Species variations in the metabolism of xenobiotics with particular reference to the marmoset (*Callithrix jacchus*)

by

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Synopsis

Considerable species variations exist in the metabolism of xenobiotics, consequently one of the most difficult problems in examining the safety of a drug for human use lies in finding a suitable animal model in which the toxicity and metabolism of the drug can be usefully examined. This thesis describes an investigation of some pathways of xenobiotic metabolism in the marmoset (*Callithrix jacchus*), a small primate known to be suitable as a model for man in the study of teratogenic effects of drugs but for which few detailed studies of drug metabolism have been reported. The biotransformations studied were mainly conjugation reactions involving the combination of xenobiotics with glycine, glucuronic acid and acetic acid. Mercapturic acid formation which involves an initial conjugation with glutathione was also examined. During this work techniques were devised for the oral dosing of the marmosets, for housing the animals under study in conditions allowing the separate collection of urine and faeces and for the collection of small serial samples of blood. The regular breeding by the adults made it possible to study drug metabolism in the neonates, novel methods being developed for this study.

Examination of the fate of ^{14}C-labelled benzoate showed that the availability of glycine was probably the limiting factor in hippuric acid synthesis in the adult marmoset, the proportion of the dose excreted as benzoylglucuronide increasing with increasing dose level; considerable individual variation was observed. In contrast to the marmoset, availability of glycine did not limit hippuric acid synthesis by the mature rat at the dose levels studied. Relatively small proportions of ^{14}C-labelled aspirin

were excreted as salicyluric acid by either species, the extent of this reaction being variable also. By the use of high pressure liquid chromatography the excretion of small amounts of unchanged drug by man and marmoset after the oral administration of aspirin at the same dose level was detected.

Acetylation of p-aminobenzoic acid, sulphadiazine and sulphadimidine was effected by the marmoset. The major metabolite of p-aminobenzoate was p-acetamidobenzoate; less p-aminohippuric acid was excreted than by similarly dosed rats or than reported for man. The extent of acetylation of sulphadiazine was similar to that reported for man and was found to be greater than that observed in the rat and less than that in the rabbit. The proportion of administered sulphadimidine excreted as the acetyl derivative and the proportion of this metabolite found in the blood indicated that all the marmosets in the colony corresponded to 'fast acetylators' in human populations.

Benzylmercapturic acid was excreted by marmosets as a major metabolite of benzyl chloride and 2-hydroxybutylmercapturic acid was detected in the urine of marmosets to which 1,2-epoxybutane had been dosed. Wide inter-individual variation in the conversion of benzyl chloride to hippuric acid by the marmoset was observed while traces of metabolites arising from the deamination of benzylcysteine also appeared to be excreted by this species. Pentylcysteine was extensively acetylated and excreted as pentylmercapturic acid. Some evidence of the formation of mercapturic acid sulphoxides by the marmoset was obtained.

A short study of the metabolic fate of orally administered quinic acid and cyclohexanecarboxylic acid suggested that the

marmoset behaved as a typical New World monkey in its inability to aromatize the former compound but to metabolise the latter to hippuric acid. In this respect the marmoset is different from the higher primates, including man, in which the ability to reduce quinic acid is attributed to the action of the gut micro-flora.

The metabolic fates of benzoic acid and p-aminobenzoic acid were also studied in neonatal marmosets and rats. Hippuric acid synthesis was strongly dose dependent in the young of both species this being more marked in the marmoset where at high dose levels benzoylglucuronide became the major metabolite excreted. In this species the availability of glycine for conjugation with benzoate was clearly limiting. In both species glucuronidation was significantly more important in the neonates than in the adults. Evidence that the liver of the neonatal marmoset possessed UDPGT activity towards bilirubin was obtained. The metabolic fate of p-aminobenzoate in the developing marmoset followed an opposite trend to that in either the rat or to that reported for man, the overall results suggesting that the observed metabolic profiles were governed mainly by the acetylase system.

The results obtained indicate that the marmoset possesses the ability to carry out the Phase II reactions studied although the relative importance of these reactions in the metabolic fate of a particular drug may not be the same as in other species, including man.

to my family

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Contents

CHAPTER 1

Introduction

Chapter 1 - Introduction

Today organic compounds which are foreign to the animal body in the sense that they are neither utilised for the production of energy nor for synthesis, are employed on a vast scale in the service of mankind. Thus synthetic organic compounds are used as drugs in the treatment of illness, as food additives, as emulsifiers in drink manufacture, as pesticides in agriculture, as dyes, as cleansing agents and as cosmetics. All these foreign compounds (xenobiotics) are potentially toxic to man and it is essential that before any such compound is manufactured and released for general use, its toxicity should be evaluated so that safety levels for its use can be clearly stated. The tests involved in establishing whether there is an acceptable level at which a compound can be safely used are normally carried out first on laboratory animals such as the rat, rabbit, guinea pig, dog, cat and occasionally non-human primates and it is on the evidence obtained from these investigations that the final decision to proceed to examine the effect of the compound in man depends. However scientists concerned in extrapolating the results from animal experiments to man are faced with the problem of species variations in both the therapeutic and toxic action of foreign compounds.

It is becoming increasingly recognised that the effect of a drug, therapeutic and/or toxic, may be due not only to the parent drug administered but to its metabolites and a study of the metabolic profile of the drug in various animal species may indicate the basis of any observed species variations in its effects. The ultimate aim of such studies is to find an animal in which the absorption, distribution, excretion and the rate

and route of metabolism of the substance are closely similar to those in man. New regulations for drug safety require information on the metabolic fate of the drug in man and in at least two other animal species, and that toxicity studies have been carried out in animals in which the drug has been shown to have a similar metabolic profile to that in man. Knowledge so far accumulated indicates that the existence of a perfect animal model for man is very unlikely, so the search must continue for the species which most closely resembles man in its metabolism of foreign compounds.

Although this is the main reason for studying species variations in the metabolism of xenobiotics, such studies are also interesting from an academic point of view for variations in the metabolic fate of a xenobiotic in different species may reveal biochemical relationships between the species which can be of importance in the problem of taxonomy. This applies particularly to primate taxonomy since some pathways of xenobiotic metabolism occur only in primate species and in some cases only in particular groups of primates and these can be correlated with the evolutionary status of the groups. Williams, (1977) coined the self-explicit term 'pharmacozootaxonomy' for the relationship between xenobiotic metabolism and taxonomic classification. Studies on species variations in the metabolism of xenobiotics are furthermore of academic interest in that they may lead to the discovery of previously unknown biochemical pathways peculiar to one or a limited number of species. In this connection studies of the metabolism in the more exotic animal species are of interest and it may well be that such investigations could have some importance to clinical studies. A recent WHO report (1975) on guidelines for the

evaluation of drugs for use in man considered that one future research need for the development of efficacious and safe drugs is the use of new or seldom used species of test animal.

Several other factors, apart from species, help determine the overall metabolic profile of a xenobiotic, and when studying the effects of species variations these other factors must be recognised and controlled as far as possible. These factors are listed later in Table 1.4 and the extent to which any one of them influences the apparent metabolic fate of a drug is itself species dependent. A detailed discussion of how these factors can or cannot be controlled between species is contained in a later section.

The fate of foreign compounds in animals

In general when a foreign compound enters the body it may be disposed of in three ways:

(i) It may be excreted unchanged.

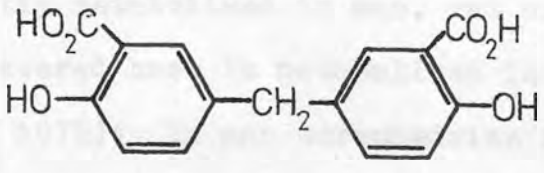
(ii) It may undergo chemical reactions which do not involve enzymes.

(iii) It may be metabolised by appropriate enzyme systems.

The physical and chemical properties of the compound determine the extent to which it is distributed among these three possibilities, bearing in mind that the fate of any one compound will probably be a result of the operation of more than one of the possibilities. Furthermore the metabolic fate of a compound can vary depending on its route of administration to the animal. Orally administered strong acids and strong bases are poorly absorbed from the gut and consequently large proportions of the dose appear unchanged in the faeces of the animal. Compounds which are absorbed from the gut encounter the

xenobiotic metabolising enzymes of the gut wall, an environment not encountered by compounds administered either by intravenous or intraperitoneal injection.

Polar, water-soluble compounds are excreted by the kidney and are not re-absorbed by the kidney tubules in contrast to the less polar more lipophilic compounds. Consequently many polar compounds, acids and bases are excreted unchanged. However a few cases are known where relatively non-polar compounds are excreted unchanged, or are only metabolised to a limited extent by animals. Very few compounds are known that are not metabolised at all in various animal species, but some are excreted mainly unchanged with only a small degree of metabolism. One would not expect species variations to occur in the fate of foreign compounds that are not metabolised at all. It is however difficult to establish that a compound is excreted entirely unchanged since the accuracy of the statement is dependent on the sensitivity of the methods employed for the detection of the compound and its metabolites. It may be that the compound is poorly metabolised and that the methods of detection used are not sufficiently sensitive to detect the low concentrations of the metabolites formed or are not specific and do not distinguish between the unchanged compound and its metabolites. 5,5'-Methylenedisalicylic acid (pKa 3.5) (I) was found by Davison and Williams (1968) to be excreted entirely unchanged by the mouse, rat, guinea pig, hamster, chicken, rabbit and rhesus monkey; so far this seems to be the best



(I)

example of a compound which is excreted entirely unchanged.

Some compounds undergo non-enzymic chemical reactions within the animal body. A compound may be unstable under physiological conditions and decompose or it may react spontaneously with a normal chemical constituent of the body. An excellent example of a compound undergoing spontaneous change within the body is thalidomide which has a half-life of about 5h at pH 7.4 in phosphate buffer at 37°C and undergoes hydrolysis at physiological pH values to give some twelve products (Schumacher et al., 1965; Williams, 1968).

One would not expect to find species differences in the fate of compounds undergoing such spontaneous changes and indeed the fate of thalidomide is much the same in several animal species. However any further metabolism of the breakdown products may be species dependent and this must always be considered. If a normal body constituent is involved in the spontaneous reaction of a xenobiotic then the relative amounts of the normal constituent within different species may result in a species variation in the metabolic fate of the administered compound. In cases where the normal body constituent is present in limiting quantities, a situation which itself may also vary with species, the metabolic fate of the administered compound may well be dependent on the dose level.

Most foreign compounds on entering the body are metabolised, although the extent to which this occurs varies over a wide range and is strongly species dependent. For example cyclohexylamine is only slightly metabolised in man, rat and guinea pig but 30% of the administered base is metabolised in the rabbit, (Renwick and Williams, 1972); in man norephedrine is only slightly metabolised but in the rat 30%, and in the rabbit 90% of the

administered dose is metabolised, (Sinsheimer et al., 1973); in man and the rhesus monkey 4-nitrophenylacetic acid is excreted largely unchanged but in the rat 61% of the dose is excreted conjugated with glycine (James et al., 1972a).

Metabolism increases the rate of excretion of a xenobiotic since in this process lipophilic compounds are converted into more polar, water-soluble compounds which are readily excreted by the kidney in the urine and under certain circumstances by the liver in the bile. The importance of metabolism is seen in the case of the organohalogenic pesticides which are only poorly metabolised and which accumulate in the tissues e.g. DDT (Hayes, 1965; Hart et al., 1972).

The therapeutic action of many xenobiotics is terminated by their conversion to more polar, water-soluble compounds which are usually physiologically inactive and which are more readily excreted than the parent compound. In certain cases however, metabolism may also convert a pro-drug into a pharmacologically active substance, and similarly metabolism can also change the pharmacological activity of a drug, converting it from a compound with one kind of activity to another substance with a different pharmacological activity. Table 1.1 contains examples of how the pharmacological activity of a substance can be altered by its metabolism.

Outline of xenobiotic metabolism

The metabolism of xenobiotics is usually considered to occur in two phases (Williams, 1959). In the first phase the xenobiotic undergoes one or more asynthetic reactions which may be classified as hydrolyses, reductions, or oxidations. These reactions introduce into the xenobiotic, or expose within its

Drug	Metabolic reaction	Metabolite	Pharmacological consequence
DEACTIVATION			
Phenobarbitone	aromatic hydroxylation	4-hydroxy phenobarbitone	Loss of hypnotic activity
Chlorcyclizine	N-oxidation	chlorcyclizine N-oxide	Loss of antihistamine activity
ACTIVATION			
Prontosil	azo reduction	sulphanilamide	Becomes antibacterial
Prednisone	cyclic ketone reduction	prednisolone	Becomes corticosteroid immuno-suppressive
CHANGE IN ACTIVITY			
Iproniazid	N-dealkylation	isoniazid	Antidepressive ⟶ antitubercular
Codeine	O-demethylation	morphine	Analgesic ⟶ narcotic

Table 1.1 The effect of metabolism on the pharmacological activity of various medicinal drugs

(Examples from Gorrod and Beckett 1978)

structure, biochemically reactive groups such as hydroxyl, carboxyl, amino and sulphydryl groups. Many of the Phase I reactions are carried out by enzymes situated in the endoplasmic reticulum of the liver cell.

In Phase II of xenobiotic metabolism the Phase I products, having acquired biochemically reactive groups, undergo synthetic reactions which are carried out by enzyme systems located mainly in the liver cell but which also occur in other tissues such as the kidney, the intestinal mucosa and the lung. During the Phase II reactions the xenobiotic is conjugated through its biochemically reactive group(s) with a normal physiological molecule, the resulting conjugate being relatively more polar than the parent xenobiotic and therefore more readily excreted from the body. In many cases the xenobiotic may possess at least one biochemically reactive group within its parent structure and may undergo Phase II reactions directly. In general it is likely that a xenobiotic is distributed along all the possible metabolic pathways, the extent to which a particular pathway is followed being dependent upon the affinities of the enzymes for the particular substrate, the relative amounts of the enzymes present and the availability of the endogenous compound for conjugation with the xenobiotic. With some complex molecules many metabolites may be formed as in the case of chlorpromazine where more than 35 metabolites have been detected (Turano et al., 1973).

The common conjugating mechanisms in mammals are those utilising glucuronic acid, amino acids (in particular glycine, glutamine and taurine), acetate, sulphate and glutathione. Conjugation with phosphate, methyl groups and the conversion of cyanide to thiocyanate also occur (Williams and Millburn, 1975).

In some avian species, ornithine conjugation replaces glycine conjugation while in insects glucose conjugation replaces that with glucuronic acid. Table 1.2 is a summary of the types of Phase II reactions and the biochemically reactive groups with which they occur. Conjugation with glucuronic acid is the most widespread and versatile Phase II reaction probably because the glucuronic acid moiety can be transferred to many different biochemically reactive groups and is itself readily available from a variety of carbohydrate sources within the animal.

Phase II reaction	Biochemically reactive groups with which reaction occurs
Conjugation with glucuronic acid	hydroxyl, carboxyl, amino, sulphamido and sulphydryl groups.
Conjugation with sulphate	hydroxy compounds (usually phenolic) and aromatic amines.
Conjugation with amino acids	carboxylic acid groups (in particular those contained in a molecule which has a heterocyclic or carbocyclic aromatic system within its structure).
Conjugation with acetate	aromatic and aliphatic amino groups, sulphonamido and hydrazine groups, and α-amino groups of S-substituted cysteines.
Conjugation with methyl groups	hydroxyl, sulphydryl amino or substituted amino groups.
Conjugation with glutathione	aromatic hydrocarbons, aryl, alkyl and aralkyl halides, nitro compounds, epoxides, esters and unsaturated carboxylic acids.

Table 1.2 The group specificity of the various Phase II reaction types

Conjugation reactions are biosynthetic and involve the formation of an activated intermediate from either the xenobiotic or the endogenous conjugating agent. Conjugation reactions which involve an amino acid as the endogenous compound usually proceed via an activated xenobiotic intermediate and appear to be restricted to the liver and/or kidney (type (i) reaction), whereas glucuronidation, sulphation, acetylation and methylation reactions proceed via an activated conjugating agent intermediate and occur not only in the liver and kidney but also in the lung and intestine (type (ii) reaction). Both of these reaction types are employed in the metabolism of benzoic acid as follows,

Reaction type (i) Hippuric acid formation involving the activation of benzoic acid.

$$\underset{\text{benzoic acid}}{C_6H_5COOH} + ATP \xrightarrow{\text{Acylthiokinase}} \underset{\text{Adenyl benzoate}}{C_6H_5CO.AMP} + P_2O_7^{4-}$$

$$C_6H_5CO.AMP + \underset{\text{Coenzyme A}}{CoASH} \xrightarrow{\text{Acylthiokinase}} \underset{\text{benzoyl Coenzyme A}}{C_6H_5CO.SCoA} + AMP$$

$$C_6H_5CO.SCoA + \underset{\text{glycine}}{H_2NCH_2COOH} \xrightarrow{\text{N-acyltransferase}} \underset{\text{hippuric acid}}{C_6H_5CONHCH_2COOH} + CoASH$$

Reaction type (ii) Benzoylglucuronide formation involving the activation of glucuronic acid.

$$\text{Glucose-1-phosphate} + UTP \xrightarrow{\text{Uridyl transferase}} \underset{\substack{\text{uridine diphosphate}\\ \alpha\text{-D-glucose}}}{UDPG} + P_2O_7^{4-}$$

$$UDPG + 2NAD^+ \xrightarrow{\text{UDPG dehydrogenase}} \underset{\text{UDP-D-glucuronic acid}}{UDPGA} + 2NADH + 2H^+$$

$$UDPGA + C_6H_5COOH \xrightarrow[\text{transferase}]{\text{UDP glucuronyl}} \underset{\text{benzoylglucuronide}}{C_6H_5CO.C_6H_9O_6} + UDP$$

In both sequences, a transferase enzyme is used for the

final step. Table 1.3 is a summary of both reaction types, including the activated intermediates and transferase enzymes employed.

Reaction	Activated Intermediate	Transferase enzyme
Type (i) reactions		
Glycine conjugation	Aroyl CoA	Glycine acyl transferase
Glutamine conjugation	Arylacetyl CoA	Glutamine acyl transferase
Ornithine conjugation	Aroyl CoA	Ornithine acyl transferase
Taurine conjugation	Arylacetyl CoA	Taurine acyl transferase
Type (ii) reactions		
Glucuronic acid conjugation	UDP-glucuronic acid	UDP-glucuronyl transferase
Glucose conjugation	UDP-glucose	UDP-glucose-glucosyl transferase
Sulphate conjugation	3'-Phosphoadenosine-5'-phosphosulphate	Sulphotransferase
Acetyl group conjugation	Acetyl CoA	Transacetylase
Methyl group conjugation	S-adenosyl-methionine	Methyltransferase

Table 1.3 Summary of the Phase II conjugation mechanisms

(Information taken from Williams, 1974)

As may be seen from Table 1.3 the different amino acid conjugation systems have different substrate specificities. This is an important aspect of amino acid conjugation and will be mentioned in later sections. Conjugation of arylacetic acids with taurine was discovered as recently as 1972 (James et al.b), and consequently the mechanism of this reaction is not definitely

established although it would appear logically to follow the mechanisms already established for the other amino acids.

Conjugation with glutathione follows neither type (i) nor type (ii) reaction sequences. Specific glutathione-S-transferases are known which catalyse the conjugation of electrophilic compounds with the nucleophilic glutathione. Some compounds, for example aromatic hydrocarbons, are converted to epoxides which then combine with glutathione, the reaction being catalysed by glutathione-S-epoxide transferase although the conjugation may to some extent occur spontaneously.

The detoxication of cyanide to thiocyanate appears to be the result of a direct reaction between cyanide and thiosulphate, catalysed by the enzyme thiosulphate sulphurtransferase (rhodanese). This reaction does not involve an activated intermediate although PAPS (3'-phosphoadenosine-5'-phosphosulphate) may be involved in the formation of the thiosulphate used in the reaction (Roy and Trudinger, 1970). The metabolism of cyanide is a classic example of detoxication since a 200 fold decrease in toxicity is achieved by converting cyanide to thiocyanate. The occurrence of rhodanese throughout nature is widespread and the enzyme is thought to have developed in response to the high cyanide content in the atmosphere at the time of early life forms. Today the detoxication of cyanide is only of use for the small amounts of cyanide that may be encountered in the diet. Rhodanese will only convert the cyanide ion to thiocyanate and has no activity towards unhydrolysed organic cyanides.

Metabolism of 2-naphthylamine

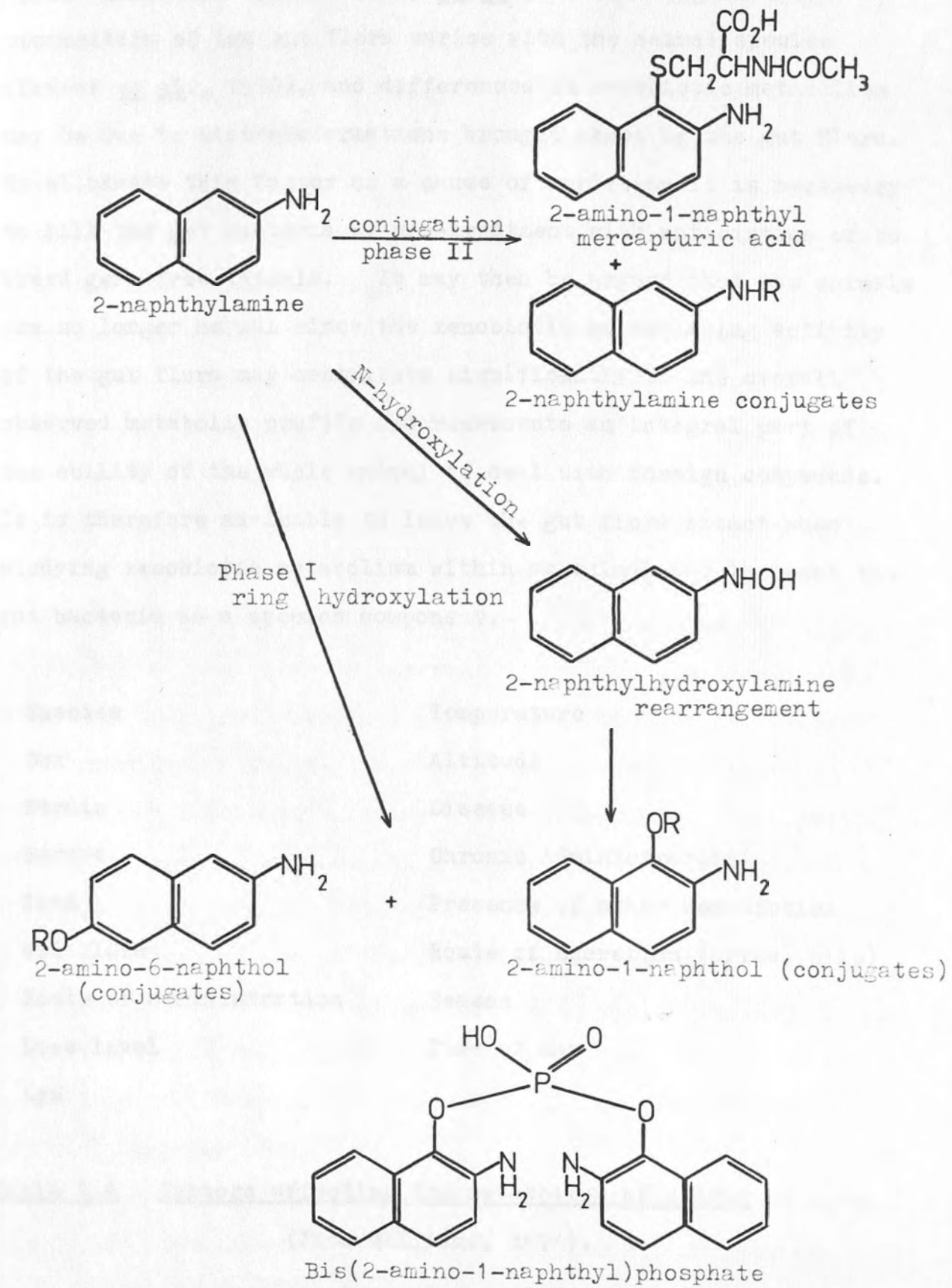
The complete metabolism of 2-naphthylamine in the animal

body provides good examples of both Phase I and Phase II reactions, many of which have been described above (Fig 1.1).

Species variation in the factors affecting the overall pattern of drug metabolism.

The complete study of the metabolism of a xenobiotic takes into account the absorption, distribution, excretion, protein binding and membrane permeability of the compound as well as its biotransformation. Any or all of these factors may vary with species and the combined result of the interaction of these processes constitutes the metabolic profile of the xenobiotic in a particular species. Most studies concerned with species variations in xenobiotic metabolism have reported the differences observed in the metabolites excreted, less attention having been paid to the possible differences in the underlying biochemical pathways which would result in the observed in vivo results.

The fate of a foreign compound may be affected by factors influencing either the asynthetic or synthetic phases of drug metabolism. Of these factors, which are listed in Table 1.4, probably the greatest effect is due to species variations although when examining the metabolism of any xenobiotic the other factors listed must be controlled wherever possible. Most of the factors listed in Table 1.4 can be standardised within experiments but some are difficult to control. For example in studying the metabolism of a xenobiotic in a herbivorous and a carnivorous species of animal it is obviously not possible to maintain the animals on the same diet. The importance of the dietary factor, in affecting the routes of xenobiotic metabolism, has been reported by various workers,



(R=$C_6H_9O_6$, SO_3H or $COCH_3$)

Fig 1.1 **Metabolic fate of 2-naphthylamine**

(Basu et al., 1971; Woodcock and Wood, 1971; Dickerson et al., 1976; Campbell, 1977; Conney et al., 1977). Again the composition of the gut flora varies with the animal species (Drasar et al., 1970), and differences in xenobiotic metabolism may be due to biotransformations brought about by the gut flora. To eliminate this factor as a cause of variation it is necessary to kill the gut bacteria by pre-treatment with antibiotics or to breed germ-free animals. It may then be argued that the animals are no longer normal since the xenobiotic metabolising activity of the gut flora may contribute significantly to the overall observed metabolic profile and represents an integral part of the ability of the whole animal to deal with foreign compounds. It is therefore advisable to leave the gut flora intact when studying xenobiotic metabolism within an animal and to treat the gut bacteria as a species component.

Species	Temperature
Sex	Altitude
Strain	Disease
Stress	Chronic Administration
Diet	Presence of other xenobiotics
Gut flora	Route of excretion (urine, bile)
Route of administration	Season
Dose level	Time of day
Age	

Table 1.4 Factors affecting the metabolism of a drug (From Williams, 1974).

The influence of sex on the metabolism of xenobiotics is itself species dependent being manifested to a marked extent only in the rat (Inscoe and Axelrod, 1960; Kato et al., 1962; Kato and Gillette, 1965; Macleod et al., 1972) and mouse (Catz and Yaffe, 1967), whereas only minor sex differences have been reported in man (Guidicelli and Tillement, 1977). Furthermore this effect appears to be age dependent also, being absent in immature rats (Macleod et al., 1972). An example of the influence of sex on the metabolism of biphenyl in the rat was reported by Rahman (1970). In male rats the increase in hepatic biphenyl 4-hydroxylase activity induced by β-ionone given at a dose level of 100mg/kg body weight for three days, paralleled the increase in hepatic cytochrome P_{450}. However, although the increase in hepatic cytochrome P_{450} content observed in the female rat was similar to that in the male, the hepatic biphenyl 4-hydroxylase activity was increased to a much greater extent even at a lower dose level of β-ionone. The sex differences have since been attributed to the greater induction of NADPH-cytochrome c reductase activity in the female rat. Similar sex differences have been shown in the metabolism of amphetamine (Dingell et al., 1973; Williams, 1974).

The effect of age on the metabolism of xenobiotics is very important since the enzymes concerned with the metabolism of xenobiotics undergo developmental changes, particularly in the early life of the animal. This will be discussed in more detail in a later section.

The metabolism of a xenobiotic depends on the enzyme make-up within the tissues and variations in the enzymic complement between species is the main reason for many observed species variations in the overall metabolism of foreign compounds. In

some cases these differences are quantitative, in others qualitative, depending on whether different amounts of the same metabolite are produced in different species or whether a metabolite is peculiar to a limited number of species only. Furthermore the enzyme activity can also be affected by other factors including the presence or absence of a specific enzyme inhibitor, the activity of any enzyme competing for the same substrate and the activity of any enzyme reversing the biotransformation reaction. In most cases the pattern of urinary metabolites is governed by the relative extent of the enzymic reactions responsible for converting the xenobiotic into these metabolites. Many of the overall reactions involve several steps requiring more than one enzyme and hence species differences may be due to the number of enzymes that catalyse the metabolism of a xenobiotic, their activity and their relative amounts.

Two examples where species differences occur in normal body processes as a direct result of the absence of enzyme activity are the synthesis of L-ascorbic acid and the production of allantoin as the end product of purine metabolism. The absence of the enzyme L-gulonolactone oxidase in man, monkeys and in evolutionary distant animals such as the guinea pig, the Indian fruit bat and the red-vented bulbul explains the inability of these animals to synthesise L-ascorbic acid. Similarly man, monkeys, birds and reptiles excrete uric acid as the end product of purine metabolism since they lack the enzyme uricase necessary for the conversion of uric acid to allantoin (Truszkowski and Goldmanowna, 1933), the excretory product in other animal species (see Williams, 1974).

Species variations in the metabolism of xenobiotics

When discussing examples of species variations in the metabolism of xenobiotics it is difficult to deal with Phase I and Phase II reactions as separate sections since many molecules undergo metabolism by both types of reaction, either at the same centre or at different centres within the molecule. However some examples of species variations in the metabolism of xenobiotics which are known to be essentially metabolised by either Phase I or Phase II reactions only are given below.

Species differences in Phase I reactions

Species variations in the oxidative, reductive and hydrolytic reactions of xenobiotic metabolism are widespread and often unpredictable. From all the data so far accumulated concerning species differences in these reactions, it is still not possible to discern any well-defined pattern. All that can be tentatively concluded is that two structurally related compounds that are metabolised by similar Phase I reactions are likely to have a similar metabolic fate within one particular species. Any such similarity is much more likely to be qualitative rather than quantitative. It appears that any one compound undergoing Phase I metabolism is likely to show quantitative and/or qualitative differences in its metabolic fate in two different species of test animal. In many cases such species differences in Phase I metabolic processes can be shown by *in vitro* as well as by *in vivo* studies.

Metabolism of amphetamine and related compounds

Amphetamine (1-amino-2-phenylpropane) is metabolised essentially by a combination of aromatic hydroxylation via an

epoxide intermediate and oxidative deamination to benzyl methyl ketone which, in the majority of animal species studied, undergoes further side chain oxidation to benzoic acid which is then further metabolised by Phase II reactions to hippuric acid and benzoylglucuronide (Dring et al., 1970; Caldwell et al., 1972) (Fig 1.2). In the rabbit however large quantities of an acid-labile conjugate of the enolic form of benzyl methyl ketone are excreted together with appreciable amounts of conjugated phenylpropan-2-ol (Dring et al., 1970).

The aromatic hydroxylation reaction is specific for the para position producing 4-hydroxyamphetamine which may be excreted in the urine conjugated with either glucuronic acid or sulphate. The extent to which aromatic hydroxylation and oxidative deamination occur can be determined by the measurement of 4-hydroxyamphetamine in the urine together with the amount of deaminated metabolites. In both cases any conjugates must also be taken into account. The relative importance of aromatic hydroxylation and oxidative deamination of amphetamine varies with species as shown in Table 1.5.

It is evident that aromatic hydroxylation which is the major route of amphetamine metabolism in the rat, is a minor route in the rabbit and in primates including man, and is absent from the guinea pig. In those animals in which aromatic hydroxylation is a minor metabolic route, oxidative deamination is the predominant route of amphetamine metabolism, except in the case of the marmoset. In both man and the marmoset, and to a lesser extent in the rhesus monkey, the major excretory product of amphetamine is the unchanged drug. Therefore the metabolic profile of amphetamine in man is best paralleled by that in the marmoset.

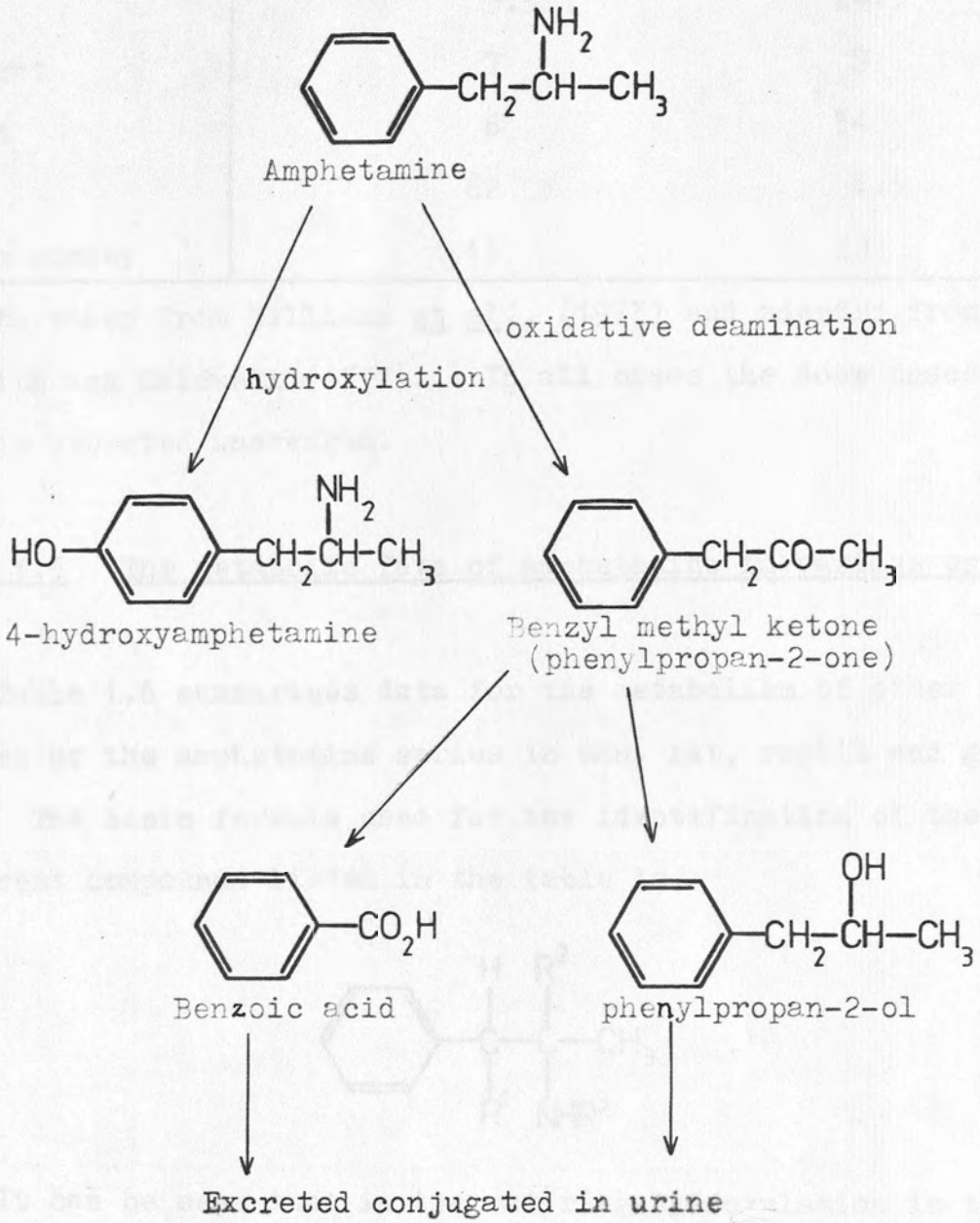


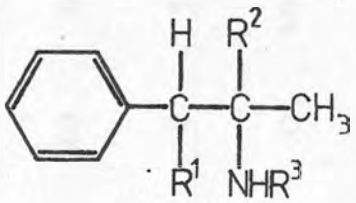
Fig 1.2 Metabolic fate of amphetamine

Species	Percentage of dose that undergoes hydroxylation	oxidative deamination
Guinea pig	0	63
Man	3.5	24.5
Marmoset	7	9
Rabbit	6	54
Rat	62	4.5
Rhesus monkey	11	33

Data taken from Williams et al., (1973) and adapted from Smith and Caldwell (1977). In all cases the dose unaccounted for was excreted unchanged.

Table 1.5 The metabolic fate of amphetamine in various species

Table 1.6 summarises data for the metabolism of other members of the amphetamine series in man, rat, rabbit and guinea pig. The basic formula used for the identification of the different compounds listed in the table is,



It can be seen that in the rat ring hydroxylation is the major metabolic reaction for all the compounds listed and that oxidative deamination is the major reaction in the guinea pig and rabbit. In man however, the relative importance of the two reactions varies but it appears that the presence of an α-hydroxyl group excludes any further hydroxylation of the molecule.

Table 1.7 shows the metabolic fate of norephedrine (II),

Derivative	Percentage of the dose which is excreted as hydroxylated metabolites (H) or deaminated metabolites (D) in the species listed							
	MAN		RAT		RABBIT		GUINEA PIG	
	H	D	H	D	H	D	H	D
Amphetamine ($R^1=R^2=R^3=H$)	2	24	60	3	6	54	0	62
Methamphetermine ($R^1=R^2=H, R^3=CH_3$)	18	6	53	4	-	-	0	74
Norephedrine ($R^1=OH, R^2=R^3=H$)	0	3	28	1	3	76	-	-
Ephedrine ($R^1=OH, R^2=H, R^3CH_3$)	0	10	14	3	11	91	1	-
Pondinil ($R^1=R^2=H, R^3=Cl(CH_2)_2$)	37	-	58	-	6	-	15	-
Mephentermine ($R^1=H, R^2=R^3=CH_3$)	-	-	32	-	5	-	-	-

- data not available

Data from Williams et al. (1973) adapted as in Williams (1974).

Table 1.6 Metabolism of amphetamine and some amphetamine derivatives in various species.

Compound	metabolite	guinea pig	man	marmoset	rat	rhesus monkey
Norephedrine	unchanged	-	79	70	44	-
	ring hydroxylated	-	1	1	25	-
	deaminated conjugates	-	2	2.5	2	-
Phenmetrazine	unchanged	6	19	31	9	-
	ring hydroxylated	0	22	13	44	-
	Lactam derivative	50	20	11	6	-
Chlorphentermine	unchanged	27	22	22	24	20
	hydroxylamine	25	15	0	0	63
	Conjugate	0	0	30	7	0

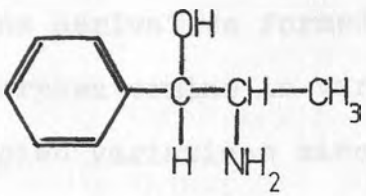
Figures represent the percentage of the dose excreted in each form

- data not available

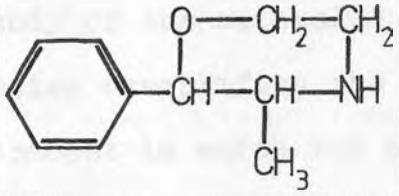
Data adapted from Smith and Caldwell (1977) using reports of Williams et al. (1973); Caldwell et al. (1975a, 1977).

Table 1.7 Metabolic fate of norephedrine, phenmetrazine and chlorphentermine in various species

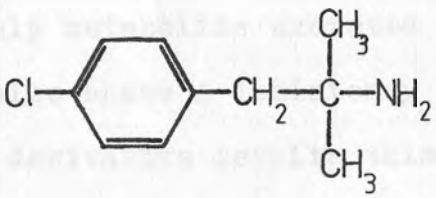
phenmetrazine (III) and chlorphentermine (IV) in man, rat, guinea pig and two species of primate.



II



III



IV

Norephedrine is metabolised by the same pathways as amphetamine (Williams et al., 1973), and the data show that as with amphetamine its metabolic fate in the marmoset and man are similar. Aromatic hydroxylation is the main reaction occurring in the rat, again paralleling the situation found with amphetamine in this species.

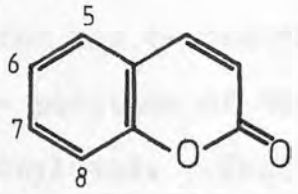
Phenmetrazine can undergo aromatic hydroxylation or oxidation at the methylene carbon α - to the nitrogen atom, giving rise to a lactam derivative namely 5-methyl-6-phenyl-morpholin-3-one. The metabolic fate of phenmetrazine is again similar in the marmoset and man, although slightly less of the dose is metabolised in the marmoset. In the rat, ring hydroxylation is again the predominant reaction, but no such reaction is seen in the guinea pig, a situation encountered with both amphetamine and methamphetamine (Table 1.6).

The p-chloro atom of chlorphentermine excludes ring hydroxylation in this position, and furthermore the lack of a

proton in the substituted side chain excludes any oxidative deamination. The major metabolic route available for this molecule is N-oxidation followed by conjugation of the hydroxylamine derivative formed. A study of the metabolic profile of chlorphentermine in various species exemplifies the problem of species variations since the marmoset in which the metabolism of amphetamine and several of its derivatives resemble that in man, shows a defect in N-oxidation and forms no hydroxylamine derivative, the only metabolite excreted by man. It is interesting that the rat also shows a deficiency in its ability to form the hydroxylamine derivative despite this species' large capacity to carry out aromatic hydroxylation.

Thus in comparing the metabolism of amphetamine and related compounds in various species it is possible to distinguish various trends in Phase I metabolism e.g. the high degree of aromatic hydroxylation in the rat; to show similarities in the metabolic profiles of various compounds in different species e.g. man and the marmoset; and to show how these similarities can suddenly disappear as a result of a slight alteration in the structure of the molecule. The whole study illustrates how structurally related compounds undergoing Phase I metabolism may be expected to have similar metabolic profiles in any one species, but that any one compound studied in more than one species may give rise to wide variations in both the quantitative and qualitative spectrum of metabolites. The differences which occur are at present unpredictable and arise as a result of minor structural changes within the molecule.

The anticoagulant coumarin (V) is a further example of a compound in the Phase I metabolism of which wide and pronounced species differences occur. Biotransformation proceeds ex-



V

clusively via hydroxylation, in the aromatic ring system four positions namely the 5-, 6-, 7-, and 8- positions being known to undergo hydroxylation in various species. In man 90% of a coumarin dose is converted to 7-hydroxycoumarin (Shilling et al., 1969) whereas in the rat the corresponding figure is only 0.3%, hydroxylation of the other three positions also being equally low (Kaighen and Williams, 1961). The rabbit on the other hand converts 13% of a coumarin dose to 7-hydroxycoumarin, the other three positions also being hydroxylated to some extent (Kaighen and Williams, 1961). Therefore coumarin and amphetamine, although both undergoing aromatic hydroxylation, show completely different trends of metabolism in the species studied. With amphetamine aromatic hydroxylation in the rat far surpasses that in the rabbit and man, but with coumarin aromatic hydroxylation in the 7- position of the molecule is 300 times greater in man than in the rat, while in the rabbit all four positions known to undergo aromatic hydroxylation in the coumarin molecule, do so to a greater extent than in the rat. Creaven et al., (1965a) and Creaven and Williams (1963) demonstrated that the species differences in the metabolism of coumarin can be shown in vitro since liver preparations from man, cat, guinea pig, rabbit, pigeon and coypu but not from rat and mouse convert coumarin to its 7-hydroxy derivative.

The species differences observed in the aromatic hydroxylation of drugs are probably due to different enzyme levels within the tissues. In the case of amphetamine, the hydroxylation that

occurs is specific for the 4- position of the molecule, while with coumarin the 7- position of the molecule appears to be preferentially hydroxylated. Thus it is probable that the species variation seen in the hydroxylation of these compounds is due to differing levels of hydroxylating enzymes which catalyse hydroxylation reactions at particular positions in the molecules. The observed differences in the degree of hydroxylation of amphetamine and coumarin in the rat indicate that the reactions are catalysed by different enzyme systems.

Creaven et al. (1965b) have shown that the extent of 2- and 4-hydroxylation of biphenyl depends on species and age. This is an example of a species variation occurring as a result of the presence of two sites within a molecule, both known to be hydroxylated to a significant extent. Furthermore the enzymic basis of these orientated hydroxylations is probably different since the 4-hydroxylation of biphenyl in rats and hamsters is preferentially stimulated by phenobarbitone whereas the 2-hydroxylation is preferentially stimulated by carcinogens in general, including aromatic hydrocarbons such as 3-methylcholanthrene (Creaven and Parke, 1966). Recent work has suggested that phenobarbitone exerts its stimulatory effect on cytochrome P_{450}, whereas 3-methylcholanthrene does so by inducing cytochrome P_{448} in the tissues. Parke and Rahman (1970) reported that the increase in the 4-hydroxylation of biphenyl following the administration of safrole and isosafrole was true enzymic induction, inhibited by actinomycin D, whereas the 2-hydroxylation was an activation not dependent on protein synthesis. The increase in biphenyl 2-hydroxylase activity brought about by induction, is now thought to be a biphasic reaction, the initial stimulation occurring within 0-15 min of treatment with the

inducing agent probably representing a conformational change in the cytochrome P_{450} to cytochrome P_{448}, followed later by true enzymic induction. These observations suggest that two different enzyme systems are involved in the hydroxylation of biphenyl in the animal body, a suggestion which is further strengthened by the observation that the 2-hydroxylation of biphenyl has a pH optimum of 7.8, whereas that for the 4-hydroxylation is 7.4 (Bridges and Burke, 1971). The relative importance of the two hydroxylase systems in different species is undoubtedly responsible for the overall species differences in the metabolism of biphenyl reported by Creaven et al. (1965b).

Trends in the Phase I metabolism of xenobiotics in different species.

The large degree of species variation seen in Phase I metabolic reactions makes it difficult to reach any general conclusions, but a few tentative conclusions can be drawn from the metabolic profiles of amphetamine and related compounds, coumarin and biphenyl in various species. For instance amphetamine and norephedrine, which are structurally very similar, have a similar metabolic fate within a particular species, possibly implicating the involvement of a common enzyme or enzymes in their metabolism. Thus the species differences seen in the metabolism of norephedrine would be expected to be similar to those seen with the metabolism of amphetamine. However these similarities are lost when the structure of the molecule is changed so that a different enzyme system is used in its metabolism, as seen in the case of chlorphentermine. A comparison of the metabolic fate of amphetamine and coumarin in the same species indicates that although both molecules are

metabolised by aromatic hydroxylation, their different structures necessitates the use of at least two different enzymes resulting in totally different metabolic profiles. That such enzymes have vastly different activities and properties is shown by the detailed investigations of biphenyl 2- and 4-hydroxylase.

Species differences in Phase II metabolism

Compared with the variety of Phase I reactions relatively few types of conjugation reactions are known to occur in mammals. Each type of Phase II conjugation reaction occurs with a limited number of biochemically reactive groups (see Table 1.2) and consequently patterns of species variations in Phase II reactions might be expected to be more easily discernible than with the more numerous types of Phase I reactions. In the field of comparative metabolism of xenobiotics, great interest focuses around the Phase II reactions since certain of these reactions are peculiar to a limited number of species while others are known to occur in all except a limited number of species. It is known for example that the cat has a low capacity to conjugate various substrates with glucuronic acid while the pig has a limited ability to conjugate various substrates with sulphate.

Glucuronide formation in the cat

Despite the fact that glucuronide formation is the most common and versatile Phase II reaction in mammals, the cat seems unable to synthesise more than trace amounts of glucuronic acid conjugates from a range of xenobiotic substrates. Hartiala (1955), using tissue slices from various animal species, first showed that cat tissues did not convert o-aminophenol to o-aminophenylglucuronide. These *in vitro* studies were later

confirmed by the *in vivo* investigations carried out by Robinson and Williams (1958) using a range of xenobiotic substrates. The reason for the low capacity of the cat to conjugate various xenobiotic substrates with glucuronic acid was shown to be due to a lack of the transferase enzyme, UDP-glucuronyltransferase (Dutton and Greig, 1957) and not to a deficiency of the activated intermediate UDP-glucuronic acid nor to the occurrence of any natural inhibitor.

Using liver preparations from a number of species, Lathe and Walker (1958) confirmed the poor glucuronidation of *o*-aminophenol in the cat, but showed that the liver preparations from this animal could form bilirubin glucuronide normally, indicating that more than one form of UDP-glucuronyltransferase may be present in the tissues and that the cat is probably deficient in at least one of these forms. Similar conclusions were drawn from the work of Zakim *et al.* (1973) and Vessey *et al.* (1973) where four forms of UDP-glucuronyltransferase were implicated. Since the work of Hartiala, many workers have confirmed the cats' low capacity for forming phenolic glucuronides (Yeh *et al.*, 1971; Capel *et al.*, 1972, 1974a; Miller *et al.*, 1973; Smith *et al.*, 1973). As a result of this low capacity, cats are much more susceptible to the toxic effects of phenols than are other species (see Spector, 1956). As well as bilirubin glucuronide the cat is able to synthesise phenolphthalein glucuronide (Capel *et al.*, 1974a)and also glucuronide conjugates of iopanoic acid, tyropanoic acid and bunamiodyl (McChesney, 1964). The cat can also conjugate various arylacetic acids with glucuronic acid but this will be discussed in more detail later. Other members of the Feloidea also have a limited capacity to form various glucuronide conjugates but this will

also be considered in a later section.

Sulphate conjugation in the pig

The inability of the cat to form significant quantities of glucuronide conjugates from various phenolic compounds results in administered phenols being excreted mainly as sulphate esters in this species. In the pig, the opposite situation is seen since this animal is unable to form significant quantities of sulphate conjugates from a variety of administered phenols (Capel et al., 1972; Capel, 1973; Capel et al., 1974a) which consequently are excreted as glucuronic acid conjugates in the urine. However, as with glucuronide formation in the cat, the extent of sulphate conjugation in the pig varies with the administered phenol good examples being 1-naphthol and 2-naphthol (see page 38). Thus the pig, like the cat is probably deficient in one or more of the forms of a transferase enzyme in this case sulphotransferase (Spencer, 1960, and McEvoy and Carroll, 1971, have described various sulphotransferases and their acceptors).

Acetylation of aromatic amines and hydrazines in the dog

Acetylation is a major route of biotransformation of compounds containing amino groups, and five types of such groups are known to be acetylated in mammals. These are the aromatic and aliphatic amino groups, the sulphonamide group, the hydrazine group and the α-amino group of S-substituted cysteines. The dog and fox are unable to acetylate the aromatic amino group (Boyer et al., 1956; Bridges and Williams, 1963) or the hydrazine group (see Williams, 1967). Furthermore, Weber and Cohen (1967) presented evidence suggesting that the N^4-amino group (i.e. the aromatic amino group) of sulphanilamide and the

amino group of isonicotinic acid hydrazide were acetylated by a common N-acetyltransferase enzyme, known as arylamine N-acetyltransferase, which is absent in the dog. Evidence was obtained earlier that the apparent inability of the dog to acetylate aromatic amines was due to the presence of an active deacetylase system (Krebs et al., 1947; Brodie and Axelrod, 1948; Smith and Williams, 1948b; Bray et al., 1950a). Such a deacetylase system was not found in the rabbit (Smith and Williams, 1948a; Bridges and Williams, 1963), an animal with a high capacity to acetylate aromatic amines (Bridges and Williams, 1963). Evidence has also been obtained that dog liver contains a thermolabile, non-diffusable inhibitor of N-acetyltransferase (Liebman and Anaclerio, 1962).

The formation of mercapturic acids in the guinea pig

The guinea pig, compared with the rabbit and the rat, excretes a small proportion of a mercapturic acid precursor as mercapturic acid (Bray et al., 1959a and b). It was suggested (Bray et al., 1959b) that since only a small proportion of S-substituted cysteines administered to the guinea pig was acetylated and excreted as mercapturic acid, that this species was defective in its ability to acetylate the S-substituted cysteines. Furthermore Bray and James(1960) showed that the guinea pig did not deacetylate administered mercapturic acids indicating that the low excretion of mercapturic acids by this species was not due to the presence of an abnormally high deacetylase activity. More recent work in this laboratory (unpublished) has indicated that a defect in the ability of the guinea pig to acetylate S-substituted cysteines may not be the whole explanation for this animals' low capacity to form and

excrete mercapturic acid conjugates, but that the mercapturic acid formed may to a large extent be further metabolised and excreted as a sulphoxide.

Examples of species differences in Phase II reactions

The metabolism of phenol and related compounds

The metabolism of phenolic compounds proceeds mainly by Phase II reactions, the phenolic group being conjugated with either glucuronic acid or with sulphate; more rarely the phenolic hydroxyl groups undergo methylation. The extent to which ether glucuronide and sulphate ester formation occurs is usually high, other reactions contributing to a minor extent in the overall metabolism of a phenolic compound. The relative proportions of glucuronide and sulphate conjugates excreted in the urine after the administration of a phenol is both species and dose level dependent (Williams, 1938; Bray *et al.*, 1952a,b; Capel *et al.*, 1972; Mehta *et al.*, 1978).

Baumann and Preusse (1879) first reported the *in vivo* conjugation and oxidation of phenol, the metabolites of which are now known to be phenylglucuronide, phenylsulphate and conjugated catechol and quinol. In a quantitative study of the metabolism of phenol, Parke and Williams (1953) showed that in the rabbit 50% of a 0.53mmol/kg body weight oral dose of [^{14}C]phenol was excreted as phenylglucuronide and 45% as phenylsulphate; conjugated quinols accounted for up to 10% of the dose, and conjugated catechols for up to 1%. More recently a comprehensive quantitative study of the metabolism of [^{14}C]phenol in man and 18 other species was reported by Capel *et al.* (1972). Some of the results obtained for the mammalian species are shown in Table 1.8.

Species	Dose (mmol/kg)	Percentage of excreted dose in 24h as				
		phenyl-sulphate	quinol sulphate	phenyl-glucuronide	quinol glucuronide	
Man (3M)	1.1×10^{-4}	77	1	16	tr	
Old World primates						
Rhesus monkey (2F)	0.53	65	-	35	-	
Cynomolgus monkey	0.27	80	-	12	-	(1)
New World primates						
Squirrel monkey (3F)	0.27	7	-	68	25	
Capuchin (1F)	0.27	14	-	65	21	
Non-primates						
Cat (3F)	0.27	87	13	-	-	
Pig (3F)	0.22	-	-	100	-	
Fruit bat (2F)	0.27	10	-	90	-	
Rabbit (3F)	0.27	45	9	46	-	
Rat (3F)	0.27	54	1	42	2	

Data from Capel et al. (1972) except (1) - Smith and Williams (1974)

- means not detected, F - female, M - male.

Table 1.8 The metabolism of [^{14}C]phenol in various animal species

The extent of glucuronic acid and sulphate conjugation of both phenol and quinol in those primate species studied is very interesting from a taxonomic point of view. It may be seen that in man and the more closely related Old World monkeys, sulphate conjugation predominates over glucuronide formation, whereas in the more distantly related New World monkeys glucuronide formation is the main metabolic pathway. The metabolism of 1-naphthol, administered at a dose level of 0.07mmol/kg body weight, in various primate species showed a similar trend (Mehta et al., 1978). In the rhesus and cynomolgus monkeys 1-naphthol was excreted mainly as its sulphate ester whereas in the tamarin and capuchin monkey it was excreted mainly as the glucuronic acid conjugate.

Williams, (1938) showed that in the rabbit the extent to which phenol was conjugated with sulphate decreased with increasing dose levels and similar dose dependent effects have been reported for salicylamide in man (Levy and Matsuzawa, 1967). George et al. (1974) found that the percentage of isoprenaline conjugated with sulphate by isolated loops of dog gut decreased dramatically if the dogs were pre-treated with salicylamide. By feeding sulphate precursors such as L-cystine and sodium sulphite to rabbits, the proportion of a 2.66mmol/kg body weight dose of phenol excreted conjugated with sulphate was increased from 15 to 30%, the glucuronide conjugation being reduced accordingly (Williams, 1938; Porteous and Williams, 1949; Bray et al., 1952a and b).

The marked capacity of Old World primates to form phenylsulphate is emphasised by the results obtained for the metabolism of phenol in the rhesus monkey (Table 1.8); although the dose level of administered phenol was twice that given to the other

species studied and five thousand times that administered to man, the percentage of the dose excreted as the sulphate ester was not substantially lower than that excreted in this form by man and the cynomolgus monkey at the lower dose levels employed.

The inability of the cat to form significant quantities of glucuronide conjugates is reflected in the results for the metabolic fate of phenol in this animal. The data for the cat show that phenol, together with any quinol that may be formed from the administered phenol, is excreted conjugated exclusively with sulphate. However a detailed analysis of the results showed that the cat did in fact excrete trace amounts of phenylglucuronide indicating that this animal has a low capacity to form phenylglucuronide rather than an absolute inability to do so. Other members of the Feloidea have also been shown to have a low capacity to conjugate phenol with glucuronic acid (French et al., 1974; Caldwell et al., 1975b). The limited capacity of the sulphate conjugation mechanism together with the low capacity of the cat to conjugate various substrates with glucuronic acid may account for the increased toxicity of phenols in this species (Miller et al., 1973). Capel et al. (1972) reported that up to 8% of the phenol administered to cats was excreted as an unidentified metabolite, later identified as the phosphate conjugate, phenyl dihydrogen phosphate (Capel et al., 1974b).

Investigations into the fate of phenol in the pig revealed the relative inability of this animal to form sulphate esters since not more than trace amounts of phenylsulphate were excreted. Capel et al. (1972) using pig liver preparations showed that phenol was conjugated with glucuronic acid at a much faster rate than with sulphate, but that under the same conditions rat liver

preparations conjugated phenol with glucuronic acid and sulphate with equal facility. These *in vitro* results agree with the *in vivo* results for the metabolism of phenol in the two species.

The excretion of a high percentage of administered phenol as phenylglucuronide in the fruit bat is interesting. The high percentages of doses of α-naphthylacetic acid (Dixon *et al.*, 1974, 1977a), diphenylacetic acid (Dixon *et al.*, 1977c) and benzoic acid (Bridges *et al.*, 1970; Idle *et al.*, 1975), excreted as ester glucuronides in this species were attributed to a limited capacity to conjugate these compounds with an amino acid. Although only trace amounts of benzoic acid administered to the fruit bat were excreted as hippuric acid, more was excreted as benzoylglutamate, while phenaceturic acid was excreted in significant amounts as a metabolite of phenylacetic acid (Ette *et al.*, 1974). Except for phenylacetic acid, glucosiduronic acid formation was the major pathway followed regardless of the type of amino acid conjugate formed. With phenol however, where amino acid conjugation is not an alternative pathway, the high percentage of the administered dose excreted as phenylglucuronide by the fruit bat, coupled with the low excretion of phenylsulphate, suggests that this species also has a higher capacity to conjugate various substrates with glucuronic acid than with sulphate.

The metabolism of [1-^{14}C]-1-naphthol and [8-^{14}C]-2-naphthol, administered by intraperitoneal injection, has been studied extensively in the cat and pig (Capel *et al.*, 1974a). Both these compounds were excreted almost entirely as sulphate esters in the cat, not more than trace amounts of glucuronic acid conjugates being detected. With 2-naphthol, approximately 20% of the sulphate conjugates excreted by the cat could not be accounted for as 2-naphthylsulphate but appeared to be an unidentified

hydroxynaphthylsulphate ester. 2-Naphthol injected into the pig was excreted as 2-naphthylglucuronide and 2-naphthylsulphate in the ratio of 15:1. However, when 1-naphthol was administered by the same route to the pig, the ratio of 1-naphthylglucuronide to 1-naphthylsulphate excreted in the urine was 2:1. Since the 24h recovery of both doses were high (84 and 81% respectively) the results suggest that the pig can form substantial amounts of 1-naphthylsulphate but not of 2-naphthylsulphate. These results are evidence for the existence of more than one form of sulphotransferase in mammalian tissues and indicate that in the pig the activity of at least one of these forms is low.

A study of the metabolism of 1-naphthol in the marsupial opossum, Trichosuros vulpecula (Roy, 1963) showed that the major metabolite excreted in the urine was 1-naphthylglucuronide, only traces of 1-naphthylsulphate being detected. Furthermore in vitro experiments showed that opossum liver synthesised 4-nitrophenylsulphate only very slowly and no synthesis of steroid sulphates was detected under the same conditions used for their assay with liver preparations from eutherian mammals. It would appear that the opossum, like the pig, has a low capacity to form sulphate esters, but that unlike the pig it does not possess the sulphotransferase necessary for the formation of 1-naphthylsulphate.

There is evidence (unpublished - see Hirom et al., 1977a) that in the cat the low capacity to form 1-naphthylglucuronide is compensated for by the presence of a sulphate ester synthetase system, the activity of which is higher in the cat than in the rat which excretes approximately equal quantities of 1-naphthylglucuronide and 1-naphthylsulphate (Capel et al., 1974a). The apparent Michaelis constant (Km) for the glucuronidation of

1-naphthol by rat liver preparations was found to be 12μM, whereas the corresponding value for cat liver preparations was approximately 3000μM. For sulphation, using the same substrate, the apparent Km values for the rat and cat were 20μM and less than 1μM respectively indicating the greater affinity of cat liver sulphotransferase for 1-naphthol compared with that of the rat.

The metabolism of phenacetin (p-ethoxyacetanilide) in various mammalian species resembles that of phenolic compounds, since the major metabolic reaction of this compound in many species involves oxidative de-ethylation to p-acetamidophenol (paracetamol) which then undergoes Phase II conjugation to form p-acetamidophenylglucuronide and p-acetamidophenylsulphate (Brodie and Axelrod, 1949; Smith and Williams, 1949a,b; Jagenberg and Tocyko, 1964; Smith and Timbrell, 1974) (see Figure 1.3). Deacetylation of phenacetin can also occur and the extent of this reaction is also species dependent.

Capel et al. (1974a)showed that the cat excreted large quantities of administered [1-^{14}C-acetyl] phenacetin as p-acetamidophenylsulphate with only small amounts of p-acetamidophenylglucuronide, whereas the pig excreted large quantities of the same dose as p-acetamidophenylglucuronide, but only small quantities as p-acetamidophenylsulphate, results which are in agreement with the limited capacity of the cat and pig to form glucuronide and sulphate conjugates respectively. Also using [1-^{14}C-acetyl]phenacetin Smith and Timbrell, (1974) showed that glucuronide conjugation of p-acetamidophenol was the dominant pathway of metabolism in the guinea pig, ferret and rabbit, whereas sulphate conjugation was the main pathway in the rat. A comparison of these results with those obtained by Capel et al.

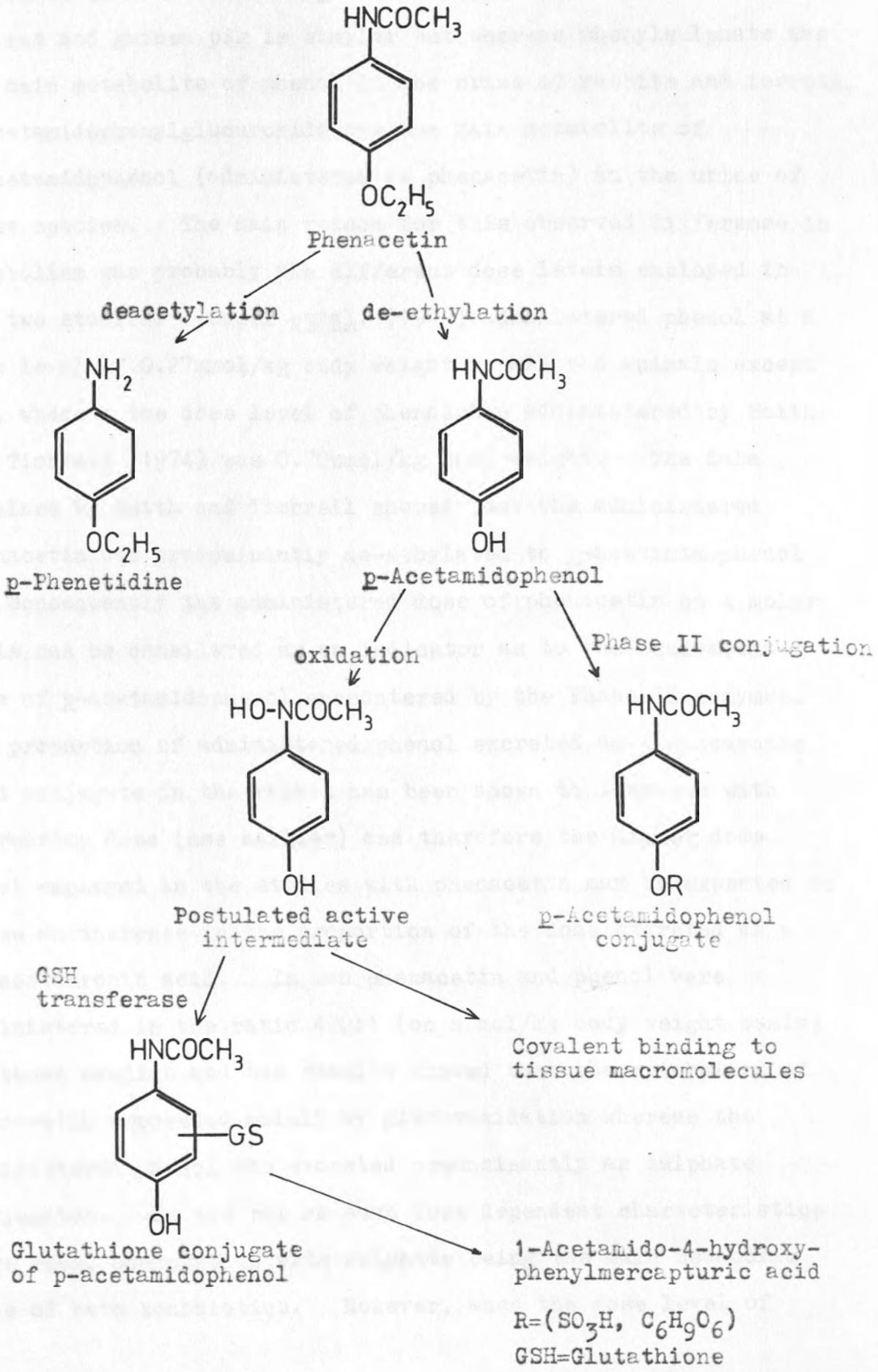


Fig 1.3 Pathway of Phenacetin metabolism

(1972) for the metabolism of phenol in these species shows that the Phase II metabolism of p-acetamidophenol and phenol in both the rat and guinea pig is similar but whereas phenylsulphate was the main metabolite of phenol in the urine of rabbits and ferrets, p-acetamidophenylglucuronide was the main metabolite of p-acetamidophenol (administered as phenacetin) in the urine of these species. The main reason for this observed difference in metabolism was probably the different dose levels employed in the two studies. Capel et al. (1972) administered phenol at a dose level of 0.27mmol/kg body weight in all the animals except man, whereas the dose level of phenacetin administered by Smith and Timbrell (1974) was 0.70mmol/kg body weight. The data obtained by Smith and Timbrell showed that the administered phenacetin was predominantly de-ethylated to p-acetamidophenol and consequently the administered dose of phenacetin on a molar basis can be considered as an indicator as to the equivalent dose of p-acetamidophenol encountered by the Phase II enzymes. The proportion of administered phenol excreted as a glucuronic acid conjugate in the rabbit has been shown to increase with increasing dose (see earlier) and therefore the higher dose level employed in the studies with phenacetin may be expected to cause an increase in the proportion of the dose excreted as a glucosiduronic acid. In man phenacetin and phenol were administered in the ratio 420:1 (on a mol/kg body weight basis) in these studies and the results showed that the metabolism of phenacetin proceeded mainly by glucuronidation whereas the administered phenol was excreted predominantly as sulphate conjugates. In the rat no such dose dependent characteristics were seen, conjugation with sulphate being the main metabolic fate of both xenobiotics. However, when the dose level of

phenacetin administered to the rat was increased to 11.17mmol/kg body weight, there was a change in the pattern of conjugation of p-acetamidophenol away from sulphate conjugation towards glucuronic acid conjugation (Smith and Timbrell, 1974) indicating that more dramatic changes in dose level are required to alter the conjugation patterns of certain phenols in this species. It was also shown by Smith and Timbrell (1974) that at a higher dose level of phenacetin, a greater proportion of the administered dose was deacetylated to phenetidine thus decreasing the relative proportion of the dose being metabolised via p-acetamidophenol.

p-Acetamidophenol may also be metabolised to a mercapturic acid derivative (Jagenburg and Toczko, 1964) as shown in Fig 1.3. Although this is a minor pathway of metabolism it is very important from a toxicological point of view since at high dose levels of paracetamol (p-acetamidophenol) the glutathione levels of the liver become depleted and the postulated N-hydroxylated intermediate binds to tissue macromolecules resulting in hepatic necrosis. The susceptibility of different species to paracetamol induced liver necrosis depends on their capacity to conjugate the dose with glutathione thereby deactivating the active intermediate. Hence in mice the proportion of a dose of paracetamol that is excreted as the mercapturic acid is about 10% when non-toxic doses of the drug are administered, this proportion decreasing as the dose level is increased (Jollow et al., 1974). On the other hand a relatively high dose of 300mg/kg body weight administered to rats does not significantly decrease the hepatic glutathione levels (Davis et al., 1974) and the proportion of the dose that is excreted as the mercapturic acid derivative is only about 4% when low doses are given and

does not significantly change when the dose is increased (Jollow et al., 1974). These differences in the capacity of mice and rats to form the mercapturic acid derivative explains why less than 10% of rats dosed with paracetamol at a dose level of 1500mg/kg body weight showed signs of liver necrosis whereas all mice, dosed at a level of 750mg/kg body weight, developed hepatic necrosis (Mitchell et al., 1973).

Although conjugation with glucuronic acid and/or sulphate is the main metabolic reaction undergone by many phenolic hydroxyl groups, a third type of reaction, O-methylation also occurs with some compounds. The phenols undergoing methylation are usually polyphenols, with at least two of the hydroxyl groups being vicinal. The extent of O-methylation of 4-hydroxy-3,5-diiodobenzoic acid to 3,5-diiodo-4-methoxy benzoic acid has been shown to be species dependent (Wold et al., 1973), and shows a remarkable taxonomic trend. Administered as the n-butyl ester to man, the rhesus and cynomolgus monkeys (Old World primates) and capuchin and squirrel monkeys (New World primates), 4-hydroxy-3,5-diiodobenzoic acid was excreted in the urine partly as 3,5-diiodo-4-methoxy benzoic acid and its glycine conjugate, whereas the rat and rabbit excreted neither the methoxy derivative nor its glycine conjugate, the only metabolites present in the urine being the unchanged acid and its glycine conjugate. Therefore the ability to methylate 4-hydroxy-3,5-diiodobenzoic acid appears, so far, to be present in man, Old World and New World primates and not in rats and rabbits. However, only when studies have been conducted into the metabolism of 4-hydroxy-3,5-diiodobenzoic acid in prosimian species can it be seen whether the ability to methylate this compound is a characteristic of all primates.

Tomita et al. (1964) showed that preparations of liver and kidney from both rabbit and rat were able to methylate 4-hydroxy-3,5-diiodobenzoic acid using S-adenosylmethionine as the methyl donor. This observation suggests that in vivo the enzyme may not be accessible to the substrate or that in the rabbit and rat tissues there exists an active O-demethylase enzyme system, which is not active under the in vitro conditions used by Tomita et al. The observed species variations in the excretion of 3,5-diiodo-4-methoxybenzoic acid might therefore be reflecting species variations in the net balance of O-methylation and O-demethylation as well as some differences in membrane permeabilities.

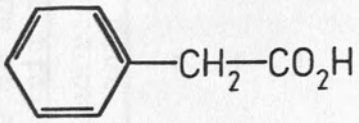
Comparative metabolism of arylacetic acids

Arylacetic acids are metabolised in vivo by conjugation of the carboxylic acid group with either glucuronic acid, to form an ester glucuronide, or with an amino acid. Amino acid conjugation of carboxylic acid groups occurs in particular when such groups are present in molecules whose structure incorporates a carbocyclic or heterocyclic aromatic system. The particular amino acid utilised depends not only on the animal species in which the study is made, but also on the structure of the acid administered. Thus benzoic acid is conjugated with glycine and glucuronic acid in the majority of mammalian species studied (Bridges et al., 1970), whereas phenylacetic acid is conjugated with glycine, glutamine, taurine and glucuronic acid, the combination of endogenous compounds used depending on the species (James et al., 1972b). Both these acids also undergo conjugation with ornithine in some avian species, a metabolic process not seen in other animal species. The metabolism of benzoic acid in various animal

species is described in a later chapter.

Extensive comparative studies have been carried out on the metabolism of five arylacetic acids namely phenylacetic acid (VI), indol-3-ylacetic acid (VII), 1-naphthylacetic acid (VIII), hydratropic acid (IX) and diphenylacetic acid (X) (Fig 1.4). Great interest centres around the metabolic fate of arylacetic acids in mammals because of their conjugation with glutamine. The conjugation of a xenobiotic with glutamine was first observed by Thierfelder and Sherwin (1915) who reported that the main urinary metabolite of phenylacetic acid in man was phenacetylglutamine. Sherwin (1917) was unable to detect phenacetylglutamine as a metabolite of phenylacetic acid in the rhesus monkey, although this metabolite was detected in the urine of chimpanzees (Power, 1936). These early observations suggested that the ability to convert phenylacetic acid to its glutamine conjugate was present only in anthropoid apes, a theory which was dispelled by the work of James et al. (1972b). These authors conducted a comprehensive study of the metabolism of [carboxy-^{14}C]phenylacetic acid in man, fourteen species of subhuman primates and eleven non-primate species. Table 1.9 contains a selection of their results.

In the primates studied, the degree of conjugation of phenylacetic acid with glutamine and glycine was correlated with the evolutionary status of the species. Thus man excreted only phenacetylglutamine, Old World monkeys phenacetylglutamine plus small quantities of phenaceturic acid, New World monkeys phenacetylglutamine together with significant quantities of phenaceturic acid, while the prosimians excreted phenaceturic acid only. Phenacetylglutamine was not excreted by non-primate species in this study but more recent studies (Idle et al., 1976;



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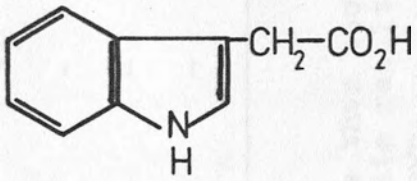

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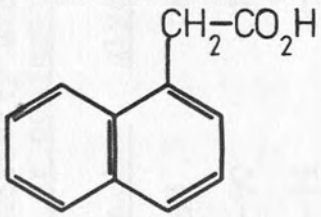

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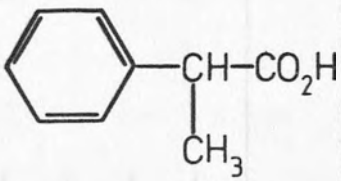

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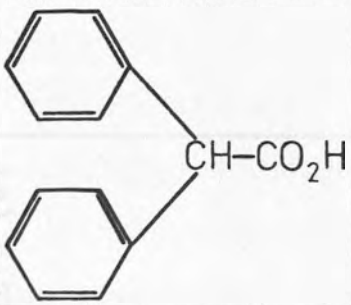


X

Fig 1.4 The structures of various arylacetic acids

Species	Percentage of radioactivity excreted in the urine as unchanged phenylacetic acid or conjugated with the amino acid shown					
	phenylacetic acid	glutamine	glycine	taurine	glucuronic acid	ornithine
Man	-	93	tr	6	-	-
Old World Monkeys (8)	5-65	22-90	1	1-26	-	-
New World Monkeys (4)	4	33-79	1-19	0.4-44	-	-
Prosimii (2)	0,10	-	87,80	13,10	-	-
Cat	1	-	98	1	-	-
Ferret	3	-	63	21	-	-
Hen	-	-	6	-	-	80
Pigeon	-	-	59	38	-	-
Rat	-	-	99	1	-	-

- not detected.
Figures in parentheses show the number of species studied. Where more than two species are studied the range of the results are shown; for two species the separate results are given. In all other cases the average results for a particular species are shown.

Table 1.9 The metabolism of carboxy [^{14}C] phenylacetic acid in various species

Data from James et al. (1972b)

Hirom et al., 1977b) showed that traces of phenacetylglutamine were excreted by the ferret which had been dosed with phenylacetic acid. 4-Chlorophenacetylglutamine was also detected as a metabolite of 4-chlorophenylacetic acid in the cat (unpublished, see Hirom et al., 1977a), and ferret (Idle et al., 1978). Therefore the conjugation of phenylacetic acid with glutamine shows a taxonomic variation, being a major metabolic reaction only in man, the anthropoid apes, Old World and New World primates.

The work of James et al. (1972b) demonstrated for the first time the conjugation of a xenobiotic with taurine. Phenacetyltaurine was present in the urine of all the species studied apart from the hen and vampire bat, although the percentage of the dose excreted in this form was found to be variable and unpredictable. Significant quantities were found in the urine of some Old World and New World monkeys, the pigeon, the ferret and the bushbaby. The relatively high excretion of phenacetyltaurine by the ferret may be a compensatory mechanism for the limited capacity of this species to utilise glycine for conjugation processes. Hirom et al. (1977b) showed that in the ferret, the proportion of administered phenylacetic acid excreted as phenaceturic acid decreased with an increase in dose level while the proportion excreted as phenacetyltaurine increased. By contrast, at a high dose level (400mg/kg body weight) the rabbit excreted the majority of administered phenylacetic acid as phenaceturic acid, no phenacetyltaurine being detected. James et al. (1972b) did not detect the excretion of a glucuronide conjugate by ferrets or rabbits which had been given phenylacetic acid at a dose level of 80mg/kg body weight but at a higher dose (400mg/kg body weight), Hirom et al. (1977a,b) showed that small amounts of phenacetylglucuronide were present in the urine of both species indicating

that the conjugation of phenylacetic acid with glucuronic acid may become increasingly important at higher dose levels.

The conjugation of carboxylic acids with ornithine is unique to certain avian species. The conversion of benzoic acid to ornithuric acid (N^2:N^5-dibenzoylornithine) in the hen was discovered by Jaffe (1877) and later Totani (1910) reported a similar conjugation of phenylacetic acid in the same species. The results of James et al. (1972b) showed that phenacetylornithuric acid was the major metabolite of phenylacetic acid excreted by the hen, but that no such metabolite was excreted by the pigeon. Similar results have been obtained for the metabolism of benzoic acid (Bridges et al., 1970) and indicate how the conjugation of xenobiotics with ornithine is peculiar to only certain species of birds.

The comparative pattern of metabolism of indol-3-ylacetic acid was shown to be similar to that for phenylacetic acid (Patel and Crawford, 1963; Bridges et al., 1974). Conjugation of indol-3-ylacetic acid with glutamine was confined to man, Old World and New World monkeys although the extent to which it occurred was less than that for phenylacetic acid. In general, the extent of glycine conjugation was less than that observed with phenylacetic acid in most species, with the exception of some New World monkeys. The lower excretion of both glutamine and glycine conjugates of indol-3-ylacetic acid as compared with phenylacetic acid was compensated by a larger proportion of the administered acid being excreted unchanged. The excretion of the taurine conjugate was again found to be variable, being high in some New World monkeys, the ferret and the pigeon. The significant quantities of indol-3-ylacetyltaurine excreted in the urine of the green monkey, a species of Old World monkey, possibly

suggests that taurine conjugation within this species is structurally dependent since only a small amount of administered phenylacetic acid was excreted conjugated with taurine (James et al., 1972b).

Perhaps the most significant result from the work with indol-3-ylacetic acid was the excretion by man of a large proportion of the administered dose conjugated with glucuronic acid, the actual percentage varying widely between subjects. No conjugation of indol-3-ylacetic acid with glucuronic acid was detected in any of the other species studied. However the dose level of indol-3-ylacetic acid in man was less than 10mg/kg body weight compared with 100mg/kg body weight in the other species studied and this difference may be responsible in part for the appearance of the glucuronide conjugate in the urine of man. If this were so then it would be in direct contrast to the results already mentioned for the metabolism of phenylacetic acid in the rabbit and ferret where the glucuronide conjugate is only detected in the urine at high dose levels. This problem of the effect of dose level on the metabolism of indol-3-ylacetic acid can only be solved when comparable dose levels are studied in man and other species. Furthermore, the dose was administered orally to man and by intraperitoneal injection to the other species. It is now accepted that the gut wall has a high capacity to conjugate certain substrates with glucuronic acid and it may well be that indol-3-ylacetic acid is one such substrate. Therefore comparable results can only be obtained if the compounds under study are administered by the same route.

The metabolism of 1-naphthylacetic acid in various species has been investigated by Dixon et al. (1974, 1977a) whose results are summarised in Table 1.10.

Species	% of dose excreted unchanged or conjugated with the agent listed				
	unchanged	glutamine	glycine	taurine	glucuronic acid
PRIMATES					
Man (2M)	-	-	-	7.8,3.4	88,94
Rhesus Monkey (1F,1M)	12,6	-	-	3.5, 1	83,94
Cynomolgus Monkey (1F)	35	2.6	-	3.5	29
Squirrel Monkey (2F)	13,7.3	2.0,0.9	7.5,5.0	7.5,5.0	9.4,3.7
Capuchin (2F)	16,4.5	0.9,3.0	2.2,8.8	14,15	2.2,13
Marmoset (1M)	5.4	1.6	1.8	9.0	49
Bushbaby (1M,1F)	3.9,10.5	-	21,4	4.6,7.5	11,3.9
NON-PRIMATES					
Cat (2F,1M)	1.8	-	37	25	-
Rabbit (3F)	6.2	-	4.5	-	71
Rat (3F)	17	-	15	-	33
Fruit Bat (1F,1M)	6.6,2.0	-	-	-	62,56

- not detected.
Where more than two animals were used, the average results are recorded.
M Male, F Female
Data from Dixon et al. (1977a)

Table 1.10 The comparative metabolism of 1-Naphthylacetic acid

The pattern of metabolism seen with phenylacetic acid and indol-3-ylacetic acid was not observed in the case of 1-naphthylacetic acid, since this compound showed very little conjugation with glutamine in all the species studied, the main metabolite in the urine being the glucuronic acid conjugate. In this study, all the animals were given the dose orally so no differences due to the route of administration arose. In all species, except the bushbaby and the cat, the glucuronide was the major metabolite excreted in the urine in contrast to the metabolism of indol-3-ylacetic acid where only man excreted the glucuronide. Only the cat was unable to conjugate 1-naphthylacetic acid with glucuronic acid indicating yet again this animals low capacity to form glucuronides from a variety of substrates. The inability of other members of the Feloidea to conjugate 1-naphthylacetic acid with glucuronic acid had previously been demonstrated (French et al., 1974). The extent to which 1-naphthylacetic acid was conjugated with taurine in the various species was again variable. It was again high in the ferret and also in the cat, where it may be compensating for the low capacity of this species to conjugate the dose with glucuronic acid. However the degree of taurine conjugation with 1-naphthylacetic acid in other members of the Feloidea (French et al., 1974) was low suggesting that taurine conjugation may not be a compensatory mechanism. The extent to which 1-naphthylacetic acid was excreted conjugated with glycine varied with species but appeared to be significant only in New World monkeys, prosimians and non-primates. The Indian fruit bat was the only non-primate species investigated which did not conjugate 1-naphthylacetic acid with glycine indicating again this animals low capacity to conjugate certain carboxylic acids with glycine, glucuronidation being the major

pathway of metabolism.

The data reported for the metabolism of 1-naphthylacetic acid in the rat was obtained from urinary analysis only (Dixon et al., 1977a) whereas a complete study of the metabolism of this compound in the rat should include investigations of the biliary excretion of the conjugates. Dixon et al. (1977a) demonstrated that at a dose level of 5mg/kg body weight the urine contained, as the major excretory product, 1-naphthylaceturic acid (88% of the dose excreted). It is generally recognised that for significant biliary excretion (>10% of the dose) of an aromatic anion to occur in the rat, the molecular weight should be in the region of, or in excess of, 325 ± 50 (Millburn et al., 1967). The molecular weight of 1-naphthylaceturic acid (246) is below the threshold requirement, whereas that of 1-naphthylacetyl-glucuronide (361) is above this requirement so that this conjugate is likely to be excreted in rat bile in appreciable amounts. The values in Table 1.10 for the proportions of a 100mg/kg body weight dose excreted as the glycine or glucuronic acid conjugates in the urine of the rat show that the proportion of the dose excreted conjugated with glycine is less than that conjugated with glucuronic acid, in contrast to the results mentioned above for a dose level of 5mg/kg body weight. Dixon et al. (1977a) showed that for the rat, the proportion of the dose excreted in the urine conjugated with glycine decreased with increasing dose level whereas the proportion conjugated with glucuronic acid increased. This relationship was not seen in the bile presumably because the extent of 1-naphthylaceturic acid excretion by this route is severely limited by its relatively low molecular weight.

Dixon et al. (1977b) investigated the metabolism of

[methyl-^{14}C]2-phenylpropionic acid (hydratropic acid) in man, the rhesus monkey, the cat, the rabbit and the rat. In all these species, 2-phenylpropionylglucuronide was the major metabolite excreted in the urine over a 24h period. The cat also excreted small amounts of both the glycine and taurine conjugates possibly indicating that although glucuronide formation from 2-phenylpropionic acid occurs in the cat, it may not be as efficient as in the other species studied. In the rat, 20-30% of the dose was excreted in the bile but the pattern of the excretory products was the same as that in the urine.

The metabolism of [carboxy-^{14}C]diphenylacetic acid in man, six species of sub-human primates and four species of non-primates including the cat, was shown to proceed exclusively via glucuronic acid conjugation (Dixon *et al.*, 1977c) only trace amounts of glycine and taurine conjugates being detected in the urine of some lower primates and non-primate species. As in the case of 2-phenylpropionic acid, the rat was shown to excrete significant amounts of both the unchanged acid and its glucuronide in the bile; 2-phenylpropionic acid and diphenylacetic acid are therefore two examples of compounds which are excreted extensively as glucosiduronic acids by the cat.

The data presented above for the metabolism of various arylacetic acids in different species demonstrates not only species differences in the metabolism of a particular compound, but also how such species differences may alter or disappear with the modification of the structure of the arylacetic acids investigated. In general it would appear for all species studied, that as the structure of the arylacetic acid becomes more complex there is a general movement away from amino acid conjugation towards conjugation with glucuronic acid. The point at which

glucuronic acid conjugation takes over from amino acid conjugation as the major pathway of metabolism, occurs, for the majority of species, between indol-3-ylacetic acid and 1-naphthylacetic acid. The whole problem of the relationship between the type of conjugation involved in the metabolism of arylacetic acids and the structure and chemical properties of the acid was studied by Dixon et al. (1976, 1977d). The two most important points, offering explanations for the observed in vivo results to emerge from these studies were:

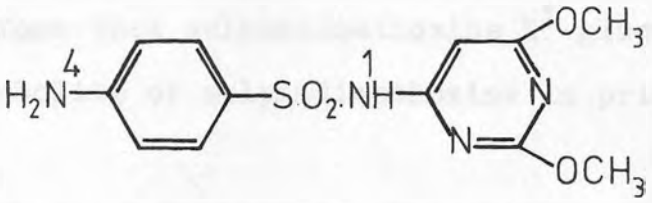
i) The substitution of the α-methylene group of phenylacetic acid (as in the cases of hydratropic acid and diphenylacetic acid) results in loss of specific binding to mitochrondria, the sites of amino acid conjugation (Schachter and Taggart, 1954), and an inability to undergo glycine conjugation both in vivo and in vitro.

ii) 1-Naphthylacetic acid, hydratropic acid and diphenylacetic acid exhibit an extensive non-specific entrapment by isolated rat liver microsomes, the site of glucuronic acid conjugation (Dutton, 1966a).

In conclusion it can be seen that phenylacetic acid, indol-3-ylacetic acid and 1-naphthylacetic acid show marked species variations in their metabolism, differences which can be related to the evolutionary status of the species. The species differences observed in the metabolism of the three compounds are not the same partly because the metabolism of the compounds is influenced by their detailed chemical structure. The substitution of the α-methylene group of phenylacetic acid in general abolishes any qualitative species differences seen in the metabolism of the parent compound and its derivatives which are not substituted in this position.

Species differences in the metabolism of sulphadimethoxine

Sulphadimethoxine (2,4-dimethoxy 6-sulphanilamidopyrimidine), a member of the sulphonamide group of anti-bacterial drugs, is metabolised in most animal species by a combination of N^4-acetylation and N^1-glucuronidation (Fig 1.5); N^4-glucuronidation and sulphation also occur to a minor extent in some species, the product of the latter biotransformation being a sulphamate derivative.



Sulphadimethoxine

N^4-Acetylsulphadimethoxine

Sulphadimethoxine N^4-sulphate

Sulphadimethoxine N^4-glucuronide

Sulphadimethoxine N^1-glucuronide

Fig 1.5

The degree to which sulphadimethoxine is excreted in the urine as its N^4-acetyl derivative or its N^1-glucuronide conjugate has been reported for numerous species including man (Bridges et al., 1965, 1966, 1968, 1969a; Adamson et al., 1966, 1970a; Uno et al., 1967; French et al., 1974). Adamson et al. (1970a) studied the metabolism of sulphadimethoxine in man, eight species of sub-human primates and nine non-primate species. The main metabolite of the drug in the urine of man, rhesus monkey, baboon, squirrel monkey, capuchin monkey, bushbaby, slow loris

and treeshrew was sulphadimethoxine N^1-glucuronide. In the African green monkey, although the main metabolite was N^4-acetylsulphadimethoxine, the N^1-glucuronide was also a major metabolite. In dog, rat, mouse, guinea pig, Indian fruit bat and the hen, sulphadimethoxine N^1-glucuronide was a minor metabolite excreted in urine, whereas in the cat, ferret and rabbit this metabolite was not detected. However Uno et al. (1967) detected small but significant amounts of sulphadimethoxine N^1-glucuronide in the urine of rabbits dosed with sulphadimethoxine. It appears therefore that sulphadimethoxine N^1-glucuronide is the main urinary metabolite of sulphadimethoxine in primate species only.

All the species studied, except the dog, excreted some N^4-acetylsulphadimethoxine which was a major metabolite in the green monkey, the rabbit and the guinea pig. The absence of any detectable quantities of N^4-acetylsulphadimethoxine in the urine of the dog is further evidence for this animals low capacity to acetylate aromatic amines. Of all the non-primate species studied the dog excreted the highest proportion of the administered sulphadimethoxine as the N^1-glucuronide conjugate, suggesting that in this species the process of N^1-glucuronidation may be a compensatory mechanism for N^4-acetylation. The absence of sulphadimethoxine N^1-glucuronide in the urine of cats dosed with sulphadimethoxine indicates that this animal is unable to form significant quantities of N-glucuronide conjugates and that the low capacity of the cat to conjugate many substrates with glucuronic acid is not only limited to the formation of O-glucuronides. French et al. (1974) have shown that other members of the Feloidea Superfamily, namely the lion, the civet and the genet were unable to conjugate sulphadimethoxine with glucuronic acid.

The observation that sulphadimethoxine N^1-glucuronide is the major urinary metabolite of sulphadimethoxine in primate species only, can be used to verify the classification of animal species. Thus the treeshrew (Tupaia), an animal at the foot of the primate evolutionary scale as defined by Napier and Napier (1967) (see Fig 1.6) is sometimes classified as a primate and sometimes as a non-primate. From its ability to excrete sulphadimethoxine N^1-glucuronide as the main metabolite of sulphadimethoxine the treeshrew should be classified as a primate. That N^1-glucuronide formation occurs with a limited number of sulphanilamidopyrimidines has been reported (Bridges et al., 1966, 1969a and b; Walker and Williams, 1972), and consequently care must be taken to ensure that the particular sulphanilamido-pyrimidine under study is capable of undergoing N^1-glucuronidation before any taxonomic conclusions are drawn from the results obtained.

The observed urinary excretion of sulphadimethoxine N^1-glucuronide by the rat is not a true indication of this animals ability to carry out the N^1-glucuronidation of sulphadi-methoxine since the physico-chemical properties of the conjugate are such that in the rat considerably more of the metabolite is excreted in the bile than in the urine (7% and 1% of the dose respectively) (Adamson et al., 1970a).

The excretion of sulphadimethoxine N^4-glucuronide and N^4-sulphate as minor metabolites of sulphadimethoxine has received relatively little attention. Bridges et al. (1968) investigated the metabolism of several methoxy-6-sulphanilamidopyrimidines in various species but the combined excretion of the N^4-glucuronide and N^4-sulphate derivatives did not exceed 11% of the dose in any of the species studied. While Adamson et al. (1970a) did not detect sulphadimethoxine N^4-glucuronide in the urine of rabbits

dosed with sulphadimethoxine, Uno et al. (1967) reported that 13% of a low dose of sulphadimethoxine was excreted in the urine of rabbits as the N^4-glucuronide conjugate. The instability of N-glucuronide conjugates formed from primary amines and the observation that such conjugates can be formed spontaneously in the urine (Bridges et al., 1968) probably accounts for the variations in the above results. However, overall insufficient data is available for conclusions to be drawn about species differences in the formation of both sulphadimethoxine N^4-glucuronide and sulphadimethoxine N^4-sulphate.

Species differences in the formation of mercapturic acids

Only one comparative study has been carried out of species variations in the formation of mercapturic acids (see Smith and Williams, 1974). Using chlorobenzene, French et al. (unpublished) showed that man, the rhesus monkey, the squirrel monkey, the capuchin monkey together with a number of non-primate species excreted varying amounts of p-chlorophenylmercapturic acid. The percentage of the dose excreted in the urine by non-human primates as the mercapturic acid was fairly constant between 40 and 50%, but that for the non-primate species varied widely between 21 and 65%. The figure for man was 19% but in this case the dose level was considerably lower than in the other species studied making a true comparison with the other species difficult. However the excretion of significant amounts of p-chlorophenylmercapturic acid after a dose of chlorobenzene in man is interesting since previous studies (Boyland and Simms, 1958; Wainer and Lorincz, 1963) have indicated that mercapturic acid formation in man was low.

In conclusion it is possible to discern species patterns in

the Phase II reactions of drug metabolism. In some cases these patterns show a remarkable taxonomic trend particularly amongst primates. These patterns observed in the conjugation reactions appear to be dependent, in many cases, on the dose level of the xenobiotic used and on its route of administration.

Neonatal development of xenobiotic metabolising enzyme systems

Growth to maturity is accompanied by changes in the activities of various enzyme systems, changes which are not nearly so apparent as the more striking morphological changes. The developmental patterns of enzymes concerned with intermediary metabolism have been studied in some detail (Driscoll and Hsia, 1958; Greengard, 1971; Walker, 1971), the levels of these enzymes increasing suddenly within periods of hours or days around the time of birth or weaning. Three main 'clusters' of enzymes have been described for the mammalian liver; the late foetal cluster, the neonatal cluster and the late suckling cluster (around the time of weaning), the development of these clusters being associated with major changes in diet or environment.

The detoxication of xenobiotics has been shown to be affected by enzyme developmental changes. The specific activities of many drug metabolising enzymes are known to be low in the foetus and neonate, and to undergo characteristic developmental patterns between birth and maturity (for reviews see Done, 1964; Hanninen, 1975), and the increased sensitivity of the foetus and neonate to many xenobiotics has been attributed to their low drug metabolising capacities (Done, 1964) an early example being the high toxicity of chloramphenicol in the premature human infant (Weiss et al., 1960). If however biotransformation is required before the toxic effects of a xenobiotic are manifested the low xeno-

biotic metabolising capacity of the foetus and neonate may afford some protection against the potential toxicity of that compound.

The earliest studies of xenobiotic metabolism in the foetus and newborn were carried out by Jondorf et al. (1958) and Fouts and Adamson (1959). Jondorf et al., showed that newborn mice, when injected with amidopyrine, phenacetin, or hexobarbitone, still contained most of the dose in the unmetabolised form three hours after administration but that three week old mice contained much less of the unchanged drug after a similar period. This suggested that three week old mice had a greater capacity to metabolise these drugs than had newborn mice. These workers also demonstrated that liver preparations from newborn guinea pigs were virtually unable to carry out the N-demethylation of aminopyrine, the O-dealkylation of phenacetin, the side chain oxidation of hexobarbital or the conjugation of phenolphthalein with glucuronic acid. The enzyme activities required for these reactions appeared in the first week of life and increased until the guinea pigs were about eight weeks old. Fouts and Adamson, (1959) showed that newborn rabbits were virtually unable to metabolise acetanilide, aminopyrine, amphetamine, chlorpromazine, hexobarbital and p-nitrobenzoic acid. Since these early studies many laboratory animal species including the rat (Klinger et al., 1968; Eling et al., 1970; Henderson, 1971; Wilson and Frohman, 1974), rabbit (Fouts and Adamson, 1959; Rane et al., 1973; Vainio, 1975), mouse (Pomp et al., 1969; Stålhandske et al., 1969), guinea pig (Jondorf et al., 1958; Kuenzig et al., 1974), hamster (Nebert and Gelboin, 1969), swine (Short and Davis, 1970; Short et al., 1972) and ferret (Ioannides and Parke, 1975) have been shown to be deficient in hepatic microsomal xenobiotic oxidising

activity during early development and to have a low capacity to metabolise xenobiotics until a few days after birth.

The pattern of development followed by the xenobiotic metabolising systems from the foetal period to maturity in various species has received much attention (Kato et al., 1964; Heinrich and Klinger, 1968; Yaffe et al., 1968; Gram et al., 1969; Berte et al., 1970; Short and Davis, 1970; Basu et al., 1971; Henderson, 1971; Uehleke et al., 1971; Fouts and Devereux, 1972; Kuenzig, 1972; Macleod et al., 1972; Short et al., 1972; Rane et al., 1973; Yeary et al., 1973). This work, carried out using tissue preparations, suggested that although there were individual variations between species and substrates, the development of the hepatic xenobiotic metabolising enzyme systems in the animals studied were similar. The most rapid phase of development occurred in general between the first 20 and 40 days after birth regardless of the length of the gestational period, the size of the species, the degree of maturity at birth, or the life span of the species. Two notable exceptions to this general trend of development are known. Firstly the maturation of xenobiotic oxidative systems in the guinea pig is very rapid occurring within a few days of birth; this may be attributed to the high degree of development of this species at birth; secondly the ability to synthesise glucuronides in the rat is not low at birth and may increase substantially within a few days.

More recent studies using less common laboratory animals have shown that the xenobiotic oxidation capacity of the stump-tail monkey during early gestation is considerable (Dvorchick et al., 1974). Furthermore Quattropani et al. (1975) reported the presence of smooth endoplasmic reticulum in the foetal liver

of the stumptail monkey which is in agreement with its capacity to carry out the oxidative reactions since it has been shown that in adult liver, xenobiotic oxidising enzymes are associated with the smooth endoplasmic reticulum (Remmer and Merker, 1963; Conney, 1967; Remmer, 1972). The presence of benzo-[a]-pyrene hydroxylase in the foetal tissue of the pig-tailed monkey was also shown (Juchau and Pedersen, 1973).

Fouts (1973) emphasised the importance of extra-hepatic xenobiotic metabolism and showed that the microsomal xenobiotic oxidising capacity of rabbit lung increased gradually with age and did not exhibit the sudden burst in activity observed with the corresponding hepatic development. Furthermore the time taken to reach adult enzyme levels was considerably longer for the lung than for the liver. These observations suggest that the factors governing the development of the xenobiotic metabolising enzyme systems of the lung and liver are different, or that the same factors have a much more pronounced effect in the liver.

Human foetal liver has been shown to contain cytochrome P_{450} and certain xenobiotic metabolising enzymes as early as the first half of gestation (Yaffe et al., 1970; Pelkonen et al., 1971c; Rane et al., 1973) observations which suggest that the human foetus may be able to metabolise certain xenobiotics and that the development of these enzymes in the human may be different from that in experimental animals. Due to the socio-legal difficulties encountered in obtaining foetal tissue throughout gestation, relatively few reports exist on the activities of the Phase I enzymes in the human foetus (Pelkonen et al., 1969, 1971a, b; Arvela et al., 1970; Yaffe et al., 1970; Juchau, 1971).

The human newborn is able to metabolise various substrates (O'Donaghue, 1971; Kanto et al., 1973; Krauer et al., 1973; Sereni et al., 1973; Rane et al., 1974; Horning et al., 1975; Mandelli et al., 1975). However in those studies where the xenobiotic is transplacentally transferred after administration to the mother, it was not completely certain whether a particular metabolite was formed in the newborn rather than in the mother. Even so the studies strongly suggest that foetal metabolism of these compounds may be significant in vivo. Fouts (1973) suggested that the levels of the xenobiotic metabolising enzymes in the human foetus and newborn were due to the exposure of the mother to various inducers throughout pregnancy. It has been shown (Basu et al., 1971; Chadwick et al., 1975; Mathur et al., 1975) that the xenobiotic metabolising enzymes of young animals show a greater response to inducers than do their adult counterparts and there is evidence that the induction of these enzyme systems can occur in humans during the last period of gestation and shortly after birth (Sereni et al., 1973).

Although the development of the enzyme systems responsible for the conjugation reactions have in general received less attention than those concerned with Phase I type metabolic reactions, considerable work has been carried out on the development of the enzyme system responsible for glucuronide formation. One of the reasons for this interest is that a low capacity to conjugate bilirubin with glucuronic acid is a possible cause of unconjugated hyperbilirubinaemia frequently seen in humans as jaundice of the newborn (Brown and Zuelzer, 1958).

Karunairatnam et al. (1949) showed that liver slices from newborn mice had a low capacity to conjugate o-aminophenol with glucuronic acid, and that adult levels of conjugation were not

attained until the end of the first postnatal month. Since this initial report, many detailed studies have been carried out in laboratory animals to confirm the impairment of glucuronide synthesis in the neonate. These investigations have involved many animal species including the chick (Dutton, 1963), guinea pig (Brown and Zuelzer, 1958; Jondorf et al., 1958; Dutton, 1959, 1963; Gartner and Arias, 1963), mouse (Dutton, 1959, 1963 and 1966b),pig (Short and Davis, 1970; Short et al., 1972), rabbit (Hartiala and Pulkkinen, 1955; Flint et al., 1964; Yaffe et al., 1968) and rat (Lathe and Walker, 1958; Inscoe and Axelrod, 1960; van Leusden et al., 1962; Stevens, 1962; Arias et al., 1963; Gartner and Arias, 1963; Dutton et al., 1964; Dutton, 1966b; Halac and Sicignano, 1969; Berte et al., 1970; Basu et al., 1971; Henderson, 1971; Yeary et al., 1973). These investigations indicated that for all the animal species studied, with the possible exception of the rat, an impairment of the enzyme UDP-glucuronyltransferase (UDPGT) (EC.2.4.1.17) was common to the neonates. Flint et al. (1964) showed the presence of low hepatic levels of UDPGT in neonatal rabbits, hamsters and rats, and concluded that it was the low levels of this enzyme and not any deficiency in the hepatic content of UDP-glucuronic acid (UDPGA) which was responsible for the low level of glucuronidation in those animals. However it has been reported that compared to adult levels, the foetal level of hepatic UDPGA is low in many mammals (Dutton, 1959; Flodgaard and Brodersen, 1967; Flodgaard, 1968; Zhivkov et al., 1975), but high in embryo chick (Dutton and Ko, 1966; Fyffe and Dutton, 1975), suggesting that the subsequent development which occurs much more rapidly in the hatched chick than in mammalian neonates is related to the supply of UDPGA (Dutton and Burchell, 1974;

Fyffe and Dutton, 1973, 1975). Furthermore the activity of hepatic UDP-glucose dehydrogenase has been found to reflect the levels of UDPGA in the foetal tissues, being low in foetal mammals (Brown and Zuelzer, 1958; Fyffe and Dutton, 1975), and high in embryo chick (Dutton and Burchell, 1974; Fyffe and Dutton, 1973, 1975). Brown and Zuelzer (1958) reported that the activity of both UDP-glucose dehydrogenase and UDPGT was low in foetal guinea pig liver but increased gradually during the first few days of life, an observation which probably explains the low levels of hepatic UDPGA found in mammalian foetuses but the higher levels found in the corresponding neonates. Overall the results suggest that mammalian neonatal development of glucuronidation is governed by the activities of both UDP-glucose dehydrogenase and UDPGT around the time of birth, but that after a few days when the activity of UDP-glucose dehydrogenase is such that the supply of UDPGA is no longer rate limiting, the observed development of glucuronidation is related to the activity of UDPGT only. Although the factors governing the natural perinatal development of glucuronidation and UDPGT are not known, Wishart and Dutton (1977) have presented evidence that in foetal rat tissue the precocious development of UDPGT and of overall glucuronidation can be brought about both in culture and *in utero* by the long-acting synthetic glucocorticoid dexamethasone indicating that perinatal development of glucuronidation and UDPGT activity towards certain substrates may be brought about *in vivo* by glucocorticoids. More recently Dutton *et al.* (1978) have presented further evidence that fetal adrenocorticotrophin and 11β(OH)-corticosteroids are triggers for UDPGT activity towards *o*-aminophenol in the rat.

Although it is generally accepted that foetal and newborn

animals of various species have a low capacity to form glucuronide conjugates from a variety of substrates, the situation in the rat is confused. While some reports indicate that the newborn rat has a relatively poor ability to synthesise glucuronic acid conjugates, there is an increasing body of evidence which suggests that the development of UDPGT in the rat exhibits what is termed the 'overshoot' phenomenon, whereby infant levels of UDPGT activity rise temporarily above adult levels. Such an increase is typical of an hormonal induced rise in enzymic activity (see Greengard, 1971) and has been shown to occur for UDPGT in the rat using a variety of substrates (Gartner and Arias, 1963; Dutton, 1964, 1966b; Tomlinson and Yaffe, 1966; Halac and Sicignano, 1969, Henderson, 1971; Sanchez and Tephly, 1974; Lucier et al., 1975). The peak level of activity attained, together with the time at which the peak occurs varies with substrate, strain and to some degree with the report.

Using rats of the Wistar strain, Henderson (1971) reported that the hepatic activity of p-nitrophenol UDPGT (PNP-UDPGT) was low at birth, but that one week post partum the activity had risen to three times the adult level. On the other hand Sanchez and Tephley (1974) using rats of the Sprague-Dawly strain, reported that the activity of hepatic PNP-UDPGT was four times the adult level at one day of age. Whether or not these observed differences in the pattern of PNP-UDPGT development are due to the strain of rat used is not certain, since both groups of workers reported that the activity of hepatic UDPGT using morphine as the substrate was similar in one day old and adult rats. Similar results for the activity of morphine UDPGT in neonatal and adult rats have recently been reported (Yeh, 1978).

Kupferberg and Way (1963) noted differences in the ability of 16 and 32 day old rats to conjugate and excrete morphine but concluded that the increased toxicity of this drug in the 16 day old rats as compared to the 32 day old rats could best be correlated with differences in the permeability of the brain to morphine. Hence the developmental patterns in glucuronidation seem to vary with the substrate which suggests that different substrate-specific types of UDPGT reported by Lathe and Walker (1958) and Yaffe et al. (1968) each have their own particular characteristic pattern of development.

Using p-nitrophenol (PNP) as substrate, Dutton et al. (1964), Henderson (1971) and Lucier et al. (1975) have shown that in the rat there occurred a peak in UDPGT activity, above adult levels, about 4 days post partum. Furthermore the overall patterns of development for this enzyme, as reported by these different authors, were in good agreement. A similar pattern of development for PNP-UDPGT has also been reported for the rabbit (Tomlinson and Yaffe, 1966). Dutton et al. (1964) reported that the peak in activity of rat hepatic UDPGT using o-aminophenol as substrate occurred 4 days post partum although Gartner and Arias (1963) claimed that such a peak occurred one day post partum. Halac and Sicignano (1969) studied the development of hepatic UDPGT in rats using PNP and bilirubin as substrates. A peak in activity of UDPGT towards both substrates occurred when the rats were about 18 days old. The pattern of development of PNP-UDPGT reported by these authors differed from that outlined above in that there was a sharp drop in enzyme activity between 1 and 4 days post partum followed by a gradual rise to a peak of activity around 18 to 20 days post partum. Therefore while some authors report a peak in the activity of hepatic PNP-

UDPGT in the rat at 4 days post partum, others claim that a trough in activity occurs at this time. No such drop in the activity of bilirubin UDPGT between 1 and 4 days post partum was observed by Halac and Sicignano (1969) who reported a gradual increase in the activity of this enzyme from the low level at birth to a peak at 18 days post partum, after which the activity fell off to adult levels. In some of the newborn rats studied, no hepatic bilirubin-UDPGT was detected within the first 10h of life. Grodsky et al. (1958) did not detect bilirubin-UDPGT activity in foetal rat tissue and found that maximal levels of activity were not attained until 35-40 days after birth. Wong (1972) demonstrated a deficiency in hepatic bilirubin-UDPGT activity in young rats and reported that there was a gradual increase in the activity of this enzyme until 50% of the adult level of activity was reached within 4 weeks of birth. However Henderson (1971) had reported that the hepatic bilirubin-UDPGT activity in 4 day old rats was more than twice that seen in 60 day old animals indicating a much more rapid rate of development of this enzyme system.

Similar patterns of development for bilirubin-UDPGT as those reported for the rat, have also been reported for the mouse (Krasner et al., 1973), and the rabbit (Tomlinson and Yaffe, 1966) while other investigations have shown low bilirubin-UDPGT activity in the newborn of the guinea pig (Brown and Zuelzer, 1958; Gartner and Arias, 1969), mouse (Catz and Yaffe, 1968) and rabbit (Flint et al., 1964; Yaffe et al., 1968). Low bilirubin-UDPGT activities have also been found in tissues taken from human foetuses (Dutton, 1959) and from short-lived premature infants (Lathe and Walker, 1958). Catz and Yaffe (1962, 1968) have indicated that UDPGT enzymes can be induced in mammals as for

other microsomal drug metabolising enzymes, a phenomenon which has been used in the relief of hyperbilirubinaemia in infants by the administration of barbiturates to the expectant mother, to the newborn infant or to both (Crigler and Gold, 1966; Yaffe et al., 1966; Maurer et al., 1968; Trolle, 1968; Vest et al., 1970). An inverse relationship between the serum bilirubin levels and the ability to convert acetanilide to N-acetyl p-aminophenyl glucuronide in full term and premature infants was shown by Vest (1958) an observation further implicating the role of glucuronidation in the prevention of hyperbilirubinaemia.

The glucuronidation of substrates other than bilirubin in the human neonate, has been less extensively studied than in the neonates of other species. Vest and Rossier (1963) using acetanilide and N-acetyl p-aminophenol as the administered compounds showed that human neonates formed ether glucuronides at a reduced rate. Vest and Salzberg (1965) in a study on the metabolism of p-aminobenzoic acid in human neonates and children showed that only a small amount of the dose was excreted conjugated with glucuronic acid by the newborn infant. Both reports showed that although the rate and extent of glucuronidation in human newborns was low, the reaction did occur. As mentioned earlier, this ability may be due to the induction of the foetal and also the neonatal enzymes by the exposure of the expectant mother and/or the newborn infant to inducing agents present in the environment.

The glucuronidation of a few substrates has been studied in human foetal tissue and the results show very low or absent UDPGT activity with respect to PNP (Pelkonen et al., 1971a; Rane et al., 1973), o-aminophenol (Dutton, 1959), 1-naphthol (Rane et al., 1973), and 4-methylumbelliferone (Hirvonen, 1966;

Rane et al., 1973). On the other hand conjugation with glutathione is fairly well developed in the human foetal tissues (Chasseaud, 1974) while Irjala (1972) reported that the rates of formation of salicyluric acid in foetal and adult tissues were similar.

Apart from glucuronidation the only other conjugation reaction that has been studied to any appreciable extent in neonatal animals is the conjugation of benzoic acids with glycine to form hippuric acids. Bridges et al. (1970) reported that in adult man and many mammalian species, benzoic acid is almost entirely excreted as hippuric acid whereas Vest (1959) reported that the formation of hippuric acid was less efficient in the human infant than in the adult. The studies of Vest and Rossier (1963) and Vest and Salzberg (1965) showed that human infants conjugated p-aminobenzoic acid only very slowly with glycine, the excretion of the dose being maintained by the formation of acetylated derivatives. This is interesting since Fichter and Curtis (1956) indicated that newborn and premature infants had a poorly developed ability to acetylate the aromatic amino group of sulphonamides.

The enzymes responsible for catalysing the synthesis of hippuric acid and p-aminohippuric acid were characterised by Schacter and Taggart (1953, 1954) but their activity in rat liver was undetectable until the age of about 6 days (Brandt, 1964), adult levels of activity not being reached until approximately 30 days after birth. The pattern of activity during development, of glycine N-acyltransferase (EC.2.3.1.13) has been shown to resemble closely the activity of the whole p-aminohippurate synthesising system (Brandt, 1966) suggesting that the activity of this enzyme is the rate limiting factor in p-

aminohippuric acid synthesis by neonates. The development of the hippuric acid synthetase system in the mouse follows a similar pattern to that in the rat (Gorodischer et al., 1971) and it has also been shown (Irjala, 1972) that liver from foetal and neonatal rabbits has a very limited capacity to synthesise p-aminohippuric acid.

The wide variations observed in the developmental patterns of the enzyme systems responsible for both Phase I and Phase II reactions of xenobiotic metabolism makes it difficult to state any well-defined outlines for this development, but in general it appears that in the majority of mammalian species, the activities of the drug metabolising enzymes are low in foetal and newborn animals and rise to adult levels within a few weeks of birth. However, the neonatal rat appears to have a large capacity to form glucuronides from certain aglycones, and in general the human foetus and neonate appear to have higher levels of drug metabolising enzyme systems than have the other species studied.

Animal species used in the study of xenobiotic metabolism

The vast number of existing animal species are divided into 17 phyla of which only two, namely the Arthropoda whose members include insects and spiders, and the Chordata which includes fish, amphibia, birds, reptiles and mammals, have been used to any significant extent in the study of xenobiotic metabolism. It is however significant that these two phyla contain animal species that are of interest to man from both an agricultural and a medical point of view (Williams, 1967). An idea of the number of extant animal species can be gained by considering that one class of the Chordata alone, the mammalia, contains over 4200

species including 1729 species of rodent and over 200 species of primates (see Williams, 1974), and that there are about twice as many species of bird as mammals and approximately 23,000 species of fish. Furthermore over 90% of all animals are invertebrates, 75% of them insects. The most common animals used in studying xenobiotic metabolism are rats, rabbits, guinea pigs, cats, dogs, and occasionally monkeys, although the type of xenobiotic under study may necessitate the use of other species, such as insects in the development of insecticides. In the development of selective weedkillers, xenobiotic metabolism in different plant species becomes important and the possible toxic effects of herbicides in man and animals must also be investigated.

The use of non-human primates in the study of xenobiotic metabolism

It is likely that the best animal model for studying drug metabolism in man would be found among those species most closely related to man. For this reason the fate of foreign compounds in non-human primates has been investigated. The 200 known species of primates are divided into the sub-orders Anthropoidea and Prosimii, each of which is further sub-divided into six families. The Anthropoidea include man, the Greater and Lesser apes such as the chimpanzee and gorilla, the Old World monkeys for example the rhesus monkey and the baboon, and lastly the New World monkeys which include tamarins and marmosets. The Prosimii include the Tarsiers, Lorises, Lemurs and Treeshrews. Napier and Napier (1967) suggested the primate species could be organised into a series according to their increasing complexity of structural and behavioural organisation (Fig 1.6). Table 1.11 shows a simplified classification of the primate species and indicates those species which have been used in the study of

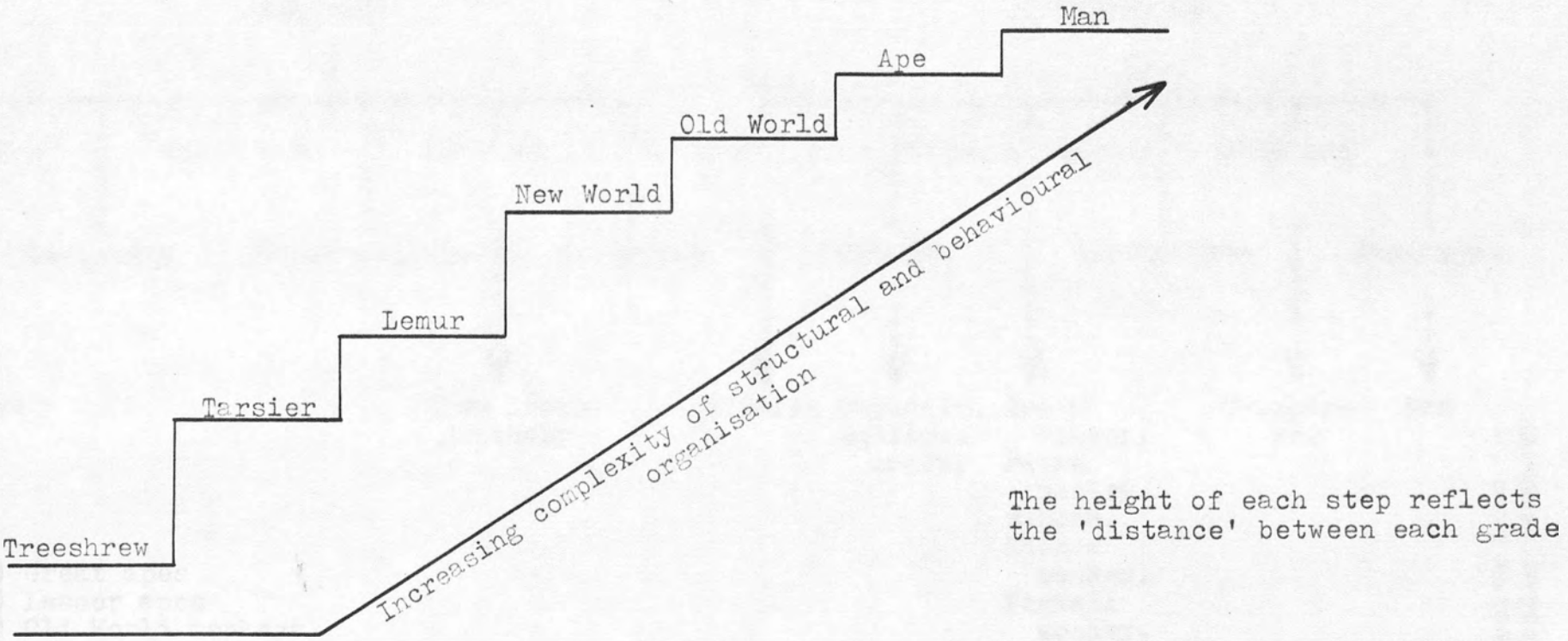


Fig 1.6 Successive grades of primate organisation (From Napier and Napier, 1967).

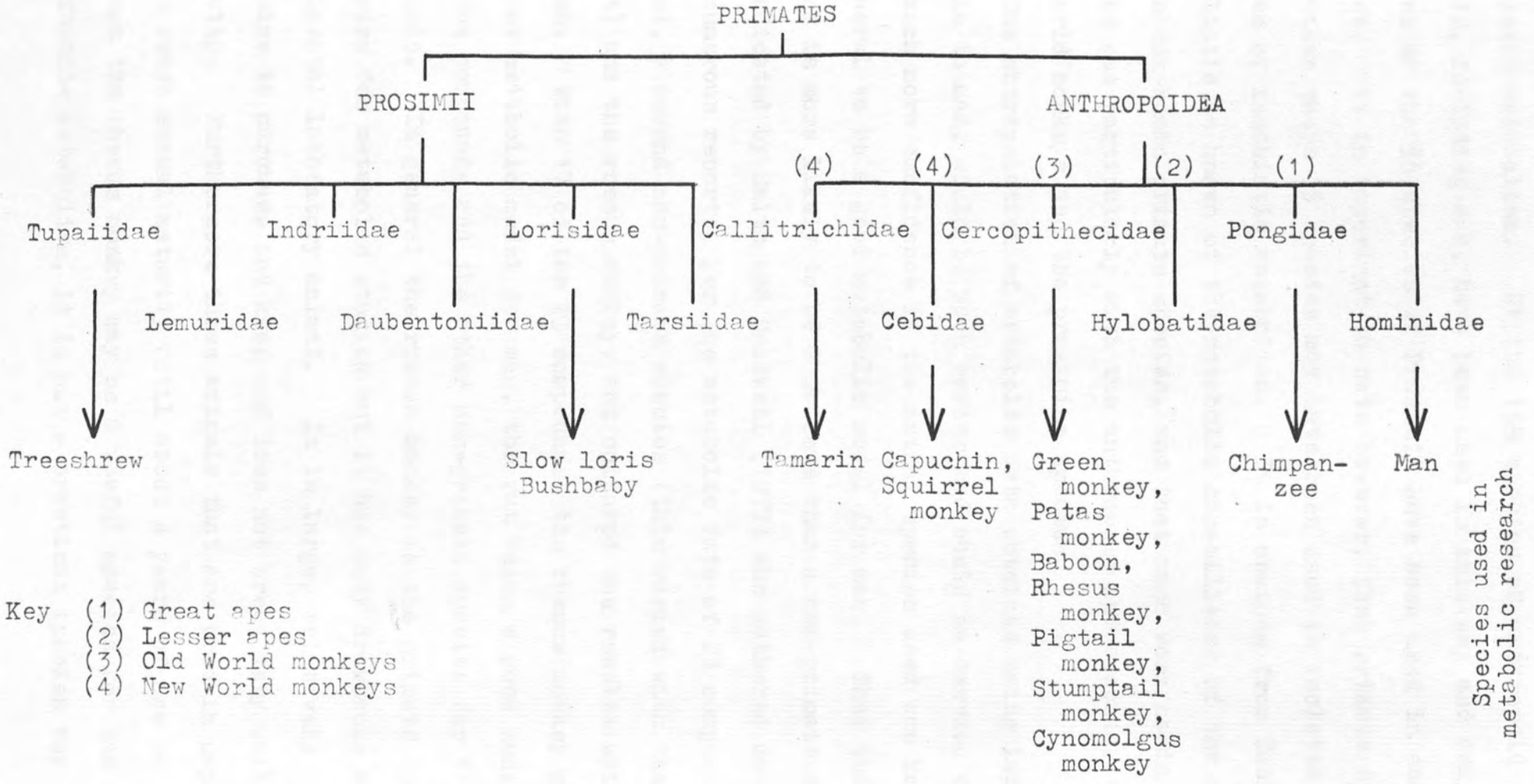


Table 1.11 Classification of primates used in drug metabolism research (Taken from Napier and Napier, 1967; Smith and Caldwell, 1977).

xenobiotic metabolism. Of the 159 species of anthropoid primates only 12, including man, have been used in this way and only 3 species of the 55 species of Prosimii have been used in such studies. It is important to note however, that primate species other than these 15 species may have been used in isolated studies of xenobiotic metabolism. It is obvious from Table 1.11 that little is known of the metabolic capabilities of the majority of the non-human primate species, and that much work needs to be carried out particularly with the anthropoid ape species, the New World monkeys and the prosimian species.

The extrapolation of metabolic data obtained using laboratory animals to man, would be much easier and could be carried out with much more confidence if the animal species used was known in general to be a good metabolic model for man. That this species is more likely to be a primate than a non-primate species was indicated by Smith and Caldwell (1977) who gathered data from numerous reports, for the metabolic fate of 23 compounds in the rat, a second non-primate species (this varied with the report) and the rhesus monkey, and compared the results with those for man. With 17 of the 23 compounds, the rhesus monkey provided the best metabolic model for man, the rat being a good model for 4 of the compounds and the other non-primate species for 5 compounds. In general the rhesus monkey is the primate species of choice for metabolic studies but it has many drawbacks as an experimental laboratory animal. It is large, relatively expensive to purchase and keep and does not breed very well in captivity. Furthermore those animals that are bred in captivity do not reach sexual maturity until about 4 years of age so although the rhesus monkey may be a useful species for the study of infantile metabolism, it is not a practical species for

establishing self-sustaining colonies on the scale used in the average laboratory. This means that the regular purchase of large numbers of these animals for medical research is necessary. However the main source of supply for the rhesus monkey is the Indian sub-continent which from April 1978, has effectively stopped the export of these animals for scientific research (Wade, 1978). There exists as a result, a demand amongst those companies which develop and market products for human use, as well as amongst research institutions, for a new laboratory species of non-human primate which can overcome the present problems outlined above. A strong candidate to fill this position is the New World monkey Callithrix jacchus, the common marmoset, which has already been shown to be an excellent animal model for teratogenic studies in man (Poswillo et al., 1972). However the xenobiotic metabolising capabilities of this animal have received relatively little attention.

The aim of the work presented in this thesis was to obtain detailed information about the fate of foreign compounds in the marmoset in order to compare the extent to which the known pathways of drug metabolism occurred with the extent to which they operated in other species, including man.

CHAPTER 2

Materials and Methods

Chapter 2 - Materials and Methods

Materials

The materials used in each study are described at the beginning of the relevant results chapter.

Animals

The rats used were of the Birmingham Wistar strain. Adult rats (250-300g) were fed on Heygates Oxoid pasteurised or modified cube diet with water *ad libitum* and maintained in conditions of constant temperature and light cycle. For neonatal studies litters were bred from previously unmated females. *In vivo* studies in the adult were carried out on female rats whereas males and females were used in the neonatal studies and for *in vitro* experiments.

The rabbits used were does of the New Zealand white strain. They were fed on modified Oxoid diet 18 (Styles Ltd., Bewdley) with water *ad libitum* and maintained in conditions of constant temperature and light cycle.

Common 'cotton-eared' marmosets, *Callithrix jacchus* were used. The animals were members of the Birmingham colony which was founded by one breeding pair in 1973. Since that date two feral animals have been imported (Shamrock Farms G.B. Ltd) and introduced into the colony in order to strengthen the genetic strain (see Fig 2.1). In their early attempts to set up marmoset colonies, Fitzgerald (1935) and Lucas *et al*. (1927, 1937) encountered many difficulties which are only to be expected with a new species of tropical primate. The animals were found to be susceptible to respiratory infections, parasites and dietary deficiencies. Eventually some of these problems

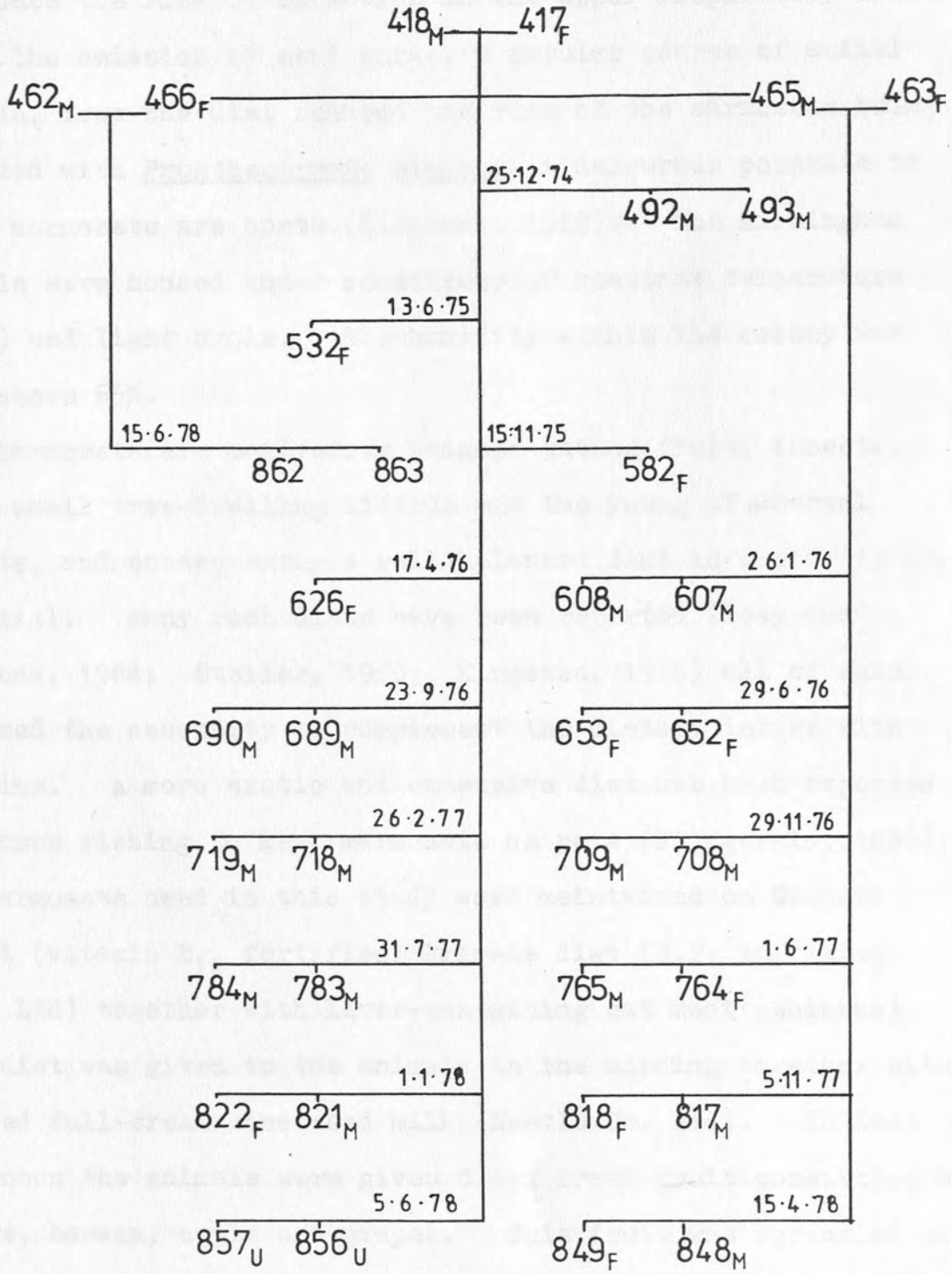


Fig 2.1 The family structure of the Birmingham marmoset colony.

Key: Numbers are code numbers of each individual
M - male F - female U - sex undetermined.

were solved; maintenance of the humidity above 60% was found to reduce the risk of infection in the upper respiratory tract, while the omission of meal worms, a popular source of animal protein, from the diet reduced the risk of the marmosets being infected with Prosthenorchus elegans, a dangerous parasite to which marmosets are hosts (Kingston, 1969). The Birmingham animals were housed under conditions of constant temperature (75°F) and light cycle. The humidity within the colony was kept above 65%.

Marmosets are omnivorous animals eating fruit, insects, eggs, small tree-dwelling lizards and the young of arboreal rodents, and consequently a well balanced diet in captivity is essential. Many such diets have been reported (Levy and Artecona, 1964; Stellar, 1960; Kingston, 1969) all of which stressed the necessity to supplement the dietary intake with vitamins. A more exotic and expensive diet has been reported for those wishing to keep marmosets as pets (Fitzgerald, 1935). The marmosets used in this study were maintained on Coopers Mazuri (vitamin B_{12} fortified) Primate diet (B.P. Nutrition, U.K., Ltd) together with liver-containing cat meat (Whiskas). This diet was given to the animals in the morning together with diluted full-cream condensed milk (Nestlé Co. Ltd). In late afternoon the animals were given diced fresh fruit consisting of orange, banana, apple and grapes. This fruit was sprinkled with a vitamin-mineral supplement, SA 37 (Intervet Lab. Ltd., Cambridge). They were allowed water ad libitum. Animals of both sexes were used in all experiments; a rota system was applied so that no animal was used too frequently in a given period of time. Neonatal animals were used when available from the three regular breeding pairs within the colony.

Dosing and collection of urine

Rats were dosed via a stomach tube made of polythene or transparent non-toxic vinyl. The external diameter of the tube was 3.0mm for the adult and 0.8mm for the neonatal animals. The form of the dose administered, together with the dosing vehicle, was the same as that for the marmoset, including Cytacon (Glaxo Lab. Ltd), a vitamin B_{12}-containing blackcurrant syrup, where necessary (see below). Adult rats were housed in cages designed for the separate collection of urine and faeces. Control 24h-urine samples were collected from animals to which the dosing vehicle only had been administered.

Rats aged 7-11 days were kept in an incubator at 30°C after dosing. Rats in this age group do not urinate without stimulation and so the urine was collected at two hourly intervals by exerting a gentle pressure on the lower abdomen of the animal, the droplets which formed being collected with a Pasteur pipette. After each urine collection the rats were fed a solution (0.3ml) of glucose (2% w/v) in aqueous cows milk (40% v/v).

Adult rabbits were dosed via a rubber stomach tube (external diameter 4.0mm), the dose form and vehicle being the same as that for the rat and marmoset. The animals were housed in large cages designed for the separate collection of urine and faeces.

Adult marmosets were dosed orally by placing the tip of a disposable syringe (1.0ml capacity) containing the dose firmly between the back teeth of the animal and slowly ejecting the dose to the rear of the throat. In the early work milk was used as the dosing vehicle but later Cytacon was found more suitable. Compounds were administered to the animals either as aqueous solutions mixed with Cytacon (0.5ml) or as suspensions

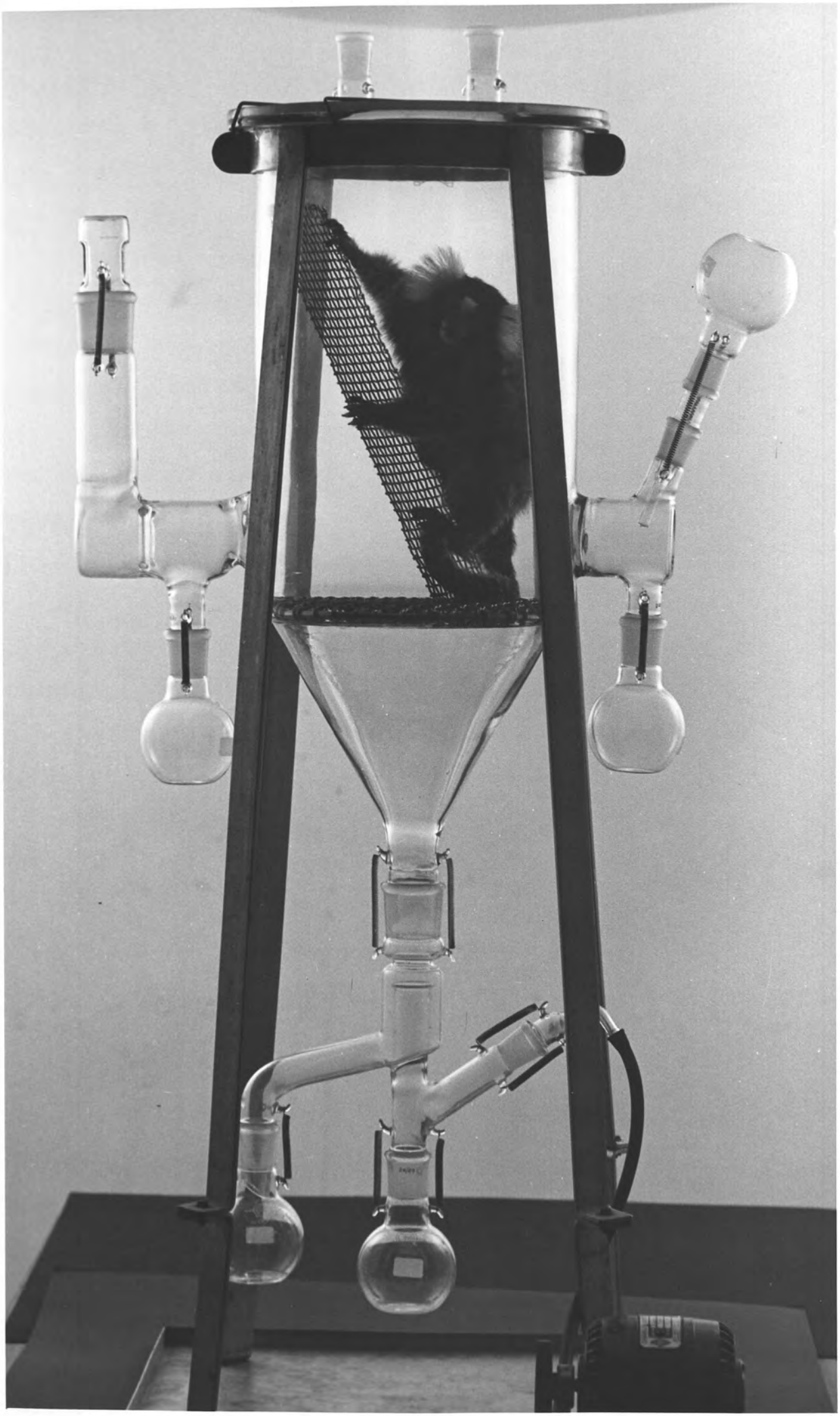
in diluted Cytacon. In the initial experiments the adult animals, after dosing, were housed in large cages which permitted the separate collection of urine and faeces. To avoid loss of urine, each cage was surrounded by a polythene sheet which was supported about 1" away from the cage with its lower edge within the metal funnel upon which the cage stood during the experiment. In later experiments the adult animals were housed in all-glass metabolism cages (R.B. Radley & Co. Ltd, Sawbridgeworth) which had a living chamber height of 12". Air was drawn through these glass cages using an electric pump (Charles Austen Pumps Ltd., Weybridge). A piece of steel gauze was placed inside the glass cages to act as a climbing frame for the animal, allowing it a greater freedom of movement. These glass cages allowed the separate collection of urine and faeces (Plate 1). The glass cages were designed originally for rodent use and although the marmosets could be fed while they were housed in them, they had great difficulty in obtaining water. Consequently the animals were removed from the glass cages at intervals and given a drink of diluted Cytacon.

Dose administration and urine collection in neonatal marmosets

The prolific breeding capabilities of the common marmoset, Callithrix jacchus have been recognised for a long time (Lucas et al., 1927; Fitzgerald, 1935; Kingston, 1969, 1972), the incidence of multiple births, especially twins, being high (Wislocki, 1939; Kingston, 1969). The breeding pairs within the Birmingham colony gave birth to either triplets or twins approximately every 5 to 6 months. Where triplets were born the weaker animal usually died within a few days due to the inability of the mother to feed more than two offspring. There-

Plate 1.

The apparatus used for the separate collection of urine and faeces from marmosets under study.



fore by day four post partum the mother was carrying only two babies and to avoid causing undue anxiety with the possibility of a total rejection of the young by the mother the twins were used on successive days so that the parents always had one baby to look after. Our experience in attempting to hand rear baby marmosets, usually the rejected triplet, showed that they died during the long over-night period. Consequently in order to avoid losing the healthy offspring, the neonatal experiments were carried out for a maximum period of six hours, usually between the times of 10am and 4pm. The baby animals were dosed in a similar fashion to that described above for the adult animals except that in all cases diluted milk was used as the dosing vehicle. After dosing, the baby marmosets were placed on filter papers supported by a wire disc in the bottom of a small glass desiccator. The top, which had a large hole left open to allow adequate air circulation, was then fitted to the desiccator which was lagged and kept away from draughts.

It was discovered that like baby rats, baby marmosets do not urinate without stimulation of some kind. Three methods of stimulation were found to be suitable for these animals.

(i) The exertion of gentle pressure on the lower abdomen, a similar procedure to that applied in the rat.

(ii) Gently touching the genitalia with the tip of a 'rounded-off' Pasteur pipette.

(iii) Touching the genitalia with a piece of dampened tissue onto which the urine was directly absorbed.

Method (iii) was by far the most successful probably because it mimicked the natural method of stimulation used by the parents. The disadvantage lay in the necessity to extract the urine from the tissue. Urine was collected every two hours

after dosing. At 2 and 4h after dosing, the baby animals were given a drink of diluted condensed milk (0.4ml), and after 6h they were returned to their parents who, in no instances, showed any signs of rejecting them (see Kingston, 1972).

Chromatography

Paper chromatography was carried out on Whatman 3MM paper. Chromatograms were developed for about 15h by the descending method or for about 24h by the ascending method. The choice of technique depended upon the compound under study. Thin layer chromatography was carried out on either glass plates coated with a layer of silica gel G (E. Merck A-G, Darmstadt, W. Germany) 0.3mm thick or on aluminium sheets pre-coated with silica gel $60F_{254}$ (E. Merck A-G) 0.2mm thick.

Solvents

The solvents listed below were used

A - Butan-1-ol-ethanol-water-acetic acid (30:10:10:1 by vol)

B - Chloroform-cyclohexane-acetic acid (8:2:1 by vol)

C - Benzene-acetone-acetic acid (6:2:1 by vol)

D - Benzene-acetone-acetic acid (2:2:1 by vol)

E - Butan-1-ol-ethanol-aq. NH_3 (sp.gr. 0.88)-water (10:10:1:4 by vol)

F - Butan-1-ol-acetic acid-water (4:1:2 by vol)

G - Ethanol-water (4:1 v/v)

H - Propan-2-ol-water (3:1 v/v)

I - Butan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (10:1:1 by vol)

J - Butan-1-ol-water-benzene-acetic acid (6:6:1:2) (prepared by shaking the mixture for 5 min and using the upper layer which separated).

K - Butan-1-ol-acetic acid-butylacetate-water (120:25:10:50 by vol)

L - Butan-1-ol-acetic acid-water (4:1:1 by vol)

M - Propan-2-ol-water-aq. NH_3 (sp.gr. 0.88) (20:1:2 by vol)

N - Butan-1-ol-ethanol-acetic acid-water (10:10:1:4 by vol)

O - Benzene-diethylether-acetic acid-methanol (120;60:18:1 by vol)

P - Butan-2-one saturated with aqueous 2M-NH_3 (prepared by shaking the ketone with aqueous ammonia (2:1 v/v) for 2h and using the upper layer which separated).

The following detecting reagents were used

I - Naphthoresorcinol for the detection of glucuronides (Elliot et al., 1959). Chromatograms were sprayed with a solution of 2% (w/v) 1,3-dihydroxynaphthalene in 33% (w/v) aqueous TCA followed by heating for 5 min at 120°C. Glucuronic acid conjugates show up as blue spots on a pink background.

II - Diazotised-p-nitroaniline for the detection of phenols. A mixture of 3% (w/v) p-nitroaniline in 8% (w/v) HCl (25ml) and 5% (w/v) sodium nitrite (1.5ml) was sprayed onto the chromatograms, followed by 20% (w/v) sodium carbonate. Phenols show up as coloured spots on a light yellow background.

III - 3% (w/v) Ferric chloride in ethanol
Phenols show up as deep coloured spots on a yellow background.

IV - Ferric chloride/Ferricyanide for the detection of phenols (Barton et al., 1952). A mixture of equal volumes of 5% (w/v) aqueous ferric chloride and 5% (w/v) aqueous potassium ferricyanide was sprayed onto the chromatograms. Phenols show up as blue spots on a yellow background.

V - Ammoniacal silver nitrate for the detection of dihydroxybenzoic acids (Bray et al., 1950b). Aqueous 0.1M silver

nitrate (25ml) was added to aqueous ammonia (sp.gr. 0.88, 8ml) and the volume adjusted to 50ml with water. Dihydroxybenzoic acids show up as coloured spots on a beige background.

VI - The rhodizonate reagent of Schneider and Lewbart (1956) was used for the detection of sulphates which show up as yellow spots against an orange/pink background.

VII - 10% (w/v) p-Dimethylaminobenzaldehyde in acetic anhydride (Gaffney et al., 1954). Both papers and thin layer plates were sprayed with the reagent and then heated for 8-10 min at 120°C. Hippuric acid showed up as a bright orange spot on a pale yellow background.

VIII- p-Dimethylaminocinnamaldehyde for the detection of aromatic amines and their conjugates (Bridges et al., 1965). Chromatograms were sprayed with a solution of 0.2% (w/v) p-dimethylaminocinnamaldehyde in 2M HCl, diluted 1:4 (v/v) before use with ethanol. Aromatic amines showed up immediately as pink/purple spots on a very pale pink background. With compounds in which the amino group was conjugated the colour developed slowly (e.g. 6-24h for N-acetyl conjugates).

IX - Chloroplatinic acid reagent (Toennies and Kolb, 1951). This reagent was used as modified by Barnsley et al., (1964). Compounds containing divalent sulphur showed up as the paper dried, as white areas on a pink background. Sulphoxide derivatives appeared as yellow spots after 8-24h.

X - Potassium dichromate - silver nitrate reagent (Knight and Young, 1958). This reagent was used for the detection of divalent sulphur-containing compounds which show up as cream or yellow areas on a russet coloured background.

XI - Ninhydrin reagent (Moffat and Lytle, 1959).
This was used to detect S-substituted cysteine derivatives which show up as dark bluish-grey spots on a pale background.

XII - 3% (w/v) aqueous selenium dioxide for the detection of phenols (Mitchell and Waring, 1978). Chromatograms were sprayed with the solution and then heated for 15-20 min at 120°C. Phenols appeared as brown spots on a pale background.

Gas-Liquid Chromatography

Gas-liquid chromatography was carried out using a Pye Series 104, dual column chromatograph with a flame ionisation detector (FID). The columns consisted of acid washed silanized Chromosorb W, 30-60 mesh, coated with 15% (w/w) high vacuum silicone grease or acid washed silanized Chromosorb W, 60-80 mesh coated with 5% (w/w) OV101. In both cases the carrier gas used was argon at a flow rate of 40ml/min. The temperature conditions employed with each column are described in the relevant results chapters.

High Pressure Liquid Chromatography

Reverse-phase high pressure liquid chromatography was carried out using two high pressure solvent pumps (Waters Associates Inc., Milford MA) controlled by a Waters Associates Model 660 solvent programmer. The column used was a μ Bondapak C_{18} column of dimensions 30cm x 4mm diameter (Waters Associates), the packing consisting of a monomolecular layer of octadecyltrichlorosilane bonded to fully porous rigid beads of less than 10 microns diameter (μ Porasil). Metabolites were detected

using a CE 212 Variable wavelength UV monitor (Cecil Instruments Ltd., Cambridge), connected to a Servoscribe 1S chart recorder. The eluting solvents used were methanol (HPLC grade, Rathburn Chemicals Ltd., Walkerburn) and a solution of acetic acid (1% v/v) in glass distilled water, at a combined flow rate of 2ml/min. The solvent program used is described in the relevant results chapter.

Mass Spectrometry

Mass spectra of standard compounds and isolated metabolites were determined by Dr. J.R. Majer of the Department of Chemistry, University of Birmingham, using an A.E.I MS9 high resolution instrument.

Liquid Scintillation Counting

All quantitative determinations of radioactivity were carried out using a Philips automatic liquid scintillation analyser. The results were corrected for quench by the external standard ratio (ESR) method. All counting was carried out at 11°C for 10 min or until 10^4 counts had been recorded. Aliquots (0.05-1.0ml) of urine, standard solutions or aqueous faecal extracts were made up to a volume of 1ml with water and mixed with a scintillation cocktail (10ml) for counting. The scintillation cocktail used was similar to that described by Patterson and Greene(1965) and consisted of a solution of 2,5-diphenyloxazole (PPO) (4.0g/l) and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) (0.12g/l) in toluene, which was mixed with triton X-100 (2:1 v/v).

Determination of radioactivity in blood samples

Blood samples taken from the tail vein of marmosets, were placed in pre-weighed, stoppered scintillation vials and the sample weight determined. Soluene-350 tissue solubilizer (0.5ml) (Packard Inst. Ltd) was then added and the samples digested for 48h at 40°C. The contents were de-colourised by the addition of hydrogen peroxide (100vols). The scintillant was then added and the radioactivity counted as described above.

Measurement of radioactivity by combustion

The measurement of radioactivity in freeze-dried faeces was carried out by the exhaustive combustion of an aliquot (200mg) in a Harvey Biological Oxidiser (ICN Tracer Lab. Ltd., Horsham, Surrey), for 4 min. The $^{14}CO_2$ so produced was trapped in 15ml of a scintillation cocktail containing the following components per litre: toluene, 430ml; methanol, 300ml; 2-phenethylamine, 270ml; PPO, 5g; dimethyl POPOP, 0.5g; (Peterson, 1969). The samples were counted as described above and the results corrected for quenching by the channels ratio method.

The distribution of labelled metabolites on chromatograms and their quantification

Samples of urine or urine extracts containing radioactive compounds were applied to paper and/or to thin layer plates. The chromatograms were developed in a suitable solvent and the distribution of labelled metabolites was determined using a Packard radiochromatogram scanner model 7200.

The quantification of labelled metabolites on paper chromatograms was carried out by cutting the paper into strips either ½" or 1" wide, depending on the resolution, starting

below the origin and proceeding to the solvent front. Each small strip was then cut in 5 or 6 further pieces which were placed in the bottom of a scintillation vial; 90% methanol (1ml) was then added to the vial which was stoppered and allowed to stand for at least 24h to allow an efficient elution of the radioactive components from the paper. The scintillant (10ml) was then added and the stoppered vials were thoroughly shaken. After keeping for 48h, with occasional shaking, the radioactivity was counted. A similar technique was employed for the quantification of the labelled metabolites separated by thin layer chromatography, except that in this case, the silica surface was scraped off the plate in ½" or 1" sections. Where possible the extraction efficiencies were checked using authentic compounds. The distribution of radioactivity along the chromatograms was plotted in the form of histograms.

Autoradiography

Dried chromatograms containing labelled metabolites were exposed to X-ray film (Kodirex - Kodak Ltd., London) for periods of up to 3 months prior to development with a '19B' developer solution.

Determination of Bilirubin-UDPGT activity

The activity of bilirubin-UDPGT in liver and kidney homogenates from rats and marmosets was estimated by the method of Van Roy and Heirwegh (1968).

Protein determinations

These were carried out using the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Glucuronide estimation

Glucosiduronic acid was measured using the modified carbazole reaction as described by Bitter and Ewins (1961).

Sulphate determination

The sulphate content of urine samples was measured using the titrimetric method of Morrison (1973).

The determination of aromatic amines

Primary aromatic amines were determined by the colorimetric method of Bratton and Marshall (1939) using N-(1-naphthyl)-ethylenediamine dihydrochloride as the coupling agent.

CHAPTER 3

The metabolism of benzoic acid

Chapter 3 - The metabolism of benzoic acid

Introduction

Conjugation of ingested benzoic acid with glycine to form hippuric acid was the first detoxication mechanism discovered (Keller, 1842). Investigations since then have shown that in the majority of mammals examined, administered benzoic acid is excreted as hippuric acid and benzoylglucuronide; in some species (e.g. rats and guinea pigs) small amounts of hydroxybenzoic acids are also excreted (Acheson and Gibbard, 1962). In 1877, Jaffe discovered that the hen converted benzoic acid to ornithuric acid (N^2:N^5-dibenzoylornithine) a conversion now known to occur in many species of birds and reptiles. The conjugation of aromatic acids with arginine and glutamine in arachnids, and with agmatine in the scorpion was reported by Hitchcock and Smith (1964, 1966). It is considered that the arginine and glutamine conjugates are primary metabolites and that the agmatine and glutamic acid conjugates are formed from these by further metabolism. A glutamic acid conjugate has also been found in the urine of Indian fruit bats which had been dosed with benzoic acid (Idle *et al.*, 1975).

In mammalian species the proportion of administered benzoic acid excreted conjugated with glycine or glucuronic acid varies not only with species but also with the dose level used and the age of the animal.

Species variations in the metabolic fate of benzoic acid

Bridges *et al.* (1970) studied the metabolic fate of [^{14}C] benzoic acid in man and 20 other species of animal. The results relevant to the present study, taken from their report, are shown

in Table 3.1.

Species	Dose level (mg/kg body wt.)	Percentage of radioactive components excreted in urine as			
		Benzoic acid	Hippuric acid	Benzoyl-glucuronide	Benzoyl-ornithine
Man	1	0	100	0	0
Rhesus monkey	20	0	100	0	0
Squirrel monkey	50	16	82	tr	0
Capuchin+	50	0	100	tr	0
Rabbit	49	0	100	0	0
	200	tr	98	2	0
Rat	50	tr	99	tr	0
Mouse	56	tr	95	5	0
Cat	51	tr	100	0	0
Dog	51	0	82	18	0
Ferret	50	9	70	22	0
	198	9	47	44	0
	400	22	30	49	0
Chicken	50	22	21	3	54
Pigeon	50	15	84	1	0
Turtle+	50	10	16	2	72
Gecko	19	3	6	6	85
Indian fruit bat++	50	12,30	tr	88,70	0

Data taken from Bridges et al. (1970)
Where three or more animals were used the results are expressed as averages. Where only one (+) or two animals (++) of a species were used the individual results are given. Animals of mixed sexes were used.

Table 3.1 Metabolites of [^{14}C]benzoic acid in urine of various species

This comparative study is particularly useful since in all

but three species the dose level was standardised. Thus at a dose level of 50mg/kg body weight administered benzoic acid was excreted by rodents, the cat, the rabbit and the capuchin monkey almost exclusively as hippuric acid whereas in the dog and ferret significant quantities of benzoylglucuronide were excreted; small but significant quantities of this metabolite were also excreted by mice.

In the Indian fruit bat benzoylglucuronide was the major metabolite excreted accounting for not less than 70% of the radioactive components excreted in the urine. The data in Table 3.1 suggest that in this species the remainder of the administered dose was excreted unchanged, but Idle et al. (1975) later showed that apart from benzoylglucuronide the only other product excreted by this species was benzoylglutamate. As mentioned earlier, the Indian fruit bat also excretes 1-naphthylacetic acid (Dixon et al., 1974, 1977a) and diphenylacetic acid (Dixon et al., 1977c) chiefly as their glucosiduronic acids.

With the exception of the cat, the carnivorous species studied excreted substantial quantities of administered benzoic acid as benzoylglucuronide whereas those herbivorous species studied excreted a similar dose almost entirely as hippuric acid. The results reported for the metabolic fate of benzoic acid in Indian cattle (Gupta, 1932), the goat (Ringer, 1911), and sheep (Magnus-Levy, 1907), fit into this general pattern of metabolism. Williams (1959) suggested that the metabolic fate of benzoic acid in omnivorous species might occupy an intermediate position between that in herbivorous and carnivorous animals. Later results did not fit into this simple scheme. Of those species listed in Table 3.1 only the primates can be classed as omnivorous, and of these only two have been studied at a dose level which is

comparable to that in the other species. These two species, the squirrel monkey and the capuchin monkey, both require omnivorous diets when kept in captivity (Napier and Napier, 1967) and the results indicate that both excreted traces of administered benzoic acid as benzoylglucuronide and can hardly be described as occupying the intermediary position suggested. It is now firmly established that the effect of the diet on the metabolic fate of benzoic acid in various species is mediated through the dietary supply of glycine for hippuric acid formation.

In the chicken, in which glycine is an essential amino acid, the major metabolite of administered benzoic acid was ornithuric acid whereas hippuric acid was the main metabolite in the pigeon. Baldwin et al. (1960) studied the metabolic fate of benzoic acid in different avian species and showed that in the Galliformes and Anseriformes ornithuric acid, and in the Columbiformes hippuric acid, were the only amino acid conjugates excreted. In all these Orders varying quantities of the administered benzoic acid were excreted as benzoylglucuronide. The Passeriformes and Psittaciformes on the other hand excreted no amino acid conjugates of benzoic acid, only benzoylglucuronide. Ornithuric acid was also the major metabolite excreted by reptiles dosed with benzoic acid (Table 3.1) which, when considered with the avian results, suggests that the conjugation of benzoic acid with ornithine may be correlated with the excretion of uric acid as the end product of amino acid metabolism. Those reptiles which have the most uricotelic nitrogen metabolism (e.g. lizards such as the gecko) would be expected therefore to use mainly ornithine for detoxication but more primitive reptiles such as the turtle which are partly ureotelic and partly uricotelic would use both ornithine and glycine for amino acid conjugation

as indicated in Table 3.1.

Effect of dose level on the metabolic fate of benzoic acid in different species

Bridges et al. (1970) (see Table 3.1) showed that by increasing the dose level of administered benzoic acid in the ferret, the proportion of the excreted dose conjugated with glucuronic acid increased while that conjugated with glycine decreased. Since the recoveries of the different doses were similar, these data suggest that the ability of the ferret to form hippuric acid is limited by the supply of glycine, while the ability to form benzoylglucuronide is under no such limitations. In the rabbit no significant difference in the metabolic fate of benzoic acid was seen at the different dose levels employed. The increased importance of benzoylglucuronide formation at higher dose levels of administered benzoic acid has been shown in sheep (Magnus-Levy, 1907), in the pig (Csonka, 1924), and in man (Magnus-Levy, 1907; Dakin, 1909-10; Quick, 1931).

Csonka (1924) showed that in the pig the proportion of a high dose of benzoic acid excreted as benzoylglucuronide could be decreased by feeding glycine or a glycine-rich protein such as gelatin, suggesting that the supply of glycine for hippuric acid synthesis was the main factor in determining the metabolic fate of benzoic acid in this species. Furthermore the work of Griffith and Lewis (1923) had shown that the simultaneous administration of glycine and a very large dose of sodium benzoate (1g/kg body weight) to the rabbit caused an increase in the rate of hippuric acid excretion above that seen when sodium benzoate was administered alone. Bray et al. (1951a) calculated

that the excretion of hippuric acid by rabbits which had received sodium benzoate, took place at a constant rate varying with different animals from 115-166mg/h and showed that the administration of glycine increased this rate. Simkin and White (1957a, b) showed that the administration of benzoic acid to the rat and rabbit caused a decrease in the concentration of free glycine in the blood of both species and in the liver of the rat (the liver of the rabbit was not studied). Quick, (1931) reported that the rate of glycine mobilisation was species dependent and calculated that in the dog the rate was 3.5, in man, 9, in the pig, 15 and in the rabbit 24mg/kg/h while Bray et al. (1951a)reported that the formation of benzoyl-glucuronide in the rabbit followed the kinetics of a first-order reaction with a velocity constant of $0.08h^{-1}$. Using these values, together with derived equations, Bray et al. (1951a) were able to calculate the theoretical percentage of benzoic acid that might be expected to be excreted as benzoylglucuronide in different animal species at a given dose level. These percentages correlated well with the experimentally determined values which showed that in those species where the rate of glycine mobilisation was highest the percentage of an administered dose of benzoic acid (500mg/kg body weight) excreted as benzoyl-glucuronide was lowest.

Bray et al. (1952b)derived a mathematical model to explain the metabolic fate of benzoic acid at different dose levels. They showed that in general where a dose is metabolised to two products only (e.g. hippuric acid and benzoylglucuronide in the case of benzoic acid) the proportion of that conjugate formed by a first-order reaction only (i.e. benzoylglucuronide), increases with increasing dose levels while that formed by zero-

order reaction (i.e. hippuric acid) decreases. This only applies at dose levels above a critical value and not at very low dose levels where, for instance, glycine conjugation can adequately cope with the whole of the administered compound.

Therefore both the experimental and derived kinetic models suggest that at a given dose level of administered benzoate, the proportions of the dose excreted as hippuric acid and benzoylglucuronide by different animal species is dependent upon the availability of free glycine for conjugation in each species and that in any one species, the proportion of a relatively large dose of benzoic acid excreted conjugated with glucuronic acid increases with increasing dose level.

Effect of age on the metabolic fate of benzoic acid

Whereas many adult mammalian species conjugate benzoic acid almost entirely with glycine (Bridges *et al.*, 1970), Vest (1959) reported that the human infant was less able to carry out this conjugation reaction. Alimova (1958) reported the detection of hippuric acid in the urine of both full-term and premature infants from the second post natal day onwards and that throughout the first year of life urinary hippuric acid gradually increased with age.

Baines (1975) studied the metabolism, *in vivo*, of sodium [^{14}C]benzoate in 5 and 10 day old rats and found that hippuric acid constituted a markedly lower proportion of the radioactive components in the urine of the young rats than in their adult counterparts. Furthermore two labelled unidentified metabolites, one of which was not detected in the urine of adult rats, were excreted by these young rats.

This chapter describes the investigations of the metabolic

fate of sodium [^{14}C]benzoate in the marmoset at different dose levels. The metabolic profile of the compound was also examined in the neonatal marmoset and compared with that found in similarly dosed neonatal rats.

Materials and Methods

Materials

Sodium [carboxyl-^{14}C]benzoate

Two ampoules of [carboxyl-^{14}C]benzoic acid (100μCi; 465μCi/mg) were obtained from the Radiochemical Centre Ltd. The contents of the ampoules were diluted with unlabelled benzoic acid as follows:

(i) A solution of unlabelled sodium benzoate was prepared by adding benzoic acid (2.00g) to water (8ml) followed by an equimolar quantity of $NaHCO_3$. Aliquots of this solution were used to transfer the contents of one ampoule to a standard flask; the pH was adjusted to 7.0 and the volume to 10ml. The activity of this solution was 7.1μCi/ml (0.036μCi/mg benzoic acid) giving a radioactive recovery from the ampoule of 71%.

(ii) A solution of unlabelled sodium benzoate was prepared as described above using 50mg of benzoic acid in a final volume of 10ml. An aliquot (3.0ml) of this solution was added to the second ampoule and allowed to stand overnight. After transferring the sample to another container, a further aliquot (1.0ml) of the unlabelled solution was used to wash out the ampoule. The extracts were then combined. The final activity of the solution was 27.8μCi/ml (5.56μCi/mg benzoic acid) giving a radioactive recovery from the ampoule of 111%.

Using these solutions of differing specific activity suitable quantities of radioactivity could be administered to both adult and neonatal animals at dose levels of benzoic acid ranging from 1 to 100mg/kg body weight.

Benzoyltaurine (sodium salt) was a kind gift from Dr. P. Millburn. The melting point of the sample supplied was

280-285°C.

All other chemicals used in this study were freely available from the laboratory stores.

Methods

Administration of dose

Sodium [^{14}C]benzoate was administered as an aqueous solution either mixed with Cytacon or milk as described in the general methods (p.81). The dose levels reported in this chapter, unless otherwise specifically stated, refer to the amount (mg) of benzoic acid administered per kg body weight regardless of whether the compound was administered as the free acid or as its sodium salt.

Chromatography

Paper chromatography was carried out using the descending technique in solvents A, E and F while glass plates coated with a thin layer of silica gel G (see p.85) were developed in solvents A, B, C and E. Aluminium backed 60F_{254} thin layer chromatography plates, used for the detection of benzoyltaurine were developed in solvent D. The chromatographic properties of metabolites and reference compounds as determined in these different systems are shown in Table 3.2. The use of either solvents A and B to develop glass silica gel G-coated thin layer plates gave a good resolution of benzoylglucuronide, hippuric acid and benzoic acid and these two systems were used for the quantitative work (see p.91 and 106).

Identification of metabolites

a) Hippuric acid was detected on chromatograms by its

Compound	Descending paper chromatography in solvent A	E	F	Thin layer chromatography in solvent A(a)	B(a)	C(a)	E(a)	D(b)
Reference compounds								
Hippuric acid	0.83	0.55	0.85	0.70	0.17	0.51	0.57	0.51
Benzoic acid	0.89	0.58	0.88	0.85	0.87	0.89	0.62	0.79
Benzoyltaurine	ne	ne	ne	ne	ne	ne	ne	0.15
Urinary metabolites								
+Benzoylglucuronide	0.55	0.61	0.00	0.42	0.00	nd	0.45	0.16
Hippuric acid	0.85	0.57	0.83	0.71	0.17	0.51	0.59	0.50
++Benzoic acid	0.92	0.59	0.89	0.85	0.87	0.86	0.62	0.79

(a) Glass plates coated with silica gel G

(b) Aluminium plates pre-coated with silica gel $60F_{254}$

+ detected by reagent I and liquid scintillation counting

++ detected by co-chromatography

ne - not examined nd - not detected.

Table 3.2 Chromatographic properties (R_F values) of reference compounds and metabolites of benzoic acid

characteristic colour reaction with detecting reagent VII. It was identified in the urine of animals dosed with sodium [^{14}C]-benzoate by its R_F value obtained by co-chromatography using an authentic sample of hippuric acid.

b) Benzoylglucuronide was detected by reagent I. Where more than one positive spot appeared, due to the presence of normal urinary glucuronides, the benzoylglucuronide was identified by radiochromatogram scanning and the extraction of the spray positive areas of the chromatogram (usually thin layer) for liquid scintillation counting. Further identification of benzoylglucuronide was obtained by digesting the urine samples from dosed animals with β-glucuronidase type H-1 (Helix pomatia; Sigma Chemical Co.Ltd.) in an equal volume of 0.4M sodium acetate buffer pH 5.0 at 37°C for 24-36h. A control digest containing D-saccharic acid-1,4-lactone, a potent inhibitor of the enzyme β-glucuronidase (Levvy, 1952) was incubated at the same time. The disappearance on β-glucuronidase treatment of the naphtho-resorcinol positive spot, together with a loss of radioactivity in the region of the plate that gave a positive reaction with this reagent before digestion and a corresponding increase in the quantity of labelled benzoate, confirmed the identification of benzoylglucuronide.

c) Benzoic acid was identified by its R_F value obtained by radiochromatogram scanning and liquid scintillation counting. Further evidence for the presence of [^{14}C]benzoate in the urine of dosed animals or in β-glucuronidase digests was obtained by preparing autoradiographs of samples of urine and β-glucuronid-ase-treated urine using sodium [^{14}C]benzoate as the reference compound. Co-chromatography of the urine samples with sodium [^{14}C]benzoate was carried out routinely.

Detection of benzoyltaurine

Benzoyltaurine when chromatographed on aluminium backed $60F_{254}$ thin layer plates in solvent D, appeared as a blue quenching spot on a yellow fluorescent background when the plates were viewed under light of wavelength 254nm. The R_F value in this solvent was very similar to that of benzoyl-glucuronide (Table 3.2) (see also Idle et al., 1975) and it was necessary to treat the urine with β-glucuronidase to hydrolyse any excreted benzoylglucuronide before attempting the identification of benzoyltaurine. After such treatment of the urine from dosed animals no quenching spot was observed in the area occupied by benzoylglucuronide in the untreated urine.

The gas-chromatographic method described by Kirkland (1960) for the separation of sulphonic acids as their sulphonylchloride derivatives was also tried for the identification of benzoyl-taurine. The reference compounds benzene sulphonic acid, toluene p-sulphonic acid and benzoyltaurine were converted to the corresponding sulphonylchlorides and the derivatives were applied to the gas-liquid chromatographic column described by Kirkland (1960) except that a 15% rather than a 20% column coating was used; a sample of authentic toluene p-sulphonyl-chloride was also examined. The retention times for benzene sulphonylchloride, toluene p-sulphonylchloride, prepared and authentic samples, and benzoyltaurine sulphonylchloride were 2.0, 3.6, 3.7 and 26 minutes respectively. No success however was achieved in extracting an authentic sample of benzoyltaurine which had been applied to the thin layer plate described above so the method was of no use for the detection of benzoyltaurine which might have been present in the urine only in traces as a metabolite of benzoic acid.

Quantification of metabolites

For every urine sample collected from animals which had received sodium [^{14}C]benzoate three identical silica gel G thin layer chromatography plates were run in solvent A and a similar set in solvent B. The radiochemical distribution on each plate was determined by radiochromatogram scanning. One plate from each set was then used to detect hippuric acid, a second to detect benzoylglucuronide and the third plate was used to quantify the metabolites as follows.

Urinary metabolites of sodium [^{14}C]benzoate separated by chromatography on glass plates coated with a thin layer of silica gel G and developed in either solvent A or B were quantified by the procedure described in the general methods (p.91). Those thin layer plates run in solvent A were scraped into 0.5cm sections starting 1.0cm below the origin and proceeding to the solvent front (for all plates this was about 15cm above the origin). Those thin layer plates developed in solvent B were scraped into 0.5cm sections starting again 1.0cm below the origin and proceeding up to 5cm above the origin. Above this point, the remainder of the plate was scraped into 1.0cm sections since only benzoate was present in this region.

Results

The metabolic fate of sodium [^{14}C]benzoate in the adult rat and marmoset

The proportions of the radioactive components in the urine of animals dosed with sodium [^{14}C]benzoate as determined in solvents A and B were in good agreement as indicated by the histograms obtained in a typical experiment (Figs 3.1 and 3.2). Table 3.3 is a summary of the data obtained for the distribution of radioactive components in the urine of adult rats and marmosets to which sodium [^{14}C]benzoate had been administered at three different dose levels. The values shown are averages of the results from both chromatographic systems. When the benzoate was administered at a dose level of 1mg/kg body weight, the urine was collected for 6h after dosing since Bridges *et al.* (1970) reported that 97% of a similar dose in man was excreted within 4h. This shorter collection period had the advantage of reducing the time in which any benzoylglucuronide might hydrolyse.

The results show that the rat excreted administered sodium [^{14}C]benzoate almost entirely as hippuric acid even at dose levels of 100mg/kg body weight. The relative proportions of the radioactive compounds excreted were not significantly different over the range of dose levels employed.

The marmoset, even at the low dose level of 1mg/kg body weight, excreted an appreciable proportion of the dose conjugated with glucuronic acid, the major metabolite being hippuric acid with only small amounts of unchanged benzoate. At a dose level of 40mg/kg body weight a wide variation in the proportions of the radioactive components excreted was evident. In all the animals

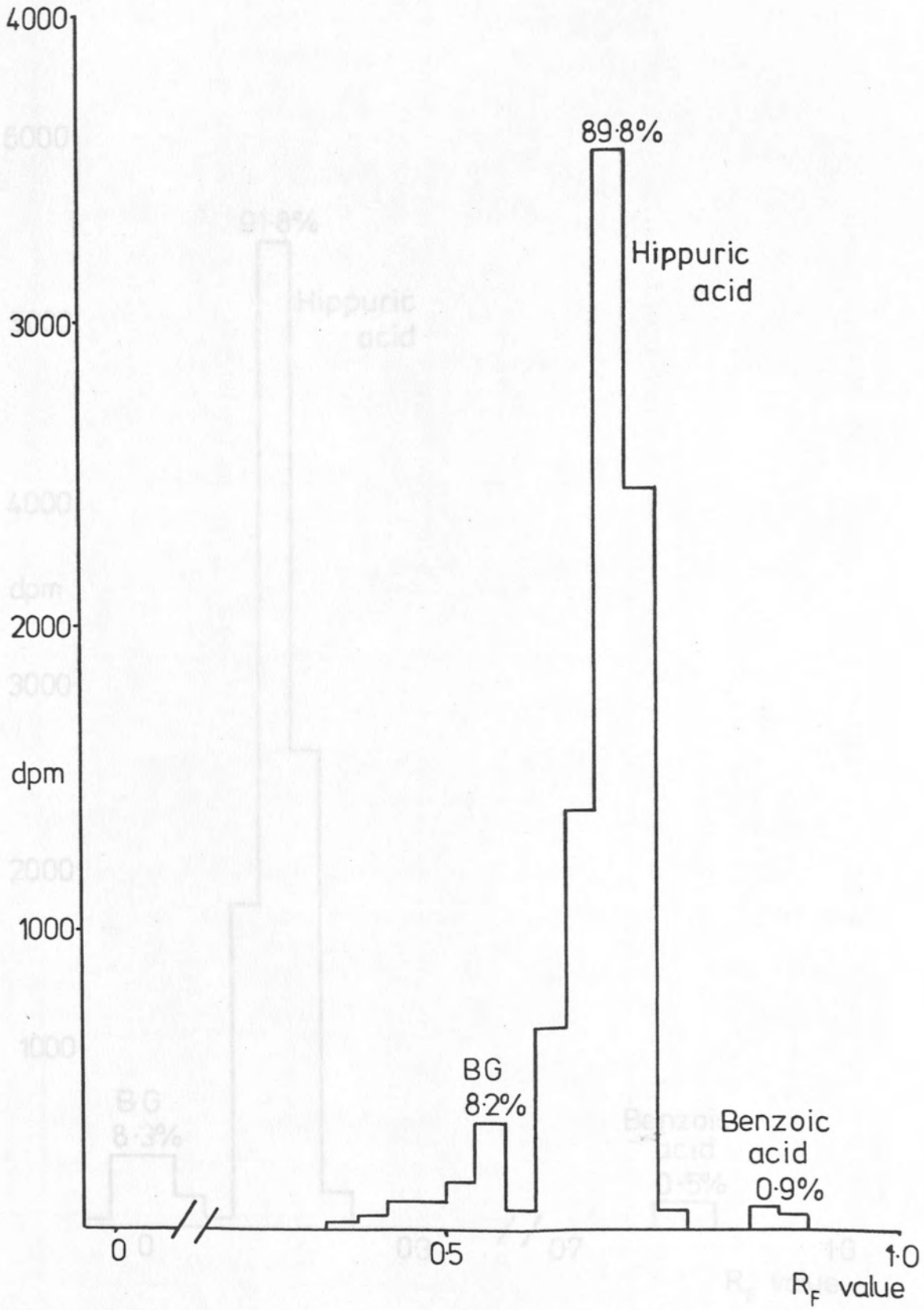


Fig 3.1 Distribution of radioactivity along a silica gel coated thin layer chromatography plate developed in solvent A of urine from a marmoset dosed with sodium [^{14}C]benzoate. (BG = benzoylglucuronide)

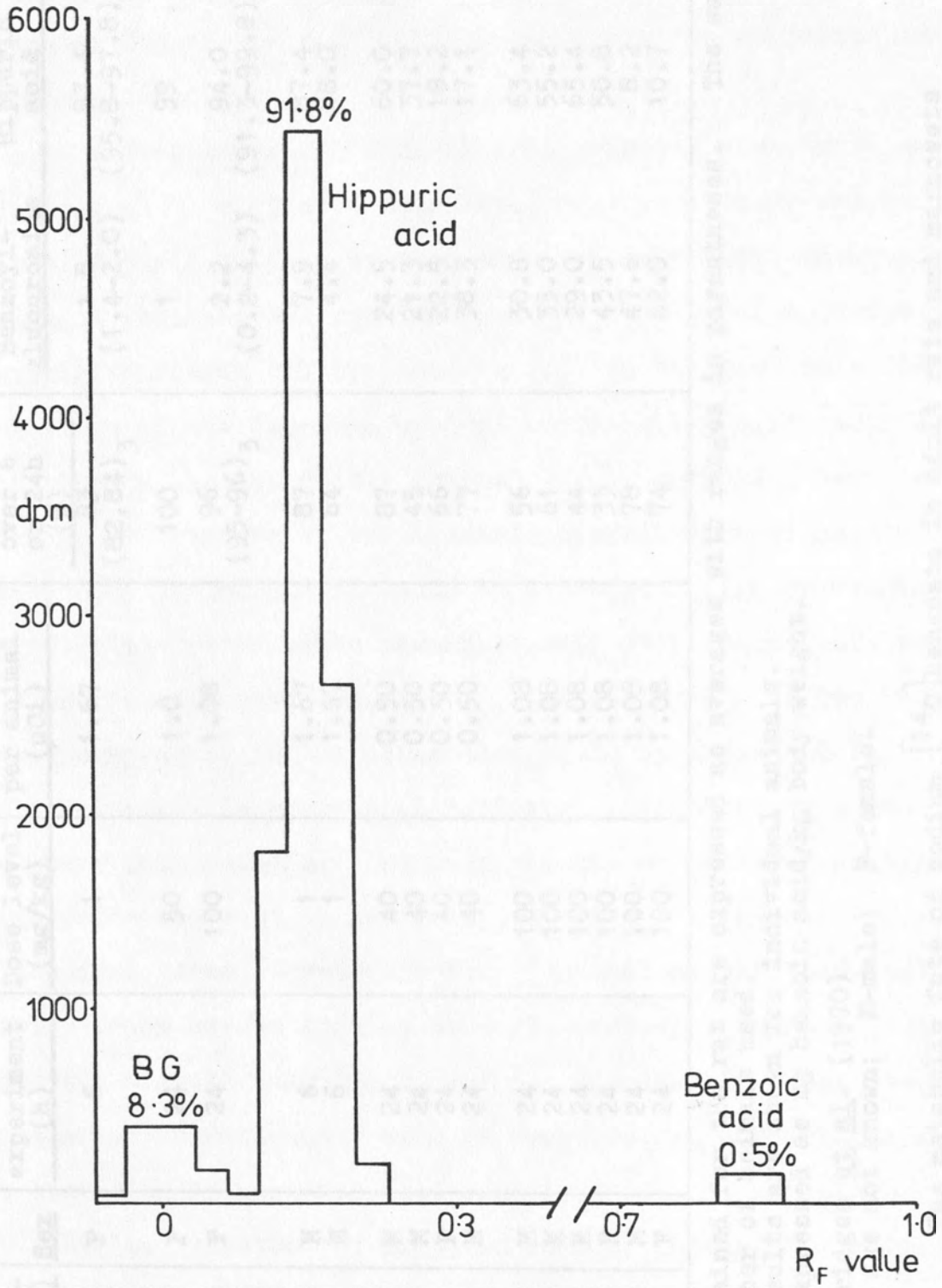


Fig 3.2 Distribution of radioactivity along a silica gel coated thin layer chromatography plate developed in solvent B of urine from a marmoset dosed with sodium [^{14}C] benzoate.
(BG = benzoylglucuronide)

Species	Age (years-months)	Sex	Duration of experiment (h)	Dose level (mg/kg)	Radioactivity per animal (μCi)	Dose recovered over 6 or 24h (%)	Percentage of radioactive components in urine present as:- Benzoyl-glucuronide	Hippuric acid	Benzoic acid
Rat	A	F	6	1	1.67	83 $(82,84)_3$	1.8 (1.4-2.0)	97.4 (95.8-97.8)	0.3 (0.2-1.2)
	A[a]	F	24	50	1.0	100	1	99	tr
	A	F	24	100	1.08	96 $(95-96)_3$	2.2 (0.2-4.3)	94.0 (91.3-99.8)	3.1 (0.0-5.6)
Marmoset	A	M	6	1	1.67	87	7.9	87.4	3.0
	3-9	M	6	1	1.67	64	4.4	88.0	2.4
	A	M	24	40	0.50	87	24.5	60.0	14.5
	3-9	M	24	40	0.50	45	21.3	37.7	38.6
	2-8	F	24	40	0.50	66	22.5	18.2	57.7
	1-8	M	24	40	0.50	77	38.5	17.1	44.4
	A	M	24	100	1.08	56	30.8	63.4	2.6
	3-11	M	24	100	1.08	61	35.0	55.2	7.5
	1-0	M	24	100	1.08	44	29.0	65.4	5.6
	1-6	F	24	100	1.08	33	43.5	50.8	5.7
	3-2	M	24	100	1.08	78	47.6	8.2	44.2
	2-8	F	24	100	1.08	74	42.0	10.7	47.0

The results obtained for the rat are expressed as averages with ranges in parentheses. The subscript denotes the number of animals used.
The marmoset results are shown for individual animals.
All doses are expressed as mg benzoic acid/kg body weight.

[a]Results from Bridges et al. (1970).
A-adult animal, age not known; M-male; F-female.

Table 3.3 The metabolic fate of sodium [^{14}C]benzoate in adult rats and marmosets

a substantial proportion of the dose was excreted as benzoyl-glucuronide but the percentage excreted as hippuric acid showed wide variation. The radioactive component in the urine not accounted for as the glucuronic acid or glycine conjugate was unchanged benzoic acid.

At a dose level of 100mg/kg body weight a similar situation was observed to that at a dose level of 40mg/kg body weight. Four of the six animals studied excreted only small amounts of unchanged benzoate the remainder of the dose being excreted as benzoylglucuronide and hippuric acid. In these animals the proportion of the dose excreted as benzoylglucuronide was higher than that at the lower dose levels. This metabolic profile could be referred to as the 'normal' distribution of metabolites expected. Two of the six animals excreted a high percentage of the administered benzoate unchanged only relatively small amounts of hippuric acid being excreted. The proportion of the radio-active components in the urine that could be accounted for as benzoylglucuronide was not significantly different from that for the other four animals. However, if the quantity of benzoyl-glucuronide excreted in the urine of these six animals is related to the dose administered, the four 'normal' animals excreted on average 16.3% of the dose as benzoylglucuronide whereas in the other two animals this figure was 34.1% indicating that benzoyl-glucuronide formation may well be compensating in part for the low excretion of hippuric acid seen in these two animals.

The low excretion of hippuric acid by some of the animals mentioned in Table 3.3 cannot be correlated with either age or sex. Furthermore the animals came from two different sets of parents and consequently the differences were unlikely to be genetic. That previous dosing of these animals had resulted in

an impairment of their glycine conjugating systems seems unlikely. This effect would be expected to be most prominent in those animals which had previously been dosed with xenobiotics the metabolism of which involved substantial glycine conjugation. The two most frequently dosed animals in this respect were two male animals (Birmingham Code Nos 462 and 465) which were dosed three times with sodium [^{14}C]benzoate, once at each dose level, but at two of these dose levels (i.e. 1 and 100mg/kg body weight) these two animals excreted very little unchanged benzoate while at the third dose level studied they excreted less free benzoate than the other two animals studied.

Confirmation of the identification of the labelled metabolites excreted was furnished by the results contained in Table 3.4 which show the relative proportions of the radioactive components in the β-glucuronidase-treated urine from both adult and neonatal rats and marmosets. The result for one adult marmoset, which excreted only small quantities of hippuric acid after it had been given labelled benzoate at the highest dose level used, support the values obtained for the relative proportions of labelled components in the untreated urine. Figures 3.3 and 3.4 show the change in the radiochemical distribution in the urine, as determined in solvents A and B respectively, when the urine used to obtain Figs 3.1 and 3.2 was digested with β-glucuronidase. When the urine was subjected to β-glucuronidase digestion in the presence of D-saccharic acid-1,4-lactone the radiochemical distribution was the same as that in the untreated urine (Figs 3.1 and 3.2).

Metabolic fate of sodium [^{14}C]benzoate in neonatal animals

Table 3.5 is a summary of the data obtained for the

Species	Age	Dose level (mg/kg)	Percentage of total metabolites excreted in urine present as:- Urine + β-glucuronidase			Urine or urine + β-glucuronidase + D-saccharic acid-1,4-lactone		
			BG	HA	BA	BG	HA	BA
Rat	Adult	100	0.1	91.2	7.8	1.6	95.7	2.7
	10 day[a]	100	3.9	73.8	20.2	+23.1	76.4	0.5
		100	0.0	79.2	17.6	+19.7	75.9	0.0
	10 day[b]	100	1.3	78.5	17.2	17.5	78.0	0.3
		100	1.4	68.6	23.7	25.8	69.0	1.2
Marmoset	Adult	100	18.1	65.5	14.5	30.4	65.8	3.7
		100	9.5	10.5	70.8	38.0	9.5	43.4
	Adult	1	1.4	88.1	9.9	7.2	88.7	3.8
	6 day	100	4.4	64.1	31.1	+39.8	60.2	0.0
	6 day	100	5.6	39.1	35.3	+61.8	38.2	0.0
	7 day	100	8.5	32.2	59.2	+68.2	31.8	0.0
	5 day	1	1.1	89.4	7.9	+8.3	90.8	0.7
	6 day	1	0.8	93.5	4.2	+5.4	93.1	0.0

[a]Duration of experiment 24h; [b]Duration of experiment 6h.

+The control results for these animals represent the metabolite distribution in untreated urine.
HA=Hippuric acid; BA=Benzoic acid; BG=Benzoylglucuronide.

Table 3.4 Effect of β-glucuronidase on the metabolite distribution of animals dosed with sodium [^{14}C]benzoate at various levels

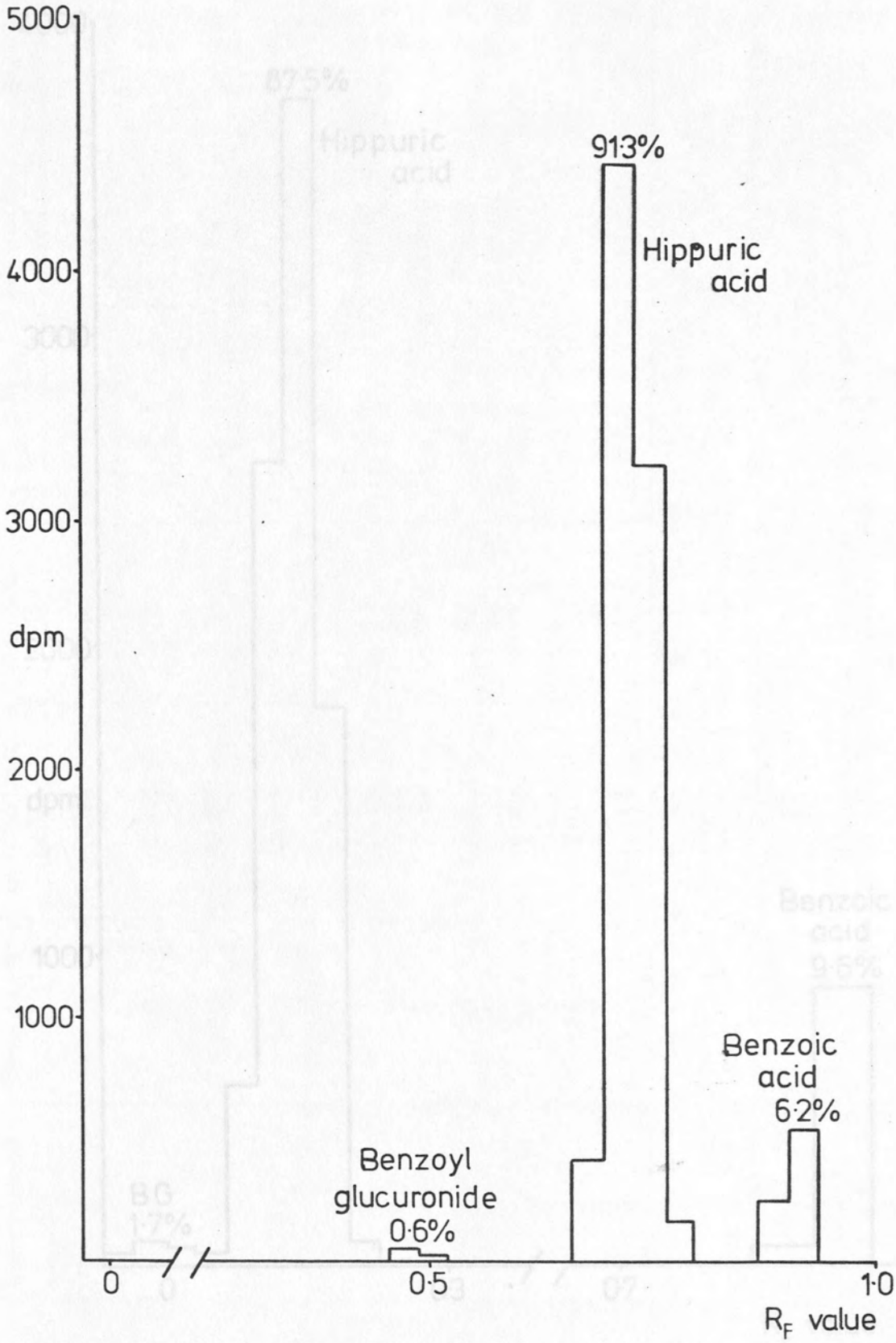


Fig 3.3 Distribution of radioactivity on a silica gel coated thin layer chromatography plate developed in solvent A of β-glucuronidase treated urine from a marmoset dosed with sodium [^{14}C] benzoate.

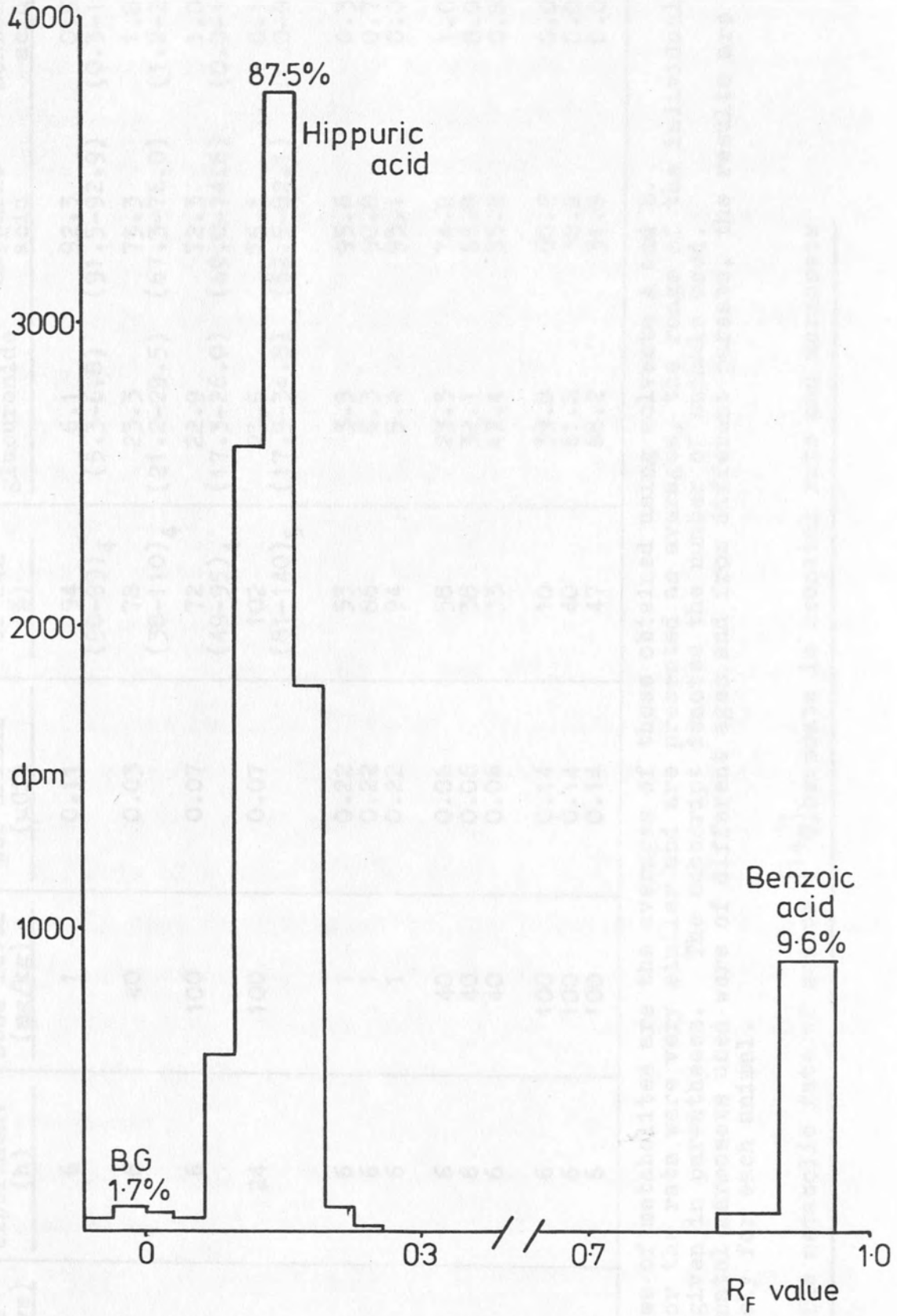


Fig 3.4 Distribution of radioactivity along a silica gel coated thin layer chromatography plate developed in solvent B of β-glucuronidase treated urine from a marmoset dosed with sodium [^{14}C] benzoate.
(BG = benzoylglucuronide)

Species	Age (days)	Duration of experiment (h)	Dose level (mg/kg)	Radioactivity per animal (μCi)	Recovery of dose over 6 or 24h (%)	Percentage of radioactive components in urine present as:-		
						Benzoyl-glucuronide	Hippuric acid	Benzoic acid
Rat	9	6	1	0.11	94 (90-99)$_4$	6.1 (5.3-6.8)	92.3 (91.5-92.9)	0.5 (0.3-1.1)
	9	6	40	0.03	78 (38-110)$_4$	23.3 (21.2-29.5)	73.3 (67.3-76.0)	1.8 (1.2-2.5)
	10	6	100	0.07	72 (49-95)$_4$	22.9 (17.3-26.0)	72.3 (69.0-74.6)	1.0 (0.0-1.9)
	10	24	100	0.07	102 (81-140)$_5$	23.5 (17.9-34.8)	75.1 (62.5-82.1)	0.1 (0.0-0.5)
Marmoset	5	6	1	0.22	53	3.5	95.6	0.3
	5	6	1	0.22	86	8.3	90.8	0.7
	6	6	1	0.22	94	5.4	93.1	0.0
	4	6	40	0.06	58	23.3	74.2	1.0
	4	6	40	0.06	38	32.1	66.8	0.0
	5	6	40	0.06	33	42.4	55.8	0.8
	6	6	100	0.14	10	39.8	60.2	0.0
	6	6	100	0.14	40	61.8	38.2	0.0
	7	6	100	0.14	47	68.2	31.8	0.0

The percentages of metabolites are the averages of those obtained using solvents A and B.
The results for the rats were very similar and are presented as averages, the range of the individual values being given in parentheses. The subscript denotes the number of animals used.
Since the neonatal marmosets used were of different ages and from different parents, the results are given separately for each animal.

Table 3.5 The metabolic fate of sodium [^{14}C]benzoate in neonatal rats and marmosets

proportions of benzoylglucuronide, hippuric acid and unchanged benzoate excreted when sodium [^{14}C]benzoate was administered to neonatal rats and marmosets at different dose levels. At all the dose levels examined, benzoylglucuronide constituted a larger proportion of the radioactive components excreted in the urine of 9 and 10 day old rats than it did in the urine of adult animals. In the neonatal rats the metabolic fate of labelled benzoate exhibited a dose dependent pattern not evident in the adults, the proportion of the dose excreted conjugated with glucuronic acid being much smaller at the lowest dose level than at the two higher dose levels examined. The proportion of the radioactive components in the urine present as benzoylglucuronide were not significantly different at the two higher dose levels studied. The proportion of the metabolites excreted in the urine at a dose level of 100mg/kg body weight in these neonatal animals appeared to be the same whether the urine analysed was collected for 6h or 24h after dosing.

The fate of sodium [^{14}C]benzoate in the neonatal marmoset was clearly dose dependent and at the 100mg/kg body weight dose level the glucosiduronic acid was the main urinary metabolite. At the lowest dose level studied the metabolic fate of sodium [^{14}C]benzoate in the neonate was very similar to that in the adult marmoset.

Plates 2 and 3 are autoradiographs showing the distribution of labelled metabolites in the urine of a neonatal marmoset to which sodium [^{14}C]benzoate had been administered at a dose level of 40mg/kg body weight. Plates 2 and 3 represent the results obtained when the urine was chromatographed on glass, silica gel G-coated, thin layer plates in solvents A and B respectively. Neonatal marmoset urine was chosen for its relatively high

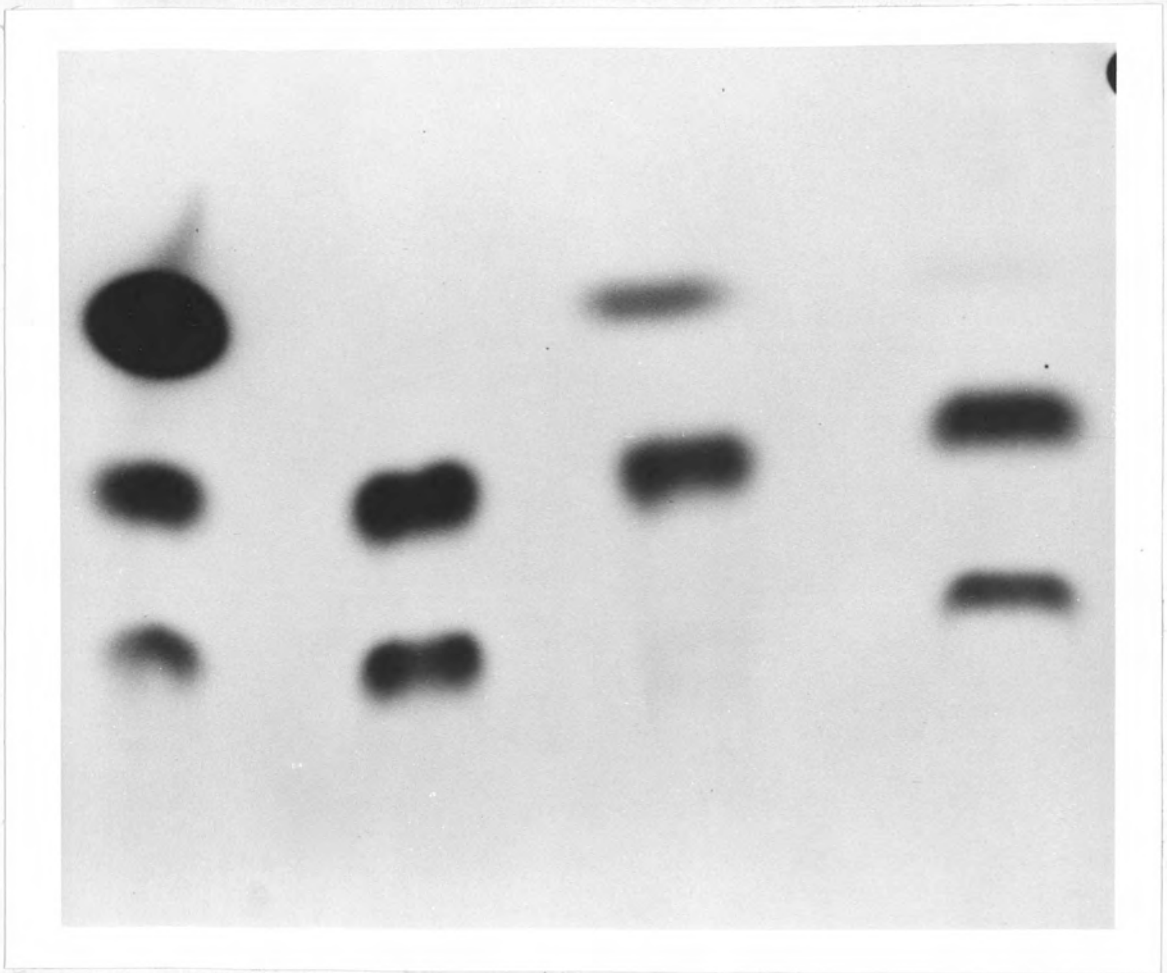


Plate 2 Autoradiograph taken from a thin layer chromatogram developed in solvent A of the urine from a neonatal marmoset which had been dosed with sodium [^{14}C]benzoate. The effect of β-glucuronidase on the distribution is also demonstrated.

BA = Benzoic acid, HA = Hippuric acid, BG = Benzoylglucuronide.

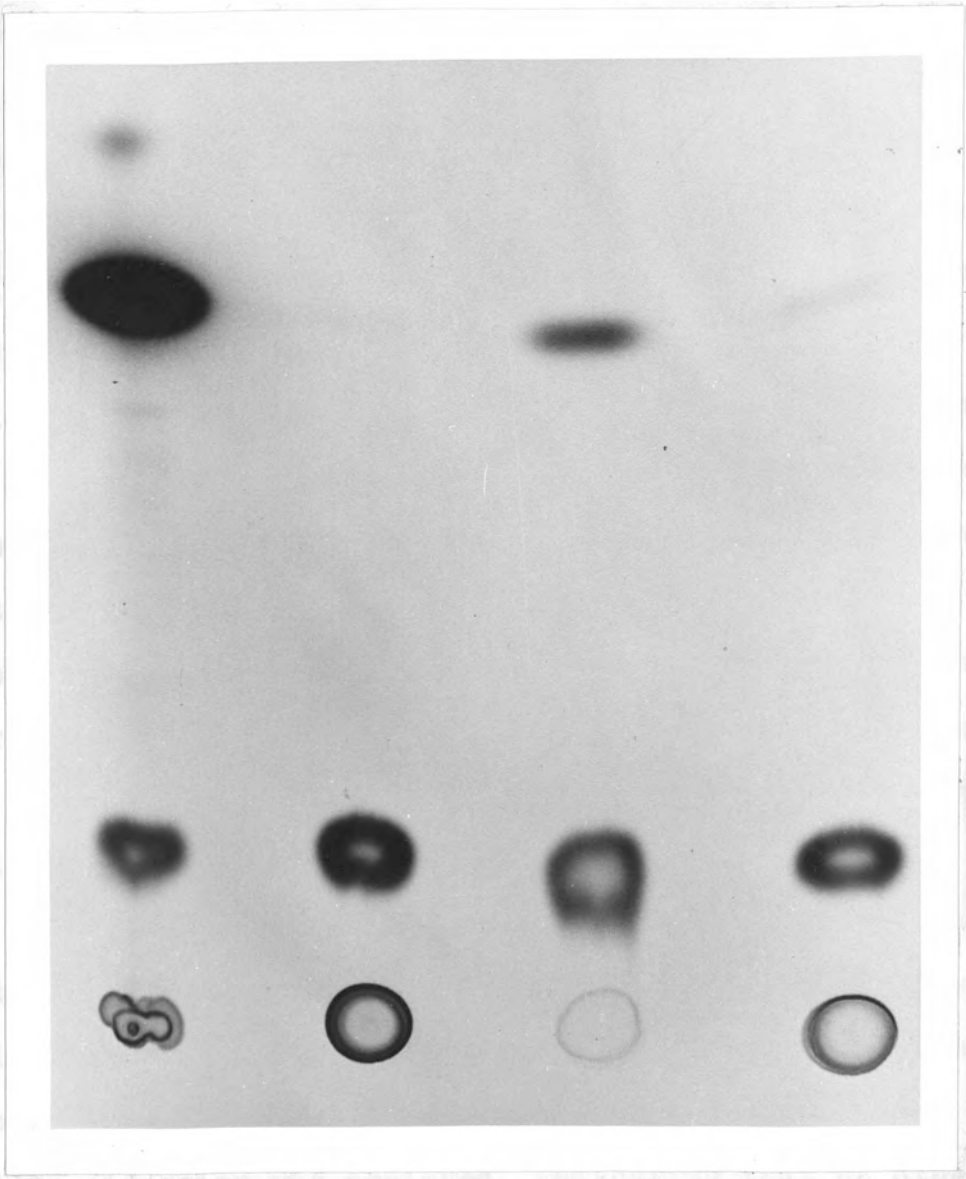


Plate 3. Autoradiograph taken from a thin layer chromatogram, developed in solvent B, of the urine from a neonatal marmoset which had been dosed with sodium [^{14}C] benzoate. The effect of β-glucuronidase on the distribution is also demonstrated.

BA = Benzoic acid, HA = Hippuric acid, BG = Benzoylglucuronide.

content of benzoylglucuronide which enabled the effect of β-glucuronidase both in the presence and absence of the inhibitor D-saccharic acid-1,4-lactone to be clearly demonstrated.

The effect of the dose level on the metabolic fate of sodium [^{14}C]benzoate in the neonate is illustrated in Plate 4 which shows an autoradiograph taken from a thin layer chromatography plate developed in solvent A to which samples of urine from neonatal rats and marmosets, which had been dosed with labelled benzoate at levels of both 1 and 40mg/kg body weight, were applied. Two identical thin layer plates were prepared simultaneously and the metabolites identified by spraying one with reagent I and the other with reagent VII. The increase in the proportion of the dose excreted conjugated with glucuronic acid at the higher dose level is clearly visible for both species.

As shown by the results in Table 3.4, β-glucuronidase treatment of the urine collected from neonatal animals which had received sodium [^{14}C]benzoate caused a substantial decrease in the benzoylglucuronide content of the urine and in some cases complete digestion was achieved, indicating that no other metabolite of sodium [^{14}C]benzoate, such as benzoyltaurine, was running at an R_F value similar to that of benzoylglucuronide in either solvent A or B.

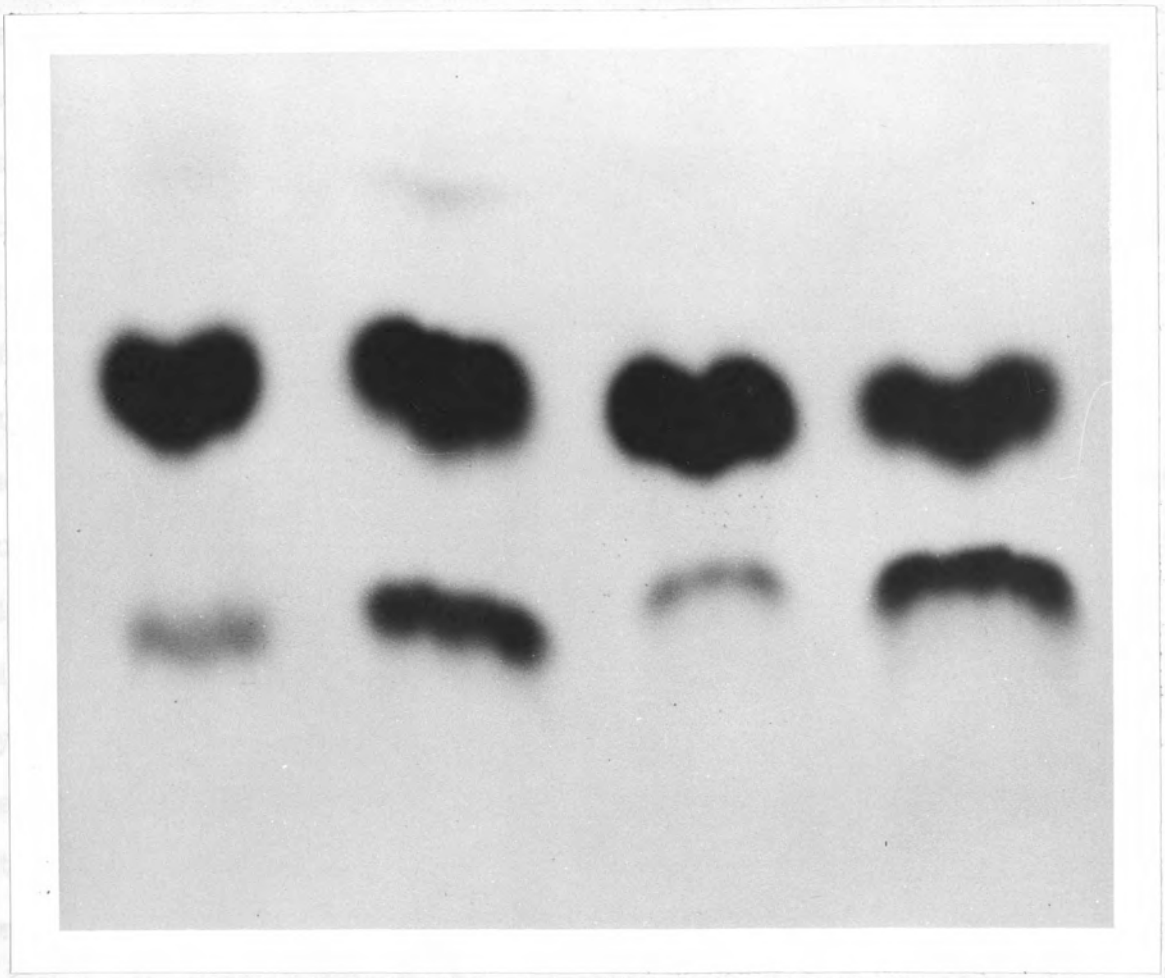


Plate 4 Effect of dose level on the metabolic profile of sodium [^{14}C]benzoate in neonatal rats and marmosets as depicted by an autoradiograph taken from a thin layer chromatogram developed in solvent A.

BA = Benzoic acid, HA = Hippuric acid, BG = Benzoylglucuronide. Figures in parentheses indicate the dose level in mg/kg body weight.

Discussion

The results show that the metabolic fate of sodium [^{14}C]-benzoate over the range of dose levels tested is strongly dose dependent in the adult marmoset but not in the rat. In the marmoset as the dose level is increased, the excretion of benzoylglucuronide increases while the proportion of the radioactive components in the urine present as hippuric acid decreases. The amounts of glycine used to support the synthesis of the quantity of hippuric acid found to be excreted in the urine of the adult animals at different dose levels were calculated and are given in Table 3.6. The experiments at the lower dose level were carried out over 6h while those at the higher dose levels were carried out for 24h which makes it difficult to compare the rates of glycine utilisation for the synthesis of hippuric acid since the majority of the recovery recorded for the 24h experiments will undoubtedly have been attained in a much shorter time period. By using the total recoveries obtained for the neonatal rats (Table 3.5) which were dosed with sodium [^{14}C]benzoate at a dose level of 100mg/kg body weight and studied over both 6h and 24h it can be calculated that the observed 24h excretion probably occurred over a period of 9h. The rates of glycine utilisation have therefore been calculated assuming that the dose was largely excreted within this period.

In the adult marmoset much less glycine appears to be available for conjugation with benzoic acid than in similarly dosed rats. The highest rate of glycine utilisation observed in the adult marmoset is 2.4mg/kg body weight/h, obtained at the highest dose level, whereas that in the rat was 6.6mg/kg body weight/h. Even at the highest dose level employed, the rat

Species	Duration of Experiment (h)	Dose level (mg/kg)	Recovery of dose (%)	Percentage of total metabolites as Hippuric acid	Rate of glycine utilisation (mg/kg body wt/h)
Rat	6	1	82	98.0	0.08
	6	1	84	96.9	0.08
					average = 0.08
	24	50	100	99	3.38
	24	100	95	91.4	5.94
	24	100	96	99.8	6.56
	24	100	96	93.4	6.10
					average = 6.20
Marmoset	6	1	64	87.0	0.06
	6	1	87	88.2	0.08
					average = 0.07
	24	40	87	60.1	1.42
	24	40	45	37.7	0.46
	24	40	66	18.2	0.33
	24	40	77	17.1	0.36
					average = 0.64
	24	100	56	63.4	2.42
	24	100	61	55.2	2.29
	24	100	43	65.4	1.90
	24	100	33	50.8	1.15
	24	100	78	8.2	0.43
	24	100	74	10.7	0.54
					average = 1.46

Table 3.6 Rates of glycine utilisation for the formation of hippuric acid by adult animals

excreted administered benzoate almost entirely as hippuric acid no limitation in the amount of glycine available being encountered at the dose levels examined. Arnstein and Neuberger (1951) reported that the quantity of glycine immediately available for conjugation of small doses of benzoate in the rat was about 10mg/100g body weight while Simkin and White (1957a) found that the capacity of the rat to provide glycine for the conjugation of benzoate was 36μmol/100g body weight which corresponds to 27mg/kg body weight/h, so that even at the highest dose level of benzoate used in the present study, the amount of glycine used was well below the maximum amount available. Simkin and White (1957a) further reported that the concentration of free glycine in rat liver was 10.7μmol/100g body weight whereas the rate of hippuric acid synthesis was 36μmol/100g body weight which suggested that the liver must withdraw free glycine from the remaining viscera via the plasma for conjugation purposes.

In the marmoset the low rate of glycine utilisation for the conjugation of the administered benzoate appears to be compensated by the increase in glucosiduronic acid synthesis. In those marmosets excreting very small amounts of hippuric acid, this compensation may not be complete.

The ability of the human liver to conjugate orally administered benzoic acid with glycine was formerly used as a clinical test of liver function. The test was originally reported by Quick (1933a) and later modified by Probstein and Londe (1940). Various figures are available as guidelines in this test but in general a subject with a normally functioning liver was found to excrete 3.0g of benzoic acid as hippuric acid (4.4g) in the urine 4h after the ingestion of a 6g dose of

sodium benzoate. The normal range varies from 85-110% of this amount (i.e. from 2.6-3.3g benzoic acid). In a 70kg man the dose administered is equivalent to 86mg sodium benzoate/kg body weight and the normal range of glycine utilisation is 5.6-7.2mg/kg body weight/h which is considerably greater than that found in the marmoset at a similar dose level.

When sodium [^{14}C]benzoate was administered to adult rats and marmosets at a dose level of 1mg/kg body weight, the rates of glycine utilisation for the synthesis of hippuric acid were very similar and clearly not rate limiting in either animal. At this dose level, the rat excreted the administered dose almost entirely as hippuric acid, a similar situation to that in man as reported by Bridges et al. (1970). However a significant quantity of this dose was excreted as the glucosiduronic acid by the marmoset which suggests that in this species the glucuronide synthetase system may have an activity greater than that in either man or the rat. At this dose level all the results were obtained using 6h excretions and consequently they all are directly comparable and contain no 'time approximations'.

Hippuric acid is excreted as a normal metabolite by man, marmoset and the rat, the average daily excretion being about 700, 4 and 13mg respectively; these values correspond to an average daily output of glycine of 4, 5 and 22mg/kg body weight. This normal excretion of hippuric acid has not been considered in the discussion of the utilisation of glycine to support the synthesis of hippuric acid on administration of benzoate to the marmoset and rat reported in the present results. It is evident that the difference in the normal daily excretion represents a greater utilisation of glycine in the rat than in the marmoset and emphasises the superior ability of the rat to

conjugate benzoic acid with glycine.

The metabolic fate of sodium [^{14}C]benzoate in both neonatal rats and marmosets is strongly dose dependent. The rates of glycine utilisation calculated from the results in Table 3.5 are given in Table 3.7.

Marmosets aged 4-7 days have a limited capacity to utilise glycine for hippuric acid formation which is reached below a dose level of benzoic acid of 40mg/kg body weight. At this dose level the benzoylglucuronide excreted represents 33% of the metabolites excreted and at the 100mg/kg body weight dose level this proportion rises to an average of 57%. Although in the adult marmoset the rate of glycine utilisation is low, it is not limited at the 40mg/kg body weight dose level since higher rates are obtained at the higher dose level. This difference in the availability of glycine between neonatal and adult marmosets may account for the sharp increase in the proportion of the radioactive components excreted in the urine of the neonate which are present as benzoylglucuronide when the dose level is increased from 40 to 100mg/kg body weight, and the less marked increase observed, in general, in the adults. The two adult marmosets which excreted very low quantities of a 100mg/kg body weight dose of labelled benzoate as hippuric acid excreted more of the dose as benzoylglucuronide compared with the 'typical' dosed animals underlining the inverse relationship between the availability of glycine for the conjugation of benzoic acid and the proportion of the dose excreted conjugated with glucuronic acid.

These results indicating the relatively high ability of the neonatal marmoset to synthesise glucosiduronic acids may be contrasted with the situation in the human neonate which has

Species	Age (days)	Dose level (mg/kg)	Recovery of dose (%)	Percentage of total metabolites as hippuric acid	Rate of glycine utilisation (mg/kg body wt/h)
Rat	9	1	90	92.3	0.09
	9	1	98	92.4	0.09
	9	1	99	91.5	0.09
	9	1	91	92.9	0.09
					average = 0.09
	9	40	38	67.3	1.04
	9	40	65	74.9	2.00
	9	40	110	74.9	3.38
	9	40	100	76.0	3.10
					average = 2.38
	10	100	72	74.5	5.48
	10	100	72	74.2	5.50
	10	100	49	72.8	3.66
	10	100	95	67.7	6.56
					average = 5.30
Marmoset	5	1	53	95.6	0.05
	5	1	86	90.8	0.08
	6	1	94	93.1	0.09
					average = 0.07
	4	40	58	74.2	1.77
	4	40	38	66.8	1.02
	5	40	33	55.8	0.76
					average = 1.18
	6	100	10	60.2	0.62
	6	100	40	38.2	1.55
	7	100	47	31.8	1.50
					average = 1.22

Table 3.7 Rates of glycine utilisation for the formation of hippuric acid by neonatal animals

been shown to conjugate N-acetyl p-aminophenol with glucuronic acid at a much slower rate than the adult (Vest, 1958; Vest and Streiff, 1959; Vest and Rossier, 1963; Vest, 1965).

At the 40mg/kg body weight dose level the rate of glycine utilisation for conjugation of the administered benzoate was lower in neonatal rats than in animals of the same age to which benzoate was administered at a dose level of 100mg/kg body weight. At both dose levels significant amounts of benzoylglucuronide were excreted possibly reflecting the relatively high activity of the transglucuronylase enzyme in this species which may be an example of the 'overshoot' phenomenon (see p.67). Even at the lowest dose level when the glycine utilised was far below that available, significant amounts of benzoate were excreted conjugated with glucuronic acid, the actual amounts excreted by both neonatal rats and marmosets being approximately 60µg/kg body weight/h.

Baines (1975) reported that neonatal rats excreted two unidentified metabolites of sodium [^{14}C]benzoate, administered at a dose level of 40mg/kg body weight. These were designated 'X' and 'Y' neither of which appeared to be unchanged benzoate nor benzoylglucuronide. The present results show conclusively that metabolite 'Y' corresponds to benzoylglucuronide but no labelled metabolite corresponding to 'X' was detected in the urine of the dosed rats examined.

CHAPTER 4

The comparative metabolism of *p*-aminobenzoic acid

Chapter 4 - The comparative metabolism of p-aminobenzoic acid

Introduction

p-Aminobenzoic acid (PAB) is metabolised by acetylation of the amino group and conjugation of the carboxyl group with either glycine or glucuronic acid. Metabolites in which both biochemically reactive groups are conjugated also occur (Fig 4.1). As with benzoic acid PAB undergoes conjugation with ornithine in some reptilian species (Smith, 1957).

Many of the investigations of the metabolism of PAB have concentrated on its acetylation which has been demonstrated in man (Strauss et al., 1941; Smith et al., 1946; Tabor et al., 1947, 1948, 1951; Vest and Rossier, 1963; Drucker et al., 1964; Vest and Salzberg, 1965), rabbit (Harrow et al., 1933; Bray et al., 1948a; Smith and Williams, 1948; Venkataraman et al., 1950; Terp, 1951), rat (Riggs and Christensen, 1951; Riggs and Hegsted, 1951), and guinea pig (Terp, 1951), but not in the dog (Terp, 1951).

Fewer comprehensive studies of the metabolism of PAB in different species have been reported. Tabor et al. (1951) found that PAB (6g) administered to man was excreted in urine mainly as p-aminohippurate (PAH) and p-aminobenzoylglucuronide (PABG), with small amounts of PAB and acetylated derivatives of PAB, PAH and PABG. Vest and Salzberg (1965) investigating the development of the conjugating reactions responsible for the metabolism of PAB in humans, showed that an 11 year old child, who had received sodium p-aminobenzoate at a dose level of 100mg/kg body weight, excreted 50% of the total dose recovered in the urine in 24h in the form of PAH; p-acetamidobenzoic acid (PAAB) accounted for a further 27%, glucosiduronic acids for 18%, p-acetamidohippuric

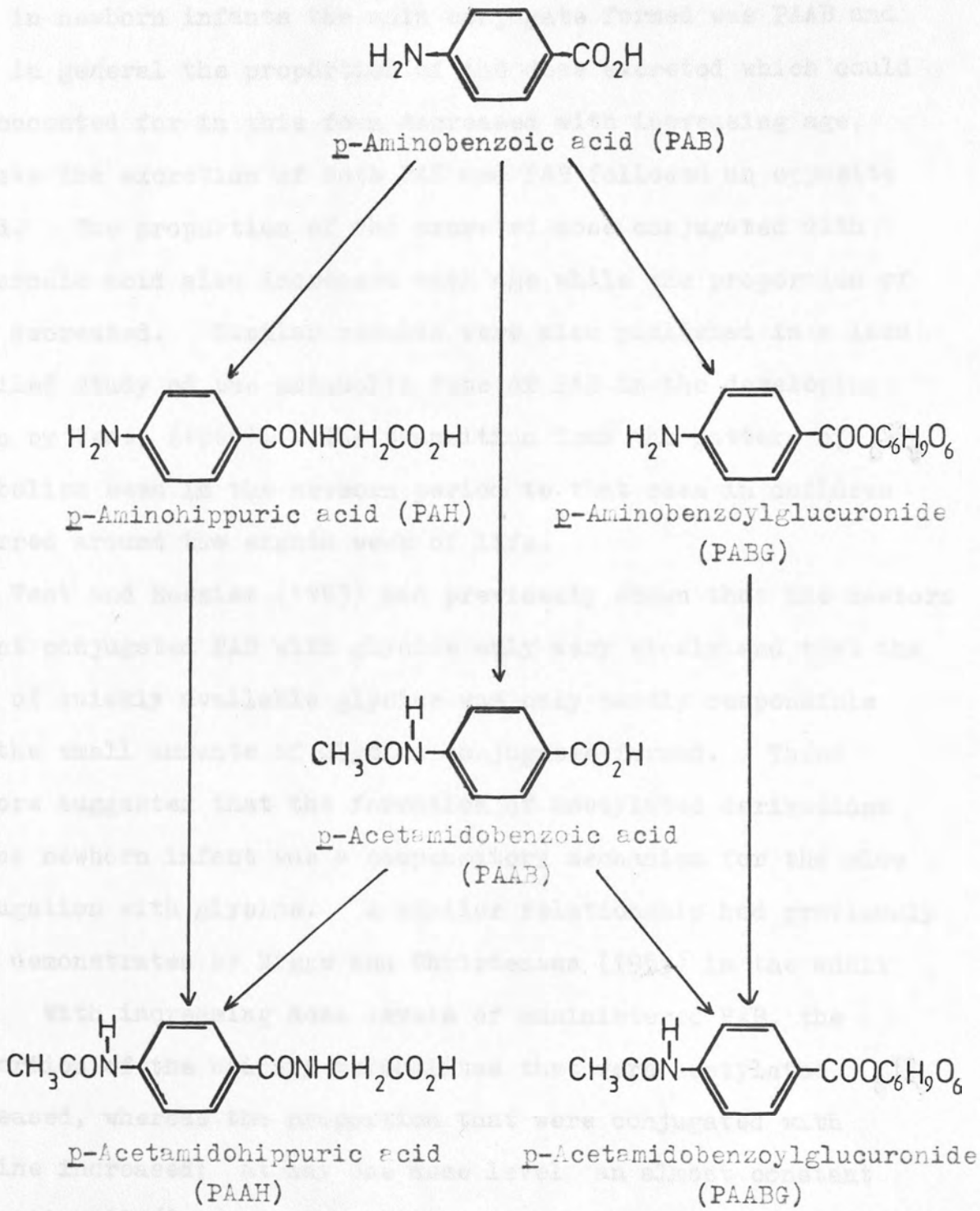


Fig 4.1 The metabolic fate of p-aminobenzoic acid

acid (PAAH) for 9% and free PAB for 8%. Similar investigations carried out in three newborn premature infants of 2 to 6 days of age, two newborn full-term infants, two 5 and 8 week old infants and three children between the ages of 8 and 10 years, indicated that in newborn infants the main conjugate formed was PAAB and that in general the proportion of the dose excreted which could be accounted for in this form decreased with increasing age, whereas the excretion of both PAH and PAB followed an opposite trend. The proportion of the excreted dose conjugated with glucuronic acid also increased with age while the proportion of PAAH decreased. Similar results were also published in a less detailed study of the metabolic fate of PAB in the developing human by Vest, (1965). The transition from the pattern of metabolism seen in the newborn period to that seen in children occurred around the eighth week of life.

Vest and Rossier (1963) had previously shown that the newborn infant conjugated PAB with glycine only very slowly and that the lack of quickly available glycine was only partly responsible for the small amounts of glycine conjugates formed. These authors suggested that the formation of acetylated derivations by the newborn infant was a compensatory mechanism for the slow conjugation with glycine. A similar relationship had previously been demonstrated by Riggs and Christensen (1951) in the adult rat. With increasing dose levels of administered PAB, the proportion of the urinary metabolites that were acetylated decreased, whereas the proportion that were conjugated with glycine increased; at any one dose level an almost constant proportion (91%) of the PAB was conjugated in one position or the other. The administration of glycine to these animals stimulated acetylation rather than glycine conjugation, an

observation also made by Venkataraman et al. (1950) in experiments using the rabbit. Riggs and Hegsted (1951) reported that in the rat and rabbit the acetylation of PAB did not exceed 75-80% irrespective of the dose level employed.

The development of the glycine N-acyltransferase system in the rat and mouse liver has been studied in detail (Brandt, 1964, 1966; Gorodischer et al., 1971). Using rat liver homogenates and mitochondrial preparations, Brandt (1964, 1966) was unable to detect the activity of this enzyme, using PAB as substrate, in newborn animals; activity was detected when the animals were between 6 and 10 days of age, after which it rose to a peak value at about 30 days. Similar results were obtained by Gorodischer et al. (1971) using mouse liver preparations, although this species differed from the rat in that glycine N-acyltransferase activity was present on the first day of life.

This chapter describes an investigation of the comparative metabolism of PAB in the adult and neonatal marmoset and rat.

Materials

PAB, PAAB and PAH were all available from the laboratory stores. PAAH was synthesised by heating PAH under reflux for 30min with an acetylating mixture consisting of equal volumes of acetic acid and acetic anhydride. The residue obtained on evaporation of the reaction mixture under reduced pressure had a melting point of 209-213°C, in agreement with that given in the literature (Miyatake and Kaya, 1952). On chromatography in solvent L on an aluminium-backed $60F_{254}$ plate, the product gave only a single spot when viewed under UV light. Co-chromatography with PAH demonstrated that the R_F value of the product was different from that of PAH. The product gave no colour in the Bratton and Marshall (1939) test for aromatic amines.

Methods

Administration of dose

PAB was administered to all animals as a solution of its sodium salt in water at a dose level equivalent to 100mg PAB/kg body weight. The dose was mixed with Cytacon for administration to adult animals or with diluted milk for administration to the neonates.

Collection of urine

a) From dosed rats

At each age studied, twelve neonatal rats were dosed and divided into three groups of four animals. A control group was also studied. Each group was placed on a layer of filter paper in a beaker which was housed in an incubator (p.81). Urine was

collected as previously described at intervals up to 6h after the administration of the dose. The urine from each group was pooled and made up to 1.0ml with water. The urine from adult rats was collected at 6h and 24h after the administration of the dose.

b) From dosed marmosets

After the administration of the dose urine was collected for 6h from neonates (p.84) and at 6h and 24h from adults.

The filter paper on which the neonatal animals were placed and the tissues used in collecting urine from the neonatal marmosets were extracted with a solution of ammonia (5% w/v) and the amount of metabolites in these extracts also determined. The extraction efficiency of metabolites from the absorbent materials was 97%.

Identification and quantification of metabolites

The colorimetric method of Bratton and Marshall (1939) was used to determine the free aromatic amino compounds excreted in the urine of dosed animals. Samples of urine which had been heated at 100°C for 1h with 0.2M HCl to hydrolyse any N-acetylamino compounds were also analysed by the same method to give the total amount (free + acetylated) of aromatic amino compounds present. The difference between the free and 'total' amino compounds corresponds to the acetylated material present. The absorbance, at 550nm, of the colour produced in the Bratton and Marshall reaction, was used to determine the amount of aromatic amino compounds present by reference to a calibration curve prepared from known amounts of PAB.

The separation, identification and quantification of the individual metabolites were carried out by the method of Davis

and Yeary (1977) in which the metabolites were separated by chromatography, the zones containing the metabolites eluted and the amino compounds present in the eluate determined before and after acid hydrolysis using the Bratton and Marshall method. Calibration curves for PAB and PAH were not significantly different and the PAB calibration curve was used for the determination of all the metabolites.

The efficiency of extraction of PAB, PAAB, PAH and PAAH from the chromatograms was determined by application of known amounts to the plate and measurement of the material recovered in the eluate. The recoveries of PAB, PAAB, PAH and PAAH were 78%, 90%, 75% and 80% respectively.

The R_F values of the reference compounds in solvent L, detected by their absorbance in UV light, are given in Table 4.1. It may be seen that the R_F values for PAB and PAAB are the same and it was found that PAH and PAAH (R_F values 0.55 and 0.63 respectively) tended to overlap.

Proof of identity of metabolite 4 as PABG was obtained by digesting the urine samples from dosed animals with β-glucuronidase type H - 1 (Helix pomatia) in an equal volume of 0.4M sodium acetate buffer pH 5.0 at 37°C for 16h. A control digest containing D-saccharic acid-1,4-lactone was carried out at the same time. The disappearance on β-glucuronidase treatment of the quenching spot with an R_F value of 0.30 together with a loss of aromatic amino group content in this zone of the plate and a corresponding increase in the aromatic amino group content in the zone of R_F 0.80 confirmed the identification.

Compound	R_F value in solvent L
Reference compounds	
p-Aminobenzoic acid (PAB)	0.84
p-Acetamidobenzoic acid (PAAB)	0.84
p-Acetamidohippuric acid (PAAH)	0.63
p-Aminohippuric acid (PAH)	0.55
Urinary metabolites	
Metabolite 1 (PAB + PAAB)	0.80
Metabolite 2 (PAAH)	0.60
Metabolite 3 (PAH)	0.52
Metabolite 4 (PABG)	0.30

Chromatography was carried out on aluminium sheets pre-coated with a layer of silica gel $60F_{254}$ (0.2mm thick).
PABG p-Aminobenzoylglucuronide.

Table 4.1 Chromatographic properties of reference compounds and metabolites of p-aminobenzoic acid

Results

Four zones which absorbed UV light were detected on chromatograms of samples of the urine of dosed animals. These occurred at R_F values 0.80, 0.60, 0.52 and 0.30 and are described as metabolites 1-4. These quenching areas were not detected in urine from control animals.

Metabolite 1 was shown to be a mixture of PAB and PAAB since the acid-hydrolysed eluate of this zone showed an increase in colour in the Bratton and Marshall test as compared with the unhydrolysed eluate.

Metabolites 2 and 3 corresponded to PAAH and PAH respectively. The zones containing these two metabolites were not completely separated and again the amounts of the individual metabolites present were determined by application of the Bratton and Marshall test before and after acid hydrolysis of the eluate of the two combined zones containing metabolites 2 and 3.

Eluted metabolite 4 also gave a colour in the Bratton and Marshall test showing the presence of an aromatic amino group which was not usually increased when the acid-treated eluate was examined. This spot was identified as PABG by the methods outlined on p.135.

The quantitative results for adult marmosets and rats showing the recoveries of the dose, the degree of acetylation of the dose and the relative proportions of the metabolites present in the urine are given in Table 4.2.

It may be seen that the proportion of the dose excreted in the acetylated form was much greater in the marmoset than in the rat and that this was due almost entirely to the excretion of a greater proportion of the dose as PAAB by the marmoset as

Species	Dose recovered (%) over 6h	Degree of acetylation in 6h urine sample		Percentage of total metabolites excreted in 6h urine present as				
		(i)	(ii)	PABG	PAH	PAAH	PAB	PAAB
Rat	84	23.9	20.1	6.7	69.4	4.5	0.0	19.2
	89	18.6	16.5	7.8	65.9	7.2	2.5	16.4
Marmoset	38	66.9	25.4	7.0	10.4	nd	9.9	72.7
	56	59.4	33.3	18.9	4.9	nd	17.2	54.5
	77	80.8	62.2	7.3	11.6	5.0	1.7	75.3

(i) Expressed as a percentage of the dose recovered in the 6h sample.

(ii) Expressed as a percentage of the dose administered.

All animals were dosed with sodium p-aminobenzoate at a level equivalent to 100mg PAB/kg body weight.

nd not detected.

Table 4.2 The metabolic fate of p-aminobenzoic acid in adult rats and marmosets

compared with the rat. PAH was the major metabolite excreted by the rat but represented less than an average of 10% of the metabolites excreted by the marmoset. About 6% of the metabolites excreted by the rat corresponded to PAAH but this metabolite was only excreted by one of the adult marmosets examined. PAB was excreted unchanged by both species but to a greater extent in the marmoset than in the rat. PABG was excreted by both species and the determination of the metabolites present in the urine of dosed marmosets and rats after treatment with β-glucuronidase both in the presence and absence of the enzyme inhibitor D-saccharic acid-1,4-lactone is shown in Table 4.3. In experiments with adult animals, the expected increase in PAB on β-glucuronidase treatment was found in three cases but in one of the experiments the increase in PAB was higher than expected and cannot be explained.

The quantitative results showing the recovery of the dose, the extent of acetylation and the relative amounts of individual metabolites excreted by 7 and 11 day old rats and 4, 5, 10 and 11 day old marmosets which had been dosed with PAB are given in Table 4.4.

It may be seen that in the rats aged 7 days approximately equal amounts of PABG, PAAB and PAH were excreted. In rats aged 11 days the proportions of these metabolites were markedly different; much less PABG was present and the proportion of PAH was slightly greater than that of PAAB. At neither age was unchanged PAB excreted.

The recoveries of the doses in marmosets aged 4 or 5 days were very low; as in the rat about one third of the metabolites excreted were acetylated; in contrast to the rat the relative proportion of PAH excreted was small and that of PABG was rather

Species	Age	Percentage of metabolites present in 6h urine present as:- (i) after treatment with β-glucuronidase					(ii) after treatment with β-glucuronidase + D-saccharic acid-1,4-lactone				
		PABG	PAH	PAAH	PAB	PAAB	PABG	PAH	PAAH	PAB	PAAB
Rat	Adult	0.0	51.3	4.8	22.1	20.1	6.8	69.0	4.8	0.0	19.2
	Adult	0.0	68.1	2.0	10.4	19.5	7.0	66.1	7.2	2.5	16.0
Marmoset	Adult	0.0	3.7	nd	35.7	60.2	15.2	5.1	nd	18.6	61.0
	Adult	0.0	8.9	nd	8.0	76.3	8.3	9.2	5.0	0.7	76.1
Marmoset	5 day	0.0	7.8	nd	23.3	75.0	14.3	2.9	nd	23.9	59.0

nd = not detected

Table 4.3 Effect of β-glucuronidase digestion on the distribution of metabolites of PAB in adult and neonatal animals

Key Table 4.4

(i) Expressed as a percentage of the dose recovered in 6h.

(ii) Expressed as a percentage of the dose administered.

All animals were dosed with sodium p-aminobenzoate at a dose level equivalent to 100mg PAB/kg body weight.

The rat results are averages of 4 animals.

The marmoset results are reported for individual animals.

(a) This zone contained an acetylated metabolite.

(b) This zone was totally acetylated.

+ Parents 462, 466
++ Parents 417, 418
+++ Parents 463, 465

See Fig 2.1

Species	Age (days)	Dose recovered (%) in 6h	Degree of acetylation in 6h urine sample		Percentage of total metabolites excreted in 6h urine present as:			
			(i)	(ii)	PABG	PAH	PAB	PAAB
Rat	7	31	35.4	11.0	28.8	35.7	0.0	35.5
		27	35.8	7.3	(a) 36.5	30.1	0.0	33.4
		56	30.8	16.9	35.4	32.6	0.0	32.0
	11	9	33.9	2.0	3.6	56.7	0.0	39.8
		22	41.0	6.4	8.4	46.7	0.0	44.8
		17	37.4	4.0	8.5	48.7	0.0	42.8
Marmoset	4^{+}	10	37.5	3.8	9.8	9.8	43.6	37.0
	4^{++}	10	39.1	3.9	5.6	9.4	21.9	54.3
	5^{+}	10	33.4	3.3	22.6	7.4	34.4	35.5
	5^{++}	10	48.1	4.8	(b) 10.3	3.3	30.2	57.4
	10^{+++}	51	68.6	35.0	12.6	3.9	3.1	80.9
	11^{+++}	62	71.5	44.3	6.6	3.3	6.0	84.4

Table 4.4 Metabolic fate of p-aminobenzoic acid in neonatal rats and marmosets

less than in the rat aged 7 days. These marmosets excreted approximately one third of the dose as unchanged PAB. Marmosets aged 10 and 11 days excreted the dose more rapidly. Unchanged PAB constituted not more than 6% of the excreted products and PAH less than 5%. The relative amount of PABG was not significantly different from that excreted by the younger animals but the relative proportion of PAAB was higher being above 80% of the metabolites excreted.

Incubation of a sample of the urine from a 5 day old marmoset, which had been dosed with PAB, with β-glucuronidase both with and without D-saccharic acid-1,4-lactone, was carried out and the relative amounts of the metabolites present in the urine are given in Table 4.3. The results show that the glucuronide was hydrolysed without any increase in the relative amount of PAB but with an increase in PAAB suggesting that the double conjugate N-acetyl p-aminobenzoylglucuronide constituted the glucuronic acid conjugate in this marmoset. This was also suggested from the results shown in Table 4.4 for this animal in which an increase in aromatic amino compound occurred on acid hydrolysis of "PABG".

Discussion

The results show that in the adult marmoset a greater proportion of administered PAB is acetylated than in the rat. The major metabolite in the rat is the glycine conjugate PAH. In both species PABG is excreted.

The development of the conjugating systems responsible for the metabolism of PAB as determined by the study of the metabolic fate of administered PAB in animals of different ages is completely different in the rat and marmoset. In the rat conjugation of PAB with glycine becomes increasingly important and appears to approach adult levels when the animals are 11 days old. The conjugation of PAB with glucuronic acid becomes less important falling to adult levels by 11 days of age. No marked change in the proportion of the dose acetylated occurs between 7 and 11 days but the extent of acetylation is greater in young than in adult rats.

The metabolic fate of PAB is similar in both 4 and 5 day old marmosets but different from that in 10 and 11 day old marmosets by which time the metabolic profile of PAB closely resembles that in the adult animals. In the neonatal period between 5 and 10 days post partum, the acetylation of PAB becomes increasingly important whereas there is little change in the relative activities of the other conjugating systems. With this increase in the proportion of the dose excreted as PAAB the amount of unchanged PAB excreted falls.

Table 4.4 shows that the metabolic fate of PAB was investigated in two 4 day old and two 5 day old marmosets one of each pair being of different parentage. The offspring from parents No. 462 and No. 466 were always smaller than those from

the other two sets of parents (see Fig 2.1) and at the age of 5 days their weight was, on average, only 65% of that of 5 day old offspring from the other parents. These smaller animals when compared with the larger offspring from parents No. 417 and No. 418 excreted less of the administered PAB in the acetylated form and more in the unchanged form reflecting the developmental pattern outlined above.

The overall results for the metabolic fate of PAB in the neonatal rat and marmoset suggest that whereas in the young rat the metabolic fate of PAB is determined by the developmental changes in the relative activities of the enzyme systems responsible for the conjugation of PAB with glycine, glucuronic acid and acetate, in the young marmoset only the development of the acetylation system appears to govern the metabolic fate of administered PAB.

Using PAB as substrate, Brandt (1964, 1966) showed that glycine N-acyltransferase activity was present in the livers of rats at about 6 days of age and continually increased thereafter until the rats were about 30 days of age. The *in vivo* results reported here for the conjugation of administered PAB with glycine support these early *in vitro* experiments. As with administered benzoic acid (see chapter 3) glucosiduronic acid formation from PAB is much more important in the neonatal rat than it is in the adult, which may be the result of the 'over-shoot' phenomenon mentioned earlier (p.67). Glucuronidation accounts for a greater proportion of the dose in 7 day old rats than in those aged 11 days suggesting that the relative activity of the enzyme systems catalysing the conjugation of PAB with glucuronic acid and glycine changes during this period. In view of the ability of the young rat to synthesise hippuric acid from

administered benzoate as described in the last chapter it is unlikely that the availability of glycine is rate limiting in the metabolism of PAB at the dose level used. The small proportion of the dose of PAB conjugated with glycine in the young rat as compared with the formation of hippuric acid from benzoate may be due to the lower affinity of the glycine N-acyltransferase for PAB as compared with its affinity for benzoic acid or different enzymes may be involved. A further explanation might be the competition of the alternative acetylation pathway for PAB.

In chapter 3 it was reported that in neonatal marmosets the rate of glycine utilisation in the formation of hippuric acid became rate limiting when benzoic acid was administered at or below a dose level of 40mg/kg body weight. While such a limitation may partly determine the metabolic fate of PAB, an alternative pathway of metabolism, N-acetylation, is available and appears to be active in 4 and 5 day old marmosets and to reach adult levels of activity by 10 days of age. The high activity of the acetylation system in the young marmoset is emphasised by the observation that a 5 day old marmoset studied excreted no PABG, only the acetylated double conjugate N-acetyl p-aminobenzoylglucuronide.

Vest (1965) and Vest and Salzberg (1965) reported that the development of the conjugating systems responsible for the metabolism of PAB in the human were manifested by an increase in the formation of both PAH and PABG and a decrease in PAAB formation. A detailed study of the results obtained by Vest and Salzberg shows that the decrease in the proportion of the metabolites excreted as PAAB was not completely compensated for by the formation of PAH and PABG and that between the ages of 8

weeks and 8.5 years the substantial decrease in the excretion of PAAB was accompanied by an increase in the proportion of the dose that was excreted unchanged. This suggests that it is not so much an increase in the activity of the PAH- and PABG-synthetase systems that is responsible for the developmental changes observed in the metabolic fate of PAB in humans, but a decrease in the activity of the N-acetylase system. However, whatever changes in enzyme activity are involved, the metabolic fate of PAB in both the rat and man follows a similar developmental pattern, with the exception of a possible 'overshoot' in the activity of the PAB-UDPGT system in the very young rat.

The change in the metabolic profile of PAB with age is very different in the marmoset from that in man. The most marked difference is the increase in the ability to acetylate PAB which clearly increases with age whereas in man the proportion of administered PAB which is acetylated decreases with age.

CHAPTER 5

The activity of hepatic and renal bilirubin-UDPGT in the marmoset

Chapter 5 - The activity of hepatic and renal bilirubin-UDPGT in the marmoset

Introduction

The results described in chapters 3 and 4 showed that both neonatal rats and marmosets excreted a higher proportion of administered benzoic acid and PAB as their glucosiduronic acids than did their adult counterparts. Furthermore, glucuronidation appeared to be a more important biotransformation reaction in marmosets than it was in rats. In view of these findings, experiments were carried out to determine the activity of UDPGT in the liver and kidney of the marmoset and to compare it with that in the rat using bilirubin as substrate; the results are now reported.

Materials and Methods

Materials

UDPGA (ammonium salt), bilirubin (from bovine gall stones) and bovine serum albumin were purchased from Sigma Chemical Co. Ltd. Ethylanthranilate was purchased from Eastman Organic Chemicals (Rochester N.Y.) and methyl n-propyl ketone from BDH Chemicals Ltd., Poole.

All the other materials used in this study were available from the laboratory stores.

Methods

Animals

As the number of marmosets in the colony was small and each animal valuable no marmoset was deliberately killed to provide tissue for the determination of bilirubin-UDPGT activity. Assays were carried out using the tissues of animals which had died from natural causes or which were killed after sustaining physical injury. The marmosets in this colony nearly always gave birth to triplets one of which was abandoned either at birth or after a few days. Neonates abandoned at birth were invariably discarded overnight so that these animals had been dead for some hours before the tissues were obtained. The animals which were rejected after a few days were ill-treated by the mother and when this was detected the young animal was taken and killed. Two young males injured each other fighting and had to be killed. All animals were killed by exposure to CO_2, their liver and kidneys removed and stored at $-20^{o}C$ until the assays could be carried out.

For each determination of the enzymic activity of the

tissues of a marmoset, rats of the same sex and comparable maturity were killed by exposure to CO_2 and the bilirubin-UDPGT activity of their tissues determined at the same time. In some cases this was not possible and then fresh tissue from an adult rat was included as a check that the assay was satisfactory.

Preparation of homogenates

Homogenates of liver and kidney (10% w/v) were prepared using ice-cold 0.25M sucrose in 0.001M EDTA (pH 7.4) by rapid homogenisation using a teflon head. An equal volume of a 0.4% (w/v) solution of Lubrol in 0.25M sucrose was then added. The rate of bilirubin conjugation with glucuronic acid by an aliquot (0.2ml) of this homogenate was then assayed by the method of Van Roy and Heirwegh (1968), using an incubation period of 30min. A control digest to which no UDPGA or $MgCl_2$ was added was also incubated.

Measurement of the rate of bilirubin conjugation

In the method employed conjugated bilirubin was converted to an azo pigment the absorbance of which was read against a solvent blank at 530nm using a Unicam SP1800 spectrophotometer. Subtraction of the absorbance of the control from that of the test gave a value equivalent to the amount of conjugated bilirubin formed by 10mg of tissue in 30min.

Protein determinations were carried out on the homogenates by the method of Lowry *et al.* (1951).

Results

The activities of hepatic bilirubin-UDPGT found in the abandoned neonatal marmosets ranged from 0.7 - 12.4µg bilirubin conjugated/h/mg protein and exhibited no obvious trend with age. The wide variability in these results was probably due to the condition of the tissues used in the assays. Beyond showing that the enzyme was present at birth these results are not considered of value and are not included in Table 5.1.

The results obtained using tissues dissected from animals immediately after they were killed are summarised in Table 5.1. It can be seen that the specific activity of hepatic bilirubin-UDPGT does not vary significantly over the range 24 days to adult in the rat. The results for the corresponding hepatic enzyme activity in the marmoset were lower than in the rat being on average 79% of the value found for the rat liver.

The specific activity of renal bilirubin-UDPGT in the marmoset was found to be 14% of that in the liver while that of the rat was 21% of the level in the liver. The results indicate that in both species the activity of this enzyme developed more slowly in the kidney than it did in the liver.

Species	Sex	Age	Organ	Activity of bilirubin-UDPGT expressed in terms of µg bilirubin conjugated/h :- g tissue	organ	mg protein
Rat	M	A	L	3500	51850	21.9
	M	A	L	2350	35110	12.5
	F	A	L	2930	30590	15.3
	F	8w	L	3434	23540	17.5
	M	7w	L	2723	26524	14.7
	M	24d	L	2620	7070	15.3
Marmoset	M	29w	L	1435	15510	10.2
	M	29w	L	932	10870	6.3
	F	8w	L	2205	9116	13.2
	M	4w	L	3480	9990	21.6
	U	5d	L	1480	1990	9.9
Rat	M	A	K	858	2673	5.5
	M	A	K	474	1330	3.0
	F	A	K	488	1247	2.8
	F	8w	K	266	381	1.8
	M	7w	K	784	1570	5.5
	M	24d	K	74	56	0.6
Marmoset	M	29w	K	326	525	2.7
	M	29w	K	518	1010	4.3
	F	8w	K	59	50	0.4
	M	4w	K	44	210	0.4
	U	5d	K	59	10	0.5

M - male F - female U - sex undetermined

A - adult w - weeks d - days

L - liver K - kidney

Table 5.1 Activities of hepatic and renal bilirubin-UDPGT in rats and marmosets of various ages.

Discussion

It is probable that the levels of bilirubin-UDPGT activity found in the marmoset represent minimum values. The animals used were all in a relatively poor condition with the exception of the four-week old animal which was killed immediately after it was found with an almost severed hand but was otherwise healthy; this animal had the highest level of bilirubin-UDPGT activity found in the liver of all the marmosets examined.

Black and Billing (1969) reported that the average rate of conjugation of bilirubin in the human liver was 1100 ± 280µg conjugated/g liver/h which is similar to that rate obtained in this study using the 29-week old marmosets. The results obtained for healthy rats of different ages were higher than those obtained for the marmoset but it may be tentatively concluded that the bilirubin-UDPGT activity is of the same order in the marmoset as it is in the rat.

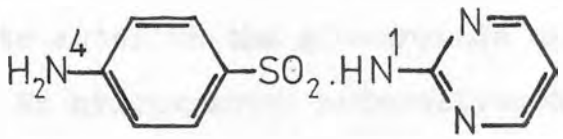
CHAPTER 6

The comparative metabolism of sulphadiazine

Chapter 6 - The comparative metabolism of sulphadiazine

Introduction

Sulphadiazine (2-sulphanilamidopyrimidine) (I) is a member of the sulphonamide group of antibacterial drugs. It is known that in order to maintain therapeutic concentrations in the blood,



(I)

sulphonamides must usually be administered to a patient several times a day. However sulphadiazine is readily absorbed but not rapidly excreted resulting in the maintenance of high blood levels of the drug for a considerable time after a single dose (Sadusk and Tredway, 1941). Consequently the maintenance of therapeutic blood levels of sulphadiazine can be achieved by less frequent administration of 'booster-doses' than are required for other sulphonamides. Recently studies have been carried out to test the suitability of sulphadiazine in combination with trimethoprim in the treatment of urinary tract infections (Männistö et al., 1973; Tuomisto et al., 1977; Seppänen and Wilen, 1978).

Sulphadiazine may be expected to undergo four main metabolic transformations consisting of N^4-acetylation, -glucuronidation, -sulphation, and N^1-glucuronidation (see p.56). However since N^1-glucuronide formation, a reaction characteristic of primate species only, occurs with a limited number of sulphanilamido-pyrimidines, it may not take place with sulphadiazine (Bridges et

al., 1966, 1969a,b; Walker and Williams, 1972).

Welch et al. (1943) in a study of the comparative metabolism of sulphamethazine, sulphamerazine and sulphadiazine in human volunteers reported that of these three drugs sulphadiazine was the least acetylated. This result was confirmed by Gilligan (1945) who studied the metabolic fate of a number of sulphonamides in patients undergoing sulphonamide therapy. In those patients treated with sulphadiazine there was no appreciable increase in either the sulphate ester or the glucuronide excretion over control levels. No hydroxylated metabolites of sulphadiazine were excreted by these patients. Similar results to these for man were obtained for the rabbit (Smith and Williams, 1948a). By administering N^4-acetylsulphadiazine to rabbits Smith and Williams (1948a)were able to show that the relatively low excretion of sulphadiazine as its N^4-acetyl derivative as compared with other sulphonamides, was not due to an active deacetylase system. However Shaffer and Bieter (1950) demonstrated the presence of an active N-deacetylase system in the kidney of the chick.

In 1963, Uno et al.reported the presence of unchanged sulphadiazine, sulphadiazine N^4-glucuronide, sulphadiazine N^4-sulphate, N^4-acetylsulphadiazine and sulphanilamide in the urine of humans after the oral administration of sulphadiazine. Later Uno and Sekine (1966) determined these metabolites in the 48h urine of humans dosed with sulphadiazine (2g) and found 43% of the dose was excreted unchanged, 36.5% in the acetylated form, 0.7% as the N^4-sulphate, 0.5% as the N^4-glucuronide and 0.3% as sulphanilamide. Madsen (1966) reported similar results for the excretion of the unchanged and acetylated drug but found that 4% of the dose excreted was conjugated with glucuronic acid. These reports indicate that in man, sulphadiazine is metabolised mainly

to its N^4-acetyl derivative, the other possible conjugates appearing in the urine in only trace amounts.

Atef and Nielsen (1975) investigated the metabolism of sulphadiazine in goats and found that like man, these animals excreted a large proportion of the dose in the unchanged form, but that unlike man little N^4-acetylsulphadiazine was excreted in the urine. The major metabolite of sulphadiazine detected in the urine of this species was the hydroxylated product 2-sulphanilamido-4-hydroxypyrimidine. Bray *et al.* (1951b)had previously reported the presence of small quantities of an oxidation product of sulphadiazine in the urine of rabbits to which sulphadiazine had been administered. This metabolite appeared to be oxidised in the 3-position of the benzene ring and not in the pyrimidine ring as reported for the goat. Nielsen (1973a, b) reported that sulphadimidine (4,6-dimethyl-2-sulphanilamidopyrimidine) was metabolised in goats and cows mainly by oxidative reactions which together with the results mentioned above for sulphadiazine suggest that the oxidation of sulphonamides is an important metabolic reaction in ruminants only.

This chapter describes investigations into the metabolic fate of both labelled and unlabelled sulphadiazine in the adult marmoset, rat and rabbit.

Materials

[^{35}S] Sulphadiazine (2.3mCi; 29.8μCi/mg) was obtained from the Radiochemical Centre Ltd., the reference date from which all decay was calculated being 6th September 1976. Because of the short half-life (87.1 days) of sulphur-35, the labelled sulphadiazine was diluted in small batches with an appropriate quantity of unlabelled sulphadiazine (Sigma Chemical Co. Ltd., Surrey) shortly before use so that doses of suitable specific activity could be administered. The procedure for diluting the labelled sulphadiazine involved dissolving the required quantities of both labelled and unlabelled sulphadiazine in a mixture of aqueous ammonia (sp. gr. 0.88) and ethanol (1:10 v/v) and mixing the solution thoroughly for 10min. The solvents were then removed at 40°C under reduced pressure and the product dried in a vacuum desiccator.

The mass spectrum of the diluted [^{35}S] sulphadiazine is shown in Figure 6.1. It exhibits no top mass ion (M^+) peak at 250, its molecular weight, but instead the fragmentation pattern is dominated by two ion peaks at $^m/_e$ 185 and 186. The peak at $^m/_e$ 186 corresponds to M^+-64 representing the loss of SO_2 from the molecule. Similar fragmentation patterns with the expulsion of SO_2 have previously been described for sulphadiazine (Atef and Nielsen, 1975) and for sulphonylureas (Budzikiewicz et al., 1967). Mass measurement of the peak at $^m/_e$ 186 gave a value of 186.0891 ± 0.005 whereas that for $C_{10}H_{10}N_4$ (M^+-SO_2) requires 186.0905. Other peaks in the fragmentation pattern of the diluted [^{35}S]-sulphadiazine occur at $^m/_e$ values of 108, 92 and 65 which can be accounted for by the proposed breakdown of sulphanilamide (Budzikiewicz et al., 1967) (Fig 6.2). The peaks at $^m/_e$ 172 and

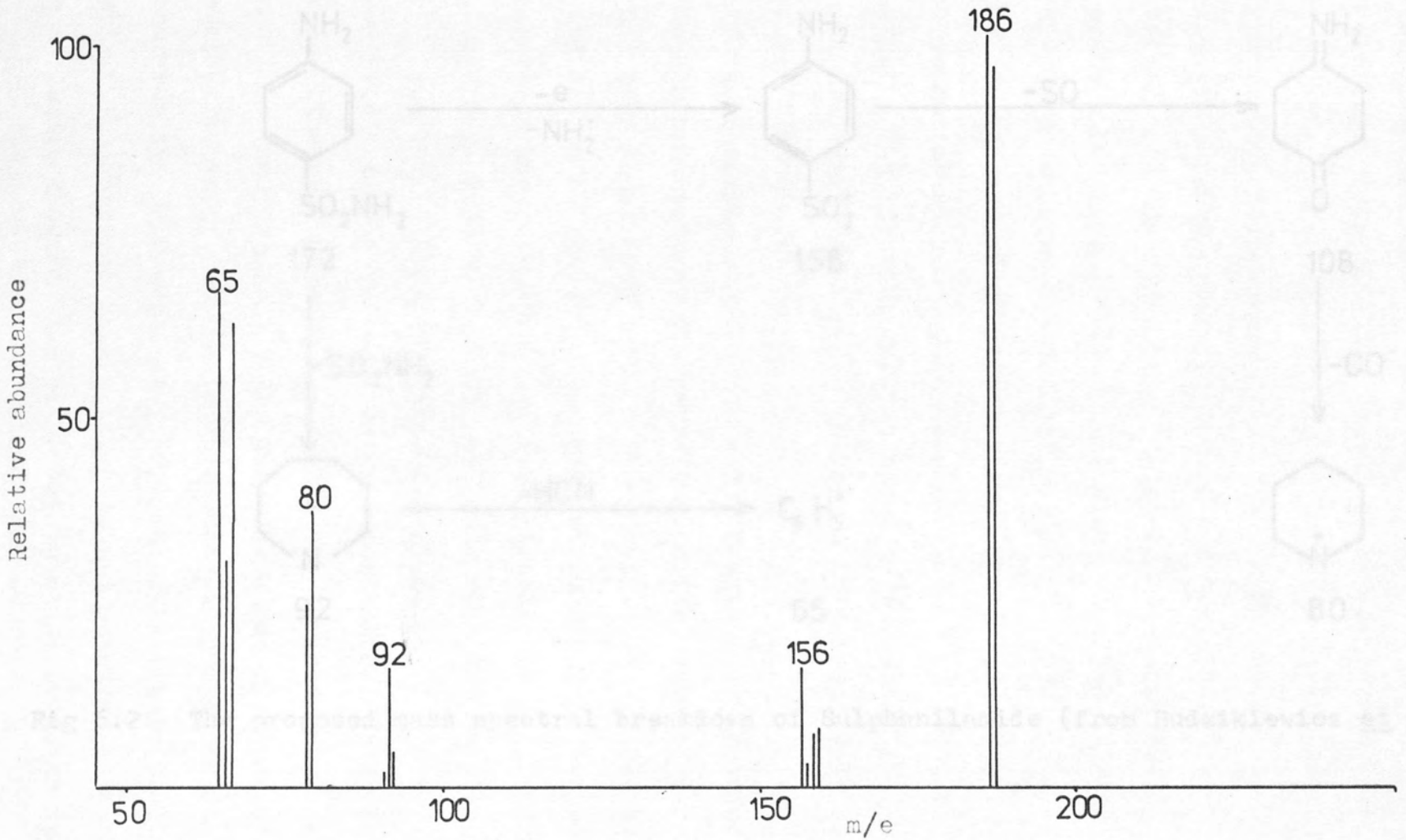


Fig 6.1 Mass spectrum of [^{35}S] sulphadiazine following its dilution in ammoniacal ethanol.

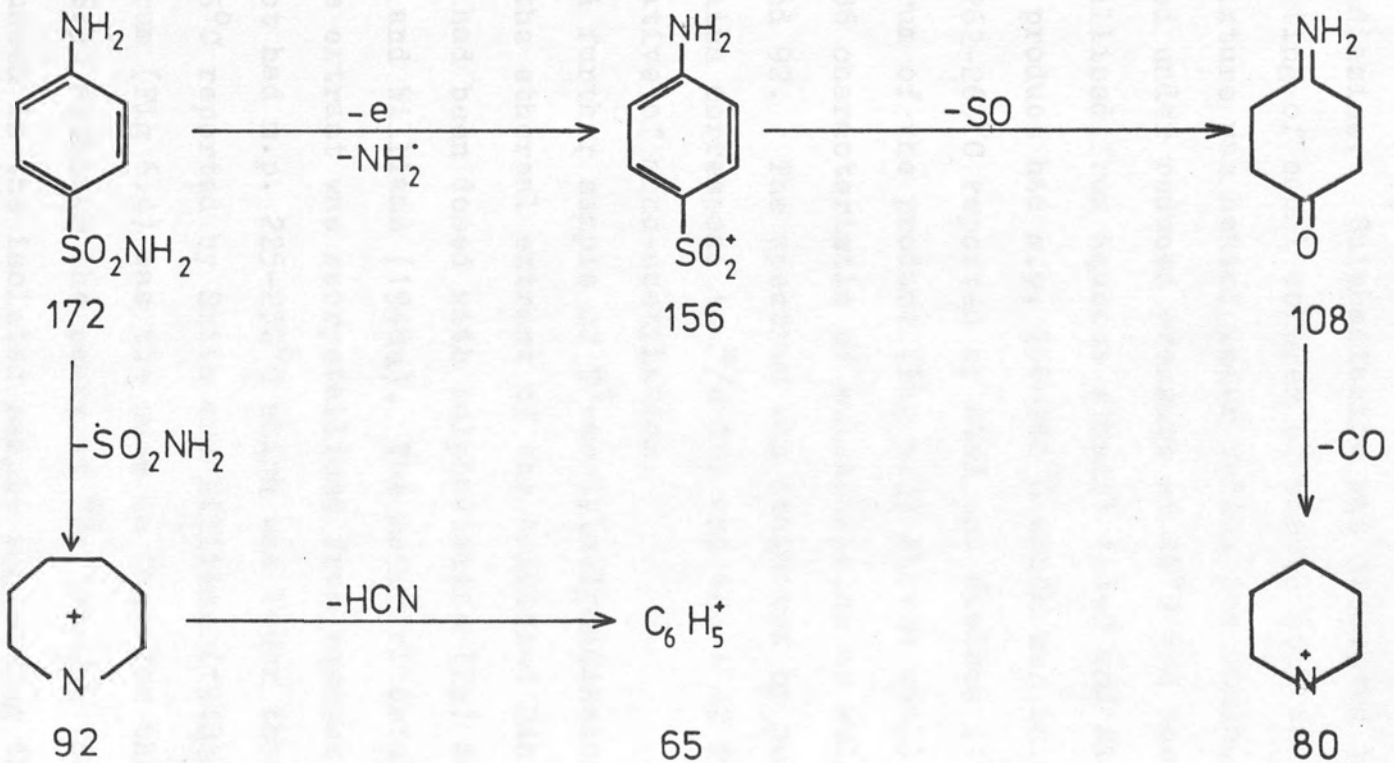


Fig 6.2 The proposed mass spectral breakdown of Sulphanilamide (from Budzikiewicz et al. (1967)).

156 are not seen with sulphadiazine since the sulphur atom has been lost from the molecule.

N^4-Acetylsulphadiazine was prepared by the acetylation of sulphadiazine. Sulphadiazine was dissolved in a solution consisting of equal volumes of acetic acid and acetic anhydride. The mixture was heated under reflux for 30min, the solvents removed under reduced pressure at 40°C and the product re-crystallised from aqueous ethanol (Atef and Nielsen, 1975). The final product had m.p. 258-260°C which was in close agreement with that 262-263°C reported by Atef and Nielsen (1975). The mass spectrum of the product (Fig 6.3) showed small peaks at $^m/_e$ 185 and 186 characteristic of sulphadiazine as well as those at $^m/_e$ 108 and 92. The spectrum was dominated by peaks at $^m/_e$ 227 and 228 which correspond to $^m/_e$ 185 and 186 + 42 respectively and are indicative of mono-acetylation.

A further sample of N^4-acetylsulphadiazine was isolated from the ethereal extract of the acidified 24h urine of a rabbit which had been dosed with sulphadiazine (2g) as described by Smith and Williams (1948a). The material obtained by evaporation of the extract was recrystallised from aqueous ethanol. The product had m.p. 225-230°C which was lower than the melting point of 245°C reported by Smith and Williams (1948a). Its mass spectrum (Fig 6.4) was the same as that for the synthetic material (Fig 6.3) although the peaks at $^m/_e$ 185 and 186 were more pronounced in the isolated sample suggesting the presence of some free sulphadiazine. This was also indicated by the results obtained from the HPLC analysis of the material (Table 6.4) and may explain the lower melting point observed compared with that of the synthetic material and with that reported by Smith and

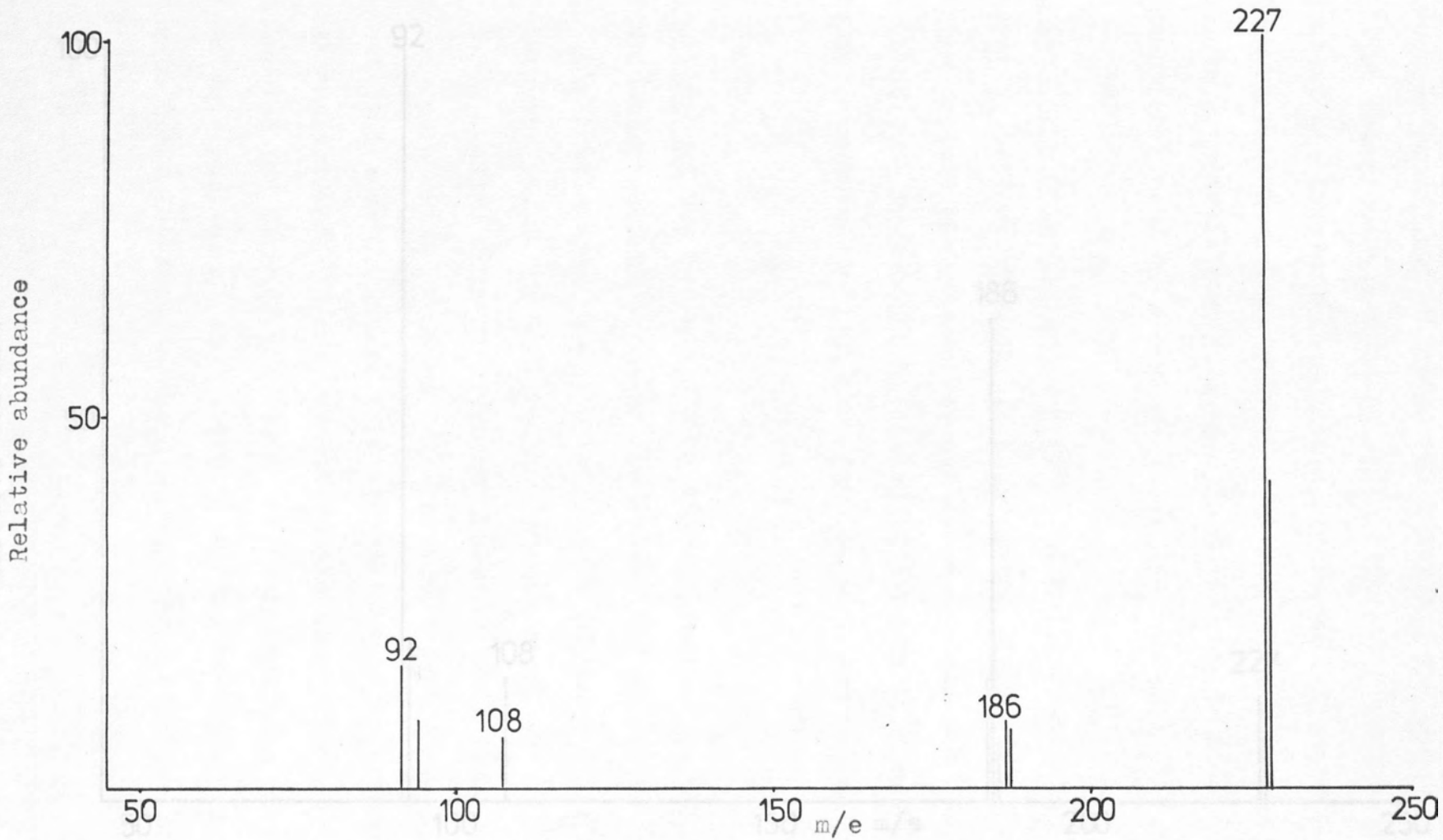


Fig 6.3 The mass spectrum of synthetic N^4-acetylsulphadiazine.

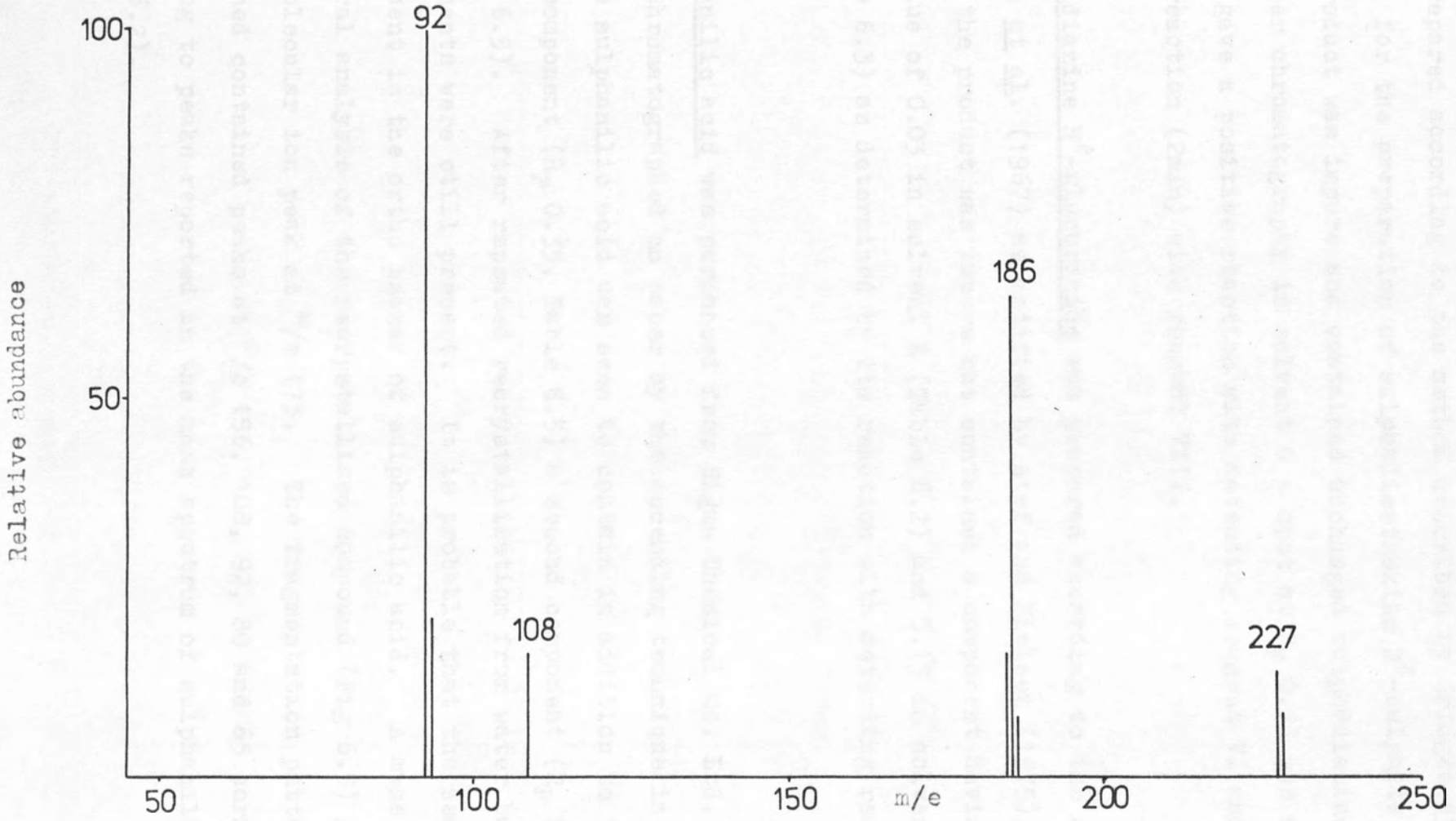


Fig 6.4 The mass spectrum of N^4-acetylsulphadiazine isolated from the urine of rabbits which had been dosed with sulphadiazine (2g).

Williams (1948a)for their isolated compound.

Sulphadiazine N^4-sulphate. The potassium salt of this compound was prepared according to the method described by Bridges et al. (1968) for the preparation of sulphadimethoxine N^4-sulphate. The product was impure and contained unchanged sulphadiazine. On paper chromatography in solvent G a spot at R_F 0.22 was seen which gave a positive reaction with detecting reagent VI and a slow reaction (2min) with reagent VIII.

Sulphadiazine N^4-glucuronide was prepared according to the method of Uno et al. (1967) as modified by Atef and Nielsen (1975). Again the product was impure but contained a component having an R_F value of 0.03 in solvent E (Table 6.2) and 0.19 in solvent G (Table 6.3) as determined by its reaction with detecting reagent VIII.

Sulphanilic acid was purchased from Sigma Chemical Co. Ltd. When chromatographed on paper by the ascending technique in solvent G, the sulphanilic acid was seen to contain in addition to the main component (R_F 0.35, Table 6.5) a second component (R_F 0.47, Table 6.5). After repeated recrystallisation from water both components were still present. It is probable that the second component is the ortho isomer of sulphanilic acid. A mass spectral analysis of the recrystallised compound (Fig 6.5) gave the molecular ion peak at $^m/e$ 173. The fragmentation pattern obtained contained peaks at $^m/e$ 156, 108, 92, 80 and 65 corresponding to peaks reported in the mass spectrum of sulphanilamide (Fig 6.2).

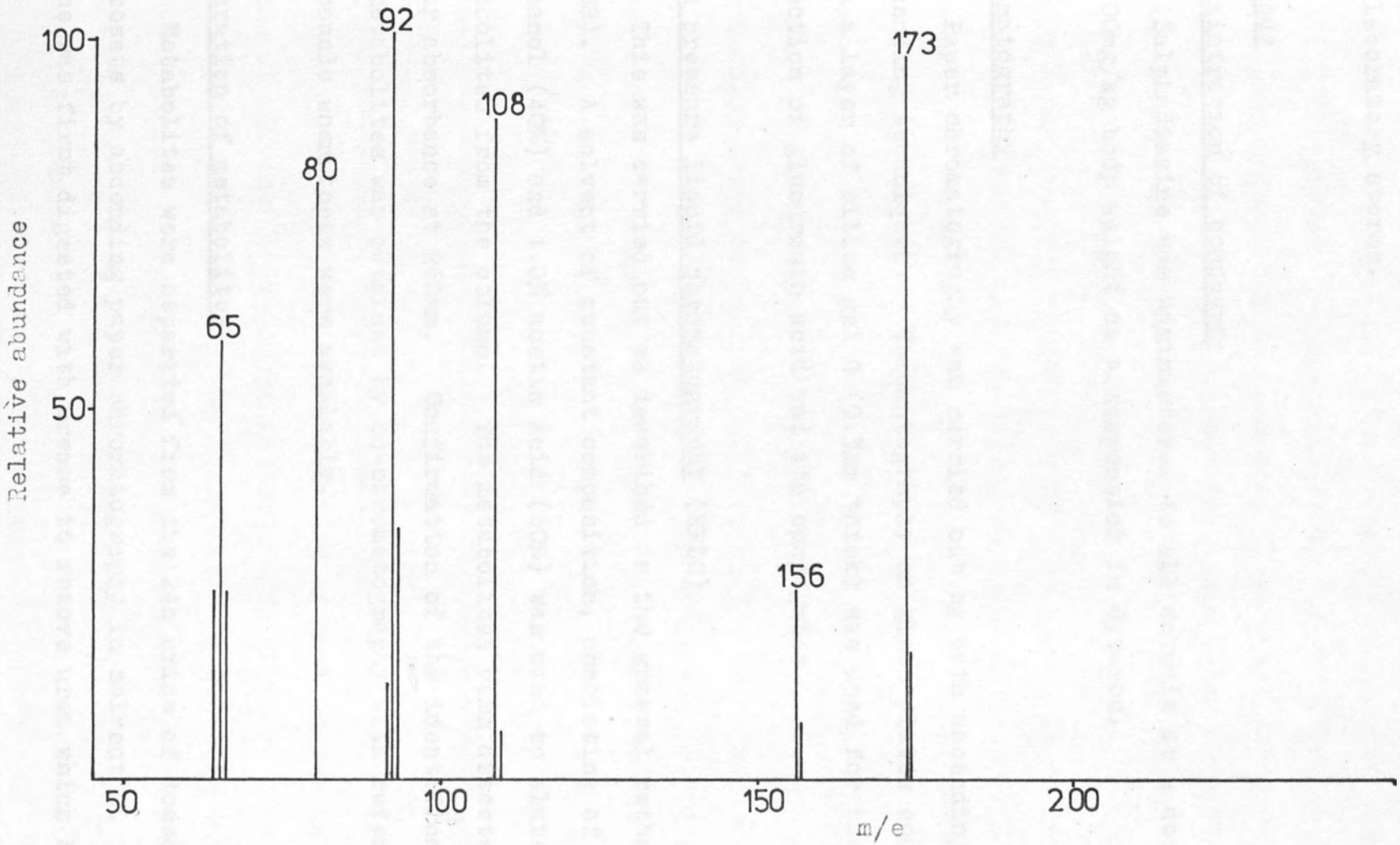


Fig 6.5 Mass spectrum of recrystallised sulphanilic acid.

The enzyme preparations used were all obtained from Sigma Chemical Co. Ltd., Surrey.

All other chemicals used in this study were available from the laboratory stores.

Methods

Administration of compound

Sulphadiazine was administered to all animals at a dose level of 100mg/kg body weight as a suspension in Cytacon.

Chromatography

Paper chromatography was carried out by both ascending and descending techniques. Chromatography on glass plates coated with a layer of silica gel G (0.3mm thick) was used for the detection of glucuronic acid and its conjugates.

High pressure liquid chromatography (HPLC)

This was carried out as described in the general methods (p.88). A solvent of constant composition, consisting of methanol (40%) and 1.0% acetic acid (60%) was used to elute the metabolites from the column. The metabolites were detected by their absorbance at 260nm. Confirmation of the identification of metabolites was obtained by co-chromatography with reference compounds where these were available.

Separation of metabolites

Metabolites were separated from the 24h urine of dosed marmosets by ascending paper chromatography in solvent G. The urine was first digested with urease to remove urea which had a

similar R_F value to one of the metabolites of sulphadiazine (Table 6.3). Digestion with urease type III (from Jackbeans) was carried out at 30°C. The pH of the digest was maintained at 7.0 by the addition of 2M HCl at intervals. The treated urine was applied as a streak to a 9" wide chromatogram which was developed in solvent G. A sample strip was cut from the dried chromatogram and sprayed with reagent VIII. The zones corresponding to the located metabolites (G_1 and G_2) were cut out and eluted with methanol. Metabolite G_1 (Table 6.3) contained two overlapping components one of which, the lower R_F component (G_{1b}), gave an immediate colour with reagent VIII while the second component (G_{1a}) which had the higher R_F value, developed the colour in about 12h. The two metabolites were eluted separately. The extracts were evaporated under reduced pressure at room temperature and the residues containing the partially purified metabolites were used for their identification. Control 24h urine samples, from animals which had received Cytacon only, were treated in the same way, and extracts prepared from the zones with R_F values corresponding to those of the different extracts prepared from the urine of dosed animals.

Determination of unchanged sulphadiazine and its metabolites excreted in urine.

Aromatic amino and N-acetylamino compounds

These were determined by the method of Bratton and Marshall (1939) as previously described (p.134). Calibration curves were prepared with sulphadiazine and the absorbance was determined at 550nm. Similar determinations were carried out on the urine collected from undosed animals and from animals which had received Cytacon only.

In experiments using [^{35}S]sulphadiazine the recovery of the dose in urine was measured by liquid scintillation counting as previously described (p.89). The individual radioactive components in the urine were separated by ascending paper chromatography in solvents E and G and the distribution of radioactivity along the chromatograms measured as previously described (p.90) using 1cm sections. The average recovery of the radioactivity applied to the chromatograms was 93%.

Determination of dose in faeces

The 24h faeces of dosed animals were lyophilized and ground to a fine powder which was extracted with 0.2M HCl. Aliquots of the extract were used for measurement of radioactivity and of material containing aromatic amino compounds. The recovery of sulphadiazine added to a sample of dried faeces was 98% as determined by the method of Bratton and Marshall (1939).

Blood levels of sulphadiazine and its metabolites

The levels of radioactivity in blood samples taken from marmosets at intervals after the oral administration of [^{35}S]-sulphadiazine were determined by the method previously described (p.90).

Results

The R_F values of the reference compounds and of the three main urinary constituents excreted by dosed animals and not seen in normal urine are summarised in Table 6.1. In some systems sulphadiazine and N^4-acetylsulphadiazine overlapped on the chromatograms but the two compounds were distinguished because the sulphadiazine gave an immediate reaction with reagent VIII while the N^4-acetyl derivative reacted slowly, the colour developing in about 12h. Better separations of sulphadiazine and its metabolites were achieved by ascending chromatography in solvents E and G; R_F values of minor metabolites in addition to those shown in Table 6.1 are given in Tables 6.2 and 6.3 respectively. Chromatography, in these two solvents, of a sample of urine from animals to which Cytacon alone had been administered and from undosed animals, indicated that the metabolites detected by reagent VIII in the urine of dosed animals were not due to the dosing vehicle.

Identification of unchanged sulphadiazine and its metabolites in the urine of dosed animals.

Sulphadiazine

Unchanged sulphadiazine was detected in the urine of all dosed animals by ascending paper chromatography (Metabolite 1, Table 6.1; E_2, Table 6.2; G_1, Table 6.3). A sample of the material separated from the urine of a dosed marmoset (p.165; extract G_{1b}) had the same retention time as sulphadiazine on HPLC (Table 6.4). The aromatic amine content of this extract increased slightly on acid hydrolysis as determined by the method of Bratton and Marshall (1939) which indicated that it was

Compound	R_F value in the solvent listed							
	E[b]	F[a]	G[b]	H[b]	I[a]	I[b]	J[b]	K[a]
Urinary metabolites								
1	0.26	0.77	0.77	0.79	0.09	0.09	0.77	0.81
2	0.45	nd	0.82	nd	0.17	0.17	0.82	nd
3	nd	0.62	0.63	0.62	nd	nd	nd	nd
Reference compounds								
Sulphadiazine	0.28	0.79	0.77	0.79	0.07	0.09	0.78	0.80
N^4-Acetylsulphadiazine	0.45	ne	0.82	0.79	0.16	0.17	0.83	0.84

All chromatograms were developed on Whatman 3MM paper either by the descending (a) or ascending (b) technique.

All reference compounds and metabolites were detected using reagent VIII. Metabolites 1 and 3 and sulphadiazine gave an immediate purple colour with this reagent, white metabolite 2 and N^4-acetylsulphadiazine developed the colour after about 12h.

nd - not determined ne - not examined

Table 6.1 Chromatographic properties of reference compounds and major metabolites of sulphadiazine

Compound	R_F value	Colour with reagent VIII	Time to develop colour	Reaction with reagent I	Species in urine from which metabolite was detected
Metabolite					
E_1	0.45	pu	8-24h	-	M, Ra, Rb
E_2	0.26	pu	imm	-	M, Ra, Rb
E_3	0.16	pi.fl	imm	-	M, Ra, Rb
E_4	0.10	pi.fl	imm	-	M, Ra
E_5	0.03	pi.fl	imm	-	M, Ra, Rb
Reference compounds					
N^4-Acetylsulphadiazine	0.45	pu	8-24h	-	-
Sulphadiazine	0.28	pu	imm	-	-
Sulphadiazine N^4-glucuronide	0.03	pi.fl	imm	bl/br	-

Colour codes: pu, purple; pi.fl, pink fluorescence (seen under UV light); bl/br, blue brown.
imm - immediate colour development.
Species: M, marmoset; Ra, rat; Rb, rabbit.

Table 6.2 Detailed chromatographic properties of urinary metabolites and reference compounds as determined by ascending chromatography in solvent E

Compound	R_F value	Colour with reagent VIII	Time to develop colour	Colour reaction with reagents					Species in urine from which metabolite was detected
				I	II	IV	VI	XII	
Metabolite									
G_1	0.77-0.82	pu	imm[+]	-	red	bl	-	or	M, Ra, Rb
G_2	0.63	pu	imm	-	-	-	-	-	M, Ra
G_3	0.27	pi	imm	-	-	-	-	ye	M
G_4	0.23	pi.fl	2min	-	-	bl	-	-	Ra, Rb
G_5	0.19	pi.fl	1min	-	-	-	-	-	M, Ra
Reference compounds									
N^4-Acetylsulphadiazine	0.82	pu	8-24h	-	red	bl	-	or	
Sulphadiazine	0.77	pu	imm	-	red	bl	-	or	
Sulphadiazine N^4-sulphate	0.22	pi	2min	-	ne	ne	ye	ne	
Sulphadiazine N^4-glucuronide	0.18	pi.fl	imm	bl/br	ne	ne	-	ne	
Normal metabolite urea	0.53	pi	imm	-	-	-	-	-	

Colour codes: pu, purple; pi, pink; pi.fl, pink fluorescence (seen under UV light); bl/br, blue-brown; bl, blue; or, orange; ye, yellow.
imm, immediate colour development. [+]This spot developed an immediate purple colour on spraying with reagent VIII. After 12h a purple cap appeared.
ne, not examined.
Species: M, marmoset; Ra, rat; Rb, rabbit.

Table 6.3 Detailed chromatographic properties of urinary metabolites and reference compounds as determined by ascending chromatography in solvent G

Sample	Retention time (min)	
	Main peak (s)	Subsidiary peak (s)
Reference Compounds		
Sulphadiazine	2.00	-
N^4-Acetylsulphadiazine (a)	2.55	-
N^4-Acetylsulphadiazine (b)	2.60	2.00
Extracts		
CG_{1a}	1.55	-
CG_{1b}	1.55	-
CG_2	1.50	1.70
G_{1a}	2.65	1.55
G_{1b}	2.00	2.65, 1.55
G_2	1.55	1.70, 1.85, 2.00
G_{1a} + N^4-acetylsulphadiazine	2.60	1.55, 2.00
G_{1b} + N^4-acetylsulphadiazine	2.05, 2.65	-
G_{1b} + sulphadiazine	2.05	2.65, 1.55

(a) synthetic compound
(b) isolated from rabbit urine
CG_2 etc - extract from control urine with R_F value equivalent to that of metabolite G_2 etc.

Table 6.4 HPLC analysis of methanolic extracts of urinary metabolites isolated from paper chromatograms developed in solvent G.

slightly contaminated with N^4-acetylsulphadiazine (Metabolite G_{1a}). A similar contamination was also suggested by the HPLC result. The mass spectrum of the isolated metabolite was identical with that of authentic sulphadiazine (Fig 6.1).

N^4-Acetylsulphadiazine

This metabolite was identified on chromatograms by its R_F value and by the colour given with reagent VIII which developed slowly (Metabolite 2, Table 6.1; E_1, Table 6.2; G_1, Table 6.3). A sample of this metabolite separated by preparative paper chromatography (p.165; extract G_{1a}) had the same retention time on HPLC as a synthetic sample of N^4-acetylsulphadiazine (Table 6.4). Analysis of the extract by the Bratton and Marshall (1939) method, before and after acid hydrolysis, showed that the separated metabolite contained 16% free amino compound.

Sulphadiazine N^4-glucuronide was identified in the urine of dosed animals by its R_F value in solvents E and G. (Metabolite E_5, Table 6.2; G_5, Table 6.3), using detecting reagent VIII. Since N^4-glucuronide conjugates of sulphonamides can form spontaneously in the urine (Bridges et al., 1968) this metabolite may be an artifact.

Sulphadiazine N^1-glucuronide

Sulphadiazine N^1-glucuronide (metabolite G_2, Table 6.3) was tentatively identified in the urine of dosed animals by comparison of chromatograms of samples of urine before and after digestion with β-glucuronidase (Table 6.5) when metabolite G_2 was almost completely destroyed and an aromatic amino compound, R_F 0.36 was produced. Two types of β-glucuronidase digestion were used in

Metabolite	R_F in solvent G as determined by reagent VIII		
	Untreated urine	Urine + enzyme[a]	Urine + buffer[b] only
G_1	0.76-0.81	0.75-0.81	0.76-0.81
G_2	0.64	0.62[c]	0.66[d]
G_3	nd	nd	nd
G_4	0.21	0.23	0.23
G_5	0.19	0.19	nd
breakdown product(s)[e]	-	0.36	0.36;[f] 0.42
Reference compounds			
N^4-Acetylsulphadiazine	0.80	—	—
Sulphadiazine	0.76	—	—
Sulphadiazine N^4-glucuronide	0.19	—	—
Sulphanilic acid[e]	0.35, 0.47	—	—

(a) β-glucuronidase type L-1; (b) Sodium acetate buffer 0.2M, pH 3.8; (c) Barely detectable
(d) Faint colour; (e) Both spots developed purple colour immediately on spraying;
(f) Main metabolite.

Table 6.5 The effect of β-glucuronidase digestion on the chromatographic pattern of metabolites seen in solvent G

this study:

(i) β-glucuronidase type H-1 (from Helix pomatia) was used to digest samples of urine mixed with an equal volume of 0.4M sodium acetate buffer pH 5.0

(ii) β-glucuronidase type L-1 (from Patella vulgata) was used to digest samples of urine in an equal volume of 0.2M sodium acetate buffer pH 3.8.

Both types of digest were incubated at 37°C for 12-24h. Control digests prepared as above but with the addition of D-saccharic acid-1,4-lactone were incubated at the same time.

Samples of the metabolite tentatively identified as sulphadiazine N^1-glucuronide by the above procedure were separated by preparative paper chromatography and further examined to determine the extent of acetylation and the glucuronic acid content. There was no increase in the aromatic amine content of the extract on acid hydrolysis as determined by the Bratton and Marshall (1939) test, indicating that metabolite G_2 was not acetylated. A further sample of the metabolite when subjected to the carbazole test for glucosiduronic acids (p. 92), gave a faint pink colour which was not detected with the corresponding extract from normal urine. None of the other extracts gave this positive reaction. Samples of all the methanolic extracts were evaporated, the residue dissolved in 0.2M sodium acetate buffer pH 5.0 (0.5ml) and subjected to digestion with β-glucuronidase (type H-1) for 12h at 37°C. Samples of the digests, together with samples of authentic glucuronic acid, sulphadiazine and sulphanilic acid, were chromatographed on thin layer plates in solvent G and the components detected using reagents I and VIII. The results are shown in Table 6.6. Only metabolite G_2 broke down under β-glucuronidase digestion to yield glucuronic acid; this did not

Sample	Results using detecting reagent I		Results using detecting reagent VIII	
	R_F	colour	R_F	colour
CG_{1a}	-	-	-	-
+ E	0.85	bl/t	-	-
CG_{16}	-	-	-	-
+ E	0.80	bl/t	-	-
CG_2	-	-	-	-
+ E	0.84	bl/t	-	-
G_{1a}	-	-	0.88	pi/pu
+ E	0.86	bl/t	0.87	pi/pu
G_{1b}	-	-	0.82	pi/pu
+ E	0.86	bl/t	0.85	pi/pu
G_2	-	-	0.82	pi/pu
+ E	0.38	bl	0.78	pi/pu
	0.86	bl/t		
glucuronic acid+	0.45	bl	-	-
E + buffer blank	0.80	bl/t	-	-
Sulphadiazine	-	-	0.83	pu
Sulphanilic acid	-	-	0.85	pi/pu

E - β-glucuronidase enzyme type H-1
Colour codes:- bl/t, blue tinge; bl, blue; pu, purple; pi/pu, light purple colour (pinkish)
+Chromatographed in a buffer/enzyme mixture
CG_2 etc:- extract from control urine with an R_F value equivalent to that of metabolite G_2 etc.

Table 6.6 Thin layer chromatographic analysis in solvent G of the effects of β-glucuronidase on extracts isolated from marmoset urine after paper chromatography in solvent G.

occur with the corresponding extract from control urine. (The faint colour seen with reagent I at R_F 0.83 ± 0.02 was also seen in control digests of enzyme and buffer only). Metabolite G_2 appears to break down under β-glucuronidase treatment to yield sulphanilic acid (see later) which has an R_F value similar to that of metabolite G_2 in this chromatographic system. This explains why on treatment of metabolite G_2 with β-glucuronidase no change was observed in the position of the aromatic amino compounds on the chromatogram.

Sulphadiazine N^4-sulphate was detected on chromatograms by its reaction with detecting reagent VIII (metabolite G_4, Table 6.3). The presence of $[^{35}S]$sulphadiazine N^4-sulphate in the urine of dosed animals was confirmed by comparing the distribution of labelled metabolites along chromatograms of the urine before and after it had been digested with an arylsulphatase preparation (from Helix pomatia). The digestion was carried out at 37°C for 12h with an equal volume of 0.075M sodium acetate buffer pH 5.5 to which was added D-saccharic acid-1,4-lactone to inhibit the endogenous β-glucuronidase activity present in the urine (see later) and the β-glucuronidase activity which was present in the arylsulphatase preparation. The results in Table 6.3 show that when the a urine sample from a dosed animal was chromatographed in solvent G, the area occupied by sulphadiazine N^4-sulphate also gave a positive reaction with detecting reagents IV and XII for the detection of phenolic compounds. These results cannot however be used as evidence for the presence of a phenolic metabolite of sulphadiazine since these detecting reagents also gave a positive reaction with both sulphadiazine and N^4-acetylsulphadiazine.

The species in which unchanged sulphadiazine and its metabolites were detected are shown in Tables 6.2 and 6.3.

Determination of sulphadiazine and its metabolites excreted in the urine of dosed marmosets, rats and rabbits.

The quantitative results obtained for the recovery of the dose and the degree of acetylation of sulphadiazine in the three species are summarised in Table 6.7. The radiochemical recoveries after the administration of [^{35}S] sulphadiazine to the animals are also included in this table and show in general, within experimental error, good agreement with those recoveries obtained by the method of Bratton and Marshall (1939).

The urinary distribution of the radioactivity between the sulphadiazine excreted unchanged and its metabolites was determined as previously described (p.90). Figures 6.6 and 6.7 are examples of the histograms obtained using solvents E and G respectively. The percentage of radioactive components excreted that were accounted for as N^4-acetylsulphadiazine (metabolite E_1) as determined in solvent E by this method are included in Table 6.7 and show excellent agreement with the results obtained by the method of Bratton and Marshall (1939) for the proportion of N^4-acetylsulphadiazine present in the urine. Chromatography in solvent E of the extract corresponding to sulphadiazine N^1-glucuronide (metabolite G_2) isolated from marmoset urine, showed that in this solvent metabolite G_2 had a similar R_F value to that of authentic sulphadiazine so that it will be present in metabolite E_2.

The proportion of the total urinary metabolites excreted in 24h urine samples after the administration of [^{35}S] sulphadiazine to the different species as determined in solvents E and G are

Species	Recovery of dose (%)(c) (a)	(b)	Percentage of radioactive components excreted in acetylated form: Of dose (a) administered	Of dose (a) excreted	Of dose (b) excreted
Rat	61	-	6.8	12.1	-
	76	-	9.3	13.1	-
	93	-	18.8	25.9	-
	78	-	10.2	20.5	-
	92	82	6.0	11.7	ne
	87	82	10.9	16.4	ne
	87	77	10.1	18.4	ne
	80	80	6.8	11.8	ne
	92	78	7.5	13.3	15.5
	82	83	10.7	14.5	14.4
	61	61	9.9	17.7	17.8
	84	82	11.2	17.2	18.7
	81	77	11.5	16.7	17.3
average	81	78	10.0	16.1	16.7
Marmoset	32	-	11.3	37.3	-
	32	-	7.3	31.0	-
	26	-	6.8	30.2	-
	29	-	4.1	21.8	-
	47	46	3.9	27.7	ne
	44	40	5.5	27.8	ne
	62	61	14.7	26.7	29.3
	53	53	21.6	50.1	35.8
	59	76	17.3	34.0	40.0
	72	70	16.1	30.1	31.6
	80	ne	23.7	29.6	33.4
average	48.7	58	12.0	31.5	34.0
Rabbit	79	71	26.8 (50.1)	56.9	58.2
	88	67	51.0 (56.9)	63.8	66.4
	84	69	38.9 (53.5)	60.4	62.3

(a) Determined by method of Bratton and Marshall (1939)

(b) Determined by liquid scintillation counting

(c) Total urinary and faecal recoveries were determined for a 24h period after dosing in the marmoset, and a 48h period in the rat and rabbit.

ne - not examined

Figures in parentheses indicate the 48h excretion figures for the rabbits studied.

Table 6.7 Degree of acetylation of sulphadiazine in 24h urine samples of the marmoset, rat and rabbit.

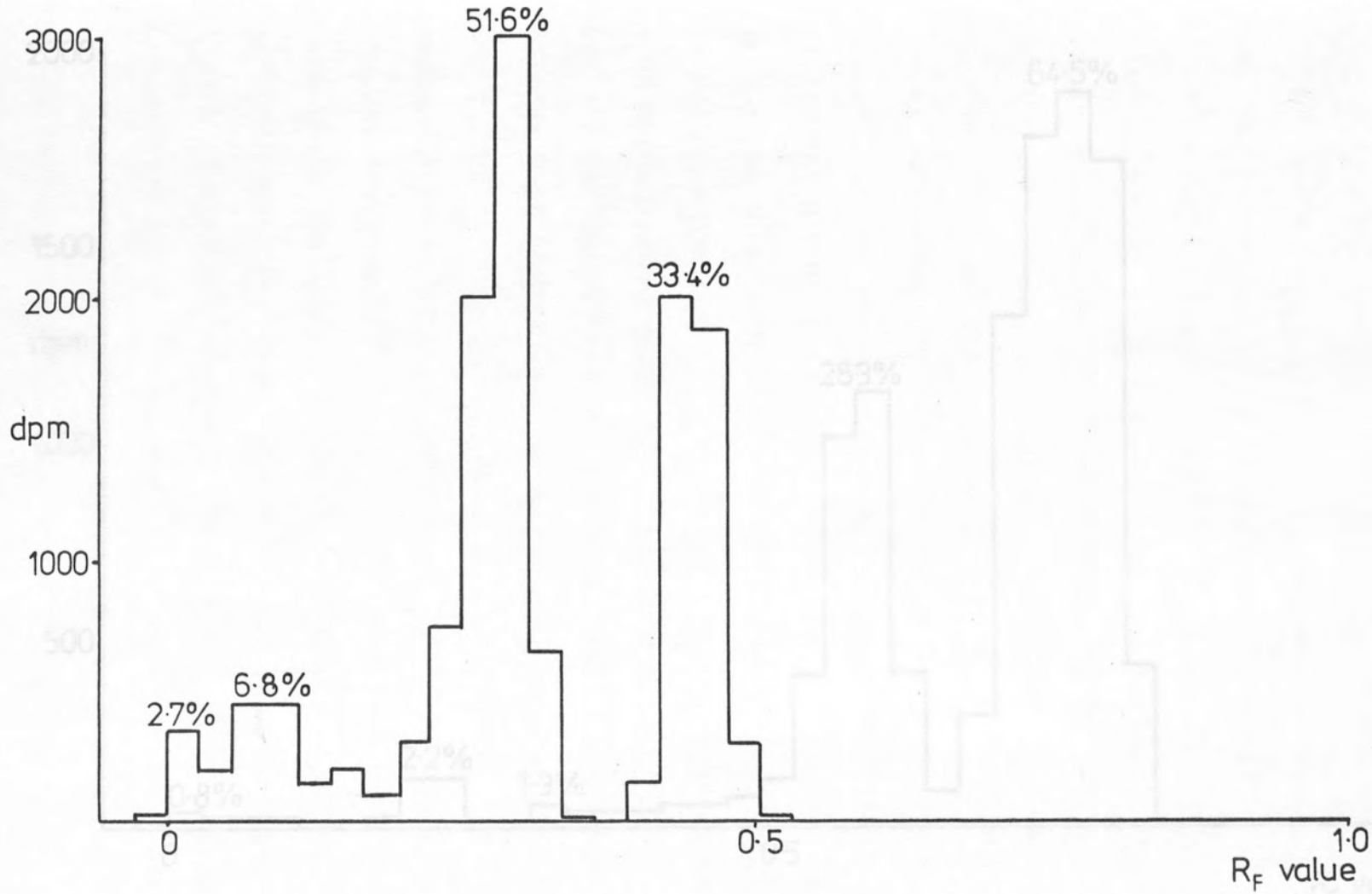


Fig 6.6 Distribution of radioactivity along a paper chromatogram developed in solvent E of urine from a marmoset dosed with [^{35}S] sulphadiazine.

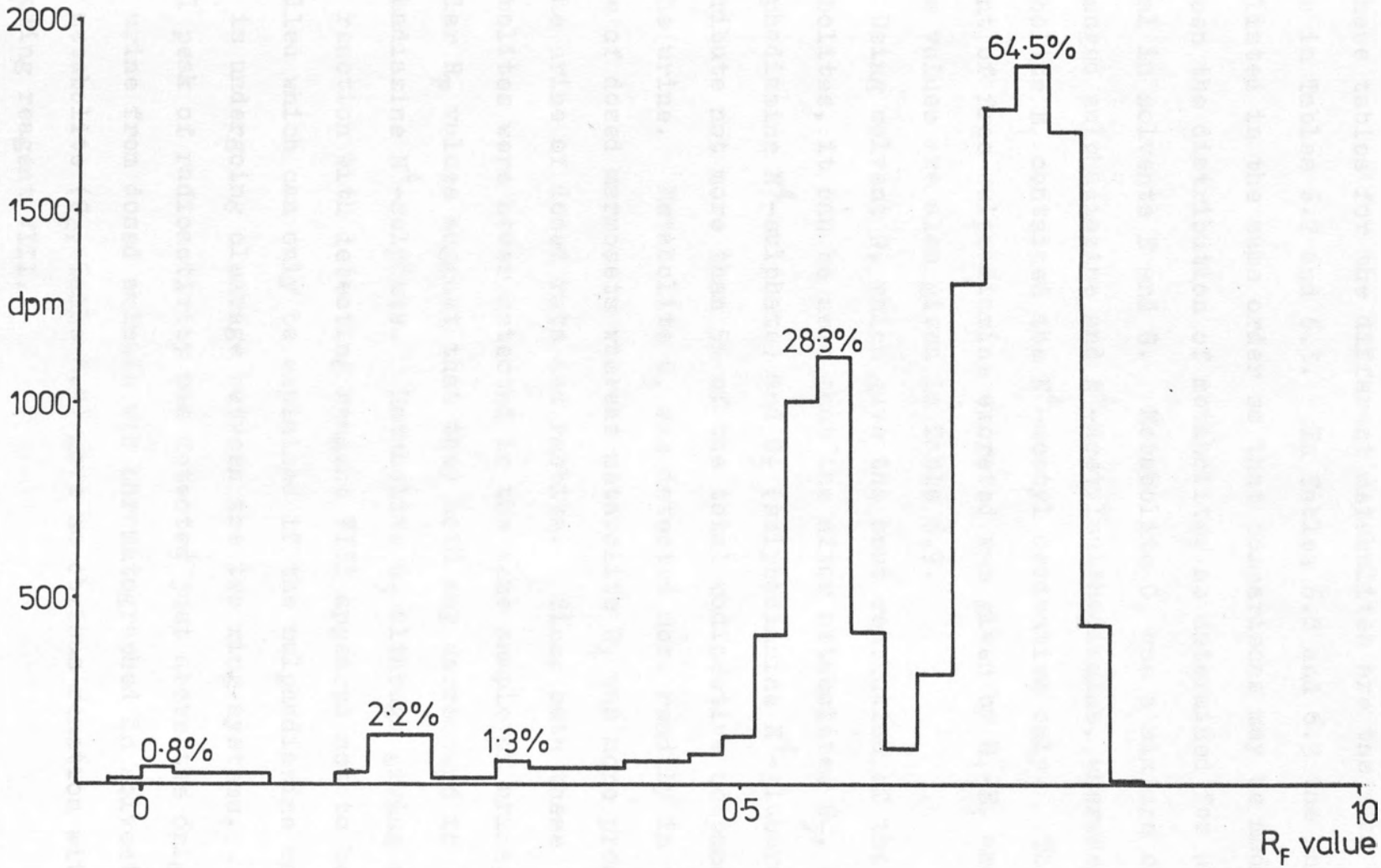


Fig 6.7 Distribution of radioactivity along a paper chromatogram developed in solvent G of urine from a marmoset dosed with [^{35}S] sulphadiazine.

shown in Tables 6.8 and 6.9 respectively. The code numbers used in these tables for the different metabolites are the same as those in Tables 6.2 and 6.3. In Tables 6.8 and 6.9 the animals are listed in the same order so that comparisons may be made between the distribution of metabolites as determined for any one animal in solvents E and G. Metabolite G_1 was a mixture of unchanged sulphadiazine and N^4-acetylsulphadiazine, whereas metabolite E_1 contained the N^4-acetyl derivative only. The amount of free sulphadiazine excreted was given by G_1-E_1 and these values are also given in Table 6.9.

Using solvent G, which gave the best resolution of the metabolites, it can be seen that the minor metabolites G_3, G_4 (sulphadiazine N^4-sulphate) and G_5 (sulphadiazine N^4-glucuronide) contribute not more than 5% of the total radioactive components in the urine. Metabolite G_3 was detected more readily in the urine of dosed marmosets whereas metabolite G_4 was more prominent in the urine of dosed rats and rabbits. Since both these metabolites were never detected in the same sample of urine, their similar R_F values suggest that they both may correspond to sulphadiazine N^4-sulphate. Metabolite G_5 although giving a weak reaction with detecting reagent VIII appeared not to be labelled which can only be explained if the sulphadiazine molecule is undergoing cleavage between the two ring systems. A small peak of radioactivity was detected just above the origin when urine from dosed animals was chromatographed in solvent G. This metabolite (G_6, Table 6.9) gave no obvious reaction with detecting reagent VIII.

Chromatograms developed in solvent E, although providing a good separation of sulphadiazine and N^4-acetylsulphadiazine did not give a very good resolution of the minor metabolites in the

Key Table 6.8

Metabolite codes (eg E_1) refer to the metabolites in Table 6.2. Figures in parentheses denote the R_F value used to identify the metabolites.

[a] Radiochemical recovery of dose (see Table 6.7).

[b] The R_F value of these radioactive peaks corresponded to neither metabolite E_3 or E_4 exactly.

[c] The R_F value of this radioactive peak corresponded to neither metabolite E_4 or E_5 exactly.

nd not detected

Species	Recovery of dose[a]	Radioactivity of metabolites E_1-E_5 expressed as a percentage of the total radioactivity present				
		E_1 (0.45)	E_2 (0.26)	E_3 (0.16)	E_4 (0.10)	E_5 (0.03)
Rat	78	15.5	75.3	nd	nd	9.3
	82	18.7	53.9	nd	11.5[b]	11.5
	77	17.3	55.1	nd	7.9[b]	8.5
	83	14.4	75.6	2.2	nd	3.9
	61	17.8	67.2	4.1	nd	5.2
Average	76	16.7	65.4	1.3	3.9	7.7
Marmoset	70	31.6	54.6	nd	9.4	4.0
	53	35.8	51.4	nd	11.6[c]	nd
	61	29.3	54.4	nd	nd	9.6
	76	40.0	38.7	nd	12.3	5.0
	80	33.4	51.6	nd	6.8	2.7
Average	68	34.0	50.1	-	8.0	4.3
Rabbit	71	58.2	35.1	nd	nd	6.8
	67	66.4	25.7	nd	nd	4.1
Average	69	62.3	30.4	-	-	5.5

Table 6.8 The relative proportions of the radioactive components present in the 24h urine excreted by animals dosed with [^{35}S] sulphadiazine as determined in solvent E.

Key Table 6.9

Metabolite codes (eg G_1) refer to the metabolites in Table 6.3. Figures in parentheses denote the R_F values used to identify the metabolites.

[a] Not detected by reagent VIII and therefore is not included in Table 6.3.

[b] Obtained by subtracting the proportion of metabolite E_1 (Table 6.8) from that of metabolite G_1.

[c] The urine from these animals was not analysed in solvent E (Table 6.8).

[d] Average of 5 animals only.

nd not detected.

Species	Radioactivity of metabolites G_1-G_6 expressed as a percentage of the total radioactivity present						Unchanged sulphadiazine[b]
	G_1 (0.80)	G_2 (0.63)	G_3 (0.27)	G_4 (0.23)	G_5 (0.19)	G_6 (0.00)[a]	
Rat	93.2	5.3	nd	nd	nd	1.5	77.7
	78.7	11.0	3.0	nd	nd	2.1	60.0
	71.8	14.5	nd	2.7	1.7	2.1	54.5
	91.4	4.0	nd	1.2	nd	1.0	77.0
	85.8	7.1	nd	1.8	nd	1.6	68.0
	78.8[c]	10.8	3.6	nd	nd	1.8	-
	79.3[c]	8.6	4.1	nd	nd	1.3	-
Average	82.7	8.8	1.5	0.8	-	1.6	67.4[d]
Marmoset	69.2	23.9	nd	1.3	nd	0.8	37.6
	75.4	18.6	2.9	nd	nd	nd	39.6
	65.9	21.5	3.3	nd	nd	2.1	36.6
	72.6	19.8	4.4	nd	nd	nd	32.6
	64.5	28.3	1.3	2.2	nd	0.8	31.1
Average	69.5	22.4	2.4	0.7	-	0.7	35.5
Rabbit	84.3	nd	4.1	nd	nd	6.9	26.1
	83.9	nd	nd	4.6	nd	4.6	17.5
Average	84.1	-	2.1	2.3	-	5.8	21.8

Table 6.9 The relative proportions of the radioactive components present in the 24h urine excreted by animals dosed with [^{35}S]sulphadiazine as determined in solvent G.

low R_F value region.

Figures 6.8, 6.9, 6.10 and 6.11 show the effect of β-glucuronidase (type H-1) on the distribution of labelled metabolites as shown by chromatography in solvent G. The urine samples used in these experiments were from the same 24h sample used to obtain Figures 6.6 and 6.7. When the urine was incubated with an equal volume of 0.4M sodium acetate buffer pH 5.0 containing D-saccharic acid-1,4-lactone, either alone or with the enzyme, no change in the distribution of labelled metabolites was observed as compared with the untreated urine. When the inhibitor was omitted from the incubation, the concentration of metabolite G_2 was severely diminished whether or not the enzyme was added. Under these conditions the decrease in the amount of metabolite G_2 was not accompanied by a corresponding increase in sulphadiazine but instead two labelled break down products were formed, the main one having an R_F value of 0.33 as determined by radiochemical counting (see Figs 6.10 and 6.11). Similar results were obtained for the effect of β-glucuronidase type L-1 at pH 3.8 on the distribution of metabolites of sulphadiazine as detected by reagent VIII (Table 6.5). In both cases the main break down product had an R_F value equivalent to the major component observed in the authentic sulphanilic acid sample (Table 6.5).

That the breakdown of metabolite G_2 on incubation of the urine sample with buffer alone was inhibited by D-saccharic acid-1,4-lactone suggests that the urine of marmosets contains an active β-glucuronidase.

When sulphanilic acid was administered to the animal, the urine from which had been used to obtain Figures 6.6 - 6.11, at a dose level of 100mg/kg body weight no metabolite corresponding

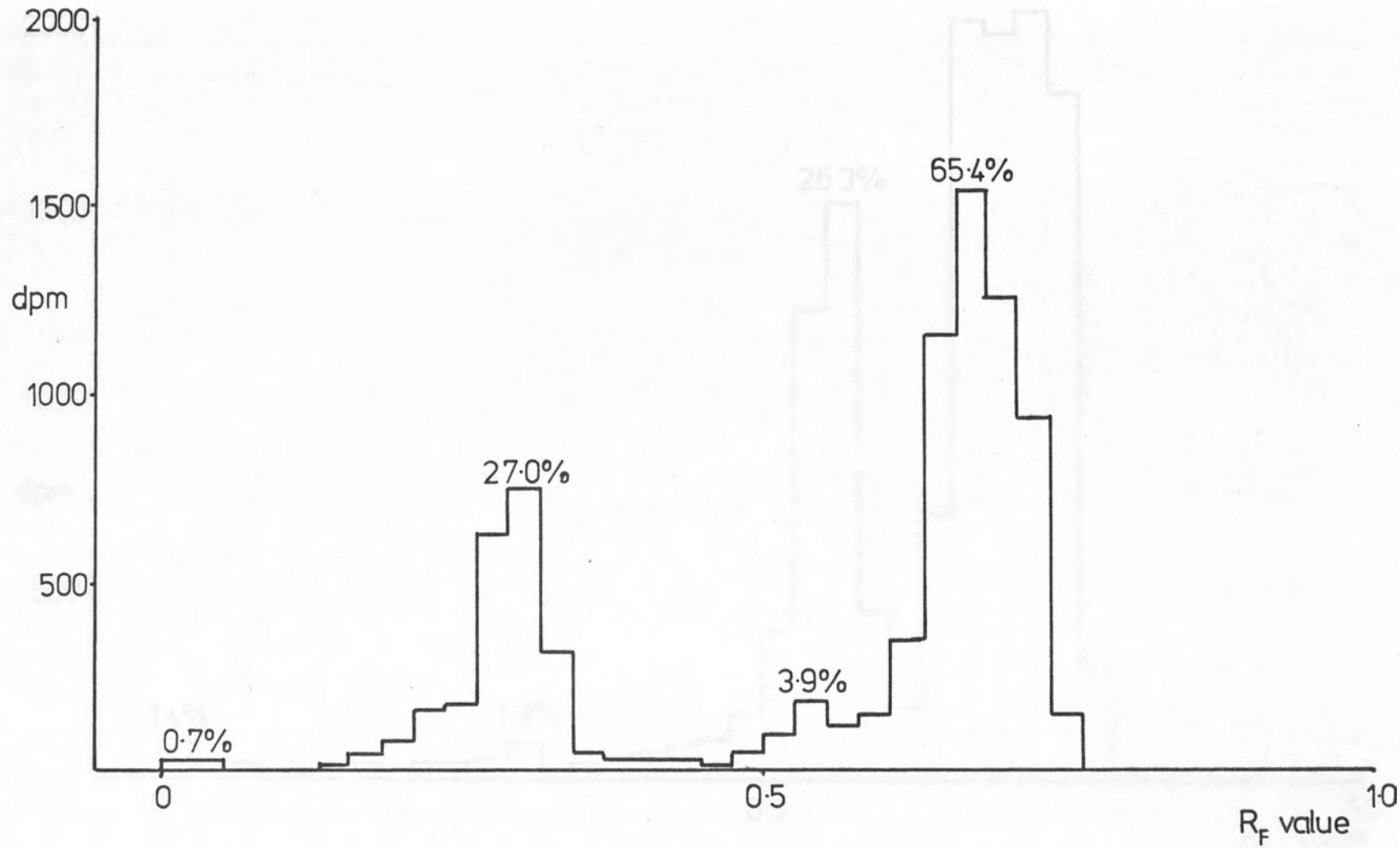


Fig 6.8 Distribution of radioactivity along a paper chromatogram developed in solvent G of [^{35}S]sulphadiazine dosed marmoset urine which was incubated with 0.4M acetate buffer (pH 5.0) only.

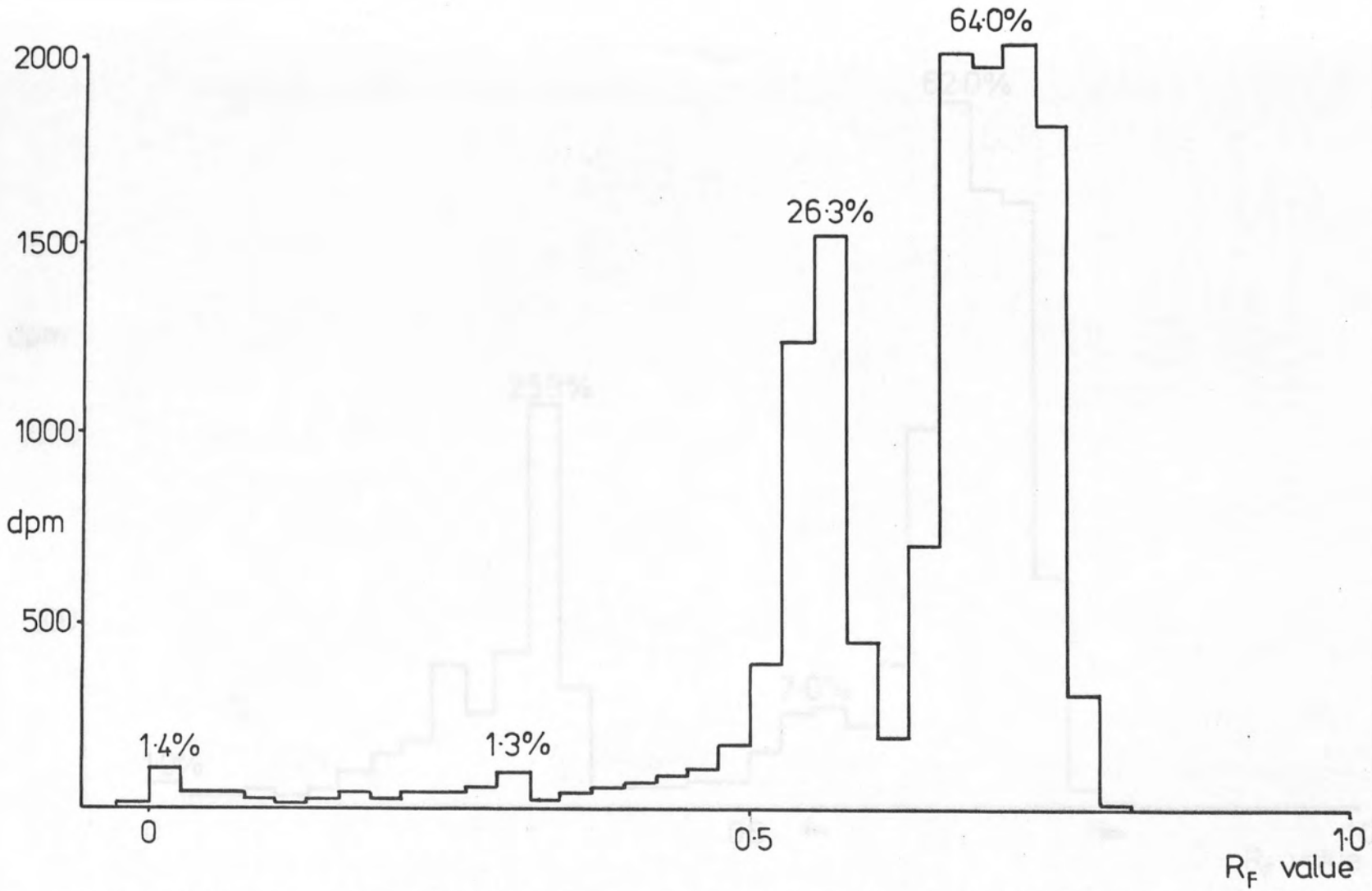


Fig 6.9 Distribution of radioactivity along a paper chromatogram developed in solvent G of [^{35}S] sulphadiazine dosed marmoset urine incubated with 0.4M acetate buffer (pH 5.0) in the presence of D-saccharic acid-1,4-lactone.

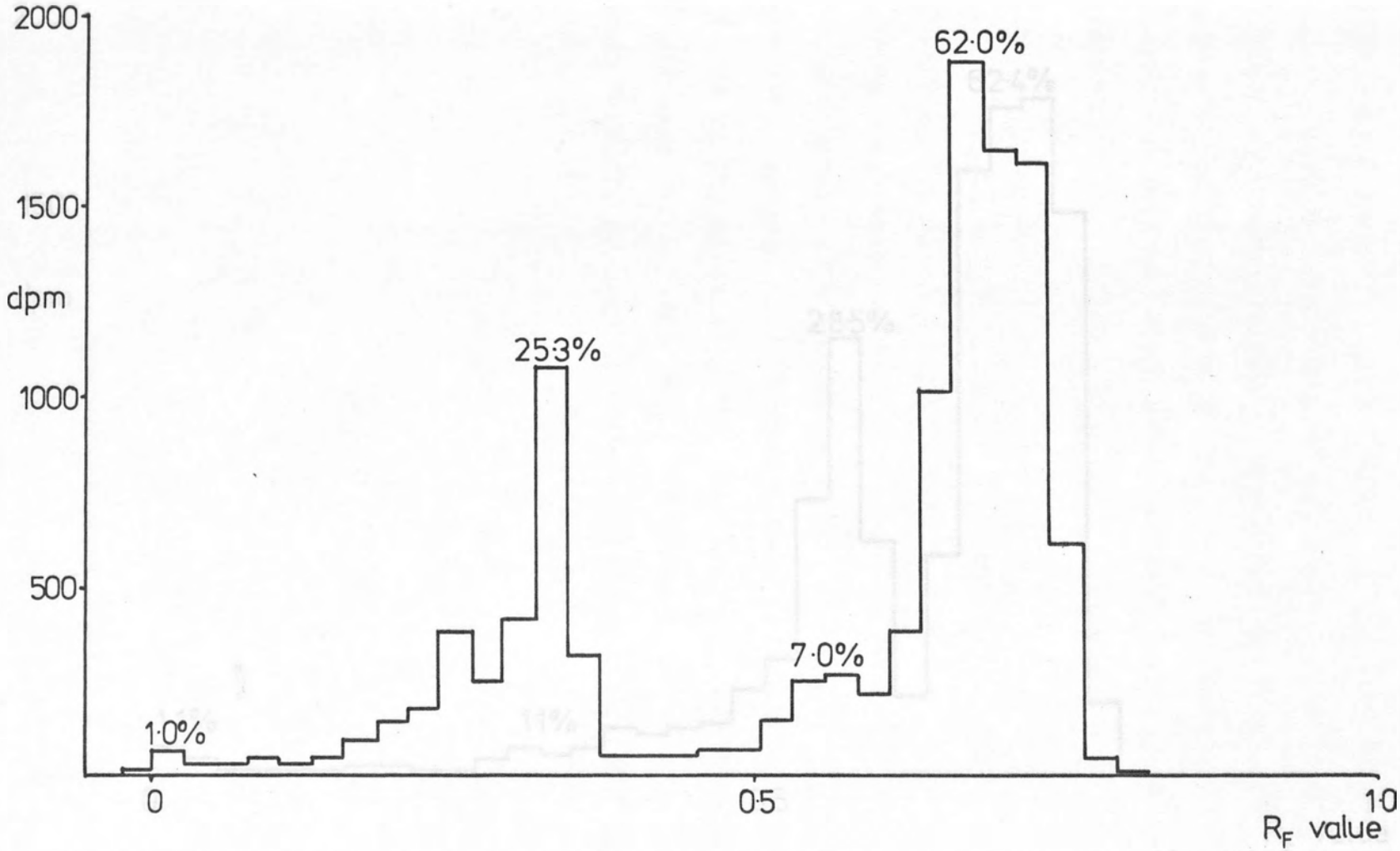


Fig 6.10 Distribution of radioactivity along a paper chromatogram developed in solvent G of β-glucuronidase-treated urine from a marmoset dosed with [^{35}S]sulphadiazine.

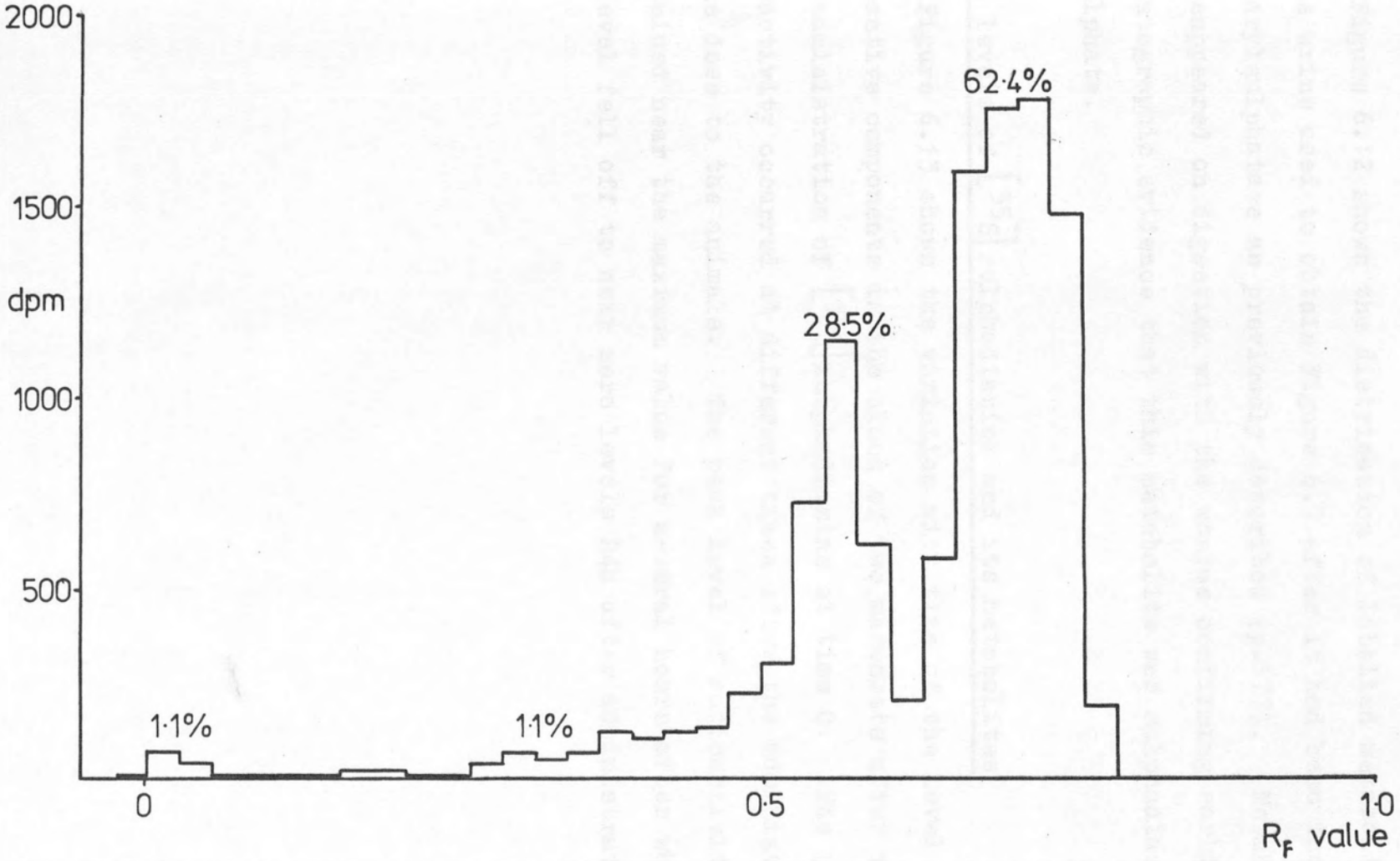


Fig 6.11 Distribution of radioactivity along a paper chromatogram developed in solvent G of β-glucuronidase treated urine (in the presence of D-saccharic acid-1,4-lactone) from a marmoset dosed with [^{35}S] sulphadiazine.

to G_2 was detected by reagent VIII.

Figure 6.12 shows the distribution of labelled metabolites in the urine used to obtain Figure 6.7 after it had been incubated with arylsulphatase as previously described (p.177). Metabolite G_3 disappeared on digestion with the enzyme confirming earlier chromatographic evidence that this metabolite was sulphadiazine N^4-sulphate.

Blood levels of $[^{35}S]$ sulphadiazine and its metabolites

Figure 6.13 shows the variation with time of the level of radioactive components in the blood of two marmosets after the oral administration of $[^{35}S]$ sulphadiazine at time 0. The peak of radioactivity occurred at different times after the administration of the dose to the animals. The peak level of radioactivity was maintained near the maximum value for several hours after which the level fell off to near zero levels 24h after administration.

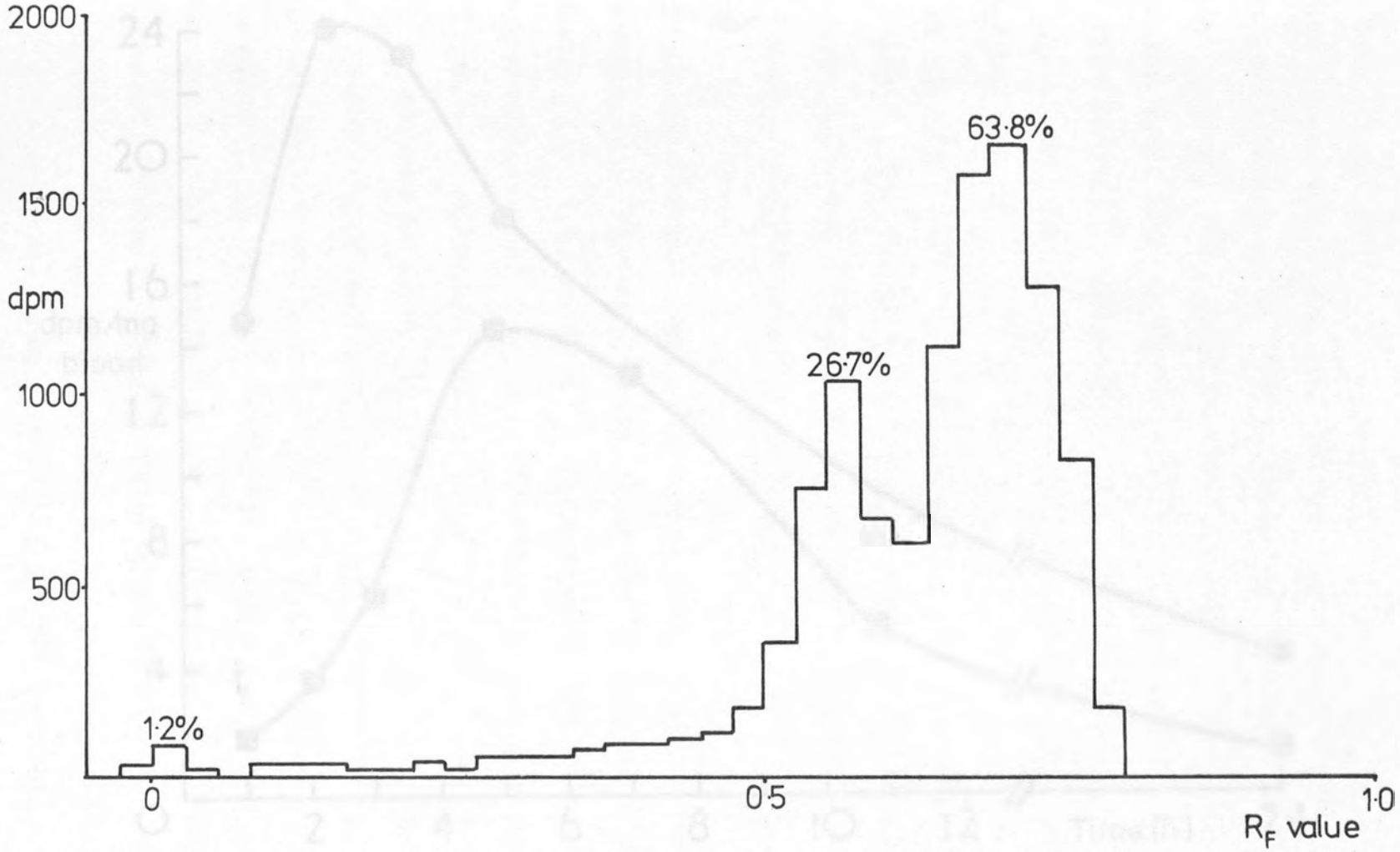


Fig 6.12 Distribution of radioactivity along a paper chromatogram developed in solvent G of sulphatase-treated urine from a marmoset dosed with [^{35}S]sulphadiazine.

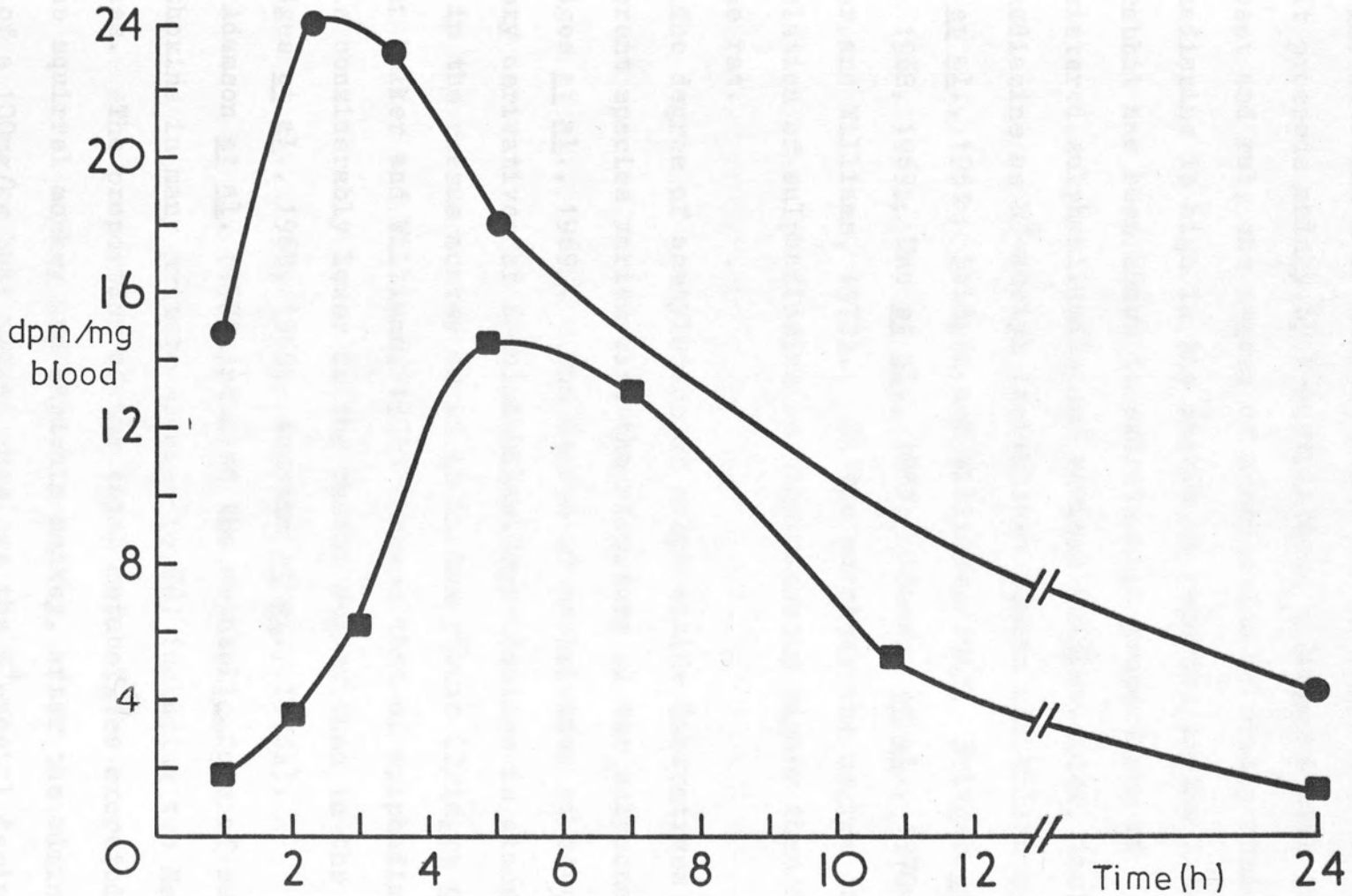


Fig 6.13 The levels of radioactive components in the blood of two marmosets at various time intervals following the oral administration of [^{35}S] sulphadiazine.

Discussion

The metabolism of sulphadiazine in the marmoset, rat and rabbit proceeds mainly by N-acetylation. Compared with the marmoset and rat, the degree of acetylation of orally administered sulphadiazine is high in the rabbit as reported in the literature. The rabbit has been shown to excrete high proportions of administered sulphanilamide and various sulphonamides, including sulphadiazine as N^4-acetyl derivatives (Smith and Williams, 1948a; Bray et al., 1951b; Bridges and Williams, 1963; Bridges et al., 1966, 1968, 1969b; Uno et al., 1967; Adamson et al., 1970a; Walker and Williams, 1972). In the marmoset the degree of acetylation of sulphadiazine is significantly higher than it is in the rat.

The degree of acetylation of sulphonamide derivatives in different species varies with the structure of the sulphonamide (Bridges et al., 1969a). The degree of acetylation of several methoxy derivatives of 6-sulphanilamidopyrimidines is almost as high in the rhesus monkey as it is in the rabbit (Bridges et al., 1969a; Walker and Williams, 1972) whereas that of sulphadimethoxine is considerably lower in the rhesus monkey than in the rabbit (Bridges et al., 1968, 1969b; Adamson et al., 1970a).

Adamson et al. (1970a)studied the metabolic fate of sulphadimethoxine in many primate species (p.56) including two New World species. The proportion of the total metabolites excreted in 24h by the squirrel monkey and capuchin monkey, after the administration of a 100mg/kg body weight dose, as the N^4-acetyl derivative were 37% and 2% respectively. The results obtained in this work suggest that the degree of acetylation of sulphadiazine in the marmoset is similar to that of sulphadimethoxine in the squirrel

monkey.

Uno and Sekine (1966) reported that 36.5% of administered sulphadiazine (2g) was excreted in the acetylated form in the urine of humans over 48h. This was equivalent to 45% of the total metabolites excreted in this period. Although the dose level used in this study was less than that used with the marmoset, there are clearly similarities between the degree of sulphadiazine acetylation in the marmoset and man.

The proportion of administered sulphadiazine found to be excreted unchanged in the present investigation was highest in the rat and lowest in the rabbit. A major metabolite, G_2, was excreted by both rats and marmosets but not by rabbits to which sulphadiazine had been administered. This metabolite accounted for a greater proportion of the total metabolites excreted by the marmoset than by the rat.

Although the identity of metabolite G_2 has not been definitely established it is probably a conjugate of glucuronic acid in which the aglycone contains a free amino group. Since hydroxylation of sulphadiazine has been reported (Bray et al., 1951b; Atef and Nielsen, 1975) it is possible that metabolite G_2 could be either an O- or an N^1-glucuronide. Digestion of this metabolite with either type L-1 or H-1 β-glucuronidase appeared to release sulphanilic acid, but this compound is unlikely to be the aglycone since it was not found to be metabolised to metabolite G_2 by the marmoset. Bridges et al. (1965) reported that sulphadimethoxine N^1-glucuronide was slowly broken down by β-glucuronidase (type L-1) to sulphadimethoxine and glucuronic acid, whereas acid hydrolysis of the conjugate yielded sulphanilic acid, barbituric acid and glucuronic acid showing that it is possible for sulphanilic acid to be formed as a result of a non-enzymic

hydrolysis of the N^1-glucuronide of a sulphonamide.

Since the break down of metabolite G_2 by β-glucuronidase was inhibited by D-saccharic acid-1,4-lactone there must be a specific binding of the enzyme to the molecule and therefore the release of sulphanilic acid is much more likely when the glucuronic acid moiety is attached in the N^1-position than if it were attached through a ring hydroxyl group.

That N^1-glucuronide formation only occurs with a limited number of sulphanilamidopyrimidines has already been mentioned (p.154). The conclusions drawn by Walker and Williams (1972) as to the structural requirements for N^1-glucuronidation suggest that sulphadiazine would not undergo such a reaction. Bridges et al. (1966, 1969a), Adamson et al. (1970a) and Walker and Williams (1972) reported that in general where N^1-glucuronide formation did occur with a particular sulphanilamidopyrimidine, it did so to a major extent only in man and rhesus monkey, to a minor extent in the rat and did not occur in the rabbit. Assuming that the tentative identification of metabolite G_2 as an N^1-glucuronide is correct the results obtained in the present study are in accord with these reports for the rat and rabbit; the N^1-glucuronide of sulphadiazine was a significant metabolite excreted by the marmoset. If metabolite G_2 is sulphadiazine N^1-glucuronide its low excretion in the urine of dosed rats may not be a true indication of the capability of this species to form it since its high molecular weight (427) would permit its excretion in the bile as in the case of sulphadimethoxine (Adamson et al., 1970a).

Uno and Sekine (1966) did not report the presence of sulphadiazine N^1-glucuronide as a metabolite of sulphadiazine in man, while Gilligan (1945) had previously reported that the administration of sulphadiazine to humans caused no increase in

the excretion of total glucuronic acid above control levels. A comparison of these results with those reported here, may suggest that there are major differences in the metabolic fate of sulphadiazine between man and the marmoset.

The excretion of sulphadiazine N^4-sulphate as a minor metabolite of sulphadiazine in the marmoset has been shown in this work and this result is similar to that obtained by Uno and Sekine (1966) for the excretion of sulphadiazine N^4-sulphate in the urine of humans to whom sulphadiazine had been administered.

A detailed analysis of the radiochemical distribution along chromatograms of urine from dosed animals, developed in solvent G, indicates the presence of a minor metabolite (G_6, Table 6.9) which was not detected by reagent VIII. The absence of such a reaction may be due to the low concentration of this metabolite in the urine.

Peak blood levels of [^{35}S]sulphadiazine and its metabolites were observed in the marmosets studied at 2.5h and 5.0h after dosing. After this peak the levels of radioactivity fell off to near-zero by 24h. Goodwin *et al.* (1942) showed that the blood levels of both free sulphadiazine and total sulphadiazine reached a plateau 6h after the administration of a single 5g oral dose to human subjects. This plateau level was maintained for a period of 6h after which there was a gradual decrease in the blood levels until near-zero levels were achieved 3 days after dosing. Welch *et al.*(1943) reported that when sulphadiazine was administered to human subjects at a dose level of 50mg/kg body weight peak blood levels of both free and total drug were reached 4h after dosing, after which the levels fell off to reach near-zero between 3 and 4 days. The rates of absorption of sulphadiazine from the gastrointestinal tract appear therefore to

be similar in man and the marmoset, but the rate of elimination of the dose from the blood appears to be much faster in the marmoset than it is in man.

CHAPTER 7

Acetylation of sulphadimidine by the marmoset

Chapter 7 - Acetylation of sulphadimidine by the marmoset

Introduction

The rate of inactivation of certain amino group-containing compounds is an individual characteristic, highly variable from person to person and known to be under genetic control. The inter-individual differences are due to the activities of hepatic N-acetyltransferase, the enzyme responsible for the acetylation of amino groups. Acetylator polymorphisms were originally discovered in man as a result of studies of the metabolism of isoniazid in both healthy volunteers and in patients suffering from tuberculosis. Slow inactivation of isoniazid by means of acetylation is a Mendelian autosomal recessive character, while rapid inactivation is an autosomal dominant character (Knight et al., 1959; Evans et al., 1960; Sunahara et al., 1961). The frequency of the acetylator phenotypes exhibits large inter-ethnic variability (Armstrong and Peart, 1960; Dufour et al., 1964; Scott et al., 1969; Rao et al., 1970; Lunde et al., 1977).

Much of the early work on acetylation polymorphisms was carried out using isoniazid. However Evans and White (1964) and Peters et al., (1965) showed that similar polymorphisms were also apparent in the acetylation of the sulphonamide, sulphadimidine (sulphamethazine). The ease of phenotyping individuals using a single test dose of sulphadimidine (Evans, 1969; Eze and Evans, 1972) has resulted in the replacement of isoniazid by this compound as the most widely used drug for the determination of acetylator phenotypes.

Several other amino compounds also exhibit an acetylation polymorphism in man, including dapsone (Gelber et al., 1971) sulphapyridine (Schröder and Evans, 1972) and procainamide (Gibson

et al., 1975; Karlsson and Molin, 1975). The rabbit has also been shown to exhibit acetylation polymorphisms with respect to sulphadiazine (Frymoyer and Jacox, 1963a,b) and sulphadimidine (Hearse and Weber, 1973). Using rabbits which had been phenotyped with sulphadimidine, Hearse and Weber (1973) demonstrated the possible existence of more than one N-acetyltransferase. One type of this N-acetyltransferase appeared to be present in the liver and gut and was responsible for the polymorphism trait exhibited in vivo, while a second N-acetyltransferase, found in extra-hepatic tissues, exhibited no polymorphic characteristics.

In primate species there exist wide inter-individual differences in the extent of acetylation of isoniazid but only in the mangabey and African green monkey have there been any indications suggesting the existence of a polymorphism in acetylation (Goedde et al., 1964; Peters et al., 1965).

Using sulphadimidine, the acetylator phenotype of an individual can be determined by measuring the degree of acetylation of sulphadimidine in the urine or plasma of the subject. Evans (1969) observed that the percentage of N^4-acetylsulphadimidine in serum appeared to be a better phenotype discriminant than the same constituent in urine. Hoo et al. (1977) also reported that a distinct difference between fast and slow acetylators of sulphadimidine could be demonstrated by measuring the proportion of acetylated drug in a blood sample taken 2h after dosing but that considerable overlap existed with urine samples. However Rao et al. (1970) concluded that when either sulphadimidine or isoniazid was used to determine the acetylator phenotype of patients, the urine test was preferable to the blood test, while more recently Tulseth and Landmark (1977) reported that it was possible to distinguish fast and slow acetylators by measuring the percentage

of N^4-acetylsulphadimidine in a single serum or urine sample. Furthermore these authors also reported that the amount of sulphadimidine administered in the test was not critical and that differences between slow and fast acetylators could be demonstrated in plasma samples taken at any time between 0.5 and 24h after the administration of the compound, and in urine samples up to 8h after dosing.

This chapter gives an account of the acetylation of sulphadimidine by the marmoset. The members of two families of these animals were examined.

Materials and Methods

Materials

Sulphadimidine tablets were obtained from the Area Pharmaceutical Service, United Birmingham Hospitals. Each tablet weighed 600mg and contained 500mg of sulphadimidine. Four tablets were crushed up to form an evenly mixed powder for dosing.

All the other chemicals used in this study were available from the laboratory stores.

Methods

Administration of dose

The tablet preparation was administered to each marmoset in the colony as a suspension in Cytacon at a dose level of 60mg/kg body weight, equivalent to 50mg sulphadimidine/kg body weight. After determining the quantity of the doses which remained in the syringes after dosing, the average dose of sulphadimidine administered to each animal was found to be 45mg/kg body weight.

Collection of urine

Urine samples were collected at 2, 4, 6 and 24h after the administration of the dose.

Collection of blood samples

Triplicate samples of blood were collected approximately 4h after the administration of the dose as described previously (p 90) and placed onto pieces of filter paper according to the method of Weber and Brenner (1974).

Determination of the degree of acetylation in urine and blood samples

(a) Urine The percentage of free and acetylated drug excreted in each urine sample was determined by the Bratton and Marshall (1939) method, as previously described (p 134). A calibration curve constructed from known quantities of sulphadimidine was used.

By repeating the experiments on four animals the reproducibility of the results was established.

(b) Blood The percentage of sulphadimidine that was present in the plasma in the acetylated form was determined by the method of Weber and Brenner (1974).

Results

Figure 7.1 shows a typical excretion curve obtained for free, total and hence acetylated metabolites of sulphadimidine in the urine of a dosed marmoset. In the first 2h after the administration of the dose, a slight lag phase was seen in the excretion of sulphadimidine and its N^4-acetyl derivative and so the 2h urine sample was not used in determining the acetylator status of the animals.

Tables 7.1 and 7.2 are summaries of the data obtained for the proportion of the dose excreted as N^4-acetylsulphadimidine and the rates of excretion of the acetylated derivative in the urine of marmosets from family I (parents Nos 417 and 418) and family II (parents Nos 463 and 465) respectively (see Fig 2.1). The rates of excretion of N^4-acetylsulphadimidine were calculated for the period 2-6h after dosing from the cumulative curves for the excretion of N^4-acetylsulphadimidine. The rates of excretion of N^4-acetylsulphadimidine were taken as being equivalent to the rate of acetylation of the drug. Table 7.2 also contains the results obtained using marmoset No 462, a feral male, which had not started breeding at the time of this study.

The proportions of the dosed sulphadimidine present as N^4-acetylsulphadimidine in the urine samples collected at 4h and 6h after the administration of the dose were very similar in all the animals studied. The average results for the proportion of the dose excreted as N^4-acetylsulphadimidine in the 4h and 6h urine samples, together with the rate of acetylation of sulphadimidine are slightly higher for family I than for family II but the difference is not statistically significant. When averaging the rates of acetylation per animal for either family, the results obtained using animals Nos 764, 765, 783 and 784 were not included

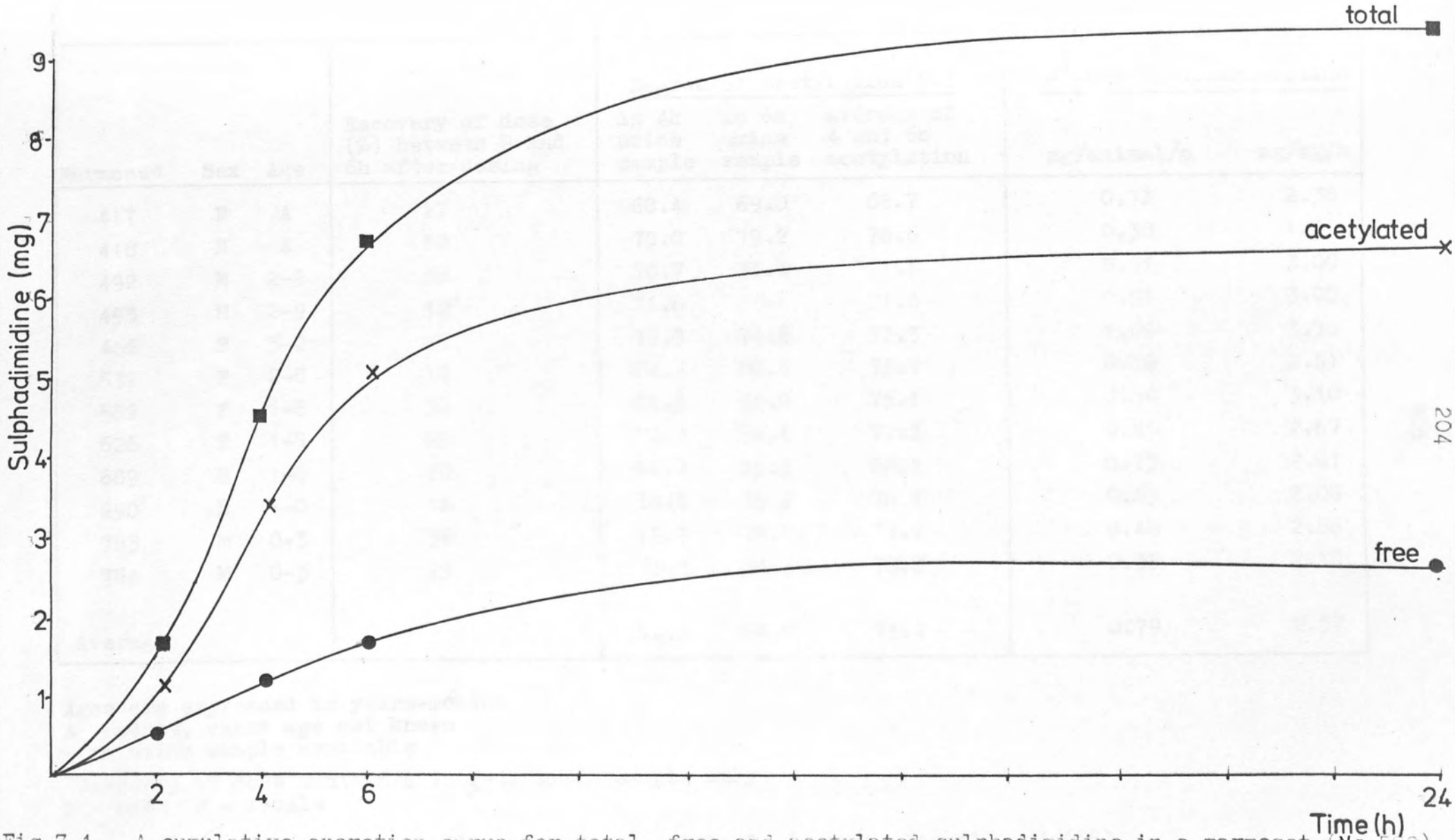


Fig 7.1 A cumulative excretion curve for total, free and acetylated sulphadimidine in a marmoset (No 582)

Marmoset	Sex	Age	Recovery of dose (%) between 2 and 6h after dosing	Degree of acetylation (%)			Rate of excretion of N^4-acetylsulphadimidine	
				in 4h urine sample	in 6h urine sample	average of 4 and 6h acetylation	mg/animal/h	mg/kg/h
417	F	A	27	68.4	69.0	68.7	0.72	2.38
418	M	A	12	78.0	79.2	78.6	0.38	1.25
492	M	2-9	33	70.7	71.4	71.1	0.91	3.00
493	M	2-9	12+	71.8	-	71.8	0.91	3.00
466	F	3-2	25	72.3	74.2	73.3	1.06	3.50
532	F	2-8	14	72.7	80.6	76.7	0.76	2.51
582	F	1-8	32	74.2	76.0	75.1	0.94	3.10
626	F	1-5	20	70.3	74.1	72.2	0.81	2.67
689	M	1-0	20	66.9	69.3	68.1	0.73	2.41
690	M	1-0	16	78.2	79.2	78.7	0.63	2.08
783	M	0-3	36	72.0	75.8	73.9	0.48	2.88
784	M	0-3	25	71.4	74.2	72.8	0.35	2.10
Average				72.2	74.8	73.4	0.79	2.57

Ages are expressed in years-months
A - adult, exact age not known
- no urine sample available
+Recovery of dose contained in 2-4h urine sample only
M - male F - female

Table 7.1 Acetylation of sulphadimidine in family I (parents Nos 417 and 418)

Marmoset	Sex	Age	Recovery of dose (%) between 2 and 6h after dosing	Degree of acetylation (%) in 4h urine sample	Degree of acetylation (%) in 6h urine sample	Degree of acetylation (%) average of 4 and 6h acetylation	Rate of excretion of N^4-acetylsulphadimidine mg/animal/h	Rate of excretion of N^4-acetylsulphadimidine mg/kg/h
463	F	A	31	61.2	59.4	60.3	0.75	2.48
465	M	3-3	19	69.6	57.8	63.7	0.48	1.58
607	M	1-8	28	62.9	65.0	63.9	0.45	1.49
608	M	1-8	31	60.8	60.9	60.9	0.70	2.31
652	F	1-3	23	70.5	68.1	69.2	0.91	3.00
653	F	1-3	21	67.6	74.1	70.8	0.85	2.80
708	M	1-0	12	67.2	65.9	66.6	0.44	1.58
709	M	1-0	14+	-	63.9	63.9	0.54	1.94
764	M	0-4	30	67.4	73.4	70.4	0.42	2.52
765	M	0-4	25	64.0	61.8	62.9	0.32	1.92
Average				65.7	65.0	65.3	0.64	2.16
462	M	A	17	68.9	69.7	69.3		

Ages are expressed in years-months
A - adult, exact age not known
- no urine sample available
+Recovery of dose contained in 4-6h urine sample only
M - male F - female

Table 7.2 Acetylation of sulphadimidine in family II (parents Nos 463 and 465)

since these animals were very young and received less than half the dose administered to adult animals. However the results obtained using these animals were included when averaging the rates of acetylation expressed per kg body weight since on a mg/kg body weight basis the dose administered to these young animals was the same as that given to the adults.

Table 7.3 shows the average rates of acetylation of sulphadimidine in male and female animals both from the separate families and from both families combined. In all cases the average rate of acetylation of sulphadimidine is higher in female animals than in males but the values are not significantly different, the difference in the average values being less than the sum of their standard deviations.

The degree of acetylation of sulphadimidine in the blood of three animals was determined and the results are given in Table 7.4. Blood samples were taken from these animals at the same time as they were being used for the excretion studies and so the degree of acetylation in the blood and urine of these animals may be compared. It may be seen that the percentage of the total drug present in the acetylated form is lower than that in the corresponding urine indicating that the acetylated drug is preferentially excreted.

Family	Average acetylation rates (mg/kg/h) ± S.D.	
	Females	Males
I	2.83 ± 0.46	2.39 ± 0.64
II	2.76 ± 0.26	1.91 ± 0.39
I + II	2.80 ± 0.38	2.15 ± 0.57

Table 7.3 Average acetylation rates of sulphadimidine in male and female marmosets

Marmoset	Time of sampling after dose administration h min	Percentage of metabolites present in acetylated form
493	3 - 50	35.5
532	3 - 40	37.9
582	4 - 00	25.5

Table 7.4 Degree of sulphadimidine acetylation in blood of adult marmosets

Discussion

Although the rates of acetylation of sulphadimidine found for all the animals examined ranged from 1.25 - 3.50mg/kg/h the values did notfall into distinct groups; the percentage of the dose excreted as the acetyl derivative varied from 60.3% to 78.7% and again no dividing line in these values was discernible. It is unlikely therefore that acetylation dimorphism is present in these animals. The extension of this conclusion to this species of marmoset in general can only be tentative for two reasons
(i) The number of animals used in this study was only small.
(ii) Although two families were used, one parent of family II (i.e. No 465) was an offspring of family I (see Fig 2.1) and hence the families were genetically related.

If a polymorphism does exist in marmosets, regarding the acetylation of sulphadimidine then the high degree of acetylation observed in the marmosets studied, together with the consistency of these results, suggest that the minimum genetical requirements for both sets of parents would be for one parent to be homozygous dominant and the other heterozygous. If the gene responsible for a high degree of sulphadimidine acetylation is not dominant over that for a low degree of acetylation then both sets of parents examined would appear to be homozygous with respect to the gene for high acetylation since no results were obtained which could be attributed to an intermediate degree in the acetylation of sulphadimidine.

Table 7.5 is a summary of some of the results obtained by other workers for the bi-modal distribution of sulphadimidine acetylation in man. The results, taken from the different reports, are similar and a comparison with the results obtained here for the marmoset suggest the acetylation of sulphadimidine

Dose level (mg/kg body weight)	Percentage of urinary metabolites present in acetylated form		Reference
	Slow acetylators	Fast acetylators	
40[+]	40 - 55	65 - 85	Evans and White (1964)
47 (160[+])	30 - 60	70 - 95	Evans (1969) - 2 groups
40	40 - 70	80 - 100	
44	40 - 60	65 - 95	Rao et al. (1970)
40	40 - 55	75 - 90	Eze and Evans (1972)
47	29 - 53	69 - 93	Tulseth and Landmark (1977)

[+]Dose level expressed per kg active body mass.

Table 7.5 Published data for the bi-modal distribution of sulphadimidine acetylation in humans

occurs to a similar degree in these animals as it does in those humans who are classified as fast acetylators. The results obtained by Rao et al. (1970) may be considered guidelines for the phenotyping of individuals for the acetylation of isoniazid. Hence if more than 70% of the metabolites of sulphadimidine present in the urine 6h after the administration of a dose of 44mg sulphadimidine/kg body weight are accounted for as N^4-acetylsulphadimidine then the subject may be classified not only as a fast acetylator of sulphadimidine but also as a fast inactivator of isoniazid.

From the results of Schröder and Evans (1972) it is possible to calculate that the rate of acetylation of sulphadimidine in man after a 10mg/kg body weight dose is 0.63mg/kg/h in slow acetylators and 1.39mg/kg/h in fast acetylators. An investigation into the rate of acetylation of orally administered sulphadimidine in a class experiment involving 27 students in this department, showed that at a dose level of approximately 15mg/kg body weight the rates of acetylation varied from 0.11 to 1.37mg/kg/h, the majority of the subjects (60%) acetylating the drug at a rate of between 0.14 and 0.53mg/kg/h. While these results are not entirely reliable they support the conclusion that the rates of acetylation of sulphadimidine in the marmoset, expressed per kg body weight as given in Tables 7.1 and 7.2, are considerably higher than the rate of acetylation reported for man.

The proportion of sulphadimidine present as the N^4-acetyl derivative in the blood of dosed marmosets is much lower than that in the corresponding urine. Similar results have been reported for man (Evans, 1969; Rao et al., 1970; Eze and Evans, 1972). If more than 25% of the plasma metabolites of administered sulphadimidine (44mg/kg body weight) are present as N^4-acetyl-

sulphadimidine 6h after the administration of the dose, then the human individual is classified as a fast acetylator (Rao et al., 1970). The results reported here for the degree of acetylation of sulphadimidine in the plasma of marmosets indicates that these animals acetylated the drug to a similar degree as humans classified as fast acetylators, which is in agreement with the urinary results obtained.

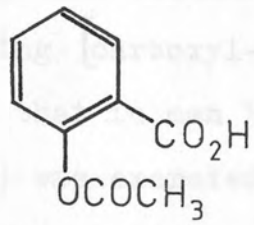
CHAPTER 8

The comparative metabolism of aspirin

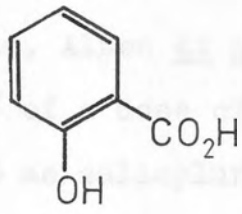
Chapter 8 - The comparative metabolism of aspirin

Introduction

Aspirin (acetylsalicylic acid; I), a powerful analgesic and antipyretic has probably been the most widely used drug for many years. Acetylsalicylic acid is easily hydrolysed in



(I)



(II)

solution especially at pH values above 7, and in the animal body is readily converted to salicylic acid (Hill, 1902; Lester et al., 1946; Smith et al., 1947; Williams, 1959; Leonard, 1962; Smith and Smith, 1966; Rowland et al., 1967; Davison, 1971). The work of Mandel et al. (1954) and Leonard (1962) showed that the drug was absorbed in its unhydrolysed form from the gastrointestinal tract and that hydrolysis occurred after absorption, but Rowland et al. (1967) concluded that appreciable hydrolysis occurred in the gastrointestinal fluids and/or in the gut wall.

Because of the susceptibility of acetylsalicylic acid to hydrolysis, the metabolism and excretion of this compound becomes almost equivalent to that of salicylic acid (II); conjugation with glycine to form salicyluric acid and with glucuronic acid to form acyl and phenolic glucuronides occurs (Quick, 1933b; Alpen et al., 1951; Robinson and Williams, 1956). A small fraction of salicylic acid is further hydroxylated to gentisic

acid (2,5-dihydroxybenzoic acid) while the excretion of small amounts of the oxidation products 2,3-dihydroxy and 2,3,5-trihydroxybenzoic acids has also been reported (Bray et al., 1950b; Dumazart and Ouachi, 1954).

Despite the widespread use of aspirin there is relatively little published data on its metabolic fate in different animal species, and those reports that have been published are mainly concerned with the metabolic fate of salicylic acid. In a study using [carboxyl-^{14}C]salicylic acid, Alpen et al. (1951) reported that in man between 10 and 85% of a dose of salicylic acid (1g) was excreted unchanged, 0-50% as salicyluric acid, 1% or less as gentisic acid, 12-30% as an ether glucuronide and 0-10% as an ester glucuronide. In general a low excretion of free salicylic acid was accompanied by a high excretion of salicyluric acid. Hoffman and Nobe (1950) demonstrated the importance of urinary pH on the renal excretion of salicylic acid derivatives during acetylsalicylic acid therapy, but the wide variations in the proportions of the various metabolites excreted by the different subjects reported by Alpen et al. (1951) could not be correlated with urinary pH. More recently Levy et al. (1972) showed that an average of 50% of administered salicylate (3g) was excreted as salicyluric acid in the urine of four healthy human subjects, 14% of the dose was excreted as unchanged salicylate, 20% as salicylphenylglucuronide[a], 10% as salicylacyl-glucuronide[b] and 3% as gentisic acid. The wide inter-individual variations in the results observed by Alpen et al. (1951) were not encountered by Levy et al. (1972), suggesting that the use by these earlier authors of patients suffering from malignant growths may have been responsible for the variations observed.

Alpen et al. (1951) also showed that in the dog, 50% of a

a- o-Carboxyphenylglucuronide b- o-Hydroxybenzoylglucuronide

dose of salicylic acid (1g) was excreted unchanged, 25% conjugated with glucuronic acid, 10% conjugated with glycine and 4-5% was excreted as gentisic acid. In this species only the ether-type glucosiduronic acid was excreted. In the rat, the same metabolites were formed as those described above for man and the dog (Williams, 1959).

Bray et al. (1948b) reported that in the rabbit a large proportion (85%) of administered salicylic acid was excreted as ether-soluble acid which contained very little salicyluric acid, 3-4% was excreted as salicylacylglucuronide, 5-14% as salicylphenylglucuronide and 4-5% as gentisic acid. Roseman and Dorfman (1951) administered sodium gentisate to man and mouse and found that the dose was excreted unconjugated in the urine of man but that in the mouse up to 50% of the dose may be further metabolised.

Although the hydrolysis of aspirin in the body occurs relatively quickly, appreciable levels of an oral dose to man can be detected in plasma up to 2h after its administration (Lester et al., 1946; Mandel et al., 1954; Leonard, 1962; Rowland et al., 1967). Cummings and King (1966) using a thin layer chromatographic procedure showed that an average of 1.5% of a 12mg/kg body weight oral dose of acetylsalicylic acid in human male subjects was excreted unchanged in the urine within 1.5h of dosing, but that after this time very little unchanged acetylsalicylic acid was excreted. This was the first report of the urinary excretion of unchanged acetylsalicylic acid.

This chapter gives an account of an investigation of the metabolic fate of acetylsalicylic acid in the marmoset which is compared with that in the rat. The excretion of unchanged aspirin by man is also described.

Materials and Methods

Materials

Acetyl [carboxyl-^{14}C] salicylic acid (250μCi, 10-20mCi/mmol) was obtained from the Radiochemical Centre Ltd. and diluted with unlabelled acetylsalicylic acid (British Drug Houses Ltd., Poole) by removing the contents of the ampoule with small volumes of hot benzene (3ml) and transferring the solution to a small round flask. Unlabelled acetylsalicylic acid (250mg) was then added and the contents heated under reflux. Sufficient benzene was added dropwise until all the residue had dissolved. The crystals which separated on cooling were filtered, washed with cold benzene (2ml) and petroleum ether (b.pt range 60-80°C; 10ml) then dried under vacuum. The specific activity of the acetyl-[carboxyl-^{14}C] salicylic acid obtained was 8.89μCi/mg giving a recovery of the nominal activity supplied of 78%.

When chromatographed on glass plates coated with a thin layer of silica gel G in solvent B and on aluminium sheets pre-coated with a layer of silica gel $60F_{254}$ in solvent O (p 85) the product was shown to be radiochemically pure as determined by liquid scintillation counting and radiochromatogram scanning. However an autoradiograph prepared from an aluminium pre-coated plate, developed as above, indicated the presence of a small amount of [carboxyl-^{14}C] salicylic acid in the sample (Plate 5).

A further sample of acetyl [carboxyl-^{14}C] salicylic acid which had been diluted by the method described above was also available in the laboratory. The specific activity of this sample was 0.12μCi/mg and its radiochemical purity was 99.8% as determined by the above methods. The different specific activities permitted the administration of suitable quantities of radio-

activity to the animals at the different dose levels of acetylsalicylic acid employed.

[Carboxyl-^{14}C]salicylic acid was prepared by alkaline hydrolysis of acetyl [carboxyl-^{14}C]salicylic acid and was used as a reference compound in the preparation of chromatograms from which autoradiographs were made.

Methods

Administration of dose

Acetyl [^{14}C]salicylic acid was administered to rats and marmosets at dose levels of 100 and 13mg/kg body weight. At the higher dose level the dose was administered as its sodium salt with either milk or Cytacon as the dosing vehicle (p 81). The labelled dose was suitably diluted with unlabelled aspirin so that the radioactivity administered at this dose level was 4.5μCi/kg body weight. At the lower dose level acetyl [^{14}C]-salicylic acid of high specific activity (0.89μCi/mg) was administered as a suspension in Cytacon.

Human volunteers took three tablets of soluble aspirin (Queen Elizabeth Hospital Pharmacy) each containing 300mg of acetylsalicylate, approximately equivalent to a dose of 13mg/kg body weight.

Collection of urine

For investigations in which acetyl [^{14}C]salicylic acid was administered to rats and marmosets at the higher dose level urine samples were collected 24h after dosing. In experiments where the lower dose level was employed urine was collected from the dosed animals after a period of 6h and then after 24h.

In the study of the excretion of unchanged acetylsalicylic

acid by the marmoset and man, urine samples were collected 1.5h, 3.0h and 4.5h after the administration of the dose.

Chromatography

Chromatography was carried out using the following methods:- (i) glass plates coated with a thin layer of silica gel G were developed in solvents B and M; (ii) descending paper chromatography in solvents E and N; (iii) aluminium sheets pre-coated with silica gel $60F_{254}$ in solvent O as described by Cummings and King (1966). The metabolites were identified by comparison with reference compounds. In methods (i) and (ii) the compounds were detected by their radioactivity and/or by their reaction with the detecting reagents listed in Table 8.1. In method (iii) the metabolites and reference compounds were detected by their radioactivity and/or their absorption in UV light.

Glucosiduronic acids, resulting from the administered dose were located on non-fluorescent chromatograms by the reaction with detecting reagent I. The presence of these labelled metabolites was confirmed by digesting the urine from dosed animals with β-glucuronidase (type H-I or L-I) as previously described (p 175) and observing the change in the radiochemical distribution on the chromatograms as compared with that observed for the untreated urine.

To distinguish between labelled salicylacyl and labelled salicylphenyl glucuronides on chromatograms a sample of urine, collected from a marmoset which had received acetyl [^{14}C]salicylic acid, was adjusted to pH 10-11 with 0.1M NaOH and kept at 50°C for 45min. Under these conditions salicylacyl but not salicylphenyl glucuronide would be expected to hydrolyse. Chromatography of samples of the urine before and after this mild alkaline

hydrolysis showed which glucuronide had been destroyed.

Recovery of dose

(a) In urine

The urinary recovery of administered acetyl[^{14}C]salicylic acid was determined by liquid scintillation counting (p 89).

(b) In faeces

The quantity of radioactive components excreted in the faeces of dosed animals was determined by the combustion method (p 90).

Detection and determination of unchanged acetylsalicylic acid in the urine of dosed marmosets and humans

The excretion of unchanged acetylsalicylic acid in the urine of dosed marmosets and humans was investigated.

A GLC method based upon that described by Rance et al. (1975) for the detection of aspirin in plasma was explored using a 5% OV-101 column coating, but although salicylic acid was detected in the urine extracts, unchanged aspirin was not. The following HPLC method was therefore developed.

After administration of acetylsalicylic acid (13mg/kg body weight) to the marmoset, urine samples were collected at intervals up to 4.5h after the dose was given; each sample was diluted to 20ml with water, acidified to pH 1.0 and extracted manually with ether (3 x 40ml). The combined extracts from each sample were evaporated to dryness under reduced pressure at room temperature and the residue dissolved in methanol (0.5ml). The methanolic extract was quickly filtered through a Fluoropore filter (Millipore Corp., Mass.) of pore size 0.5μm and an aliquot of the filtrate applied to the HPLC column (p 88). A solvent

gradient was used to elute the metabolites as shown in Fig 8.1. The metabolites were detected by their absorption at 260nm. The identification of the acetylsalicylic acid peak was based upon a comparison of the retention time with that of a reference sample of aspirin and was confirmed by co-chromatography. The amounts of aspirin present in the samples were determined by measuring the areas under the peaks and comparing them with the areas produced by known amounts of aspirin. By this method the recovery of acetylsalicylic acid added to normal marmoset urine was 82%.

Urine samples collected from dosed humans were similarly extracted and the amount of unchanged aspirin determined.

Distribution of labelled metabolites in the urine of dosed rats and marmosets

This was carried out by the method of chromatography and liquid scintillation counting of the fragmented chromatograms (p 90). Most of the quantitative work, particularly at the high dose level, was carried out using descending paper chromatography in solvents E and N. In all cases parallel chromatograms were developed so that the labelled metabolites could be identified using detecting reagents I, II and III.

When the lower dose was administered to marmosets and rats the urine was collected 6h after the dose was given. Samples of the urine were chromatographed on aluminium sheets pre-coated with silica gel $60F_{254}$ in solvent O, and the distribution of radioactivity determined by radiochromatogram scanning and liquid scintillation counting of the scraped-off coating from 0.5cm sections of the plates. With these chromatograms the metabolites were located by their quenching of UV light before the plates

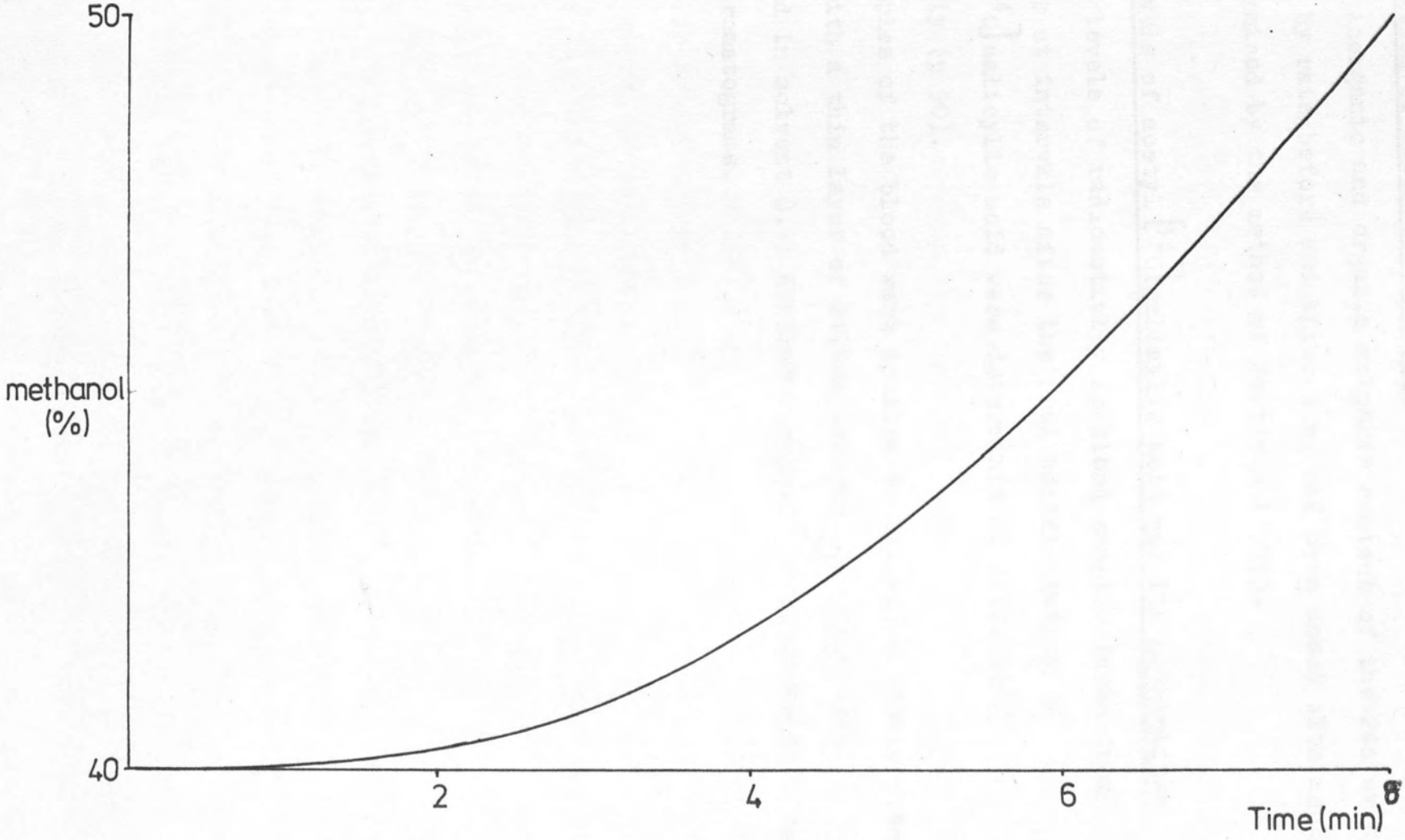


Fig 8.1 The methanolic solvent gradient used in the determination of unchanged acetylsalicylic acid in the urine by HPLC.

were scraped.

Determination of sulphate excretion

The inorganic and organic sulphate content of the 24h urine excreted by rats before and after they had been dosed with aspirin was determined by the method of Morrison (1973).

Blood levels of acetyl [^{14}C]salicylic acid and its metabolites

The levels of radioactivity in blood samples taken from marmosets at intervals after the oral administration of acetyl [^{14}C]salicylic acid were determined as described previously (p 90).

Samples of the blood were applied to aluminium sheets pre-coated with a thin layer of silica gel $60F_{254}$, which were developed in solvent O. Autoradiographs were prepared from the dried chromatograms.

Results

Identification of metabolites

The chromatographic properties of the reference compounds and of the main urinary metabolites excreted by dosed animals and not seen in normal urine are summarised in Table 8.1.

Aspirin and salicylic acid were only clearly separated by chromatography on the aluminium-backed plates in solvent O. Unchanged aspirin was not detected in samples of the urine of dosed animals using this system. Salicylic, salicyluric and gentisic acids were clearly separated by this procedure and gave characteristic colours when viewed under UV light. Plate 5 is an autoradiograph of a chromatogram, developed in solvent O of samples of urine from three marmosets which had been dosed with acetyl [^{14}C]salicylic acid.

Metabolite 1 (Table 8.1) detected in the urine of all dosed animals was identified as salicylic acid by its R_F values and by the reaction given with the detecting agents shown in Table 8.1. Similarly metabolite 2, excreted by dosed rats and marmosets was identified as salicyluric acid and metabolite 3 (detected by reagent II in urine of two dosed rats) as gentisic acid. Metabolites 4 and 5 detected in the urine excreted by dosed rats and marmosets gave a positive reaction with the naphthoresorcinol reagent (reagent I) indicating that they were glucosiduronic acids. Their further examination is described later.

2,3-, 2,4- and 2,6-dihydroxybenzoic acids were not detected by reagent V in the urine of either marmosets or rats which had been dosed with acetylsalicylic acid. When parallel chromatograms were developed in solvents E and N, one containing reference dihydroxybenzoic acids and the other urine from either marmosets

Compound	R_F in solvent				Colour with detecting agent				R_F in solvent	Colour of quench under uv light
	B[a]	M[a]	E[b]	N[b]	I	II	III	V	O[c]	
Reference compounds										
Acetylsalicylic acid[d]	0.82	0.67	0.65	nd	-	yellow	-	ne	0.85	d/b
Salicyclic acid	0.86	0.65	0.65	0.90	-	yellow	blue	ne	0.89	l/b
Gentisic acid	0.32	0.38	0.43	0.80	-	beige	blue	beige	0.77	l/b
Salicyluric acid	0.26	0.28	0.22	0.82	-	red	purple	ne	0.38	violet
2,3-dihydroxybenzoic acid	ne	ne	0.50	0.80	-	ne	ne	grey	ne	-
2,4-dihydroxybenzoic acid	ne	ne	0.39	0.80	-	ne	ne	orange	ne	-
2,6-dihydroxybenzoic acid	ne	ne	0.70	0.60	-	ne	ne	yellow	ne	-
Urinary metabolites										
Metabolite 1	0.86	0.72	0.63	0.85[e]	-	yellow	blue	ne	0.89	l/b
2	0.21	0.26	0.24	0.85	-	red	purple	ne	0.37	violet
3	nd	0.36	0.44	nd	-	beige	blue	nd	nd	-
4	nd	0.08	0.05	0.24	b1	-	-	ne	0.00	d/b
5	-	-	-	0.10	b1	-	-	ne		

a Chromatography carried out on glass thin layer plates
b Chromatography carried out by descending technique on paper
c Chromatography carried out on aluminium backed fluorescent thin layer plates
d Acetylsalicylic acid does not react with the phenolic sprays in its unhydrolysed form
e Can be distinguished by the mixtures of colours given with reagent II
l/b - light blue, d/b - dark blue nd - not detected, ne - not examined.

Table 8.1 Chromatographic properties of reference compounds and metabolites of acetylsalicylic acid

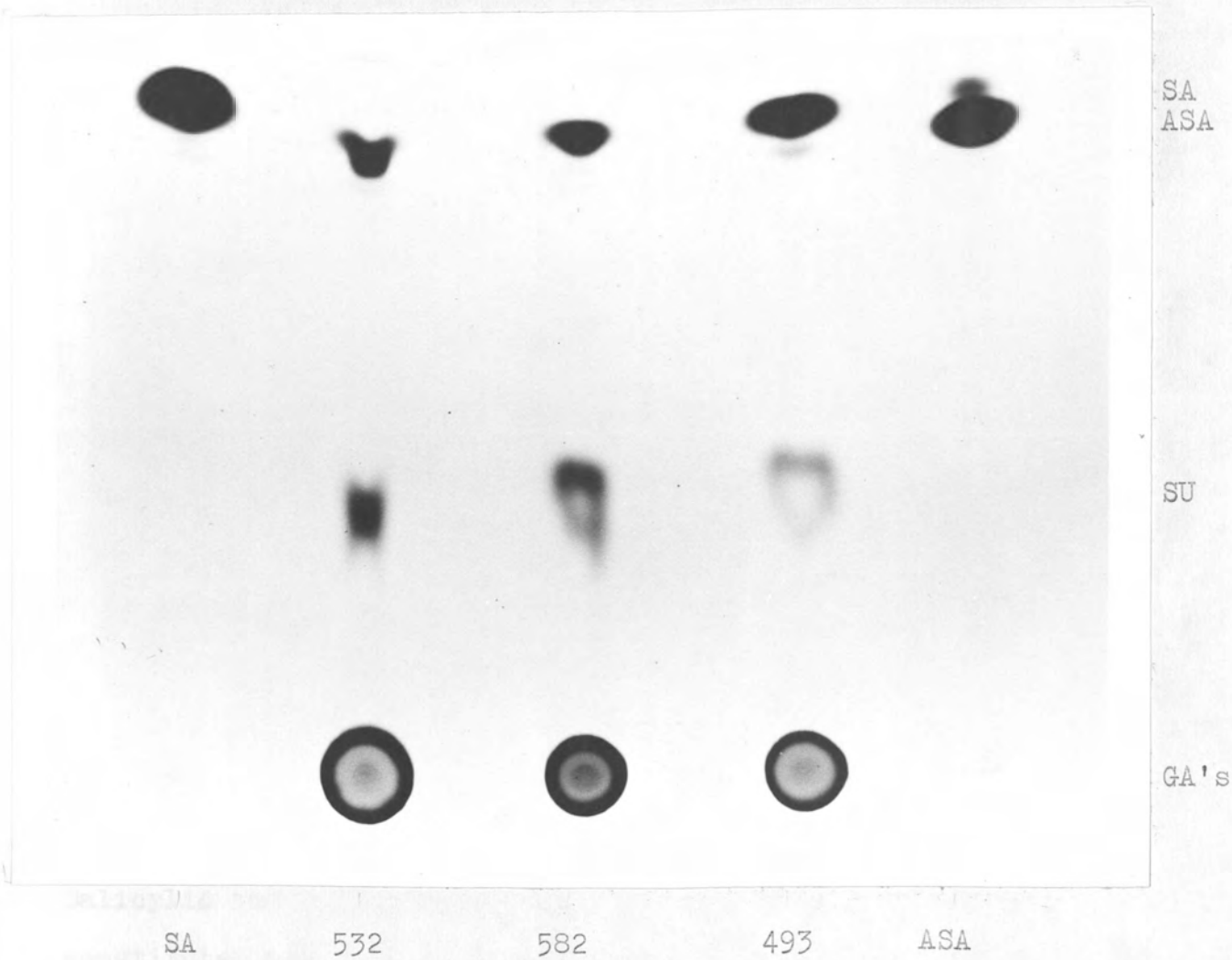


Plate 5 Autoradiograph taken from an aluminium-backed thin layer chromatogram developed in solvent O, showing the radio-metabolite distribution in the urine of three marmosets (Code numbers as shown) which had been dosed with acetyl [^{14}C] salicylic acid.

SA = Salicylic acid, ASA = acetylsalicylic acid,
SU = salicyluric acid, GA's = glucosiduronic acids.

or rats which had been dosed with acetyl [^{14}C] salicylic acid, no radioactive metabolite was detected in the urine of the dosed animals which corresponded to any of the reference compounds.

Determination of the relative amounts of radioactive metabolites in the urine of dosed animals

Figures 8.2 and 8.3 are typical histograms showing the radiochemical distribution along paper chromatograms of samples of urine from a dosed marmoset, developed in solvents E and N respectively. In solvent E, radiochemical peaks were apparent corresponding to salicylic acid, salicyluric acid and metabolite 4 (Table 8.1) a glucosiduronic acid. In some experiments a small peak corresponding to gentisic acid was seen. In some cases the peak attributed to salicyluric acid appeared to consist of two separate components, especially when the lower dose level was employed where the specific activity of the administered compound was relatively high.

In solvent N, four labelled metabolites were detected. Salicylic and salicyluric acids were not separated and constituted the large radiochemical peak at high R_F value. Two peaks of low R_F values were evident and correspond to metabolites 4 and 5 (Table 8.1). A fourth radioactive area was also detected; this was diffuse and had an average R_F value of 0.57 (metabolite X; Table 8.3). It was not detected by either detecting reagents I, II, III or V. In a few experiments this peak appeared to consist of two components. The further examination of this material is described later.

In Tables 8.2 and 8.3 the recoveries of the doses are given and the relative proportions of the metabolites expressed as percentages of the total metabolites excreted in the urine of

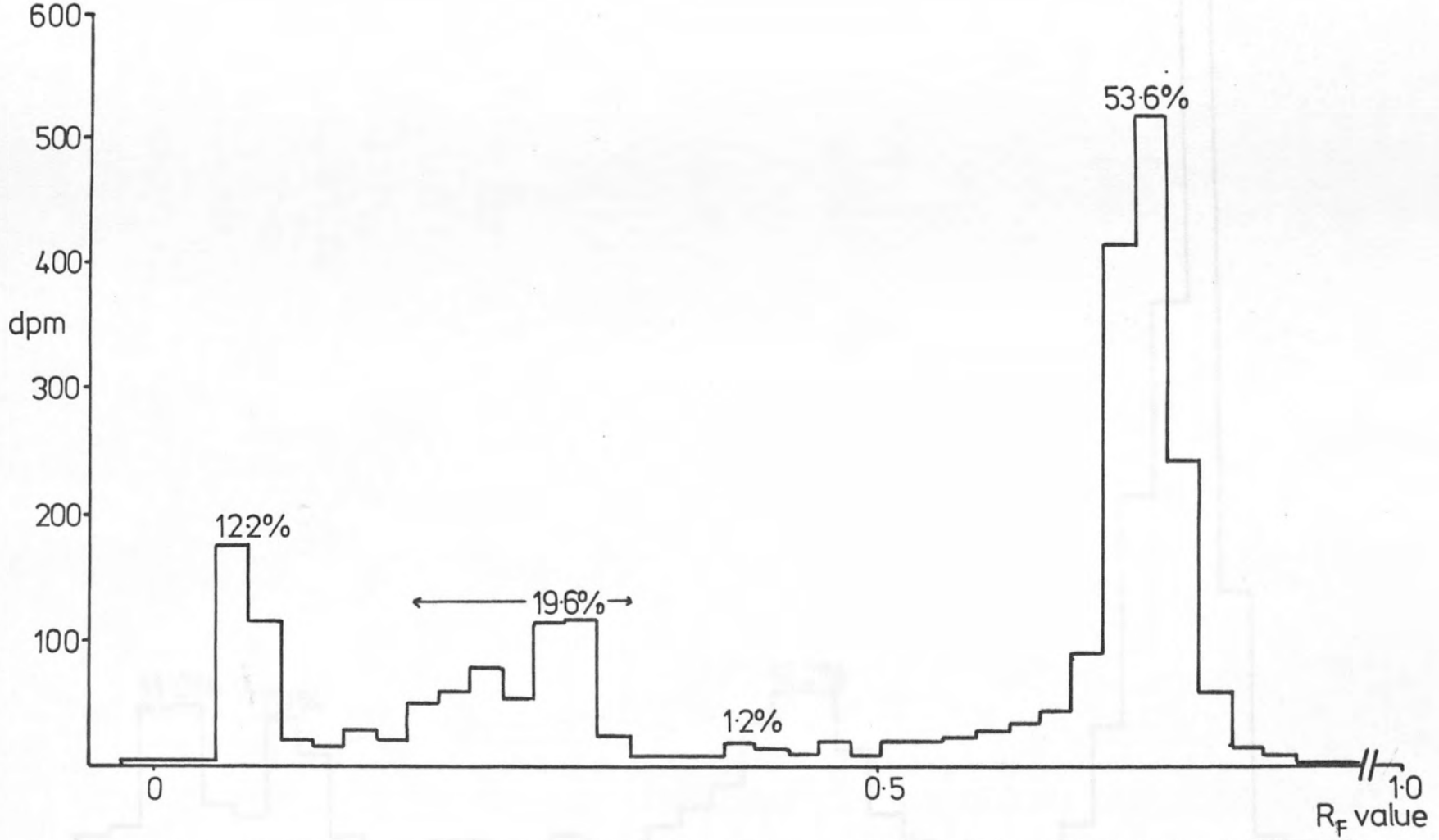


Fig 8.2 Distribution of radioactivity along a chromatogram developed in solvent E of 24h urine from a marmoset which had been dosed with acetyl [^{14}C] salicylic acid.

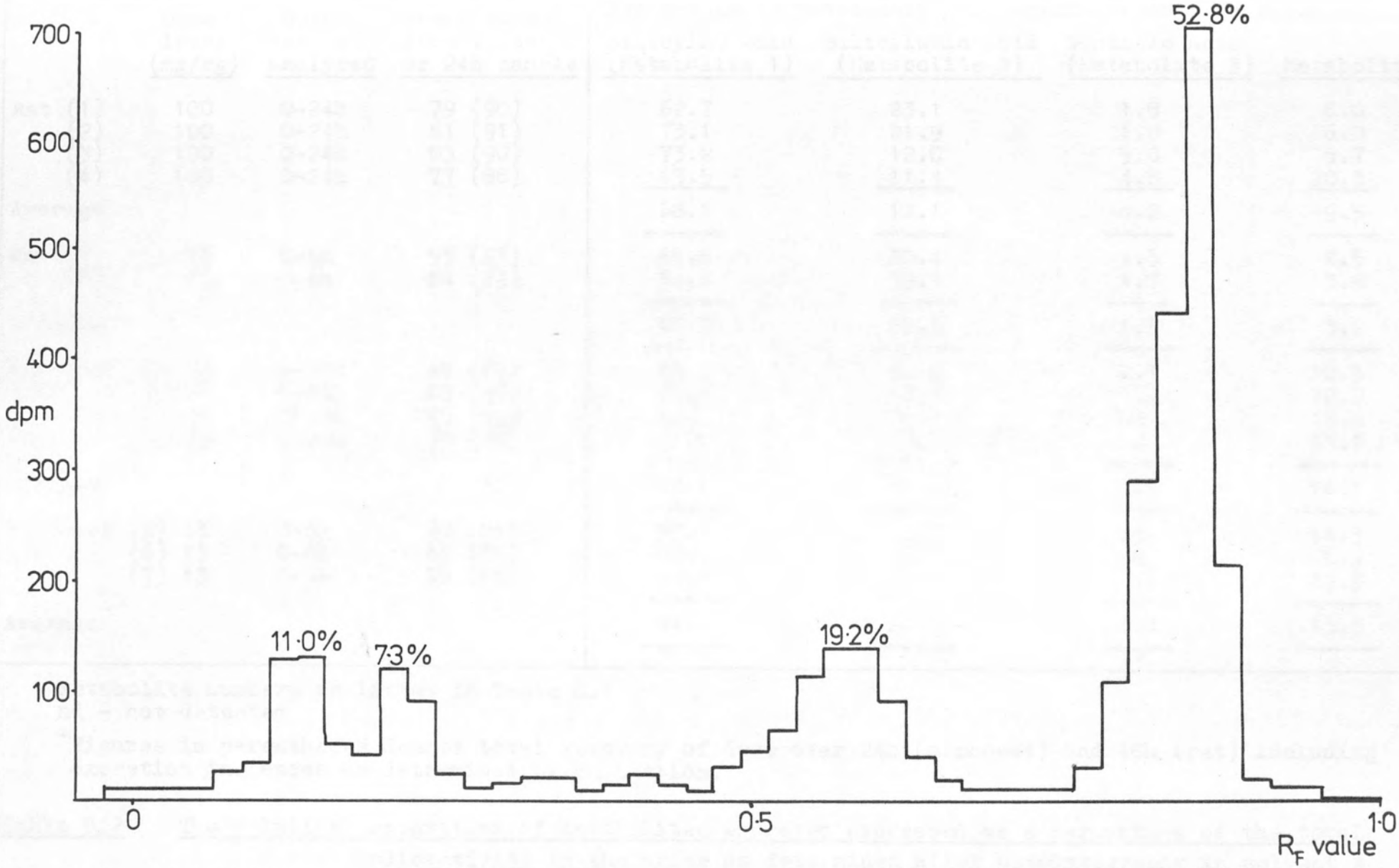


Fig 8.3 Distribution of radioactivity along a chromatogram developed in solvent N of 24h urine from a marmoset which had been dosed with acetyl [^{14}C] salicylic acid.

	Dose level (mg/kg)	Urine sample analysed	Recovery of[+] dose in 6h or 24h sample	Percentage of radioactive components present in urine as			
				Salicylic acid (Metabolite 1)	Salicyluric acid (Metabolite 2)	Gentisic acid (Metabolite 3)	Metabolite 4
Rat (1)	100	0-24h	79 (90)	62.7	23.1	1.8	6.0
(2)	100	0-24h	81 (91)	73.1	21.9	1.0	6.0
(3)	100	0-24h	83 (90)	73.2	12.0	9.0	5.7
(4)	100	0-24h	77 (88)	63.5	11.4	4.8	20.3
Average				68.1	17.1	4.2	9.5
Rat (5)	13	0-6h	55 (93)	65.8	30.4	1.3	2.5
(6)	13	0-6h	54 (79)	54.8	39.7	1.7	3.8
Average				60.3	35.1	1.5	3.2
Marmoset (1)	100	0-24h	49 (60)	55.3	25.6	2.1	10.3
(2)	100	0-24h	22 (46)	53.6	19.6	1.2	12.2
(3)	100	0-24h	45 (63)	67.3	14.1	nd	18.6
(4)	100	0-24h	55 (72)	72.0	7.2	nd	15.1
Average				62.1	16.6	0.8	14.1
Marmoset (5)	13	0-6h	62 (81)	57.1	25.3	nd	14.5
(6)	13	0-6h	62 (68)	88.2	6.7	nd	6.2
(7)	13	0-6h	78 (85)	47.3	28.3	5.0	19.3
Average				64.2	20.1	1.7	13.3

Metabolite numbers as listed in Table 8.1
nd - not detected

[+]Figures in parentheses denote total recovery of dose over 24h (marmoset) and 48h (rat) including excretion in faeces as determined by combustion.

Table 8.2 The relative proportions of metabolites excreted expressed as a percentage of the total radioactivity in the urine as determined after chromatography in solvent E

	Dose level (mg/kg)	Urine sample analysed	Recovery of[+] dose in 6h or 24h sample	Percentage of radioactive components present in urine as SA+SU+GA (Metabolites 1+2+3)	Unidentified (X) R_F = 0.57	Metabolite 4	Metabolite 5
Rat (1)	100	0-24h	79 (90)	77.7	15.1	6.1	nd
(2)	100	0-24h	81 (92)	79.2	13.6	2.8	4.0
(3)	100	0-24h	83 (90)	72.5	18.2	2.8	6.2
(4)	100	0-24h	77 (88)	78.4	15.8	nd	7.8
Average				77.0	15.7	2.9	4.5
Rat (5)	13	0-6h	55 (93)	85.4	17.7	1.0	0.9
(6)	13	0-6h	54 (79)	79.1	17.8	2.0	1.2
Average				82.3	17.8	1.5	1.1
Marmoset (1)	100	0-24h	49 (60)	46.2	34.1	11.9	5.0
(2)	100	0-24h	22 (46)	52.8	19.2	7.3	11.0
(3)	100	0-24h	45 (63)	76.8	15.0	nd	8.3
(4)	100	0-24h	55 (72)	74.7	14.3	8.6	nd
Average				62.6	20.7	9.3	8.1
Marmoset (5)	13	0-6h	62 (81)	59.0	26.3	13.7	1.0
(6)	13	0-6h	62 (68)	90.8	4.2	5.0	nd
(7)	13	0-6h	78 (85)	57.2	27.1	4.6	nd
Average				69.0	19.2	7.8	0.3

Metabolite codes as listed in Table 8.1
nd - not detected; SA - Salicylic acid, GA - Gentisic acid, SU - Salicyluric acid

[+]Figures in parentheses are total recoveries over 24h (marmoset) and 48h (rat) including faecal excretion as determined by combustion.

Table 8.3 The relative proportions of metabolites excreted expressed as a percentage of the total radioactivity in the urine as determined by chromatography in solvent N

dosed rats and marmosets as determined in solvent systems E and N.

The results obtained using solvent E indicated that at the higher dose level the conversion of salicylic acid to gentisic acid occurred to a greater extent in the rat than in the marmoset, while the formation of the metabolite of low R_F value, tentatively identified as a glucosiduronic acid, was more evident in the marmoset. In both species the formation of the metabolite(s) with an R_F value equivalent to that of salicyluric acid occurred to a similar extent at the higher dose level but the relative proportion of this metabolite(s) in the urine was greater in the rat than in the marmoset after the administration of the dose at the lower level. At the lower dose level the average excretion of gentisic acid by rats and marmosets were similar. As with the higher dose level, the formation of the metabolites of lower R_F value was more evident in the marmoset than in similarly dosed rats.

The quantitative results obtained using solvent N (Table 8.3) show that the formation of the unidentified metabolite mentioned above (metabolite X) was more evident in the marmoset than in the rat. The two minor metabolites 4 and 5 (Table 8.1) were clearly visible in this solvent.

<u>The effect of incubation with β-glucuronidase on the distribution of radioactive components in the urine</u>

The distribution of the labelled components in the urine of dosed rats and marmosets after treatment with β-glucuronidase type L-I and H-I is given in Table 8.4. In solvent E, metabolite 4 (Table 8.1, 8.2) was little affected by this treatment, whereas metabolite 2, initially identified as salicyluric acid was almost completely destroyed. In all the digestions carried out, the

Animal	Percentage of radioactive components present in urine after β-glucuronidase++ treatment			
	Metabolite 1 (E)	Metabolite 2 (E)	Metabolite 3 (E)	Metabolite 4 (E)
Rat (1)+	75.8 (62.7)	5.3 (23.1)	9.4 (1.8)	7.3 (6.0)
(6)	88.7 (54.8)	nd (39.7)	1.5 (1.7)	9.6 (3.8)
Marmoset (1)	79.0 (55.3)	7.8 (25.6)	1.8 (2.1)	9.2 (10.3)
	Metabolite 1 (N)	Metabolite X (N)	Metabolite 4 (N)	Metabolite 5 (N)
Rat (1)	90.6 (77.7)	4.1 (15.1)	2.1 (6.1)	nd (nd)
(2)	98.5 (79.2)	nd (13.6)	1.5 (2.8)	nd (4.0)
Marmoset (1)	81.1 (46.2)	13.8 (34.1)	3.0 (11.9)	nd (5.0)

+Animal numbers correspond to those in Tables 8.2 and 8.3

++Preparation H-I or L-I
(E) - As determined in solvent E
(N) - As determined in solvent N
Percentages in parentheses denote the values observed before digestion with β-glucuronidase
nd - Not detected.

Table 8.4 Effect of β-glucuronidase on the metabolite distribution in the urine of dosed rats and marmosets as determined in solvents E and N

reduction in the labelled components was balanced by an increase in salicylic acid, except in the case of one rat, where an increase in gentisic acid concentration was observed suggesting the presence of a glucuronide conjugate of gentisic acid. These results strongly support the previous suggestion that two labelled components constituted the peak of radioactivity in solvent E, attributed initially to salicyluric acid, and further suggest that the second component of this peak was a glucosiduronic acid. A sample of authentic salicyluric acid was dissolved in 0.2M sodium acetate buffer pH 3.8 and subjected to digestion with β-glucuronidase type L-I at 37°C for periods of up to 48h. By using the GLC method described previously (p 219) it was shown that such treatment did not result in the formation of salicylic acid (retention time 12min).

The results in Table 8.4 for solvent N indicated that metabolite 5 (Tables 8.1 and 8.3) was a glucosiduronic acid but that metabolite 4 which was only partially digested by the β-glucuronidase preparation may have been a sulphate ester which was partially hydrolysed by the sulphatase impurities in the β-glucuronidase used. No increase in the excretion of sulphate esters on the administration of aspirin to rats was detected (Table 8.5) but metabolite 4 (Table 8.3) was present in only small amounts which the method of Morrison (1973) may not be sufficiently sensitive to detect.

The unidentified metabolite X of R_F value 0.57 in solvent N (Table 8.3) was broken down significantly by β-glucuronidase treatment which suggested it was a glucosiduronic acid. After mild alkaline hydrolysis of the urine collected from a marmoset which had been dosed with acetyl [^{14}C] salicylic acid, an analysis of the radiochemical distribution along chromatograms developed

Animal	Form of sulphate excreted	Sulphate (mg) excreted either free or in conjuged form			
		Control day 1	Control day 2	Dosed day 1	Dosed day 2
Rat (1)	Total	65.0	62.6	55.9	49.3
	Inorganic	51.0	60.8	51.7	45.7
	Ethereal	13.7	1.8	4.1	3.6
Rat (2)	Total	68.1	47.2	65.4	57.5
	Inorganic	62.1	48.0	61.3	55.2
	Ethereal	6.1	-	4.1	2.3
Rat (3)	Total	58.2	60.2	50.8	49.9
	Inorganic	52.4	60.3	47.2	45.8
	Ethereal	5.7	-	3.6	4.1

- no increase observed.

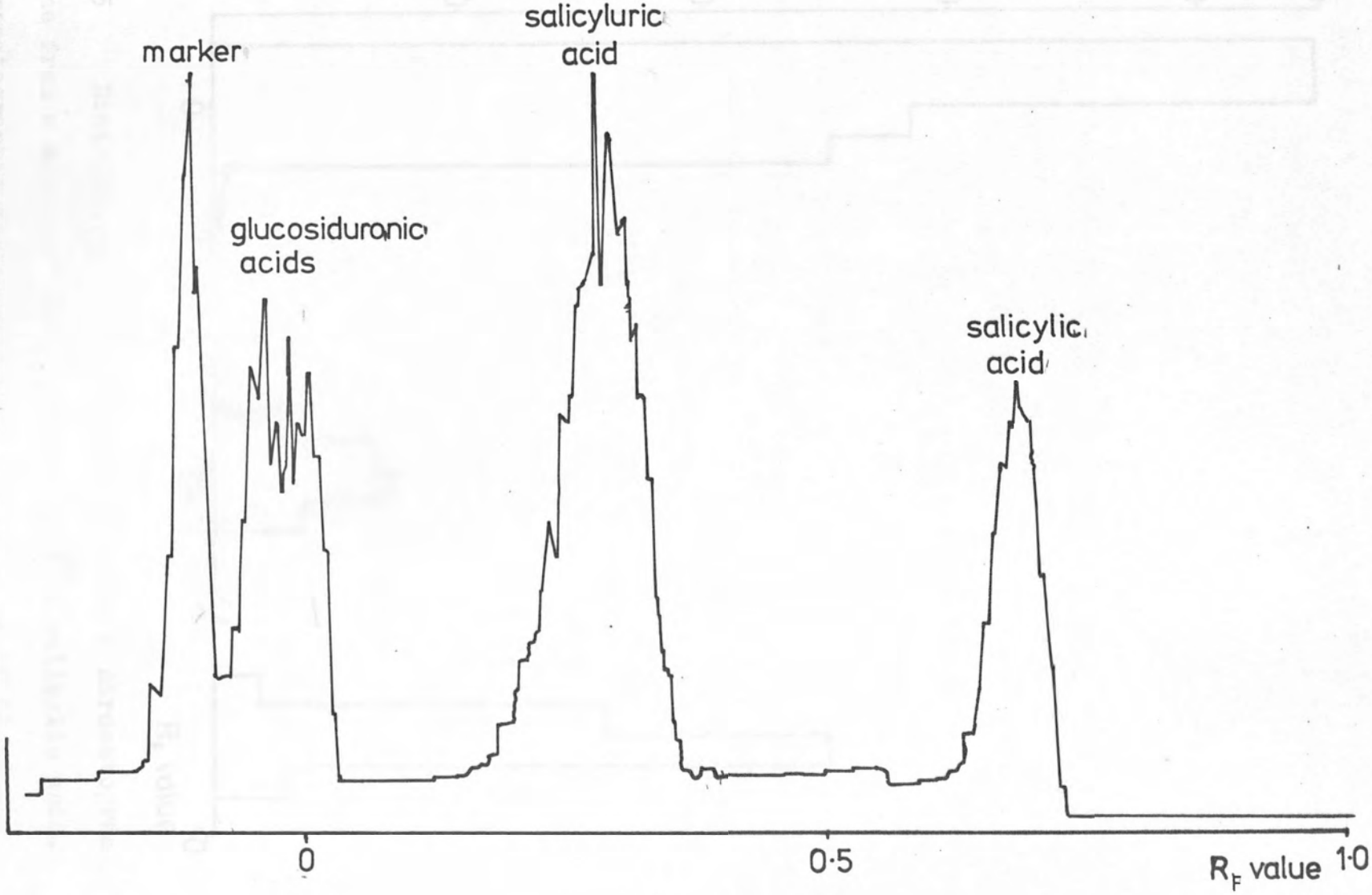
Table 8.5 Effect of the administration of acetylsalicylic acid (100mg/kg body weight) on free and ethereal sulphate excretion in rats

in solvent N by radiochromatogram scanning and fragmentation analysis (p 90) showed that the diffuse peak corresponding to metabolite X was entirely destroyed. Metabolite 5 was unaffected and the proportion of metabolite 4 was much reduced. It is therefore probable that metabolite 5 was salicylphenylglucuronide and metabolite X was salicylacylglucuronide. In both species studied the proportion of the metabolites excreted in the urine that were present as salicylphenylglucuronide increased significantly on increasing the dose level from 13 to 100mg/kg body weight, whereas the proportion of salicylacylglucuronide was unaffected. In no instance did metabolite X give a positive reaction with detecting reagent I for glucosiduronic acids. The chromatographic properties of this metabolite were such that the peak of radioactivity observed in solvent N was always diffuse, being on average 8cm in length and consequently the concentration of the metabolite per unit area of chromatogram may well have been too low to give a positive reaction with detecting reagent I.

Figure 8.4 shows a typical radiochromatogram scan when the urine from a dosed marmoset was chromatographed on aluminium backed thin layer chromatography plates in solvent O while Fig 8.5 shows a typical histogram obtained when the radioactivity contained in 0.5cm sections of the silica was determined by liquid scintillation counting. Only three quenching spots were seen on the chromatograms when they were viewed under light of wavelength 254nm; they corresponded to salicylic acid, salicyluric acid and the glucosiduronic acids (see Table 8.1 and Plate 5). Proof of the identity of the glucuronic acid conjugates was obtained by digesting the urine with β-glucuronidase (type H-I) when the quenching spot on the origin

Fig 8.4

Distribution of radioactivity along an aluminium thin layer chromatography plate coated with silica gel $60F_{254}$ and developed in solvent 0, of [^{14}C]acetylsalicylic acid dosed (13mg/kg body weight) marmoset urine as determined by radiochromatogram scanning.


marker
salicyluric acid
glucosiduronic acids
salicylic acid
0
0·5
1·0
R_F value

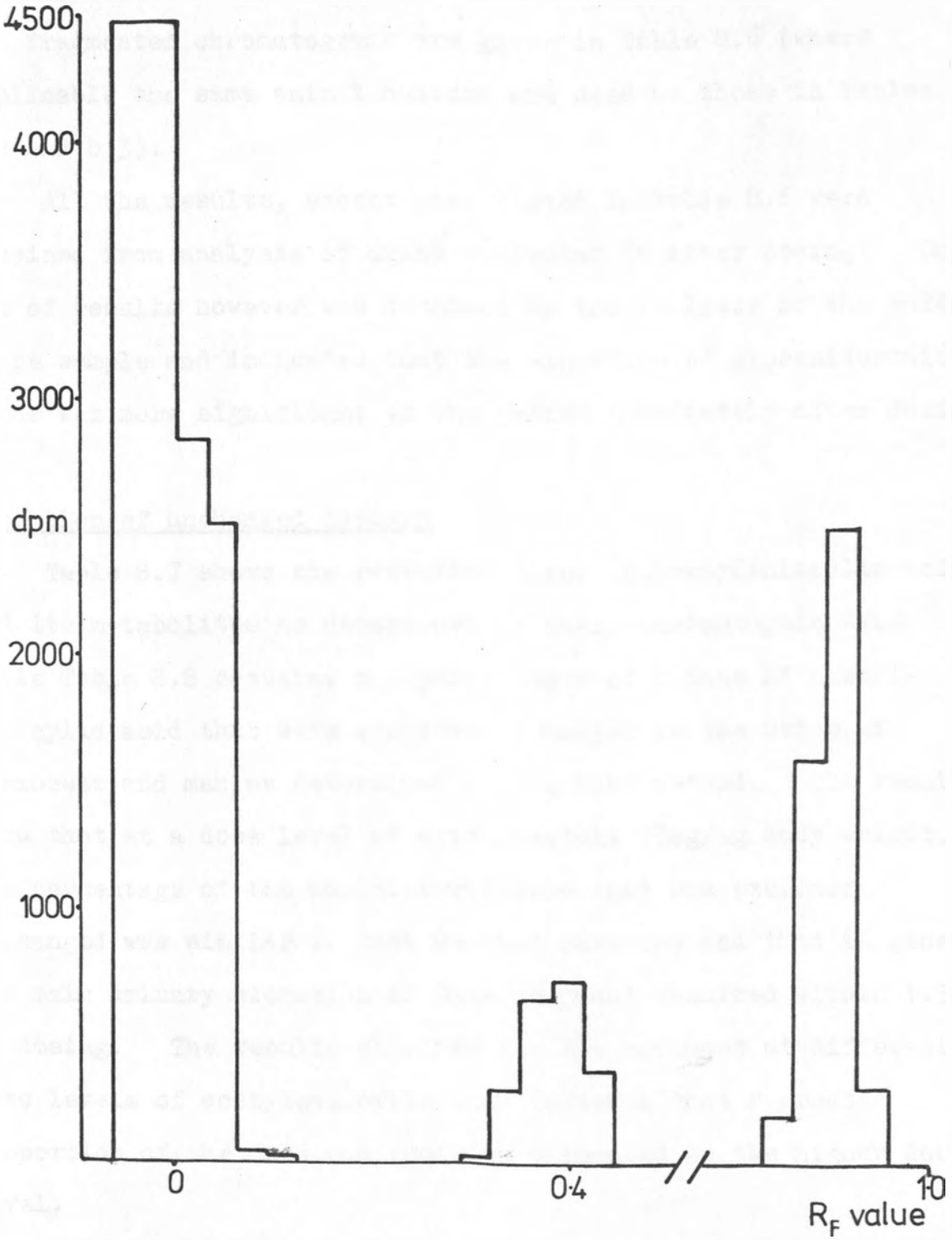


Fig 8.5 Distribution of radioactivity along a chromatogram of urine from a marmoset dosed with acetyl [^{14}C] salicylic acid. The chromatographic system employed consisted of an aluminium plate pre-coated with a thin layer of silica gel $60F_{254}$, developed in solvent O.

of the plate disappeared. The relative proportions of the various urinary metabolites of acetyl [^{14}C]salicylic acid in rats and marmosets as determined by liquid scintillation counting of the fragmented chromatograms are given in Table 8.6 (where applicable the same animal numbers are used as those in Tables 8.2 and 8.3).

All the results, except one, listed in Table 8.6 were obtained from analysis of urine collected 6h after dosing. One set of results however was obtained by the analysis of the 6-24h urine sample and indicates that the excretion of glucosiduronic acids was more significant in the period immediately after dosing.

Excretion of unchanged aspirin

Table 8.7 shows the retention times of acetylsalicylic acid and its metabolites as determined by co-chromatographic HPLC while Table 8.8 contains the percentages of a dose of acetylsalicylic acid that were excreted unchanged in the urine of marmosets and man as determined by the HPLC method. The results show that at a dose level of approximately 13mg/kg body weight, the percentage of the administered dose that was excreted unchanged was similar in both man and marmoset and that in general the main urinary excretion of this compound occurred within 1.5h of dosing. The results obtained for the marmoset at different dose levels of acetylsalicylic acid indicate that a greater proportion of the dose was excreted unchanged at the higher dose level.

Blood levels of acetyl [^{14}C]salicylic acid and its metabolites

Figure 8.6 shows the variation with time in the level of radioactive components present in the blood in two marmosets

Animal	Urine sample analysed	Dose recovered[+] in urine sample	Percentage of radioactive components in urine present as		
			Salicylic acid	Salicyluric acid	Glucosiduronic acids
Rat (5)	0-6h	55 (93)	56.9	23.4	18.8
(6)	0-6h	54 (79)	43.3	29.1	25.4
(7)	0-6h	52 (95)	81.9	5.4	12.5
(8)	0-6h	61 (97)	73.6	12.3	13.6
Average			63.9	17.6	17.6
Marmoset (5)	0-6h	62 (81)	30.8	16.2	51.5
(6)	0-6h	62 (68)	64.6	2.5	32.6
(6)	6-24h	3	74.6	1.9	23.4
(7)	0-6h	78 (85)	60.7	11.3	28.0
Average			52.0	10.0	37.4

[+]Figures in parentheses are total recoveries over 24h (marmoset) and 48h (rat) including the faecal excretion as determined by combustion.

Table 8.6 The relative proportions of metabolites excreted expressed as a percentage of the total radioactivity in the urine as determined by chromatography on aluminium backed thin layer fluorescent chromatography plates developed in solvent O.

Compound	Retention time (min)
Gentisic acid	2.4
Salicyluric acid	2.9
Acetylsalicylic acid	4.5
Salicylic acid	6.2

Table 8.7 Retention times of metabolites of acetylsalicylic acid as determined by HPLC

Species	Sex	Dose (mg/kg)	Percentage acetylsalicylic acid excreted unchanged in urine sample listed		
			1.5h	3.0h	4.5h
Marmoset	F	13.0	tr	0	0
	M	15.0	1.1	tr	0
	M	77.0	3.4	1.2	0
Human	F	7.0	2.1	nd	nd
	F	13.0	1.4	0.2	0
	M	13.0	0.5	0.7	0.2
	F	16.0	5.4	0.2	0
Human[+]	M	12.0	0-2.6	0	nd

M - Male F - Female
tr - trace nd - not determined
[+]Results obtained by Cummings and King (1966) using six male subjects.

Table 8.8 Excretion of unchanged acetylsalicylic acid as determined by HPLC

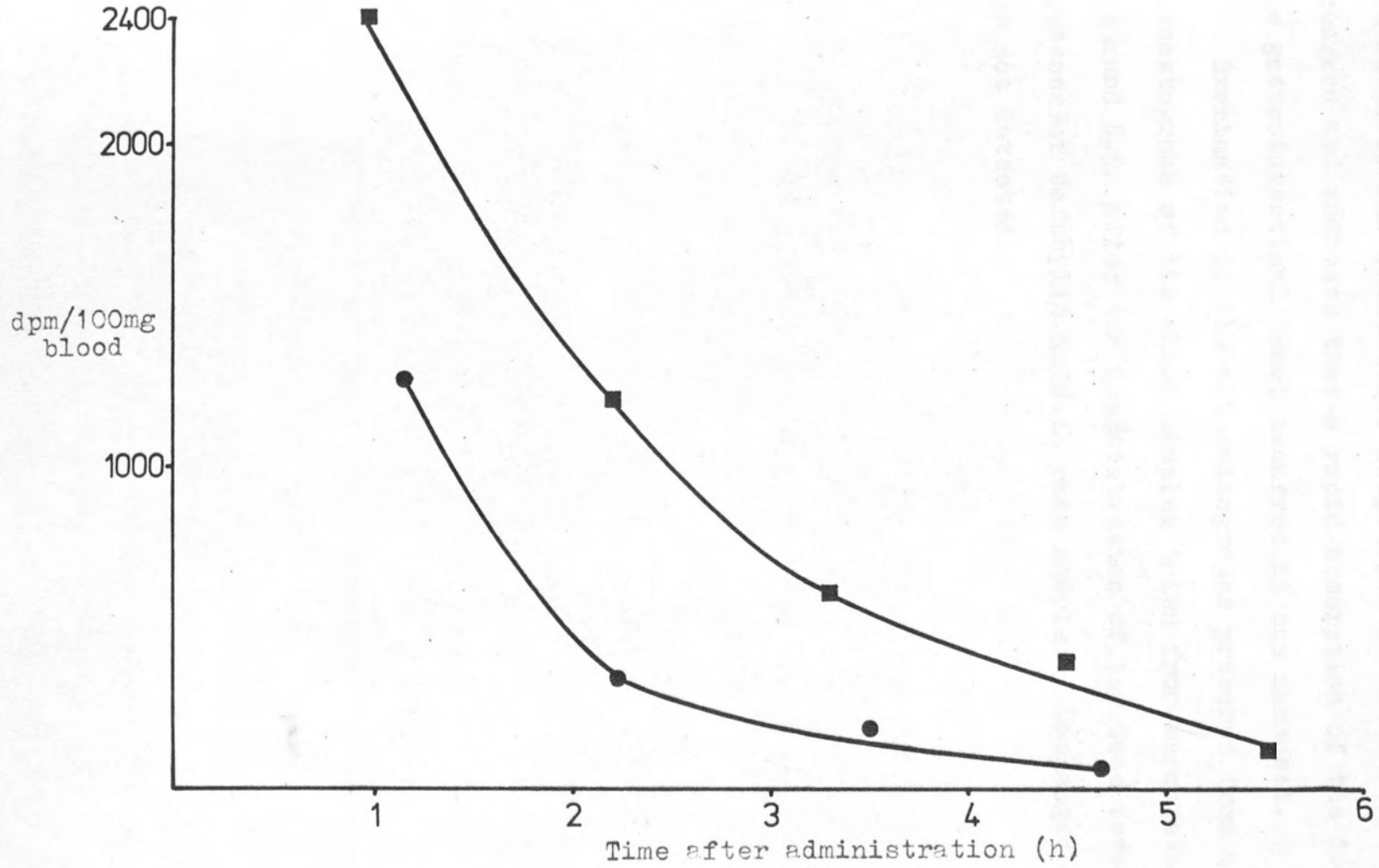


Fig 8.6 Variation in the blood level of radioactive components with time, in two marmosets following the administration of acetyl [^{14}C] salicylic acid (13mg/kg body weight).

after the administration of acetyl [^{14}C]salicylic acid. In both experiments the first blood sample was taken after the peak in the level of the radioactive components in the blood had occurred and indicate that a rapid absorption of the dose from the gastrointestinal tract occurred in the marmoset.

Examination of the autoradiographs prepared from the chromatograms of the blood samples taken from marmosets at 1.0h, 2.25h and 5.5h after the administration of the dose revealed the presence of salicylic acid in each sample. Unchanged aspirin was not detected.

Discussion

The metabolic products excreted in the urine after the administration of acetylsalicylic acid are similar in the marmoset and rat. There are however variations in the relative proportions of the different metabolites excreted; glucosiduronic acid formation occurs to a greater extent in the marmoset than in the rat while the reverse appears to be true for glycine conjugation at the lower dose level. The proportion of administered acetylsalicylic acid that is excreted as salicyluric acid is variable in both species studied. A similar situation has already been reported for the marmoset, but not the rat, using benzoic acid as the dose (Chapter 3). It is interesting that the marmoset which excreted very little of the administered aspirin as salicyluric acid (M6) was the animal which excreted only 8.2% of the total metabolites of a 100mg/kg body weight dose of benzoate as hippuric acid (Chapter 3). Over the 24h period after the administration of acetylsalicylic acid the quantity of glycine utilized by this animal for the synthesis of salicyluric acid was only 0.1mg/kg body weight. In a similar time period this animal when challenged with a 100mg/kg body weight dose of [^{14}C]benzoate utilized 3.9mg of glycine/kg body weight in the synthesis of hippuric acid which suggests that the low proportion of administered acetyl[^{14}C]salicylic acid excreted conjugated with glycine is probably due to some factor other than a deficiency in the supply of glycine available for conjugation.

Both the rat and marmoset excrete administered acetylsalicylic acid as salicylacyl and salicylphenyl glucuronides. In both species salicylacylglucuronide constitutes the major proportion of the total glucosiduronic acids excreted. The proportion of the total metabolites accounted for by this component appears not

to vary with the dose level employed although the proportion of salicylphenylglucuronide excreted in the urine appears to be slightly dose dependent. Alpen et al. (1951) reported that in the dog salicylphenylglucuronide was the only glucosiduronic acid formed from administered salicylate while the excretion of administered salicylate as salicylphenylglucuronide has been shown to be more important than salicylacylglucuronide in the rabbit (Bray et al., 1948b) and in man (Levy et al., 1972). The present results suggest that in the rat and marmoset more acyl than phenyl glucuronide is formed, emphasising the high ability of the marmoset to form ester glucosiduronic acids.

Both the rat and marmoset excreted a minor metabolite tentatively identified as a sulphate ester. The concentration of this metabolite in the urine was low and no increase in the excretion of sulphate esters after the administration of unlabelled acetylsalicylic acid to the rat was found; no ester sulphate formation was reported for the rabbit (Bray et al., 1948b) which had been dosed with salicylic acid.

The only other minor metabolite detected in the urine of rats and marmosets which had been dosed with acetyl [^{14}C]salicylic acid was gentisic acid. In the marmoset this metabolite appeared to be excreted unconjugated while in the rat evidence was obtained that some conjugation of gentisic acid with glucuronic acid occurred. In the marmoset the proportion of the administered dose that was excreted as gentisic acid was only small; similar results have been reported for man (Alpen et al., 1951; Levy et al., 1972). Sodium gentisate, on administration to man, is excreted unchanged and not conjugated with any endogenous molecule (Roseman and Dorfman, 1951).

After the administration of acetylsalicylic acid at a dose

level of 43mg/kg body weight to man, Levy et al. (1972) reported that salicyluric acid accounted for 50% of the metabolites excreted which is considerably higher than that found in the present study for the metabolic fate of acetyl [^{14}C]salicylic acid in rats and marmosets at dose levels of either 13 or 100mg/kg body weight. Furthermore the proportions of metabolites excreted that were accounted for as salicyluric acid after the administration of acetyl [^{14}C]salicylic acid to the rat and marmoset did not appear to be significantly affected by the dose level and clearly the availability of glycine was not the limiting factor in the synthesis of salicyluric acid. Davison (1971) reported that glycine administration in man did not modify salicyluric acid excretion after the administration of salicylic acid, but did cause an increase in hippurate formation following benzoate administration. The limitation was not in the supply of glycine but in another factor. It was suggested by this author that different enzymes may be operative in the synthesis of hippurate and salicylurate, or that the affinities of the two substrates for the conjugating enzyme may vary markedly. A low excretion of salicyluric acid has also been reported for the rabbit (Bray et al., 1948b) following the administration of salicylic acid. The results indicate that the formation of salicyluric acid from administered salicylate may be a more important biotransformation reaction in man than in either New World primates or non-primate species.

The pH of the marmoset urine both before and after the administration of sodium acetylsalicylate at a dose level 100mg acetylsalicylate/kg body weight or as the free acid at a level of 13mg/kg body weight varied between 6.9 and 7.1. This is higher than the average pH of human urine (6.0) and may

explain to some extent why the proportions of the administered dose of aspirin excreted as salicylic acid is higher in the marmoset than in man (see Hoffmann and Nobe, 1950).

The proportion of a 13mg/kg body weight dose of acetylsalicylic acid that is excreted unchanged within a short period after dosing is similar in the marmoset and man. The results obtained for man in the present study indicate that female subjects excrete a higher proportion of the administered acetylsalicylic acid unchanged than do males which may possibly be correlated with the reported lower levels of plasma aspirin esterase found in females as compared with males (Menguy et al., 1972).

In the marmoset, peak plasma levels of radioactivity are attained within one hour of the oral administration of acetyl [^{14}C]salicylic acid (13mg/kg body weight). Peak plasma levels of both unchanged acetylsalicylic acid and salicylate have been found to occur within 40min of the administration of acetylsalicylic acid (0.65g) to human subjects (Rowland et al., 1967). Leonard (1962) obtained similar results when the dose was administered as its sodium salt, but this author also showed that when the dose was administered as the free acid the peak level of total plasma salicylate was not reached within a period of one hour after the dose was given. The administration of acetylsalicylic acid as its sodium salt had previously been shown to increase its rate of absorption from the human gut as determined by the time taken for peak plasma levels of salicylate to be reached (Lester et al., 1946). In the present study the dose was administered as the free acid to the marmoset, and at a dose level which was higher than that used by either Leonard (1962) or Rowland et al. (1967) suggesting that the short time taken to reach peak plasma levels in the marmoset indicates a rapid rate

of absorption of acetylsalicylic acid from the gut. That the peak plasma levels of radioactivity attained in the marmoset are not maintained for any length of time indicates a rapid rate of metabolism of aspirin in this species.

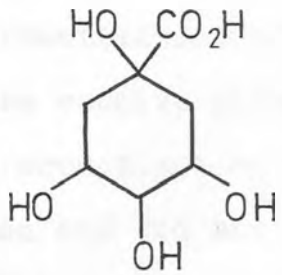
CHAPTER 9

The comparative metabolism of quinic acid

Chapter 9 - The comparative metabolism of quinic acid

Introduction

The increased excretion of hippuric acid by man after the ingestion of quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid; I), a compound occurring in tea, coffee, fruits and vegetables was first reported by Lautemann (1863). This early



(I)

observation has since been confirmed by several authors (Quick, 1931; Beer et al., 1951; Bernhard et al., 1955; Cotran et al., 1960; Adamson et al., 1970b).

Beer et al. (1951) reported that little or no aromatization of orally administered quinic acid occurred in rabbits and rats and that subcutaneously administered quinic acid was not aromatized by the cat or guinea pig. Bernhard et al. (1955) and Cotran et al. (1960) however, found that a substantial proportion of orally administered quinic acid was aromatized in the guinea pig which suggested a possible role of the gut flora in the aromatization process.

A comprehensive study of the aromatization of quinic acid in various animal species, including man, was reported by Adamson et al. (1970b). These authors found that orally administered quinic acid was extensively aromatized (20-70% of the dose) in man, rhesus monkey, baboon and green monkey but that

little aromatization occurred (0-11% of the dose) in the squirrel, spider and capuchin monkeys, bush baby, slow loris and treeshrew. In the dog, cat, ferret, rabbit, rat, mouse, guinea pig, hamster, lemming, fruit bat, hedgehog and pigeon oral quinic acid was not significantly aromatized (0-5% of the dose). Further studies showed that injected quinic acid was not converted to hippuric acid by the rhesus monkey but was largely excreted unchanged; pre-treatment with neomycin to suppress the gut flora considerably suppressed the aromatization of oral quinic acid (Adamson et al., 1970b). These results clearly indicated that an essential step in the aromatization of quinic acid was effected by the gut flora of man and Old World monkeys, but not by the gut flora of New World monkeys, Prosimians and non-primate species. Furthermore, the same authors demonstrated that orally administered shikimic acid (trihydroxycyclohex-1-enecarboxylic acid) was metabolised to hippuric acid by the rhesus monkey but not by the rat.

The results reported by Adamson et al. (1970b) for the degree of aromatization of quinic acid by the rat do not agree with those previously reported by Asatoor (1965) who found that orally administered quinic acid was aromatized by the rat, but as with the results obtained by Adamson et al. (1970b) using the rhesus monkey, the aromatization was suppressed in neomycin pre-treated animals.

The mechanism by which orally administered quinic acid is converted to hippuric acid has been the subject of many investigations. Davis and Weiss (1953) showed that coliform bacteria could aromatize quinic acid, whereas Mitoma et al. (1958) found no aromatization of quinic acid by human or guinea pig liver homogenates. These authors also found that guinea pig

liver homogenates were capable of carrying out the aromatization of cyclohexanecarboxylic acid. These results suggested that either administered quinic acid was aromatized to benzoic acid by the gut flora and then conjugated with glycine in the liver to form hippuric acid, or that the formation of benzoic acid occurred by the aromatization of cyclohexanecarboxylic acid in the liver. Some support for the former theory came from work by Asatoor (1965) and Indahl and Scheline (1973) which showed that the incubation of quinic acid with rat gastrointestinal micro-organisms resulted in the formation of benzoic acid under both aerobic and anaerobic conditions.

Early work on the metabolism of cyclohexanecarboxylic acid (Beer et al., 1951; Bubior and Bloch, 1966) showed that this compound was aromatized by mammalian species and more recent reports have shown that hippuric acid is a major metabolite of cyclohexanecarboxylic acid in the rat. Brewster et al. (1977a) demonstrated that intraduodenally administered sodium cyclohexylcarboxylate was metabolised to hippurate, hexahydrohippurate, 3,4,5,6-tetrahydrohippurate and benzoyl and cyclohexylcarbonyl β-glucuronides in the rat, while using a liver perfusion technique Brewster et al. (1977b) were able to show that the formation of the various hippuric acids occurred in the liver of this species.

The observation that the oral administration of shikimate to the rhesus monkey resulted in the increased excretion of hippuric acid (Adamson et al., 1970b) suggested that shikimic acid may lie on the pathway of the conversion of quinic acid to hippuric acid. Brewster et al. (1976) investigated the metabolic fate of shikimic acid in the rat and showed that cyclohexanecarboxylic acid was the major product following the anaerobic

metabolism of shikimic acid by rat gastrointestinal micro-organisms. Furthermore the observation that the metabolism of shikimate in the rat was suppressed by pre-treatment with neomycin and that shikimic acid was not metabolised by animal tissues (Brewster et al., 1978) suggested that the conversion of shikimic acid to cyclohexanecarboxylic acid by the gut flora was an important step in the overall metabolism of shikimic acid and closely related quinic acids. Brewster et al. (1978) have shown that the metabolites of shikimic acid excreted by the rat are the same as those excreted after the administration of cyclohexane-carboxylic acid (Brewster et al., 1977a) further implicating the role of cyclohexanecarboxylic acid as an intermediate in the metabolism of shikimic acid.

Based on the evidence so far accumulated a series of bio-transformations can be postulated for the conversion of orally administered quinic acid to hippuric acid involving an initial reduction (possibly via shikimate) to cyclohexanecarboxylic acid by the gastrointestinal micro-organisms and the aromatization of this intermediate to benzoic acid in the liver (Fig 9.1). The formation of hexahydrohippurate and 3,4,5,6-tetrahydrohippurate either after the intraduodenal administration of cyclohexane-carboxylic acid or during perfusion of the liver with this compound (Brewster et al., 1977a,b) suggests that the various co-enzyme A intermediates involved in the hepatic aromatization of cyclohexanecarboxylic acid are able to undergo conjugation with glycine, a reaction which prevents any further aromatization of the ring system.

In this chapter an investigation of the ability of adult marmosets to convert orally administered quinic acid and sodium cyclohexylcarboxylate to hippuric acid is described.

Key: I = Quinic acid

II = Shikimic acid

III = Dihydroshikimic acid

IV = Cyclohexanecarboxylic acid

V = Hexahydrohippuric acid

VI = 3,4,5,6-tetrahydrohippuric acid

VII = Hippuric acid

Metabolites V → VII are excreted in the urine

Fig 9.1 Postulated pathway for the conversion of orally administered quinic acid to hippuric acid in the mammal.

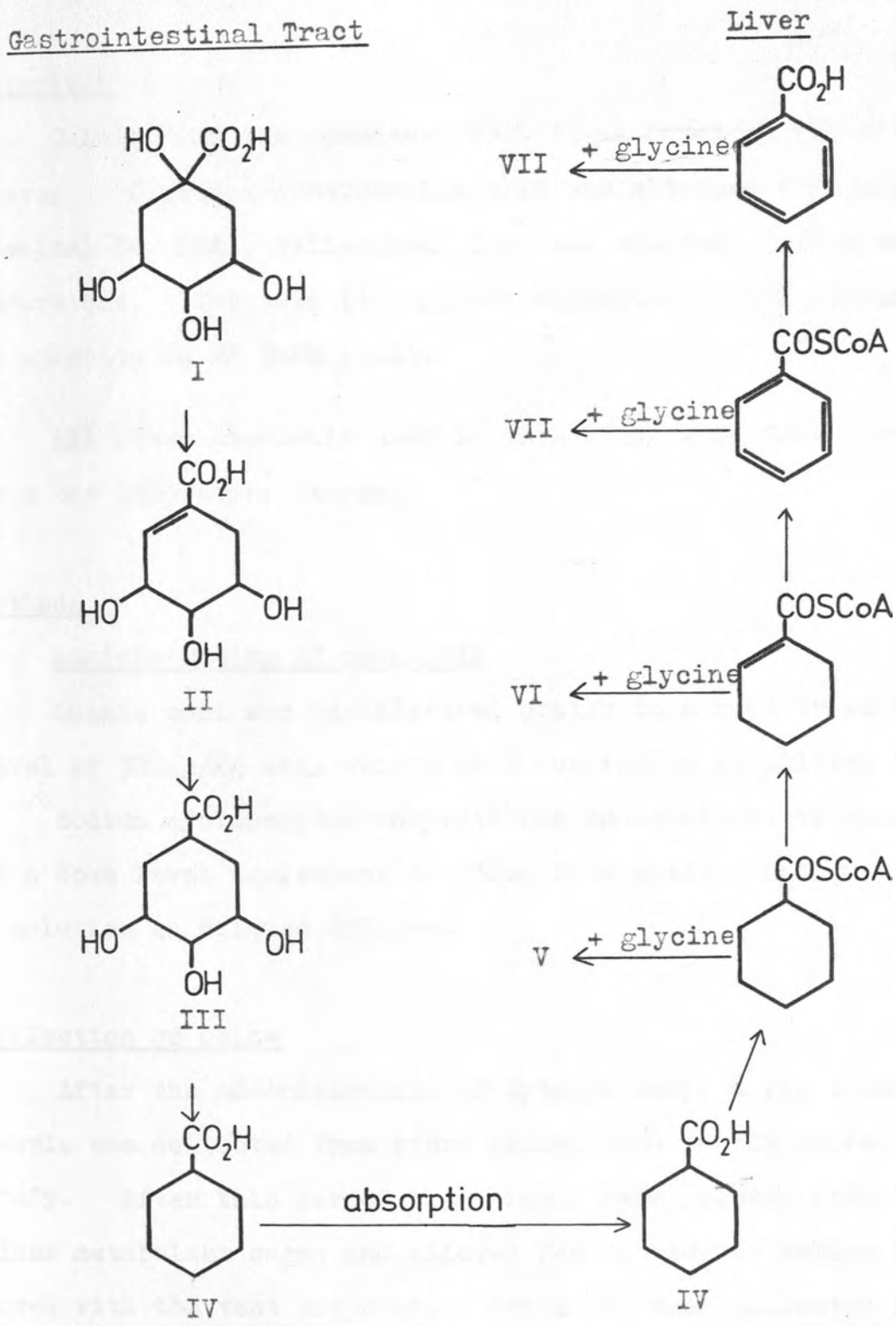

Gastrointestinal Tract
Liver
HO
CO2H
HO
OH
OH
I
CO2H
HO
OH
OH
II
CO2H
HO
OH
OH
III
CO2H
IV
absorption
CO2H
IV
CO2H
VII
+ glycine
COSCoA
VII
+ glycine
COSCoA
VI
+ glycine
COSCoA
V
+ glycine

Fig. 9.1

Materials and Methods

Materials

Quinic acid was purchased from Sigma Chemical Co. Ltd., Surrey. Cyclohexanecarboxylic acid was obtained from Aldrich Chemical Co. Ltd., Gillingham, Kent and was redistilled twice before use. The acid (1.28g) was converted to its sodium salt by solution in 2M NaOH (5ml).

All other chemicals used in this study were freely available from the laboratory stores.

Methods

Administration of compounds

Quinic acid was administered orally to marmosets at a dose level of 300mg/kg body weight as a suspension in diluted Cytacon.

Sodium cyclohexylcarboxylate was administered to marmosets at a dose level equivalent to 130mg free acid/kg body weight as a solution in diluted Cytacon.

Collection of urine

After the administration of Cytacon only, a 24h normal urine sample was collected from every animal used in the course of this study. After this period the animals were removed from the all-glass metabolism cages and allowed 24h to recover before being dosed with the test compound. Urine was then collected for a further 24h period.

Concentration of urine samples

The 24h urine samples were lyophilized and the residue extracted with a known volume of methanol (5-10ml). The extracts

were then centrifuged using stoppered tubes and the hippuric acid content of the supernatant determined as outlined below.

Estimation of hippuric acid

Using an Agla syringe duplicate volumes of the methanolic extracts were applied to a large (46cm wide) sheet of chromatography paper. Duplicate aliquots of a standard methanolic solution of hippuric acid (2mg/ml) were also applied to the paper to provide a calibration curve over the range 20-80µg hippuric acid. The chromatogram was developed in solvent E by the descending technique and the hippuric acid located using detecting reagent VII. The bright orange spots corresponding to hippuric acid were cut out and eluted with methanol (4ml) for 15min in stoppered tubes in the dark. The optical density of each eluate was measured at 460nm using a Unicam SP1800 spectrophotometer and the quantity of hippuric acid in the urinary extracts determined from the calibration curve.

Results

The results in Table 9.1 show the quantity of hippuric acid excreted by four marmosets following the administration of quinic acid. Only two of the animals studied showed any increased excretion of hippuric acid when quinic acid was administered, and in both cases the observed increase was small.

Table 9.2 shows the effect of the administration of sodium cyclohexylcarboxylate upon hippuric acid excretion in the marmoset. It may be seen that two of the four animals studied converted a substantial proportion of the administered dose to hippuric acid while with the other two animals no increased hippuric acid output was observed.

Marmoset	Hippuric acid excretion (mg/day)		Degree of aromatization (% of dose administered)
	After administration of dosing vehicle only	After administration of quinic acid (100mg)	
689	4.4	6.1	1.8
532	4.6	8.4	4.1
690	3.2	3.0	0.0
582	4.3	3.5	0.0

Table 9.1 Effect of the administration of quinic acid on the urinary hippuric acid excretion in adult marmosets

Marmoset	Hippuric acid excretion (mg/day)		Degree of aromatization (% of dose administered)
	After administration of dosing vehicle only	After administration of sodium cyclohexylcarboxylate (equivalent to 43mg free acid)	
462	3.9	1.0	0.0
764	4.3	30.8	44.1
765	2.9	1.2	0.0
784	8.8	46.3	62.4

Table 9.2 Effect of the administration of sodium cyclohexylcarboxylate on the urinary hippuric acid excretion in adult marmosets

Discussion

The low capacity of the marmoset to aromatize orally administered quinic acid is a characteristic of New World monkeys and in this respect this species fits into the general taxonomic trend reported by Adamson *et al.* (1970b).

The conversion of sodium cyclohexylcarboxylate to hippuric acid has been demonstrated in two marmosets although two other marmosets did not carry out this process. This was a surprising result since in all the studies the experimental conditions were kept constant. Unfortunately it was not possible to carry out further work using marmosets at this time and so the indication obtained that the marmoset shares with other animal species the ability to aromatize cyclohexanecarboxylic acid could not be confirmed.

CHAPTER 10

Mercapturic acid formation in the marmoset

Chapter 10 - Mercapturic acid formation in the marmoset

Introduction

The formation of mercapturic acids in the marmoset has not been reported and it was of interest to establish to what extent this pathway of xenobiotic metabolism occurred in this species.

Mercapturic acid formation is initially a Phase II reaction involving the conjugation of a xenobiotic with glutathione to give an S-substituted glutathione derivative. The two terminal amino acids are then successively removed from the conjugate, firstly glutamic acid and then glycine, the reactions being catalysed by the enzymes γ-glutamyltranspeptidase and cysteinylglycinase respectively. The conjugate remaining, an S-substituted cysteine, then undergoes N-acetylation to yield the mercapturic acid.

The S-substituted cysteines may be metabolised with the formation of products other than mercapturic acids. The possible reactions were summarised by James and Needham (1973) in an investigation of the metabolism of S-pentylcysteine. The main reactions in addition to acetylation involved oxidative deamination with the formation of S-pentylthiolactic and S-pentylthiopyruvic acids. With this particular substituted cysteine hydroxylation of the pentyl residue may occur although this is more evident in the metabolism of 1-bromopentane the precursor of pentylcysteine (Grasse and James, 1972) and the terminal methyl group may be oxidised to a carboxylic acid group (James and Needham, 1973). Mercapturic acids may also be excreted as the corresponding sulphoxides.

The initial reaction with glutathione may often be detected by a decrease in the level of hepatic glutathione (Barnes *et al.*

1959). Thus the administration of methyl iodide to the rat leads to a marked decrease in hepatic glutathione and a high proportion of the dose is converted to the S-methylglutathione derivative which is excreted in the bile (Johnson, 1966). Reabsorption occurs and methylcysteine is eventually formed. This compound undergoes several biotransformations and the amount of methylmercapturic acid excreted represents less than 0.5% of the dose (Barnsley and Young, 1965). Thus the excretion of even small amounts of mercapturic acid may indicate that a considerable proportion of the dose has entered the glutathione detoxication pathway.

Considerable species differences exist in the extent of mercapturic acid formation. Thus in the rat large proportions of doses of different precursors are excreted as mercapturic acids (Bray et al., 1958). In the rabbit a considerable proportion of the dose of some precursors is represented by deaminated products of the substituted cysteine the amount of mercapturic acid excreted being correspondingly less (James and Needham, 1973). The guinea pig is notable for its apparent inability to excrete more than a small proportion (usually less than 10% of the dose) of known mercapturic acid precursors as mercapturic acids (see p 32). This has been attributed to a poor ability to acetylate the S-substituted cysteines but it seems likely that this is not the whole explanation as recent work using benzyl chloride in this laboratory (James S.P. unpublished results) has shown that the small amounts of benzylmercapturic acid excreted are accompanied by larger amounts of the corresponding sulphoxide.

Mercapturic acid formation is considered to be of relatively little importance in man (e.g. Dutton, 1978); few investigations

have been carried out in man possibly due to the toxic properties of many mercapturic acid precursors. However the protective role of glutathione in the prevention of hepatic necrosis following the administration of phenacetin and paracetamol has already been discussed (p 42). Wainer and Lorincz (1963) reported that mercapturic acid formation from orally administered bromobenzene was very low in infants while French et al. (unpublished observations: see Smith and Williams, 1974) have reported that 19% of administered chlorobenzene was excreted as p-chlorophenylmercapturic acid by human subjects.

Considerable interest in the 'glutathione pathway' arises from the finding that some environmental hydrocarbons are metabolised to epoxides which may be converted to diols by the action of epoxide hydrase or conjugated with glutathione in a reaction catalysed by glutathione-S-epoxide transferase (Boyland and Williams, 1965).

This chapter describes an investigation of the formation of mercapturic acids by marmosets dosed with [^{14}C]benzyl chloride or 1,2-epoxybutane and of the acetylation of S-pentylcysteine. The metabolic fate of [^{14}C]benzyl chloride was also studied in the rat for comparison with the marmoset.

Materials and Methods

Materials

[Methylene-^{14}C]benzyl chloride (500μCi; 87μCi/mg) was purchased from the Radiochemical Centre Ltd. and diluted with unlabelled benzyl chloride (1g) at 0°C. The specific activity of the diluted material was 0.79μCi/mg. S-Benzyl-L-cysteine (benzylcysteine), N-acetyl-S-benzylcysteine (benzylmercapturic acid) and N-acetyl-S-benzylcysteine sulphoxide (benzylmercapturic acid sulphoxide) were all available in the laboratory.

1,2-Epoxybutane was purchased from Fluorochem Ltd., Glossop, Derbyshire. N-Acetyl-S-[2-hydroxybutyl]-L-cysteine (2-hydroxybutylmercapturic acid), dicyclohexylamine salt was available in the laboratory. It had been synthesised by the method described by James and White (1967) for the preparation of N-acetyl-S-β-hydroxyphenethyl-L-cysteine.

S-Pentyl-L-cysteine (pentylcysteine), N-acetyl-S-pentyl-L-cysteine (pentylmercapturic acid) and N-acetyl-S-pentyl-L-cysteine sulphoxide (pentylmercapturic acid sulphoxide) were all available in the laboratory.

Methods

Administration of doses

[^{14}C]Benzyl chloride was administered to rats and marmosets at a dose level of 30mg/kg body weight. The rats were given the dose mixed with Cytacon by the method previously described (p 81). The pungent smell of this compound led to dosing difficulties with the marmoset and as a result the usual dosing technique was modified. The method found most successful was carried out by allowing the marmoset to drink diluted Cytacon

from a syringe as previously described (p 81) and then injecting the dose to the rear of the throat using a Pasteur pipette. The quantity of [^{14}C]benzyl chloride remaining in the pipette was estimated by liquid scintillation counting.

1,2-Epoxybutane was administered to marmosets at a dose level of 100mg/kg body weight by the method described above for [^{14}C]benzyl chloride. In this case the residual dose was not determined.

Pentylcysteine was administered to marmosets at a dose level of 115mg/kg body weight as a suspension in Cytacon.

Collection of urine samples

Urine samples were collected at 24 and 48h after the doses were given. No normal sulphur-containing metabolites corresponding to the metabolites of the doses were present in control urine samples.

Chromatography

Paper chromatography was carried out by the descending technique in solvents E and P. In the studies of the metabolism of [^{14}C]benzyl chloride and the acetylation of pentylcysteine, chromatograms were developed in solvents E and P for periods of 12 and 24-30h respectively. In the investigations with 1,2-epoxybutane, chromatograms were developed in solvent E for 10h.

Quantitative methods

Recovery of dose

The quantity of radioactive components excreted in the urine of animals dosed with [^{14}C]benzyl chloride was determined by liquid scintillation counting (p 89). The radiochemical content

of freeze dried faecal samples was determined by the combustion method (p 90).

Quantification of metabolites

The relative proportions of labelled metabolites excreted in the urine of animals dosed with [^{14}C]benzyl chloride were determined by liquid scintillation counting following chromatography in solvents E and P (p 90). The chromatograms were cut up into strips of width 0.5cm starting 1.0cm below the origin and proceeding to the end of the chromatogram.

Determination of unlabelled mercapturic acids by planimetry

The percentages of doses of 1,2-epoxybutane and pentylcysteine excreted as the corresponding mercapturic acids were estimated by the method of planimetry (Bray *et al.*, 1964) after separation by paper chromatography. Calibration curves were constructed using the appropriate mercapturic acid. Aliquots of the standard solutions (the dicyclohexylamine salt of 2-hydroxybutylmercapturic acid or pentylmercapturic acid) were applied to the chromatograms and suitable quantities of urine from a marmoset which had been dosed with Cytacon only were then applied to the spots of the reference compounds. The chromatograms were developed in either solvent E or P and the areas corresponding to the mercapturic acid visualised with the chloroplatinate reagent, detecting reagent IX. The areas of the spots were measured with a planimeter and the concentration of the mercapturic acid in the urine sample determined by comparison with the standard curve.

Determination of pentylcysteine

The method used was described by Grasse (1971) and was based

upon the method of Suga et al. (1967). After the administration of pentylcysteine to the marmoset, the residual dose was estimated by transferring the contents of the syringe and specimen tube into which the dose was weighed, to a volumetric flask (5ml capacity) using aqueous ammonia (10% v/v) and the contents diluted to the mark. Aliquots of this solution were then applied to a thin layer silica gel G plate (p 85). A standard curve was prepared ranging from 25-100µg of pentylcysteine by the application of aliquots of a solution of pentylcysteine (5mg/ml) in aqueous ammonia (10% v/v). After developing in solvent L the chromatogram was sprayed with a solution of 0.5% ninhydrin in acetone/butan-1-ol (1:1 v/v). After heating at 120°C for 5min the areas corresponding to pentylcysteine were scraped off the plate and transferred to stoppered test tubes each containing cupric sulphate (8mg). Aqueous ethanol (80% v/v; 4ml) was then added and after mixing, the tubes were left to extract in the dark for 30min. The absorbance of the extract was determined at 540nm using a Unicam SP1800 spectrophotometer, and the amount of pentylcysteine in the unknown solutions determined by reference to the standard curve.

Results

The R_F values obtained for the urinary metabolites of [^{14}C] benzyl chloride, together with those of reference compounds as determined by their reactions with different detecting reagents, are given in Table 10.1. With those chromatograms developed in solvent P, no solvent front remained on the paper and so all R_F values were calculated relative to benzylmercapturic acid which was taken as 1.0. The presence of benzylcysteine and benzylmercapturic acid sulphoxide in the urine of dosed animals could only be determined following co-chromatography with authentic samples and radiochromatogram scanning since their concentration in the urine appeared to be too low for detection by the detecting reagents used. Solvent E failed to resolve hippuric acid and benzylmercapturic acid sulphoxide and did not give an absolute resolution of benzylcysteine and benzylmercapturic acid in many instances. For this reason solvent P was used for the majority of the quantitative estimations carried out. Figure 10.1 shows a typical histogram obtained when the radioactivity contained in 0.5cm sections of paper chromatograms developed in solvent P was determined by liquid scintillation counting.

Tables 10.2 and 10.3 show the percentages of the metabolites of [^{14}C] benzyl chloride present in the urine of dosed rats and marmosets as determined in solvents E and P respectively. Table 10.3 also indicates the percentage of the administered dose which was recovered in the urine of rats and marmosets during the first 24h period after dosing. Very little of the dose (less than 1%) was recovered from either species in the second 24h period or in the faeces. Figures 10.1 and 10.2 show typical histograms from which the figures in Tables 10.2 and 10.3 were

Compound	R_F value in solvent E	R_F value in solvent P^+	Reaction with detecting reagent listed VII	IX	X	XI
Reference compounds						
Benzylmercapturic acid	0.63	1.0	-	pale spot	yellow	-
Benzylcysteine	0.55	0.71	-	pale spot	pale cream	blue/grey
Benzylmercapturic acid sulphoxide	0.45	0.23	-	yellow[a]	nd	-
Hippuric acid	0.50	0.31	orange	-	-	-
Urinary metabolites						
Benzylmercapturic acid	0.66	1.0	-	pale spot	yellow	-
Benzylcysteine[b]	0.53	0.65	-	pale spot	pale cream	blue/grey
Benzylmercapturic acid sulphoxide[b]	0.40	0.24	-	yellow[a]	nd	-
Hippuric acid	0.47	0.30	orange	-	-	-

[+]Rate of movement of spots relative to that of mercapturic acid which is taken as 1.0

[a]Developed colour after 6h

[b]Identified in urine by co-chromatography with unlabelled reference compound and radiochromatogram scanning

nd - not detected.

Table 10.1 Chromatographic properties of reference compounds and urinary metabolites of [^{14}C]benzyl chloride

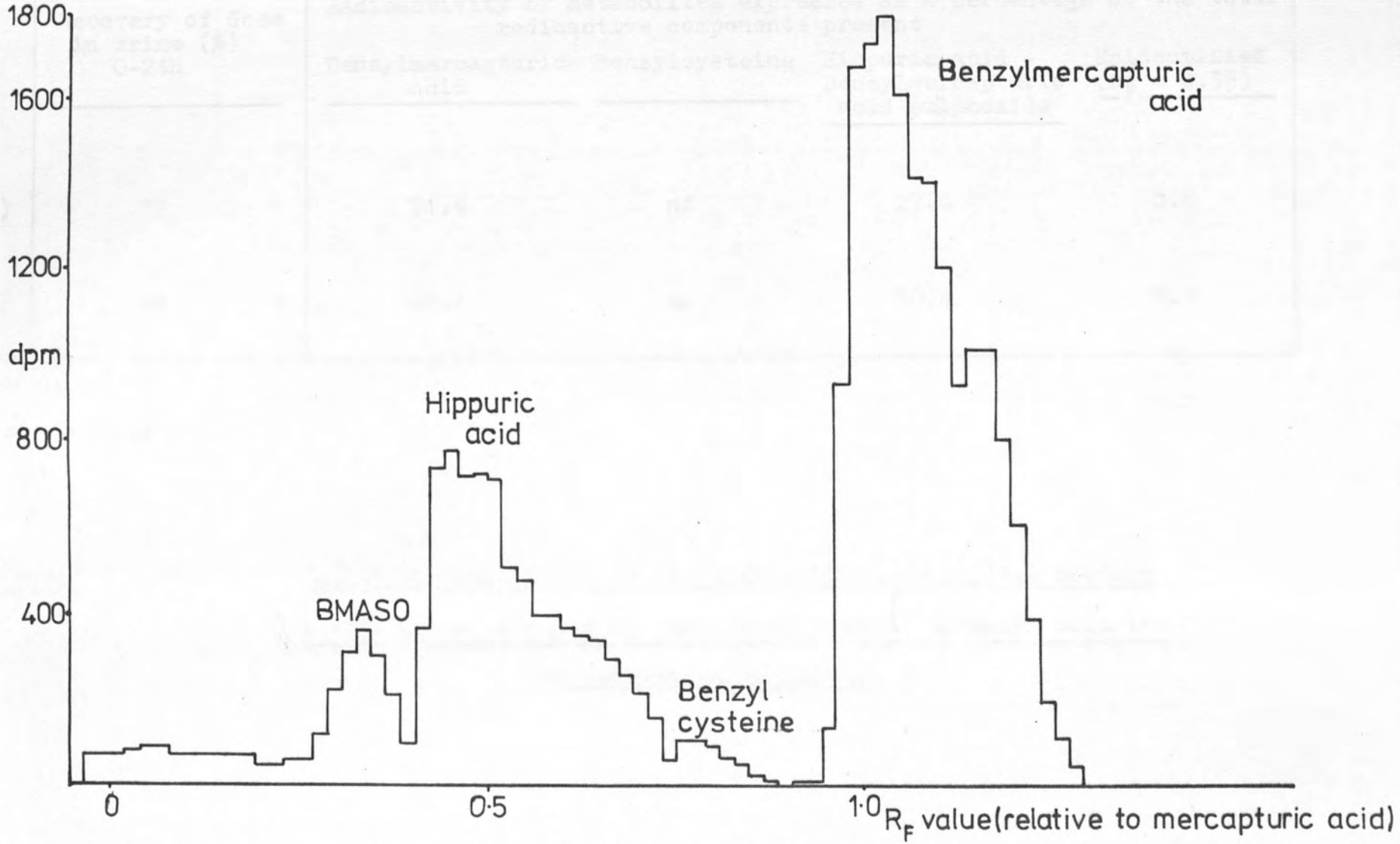


Fig 10.1 Distribution of radioactivity along a chromatogram, developed in solvent P, of urine from a rat dosed with methylene [^{14}C] benzyl chloride.

BMASO = Benzylmercapturic acid sulphoxide

Animal	Recovery of dose in urine (%) 0-24h	Radioactivity of metabolites expressed as a percentage of the total radioactive components present			
		Benzylmercapturic acid	Benzylcysteine	Hippuric acid + Benzylmercapturic acid sulphoxide	Unidentified (R_F = 0.35)
1)	72	71.4	nd	27.8	0.8
2)	82	68.4	nd	30.4	0.2

nd - not detected

Table 10.2 Relative proportions of the radioactive metabolites present in 24h urine excreted by rats dosed with [^{14}C] benzyl chloride as determined in solvent E

Animal	Recovery of dose in urine (%) 0-24h	Percentage of dose excreted as benzyl-mercapturic acid	Radioactivity of metabolites expressed as a percentage of the total radioactivity present					
			Unidentified (R_F 1.20)	Benzyl-mercapturic acid	Benzyl-cysteine	Hippuric acid	BMASO	Unidentified (R_F 0.05)
Rat+ 1	72	43.4	nd	68.6	1.2	23.3	5.7	1.2
2	82	53.1	nd	64.7	1.2	24.5	5.4	1.1
Average	77	48.3	nd	66.7	1.2	23.9	5.6	1.2
Marmoset								
No 652	52	36.3	tr	69.9	7.6	nd	16.2	6.4
689	71	35.6	nd	50.2	5.3	24.4	12.4	1.2
690	52	25.4	0.8	48.9	30.3	nd	10.5	2.8
784	48	35.7	tr	74.4	9.8	4.8	9.1	1.9
Average	56	33.3	0.2	60.9	13.3	7.3	12.1	3.1

+same number code used as in Table 10.2
tr - trace
nd - not detected
BMASO - Benzylmercapturic acid sulphoxide

Table 10.3 The relative proportions of radioactive metabolites present in 24h urine excreted by animals dosed with [^{14}C]benzyl chloride as determined in solvent P

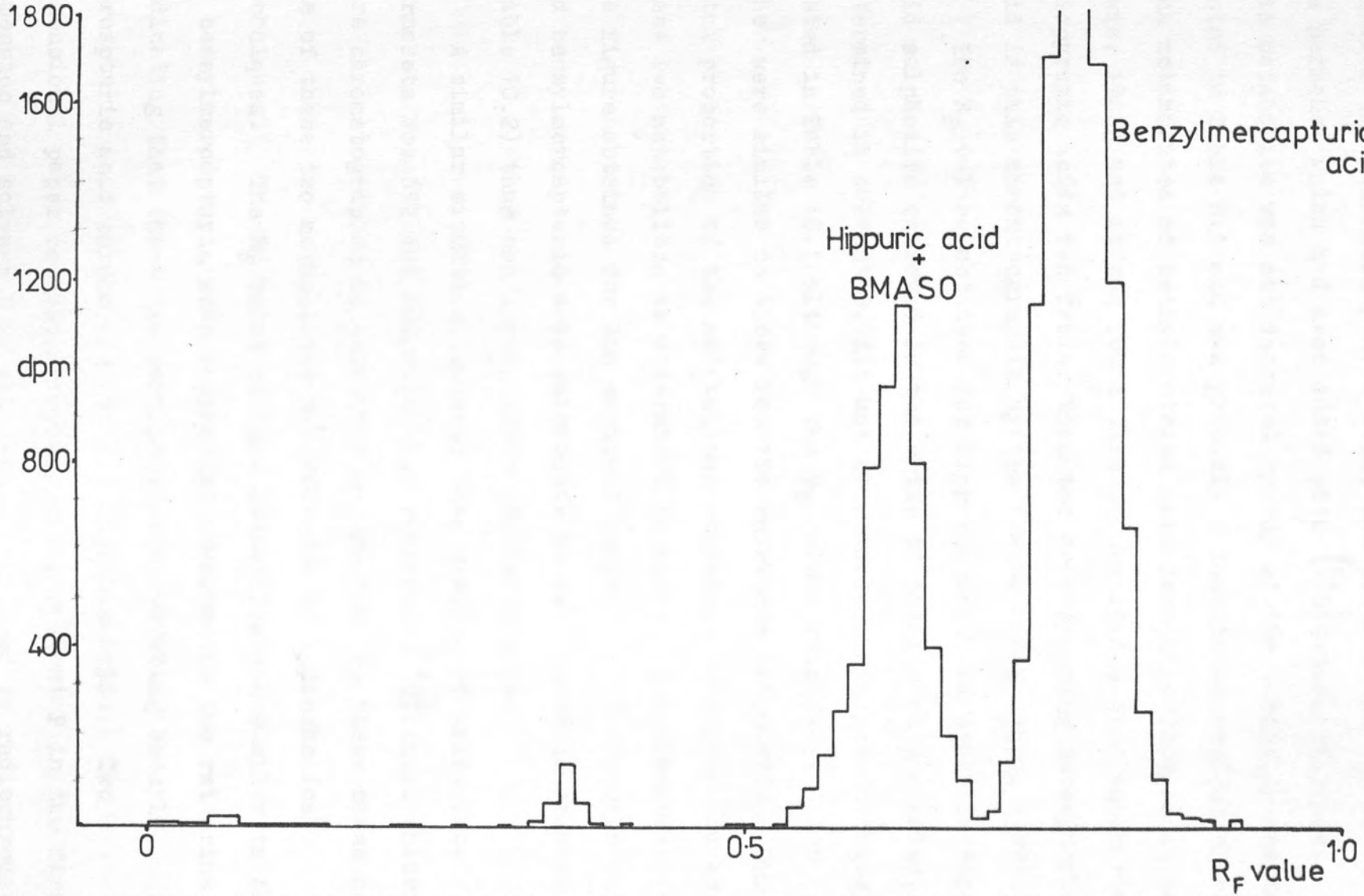


Fig 10.2 Distribution of radioactivity along a chromatogram, developed in solvent E, of urine from a rat dosed with methylene [^{14}C] benzyl chloride.

BMASO = Benzylmercapturic acid sulphoxide

obtained. A labelled metabolite having an R_F value of 1.2 relative to benzylmercapturic acid, was detected in the urine of one marmoset which had been dosed with [^{14}C]benzyl chloride. This metabolite was not detected by any of the detecting agents listed in Table 8.1 and was probably a deaminated product since such metabolites of benzylcysteine have been described (Shen and Lewis, 1946) and it was found that S-substituted thiolactic and thiopyruvic acids ran faster than the corresponding mercapturic acid in this chromatographic system (James and Needham, 1973).

The R_F values obtained for hippuric acid and benzylmercapturic acid sulphoxide excreted in the urine of the two rats studied, as determined in solvent P, did not correspond exactly with those listed in Table 10.1 although the R_F values relative to each other were similar to those for the reference compounds. The total proportion of the metabolites excreted accounted for by these two metabolites as determined in solvent P, corresponded to the figure obtained for the combined proportion of hippuric acid and benzylmercapturic acid sulphoxide as determined in solvent E (Table 10.2) thus confirming their identification.

A similar situation occurred when samples of urine from marmosets Nos 652 and 690, which had received [^{14}C]benzyl chloride, were chromatographed in solvent P except that in these cases only one of these two metabolites was detected by radiochemical techniques. The R_F value of this metabolite was similar to that of benzylmercapturic acid sulphoxide observed in the rat urine indicating that these two marmosets were excreting benzylmercapturic acid sulphoxide and not hippuric acid. Two dimensional paper co-chromatography using solvent P in the first dimension and solvent E in the second, followed by radiochromatogram scanning, failed to detect labelled hippuric acid in the

urine. Determination of the 24h excretion of hippuric acid by marmoset No. 652 before and after the administration of [^{14}C]benzyl chloride showed that no increased excretion of hippurate occurred. Furthermore, when a sample of the urine of the dosed animal was heated with HCl (final concentration 6M) under reflux for 6h, subsequent extraction of the hydrolysate with ether and evaporation of the extract at room temperature and atmospheric pressure gave a residue which was not radioactive. Under these conditions any labelled benzoic acid formed from labelled hippuric acid should have been present in the extract. The residue from the ethereal extract and a sample of the aqueous solution were both examined by chromatography in solvent P. The aqueous solution contained increased amounts of benzylcysteine which was not extracted from the strongly acidic solution.

The results in Table 10.2 indicate the presence of a minor metabolite in the urine of dosed rats which had an R_F value of 0.35. This metabolite was tentatively identified as benzyl-glucuronide by comparison with a sample of benzylglucuronide which had been isolated, by chromatography, from the urine of neonatal rats to which [^{14}C]benzoate had been given at a dose level of 100mg/kg body weight (Baines et al., 1978). An unidentified metabolite of low R_F value in solvent P was also detected in the urine of both rats and marmosets which had been dosed with [^{14}C]benzyl chloride.

The amount of 2-hydroxybutylmercapturic acid excreted by each of two marmosets which had been dosed with 1,2-epoxybutane represented 1.1% of the dose. The results are minimum values since not all the dose was received by the marmosets, but the remaining undosed material was not determined. Furthermore the volatility of 1,2-epoxybutane (b.pt 63°C) will probably result in

a significant amount of the administered dose being exhaled.

The chromatographic properties of the metabolites of pentylcysteine excreted in the urine of dosed marmosets as determined in solvents E and P using three detecting reagents are summarised in Table 10.4. The results show that orally administered pentylcysteine was mainly excreted as pentylmercapturic acid by the marmoset but that traces of pentylmercapturic acid sulphoxide were also formed; no unchanged pentylcysteine appeared to be excreted by either animal studied. No metabolites with a greater mobility on chromatography in solvent P than pentylmercapturic acid were detected so that deamination of pentylcysteine does not occur to any significant extent. At a dose level of 115mg/kg body weight two marmosets excreted 44.7% and 33.7% of the dose as pentylmercapturic acid.

Compound	R_F value in solvent E	R_F value in solvent P^+	Reaction with detecting agent listed IX	X	XI
Reference compounds					
Pentylmercapturic acid	0.73	1.00	pale spot	yellow	-
Pentylcysteine	0.69	0.92	pale spot	yellow	grey
Pentylmercapturic acid sulphoxide	0.59	0.35	pale spot[a]	-	-
Urinary metabolites					
Pentylmercapturic acid	0.72	1.00	pale spot	yellow	-
Pentylmercapturic acid sulphoxide	0.59	0.39	pale spot[a]	-	-

[+]Rate of movement of spots relative to that of mercapturic acid which is taken as 1.0

[a]Developed colour after 6h.

Table 10.4 Chromatographic properties of reference compounds and urinary metabolites of pentylcysteine in solvents E and P

Discussion

The results show that the marmoset is able to synthesise mercapturic acids from two types of mercapturic acid precursors, benzyl chloride and 1,2-epoxybutane and that it readily acetylates pentylcysteine.

In both the marmoset and the rat the main urinary metabolite of [^{14}C]benzyl chloride, administered at a dose level of 30mg/kg body weight, is benzylmercapturic acid which accounts for an average of 33.3% of the administered dose in the marmoset and 48.3% in the rat. The percentage of the total urinary metabolites of benzyl chloride that are accounted for by benzylmercapturic acid are however similar in both species. Barnes et al. (1959) reported that at a dose level of 200mg/kg body weight, only 27% of administered benzyl chloride was excreted as benzylmercapturic acid by the rat, indicating that the degree of conversion of administered benzyl chloride to its corresponding mercapturic acid may be dose dependent. The conversion of benzyl chloride to benzylmercapturic acid sulphoxide appears to be a more important metabolic biotransformation in the marmoset than it is in the rat.

The excretion of benzylcysteine after the administration of benzyl chloride is more evident in the marmoset than it is in the rat and one marmoset examined excreted considerable quantities of this metabolite. The deamination of benzylcysteine appears to be a minor metabolic reaction in the marmoset but was not detected in the rat. The conversion of benzyl chloride to hippuric acid is variable in the marmoset and in three of the animals examined the formation of hippuric acid accounted for less than 5% of the labelled metabolites excreted. In the marmoset which did carry out this synthesis from benzyl chloride,

the amount of hippuric acid formed was similar to that synthesised by similarly dosed rats. Benzoylglucuronide was tentatively identified as a minor metabolite of benzyl chloride in the rat but was not detected in the urine of dosed marmosets.

With 1,2-epoxybutane at a dose level of 100mg/kg body weight, mercapturic acid formation by the marmoset accounts for only 1.1% of the administered dose. James et al. (1968) reported that at a dose level of 180mg/kg body weight the rat excreted 11% of the administered epoxide as 2-hydroxybutylmercapturic acid whereas the rabbit at a dose level of 137mg/kg body weight excreted 4% of the dose in this form.

Pentylcysteine is readily acetylated to pentylmercapturic acid by the marmoset and some pentylmercapturic acid sulphoxide is also formed by this species. The percentage acetylation of the dose may be compared with those percentages found for other species which are summarised in Table 10.5 from the results reported by James and Needham (1973). The degree of acetylation of pentylcysteine by the marmoset is similar to that in the rat. No evidence for the deamination of pentylcysteine by the marmoset was obtained in the present study and in this respect the marmoset resembles the rat but differs from the rabbit and mouse (James and Needham, 1973).

A comparison of the capabilities of the marmoset and man to convert various mercapturic acid precursors to their corresponding mercapturic acids is difficult since little work has been carried out on mercapturic acid formation in man. As mentioned earlier (pages 59 and 260) man converts a significant proportion (19%) of chlorobenzene to p-chlorophenylmercapturic acid (French et al. unpublished observations) but as pointed out by Smith and Williams (1974) the dose level used in this study was considerably lower

Species	Dose level mmol/kg body weight	Percentage of dose excreted as pentylmercapturic acid
Guinea pig	1.00	2.1 (1.0 - 3.7)^{5}
Hamster	1.10	73.4 (59 - 89)^{18}
Mouse	1.10	14.1 (8.7 - 26)^{62}
Rabbit	0.75	11.9 (7.0 - 22)^{7}
Rat	0.79	46.5 (35 - 54)^{18}
Marmoset	0.60	39.2 (33.7 - 44.7)^{2}

Range of results are in parentheses; the number of animals used is indicated by the superior figures.

+Data from James and Needham (1973) except that for the marmoset which is the result from the present work.

Table 10.5 The degree of acetylation of pentylcysteine in various species+

than that employed with the other species studied. That lower dose levels of mercapturic acid precursors may result in a greater proportion of the dose being excreted as the mercapturic acid derivative has already been suggested and it is possible that the marmoset has a greater ability than man to carry out mercapturic acid synthesis.

CHAPTER 11

Final Discussion

Chapter 11 - Final Discussion

While the existence of a perfect animal model for the study of drug metabolism in man seems unlikely the best model might be expected to be found amongst those species most closely related to man. The Greater and Lesser Apes are one step down from man on the evolutionary scale (see Table 1.11) but there are many factors which make the use of these species impracticable. The animals are difficult to obtain, especially in large numbers, extremely expensive to purchase and their maintenance cost is high; they are also difficult to handle. With the rhesus monkey, the species of Old World monkey most commonly used in the study of drug metabolism, these problems are substantially reduced but their export from India is now severely limited (Wade, 1978). The New World monkeys, the next Family of primates down the evolutionary scale, includes the common marmoset, Callithrix jacchus shown by Poswillo et al. (1972) to be an excellent model for the study of teratogenesis in man.

The marmoset is a convenient size for use as a laboratory animal and, as reported by Stevenson (1977), was found in this study to be relatively easy to handle. Compared with larger primates it is not expensive to maintain and it adapts easily to experimentation. The supply of feral animals is restricted but the animal breeds readily in captivity, a breeding pair giving birth to twins or triplets every five months. Thus once a small nucleus of animals has been obtained a colony of useful size can easily be established in three to four years (see Fig 2.1).

In the present study the metabolism of several xenobiotics has been investigated in the marmoset. Oral dosing was found to be satisfactory and the dosed animals could be housed in glass

metabolism cages similar to those originally designed for use with the rat, but with extra height to allow the animal greater freedom of movement.

Some disadvantages have been encountered with the use of the marmoset in metabolic studies. In the majority of cases the recovery of the administered dose was lower than in similarly dosed rats. Since, in most of these cases, only a small proportion of the dose was excreted during the second 24h period after dosing it appears that the low recovery is due to the practical difficulty of obtaining a complete collection of urine. The movement of these animals around the glass metabolism cages results in some of the urine which has been excreted onto the walls of the living chamber, being adsorbed onto the animals' bushy fur. Attempts were made to wash the urine from the tails of the animals under study but this was accomplished with varying degrees of success. Other workers have also reported that dose recoveries are variable from primate species while Mehta *et al.* (1978) reported a relatively low recovery of 1-naphthol from New World monkeys.

The small size of the marmoset means that it is not possible to take large blood samples from any one animal at regular intervals. In view of this limitation the use of the marmoset in experiments designed to determine blood levels of drugs and/or metabolites is only possible where sensitive methods of assay are available. In the present study, successful sampling of the blood was obtained from the tail vein of the animals although the quantity obtained by this method was only small due partly to the collapse of the blood vessel but more especially to the remarkable rate at which marmoset blood was found to clot.

Previous studies of xenobiotic metabolism in the marmoset

have shown that the metabolic fate of some compounds is similar to that in man, while that of others is very different in the two species. Thus amphetamine and norephedrine undergo both deamination and hydroxylation in man and the tamarin (sp. Saguimus oedipus - of the same Family as the marmoset) while both these species also carry out lactam formation and ring hydroxylation of phenmetrazine; however the conversion of chlorphentermine to its hydroxylamine derivative occurs in man but not in the tamarin (Williams et al., 1973; Caldwell et al., 1975a; 1977). Conjugation of phenols in man occurs mainly by sulphation (Capel et al., 1972; Mehta et al., 1978) whereas in the tamarin (sp. Saguimus oedipus) glucuronidation is the main biotransformation. The metabolic fate of arylacetic acids in the marmoset is typical of that in New World monkeys (see p 44-55) the extent to which phenylacetic acid is conjugated with glutamine being less in the marmoset than in man, while the extent of conjugation with glycine is greater in the non-human primate.

The ability of the marmoset (sp. Callithrix jacchus)to carry out the hydroxylation of Δ^8 and Δ^9-tetrahydrocannabinol was reported by Just et al. (1975), a biotransformation previously reported to occur in man (Lemberger et al., 1971).

In the present study compounds containing different biochemically reactive groups were administered to the marmoset and the extent to which this species carried out Phase II reactions was measured. Throughout the study the observed metabolic fate of the various xenobiotics in the marmoset was compared with that in man where data was available and for this reason the dose levels employed were always similar to those used when the metabolic fate had been investigated in man or when the drug was used therapeutically.

The results obtained suggest that the marmoset conjugates xenobiotics less readily with glycine than does man or the rat. This was evident from the experiments using benzoic acid as the test compound. The fate of this acid was found to be dose dependent in the adult marmoset, a situation not observed with the rat up to a dose level of 100mg/kg body weight. In the case of the marmoset, as the dose level was increased the proportion of the metabolites excreted accounted for as hippuric acid decreased while the proportion of benzoylglucuronide increased suggesting that the low conjugation with glycine may have been due partly to a low availability of glycine.

At a dose level of 1mg/kg body weight, administered benzoic acid was excreted by the marmoset and rat almost exclusively as hippuric acid, a similar situation to that shown by Bridges et al. (1970) to occur in man. As the dose level was increased and the relative importance of hippuric acid formation by the marmoset decreased, the capacity of the glucuronidating system appeared to be insufficient to compensate completely, particularly at a dose level of 100mg/kg body weight, and significant quantities of the dose were excreted unchanged. Quick (1931) reported that between 7 and 12% of a 5.0g dose of benzoic acid in man was excreted conjugated with glucuronic acid which when considered together with the results of Bridges et al. (1970) confirm the observation of Davison (1971) that the fate of this compound in man is dose dependent. The metabolic fate of benzoic acid has also been shown to be dose dependent in the dog and ferret (Bridges et al., 1970) in which species the relative importance of glucuronidation increases with increasing dose level.

Bridges et al. (1970) studied the metabolic fate of benzoic acid (50mg/kg body weight) in the capuchin and squirrel monkeys;

in the former species the administered dose was excreted almost entirely as hippuric acid whereas in the latter species a significant proportion of the dose was excreted unchanged. Neither species excreted more than trace amounts of benzoyl-glucuronide so that the high capacity of the marmoset to conjugate benzoic acid with glucuronic acid is not a characteristic shared by other species of New World monkeys.

The metabolic fate of aspirin however did not appear to be dose dependent in the marmoset. As described for man considerable inter-individual variation in the metabolism of this drug was observed. The conjugation of the drug with glycine was, as in the case of benzoic acid, found to be highly variable in the marmoset while some variation was also exhibited by the rat. In those animals excreting only a small proportion of the administered acetylsalicylic acid as salicyluric acid, the proportion of the metabolites accounted for as glucuronic acid conjugates was not significantly different from that observed in those animals excreting a higher proportion of the dose as salicyluric acid. Hence glucuronidation did not appear to occur simply as a compensatory mechanism for the low conjugation of salicylic acid with glycine. In one instance where the metabolic fate of both benzoic acid and acetylsalicylic acid was studied in the same marmoset, the animal excreted only a small percentage of the total urinary metabolites as glycine conjugates. However the quantity of glycine utilised by this animal for the synthesis of salicyluric acid was less than that utilised to synthesise hippuric acid indicating that a low availability of glycine did not account for the small extent of salicyluric acid formation. Furthermore Davison (1971) reported that in man the extent of salicyluric acid formation was not increased by the simultaneous

administration of glycine. These results suggest that the conjugation of benzoic acid and salicylic acid with glycine is carried out by different glycine N-acyltransferase enzymes or that salicylic acid is a poorer substrate for the enzyme than is benzoic acid (see Davison, 1971).

The proportion of a 13mg/kg body weight dose of acetylsalicylic acid excreted by the marmoset conjugated with glucuronic acid is similar to that of a 43mg/kg body weight dose in man as reported by Levy et al. (1972), whereas the proportion of the metabolites excreted accounted for as salicyluric acid is five times greater in the human emphasising the absence of any relationship between the conjugation of salicylic acid with glycine and glucuronic acid. The results obtained with the marmoset suggested that the major fraction of the glucosiduronic acids derived from aspirin consisted of the ester glucuronide whereas in the rabbit (Bray et al., 1948b)and man (Levy et al., 1972) the phenolic glucuronide is the major glucuronide formed.

Both man and the marmoset excreted a small quantity of administered aspirin as gentisic acid. In the rat a greater proportion of the administered dose was metabolised in this way and evidence was obtained that in this species some gentisic acid was excreted conjugated with glucuronic acid.

Using HPLC it was shown that man and the marmoset both excreted a small amount of unchanged aspirin in the urine within 1.5h of the administration of the compound at the same dose level. At higher dose levels the marmoset excreted a greater proportion of the dose unchanged probably reflecting higher plasma levels of the unmetabolised drug. Peak plasma levels of radioactivity were reached within one hour of the administration of [^{14}C]acetylsalicylic acid to the marmoset.

The marked ability of the marmoset to acetylate aromatic amino groups was shown using p-aminobenzoic acid, sulphadimidine and sulphadiazine. Using sulphadimidine it was found that all the adult animals in the colony corresponded to 'fast-acetylators' in human populations, based upon the proportion of the drug excreted as the acetylated derivatives; the relative proportions of the acetyl derivative as compared with the total drug in the blood, investigated in three animals, was in agreement with this finding.

The high ability of the marmoset to acetylate aromatic amines was also seen in the fate of administered p-aminobenzoic acid. The small proportion of this drug excreted as p-aminohippurate could be due partly to the low availability of glycine as described above for benzoic acid but the small proportion of the dose excreted as p-aminobenzoylglucuronide suggests that unlike the situation with benzoic acid, glucuronidation is not compensating, even fractionally, for the low glycine conjugation. In this case it seems probable that a high activity of the N-acetylase enzyme system, as compared with that of the glycine N-acyltransferase and transglucuronylase systems, results in a large proportion of the dose being excreted in the acetylated form.

The conjugation of sulphadiazine with glucuronic acid by animal species occurs through the N^1 and N^4 nitrogen atoms. The extent to which N^1-glucuronide formation occurred in the marmoset was greater than that in the rat although the difference was not nearly so great as that reported between primates and non-primates for sulphadimethoxine (Adamson et al., 1970a). It is possible that the excretion of sulphadiazine as its N^1-glucuronide is relatively low in the marmoset because of the operation of an

active N-acetylase system resulting in the excretion of a large proportion of the administered drug in the acetylated form. The extent of acetylation of sulphadiazine by the marmoset was about half that effected by the rabbit, twice that which occurs in the rat and slightly less but of the same order as that reported for man by Uno and Sekine (1966). Economic considerations prohibited the investigation of the excretion of sulphadiazine and/or its metabolites in the bile of the marmoset but it seems likely that the extent of the biliary excretion of this compound would be low since in the rat less than 2% of a dose of sulphadiazine is excreted by this route (Smith, 1973).

Following the oral administration of [^{35}S]sulphadiazine to the marmoset, peak blood levels of radioactivity were attained within 5h of dosing. This peak in plasma radioactivity was maintained for approximately 2h and was followed by a sharp decrease in the plasma content of the drug and/or its metabolites which may indicate a rapid rate of metabolism of the drug. This rate is greater than that suggested by similar studies reported for man (Goodwin *et al.*, 1942; Welch *et al.*, 1943) so that the relatively prolonged high blood levels responsible for the long-acting therapeutic effect in man are not seen in the marmoset.

Acetylation of the aliphatic amino group of pentylcysteine was also extensive in the marmoset, almost 40% of a 100mg/kg body weight dose of the compound being excreted as pentylmercapturic acid together with traces of pentylmercapturic acid sulphoxide. The deamination of pentylcysteine to pentylthiolactic and pentylthiopyruvic acids was not detected in the marmoset which is interesting since this species has been shown to deaminate both amphetamine and norephedrine to a significant extent (Caldwell *et al.*, 1977). This substrate dependence in the deamination of

xenobiotics is not seen in the rabbit which has been shown to deaminate pentylcysteine, amphetamine and norephedrine (Dring et al., 1970; James and Needham, 1973; Williams et al., 1973).

The ability of the marmoset to synthesise mercapturic acids was demonstrated using two known mercapturic acid precursors, benzyl chloride and 1,2-epoxybutane. When the former compound was administered to the marmoset the amount of benzylmercapturic acid excreted was similar to that excreted by similarly dosed rats; significant amounts of benzylcysteine and benzylmercapturic acid sulphoxide were also excreted. Some exidence was obtained that deamination of benzylcysteine occurred to a limited extent in the marmoset. Benzylthiopyruvic acid was detected as a metabolite of benzylcysteine in the rat (Shen and Lewis, 1946) but was not seen in the present study.

The excretion of hippuric acid by marmosets dosed with benzyl chloride is variable, the percentage of the total urinary metabolites of [^{14}C]benzyl chloride, administered at a dose level of 30mg/kg body weight, in this form ranged from 0-24.4%. In those animals excreting very little or no benzyl chloride as hippuric acid the excretion of benzoic acid was not detected suggesting that the availability of glycine did not limit the formation of hippuric acid.

It is likely that the marmoset possesses the ability to detoxicate epoxides by conjugation with glutathione and subsequent conversion of the conjugate to a mercapturic acid as shown by the metabolism of 1,2-epoxybutane to 2-hydroxybutylmercapturic acid. The extent of this conversion was low being of a similar order to that observed for the rat (James et al., 1968). The formation of a mercapturic acid by marmosets dosed with β-bromostyrene by a pathway which probably involves the intermediate formation of an

epoxide has been reported by Dawes et al. (1978). Scanty evidence exists for the ability of humans to form mercapturic acids and the extent to which this pathway is followed in man and the marmoset cannot be compared at present.

The results obtained using quinic acid suggested that the marmoset is a typical New World monkey in its inability to aromatize this compound (see Adamson et al., 1970b). However like non-primate species which are also unable to aromatize quinic acid it is possible that marmosets are able to aromatize cyclohexylcarboxylate. The experiments carried out using sodium cyclohexylcarboxylate must be regarded as a preliminary study only, but the results obtained with quinic acid suggest that the gut flora of the marmoset resembles that of other New World primate species and has a low capacity to reduce quinic acid.

A comparative investigation of the in vivo metabolism of two compounds in neonatal marmosets and rats was carried out. It was found that the metabolic fate of [^{14}C]benzoic acid in neonatal rats and marmosets was quantitatively very different from that in the adult animals of these species. In the neonatal animals of both species the metabolic fate of sodium [^{14}C]benzoate was strongly dose dependent, the relative importance of benzoylglucuronide formation being much greater in the neonates than in the adults of either species. At a dose level of 100mg/kg body weight, benzoylglucuronide was found to be the major metabolite of administered benzoate in the neonatal marmoset and the availability of glycine was shown to be clearly limiting.

Bilirubin-UDPGT activity was detected in the liver of neonatal marmosets although the activity was lower than that found in the liver of adult rats and marmosets. It is recognised that the tissue used had not been obtained until some hours after

death and that the enzyme levels found were likely to be lower than those present in fresh tissue. In the human infant it is probable that bilirubin-UDPGT is not fully developed at birth which may to some extent be responsible for the physiological jaundice of the newborn.

The development of the Phase II enzyme systems responsible for the metabolism of p-aminobenzoate follow different trends in the rat, marmoset and man. In the rat the extent of the formation of p-aminohippurate increased with age while the relative extent of glucuronidation decreased. The percentage of the total urinary metabolites that were acetylated increased with age although the extent of this change was less than the increase in glycine conjugation. In the marmoset, comparatively little change in the extent to which p-aminobenzoate was conjugated with glucuronic acid occurred throughout development, although a slight increase was detected. Similarly the relative importance of p-aminohippurate formation did not change significantly throughout development although a slight decrease in its excretion was observed. The main changes in the metabolic fate of p-aminobenzoate in the developing marmoset occurred in the proportion of the doses which were excreted unchanged or as the N-acetyl derivative. During early development there was a substantial increase in the percentage of the total metabolites excreted that was accounted for by p-acetamidobenzoate with a corresponding decrease in the percentage of unchanged p-aminobenzoate. In developing children, Vest and Salzberg (1965) showed that the activity of the acetylating system decreased with age and that an increased excretion of unchanged p-aminobenzoate occurred. Hence in man and marmoset the metabolic fate of p-aminobenzoate in the neonate appears to be controlled mainly by developmental changes

in the activity of the acetylase, or possibly a de-acetylase, enzyme system, changes which are directionally opposite in the two species.

Therefore although no qualitative differences have been observed in comparing those biotransformations of xenobiotics involving synthetic reactions in the marmoset, man and rat marked quantitative differences occur; these are particularly evident in the neonates of these species. While the marmoset is undoubtedly a useful primate species for the study of the metabolic fate of xenobiotics, the frequently quoted lines from Alexander Pope's 'An Essay on Man' must always be borne in mind,

"Know then thyself, presume not God to scan,
The proper study of mankind is man."

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METABOLISM OF [^{14}C]-BENZOIC ACID IN THE DEVELOPING RAT

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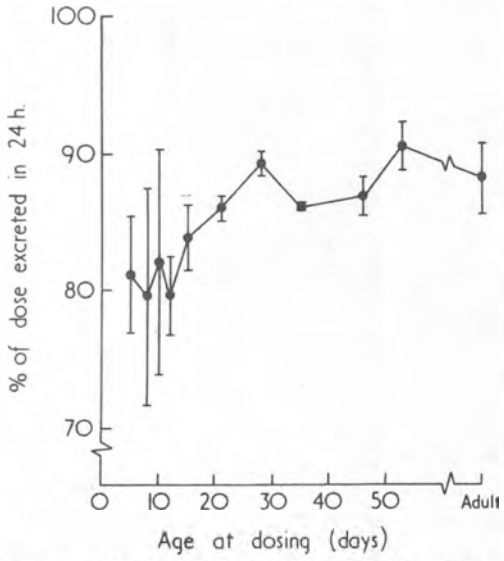
It is generally recognised that the level of activity of drug-metabolizing enzymes is low in the neonate as compared with the adult. Glycine N-acyltransferase (EC 2.3.1.17) activity was not detected in mitochondria from neonatal rat liver using *p*-aminobenzoic acid as substrate; the activity increased with age reaching 10% of the adult value at 10 days after (1). By contrast hepatic UDP-glucuronyl transferase (EC 2.41.17) activity is relatively high in the neonatal rat, falling to adult levels after about 10 days (2). The extent to which these enzymes are involved in the metabolism of benzoic acid in rats of different ages has been investigated by quantitation of the metabolites excreted in urine.



Percentage of radioactivity of administered benzoate excreted in urine in 24 h after dosage. The results are expressed as a percentage of the dose and vertical bars represent ± S.E.M. for groups of animals numbering 2–5.

Methods: [Ring-U-^{14}C]-labelled benzoic acid, in solution as the sodium salt, was administered to female rats (Wistar strain) aged from 5 days to adult, at a dose level of 0.33 mmol (4.6 μCi) kg^{-1} and the radioactivity of the 24 h urine determined by scintillation counting. The procedure followed for dosing the rats and the collection of urine samples has been described (3). The distribution of the radioactivity between benzoic acid, hippuric acid and benzoyl glucuronide was determined by chromatographic examination of aliquots of the urine of dosed rats on Whatman 3 MM paper or on thin layer plates coated with silica-gel-G (300 μm thick) (Merck, A.G., West Germany). Solvents used were butan-1-ol:ethanol:acetic acid:water (30:10:1:10 by vol), in which benzoic acid, hippuric acid and benzoyl glucuronide had R_F values 0.91, 0.82 and 0.51, respectively, on paper and 0.85, 0.70 and 0.42 on tlc, and chloroform:cyclohexane:acetic acid (8.2:1, by vol) in which the corresponding R_F values on tlc were 0.87, 0.17 and 0.00. Urine samples were also incubated for 24 h with limpet β-glucuronidase (Sigma Chemical Co Ltd) to hydrolyse benzoyl glucuronide to benzoic acid and then similarly examined. The radioactivity on the chromatograms were scanned and the chromatograms then fragmented into 0.5 or 1.0 cm zones, the radioactivity of each being measured by scintillation counting.

Relative amounts of radioactive compounds in 24 h urine of rats dosed with [^{14}C]-benzoate.

Age of rat		Dose (mmol kg^{-1})	% of urinary activity: Benzoic acid	Hippuric acid	Benzoyl glucuronide
Adult	(a)	0.33	ND	98.0	1.6
	(b)	2.5	3.5 (5.6)	94.2 (93.8)	2.3 (0.7)
5 days*		0.33	ND	64.2	18.5
10 days	(a)*	0.33	ND	52.7	20.7
	(b)	0.83	0.5	77.8	21.7
	(c)	0.83	0.5 (19.4)	76.4 (76.6)	23.1 (4.0)
	(d)	0.83	ND	82.1	17.9
	(e)	0.83	0.4	62.5	34.8
	(f)	0.83	ND (16.8)	75.9 (81.4)	19.7 (ND)

*Figures in parentheses are the values found after β-glucuronidase hydrolysis. ND = not determined. *In the urine of these two animals only an unidentified radiometabolite was detected.*

Results and discussion: The total percentage of the dosed [^{14}C]-benzoate excreted in urine in 24 h is shown in the figure. The distribution of the radioactivity is shown in the table which includes also the effect of hydrolysis with β-glucuronidase. Whereas, as has been shown by other workers (4), adult rats excrete benzoate almost entirely as hippuric acid, in neonatal rats about 20% of the radioactivity in the urine of dosed animals corresponds to benzoyl glucuronide. Although the level of hepatic glycine-N-acyltransferase is low in the ten-day old rat a considerable proportion of the dosed benzoate is converted to hippuric acid. It is therefore possible that extra-hepatic tissues contribute to this synthesis.

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