

PROPERTIES OF OXIDATION POLYMORPHISM IN MAN
WITH PARTICULAR REFERENCE TO THE DISPOSITION
OF DEBRISOQUINE, PHENACETIN AND PHENYTOIN

Timothy Peter Sloan

Being a thesis submitted for the degree of
Doctor of Philosophy in the University of London

Department of Biochemical and Experimental Pharmacology
St. Mary's Hospital Medical School
Paddington
London W2 1PG

JUNE 1980

".....it is rather surprising that intensive studies of visual perception have rarely identified examples of polymorphism, but a possible explanation is that such studies have involved refining figures until they are 'strong'; that is perceived by all subjects in all conditions. If figures are, instead, refined to maximise discrimination, then it is possible that polymorphisms of visual perception will be found to be as general as polymorphisms of other characters."

Fraser A, Wilcox KJ. Nature 281: 566 (1979).

FOREWORD

The work described in this thesis is concerned with polymorphic drug oxidation, a feature of drug metabolism first described by members of this department in 1977. I would like to express my thanks at the outset to both Prof R.L. Smith and Dr. J.R. Idle for the opportunity to work in a new and stimulating field of biochemical pharmacology. My particular thanks to Dr. Idle for his unstinted help throughout the course of this work and during the writing of this thesis.

I would also like to thank the Medical Research Council, whose financial support made my participation in this work possible. The studies involving intravenous drug administration could not have been performed without the skill and supervision of Dr. R. Lancaster, whose help on this and many other counts I gratefully acknowledge.

The population studies of phenacetin metabolism were conducted in collaboration with Miss H.W. Devonshire, who kindly gave her permission for the reproduction of those data here.

My thanks also to the many who participated in the in vivo metabolism studies described here, particularly Messrs. Lawrence Wakile, John O'Gorman, James Ritchie and Andrew Zangouras for their co-operation on many occasions. My particular thanks to Mr. Wakile for his keen interest in my safety in the laboratory at all times!

I would like to take this opportunity to thank my parents for their unfailing support throughout my education.

Finally, I would like to thank Sue for her good-humoured tolerance over recent months and, not least, for her typing of this thesis.

ABSTRACT

1. The contribution of genetic factors to inter-individual variation in drug metabolism and the methods involved in their study have been reviewed.
2. The elimination kinetics of debrisoquine, a drug whose oxidative metabolism is known to be polymorphic, have been studied in volunteers of known oxidation phenotype. Subjects of the poor metaboliser phenotype (PM) displayed saturation kinetics with respect to pre-systemic alicyclic 4-hydroxylation of debrisoquine, resulting in higher peak and mean plasma concentrations of unchanged drug following an oral dose than were observed in extensive metaboliser (EM) subjects.
3. The 4-hydroxylation of debrisoquine has been studied in a population of Ghanaian volunteers and found to display polymorphism. The frequencies of the genes giving rise to the EM and PM traits were similar to those found in a comparable Caucasian population. The ethnic groups differed in that a third mode was observed in the Ghanaian frequency distribution, apparently corresponding to heterozygotes.
4. In both Caucasian and Ghanaian populations, the aromatic hydroxylation of debrisoquine paralleled alicyclic 4-hydroxylation, and is thus controlled by the same gene locus.
5. The metabolism of phenytoin has been studied in Caucasian volunteers of known oxidation phenotype. PM subjects displayed significantly slower elimination of this drug as the 4-hydroxylated metabolite than EM subjects. The formation of the same metabolite has been studied in Ghanaian volunteers of known oxidation phenotype. Marked inter-ethnic differences were observed, with both Ghanaian phenotypes excreting only one-third of the amount of metabolite observed in their Caucasian counterparts.

6. The metabolism of phenacetin has been studied in both a random Caucasian population and in selected individuals of known oxidation phenotype. The PM subjects studied were observed to be relatively deficient with respect to O-de-ethylation, whilst displaying a concomitant increase in the excretion of aromatic 2-hydroxylation products. The phenomenon of phenacetin 'rubruria', apparently related to enhanced 2-hydroxylation in the PM phenotype, has been investigated.

7. The theoretical basis of existing methods for studying inter-individual variation in metabolic oxidation capacity are discussed.

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CHAPTER ONE

INTRODUCTION

- 1.1. Introductory remarks
- 1.2. Scope of the present work

1.1. Introductory remarks:

'Pharmacogenetics', a term coined by Vogel (1959), deals with the contribution of genetic factors to the considerable inter - individual differences long known to exist in drug disposition and responsiveness. In recent years, the field has been the setting for considerable debate on two main fronts. The first aspect is essentially a 'nature versus nurture' argument similar to that waged over the 'inheritance' and development of intelligence in the earlier years of this century: to what extent is the variable under study the result of an individual's genetic constitution and to what extent the result of his environment? The second aspect concerns the nature of transmission of hereditary factors where the presence and significance of such factors has been demonstrated: is the factor the end - product of the expression of many genes of small, additive effect ('polygenic'), or the result of the expression of a single gene ('polymorphic' or 'monogenic')? The interactions of genetic factors, their possible modes of transmission and the environment can give rise to a number of population 'structures', summarized briefly below: (see also Figure 1.1)

1. Multifactorial control (Figure 1.1a): population studies show the variable in question to have a unimodal, Gaussian distribution. This structure will be seen regardless of whether the population variance (V_p) is due mainly to environmental factors (low 'heritability') or mainly to small, additive gene effects (high 'heritability'). The meaning of the term 'heritability' and methods for its assessment are discussed more fully below.
2. Genetic polymorphism, or monogenic inheritance (Figures 1.1b & c): in this situation the population is resolved into distinct modes or 'types' with respect to the variable under study: this occurs

Figure 1.1a: A unimodal population structure resulting from multifactorial control of interindividual variability.

Figure 1.1b: A bimodal population structure resulting from monogenic inheritance. The larger phenotype (P_1) comprises individuals both homo- and heterozygous for the allele giving rise to a dominant trait, the smaller (P_2) comprises individuals homozygous for the allele giving rise to the recessive trait; both alleles exert their effect at the same gene locus, and in this instance dominance is 100%.

Figure 1.1c: A trimodal population structure resulting from monogenic inheritance. The alleles considered are identical to those portrayed in Figure 1.1b; here the effect of reducing dominance to zero is shown. The mean value of the trait as displayed by heterozygous individuals (P_2) now lies midway between the mean values of the two homozygous groups (P_1 and P_3).

In each instance the terms V_p and σ refer to the phenotype variance and standard deviation respectively. In the case of multifactorial inheritance the population variance and phenotype variance are identical since the population consists of a single 'phenotype'.

Figure 1.1a:

Unimodal variation

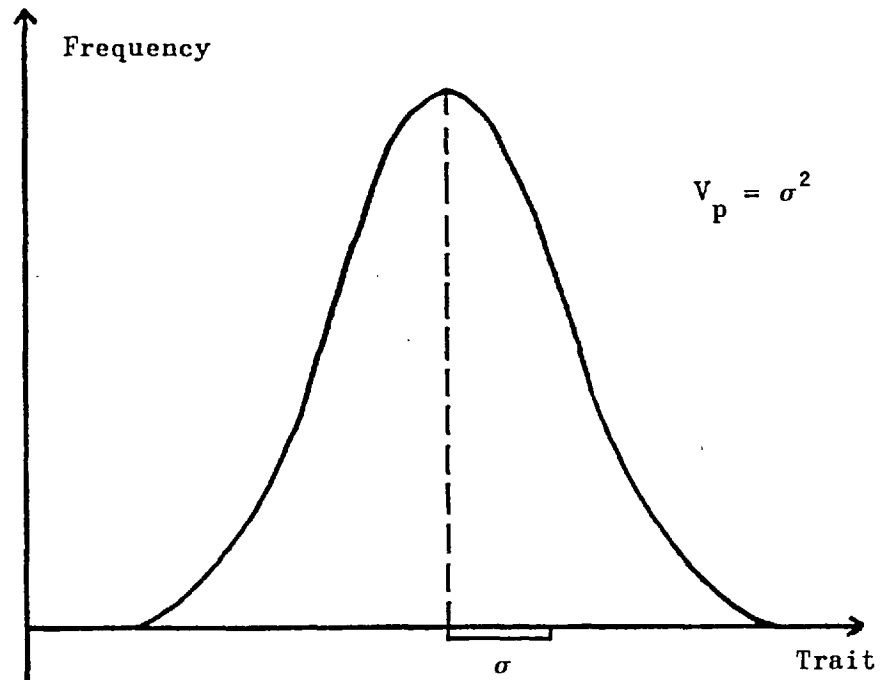


Figure 1.1b:

Bimodal variation

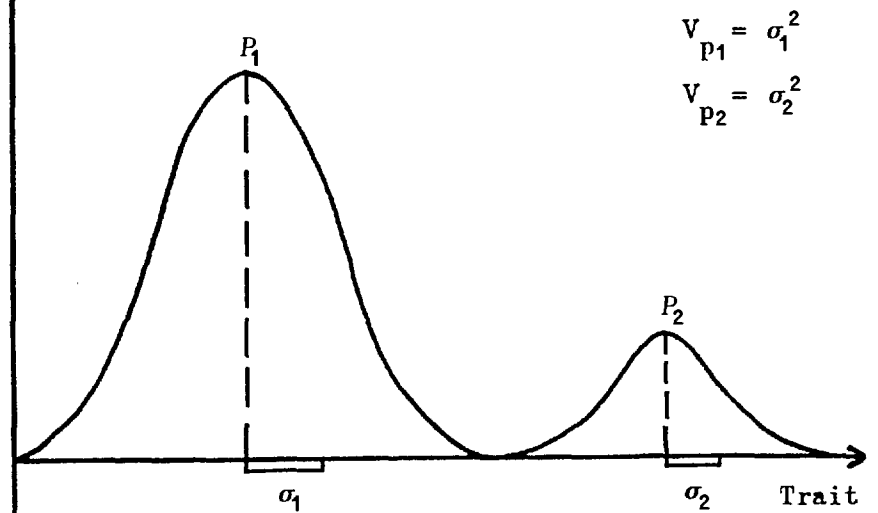
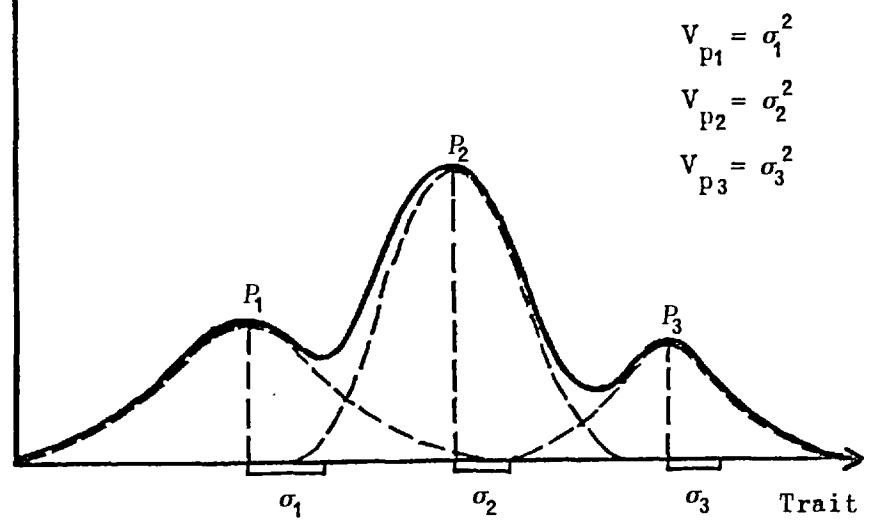


Figure 1.1c:

Trimodal variation



where a gene product, usually an enzymic protein, controls a rate - limiting step in the process under consideration. The number of modes seen in the population depends on the degree of dominance operating within the single gene effect. The simplest situation is considered in Figure 1.1b; an autosomal recessive character is displayed by 20 % of a hypothetical study population, and this character is subject to 100 % dominance. Any individual possessing the allele giving rise to the dominant trait will display that trait regardless of genotype, thus there will be no resolution of homo- and heterozygous individuals. Figure 1.1c shows a situation where dominance is incomplete, set here at zero. Heterozygous individuals are now partially resolved from the two homozygous genotypes and the overall population structure is trimodal.

It should be emphasized that these modes of inheritance are not mutually exclusive; it is quite conceivable that a trait may be polymodally distributed in a population, with a primarily monogenic mode of inheritance, whilst the degree of intra - phenotype variation (V_{P_1} , V_{P_2} and V_{P_3} in Figures 1.1b & c) is determined by polygenic factors; in other words a single gene of rate - limiting significance superimposed on a background of small additive gene effects.

Early studies of the relative contributions of environmental and genetic factors to inter - individual differences in drug metabolism were based on a methodology introduced by Galton (1875) and developed by Neel and Schull (1954). This approach is based on the comparison of a trait as displayed by identical (monozygotic) and fraternal (dizygotic) twins. Monozygotic twins have a common genetic constitution, whereas fraternal twins have, on average, only half their genes in common, thus intra - twin differences that are less in the former than

the latter will indicate the predominance of genetic factors over environmental. A quantitative assessment of this predominance (usually termed 'heritability') is given by the quotient:

$$(V_f - V_I) / V_f$$

where V_f and V_I are the variances within pairs of fraternal and identical twins respectively (Penrose, 1968). This approach has demonstrated predominantly genetic control over the rate of elimination from plasma of antipyrine (Vesell and Page, 1968a), phenylbutazone (Vesell and Page, 1968b), bishydroxycoumarin (Vesell and Page, 1968c), nortriptyline (Alexanderson et al., 1969), ethanol (Vesell et al., 1971) and halothane (Cascorbi et al., 1971).

A second approach to the 'heritability' question in general was proposed by Fisher (1918) and applied to drug disposition in particular by Whittaker and Evans (1970): in this instance the heritability is determined by assessing the degree of regression of mean offspring values of a given trait on mid - parent values; family studies of nortryptiline (Åsberg et al., 1971) and phenylbutazone (Whittaker and Evans, 1970) disposition confirmed the predominance of genetic factors suggested by twin studies.

Whilst either methodology will yield information as to the relative importance of inherited and environmental factors, neither will give any indication of the mode of inheritance of the genetic component. Indeed, it can be argued that both methodologies are only valid where it can be assumed that the trait in question has a unimodal, Gaussian distribution in the general population, since both rely on parameters (variances and / or regression coefficients) which are only applicable to this form of distribution.

A more direct approach is to simply perform population studies and inspect the form of frequency distribution obtained. Unfortunately,

this methodology too has its drawbacks. First there is the problem of reproducibility, both intra - individual and inter - study. From 1975 - 5, Kellerman et al., (1973a & b, 1975) published three studies demonstrating firstly that antipyrine and phenylbutazone half - lives and arylhydrocarbon hydroxylase (AHH) inducibility in cultured mitogen - stimulated lymphocytes each had a trimodal distribution in healthy subjects, and that strong correlations could be drawn between any two of these three 'indices' of carbon oxidation capacity. The second study of AHH inducibility in 50 patients with bronchial carcinoma, 46 patients with tumours arising from other sites and 85 healthy controls, led to the conclusion that the 'high' and 'intermediate' modes of inducibility were 36 and 16 times more susceptible to bronchial carcinoma respectively than was the 'low' inducibility mode. The implication that carbon oxidation is controlled by two alleles acting at a single gene locus was clear, and the data gave an excellent fit to the Hardy - Weinberg equilibrium. However, widespread reporting of poor reproducibility for the AHH determination (Nebert and Atlas, 1976 inter al.) and Vesell's claim (1972) that the plasma half - life of antipyrine has a unimodal, Gaussian distribution in the general population have led to the monogenic hypothesis being regarded with considerable scepticism.

A second problem in population studies is exemplified by the work of Kalow and Genest (1957) on plasma pseudocholinesterase. Shortly after the introduction of the muscle relaxant suxamethonium in 1952, a number of otherwise healthy patients displayed hypersensitivity to the drug, expressed as prolonged paralysis and apnoea. Lehmann and Ryan (1956) studied the families of five hypersensitive probands and concluded that the phenomenon was the result of an atypical (low activity) plasma pseudocholinesterase inherited as an autosomal recessive trait.

However, when plasma pseudocholinesterase activity was studied in a random population, a unimodal distribution was found with atypical individuals aggregated in the lower tail. It was not until Kalow and Genest (1957) utilized dibucaine as a differential inhibitor of the normal and atypical plasma enzymes that the existence of three distinct phenotypes could be demonstrated, in other words the population structure obtained with respect to this trait depended primarily on the methodology applied to its study.

These problems notwithstanding, a number of significant pharmacogenetic traits displaying polymorphism have been identified in the last thirty years: these traits and their clinical consequences are summarized in Table 1.1. Two other traits should be mentioned in the context of the present work, though neither has yet been observed with sufficient frequency to be termed a polymorphism per se. The first is deficient aromatic 4-hydroxylation of phenytoin associated with increased phenytoin toxicity (Kutt et al., 1964), and the second deficient 0-deethylation of phenacetin, associated with increased drug - induced methaemoglobinaemia (Shahidi, 1967). The former defect is considered in detail in Chapter Four and the latter in Chapter Five.

The polymorphism of debrisoquine oxidation (Mahgoub et al., 1977) is unique in that it is the only polymorphism of carbon oxidation described to date. The metabolism of this compound is shown in Figure 1.2; it has been established that some 10 % of the British white Caucasian population is deficient with respect to the alicyclic 4-hydroxylation pathway. Two phenotypes have been defined in terms of the quotient:

$$\frac{\% \text{ dose excreted unchanged as debrisoquine}}{\% \text{ dose excreted as 4-hydroxydebrisoquine}}$$

in a bulked 0 - 8 h urine sample, the 'metabolic ratio' (MR). The major phenotype, 'extensive metabolisers' (EM), comprise 90 % of the

Table 1.1: Genetic polymorphisms of recognized pharmacological significance.

* D or R refers to a dominant or recessive trait respectively where this has been ascertained.

** The phenotypes listed are restricted to those for which a significant clinical consequence of their genetic constitution has been shown to occur.

Compiled from: Vesell (1975)

Evans (1977)

Ritchie et al. (1980)

<u>Trait</u> *	<u>Alleles known</u>	<u>Phenotypes</u> **	<u>Clinical consequences</u>
Rapid Acetylation (D)	2 autosomal	Rapid (60 %)	Isoniazid toxicity (Hepatitis)
Slow Acetylation (R)		Slow (40 %)	Isoniazid toxicity (Peripheral neuropathy) Hydrallazine toxicity (SLE-like syndrome)
Atypical plasma pseudocholinesterase (R)	4 autosomal	Atypical (2 %)	Suxamethonium sensitivity (apnoea)
Atypical liver alcohol dehydrogenase	23 autosomal	Rapid metaboliser	Enhanced ethanol metabolism
Glucose - 6 - phosphate dehydrogenase (R)	Many - all X - linked	G6PD deficient (up to 25 %)	Drug induced haemolysis especially after non - narcotic analgesics, sulphonamides, antimalarials and some antibacterials.
Hyper - responsiveness of ocular pressure to topical steroids (R)	2 autosomal	Hyper-responsive	Beta - or dexamethasone induced intraocular hypertension.
Methaemoglobin reductase deficiency (R)	2 autosomal	Deficient (2 %)	Dapsone / antimalarial induced methaemoglobinaemia and cyanosis.
Debrisoquine alicyclic oxidation (R)	2 autosomal	Poor metabolisers (10 %)	Enhanced hypotensive responsive- ness to debrisoquine.
Sparteine metabolism (R)	2 autosomal	Poor metabolisers (10 %)	Prolonged plasma $t_{1/2}$ of sparteine.

Figure 1.2: The metabolism of debrisoquine in man; the figures in parentheses indicate the proportion of a 10 mg oral dose excreted in urine (0 - 8h) in a given form.

(Idle et al., 1979a)

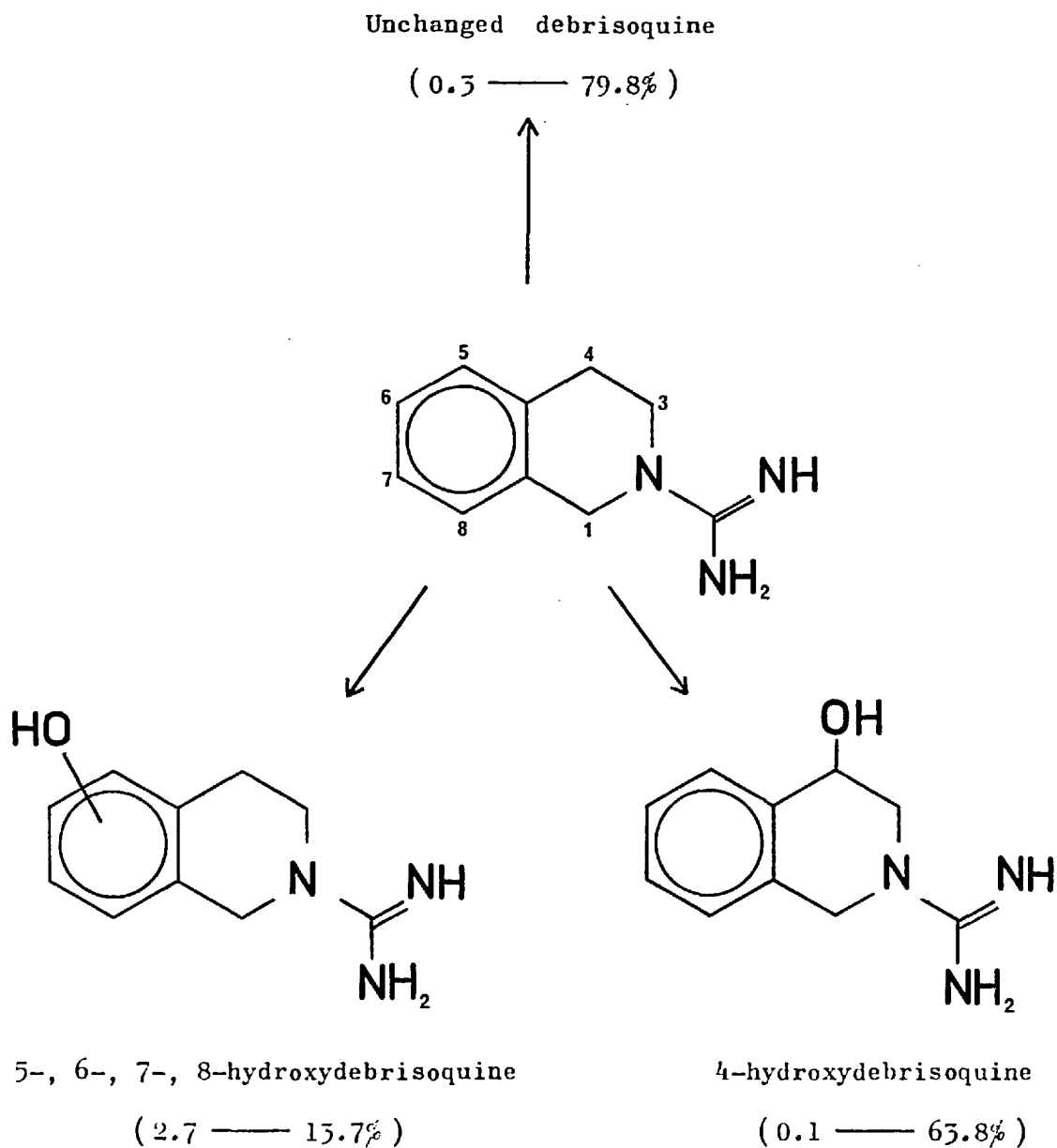
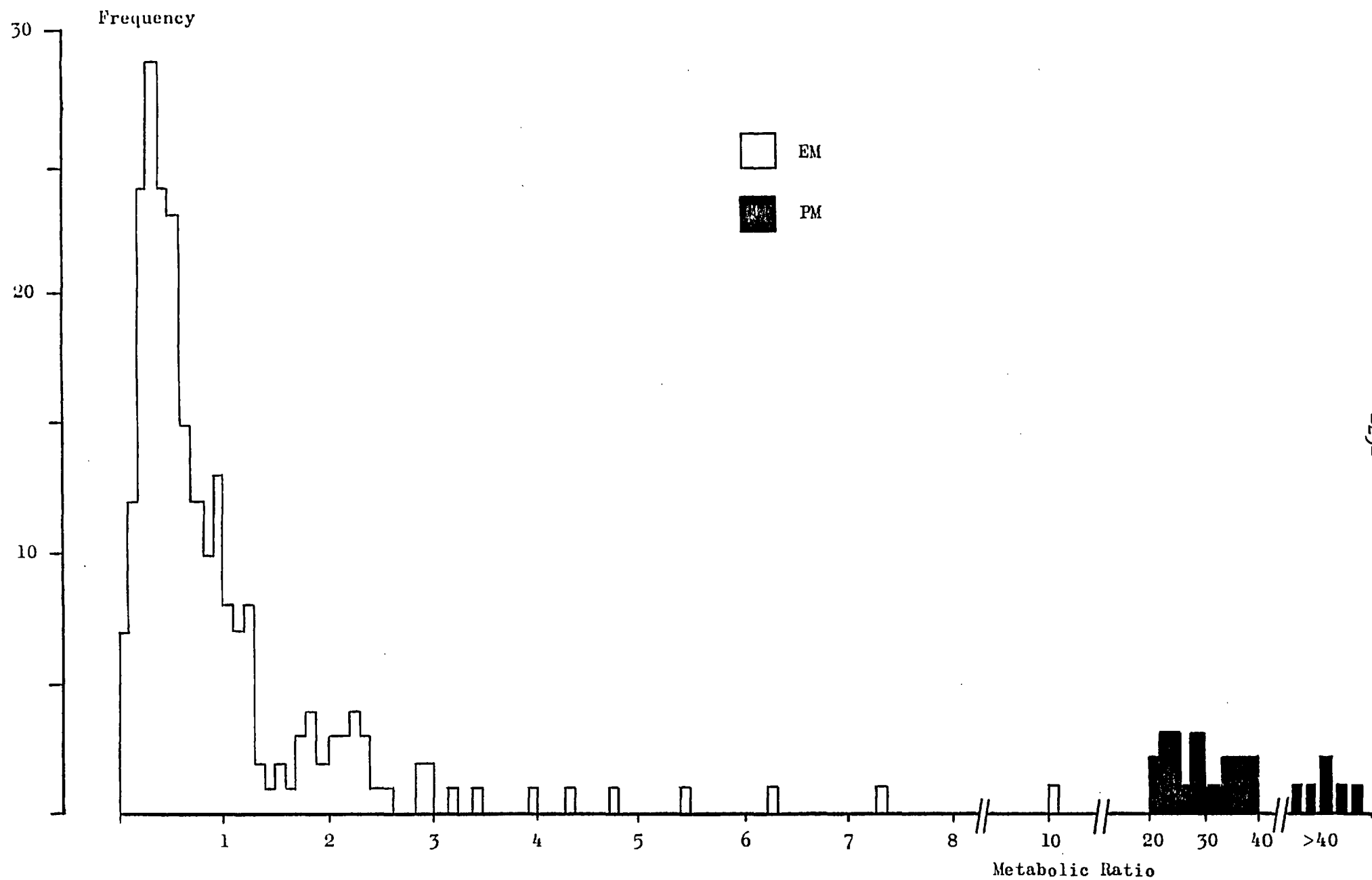


Figure 1.3: Frequency distribution of the debrisoquine metabolic ratio in 258 unrelated British white Caucasians.
(Evans et al., 1980).



population, and have MR values in the range 0.01 - 10; 'poor metabolisers' (PM) comprise 10 % of the population, displaying MR values in the range 20 - 200. The distribution of MR values in 258 unrelated British white Caucasians is shown in Figure 1.3.

The potential significance of polymorphic carbon oxidation has been stressed by Kellermann et al. (1973b) in that the activation of a variety of chemical carcinogens is thought to depend on oxidative attack of carbon centres (Miller and Miller, 1974), and hence genetically determined oxidation status may well provide a predisposition to (or indeed, protection from), those forms of carcinoma whose aetiology involves oxidative activation.

Consideration of this fact and the 'clinical consequences' section of Table 1.1 emphasizes that the debate over the relative importance of genetic factors and the environment in the determination of drug metabolizing capacity is not merely academic, nor is the search for polymorphic variation in this field an esoteric exercise. Unlike his environment, an individual's genetic constitution is immutable, and the investigation of that constitution may potentially yield many predictive markers as to the likely effectiveness, toxicity and carcinogenicity of drugs and other foreign compounds.

1.2. Scope of the present work:

In 1969, the NAS Drug Research Board Committee on Problems of Drug Safety reported on the applications of data derived from studies of drug metabolism to the evaluation of new drugs. The field of pharmacogenetics came within the scope of their deliberations, and it was concluded that extensive exploration of pharmacogenetic variations in man was needed, with the following being listed as "urgent needs":

A. The relationship of known pharmacogenetic examples to the metabolism

of other drugs should be investigated.

- B. Improved methods are required to detect pharmacogenetic traits so that more extensive studies can be made.
- C. The possibility that persons who metabolize one drug slowly may also metabolize other drugs slowly should be clarified.
- D. The relative role of genetic and environmental factors in determining individual differences in drug metabolism should be further explored.

Elsewhere in their report the Committee also noted that extensive inter - ethnic variation occurred in the then known pharmacogenetic polymorphisms.

The present work was undertaken to answer at least some of these questions in relation to the only drug oxidation unambiguously shown to display genetic polymorphism in man, the alicyclic 4-hydroxylation of debrisoquine.

The primary objectives may be summarized as follows:

1. An examination of the relationship between debrisoquine oxidation status and the capacity to effect the other two carbon oxidation reactions suspected of being subject to a single gene defect, namely the O-de-ethylation of phenacetin and the aromatic 4-hydroxylation of phenytoin.
2. An examination of the pharmacokinetic consequences of defective carbon oxidation.
3. An examination of inter - ethnic variation in gene frequency and gene expression with respect to polymorphic carbon oxidation.

In addition to the above it was anticipated that these studies would provide scope for a critical assessment of current 'pharmacogenetic methodology'. In recent years, a number of authors have expressed surprise at the scarcity of polymorphisms of carbon oxidation:

(Scott and Poffenbarger, 1979; Eichelbaum et al., 1980). However, the bulk of studies into variation of carbon oxidation published to date have been based on either plasma elimination half - life or plasma clearance of the substrate under study rather than on the rate of appearance of metabolites. It has recently been pointed out (du Souich et al., 1979) that the use of metabolite kinetics has improved resolution of acetylator phenotypes, and it seems reasonable to suppose that application of such an approach to carbon oxidation will yield a more reliable assessment of the population genetic structure with respect to this, the most commonly encountered Phase I biotransformation pathway.

CHAPTER TWO

HYDROXYLATION PHENOTYPE AND THE PHARMACOKINETICS OF DEBRISOQUINE

2.1. Introduction

2.2. Materials and methods:

2.2.1. Subjects and analytical methods

2.2.2. Treatment of data

2.3. Results:

2.3.1. The elimination of debrisoquine after oral dosing

2.3.2. The formation of 4-hydroxydebrisoquine after oral dosing

2.3.3. The elimination of 4-hydroxydebrisoquine

2.3.4. Plasma levels of debrisoquine after oral dosing

2.3.5. Plasma levels of debrisoquine after intravenous dosing

2.4. Discussion

2.1. Introduction:

The usefulness of debrisoquine as an anti-hypertensive agent in recent years has been somewhat restricted by the wide variation in the daily dose required to control the blood pressure of different individuals, (10 - 360 mg/day, Silas et al., 1977). This inter-individual variation has been rationalized by the discovery of the polymorphic nature of debrisoquine metabolism, as previously described (pp. 20-26, and Mahgoub et al., 1977).

A study by Silas et al., (1978) of debrisoquine disposition in hypertensive patients receiving multiple doses of the drug suggested that hypotensive responsiveness was related to the concentration of debrisoquine in plasma, but this study was not concerned with the influence of oxidation phenotype on the magnitude of the plasma levels obtained. It has subsequently been demonstrated by Idle et al. (1978) that subjects of the PM phenotype display marked postural hypotension in response to oral doses of debrisoquine which have no discernible effect on the blood pressure of EM subjects.

Scope of the present study:

The studies of debrisoquine pharmacokinetics reported here were undertaken to establish the qualitative and quantitative influences of the hydroxylation defect on the elimination of the drug from plasma, and to its effect on the systemic availability of a single oral dose.

It would be anticipated that defective metabolic inactivation would increase availability and impair elimination, and thus correlate with enhanced hypotensive responsiveness.

The studies of the urinary elimination of debrisoquine and 4-hydroxydebrisoquine were undertaken for a number of reasons. There is an at least theoretical possibility that the defect observed results from an

inability to excrete rather than form 4-hydroxydebrisoquine. Furthermore, it is possible that measurement of drug and metabolite in a single bulked urine sample taken over an arbitrary time period may disguise the true quantitative variation between phenotypes. Both these possibilities may be examined from urinary elimination data which firstly indicate whether formation or elimination is rate-limiting for the appearance of 4-hydroxydebrisoquine in urine, and secondly permit estimation of apparent first order metabolite formation rate constants. Since these are estimated from integration over an indefinite time period they will provide the most objective criterion for assessment of inter-phenotype variation in hydroxylation capacity.

2.2. Materials and methods

2.2.1. Subjects and analytical methods

Plasma pharmacokinetics: Three subjects were studied following intravenous dosing, one homozygous poor metabolizer, one homozygous extensive metabolizer and one individual, phenotypically extensive, but known to be genotypically heterozygous from family studies.

A total of seven subjects were studied after oral dosing with debrisoquine, three of whom were poor metabolizers, and four extensive.

Urinary elimination: Urinary data was obtained from all the subjects participating in both intravenous and oral dosing studies. In addition two other poor and one other extensive metabolizer were studied after oral dosing, thus the totals for studies of urinary elimination following a single oral dose were EM, $n = 5$ and PM, $n = 5$.

Protocol:(I.V. dosing): Three subjects each received 12.8 mg

debrisoquine hemisulphate, equivalent to 10 mg free base, injected in 5 ml isotonic saline as a 2 min infusion. The dose was sterilised prior to injection by Millipore filtration. Venous blood samples were taken at 2.5, 5, 10, 20, 30, 45, 60, 90, 120, 150 and 180 min during the first three hours post-dosing, then hourly to 8 h. In all instances blood samples were collected from an indwelling catheter inserted in the cephalic vein of the forearm. The blood collected was placed in tubes containing lithium heparin impregnated granules, and centrifuged immediately, (1000 x g, 10 min). Plasma was stored at -20°C prior to analysis. Urine samples were obtained half-hourly in the first three hours post-dosing, then hourly to 8 h. Urine sampling was then continued ad libitum for a minimum of 24 and a maximum of 48 h. A 20 ml aliquot of each measured urine sample was stored at -20°C prior to analysis.

Protocol:(Oral dosing): Each subject received the free-base equivalent of 10 mg debrisoquine given orally as Declinax (Roche) tablets. Venous blood samples were obtained by venepuncture at 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h post-dosing and treated in the manner described above. Urine samples were obtained hourly for eight hours post-dosing. Three of the PM subjects studied provided additional urine samples passed ad libitum for periods of 24-48 h post-dosing. All urine samples were treated in the manner described above.

Analysis: Debrisoquine (D) and 4-hydroxydebrisoquine (4HOD) were assayed in plasma and urine using modifications of the method of Erdtmansky and Goehl (1975) for guanidine compounds in biological fluids. Derivatisation of the guanidino- group with hexafluoroacetylacetone (HFAA) yields readily extractable pyrimidines suitable for electron capture detection gas chromatography. The assay procedures for plasma and urine were essentially similar, differing only in the

relative volumes of biological fluid and reagents employed.

Plasma analysis: Screw-top septum vials (15 ml) were used for derivatization. To each vial was added 0.4 ml test or standard (5 - 100 ng/ml D or 4HOD) plasma followed by 25 ng 7-methoxyguanoxan in 50 μ l distilled water as internal standard. The plasma was buffered with 0.6 ml 1M sodium bicarbonate, followed by the addition of benzene (3.0 ml) and HFAA (0.3 ml). The vials were heated in an aluminium heating block at 100°C for 1 h. After heating 3M NaOH (10 ml) was added to hydrolyse excess HFAA. The samples were mixed by vortex agitation for 10 sec, then centrifuged for 30 min at 1000 x g; 1 - 5 μ l of the organic phase was taken for ECD--GLC analysis. The limit of sensitivity of the assay was 5 ng/ml and calibration was curvilinear over the stated range (Figs 2.1a & b). The coefficient of variation did not exceed 10 % for any of the calibration standards.

Urine analysis: The experimental procedure was identical to that described for plasma analysis, other than that the volumes of urine and reagent employed were as follows: 1M sodium bicarbonate (0.1 ml), urine (0.1 ml), benzene (1.0 ml), HFAA (0.05 ml) and 3M sodium hydroxide (5.0 ml). The amount of internal standard employed was 0.5 μ g in 0.05 ml distilled water. Calibration was linear over the range 0.5 - 10 μ g/ml with respect to both debrisoquine and 4-hydroxydebrisoquine (Figs 2.2a & b).

Debrisoquine and 4-hydroxydebrisoquine were the gift of Roche Products Ltd. and 7-methoxyguanoxan was the gift of Pfizer Ltd. HFAA was obtained from Koch-Light Ltd. All other reagents were AnalaR reagent grade, used without further purification.

The methods employed a Packard Model 417 gas-liquid chromatograph,

Figure 2.1a: Typical GLC trace obtained from derivatized plasma of volunteer receiving 10 mg debrisoquine p.o.

Figure 2.1b: Calibration curves for debrisoquine and 4-hydroxydebrisoquine added to blank human plasma. (Points represent mean $\pm$ S.D. for three determinations.

D = debrisoquine

4HOD = 4-hydroxydebrisoquine

7CH₃OG = 7-methoxyguanoxan (Internal standard)

HFAA = hexafluoroacetylacetone

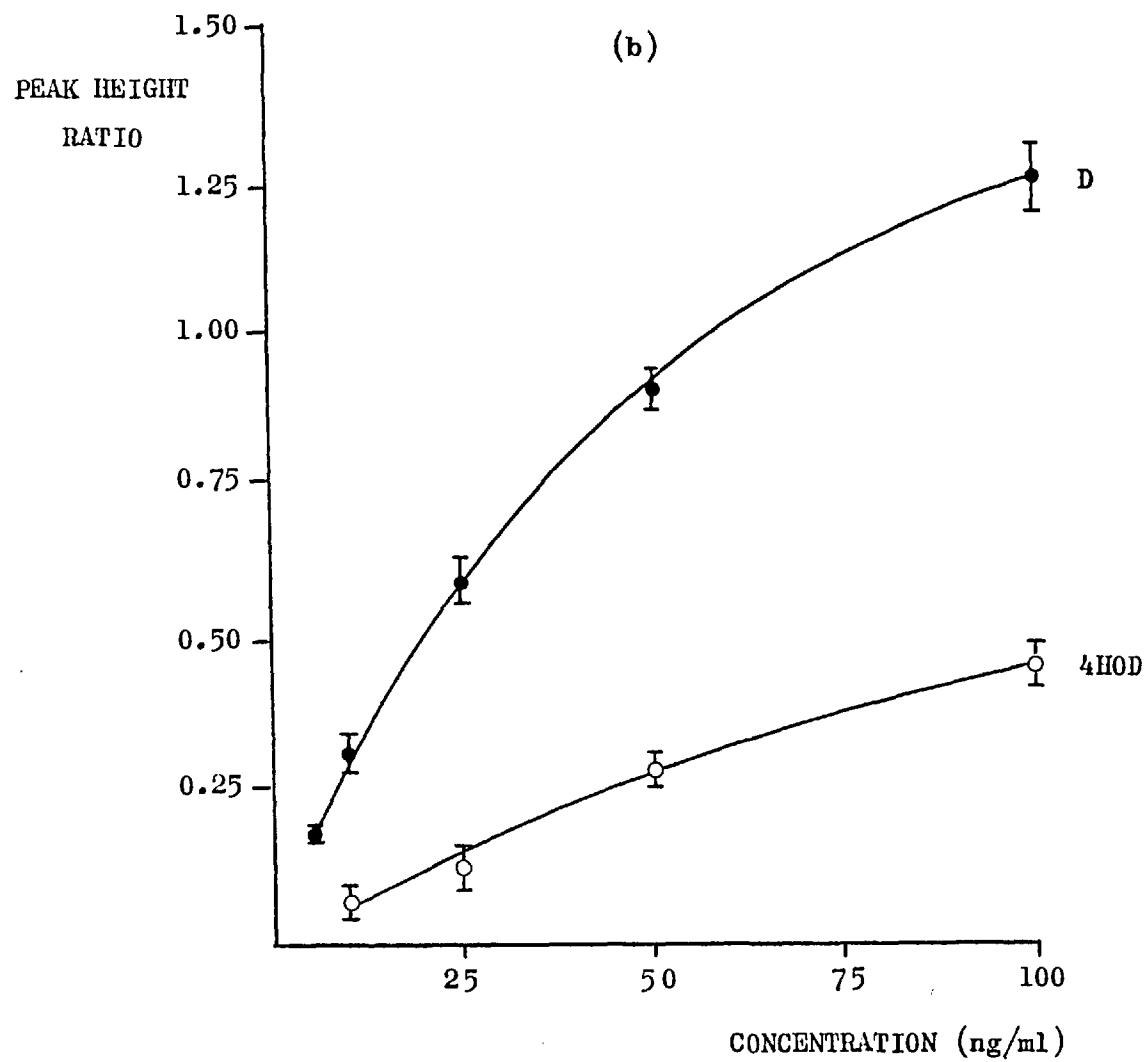
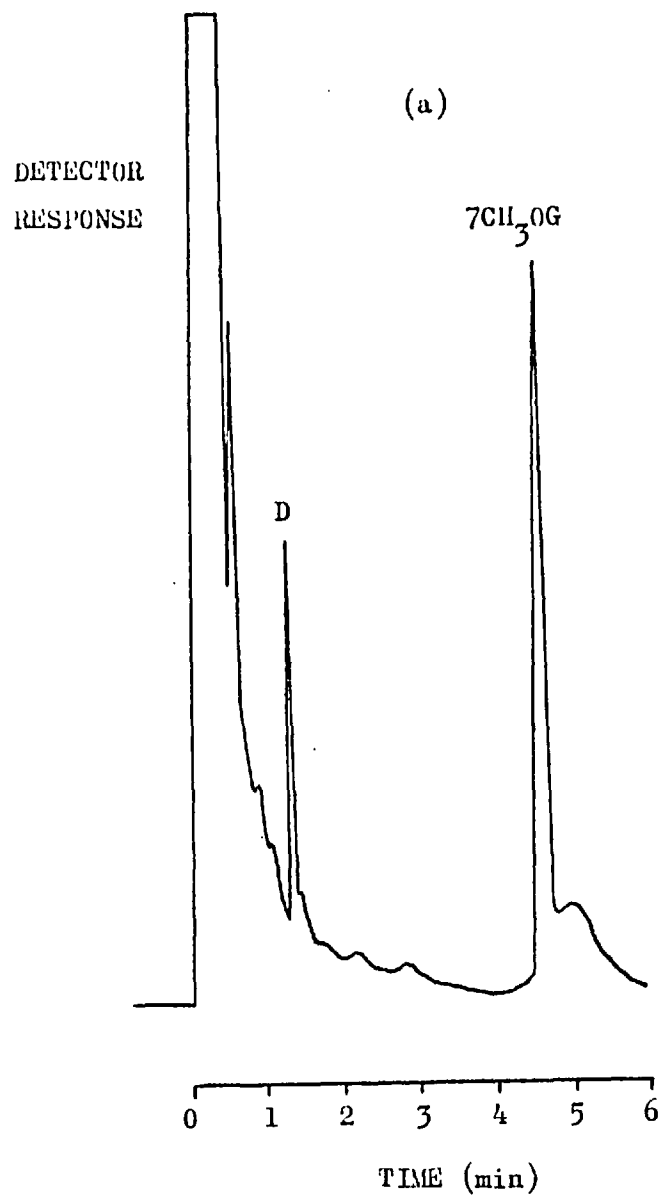
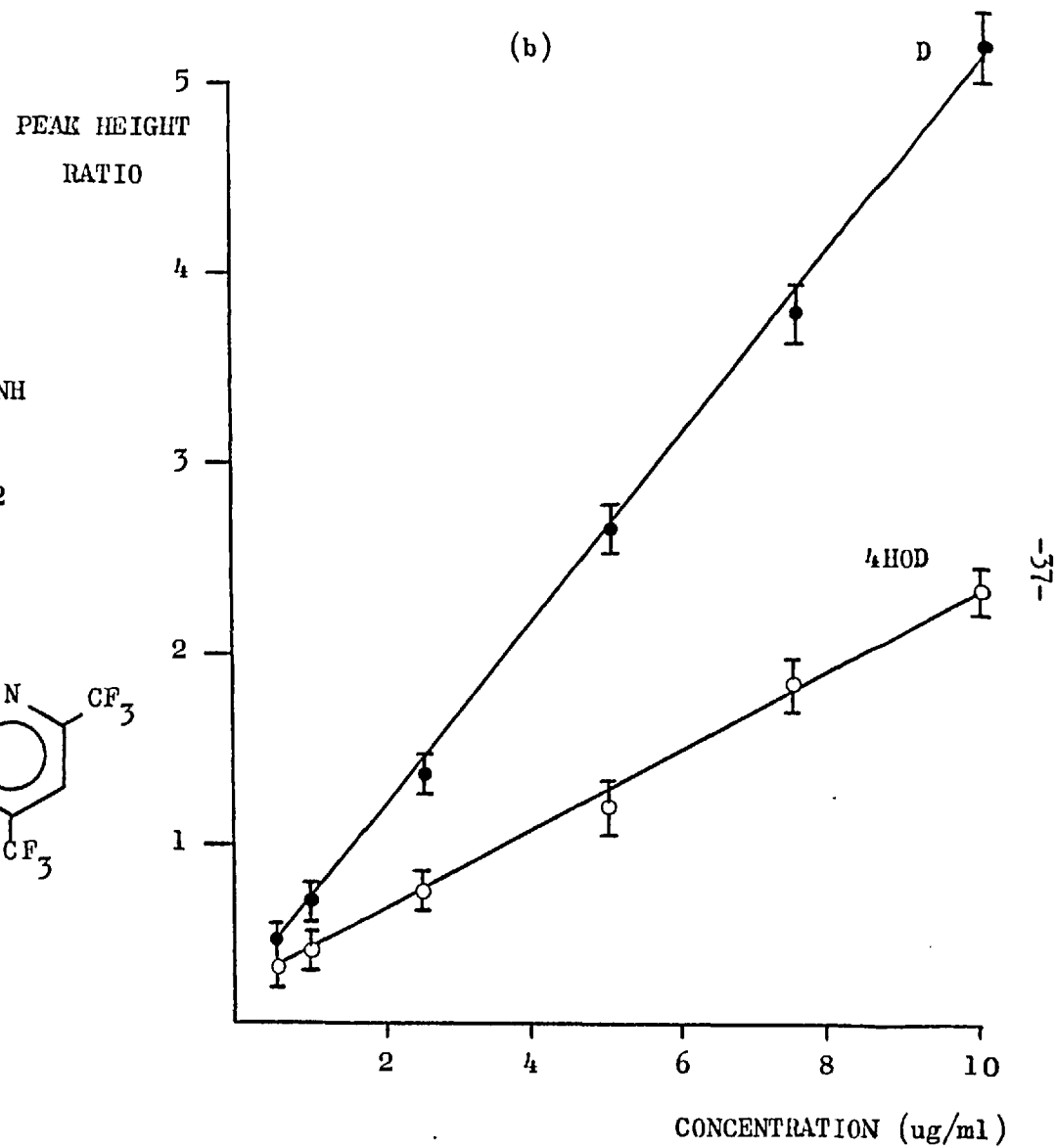
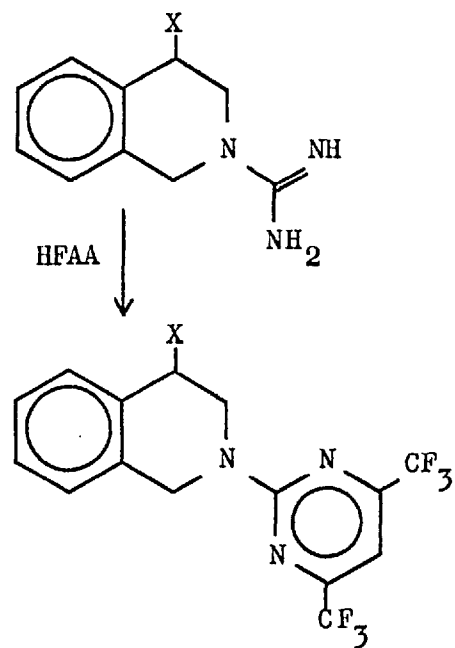
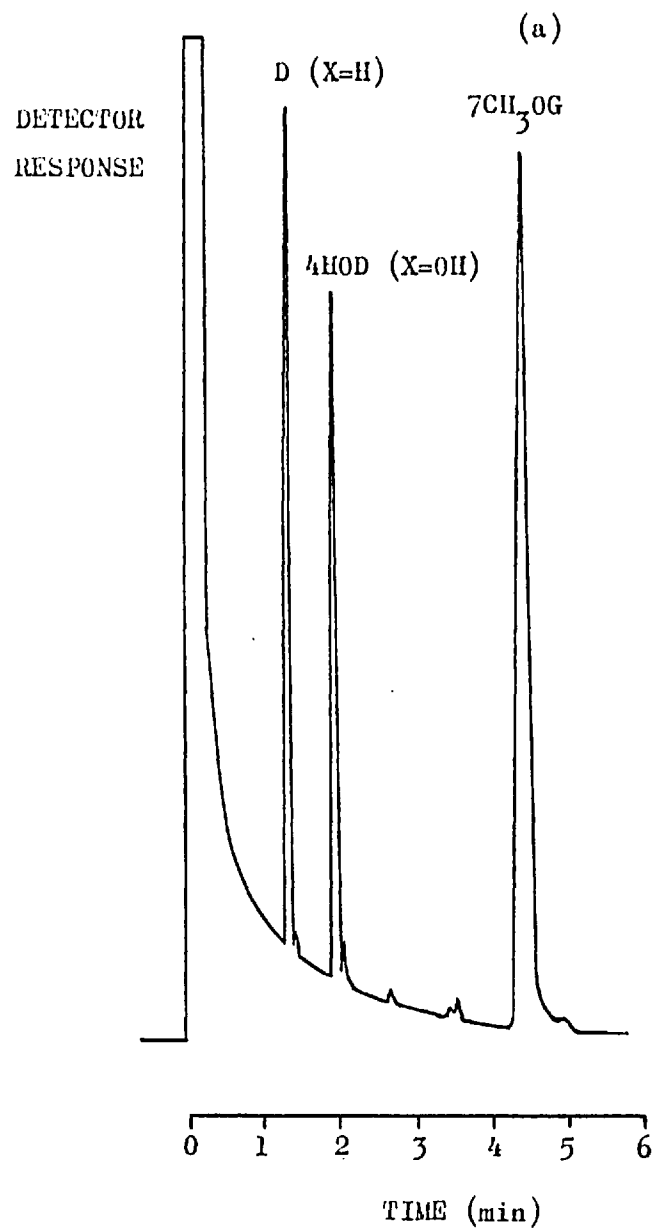


Figure 2.2a: Typical GLC trace obtained from derivatized urine of volunteer receiving 10 mg debrisoquine p.o.

Figure 2.2b: Calibration curves for debrisoquine and 4-hydroxy-debrisoquine added to blank human urine. (Points represent mean $\pm$ S.D. for five determinations).



equipped with a tritium - scandium electron - capture detector (75 mCi). The 1.8 m column, i.d. 3 mm, was packed with 3 % OV - 1 on Chromosorb WHP 80 - 100 mesh. The carrier gas (95 % argon, 5% methane) flow rate was 60 ml/min. The oven, detector and injection port temperatures were 195°C, 235°C and 250°C respectively.

2.2.2: Treatment of data:

Plasma pharmacokinetics: Following intravenous dosing, log plasma concentration - time curves were plotted for each subject. The decline in debrisoquine concentration with time was found to be biexponential, and these data were thus analysed according to a two compartment open model, assuming first order drug transfer and elimination. Initial estimates of the hybrid rate constants α and β and the intercept terms A' and B' were obtained by least squares regression analysis and the method of residuals. The intercept terms were then corrected for the duration of the infusion given (0.033 h), as described by Loo and Riegelman (1970). The corrected intercept terms A and B were calculated thus:

$$A = \frac{0.033}{1 - e^{-0.033\alpha}} \cdot A' \quad (1)$$

$$B = \frac{0.033}{1 - e^{-0.033\beta}} \cdot B' \quad (2)$$

The first order constants describing drug distribution and elimination were calculated from A , α , B , and β in the usual manner (Greenblatt and Koch-Weser, 1975). Renal clearance over the 8 h period following dosing was calculated from the expression:

$$\text{Renal Clearance} = \frac{\bar{C}_p}{\bar{C}_u} \cdot \bar{Q}_u \quad (3)$$

Where $\bar{C}_p$ = Mean debrisoquine plasma concentration (0 - 8 h)

Calculated as:

$$\frac{\frac{A}{\alpha} + \frac{B}{\beta} - \frac{D}{\beta}}{8} \quad (4)$$

Where D is the debrisoquine plasma concentration at 8 h post - dosing and

$\bar{C}_u$ = Mean debrisoquine urine concentration (0 - 8 h)

Calculated as:

$$\frac{\text{Total debrisoquine excretion 0 - 8 h}}{\text{Total urine volume 0 - 8 h}} \quad (5)$$

and $\bar{Q}_u$ is mean urine flow rate (0 - 8 h)

Calculated as:

$$\frac{\text{Total urine volume 0 - 8 h}}{8 \times 60} \quad (6)$$

Following oral dosing, the decline in debrisoquine plasma concentration with time was monoexponential over the period studied after a brief absorption phase. For each subject studied the following parameters were calculated: the peak debrisoquine plasma concentration, being simply the highest recorded concentration; the mean debrisoquine plasma concentration over the 0 - 8 hour study period, as calculated from equation (7):

$$\bar{C}_{0-8} = \text{AUC}_{0-8} / 8 \quad (7)$$

where the 0 - 8 h area under the plasma concentration - time curve was estimated by the trapezoidal rule; the total area under the plasma - concentration - time curve ($t = 0 \rightarrow t = \infty$), as calculated from equation (8):

$$\text{AUC}_{0-\infty} = \text{AUC}_{0-8} + \frac{D}{K_{el}} \quad (8)$$

where D is the plasma concentration of debrisoquine and K_{el} is the terminal slope of a semi-logarithmic plot of debrisoquine plasma concentration against time ($\ln C$ vs. t), and finally the elimination half-life, calculated from equation (9):

$$t_{\frac{1}{2}} = \frac{\ln 2}{K_{el}} \quad (9)$$

The plasma debrisoquine levels obtained after oral dosing were analysed in this way because it is generally recognized that the plasma clearance of drugs undergoing presystemic metabolism is determined by hepatic blood flow (Raaflaub and Dubach, 1975) rather than metabolic capacity per se, thus inter-individual differences in the ability to perform alicyclic 4-hydroxylation of debrisoquine will be reflected in the amount of drug escaping 'first - pass' metabolism, i.e. in the peak plasma concentration and systemic availability rather than in the terminal half-life or apparent metabolic clearance rate.

Urinary elimination kinetics: Following intravenous dosing log excretion rate - time curves were plotted for each of the three subjects studied with respect to both debrisoquine and 4-hydroxydebrisoquine. In all cases, the decline in excretion rate with time was apparently biexponential, but too erratic to permit estimation of elimination parameters in the usual fashion (Cummings et al., 1967). Accordingly, the data were utilised only for the calculation of ultimate urinary recoveries of debrisoquine and 4-hydroxydebrisoquine and of renal clearance of debrisoquine. Following oral dosing, the five EM subjects yielded parallel log - linear excretion rate curves with respect to both debrisoquine and 4-hydroxydebrisoquine. These data were therefore analysed by the methods of Cummings et al., (1967), with the following modification:

$$\text{Given that: } \ln[D_{u\infty} - D_u] = \ln \frac{k_d \cdot \text{Dose}}{K} - Kt \quad (10)$$

$$\text{and: } \ln[M_{u\infty} - M_u] = \ln \frac{k_u \cdot k_f \cdot \text{Dose}}{Kk_u - K^2} - Kt \quad (11)$$

Where: D_u = Cumulative excretion of debrisoquine at time t .

$D_{u\infty}$ = Ultimate excretion of debrisoquine at $t = \infty$.

M_u = Cumulative excretion of 4-hydroxydebrisoquine at time t .

$M_{u\infty}$ = Ultimate excretion of 4-hydroxydebrisoquine at time $t = \infty$.

K = Overall elimination rate constant.

k_d = Apparent first - order constant describing elimination of unchanged debrisoquine in urine.

k_f = Apparent first - order constant describing formation of 4-hydroxydebrisoquine from debrisoquine.

k_u = Apparent first - order constant describing elimination of 4-hydroxydebrisoquine in urine.

It will be seen that K may be obtained from a semilogarithmic 'Sigma - minus' plot: $\ln[D_{u\infty} - D_u]$ vs. t , where t is the end point of each urine collection period. In the past it has been usual to estimate $D_{u\infty}$ by extrapolation to a 'negligible' excretion rate, but in fact a simpler and more accurate procedure is to evaluate the two integrals:

$$\int_0^{t'} dD_u / dt \cdot dt \quad \text{and} \quad \int_{t'}^{\infty} dD_u / dt \cdot dt$$

where t' is the full period over which urine was collected. The former integral may be evaluated as the observed recovery of D from $0 - t'$, and the latter according to equation (12).

$$\int_{t'}^{\infty} dD_u / dt \cdot dt = \frac{\text{Excretion rate at } t'}{K'} \quad (12)$$

This method requires only a small extrapolation from the midpoint of the final period to the end of that period (t'); K' is determined as the terminal slope of the semi-logarithmic plot $\ln dD_u / dt$ against time. Equation (13) now yields a value for $D_{u\infty}$:

$$D_{u\infty} = \text{Observed recovery} + \frac{\text{Excretion rate at } t'}{K'} \quad (13)$$

$M_{u\infty}$ was calculated in an identical manner from the metabolite excretion rate plot. In turn, $D_{u\infty}$ and $M_{u\infty}$ may be used to estimate k_d and k_f from equations (14) and (15) respectively:

$$k_d = K \cdot \frac{D_{u\infty}}{\text{Dose}} \quad (14)$$

$$\text{and} \quad k_f = K \cdot \frac{M_{u\infty}}{\text{Dose}} \quad (15)$$

It should be stressed that these equations yield minimum estimates of k_d and k_f since they assume quantitative absorption of the dose. In addition, k_u was evaluated for each EM subject using a modified version of the method of Martin (1967). This method is derived from the simple first - order statement (assuming no further metabolism of M):

$$\text{Rate of metabolite excretion} = k_u \cdot M_B$$

M_B is the 'body load' of metabolite, given by

$$M_B \text{ at } t = \text{Dose} \frac{k_f}{K} (1 - e^{-Kt}) - M'_{ut} \quad (16)$$

where M'_{ut} is the cumulative excretion of metabolite at the end, rather than the mid-point of the collection period, estimated in each case by the appropriate interpolation. It can be seen from equation (15) above that equation (16) simplifies to:

$$M_B \text{ at } t = M_{u\infty} (1 - e^{-Kt}) - M'_{ut} \quad (17)$$

All k_u values quoted were obtained as the slopes of the plot: observed 4-hydroxydebrisoquine excretion rate vs. calculated 4-hydroxydebrisoquine body load, determined by least - squares regression analysis.

In contrast, the five PM subjects studied deviated markedly from first - order linearity with respect to 4-hydroxydebrisoquine excretion. In this instance a statistical analysis based on the approach of Levy et al. (1972) was employed. If it can be assumed that formation and not excretion is the rate - limiting step for the appearance of 4-hydroxydebrisoquine in urine then it is possible to apply one of the standard linearized forms of the Michaelis-Menten equation to non - first - order urinary excretion rate data thus: (the 'Hanes' plot).

$$\frac{D}{V} = D \cdot \left[\frac{1}{V_{\max}} \right] + \frac{K_m}{V_{\max}} \quad (18)$$

where: D = The 'body load' of debrisoquine.

V = The observed excretion rate of 4-hydroxydebrisoquine in urine.

$V_{\max}$ = The maximal excretion rate of 4-hydroxydebrisoquine in urine.

K_m = The body load of debrisoquine yielding a 4-hydroxydebrisoquine excretion rate of 50 % maximum.

D at time t was calculated from equation (19):

$$D = \text{Dose} \cdot e^{-Kt} \quad (19)$$

where K was estimated from excretion of unchanged debrisoquine.

It is appreciated that some small error will be incurred in the estimation of D from such a first - order assumption, however the overall elimination of debrisoquine in PM subjects was well described by first - order kinetics and the saturated pathway never accounted

for more than 1 - 2 % of that elimination. This approach yields estimates of $V_{\max}$ and K_m for debrisoquine 4-hydroxylation in poor metabolisers, which in turn may be manipulated to yield a value of k_f for comparison with the value obtained for EM subjects, since:

$$k_f = \frac{V_{\max}}{K_m} \quad (20)$$

This approach was actually used for data derived from both phenotypes to examine the extent of deviation from first - order kinetics displayed by each group. For both EM and PM subjects the slope of the D / V vs. D plot was subjected to the appropriate statistical test to determine or not it significantly exceeded zero. Where the difference from zero proved significant it was assumed that appreciable saturation of the 4-hydroxylation pathway had occurred and the appropriate Michaelis - Menten parameters were calculated; where the slope was not significantly greater than zero, an independent estimate of k_f was obtained from the reciprocal of the mean substrate / metabolite excretion rate ratio ($\overline{D/V}^{-1}$), since under first - order conditions equation (18) contracts to:

$$\begin{aligned} \frac{D}{V} &= \frac{K_m}{V_{\max}} \\ &= \frac{1}{k_f} \end{aligned} \quad (21)$$

2.3: Results

2.3.1.: The elimination of debrisoquine after oral dosing.

The rates of urinary excretion of debrisoquine and 4-hydroxydebrisoquine in the two phenotypes following a 10 mg oral dose are shown in Table 2.1. Peak rates of excretion of both metabolite and unchanged drug were approximately co-incidental in all subjects, occurring between 1.5 - 2.5 hours following the oral administration. The EM subjects studied displayed parallel log - linear excretion patterns with respect to debrisoquine and 4-hydroxydebrisoquine, as exemplified by the 'Sigma - minus' plots shown in Figure 2.3a. The levels of excretion of unchanged debrisoquine displayed by the five PM subjects studied were consistently 2 - 3 times higher than those of the EM subjects, though the pattern of decline was again log - linear, and essentially parallel to that displayed by the dominant phenotype. In contrast, the excretion rates of 4-hydroxydebrisoquine displayed by the PM subjects resulted in a non - linear 'Sigma - minus' plot (Figure 2.3b), suggesting deviation from first - order kinetics. These data are examined further in section 2.3.2, (vide infra). The kinetic parameters derived from the urinary excretion rates of drug related material are summarized in Table 2.2. It can be seen that the oxidation defect is not reflected in the parameters of overall elimination (K or $t_{1/2}$), but that the PM subjects have a significantly higher capacity to excrete unchanged debrisoquine, as manifested in the k_d values, which exceed those of the EM subjects by a factor of approximately 2.4.

Table 2.1: The excretion of debrisoquine (D) and 4-hydroxydebrisoquine (4HOD) in EM (n = 5) and PM (n = 5) subjects following a 10 mg oral dose. (Mean $\pm$ S.D.)*

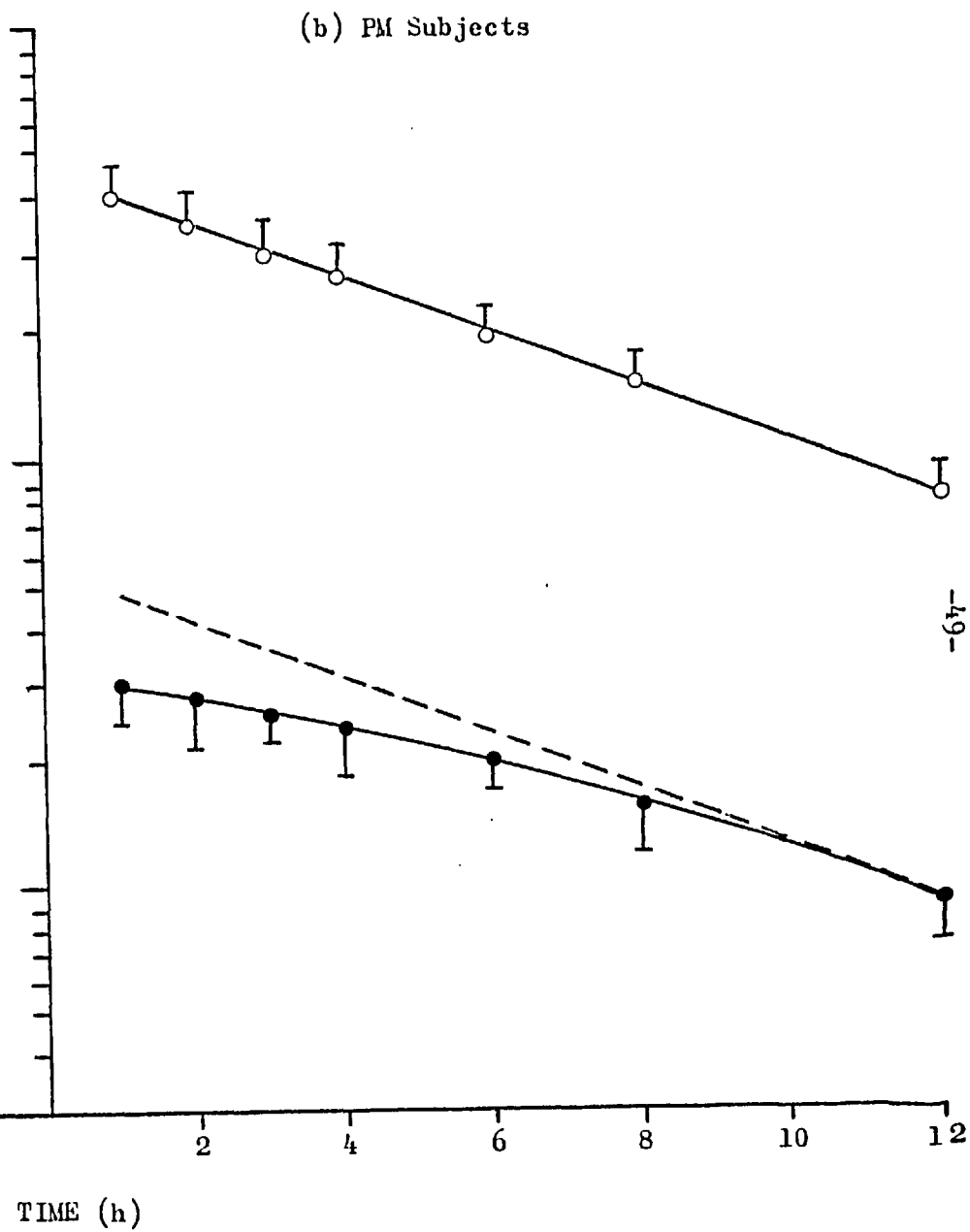
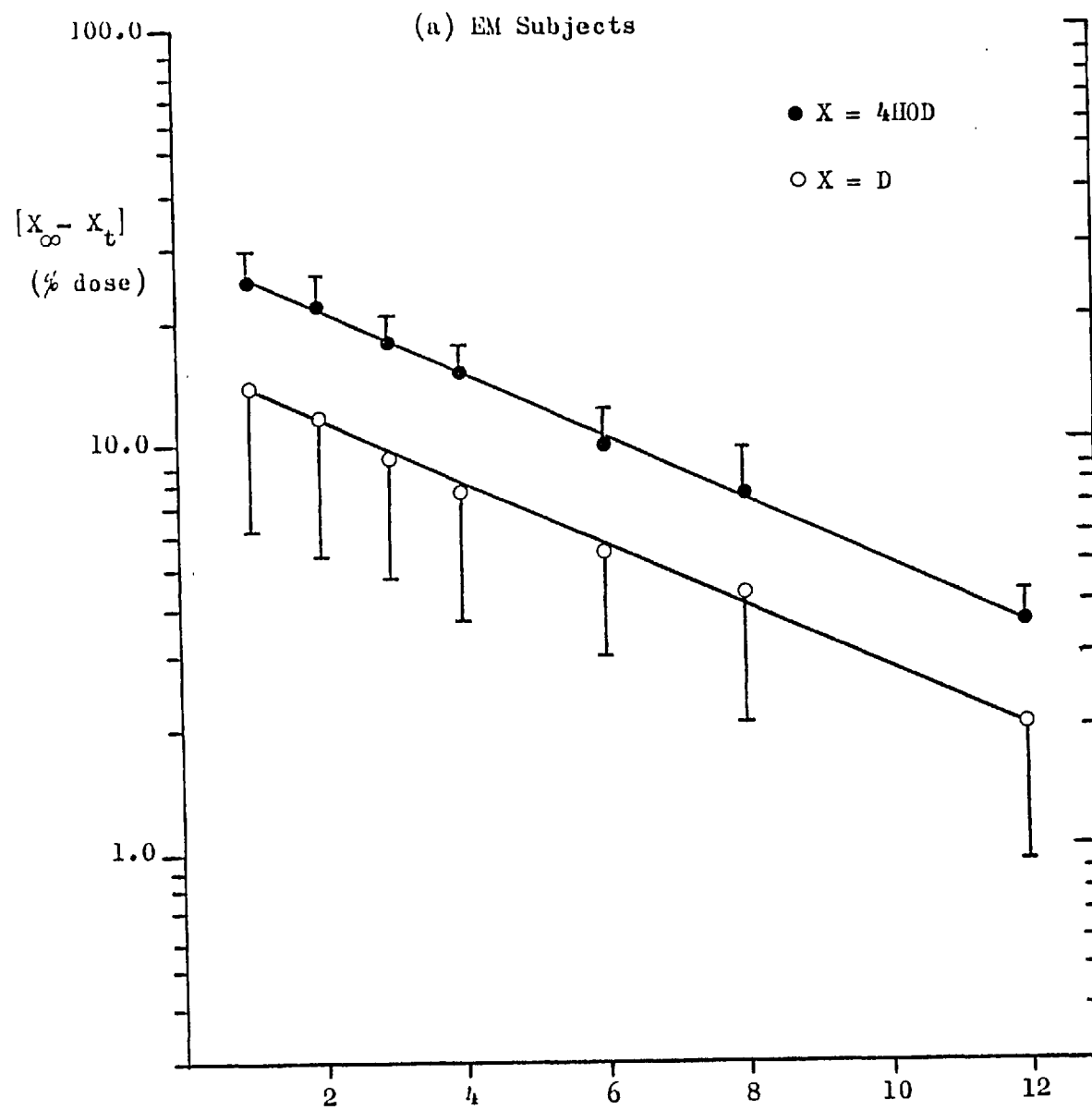
Time (h)	EM Subjects		PM Subjects	
	Excretion Rate (% dose / h)		Excretion Rate (% dose / h)	
	D	4HOD	D	4HOD
0.5	1.74 $\pm$ 1.69	0.81 $\pm$ 0.74	1.73 $\pm$ 0.91	0.12 $\pm$ 0.10
1.5	2.52 $\pm$ 1.39	4.02 $\pm$ 1.97	6.16 $\pm$ 2.61	0.26 $\pm$ 0.12
2.5	2.32 $\pm$ 1.71	4.09 $\pm$ 0.98	5.74 $\pm$ 1.04	0.22 $\pm$ 0.08
3.5	1.69 $\pm$ 1.08	3.51 $\pm$ 0.97	3.82 $\pm$ 0.90	0.19 $\pm$ 0.08
5.0	0.91 $\pm$ 0.46	2.09 $\pm$ 0.27	3.46 $\pm$ 1.43	0.21 $\pm$ 0.08
7.0	0.71 $\pm$ 0.39	1.42 $\pm$ 0.35	2.30 $\pm$ 0.75	0.17 $\pm$ 0.05
10.0	0.57 $\pm$ 0.30	0.94 $\pm$ 0.18	1.81 $\pm$ 0.41	0.15 $\pm$ 0.04
% Dose in 12 hours	13.10 $\pm$ 8.20	23.60 $\pm$ 3.60	35.80 $\pm$ 8.10	2.10 $\pm$ 0.40

* Raw data tabulated in Appendix A.

Table 2.2: The principle parameters describing the elimination of debrisoquine in EM (n = 5) and PM (n = 5) subjects.

	EM	PM
K (h ⁻¹)	0.19 ± 0.03	0.16 ± 0.02 (N.S.)
t _{1/2} (h)	3.8 ± 0.7	4.5 ± 0.5 (N.S.)
k _d (h ⁻¹)	0.029 ± 0.021	0.070 ± 0.018 (2P < 0.01)

Figure 2.3: Semi - logarithmic 'Sigma - minus' plots for debriso-
quine (D) and 4-hydroxydebrisoquine (4HOD) in EM (a)
and PM (b) subjects; the broken line in plot (b) is
that expected from first - order considerations based
on the observed 12 h value for 4-hydroxydebrisoquine
excretion. (Each point represents mean $\pm$ S.D. for
five individuals.)



2.3.2: The formation of 4-hydroxydebrisoquine after oral dosing.

In order to obtain values for the apparent first - order rate constants determining 4-hydroxylation of debrisoquine in the two oxidation phenotypes, urinary excretion data were subjected to the analyses described in 2.2.2 (vide supra). Data from both phenotypes were used to obtain the Hanes plots shown in Figure 2.4; the 'Sigma - minus' approach was only applied to the urinary excretion rate data obtained from EM subjects, since this treatment requires a first - order assumption. The Hanes plot derived from the PM subjects studied yielded a slope of 5.167 with an associated variance of 1.390; the t - value derived from these parameters is significant at the 0.1 % level, and thus is strongly suggestive of deviation from first - order kinetics with respect to 4-hydroxylation in the PM phenotype. In contrast, the Hanes plot derived from the EM subjects studied yielded a slope of 0.017 with an associated variance of 0.066; in this instance the derived t - value is not significant, and hence there is no evidence of deviation from first - order kinetics in the EM phenotype. The parameters describing oxidation of debrisoquine in the two groups are summarized in Table 2.3, whilst the two estimates of k_f obtained for the five EM subjects by the Hanes and 'Sigma - minus' methods are compared in Table 2.4. It can be seen that the PM phenotype has significantly less ability to perform 4-hydroxylation whichever comparator is used. The merits and limitations of the kinetic approaches used and the applications of the first - order formation constant in the study of interindividual differences in hydroxylation capacity will be discussed.

Figure 2.4: Hanes plots relating the rate of excretion of 4-hydroxydebrisoquine (V) to the concomittant body load of debrisoquine (D). In each instance the points represent data pooled from five individuals of each oxidation phenotype.

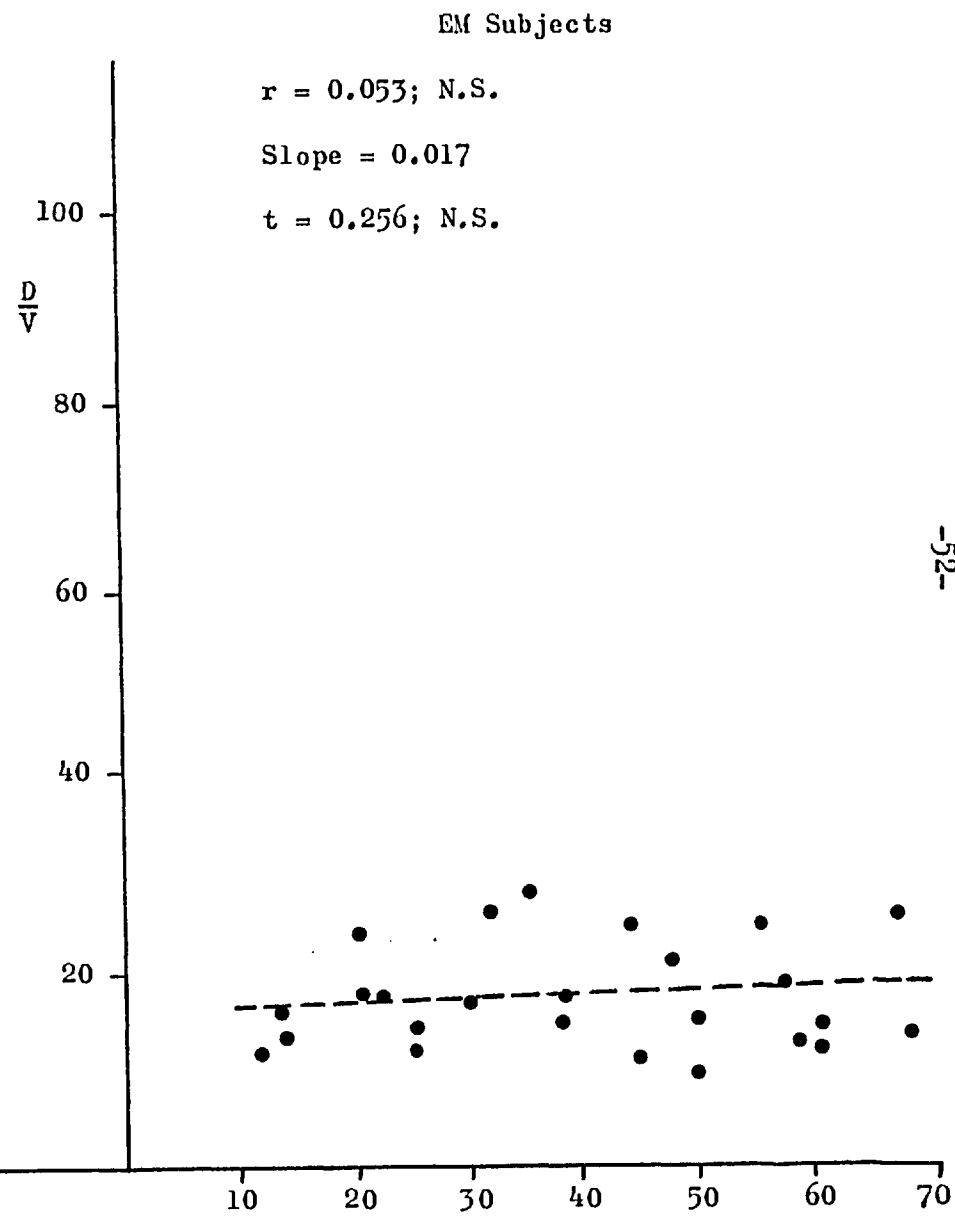
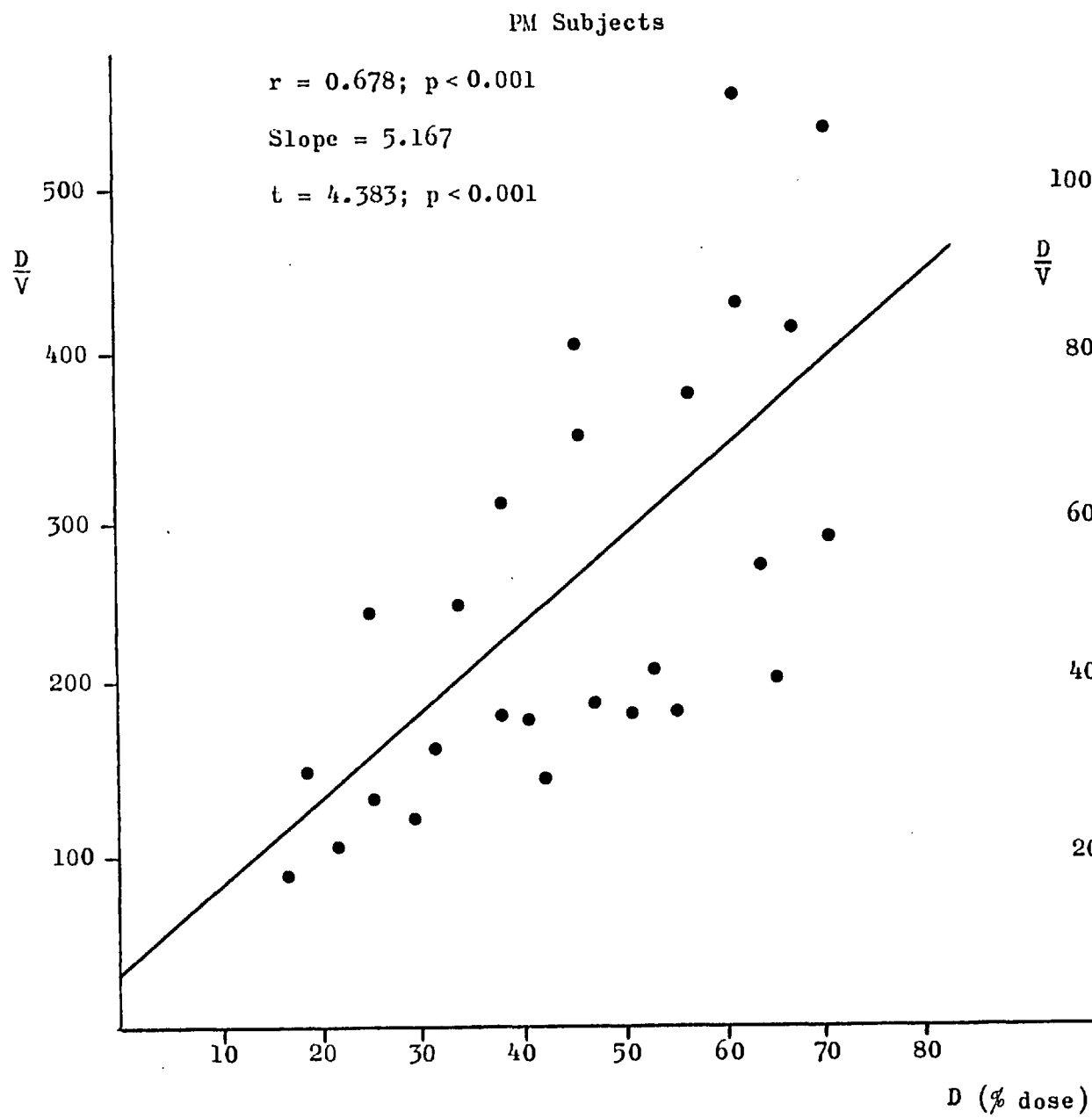


Table 2.3: The principle parameters describing the formation of 4-hydroxydebriso-
quine in EM (n = 5) and PM (n = 5) subjects.

	EM	PM
$V_{\max}$ (mg / h) **	No deviation from first - order kinetics	0.019 ± 0.004
K_m (mg) **	No deviation from first - order kinetics	1.11 ± 0.28
k_f (h ⁻¹)	0.060 ± 0.015	0.017 ± 0.005 (2P < 0.001)

** Corrected to debrisoquine equivalents

Table 2.4: Values for the first - order rate constant determining the formation of 4-hydroxydebrisoquine in subjects of the EM phenotype determined by (i) the Hanes plot* and (ii) the 'Sigma - minus' plot.^φ

Subject no.	k _f determined by:	
	Method (i)	Method (ii)
1	0.038	0.042
2	0.052	0.041
3	0.072	0.058
4	0.067	0.055
5	0.073	0.077
Mean ± S.D.	0.060 ± 0.015	0.053 ± 0.012

*Levy et al. (1972)

^φCummings et al. (1967)

2.3.3: The elimination of 4-hydroxydebrisoquine.

It can be seen from Figure 2.3 that in the instance of the EM subjects studied, excretion of 4-hydroxydebrisoquine paralleled that of debrisoquine over the entire period of the study. An extended study (24 - 48 h post - dosing) in three of the PM subjects indicated that here also 4-hydroxydebrisoquine excretion paralleled that of debrisoquine over period 12 - 48 h post - dosing. These data are shown in Figure 2.5. It may thus be stated with some confidence that excretion is not rate - limiting for the appearance of 4-hydroxydebrisoquine in urine for either phenotype. An apparent first - order rate constant for the elimination of this metabolite (k_u) was calculated for the EM subjects studied in the manner previously described. This calculation could not be performed for the PM subjects studied because of the pronounced deviation from log - linearity of metabolite excretion in the 10 hours immediately following dosing. It can be seen from Figure 2.6 that there is a linear first - order relationship between 4-hydroxydebrisoquine 'body - load' and its excretion rate in urine. The rate constants derived from the five individuals studied are tabulated in Table 2.5, together with comparative values for overall elimination of debrisoquine. It may be concluded that for equivalent body loads of debrisoquine and its major metabolite, elimination of the latter will occur approximately seven times faster than for debrisoquine itself.

Figure 2.5: Urinary excretion rates of debrisoquine (D) and 4-hydroxydebrisoquine (4HOD) over an extended study period (24 - 48 h) following oral administration of 10 mg debrisoquine to three PM subjects.

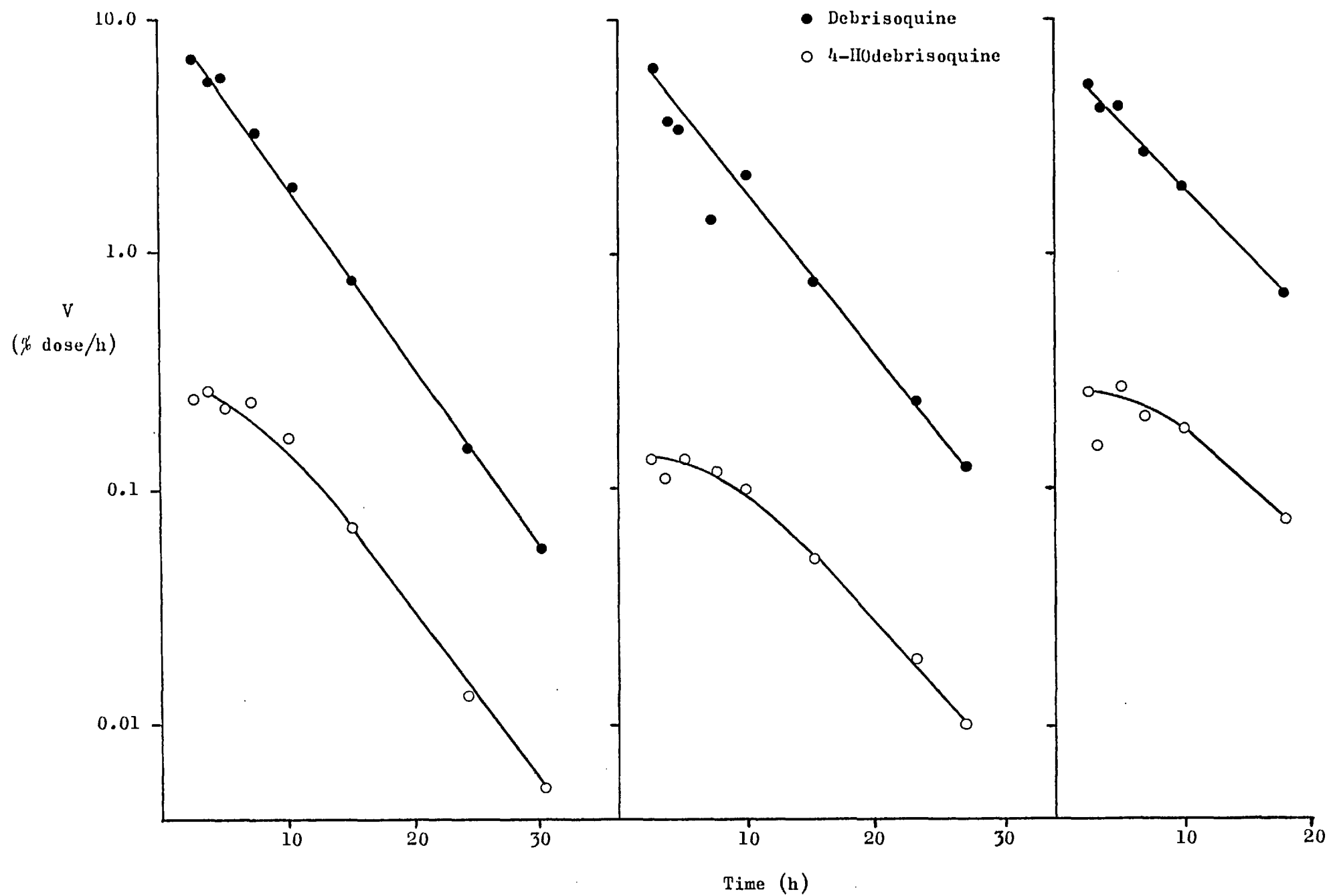


Figure 2.6: Excretion rates of 4-hydroxydebrisoquine (V) plotted against calculated 'body - loads' of 4-hydroxydebrisoquine (M_B) for the corresponding post - dosing time - values. Points represent data pooled from five EM subjects.

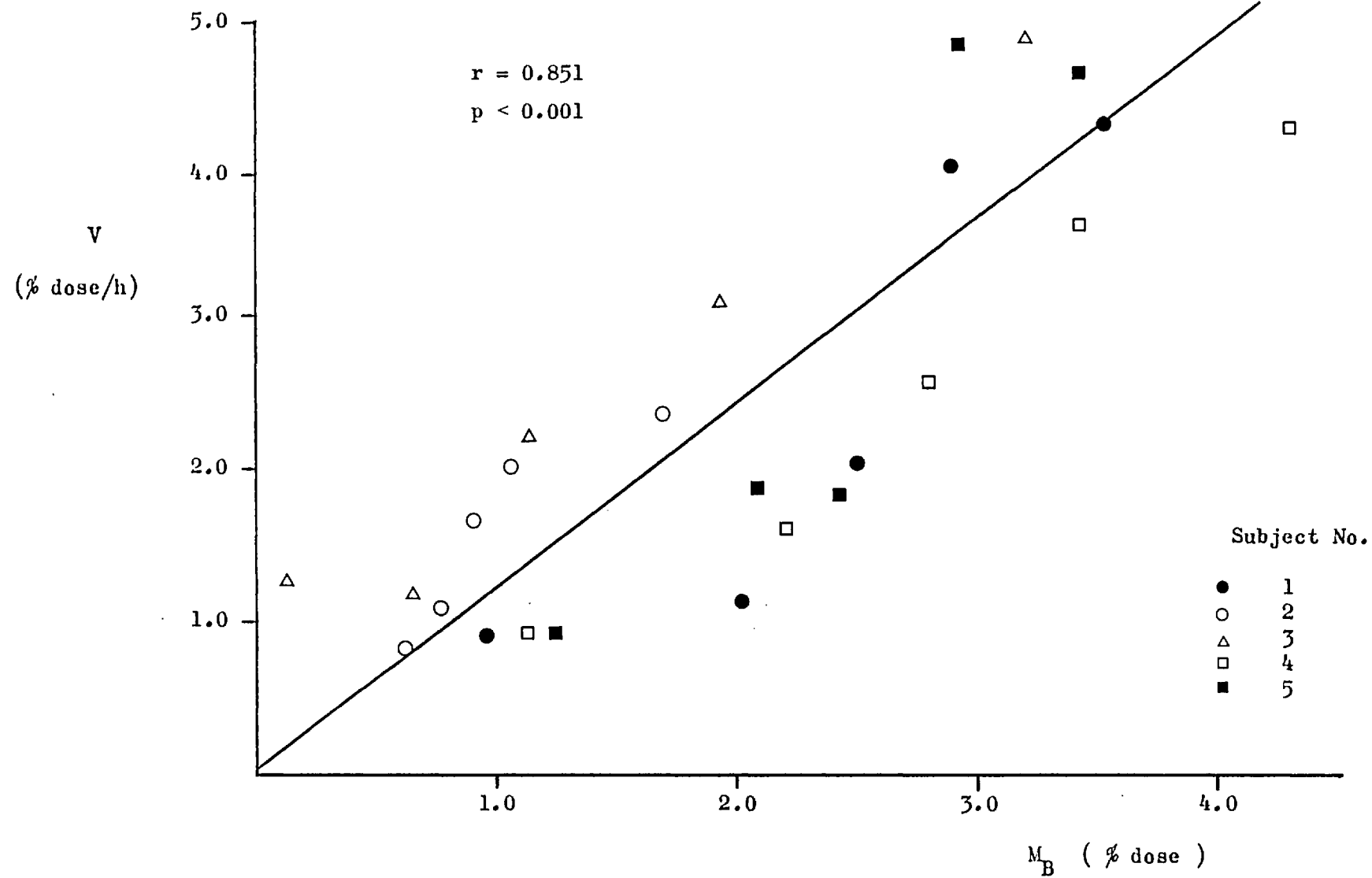


Table 2.5: Comparison of overall - elimination rate constants for debrisoquine and 4-hydroxydebrisoquine in five EM subjects following a 10 mg oral dose of debrisoquine.

Subject	Rate constant for overall elimination of		
	Debrisoquine (K)	4-hydroxydebrisoquine (k_u)	$\frac{k_u}{K}$
	(h ⁻¹)	(h ⁻¹)	
1	0.22	1.20	5.45
2	0.16	1.57	9.81
3	0.15	1.36	9.07
4	0.20	1.01	5.05
5	0.22	1.36	6.18
Mean $\pm$ S.D.	0.19 $\pm$ 0.03	1.30 $\pm$ 0.21	7.11 $\pm$ 2.18

2.3.4: Plasma debrisoquine levels after oral dosing.

The plasma concentrations of debrisoquine attained in the two phenotypes following oral dosing are shown in Table 2.6 and Figure 2.7. The PM subjects attained significantly higher ($2P < 0.001$) plasma levels at all time points following 1 h post - dosing. The parameters derived from these data are summarized in Table 2.7. It is evident that the three PM subjects studied were exposed to more than three times the amount of unchanged debrisoquine compared to EM subjects, irrespective of whether this inter - phenotype difference is expressed in terms of peak or mean plasma concentration or area under the plasma concentration - time curve. No significant inter - phenotype differences were discernible in either elimination half - life or renal clearance of debrisoquine. Interestingly, despite the fact that some subjects displayed 4-hydroxydebrisoquine concentrations in urine some 4 times that of debrisoquine itself, at no stage and in no subject was 4-hydroxydebrisoquine detectable in plasma. The assay used for the metabolite had limits of sensitivity of 5 ng / ml when the compound was added in known amounts to blank human plasma.

Table 2.6: Plasma debrisoquine levels in EM (n = 4) and PM (n = 3) subjects following
10 mg p.o. (Mean $\pm$ S.D.) *

Time (h)	Plasma debrisoquine concentration (ng / ml)	
	EM subjects	PM subjects
0.5	Not detected	10.3 $\pm$ 0.8
1.0	14.7 $\pm$ 7.2	30.3 $\pm$ 7.2
1.5	21.4 $\pm$ 3.7	72.3 $\pm$ 11.9
2.0	15.2 $\pm$ 2.3	63.2 $\pm$ 6.4
3.0	12.0 $\pm$ 3.6	44.4 $\pm$ 6.9
4.0	9.6 $\pm$ 3.5	35.9 $\pm$ 2.5
6.0	4.9 $\pm$ 1.1	28.6 $\pm$ 2.1
8.0	3.6 $\pm$ 1.1	16.5 $\pm$ 4.1

* Raw data tabulated in Appendix A

Figure 2.7: Plasma concentrations of debrisoquine in EM ($n = 4$) and PM ($n = 3$) subjects following oral administration of 10 mg debrisoquine. (Points represent mean $\pm$ S.D. in each instance.)

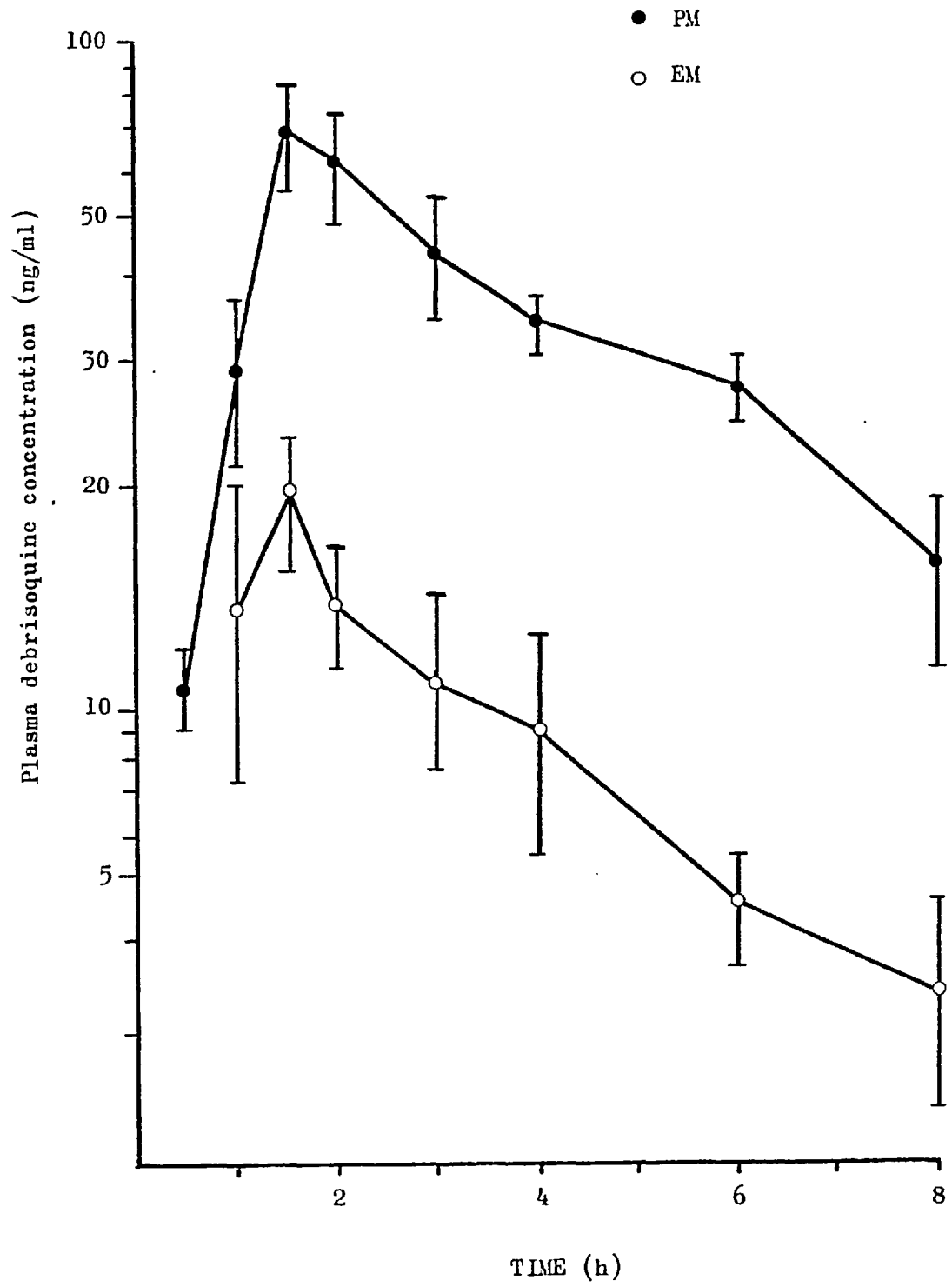


Table 2.7: Pharmacokinetic parameters derived from plasma levels of debrisoquine in EM (n = 4) and PM (n = 3) subjects following 10 mg p.o. (Mean $\pm$ S.D.)

Parameter	EM Subjects					PM Subjects				
	1	2	3	4	Mean $\pm$ S.D.	1	2	3	Mean $\pm$ S.D.	
Peak plasma concentration (ng / ml)	19.3	25.2	23.5	19.5	21.9 $\pm$ 2.9	85.4	70.6	62.0	72.7 $\pm$ 11.8	
Mean plasma concentration (ng / ml; 0-8h)	11.9	10.8	11.4	11.7	11.5 $\pm$ 0.7	42.0	37.3	33.7	37.7 $\pm$ 4.2	
AUC _{0 - ∞} (ng / ml min)	112.2	95.4	107.5	108.2	105.6 $\pm$ 7.0	396.7	353.7	363.8	371.4 $\pm$ 22.4	
Elimination half - life (h)	3.5	2.3	2.9	3.2	3.0 $\pm$ 0.5	3.3	2.9	3.7	3.3 $\pm$ 0.4	
Renal clearance (ml / min)	133.6	167.1	188.2	122.4	152.8 $\pm$ 30.3	132.0	140.6	138.5	137.0 $\pm$ 4.5	

2.3.5: Plasma debrisoquine levels after intravenous dosing.

The plasma concentrations of debrisoquine attained in the three individuals of different genotype studied are shown in Figure 2.8; the biexponential function describing the decline in concentration is given for each subject. The derived pharmacokinetic parameters peculiar to intravenous administration are listed in Table 2.8, whilst the parameters for which comparison can usefully be made with those obtained after oral administration are listed on Table 2.9. Whilst these data are too few for rigorous statistical analysis, it would appear that increasing 4-hydroxylation capacity results in a high degree of drug sequestration, as judged by the ratio between the distribution micro-constants (k_{12} / k_{21}) and the apparent steady state volume of distribution ($V_D(SS)$). In turn, high metabolic capacity (subject 1) is also associated with relatively low irreversible elimination from the central compartment (k_e) and a longer elimination half - life. The single PM subject studied displayed the largest value for oral bioavailability and also the fastest overall elimination as judged by k_e and plasma and renal clearance values. The proportion of the intravenous dose excreted unchanged was in reasonable agreement with that predicted from the proportion of total plasma clearance accounted for by renal clearance in all three subjects.

Figure 2.8: Plasma concentrations of debrisoquine in homozygous and heterozygous EM subjects (1 & 2 respectively) and a homozygous PM subject (3) following intravenous administration of 10 mg debrisoquine. In each instance the broken line represents the plasma concentrations of debrisoquine attained after oral dosing in the same individual.

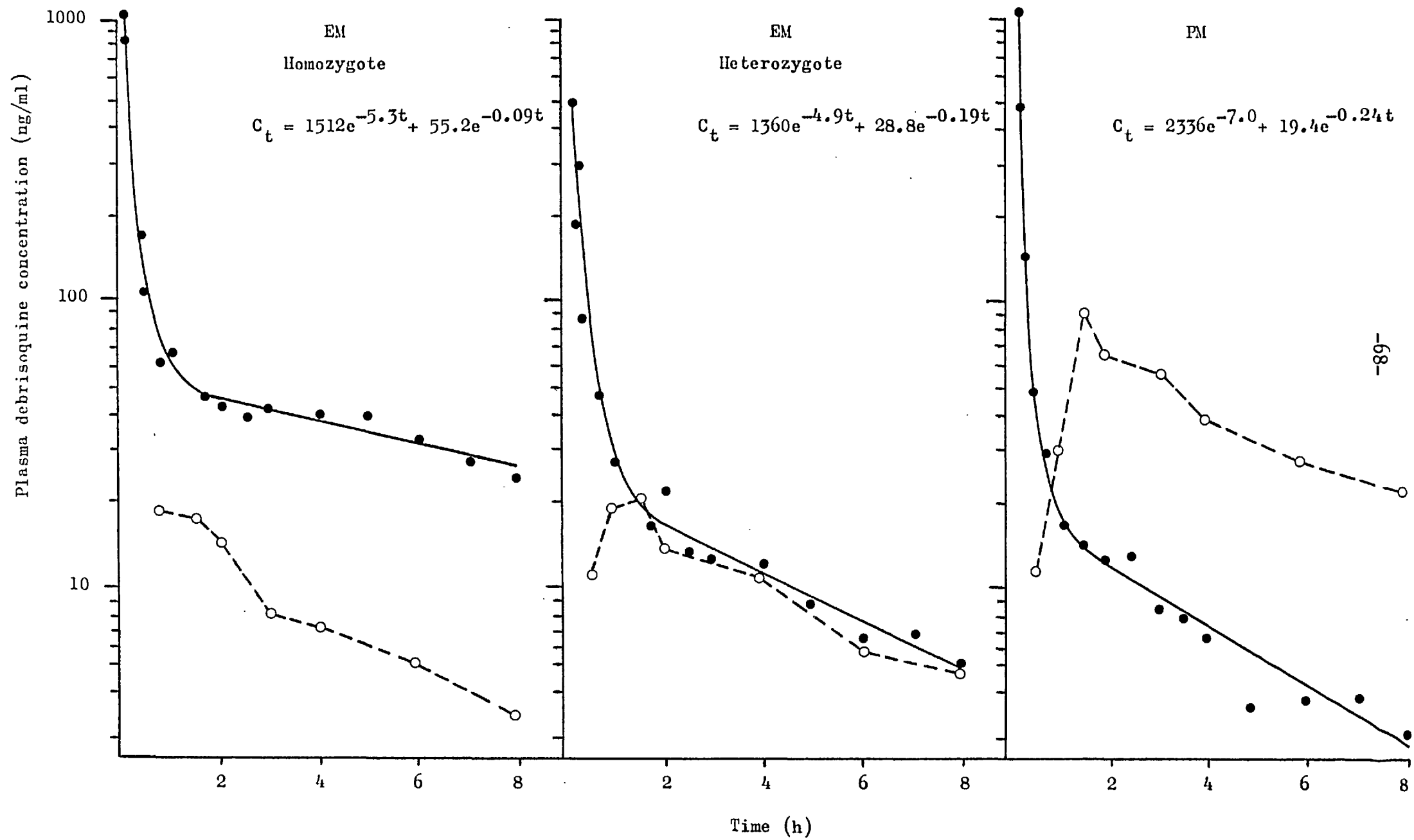


Table 2.8: kinetic parameters derived from plasma and urine levels of debrisoquine in homozygous and heterozygous EM subjects (1 & 2 respectively) and a homozygous PM subject (3) following intravenous administration of 10 mg debrisoquine.

	Subject 1	Subject 2	Subject 3
$k_e \text{ (h}^{-1}\text{)}$	1.79	3.21	5.40
$k_{12} \text{ (h}^{-1}\text{)}$	3.36	1.87	1.50
$k_{21} \text{ (h}^{-1}\text{)}$	0.28	0.28	0.31
k_{12} / k_{21}	12.21	6.60	4.83
Plasma clearance (ml / min)	190.0	385.0	401.0
<u>Renal clearance</u> Plasma clearance	48.5 %	62.9 %	87.3 %
% i.v. dose ex- creted unchanged	34.0 %	57.9 %	82.2 %
$V_D(\text{area})$ (l / kg)	1.4	1.6	1.4
$V_D(\text{SS})$ (l / kg)	0.91	0.78	0.36

Table 2.9 : Kinetic parameters derived from plasma and urine levels of debrisoquine in homozygous and heterozygous EM subjects, (1 & 2 respectively) and a homozygous PM subject (3) following intravenous administration of 10 mg debrisoquine.

	Subject 1		Subject 2		Subject 3	
	i.v.	Oral	i.v.	Oral	i.v.	Oral
Peak plasma D concentration (ng / ml)	1442	19.3	1006	23.5	2106	85.4
Mean plasma D concentration (ng / ml)	77.7	11.9	49.8	11.4	49.1	42.0
Renal clearance (ml / min)	92.2	133.6	242.5	188.2	350.1	132.0
AUC _{0 - ∞} (ng / ml .h)	877.3	112.2	432.9	107.5	415.6	396.7
Bioavailability	-	12.7 %	-	24.8 %	-	95.3 %
Terminal t _{1/2} (h)	7.5	3.5	3.7	2.9	3.0	3.3
Metabolic ratio	3.1	0.6	9.5	1.4	100	21.3

2.4: Discussion

The results obtained from a detailed examination of the elimination kinetics of debrisoquine and its major metabolite, 4-hydroxydebrisoquine point to a number of conclusions which may be summarized as follows:

1. The 4-hydroxylation of debrisoquine occurs pre-systemically, as judged by the coincidence of peak excretion rates of drug and metabolite in urine displayed by both phenotypes.
2. This metabolic pathway is readily saturable in individuals of the PM phenotype at an oral dose level of 10 mg, as judged by the highly significant deviation from first - order kinetics displayed by these individuals with respect to metabolite formation.
3. The defect is genuinely one of oxidation capacity, since it can be demonstrated that formation and not excretion of 4-hydroxydebrisoquine is rate - limiting for its appearance in urine, as judged by the parallelism of the terminal portions of the drug and metabolite urinary excretion rate plots obtained from both oxidation phenotypes.

The immediate consequences of this oxidation defect are seen in the plasma levels of unchanged debrisoquine displayed by the PM phenotype after an oral dose. Saturation of the pre-systemic inactivation pathway in these individuals results in an exposure to unchanged drug between three and four times higher than that seen in their EM counterparts. It can readily be seen why the PM individual is significantly more susceptible to the hypotensive effects of debrisoquine (Idle et al., 1978). A prime objective of this study was to obtain an estimate of the absolute degree of variation existing between the two oxidation phenotypes. In the event the difference

between the apparent first - order rate constants for the formation of 4-hydroxydebrisoquine was approximately three-fold:

$$\text{EM } k_f = 0.060 \pm 0.015 \text{ h}^{-1}$$

$$\text{PM } k_f = 0.017 \pm 0.005 \text{ h}^{-1}$$

There is, however, a considerable body of evidence to suggest that the actual difference in capacity is very much higher. Dose - dependancy studies performed by Mahgoub (1979) demonstrated that whilst an EM subject displayed a constant metabolic ratio and no hypotensive response to oral doses of debrisoquine in the range 10 - 60 mg, a PM individual subjected to a similar regime showed a marked hypotensive response following an oral dose of 20 mg, then prolonged and severe orthostatic hypotension, with a concomittant ten-fold increase in metabolic ratio after a 40 mg dose. In the light of these findings the theoretical kinetic basis of drug metabolism studies in general bears consideration.

In conventional terms, it is considered that the first - order rate constant k_f describes the rate of metabolism (V) of a vanishingly small amount of substrate (C), formally expressed as:

$$V \rightarrow k_f \cdot C \text{ as } C \rightarrow 0.$$

Strictly, this is the only situation, albeit hypothetical, where a first - order assumption is justified. However, it is held that the first - order constant, or more commonly the half - life derived from it, is a direct measure of metabolic capacity since k_f is also an approximation of the ratio $V_{\max} / K_m$, thus:

$$k_f = \frac{V_{\max}}{K_m} \quad (22)$$

It is assumed that the affinity of the enzyme for its substrate (K_m) is a constant, and so any variation observed in k_f is a direct quantitative reflection of variation in capacity ($V_{\max}$).

Using this reasoning, it is possible to examine the hypothesis that poor and extensive metabolisers of debrisoquine share a common enzymic protein which varies in amount between genotypes, but not, by definition, in affinity. Re-arranging equation (22);

$$V_{\max} = k_f \cdot K_m \quad (23)$$

The observed K_m value, now assumed to be a constant for both oxidation phenotypes, was 1.11 ± 0.28 mg; the observed k_f value in the EM phenotype was 0.060 ± 0.015 h⁻¹, thus the hypothetical maximal rate of 4-hydroxydebrisoquine production which can be expected in the EM phenotype is 0.066 ± 0.017 mg / h. In fact, rates of 4-hydroxydebrisoquine production in excess of 1.5 mg / h, twenty times the predicted value, were commonly observed by Mahgoub (1979) in the course of dose - dependancy studies. It can only be concluded that the enzymic protein performing 4-hydroxylation of debrisoquine in the PM phenotype differs qualitatively as well as quantitatively from that existing in the EM phenotype. This aspect of the current work is discussed in more general terms in Chapter 6 (vide infra). A notable feature of the studies undertaken of levels of drug - related material on plasma was the failure to detect 4-hydroxydebrisoquine in any of the individuals studied. From urinary levels in the EM subjects, it would have been predicted that peak levels of some 60 - 80 ng / ml 4-hydroxydebrisoquine should be observed. This was almost certainly not a methodological problem, since a standard concentration of 10 ng / ml was readily detectable regardless of whether the standard was prepared by spiking blank plasma or whole blood.

Two constructions could be placed on this observation based on pharmacokinetic considerations, though it should be stressed that the two are not mutually exclusive.

It has been demonstrated that the metabolic 4-hydroxylation of debrisoquine apparently occurs pre-systemically, so for the purposes of the current argument, an EM subject may be considered as receiving simultaneous oral doses of debrisoquine and its primary metabolite. It is thus feasible that the metabolite may exhibit either a different clearance rate or a different volume of distribution from debrisoquine itself, or a combination of the two. The numerical relationship between these parameters is shown in equation (24):

$$Cl_{4HO} = V_{D4HO} \cdot k_u \quad (24)$$

where:

Cl_{4HO} = Clearance of 4-hydroxydebrisoquine

V_{D4HO} = Volume of distribution of 4-hydroxydebrisoquine

k_u = Apparent first - order rate constant for the elimination of 4-hydroxydebrisoquine.

As far as is known 4-hydroxydebrisoquine does not undergo further metabolism (Silas et al., 1978, Idle et al., 1979), so the above equation refers only to renal clearance of this compound. The calculated k_u values for the EM subjects studied are listed in Table 2.5 (vide supra), and indicate that for a given body load, the elimination of 4-hydroxydebrisoquine will occur some seven times more rapidly than that of debrisoquine itself. Given that the renal clearance of debrisoquine in EM subjects is approximately 150 ml / min, it follows that 4-hydroxydebrisoquine will have a renal clearance of some 1000 ml / min if it has the same volume of distribution as its parent compound. This would seem unlikely as renal clearance is generally considered to be perfusion limited, normal renal plasma flow being some 600 - 700 ml / min (Rowland et al., 1973).

The second factor in equation (24), namely the metabolite's volume of distribution may also be significant. If 4-hydroxydebrisoquine were to be sequestered from plasma by, say the adrenergic neurone, it could potentially have a higher volume of distribution than debrisoquine itself, with a concomittantly lower observed plasma concentration than that which would have been anticipated from urinary levels. Both of the possibilities outlined above could be tested by administration of 4-hydroxydebrisoquine, preferably by infusion to steady state, to permit direct estimation of its actual renal clearance and apparent volume of distribution, but such an exercise was beyond the scope of the present study. The failure of 4-hydroxydebrisoquine to acheive plasma concentraions in excess of 5 ng / ml following a 10 mg oral dose of its parent compound is an intriguing observation. Whilst the reasoning above is obviously speculative, it is felt that the phenomenon may have some relevance to the pharmacology of debrisoquine itself, and thus merits further investigation.

The final aspect of this study, an investigation into the plasma levels of debrisoquine attained in individuals representative of the three oxidation genotypes following an intravenous dose of debrisoquine, demonstrated that the disposition of the drug shows a considerable dependance on its route of administration. The logistical difficulties encountered meant that intravenous studies could only be performed in a very limited number of people, leaving no scope for rigorous statistical analysis of these data for significant trends or relationships. This not withstanding, the results as they stand suggest an inverse relationship between metabolic capacity and the ability to eliminate intravenously administered debrisoquine from plasma. The homozygous EM subject showed a high degree of sequest-

-ration, as judged by a k_{12} / k_{21} ratio of 12.21 and an apparent steady state volume of distribution of 0.91 l / kg, and this resulted in a relatively low plasma clearance (190.0 ml / min) and a lengthened $t_{1/2\beta}$ (7.5 h). In contrast, the PM subject studied showed less sequestration ($k_{12} / k_{21} = 4.83$, $V_D(SS) = 0.36$ l / kg) and faster gross elimination (Plasma clearance = 401 ml / min, $t_{1/2} = 2.9$ h). This would suggest that the process of oxidation requires a physically longer time than does active renal clearance on a molecule - for - molecule basis, and that the gross elimination parameters are determined by the fastest pathway available rather than the sum of all the possible elimination pathways. Comparison of the oral and intravenous data obtained from these three subjects indicates that an oral dose of debrisoquine is almost quantitatively bioavailable in the PM phenotype, again indicating that the 4-hydroxylation of this drug is performed by a pre-systemic pathway which is subject to an inborn error in 5 - 10 % of the British white Caucasian population, such that the capacity of these affected individuals to effect metabolic inactivation is saturable at doses considerably below those in normal therapeutic use.

CHAPTER THREE

INTER - ETHNIC VARIATION IN GENOTYPE EXPRESSION - DEBRISOQUINE
METABOLISM AMONG GHANAIANS AND CAUCASIANS

3.1. Introduction

3.2. Subjects and methods

3.3. Results:

3.3.1. Debrisoquine alicyclic hydroxylation

3.3.2. Debrisoquine aromatic hydroxylation

3.4. Discussion

3.1. Introduction:

In 1940 E.B. Ford defined genetic polymorphism as "the occurrence together in the same habitat of two or more discontinuous forms or 'phases' of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation." This succinct statement excludes several familiar types of variation - the various ethnic groups of Man, for example do not constitute a polymorphism since interbreeding results in intermediate and variable hybrid populations.

An authentic genetic polymorphism arises initially by mutation; its perpetuation as a 'balanced' or 'stable' polymorphism depends on the mutant gene being at an advantage compared with other alleles under certain circumstances (Clarke, 1970). As Ford (1965) points out any mutant gene which begins to be favoured must exist almost exclusively in the heterozygous state during the early stages of its increase since there is little probability of matings between the rare homozygotes. This implies that where a polymorphism is stable its maintenance depends on the heterozygote possessing some selective advantage over both homozygotes.

This principle is well illustrated by consideration of one of the few polymorphic systems where the selective pressures creating heterozygous advantage are understood. Some 20 % of the indigenous East African population carry the Hb^S gene, which controls the formation of sickle cell (S) haemoglobin. Homozygous Hb^S / Hb^S individuals generally die in infancy from sickle cell anaemia; the heterozygote (Hb^A / Hb^S) however, is resistant to infection by malignant tertian malaria, and thus is at an advantage compared to homozygous Hb^A / Hb^A individuals even though he displays the sickle cell trait (Allison, 1954).

It is obvious that the degree of selection pressure operating on any given genotype will vary with habitat, and most polymorphic systems display marked inter - ethnic variability. This is certainly the case with the well studied polymorphisms affecting drug metabolism. Five such characteristics and the degree of inter - ethnic variability which has been encountered in the study of each are summarized in Table 3.1. It can be seen that in every instance the frequency of the gene in question shows statistically significant inter - ethnic variation. Plasma paroxonase is worthy of particular mention in this respect. The activity of this enzyme is clearly bimodal in British and Indian subjects, with similar antimodes, but in African and Oriental subjects unimodal distributions were observed (Playfer et al., 1976). In a population study of 258 unrelated British white Caucasians, Price - Evans et al. (1980) estimated the frequency of the gene determining defective alicyclic 4-hydroxylation of debrisoquine at 29.8 %. The observed frequency of the homozygous recessive PM character (D^L / D^L) was 8.9 %. Studies of the families of probands suggested that the mean metabolic ratio obtained from a group of known heterozygous (D^H / D^L) individuals was significantly displaced from the equivalent mean value calculated for the dominant D^H / D^H genotype. This finding strongly suggests that dominance by the D^H allele is incomplete, and in fact the authors estimated the degree of dominance at 30 %.

Scope of the present work:

The data presented here are part of a larger study of inter - ethnic variability in debrisoquine metabolism. It can be seen from Table 3.1 that the D^L gene is apparently ubiquitous, but variable in frequency, In the present work a group of Ghanaian volunteers was compared with

Table 3.1.: Inter - ethnic variability in various aspects of drug metabolism displaying genetic polymorphism. *

<u>Polymorphic Characteristic</u>	<u>Population</u>	<u>Gene Frequency</u>	<u>95 % Confidence Limits for Recessive Phenotype</u>
<u>Slow acetylation of isoniazid</u>	Caucasian	0.765	60.6 % - 56.4 %
	Negroid	0.740	63.5 % - 46.6 %
	Eskimo	0.321	13.2 % - 7.8 %
	Japanese	0.344	13.2 % - 10.5 %
	Chinese	0.469	25.2 % - 19.0 %
<u>Low plasma pseudocholinesterase</u>	Caucasian	0.016	n / a
	'Pacific and Asian'	0	-
<u>Low plasma paraoxanase</u>	Caucasian	0.703	n / a
	Indian	0.463	32.1 % - 12.9 %
<u>Atypical liver alcohol dehydrogenase</u>	Caucasian	0.05	n / a
	Japanese	0.61	n / a
<u>Defective hydroxylation of debrisoquine</u>	Caucasian	0.298	12.7 % - 5.7 %
	Nigerian	0.285	13.6 % - 4.0 %
	Egyptian Arab	0.118	5.5 % - 0.1 %
	Saudi Arab	0.099	3.8 % - 0.1 %
	Chinese	0.171	11.3 % - 0.1 %

* Data pooled from Ellard (1976), Price - Evans (1977) and Islam et al. (1980)

a British white Caucasian population. Two properties of the population profiles of debrisoquine metabolism were considered to be of interest. Firstly, the observed phenotype frequencies and derived gene frequencies for the reasons outlined above, and secondly the degree of homogeneity or otherwise displayed by the EM phenotype. Since dominance of D^L by D^H has been shown to be incomplete it is at least theoretically possible that the dominant genotype may display statistical heterogeneity, with heterozygosity emerging as a separate trait.

A subsidiary feature of this study was an examination of aromatic debrisoquine hydroxylation. This pathway yields four phenolic metabolites in proportions and amounts subject to wide inter - individual variation (for a fuller description of debrisoquine metabolism in man, vide supra, Chapter 1). It was felt that this study provided an opportunity to assess whether or not aromatic hydroxylation of debrisoquine was affected by the D^L locus, and whether or not this aspect of debrisoquine metabolism was also subject to inter - ethnic variation.

3.2. Subjects and methods:

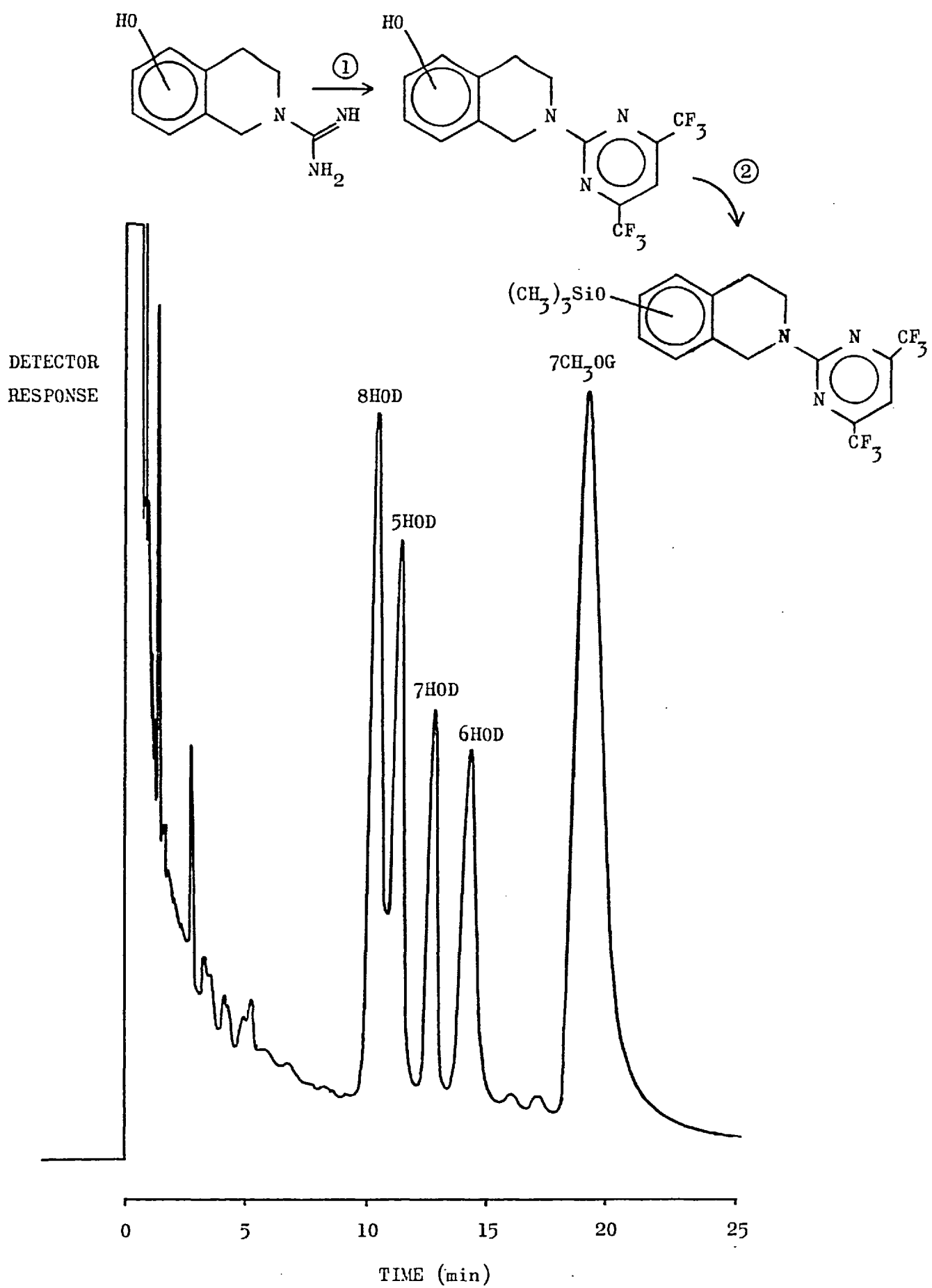
The Caucasian subjects studied were 140 apparently healthy members of the staff and students of St. Mary's Hospital Medical School, London, and volunteer members of the general public drawn from the Ealing area. The Ghanaian volunteers were 80 apparently healthy members of the staff and students of the University of Ghana Medical School and Hospital, Accra, Ghana. After an overnight fast and having emptied their bladders, each subject was given a single Declinax tablet (Roche products) containing 12.8 mg debrisoquine sulphate (equivalent to 10 mg free base). Urine was collected in bulk for 8 h post - dosing. Following the Ghanaian study the samples were frozen and transported to London for analysis. All samples were analysed for debrisoquine and 4-hydroxydebrisoquine by electron - capture gas chromatography in the manner previously described (Chapter 2, vide supra). Individuals were phenotyped on the basis of the metabolic ratio (MR):

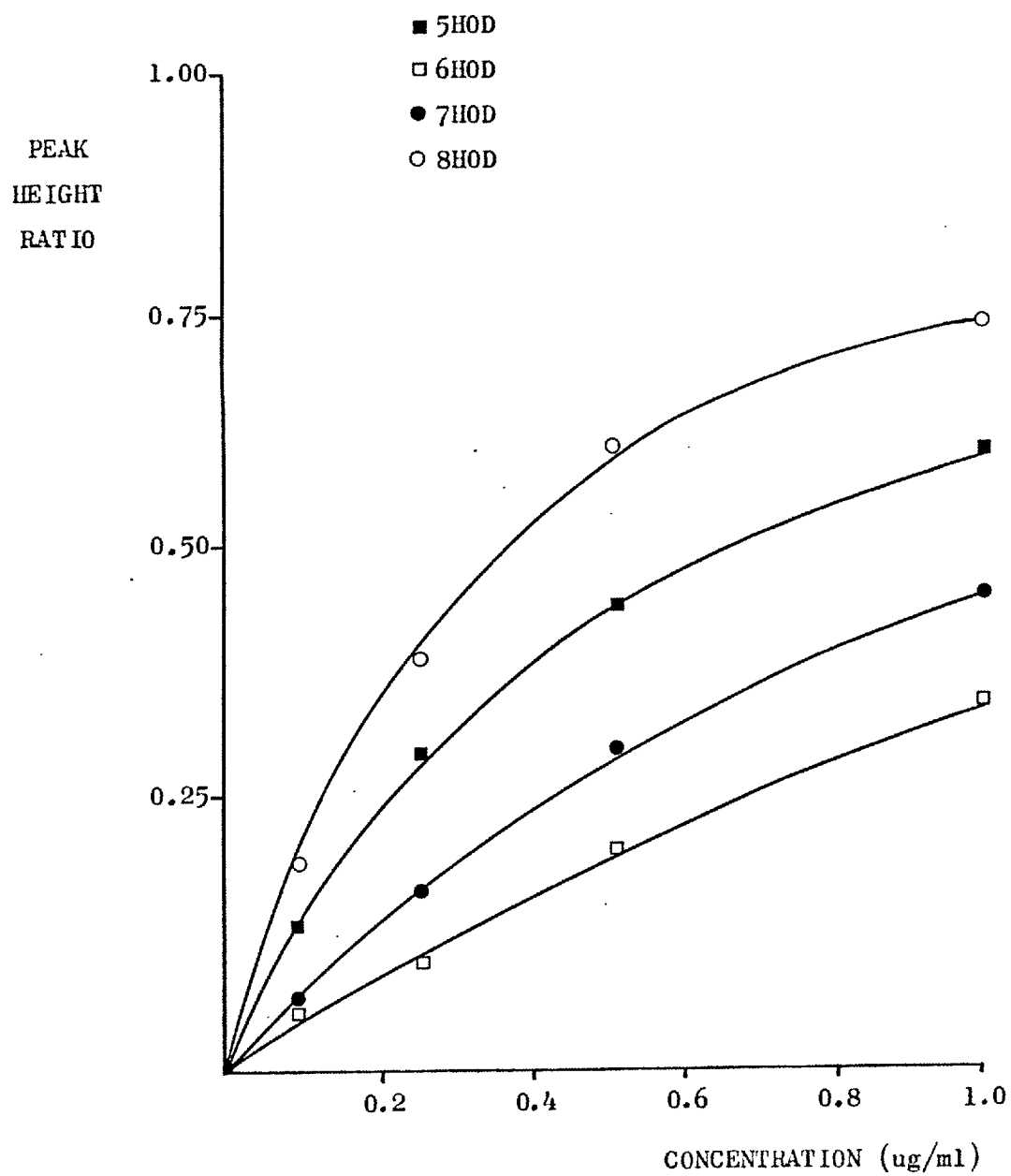
$$\frac{\% \text{ dose excreted unchanged } 0 - 8 \text{ h}}{\% \text{ dose excreted as 4-hydroxydebrisoquine } 0 - 8 \text{ h}}$$

Allocation to phenotype was based on the antimodal value calculated by Price - Evans et al. (1980), such that EM : MR < 12.6 and PM : MR > 12.6.

For the study of debrisoquine aromatic hydroxylation the urines of 17 Ghanaian and 17 Caucasians subjects were further analysed for their content of 5-, 6-, 7- and 8-hydroxydebrisoquine. In this instance urine (0.5 ml) was derivatized with hexafluoroacetylacetone in the manner previously described. The bis-(trifluoromethyl)-pyrimidines thus formed were further derivatized with N-,O-bis-(trimethylsilyl)-acetamide (20 % in 100 ul benzene; Phase Separation Ltd.) after

Figure 3.1: Derivatisation and assay of the phenolic metabolites of debrisoquine. The two stage procedure involves derivatisation with hexafluoroacetylacetone (①), followed by secondary derivatisation with N-, O-bis(trimethylsilyl)-acetamide (②).





extraction into benzene (2 ml) and removal of the extracting solvent under the conditions previously described, save that the column temperature was reduced to 165 °C and the carrier gas flow rate to 40 ml / min. Calibration was curvilinear for each phenol in the range 0.05 - 1 ug / ml, being achieved by addition of known amounts of the authentic metabolites to blank human urine (Fig. 3.1).

Methods of statistical analysis:

Observed phenotype frequencies and estimates of gene and genotype frequencies were calculated on the basis of the Hardy - Weinberg Equilibrium. Given that there is no strong selection pressure against either the D^H or the D^L allele and that their frequencies are p and q respectively, then:

$$p + q = 1, \quad \text{or } 100 \% \text{ of the population.}$$

If mating is random, by algebraic manipulation:

$$p^2 + 2pq + q^2 = 1$$

where p^2 is the frequency of the D^H / D^H homozygote, $2pq$ the heterozygote frequency, and q^2 is the frequency of the D^L / D^L homozygote.

The standard error of the frequency of the recessive gene estimate has been shown to be:

$$SE_q = \left(\frac{(1 - q^2)}{4n} \right)^{\frac{1}{2}} \quad (\text{Emery, 1971})$$

where n is the number of individuals studied. This equation differs slightly from the conventional equation for the standard error of a proportion since q is not a directly observable parameter, it must be estimated from the observed PM frequency, q^2 . The standard error of the observed frequency of the PM phenotype (q^2) is given by:

$$SE_{q^2} = \left(\frac{q^2(1 - q^2)}{n} \right)^{\frac{1}{2}}$$

The 95 % confidence limits for the true frequency of the D^L allele in a population were calculated from:

$$\text{Upper limit: } q + 1.96 (SE_q)$$

$$\text{Lower limit: } q - 1.96 (SE_q)$$

The 95 % confidence limits for the true frequency of the PM phenotype were calculated from:

$$\text{Upper limit: } [q + 1.96 (SE_q)]^2$$

$$\text{Lower limit: } [q - 1.96 (SE_q)]^2$$

The homogeneity of the EM phenotype in both populations was examined by χ^2 analysis of the observed deviation of the probit - transformed cumulative frequencies from the linearity predicted for a normally distributed population, after the method of Finney (1952).

Since the data obtained from both populations displayed bimodal (i.e. non - normal) distribution the significance of apparent differences or correlations between observed variables were tested by distribution - free methods as follows:

(i) Comparison of variables:

If the measured variable parameters observed in two populations are pooled (on a null difference hypothesis) and ranked by magnitude, Wilcoxon (1945) has shown that the probability of the sum of the ranks displayed by either population (R) attaining any particular value has a standard normal distribution about μ_R with variance σ_R^2 , where:

$$\mu_R = n_1 [n_1 + n_2 + 1] / 2$$

$$\text{and: } \sigma_R^2 = n_1 n_2 [n_1 + n_2 + 1] / 12$$

n_1 and n_2 being the number in the sub-group whose ranks have been summed and the remainder of the population respectively. The exact probability of the observed R values in this study were obtained by reference to conventional ordinate and probability (2P) tables.

(ii) Correlation of variables:

Variables from the two ethnic groups were correlated by calculation of the rank correlation coefficient ($r(S)$), as follows:

$$r(S) = 1 - \left[\frac{6 \sum (D_1 - D_2)^2}{n^3 - n} \right] \quad (\text{Kendall, 1970})$$

where D_1 and D_2 are the respective ranks of the two variables under examination displayed by the same individual. The probability associated with $r(S)$ is readily obtained, since:

$$r(S) \cdot [n - 1]^{\frac{1}{2}}$$

has a standard normal distribution.

3.3. Results:

The frequency distribution histograms for the debrisoquine metabolic ratio in the Ghanaian and Caucasian study populations are shown in Figure 3.1. Both distributions are clearly polymodal, with 12 poor metabolisers being identified in the Caucasian population and 5 in the Ghanaian population. The observed homozygous D^L / D^L frequencies and the computed frequencies for the other genotypes, with appropriate confidence limits, are listed in Table 3.2. No significant inter-ethnic differences were seen in these parameters, and neither population differed significantly from the original Caucasian population study of debrisoquine metabolism performed by Evans et al. (1980).

Inspection of the distribution of EM metabolic ratios obtained in the Ghanaian subjects suggested a heterogeneity not obvious in the equivalent Caucasian data. This was borne out by a probit analysis of the two groups (Figure 3.2). The metabolic ratio distribution of the Ghanaian EM subjects was significantly heterogeneous ($\chi^2 = 110$, $p < 0.001$), whilst that of the Caucasian subjects was homogeneous, ($\chi^2 = 29.3$, $p > 0.1$). The distribution of probit values in the Ghanaian group can be seen to be biphasic, suggesting the presence of two resolved normal distributions with an approximate antimodal value of metabolic ratio 1.9, as estimated from the point of inflection of the probit curve.

The median metabolic ratio of the Ghanaian subjects was 5.1, against a median for the Caucasian subjects of 2.2. The rank sum obtained from the Ghanaian EM metabolic ratios was 979½, against a null expectation of 7612.5 ($\sigma_R = 401.9$, $2p = 0.00009$), thus the difference between the medians was significant. The Ghanaian EM rank - order distribution (Figure 3.5) confirmed the conclusions drawn from the probit analysis as it is clearly bimodal.

Figure 3.2 : Frequency (f) distribution histograms for the debriso-
quine metabolic ratio in Caucasian (n = 151) and
Ghanaian (n = 80) study populations. The antimodal
value (MR = 12.6) was calculated by Evans et al. (1980)

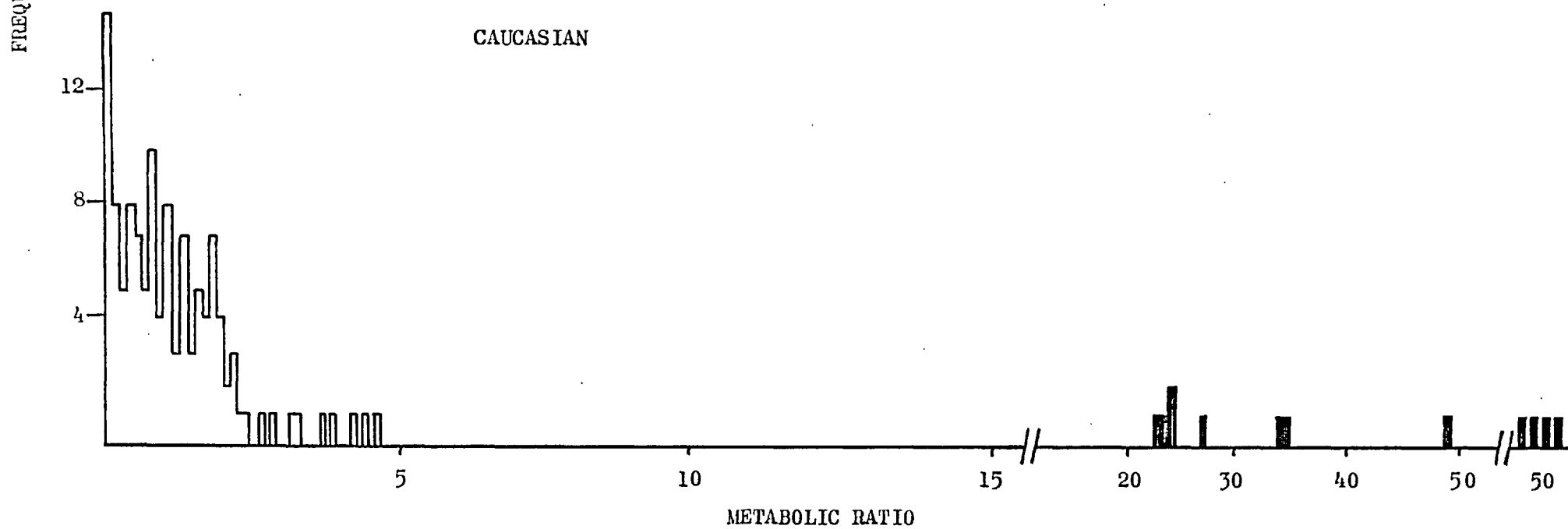
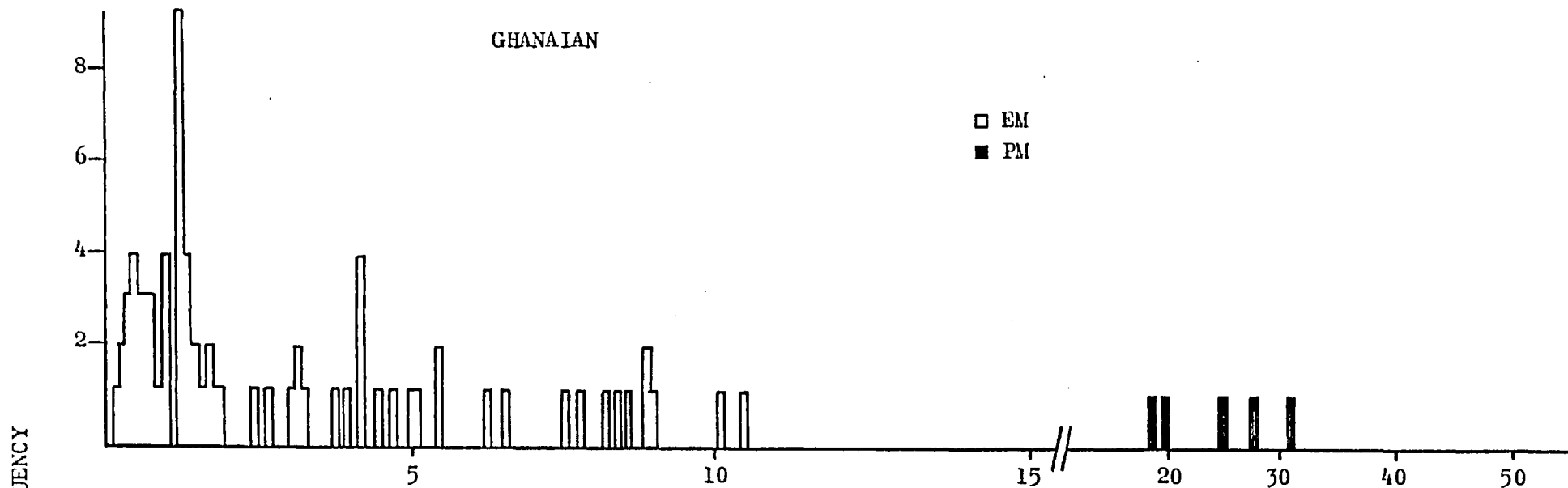


Table 3.2: Gene frequencies and Genotype frequencies for the D^H / D^L loci in Ghanaian (n = 80) and Caucasian (n = 140) study populations; figures in parentheses indicate the number of individuals corresponding to the observed and computed frequencies.

PM frequency (q^2)		95 % Confidence limits (PM)		Expected ($2pq$) Heterozygote frequency		Expected (p^2) Homozygous EM frequency	
Caucasian	Ghanaian	Caucasian	Ghanaian	Caucasian	Ghanaian	Caucasian	Ghanaian
		Upper	Upper	41.3 %	37.5 %	50.1 %	56.3 %
8.6 %	6.3 %	13.8 %	12.7 %	(57.9/140)	(30/80)	(70.1/140)	(45/80)
(12/140)	(5/80)	Lower	Lower				
q = 29.2 %	q = 25.0 %	4.5 %	2.1 %				

Figure 3.3: Probit analysis of the frequency distribution (f)
histogram for Ghanaian (n = 75) and Caucasian (n = 139)
EM subjects.

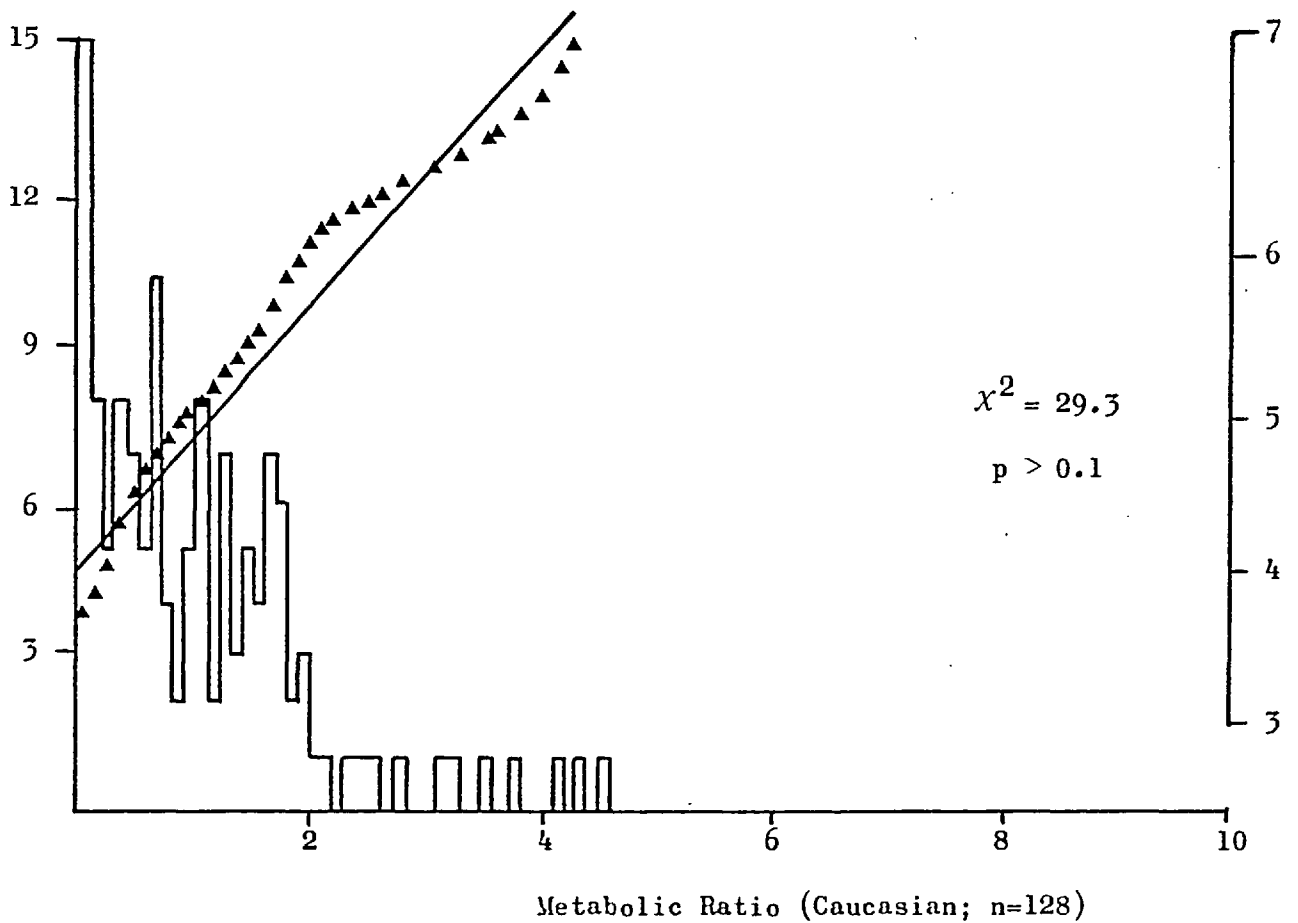
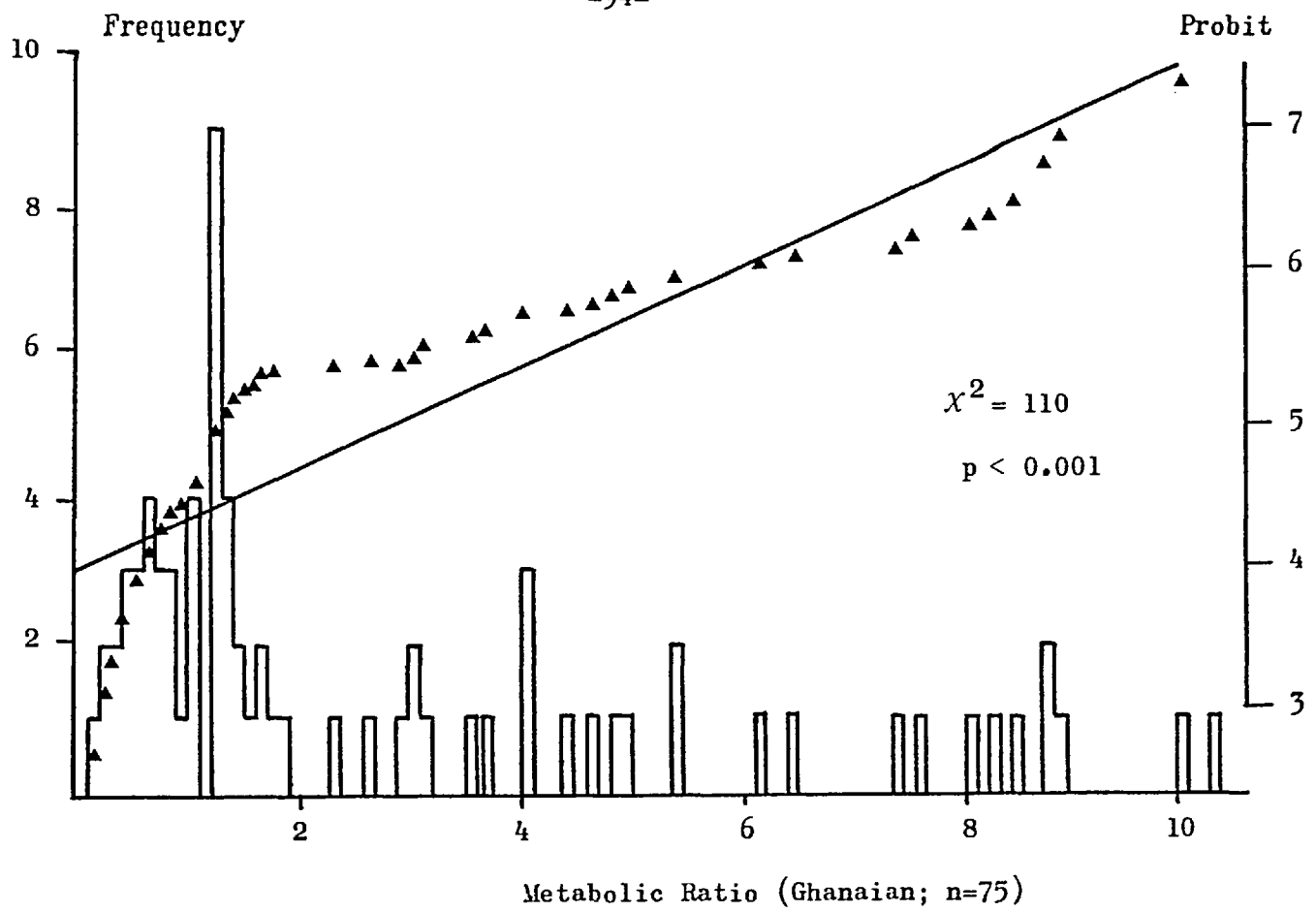
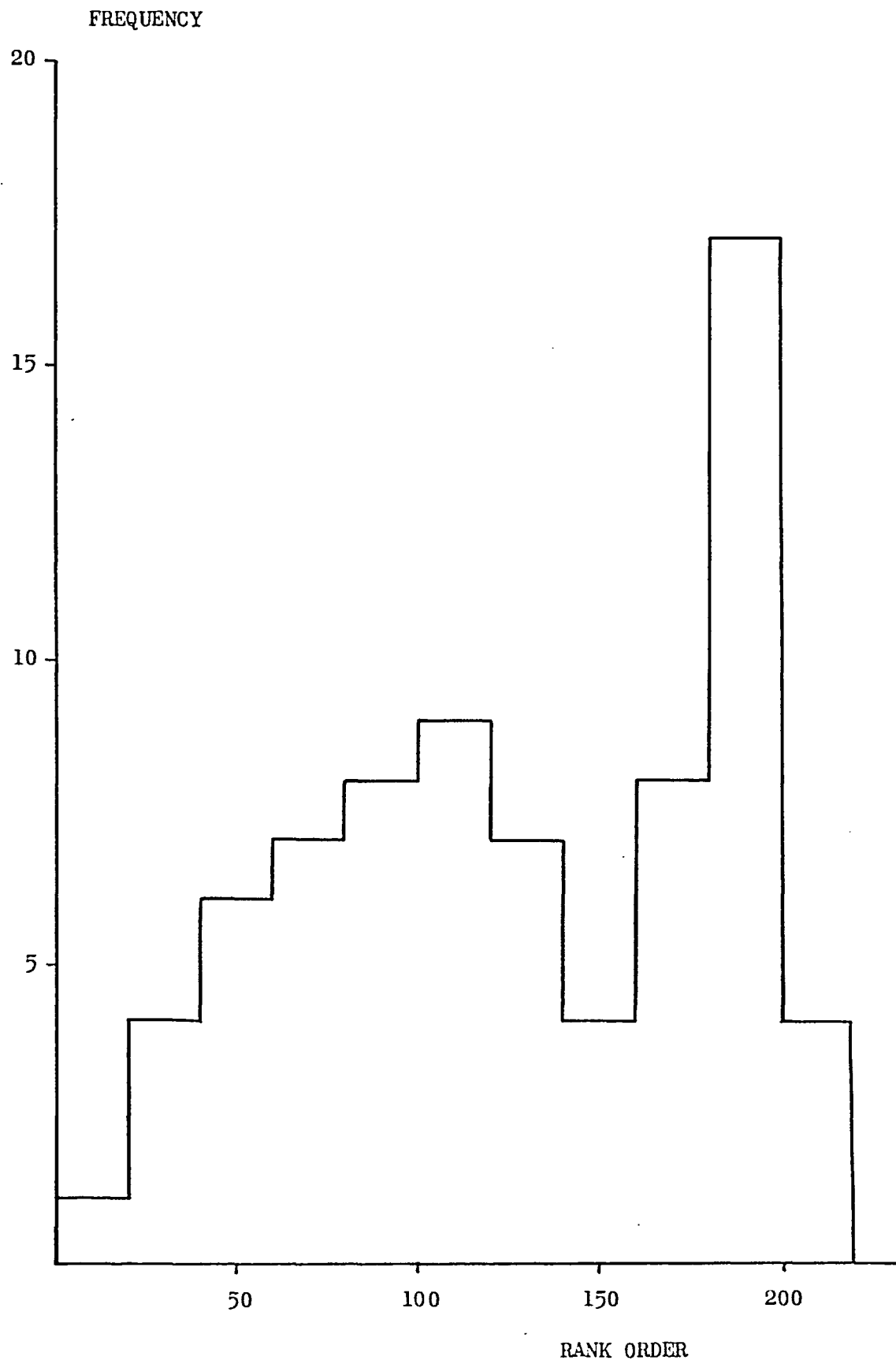


Figure 3.4: Rank - order distribution for Ghanaian EM subjects
(n = 75); Rank - order values were obtained after pooling
of data obtained from both Caucasian and Ghanaian subjects,

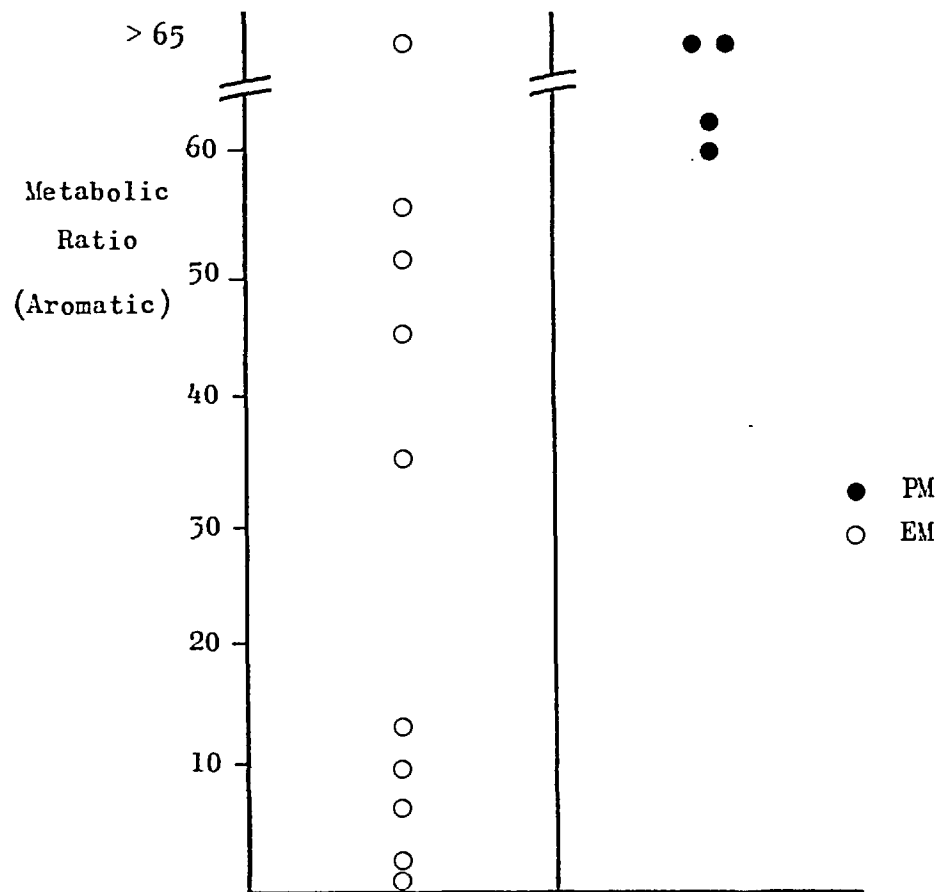


3.3.2: Debrisoquine aromatic hydroxylation:

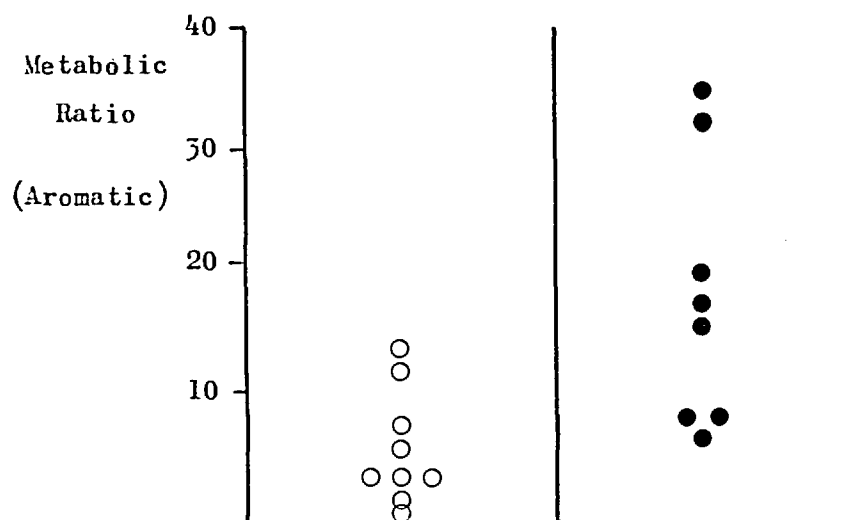
The distributions of metabolic ratios with respect to aromatic hydroxylation of debrisoquine in the two ethnic groups is shown in Figure 3.4. The rank sum for the 4 Ghanaian PM subjects with respect to aromatic hydroxylation was 56, against a null expectation of 36 ($\sigma_R = 8.8$, $2p = 0.030$). The equivalent rank sum for the 8 Caucasian PM subjects studied was 102, against a null expectation of 72 ($\sigma_R = 10.4$, $2p = 0.0063$), indicating that aromatic hydroxylation was significantly lower in the PM phenotype, irrespective of the ethnic group in which this characteristic is studied.

Analysis of data from both phenotypes showed a strong positive rank correlation between the abilities to perform aromatic and alicyclic hydroxylation in both ethnic groups (Caucasian, $r_{(S)} = 0.82$, $p = 0.0018$; Ghanaians, $r_{(S)} = 0.87$, $p = 0.0009$), Figure 3.5a and b.

Figure 3.5: Distribution of the aromatic hydroxylation metabolic ratio in Caucasian (EM, n = 9; PM, n = 8) and Ghanaian (EM, n = 13; PM, n = 4 subjects selected from the original population survey.



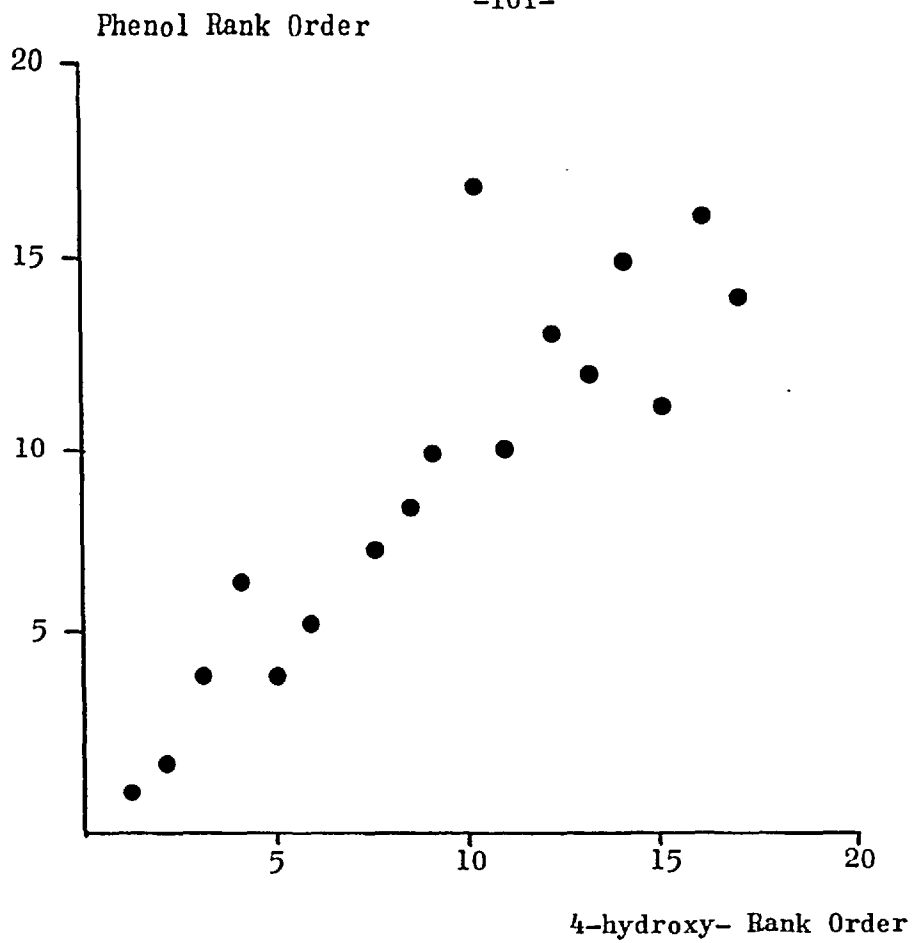
GHANAIAN



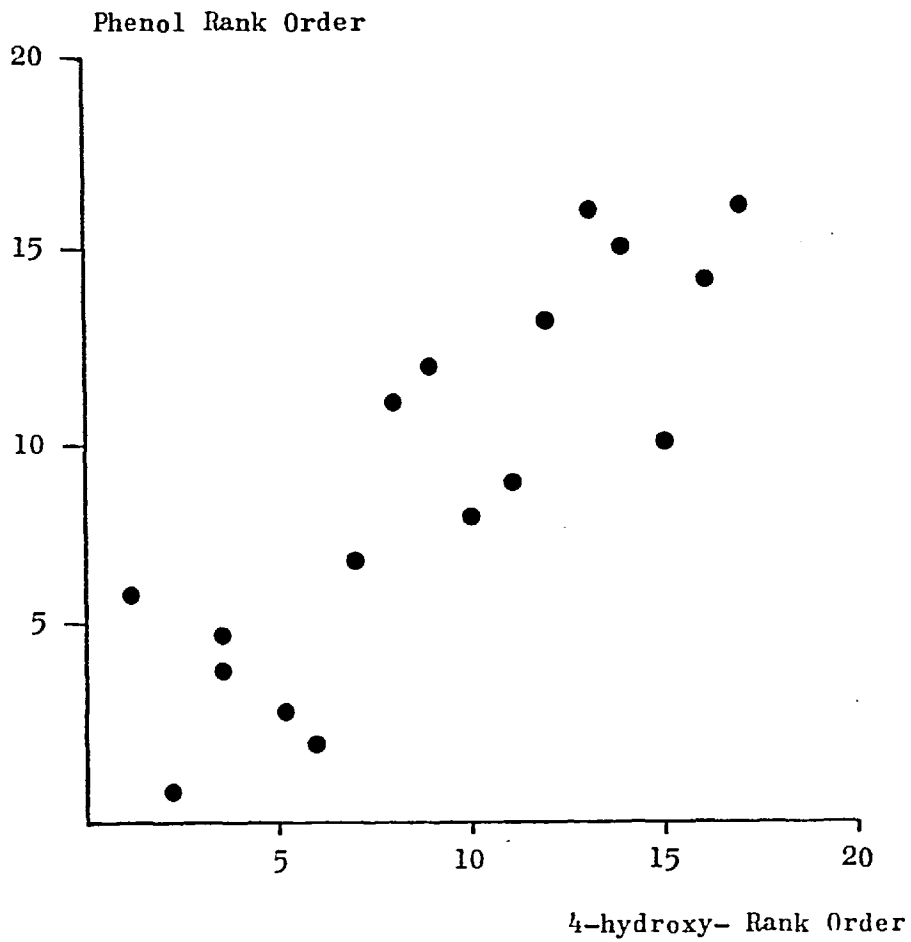
CAUCASIAN

Figure 3.6a: Rank correlation of debrisoquine alicyclic 4-hydroxylation and debrisoquine aromatic hydroxylation in 17 Caucasian subjects.

Figure 3.6b: Rank correlation of debrisoquine alicyclic 4-hydroxylation and debrisoquine aromatic hydroxylation in 17 Ghanaian subjects.



(a) GHANAIAN



(b) CAUCASIAN

3.4: Discussion:

The conclusions drawn from the population studies described may be summarized as follows:

1. The alicyclic 4-hydroxylation of debrisoquine displays genetic polymorphism in a random sample of 80 Ghanaian volunteers. The observed frequency of the homozygous D^L / D^L (PM) phenotype and the calculated frequencies of the D^H / D^H and D^H / D^L (homo- and heterozygous EM) genotypes did not differ significantly from those found in this and previous studies of random samples drawn from the British white Caucasian population.
2. The Ghanaian EM phenotype displayed significant heterogeneity - a feature not seen in Caucasian study populations.
3. The ability to perform aromatic hydroxylation of debrisoquine showed significant inter - phenotype differences (EM PM) in both ethnic groups studied, and was positively correlated with the ability to perform alicyclic 4-hydroxylation, indicating that both reactions are under the control of the D^H / D^L locus.

Consideration of these data and those of Islam et al., (1980) (Table 3.1, vide supra) suggests that the D^L allele is widespread in occurrence and relatively stable in its frequency in habitats as disparate as Accra, Liverpool and Hong Kong. This would indicate that the mutation is 'old' in anthropological terms, having become stable before the now extensive geographical and cultural isolation of the different ethnic groups studied had occurred. A second implication of these findings is that there is some advantage associated with the heterozygous D^H / D^L genotype, the rationale for this conclusion having been discussed in the introductory remarks prefacing this chapter. It would be tempting to hypothesize that such an advantage may arise from the grossly reduced

ability of the PM phenotype to eliminate toxic xenobiotic compounds requiring oxidation for their removal, combined with the enhanced ability of a homozygous EM individual to activate environmental carcinogens, again by metabolic oxidation. However, it must be remembered that mortality from such neoplastic diseases occurs mainly after the reproductive period is over - and so their relevance to arguments of 'fitness' and 'selective pressure' is limited - but this may not always have been so.

The true nature of any advantage associated with the D^H / D^L heterozygote is obviously a subject for speculation at this stage, but light may be thrown on the problem by studies of metabolic ratio distributions in individuals surviving for longer than the prevailing life expectancy in a given ethnic group or habitat. It would be of particular value to study the ethnic groups at the extremes of the gene frequency range in this respect.

It was further observed in the study that the Ghanaian EM phenotype was heterogeneous, apparently containing two sub - groups distributed around an antimode of metabolic ratio 1.8. The number of individuals displaying metabolic ratios lower than the antimode was 43, the number displaying greater 31. These two figures correspond very closely with the expected numbers of homozygous and heterozygous EM subjects (45 and 30 respectively) and it can thus be reasonably concluded that heterozygosity is expressed as a separate trait in the Ghanaian population. Evans et al. (1980) calculated the degree of dominance of D^H over D^L by estimation of the displacement of the mean $\log_{10}$ MR value displayed by known heterozygotes from the midpoint of $\log_{10} \overline{MR}$ (homozygous EM) and $\log_{10} \overline{MR}$ (PM). If a similar calculation is performed on the data from the present study, the dominance of D^H over D^L in the Ghanaian study population is apparently only 4 - 5 %, ($\log_{10} \overline{MR} (D^H / D^L) = 0.68$,

Inter - homozygote midpoint = 0.72). This inter - ethnic difference in phenotype characteristics is presumably due to inter - ethnic differences in the genetic background against which the D^H / D^L alleles are expressed. The functional result of this difference in gene expression is that the Ghanaians as a group are less effective hydroxylators of debrisoquine than Caucasians, and possibly certain other drugs (Idle and Smith, 1979). This finding would imply that dosage regimens based on studies of drug disposition in Caucasian subjects are not necessarily universally applicable.

The final aspect of this study was the demonstration of the parallel pharmacogenetics of the aromatic and alicyclic hydroxylation of debrisoquine. It has already been demonstrated (Idle and Smith, 1979) that the aromatic hydroxylation of another mono - substituted guanidine, namely guanoxan, is also under the control of the D^H / D^L locus. The significance and implications of the fact that examples of aliphatic and aromatic carbon oxidation, conventionally considered to proceed by different mechanisms (Jerina and Daly, 1977), apparently share the same rate - limiting step will be discussed more fully in Chapter Six.

CHAPTER FOUR

HYDROXYLATION PHENOTYPE AND THE DISPOSITION OF DIPHENYLHYDANTOIN

4.1. Introduction

4.2. Materials and methods

4.3. Results:

4.3.1. Panel studies of diphenylhydantoin disposition in Caucasian subjects.

4.3.2. Panel studies of diphenylhydantoin disposition in Ghanaian subjects.

4.4. Discussion

4.1. Introduction:

5, 5 -Diphenylhydantoin (phenytoin) was introduced as an anti-convulsant by Merritt and Putman (1938). It is effective in the treatment of grand mal and psychomotor seizures, but of little use in the management of petit mal variants. The drug may be used alone but it is more commonly found in combination with phenobarbitone or primidone.

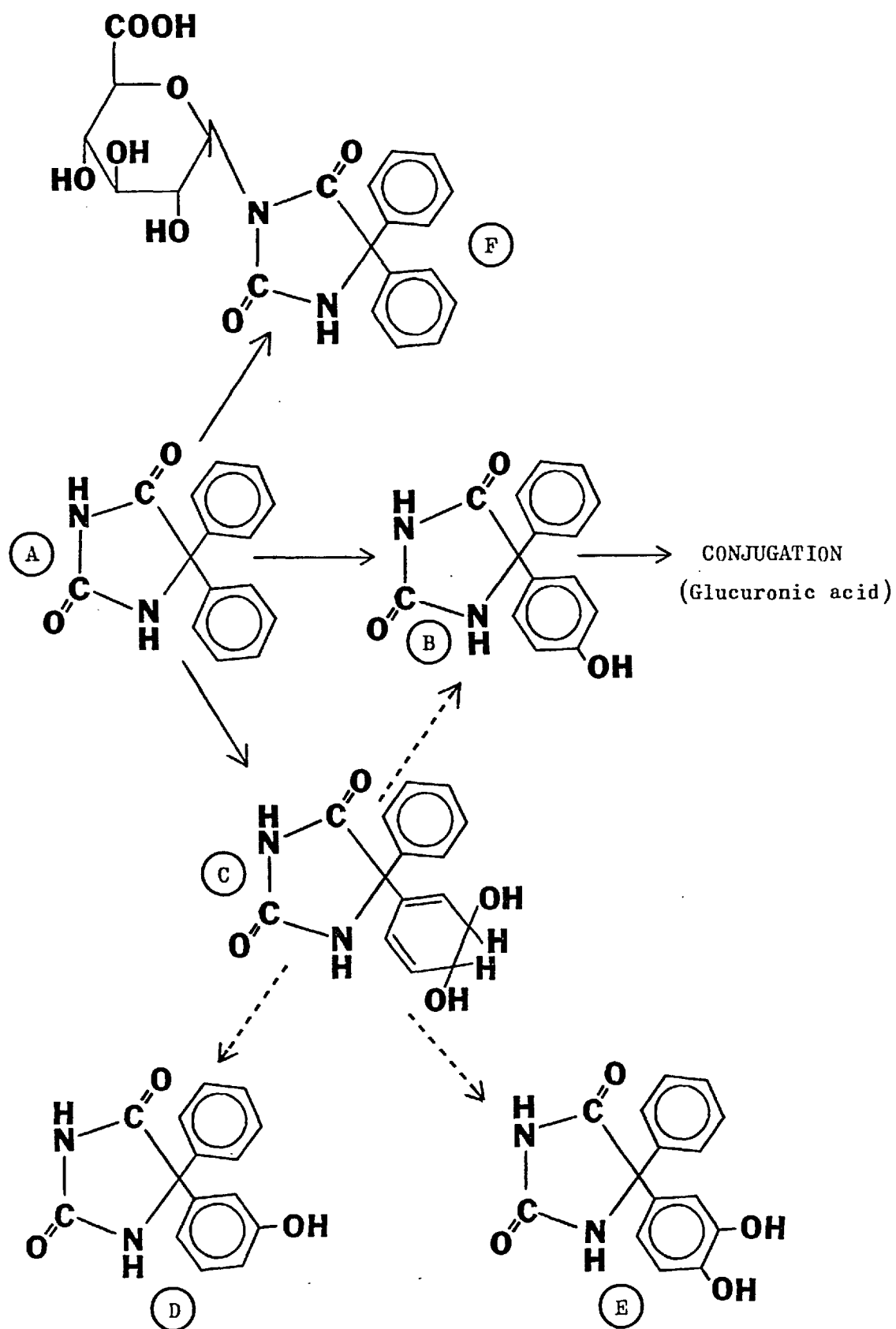
Phenytoin is a good example of a drug with a narrow therapeutic window; seizures are well controlled with plasma drug levels in the range 10 - 20 ug / ml, but above the upper limit of this range the easily recognizable symptoms of phenytoin intoxication, nystagmus, ataxia and lethargy become apparent (Kutt and McDowell, 1968). In the majority of patients stable plasma phenytoin levels and seizure control without side effects are achieved on a daily dose of 300 - 400 mg. However, there are now many reports in the literature of 'deficient' or 'hypo-' metabolizers of the drug, a phenomenon first described by Kutt et al. (1964b). Since this time similar observations have been made by Glazko et al. (1969), Gerber et al. (1972), Eadie et al. (1976) and Vasko et al. (1980). There is general agreement that defective metabolism of phenytoin is an inherited trait, and there is considerable kinship data available to support this supposition (Kutt et al., 1964b; Vasko et al., 1980). Typically, a defective metaboliser of phenytoin will display unusually high plasma drug levels (5 - 6 times normal, Kutt et al., 1964b) when given the standard therapeutic dose, with concomittant symptoms of intoxication.

The metabolism of phenytoin in man has been extensively studied, and is summarized in Figure 4.1. The 4-hydroxy-, dihydrodiol and N³-glucuronide (metabolites B, C and F respectively) are well authenticated in vivo biotransformation products. It is to be presumed that the

Figure 4.1: The metabolism of phenytoin in man.

- (A) 5, 5 -diphenylhydantoin (phenytoin)
- (B) 5-(4'-hydroxyphenyl)-5 -phenylhydantoin (4-hydroxyphenytoin;
HPPH Butler, 1957)
- (C) 5-(3',4'-dihydroxy-1',5'-cyclohexadien-1-yl)-5 -phenyl-hydantoin
(Horning et al., 1971)
- (D) 5-(3'-hydroxyphenyl)5 -phenylhydantoin (3-hydroxy-phenytoin;
Atkinson et al., 1970)
- (E) 5-(3',4'-dihydroxyphenyl)-5 -phenylhydantoin (Horning et al., 1971)
- (F) 5, 5 -diphenylhydantoin N³ glucuronide (Smith et al., 1977)

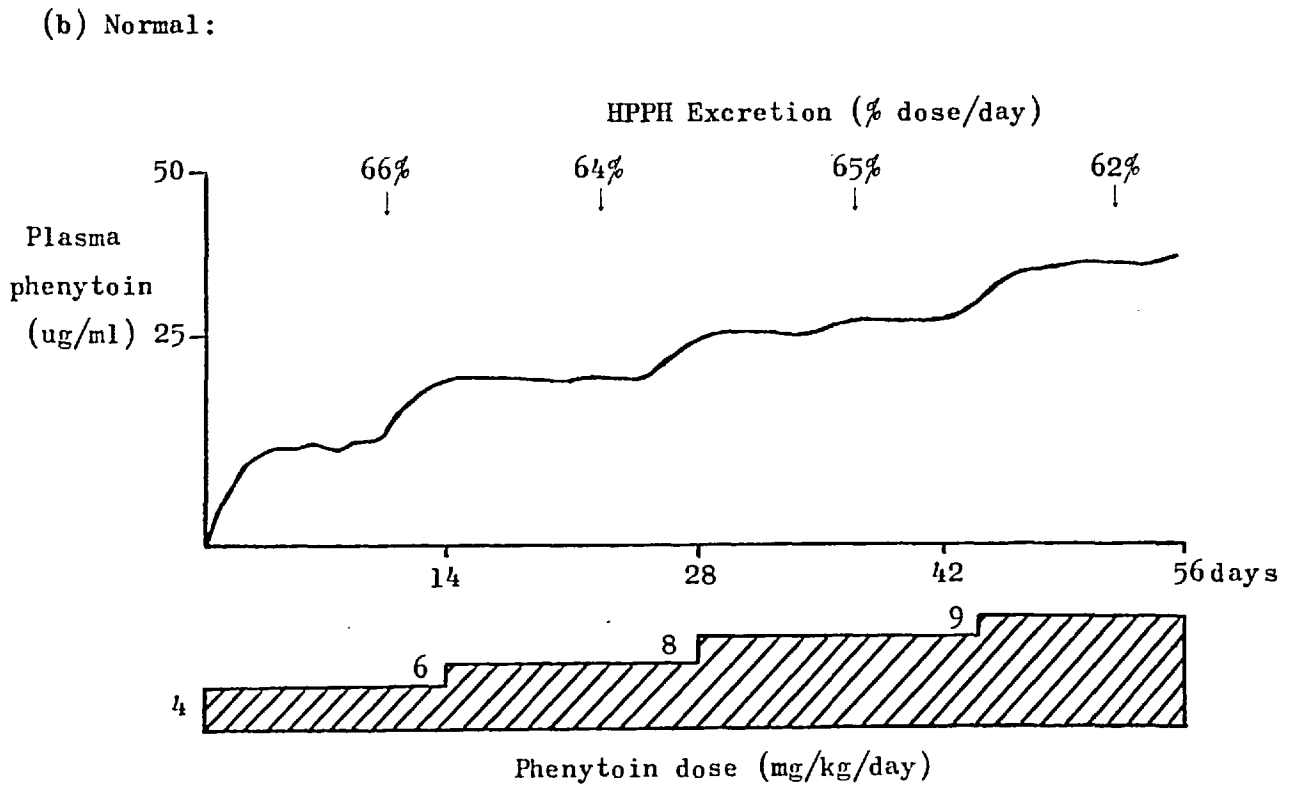
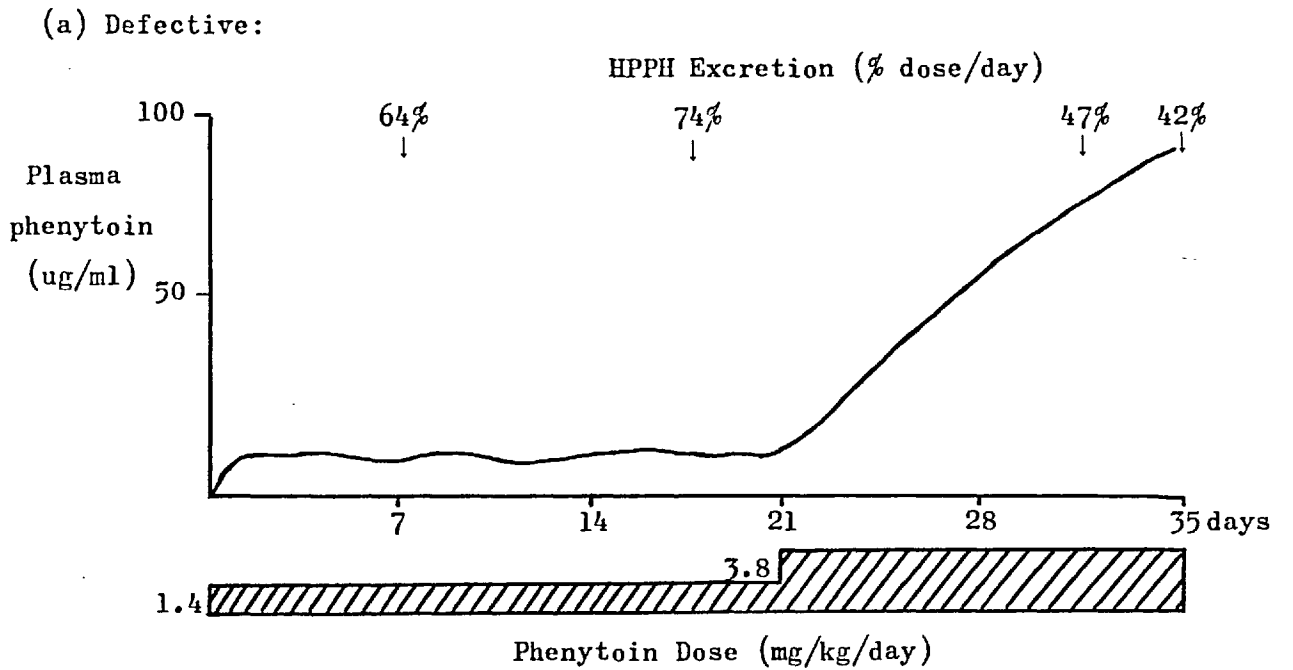
Pathways indicated by broken lines may be either authentic in vivo biotransformation pathways, or the result of in vitro rearrangement.



the dihydrodiol arises from enzymic hydration of an intermediary epoxide (Chang et al., 1970). It has been pointed out by Atkinson et al. (1970) that 3-hydroxyphenytoin and possibly the catechol metabolite (D and E respectively) may arise from in vitro re-arrangement of C in acid conditions.

This notwithstanding, 75 - 90 % of a given dose of phenytoin, regardless of route of administration, can be accounted for as 4-hydroxyphenytoin and its glucuronic acid conjugate, and the evidence available strongly suggests that the widely reported defect of phenytoin metabolism is a defect of this pathway, and that there is no alternative metabolic pathway available. This is exemplified by the data of Kutt et al. (1964b) which is summarized diagrammatically in Figure 4.2. In the case of the normal subject, metabolite excretion remains proportional to dose and steady - state plasma concentration of phenytoin over the entire dose range studied; first - order kinetics are obeyed. At a low dose the defective metaboliser is indistinguishable from the normal; HPPH excretion is comparable and plasma phenytoin levels are at steady - state; when the dose is increased beyond what the authors describe as this subject's "congenitally low ceiling for metabolizing the drug" accumulation of unchanged drug in plasma occurs and HPPH excretion as a function of dose starts to decline; that is, non - first - order kinetics. In fact, Gerber and Wagner (1972) have pointed out that the disposition of phenytoin in many subjects is better described by Michaelis - Menten, rather than first - order kinetics. It is felt that the fact that resolution of normal and defective metabolizers is essentially dose - dependant is of considerable significance; this feature is discussed more fully in Chapter Six.

Figure 4.2: Plasma levels of phenytoin and HPPH excretion in
'normal' and 'defective' metabolisers of phenytoin.
(Kutt et al., 1964b)



Scope of the present work:

The data reported here are derived from the application of a 'panel' approach to the study of phenytoin metabolism to ascertain whether or not the metabolic oxidation of debrisoquine and phenytoin are under parallel genetic control. The panels of volunteers were selected on the basis of the predetermined debrisoquine metabolic ratio (Chapter Three and Mahgoub et al., 1977). Individuals representing extremes of the debrisoquine oxidation status were deliberately chosen (EM subjects, $MR < 1$, PM subjects $MR > 20$) and challenged with a single oral dose of phenytoin. The simple hypothesis is proposed; do individuals with a reduced capacity to hydroxylate debrisoquine also possess a reduced capacity to hydroxylate phenytoin? In other words, are both defects manifestations of possession of the D^L / D^L genotype? This approach has already demonstrated that the aromatic hydroxylation of debrisoquine (Chapter Three, vide supra) and guanoxan (Mahgoub, 1979) are under the control of the D^H / D^L locus, whereas that of acetanilide (Wakile et al., 1979) is under independent control.

4.2: Subjects, materials and methods:

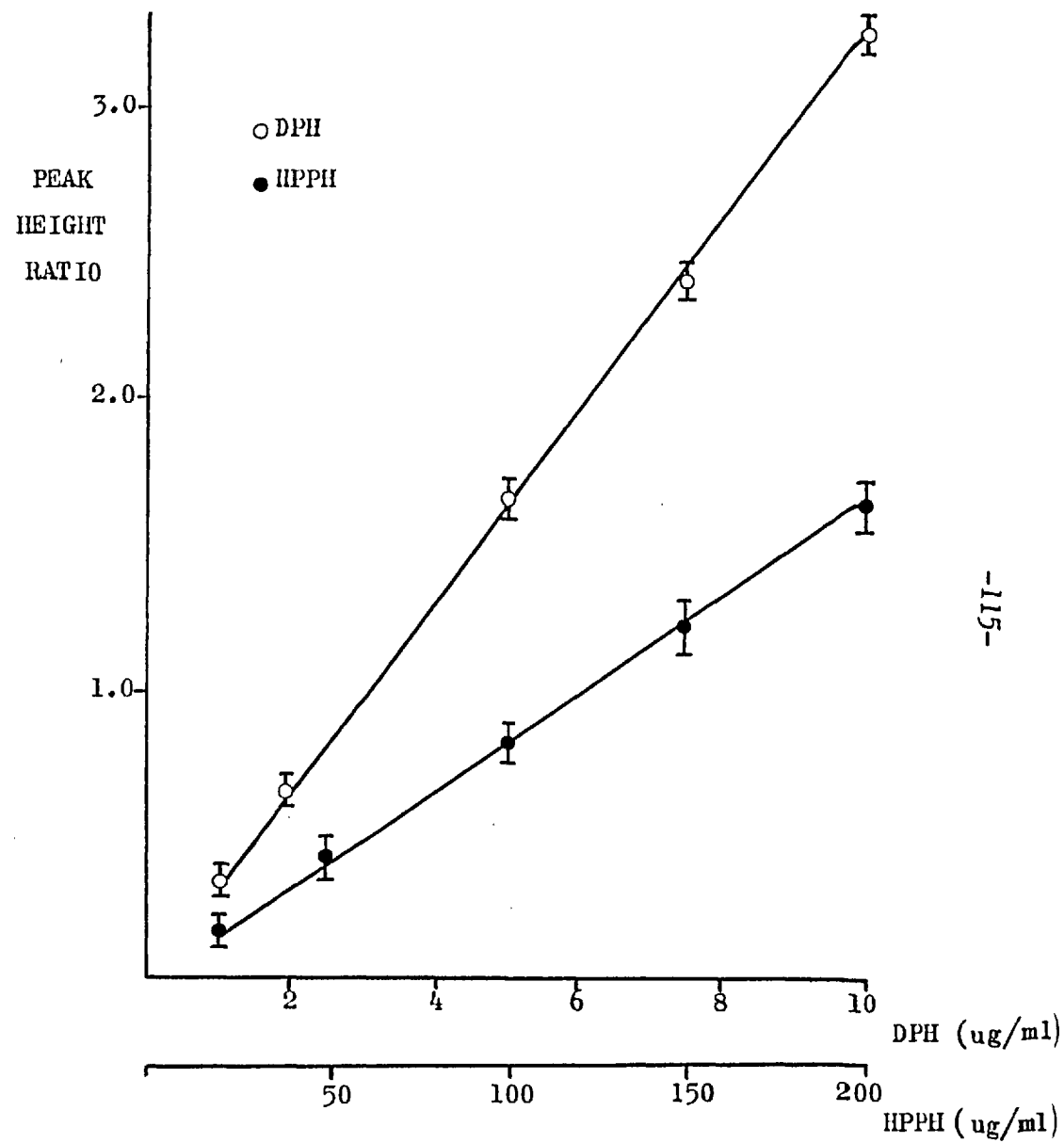
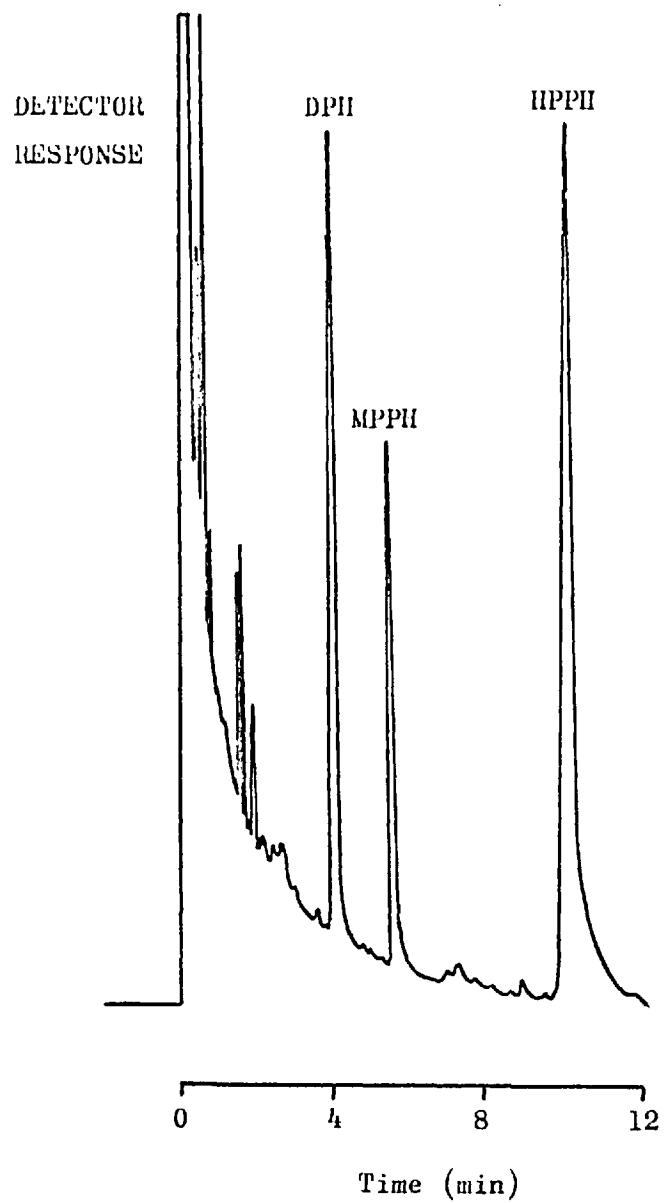
The Caucasian subjects studied were selected on the basis of their oxidation phenotype as determined by Mahgoub (1979). Five subjects with metabolic ratios (MR) of less than 12.6 (phenotypically EM), and six subjects with MR > 12.6 (phenotypically PM) each received 200 mg diphenylhydantoin (2 x 100 mg sodium phenytoin, 'Epanutin', Parke - Davis) by mouth following a light breakfast. Urine for phenytoin and 5-p-hydroxyphenyl-5 -phenylhydantoin (4-hydroxyphenytoin, HPPH) determination was collected over four sequential 12 h intervals after dosing.

The Ghanaian subjects studied were selected on the basis of their oxidation phenotype as determined in the course of the population study described in Chapter Three (vide supra). Four EM and four PM subjects followed an identical protocol to that described for the Caucasian subjects save that urine was collected over six sequential 8 h intervals after dosing. These urine samples were frozen after collection and transported from Accra to London by air for determination of their phenytoin and HPPH content in the manner to be described.

Urinary concentrations of phenytoin and HPPH were determined simultaneously from a single sample by a modification of the gas - liquid chromatography method of Kupferberg (1970). Following the addition of 5-(p-methylphenyl)-5 -phenylhydantoin (5 ug in 50 ul methanol) as internal standard, urine (1 ml) was incubated with 6 M HCl (2 ml) for 1 h at 100 °C to effect hydrolysis of conjugated material. After cooling the samples were extracted with dichloromethane (10 ml). The organic layer was removed by means of phase - separation paper (Whatman 1PS 15 cm) and then shaken with saturated lead acetate solution (1 ml); the aqueous and organic phases were again separated in the manner

previously described. The dichloromethane phase was evaporated under reduced pressure and the dry residue redissolved in 50 μ l 10 % trimethylanilinium hydroxide ('Methelute', Pierce, Rockford, Ill.) in methanol; 1 - 4 μ l of this solution were injected on the GLC. The method employed a Packard Model 417 Becker gas - liquid chromatograph fitted with a hydrogen flame - ionization detector. The 1.8 m column (i.d. 2 mm) was packed with 3 % OV-17 on Chromosorb W(HP) 80 - 100 mesh; carrier gas flow (oxygen - free nitrogen) was 50 ml / min, air flow 40 ml / min and hydrogen flow 20 ml / min. The injection port, column and detector temperatures were 300 °C, 240 °C and 270 °C respectively. Calibration was achieved by adding known amounts of 5,5'-diphenylhydantoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (1 - 10 μ g / ml and 20 - 200 μ g / ml respectively, Sigma Chemical Co.) to aliquots of blank human urine. Calibration was linear with respect to both compounds over the measured range (Figure 4.3.) Metabolic data from the two phenotypes were compared using both conventional parametric methods and the distribution - free methods described in Chapter Three (vide supra).

Figure 4.3: GLC trace and calibration for diphenylhydantoin (DPH), and 4-hydroxyphenytoin (HPPH), with 4-methylphenytoin (MPPH) employed as internal standard. Points represent Mean $\pm$ S.D. for 4 determinations.



4.3: Results:

4.3.1: Diphenylhydantoin disposition in phenotyped panels of Caucasian subjects.

Cumulative excretion of HPPH following administration of a single oral dose of 200 mg diphenylhydantoin to 11 Caucasian subjects of previously determined debrisoquine oxidation phenotype (EM, n = 5; PM, n = 6) is summarized in Table 4.1. The PM subjects excreted significantly less metabolite 24 - 48 h post - dosing. The individuals studied were further characterized in terms of an apparent first - order metabolite formation rate constant ($k_{f \text{ HPPH}}$) and a half - time for metabolite excretion (t_{50} : The time required for elimination of half the administered dose as HPPH). In some instances, the latter quantity was not achieved during the study period, and was thus estimated by extrapolation of the plot: cumulative excretion versus $\ln t$, which is linear under first - order conditions. These two kinetic parameters are summarized in Table 4.2. The median first - order formation rate constants in the two phenotypes were EM: 0.030 h^{-1} , PM: 0.018 h^{-1} ; the PM subjects yielded a rank - sum of 50.5 (Null expectation 36.0 ± 5.5 , $2p = 0.008$). The median t_{50} values in the two phenotypes were EM : 46.5 h, PM : 81.0 h; the PM subjects yielded a rank sum of 49.0 (Null expectation 36.0 ± 5.5 , $2p = 0.018$). Thus in both instances, the parameters indicate that PM subjects metabolized diphenylhydantoin at a slower rate than those of the EM phenotype. Reanalysis of these data assuming parametric criteria and comparing the means listed in Table 4.2 reinforced this conclusion ($k_{f \text{ HPPH}}$: EM > PM, $2p < 0.001$; t_{50} : EM < PM, $2p < 0.005$).

Excretion of unchanged phenytoin in urine displayed no significant interphenotype differences (EM: $1.6 \pm 0.5 \%$ dose / 48 h; PM: $1.7 \pm 0.7 \%$ dose / 48 h); 5-hydroxyphenytoin could not be detected in the

Table 4.1: Cumulative excretion of 4-hydroxyphenytoin by EM (n = 5) and PM (n = 6) subjects following a 200 mg oral dose of diphenylhydantoin, (Mean $\pm$ S.D.)

<u>Time post - dosing (h)</u>	<u>EM (n = 5) (% dose)</u>	<u>PM (n = 6) (% dose)</u>
12	11.5 $\pm$ 7.1	9.9 $\pm$ 4.3
24	29.0 $\pm$ 10.5	22.5 $\pm$ 7.9
36	43.7 $\pm$ 11.2	31.0 $\pm$ 7.9 *
48	52.8 $\pm$ 10.7	36.9 $\pm$ 7.0 **

* PM significantly lower than EM, 2p < 0.05

** PM significantly lower than EM, 2p < 0.01

Table 4.2: Elimination parameters for diphenylhydantoin in EM (n = 5) and PM (n = 6) panels following an oral dose of 200 mg.

	EM						PM						
	1	2	3	4	5	Mean	1	2	3	4	5	6	Mean
						$\pm$							$\pm$
Debrisoquine MR	0.5	0.2	0.3	0.7	0.7	S.D.	24.5	26.4	19.9	26.0	28.4	49.8	S.D.
$*k_f$ HPPH (h^{-1})	0.032	0.036	0.038	0.024	0.021	0.030	0.021	0.016	0.016	0.011	0.017	0.015	0.016
						$\pm$							$\pm$
						0.007							0.003
$^+t_{50}$ (h)	33.0	35.9	38.1	54.5	60.1	44.3	54.7	81.4	81.7	109.0	56.2	106.0	81.5
						$\pm$							$\pm$
						12.1							23.3

* Apparent first - order rate constant for the formation of 4-hydroxyphenytoin.

$^+$ Half - time for the urinary elimination of 200 mg diphenylhydantoin (p.o.) as 4-hydroxyhydantoin.

urine of any of the subjects studied.

Intra - individual variability in phenytoin metabolism was evaluated in three EM and a single PM subject; these data are listed in Table 4.3. It can be seen that in no instance did intra - individual variation exceed inter - phenotype variation.

Significant rank correlations were found between the pre-determined debrisoquine metabolic ratio and the two kinetic parameters employed in the study, k_f HPPH and t_{50} ($r_{(S)} = 0.83$, $p = 0.004$; $r_{(S)} = 0.74$, $p = 0.010$ respectively, cf. Figures 4.4a and b).

4.3.2: Panel studies of diphenylhydantoin disposition in Ghanaian subjects.

Cumulative excretion of HPPH following administration of a single oral dose of 200 mg diphenylhydantoin to 8 Ghanaian subjects of previously determined debrisoquine oxidation phenotype (EM, $n = 4$; PM, $n = 4$) is summarized in Table 4.4. There was a small but significant interphenotype difference (PM < EM) in the 24 - 48 h post - dosing period. However, as can be seen from Figures 4.5 and 4.6, the inter - ethnic differences in phenytoin disposition were more marked than those between the phenotypes. The Ghanaian EM subjects produced some 3 times less the amount of HPPH during the study period than their Caucasian counterparts, and the Ghanaian PM subjects likewise produced some 2.8 times less than Caucasian PM subjects. It was not possible to derive values for k_{fHPPH} and t_{50} for the Ghanaian study group since, as can be seen from Figure 4.6, no individual from this group, EM or PM, displayed a terminal linear phase with respect to urinary excretion of HPPH. In neither phenotype was the rate of excretion of metabolite significantly lower at 42 h post - dosing than it was in the 'peak' period of 12 - 20 h post - dosing. In

Table 4.3: Reproducibility of kinetic parameters describing the elimination of diphenylhydantoin in 3 EM subjects and a single PM subject.

Subject	k_f HPPH (h^{-1})	t_{50} (h)	Unchanged Diphenylhydantoin (% dose / 48 h)
EM (1)	0.031	31.0	1.3
	0.032	35.0	0.8
EM (2)	0.025	35.3	1.9
	0.046	36.5	1.5
EM (3)	0.036	42.0	2.2
	0.039	36.0	1.6
PM (1)	0.019	61.4	2.1
	0.023	48.0	1.8

Figure 4.4a: Rank correlation between debrisoquine metabolic ratio (MR) and apparent first - order formation rate constant for 4-hydroxyphenytoin (k_f HPPH) in 11 subjects (EM, n = 5; PM, n = 6)

Figure 4.4b: Rank correlation between debrisoquine metabolic ratio (MR) and half - time for elimination of phenytoin as 4-hydroxyphenytoin (t_{50}) in 11 subjects (EM, n = 5; PM, n = 6)

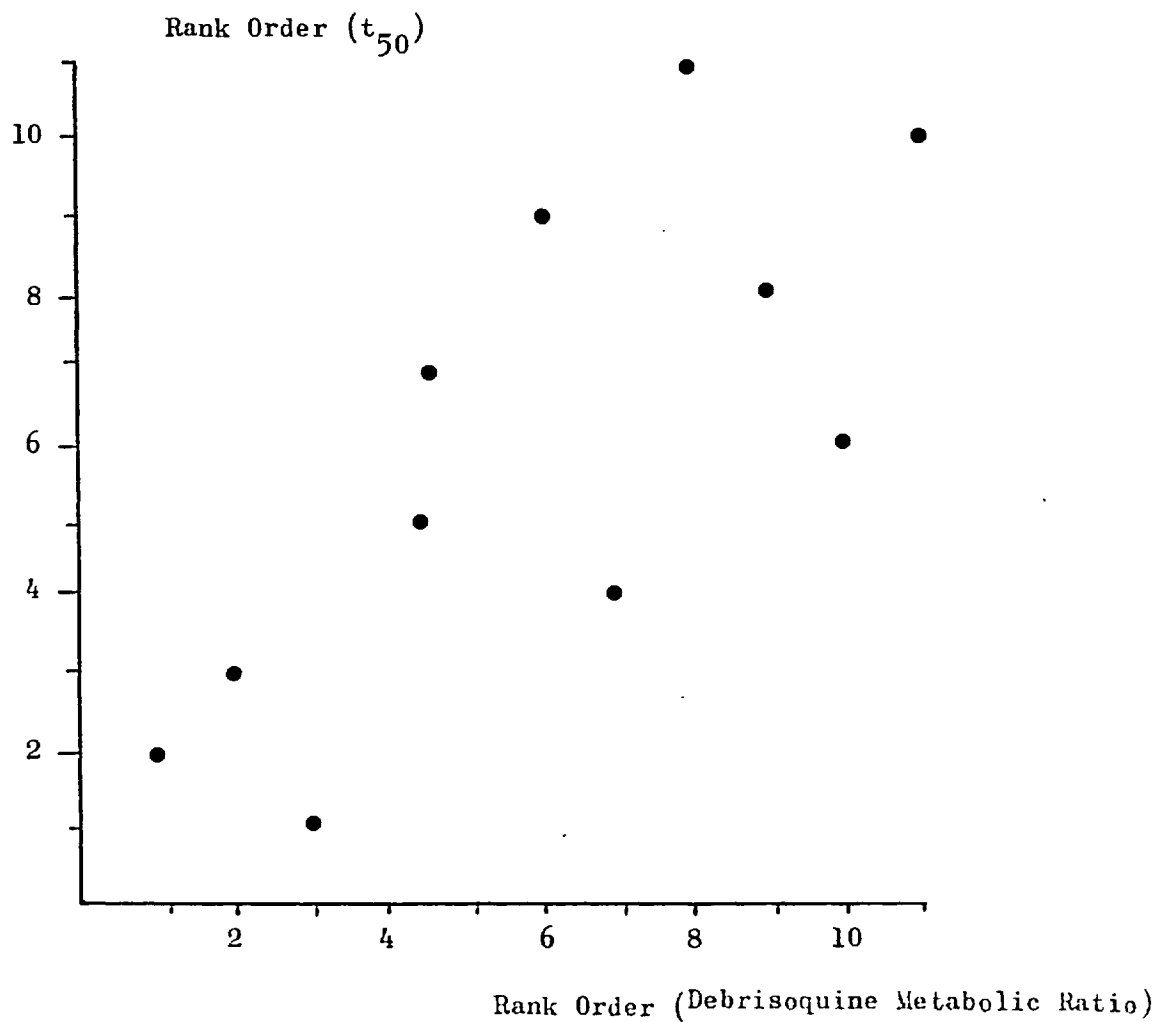
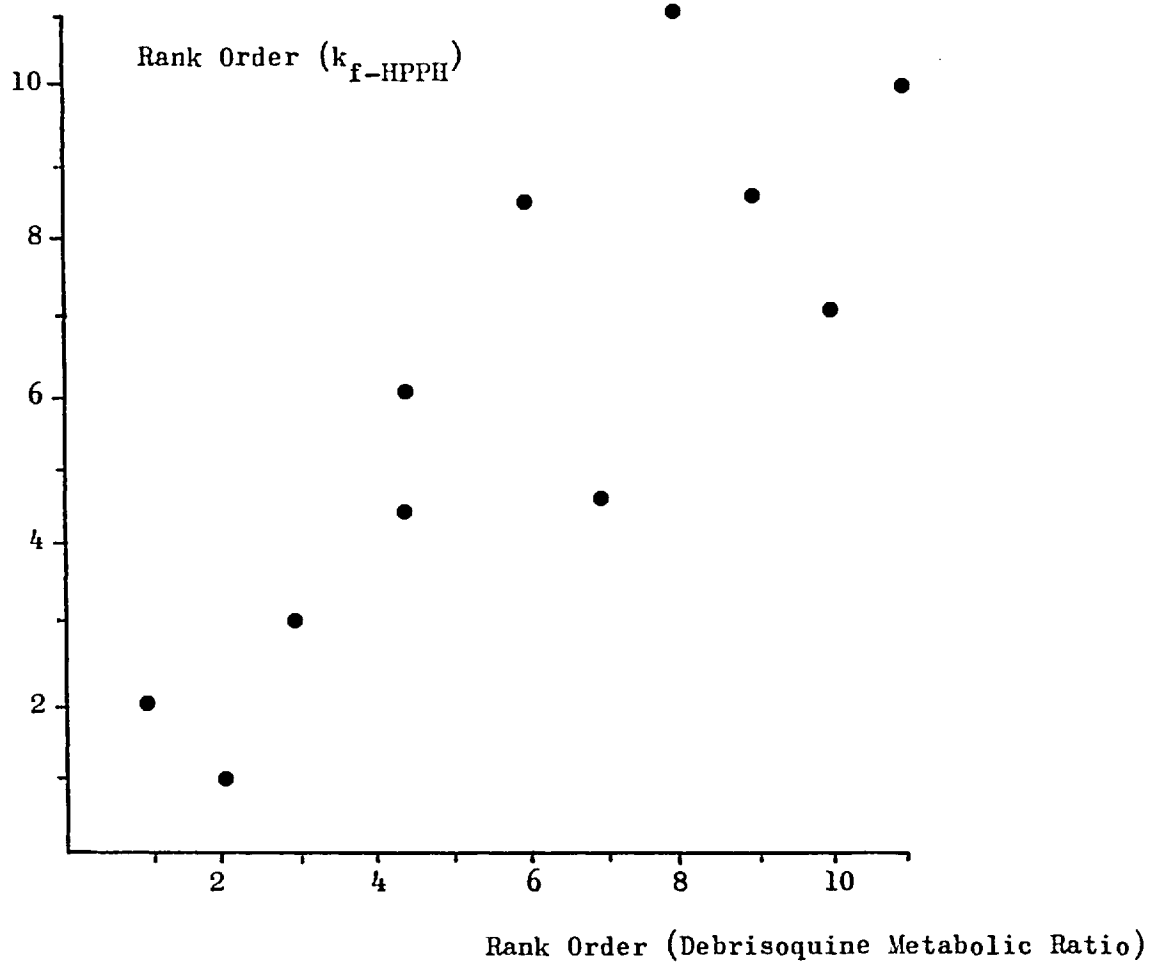


Table 4.4: Cumulative recovery of HPPH in Ghanaian subjects of known debrisoquine oxidation phenotype (EM, n = 4; PM, n = 4) following a 200 mg oral dose of diphenylhydantoin.

Debrisoquine M.R.	EM					PM				
	1	2	3	4	Mean ± S.D.	1	2	3	4	Mean ± S.D.
Time (h)	Cumulative % dose					Cumulative % dose				
8	3.0	1.3	1.2	2.5	2.0 ± 0.9	1.6	1.1	1.0	2.5	1.6 ± 0.7
16	6.0	4.5	3.5	5.6	4.9 ± 1.1	4.5	3.3	3.1	5.2	4.0 ± 1.0
24	12.2	7.0	6.6	9.5	8.8 ± 2.6	7.0	5.3	5.2	9.2	6.7 ± 1.9
32	14.3	12.0	9.3	13.1	12.2 ± 2.1	8.3	7.5	7.3	10.4	8.4 ± 1.4*
40	16.3	14.8	12.7	16.3	15.0 ± 1.7	10.5	9.1	9.6	14.2	10.9 ± 3.3*
48	17.0	18.6	14.3	19.0	17.2 ± 2.1	12.7	10.2	11.9	17.4	13.1 ± 3.1*

* PM significantly lower than EM 2p < 0.05

Figure 4.5: Cumulative recovery of HPPH in 11 Caucasian (EM, n = 5; PM, n = 6) and 8 Ghanaian (EM, n = 4; PM, n = 4) volunteers following a 200 mg oral dose of diphenylhydantoin. (All points indicate Mean $\pm$ S.D.)

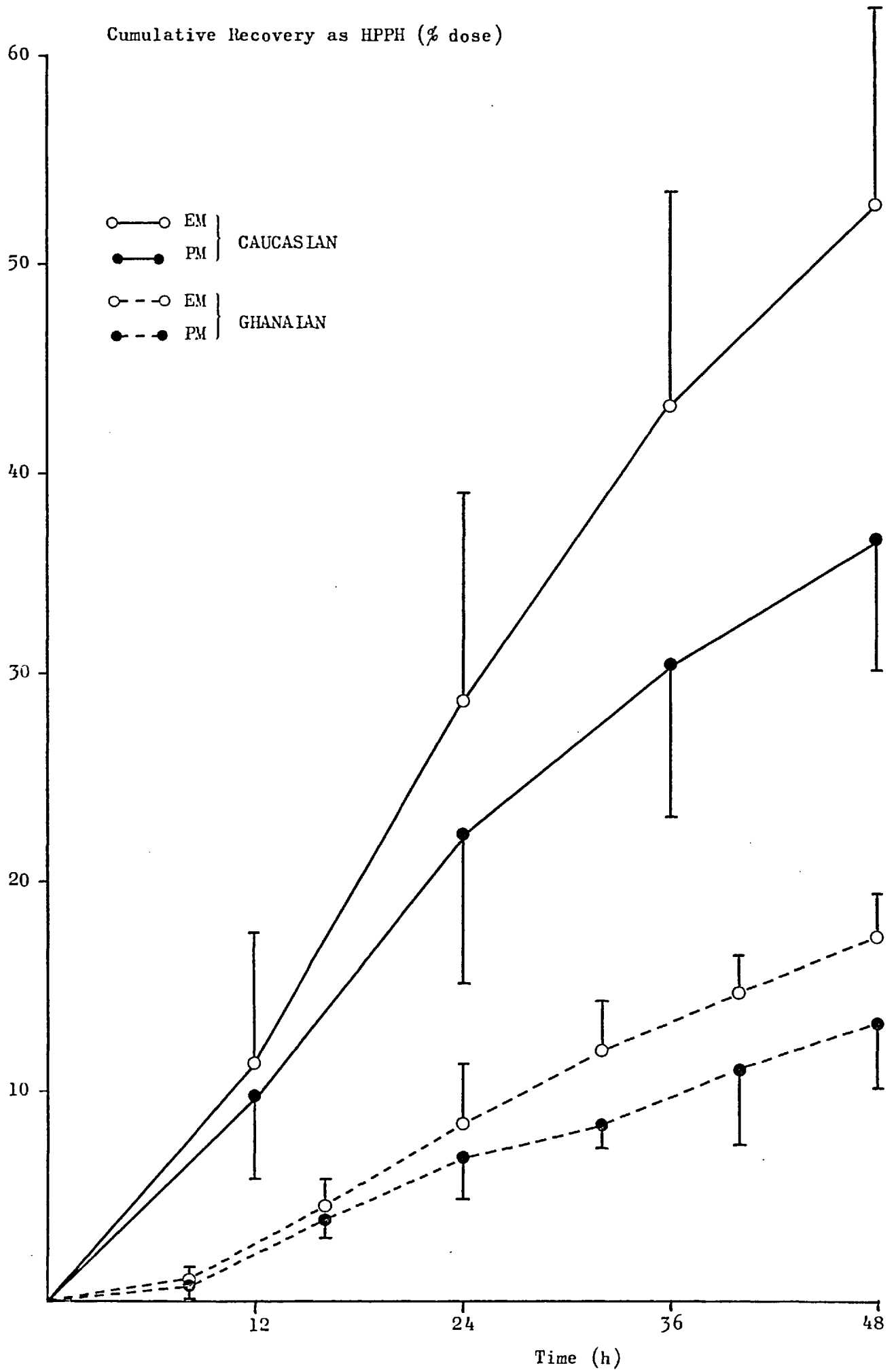
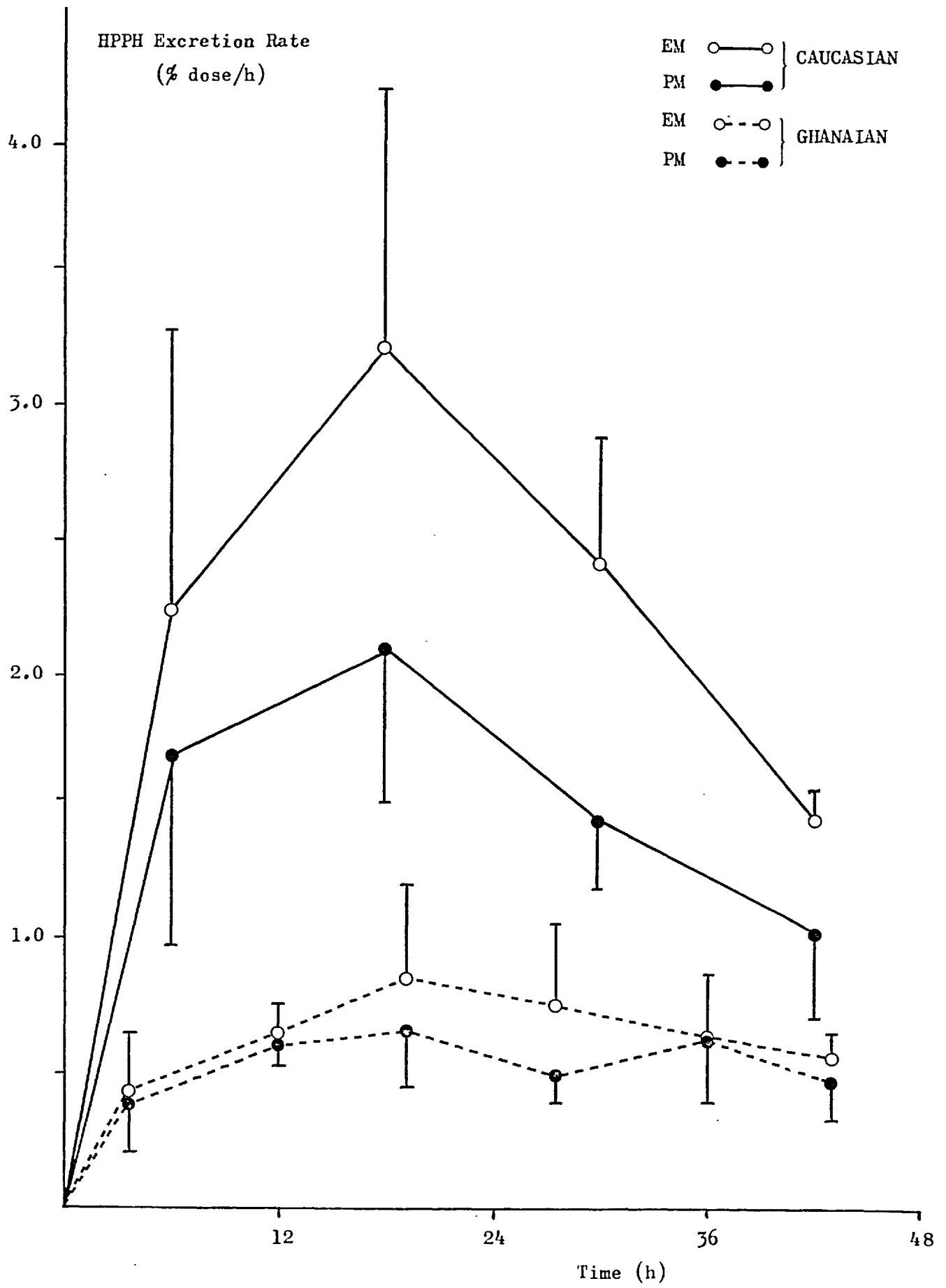


Figure 4.6: Urinary excretion rates of HPPH in 11 Caucasian (EM, n = 5; PM, n = 6) and 8 Ghanaian (EM, n = 4; PM, n = 4) volunteers following a 200 mg oral dose of diphenylhydantoin. (All points indicate Mean $\pm$ S.D.)



contrast all the Caucasian subjects studied, of either phenotype, displayed a terminal log - linear decline in rates of metabolite excretion in the period 18 - 42 h post - dosing. (EM $t_{\frac{1}{2}}$: 17.2 ± 4.0 h; PM $t_{\frac{1}{2}}$: 23.8 ± 3.5 h).

As with the Caucasian study group, there were no significant inter-phenotype differences in excretion of unchanged phenytoin (EM : 1.2 ± 0.6 % dose / 48 h; PM : 1.3 ± 0.5 % dose / 48 h); these values were lower, but not significantly so, than those displayed by the Caucasian phenotypes. Once again, no 3-hydroxyphenytoin could be detected in the urine of any of the Ghanaian study group.

4.4: Discussion:

The findings of the present study of the disposition of phenytoin may be summarized briefly as follows:

1. Subjects of predetermined EM or PM phenotype with respect to debrisoquine oxidation displayed significantly different capacities to perform the aromatic hydroxylation of phenytoin and thus phenytoin hydroxylation would appear to be polymorphic. This has been demonstrated both as a reduced capacity on the part of PM subjects and as an overall correlation across the two phenotypes.
2. The inter - phenotype patterns of debrisoquine and phenytoin metabolism were qualitatively similar but quantitatively different. Typically, a PM subject differs from his EM counterpart by one or two orders of magnitude with respect to debrisoquine oxidation, but the inter - phenotype differences with respect phenytoin oxidation were only 2 - 3 fold, and resolution of the phenotypes at the dose employed was incomplete.
3. Studies of Ghanaian volunteers revealed inter - ethnic differences in phenytoin metabolism which were more pronounced than those seen between the two phenotypes. Inter - phenotype differences were seen in this study (PM < EM by a factor of 1.5 - 1.5) but these were overshadowed by the three - fold differences displayed by both Ghanaian phenotypes with respect to their Caucasian counterparts.

These data would suggest that the alicyclic hydroxylation of debrisoquine and the aromatic hydroxylation of phenytoin display parallel pharmacogenetics. Confirmation of such a supposition could be achieved by cross - over studies of debrisoquine metabolism in

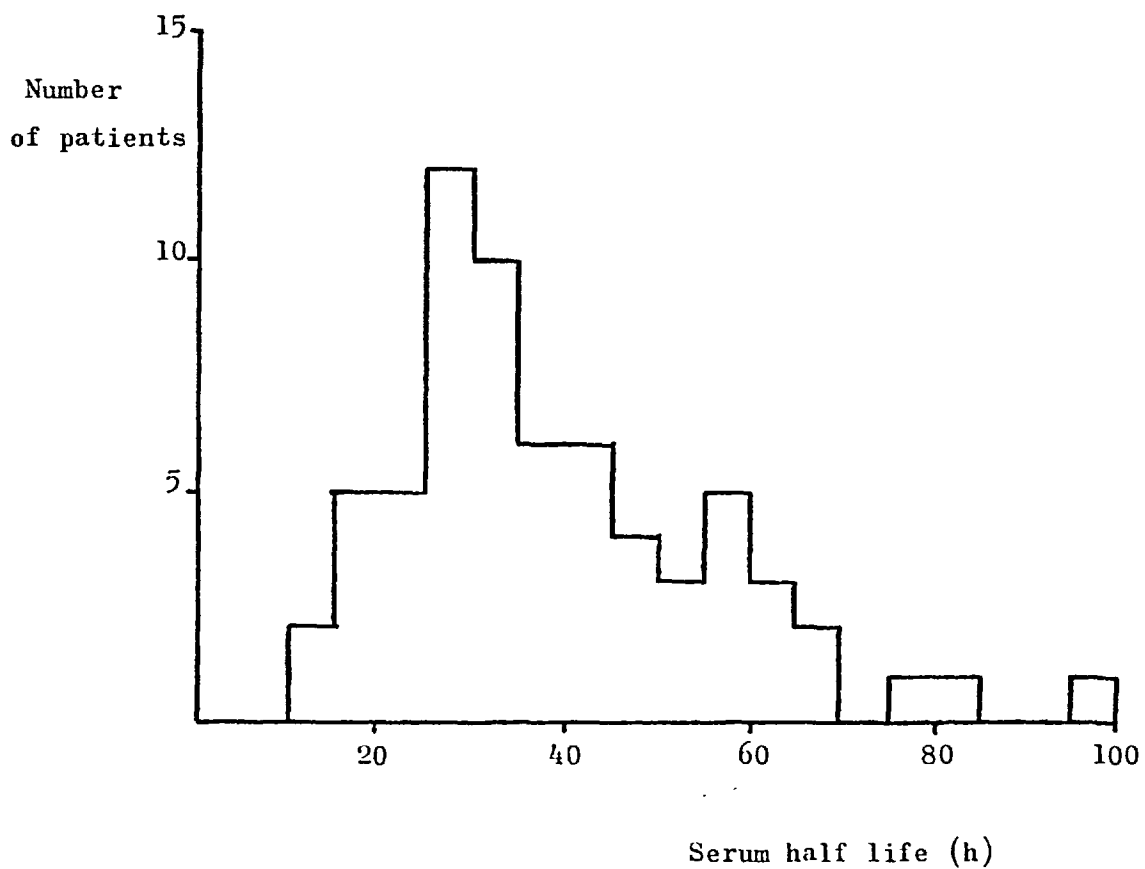
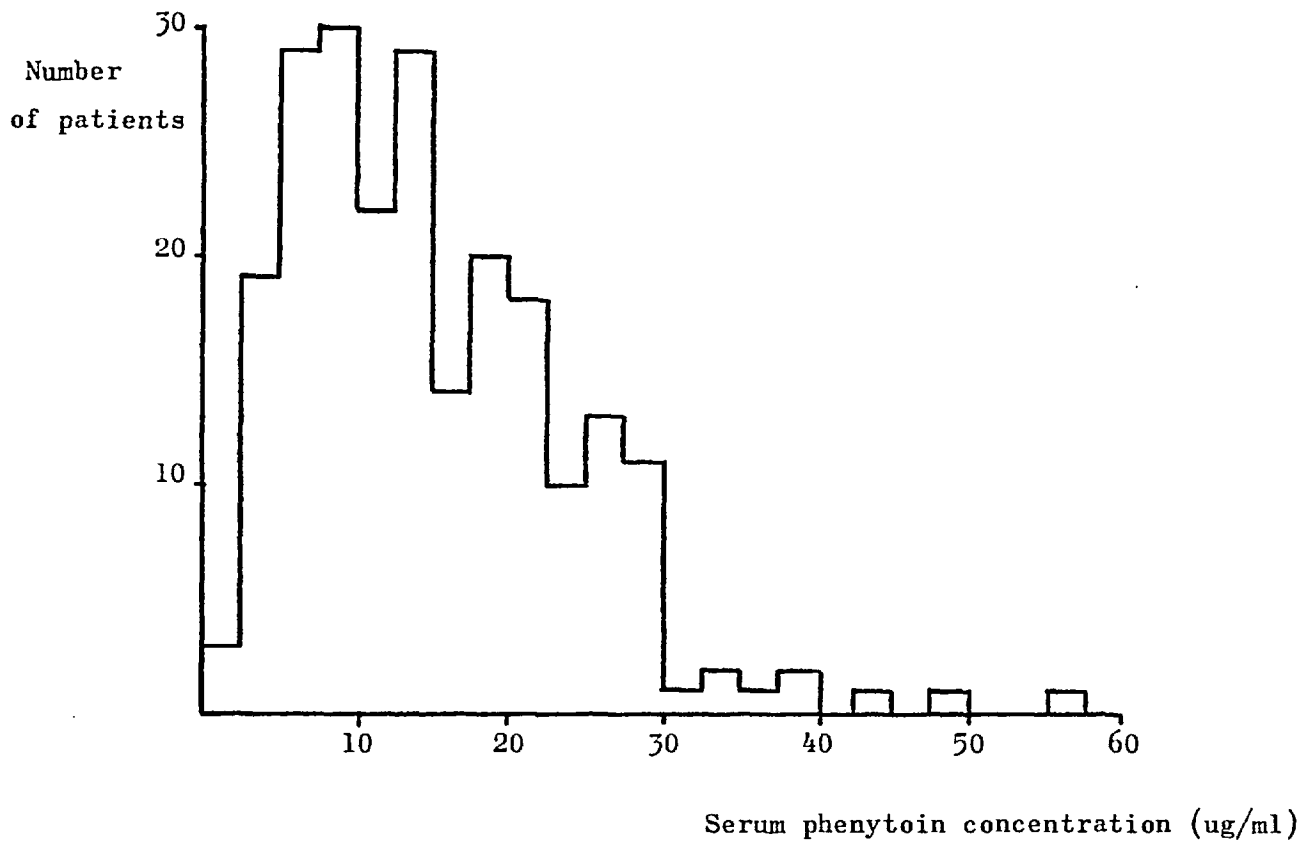
the 'defective' metabolisers of phenytoin and their families as identified by Kutt et al. (1964a) and Vasko et al. (1980).

These data imply that both metabolic oxidations are controlled by the D^H / D^L locus and hence some 8 % of the population may display defective hydroxylation of phenytoin. Examination of the available literature provides additional evidence, albeit circumstantial, in support of this conclusion. Population studies of steady - state serum phenytoin concentration in epileptic patients (Richens and Houghton, 1977) and of steady - state phenytoin half - life (Richens, 1979) have both recently been published. The results of the former study, on 218 patients, are shown in Figure 4.7a. The modal value observed was 10 ug / ml and the median 30 ug / ml. The full range of serum phenytoin concentrations encountered was 2.5 - 55 ug / ml. Such data are not compatible with the unimodal normal distribution which results from multifactorial control of phenytoin metabolism. A similar pattern was found in a smaller study (n = 66) of steady - state phenytoin half - life; in this instance the observed mode was 25 - 30 h, the median 58 h and the range of variability 15 - 100 h (Figure 4.7b).

The consequences of the defect for those individuals displaying it differ in the case of phenytoin from those seen with debrisoquine. In the instance of debrisoquine unmetabolized drug is cleared rapidly into urine, thus whilst the peak plasma concentration is higher in the PM phenotype, the persistence of a single dose shows no inter - phenotype differences. In the instance of phenytoin no such alternative elimination pathway is available and hence the drug would be expected to persist longer in the PM phenotype. This conclusion is borne out by the observed t_{50} values (EM: 44.3 ± 12.1 h; PM: 81.5 ± 23.3 h) and terminal elimination half - lives (EM: 17.2 ± 4.0 h;

Figure 4.7: The distribution of serum phenytoin concentrations in 218 patients receiving a maintenance dose of 300 mg / day (From: Richens and Houghton, 1977)

Figure 4.7b: Serum half - life of a tracer dose of phenytoin administered to 66 patients receiving phenytoin maintenance therapy. (From: Richens, 1979)



PM: 23.8 ± 3.5 h). It is appreciated that the latter values are derived from excretion of a metabolite, but Eadie et al. (1976) have demonstrated that the appearance of HPPH in urine is "formation - limited" regardless of phenytoin oxidation status.

The principal problem in interpreting the elimination kinetics of phenytoin is that its metabolism is known to be dose - dependant in the vast majority of the population. In essence, the 'defective' hydroxylator has a steeper plasma phenytoin concentration / ingested dose - response curve than the normal hydroxylator. Thus the differences seen in apparent first - order formation constants and terminal half - life are absent certainly indicative of different degrees of saturation in the EM and PM phenotypes. An estimate of the true inter - phenotype difference, i.e. that in maximal metabolic capacities, could only be achieved at higher doses; such a study for experimental purposes is obviously precluded on toxicological grounds.

At first sight, it seems difficult to reconcile these findings with previous studies of phenytoin metabolism. Debrisoquine is metabolised pre-systemically by oxidation of a saturated carbon centre, whereas phenytoin is apparently metabolized by oxidation of an aromatic carbon with no appreciable 'first - pass' hepatic extraction. However, recent studies by Gugler et al. (1976) and Butler et al. (1976) suggest that the metabolism of phenytoin is considerably more complex than has previously been appreciated. In the first of these studies, the bioavailability of phenytoin after oral administration was determined by simultaneous measurement of AUC_0 / AUC_{IV} ratios and the comparison of HPPH excretion after the same oral and intravenous doses. The authors found that the AUC ratios method consistently under-estimated bioavailability as judged from metabolite excretion. The

extent of this underestimation varied considerably between the individuals studied, the range being 0 - 25 %, mean: 10.4 ± 8.6 %. These data are consistent with variable 'first - pass' oxidation of 10 - 15 % of the oral dose.

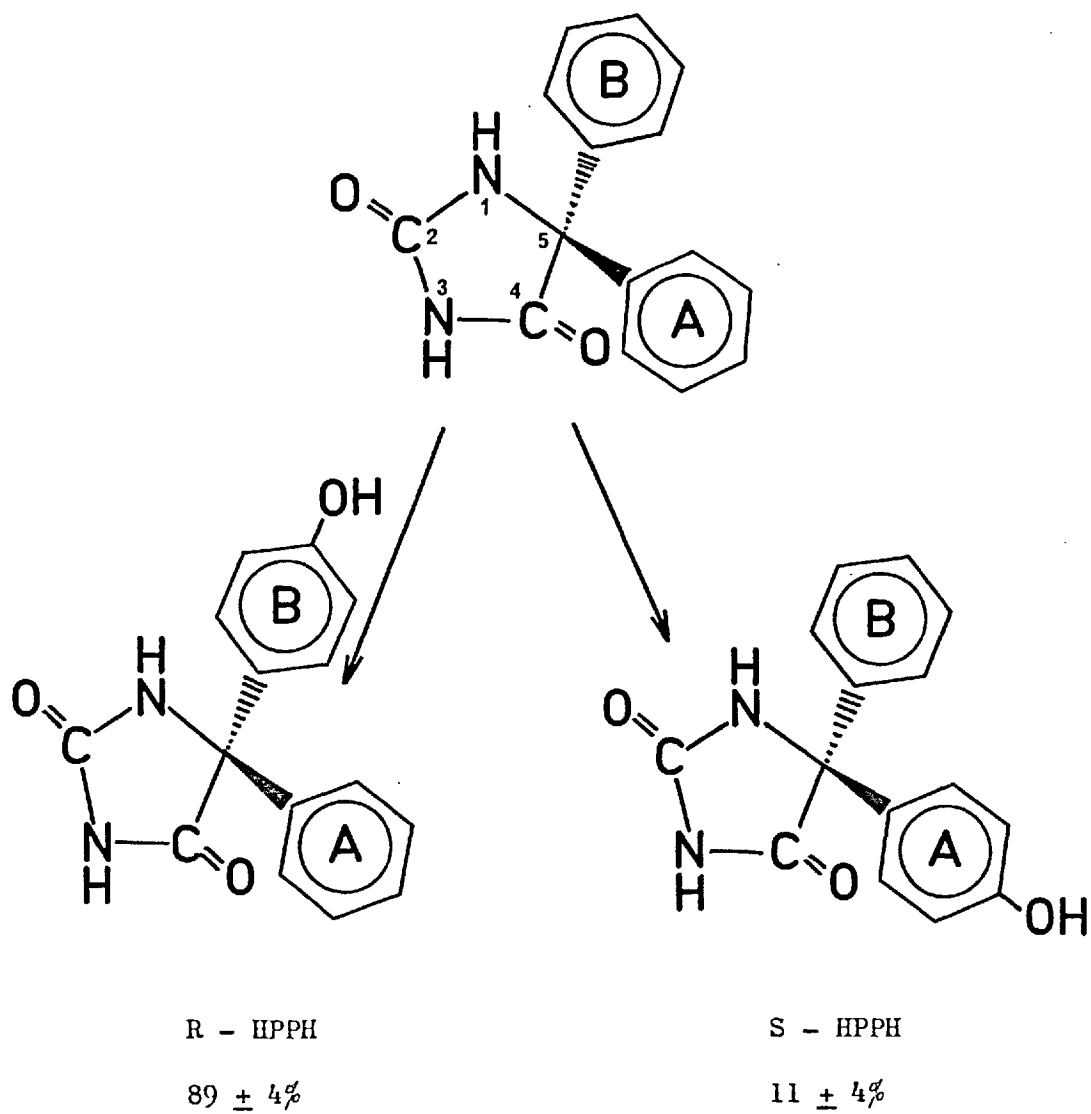
The second study yielded unambiguous evidence that the enzyme system responsible for the 4-hydroxylation of phenytoin is in fact heterogeneous. Introduction of a hydroxyl group in one of the phenyl rings of the phenytoin molecule converts carbon-5 of the hydantoin ring to a chiral centre. In the nomenclature of Hanson (1966) this carbon is termed a "pro-chiral" centre (Figure 4.8).

The authors reported that human patients excreted the isomers in the proportion R-HPPH: 89 ± 4 %, S-HPPH: 11 ± 4 %. These data were obtained after enzymic cleavage of conjugates, and at no stage were the metabolites in contact with strong acid. The authors concluded that their observations could only be explained by "...a minimum of two functional types of hydroxylating enzymes differing in stereospecificity and perhaps also in mechanism of reaction."

The values for apparent 'first - pass' metabolism (10.4 ± 8.6 %), production of S-HPPH (11 ± 4 %) and the observed interphenotype differences in HPPH excretion seen in the present study (15.2 ± 3.5 %) are similar. The hypothesis that 4-hydroxyphenytoin is produced by concurrent oxidation reactions performed by functionally distinguished enzymes, one of which is deficient or absent in the PM phenotype is attractive and consistent with the data outlined above. The proposition is speculative at this stage, but could be further investigated by the study of interphenotype patterns of phenytoin plasma concentration and examination of the optical rotations of the 4-hydroxyphenytoin excreted. It is provocative to note that the alicyclic 4-carbon of the debrisoquine molecule is also a 'pro-chiral' centre, and the

Figure 4.8: Stereospecificity of phenytoin 4-hydroxylation in man.

(From: Butler et al., 1976)



PM subjects studied with respect to debrisoquine oxidation displayed different substrate affinities in addition to different oxidation capacities in comparison to the EM phenotype. The kinetic implications of co-existing multiple forms of hydroxylating enzymes producing nominally identical metabolites are discussed more fully in Chapter Six.

The significant inter - ethnic differences seen in phenytoin disposition can be explained in two principal ways. The pattern of grossly reduced 4-hydroxyphenytoin excretion observed could be the consequence of either defective absorption or defective metabolism. Malabsorption of phenytoin has been reported (Kutt et al., 1966) but was apparently an idiosyncrasy of a single patient. Reduced absorption due to dietary factors cannot be precluded.

The failure of excretion rates of HPPH to reach a terminal log - linear decline in the Ghanaian subjects would be consistent with reduced metabolic capacity, but this factor is not descisive in the absence of plasma levels of unchanged phenytoin.

From the therapeutic viewpoint it is obviously important to establish whether reduced absorption capacity or reduced oxidation capacity is the primary factor resulting in these observations, since their implications are diametrically opposed. In the extreme cases, malabsorption will lead to failure of seizure control and defective oxidation will lead to accumulation of phenytoin in plasma, and hence toxicity.

It is anticipated that this question will be resolved by a proposed study of both plasma phenytoin levels and HPPH excretion in Ghanaian subjects of both oxidation phenotypes.

CHAPTER FIVE

HYDROXYLATION PHENOTYPE AND THE DISPOSITION OF PHENACETIN

- 5.1. Introduction
- 5.2. Materials and methods
- 5.3. Results:
 - 5.3.1. A population study of phenacetin metabolism.
 - 5.3.2. The effect of dose; the metabolism of phenacetin
in a phenotyped panel.
 - 5.3.3. Phenacetin rubruria; studies with [^3H]phenacetin.
- 5.4. Discussion

5.1. Introduction:

Phenacetin was introduced into clinical use as a non - narcotic analgaesic and antipyretic in 1887. Within twenty years it had become the commonest component of proprietary analgaesic mixtures, its most usual companions being acetylsalicylic acid and caffeine. The effects of prolonged phenacetin consumption were not to become apparent until the years immediately following the Second World War (Spühler and Zollinger, 1953), and since this time the drug, its abuse and toxic effects have been the subject of much controversy and two major reviews (Smith, 1958; Carro-Ciampi, 1978).

The compound has variously been implicated in the onset of cyanosis caused by methaemoglobin formation (Smith, 1958), chronic haemolytic anaemia (Dacie, 1967), chronic interstitial nephritis (Spühler and Zollinger, 1953), chronic pyelonephritis (Bengtsson et al., 1968), renal pelvic carcinoma (Angervall et al., 1969) and renal papillary necrosis (Clausen and Pedersen, 1961). It should be emphasized that phenacetin has rarely, if ever, been administered alone, and it has been incriminated in the aetiology of 'analgaesic nephropathy' on the basis that it is a 'common denominator' amongst the most frequently abused analgaesic mixtures. Despite intensive studies in recent years a satisfactory animal model for the toxicological picture as seen in man has yet to emerge (Rosner, 1976), and a number of authors have implicated acetylsalicylic acid and caffeine as either the primary nephrotoxic agent or as secondary agents which enhance phenacetin nephrotoxicity by modifying its metabolism, (Thomas et al., 1972 ; Prescott, 1965). In as far as any consensus can be said to exist on the topic, it would seem that the most likely culprits are certain phenacetin metabolites, namely p-phenetidine and

its N- and 2- hydroxylated derivatives, (Fig. 5.1). The evidence implicating these compounds in the haemo- and renal toxicity of phenacetin - containing analgaesic mixtures is summarized below:

1. All three compounds have been detected in human urine following oral doses of phenacetin (Brodie and Axelrod, 1949; Uehleke, 1969), and all three are capable of oxidising haemoglobin in vitro (Uehleke, 1973).
2. Proximal tubular necrosis could be produced in rats following intravenous administration of 2-hydroxyphenetidine (Calder et al., 1973).
3. The production of 2-hydroxyphenetidine is disproportionately increased with successively higher doses (Raaflaub and Dubach, 1969) and by concomittant administration of aspirin, caffeine and codeine (Thomas et al., 1972).
4. In alkaline solution, 2-hydroxyphenetidine forms a lipid - soluble, strongly coloured condensation product, 3-amino-7-ethoxyphenoxazone. Reber and Studer (1975) isolated a similarly coloured drug-related compound from the kidneys of cats several months after administration of ³H phenacetin.

Because of the apparent 'selectivity' of analgaesic nephropathy in a population of phenacetin abusers, both Gault et al., (1974) and Thomas et al., (1972) have speculated that susceptibility to haematological and renal toxicity following phenacetin ingestion may have a genetic basis. The work of Shahidi (1967) has already been alluded to in general terms, but it is worth re-iterating here that this author observed two sisters who displayed defective metabolism of phenacetin with respect to O-de-ethylation capacity. These two individuals produced 50 % and 49 % of a 30 mg / kg oral dose of phenacetin as paracetamol in 24 h together with 54 % and 16 % as 2-hydroxyphenet-

Figure 5.1: The metabolism of phenacetin in man.

Figures in parentheses indicate the proportion of the dose found in a 24 h human urine after oral administration of ca.2 g. Broken lines indicate possible routes of formation where more than one possibility exists.

(Modified from Uehleke, 1969, and Welch and Gillespie, 1973)

I = phenacetin

II = 2-hydroxyphenacetin

III = 2-hydroxyphenetidine

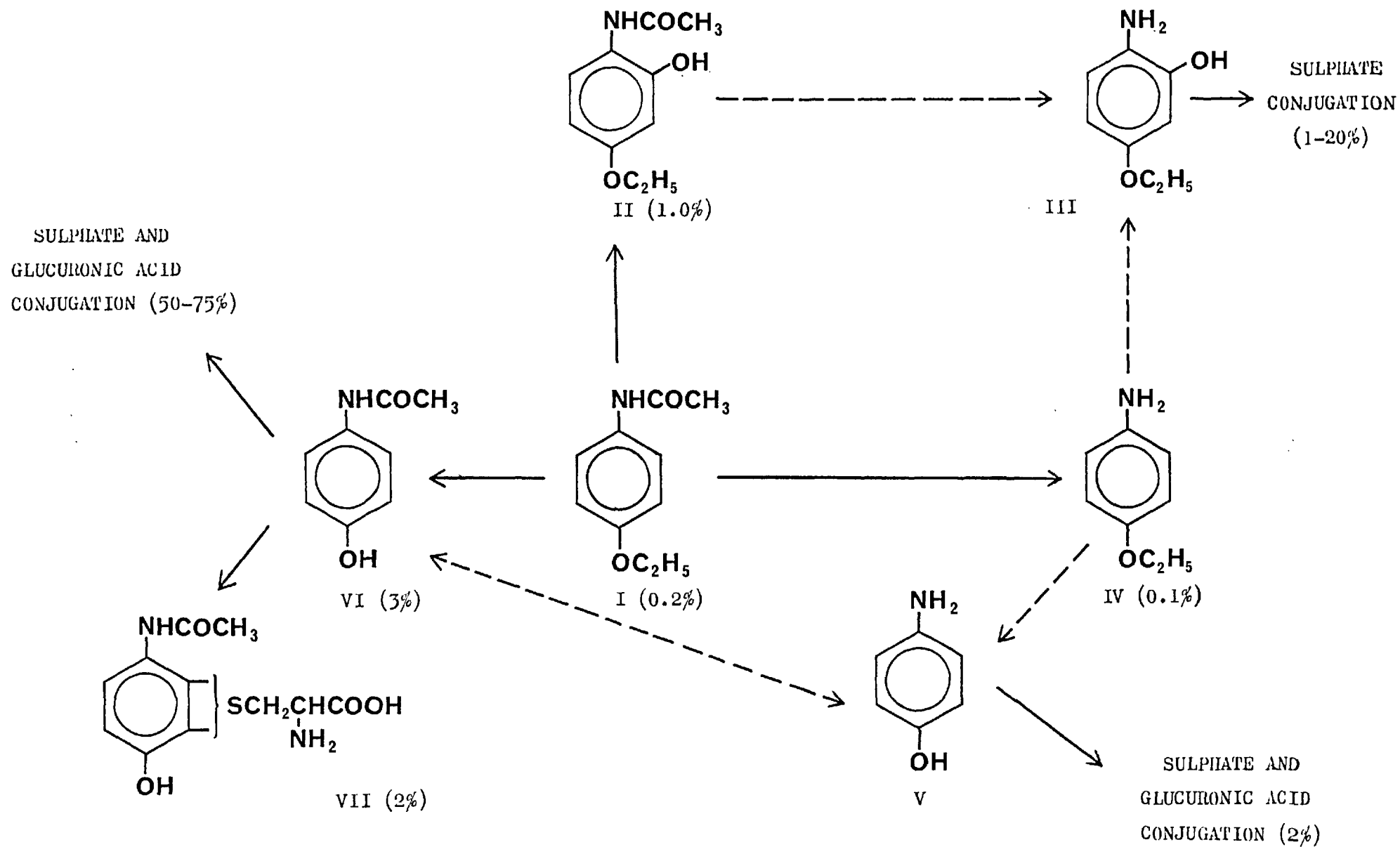
IV = p-phenetidine

V = p-aminophenol

VI = N-acetyl-p-aminophenol, (paracetamol)

VII = S-(1-acetamido-4-hydroxyphenyl)-cysteine

(N-oxidation products of I and IV have been reported in trace amounts by Uehleke, 1969)



idine. The range encountered in ten control subjects was paracetamol 62 - 90 %, 2-hydroxyphenetidine 2.5 - 8 %. The toxicological consequences of this defect were manifested in a maximum rise of methaemoglobin of 5.4 - 11.4 % in the affected siblings, against a control range of 0.6 - 2.8 % (Shahidi and Hemaïdan, 1969). There is thus considerable evidence that whenever O-de-ethylation capacity is reduced, whether by virtue of an inherited defect or the presence of what are in effect competing substrates, the production of a potentially toxic alternative metabolite, 2-hydroxyphenetidine, is enhanced.

Scope of the present study:

The evidence that the oxidation of debrisoquine and diphenylhydantoin are in the control of the same single gene has been presented and discussed (vide supra, Chap 2 & 4). In 1979, Kitchen et al. demonstrated that the O-de-methylation of p-methoxyamphetamine was apparently likewise determined. It was thus of immediate interest to ascertain whether or not O-de-ethylation of phenacetin was similarly effected in PM subjects. A serendipitous observation made early in phenacetin metabolism studies had suggested that this might be the case. It was seen that the urine of PM volunteers who had received oral doses of phenacetin turned dark red on incubation with sulphatase / β -glucuronidase; the term phenacetin 'rubruria' was coined for this phenomenon. It transpired that this was not a novel observation; in fact, it had first been reported by Müller (1888), a year after phenacetin's introduction into clinical use. Pigmented urine following phenacetin ingestion was more thoroughly investigated by Gault and Shahidi in 1971. The authors concluded firstly that the degree of pigmentation seen in

volunteers' urines following oral doses of 2 g phenacetin was subject to wide inter - individual variation, and secondly that it was at least in part due to the presence of 2-hydroxyphenetidine. It therefore seemed possible that the PM subjects alluded to above as displaying 'rubruria' were producing significantly more 2-hydroxyphenetidine than the EM subjects studied, none of whom displayed noticeable urine pigmentation.

It was thus determined to investigate the following:

1. The distribution of capacity to metabolize phenacetin, expressed in terms of O-de-ethylation and total 2-hydroxylation, in a random population.
2. The distribution of phenacetin 'rubruria' in a random population, and its correlation, if any, with 2-hydroxylation.
3. The effect of debrisoquine hydroxylation phenotype on O-de-ethylation capacity, by metabolism studies in panels of phenotyped volunteers.
4. The effect of dose on resolution between phenotypes if inter-phenotype differences were observed.

In the previous situations considered, i.e. the effect of oxidation phenotype on the elimination kinetics of debrisoquine and diphenylhydantoin, it has been seen that the major implication of the defect for those subjects displaying it has been one of enhanced blood levels of parent compound; the situation with phenacetin has an extra dimension. If the major inactivation pathway is relatively ineffective, then it is probable that more parent compound will be available for a normally minor alternative pathway, which, in this instance, may well enhance the acute and chronic toxicity of the drug in question.

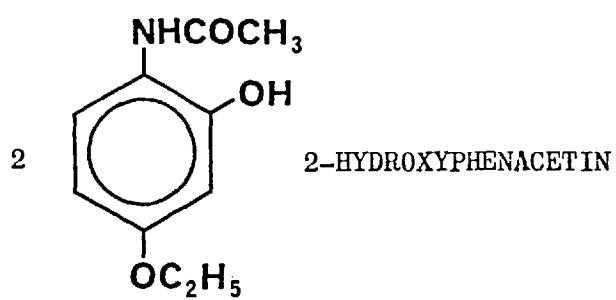
5.2. Materials and Methods:

Subjects and protocols: In the population studies, 51 volunteers drawn from the staff and students of St Mary's Hospital Medical School each received 500 mg phenacetin orally after a light breakfast and voiding their bladders. A bulked urine sample was collected from each subject for the period 0 - 8 h post - dosing. A 20 ml aliquot of the measured final volume was stored at -20 °C prior to analysis for its content of paracetamol, total 2-hydroxylation products and degree of phenacetin rubruria. Of the original 51 volunteers, it eventually proved possible to phenotype 22 with respect to their debrisoquine 4-hydroxylation capacity, and data from these individuals was compared on a panel basis. In the second study, five subjects previously phenotyped as EM and seven subjects previously phenotyped as PM each received 1000 mg phenacetin orally after a light breakfast and voiding their bladders. Sequential urine samples were obtained from each subject at hourly intervals to 8 h post - dosing, and a 20 ml aliquot of each measured volume was treated as described above. It was not possible to include subjects from the original 500 mg population study in the 1000 mg panel study, and so the degree of intra - individual dose - dependency could not be assessed.

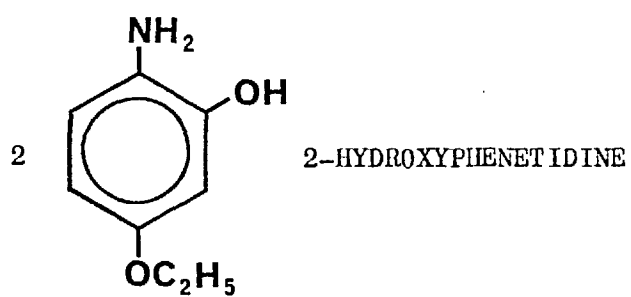
Determination of 2-hydroxylation products:

2-Hydroxylation products in urine were determined by the modification of a published assay for 2-hydroxyphenetidine (Shahidi and Hemaïdan, 1969). Urine (0.1 - 0.5 ml) was heated with 5 ml 2M HCl for 2 h at 100 °C, thus effecting deconjugation of all conjugated material and de-acetylation of any 2-hydroxyphenacetin to 2-hydroxyphenetidine. The urine was then neutralized with solid sodium bicarbonate and made alkaline (pH 8.5 - 9.0) with 10 % ammonia

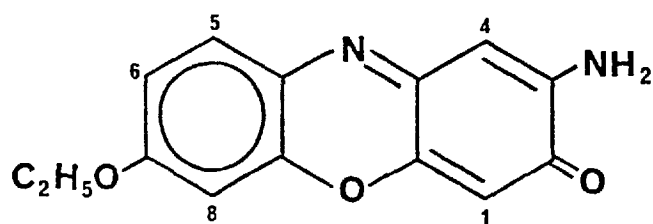
Figure 5.2: Hydrolysis and condensation reactions in the assay of
2-hydroxyphenacetin and 2-hydroxyphenetidine.



HYDROLYSIS (ACID)



CONDENSATION (ALKALI)



3-AMINO-7-ETHOXYPHENOXAZONE

Figure 5.3: Absorption spectrum of 3-amino-7-ethoxyphenoxazone (a)
and calibration curve obtained from addition of known
amounts of 2-hydroxyphenacetin to urine (b).

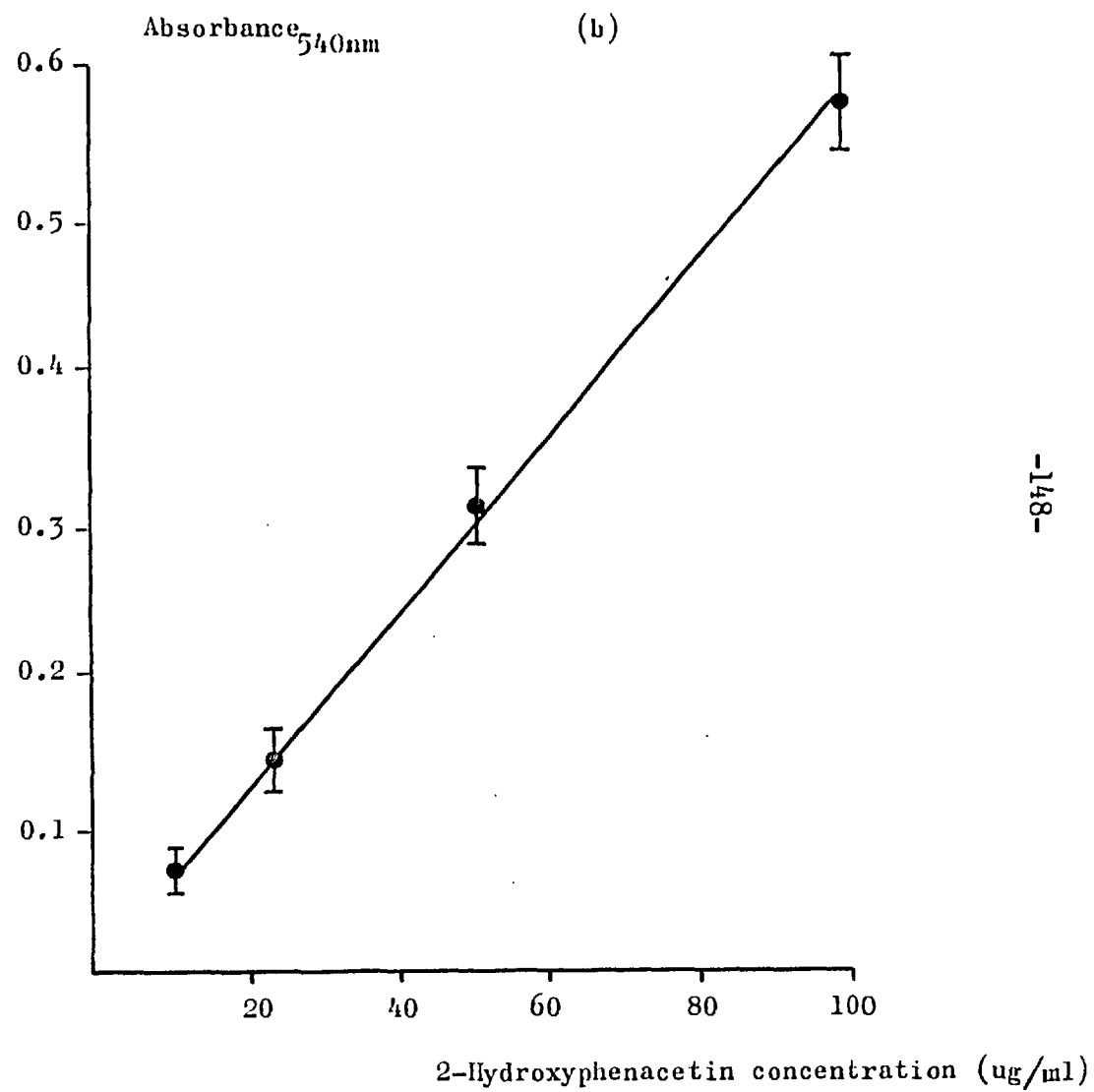
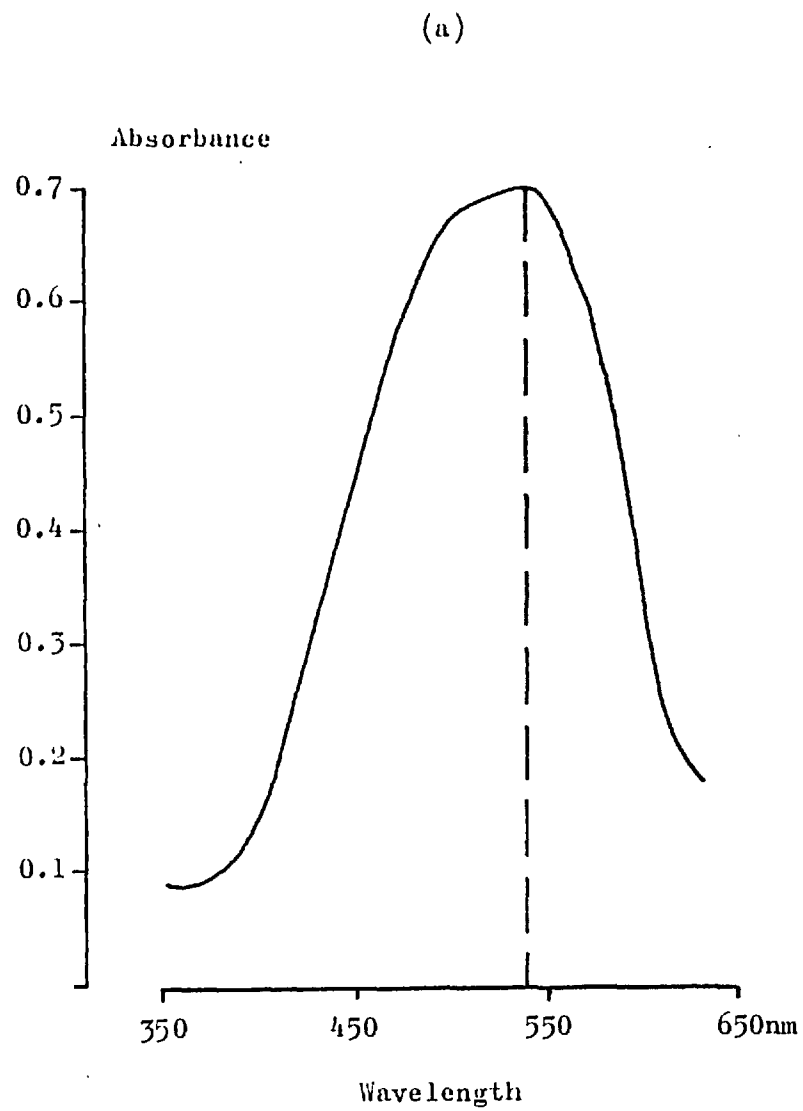
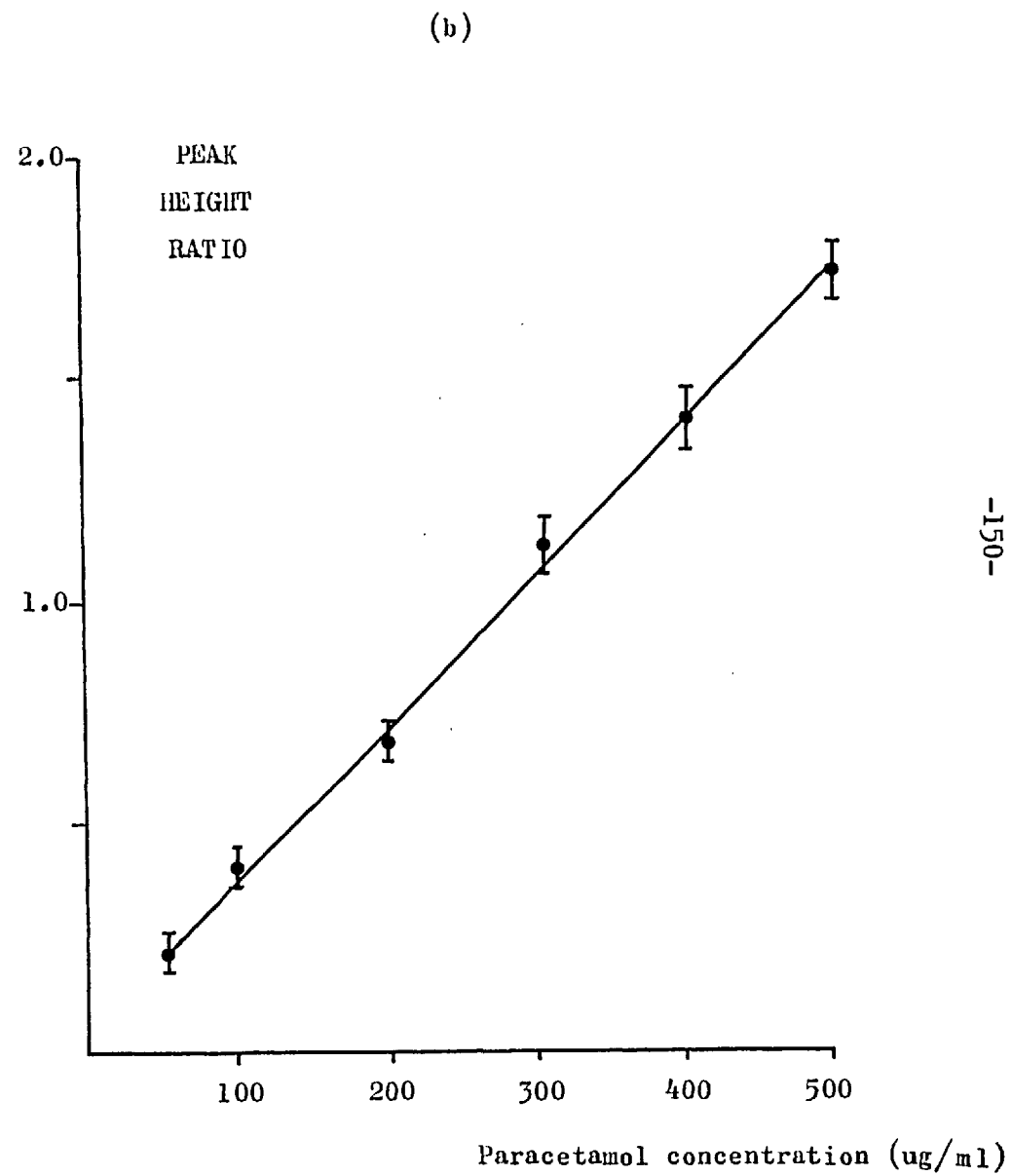
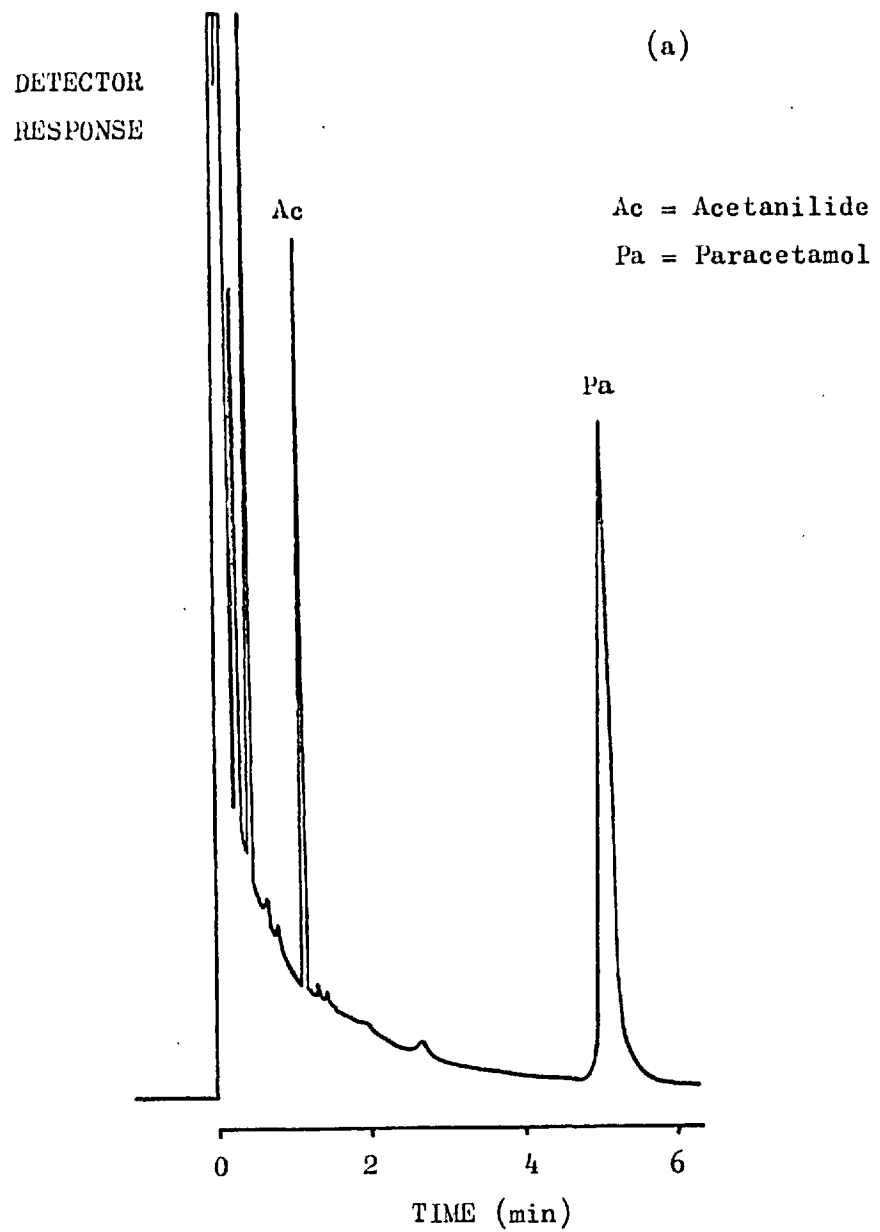


Figure 5.4: Typical G.L.C. trace of paracetamol in urine following ingestion of phenacetin (a); calibration curve obtained by addition of known amounts of paracetamol to urine (b).



solution (2 ml), resulting in the condensation of two 2-hydroxy-phenetidine molecules to form 3-amino-7-ethoxyphenoxazone (Fig.5.2.). This yellow coloured derivative was then extracted into 2 x 10 ml ethyl acetate. The organic layer was removed and evaporated under reduced pressure. The dry residue was dissolved in methanol (0.1 ml) followed by 1 M HCl (3.0 ml). The absorbance of the resulting purple - red phenoxazone. HCl complex was read at 540 nm against a blank prepared by identical treatment of a urine sample obtained from each subject prior to phenacetin administration. A standard curve was prepared from known amounts of 2-hydroxyphenacetin added to blank urine and treated in the manner described. Calibration was linear over the measured range (10 - 100 μg / ml), and the coefficient of variability did not exceed 10 % for any of the standard concentrations, (Fig. 5.3a & b).

Determination of paracetamol and 'rubruria':

Paracetamol in urine was estimated following hydrolysis of conjugates with β -glucuronidase (10,000 units / sample) and sulphatase (1,500 units / sample). Urine (0.2 - 1.0 ml) was incubated with enzyme and 0.2 M sodium acetate buffer, pH 5.0 (4 ml), for 48 h at 37 °C. Following incubation, acetanilide (50 μg in 0.05 ml methanol) was added as internal standard. The enzymic protein in each sample was then precipitated by addition of 10 % trichloroacetic acid (3 ml) followed by centrifugation at 1500 g for 30 min. Each sample was then neutralized with solid sodium bicarbonate and centrifuged for a further 20 min at 1500 g. An aliquot of the supernatant (1 ml) was then read at 450 nm against a pre-dose urine blank passed through an identical procedure, and this absorbance recorded as a measure of phenacetin 'rubruria'. The remainder of the supernatant was then extracted with ethyl acetate (2 x 10 ml) by gentle manual

shaking. Following centrifugation to effect phase separation, the organic layer was removed and evaporated under reduced pressure. The dry residue was derivatized by addition of bis-(trimethylsilyl)-trifluoroacetamide catalysed by 1 % trimethylchlorosilane, of which 2 μ l was analysed by gas - liquid chromatography. Standards in the range 50 - 500 μ g / ml were prepared by addition of the appropriate amount of paracetamol to a mixture of enzyme, buffer and blank pre-dose urine. These were incubated and assayed in the manner previously described. Calibration was linear over the range employed, and the coefficient of variation did not exceed 7 % for any standard concentration, (Fig. 5.4 a & b).

Preparation of [^3H]Phenacetin and further studies on phenacetin
rubruria:

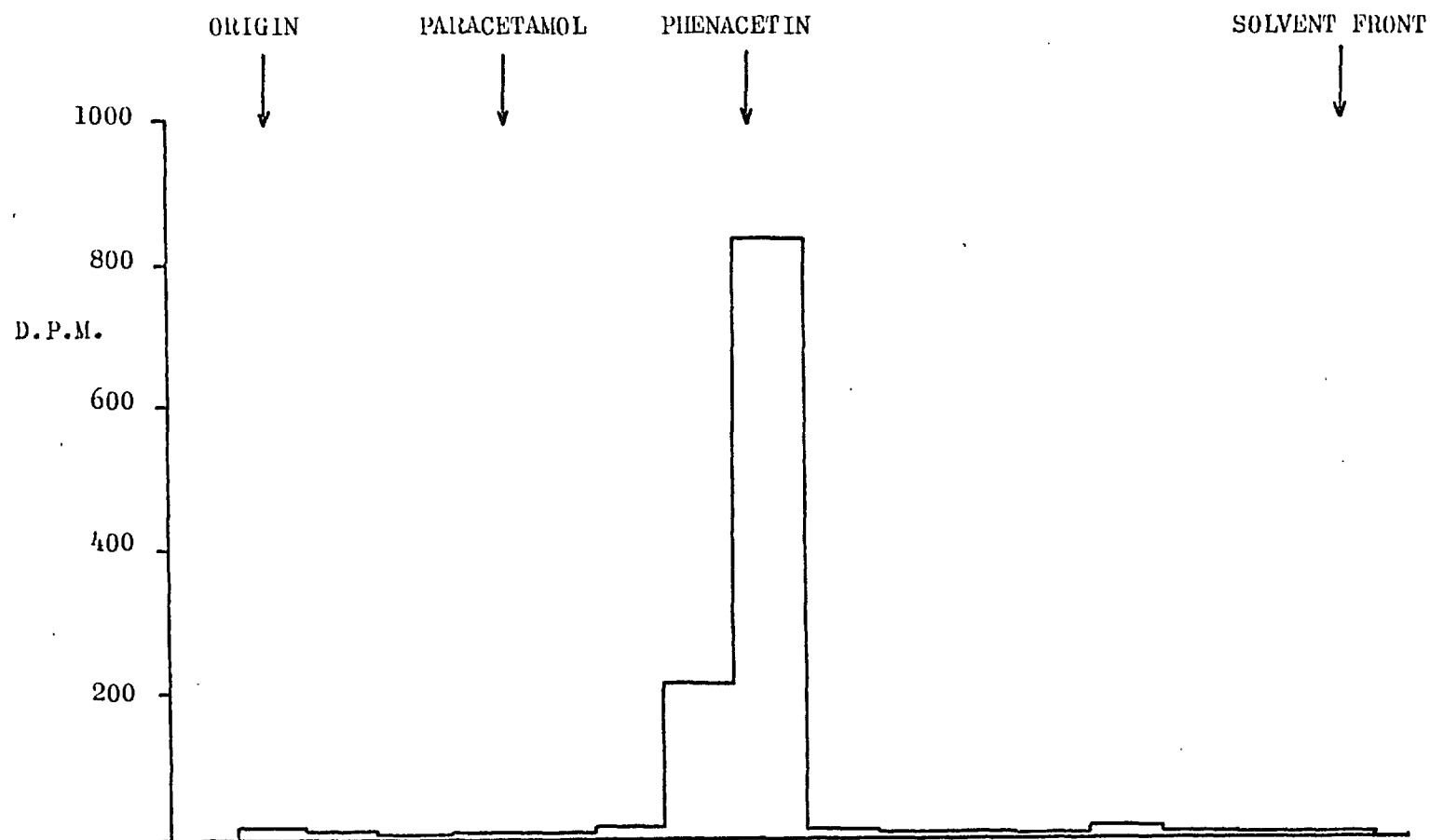
[^3H]Phenacetin was prepared from [$\text{G-}^3\text{H}$]p-hydroxyacetanilide ([$\text{G-}^3\text{H}$]-paracetamol) in the following manner.

Clean sodium metal (1.55 g) was added to absolute ethanol (40 ml) followed by unlabelled paracetamol (10 g) and 100 μCi [$\text{G-}^3\text{H}$]-paracetamol (approx. 4.5 μg in 50 μl absolute ethanol) after cooling. After addition of ethyl iodide (8 ml), the resultant solution was boiled under reflux for 1 h. The reaction was terminated by the addition of distilled water (100 ml). The crude precipitate was redissolved in 40 % aq. ethanol (200 ml) and allowed to recrystallize at 4 $^{\circ}\text{C}$ over a period of 48 h. The resulting product was purified by five recrystallizations to constant specific activity (4.74 μCi / g) and its radiochemical purity was assessed by thin - layer chromatography of 100 μl of a stock solution (10 mg product / ml absolute ethanol) spotted on Merck silica gel 60 F₂₅₄ plates (5 x 20 cm), developed in benzene - methanol - glacial acetic acid 45:8:4 v/v. Phenacetin and paracetamol were well separated (R_f : 0.48 and 0.22 respectively) and 96 % of the applied radioactivity ran as phenacetin with no detectable paracetamol contamination even following addition of 1 mg paracetamol to the spotting solution as cold carrier (Fig.5.5).

The melting point of the final product was 135 - 136 $^{\circ}\text{C}$ (authentic phenacetin 136 $^{\circ}\text{C}$), and its mass spectrum was identical to that obtained from the pure compound.

A single PM volunteer who had proved markedly rubruric in previous studies received 1 g [^3H]phenacetin (4.74 μCi) orally and produced hourly urines for the first 6 h post - dosing and then at 8, 12, 20.5, 21.5 and 24 h. Urines were analysed for their paracetamol and 2-hydroxyphenetidine content in the manner previously described, and

Figure 5.5: Radiochromatography of [^3H]phenacetin following synthesis described in text. Positions of authentic reference compounds are indicated.



also for total radioactivity by liquid scintillation spectrometry. A pooled 50 ml sample of the most notably pigmented urine passed during the study was incubated with β -glucuronidase (75,000 units) and sulphatase (10,000 units) for 72 h at 37 °C. Following incubation, dipotassium hydrogen phosphate (10 gm) was added to the sample. which was then neutralized with 6 M HCl and extracted with acetone (2 x 10 ml). The acetone extracts were combined and the solvent removed under reduced pressure. Residual water was removed in vacuo over P₂O₅. The dry residue was dissolved in methanol (1 ml), and 100 μ l aliquots were analysed by thin - layer chromatography. Any pigmented band thus detected was eluted from the silica gel and its ³H content, estimated by liquid scintillation spectrometry.

Preparation of 3-amino-7-ethoxyphenoxazone.

3- Amino-7-ethoxyphenoxazone was prepared from 2-hydroxyphenacetin by a modification of Shahidi and Hemaïdan (1969). 2-Hydroxyphenacetin (10 mg) was dissolved in methanol (0.2 ml) and boiled under reflux with 6 M HCl (5 ml) in a 15 ml screw - top septum vial for 2 h. After cooling the solution was neutralized with solid NaHCO₃ then made alkaline (pH 8.5 - 9.0) with 10 % aq. ammonia (2 ml). The resulting condensation product, 3-amino-7-ethoxyphenoxazone (Fig. 5.2 vide supra) was exhaustively extracted into ethylacetate (5 x 5 ml). After removal of the pooled solvent, the crude product was reconstituted in methanol (0.2 ml), and purified by preparative thin - layer chromatography on Merck silica gel 60F₂₅₄ plates (20 x 20 cm), developed in benzene - methanol - acetic acid 45:8:4 v/v. The R_f of 3-amino-7-ethoxyphenoxazone in this system was 0.75.

All absorbance determinations and visible spectra were performed using a Cecil CE 595 double - beam digital spectrophotometer interfaced with a CE 500 control - record module.

Gas - chromatographic procedures employed a Packard Model 417 Becker gas - liquid - chromatograph equipped with a flame - ionisation detector. Samples (1 - 3 μ l) were injected onto a 10 % SE-30 on Chromosorb W.HP (80 - 100 mesh) column (1.85 m x 2 mm i.d. glass). GLC conditions were: injection port temperature, 250 °C, column, 240 °C and detector 270 °C; air 40 ml / min, hydrogen 25 ml / min and nitrogen 50 ml /min.

Radiochromatographic procedures employed a Packard model 7201 radio-chromatogram scanner. Tritium was quantified in biological samples using a Packard model 3385 Tri-Carb liquid scintillation spectrometer. Samples (0.1 - 1 ml) were counted in a cocktail (10 ml) consisting of Triton - X-100 (Koch - Light laboratories Ltd.) 33.3 %, toluene, 66.6 %, 2,5-diphenyloxazole (55 mg / 10 ml) and 1,4-di-2-(5-phenyloxazolyl)-benzene (1 mg / 10 ml). The latter reagents were obtained from Fison's Scientific Ltd.

Mass spectra were obtained using a Varian model CH 5 Mass spectrometer interfaced with a Varian model 620 i digital computer. Samples were analysed using a direct inlet probe (temp. 50 ° - 200 °C); the ionization energy was 70 eV and the ion source temperature was 120 °C.

2-Hydroxyphenacetin was the gift of Dr. R.M. Welch (Wellcome Research Laboratories, Research Triangle Park, N.C.).

[G-³H]-paracetamol was obtained from New England Nuclear Inc., St Louis, Mo. Phenacetin, paracetamol and sulphatase were obtained from the Sigma Chemical Co., St Louis, Mo. β -Glucuronidase ("Ketodase") was obtained from Warner - Chilcott Ltd.. All other reagents were AnalaR, reagent grade used without further purification.

Statistical analyses:

Population data were subjected to probit transformation and tested for heterogeneity in the manner previously described (Chap.3, vide supra). Panel data were analysed using the distribution - free tests previously described. Where parametric assumptions were not precluded a conventional Student's t-test was employed.

5.3: Results:

5.3.1: A population study of phenacetin metabolism

The frequency distributions of the various aspects of phenacetin metabolism under study are shown in Figure 5.6a - d. Three of these four aspects, namely rubruria ($\chi^2 = 61.0$, $p < 0.0005$), total 2-hydroxylation ($\chi^2 = 35.2$, $p < 0.001$) and the ratio between paracetamol production and total 2-hydroxylation ($\chi^2 = 32.6$, $p < 0.0005$) showed a highly significant degree of heterogeneity. In contrast, total paracetamol production 0 - 8 h post - dosing did not differ significantly from a standard normal distribution ($\chi^2 = 11.1$, $p > 0.5$). Of the 22 participants in the population study who were later phenotyped with respect to debrisoquine 4-hydroxylation capacity, 17 were found to be EM, with metabolic ratios in the range 0.4 - 2.9 (median 0.9) and 5 were found to be PM, with metabolic ratios in the range 21.0 - 35.0 (median 28.6).

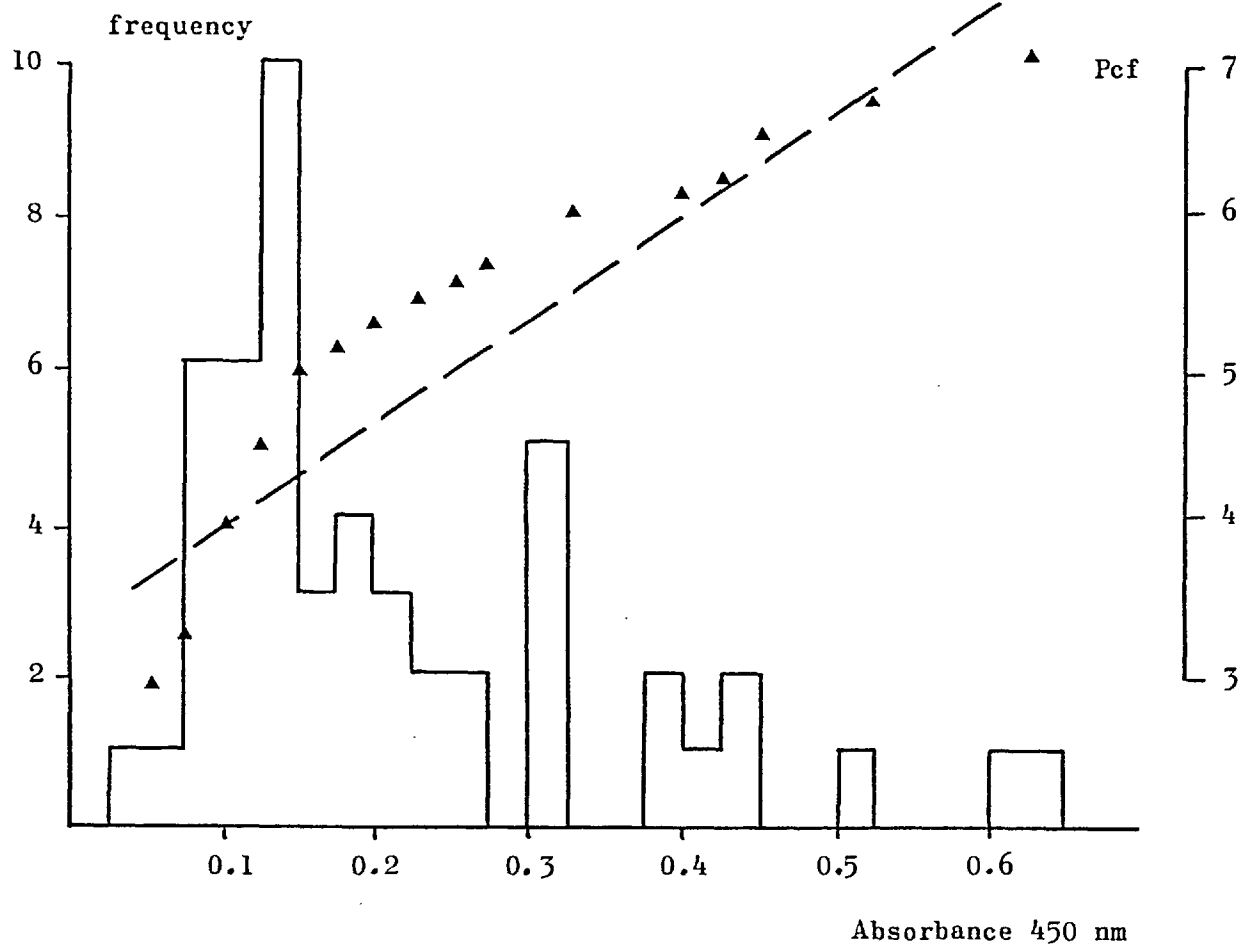
Panel - based comparison of the two phenotype's metabolism of phenacetin is shown diagrammatically in Figure 5.7a - d. These data were examined both with and without parametric assumptions, and the statistical findings are summarized in Table 5.1. Either statistical approach led to similar conclusions. Rubruria and 2-hydroxylation of phenacetin following a 500 mg oral dose show significant inter - phenotype differences, whereas paracetamol production and the ratio between paracetamol production and total 2-hydroxylation do not. The significance of the aspects of phenacetin metabolism displaying an interphenotype difference was not test - dependant.

Figure 5.6a: Frequency (f) distribution of phenacetin 'rubruria' in a random sample of 51 volunteers following a 500 mg oral dose.

Figure 5.6b: Frequency (f) distribution of phenacetin 2-hydroxylation products in the same random sample.

In each instance the probit of the cumulative frequency (Pcf) is superimposed on the diagram. The broken line is that expected for a homogenous population with a standard normal distribution, calculated by least squares regression analysis of the observed data.

(a) RUBRURIA



(b) 2-HYDROXYLATION

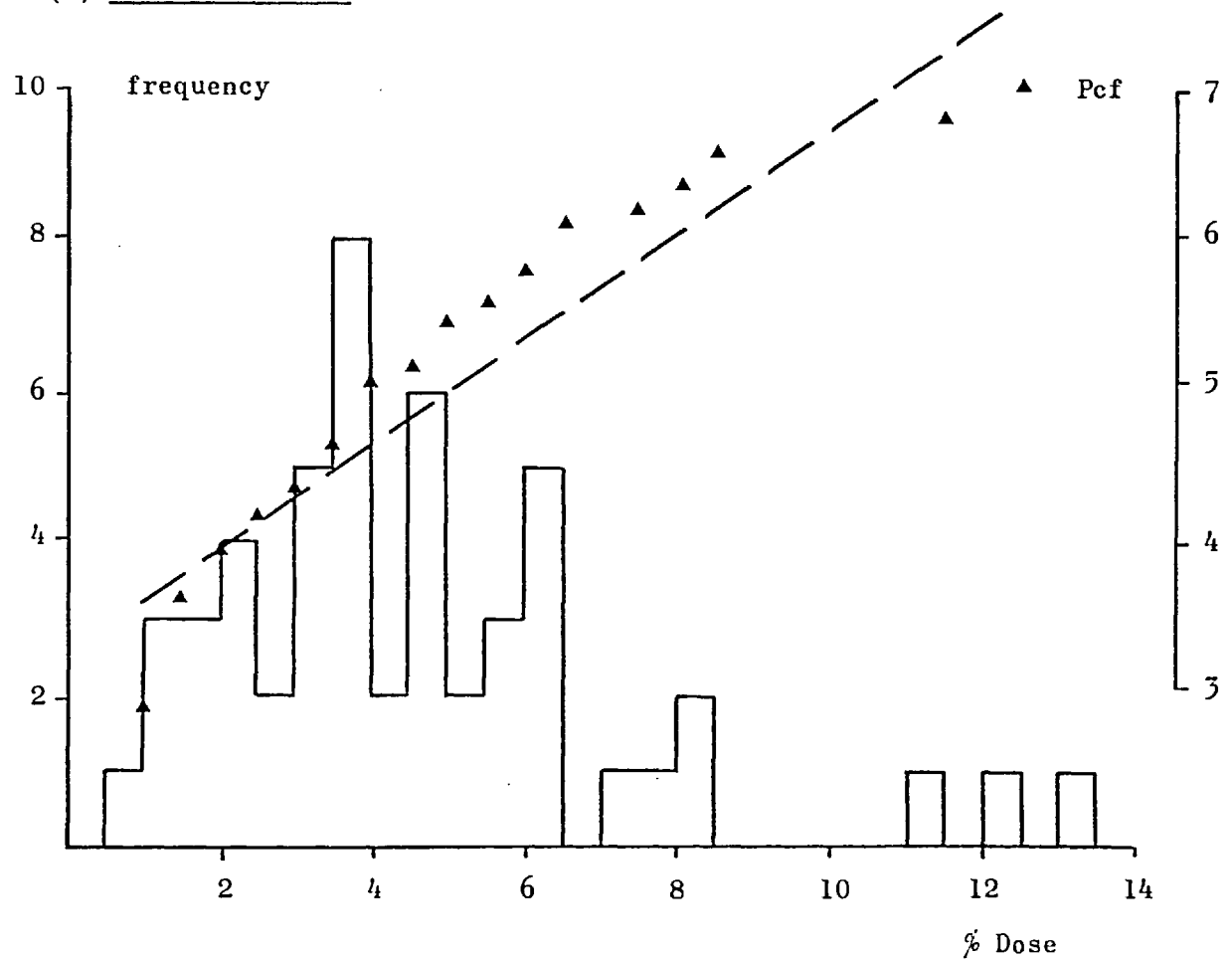
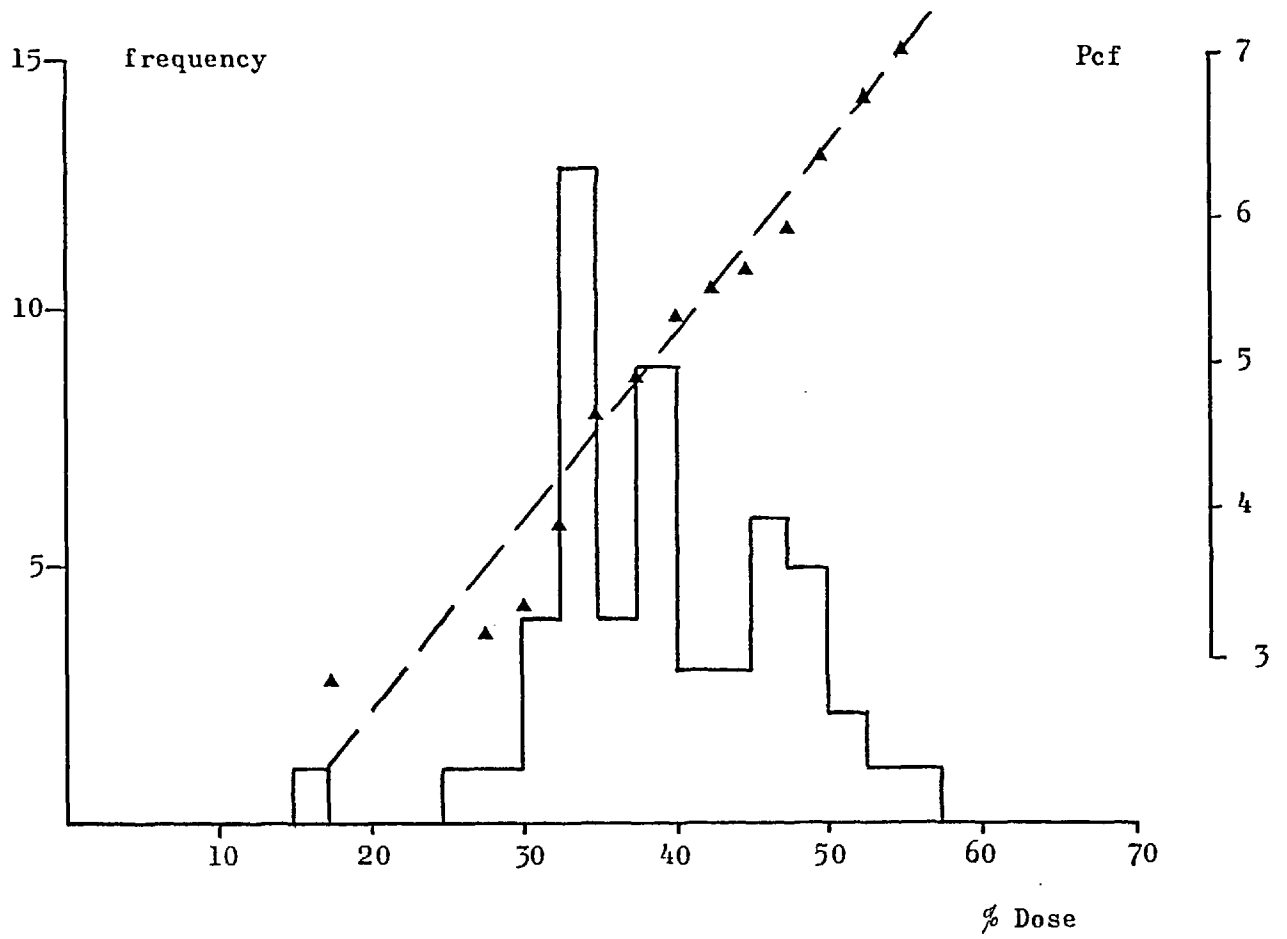


Figure 5.6c: Frequency (f) distribution of paracetamol production
(0 - 8 h` post - dosing) in the same random sample.

Figure 5.6d: Frequency (f) distribution of the paracetamol (P) /
2-hydroxylation products (2HOP) in the same random
sample.

(c) PARACETAMOL



(d) 0-de-ETHYLATION:2-HYDROXYLATION RATIO

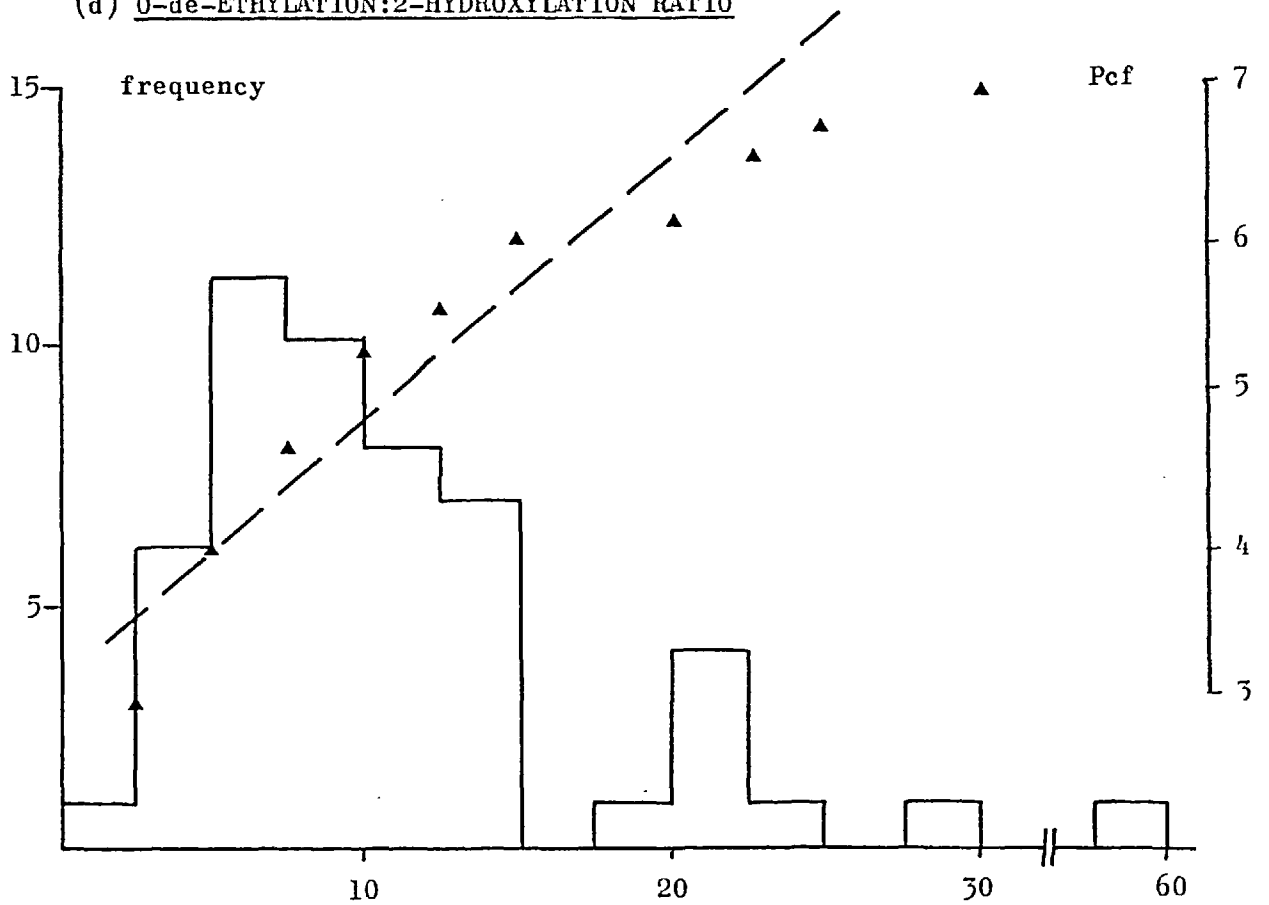
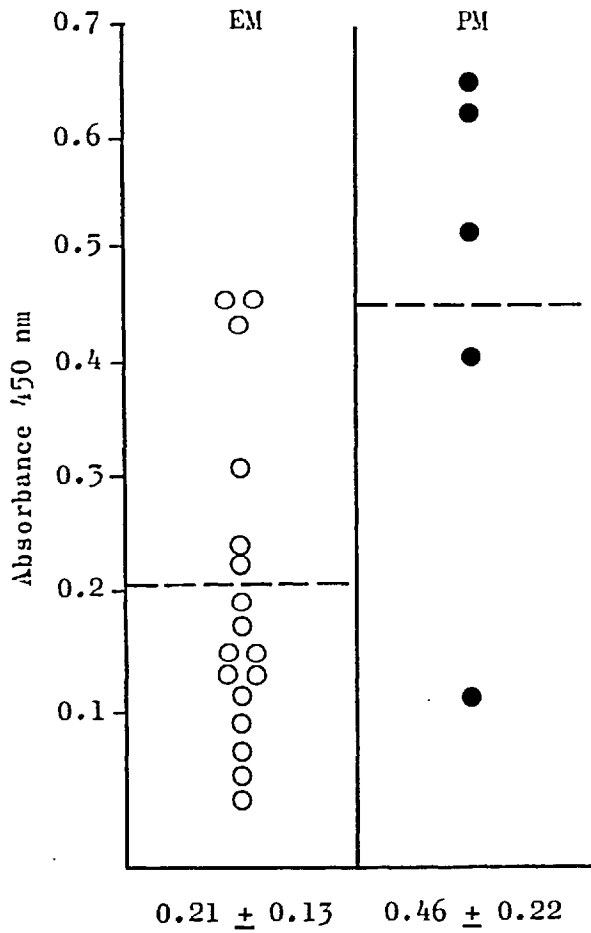
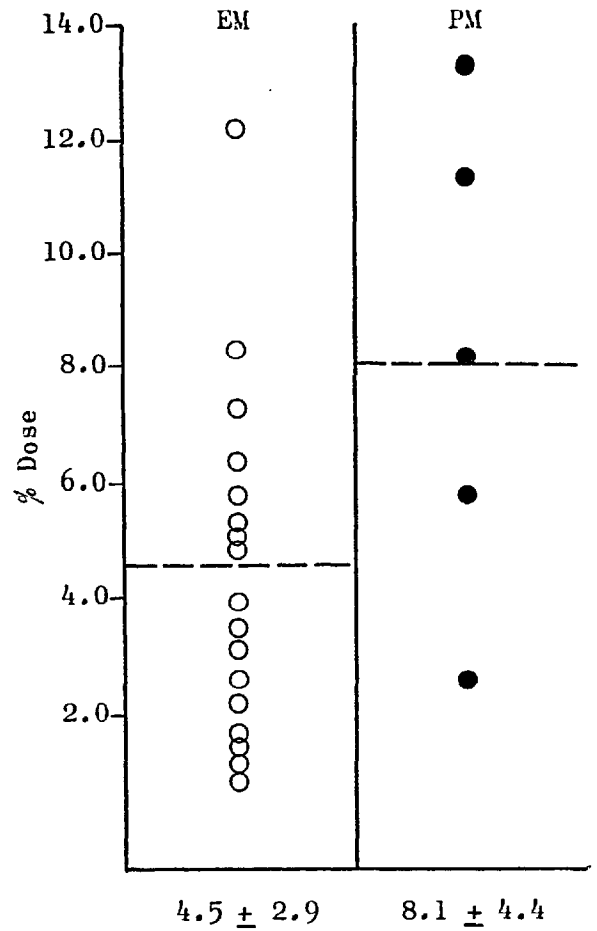


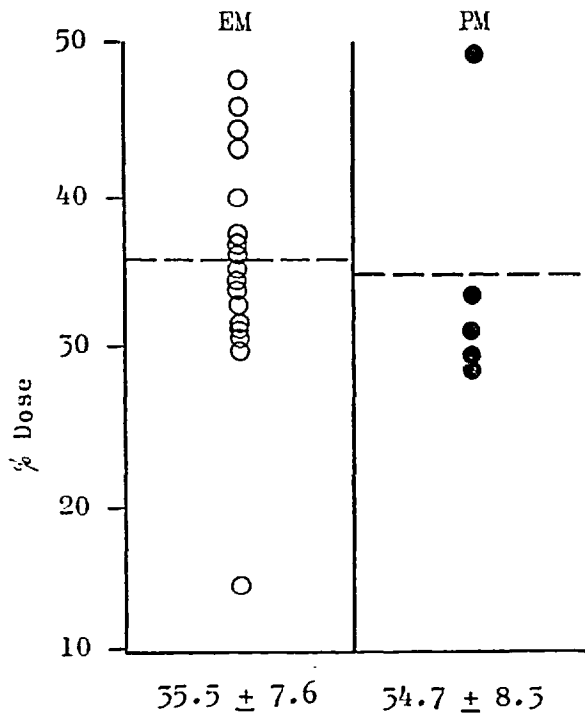
Figure 5.7: Distributions of rubruria (a), total 2-hydroxylation (b), paracetamol (c) and paracetamol / 2-hydroxylation products ratio (d) in 22 individuals (EM, n = 17; PM, n = 5) phenotyped with respect to debrisoquine oxidation status after participating in the original population study of phenacetin disposition.



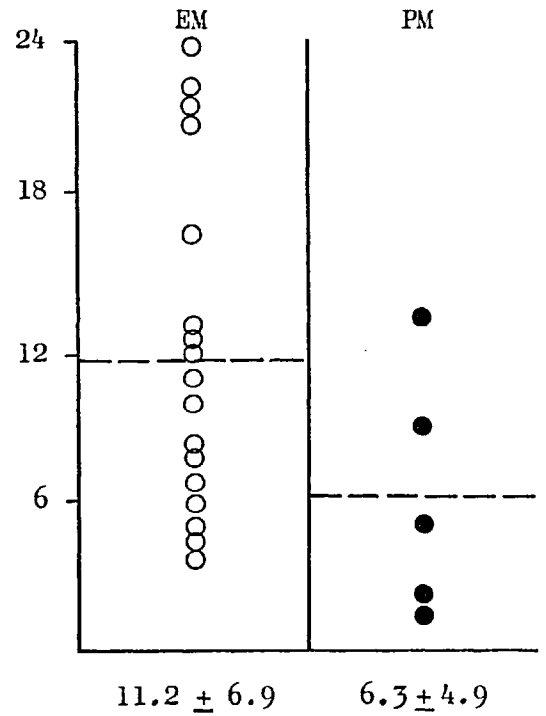
(a) RUBRURIA



(b) TOTAL 2-HYDROXYLATION



(c) PARACETAMOL



(d) $\frac{0\text{-de-ETHYLATION}}{2\text{-HYDROXYLATION}}$

Table 5.1: Panel - based comparison of phenacetin metabolism in EM (n = 17) and PM (n = 5) subjects.

Statistical Assumptions	Distribution-free		Parametric		
	PM rank sum	P	Mean $\pm$ S.D. (EM)	Mean $\pm$ S.D. (PM)	P
Rubricaria	84.5	0.017	0.21 $\pm$ 0.13	0.46 $\pm$ 0.22	0.0025
Total 2-hydroxylation	80.5	0.036	4.5 $\pm$ 2.9	8.1 $\pm$ 4.4	0.025
P / 2HOP	38.5	0.068	11.2 $\pm$ 6.9	6.3 $\pm$ 4.9	N.S.
Total Paracetamol	42.5	0.119	35.5 $\pm$ 7.6	34.7 $\pm$ 8.3	N.S.

5.3.2: The effect of dose; the metabolism of phenacetin in a phenotyped panel.

The cumulative excretion of the phenacetin metabolites under-study in EM (n = 5) and PM (n = 7) volunteers after a 1000 mg oral dose of phenacetin is summarised in Table 5.2. PM Subjects excreted significantly more total 2-hydroxylation products ($2p < 0.01$) and significantly less paracetamol ($2p < 0.01$) at all time points subsequent to 4 h post - dosing. The inter - phenotype differences in phenacetin disposition as exemplified by comparison of metabolite urinary excretion rates are shown in Figures 5.9a and 5.9b. In the 4 h immediately following dosing, the PM subjects displayed lower rates of paracetamol excretion, whereas their excretion of 2-hydroxylation is consistently higher and prolonged when compared to that of EM subjects. A further notable feature of these data is that excretion of 2-hydroxylation products and paracetamol was asynchronous. Peak excretion of 2-hydroxylation products occurred consistently between 1.5 and 3.5 h irrespective of phenotype, whereas peak excretion of paracetamol always occurred between 3.5 and 4.5, again irrespective of phenotype. This factor is illustrated in Figure 5.10. In the EM subjects studied, 2-hydroxylation products initially accounted for 35 ± 20 % of total excretion, falling to an approximately constant 3 ± 1 % from 4 - 6 h post - dosing, after which time no 2-hydroxylation products could be detected in the urine of EM subjects. In contrast, 2-hydroxylation accounted for some 60 ± 20 % of the metabolic products excreted by PM subjects in the first hour post - dosing, falling to 20 ± 9 % at 4 h, and still detectable as 8 ± 3 % of drug - related excretion products at 8 h post - dosing.

The metabolite formation rate - constants derived from these data are summarized in Table 5.3. It can be seen that paracetamol

Table 5.2: Cumulative excretion of paracetamol and 2-hydroxylation products in EM (n = 5) and PM (n = 7) subjects following a 1000 mg oral dose of phenacetin (% dose, Mean $\pm$ S.D.)

Time (h)	Paracetamol	2HOP	Paracetamol	2HOP
1	2.1 $\pm$ 1.5	1.1 $\pm$ 0.6	0.9 $\pm$ 0.4	1.5 $\pm$ 1.0
2	8.3 $\pm$ 3.4	2.1 $\pm$ 0.8	3.8 $\pm$ 1.8	3.3 $\pm$ 1.6
3	16.6 $\pm$ 4.0	3.0 $\pm$ 1.3	9.2 $\pm$ 2.3	5.2 $\pm$ 2.6
4	28.1 $\pm$ 3.4	3.6 $\pm$ 1.9	15.6 $\pm$ 1.9	6.7 $\pm$ 3.2
5	39.5 $\pm$ 3.3	3.7 $\pm$ 1.5	21.3 $\pm$ 1.3	7.7 $\pm$ 3.6
6	48.6 $\pm$ 2.7	3.7 $\pm$ 1.5	25.9 $\pm$ 1.7	8.4 $\pm$ 3.7
7	51.9 $\pm$ 6.4	3.8 $\pm$ 1.6	30.5 $\pm$ 2.1	8.9 $\pm$ 3.6
8	58.1 $\pm$ 3.8	3.8 $\pm$ 1.7	34.7 $\pm$ 2.5	9.3 $\pm$ 3.7

Table 5.3: Kinetic constants governing paracetamol formation and 2-hydroxylation, and the ratios between 0-de-ethylation and 2-hydroxylation products in EM (n = 5) and PM (n = 7) subjects following a 1000 mg oral dose of phenacetin.

Parameter	EM Mean $\pm$ S.D.	PM Mean $\pm$ S.D.
k_f paracetamol (h^{-1})	$0.251 \pm 0.088 \text{ h}^{-1}$	$0.092 \pm 0.015 \text{ h}^{-1}$
k_f 2-hydroxyproducts (h^{-1})	$0.015 \pm 0.009 \text{ h}^{-1}$	$0.017 \pm 0.006 \text{ h}^{-1}$
0-de-ethylation		
2-hydroxylation	18.1 ± 10.6	4.2 ± 1.5

Figure 5.9: Semilogarithmic excretion rate plots for 2-hydroxylation products (2HOP) and paracetamol (P) for EM (n = 5) and PM (n = 7) subjects following a 1000 mg oral dose of phenacetin. (All points Mean $\pm$ S.D.)

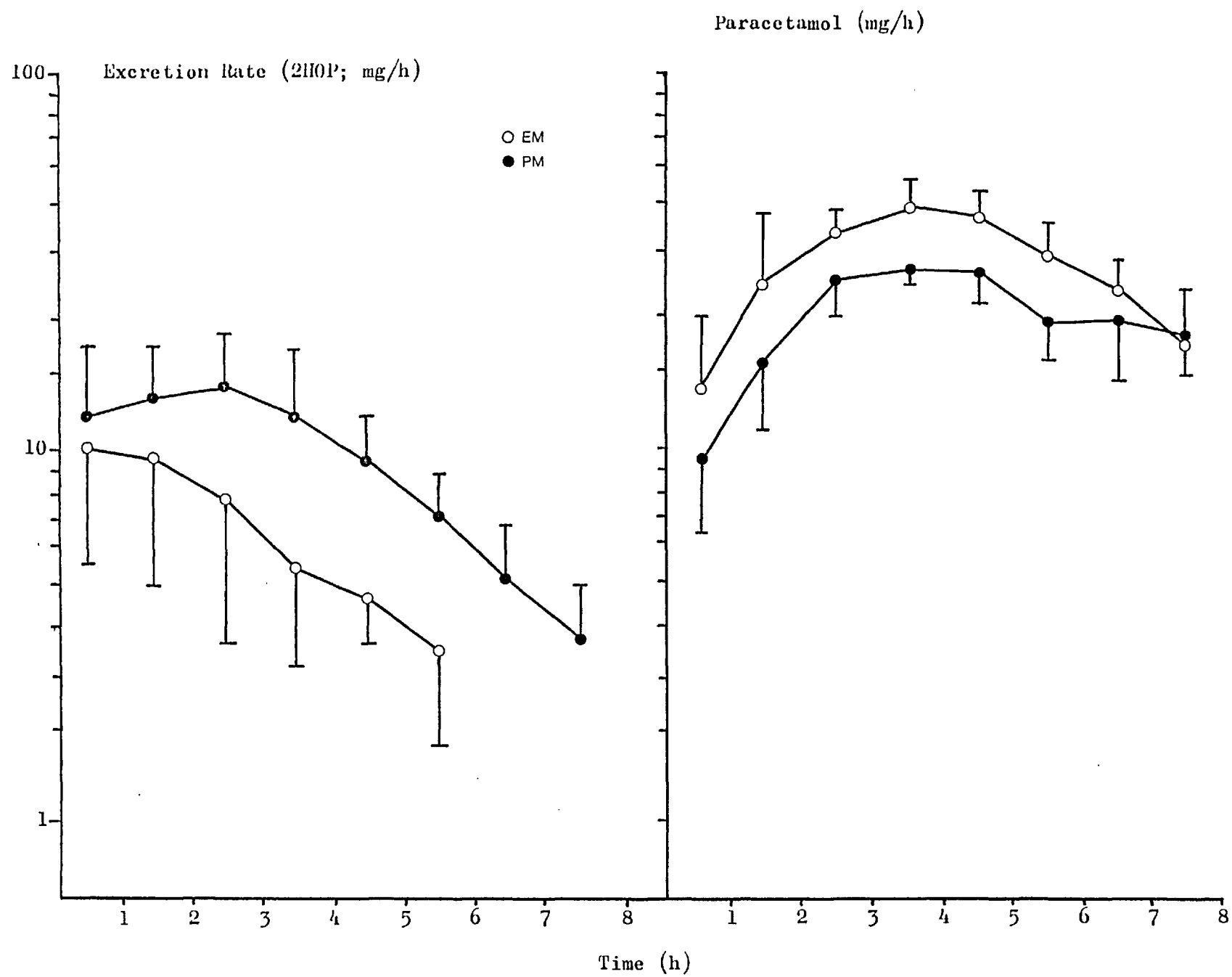
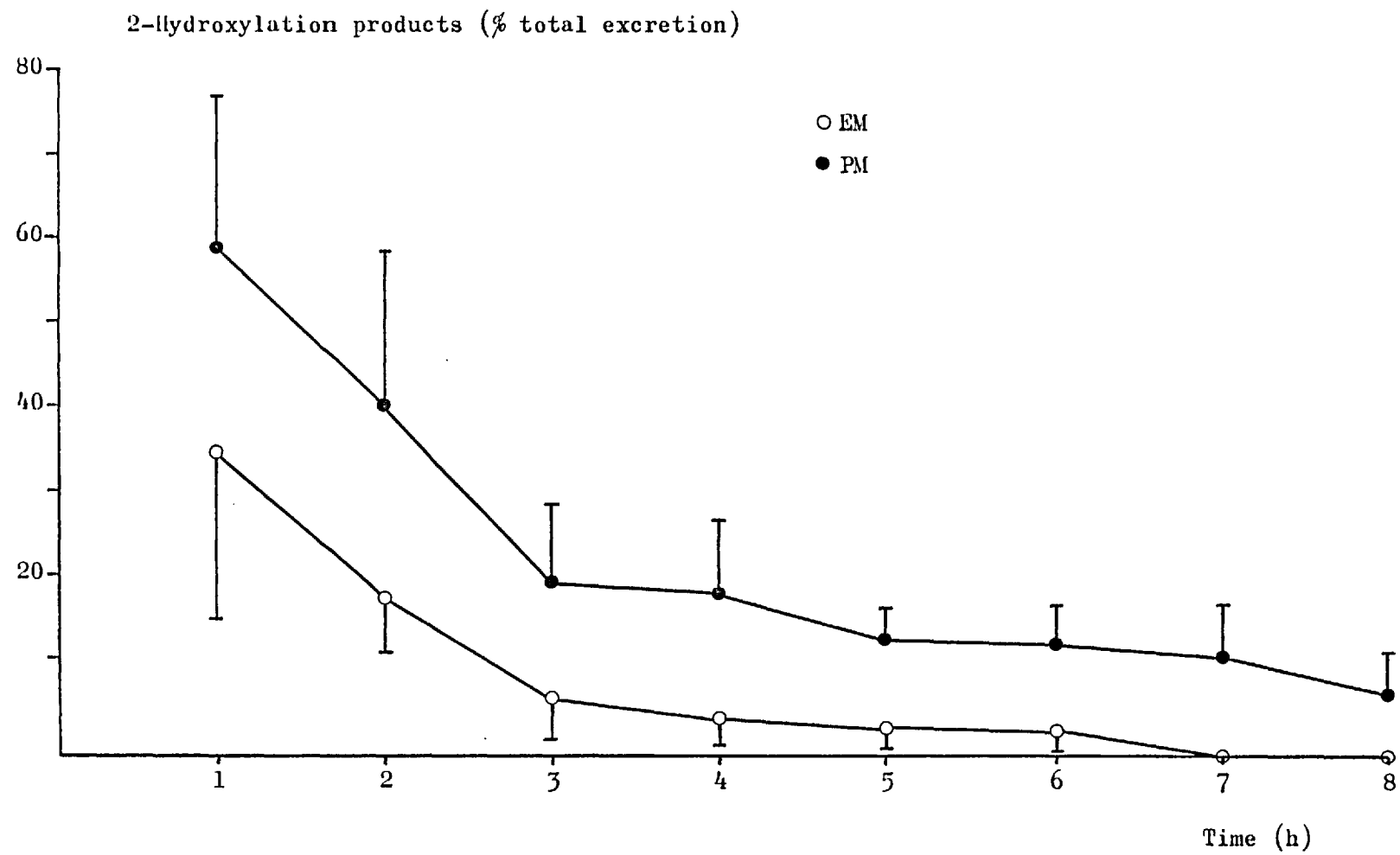


Figure 5.10: 2-Hydroxylation expressed as a proportion of total excretion products in EM (n = 5) and PM (n = 7) subjects following a 1000 mg oral dose of phenacetin (All points Mean $\pm$ S.D.)



formation is significantly slower in the PM phenotype, whereas the apparent first - order rate constant governing 2-hydroxylation displays no significant interphenotype difference. It can further be seen that the phenotypes differ markedly with respect to the ratio between paracetamol and total 2-hydroxylation products, in other words in their relative capacities to perform aliphatic α -carbon oxidation and aromatic ortho- or 2-carbon oxidation.

5.3.3: Phenacetin rubruria; studies with [^3H] phenacetin.

The degree of inter- and intra- individual correlation between rubruria and excretion of 2-hydroxylation products is shown in Figure 5.11. The degree of inter - individual correlation was assessed from data obtained from the initial population study. Total 2-hydroxylation products were ranked and correlated on a distribution - free basis with the degree of rubruria displayed in the same 0 - 8 h bulked urine sample. The correlation found ($r_{(S)} = 0.09$, $p = 0.165$) was not significant. The degree of intra - individual correlation was assessed from serial urine data obtained during the panel studies. The rubruria displayed in any given serial urine sample was correlated on a distribution - free basis with the excretion rate of total 2-hydroxylation products appropriate to that sample. In this instance a significant correlation was found: ($r_{(S)} = 0.53$, $p = 0.008$). The results of studies with [^3H] -phenacetin in a single individual of the PM phenotype are summarized in Table 5.4. There were no major discrepancies between metabolite and radioactivity excretion. The urines passed during this study are shown in Fig. 5.12. The absorbance spectra of rubruric urine under various conditions and extracts thereof are shown in Fig. 5.5, together with spectra obtained from synthetic 5-amino-7-ethoxyphenoxazone. It can be seen that rubruric

Figure 5.11: Intra- and inter-individual correlations between phenacetin rubruria and the excretion of 2-hydroxylation products.

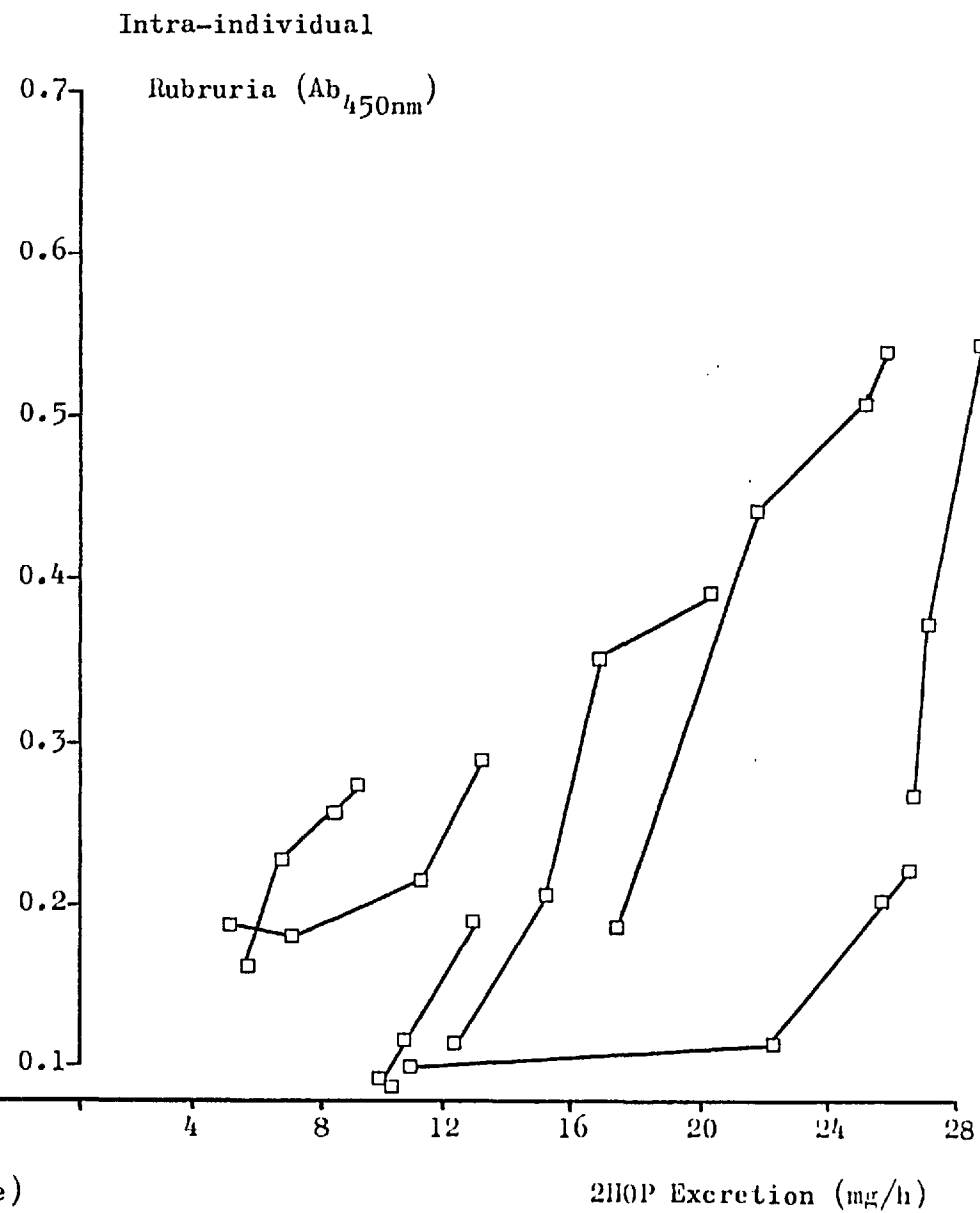
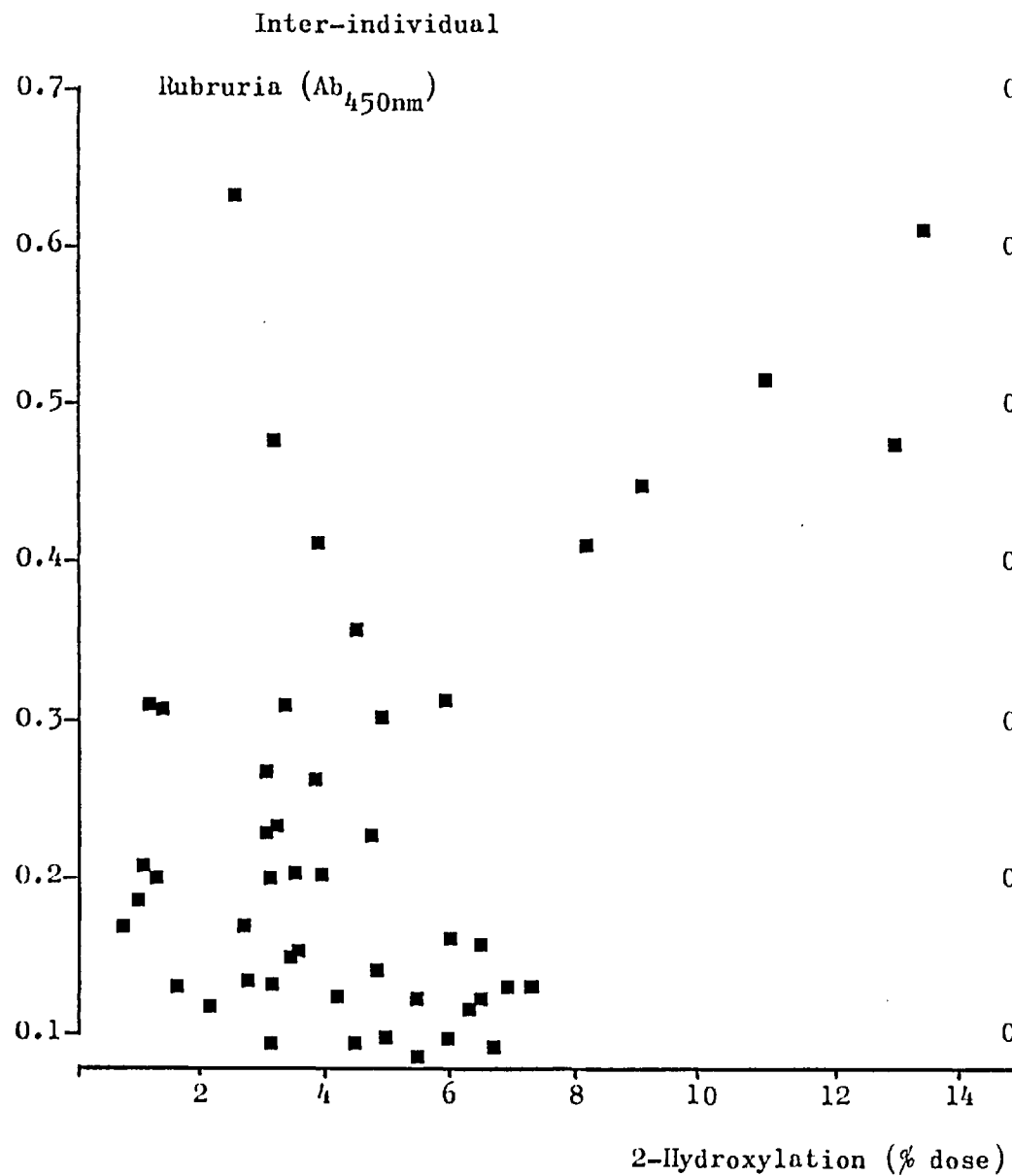


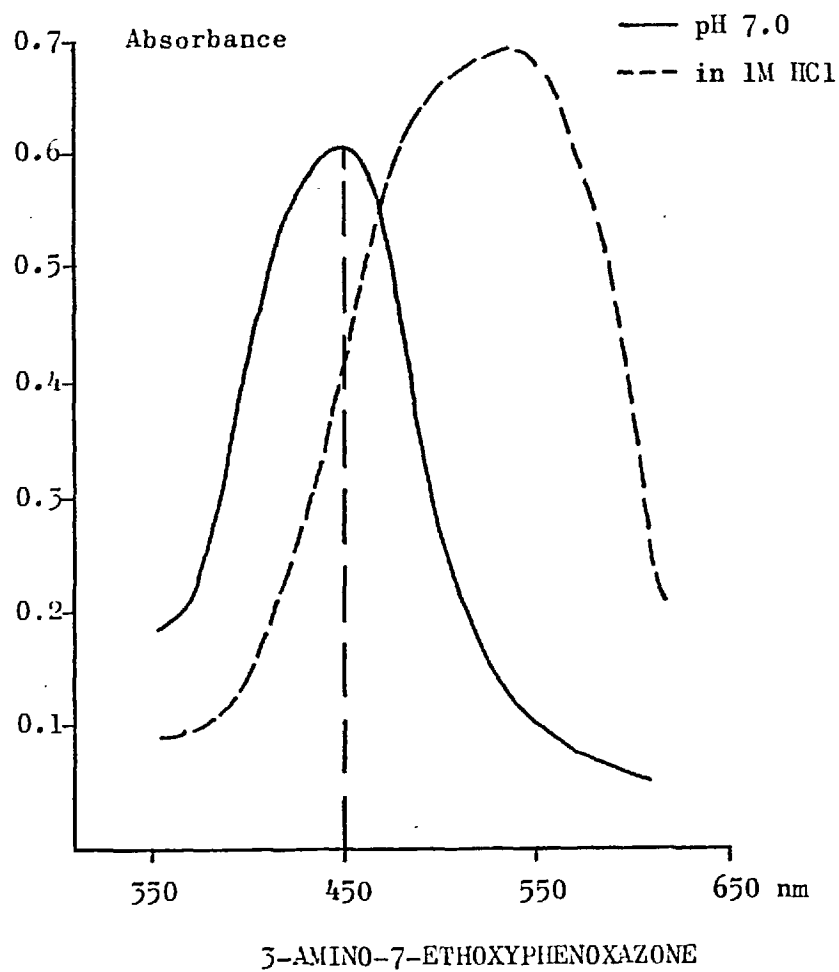
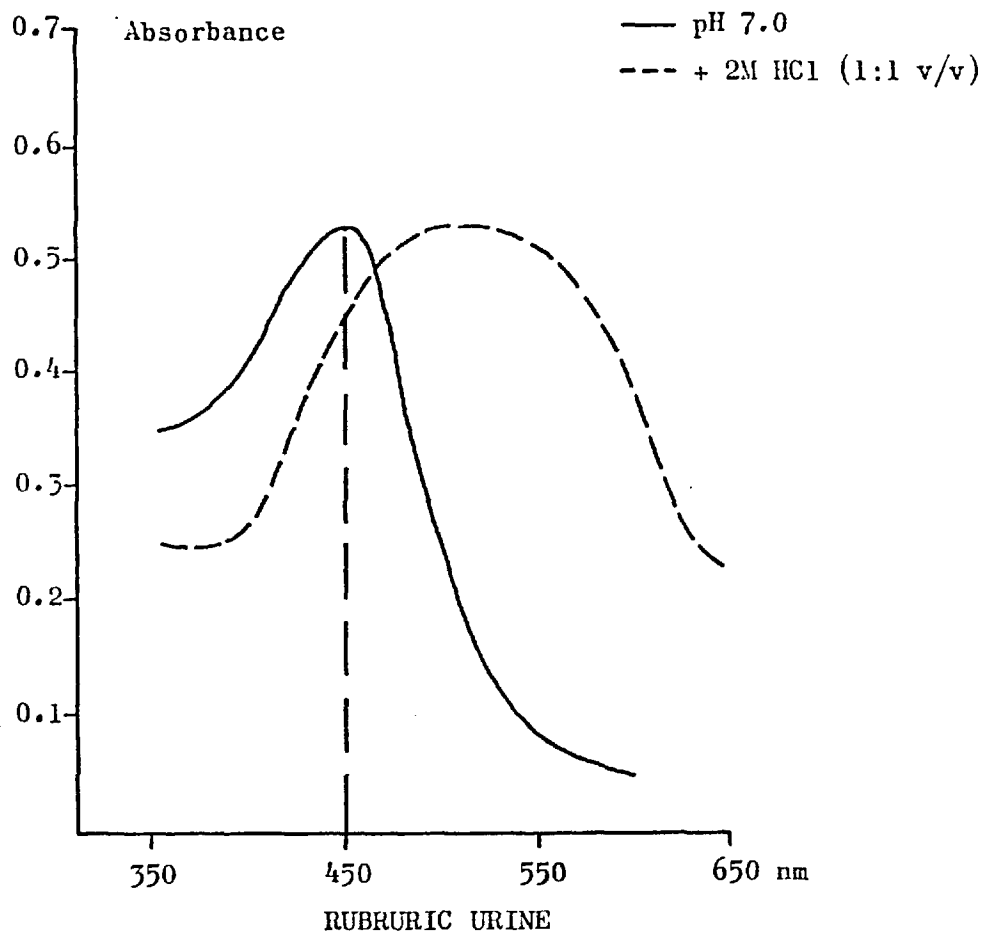
Figure 5.12: Phenacetin Rubruria; the urine is shown following incubation with sulphatase/ β -glucuronidase; for details see text.



Table 5.4: Excretion of phenacetin metabolites and radioactivity by a PM subject following a single oral dose of [^3H] -phenacetin; (1000 mg, 4.74 uCi)

Time (h)	Cumulative Recovery (% dose) as		
	Paracetamol	2-hydroxylation products	^3H
1	1.2	1.1	3.4
2	4.1	3.8	9.8
3	11.5	6.5	17.9
4	17.6	8.8	27.9
5	23.7	10.1	35.6
6	27.7	10.8	40.8
8	35.0	11.7	47.0
12	45.0	12.7	54.8
20.5	54.9	13.8	62.7
21.5	55.3	13.9	65.7
24.0	55.8	13.9	67.1

Figure 5.13: Absorption spectra of rubruric urine and synthetic
3-amino-7-ethoxyphenoxazone.



urine at neutral pH displays maximal absorbance at 450 nm, as does an ethyl acetate extract thereof. Addition of 1M HCl to the urine caused a broadening of the range of absorbance, with a plateau in the region 480 - 520 nm. Synthetic 3-amino-7-ethoxyphenoxazone absorbs maximally at 450 nm in either organic solvent or alkaline aqueous solution. The phenoxazone.HCl formed on addition of 1M HCl absorbs maximally at 540nm.

The results of thin-layer radiochromatography performed on acetone extracts of rubruric urine are summarized in Table 5.5 and Fig. 5. Four radioactive compounds (A - D) were identified, two of which (C and D) corresponded to authentic paracetamol and 3-amino-7-ethoxyphenoxazone respectively. Compound B was shown to possess both amine and phenolic properties, and was almost certainly 2-hydroxyphenetidine. Attempts to synthesize an authentic standard for co-chromatography were unsuccessful owing to the compound's inherent instability. A fourth compound (A), which was deep purple in colour without any spray reagent treatment was also seen. Attempts to harvest sufficient of this compound for farther analysis were unsuccessful. It was noted that the relative proportions of applied radioactivity running as paracetamol and 2-hydroxylation products (76.8 % and 16.1 % respectively) did not correspond well with that predicted from assay of these metabolites in the urine samples employed (62 % and 38 % respectively). Possible interpretations of this observation will be discussed.

Figure 5.14: Radiochromatography (TLC) of an extract of rubruric urine. Details of chromatographic procedures in text and Table 5.5.

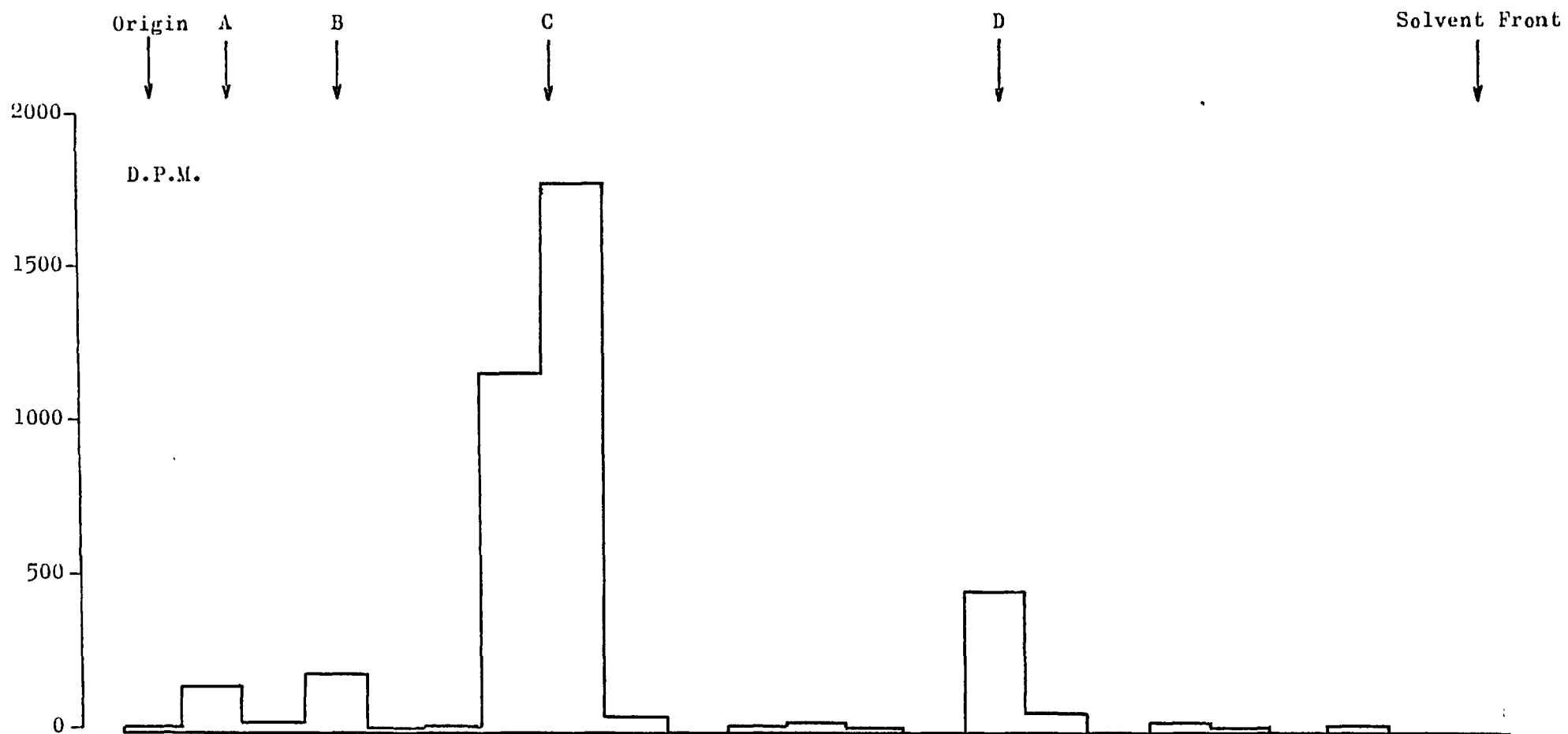


Table 5.5: Thin - layer chromatography * of rubruric urine obtained from a PM subject receiving 1000 mg (4.74 uCi) [³H] phenacetin. Analysis performed after enzymic hydrolysis of conjugates.

Compound R _f	% Applied Radioactivity	Corresponding Standard	Remarks **
0.05 (A)	2.5 %	-	Purple colour
0.12 (B)	4.2 %	-	+ve amine (strong) +ve phenol (weak)
0.30 (C)	76.8 %	Paracetamol	+ve phenol
0.68 (D)	11.9 %	3-amino-7-ethoxyphenoxazone	Yellow colour +ve amine

* T.L.C. system: Benzene - methanol - glacial acetic acid 50:8:4 v/v.

** Spray reagents: Amines: p-dimethylaminobenzaldehyde in ethanol (5% w/v) ; Amines give yellow spot.
Phenols: p-nitroaniline (250 mg) in ethanol / 1M HCl (1/1 v/v; 25 ml), with sodium nitrite (100 mg/10 ml) added prior to use; Phenols give yellow spot turning blue/purple on overspraying with 0.05M KOH in ethanol.

5.4: Discussion:

The conclusions which may be drawn from population and panel studies of phenacetin disposition are summarized below:

1. Certain aspects of phenacetin metabolism namely 2-hydroxylation and production of phenacetin 'rubruria' are heterogeneously distributed in the general population, implying that phenacetin metabolism displays genetic polymorphism.
2. Significant differences were observed in mean and median values of 2-hydroxylation and rubruria between individuals of known debrisoquine oxidation phenotype, indicating that the heterogeneity detected in a random population with respect to these aspects of phenacetin disposition is due to the fact that the oxidative metabolism of both substrates is under the control of the same gene.
3. At a higher dose resolution between oxidation phenotypes is more distinct, with PM subjects displaying significantly lower initial rates of paracetamol production combined with enhanced production of 2-hydroxylated metabolites.
4. Phenacetin 'rubruria' appears to be a genetically controlled trait related predominantly but not exclusively to the enhanced production of 2-hydroxylated metabolites seen in PM subjects.

It is generally recognised that O-de-ethylation of phenacetin to paracetamol occurs pre-systemically (Raaflaub and Dubach, 1975). This fact, in conjunction with the kinetic data derived from the present study suggests an explanation for the observed findings. The apparent formation rate constants governing 2-hydroxylation displayed no significant inter - phenotype difference, thus the PM subjects do not possess a higher capacity to perform this reaction per se. The excretion patterns seen can only be rationalized in terms of the 'body loads' of

phenacetin presented to the alternative pathways. The data indicate that PM subjects have a significantly lower capacity to perform O-de-ethylation than EM subjects, as judged by the apparent first - order formation rate constants ($0.092 \pm 0.015 \text{ h}^{-1}$ as opposed to $0.251 \pm 0.088 \text{ h}^{-1}$). This almost certainly represents deviation from first - order kinetics in the PM phenotype, since progressive saturation leads to progressive under - estimation of the first - order rate constant. It is therefore concluded that phenacetin O-de-ethylation is saturable in the PM phenotype at the dose levels studied, and that the 'body load' of phenacetin in excess of O-de-alkylation capacity becomes available for an alternative route of biotransformation, namely aromatic 2-hydroxylation. Enhanced excretion of products derived from this latter pathway arises from presentation of higher levels of substrate to a pathway of the same intrinsic activity as that possessed by the EM phenotype.

If this is the case, a further consequence of genetically determined defects of carbon oxidation becomes apparent. In the two previous examples considered it has been suggested that the primary consequences of defective oxidation of debrisoquine and phenytoin are increased pharmacological effects due to higher or more persistent plasma levels of the unmetabolized compound.

In contrast, defective O-de-ethylation of phenacetin is reflected in an increase in the significance of a normally minor alternative metabolic pathway. The relevance of the fact that the products of this pathway are of demonstrated toxicity is obvious, and has been underlined by the work of Shahidi and Hemaiden (1969). It may well be that the defect of carbon oxidation displayed by some 8 % of the white Caucasian population represents a genetic predisposition to the haemo- and nephrotoxic effects of phenacetin.

The evidence produced by this study suggest that phenacetin 'rubruria' occurs mainly as a result of the excretion of 2-hydroxylation products in urine, this finding being in broad agreement with that of Gault and Shahidi (1971).

The major coloured compound found in rubruric urine was 3-amino-7-ethoxyphenoxazone, a product of the condensation of 2 molecules of 2-hydroxyphenetidine. The absorption spectra of this compound under a variety of conditions were identical to that displayed by rubruric urine and extracts thereof. It should be stated that trace amounts of a second coloured, but relatively polar drug - related compound were observed, but unfortunately this metabolite could not be isolated in sufficient amounts for more definitive analysis.

The relationship between rubruria and excretion of 2-hydroxylation products in consecutive serial urine samples passed by the same individual gave rise to a significant (intra-individual) correlation. However no such correlation could be demonstrated between different individuals in the population study. It should be noted that the assay used for determination of 2-hydroxylated material involved an acid hydrolysis of any 2-hydroxyphenacetin present which contributes to the final figure. In contrast, rubruria apparently results only from material which has undergone both N-deacetylation and aromatic 2-hydroxylation. The N-deacetylation of phenacetin is known to display wide inter-individual variation (Smith, 1958), but this variation is quite possibly independent of the variation in oxidation capacity studied here. Such independent variability would obscure the inter-individual relationship between rubruria and 2-hydroxylation. This aspect of the study merits further investigation, since extensive

N-deacetylation capacity superimposed on enhanced 2-hydroxylation related to oxidation status may well constitute a further pre-disposing factor to the toxic effects of phenacetin.

CHAPTER SIX

SUMMARY OF RESULTS AND CONCLUDING REMARKS

6.1. Summary of results

6.2. Concluding remarks

6.1. Summary of results:

In the course of the present work, a number of properties of the debrisoquine hydroxylation polymorphism have been studied; evidence has been presented to support a number of conclusions, summarized briefly below:

1. The defective hydroxylation of debrisoquine is a ubiquitous trait amongst ethnic groups otherwise distinct in terms of culture and geographical location. The relative lack of inter-ethnic variability in gene frequency compared with other aspects of drug metabolism displaying polymorphism is indicative of a 'stable' polymorphism in which the heterozygous state is advantageous.
2. The D^H / D^L locus also controls the metabolism of certain other substrates, namely the aromatic 5-, 6-, 7- and 8- hydroxylation of debrisoquine, the aromatic 4-hydroxylation of diphenylhydantoin and the aliphatic α -carbon oxidation of phenacetin preliminary to O-de-ethylation. Aromatic 2-hydroxylation of phenacetin was shown to be independant of the D^H / D^L locus.
3. The consequences of the PM (D^L / D^L) trait for the individual displaying it were investigated:
 - a. In the instance of debrisoquine itself, any drug escaping the pre-systemic metabolic pathway displaying the genetically-determined variation in capacity, is readily cleared by the kidney. Accordingly, the PM subject displays higher peak and mean plasma concentrations of unchanged drug, but there is no inter-phenotype variation in terminal half-life or renal clearance.
 - b. In the instance of diphenylhydantoin some pre-systemic

metabolism occurs, but unchanged drug escaping into the systemic circulation still requires para - hydroxylation for elimination, as no major alternative metabolic or excretory pathway is available. Accordingly, the PM subject was found to display a longer half - time for metabolite excretion, presumably an indication of persistence of the unchanged drug in plasma.

- c. In the instance of phenacetin, pre - systemic O-de-ethylation is defective in the PM phenotype, but an alternative pathway not subject to the defect is available for the excess substrate. Accordingly, the PM subject displays a level of overall elimination of drug related material similar to his EM counterpart, but he produces a significantly higher proportion as the toxic 2-hydroxylation products, and a significantly lower proportion as the relatively less toxic paracetamol.

It is concluded that the 'single - gene' defects previously known to exist in the oxidative metabolism of diphenylhydantoin and phenacetin are actually different manifestations of possession of the homozygous D^L / D^L genotype, and thus exist at far higher frequencies in the population than has previously been suspected. Since there is evidence that this defect may have potentially serious pharmacological and toxicological implications for the affected individual, it is suggested that the use of panels phenotyped with respect to debrisoquine oxidation status may have a usefulness in new drug development. A single oral dose of debrisoquine yields unambiguous resolution of oxidation phenotypes, and the observation of significant inter - phenotype differences in the disposition of any novel substrate may well give advance warning of potential problems with the compound in more widespread

clinical use.

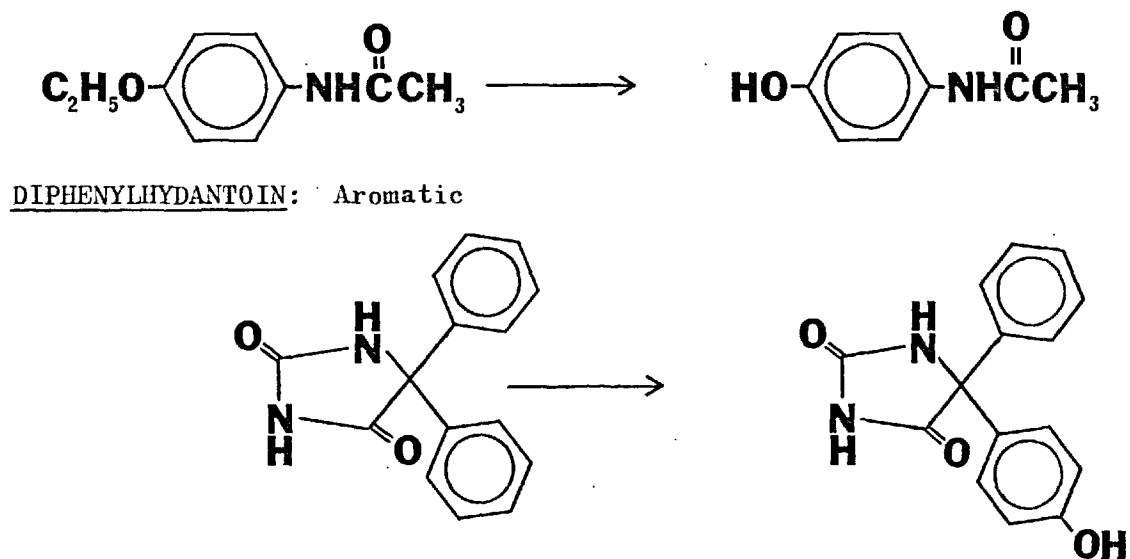
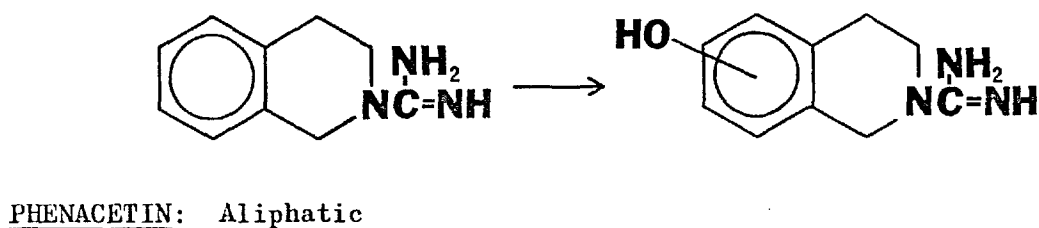
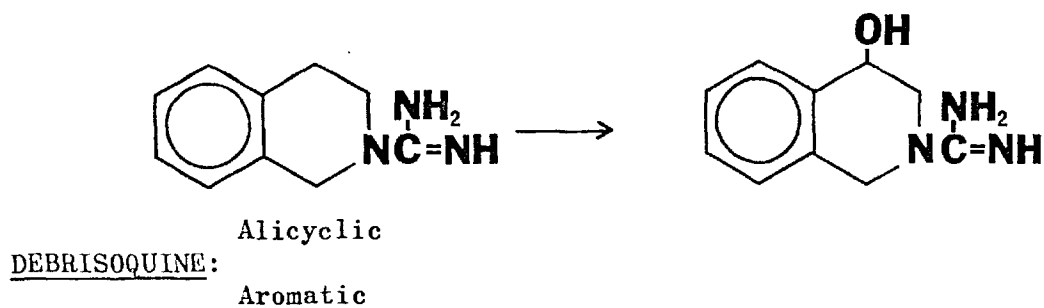
6.2: Concluding remarks:

The preceding section has described specific consequences of defective oxidation of specific substrates; it is felt that the information yielded by the present studies has more general implications for the field of carbon oxidation.

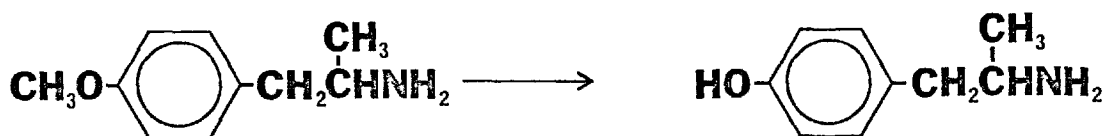
The oxidative metabolic pathways apparently under the control of the D^H / D^L locus are shown in Figure 6.1a; the pathways shown in Figure 6.1b have been demonstrated to be independant of the locus. It can readily be seen that both categories contain both 'aliphatic' and 'aromatic' hydroxylation reactions, though these terms are usually used to demarcate separate enzymic oxidation mechanisms (Jerina and Daly, 1977). The detailed mechanisms of 'aliphatic hydroxylation' have yet to be elucidated, but the reaction is generally considered to proceed by direct insertion of activated molecular oxygen into the saturated substrate (Daly, 1971). This mechanism is shown in Figure 6.2 with particular reference to the oxidation of S(+)- α -[2H]-ethylbenzene (McMahon et al., 1969). This study demonstrates two features generally considered to be diagnostic of a reaction proceeding by oxygen insertion:

- a. Retention of configuration: the retention of absolute tetrahedral configuration during alcohol formation is characteristic of oxygen insertion. In the example quoted, this is manifested as stereospecificity; the deuterated α -carbon is assymmetric, and oxidation of the R(-)-enantiomer occurs at a significantly slower rate.
- b. Isotope effects: the aforementioned is in fact a stereospecific isotope effect, such that the rate of hydroxylation at a carbon - hydrogen bond is greater than the carbon - deuterium analogue

Figure 6.1a: Hydroxylation reactions controlled by the D^H/D^L locus



4-METHOXYAMPHETAMINE: Aliphatic (Kitchen *et al*, 1979)



GUANOXAN: Aromatic (Mahgoub, 1979)

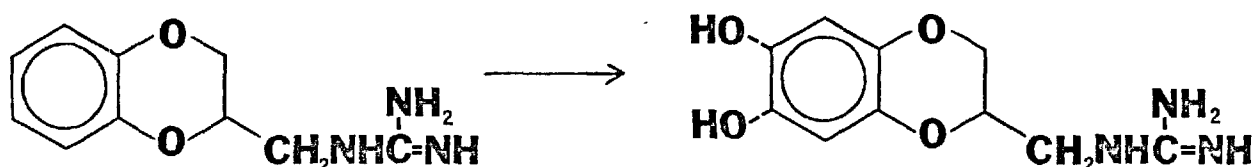
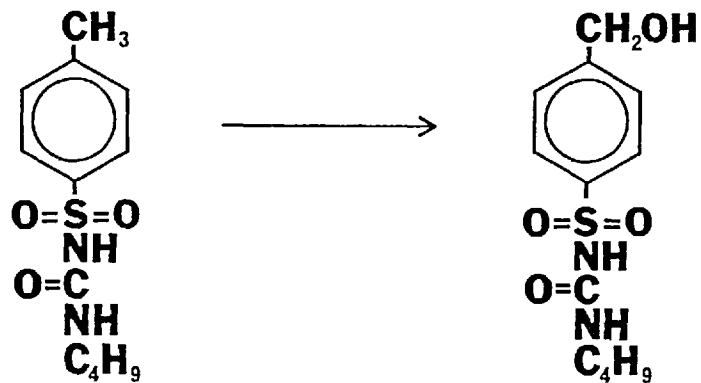
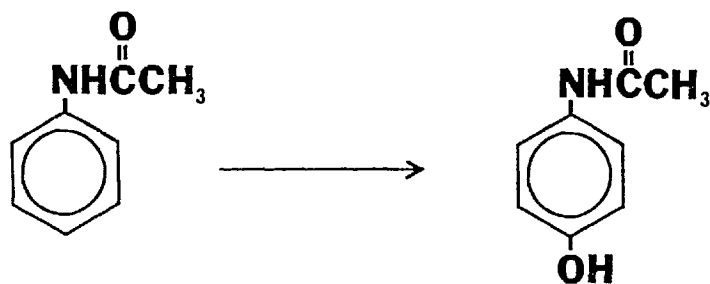


Figure 6.1b: Hydroxylation reactions not controlled by the D^H/D^L locus

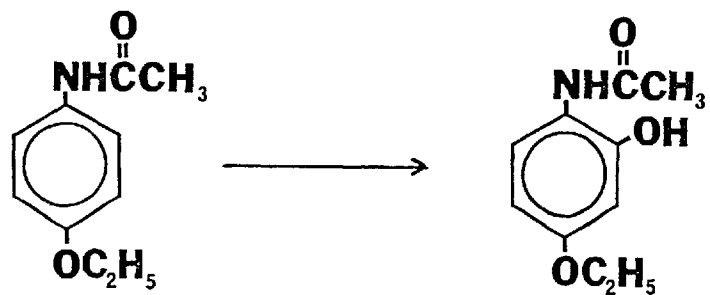
TOLBUTAMIDE: Aliphatic (Idle et al, 1979)



ACETANILIDE: Aromatic (Wakile et al, 1979)



PHENACETIN: Aromatic



(k_H / k_D). Significant isotope effects have been reported both in alcohol formation and in the aliphatic hydroxylations thought to precede N- and O-dealkylation reactions (Tomaszewski et al., 1975; Gorrod and Temple, 1976).

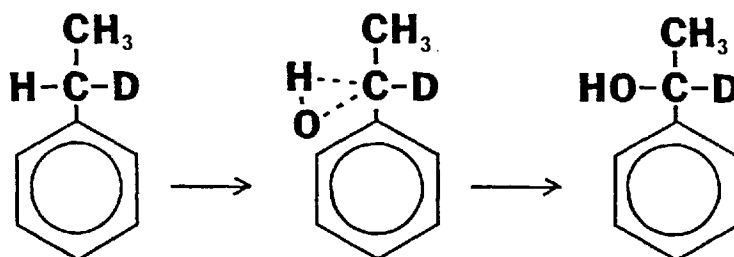
In contrast, the majority of phenolic metabolites are thought to be formed by an addition reaction yielding an arene oxide, followed by spontaneous isomerization to the phenol (Figure 6.3). The essential distinction is that this process is not accompanied by an isotope effect (Tomaszewski et al., 1975). In general terms, this criterion holds good, although a few particular aromatic hydroxylations have been shown to display marked isotope effects, namely the 6-hydroxylation of 6-[2H]-zoxazolamine (Tanabe et al., 1970) and 3-hydroxylation of nitrobenzene and methylphenylsulphone (Tomaszewski et al., 1975).

It has been pointed out by Atlas and Nebert (1977), that a pharmacogenetic polymorphism results from a defect of a rate - limiting step; in other words substrates displaying parallel pharmacogenetics must necessarily interact with the same gene product. It is thus arguable that classification of hydroxylation reactions in terms of 'controlled' or 'not controlled by the D^H / D^L locus' is a more rational criterion than those outlined above since it is based on direct observation of whether or not the compound in question is a substrate for the enzyme protein produced at that locus.

In conclusion, it is appropriate to return to the question posed in the first chapter; are there "surprisingly few" polymorphisms of carbon oxidation? Or does current methodology militate against their detection? Two final aspects of the current work will be considered and the proposition made that unambiguous information regarding the disposition

Figure 6.2: Stereospecific hydroxylation of S(+)- α -[^2H]ethylbenzene
(McMahon et al, 1969)

S(+)-enantiomer:



R(-)-enantiomer:

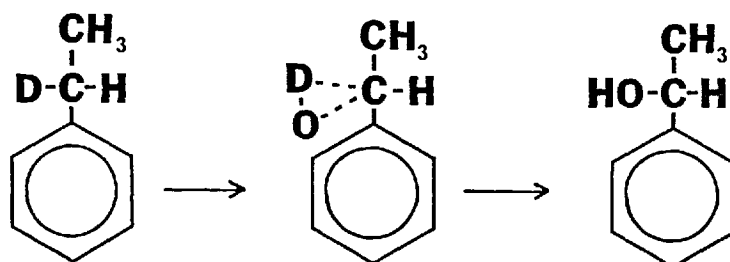
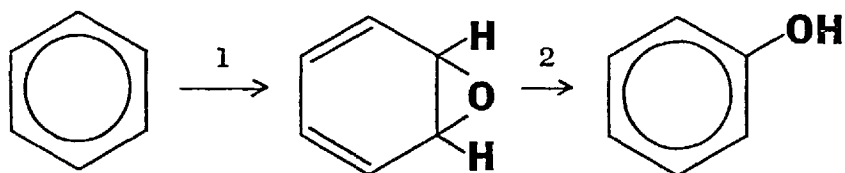


Figure 6.3: Aromatic hydroxylation; direct addition (1), followed
by spontaneous isomerisation (2).



of a drug and the way in which that disposition varies within a population can only be obtained by a very particular methodological approach.

1. The study population and statistical assumptions:

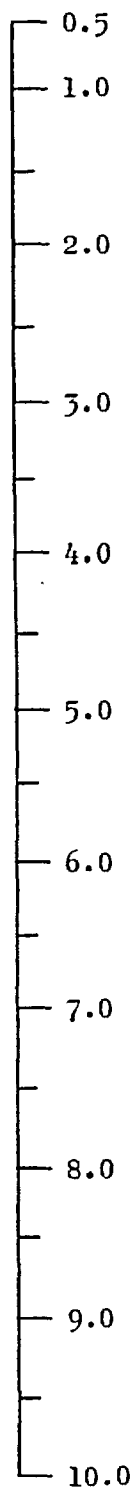
The commonest form of drug disposition study encountered in reviewing relevant literature is one in which 6 - 10 volunteers receive a single dose of the compound in question and the drug's elimination parameters are then quoted in terms of the group mean $\pm$ the standard deviation. It is appreciated that in many instances this is the only feasible approach; however, in the present context the calculation of mean $\pm$ standard deviation has a very particular connotation: it assumes that the study sample is drawn from a normally distributed unimodal population, that is, it is assumed at the outset that the metabolism of the drug under study is under polygenic control.

The nomogram shown in Figure 6.4 relates the homozygous recessive genotype and heterozygote frequencies for a hypothetical defect of drug metabolism with the study populations required in order to be 95 % and 99 % confident of detecting an individual homozygous for the defect. It can be seen that to be 99 % confident of detecting a defect of 1 % incidence (heterozygote frequency 18 %) would entail the study of 1085 unrelated subjects. Inversion of this argument demonstrates that for a study population of 10 unrelated individuals a defect could be present in 38 % of the general population (heterozygote frequency 47 %), and the failure to observe the defect in those 10 subjects is not statistically significant.

It is thus suggested that whenever feasible as large a population as possible should be studied and the raw data obtained therefrom tested for heterogeneity and otherwise analyzed by non - parametric statistical methods.

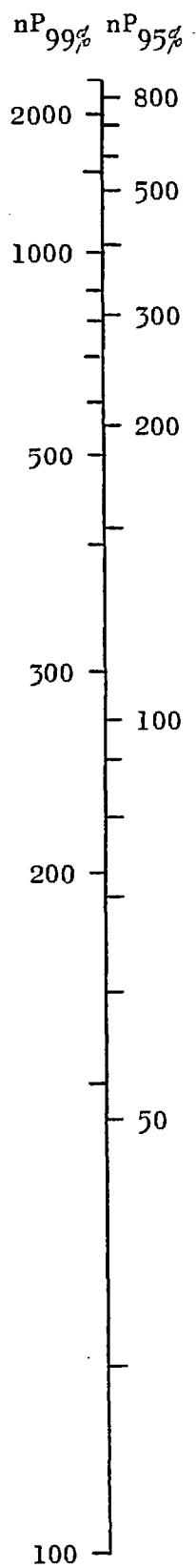
Figure 6.4: A nomogram relating recessive phenotype frequency (q^2), calculated heterozygote frequency ($2pq$) and the minimum study populations required for 95 % and 99 % confidence of detecting the homozygous recessive trait.

Homozygous
recessive
frequency
(% population)



-198-

Heterozygote
frequency
(% population)



2. Pharmacokinetic considerations:

The parameters almost universally employed in the estimation of capacity to metabolize a drug are either the rate of clearance of that drug from plasma or its elimination half - life ($t_{1/2}$). Occasionally the parameter from which $t_{1/2}$ is derived, namely the first - order elimination rate constant (K) is used. These are essentially dependant variables, since:

$$(i) t_{1/2} = 0.693 / K$$

$$(ii) \text{Clearance} = K \text{ Volume of distribution .}$$

A more crucial property of K and plasma clearance is that they are additive, that is:

$$(i) \text{Plasma clearance} = \text{Metabolic clearance} + \text{Renal clearance}$$

$$(ii) K = k_f + k_d$$

where k_f is the first - order rate constant governing metabolite formation, and k_d that governing excretion of unchanged drug. If three metabolites were formed then:

$$K = k_{f1} + k_{f2} + k_{f3} + k_d$$

The logical consequences of this situation are shown in Table 6.1.

The fate of a hypothetical drug is described in a "normal" and "defective" individual. The compound is cleared by four competing first - order pathways, each accounting for 25 % of total elimination in the "normal" subject. In the "defective" metaboliser the contribution of one of the pathways has been reduced by a factor of ten. If volume of distribution is constant it can be seen that the three parameters of overall elimination reflect the true inter-individual variation of ten - fold as a factor of ca. 1.2, a considerable underestimate. This model's predictions are borne out by the findings of the present studies - of the three substrates studied only diphenylhydantoin revealed the defect in its overall elimination parameters -

Table 6.1: The metabolism of a hypothetical compound cleared by four competing first - order pathways; in the 'defective' metaboliser the contribution of pathway (2) has been reduced by a factor of ten. (The plasma clearance quoted assumes a volume of distribution of 50 l in both subjects.)

<u>Metabolic Pathway</u>		<u>Normal (% Dose)</u>	<u>Defective (% Dose)</u>
(1)	$k_f = 0.025 \text{ h}^{-1}$	25.0	32.1
(2)	$k_f = 0.025 \text{ h}^{-1}$ (normal)	25.0	3.2
	$k_f = 0.0025 \text{ h}^{-1}$ (defective)		
(3)	$k_f = 0.025 \text{ h}^{-1}$	25.0	32.1
(4)	$k_f = 0.025 \text{ h}^{-1}$	25.0	32.1
	K	0.100 h^{-1}	0.078 h^{-1}
	$t_{1/2}$	6.9 h	8.9 h
	Clearance	83 ml min^{-1}	65 ml min^{-1}

and this was the only substrate for which no alternative elimination pathway existed. The model demonstrates that where more than one pathway exists only quantitation of metabolites and the ratios between them will give an accurate measure of "true" inter - individual variation in metabolic capacity. It could be argued that the figures used were selected to best illustrate a subjective argument, but they are not necessarily unrealistic. In 1979, over 700 publications referred to antipyrine clearance or half-life as an index of hepatic microsomal enzyme activity; antipyrine is eliminated by at least six competing pathways, none of which accounts for more than 30% of an oral dose (Breimer et al., 1979).

There is, however, an aspect of the current work which raises more serious doubts about the use of first - order elimination parameters in studies of inter - individual variation in drug metabolism.

The first - order elimination constant and the half - life and clearance derived from it can generally be assumed to reflect absolute elimination capacity since, for elimination by a single metabolising enzyme:

$$K = \frac{V_{\max}}{K_m}$$

where $V_{\max}$ reflects the amount of enzyme present, and K_m the affinity of the enzyme for its substrate. The affinity is constant, so simple increases or decreases in the amount of enzyme present will alter only $V_{\max}$, and cause a directly proportional change in the first - order constant. This assumption is readily borne out by in vitro characterization of changes in microsomal enzymes following pretreatment with an enzyme inducer. Such data are shown in Table 6.2. The authors demonstrate that the induced enzyme has the same K_m but a higher $V_{\max}$, sim-

Table 6.2: The effects of Chlordane or dichlorodiphenyltrichloroethane (DDT) on the kinetics of in vitro diphenylhydantoin metabolism by rat liver microsomes. (Data of Kutt and Fouts, 1970).

Pretreatment	$V_{\max}$ (nmol DPH / mg / h)	K_m (μ M)	k_f^* (h ⁻¹)
Control	39.3 $\pm$ 8.1	27 $\pm$ 4	1.43 $\pm$ 0.14
Chlordane (30 mg / kg for 3 days)	59.4 $\pm$ 11.5	30 $\pm$ 4	1.97 $\pm$ 0.18
Control	38.0 $\pm$ 6.6	28 $\pm$ 4	1.35 $\pm$ 0.06
DDT (50 mg / kg for 3 days)	63.9 $\pm$ 8.1	32 $\pm$ 4	2.07 $\pm$ 0.10

* Calculated from authors' data.

plistically, there is more of it there. It can readily be shown that the first - order rate constant and half - life will change in direct proportion to the increase in metabolizing capacity.

Can this logic be inverted when considering decreased metabolic capacity owing to a genetic defect?

It should be stated at the outset that a consistent feature of the metabolic defects under consideration is that the degree of resolution between the "normal" and "defective" individual shows a marked dose - dependancy, as illustrated by the data in Table 6.3. Both Kutt et al. (1964) and Gerber et al. (1972) observed that "defective" subjects could metabolize low doses of diphenylhydantoin in an identical manner to "normal" subjects, though the defect became apparent with increasing doses of the drug. Similarly, Mahgoub (1979) demonstrated that the degree of resolution between poor and extensive metabolisers of debrisoquine increases with increasing dose.

Table 6.4 shows a series of models devised in an attempt to explain this phenomenon of dose - dependant phenotype resolution.

Model 1: Single enzyme systems: in "defective" subject 1 the simplest situation is considered. The enzyme present is identical to that of the "normal" subject (K_m constant), but reduced in amount (V_{max}) by a factor of ten. It can be seen phenotype resolution in this situation is independant of dose, as judged by the calculated rates of metabolism of an exponentially increasing series of substrate concentrations. The data for "defective" subject 2 shows that the dose - dependancy effect can only be acheived by combining decreased amount with increased affinity (K_m decreased).

Model 2: An alternative scheme is shown which also predicts dose - dependant resolution of phenotypes. In this instance, both individuals possess an identical amount of a high affinity - low capacity enzyme

Table 6.3: The effect of dose on the resolution of 'normal' and 'defective' individuals with respect to the hydroxylation of diphenylhydantoin (DPH) and debrisoquine.

<u>Diphenylhydantoin</u> [*]			<u>Debrisoquine</u> [†]		
<u>Urinary output of pHO-DPH (% dose)</u>			<u>Metabolic Ratio</u>		
Dose (mg / p.o.)	'Normal' (n = 5)	'Defective' (n = 2)	Dose (mg / p.o.)	EM (n = 4)	PM (n = 3)
120	-	69 ± 7	1	-	11.5 ± 5.3
300	66 ± 5	44 ± 3	10	0.8 ± 0.4	20.7 ± 0.7
400	66 ± 5	-	20	0.6 ± 0.3	47.7 ± 11.9
500	64 ± 5	-	40	0.8 ± 0.7	-

* Data from: Kutt et al. (1964)

Gerber et al. (1972)

† Data from: Mahgoub (1979)

Table 6.4: Models of enzyme deficiency resulting in dose - dependant resolution of "normal" and "defective" phenotypes. (All units arbitrary)

Model 1: Single enzyme; variable amount / affinity (see text for explanation of components)

S*	Normal	Defective 1	Resolution	Defective 2	Resolution
	V _{max} : 100 K _m : 1000	V _{max} : 10 K _m : 1000		V _{max} : 10 K _m : 100	
	V [†]	V		V	
1	0.1	0.01	10	0.1	1.0
3	0.3	0.03	10	0.3	1.0
10	1.0	0.1	10	0.9	1.1
30	2.9	0.3	10	2.3	1.3
100	9.1	0.9	10	5.0	1.8
300	23.1	2.3	10	7.5	3.2
1000	50.0	5.0	10	9.1	5.5

*S = concentration of substrate

†V = rate of metabolism; Resolution = Normal V / Defective V.

Table 6.4; Continued

Model 2: Multiple enzyme forms, variable amounts of one component

S	Normal	Defective	Resolution
	$V_{\max_1} : 100 ; V_{\max_2} : 1000$ $K_{m_1} : 1000 ; K_{m_2} : 100000$	$V_{\max_1} : 100 ; V_{\max_2} : 100$ $K_{m_1} : 1000 ; K_{m_2} : 100000$	
	V	V	
10	1.1	1.0	1.1
30	3.2	2.9	1.1
100	10.1	9.2	1.1
300	26.1	23.4	1.1
1000	59.9	51.0	1.2
3000	104.1	77.9	1.3
10000	181.8	100.0	1.8
30000	327.5	119.8	2.7
100000	599.0	149.0	4.0

$(V_{\max_1}; K_{m_1})$ in combination with variable amounts of a low - affinity - high capacity enzyme $(V_{\max_2}; K_{m_2})$; here again phenotype resolution increases with substrate concentration.

Regardless of which of these models is the more adequate description of the "defective" metaboliser, it is obvious from Table 6.3 that dose is a crucial factor in the investigation of inter - individual variation in drug metabolism. Considering the situation at its simplest, a population study of diphenylhydantoin metabolism conducted at a dose level of 120 mg would completely mask the presence of a metabolic defect which is all too obvious at higher doses.

The logical consequence is that studies which seek to evaluate individuals in terms of first - order elimination (that is, minimal saturation) characteristics, may ultimately be self - defeating. The experience of diphenylhydantoin and debrisoquine suggests that at best, first - order parameters will minimize phenotype resolution, and at worst, fail to distinguish between phenotypes at all.

The general conclusion drawn from the present work is that realistic assessment of the range and nature of inter - individual variation in drug metabolism capacity will necessarily involve the study of metabolite excretion patterns in large populations at more than one dose level; of these factors dose appears to be the most important. The present studies have demonstrated that where discontinuous variability exists the term 'dose' has two components: the 'extrinsic dose', or physical quantity ingested, and the 'intrinsic dose', the ratio between ingested compound and the genetically determined capacity to effect its metabolic inactivation.

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APPENDICES

APPENDIX A:

- (i) Excretion rates: unchanged debrisoquine; EM subjects.
- (ii) Excretion rates: 4-hydroxydebrisoquine; EM subjects.
- (iii) Excretion rates: unchanged debrisoquine; PM subjects.
- (iv) Excretion rates: 4-hydroxydebrisoquine; PM subjects.
- (v) Debrisoquine plasma levels: oral dosing; EM subjects.
- (vi) Debrisoquine plasma levels: oral dosing; PM subjects.
- (vii) Debrisoquine plasma levels: intravenous dosing.
- (viii) Metabolic ratios: Caucasian subjects.
- (ix) Metabolic ratios: Ghanaian subjects.

APPENDIX B:

- (i) Excretion of 4-hydroxyphenytoin; Caucasian subjects.
- (ii) Excretion of 4-hydroxyphenytoin; Ghanaian subjects.
- (iii) Phenacetin metabolism; population study.
- (iv) Phenacetin metabolism; panel study.

APPENDIX A

(i) Excretion rates: unchanged debrisoquine; EM subjects (% dose/h).

Time*	Subject				
(h)	1	2	3	4	5
0.5	n.d.	1.61	n.d.	3.49	0.12
1.5	1.69	1.85	1.62	4.93	2.52
2.5	1.55	1.27	2.60	5.20	1.18
3.5	1.36	1.17	1.32	3.60	1.00
5.0	0.74	0.70	0.95	1.69	0.49
7.0	0.50	0.63	0.61	1.37	0.39
10.0	0.56	0.45	0.75	1.01	0.28

(ii) Excretion rates: 4-hydroxydebrisoquine; EM subjects (% dose/h).

Time	Subject				
(h)	1	2	3	4	5
0.5	0.19	2.00	0.95	0.22	0.12
1.5	4.10	2.95	2.44	3.21	7.38
2.5	4.32	2.41	4.81	4.18	4.75
3.5	4.01	2.25	3.15	3.29	4.84
5.0	2.04	1.75	2.24	2.47	1.97
7.0	1.15	1.18	1.18	1.66	1.91
10.0	0.90	0.83	1.25	0.92	0.82

*Mid-point of the urine collection period; each subject received 10 mg debrisoquine p.o. at time = 0.

(iii) Excretion rates: unchanged debrisoquine; PM subjects (% dose/h).

Time	Subject				
(h)	1	2	3	4	5
0.5	1.51	0.70	-	2.90	1.82
1.5	10.22	5.72	3.17	6.66	5.03
2.5	7.35	6.20	5.36	4.85	4.95
3.5	5.23	3.47	4.17	3.10	3.12
5.0	5.45	3.14	4.23	2.74	1.74
7.0	3.30	1.32	2.72	2.16	2.00
10.0	1.92	2.33	2.00	1.42	1.36
15.0	0.76	0.76	-	-	-
18.0	-	-	0.70	-	-
23.0	-	0.24	-	-	-
24.0	0.15	-	-	-	-
27.0	-	0.13	-	-	-
30.0	0.05	-	-	-	-

(iv) Excretion rates: 4-hydroxydebrisoquine; PM subjects (% dose/h).

Time	Subject				
(h)	1	2	3	4	5
0.5	0.02	0.06	-	0.18	0.22
1.5	0.23	0.14	0.22	0.26	0.45
2.5	0.23	0.13	0.25	0.32	0.16
3.5	0.25	0.11	0.14	0.29	0.15
5.0	0.22	0.14	0.27	0.29	0.11
7.0	0.23	0.12	0.20	0.18	0.13
10.0	0.17	0.10	0.18	0.12	0.19
15.0	0.07	0.05	-	-	-
18.0	-	-	0.06	-	-
23.0	-	0.02	-	-	-
24.0	0.013	-	-	-	-
27.0	-	0.010	-	-	-
30.0	0.005	-	-	-	-

(v) Debrisoquine plasma concentrations: oral dosing; EM subjects (ng/ml).

Time (h)	Subject*			
	1	2	3	4
0.5	-	-	-	-
1.0	5.8	19.3	12.0	21.5
1.5	19.5	17.2	25.2	23.5
2.0	14.8	12.1	16.2	17.6
3.0	-	8.0	16.1	10.0
4.0	13.7	6.8	13.3	6.5
6.0	4.5	4.8	3.7	-
8.0	3.2	3.1	-	5.3

*Subject numbering as per (i) and (ii); subject 5 not studied.

(vi) Debrisoquine plasma concentrations: oral dosing; PM subjects (ng/ml)

Time (h)	Subject*		
	3	4	5
0.5	11.2	9.9	9.8
1.0	27.9	24.6	38.3
1.5	85.4	69.5	62.0
2.0	59.6	70.6	59.3
3.0	52.3	39.6	41.4
4.0	38.2	33.3	36.2
6.0	27.0	31.0	27.9
8.0	19.9	12.1	17.6

*Subject numbering as per (iii) and (iv); subjects 1 & 2 not studied.

(vii) Debrisoquine plasma concentrations: intravenous dosing (ng/ml).

Time (h)	Subject*		
	EM(2)	EM(4)	PM(3)
0.06	1058.0	516.0	-
0.09	-	300.2	1142.0
0.13	809.0	191.0	-
0.17	-	189.1	-
0.22	-	-	491.0
0.25	-	-	146.0
0.33	167.0	-	-
0.37	-	85.3	-
0.40	-	-	50.5
0.50	105.1	47.4	-
0.67	-	-	31.5
0.75	61.0	47.9	-
1.00	69.9	28.1	16.0
1.33	-	-	14.3
1.50	46.4	-	-
1.67	-	16.2	-
2.00	41.0	22.0	12.8
2.50	38.8	13.1	14.0
3.00	40.7	11.9	8.5
3.50	-	-	7.5
4.00	44.0	12.1	6.8
5.00	43.5	9.0	3.8
6.00	32.0	7.2	4.0
7.00	26.6	6.7	5.1
8.00	22.0	4.9	3.0

*Genotypes, determined from family studies as follows:

EM(2): D^H/D^H

EM(4): D^H/D^L

PM(3): D^L/D^L

Subject numbering as per preceding tables.

(viii) Metabolic ratios: Caucasian subjects.

<u>SUBJECT</u>	<u>DEBRISOQUINE*</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
EB	0.5	23.1	23.6	0.02
EK	0.4	11.9	12.3	0.03
GW	0.5	16.0	16.5	0.03
EKE	0.5	14.0	14.5	0.03
BP	1.3	26.7	28.0	0.05
AZ	1.4	10.5	11.9	0.1
SD	1.4	16.7	18.1	0.1
JP	1.3	16.8	18.1	0.1
VB	3.5	24.6	28.1	0.1
JG	2.9	20.8	23.7	0.1
MDT	3.7	29.0	32.7	0.1
SJH	0.4	4.1	4.5	0.1
BJL	2.7	19.4	22.1	0.1
VSB	2.1	26.7	28.8	0.1
SH	0.3	2.5	2.8	0.1
MJRL	5.5	26.2	31.7	0.2
PALK	5.3	22.4	27.7	0.2
JCR	3.1	19.8	22.9	0.2
PBS	3.5	19.6	23.1	0.2
SH	4.6	24.0	28.6	0.2
RNM	4.1	23.5	27.6	0.2
JC	5.3	25.5	30.8	0.2
AM	4.9	18.6	23.9	0.2
AMW	5.1	17.9	23.0	0.3
KL	7.4	23.9	31.3	0.3
SW	0.6	2.0	2.6	0.3
PM	8.1	24.8	32.9	0.3
RLF	5.2	19.7	24.9	0.3
ADH	9.8	23.0	32.8	0.4
MBS	8.7	21.5	30.2	0.4
LZ	10.7	30.4	41.1	0.4
SD	5.6	13.1	18.7	0.4
SGL	2.8	8.1	10.9	0.4
JH	8.0	18.0	26.0	0.4

*All tabulated figures refer to % dose excreted in urine (0-8h).

<u>SUBJECT</u>	<u>DEBRISOQUINE</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
DJB	9.7	25.5	34.2	0.4
RAH	9.2	24.1	33.3	0.4
RT	11.0	21.4	32.4	0.5
BM	0.8	1.5	2.3	0.5
MM	9.5	19.7	29.2	0.5
MSH	11.1	22.0	33.1	0.5
SAS	9.8	18.1	27.9	0.5
YHC	8.3	17.3	25.6	0.5
SPF	6.5	13.7	20.2	0.5
MDJ	13.1	22.7	35.8	0.6
PST	10.1	17.7	29.0	0.6
PJT	15.1	25.4	40.5	0.6
MT	11.1	19.3	30.4	0.6
HRG	6.1	10.8	16.9	0.6
DNHJ	10.8	16.4	27.2	0.7
PLH	13.5	20.7	34.2	0.7
MCFJ	13.3	18.3	31.6	0.7
CJHo	16.3	21.9	38.2	0.7
CJHe	11.3	16.0	25.3	0.7
JS	7.7	11.7	19.4	0.7
DJS	7.7	11.3	19.0	0.7
SPB	14.9	21.3	36.2	0.7
TACH	8.4	13.9	22.0	0.7
NV	15.0	22.3	37.3	0.7
VB	11.6	14.4	26.0	0.8
LFS	14.4	18.9	33.3	0.8
JR	15.2	19.3	34.5	0.8
MSP	12.8	13.6	26.0	0.9
AWW	4.3	4.9	9.2	0.9
MCW	20.7	21.2	41.9	1.0
CDY	18.9	18.8	37.7	1.0
ST	14.7	14.6	29.3	1.0
PN	17.3	16.6	33.9	1.0
RJG	11.1	10.8	21.9	1.0
JJL	15.9	14.0	29.9	1.1
AJ	25.1	22.1	47.2	1.1

<u>SUBJECT</u>	<u>DEBRISOQUINE</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
AN	15.9	14.0	29.9	1.1
MB	16.1	15.4	31.5	1.1
NO	13.8	12.4	26.2	1.1
RMP	15.1	14.2	29.3	1.1
PTB	16.8	14.9	31.7	1.1
MET	14.9	12.8	27.7	1.1
PGD	17.4	14.8	32.2	1.2
AB	21.9	17.1	38.0	1.2
DG	10.5	8.3	18.8	1.3
PRE	14.6	11.5	26.1	1.3
PKM	14.5	11.4	25.9	1.3
HCML	2.4	1.8	4.2	1.3
SD	11.9	9.5	21.3	1.3
RB	11.3	8.3	19.6	1.3
BB	22.6	16.8	39.4	1.3
CM	1.7	1.3	3.0	1.4
NC	17.0	12.0	29.0	1.4
TC	2.3	1.7	4.0	1.4
EACJ	12.6	8.6	21.2	1.5
JAB	12.9	8.7	21.6	1.5
JH	8.7	6.0	14.7	1.5
DE	5.7	3.7	9.4	1.5
MD	7.8	5.4	13.2	1.5
VF	4.9	3.1	8.0	1.6
EE	21.9	13.5	35.4	1.6
ST	9.7	6.2	15.9	1.6
SYC	4.7	3.2	7.9	1.6
LL	12.3	7.2	19.5	1.7
DMR	7.4	4.0	11.4	1.7
KC	19.2	12.1	31.3	1.7
LT	15.1	8.9	24.0	1.7
CRA	9.1	5.4	14.5	1.7
NG	2.0	1.1	3.1	1.8
BC	41.5	22.6	64.1	1.8
EC	9.6	5.2	14.8	1.8
SN	19.9	10.8	31.7	1.8

<u>SUBJECT</u>	<u>DEBRISOQUINE</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
WSE	23.1	12.8	35.9	1.8
RE	18.8	10.3	29.1	1.9
NW	8.3	4.5	12.8	1.9
KC	7.0	4.1	11.1	1.9
NB	32.6	16.5	49.1	2.0
ARM	11.9	5.7	17.6	2.0
ECr	15.3	8.1	23.4	2.0
PBN	16.7	7.9	24.6	2.1
SLD	10.0	4.5	14.5	2.2
CAB	17.8	7.3	25.1	2.4
CJ	28.4	11.7	40.1	2.5
CJM	1.8	0.7	2.5	2.6
RC	4.7	1.7	6.4	2.8
MG	14.3	4.6	18.9	3.1
PAS	16.5	5.1	21.6	3.2
OH	14.9	4.3	19.2	3.5
CAL	24.3	6.6	30.9	3.7
CG	4.5	1.1	5.6	4.1
VT	5.4	1.3	6.7	4.3
SML	5.2	1.1	6.3	4.6
JMW	36.2	1.6	37.8	22.6
LAE	20.6	0.9	21.5	23.2
EMH	19.4	0.8	20.2	24.3
JOG	19.9	0.8	20.7	24.5
ASCR	37.6	1.4	39.0	26.8
JEC	48.2	1.7	49.9	28.4
AMS	17.3	0.5	17.8	34.6
MPS	31.9	0.9	32.8	35.5
RG	32.4	0.5	32.9	64.8
SBJ	33.6	0.4	34.0	84.1
KBLR	8.3	0.1	8.4	84.8
BW	61.6	0.3	61.9	205.4

(ix) Metabolic ratios: Ghanaian subjects.

<u>SUBJECT</u>	<u>DEBRISOQUINE</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
44	2.3	10.1	12.4	0.2
73	5.1	18.8	23.9	0.3
31	4.5	13.7	18.2	0.3
47	5.9	16.2	22.1	0.4
51	11.1	27.4	38.5	0.4
10	6.0	11.5	17.5	0.5
19	3.4	6.4	9.8	0.5
59	8.4	17.9	26.3	0.5
6	7.4	11.9	19.3	0.6
28	11.1	17.3	28.4	0.6
60	7.1	11.2	18.3	0.6
42	7.0	10.6	17.6	0.7
78	12.1	16.8	28.9	0.7
79	1.6	2.1	3.7	0.7
27	5.5	7.6	13.1	0.7
8	7.8	10.0	17.8	0.8
57	6.2	7.4	13.6	0.8
69	8.0	10.5	18.5	0.8
80	8.7	9.3	18.0	0.9
41	7.9	9.3	17.2	0.9
9	10.3	11.3	21.6	0.9
74	14.8	14.7	29.5	1.0
40	6.8	6.0	12.8	1.1
75	7.5	6.6	14.1	1.1
64	6.2	5.5	11.7	1.1
58	9.6	9.1	18.7	1.1
5	50.4	37.9	88.3	1.3
7	3.7	2.8	6.5	1.3
11	11.5	8.8	20.3	1.3
13	20.4	15.2	35.6	1.3
15	4.0	3.0	7.0	1.3
18	11.3	8.6	19.9	1.3
21	9.7	7.4	17.1	1.3
22	7.9	6.0	13.9	1.3
66	14.0	10.8	24.8	1.3

<u>SUBJECT</u>	<u>DEBRISOQUINE</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
12	17.1	12.2	29.3	1.4
24	43.6	31.2	74.8	1.4
52	18.4	13.5	29.2	1.4
72	25.8	19.0	44.8	1.4
52	16.6	10.9	27.5	1.5
46	23.8	15.9	39.7	1.5
71	9.5	5.8	15.1	1.6
76	17.5	9.1	26.6	1.7
70	2.4	1.4	3.8	1.7
61	11.6	6.5	18.1	1.8
62	4.4	2.3	6.7	1.9
65	12.9	5.6	18.1	2.3
29	10.8	4.1	14.9	2.6
55	21.4	7.4	28.8	2.9
37	13.4	4.4	17.8	3.0
39	13.5	4.4	17.9	3.0
16	15.5	5.0	20.5	3.1
53	9.9	2.8	12.7	3.5
23	15.9	4.4	20.5	3.6
14	16.5	4.1	20.6	4.0
25	49.5	12.4	61.9	4.0
38	6.5	15.2	21.7	4.0
20	18.0	4.1	22.1	4.4
3	26.3	5.7	32.0	4.6
68	15.8	3.3	19.1	4.8
4	6.8	1.4	8.2	4.9
35	37.0	6.8	43.8	5.4
56	2.5	0.5	3.0	5.4
33	26.4	4.3	30.7	6.1
63	7.7	1.2	9.9	6.4
67	18.8	2.5	21.3	7.4
26	23.6	3.1	26.7	7.6
1	50.4	6.2	56.6	8.0
49	14.7	1.8	16.5	8.2
48	9.2	1.1	10.3	8.4
34	31.2	3.6	34.8	8.7
43	26.1	3.0	29.1	8.7

<u>SUBJECT</u>	<u>DEBRISOQUINE</u>	<u>4-HYDROXYDEBRISOQUINE</u>	<u>RECOVERY</u>	<u>METABOLIC RATIO</u>
36	37.0	4.2	41.2	8.8
54	27.1	2.8	29.9	9.9
17	24.8	2.4	27.2	10.3
50	9.3	0.5	9.8	19.8
77	22.8	1.1	23.9	21.7
45	10.9	0.4	11.3	25.6
2	22.1	0.8	22.9	27.6
30	56.9	1.8	58.7	31.6

APPENDIX B

(i) Excretion of 4-hydroxyphenytoin: Cumulative % dose.

CAUCASIAN EM SUBJECTS

t (h)	1		2		3		4	5
	(i)	(ii)	(i)	(ii)	(i)	(ii)		
12.0	18.2	24.6	19.7	17.6	8.7	13.3	7.7	3.3
24.0	36.9	42.9	35.8	34.4	36.2	36.6	22.8	13.5
36.0	51.6	54.0	54.2	49.2	50.0	49.2	34.4	29.1
48.0	61.1	59.3	62.3	60.2	58.1	54.8	42.2	40.1

CAUCASIAN PM SUBJECTS

t (h)	1		2	3	4	5	6
	(i)	(ii)					
12.0	14.9	7.8	8.8	3.5	9.8	8.0	14.5
24.0	31.9	25.7	20.5	26.6	11.4	15.9	28.5
36.0	41.0	42.9	28.5	36.4	19.2	26.3	34.8
48.0	44.2	50.8	33.8	42.0	25.0	35.7	40.9
60.0	n.s.*	n.s.	n.s.	n.s.	32.4	42.7	n.s.
72.0	n.s.	n.s.	n.s.	n.s.	35.9	45.5	n.s.
92.0	n.s.	n.s.	n.s.	n.s.	n.s.	51.4	n.s.

*n.s.: indicates subject not studied over given time period.

(ii) Excretion of 4-hydroxyphenytoin: Cumulative % dose.

GHANAIAN EM SUBJECTS

t (h)	1	2	3	4
8.0	3.0	1.3	1.2	2.5
16.0	6.0	4.5	3.5	5.6
24.0	12.2	7.0	6.6	9.5
32.0	14.3	12.0	9.3	13.1
40.0	16.3	14.8	12.7	16.3
48.0	17.0	18.6	14.3	19.0

GHANAIAN PM SUBJECTS

t (h)	1	2	3	4
8.0	1.6	1.1	1.0	2.5
16.0	4.5	3.3	3.1	5.2
24.0	7.0	5.3	5.2	9.2
32.0	8.3	7.5	7.3	10.4
40.0	10.5	9.1	9.6	14.2
48.0	12.7	10.2	11.9	17.4

(iii) Phenacetin metabolism; population study.

<u>SUBJECT</u>	<u>METABOLIC RATIO</u> (where known)	<u>PHENOTYPE</u>	<u>PARACETAMOL</u> (% dose 0-8h urine)	<u>2-HO-PRODUCTS</u>	<u>RUBRURIA</u> (Ab ₄₅₀)
1	0.8	EM	47.6	4.0	0.21
2			43.6	3.2	0.32
3			36.9	5.0	0.31
4			37.4	4.9	0.22
5	22.0	PM	31.4	11.4	0.52
6	0.9	EM	37.2	3.6	0.47
7			40.6	4.9	0.28
8	28.6	PM	29.4	13.2	0.62
9	35.0	PM	33.6	8.1	0.41
10	0.4	EM	43.4	12.3	0.46
11			36.5	0.7	0.17
12			45.6	3.7	0.23
13	1.8	EM	36.6	5.0	0.09
14			40.2	3.6	0.23
15	0.4	EM	43.0	8.4	0.45
16			36.1	1.4	0.31
17			31.6	3.3	0.20
18			35.9	1.7	0.20
19	1.0	EM	32.5	1.6	0.21
20	0.4	EM	14.8	1.4	0.18
21	1.2	EM	45.4	3.6	0.23
22			30.2	3.9	0.27
23			23.5	6.3	0.16
24	0.4	EM	35.8	1.5	0.31
25			31.9	2.7	0.13
26	0.5	EM	39.9	2.1	0.12
27			45.5	6.0	0.15
28	21.3	PM	29.8	2.2	0.64
29			48.4	5.9	0.31
30			30.2	3.6	0.42
31			37.7	3.1	0.13

<u>SUBJECT</u>	<u>METABOLIC RATIO</u>	<u>PHENOTYPE</u>	<u>PARACETAMOL</u>	<u>2-HO-PRODUCTS</u>	<u>RUBRURIA</u>
32	0.8	EM	35.8	1.7	0.13
33			29.2	3.2	0.08
34	0.6	EM	34.6	7.7	0.14
35(i)	21.0	PM	48.4	4.3	0.08
35(ii)	21.0	PM	50.2	7.1	0.14
36			44.1	2.4	0.05
37			46.0	6.4	0.08
38	0.8	EM	32.0	5.4	0.09
39			54.6	6.4	0.14
40			37.8	6.0	0.09
41			26.4	6.2	0.12
42			34.6	4.8	0.14
43			30.6	3.5	0.20
44			32.4	4.1	0.12
45	2.9	EM	32.4	2.7	0.15
46	0.9	EM	29.8	4.6	0.12
47	1.1	EM	32.5	6.3	0.13
48			31.5	2.3	0.17
49	0.7	EM	30.3	5.4	0.07
50			40.5	3.6	0.15

(iv) Phenacetin metabolism; panel study; EM subjects - paracetamol excretion (phenacetin equivalents, mg).

t (h)	1	2	3	4	5
1.0	16.8	15.2	8.4	41.7	27.9
2.0	64.5	37.8	44.6	98.1	50.9
3.0	80.1	72.8	88.9	91.7	80.5
4.0	120.5	154.5	111.4	104.1	84.2
5.0	94.7	121.8	101.6	103.3	} 175.5
6.0	84.1	101.4	101.2	75.1	
7.0	45.2	71.4	58.4	58.9	
8.0	34.8	32.8	33.9	45.6	41.1

EM subjects - 2-hydroxylation products (phenacetin equivalents, mg).

t (h)	1	2	3	4	5
1.0	10.9	3.0	16.0	6.4	18.0
2.0	11.0	11.0	14.1	5.6	9.5
3.0	9.8	2.1	15.8	5.6	3.6
4.0	2.1	2.0	9.0	4.7	3.6
5.0	2.0	—*	3.0	2.5	} 8.6
6.0	1.8	—	—	0.8	
7.0	—	—	—	—	
8.0	—	—	—	—	—

*- indicates metabolite not detected.

(v) Phenacetin metabolism; panel study; PM subjects - paracetamol excretion (phenacetin equivalents, mg).

t (h)	1	2	3	4	5	6	7
1.0	7.8	6.8	15.6	2.7	12.3	8.9	9.0
2.0	20.2	33.8	46.6	5.3	28.9	25.8	45.4
3.0	31.5	63.8	51.8	57.2	73.7	64.2	33.7
4.0	75.7	61.0	66.9	67.0	61.4	47.2	67.7
5.0	68.1	43.4	45.6	74.5	60.9	71.0	77.6
6.0	52.5	33.6	44.4	25.1	39.6	46.7	40.7
7.0	44.7	35.7	32.6	69.7	73.2	78.6	31.3
8.0	42.6	28.3	36.2	67.1		44.0	31.3

PM subjects - 2-hydroxylation products (phenacetin equivalents, mg).

t (h)	1	2	3	4	5	6	7
1.0	22.1	5.0	10.1	19.2	10.9	31.6	7.1
2.0	25.0	12.2	12.3	12.0	27.3	26.0	10.3
3.0	24.8	11.3	10.2	15.1	26.5	36.5	10.0
4.0	17.0	7.0	9.8	16.2	23.4	25.3	8.1
5.0	12.1	6.1	6.1	14.0	12.5	14.0	7.0
6.0	9.8	4.9	8.1	2.1	7.8	7.0	5.1
7.0	10.0	6.0	4.3	7.2	8.6	3.0	4.9
8.0	6.0	3.1	4.0	1.9		1.8	4.0

PM subjects - Rubruria Ab₄₅₀; measured 0-4h only.

t (h)	1	2	3	4	5	6	7
1.0	0.45	0.18	0.07	0.38	0.10	0.54	0.25
2.0	0.55	0.29	0.19	0.11	0.23	0.38	0.27
3.0	0.48	0.21	0.12	0.21	0.20	0.62	0.25
4.0	0.19	0.17	0.10	0.35	0.12	0.27	0.16

PUBLICATIONS

The following have been published in connection with the work described in this thesis:

1. Smith RL, Idle JR, Mahgoub A, Sloan TP, Lancaster R.
Genetically determined defects of oxidation at carbon centres of drugs.
Lancet i: 943 (1978).
2. Sloan TP, Mahgoub A, Lancaster R, Idle JR, Smith RL.
Polymorphism of Carbon Oxidation of drugs and clinical implications.
Brit Med J 2: 655 (1978).
3. Sloan TP, Idle JR, Lancaster R, Smith RL.
Alicyclic hydroxylation phenotype status and the elimination kinetics of debrisoquine.
Proc 7th Int Congr Pharmacol: 2866 (1978).
4. Idle JR, Sloan TP, Smith RL, Wakile LA.
Application of the phenotyped panel approach to the detection of polymorphism of drug oxidation in man.
Brit J Pharmacol 66: 430P (1979).
5. Wakile LA, Sloan TP, Idle JR, Smith RL.
Genetic evidence for the involvement of different oxidative mechanisms in drug oxidation.
J Pharm Pharmacol 31: 350 (1979).
6. Woolhouse NM, Andoh B, Mahgoub A, Sloan TP, Idle JR, Smith RL.
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Clin Pharmacol Therap 26: 584 (1979).
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J Med Genet 17: 102 (1980).

9. Andoh B, Idle JR, Sloan TP, Smith RL, Woolhouse NM.

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The influences of changes in dietary fat on the clearance of antipyrine and the 4-hydroxylation of debrisoquine.

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In press:

- Danhof M, Breimer DD, Idle JR, Sloan TP, Smith RL.

Antipyrine metabolite formation in extensive and poor metabolisers of debrisoquine.

Proc 1st World Confr Clin Pharmacol: (1980).

- Hetzel MR, Law M, Keal EE, Sloan TP, Idle JR, Smith RL.

Is there a genetic component in Bronchial Carcinoma in smokers?

Thorax: (1980).

ADDENDUM

REFERENCE COMPOUNDS:

DEBRISOQUINE SULPHATE: (3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate; Declinax[®]) (m.p. 274-276°C) and its phenolic derivatives (5-; m.p. 298-300°C, 6-; m.p. not determined, 7-; m.p. 303°C, 8-; 302-304°C) were the gift of Roche Products Ltd., U.K.

4-HYDROXYDEBRISOQUINE: The synthesis of the material employed in these studies is described by Idle *et al.*, (1978a). Its m.p. (253-254°C), infra red spectrum (strong absorptions at 3.0 (O-H), 3.1 (N-H), 6.0 (C=N), 6.3 (C=N), 9.2 (O-H) μ m.) and proton n.m.r. spectrum were identical to those of an authentic sample provided by Roche Products Ltd., U.K.

PHENYTOIN: (5-, 5-diphenylhydantoin) (m.p. 294-296°C) was obtained from the Sigma Chemical Co., St. Louis. 5-(4'-Methylphenyl)-5-phenylhydantoin (m.p. 225-226°C) and 5-(4'-hydroxyphenyl)-5-phenylhydantoin (m.p. 320°C dec.) were obtained from the Aldrich Chemical Co. Inc., Milwaukee. 5-(3'-Hydroxyphenyl)-5-phenylhydantoin (m.p. 215-217°C) was the gift of Dr. A.J. Glazko.

PHENACETIN: (4-Acetophenetidine) (m.p. 133-135°C) and paracetamol (m.p. 171°C) were obtained from the Sigma Chemical Co., St. Louis. Acetanilide (m.p. 113-115°C) was obtained from the Aldrich Chemical Co. Inc., Milwaukee.

2-Hydroxyphenacetin (m.p. 146-148°C) was the gift of Dr. R.M. Welch, Wellcome Research Laboratories, Research Triangle Park, N.C., U.S.A.

4-[³H(G)]-Hydroxyacetanilide ([³H]-paracetamol) specific activity: 3.4 Ci mmol⁻¹, was obtained from New England Nuclear, Boston, Mass.

Polymorphism of carbon oxidation of drugs and clinical implications

T P SLOAN, A MAHGOUB, R LANCASTER,
J R IDLE, R L SMITH

British Medical Journal, 1978, 2, 655-657

Summary and conclusions

Eight volunteers previously phenotyped for their ability to hydroxylate debrisoquine (four extensive metabolisers (EM), four poor metabolisers (PM)) were investigated for their metabolic handling of guanoxan and phenacetin. All three drugs are oxidised at carbon centres. Oxidative dealkylation of phenacetin was determined by measuring the rate of formation of paracetamol.

The EM subjects excreted mostly metabolites of guanoxan (mean 29% of dose), whereas the PM group excreted large amounts of unchanged drug (48% of dose). The rate of formation of paracetamol was noticeably slower in the PM group, and, when analysed by minimum estimates of apparent first-order rate constants, the difference between the two phenotypes was significant. Thus the hydroxylation defect shown for debrisoquine metabolism carries over to the oxidative metabolism of phenacetin and guanoxan.

Some 5% of the population are genetically defective hydroxylators of drugs. Thus methods for evaluating the metabolism of new drugs in respect of usage and side effects need to be revised.

Department of Biochemical and Experimental Pharmacology,
St Mary's Hospital Medical School, London W2 1PB

T P SLOAN, BSC, postgraduate student

A MAHGOUB, MB, CHB, postgraduate student (now lecturer in clinical pharmacology, University of Alexandria)

R LANCASTER, MRCP, PHD, consultant and honorary senior lecturer in clinical pharmacology

J R IDLE, BSC, PHD, lecturer in biochemical pharmacology

R L SMITH, PHD, DSC, professor of biochemical pharmacology

Introduction

Drug metabolism is a major determinant of the therapeutic and toxic responses to many drugs. Metabolism of a drug, however, may be highly variable, and this is often the source of major interpatient variations in response to drugs. A person's metabolic capacity depends on the interaction of various genetic, environmental, physiological, and possibly pathological factors. Some drug metabolism reactions are mainly under genetic control and exhibit polymorphism in the population. Examples are the polymorphic acetylation of various amino compounds such as sulphonamides and hydrazines. The therapeutic implications of polymorphic acetylation of drugs have been discussed.^{1,2} The hydrolysis of certain ester drugs such as succinylcholine (suxamethonium) and paroxon is also mainly under genetic control and exhibits polymorphism.^{3,4}

The most common metabolic reaction in the metabolism of drugs is oxidation at a carbon centre: this may be aliphatic, alicyclic, or aromatic. Despite the prevalence of carbon oxidation in drug metabolism no examples of polymorphism of such reactions have been described, though several have a genetic component, which in some cases has been quantified.^{5,6} We have reported the occurrence in Caucasians of a polymorphism in respect of the carbon oxidation of the antihypertensive drug debrisoquine.⁷ Some people (about 5% of the population) have a reduced ability to effect the alicyclic hydroxylation of this drug and, furthermore, are more sensitive to its hypotensive effects.⁸ Apparently this 4-hydroxylation of debrisoquine is under the control of the alleles at a single autosomal locus and defective alicyclic hydroxylation is a recessive character.⁷

Because of the importance of carbon oxidation as a determinant of drug response it became necessary to know whether the alleles responsible for the polymorphic metabolism of debrisoquine also control the oxidation of other drugs. We therefore examined the oxidative metabolism of two model substrates—namely, phenacetin and guanoxan—in subjects previously phenotyped for their ability to hydroxylate debrisoquine. Phenacetin, guanoxan, and debrisoquine each undergo three different types of carbon oxidation—namely, oxidative dealkylation, aromatic hydroxylation, and alicyclic hydroxylation.

Investigation and results

GUANOXAN

Guanoxan (2-guanidinomethyl-1, 4-benzodioxan) is an antihypertensive drug chemically and pharmacologically related to debrisoquine and guanethidine. Jack *et al* showed that it undergoes oxidative metabolism to give phenolic metabolites.⁹ Their study included a woman who excreted only the unchanged drug with no evidence of phenolic metabolite formation. Apparently she was not further investigated.

We studied the metabolism and elimination of guanoxan in a panel of eight volunteers, four of whom were phenotyped as extensive metabolisers (EM) and four as poor metabolisers (PM) in respect of debrisoquine 4-hydroxylation. Each received a single tablet of Envacar containing 10 mg guanoxan sulphate. Urine samples were collected hourly for up to eight hours after administration. These samples were then analysed for guanoxan and its phenolic metabolites by gas chromatography using a modification of an assay for guanidine drugs.¹⁰

Table I shows the pattern of urinary elimination of guanoxan by the eight volunteers. The metabolic ratio was calculated as: % dose

TABLE 1—*Hydroxylation of guanoxan and phenotypes of eight volunteers*

Subject	Sex	Hydroxylation phenotype*	Characteristic and % of dose excreted in urine in 8 hours		Metabolic ratio
			Unchanged	Hydroxylated metabolites†	
1	M	PM	31.0	4.2	7.4
2	M	PM	48.0	6.2	7.7
3	M	PM	60.0	7.6	7.9
4	F	PM	53.0	6.7	7.9
5	M	EM	1.3	36.0	0.04
6	M	EM	1.9	25.0	0.08
7	M	EM	1.2	26.0	0.05
8	M	EM	1.5	29.0	0.05

*Determined by metabolic ratio for 4-hydroxylation of debrisoquine. † PM = Poor metabolisers. EM = Extensive metabolisers.

†Consisted of about equal amounts of 6- and 7-hydroxyguanoxan.

excreted as unchanged guanoxan/% dose excreted as hydroxylated metabolites. There was a pronounced difference between the two phenotypes in the metabolic disposition of guanoxan. The four EM subjects excreted the drug mostly as 6- and 7-hydroxyguanoxan (mean $29.0 \pm \text{SD } 5.0\%$ of dose) with only small amounts of the unchanged drug ($1.5 \pm 3.0\%$). In contrast, the PM group excreted large amounts of the unchanged drug ($48.0 \pm 12.4\%$) with only small amounts of the metabolites ($6.2 \pm 1.4\%$). The mean metabolic ratio for the EM phenotype was 0.06 ± 0.02 , and for the PM phenotype 7.8 ± 0.2 .

The pharmacogenetics of hydroxylation of guanoxan and debrisoquine in these subjects appeared to be the same. The results also show that the aromatic hydroxylation of guanoxan is polymorphic and that there are at least two phenotypes, one of which is characterised by a reduced ability to metabolise the drug.

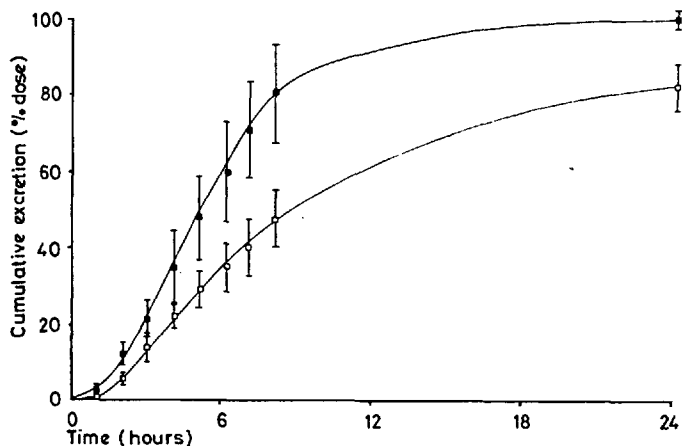
PHENACETIN

Phenacetin is metabolised along several pathways, the main one being oxidative de-ethylation to paracetamol, which is excreted in a conjugated form.¹¹ The rate of oxidative dealkylation of phenacetin taken by mouth was investigated in the same panel of volunteers by measuring the rate of formation of paracetamol. This was assessed by determining the rate of elimination of paracetamol conjugates by colorimetry.

Each subject took a gelatine capsule containing 500 mg phenacetin.

Urine samples were collected hourly for eight hours, an 8-24-hour collection then being made and the samples bulked. The samples were then analysed for paracetamol by colorimetry after enzymic deconjugation.¹²

The figure shows the rate of formation and elimination of paracetamol in the EM and PM subjects. The rate of formation in the PM phenotype group was noticeably slower than in the EM group.



Cumulative urinary excretion of paracetamol in extensive metabolisers (■) and poor metabolisers (□) after single oral dose of 500 mg phenacetin.

To qualify this difference minimum estimates of the apparent first-order rate constants for the formation of paracetamol (K_f paracetamol) were calculated from the urinary data (table II).^{13, 14} The mean value of the rate constant for the PM phenotype was $0.09 \pm \text{SD } 0.024$, and for the EM phenotype 0.277 ± 0.064 . This difference was significant ($P < 0.005$). Thus the rate of oxidative de-ethylation of phenacetin in the EM subjects was about three times faster than in the PM subjects.

Discussion

We have shown that the genetically determined hydroxylation polymorphism described for debrisoquine also controls the oxidative metabolism of guanoxan and phenacetin. Thus the same genetic defect appears to influence metabolic oxidative processes at such dissimilar carbon centres as the aromatic 6- and 7-carbon centres of guanoxan, the alicyclic 4-carbon centre of debrisoquine, and the aliphatic α -carbon centre of phenacetin. Hence the defect apparently influences some component common to the processes concerned in the oxidation of

several dissimilar centres. Whether the defect influences oxidation at non-carbon functions in drugs, such as nitrogen and

TABLE II—*De-ethylation of phenacetin and hydroxylation phenotypes of eight volunteers*

Subject:	1	2	3	4	5	6	7	8
Hydroxylation phenotype ..	PM	PM	PM	PM	EM	EM	EM	EM
K _f paracetamol/hour*	0.07	0.09	0.13	0.09	0.36	0.25	0.21	0.30

*First-order rate constant for formation of paracetamol—see text.
PM = Poor metabolisers. EM = Extensive metabolisers.

sulphur centres, is unknown and is under investigation, but it certainly seems plausible.

The main implication of our findings is that the metabolism of a whole range of drugs that are metabolised by oxidation at carbon centres may be defective in some people. The question therefore arises of the possible consequences of this. The first point to consider is the size of the group. Our studies with debrisoquine metabolism suggest that among Caucasians the prevalence of the defect is about 5%. Thus a town with a population of about 200 000 would be expected to include about 10 000 people defective in their capacity to metabolise drugs. Furthermore, a GP with a list of, say, 3000 might expect to have around 150 defective hydroxylators. Clearly the prevalence of the defect is too high to be ignored. The second question is whether the defect alters people's responsiveness to drugs. Little information on this is available at present, but existing data suggest that it does. We, for example, showed that people with an oxidation defect for debrisoquine are much more sensitive to the hypotensive effects of this drug than are extensive metabolisers.⁸ Some of the poor metabolisers reported severe side effects when given therapeutic doses of the drug. Shahidi studied the pedigree of two sisters with a faulty de-ethylation of phenacetin similar to that described by us and found that they were exceptionally sensitive to the methaemoglobin-producing effects of the drug.¹⁵ In our view many of the side effects reported for drugs will be found to have their origins in a genetically determined impaired ability to oxidise drugs.

The third point to consider is the practical implications of these findings in the development of new drugs and the use of drugs. So far as metabolic studies on man are concerned contemporary practice is singularly ineffective in detecting inter-individual variations in drug metabolism. This is because the number of subjects studied is usually small (three to six) and therefore rarely permits the detection of pronounced intersubject variations. Also, current practice in using pharmacokinetic analyses of drug disposition may disguise important aspects of intersubject variation in handling drugs. This is because of the common use of hybrid pharmacokinetic variables—for example,

plasma elimination $t_{1/2}$, whole-body clearance rates, etc—without defining their major components—for example, metabolism—which may show pronounced interindividual variation without this necessarily being clearly evident when subjects are evaluated in terms of hybrid variables. This is exemplified in the case of debrisoquine itself. The first-order rate constant for the overall elimination of debrisoquine (K) did not differ significantly between the extensive and poor metabolising phenotypes—EM ($n=4$): $K=0.20 \pm 0.04/h$; PM ($n=4$): $K=0.16 \pm 0.13/h$ —whereas the respective rates of formation of the pharmacologically inactive 4-hydroxy metabolite (K_F) did differ significantly—EM: $K_F=0.05 \pm 0.02/h$; PM: $K_F=0.0015 \pm 0.0007/h$; $P < 0.025$.

In our view the present practice for the metabolic evaluation of new drugs needs to be revised. It is important to define the metabolic variability that may be encountered for a particular drug and to evaluate its implications in terms of drug use and possible side effects. The problems of such an approach are not insuperable. We advocate that information of this sort would be best obtained by studying drug disposition and effects in panels of subjects previously phenotyped for oxidation status. Techniques for ascertaining this have been described.⁷

TPS is in receipt of an MRC studentship. AM is grateful to the EAR Educational Bureau for a scholarship. We thank the Wellcome Trust for a grant for interdisciplinary research, and Pfizer Ltd for the samples of guanoxan and metabolites.

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(Accepted 5 July 1978)

DEBRISOQUIN HYDROXYLATION
POLYMORPHISM AMONG
GHANAIAANS AND CAUCASIANS

N. M. WOOLHOUSE, PH.D.,
B. ANDOH, DR.MED.,
A. MAHGOUB, PH.D.,
T. P. SLOAN, B.Sc.,
J. R. IDLE, PH.D.

and

R. L. SMITH, D.Sc.

Accra, Ghana, and London, England

Department of Biochemistry, University of Ghana Medical
School, and Department of Biochemical and Experimental
Pharmacology, St. Mary's Hospital
Medical School, Paddington

Reprinted from

CLINICAL PHARMACOLOGY
AND THERAPEUTICS
St. Louis

Vol. 26, No. 5, pp. 584-591, November, 1979

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(Printed in the U. S. A.)

Debrisoquin hydroxylation polymorphism among Ghanaians and Caucasians

The alicyclic and aromatic hydroxylation of debrisoquin was studied in Ghanaians. As in a previously studied Caucasian population, the alicyclic 4-hydroxylation of debrisoquin in Ghanaians was polymorphic. Three phenotypes were observed: homozygous extensive metabolizers (58%), heterozygous extensive metabolizers (36%), and homozygous poor metabolizers (6%). In contrast, British Caucasians are primarily monomorphic extensive metabolizers (92%) and homozygous poor metabolizers comprise 8% of the population. Urinary recovery of the drug and its hydroxylated metabolites was significantly less in the Ghanaian subjects. In both Ghanaian and British populations, aromatic hydroxylation producing 5-, 6-, 7-, and 8-hydroxydebrisoquin was shown to parallel the alicyclic 4-hydroxylation of debrisoquin, and thus to be controlled by the same gene locus. Debrisoquin is advocated as a tool for uncovering polymorphism in drug oxidation and its interethnic variations.

N. M. Woolhouse, Ph.D., B. Andoh, Dr.Med., A. Mahgoub, Ph.D., T. P. Sloan, B.Sc., J. R. Idle, Ph.D., and R. L. Smith, D.Sc. *Accra, Ghana, and London, England*
Department of Biochemistry, University of Ghana Medical School, and Department of Biochemical and Experimental Pharmacology, St. Mary's Hospital Medical School, Paddington

Debrisoquin (Declinax) is 3,4-dihydro-2 (1*H*)-isoquinolinecarboxamidine sulfate (Fig. 1) and was first introduced into the United Kingdom in 1966 as an orally active adrenergic blocking agent for therapy in hypertension. Its prescription has however been complicated by a large interpatient variation (40-fold) in optimal dose requirement.^{1, 12} We have shown that variation in response to debrisoquin is largely due to genetically determined variations in metabolism.⁴ Debrisoquin is metabolized and inactivated in man mainly by alicyclic 4-hydroxylation, with a small proportion oxidized in the

aromatic ring to yield 4 phenolic metabolites, namely, 5-, 6-, 7-, and 8-hydroxydebrisoquin.³ The extent to which the drug is hydroxylated in the 4-position in Caucasian subjects is controlled by a single autosomal gene locus and demonstrates polymorphism.⁸ There are 2 apparent phenotypes within the population: The first small recessive phenotype (8% of Caucasians) is characterized by an almost total inability to 4-hydroxylate the drug and is designated the poor metabolizer (PM) phenotype. The remaining 92% comprise a separate and dominant extensive metabolizer (EM) phenotype in which there is 10% to 90% 4-hydroxylation. It is this polymorphism that largely determines the response to debrisoquin, PM subjects being more responsive than EM subjects to the hypotensive effect of a single, oral 20-mg dose.⁴

One of the main features of polymorphism in general is that measurable interethnic differ-

Supported by a grant for interdisciplinary research from the Wellcome Trust.

Received for publication March 10, 1979.

Accepted for publication May 18, 1979.

Reprint requests to: J. R. Idle, Ph.D., Department of Biochemistry and Experimental Pharmacology, St. Mary's Hospital Medical School, Paddington, London W2 1PG, United Kingdom.

ences in gene frequency are shown. This is no less the case with polymorphism of drug metabolism—for example, with the polymorphic acetylation of isoniazid and certain sulfonamides in which there is marked global heterogeneity.¹⁰ Despite the importance of metabolic oxidation to processes of drug disposition, responsiveness, and toxicity, little is known of the interethnic distribution of drug oxidation capacity. In this article we describe polymorphic hydroxylation of debrisoquin in Ghanaian subjects and compare the findings with our data for Caucasians⁸ and Egyptians.⁹

Plan of investigation

Our subjects were 80 apparently healthy Ghanaians, male and female, who were members of staff and students of the University of Ghana Medical School and Hospital, Accra, Ghana. After an overnight fast and having emptied their bladders, each subject was given a single tablet containing 12.8 mg debrisoquin sulfate (equivalent to 10 mg free base). Urine was collected up to 8 hr postdosing. Samples were then frozen and transported to London for analysis of debrisoquin and its hydroxylated metabolites by electron-capture gas chromatography.³ From these data the metabolic ratio⁸ (MR) in the 0- to 8-hr urine was calculated as follows:

$$\frac{\% \text{ Dose excreted as unchanged debrisoquin}}{\% \text{ Dose excreted as 4-hydroxydebrisoquin}}$$

A similar ratio was calculated for the total phenolic metabolites, 5-, 6-, 7-, and 8-hydroxydebrisoquin, for 17 Ghanaian subjects and 17 Caucasian subjects who had taken part in an earlier study.⁸ Phenotype assignment was made on the basis of the criteria proposed by Price Evans et al.,¹¹ namely, PM MR > 12.6 and EM MR < 12.6.

Results

Debrisoquin alicyclic 4-hydroxylation.

Table I lists details of the debrisoquin study of 80 Ghanaian subjects. Mean 0- to 8-hr urinary recovery of debrisoquin and 4-hydroxydebrisoquin was 24.0% compared with 40.6% in a Caucasian population.⁸ These data were not normally distributed and thus the 2 means could

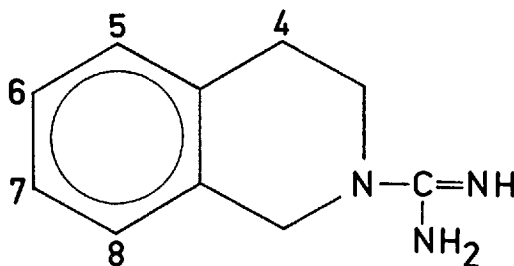


Fig. 1. Structure of debrisoquin.

not be compared by parametric tests such as Student's *t* test, but these 2 population samples were compared using chi-square analysis of the number of Ghanaians whose urinary recovery fell below (72 of 80) or above (8 of 80) the mean Caucasian recovery (40.6%). Such analysis clearly showed that urinary recovery of drug with metabolite was low more frequently in the Ghanaian subjects ($\chi^2 = 51.2$, $p < 0.001$).

The MR was calculated for each subject from the percentage excretion of drug and metabolite. The frequency distribution histogram of these data, plotted as log MR against MR frequency, is shown in Fig. 2 along with a similar histogram for 229 British Caucasian subjects for comparison. As in Caucasians,⁸ the 4-hydroxylation of debrisoquin in Ghanaians was polymorphic, indicating that the previously described single gene defect of debrisoquin metabolism is observable in the Ghanaian population. From our studies of both Caucasian⁸ and Egyptian⁹ families, it was shown that phenotype PMs have MR values exceeding 18 ($\log \text{MR} > 1.25$); 5 such individuals were identified, a PM phenotype frequency of 6.3%. Frequencies of the EM E allele (*p*), the PM (or nonmetabolism) N allele (*q*), the dominant homozygous extensive EE genotype (*p*²), the heterozygous EN genotype (2*pq*), and the recessive homozygous poor NN genotype (*q*²) have thus been derived from the Hardy-Weinberg expression $p^2 + 2pq + q^2 = 1$, and are listed in Table II. Between Ghanaian and Caucasian study groups neither allele nor genotype frequency was different as judged by Student's *t* test. However, the distribution of MR in Ghanaian subjects

Table I. Age, sex, and metabolic data for 80 healthy Ghanaian subjects

Subject no.	Sex	Age (yr)	% Dose eliminated in 0-8 hr			Metabolic ratio
			Debrisoquin	4-Hydroxydebrisoquin	Recovery	
1	M	21	50.4	6.2	56.6	8.0
2	M	22	18.8	1.7	20.5	11.1
3	M	25	26.3	5.7	32.0	4.6
4	M	22	6.8	1.4	8.2	4.9
5	F	22	50.4	37.9	88.3	1.3
6	M	22	7.4	11.9	19.3	0.6
7	M	23	3.7	2.8	6.5	1.3
8	M	24	7.8	10.0	17.8	0.8
9	F	22	10.3	11.3	21.6	0.9
10	M	21	6.0	11.5	17.5	0.5
11	M	23	11.5	8.8	20.3	1.3
12	M	22	17.1	12.2	29.3	1.4
13	F	21	20.4	15.2	35.6	1.3
14	M	24	16.5	4.1	20.6	4.0
15	M	23	4.0	3.0	7.0	1.3
16	M	22	15.5	5.0	20.5	3.1
17	M	23	24.8	2.4	27.2	10.3
18	M	22	11.3	8.6	19.9	1.3
19	M	23	3.4	6.4	9.8	0.5
20	M	22	18.0	4.1	22.1	4.4
21	M	20	9.7	7.4	17.1	1.3
22	M	21	7.9	6.0	13.9	1.3
23	M	21	15.9	4.4	20.3	3.6
24	M	24	43.6	31.2	74.8	1.4
25	M	22	49.5	12.4	61.9	4.0
26	M	24	23.6	3.1	26.7	7.6
27	M	22	5.5	7.6	13.1	0.7
28	M	22	11.1	17.3	28.4	0.6
29	M	23	10.8	4.1	14.9	2.6
30	M	23	56.9	1.8	58.7	31.6
31	M	52	4.5	13.7	18.2	0.3
32	M	34	16.6	10.9	27.5	1.5
33	M	23	26.4	4.3	30.7	6.1
34	F	20	31.2	3.6	34.8	8.7
35	M	24	37.0	6.8	43.8	5.4
36	M	21	37.0	4.2	41.2	8.8
37	M	22	13.4	4.4	17.8	3.0
38	M	22	6.5	15.2	21.7	4.0
39	M	24	13.4	4.4	17.8	3.0

showed a novel characteristic. EM ratios in Caucasian (Fig. 2) and Egyptian⁹ subjects are apparently normally distributed. Inspection of the Ghanaian EM group (93.7% of the population) reveals intraphenotype polymorphism. Analysis of these data by probit transformation strongly supported this observation. The systematic deviation from linearity shown in Fig. 3 is characteristic of a trimodal distribution of Ghanaian EM MRs which is heterogeneous ($\chi^2 = 110$, $p < 0.001$). Thus, the alicyclic

4-hydroxylation of debrisoquin in Ghanaians is polymorphic with 3 distinguishable modes.

Debrisoquin aromatic hydroxylation. Four phenolic metabolites of debrisoquin, occurring in small but variable amounts, have been described.³ It has been questioned³ whether or not aromatic hydroxylation of debrisoquin to yield these metabolites is under the control of the same gene locus as governs the alicyclic 4-hydroxylation process. We measured 5-, 6-, 7-, and 8-hydroxydebrisoquin in 17 members of the

Table I.—Cont'd.

Subject no.	Sex	Age (yr)	% Dose eliminated in 0-8 hr			Metabolic ratio
			Debrisoquin	4-Hydroxydebrisoquin	Recovery	
40	M	20	6.8	6.0	12.8	1.1
41	F	21	7.9	9.3	17.2	0.9
42	M	21	7.0	10.6	17.6	0.7
43	M	23	26.1	3.0	29.1	8.7
44	M	20	2.3	10.1	12.4	0.2
45	M	23	10.9	0.4	11.3	25.6
46	M	23	23.8	15.9	39.7	1.5
47	M	22	5.9	16.2	22.1	0.4
48	M	65	9.2	1.1	10.3	8.4
49	M	23	14.7	1.8	16.5	8.2
50	M	35	9.3	0.5	9.8	19.8
51	M	44	11.1	27.4	38.5	0.4
52	M	40	18.4	13.5	29.2	1.4
53	M	28	9.9	2.8	12.7	3.5
54	M	40	27.1	2.8	29.9	9.9
55	M	27	21.4	7.4	28.8	2.9
56	M	37	2.5	0.5	3.0	5.4
57	M	32	6.2	7.4	13.6	0.8
58	M	21	9.6	9.1	18.7	1.1
59	M	21	8.4	17.9	26.3	0.5
60	M	24	7.1	11.2	18.3	0.6
61	M	22	11.6	6.5	18.1	1.8
62	M	18	4.4	2.3	6.7	1.9
63	M	20	7.7	1.2	8.9	6.4
64	M	22	6.2	5.5	11.7	1.1
65	M	22	12.9	5.6	18.1	2.3
66	M	23	14.0	10.8	24.8	1.3
67	M	22	18.8	2.5	21.3	7.4
68	M	22	15.8	3.3	19.1	4.8
69	F	20	8.0	10.5	18.5	0.8
70	M	30	2.4	1.4	3.8	1.7
71	M	39	9.3	5.8	15.1	1.6
72	M	22	25.8	19.0	44.8	1.4
73	M	22	5.1	18.8	23.9	0.3
74	M	22	14.8	14.7	29.5	1.0
75	M	22	7.5	6.6	14.1	1.1
76	M	23	17.5	9.1	26.6	1.7
77	M	24	22.8	1.1	23.9	21.7
78	M	24	12.1	16.8	28.9	0.7
79	M	40	1.6	2.1	3.7	0.7
80	M	46	8.7	9.3	18.0	0.9

Table II. Gene and phenotype frequencies for the polymorphism in alicyclic 4-hydroxylation of debrisoquin in Ghanaians and British Caucasians

	Frequency				
	<i>p</i> (calc.)*	<i>q</i> (calc.)	<i>p</i> ² (calc.)	2 <i>pq</i> (calc.)	<i>q</i> ² (observed)
Ghanaian	0.749	0.251	0.561	0.376	0.063
Caucasian	0.719	0.281	0.517	0.404	0.079

*Calculated.

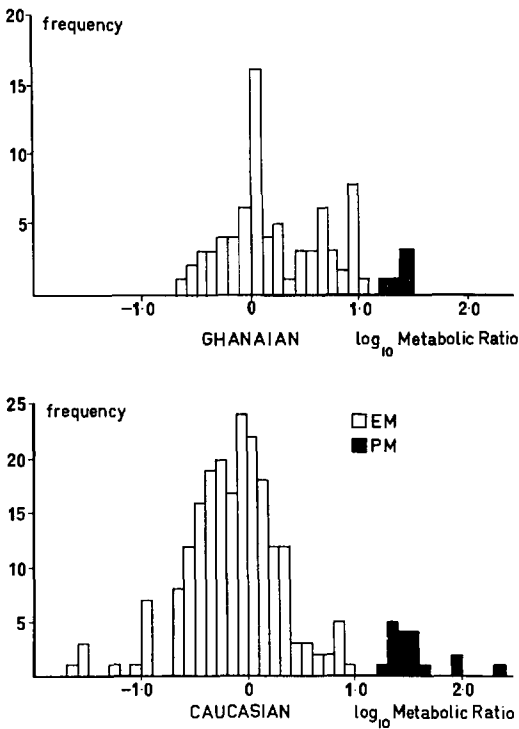


Fig. 2. Semilog frequency distribution histograms of debrisoquin 4-hydroxylation (metabolic ratio) in Ghanaians and Caucasians.

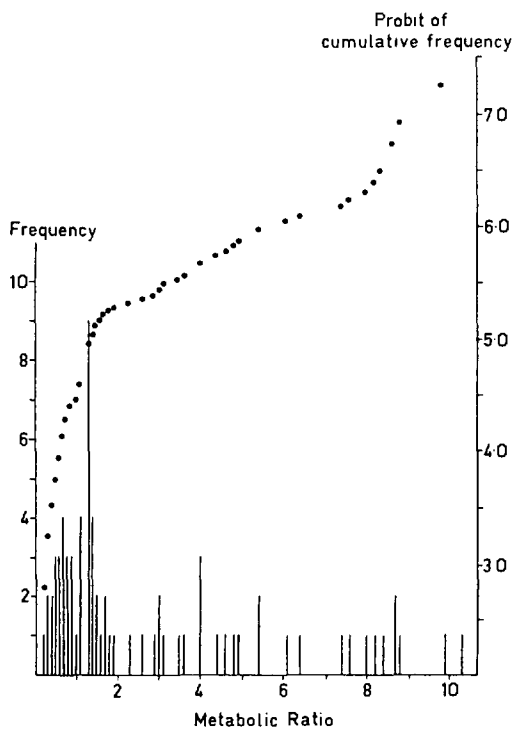


Fig. 3. Probit analysis of the frequency distribution histograms for Ghanaian EMs.

Table III. Rank correlation between debrisoquin alicyclic 4-hydroxylation and debrisoquin aromatic hydroxylation in representative EMs and PMs from Ghanaian and Caucasian populations

Ghanian subjects				Caucasian subjects			
4-Hydroxydebrisoquin		Phenols		4-Hydroxydebrisoquin		Phenols	
Metabolic ratio	Rank No.	Metabolic ratio	Rank No.	Metabolic ratio	Rank No.	Metabolic ratio	Rank No.
EM	0.2	1	1.7	1	EM	0.3	6
	0.3	2	4.2	2		0.4	1
	0.4	3.5	6.1	3		0.6	4
	0.4	3.5	10.4	5		0.6	5
	0.5	5	12.1	6		0.7	3
	0.7	6	6.8	4		0.8	2
	1.1	7	36.0	7		1.7	7
	1.3	8	46.6	8		2.5	11
	7.6	9	57.6	10		3.8	12
	8.0	10	420.0	17	PM	19.1	8
PM	8.8	11	52.1	9		19.3	9
	9.9	12	123.0	13		20.9	13
	10.3	13	118.0	12		21.1	17
	19.8	14	232.0	15		21.6	15
	25.6	15	61.0	11		25.2	10
	26.2	16	247.0	16		26.1	14
	31.6	17	158.0	14		27.1	16

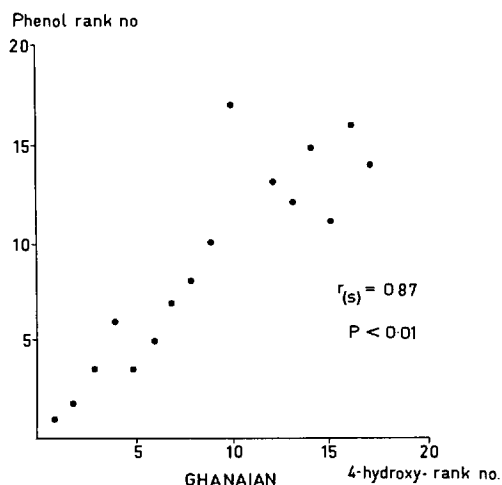


Fig. 4. The rank correlation between debrisoquin alicyclic 4-hydroxylation and debrisoquin aromatic hydroxylation metabolic ratios for Ghanaians.

Ghanaian study group. The phenol metabolic ratio, defined as dose excreted as unchanged debrisoquin/% dose excreted as total phenols, was calculated for these subjects and is listed in Table III together with comparative data for 17 Caucasian subjects. The 4-hydroxydebrisoquin metabolic ratio and the phenol metabolic ratio were correlated (Spearman rank correlation⁷) since the samples contain polymorphically distributed data and cannot therefore be analyzed by a parametric least squares method. Plots of phenol rank number for both Ghanaians (Fig. 4) and Caucasians (Fig. 5) demonstrate correlations between the alicyclic and aromatic hydroxylation processes in both populations (Spearman rank correlation coefficients, $r_s = 0.87$ for Ghanaian and 0.82 for Caucasian). Thus, decreased ability to perform the aromatic hydroxylation of debrisoquin is linked to decreased ability to carry out the alicyclic hydroxylation of the drug.

Discussion

The alicyclic 4-hydroxylation of debrisoquin in Ghanaians was found to be polymorphic (see Fig. 2). Probit analysis of the frequency distribution histogram revealed that the polymorphism in the Ghanaian population is trimodal. Both the first mode (MR 0-2) and the second mode (MR 2-10) correspond to the phenotypic EMs of debrisoquin in our previous studies.^{8, 9}

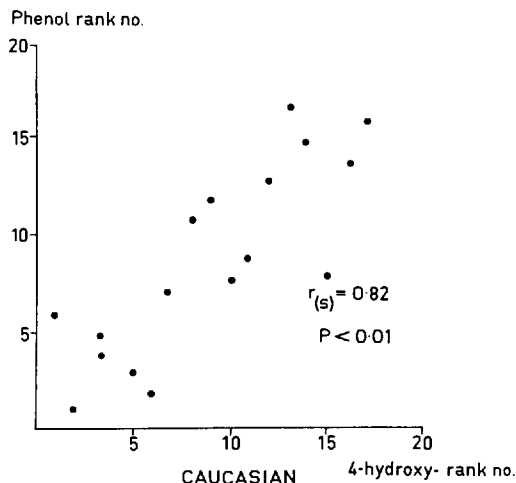


Fig. 5. The rank correlation between debrisoquin alicyclic 4-hydroxylation and debrisoquin aromatic hydroxylation metabolic ratios for Caucasians.

The third mode (MR 20-32) corresponds to the PM phenotype seen in Caucasians⁸ and Egyptians.⁹ We postulate here that the resolution of EM subjects into 2 modes arises from metabolic differences between the homozygous (EE) and heterozygous (EN) EM states. Such a situation could not arise if the EM trait were truly dominant, but in certain instances, for example sickle cell anemia, 1 allele does not completely dominate its variant allele and so the heterozygotes are expressed as an independent trait.¹⁴ Although this does not appear to be the case for debrisoquin 4-hydroxylation in Caucasians, it seems likely to be so in the Ghanaian study group. This postulate may be supported by use of the Hardy-Weinberg law. On the basis of the observation that 6.3% of the Ghanaians were PMs, i.e., $q^2 = 0.063$ (see Table III), the expected frequency of both homozygous EM subjects (p^2) and heterozygotes ($2pq$) can be calculated. This results in expected values of 0.561 for homozygous and 0.376 for heterozygous EM subjects which compare well with observed frequencies of 0.575 for mode 1 and 0.363 for mode 2 in the population histogram. It is concluded that the genetically determined PM phenotype (NN phenotype) is not totally recessive in the Ghanaian population and therefore 2 more functionally distinct modes are seen corresponding to homozygous EMs (EM phenotype, EE genotype) and heterozygous intermediate

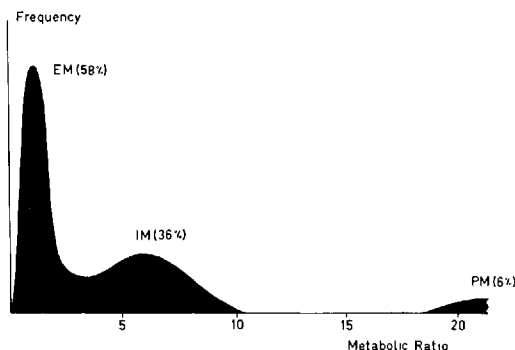


Fig. 6. Stylized frequency distribution histogram of the polymorphic 4-hydroxylation of debrisoquin among Ghanaians showing the 3 apparent phenotypes: EM, homozygous EMs; IM, intermediate (heterozygous) metabolizers; PM, poor metabolizers. Frequency of each phenotype in parentheses.

metabolizers (IM phenotype, EN genotype). These findings have been summarized in Fig. 6, which shows the spatial relationship between frequency and metabolic ratio in the 3 apparent phenotypes.

A second point of interest from this study is that the Ghanaian subjects had less 0- to 8-hr urinary recovery (24%) of debrisoquin and 4-hydroxydebrisoquin than the Caucasian subjects (41%). It was also our finding that this recovery was low in Egyptian subjects⁹ (16%) in whom it was related to the eating habits during the month of Ramadan, when they were investigated. By the same token, decreased gastrointestinal rate of debrisoquin absorption may have given rise to lowered urinary recoveries, as seemed to be the case in Egypt.

The fact that using the same technique, heterozygotes are detectable among Ghanaians but not among British Caucasians and Egyptians, requires comment. It does not appear to be a methodologic problem associated with differences in urinary recovery of the drug. Urine samples were collected under carefully controlled conditions and only those samples that represented a genuine 0- to 8-hr collection were used for analyses (incomplete collections were rejected). The metabolic ratio and phenotype assignment are largely independent of the amount of drug absorbed and excreted.^{8, 11} Furthermore, the correlation coefficient for urinary recovery against metabolic ratio for the EM sub-

jects shown in Table I is very low ($r = 0.16$; $p > 0.1$, $n = 75$).

It seems likely therefore that the reason why the heterozygotes are detectable among Ghanaians and not among English Caucasians and Egyptians could be due to a difference in genetic background against which the debrisoquin hydroxylation genes are expressed. Final proof will have to come from family studies among Ghanaians.

Finally, it has been shown that the formation of phenolic metabolites, 5-, 6-, 7-, and 8-hydroxydebrisoquin by aromatic hydroxylation parallels the polymorphic alicyclic 4-hydroxylation process and is thus under the same genetic control. This is not surprising, since we have shown that the aromatic hydroxylation of both guanoxan¹³ and phenytoin⁶ is polymorphic and controlled by the same gene that determines debrisoquin 4-hydroxylation.

This investigation has demonstrated some important differences between the West African and British populations which bear on the use of drugs in these different ethnic groups. On average Ghanaians were poorer hydroxylators of debrisoquin than Caucasians and presumably they will be poorer hydroxylators of other drugs, the metabolism of which is controlled by the same gene locus. This list is by no means exclusive, but includes the aromatic hydroxylation of guanoxan,¹³ the *O*-deethylation of phenacetin,¹³ the aromatic hydroxylation of phenytoin,⁶ the *S*-oxidation of metiamide,⁵ and the *O*-demethylation of 4-methoxyamphetamine.*

To properly assess the possible toxicologic and therapeutic implications of the use of drugs in non-Caucasian populations, we would advocate the development of probes such as debrisoquin to measure oxidative phenotype frequencies and their global heterogeneity.

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**A FAMILY AND POPULATION STUDY OF THE GENETIC
POLYMORPHISM OF DEBRISOQUINE OXIDATION IN A WHITE
BRITISH POPULATION**

BY

D A PRICE EVANS, A MAHGOUB, T P SLOAN, J R IDLE, AND R L SMITH

Reprinted from Journal of Medical Genetics, April 1980, Vol. 17, No. 2, 102

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JOURNAL OF MEDICAL GENETICS

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LONDON

BRITISH MEDICAL ASSOCIATION

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A family and population study of the genetic polymorphism of debrisoquine oxidation in a white British population

D A PRICE EVANS, A MAHGOUB, T P SLOAN, J R IDLE,
AND R L SMITH

From the Department of Medicine, University of Liverpool, PO Box 147, Liverpool L69 3BX; and the Department of Biochemical and Experimental Pharmacology, St Mary's Hospital Medical School, Paddington, London W2 1PG.

SUMMARY A population survey of 258 unrelated white British subjects showed a polymorphism for the 4-oxidation of debrisoquine. 'Extensive metabolisers' (EM) and 'poor metabolisers' (PM) are recognisable, 8.9% of the population being PM. Nine pedigrees ascertained through PM probands show that the PM phenotype is an autosomal Mendelian recessive character. The EM phenotype is dominant and the degree of dominance has been estimated at 30%. PM subjects are more prone to hypotension during debrisoquine therapy. The alleles controlling this polymorphism appear to control the oxidation of other drugs.

The antihypertensive drug, debrisoquine, undergoes metabolic hydroxylation in man. The formation of the major metabolite, namely 4-hydroxy-debrisoquine, displays polymorphism in the population.¹ In this study, two well-resolved phenotypes were seen. The first was characterised by persons who 4-hydroxylated the majority of the drug and were thus designated extensive metabolisers (EM phenotype, about 95% of the white British population). For these subjects, the metabolic ratio (MR), defined as % of dose as debrisoquine/% of dose as 4-hydroxy-debrisoquine excreted in the 0 to 8 hour urine, was of the range 0.01 to 9. In the second phenotype (poor metabolisers, PM, about 5% of British whites), MR values greater than 20 were observed, and thus these subjects had a relative inability to carry out debrisoquine 4-hydroxylation. The few families studied suggested strongly that PM subjects were autosomal recessives.

In this paper, we describe a more extensive population study of white British subjects, together with further family data which uphold the resolution of the population into EM and PM phenotypes and confirm that the PM phenotype is an autosomal recessive trait.

Received for publication 6 June 1979

Methods

VOLUNTEER SUBJECTS

Subjects were studied in both London and Liverpool. The volunteers comprised 97 healthy staff and students from the University of Liverpool Medical School and 161 from St Mary's Hospital Medical School, part of whom were studied by Mahgoub *et al.*¹

FAMILY MEMBERS

The probands for the family studies were the poor metabolisers identified in the London and Liverpool population studies.

PHENOTYPING PROCEDURE

As described by Mahgoub *et al.*,¹ a single 10 mg Declinax tablet (Roche) (equivalent to 12.8 mg debrisoquine sulphate) was ingested in the fasting state by volunteer subjects who had not been exposed to any recent drug medication. The bladder was emptied before dosing and thereafter all urine was collected up to 8 hours. Urine volume was recorded and an aliquot stored at -20°C to await analysis.

ANALYTICAL PROCEDURE

Urine was analysed for debrisoquine and 4-hydroxy-debrisoquine by electron-capture gas-chro-

matography after derivatisation with hexafluoroacetylacetone as described by Idle *et al.*² From these data the metabolic ratio (MR) was calculated from the expression:

$$\left(\frac{\% \text{ of dose as debrisoquine}}{\% \text{ of dose as 4-hydroxy-debrisoquine}} \right)$$

excreted in the 0 to 8 hour urine. The transformation $\log_{10}$ MR was distributed in an approximately normal manner and was used for computation.

Results

The variable log MR was found to be reproducible in eight EM and nine PM subjects on whom complete repeat tests were performed on two separate occasions. Fig 1 shows a scattergram of the first estimate of log MR plotted against the second estimate of log MR. The non-parametric Spearman rank correlation gives $r_s = 0.88$, $p < 0.001$.

There was no association of either phenotype with age or sex. The urinary volumes of both phenotypes were within the same range and MR was not dependent on urine volume within either phenotype. There was no heterogeneity in MR values between the London and the Liverpool samples. The distribution of log MR in 258 unrelated white British subjects is shown in fig 2. Two phenotypes are apparent in the population with an antimode of 1.10. Poor metabolisers are thus defined as subjects with $\log \text{MR} > 1.10$. Twenty-three phenotypically poor metabolisers were identified, 8 of 97 subjects tested in Liverpool and 15 of 161 subjects tested in London. The PM phenotype frequency in the total sample was thus 0.0891, approximately twice that found by Mahgoub *et al.*¹ in a smaller sample.

Families of the probands derived from among the 23 PM subjects in fig 2 were phenotyped as described for the unrelated volunteers and the results are shown in the table. Metabolic ratios greater than 12.6 ($\log \text{MR} > 1.10$) are indicative of poor meta-

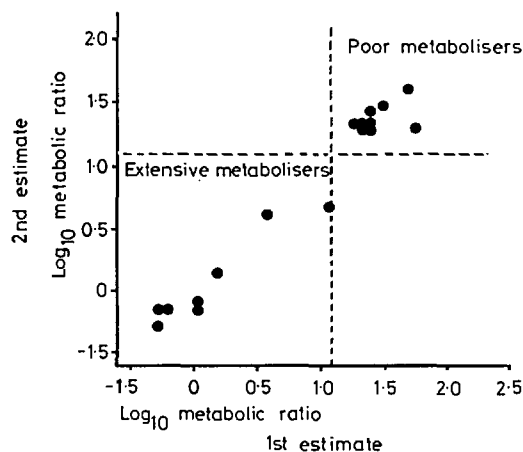


FIG 1 Repeat phenotyping tests carried out on separate occasions in unrelated people of both phenotypes

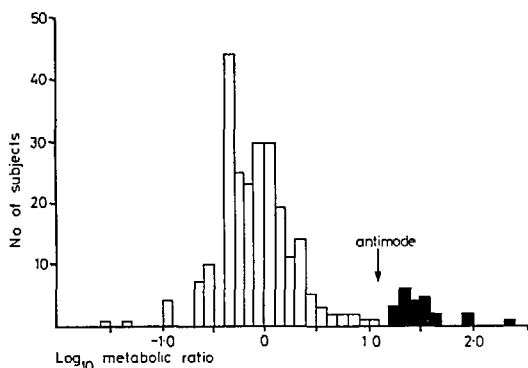


FIG 2 Frequency distribution of $\log_{10}$ metabolic ratio in 258 unrelated white British subjects

bolisers. In pedigrees 1 to 5, all PM offspring had a PM parent (either sex), but for pedigrees 6 to 9, both parents of PM offspring were extensive metabolisers (EM). This clearly shows that PM is an

TABLE $\log_{10}$ urinary metabolic ratios in 9 pedigrees ascertained through probands who are poor metabolisers

Pedigree no	Parents		Offspring				
	Father	Mother	1	2	3	4	5
1	1.48	→1.52	0.15 M	0.18 M	0.42 M	0.11 M	0.15 M
2	→1.29	1.78	1.78 M	0.58 M	1.95 M	—	—
3	1.32	0.23	1.43 M	→1.33 M	0.40 M	—	—
4	1.40	Not available	→1.32	1.90	—	—	—
5	2.29	0.04	→1.48 M	1.88 M	—	—	—
6	0.28	0.48	→1.41	1.32 M	0.32 M	1.84 M	—
7	0.48	0.34	→1.59	1.26	—	—	—
8	0.63	1.00	→1.30 M	—	—	—	—
9	0.72	0.04	→1.32 M	0.77 M	0.64 M	—	—

→ proband; M indicates male offspring.

autosomal recessive character. An estimate can, therefore, be made of the frequency of the allele controlling poor metabolism ($0.2986 \pm \text{SE } 0.0297$)³ and the allele controlling extensive metabolism (0.7014). Thus, the expected genotype frequencies are homozygous PM 0.0891 , heterozygous EM 0.4189 , and homozygous EM 0.4920 .

An estimate of the degree of dominance can be made from the extent of displacement of the mean observed log MR value for heterozygotes (positively identified from family pedigrees) from the mid-point between the mean log MR values for the two homozygous states. From the data given in the table and from Mahgoub *et al.*,¹ it is possible to recognise 21 heterozygotes who have a mean value of 0.27 ± 0.31 (log MR $\pm$ SD). Accordingly, the mean value (x) for homozygous dominants can be calculated from the expected number of homozygous (127) and heterozygous (108) subjects who comprise the extensive metabolisers (235) in fig 2 as follows:

Sum of values for 235 extensive metabolisers	Sum of values for 127 homozygotes	Sum of values + for 108 heterozygotes
-22.58	$= 127x$	$+ 108 \times 0.27$

The estimated mean value (x) for homozygous dominants is therefore 1.5926 (mean MR = 0.39). The observed mean value for the homozygous recessives (23 in fig 2 plus the eight non-probands in the table plus an additional one from the extended family B of Mahgoub *et al.*,¹ making 32 in all) is 1.53 (mean MR = 33.9). The mid-point between the two homozygous means is thus estimated as 0.56 . So the displacement of the observed heterozygous value from the expected mid-point value (expected for zero dominance) is 0.29 , which gives an approximate dominance of 30%.

Discussion

Two debrisoquine oxidation phenotypes were characterised, first a Mendelian autosomal recessive character (PM phenotype = 8.9% of population) with metabolic ratios greater than 12.8 (log MR > 1.10), and secondly a dominant character (EM phenotype 91.2%) comprising both homozygous dominants and heterozygotes in whom a strong (about 30%) dominance effect is observed.

The calculation of dominance gives a clearer picture of the genetic structure of the white British population. There is a practical significance to this finding because if the homozygous EM genotype and the heterozygote had the same distribution they would probably be at equal risk of adverse reactions.

However, since the heterozygotes have a smaller metabolic capacity than the homozygotes it is probable that they are more prone to adverse effects of drugs undergoing C-oxidation.

The biochemical basis of the debrisoquine polymorphism is unknown, but it is possibly concerned with a hepatic mono-oxygenase enzyme which may or may not involve the haemoprotein cytochrome P450 which is considered central to the processes of drug oxidation. As encountered with most polymorphisms of drug metabolism, the natural or endogenous substrate(s) remains unknown. The clinical importance of the debrisoquine polymorphism has been stressed by Sloan *et al.*⁴ and is reiterated here. The oxidative metabolism of other drugs appears to be under the control of the same alleles as debrisoquine hydroxylation. These drugs include guanoxan and phenacetin,⁴ phenytoin,⁵ metiamide,⁶ and 4-methoxyamphetamine⁷; other drugs may be added to this list with the passage of time. In addition, hypotension is produced by a standard 20 mg therapeutic dose of debrisoquine in poor metabolisers, but not in extensive metabolisers.⁸ It would appear, therefore, that the alleles controlling debrisoquine metabolism, and the resulting phenotype of a subject, are of considerable medical importance.

We gratefully acknowledge the invaluable assistance of Mrs A Howard Jones SRN, Nuffield Unit of Medical Genetics, Department of Medicine, University of Liverpool, for carrying out tests on probands and family members. AM was supported by the Egyptian Education Bureau, and TPS is in receipt of an MRC Studentship. We thank the Wellcome Trust for a grant for interdisciplinary research. The study was dependent on the co-operation and goodwill of probands and family members and the authors thank them.

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Requests for reprints to Professor D A Price Evans, Department of Medicine, University of Liverpool, PO Box 147, Liverpool L69 3BX.