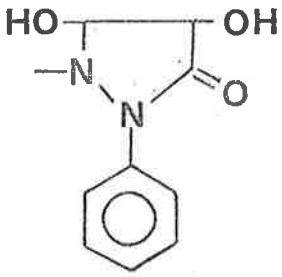


VI

through the liver, its elimination is not greatly affected by changes in hepatic blood flow (Branch et al., 1974). Thus the metabolic clearance rate of antipyrine will not be significantly influenced by factors that are likely to alter distribution or hepatic blood flow. Consequently the rate of disappearance of antipyrine from the plasma reflects its intrinsic hepatic clearance rate which is a measure of the activity of the enzymes that metabolize antipyrine. It is on this basis that antipyrine has been used in the past as a measure of the activity of the mixed function oxidase enzymes.

The validity of using antipyrine elimination as a test of its rate of metabolism has been verified by Huffman et al. (1974). These workers showed a correlation between the appearance of 4-hydroxyantipyrine in urine and the disappearance of antipyrine in plasma. This result was verified by Kellermann and Luyten-Kellermann (1978) who also found good correlations between antipyrine half-life and the urinary excretion half-lives of N-demethylantipyrine and 3-carboxymethylantipyrine in man.

One property of antipyrine, viz. its even distribution throughout total body water, can be exploited for measuring antipyrine levels in body fluids. It has recently been shown by several groups that antipyrine levels in saliva are very similar to levels in plasma. Since antipyrine is not appreciably bound to proteins and has even distribution, on a theoretical basis, salivary concentrations should reflect the unbound concentration in plasma. Reported mean ($\pm$ SD) saliva/plasma concentration ratios include 1.00 ± 0.05 (Welch et al., 1975), 0.92 ± 0.04 (Vesell et al., 1975),

0.89 ± 0.14 (Van Boxtel et al., 1976), 0.92 ± 0.02 (Fraser et al., 1976) and 1.02 ± 0.09 (Harman et al., 1977).

The utility of using saliva sampling is that it enables the study of a wider range of subjects. The restrictions associated with an invasive technique such as repeated venipuncture sampling are not attendant and the subject can carry out the test with minimal interruption to his/her daily routine.

The aim of this thesis was to assess the usefulness of antipyrine metabolism as an index of hepatic mixed function oxidase activity in man. The ease of salivary sampling of antipyrine concentration in the body was exploited in determining the effects of various disease states, drug treatments and environmental chemicals on antipyrine elimination kinetics.

C H A P T E R O N E

MEASUREMENT OF ANTIPYRINE ELIMINATION IN A CONTROL POPULATION

INTRODUCTION

Antipyrine has been commonly measured using the spectrophotometric method of Brodie et al. (1949). This method, which involves the conversion of antipyrine to 4-nitrosoantipyrine with sodium nitrite, has been criticized on several grounds, both in the technique and because of interference by other compounds. Variations have been noted in the time required to reach maximum colour formation after the addition of nitrite (Kraybill et al., 1953; Ikkos et al., 1954) and this is thought to be due to temperature differences and nitrite concentration (Davidson and MacIntyre, 1956; Zak, 1957). Edwards (1959) found high blank values in anuric patients and thought that this was probably due to drugs that had accumulated in the body. Hence the spectrophotometric technique would seem unsatisfactory when studying groups that are taking medication which may influence the assay. In the present studies therefore, a gas chromatographic method was used to determine antipyrine levels and this proved to be specific and sensitive. Since antipyrine levels in saliva have been shown to be similar to those in plasma (Harman, Priestly & Frewin, 1977) the decline in antipyrine concentration with time in the body could be monitored by measuring the amount of this drug in saliva samples.

METHOD

1. Subjects

Twenty-five healthy subjects, nineteen male and six female, volunteered to act as controls in these experiments. None of these were taking any medication at the time of the study. The ages of the males ranged from 21 to 69, mean (±SD) 29 ± 12. The females were either 20 or 21 years old.

2. Protocol

After an overnight fast, each subject received antipyrine in a dose of 10 mg/kg body weight taken orally in water. Saliva samples (4 ml) were obtained prior to and approximately 3, 6, 9, 12, 24 and 36 hours after the drug was administered, the exact sampling time being recorded and used in subsequent calculations. These were centrifuged to remove mucous and particulate matter from the supernatant and stored at -15^{o}C until analysed.

3. Antipyrine Assay

Antipyrine levels were measured in duplicate saliva samples using a gas chromatographic technique. To 1 ml saliva in a 15 ml glass extraction tube containing 10ug phenacetin (internal standard) was added 0.2 ml 5 M NaOH followed by 5 ml chloroform. The tube was stoppered and shaken for 15 minutes on a mechanical shaking device. After centrifugation the upper aqueous phase was aspirated and the chloroform evaporated by heating at 80^{o}C in a Dri-Block heater. The residue was dissolved in 25ul acetone and 1 - 3ul injected into the gas chromotograph.

A column packing of 3% Carbowax 20M coated on Varoport 30 support was prepared by the rotary evaporator procedure described by McNair and Borelli (1968) and packed into a coiled glass column, 1.7 m x 3 mm (id). The injector and the flame ionization detector of the gas chromatographic equipment (Becker, model 409) were maintained at 250°C. The temperature of the column was adjusted between 190° and 210°C to give an antipyrine retention time of approximately 5 min. Carrier gas was oxygen-free, high purity nitrogen delivered at a rate of 50 ml/min.

4. Calculations

The rate of antipyrine elimination was determined from the slope of the least-squares, linear regression line of semilogarithmic transformations of the saliva concentrations versus time. Analysis was performed on a PDP-11/40 digital computer. From the slope of this line and its intercept with the y-axis (apparent initial concentration, C_o) the following were derived:

$$k_e = \text{slope} / 2.303$$

$$V_d = \text{dose}^* / C_o$$

$$T_{1/2} = 0.693 / k_e$$

$$MCR = V_d \cdot k_e$$

where k_e = elimination rate constant

V_d = apparent volume of distribution

$T_{1/2}$ = half-life

MCR = metabolic clearance rate

*the dose assumes 100% bioavailability and compliance (see later discussion). All means in this and subsequent chapters are expressed with their standard deviation.

RESULTS

1. Gas Chromatographic Method

Using the conditions described, no interfering peaks were found in blank saliva extracts. Both phenacetin and antipyrine produced sharp, symmetrical peaks at retention times of 3 and 5 min respectively (Figure 1.1). A linear relationship, passing through the origin, was found when peak height ratios of antipyrine to phenacetin were plotted against antipyrine concentration in the range 1 to 20 ug/ml (Figure 1.2). The coefficient of variations of the method ranged from 3.1 to 6.6% when replicate samples were analysed at various antipyrine concentrations added to blank saliva (Table 1.1). The limit of sensitivity of the assay was approximately 0.3 ug/ml.

2. Antipyrine Elimination Kinetics

Antipyrine half-lives in the 19 male volunteers ranged from 7.1 to 16.5 hr and the corresponding values in the six females were 6.2 to 12.9 hr. The individual values for half-life, clearance and apparent volume of distribution are shown in Table 1.2 and comparisons between males and females in these parameters are made in Table 1.3. The values for half-life were less and the values for MCR and V_d greater in the females than in the males ($P < 0.025$, U-test, one-tail). There was a significant rank correlation between half-life and clearance in the 25 subjects ($r_s = -0.75$, $P < 0.01$).

Figure 1.1 Chromatograms of extracts of blank saliva (A) and saliva from a subject who received a dose of antipyrine (B).

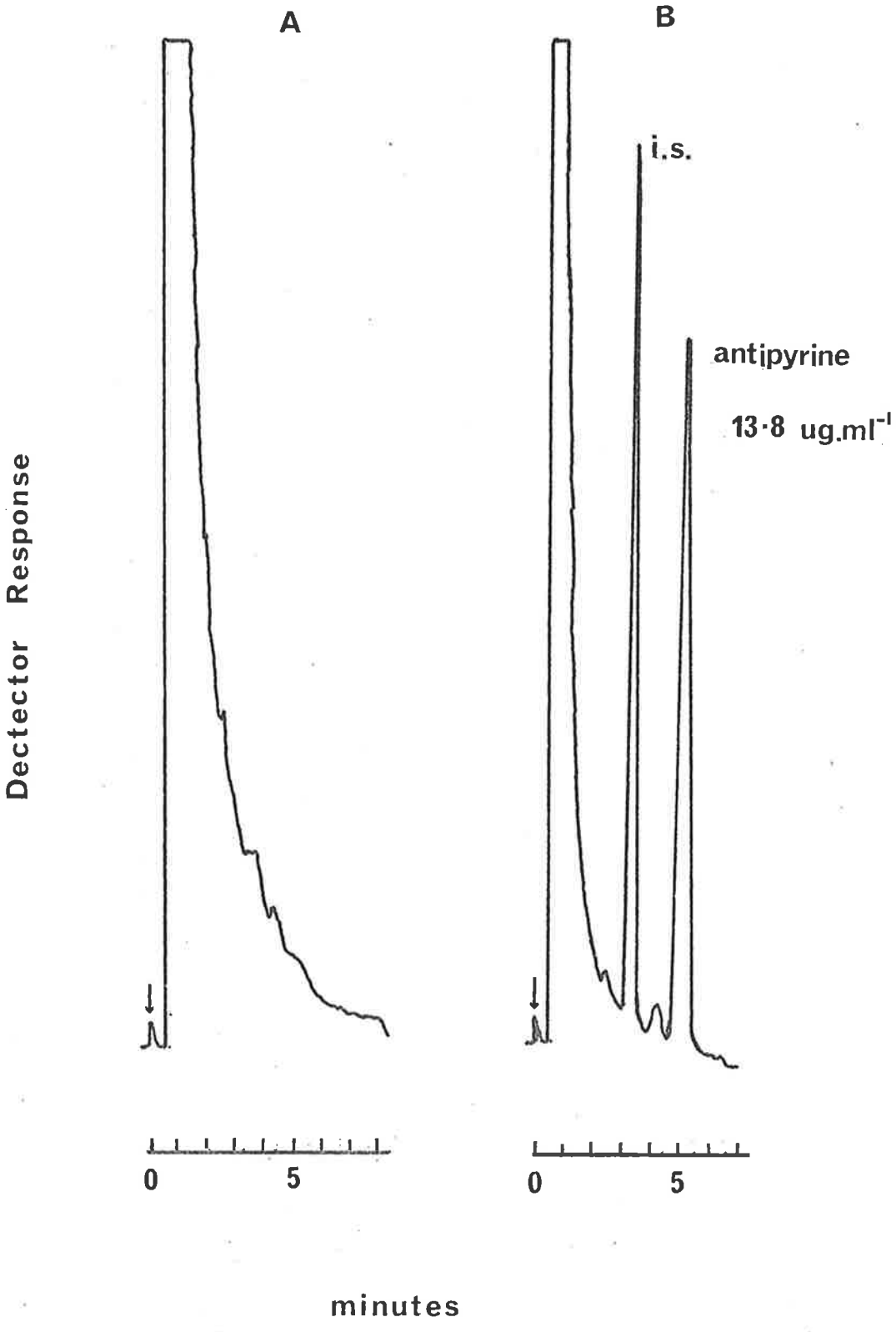

A
B
i.s.
antipyrine
13·8 ug.ml⁻¹
Dectector Response
0
5
0
5
minutes

Figure 1.2 Standard curve of peak height ratio of antipyrine to phenacetin vs. antipyrine concentration in the range 0 - 20 ug/ml.

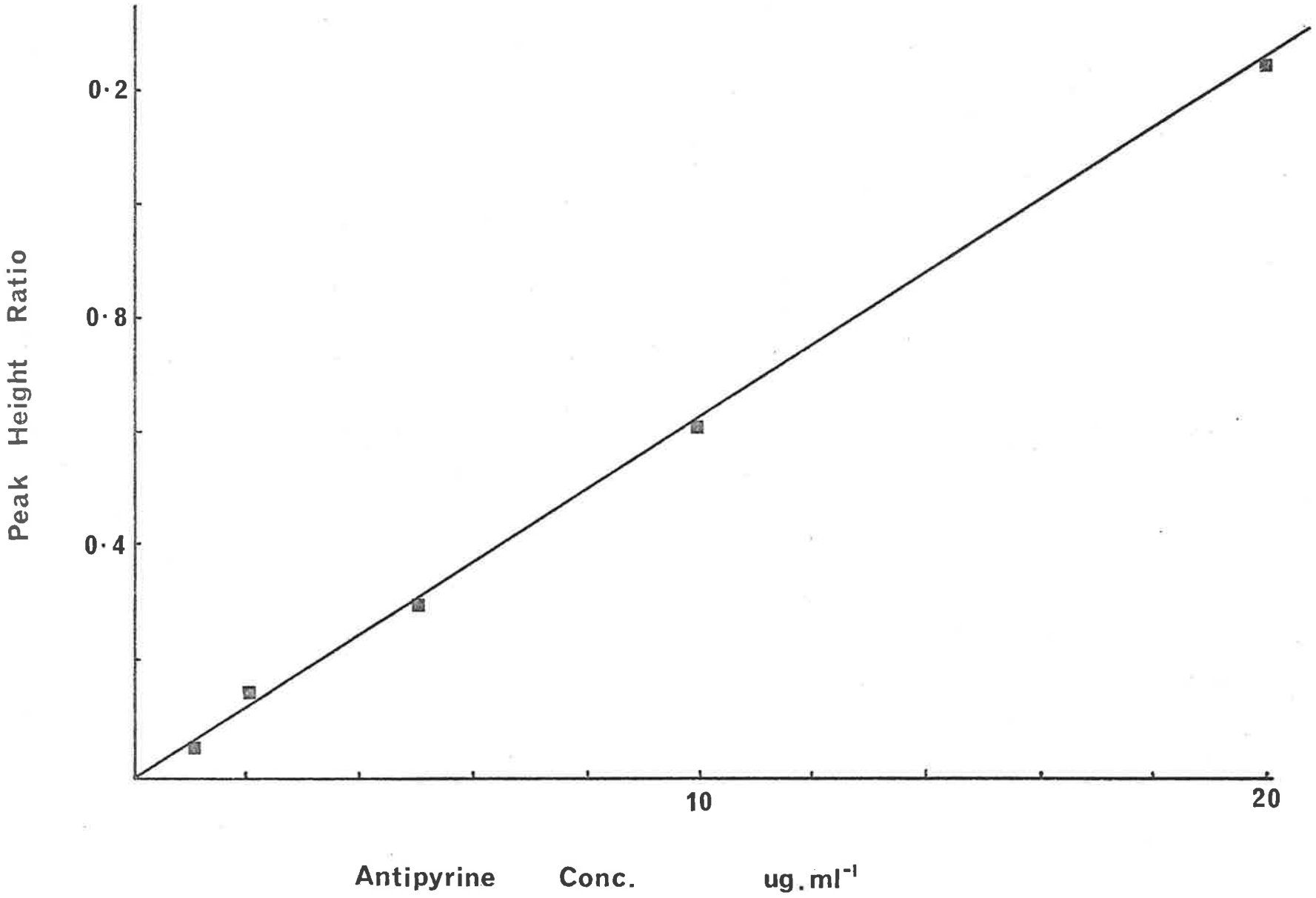

Peak Height Ratio
0·2
0·8
0·4
10
20
Antipyrine Conc. ug.ml⁻¹

Table 1.1 Peak height ratio of antipyrine to phenacetin when antipyrine was assayed by gas chromatography at various concentrations as described in Methods.

Antipyrine concentration (ug/ml)	Mean Peak Height Ratio (n=5)	Coefficient of variation
5	0.29	6.6%
10	0.54	3.1%
15	0.83	5.0%
20	1.07	3.7%

Table 1.2 Kinetics of antipyrine elimination in 19 male and 6 female volunteers

Subject	Sex	Age (yr)	Half-life (hr)	MCR (ml/min/kg)	Vd (L/kg)
1	M	21	16.5	0.501	0.717
2	M	22	10.1	0.812	0.707
3	M	35	11.5	0.870	0.864
4	M	22	12.5	0.636	0.687
5	M	21	8.0	0.761	0.528
6	M	21	12.9	0.841	0.938
7	M	22	11.5	0.635	0.635
8	M	25	9.9	0.823	0.703
9	M	25	9.5	0.828	0.681
10	M	28	10.7	0.782	0.724
11	M	34	14.3	0.766	0.776
12	M	30	11.3	0.680	0.665
13	M	26	13.4	0.627	0.727
14	M	25	10.3	0.775	0.691
15	M	25	7.9	0.810	0.554
16	M	27	7.1	1.171	0.720
17	M	47	7.7	1.355	0.903
18	M	69	12.6	0.519	0.566
19	M	37	14.1	0.615	0.750
20	F	20	6.2	1.761	0.939
21	F	21	9.7	1.060	0.886
22	F	21	8.5	1.951	1.434
23	F	20	6.4	1.870	1.042
24	F	21	12.9	0.789	0.878
25	F	20	8.8	0.902	0.686

Table 1.3 Comparisons of half-life, metabolic clearance (MCR) and apparent volume of distribution (V_d) in male and female volunteers shown with approximate 98% non-parametric confidence limits. Differences were assessed using a U-test (two-tailed).

	Half-life (hr)	MCR (ml/min/kg)	V_d (ml/kg)
Males	11.3	0.775	707
n=19	9.5 - 12.9	0.635 - 0.828	665 - 750
Females	8.7	0.980	912
n=6	6.4 - 8.5	0.902 - 1.870	878 - 1042
Males vs Females	$P < 0.025$	$P < 0.025$	$P < 0.025$

DISCUSSION

There are now many reports of values for antipyrine half-life in healthy volunteer groups in the literature. In 16 such studies where moderate numbers of subjects were examined, the mean values range from 10.0 hr to 14.6 hr and the standard deviation is usually between 2 to 4 hr. The values obtained in the present study were 10.5 ± 2.6 hr which is at the lower end of this range. However the value for the parameter in the six females studied was 8.8 ± 2.5 hr, a figure lower than the 10.6 ± 2 hr reported by O'Malley et al. (1971) in a group of 23 females. However since the females in the present study fell into a very narrow age span it is more appropriate to compare them to a group of nursing students studied by Bakke et al. (1977) whose ages ranged from 19 to 23 yr and whose antipyrine half-lives ranged from 6.1 to 12.8 hr. This is very similar to the range of 6.2 to 12.9 hr obtained for the six females in this study. The mean half-life in the 19 males was 11.1 ± 2.5 hr and this is consistent with values obtained in studies of other male populations, namely 11.5 ± 1.9 hr (Vesell and Page, 1968) 11.7 ± 2.2 (Kellermann and Luyten-Kellermann, 1977) and 11.3 ± 1.3 (Lindgren et al., 1974). The finding that half-lives in females were less than those in males is in agreement with the study of O'Malley et al. (1971); however, the age distributions in the present study do not match, the females being younger than the males. This is an important consideration as Vestal et al. (1975) have shown that the rate of antipyrine metabolism decreases with age.

It could be argued that half-life is influenced by

distribution factors as well as the rate of metabolic activity. Antipyrine has low protein binding (Brodie et al., 1950) and distributes evenly throughout total body water, hence the only conceivable effect distribution could have on half-life would be an alteration in the body water content, for example, oedema formation. Reidenberg and Vesell (1975) found a decrease in volume of distribution of antipyrine but unchanged half-life and clearance in obese subjects during fasting. However this is not surprising since it is well known that there is a substantial fluid loss during the first week of fasting (Gamble et al., 1923; Haag et al., 1967). Bakke et al. (1977) found normal volumes of distribution in subjects with anorexia nervosa. Apparent volume of distribution of antipyrine is not changed by phenobarbitone pretreatment (Roberts et al., 1976), chronic vitamin C administration (Houston, 1977), delta-9-tetrahydrocannabinol (Benowitz and Jones, 1977), iron deficiency (Langman and Smithard, 1977), diet (Kappas et al., 1976), disulfiram administration (Freundt, 1978), oral contraceptive steroids (Homeida et al., 1978), chronic liver disease (Branch et al., 1976), acute viral hepatitis (Burnett et al., 1976) or in patients with lung cancer (Tschanz et al., 1977) all of which have been shown to alter antipyrine metabolism. Further to this, apparent volume of distribution is unchanged in chronic renal failure (Lichter et al., 1973), a condition that may be expected to alter body water. Hence it seems unlikely that the volume of distribution of antipyrine alters significantly even under severe pathological conditions. This being the case, antipyrine half-life is an accurate estimate of the rate of its metabolism by the liver mixed function oxidase enzymes.

Metabolic clearance rate is considered to be a more precise estimate of hepatic enzyme activity as it is independent of alterations in distribution. However, estimates of metabolic clearance require two basic assumptions regarding the dose. These assumptions are that both bioavailability of, and patient compliance with the dosage form are known. In this study bioavailability is assumed to be 100%. This is based on the study of Andreasen and Vesell (1974) who found that plasma levels of antipyrine after intravenous administration were similar to those after oral dosing in aqueous solution. Total bioavailability of antipyrine is not surprising since it is both highly lipid and water soluble and would be expected to be readily absorbed across membranes. However, dose compliance in these experiments cannot be determined in many cases as some of these experiments were performed where dosing was unsupervised.

The estimates of apparent volume of distribution in some of the males and most of the females as seen in Table 1.2 are unusually high, some being of the order of one litre/kg. In a study of 307 male subjects reported by Vestal et al. (1975) where antipyrine was administered by intrvenous infusion, the value for this parameter was 0.545 ± 0.060 L/kg. The variation in this study of 11% is far less than that seen in the present study, where antipyrine was administered orally. Since antipyrine is both water and lipid soluble, is evenly distributed throughout total body water (Soberman et al.,1949) and is completely absorbed from the gastrointestinal

tract (Andreasen and Vesell, 1974), the most likely explanation for these findings is that the subjects did not take the entire dose of antipyrine. Many of the subjects, and in particular, all the females, performed the study on a domiciliary basis where dosing was, in the main, unsupervised. Although the subjects were given clear instructions to take the entire dose, in some cases there was direct evidence (antipyrine crystals left in the container in which they were provided) that they did not. The most likely explanation is that these subjects either did not dissolve all the antipyrine crystals in water or, since antipyrine has such a bitter taste, did not drink all the antipyrine solution. This would result in the theoretical dose being higher than the actual amount taken and the estimate of apparent initial concentration (extrapolated y-intercept) would be low. Hence estimates of V_d and MCR would be higher than expected. The foregoing suggests that in studies where oral dosing is used and dose administration cannot be supervised, half-life is likely to be a better estimate of antipyrine metabolism than clearance as it is independent of dose compliance problems.

For this reason in subsequent chapters only half-life estimates were used in studies where dosing was unsupervised. It should be noted that many previous studies (Andreasen and Vesell, 1974; Branch et al., 1976; Benowitz and Jones, 1977; Meredith et al., 1977; Freundt, 1978; Homeida et al., 1978; Pirttiaho et al., 1978; Fraser et al., 1979; Kellermann and Luyten-Kellermann, 1979) values for clearance and volume of distribution have been reported where an aqueous solution of antipyrine was used as the dose formulation.

C H A P T E R T W O

EFFECT OF LIVER AND KIDNEY DISEASE ON ANTIPYRINE METABOLISM

INTRODUCTION

Alteration in the activity of the microsomal mixed function oxidase enzymes can occur under the influence of several pathological or abnormal physiological states. These changes in enzyme activity are reflected by changes of drug metabolism *in vivo*. This may be important clinically, where overdosing or ineffective treatment may occur if these changes are not taken into account. In the present study, the effects of two pathological situations viz. chronic liver disease and chronic renal dysfunction, on the activity of these enzymes were examined.

Liver disease consists of several disturbances to hepatic functions, including damage to the liver cell, changes in plasma protein synthesis, reduction in liver blood flow and the formation of extra- and intrahepatic shunts. These changes may lead to alterations in the liver's ability to handle drugs, yet the evidence relating to the effect of liver disease on drug metabolism is conflicting. Table 2.1 summarises literature reports of the effects of liver disease on drug disposition. In some, the rate of metabolism is depressed while in others, it is unaffected.

In kidney disease, the elimination of some compounds such as water soluble drugs, is markedly impaired. However the effect of this disorder on lipid soluble drugs that are largely metabolized is unclear. Previous studies have shown that for some drugs of this type the elimination rate is reduced, in some increased, while in

Table 2.1 Effect of chronic liver disease on the elimination of drugs. I. No difference in elimination compared to a normal population. II. Elimination rate is decreased compared to control.

I NORMAL

drug	reference
aminopyrine	Brodie et al. (1959)
dicoumarol	"
antipyrine	"
phenylbutazone	Hvidberg, Andreason and Ranek (1974)
chlorpromazine	Maxwell et al. (1972)
tolbutamide	Nelson (1964)
pentobarbitone	Held et al. (1970)
oxazepam	Shull et al. (1976)

II DECREASED

antipyrine	Branch, Herbert and Read (1973) Andreason et al. (1974)
prednisolone	Powell and Axelson (1972)
carbenicillin	Hoffman, Cestero and Bullock (1970)
lidocaine	Thomson, Rowland and Melmon (1971)
chloramphenicol	Kunin, Glazko and Finland (1959)
rifampicin	Acocella et al. (1972)
isoniazid	"
amylobarbitone	Mawer, Miller and Turnberg (1972)

others it is normal (Table 2.2.). In the case of such drugs as antipyrine, tolbutamide and chloramphenicol, different workers have found conflicting results.

The aim of studies reported in this chapter was to assess the influence of kidney and liver disease on mixed function oxidase activity in the face of complications such as changes in protein binding, blood flow, etc., which may also contribute to altered drug disposition. Antipyrine would seem to be an ideal drug to use for these types of studies. It is almost completely metabolized in the liver, is less than 10% plasma protein bound and has a low clearance rate. Hence it is largely unaffected by changes in protein binding, liver blood flow or renal function and provides an index of the activity of the enzymes that oxidize it.

Table 2.2. Effect of chronic renal disease on the drug elimination.

I. Drugs whose elimination is not different from controls.

II. Elimination rate is decreased compared to controls.

III. Elimination rate is increased compared to controls.

drug	reference
I. NORMAL	
chloramphenicol	Kunin, Glazko and Finland (1959)
tolbutamide	Glogner, Lange and Pfab (1968)
phenacetin	Prescott (1969)
isoniazid	Mitchell and Bell (1957) Bowersox et al, (1973)
sulphadimidine	Fine and Summer (1975)
antipyrine	Lichter, Black and Arias (1973)
lidocaine	Thomson et al. (1971)
pentobarbitone	Reidenberg et al. (1976)
II. DECREASED	
chloramphenicol	Suhrland and Weisburger (1963)
tolbutamide	Ueda et al. (1963)
sulphisoxazole	Reidenberg et al. (1968)
III. INCREASED	
diphenylhydantoin	Letteri et al. (1971)
antipyrine	Maddocks, Wake and Harber (1975)
propranolol	Thompson, Joekes and Foulkes (1972)

METHODS

1. Subjects

A total of 17 subjects were studied. They were grouped as follows:

(a) Six male hospitalized patients, aged from 49 to 56 years, mean 53 ± 2 (±SD) years, clinically diagnosed as having chronic liver disease;

(b) Five hospitalized patients (four male, one female), aged 21 to 61 years, mean 48 ± 16 years, clinically diagnosed as having chronic renal failure;

(c) Six healthy, unmedicated male volunteers aged 30 to 69 years, mean 42 ± 14 years, selected from the control population in Chapter I, such that they provided a matched control group with ages as close as possible to the hospitalized patients. The half-life of antipyrine in this group did not differ from the 25 control subjects studied as a whole ($P > 0.1$, t-test, two-tail).

The only known enzyme inducing drug taken by any of the patients was spironolactone, which has been shown to increase its own metabolism (Abshagen et al., 1977).

2. Protocol

The determination of antipyrine elimination kinetics was the same as described in Chapter I. Plasma samples from the patients with liver disease were assayed for total and conjugated bilirubin, alkaline phosphatase, lactic dehydrogenase, aspartate transaminase and albumin by routine Technicon autoanalyser techniques. Plasma and urine from the patients with renal disease was assayed for creatinine and plasma was assayed for urea.

RESULTS

The half-life of antipyrine in the control group of 6 volunteers ranged from 7.7 to 14.3 hr, median 12 hr. The metabolic clearance rate (MCR) of antipyrine ranged from 0.519 to 1.355 ml/min/kg body weight. MCR and half-lives of antipyrine and relevant biochemical data from the six patients with liver disease and the five uraemic patients are shown in Tables 2.3 and 2.4 respectively. The MCR in the six patients with liver disease was less than that in the control group and half-lives longer (U-test, $P<0.001$, one-tail). In the five uraemic patients neither MCR nor half-lives were significantly different from controls. (U-test, $P>0.1$, two-tail). These comparisons are illustrated in Figs. 2.1 and 2.2. Apparent volumes of distribution in both patients with renal disease and liver disease did not differ from control values (U-test, $P>0.1$, two-tail). The biochemical indices of liver function in the six patients with liver disease indicate marked impairment of liver function. There was no rank correlation between either antipyrine clearance or half-life and any of these indices. The patients suffering from renal disease were characterized by high plasma creatinine levels and plasma urea levels and low creatinine clearance rates.

Table 2.3. Biochemical data pertaining to hepatic function, antipyrine kinetics and drug therapy of the 6 patients with liver disease

Subject	Age	Sex	Conj. bilirubin (umol/l) (4)*	Total bilirubin (umol/l) (24)*	Alk. phosphatase (U/l) (95)*	LDH† (U/l) (300)*	AST‡ (U/l) (40)*	Albumin (g/l) (43-53)	Antipyrine clearance (ml/min/kg) (0.5-1.9)	Antipyrine half-life (hr) (6-16)	Drug Therapy
A Alcoholic cirrhosis	56	M	85	185	343	308	192	24	0.427	18.4	Vitamins and minerals "Gaviscon", "Slow K", "Mylanta"
B Alcoholic cirrhosis	56	M	56	187	147	388	104	22	0.125	66.7	Oral neomycin, lactulose, thiamine, prothrombinex
C Alcoholic cirrhosis	54	M	8	30	92	147	20	38	0.266	31.0	Spironolactone, nitrazepam, vitamin K, "Slow K", digoxin, tolbutamide, frusemide, "Mylanta"
D Alcoholic cirrhosis	49	M	25	71	99	294	40	23	0.099	73.2	Spironolactone, nitrazepam
E Obstructive jaundice	51	M	61	100	258	240	42	40	0.402	15.5	Salbutamol, "Brondecon", chlorthalidone
F Alcoholic cirrhosis	55	M	67	169	169	219	60	32	0.140	49.7	Spironolactone, multivitamins

* Upper limit of normal range † Lactic dehydrogenase ‡ Aspartate transaminase

Table 2.4 Biochemical data, antipyrine kinetics and drug regimes in the five patients with chronic renal disease

	Subject	Age (yr)	Sex	Plasma creatinine (mmol/l) 0.05–0.12	Creatinine clearance (ml/min) (90–180)	Urea (mmol/l) (3–8)	Antipyrine clearance (ml/min/kg) (0.5–1.9)	Antipyrine half-life (hr) (6–16)	Drug Therapy
1	SLE, nephrotic syndrome	61	M	0.31	21	17.0	0.483	18.6	Frusemide, prazosin, propranolol, warfarin, dipyridamole
2	Chronic RF, polycystic disease	58	M	1.00	6	40.0	1.211	7.2	Ethacrynic acid, "Slow K", prochlorperazine, "Amphojel" duplex, calcium Sandoz, digoxin
3	Chronic RF hypertension polycystic disease	48	M	0.58	17	23.5	0.909	9.6	Frusemide, "Slow K", hydrallazine, propranolol, methyldopa
4	Chronic RF, hypertension	21	M	0.55	20	14.2	0.689	12.7	Propranolol, frusemide, "Slow K", "Amphojel", methyldopa, prazosin
5	Chronic RF, analgesic nephropathy	54	F	0.74	4	22.4	0.610	10.7	"Amphojel", sodium bicarbonate

Figure 2.1 Values of antipyrine half-life in patients with chronic liver and renal disease and in 6 control subjects. The mean value of each group is indicated by a bar.

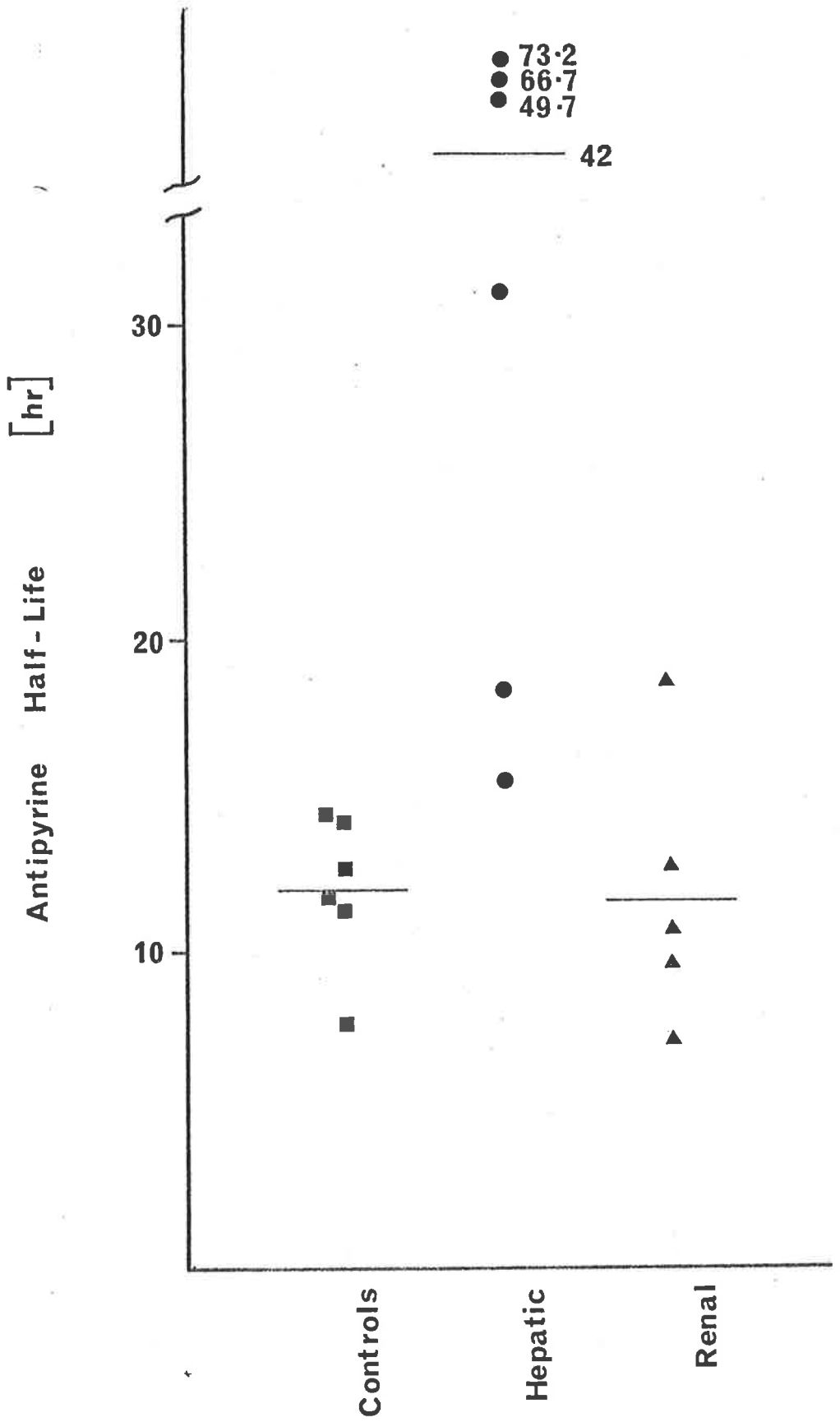
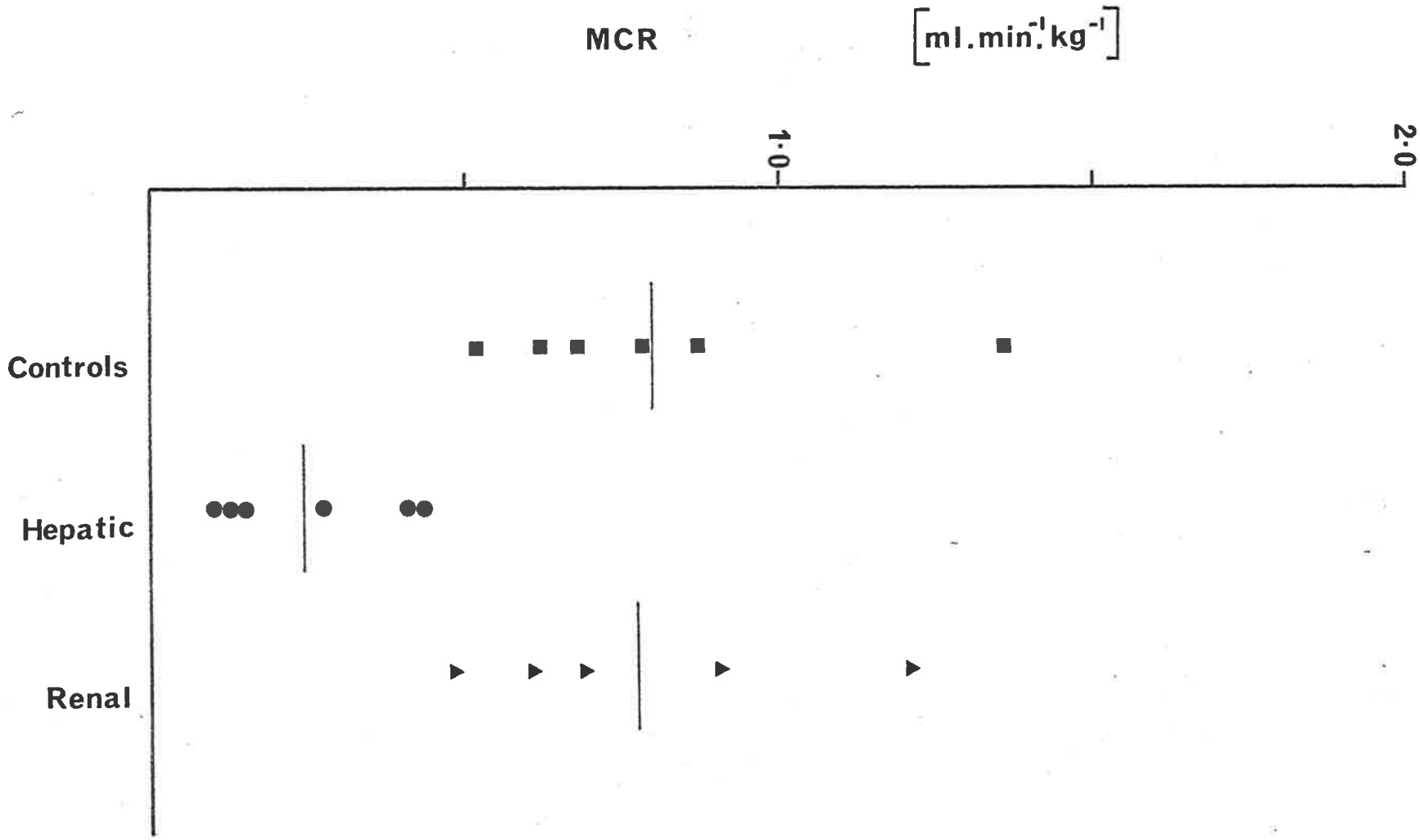

Antipyrine Half-Life [hr]
73·2
66·7
49·7
42
30
20
10
Controls
Hepatic
Renal

Figure 2.2 Value of antipyrine clearance in patients with chronic liver and renal disease and in 6 control subjects. The mean value of each group is indicated by a bar.


MCR
[ml.min⁻¹.kg⁻¹]
1·0
2·0
Controls
Hepatic
Renal

DISCUSSION

The decreased rate of antipyrine elimination in patients with chronic liver disease is consistent with results obtained in two previous studies (Branch et al., 1973, Andreasen et al., 1974) and indicates that this disease state causes decreased activity of drug metabolizing enzymes. Since the present study was completed, these conclusions have been further substantiated in more recent investigations and now there seems to be little doubt that hepatic metabolism of many drugs in man is depressed in chronic liver disease. Greisen and Andreasen (1976), Branch et al. (1976) and Forrest et al. (1977), found decreased metabolism of antipyrine in patients with liver disease of various aetiologies and Burnett et al. (1976) had similar findings in patients with acute viral hepatitis. Other drugs, too, have been shown to have a decreased rate of elimination. These include aminopyrine (Hepner and Vesell, 1977; Bircher et al., 1976), hexobarbital (Zilly et al., 1978), diazepam (Hepner et al., 1977), lignocaine and paracetamol (Forrest et al., 1977). However the elimination of antipyrine in patients with obstructive jaundice was found to be unchanged (Elfstrom and Lindgren, 1974). Interestingly, subject E from Table 2.1 was diagnosed as having obstructive jaundice and his half-life of 15.5 hr was the closest to the normal range of values in that group.

Several previous studies have found significant correlations between the hepatic metabolism of drugs used as indices of mixed function oxidase activity and certain biochemical liver function tests.

Serum albumin and prothrombin index have been shown to correlate with the metabolism of antipyrine (Branch et al., 1973; Andreasen et al., 1974; Forrest et al., 1977), hexobarbital (Zilly et al., 1978), phenylbutazone (Levi et al., 1968) and amylobarbitone (Mawer et al., 1972). Galactose elimination capacity has been shown to correlate with antipyrine (Andreasen et al., 1974) and phenylbutazone metabolism (Hvidberg et al., 1974). No correlation was found between either antipyrine half-life or clearance and any of the liver function tests performed in this study. However, good correlations are difficult to obtain in small sample sizes such as the one in this study.

The mechanism for the reduced metabolism of antipyrine in the patients with chronic liver disease may be due to a number of potentially rate limiting processes which might be altered to affect the resulting clearance of the drug. Total hepatic clearance has been proposed as a useful index of the efficiency of the liver to remove a drug. This parameter is defined as:

$$C_H = Q \ f_B \ Cl_{in} \ / \ (Q + f_B \ Cl_{in}) = Q \ E$$

where Q = liver blood flow, f_B = fraction of unbound drug in the blood, Cl_{in} = intrinsic hepatic clearance and E = extraction ratio (Rowland et al., 1973).

As can be seen in Table 2.3, patients with chronic liver disease are characterized by a reduced plasma albumin concentration and this may be expected to result in changes in plasma protein

binding leading to alterations in the value of f_B and hence, clearance, C_H. A reduction in binding would lead to an increase in f_B and for drugs with a low extraction ratio, such as antipyrine, an increase in total clearance. However, this was not found to be the case for antipyrine. Since this compound has low plasma protein binding in man (Brodie and Axelrod, 1950) and since there was no difference in apparent volumes of distribution between cirrhotics and controls it is not likely that altered distribution of antipyrine was a cause of its altered clearance.

Alterations in hepatic blood flow, Q, can produce changes in clearance of a drug. This may be an important consideration in cirrhotic patients as blood flow to the liver can be impaired and this could lead to a reduction in total hepatic clearance (Caesar et al., 1961). The clearance of lidocaine has been shown to be reduced in patients with reduced cardiac output and with cirrhosis and this decrease was associated with a reduced hepatic blood flow (Thomson et al., 1973; Prescott et al., 1976). However, Pessayre et al. (1978) demonstrated that while liver blood flow was important in the extraction of d-propranolol in subjects with normal liver function, in cirrhotic patients d-propranolol is no longer a highly extracted drug and its hepatic clearance depends not only on liver blood flow, but predominantly, on the ability of the liver to remove the drug from the blood. This is consistent with the findings with antipyrine in the present study. As antipyrine is a "low clearance" drug with an extraction ratio of approximately 0.05, its hepatic clearance is largely independent of the liver blood flow (Branch et al., 1974).

Hence decreased clearance of this drug must be due to decreased intrinsic hepatic clearance which is a function of the metabolic activity of the liver itself.

There could be a reduced capability of each cell to eliminate the drug due to a reduction in the amount of enzyme in the cell or alternatively chronic liver disease may result in a reduced mass of normally functioning cells. Villeneuve et al. (1978) found that in isolated hepatocytes from rats with experimentally induced cirrhosis, the V_{max} for aminopyrine, hexobarbitone and propranolol were reduced without a change in the K_m values. This means that although the capacity of the liver cells to metabolize these compounds had been reduced, the affinity of each substrate and the enzyme(s) involved in its metabolism remained unchanged. This indicates that the decreased metabolism was not due to alterations in the quality of the enzyme itself, but rather to a reduction in the total amount of enzyme. However this does not help distinguish between the latter two possibilities mentioned above, i.e., whether the decreased metabolism is due to a reduced number of cells or reduced amount of enzyme/cell. The lower rates of antipyrine metabolism found in the patients with chronic liver disease in the present study are consistent with either of these explanations.

But what of the findings that the metabolism of some drugs is apparently unaffected by liver disease? There are at least two possible explanations for these inconsistencies:

(a) The difficulty in quantitating hepatic functional impairment. Procedures for quantitating liver function are relatively crude. Although useful as an initial screen, biochemical liver function tests rarely point to a precise diagnosis (Editorial, Brit. Med. J., 1977). Also, impaired drug metabolism has been found in certain types of compensated liver disease without concomitant evidence of liver dysfunction as judged from clinical or biochemical data (Zilly et al., 1978), and to further complicate the situation in certain types of liver disease drug metabolism was found to be normal, as for example, in obstructive jaundice (Elfstrom and Lindgren, 1974).

(b) The use of half-life estimates as the quantitative index of the changes in drug metabolism. Many of these studies use half-life as their index of the rate of drug metabolism. Although this is a valid assumption for certain drugs (e.g. antipyrine), it is not valid for all drugs, since half-life is dependent upon both systemic clearance of the drug and its distribution in the body:

i.e. $T_{\frac{1}{2}} \propto V_d \ / \ MCR$

Thus half-life can be altered by a number of factors. If liver disease acts to alter clearance it may also act to alter distribution in the opposite direction resulting in little change in half-life. The use of clearance estimates

would overcome these types of difficulties.

Antipyrine metabolism was found to be normal in the patients with chronic renal disease. This finding is in agreement with that of Lichter et al. (1973) who found that half-life was normal or occasionally enhanced and with that of Reidenberg et al. (1976) who demonstrated that pentobarbitone clearance was normal in uraemic patients. Tolbutamide and phenacetin have also been shown to have normal rates of metabolism in these patients (Glogner et al., 1968; Prescott, 1969). Others have shown that drug elimination is enhanced in renal disease. Maddocks et al. (1975) found shorter half-lives of antipyrine in uraemic patients while phenytoin elimination was also shown to be increased in these patients (Letteri et al., 1971; Oder-Cederlof and Borgia, 1974). Some studies have shown that drugs such as chloramphenicol (Suhrland and Weisburger, 1963) and tolbutamide (Ueda et al., 1963) have prolonged elimination.

In a study in rats with experimentally induced renal failure Black and Arias (1975) found that hepatic cytochrome P450 and benzo(a)-pyrene and aminopyrine metabolism were decreased and ketamine narcosis time and zoxazolamine paralysis time were increased. These changes were thought to be due to damage to liver cell organelles and support studies showing decreased metabolism of drugs in chronic renal disease. But again, many of these drugs may not be good indices of the activity of drug metabolizing enzymes as their rate of elimination may reflect changes in factors other than metabolism, such as alterations in distribution. Alternatively the effect may change with the severity

of the disease. Whatever the reasons for the conflicting evidence, predictions as to the effect of renal disease on the metabolism of drugs in the liver are difficult to make and further evidence is required to elucidate this effect in humans.

C H A P T E R T H R E E

EFFECT OF LONG-TERM DRUG THERAPY ON THE ELIMINATION KINETICS OF ANTIPYRINE - ANTICONVULSANTS AND ANTIPSYCHOTICS

INTRODUCTION

A variety of therapeutic agents have been shown to increase the activity of the microsomal drug metabolizing enzymes during long-term therapy. These include barbiturates such as barbitone (Dayton et al., 1961) and phenobarbitone (Cucinell et al., 1965), anticonvulsants such as primidone and phenytoin (Perucca, 1978), antiinflammatory agents such as phenylbutazone (Chen et al., 1962) and steroids such as spironolactone (Abshagen et al., 1977). The extent of the induction of the enzymes is often hard to predict and hence the clinical significance of administering these types of compounds is often unclear. However changes in the rate of drug metabolism may necessitate modification of drug dosages and for these reasons it is important to identify those pharmacological agents that induce these enzymes and their relative inductive potency.

In epileptic patients, a knowledge of the degree of enzyme induction may be useful in identifying those patients at risk from metabolic bone disease and folate deficiency, in assessing drug compliance and in predicting anticonvulsant dose/serum relationships.

Although the mechanism of folate deficiency in epileptics is unclear, a significant inverse correlation has been found between the degree of enzyme induction of drug metabolizing enzymes as determined by D-glucaric acid excretion, and serum and red cell folate (Maxwell et al., 1972a). On the other hand, alternative mechanisms, such as reduced absorption, have been proposed (Reynolds, 1972).

Osteomalacia is also commonly found in epileptics on anticonvulsant drug therapy (Dent et al., 1970; Genruth et al., 1972; Marsden et al., 1973). Vitamin D undergoes hepatic microsomal metabolism to 25-hydroxycholecalciferol which in turn is metabolized in kidney mitochondria to 1,25-dihydroxycholecalciferol. This is believed to be the active form of the vitamin (Kodicek, 1974). It has been suggested that enzyme induction by anticonvulsant drugs stimulates alternative pathways of vitamin D metabolism in the liver leading to inactive metabolites of the vitamin (Hahn, 1976).

Problems with dose compliance are well recognised in epileptics. A simple test for enzyme induction would be a useful qualitative compliance test for patients taking known enzyme inducing agents. If a correlation could be found between antipyrine metabolism and phenytoin plasma levels, antipyrine elimination kinetics may also assist in differentiating between non-compliers, and patients who metabolize the anticonvulsant drug at a fast rate resulting in low serum levels.

An attempt was made in this study to differentiate some of these factors in a group of epileptics on the basis of the degree of enzyme induction as determined by antipyrine elimination kinetics. In addition the study examined antipyrine elimination in psychotic patients on prolonged therapy with either chlorpromazine or fluphenazine decanoate to compare the relative inductive effects of these two therapies. Chlorpromazine has been shown to cause enzyme induction in animals (Kato and Vasanelli, 1962; Kato and Chiesara, 1962; Stevenson et al., 1972), however conflicting results have been

obtained in man with both normal (Stevenson et al., 1972) and increased metabolism of antipyrine having been reported (Loga et al., 1975). Fluphenazine decanoate is thought not to be an inducing agent in man (Majumbar and Kakad, 1978).

METHODS

1. Subjects

A total of 47 subjects were involved in this study. They included:

(a) Twenty-four epileptic patients, fifteen male and fourteen female, aged 17 to 56 years, 31± 10 (mean ± SD), who had been receiving long-term anticonvulsant drug therapy for at least 4 years.

(b) Six psychotic patients, four males and two females, aged 22 to 37 years, 32± 6 yr (mean± SD), who were receiving long-term chlorpromazine therapy.

(c) Twelve psychotic patients, seven males and five females, aged 17 to 49 years, 34± 12 yr(mean± SD), who were receiving long-term fluphenazine decanoate therapy.

2. Analysis

The elimination kinetics of antipyrine were determined as described in Chapter I. Plasma samples from the epileptic patients were taken just prior to the administration of a phenytoin dose close to the time of antipyrine administration. The plasma was assayed for phenytoin concentration by an enzyme immunoassay (EMIT, Syva b). Nonparametric confidence limits in this and consequent chapters were determined as described by Colquhoun (1971).

RESULTS

1. Epileptics

The antipyrine half-lives in the 24 epileptic patients ranged from 2.6 to 11.7 hr, median 5.2 hr, with 98% non-parametric confidence limits of 4.2 and 6.8 hr, and these were shorter than values found in the 25 normal volunteers studied in Chapter I, which ranged from 6.2 to 16.5 hr, median 10.3 hr, with 98% non-parametric confidence limits 8.8 and 12.5 hr. (U-test, $P<0.01$, one-tail). This difference is illustrated in Figure 3.1. Six patients examined had phenytoin induced osteomalacia which was confirmed by bone biopsy (Table 3.1). Half-lives ranged from 3.1 to 11.7 hr, median 6.0 hr, and were not different from the values obtained in the remainder of the epileptic group (U-test, $P>0.1$, two-tail). Six epileptics were on phenytoin therapy alone (Table 3.2). Half-lives in this group ranged from 3.0 to 7.9 hr, median 4.7 hr and were not different from fourteen patients who were receiving phenytoin plus a barbiturate (U-test, $P>0.1$, two-tail). This latter group had values which ranged from 2.6 to 11.7 hr, median 5.2 hr and are shown in Table 3.3. The comparison of these two subgroups showed that an additional inducing drug did not further enhance antipyrine metabolism. In the epileptic group as a whole there was no significant correlation between antipyrine half-life and phenytoin dose ($r_s = 0.34$, $P>0.05$) or between half-life and phenytoin serum levels ($r_s = 0.12$, $P>0.05$).

Figure 3.1 Antipyrine half-lives in 25 control and 24 epileptic subjects. The median value in each group is indicated by a bar.

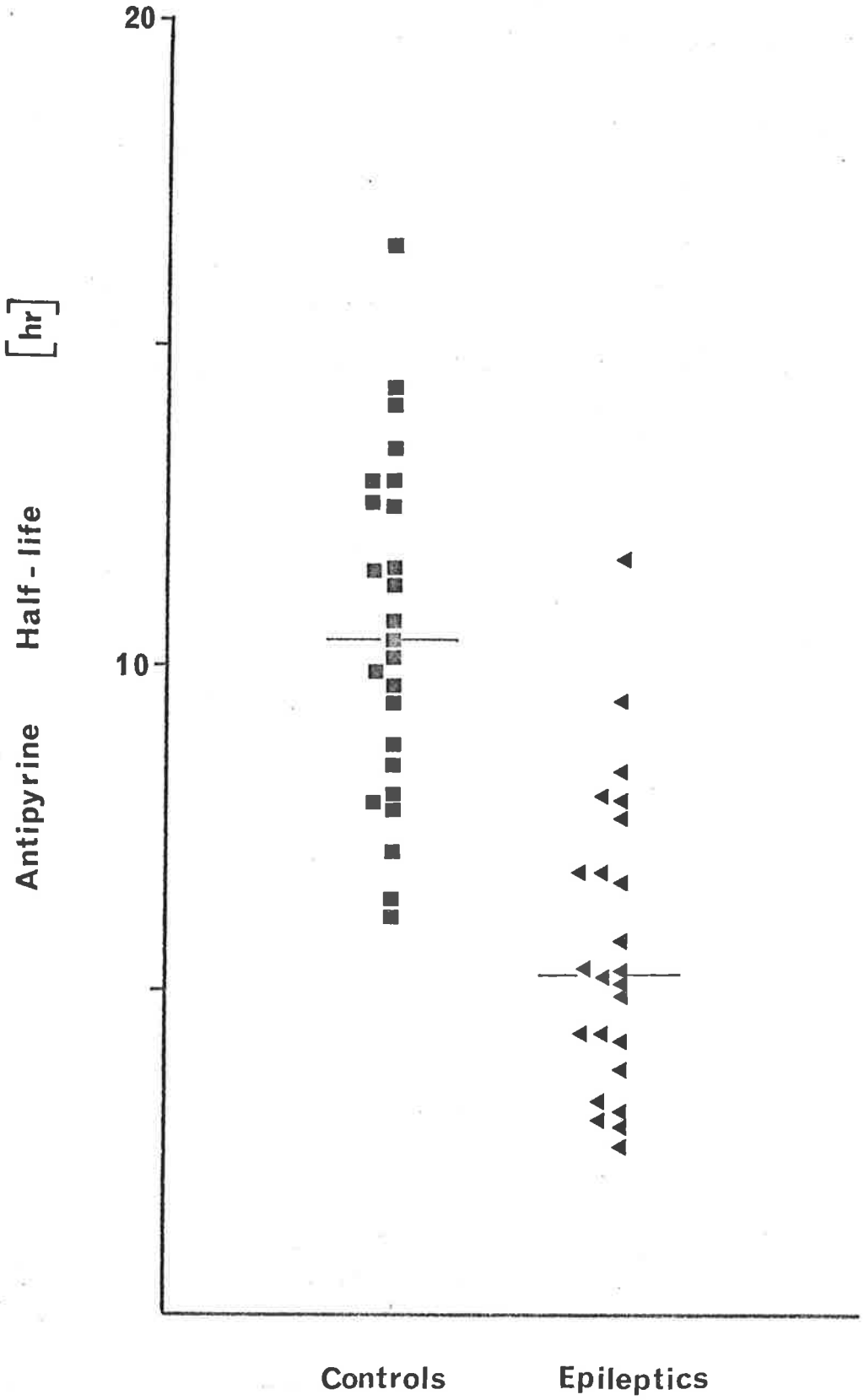

20
10
Antipyrine Half-life [hr]
Controls
Epileptics

Table 3.1 Antipyrine half-lives in 6 epileptic patients with drug induced osteomalacia

Subject	Age	Sex	Phenytoin dose (mg/day)	Other Treatment	Phenytoin Level (umol/l)	Antipyrine Half-Life (hrs)
RD	32	M	230	Carbamazepine	22	5.3
JM	30	F	200	Primidone	22	8.4
MM	29	F	400	Primidone	73	5.2
ES	37	M	300	Ethotoin Carbamazepine	40	6.8
RR	33	F	400	Primidone Carbamazepine	66	11.7
BE	52	F	300	-	22	3.1
MEAN			310		41	6.8

Table 3.2 Antipyrine half-lives in 6 epileptic patients receiving phenytoin therapy alone

Subject	Age	Sex	Dose Phenytoin (mg/day)	Phenytoin Level (umol/l)	Antipyrine Half-Life (hr)
RW	25	M	450	36	5.8
GM	31	F	600	91	5.1
JP	32	F	300	50	3.0
BE	52	F	300	22	3.1
WB	24	M	300	108	4.3
PM	56	F	300	17	7.9
MEAN			375	60	4.9

Table 3.3 Antipyrine half-lives in 14 epileptic patients receiving phenytoin plus a barbiturate

Subject	Age	Sex	Phenytoin dose (mg/day)	Other Treatment	Phenytoin Level (umol/l)	Antipyrine Half-Life (hrs)
JM	30	F	200	Primidone	22	8.4
MM	29	F	400	Primidone	73	5.2
RR	33	F	400	Primidone Carbamazepine	66	11.7
PL	26	M	500	Primidone	14	2.9
KJ	25	F	400	Primidone	25	9.5
JC	25	F	330	Phenobarbital Carbamazepine	58	4.2
MA	30	F	460	Primidone Sulthiame	41	6.7
ER	31	M	330	Primidone Sulthiame	58	4.9
DC	17	F	200	Primidone	74	3.8
PC	35	F	400	Phenobarbital	69	4.3
EC	18	F	400	Primidone	43	7.7
IR	50	M	300	Phenobarbital Carbamazepine	64	5.3
NC	18	M	200	Ethosuximide Primidone	83	2.6
EB	29	M	200	Primidone	17	6.8
MEAN			330		51	6.0

2. Psychotic patients

The half-life of antipyrine in the six patients receiving chlorpromazine ranged from 5.2 to 12.3 hr, median 6.2 hr and these were shorter than in controls from Chapter I. (U-test, $P<0.05$, two-tail). However the values for this parameter in the twelve patients receiving fluphenazine decanoate therapy, which ranged from 6.5 to 16.5 hr, median 9.8 hr, were not different from control values (U-test, $P>0.1$, two-tail). These values are shown in Tables 3.4 and 3.5 and the comparison to control values is illustrated in Figure 3.2.

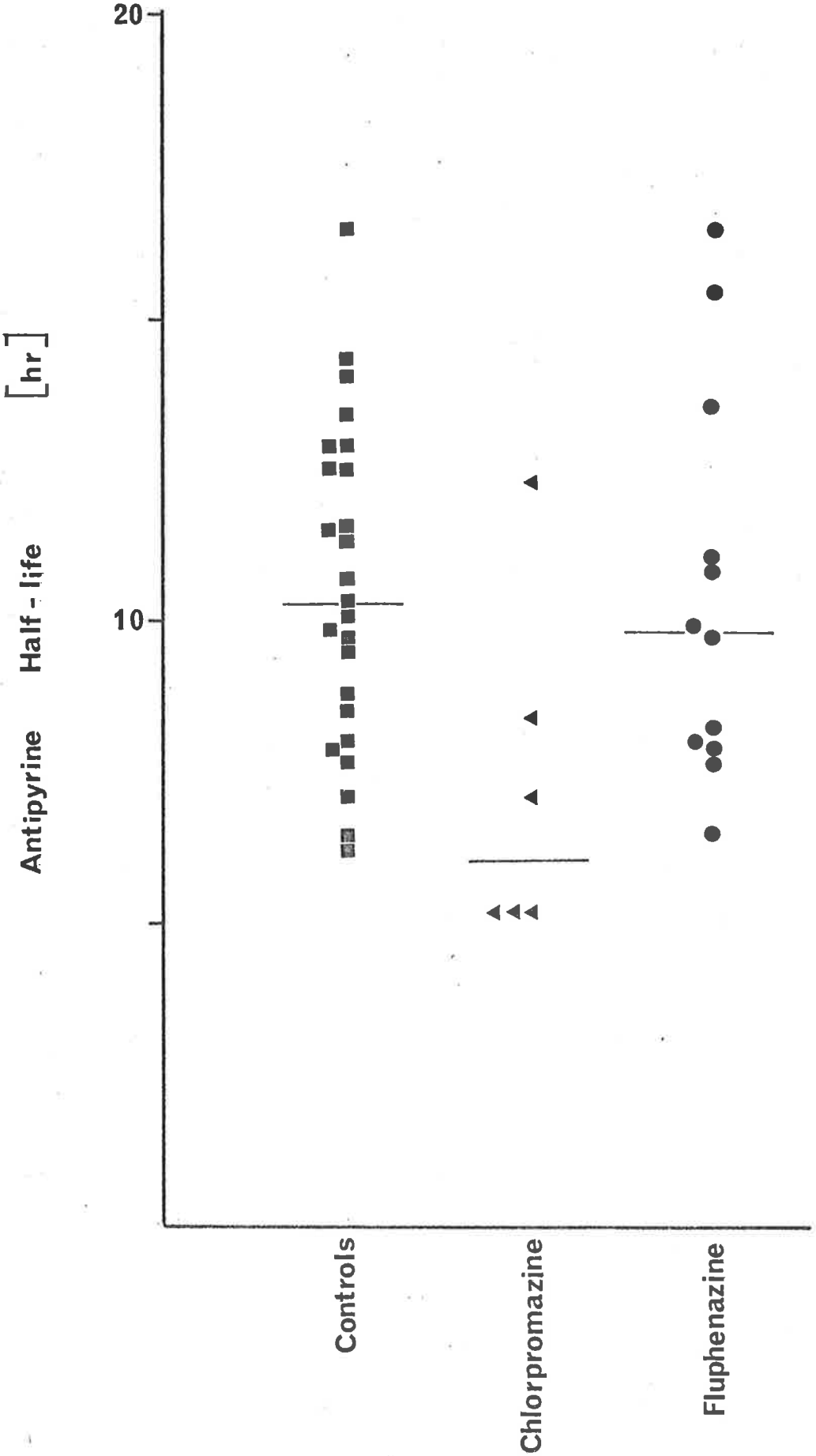
Table 3.4 Antipyrine half-lives in psychotic patients receiving long-term fluphenazine decanoate therapy (25 mg / I.M. every 4 weeks)

Subject	Age	Sex	Antipyrine Half-Life
1	34	M	6.5
2	18	F	7.7
3	31	M	7.9
4	25	M	8.0
5	17	F	8.3
6	29	F	9.8
7	27	M	9.9
8	49	M	10.9
9	47	M	11.1
10	43	M	13.6
11	38	F	15.5
12	48	F	16.5
MEAN $\pm$ SD	34 $\pm$ 12		10.4 $\pm$ 3.4

Table 3.5 Antipyrine half-lives in psychotic patients receiving long-term chlorpromazine therapy

Subject	Age	Sex	Antipyrine Half-life (hr)	Dose (mg/day)
1	34	F	5.2	150
2	37	M	5.2	400
3	22	M	5.2	450
4	30	M	7.1	500
5	24	M	8.4	450
6	34	F	12.3	700
MEAN $\pm$ SD	30 $\pm$ 6		7.2 $\pm$ 2.8	440 $\pm$ 180

Figure 3.2 Antipyrine half-lives in psychotic patients receiving either long-term chlorpromazine or fluphenazine decanoate therapy and 25 control subjects. The median values in each group are indicated by a bar.


Antipyrine Half - life [hr]
20
10
Controls
Chlorpromazine
Fluphenazine

DISCUSSION

1. Epileptics

This study confirms earlier reports that anticonvulsant drug therapy causes a reduction of antipyrine half-life in epileptics (Petruch et al., 1974). Antiepileptic agents have been shown to increase the metabolism of a variety of drugs. Among these include warfarin (Cucinell et al., 1965), cortisol (Choi et al., 1971), steroid contraceptives (Stockley, 1976) and paracetamol (Perucca and Richens, 1979). As well as increased drug metabolism, increases have been found in D-glucaric acid excretion (Hunter et al., 1971; Sotaneimi et al., 1974) plasma gamma glutamyltranspeptidase (Rosalski, 1976), 6-beta-hydroxycortisol excretion (Werk et al., 1964) and cytochrome P450 content in liver biopsy samples (Sotaniemi et al., 1978). This evidence clearly shows that anticonvulsant drug therapy causes induction of the hepatic mixed function oxidase enzymes.

All the epileptics in this study were on phenytoin therapy and many were also taking other anticonvulsants such as phenobarbitone and carbamazepine which have been shown to have enzyme inducing properties (Vesell and Page, 1969; Luhdorf et al., 1977). However patients receiving phenytoin plus a barbiturate did not show an additional decrease in antipyrine half-life below that found in patients on phenytoin monotherapy. Mean phenytoin dosage and plasma levels were comparable in these two groups. Phenobarbitone has been shown to lower the plasma phenytoin levels (Buchanan et al., 1969), however other studies have noted an indefinite effect of barbiturate

on phenytoin levels (Kristensen et al., 1969; Kutt, 1971). Lack of apparent additional inductance by barbiturate in this study may be due either to maximal or near maximal induction of the mixed function oxidases by phenytoin at the doses prescribed or antipyrine elimination not being a sufficiently sensitive test to measure quantitatively the extent of enzyme induction.

The incidence of rickets and osteomalacia is high in patients with epilepsy (Dent et al., 1970). The condition is thought to be associated with the increased breakdown of vitamin D by anticonvulsant drug therapy. The precise mechanism is yet to be determined (Richens and Rowe, 1970). No difference was found in antipyrine half-life in the group with osteomalacia when compared to epileptic patients without known bone disease. From these results it does not seem that antipyrine elimination can be employed to differentiate patients at risk from bone disease. There may be a number of reasons for this:

(a) Antipyrine elimination may not be a sufficiently sensitive quantitative test of the extent of enzyme induction;

(b) Although it is very likely that enzyme induction is a factor producing reduced vitamin D status, other factors such as a low dietary intake of vitamin D and inadequate exposure to sunlight may play an additional role in development of frank osteomalacia;

(c) The rate of metabolism of antipyrine in any individual

does not necessarily reflect the rate of metabolism of vitamin D.

The latter point may also be true for antipyrine and phenytoin metabolism. Davies et al. (1973) found a weak, but significant correlation between the clearances of these two drugs but other workers have failed to find a correlation between their half-lives (Davies and Thorgeirsson, 1971; Brien et al., 1975). In this study no correlation was found between antipyrine elimination and either the steady-state levels of phenytoin or the dose of phenytoin. Hence it seems that antipyrine elimination is of little value in predicting the rate of phenytoin metabolism, or the extent of enzyme induction, in patients receiving long-term anticonvulsant therapy.

2. Psychotic patients

The shorter half-lives of antipyrine in patients receiving long-term chlorpromazine therapy supports evidence presented by Loga et al. (1975), that this drug causes induction of the hepatic mixed function oxidase enzymes in man. They found a reduction in mean half-life from 11.7 hr to 8.5 hr after chlorpromazine therapy (100 mg) 8 hourly for 3 weeks. This was further reduced by addition of orphenadrine (100 mg) or phenobarbitone (50 mg) 8 hourly for 3 weeks. In the present study the median half-life was reduced 40% and is comparable to the reduction in means in the study of Loga et al., which was 38%. It is interesting that the addition of another inducing agent in the latter study caused a further reduction in half-life of antipyrine. This effect could not be seen in the

epileptics in the present study when comparing groups on phenytoin monotherapy and phenytoin plus barbiturate. This may reflect the fact that chlorpromazine is not as potent an inducing agent at these doses as anticonvulsant drugs. In another study Stevenson et al. (1972) were unable to find any evidence of enzyme induction, using antipyrine half-life, in ten elderly schizophrenics given chlorpromazine in a dosage range of 150 to 600 mg daily for a minimum of 2 months. The mean age of these subjects 61 $\pm$ 9 years which is greater than in the subjects in the present study and that of Loga et al.. Stevenson et al. pointed out the possibility that drug metabolism in elderly subjects may not be as readily inducible.

Fluphenazine decanoate did not cause an increase in antipyrine elimination compared to controls. This is in agreement with findings of Latham et al. (1974) who found normal D-glucaric acid excretion and quinine half-lives in six male patients treated with this agent and with Majumbar and Kakad (1978) who found normal plasma gamma-glutamyl transpeptidase activity in twenty-eight schizophrenic patients treated with this phenothiazine. This particular drug is administered by intramuscular injection and because it is released very slowly from the site of injection the resulting plasma levels are very low, usually less than 1 ng/ml (Curry et al., 1979). These levels may not be sufficient to cause any significant inducing effect.

CHAPTER FOUR

IMPAIRMENT OF HEPATIC DRUG METABOLISM IN ALCOHOLICS

INTRODUCTION

As discussed in Chapter II severe liver disease causes impairment of hepatic drug metabolizing enzymes, but there is uncertainty as to the effects of lesser degrees of hepatic impairment on this particular function. It is known that acute hepatitis reduces antipyrine metabolism to a lesser degree than chronic cirrhosis or chronic active hepatitis, and antipyrine metabolism is normal or slightly reduced in obstructive jaundice (Branch et al., 1973; Elfstrom and Lindgren, 1974). One of the major causes of liver disease in the community is alcoholic cirrhosis. Five of the six patients with liver disease in Chapter II suffered from this form of the disease and it was associated with a marked impairment of drug metabolism.

Ethanol is metabolized by the hepatic mixed function oxidase enzymes and it can also have effects on the metabolism of other compounds. These effects appear to differ depending on whether it is administered acutely or chronically. Acute ethanol administration to volunteers has been found to inhibit metabolism of a number of drugs, including meprobamate, pentobarbitone (Rubin et al., 1970) and tolbutamide (Carulli et al., 1971) and this is thought to be due to inhibition of the mixed function oxidase enzymes. On the other hand chronic administration has been found to stimulate metabolism. Antipyrine (Vesell et al., 1971), meprobamate, pentobarbitone (Misra et al., 1971), phenytoin, tolbutamide and warfarin elimination (Kater et al., 1969) were found to be increased on chronic ethanol administration. However, ethanol has

been found to affect cell membrane permeability, cardiac output, tissue blood flow and protein synthesis (Rubin and Lieber, 1967; Tobin and Mezey, 1971; Rothschild et al., 1975; Isselbacher, 1977). Thus mechanisms exist to explain changes in drug disposition other than those involving alterations in drug metabolism. These factors are unlikely to influence antipyrine disposition, as this is largely unaffected by changes in blood flow and protein binding. Hence antipyrine elimination will reflect its rate of metabolism and will not be affected by any potential alterations to distribution or blood flow produced by ethanol.

In the present study the elimination kinetics of antipyrine were examined in a group of alcoholics. Since chronic ethanol ingestion appears to enhance drug metabolism, yet long-term abuse leads to cirrhosis and decreased metabolic activity, it was of interest to examine the activity of drug metabolizing enzymes in a group of chronic alcoholics whose liver function had not been seriously affected by their intemperance. By undertaking such a study it was hoped to evolve the relationship between antipyrine handling and the various parameters of hepatocellular function.

METHODS

1. Subjects

Nineteen male patients, aged 23 to 66 years (mean ± SD 43 ± 11), who were under the management of an Alcohol and Drug Addicts Treatment Unit, were studied. Each one of them had had a drinking problem for more than 8 years. Although an accurate figure for daily alcohol consumption could not be obtained from the subjects, it was estimated that this value ranged from 100-1560 g. Most of the alcohol was taken in the form of beer, fortified wines or spirits. One subject had been taking 200 mg phenytoin per day for epilepsy. The only other medication administered to the subjects was chlormethiazole (Hemineurin), the maximum daily dose of this agent being 3200 mg. Control data was obtained from nineteen healthy male volunteers, aged 21 to 69 years (mean ± SD 29 ± 12) who were not on any medication at the time of the study. These control subjects consumed alcohol occasionally on a social basis.

2. Protocol

Antipyrine was administered to each patient in a dose of 10 mg/kg body weight and elimination kinetics determined as described in Chapter I. A 10 ml blood sample was taken from each alcoholic prior to drug administration and alkaline phosphatase, lactate dehydrogenase, aspartate transaminase, bilirubin, albumin and globulin were determined in plasma by standard Technicon auto-analyser procedures.

3. Calculations

The Mann-Whitney U-test for unrelated samples and the Spearman rank correlation test were used for statistical analysis of the data. Multiple regression was performed using the SPSS Program (Subprogram Regression).

RESULTS

Table 4.1 shows the relevant clinical and biochemical data obtained in the nineteen alcoholic patients. The values for the three parameters used to evaluate kinetics of antipyrine measured in the saliva of the alcoholics were compared with those obtained from nineteen male controls. Half-lives in the alcoholic subjects ranged from 7.3 to 38.0 hr (median = 15.2) and were greater than in controls which ranged from 7.1 to 16.5 hr (median = 11.3, $P<0.05$, two-tail). The values for metabolic clearance rate were less and volume of distribution were greater in the alcoholics (U-test, $P<0.05$, two-tail). These differences are illustrated in Figs. 4.1, 4.2 and 4.3. Inclusion of Subject 5 (who was receiving phenytoin, a known enzyme inducing drug) in the analysis, did not bias these differences.

The lower value for clearance rate in the chronic alcoholics is consistent with the prolonged half-life in this group. The mechanism of increased volume of distribution is uncertain. It may be an artifact due to problems of dose compliance as discussed in Chapter I or it may be that alcoholics have an increased body water content. However this difference, in relative terms, was less than the difference in clearance values between alcoholics and controls and since half-lives were longer and clearance rates less in the alcoholics it can be concluded that these differences are due to changes in antipyrine metabolism. Significant rank correlations were found for both antipyrine half-life and clearance when compared

Table 4.1 Pharmacokinetic data for antipyrine and the results of hepatic function tests in the nineteen alcoholic subjects

Subject	$T_{½}$ (h)	MCR (ml/min/kg)	V_d (ml/kg)	AST (IU/l)	LDH (IU/l)	ALP (IU/l)	Conj Bili (umol/l)	Total Bili (umol/l)	Alb (g/l)	Glob (g/l)
1	7.3	1.068	675	18	195	136	2	10	49	27
2	7.4	1.249	800	15	188	88	1	16	45	33
3	8.4	1.146	834	9	193	78	1	6	51	20
4	9.1	0.963	755	11	225	43	1	5	58	13
5*	10.0	0.990	857	18	231	82	1	7	48	22
6	10.5	0.579	528	43	223	60	2	16	51	31
7	11.9	0.767	793	23	198	102	2	10	34	39
8	14.0	0.512	619	29	197	53	2	28	46	31
9	15.0	0.536	696	67	270	77	1	9	41	36
10	15.2	0.585	768	17	229	99	2	13	36	39
11	16.7	0.473	685	63	257	75	3	20	40	34
12	16.8	0.536	779	50	208	87	2	8	46	34
13	19.1	0.558	923	170	283	137	10	35	42	45
14	21.1	0.453	830	39	217	73	6	27	40	40
15	21.7	0.473	890	39	204	151	3	20	41	37
16	22.1	0.381	730	92	313	100	6	22	40	42
17	26.2	0.488	1107	32	252	76	5	19	44	36
18	29.2	0.436	1102	92	261	106	3	16	40	40
19	38.0	0.301	989	49	253	74	2	9	40	25
Normal range				5-40	140-300	25-95	1-4	6-24	34-44	24-42

$T_{½}$ - half life; CLR - clearance; V_d - apparent volume of distribution; AST - aspartate transaminase; LDH - lactate dehydrogenase; ALP - alkaline phosphatase; Bili - bilirubin; Alb - plasma albumin; Glob - plasma globulins.

* Received phenytoin, 200 mg/day

Figure 4.1 Values for antipyrine half-lives in 19 alcoholics compared to 19 control subjects. The median values in each group are indicated by a bar.

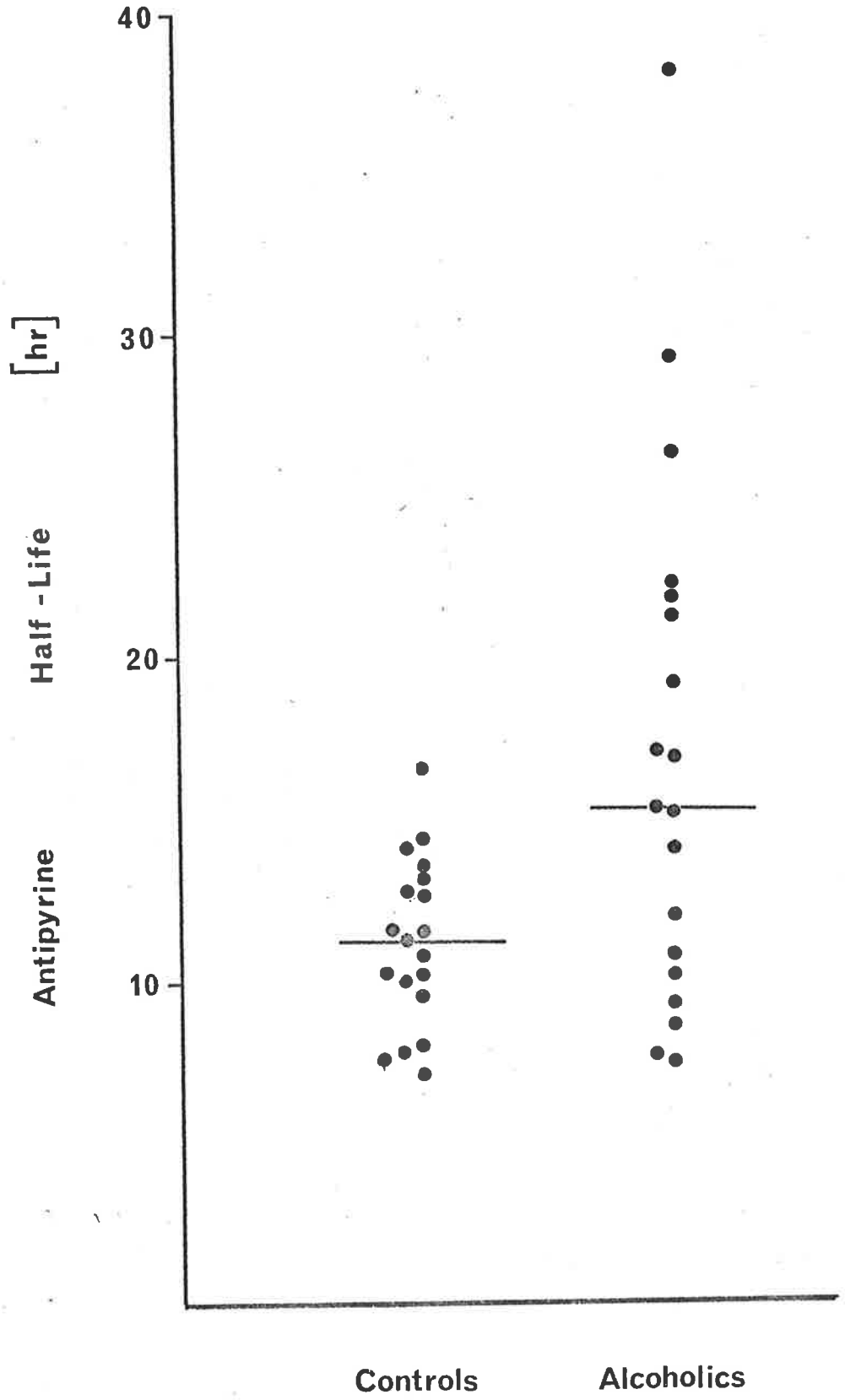

40
30
20
10
[hr]
Half - Life
Antipyrine
Controls
Alcoholics

Figure 4.2 Values for antipyrine volume of distributions in 19 alcoholics compared to 19 control subjects. The median values in each group are indicated by a bar.

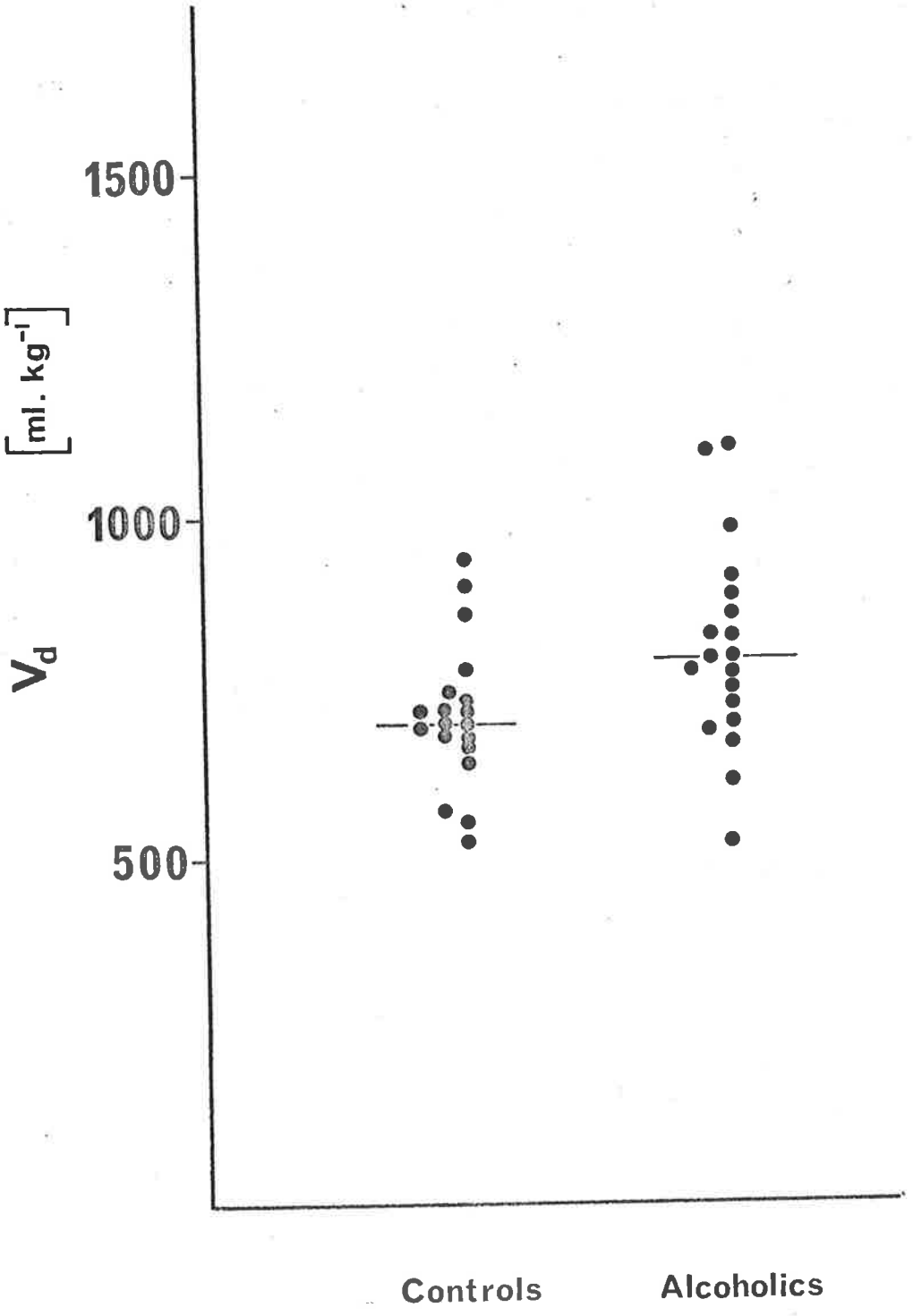
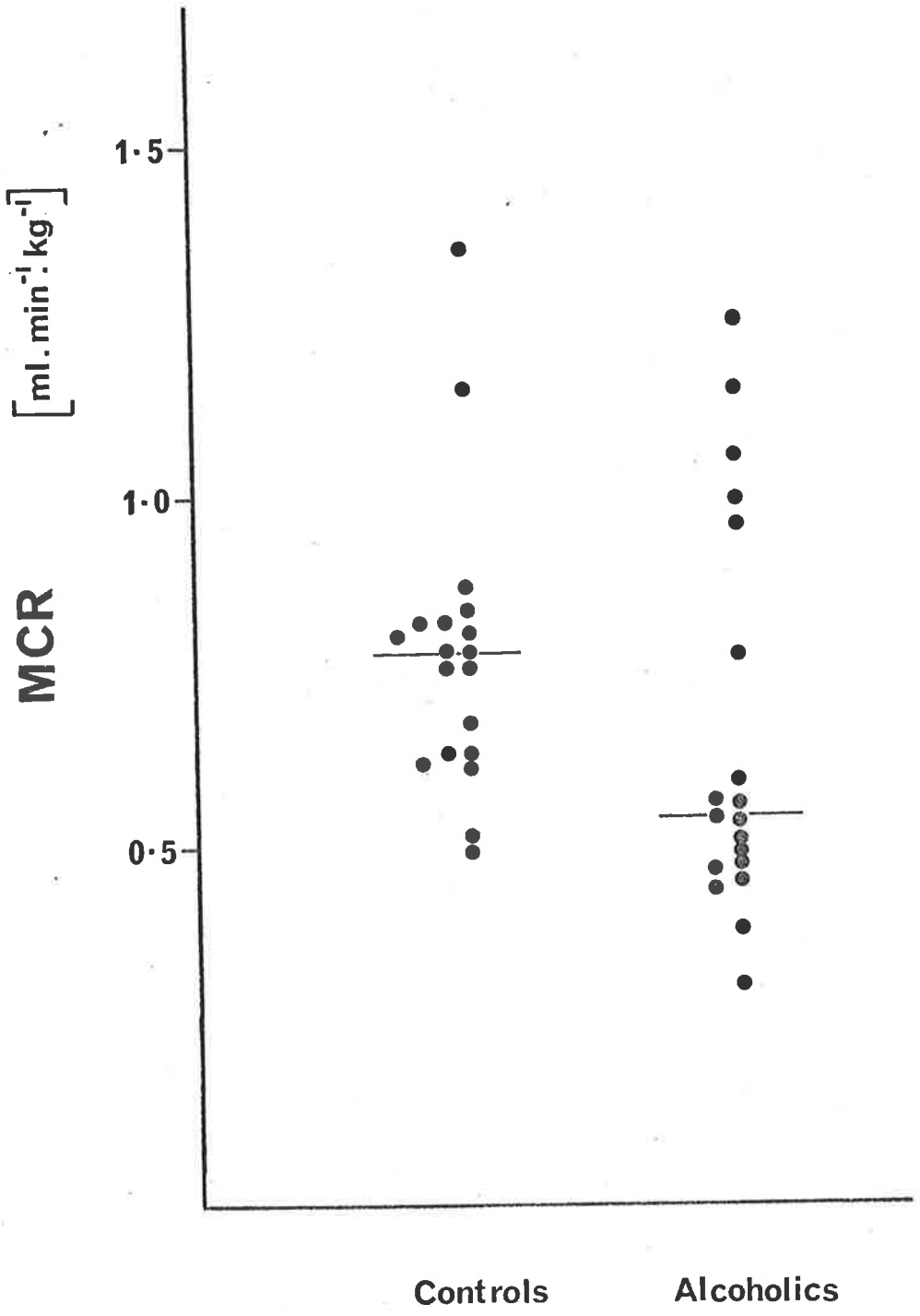

1500
1000
500
V_d [ml. kg^{-1}]
Controls
Alcoholics

Figure 4.3 Values for antipyrine metabolic clearance rate in 19 alcoholics compared to 19 control subjects. The median values in each group are indicated by a bar.


MCR
[ml.min⁻¹.kg⁻¹]
1·5
1·0
0·5
Controls
Alcoholics

with lactate dehydrogenase, aspartate transaminase activities, conjugated bilirubin, albumin and globulin (Table 4.2). An increase in total bilirubin correlated with an increase in half-life, but not with reduced clearance. Multiple regression was used to relate these biochemical liver function tests in combination as predictors of antipyrine half-life. Aspartate transaminase activity was found to contribute 35%, globulin level 13% and albumin level 6% to the variance explained when antipyrine half-life was significantly predicted ($F = 6.10$ with 3, 15 df, $P = 0.006$) by the following equation: antipyrine half-life = 56.8 + 18.7 (log aspartate transaminase activity) - 1.0 (albumin level) - 0.7 (globulin level). The coefficients were all significant at $P < 0.05$. The other tests contributed little to the variance explained and their regression coefficients were not statistically significant. These variables were therefore excluded from the regression analysis.

Table 4.2 Spearman rank correlations between antipyrine half-life and clearance and various biochemical parameters in the nineteen alcoholic subjects

	AST	LDH	ALP	Conj Bili	Total Bili	Alb	Glob
Half-Life	0.70	0.63	0.09*	0.67	0.57	-0.59	0.57
Clearance	-0.72	-0.54	-0.01*	-0.80	-0.39*	0.58	-0.48**

*$P > 0.05$; **$0.05 > P \geq 0.02$

AST - aspartate transaminase; LDH - lactate dehydrogenase; ALP - alkaline phosphatase; Bili - Bilirubin; Alb - plasma albumin; Glob - plasma globulins.

DISCUSSION

Sotaniemi, Ahlqvist, Pelkonen, Pirttiaho & Luoma (1977) found that the ability of the liver to metabolize drugs in alcoholics was related to ethanol-induced changes in the liver. It was confirmed by liver biopsy that changes in antipyrine clearance were related to a reduction in cytochrome P450 content. Hence it appeared that the rate of drug metabolism decreased as the amount of hepatic injury increased. These findings substantiated those of Schoene, Fleischmann, Remmer & von Olderhausen (1972), who found that needle biopsy samples from mild and moderate hepatitis patients of various aetiologies, some of whom were alcoholics, had normal values of cytochrome P450 content, NADPH-cytochrome P450 reductase, aminopyrine-N-demethylase and p-nitroanisole-O-demethylase activities, but that these were decreased in severe hepatitis and cirrhosis.

Nine of the nineteen alcoholics in this study had antipyrine half-lives above the normal range of the controls. This suggests an impaired capacity of the liver to metabolize antipyrine in these individuals. The rise in antipyrine half-life was found to correlate with elevation of lactate dehydrogenase, aspartate transaminase, conjugated bilirubin, total bilirubin, and globulin, and inversely correlate with albumin (Table 4.2). Thus it appears that, as liver damage progresses, the activity of the hepatic microsomal enzymes decreases concomitantly. These findings are consistent with those of Sotaniemi et al. (1977) and were confirmed to some extent by the multiple regression analysis, which showed that aspartate transaminase activity, albumin and globulin levels

could be used to predict antipyrine half-life in these subjects.

In the present study it was not possible to determine the severity of liver damage by needle biopsy sampling. However, the results of the biochemical tests of liver function in these alcoholics indicate an apparently mild degree of hepatic impairment in some. The values of aspartate transaminase and alkaline phosphatase activities were, in appoximately 50% of cases, either outside or at the upper end of the 95% confidence limits of the normal range. These values are considerably less than those found in a group of subjects with severe alcoholic cirrhosis in which antipyrine metabolism was markedly impaired (Chapter II). One explanation for the present findings is that our alcoholic subjects are suffering from more severe hepatic impairment than the biochemical tests suggest. However, these results contrast with those of Mezey (1976), who found evidence of enzyme induction in alcoholics, and Kater, Roggin, Tobin, Zieve and Iber (1969), who demonstrated that alcoholic patients had an increased rate of drug metabolism.

Schuppel and Steinhilber (1973) have shown that an acute ethanol load of 1.4 ml/kg as 25% - v/v ethanol produced an acute reversible inhibition of antipyrine metabolism in man that was found to last for up to 6 hours. Antipyrine elimination kinetics in the alcoholics in this present study were investigated during a period of "drying-out" on the day following admission to the clinic. Since alcoholics have been shown to clear ethanol from the body at least as fast as normal controls (Ugarte et al., 1977) the ethanol levels during the antipyrine study performed would be

very low and an acute interaction between ethanol and antipyrine is an unlikely explanation for the present findings. Another possible interaction is that between antipyrine and chlormethiazole. The latter was used acutely to treat withdrawal symptoms in the alcoholics. The possibility of chlormethiazole inhibiting antipyrine metabolism is discussed in Chapter VII.

One aspect of this study which proved difficult to control was the nutritional status of the alcoholics. Diet has now been shown to influence drug metabolism (Kappas et al., 1976) and must be considered in analysing the results obtained.

It is interesting that all the alcoholics had serum albumin levels which were either within the standard range or above it, the highest value for this parameter being recorded in those subjects with relatively normal hepatic function. The mechanism for this increase in albumin levels is unclear and needs further evaluation.

It is worth noting that the subjects studied were being treated for alcoholism, not liver disease, and hence it is tempting to speculate that there may be a significant proportion of alcoholics in the community with severely impaired hepatic drug metabolizing capacity and undiagnosed liver disease. Measurement of the elimination kinetics of antipyrine in saliva may therefore provide a useful means of determining incipient liver disease in a population of alcoholics.

C H A P T E R F I V E

ENHANCED DRUG METABOLISM IN ANAESTHETISTS EXPOSED TO VOLATILE ANAESTHETIC AGENTS

INTRODUCTION

Lipid soluble anaesthetic agents have been shown to be metabolized by the hepatic mixed function oxidase enzymes in many animals, including man (Brown, 1976). Furthermore, metabolism of these compounds can be enhanced by inducers of mixed function oxidase activity. Phenobarbitone pretreatment of rats has been shown to increase the metabolism of sevoflurane (Cook et al., 1975), methoxyfluane and isoflurane (Mazze et al., 1974). In man, unusually high levels of fluoride after enflurane anaesthesia have been associated with enzyme-inducing drugs (Cousins et al., 1976).

It has been demonstrated in animals that some anaesthetic agents are themselves capable of enzyme induction. Diethyl ether (Brown and Sagalyn, 1974) and nitrous oxide (Remmer, 1962) have been shown to cause enzyme induction in rats. Similarly hexobarbitone sleeping time was shortened in rats exposed to sub-anaesthetic concentrations of ether, halothane and enflurane, but not nitrous oxide (Linde and Berman, 1971).

In man, the evidence for enzyme induction caused by anaesthetic agents is not conclusive. Berman et al. (1976) suggested that an increase in the ratio of 6-beta-hydroxycortisol to 17-hydroxy-corticosteroids in the urine of patients after enflurane-induced anaesthesia was evidence of enzyme induction. Two attempts have been made to show that enzyme induction has occurred in subjects chronically exposed to anaesthetics in a work environment. In a

study by Cascorbi et al. (1970), four of five anaesthetists excreted more radioactivity in 2 hours after intravenous injection of C^{14} halothane than did four pharmacists chosen as controls. In a study by Wood et al. (1974), twenty-three subjects comprising 17 anaesthetists and 6 operating theatre technicians were matched by age and sex to 23 previously studied controls. The difference in mean antipyrine half-life in plasma in these two groups was just statistically significant ($P=0.05$) with a one-tail t-test. However, the difference was much smaller when the anaesthetists alone were compared to the controls.

Inter-subject variation in antipyrine half-life makes changes in metabolism difficult to demonstrate. One way to limit the variability is to study the intra-subject changes in metabolism in anaesthetists, during periods of exposure and non-exposure to anaesthetic agents in an otherwise similar working environment. This approach has the advantage that genetic and many environmental factors which may contribute substantially to inter-subject variability in metabolism may be discounted in the results (Vesell and Page, 1968; Stevenson, 1977).

In the present study, antipyrine elimination kinetics were used to assess metabolic activity in anaesthetists during sequential periods of exposure and non-exposure to anaesthetics, both on an intra- and intersubject basis.

METHODS

Eight anaesthetists from the Royal Adelaide Hospital (7 males, 1 female) aged 26 to 33 years, took part in this study. They were each studied at the end of two periods. During one period the subjects were undertaking routine anaesthetic duties and were exposed to anaesthetic agents (principally halothane and nitrous oxide). Scavenging of the operating theatres was not in use at this time and spot sampling (infrared) revealed typical concentrations of about 200 ppm nitrous oxide and 5 ppm halothane. During the other period the subjects were working on a four week assignment in the intensive care unit (I.C.U.) where no anaesthetic agents were used. The pattern of hours and duty were similar in both situations. In five subjects, the study was performed during the I.C.U. period first; in three the anaesthetic period. Subjects were not studied until they had been working at their assigned duties for at least 3 weeks. Another 13 male anaesthetists provided data from only one of the periods. Group comparisons were made with the male control subjects. Antipyrine elimination kinetics were determined as described in Chapter I. Statistical analysis of the difference between paired data was performed using the Wilcoxon matched-pairs signed-ranks test. Differences between grouped data were analysed by Kruskal-Wallis one-way analysis of variance. All means are given with their standard deviation.

RESULTS

The values for half-lives, clearances and apparent volumes of distribution for the two situations are shown in Table 5.1. The results of antipyrine concentration versus time in one subject, as an example, are shown in Figure 5.1. A within subject comparison revealed that half-life was shorter ($P=0.01$, one-tail) and clearance values higher ($P<0.05$, one-tail) when the anaesthetists were working in the operating theatre environment. This change represents a 17% reduction in mean half-life and a 16% increase in mean clearance while working with volatile anaesthetic agents. There was no change in apparent volumes of distribution in the two work situations ($P>0.1$, two-tail). The difference produced in half-life is illustrated in Figure 5.2.

Previous studies (Cascorbi et al., 1970; Wood et al., 1974) aimed at showing enzyme induction in anaesthetists, have compared data from exposed subjects with those of unexposed control subjects. Table 5.2 shows data obtained from the larger group of anaesthetists studied in theatre or intensive care unit, from which the sample of 8 studied under both conditions was drawn. These were compared using a one-way analysis of variance with a control group of 19 male volunteers not associated with anaesthetic practice. The mean age of this group was similar to that of the anaesthetists but the distribution of age was not. The groups were not found to be different in their ability to metabolize antipyrine (Table 5.2). The reproducibility of antipyrine half-life determinations in control individuals was assessed in 9 of the subjects studied in Chapter I. The initial

Table 5.1 Salivary antipyrine pharmocokinetic data of 8 anaesthetists studied in both the operating theatre and the intensive care unit

			Theatre			ICU		
Subject	Age	Sex	$T_{½}$ (h)	V_d (ml/kg)	MCR (ml/min/kg)	$T_{½}$ (h)	V_d (ml/kg)	MCR (ml/min/kg)
1	31	M	7.1	429	0.694	12.2	597	0.567
2	27	M	7.5	694	1.062	10.3	601	0.675
3	30	F	7.9	510	0.745	8.7	572	0.762
4	35	M	10.0	579	0.670	12.2	724	0.680
5	31	M	10.4	564	0.627	12.7	667	0.608
6	27	M	10.5	744	0.815	14.5	784	0.624
7	29	M	12.9	765	0.684	11.8	691	0.675
8	33	M	19.7	617	0.362	21.7	565	0.301
Mean			10.8	612	0.707	13.0	650	0.612
S.D.			4.0	116	0.915	3.9	79	0.318

Figure 5.1 Disappearance of antipyrine from saliva of a representative anaesthetist under conditions of anaesthetic exposure (AN) and non-exposure (I.C.U.).

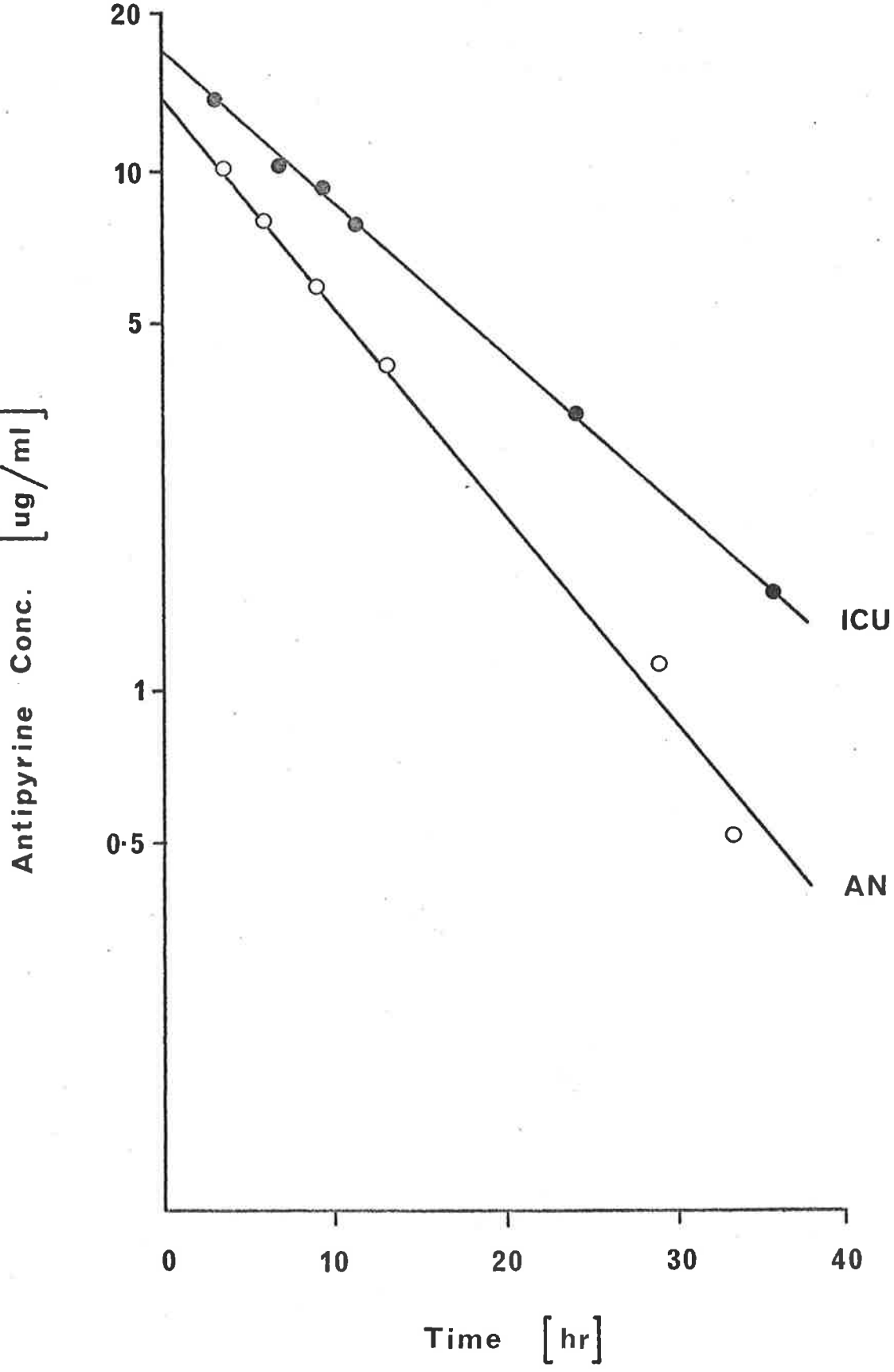

20
10
5
1
0·5
Antipyrine Conc. [ug/ml]
ICU
AN
0
10
20
30
40
Time [hr]

Figure 5.2 Change in salivary antipyrine half-life in 8 anaesthetists from a work environment with anaesthetic exposure (AN) to a non-exposed environment (I.C.U.).

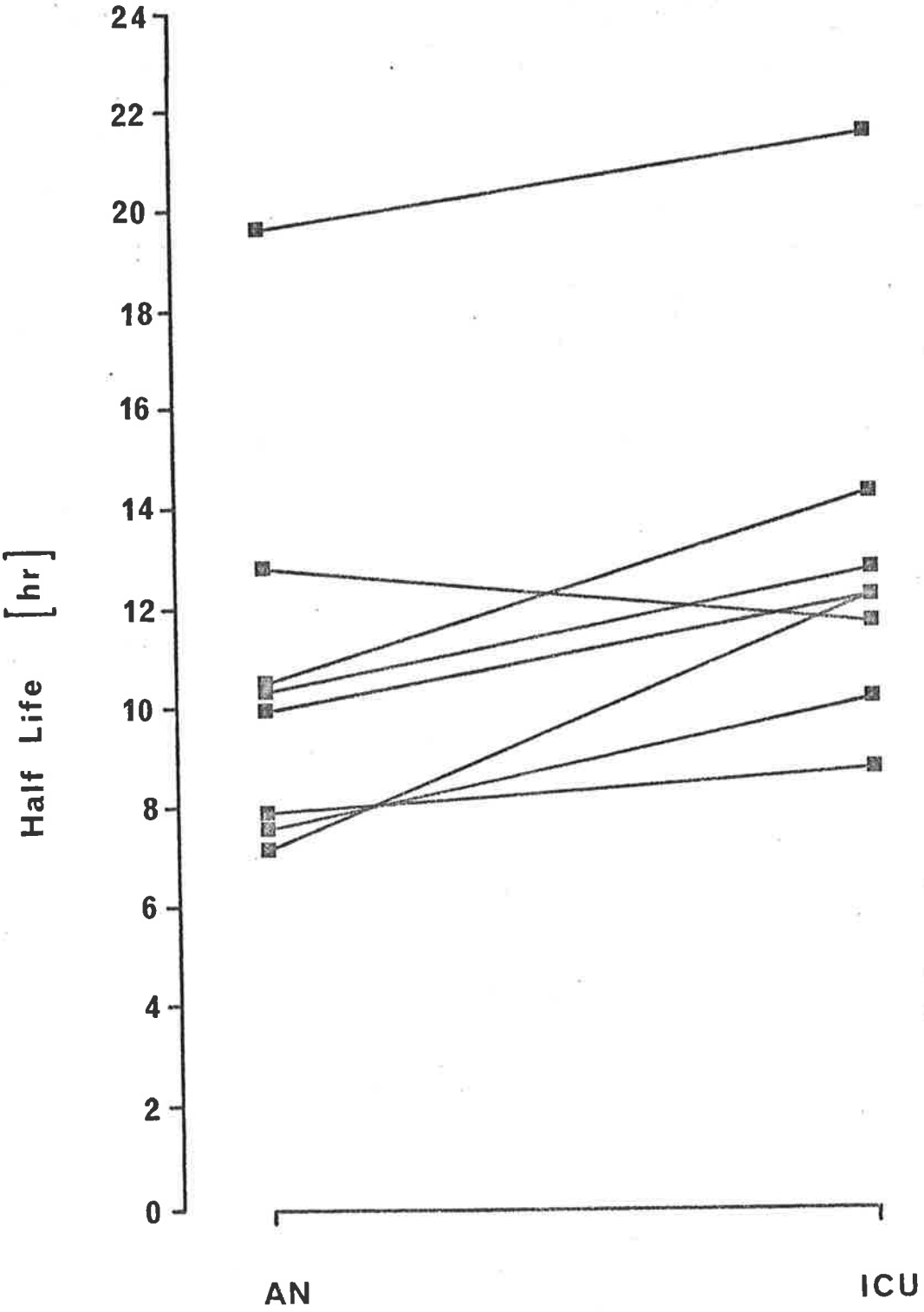

24
22
20
18
16
14
12
10
8
6
4
2
0
Half Life [hr]
AN
ICU

Table 5.2 Antipyrine pharmacokinetic data of 21 anaesthetists and 19 control subjects

	Half-life	MCR (ml/min/kg)	V_d (ml/kg)	n	Age
Anaesthesia	11.7±4.0	0.747±0.257	706±173	18	34±5
I.C.U.	13.3±3.4	0.629±0.130	692±102	11	31±4
Controls	11.2±2.5	0.779±0.204	712±107	19	29±12
H	3.89	4.03	0.17		
Level of sig.	0.2>P >0.1	0.2>P >0.1	0.7>P >0.5		

half-lives of 11.7 $\pm$ 2.8 hr were not changed when reassessed after a period of between 3 months and 3 years in the same subjects, the values in the subsequent study being 12.8 $\pm$ 2.6 hr. ($P > 0.05$, two-tail). This confirms previous reports that antipyrine half-lives are reproducible upon re-examination in the same individuals (Lindgren et al., 1974; Kellermann et al., 1976; Pantuck et al., 1979).

DISCUSSION

This study shows that the same subject, in given working conditions, can be expected to increase his hepatic metabolic activity approximately 16% for antipyrine when exposed to trace levels of anaesthetic agents. It is of interest that this increase was obscured when group comparisons were made because of the wide variability between subjects. This variability in drug metabolism has been shown to be largely due to genetic factors (Vesell and Page, 1968) and hence a large inter-individual variation in rates of metabolism results. This variation is compounded by environmental factors which can influence the activity of hepatic microsomal enzymes. Such factors as diet (Kappas et al., 1976), smoking habits (Hart et al., 1976) and environmental chemicals (Kolmodin et al., 1969) can produce further between-subject variability. This may explain the difficulty encountered for example, in studies by Wood et al. (1974), in attempting to determine a change in hepatic enzyme activity using modest sample sizes. However, when using a subject as his/her own control, variability due to genetic factors and to some extent environmental factors, is eliminated.

The change in metabolic activity is small compared with that expected after moderate doses of anticonvulsants as seen in Chapter III. It may be that longer exposure to anaesthetics would produce a greater degree of enzyme induction, however, this has not been demonstrated in the present study.

The identity of the anaesthetic agent responsible for the

effect has not been determined. Brown and Sagalyn (1974) found that N_2O did not effect hexobarbitone sleeping time in rats but Remmer (1962) found, in the same species, that this agent had inducing properties. Linde and Berman (1971) have claimed that halothane enhances hexobarbitone metabolism but other evidence suggests that halothane has no inducing properties (Remmer, 1972).

Thus it is not possible to discriminate between the two agents, viz. halothane or nitrous oxide, as to which is causing the inducing effect.

The clinical significance of the present findings is small in terms of the potential need to alter the dose of any drug given to exposed anaesthetists. However, this small degree of enzyme induction contributes to the variability in drug response in man as do such factors as smoking (Hart et al., 1976), dietary consitituents and environmental chemicals (Conney et al., 1977).

Volatile anaesthetic agents, such as halothane, have been shown to be metabolized to reactive species that covalently bind to cellular macromolecules. Induction of the hepatic mixed function oxidase enzyme system has been shown to enhance this binding (Sipes and Brown, 1976). It has been suggested that tissue damage may be mediated by these reactive metabolites (Mitchell et al., 1973). There have been reports suggesting an increased incidence of cancer (Corbett et al., 1973) and spontaneous abortion and birth defects (Cohen et al., 1971; Corbett et al., 1974) in persons occupationally exposed to volatile anaesthetic agents and it has been

speculated that a possible mechanism of toxicity is via reactive metabolites of these agents (Cascorbi, 1977). However the long-term effects of the degree of enzyme induction found in the present study in relation to the development of certain types of disease states has yet to be established.

C H A P T E R S I X

INDUCTION OF MICROSOMAL DRUG METABOLISM IN MAN AND IN THE RAT BY EXPOSURE TO PETROLEUM

INTRODUCTION

Exposure to environmental chemicals may produce alterations in physiological systems which can lead to changes in absorption, distribution, metabolism and excretion of drugs and other compounds. In particular, animal studies have shown that environmental exposure to numerous substances can effect the activity of hepatic microsomal mixed function oxidases (Conney, 1967; Remmer, 1972).

Some compounds, such as organophosphate insecticides, may inhibit the hydroxylation of drugs and steroids when given chronically (Rosenberg and Coon, 1958; Welch et al., 1967), but by far the majority of these compounds cause induction of this enzyme system (Conney, 1967; Remmer, 1972). Compounds in this category include insecticides such as DDT, chlordane and dieldrin, and environmental carcinogens such as 3-methylcholanthrene and 3,4 benzo(a)pyrene. Recently, polychlorinated and polybrominated biphenyls, compounds widely used in industry, have been shown to have enzyme inducing properties in animals (Alvares et al., 1973). In man it has been demonstrated that chlorinated hydrocarbon insecticides increase antipyrine metabolism in workers exposed to these compounds (Kolmodin et al., 1969). Hence antipyrine elimination kinetics may be a useful test for detecting chemicals in the environment that can alter the activity of hepatic microsomal enzymes.

In the present study, the effects of chronic petrol exposure

on the activity of the enzymes was assessed. Petrol is one of the most commonly used solvents throughout the world, yet very little is known of the effects of long-term exposure. Studies that have been done are usually concerned with the levels of exposure to constituents of petrol, such as lead. Since petrol consists of a wide variety of lipid soluble compounds, many of these may be capable of inducing enzyme activity. The kinetics of antipyrine elimination were determined *in vivo* in petrol station workers occupationally exposed to this agent and *in vitro* enzyme activities were determined in 10,000 x g hepatic microsomal supernatant preparations from rats exposed to petrol vapour for three weeks.

METHODS

Human Study

1. Subjects

Nineteen male petrol station workers, aged 16 to 50 years, mean 28 ± 10 were examined in this study. All had worked as driveway attendants and/or mechanics for more than one year and were not taking any medication at the time of the study. Thirteen of these workers participated in a study of blood and urine parameters in an attempt to find a measure of exposure to petrol. On the morning prior to the study, a 20 ml venous blood sample was collected from each of the subjects. On the following morning, the third day of a six day working week, they began a 24 hour urine collection into plastic bottles containing 50 ml of 2 M nitric acid as a preservative. At this same time they took an oral dose of antipyrine (10 mg/kg) and collected saliva samples as described in Chapter I. The remaining six workers participated in this latter part of the study only. Antipyrine analysis was performed as described in Chapter I.

2. Urine Analysis

(a) Phenol

Phenol levels in urine were determined by the distillation method of Schmidt (1949). This method consists of determining the total phenolic value in urine (phenol and p-cresol) by two separate colourimetric techniques and then solving values for the individual phenolic components by simulteneous equations. Control values in normal male

volunteers were obtained from 10 male subjects who worked in this University.

(b) Lead

Urinary lead level analysis was performed at the Institute of Medical and Veterinary Science, Frome Road, Adelaide.

3. Blood Analysis

(a) Aminolaevulinic Acid Dehydratase (ALA-D) activity:

ALA-D activity was determined in whole blood as follows: to 1.3 ml of 0.2% Triton X-100 in 0.05 M phosphate buffer (pH 7) in a Thunberg tube was added 0.2 ml whole blood. To the side-arm was added 0.1 ml of 20mM aminolaevulinic acid in phosphate buffer. The tube was evacuated with a water aspirator and the solutions mixed and incubated at $37^{o}C$ in a shaking water bath (Gallenkampf). After 30 min., 1 ml of 10% $HgCl_2$ in 10% trichloroacetic acid was added to stop the reaction. This mixture was centrifuged for 5 min. at 3000 rpm and 1.5 ml of clear supernatant added to 1.5 ml of modified Erhlich's reagent (see Chakrabarti et al., 1975) in a 10 ml test-tube. After 15 min. the resultant colour was read at 553 nm in a Unicam SP1800 Ultraviolet Spectrophotometer with a 1 cm light path against a blank (ALA added after incubation). The amount of porphobilinogen formed was calculated using the method of Granick et al.,

(1973), taking the molar absorbance of the porphobilinogen/ Errlich pyrrole as 6.1×10^4 L $mole^{-1}$ cm^{-1} and correcting for dilution and haematocrit. The blood haematocrit was measured with a micro-capillary centrifuge (Hawksly, London).

(b) Blood Lead

One ml of whole EDTA blood was mixed with 4 ml of 0.25% Triton X100 and the analysis carried out as for urinary lead levels.

(c) Blood biochemistry

Blood biochemical analysis was performed using standard Technicon autoanalyser techniques. Parameters measured, included sodium, potassium, chloride, bicarbonate, osmolarity, glucose, urea, creatinine, urate, phosphate, calcium, albumin, globulins, cholesterol, conjugated and total bilirubin, alkaline phosphatase activity, lactic dehydrogenase, aspartate transaminase and creatinine kinase.

Animal Studies

1. Animals

Male Porton rats, weighing 100-150g were obtained from this University's animal breeding house. While not in the exposure chambers they were kept in a 12 hour light/dark cycle and allowed food and water ad libitum. Six rats were exposed to a metered concentration of 5 mg/L petrol vapour for 8 hours/day. Six control rats received the same air source without petrol vapour.

2. Apparatus

Perspex exposure chambers of approximately 40 L capacity were supplied with an airflow of 4 L/min from a respiratory pump (C.F. Palmer, London). The air flow from this pump was split, with half flowing to the chamber housing the control rats and the other passing to the top of a petrol vapourizing chamber (Figure 6.1). This consisted of a water-heated, vertical 22 x 4 cm Pyrex tube. The middle 16 cm had a glass spiral tube through which water at 40^{o}C was pumped. Petrol (commercial grade, 50% super: 50% standard) was introduced into the top of the spiral at a rate of 20 mg/min and allowed to evaporate on its surface. The resultant vapour was carried off by the air stream through a tube at the base, passing to the chamber housing the exposed rats (Figure 6.2). Petrol flow was controlled using a variable speed peristaltic pump (Desaga, Heidelberg).

3. In vitro microsomal enzyme activities

After 21 days exposure, rats were killed by decapitation and 2 ml blood samples collected into heparinized tubes. The livers were perfused in situ with ice-cold saline via the portal vein and placed in 20 ml of ice-cold 0.25M sucrose / 0.05M Tris (pH 7.4). All subsequent operations were carried out at 0-4^{o}C. The livers were then weighed and homogenized using an Ultra Turrax blender. The homogenate was centrifuged for 20 min. at 10000 x g and the resulting supernatant decanted and the volume adjusted to 400 mg liver/ml with the sucrose / Tris buffer. The incubation mixture consisted of 0.5 ml of the supernatant preincubated for 2 min. before addition to 2.5 ml of Tris buffer.

Figure 6.1 Schematic diagram of the heated glass coil used to vapourize the petrol. Petrol (20 mg/min) was fed onto the top of the heated (40^{o}C) coil and the resultant vapours carried out the base of the column by an air stream (4 L/min) to the exposure chamber.

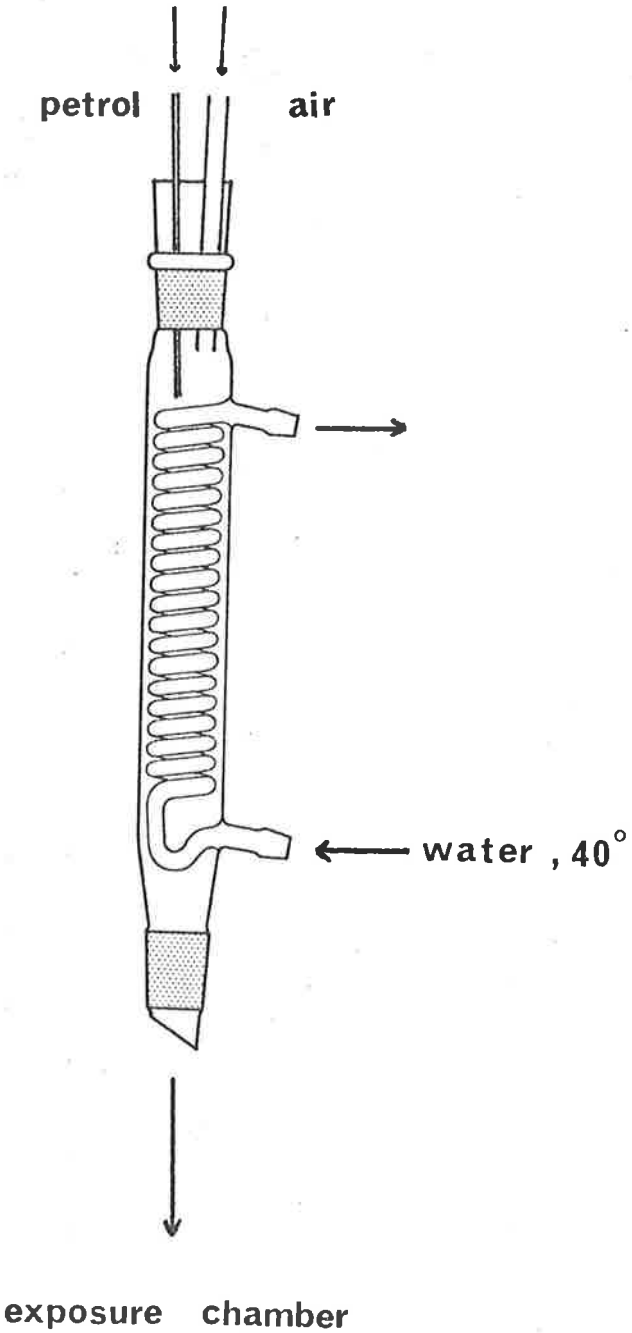
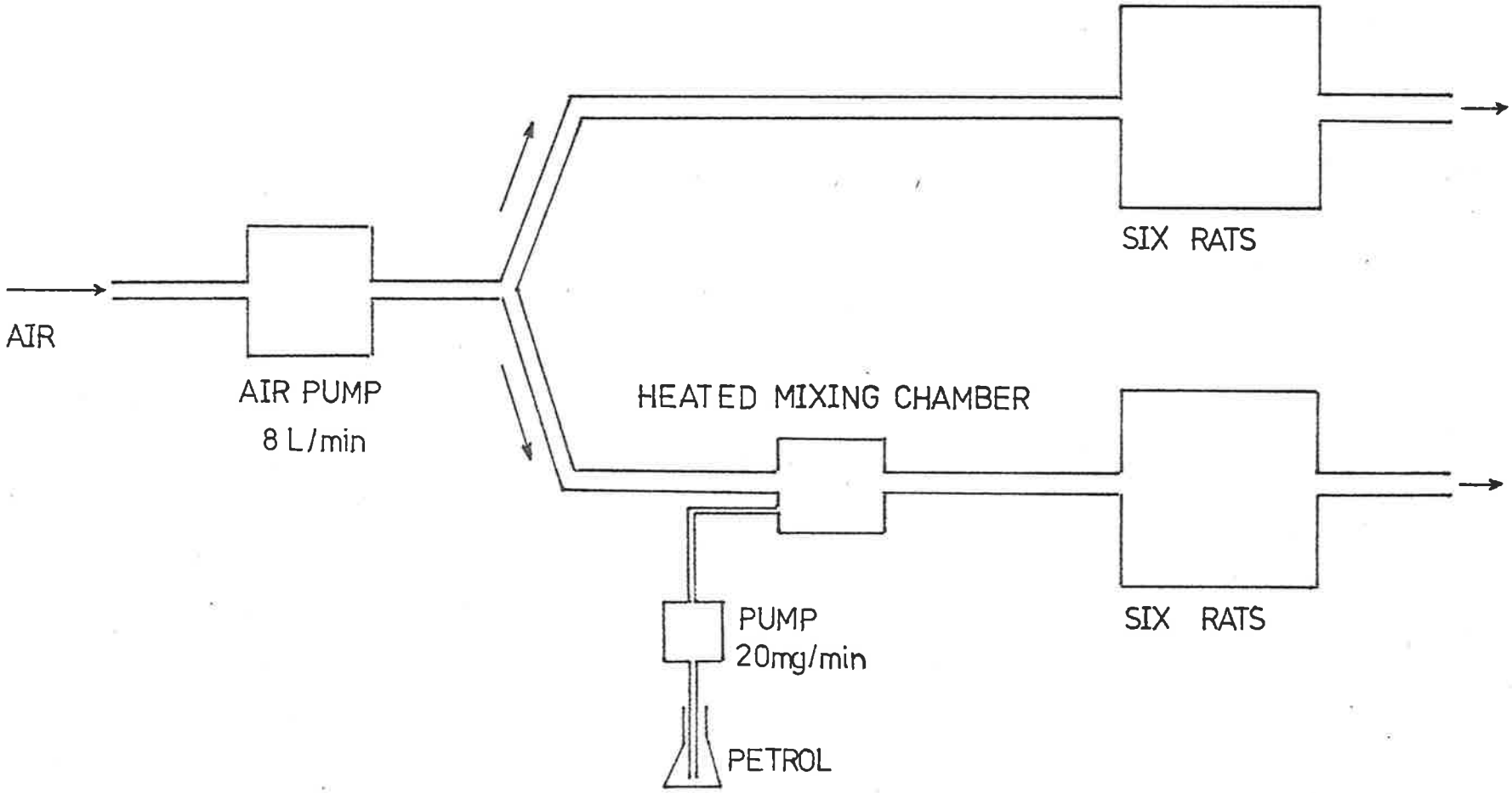

petrol
air
water , 40°
exposure chamber

Figure 6.2 Schematic diagram of the system in which rats were exposed to petrol vapour. An air stream from a common source was split, one stream passing to a chamber housing six control rats, the other passing to the vapourizing chamber (see Figure 6.1). After petrol vapour had been mixed with this air stream it passed to six rats in an exposure chamber. Air passing out of the chambers was exhausted in a fume hood. Both chambers were of approximately 40 L volume.


AIR
AIR PUMP
8 L/min
SIX RATS
HEATED MIXING CHAMBER
SIX RATS
PUMP
20mg/min
PETROL

This 3 ml contained 5mM Mg Cl_2, 0.33 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 50 mM Tris buffer (pH 7.4) and substrate. The incubations were carried out in a metabolic shaker bath at 37°C under air at 80 oscillations per min. with a glass marble added to each tube to facilitate mixing. Substrate concentrations were 5mM for aminopyrine, ethylmorphine, aniline and antipyrine and 80uM for benzo(a)pyrene (added as 15 ul of a 4 mg/ml solution in acetone). Demethylase activities and aniline-p-hydroxylase activity were determined as described by Schenkman et al. (1967) except that semicarbazide (5mM) was added to trap formaldehyde formed during demethylation. Benzo(a) pyrene hydroxylase activity was determined using a modification of the method of Nebert and Gelboin (1968). The incubation was stopped by the addition of 3 ml ice-cold acetone and 3 ml of this mixture was extracted with 10 ml n-hexane in a 20 ml glass extraction tube for 10 min. and then centrifuged for 5 min. at 3000 rpm. Three ml of the organic phase was back extracted into 3 ml 1M NaOH and the fluorescence of the aqueous phase read at 522 nm with the excitation at 396 nm in a spectrofluorimeter (Farrand Optical Co., New York). In the blank, substrate was added after incubation. Antipyrine metabolism was determined as the amount of water-soluble metabolites produced during incubation. The antipyrine used as substrate was spiked with (N-methyl-^{14}C) antipyrine (2.2×10^5 dpm/vial). The reaction was stopped with 0.6 ml 5 M NaOH and 3 ml of this mixture was extracted with 10 ml chloroform for 30 min. Unchanged antipyrine only was extracted into the organic phase (Bakke et al., 1974) leaving the metabolites in

the aqueous phase. After centrifugation for 30 min. at 3000 rpm, 2 ml of the aqueous medium was transferred to a counting vial containing 10 ml of scintillant (0.17g 1,4-bis (2(4-methyl-5-phenyl-oxazolyl)) benzene, 5.3g 2,5-diphenyloxazole, 333 ml Triton X100, 666 ml toluene) and the activity determined in a liquid scintillation counter (Packard, Model 3310). In the blank, substrate was added after incubation. The amount of antipyrine metabolized, allowing for volume corrections, was determined as follows:

antipyrine metabolized = ((dpm in aqueous phase - dpm in blank) /total dpm added) x initial amount in incubation vial

Incubation times were 10 min. for ethylmorphine and benzo(a)pyrene, 15 min. for aminopyrine and aniline and 30 min. for antipyrine.

Aniline aminotransferase (ALAT) activity and bilirubin content of rat plasma were assayed by standard Technicon autoanalyser techniques.

4. Analysis of data

Comparison between groups was made with the Mann-Whitney U-test. The Spearman rank test was used to test correlation, and the classification measurements were made using chi-squared analysis (Colquhoun, 1971).

RESULTS

Antipyrine metabolism in petrol station workers

Antipyrine, 10 mg/kg, was given as a single oral dose to each petrol station worker and the elimination rate of antipyrine determined. As illustrated in Figure 6.3, the half-lives ranged from 4.2 to 17.9 hr, median 9.7 hr with 98% confidence limits of 8.4 and 10.8 hr. These values were shorter than in the 19 male control subjects studied in Chapter I where values ranged from 7.1 to 16.5 hr, median 11.3 hr with 98% confidence limits of 9.5 and 12.9 hr ($P < 0.05$, one-tail).

Parameters of Exposure

1. Urinary phenol

The 24 hr urinary excretion of phenol in the 13 petrol station workers who participated in the blood and urine collections ranged from 4.8 to 34.1 mg, median 12.2 mg with 98% confidence limits of 6.6 and 21.0 mg. These values were not different from those found in 10 male volunteers whose values ranged from 5.7 to 15.6 mg, median 8.8 mg with 98% confidence limits of 5.9 and 14.0 mg ($P > 0.05$, one-tail). These results are shown in Figure 6.4. The temperature range during the day of the urine collection was 13-19^{o}C. The values obtained in the control subjects are very similar to those found in urine from normal subjects by Schmidt (1949). There was no rank correlation between antipyrine half-life and phenol excretion ($r_s = 0.29$, $P > 0.05$)

Figure 6.3 Antipyrine half-lives in petrol station workers and control subjects. The median value of each group is shown by a bar.

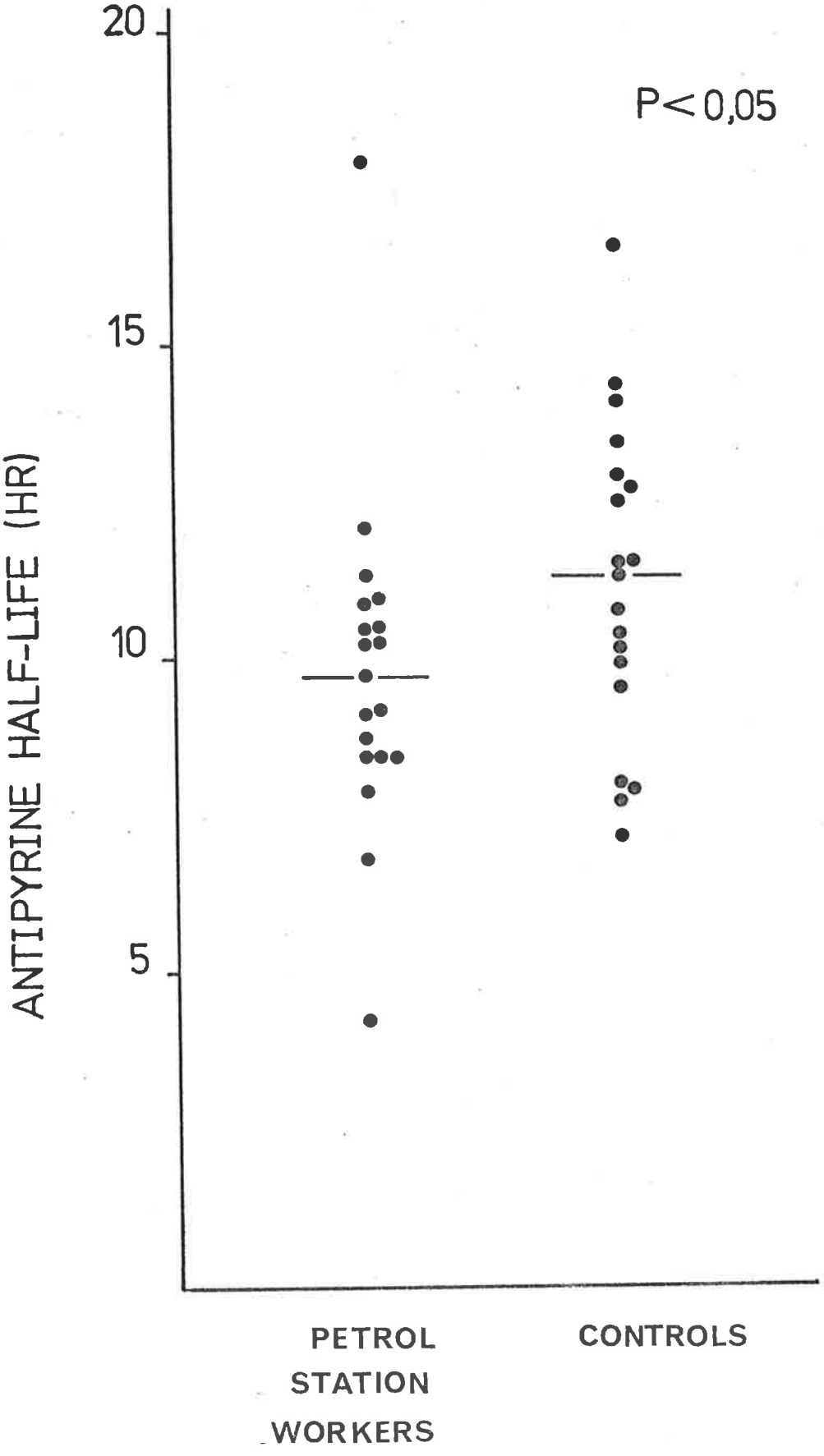
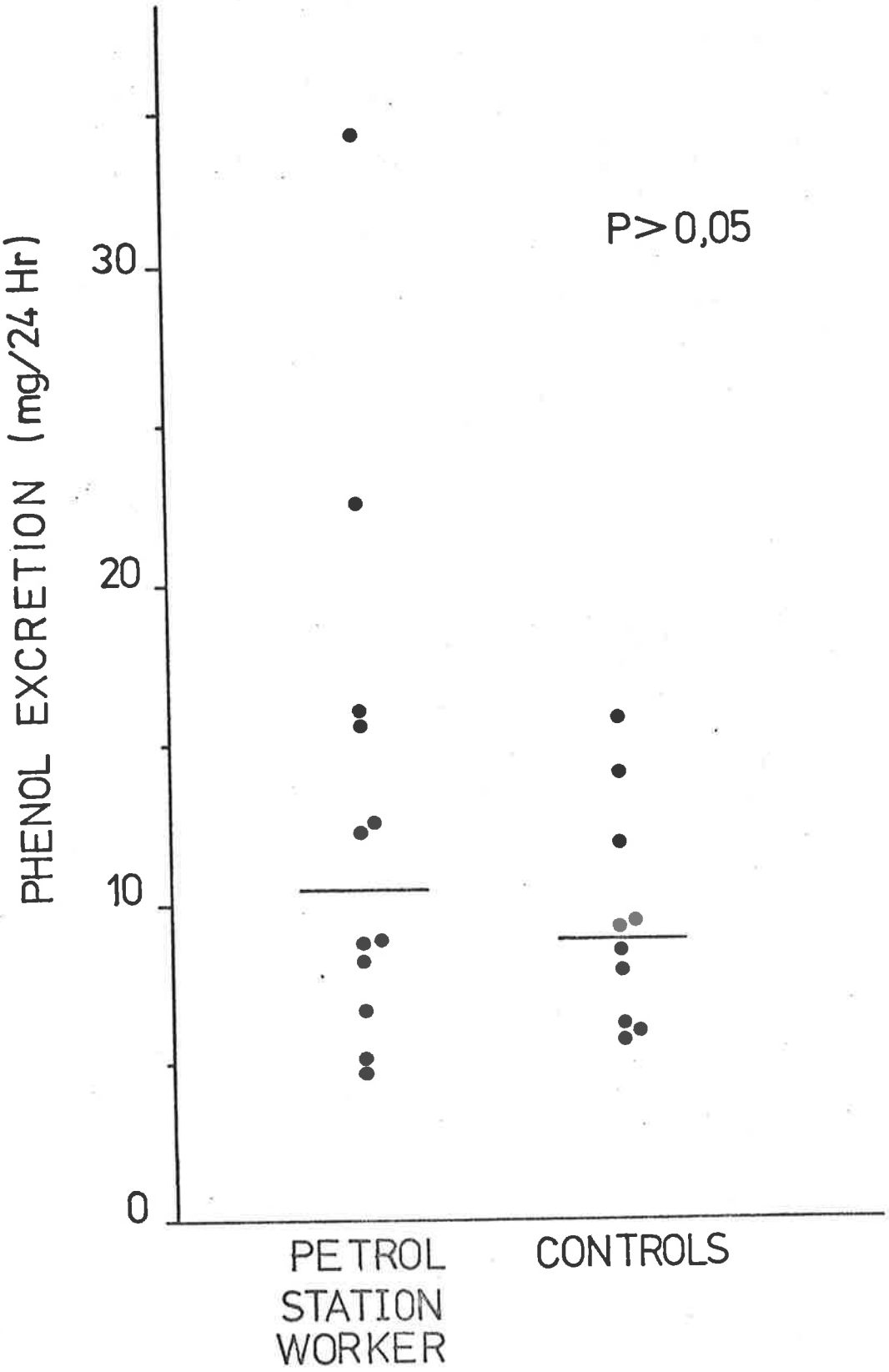

20
15
10
5
ANTIPYRINE HALF-LIFE (HR)
P< 0,05
PETROL STATION WORKERS
CONTROLS

Figure 6.4 The amount of phenol excreted in 24 hr urine samples from 13 petrol station workers and 10 controls. The median value of each group is represented by a bar.


PHENOL EXCRETION (mg/24 Hr)
30
20
10
0
P>0,05
PETROL STATION WORKER
CONTROLS

2. Lead content

Values for blood and urinary lead and blood ALA-D activity in the 13 workers are shown in Table 6.1. The urinary lead content in the 24 hr urine samples of the petrol station workers ranged from 0.04 to 0.20 umol /24 hr, median 0.07 umol with 98% confidence limits of 0.06 and 0.17 umol. These values are all well within the range for normal non-exposed workers quoted by Green et al. (1976) which was 0.10 - 0.50 umol/24 hr. The blood lead content in the workers studied here ranged from 0.79 to 1.56 uM, median 0.95 uM, with 98% confidence limits of 0.80 and 1.15 uM. These values are well below the limit for non-exposed subjects which is 1.7 uM (Green et al., 1976). The levels of ALA-D activity in the petrol station workers were consistent with these findings of normal values for blood and urinary lead. ALA-D activity values ranged from 4.25 to 16.9 nmole porphobilinogen formed / min / ml RBC with a median of 11.8 nmole formed and 98% confidence limits of 7.5 and 15.8 nmole formed. These values are consistent with values found in normal subjects in a study by Meredith et al. (1978), who found a mean value of 13.5 nmole porphobilinogen formed / min / ml RBC in 37 subjects with no known exposure to lead. There was no significant rank correlations between half-life and either urinary lead (r_s = 0.14, $P>0.05$), blood lead (r_s = 0.40, $P>0.05$) or ALA-D (r_s = 0.19, $P>0.05$). A significant correlation was found between blood lead levels and ALA-D activity (r_s = 0.48, $P<0.05$) and this result is consistent with previous findings (Meredith et al., 1978; Chakrabarti et al., 1975; Lauwarys et al., 1973) that a correlation exists between these two parameters.

Table 6.1 Values for plasma and urinary lead levels and plasma ALA-D activity in thirteen petrol station workers

Subject	Lead Level		ALA-D[c]
	Blood[a]	Urine[b]	
1	1.04	0.08	7.5
2	0.80	0.06	14.5
3	0.79	0.04	15.8
4	0.91	0.15	11.9
5	1.15	0.07	7.1
6	0.82	0.07	11.9
7	0.95	0.06	16.9
8	0.79	0.12	16.6
9	1.56	0.20	11.8
10	1.42	0.12	4.3
11	1.03	0.05	15.9
12	1.15	0.07	10.4
13	1.03	0.17	13.6
Mean	1.03	0.10	11.6
S.D.	0.24	0.05	3.8
Normal range of controls	<1.93	(0.10-0.50)	(7.9-19.1)

(a) uM

(b) umole/24 hr

(c) nmole porphobilinogen formed / min / ml RBC

These results indicate that the petrol station workers did not appear to accumulate lead to a greater extent than that expected in a normal population group.

3. Blood Biochemistry

No abnormalities were revealed by the biochemical tests of blood chemistry in the 13 workers.

4. Social History

An attempt was made to relate antipyrine metabolism in the 19 workers studied with various factors some of which have been shown previously to alter metabolism of this drug. They included age, work duration, smoking habits, alcohol and caffeine intake. These are listed in Table 6.2. No correlations were found between half-life and age (r_s = 0.1, $P>0.1$) or work duration (r_s = 0.06, $P>0.1$). Using chi-squared analysis, no relationships were found between half-life and alcohol consumption, cigarette smoking or caffeine intake (Table 6.3).

Animal Study

As shown in Table 6.4, all hepatic microsomal enzyme activities measured in the 10000 x g microsomal supernatant preparations showed increases in the petrol exposed rats compared to controls. Aminopyrine N-demethylase activity was increased 65%, ethylmorphine N-demethylase 79%, antipyrine metabolism 54%, benzo(a)-pyrene hydroxylase activity 45% and aniline hydroxylase 57% in the exposed rats. All these increases were highly significant

Table 6.2 Antipyrine half-lives and age, work duration and parameters of social drug intake in the nineteen petrol workers

Subject	Half-life	Age	Work Duration	Alcohol* Intake	Caffeine** Intake	Smoking***
1	4.2	20	4	0	1	0
2	6.8	16	1	1	2	1
3	7.8	28	11	1	1	1
4	8.4	33	18	2	2	0
5	8.4	47	33	0	2	1
6	8.4	22	7	1	1	0
7	8.7	19	3	2	1	1
8	9.1	23	7	1	1	0
9	9.1	42	20	1	2	1
10	9.7	19	3	1	1	0
11	10.2	17	2	0	0	0
12	10.2	42	25	0	2	0
13	10.4	27	10	1	1	0
14	10.5	50	10	2	2	1
15	10.8	30	12	2	1	2
16	10.9	20	2	0	1	0
17	11.3	21	4	1	1	1
18	12.1	27	10	0	1	0
19	17.9	30	15	1	2	0

* 0 - no alcohol; 1 -$\leq$ 1 bottle beer/day or equivalent; 2 -$>$1 bottle beer/day

** 0 - no coffee or tea; 1 -$\leq$ 5 cups coffee or tea/day; 2 -$>$5 cups coffee or tea/day

*** 0 - non-smoker; 1 -$\leq$ 20 cigarettes/day; 2 -$>$20 cigarettes/day

Table 6.3 Relationship between antipyrine half-life and parameters of social drug intake in the nineteen petrol station workers

Habit Classification	Percent of group	Mean Half-life	Chi-square
Smoking			
0	58	10.1	0.50
1	37	8.9	$P > 0.5$
2	5	10.8	
Alcohol			
0	32	9.3	0.36
1	47	10.0	$P > 0.8$
2	21	9.5	
Caffeine			
0	11	10.5	0.44
1	53	9.3	$P > 0.8$
2	37	10.1	

Table 6.4 Enzyme activities in 10,000 x g hepatic microsomal supernatant preparations from control and petrol exposed rats. The liver to body weight ratio in both groups is also shown. All increases in petrol exposed rats are significant ($P < 0.01$).

	Control	Petrol Exposed	Exposed/control
Aminopyrine[a] N-demethylase	74 ± 7	122 ± 11	1.65
Ethylmorphine[a] N-demethylase	75 ± 10	134 ± 21	1.79
Antipyrine metabolism[b]	35 ± 2	54 ± 5	1.54
Aniline hydroxylase[c]	21 ± 1	33 ± 3	1.57
Benzo(a)pyrene hydroxylase[d]	42 ± 3	61 ± 8	1.45
Liver/Body weight ratio	0.059 ± 0.001	0.067 ± 0.002	1.14

a. nmole HCHO/min/g

b. nmole ^{14}C/min/g

c. nmole PAP/min/g

d. relative fluoresence units

($P<0.001$, two-tail). The liver/body weight ratio was also increased in the petrol exposed rats ($P<0.01$, two-tail) although body weights in the two groups were not different ($P>0.1$, two-tail), nor was the increase in body weight over the 21 day exposure (89% in control vs. 75% in petrol exposure).

There was no difference in plasma alkaline phosphatase and bilirubin levels between control and exposed rats ($P>0.1$, two-tail) indicating an absence of any overt liver damage that may have been caused by petrol exposure. The values for bilirubin were 0.45 ± 0.33 mg/100 ml in controls and 0.28 ± 0.18 in exposed rats and the levels for (ALAT) activity were 45.7 ± 11.3 I.U./L in controls and 46.2 ± 12.0 I.U./L in exposed rats.

DISCUSSION

Man is becoming increasingly exposed to a wider range of environmental chemicals, many of which have been shown in animals to stimulate their own metabolism or the metabolism of other compounds. Hence the possibility exists that workers, who come in contact with sufficient amounts of certain compounds, may have alterations produced in their bodies of the activity of microsomal mixed function oxidases. In one instance, with workers exposed to halogenated hydrocarbon insecticides, a change in the activity of these enzymes has been noted. In a study by Kolmodin et al. (1969), workers in a pesticide factory, exposed to large amounts of DDT and lindane were found to metabolize antipyrine faster than a group of control subjects. These findings were supported by evidence from Poland et al. (1970) who found that phenylbutazone was metabolized faster than normal, and the urinary output of 6-beta-hydroxycortisol was greater in workers in a factory manufacturing DDT than in a control group.

In this study, antipyrine metabolism in a group of petrol station workers was enhanced when compared to a group of normal volunteers. This may indicate that a chemical or chemicals to which these workers are exposed may be causing this effect. One obvious candidate as a potential inducing agent is petrol. Petrol consists of a hetergeneous mixture of hydrocarbon molecules with the number of carbon atoms ranging from four to twelve per molecule. Hence it is useless to speculate which compounds or types of compounds

are the potential inducing agents in this mixture. However, previous studies have shown the possibility of petroleum acting as an enzyme inducing agent. Payne and Penrose (1975) found increased aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity in microsomal preparations of fish exposed to a 1 ppm emulsion of crude oil in water. More indirect evidence was produced by Kala et al. (1978) who demonstrated that when gasoline was given intraperitoneally to rats in daily doses of 3 ml, there was an increase in alkaline phosphatase activity after 2 days treatment. Further to these studies, it has been shown that halogenated benzenes, compounds found in petrol, increase cytochrome P450 content in the liver of rats given daily doses of 20 mg/kg p.o. (Carlson, 1978). Evidence that induction may occur in man comes from a study by Raitcheva and Zlatarev (1970) who found increased serum activity of glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase and ornythil-carbamyl transferase in oil-tanker fleet workers. The latter activity was chosen as a typical hepatic enzyme, however, it is questionable whether it reflects changes that occur in the membrane bound enzymes in the liver. Hence this is only indirect evidence, at best, that the activity of the mixed function oxidase enzymes can be induced by petroleum in man.

In an attempt to clarify the situation with respect to petrol and its enzyme inducing properties, rats in this study were exposed to petrol vapour over a period of twenty-one days and hepatic microsomes prepared. Evidence of enzyme induction

was found for the activities of enzymes of five substrates. These were aminopyrine, ethylmorphine and antipyrine whose enzyme activities are associated with cytochrome P450 and benzo(a)pyrene and aniline which are associated with metabolism linked to cytochrome P448. Hence the effect seems to be that of generalized induction of the microsomal mixed function oxidases. This is not surprising, since the possibility exists that a number of compounds could be causing this effect. For the same reason it is not possible to determine the potency of the inducing agent or agents as it could be a small number of very potent compounds at low concentration or the inductive effect seen may be a general response of the liver when presented with a reasonably large dose of organic compound of any type.

If petrol is the agent producing the change in antipyrine metabolism in petrol station workers, it would be useful to have a parameter of exposure to petrol in these subjects. However, attempts to find one proved unsuccessful. In previous studies, phenol output in the urine has been used (Docter and Zielhuis, 1967; Parkinson, 1971; Sherwood, 1972; Pandya et al., 1975). This method is based on the metabolism of benzene in the liver producing phenol which is then conjugated and excreted. The major problem with this method is that the benzene content of petrol depends on the source of the crude oil from which the petrol is refined. In the study by Pandya et al. (1975), the benzene content was quoted as 10-17% of petrol, but Sherwood (1972) quoted a figure at 5%. The benzene content of petrol in Adelaide is about 3% (B. O'Neil, personal communication). Hence

the sensitivity of the method will depend largely on the benzene content of the petrol. In this study the only difference seen in the pattern of phenol excretion in the petrol station workers was that it was more variable than found in the controls, however, as a group, the amount excreted did not differ from control values.

Since tetraethyl lead is added to petrol as an "anti-knock" agent, increases in body lead levels might be expected in petrol station workers. Lob (1975) found blood lead levels ranging from 0.80 to 2.95 uM, mean 1.30 uM in office workers and values of 1.11 to 2.85 uM, mean 1.84 uM in garage attendants. Moore et al. (1976) found that the level in petrol vendors was 1.58 $\pm$ 0.08 (SE) uM and this was greater than in a control population of public servants and university personnel which was 0.69 $\pm$ 0.03 uM. In the workers studied in the present experiment the mean value was less than that found for office workers in Lob's study and the higher values were less than the upper 95% confidence limits expected in non-exposed workers (Green et al., 1976). Hence blood lead levels did not differentiate the petrol station workers in this study from the normal non-exposed population. It is supported by findings that ALA-D activity in blood and urinary lead excretion were normal in these workers.

Although the metabolism of antipyrine has been found to be primarily under the control of genetic factors (Vesell and Page, 1968), other factors, such as age, sex (Vestal et al., 1975) and smoking (Hart et al., 1976) have been shown to alter its rate of

metabolism. With this in mind, some personal and social parameters were examined in the 19 petrol station workers in an attempt to explain the difference in antipyrine metabolism on the basis of environmental factors. In this group there was no relationship between antipyrine half-life and age, work duration, smoking or alcohol and caffeine intake. Furthermore, the distributions found for both smoking and caffeine intake were very close to those found by Vestal et al. (1975) in 307 healthy male North Americans. This is evidence that the habits of this group of workers in terms of smoking and drinking are not radically different from the general population and that the altered metabolism of antipyrine in this group cannot be explained in terms of differences in these tabulated social habits.

Since antipyrine metabolism shows a 2-3 fold variation in the general population, small changes in the activity of the enzymes that metabolize this compound are difficult to demonstrate when comparing two heterogeneous subgroups. This was illustrated in the study with the anaesthetists (Chapter V) where the inductive effect of anaesthetic gases was shown if intra-individual comparisons were made but not when anaesthetists were compared with a control population. Hence the inductive effect seen in the petrol station workers is likely to be substantial. The decrease in half-life over the control values was 17% and this is smaller than the 47% decrease seen in workers exposed to insecticides studies by Kolmodin et al. (1969). However such comparisons are confused by the fact that in the latter study the mean half-life of the control population was

13 hours compared to the 11.3 hours in this study. This inter-individual variation makes relationships between degree of shortening of antipyrine half-life and the potency of the inducing agent difficult to interpret.

In conclusion, the results indicate that petrol vapour is a moderately potent inducer of microsomal mixed function oxidases in rats, and that occupational exposure to petroleum may result in enhanced microsomal drug metabolism.

CHAPTER SEVEN

DRUG INTERATIONS WITH ANTIPYRINE METABOLISM IN ISOLATED RAT HEPATOCYTES

INTRODUCTION

It is well known that, when a combination of drugs is given to an individual, drug interactions influencing absorption, distribution, metabolism and excretion may lead to deleterious or toxic side effects. For a large number of lipid soluble drugs and chemicals metabolism becomes a key site of interaction and may involve the microsomal mixed function oxidase enzyme system. Since this enzyme system is implicated in metabolic pathways that may be common for many different compounds, the possibility exists for potential drug interactions to occur when substrates compete for enzyme sites.

However, many factors other than the rate of enzymic degradation of a compound can influence its rate of metabolism *in vivo*. For example, the increased half-life of lidocaine caused by propranolol is due primarily to a reduced hepatic blood flow produced by propranolol (Branch et al., 1973(a)). In order to eliminate such variables from the study of interactions at the site of metabolism, *in vitro* systems are used. Most studies of this nature have been performed using subcellular fractions. These are formed from homogenized tissues and the microsomes so derived may contain fragments of both rough and smooth endoplasmic reticulum and other membranes such as components of the electron transport system, plasma membrane and Golgi bodies. These systems also require the addition of cofactors such as NADPH to maintain adequate rates of metabolism. Hence these types of studies are of limited value in predicting metabolic rates and rate-limiting

steps in the metabolism of a drug *in vivo*.

It has been recently shown that drug metabolism in isolated hepatocyte preparations may more closely resemble the *in vivo* situation even more so than that found in microsomal preparations. Billings et al. (1977) found that the rate of butamoxane hydroxylation in isolated rat hepatocytes was identical to that in the perfused liver, but slower than that found in microsomes. Similarly, Yih and van Rossum (1977) found that heptabarbital metabolism in the hepatocyte preparation was similar to values obtained with isolated perfused liver, but the values obtained with 9000 x g liver supernatant preparations were not.

The preparation of isolated hepatocytes is usually based on the method of Berry and Friend (1969) or one of its many modifications. The cells isolated by these methods have been found to retain the major metabolic properties of the intact tissue. A number of drugs have been shown to be metabolized and these include benzo(a)pyrene (Cantrell and Bresnick, 1972), alprenolol (Grundin, 1975), antipyrine, dansylamide and quinine (Hayes and Brendell, 1976), ethylmorphine (Erickson and Holtzman, 1976), aminopyrine (Stewart and Inaba, 1979) and phenacetin (McLean, 1978). Conjugation reactions have also been found (Siliciano et al., 1977).

The aim of the present investigation was to examine the metabolism of antipyrine in isolated rat hepatocytes in the presence of inhibitory concentrations of three agents: SKF525-A, phenobarbitone and chlormethiazole. The first is a known potent

inhibitor of type I drug metabolism and the latter two were included as they were used as therapeutic agents in studies in previous chapters, namely the epileptics and alcoholics. The usefulness of the system was assessed in terms of determining potential acute drug interactions at the cellular level.

METHODS

1. Animals

Male Porton rats (190-250g) given food and water *ad libitum* were used in these experiments. Rats pre-treated with phenobarbitone were given a daily intraperitoneal dose of 80 mg/kg in saline for the 3 days preceding cell preparation.

2. Hepatocyte preparation

The procedure used was based on that developed by Seglen (1973) with some modifications. Rats were anaesthetized with ether and the abdomen opened by midline incision. Connective tissue between the liver and stomach was divided and the oesophagus and associated blood vessels ligated and cut. The animal was injected with approximately 1000 IU of heparin via the inferior vena cava and after two minutes the portal vein was cannulated using a bevelled plastic cannula. The inferior vena cava was then cut and the liver perfused *in situ* with a calcium-free modified Krebs-Hensleit perfusion buffer (pH 7.4 heated to 37^{o}C and gassed with carbogen (95% O_2 : 5% CO_2). The perfusion pressure was maintained using a constant pressure head of 20 cm while the liver was excised. The composition of the perfusion buffer is shown in Appendix 1. After excision the liver was transferred to a humidified cabinet thermostatically maintained at 37^{o}C and perfused under recirculatory conditions with the same gassed perfusion medium. As soon as adequate flow through the liver was achieved (greater than 35 ml/min), 10 ml of a concentrated collagenase solution was added to give a final perfusate concentration of 70 IU/ml and a perfusate

volume of 90 ml. After adequate digestion of the intercellular matrix (usually 10-15 min), the liver was transferred to a beaker containing 25 ml of fresh collagenase buffer and disrupted with blunt dissection. The volume was adjusted to 60 ml with the collagenase buffer from the recirculatory system and transferred to 2 x 250 ml flasks and incubated for 10 min in a metabolic shaker bath (Gallenkamp) at 80 oscillations per min at 37^{o}C under constant gassing with carbogen. A concentrated solution of bovine serum albumin (BSA) was added to the cell suspension to a final concentration of 1.2% (w/v) and filtered through two layers of nylon mesh (250 u, 61 u). The resulting filtrate was centrifuged at 50 x g for one minute and the pellet washed in perfusion buffer containing 1.2% BSA. The washing and centrifugation steps were repeated, once with the medium and once with the incubation buffer, the composition of which is described in Appendix 1. The resulting cell pellet was weighed and suspended in incubation buffer (pH 7.4) under carbogen to a final concentration of 31.5 mg (wet weight) cells/ml. A 0.2% (w/v) Trypan Blue solution in normal saline was used to determine the integrity of the cell membrane. Cells excluding Trypan Blue were defined as viable.

3. Incubations

Cell suspensions were incubated in glass pill vials (25mm base diameter) under constant gassing with carbogen in a metabolic shaking bath (100 oscillations per minute) at 37^{o}C. Cells were pre-incubated for 5 min at 37^{o}C and the drug metabolic reactions started by adding 2 ml of cell suspension to the pill vial containing

the appropriate amount of antipyrine and inhibitor, which had been added previously as concentrated solutions in 0.1 ml of incubation buffer. A constant amount of (N-methyl-C^{14})-antipyrine (2.2×10^5 dpm) was added to each incubation vial. Hence the final volume was 2.1 ml and the final cell concentration was 30 mg/ml. The reactions were stopped by the addition of 0.4 ml 5 M NaOH after the appropriate incubation time. The range of antipyrine concentrations used was 0.25 - 20mM. Inhibitor concentrations ranges were: SKF525-A, 0.5 - 5um; phenobarbitone, 3.3 - 20mM; chlormethiazole ethane-disulfonate, 0.01 - 1 mM.

4. Determination of antipyrine metabolism

The amount of antipyrine metabolized was determined essentially by the method of Bakke et al. (1974). Two ml of alkalized incubation mix were transferred to a 15 ml glass extraction tube containing 6 ml chloroform and extracted for 30 min with gentle shaking on a mechanical shaking device. Only unchanged antipyrine is extracted into the chloroform phase (Bakke et al., 1974), leaving the water soluble metabolites in the aqueous phase. After centrifugation for 30 min at 3000 rpm, 1.5 ml of the aqueous phase was transferred to a plastic vial containing 10 ml of scintillant (0.17 g 1,4 - bis (2 (4-methyl-5-phenyl-oxazolyl)) benzene, 5.3 g 2,5-diphenyl-oxazole, 333 ml Triton X100, 666 ml toluene) and the activity counted in a scintillation spectrophotometer (Packard, Model 3310). The amount of antipyrine metabolized was calculated from the net dpm appearing corrected for the blank dpm (NaOH added before incubation),

by the following formula:

$$\text{amount metabolized} = \frac{(\text{dpm appearing} - \text{dpm blank}) \times \text{initial amount}}{\text{dpm added to the incubate}}$$

5. Determination of N-demethylated antipyrine

The above method does not measure the N-demethylation pathway as in this situation, the radioactive carbon atom is lost to the atmosphere as CO_2. The rate of antipyrine N-demethylase activity was determined from the amount of $^{14}CO_2$ released from hepatocyte suspensions using the method of Stewart and Inaba (1979) except the filter paper wick was placed in a side-arm instead of a centre-well.

6. Measurement of intracellular potassium (K^+) concentration

Baur et al. (1975) found that retention of K^+ was a sensitive index of cell membrane viability. For estimation of intracellular K^+ an 0.5 ml aliquot of cell suspension was centrifuged for one minute at 50 x g and the resulting cell pellet resuspended in one ml of 3% perchloric acid. This was centrifuged for 10 min at 3500 rpm and a suitably diluted portion of the resulting supernatant was used to estimate K^+ with an Eel Model 150 Clinical Flame Photometer. The value was corrected for an extracellular space in the pellet of 16%.

7. Measurement of Cytochrome P-450

The cytochrome P-450 content of hepatocytes was determined essentially by the method of Omura and Sato (1964). Six ml of cell suspension was centrifuged at 50 x g for 1 min and the cell pellet

resuspended in 6 ml 0.1 M phosphate buffer (pH 7.4). Cytochrome P-450 was determined from the carbon monoxide difference spectrum of dithionite-reduced cells assuming an extinction coefficient of $9.1 \times 10^{-2} M^{-1} cm^{-1}$.

8. Data Analysis

(a) Determination of K_m, V_{max}

Metabolism was assumed to follow Michaelis-Menten kinetics where

$$v = V_{max} \cdot s / (K_m + s)$$

where v = velocity of the reaction (rate of product formation)

s = substrate concentration

V_{max} = maximum velocity

K_m = Michaelis constant (equal to the substrate concentration at half maximum velocity)

Estimates of K_m and V_{max} were obtained by fitting the data to this equation by a nonlinear least-squares procedure based on Newton's method (Lambert, 1973). The equation

$$v_i = V s_i / (s_i + K)$$

where v_i = rate of reaction, s_i = substrate concentration, $V = V_{max}$ and $K = K_m$ and K, V are unknowns is solved for

$$F = \sum_i (v_i - V s_i / (s_i + K))^2 \quad - - - - 1.$$

where V, K are to be found so that F is minimized and v_i, s_i are the individual data points.

From 1.

$$F = \sum_i \left(v_i^2 + \frac{s_i^2 V^2}{(s_i + K)^2} - \frac{2 v_i s_i V}{s_i + K} \right)$$

A minimum value of F corresponds to

$$F_v' = 0 \quad \text{and} \quad F_k' = 0$$

where F_v' = first partial derivative of F with respect to V

and

F_k' = first partial derivative of F with respect to K

Thus

$$F_v' = \sum_i \left(\frac{2 s_i^2 V}{(s_i + K)^2} - \frac{2 v_i s_i}{s_i + K} \right) = 0 \text{ - - - - 2.}$$

and $$F_k' = \sum_i \left(\frac{2 v_i s_i V}{(s_i + K)^2} - \frac{2 s_i^2 V^2}{(s_i + K)^3} \right) = 0 \text{ - - - - 3.}$$

Equations 2 and 3 represent two equations in two unknowns, K, V.

The sum of the left hand side of both these equations is a function of K, V

Denote L.H.S. equation 2. by P (K, V)

L.H.S. equation 3. by Q (K, V)

These two equations can then be solved by Newton's method as follows:

$$P_k' = \sum_i \left(\frac{v_i s_i}{(s_i + K)^2} - \frac{2 s_i^2 V}{(s_i + K)^3} \right)$$

$$P_v' = \sum_i \left(\frac{s_i^2}{(s_i + K)^2} \right)$$

$$Q_k' = \sum_i \left(\frac{6 s_i^2 V^2}{(s_i + K)^4} - \frac{4 v_i s_i V}{(s_i + K)^3} \right)$$

$$Q_v' = \sum_i \left(\frac{2 v_i s_i}{(s_i + K)^2} - \frac{4 s_i^2 V}{(s_i + K)^3} \right)$$

Initial estimates of K, V i.e. K_o, V_o are improved as follows:

$$\begin{bmatrix} K_1 \\ V_1 \end{bmatrix} = \begin{bmatrix} K_o \\ V_o \end{bmatrix} - a \begin{bmatrix} P_k' & P_v' \\ Q_k' & Q_v' \end{bmatrix}^{-1} \begin{bmatrix} P \\ Q \end{bmatrix}$$

where a is a damping factor which may be required if the convergence becomes unstable. Iteration is repeated on the improved estimates until the change in both K_n, V_n is less than 0.5%.

The variance was determined from the following equation:

$$\text{variance} = \sum_i (w_i - v_i)^2 / (n - 2)$$

where w_i = computed value of reaction rate at each concentration, s_i

n = number of concentration points

These equations were solved on a PDP 11/40 computer by a programme written in Fortran language.

(b) Determination of inhibitor constants (K_i)

For the competitive cases, K_i values were determined from the following equation:

$$K_i = i / (K_p / K_m - 1)$$

where i = inhibitor concentration, $K_p = K_m$ for inhibited reation.

For the mixed-type inhibition, K_i values were determined (assuming partially competitive, purely non-competitive inhibition) from the equation:

$$K_i = (i K_m / K_p) / (V_{max} / V_p - 1)$$

where $V_p = (1 + i K_m / K_i K_p) / V_{max}$ (Dixon and Webb, 1964).

RESULTS

1. Cell Preparation

In 25 experiments in untreated rats the yield of hepatocytes was 4.6±0.08g wet weight cells/rat (weighing 210 ± 30 g). The corresponding values found in phenobarbitone rats was 5.1 ± 1.3 g wet weight/rat (weighing 220 ± 20 g).

Trypan Blue exclusion tests of viability were performed soon after preparation of each cell batch. Values were all equal to or greater than 90% with some as high as 98%. The mean value obtained was 94 ± 2%.

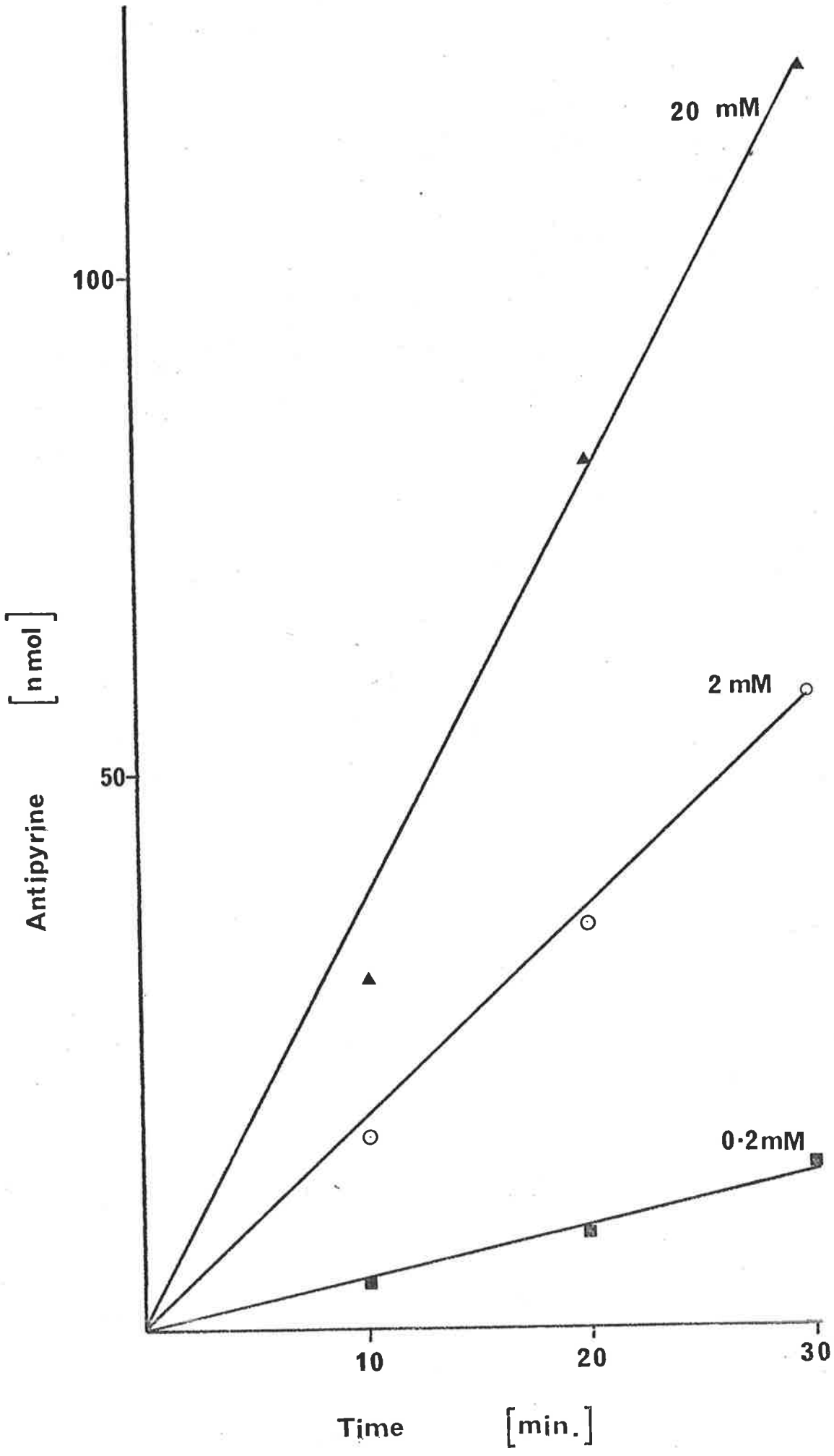
2. Linearity of Metabolism

Estimates of initial velocity assume that the reaction rate is constant over the incubation period. The appearance of metabolites was linear at initial antipyrine concentrations of 0.2, 2.0 and 20 mM over a 30 min incubation period (Figure 7.1). The percentage of the initial amount of substrate metabolized in this time were 7.4, 2.9 and 0.8% respectively.

3. Effect of Incubation on Cell Viability

Cells incubated for 30 min in the absence of any substrate had similar viability to non-incubated cells maintained at room temperature as assessed by intracellular K^+ ($P > 0.1$, U-test). There was no difference in intracellular K^+ levels in cells incubated for 30 min with maximal concentrations of antipyrine and either SKF525-A or chlormethiazole ($P > 0.05$, U-test) and cells incubated in the

Figure 7.1 The linear appearance of water soluble ^{14}C - labelled antipyrine metabolites in cell suspension incubated with (^{14}C - N - methyl)-antipyrine over a period of 30 min. Initial antipyrine concentrations were 0.2, 2.0 and 20.0 mM.


20 mM
2 mM
0·2mM
100
50
Antipyrine [n mol]
10
20
30
Time [min.]

absence of drugs. However there was a small decrease in this parameter in cells incubated with 20mM antipyrine and 20mM phenobarbitone ($P < 0.05$, U-test). These data are shown in Table 7.1. The mean value of K^+ in the cells before incubation was 71 $\pm$ 15 umole/g wet weight. The values for this parameter reported in the literature range from as low as 21 umole/g wet weight (Dubinsky and Cockrell, 1974) to 100 umole/g wet weight (Barnabei et al., 1974). The latter value was found using the hepatocyte preparation technique of Seglen (1972). When these authors used the method of Berry and Friend (1969) to prepare the cells they found K^+ values of about 50 umole/g wet weight. Hence it appears that the preparative procedure may markedly influence the K^+ levels measured. However, since differences in K^+ levels are made on a within-experiment basis in the present study, this variation is not critical.

4. Effect of SKF525-A on antipyrine metabolism

Figure 7.2 shows the inhibitory effect of SKF525-A, up to concentrations of 5 uM on the apparent K_m of antipyrine metabolism. The K_m was increased with increasing concentrations of SKF525-A ($F = 70.2$, 4, 15 df, $P < 0.01$) but the V_{max} remained unchanged ($F = 0.36$, 4, 15 df, $P > 0.1$). Table 7.2 shows the values of K_m and V_{max} at the various inhibitor concentrations. These results are consistent with SKF525-A acting as a competitive inhibitor of antipyrine metabolism at the range of concentrations used, with an apparent K_i value of 1.2 $\pm$ 0.43 uM. Figure 7.3 shows the computed fit of the Michaelis-Menton equation to a typical set of data.

Table 7.1 Intracellular K^+ ion concentrations after 30 min incubations of cell suspensions in the absence of substrates, and in the presence of maximum concentrations of antipyrine and either SKF525-A, phenobarbitone or chlormethiazole.

Incubation Conditions	SKF525-A	Phenobarbitone	Chlormethiazole
Without added substrates	69 ± 17[b] n = 16	80 ± 24 n = 14	103 ± 5 n = 6
With added[a] substrates	66 ± 19 n = 17	64 ± 7[c] n = 13	93 ± 4 n = 6

a. Substrate concentrations ; antipyrine 20mM; SKF525-A 5 uM, phenobarbitone 20mM, chlormethiazole 1mM

b. meq / l (mean ± SD)

c. significantly less than without added substrates ($P < 0.05$)

Figure 7.2 Computer simulated plots of velocity of antipyrine metabolism (nmole/min/30 mg cells) vs. antipyrine concentration in the presence of varying amounts of SKF525-A. The values of K_m and V_{max} were derived from the mean values of four experiments. The curves are consistent with competitive inhibition of antipyrine metabolism by SKF525-A.

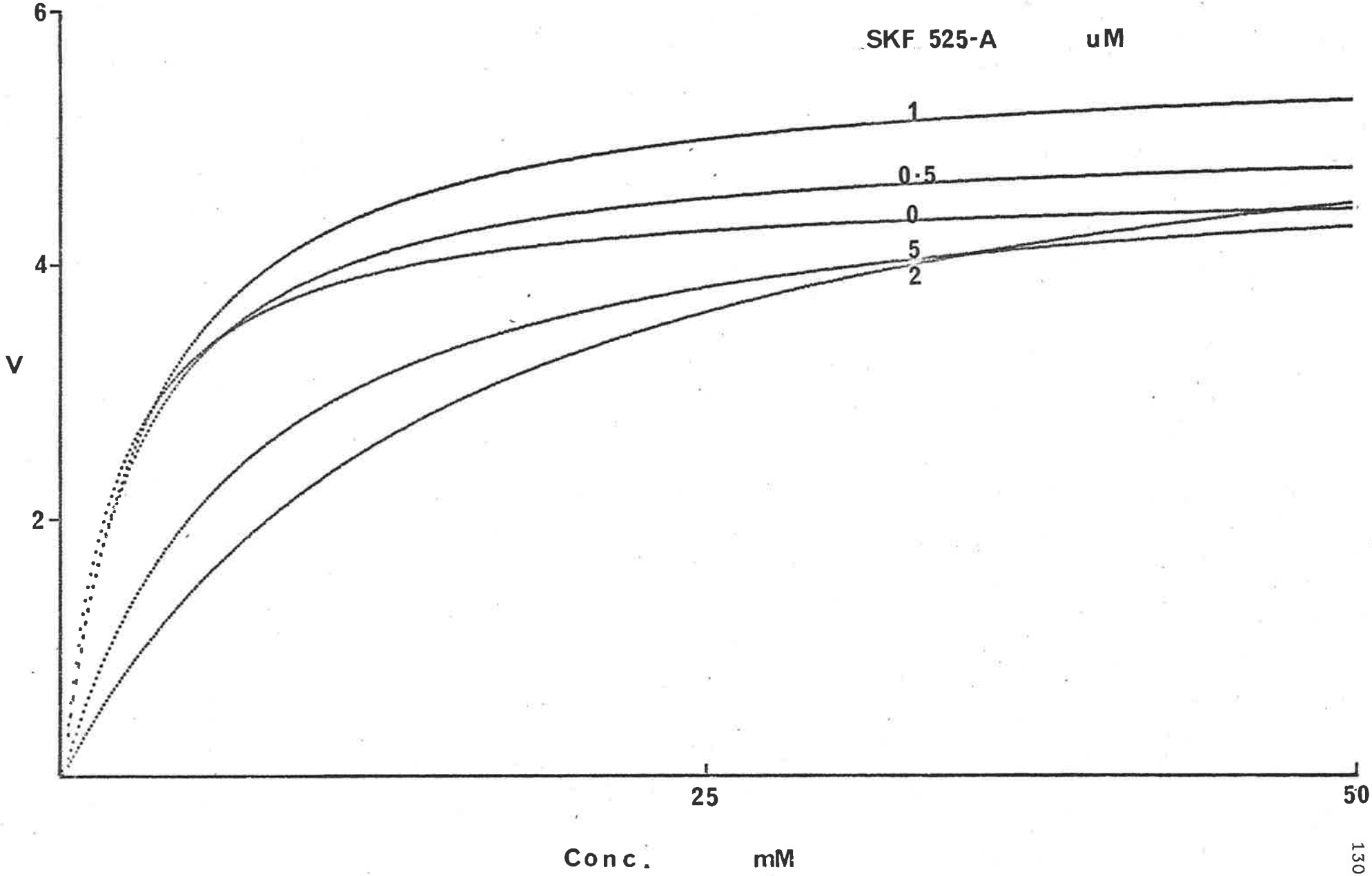

SKF 525-A uM
1
0·5
0
5
2
6
4
2
V
25
50
Conc. mM

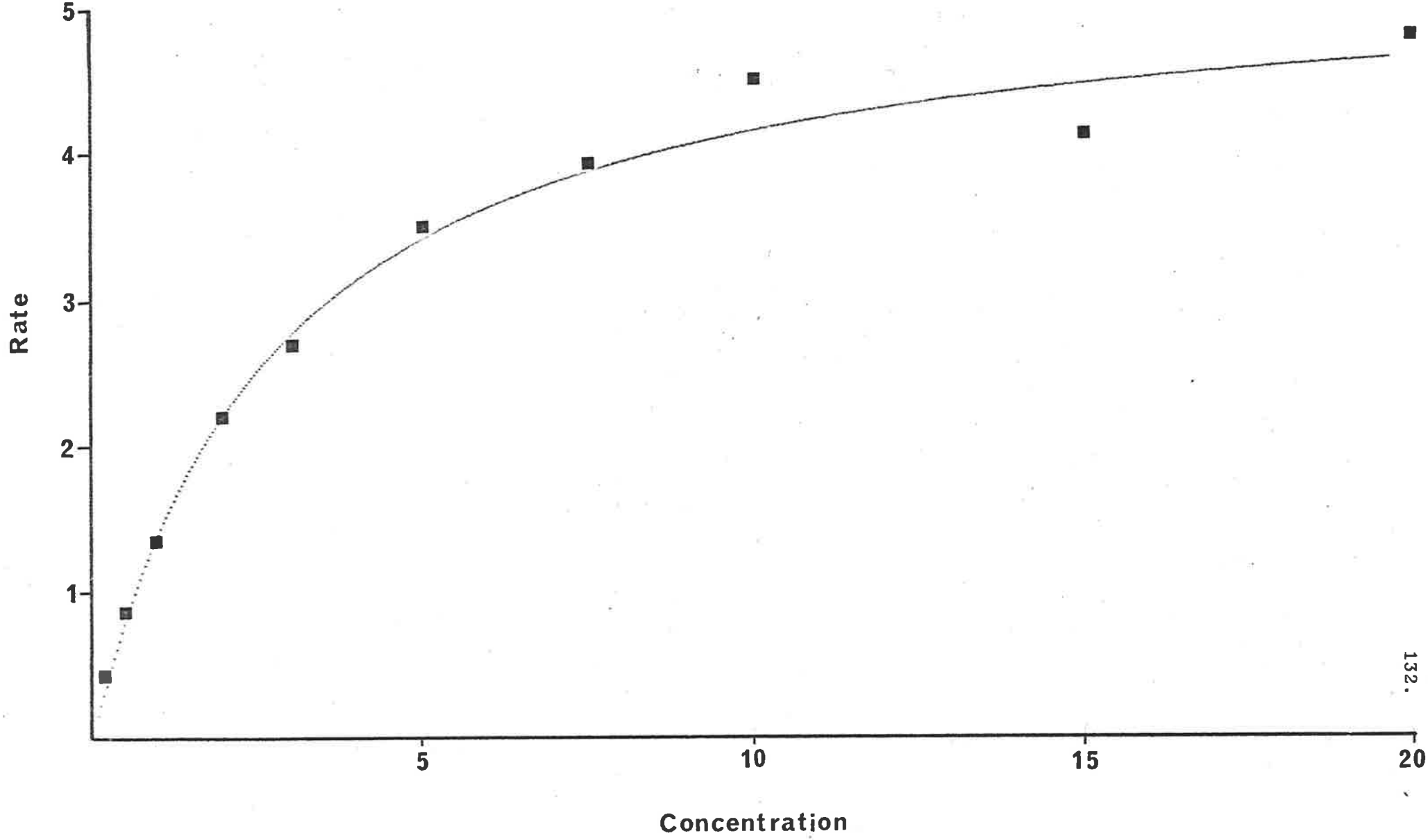
Table 7.2 Values for K_m and V_{max} of antipyrine metabolism in incubates of isolated rat hepatocytes in the presence of varying amounts of SKF525-A. The results are the mean (± SD) of four experiments.

	SKF525-A concentration (uM)				
	0	0.5	1.0	2.0	5.0
K_m	2.12[a] ±0.62	2.86 ±0.46	3.38 ±0.98	7.04 ±2.10	15.22 ±1.56
V_{max}	4.16[b] ±0.34	4.53 ±0.89	5.09 ±1.87	4.41 ±1.89	5.26 ±2.05

a. mean ± SD ; units - mM

b. mean ± SD ; units - nmole/min/30mg wet weight cells

Figure 7.3 Computed curve fitted by Newton's method to experimental data. Concentration is in units of mM, reaction rate in units of nmole/min/30 mg cells. In this example the K_m = 2.84 mM, V_{max} = 5.42 nmole/min/30 mg, Variance = 0.36, S.E. = 0.19.


Rate
5
4
3
2
1
5
10
15
20
Concentration

5. Effect of phenobarbitone on antipyrine metabolism

Hepatocytes isolated from rats pretreated with the microsomal enzyme inducer, phenobarbitone, metabolized antipyrine faster than control cells. The V_{max} from cells of treated rats was 12.61 ± 10.14 nmol/min/30 mg wet weight cells and this was greater than that in control cells which was 6.65 ± 1.07 nmol/min/30 mg wet weight cells ($P < 0.05$, U-test, one-tail, df=14). Pretreatment did not alter the apparent K_m value of antipyrine metabolism ($P > 0.1$, U-test, two-tail) The K_m in the control cells was 2.54 ± 0.68 mM and in the pretreated cells 2.58 ± 1.58 mM. This is illustrated in Figure 7.4.

Cytochrome P450 levels were measured in cells from each of four control and pretreated rats. Values from pretreated animals were 1.26 ± 0.26 nmol P450/30 mg wet weight cells and these were greater than in control rats which had levels of 0.68 ± 0.19 nmol P450/30 mg wet weight cells. The increase in the mean rate of metabolism of antipyrine over control values produced by pretreatment was 90% and this was almost the same as the increase seen in cytochrome P450 content which was 85%.

The concentration of phenobarbitone required to inhibit antipyrine metabolism was more than 1000 fold greater than required with SKF-525A. Figure 7.5 shows that increasing concentrations of phenobarbitone increased the apparent K_m of antipyrine ($F = 4.64$; 4,25 df; $P < 0.01$), but decreased the V_{max} ($F = 2.82$; 4,25 df; $P < 0.05$). These values are shown in Table 7.3. Hence it appears that phenobarbitone inhibits antipyrine by a combination of competitive and non-competitive (mixed) inhibition. The apparent K_i was approximately 13.1 ± 2.3 mM.

Figure 7.4 Effect of phenobarbitone (PB) pretreatment on the metabolism of antipyrine in isolated rat hepatocytes. The rate of antipyrine metabolism (nmole/min/30 mg cells) was increased without a change in the K_m. The curves are computer simulations calculated from V_{max} and K_m values described in the text.

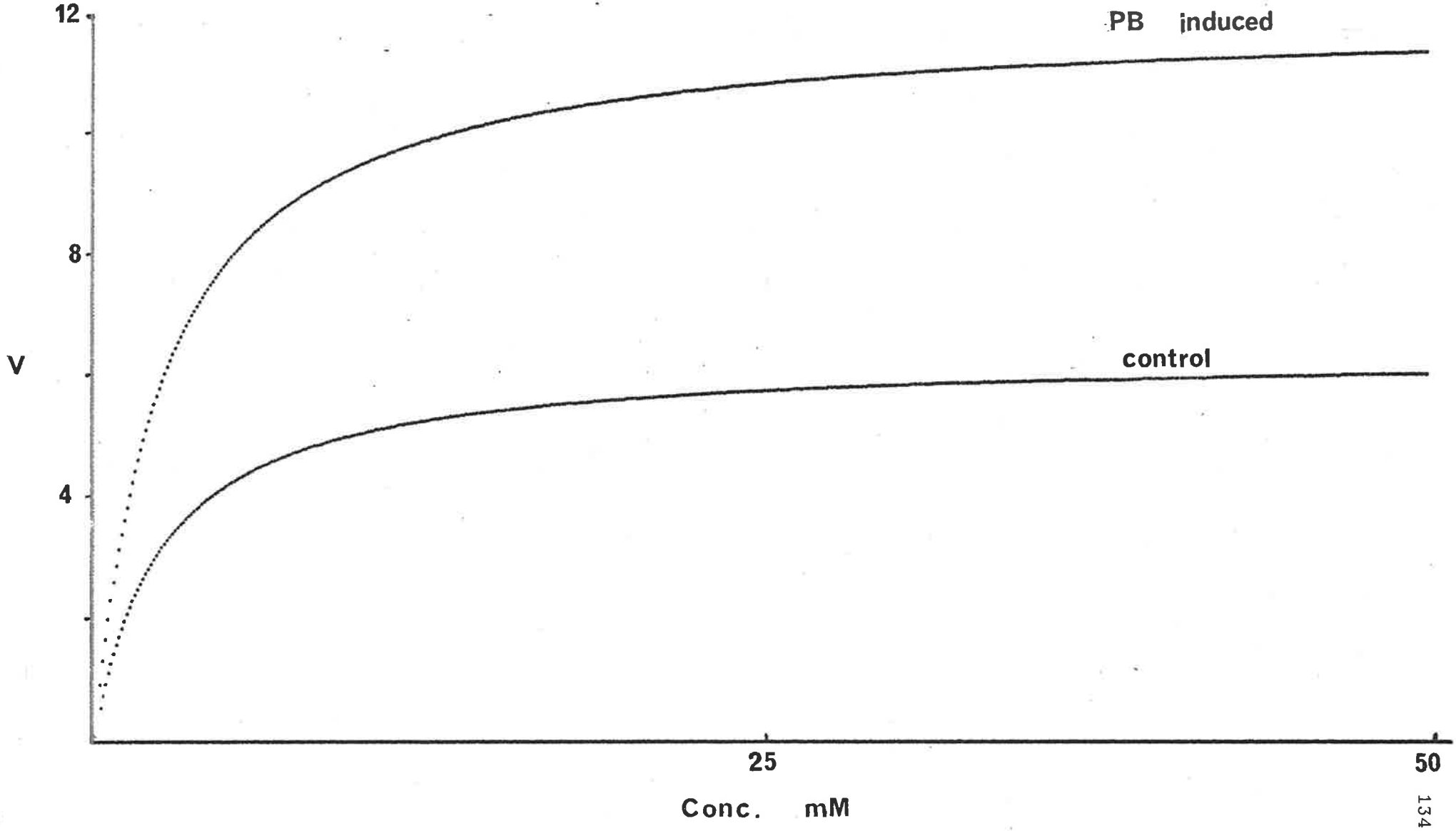

12
8
4
V
PB induced
control
25
50
Conc. mM

Figure 7.5 Computer simulated plots of velocity of antipyrine metabolism (nmole/min/30 mg cells) vs. antipyrine concentration in the presence of varying concentrations of phenobarbitone. The mean values for K_m and V_{max} were derived from the mean values of eight experiments. The curves are consistent with a mixed-type inhibition of antipyrine metabolism by phenobarbitone.

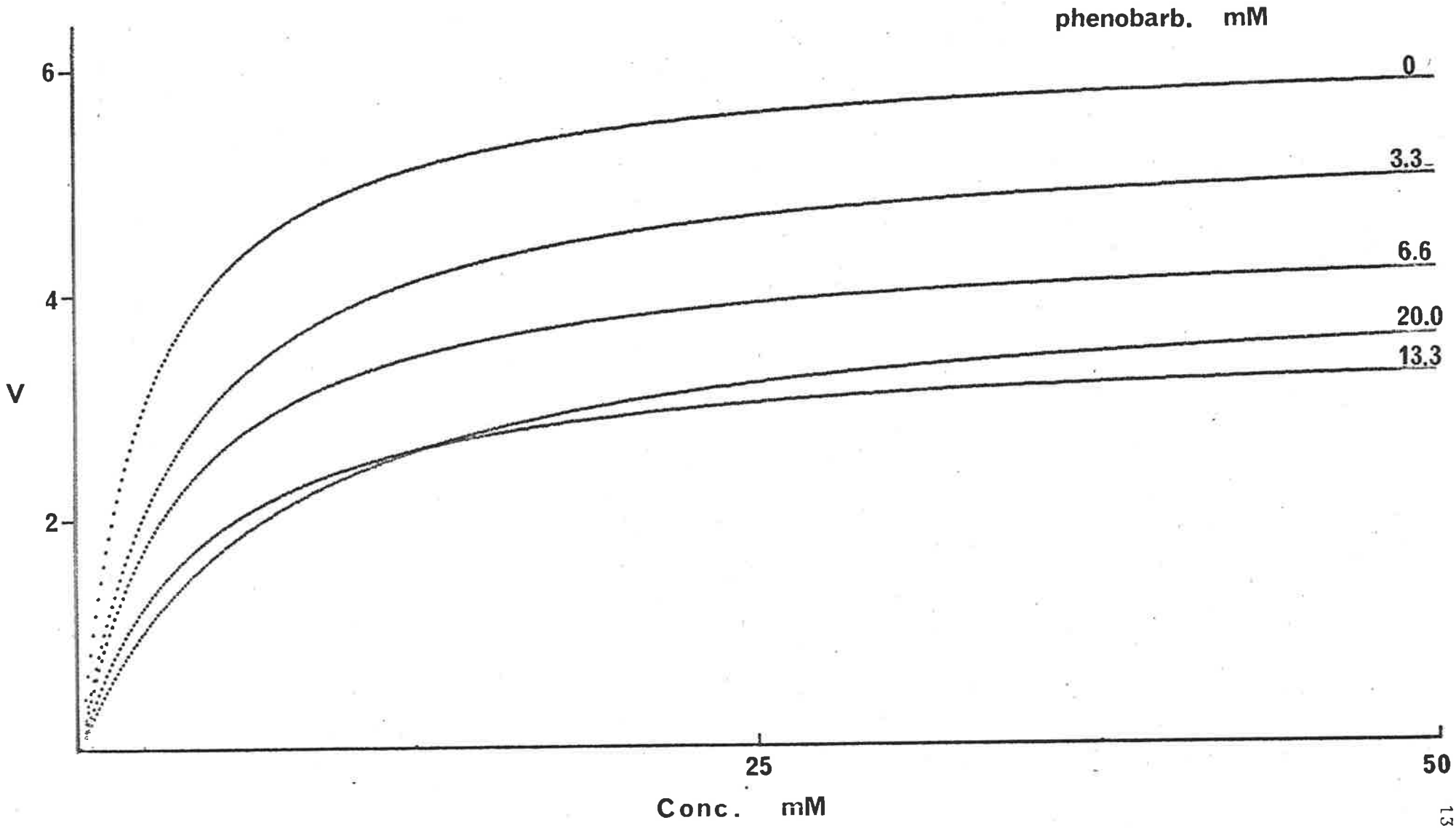

phenobarb. mM
0
3.3
6.6
20.0
13.3
6
4
2
V
25
50
Conc. mM

Table 7.3 Values of K_m and V_{max} of antipyrine metabolism in incubates of isolated rat hepatocytes in the presence of varying amounts of phenobarbitone. The results are the mean (± SD) of 8 experiments.

	Phenobarbitone concentration (mM)				
	0	3.3	6.6	13.3	20
K_m	2.54[a] ±0.68	3.93 ±1.39	3.77 ±1.17	4.49 ±3.10	7.20 ±3.51
V_{max}	6.65[b] ±1.09	5.84 ±1.83	4.85 ±1.74	3.85 ±1.88	4.45 ±2.58

a. mean ± SD ; units - mM

b. mean ± SD ; units - nmole/min/30 mg wet weight cells

6. Effect of chlormethiazole on antipyrine metabolism

Chlormethiazole ethanedisulphonate was found to competitively inhibit antipyrine metabolism in the concentration range 0.01 - 1.0 mM (Figure 7.6). The K_m of antipyrine metabolism was increased with increasing chlormethiazole concentration ($F = 6.41$; 4, 15 df, $P < 0.01$), but the V_{max} remained unchanged ($F = 0.41$; 4, 15 df, $P > 0.05$). These values are shown in Table 7.4. The apparent K_i calculated from these results was 0.12 $\pm$ 0.07 mM.

7. Antipyrine N-demethylase activity

When antipyrine (1 mM) was incubated for 30 min, 14% of the radioactivity associated with metabolites could be accounted for as $^{14}CO_2$. The amount of antipyrine N-demethylated was calculated as 0.128 $\pm$ 0.006 nmoles/min/30 mg. wet weight cells (n = 4). This pathway was inhibited roughly to the same extent as production of all other metabolites. Chlormethiazole (0.5 mM) inhibited N-demethylation by 62% and inhibited the production of the other metabolites by 77%. Similarly SKF525-A (2 uM) inhibited N-demthylation by 10% and production of the other metabolites by 11%.

Figure 7.6 Computer simulated plots of velocity of antipyrine metabolism (nmole/min/30 mg cells) vs. antipyrine concentration in the presence of different concentrations of chlormethiazole. The plots were derived from K_m and V_{max} values obtained from four experiments. The curves are consistent with competitive inhibition of antipyrine metabolism by chlormethiazole.

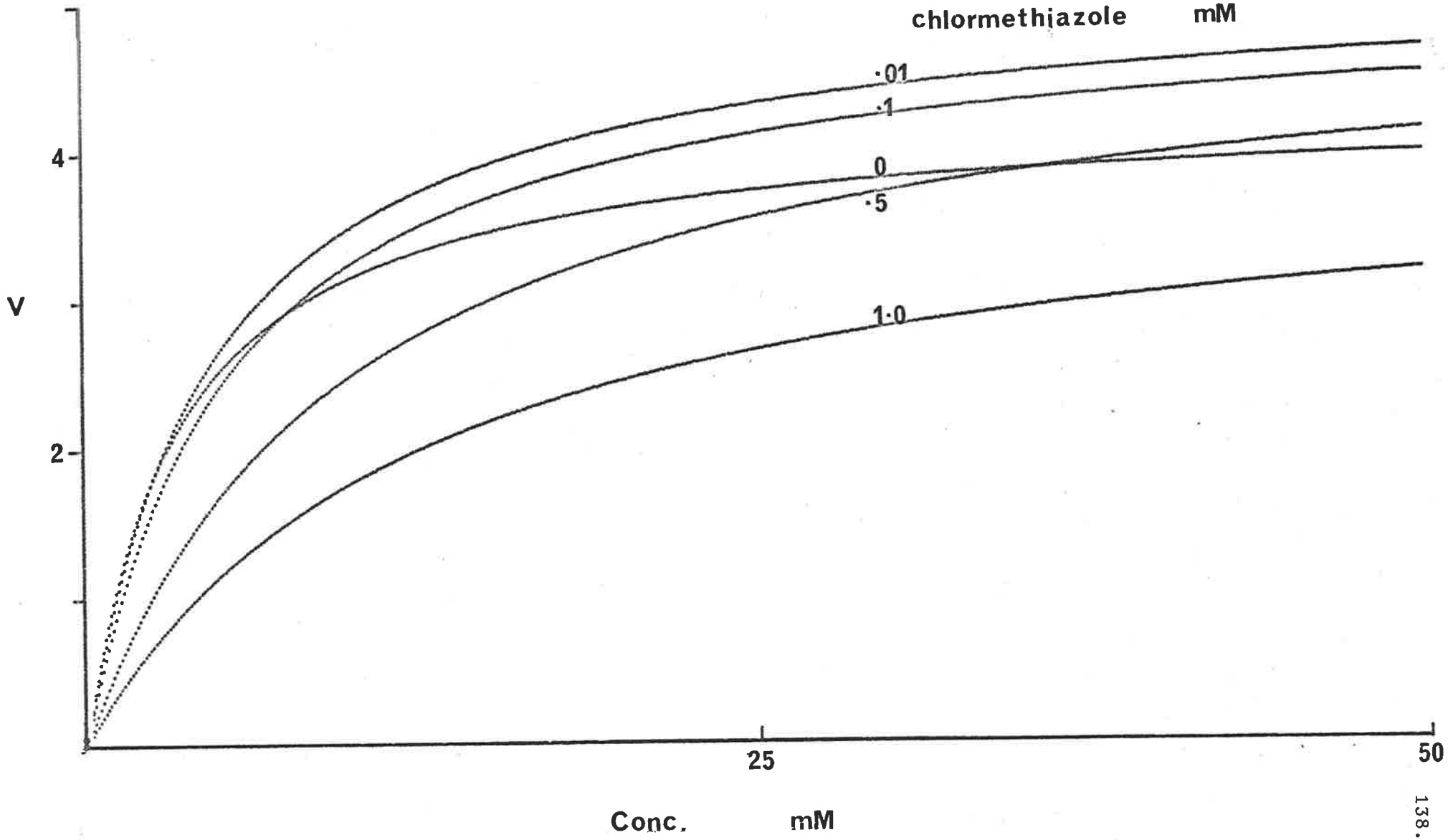

chlormethiazole mM
·01
·1
0
·5
1·0
V
4
2
25
50
Conc. mM

Table 7.4 Values of K_m and V_{max} of antipyrine metabolism in incubates of isolated rat hepatocytes in the presence of varying amounts of chlormethiazole. The results are the mean (±SD) of 4 experiments.

	Chlormethiazole concentration (mM)				
	0	0.01	0.1	0.5	1.0
K_m	3.41[a] ±1.78	4.65 ±2.25	5.35 ±2.69	9.68 ±2.96	12.33 ±4.47
V_{max}	4.24[b] ±1.85	5.13 ±1.75	5.00 ±1.82	4.92 ±1.43	3.95 ±1.22

a. mean ± SD ; units - mM

b. mean ± SD ; units - nmole/min/30 mg wet weight cells

DISCUSSION

The Michaelis-Menten theory of enzyme action has been used to interpret the results of many previous studies on microsomal fixed function oxidase activity. The types of activities studied include O-dealkylation (Lin et al., 1973), N-dealkylation, N-oxygenation (Willi and Bickel, 1973) and C-oxygenation (Archakov et al., 1974), and are typically performed in either rat or rabbit microsomal preparations. The value of these types of studies is that they provide information as to the affinity of the enzyme for the substrate (reciprocal of K_m) and a measure of the maximal velocity constant (V_{max}). These *in vitro* studies are not aimed at determining absolute values for K_m and V_{max}, but are used as guides for estimating the effects of various drugs or chemicals on the enzyme system and to determine the relative inhibitory or stimulatory effects they may possess.

Many previous estimates of K_m and V_{max} have been made by graphical means from transformations of the Michaelis-Menten equation. However these methods, such as double-reciprocal plots, suffer from inaccuracies due to weighting problems with the data (Cleland, 1967). More recently methods that involve direct fitting of the data to the curve by non-linear optimization have been developed, such as the method of Wilkinson (1961). Most of these methods involve the application of a similar sequence of events i.e. one makes an initial estimate or guess, determines, according to a fixed rule, a direction of movement and then moves in that direction to a (relative) minimum

of the objective function (in this case, the Michaelis-Menten equation) on that line. At the new point a new direction is determined and the process repeated until the solution is reached. One such method, used in this study, is Newton's method and although it suffers from instability in convergance in some situations, it is popular because it converges rapidly when it is close to a solution.

In the present study, the metabolism of antipyrine appeared to follow a first-order, one enzyme-one substrate model. However, it is known that antipyrine undergoes several different oxidation reactions to yield metabolites such as 4 - OH antipyrine, 3-carboxyantipyrine and norantipyrine. Hence the values of K_m and V_{max} obtained do not reflect the result from one specific metabolic pathway, but are hybrids of a number of individual constants of different reactions (Porter et al., 1976).

The relative inhibitory effects of different compounds on the metabolism of antipyrine can be demonstrated in isolated hepatocytes. SKF525-A was shown to competitively inhibit antipyrine metabolism. The K_i of 1.2 uM compares favourably with values found in previous studies using rat microsomes, namely 6 uM for ethylmorphine N-demethylase inhibition (Anders and Mannering, 1966) and 3 uM for the inhibition of butynamine N-demethylase (McMahon, 1962). This inhibitory effect may also occur in vivo; however, it is likely that a more complicated situation exists in the whole animal

than simple substrate competition for the active site on the enzyme. A number of theories have been presented to account for the inhibitory action of SKF525-A *in vitro*, including uncoupling of NADPH oxidation (Netter, 1962) and blocking of drug entry across membranes (Brodie, 1962). There is ample evidence that SKF525-A can act as an alternative substrate (McMahon, 1962; Rubin et al., 1964; Anders et al., 1966) causing competitive inhibition and this theory is supported by the findings in this study. It has also been shown that SKF525-A binds strongly to the cytochrome P450 type I binding site (Schenkman et al., 1967). Sasame and Gillette (1970) found that nicotinamide, at low concentrations, competitively inhibited ethylmorphine N-demethylase, but the inhibition was non-competitive at high inhibitor concentrations. They suggested that this was evidence for two enzymes that metabolize ethylmorphine. After the loss of the type I site due to binding of SKF525-A, the other site may act differently to the effects of the inhibitor. Paradoxically, Kamataki and Kitagawa (1973) found that aminopyrine was competitively inhibited in the concentration range 2 - 10 mM but inhibition was non-competitive at a lower concentration range of 0.75 - 2 mM. Hence the mechanism of inhibition by SKF525-A has yet to be clearly defined. To further complicate the situation *in vivo*, it has been shown that metabolites of SKF525-A have an inhibitory effect (Anders et al., 1966) and that the inhibitor itself produces stimulation of drug metabolism after an initial inhibitory phase (Cook et al., 1954; Kato et al., 1962). Thus the acute inhibitory effect on drug metabolism found in the isolated hepatocytes in this study may bear little relationship to the effects the

inhibitor produces in the whole animal. This highlights some of the limitations of *in vitro* methods in attempting to extrapolate to the *in vivo* situation. Notwithstanding, the acute inhibitor effect of SKF525-A may serve as a useful yardstick to judge to potency of certain other compounds as inhibitors of microsomal mixed function oxidases.

Phenobarbitone is unlikely to be an inhibitor of antipyrine metabolism at concentrations that occur in the body. The K_i of 13.1 mM suggests that very large concentrations are required to appreciably alter the K_m of antipyrine metabolism. The occurrence of membrane leakage of cellular K^+ may suggest that these high phenobarbitone concentrations cause membrane damage and this could partly account for the decreased V_{max} obtained with increasing amounts of phenobarbitone.

Chlormethiazole concentrations required to achieve appreciable inhibition were far higher than those expected in plasma when chlormethiazole is given therapeutically. Peak concentrations after oral dosing in patients with alcohol withdrawal symptoms in one study ranged from 0.35 to 13.92 uM after a 768 mg dose and the half-lives ranged from 3.5 to 4.7 hr. The highest dose given to the alcoholics studied in Chapter IV was equivalent to 1 gram chlormethiazole base, hence the possibility of chlormethiazole causing the increased antipyrine half-lives seen in the alcoholics is unlikely. Once again, care must be taken in interpolating from

rat to man as the possibility exists that the K_i in man may be lower than the 120 uM found in rats. However, indirect evidence in man suggests that chlormethiazole does not affect drug metabolizing enzymes (Majumbar, 1978) and the findings in the rat hepatocytes lend support to those findings.

The increased V_{max} of antipyrine metabolism in rats pretreated with phenobarbitone was related to an increase in the amount of cytochrome P450 content of the hepatocytes. Since no difference was found between the K_m values obtained in cells of control and pretreated rats, it is likely the increased metabolism of antipyrine is due to an increase in the total amount of enzyme rather than increased affinity of enzyme for the substrate.

The method of measuring the rate of appearance of water soluble metabolites as an index of antipyrine metabolism does not quantitate the N-demethylation pathway of metabolism because the radioactive carbon is lost as $^{14}CO_2$. This pathway was found to account for 14% of the total antipyrine metabolized in a 30 min incubation. The N-demethylation was inhibited by SKF525-A and chlormethiazole, hence the decreased rate of appearance of water soluble metabolites by these inhibitors cannot be explained in terms of a compensatory increase in the rate of antipyrine N-demethylation.

Krebs et al. (1974) have found that ATP and intracellular K^+ levels in isolated hepatocytes are similar to those in vivo. These authors also found that rates of oxygen consumption, glucose synthesis from lactate, urea formation in the presence of excess

ammonia and ketone body formation from oleate were almost identical in perfused liver and isolated liver cells. Johnson et al. (1972) found that the isolated cells respond to glucagon and to insulin (i.e. the effects on glycogen storage and gluconeogenesis respectively) in much the same way as the perfused liver. Hence, biochemically, the isolated liver cell preparation has many similar functions when compared to the perfused liver and therefore may be superior to other in vitro liver preparations. For example, liver slices have been shown to have an inferior functional state to isolated perfused liver which may be related to cell damage at the perimeters and lack of an adequate oxygen supply to the inner layers (Johnson et al., 1972). Cultured liver cells suffer from problems of de-differentiation of specific hepatic functions and these can differ from those in vivo (Bissell et al., 1973; Jeejeebhoy and Phillips, 1976). In subcellular fractions the spatial arrangement of the components of the membrane may be altered or a second or follow-up pathway may be lost (Yih and Van Rossum, 1977). As discussed in the Introduction, metabolism of drug in the isolated liver cells more closely resembles the in vivo situation than does metabolism in subcellular fractions. Hence events occurring in the isolated hepatocyte may more closely parallel the in vivo situation than these other preparations. In addition, the isolated hepatocytes have the advantage over the isolated perfused liver in that a far greater amount of information can be obtained from one liver. Whereas the perfused liver is limited to one rate determination at a time, in the isolated liver cells 60 to 70 separate

rate determinations may be made at the same time from one liver. The results of this study therefore suggests that suspensions of isolated rat liver cells may be useful in examining acute drug interactions at the site of metabolism that may potentially occur in the hepatocyte. Since it has recently been demonstrated that viable liver cells can be isolated from human liver slices (Bellemann et al., 1977; Nau et al., 1978) the possibility exists that human liver biopsy samples may be available in the future for these types of enzyme kinetic studies.

GENERAL DISCUSSION

A general index of hepatic mixed function oxidase activity would be a valuable aid in pharmacological studies. If such an index could quantitate an individual's metabolic activity, then dosage regimes could be tailored to optimize that patient's drug therapy. This would not be feasible unless the correlation between the metabolism of the general test drug and the therapeutic agent was high. Since the predictive nature of a correlation is related to r^2, a correlation coefficient of, say, 0.80 would only give a predictive power of about 65%. This would be of little use in the clinical situation where correlations between test drug and therapeutic agent would need to be very close to unity.

It has been suggested that since antipyrine elimination does not always correlate with the elimination of other compounds, or other tests of drug metabolizing ability, it is not a good index of hepatic mixed function oxidase activity (Sjoqvist and von Bahr, 1973; Stevenson, 1977). There have been reports indicating a lack of correlation between antipyrine half-life and the half-lives of phenylbutazone and phenytoin (Davies et al., 1973), glutethimide and amobarbital (Kadar et al., 1973), aminopyrine and phenacetin (Vesell et al., 1975). Brien et al (1975) found very poor correlation between phenytoin half-life and antipyrine half-life whereas amobarbital, glutethimide and sulfinpyrazone half-lives correlated well with phenytoin elimination. These authors suggested that antipyrine may have a different fate to these other drugs. If this were the case, it would imply that antipyrine metabolism is under the control of different rate limiting steps.

Nevertheless, other workers have found good correlations between the metabolism of antipyrine and other drugs in man. Antipyrine clearance or half-life has been found to significantly correlate with phenylbutazone half-life after multiple dosing (Davies and Thorgeirsson, 1971), phenytoin clearance (Davies et al., 1973), theophylline and acetanilide clearance (Kellermann and Luyten-Kellermann, 1978(a)), amylobarbitone half-life and clearance (Boobis et al.,1979), phenobarbitone half-life (Kellermann and Luyten-Kellermann, 1977), paracetamol and lignocaine half-lives (Forrest et al., 1977), imipramine clearance (Gram et al., 1976), oxazepam half-life and clearance (Kellermann and Luyten-Kellermann, 1979) and to diazepam and aminopyrine breath tests (Hepner et al., 1977).

It is interesting that the rate of antipyrine metabolism, which is dependent on the activity of the mixed function oxidases, correlates with oxazepam metabolism. Oxazepam is cleared almost entirely by conjugation to glucuronic acid. This may indicate a common rate limiting step for metabolism by the mixed function oxidase enzymes and the conjugating system in the liver. This would be consistent with D-glucaric acid excretion being an index of mixed function oxidase activity. However a correlation between two variables does not necessarily imply a relationship exists between them.

The use of half-life of a drug as a measure of the rate of drug metabolism may contribute to the poor correlations often found when comparing the elimination of two drugs. Half-life is influenced by factors that can alter distribution as well as those that can influence the rate of metabolism. Better correlations may be obtained

when drug clearance rates are compared, as these are independent of changes in distribution. An example of this is in the case of phenytoin. When phenytoin half-life was compared to antipyrine half-life, a very poor correlation (r = 0.07) was obtained. However when the clearances of those two compounds were compared the correlation was far better (r = 0.66) (Davies et al., 1973). In a recent study, Sotaniemi et al. (1979) found that the clearances of propranolol and antipyrine showed good correlation (r = 0.64) but the correlation between half-lives was not as strong (r = 0.34).

Further evidence for the utility of antipyrine metabolism as an index of mixed function oxidase activity can be found when antipyrine metabolism *in vivo* is compared to *in vitro* parameters of drug metabolism. Kalamegham et al. (1979) found a significant correlation between antipyrine elimination from the body and benzo(a)pyrene hydroxylase activity in microsomes prepared from human liver biopsy samples, but not with aniline hydroxylase or aminopyrine N-demethylase activities. Total cytochrome P450 content in human liver biopsy samples has been shown to correlate with antipyrine elimination kinetics in alcoholics (Sotaniemi et al., 1977) and epileptics (Pirttiaho et al., 1978). In animal studies antipyrine half-life had inverse correlation with hepatic microsomal aniline hydroxylase and ethylmorphine N-demethylase activities in dogs, but no relationship was found with cytochrome P450 content, cytochrome c reductase activity or NADPH oxidase activity (Vesell et al., 1973). Statland et al. (1973) found good correlation between antipyrine half-life and antipyrine hydroxylase in rabbits.

Relationships between antipyrine metabolism and that of other drugs have been found in vitro. Kapitulnik et al. (1977) found significant correlations between antipyrine hydroxylase and benzo(a)-pyrene hydroxylase, zoxazolamine hydroxylation, courmarin hydroxylase, hexobarbitone hydroxylase and 7-ethoxycoumarin O-dealkylation in human liver samples obtained by biopsy sampling.

Hence there is considerable evidence to suggest that antipyrine metabolism is a reasonable index of the rate of metabolism of many other drugs. The correlations, in most cases, are not high enough to enable one, knowing the rate of antipyrine metabolism, to then make predictions as to the rate an individual will metabolize other drugs. However, the correlations do suggest that the enzymes that metabolize these compounds are under a degree of common control.

Factors that alter antipyrine elimination also alter the elimination of other drugs in the same direction. For example, in patients with hyperthyroidism, antipyrine, propylthiouracil and methimazole elimination were all increased; and in hypothyroidism the elimination of these drugs was decreased (Vesell et al., 1975(a)). Other examples include the stimulation of both antipyrine and phenacetin metabolism by a diet rich in brussels sprouts and cabbage (Pantuck et al., 1979), increased antipyrine and theophylline metabolism by a protein rich diet (Kappas et al., 1976) and decreased elimination of antipyrine, lignocaine and paracetamol in chronic liver disease (Forrest et al., 1977). Although the extent of the changes produced in the metabolism of each drug was not always the same, the direction of the change was. Hence antipyrine elimination kinetics would seem

to be a good qualitative index of the activity of the mixed function oxidase enzymes. Since the substrate specificities for these enzymes appears to vary between individuals, the use of one test drug is of little value as a quantitative test of activity.

Despite the foregoing, antipyrine elimination has been used in clinical situations as an aid in diagnosis. Fraser et al. (1976) reported a short antipyrine half-life (5 hr) in a patient with osteomalacia and who was receiving butabarbitone and spironolactone. These two drugs are known to induce hepatic microsomal enzymes and the authors suggested that enzyme induction, indicated by the short half-life, coupled with a reduced vitamin D intake, was the cause of osteomalacia. The therapy was replaced with nitrazepam, which does not induce microsomal enzymes, and a vitamin D supplement. After three weeks, the half-life had increased from 5 to 18 hr. The authors concluded that measurement of antipyrine metabolism had a place in monitoring the degree of enzyme induction in an individual. Whiting et al. (1976) also found clinical complications arising from enzyme induction. In one case an epileptic patient had low phenytoin levels after the addition of phenobarbitone and rifampicin therapy and this resulted in an increased incidence of fitting. The antipyrine half-life was found to be 5 hr which was suggestive of enzyme induction. By increasing the phenytoin dose they achieved adequate plasma levels and a cessation of fits.

The major complication in interpreting antipyrine half-life data in this manner is the large interindividual variation in a population. The range in the controls studied in Chapter I was 7 to

16 hr. Therefore a half-life of 5 hr in an epileptic on anticonvulsant drug therapy may not necessarily indicate a marked degree of enzyme induction if that subject had an uninduced level of, say, 7 hr rather than 16 hr. In the epileptics studied in Chapter III antipyrine half-lives ranged from approximately 3 to 11 hr and this overlaps the range found in the controls. This variation makes it difficult to identify enzyme induction in an individual.

It has been shown that multiple forms of cytochrome P450 exist in microsomes isolated from animals (Comai and Gaylor, 1973). Thomas et al. (1976) have provided evidence that there may be at least six forms of cytochrome P450 in rat liver microsomes. Gustafsson and Ingelman-Sundberg (1976) reported that the different forms have different types of regulation and catalytic ability. Moreover the type and content of each can be altered in animals by pretreatment with a variety of agents (Haugen et al., 1975; Thomas et al., 1976(a)). It has been suggested that this may help explain differences in individuals with regard their ability to metabolize various drugs (Lu, 1976). The different forms of this terminal oxidase may have different substrate specificities and variation in the proportion of each form could explain how such factors as species difference, age and sex can influence the rate of drug metabolism. It may also help explain the large inter-individual differences in drug metabolism found in a population.

From the foregoing, the use of antipyrine as an indicator of the level of intrinsic activity of <u>all</u> hepatic mixed function oxidase

enzyme activities may not be valid. However it seems to be a good index of the direction in which a factor will alter the rate of drug metabolism without necessarily providing information as to what extent the rate will change.

The value of using the rate of antipyrine metabolism as an index of mixed function oxidase activity lies in it being sensitive to small changes in the activity of these enzymes when comparing different populations. This can best be appreciated when groups are compared in an intrasubject basis as was the case with the anaesthetists studied in Chapter V. When analysed as a group, comparing controls and anaesthetists (in two different work environments), no differences were found in the rate of antipyrine metabolism. However when the anaesthetists were compared in the two work environments using each subject as his/her own control a small difference in the rate of antipyrine metabolism was observed. This method eliminates the large interindividual differences produced by genetic and environmental factors which tend to mask small differences in metabolism between two populations.

Notwithstanding, small changes in antipyrine metabolism were observed when comparing two heterogeneous groups. In the alcoholics in Chapter IV an increase in antipyrine half-life was observed. In this group the biochemical tests did not indicate marked impairment of liver function as was the case in the patients with alcoholic cirrhosis studied in Chapter II. This may indicate that antipyrine metabolism is potentially a sensitive index of damage to the liver for despite the fact that these alcoholics had had a long-term drinking problem, they had not been diagnosed as having frank liver disease. It seems

that a decrease in the activity of hepatic microsomal enzymes may precede the development of overt symptoms of alcoholic liver disease.

Further evidence of the sensitivity of the antipyrine test was obtained in the study with the petrol station workers in Chapter VI. The half-life of antipyrine was decreased in workers occupationally exposed to petrol. Although this is not direct evidence that petrol exposure produced this increase in metabolism, when rats were exposed to petrol vapour for three weeks, there was induction of hepatic microsomal enzyme activity, indicating that some component of petrol had inducing properties. The tests designed to measure the exposure to petrol (lead levels and phenol excretion) did not differentiate between the workers and controls, yet a difference in the rate of antipyrine metabolism was observed. This may indicate that although exposure levels to petrol were low, the antipyrine test was able to detect the small difference produced in mixed function oxidase activity.

The consequences of induction or inhibition of mixed function oxidase enzymes are not always clear. Enzyme induction has been implicated in folate deficiency, vitamin D deficiency and the development of osteomalacia in epileptics on anticonvulsant therapy, and this was discussed in Chapter III. Toxicity may arise in cases such as in patients with alcoholic cirrhosis where the capacity of the liver to metabolize drugs is impaired. However, in most cases the physician will be aware of the problem and simply titrate the therapy to achieve the desired response.

The effects of lesser degrees of enzyme induction are more difficult to interpret. In many situations the value of the anti-

pyrine test of hepatic microsomal enzyme activity may be of scientific interest only. The small increase in the enzyme activity in the anaesthetists when exposed to volatile anaesthetic agents, for instance, probably means little in terms of having to alter dosage regimes when treating these subjects.

One known toxic consequence of enzyme induction is the production of reactive metabolites. Some substances are chemically innocuous until converted by oxidation into metabolites which can bind covalently to tissue macromolecules. Included in these types of compounds are halogenated hydrocarbons, such as bromobenzene and halothane, paracetamol, and polycyclic hydrocarbons which are present in our environment in the air, in food and cigarette smoke (Mitchell et al., 1973; Breckenridge and Roberts, 1976). Many of these compounds are converted by the mixed function oxidase enzymes to highly reactive, unstable epoxides which then react with glutathione to produce an inactive complex (Mitchell et al., 1973). Pretreatment of animals with phenobarbitone has been shown to stimulate the metabolism of these compounds and to produce necrosis in liver cells (Mitchell et al., 1971). It is thought that the enzyme induction produces sufficiently high levels of epoxide to deplete glutathione stores, leaving the epoxide to react with tissue macromolecules. This can lead to genetic mutations and cancer formation (Miller, 1970).

These reactions are only seen in animals pretreated with an inducing agent which will markedly increase the activity of microsomal enzymes.

These epoxides are more than likely formed in humans and under

normal conditions would be inactivated by glutathione. It is unknown whether the degree of long-term enzyme induction seen in many of the groups in this thesis would be sufficient to increase the level of these reactive metabolites so as to predispose them to a greater risk of tissue damage or cancer.

In conclusion, the rate of elimination of antipyrine from the body is a useful qualitative index of drug metabolism and is sensitive to factors that alter the activity of hepatic microsomal mixed function oxidase enzymes.

APPENDIX I

1. Composition of liver perfusion medium

Na Cl	96 mM
K Cl	1.4 mM
Mg SO_4	0.74 mM
K H_2 PO_4	2.5 mM
Na HCO_3	30 mM
Na gluconate	21.7 mM

2. Composition of incubation medium

As for the perfusion medium, plus

Ca Cl_2	1.6 mM
glucose	5.5 mM
bovine serum albumin	1.2% (w/v)

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