EFFECTS OF THE ENVIRONMENT AND DISEASE ON DRUG METABOLISM IN MAN

by

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TO MY WIFE MAUREEN

ABSTRACT

The effects of environmental factors on antipyrine metabolism were investigated in 131 London factory and office workers and in 49 Gambian villagers. There were 109 females and 71 males between 18 and 60 years of age. Saliva half-life of antipyrine was measured over 32 hours after a 600 mg dose and correlated with nutritional indices and use of social drugs. Saliva half-life of paracetamol was measured in 40 of the London subjects. Indices of nutritional status, i.e. body weight, height, ponderal index, albumin and haemoglobin were reduced in Gambians compared with London Caucasians, while there were considerable differences in the use of social drugs in the three ethnic groups.

Multiple regression analysis showed that in the Gambian sample four factors were statistically significant independent predictors of antipyrine half-life, accounting for 36% of the variation. Half-life was shorter in women, decreased with an increase in height in men and was prolonged by cola nut consumption. In women it increased with haemoglobin.

In the London sample environmental factors explained 49% of the variation in antipyrine half-life. Half-life was longer in vegetarians than in non-vegetarians, shorter in cigarette smokers and prolonged in users of the steroid oral contraceptive. Alcohol, coffee and tea were not significantly correlated with antipyrine half-life. Paracetamol

half-life was prolonged by a vegetarian diet, use of the contraceptive pill and an increase in serum globulin and decreased with an increase in ponderal index. The vegetarian diet was characterised by calorie, protein and specific nutrient deficiencies. These data indicate that both diet and socially used drugs can have important effects on drug metabolism, with important implications for therapy and toxicity in man.

Studies were made of drug elimination *in vivo* and enzyme activity in liver biopsy samples from the same subjects. In eleven patients hospitalised for alcohol withdrawal, amylobarbitone clearance and urinary 3'-OH amylobarbitone were measured and amylobarbitone hydroxylase activity assayed in 9000 g supernatant prepared from 20 mg. needle biopsies of liver. There were wide variations in half-life and clearance which were prolonged compared with values for healthy adult males. Urinary 3'-OH amylobarbitone increased with an increase in clearance but clearance correlated poorly with *in vitro* activity. A highly significant correlation was seen between urinary 3'-OH amylobarbitone and amylobarbitone 3'-hydroxylase activity.

In six patients studied twice, on admission to hospital and four weeks later, there was no consistent change in ability to oxidise amylobarbitone. In two patients there was no change, in three activity increased and in one it decreased. Nutritional deficiency and enzyme induction were two relevant factors in these patients.

These findings indicate that for some drugs measurement of metabolite formation may be preferable to measurement of elimination of the parent drug when studying comparative metabolising capacity.

PUBLICATIONS

Some of the work presented in this thesis has been published:

1. Fraser, H.S., Mucklow, J.C., Murray, S. and Davies, D.S. (1976) Assessment of antipyrine kinetics by measurement in saliva. Brit. J. Clin. Pharmac., 3, 321-325.
2. Fraser, H.S., Williams, Faith M., Davies, D.L., Draffan, G.H. and Davies, D.S. (1976) Amylobarbitone hydroxylation kinetics in small samples of rat and human liver Xenobiotica, 6, 465-472.
3. Fraser, H.S., Bulpitt, C.J., Kahn, Clare, Mould, G., Mucklow, J.C. and Dollery, C.T. (1976) Factors affecting antipyrine metabolism in West African villagers. Clin. Pharmacol. Ther., 20, 369-376.

The following papers have been presented and are in press:

1. Fraser, H.S., Davies, D.S. and Neale, G. Correlation of amylobarbitone metabolism *in vivo* with amylobarbitone hydroxylation kinetics in needle biopsies of liver in alcholics. 37th Annual Meeting of the British Society of Gastroenterology, September, 1976.
2. Fraser, H.S., Mucklow, J.C., Bulpitt, C.J., Kahn, Clare, Mould, G. and Dollery, C.T. Environmental effects on antipyrine half-life in man. Fourth Deer Lodge Conference in Clinical Pharmacology, Montana, U.S.A. September, 1976, in Clin. Pharmacol. Ther.

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INTRODUCTION

a) INTER-INDIVIDUAL VARIATION IN DRUG METABOLIC RATES

The treatment of human disease is largely dependent on the rational use of drugs. Success depends not only on selection by the doctor of the appropriate drug for each condition, but on the route, formulation, dose and frequency of administration. The oral route is most practical in the majority of situations and for long term use, and most oral preparations are well absorbed. Often optimal results are not achieved because the dose regime is not the most appropriate.

The main reason for this is the limited dose range employed by many doctors, in contrast with the wide patient variation in the dose response curve. A large number of factors have been shown to contribute to this variation. They include: variable compliance with therapy, pharmacokinetic differences, variation in receptor sensitivity, disease variables and placebo effects. Pharmacokinetic differences are due to variation in absorption, first pass metabolism, distribution in body compartments and elimination by renal, metabolic or other routes. Of these metabolic variation (biotransformation) is one of the most important.

Variation in drug metabolism and drug response in animals

Early animal experiments in Brodie's laboratory first suggested

that species differences in drug response may be related to differences in drug metabolism. Quinn, Axelrod and Brodie (1958) found a 26-fold difference in the duration of action of hexobarbitone in four animal species (Table I). They measured serial plasma concentrations in mice, rabbits, rats and dogs after intravenous injection of the barbiturate and found a close correlation between plasma half-life and duration of action, as indicated by sleeping time. The *in vitro* activity of microsomal fractions prepared from the livers of these animals also correlated with plasma half-life and sleeping time. Thus dogs, who slept longest after hexobarbitone administration, had the longest half-life and the lowest enzyme activity *in vitro*. Furthermore, the plasma level on awakening showed only a four-fold range compared with the 26-fold range in sleeping time.

This suggested that differences in metabolism rather than receptor sensitivity were mainly responsible for the species difference in drug response.

TABLE I

Species differences in the duration of action, plasma half-life and enzyme activity of hexobarbitone (Data of Quinn, Axelrod and Brodie, 1958)

Species	Duration of action (min)	Plasma half-life (min)	Enzyme activity (µg/g/hr)	Plasma level on awakening (µg/ml)
Mouse	12	19	598	89
Rabbit	49	60	196	57
Rat	90	140	134	64
Dog	315	260	36	19

Species differences in plasma half-life of many other drugs have been shown in animals, e.g. phenylbutazone, oxyphebutazone, antipyrine, pethidine and ethylmorphine and have been reviewed by Brodie and Reid (1967) and Burns (1968). Interstrain differences have also been shown within animal species. For example, the duration of action of hexobarbitone varies two to three fold between in-bred strains of mice (Jay, 1955). The strain which was not in-bred showed much greater variability between animals than the in-bred strains as one would expect for a trait under genetic control.

In spite of the apparent importance of genetic control, however, hexobarbitone metabolism in mice was also shown to be susceptible to environmental conditions (Vessell, 1968). Thus age, sex, litter size, type of bedding, ambient temperature, time of day of drug dosing, degree of crowding and painful stimuli all had significant effects on the rate of hexobarbitone metabolism.

Inter-individual variation in drug metabolism and drug response in man.

Drug oxidation rates in man are usually much slower than in animals. For example, the mean half-life of phenylbutazone is 12 times as long as in dogs and that of antipyrine is seven times as long (Burns, 1968). The inter-individual variation in man is even greater for some drugs. For example Hammer and

Sjoqvist (1967) reported a thirty-six fold variation in the steady state plasma level of desmethyl imipramine in 11 psychiatric patients receiving the same dose. Loeser (1961) found a twelve-fold variation in steady state plasma concentrations of phenytoin in patients on the same dose. Vessell and Page (1968) reported a ten-fold range of dicoumarol (bishydroxycoumarin) half-lives in normal volunteers and ranges of three to ten-fold are widely reported for the half-lives of other oxidised drugs.

A relationship between steady state plasma concentration and drug response has now been shown for several widely used drugs whose elimination is dependent on oxidation; for example, phenytoin (Lund, 1974) and lignocaine (Harrison and Alderman, 1971). In the case of phenytoin the steady state concentration associated with optimal therapeutic effect covers a relatively narrow range (10-25 µg/ml), and hydroxylating capacity becomes saturated within this range (Houghton and Richens, 1974). With a few drugs, for example warfarin, in addition to wide variation in oxidation rates there is a six-fold variation in the plasma concentration required to produce a particular anticoagulant response (Breckenridge and Orme, 1971).

In both cases recognition of the wide inter-individual variation is necessary to achieve the best therapeutic response. While the pharmacological effect of an anticoagulant can be directly measured, steady state plasma or saliva levels of phenytoin and

several other drugs provide a valuable guide to therapy. Discussion of the factors influencing the inter-individual variation in drug levels requires consideration of the principles of pharmacokinetics and drug metabolism.

b) PHARMACOKINETIC CONSIDERATIONS

The oral route is the most practical and acceptable for medication in most situations and particularly for chronic conditions. Good absorption from the gastrointestinal tract is therefore an essential feature of drugs administered orally. Most zenobiotic substances cross cell membranes by passive diffusion and absorption is favoured by a low degree of ionisation and a high lipid/water partition of the non-ionised form of the drug. As the enterocytes can be considered as lipid barriers, lipid-soluble drugs with a low degree of ionisation are absorbed more readily than lipid-insoluble, high ionised drugs.

Conversely renal excretion of a lipid-soluble drug is limited as the drug is readily reabsorbed across the tubular epithelium. Only polar, less lipid-soluble compounds are unable to diffuse back and are therefore excreted. The body is able to transform foreign substances into more polar, less lipid-soluble compounds. Without such mechanisms many highly protein-bound, lipid-soluble drugs could only be disposed of very slowly, over weeks, months or even a lifetime (Butler, 1958).

Drug metabolism

Pathways of drug metabolism are numerous and complex, but the principle routes were defined by Williams (1959, 1971) and are described in detail in textbooks. They can be divided into two phases. Phase I reactions comprise oxidations, reductions and hydrolyses, and can result in:

(i) the inactivation of the drug

(ii) conversion of an inactive compound into an active drug

(iii) conversion of an active drug into an active metabolite

(iv) conversion of a stable drug into a toxic metabolite

Phase II reactions are synthetic and usually convert active compounds into inactive excretory products.

Phase I reactions usually introduce or unmask polar groups in the molecule prior to the synthetic phase II reactions. Drugs containing functional groups such as OH, COOH or NH_2 can undergo phase II reactions directly; for example paracetamol (p-acetamidophenol) is conjugated to form p-acetamidophenyl glucuronide and sulphate. Its precursor phenacetin is a neutral drug and must first undergo oxidative de-ethylation, a phase I reaction, to form the weakly acidic paracetamol, and then the phase II reactions. The glucuronide

and sulphate are inactive, almost completely ionised and therefore water soluble, and rapidly excreted by the kidney.

The enzymes catalysing biotransformations are found mainly in the liver, but also in lung, intestine, kidney and skin. Most phase I reactions are catalysed by enzymes located in hepatic smooth endoplasmic reticulum (SER), although hydrolyses of esters and amides can occur in plasma and liver. Homogenisation of liver tissue disrupts the SER giving rise to vesicles, the "microsomes" which can be separated from the rest of the homogenate by high speed centrifugation. The microsomal enzymes are classified as mixed function oxygenases, and although they also carry out reduction of azo-and nitro-compounds these reactions are of secondary importance in drug metabolism. Most drugs are metabolised by a small number of reactions, the most common being oxidation (Gillette, 1963). Oxidative reactions include aromatic and aliphatic hydroxylation, deamination, O-dealkylation and N-dealkylation, N-oxidation and sulphoxidation. The enzyme system is of low specificity and is shared by many endogenous compounds, for example, steroid hormones, vitamin D, cholesterol and fatty acids.

The microsomal oxidising enzyme system

Studies in the nineteen fifties by Axelrod (1955 (a) and (b)) and Brodie, et al. (1955), showed that most of the drug oxidising activity present in the whole liver homogenate could be obtained in

a re-constituted system containing the microsomal fraction and soluble supernatant or the microsomal fraction and the co-factors NADPH and oxygen. Klingenberg (1958) and Garfinkel (1958) described the presence of a carbon monoxide binding pigment in liver microsomes. Omura and Sato (1964) showed it to be a cytochrome which when combined with carbon monoxide in the reduced state had an absorption peak at a wavelength of 450 mμ . It was called cytochrome P-450, and has been shown to be the final oxygen-activating enzyme in the microsomal oxidising enzyme system.

More recently evidence has accumulated to suggest that there are multiple forms of the cytochrome. Most of the evidence is based on animal studies with various enzyme inducing agents. These fall into one of two classes (Conney, 1967) characterised by the substrates whose metabolism they induce, and by their effects on cytochrome P-450. The groups of inducers of which phenobarbitone is the prototype enhances the metabolism of a wide variety of substrates but is a poor inducer of the aryl hydrocarbon hydroxylases. In contrast 3, 4 benzpyrene, 3-methyl cholanthrene and other polycyclic hydrocarbons stimulate the metabolism of only a few substrates but are potent inducers of aryl hydrocarbon hydroxylase. Both types of inducer increase cytochrome P-450 content but the polycyclic hydrocarbons also induce the synthesis of another cytochrome, P-448, which is not present in the microsomes of control or phenobarbitone treated rats. Alvares, Bickers and Kappas (1973) and Alvares (1976) have recently described a new type of inducer the polychlorinated

biphenyls, which induce haemoprotein with a mixture of the properties of P-448 and P-450,and suggested that it may be a mixture of the two cytochromes or a catalytically distinct cytochrome.

While the number of forms of cytochrome"P-450" is not yet resolved, the following reactions are thought to take place. Oxidised cytochrome P-450 reacts with the substrate to form a substrate-cytochrome complex. This is reduced by an electron from the flavoprotein NADPH-cytochrome c reductase, either directly or indirectly through a still unidentified carrier. The reduced cytochrome-substrate complex then combines with molecular oxygen to form an "active" oxygen complex. This in turn breaks down to give oxidised drug and oxidised cytochrome P-450. There are about 20 molecules of cytochrome P-450 per molecule of NADPH-cytochrome c reductase.

The only rate-limiting step identified in the overal oxidation of a drug is the reduction of the cytochrome P-450-drug complex. Species differences in the P-450 reductase activity (but not P-450 concentration) have been shown to parallel differences in rates of N-demethylation of ethylmorphine (Davies, Gigon and Gillette, 1969). In man however, in vitro rates of N-demethylation of ethylmorphine in microsomal preparations from wedge biopsies of liver did not correlate with antipyrine half-lives in vivo (Davies, Thorgeirsson, Breckenridge and Orme, 1973). In another study of in vitro and in vivo metabolism antipyrine and quinine half-lives gave no clear

correlation with P-450 content or cytochrome c reductase (May, Helmstaedt, Bustgens and McLean, 1974). Thus, although induction of enzyme synthesis is associated with increased metabolism, neither content nor activity of any single component of the system comprises a critical rate-limiting step in man.

Drug conjugation

In conjugation reactions a drug is coupled to a conjugation agent provided by the body from intermediary metabolic routes. The commonest conjugating agent is glucuronic acid which can combine with all of the commonly reactive groups (Dutton, 1956) and provides a metabolic route for endogenous and foreign compounds. Other important conjugation reactions are acetylation, sulphation, methylation, glycine conjugation and mercapturic acid synthesis.

Conjugation occurs in two stages. Either the conjugating agent or the drug is "activated" to form part of an "activated" nucleotide which is then transferred to the other component of the conjugated product. For example, ezymes in the soluble fraction of the liver catalyse the synthesis of uridine diphosphate glucuronic acid (UDPGA). The second step is effected by a glucuronyl transferase in the microsomal fraction of the liver (Dutton, 1956). Endogenous glucuronides (e.g. bilirubin and thyroxine) and foreign compounds appear to share the same pathways, although there is some evidence

of specificity of glucuronyl transferases (Gillette, 1963).

It has been suggested that if there is a common rate-limiting step for oxidation, it should be possible to classify individuals according to their ability to oxidise drugs by measuring the rate of oxidation of a single prototype drug. Attempts to find a drug or drugs which might serve as markers of oxidation have led to extensive studies of metabolising ability in groups of individuals (Davies and Thorgeirsson, 1971; Alexanderson, 1972; Kadar, Inaba, Endrenyi, Johnson and Kalow, 1973; Smith and Rawlins, 1974). In all of these studies, measurements of plasma half-life or clearance were employed as the index of oxidation. Use of these values has provided valuable information about variation in drug metabolism in man and about the factors controlling this variation. Their use is based on the following considerations:

The one compartment-model

The most common approach to the pharmacokinetic study of drugs is to depict the body as a system of compartments. The simplest model is the one compartment-model in which the body is assumed to be a homogeneous unit, in which drugs are uniformly distributed. This model is useful for analysis of plasma and urine concentrations when drugs are very rapidly distributed.

Usually drug absorption and elimination can be described

by linear or "first-order" kinetics, e.g. the rate of biotransformation is proportional to the amount of drug in the body. Although this assumption is not strictly true, as metabolism involves enzymes with limited capacities, at the usual drug concentrations in the body first order processes are usual. Only substrates at very high concentrations such as ethanol or a few drugs, for example, diphenylhydantoin, show zero-order kinetics.

When a drug is given intravenously and distributed very rapidly in the body it confers on the body the pharmacokinetic characteristics of a one-compartment-model. If drug elimination is proportional to concentration a plot of drug concentration as a function of time shows an exponential decay and a plot of log. concentration versus time yields a straight line. The time taken for drug concentration to decrease by half is known as the biologic half-life, elimination half-life, or, if it is obtained by measurement of plasma concentrations, the plasma half-life ($T\frac{1}{2}$). This allows elimination rate to be expressed in two ways, by the half-life or the proportionality constant (elimination rate constant) K_{el}. The two are related as follows:

Since:	$\text{Rate} \propto C$	(1)
In differential form:	$dc/dt = -K_{el}C$	(2)

where C = drug concentration

t = time

K_{el} = the proportionality constant or "apparent" first

order elimination rate constant (units = t^{-1}).

Integrating equation (2) gives

$$C = Co.e^{-K_{el}t} \qquad (3)$$

Taking natural logarithms: $$\text{Ln } C = \text{Ln } C_o - K_{el}t \qquad (4)$$

Converting to the base of 10: $$\text{Log } C = \text{Log } C_o - \frac{K_{el}t}{2.303} \qquad (5)$$

which is the equation of a straight line with intercept log C_o and slope $-\frac{K_{el}}{2.30}$

It can also be shown by substituting for C_o and $C = \frac{C_o}{2}$ in equation (5)

that $T\frac{1}{2} = \frac{0.693}{K_{el}}$

Many drugs are eliminated by more than one route e.g. metabolism to one or more metabolites and excretion of unchanged drug in the kidney or bile. Each process has its own first order rate constant (K_1, K_2, K_3, etc.) and the overall rate constant is given by:

$$K_{el} = k_1 + k_2 + k_3 \text{ etc.}$$

or $$k_{m_1} + k_{m_2} + k_r$$

where k_{m_1} and k_{m_2} represent rate constants for formation of metabolite 1 and 2 and k_r represents the rate constant for elimination of unchanged drug via the kidney.

The apparent volume of distribution of a drug (V_d) can be established from the relationship:

$$V_d = \frac{\text{Total drug in body}}{\text{Concentration in plasma}}$$

Back extrapolation of the log. concentration versus time plot enables an estimate of initial concentration C_o to be made, and then $V_d = \frac{\text{Dose}}{C_o}$, if the whole dose is absorbed. In the case of antipyrine which is uniformly distributed throughout the body and is almost completely absorbed, V_d gives a good estimate of total body water. For many drugs, because of binding to protein or sequestration in tissues V_d is only an apparent volume of distribution..

The steady state plasma concentration (C_{ss}) of a drug is determined by the half-life and volume of distribution as well as the dose and dosage interval. Thus:

$$C_{ss} = \frac{\text{Absorbed dose}}{V_d} \times \frac{\text{Half-life}}{\text{Dose interval}} \times 1.44$$

(Wagner, Northam, Alway and Carpenter, 1965).

It is immediately apparent why inter-individual variation in

drug oxidation can result in a very wide range of steady state plasma concentrations, resulting in both treatment failure and toxicity for different individuals given the same dose.

Distribution of drugs from plasma into tissue may be slow, and depends on factors such as tissue protein binding and vascular perfusion of tissues into which a drug is taken up. Pharmacokinetic analysis then requires a model with two or more compartments and elimination is bi-exponential or multi-exponential. Analysis of multi-compartment systems is described by Mayersohn and Gibaldi (1971).

For practical purposes elimination can often be analysed using a single compartment model if both the absorption phase and distribution phase are relatively short compared to elimination. Drug concentrations can then be measured after these phases are completed.

Estimation of the volume of distribution V_d permits clearance of the drug to be calculated from the log. concentration/time profile. Thus:

$$\text{Clearance} = V_d K_{el} = \frac{V_d \times 0.693}{T\frac{1}{2}}$$

It is apparent from this relationship that changes in distribution volume can affect K_{el} and half-life even if clearance is unchanged, and this is one reason why half-life is a less satisfactory

measurement of metabolic activity than clearance for some drugs.

Physiological considerations

This approach has provided useful knowledge about the importance of inter-individual variation, route of administration, drug interactions and disease in drug therapy. However, it takes no account of a number of biological factors which are known to affect the time course of drugs in the body. Wilkinson and Shand (1975) have developed a physiological approach to hepatic drug clearance which takes into account changes in hepatic blood flow, protein binding of drugs and the inherent enzyme activity of the liver. Conventionally the ability of an organ to remove a drug from the perfusing blood (clearance) is expressed as the volume of blood from which drug is completely removed in unit time. It is thus dependent on organ blood flow and the extraction ratio. Wilkinson and Shand's approach is based on the assumption that the elimination process is first order but clearance is perfusion limited. They introduced the term "intrinsic clearance" ($Cl_{intrinsic}$) to indicate the maximal ability of the liver to irreversibly remove drug by all pathways in the absence of any limitation of blood flow. Accordingly

$$Cl_H = QE = Q \left(\frac{Cl_{intrinsic}}{Q + Cl_{intrinsic}} \right)$$

where Cl_H = hepatic clearance

Q = hepatic blood flow

E = extraction ratio

Rearrangement gives an empirical definition of intrinsic clearance:

$$Cl_{intrinsic} = \frac{QE}{1 - E}$$

Using these equations it was possible to predict the effects of the variables intrinsic clearance and hepatic blood flow on hepatic clearance. They also showed the importance of protein binding on hepatic clearance of drugs with a high intrinsic free clearance.

Wilkinson and Shand have suggested that this approach provides a basis for the classification of drugs as high or low extraction compounds, each type having clearly definable pharmacokinetic properties. If a drug has a low extraction ratio, due to a small intrinsic clearance relative to hepatic blood flow, hepatic clearance and elimination half-life will be independent of changes in hepatic blood flow, but very sensitive to changes in intrinsic clearance, whether due to enzyme induction, inhibition or other mechanism. There will be only a small first pass metabolism and most of the absorbed dose will reach the systemic circulation. Antipyrine is a prototype of such a drug so that elimination half-life after oral or intravenous administration reflects its metabolic rate if there is no change in volume of distribution. On the other hand for a drug with a high extraction ratio hepatic clearance will predominantly reflect liver blood flow rather than drug metabolising activity. Propranolol is a prototype of such a drug. Between these two extremes clearance would in theory be partly dependent on liver blood flow and partly on drug metabolising activity. In practice while low and high extraction drugs have been described there is little data

relating to medium extraction compounds. Protein binding provides a further complicating factor but seems to be important only with drugs which are very highly protein bound, e.g. warfarin.

Most investigations of inter-individual variation in drug elimination have employed plasma half-lives. The above considerations suggest that unless, like antipyrine, a drug has a low hepatic extraction ratio and low protein binding, half-life is not the best index of metabolic activity. This may partly explain poor correlations seen when examining co-variance of drugs. For example, Davies, Thorgeirsson, Breckenridge and Orme (1973) showed that phenytoin half-life and clearance in healthy volunteers were not significantly correlated. As a result phenytoin half-life did not show a significant correlation with antipyrine half-life, but if the results were expressed in terms of clearance, then a significant correlation was seen. Another drug used to study covariance of metabolism and currently being used as a tool to investigate genetic control of oxidation rates is amylobarbitone (Kalow and Inaba, 1975) and Inaba, Tang, Endrenyi and Kalow (1976) have shown that repeated measurements of half-life in a single individual show greater variation than clearance values.

c) GENETIC FACTORS AFFECTING DRUG METABOLISM IN MAN

Before the development of drug assays sensitive enough to measure therapeutic drug levels in plasma it had been recognised that

a number of abnormal drug responses were due to gene defects (Motulsky, 1957). Most of these inborn errors were shown to result from disorders transmitted by Mendelian dominants or recessives, and demonstrated polymorphism (Kalow, 1962); that is, a frequency distribution curve of a large enough population sample shows discrete curves corresponding to different genotypes. These curves are described as multimodal or discontinuous. Several instances of polymorphic control of a drug metabolic pathway have been identified, the best known example being that of isoniazid acetylation (Evans, Manley and McKusick, 1960). Rapid inactivation is inherited as a dominant character which results in high levels of acetyl transferase.

In contrast the metabolic rates of most oxidised drugs follow a continuous unimodal distribution (Vessell, 1972). Such curves are typical of variables which are multifactorially determined.

In animals inter-species and inter-strain variations of metabolic rates are marked, in contrast with small intrastrain variance, suggesting genetic control (Jay, 1955). Despite the evidence for genetic control, mice were shown to be susceptible to a wide variety of environmental influences, on the basis of measurements of hexobarbitone sleeping time in vivo and drug oxidising activity in vitro. For example, softwood (red cedar) beddings decreased hexobarbitone sleeping time by 65% and increased hexobarbitone oxidase activity three fold. This effect was attributed to induction of

microsomal enzymes by terpenes in these woods. Conversely a reduction in ambient temperature from 25–20° reduced enzyme activity and prolonged sleeping time (Vessell, 1968).

Two approaches were used to assess genetic and environmental influences on drug oxidation in man: Twin studies and family studies.

Twin studies

Following the demonstration of significant environmental effects on oxidation in animals, Vessell and his co-workers employed the twin method of Sir Francis Galton (1875) to investigate the relative contribution of genetic and environmental factors in man. They studied healthy adult Caucasian twins living in Washington, D.C. and chose four oxidised drugs, phenylbutazone, antipyrine, bishydroxycoumarin and ethanol, which are almost completely metabolised. Each twin received a single oral dose of each drug at intervals of several months and plasma concentration/time profiles were followed. Attempts were made to maintain the environments of the volunteers unchanged from their usual pattern, with the provision that no drugs should be taken. A history of smoking, coffee and alsochol consumption was taken.

It was found that for all drugs there was less inter-individual variation between identical than fraternal twins. The contribution of heredity to variation in half-life was estimated by comparison

of within pair variance of the fraternal and identical twins (Neel and Schull, 1954; Osborne and DeGeorge, 1959). Thus genetic contribution to variance =

$$\frac{\left(\begin{array}{c}\text{Variance within pairs of}\\ \text{fraternal twins}\end{array}\right) - \left(\begin{array}{c}\text{Variance within pairs of}\\ \text{identical twins}\end{array}\right)}{\text{(Variance within pairs of fraternal twins)}}$$

The variance of each set of twins was calculated from the formula:

$$\text{Variance} = \frac{(\text{Difference between twins})^2}{2n}$$

Theoretically values derived from this formula can range from 0, indicating no significant hereditary contribution and 1, indicating virtually complete hereditary control. The values obtained for all four drugs were between 0.97 and 0.99, suggesting virtually complete genetic control.

For some sets of identical twins intrapair differences in half-life were less than those for some sets of fraternal twins. Intraclass coefficients were calculated for identical and fraternal twins from the formula:

$$r = \sum \frac{(x_1 - a)\,(y_1 - a)}{ns^2}$$

where x_1 and y_1 are the half-lives for each individual of a twinship, a is the mean half-life for identical or fraternal twins and s^2 the variance. The theoretical values on the basis of complete genetic control should be 1 for identical twins and 0.5 for fraternal twins, having half their total genes in common. For phenylbutazone, antipyrine, bishydroxycoumarin and ethanol intraclass correlation coefficients were 0.83, 0.85, 0.85 and 0.82 for identical twins and 0.33, 0.47, 0.66 and 0.38 for fraternal twins. The Holzinger index (Holzinger, 1929) combines these coefficients to estimate the genetic component. Thus:

$$\text{Genetic component} = \frac{(r_I - r_F)}{(1 - r_F)}$$

Using this formula the estimated genetic component for the four drugs was 0.75 (phenylbutazone), 0.72 (antipyrine), 0.56 (bishydroxycoumarin) and 0.71 (ethanol). These values are considerably lower than the estimates obtained using the first method of analysis.

It has been acknowledged that the estimate of heritability derived from comparison of within pair variances is an overestimate (Vessell, 1972) because negligible environmental differences between twinships are assumed. Vessell's tables showed that there were marked intraclass differences in the use of cigarettes, alcohol and coffee and marked differences between fraternal twinships, particularly with respect to coffee. These differences could therefore explain the discrepancy between estimates of 97% to 99% using the

first method of analysis and estimates of 56% to 75% using the Holzinger index. The latter would infer that 25 to 44% of the variance was determined by environmental factors. Alexanderson, Price Evans and Sjoqvist (1969) calculated a value of 0.98 for nortriptyline from twin data, using the former method of estimation.

Family studies

Whittaker and Price Evans (1970) performed a family study to assess the genetic component of variance of phenylbutazone half-lives. Their measurement of heritability was based on the model of polygenic inheritance described by Fisher (1921) and Falconer (1960). Thus

$$V_P = V_G + V_E$$

where V_P = phenotypic variance observed

V_G = genetic component of variance

V_E = environmental component of variance

$V_G \simeq V_A$ = variance due to additive effects of genes, assuming no variance due to interactions or dominance effects.

Then V_A / V_P = the proportion of variance due to the genetic component, or "heritability". It is estimated by the regression coefficient of mean offspring values on midparent values.

Whittaker and Evans found a significant regression of mean offspring value on midparent value ($r = 0.74$, $p < 0.001$) for 27 families. However, when one family with very high values was excluded the correlation was much weaker ($r = 0.53$, $p < 0.01$). Furthermore there was a significant correlation between spouses, suggesting a shared environmental contribution. Whittaker and Evans gave all subjects 1.2 mg/kg of phenobarbitone nightly for three nights in order to subject all individuals to a uniform inducing agent, which they argued would reduce the environmental differences between subjects and reduce the environmental component of variance. This device shortened the half-lives only modestly, but brought the distribution curve to a normal curve, and increased the estimate of heritability to 0.65. This infers an environmental component of 0.35 or 35%.

d) ENVIRONMENTAL FACTORS AFFECTING DRUG METABOLISM IN MAN

Drugs

During the 1960s it was recognised that the phenomenon of enzyme induction by drugs and other foreign substances could have significant effects in man (Conney, 1967). A number of clinical studies demonstrated that drugs of the phenobarbitone class of inducers can produce clinically important interactions with other drugs whose oxidation they increase. Such interactions are of greatest

importance and often life-threatening with drugs which require regulation within narrow limits, e.g. anticoagulants, antiepileptics and hypoglycaemics. For example, the elimination rate of the anticoagulant warfarin is induced in man by phenobarbitone (Robinson and MacDonald, 1966), amylobarbitone, chloral phenazone and antipyrine (Breckenridge and Orme, 1971) and dextropropoxyphene (Orme, Breckenridge and Cook, 1975) but not by the benzodiazepine groups of drugs (Orme, Breckenridge and Brooks, 1970). Induction of warfarin metabolism has been shown by measurement of therapeutic response (thrombotest), changes in steady state concentrations and the plasma half-life, and by showing an increase in urinary excretion of (^{14}C)-labelled warfarin metabolites (Breckenridge, Orme, Thorgeirsson, Davies and Brooks, 1971).

By performing dose-response studies in rats with several barbiturates and antipyrine at six dose levels, Breckenridge and Orme showed that induction was dose-related, phenobarbitone being the most potent of the drugs studied, with a dose-response curve far to the left of the other drugs. Long half-life and high liver:plasma ratio seemed to be related to potency as an inducing agent. Whitfield, Moss, Neale, Orme and Breckenridge (1973) also found evidence of dose-dependence in man.

Inhibition of metabolism by drugs which share the cytochrome P-450 enzyme system also occurs in clinical practice. The number of examples is smaller than for interactions due to induction, and

it is usually important only with drugs used in large doses or with a narrow therapeutic range. Increased steady state levels, drug half-life and therapeutic effect have been demonstrated, showing inhibition of tolbutamide by dicoumarol, chloramphenicol and sulphaphenazole. Diphenylhydantoin is inhibited by a number of drugs, while warfarin metabolism can be inhibited by drugs and alcohol (Breckenridge and Orme, 1971). Alcohol and some drugs can act both as inducers and inhibitors.

Environmental chemicals

Simultaneously with these observations in man the effects of non-drug inducing agents in the environment were investigated. A number of environmental chemicals, particularly insecticides and industrial toxins had been shown to affect metabolism in animals and man (Conney and Burns, 1972). The first controlled investigation of the effects of such substances on exposed subjects was that of Kolmodin, Azarroff and Sjoqvist (1969). They compared antipyrine half-lives in 26 males working in an insecticide factory with the half-lives of 33 male and female office workers in the same firm. They found a mean value of 7.7 ± 2.6 (S.D.) hours in the factory workers (a four-fold range) compared with 13.1 ± 7.5 hours in the office workers (a seven-fold range). The chief insecticide concerned was lindane, but the chlorinated hydrocarbons, DDT, chlordone, endrin and dieldrin are all potent inducers, and high concentrations can accumulate in exposed workers, e.g. 15-30 µg/g

for DDT and its metabolite DDE. DDT factory workers with DDT and DDE levels twenty times those of controls eliminated antipyrine and phenylbutazone faster than controls, while urinary 6 β OH-cortisol was 50% higher than in controls (Poland, et al., 1970).

O'Malley, et al. (1973) have demonstrated that operating theatre personnel exposed to a variety of volatile chemical agents, have 21% faster antipyrine clearance than sex and age-matched controls ($p < 0.05$). On the other hand, exposure to lead in industry has been shown to prolong antipyrine half-life, although only clinically overt poisoning produces a degree of impairment likely to be clinically significant (Alvares, et al., 1975; Alvares, et al., 1976). In industrial areas atmospheric pollutants which appear to be related to both chronic disease (bronchitis and lung cancer) and epidemics of deaths from respiratory disease (Ayres & Buehler, 1970) could, at least in theory, have effects on liver microsomal enzymes.

Socially used drugs

The socially used drugs cigarettes, alcohol and the contraceptive pill have been shown recently to have significant effects on drug metabolism. Cigarette smoke contains 3-methyl cholanthrene, 3-4 benzpyrene and other polycyclic hydrocarbons. These substances, nicotine itself and caffeine are drug metabolising enzyme inducers in animals (Wenzel and Broadie, 1966; Yamamoto, Nagai, Kimuia, et al., 1966;

Mitoma, Sorich and Neubauer, 1968). The activity of benzpyrene hydroxylase is increased in the lungs, livers, intestines and placentas of human cigarette smokers (Welch, Harrison, Conney, Poppers & Finster, 1968). Indirect evidence of enzyme induction in man comes from a study of pentazocine requirements for supplementing nitrous oxide anaesthesia in surgical patients (Keariszanto and Pomeroy, 1971). Subjects who smoked and those from an urban environment required higher doses of pentazocine than non-smokers or country-dwellers, suggesting that both smoking and atmospheric pollution may be related to drug metabolic rates.

Surveillance of unwanted drug side effects has provided evidence that some of these are less frequent and less severe in smokers (Jick, 1974). To date it has been shown that smoking reduces the efficacy of propoxyphene analgesia (Boston Collaborative Drug Surveillance Program, 1973a) and reduces the incidence of drowsiness associated with the use of chlordiazepoxide and diazepam (Boston Collaborative Drug Surveillance Program, 1973b). Preliminary data (Jick, 1974) suggested that smoking reduced the incidence of drowsiness associated with chlorpromazine use. The most recent report on patients in 22 hospitals in 7 countries indicates that smokers have s six-fold decrease in drowsiness (16.2%) compared to non-smokers (2.8%) (Miller, 1976). It has been postulated that the reduction in drowsiness was due to induction of metabolism by cigarette smoking, but drug levels were not measured.

More direct evidence of acceleration of drug metabolism by smoking in man has been shown by a number of studies. Thus cigarette smokers have faster elimination rates both for nicotine (Beckett & Triggs, 1967; Beckett, Gorrod & Jonner, 1971) and for other drugs, e.g. theophylline and antipyrine. Theophylline half-life in 10 young adult male smokers was 4.14 ± 1.24 hours compared with 7.19 ± 1.83 hours in 14 male controls of similar age ($p < 0.001$) (Jenne, Wyse, McHugh & McDonald, 1975). Antipyrine clearance was increased by 25% in moderate smokers in the study of Vestal, et al. (1975) but heavy smoking produced no further increase. An increase of 23% was seen in the antipyrine half-life of 8 subjects who stopped smoking and were restudied two months later (Hart, Farrell, Cooksley and Powell, 1976).

Caffeine does not appear to be an inducing agent in man at the doses used socially. In Vessell's study of 14 pairs of identical and fraternal twins (Vessell, 1972) there was no significant effect of coffee or smoking on antipyrine, phenylbutazone or bishydroxycoumarin half-lives. Mitoma, et al. (1968) calculated on the basis of metabolic weight that the amount of coffee required to show induction in the rat was equivalent to 8 cups daily for a 70 Kg man. In the study of Vestal, Norris, Tobin, Cohen, Shock and Andres (1975) on 309 men, an apparent correlation of coffee consumption with a decrease in half-life was shown by multiple regression analysis to be due to the higher coffee consumption

by younger people, and was an effect of age.

The third widely used social drug, alcohol, increases drug oxidation rates in man in some studies but had no effect in others. The effects of alcohol on drug metabolism depend on several factors: (i) the size of the dose, (ii) the duration of exposure (iii) the presence of liver damage and (iv) the presence of alcohol in the body at the time of the study. Modest use of alcohol in man shows no significant relationship with antipyrine metabolism in large samples (Vestal, et al., 1975), but at higher doses (200 g per day and over) all subjects have shortened half-lives of antipyrine and tolbutamide (Iber, 1976). These values became prolonged between 4 and 10 weeks after withdrawal.

Non-ethanol constituents of alcoholic beverages also appear to be important as a higher dose (460 g/day) of pure ethanol is required to produce significant changes in drug elimination (Iber, 1976).

With changes in social customs other drugs have become more widely used, particularly marihuama, amphetamines and "hard drugs" Chronic use of marihuana has been shown to double the elimination rate of its active component, Δ -9-tetra-hydrocannabinol, most of which is oxidised prior to elimination (Lemberger, et al., 1971). Its potential for inducing therapeutic agents is not yet established.

Another agent increasingly used by healthy people is the steroid

oral contraceptive. Evidence is accumulating that this significantly slows the elimination of antipyrine (O'Malley, Stevenson & Crooks, 1972; Carter, et al., 1974; Carter, et al., 1975) by increasing the volume of distribution (after long term use) and apparently decreasing the rate of metabolism.

Diet

Finally, another major source of environmental influences on drug metabolism is the diet. Animals are known to be particularly sensitive to both calorie and protein deprivation, although effects depend on both the sex of the animal and the nature of the experiment. For example, Kato and Gillette (1965) have shown that barbiturate oxidation is 3 times greater in normally fed adult male rats than in normally fed female rats. After starvation for 72 hours barbiturate hydroxylation increased by 50% in the liver microsomal fraction of the female rats, while it decreased by 50% in the males. *In vivo* studies support this sex difference (Kato, Chiesara & Vassanelli, 1962). Protein deprivation, on the other hand, reduces the concentration of cytochrome P-450 per unit of liver weight to one third that of controls (Marshall & McLean, 1962) and reduces the oxidation *in vitro* of a variety of substrates (Campbell & Hayes, 1974). It also increases the toxicity of oxidised drugs *in vivo* and the toxicity of pesticides (Lee, Harris & Trowbridge, 1964). Drugs whose toxicity arises from oxidation to reactive intermediates are less toxic in protein

deficient rats; for example, carbon tetrachloride (McLean & McLean, 1966) and paracetamol (McLean & Day, 1975).

Variations in quality and quantity of dietary fat also affect drug oxidation. Deficiency of animal fat leads to low cytochrome P-450 levels in the rat and impaired phenobarbitone induction of P-450 (Marshall and McLean, 1971). In view of the wide variation in quantity and type of dietary fat these observations could be relevant to man.

Animal experiments are complicated by the sensitivity of the rat to the experimental situation, e.g. feeding patterns, bedding, temperature, crowding and stress (Vessell, 1968). Despite the wealth of animal experiments there is little information on effects of protein deficiency states, chronic protein-calorie malnutrition or dietary manipulation on drug metabolism in man. An early but neglected report is that of Wharton & McChesney (1970) who showed a modest impairment of chloroquine metabolism in malnourished infants.

Recently attention has focused on the effects of inducing agents present in certain dietary constituents. A number of vegetables, e.g. brussel sprouts, cabbage, turnips, broccoli, cauliflower and spinach, induce benzo(a)pyrene in rat small intestine (Wattenberg, 1971; 1972). Inducing agents also occur in charcoal-broiled beef in very high concentrations (Lijinsky and Shubik, 1964). It has now been shown in controlled studies

(Conney, 1976) that the latter can result in a 20 to 50% reduction in antipyrine and theophylline half-lives in healthy human volunteers.

Importance of sample selection

Most studies of inter-individual oxidation have been performed on patients or laboratory workers. Many drugs are reported to show a range of 3-10 fold, but much depends on the sample chosen for study. Patients are likely to be subject to more pharmacokinetic variables related to the disease state, as well as effects of other drugs. Laboratory workers are a small selected group and unrepresentative of the general population. Medical personnel for example, are likely to be non-smokers. The importance of the sample may be illustrated by considering the literature on rates of antipyrine half-life in Table II. This may vary from as low as 5.3 ± 1.2 (S.D.) hours in barbiturate addicts and 7.7 ± 2.6 in insecticide factory workers to as high as 17.4 ± 6.8 hours in geriatric patients. There are no community-based samples of healthy adults in the literature.

e) AIMS AND SCOPE OF THESIS

The primary aim of this work was to investigate variation in drug metabolism in healthy men and women using a community based

TABLE II

REPORTED VALUES FOR ANTIPYRINE HALF-LIVES IN DIFFERENT TYPES OF VOLUNTEERS

AUTHORS	MEAN AGE	NUMBER & SEX	SOURCE OF SAMPLE	MEAN ± SD
O'Malley, et al., (1971)	76	19 M & F	Geriatric patients	17.4 ± 6.8
Liddell, et al., (1975)	78.9	26 M & F	Geriatric patients	16.8 ± 5.9
	27.2	26 M & F	Medical personnel	12.5 ± 3.2
Vestal, et al., (1975)	68.7	84 M	Healthy volunteers in study of ageing	14.8 ± 5.9
	49.9	150 M		13.8 ± 5.8
	33.0	73 M		12.7 ± 4.3
O'Malley, et al., (1971)		34 M		13.4 ± 3.7
		27 F		10.3 ± 2.1
Fraser, et al., (1972)	32	10 M	Medical & lab. personnel	12.8 ± 1.4
Lindgren, et al., (1974)	25.9	14 M, 2 F	Medical & lab. personnel	11.1 ± 1.3
Kolmodin, et al.	-	15 M, 18 F	Office workers	13.1 ± 7.5
		26 M	Insecticide factory workers	7.7 ± 2.6
Ballinger, et al.	-	8	Barbiturate addicts	5.3 ± 1.2

sample. In view of the apparently large genetic component of variance shown for antipyrine half-life it was decided to sample subjects of the three major ethnic groups. Because of the experimental evidence in animals that diet plays an important part in drug metabolic rates we set out to obtain some subjects who might be sub-optimally nourished.

We obtained subjects from two sources:

(i) A rural West African village, where the people belonged to a single tribe, and the diet was vegetarian (chiefly cereals) and sub-optimal (49 subjects).

(ii) Four London industrial firms, whose employees were Caucasians, eating a normal British diet and Asian emigrants, many of whom ate a vegetarian diet (131 subjects).

As the study progressed a possible contribution of socially used drugs was recognised and a history of their use was obtained. When the contribution of diet became clear a sub-sample of subjects had detailed dietary assessments made.

We chose antipyrine because of its complete absorption when given orally, low (10%) protein binding,distribution in the total body water and ease of measurement. We chose paracetamol as a marker of conjugation. Because of the obvious difficulty in

obtaining serial plasma samples we first developed and assessed techniques for the use of saliva samples to determine the half-lives of both drugs.

Antipyrine oxidation can be assessed by measurement of half-life for reasons mentioned above. For many other drugs this is not a valid index of oxidation and while clearance is more valid, it does not take account of different metabolic routes. It is often important to know the rates of individual pathways, particularly if some pathways produce toxic metabolites, for example paracetamol, isoniazid and furosemide. These pathways can be affected by a variety of environmental influences, e.g. diet, with important clinical consequences. Thus measurement of product formation should provide the most valuable information about a drug's metabolism.

We therefore set out in the second part of these studies to assess oxidation of a common drug, amylobarbitone, by the classical clearance method, by measurement of product formation (3'-hydroxyamylobarbitone) <u>in vivo</u> and by measurement of product formation <u>in vitro</u>, using needle biopsy samples of human liver. It was possible to obtain enough data on each biopsy using a sensitive radioassay to estimate Michaelis-Menton kinetic parameters.

CHAPTER II

Assessment of antipyrine and paracetamol kinetics by measurement in saliva

(a) Antipyrine kinetics in saliva

- (i) Subjects and sample collection
- (ii) Methods
- (iii) Results

(b) Paracetamol kinetics in saliva

- (i) Subjects and sample collection
- (ii) Methods
- (iii) Results

(c) Concurrent administration of antipyrine and paracetamol

- (i) Results
- (ii) Discussion

(a) ANTIPYRINE KINETICS IN SALIVA

(i) Subjects and sample collection

Ten healthy male volunteers aged between 26 and 36 (mean 32) years took part in the study. All were medical research workers in the Department of Clinical Pharmacology at the Royal Postgraduate Medical School. None was taking any medication and only one (A.B.) was a smoker (20/day). After an overnight fast, whole blood (10 ml) was obtained by venepuncture and 3–6 ml mixed saliva were collected. The flow of saliva was stimulated by chewing a small piece of Parafilm (Gallenkamp) for 3–5 minutes. No subject had difficulty producing adequate samples although flow rates varied 3–5 fold. Antipyrine (phenazone tablets B.P., 600 mg) was taken with water (100 ml) and further blood and saliva samples were obtained after 3, 5, 8, 24 and 32 hours. Subjects were asked to rinse the mouth with water after each meal or drink in between sampling times. Blood samples were placed in heparinized tubes and centrifuged. Plasma and saliva samples were stored frozen until analysis.

(ii) Methods

Extraction Procedure

Antipyrine was extracted and assayed by a modification of the gas-chromatographic method described by Lindgren, et al. (1974). Plasma and saliva samples were treated in an identical manner.

An aqueous solution (100 µl) containing 4-methylantipyrine (150 µg/ml), as an internal standard, was added to plasma (1 ml)

or saliva (1 ml) in a glass-stoppered 15 ml pyrex centrifuge tube. The sample was adjusted to pH 1.0 with 5 N HCl (75 µl), extracted with toluene (2 ml) by brief whirlmixing and centrifuged for 10 min. at 2000 rev/minute. The toluene phase was discarded after rapid freezing of the aqueous phase in solid CO_2/acetone. In the course of these studies it was established that extraction with toluene was unnecessary when analysing saliva. The aqueous phase was alkalinized to pH 11.0 with 0.5 N NaOH (1 ml), extracted with dichloromethane (6 ml) by brief whirlimixing (vortex 2-3 s gives 60% recovery) and centrifuged for 10 min. at 2000 rev/minute. After removal of the aqueous phase, the organic phase was transferred to a clean pyrex tube. The solvent was evaporated under nitrogen and the residue dissolved in methanol (30 µl). An aliquot of the resulting solution (0.5-1.5 µl) was injected into the gas-chromatograph. All extractions were carried out in duplicate.

Standard curve

A standard aqueous solution of antipyrine (1mg/ml) was used to prepare 1, 2, 5, 10 and 15 µg/ml standards in drug-free human plasma or saliva. All standard samples were made up and analysed in duplicate along with unknown samples at each assay. Standard curves obtained using saliva were identical with those using plasma.

Gas Chromatography

The instrument used was a Pye Unicam series 104 chromatograph

equipped with a hydrogen flame-ionization detector and a Servoscribe strip-chart recorder.

A glass column 5' x ¼" packed with a mixture of 0.5% Carbowax 20M and 0.5% SE 30 on Gas-Chrom Q, mesh 80-100 (Phase Separations Ltd., Queensferry, Flintshire, U.K.) was conditioned by placing in an oven at 50°C for 1 h and then by raising the oven temperature by 1°C/min from 50°C to 230°C for 1 hour. The flow of carrier-gas (nitrogen) was 20 ml/min throughout conditioning.

The conditions employed during chromatography were: injector temperature 230°C, column temperature 220-230°C, detector temperature 240°C and nitrogen flow 50 ml/minute. Retention times using these conditions were 2.1-2.8 min for antipyrine and 3.1-3.9 min for 4-methyl antipyrine (Fig. 1). There were no interfering peaks. As the column aged, retention times increased slightly, requiring an increase in column temperature from 220 to 230°C to maintain constant retention times. Antipyrine concentration was obtained from the standard curve after measurement of peak height ratios.

Concentration-flow relationship

It was considered important to assess the effect of salivary flow on the salivary concentration of antipyrine within

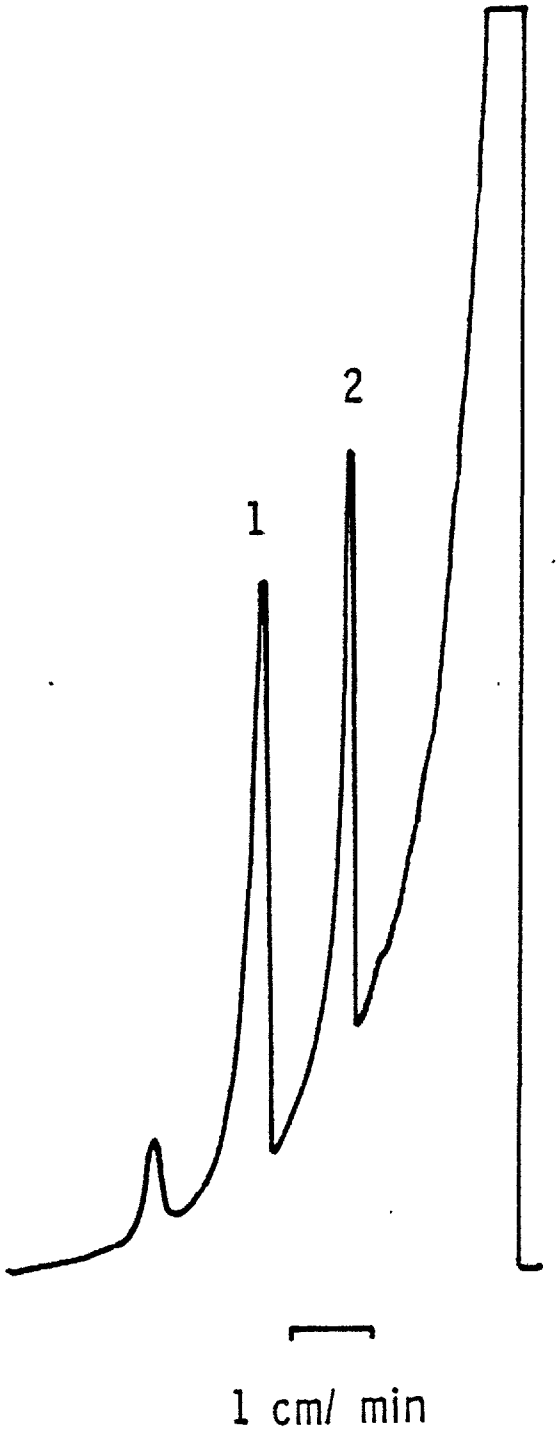


Figure 1

Gas chromatograph trace showing antipyrine and 4 methyl antipyrine peaks from extract of saliva containing 10 µg/ml of each

1 = 4 methyl antipyrine. RT = 3.2 min

2 = antipyrine. RT = 2.2 min

the range of flow rates convenient for collection. Three different flow rates were obtained in one subject (JM) using different stimuli: (i) Mouth and tongue movements alone for 3 min, (ii) 1 drop of 10% citric acid solution on the tongue followed by mouth and tongue movements for 1 min, (iii) 3 drops of 10% citric acid on the tongue followed by mouth and tongue movements for 1 minute. Six samples were collected consecutively by each method and the concentration of antipyrine in each sample was compared with that in a plasma sample obtained between the third and fourth saliva samples. Collections were timed at 3, 4 and 6 h after a 600 mg dose and saliva volume was assessed by weighing receptacles before and after receiving each sample.

(iii) Results

Simultaneous antipyrine concentrations in saliva and plasma for all samples in the ten subjects are shown as a scatter diagram in Figure 2. There was very close correlation ($r = 0.96$) and the ratio of saliva to plasma concentration was 0.92 ± 0.02 (s.e. mean).

Mean antipyrine half-life in saliva was $12.58 \text{ h} \pm 0.59$ (s.e. mean) and mean plasma half-life $12.78 \text{ h} \pm 0.43$ (s.e. mean). Table 1 shows half-lives, apparent volumes of distribution and total body clearance rates derived from plasma and saliva data

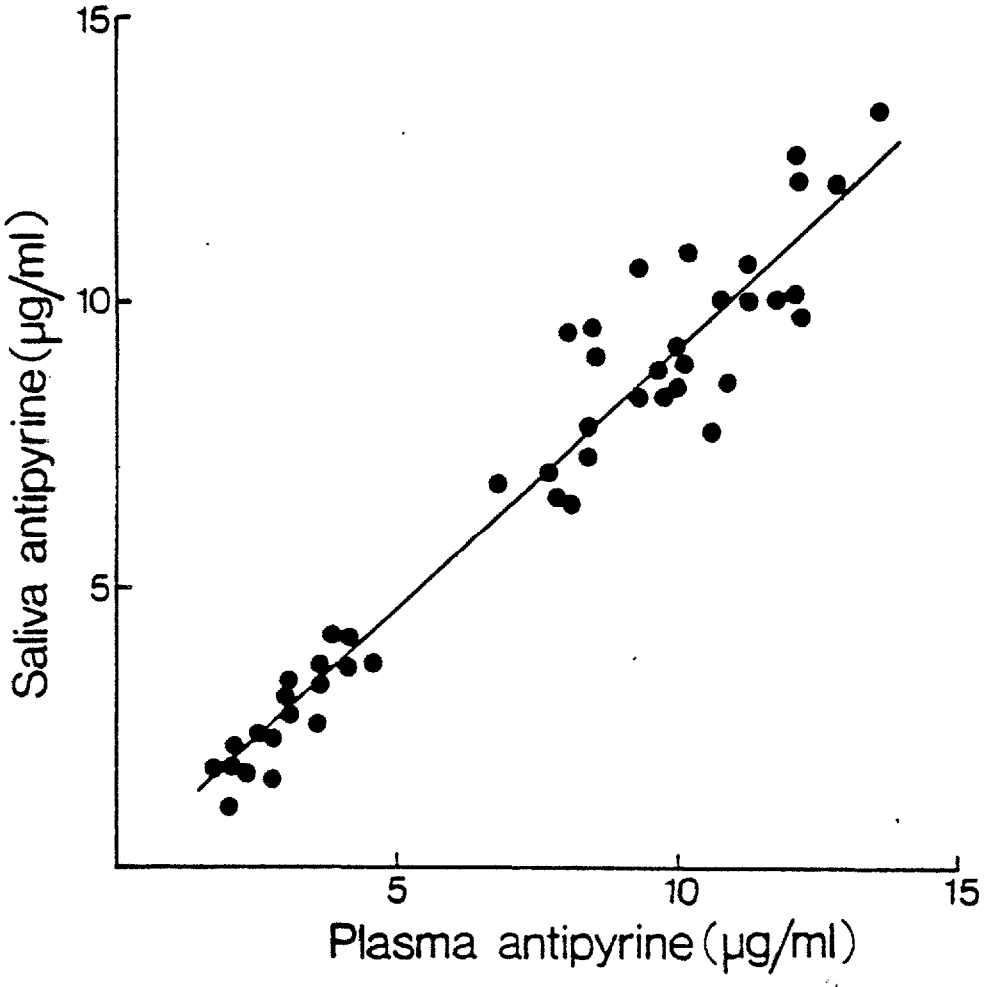


Figure 2

Correlation between saliva and plasma antipyrine concentration in 10 subjects.

Table I

Kinetic parameters derived from plasma and saliva antipyrine concentrations in 10 subjects with means and standard error of means

	Half-life (hr))		Apparent volume of distribution* (l/kg))		Total body clearance+ (ml/min)	
Subject	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva
A.B.	11.6	11.6	0.79	0.70	51.8	46.7
F.K.	14.6	13.2	0.56	0.61	32.2	39.8
M.O.	13.9	13.4	0.66	0.59	38.5	35.9
R.M.	12.5	14.0	0.59	0.66	46.7	46.8
F.F.	11.4	11.9	0.62	0.74	43.2	49.3
P.B.	13.3	13.6	0.63	0.70	39.8	43.2
J.M.	12.7	11.3	0.58	0.58	35.0	35.8
H.F.	11.4	9.19	0.59	0.69	45.6	65.6
M.C.	11.4	11.5	0.49	0.58	38.6	45.0
K.H.	14.9	16.0	1.00	0.99	37.0	39.5
Mean	12.8	12.6	0.60	0.64	40.8	44.8
S.E.M.	0.43	0.59	0.03	0.02	1.88	2.74

$$*Vd = \frac{\text{Dose}}{C_0}$$

$$+\text{Clearance} = \frac{Vd \times 0.693}{t_{\frac{1}{2}}}$$

assuming a one compartment model (Davies, et al., 1973). Comparison by paired t-test showed no significant difference between plasma and saliva-derived perameters. Standard deviations of all duplicate analyses (Table II) varied between 0.22 and 0.61 µg/ml and the accuracy of both plasma and saliva determinations was comparable with that reported by Lindgren, et al. (1974).

Mean salivary flow rates, obtained by the method described, were 2.03 ± 0.07 (s.e. mean), 4.56 ± 0.13 and 6.14 ± 0.18 ml/min and mean saliva antipyrine concentrations expressed as a percentage of plasma concentration at these flow rates were respectively 94.5% ± 0.83 (s.e. mean), 95% ± 2.67 and 91% ± 1.58. With one exception, saliva concentration was always lower than plasma concentration.

(b) PARACETAMOL KINETICS IN SALIVA

(i) Subjects and sample collection

Three healthy adults, members of the Department of Clinical Pharmacology at the Royal Postgraduate Medical School, received 1.5 g of paracetamol at 9.00 a.m. after an overnight fast, and accompanied by 100 ml of water. Nothing else was taken by mouth until the first sample of saliva was collected at two hours, as described for antipyrine ((a) (i)) and three further samples

Table II

Standard deviation of duplicate analyses of plasma and saliva

Standard deviation from the formula $s = \sqrt{\frac{d^2}{2n}}$ where d = the difference between each pair of duplicates and n = the number of pairs of observations

Coefficient of variation $= \frac{s}{x} \times 100$

	Plasma		Saliva	
Antipyrine concentration (µg/ml)	Standard deviation (µg/ml)	Coefficient of variation (%)	Standard deviation (µg/ml)	Coefficient of variation (%)
< 5.1	0.41	13.6	0.33	11.8
5.1 - 10.0	0.43	5.0	0.44	5.3
> 10.0	0.61	5.2	0.22	1.8
All Values	0.48	6.6	0.38	5.6

were taken at 3, 5 and 8 hours. In two subjects plasma and saliva samples were taken at the same times. On another occasion antipyrine 600 mg and paracetamol 1.5 g were taken concurrently and samples collected at 2, 3, 5, 8, 24 and 32 hours. The 3, 5 and 8 hour samples were divided into two portions and the half-lives of both drugs were determined.

(ii) Methods

Samples were frozen, after separating the aqueous phase from the mucopolysaccharide debris and discarding the latter, until analysis. Paracetamol analysis was based on the glc assay of Prescott, 1971. Paracetamol and an internal standard are silylated for detection with a flame ionisation detector. Plasma and saliva were treated in an identical manner. One ml of phosphate buffer (pH 10) was added to 2 ml of plasma or saliva (in duplicate) and thoroughly mixed with 5 ml of ethyl acetate containing 5 µg/ml of N-butylamino phenol (N-BAP) as internal standard. After centrifugation, 4 ml of the organic solvent (upper layer) was removed into a clean tube and evaporated to dryness under nitrogen. The residue was reconstituted in 700 µl of ethyl acetate and transferred to a 1.7 ml screw-capped phial, evaporated again and redissolved in 20 µl of pyridine. The silylating agent, bis-trimethyl silyl acetamide (BSA) (10 µl) was then added, the phial rotated and allowed to stand for 30 minutes

before injecting 1 µl onto the glc column.

A standard aqueous solution of paracetamol was used to prepare standards over the range 2.5 - 40 µg/ml plasma or saliva, using blank specimens of H.F. and J.M. Calibration of paracetamol to N-BAP peak heights against paracetamol concentration was used to read off the concentrations of paracetamol in samples.

The gas chromatograph was the same instrument as used for antipyrine assays (Pye Unicam 104 with FID). The column was a five foot x ¼ inch glass column packed with 3% OV 17.

Conditions were: oven 170°C, detector 230°C, injector 220°C. Nitrogen flow was 50 mg/min and retention times under these conditions were: paracetamol 2.1 min and N-BAP 3.5 min (Fig. 3). There were no interfering peaks, although extracts from plasma produced a number of peaks with retention times of 12-18 minutes requiring column heating to 230° after every other injection. This was not a problem with saliva samples.

Half-lives were estimated by the method of least squares, using the mean of duplicate analyses.

(iii) Results

Standards produced a linear standard curve which passed

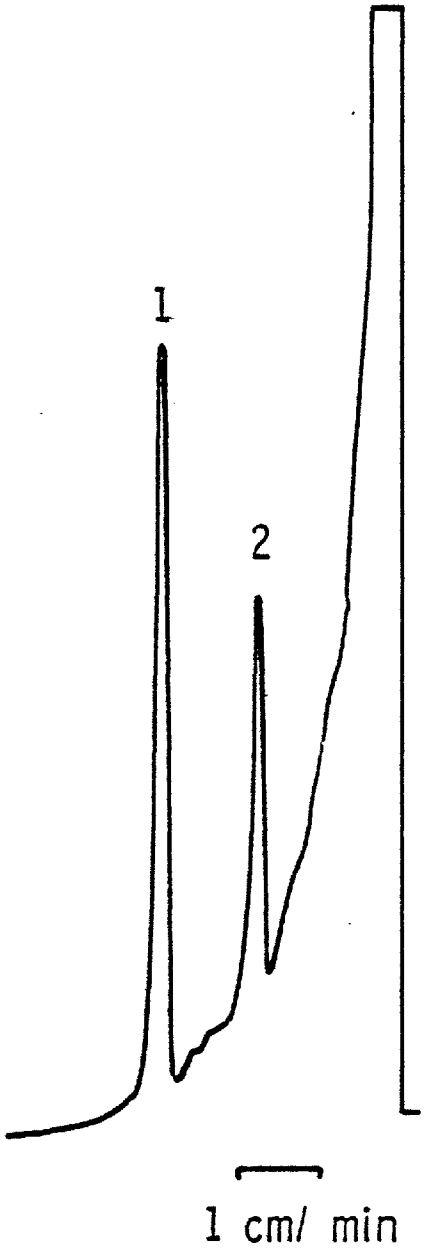


Figure 3

Gas chromatograph trace showing paracetamol and N-BAP peaks from extract of saliva containing 10 µg/ml paracetamol and 25 µg/ml N-BAP as internal standard

1 = N-BAP. RT = 3.5 min

2 = Paracetamol. RT = 2.1 min

through the origin. Extracts were stable for up to 72 hours if left at 4^{o} prior to silylation. Over longer periods paracetamol concentration fluctuated more than 10%.

Plasma half-lives for four subjects ranged from 1.63 to 3.09 hours. These values were similar to those reported by other workers for healthy volunteers. Average values of 1.9 to 2.2 hours were observed in 5 studies of 39 healthy adults, quoted by Levy, Khanna, Soda, Tsuzuki and Stern, 1975.

Saliva and plasma half-lives differed by 7% and 6% (Table III). The 95% confidence interval was smaller for both saliva estimations than the corresponding plasma estimations. Figures 4 and 5 show log. concentration/time profiles for H.F. and F.K.

(c) CONCURRENT ADMINISTRATION OF ANTIPYRINE AND PARACETAMOL

(i) Results

Saliva estimations of paracetamol half-life given separately and with antipyrine were not significantly different (paired t test) nor were saliva estimations of antipyrine given separately and with paracetamol (Table IV).

TABLE III

HALF-LIFE OF PARACETAMOL IN TWO SUBJECTS DETERMINED FROM SIMULTANEOUS PLASMA AND SALIVA SAMPLES

	Half-life from plasma		Half-life from saliva	
Subject	Hours	95% confidence interval	Hours	95% confidence interval
1.	2.83	0.49	2.63	0.28
2.	2.91	0.96	3.09	0.52

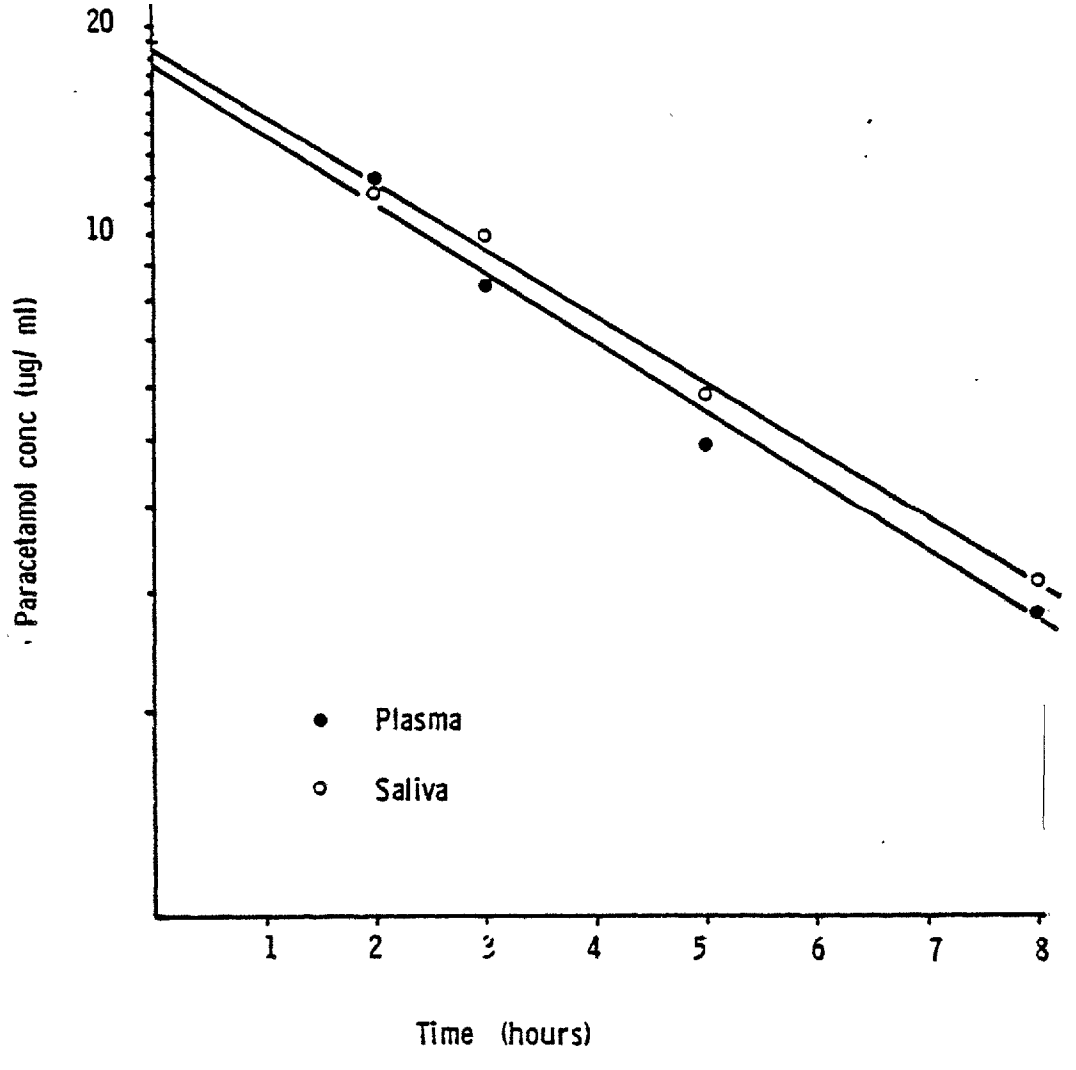


Figure 4

Paracetamol half-life of F.K. in plasma and saliva on the same occasion, after 1.5 g of paracetamol

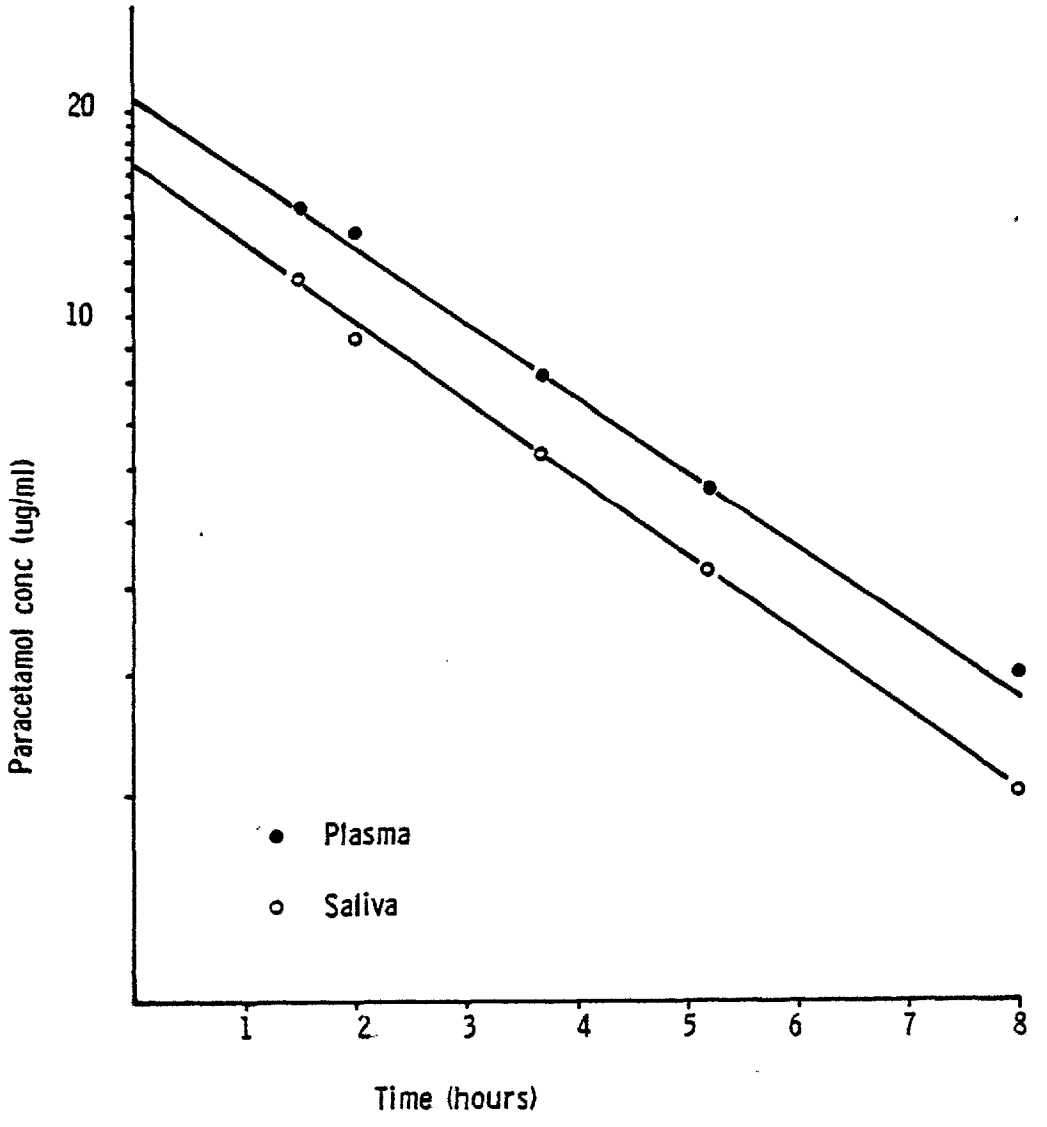


Figure 5

Paracetamol half-life of H.F. in plasma and saliva on the same occasion, after 1.5 g of paracetamol

TABLE IV

a) Half-life of paracetamol in three subjects given paracetamol alone and paracetamol + antipyrine

b) Half-life of antipyrine in two subjects given antipyrine alone and paracetamol + antipyrine

Subject	Half-life of paracetamol (given alone)		Half-life of paracetamol (given with antipyrine)	
	Hours	95% confidence interval	Hours	95% confidence interval
1.	1.83	0.11	2.55	0.27
2.	1.63	0.25	2.00	0.05
3.	3.09	0.52	3.12	0.54

Subject	Half-life of antipyrine (given alone)		Half-life of antipyrine (given with paracetamol)	
	Hours	95% confidence interval	Hours	95% confidence interval
1.	14.8	1.9	13.7	4.8
2.	9.3	3.4	10.0	1.1

(ii) Discussion

The close correlation between the concentrations of antipyrine in saliva and plasma is not unexpected, considering the low pKa (1.4) of this basic drug and the small extent to which it is bound to plasma proteins (10%). Welch et al. (1975) reported exact correlation in 4 subjects followed over 24 hours. Killmann and Thaysen (1955) showed in a study of different sulphonamides that the relationship between saliva and plasma concentrations depended upon the pKa of the particular drug and the pH of both plasma and saliva. Matin, et al. (1974) showed with tolbutamide that the measured ratio of saliva to plasma concentration agreed with that predicted from the formula for acidic drugs:

$$R = \frac{1 + 10^{(pHs-pKa)}}{1 + 10^{(pHp-pKa)}} \times \frac{fp}{fs}$$

where R = ratio of saliva concentration : plasma concentration

pHs = pH of saliva

pHp = pH of plasma

pKa = pKa of drug

fp = fraction of drug unbound in plasma

fs = fraction of drug unbound in saliva

This formula would also be applicable to paracetamol, a very weakly acidic drug with a pK of 9.5

The formula for basic drugs such as antipyrine is:

$$R = \frac{1 + 10^{(pKa-pHs)}}{1 + 10^{(pKa-pHp)}} \times \frac{fp}{fs}$$

fs for most drugs = 1 and where pKa << pHs as in the case of antipyrine, $R \simeq fp$.

The observed value of R for antipyrine from our results was 0.92 (S.E. ± 0.02) which is in good agreement with the predicted value of .90, the fraction of unbound antipyrine in plasma (Soberman, et al., 1949).

The flow and composition of unstimulated saliva exhibit a circadian rhythm but no such rhythm is observed when flow is stimulated (Dawes & Ong, 1973). The composition of saliva is, moreover, dependent upon flow rate and not upon the nature of the stimulus (Dawes & Jenkins, 1964). The results suggest that there is no significant variation in antipyrine concentration within the range of saliva flow-rates expected in healthy subjects salivating in response to simple mechanical or sapid stimuli.

The ratio of saliva to plasma (R) for paracetamol was consistent for each subject at all data points, but in one subject it was greater than unity (1.11 ± 0.13) and in the other it was

less (0.74 ± 0.06) (Figures 4 & 5). There is no obvious explanation for this interindividual difference in ratio. Glynn and Bastain (1973) reported close correlation between saliva and plasma concentrations and interpretation of their graphs shows that mean saliva : plasma ratios were slightly greater than unity for most data points (1.19 ± 0.16).

As only unbound drugs can diffuse into saliva, the ratio of saliva : plasma (R) depends on protein binding as well as on drug pKa and plasma and saliva pH. Variations in protein binding therefore provide a source of interindividual variation in R which is insignificant in the case of antipyrine. The results demonstrate that the plasma half-lives of antipyrine and paracetamol are predictable from the half-lives of the drugs in saliva. In addition, for antipyrine, the saliva data permits calculation of apparent volume of distribution and total body clearance because the ratio of saliva to plasma concentration is close to one. Prediction of other pharmacokinetic parameters for paracetamol and other drugs more highly bound to plasma proteins would require correction for the saliva-to-plasma concentration ratio, which may be more variable than for antipyrine.

Antipyrine is a valuable marker in pharmacokinetic studies of drug oxidation. Paracetamol has not been used to any extent as a tool in studying interindividual variation in metabolism,

but its ease of measurement in saliva make it an equally useful tool for studying variation in drug conjugation rates. Repeated plasma sampling, which is necessary in order to determine plasma half-lives, is unpleasant for the subject and inconvenient for the investigator, especially when carrying out investigations with large numbers of subjects. The sampling procedure can be simplified considerably by the use of saliva and this has made possible the extensive population studies described in Chapter III. Such a study would have been impossible both in London and Gambia without a non-invasive technique.

There are other applications for the use of saliva half-lives. For example such a non-invasive technique would solve one of the major practical problems which has deterred investigators from studying drug kinetics in children. It would also be more acceptable to the elderly, ill or unco-operative patient in hospital, where, for example, changes in antipyrine half-life may given useful clinical information (Fraser, Kahn and Thompson, 1976). The simplicity of sample collection allows patients or para-medical personnel to supervise the sampling. Finally, saliva samples would be ideal for assessing drug compliance as they could be collected by non-medical personnel at specified times in the patients' homes.

CHAPTER III

Studies of antipyrine metabolism in healthy adult Gambians and Londoners

(a) Gambia Study

- (i) Subjects and sample collection
- (ii) Methods
- (iii) Results

(b) London Study

- (i) Subjects and sample collection
- (ii) Methods
- (iii) Results

(c) Comparison of antipyrine half-life in Londoners and Gambians

(d) Discussion

- (i) Gambians
- (ii) Londoners
- (iii) General discussion

(a) GAMBIA STUDY

(i) Subjects and sample collections

The study was carried out in the village of Keneba (population 800) in Gambia, West Africa (Figure 1). The village is situated 100 miles inland from the capital Banjul and close by is a field station of the Medical Research Council of Great Britain, which served as a base. The community is agricultural and has a subsistence economy based on groundnuts (peanuts), the only cash crop. The diet is almost completely vegetarian and rice, millet, sorghum and groundnuts are the staple items. Alcohol is taboo for religious reasons,but there is mild use of tobacco and the majority of the villagers chew the cola nut, which contains caffeine, for its stimulant effects.

The villagers are all of the same tribe (Mandinkos) and are Mohammedans and therefore polygamous. Extended family units of an elder, his wives and children and their families live as a commune in "compounds" consisting of several houses grouped around an enclosure or "yard". The subjects studied belonged to three such compounds which were assessed as being of average wealth, more than average wealth and less than average wealth. The protocol was explained through an interpreter to the village chief who in turn explained it to the villagers and asked for their

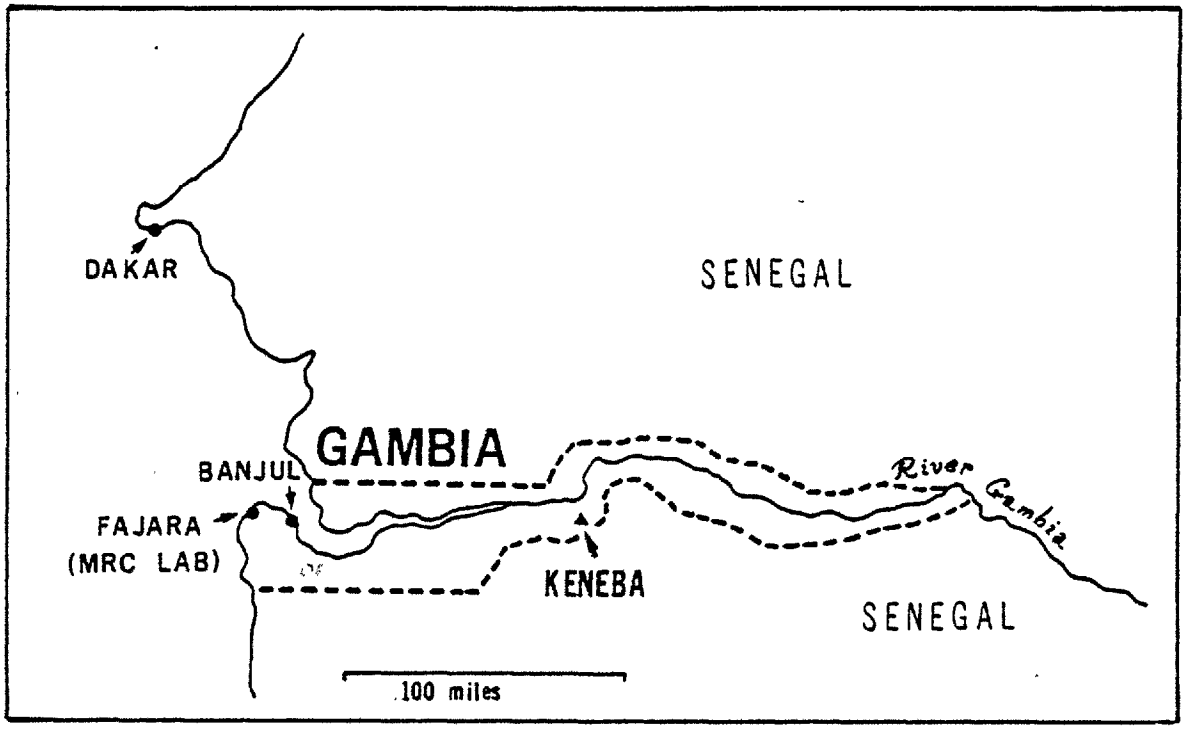


Figure 1

Map of West African cost showing Gambia and location of Keneba

co-operation.

As the project was performed in March, 1975, before the season of heavy rains and agricultural activity, no work was being done in the village and it was possible to study all adult members of the selected compounds except four. These were excluded because of pregnancy (2), febrile illness (1) and long-standing paraplegia and old age (1). Subjects were uniformly distributed between the ages of 20 and 60 (Table I) and were not suffering from any known illness. In response to enquiry about their health, three of 55 complained of headaches, two of tiredness and one of backache, but all looked fit and agreed to take part.

Subjects were asked to have no food or drink on the morning of the study. They were brought from the village to the field station by land rover at 9 a.m. and given two 300 mg tablets of antipyrine B.P. with 100 ml of water. Saliva samples were collected at 3, 5, 8, 24 and 32 hours as described in chapter II (a) (Fraser, Mucklow, Murray and Davies, 1976). They were allowed to stand for 30 minutes and then the aqueous portion was decanted from the heavier mucopolysaccharide, epithelial cell debris and solid particles. The latter consisted of the bright orange residue of cola nuts, which was seen in one or more specimens of all regular users, in spite of pleas for abstention during the study. The samples of three subjects were discarded because of gross contamination, and

TABLE I

DISTRIBUTION BY AGE AND SEX OF 49 GAMBIAN SUBJECTS

AGE GROUP	MALES	FEMALES	TOTAL
21–30 years	5	7	12
31–40 years	8	5	13
41–50 years	7	6	13
51–60 years	7	4	11
TOTAL	27	22	49
Mean age	44.5	39.1	42.0
S.D.	13.5	11.5	12.8

three subjects were unable to produce an adequate sample because of gingivitis with bleeding. Satisfactory samples were collected from 27 men and 22 women.

After collection of the last specimen, a 10 ml blood sample was taken from each patient into a plain tube for biochemical estimations. The clotted blood was centrifuged and the serum and saliva samples were stored at $-20^{o}C$ in five ml stoppered plastic vials, transferred later to a $-70^{o}C$ freezer for 24 hours, and transported frozen to the laboratory at the Royal Postgraduate Medical School for subsequent analysis.

The village of Keneba has been the subject of longitudinal epidemiological study since 1950 (McGregor and Smith, 1952). From the up-to-date records it was possible to obtain the age, height, weight and haemoglobin level recorded in the previous month, and a history of significant past illnesses. The weight and height were used to calculate a ponderal index (Wt/Ht^2), as an index of relative adiposity (Gouldbourt and Medalie, 1974). The daily cola nut consumption was obtained by direct questioning with the aid of the interpreter.

(ii) Methods

The concentration of antipyrine in the saliva samples was

determined as described in chapter II (a). There were no interfering peaks although extraction of samples from cola nut users yielded peaks with an identical retention time (RT) to that of caffeine (0.67 x RT for antipyrine) (Figure 2). As these peaks were often enormous in relation to antipyrine, the recovery of ^{14}C antipyrine from saliva was tested in the presence of caffeine concentrations of 10 and 100 µg/ml. The latter produced peaks of the same order as those from heavy cola nut users. There was no difference in recovery between the samples with high and low caffeine concentrations.

Serum samples were analysed on a Technicon SMA Plus by Mr. Geoff Mould, Department of Chemical Pathology, Royal Postgraduate Medical School. The following variables were measured: Total protein, albumin, calcium, inorganic phosphate, cholesterol, uric acid, bilirubin alkaline phosphatase, glutamic oxaloacetic transaminase (SGOT), urea, creatinine and sodium. Serum was also examined for the presence of hepatitis-B surface antigen (HBsAg).

Antipyrine half-lives were estimated by the method of least squares. The apparent volume of distribution (Vd) was calculated from the dose and the estimated initial concentration (C_o), using the relationship $V_d = Dose/C_o$ and assuming 100% absorption of an oral dose (Brodie and Axelrod, 1950; Andreasen and Vesell, 1975).

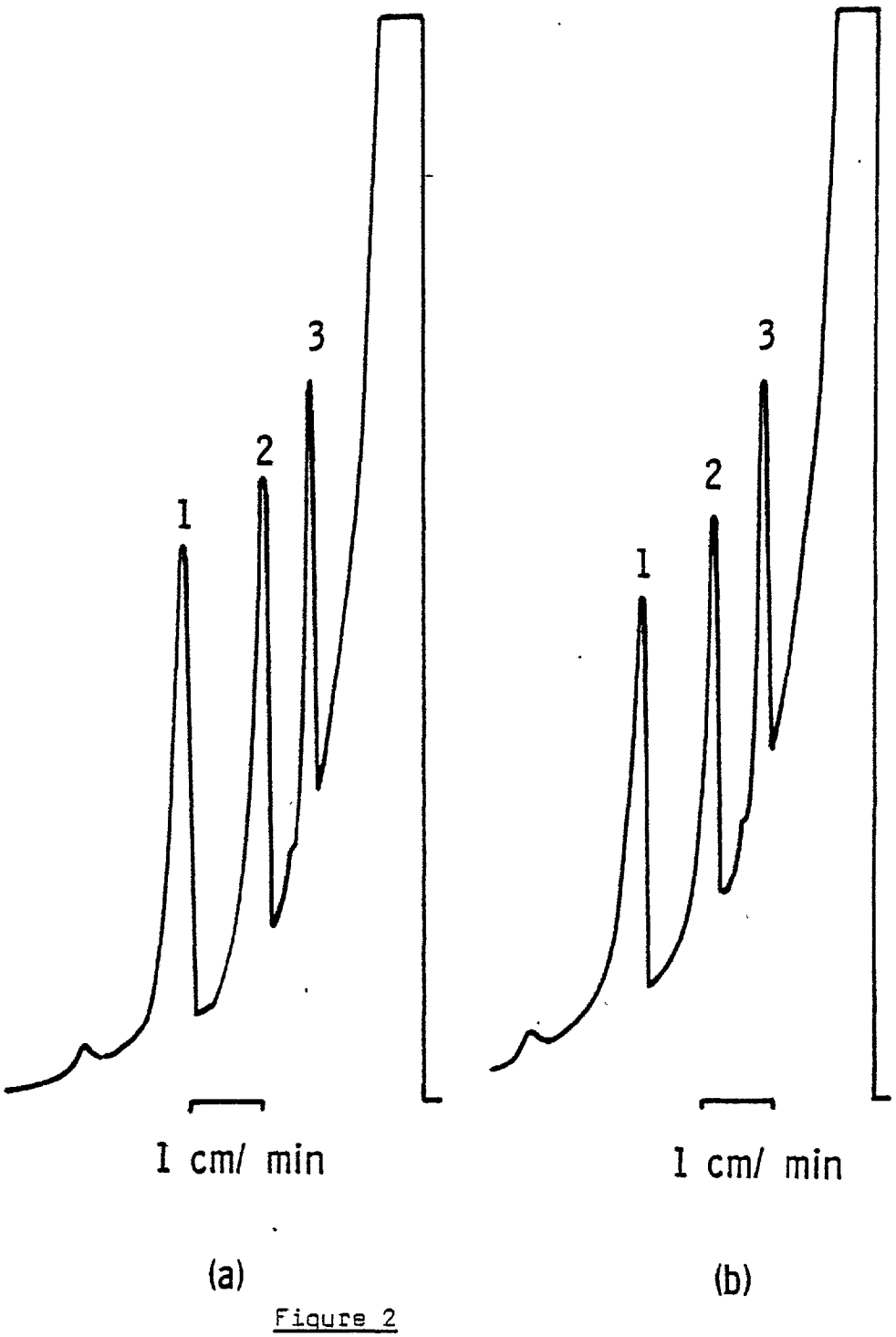


Figure 2

Gas chromatograph traces showing caffeine peak and peak obtained from extract of saliva contaminated with cola nut

a) Extract from contaminated saliva spiked with antipyrine and 4 methyl antipyrine (10 µg/ml of each)

1 = 4 methyl antipyrine RT = 3.2 min
2 = antipyrine RT = 2.2 min
3 = contaminating peak RT = 1.5 min

b) Extract from saliva spiked with antipyrine, 4 methyl antipyrine and caffeine (10 µg/ml of each)

1 = 4 methyl antipyrine RT = 3.2 min
2 = antipyrine RT = 2.2 min
3 = caffeine RT = 1.5 min

Multiple regression analyses of half-life as dependent variable on nine observed independent variables were carried out using a stepwise regression program (Biomedical Computer Program BMD 02R). The significance of differences between males and females was tested using Student's 't' test.

(iii) Results

The results of profile and electrolyte estimations are shown in Table II, with the normal values obtained in our laboratory. Albumin levels were low, with 7 of 49 (14%) more than two standard deviations less than the London mean, while globulin levels (total protein minus albumin) were high, with 41 of 49 (82%) more than two standard deviations greater than the London mean. Ten subjects had a serum cholesterol of less than 140 mg/100 ml (non-fasting). Liver function tests showed minor abnormalities in 7 subjects, one having two abnormalities. Serum bilirubin was raised in one subject (1.3 mg/100 ml), alkaline phosphatase in 5 (154-167 U/l) and SGOT in two (54 and 69 U/l). Only one urea value exceeded 40 mg/100 ml (41 mg/100 ml) and was associated with a normal creatinine of 1.3 ng/100 ml. Although 4 subjects were HBsAg-positive, none had abnormal liver function tests.

Figure 3 shows the distribution of antipyrine half-lives in 49 subjects. The curve is that of a continuous unimodal distribution with a mean of 13.6 hours ± 4.1 (SD). The males tended

Table II

Biochemical profile, urea, creatinine and electrolytes for 49 subjects and normal laboratory values (Hammersmith Hospital)

		Subjects Mean ± S.D.	Normal laboratory values Mean ± 2 S.D.
Total Protein	(g/100 ml)	7.6 ± 0.8	7.2 ± 0.7
Albumin	(g/100 ml)	3.9 ± 0.4	4.5 ± 1.0
Globulin	(g/100 ml)	3.8 ± 0.6	2.7 ± 0.7
Calcium	(mg/100 ml)	9.3 ± 1.0	9.5 ± 1.0
Inorg. P.	(mg/100 ml)	3.6 ± 0.7	3.5 ± 1.0
Cholesterol	(mg/100 ml)	169 ± 34	190 ± 50
Uric acid	(mg/100 ml)	4.6 ± 0.9	4.5 ± 2.5
Bilirubin	(mg/100 ml)	0.4 ± 0.2	0.5 ± 0.4
Alk. Phos.	(i.u./L)	83 ± 26	80 ± 50
SGOT	(i.u./L)	27 ± 9	24 ± 17
Urea	(mg/100 ml)	25 ± 6	28 ± 13
Creatinine	(mg/100 ml)	0.8 ± 0.2	1.0 ± 0.4
Sodium	(mN)	146 ± 1	143 ± 7

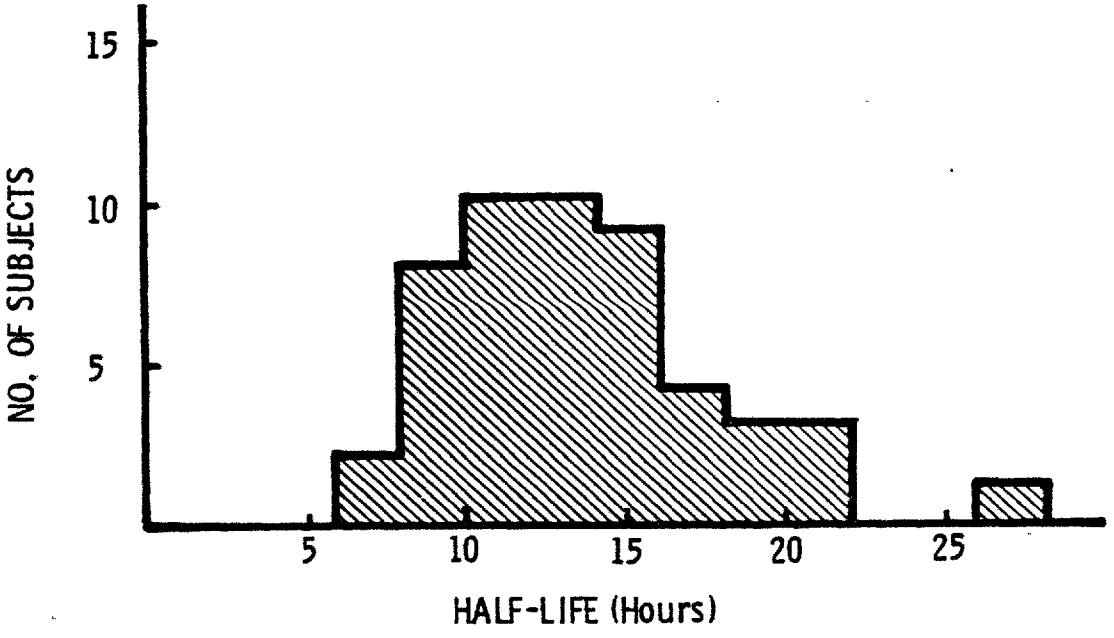


Figure 3

Frequency distribution of antipyrine half-lives in 49 Gambians

to have longer half-lives (14.5 ± 4.5 hours) than the females (12.5 ± 3.2 hours) but the difference did not reach a significant level ($0.10 > p > 0.05$).

Means and standard deviations of half-life, apparent volume of distribution and 9 observed variables are shown in Table III. First order correlations are shown in Table IV. The females had a lower mean body weight, height, haemoglobin, cola nut consumption and antipyrine half-life than the males. In males height and ponderal index were significant predictors ($p < 0.05$ and $p < 0.01$ respectively) of half-life by the equation:

$$T\frac{1}{2} = 33.7 - 24.9\ (\text{Height}) + 1.3\ (\text{Ponderal Index})$$

accounting for 40% of the variation ($R^2 = 0.40$) and showing a non-linear negative correlation between half-life and height. In females only haemoglobin (Hb) was a significant ($p < 0.05$) predictor with a regression equation:

$$T\frac{1}{2} = 1.32\ (\text{Hb}) - 3.61$$

The correlation coefficient was 0.66 and haemoglobin alone accounted for 44% of the variation.

Because of the sex dependence of these predicting variables,

TABLE III

Values of half-life, apparent volume of distribution (V_d) and eight other variables analysed for males only, females only and for all subjects

Variable	Units	Males only (n = 27)	Females only (n = 22)	All subjects	Difference between males and females
Age	years	44.5 ± 13.5*	39.1 ± 11.5	42.0 ± 12.8	N.S.
Weight	Kg	58.3 ± 5.2	51.6 ± 5.7	55.3 ± 6.3	$P < 0.001$
Height	cm	168 ± 5	159 ± 4	164 ± 7	$P < 0.001$
Haemoglobin	g/100 ml	14.0 ± 2.3	12.2 ± 1.6	13.2 ± 2.2	$P < 0.001$
Albumin	g/100 ml	3.85 ± 0.40	3.72 ± 0.43	3.72 ± 0.41	N.S.
Globulin	g/100 ml	3.82 ± 0.59	3.71 ± 0.55	3.77 ± 0.57	N.S.
Cola nuts	Nuts/day	1.22 ± 1.17	0.82 ± 0.78	1.04 ± 1.02	N.S.
Ponderal Index+	g/cm^2	2.05 ± 0.13	2.03 ± 0.20	2.04 ± 0.16	N.S.
V_d	l/Kg	0.64 ± 0.12	0.75 ± 0.14	0.71 ± 0.15	$P < 0.01$
Half-life	hours	14.6 ± 4.5	12.5 ± 3.2	13.6 ± 4.1	$0.1 > P > 0.05$

* Mean and S.D.

+ Ponderal index = weight ÷ $Height^2$

TABLE IV

First-order correlation matrix for half-life, volume of distribution and nine other variables

Variable	Sex	Age	Wt	Ht	Hb	Alb	Glob	CN	$T_{\frac{1}{2}}$	V_d	PI
Sex +	1.00	-.21	-.53**	-.68**	-.41**	-.16	-.10	-.20	-.26	-.14	-.07
Age		1.00	-.03	.07	-.12	-.04	-.06	.53**	.18	.12	-.12
Weight			1.00	.73**	.26	.13	-.01	.16	.10	-.08	.68
Height				1.00	.37*	.13	.11	.01	-.01	-.07	-.01
Haemoglobin					1.00	.24	-.11	.06	.17	.24	-.03
Albumin						1.00	.45**	.22	.14	.23	.08
Globulin							1.00	-.11	-.17	.10	-.11
Cola Nuts								1.00	.40**	-.04	.21
Half-life									1.00	.28	.12
V_d										1.00	-.07
Ponderal Index											1.00

+ For purpose of computation, men given value of 1 and women given value of 2

* $p < 0.05$

** $p < 0.01$

interaction terms were introduced in the combined analysis. These terms were: (Sex x Haemoglobin (Hb)), (Sex x Weight), (Sex x Height) and (Sex x Ponderal Index (PI)). Multiple regression analysis on the whole sample revealed the following regression equation:

$$T\frac{1}{2} = 36.01 - 19.13\ (Sex) - 1.66\ (Hb) + 1.17\ (Cola\ nuts/day) + 1.39\ (Sex \times Hb)$$

Sex and cola nuts consumed were significant predictors ($p < 0.01$) of half-life, as well as haemoglobin ($p < 0.05$) and the (Sex x Haemoglobin interaction term ($p < 0.05$) (Figures 4 and 5). Sex explained 7% of the variation, cola nut consumption an additional 12% and the haemoglobin plus interaction term an additional 9%. Together 29% of the variation in the whole sample was explained. ($R = 0.54$ and $R^2 = 0.29$).

If the height term and (Sex x Height) interaction terms were included in equation (3) the effect of height in combination was significant at the 5% level. The final equation was:

$$T\frac{1}{2} = 141.2 - 78\ (Sex) - 64.6\ (Height) - 1.5\ (Hb) + .94\ (Cola\ nuts/day) + 1.3\ (Sex \times Hb) - 36.2 \times (Sex \times Height)$$

In this equation the regression coefficient for cola nut consumption was significant at the 10% level. The sex effect

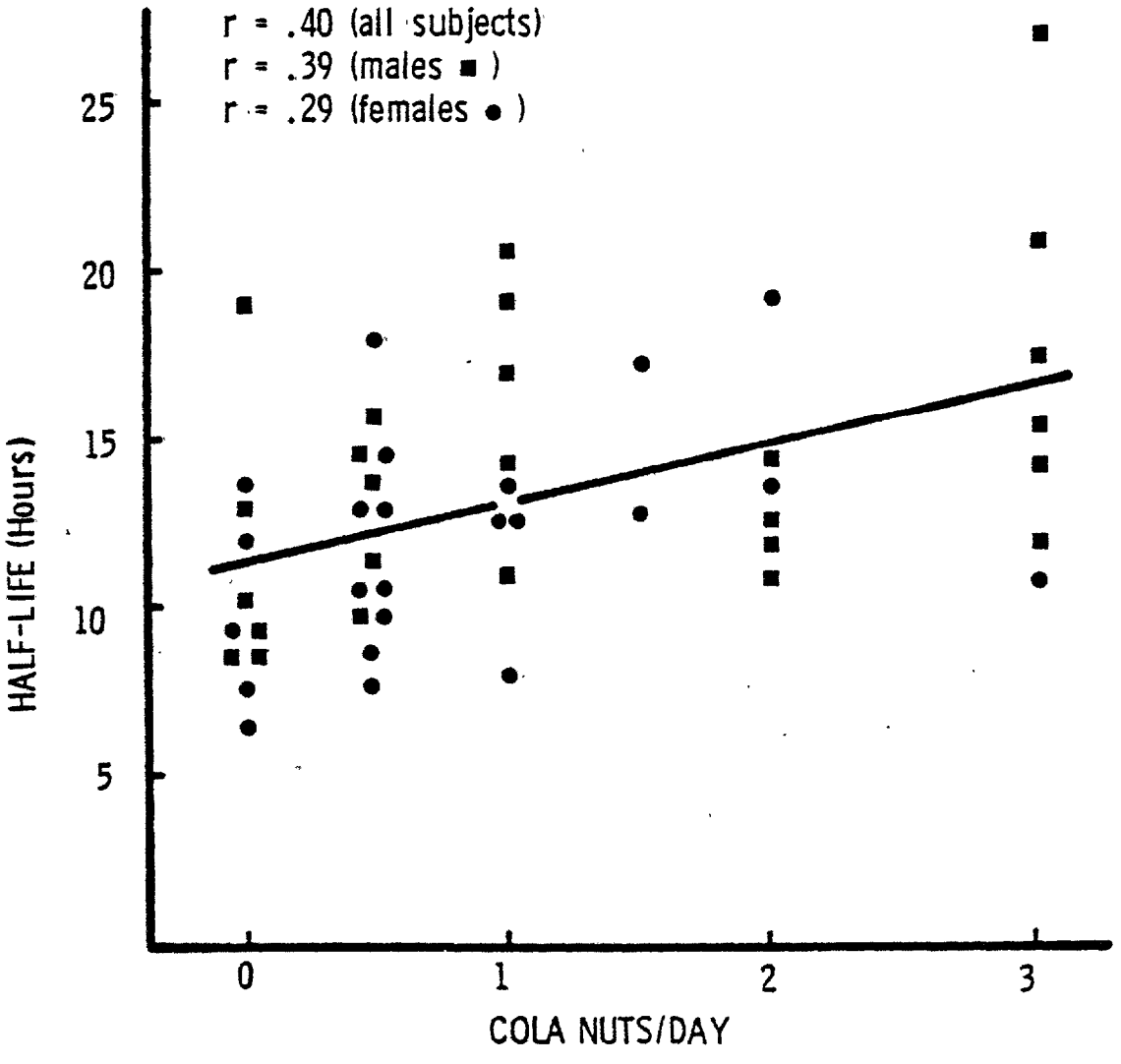


Figure 4

Relationship between antipyrine half-life and cola nut consumption in 49 Gambians

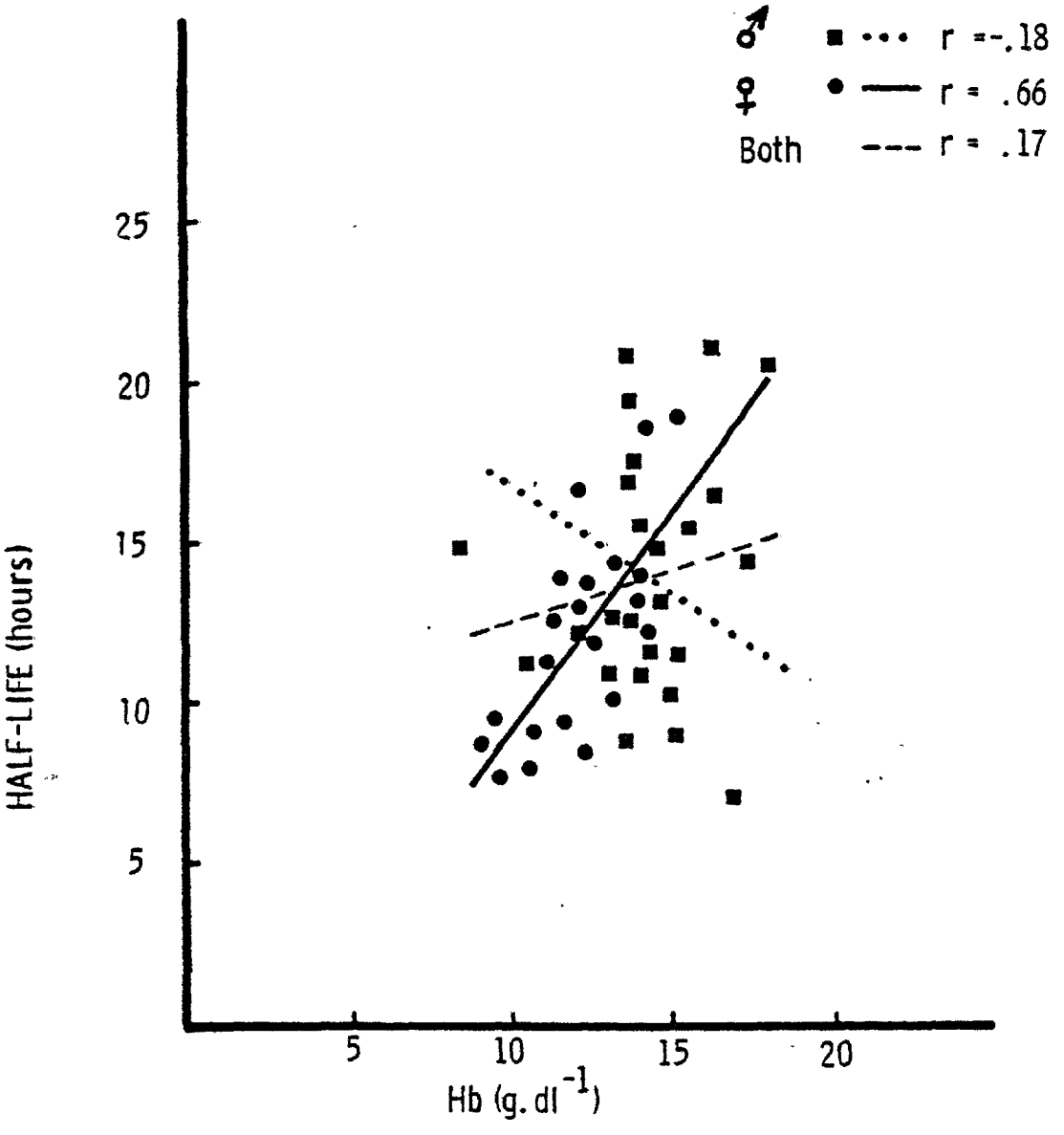


Figure 5

Relationship between antipyrine half-life and haemoglobin in 49 Gambians

and the haemoglobin and (Sex x Hb) effect were unaltered ($p < 0.05$). Altogether 35% of the variation was explained, as the height and interaction term accounted for an additional 7%.

The effect of cola nut consumption was independent of the sex of the subject. The average antipyrine half-life for men and women who did not consume cola nuts was 11.6 and 11.0 hours respectively, rising to an average of 15.7 and 14.7 hours when more than one nut was eaten per day.

(b) LONDON STUDY

(i) Subjects and sample collection

The London study was carried out in four industrial firms in North-West London. An ideal community-based sample, e.g. using the electoral register as the sampling frame, was impractical for such a study. The performance of a drug study involving collection of samples over two days required as a source of subjects an organisation where a large number of people worked together. Letters were therefore written to the personnel managers of twenty-five large London firms, most of which had headquarters or factories in North-West London, as well as to London Transport and British Rail, requesting co-operation. Four firms agreed to allow an approach to be made to their employees. These were: The National Westminster Bank Stationary Services (managerial, clerical and printing staff);

Elizabeth Arden Ltd. (Mangerial, clerical and cosmetics assembly line workers); Smith Industries (Managerial, clerical and computer operating staff); and Smiths Foods Ltd. (packaged food assembly line workers).

Employees were circularised by the personnel manager with a brief circular explaining the nature and purpose of the study, followed by a talk to provide further explanation and answer questions. For ethical reasons a random sample of employees could not be made. Volunteers were therefore invited to indicate their willingness to participate by giving signed, informed consent. Individuals suffering from liver, heart or thyroid disease, diabetes or other chronic illness were excluded, as well as anyone with a history of jaundice. Regular medication, particularly sedatives was also a contraindication to inclusion in the study. The steroid oral contraceptive (SOC) pill was considered a socially used drug, and its use was recorded.

In order to obtain a sample with as uniform sex and age distribution as possible from each firm volunteers were classified according to sex and age (18-30, 31-40, 41-50 and 51-60 years of age). There was an excess of younger people, particularly females and random selection was made from the Caucasian volunteers in the 18-30 and 31-40 age groups. All non-caucasian volunteers were accepted in an attempt to obtain an equal sample of all races. All volunteers over the age of 40 were accepted as long as they met the criteria

of good health and non-medication. The distribution by age and sex is shown in Table V. There was a 2:1 ratio of females to males, with a predominance of younger people. The mean age for males and females was similar, 34.6 and 34.0 years respectively. This distribution reflected the age and sex distribution of the employees of the four factories.

The 216 volunteers between the ages of 18 and 60 from the four firms were thus reduced to 141 who were selected for study. There were 57 Asians, 78 Caucasians and 6 Negroes. The latter were excluded from analysis because of the small number. Four subjects failed to complete the study, leaving a total of 131 subjects. They covered all social classes from manager to messenger and were equally divided between office workers and factory workers. Distribution by firm and ethnic origin is shown in Table VI. Each firm provided between 24 and 43 successfully studied subjects. Thirty eight of 57 Asians (67%) were from one firm (Smiths Foods Ltd.) and were all factory workers.

Weights and heights of all subjects were recorded. A history was obtained of socially used drugs, specifically cigarettes, alcohol, coffee and tea and the SOC pill. Subjects were classified as smokers or non-smokers, pipe-smokers being considered by convention as non-smokers (Vestal, Norris, Todin, Cohen, Schook and Andres, 1975), and the number of cigarettes per day was recorded. Alcohol consumption was scored as drinks per week, where half a

TABLE V

DISTRIBUTION BY AGE AND SEX OF 131 LONDON SUBJECTS

AGE GROUP (years)	MALES (number)	FEMALES (number)	TOTALS
18–30	21	32	53
31–40	9	29	38
41–50	9	17	26
51–60	5	9	14
TOTALS	44	87	131
Mean Age	34.6	34.0	34.2
S.D.	11.6	11.4	11.5

TABLE VI

DISTRIBUTION BY FIRM AND ETHNIC ORIGIN OF 131 LONDON SUBJECTS

FIRM	Males		Females		TOTALS
	Caucasians	Asians	Caucasians	Asians	
National Westminster Bank Stationary Services	12	0	20	11	43
Elizabeth Arden Ltd.	11	1	9	3	24
Smiths Industries	7	3	15	1	26
Smiths Foods Ltd.	0	10	0	28	38
TOTALS	30	14	44	43	131

Total Caucasians 74 (56%)
Total Asians 57 (44%)
Total Subjects 131

pint of beer was equated with a standard measure of spirit, sherry or glass of wine and equal to approximately 12 g of alcohol (Laurence, 1973). A coffee/tea score of cups of coffee/day + 0.6 x cups of tea per day was obtained for all London subjects[1]. This was based on the estimate that the caffeine content of an average cup of tea is 60% of that of coffee (70 mg and 90-120 mg respectively) (Nagy, 1974). Use of the SOC pill was recorded but no details of the preparation used.

Diet was classified as vegetarian or non-vegetarian. All vegetarians except one were Asian, while eight Asians were non-vegetarian. Some subjects (Asians) who ate meat only occasionally were classified as vegetarians. These were members of families whose only meat dish was a single chicken on a Sunday, and the quantity per person was minimal. The vegetarian diets varied considerably in the quantity of egg and dairy products consumed but no attempt was made to assess this or to further classify the diet of the entire sample. However a sub-sample of seven vegetarians was selected on the basis of a good command of English, and a

Footnote

1. When the protocol was designed coffee and tea consumption was not included because of the observation of Vessell (1968) that they were unrelated to antipyrine half-life. Because of the observed effects of cola nut consumption among the Gambians, a history of coffee and tea consumption was subsequently included in the protocol. By that time the study in the first London factory (National Westminster Bank Stationary Services) had been completed and 11 employees transferred or left. Their values were therefore set to the mean of the whole sample.

detailed dietary history obtained by Ms. Sue Rendle, a dietician on the staff of Hammersmith Hospital. Seven age and sex-matched non-vegetarians were also interviewed and dietary histories obtained. Diets were analysed for total calories, protein, carbohydrate, fat (totals and polyunsaturated and cholesterol), iron, folate and vitamin B_{12} content using the tables of McCance and Widdowson (1960).

On arrival at work after an overnight fast each subject received 600 mg of antipyrine and 1.5 g of paracetamol orally, accompanied by 150 ml of drinking water. Saliva samples were collected at 2, 3, 5, 8, 24 and 32 hours, using parafilm as a stimulus, and allowed to stand for 30-60 minutes. The aqueous phase was then decanted, divided into two portions, and frozen until analysis. Samples at 2, 3, 5 and 8 hours were analysed for paracetamol and the 3, 5, 8, 24 and 32 hour samples for antipyrine.

Blood samples (12 ml) were taken by venipuncture on the second day of the study for profile, electrolyte and haemoglobin estimations. Nine subjects declined blood samples and their values for albumin, globulin and haemoglobin were set to the mean for males or females in the subsequent analyses.

(ii) Methods

Antipyrine and paracetamol were analysed by glc methods as

described in Chapter II.

Profile and electrolyte estimations were performed on the day of sampling by Mr. Geoff Mould, Department of Chemical Pathology, Royal Postgraduate Medical School. Haemoglobin estimations were performed on the following morning in the Haematology laboratory by courtesy of Dr. Kate Murray.

Estimation of antipyrine and paracetamol half-life and multiple regression analyses of antipyrine and paracetamol half-life on observed variables were performed as described above (Chapter III, a (ii)).

(iii) Results

Nutritional Indices

Anthropometric and biochemical indices of nutritional status are shown in Table VII for the London subjects, broken down according to sex and ethnic origin. Males were taller and heavier than females in both races, but ponderal indices were not significantly different. Caucasian males and females were taller than Asian males and females but again ponderal indices showed no difference. Women had a lower haemoglobin than men ($p < 0.001$) and Asian women had a significantly lower haemoglobin than Caucasian women ($p < 0.001$). Serum albumin was also significantly lower in Asian women than in

TABLE VII

NUTRITIONAL INDICES* OF 131 LONDONERS, BY SEX AND ETHNIC ORIGIN

	Males		Females	
	Caucasians (n = 30)	Asians (n = 14)	Caucasians (n = 44)	Asians (n = 43)
Weight (Kg)	73.5	65.5	61.6	60.3
Height (cm)	174	168	162	158
Ponderal Index (Wt/Ht^2: g/cm^2)	2.43	2.32	2.35	2.42
Albumin (g/dl)	4.69	4.52	4.65	4.39
Globulin (g/dl)	2.77	3.07	2.83	3.04
Haemoglobin (g/dl)	15.0	13.3	14.7	11.7

* Analysed using a general multivariate tabulation programme (Multitab).

other groups ($p < 0.02$) and globulins were higher in Asians than in Caucasians ($p < 0.001$).

Social Drug Use

The use of social drugs differed considerably in the two ethnic groups in London as shown in Table VIII. Asians used all of the drugs documented to a less extent than Caucasians. The percentage of male Asian smokers (50%) was similar to that of male and female Caucasians (46 and 52% respectively) but mean cigarettes smoked per day (5.9) was almost half that of the Caucasians, while no Asian women smoked. Similarly alcohol consumption by Asian women was negligible and by Asian men 20% of that consumed by Caucasian men. The coffee/tea consumption of Asians was half that of Caucasians, one of whom drank 20 cups of tea per day. Only one Asian woman used the contraceptive pill.

Antipyrine half-life and volume of distribution

Antipyrine half-lives and volumes of distribution are shown in Table IX. The half-lives of the whole sample were 12.8 ± 4.4. (S.D.) hours. Asians had significantly longer half-lives (15.6 ± 4.2 hours) than Caucasians (10.6 ± 3.1 hours, $p < 0.001$). There was no significant difference between half-lives of males and females. Volumes of distribution of antipyrine were significantly greater for males than females and for Caucasians than Asians

TABLE VIII

SOCIAL DRUG USE AMONG 131 LONDON SUBJECTS, BY SEX AND ETHNIC ORIGIN

	Males		Females	
	Caucasians (n=30)	Asians (n=14)	Caucasians (n=44)	Asians (n=43)
Smoker	46%	50%	52%	0
Number of cigarettes per day	9.8	5.9	10.6	0
Alcoholic drinks per week	10.1	2.1	7.1	0.05
Coffee/tea index per day	5.4	2.8	5.2	2.2
Use of SOC pill	-	-	26%	2%

TABLE IX

ANTIPYRINE HALF-LIVES AND VOLUME OF DISTRIBUTION FOR 74 CAUCASIANS, 57 ASIANS AND ALL 131 LONDON SUBJECTS

	Caucasians	Asians	All Subjects
Half-life (hours)			
Mean	10.6	15.6*	12.8
S.D.	3.1	4.2	4.4
Volume of distribution (l/kg)			
Mean	0.58	0.53*	0.56
S.D.	0.08	0.08	0.08

* Significance of difference between Asians and Caucasians : $p < 0.001$

($p < 0.001$). Full tables of half-life, volume of distribution and all 15 observed variables are given in the Appendix, according to race and sex.

Diet

Because of the differences between Caucasians and Asians with respect to diet and use of social drugs, the data was further examined for effects of these factors. Figure 6 shows frequency distribution curves for vegetarians and non-vegetarians. Both are continuous unimodal curves, with means of 16.2 and 10.7 hours respectively. The curve for the non-vegetarians is slightly skewed towards higher values.

Smoking

Figure 7 shows the distribution of antipyrine half-lives for male Caucasian smokers and non-smokers, factors of race, diet and sex being eliminated in this comparison. The half-life was 28% shorter in smokers than in non-smokers, and the variance was reduced.

Smoking and the contraceptive pill

In the female Caucasians the situation was more complex because 26% were using the SOC pill. In figure 8 half-lives are plotted according to both smoking status and use of the pill. In

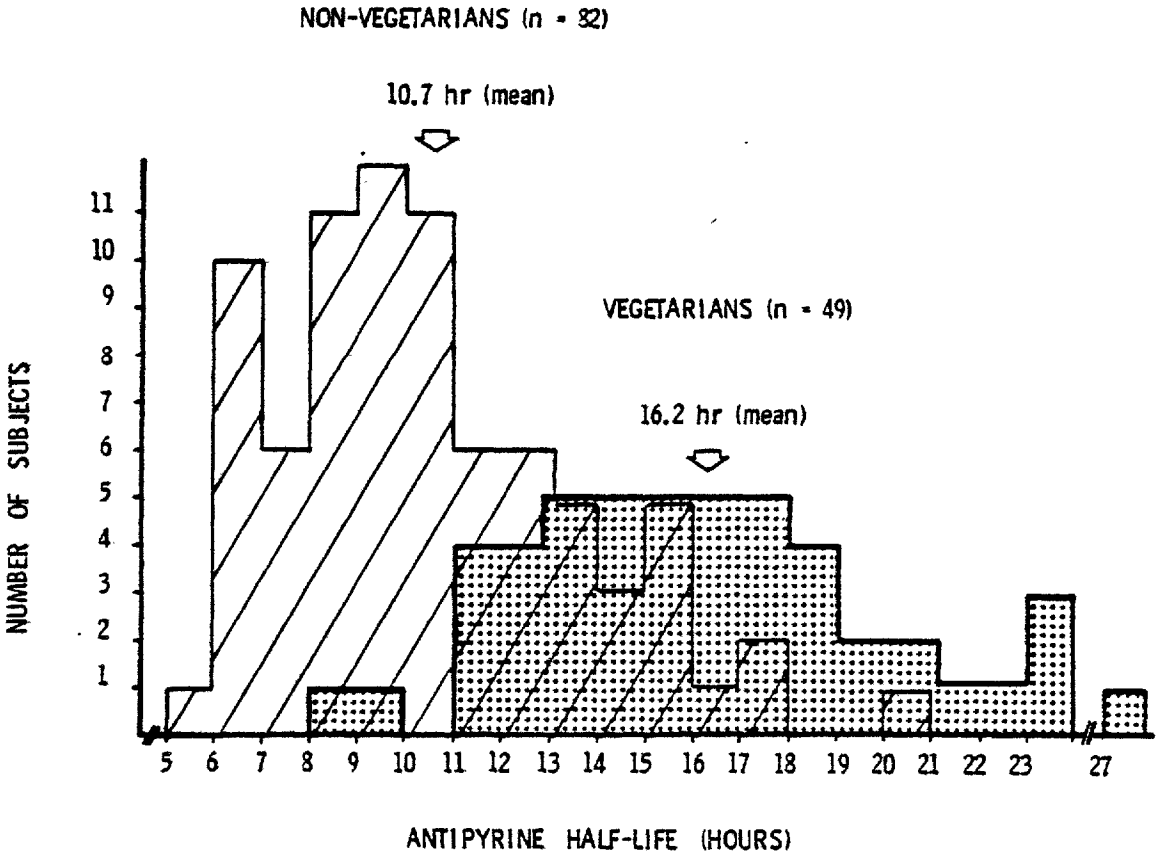


Figure 6

Frequency distribution of antipyrine half-lives in vegetarian & non-vegetarian Londoners

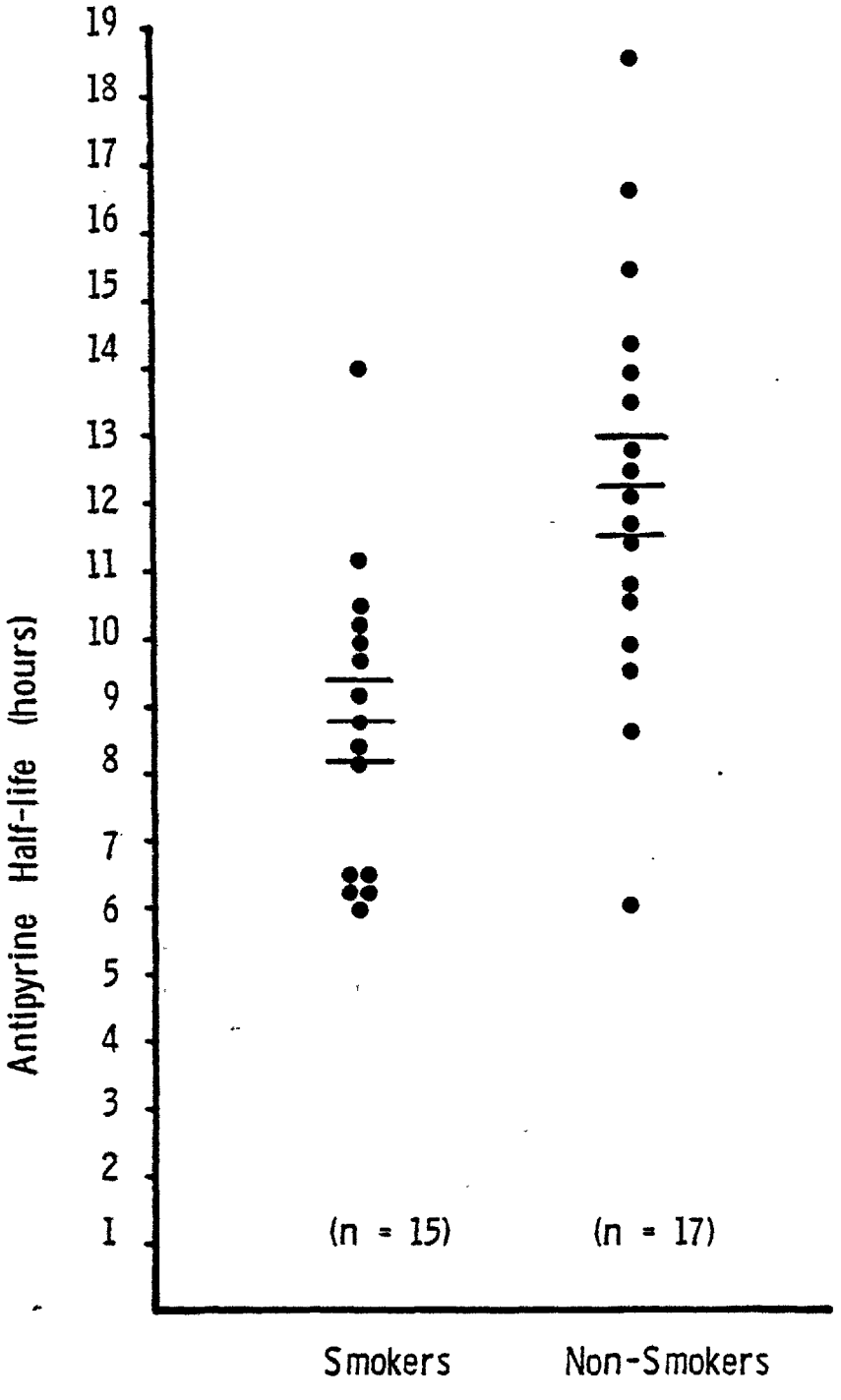


Figure 7

Antipyrine half-life in male caucasian cigarette smokers and non-smokers

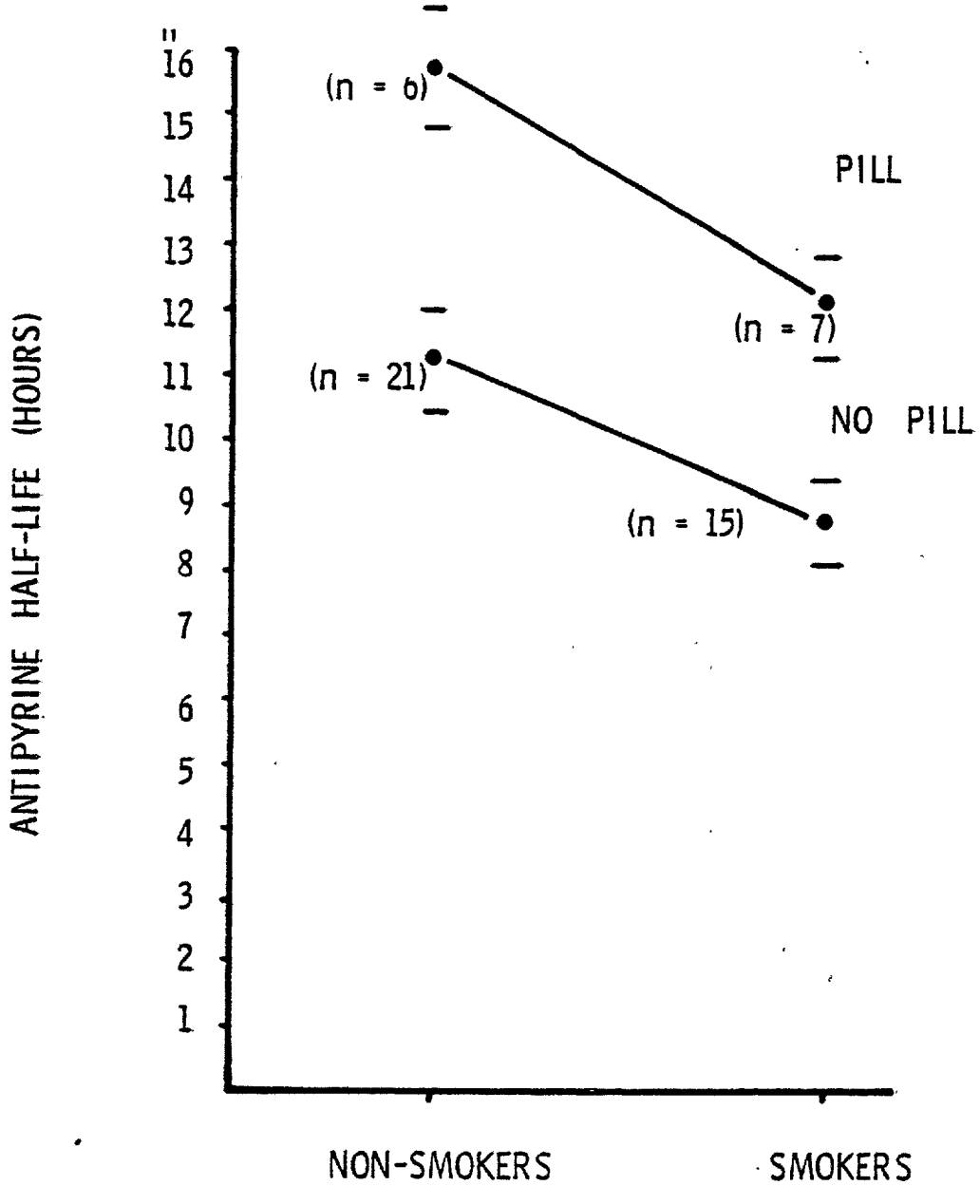


Figure 8

Antipyrine half-life in non-vegetarian women, in relation to smoking habits and use of the contraceptive pill

TABLE X

FIRST ORDER CORRELATION MATRIX FOR HALF-LIFE OF ANTIPYRINE, VOLUME OF DISTRIBUTION (Vd), AND 16 VARIABLES IN 131 LONDONERS

	SEX	AGE	WT	HT	HB	ALB	GLOB	$T_{\frac{1}{2}}^{A}$	Vd	RACE	Sm	//	ALC	DIET	C/T	PILL	$T_{\frac{1}{2}}^{P}$	PI
SEX	1.00	-.03	-.44*	-.68*	-.60*	-.39*	.08	.11	-.44*	.17	-.21	-.14	-.21	.19	-.16	.25	.13	-.01
AGE		1.00	.30*	.10	-.02	-.09	.12	-.07	-.07	-.21	-.06	-.09	-.12	-.19	.15	-.23	-.13	.28
WEIGHT (WT)			1.00	.56*	.32*	.21	.04	.00	.09	-.22	.09	.16	.20	-.17	.23	-.14	-.29*	.78*
HEIGHT (HT)				1.00	.49*	.40*	-.19	-.13	.51*	-.39*	.25	.22	.33*	-.34*	.28	-.05	-.21	-.09
HAEMOGLOBIN (HB)					1.00	.32*	-.24	-.29*	.33*	-.38*	.32*	.27	.27	-.36*	.30*	-.07	-.12	.00
ALBUMIN (ALB)						1.00	-.03	.00	.31*	-.24	.19	.11	.20	-.23	.03	.14	-.15	-.06
GLOBULIN (GLOB)							1.00	.34*	-.18	.35*	-.27	-.22	-.33*	.38*	-.14	.09	.10	.22
HALF-LIFE ($T_{\frac{1}{2}}^{A}$)								1.00	-.02	.57*	-.42*	-.38*	-.29*	.62*	-.47*	.11	.20	.11
V_d									1.00	-.29*	.24	.19	.19	-.19	.20	-.05	-.17	-.29*
RACE										1.00	-.40*	-.41*	-.43*	.86*	-.60*	-.20	.31*	.03
SMOKER (Sm)											1.00	.85*	.40*	-.39*	.42*	.12	-.16	-.09
NO. OF CIGARETTES (//)												1.00	.37*	-.38*	.53*	.14	-.17	.02
ALCOHOL (A C).													1.00	-.37*	.21	.12	-.10	-.02
DIET														1.00	-.56*	-.12	.29*	.07
COFFEE/TEA(C/T)															1.00	.06	-.16	.07
PILL																1.00	.12	-.12
HALF-LIFE OF PARACETAMOL ($T_{\frac{1}{2}}^{P}$)																	1.00	.20
PONDER INDEX (PI)																		1.00

* DENOTES SIGNIFICANCE ($p < 0.001$)

women who used the pill and those who did not, cigarette smoking shortened the half-life by almost 25% (24 and 22% respectively). Conversely the effect of the pill was the same in both smokers and non-smokers, i.e. it was associated with a 40% prolongation of antipyrine half-life. These effects negated one another; women who smoked and took the pill had a similar half-life to those who neither smoked nor took the pill.

Multiple regression analysis

First order correlation coefficients for antipyrine with 8 observed variables reached a significant level ($p < 0.01$). These variables were: vegetarian diet ($r = 0.62$), race ($r = 0.57$), coffee/tea index ($r = -0.47$), smoking ($r = -0.42$), cigarettes smoked per day ($r = -0.38$), globulin ($r = 0.34$), alcohol consumption ($r = -0.29$) and haemoglobin ($r = -0.29$). However all of these variables showed a significant correlation with race and with each other as seen in Table X which lists all first order correlations for 131 London subjects. Multiple regression analyses were performed with antipyrine half-life as dependent variable on the observed variables of diet, social drugs, race, nutritional indices, age and sex. The sex interaction terms for haemoglobin, weight, height and ponderal index, which were introduced in the analysis of the Gambian data, were also used here. Analysis was performed on all London subjects and separately on Asians, Caucasians, all males and all females.

As before the variable with the largest first order correlation coefficient (vegetarian diet, r = 0.62) was entered first in the regression equation. Having corrected for diet, race was no longer significant. The variable with the largest F value (smoking status) was automatically entered next in the multiple regression equation and so on. Thus pill status was entered at the third step and body weight at the fourth.

For the whole sample only these four variables were found to be independent predictors of antipyrine half-life. The multiple regression equation relating them is:

$$T\frac{1}{2} = 0.952 + 5.14 \times \text{Diet} + 3.27 \times \text{Pill status} + 0.063 \times \text{Weight} - 2.2 \times \text{Smoking status}$$

where $T\frac{1}{2}$ = half-life, and for computation:

non-smokers = 1, smokers = 2

non-vegetarians = 1, vegetarians = 2

and non-users of the pill = 1, users of the pill = 2

Thus half life increased with increase in weight, a vegetarian diet and use of the pill, but decreased with cigarette smoking. Smoking status alone was the important factor and not the number of cigarettes smoked. The variance explained by these four factors was 49% ($R = 0.70$, $R^2 = 0.49$). The factor making the greatest contribution to the variance was the diet (37%), while smoking contributed 5%, the pill 4% and body weight 3%. The F values were

65.9, 11.8, 12.6, and 5.6 respectively and all were significant at the 1% level.

Multiple regression analysis of the Asian sample identified three factors as independent predictors of half-life ($p < 0.01$). Again diet was the most important, accounting for 12% of the variance. The other two factors were coffee/tea consumption and height, accounting for 6.6% and 7.4% respectively. The total variance explained was 26% ($R = 0.51$, $R^2 = 0.26$) and the multiple regression equation was:

$$T\tfrac{1}{2} = -16.3 + 17.1 \times Ht + 3.7 \times Diet - 1.1 \times \text{coffee/tea index}$$

Multiple regression analysis of the Caucasian sample also identified smoking and the pill as independent predictors of antipyrine half-life ($p < 0.01$). The equation was:

$$T\tfrac{1}{2} = -0.9 + 3.0 \times \text{pill status} + 2.6 \times \text{Albumin} - 2.5 \times \text{smoking status}$$

and $R = 0.60$.

The total variance explained was 36%, the individual contributions being: smoking 17%, use of the SOC pill 14% and serum albumin 5%.

Analysis by sex yielded similar results. Thus for men only

two factors were independet predictors of half-life ($p < 0.01$) and these were, once again, diet and smoking, accounting for 42% and 14% respectivel and together explaining 56% of the variance. For all women diet, use of the pill and smoking were most important, accounting for 35%, 6% and 3.5% of the variance respectively. Age and serum albumin also reached the 0.01 level of significance, each accounting for 2.5% of the variance, and all five factors explained 50% of the variance.

The contribution to the total variance of the first variable entered in the multiple regression equation is always an overestimate in relation to the other significant variables. It is important to note that diet always showed the largest first order correlation, and whether entered first in the equation or not always explained the largest proportion of variance.

Diet

Dietary analysis of seven female vegetarians (mean age 21) and seven age-matched female non-vegetarians (mean age 23) is summarised in Table XI and shown in detail in the appendix. The vegetarians had a significantly lower estimated daily intake of calories, protein, fat and vitamin B_{12} than the non-vegetarians. Iron and folate intake were also lower, but the differences were not significant.

The greatest differences in dietary content between the two

TABLE XI

DIETARY ANALYSIS OF SEVEN FEMALE VEGETARIANS AND SEVEN AGE-MATCHED FEMALE NON-VEGETARIANS

	Vegetarians	Non-Vegetarians	Significance (Students t test)	RDA‡
Total Calories*	1329 ± 189+	1713 ± 185	p<0.005	2100
Protein (g/day)	35.1 ± 9.2	70.0 ± 10.7	p<0.001	30
Carbohydrate (g/day)	186 ± 45	180 ± 48	NS	
Total fat (g/day)	56.7 ± 15.2	83.2 ± 22.5	p<0.025	
Polyunsat.Fat (g/day)	3.5 ± 2.0	3.4 ± 1.8	NS	
Cholesterol (g/day)	0.13 ± 0.11	0.37 ± 0.16	p<0.01	
Iron (mg/day)	8.4 ± 2.3	10.7 ± 2.6	NS	10
Folate (µg/day)	29.2 ±	32.8 ±		190
Vitamin B_{12} (µg/day)	1.01 ± 0.50	4.90 ± 2.60	p<0.005	2

* Daily intake for each subject averaged from a 7 day diet history i.e. 5 week days plus Saturdays and Sunday

\+ Mean ± SD

‡ Recommended daily allowance (Food and Nutrition Board, 1974)

groups were in protein and vitamin B_{12}. The mean vegetarian daily intake of protein was half that of the non-vegetarians and the B_{12} intake was 20% of that of the non-vegetarians. The mean calorie, iron, folate and B_{12} intake for the vegetarians was below the recommended daily allowances (RDA) of the Food and Nutrition Board of the FAO-WHO, 1974. Indeed all seven subjects had a B_{12} intake of less than 2 µg/day, while two subjects had estimated protein intakes of 22.3 and 22.7 g/day compared to the RDA of 30 g/day. Figure 9 shows that antipyrine half-life increases with a decrease in dietary protein intake ($r = 0.74$, $p < 0.005$).

The mean estimated calorie and folate intake of the non-vegetarians was also less than the RDA, while iron intake was border line. It may be significant that these subjects were all young women, six out of seven being unmarried and probably figure conscious. Both groups of subjects tended to avoid canteen lunches and eat only packaged crisps at lunchtime.

Albumin levels were not significantly different for the two groups but haemoglobin levels were lower in the vegetarians (11.6 ± 1.7) (S.D.) g/dl than the non-vegetarians (13.4 ± 0.6) g/dl ($p < 0.025$). This suggests that the apparent dietary deficiencies of haematinic factors (iron, folate and B_{12})were of clinical importance.

Further evidence of significant iron deficiency was provided by a low MCV (mean corpuscular volume) and a low MCHC (mean corpuscular

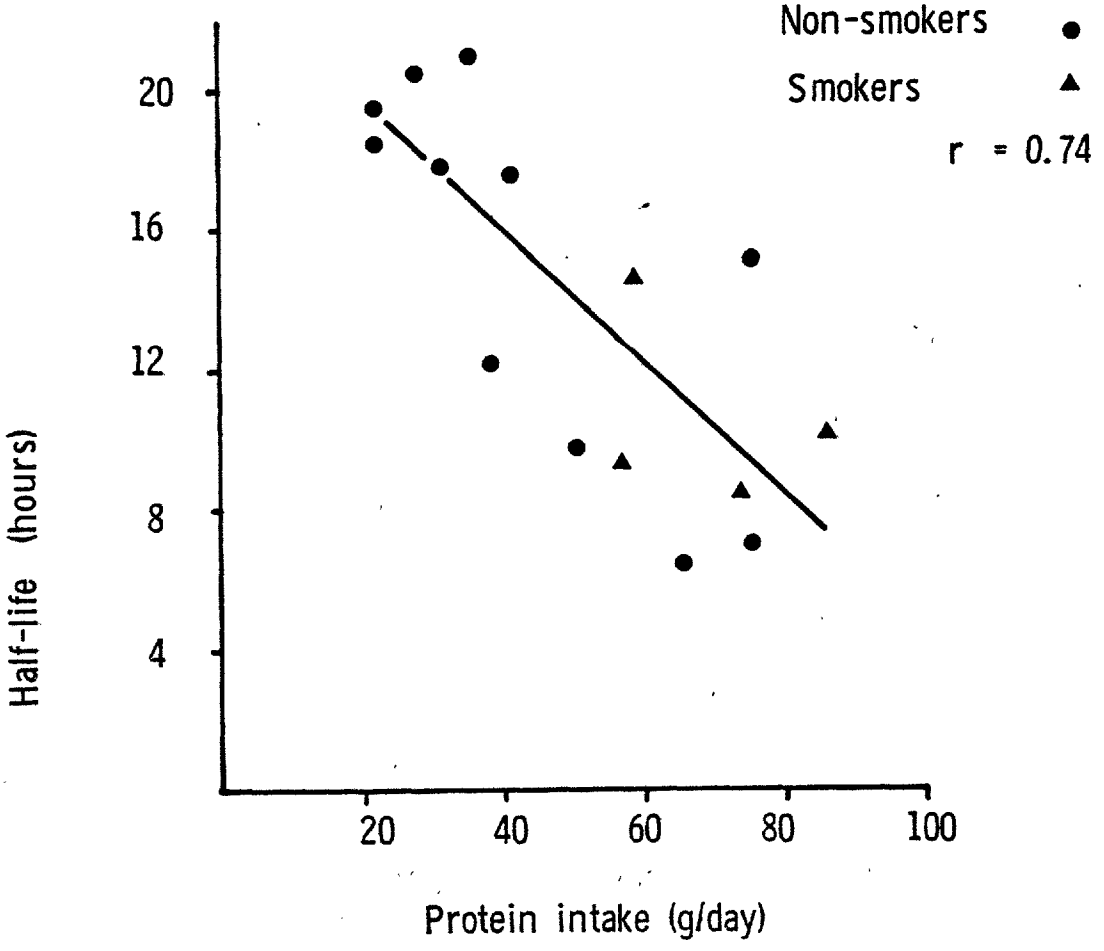


Figure 9

Correlation of antipyrine half-life and estimated dietary protein intake in 14 female Londoners

haemoglobin content) in the three vegetarians with the lowest haemoglobins.

(c) COMPARISON OF ANTIPYRINE HALF-LIFE IN LONDONDERS AND GAMBIANS

Indices of nutritional status of Londoners and Gambians are summarised in Table XII for the three ethnic groups. Like the Asians, the Gambians had a lower body weight, height, albumin and haemoglobin than the Caucasians. There was a smaller difference in height, however, and a greater difference in body weight, with the result that the ponderal index also showed a highly significant difference ($p < 0.001$). The globulin was not only higher in Gambians than Caucasians but significantly higher than in the Asians.

Use of social drugs differed considerably in the three groups, the Gambians resembling the Asians in their minimal use of all agents except for the cola nut. Most of them (men and women) smoked an occasional pipe of a local tobacco-like leaf, while one subject smoked pipe tobacco regularly and four smoked cigarettes (20, 10, 8 and 8 cigarettes per day respectively). As the antipyrine half-life of these subjects did not appear to differ from the others smoking was not included in the multiple regression analysis.

Table XIII shows the antipyrine half-life and volumes of distribution for all subjects studied according to ethnic origin.

TABLE XII

NUTRITIONAL INDICES OF LONDONERS AND GAMBIANS, BY ETHNIC ORIGIN

	LONDONERS			GAMBIANS	
	Caucasians (n=74)	Asians (n=57)	Significance of difference	(n=49)	Significance* of difference
Weight (Kg)	66.5 ± 10.1+	61.6 ± 11.2	p<0.001	55.3 ± 6	p<0.001
Height (cm)	167 ± 9	160 ± 7	p<0.001	164 ± 7	p<0.05
Ponderal Index (Wt/Ht^2: g/cm^2)	2.38 ± 0.28	2.40 ± 0.41	N.S.	2.04 ± 0.16	p<0.001
Albumin (g/dl)	4.59 ± 0.28	4.46 ± 0.22	p<0.01	3.79 ± 0.41	p<0.001
Globulin (g/dl)	2.81 ± 0.29	3.04 ± 0.32	p<0.001	3.77 ± 0.57	p<0.001
Haemoglobin (g/dl)	13.9 ± 1.3	12.5 ± 2.1	p<0.001	13.2 ± 2.2	p<0.05

*Significance of difference from Caucasians

+Mean ± S.D.

TABLE XIII

ANTIPYRINE HALF-LIFE AND VOLUMES OF DISTRIBUTION FOR ALL 180 SUBJECTS, BY ETHNIC ORIGIN

	Caucasians (n = 74)	Asians (n = 57)	Gambians (n = 131)
Half-life (hours)			
Mean	10.6	15.6	13.6
S.D.	3.1	4.2	4.1
Volume of Distribution			
Mean	0.58	0.53	0.71
S.D.	0.008	0.08	0.15

Antipryien half-life was longest in the Asians (15.6 ± 4.2 (S.D. hours) and shortest in the Caucasians (10.6 ± 3.1 hours). Half-lives of both the Asians and the Gambians (13.6 ± 4.1 hours) were significantly longer than the Caucasians ($p < 0.001$). The Asians also had significantly longer half-lives than the Gambians ($p < 0.01$).

Volumes of distribution were also significantly different for the three groups ($p < 0.001$). The Gambians had the largest volumes of distribution when expressed in litres per kilogram body weight (0.71 ± 0.15 l/kg) while the Asians had the smallest (0.53 ± 0.08 1/Kg).

When multiple regression analysis was performed on the whole file of 180 subjects (Londoners and Gambians) five variables were found to be independent predictors of antipyrine half-life. These were the same as for the whole London sample, with the addition of cola nut consumption. Together they explained 40% of the variance ($r = 0.63$). The individual contributions to the variance were: diet 24%, use of the pill 7%, cola nut consumption 4%, and smoking and body weight 2.5% each. The final multiple regression equation was:

$$T\tfrac{1}{2} = -2.0 + 0.07 \times \text{body weight} + 1.49 \times \text{cola nuts/day} + 5.25 \times \text{diet} + 3.45 \times \text{pill status} - 0.09 \times \text{cigarettes smoked per day}$$

(d) DISCUSSION

(i) Gambians

The village of Keneba was chosen for this study because it satisfied the desired environmental, nutritional and ethnic criteria, and because of the Medical Research Council field station which served as a base. The population is Negro, of the Mandinka tribe, and as marriage partners are usually chosen from within the village the sample was relatively homogeneous genetically. Secondly, the nutritional status of the subjects is suboptimal because of dependence on a vegetarian diet, which frequently becomes scarce between June and October of each year (Thompson, Billewicz, Thompson and McGregor, 1966) and causes weight loss of 6–8 pounds in both males and females. Thirdly, alcohol and the atmospheric pollutants of the developed countries are lacking. Finally the health status of the community is very different from western communities. The incidence of chronic infestation with malarial and helminthic parasites is extremely high, infant mortality is high and life expectancy is relatively short. All of these factors might be expected to have some effect on rates of drug metabolism.

The data obtained confirm the picture of suboptimal nutrition of a mild degree. Body weight, height, ponderal indices and serum albumin were all low compared to the reported values for Europeans and North Americans (Documenta Geigy Scientific Tables, 1962; Gouldbourt

and Medalie, 1974). Haemoglobin, as expected, was lower in women of child hearing age than in the older women. Globulin levels were strikingly elevated, as reported by McGregor, et al., and are due largely to elevation of malaria-induced immunoglobulins IgG and IgM (McGregor, Rowe, Wilson and Billewicz, 1970).

No subjects had significant impairment of liver function, although 14% had minor abnormalities of liver function tests. Four subjects (8.2%) were sero-positive for HBsAg, a figure similar to those reported both in Gambia and elsewhere in West Africa (Ree, 1975). Neither these subjects nor the 7 with biochemical abnormalities had significantly different antipyrine half-lives, suggesting that liver disease was not an important factor in this sample.

There were four significant independent predictors of antipyrine half-life: cola nuts, sex, haemoglobin in women and height in men. Of these, cola nut consumption was the most important factor. The daily consumption of 3 cola nuts prolonged the antipyrine half-life by approximately 3.5 hours.

On the basis of a caffeine content of 2% by weight (Goodman and Gilman, 1975) an average nut would be equivalent to 3 cups of coffee, and 3 nuts represents a very high daily intake. It is possible that the caffeine or some other unidentified constituent of cola nuts competes with antipyrine for oxidation by the microsomal

enzyme system as occurs with some drugs which share this metabolic pathway. As consumption of cola nuts continued during measurement of antipyrine half-life this could have resulted in inhibition of antipyrine metabolism. In view of their widespread use as a stimulant in West Africa, this problem merits further study.

The effect of sex on antipyrine half-life confirms the report of O'Malley, Crooks, Duke and Stevenson (1971) in 61 healthy caucasians, aged 20-50. The effect of age has also been demonstrated by O'Malley, et al., Vestal, et al., and Lidell, Williams and Briant (1975) but although apparent in our sample it did not reach levels of statistical significance. Our sample was drawn from subjects under the age of 60 years and it appears that significant differences are only seen if the geriatric age group is examined.

The relationship between haemoglobin and half-life is more difficult to explain. In anaemia, cardiac output and liver blood flow are increased and hence clearance of a drug by the liver could be accelerated. In the case of antipyrine, with a low hepatic extraction ratio and a relatively long half-life of 13 hours, this is unlikely to be a significant factor. The effect may be a hormonal one analogous to that of starvation in rats, where oxidation is increased in the female only. In this context it may be noted that in Keneba, as in most of Gambia, women work actively during pregnancy. There is increased energy expenditure and weight loss in the "hungry season", and no dietary privileges

are received in times of shortage. Although the study was performed midway between harvests and there was no current food shortage, the low values of the nutritional indices weight, height, ponderal index and albumin support the concept of suboptimal nutrition. It is recognised that the portal circulation and particularly insulin play a role in maintaining hepatocyte integrity and in hepatic regeneration (Editorial, Lancet, 1975). It would not therefore be surprising if suboptimal nutrition affected drug metabolism by an effect on liver mass or directly on enzyme activity.

(ii) Londoners

The London sample was designed as the nearest practical approximation to a community based sample of healthy adults in an industrial city. It was anticipated that it would differ environmentally in many ways from the Gambian sample. The results confirmed this, but also demonstrated that within the London industrial sample there were two sub-groups whose different cultures resulted in differences of a number of the environmental factors investigated. Several of these environmental factors were shown to be associated with differences in antipyrine half-life.

These two cultural sub-groups were the vegetarian Asians and the non-vegetarian Caucasians. The majority of Asians were vegetarians (86%), non-smokers, teetotallers and modest users of coffee and tea. A minority of Asians had more westernised social habits, and four of the eight who were non-vegetarian also smoked

and/or drank alcohol.

There is no reason to believe that the incidence of social drug use among the Caucasian, non-vegetarian subjects is atypical of industrial workers in London or other Western cities. The figure of 46% male smokers contrasts with the one smoker among ten medical research workers studied in the assessment of antipyrine kinetics in saliva (Chapter II), and emphasises the high selectivity of many drug studies with volunteers. The high incidence of smokers among female Caucasians (52%) and of users of the pill (26%) reflects the greater number of young women in the sample and emphasises the differences in environmental influences due to contrasting cultural patterns in Caucasian and Asian women.

The different diets of London Caucasians, Asians and the Gambians appeared to be responsible for significant differences in nutritional status. The mean values of the anthropometric indices, serum albumin and haemoglobin for the three samples showed strong correlation. Thus the Caucasians had the highest serum albumin and haemoglobin and a high ponderal index, while the Gambians had the lowest serum albumin, a low haemoglobin, and the lowest ponderal index. The Gambians also had the highest volume of distribution of antipyrine (0.71 ± 0.15 l/Kg) compared with 0.58 ± 0.08 l/Kg for the Caucasians and 0.53 ± 0.08 for the Asians. Since antipyrine is distributed uniformly throughout the body water the volume of distribution is inversely proportional to body fat. Thus volume of

distribution correlates inversely with pondral index (weight/height^2). This correlation was also seen as a first order correlation coefficient of -0.36 ($p < 0.001$) for the whole file of 180 subjects. The validity of using anthropometric indices, serum albumin and haemoglobin as indices of nutritional status was shown by the significant positive correlations ($p < 0.005 - p < 0.001$) between body weight, ponderal index, albumin and haemoglobin.

The antipyrine half-life was significantly longer in Asians than in Caucasians. Multiple regression analysis showed that this difference was due to a number of environmental factors associated with the Asian and Caucasian cultures and not with race per se. However separate analyses by race and sex confirmed that these environmental factors operated within sub-groups as well as for the whole sample.

Some of these factors have only recently been shown to be capable of altering drug oxidation in man i.e. cigarette smoking, use of the contraceptive pill and dietary manipulation. Cigarette smoking has been shown to shorten antipyrine half-life in age and sex-matched subjects and in subjects who stopped smoking and were later re-tested, thereby acting as their own controls (Hart, Farrell, Cooksley and Powell, 1976). It was shown to explain 12% of the variance in antipyrine half-life in a sample of 307 male subjects (Vestal, et al., 1975). In both of these studies the effect appeared to be one of induction as half-life was shortened and

metabolic clearance rate increased by 18 to 28%. In the present study the effect was of similar magnitude, shortening the half-life by 28% in male Caucasian smokers and by 24% in female smokers. Cigarette smoking has also been shown to accelerate by almost two-fold the elimination of theophylline (Jenne, Nagasawa, McHugh, MacDonald and Wyse, 1975) which is probably a prototype of a drug metabolised by the cytochrome P-448 system. Thus its metabolism is increased in vitro by pretreatment of rats with the polycyclic hydrocarbon 3-methylcholanthrene (Lohmann and Miech, 1976). In man pre-treatment with phenobarbitone does not alter theophylline eliminate rate (Piafsky, et al., 1975) while it markedly accelerates that of antipyrine (Vessell, Passanti and Greene, 1970). Since cigarette smoke contained 3-methylcholanthrene, 3, 4 benzpyrene and other polycyclic hydrocarbon-type inducing agents, the greater effect of smoking on theophylline elimination compared with its effect on antipyrine elimination is consistent with the concept of two haemoproteins, the cytochrome P-448 and cytochrome P-450, involved in the metabolism of theophylline and antipyrine.

The effect of the steroid oral contraceptive pill in prolonging antipyrine half-life by 40% confirmed the findings in a small number of women studied before and after use of the pill (Carter, et al., 1974; Carter, et al., 1975). In the latter studies chronic use appeared to prolong antipyrine half-life by increasing the volume of distribution while short-term therapy (3 months) had no effect on volume of distribution and appeared to inhibit metabolism. It is of

interest that the prolongation of antipyrine half-life by use of the pill and the shortening by cigarette smoking appeared to negate each other. Thus Caucasian women who smoked and took the pill had similar half-lives (12.0 ± 2.0 (S.D.) hours) to those who used neither (11.2 ± 3.7 hours). Conversely the difference between the half-lives of pill-taking non-smokers (15.7 ± 2.3 hours) and non-pill taking smokers (8.9 ± 2.0 hours) was 79%, or almost two-fold, and therefore likely to be clinically as well as statistically significant.

(iii) General Discussion

Consumption of a vegetarian as opposed to a non-vegetarian diet has been shown to be the single most important determinant of antipyrine half-life. This classification of diets was of course a major over-simplification for several reasons; firstly Gambian and Asian vegetarian diets are very dissimilar both in their major protein and non-protein constituents; secondly, factors such as total protein or calorie intake, which have been shown to affect both microsomal enzyme content and drug metabolic rates in rats (Marshall and McClean, 1962; Campbell and Hayes, 1974; Kato and Gillette, 1965) may vary widely within the classification used; thirdly a wide range of vitamins and minerals can affect metabolic rates in animals; fourthly many vegetarians in our London sample ate and drank liberal amounts of dairy products while others did not. On the other hand, classification of occasional meat-eaters as vegetarians could have blurred differences between omnivores and pure vegetarians. However, in spite of these drawbacks this study suggests that the multitude of dietary factors affecting drug metabolism in the rat may be relevant to man.

A recent report of Alvares, Anderson, Conney and Kappas (1976) showed that both antipyrine and theophylline half-life were longer (90% and 50% respectively) on a high carbohydrate, low protein diet - than on a low carbohydrate, high protein diet. In the limited sub-sample of 14 in the present study whose dietary history was taken in detail it is note-worthy that the four longest antipyrine half-lives (21.1, 20.5, 19.4,and 17.8 hours) were associated with the four lowest estimated protein intakes of 35.6, 27.7, 22.3 and 31.2 G per day. In all 14 subjects there was a negative correlation of half-life with protein intake ($r = -0.74$, $p < 0.005$). This supports the findings of Alvares, et al. Our diet histories also showed that a low calorie, fat and protein intake were features of the diets of seven vegetarians studied, as well as low iron and vitamin B_{12} intake, while one subject had a very low folate intake. It is impossible to relate any single dietary factor to antipyrine half-life in such a small sample, but further study seems to be indicated.

Body weight explained 3% of the variation of half-life in the whole London sample. This is a relatively small proportion and is probably explained by the relationship between body weight and volume of distribution of antipyrine, as half-life is proportional to volume of distribution. In the Gambian subjects, whose variation in body weight was less, it was not a significant factor.

The proportion of the variance explained by environmental factors varied considerably in our samples. Vessell showed in his twin

studies that genetic factors appeared to explain most of the individual variation in antipyrine half-life. However estimates based on comparison of within pair differences in variance of fraternal and identical twins gave a much larger figure (98%) than other methods of analysis of the same data. The latter gave values similar to that derived from family studies of 65% (Whittaker and Evans, 1970). Twin studies do not take into account the greater intra-pair environmental differences of fraternal twins compared with identical twins, which are widely recognised and are apparent from Vessell's published tables, showing smoking habits, coffee and alcohol consumption.

This means that other important environmental factors such as the contraceptive pill and dietary fads or deficiencies may have been similar in identical twins and dissimilar in fraternal twins. This could explain the discrepancy between Vessell's conclusions and our own.

If environmental factors are indeed capable of influencing drug metabolism in man it follows that the greater the number and potency of such factors which can be identified in a community the greater will be the proportion of inter-individual variation which can be explained. This can be seen to be true in our samples of Gambian and London subjects. The only heavily used social drug among the Gambians was the cola nut, and cola nut consumption and haemoglobin explained 19% of the variance. In the London Caucasians two widely used social drugs, the SOC pill and cigarettes, explained

36% of the variance. In the Asian sample, where these drugs were not used, diet and coffee and tea consumption accounted for 19% of the variance. When the Asian and Caucasian samples were combined for analysis the larger sample included a larger number of identified significant environmental factors, and 49% of the variance was explained by the four factors diet, smoking, use of the SOC pill and body weight.

Thus the amount of inter-individual variation in any sample of subjects which is explained by environmental factors will depend both on the sample and on the recognition of the important environmental factors. It follows from this statement that the proportion of the variance not explained by environmental factors is not necessarily due to hereditary determinants but may be partially due to other unrecognised environmental factors.

There are no comparable community based studies of antipyrine half-life, but in recent years the drug has been widely used to investigate inter-individual variation of oxidation rates, and Table I, Chapter I, summarises most of the larger or most pertinent studies. Mean values of antipyrine half-life range from 17.4 hours for a geriatric sample to 5.3 hours for a group of barbiturate addicts. The large interindividual variation seen in some studies e.g. Kolmodin, et al., 1969; S.D. of 7.5 hours in office workers, contrasts with the small variation seen in some samples e.g. Lindgren, et al., 1974; S.D. of 1.3 hours and Fraser, et al., 1976; S.D. of 1.4 hours, in medical students and research workers.

After treatment with phenobarbitone, the mean half-life of antipyrine is shortened and the inter-individual variation is reduced (Vessell, 1972). Similarly in these studies short mean half-lives are associated with small variances. This is consistent with the effects of environmental inducing agents in samples of subjects with the shortest half-lives.

A feature in common for our Caucasian, Asian and Gambian samples as well as for previously reported studies is a unimodal distribution curve. Kellerman, et al. (1976) have recently reported conflicting results. They have found a correlation between antipyrine half-lives in vivo and benzo(a)pyrene hydroxylase inducibility in lymphocyte cultures from the same individuals, suggesting that the two enzymes were identical. On the basis of both measurements the population sampled fell into 3 distinct groups, suggesting genetic control by simple Mendelian inheritance. However, Kellerman, et al. did not take into account any of the environmental factors shown here to be significant. It would be possible for example for a group of smokers (mean half-life 8 hours) and a group of non-smoking contraceptive pill takers (mean half-life 15 hours) to give a false impression of polymorphism in a sample of young people. Kellerman's= findings therefore require confirmation, with identification of environmental influences.

Factors such as age and sex, which have been shown to be related to antipyrine half-life in man (O'Malley, et al., 1971; Liddell, et al., 1975) have now been shown by multiple regression

analysis to be linked with social habits. These social habits and other environmental factors appear to be able to account for as much as 50% and possibly more of the variance in antipyrine half-life, depending on the sample being investigated. The studies described here, together with those summarised in Table I, Chapter I, suggest that for the community as a whole, including prescribed drug takers, addicts and the elderly, more of the inter-individual variation may be environmental than genetic in origin.

The effects of single factors shown to contribute to variance of antipyrine half-life are of the order of 25-50% which means that individually they may not be of great clinical importance. A combination of two factors however may produce more than two-fold changes in groups of people: for example, smoking Caucasians (mean half-life 8 hours) and non-smoking vegetarians and non-smoking pill takers (mean half-life 15 hours). Effects of this magnitude are likely to be important clinically (See Chapter VII).

CHAPTER IV

PARACETAMOL METABOLISM IN HEALTHY ADULTS

(a) Subjects and sample collection

(b) Methods

(c) Results

(d) Discussion

(i) Subjects and sample collection

All subjects from the National Westminster Bank received paracetamol and antipyrine as described in Chapter III, b (i). Forty subjects had complete collections and analysis of four saliva samples at 2, 3, 5 and 8 hours. The dstribution of these subjects by age and sex is shown in Table I. There were 30 Caucasian and 10 Asian subjects.

(ii) Methods

Paracetamol was analysed as described in Chapter II b and half-lives were estimated by the method of least squares. Multiple regression analysis was performed for paracetamol as dependent variable on the same observed variables as described in III b for antipyrine using the Biomedical Computer Program BMD 02R for stepwise regression. Samples collected from subjects at Elizabeth Arden Ltd., Smiths Industries and Smiths Foods Ltd. will be analysed at the Royal Postgraduate Medical School at a later date and the results reported together with those described here.

(iii) Results

Means and standard deviations for paracetamol half-life and observed variables are listed in Table II. The paracetamol half-life for the 40 subjects was 2.80 ± 0.81 (S.D.) hours with a three-fold

TABLE I

DISTRIBUTION OF SUBJECTS BY AGE, SEX AND ETHNIC ORIGIN

	MALES	FEMALES
18–30 years	6	16*
31–40 years	2	6+
41–50 years	2	3
51–60 years	2	3
TOTALS	12	28

* Includes 8 Asians

+ Includes 2 Asians

TABLE II

PARACETAMOL HALF-LIFE AND 17 VARIABLES FOR 40 LONDON SUBJECTS

VARIABLE	UNITS	MEAN	S.D.
SEX		1.70	0.46
AGE	Years	31.3	12.5
WEIGHT	Kg	61.9	12.1
HEIGHT	Cm	164	10
HAEMOGLOBIN	G/dl	13.5	1.9
ALBUMIN	G/dl	4.42	0.26
GLOBULIN	G/dl	2.63	0.24
RACE *	-	1.50	0.88
SMOKING *	-	1.43	0.50
CIGARETTES	/day	9.1	12.9
ALCOHOL	Drinks/week	6.2	9.8
DIET *	-	1.15	0.36
COFFEE/TEA	Cups/day		
PILL *	-	1.05	0.22
PONDERAL INDEX	G/cm^2	2.29	0.28
V_d (Antipyrine)	L/Kg	0.56	0.10
Antipyrine half-life	Hours	10.3	4.0
Paracetamol half-life	hours	2.80	0.81

* Race: Caucasians = 1, Asians = 3

Smoking: Non-smokers = 1, smokers = 2

Diet: Non-vegetarians = 1, vegetarians = 2

SOC pill: Non-pill takers = 1, pill-takers = 2

range. The frequency distribution curve is shown in figure 1. It is a continuous unimodal curve, with three outlying values of 4.6, 5.0 and 5.4 hours, the mean of which is almost twice the sample mean. All three values lie more than two standard deviations above the sample mean, and all three values were from vegetarian subjects. Three other vegetarian subjects in the sample had half-lives of 3.0, 3.2 and 3.4 hours. The half-lives of the six vegetarians (4.16 ± 0.96 (S.D.)) were significantly longer ($p < 0.01$) than those of the non-vegetarians (2.68 ± 0.40). When the results were expressed as clearance the subjects with the longest half-life had the lowest clearance and the clearance of the six vegetarians (147 ± 49 ml/min) was also significantly different ($p < 0.01$) from that of the non-vegetarians (2.52 ± 52 ml/min).

Four variables showed a significant first order correlation coefficient with paracetamol half-life at the 1% level. The strongest correlation was with diet, where as before a non-vegetarian diet was given a value of 1 and a vegetarian diet a value of 2 ($r = 0.70$, $p < 0.001$). The other significant correlations were with race ($r = 0.64$, $p < 0.001$); body weight ($r = -0.47$, $p < 0.005$) and ponderal index ($r = -0.44$, $p < 0.005$) (Table III).

However, ponderal index, body weight and race were all significantly correlated and of these variables multiple regression analysis confirmed that only diet and ponderal index were significant independent predictors of paracetamol half-life ($p < 0.01$).

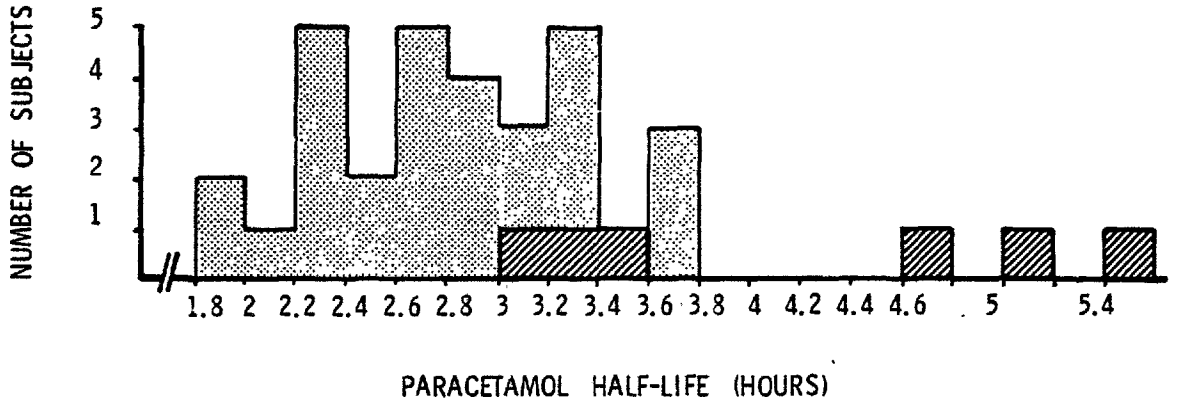


Figure 1

Frequency distribution of paracetamol half-lives:
34 non-vegetarians (stippled) and 6 vegetarians (hatched)

TABLE III

FIRST ORDER CORRELATION MATRIX FOR HALF-LIFE OF PARACETAMOL, HALF-LIFE AND VOLUME OF DISTRIBUTION (Vd) OF ANTIPYRINE AND 15 VARIABLES IN 40 LONDONERS

	SEX	AGE	WT	HT	HB	ALB	GLOB	$T\frac{1}{2}^{A}$	Vd	RACE	SM	//	ALC	DIET	C/T	PILL	PI	$T\frac{1}{2}^{P}$
SEX	1.00	-.22	-.65*	-.67*	-.62*	-.29	.06	.01	-.44+	.38+	-.21	-.37	-.32	.28	-.34	.15	-.33	.24
AGE		1.00	.29	.22	-.01	-.10	.14	-.25	.06	-.33	.15	-.01	-.02	-.36	.26	-.19	.23	-.22
WEIGHT (WT)			1.00	.79*	.49*	.30	.08	-.19	.30	-.54*	.28	.33	.40+	-.44+	.35	-.07	.76*	-.47*
HEIGHT (HT)				1.00	.40+	.25	-.10	-.14	.36	-.43	.27	.35	.41+	-.34	.20	-.13	.22	-.32
HAEMOGLOBIN (HB)					1.00	.25	-.04	-.14	.33	-.37	.20	.27	.35	-.15	.25	-.01	.35	-.22
ALBUMIN (ALB)						1.00	-.07	.07	.30	-.24	.26	.27	.27	-.30	.07	-.10	.21	-.28
GLOBULIN (GLOB)							1.00	-.06	-.03	.10	-.18	-.19	-.26	.10	.06	.02	.22	.26
HALF-LIFE ($T\frac{1}{2}^{A}$) OF ANTIPYRINE								1.00	-.03	.54*	-.43	-.35	-.12	.66*	-.58*	.26	-.20	.38+
Vd OF ANTIPYRINE									1.00	-.35	.31	.27	.15	-.17	.12	-.31	.08	-.25
RACE										1.00	-.50	-.41+	-.36	.73*	-.50*	.13	-.44+	.64*
SMOKER (SM)											1.00	.83*	.37+	-.36	.38+	.04	.16	-.27
NO. OF CIGARETTES (//)												1.00	.36	-.30	.41+	-.07	.17	-.26
ALCOHOL (ALC)													1.00	-.27	.08	.02	.21	-.16
DIET														1.00	-.46*	.23	-.38+	.70*
COFFEE/TEA (C/T)															1.00	-.17	.36	-.31
PILL																1.00	.05	.32
PONDERAL INDEX (PI)																	1.00	-.44*
PARACETAMOL HAEF-LIFE ($T\frac{1}{2}^{P}$)																		1.00

* Denotes significance ($p < 0.01$)

\+ " " ($p < 0.02$)

Paracetamol half-life decreased with an increase inponderal index and was longer in subjects with a vegetarian diet. After correction for the effect of diet on paracetamol half-life the correlation with race was no longer significant. Similarly after correcting for ponderal index there was no significant correlation of half-life with body weight. However use of the contraceptive pill and high globulin levels were also associated with a prolonged half-life. The final multiple regression equation was:

$$T\frac{1}{2} = 0.33 + 1.13 \times \text{Diet} + 0.95 \times \text{globulin} + 0.78 \times \text{Pill Status} - 0.09 \times \text{ponderal index.}$$

The total variance explained was 64% (R = 0.80) and the individual contributions to the variance of these four factors were: diet 49%, globulin 6%, use of the pill 5% and ponderal index 4%.

(iv) Discussion

Reported half-lives of paracetamol for small groups of healthy adults range from 1.6 to 3.6 hours (Table IV). There is a consistently small range of values in contrast to the large inter-individual variation in the half-lives of drugs eliminated predominantly by oxidation. Two groups of subjects with prolonged paracetamol half-lives are infants and patients with liver damage due to paracetamol overdose. Neonates of 2-3 days have half-lifes

TABLE IV

REPORTED VALUES FOR PARACETAMOL HALF-LIFE IN MAN

Authors	Number of Subjects	Paracetamol Half-life (hours)	Range or SD
Careddu, et al., 1961	10 children	2.2	SD = 0.3
Nelson and Morioka, 1963	5 adults	1.9	1.6 - 2.8
Cummings, et al., 1967	4 adults	2.2	2.0 - 2.6
Levy and Regardh, 1971	5 adults	2.2	1.6 - 2.8
McGilveray and Mattok, 1972	5 adults		2.0 - 2.7
Prescott, et al., 1971	17 adults	2.0	SE = 0.1
Prescott and Wright, 1973 (patients with a paracetamol overdose)	18 adults without liver damage	2.9	SE = 0.3
	23 adults with liver damage	7.2	SE = 0.7
Levy, et al., 1975	12 neonates	3.5	2.2 - 5.0 (SD = 0.85)
Miller, et al., 1976	3 neonates	4.9	SE = 0.9
	7 children (3-9 years)	4.5	SE = 0.6
	4 children (12 years)	4.4	SE = 0.7
	4 adults	3.6	SE = 0.1

of 3.5 ± 0.85 hours (S.D.) (Levy, 1975), and 4.9 ± 1.5 hours (S.D.) (Miller, et al., 1976). Patients with paracetamol overdose and liver damage had a three to four fold prolongation of half-life compared to other reported adult values, while 18 cases without liver damage had normal values (2.9 ± 0.3 hours) (Prescott and Wright, 1973). There are no community based studies of paracetamol metabolism in man.

The results described in this thesis suggest that the inter-individual variation in paracetamol elimination rates in normal adults is greater than hitherto recognised. In forty healthy subjects there was a three-fold range of half-lives (1.8 to 5.4 hours). Use of the technique of stepwise multiple regression analysis suggests that 64% of the variation can be explained by four measured variables, and that variation is predominantly under environmental control. The mechanism of these effects is not clear as the metabolism of paracetamol is complex.

Paracetamol is metabolised chiefly by conjugation to the glucuronide (two thirds) and sulphate (one third). The enzymes responsible for sulphation are situated in the soluble fraction of the liver (Robbins and Lipmann, 1957). Glucuronidation is dependent on the availability of uridine diphosphate glucuronic acid (UDPGA), formed in the soluble fraction, and on the activity of the glucuronyl transferase in the microsomal fraction (Dutton, 1956). Several situations have been shown to affect the pattern of

paracetamol metabolism, including age, diet and inducing drugs.

The half-life in neonates is prolonged, apparently because of reduced glucuronidation, the elimination rate constant for the glucuronide being one fifth the adult value (Levy, 1975). This is partly compensated by an increase in sulphation, the infants having higher rate constants than adults for sulphate formation.

In the rat the diet affects the relative rates of glucuronidation and sulphation (Woodcock and Wood, 1971). Thus a protein deficient diet produces an increase in the activity of glucuronyl transferase with no significant change in sulphotransferase, despite a reduction in soluble and microsomal protein in the liver. This is in marked contrast to the effects of protein deficiency in decreasing microsomal oxidation in the rat (McLean and McLean, 1966; Kato, Oshima and Tomizawa, 1968). The reason for the increased glucuronyl transferase activity in protein deficiency is not known.

A minor pathway of paracetamol metabolism is that of oxidation by the cytochrome P-450 system to a reactive metabolite (Mitchell, et al., 1973). The diet also affects the toxicity of this metabolite, but the mechanisms are complex. Although protein deficiency reduces cytochrome P-450 and increases glucuronyl transferase activity, the feeding of low protein diets potentiates paracetamol toxicity (McLean and Day, 1975). This has been attributed to the deficiency of liver glutathione associated with the low protein diet. Thus

the reactive metabolite may exhaust the supply of glutathione and bind to tissue macromolecules, causing liver cell necrosis.

Paracetamol toxicity is affected by drugs both in animals and man. Pre-treatment with phenobarbitone, an inducer of microsomal enzymes, increased the incidence and severity of hepatic necrosis in mice, while both phenobarbitone and 3 methylcholanthrene increased toxicity in the rat (Mitchell, Jollow, Potter, Davis, Gillette and Brodie, 1973). Piperonyl butoxide, an inhibitor of microsomal oxidation protected against necrosis. In man prior barbiturate medication increases hepatic necrosis following paracetamol overdose (Wright and Prescott, 1973). Experimental and clinical evidence are consistent with the hypothesis that paracetamol toxicity results from oxidation to a toxic metabolite, and can be affected by diet and drugs.

The factory based study described here suggests that the overall elimination rate of paracetamol may be influeced by environmental factors. In view of the known effects of diet and drugs on toxicity referred to above it is possible that other environmental factors may affect toxic and non-toxic pathways. An important limitation of this study is the relatively small number of subjects studied to date (40) in contrast with 180 subjects for antipyrine half-life.

In using multiple regression analysis the number of predictor variables should be considerably less than the sample size (Armitage, 1971) and the use of this type of analysis with a comparatively

small sample may be unreliable. Assessment of the role of environmental factors on paracetamol elimination should therefore await results on a larger number of subjects, which will be forthcoming, from the same sampling frame. Paracetamol is a good example, however, of a drug where alteration of the rate of a specific, possibly toxic pathway by an environmental agent may be of greater importance than alteration of the overall elimination rate.

CHAPTER V

AMYLOBARBITONE HYDROXYLATION KINETICS IN LIVER NEEDLE BIOPSIES OF PATIENTS WITH ALCOHOLIC LIVER DISEASE

a) Patients

(i) Indications for study and protocol
(ii) Case histories
(iii) Clinical and laboratory features of liver disease and nutritional deficiency

b) Methods

(i) Amylobarbitone hydroxylation assay
(ii) Preliminary studies with rat liver biopsies
(iii) Preliminary studies with human surgical biopsies
(iv) Benzo(a)pyrene hydroxylase

c) Results of needle biopsy studies

(i) Amylobarbitone hydroxylase activity
(ii) Benzo(a)pyrene hydroxylase activity

d) Discussion

(i) Amylobarbitone hydroxylase activity

(ii) Benzo(a)pyrene hydroxylase activity

a) PATIENTS

(i) Indications for study and protocol

All patients studied were believed to be alcoholics who either desired admission to hospital for "drying out" (7 patients) or were admitted as emergencies due to complications of heavy alcohol consumption (4 patients). Six of those wishing admission for "drying out" were referred to the Hammersmith Hospital (H.H.) Liver Clinic run by Dr. Graham Neale and Dr. Carol Seymour, over the period January, 1975 to July, 1976. The other presented himself to the Casualty Department with a story of alcoholism and drug addiction but was subsequently diagnosed as a psychopath and malingerer.

The World Health Organisation description of alcoholism (1951) fitted all patients except the last mentioned (S.W.) and one of the emergency cases (M.W.), whose admission was precipitated by the combination of a heatwave and only moderate alcohol consumption (80g/day). The history of alcohol consumption could only be obtained from the patient himself or herself in seven patients as they were unmarried or lived alone. In four patients corroboration was obtained from the wife or close relation. Table I shows the age and sex, alcohol consumption, smoking habits and drug use of all 11 patients.

TABLE 1

AGE, WEIGHT, ALCOHOL CONSUMPTION, DRUG USE AND SMOKING HABITS OF PATIENTS

PATIENT	AGE	WT.	ALCOHOL/DAY (G)	CIGS/DAY
P.M.!	68	46.2	130	20
F.B.	65	53.5	185	0
D.M.	56	77.0	200	0
S.W.*	35	70.0	130	15
R.B.	50	88.6	520	10
H.N.	55	70.0	390	10
T.G.	48	54.0	330	20
E.P.	69	53.1	(260)	0
A.D.+!	30	52.2	130	10
M.W.	62	44.0	80	15
C.M.≠	50	52.0	260	20

* Intermittent use of barbiturates (unreliable history)

\+ Librium 10 mg t.d.s. for 6 months

≠ Phenobarbitone 100 mg t.d.s. for 1 week

() Until 3 weeks before admission when intake stopped

! Female

The initial protocol, approved by the Ethics Committee of Hammersmith Hospital (June, 1974) is shown in Table II. The BSP excretion was done on the first three patients only. After studying the first subject, P.M., who showed evidence of nutritional deficiency, serum iron, serum and red cell folate and serum B_{12} were also measured. All patients had the purpose of the study explained to them after their admission to hospital, and the protocol described. It was explained that their stay in hospital should be of a minimum of three and preferably four weeks duration to ensure complete abstinence from alcohol, a full diet, vitamin supplements and supportive psychiatric, medical and social care, including Alcoholics Anonymous support and attempts at rehabilitation. It was also explained that participation in the study would in no way affect their receiving treatment. The first seven patients gave written informed consent to the full protocol, but the repeat study was not done on S.W. when he was seen to have a normal biopsy and a diagnosis of malingering was made. Consent to the study was given by the only relative of E.P., who lived with and care for him, as he suffered from Korsakoff's psychosis. The last three patients, A.D., M.W. and C.M. were studied only once.

Sodium amylobarbitone 200 mg orally was given at 10 p.m. on the evening of the second or third day in hospital, as soon as clinical and laboratory assessment was completed. If prothrombin

TABLE II

PROTOCOL FOR STUDY OF AMYLOBARBITONE METABOLISM IN PATIENTS WITH ALCOHOLIC LIVER DISEASE

1. Points from History

   (a) Specific attention to alcohol intake, if possible with confirmation from husband/wife and other relative.

   (b) Recent drug therapy.

2. Clinical Signs

   (a) Liver size (measured in cm below right costal margin to mid clavicular line.

   (b) Liver size measured by liver scan (not carried out).

   (c) Stigmata of liver disease.

   (d) Any features of hepatic encephalopathy.

3. Laboratory Tests

   (a) Routine haematology incl. PT, PTT and platelets.

   (b) ELUC, PROFILE, which would include SGOT, Alk. Phos., Albumin and globulin.

   (c) GGT (Gamma glutamyl transpeptidase).

   (d) BSP excretion.

4. Half-life Measurement - as soon as lab. investigations cleared

   (a) If possible have patient on no other drug therapy, particularly potential enzyme inducers. If necessary diazepam or nitrazepam.

   (b) Informed consent.

   (c) Amylobarbitone 200 mg given orally at 10 p.m. Venous blood (10 ml in a heparinised (profile) tube) taken by venepuncture at 0, 10, 18, 24, 34 and 42 hours - i.e. 8 a.m., 4 p.m., 10 p.m., 8 a.m. and 4 p.m.

   (d) Urine collections from 0-24 and 24-48 hours.

5. Liver Biopsy

   Portion for amylobarbitone kinetics put into vial containing 0.02 M Tris-KCl on ice and sucrose Imidazole for other enzyme studies.

6. Repeat 4. and 5. after appropriate hospital stay

7. Repeat lab. investigations weekly.

time was elevated a three day course of vitamin K (10 mg daily) was given. The liver biopsy was carried out 60 hours after the dose of amylobarbitone, when plasma concentrations were 20-100 times lower than the lowest incubation substrate concentration.

(ii) Case Histories

1. P.M.: A 68 year old spinster who had lived alone for six years. She had lived on canned meats, bread, marmalade and tea, rarely cooked and never ate fresh fruit and vegetables.. She had been drinking a half bottle of gin/day for several years and smoked 20 cigarettes/day. She had become unable to go out, spent all day in bed and was admitted to H.H. after falling asleep with a lit cigarette and causing small, superficial burns on her head, hair and chest.

She was feeble, slow old lady, looking older than 68, with no interest in the environment and impaired memory for recent events. She was grossly underweight and had an enlarged liver, 1.5 cm below the right costal margin (RCM), but no jaundice or other evidence of liver decompensation. There was impairment of light touch and pinprick below both ankles, an unsteady gait, heel/knee/shin ataxia and dysgraphia attributed to a combination of alcohol and vitamin deficiency.

Laboratory investigations showed a low serum albumin of

3.5 g/100 ml and a mild elevation of bilirubin (1.3 mg/100 ml) but no other liver function abnormality. Serum B_{12} was very low (60 ng/ml, normal range 160 - 925) as were serum folate (0.7 µg/ml, normal range 260 - 600) and red cell folate (176 µg/ml, normal range 260 - 600). Liver biopsy showed a number of double cell plates and a non-specific triaditis but no diagnostic features of alcoholic liver disease. After her biopsy and in vivo study of amylobarbitone metabolism she showed steady improvement in mental function and ataxia. Her second study was performed after four weeks of vitamin supplements and full hospital diet and at discharge she had gained 4 kg and was rehabilitated.

2. F.B.: A 65 year old retired house painter, widowed and living alone for five years. Past history included diagnosis of duodenal ulcer (1965), Meniere's disease (1968), ulcerative colitis diagnosed in 1971 on biopsy, treated with a left hemicolectomy in 1972 and a colo-rectal anastomosis. Crohn's disease was diagnosed on history, but had been inactive since 1973.

His alcohol intake had increased steadily to a bottle of whisky every 1-2 days over the last two years. He had fallen several times recently while intoxicated and on the last occasion was seen by the general practitioner and admitted to H.H.

His diet consisted almost entirely of pub sausages, crisps and fish and chips and he weighed only 53.3 kg. There were no

other abnormal clinical features except a 2 cm enlargement of the liver below the RCM. A dietary history confirmed a deficient diet, particularly in vitamins B and C and iron, and blood tests showed albumin, haemoglobin and folate to be low, with features of iron deficiency. The liver biopsy on day 5 showed severe fatty infiltration which was still present in moderate amounts four weeks later. During this period he gained 6.5 kg and haemoglobin and serum iron rose.

3. D.M.: A 55 year old business man, happily married (2nd) for three years. He had been drinking one bottle of whisky daily for 3-4 years and changed to wine (2 daily) two years previously, when he first recognised many features of alcoholism. He had developed ascites eight months before admission and it had not responded to bendrofluazide.

On examination he was an emotional, obese man with symptoms and signs of alcohol withdrawal (tremor, conjunctival hyperaemia and depression) and features of liver disease - spider naevi, palmar erythema, Dupuytren's contracture and hepatomegaly of 4 cm below the RCM, with signs of ascites, but no encephalopathy. There was elevation of serum transaminase, gamma glutamyl transpeptidase, bilirubin, prothrombin time and BSP excretion, and a low red cell folate. The first liver biopsy was performed after 3 days of vitamin K had corrected the prothrombin time. It showed moderate

fat infiltration and features of alcoholic hepatitis (thickening of cell plates, increased fibrous tissue in portal tracts, degenerating hepatocytes with alcoholic hyaline and infiltration of inflammatory cells and mild siderosis). After four weeks in hospital the liver enzymes were higher, suggesting increased tissue damage and the repeat biopsy showed evidence of portal hypertension and the development of cirrhosis.

4. S.W.: A 35 year old peripatetic and petty criminal who was a casual drug user (heroin intravenously for two years in the past, and more recently barbiturates intermittently). He drank intermittently (1 bottle of sherry, wine or whisky every few days) to intoxication, combining it with several barbiturate tablets, collapsing on the sidewalk, and so engineering admission to hospital. On this occasion he presented under the influence of alcohol and requested admission for drying out.

He gave a convincing story of severe withdrawal symptoms with hallucinations on attempting withdrawal in the previous week and was prescribed stelazine and propranalol by the admitting resident. Physical examination was normal except for a palpable liver edge and sclerosed arm veins from "mainlining". A liver biopsy showed only mild triaditis and as liver function tests were normal further studies were unjustified.

5. R.B.: A 50 year old publican, married with two children.

He was the son of publicans and a life-long heavy drinker, increasing to at least two bottles of brandy per day for the last two years. He had had multiple admissions to H.H. for nausea, vomiting and haematemesis (1965), and withdrawal of alcohol. He was "dry" for eight months in 1973. Abdominal distension was first noted in 1972 and jaundice occasionally since.

He was an obese slightly jaundiced man with a severe tremor and anxiety state. There were many spider naevi, small bruises and petechiae, liver enlargement of 15 cm below the RCM and mild ascites, but no features of encephalopathy. All serum liver enzymes were elevated, and he required a course of vitamin K to correct his elevated prothrombin time. Both his initial and final biopsy showed alcoholic hepatitis and severe cirrhosis while the moderate steatosis seen initially had cleared after four weeks. The ascites cleared in 10-14 days without specific treatment.

6. H.N.: A 56 year old long-standing passive alcoholic, unemployed, former clerk, single and living in a hostel run by the Cyrenians, a caring organisation. He had drunk 1½ bottles of whisky daily, often with wine, beer or methylated spirit as well, for over 20 years. He was anxious, emotional and tremulous, and had nightmares and minor auditory hallucinations after withdrawal. Apart from enlargement of the liver to 5 cm below the RCM there were no other features suggestive of liver disease. Biochemical and haematological indices were normal, and liver biopsy showed

only mild steatosis initially, clearing within four weeks.

7. T.G.: A 49 year old unemployed emigrant (Irish) building-worker, single, living in a hostel run by the Cyrenians. He had been a life-long heavy drinker and had been drinking 10-20 quarts of cider daily often with additional spirits, for five years. He smoked heavily (20-40 rolled cigarettes daily) and his past history included a bleeding gastric ulcer treated with a partial gastrectomy (1973) and chronic bronchitis.

He showed severe withdrawal symptoms, requiring medication with diazepam and chlormethiazole (Heminevrin), but the liver was not palpable and there were no stigmata of liver disease. The only abnormal liver function test was the gamma glutamyl transpeptidase. A serum B_{12} of 110 pg/ml and border-line folate confirmed the history of a poor diet. Surprisingly the first liver biopsy was entirely normal while the repeat biopsy showed hyperplasia.

8. E.P.: A 69 year old retired engineer, widowed and looked after by his sister-in-law. He was described by his general practitioner as a chronic alcoholic for 20 years, drinking a bottle of whisky daily, until a few weeks before admission. He had become ataxic, taken to his bed and continued drinking but stopped eating. For about two weeks before admission he drank no alcohol because of continuous vomiting.

On admission as an emergency he showed all the features of Wernicke-Korsakoff's psychosis. In addition he was dehydrated and cachectic, weighing 53 kg, had marked asterixis and responded to a dose of 100 mg of chlormethiazole prescribed by the admitting officer by becoming comatose for 2-3 hours. He was profoundly hypokalaemic (K^+ 1.7 meq/l), hypomagnesemic (Mg^{++} 0.8 meq/l) and haemoconcentrated despite a severe non-specific anaemia (Hb 8.8 g/100 ml on admission and 7.8 g/100 ml after rehydration). Serum transaminase was 32 iu/l on admission and rose progressively over a week to 58 iu/l. Bilirubin was 1.4 mg/100 ml. Red cell transketolase was 35 iu/l after a dose of parentrovite (normal = 35-90 iu/l). Liver biopsy showed moderate steatosis and perivenous hepatocyte atrophy indicating hypoperfusion. His coma may be explicable on the basis of the combination of hypomagesemia and hypokalaemia, a high production of ammonia associated with the latter (Sherlock, 1968) and a hypoperfused liver.

Oespite correction of his metabolic and nutritional deficiencies he showed little improvement in dementia or ataxia. The repeat biopsy showed marked hyperplasia.

9. A.O.: A 30 year old single female stenographer. She had been alcoholic for five years, "dry" for 18 months and drinking a half bottle of whisky again for one month. She complained of epigastric pain and had withdrawal symptoms and a tender liver, 2 cm below the RCM. Laboratory tests were all normal however, and her liver biopsy

(1 only) showed inflammatory cells and steatosis but was too small a portion to be diagnostic of hepatitis.

10. M.W.: A 62 year old male domestic cleaner, single and living alone. He claimed to be only a moderate drinker (4 pints of beer daily) and was admitted to H.H. during a severe heat spell (T. 33°C) as an emergency following an acute confusional state. He was confused and obstreporous, dehydrated (weight 55.5 kg), pyrexial (T. 39°C by general practitioner, 38°C in casualty) and flushed, with a pulse of 136/min. He was having hallucinations of his landlady's presence when first seen. The liver edge was palpable.

Serial haemoglobin and urea values confirmed the picture of dehydration, and 48 hour infusions of fluid and electrolytes restored his cardiovascular and mental state to normal. At this time he gave a history of progressive weakness, anorexia and confusion over several days, but continued drinking beer. His transaminase, bilirubin and gamma glutamyl transpeptidase were raised, and his liver biopsy (1 only) showed steatosis and alcoholic hepatitis.

His clinical state was attributed to heat stroke/ heat pyrexia precipitated by the combination of the heat wave and beer drinking.

11. C.M.: A 50 year old married, unemployed male clerk. He had been alcoholic for over 20 years, since serving in the navy,

drinking at least 1 bottle of gin daily. He was being treated with phenobarbitone 100 mg t.d.s. for one week following a grand mal fit.

He was an anxious tremulous man, smelling heavily of alcohol on admission. He had many spider naevi and liver enlargement of 4 cm below the RCM. All serum liver enzymes were elevated and he had a folate deficiency anaemia. Liver biopsy (1 only) showed steatosis, alcoholic hepatitis and cirrhosis.

(iii) Clinical and laboratory features of liver disease and nutritional deficiency

Liver histology, liver function tests and haematological indices of nutritional deficiency are shown in Table III. Admission values and values shortly before discharge are both shown for patients studied twice. In Table IV the patients are arranged in order of histological abnormality of their liver biopsies. It is apparent that they fall into 3 groups. The first group of 4 subjects, A.D., S.W., T.C. and P.M. had non-specific changes only. The second group of 3 subjects, H.N., F.B. and E.P. had moderate or severe steatosis. The third group of R.B., D.M. and C.M. had steatosis, alcholic hepatitis and cirrhosis, and were the only three to show unequivocal evidence of impairment of liver function. One patient, M.W., with steatosis and alcoholic hepatitis, had elevation of transaminase and gamma glutamyl

LIVER FUNCTION TESTS, HAEMATOLOGICAL INDICES
AND LIVER HISTOLOGY OF 11 ALCOHOLIC PATIENTS

	Patient	Hepato-megaly (cm)	P.T.+ (secs)	SGOT (I.U./L)	Alk.P (I.U./L)	GGT (I.U./L)	Bil. (mg/100 ml)	BSP % at 45 min	Alb. (g/100)	Hb. (g/100 ml	Folate	Fe	B_{12} (pg/100 ml)	Histology+
1.	P.M.	1.5	12/12	35	57	19	1.3	3	3.5	13.9	D	N	60	N
			-	52	54	11	0.6	3	3.6	14.0				
2.	F.B.	2	12/12	18	77	14	0.6	2.5	3.4	11.7	D	D	N	S
			-	15	77	9	0.8	2.5	3.4	12.2				
3.	D.M.	3	15/12	73	103	182	1.5	22	4.0	13.8	D	N	N	S H
			12/12	36	253	213	1.2	17	4.3	13.2				C
4.	S.W.	1	12/12	31	64	15	0.8	-	4.4	14.3	N	N	N	N
5.	R.B.	15	14/12	75	338	560	2.4	-	3.9	15.0	B	N	N	S H
			15/12	49	229	196	1.0	-	3.9	14.0				C
6.	H.N.	5	12/12	37	83	17	0.7	-	4.1	14.6	N	N	N	S
			-	39	66	-	0.4	-	4.0	14.7				
7.	T.G.	0	12/12	40	124	31	0.4	-	4.1	15.2	B	N	110	N
			-	14	62	28	0.3	-	4.0	-				
8.	E.P.	0	12/12	32	34	18	1.4	-	3.7	8.1	D	N	N	S
				58	51	35	0.5	-	3.7	10.2				
9.	A.D.	2	12/12	44	42	18	0.5	-	3.6	12.3	N	N	N	S
10.	M.W.	1	12/12	178	67	33	2.6	-	4.5	12.9	N	N	N	S H
11.	C.M.	4	12/12	92	141	360	0.9	-	4.4	12.4	D	N	N	S H C

Table III

(see attached sheet for key)

* Hepatomegaly measured in cm. below R.C.M.

\+ P.T. = Prothrombin time

SGOT = Serum glutamic oxaloacetic transaminase (7–40 I.U./L)

Alk.P = Alkaline Phosphatase (30–130 I.U./L)

GGT = Gamma glutamyl transpeptidase (5–25 I.U./L)

Bil = Bilirubin (1–9 mg/100 ml)

Alb = Albumin (3.5–5 g/100 ml)

BSP = Bromsulphthalein Excretion (< 5% at 45 min)

Hb = Haemoglobin (g/100 ml)

Fol = Serum & RBC folate, normal (N), deficient (D) or borderline (B)

Fe = Normal (N) or iron deficient (D) assessed on blood film, serum iron and total iron binding globulin or transferrin

B_{12} = (Vitamin B_{12}) (160–925 pg/ml)

‡ N = Normal or non-specific changes

S = Steatosis

H = Alcoholic hepatitis

C = Cirrhosis

transpeptidase.

A further distinction can be drawn between the patients. There was a high incidence of nutritional deficiencies as indicated by the serum albumin and haemoglobin, serum and red cell folate, iron and B_{12} analyses. These deficiencies correlated closely with a history of a clearly inadequate diet and only four subjects, A.D., M.W., S.W. and H.N. gave history of eating a full diet.

b) METHODS

(i) Amylobarbitone hydroxylation assay

Amylobarbitone hydroxylation was estimated by measuring the formation of ^{14}C-labelled product obtained by incubating (2-^{14}C) amylobarbitone with 9000 g supernatant. The side chain-hydroxylated metabolite was extracted with ether and separated by thin layer chromatography (tlc). As a preliminary to working with human needle biopsies of 20-30 mg weight, studies were carried out with 20 mg portions of rat liver and human wedge biopsies obtained at laparotomy, using identical microscale (50 µl) incubations.

Chemicals

Sodium amylobarbitone was obtained from Eli Lilly and Co. Ltd.

(Indianapolis, Indiana, U.S.A.).

(2-^{14}C) amylobarbitone (5.7 μCi/μmol) was purchased from the Radiochemical Centre, Amersham.

Hydroxyamylobarbitone was supplied by the Lilly Research Centre Ltd. (Erl Wood Manor, Windlesham, Surrey, U.K.).

NADP and sodium isocitrate were obtained from Sigma Chemical Co. (London) Ltd. and isocitrate dehydrogenase from= Boehringer Corporation (London) Ltd. Other reagents were analytical grade.

The (2-^{14}C) amylobarbitone was purified by a modification of the method of Kuntzman, et al. (1967). The compound (5.0 mg) was dissolved in 0.2 ml ethanol and made up to 2 ml with redistilled petroleum ether (boiling range 80-100^{o}C). After centrifugation the petroleum ether layer was immediately transferred to a fresh tube and evaporated under nitrogen to a volume of about 0.4 ml. Several drops of diethyl ether were added to redissolve precipitated amylobarbitone, and a back extraction was then carried out into a concentrated aqueous solution of unlabelled sodium amylobarbitone. The aqueous layer was removed after centrifugation and duplicate aliquots taken for counting of radioactivity. Comparison with the activity of aliquots from the original aqueous sample gave an estimate of 90% extraction

of the ^{14}C-labelled drug. The purified extract was diluted to give the desired concentration of 12 mM sodium amylobarbitone with a final specific activity of 1.45 μCi/μmol and 99.03% purity. Despite purification, it was necessary to run blank incubations at each substrate concentration because of the residual impurities which chromatographed as hydroxyamylobarbitone.

Preparation of 9000 g supernatant

Liver needle biopsies from 11 patients (7 studied twice) were obtained personally with a 1.9 mm diameter Menghini needle and with a local anaesthetic only (5–10 ml of 1 or 2% lignocaine). Each biopsy was divided into three portions; 5 mg was placed in 2 ml of 0.25 M sucrose buffered in 3 mM Imidazole (pH 7.2) for benzpyrene hydroxylase analysis, 5–10 mg sent for histology and at least 20 mg retained for amylobarb hydroxylase assay. This portion was placed at once in 0.02 M tris–1.15% KCl buffer (ph.7.4) on ice, and transported to the laboratory. Only one biopsy provided insufficient tissue for amylobarb hydroxylase activity to be determined (10 mg). Benzpyrene hydroxylase activity was determined with the first eight subjects' biopsies but not with the last three. Only one patient complained of right shoulder-tip pain and there were no other eventualities arising from biopsy.

The biopsy sample was blotted on filter paper, weighed and transferred to a 0.1 ml all-glass Jencon homogeniser. Homogenisation

was carried out by hand within 15-20 minutes of obtaining the biopsy, using a standard procedure of 12 vertical strokes, each followed by a rotatory stroke. The volume was made up to 200 µl with 0.02 M Tris-1.15% KCl buffer (pH 7.4) and 9000 g supernatant was prepared by centrifugation at 12,000 rpm for 20 minutes in a refrigerated MSE Superspeed 50 centrifuge, using pre-chilled Teflon microcentrifuge tubes of 250 µl capacity.

Incubations

Incubations were carried out in a final volume of 50 µl in 3 ml tapered quickfit tubes. The final composition of the incubation mixture was: 0.02 M Tris buffer (pH 7.4); magnesium chloride 4 mM, NADP 0.4 mM; sodium isocitrate 9.6 mM; isocitrate dehydrogenase 0.2 units per ml; 9000 g supernatant 1-1.5 mg protein per ml and (2-^{14}C) amylobarbitone 0.3 - 4 mM. Eight substrate concentrations were used and 0.02 M Tris-KCl replaced 9000 g supernatant in a blank incubation at each substrate concentration.

The 9000 g supernatant was added last, and the stoppered tubes were transferred to a 37^{o}C water bath. They were incubated with shaking for 15 minutes. The reaction was stopped by removal to ice and addition of 10 µl of 4 M-NaH_2PO_4 after which the incubations were stored deep frozen until analysis. Time from biopsy to incubation was approximately 3 hours.

Protein determination

The protein content of the 9000 g supernatant was measured by the method of Lowry, et al. (1951). For the 20 mg biopsy samples it was modified to permit measurement of 1 mg protein in 9000 g supernatant. Measurements of activity were subsequently related to the 9000 g protein content and to the liver wet weight per incubation.

Thin-layer chromatography

Each 50 µl incubation, to which 20 µg unlabelled hydroxyamylobarbitone was added as carrier and marker, was extracted three times with 1 ml of heptane/1.5% isoamyl alcohol, and then with 1 ml ether. The ether extract was taken to dryness and reconstituted in 50 µl methanol; 40 µl of this was spotted on a silica gel F_{254} (250 µm thick) plate with an additional spot of hydroxyamylobarbitone as marker on each plate. The plates were developed in benzene-chloroform-ethanol-acetone (80:10:10:5, by volume) for 15 cm. The hydroxyamylobarbitone was located under u.v. light (254 nm) at R_f 0.2 and confirmed with a Packard Model 7201 radiochromatogram scanner. The corresponding bands in the extractions were scraped onto foil, shaken with 1 ml of pH 5 acetate buffer and 10 ml "Instagel" (Packard) added. The samples were counted in a Packard Scintillation counter to a constant standard deviation (1%).

In order to assess recovery of hydroxy (2-^{14}C) amylobarbitone through the extraction and tlc, a rat microsomal preparation was incubated with (2-^{14}C) amylobarbitone and the metabolite formed was extracted, separated by tlc, redissolved and aliquots counted. Six aliquots of hydroxy (2-^{14}C) amylobarbitone (2000 dpm) were then taken through the extraction and tlc separation. Recovery was 57 ± 7.5% (S.D.). Reproducibility of measurement for incubation followed by extraction and tlc separation at 0.1 nmole/extract was 5.8% (coefficient of variation) for 6 incubations at 1 mM (2-^{14}C) amylobarbitone.

Hydroxylation of amylobarbitone was linear with time to 20 minutes with male rat liver and human biopsy liver obtained at laparotomy.

(ii) Preliminary studies with rat liver biopsies

The variance of the method was assessed with a 9000 g supernatant from a 26 mg liver biopsy (obtained with a cork borer) from an adult male Wistar rat (weight 239 g). Six incubations were processed as described above, at a substrate concentration of 2 mM, and the activity was 0.172 ± 0.004 (SEM) nmoles/100 µg protein/15 min incubation, with a coefficient of variation ($\frac{S.D.}{\bar{x}} \times 100$) of 5.8%.

Portions of liver (20-25 mg) were taken with a cork borer from six adult male Wistar rats of mean weight 242 ± 11 (SEM) g

immediately after sacrifice and exsanguination. They were blotted on filter paper, weighed and placed in 0.02 M Tris-1.15% KCl buffer and then treated exactly as described above. Incubations were done at eight substrate concentrations (0.3 - 2 mM).

The results are illustrated by graphical plots of velocity (v) versus substrate concentration (s) (Figure 1) and 1/v versus 1/s (Lineweaver-Burk plot) in Figure 2. Table IV summarises the Michaelis-Menten kinetic parameters (Vmax and apparent Km) obtained using the method of least squares described by Davies, Gigon and Gillette (1968). The maximum velocity (Vmax) of the reaction for the six rat biopsies was 0.38 ± 0.06 nmoles hydroxyamylobarb/100 µg of 9000 g protein/15 min incubation. The apparent Km was 0.75 ± 0.08 mM. In Table IV the Vmax has also been expressed per mg liver per incubation, per hour incubation time, to permit comparison on a liver weight basis with the human subjects.

These values for Km are three times higher than those reported for large scale rat microsomal preparations by Sitar and Mannering (1973) and Fraser, Williams, Davies, Draffan and Davies (1976). The former used 5 ml incubations containing 5 mg of microsomal protein (1 mg/ml). The latter used 3 ml incubations with 4.5 mg of microsomal protein (1.5 mg/ml). In this study the incubation contained 100 µg 9000 g protein in 50 µl (2 mg/ml) but the microsomal protein concentration comprises only a small proportion of this. This may provide an explanation for the higher

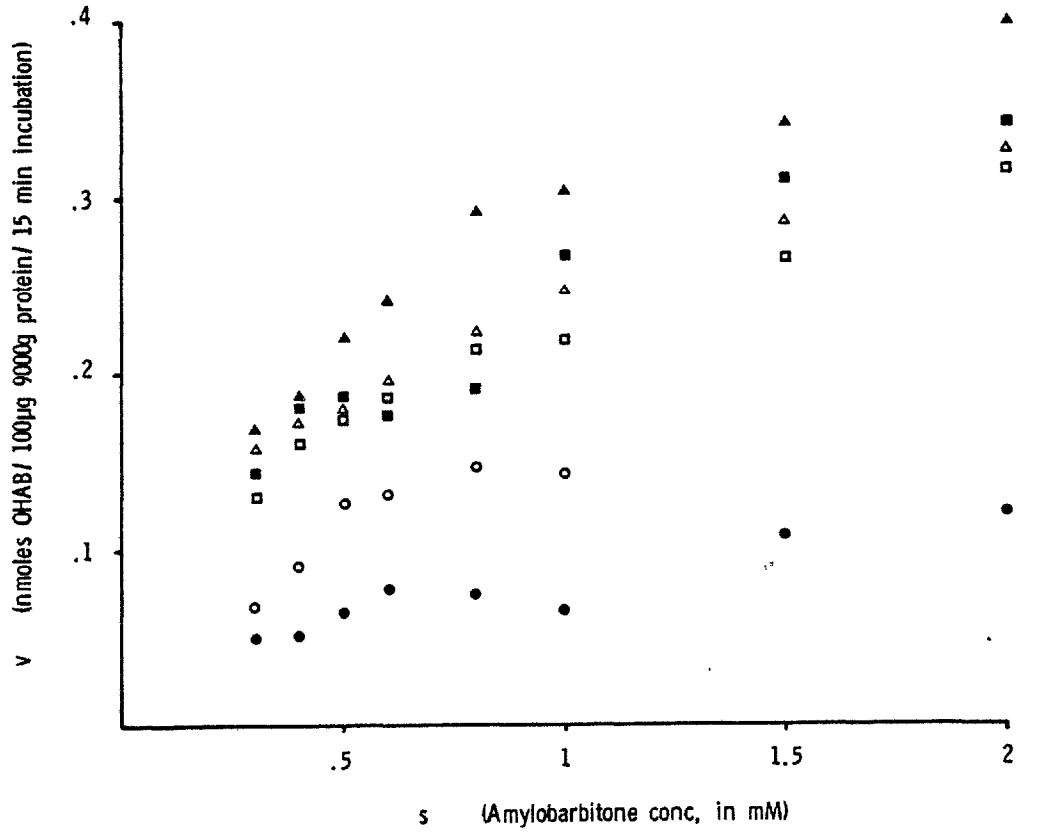


Figure 1

Kinetics of amylobarbitone hydroxylation in 20 mg biopsies from 6 rats: plot of velocity (v) versus substrate concentration(s)

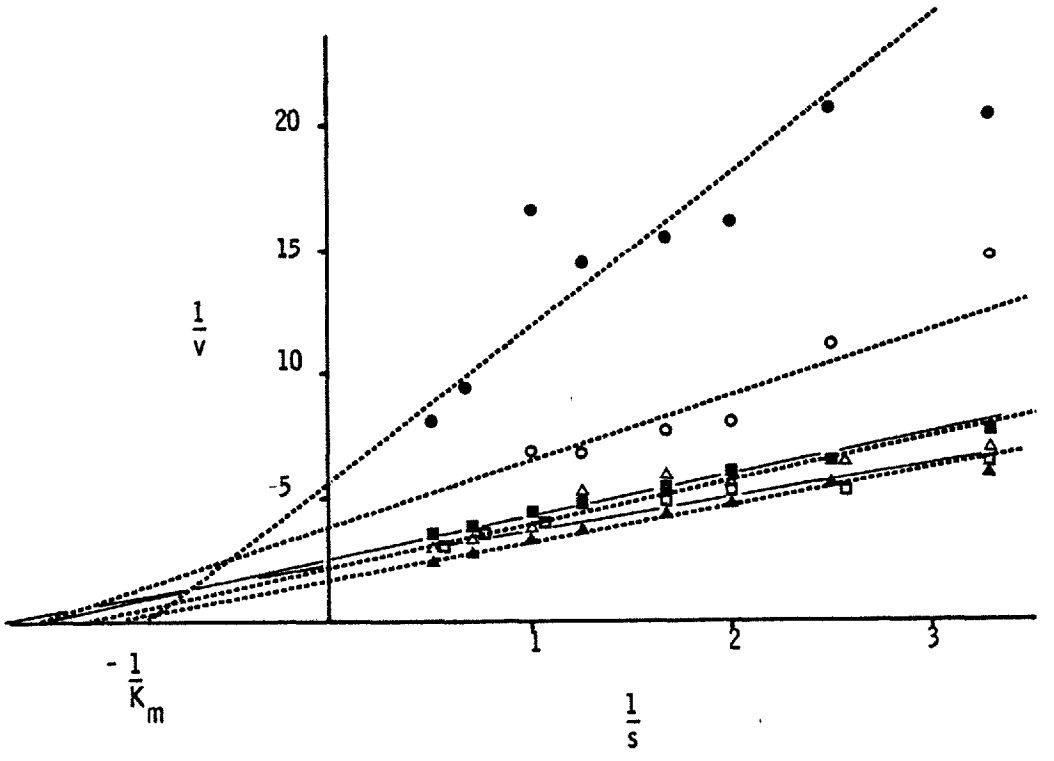


Figure 2

Kinetics of amylobarbitone hydroxylation in 20 mg biopsies from 6 rats: Lineweaver Burk plot

TABLE IV

KINETICS OF AMYLOBARBITONE HYDROXYLATION BY 9000 G SUPERNATANT FROM 20–30 MG LIVER BIOPSIES OF SIX RATS

Rat	Body Wt. (g)	Biopsy Wt. (mg)	V max (nmoles OHA /100 µg 9000G protein/15 min)	V max (nmoles OHA /mg liver/h	Km (mM)
1	210	26.0	0.18	0.43	1.08
2	220	27.0	0.55	1.31	0.76
3	268	23.5	0.48	1.13	0.85
4	276	18.0	0.25	0.77	0.64
5	245	17.5	0.40	1.260	0.55
6	231	24.5	0.40	1.18	0.65
Mean	242		0.38	1.01	0.75
SEM	±11		±0.05	±0.16	±0.08

Km values in the 9000 g preparations. The high concentration of cytosol protein relative to microsomal protein may permit a high degree of non-specific substrate binding (amylobarbitone is about 50% bound to plasma proteins). In Table V these values are compared with those for the microsomal preparations cited above. Vmax is expressed as nmoles OHAB/mg protein/hour, although they are not directly comparable because of the uncertain proportion of microsomal protein in the 9000 g supernatant.

(iii) Preliminary studies with human surgical biopsies

Wedge biopsies of liver were obtained from two patients undergoing elective cholecystectomy for gall bladder disease. Both patients had normal liver function tests at the time of laparotomy and minor non-specific changes were seen on light microscopy. The biopsies weighed 465 and 120 mg respectively after removal of a portion for histology. They were placed in 0.02 M Tris-KCl buffer on ice in the operating theatre and sub-divided within 10 minutes into 20-30 mg portions. These were weighed and processed in identical fashion to the needle biopsies.

Care was taken to obtain the sub-biopsies from the innermost portion of liver and not from the capsular surface as inclusion of capsular tissue could be a source of intra-individual variation in activity of the mixed function oxidase. This was difficult with the second, smaller biopsy.

TABLE V

COMPARISON OF AMYLOBARBITONE HYDROXYLATION KINETICS BY MICRO-INCUBATIONS OF 9000G SUPERNATANT WITH DATA OBTAINED BY MACRO-INCUBATIONS OF MICROSOMAL PREPARATIONS

PREPARATION	V MAX * (nmoles OHAB /mg protein/h)	Km (mM)
9000 G	15.2 ± 2.2	0.75 ± 0.08
Microsomal Prep. (Sitar & Mannering, 1973)	85 ± 7	0.24 ± 0.09
Microsomal prep. (Fraser, Williams, Davies, Draffan & Davies, 1973)	69 ± 13	0.23 ± 0.03

* All values are means ± SEM

The results for both patients are summarised in Table VI. The values for Vmax were 0.026 and 0.120 nmoles OHAB/100 µg 9000 g protein/15 min incubation. The first biopsy showed a two-fold range of values between the four sub-biopsies and the second biopsy showed a three-fold range. The data points for the second biopsy are shown in figures 3 and 4.

The Km values were 0.40 and 1.5 mM with no significant variation in the first set of sub-biopsies but an eight-fold range in the second set. This could be a result of unrepresentative subdivision of the wedge in the vicinity of the capsule, as the second biopsy weighed only 120 mg. This should be less of a problem with needle biopsies as the needle penetrates 3 or more cm into the liver and capsular tissues represent a very small proportion of the whole sample.

It can be seen from Table VI that if the Vmax is expressed as nmoles OHAB/100 µg 9000 g protein/15 min there is a 4.5-fold difference in activity between the first subject (mean 0.026) and the second (mean 0.120). If expressed as nmoles OHAB/mg liver/ hour, because of the different concentration of 9000 g protein (and by inference microsomal protein) between the two subjects' biopsies this inter-individual difference is reduced to three-fold.

Furthermore the biopsy with the greatest Vmax has the

KINETICS OF AMYLOBARBITONE HYDROXYLATION BY 9000 G
SUPERNATANT FROM WEDGE BIOPSIES OF HUMAN LIVER OBTAINED AT LAPAROTOMY

PATIENT	SUB-BIOPSY WT. (mg)	9000 G PROTEIN /mg LIVER	V MAX nMOLES OHAB /100 µg 9000 G PROTEIN/15 min	V MAX (nMOLES OHAB) /mg LIVER/hr	Km (mM)	V MAX/Km
1	21.5	81.4	0.028	0.092	0.46	0.20
	28	85.5	0.027	0.092	0.38	0.24
	28	86.9	0.030	0.104	0.39	0.26
	21.5	71.5	0.017	0.048	0.37	0.13
			0.026* ±	0.084 ±	0.40 ±	0.21 ±
2	27	51.0	0.206	0.420	3.3	0.128
	29	70.9	0.080	0.227	0.8	0.284
	23	73.9	0.070	0.207	0.4	0.517
			0.120 ±	0.255 ±	1.5 ±	0.310 ±

* All values are Means ± SEM

TABLE VI

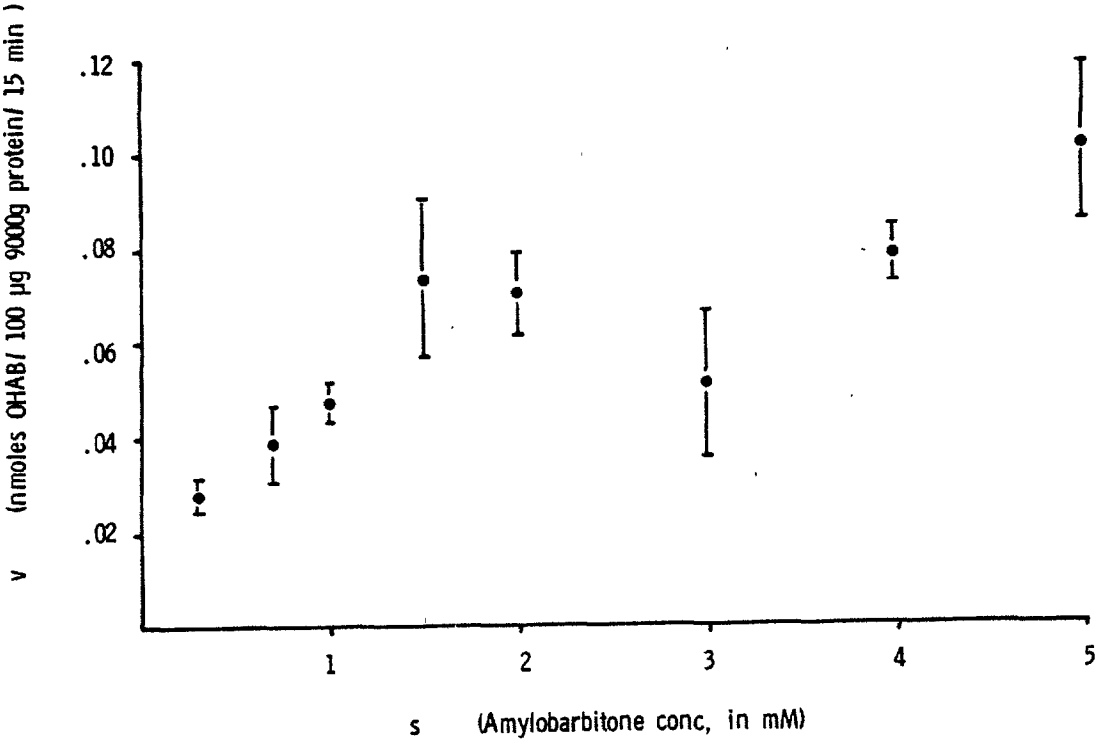


Figure 3

Kinetics of amylobarbitone hydroxylation in a wedge biopsy of human liver (n = 3 sub-sample of 23, 27 and 29 mg) Mean and S.D.

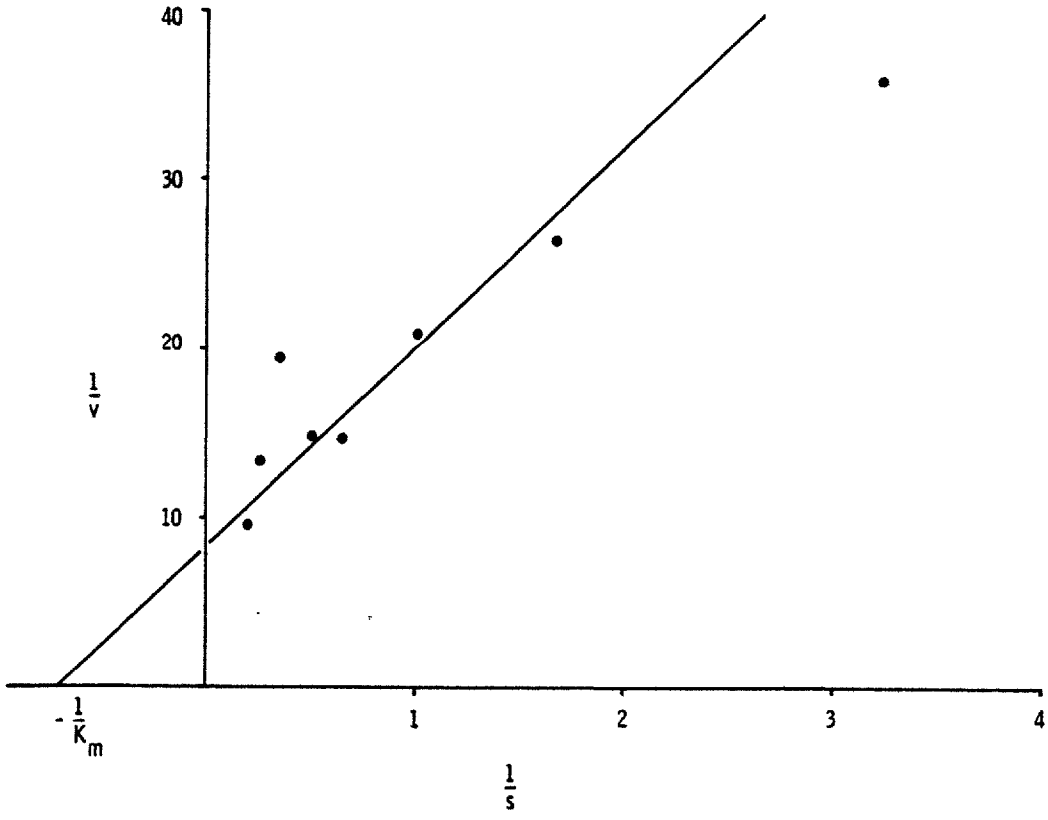


Figure 4

Kinetics of amylobarbitone hydroxylation in a wedge biopsy of human liver: Lineweaver-Burk plot. Each point is mean of 3 data points

highest Km, and by expressing the activity as Vmax/Km, the difference between the two biopsies is shown to be further reduced to 1.5-fold (last column, Table VI). The importance of expressing activity in terms of both Vmax and Km is stressed by Gillette (1971) and Adamson and Davies (1973) because of the low substrate concentrations usually found in vivo. Since:

$$v = \frac{Vmax \times s}{s + Km}$$

where v = velocity and s = substrate (drug) concentration, when the substrate concentration is much lower than Km or $s \ll Km$

$$v = \frac{Vmax \times s}{Km}$$

i.e. the velocity of the reaction is directly proportional to Vmax and inversely proportional to Km. Thus a two-fold increase in Vmax associated with a two-fold increase in Km would result in no change of velocity. When Vmax is calculated as the total amount of drug metabolised by the liver per unit time, the ratio Vmax/Km is proportional to the first-order elimination rate constant for metabolism in vivo (Gillette, 1971) and has been termed the intrinsic clearance of the drug (Wilkinson and Shand, 1975).

(iv) Benzo(a)pyrene hydroxylase

Benzo(a)pyrene hydroxylase was assayed in approximately 5 mg portions of needle biopsies from 7 of the patients (4 patients biopsied twice). The liver sample was stored frozen in 2 ml of 0.25 M sucrose buffered in 3 mM Imidazole (pH 7.2) until analysis. They were assayed by the method of Hayakawa and Udenfriend, using generally tritiated benzo(a)pyrene as substrate and measuring the tritiated water liberated after oxidation and rearrangement of benzo(a)pyrene oxide to the phenol. The assays were performed by Mr. John Tilleray of the Department of Cell Biology, at the Royal Postgraduate Medical School.

c) RESULTS OF NEEDLE BIOPSY STUDIES

(i) Amylobarbitone hydroxylase activity

The biopsy results of each subject are illustrated in figures 5-17. Activity of amylobarbitone hydroxylase is plotted as v versus s, where v = the velocity of the oxidation reaction in nmoles ^{14}C-hydroxyamylobarbitone (OHAB) formed/incubation of 100 µg 9000 g protein in 15 minutes, and s = substrate concentration in mM. Michaelis-Menton analysis was performed using the method of least squares (Davies, Gigon and Gillette, 1968). The estimates of maximum velocity (Vmax) and apparent Michaelis constant (Km) are

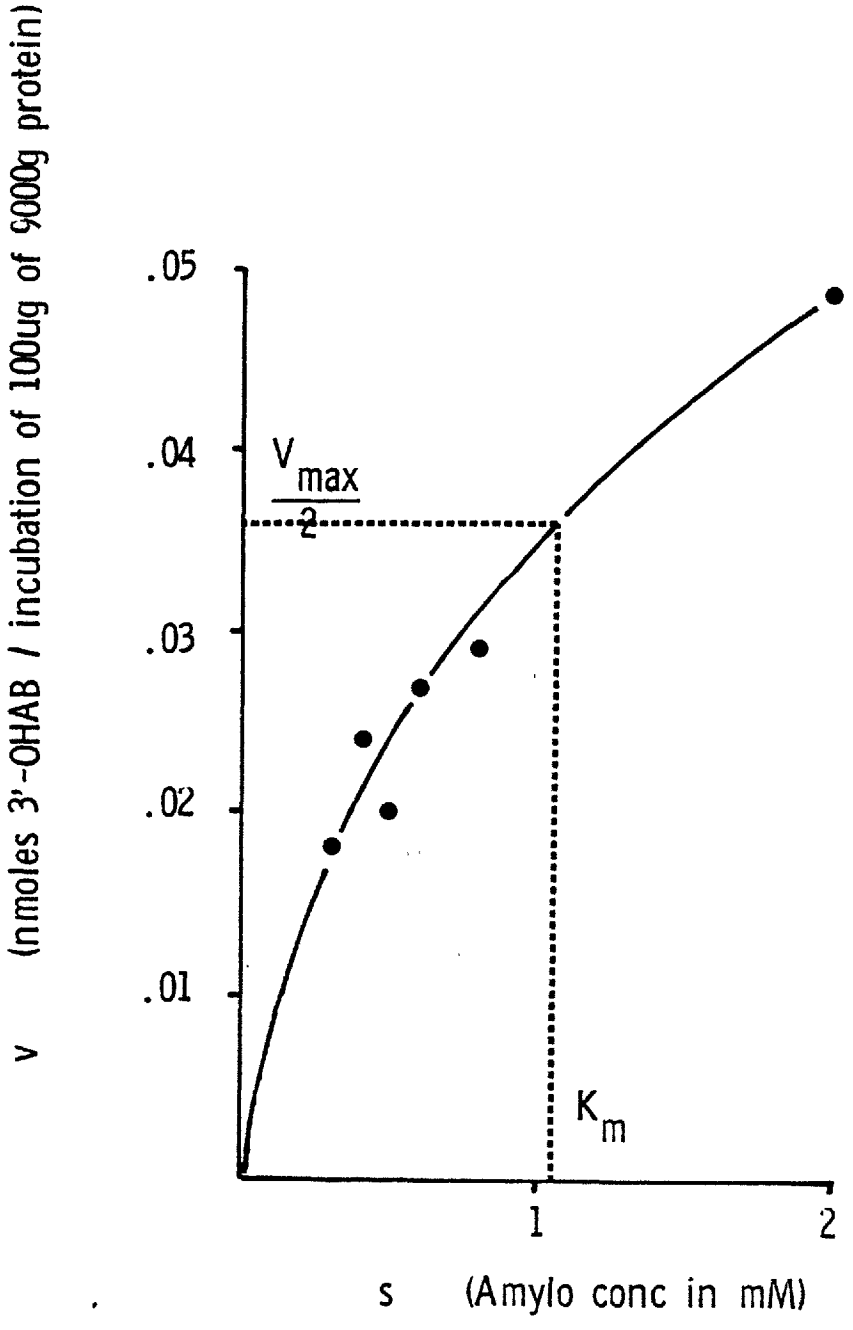


Figure 5

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsy of P.M.

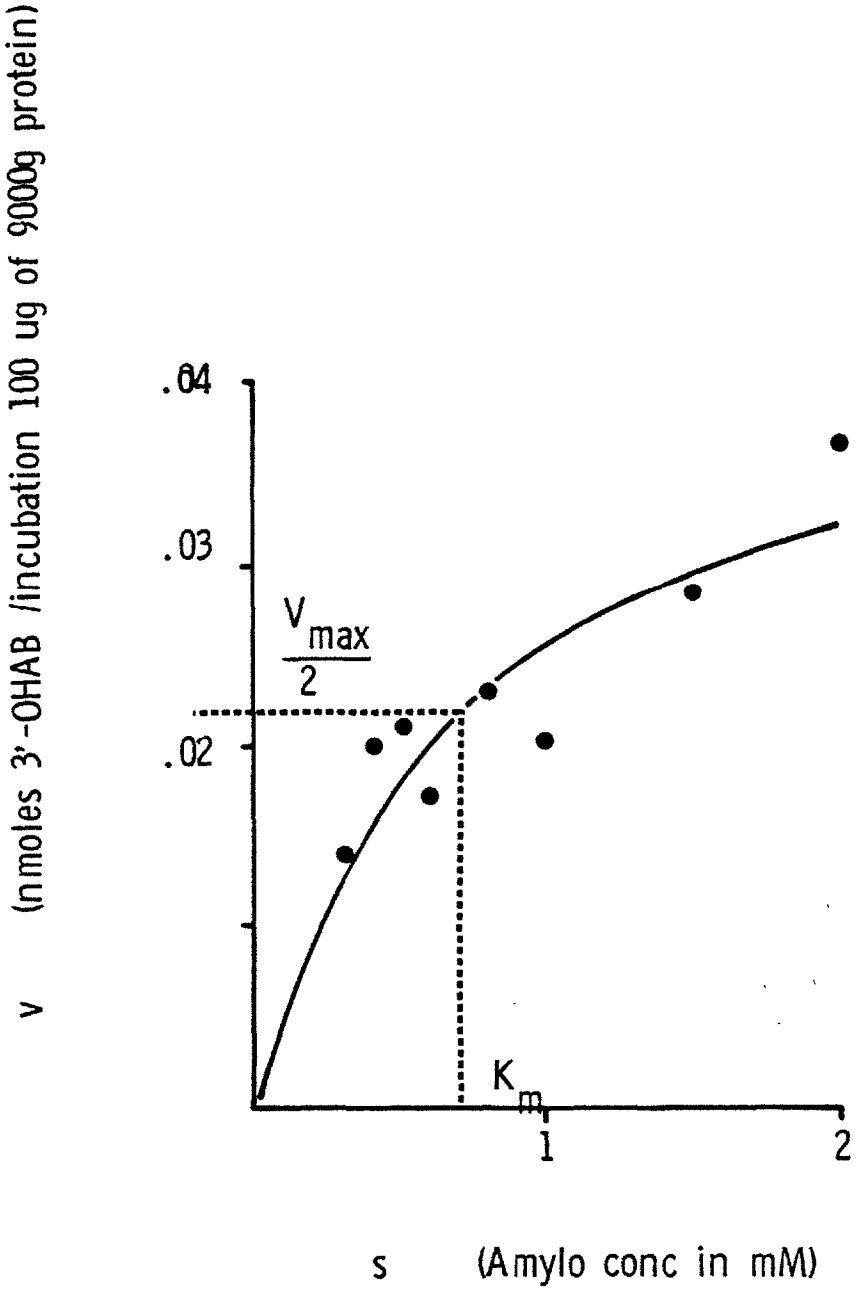


Figure 6

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsy of S.W.

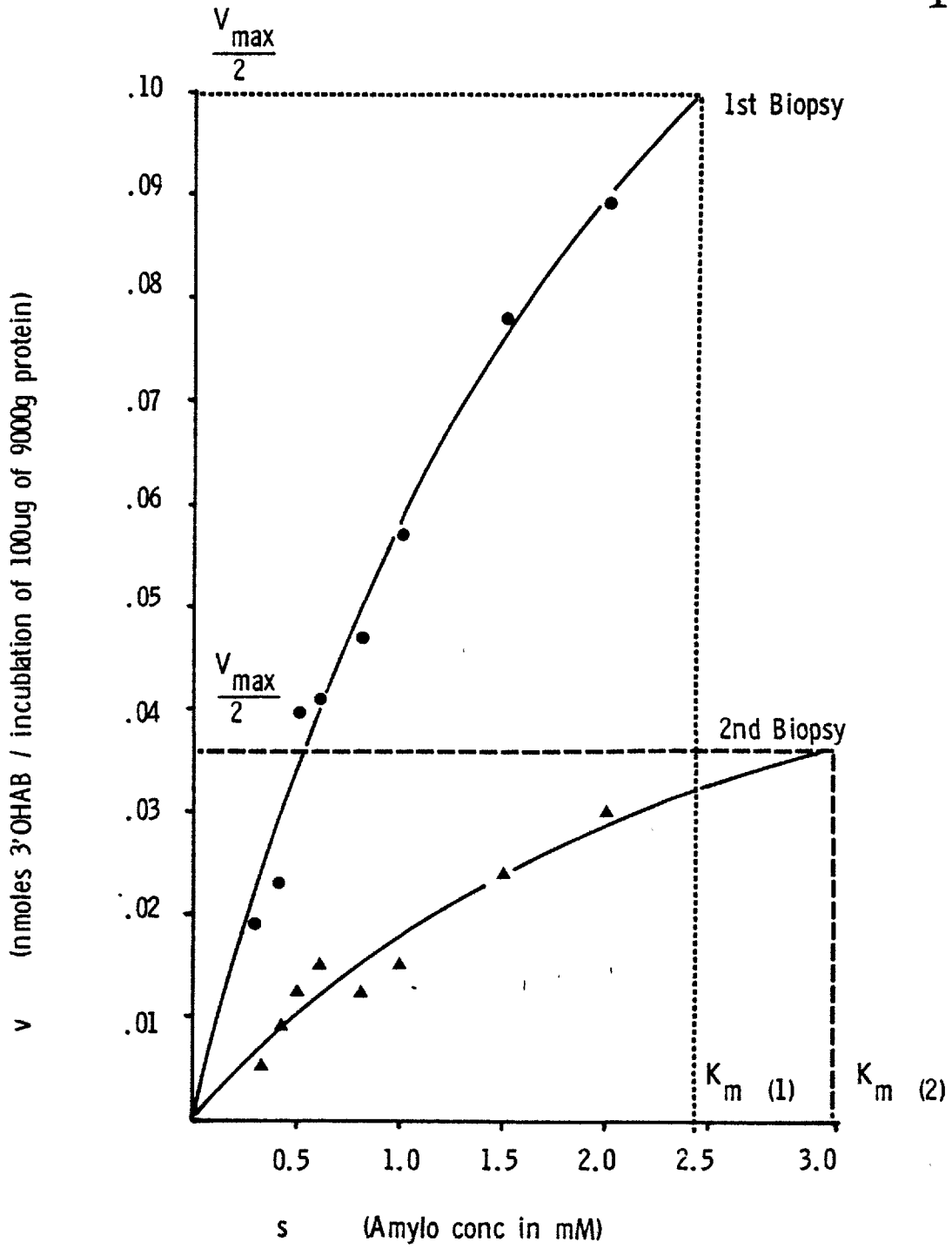


Figure 7

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of D.M., four weeks apart

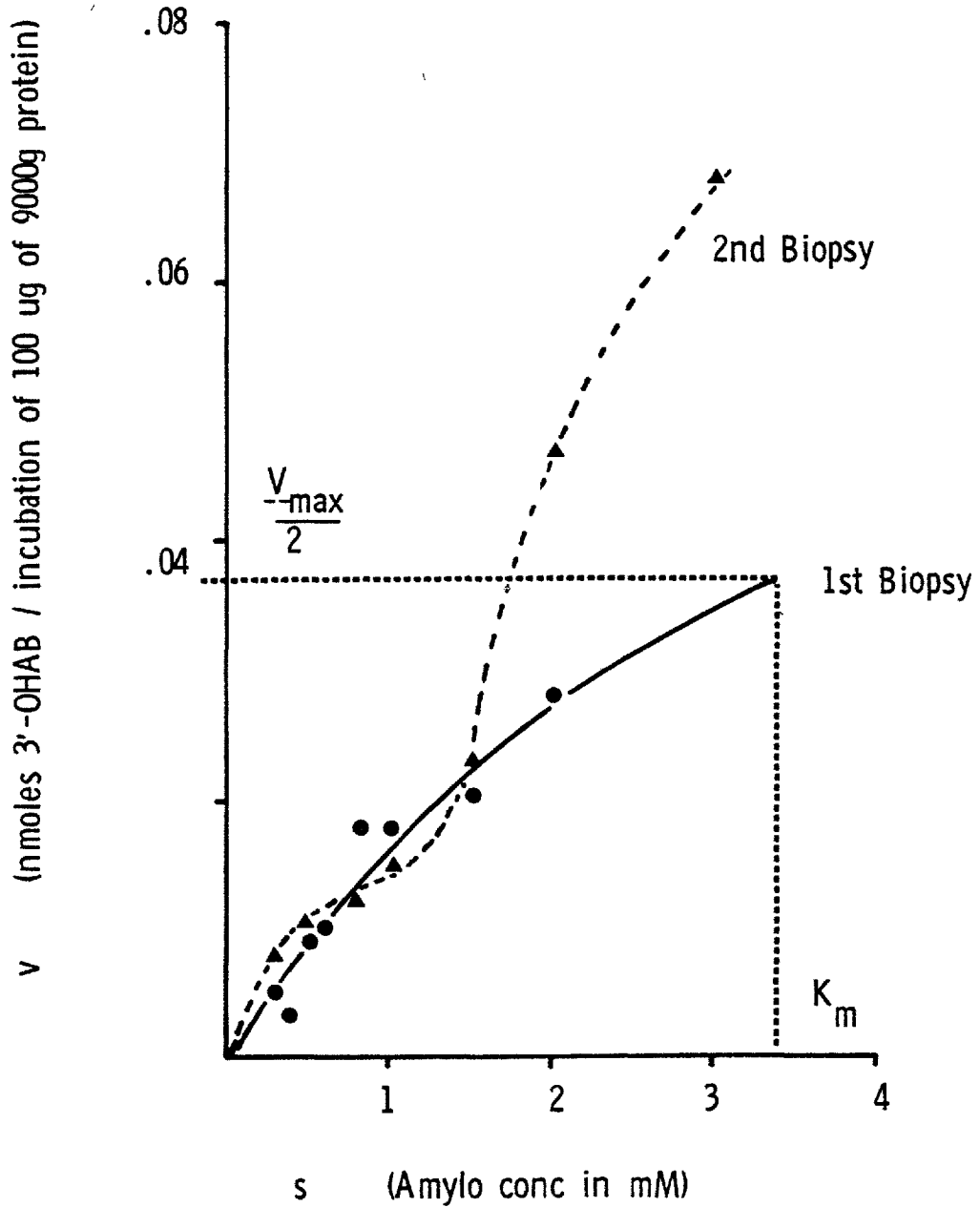


Figure 8

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of R.B., four weeks apart

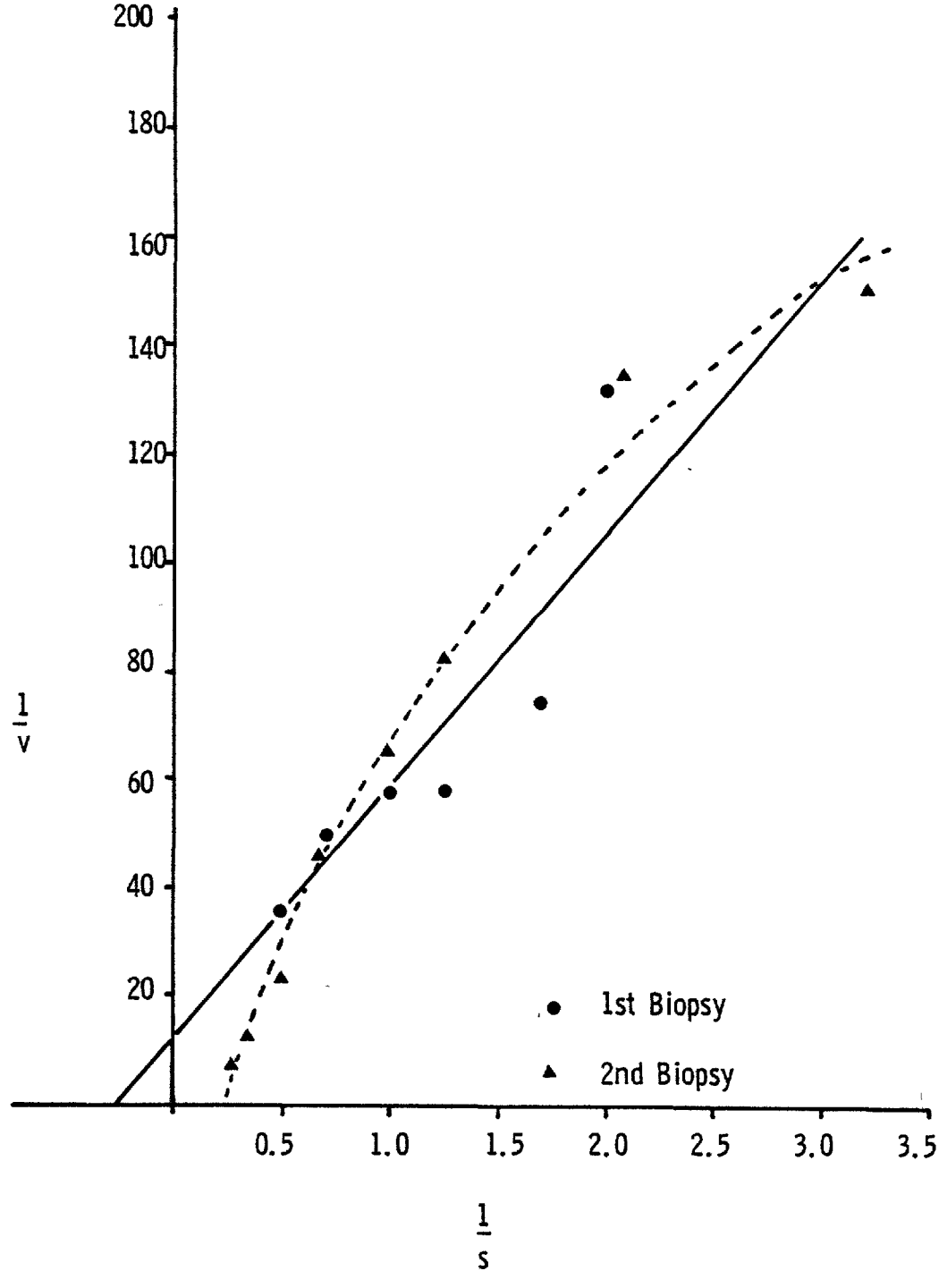


Figure 9

Lineweaver-Burk plots of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of R.B.

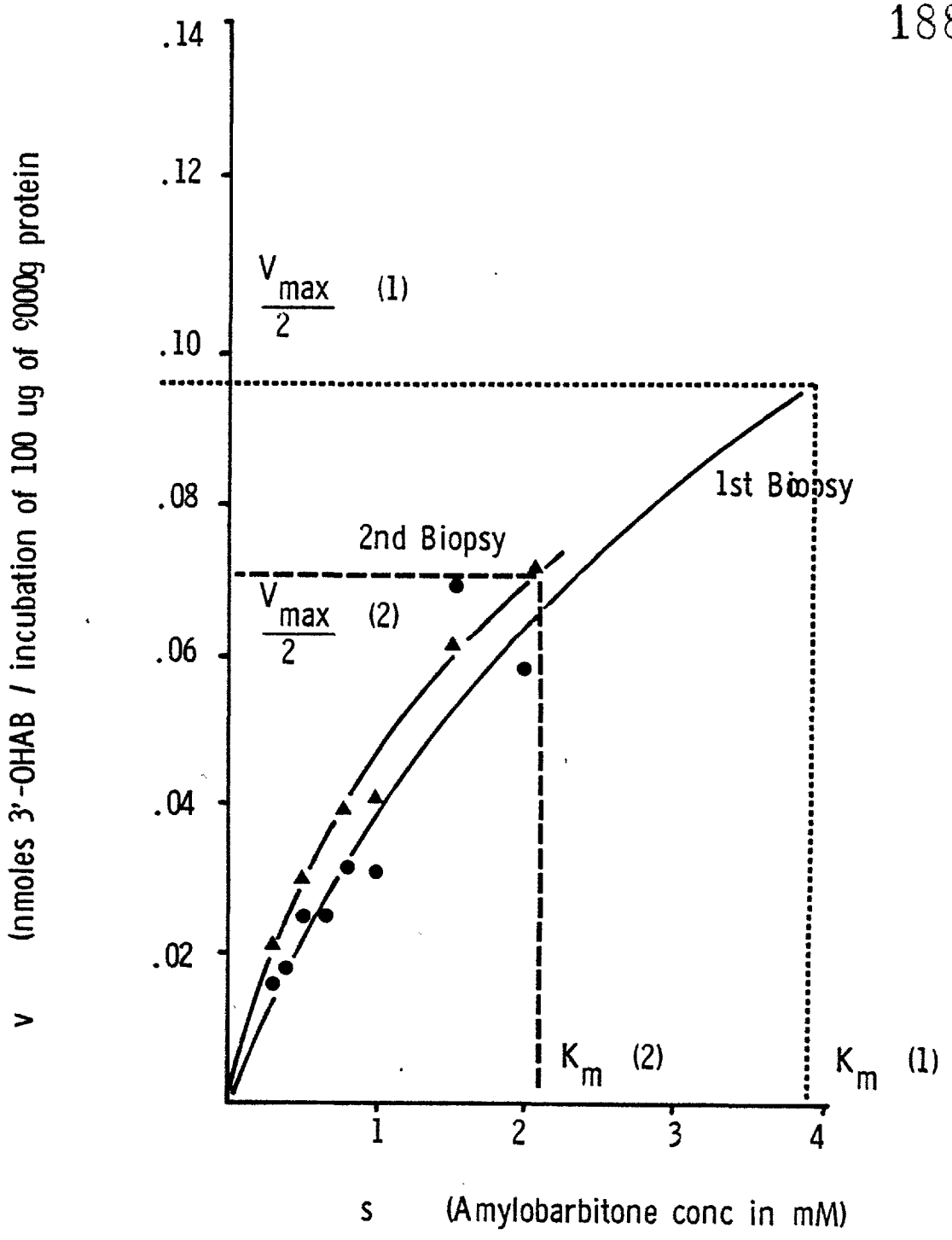


Figure 10

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of H.N., four weeks apart

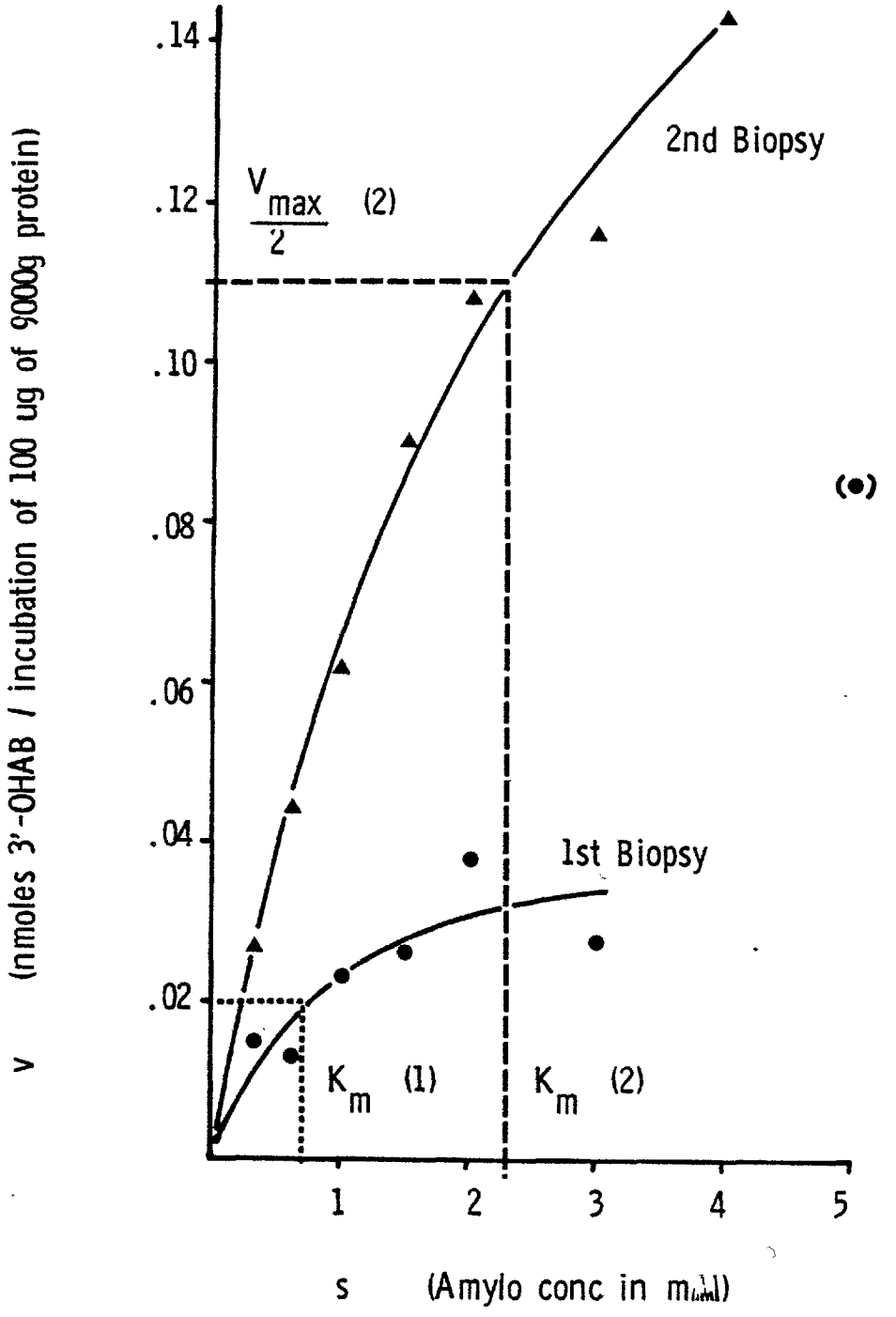


Figure 11

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of T.G., four weeks apart

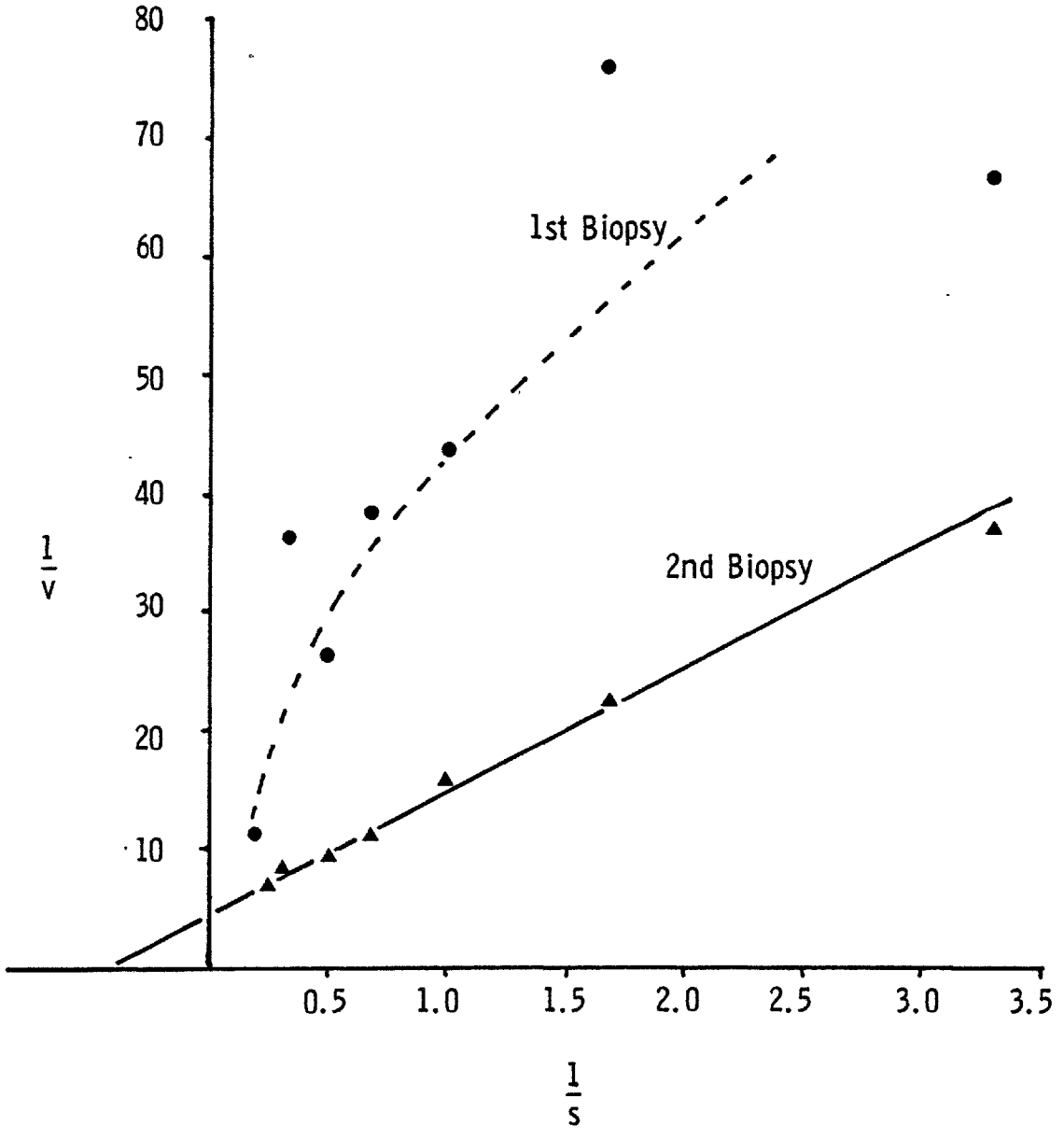


Figure 12

Lineweaver-Burk plot of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of T.G.

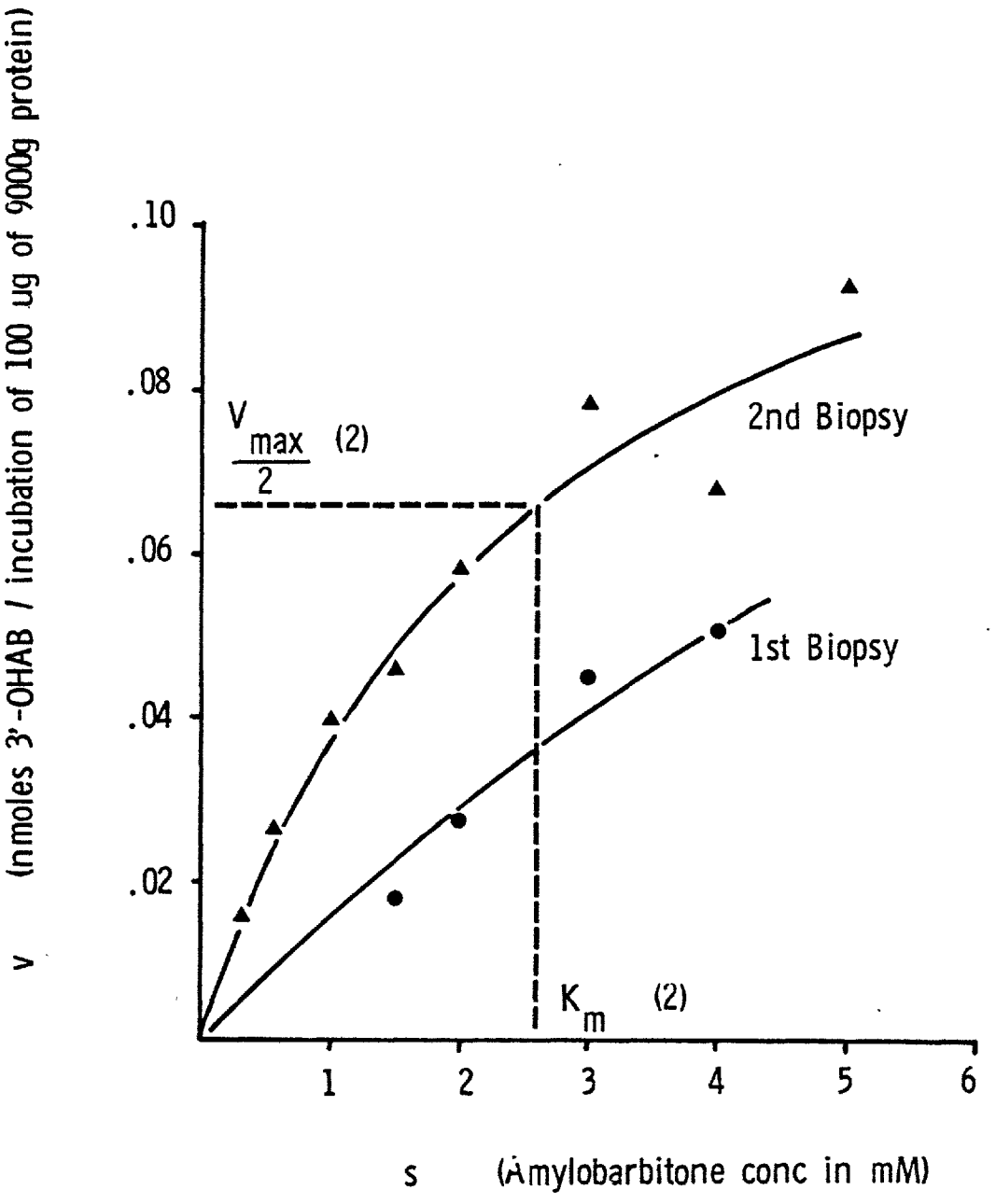


Figure 13

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of E.P., four weeks apart

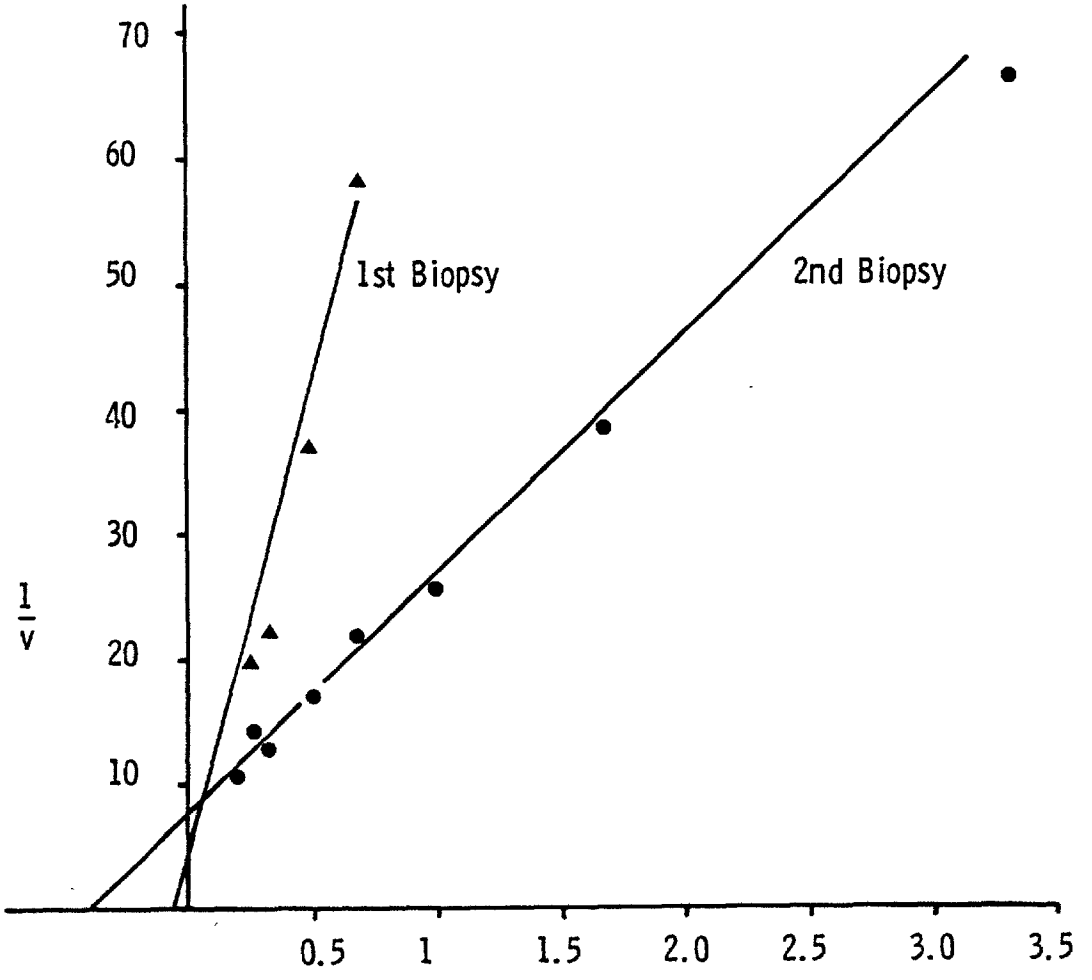


Figure 14

Lineweaver-Burk plot of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsies of E.P.

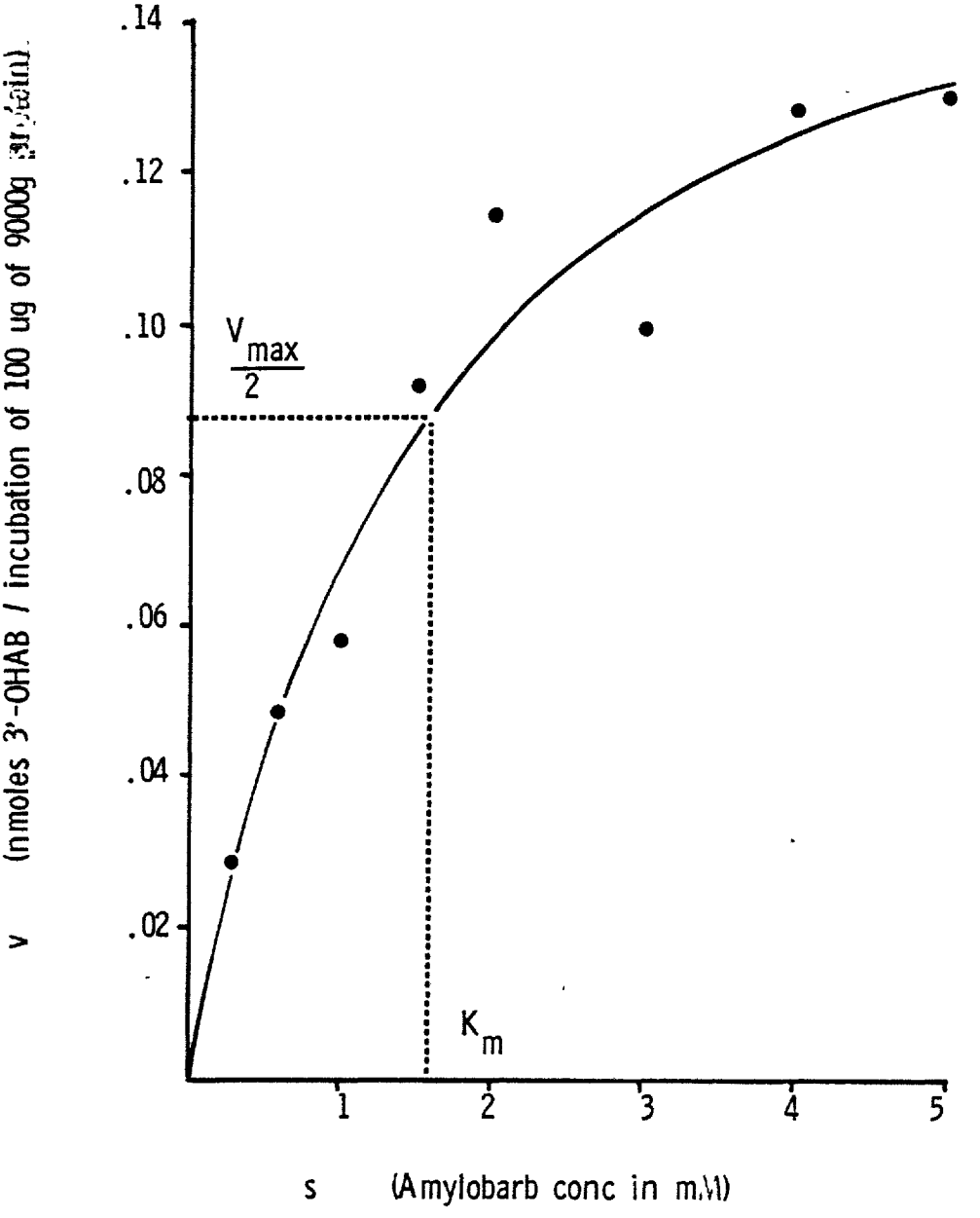


Figure 15

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsy of A.D.

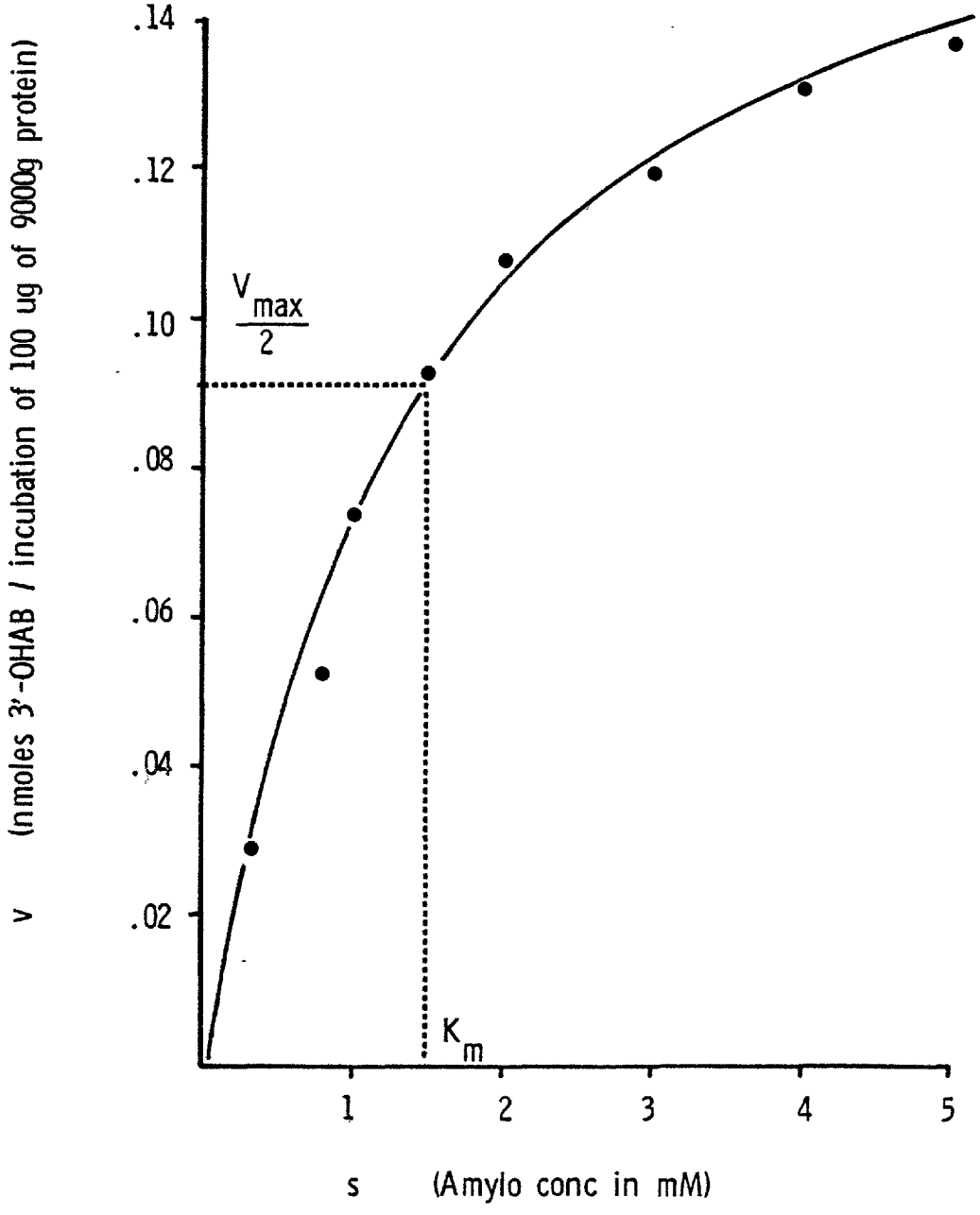


Figure 16

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsy of M.W.

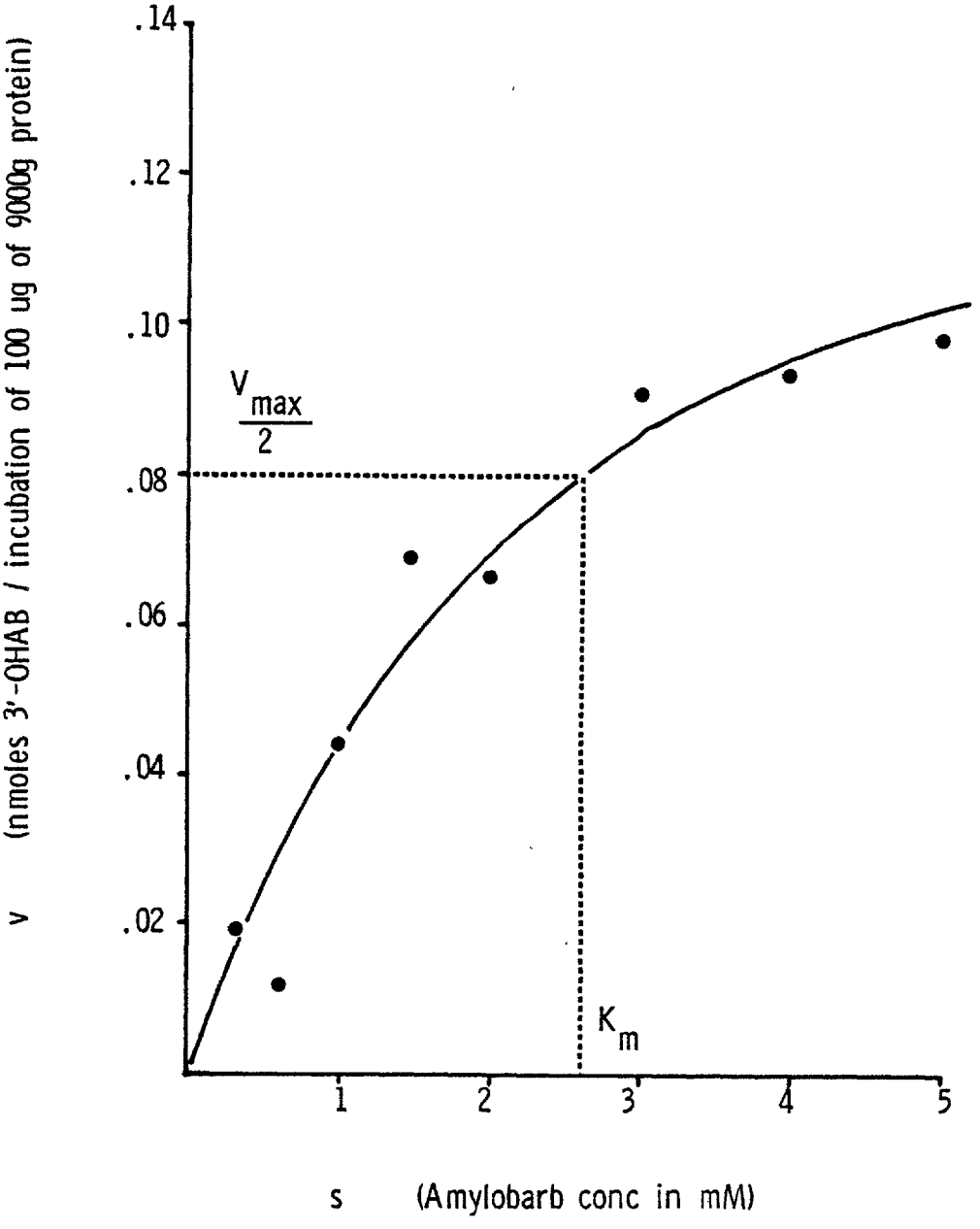


Figure 17

Kinetics of amylobarbitone hydroxylation in 9000 g supernatant of liver biopsy of C.M.

indicated on the v versus s plots and "best fit hyperbolic" curves are drawn by substituting for Vmax and Km in the Michaelis Menten equation $v = \frac{Vmax.s}{Km + s}$. Linear plots (Lineweaver-Burk) are illustrated for two subjects, R.B. and T.G., one each of whose biopsies showed anomalies.

For all biopsy samples Vmax was 0.14 ± 0.02 (SEM) nmoles OHAB/100 µg 9000 g protein/15 min and Km 2.2 ± 0.3 mM. Individual values are listed in Table VII with the standard errors of the estimates. Amylobarbitone hydroxylation by the 9000 g preparations of seven subjects fitted Michaelis-Menton-Kinetics closely with small standard errors for determination of Vmax (i.e. subjects P.M., S.W., D.M. (both biopsies) H.N. (both biopsies), A.D., M.W. and C.M.). In one subject, P.M., the repeat biopsy was small and because of the limited tissue available for the assay (10 mg) only five incubations were done, with 50 mg of 9000 g protein instead of 100 µg/incubation, i.e. half the protein concentration used with the other biopsies. Readings were less than twice blank, i.e. not high enough above blank readings to provide reliable estimates of Vmax and Km.

In the preparations from both biopsies of another subject, F.B., readings were less than twice blank readings even at the highest substrate concentrations. This subject had an amylobarbitone clearance of 0.24 and 0.72 ml/min/Kg at the first and second study respectively. His urine collections were incomplete on both occasions

TABLE VII

V MAX AND KM VALUES FOR 9000 G SUPERNATANT PREPARATIONS FROM ALL PATIENTS BIOPSIES

	Patient		V max (nmoles OHAB /100 µg/9000 G protein/15 min	Km (mM)
1.	P.M.		0.073 ± 0.011*	1.05 ± 0.30
2.	F.B.		-	-
3.	S.W.		0.044 ± 0.008	0.71 ± 0.31
4.	D.M.	(a)	0.199 ± 0.033	2.44 ± 0.62
		(b)	0.073 ± 0.029	3.11 ± 1.76
5.	R.B.	(a)	0.075 ± 0.040	3.43 ± 2.55
		(b)	Not measurable	Not measurable
6.	H.N.	(a)	0.193 ± 0.143	3.90 ± 3.87
		(b)	0.142 ± 0.030	2.14 ± 0.73
7.	T.G.	(a)	0.040 ± 0.010	0.72 ± 0.54
		(b)	0.220 ± 0.020	2.30 ± 0.42
8.	E.P.	(a)	0.307+ ± 0.900	49.00+ ± 196
		(b)	0.132 ± 0.021	2.58 ± 0.86
9.	A.D.		0.174 ± 0.020	1.52 ± 0.44
10.	M.W.		0.182 ± 0.005	1.51 ± 0.11
11.	C.M.		0.155 ± 0.029	2.57 ± 0.99
			0.144 ± 0.020‡	2.15 ± 0.28‡

* Standard error of estimate

\+ Omitted in calculation of means and SEM because of inaccuracy of prediction of V max and Km

‡ Mean & SEM

but there was a significant amount of side chain-hydroxyamylobaritone present.

Three patients' biopsies showed significant deviations from Michaelis-Menton kinetics. All three had been studied twice.

R.B. (Figure 8)

Data from the first biopsy gives a moderately good fit to the Michaelis Menton equation but with the second biopsy there is a definite inflexion between substrate concentrations of 1 and 2 mM which does not permit Vmax and Km to be calculated. This can be clearly seen in the Lineweaver-Burk plot of 1/v versus 1/s (Figure 9) where activity curves downward at the higher concentrations.

T.G. (Figure 11)

Similarly in the data from the first biopsy of T.G., the 0.3-3 mM data points provide a fair fit, but the 5 mM point shows an activity of a different order, i.e. three times higher than the 2 and 3 mM points. Again this is shown in the Lineweaver-Burk plot as a curve downward at the higher concentrations. Data from the second biopsy however, provides a perfect fit.

E.P. (Figure 13)

Data from the second biopsy provides a good fit but the first biopsy shows a very small and almost linear increase with substrate concentration. Unfortunately the few data points cover such a narrow range compared to the large predicted Vmax and apparent Km, and intrinsic activity is so low that these estimations must be much less accurate than those of the second biopsy.

It can be seen from Table VII that there was wide inter-individual variation in Vmax and in apparent Km (six to seven fold) among our subjects. There were also considerable differences in Vmax in some subjects between first and second biopsies. Table VIII lists the individual values of another variable, 9000 g protein yield per mg of liver tissue. This covered a two-fold range, from 27.4, 37.5 and 33.3, and 35 and 44.1 µg/mg liver for the patients with cirrhosis to 62.2 µg/mg liver. The latter value was associated with the highest activity of all. In order to take protein content into account Vmax was therefore expressed as nmoles OHAB per mg liver/hour (Table IX). As discussed in section V (b) activity of amylobarbitone hydroxylase was expressed as Vmax/Km and these values are also shown in Table IX. The importance of assessing activity in this way is illustrated by considering the biopsies of H.N. (Figure 10). The first biopsy had a 45% higher Vmax (0.37 nmoles OHAB/mg/hr) than the second biopsy (0.27 nmoles OHAB/mg liver/ hour), and an estimate of activity at supra-maximal substrate concentration would suggest that oxidising activity of the first biopsy was greater. However, the Km of the first biopsy was almost twice as high as that of the second and therefore the intrinsic activity of the first biopsy was lower. This would be of importance in vivo where drug concentrations are much lower than the Km.

(ii) Benzo(a)pyrene hydroxylase activity

Table X lists the benzo(a)pyrene hydroxylase activities of 11 biopsy samples, at a single substrate concentration of 0.1 µmole

TABLE VIII

9000 G PROTEIN CONTENT OF PATIENTS' BIOPSIES

	PATIENT	9000 G PROTEIN/MG LIVER (µg)	HISTOLOGY
1.	P.M.	48.3 44.0	Non-Specific
2.	F.B.	42.0 35.0	Severe Steatosis
3.	S.W.	49.1	Non-Specific
4.	D.M.	44.1 35.0	Steatosis, Hepatitis & Cirrhosis
5.	R.B.	37.0 33.0	Steatosis, Hepatitis & Cirrhosis
6.	H.N.	48.1 47.0	Steatosis
7.	T.G.	43.1 43.2	Non-Specific
8.	E.P.	57.2 53.0	Steatosis
9.	A.D.	41.5	Steatosis
10.	M.W.	62.5	Steatosis & Hepatitis
11.	C.M.	27.4	Steatosis, Hepatitis & Cirrhosis
	MEAN ± SEM	43.9 ± 2.0	

TABLE IX

VMAX, KM AND INTRINSIC ACTIVITY OF
9000 G SUPERNATANT OF ALL PATIENTS BIOPSIES

PATIENT	VMAX (nMOLES OHAB /100 µG 9000 G PROTEIN/15 MIN (n = 13)	VMAX (nMOLES OHAB /MG LIVER/HOUR (n = 13)	KM (mM)	INTRINSIC ACTIVITY (VMAX/KM; nMOLES OHAB/MG LIVER/ HR/mM)	% CHANGE IN VMAX/ KM
1. P.M.	0.073	0.140	1.05	0.133	
2. F.B.	-	-	-	-	
3. S.W.	0.044	0.086	0.71	0.121	
4. D.M. a)	0.199	0.351	2.44	0.144	
b)	0.073	0.102	3.11	0.033	- 77%
5. R.B. a)	0.075	0.113	3.43	0.033	
b)	not measurable		not measurable		
6. H.N. a)	0.193	0.371	3.90	0.095	
b)	0.142	0.267	2.14	0.125	+ 32%
7. T.G. a)	0.040	0.069	0.72	0.096	
b)	0.220	0.380	2.30	0.165	+ 72%
8. E.P. a)	0.307*	0.703	49.00*	0.029*	
b)	0.132	0.280	2.58	0.109	+267%
9. A.D.	0.174	0.280	1.52	0.185	
10. M.W.	0.182	0.453	1.51	0.300	
11. C.M.	0.155	0.170	2.57	0.066	
	0.144±0.020+	0.269±0.047+	2.15±0.28+	0,117±0.019+	

* Omitted in calculation of means and SEM

\+ Mean & SEM

TABLE X

BENZPYRENE HYDROXYLASE ACTIVITIES OF MICROSOMAL PREPARATIONS FROM 11 BIOPSIES OF SEVEN PATIENTS

			Benzpyrene hydroxylase activity (nmoles/mg protein/min)
1.	P.M.	(a)	0.321
4.	D.M.	(a)	0.392
		(b)	0.429
5.	R.B.	(a)	0.563
6.	H.N.	(a)	0.427
		(b)	1.286
7.	T.G.	(a)	0.582
		(b)	0.520
8.	E.P.	(a)	0.345
		(b)	0.904
9.	A.D.		0.551
		Mean	0.575
		SEM	0.086

tritiated benzo(a)pyrene. The mean activity was 0.58 ± 0.09 (SEM) nmoles/mg protein/min with a four-fold range.

d) DISCUSSION

(i) Amylobarbitone hydroxylase activity

The data presented here represents the first known study of apparent kinetic constants for metabolism of any drug by human liver needle biopsy preparations. The metabolism of amylobarbitone to 3'-hydroxyamylobarbitone has been followed by measuring product formation at eight substrate concentrations, using a 9000 g preparation from 20 mg of liver. There is no published kinetic data for amylobarbitone metabolism in man, but comparison with studies in the rat suggests that amylobarbitone, like many other drugs, is more slowly metabolised in man than in other species. Thus, Sitar and Mannering (1973) obtained a value for Vmax in the rat of 85 nmol/mg microsomal protein/hour or about 4 nmol/mg liver, which is ten times as great as the mean value in this study. This is consistent with the relative metabolising rates of other barbiturates. For example *in vitro* metabolism of pentobarbitone by whole liver homogenate is five times greater in the male rat than in man (Kuntzman, et al., 1966), while the half-life of pentobarbitone is ten times as long in man as in the male rat (Thorgeirsson, 1971; Breckenridge, et al., 1971).

The apparent Km values for human 9000 g preparations were of the same order as those obtained with rat 9000 g preparations although the mean value was more than twice that for the rat. Lower values were obtained with rat liver microsomes (Fraser, Williams, Draffan and Davies, 1976). Rubin, Tephly and Mannering (1964) also observed a much higher Km (five-fold) for hexobarbital metabolism with rat 9000 g supernatant than with the microsomal fraction (Sitar and Mannering, 1973). This may result from non-specific binding of amylobarbitone to cytosol protein in the 9000 g supernatant.

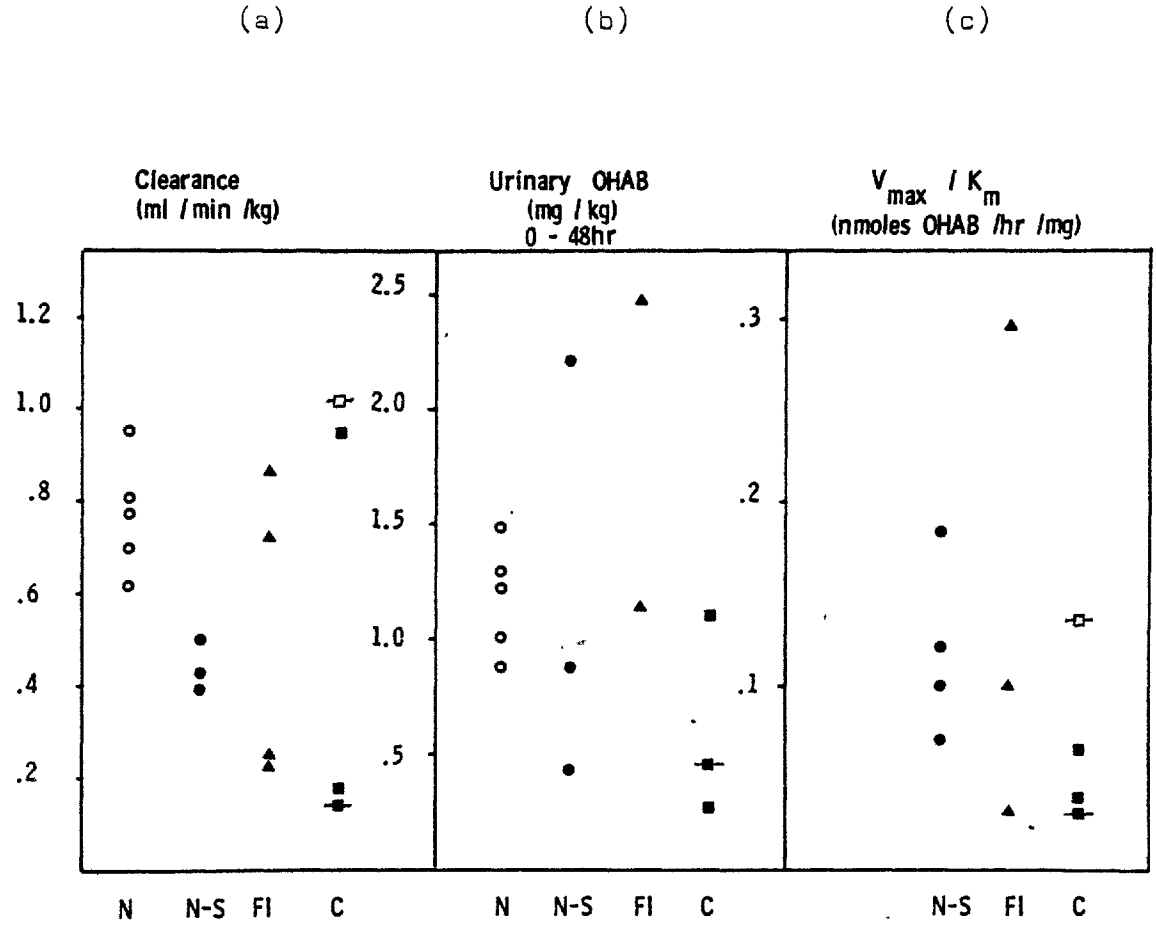
Although there is no comparable published data in man, work with dog and human liver microsomes is currently in progress in Kalow's laboratory in Toronto (Currie, et al., unpublished data). Apparent Km and Vmax for microsomal preparations from two human surgical liver biopsies were in the same range as those described here. The values for two surgical biopsies examined in this thesis were also in the same range as those for the needle biopsies, which showed a ten-fold variation in Vmax and Vmax/Km.

Despite the wide range of amylobarbitone hydroxylation capacity in vitro of the patients studied there was no clear cut relationship with liver damage. At the outset of the study it was anticipated that patients with liver damage would act as their own controls after a period of hospital in-patient treatment, with alcohol withdrawal, vitamin supplementation and a full diet. Such

comparative data could provide valuable information if there were correlations between liver damage and drug oxidation. The patients who consented to the study were a heterogeneous group, ranging from those with no specific histological changes (S.W.) to three with cirrhosis. Figure 18(c) shows that if patients were classified according to histological features (non-specific change, fatty infiltration or cirrhosis) there was no clear relationship with amylobarbitone hydroxylation activity (Vmax/Km). There was a trend for the patients with cirrhosis to have lower activity but numbers were too small for conclusions to be drawn. Table X also shows that of four patients whose biopsies were repeated three showed an increase in activity (32%, 72% and 267%) while one showed a decrease (-77%).

Correlation of clinical function with *in vitro* and *in vivo* measurements of metabolism will be considered in more detail in Chapter VI (c).

While the data from most of the biopsies fitted Michaelis-Menten Kinetics, there were several which showed deviations. Both preparations from the biopsies of one subject, F.B., had no apparent activity, although the urine contained significant amounts of side-chain hydroxyamylobarbitone. This suggests that the absence of activity in his preparation was an artefact of the technique rather than a defect in the microsomal enzymes. However, the accompanying preparation of rat 9000 g had normal activity. A more likely explanation may be that another constituent of the 9000 g preparation



-□- = D.M. on admission

-■- = D.M. four weeks later, after development of cirrhosis

Figure 18

Relationship between clearance, urinary OHAB and V_{max}/K_m and histology of liver

(N = Normals, N-S = Non-specific changes, FI = Fatty infiltration, C = Cirrhosis)

may compete with amylobarbitone for receptor sites. F.B.'s biopsy showed the most marked fatty infiltration of all subjects and it has been reported that the fatty layer on the top of a 9000 g rat liver supernatant is a potent inhibitor of microsomal oxidation of diphenylhydantoin (Kutt and Venebely, 1970; Gabler and Hubbard, 1972). With a 9000 g supernatant of less than 200 µl, the fatty layer is not sufficiently clearly seen to make separation feasible without loss of a considerable proportion of the supernatant.

There is no obvious explanation for the anomalies seen in the kinetics of three subjects' 9000 g preparations (R.B., T.G. and E.P.).

It may be concluded that despite the complexity of the cytochrome P-450 system, Michaelis-Menten analysis can be used to study the kinetics of drug oxidation in man. Furthermore, the small sample of tissue (20 mg) obtained from a needle biopsy is adequate for such studies. It is therefore possible to assess both *in vitro* and *in vivo* metabolising capacity in the same subjects, if a liver needle biopsy is indicated on clinical and ethical grounds. There are no reported studies in man of *in vitro* and *in vivo* metabolising capacity for the same drug. In Chapter VI the *in vivo* estimations of amylobarbitone side-chain hydroxylation are described and compared with the enzyme activity *in vitro* for the same subject.

(ii) Benzo(a)pyrene hydroxylase activity

Figure 19 illustrates the relationship between benzo(a)pyrene hydroxylase activity and amylobarbitone hydroxylase activity. Bearing in mind the limitations of the measurement of benzpyrene hydroxylase activity at a single concentration in contrast to measuring Vmax and Km, it can be seen that although there is a positive correlation ($r = \pm 0.17$) it does not reach a significant level. This result is perhaps not surprising in view of the body of evidence indicating that 3, 4 benzo(a)pyrene is metabolised by cytochrome P-448, a distinct haemoprotein from P-450, both in spectral properties, the substrates which induce it and the substrates it metabolises (Lu, Kuntzman, West, Jacobson and Conney, 1972; Estabrook, 1971).

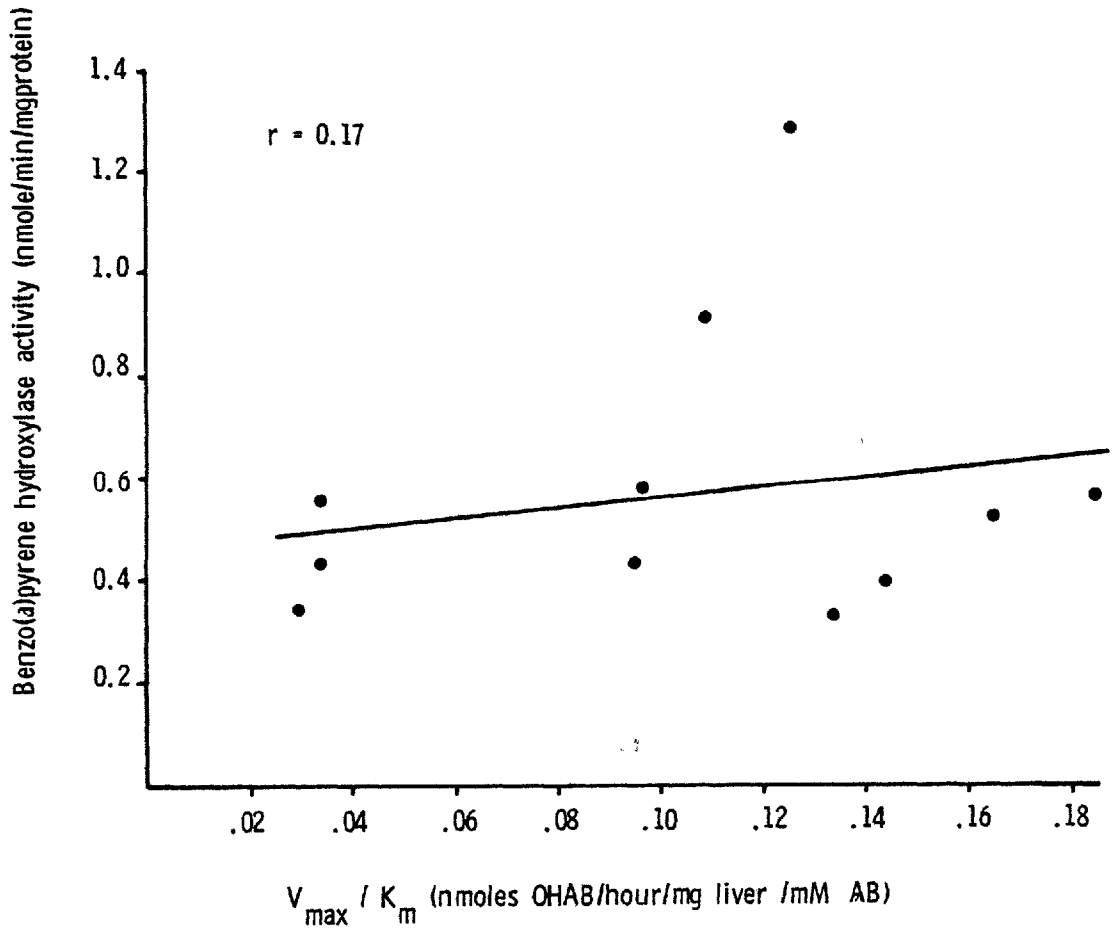


Figure 19

Correlation between benzo(a)pyrene hydroxylase activity and amylobarbitone hydroxylase activity for 11 needle biopsies of liver

CHAPTER VI

Amylobarbitone metabolism *in vivo*: clearance and urinary 3'-hydroxyamylobarbitone formation

a) Subjects and sample collection
   - (i) Patients
   - (ii) Controls

b) Methods
   - (i) Analysis of amylobarbitone in plasma
   - (ii) Analysis of 3'-hydroxyamylobarbitone in urine

c) Results
   - (i) Amylobarbitone clearance
   - (ii) Urinary 3'-hydroxyamylobarbitone
   - (iii) Correlations with *in vitro* activity

d) Discussion
   - (i) Amylobarbitone clearance
   - (ii) Urinary 3'-hydroxyamylobarbitone
   - (iii) Correlation of *in vivo* measurements of amylobarbitone metabolism
   - (iv) Correlation of *in vivo* and *in vitro* measurements of amylobarbitone metabolism
   - (v) Correlation of amylobarbitone metabolism with liver damage
   - (vi) Amylobarbitone metabolism and nutrition
   - (vii) Amylobarbitone metabolism and drugs

CHAPTER VI

a) SUBJECTS AND SAMPLE COLLECTION

(i) Patients

All 11 patients described in Chapter V had measurements of amylobarbitone half-life and clearance made, with the exception of S.W. This patient's long standing drug addiction with frequent intravenous injections of heroin had sclerosed all potentially accessible peripheral veins and repeated plasma sampling was therefore not practical. Seven patients had the estimations repeated after four weeks of medical, psychiatric, dietary and supportive care in hospital.

Blood samples (10 ml) were taken prior to an oral dose of amylobarbitone (200 mg, Eli Lilly & Co. Ltd.) at 10 p.m., and at 10, 16, 22, 34 and 42 hours in the first eight subjects. An additional sample was taken at 60 hours in the last three subjects after several subjects were seen to have a half-life greater than the original sampling time. Plasma was stored frozen until analysis of amylobarbitone.

All 11 patients had urine collected for 48 hours after the dose of amylobarbitone. Twelve hour collections were obtained and stored in a refrigerator until taken to the laboratory,

measured and 20 ml aliquots stored frozen until analysis. All patients had collections made and the study was repeated in seven, but two patients failed to provide complete collections in more than one collection period and they had to be excluded from analysis. In two further patients the first sample was omitted from the collections. It was therefore possible to include those patients' 24 - 48 hour urines in the subsequent analyses but necessary to omit the 0 - 24 hour collections. Aliquots from 12 hour collections were analysed for total 3'-OH amylobarbitone.

(ii) Controls

Three healthy adult male medical research workers aged 27, 29 and 31, members of the Department of Clinical Pharmacology of the Royal Postgraduate Medical School, received an oral dose of amylobarbitone (200 mg) at 10 p.m. and had blood and urine collections made over the same period as the patients, for plasma amylobarbitone and urinary 3'-OH amylobarbitone analysis. None of the three subjects were smokers and all three drank alcohol only occasionally and were asked to refrain for 24 hours before and during the study. None had taken drugs for four weeks prior to the study. Two subjects had the study repeated after a three month interval to check reproducibility of half-life and clearance.

b) METHODS (i)

Analysis of amylobarbitone in plasma

The pharmacokinetic properties of amylobarbitone have only recently been studied because of the lack of sufficiently sensitive assays to follow plasma concentrations after a hypnotic dose. The first sensitive and specific glc method (Balasubramaniam, Mawer and Rodgers, 1969) utilised thin layer chromatography to purify the extracts obtained with ethyl ether.

Other glc methods have been developed, claiming greater precision and speed. The method of Inaba and Kalow (1972) and Inaba and Kalow (1975) requires multiple extractions and the extract is injected directly onto a column packed with SE - 30 on a coarse (60-80 mesh) solid support, saturated with 10 µg of amylobarbitone. Ehrnebo, Agurell and Boreus (1972) used a single extraction with ether and on column "flash methylation" with trimethylanilinium hydroxide. In preliminary studies neither method yielded consistent results and it was decided to utilise a modification of the recently described rapid esterification method of Greeley (1974).

In this method the amylobarbitone, which is acidic, is converted to a soluble salt with the organic base tetramethyl ammonium hydroxide in a highly polar solvent system of anhydrous N, N-dimethyl formamide. The soluble salt formed reacts with an excess

of methyliodide, forming the N,N-dimethylated barbiturate derivative. The reaction is mild but fast and is complete at room temperature within minutes. This method produced considerably less tailing of peaks than on column "flash methylation".

Extraction procedure

To 1 ml of plasma (in duplicate) was added 1 µg of butobarbitone (in methanol) as internal standard. It was buffered with 0.2 ml of 4 M NaH_2PO_4 and mixed thoroughly. Amylobarbitone and butobarbitone were extracted into 10 ml of hexane (analytical grade) with 1.5% isoamylalcohol. Yield was 61% (mean of six determinations at 1 µg/ml of (2^{14}-C)-amylobarbitone[1].

The organic layer was transferred to a clean quick-fit tapered tube, evaporated under nitrogen, washed down with methanol and re-evaporated to dryness. The residue was dissolved in 400 µl of anhydrous N, N dimethyl formamide (Aldrich Chemical Co. Incorp.) and re-distilled before use.

Footnote:

1. Higher yields were obtained using ethyl acetate, ether, toluene and heptane, but the use of solvents with higher extraction ratios consistently yielded interfering peaks from blank plasma on glc. These were presumably due to extraction of non-polar endogeous compounds. Although other workers (Ehrnebo, et al., 1972; Inaba and Kalow, 1972) have not reported this to be a problem, at concentrations of amylobarbitone less than 1 µg/ml it appeared likely to affect peak height measurements. With hexane interfering peaks posed less of a problem.

Trimethyl anilinium hydroxide, 100 µl (Phase Separations, Ltd) was added and mixed well. Methyl iodide (15 µl) was added in a fume cupboard and mixed again. After 10 minutes at room temperature water (0.5 ml) was added and mixed. The methylated derivatives were extracted into 3 ml of dichloroethane (analytical grade). The upper aqueous layer was removed by suction using a pasteur pipette, and the lower organic layer remaining evaporated to dryness. The residue was dissolved in 30 µl of methanol for injection onto the glc column.

Gas Chromatography

The instrument used was a Varian 1400 gas chromatograph with a hydrogen flame ionisation detector (FID). A 12 foot x 1/8 inch Pyrex glass column was used, packed with 3% OV-1 on Gas-Chrom Q (100 - 120 mesh). This column gave excellent resolution of peaks (Figure 1a) and resolved most of the problems found when shorter columns were tried. The conditions were: injector temperature 240°, detector temperature 270° and oven temperature 200°. Carrier gas flow was 20 ml/min, (nitrogen) and at this flow rate retention times were: butobarbitone 2.7 minutes and amylobarbitone 3.1 minutes. Because of large peaks with longer retention times (6-20 minutes) the oven temperature had to be raised between injections to 240° for 8-10 minutes.

After analysis of 18 half-lives with the FID an interfering

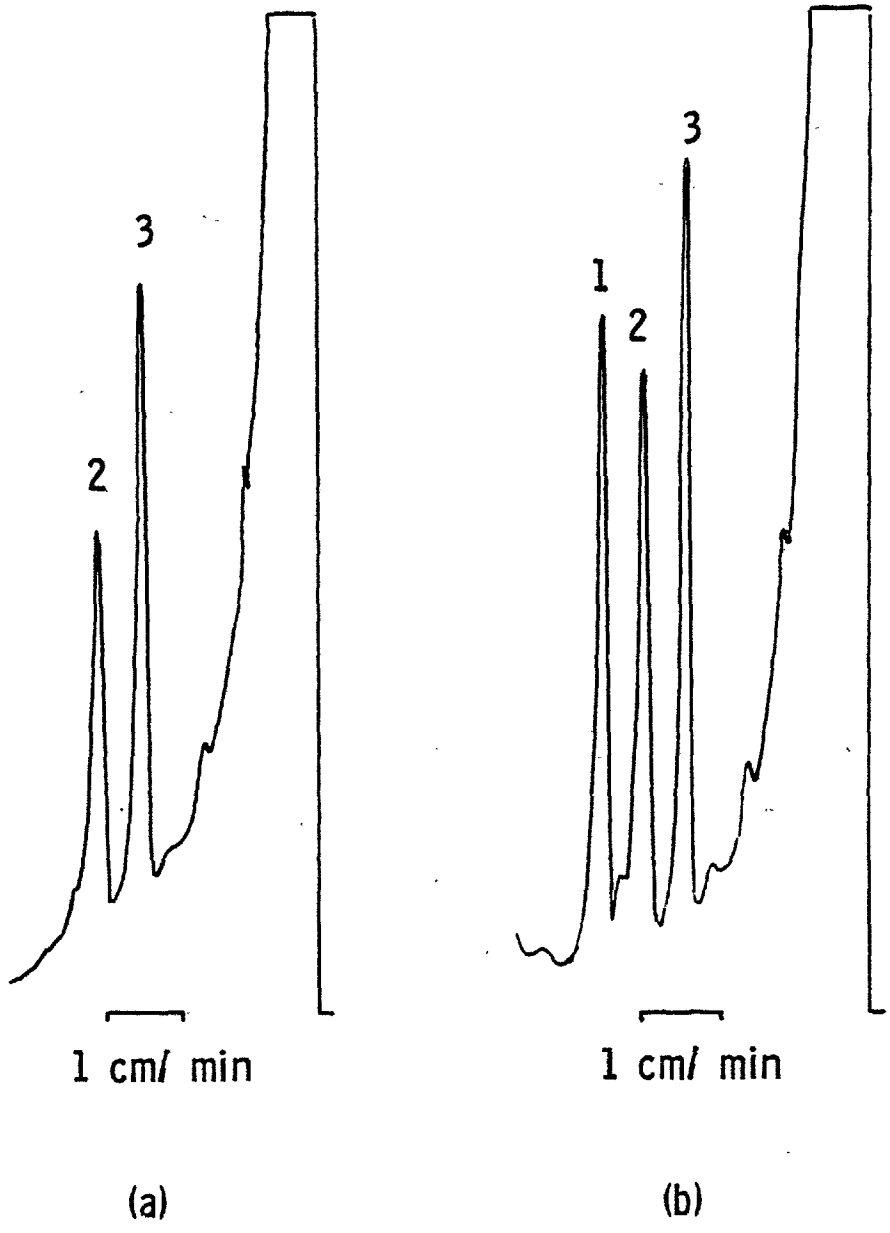


Figure 1(a)

Gas chromatograph traces showing amylobarbitone and butobarbitone peaks from extracts of plasma

a) Blank, containing butobarbitone only (1 µg/ml)

b) Standard, containing 1 µg/ml each of butobarbitone and amylobarbitone

1	Amylobarbitone	RT = 3.1 min
2	Butobarbitone	RT = 2.7 min
3	Unidentified	RT = 2.2 min

peak contaminated all extractions of control subject H.F.'s second half-life (See below). These samples were analysed by single ion monitoring gas chromatography mass spectrometry (GC-MS) by Miss Beth Neil (R.P.M.S.). Subsequent half-lives (3) were analysed using a Hewlett-Packard gas chromatograph (5750 Research chromatograph) with a nitrogen detector. The column was a four foot x ¼" glass column, packed with 3% OV-1 on Gas Chrom Q (100 - 120 mesh). Column conditions were: injector temperature 230°, oven temperature 190°, detector temperature 400°. Hydrogen flow was 28 ml/min and oxygen 180 ml/min. The carrier gas was helium, and at a flow rate of 60 ml/min the retention times were: butobarbitone 2.5 min and amylobarbitone 3.0 min.

Standards

Standards were prepared in two large batches three months apart, using fasting blank plasma from H.F. and stored frozen until used. Blank samples and concentrations of 0.5, 1.0, 2.0 and 3.0 µg/ml of amylobarbitone were prepared by adding aliquots of amylobarbitone in methanol to large volumes of plasma containing 1 µg/ml of butobarbitone. Aliquots of 2.5 ml were then stored for use in duplicate as required.

Standard curves

Peak height ratios of amylobarbitone: butobarbitone were

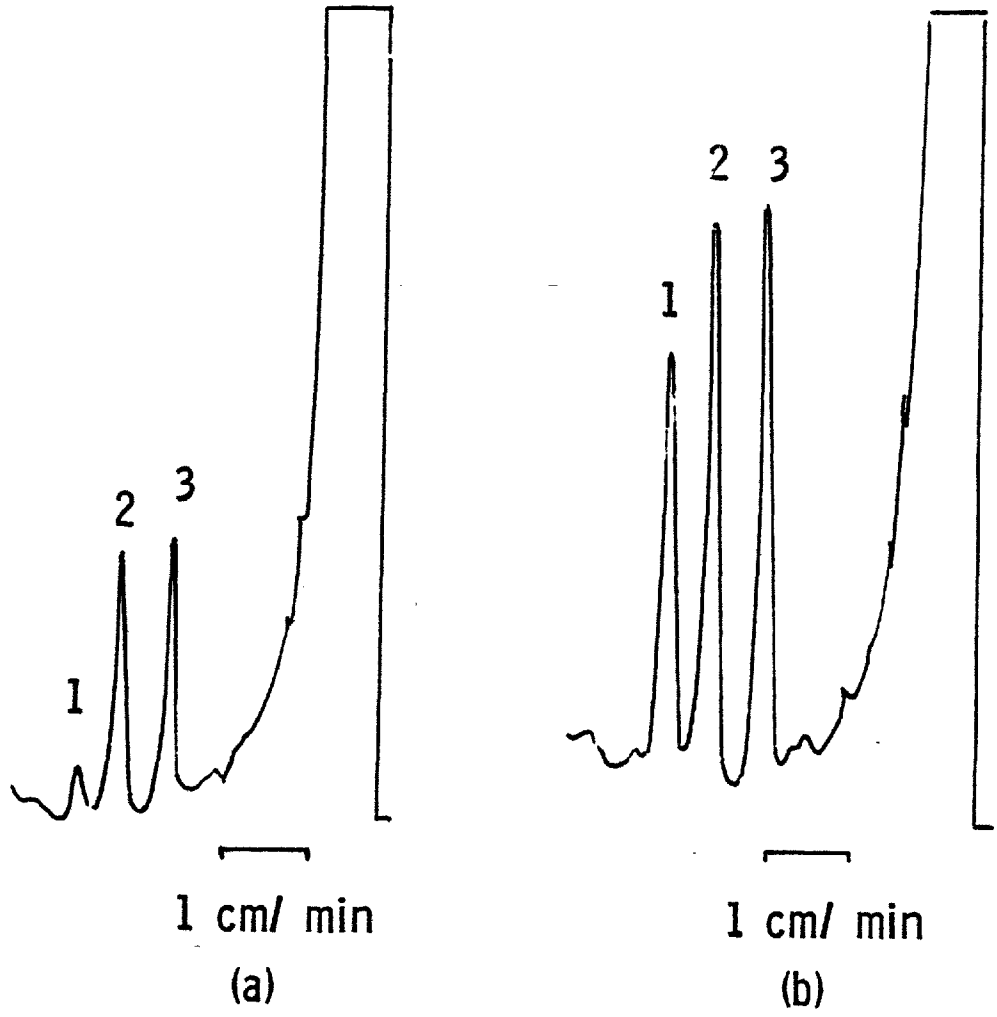


Figure 1(b)

Gas chromatograph traces showing butobarbitone and contaminating peak from extracts of plasma containing butobarbitone only (1 µg/ml)

a) Trace from patient R.M. showing small contaminating peak

b) Trace from H.F. (second study) showing large contaminating peak

1	Contaminating peak	RT = 3.2 min
2	Butobarbitone	RT = 2.7 min
3	Unidentified	RT = 2.2 min

used to obtain standard curves and amylobarbitone concentrations were read from the standard curves. Standard curves were linear but did not pass through the origin (Figure 2), because of a consistent small peak occurring 0.2 min after the amylobarbitone peak (Figure 1b). In the samples of H.F.'s second half-life a consistently large peak produced unacceptable variations in standards and duplicate samples. The extracts were examined by GC-MS, (Miss Beth Neil) and the interfering peak was identified as a long chain hydrocarbon derivative with a chain-length of approximately 18 carbon atoms. Its source was also traced by the use of GC-MS to the plastic storage sample tubes, which had been thawed on that occasion in a water bath which was inadvertently switched on (approx. 40°). Analysis of these samples was performed by GC-MS (Figure 7) and the subsequent three half-lives were analysed with the nitrogen detector. The latter produced pure barbiturate peaks (Figure 3) and standard curves passed through the origin (Figure 4).

Calculations

Amylobarbitone half-lives were estimated by computer using the least squares method assuming a one compartment model between 10 and 60 hours. Clearance was calculated from the relationships:

$$\text{Clearance} = \frac{Vd \times 0.693}{T\frac{1}{2}}$$

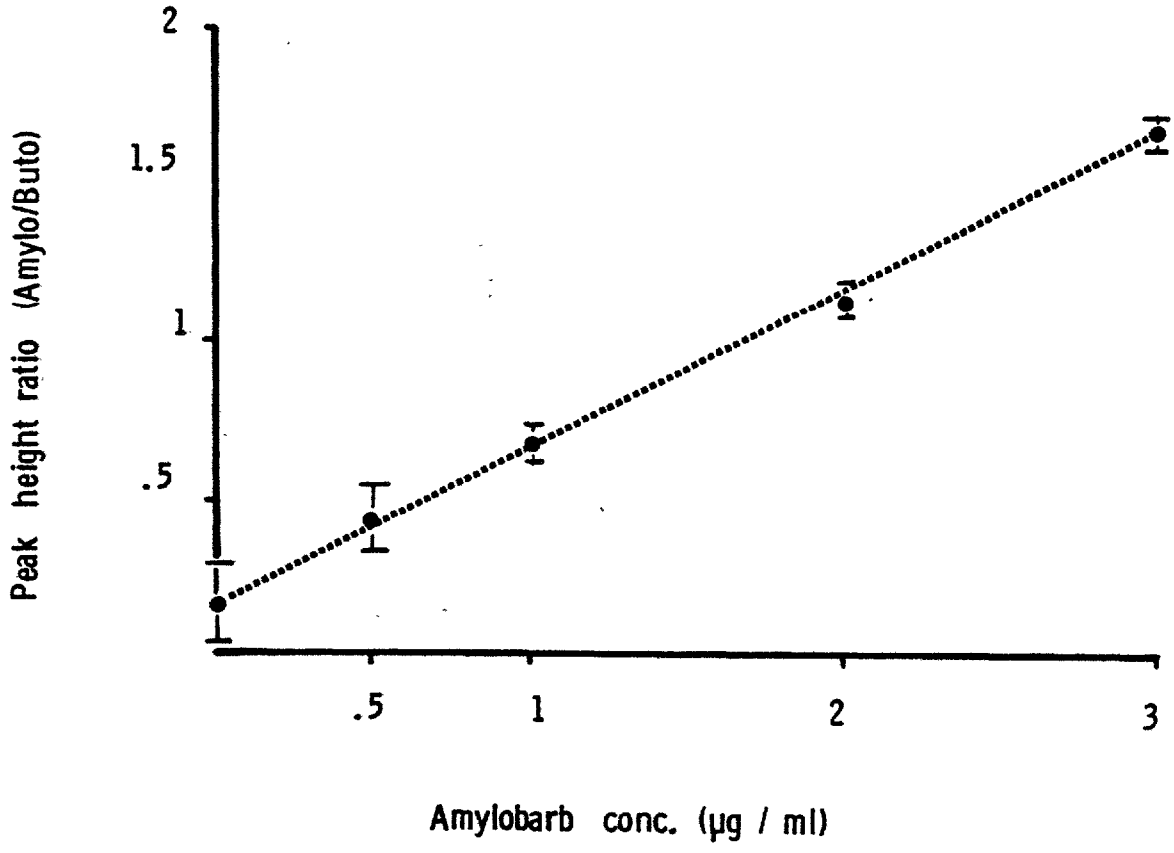


Figure 2

Standard curve for glc assay of amylobarbitone with the flame ionisation detector, using butobarbitone as the internal standard (n = 10, mean ± SD)

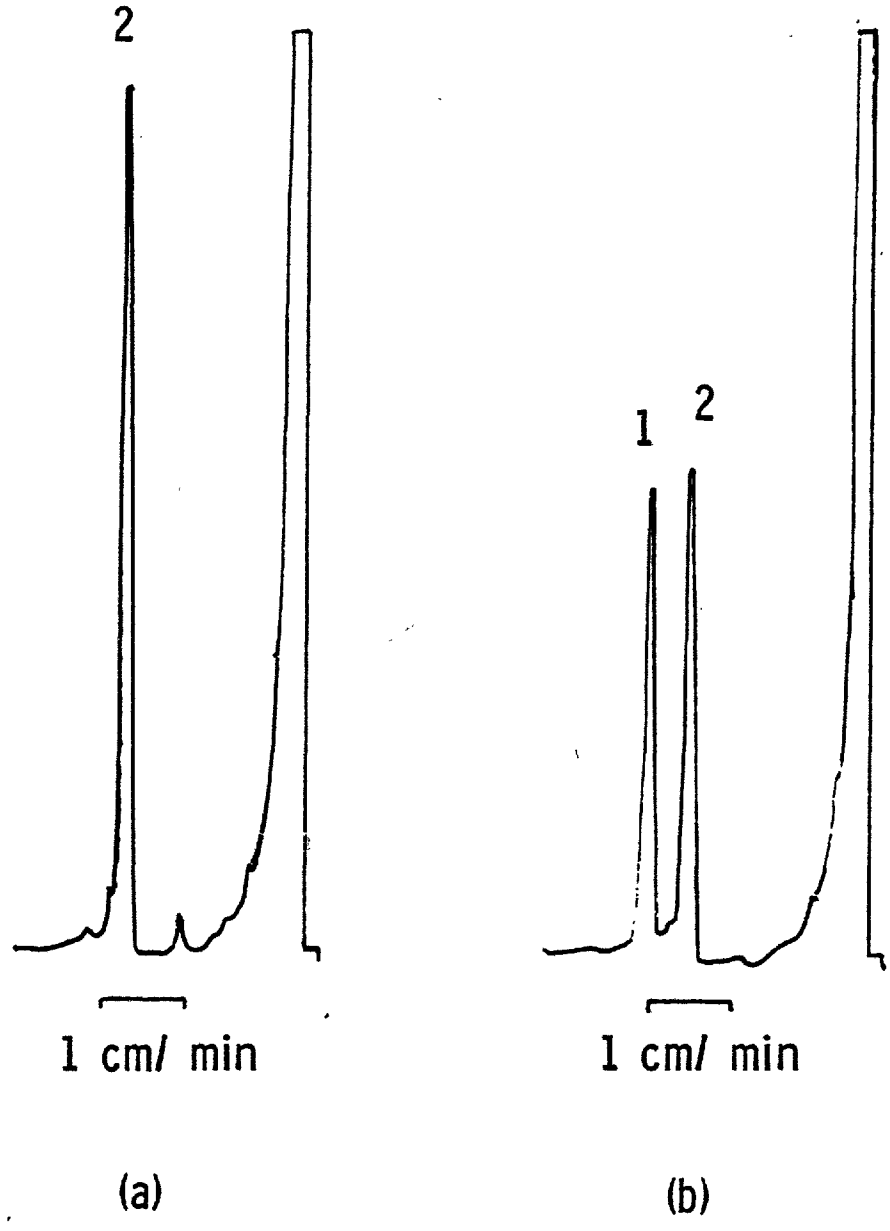


Figure 3

Gas chromatograph traces (using the nitrogen detector) showing amylobarbitone and butobarbitone peaks from extract of plasma containing (a) 1 µg/ml butobarbitone and (b) 1 µg/ml each of butobarbitone and amylobarbitone

1 amylobarbitone RT = 3.0 min

2 butobarbitone RT = 2.5 min

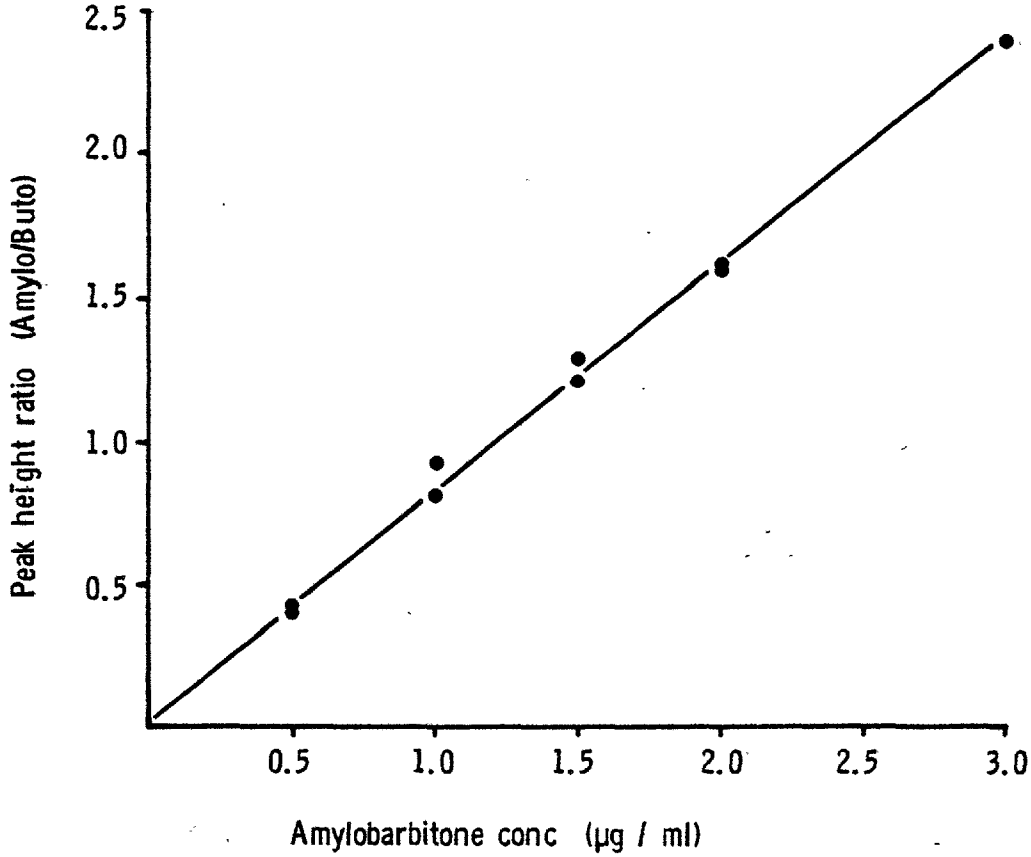


Figure 4

Standard curve for glc assay of amylobarbitone using the nitrogen detector, with butobarbitone as internal standard

where Vd = volumes of distribution (= Dose/Co)

$T\frac{1}{2}$ = half life

and Co = estimated initial plasma concentration

METHODS (ii)

Analysis of 3'-hydroxyamylobarbitone in urine

Extraction

Urine samples were analysed in duplicate for 3'-OH amylobarbitone by a modification of the method of Grove and Toseland (1970). Aliquots of urine (3 ml) were spiked with 60 µg of hydroxypentobarbitone (Eli Lilly and Co. Ltd,, 60 µl of a 1 mg/ml aqueous solution) as internal standard to give a concentration of 20 µg/ml. Florisil columns (30-60 U.S. mesh) were prepared in pasteur pipettes and the urine run through into large pyrex tubes. After alkalinising with 1 ml of 1N NaOH an excess of ammonium sulphate was added to saturate and adjust the pH to 9.0. The hydroxybarbiturates were extracted into 10 ml of ether, by vortex mixing for 15 seconds, centrifuging and removing the upper ether layer into a clean tapered tube. This was evaporated to dryness, reconstituted in 100 µl of methanol and methylated with diazomethane (0.5 ml) in ether for 30 minutes. The diazomethane was evaporated and the residue dissolved in 30

µl of methanol for injection. The diazomethane did not have to be prepared freshly as long as it retained its bright lemon yellow colour. Methylation appeared to be complete on all occasions as indicated by pure peaks (Figure 5) and linear and reproducible standard curves passing through the origin (Figure 6).

Gas Chromatography

The methylated derivatives were injected on a 5 foot x 1/8 inch 3% OV-1 column, using the Varian 1400 gas chromatograph. Conditions were: injection temperature 220°, oven temperature 200° and detector temperature 260°. Carrier gas flow (nitrogen) of 60 ml/min with these conditions gave retention times of 2.65 min for hydroxyamylobarbitone and 3.2 min for hydroxypentobarbitone.

Standards

Standards at 5, 10, 20 and 40 µg/ml were prepared in batches with aqueous hydroxyamylobarbitone (Eli Lilly and Co. Ltd) hydroxypentobarbitone and blank urine of H.F. Three ml aliquots were stored frozen until required and analysed in duplicate with each assay of patient or control samples. Peak height measurements of OH'-amylobarbitone: OH'-pentobarbitone were used to obtain standard curves (Figure 6) for interpretation of OH'-amylobarbitone concentration and total OH'-amylobarbitone for 0-24 hours and 24-48 hours calculated from the urine volumes.

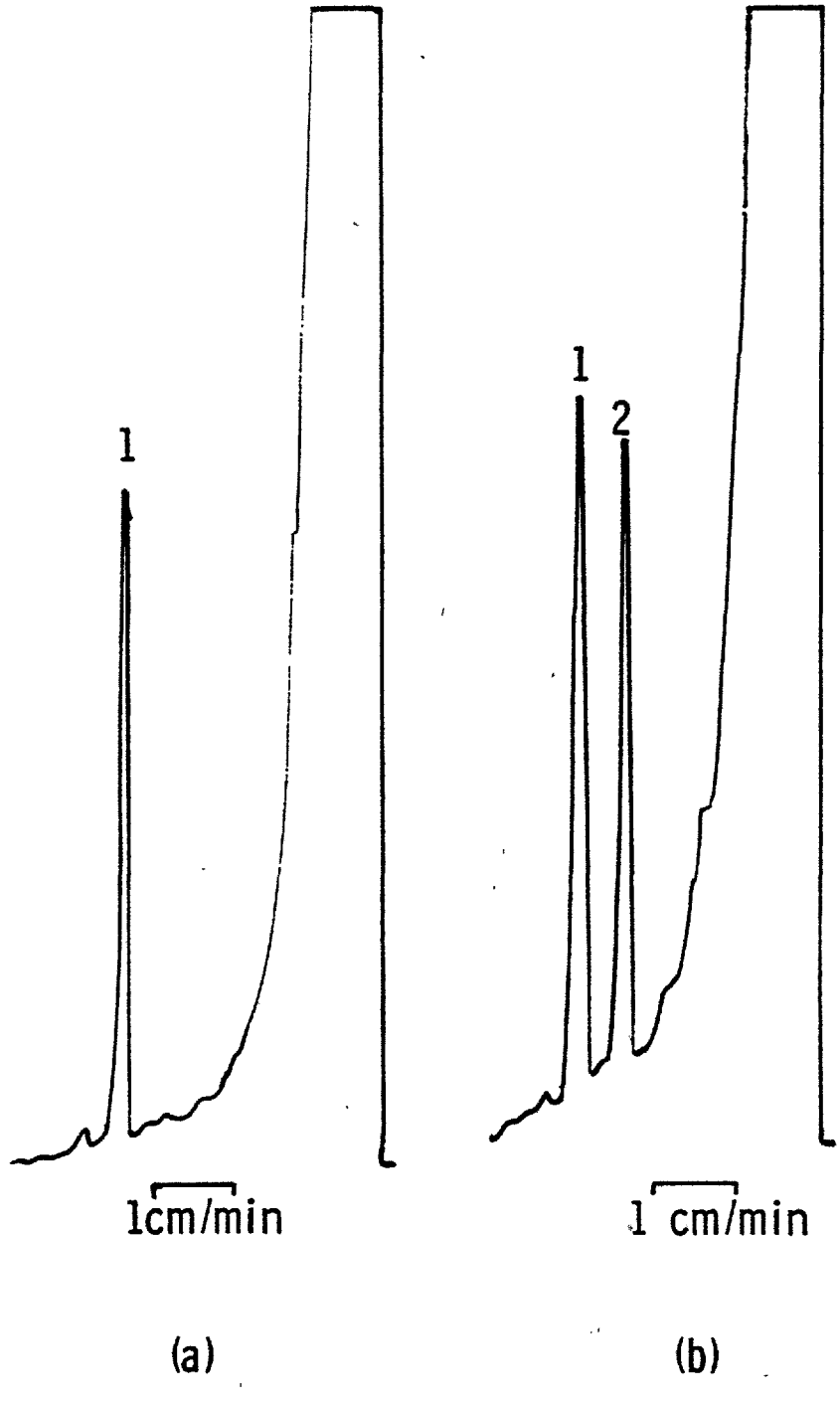


Figure 5

Gas chromatograph trace showing peaks of OH-amylobarbitone and OH-pentobarbitone extracted from plasma containing:

(a) OH-pentobarbitone 20 µg/ml
(b) OH-amylobarbitone and OH-pentobarbitone, 20 µg/ml each

1 OH-pentobarbitone RT 3.2
2 OH-amylobarbitone RT 2.65

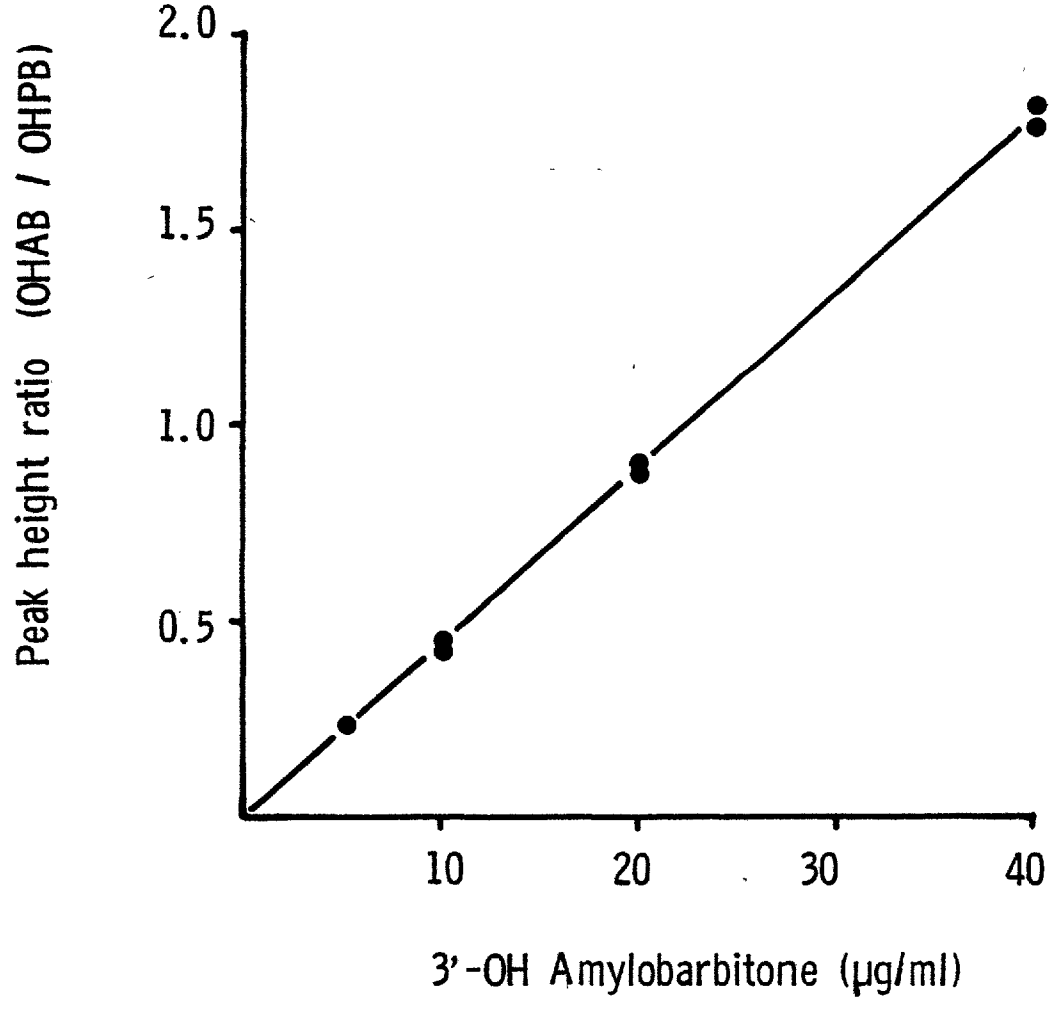


Figure 6

Standard curve for g.l.c. assay of OH-amylobarbitone, with OH-pentobarbitone as internal standard

Comparisons between healthy controls and patients were made using Students t-test.

c) RESULTS

(i) Amylobarbitone clearance

Table 1 summarises the half-lives, elimination rate constants, clearance in ml/min and ml/min/kg body weight for healthy controls and patients (all estimations, including both values for patients studied twice). Amylobarbitone half-life of healthy controls (16.1 ± 5.9 (S.D.) hours) and clearance (54.1 ± 7.9 ml/min) differed from values for patients (37.2 ± 26 hours and 30.00 ± 18 ml/min respectively, $0.05 < p < 0.1$ and $p < 0.02$). Individual values of all subjects and patients are listed in the Appendix Table IV Figure 7 illustrates the log. concentration/time profiles of one control subject (H.F.) on two occasions, analysed by glc and GC-MS.

(ii) Urinary 3'-hydroxyamylobarbitone

Table II summarises the urinary 3'-OH amylobarbitone results for controls and patients, over 0-24 hours, 24-48 hours and 0-48 hours, expressed as total 3'-OH amylobarbitone in mg, in mg/kg body weight and as a percentage of dose excreted in 48 hours. Again individual values are listed in the Appendix Table V. Urinary 3'-OH amylobarbitone of controls over 48 hours (85.9 ± 20.1 mg

TABLE I

AMYLOBARBITONE HALF-LIVES, ELIMINATION RATE CONSTANTS (K_{el}) AND CLEARANCE IN HEALTHY CONTROLS AND ALCOHOLIC PATIENTS (ALL ESTIMATIONS, MEANS AND S.D.)

	Half-life (hours)	K_{el} ($hours^{-1}$)	Clearance (ml/min)	Clearance (ml/min/kg)
Controls (n = 5	16.1 ± 5.0	0.048 ± 0.021	54.1 ± 7.9	0.76 ± 0.13
Patients (n = 17)	43.1 ± 29.8	0.024 ± 0.016	30.1 ± 18.4	0.52 ± 0.23
P (Students t test)	$0.05<p<0.1$	<0.02	<0.02	$0.05<p<0.1$

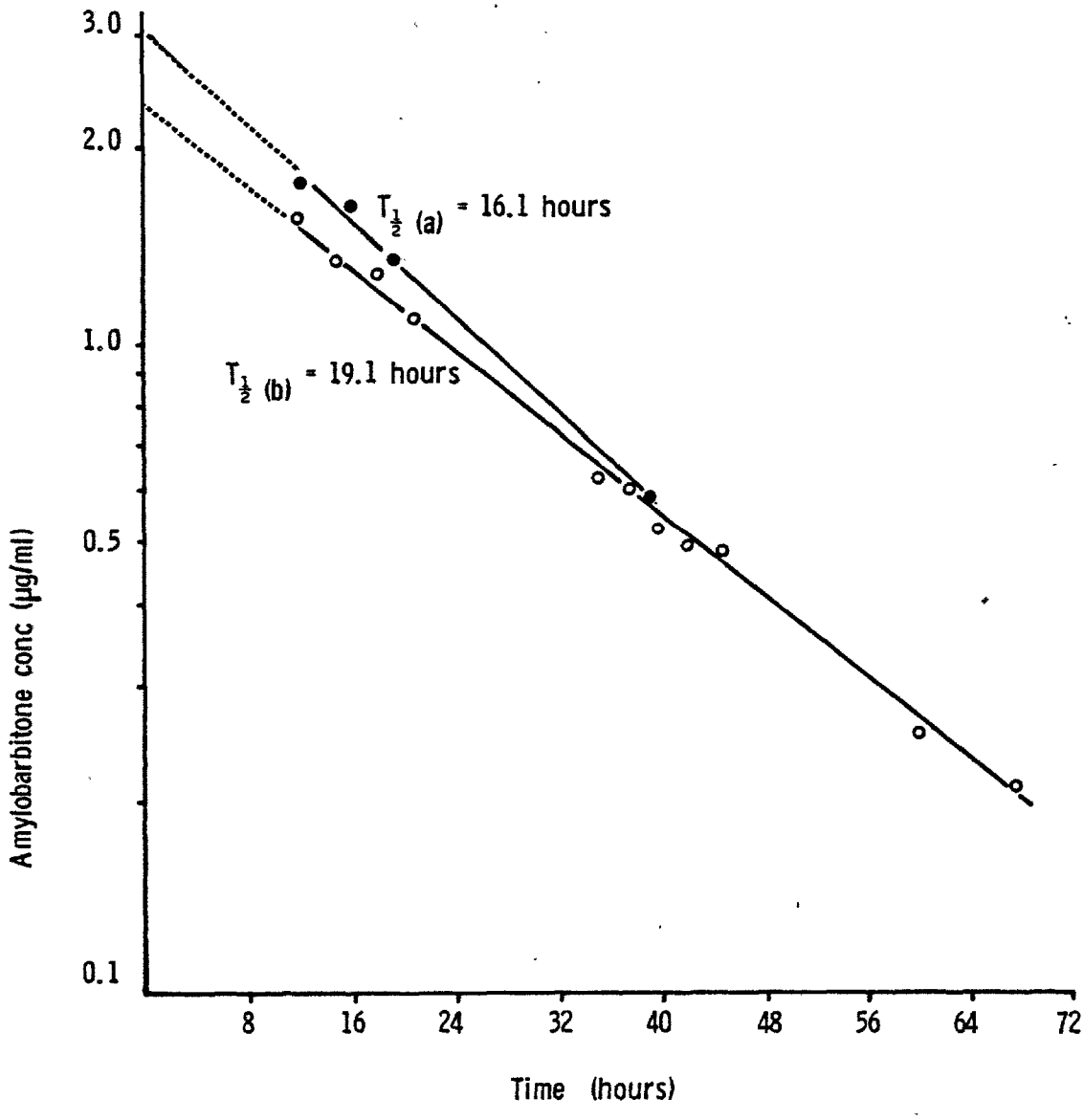


Figure 7

Amylobarbitone half-life of H.F. on two occasions, estimated by g.l.c. (a) and by GC-MS (b)

TABLE II

URINARY 3'-OH AMYLOBARBITONE (3'-OHAB) EXCRETION IN 24 HOURS, 24-48 HOURS AND 0-48 HOURS IN CONTROLS AND PATIENTS (ALL ESTIMATIONS, MEANS AND S.D.)

	3'OHAB 0-24 hours (mg)	3'-OHAB 24-48 hours (mg)	3'-OHAB 0-48 hours (mg)	3'-OHAB 0-48 hours (mg/kg)	3'-OHAB 0-48 hours % of amylobarb. dose
Controls	48.3 ± 11.6	37.5 ± 9.2	85.9 ± 20.2	1.15 ± 0.26	43 ± 10
Patients	31.2 ± 21.4	26.3 ± 14.2	57.1 ± 34.7	0.99 ± 0.76	28 ± 17
P (Students'	N.S.	N.S.	<0.05	N.S.	0.05<p<0.1

or 43% of the dose) was greater than urinary 3'-OH amylobarbitone for patients (57.1 ± 34.7 mg or 28.5%) but did not quite reach the 5% level of significance.

Urinary excretion of 3'-OH amylobarbitone in the first 24 hours was consistently greater than in the second 24 hours in the controls, but the relationship was variable in patients. Total body clearance correlated positively with urinary 3'-OH amylobarbitone over 24 hours ($r = 0.63$), 24 - 48 hours ($r = 0.64$) and 0 - 48 hours ($r = 0.63$) (Figure 8). All correlations were highly significant ($p < 0.01$).

(iii) Correlation with in vitro activity

In figure 9 and 10 these in vivo measurements of amylobarbitone metabolism in patients are correlated with the in vitro measurements obtained with needle biopsies and described in Chapter V. Clearance (ml/min) showed a positive correlation with the index of in vitro activity V max/km ($r = 0.42$) but this was not significant ($p < 0.1$) (figure 9). If clearance was expressed as ml/min/kg to take body weight and total liver mass into account, the correlation was improved ($r = 0.50$) but did not quite reach the 5% level of significance.

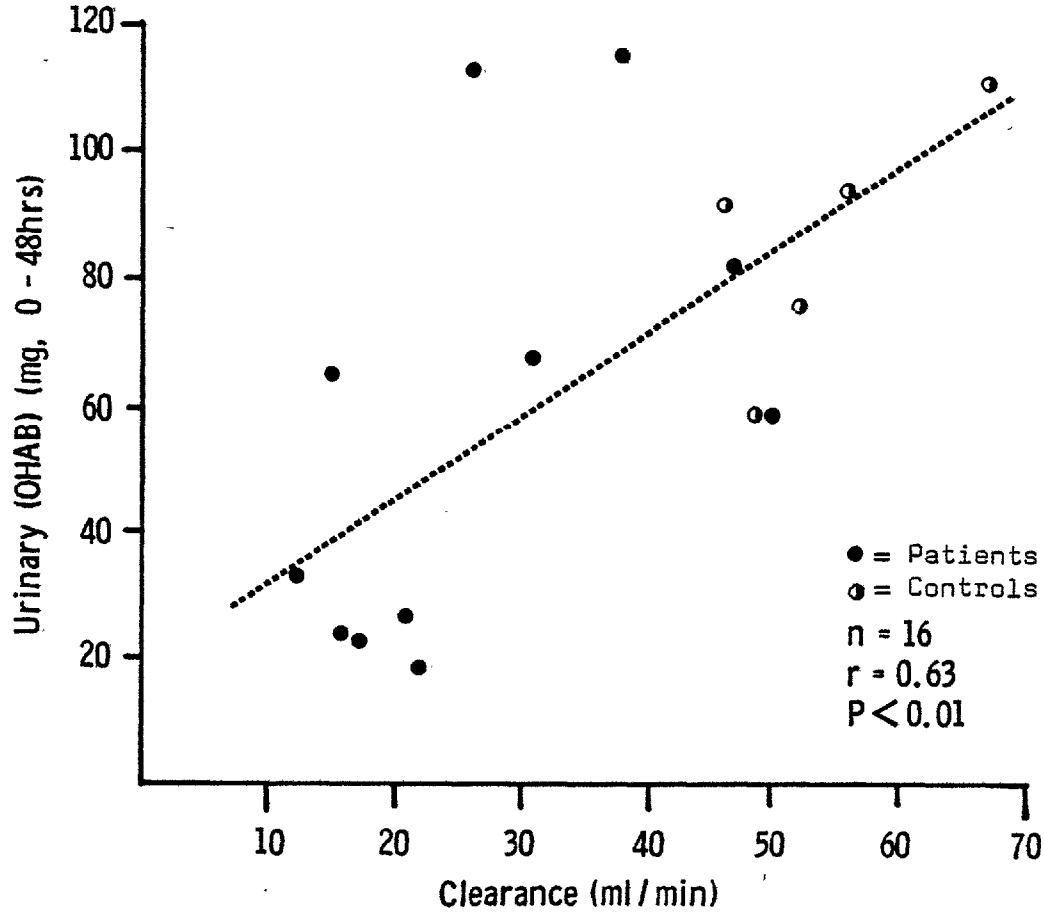


Figure 8

Correlation between urinary hydroxyamylobarbitone (OHAB) and clearance of amylobarbitone.

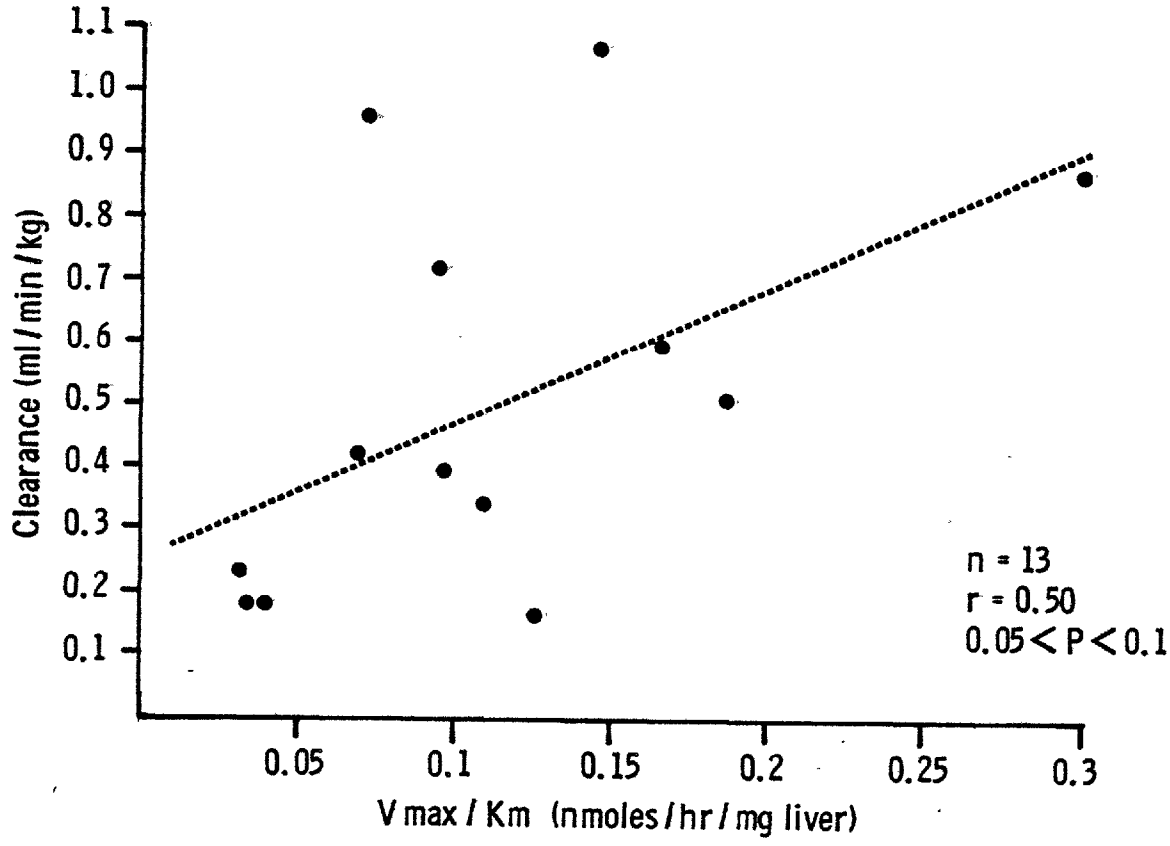


Figure 9

Correlation between clearance of amylobarbitone and amylobarbitone hydroxylase activity (V max/Km) in needle biopsy samples of liver

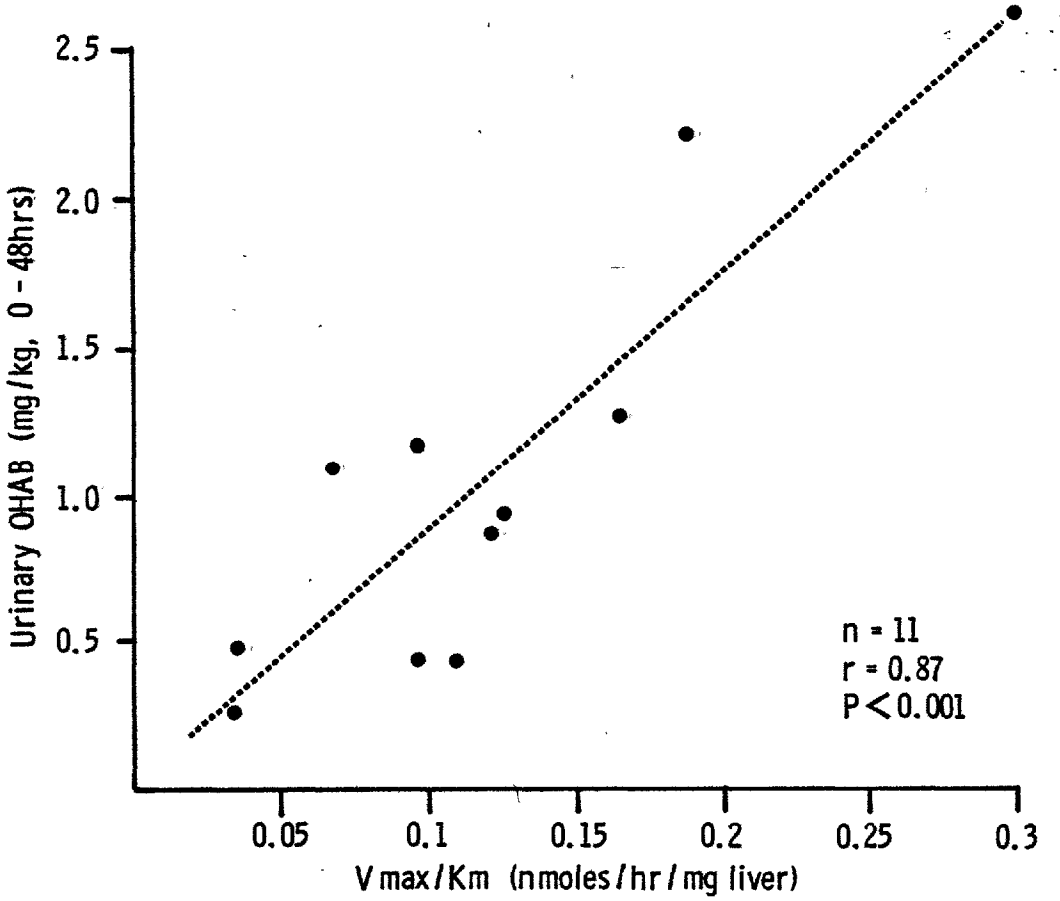


Figure 10

Correlation between urinary OHAB (0-48 Hrs) and amylobarbitone hydroxylase activity (V max/Km) in needle biopsy samples of liver

Figure 11 shows the correlation of urinary 3'-OH amylobarbitone also expressed in relation to body weight, in 48 hours, with V max/km. There was a correlation coefficient r of 0.87 which was highly significant ($p < 0.001$).

Figure 11, Chapter V, shows the relationship between *in vivo* and *in vitro* measurements and the histological features of the patients' liver biopsies, as shown in Table III, Chapter V. There is no consistent relationship for any of these indices. It is apparent that most values for clearance and urinary 3'-OH amylobarbitone fall below the values for controls, but there is a wide scatter, a few patient values being in the normal range, and two having values of urinary 3'-OH-amylobarbitone far in excess of the controls. One of these had been taking phenobarbitone prior to admission. One patient with cirrhosis had consistently low values for all indices, one had intermediate values and one had very high values on admission with a six fold fall in clearance and a four fold fall in V max/km when the study was repeated. (Figure 11 a and c). On admission the biopsy showed steatosis and hepatitis but no cirrhosis, while the second biopsy showed the development of cirrhosis.

d) DISCUSSION

(i) Amylobarbitone clearance

Half-lives of amylobarbitone in three healthy adults (16.1 hours) (5 estimations) were similar to values of 21 hours reported by Mawer, et al. (1972) and to values of 17 hours for their patients with chronic liver disease but no abnormality of liver function. One subject, H.F., had values of 16.6 and 19.1 hours on two occasions while another showed an increase from 8.1 to 16.1 hours, with a fall in the elimination rate constant. This subject had completed a five day course of phenylbutazone 6 weeks before the study and although it is customary to observe only a 4 week interval between studies of comparative drug elimination this interval may be too small to overcome the inducing effects of phenylbutazone on the P-450 system.

Estimation of clearance rather than half-life is important in view of inter-individual differences in volume of distribution for amylobarbitone. Protein binding differs from subject to subject and varies with albumin concentration, while body weight changed as much as 16% over 4 weeks in some patients (M.W., F.B.). All kinetic parameters, half-life, elimination rate constant and clearance indicated significantly slower metabolism of amylobarbitone in alcoholic patients than in controls. This is consistent with findings in patients with liver disease and

abnormal serum albumin and prothrombin time (Mawer et al.) but surprising in view of the fact that only three patients had cirrhosis, only two of these had a delayed prothrombin time and none of them had a low serum albumin. Two subjects with a low serum albumin did have very low amylobarbitone clearance, but normal prothrombin times and no other evidence of liver dysfunction. As they were also grossly underweight, both had low serum and red cell folate and normal blood urea while P.M. had a very low serum B_{12}, the low albumin was almost certainly related to dietary deficiency and not liver or renal impairment.

(ii) Urinary 3'-hydroxyamylobarbitone

Urinary 3'-OH amylobarbitone was also significantly reduced in alcholic patients, although the difference was not as great ($p < 0.05$). Again results were comparable with the data of Mawer, et al.

(iii) Correlation of in vivo measurements of amylobarbitone metabolism

As figure 8 shows, and again as was shown by Mawer, et al, amylobarbitone clearance and urinary excretion of 3'-OH amylobarbitone show a significant correlation ($r = 0.63$, $p < 0.01$). However the covariance for patients ($r = 0.49$) is not as good as for the combined values of patients and healthy controls, and does

not reach statistical significance ($0.05 < p < 0.1$). This suggests that a large proportion of the variation in clearance is determined by variation in some other elimination pathway than oxidation to 3'-OH amylobarbitone.

(iv) Correlation of in vivo and in vitro measurements of amylobarbitone metabolism

Figure 9 shows that while amylobarbitone clearance in vivo does increase with an increase in the estimated V max/km for 3'-OH amylobarbitone formation in vitro, the correlation is poor ($0.05 < p < 0.1$). Thus clearance does not correlate closely with formation of 3'-OH amylobarbitone formation either in vivo or in vitro. If clearance of a drug is used as an index of its oxidation it is essential that it should be shown to correlate closely with metabolite formation.

Figure 10 shows that there is very close correlation between measurements of 3'-OH amylobarbitone formation in vivo and in vitro in the same individual ($r = 0.87$, $p < 0.001$). Thus, in vivo and in vitro measurements do correlate if the product of metabolism is measured in both instances. The poor correlation of in vivo clearance with 3'-OH amylobarbitone formation either in vivo or in vitro suggests a significant additional pathway for elimination. No significant amounts are excreted unchanged in the urine, and 3'-OH amylobarbitone was identified as the major metabolite by Maynert (1965). However, only 30-50% of the total dose is excreted

in this form (Freudenthal and Carroll, 1973) as findings in the patients described here confirm.

Tang, Inaba and Kalow (1975) have identified another major metabolite, N-hydroxyamylobarbitone (Figure 11). This metabolite represents between 1% and 40% of the metabolism of amylobarbitone in more than 30 healthy adult subjects studied since the original report (Kalow, 1976, personal communication). The proportion of N- to C-hydroxylation is constant for each subject over 72 hours, and in subjects studied twice it appears to be the same on both occasions. The most likely explanation for the poor correlation between amylobarbitone clearance and 3'-OH amylobarbitone formation is that there is considerable inter-individual variation among our subjects, as in Kalow's, of this second major metabolite, N-'OH amylobarbitone.

This conclusion has important implications for studies of comparative drug elimination in man. Conflicting results in comparing drug metabolic rates using estimation of clearance (Kalow, 1976) may be due to the inability to take account of more than one pathway of drug metabolism. Clearance would only be a reliable index of drug oxidation if either (i) by far the greatest proportion of the drug's metabolism formed only one oxidation product, or (ii) the proportion oxidised to a major product was constant for all subjects. This has been shown not to be the case for amylobarbitone, and it means that studies of inter-individual

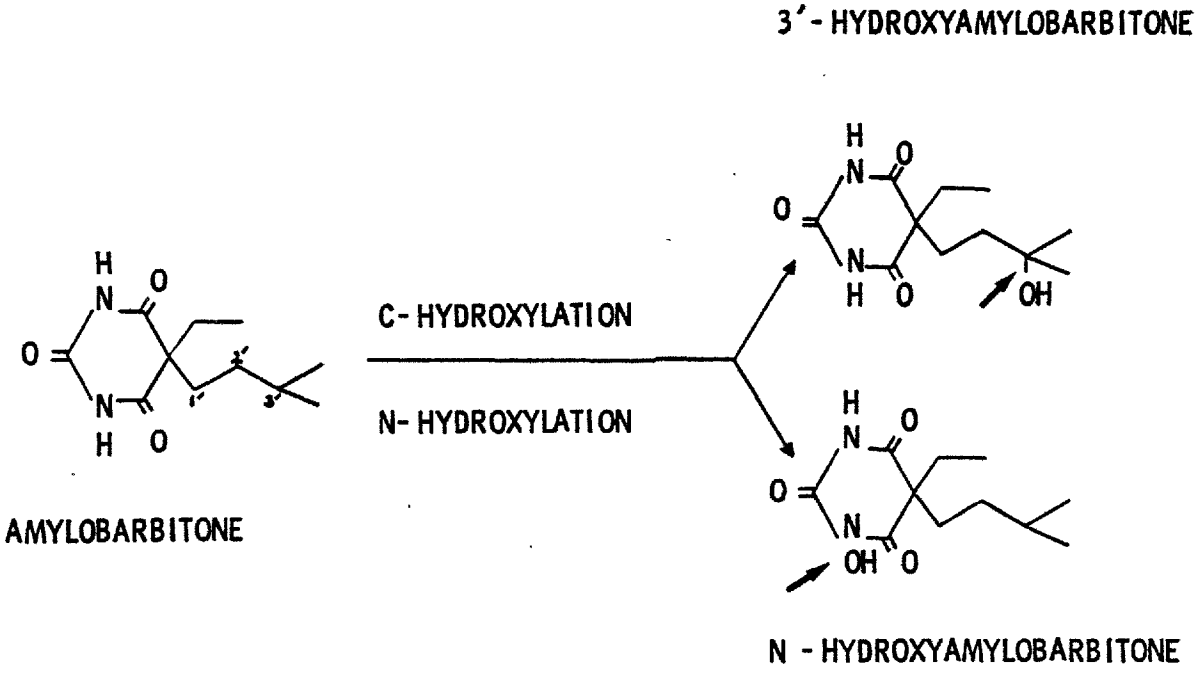


Figure 11

Oxidation of amylobarbitone to 3'-OH amylobarbitone and N-hydroxyamylobarbitone

variation and of comparative metabolic rates will only be meaningful if metabolites are measured.

A further implication of these findings is the possibility of environmental effects on metabolic pathways yielding toxic metabolites. It is well established in animals that a protein deficient diet, for example, protects young male rats from the hepatotoxic effects of carbon tetrachloride and dimethyl nitrosamine, compounds whose toxicity is attributed to toxic metabolites (McLean and McLean, 1966; McLean and Verschuuren, 1969). In contrast a protein deficient diet increases the toxicity of paracetamol. Although cytochrome P-450 levels are reduced, reduction of glutathione is more critical, and the toxic metabolites react with cellular macromolecules (McLean and Day, 1975).

It is possible that diet and other environmental factors such as alcohol and cigarette smoking, both inducing agents, may induce one pathway more than the other. N-hydroxylation is an unusual biochemical oxidation and can lead to compounds of greater reactivity and toxicity than the parent compounds (Weisburger, 1973). Environmental influeces may thus not only affect drug levels by induction, inhibition or enzyme or co-factor depletion, but may affect toxocity by similar mechanisms to those concerned with paracetamol and dimethyl nitrosamine toxicity.

(v) Correlation of amylobarbitone metabolism with liver damage

As shown in figure 11 neither amylobarbitone clearance nor 3'-OH amylobarbitone formation *in vivo* or *in vitro* correlate consistently with histological changes in the liver. This is perhaps not surprising as previous studies of amylobarbitone clearance in liver disease (Mawer, et al., 1972) have shown that clearance is not impaired unless liver damage is so severe as to produce changes in prothrombin time and serum albumin. Most of our patients (7 of 11) had steatosis, but only three had cirrhosis, and only two an abnormal prothrombin time. Other workers have found that drug metabolism is impaired only in severe liver disease. For example Schoene, et al. (1972) found demethylation rates *in vitro* to be normal in mild liver damage but reduced in severely damaged livers. Branch, et al. (1973) found that antipyrine half-lives showed the greatest prolongation in patients with decompensated liver disease.

There are other possible reasons for poor correlation of amylobarbitone C-hydroxylation with liver pathology, e.g. the inducing effects of alcohol and cigarette smoking. Gamma glutamyl transpeptidase was grossly elevated in the three patients with cirrhosis, but only mildly elevated in others and it is not a reliable index of enzyme induction. Alcohol is a known enzyme inducing agent (Mezey and Tobin, 1971; Lieber, 1973; Kalant, et al., 1976) and induction is dose related (Iber, 1976). Alcoholics taking more than 200 g of alcohol daily are invariably induced, as indicated by a shortening

of antipyrine and tolbutamide half-lives over 4 to 10 weeks after alcohol withdrawal (Iber, 1976). Although there was no significant difference in any indices between drinkers of less than 200 g or 200 g and more in our study, one patient (D.M.) showed changes explicable only by induction of the microsomal enzymes on admission to hospital. Clearance, urinary 3'-OHAB and *in vitro* formation of 3'-OHAB all showed a 4-5 fold reduction over four weeks, accompanied by the development of cirrhosis.

(vi) Amylobarbitone metabolism and nutrition

Another factor of relevance in alcoholic patients is their poor nutritional state. Five patients had low folate levels (red cell or red cell and serum). Two had low vitamin B_{12} levels. Two had low albumin, two a low haemoglobin and one iron deficiency. Four (P.M., F.B., T.G. and E.P.) were underweight and gained more than 5 kg during four weeks of hospitalisation.

It is not possible to isolate these individual deficiencies and relate them to amylobarbitone metabolism, but the results in patients studied twice are of interest (see Table X, Chapter V). Only one patient, (D.M.) discussed above, showed a fall in V max/km (77%). Six other patients were studied twice. One biopsy (P.M.) was too small for accurate results. Another (F.B.) had no activity on either occasion. A third (R.B.) did not follow Michaelis-Menten kinetics. The other three, however, all showed an increase in activity after four weeks on a full diet with vitamin supplementation (H.N., 32%, T.G., 72% and E.P. 267%).

This increase may have been due to a number of factors, but as none of these patients showed chronic liver damage and all showed general or specific nutritional deficiencies on admission, it is possible that improvement of nutritional status may have been responsible. In view of the effects of dietary deficiencies on oxidation in animals and the effects on antipyrine described in Chapter III, this problem merits further study.

(vii) Amylobarbitone metabolism and drugs

One patient (C.M.) had been taking phenobarbitone 100 mg t.d.s. for 1 week prior to admission. This may have contributed, by inducing the P-450 system, to his high clearance. No other subject was on inducing agents, although T.G. and A.D. were receiving diazepam 10 mg three times daily. This has been shown not to be an inducing agent. (Orme, Breckenridge and Brooks, 1972).

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

a) Community based studies of variation in drug metabolism

b) In vivo/in vitro studies of amylobarbitone metabolism

c) Conclusions

The studies described in this thesis have revealed a number of important new observations related to drug metabolism in man.

a) COMMUNITY BASED STUDIES OF VARIATION IN DRUG METABOLISM

The first part of the thesis describes the environmental influences on drug metabolisn in 180 healthy adults from two community-based samples in London and West Africa. The non-invasive technique of estimating drug half-life in saliva has been used to measure the half-lives of antipyrine and paracetamol as markers of drug oxidation and conjugation. We have shown both to give reliable estimates of the plasma half-lifes.

It has been shown that factors known to be important determinants of drug metabolic rates in animals are also relevant to man. Thus differences in diet appear to be significant determinants of antipyrine and paracetamol half-life. This may be a reflection of either major of minor dietary constituents. Vegetarian diets, which prolong drug elimination, are known to be low in animal fat and protein. There is epidemiological evidence of protein and calorie deficiency in the diet in the village of Keneba, Gambia, and the albumin levels and ponderal indices of the subjects support this. Detailed dietary histories of London vegetarians suggest that micro-nutrients (vitamin B_{12} and iron) are deficient as well as calorie, protein and fat intake. One or more of these factors may be related to the observed prolongation of drug half-lives. The possibility of an ethnic component cannot be entirely ruled out on the present evidence

and further studies of Asians with differing diets are planned.

These community based studies are consistent with animal studies, which have shown that a large number of dietary factors can affect drug metabolism. They indicate the need for the next phase of investigation, controlled clinical studies, in which effects of single dietary constituents are examined. Several common clinical situations provide relevant opportunities. Subjects requiring prolonged special diets, for example, polyunsaturated fat diets, could be investigated before and after instituting the diet. Fasting obese patients in hospital for the purpose of weight reduction provides another such valuable opportunity. Short periods of fasting have been shown not to alter antipyrine half-lives significantly (Reidenberg and Vessell, 1975). However, in animals metabolic changes associated with short term nutritional deficiencies cannot be extrapolated to more chronic situations, and this may be true for man. Longer periods of calorie deprivation under observation are often considered justified in the grossly obese for clinical reasons, and could provide valuable information on drug metabolising capacity.

We have shown that in addition to diet indices of body habitus and haemoglobin are related to antipyrine oxidation while both diet and ponderal index are related to paracetamol conjugation. In the sub-sample of 14 subjects whose dietary histories were taken low protein intake prolonged antipyrine half-life. Diet histories may

be subject to inaccuracies, for example, figure conscious young ladies may underestimate their food intake, but support for this finding comes from recent work by Alvares, et al. (1976) and Conney (1976), who showed that a low protein, high carbohydrate diet prolongs theophylline half-life by 80% and antipyrine half-life by 50%. It is conceivable that in man, as in the rat, protein deficiency may decrease the rate of drug oxidation by a decrease in cytochrome P-450 or perhaps a decrease in total liver cell mass.

The effects of cigarette smoke, which contains inducing agents, and of the steroid oral contraceptive pill, a substrate for the microsomal enzyme system, confirm the recent findings in controlled studies cited previously. Although the reduction of antipyrine half-life by smoking and prolongation by the contraceptive pill are relatively minor effects in individual subjects, the application of multiple regression analysis to large samples shows them to be highly significant. Individually they may not be of clinical importance, but a combination of two or more factors may produce a two-fold or greater difference in antipyrine half-life. For example, non-contraceptive pill-taking smokers had half-lives of 6-10 hours while non-smoking pill-takers had half-lives of 14-17 hours. Similarly, prolongation of antipyrine half-life by a vegetarian diet (mean 16.2 hours) appears to be further compounded by use of the contraceptive pill, as two of the longest half-lives of 20.5 and 19.4 hours were those of pill-taking vegetarians.

Forty-nine percent of the variation in antipyrine half-life in 131 Londonders has been explained by environmental factors. It is possible that other, as yet unrecognised factors, are responsible for a further proportion. For example, Conney and others (1976) have recently demonstrated that a diet containing daily charcoal broiled steaks (which contain large amounts of polycyclic hydrocarbons) will shorten antipyrine and theophylline half-life by 20 and 50% respectively. It also appears to increase the metabolism of phenacetin in the gut, as the ratio of paracetamol to phenacetin increases, the area under the curve falls and the phenacetin half-life is unchanged. As other dietary constituents, e.g. members of the Brassicacae family including broccoli, turnips, cabbage and brussel sprouts, are also rich in inducing agents, (Wattenberg, Page and Leong, 1968; Wattenberg, 1971; Pantuck, Hsiao, Loub, Wattenberg, Kuntzman and Conney, 1976) it may be postulated that even more than 50% of inter-individual variation may be due to environmental influences.

Recognition of the effects of environmental factors on drug oxidation in man has several important implications:

(i) Therapeutic implications

Important sources of variation in drug response can be identified by the physician. If extrapolation from antipyrine to other drugs is valid, two-fold differences in plasma levels might be anticipated by careful history taking. For example if a

vegetarian non-smoker and a non-vegetarian smoker were given the same dose of a drug metabolised by the cytochrome P-450 system, the vegetarian might be expected to have plasma levels twice as high as the non-vegetarian smoker. He would therefore, be more likely to manifest toxic symptoms. It is possible that for some drugs the predictable changes may be greater, and there is evidence that this is the case for theophylline, a drug metabolised by cytochrome P-448 (Jenne, Nagasawa, McDonald and Wyse, 1975; Alvares, Anderson, Conney and Kappas, 1976)

(ii) Implications for regulatory bodies

In view of the very varied diet indifferent parts of the world, recommendations of drug doses for non-European countries may need modification. The most outstanding differences in diet are the lower calorie intake and the excess of vegetable as opposed to animal fat in the developing countries. Both overall elimination rates and variation in metabolic routes may be altered by these factors.

(iii) Carcinogenicity

Drugs and environmental toxins may be carcinogenic directly or as a result of metabolism to carcinogenic metabolites. Diet and inducing agents have been shown to alter carcinogenicity in animal experiments, and epidemiological studies implicate diet in the

aetiology of many forms of cancer. Likely mechanisms include alteration of rates of individual metabolic pathways and overall elimination rates of carcinogens.

b) IN VIVO/IN VITRO STUDIES OF AMYLOBARBITONE METABOLISM

The second aim of this study was to examine the correlation between *in vivo* and *in vitro* indices of oxidation in man using the same substrate. It has been shown that 20 mg needle biopsies can be used to estimate the kinetic parameters of amylobarbitone C-hydroxylation with 9000 g supernatant. There are no reports in the literature of kinetic data derived from needle biopsies of human liver.

Using the expression V max/Km derived from the in vitro assay, a highly significant correlation has been shown with 3'-OH amylobarbitone formation *in vivo* (in a 48 hour urine collection). The correlation with amylobarbitone clearance was poor. As the same metabolite, 3'-OH amylobarbitone was being measured *in vitro* and in the urine, a correlation between these two measurements would be expected. The failure of clearance to correlate with 3'-OH amylobarbitone formation *in vitro* may be due to variation in the production of another metabolite, the N-hydroxy derivative described by Tang, Inaba and Kalow (1975). As they have shown that the proportion of this metabolite varies from 1-40% in healthy individuals[1], but is constant over 72 hours in the same individual, it is apparent that

[1]Personal communication

clearance of amylobarbitone is not an accurate index of a single major metabolic pathway but the sum of metabolism by two distinct pathways whose relative importance varies from one individual to another.

This conclusion has an important bearing on recent attempts to correlate the half-lives (or clearances) of different drugs sharing the microsomal oxidising enzyme system. Kadar, et al., 1973, for example, showed that sulphinpyrazone, amylobarbitone and glutethimide half-lives correlated with each other, and Brien, Inaba and Kalow (1975) have shown that diphenyl hydantoin half-life correlated significantly only with amylobarbitone, while antipyrine did not correlate significantly with either.

It is now clear, however, that unlike antipyrine, whose 4-OH metabolite correlates closely with antipyrine half-life ($r = 0.89$, $p < 0.001$, Huffman, Shoeman and Azarnoff, 1974), amylobarbitone disappearance from plasma does not correlate sufficiently closely with formation of one major metabolite to justify use of half-life or clearance in comparative studies. This may explain some of the anomalies in the literature of comparative drug oxidation capacity in man. Measurement of urinary metabolite formation may be expected to produce much more meaningful correlations with drugs undergoing similar oxidation reactions.

The subsidiary aim of the studies done in alcoholic patients was to relate their <u>in vivo</u> and <u>in vitro</u> metabolic rates to other indices

of liver function and to alcoholic intake and to follow changes in these metabolic indices with recovery. This proved most complex as oxidising capacity showed only a poor correlation with liver damage. Only three patients had chronic liver damage on histological criteria, only two of these had an abnormal prothrombin time (the only clinically used liver function test shown to be consistently significantly related to drug oxidation) and only two of the three had a very low oxidising capacity. However, the majority had both hepatomegaly and fatty infiltration, features associated with a chronic high alcohol intake. As the majority of patients had an alcohol intake of over 200 g/day, a high incidence of induction of microsomal enzymes would be expected. Yet only one subject had very rapid amylobarbitone metabolism on admission (in vivo and in vitro) falling over four weeks.

A consistent finding was a history of a grossly deficient diet and deficiencies of vitamin B_{12} and folate. In such patients metabolising capacity increased after four weeks of vitamin treatment and a full diet. These few subjects suggest another possible dietary cause of impaired drug oxidation and a need for further studies. Furthermore, both alcohol and other socially used drugs and dietary factors may alter not only rates of a single metabolic pathway but the proportions of a drug metabolised by different routes. This could affect the toxicity and carcinogenicity in man of a drug with a minor pathway to a toxic metabolite.

c) CONCLUSIONS

1. Measurements of antipyrine and paracetamol half-lives in saliva provide a valuable method of investigating variation in drug metabolism in community based samples.

2. Environmental factors accounted for 49% of the variation in antipyrine half-life among 131 office and factory workers in London and 21% of the variation among 49 Gambian villagers.

3. The major factors were diet and socially used drugs. A vegetarian diet, the contraceptive pill and cola nut consumption prolonged the half-life while cigarette smoking shortened it.

4. Environmental factors accounted for 64% of the variation in paracetamol half-life among 40 Londoners and again diet and use of the pill were important.

5. A low estimated dietary protein intake was associated with prolongation of antipyrine half-life.

6. These findings have important implications for clinical practice, use of drugs in developing countries and risks of carcinogenicity.

7. Needle biopsy samples of human liver can provide accurate information on the kinetics of oxidation by the cytochrome P-450 enzyme system.

8. Clearance of amylobarbitone correlated poorly with urinary 3'-hydroxyamylobarbitone and not at all with *in vitro* estimation of V max/Km for amylobarbitone C-hydroxylation in needle biopsy samples.

9. *In vitro* estimation of V max/Km for amylobarbitone C-hydroxylation correlated closely with *in vivo* measurement of urinary 3'-hydroxyamylobarbitone.

10. *In vivo* and *in vitro* measurements of amylobarbitone metabolism showed a poor correlation with liver damage in alcoholic patients.

11. These findings indicate the importance of measuring metabolite formation in preference to elimination of the parent drug when studying comparative drug metabolising capacity in man.

APPENDIX

APPENDIX TABLE I

ANTIPYRINE HALF-LIFE, VOLUME OF DISTRIBUTION (V.D.), PONDERAL INDEX AND 15 OBSERVED VARIABLES FOR 44 MALES, 87 FEMALES AND ALL 131 LONDON SUBJECTS

Variable	Units	Males (n=44)	Females (n=87)	All Subjects (n=131)
Sex[1]				1.66 ± 0.47
Age	years	34.6 ± 11.6	34.0 ± 11.4	34.2 ± 11.4
Weight	Kg	71.0 ± 8.9	61.0 ± 10.2	64.3 ± 10.9
Height	cm	172 ± 6	160 ± 7	164 ± 8
Haemoglobin	g/dl	14.9 ± 1.1	12.5 ± 1.6	13.3 ± 1.8
Albumin	g/dl	4.68 ± 0.23	4.46 ± 0.25	4.54 ± 0.26
Globulin	g/dl	2.86 ± 0.29	2.93 ± 0.34	2.91 ± 0.32
Race[2]		1.64 ± 0.94	1.99 ± 1.01	1.87 ± 0.99
Smoking[3]		1.48 ± 0.51	1.26 ± 0.44	1.34 ± 0.47
Cigarettes	/day	8.5 ± 11.2	5.31 ± 10.2	6.41 ± 10.6
Alcohol	drinks/week	7.56 ± 8.51	3.61 ± 9.14	4.93 ± 9.09
Diet[4]		1.25 ± 0.44	1.45 ± 0.50	1.38 ± 0.49
Coffee/tea	cups/day	4.55 ± 2.08	3.75 ± 2.45	4.02 ± 2.36
Pill[5]			1.17 ± 0.37	1.11 ± 0.31
Ponderal Index[+]	/cm^2	2.39 ± 0.27	2.38 ± 0.38	2.39 ± 0.47
V_d	l/kg	0.61 ± 0.07	0.53 ± 0.08	0.56 ± 0.08
Half-life	hours	12.1 ± 4.0	13.1 ± 4.5	12.8 ± 4.4

* mean and S.D.

+ Weight + Height^2

Superscripts:

1. Males = 1, Females = 2
2. Caucasians = 1, Negroes = 2, Asians = 3
3. Non-smokers = 1, Smokers = 2
4. Non-vegetarians = 1, Vegetarians = 2
5. Non-pill takers = 1, Pill takers = 2

APPENDIX TABLE II

ANTIPYRINE HALF-LIFE, VOLUME OF DISTRIBUTION (V.D.) PONDERAL INDEX AND 15 OBSERVED VARIABLES FOR 74 CAUCASIANS, 57 ASIANS AND ALL 131 LONDON SUBJECTS

Variable	Units	Caucasians (n = 74)	Asians (n = 57)	All Subjects (n = 131)
Sex		1.59 ± 0.49	1.75 ± 0.43	1.66 ± 0.47
Age	Years	36.3 ± 12.3	31.5 ± 9.9	34.2 ± 11.4
Weight	Kg.	66.5 ± 10.1	61.6 ± 11.3	64.3 ± 10.9
Height	Cm	167 ± 9	160 ± 6	164 ± 8
Haemoglobin	g/dl	13.9 ± 1.3	12.5 ± 2.1	13.3 ± 1.8
Albumin	g/dl	4.59 ± 0.28	4.47 ± 0.22	4.54 ± 0.26
Globulin	g/dl	2.81 ± 0.29	3.04 ± 0.32	2.91 ± 0.32
Race				1.87 ± 0.99
Smoking		1.5 ± 0.50	1.12 ± 0.33	1.34 ± 0.47
Cigarettes	/day	10.2 ± 12.3	1.46 ± 4.46	6.4 ± 10.6
Alcohol	drinks/week	8.3 ± 10.8	0.54 ± 1.96	4.93 ± 9.09
Diet		1.01 ± 0.12	1.86 ± 0.35	1.38 ± 0.49
Coffee/tea	cups/day	5.26 ± 2.28	2.33 ± 1.17	4.02 ± 2.86
Pill		1.16 ± 0.37	1.04 ± 0.19	1.11 ± 0.31
Ponderal Index	g/cm^2	2.38 ± 0.28	2.40 ± 0.42	2.39 ± 0.47
V_d	l/kg	0.58 ± 0.08	0.53 ± 0.08	0.56 ± 0.08
Half-life	hours	10.6 ± 3.1	15.6 ± 4.2	12.8 ± 4.4

TABLE III

DIETARY ANALYSIS FOR SEVEN FEMALE VEGETARIANS AND SEVEN AGE-MATCHED FEMALE NON-VEGETARIANS

Subject	Cals. per day	Protein g/day	Carb. g/day	Fat g/day	Polyun- g/day	Chol. g/day	Iron mg/day	Folate µg/day	B_{12} µg/day
VEGETARIANS									
J.Q.	1461	22.3	273	30.9	4.51	0.01	8.4	23.9	1.0
H.T.	1299	27.7	157	62.3	1.49	0.07	7.6	14.4	1.0
S.P.	1328	31.2	187	50.5	2.97	0.08	8.3	29.0	0.7
H.B.	1435	35.6	176	65.4	1.73	0.07	7.8	32.3	1.2
H.S.	1267	37.3	140	62.0	7.48	0.30	10.1	45.0	0.5
H.P.	1712	41.3	211	78.1	2.73	0.11	7.9	31.8	1.3
R.K.	1269	50.2	160	47.5	3.37	0.27	12.2	28.0	1.9
Mean	1396	35.1	186	56.7	3.47	0.13	8.4	29.2	1.01
S.D.	159	9.2	45	15.2	2.04	0.11	2.3	9.3	0.50
NON-VEGETARIANS									
M.E.	1686	56.3	164	89.5	6.06	0.32	8.3	41.4	2.4
S.K.	1918	58.0	274	65.7	0.83	0.10	7.1	20.5	2.9
C.D.	2031	70.0	170	118.9	4.59	0.42	10.8	22.9	2.7
L.W.	1762	73.2	135	103.1	3.92	0.48	14.2	29.1	9.0
D.B.	1740	75.4	183	78.6	3.66	0.36	12.7	45.9	7.1
M.T.	1316	75.6	134	53.0	2.53	0.61	12.8	44.6	6.5
B.T.	1791	86.3	197	73.3	1.85	0.30	9.3	25.0	3.7
Mean	1747	70.0	180	83.2	3.35	0.37	10.7	32.8	4.90
S.D.	224	10.7	48	22.5	1.76	0.16	2.6	10.9	2.60
P	<0.005	<0.001	N.S.	<0.025	N.S.	<0.01	N.S.	N.S.	<0.05
RDA*	2100	30					10	190	2

* Recommended dietary allowance WHO 1974

APPENDIX TABLE IV

AMYLOBARBITONE HALF-LIVES, ELIMINATION RATE CONSTANTS (K_{el}) AND CLEARANCE IN HEALTHY CONTROLS AND ALCOHOLIC PATIENTS

(ALL ESTIMATIONS)

	SUBJECT	HALF-LIFE (hours)	K_{el} ($hours^{-1}$)	CLEARANCE (ml/min)	CLEARANCE (ml/min/kg)
a)	Controls:				
	H.F. (a)	16.1	0.043	45.6	0.61
	(b)	19.1	0.036	52.1	0.69
	J.M.	21.3	0.033	50.2	0.76
	N.B. (a)	8.1	0.085	66.5	0.95
	(b)	16.1	0.043	56.1	0.79
	Mean	16.1	0.048	54.1	0.76
	S.D.	5.0	0.021	7.9	0.15
b)	Patients:				
	P.M. (a)	33.3	0.021	19.3	0.42
	(b)	14.2	0.050	45.0	0.97
	F.B. (a)	78.5	0.010	12.9	0.24
	(b)	16.0	0.043	42.8	0.72
	S.W.	-	-	-	-
	D.M. (a)	12.1	0.057	82.6	1.07
	(b)	118.4	0.006	12.0	0.17
	R.B. (a)	50.6	0.014	15.6	0.18
	(b)	58.3	0.012	22.3	0.26
	H.N. (a)	29.3	0.024	47.0	0.72
	(b)	79.7	0.009	14.9	0.16
	T.G. (a)	40.2	0.017	40.2	0.39
	(b)	21.3	0.033	21.3	0.59
	E.P. (a)	46.7	0.015	11.9	0.23
	(b)	71.3	0.010	16.6	0.34
	A.D.	24.2	0.029	26.2	0.50
	M.W.	17.9	0.039	38.4	0.87
	C.M.	20.3	0.034	49.8	0.96
	Mean	43.1	0.024	30.5	0.52
	S.D.	29.8	0.016	19.1	0.31
P (Students t test)		$0.05<p<0.1$	<0.02	<0.02	$0.05<p<0.1$

APPENDIX TABLE V

URINARY 3'OH AMYLOBARBITONE (3'-OHAB) EXCRETION IN 0-24 HOURS, 24-48 HOURS AND 0-48 HOURS IN CONTROLS AND PATIENTS (ALL ESTIMATIONS)

SUBJECT	TOTAL 3'-OHAB 0-24 HOURS (MG)	TOTAL 3'-OHAB 24-48 HOURS (MG)	TOTAL 3'-OHAB 0-48 HOURS (MG)	3'-OHAB 0-48 HOURS (MG/KG)	3'-OHAB 0-48 HOURS % OF AMYLOBARB. DOSE
a) Controls					
H.F. (a)	54.9	36.7	91.7	1.21	46
(b)	43.2	31.7	74.9	0.99	37
J.M.	32.0	25.9	57.9	0.82	29
N.B. (a)	62.7	48.0	110.7	1.48	55
(b)	48.7	45.6	94.3	1.25	47
Mean	48.3	37.5	85.9	1.15	42.8
S.D.	11.6	9.2	20.2	0.26	10.0
b) Patients					
P.M. (a)	-	-	-	-	-
(b)	-	-	-	-	-
F.B. (a)	-	-	-	-	-
(b)	-	-	-	-	-
S.W.	37.3	23.8	61.1	0.87	31
D.M. (a)	-	43.7	-	-	-
(b)	18.5	16.2	34.7	0.48	17
R.B. (a)	11.5	11.2	22.7	0.26	11
(b)	8.8	10.4	19.1	0.22	10
H.N. (a)	39.5	42.6	82.0	1.17	41
(b)	30.3	35.0	65.3	0.93	33
T.G. (a)	12.5	10.4	22.8	0.42	11
(b)	35.3	33.1	68.4	1.27	34
E.P. (a)	-	18.0	-	-	-
(b)	6.0	14.6	20.6	0.42	10
A.D.	71.1	44.5	115.5	2.21	58
M.W.	66.4	48.5	114.9	2.61	57
C.M.	37.6	20.1	57.7	1.11	29
Mean	31.2	26.3	57.1	0.99	28.5
S.D.	21.4	14.2	34.7	0.76	17.4
P (Students t test)	NS	NS	$0.05 < p\ 0.1$	NS	$0.05 < p\ 0.1$

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