GENETIC POLYMORPHISM OF DRUG OXIDATION IN THE RAT

by

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This work is dedicated to

my parents

for their encouragement and patience

ABSTRACT

1. Debrisoquine hydroxylation was investigated in 122 animals from ten strains of rat. A bimodal frequency distribution of metabolic ratio (urinary debrisoquine/4-hydroxydebrisoquine) was obtained. All female DA rats (n=21) exclusively constituted a mode (metabolic ratios 1.2-5.3), with no female DA rat found in the second mode (metabolic ratios 0.07-0.7). By analogy with the human polymorphism, these two modes were designated poor metabolizer (PM) and extensive metabolizer (EM) phenotypes respectively.

2. Breeding studies demonstrated that the PM character is a Mendelian recessive.

3. The female DA rat hydroxylation deficiency was neither inducible by phenobarbitone nor by 20-methylcholanthrene, but was found to be sex hormone-dependent.

4. Female DA (PM) and Lewis (EM) rats were chosen as model strains for further drug investigation. Phenacetin O-deethylation, phenformin 4-hydroxylation,

and sparteine 2,3-dehydrogenation were all impaired in PM rats. Phenacetin 2-hydroxylation and acetanilide 4-hydroxylation were enhanced in PM rats. Tolbutamide metabolism showed no inter-phenotype differences. Hexobarbitone sleeping time was longer in PM than in EM rats.

5. Hepatic-derived cytochrome P-450 from EM rats gave a type I difference spectrum with debrisoquine which was virtually absent for the corresponding PM P-450.

6. Nineteen PM (female DA) and seventeen EM (female Fischer) rats were fed aflatoxin B_1 (4ppm in diet) for 4 months, culled after a further 12 months and the livers examined histologically. All EM rats had hepatocellular carcinoma, whilst PM rats showed mainly degenerative foci with no carcinoma.

7. It is concluded that the rat polymorphism parallels its human counterpart well and represents an opportunity for the study of genetically variable drug metabolism and toxicity in man. Additionally, it may be possible by using the described model strains and their tissue preparations to identify new polymorphically metabolized drug candidates and their pharmacological and toxicological properties, as an alternative to direct human investigation.

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CHAPTER ONE

Introduction

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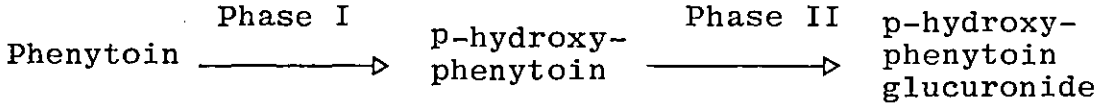
1.1 DRUG METABOLISM

Over the past three decades, the importance of metabolic studies as an integral part of the safety evaluation of drugs has been increasingly emphasized. Moreover, enhanced understanding of the value of pharmacokinetics has led to the recognition that metabolic and pharmacokinetic data are not auxiliary information, but a vital part of the characteristics of a given compound and are indispensable when it comes to designing protocols for the safety evaluation of new compounds (Wodicka et al., 1978).

Nearly all drugs undergo metabolic transformation during their passage through the liver and other organs, and one characteristic of drug metabolism is that it renders molecules more polar, so that they may be excreted more easily. By this mechanism, the process of metabolism acts to protect the body against the accumulation and undesirable sustained effects of drugs. This is not always the case however; metabolic products occasionally have greater pharmacological activity than the parent drug (as in morphine formed from codeine), or even quite different pharmacological effects (as in, isoniazid from

iproniazid) or creation of pharmacological activity (prontosil gives rise to sulphanilamide).

Drugs and other foreign compounds can be conveniently thought of as being metabolized by two phases of reaction (Williams, 1959). The first (Phase I) are reactions in which the drug undergoes one or more of a wide variety of oxidations, reductions and hydrolyses, usually resulting in the introduction or modification of specific chemical groups which increase the polarity of the molecule. Conjugation (Phase II) occurs subsequently and involves linkage of the drug itself, or of a primary metabolite produced by Phase I reactions, with endogenous compounds such as glucuronic acid, sulphuric acid and amino acids, or a group such as methyl or acetyl. The products usually, but not always, exhibit significantly enhanced water-solubility. Most foreign compounds are metabolized, at least to some extent, by both phases of reaction. For example, phenytoin (diphenylhydantoin) undergoes oxidative transformation into p-hydroxyphenytoin which is subsequently conjugated largely with glucuronic acid.



(major urinary metabolite)

It has been found that most drugs are metabolized mainly in the liver. The soluble fraction of liver homogenates contain enzymes that catalyze the oxidation of ethanol, the reduction of ketones, the formation of sulphates, methyl and glutathione derivatives and the formation of cofactors that are required for glucuronic acid conjugation. The mitochrondria contain enzymes which catalyze the oxidation of moncamines and diamines, the formation of acetyl derivatives and glycine and glutamine conjugates (see Williams and Millburn, 1975).

However, most drugs are metabolized by enzymes localized in hepatic microsomes, a fraction derived from the endoplasmic reticulum of the hepatic parenchymal cells. These microsomes contain a hemoprotein known as cytochrome P-450 which acts as the terminal oxidase for a variety of oxidative reactions which drugs undergo. The term P-450 refers to the ability of the reduced form of the hemo-protein to react with carbon monoxide, yielding a complex with an absorption peak at 450nm (Garfinkel, 1958; Klingenberg, 1958; Omura and Sato, 1962).

The major microsomal enzyme system is mixed function oxidase (Mason, 1957) or monooxygenase (Hayaishi, 1969) whose main metabolic function is to participate in the

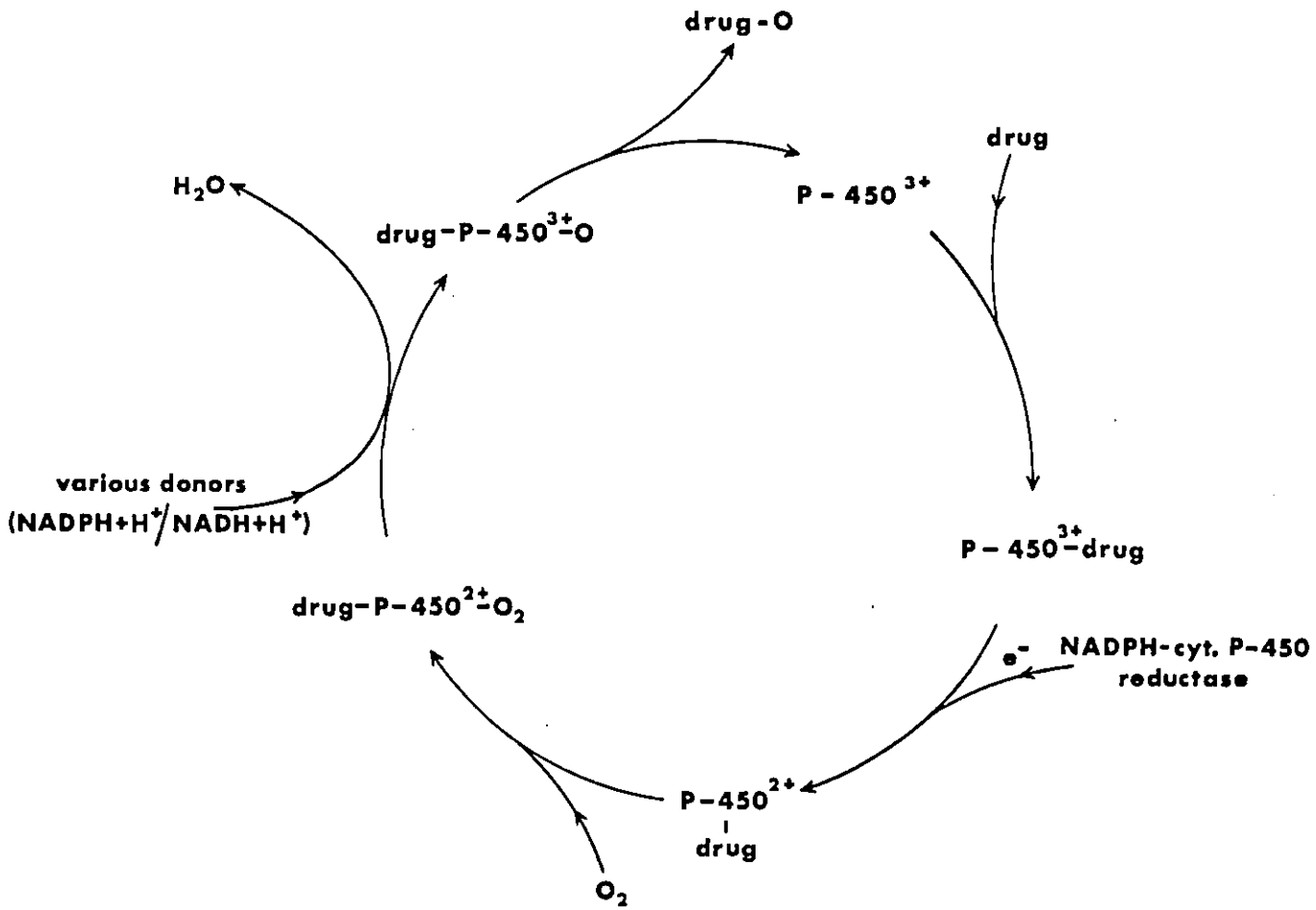
oxidation of a number of compounds such as steroids, fatty acids, drugs and carcinogens (Cooper et al., 1965; Kuntzman, 1969). In vitro, these microsomal enzyme systems require NADPH, O_2 and Mg^{++} in order to carry out this function (Gillette et al., 1957).

The interaction of drug and monooxygenase has been formulated in the following terms (Figure 1.1.) (Hildebrandt and Estabrook, 1971).

The terminal oxidase of this system consists of cytochrome P-450 which occurs in an unknown number of different varieties with overlapping specificities. However, interaction of substrate with oxidized cytochrome P-450 has shown to give rise to two distinct types of spectral changes, namely, Type I and Type II spectra. This indicates that either there exist two different binding sites or there are two types of cytochrome P-450 (Remmer et al., 1966; Imai and Sato, 1966). Indeed it has subsequently been demonstrated that the reduction of the cytochrome P-450 substrate (Type I) complex is the rate-controlling step in the oxidation of Type I compounds (Gigon et al., 1968; 1969).

Figure 1.1 Mechanism of cytochrome P-450 enzyme system

(Hildebrandt and Estabrook, 1971)



Many factors might affect the activity of the mono-oxygenase enzymes such as, species, sex (which will be discussed later) and the presence of certain inducers and inhibitors. The first inducers (stimulators) of enzyme activity to be discovered were the polycyclic hydrocarbons (Brown et al., 1954). Conney and Burns (1959) and Remmer (1959) found that phenobarbitone and a number of other common drugs also act as inducers, while other compounds such as SKF 525-A, have been found to act as both competitive and non-competitive hepatic monooxygenase inhibitors.

Variation between individuals in drug oxidation is due to differences in enzyme activity, which is the outcome of the interaction of many factors; physiological (such as liver blood flow), pathological (such as liver disease), environmental (such as diet and smoking) and genetic. It is most likely that the largest determinant of variability is the genetic component, since genetic differences in the formation of an enzyme can lead to variation in its substrate affinity, for example, with the haemoglobins or glucose-6-phosphate dehydrogenases (Brewer, 1972; Motulsky et al., 1971).

1.2 PHARMACOGENETICS

The science which deals with the contribution of genetic factors to the considerable inter-individual differences in drug disposition and responsiveness is known as pharmacogenetics, a term first introduced by Vogel in 1959.

It has been known for a long time that there is great variability in the way different human beings respond to certain drugs (Williams, 1956): but it is only within the past two or three decades that genetic differences in people have been seriously studied as a cause for individual variability in drug effects.

Hereditary variations in pharmacological responsiveness are not confined to drugs but may also occur in response to environmental chemicals; the term "Ecogenetics" has been used in order to broaden the concept and includes, for instance, the diarrhoea caused by milk in most people with lactase deficiency (Holzel et al., 1959).

The morphological and biochemical make up of a biological system is determined by the hereditary of its constituent components. The basic units of inheritance are the genes,

which are submicroscopic entities located at various areas on the chromosomes. Those genes which are located at the same points on paired chromosomes are known as alleles (see Ford, 1979).

Genetically transmitted variations in drug response arise mainly from stable mutations of DNA, giving rise to structural alterations in a protein that acts directly on drug absorption, distribution, metabolism, interaction with receptor sites, excretion, or a combination of these. Such a protein would be one in which the normal amino acid sequence, which identifies the protein, is disrupted by the presence of an incorrect amino acid somewhere along the sequence. If the mutant protein constitutes a target (or receptor) for a drug or foreign compound, then the protein may exhibit a greater, or less, or even an absence of affinity for the foreign chemical.

However, such genetic differences may go undetected until the mutant or variant system is suitable challenged, for example, by a drug, assuming these enzymes to be involved in metabolic alteration of that drug. The aims in pharmacogenetic research are not only to identify new

examples of inherited adverse drug reactions and unusual drug effects, but to study the mode of transmission (dominant or recessive; autosomal or sex-linked), the frequency of the genes in the population, the biochemical basis for the unusual drug effect and the clinical consequences (Motulsky, 1957; Vogel, 1959).

Inherited differences in drug metabolism and drug response support the generalization that it is reasonable to expect that a person has a particular 'pharmacological individuality' similar to his 'biochemical individuality' due to his unique genetic make up (Williams, 1956; LaDu, 1972).

Two useful terms may therefore be introduced at this point. A genotype is defined as the genetic constitution of an individual at one or more loci; a phenotype is an individual judged by its 'character' in the widest sense, including such consideration anatomy, appearance, chemistry and physiology. Therefore, the phenotype is the product both of hereditary and of the environment (Ford, 1979; Bodmer and Cavalli Sforza, 1976).

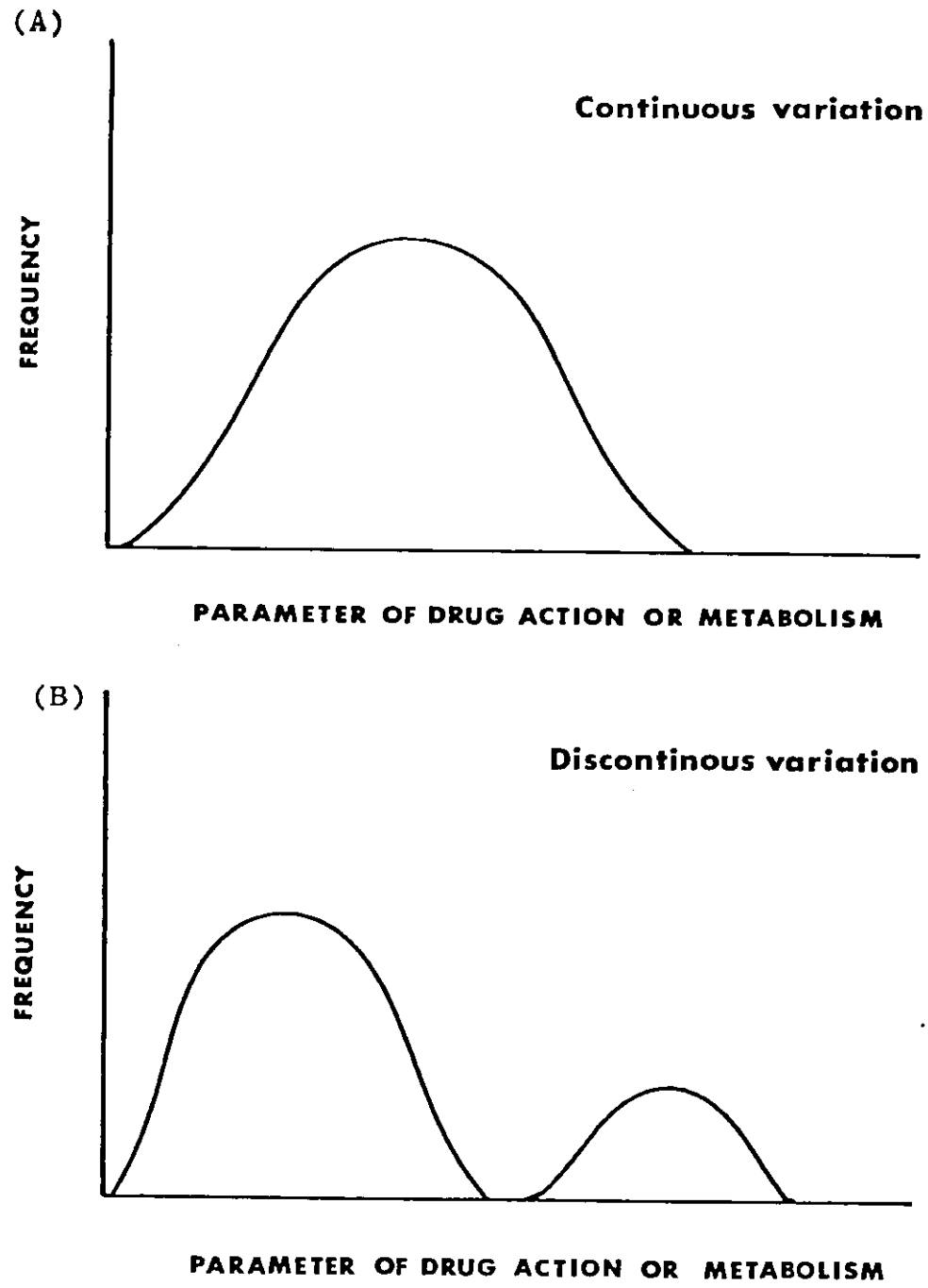
Variation between individuals in their metabolism of drugs can be studied by techniques drawn from genetics, biochemistry and pharmacology. The extent to which genetic factors determine drug metabolism or responsiveness is investigated by means of population, family and twin studies.

Population studies involve administering a fixed dose of a drug to a large number of individuals and then measuring either the response to the drug or some pharmacokinetic characteristic such as plasma half-life (Vesell, 1973), steady-state plasma concentration (Evans et al., 1960; Alexanderson et al., 1969) or rate of urinary metabolite excretion (Evans and White 1964, Wakile et al., 1979). From these data a frequency distribution histogram is constructed.

Figure 1.2 shows the two most common patterns of frequency distribution histogram, named continuous curves (A) and discontinuous curves (B).

A bimodal distribution of the frequency histogram implies genetic polymorphism. The latter term has been defined by Ford (1940) as "a type of variation in which

Figure 1.2 The types of variability of frequency distribution histograms



individuals with sharply distinct qualities co-exist as normal members of a population". However, this bimodal distribution may be under the control of a single gene, and then each mode may represent a different phenotype.

The second type of variation, Gaussian distribution, has four possible interpretations. Firstly, the population may be genetically similar with respect to the genes controlling the response being measured. Secondly, the population may be under polygenic or multifactorial control, in which case, the response is regulated by a number of genes, and a number of polymorphisms would be mutually overlapping. Thirdly, inadequate methods may have been used to separate inherently different populations from one another. Fourthly, the population may be under genetic and environmental influence. In early studies, the causes of variation among individuals could be separated into their environmental and inheritable components through the use of human twins. This approach, first introduced by Galton (1875) and developed by Neel and Schull (1954) permits determination of the extent to which such individual variations are genetically controlled. Many drugs have been studied using twins. Examples of these include phenylbutazone (Vesell and

Page, 1968a) antipyrine (Vesell and Page 1968b), bis-hydroxycoumarin (Vesell and Page 1968c), nortriptyline (Alexanderson et al., 1969), ethanol (Vesell et al., 1971) and halothane (Cascorbi et al., 1971).

This approach is based on the measurement of the variation in drug response in fraternal (dizygotic) and identical (monozygotic) twins. Monozygotic twins have a common genetic constitution, whereas fraternal twins, like all other siblings, have on average, only half their genes in common. This enables the genetic contribution to such variation to be determined, under the assumption that environmental factors will be similar for each pair of twins. When the intra-twin variance is less in the identical twins than in the fraternal, this will indicate the predominance of genetic over environmental factors.

Pharmacogenetic disorders in man have been reviewed by different authors (Kalow, 1962, 1971; Evans, 1963, 1977; Motulsky, 1964, 1971; Vesell, 1969, 1972, 1973; LaDu, 1972; Goldstein et al., 1974; Mahgoub, 1978 and Sloan, 1980) who went into greater detail than is possible here.

A genetic polymorphism in the oxidation of several drugs with clinical consequences will be discussed in Chapter Two.

There are many ethical and technical problems in performing pharmacogenetic studies and these can be summarized as follows:

1. When the target of the study is variation in drug metabolism, it is necessary to study healthy people. Many pathological conditions may affect the rate of metabolism. Therefore, checking the health of each individual is important.

2. Ethical problems may be raised in these instances by local Ethics Committees.

3. Different environmental conditions may cause variations in the phenotype of the subjects used.

4. Size of test population may also be a problem if the mean frequency of a certain trait is 1:1000, then it may remain undetected until a great many volunteers have been screened.

5. In population studies, a large number of samples is obtained, which necessitates a simplified method of analysis.

6. Family studies in pharmacogenetics require the parent and/or offspring, if they are available, of the individual who shows a rare phenotype of genetic polymorphism. This form of investigation has become encumbered with a variety of administrative regulations and restrictions.

7. In twins studies, there are difficulties in obtaining twins, and these are increased where there is no system of twin registration.

This latter problem can be resolved from a study of the blood groups and other more specific biochemical/immunological markers for confirming the identity of the twins. However, the assumption that "monozygotic human twins have identical inheritance" has been challenged by Storrs and Williams (1968) on the basis of large differences in 20 parameters among monozygotic twins.

Twin studies have several further disadvantages including their inability to establish the mode of inheritance of genetically controlled traits and their assumption of an environmental equality in all subjects, identical as well as fraternal; identical twins make more similar choices and have more tastes in common than fraternal twins in eating, drinking and even in choice of tooth paste (Vesell, 1972).

Whilst twin studies may be of value in polygenic situations family studies are still required in investigating the mode of inheritance of phenotypes and population studies are a good approach in detecting pharmacogenetic disorders relating to certain drugs and in quantifying their frequency in the population.

1.3 THE EXTRAPOLATION OF ANIMAL DATA TO MAN - THE PROBLEM OF PHARMACOGENETIC VARIATION

Before the introduction of a new drug for human use and during the various phases of its clinical development, animal studies are conducted to provide some assurance that the drug should be safe for use in man. Therefore, population studies in animals may be a good way to detect pharmacogenetic disorders in certain species which may be extrapolated to man and also to identify a genetic polymorphism in drug action that would have been difficult to demonstrate otherwise. Moreover, in experimental animals, the effect of environmental conditions on drug disposition can be carefully measured because these factors can be stringently controlled.

It has been suggested that inter-species differences in drug metabolism must be considered in the extrapolation to man of pharmacological and toxicological data obtained in experimental animals. But the wide inter-species differences in renal and biliary excretion, binding to plasma proteins and tissue distribution, lead to the conclusion that the study of strain differences will be a reasonably good approach for pharmacogenetic studies.

In contrast to man, animals live in different colonies, and within each colony the animals are related to each other and may all belong to the same strain. All of these animals are living under the same conditions, so mutation is likely to be uniform in all the animals, and uniform genetic make-up and response would be expected. At this point, the question arises as to which kind or group of animals is most convenient for pharmacogenetic studies.

Many species of animal are divided into two categories, namely inbred and outbred. At this point, it is necessary to define these terms. Inbreeding may be defined as the probability that two genes at a single locus in an individual are alike by descent (Falconer, 1960). But by international agreed convention (Festing and Straats, 1973; Staats, 1968), an inbred strain is one that has been maintained by brother x sister mating (or its genetic equivalent) for more than 20 generations with all animals in generation 20 being descended from a single pair. In such strains all animals are virtually genetically identical (isogenic) and the probability of the two genes at a locus being alike by descent is more than 0.986.

The term 'outbred' on the other hand, refers to animals of random-bred stock, who have been subjected to no deliberate inbreeding and whose genetic nature depends simply on their previous genetic history; some of these are genetically variable, while others are nearly isogenic due to previous inbreeding.

Festing (1975) has described how to choose animals within a species when the aim of a study is to investigate a genetic aspect of drug toxicity. He concluded that the inclusion of a range of different strains in one study gives a broader base for extrapolation to man, making it possible to study the pattern of drug response and of adverse effects and to correlate this with biochemical or physiological data obtained in the same experiment or from the published literature. Moreover, he described some of the advantages of using inbred strains in the evaluation of the safety of drugs as follows:-

1. Different inbred strains of animals provide a substantially wider range of genotypes than are present in a single outbred stock, and the pharmacological response to a drug in a number of different strains would be more valuable than a response based only on a single stock.

2. Wide variation within a strain may suggest that the pharmacological response of a drug is under polygenic rather than monogenic control.

3. Inbred strains can be identified by their biochemical and immunological characteristics.

4. Inbred strains are generally more stable than outbred stock, and this genetic constancy is important since it makes it possible to collect background information for future use. Therefore, some information may already be available on the characteristics of inbred strains.

5. If there is a difference between the extent of adverse effects shown by different strains and if this can be correlated with their rate of metabolism, then a causal relationship may possibly be established between adverse effect and metabolic rate and this relationship may be extrapolated to man.

Pharmacogenetic disorders in animals analogous to those in humans have been described. For example, acatalasia (in which the production of catalase, the enzyme responsible for inactivation of hydrogen peroxide, is deficient or absent) occurs with a similar pattern of inheritance in certain breeds of dogs and guinea pig (Allison et al., 1957; Rader, 1960). Acatalasia causes oral ulceration,

loss of teeth and discoloration of the blood to brownish black when it comes into contact with hydrogen peroxide. Another example is N-acetylation of isoniazid, in which hepatic and jejunal N-acetyltransferase in polymorphic in man (Evans and White, 1964) and rabbit (Frymoyer and Jacox, 1963a,b), as well as in the mouse (Tannen and Weber, 1979). In man, slow and rapid acetylators are subject to differential drug toxicity depending upon the acetylator phenotype (Woosley et al., 1978). Slow acetylators were found to be more liable to develop peripheral neuropathy and showed a systemic lupus erythematosus-like syndrome with hydralazine. A/J mice are characterized by a slow acetylator phenotype and a predisposition to develop spontaneous and drug induced antinuclear antibodies (Teague and Friou, 1969) similar to slow acetylator humans. Therefore, A/J mice are a good genetic animal model (Tannen and Weber, 1980). Moreover, breeding experiments with C57BL/6J mice (representing the rapid acetylator strain) showed that the traits are inherited in a simple Mendelian manner consistent with a model of two codominant alleles. Another animal model used for the human isoniazid acetylator polymorphism is the rabbit (Frymoyer and Jacox, 1963a,b). Other substrates have also shown polymorphism in their N-acetylation, these include procainamide, sulphamethazine, phenelzine

and arylamine carcinogens, such as aminofluorene and benzidine (Weber, 1973; Glowinski et al., 1978).

A third example relates to the enzyme hepatic UDP-glucuronyl transferase which catalyses the glucuronidation of endogenous compounds such as bilirubin and also foreign compounds (Dutton and Burchell, 1977). The defect in the glucuronide formation of bilirubin was observed in the Gunn rat and is similar to the inherited human metabolic disorder known as congenital familial non-hemolytic jaundice (Crigler-Najjar Syndrome and Gilbert's Syndrome) which is associated with reduced glucuronyl transferase activity (Gunn, 1938). Therefore, the Gunn rat provides a good animal model for this human metabolic disorder (Cornelius and Arias, 1972). Moreover, non-jaundiced litter mates of icteric rats also have reduced glucuronide forming ability, though the defect is less severe. Cross-breeding of non-icteric litter mates produced some offspring that were jaundiced. Such rats are thus carriers of the defective trait.

Several other pharmacogenetic phenomena will be described later (see Table 2.12). However, the occurrence in vivo of a genetic polymorphism in drug oxidation has never previously been described for laboratory animal species.

The background to the human polymorphism in drug oxidation together with the objectives of the work described in this thesis are given in Chapter Two.

CHAPTER TWO

The development and definition of an animal model for polymorphic drug oxidation.

Contents

2.1 INTRODUCTION

Recently, several examples of genetic polymorphism of human drug oxidation have been described. Thus, indices of the oxidative metabolism of debrisoquine (Mahgoub et al., 1977), sparteine (Eichelbaum et al., 1979a) and phenformin (Idle and Islam, 1981) have been shown to be bimodally distributed in various human populations. Available data suggested that genetic polymorphism of human drug oxidation is not an occasional phenomenon.

Debrisoquine metabolism is possibly the best studied example in this respect. Its metabolism is under genetic control, the predominant 4-hydroxylation pathway being regulated by a single autosomal gene which is allelomorphic, the recessive poor metabolizer trait (PM) being characterized by an almost total inability to effect 4-hydroxylation in some 9% of the British white population (Mahgoub et al., 1977; Evans et al., 1980).

Autosomal dominants comprise the rest of the population, with varying degrees (10-99%) of hydroxylation ability. Such subjects constitute the so called 'extensive metabolizer (EM) phenotype'.

This polymorphism influences the metabolic disposition of a number of other drugs besides debrisoquine, and this has been extensively reviewed (Idle and Smith, 1979; Smith and Idle, 1981) together with its possible toxicological implications (Ritchie et al., 1980). Briefly, relative to EM subjects, PM volunteers have been shown to be defective oxidizers of phenacetin (Sloan et al., 1978; Kong et al., 1982), guanoxan (Sloan et al., 1978), phenytoin (Sloan et al., 1981) metiamide (Idle et al., 1979a), 4-methoxy-amphetamine (Kitchen et al., 1979), S-carboxymethylcysteine (Waring et al., 1981) and nortriptyline (Bertilsson et al., 1980). Polymorphisms of sparteine oxidation (Harmer et al., 1982) and phenformin hydroxylation (Oates et al., 1982) certainly seem to be related to the deveisoquine oxidation polymorphism, if only genetically linked rather than coincidental. The PM phenotype would therefore seem to be considerably disadvantaged versus the EM phenotype, inasmuch that the impaired oxidation status can lead to drug accumulation and a higher prevalence to drug-related toxicity. Postural hypotension caused by debrisoquine (Idle et al., 1978), methaemoglobinaemia induced by phenacetin (Kong et al., 1982) and the lactic acidosis associated with phenformin (Idle et al., 1981a) can all be demonstrated

experimentally in PM subjects at drug doses which have little or minimal effect in individuals of the EM phenotype for whom oxidation is uncompromised.

A second interesting approach to drug toxicity related to this oxidative polymorphism has been to phenotype, using debrisoquine, patients presenting with untoward drug side-effects or simply inappropriate plasma levels of certain drugs. Recently, Shah et al. (1982a) demonstrated that 10/20 patients studied with perhexiline (pexid) associated neuropathy were phenotypically PM, 7 of the remainder also exhibiting a relative impairment of drug oxidation capacity.

Alvan et al. (1982) studied two patients and two volunteers with unusually high plasma concentrations of the β-blocking drugs alprenolol, metoprolol and timolol and all were found to be of the PM phenotype. Similarly, Shah et al. (1982b) investigated a single patient with marked bradycardia prescribed only 10mg propranolol per day (frequently used up to 320mg daily) and he was also found to be of PM status.

Wide inter-individual differences in the metabolic oxidation of drugs and other chemicals in man has

been known for many years. In this context, *in vitro* experiments have provided some useful insights. For example, liver microsomes from different individuals have been shown to metabolize benzo[a]pyrene, antipyrine, hexobarbitone, coumarin and aflatoxin B_1 at quite different rates (Conney *et al.*, 1979). A ninefold difference in the metabolism of aflatoxin B_1 to mutagens was observed among 10 human liver samples, which may partly explain the individual differences in the sensitivity of people to the carcinogenic action of this substance (Conney *et al.*, 1979).

Toxicity of a variety of chemicals is undoubtedly associated with the formation of chemically reactive and electrophilic oxidative metabolites. Thus, individual variability in the pathways leading to such intermediates might be of considerable importance to recognize. With aromatic substances, an arene oxide (epoxide) is frequently implicated (see Sims and Grover 1974). Thus, benzo[a]pyrene is metabolized to its 7,8-dihydrodiol-9,10-oxide which is thought to be an ultimate carcinogen of the procarcinogenic benzo[a]-pyrene (Sims *et al.*, 1974). Aflatoxin B_1, also a potent naturally occurring mycogenic carcinogen, is thought to be activated via epoxidation to the 2,3-oxide (Swenson *et al.*, 1974). Safrole (Borchert *et*

al., 1973), vinyl chloride (Gorrod, 1979), estragole (Drinkwater et al., 1976) and acetylaminofluorene (Weisburger et al., 1964) are further examples of chemicals requiring oxidation to exert their full toxic potential.

Metabolic oxidation, as stated earlier (see Chapter One) is an important mechanism of transformation of most drugs and other xenobiotics. For many such substances to which man is exposed, oxidative metabolism represents a means of detoxication, either directly or in preparing the substance for further (Phase II) metabolism to excretory water-soluble conjugates. Such is the case for diphenylhydantoin (phenytoin) which is metabolized in man to its para-hydroxy derivative, the latter being conjugated with glucuronic acid prior to urinary excretion. In cases where the para-hydroxylation is grossly impaired, as occurs in some epileptic patients (Kutt et al., 1964), the parent drug can rapidly accumulate to toxic levels. This principle is particularly important for lipophilic drugs like phenytoin, whose elimination from the body is by metabolism to more polar products. It is easy to calculate for a drug which is only, say, 0.1% per day eliminated unchanged, that in the complete absence of metabolism, the overall elimination rate constant

K_{el} would be 0.001 day^{-1}, that is the elimination half-life ($t_{\frac{1}{2}\beta}$) would be 693 days, about 2 years. Providing first-order conditions occur throughout, a body burden of 1% of the original single dose would still be found after 13 years (6.5 $t_{\frac{1}{2}\beta}$).

Variability between subjects in their oxidative capacity cannot be overlooked in pharmacokinetic, therapeutic or toxicological terms. This problem has concerned the pharmaceutical industry, regulatory bodies, clinical pharmacologists and toxicologists, for many years (see Committee on Problems of Drug Safety, 1969). The recognition of genetic polymorphism with identifiable deficient oxidizers has partly helped in our understanding of the origins of such gross variability.

Nevertheless, assessment of the wider implications of genetic factors in drug safety is hard to make directly for a multitude of reasons. In vitro testing can provide evidence of chemical toxicity in isolated systems, but whether or not this data can be extrapolated to the intact-living animal is not always clear. Direct testing for carcinogenicity, cytotoxicity or teratogenicity is obviously impossible in man. Hence the need for live animal experiments.

If genetic factors, such as polymorphic metabolism, play a part in the assessment of drug safety, then it would be important to demonstrate their existence in non-human animal populations. Apart from ethical considerations, the use of animals has many other advantages in pharmacogenetics. Large and frequently available litters are generally obtainable which facilitate genetic investigations, especially when particular hybrids need to be bred. Additionally, numerous environmental factors affecting response to drugs can be rigorously controlled in animals with much greater ease than in man.

The aim of the work described in this Chapter was to see whether or not the genetic polymorphism of debrisoquine hydroxylation described in man also occurred in another mammalian species, namely the rat. This species was chosen since limited data available suggested that the rat hydroxylates debrisoquine (Allen et al., 1975; 1976; Angelo, 1976), whereas the mouse did not (Ritchie et al., 1980). Intuitively, it was thought easier to evaluate rat strains for a deficient metabolism rather than strains of mice.

Laboratory animal species have previously been described to exhibit genetic polymorphism with respect to drug

metabolism reactions. Thus, the acetylation of arylamines and hydrazines has been shown to exhibit polymorphisms in strains of mice and various breeds of rabbits (Frymoyer and Jacox, 1963a,b, Tannen and Weber, 1979).

2.2. MATERIALS AND METHODS

2.2.1 Animals

The animals used are shown in Table 2.1. All the animals were inbred with the exception of the Wistar rat. Animals were maintained on Labshure 41B diet with free access to water.

2.2.2 Chemicals and radiochemicals

Debrisoquine hemisulphate, 4-, 5-, 6-, 7- and 8-hydroxydebrisoquine were the gift of Roche Products Limited, Welwyn Garden City, U.K.; 7-Methoxyguanoxan was the gift of Pfizer Limited, U.K. All these compounds had previously been characterized by C, H and N analysis and by mass spectrometry.

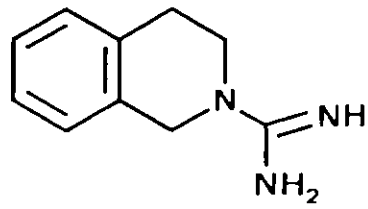
The properties of these compounds were as follows: 3,4-dihydro-2-(1H)-isoquinoline carboxamidine hemisulphate (Debrisoquine; m.p. 274-276°C, Fig. 2.1 compound I). 4-Hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate (4-hydroxydebrisoquine; m.p. 254-255°C Fig. 2.1 compound II). 5-Hydroxy-3, 4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate (5-hydroxy-debrisoquine; m.p. 298-300°C Fig. 2.1 compound III).

Table 2.1 Suppliers of rats used and some physical characteristics

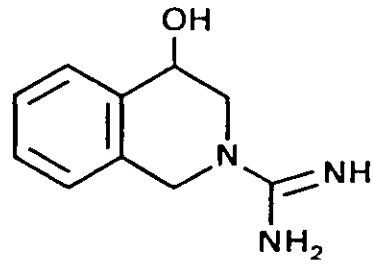
Strain	Supplier	colour of eyes	colour of hair
Wistar	SMHMS	red	white
Lewis	SMHMS	red	white
Fischer	SMHMS	red	white
A/GUS	Bantin & Kingman	black	white/black hood
PVG	Bantin & Kingman	black	brown
DA	Bantin & Kingman	black	brown
BN	SMHMS	black	dark brown
Long-Evans	Charing Cross Hospital	red	white
Sprague - Dawley	Lions Lab., Little Lions Farm, Hampshire.	red	white
WAG	OLAC	red	white

SMHMS = St. Mary's Hospital Medical School colony.

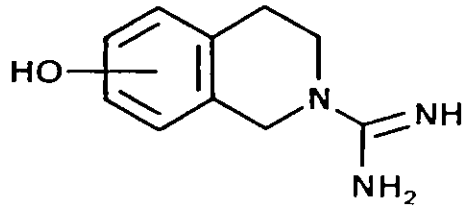
Figure 2.1 Chemical structure of debrisoquine and its authentic metabolites together with the internal standard



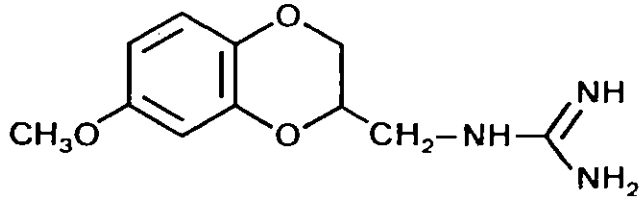
I



II



III



IV

6-Hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate (6-hydroxydebrisoquine; m.p. not determined Fig. 2.1 compound III). 7-Hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate (7-hydroxydebrisoquine; m.p. 303°C Fig. 2.1 compound III). 8-Hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate (8-hydroxydebrisoquine; m.p. 203-304°C Fig. 2.1 compound III). 7-Methoxy-2-guanidinomethyl-1,4-benzodioxan (7-methoxyguanoxan; m.p. undetermined, Fig. 2.1 compound IV).

Other compounds used: hexafluoroacetylacetone (Koch-Light Laboratories Limited, Colnbrook, U.K.). N,O-bis(trimethylsilyl)acetamide (BSA) (Pierce and Warriner Limited, Chester, U.K.). Testosterone propionate (Paines and Byrne Limited, Greenford, U.K.) estradiol benzoate (Paines and Byrne Limited, Greenford, U.K.), 20-methylcholanthrene (Sigma Chemical Company, Poole, U.K.) and phenobarbitone sodium (Sigma Chemical Company Poole, U.K.) were all purchased commercially. [^{14}C]-Debrisoquine hydrochloride was the gift of Roche Products Limited, Welwyn Garden City, U.K. and had a specific activity 2.4μCi/mg.

Reagents used for the preparation of microsomes for the binding spectra work were all obtained from commercial

sources in Switzerland. These experiments were carried out in the Department of Clinical Pharmacology, University of Bern in collaboration with Dr. A. Küpfer.

2.2.3 Dosing of animals and collection of excreta

Various experiments were performed with debrisoquine (equivalent to 5mgkg^{-1} body weight, free base), [^{14}C]-debrisoquine (equivalent to 5mgkg^{-1} body weight free base, 0.24μCi/rat) and 4-hydroxy-debrisoquine (5mgkg^{-1}). In all cases, the drugs were administered as neutral solutions in water directly into the stomach using a modified spinal needle.

Animals were housed in either metal, plastic or glass metabolism cages which separated faeces from the urine which were collected for 24h. Cages were washed thoroughly with water, which was combined with the urine to give a final volume of 50-100ml. Urine (20ml) from the final volume and was kept in plastic containers which were stored at -20°C until analysed.

2.2.4 Preparation of faeces for g.c. analysis

Faeces were homogenized in distilled water in the homogenizer to a final volume of 80ml. Following centrifugation for 10 min. at 3000 r.p.m. the supernatant was removed and kept at -20°C until analysed.

2.2.5 Preparation of faeces for ^{14}C analysis

Faeces were homogenised in distilled water in the homogenizer to a volume of 80ml. The homogenate was bleached by the following method:
Homogenate (5ml), 3M NaOH (1ml), octan-2-ol (0.5ml) and 100 vol H_2O_2 (10ml) were mixed and left to stand in a fume cupboard in 100ml conical flasks covered with aluminium foil. After two days, concentrated HCl (1ml) was added and the mixture boiled for 30 seconds, cooled and then neutralized to pH 7-8 with solid sodium bicarbonate. This neutral solution was adjusted to 50ml with water and then kept in a plastic container stored at -20°C until counted for ^{14}C.

2.2.6 Hormone treatment

Male and female rats of the DA and Lewis strain, weighing 150-180g were used. The rats were castrated (see section 2.2.10) 21 days before phenotyping with debrisoquine (see Results section) and treated with testosterone propionate ($5mgkg^{-1}$) or estradiol benzoate ($0.5mgkg^{-1}$) for ten days and further phenotyped. The two steroids were given subcutaneously (vehicle: ethyloleate). Both castrated and intact control rats were given vehicle only. In other experiments, higher doses of these steroids were given to intact male and female DA and Lewis rats as follows: testosterone propionate ($25mgkg^{-1}$) and estradiol benzoate ($2.5mgkg^{-1}$) were injected twice daily (i.p.) for four days and on the fifth day, debrisoquine (1mg) was given orally for purposes of phenotyping. Such pharmacological doses of testosterone propionate and estradiol benzoate were chosen in an attempt to overwhelm the effect of the endogenous sex steroids.

2.2.7 Induction of debrisoquine metabolism

Female rats of DA and Lewis strains, weighing about 150-160g, were used. 20-Methylcholanthrene ($80mgkg^{-1}$

in corn oil) was given to the rats in a single i.p. dose 48h before phenotyping the rats. These studies were carried out in a small laboratory dedicated to work with chemical carcinogens. Corn oil was also given i.p. to control rats. Sodium phenobarbitone (equivalent to 100mgkg^{-1} free acid) was given i.p. in saline (0.9% v/v) daily for three days and the animals phenotyped with debrisoquine on the fifth day. Saline alone was given i.p. to control rats.

2.2.8 Counting of [^{14}C]-debrisoquine

In cases where [^{14}C]-debrisoquine was administered to rats, aliquots of urine or faecal homogenates were counted for ^{14}C content as follows: urine (1ml) or faecal homogenate (2ml) were counted in duplicate in plastic inserts within glass vials containing a triton-toluene scintillation cocktail (5ml) of the following composition:-

Triton-X-100 (Koch-Light Laboratories Limited) 33%; 2,5-diphenyloxazole (PPO; Fixons Scientific Apparatus Limited) 550mg/100ml; 1,4-di-2-(5-phenyloxazoyl)-benzene (POPOP; Fisons Scientific Apparatus Limited) 10mg/100ml; toluene (May and Baker Limited) 66.6%.

The vials were then counted in a liquid scintillation spectrometer operating in the external standard mode (Packard Model 3385), after sufficient time had elapsed for cooling of the samples in the spectrometer. Efficiency of counting was generally 80-90%.

2.2.9 Analytical procedures

2.2.9.1. Determination of debrisoquine and 4-hydroxy-debrisoquine in urine and faeces

This was carried out essentially according to method described by Idle et al. (1979b). To urine or faecal homogenate (100µl and 200µl respectively) was added 7-methoxyguanoxan solution (internal standard; 50µl of a 20µg/ml solution), 1M $NaHCO_3$ (100µl), hexafluoro-acetylacetone (50µl) and redistilled benzene (1ml) and the mixture heated in a screw top septum vial at 100°C in an aluminium heating block for 1h. The samples were allowed to cool to room temperature, whereupon 3M NaOH (5ml) and benzene (2ml) were added, the mixture vortexed and then centrifuged at 2000 rpm for 5 minutes.

Benzene upper layers were analysed by gas chromatography. Samples (1µl) were injected into either a Pye Unicam GCD or a Pye Unicam 204 gas chromatograph (oven

temperature 190°C, injection port temperature 250°C) fitted with an OV-1 column (3% on chromosorb WHP; 1.83m length, 3mm internal diameter) with oxygen-free nitrogen carrier gas flow rate of 60ml min^{-1} (pressure 20 p.s.i.g.). Bis- (trifluoromethyl) pyrimidine derivatives of debrisoquine and 4-hydroxy-debrisoquine were detected using an electron-capture detector (temperature 205°C).

A typical gas chromatographic trace is shown in Fig. 2.2 and retention times (sec) of these compounds are given in Table 2.2. The electron-capture detector signal output was analysed by either DP 101 computing integrator (Pye Unicam) fitted with a calculator accessory or a Spectra Physics 4100 integrator. These devices located the peaks of interest, calculated their area above base line and gave a final output of debrisoquine and 4-hydroxydebrisoquine concentration in urine (or faeces) in $\mu g ml^{-1}$ after calibration with suitable standards in rat urine. Calibration curves were linear for both these compounds over a range 1-10.0$\mu g ml^{-1}$ (see Fig. 2.3). The coefficient of variation for each point on the calibration curve was estimated (n=3) and never exceeded 3%.

Figure 2.2 Typical chromatogram of debrisoquine and 4-hydroxydebrisoquine

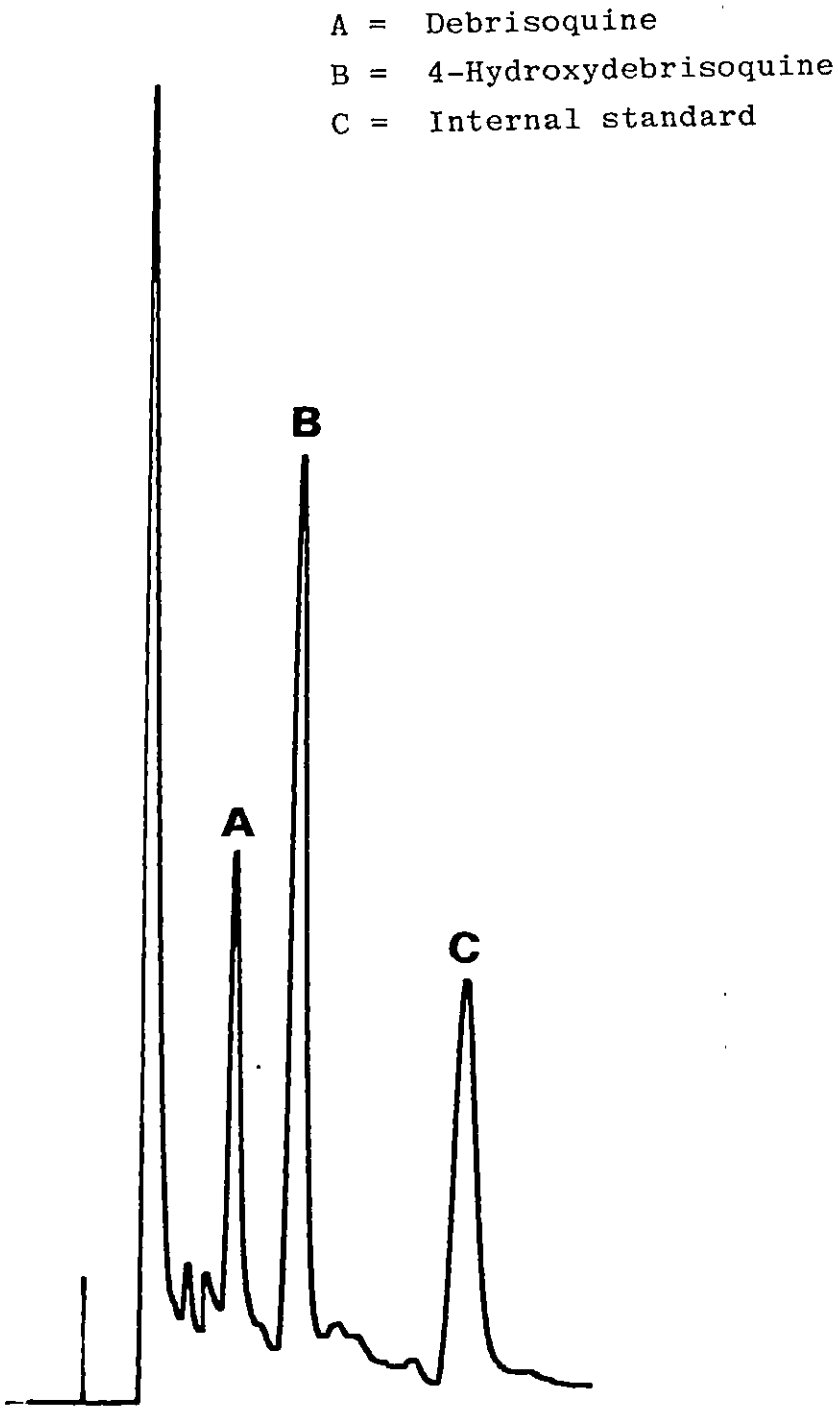
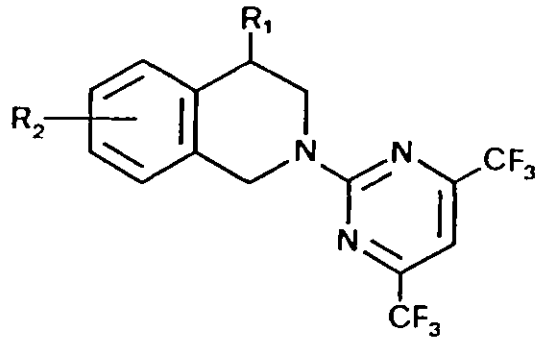
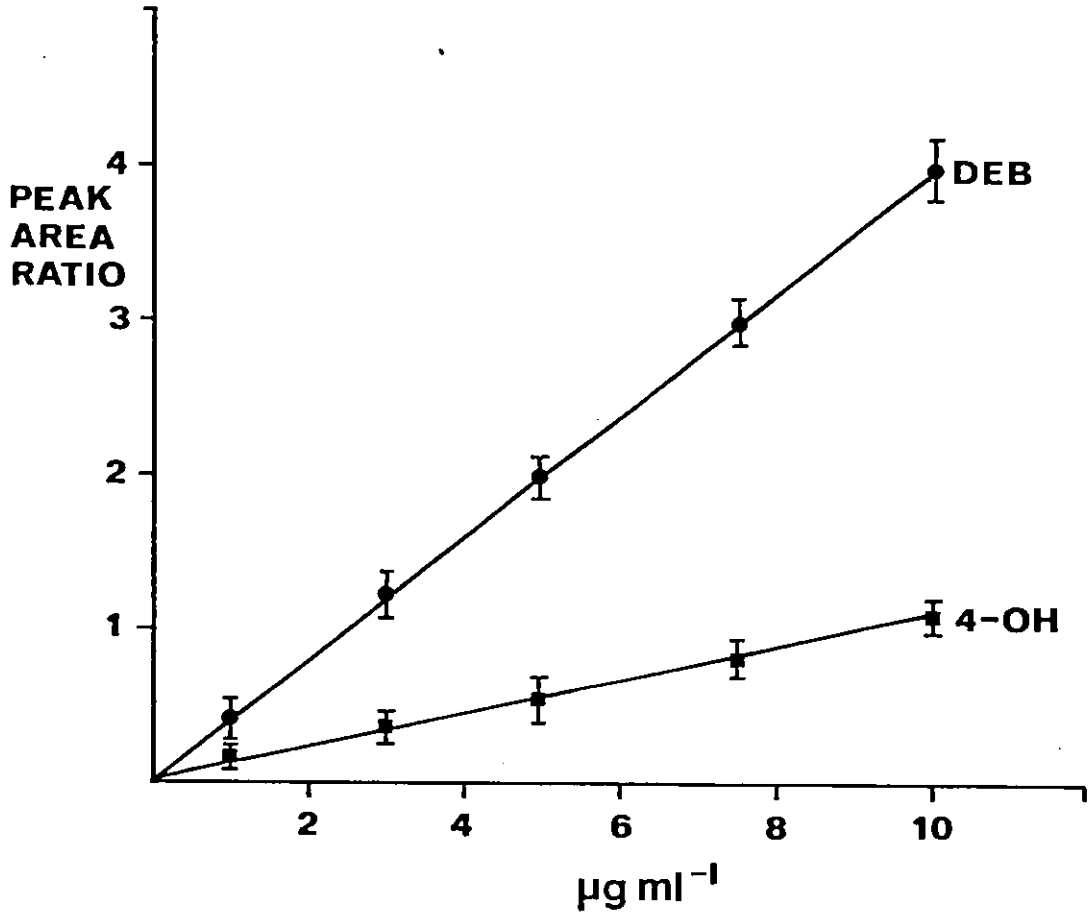


Table 2.2 Gas chromatographic characteristics of derivatized debrisoquine and its metabolites on 3% OV-1.



Compound	R_1	R_2	Retention time (sec) 190°C	160°C
Debrisoquine	H-	H-	75	-
4-Hydroxy-debrisoquine	HO-	H-	124	-
5-Hydroxy-debrisoquine	H-	$(CH_3)_3SiO$-	-	630
6-Hydroxy-debrisoquine	H-	$(CH_3)_3SiO$	-	795
7-Hydroxy-debrisoquine	H-	$(CH_3)_3SiO$	-	710
8-Hydroxy-debrisoquine	H-	$(CH_3)_3SiO$	-	577
7-Methoxy-guanoxan	-	-	267	1140

Figure 2.3 Calibration curves for debrisoquine and 4-hydroxydebrisoquine

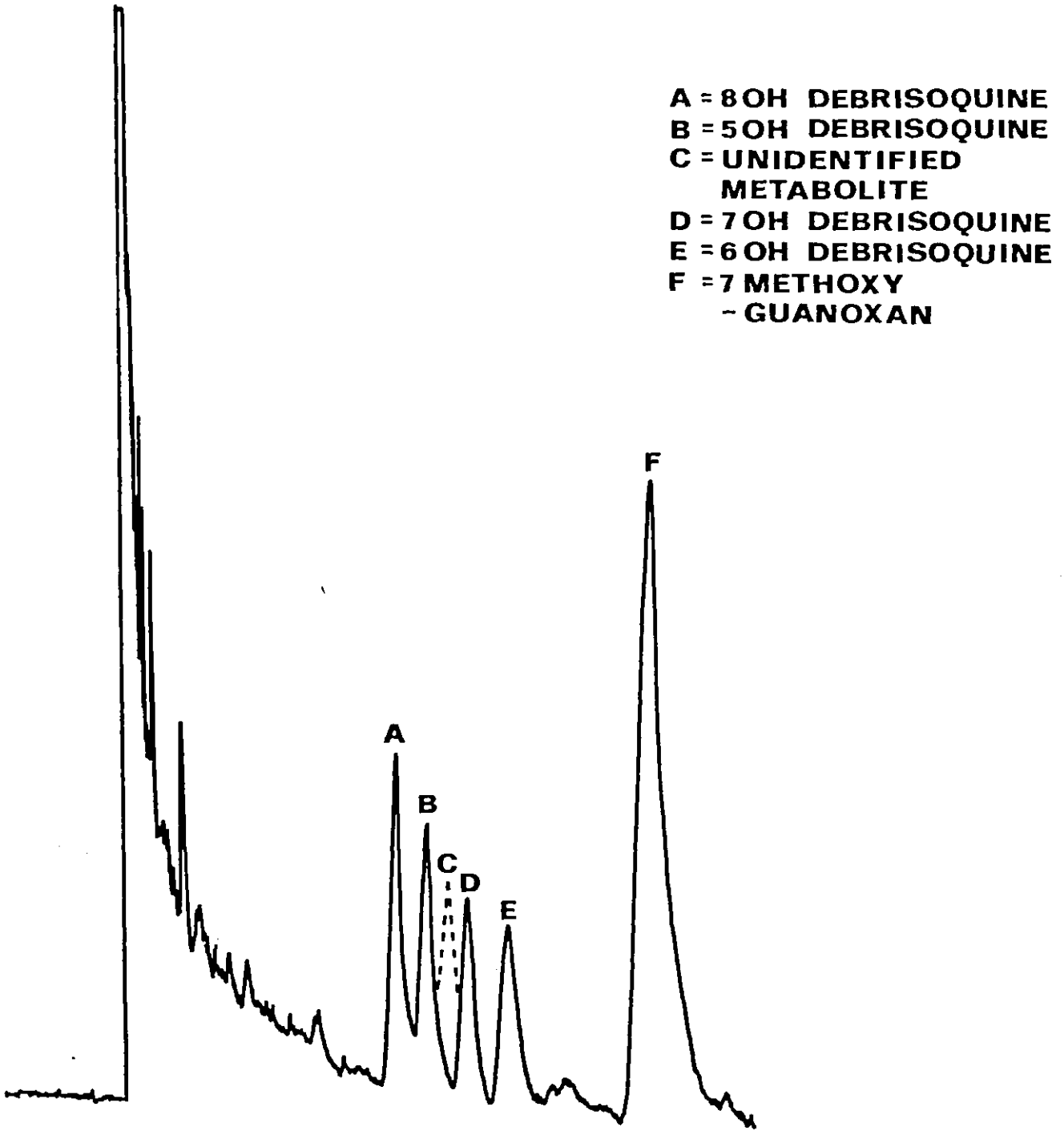


2.2.9.2 Determination of 5-, 6-, 7- and 8-hydroxy-debrisoquine in rat urine and faeces

A modification of the above assay was used for these phenolic metabolites as follows; possible conjugates in urine (1.0ml) were first hydrolysed by heating with 11.6M HCl (200µl) containing internal standard solution (100µl) for 1h at 100°C, then allowed to cool and the acid neutralized with solid sodium bicarbonate. Aliquots of the hydrolysates (500µl) were then treated as before with hexafluoroacetylacetone (100µl) and after the reaction, 3M NaOH (10ml) was added with the addition of further benzene after vortexing and centrifugation, benzene layers (200µl) and BSA (20µl) were mixed and used for gas chromatography.

Gas chromatographic conditions for this assay were: column temperature (160°C) and oxygen-free nitrogen carrier gas flow rate (50ml min^{-1}), injection port temperature (250°C). The column (1.83m length, 3mm internal diameter) was packed with 3% OV-1 on chromosorb WHP. The retention times (sec) of the TMS ether derivatives of the bis-(trifluoromethyl)-pyrimidine derivatives of the phenolic metabolites are given in Table 2.2. A typical chromatogram is shown in Fig. 2.4. Signal integration was again performed using the DP 101 computing integrator. When suitably calibrated

Figure 2.4 Typical chromatogram of the phenolic metabolites of debrisoquine



against known standards in rat urine, the equipment described gave concentrations (μgml^{-1}) of the metabolites in samples analysed. This assay was linear for all these derivatives over a range $0.2 - 1 \mu gml^{-1}$ (Fig. 2.5)

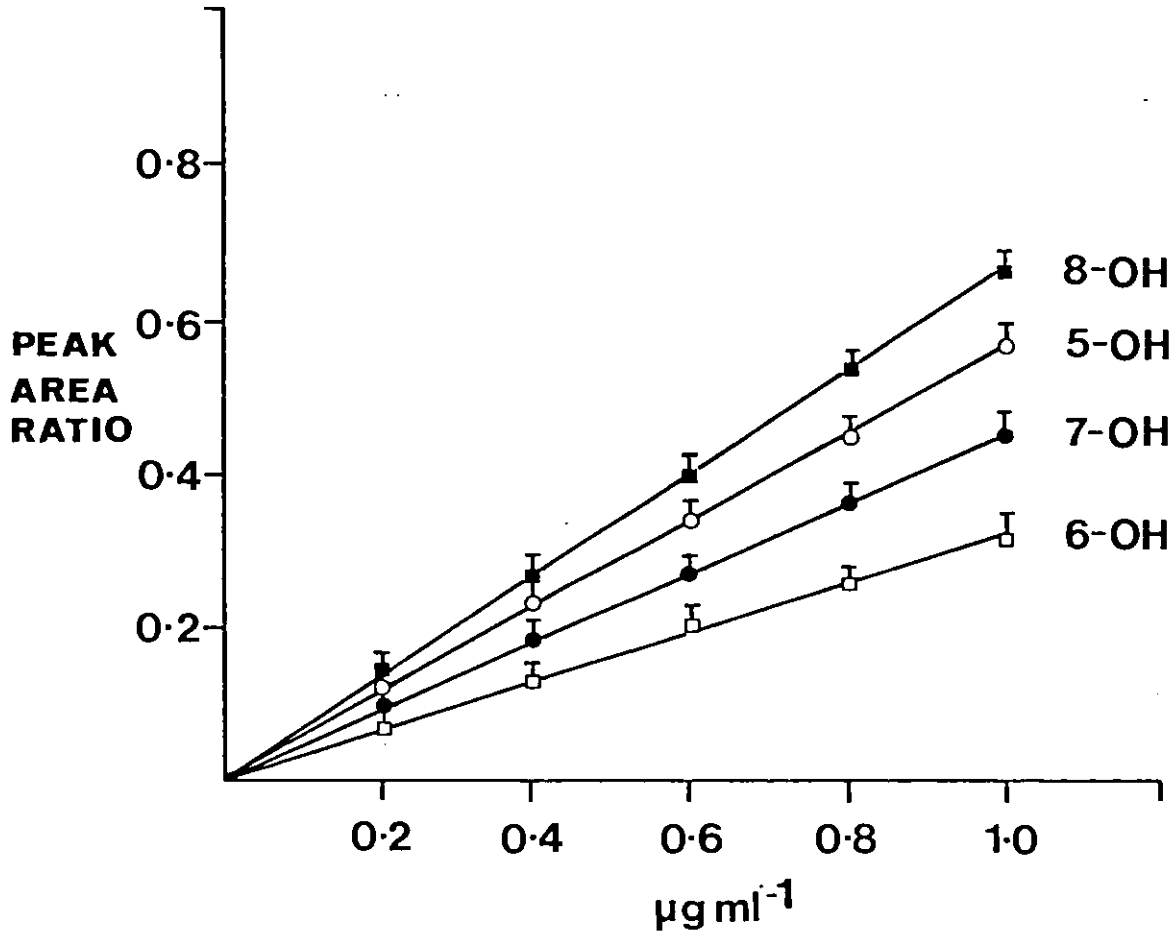
2.2.10 Surgical Procedures

Castration of 3-4 month old male and female rats was made according to the methods described by Waynforth (1980).

2.2.10.1 Orchidectomy

Orchidectomy was carried out on DA and Lewis rats. Each was anaesthetised with ether until it had lost its righting reflex. The rat was laid on its back, and was maintained under ether anaesthesia. The skin was cleaned, a small incision was made (1cm) through the skin at the top of the scrotum. After cleaning the subcutaneous connective tissues, the sacs of testes were easily seen. The testes were pulled out, a single ligature was placed around the blood vessels (spermatic blood vessels) and vas deferens. The testes were removed and the fat was pushed back into the sac. The sac incision was closed with a single suture and

Figure 2.5 Calibration curves for the phenolic metabolites of debrisoquine



the skin incision was also closed with two skin clips. The rats were followed up every day to ensure that there was no sign of inflammation. None of the orchidectomized rats showed any signs of inflammation or infection after these procedures.

2.2.10.2 Ovariectomy

The rat was anaesthetised with ether, laid on its ventral surface and a small midline dorsal skin incision made (approximately half way between the middle of the back and the base of the tail). Another muscle incision was made in order to see the ovary. The ovary was then pulled out with its Fallopian tube, uterine horn, blood vessel and fat. The ovary and Fallopian tube were removed by cutting at the junction between the Fallopian tube and the uterine horn. The other ovary was removed from the other side in the same way. The muscle incision was closed with two sutures. For more than two months after the operation, there was no sign of inflammation or infection of these overiectomized rats.

2.2.11 Preparation of hepatic microsomes

Hepatic microsomes were prepared from several

strains of female rats namely, Lewis, DA, Sprague-Dawley and Fischer. After anaesthetizing the animals with ether, the livers were perfused *in situ* with ice-cold saline through the portal vein, the livers then excised and rinsed thoroughly in ice-cold saline. The livers were then chopped into small pieces and homogenized with 5 volumes of sucrose (0.25M) using 6 up and down-strokes in a Dounce glass homogenizer. The homogenates were centrifuged at 900g for 10 min, 900g for 15 min and 19000g for 15 min in a high-speed centrifuge at 4°C.

The supernatant fraction was decanted and centrifuged at 100,000g for 1h in an ultracentrifuge.

The resultant microsomal pellet was washed in 0.15M KCl (20ml) and recentrifuged at 100,000g for 1h. The washed pellet was suspended in potassium phosphate buffer (0.1M, pH 7.4) to give a final protein concentration of $2mgml^{-1}$ corresponding to approximately 1.5nmol cytochrome P-450 ml^{-1}.

Protein was measured by a modified Lowry method (Hartree, 1972) and cytochrome P-450 concentration after its reaction with carbon monoxide (Omura and Sato, 1964).

2.2.12 Acquisition of binding spectra

In preliminary experiments, the approximate K_d values were established (the concentration at which half of the maximal spectral binding, ΔE_{max} $nmol^{-1}$ P-450 occurred) in order to determine the appropriate concentrations for the quantitative spectral binding analysis. At least 6 data points were obtained from each compound (in triplicate) in the concentration range of 0.1 X K_d to 10 X K_d. An Aminco Chance DW-2 UV/VIS spectrophotometer was employed equipped with Aminco Midan TMT Microprocessor data analyser and an Aminco X-Y plotter. Disposable polystyrene semi-micro cuvettes (1ml volume, 1cm path length) were used. The test compound (debrisoquine dissolved in water) and water only were added to the sample and reference cuvettes respectively in 5µl aliquots to reach the various drug concentrations given in the Results section, scans were made between 350-500nm wavelength in the split-beam mode. The spectra were recorded at a scanning speed of 10nm sec^{-1} with a slit width of 3.0nm band pass. After baseline subtraction by the Midian TMT Microprocessor data analyzer, the different spectra were plotted with a sensitivity of E=0.05 full scale.

Spectral binding was quantitated for types I and II difference spectra by the sum of absolute absorbances E at peak and trough wave lengths. The ΔE values were plotted against substrate concentrations in the sample cuvette and the spectral dissociation constant ($K_D \pm$ S.D.) and the ΔE max $\pm$ S.D. values were calculated (Wilkinson, 1961) according to the Michaelis-Menten equation and the number of binding sites involved was determined by graphical analysis of the Scatchard transformations of these spectral binding data.

2.3 RESULTS

2.3.1 Strain differences in debrisoquine 4-hydroxylation in the rat

Females of ten strains of rat (Wistar, Lewis, Fischer, A/GUS, PVG, DA, WAG, BN, Long Evans and Sprague-Dawley) and males of six strains (Wistar, Lewis, Fischer, PVG, DA and BN) were investigated with debrisoquine. In all cases, the 0-24h urinary excretion of debrisoquine and 4-hydroxydebrisoquine was determined after a single oral dose of $5mgkg^{-1}$ debrisoquine. In certain cases, ^{14}C-labelled drug was given to check the total excretion of drug-related material. The measurement of debrisoquine and 4-hydroxydebrisoquine in rat urine was analogous in humans (Mahgoub et al., 1977; Evans et al., 1980) whereby the so-called metabolic ratio (%dose excreted as debrisoquine/%dose excreted as 4-hydroxydebrisoquine) is determined and used as a determinant of phenotype. In man, subjects with ratios > 12.6 are phenotypically poor metabolizers (PM), whilst those with lower values are phenotypically extensive metabolizers (EM). Accordingly, the analogous metabolic ratio was determined for each individual rat. The excretion of debrisoquine, 4-hydroxydebrisoquine and the metabolic ratio for each of the 122 rats studied is given in Appendix I.

For males and females of each strain, means values (± S.D.) of these parameters of debrisoquine metabolism are shown in Table 2.3.

All rats excreted debrisoquine and 4-hydroxydebrisoquine, but the absolute amounts of each varied between strains. The total excretion (debrisoquine plus 4-hydroxydebrisoquine) of these was also found to be highly variable. For example, whilst female WAG rats excreted 44% of the dose as debrisoquine and 4-hydroxydebrisoquine in 24h, only 7% of the dose was found as these components in male Lewis rat urine (see Table 2.3). In general, male rats had lower recoveries of these components than female rats, a point which will be discussed later. Obviously, further metabolites, other than 4-hydroxydebrisoquine are produced by rats and therefore experiments were carried out using ^{14}C-labelled drug (see later) to clarify this point. Additionally, the phenolic metabolites were also determined in rat urine samples (see later).

Variability in the 4-hydroxylation of debrisoquine is well known in human populations (see Introduction, this Chapter), the origins of which would appear to be an allelomorphic gene locus regulating the metabolism by this pathway. This is best observed from a distribution histogram of the metabolic ratio (debrisoquine/4-hydroxy-debrisoquine in urine).

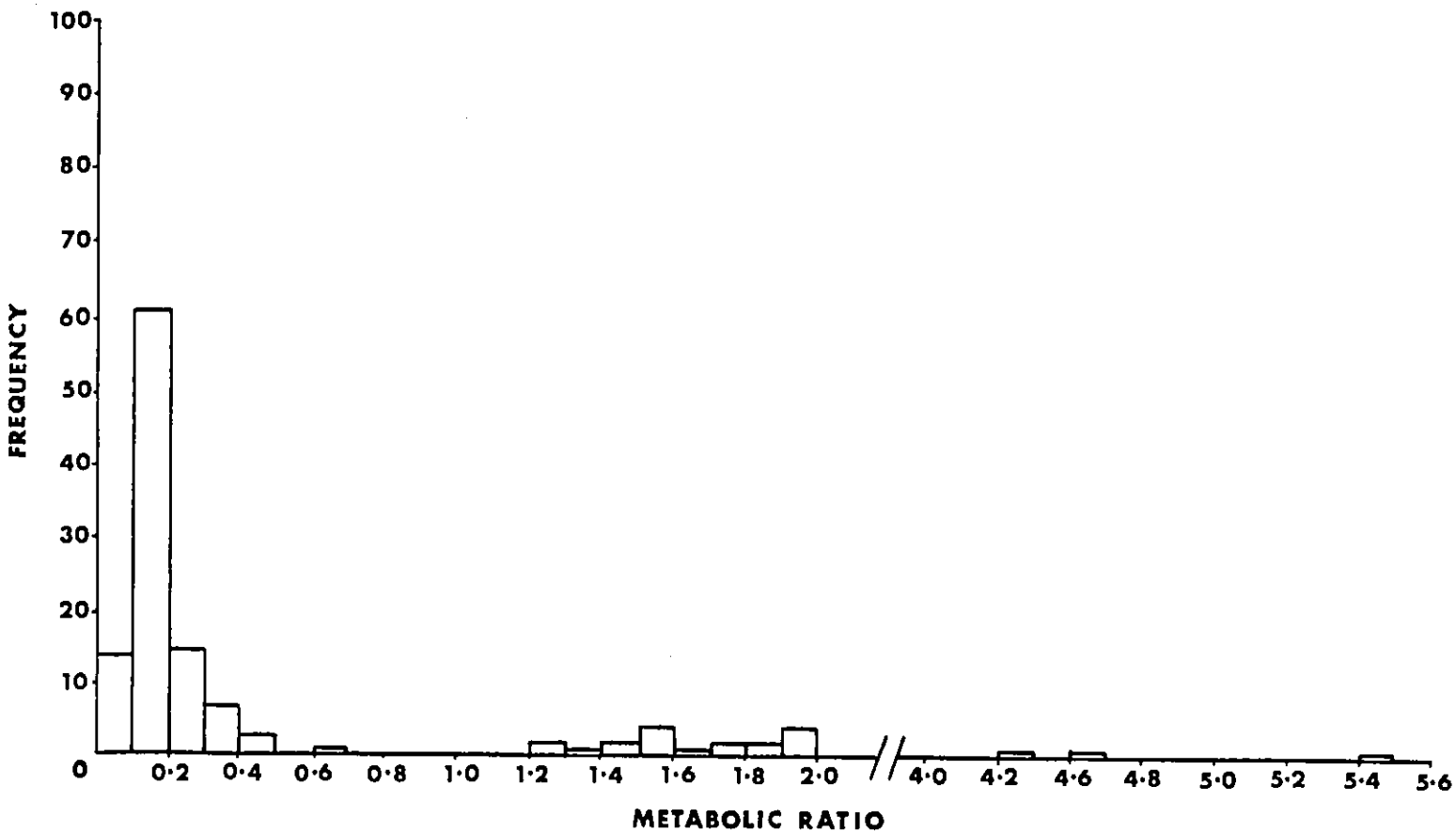
Table 2.3 Mean (± S.D.) excretion of debrisoquine, 4-hydroxydebrisoquine and the metabolic ratio for males and females of each strain studied

Strain	Sex	% dose excreted as: Debrisoquine	4-Hydroxy-debrisoquine	Total	Metabolic ratio
Wistar	F	3 ± 0.8	20 ± 4.4	23 ± 5.0	0.13 ± 0.04
	M	3 ± 0.9	12 ± 5.3	15 ± 6.1	0.23 ± 0.06
Lewis	F	6 ± 3.9	33 ± 9.3	39 ± 12.7	0.16 ± 0.07
	M	1 ± 0.0	6 ± 1.5	7 ± 1.5	0.19 ± 0.04
Fischer	F	3 ± 1.9	12 ± 4.1	15 ± 4.6	0.25 ± 0.15
	M	1 ± 0.5	7 ± 2.0	8 ± 2.3	0.17 ± 0.04
A/GUS	F	3 ± 1.2	15 ± 8.2	18 ± 8.0	0.32 ± 0.30
PVG	F	2 ± 1.3	12 ± 3.6	14 ± 4.5	0.18 ± 0.10
	M	2 ± 0.9	10 ± 7.6	12 ± 8.4	0.21 ± 0.09
DA	F	26 ± 9.1	13 ± 3.6	39 ± 10.2	2.08 ± 1.11
	M	3 ± 1.9	12 ± 1.1	15 ± 2.3	0.24 ± 0.16
WAG	F	9 ± 1.3	35 ± 8.8	44 ± 8.2	0.26 ± 0.10
BN	F	5 ± 1.6	20 ± 4.6	25 ± 5.9	0.23 ± 0.07
	M	2 ± 0.1	14 ± 2.9	16 ± 3.5	0.14 ± 0.03
Long Evans	F	3 ± 1.7	24 ± 2.5	27 ± 2.4	0.15 ± 0.08
Sprague-Dawley	F	3 ± 0.6	24 ± 2.2	27 ± 2.3	0.11 ± 0.03

Therefore, an equivalent histogram of the rat data given in Appendix I was constructed (Fig. 2.6). Metabolic ratios for individual rats of ten strains (both male and female) were plotted between 0.07 and 5.3 in increments of 0.1. Clearly, Fig. 2.6 shows a non-normal distribution of metabolic ratios for the species rat, somewhat akin to that seen in man. Two major modes are apparent. The first, containing data from 101 rats, has metabolic ratios between 0.07 and 0.67, with a modal value of 0.1 - 0.2. A second mode, distributed between 1.25 and 5.30, is also seen. Such a distribution is highly suggestive of a genetic polymorphism in debrisoquine 4-hydroxylation in the rat.

The major point of interest that emerges from the distribution given in Fig. 2.6 is that the mode characterizing relatively impaired debrisoquine 4-hydroxylation (ratios 1.25 - 5.30) comprises purely females of the DA strain. No female DA rats had metabolic ratios outside this mode and no rats from other strains fell into this mode. Thus, it might be proposed that female DA rats express a characteristic of impaired hydroxylation, as judged by the metabolic ratio even though the absolute amounts of 4-hydroxydebrisoquine are sometimes similar to those seen in other strains (see Table 2.3 and Appendix I). This apparent anomaly will be discussed later.

Figure 2.6 Frequency distribution histogram of metabolic ratio for 122 rats given debrisoquine



2.3.2 Administration of [^{14}C]-debrisoquine to rats

Since the 0-24h urinary recovery of debrisoquine and 4-hydroxydebrisoquine in the ten rat strains studied never exceeded 63% (DA female I, Appendix I) and was often considerably lower, experiments using ^{14}C-labelled drug were performed. Three rats of each female DA, female Lewis, male DA, male Lewis were administered ^{14}C-labelled debrisoquine as described.

Table 2.4 shows the 0-24h urinary recovery of ^{14}C together with the recovery of debrisoquine and 4-hydroxy-debrisoquine as estimated by gas chromatography. As can be seen, debrisoquine and 4-hydroxydebrisoquine fall short of the total urinary excretion of ^{14}C-labelled drug, often by a considerable amount. Obviously, other metabolites of debrisoquine are produced by the rat, in the order of 20-40% of the dose. The metabolism of debrisoquine in the rat has been previously studied by Allen *et al*. (1975a, b) and by Angelo (1976), both of whom cite evidence in support of phenolic and possibly dihydroxylated metabolites in rat urine. In the former case, ring-opened acidic metabolites were detected using g.c.m.s., but no proper quantitation was achieved. The results given here would agree with such previous finding. In order to test the notion that

Table 2.4 Comparison of urinary excretion (0-24h) of ^{14}C with debrisoquine and 4-hydroxy-debrisoquine as measured by gas chromatography for various rats given [^{14}C]-debrisoquine

			% dose 0-24h urinary recovery of	
Rat		Sex	*debrisoquine + 4-hydroxydebrisoquine	^{14}C
DA	1	F	33	77
	2	F	57	82
	3	F	53	81
Lewis	1	F	38	50
	2	F	46	65
	3	F	46	62
DA	1	M	14	60
	2	M	16	58
	3	M	13	48
Lewis	1	M	9	60
	2	M	7	48
	3	M	12	51

* by gas chromatography

F = female, M = male

4-hydroxydebrisoquine might be further metabolized in the rat, 6 female rats (three PVG and three BN strain) were each given 4-hydroxydebrisoquine (5mgkg^{-1} p.o.) and urine and faeces collected (vide infra).

2.3.3 Administration of 4-hydroxydebrisoquine to rats

4-Hydroxydebrisoquine (5mgkg^{-1} p.o.) was given to six female rats (three PVG and three BN strain) and urine collected for 0-24h and 24-48h, total 0-72h faeces were also collected. 4-Hydroxydebrisoquine in each sample was determined by gas chromatography. Table 2.5 gives the % dose excreted unchanged in urine and faeces for each rat. 0-24h urine contained 6-13% of the dose, 24-48h urine 1-3% of the dose and 0-72h faeces 15-29% of the dose as unchanged 4-hydroxydebrisoquine, total recovery from both routes of elimination being 29-43% as 4-hydroxydebrisoquine. These data would suggest that either 4-hydroxydebrisoquine is further metabolized by rats or that it is 'trapped' in an entero-hepatic cycle from which it is only slowly eliminated in faeces. It is thought unlikely that the missing 60-70% of the dose is unabsorbed, since faeces were collected for three days.

Table 2.5 Urinary and faecal excretion of 4-hydroxy-debrisoquine by rats given 4-hydroxy-debrisoquine (5mg/kg^{-1} p.o.)

			% dose excreted as 4-hydroxydebrisoquine in			
Rat		Sex	0-24h urine	24-48h urine	0-72h faeces	Total
PVG	1	F	8	1	22	31
	2	F	12	2	15	29
	3	F	9	2	20	31
BN	1	F	13	3	17	33
	2	F	6	1	27	34
	3	F	13	1	29	43

2.3.4 Strain differences in debrisoquine phenolic metabolite excretion in the rat

Urine samples from females of seven strains of rat which were analysed for their content of debrisoquine and 4-hydroxydebrisoquine were also analysed for the presence of phenolic metabolites (5-, 6-, 7- and 8-hydroxydebrisoquine, see Fig. 2.1) by a secondary derivatization using BSA.

In all seven strains so studied, only 6-hydroxy-debrisoquine was observed together with an unidentified metabolite which eluted at 680 sec between 5- and 7-hydroxydebrisoquine (see Table 2.2 and Fig. 2.4). Approximate concentrations of this metabolite, which is likely to be phenolic since it does not appear on gas chromatography without derivatization with BSA, were estimated from the calibration curves for the known phenolic metabolites (Fig. 2.5). Table 2.6 shows the % dose excreted in seven rat strains of 6-hydroxy-debrisoquine, with the exception of Wistar rats which produced 13-16%. Wistar also excreted larger amounts (10-14%) of the unidentified metabolite than the other strains (1-10%).

Table 2.6 Urinary excretion (0-24h) of 6-hydroxy-debrisoquine and the unidentified phenolic metabolite for females of seven strains of rat given debrisoquine (5mg/kg^{-1} p.o.)

Strain		% dose excreted as: Debrisoquine	4-Hydroxy-debrisoquine	6-Hydroxy-debrisoquine	Unidentified metabolite	Total
Wistar	1	3	21	13	10	47
	2	3	26	14	10	53
	3	3	24	16	14	57
Lewis	1	11	44	5	8	68
	2	17	55	5	5	82
	3	14	46	6	8	74
Fischer	1	7	14	6	3	30
	2	2	7	2	1	14
	3	4	10	1	1	19
A/GUS	1	4	22	6	11	43
	2	4	6	5	3	18
	3	2	17	2	1	22

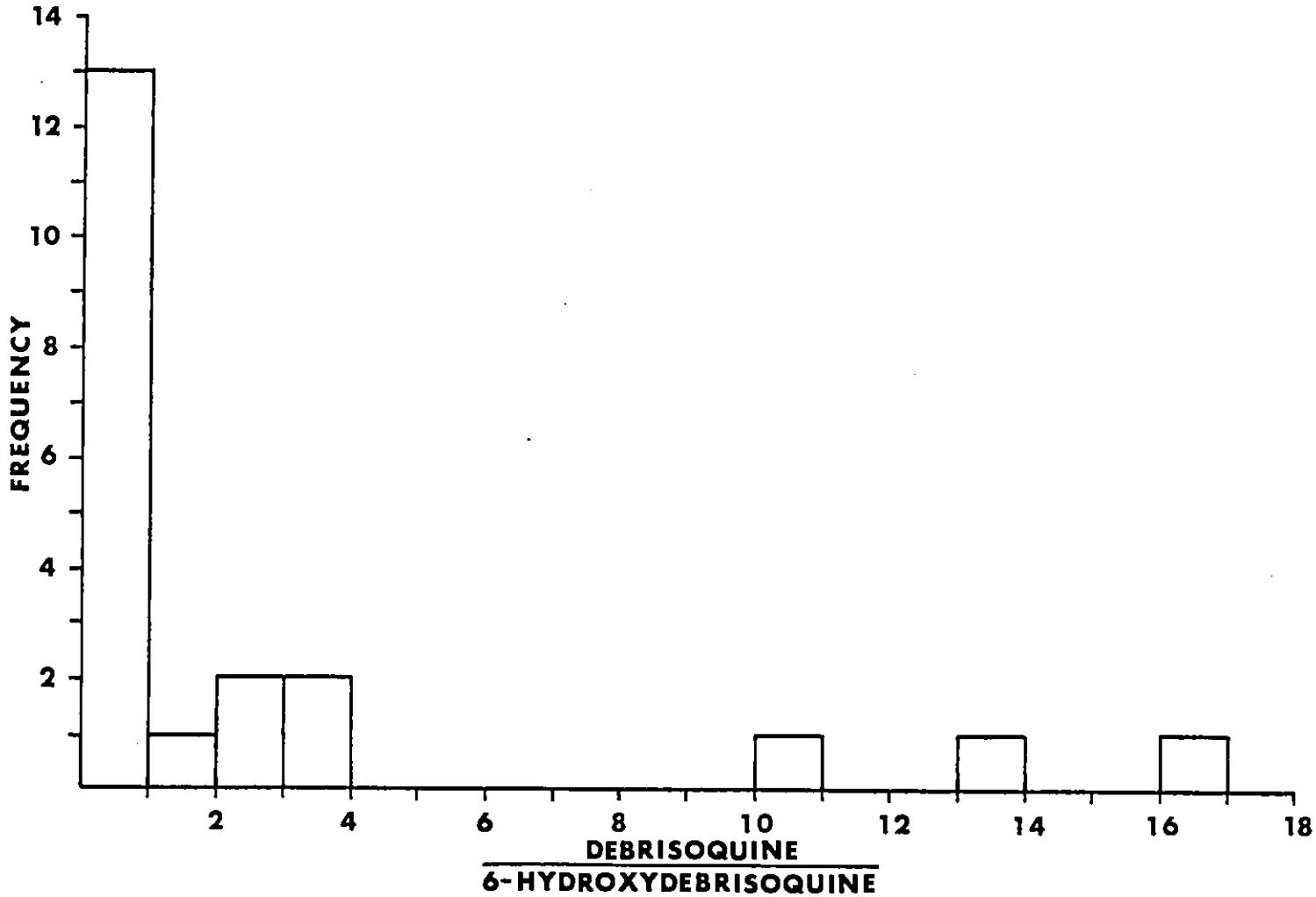
Table 2.6 (continued)

Strain		% dose excreted as: Debrisoquine	4-Hydroxy-debrisoquine	6-Hydroxy-debrisoquine	Unidentified metabolite	Total
PVG	1	2	13	7	7	29
	2	4	10	8	6	28
	3	3	20	7	6	36
BN	1	3	15	6	10	34
	2	6	17	8	10	41
	3	4	9	4	7	24
DA	1	53	10	4	5	72
	2	34	8	2	5	49
	3	31	7	3	6	47

Fig. 2.7 shows the frequency distribution of ratio debrisoquine/6-hydroxydebrisoquine for the twenty one female rats of seven strains. Two modes are apparent, as for the analogous 4-hydroxydebrisoquine ratio. The major mode was distributed from 0.2 - 4.0 and the minor mode from 10-17. Again, the high value mode comprised only female DA rats, with no DA rat appearing in the major mode.

It is interesting to speculate upon the nature of the unidentified phenolic metabolite. Both Allen et al. (1975) and Angelo (1976) give evidence in support of a dihydroxydebrisoquine metabolite in rat urine which, on mass spectrometric analysis had a fragmentation pattern analogous to 4-hydroxydebrisoquine (which readily loses H_2O to produce the base peak) and in particular, Angelo (1976) considered that this may arise from further metabolism of 4-hydroxydebrisoquine to give a 4,5-, 4,6-, 4,7- or 4,8-dihydroxydebrisoquine. Since 6-hydroxydebrisoquine seems to be the only aromatic monohydroxylation product in the rat (see Table 2.6), it is possible that 4,6-hydroxydebrisoquine, arising from a combination of the two major single pathways, is the unidentified metabolite described herein. Interestingly, strains which produce little of this metabolite (e.g. Fischer, 1-3%, Table 2.6) excrete

Figure 2.7 Frequency distribution histogram of ratio debrisoquine/ 6-hydroxydebrisoquine for 21 female rats given debrisoquine (5mgkg^{-1} p.o.)



less 6-hydroxydebrisoquine (1-6%) than say Wistar which excreted both of these metabolites in respectable amounts.

Further evidence supporting this viewpoint is that when 4-hydroxydebrisoquine was administered to rats (vide supra), the same unidentified metabolite, eluting at 680 sec in the phenol assay, was observed, confirming that it is produced metabolically from 4-hydroxy-debrisoquine. However, the exact position of the putative second hydroxyl group remains unclear.

2.3.5 Breeding studies

It has been established above that when debrisoquine is administered orally to certain inbred strains of the rat, the parameters urinary debrisoquine/4-hydroxydebrisoquine and debrisoquine/6-hydroxydebrisoquine are bimodally distributed, with only female DA rats comprising a minor mode with high values of these parameters. It is inferred from these data that this is a reflection of a relative impairment of debrisoquine 4- and 6-hydroxylation in female DA rats, which is not seen in male DA's. These data are also highly suggestive

of the existence of a genetic polymorphism in these metabolic pathways of the rat, as is the case in man. In order to test this hypothesis more carefully, two strains were chosen (Lewis and DA) which represent extensive and poor debrisoquine hydroxylation characters respectively and F_1 hybrids bred from these parental strains. Additionally, F_1 X F_1 (F_2) and F_2 X F_2 (F_3) hybrids were bred and, for reasons which will be explained, a certain back-cross was also bred.

Debrisoquine metabolism was studied in fourteen (Lewis X DA) F_1 hybrids, nine female and five male. All animals had metabolic ratios between 0.12 and 0.24 (see Appendix II), thus the DA female characteristic of a high ratio was lost in these hybrids. In the F_2 offspring of brother-sister matings within the F_1 generation, forty one animals were obtained, of which one died prior to metabolic investigation. Whilst the F_1 animals were all a uniform brown in colour, similar to parental DA's, the F_2 animals were completely heterogenous in this respect. Some of these were albino, some hooded, some brown and some black (see Appendix III). Similarly, the metabolic ratio for debrisoquine 4-hydroxylation was also heterogenous in the forty rats studied. Unlike the F_1 hybrids, five of the F_2 had ratios > 1.0 (range 1.2 - 1.8, see Appendix III);

in other words, the DA characteristic had re-emerged in the F_2 generation. More importantly, all five of these animals were female and represented 5/20 (25%) of the female F_2 animals. This is the classical pattern of Mendelian inheritance of a recessive character, whereby it disappears at F_1 and reappears in 25% of the F_2 progeny. In this case, only females (either parental DA or F_2) are affected.

The above data demonstrate that there are two phenotypes for debrisoquine 4-hydroxylation in the rat, one of which, expressed in DA female rats, is inherited in a simple Mendelian fashion and corresponds to a relatively low excretion of metabolite relative to parent drug.

F_3 Hybrids from brother-sister F_2 X F_2 matings were also investigated with debrisoquine, the results of which we give in Appendix IV. It is not unusual for the recessive character to disappear at F_3 and not re-emerge until F_{20}, whereby a new inbred strain would have been obtained (see Swank and Bailey, 1973). This seems to be the case here, where all F_3 animals are phenotypically like the parental Lewis strain (metabolic ratios 0.03 - 0.33, see Appendix IV) and are all uniform in hair colour (brown) like the parental DA strain.

In the case of hair colour, brown is obviously dominant to white as can be seen from the Lewis X DA F_1 progeny.

To test whether or not this recessive characteristic of debrisoquine metabolism is genetically peculiar to female animals, a back-cross was made between (Lewis X DA) F_3 females and DA males. Debrisoquine 4-hydroxylation in offspring of four such matings is given in Appendix V. Forty such animals were studied; twenty four female and 16 male. As these data show (see also Fig. 2.8), the recessive character re-emerges in these back-cross progeny, only in females and in each of the four separate litters. This could not have been the case unless the male DA rat, although not phenotypically like its female counterpart, carried the relative impairment of debrisoquine 4-hydroxylation.

2.3.6 Induction of debrisoquine hydroxylation in various rat strains

In order to investigate whether or not the defective 4- and 6-hydroxylation of debrisoquine in the female DA rat could be removed by induction,

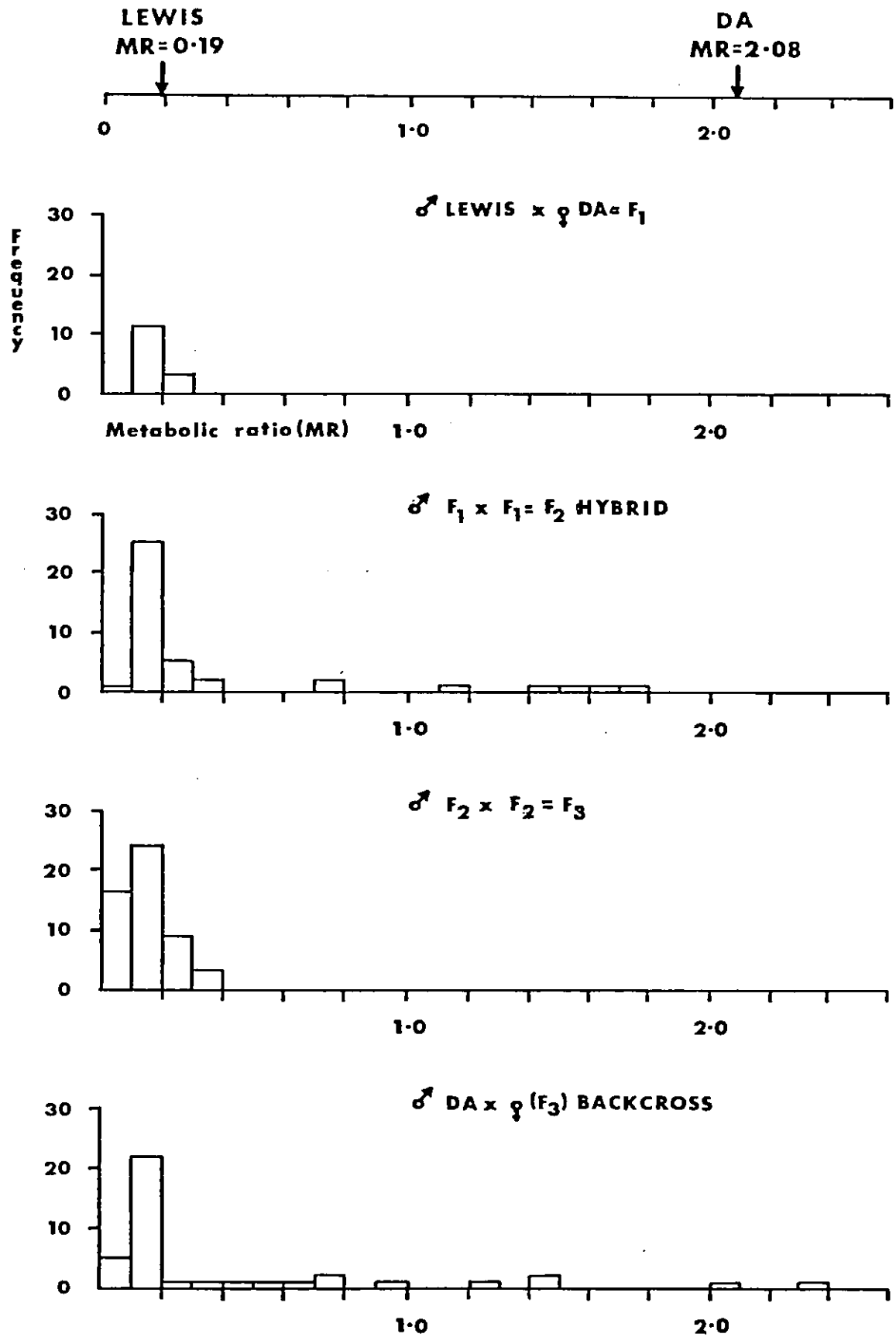


Figure 2.8 Frequency distribution histogram of metabolic ratio for various Lewis x DA hybrids and back-crosses given debrisoquine.

MR = Mean metabolic ratio from Table 2.3.

animals of the Lewis, PVG, BN and DA strain were treated with either phenobarbitone or 20-methylcholanthrene and their debrisoquine hydroxylation re-investigated.

Appendix VI shows the results of these investigations, whilst Table 2.7 gives the mean (± S.D.) results of each experiment. Phenobarbitone was given to Lewis, PVG and DA rats. None of the parameters given in Table 2.7 (% dose as drug and metabolites together with metabolic ratio) were statistically significantly altered by phenobarbitone treatment.

However, when 20-methylcholanthrene was given to Lewis, BN and DA rats, 4-hydroxydebrisoquine elimination increased in Lewis rats (39 ± 2.3 to 55 ± 7.6, $2P < 0.005$) with a concomitant decrease in metabolic ratio (0.14 ± 0.04 to 0.05 ± 0.03, $2P < 0.005$). The metabolic ratio was similarly decreased for BN and DA rats after methylcholanthrene treatment. 6-Hydroxy-debrisoquine excretion was not significantly increased ($2 P > 0.05$) in BN rats treated with methylcholanthrene, nor in those given phenobarbitone.

One additional point of interest is that when DA rats

Table 2.7 Mean (± S.D.) debrisoquine hydroxylation in females of various strains of rat before and after treatment with phenobarbitone and 20-methylcholanthrene.

Group	Mean (± S.D.) Debrisoquine	4-Hydroxy-debrisoquine	6-Hydroxy-debrisoquine	Mean (± S.D.) Metabolic ratio
Lewis control (n=5)	5.4 ± 1.5	39 ± 2.3	-	0.14 ± 0.04
Lewis PB (n=5)	4.0 ± 1.6	38 ± 2.5	-	0.11 ± 0.05
Lewis MC (n=5)	2.4 ± 1.1	55 ± 7.6	-	0.05 ± 0.03
PVG control (n=3)	3.0 ± 1.0	14 ± 4.2	7.3 ± 0.6	0.23 ± 0.10
PVG PB (n=3)	8.3 ± 2.1	18 ± 2.6	8.3 ± 1.5	0.46 ± 0.05
BN control (n=3)	4.3 ± 1.5	14 ± 4.2	6.0 ± 2.0	0.33 ± 0.12
BN MC (n=3)	9.7 ± 2.1	36 ± 11	14 ± 4.6	0.28 ± 0.03
DA control (n=5)	27 ± 6.9	16 ± 3.5	-	1.7 ± 0.18
DA PB (n=5)	24 ± 2.9	19 ± 3.6	-	1.3 ± 0.28
DA MC (n=5)	23 ± 4.1	22 ± 3.2	-	1.1 ± 0.08

All animals were female PB = phenobarbitone treated MC = methylcholanthrene treated
- = not done

were given the phenobarbitone regimen (100mgkg^{-1} i.p. once daily for three days), a number of animals died. After this, half the dose was used, which was apparently non-toxic. Therefore, data given in Appendix VI for the DA rat given phenobarbitone, is partly from animals dosed at 100mgkg^{-1} and partly for animals given 50mgkg^{-1}. It is possible that the oxidative metabolism of phenobarbitone is reduced in female DA rats, thus rendering them susceptible to the toxicity of the compound.

2.3.7 Effect of castration and sex hormone administration upon debrisoquine 4-hydroxylation in male and female Lewis and DA rats.

For these investigations, Lewis and DA rats were chosen as representative of the two rat phenotypes, extensive and poor 4-hydroxylative respectively. Since the poor hydroxylation phenotype is only expressed in female DA rats and not in males, it was of interest to see if hormonal manipulation could modify this situation.

Castration (ovariectomy, oophorectomy) of 3-4 month old female Lewis and DA rats was performed as described earlier (2.2.10). Animals were investigated for their metabolism of debrisoquine before castration, one and

three weeks after castration, and then the groups of six rats were each divided into sub-groups of three. To one sub-group was given testosterone and to the other oestradiol as described previously.

Debrisoquine 4-hydroxylation was then investigated. These data for individual rats are given in Appendix VII and the mean (± S.D.) data are given in Table 2.8. As can be seen, the various treatments had little effect upon Lewis rats, with the exception of a transient increase in excretion of the unchanged drug (4.2 ± 1.3 to 8.8 ± 2.6% $2P < 0.005$) one week after castration which returned to control values after a further two weeks. By stark contrast, castration of DA female rats increased the excretion of the metabolite at three weeks compared to controls (14 ± 3.8 to 27 ± 8.3, $2P < 0.01$) which was also reflected in a change in the metabolic ratio (1.7 ± 0.27 to 0.45 ± 0.23, $2P < 0.001$). Thus removal of the ovaries (and therefore presumably the endogenous oestrogens) was accompanied by an increase in 4-hydroxylation capacity in female DA rats, making them phenotypically like female Lewis, or indeed male DA rats. Testosterone administration decreased the metabolic ratio even further (0.25 ± 0.03, $2P < 0.001$) compared to control values. Interestingly, in the sub-group treated with oestradiol, the poor hydroxylation character re-emerged

Table 2.8 Mean (± S.D.) debrisoquine 4-hydroxylation in castrated and hormone treated female Lewis and DA rats

Group	% dose (0-24h) excreted as: % dose debrisoquine	4-hydroxy-debrisoquine	Metabolic ratio
Lewis Control, (pretreatment, n=6)	4.2 ± 1.3	29 ± 5.5	0.14 ± 0.04
Lewis Castrated, (1 week, n=6)	8.8 ± 2.6	31 ± 5.7	0.28 ± 0.07
Lewis Castrated, (3 week, n=6)	5.5 ± 1.6	29 ± 6.1	0.19 ± 0.05
Lewis Castrated, (testosterone treated n=3)	3.0 ± 1.7	30 ± 4.6	0.11 ± 0.07
Lewis Castrated, (oestradiol treated, n=3)	3.0 ± 1.0	21 ± 3.0	0.14 ± 0.04
DA control (pre-treatment, n=6)	24 ± 8.2	14 ± 3.8	1.7 ± 0.27
DA Castrated (1 week, n=6)	25 ± 2.3	22 ± 3.6	1.2 ± 0.27
DA Castrated, (3 weeks, n=6)	12 ± 6.4	27 ± 8.3	0.45 ± 0.23
DA Castrated (testosterone treated n=3)	7.3 ± 0.58	30 ± 3.0	0.25 ± 0.03
DA Castrated, (oestradiol treated, n=3)	12 ± 2.3	10 ± 1.5	1.2 ± 0.10

as judged by both excretion of metabolite (10 ± 1.5% 2 P < 0.002) and the metabolic ratio (1.2 ± 0.1, 2 P < 0.005) compared to castration only.

It would appear therefore that the poor hydroxylation character is seen only in female DA rats due to some function of sex hormones between males and females, which is further investigated below.

Male Lewis and DA rats were castrated and investigated similarly to castrated females for debrisoquine. Hormone treatment was not carried out for these castrated rats. Detailed results of these studies are given in Appendix VIII and the control male rat data in Appendix I.

Mean (± S.D.) values of excreted drug and metabolite are shown in Table 2.9. Small changes in the metabolic ratio for each strain were observed three weeks after orchidectomy compared to controls (Lewis, 0.19 ± 0.04 to 0.41 ± 0.18, 2 P < 0.001; DA, 0.14 ± 0.03 to 0.27 ± 0.06, 2 P < 0.001). These data would seem to infer that removal of endogenous testosterone in male Lewis and DA rats causes a small shift towards the poor hydroxylator (female DA-like) phenotype, which would be in agreement with the data from castrated female rats described above.

Table 2.9 Mean (± S.D.) debrisoquine 4-hydroxylation in control and castrated male Lewis and DA rats

Group	% dose (0-24h) excreted as: Debrisoquine	4-Hydroxy-debrisoquine	Metabolic ratio
Lewis controls* (n=5)	1 ± 0	6.0 ± 1.5	0.19 ± 0.04
Lewis Castrated (1 week, n=6)	1 ± 0	9.7 ± 3.3	0.12 ± 0.05
Lewis Castrated (3 weeks, n=6)	5.7 ± 2.4	15 ± 3.6	0.41 ± 0.18
DA Controls* (n=5)	4.2 ± 1.1	30 ± 3.0	0.14 ± 0.03
DA Castrated (1 week, n=6)	5.5 ± 1.1	15 ± 2.1	0.37 ± 0.10
DA Castrated (3 week, n=6)	5.5 ± 1.5	20 ± 1.5	0.27 ± 0.06

* data taken from Appendix 1.

The effect of hormonal treatment alone was investigated in intact adult Lewis and DA rats. Oestradiol was given (2.5 $mgkg^{-1}$ i.p. twice daily for four days) to five of each male and female Lewis and DA rats and testosterone was given (25$mgkg^{-1}$ i.p. daily for four days) to three of each male and female Lewis and DA rats. Debrisoquine 4-hydroxylation was investigated in these animals on the fifth day of study.

The metabolic findings in female rats were given in Appendix IX and in male rats in Appendix X. Mean (± S.D.) metabolic parameters are shown in Tables 2.10 and 2.11 for female and male rats respectively. Inspection of the data in these two tables confirms the findings of the castration experiments whereby testosterone treatment decreases the metabolic ratio in DA rats (both males and female) whilst oestradiol increases the metabolic ratio.

Interestingly, in Lewis males (Table 2.11) whilst the metabolic ratio is the same for both oestradiol and testosterone treatments, the absolute amounts of urinary components measured are markedly different. 4-Hydroxy-debrisoquine excretion in those rats treated with testosterone was only 5.7 ± 1.5% whilst for those treated with oestradiol it was 17 ± 2.7%. One possible

Table 2.10 Mean (± S.D.) debrisoquine 4-hydroxylation in intact female adult Lewis and DA rats treated with oestradiol and testosterone.

Group	% dose (0-24h) excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic ratio
Lewis (oestradiol n=5)	3.4 ± 0.60	27 ± 5.1	0.13 ± 0.02
DA (oestradiol n=5)	28 ± 5.2	14 ± 0.6	2.0 ± 0.29
Lewis (testosterone, n=3)	5.7 ± 1.2	25 ± 4.0	0.23 ± 0.02
DA (testosterone, n=3)	15 ± 2.1	18 ± 2.3	0.87 ± 0.03

Table 2.11 Mean (S.D.) debrisoquine 4-hydroxylation in intact male adult Lewis and DA rats treated with oestradiol and testosterone.

	% dose (0-24h) excreted as:		
Group	Debrisoquine	4-hydroxy-debrisoquine	Metabolic ratio
Lewis (oestradiol, n=5)	3.2 ± 0.8	17 ± 2.7	0.19 ± 0.05
DA (oestradiol, n=5)	6.8 ± 1.1	19 ± 2.6	0.36 ± 0.07
Lewis (testosterone, n=3)	1 ± 0	5.7 ± 1.5	0.19 ± 0.06
DA (testosterone, n=3)	2.3 ± 0.6	23 ± 3.0	0.10 ± 0.03

interpretation of these latter findings is that testosterone stimulates the metabolism of debrisoquine by other pathways, thus shifting the balance of metabolism away from 4-hydroxylation. This may partly explain the observations described earlier (see Appendix I) whereby male rats in general have a lower recovery of debrisoquine and 4-hydroxydebrisoquine compared to the recovery of total ^{14}C in urine, when ^{14}C-labelled drug was given (see Table 2.4). In this case, endogenous testosterone may be responsible in some way for a shift in metabolism towards other products.

In summary, therefore, the sex difference in debrisoquine 4-hydroxylation described for the DA rat in which the female expresses a poor hydroxylation phenotype which is inherited in a Mendelian recessive fashion, seems to have a hormonal basis. This phenotype is abolished in female DA rats by either castration, testosterone treatment or both.

Male DA rats, which clearly carry the gene for this phenotype (as shown by the breeding studies) cannot be made phenotypically poor hydroxylators however, by castration or oestradiol treatment. Further investigations may be required to clarify this paradox.

2.3.8 Spectral studies of the binding of debrisoquine to hepatic-derived cytochrome P-450 from Lewis and DA rats.

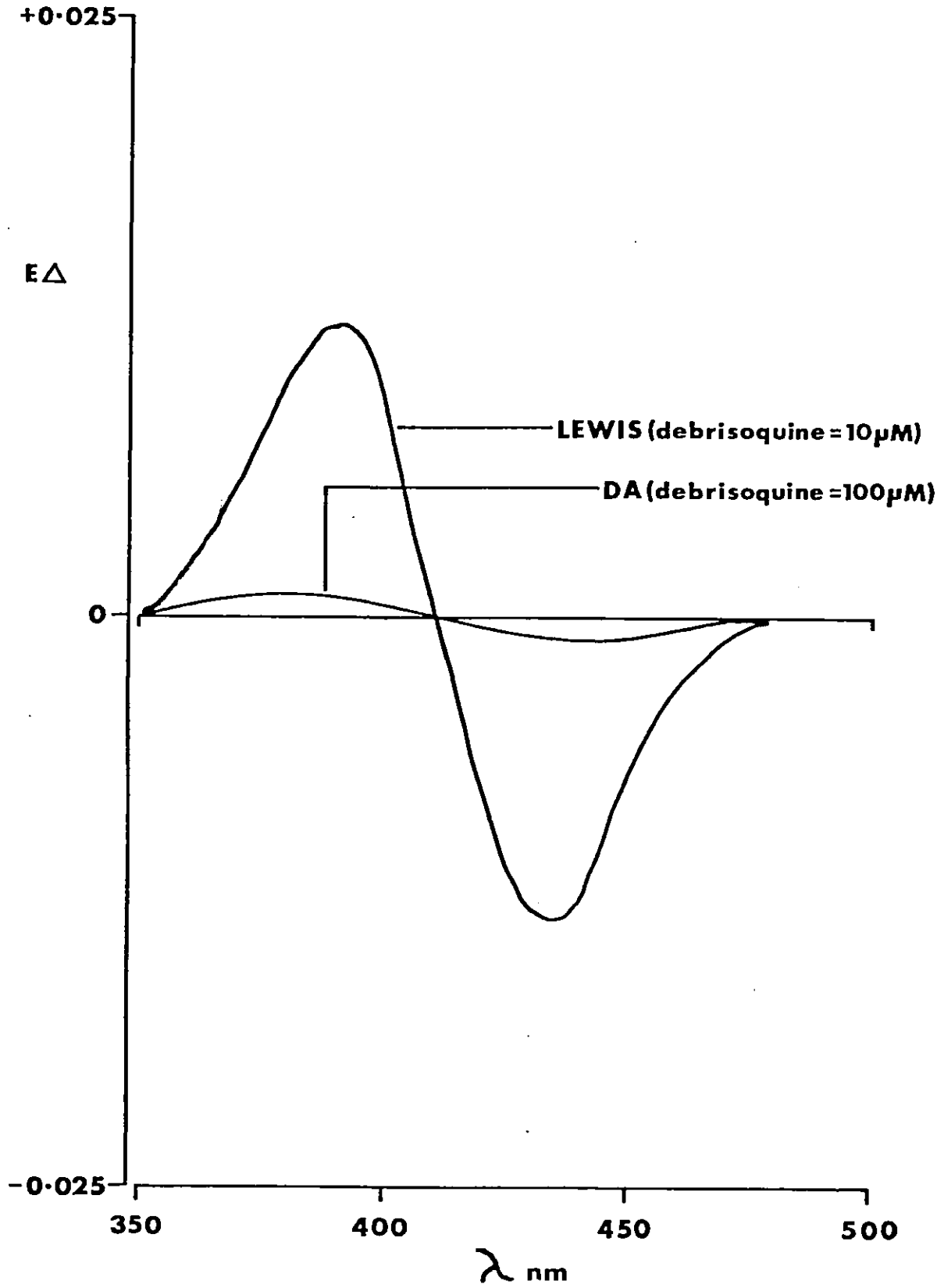
A genetic polymorphism of debrisoquine 4-hydoxylation has been observed in the rat. Extensive and poor hydroxylation characters are exemplified by female Lewis and DA rats respectively. It is possible that this single gene difference is a reflection of genetic variation in the species of cytochrome P-450 responsible for debrisoquine 4-hydroxylation. Kahn et al. (1982) have recently shown that this reaction in the rat is P-450 dependent and that DA rat microsomes catalyse the conversion at only 12.5% of the rate at which Fischer rats perform it. In more precise terms, DA P-450-related debrisoquine 4-hydroxylase may have a lower binding capacity for the substrate. In order to investigate this more fully, spectral binding studies were carried out in conjunction with Dr. A. Küpfer at the University of Bern. Hepatic microsomes from females of both strains were used to acquire binding spectra for the debrisoquine P-450 interaction.

Total cytochrome P-450, measured spectrophotometrically as its CO complex, was not significantly different between

Lewis and DA liver. Lewis microsomes gave a Type I spectrum on addition of debrisoquine with $\dot{\Delta}E_{max}$ of 6.78 $\pm$ 0.34 mol^{-1} cytochrome P-450 and a calculated dissociation constant (K_d) of 9.8 $\pm$ 1.8 M. When debrisoquine was added to DA microsomes at 10 M (Approximately K_d for Lewis microsomes), no difference spectrum was obtained. Only when the concentration was raised tenfold to 100 M, could a weak Type I spectrum be observed with ΔE_{max} of 1.6 mol^{-1} cytochrome P-450 (see Fig. 2.9).

These preliminary findings are suggestive of the metabolic defect in the DA rat occurring at, or close to, the first step in the mono-oxygenation catalytic cycle, whereby drug binds to the oxidized form of P-450. It is possible therefore that the DA rat, with a normal amount of P-450, has one form genetically modified. Such a modification would be an example of a true protein polymorphism and could be seen as analogous to the haemoglobin variants, whereby not only protein structure, but also oxygen-binding capacity is altered in genetic variants such as HbS or HbC (Winslow and French Anderson, 1978).

Figure 2.9 Difference spectra for debrisoquine-cytochrome P-450 interaction in microsomes from female Lewis and DA rats



2.4 DISCUSSION

The aim of the work described in this Chapter was to investigate whether or not the genetic polymorphism of debrisoquine 4-hydroxylation (Mahgoub *et al.*, 1977; Evans *et al.*, 1980) could be observed in another species, namely the rat. To this end, the following characteristics of the rat have been uncovered.

(i) A bimodal distribution of the ratio urinary debrisoquine/4-hydroxydebrisoquine (the 'metabolic ratio'). Thus rats can be classified as those which excrete more 4-hydroxymetabolite than parent drug [extensive metabolizer (EM) phenotype, values 0.07 - 0.70] and those which excrete less metabolite than drug [poor metabolizer (PM) phenotype, values 1.2 - 5.3].

(ii) The poor metabolizer phenotype was expressed only in female DA rats, of all rats studied. No female DA's were extensive metabolizers.

(iii) The poor metabolism trait observed in the female DA rat is transmitted by simple

Mendelian inheritance as a recessive characteristic.

(iv) Phenobarbitone treatment does not induce debrisoquine 4-hydroxylation in either extensive or poor metabolizing rats. Phenobarbitone was, however, toxic (lethal) to poor metabolizing DA females at the dose level used for induction in other rat strains ($100 mg kg^{-1}$).

20-Methylcholanthrene (20-MC) treatment increased the absolute excretion of 4-hydroxy-debrisoquine in EM Lewis females, but not in BN (EM) or DA (PM) females. However, the metabolic ratio was markedly decreased in BN and DA rats, suggesting that other pathways of metabolism were induced by 20-MC.

(v) The hydroxylation deficiency seen in the female DA rat is sex hormone dependent. Castration and testosterone treatment of female DA rats reversed the hydroxylation deficiency. In castrated female DA's treated with oestradiol drug oxidation reverted to the deficiency status.

Male DA rats could not however be made phenotypically PM by the administration of oestradiol alone.

(vi) The hydroxylation deficiency in the female DA rat may have its origins in an impaired binding of debrisoquine to (reduced affinity for) a specific form of cytochrome P-450.

The correspondence between the human and rat data is of great interest. Many studies have shown that human populations can be divided into two phenotypes, EM and PM, with respect to debrisoquine 4-hydroxylation (see Ritchie and Idle, 1982 for a review). Between 1-9%, depending upon ethnic group, are phenotypically PM.

A similar pattern has been shown in rats, admittedly using inbred strains rather than 'wild' animals. In both species, the poor metabolizers are recessive. One major discrepancy however, is that the PM character was only expressed in female animals, even in the F_2 progency of DA X Lewis crosses. In man, there is no evidence to support a sex difference in debrisoquine 4-hydroxylation (Evans et al., 1980).

Sex and strain differences in drug oxidation for several laboratory species are well known phenomena. Strain differences in metabolism, pharmacological and other measured parameters of certain drugs and other foreign compounds are shown in Table 2.12.

Probably the best studied strain differences in drug oxidation are those involving murine inducibility. Aryl hydrocarbon hydroxylase induction by the polycyclic aromatic compound, methylcholanthrene, classified the mice as responsive and non-responsive and is explained by a single autosomal gene obeying classical Mendelian segregation, the responsive allele being dominant (Gielen et al., 1972; Thomas et al., 1972a; Robinson et al., 1974; Niwa et al., 1975; Nebert et al. 1975). Hydroxylation of benzo[a]pyrene and the dealkylation of phenacetin are inducible by 20-methylcholanthrene to varying degrees in livers from different strains of mice and their metabolism is non-inducible in the liver of non-responsive mice (DBA/2A and AKR/J; Poppers et al., 1975).

Nebert and his co-workers have made a great contribution to our understanding of the genetics of P-450 induction using their mouse strains, and more detailed information is available in various reviews of this topic (see Nebert, 1979).

Table 2.12 Examples of animal species showing strains differences in metabolism and related parameters of several drugs and chemical compounds

Substrate	Species	Parameter measured	Reference(s)
Warfarin	mouse	Warfarin metabolism	Lush (1976)
Warfarin	rat	Warfarin metabolism	Jalonen and Pyorala (1970)
Chlorpromazine	mouse	Chlorpromazine resistance	Plotnikoff (1961)
Insulin	mouse	[1]Lethal dose of insulin	Chase (1950)
Antipyrine	rat	Rate of metabolism	Brodie (1959)
Hydrogen peroxide	dog, guinea pig	[2]Catalase enzyme activity	Allison et al. (1957)
Atropine	rabbit	[3]Atropine esterase activity	Sawin and Glick (1943)
Coumarin	mouse	[4]MEA	Wood and Taylor (1979)
7-Ethoxycoumarin	mouse	MEA	Lush and Andrews (1978)
Styrene oxide	mouse	Epoxide hydrolase	Lyman and Poland (1980)
Methoxyfluorane	rat	[5]Rate of metabolism	Mazze et al. (1973)
Hexobarbitone	rabbit	MEA	Cram et al. (1965)
Hexobarbitone	rat	MEA	Furner et al. (1969a)
Hexobarbitone	mouse	[6]PA and rate of metabolism	Jay (1955), Vesell (1968a)
Aniline	rat	MEA	Furner et al. (1969b)
Aniline	rabbit	MEA	Cram et al. (1965)

Table 2.12 (continued)

Substrate	Species	Parameter measured	Reference(s)
p-Nitroanisole	rat	MEA	Furner et al. (1969a)
Ethymorphine	rat	MEA	Jori et al. (1970)
Imipramine	rat	MEA	
Aminopyrine	rat	MEA	Jori et al. (1970)
Pentobarbitone	mouse	PA, sleeping time	Lush and Lovell (1978)
Zoxazolamine	mouse	PA, paralysis time	Lush (1976)
Zoxazolamine	rabbit	MEA	Cram et al. (1965)
Urethane	mouse	[7]Carcinogenicity	Matusuyama and Suzuki (1968)
Amphetamine	rabbit	MEA	
Codeine	rabbit	MEA	Cram et al. (1965)
Benzo[a]pyrene	rabbit	MEA	
Nitrobenzoic acid	rabbit	MEA	
Bilirubin	rat	Glucuronidation	Lathe and Walker (1957)
o-aminobenzoic acid	rat	Glucuronidation	Axelrod et al. (1957)
Menthol	rat	Glucuronidation	Schmid et al. (1958)
o-aminophenol	rat	Glucuronidation	Park (1968)
Androsterone	rat	Glucuronidation	Matsui and Hakozaki (1979) Matsui et al. (1979)

Table 2.12 (continued)

Substrate	Species	Parameter measured	Reference(s)
Isoniazid			
Sulphadiazine	rabbit	Metabolism	Frymoyer and Jacox (1963a,b)
Aminofluorene	mouse	Urinary acetylation product	Hein and Weber (1982)
Sulphamethazine	rat	Hepatic - NAT[8]	Tannen and Weber (1979);
Phenelzine	hamster	Blood - NAT	(1980)
Monoacetyl hydrazine			

Footnotes to Table 2.12

1. Certain strains of mice have been shown to survive 300 times the dose of insulin that would normally be lethal.

2. Deficiency of the catalase enzyme, called acatalasia, which causes oral ulceration, loss of teeth and darkening of the blood in contact with H_2O_2.

3. Assessed *in vitro*, the presence of the enzyme is controlled by a single dominant gene.

4. MEA = Microsomal enzyme activities

5. Judged by measuring free fluoride in the blood.

6. PA = Pharmacological action; hexobarbitone sleeping time.

7. Some strains showed lung cancer and the other liver tumours, whether or not this has any relation to urethane metabolism is unknown.

8. *N*-acetyltransferase

The above are a small proportion only of the vast body of literature concerning strain differences in drug oxidation in laboratory species. Nevertheless, both basal and induced activities show strain differences in several species, some of which have been shown to be under discrete genetic control. All of these examples however, involve *in vitro* determinations, whilst in intact living animals, little data have, until now, been available.

Sex differences in the ability of rats to metabolize certain drugs and other foreign compounds are also described and they have been attributed to the effects of sex hormones (Quinn *et al.*, 1958; Kato and Onoda, 1970). Sex differences in drug metabolism are not seen in all cases, and it depends upon species, strain, substrate, organ, age and many other factors related to the status of the animals. Rat is the common species which possesses a sex difference in drug metabolism. Table 2.13 shows some examples of drugs which have sex difference in the metabolism or pharmacological action or both of these.

Strain specific sex differences of drug metabolism have also been observed: Swiss-Webster mice show a sex difference in pentobarbitone sleeping time, whilst

Table 2.13 Examples of species showing sex differences in metabolism and related parameters of several drugs and foreign compounds

Substrate	Parameter measured	species showing: Sex difference	species showing: No sex difference	Reference(s)
Aflatoxin	Carcinogenicity MEA Toxicity	rat	mouse	Gurtoo and Motycka (1976)
2-Naphthylamine	MEA	rat	mouse, hamster, guinea pig, rabbit	Dewhurst (1963)
Ethylmorphine	MEA	rat	-	Castro and Gillette (1967) Davies et al. (1968) Stripp et al. (1971)
Pethidine	MEA	rat	-	Axelrod (1956)
Morphine	MEA	rat	-	Axelrod (1956)
Methadone	MEA	rat	-	Axelrod (1956)
Aminopyrine	MEA	rat	mouse, rabbit	Kato et al. (1966, 1968) Quinn et al. (1958)

Table 2.13 (continued)

Substrate	Parameter measured	species showing Sex difference	species showing No sex difference	Reference(s)
Hexobarbitone	PA	rat, mouse	rabbit, guinea pig	Kato et al., (1968)
	MEA $t_{\frac{1}{2}}$	rat		Quinn et al. (1958) Kato (1966) Noordhoek and Rumke (1969) Yaffe (1962) Catze and Yaffe (1967) Yaffe et al. (1968) Vesell (1968a)
DDT	accumulation	rat	dog, pig , monkey	Hathway (1970)
Carisprodol	PA, MEA	rat	-	Kato et al. (1962) Kato (1974)
Pentobarbitone	PA, MEA	mouse	-	Westfall et al. (1964) Kato and Gillette (1965)
Amidopyrine	MEA	rat	-	Quinn et al. (1958)
Antipyrine	$t_{\frac{1}{2}}$	rat	-	Quinn et al. (1958)
Chloroform	renal damage	mouse	-	Hewitt (1957)
Nicotine	metabolism response	man, rat	-	Beckett et al. (1971) Holck et al. (1937)

Table 2.13 (continued)

Substrate	Parameter measured	species showing: Sex difference	species showing: No sex difference	Reference(s)
Aspirin	metabolism	man	-	Menguy et al. (1972)
Heparin	metabolism	man	-	Kernohan and Todal (1966) Jick et al. (1968)
Aflatoxin	incidence of liver cancer	man	-	Peers and Linsell (1973) Shank et al. (1972 a,b)
Benzo [a]pyrene	AHH	rat	-	Gurtoo and Parker (1979) Kramer et al. (1979)

PA Pharmacological action

MEA Microsomal enzyme activities

this is not observed in other strains of mice (Vesell, 1968a). Chloroform produces nephrotoxicity more in males than in females of certain strains but not in others(Walpole and Spinks, 1958; Hewitt, 1957).

Hepatic ethyl morphine-N-demethylase activity was found greater in male mice than in female mice in one strain (BALB/CJ) and the opposite in other strains (CV1 : CD-1), other strains showing no sex difference (Brown et al., 1978). Some substrates however, do not show sex differences in rat, for example aniline hydroxylation (Kato and Gillette, 1965; El-Defrawy et al., 1974), Zoxazolamine hydroxylation (Kato and Gillette, 1965) and ethoxyresorufin-O-deethylation (Vodicnik, et al., 1981).

Benzo[a]pyrene metabolism shows no sex difference in lung and spleen in rat while it does so in liver and in kidney. Interestingly, female kidney microsomes metabolize benzo[a]pyrene faster than those from male kidney (Gurtoo and Parker, 1977).

Age is an important factor modifying sex differences. Up to the age of four weeks, there are no sex differences in the duration of action of hexobarbitone (Quinn et al. 1958).

In another study it was shown that hepatic AHH metabolism differs not only quantitatively but also qualitatively in rats of different sex and age (Wiebel and Gelboin, 1975) and the maturation of drug metabolizing ability does not occur simultaneously for all enzymes (Furner et al., 1969b).

Sex differences can be manipulated by administration of hormones. Administration of testosterone to young male rats increases rates of drug oxidation, whereas castration of males often retards the metabolism of the substrate studied. For example, castration of males eliminates the sex difference in procaine toxicity, increases the sensitivity of males to barbiturates (Hathway, 1970), decreases benzo[a]pyrene metabolism (Gurtoo and Parker, 1977), whilst not affecting the observed difference in sulphanilamide acetylation (Franz and Latta, 1957). Furthermore, castration decreases the N-demethylation of ethyl morphine which can be restored by testosterone administration (El-Dafrawy et al., 1974), decreases aminopyrine metabolism (Gurtoo and Parker, 1977) and decreases the cytochrome P-450 and b_5 levels in the liver (Ichii and Yogo, 1969). The castration of female rats however, has no effect on benzo[a]pyrene metabolism (Gurtoo and Parker, 1977) and little effect on ethyl morphine and 3-methyl-4-methylaminobenzene metabolism and on the level of

cytochrome P-450 (El-Defrawy et al., 1974).

Oestradiol administration lowers hexobarbitone metabolism in male rats (Quinn et al., 1958), aniline hydroxylation, ethylmorphine and benzo[a]pyrene metabolism in orchidectomized rats (Kramer et al., 1979; El-Defrawy et al., 1974), glucuronidation of o-aminophenol (Franz and Latta, 1957) and ethoxy-coumarin-O-deethylation in male rats. In contrast, oestradiol decreased pentobarbitone sleeping time in mice (Noordhoek and Rumke, 1969) and increased the activity of ethoxyresorufin-O-deethylation in male and female rats (Gigon et al., 1968) without any action on the activity of ethoxy coumarin-O-deethylase in female rats.

With respect to mechanistic aspects, sex differences have been rationalized in various ways. Several workers have investigated sex differences from the point of view of substrate binding to cytochrome P-450 (Schenkman et al., 1967; Castro and Gillette 1967; Davies et al., 1968), basal and substrate-stimulated rates of NADPH-cytochrome P-450 itself (El-Defrawy et al., 1974; Stripp et al., 1971). Cytochrome P-450 and NADPH-cytochrome P-450 reductase levels in hepatic microsomes from male rats are 20-30% greater than those

from females (Kato, 1966; 1968; Davies et al., 1969) but this is insufficient to explain the two-to-three fold variation in drug metabolizing activities seen for such substrates as aminopyrine and hexobarbitone.

However, the sex difference in the substrate-induced increase in cytochrome reduction closely parallels the sex difference in ethylmorphine metabolism, suggesting that only part of the cytochrome P-450 in female rat microsomes is enzymatically active (Gigon et al., 1968). An alternative hypothesis has been proposed that the basis for sex difference in microsomal drug hydroxylation resides in cytochrome P-450 rather than in NADPH-cytochrome P-450 reductase (Kato, 1974). The difference in substrate affinity between male and female animals has been explained by the presence of endogenous inhibitors in microsomal preparations from female rats (Gigon, et al., 1968). But this hypothesis was thought by other workers to be unlikely (Davies et al., 1968).

How can all the above observations help to explain the genetically-determined strain-dependent sex difference for debrisoquine 4-hydroxylation observed in the DA rat? The answer to this is by no means clear. Available

literature, examples of which are cited above, is often contradictory and somewhat confusing. Nevertheless, one possible hypothesis might be as follows: female DA rats do not lack the genetic information to synthesize the debrisoquine metabolizing P-450, since either castration or testosterone administration (or both) converts them to the extensive metabolizing phenotype (male character). Rather, this P-450 is repressed in female DA rats, derepressed by the administration of testosterone. Either the balance of endogenous oestrogens to testosterone is unique in the female DA rat or some hormonal receptor lies at the root of the genetic variation between the strains studied. Alternatively, a different P-450 not present in normal female liver is induced by testosterone, which fortuitously metabolizes debrisoquine. Obviously, much more work is required to clarify these points. In this context, the work of Vodicnik et al. (1981) is of interest. These workers, by studying the differential induction of ethoxycoumarin-O-deethylation and ethoxyresorufin-O-deethylation by oestradiol and testosterone in male and female Sprague Dawley rats, concluded that androgens and oestrogens were responsible for the inherently high levels of cytochrome P-448 in females and cytochrome P-450 in males. A

similar sex dimorphism in the ratio of P-450/P-448 maintained by sex hormones, might partly explain the observations in the DA rat.

It is interesting to speculate how the data given in this Chapter might form the basis for a model of the genetic regulation of cytochromes P-450 by sex hormones. Some clues come from the studies of Palmiter et al. (1976) on the regulation of the synthesis of specific proteins in the chick oviduct. Apparently, both ovalbumin and conalbumin synthesis is inducible by both oestrogens and progesterone via specific hormone receptors. Both $mRNA_{ov}$ and $mRNA_{con}$ synthesis is induced by the hormones after a lag period of about 0.5 - 3.0h. The various models these authors discuss are complex and the view is offered that all data cannot be explained by a cytosol receptor-hormone complex which migrates to the nucleus and binds directly at a promotor site acting as a positive effector and increasing specific mRNA transcription. Nevertheless, since a similar model is proposed to explain AHH induction by TCDD (Greenlee and Poland, 1979; Okey et al., 1979; Negishi and Nebert, 1979), it is plausible that synthesis of other mRNA's for

cytochromes P-450 is induced or repressed by sex hormones in a similar way to that described for the chick oviduct protein synthesis messengers. One might thus envisage either a cytosolic receptor for oestrogens which was a repressor of P-450 (debrisoquine 4-hydroxylase) synthesis or a testosterone receptor which acted as an effector of this enzyme synthesis, or both. In which case, the genetic polymorphism of debrisoquine 4-hydroxylation observed in intact rats, may have its origins in a variable receptor, rather than a polymorphic structural gene. Accordingly, female DA rats would have a high affinity receptor for endogenous oestrogens and thus have repressed synthesis of a specific P-450, or would have a low-affinity receptor for testosterone which in other strains acts as an effector of P-450 synthesis. Obviously, many more investigations of the uniquely observed sex-difference in metabolic oxidation are required.

In proposing the inter-strain difference in debrisoquine 4-hydroxylation as a possible metabolic model for the human situation, a number of complications can readily be envisaged. The major one of these is the fact that the poor metabolizing phenotype is only expressed in female DA rats, which is at variance with the human

data. However, irrespective of the molecular and genetic mechanisms involved, it was nevertheless thought worthy to investigate female DA and Lewis rats as possible metabolic models for the human PM and EM phenotypes. Obviously if this model was to have any validity or possible predictive value, the defect in metabolism would need to be seen for substrates other than debrisoquine, as it is in man.

Lewis rats have been chosen as an extensive metabolizing control strain, since they gave a good recovery of debrisoquine plus 4-hydroxydebrisoquine, similar to DA rats (see Table 2.3) and thus it is unlikely that alternative metabolic pathways are involved.

Accordingly, the following Chapter describes investigations of the in vivo metabolism of several other substrates in these two putative model strains.

CHAPTER THREE

The metabolism of various drugs in female DA and Lewis rats.

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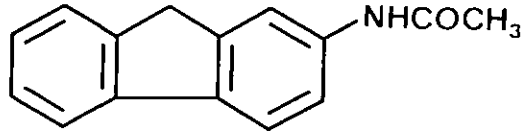
3.1 INTRODUCTION

It has been suggested that the drug-metabolizing systems may have developed in response to the special needs of terrestrial life (Brodie and Maickel, 1962). Different species of animals should show wide variation in drug metabolism; while reptiles birds and mammals have well developed oxidative drug-metabolizing systems, fish (with some exceptions) apparently lack these systems, and the aquatic amphibia are in between. These enzymes, as other enzymes and biological constituents in the body, can vary from one species to the other. Such wide differences in drug metabolism between species are not unexpected, although no rationale for these differences is apparent.

Interspecies variation in drug metabolism (route and rate of metabolism) could lead to differences in drug toxicity and pharmacological responses. Table 3.1 shows the carcinogenicity of N-2-fluorenylacetamide in relation to species differences in the formation of the proximate carcinogenic metabolite, N-hydroxy-N-2-fluorenylacetamide (Weisburger, 1964).

Table 3.1 N-Hydroxylation and carcinogenicity of N-2-fluorenylacetamide



Species	Carcinogenicity	N-hydroxylation (% dose)
Rat	+	8
Rabbit	+	21
Hamster	+	17
Dog	+	5
Guinea pig	-	0
Steppe lemming	-	trace
Man	?	9

Additionally, Table 3.2 shows pharmacological differences of hexobarbitone in different species due to variation in drug metabolizing enzyme activity. The species differences in the sleeping time response to hexobarbitone are related to interspecies differences in drug metabolizing activity.

Table 3.2 Species differences in response to and metabolism of hexobarbitone (taken from a dose of 100mgKg^{-1} (50mgKg^{-1} in dog) Quinn et al. (1958).

	Sleeping time (min)	Hexobarbitone $t_{\frac{1}{2}}$ (min)	*Enzyme activity
Mice	12 ± 8	19 ± 7	598 ± 184
Rabbits	49 ± 12	60 ± 11	196 ± 28
Rats	90 ± 15	140 ± 54	134 ± 51
Dogs	315 ± 105	260 ± 20	36 ± 30
Man	-	360	-

* $\mu g\ h^{-1}\ g^{-1}$ tissue

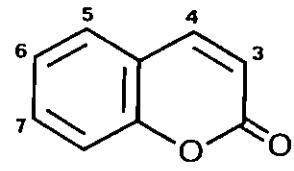
Other factors that might be responsible for inter-species variations in response to biologically active substances include rate of absorption, mode of excretion, plasma protein binding, tissue binding and the nature of tissue receptors (Smith, 1978). This latter author has described the pattern of metabolic inter-species variation as follows;

a. Competing reactions:- where the compound is metabolized to the same products, but in different amounts, as in Table 3.3, which shows the metabolism of coumarin by either ring hydroxylation (particularly in the 3- and 7-position)or by ring cleavage (Kaighen and Williams, 1961; Gangolli *et al.*, 1974).

b. species defects in common metabolic reactions:- some species appear to lack the capacity to carry out certain common metabolic reactions, particularly Phase II conjugation reactions (see Chapter One) and some examples are shown in Table 3.4.

c. Occurrence of uncommon reactions:- the occurrence of unusual reactions (restricted to some species or groups of species and also to certain compounds) which may be responsible for interspecies differences in drug metabolism. For example, the following reactions, N^1-glucuronide formation, glutamine conjugation, aromatization of quinic acid and O-methylation of 4-hydroxy-3, 5-diiodobenzoic acid are restricted to primate species (Man, New and Old World Monkey). Table 3.5 shows some of the uncommon metabolic conjugation reactions in some species.

Table 3.3 Competing reactions: Metabolism of coumarin



	% Dose excreted as	
	Hydroxy-coumarins	Ring open products
Man	68 - 92	Low
Rat	3	50
Rabbit	41	23

Table 3.4 Species defects in common metabolic reactions

Defective reaction	Species
N-Hydroxylation of aliphatic amines	Rat
Glucuronide formation	Cat, Gunn rat
Sulphate formation	Pig, opossum
Arylamine acetylation	Dog, fox
Mercapturic acid formation	Guinea-pig
Glycine conjugation	Fruit bat

Table 3.5 Some uncommon metabolite conjugation reactions

Conjugating agent		Species
Amino acids	Ornithine	Certain birds and reptiles
	Taurine	Pigeon and ferret
	Serine	Rat and rabbit
	Glycyltaurine	Cat
	Arginine	Ticks and spiders
Carbohydrates	Glucose	Insects
	Ribose	Rat and mouse
	N-Acetylglucosamine	Rabbit
Acids	Phosphate	Cat, dog and man
	Formate	Rat and dog
	Succinate	Rat and dog

Smith and Caldwell (1977) have surveyed the metabolism of twenty three drugs in several species to find a metabolic model for man and they have utilized data from the rat (rodent species), other non-primates (dog, guinea pig and rabbit) and rhesus monkey (primate species). They found that primate species are more likely, but not necessarily, to provide better metabolic models for the human than non-primate species as it is shown in Table 3.6.

From this viewpoint, the question might be asked, can primates (especially rhesus monkey) be, in practical terms, satisfactory species for the pre-clinical safety evaluation of drugs and chemical compounds? To follow any regulatory guidelines and requirements for evaluation of drugs in all aspects, like pharmaco-kinetic, pharmacodynamic, metabolic and toxicological a large number of primate animals are required. Thus many problems will be faced, such as availability, convenience and economic factors which it is not easy to obviate at this time. Investigation of small numbers of animals will give relatively little metabolic data and probably would not give an indication of any unexpected metabolic reactions for such a broad hetero-genous species as the human race (see Chapter One).

Table 3.6 Primates versus non primates as metabolic models for man

Species	Rating of species as metabolic models for man for 23 drugs[1]			
	Good[2]	Fair[3]	Poor[4]	Invalid[5]
Rat	4	2	7	8
Other non-primate	5	6	6	6
Rhesus monkey	15	4	1	0

1. Includes amphetamines (4), arylacetic acids (5), sulphonamides (4), other drugs (10).

2. Pathways similar

3. Metabolic pathways similar, but there are significant inter-species differences in amounts of metabolites produced by different pathways

4. Marked species differences in amount of metabolites produced by similar pathways

5. Metabolic pathways are quite different.

Metabolism of debrisoquine is similar in man and rat (Allen et al., 1975; Angelo, 1976). As shown in Chapter Two, screening large numbers of rats revealed a bimodal distribution of debrisoquine metabolism, and two strains of rats were suggested (DA and Lewis) as models for the poor and extensive metabolizer human phenotype respectively (see Chapter One).

As is well known, many factors, both genetic and environmental, can affect drug disposition and response, including absorption, distribution, excretion, interaction with receptor sites or a combination of these. Genetic factors may be the most important, but a number of environmental factors can play a major role as sources of variable metabolism, for example, diet, smoking, alcohol, temperature, short and long term drug administration, induction, bioavailability of pharmaceutical preparations, dose, route of administration, tolerance, vehicle, exposure to chemical compounds, sex, stress, pregnancy, malnutrition, starvation, disease, albumin concentration, age and volume of material injection (for review see Vesell, 1982).

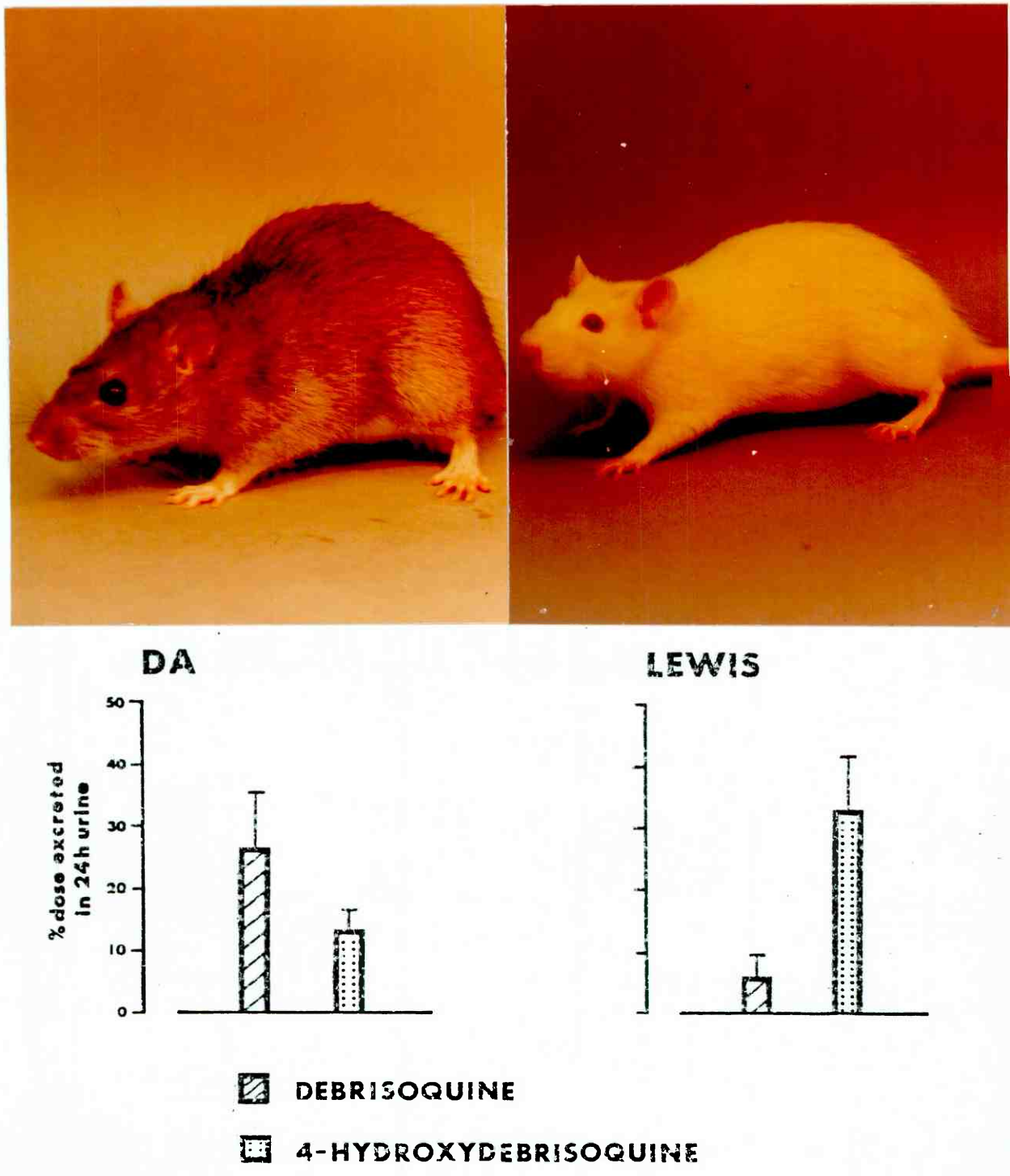
Obviously, any animal model for human drug metabolism should be subject to similar influences as man and

ideally, would have a similar anatomy, physiology, biochemistry, genetics and endocrinology as man. Patently, Drosophila would be a quite inappropriate metabolic model, even if, for certain drugs, the same metabolic pathways were found. The rat, on the other hand, is a mammal which, when compared with Drosophila, is as close to man in the above respects as the rhesus monkey.

The objective of the work described in this Chapter is to evaluate the DA and Lewis strains of rat as models, both qualitative and quantitative, for the human two known oxidation phenotypes (PM and EM) polymorphism. In pursuit of this, a comparative study of the oxidative metabolism of selected drugs in female DA and Lewis rats was undertaken. Drugs which are substrates for the human polymorphic enzyme system have been selected, including phenacetin, phenformin and sparteine (polymorphic substrates) together with tolbutamide and acetanilide (non-polymorphic substrates).

Additionally, hexobarbitone sleeping time, a commonly used indirect index of rat hepatic metabolic oxidation has been measured in these two rat strains. An outline of the metabolism of each of these compounds will be given. To recapitulate, Fig. 3.1 shows the female DA

Figure 3.1 Female DA and Lewis with an index of their capacity to metabolize debrisoquine



and Lewis rat together with an index of their capacity to metabolize debrisoquine.

3.1.1 Short outline of phenacetin, its metabolism and toxicity

In the years 1887-1888, phenacetin was introduced into clinical use as a non-narcotic analgesic antipyretic.

The antipyretic and analgesic activity of phenacetin was studied in many species of animals. In rat, phenacetin has antipyretic effects (Gilman and Barbour, 1938) and analgesic effects (Macht and Macht, 1940). In man too, it is effective as both an antipyretic (Traversa, 1891; Caldwell, 1892) and as an analgesic (Wolff et al., 1941).

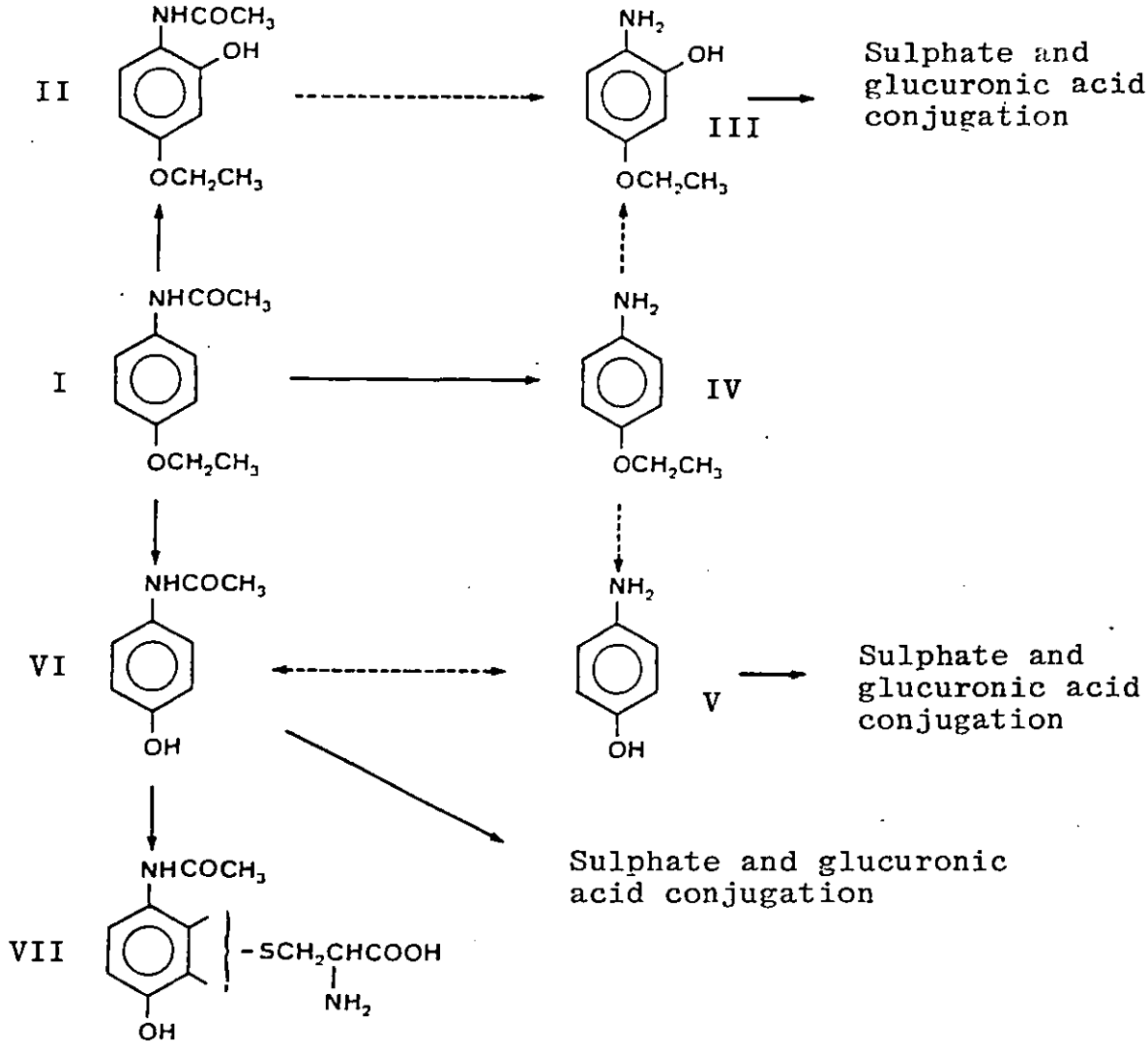
Originally, phenacetin was used alone, but it was soon used in combination with other drugs, mainly aspirin, codeine, acetanilide, antipyrine, phenobarbitone and with a variety of other preparations (see Smith, 1958).

Studies of phenacetin metabolism in man and in rat, showed that the major metabolite was paracetamol, mainly excreted in urine, conjugated with glucuronic acid and sulphate, as summarized in Table 3.7 and Fig. 3.2.

Other minor metabolites also have been detected in the urine of man and rat (Table 3.8).

Phenacetin toxicity has been studied extensively; early reports of toxicity dealt with the cyanosis which occurred after large doses (Falk, 1890; Harold, 1894; Knowles, 1894; Kebler et al., 1909). In summary, phenacetin toxicity may be divided into two categories, haemolytic and renal. The compound has variously been implicated in the onset of cyanosis caused by methaemoglobin formation (Smith, 1958) and chronic haemolytic anaemia (Dacie, 1967). Acute haemolytic anaemia may follow short term or single therapeutic doses, and this may be due to one of the following reasons, an auto-immune response, glucose-6-phosphate dehydrogenase deficiency or abnormal metabolic fate of phenacetin (Prescott, 1968).

Figure 3.2 Phenacetin metabolism



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

(Broken line indicates possible route of formation where more than one possibility exists).

Table 3.7 Metabolism of phenacetin in man and rat

Species (dose)	% dose excreted as: Phenacetin	Paracetamol (free)	Paracetamol (conjugated)	Reference
Man (2g orally)	0.2	3.3	78.0	Brodie and Axelrod (1949)
Rat ($200mgKg^{-1}$ i.p.)	0.5	3.5	58.0	Buch et al. (1967a)
Rat ($180mgKg^{-1}$ i.p.)	0.6	0.1	31.1	Nery (1971)
Rat ($125mgKg^{-1}$ orally)	0.1	4.9	57.7	Timbrell (1973)

Table 3.8 Minor metabolites of phenacetin after oral administration in man and rat

Species	Metabolite	% dose excreted	Reference
Man	2-hydroxyphenacetin	0.3	Shahidi (1968)
	3-[(5-acetamido-2-hydroxyphenyl)thio] aniline	2.0	Jagenburg and Toczko (1964)
	2-hydroxy-p-phenetidine sulphate	0.6,8.0	Raaflaub and Dubach (1969) Buch et al., (1967a)
Rat	2-hydroxyphenacetin sulphate	0.07	Buch et al., (1967a)
	3-hydroxyphenacetin	0.04	Buch et al., (1967a)
	2-hydroxy-p-phenetidine	0.5, 6.0	Dubach and Raaflaub (1969) Buch et al., (1966; 1967a)

Shahidi(1968) reported a pedigree of two sisters who exhibited a defective de-ethylation of phenacetin and who were more sensitive to the methaemoglobinaemia-producing effects of the drug. These two sisters produced 30% and 49% of phenacetin dose ($30mgKg^{-1}$) as paracetamol in 24h together with 34% and 16% as 2-hydroxyphenetidine, compared to a normal excretion of 2-8% of latter metabolite and 62-90% as paracetamol (Shahidi and Hemaidan, 1969). The large amount of 2-hydroxy-phenetidine was therefore considered to be the metabolite responsible for the haemotoxicity.

Phenacetin-induced haemolysis and methaemoglobinaemia has also been observed and investigated in experimental animals. Heyman *et al.* (1969) showed that de-acetylation is one of the metabolic transformations required for phenacetin and acetanilide to induce methaemoglobinaemia in the rat. Using the same species, Buch *et al.* (1967b) found when the metabolism of phenacetin was inhibited by barbiturates, methaemoglobinaemia was also diminished as well as p-phenetidine (deacetylation metabolite of phenacetin). These studies indicate that a deacetylation production of phenacetin is involved, which must be further metabolized before it induces methaemoglobinaemia.

Renal toxicities after phenacetin administration were also recorded, including chronic interstitial nephritis (Spuhler and Zollinger, 1953), chronic pyelonephritis (Bengtsson *et al.*, 1968), renal pelvic carcinoma (Angervall *et al.*, 1969) and renal papillary necrosis (Clausen and Pedersen, 1961).

Phenacetin in clinical use was mainly given with other drugs. A number of authors have implicated acetyl salicylic acid and caffeine either as the primary nephrotoxic agents or as secondary agents which enhance phenacetin nephrotoxicity by modifying its metabolism (Thomas *et al.*, 1972b; Prescott, 1965).

Renal toxicity studies in animals have also been done and they have indicated that phenacetin and aspirin mixtures produce papillary necrosis more conclusively than phenacetin alone.

Deethylation of phenacetin *in vitro* has been investigated using liver microsomal preparations from several species and in man, the deethylation of phenacetin by liver microsomes occurs more readily than with microsomes from the rat (Kuntzman *et al.*, 1966). Deacetylation of phenacetin has also been studied *in vitro*, using microsomes from rat

and human liver (Benoehr et al. 1966) where the metabolites are p-phenetidine and acetate (Bernhammer and Krisch, 1965).

Phenacetin metabolism shows differences in the rate of paracetamol formation in the human PM (poor metabolizer) and EM (extensive metabolizer) as judged by the measurement of k_f paracetamol (apparent first-order rate constant for metabolite formation). The mean (± S.D.) value of the PM phenotype was 0.09 ± 0.024, and for EM phenotype 0.277 ± 0.064 (Sloan et al. 1978).

From the above, it is clear that phenacetin may be a good substrate to test in the animal model (DA and Lewis rats).

3.1.2 Short outline of phenformin, its metabolism and toxicity

Phenformin (β-phenethylbiguanide) is a compound belonging to a major group of hypoglycaemic drugs biguanides. It is orally active, used in the treatment of maturity-onset diabetes. Its mechanism of

action is not clearly understood, but is absolutely different from the mechanism of action of the sulfonylureas such as tolbutamide. Phenformin does not act by stimulating secretion of insulin by the pancreas (as does tolbutamide). Hypoglycaemia is not readily induced in normal human subjects and the morphology of the β cell is uninfluenced. There are many contradictory reports about its mechanism of action, but the most likely mechanisms of action are inhibition of intestinal absorption of glucose (Berger et al., 1972) and effects on mitochrondria (Alberti and Nattras, 1977).

It is well known, from clinical and experimental studies, that biguanide drugs disturb lactate metabolism, even in the phenformin therapeutic dose range, it has been shown that phenformin can slightly raise blood lactate in some patients (Craig et al., 1960; Fajans et al., 1960; Nattras et al., 1977), but significantly increased and even more so when other factors occur in diabetic patients, like renal dysfunction, liver disease or acute alcoholosis (Assan et al., 1975; 1976; Heuclin et al., 1974).

Other biochemical effects of phenformin have been reported, such as glycogen depletion (Steiner and

Williams, 1958; Söling, 1969), inhibition of oxidative phosphorylation (Wick et al., 1958), or reduction in hepatic glyconeogenesis (Altschuld and Kruger, 1968; Cook, 1978) with patients who have high drug concentration in blood after therapeutic doses.

Alberti and Nattrass (1977) and Cohen and Woods (1976), found that lactic acidosis occurs in the presence of an abnormal high plasma level of phenformin.

The elimination of phenformin is partly renal as unchanged drug and partly metabolic as the drug metabolized by oxidation to 4-hydroxyphenformin. The relative importance of renal excretion and metabolism for the clearance of the drug varies, however, with a number of factors, including species, dose and route of administration. The conversion to the 4-hydroxy metabolite is an inactivation process as it is itself devoid of hypoglycaemic action and does not induce hyperlactic acidosis in the rat or mouse (Beckman, 1967; Holloway and Alberti, 1976).

Species variations occur in the metabolism of the drug; in mouse, rat, rabbit and man phenformin is extensively converted to its 4-hydroxy metabolite, but the guinea pig appears to be relatively impaired in this respect.

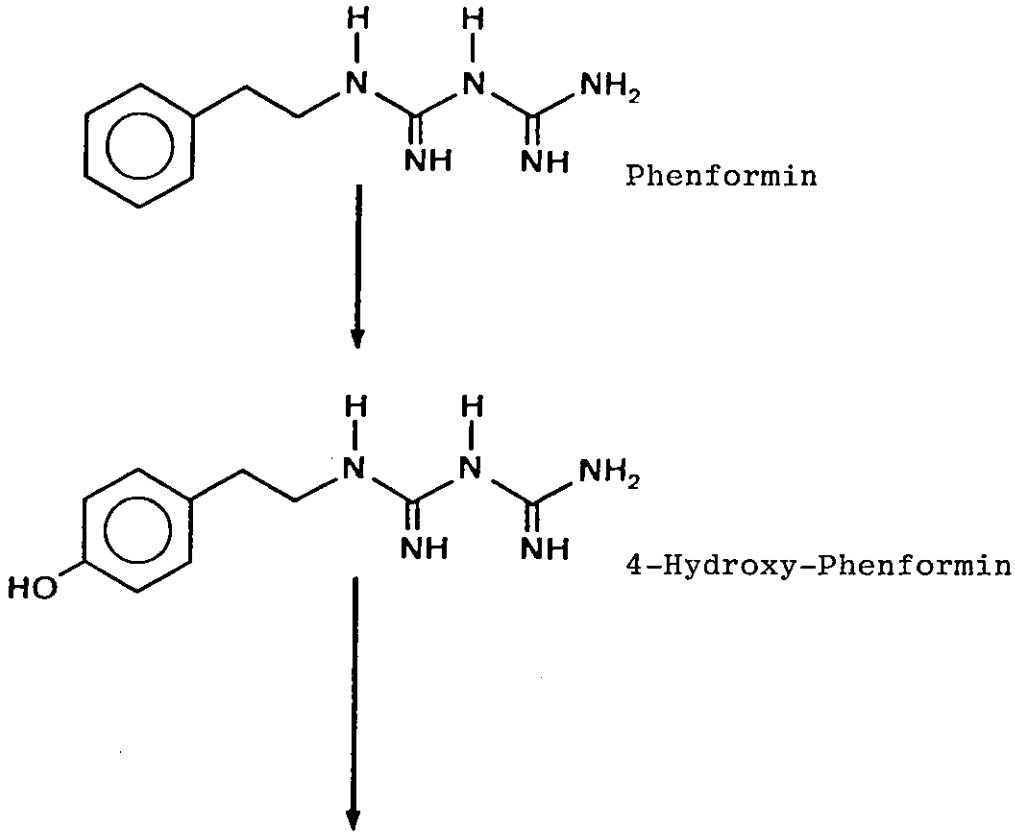
This metabolic difference appears to correlate with the greater sensitivity of the guinea pig to the hypoglycaemic effects of phenformin (Unger et al., 1957; 1960; Sterne, 1969). In the rat the metabolic 4-hydroxylation process appears to be saturated, as with high doses of the drug the proportion of the drug cleared renally as unchanged drug to that cleared as metabolite increases in favour of the former. Fig. 3.3 shows the metabolic fate of phenformin.

In man the 4-hydroxylation process exhibits genetic polymorphism, so that considerable inter-individual variation in the ratio urinary phenformin/4-hydroxy-phenformin is encountered. Evidence strongly suggests that phenformin aromatic hydroxylation is governed by the same gene locus that regulates debrisoquine oxidation. Studies suggest that individuals of the PM phenotype may be more susceptible to developing lactic acidosis in response to phenformin (Idle et al., 1981a).

3.1.3 Short outline of sparteine metabolism and toxicity

Sparteine is a naturally occurring alkaloid that was first isolated in 1851 from the flowering top

Figure 3.3 Phenformin metabolism



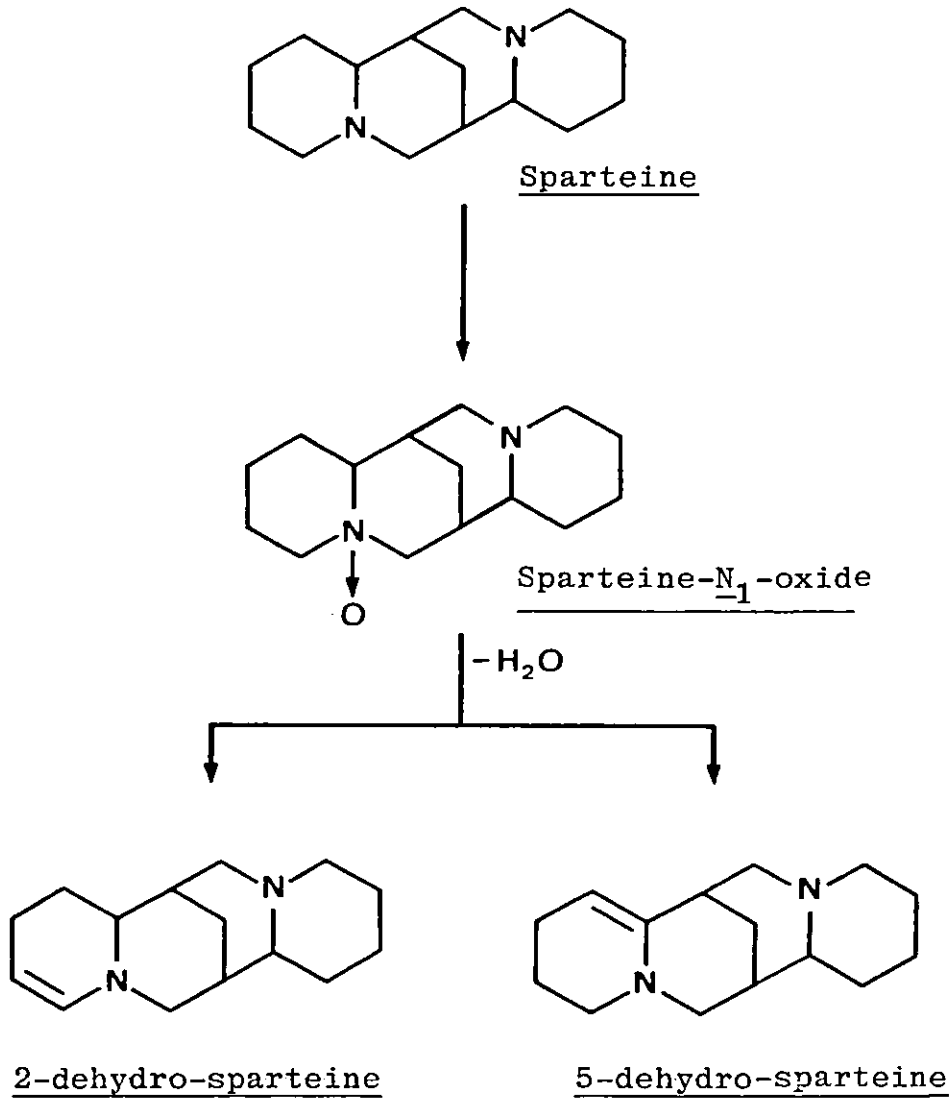
Glucuronic acid conjugation
(occurs in rat but not man)

of broom (*Sarothamnus scorparius*). It has been used as an oxytocic drug for the induction of labour at term and for management of prolonged uterine inertia. It has also been used as an antiarrhythmic drug. Its uterotonic properties are similar to those of oxytocin and the ergot alkaloids. In about 2-3% of the patients, overstimulation of the uterus resulted in the death of the foetus which was observed at doses well tolerated by the majority of patients (Goodman and Gilman, 1965).

Spiteller and Spiteller (1978) have studied sparteine metabolism in three species; man, rat and swine. They suggested that sparteine is metabolized by *N*-oxidation to form sparteine-N_1-oxide which is unstable and dehydrates to 2-dehydro and 5-dehydrosparteine (Fig. 3.4). This assumption is based on two observations, that no unchanged *N*-oxide is detected in plasma or urine, and that sparteine-N_1-oxide when added to urine can be reduced with titanous chloride to sparteine, whereas the metabolites cannot be reduced.

Sparteine metabolism shows polymorphism in human populations (Eichelbaum *et al.*, 1975a; 1979a). Five per cent of a German study population were unable to metabolize it, and the non-metabolizers were homozygous for an autosomal recessive gene.

Figure 3.4 Sparteine metabolism



In non-metabolizers, more than 99% of the dose is found in the urine unchanged and this causes a high plasma level of the unchanged drug which may be related to some side-effects like blurred vision, diplopia, dizziness, and headache (Eichelbaum et al., 1975a,b). Because only 66% of the dose recovered from metabolizing subjects, this suggested that these two dehydro-sparteine metabolites may be further metabolized or that other metabolites were being formed. An individual's capacity to 4-hydroxylate debrisoquine is closely related to the N-oxidation capacity of sparteine. It is suggested that two metabolic reactions are controlled by similar, if not identical, genetic factors (Bertilsson et al., 1980).

However, two subjects who were non metabolizers of sparteine had a normal capacity to metabolize amobarbitone to its N-hydroxymetabolite (Eichelbaum et al., 1978; Inaba et al., 1980). In vitro, rat liver microsomes have shown that N-oxidation of sparteine occurs in a cytochrome P-450-dependent reaction (Eichelbaum et al., 1979a).

3.1.4 Short outline of tolbutamide, its metabolism and toxicity

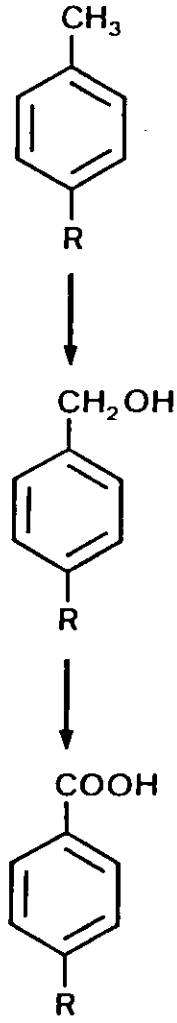
Tolbutamide is a sulfonylurea compound, with

hypoglycaemic activity. Sulfonylurea compounds act generally by stimulating or degranulating the β islet cell of the pancreas to produce insulin. They are ineffective in insulin-deficient patients. Their precise mode of action is not known. Tolbutamide has been used for treatment of diabetes since 1956. In 1970, it became clear that when tolbutamide was taken on a fixed dosage schedule for a period of a year by adults with mild, late-onset diabetes, there was a significant increased risk of cardiovascular death (University Group Diabetes Programme, 1970a,b).

This toxicity might develop from a cardiovascular susceptibility to chronic elevated blood levels of tolbutamide (Poffenbarger and White, 1975).

Metabolism of tolbutamide was studied many years ago. In these early studies, 1-butyl-3-p-carboxyphenyl-sulfonylurea (CTB) was described as the only major metabolite in man, rabbit, guinea pig and rat (Louis et al., 1956), together with a small amount of 1-butyl-3-p-hydroxymethylphenylsulfonylurea (HTB) Wittenhagen et al., 1959) (Fig. 3.5).

Figure 3.5 Tolbutamide metabolism



Tolbutamide

Hydroxymethyl-tolbutamide

Carboxytolbutamide

$R = -SO_2NHCONH(CH_2)_3CH_3$

More recent studies have been done using [^{3}H]-tolbutamide (Thomas and Ikeda, 1966) and [^{14}C]-tolbutamide (Tagg, et al., 1967). It was found that the amount of HTB is not small and it cannot be ignored in any of these species, man rabbit, guinea pig or rat.

In 1966, Thomas and Ikeda succeeded in the isolation of the metabolites in crystalline form and found that HTB is a quantatively important metabolite which accounted for 33% and CTB 67% of drug-related material excreted in urine after an oral dose of ^{3}H-labelled tolbutamide in man. The same authors described tolbutamide metabolism in rats too: in this species, 80% of the oral dose was excreted in urine, mainly as HTB. Administration of tolbutamide i.p. to rats, also shows the same pattern of metabolism (Thomas and Ikeda, 1966). Both metabolites CTB and HTB have been reported to have no hypoglycaemic activity (Fajans et al., 1956; Rusching et al., 1958). In vitro studies, using rat liver, showed that tolbutamide is hydroxylated to form HTB by a NADPH-linked microsomal drug metabolizing system. HTB is then oxidized by soluble enzymes to form CTB. Alcohol dehydrogenase and xanthine oxidase and/or aldehyde dehydrogenase may be involved in this overall conversion (McDaniel et al., 1969).

Polymorphism of tolbutamide pharmacokinetics has been reported. There was a nine-fold variation in the rate of elimination which was interpreted by the authors as variation in metabolism (Scott and Poffenbarger, 1979). They suggested that the hydroxylation of tolbutamide to form HTB as first step is under genetic control, while the cytoplasmic conversion of HTB to CTB was not the actual source of genetic variation (Scott and Poffenbarger, 1979).

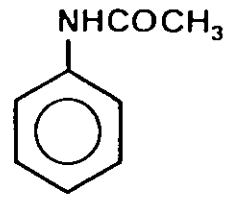
3.1.5 Short outline of acetanilide, its metabolism and toxicity

Acetanilide is considered the parent member of aniline-based weak analgesic-antipyretic drugs.

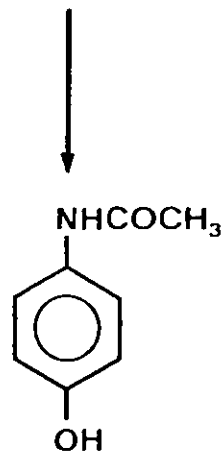
It is rapidly hydroxylated to paracetamol to which the analgesic and antipyretic effects of the drug are chiefly attributed. When acetanilide is given orally to man (2g), 82% of the dose is excreted in 24h as conjugated paracetamol, 3% as free paracetamol, 0.1% as unchanged drug and 0.04% as aniline (Brodie and Axelrod, 1948) (Fig. 3.6). The conjugates of paracetamol are

Figure 3.6 Acetanilide metabolism

(Major route of metabolism)



Acetanilide



N-Acetyl-p-aminophenol

Sulphate and glucuronic acid conjugation

glucuronic acid and sulphate (Smith, 1956; Welch et al. 1966; Cummings et al., 1967; Gault et al., 1972).

Extensive deacetylation of acetanilide was seen in an in vitro, study using rat tissue (Michel et al., 1937), which is not the same route of metabolism when whole animals are used. So, it was suggested that in vivo, de-acetylation of acetanilide occurs as the first step to give aniline, then hydroxylation and acetylation to form N-acetyl-p-aminophenol takes place. This being confirmed by the fact that aniline is metabolized to N-acetyl-p-aminophenol (paracetamol) conjugates in man (Brodie and Axelrod, 1948) and rabbit (Smith and Williams, 1949). In dogs, aniline is metabolized to p-aminophenol rather than conjugated to paracetamol.

The main toxic effect of acetanilide is the formation of methaemoglobin, which is related to the concentration of aniline in blood and is thought to be formed by oxidation of the amine (Brodie and Axelrod, 1949; Kiese, 1965).

3.1.6 Short outline of hexobarbitone metabolism and its relation to the sleeping time

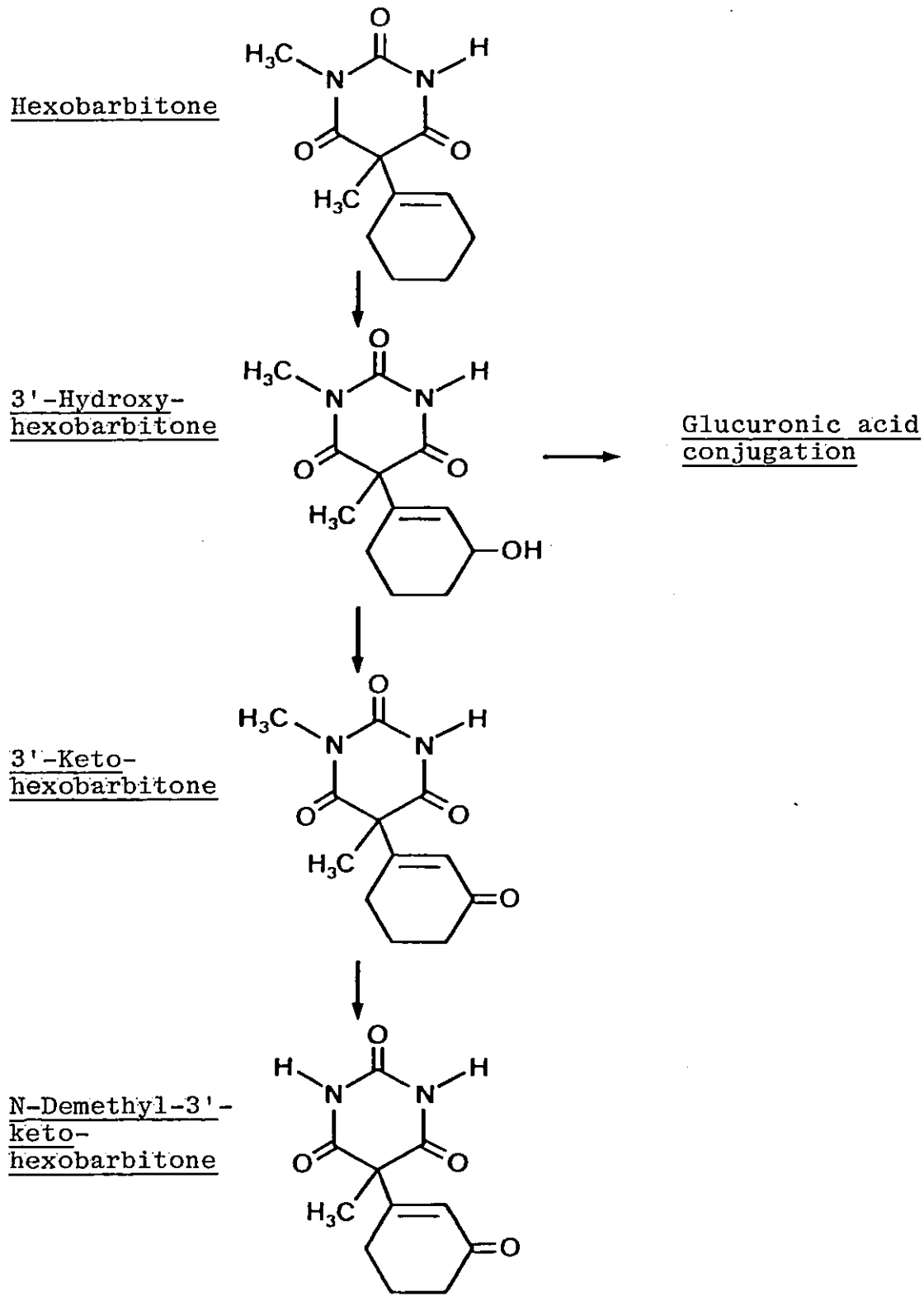
Hexobarbitone is a non-analgesic hypnotic drug, belonging to the short acting class of barbiturates. Its action is slow in both onset and recovery, so correspondingly difficult to employ in clinical use as an i.v. anaesthetic.

Bush and Weller (1972) have shown that the major metabolite of hexobarbitone is the 3'-hydroxy-derivative in many animal species, like dog, rabbit, mouse, rat and man with subsequent 3'-ketoformation, N-demethylation of the 3'-keto product and glucuronic acid conjugation of 3'-hydroxyhexobarbitone (Fig. 3.7).

The popularity of hexobarbitone as a model substrate for the liver monooxygenase system probably stems from the rapid absorption, distribution and metabolism of the drug and hypnotic activity in laboratory animals. The duration of sleeping time which reflects primary metabolism rather than tissues redistribution, in contrast to thiopentone for example, (Bush and Weller, 1972).

Figure 3.7 Hexobarbitone metabolism

(Major metabolites of hexobarbitone)



Hexobarbitone metabolism shows species differences. Quinn et al. (1958) have found a direct relationship between the biological half-life of hexobarbitone and duration of its hypnotic action in a number of species. Additionally, the in vitro activity of microsomes prepared from liver of these animals correlated with the drug metabolism rate in vivo as shown previously in Table 3.2.

Several studies have shown that there are large strain differences in hexobarbitone sleeping times in mice (Jay, 1955; Vesell, 1968a) and these differences are due to variations in the rate of metabolic hydroxylation of hexobarbitone and not to variation in brain sensitivity to the drug. The DBA strain of mice sleeps longer than the C57 strain and has higher levels of the unchanged drug indicating a faster rate of metabolism in the C57 strain (Tabakoff and Erwin, 1977). This result confirms the previous result of Belknap et al. (1973) who demonstrated that animals of the DBA strain showed markedly greater intoxication than those of C57 mice throughout a period of pentobarbitone administration. Pharmacological responsiveness of mice to hexobarbitone is altered by both genetic and environmental factors including, age, sex, litter size, bedding, pain, stimuli, ambient temperature,

animals per cage and the time of drug administration. The mice which slept longer than others had lower hexobarbitone oxidase activity in the liver microsomes and in all strains the level of hexobarbitone in the brain and in the plasma was the same (Yaffe et al. 1968; Kato, 1969). Moreover, strain differences in hexobarbitone sleeping time in mice correlated with zoxazolamine paralysis time and with differences in survival time on a warfarin diet (Lush, 1976). In vitro microsomal hexobarbitone hydroxylase activity, acetanilide hydroxylase, σ-nitro-anisole demethylase and aminopyrine demethylase and hexobarbitone sleeping time were all decreased in animals pretreated with phenobarbitone, while 20-methylcholanthrene, on the other hand, produced only slight changes in these parameters (Furner et al., 1969a).

3.2 MATERIALS AND METHODS

3.2.1 Animals

Inbred female rats (150-200g body weight) of two strains DA and Lewis (see Table 2.1) were used.

3.2.2 Chemicals and Radiochemicals

Phenacetin, paracetamol, sodium hexobarbitone, sparteine sulphate and sulphatase were obtained from Sigma Chemical Company, Poole, U.K., β-glucuronidase was obtained from Warner-Chilcott Limited, 2-hydroxy-phenacetin was a gift of Dr. R.M. Welch, Research Triangle Park, North Carolina, U.S.A., 5-dehydro-sparteine perchlorate was a gift of Professor D.S. Davies, of the Royal Postgraduate Medical School, London. Phenformin and 4-hydroxyphenformin were gifts from Dr. L.J. King, Department of Biochemistry, University of Surrey, U.K. Tolbutamide, hydroxymethyltolbutamide and carboxytolbutamide were the gifts of Hoechst Pharmaceuticals, Milton Keynes, U.K. Amberlite XAD-2 resin was commercially purchased from B.D.H. Chlorpropamide, orthocetamole, aniline, acetanilide and acetonitrile were available in our department. [G-^{3}H]-phenacetin was synthesized from [G-^{3}H]-paracetamol and was available within the department. [U-^{14}C]-Aniline hydrogen sulphate (specific activity 50μCi/98.2μg) was supplied by Amersham International Limited, Buckinghamshire, HP 79LL.

[U-^{14}C]-Acetanilide was prepared from [U-^{14}C]-aniline (Vogel, 1951) which was supplied in a sealed ampoule as follows: the ampoule opened by scratching the serration with the file, and poured into distilled water (100ml) using conc. HCl (3.6ml) and aniline (4.0ml) to wash and dissolve the trace of remaining [U-^{14}C]-aniline in the ampoule and the solution was stirred continuously until the aniline passed completely into the solution. Charcoal (3.0g) was used to decolburise the coloured solution, warmed to about 5°C and stirred for 5 minutes, then filtered at the pump. Redistilled acetic anhydride (5.2ml) was added, stirred until dissolved, and then poured into a solution (100ml) of water which contained 33.0g crystallised sodium acetate. After stirring vigorously for 30 minutes and cooling in ice, acetanilide was filtered off. The precipitate was washed with ice-cold water and recrystallized from dilute aq. methanol, drained well and dried upon filter paper in the air.

The purity of acetanilide was tested by measuring the melting point (113°C) and its radiochemical purity was assessed by thin-layer chromatography of 50µl of a solution (2mg product/ml absolute ethanol) spotted on Merck silica gel F_{254} plates (5 x 20cm), developed in ethylacetate-ether 98:2 v/v.

Acetanilide and aniline were well separated and more than 98% of the applied radioactivity ran as acetanilide, without any detectable aniline, as in Figure 3.8, using a Packard Model 7201 radiochromatogram scanner. The product had a specific activity equal to 16771 dpm mg^{-1} which was measured as described in Chapter Two, 2.2.8.

3.2.3 Dosing and collection of excreta

Table 3.9 shows the dose, solvent and route of administration of each drug which had been given to the rat. For p.o. administration, the drug was given directly into the stomach using a modified spinal needle. After dosing the animals, they were housed in either metal, plastic or glass metabolic cages (for radioactive drugs, glass cages were used) which were designed to separate faeces from the urine. Animals were allowed food and water ad libitum. Urine collections were made after washing the cages thoroughly with water. Aliquots (20ml) of the measured final volume were stored at -20°C in plastic screw-capped containers prior to analysis.

Figure 3.8 Radiochromatogram of ^{14}C -acetanilide following synthesis described in text

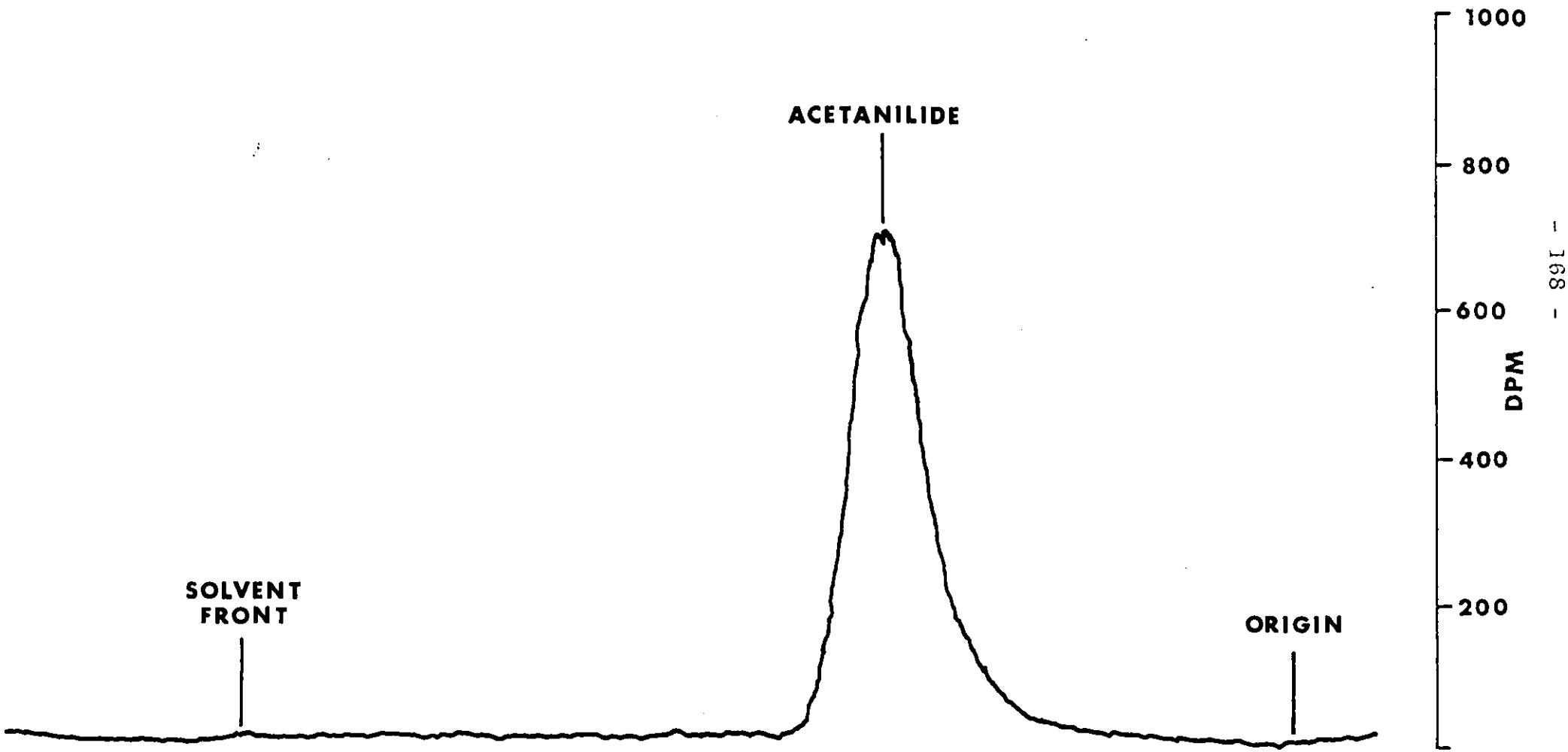


Table 3.9 Dose, route of administration and solvents used for the drugs

Drug	Dose $mgkg^{-1}$	Route of administration	Solvent
Phenacetin*	200	p.o.	75% aq. propylene-1-2-glycol
Phenformin	20	p.o.	distilled water
Sparteine	5	p.o.	distilled water
Acetanilide*	250	p.o.	75% aq. propylene-1-2-glycol
Tolbutamide	500	p.o.	suspend in water, dissolved by adding NH_4OH, pH adjusted to 7.5
Sodium hexobarbitone	180	i.p.	distilled water

* These drugs were sometimes given in a radioactive form [U-^{14}C]acetanilide as [G-^{3}H]-phenacetin

3.2.4 Radiochemical procedures

See Chapter Two, section 2.2.8.

3.2.5 Analytical procedures

3.2.5.1. Determination of 2-hydroxylation products of phenacetin

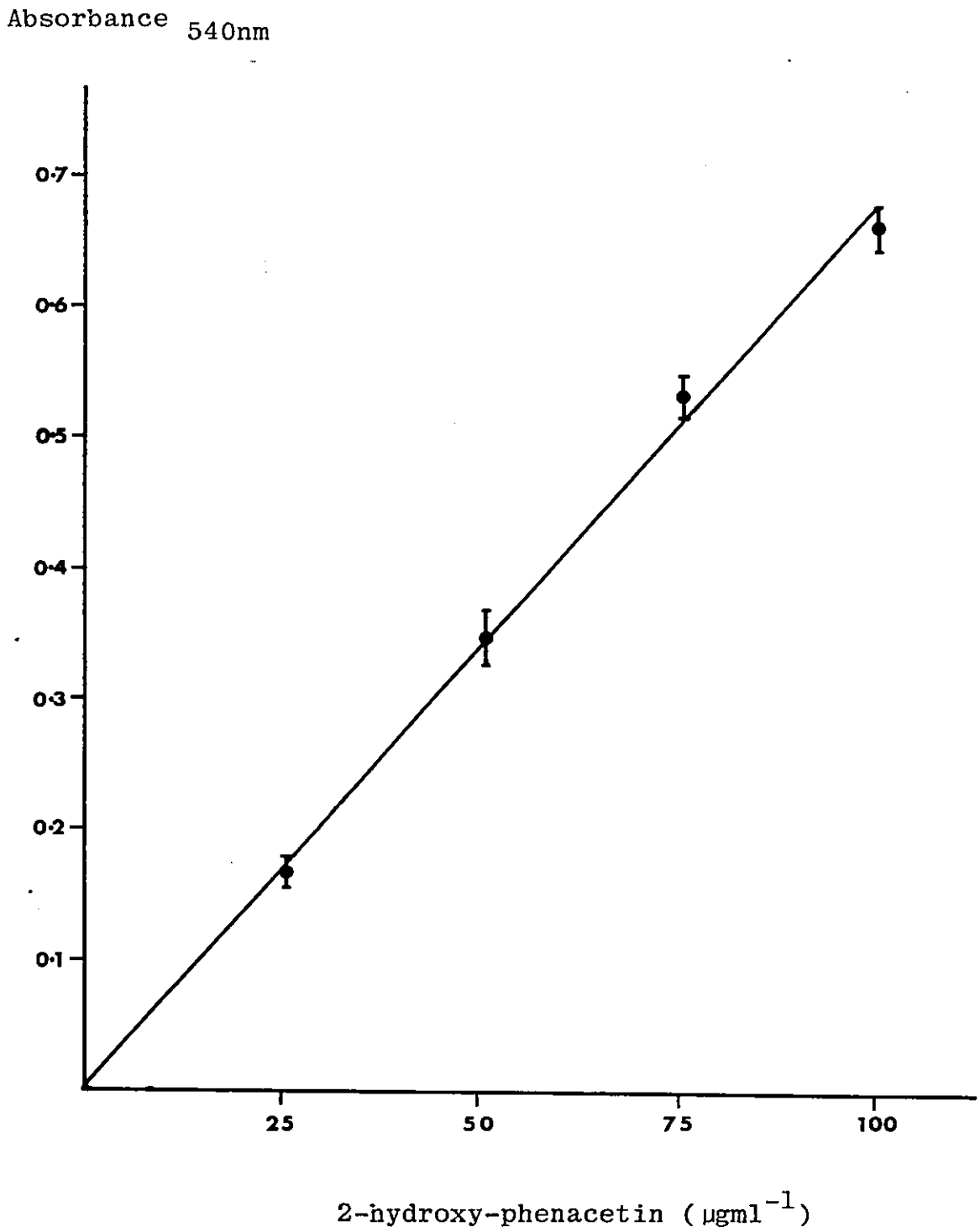
Phenacetin 2-hydroxylation products in urine were measured by a modification of the published assay (Shahidi and Hemaidan, 1969). Urine (0.1ml) was heated with 5ml 2M HCl for 2h at 100°C. This acid hydrolysis deacetylatedthe 2-hydroxy-phenacetin to produce 2-hydroxy-phenetidine and also hydrolysedits conjugates. The urine was then neutralized with solid sodium bicarbonate and 2ml of 10% ammonia solution added to condense two 2-hydroxy-phenetidine molecules to form 3-amino-7-ethoxy-phenoxazone. The solution after shaking was extracted with 2 x 5ml ethyl acetate. The organic layer was removed and evaporated in vacuo. The drug residue was dissolved in methanol (0.1ml) followed by 1M HCl (2.0ml). The absorbance of the resulting purple-red phenoxazone HCl complex was read at 546nm against a urine blank prepared by an identical treatment of a blank urine sample.

A calibration curve was prepared by using a series of known concentrations of 2-hydroxyphenacetin in blank urine, and measuring the absorbance of the solution after being treated in the same manner described as above (Fig. 3.9). Coefficient of variability did not exceed 10% for any of the standard concentrations ($25-100\mu g ml^{-1}$).

3.2.5.2. Determination of paracetamol in rat urine

Paracetamol is mainly eliminated as glucuronide and sulphate conjugates in rats. Urine samples (0.5ml) were therefore incubated with enzymes (10,000 units glucuronidase/sample, 1500 units sulphatase/sample which release the free paracetamol) and internal standard (50µg in 50µl orthoacetamole) in the presence of acetate buffer (4ml of 0.2M sodium acetate buffer, pH 5.0) for 48h at 37°C. The reaction was stopped by adding 10% of trichloroacetic acid (3ml) and spinning of the samples at 2000 rpm for 30 mins to precipitate the enzyme protein. The supernatant was neutralized with solid sodium bicarbonate and extracted with ethylacetate (5ml). After shaking thoroughly, the sample was centrifuged for 30 mins at 3000 rpm, the

Figure 3.9 Calibration curve of 2-hydroxy-phenacetin in urine



extract separated and evaporated *in vacuo* and the drug residue reconstituted in 200µl of methanol. This (2µl) was then injected onto a Pye-Unicam high-performance liquid chromatograph (h.p.l.c.) fitted with a reversed phase column (Partisil 10-ODS; 25cm) operating at 230nm and eluted with 25% acetonitrile in 0.05 M KH_2PO_4 ($2ml\ min^{-1}$).

A calibration curve was prepared using different concentrations of paracetamol and the coefficient of variability for each point never exceeded 10%. (Fig. 3.10). A typical chromatogram trace for paracetamol in rat urine is shown in Fig 3.11.

3.2.5.3 Determination of phenformin and its metabolites

4-Hydroxy-phenformin is mainly excreted as its glucuronic acid conjugates in the rat. Enzymatic hydrolysis was done as described in section 3.2.5.2., and the neutralized solution was analysed by the same procedure as described by Oates *et al*. (1980) for the extraction and measurement of the drug and its metabolite. The procedure is simple; essentially it depends on the absorption properties of Amberlite XAD-2 resin for polar aromatic compounds like phenformin and its metabolite.

Figure 3.10 Calibration curve for paracetamol

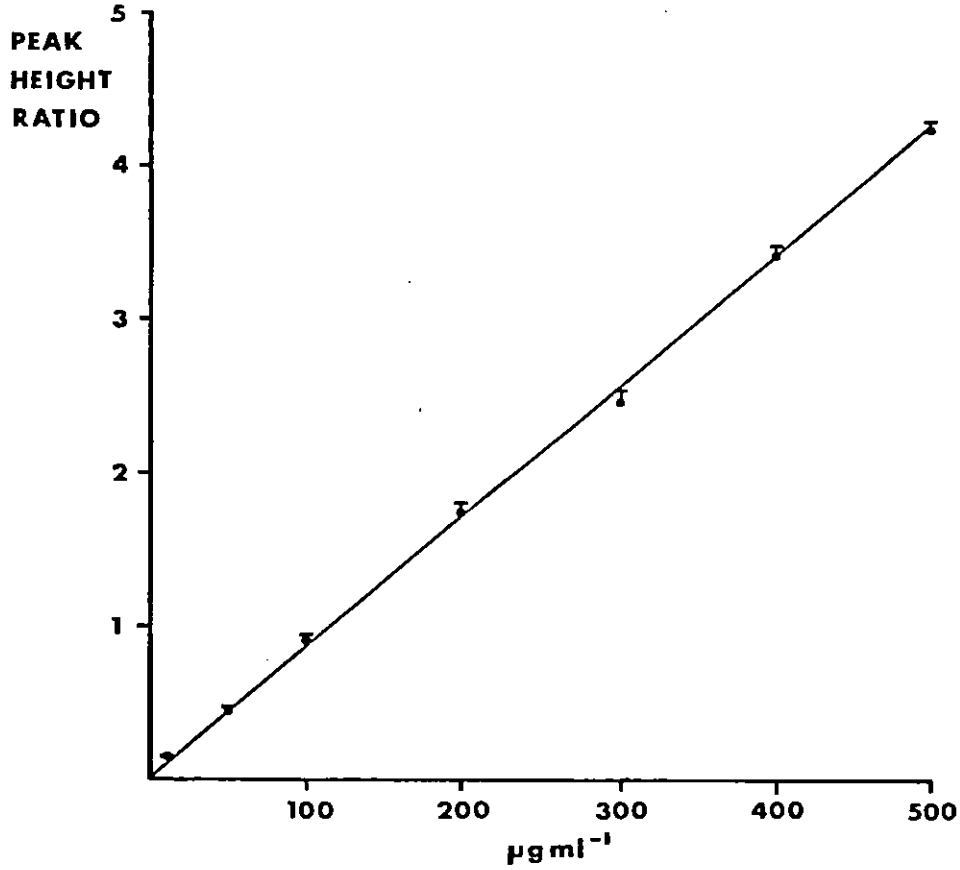
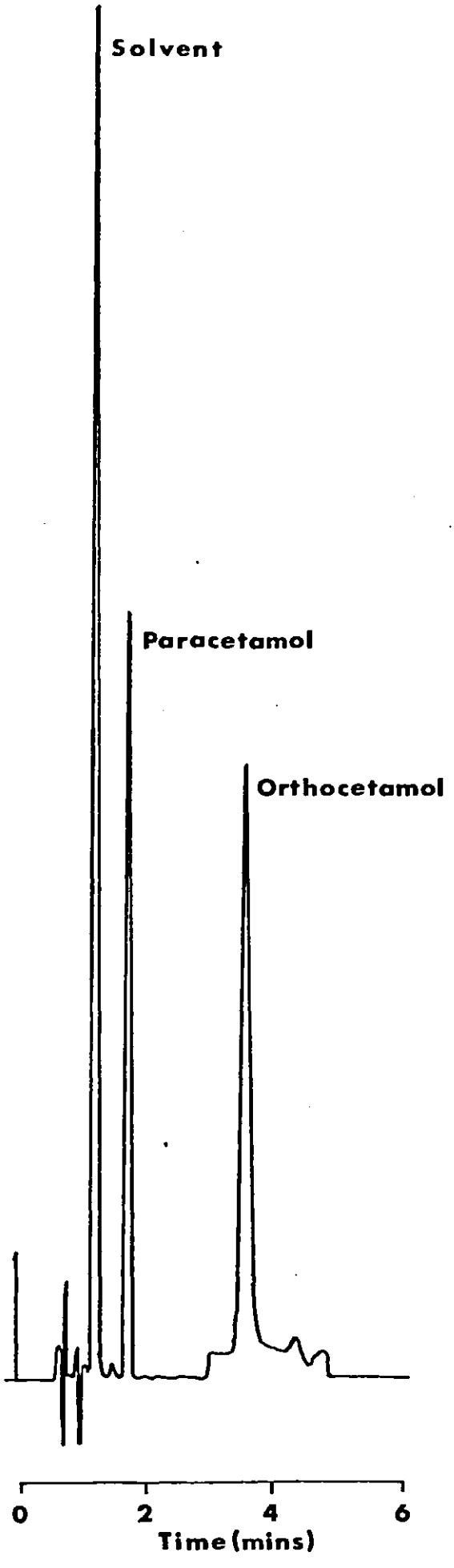


Figure 3.11 Typical trace of paracetamol in urine



Small columns (5 x 0.5cm) were packed with Amberlite-XAD-2-resin, three solvents were applied [Acetone (3ml), methanol (3ml) and water (3ml)] before extraction. Each of the columns was used for extraction of one sample. To each column was applied internal standard solution (0.5ml containing 100µg phenacetin in water) followed directly by a urine sample (2ml). The columns were washed with water (3ml), elution was carried out with methanol (3ml) and the elutes collected in 50ml ground-glass-stoppered tubes. The extracts were evaporated in vacuo at 50°C in a rotary evaporator. The drug residues were redissolved in the mobile phase (100µl) of acetonitrile in 0.05 M KH_2PO_4 (30% v/v), 5µl of which was injected onto the h.p.l.c. column. This was fitted with a column packed with a bonded reversed-phase material (Bondipak C18, particle size 10µm), through which a mobile phase was pumped (Pye Unicam LC-XPS) at ambient temperature (4ml min^{-1}).

The detection was achieved by U.V. absorption at 230nm (Pye-Unicam LC-UV). For the calibration curve (Fig. 3.12) a series of different concentrations of phenformin (2-30µg/ml urine) and 4-hydroxyphenformin (25-150µg/ml urine) and internal standard were extracted as described above. A typical chromatograph trace of phenformin and its metabolite in rat urine is shown in Figure 3.13.

Figure 3.12 Calibration curves of phenformin (A) and its metabolite (B)

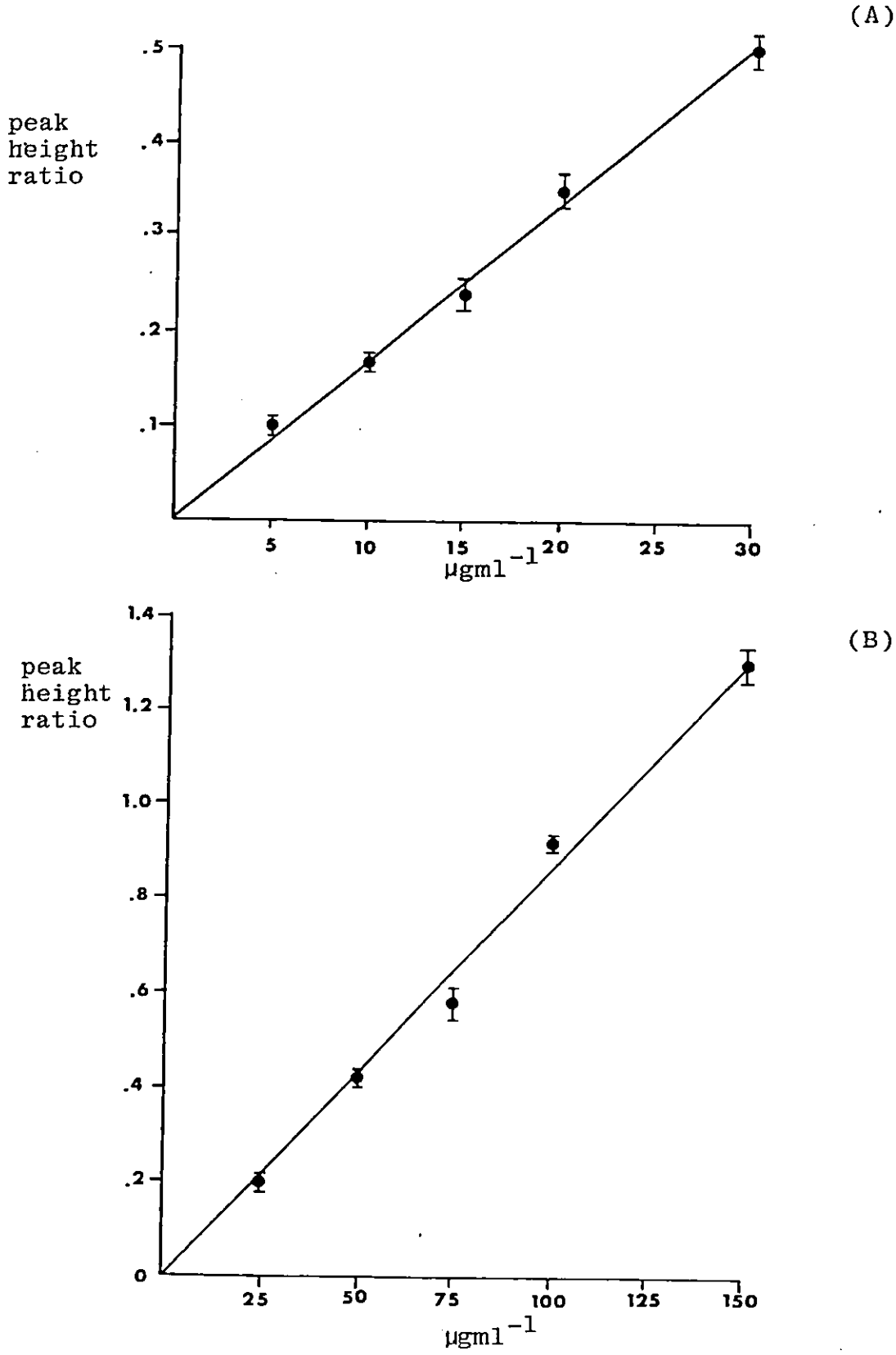
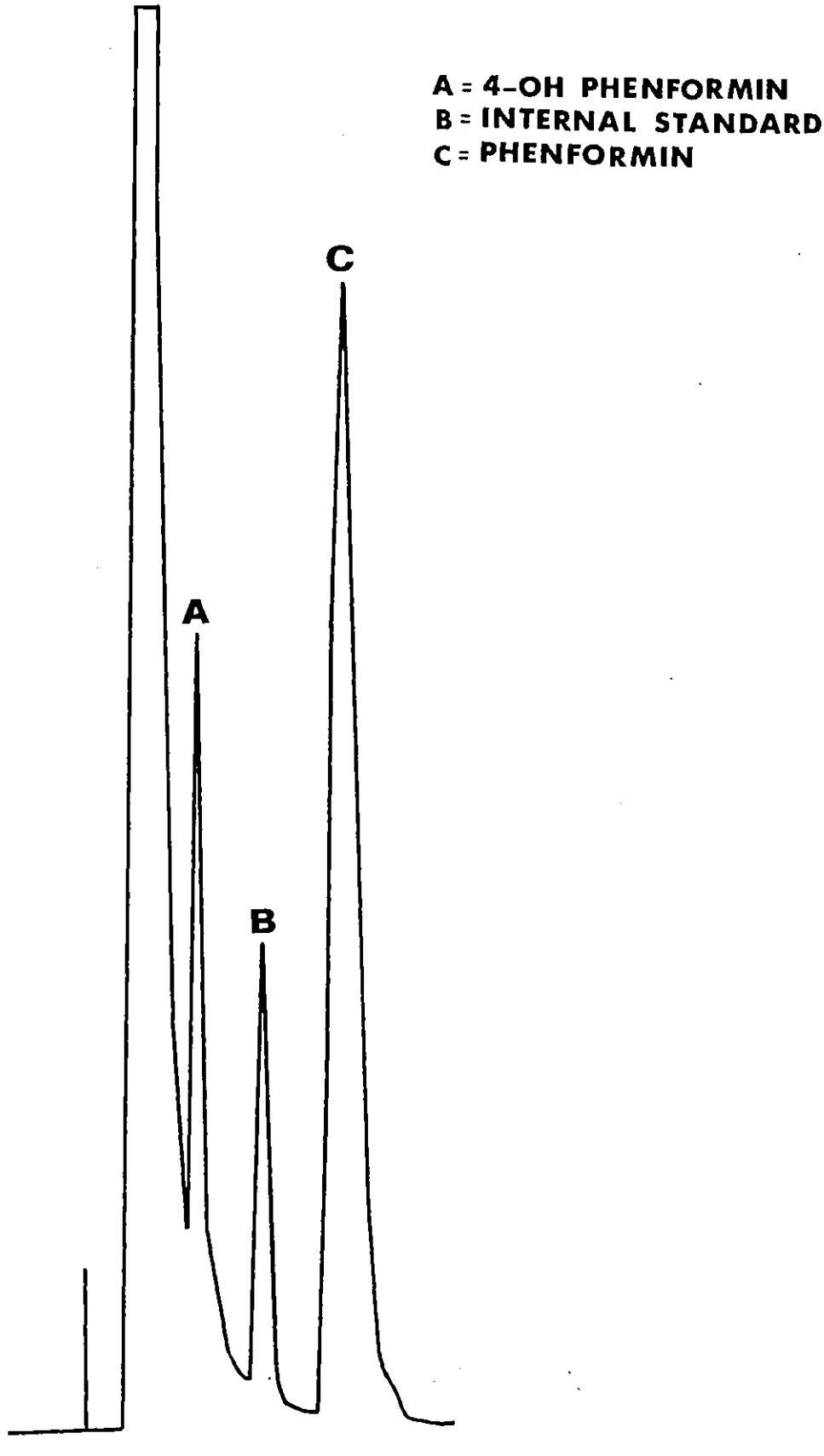


Figure 3.13 Typical trace of phenformin and its metabolite



3.2.5.4. Determination of sparteine and its metabolites

Sparteine and its metabolites in urine were determined according to the gas chromatography methods of Eichelbaum et al. (1979a) using norephedrine as an internal standard.

Urine samples (2ml) were pipetted into screw-capped tubes followed by the addition of 0.1 M HCl (2ml) containing 500µg of the internal standard norephedrine, 5 M NaOH (0.2ml) and dichloromethane (0.4ml). After capping, each tube was vortexed for a few minutes and this was followed by centrifugation at 3000 rpm for 10 minutes. The organic layer (2µl) was injected onto the g.c. column (Carbowax containing 10% KOH). A Pye-Unicam Series 204 chromatograph fitted with a flame ionisation detector used the following conditions:

Carrier gas and flow rate: Nitrogen 35ml min^{-1}
Hydrogen 25ml min^{-1}
Air 250ml min^{-1}

Column temperature 180°C
Detector temperature 240°C

A typical g.c. trace is shown in Figure 3.14. Calibration curves were constructed for sparteine (Fig. 3.15).

Figure 3.14 Typical trace of sparteine and its metabolite with internal standard

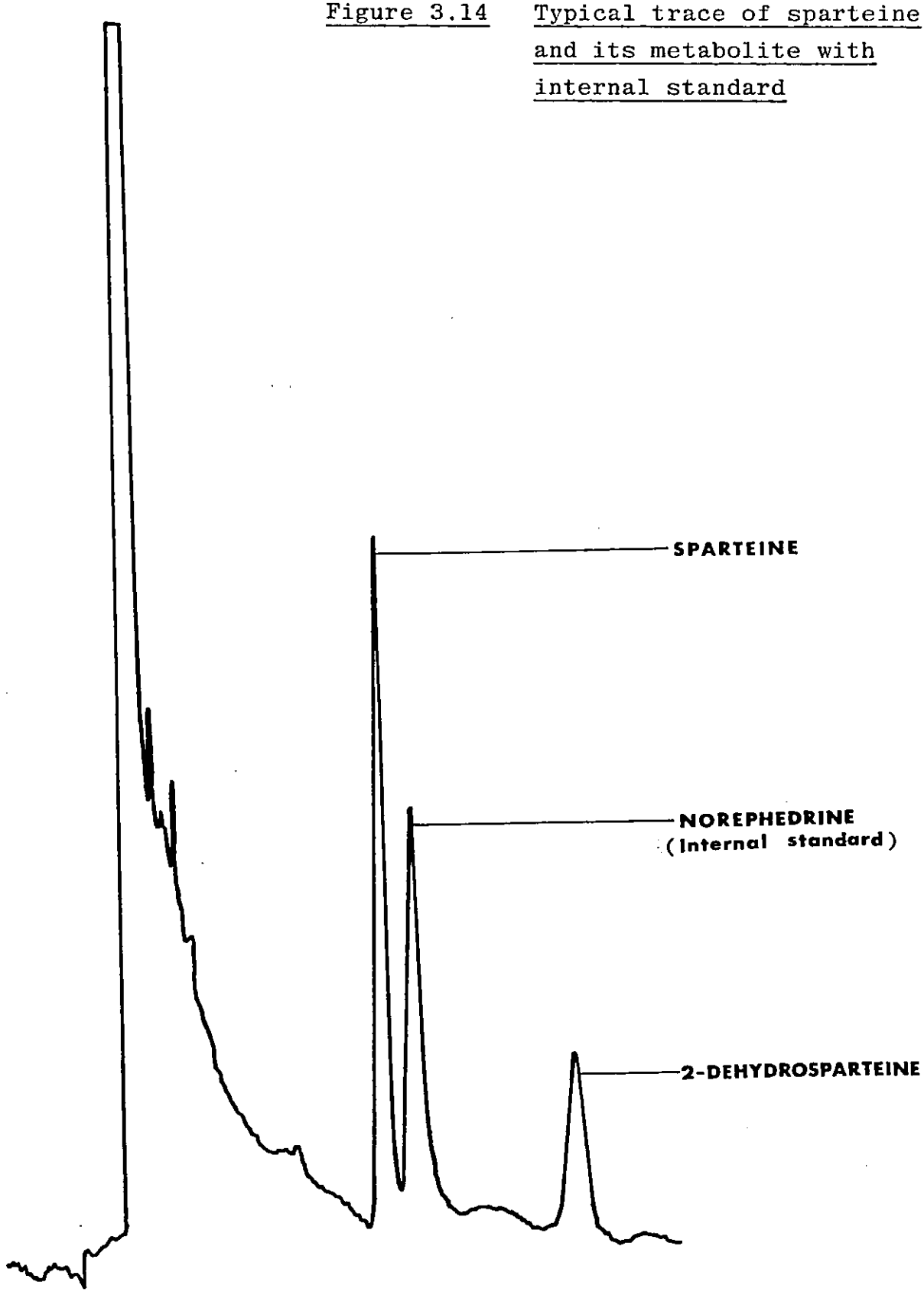
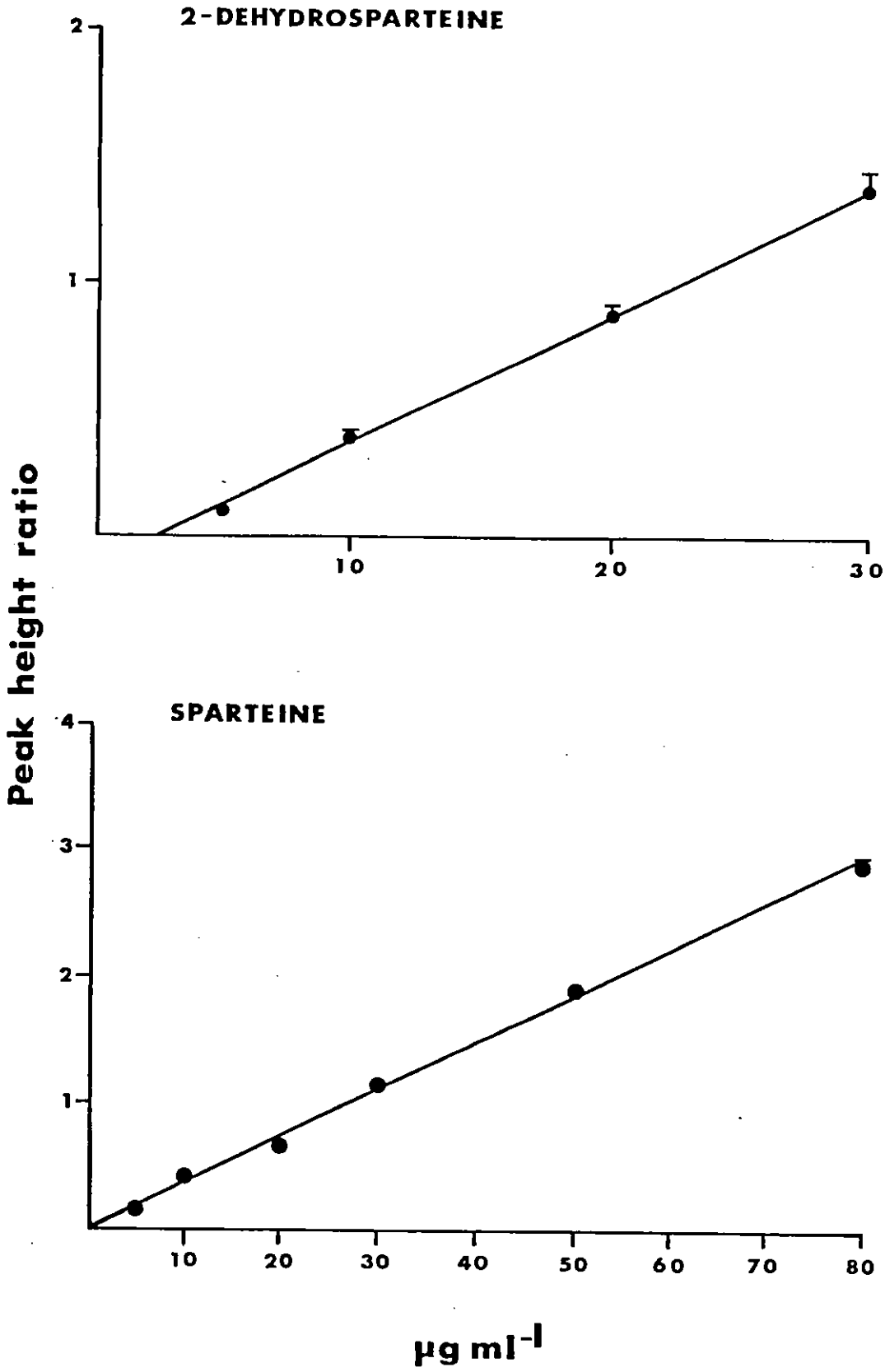


Figure 3.15 Calibration curve of sparteine and 5-hydrosparteine



For the 2-hydrosparteine metabolite in rat urine 5-dehydrosparteine (which is the only metabolite available)was used; the amount of 2-hydrosparteine was measured by estimation of 5-dehydrosparteine and correction for a difference in retention time (multiply by 1.15; retention time of 2-dehydro-sparteine/retention time of 5-dehydrosparteine). No correction was made for difference in molecular weight because sparteine sulphate and dehydrosparteine perchlorate happened to be almost identical in molecular weight. No chemical synthesis has ever been described for 2-dehydrosparteine. Figure 3.14 shows a calibration curve for 5-dehydrosparteine. The co-efficient of variation for each point on the calibration curve was estimated and never exceeded 10%.

3.2.5.5. Determination of tolbutamide and its metabolites

An h.p.l.c. analysis suitable for urine samples was employed as follows: urine (1ml), internal standard (50µg chloro-propamide/50ul 0.01 M NaOH), 3 M HCl (1ml) and ether (4ml) were added to screw-capped tubes. The tubes were shaken for 10 min and centrifuged at 2000 rpm for 10 min, the ether layers were separated and evaporated to dryness using a rotary

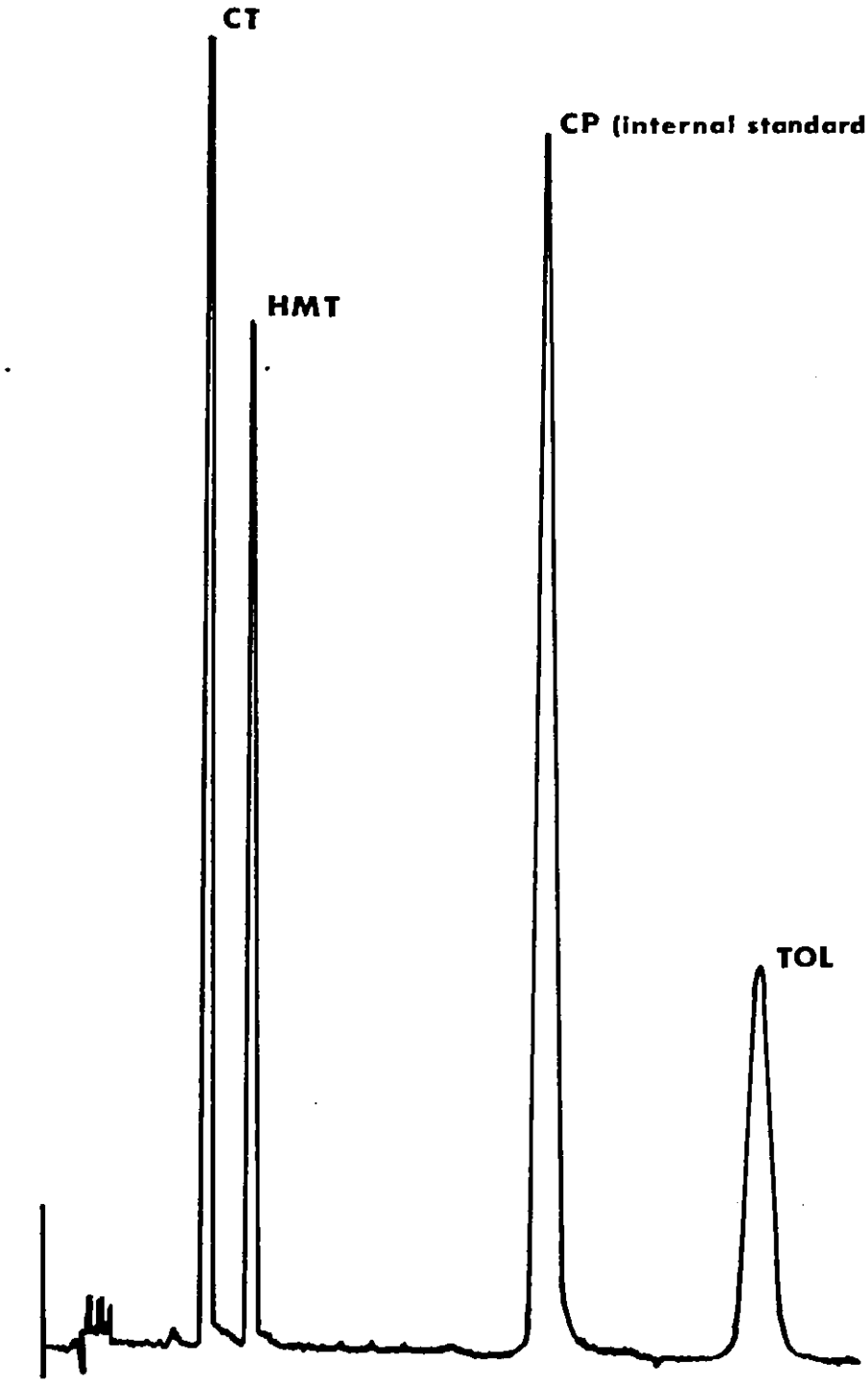
evaporator *in vacuo*, and the residues redissolved in acetonitrile (200µl), 5µl of which was injected into the h.p.l.c. which was used in a similar manner as for the analysis of phenformin with the exception that the solvent was 35% acetonitrile in 0.05% phosphoric acid (v/v) pumped at 2ml min^{-1}. The retention volume of hydroxymethyl tolbutamide was 6.2ml, carboxy-tolbutamide 7.4ml, internal standard 15.0ml and tolbutamide 19.6ml.

A typical chromatogram of tolbutamide, its metabolites and internal standard in rat urine is shown in Fig. 3.16 while Fig. 3.17 shows a calibration curve for the drug and its metabolites.

3.2.5.6. Determination of hexobarbitone sleeping time

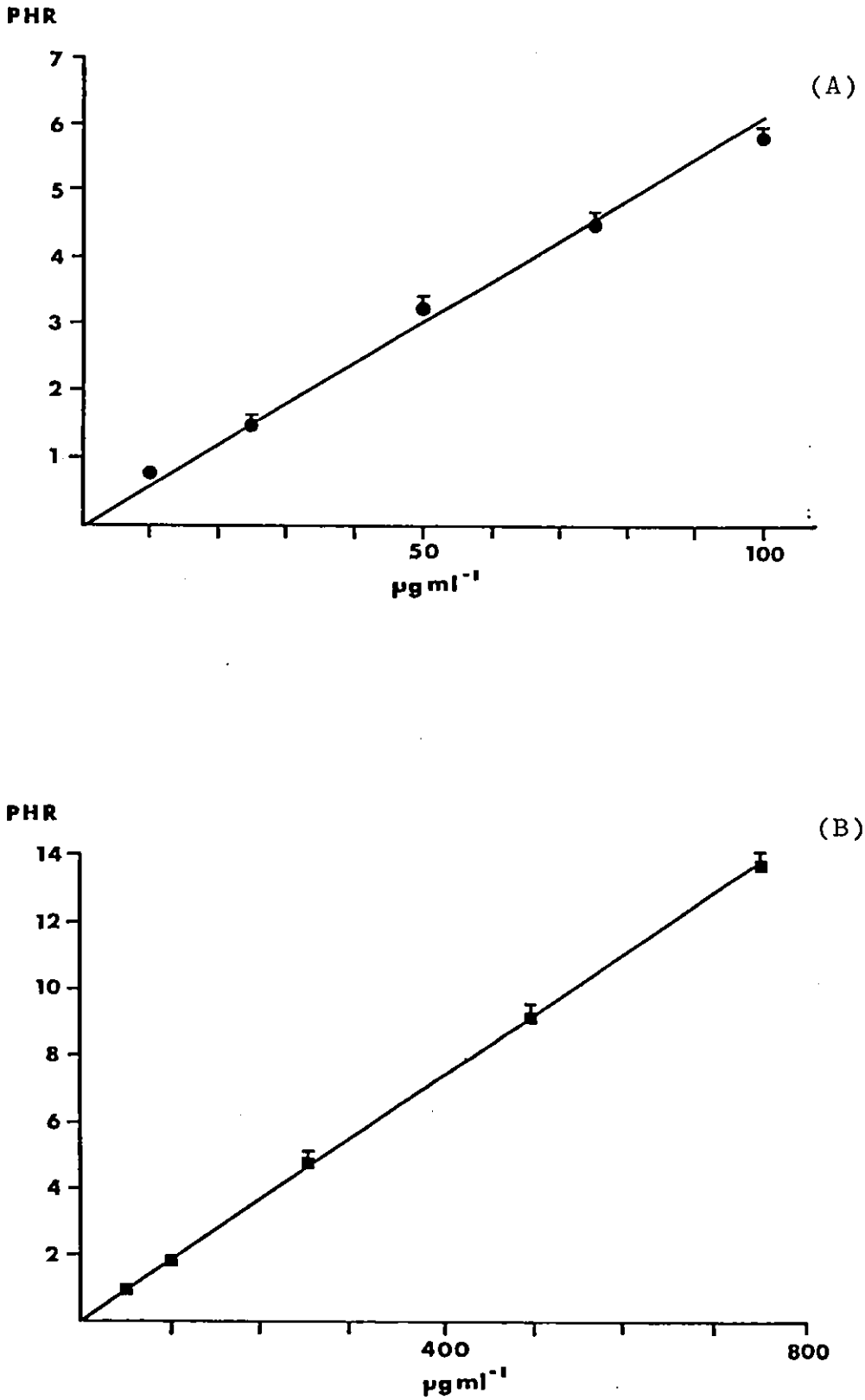
Female DA and Lewis rats were injected with sodium hexobarbitone (180mgKg^{-1} i.p.). After the animals slept, each rat was placed on its back and the sleeping time was recorded as from the time of injection until the animal had regained the ability to right itself repeatedly.

Figure 3.16 Typical trace of tolbutamide and its metabolites with internal standard



CT = Carboxytolbutamide
HMT = Hydroxy-methyltolbutamide
CP = Chlorpropamide
TOL = Tolbutamide

Figure 3.19 Calibration curve of carboxy (A) and hydroxymethyl-tolbutamide (B)



PHR = Peak height ratio

3.3 RESULTS

3.3.1 Metabolism of phenacetin in the DA and Lewis strains of rat

In a preliminary study, one female rat of each of the following strains were used; BN, Fischer 344, PVG and DA, together with one male of the DA strain. Each was given an oral dose of phenacetin ($200mgKg^{-1}$) and then urine was collected daily for three days.

Table 3.10 shows the amount of total paracetamol extracted (free, glucuronic acid and sulphate conjugates).

Thus it can be seen that the female DA rat studied excreted less paracetamol than did the other rats. Another experiment was then set up to study phenacetin metabolism more extensively in females of the Lewis and DA strains of rat. Seven female rats of both DA and Lewis strains were given an oral dose of [^{3}H]-phenacetin ($200mgKg^{-1}$, 0.5uCi) and urine was collected and analysed for total paracetamol (free and conjugated) and 2-hydroxylation products (2-hydroxyphenacetin plus 2-hydroxyphenetidine) by h.p.l.c. and colorimetry respectively. In addition, total urinary elimination of ^{3}H was determined as a measurement of overall drug elimination.

Table 3.10 % Dose excreted as total paracetamol after 200mgKg^{-1} phenacetin orally to one rat of each different strain of rat

Animal	Sex	% Dose excreted as total paracetamol in 0-24h urine	24-48h urine	48-72h urine	0-72h faeces
BN	F	62	6	1	6
Fischer	F	57	5	2	4
PVG	F	60	10	2	5
DA	F	33	8	2	5
DA	M	47	8	2	4

F = Female, M = Male

An inter-strain difference was observed in respect of both O-deethylation and 2-hydroxylation pathways (see Table 3.11). In Lewis rats, O-deethylation to paracetamol accounted for 54 ± 1.5%, whilst in DA rats there was a statistically significantly lower O-deethylation of 38 ± 2.2% ($2P < 0.001$). As a result of this, the alternative pathway of 2-hydroxylation was found to be significantly higher ($2P < 0.001$) in DA rats 13 ± 0.6% compared with the Lewis strain 7.0 ± 0.6%.

However, the percentage of total urinary recovery in 0-24h based upon colorimetric and h.p.l.c. methods was lower in the DA (Lewis 61 ± 1.0; DA 50 ± 2.5%). Moreover, the measurement of urinary ^{3}H content in 0-24h also showed a marked difference (Lewis 59 ± 6.1; DA 45 ± 6.4% dose) and once again the trend was for lower recovery in the DA strain.

3.3.2 Metabolism of phenformin in the DA and Lewis strains

Five rats of each strain (female DA and Lewis) were given an oral dose ($20mgKg^{-1}$) of phenformin. 0-24h urinary excretion of unchanged drug and its phenolic metabolite were measured by h.p.l.c. The results are summarized in Table 3.12. All rats excreted phenformin, as well as free and conjugated 4-hydroxyphenformin.

Table 3.11 Urinary excretion of phenacetin metabolites in the DA and Lewis strains

Animal	% Dose excreted 0-24h urine as			
	Paracetamol	2-hydroxylation products*	Total recovery	^{3}H
Lewis 1	52	8	60	62
2	55	7	62	52
3	54	7	61	63
Mean ± S.D.	54 ± 1.5	7 ± 0.6	61 ± 1.0	59 ± 6.1
DA 1	35	12	47	54
2	37	13	50	44
3	39	12	51	39
4	40	13	53	43
Mean ± S.D.	38 ± 2.2	13 ± 0.6	50 ± 2.5	45 ± 6.4

* Including 2-hydroxy-phenacetin and 2-hydroxy-phenetidine

Table 3.12 % Dose eliminated by DA and Lewis rats as phenformin and total 4-hydroxy-phenformin

Animal		% Dose eliminated 0-24h urine as: Phenformin	Total 4-hydroxy-phenformin	Total recovery
Lewis	1	3	51	54
	2	5	55	60
	3	5	54	59
	4	3	49	52
	5	3	52	55
Mean ± S.D.		3.8 ± 1.1	52.2 ± 2.4	56 ± 3.4
DA	1	15	40	55
	2	15	35	50
	3	15	35	50
	4	16	22	38
	5	17	32	49
Mean ± S.D.		15.6 ± 0.9	32.8 ± 6.7	48.4 ± 6.3

Several important points should be mentioned. Firstly a marked variation in the degree of phenformin metabolism was observed, only small amounts of unchanged drug being excreted in the urine of Lewis rats (3.8 $\pm$ 1.1%), whereas, by contrast, DA rats excreted larger amounts (15.6 $\pm$ 0.9%) (2 P <0.001). Secondly, there is a difference in the amount of 4-hydroxy metabolite excreted (Lewis 52.2 $\pm$ 2.4; DA 32.8 $\pm$ 6.7%) (2P < 0.001).

In addition, five female F_1 (Lewis X DA) animals were studied with phenformin. Fig. 3.18 shows the distribution of the ratio for phenformin/total 4-hydroxyphenformin in DA, Lewis and F_1 rats, just as in the case of debrisoquine. This distribution is polymorphic and the F_1 hybrid behaves like the parental Lewis rats. This is further evidence for the existence of a recessive defect in the DA female and it would appear that phenformin metabolism is under the same gene control as debrisoquine metabolism.

3.3.3 Sparteine metabolism in the DA and Lewis rat

Seven female rats of each strain (Lewis and DA) were given sparteine 25mgKg^{-1} p.o. Interestingly, unlike man, the rats excreted only sparteine and 2-hydrosparteine. No 5-hydrosparteine was detected in any urine sample.

Figure 3.18 Distribution of phenformin/total 4-hydroxyphenformin ratio in DA Lewis and F_1 female rats

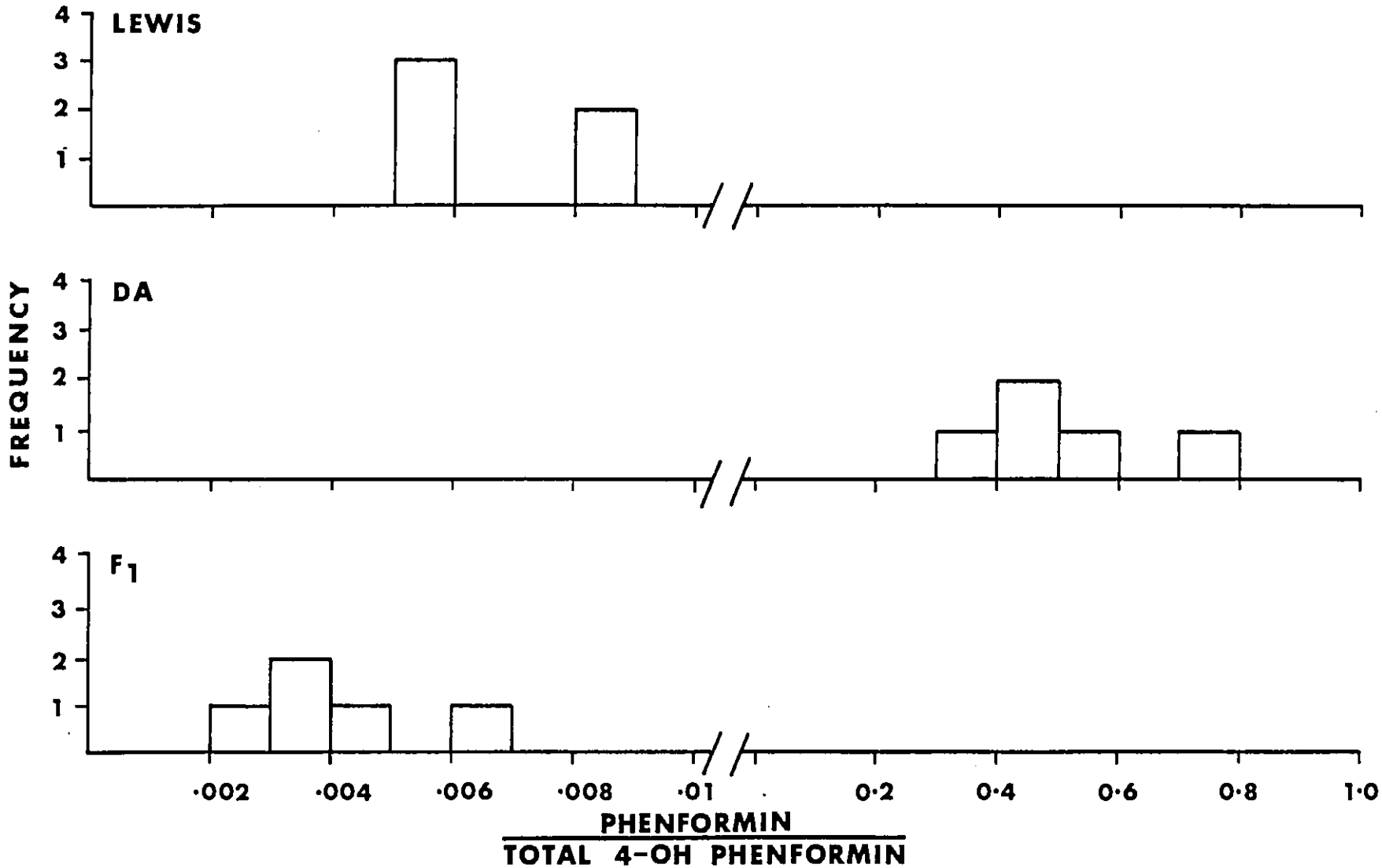


Table 3.13 shows the % dose excreted as unchanged sparteine and its 2-dehydrosparteine metabolite in these two strains. The results show that for the DA rats the excretion of unchanged drug (28.2 ± 6.8%) is higher than in the Lewis rats (7.9 ± 2.1%) (2 P < 0.001), while conversely, the metabolite excretion is higher in the Lewis (14.4 ± 1.7 than in the DA rats (6.8 ± 1.8%) (2 P < 0.001). Total recovery in Lewis rats (22.1 ± 2.1%) was lower than in DA rats (35.1 ± 7.4%) (2 P < 0.005).

3.3.4 Metabolism of tolbutamide in the DA and Lewis strains

Five rats of each strain (female DA and Lewis) were given an oral dose of tolbutamide ($500 mg Kg^{-1}$) and 0-24h urinary excretions of the two metabolites, carboxy-tolbutamide and hydroxymethyl-tolbutamide, were measured by h.p.l.c. No unchanged drug could be detected in the urine of any of these animals. The results are summarized in Table 3.14.

From Table 3.14 one sees that there are no significant differences in the total recovery of the drug carboxy-tolbutamide (CTB) and hydroxymethyltolbutamide (2 P > 0.2), nor in the amount of either CTB or HTB present in the urine

Table 3.13 Urinary excretion of sparteine and its metabolite (2-hydrosparteine) in the DA and Lewis

		% Dose excreted in 0-24h urine as:			
Animal	Number	Sparteine	2-dehydrosparteine	Total recovery	*Ratio
DA	1	23	3.9	26.9	5.9
	2	38	6.4	44.4	5.9
	3	20	5.3	25.3	3.8
	4	36	6.9	42.9	5.2
	5	27	9.2	36.2	2.9
	6	24	8.1	32.1	3.0
	7	30	8.1	38.1	3.7
Mean ± S.D.		28 ± 6.8	6.8 ± 1.8	35 ± 7.4	4.3 ± 1.3

* Ratio = % dose excreted as sparteine/% dose excreted as 2-hydrosparteine

continued...

Table 3.13 (continued)

Animal	Number	Sparteine	2-dehydrosparteine	Total recovery	*Ratio
Lewis	1	10	14	24.0	0.71
	2	12	12	24.0	1.00
	3	7.2	16	23.2	0.45
	4	7.0	16	23.0	0.44
	5	6.6	14	20.6	0.47
	6	6.0	12	18.0	0.50
	7	7.0	15	22.0	0.47
Mean ± S.D.		8.0 ± 2.2	14 ± 1.9	22 ± 2.2	0.58 ± 0.21

* Ratio = % dose excreted as sparteine/% dose excreted as 2-hydrosparteine.

Table 3.14 Urinary excretion of carboxytolbutamide and hydroxymethyltolbutamide in rat urine

Animal		% Dose excreted 0-24h urine as: Carboxy-tolbutamide	Hydroxymethyl-tolbutamide	Total recovery
Lewis	1	6	44	50
	2	3	38	41
	3	4	30	34
	4	4	37	41
	5	4	49	53
Mean ± S.D.		4.2 ± 1.1	39.6 ± 7.2	43.8 ± 7.7
DA	1	4	39	43
	2	5	53	58
	3	3	39	42
	4	5	51	56
	5	4	46	50
Mean ± S.D.		4.2 ± 0.8	45.6 + 6.5	49.8 ± 7.3

of the two strains of rat, Lewis and DA (2 P> 0.02).

Interestingly, these rats (of both strains) excreted HTB as a major metabolite while CTB is a minor product. This is in contrast to man, where the CTB is the major excretion product arising from tolbutamide metabolism.

3.3.5 Metabolism of acetanilide in the DA and Lewis rat

Five rats of each strain (female DA and Lewis were given an oral dose of [U-^{14}C] acetanilide (250mgKg^{-1}) and the 0-24h urine was collected and analysed by h.p.l.c. for paracetamol and acetanilide after enzymic deconjugation. Total recovery was assessed by counting ^{14}C in each urine sample.

From the results in Table 3.15, there is no significant difference in the total recovery of ^{14}C in these two strains of rat; DA rats excreted 77.2 $\pm$ 6.8% dose and Lewis rats 69.0 $\pm$ 6.5% dose (2 P> 0.05).

However, Lewis rats produced less paracetamol than DA rats (2 P < 0.001). The % doses of paracetamol excreted in 24h

Table 3.15 % Dose excreted by DA and Lewis rats as total paracetamol and ^{14}C

Animal		Total paracetamol excreted*	Total ^{14}C
DA	1	62	82
	2	55	69
	3	56	71
	4	63	80
	5	64	84
		60 ± 4.2	77 ± 6.8
Lewis	1	49	68
	2	40	63
	3	46	66
	4	36	80
	5	38	68
		40 ± 4.2	69 ± 6.5

* estimated by h.p.l.c.

urine of DA and Lewis were 60 ± 4.2, 40 ± 4.2 respectively. The result is of great interest because for the previous three substrates, it was Lewis rats which produced most urinary metabolites. No unchanged drug could be detected in the urine.

3.3.6 Hexobarbitone sleeping time studies

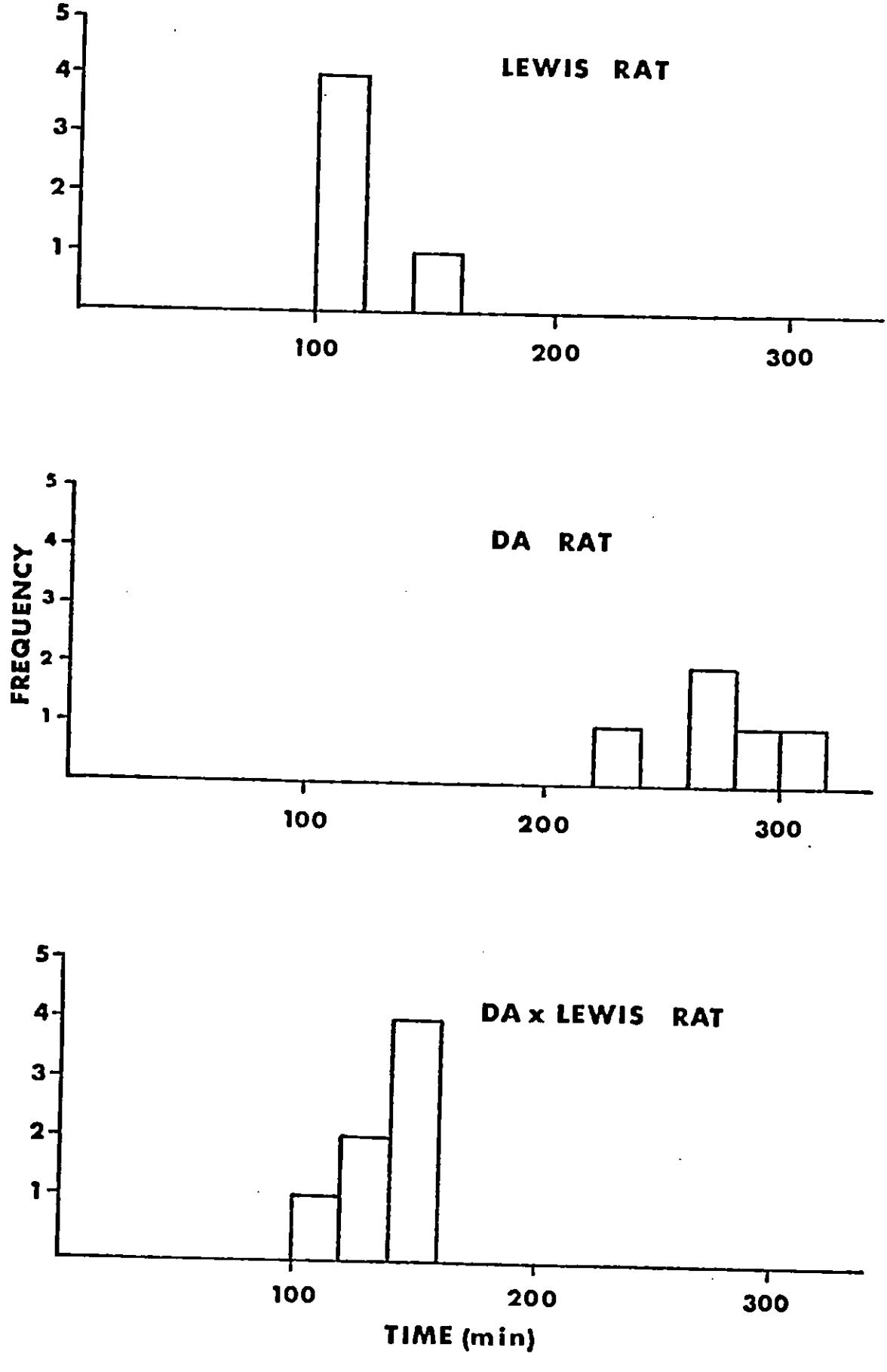
Table 3.16 shows the results for hexobarbitone induced sleeping time determinations in the two strains of rat. The data shows significant differences in response to the drug ($2P < 0.001$): the DA strain (269 ± 33 min) sleeps longer than the Lewis strain (118 ± 21 min) and there was no overlap in the sleeping times observed between these two strains.

Additionally, eight F_1 rats (DA x Lewis) were studied for hexobarbitone induced sleeping times. Fig 3.19 shows the distribution of DA, Lewis and F_1 rats, and these hybrid animals (F_1) show the same duration of sleeping time as the rats which may suggest that prolonged hexobarbitone sleeping time is a genetically determined recessive trait.

Table 3.16 Hexobarbitone sleeping times in DA and Lewis rats, dose 180mgKg^{-1} i.p.

Animals		Sleeping time (min)
Lewis	1	101
	2	108
	3	111
	4	116
	5	155
Mean ± S.D.		118 ± 21
DA	1	222
	2	260
	3	265
	4	290
	5	310
Mean ± S.D.		269 ± 33
F_1 (DA x Lewis)		
	1	105
	2	125
	3	126
	4	140
	5	141
	6	145
	7	145
Mean ± S.D.		132 ± 15

Figure 3.19 Distribution of hexobarbitone sleeping time in DA, Lewis and F_1 female rats



3.4 DISCUSSION

In the phenacetin metabolism study, the formation of two major metabolites has been evaluated, namely, total paracetamol and 2-hydroxyphenetidine. Other known minor metabolites were not measured.

The results obtained indicate that in the Lewis rats, total paracetamol formation (excreted mainly conjugated) accounts for about 54 ± 1.5% of the dose (0-24h urine collection) after phenacetin is administered orally. The results agree with those reported by Timbrell (1973), Buch et al. (1976a) and Nery (1971). 2-Hydroxyphenetidine was detected and accounted for about 7 ± 0.6% of the dose (0-24h urine collection as hydroxylation products) which is the same as in the results reported by Buch et al. (1976a).

However, the DA strain produced only 38 ± 2.2% dose as total paracetamol in 24h urine, which is a much lower value than that reported in other studies and is significantly lower than the value found for Lewis rats in this study. 2-Hydroxylation products, on the other hand, were significantly higher in the DA rat (DA 13.0 ± 0.6; Lewis 7.0 ± 0.6).

Dubach and Raaflaub (1969) and Raaflaub and Dubach (1969) have previously found that the formation of this metabolite is dose dependent in both man and rat.

When the O-deethylation process is lower in DA rats, the other minor routes, like 2-hydroxyphenetidine formation might have a good chance to proceed. This situation is the same as in human PM phenotype. The O-deethylation of phenacetin in man is polymorphic (Kong et al., 1982) and under the control of the same gene locus that determines the debrisoquine 4-hydroxylation polymorphism (Sloan et al., 1978). At higher doses of phenacetin, PM subjects display significantly lower initial rates of paracetamol formation and enhanced formation of 2-hydroxylated metabolites (Sloan, 1980). These subjects, when given phenacetin, readily develop methaemoglobin because of the greater formation of haemotoxic hydroxy phenetidine (Shahidi, 1968).

Actually, all previous phenacetin metabolism studies in rats apparently used other non-DA strains of rat, like Sprague-Dawley, Fischer or Wistar. Generally, the findings in these proposed animal models are in excellent accord both for debrisoquine (see Chapter Two) and for phenacetin metabolism with the inter-phenotypic differences for the metabolism of these two drugs in man.

Phenformin metabolism in these DA and Lewis strains was also studied. 4-Hydroxyphenformin is the major urinary

metabolite of phenformin and it is excreted largely in a conjugated form. Total recovery in the two strains (Lewis 56 ± 3.4; DA 48 ± 6.3%) agrees with the previous findings in the rat; after the administration of an oral dose ($100mgKg^{-1}$) to male Sprague-Dawley rats, phenformin 4-hydroxylation is equal to 56% of the dose (Murphy and Wick, 1968), while male Wistar rats excreted 54% of the same dose in 0-24h urine (Hall et al., 1968). However, total recovery in the DA and Lewis strains is considerably higher than the results obtained by Alkaley et al. (1979) using small doses of phenformin orally ($0.5mgKg^{-1}$) in Sprague-Dawley rats, where only 30% of the dose in 24h urine collections was detected.

These contradictory results might be explained as a result of the different analytical methods used, or of different administered doses.

The amounts of the unchanged drug and of its metabolites were also variable from one study to the other; while Murphy and Wick (1968) found that both are excreted in equal amounts. Alkaley et al. (1979) found that unchanged drug (3.8 ± 1.1) while in DA rats there is a considerably and significantly higher quantity in 0-24h urine collections (15.6 ± 0.9). The opposite is true for the amount of metabolite.

Generally, such results agree with regard to phenformin metabolism, with the inter-phenotype differences previously found for man. In these strain models, phenformin 4-hydroxylation is positively correlated with the ability to perform alicyclic 4-hydroxylation of debrisoquine; it is suggested that probably both pathways are under similar if not identical genetic control. This was confirmed by phenotyping F_1 rats (DA x Lewis) which behaved like the Lewis Strain (i.e. extensive metabolizer) rat.

It is interesting in this connection to cite a study carried out in Bern, Switzerland, with the collaboration of Dr. Kupfer, in which Lewis rat liver microsomes exhibited a clear spectral binding of phenformin (Type I) with a K_d value of 3.6 ± 0.2 (μM) and ΔEmax value 7.849 ± 0.157. In contrast, DA rat liver microsomes showed no measurable spectral binding either with phenformin or with debrisoquine at concentrations near the Lewis K_d value (see Chapter Two). However, at ten times higher concentrations, phenformin exhibits a weak type I interaction.

In the sparteine study, several points emerged. Firstly, in urine collections, only one metabolite was detected, namely 2-dehydrosparteine, which was confirmed by TLC and h.p.l.c. While in man, 5- and 2-dehydrosparteine are expected in the urine apparently as products of sparteine (Spiteller and

Spiteller, 1978). Secondly, the amount of metabolite found in the Lewis strain is significantly higher than in the DA rat. Thirdly, the excretion of unchanged parent-drug is significantly higher in DA than in Lewis. This picture of sparteine metabolism in the two strains is similar to the metabolism of other substrates mentioned earlier, namely debrisoquine, phenacetin and phenformin. Furthermore, the situation found in the Lewis and DA rats is akin to that described for the human EM and PM phenotypes respectively.

Between these two strains the ratio of % dose of unchanged sparteine/% dose of 2-dehydrosparteine in the 24h urine varies by a factor of six. It was of interest to examine the spectral binding characteristics of sparteine with a microsomes fraction obtained from female DA and Lewis rats livers. As with debrisoquine and phenformin, sparteine did not bind to DA microsomal P-450 at concentrations at which it elicited a clear type I interaction with Lewis microsomes.

The urinary and plasma pharmacokinetic studies of sparteine disposition in man would seem to suggest the presence of an unrecognised metabolite(s), possibly arising from secondary metabolism of 2- and 5-dehydrosparteine metabolites (Eichelbaum et al., 1979b). Moreover, 5-dehydrosparteine

exhibited binding spectra with liver microsomes derived from non-DA rats. Interpretation of this binding spectra seems to provide confirmation for the view that this metabolite of sparteine may be further oxidized in the rat. Unfortunately, no binding spectral study has been carried out with the 2-dehydro-metabolite.

The pattern of tolbutamide metabolism in the DA and Lewis strains of rat showed that hydroxymethyltolbutamide is a major metabolite. Only small amounts of carboxy-tolbutamide and no unchanged drug were detected. This result is in accord with previous studies using [^{3}H]-tolbutamide after oral and i.p. dosage (Thomas and Ikeda, 1966; Tagg et al., 1967). It disagrees with other studies which showed that unchanged tolbutamide was detected in rat urine or that carboxytolbutamide emerged as a major metabolite after i.p. dosage (Wittenhagan et al., 1959; Louis et al., 1956).

However, the results of tolbutamide metabolism in the rat (DA and Lewis) are unlike the finding for man, where the carboxytolbutamide and hydroxymethyltolbutamide are major metabolites (56% and 28% respectively) (Thomas and Ikeda, 1966).

It is clear therefore that there are no major inter-strain differences in the amounts of these two metabolites excreted by Lewis and DA rats. Similarly, there was no difference in tolbutamide metabolism between the human PM and EM phenotypes (Idle et al., 1979c). In another paper published by Scott and Poffenberger (1979), a polymorphism of tolbutamide pharmacokinetics was reported in a population study. Here a nine-fold variation in the rate of tolbutamide disappearance from plasma (K_{el}) was observed which could result in a high level of unchanged drug in the blood of a slow inactivator, and this variation was characterized by a trimodal frequency distribution.

Actually, tolbutamide is insoluble in water, so it is not as easily eliminated in the urine as most drugs, unless it is modified to a more polar water-soluble component. This point questions the validity of urinary data in the process of the evaluation of metabolic differences among different individuals by measuring the drug and its metabolite(s). Therefore, measuring the rate of metabolism from drug disappearance in the blood may be a good, perhaps the only, approach to detect the possible variability of tolbutamide in these two strains of rat as in man.

Moreover, McDaniel et al. (1969) have found that tolbutamide is hydroxylated in the rat by a NADPH-linked microsomal drug metabolizing system for the conversion of tolbutamide to hydroxymethyltolbutamide. It is then catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase to convert hydroxymethyltolbutamide to carboxytolbutamide. This is also true in man, where these enzymes are responsible for the conversion of hydroxymethyltolbutamide to carboxytolbutamide, this conversion not being the primary site of genetic control in tolbutamide metabolism in man (Scott and Poffenburger, 1979).

In the rat, then, hydroxymethyltolbutamide is the only major metabolite, while it would appear that aldehyde dehydrogenase or alcohol dehydrogenase do not play a major part in the metabolism of tolbutamide in rats. It may therefore be instructive to study the differences of these enzymes between man and rats for other substrates.

Interestingly, acetanilide metabolism emerged as a new point to note in relation to these proposed animal models. DA female rats produced less metabolite with most of the previously mentioned substrates; debrisoquine, phenformin, sparteine and phenacetin.
They produced significantly higher percentages of paracetamol

(60 ± 4.2% dose) than Lewis female rats (40 ± 4.2% dose) following the administration of acetanilide.

These two strains do not show large difference in total recovery of ^{14}C(77.0 ± 6.8% in DA and 69 ± 6.5% in Lewis).

Comparison between ^{14}C and total paracetamol in urine shows that a considerable amount of the dose was missing, in Lewis rats (about 30% of the dose) and about 17% of the dose in the DA strain. Additionally, there is 20-30% of the ^{14}C dose unidentified compared with the original oral dose of ^{14}C. Because there is only a small amount of ^{14}C in the urine (3-6% of the ^{14}C-dose) from the second day, it is probable that some biliary excretion occurred. It must be noted that the ^{14}C content in the faeces was not measured.

Despite the reasons for different total recovery it seems that the P-450 monooxygenase system in DA rats has a variant P-450 enzyme, which possesses a higher affinity to metabolize acetanilide to paracetamol than those in Lewis rats. DA P-450 has on the other hand, a lower affinity to metabolize phenacetin to paracetamol. In man, however, acetanilide does not

show any interphenotype differences in any of the parameters of acetanilide metabolism measured. (Wakile et al. 1979).

Inter-strain and inter-species differences in hexobarbitone sleeping time are well known. In this study, female DA rats have twice the duration of hexobarbitone induced sleeping time as Lewis rats. It is difficult to make a comparison between the duration of hexobarbitone sleeping time in the DA and Lewis strains and those mentioned in published literature. In rats, Quinn et al. (1958) have found that a dose of $100mgKg^{-1}$ hexobarbitone i.p. to female rats of several strains produced a sleeping time ($\pm$ S.D.) of 90 $\pm$ 15 min. Another study used inbred Sprague-Dawley rats ($100mgKg^{-1}$ i.p.) which had a 90 min hypnosis, but 4 years later gave a 20 min hypnosis (Brodie, 1967; Hathway, 1970).

In fact, there are many genetic and environmental factors which affect the pharmacological response to hexobarbitone (Vesell, 1968b; for review). The duration of hexobarbitone sleeping time after sodium hexobarbitone ($100mgKg^{-1}$ i.p.) in Lewis rats was 118 $\pm$ 21 and in DA rats was 269 $\pm$ 33. From these figures, it might be suggested that Lewis rats react in a similar way to the other strains while DA rats

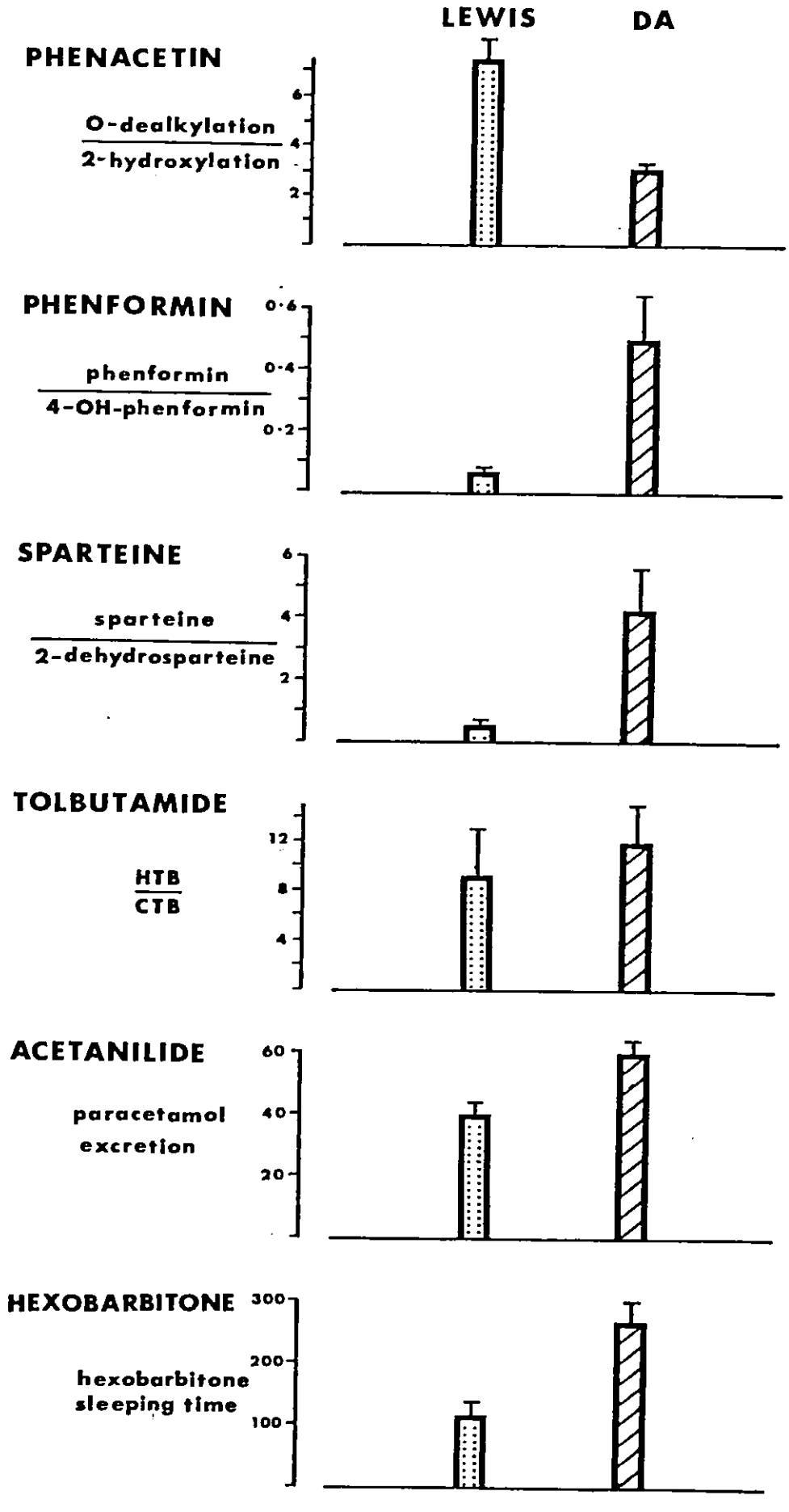
have a longer duration of hexibarbitone sleeping time. In rats as well as in other species, the variation in response to hexobarbitone mainly reflects the variability in its rate of metabolism *in vivo*. The duration of action of hexobarbitone is inversely related to the activity of the liver microsomal enzyme responsible for its metabolism (Quinn *et al.*, 1958).

Phenobarbitone might be expected to show similar variations in its metabolism and sleeping time in these model rat strains. This suggestion was confirmed by a previous chance observation when phenobarbitone was used as an inducer. At this time, two out of five DA rats died, while no death occurred among Lewis rats with a phenobarbitone-inducing dose.

The findings of the present study of the metabolism of phenacetin, phenformin, sparteine, tolbutamide and acetanilide with the hexobarbitone sleeping time may be summarized briefly as follows; (see Figure 3.20).

1. DA and Lewis rats of predetermined phenotype with respect to debrisoquine oxidation display significantly different capacities to metabolize phenacetin, phenformin and sparteine with a positive correlation to debrisoquine metabolism. Thus, their metabolism would appear to be polymorphic.

Figure 3.20 Summary of the metabolic profile of five substrates and hexobarbitone sleeping time in female DA and Lewis rats



2. These rats do not display significantly different capacities to metabolize tolbutamide.

3. These rats displayed significantly different capacities to metabolize acetanilide, where the rats (DA) which were deficient in debrisoquine metabolism, showed a high ability to metabolize acetanilide to form paracetamol.

4. Rats with known phenotype with respect to the metabolism of debrisoquine, phenacetin, phenformin and sparteine displayed significantly different sleeping times with hexobarbitone.

Therefore, all substrates studied which are polymorphically metabolized in man (phenacetin, phenformin, sparteine) are probably polymorphically metabolized in the two rat strains. Conversely, the non-polymorphically metabolized substrates (tolbutamide and acetanilide) were not deficiently metabolized in female DA rats. Interestingly, DA rats metabolized acetanilide better than Lewis rats.

Accordingly, it could be proposed that metabolic studies of drugs and other chemical compounds might more advantageously be carried out initially in female Lewis and DA rats, in order to give a broader insight into the scope of variation

of drug oxidation in man, particularly the inter-phenotypic differences which might be expected to occur in man. A proposed chain of events for investigating a new drug will be discussed later (Figure 5.1, Chapter Five).

CHAPTER FOUR

Aflatoxin B_1-induced hepatocarcinogenicity in the different rat strains.

Contents

4.1 INTRODUCTION

Aflatoxins are mycotoxic substances, the toxic metabolites produced by Aspergillus flavus. The latter may contaminate virtually every stable foodstuff in the tropics and it was identified by Sargeant et al. (1961) and Nesbitt et al. (1962) as the toxin-producing organism in peanut meal.

Mouldy peanuts and their extracts from culture media of the various moulds were found to induce liver tumours in rats (Lancaster et al., 1961) and aflatoxins were discovered to be the actual carcinogenic agent in mould-infected peanuts (Barnes and Butler, 1964; Dickens and Jones 1963; Newberne 1965 and Wogan, 1966).

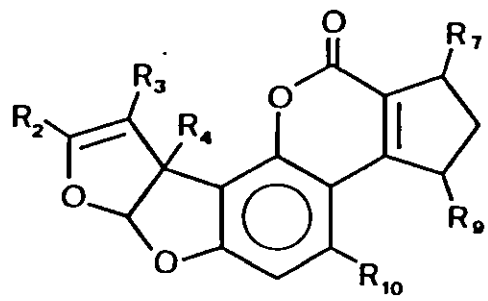
Aflatoxins are characterized by fluorescence patterns on thin-layer chromatograms, a fact which greatly facilitated their isolation. Four components were distinguished, which were designated as aflatoxin B_1, B_2 (blue fluorescence), and G_1, G_2 (green fluorescence) (Hartly et al., 1963; Asao et al., 1965).

Other components were isolated in biological fluids, namely, B_{2a} and G_{2a} (2-hydroxy-derivatives of B_2 and G_2 respectively), and M_1 and M_2 (4-hydroxy-derivatives of B_1 and B_2 respectively) (Dutton and Heathcote, 1966; Holzapfel et al., 1966). Figures 4.1 and 4.2 show the chemical structures of different varieties of aflatoxins. Carcinogenicity and toxicity studies have shown that aflatoxin B_1 is the most potent of all aflatoxins, aflatoxin G_1 is relatively active and aflatoxins B_2 and G_2 are relatively inactive in most mammalian species (Wogan et al., 1971; Wogan, 1973).

Interestingly, as Roebuck et al. (1978) showed in their study of aflatoxin B_2 metabolism in duck, rat, mouse and human liver preparations, only duck livers produced 2-8% of aflatoxin B_1 (AFB_1) among other metabolites, while rat, mouse and human liver preparations produced no detectable AFB_1. This may explain AFB_2 toxicity in the duck.

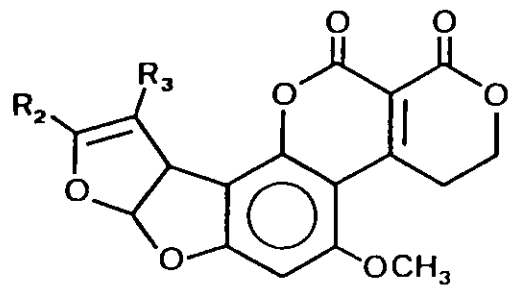
Aflatoxin B_1 is metabolized to AFM_1 in several species, and this metabolite is excreted in the milk of cows (Masri, 1967), sheep's urine (Allcroft et al., 1966), human urine (Campbell et al., 1970) and the breast milk of humans (Burton, 1971). AFM_1 is excreted in small

Figure 4.1 Structure of aflatoxin B_1 and its derivatives



Aflatoxin	R_2	R_3	R_4	R_7	R_9	R_{10}
B_1	H	H	H	=O	H	OCH_3
B_2	H_2	H_2	H	=O	H	OCH_3
B_{2a}	OH	H_2	H	=O	H	OCH_3
M_1	H	H	OH	=O	H	OCH_3
M_2	H_2	H_2	OH	=O	H	OCH_3
P_1	H	H	H	=O	H	OH
Q_1	H	H	H	=O	OH	OCH_3
Aflatoxicol	H	H	H	-OH	H	OCH_3

Figure 4.2 Structures of aflatoxin G_1 and its derivatives



Aflatoxin	R_2	R_3
G_1	H	H
G_2	H_2	H_2
G_{2a}	OH	H_2

amounts in many species of animals, generally less than 3% of the ingested dose of AFB_1; although the figure varies according to the route of administration (Purchase et al., 1973; Masri et al., 1974; Mabee and Chipley, 1973). AFM_1 has also been detected as an AFB_1 metabolite in in vitro studies (Schabort and Steyn, 1969).

Another metabolite aflatoxin P_1 has also been detected as an AFB_1 metabolite in rat urine (Wogan et al., 1967) and in the urine of rhesus monkey (Dalezois et al., 1971; Dalezois and Wogan, 1972), as well as in vitro using liver microsomes of several species (Bassir and Emafo, 1970). However, it has not yet been detected in human urine (Merrill and Campbell, 1974). Generally, it is less toxic than the parent drug AFB_1 (Buchi et al., 1973).

AFQ_1, another metabolite, was also found in the urine of monkeys after the administration of AFB_1 but was not detected in human urine (Campbell and Hayes, 1976). The toxicity of AFQ_1 has been shown to be less than that of AFB_1, both with and without microsomal activation, in vitro (Hsieh et al., 1974).

AFB_{2a} has been found in the urine of rats dosed with AFB_1 or B_2 (Dann et al., 1972) and is essentially non-toxic (Abedi and Scott, 1969; Wogan, 1973).

Aflatoxicol has been detected in the plasma of rats after oral and i.v. doses of AFB_1 but not in mice or monkeys (Wong and Hsieh, 1978); aflatoxicol is less toxic than AFB_1 (Detroy and Hesseltine, 1968; Garner et al., 1972).

Although AFB_1 is a potent hepatotoxin and hepatocarcinogen in various animal species, the type and extent of AFB_1-induced hepatic damage varies from species to species (Wogan, 1973). There is a wide range in the acute LD_{50} of AFB_1, varying from $0.3 mgkg^{-1}$ for ducklings to $16 mgkg^{-1}$ for mature female rats (Newberne and Butler, 1969).

Of domestic animals, sheep are apparently the least sensitive to AFB_1 (Allcroft, 1965), together with mice and catfish (Bassir and Emafo, 1970; Bassir and Emerole, 1975; Halver et al., 1969): ducks on the other hand show a high sensitivity to AFB_1 carcinogenicity (Carnaghan, 1965), as do rainbow trout (Sinnhuber et al., 1968). Other species have shown susceptibility to AFB_1 such as dogs (Newberne et al., 1966), monkeys (Deo et al., 1970),

swine (Barber, 1968), guinea pigs (Paterson et al., 1962), cats, rabbits and hamsters (Newberne and Butler, 1969).

Despite the differences in susceptibility, no consistent relationships were seen between sensitivity and ability to produce either of the known metabolites; AFQ, P_1, M_1 and B_{2a}. But most current literature supports the concept that microsomal enzyme activation is required before cytotoxicity can be expressed. Thus it is possible that species differences in response are attributable to a differential capacity for activation through epoxide formation and covalent binding to DNA, RNA and protein (Roebuck and Wogan, 1977).

Strain differences in AFB_1 toxicity are known in rats and mice, and the incidence of AFB_1 liver-induced tumors has been positively correlated with in vitro DNA binding studies (Wogan, 1973).

Additionally, sex differences in AFB_1 metabolism are known to exist in rats, with males producing more AFM_1, Q_1 and DNA-alkylating metabolite(s) than females in vitro and in vivo (Gurtoo and Motycka, 1976). Sex differences in toxicity after exposure to aflatoxin-contaminated food are also known in man, where there is a 2.5 - 5 fold

higher incidence in males than in females (Peers and Linsell, 1973; Shank et al., 1972a,b).

Many nutrient and non-nutrient substances influence AFB_1 toxicity (for review see Newberne, 1974). Phenobarbitone, when given continuously with AFB_1 reduced and delayed the incidence of liver tumours in rats compared to controls which were fed with AFB_1 alone, and it was suggested that phenobarbitone induced the metabolism of AFB_1 to non-carcinogenic products in liver microsomal enzymes. Cysteine (glutathione precursor), when given before AFB_1 administration, also reduced the hepatic necrosis; on the other hand, administration of diethylmaleate (depletes liver GSH) enhanced the AFB_1-induced hepatotoxicity.

Lijinsky et al. (1970) reported the covalent binding to liver DNA, RNA and proteins following administration of AFB_1 to rats, whereas no covalent binding was observed in chemical reactions. This suggests that AFB_1 requires metabolic activation.

Moreover, indirect evidence of the importance of epoxidation for the biological activity of AFB_1 has been

provided by the formation of toxic, reactive and mutagenic metabolite(s) from AFB_1 via cytochrome P-450-dependent microsomal oxidation in which the C_2-C_3 double bond of AFB_1 (Fig. 4.3) was required for the expression of these properties (Garner et al., 1972; 1973; Swenson et al., 1973).

Swenson et al. (1974) found that a large share of the $[^3H]$-AFB_1 bound to DNA and RNA in rat liver in vivo and to rRNA by human liver microsomes in vitro. This can be hydrolyzed under mildly acidic conditions to yield a compound indistinguishable from 2,3-dihydro- 2,3-dihydroxy-aflatoxin B_1.

This suggests that other possible electrophilic metabolites of AFB_1, such as 2,3-oxides of the known metabolites AFM_1, AFQ_1 and aflatoxicol, may also exist and contribute to the in vivo hepatic macromolecular binding, carcinogenicity and toxicity associated with AFB_1 administration.

Gurtoo et al. (1978) drew the following conclusions from their own work, together with work by Wong and Hsieh,(1978) and several other studies;

a. A good correlation exists between in vitro DNA binding, mutagenesis and possibly carcinogenesis by aflatoxins.

b. The intact C_2-C_3 double bond in the parent aflatoxin is necessary for microsome-mediate DNA binding, mutagenesis and in vivo carcinogenesis.

c. Monooxygenase mediated metabolism at other sites in the AFB_1 molecule essentially represents a deactivation step.

The aim of this study is to investigate whether or not the deficiency of drug oxidation of several substrates in female DA rats (as shown previously) also carries over to a deficiency in AFB_1 metabolism, particularly in the formation of the 2,3-oxide which is known to be a mutagen.

4.2 MATERIALS AND METHODS

The major part of this work has been done in the MRC Toxicology Unit in collaboration with Drs. G.E. Neal and T.A. Connors, where all the materials and chemical compounds were available.

Weanling female DA (OLAC Ltd., U.K.) and weanling female Fischer F344 rats (MRC Unit, stock were used) with average weights of 65g and 50g respectively at the beginning of the experiment.

They were fed a diet consisting of 50% peanut meal and 50% powdered MRC 41B containing 2% arachis oil.

The animals of each strain were divided into two groups: one fed with toxin-contaminated peanuts and the other (as control) fed with non-toxin-contaminated peanuts. In the toxin-fed group, a peanut meal, naturally contaminated with aflatoxin B_1 (AFB_1) (15-16PPM), was blended with a non-toxin-containing peanut meal, so that a final AFB_1 concentration of 4PPM was obtained.

The animals were housed in negative pressure isolators.

The toxic diet was discontinued after 4 months and thereafter all the animals were fed with a non-toxin-contaminated diet. The animals were then killed 14 months after the commencement of the investigation. Pathological assessement of hepatotoxicity in these rats was carried out by two consultant histopathologists, who reviewed liver sections blind.

Debrisoquine was given to the rats for phenotyping. It was administered orally to groups of three control-fed rats of both DA and Fischer strains at different times after the commencement of the feeding experiment. Urine samples were collected over 24h and assayed for the presence of debrisoquine and its 4-hydroxy metabolite according to the method described in Chapter Two, Section 2.2.

4.3 RESULTS

Table 4.1 shows the urinary excretion of the unchanged drug and of 4-hydroxydebrisoquine following the oral administration of debrisoquine to female DA and Fischer rats.

As expected, DA rats excreted more of the parent drug than its metabolite and the reverse was true for Fischer rats. The inter-strain differences were still obvious in animals that had consumed the AFB_1-containing diet for a period of one month.

Table 4.2 summarizes the pathological results in the livers of DA and Fischer rats with their corresponding controls after 14 months from the beginning of the experiment.

All Fischer rats exhibited hyperplastic nodules and 16/17 hepatocellular carcinoma. On the other hand, the DA rats all exhibited degenerative foci and 2/19 hyperplastic nodules but no hepatocellular carcinoma. All the controls for both strains showed normal histology.

Table 4.1 Comparative metabolism of debrisoquine in the female DA and Fischer strains of rat

Animal		% dose excreted as: Debrisoquine	4-Hydroxy-debrisoquine	Total recovery	Metabolic ratio
DA	1	14	12	26	1.2
	2	21	13	34	1.6
	3	18	11	29	1.6
	4*	40	22	62	1.8
	5*	27	27	54	1.0
Fischer	1	5	15	20	0.33
	2	4	20	24	0.20
	3	4	16	20	0.25
	4*	3	22	25	0.14
	5*	1	20	21	0.05

Metabolic ratio = % dose excreted unchanged/% dose as 4-hydroxydebrisoquine excreted in 0-24h urine.

*Animals were fed on the AFB_1 containing diet for one month before administration of debrisoquine.

Table 4.2 Summary of pathology of livers of DA and Fischer rats 10 months after ceasing experimental feeding of AFB_1

Experimental diet	Strain of rat	Total number of animals	Normal histology	Hyperplastic nodule	Degenerative foci	Hepatocellular carcinoma
Non-toxic	Fischer	4	4	0	0	0
Toxic	Fischer	17	0	17	2	16 + 1?
Non-toxic	DA	7	7	0	0	0
Toxic	DA	19	0	2	19	0

Toxic diet contained 4 ppm AFB_2. See Methods section.

4.4 DISCUSSION

These results show that there is a major difference between the two strains of rat in terms of their hepatic response to an experimental diet containing AFB_1. In this study none of the DA rats developed hepatocellular carcinoma at the time of culling. Although there were hepatic lesions in the DA strain, they apparently failed to progress to hyperplastic nodules and hepatocellular carcinoma as in the case of the Fischer rats. It is possible that the degree of liver damage induced by AFB_1 in the DA rats may not be sufficient to progress to carcinoma. These strain differences in AFB_1 toxicity may be due to metabolic activation/inactivation, target cell sensitivity or repair processes, the latter might be more active in DA than in the Fischer strain.

However, from the two previous chapters, it has been shown that the DA rat exhibits a genetically determined deficiency in drug oxidation of several substrates, including debrisoquine. It may be,therefore, that the female DA rat has an impaired ability to effect the metabolic oxidation and activation of carcinogens such as AFB_1.

Three points have been shown in an as yet unpublished study in collaboration with the MRC Toxicology Unit, which explained and confirmed the previous result. Firstly, liver microsomal preparations from these two strains produced AFB_1-dihydrodiol-tris complex (a parameter of the 2,3-oxide formation) with Fischer rats which were more active in this respect than DA rats. Secondly, covalent binding to DNA and protein induced by microsomal metabolism of AFB_1 was higher in the Fischer than in the DA preparations. Thirdly, Ames' tests showed that Fischer liver preparations were more active in generating mutagenic metabolites from AFB_1.

It could be therefore suggested that the female DA rats inability to effect metabolic activation and oxidation might be responsible for the observed strain difference of AFB_1 hepatotoxicity. It is admitted that other factors may be important, but it is not possible to assess their significance at this point.

The inter- and intra-species differences in carcinogenicity of other chemical substances might be explained by the same hypothesis. The importance of a host genetically determined metabolic factor in predisposing the individual to this condition was recently shown in Nigeria in a study

of polymorphic debrisoquine oxidation and human primary hepatocellular carcinoma (Idle et al., 1981b). The human poor oxidizer phenotype might be less susceptible to the toxic effect of certain ingested environmental carcinogens, such as AFB_1, where metabolic activation by oxidation is a prerequisite for the initiation process.

CHAPTER FIVE

General discussion and concluding remarks.

Contents

5.1 GENERAL DISCUSSION

As stated previously, it is well recognized that the metabolic transformation of drugs and other foreign chemicals is an important determinant of inter- and intra-species variation in their toxic, therapeutic, pharmacologic and carcinogenic actions. Much of what we know today about the pathways of xenobiotic metabolism and their quantitative variability, we owe to the sagacity and foresight of the late Professor R. Tecwyn Williams. This contribution, spanning five decades was invaluable. In one of his last review articles, Professor Williams, cognizant of the growth of human pharmacogenetics, said "can those species which show defects in certain metabolic reactions of drugs be used for comparison with those groups of human beings which show similar defects in drug metabolism?"

This proposition was never more relevant than today, and forms a major part of the hypothesis upon which this thesis is built. Human genetic polymorphism in drug oxidation, and its breadth of implication, have already been discussed (Chapter Two) and the potential value of an animal model for the two human oxidation phenotypes is beyond question. At the very least it could allow

direct investigation of, for example, the toxic effects of drugs and chemicals in the presence of a stable phenotypic drug oxidation defect, i.e. investigations which could not be carried out prospectively in man. Additionally, such a model might provide greater insight into the cytochrome P-450 complex, since it would provide a ready source of biological materials for enzymological studies in the first instance. Finally, it is conceivable that by using phenotypic animals, methods could be developed for screening potential drug candidates which might show the phenomenon of polymorphic drug oxidation in man, without recourse to human population and family studies as a first hint of investigation.

The author was thus posed the simple questions, does genetic polymorphism of drug oxidation occur in a laboratory species; does it parallel that seen in man; and is it of any direct experimental value? The investigations described in this thesis go some way towards answering this question, and the findings are summarized in the following section.

5.2 SUMMARY OF OBSERVED CHARACTERISTICS OF THE FEMALE DA RAT

Table 5.1 summarizes the observed metabolic characteristics of the female DA rat as compared to other strains.

This table of characteristics leads to certain conclusions concerning the hepatic cytochrome P-450 system of the rat. A major form of cytochrome P-450 is genetically variant in the rat. Calculations using the known extinction coefficient for cytochrome P-450, allow the conclusion to be drawn that the isozymic form which binds debrisoquine, which is variant or absent in female DA rats, constitutes about 30% of the total hepatic cytochrome P-450 in Lewis, Fischer and Sprague-Dawley rats (Kupfer et al., 1982). This variant P-450 is largely responsible for the folllowing metabolic reactions; debrisoquine 4-hydroxylation, debrisoquine 6-hydroxylation, phenacetin O-deethylation, phenformin 4-hydroxylation, sparteine metabolism to 2-hydrosparteine and, most probably, aspects of hexobarbitone and aflatoxin B_1 metabolism. This form of cytochrome P-450 does not appear to metabolize tolbutamide and, if anything in the DA rat has a greater capacity towards acetanilide 4-hydroxylation. A similar situation to that of acetanilide

Table 5.1 Metabolic characteristics of the female DA rat compared to other strains

Substrate investigates	Parameter measured	Character of female DA rat compared to other strains
Debrisoquine	4-hydroxylation (metabolic ratio)	↓
Debrisoquine	6-hydroxylation (metabolic ratio)	↓
Phenacetin	O-deethylation	↓
Phenacetin	2-hydroxylation	↑
Phenformin	4-hydroxylation	↓
Sparteine	2-dehydro-sparteine excretion	↓
Hexobarbitone	sleeping time	↑
Aflatoxin B_1	hepatocarcinogenicity	↓
Acetanilide	4-hydroxylation	↑
Tolbutamide	carboxy and hydroxy-methyl metabolites	↑↓
Debrisoquine, sparteine and phenformin	Type I binding spectra	↓

↑ = increased; ↓ = decreased; ↓↑ = no difference

hydroxylation has been observed for polymorphic acetylation in rabbit, where p-aminobenzoic acid acetylation showed no correlation with sulphadiazine and isoniazid phenotype. In fact, slow acetylator rabbits generally had a higher level of blood p-aminobenzoic acid N-acetyl transferase than did rapid acetylators (Weber et al., 1976).

Female DA and Lewis rats therefore generally match the known and supposed patterns of drug oxidation in the human PM and EM phenotypes. Debrisoquine, sparteine, phenformin and phenacetin are all polymorphically metabolized in man, whilst tolbutamide and acetanilide are not (see Ritchie and Idle, 1982, for a review). A number of interesting deviations from this parallelism were, however, observed. Tolbutamide is excreted in man mainly as the terminal metabolite carboxytolbutamide, with a small proportion of the hydroxymethyl tolbutamide precursor (Thomas and Iked, 1966). Both DA and Lewis rats excreted mainly the precursor with little of the terminal metabolite. This inter-species variation may be the result of a variation in non-P-450 enzymes which are thought to produce carboxytolbutamide from its precursor (McDaniel et al., 1969). The described metabolites of sparteine

in man are 2- and 5- dehydrosparteine (Spiteller and Spiteller, 1978). Neither DA nor Lewis rats excreted 5-dehydrosparteine. This result may simply reflect our lack of knowledge of sparteine metabolism and its genetic control in man and suggest that it is somewhat more complex than was hitherto recognized.

5.3 SPECULATION OF THE NATURE OF THE GENETICALLY DEFICIENT P-450 IN THE RAT

In spite of the parallelism between the rat strains and human phenotypes in drug oxidation, the question of the sex difference within the DA rat remains puzzling. Only female DA rats express the metabolic defect. Some success was achieved in attempting to interconvert the oxidation phenotypes of male and female DA rats using castration and hormone treatment techniques. Genetic differences in steroid receptors between rat strains may underlie the observed differences, as has already been discussed more fully in Chapter Two. Nevertheless, considerably more research effort will be required to understand why the polymorphic variable investigated in this thesis is only expressed in the female rat, whilst in man both males and females can be phenotypically PM.

Before discussing the investigational opportunities and limitations which the proposed DA/Lewis animal model might offer, it is interesting to speculate on which fraction of the P-450 complex is that which metabolizes debrisoquine and is genetically variant in the rat.

Multiple forms of cytochrome P-450 in rat liver microsomes have been purified, by using criteria such as subunit molecular weight, absorption maximum of the reduced CO complex, immunological and catalytic properties and partial amino acid sequence. From five to seven forms of cytochrome P-450 are present in rat liver microsomes. These forms were described briefly by Lu and West (1980) and they are as follows:-

1. Major phenobarbitone-inducible form, which catalyzes the metabolism of substrates such as benzphetamine, N,N-dimethylaniline, parathion and N,N-dimethylphetermine.

2. Major 20-Mc-inducible form, which catalyzes the metabolism of a variety of polycyclic aromatic hydrocarbons as well as 7-ethoxy coumarin, 7-ethoxyresorufin and zoxazolamine.

3. PCN-inducible form, which catalyzes the N-demethylation of ethylmorphine and aminopyrine.

4. P-450a, which catalyzes the hydroxylation of testosterone at the 7α-position but is a rather inactive catalyst for the metabolism of other substrates.

5. P-450b, which catalyzes the metabolism of juvenile hormones

Other forms of cytochrome P-450 have also been purified, such as P-450 I and P-450 II from untreated rats. Still others are named fraction A, fraction C and fraction D, and it is not known whether any of these fractions correspond to P-450a.

However, evidence for multiple forms of human cytochrome P-450 has only recently begun to appear, but it is still difficult to estimate the number of multiple forms.

The mechanistic basis of the genetically determined impairment of drug oxidation in man is unknown, though there are indications that it is a function of the microsomal monooxygenase system. Thus, liver microsomes obtained from a single PM subject failed to hydroxylate

debrisoquine, in contrast to preparations obtained from EM subjects. Moreover, the PM biopsy contained quantitatively 'normal' levels of cytochrome P-450 (Davies et al., 1981).

Since it has been shown that debrisoquine 4-hydroxylation is MC-inducible, but not PB-inducible in the rat, it seems likely that the variant P-450 (debrisoquine 4-hydroxylation) is one of the cytochromes which, like any hydrocarbon hydroxylase (AHH), constitutes 'cytochrome P-448'. Certainly, phenacetin O-deethylation (Poppers et al., 1975) and aflatoxin B_1 8,9-oxide formation (Yoshizawa et al., 1982) are both P-448 related pathways. Purification of cytochrome P-448 from DA and Lewis rats is required to confirm this.

5.4 IMPLICATION OF THE DA DEFICIENCY IN DRUG OXIDATION

Potentially, in spite of its shortcomings and because of its novelty, the DA/Lewis animal model offers an opportunity to the pharmacogeneticist. Historically, drug oxidation polymorphism, like most other pharmacogenetic phenomena, was discovered by serendipity. The female DA rat is no less an example, since it was the second rat strain investigated with debrisoquine in a

limited list of rat strains which it was possible to order from U.K. suppliers. The historical pathway of uncovering new candidates for polymorphic drug oxidation in man is represented in Fig. 5.1. Starting with the known characteristics of drug 'X', such as variable pharmacokinetics, sporadic dose-related adverse drug reaction or even large species differences in its metabolism, a pathway was followed involving panel investigations up to full population and family studies. Thus, the investigation of a phenotyped panel of volunteers lead to an inference that, for example, phenacetin O-deethylation is polymorphic and parallels debrisoquine hydroxylation (Kong et al., 1982). This cannot be proven until population/family studies are performed and, even then, scope is left for two independent polymorphisms, whose loci are closely linked. In this latter case, dependent upon the degree of linkage, recombinants might occur so infrequently that they are never observed in population investigations of 1-200 subjects. A second pathway can now be proposed which utilizes our knowledge of DA/Lewis drug oxidation. This pathway begins with a difference spectroscopy experiment.

Difference spectra are obtained for drug 'X' with Lewis and DA hepatic derived P-450. If little or no binding

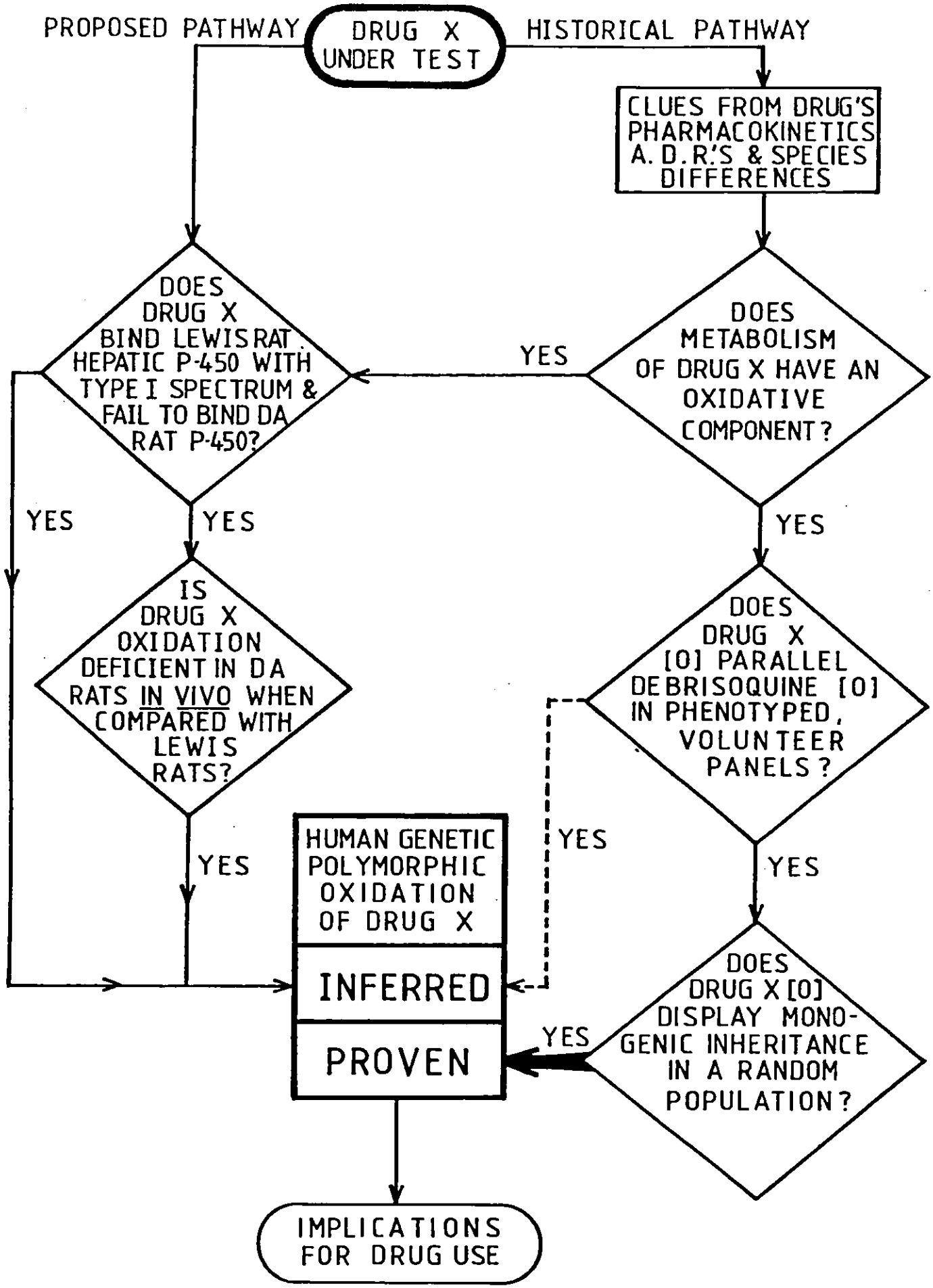


Figure 5.1 Flow diagram for uncovering genetic polymorphism of drug 'X' oxidation

O = oxidation ADR's = adverse drug reactions

(Type I spectrum) occurs with DA microsomes, under conditions whereby a reasonable interaction is seen for Lewis microsomes, then a further step can be taken down the pathway towards inferred polymorphism. The next step involves metabolism studies in DA/Lewis rats in vivo. If polymorphic oxidation is seen here, with female DA rats relatively deficient oxidizers, then we can infer polymorphism. Proof will only be obtained by reverting to the historical pathway, but further down, say at the family level. The possible advantages of this approach are that, firstly, the colorimetric experiment (which in essence is nothing more than adding drug to microsomes) is cheap and rapid, allowing many drugs and chemicals to be screened. Secondly, unnecessary human experimentation could be avoided. Thirdly, valuable inferences about genetically variable carcinogen and other toxic chemical metabolism might be obtained, which could otherwise not be obtained in man.

Undoubtedly, the debrisoquine 4-hydroxylase is only one of several genetically variant cytochromes P-450 in rat and man. The search for others and for their inter-species parallels will continue. In the meantime, enough is known about the rat/man parallelism of poly-morphic debrisoquine 4-hydroxylation to merit further

investigation of the validity, limitation and practical usefulness of the proposed animal model.

The most urgent problem for the future is to understand better the phenomenon of human variability in response to drugs and toxic substances. Clearly, the uncovering of major characterisable genetic factors as sources of such variability is a major advance in our understanding. At the same time, uncovering of animal strain models for human variability, must also be seen as a step forward. No longer should we ask which species handles or responds to the drug like man? Rather the question now is: can we define animal strains which will model for human variants? If so, we will obtain a clearer insight into the problem of human variability in response to drugs and toxic substances.

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APPENDICES

APPENDIX I

24h Urinary excretion of debrisoquine and 4-hydroxy-debrisoquine in 10 rat strains given debrisoquine (5mg/kg^{-1} p.o.)

Strain	Sex	% dose excreted as: Debrisoquine	4-hydroxy debrisoquine	[1]Total	[2]Metabolic Ratio
Wistar 1	F	3	21	24	0.14
2	F	3	26	29	0.12
3	F	3	24	27	0.13
4	F	2	18	20	0.11
5	F	3	20	23	0.15
6	F	1	14	15	0.07
7	F	3	15	18	0.20
Mean ± S.D.		3 ± 0.8	20 ± 4.4	22 ± 5.0	0.13 ± 0.04
Wistar 1	M	2	10	12	0.20
2	M	2	8	10	0.25
3	M	3	9	12	0.33
4	M	4	21	25	0.19
5	M	2	10	12	0.20
Mean ± S.D.		3 ± 0.9	12 ± 5.3	14 ± 6.1	0.23 ± 0.06

APPENDIX I (continued)

Strain	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
Lewis 1	F	11	44	55	0.25
2	F	17	55	72	0.31
3	F	14	46	60	0.30
4	F	6	40	46	0.15
5	F	7	38	45	0.18
6	F	5	42	47	0.12
7	F	6	36	42	0.17
8	F	3	38	41	0.08
9	F	5	33	38	0.15
10	F	4	30	34	0.13
11	F	6	37	43	0.16
12	F	2	27	29	0.07
13	F	4	28	32	0.14
14	F	4	21	25	0.19
15	F	3	27	30	0.11
16	F	3	22	25	0.14
17	F	2	27	29	0.07
18	F	4	28	32	0.14
19	F	4	21	25	0.19
20	F	3	26	29	0.12
21	F	3	22	25	0.14
Mean ± S.D.		6 ± 3.9	33 ± 9.3	39 ± 12.4	0.16 ± 0.07

APPENDIX I (continued)

Strain	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
Lewis 1	M	1	5	6	0.20
2	M	1	5	6	0.20
3	M	1	8	9	0.13
4	M	1	6	7	0.17
5	M	1	4	5	0.25
Mean ± S.D.		1 ± 0.0	6 ± 1.5	7 ± 1.5	0.19 ± 0.04
Fischer					
1	F	7	14	21	0.50
2	F	2	7	9	0.29
3	F	4	10	14	0.40
4	F	2	17	19	0.12
5	F	1	10	11	0.10
6	F	2	10	12	0.20
7	F	3	12	15	0.25
8	F	2	19	21	0.11
Mean ± S.D.		3 ± 1.9	12 ± 4.1	15 ± 4.6	0.25 ± 0.15

APPENDIX I (continued)

Strain	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
Fischer					
1	M	1	5	6	0.20
2	M	1	8	9	0.13
3	M	1	6	7	0.17
4	M	2	10	12	0.20
5	M	1	8	9	0.13
Mean ± S.D.		1 ± 0.5	7 ± 2.0	9 ± 2.3	0.17 ± 0.04
A/GUS 1	F	4	22	26	0.18
2	F	4	6	10	0.67
3	F	2	17	19	0.12
Mean ± S.D.		3 ± 1.2	15 ± 8.2	18 ± 8.0	0.32 ± 0.3

APPENDIX I (continued)

Strain		Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
PVG	1	F	2	13	15	0.15
	2	F	4	10	14	0.40
	3	F	3	20	23	0.15
	4	F	2	8	10	0.25
	5	F	2	9	11	0.22
	6	F	5	17	22	0.29
	7	F	1	10	11	0.10
	8	F	2	12	14	0.17
	9	F	1	12	13	0.08
	10	F	1	12	13	0.08
	11	F	1	9	10	0.11
Mean ± S.D.			2 ± 1.3	12 ± 3.6	14 ± 4.5	0.18 ± 0.1
PVG	1	M	2	18	20	0.11
	2	M	1	3	4	0.33
	3	M	1	4	5	0.25
	4	M	3	18	21	0.17
	5	M	1	6	7	0.17
Mean ± S.D.			2 ± 0.9	10 ± 7.6	11 ± 8.4	0.21 ± 0.09

APPENDIX I (continued)

Strain		Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
DA	1	F	53	10	63	5.30
	2	F	34	8	42	4.25
	3	F	31	7	38	4.43
	4	F	23	13	36	1.77
	5	F	39	21	60	1.86
	6	F	24	16	40	1.50
	7	F	23	12	35	1.92
	8	F	25	16	41	1.56
	9	F	23	12	35	1.92
	10	F	22	16	38	1.38
	11	F	39	21	60	1.86
	12	F	19	11	30	1.73
	13	F	23	12	35	1.92
	14	F	15	12	27	1.25
	15	F	25	16	41	1.56
	16	F	20	13	33	1.54
	17	F	18	11	29	1.64
	18	F	23	12	35	1.92
	19	F	15	12	27	1.25
	20	F	25	17	42	1.47
	21	F	20	13	33	1.54
Mean ± S.D.			26 ± 9.1	13 ± 3.6	39 ± 10.2	2.08 ± 1.11
DA	1	M	1	12	13	0.08
	2	M	2	13	15	0.13
	3	M	2	12	14	0.17
	4	M	5	14	19	0.36
	5	M	5	11	16	0.45
Mean ± S.D.			3 ± 1.9	12 ± 1.1	15 ± 2.3	0.24 ± 0.16

APPENDIX I (continued)

Strain		Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
WAG	1	F	10	24	34	0.42
	2	F	7	40	47	0.18
	3	F	10	37	47	0.27
	4	F	8	46	54	0.17
	5	F	8	29	37	0.28
Mean ± S.D.			9 ± 1.3	35 ± 8.8	44 ± 8.2	0.26 ± 0.10
BN	1	F	3	15	18	0.20
	2	F	6	17	23	0.35
	3	F	4	19	23	0.21
	4	F	4	22	26	0.18
	5	F	7	28	35	0.25
	6	F	3	18	21	0.17
Mean ± S.D.			5 ± 1.6	20 ± 4.6	24 ± 5.9	0.23 ± 0.07
BN	1	M	3	18	21	0.17
	2	M	2	16	18	0.13
	3	M	2	12	14	0.17
	4	M	2	14	16	0.14
	5	M	1	11	12	0.09
Mean ± S.D.			2 ± 0.07	14 ± 2.9	16 ± 3.5	0.14 ± 0.03

APPENDIX I (continued)

Strain	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Total	Metabolic Ratio
Long Evans 1	F	2	25	27	0.80
2	F	6	22	28	0.27
3	F	2	26	28	0.08
4	F	4	25	29	0.16
5	F	3	20	23	0.15
Mean ± S.D.		3 ± 1.7	24 ± 2.5	27 ± 2.4	0.15 ± 0.08
Sprague-Dawley 1	F	3	24	27	0.13
2	F	2	22	24	0.09
3	F	3	21	24	0.14
4	F	2	26	28	0.08
5	F	3	26	29	0.12
Mean ± S.D.		3 ± 0.6	24 ± 2.2	27 ± 2.3	0.11 ± 0.03

1 Total = Debrisoquine + 4-hydroxy-debrisoquine

2 Metabolic ratio = Debrisoquine/4-hydroxy-debrisoquine

F = Female; M = Male

APPENDIX II

Debrisoquine metabolism in ♂ Lewis X ♀ DA F_1 hybrids

Rat Number	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic Ratio
1	F	6	31	0.19
2	F	6	42	0.14
3	F	5	34	0.15
4	F	7	29	0.24
5	F	7	30	0.23
6	F	8	39	0.21
7	F	5	39	0.13
8	F	5	38	0.13
9	F	8	45	0.18
10	M	3	26	0.12
11	M	4	26	0.15
12	M	5	30	0.17
13	M	5	30	0.17
14	M	3	25	0.12

F = Female

M = Male

Metabolic ratio = Urinary debrisoquine/4-hydroxy-debrisoquine

All animals had brown hair and eyes similar to parental DA.

APPENDIX III

Debrisoquine metabolism and physical characteristic (in Lewis X DA) F_2 hybrids

Rat Number	Sex	Hair Colour	% dose excreted as: Debrisoquine	4-hydroxy debrisoquine	Metabolic Ratio
1	F	Bl	4	17	0.24
2	F	Bl	28	19	1.50
3	F	Br	40	24	1.7
4	F	Br	7	36	0.19
5	F	Br	5	30	0.17
6	F	Br	4	32	0.13
7	F	Br/WB	5	33	0.15
8	F	Br/WB	15	21	0.71
9	F	Br/WB	5	33	0.15
10	F	Br/WB	4	32	0.13
11	F	Br/WB	5	33	0.15
12	F	Br/WB	4	34	0.12
13	F	Br/WB	5	39	0.13
14**	F	W/BrH	-	-	-
15	F	W/BlH	26	36	0.72
16	F	W	32	21	1.52
17	F	W	4	32	0.13
18	F	W	7	34	0.21
19	F	W	5	40	0.13
20	F	W	35	20	1.8
21	F	W	20	17	1.2
22	M	Br/WB	4	11	0.36
23	M	Br/WB	3	28	0.11
24	M	Br/WB	5	34	0.15
25	M	Br/WB	3	34	0.09
26	M	Br	4	31	0.13

APPENDIX III (continued)

Rat Number	Sex	Hair Colour	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic Ratio
27	M	Br	6	56	0.11
28	M	Bl/WB	6	40	0.15
29	M	Bl/WB	5	15	0.33
30	M	Bl/WB	3	20	0.15
31	M	Bl/WB	3	16	0.19
32	M	W	3	12	0.25
33	M	W	4	13	0.31
34	M	W	3	15	0.20
35	M	W	3	20	0 15
36	M	W	3	14	0.20
37	M	W/BrH	4	20	0.20
38	M	W/BrH	5	40	0.13
39	M	W/BrH	4	21	0.19
40	M	W/BrH	3	14	0.21
41	M	W/BlH	5	47	0.11

* Bl = black, Br = Brown, Br/WB = brown with white belly, W/BrH = White with brown hood, W/BlH = White with black hood, W = White (Albino), Bl/WB = black with white belly.

** Animal died prior to metabolic investigation

Metabolic ratio = urinary debrisoquine/4-hydroxydebrisoquine

APPENDIX IV

Debrisoquine metabolism and physical characteristics in Lewis X DA F_3 hybrids

A. Mating number 1 (F_2 No. 29 X F_2 No. 2)

Rat Number	Sex	Hair Colour	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic Ratio
1	F	W	4	33	0.12
2	F	Br/WB	4	24	0.17
3	F	Br/WB	7	34	0.21
4	F	Br/WB	6	26	0.23
5	F	Bl/WB	5	20	0.25
6	F	Bl/WB	7	32	0.22
7	F	Br	5	26	0.19
8	M	W	3	39	0.08
9	M	Br/WB	2	19	0.11
10	M	Br/WB	2	20	0.10
11	M	Br/WB	1	19	0.05
12	M	Br/WB	3	30	0.10
13	M	Bl/WB	1	26	0.04
14	M	Bl/WB	1	15	0.07
15	M	Bl/WB	4	15	0.27
16	M	Bl	3	12	0.25
17	M	Bl	3	15	0.20
18	M	Br	1	14	0.07
19	M	Br	1	15	0.07

APPENDIX IV (continued)

B. Mating number 2 (F_2 No. 33 X F_2 No. 16)

Rat Number	Sex	Hair Colour	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic Ratio
1	F	W	4	37	0.11
2	F	W	6	23	0.26
3	F	W	2	16	0.13
4	F	W	3	36	0.08
5	F	W	5	30	0.17
6	F	W	4	26	0.15
7	F	W	5	29	0.17
8	F	W	3	35	0.09
9	M	W	2	16	0.13
10	M	W	4	47	0.09
11	M	W	3	30	0.10
12	M	W	6	36	0.17
13	M	W	1	31	0.03
14	M	W	2	20	0.10

APPENDIX IV (continued)

C. Mating number 3 (F_2 No. 40 X F_2 No. 20)

Rat Number	Sex	Hair Colour	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic Ratio
1	F	W	4	38	0.11
2	F	W	6	30	0.20
3	F	Br/WB	3	27	0.11
4	F	Br/WB	4	32	0.13
5	F	Br/WB	3	29	0.10
6	F	Br/WB	5	41	0.12
7	F	Br/WB	8	25	0.32
8	F	Br/WB	8	41	0.20
9	F	W	6	34	0.18
10	F	W	4	20	0.20
11	M	W	2	25	0.08
12	M	W	4	35	0.11
13	M	Br/WB	7	42	0.17
14	M	Br/WB	2	16	0.13
15	M	Br/WB	4	31	0.13
16	M	W	7	34	0.21
17	M	W	8	27	0.30
18	N	W	5	15	0.33
19	M	W	5	24	0.21

For Footnotes see Appendix III

APPENDIX V

Debrisoquine metabolism in DA X (Lewis X DA) F_3 back-crosses*

A. Mating number 1

Rat Number	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic Ratio
1	F	14	19	0.74
2	F	12	17	0.71
3	F	3	28	0.11
4	F	15	28	0.54
5	F	3	25	0.12
6	F	25	12	2.10
7	M	4	31	0.13
8	M	4	34	0.12
9	M	4	35	0.11

B. Mating number 2

1	F	2	14	0.14
2	F	3	27	0.11
3	F	3	19	0.16
4	F	33	14	2.40
5	F	12	8	1.50
6	F	4	8	0.50
7	M	6	23	0.26
8	M	2	13	0.15
9	M	3	20	0.15

APPENDIX V (continued)

C. Mating number 3

Rat Number	Sex	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic ratio
1	F	4	31	0.13
2	F	3	26	0.12
3	F	2	19	0.11
4	F	2	20	0.10
5	F	30	20	1.50
6	F	5	15	0.33
7	M	1	9	0.11
8	M	2	17	0.12
9	M	3	26	0.12
10	M	1	7	0.14
11	M	4	26	0.15
12	M	1	11	0.09

D. Mating number 4

1	F	4	27	0.15
2	F	3	30	0.10
3	F	2	20	0.10
4	F	23	18	1.30
5	F	18	19	0.95
6	F	19	29	0.66
7	M	4	33	0.12
8	M	3	15	0.20
9	M	3	21	0.14
10	M	2	27	0.07

* All matings were between male DA and female F_3 hybrids from mating number 2 (Appendix IV)

For other footnotes see Appendix III

APPENDIX VI

0-24h urinary excretion of debrisoquine and metabolites in control phenobarbitone-treated and methyl cholanthrene-treated rats.

		% dose excreted as:		
Number	Debrisoquine	4-hydroxy-debrisoquine	6-hydroxy-debrisoquine	Metabolic Ratio
Lewis (control)				
1	6	40	-	0.15
2	7	38	-	0.18
3	5	42	-	0.12
4	6	36	-	0.17
5	3	38	-	0.08
Lewis (PB)*				
1	4	38	-	0.11
2	5	41	-	0.12
3	3	36	-	0.08
4	6	35	-	0.17
5	2	40	-	0.05
Lewis (MC)*				
1	2	50	-	0.04
2	1	60	-	0.02
3	2	54	-	0.04
4	3	64	-	0.05
5	4	45	-	0.09

Appendix VI (continued)

Rat Number	% dose excreted as: Debrisoquine	4-hydroxy-debrisoquine	6-hydroxy-debrisoquine	Metabolic Ratio
PVG (control)				
1	2	15	7	0.13
2	4	17	8	0.24
3	3	9	7	0.33
PVG (PB)*				
1	9	19	8	0.47
2	6	15	10	0.40
3	10	20	7	0.50
BN (control)				
1	3	15	6	0.20
2	6	17	8	0.35
3	4	9	4	0.44
BN (MC)				
1	9	34	13	0.26
2	12	47	10	0.26
3	8	26	19	0.30
DA (control)				
1	23	13	-	1.8
2	24	16	-	1.5
3	39	21	-	1.9
4	23	12	-	1.9
5	25	16	-	1.6

APPENDIX VI (continued)

Rat Number	Debrisoquine	% dose excreted as: 4-hydroxy-debrisoquine	6-hydroxy debrisoquine	Metabolic Ratio
DA (PB)*				
1	24	15	-	1.6
2	28	20	-	1.4
3	24	24	-	1.0
4**	25	16	-	1.6
5**	20	18	-	1.1
DA (MC)*				
1	31	20	-	1.1
2	30	26	-	1.2
3	23	24	-	1.0
4	21	22	-	1.0
5	20	18	-	1.1

All animals were female.
2 see text
* PB = Phenobarbitone pre-treatment MC = 20-methylcholanthrene pre-treatment
** animals given half dose phenobarbitone, see text.
- not done

APPENDIX VII

Debrisoquine 4-hydroxylation in female Lewis and DA rats before castration, 1 week after, 3 weeks after castration and after administration of testosterone or oestradiol to the castrated rats.

		Before			1 week after castration			3 weeks after castration			after Testosterone			after oestradiol		
		+D	4	MR	D	4	MR	D	4	MR	D	4	MR	D	4	MR
Lewis	1	5	33	0.15	9	29	0.31	5	29	0.17	2	27	0.07	-	-	-
	2	4	30	0.13	8	29	0.27	8	28	0.29	2	35	0.06	-	-	-
	3	6	37	0.16	11	37	0.29	6	36	0.17	5	27	0.19	-	-	-
	4	2	27	0.07	10	27	0.37	5	30	0.17	-	-	-	4	21	0.19
	5	4	28	0.14	4	25	0.16	3	18	0.17	-	-	-	2	18	0.11
	6	4	21	0.19	11	39	0.28	6	33	0.18	-	-	-	3	24	0.13
DA	1	23	12	1.9	23	27	0.85	6	16	0.38	8	30	0.27	-	-	-
	2	22	16	1.4	24	23	1.0	11	28	0.41	7	33	0.21	-	-	-
	3	39	21	1.9	25	17	1.5	24	27	0.89	7	27	0.26	-	-	-
	4	19	11	1.7	23	25	0.92	14	41	0.34	-	-	-	11	9	1.2
	5	23	12	1.9	28	22	1.3	8	22	0.36	-	-	-	15	12	1.3
	6	15	12	1.3	28	20	1.4	10	29	0.34	-	-	-	11	10	1.1

+ D = % dose in 0-24h as debrisoquine, 4 = as 4-hydroxy-debrisoquine, MR = metabolic ratio

APPENDIX VIII

Debrisoquine 4-hydroxylation in male Lewis and DA rats 1 and 3 weeks after orchidectomy

		1 week after castration			3 weeks after castration		
		+D	4	MR	D	4	MR
Lewis	1	1	5	0.20	10	15	0.67
	2	1	9	0.11	4	17	0.24
	3	1	7	0.14	3	13	0.23
	4	1	14	0.07	6	20	0.30
	5	1	12	0.08	5	10	0.50
	6	1	11	0.09	6	12	0.50
DA	1	5	16	0.31	4	18	0.22
	2	5	16	0.31	6	20	0.30
	3	6	17	0.35	5	19	0.26
	4	4	15	0.27	8	22	0.36
	5	7	16	0.44	4	21	0.19
	6	6	11	0.55	6	21	0.29

+ for footnotes see Appendix VII

APPENDIX IX

Debrisoquine 4-hydroxylation in intact female adult Lewis and DA rats treated with Oestradiol and Testosterone

Group		% dose (0-24h) excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic ratio
Lewis (Oestradiol)	1	4	35	0.11
	2	4	27	0.15
	3	3	21	0.14
	4	3	25	0.12
	5	3	27	0.11
DA (Oestradiol)	1	22	14	1.6
	2	27	14	1.9
	3	36	15	2.4
	4	26	14	1.9
	5	30	15	2.0
Lewis (Testosterone)	1	7	29	0.24
	2	5	21	0.24
	3	5	24	0.21
DA (Testosterone)	1	16	19	0.84
	2	17	19	0.89
	3	13	15	0.87

APPENDIX X

Debrisoquine 4-hydroxylation in intact male adult Lewis and DA rats treated with Oestradiol and Testosterone

Group		% dose (0-24h) excreted as: Debrisoquine	4-hydroxy-debrisoquine	Metabolic ratio
Lewis (Oestradiol)	1	3	14	0.21
	2	4	17	0.24
	3	3	21	0.14
	4	2	15	0.13
	5	4	17	0.24
DA (Oestradiol)	1	6	16	0.38
	2	6	23	0.26
	3	8	18	0.44
	4	6	19	0.32
	5	8	20	0.40
Lewis (Testosterone)	1	1	4	0.25
	2	1	6	0.17
	3	1	7	0.14
DA (Testosterone)	1	2	26	0.08
	2	2	20	0.10
	3	3	23	0.13

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PUBLICATIONS

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