STUDIES ON THE METABOLIC CONJUGATION OF SOME ARYLACETIC ACIDS

by

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ABSTRACT

1. The fate of [^{14}C]-2-naphthylacetic acid has been studied in the rat and ferret. The major route of excretion of ^{14}C was in the urine, and the nature of the urinary metabolites has been studied by thin-layer chromatography colour reactions, selective hydrolysis and reverse isotope dilution. In addition to the unchanged acid, four conjugates of 2-naphthylacetic acid were detected, namely its glycine, glutamine, taurine and glucuronic acid conjugates.

2. The ability of 2-naphthylacetic acid to elicit both glutamine and taurine conjugation in species previously thought to be incapable of these reactions has suggested its use as a probe for these conjugations in other species. Its fate has been examined in mouse, rabbit, guinea pig, hamster and gerbil. Of these, the rabbit, ferret and hamster utilize glycine, glutamine and taurine for the conjugation of 2-naphthylacetic acid, the mouse and gerbil utilize glycine and taurine only while the guinea pig only forms the glycine conjugate. All five species were able to conjugate this acid with glucuronic acid.

3. For comparison purposes, the metabolism of [^{14}C]-1-naphthylacetic acid has been studied in mouse, guinea pig, hamster and gerbil, and in each of these, only the glycine and glucuronic acid conjugates were formed.

4. To search for possible reasons for the unusual conjugation pattern of 2-naphthylacetic acid, the effect of variation in dose size has been examined in all species, while the effect of administration of glutamine and taurine, and pretreatment with 2-naphthylacetic acid, has been studied in the guinea pig. The results show that the dose size can have a profound influence on the metabolic pattern observed, but that the unusual conjugation of

2-naphthylacetic acid in some species is apparently not the result of species differences in amino acid levels or of autoinduction of these mechanisms by the xenobiotic acid.

5. The fate of [^{14}C]-clofibric acid and of [^{14}C]-clofibrate has been studied in man and six laboratory animals using the techniques mentioned above. This aryloxyacetic acid is conjugated with glucuronic acid in all species except the cat, and with taurine in the cat, dog and ferret. The fate of clofibrate, the ethyl ester of clofibric acid, was essentially the same as that of the parent acid.

6. The results have been discussed in terms of the factors responsible for the unusual pattern of metabolic conjugation encountered for 2-naphthylacetic acid and in terms of its consequences for the predictability of conjugation patterns in general.

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INTRODUCTION

CHAPTER ONE

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Introduction

The number of xenobiotics (chemicals foreign to the energy-yielding metabolism of the body) being introduced into our environment is continually increasing. The word xenobiotic is used interchangeably with the term foreign compound. These chemicals may be used as drugs, cosmetics, detergents or food additives which are deliberately brought into contact with the animal body; or they may be in the form of compounds such as pesticides, herbicides, industrial waste products and automobile fumes which contaminate our environment. It is obviously important to understand fully the effects of these foreign compounds on biological systems, and an important aspect of this is the way in which these substances are handled by the living organism.

General Aspects of Foreign Compound Metabolism

Generally, the metabolism of foreign compounds follows a biphasic process. The so-called Phase I reactions of oxidation, reduction and hydrolysis introduce new functional groups into a lipophilic chemical. This then permits Phase II metabolism, the conjugation reactions, in which the metabolite is linked to an endogenous molecule or grouping e.g., clofibrate, the ethyl ester of clofibric acid is hydrolysed in man to clofibric acid, which is subsequently conjugated with glucuronic acid. The overall process may be simply represented thus:

$$\text{Compound} \xrightarrow{\text{Phase I}} \text{Metabolite} \xrightarrow[\text{conjugation}]{\text{Phase II}} \text{Conjugate}.$$

It must be pointed out that some compounds undergo only Phase I metabolism e.g. ethanol, debrisoquine, while others can be metabolised by conjugation directly e.g. salicylic acid, paracetamol.

The Phase I metabolic reactions can be classified chemically as oxidations, reductions or hydrolyses (Table 1.1). The major site of these reactions is in general the liver, but many extrahepatic tissues, such as the kidney and gastrointestinal tract also possess the necessary enzymes. The Phase I metabolic reactions can be subdivided into various types as is shown in Table 1.2.

During Phase I metabolism, there commonly occur changes in the biological properties of the parent compound. Most frequently, the pharmacological effect of the compound is reduced by metabolism, but there may be activation or a change in the nature of the activity. Some examples of these possibilities are shown in Table 1.3.

The Phase II reactions constitute the so called synthetic or conjugation reactions of foreign compound metabolism. These involve the addition of an endogenous substance (usually derived from an amino acid or carbohydrate) to the functional group of the foreign compound or its Phase I metabolite and this results in the foreign compound being rendered less lipophilic, more polar and easily excreted.

Energy is required for the Phase II reactions and this is supplied by the participation of 'high energy' molecules, such as nucleoside phosphates. Phase II

Table 1.1

Phase I reactions in the metabolism of foreign compounds in the liver.

Metabolic Reactions	Subcellular location of enzymes
Oxidations	
C- and N-hydroxylation	Endoplasmic reticulum*
N- and S-oxidations	" "
Dealkylation (N- and O-)	" "
Desulphuration	" "
Dehalogenation	" "
Deamination	Endoplasmic/mitochondria reticulum
Reductions	
Nitro reduction	Endoplasmic reticulum/ cytosol
Azo reduction	Endoplasmic reticulum
Aldehyde reduction	Cytosol
Hydrolyses	
De-esterification	Endoplasmic reticulum/ cytosol
De-amidation	Endoplasmic reticulum/ cytosol

* Isolated as microsomal fraction.

Table 1.2 Some examples of Phase I Metabolic Reactions

Reaction Type	Drug	Product	Ref.
Aromatic hydroxylation	Phenmetrazine	4-Hydroxyphenmetrazine	Caldwell (1976)
	Methaqualone	2-Hydroxymethaqualone	Reynolds et al. (1976)
N-Oxidation	Chlorphentermine	N-Hydroxychlorphentermine	Caldwell (1976)
	Methaqualone	Methaqualone N-oxide	Burnet et al. (1976)
N-Dealkylation	Prazepam	Desalkylprazepam	Viau et al. (1973)
	Caffeine	1,7-Dimethylxanthine	Burrows & Kestler (1974)
O-Dealkylation	Indomethacin	O-Desmethylindomethacin	Hucker et al. (1966)
Deamination	Propranolol	1-Naphthoxyacetic acid	Patterson et al. (1970)
	Amphetamine	Hippuric acid	Dring et al. (1970)
Ester hydrolysis	Halofenate	Halofenic acid	Hucker et al. (1971)
	Clofibrate	Clofibric acid	Thorp et al. (1962)
Amide hydrolysis	Procaineamide	4-Aminobenzoic acid	Dreyfuss et al. (1972)
Reduction	Warfarin	Warfarin alcohol	Lewis & Trager (1970)
	Nitrazepam	Reduced nitrazepam	Mitchard (1971)

Table 1.3

Drug Metabolism and Pharmacological activity

Drug	Metabolic Reaction	Metabolite	Pharmacological consequencies
Deactivation			
Phenobarbitone	Aromatic hydroxylation	4-Hydroxyphenobarbitone	Loss of hypnotic activity.
Chlorcyclizine	N-oxidation	Chlorcyclizine N-oxide	Loss of antihistamine activity.
Activation			
Prontosil	Azo-reduction	Sulphanilamide	Becomes antibacterial agent.
Phenacetin	O-deethylation	Paracetamol	Increased activity as analgesic.
Imipramine	N-demethylation	Desmethylimipramine	Increased activity as antidepressant.
Codeine	O-demethylation	Morphine	Analgesic converted to narcotic.
Parathion	Oxidation	Paraoxon	More active insecticide.
Change of Activity			
Iproniazid	N-dealkylation	Isoniazid	Antidepressant converted to antitubercular.

reactions can be divided mechanistically into two types, those where the endogenous molecule is activated and those where the xenobiotic is activated. Table 1.4 lists the principal conjugation reactions.

Significance of Phase II reactions

A survey of the literature on foreign compound metabolism shows that a disproportionately large effort has been expended on Phase I reactions of drug metabolism (oxidations, reactions and hydrolyses) compared to Phase II reactions or conjugations.

The relative lack of interest in conjugation reactions perhaps stems from the belief that they have less interest in term of biological activity of the compound when compared with the Phase I reactions. Thus, oxidative metabolism (a Phase I reaction) can produce chemically stable pharmacologically active or unstable reactive metabolites which may contribute towards the biological properties of the drug (see Table 1.3), while the products of conjugation are generally inactive.

However, a number of findings support the view that perhaps more attention should be paid to conjugation reactions and these are (a) their detoxication function may fail due to species defects or easy saturation (b) conjugation reactions resulting in metabolic activation may occur and (c) the metabolic conjugation can be a major determinant of drug elimination and pharmacokinetics. These points will be discussed in turn.

Table 1.4

Mechanistic Classification of the Conjugation Reactions.

Conjugation reactions	Activated intermediate
(a) Activated conjugating agent	
Glucuronic acid conjugation	Uridine diphosphate glucuronic acid (UDPGA)
Glucoside conjugation	Uridine diphosphate glucose (UDPG)
Sulphation	Adenosine-3'-phosphate-5'-phosphosulphate (PAPS)
Methylation	S-Adenosylmethionine (SAM) 5 - methyltetrahydrofolate
Acetylation	Acetyl coenzyme A (Acetyl CoA)
Cyanide detoxication	Thiosulphate
(b) Activated foreign compound	
Amino acid conjugations	Coenzyme A thioesters
Glutathione conjugation	Arene oxides, epoxides

The detoxication function of conjugation

Conjugation reactions generally lead to detoxication and elimination of foreign compounds ingested into the animal body. As already stated, many of the products of Phase I metabolism contribute to the biological activity of the compound. It therefore appears that in most cases the conjugations are the true detoxication mechanisms (Williams 1959). Their detoxication function is clearly seen by examining the toxicities of compounds metabolised by glucuronic acid conjugation in the rabbit (which is capable of glucurononic acid conjugation) and the cat (which has a well-documented defect in this pathway). Phenol, 1- and 2-naphthol, paracetamol, benzoic acid and the isomeric cresols and nitrophenols are all several times more toxic to the cat than the rabbit. It may be argued that the cat is generally more susceptible to toxic chemicals than is the rabbit and therefore these differences in relative toxicity are not due to the glucuronidation deficiency in cat. However, when the toxicities of compounds such as barbital, caffeine and strychnine (which are not conjugated with glucuronic acid) are compared in the cat and rabbit, no major species differences are observed suggesting that differences in glucuronidation are responsible for the species differences in the toxicity of the first group of compounds (Caldwell, 1979).

The detoxication function of conjugation is further illustrated by comparison of the toxicities of compounds that are normally metabolised by N-acetylation. It is

well documented that the dog unlike the rat, is unable to N-acetylate various aromatic amines. The LD_{50}s of a number of aromatic amines and hydrazines are higher (i.e. less toxic) in the rat than in the dog probably because of the species difference in ability to effect N-acetylation.

The toxic potential of the so-called reactive metabolites arises from their electrophilic nature, which can result in their covalent binding to a variety of nucleophilic centres in tissue macromolecules. Reactive metabolites have been implicated in carcinogenesis, mutagenesis and tissue necrosis induced by various compounds and the most versatile protection mechanism against these metabolites is by their conjugation with the highly reactive nucleophilic tripeptide, glutathione. This conjugation is catalysed by a family of enzymes, the glutathione S-transferases (Jerina and Bend 1977). The conjugates are either excreted as such in the bile or undergo further metabolism of the tripeptide moiety, to give mercapturic acids or related compounds which are excreted in the urine (Jerina and Bend 1977).

From the preceding discussion, it is clear that the Phase II reactions are very important detoxication processes and thus any change in the capacity of organism to carry out these reactions could have profound implications. Some species are unable to carry out certain Phase II reactions, as such, they are termed to be deficient in these reactions. The inability of the dog to N-acetylate aromatic amines, or the cat to conjugate some xenobiotics with

glucuronic acid are examples of such species defects in common metabolic reactions. (Table 1.5). These defects must be considered when attempting to extrapolate data from one species to another.

Certain Phase II reactions can be readily saturated by substrate, such as the formation of salicyluric acid and salicylphenyl-glucuronide from aspirin, reactions which are easily saturated within the normal dose range of aspirin. Their formation is thus described by Michelis-Menten kinetics rather than the more normal first-order kinetics.

Saturation of metabolic conjugation pathways can have important consequences. Thus the saturation of the glutathione conjugation mechanism leads to appearance of toxicity of compounds such as paracetamol and bromobenzene which cause hepatocellular necrosis in several species which is associated with the covalent binding of a reactive metabolite to tissue macromolecules (Mitchell et al., 1973a). Other examples include the enhanced toxicity of chloramphenicol in the human newborn (Kaufman, 1960) and increased benzoic acid toxicity in the cat, both associated with the ready saturation of the glucuronic acid conjugation of these compounds in these situations.

Metabolic activation as a result of Phase II reactions

The previous discussions show the significance of the metabolic conjugation processes as detoxication mechanisms for xenobiotics. A number of examples now exist which show that metabolic conjugation does not always result in

Table 1.5

Species Defects in Conjugation Reactions

Species	Defective reaction	Reference
Cat, lion, lynx, Civet genet	Glucuronidation	Robinson and Williams (1958); Caldwell et al. (1975)
Pig	Sulphation	Stekol (1936)
Indian Fruit Bat	Glycine	Bababunmi et al. (1973).
Dog	N-Acetylation of aromatic amine and hydrazines	Marshall (1954)
Non-primate mammals	N-Glucuronidation of sulphadimethoxine	Williams (1973)
Guinea Pig	N-Acetylation of S-substituted cysteines	Bray et al. (1959)

detoxication but rather that conjugates may occasionally be biologically active. The morphine conjugate, morphine-6-glucuronide, is a potent, long-acting analgesic and has powerful morphine-like activity on the guinea pig ileum (Mori et al., 1972). This conjugate may thus contribute to the activity of morphine in the intact animal.

The metabolic activation of carcinogenic arylamides to the ultimate carcinogen is a two step process involving first N-oxidation, giving an N-hydroxy compound which may then undergo conjugation with glucuronic acid or sulphate. The N-O-sulphates and -glucuronides of compounds such as 2-acetamidofluorene are highly reactive compounds, much more potent carcinogens than the free N-hydroxy analogues (Miller & Miller, 1977). Irving (1970) has shown that the binding of N-hydroxy-2-acetamidofluorene to the RNA of rat liver is positively correlated with the amount of sulphotransferase in the liver.

Metabolic conjugation may act as a transport system for some compounds to their site of actions. The body possesses a number of enzymes able to break down conjugates, such as β-glucuronidase, sulphatase, deacetylase and O- and N-demethylases. These enzymes may break down the conjugates to the parent drug or metabolite which may be toxic to tissues far removed from the site of their formation.

The studies of Kadlubar et al., (1977) showed that N-hydroxyarylamines are able to form 3 types of conjugates, N-glucuronides with a free hydroxyl group, N-O-glucuronides and N-O-sulphates. The N-glucuronides are chemically stable

and their involvement in carcinogenesis by N-hydroxyarylamines is due to their functioning as stable transport forms. They are broken down by β-glucuronidase in the mildly acidic environment of the urinary bladder liberating the parent N-hydroxyarylamine which causes cancer of the urinary bladder.

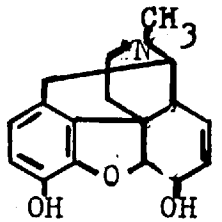
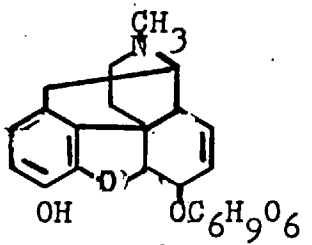
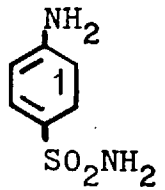
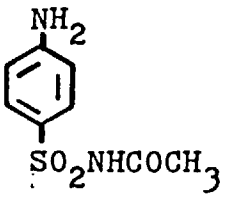
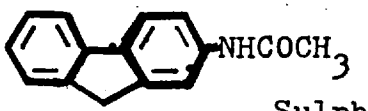
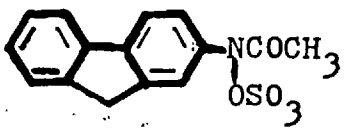
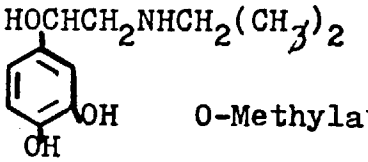
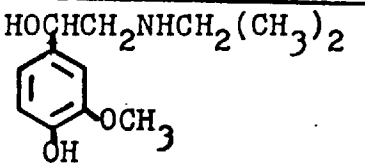
Kadlubar et al., (1977) proposed a reaction sequence for the development of both liver and bladder tumours after the administration of arylamines such as 2-naphthylamine and 4-aminobiphenyl, which is supported by the chemical and biochemical properties of the various metabolites. The arylamine is first N-hydroxylated by the hepatic microsomal enzymes and the resulting hydroxylamine can then form its N-O-sulphate, N-O-glucuronide and N-glucuronide. The N-O-sulphate, N-O-glucuronide and free hydroxylamine are able to react with liver macromolecules resulting in the initiation of tumour formation at their site of formation while the N-glucuronide, which is not reactive at the pH of the hepatocytes, is excreted in the urine. Urine in the bladder of persons on the average Western high protein diet is acidic (pH 5.5-6.0) and also contains β-glucuronidase. These two factors result in the liberation of the hydroxylamine from the conjugate and its covalent binding to the nucleic acids of bladder wall thereby initiating tumour formation (other examples are listed in Table 1.6).

Pharmacokinetic implications of Phase II metabolism

In addition to their importance for the detoxication,

Table 1.6

Some Active Metabolites

Compound	Conjugation Reaction	Conjugates formed	Pharmacological Consequences	References
 Morphine	Glucuronidation	 Morphine-6-glucuronide	More potent analgesic	Mori et.al. (1972)
 Sulphanilamide	N-Acetylation	 Sulphacetamide	Anti-bacterial agent	Williams and Milburn(1975)
 2-Acetamidofluorene	Sulphation	 N-O- Sulphate of N-Hydroxylated Acetamidofluorene	Carcinogen	Miller and Miller(1977)
 Isoprenaline	O-Methylation	 3-O-Methylisoprenaline	β Antagonist	Dollery et al(1971)

and occasionally activation, of foreign compounds, Phase II reactions have many implications for drug disposition, excretion and interactions. The principal products of conjugation reactions are mainly acidic, water-soluble compounds, which are readily eliminated from the body. Studies with a number of glucuronides provide evidence for their active secretion by the renal tubules (Sperber, 1948) and this may also be true of sulphate conjugates (Sperber, 1948). Amino acid conjugates of aromatic acids are also actively secreted by the kidney and the physiochemical properties of these conjugates indicate that they will be far less readily reabsorbed by kidney tubules than parent acids (see Caldwell et al., 1978).

There is a scarcity of quantitative data to be found in the literature showing the role of conjugation reactions in the total body clearance of compounds. Levy et al., (1972) studied salicylic acid, which is almost completely metabolised by conjugation, giving rise to both the ester and ether glucuronides as well as a glycine conjugate. The excretion half-lives of these conjugates are very much shorter than their overall elimination half lives showing that, once formed, the conjugates are rapidly cleared. The elimination half life of the unchanged drug is much longer than those of the conjugates. (Table 1.7).

Some Phase II reactions are easily saturated and once the dose administered is sufficient to saturate one of the pathways involved in drug elimination then the elimination process no longer follows first-order kinetics and new

Table 1.7

Significance of conjugation reactions in the total body clearance of salicylic acid in Man

Metabolite	Elimination $t_{\frac{1}{2}}$	Excretion $t_{\frac{1}{2}}$
Salicyluric acid	20 h	0.23 h
Salicyl ester glucuronide	9.9 h	0.49 h
Salicylphenylglucuronide	30 h	1 h
Salicylic acid	13.9 h	

Data calculated from Levy et al. (1972)

expressions involving Michaelis-Menten kinetics must be derived (Rowland, 1977). The so-called non-linearity introduced by saturation phenomena has important consequences (Wagner, 1974) and these may be summarised thus:

(i) the elimination kinetics become non-exponential in character.

(ii) the elimination $t_{\frac{1}{2}}$ increases with dose.

(iii) the pattern (quantitative and perhaps qualitative) of urinary metabolites may vary with dose.

Studies of Gerber and Wagner (1972) on phenytoin and that of Caldwell et al., (1977) on theophylline have shown that certain Phase I metabolic pathways of drugs are saturable within their therapeutic dose range, but the problem of saturation is more important for the Phase II pathways. Many examples of this can be found in the literature, such as the conjugation of paracetamol (Jollow et al., 1974), salicylate (Levy et al., 1972) salicylamide (Levy and Yamada, 1971), isoniazid (Kakemi et al., 1963), and p - aminobenzoic acid (Drucker et al., 1964). The consequences of saturation of the metabolic conjugation of such drugs may result in practical difficulties in managing dosage regimes to give optimum plasma levels.

The extensive biliary excretion of anionic compounds requires a certain molecular weight (which varies from species to species) and a lipophilic to hydrophobic balance within the molecule. This combination is most commonly achieved as a consequence of Phase II reactions and a large

number of glucuronides and glutathione conjugates are preferentially excreted in the bile (Smith, 1973). The excretion of conjugates in the bile may result in one or more of the following situations:

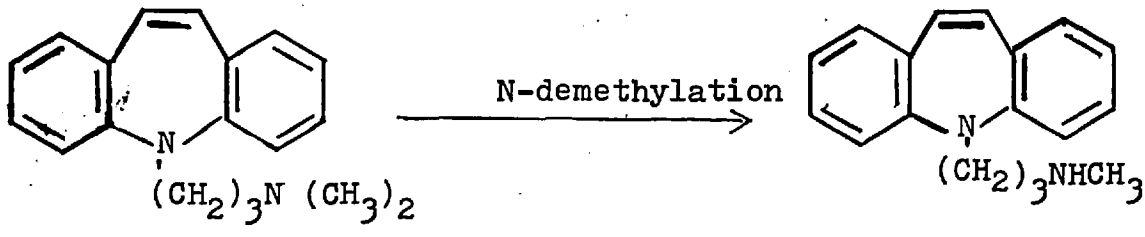
(i) hydrolysis back to parent compound.

(ii) formation by the gut flora of metabolites not normally produced in the tissues.

(iii) enterohepatic circulation of the compound or its metabolites.

(iv) local toxicity within the hepatobiliary and gastrointestinal tract systems.

The studies of Minder et al., (1971) have shown that the gut flora contributes 50% of the total N-demethylation of imipramine which occurs in the body.



Imipramine

Desmethylimipramine

The antibiotic chloramphenicol is thyrotoxic in the rat but apparently not in other species investigated. It is conjugated with glucuronic acid, and this conjugate is readily excreted in the rat bile and hydrolysed by the bacterial β-glucuronidase associated with the gut flora (Glazko et al., 1952). The gut flora has a considerable nitro reductase activity towards chloramphenicol and the arylamines so produced are absorbed and are toxic to the thyroid gland (Thomson et al., 1954). This toxicity readily manifests itself in the rat since generation of toxic arylamine is a function of the extensive biliary excretion of chloramphenicol and subsequent reduction to arylamines which to some extent is peculiar to this species.

Biliary excretion of conjugates of foreign compounds may also be a factor in the localised toxicity to the gastrointestinal trace of some drugs such as indomethacin. The ester glucuronide of indomethacin is readily excreted in bile and this is then hydrolysed by bacterial β-glucuronidase. The indomethacin liberated is an ulcerogen to the small intestine and it is the biliary excretion of the glucuronide which is an essential factor for this type of toxicity to manifest itselfs (Hucker et al., 1966).

The above discussion has indicated the significant role of Phase II reactions in the pharmacology and toxicology of xenobiotics. Phase II reactions are of great importance for the detoxication and excretion of xenobiotics and in a few cases for their activation. It is clear that any alteration in the capacity of an organism to effect these reactions will have important consequences for the

biological activity (pharmacological or toxicological), pharmacokinetics and elimination of compounds metabolised by these reactions. It is therefore necessary that more effort should be devoted to the study of Phase II reactions than has perhaps occurred hitherto.

Importance of Carboxylic Acids

The importance of carboxylic acids in medicine and agriculture cannot be over-stressed. Table 1.8 shows a list of some carboxylic acids and their various uses. Many drugs of course are transformed to carboxylic acids during the course of their metabolism. Many drugs prescribed or sold over the counter are carboxylic acids, for example, salicylic acid, indomethacin. Others are used as herbicides such as 2,4,-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, or as plant growth hormones such as p-chlorophenoxyacetic acid. The widespread use of various carboxylic acids as drugs is illustrated by the large number prescribed in England in the year 1977 alone (Table 1.9).

As stated above, some drugs are converted by Phase I metabolism to carboxylic acids. The simplest means of this is by hydrolysis of esters, as is seen in the cases of the ethyl ester of clofibric acid, a hypolipidaemic agent, to give the active aryloxyacetic acid and also halofenate, another hypolipidaemic which is also converted by hydrolysis to an active acid.

Hydrolysis is not the only way in which xenobiotics can be metabolised to carboxylic acids. This conversion can be achieved by oxidation. For example, haloperidol, a neuroleptic drug, is oxidised by the rat to 4-fluorophenyl-

Table 1.8

Carboxylic acids and their uses

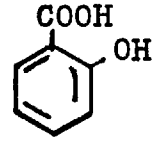
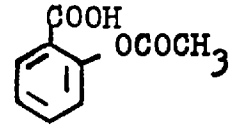
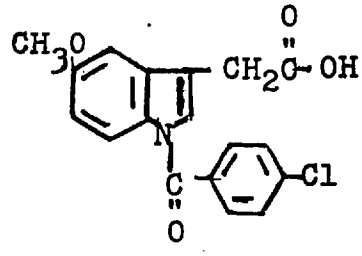
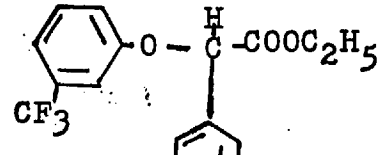
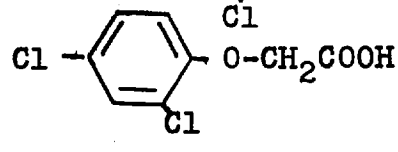
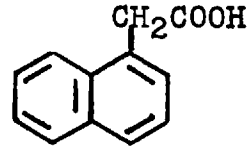
Drugs	Uses	Chemical Structure
Salicylic acid	Analgesia	
Aspirin	Analgesia	
Indomethacin	Anti-inflammatory	
Halofenate	Hypolipidaemic	
2,4-Dichlorophenoxyacetic acid	Herbicide	
1-Naphthylacetic acid	Growth hormone in plants	

Table 1.9

1977 Prescribing Figures for Drugs which are Arylacetic and Aryloxyacetic Acids in England

Drugs	EC10 Prescriptions (thousands)
Naproxen	844
Fenoprofen	431
Alclofenac	33
Indomethacin	3,026
Flufenamic Acid	11
Nalidixic Acid	156
Clofibrate	617

lacetic acid (Brown et al.,1974) while chlormezanone an anti-convulsant is oxidised to 4-chlorobenzoic acid in man (McChesney et al.,1967).

A third, more unusual, mode of conversion of xenobiotics to carboxylic acids is by aromatization, in which an aromatic compound is synthesised from a cyclic carboxylic acid, such as the synthesis of benzoic acid from quinic acid and shikimic acid (Adamson et al.,1970a) in man and Old World Monkeys. Table 1.10 shows some examples of compounds which are converted to carboxylic acids in the body by metabolic oxidation or hydrolysis.

Metabolism of Aromatic Acids

Aromatic acids have an appropriate functional group, the carboxyl group, in their structures which enables them to participate in metabolic conjugation reactions directly. As a result of this, Phase I metabolism of the carboxyl group occurs infrequently. One such reaction is decarboxylation, as in the cases of gallic acid (3,4,5-trihydroxybenzoic acid) which is converted by the gut flora to pyrogallol in the rabbit (Watanabe and Oshima 1965), and salicylic acid which is oxidised to a small extent to gentisic acid (2,5-dihydroxybenzoic acid) which is in turn decarboxylated to the corresponding catechol (Levy et al. 1972).

Studies over the past two decades have given considerable insight into the major metabolic options available to aromatic acids, and these are (i) they may be conjugated with glucuronic acid (ii) they may be conjugated with amino acids and (iii) excreted as the unchanged compound.

Table 1.10

Some xenobiotics converted to carboxylic acids.

Compound	Uses	Species	Acid produced	Method of Conversion	Reference
Propranolol	β-Blocker	Dog	Naphthoxyacetic acid	ox	a
Clofexamide	CNS stimulant	Rat	4-chlorophenoxyacetic acid	ox	b
Phenylacetylurea	Anticonvulsant	Rabbit	Phenylacetic acid	hydro	c
Mescaline	Hallucinogen	Man	3,4,5, trimethoxy-phenylacetic acid	ox	d
Haloperidol	Neuroleptic	Rat	4-fluorophenylacetic acid	ox	e
Chlormezanone	Anticonvulsant	Man	4-Chlorobenzoic acid	ox	f
Chlorophensin carbamate	Muscle relaxant	Rat	4-Chlorophenoxyacetic acid	hydro	g
Halofenate	Anticholesterol	Rat,Dog Man	Halofenic acid	hydro	h
Clofibrate	Anticholesterol	Rat, Man	Clofibric acid	hydro	i
Pyrazinamide	Tuberculostatic agent	Dog, Man	Pyrazenoic acid	hydro	j

ox = oxidation hydro = hydrolysis

P.T.O. for references

References for Table 1.10

a. Walle and Gaffney (1972)

b. Trino et al. (1972)

c. Tatsumi et al. (1967)

d. Charalampous et al. (1967)

e. Braun et al. (1967)

f. McChesney et al. (1967)

g. Buhler et al. (1967)

h. Hucker et al. (1964)

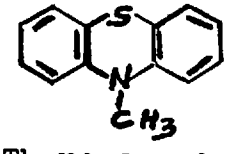
i. Thorp and Waring (1962)

j. Weiner and Tinker (1972)

In addition to these major conjugation options, there can occur two unusual conjugation reactions, methylation and the formation of an amide, and, of course, there may be Phase I and Phase II metabolism of other functional groups within the molecule. Methylation of carboxylic acids to methyl esters has been reported in the literature, for example, the acetic acid grouping of metiazinic acid, an anti-inflammatory drug is reported to undergo metabolic transformations in the rabbit and man by methylation (Populaire et al., 1969).

$$R\text{-}CH_2COOH \longrightarrow R\text{-}CH_2COOCH_3$$

R =



There can occur Phase I metabolism involving the alkyl or aryl moiety. Examples include hydroxylation of clonixin, an anti-inflammatory agent to 2'-hydroxymethylclonixin in the rat, dog, and monkey (Katchen et al.,1973) and the transformation of mefenamic acid to its hydroxymethyl derivative and to its corresponding acid in man, dog and monkey (Katchen et al.,,1973). Another carboxylic acid which undergoes Phase I metabolism is ibuprofen whose isobutyl side chain is hydroxylated to yield a tertiary alcohol or one of the geminal methyl groups is oxidised to yield an acid (Pfeifer, 1968).

Glucuronic Acid Conjugation

Many xenobiotics are metabolised in man and other mammalian species by glucuronidation. The reasons for such widespread occurrence and versatility of the glucuronic acid conjugation mechanism are as follows:

(i) The relative ease of supply of glucuronic

acid from the carbohydrate resources for conjugation.

(ii) The wide range of functional groups (e.g. OH, COOH, SH, NH) which can accept glucuronic acid.

(iii) The enzymes necessary are found in many animal tissues.

Glucuronide formation is a two step process involving first the biosynthesis of the coenzyme donor, uridine diphosphate glucuronic acid (UDPGA) and second the transfer by UDP-glucuronyl transferase, of the glucuronic acid moiety from UDPGA to the foreign compound. The enzyme is membrane bound in the microsomal fraction of the liver and many other tissues, and there is much evidence which suggests that it exists in more than one form. For example, the ability of the cat to form the glucuronides of some compounds such as phenolphthalein (Millburn, 1970), iopanoic acid (McChesney 1964), chloramphenicol (Dill et al., 1960), and its inability to conjugate many drugs extensively glucuronidated in other species may be due to the fact that the cat is deficient in one form of UDP-glucuronyl-transferase.

Studies of Wishart et al., (1978) and Del Villar et al., (1975) have isolated two forms of UDP-glucuronyltransferase and these multiple forms differ in their substrate specificities and in their regulation, at least during the perinatal period.

Amino Acid Conjugation

A wide range of aromatic acids can undergo amino acid

conjugation reactions. This involves combination of the carboxylic acid with the amino acid by formation of an amide link. The nature of the amino acid utilised can vary markedly according to species and structure of the carboxylic acid. The amino acids most commonly encountered in conjugation reactions are aliphatic and non-essential, namely glycine, glutamine, ornithine and taurine. The glycine conjugation is widespread and versatile in that it occurs in a wide range of species and can be utilised for conjugation of carboxylic acids of diverse structures. The other mechanisms are more restricted in terms of either or both species occurrence (glutamine and ornithine) or the type of the acid that can be conjugated (glutamine and taurine conjugation). Rarely, other amino acid conjugations involving serine (Rothstein and Greenberg 1957), glutamic acid (Smith,1962), arginine (Smith,1962), histidine (Jordan et al.,1970) alanine (Wallcave et al.,1974) and aspartic acid (Reif and Sinsheimer, 1975) are encountered, although their occurrence seems haphazard and appears to be peculiar to a certain combination of carboxylic acid and species (Table 1.11). A complete review of amino acid conjugation has been carried out by Caldwell et al. (1979).

The most common amino acids utilised in the conjugation of carboxylic acids are glutamine, glycine and taurine, and aspects of these reactions will be discussed in turn.

Enzymology of glycine conjugation

Quick (1931) first performed the in vitro studies of

Table 1.11

Major amino acids utilised for the metabolic conjugation of aromatic carboxylic acids.

Amino acid	Aromatic acid	Species
Glycine	Aromatic carboxylic acids	All species examined.
	Arylacetic acids	Non-primate
Glutamine	Arylacetic acids	Anthropoid species excluding Prosimiams.
Taurine	Arylacetic acids Aryloxyacetic acids	Numerous mammals including carnivores, rodents and also fish species.
Ornithine	Aromatic carboxylic acids	Birds and Reptiles
	Arylacetic acids	Birds.

glycine conjugation of aromatic acids and demonstrated the synthesis of hippuric acid from benzoic acid in liver tissue of rabbit and man and in the kidney tissue of dog, rabbit and man. Borsook and Dubnoff (1947) suggested that energy is needed for the glycine conjugation while Cohen and McGilvery (1947) showed that this energy is derived from Kreb's cycle reactions or under anaerobic conditions, it may be derived from adenosine triphosphate (ATP). Chantrenne (1951) demonstrated that glycine conjugation required the presence of Coenzyme A. Schachter and Taggart (1953) demonstrated that S-benzoyl CoA is an essential intermediate in hippuric acid synthesis and suggested the following reaction sequence for hippuric acid synthesis.

1. $R.COOH + ATP \longrightarrow R.CO.AMP + PPi$
2. $R.CO.AMP + CoA.SH \longrightarrow R.CO.S.CoA. + AMP$
3. $R.CO.S.CoA + H_2N.CH_2.COOH \longrightarrow R.CO.NH.CH_2 COOH + CoA.SH$

$R =$ 

Both reactions 1 and 2 are catalysed by acyl CoA synthetase (EC 6.2.1.2) and reaction 3 by glycine N-acyl transferase (EC 2.3.1.13). The enzymes involved in hippuric acid synthesis are all located in the mitochondria (Kielley and Schneider,1950; Leuthardt and Nielsen,1951).

Reactions 1 and 2, which result in the activation of the aromatic acid, are both catalysed by acyl CoA synthetase. This enzyme can activate C_4-C_{12} fatty acids and a range of aromatic acids (Mahler *et al.*,1953) while the reaction

between the acyl-CoA and glycine is catalysed by glycine N-acyl-transferase, which was first purified from an acetone powder of beef liver mitochondria by Schachter and Taggart (1954). Recently, glycine N-acyltransferase has been purified from human and beef liver mitochondria (Tishler and Goldman, 1970; Foreman et al., 1971).

Enzymology of glutamine conjugation

Moldave and Meister (1957) detected glutamine conjugating activity in both human liver and kidney using phenylacetic acid as substrate, but no other tissue was investigated. The levels of glutamine in other tissues suggest that glutamine conjugation activity could occur in other tissues if the enzyme is present.

The studies of Moldave and Meister (1957) on the conversion of phenylacetic acid to phenylacetylglutamine suggested that the conversion was similar to glycine conjugation involving Coenzyme A and ATP in the formation of activated derivatives of the carboxylic acid. They proposed that the synthesis proceed in three steps as follows:

1. Phenylacetic acid + ATP $\longrightarrow$ Phenylacetyl-AMP + PPi
2. Phenylacetyl-AMP + CoASH $\longrightarrow$ Phenylacetyl-S-CoA + AMP
3. Phenylacetyl-S-CoA + L-glutamine $\rightarrow$ Phenylacetylglutamine + CoASH

This enzyme (Phenacetyl-CoA.L-glutamine α-N-Phenacetyl-transferase, EC.2.31.14) is found in the soluble fractions of human liver and kidney and has been partially purified

(Moldave and Meister, 1957). Besides catalysing the condensation of phenylacetyl-CoA and glutamine, the enzyme also catalysed the reaction between benzoyl-CoA and glycine, but at a much slower rate.

Enzymology of taurine conjugation

The utilisation of taurine in the conjugation of arylacetic acids in vivo was discovered by James et al. (1971). However since then attempts in this laboratory to elucidate its mechanism have proved abortive. Lately studies of James & Bend (1978) have partially established the mechanism of taurine conjugation in fish species (Winter and Southern flounder and the dogfish shark). In their experiments, phenylacetyl-CoA and taurine were added to fish liver homogenate, and phenylacetyltaurine was formed. The N-taurine transferase has been partially purified but the mechanism has not been established. The presence of a taurine N-acyltransferase in liver suggests that the overall mechanism is similar to those of glutamine and glycine conjugation.

Factors affecting the metabolism of aromatic acids

Many factors are known to influence the activities of enzymes involved in the metabolism of drugs. In the past decade, it has been established conclusively that two main factors bear on the metabolism of aromatic acids, and these are animal species and the chemical structure of the compound. (Evans, 1972; James, 1972; Dixon et al. 1977a,b,c and Caldwell, 1978). In addition, the size of the dose may play an important role in determining the metabolism of aromatic acids.

Species variations

The basic pattern of the metabolism of xenobiotics is the same in all species, that is, they usually follow the two phase pattern outlined earlier. However, within this pattern, tremendous variation can occur, which presumably arises from interspecies variations in the amounts and activities of the enzymes involved. Species differences in the pattern of metabolism arise because of one or more of the following situations: (a) compounds may be metabolised along several competing pathways extant in all species but the relative importance of the individual pathways may vary with species, (b) occurrence of species defects in metabolism (c) occurrence of unusual conjugation reactions and these will be discussed in turn.

Metabolism along several competing pathways

The metabolism of a compound along the same pathways in a number of species is not uncommon, but the relative amounts of the various metabolites formed can vary considerably. An example is the metabolism of indolylacetic acid in the ferret, rabbit, rodents and pigeon which is by conjugation with glycine and taurine. The extent of the occurrence of these pathways varies greatly with species (Table 1.12). Glycine conjugation is the main pathway in the rabbit and rodent; in the pigeon, the major pathway is taurine conjugation while in the ferret, both pathways are utilised (Bridges et al., 1974).

Species defects in xenobiotic metabolism

Species variation in the metabolism of xenobiotics may

Table 1.12

Competing reactions as a cause of species diffences in metabolism; the fate of indolylacetic acid

Species	% Dose excreted as	
	Glycine conjugate	Taurine conjugate
Ferret	19	34
Rabbit	53	0
Rat	43	0
Mouse	32	0
Guinea Pig	53	0
Hamster	21	0
Pigeon	0	28

Data from Bridges et al., (1974).

be attributed to occurrence of the so-called species defects in metabolism. Results of studies over the past two decades have shown that some species appear to lack the ability to carry out certain common metabolic reactions, such as the inability of rat to N-hydroxylate some aliphatic amines (Caldwell, 1976), also the pigs' inability to utilise sulphate in conjugation reactions (Williams, 1973). Other examples of defects in conjugation reactions are listed in Table 1.5. From this table, one will expect variation in conjugation reactions in various species.

Occurrence of unusual conjugation reactions

Some conjugation reactions are unusual in that they may be restricted to certain species or certain compounds. Some metabolic reactions such as glutamine conjugation appears to be largely restricted to primates (James et al., 1972), as is also the O-methylation of 3,5-diiodo-4-hydroxybenzoic acid (Wold et al., 1973). Other examples are listed in Table 1.13.

Influence of chemical structure on metabolism

The studies of Bray and colleagues from 1949 to 1955 on the fate of substituted benzoic acids in the rabbit showed that the extent of amino acid conjugation of the monosubstituted benzoic acids depends on the nature of the substituent and its position on the aromatic ring. In the case of para substitution, the acids undergo principally glycine conjugation, while ortho substitution results in mainly glucuronic acid conjugation. Increase in the size of the substituent in the para position did not affect

Table 1.1

Some uncommon conjugation reactions

	Conjugating agents	Species
Amino acids	Ornithine	Birds and Reptiles
	Taurine	Pigeon and Ferret
	Serine	Rat and Rabbit
	Glycyltaurine	Cat
	Arginine	Ticks and Spiders
Carbohydrates	Glucose	Insects
	Ribose	Rat and Mouse
	N-acetylglucosamine	Rabbit
Acids	Phosphate	Cat, Dog and Man
	Formate	Rat and Dog
	Succinate	Rat and Dog

glycine conjugation but on ortho substitution there was a notable glucuronic acid conjugation (Table 1.14).

That chemical structure may influence the metabolism of arylacetic acids has been increasingly appreciated over recent years. The studies of Dixon et al. (1976, 1977a,b,c) have shown that the metabolic pattern of arylacetic acids depends on the nature of the aryl substituents and substitution on the α-methylene group adjacent to the carboxyl group. Caldwell (1978) has reviewed and classified carboxylic acids into five groups based on their chemical structure and used this to determine the influence of structure on the metabolism of carboxylic acids.

Phenylacetic acid is metabolised almost entirely by conjugation with amino acids, the particular amino acid utilised varying with species (James et al. 1972a). Similarly, simple ring substituted derivatives of phenylacetic acid, such as 4-chloro- and 4-nitro-phenylacetic acid also undergo metabolic conjugation with amino acids, the nature of which is also species dependent (James et al. 1972a). On the other hand 1-naphthylacetic acid is conjugated partly with glucuronic acid and partly with amino acids. 1-Naphthylacetic acid can be seen as substitution of an aromatic group to phenylacetic acid (see Chapter 3) with the α-methylene group unsubstituted and therefore this may be the reason for the mixed conjugation pattern of this compound. This is also the case with biphenylacetic acid which is similarly conjugated with both glucuronic acid and amino acids. However, when substituents are placed on the carbon α- to the carboxyl group

Table 1.14

Effect of substitution on the isomeric mono-substituted benzoic acid at ortho and para positions in the rabbit.

Substituent	% Dose conjugated with			
	Glycine		Glucuronic acid	
	Ortho	Para	Ortho	Para
H	83	83	15	15
F	43	43	9	9
Cl	5	63	19	21
CH_3	0	46	73	14
OH	Trace	13	13	7

data from Williams (1959).

of phenylacetic acid a dramatic change occurs in the pattern of conjugation away from amino acid conjugation to one involving almost exclusively glucuronic acid. Thus both hydratropic (α-methylphenylacetic acid) and diphenylacetic (α-phenylphenylacetic acid) acids are conjugated with glucuronic acid in almost all species rather than amino acid as occurs with the α-methylene unsubstituted arylacetic acids (Dixon et al., 1977a,b,c). Table 1.15 list other examples which shows the part played by the nature of the chemical structure. It should be noted that substitution on both the aryl and α-methylene groups usually results in conjugation mainly with glucuronic acid for example ibuprofen.

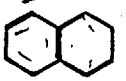
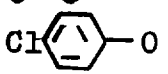
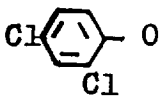
Dose Size

The kinetics of metabolism of most drugs in the body are in general first-order since their concentration at enzyme sites is generally well below Km. Thus, saturation of their metabolism does not occur and as previously stated in this chapter, there is no change in kinetics with dose size. However, it is now appreciated that some metabolic reactions are easily saturated, and when this occurs two consequences, may follow (i) the reaction kinetics change from first order to zero order and (ii) the compound may be metabolised by a secondary metabolic pathway not evident at low doses and/or excreted unchanged to a greater extent. Saturation of a particular metabolic mechanism may therefore be associated with both qualitative and quantitative changes in the pattern of metabolism of a compound and this in turn may be associated with an alteration in the pattern of renal and hepatic elimination of the drug since the metabolic pattern may be an

Table 1.15

Influence of structure on Metabolism of Aryl- and Aryloxy- acetic acids in the rat.



Acid	Ar	R'	R^2	Conjugation with Amino Acid	Conjugation with Glucuronic acid	References
Phenylacetic	C_6H_5	H	H	+++	-	James et al. (1972)
Hydratropic	C_6H_5	CH_3	H	-	+++	Dixon et al. (1977b)
Diphenylacetic	C_6H_5	C_6H_5	H	-	+++	Dixon et al. (1977c)
p-Nitrophenylacetic	P-$NO_2C_6H_4$	H	H	++++	-	James et al. (1972)
SQ 20,650	▷-C(=O)-C_6H_5	H	H	+++	++	Lan et al.(1975)
Ibuprofen	P-iso $C_4H_9C_6H_4$	H	CH_3	-	++++	Mills et al. (1973)
Biphenylacetic	P-$C_6H_5.C_6H_4$	H	H	++	+++	Wade et al. (1978)
1-Naphthylacetic		H	H	+	+++	Dixon et al.(1977)
p-Chlorophenoxyacetic		H	H	-	-	Trino et al. (1977)
2-4-Dichlorophenoxy-acetic		H	H	+	-	Grunow and Bohme (1974)
Clofibric		CH_3	CH_3	-	++++	Thorp (1962)

- = not detected. + → ++++ = arbitrary quantitation.

important factor in determining route of excretion. Some metabolic reactions appear to have a large capacity, e.g. glucuronidation; the rate of glucuronic acid conjugation of many drugs follows first-order kinetics even at large doses while the glycine conjugation mechanism appears to be a reaction of relatively limited capacity which is readily saturable by suitable substrates. It is for this reason that the metabolism of some aromatic acids is dose dependent; with increase of dose, changes in the pattern of metabolism and, sometimes, excretion occur. The limited capacity of glycine conjugation and its implications for secondary pathway metabolism and route of elimination of a compound is well illustrated by 1-naphthylacetic acid (Dixon et al. 1977a). This compound is metabolised in the rat at low doses by glycine and glucuronic acid conjugation but with increasing doses, the relative importance of glycine conjugation declines with a concomitant large increase in glucuronic acid conjugation. Thus, as the dose is increased, the acid is metabolised largely by glucuronic acid conjugation. Saturation of glycine conjugation also leads to change in the major route of elimination of the compound as well as the nature of the conjugation. The glycine conjugate is excreted preferentially via the urine, while the glucuronide has a high affinity for biliary excretion. Thus, low doses of 1-naphthylacetic acid are extensively eliminated in the urine but high doses are excreted almost exclusively via the bile.

This is also the case of the metabolism of benzoic acid in the rat (French, 1970) where it is conjugated with

glycine at low doses but with high doses it is conjugated with both glycine and glucuronic acid.

The above discussion has outlined the metabolic options open to arylacetic and aryloxyacetic acids and some of the factors which affect them. This thesis is concerned with an investigation of some aspects of the metabolic conjugation of arylacetic acids as follows:

(a) Structural isomerism; for this purpose, namely to investigate the relationship between structural isomerism and conjugation pattern, the comparative metabolism of the isomeric 1- and 2-naphthylacetic acids in various species was investigated.

(b) Dose-dependency; the influence of dose variation on the qualitative and quantitative aspects of the metabolic conjugation of 2-naphthylacetic acid was studied.

(c) The origin of the taurine moiety utilised in the in vivo conversion of 2-naphthylacetic acid to 2-naphthylacetyltaurine.

(d) That patterns of amino acid conjugation of carboxylic acids may be influenced by amino acid availability and induction phenomena.

(e) A comparative study of the metabolic fate of the hypolipidaemic α,α-disubstituted aryloxyacetic acid clofibric acid and its ethyl ester clofibrate in various species was carried out as an extension of the study of the relationship between acid structure and conjugation pattern.

CHAPTER TWO

METABOLIC CONJUGATION OF [^{14}C]-2-NAPHTHYLACETIC ACID IN THE RAT AND FERRET

CHAPTER TWO

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Introduction

Previous studies have shown that the pattern of metabolism of arylacetic acids involves two main conjugation options, that is:

i. conjugation with glucuronic acid.

ii. conjugation with various amino acids such as glutamine, taurine and glycine.

The conjugation of arylacetic acids with glucuronic acid and glycine is widespread across the animal kingdom while conjugation with glutamine is apparently restricted to primates (Table 2.1) and that with taurine occurs at random although is well developed in the carnivores (Table 2.2).

The metabolic pattern of an arylacetic acid depends on species and on the nature of the aryl substituents and substitution on on the α-methylene group (Dixon et al., 1974, 1976, 1977a,b,c). When the aryl substituent is small as in the case of phenylacetic acid, the acid is conjugated mainly with amino acids but when there are bulky substituents present as in the case of diphenylacetic acid, conjugation is mainly with glucuronic acid.

The above suggests that the metabolic pattern of arylacetic acids is possibly influenced to a greater extent by their chemical structure rather than by species considerations.

The object of the present work is to explore the possible influence of structural isomerism on the pattern of metabolic conjugation of arylacetic acids. For this purpose

Table 2.1

Glutamine conjugation of some arylacetic acids in primate species.

Compound	Species	References
Phenylacetic acid	Man, rhesus, green, cynomolgus, and squirrel monkeys, marmoset, capuchin	James *et al.* (1972)
4-Hydroxyphenylacetic acid	Man	Oakley and Seakins (1971)
4-Chlorophenylacetic acid	Man, rhesus monkey, capuchin	James *et al.* (1972b)
1-Naphthylacetic acid	Squirrel monkey, capuchin and marmoset	Dixon et al. (1977a)
Indol-3-ylacetic acid	Man, rhesus and green monkey, baboon, squirrel monkey, owl monkey and capuchin.	Bridges *et al.* (1974)

Table 2.2

Conjugation of some arylacetic acids with taurine in the ferret (a carnivore) and rat (a rodent)

Compound	% Dose conjugated with taurine in	
	Ferret	Rat
Phenylacetic acid	14[a]	1[b]
4-Chlorophenylacetic acid	8[a]	0[c]
4-Nitrophenylacetic acid	35[a]	0[c]
Hydratropic acid	38[a]	0[d]
1-Naphthylacetic acid	49[a]	0[d]
Indol-3-ylacetic acid	34[e]	0[e]

a Idle *et al.*(1978)

b James *et al.*(1972a)

c James *et al.* (1972b)

d Dixon *et al.* (1977a,b)

e Bridges *et al.* (1974)

a detailed metabolic study of the isomeric 1- and 2-naphthylacetic acids in a range of species has been made. This chapter is concerned with a detailed metabolic investigation of radiolabelled 2-naphthylacetic acid in the rat and ferret and comparison of these results with those previously obtained for the 1-isomer. It will be shown that whereas the metabolism of 1-naphthylacetic acid conforms to the 'expected' pattern that of the 2-isomer is surprisingly and remarkably different.

Materials & Methods

Potassium [^{14}C] cyanide, sp. act. 40 mCi/mmol, was purchased from Radiochemical Centre, Amersham, U.K. Proteinase (Type V from *Streptococcus griseus*) and deoxyribonuclease (DNAase) (Grade I from ox pancreas) were purchased from Sigma Chemical Co., Kingston upon Thames, Surrey. Ribonuclease was a gift from Dr. P.F.R. Little, Department of Biochemistry, St. Mary's Hospital Medical School. 2-Naphthylacetic acid, m.p. 140-142°, and 2-bromomethylnaphthalene m.p., 51-54°, were purchased from Aldrich Chemical Co., U.K. Other compounds were purchased and purified as appropriate prior to use.

[Carboxyl-^{14}C] -2-Naphthylacetic acid

2-Bromomethylnaphthalene (1.19g) and K^{14}CN (0.35 g; 3 mCi) dissolved in a mixture of ethanol (20 ml) and water (5 ml) were refluxed for 9h. After removal of the ethanol by evaporation, water (20 ml) was added and the reaction mixture extracted with diethyl ether (6 x 50 ml) until no further radioactivity was extracted. The pooled ether extracts, containing crude 2-naphthylaceto-[^{14}C] nitrile, were combined and dried (anhyd. Na_2SO_4), evaporated to dryness and the residue refluxed with 48% HBr (50 ml) for 3 h. The mixture was then extracted with ether (3 x 50 ml) and the pooled extracts re-extracted with 0.1 M NaOH (75 ml). The alkaline extract was acidified (pH 1) with conc. HCl, extracted with diethyl ether (3 x 50 ml), and these extracts combined and re-extracted with 0.1 M NaOH (75 ml). This procedure was repeated. The [carboxyl-^{14}C]-2-naphthylacetic acid was then precipitated by the cautious addition

of conc. HCl to the NaOH extract, filtered and recryst. from aq. ethanol, m.p. 143°, sp. act. 3.42 μCi/mg. This material gave a single radioactive spot with the same R_F as authentic 2-naphthylacetic acid when subjected to t.l.c. in systems A-D (Table 2.3) and was shown by reverse isotope dilution to have a radiochemical purity of 99%.

Amino acid conjugates of 2-naphthylacetic acid

The synthesis of the amino acid conjugates of 2-naphthylacetic acid was carried out in two stages, involving the preparation of 2-naphthylacetyl chloride and linking this to the amino acid by the Schotten-Baumann reaction.

2-Naphthylacetylchloride

2-Naphthylacetic acid (100 g) was dissolved in benzene (100 ml), thionyl chloride (66.6 g) added and the mixture refluxed for 4 h. The solvent and excess thionyl chloride were distilled off *in vacuo* and on cooling the residue of 2-naphthylacetylchloride solidified and was used without further purification.

2-Naphthylacetylglycine

Sodium bicarbonate (15 g) and glycine(3.75 g) were dissolved in water (50 ml) and stirred thoroughly while 2-naphthylacetylchloride (20.4 g) was added slowly in portions of 2 g. The mixture was stirred for 8 h at room temperature, filtered, and the filtrate adjusted to pH 2 with 10 M - HCl. The precipitate so formed was filtered, washed several times with ether to remove any free acid present, and recrystallised

from aqueous ethanol to give white crystals of 2-naphthylacetylglycine with m.p. 145-146°. Equivalent weight by titration (0.1 M-NaOH) 245 (requires 243). $C_{14}H_{13}O_3N$ requires C, 69.1; H, 5.4; and N, 5.8; found C, 68.8; H, 5.5; and N, 5.7. The mass spectrum of the methyl ester showed molecular ion at m/e 257 (rel. intens. 6%) with prominent ions at m/e 225 (5%, loss of $-OCH_3$), 168 (35%) 141 (40%) and 115 (10%).

2-Naphthylacetyl-L-glutamine

L-Glutamine (6 g) and sodium bicarbonate (10 g) were dissolved in water (50 ml) and stirred during the addition of 2-naphthylacetyl chloride (16.4 g) in 2 g portions. The mixture was stirred for 10 h at room temperature, filtered, and the filtrate adjusted to pH 2 with 10 M-HCl. The precipitate so obtained was washed with ether (5 x 50 ml) to remove any free acid present, and the 2-naphthylacetyl-L-glutamine was then recrystallised from water, m.p. 160-161°. Equivalent weight by titration (0.1 M-NaOH) 315 (requires 316). $C_{17}H_{18}O_4N_2$ requires C, 64.9; H, 5.7; and N, 8.9, Found C: 64.6; H, 5.8; and N, 9.0. The mass spectrum of 2-naphthylacetyl-L-glutamine methyl ester showed a small molecular ion at m/e 328 (rel. intens. 0.5%) with ions at 296 (3%, loss of $-OCH_3$), 163 (38%), 141 (47%) and 115 (33%).

2-Naphthylacetyltaurine

2-Naphthylacetyl chloride (17.4 g) was added in small portions over a period of 4 h to a well-stirred ice-cold

solution of taurine (9.2 g) dissolved in 1 M-NaOH (82 ml) and the reaction mixture left overnight. The mixture was adjusted to pH 2 with 2 M-HCl and extracted with ether (3 x 50 ml) to remove the free acid. The aqueous layer was reduced to dryness on the rotary evaporator, the residue dissolved in methanol and acetone added until crystals began to form. These were filtered, and recrystallised from methanol: acetone (2:1) to give the sodium salt of 2-naphthyl-acetyltaurine as small white crystals of its hemihydrate, m.p. 293-294°. The crystals gave a positive sodium flame test and did not effervesce when treated with sodium bicarbonate. $C_{14}H_{14}NO_4SNa \frac{1}{2} H_2O$ requires C,51.9; H, 4.3; N, 4.3; S, 9.9, and Na 7.1 Found C, 52.2; H, 4.3; N, 4.3; S, 10.0 and Na, 7.2. The mass spectrum of the methyl ester did not show a molecular ion but contained ions diagnostic of taurine conjugates at m/e 185 (rel. intens. 5%); 167 (1%) 141 (13%) and 115 (4%) (see Idle et al. 1978). The infra-red spectrum (KBr disc) showed prominent absorption bands at 3260 cm^{-1} (N-H Stretch), 1640 (amide I, C=O stretch) 1555 (amide II, C-N stretch), 1220 - 1165 (broad) and 1065 1020 (broad), both due to S=O stretch.

Mass spectrometry

Mass spectra were obtained using a Varian-MAT CH5 instrument with a Spectro-System 100 data system by direct insertion. The ionising voltage was 70 eV.

Infra-red spectroscopy

A Perkin Elmer 157 G spectrometer was used.

Animals

Female Wistar albino rats, (body weight 150-200 g) and mongrel polecat ferrets of both sexes (body weight 400-800 g) purchased from local suppliers were used. [^{14}C]-2-naphthylacetic acid (100 mg/kg) was administered dissolved in propane-1, 2-diol by intraperitoneal (i.p.) injection.

Collection of excreta

Animals were housed individually in metabolism cages appropriate to their size and urine and faeces were collected at room temperature daily over a period of 6 days. They were allowed free access to water, food being withheld for the first 24 hours to avoid contamination of the urine.

Collection of $^{14}CO_2$ in expired air

Rats dosed with [^{14}C]-2-naphthylacetic acid were placed in Metabowl cages (Jencons Ltd.) which facilitated the collection of expired air as well as urine and faeces. The expired air was drawn by a pump through a drying trap of anhydrous $CaCl_2$ and then through 2 traps each containing 100 ml of ethanolamine-2-methoxyethanol (2:1 v/v) (Jeffay and Alvarez; 1961). The air flow was adjusted so as to prevent condensation inside the Metabowls.

Storage of excreta

All excreta were stored at -20°, without pH adjustment, prior to analysis.

Surgical procedures - bile duct cannulation

Rats were anaesthetised with pentobarbitone sodium (60 mg/kg) by i.p. injection and cannulation of the bile duct was performed as described by Abou-el-Makarem et al. (1967). Following i.p. administration of [^{14}C] -2-naphthylacetic acid, bile was collected at room temperature at hourly intervals for the first 12 hours and thereafter as one fraction from 12-24 hours. The rats were housed in restraining cages, kept warm with a heating lamp and allowed free access to water. Urine was collected in a tray positioned beneath the restraining cage.

In another set of experiments, [^{14}C] -2-naphthylacetic acid was administered intraperitoneally to the rats 24 hours prior to bile duct cannulation as described above and their bile collected for the period 24-48 hours following dosing.

Chromatography

Thin layer chromatography (t.l.c.) of 2-naphthylacetic acid, its conjugates and certain amino acids was performed using plastic-backed Silica gel 60 HF_{254} (Cat. No. 5735, Merck A.G., Darmstadt, W. Germany). For the dansyl derivatives of the amino acids, non-fluorescent Silica gel plates were used (Merck Cat. No. 5748). For preparative chromatography thick layer plates (20 x 20 cm, layer thickness 1 mm) were made from Silica gel HF_{254} type 60 (Merck), and activated by heating at 110^{o} for 1 h prior to use.

In all cases, following application of the compounds, the plates were developed by the ascending technique to 12 cm from the origin in one of the following solvent systems.

A.	Benzene/acetone/glacial acetic acid	2:2:1 by vol.
B.	Chloroform/methanol/glacial acetic acid	24:8:1 by vol.
C.	Ethanol/benzene/glacial acetic acid	5:5:3 by vol.
D.	Chloroform/methanol/glacial acetic acid	15:4:1 by vol.
E.	Ethanol/water	7:3 by vol.
F.	n-Butanol/glacial acetic acid/water	4:1:1 by vol.

Systems A-D were used for chromatography of 2-naphthylacetic acid and its conjugates, system C for the dansyl derivatives of amino acids and systems E and F for amino acids. R_F values and colour reactions of reference compounds are given in Table 2.3.

Detection of compounds on chromatograms

Ultraviolet light

The compounds were seen as dark quenching spots on fluorescent plates and the dansyl amino acids were seen as intensely fluorescent, yellow spots on non-fluorescent plates when viewed under u.v. light at 254 nm (Hanovia Chromatolite).

Naphthoresorcinol spray

Chromatograms were sprayed with naphthoresorcinol in acetone (4% w/v) to which phosphoric acid (10%) was added (4:1, v/v) just before use. Glucuronides showed up as a purple spots on heating at 105° for 5 min.

Table 2.3

Chromatographic properties of 2-naphthylacetic acid, its conjugates and certain amino acids

Compounds	Solvent	R_F value						Appearance beneath u.v.	Colour reactions with	
		A	B	C	D	E	F		Ninhydrin	Isatin
2-Naphthylacetic acid		0.90	0.92	0.95	-	-	-	dark purple	-	-
2-Naphthylacetyltaurine		0.21	0.44	0.32	-	-	-	dark purple	-	-
2-Naphthylacetyl-L-glutamine		0.53	0.69	0.44	-	-	-	dark purple	-	-
2-Naphthylacetylglycine		0.76	0.87	0.77	-	-	-	dark purple	-	-
Dansylglutamic acid		-	-	0.45	-	-	-	intense blue*	-	-
Dansylglycine		-	-	0.54	-	-	-	intense blue*	-	-
Dansyltaurine		-	-	0.32	-	-	-	intense blue*	-	-
Glycine		-	-	-	0.43	-	-		purple	pink
Glutamic acid		-	-	-	0.63	0.24	-		purple	pink
Taurine		0.06	-	0.08	-	0.18	.18		purple	yellow

*These colours were highly fluorescent spots seen on the non-fluorescent plates.

Ninhydrin spray

Ninhydrin (0.2 g) was dissolved in methanol (10 ml), and sprayed on to chromatograms which were then heated with a hot air blower. The three amino acids (taurine, glutamic acid and glycine) showed as purple spots.

Isatin spray

Isatin (0.2 g) was dissolved in methanol (10 ml), and this sprayed on to chromatograms which were then heated with a hot air blower. Taurine gave a yellow spot while glycine and glutamic acid gave a pink colour.

Radiochemical techniques

The ^{14}C content of urine, bile and faeces was determined by liquid scintillation counting with Packard TriCarb liquid scintillation spectrometers (Models 3320 and 3385) using a Triton X-100-based scintillation cocktail (Triton X-100 3.33 litres, toluene 6.67 litres, PPO, 5.5 g POPOP 1 g). Counting efficiency was determined by the channels ratio method.

Rat faeces were homogenised with 2 volumes of water using an Ultra-Turrax homogeniser. An aliquot (20 ml) of the homogenate was made alkaline with 10 M NaOH (2 ml) and an equal volume of H_2O_2 solution (100 vol. %) added, along with few drops of iso-octanol to control foaming. The mixture was kept at room temperature until decolourised (generally 48 h), heated for 1 h at 60° to remove excess H_2O_2 and aliquots assayed for ^{14}C as above(Caldwell et al., 1972).

Ferret faeces were homogenised in methanol and the methanol filtered to remove undigested food particles and hair from their food. Aliquots (1.0 ml) of the filtrate were counted direct without bleaching as described above.

Reverse isotope dilution for 2-naphthylacetic acid and its amino acid conjugates

For 2-naphthylacetic acid and its glycine and glutamine conjugates 1 g of the appropriate acid, in aq. $NaHCO_3$, was added to urine samples containing 0.5-1 μCi of ^{14}C. The urine was adjusted to pH 1 with 2 M HCl and the precipitate so formed was filtered and recrystallised from the appropriate solvent (see syntheses above) to constant specific radioactivity and melting point. 2-Naphthylacetyl-glucuronide was estimated by difference following its conversion to 2-naphthylacetic acid by mild alkaline hydrolysis (see below). The difference between 'free' and 'total' naphthylacetic acid is a measure of the glucuronic acid conjugate. For 2-naphthylacetyltaurine, 1 g of the Na salt of the conjugate was added to urine and the whole reduced to dryness on the rotary evaporator. The residue was extracted with hot methanol (5 ml), filtered and the methanol extract treated with acetone (3 ml). The white crystals that separated were filtered and recrystallised from methanol-acetone (2:1, v/v) to constant specific radioactivity and melting point.

Treatment of urine and bile samples

Urine and bile were examined by t.l.c. as such and

following mild alkaline hydrolysis or treatment with β-glucuronidase.

Alkaline hydrolysis

To urine (1 ml) was added M-NaOH (1 ml) and the mixture heated at 90° for 15 min. The mixture was neutralised with 2 M - HCl prior to t.l.c.

β-Glucuronidase treatment

Aliquots (1 ml) of urine or bile were adjusted to pH 5 with glacial acetic acid and incubated with β-glucuronidase (Ketodase, General Diagnostics Co., Eastleigh, Hants) (1 ml) at 37°C for 18 h. To another tube containing the above was added the specific inhibitor saccharo-1, 4-lactone (1×10^{-4}M) to show that any hydrolysis occurring was due to β-glucuronidase. As a control, and in order to establish the activity of the enzyme, a tube was included containing phenolphthalein glucuronide (1 mg) dissolved in a sample (1 ml) of urine. At the end of the experiment this tube was made alkaline with M - NaOH, and the appearance of a deep purple colour showed that the enzyme was active.

Isolation of the amino acid metabolites of 2-naphthylacetic acid

Urine samples (5 ml) following mild alkaline hydrolysis were streaked on thick layer plates which were developed in Solvent A. Successive 1 cm x 1 cm sections were scraped off the middle of the plate and counted for ^{14}C. A histogram was plotted of ^{14}C against chromatographic mobility and the

areas of the plate containing the ^{14}C-metabolites scraped off and eluted with methanol (20 ml) which was subsequently removed by evaporation, the residues taken up in methanol (2 ml) and divided into portions, treated as below.

Acid hydrolysis

Aliquots (0.5 ml) of the above methanol solutions were taken, the methanol removed and the residue dissolved in 11.6 M HCl (2 ml). This was heated in a sealed tube for 24 h at 110°. After cooling the tubes were opened and dried over NaOH in a vacuum dessicator. The residue was dissolved in water, neutralised with M-NaOH and examined by t.l.c. Other samples were carried through this procedure and the final solution dansylated prior to t.l.c. on non-fluorescent plates in Solvent C.

Dansylation of amino acids

Small samples (500 mg) of glycine, glutamic acid and taurine in water (0.5 ml) were treated with a solution of dansyl (5-dimethylamino-1-naphthalenesulphonyl) chloride (3 mM in acetone, 0.1 ml), left at room temperature for 4 h and examined by t.l.c. on non-fluorescent plates in Solvent C.

Tissue distribution of [^{14}C] -2-naphthylacetic acid in the rat and ferret

[^{14}C]-2-naphthylacetic acid (100 mg/kg i.p.) was administered to rats and ferrets, and 24 h later they were sacrificed by cervical dislocation. Residual radioactivity in the peritoneal cavity was obtained by injecting water

in the peritoneal cavity, and this was recovered in the course of dissection and its ^{14}C content determined. The tissues were dissected, homogenised in 3 vol. water and their ^{14}C content estimated after bleaching as described previously for faeces. The carcass was dissolved in 3 volumes ethanolic sodium hydroxide (10% w/v). Aliquots were neutralised with M HCl and their ^{14}C content estimated by liquid scintillation counting.

Preparation of liver subcellular fractions

Female Wistar albino rats were dosed intraperitoneally with [^{14}C]-2-naphthylacetic acid (100 mg/kg) and sacrificed 3 h later. Their livers were removed, washed in ice-cold water, weighed and minced in 3 volumes of ice-cold 0.25 M sucrose. This was then homogenized by hand in a Teflon-glass homogenizer. The homogenate was centrifuged at 24,000 x g at 4° for 10 mins (Sorvall MSE Model RC58). This procedure sediments nuclei, unbroken liver cells, red blood cells, and mitochondria while the supernatant contains the microsomes and the soluble fraction.

Isolation of nuclei

The pellet obtained in the 24,000 x g centrifugation was resuspended in the original volume of 0.25 M ice-cold sucrose solution, this layered over an equal volume of 0.34 M sucrose and centrifuged for 10 min at 4°. The pellet, which contained the nuclei was resuspended in ice-cold 0.25 M sucrose and the above procedure repeated five times. The final nuclear

pellet was resuspended in the original volume of isotonic KCl and stored at 4°c.

Isolation of mitochondria

The supernatant from the nuclear fraction was centrifuged for 10 min at 5000 x g at 4°. The supernatant and the fluffy top of the pellet were removed, the pellet resuspended in 0.25 M sucrose which was centrifuged at 5000 x g for 10 min. at 4°. This procedure was repeated three times and the final mitochondrial pellet was resuspended in the original volume of isotonic KCl and stored at 4°.

Isolation of microsomes

The original 24,000 x g supernatant and the 5000 x g supernatant from the mitochondrial isolation were combined and centrifuged for 60 min at 100,000 x g at 4°. The fatty material at the top was discarded and the soluble fraction (supernatant) was removed, the pellet resuspended in isotonic KCl, and this was then centrifuged at 100,000 x g for 60 min. at 4°. The microsomal pellet so obtained was finally resuspended in the original volume of isotonic KCl and stored at 4°.

Determination of binding to subcellular fractions

This was carried out according to Jollow et al. (1973). To each subcellular fraction (1 ml) was added 0.9 M trichloroacetic acid (2 ml) and after mixing this was

centrifuged for 15 min. at room temperature at 1000 x g. The supernatant was removed, and its ^{14}C content determined. The pellet was resuspended in 80% methanol in water (3 ml) on a vortex mixer and centrifuged as before. The supernatant was removed and counted for ^{14}C. This procedure was repeated until no more ^{14}C could be extracted recovered into methanol. The final pellet was dissolved in M - NaOH and counted for ^{14}C after neutralization with 6 M - HCl.

Isolation of DNA from the nuclear fraction

The nuclear fraction of rat liver obtained as described was added to a mixture of 0.15M sodium chloride and 0.1M ethylene-diamine tetraacetic acid (pH 10.5) (20 ml) to the above mixture was added 20% vol/vol sodium dodecyl sulphate (SDS) (20 ml) and the mixture shaken gently until a homogeneous solution was obtained.

The solution was centrifuged at room temperature at 1500 x g for 20 min. and the organic phase discarded. To the aqueous phase was added redistilled phenol in EDTA, water, SDS shaken and centrifuged and the organic phase discarded. This procedure was repeated 3 times. A mixture of chloroform/octan-2-ol (24:1 v/v) (20 ml) was added to the final aqueous phase and shaken until a homogeneous mixture was obtained. The mixture was centrifuged at 2,000 x g for 10 min. at room temperature, and the organic phase discarded. The procedure was repeated twice. 2 volumes of 100% ethanol were added to the remaining aqueous phase to precipitate the deoxyribonucleic acid (DNA), which was

harvested by centrifugation.

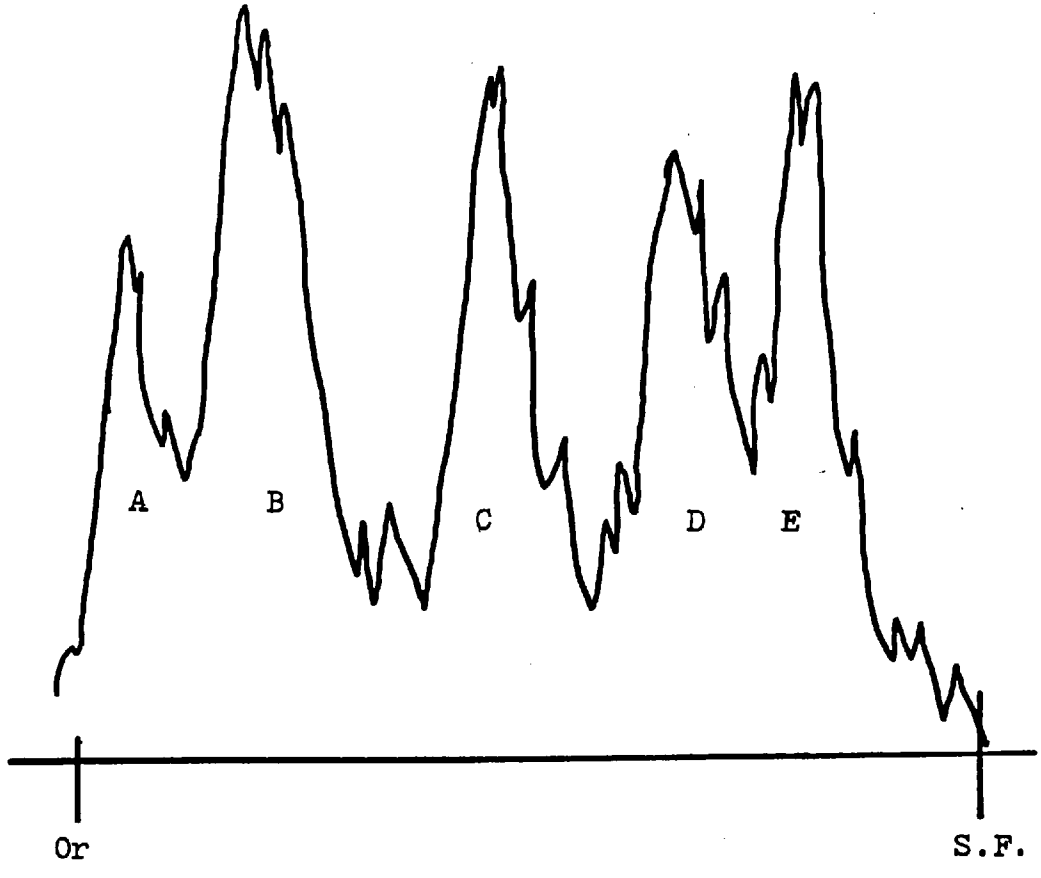
Chromatographic identification of urinary metabolites

Aliquots (0.1 ml) containing 0.5 µCi of the 0-24 h urine of rats and ferrets dosed with [^{14}C] -2-naphthylacetic acid were examined by t.l.c. in solvent A. Scanning for ^{14}C revealed five peaks with R_F values of 0.10, 0.22, 0.56, 0.78 and 0.90 (Fig 2.1). The peak R_F 0.10 gave a positive naphthoresorcinol reaction and disappeared when urine samples were treated with β-glucuronidase or M-NaOH (Fig 2.2), this disappearance not occurring when urine was incubated with β-glucuronidase and saccharo-1, 4-lactone. Its disappearance was accompanied by an increase in the size of the peak at R_F 0.92 which corresponded in R_F to 2-naphthylacetic acid. The peaks with R_F values of 0.22, 0.56, and 0.78 corresponded to 2-naphthylacetyl-taurine, -glutamine and -glycine respectively. Chromatograms of the urines developed in solvent B were also scanned for ^{14}C and this also revealed five peaks. The peak at R_F 0.07, which gave a blue colour with naphthoresorcinol disappeared when the urine was treated with β-glucuronidase or M-NaOH as described above, with an increase in the size of the peak at R_F 0.93, which corresponded to 2-naphthylacetic acid. The peaks with R_F values of 0.42, 0.69, and 0.84 correspond to 2-naphthylacetyl-taurine, -glutamine and -glycine respectively.

Larger volumes of urine (2-3 ml) were treated with M - NaOH, subjected to preparative t.l.c. on thick layer

Fig. 2.1

Radiochromatogram scan of urine of rat dosed with [^{14}C]-2-naphthylacetic acid (100 mg/kg, i.p., 16 μCi/kg) developed in Solvent A.



A = 2-Naphthylacetylglucuronide

B = 2-Naphthylacetyltaurine

C = 2-Naphthylacetylglutamine

D = 2-Naphthylacetylglycine

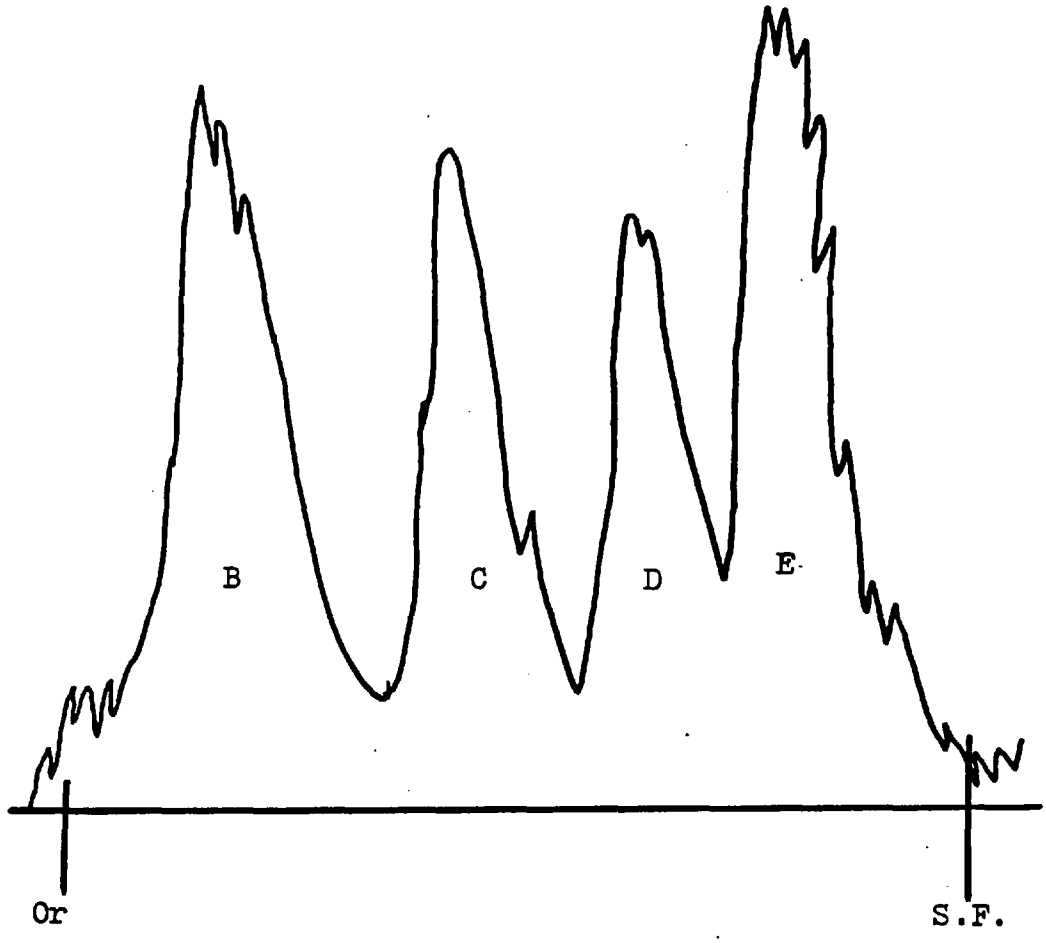
E = 2-Naphthylacetic acid.

Or = Origin

S.F. = Solvent Front.

Fig 2.2

Radiochromatogram scan of β-glucuronidase treated urine of rat dosed with [^{14}C]-2-naphthylacetic acid (100 mg/kg, i.p. 16 μCi/kg) developed in Solvent A.



B = 2-Naphthylacetyltaurine

C = 2-Naphthylacetylglutamine

D = 2-Naphthylacetylglycine

E = 2-Naphthylacetic acid.

Or = Origin

S.F. = Solvent Front.

plates, and ^{14}C bands located as described. Four ^{14}C peaks were found with R_F values of 0.32, 0.44, 0.77 and 0.95, which correspond to 2-naphthylacetyl-taurine, -glutamine and -glycine and 2-naphthylacetic acid respectively. The areas of silica gel containing each of the amino acid conjugates were scraped off the plates, eluted with methanol, and the conjugates hydrolysed as described. The final residue was examined in three ways:

i. t.l.c. in systems A and B, followed by radiochromatogram scanning.

ii. t.l.c. in systems D and E, followed by spraying with ninhydrin and isatin.

iii. dansylation followed by t.l.c. in system solvent C.

The area with R_F 0.32 solvent C on hydrolysis gave (1) single ^{14}C peaks in solvents A and B with R_F values 0.91 and 0.92 respectively, corresponding to 2-naphthylacetic acid, (2) a spot of R_F 0.18 in solvent E, which gave a purple colour with ninhydrin and a yellow colour with isatin, which corresponded to taurine. (3) t.l.c. in solvent C of the hydrolysate after dansylation showed an intensely fluorescent spot of R_F 0.33 under u.v. light, which corresponded to dansyltaurine. This peak of R_F 0.32 in solvent C was therefore assigned to 2-naphthylacetyl-taurine. Similar treatment of the hydrolysates from the area of R_F 0.44 in solvent C showed (1) ^{14}C peaks corresponding to 2-naphthylacetic acid as above (2) spots

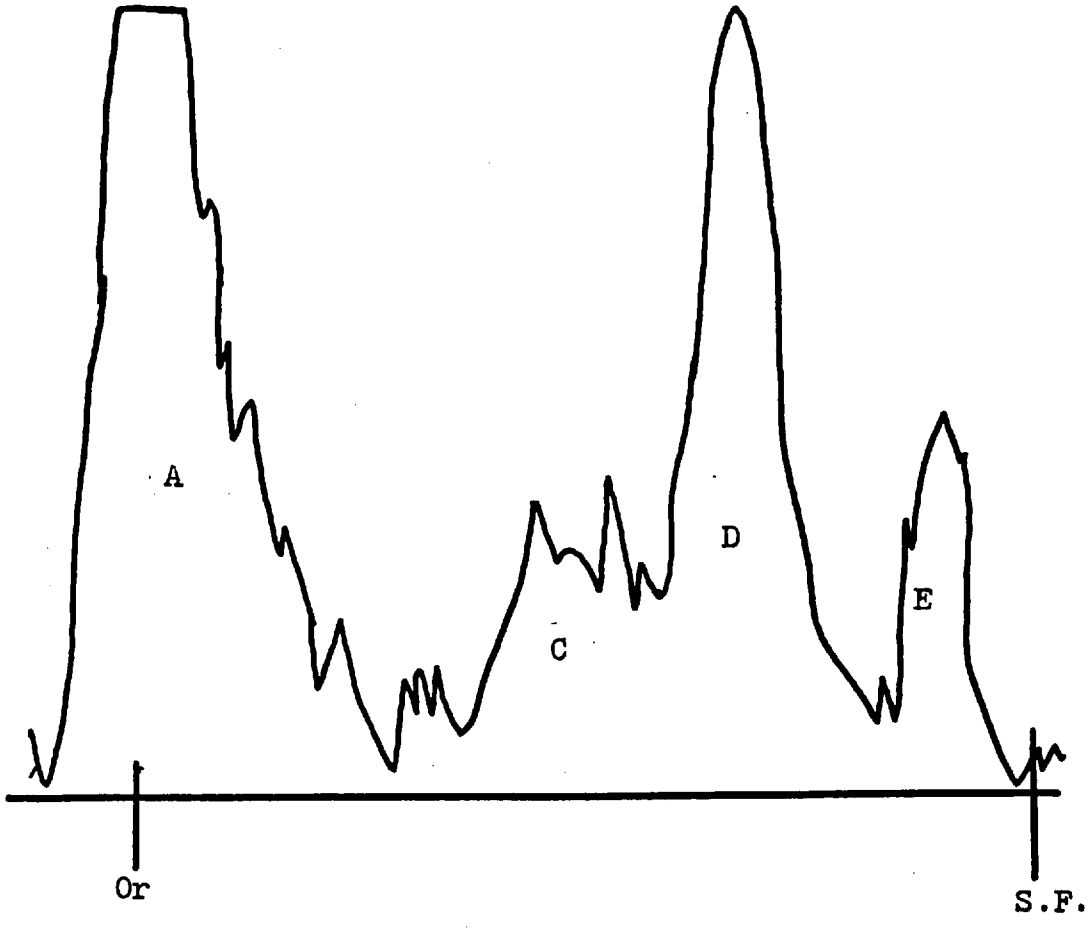
of R_F 0.63 and 0.24 in solvents E and F, which gave a purple colour with ninhydrin and pink with isatin, which corresponded with glutamic acid, (3) t.l.c. in solvent C of the hydrolysate after dansylation showed under u.v. light an intensely fluorescent spot with R_F 0.45, which corresponded to dansylglutamic acid. This peak was therefore assigned to 2-naphthylacetyl-glutamine or -glutamic acid, both of which would give rise to 2-naphthylacetic acid and glutamic acid on hydrolysis, but was confirmed as 2-naphthylacetylglutamine by reverse isotope dilution. The hydrolysate of the area with R_F 0.77 in solvent C was examined as described above and showed (1) ^{14}C peaks corresponding to 2-naphthylacetic acid as above, (2) a spot with R_F 0.40 in solvent E, which gave purple and pink colours on spraying with ninhydrin and isatin respectively, (3) after dansylation, t.l.c. in solvent C showed under u.v. light a highly fluorescent spot with R_F 0.55, which corresponded to dansylglycine. This peak was therefore assigned to 2-naphthylacetylglycine.

Chromatographic identification of biliary metabolites in the rat

Aliquots (0.1 ml) of the bile of rats dosed with [^{14}C] - 2-naphthylacetic acid was examined by t.l.c. in solvent system A. The chromatograms were scanned for ^{14}C, and four peaks were observed, with R_F values of 0.09, 0.54, 0.76 and 0.91. (Fig.2.3). The peak at R_F 0.09 disappeared when the bile was treated with β-glucuronidase or M-NaOH, with a

Fig. 2.3

Radiochromatogram scan of the bile of a rat dosed with [^{14}C]-2-naphthylacetic acid (100 mg/kg, i.p., 16 μCi/kg) developed in Solvent A.



A = 2-Naphthylacetylglucuronide

C = 2-Naphthylacetylglutamine

D = 2-Naphthylacetylglycine

E = 2-Naphthylacetic acid.

Or = Origin

S.F. = Solvent Front

corresponding increase in the peak at R_F 0.91 which corresponded to 2-naphthylacetic acid. The peak at R_F 0.09 gave a blue colour with naphthoresorcinol and was identified as 2-naphthylacetylglucuronide. The peaks at R_F 0.54 and 0.76 were corresponded in R_F to 2-naphthylacetyl-glutamine and glycine respectively.

Radiochromatogram scanning of chromatograms of bile similarly prepared in solvent system C showed four peaks with R_F values of 0.06, 0.44, 0.77 and 0.95. The peak at R_F 0.06 was identified as 2-naphthylacetyl glucuronide while those with R_F values 0.44, 0.77 and 0.95 corresponded in R_F to 2-naphthylacetyl-glutamine -glycine and 2-naphthyl-acetic acid respectively.

Results

The excretion of ^{14}C in the urine and faeces of rats and ferrets following the intraperitoneal injection of [^{14}C] -2-naphthylacetic acid is shown in Table 2.4. Over the six day period only a partial recovery (rat 60%, ferret 63% of the dose) was achieved. Most of the ^{14}C was excreted in the 0-24 h urine with progressively smaller amounts on succeeding days. The bulk of faecal ^{14}C excretion occurred in the first 3 days.

In separate experiments, less than 0.1% of the dose was found as $^{14}CO_2$ in the expired air of rats in five days after the administration of [^{14}C] -2-naphthylacetic acid.

The urinary metabolites of [^{14}C] -2-naphthylacetic

Table 2.4

The elimination of ^{14}C by the rat and ferret after intraperitoneal administration of $[^{14}C]$-2-naphthylacetic acid

$[^{14}C]$-2-Naphthylacetic acid was dissolved in propane-1, 2-diol and administered to animals as described. Values are means of 3 animals with ranges in parentheses

Species	Rat	Ferret
Urine	% ^{14}C dose excreted in	
Day 0 - 1	35.4(28.4-41.9)	29.8(27,0-32.4)
1 - 2	8.2((2.8-19.1)	6.0(5.7-6.4)
2 - 3	1.6(0.7-2.3)	3.2(1.7-4.4)
3 - 4	0.8(0.5-1.2)	2.6(2.5-2.8)
4 - 6	0.9(0.5-1.6)	
Total in urine	46.9(40.5-52.6)	41.6(39.6-47.0)
Faeces		
Day 0 - 3	11.7(6.2-18.1)	13.3(11.7-15.9)
3 - 6	1.2(0.5-2.6)	8.0(6.8-9.4)
Total in faeces	12.9(6.8-18.6)	21.3(20.3-22.7)
Total ^{14}C recovered in urine and faeces	59.8(54.5-59.1)	62.9(61.2-67.4)

acid were identified by comparison of their chromatographic properties with those of authentic samples, colour reactions, dansylation and reverse isotope dilution. In addition to unchanged 2-naphthylacetic acid, four conjugates were detected, namely, its glucuronic acid, glycine, glutamine and taurine conjugates and their identities confirmed by reverse isotope dilution. Quantitative data are presented in Table 2.5. The major urinary metabolite was 2-naphthylacetyltaurine (10% of dose in rat and 15% in ferret) with the other conjugates present in smaller amounts. The free acid was also excreted in the urine.

The elimination of ^{14}C in the bile after the administration of [^{14}C]-2-naphthylacetic acid to bile duct cannulated rats is shown in Table 2.6 Over 24 h period, some 59% of the dose was excreted in the bile with a further 16% in the urine. Most of the ^{14}C in the bile was recovered in the first 10 h after administration with a much smaller amount excreted in last 14 h.

Analysis of the bile with the techniques already described revealed the presence of four radioactive excretion products which were identified as 2-naphthylacetyl-glucuronide, -glutamine, -glycine and 2-naphthylacetic acid, (Table 2.7), the major biliary metabolite being 2-naphthylacetylglucuronide. The urine volume was too small to permit analysis. In other experiments, rats were dosed with [^{14}C]-2-naphthylacetic acid by intraperitoneal injection (100 mg/kg) 24 h prior to cannulation of the common bile duct, and their bile collected for 24 h, that is, 24-48 h after dosing.

Table 2.5

Urinary Metabolites of [^{14}C]-2-Naphthylacetic Acid in Rat and Ferret

[^{14}C]-2-Naphthylacetic acid (100 mg/kg; 16 μCi/kg) was administered to the animals and their excreta collected. The 0-24 h urine was analysed for metabolites as described in the text. Results quoted are the means of at least 3 animals with ranges in parentheses

Compound	% dose excreted in that form by Rat	Ferret
2-Naphthylacetic acid	5.2(2.1-7.8)	1.4(1.1-1.5)
2-Naphthylacetylglycine	8.6(7.9-9.6)	5.4(4.9-5.7)
2-Naphthylacetyltaurine	10.2(8.8-12.8)	14.7(14.2-15.0)
2-Naphthylacetylglutamine	5.7(5.1-6.2)	6.3(5.5-7.1)
2-Naphthylacetylglucuronide	5.6(4.9-6.2)	5.4(5.2-5.5)
Total of above metabolites	35.3(28.2-41.9)	33.2(31.0-35.5)
Total ^{14}C excreted in 0-24 h urine	35.4(28.4-41.9)	33.4(31.1-35.6)

Table 2.6

Biliary Excretion of [^{14}C]-2-Naphthylacetic Acid in the Rat

Bile duct-cannulated rats were dose intraperitoneally with [^{14}C]-2-naphthylacetic acid and bile and urine collected for 24 h. Results quoted are the means of three animals with ranges in parentheses

		% Dose excreted
Bile	0-1h	10.1(5.2-12.6)
	1-2	8.1(5.1-9.6)
	2-3	8.3(6.4-9.6)
	3-4	7.9(4.1-11.2)
	4-5	5.6(3.5-7.4)
	5-10	14.9(10.5-22.6)
	10-24	3.2(1.8-4.5)
	Total	58.1(47.6-65.2)
Urine	0-24h	15.8(13.9-25.7)
Total	Urine + bile	73.9(61.5-90.9)

Table 2.7

Biliary Metabolites of [^{14}C] -2-Naphthylacetic Acid in Rat.

Bile-duct cannulated rats were injected i.p. with [^{14}C]-2-naphthylacetic acid (100 mg/kg; 16 µCi/kg) and the bile and urine collected for 24 h and analysed for ^{14}C and metabolites as described in the text. Figures represent the means of 3 animals with ranges in parentheses

	% dose excreted in that form in	
Compound	Urine	Bile
2-Naphthylacetic acid	-	4.3(2.4-5.9)
2-Naphthylacetylglycine	-	11.8(5.9-13.5)
2-Naphthylacetyltaurine	-	n.d.
2-Naphthylacetylglutamine	-	5.0(2.8-5.3)
2-Naphthylacetyl glucuronide	-	37.1(27.7-42.5)
Total of above metabolites	-	58.2(43.1-65.0)
Total ^{14}C excreted in 0-24h	15.8(7.9-25.7)	58.2(43.0-66.2)

N.D. = Not detected.

These animals excreted 2.0% (1.9-2.9%) of the administered dose in the bile during this period, the nature of which was not investigated.

Tissue Distribution

The tissue distribution of ^{14}C 24 h after the intraperitoneal injection of [^{14}C]-2-naphthylacetic acid to rats and ferrets is shown in Table 2.8. In both species some 94% of the dose was accounted for, the bulk being present in the tissues of the animal. The levels of ^{14}C in the liver, heart, lung, brain, kidney and gut wall were very similar in both rat and ferret. The main difference between the two species was in the ^{14}C content in the gut contents and carcass. Some 54% of the dose was present in the gut contents of the rat compared with 6% in the ferret while on the other hand, the rat carcass contained 15% of the dose compared to 47% in the ferret. The remainder of the ^{14}C was excreted in the 0-24 h urine.

The results of the tissue distribution studies suggested that 2-naphthylacetic acid has a high affinity for the tissues, and this was studied in more detail in the liver.

Table 2.9 shows the subcellular distribution of ^{14}C in rat liver after the administration of [^{14}C]-2-naphthylacetic acid. Of the total liver ^{14}C content, the bulk was present in the cytosol, with smaller amounts present in the nuclei, microsomes and mitochondria. When

Table 2.8

Tissue distribution of ^{14}C in rat and ferret 24h after the i.p. injection of [^{14}C]-2-naphthylacetic acid

Rats and ferrets were injected with [^{14}C]-2-naphthylacetic acid (100 mg/kg i.p.), kept in metabolism cages for 24h and then killed. After dissection, the ^{14}C content of each organ was determined as described in the text. Values given are means of 3 rats with ranges in parentheses and of 2 ferrets with individual values in parentheses

	Rat		Ferret	
Organ	% dose in organ	% dose/g tissue	% dose in organ	% dose/ tissue
Liver	0.8(0.7-1.1)	0.12	2.1(2.1,2.2)	0.13
Heart	0.1(0.05-0.11)	0.13	0.1(0.1,0.1)	0.04
Lung	0.1(0.08-0.1)	0.09	0.2(0.2,0.2)	0.05
Brain	0.16(0.13-0.18)	0.12	0.1(0.1,0.1)	0.02
Kidney	0.23(0.17-0.3)	0.15	0.34(0.34,0.35)	0.14
Gut wall	7.6(6.1-9.1)	0.63	3.0(3.0,3.0)	0.10
Gut contents	53.9(50.9-55.4)	-	3.7(3.6,3.7)	-
Carcass	15.0(14.2-16.0)	0.10	47.2(45.1,49.3)	0.12
Blood*	n.d.	-	4.5(4.3,4.7)	-
Urine (0-24h)	16.1(15.1-16.9)	-	32.8(32.0,33.5)	-
Total	94.0(90.1-98.2)	-	94.0(90.9,97.1)	-

*Blood volume taken as 8% of body weight.

n.d. = not determined.

Table 2.9

Subcellular distribution of ^{14}C in the liver of rats given [^{14}C]-2-naphthylacetic acid (100 mg/kg) i.p. 3 h earlier, before and after TCA/methanol treatment.

Figures shown represent the means of three animals, with ranges in parentheses.

Fraction	% Dose	% Dose left after TCA/methanol treatment
Nuclei	1.40(1.02-1.80)	0.5(0.3-0.6)
Mitochrondria	0.27(0.20-0.30)	0.02(0.015-0.0025)
Microsomes	1.11(0.70-1.10)	0.2(0.10-0.3)
Cytosol	4.00(2.92-4.80)	0.3(0.2-0.3)
Total	6.79(4.84-8.00)	1.02(0.61-1.22)

the nature of the binding of ^{14}C to the various subcellular fractions was investigated by the TCA/methanol procedure, it was seen that the ^{14}C was only weakly associated with the mitochondria, microsomes and cytosol, but some 38% of the nuclear ^{14}C was not removed by this method. This suggests that ^{14}C is very strongly bound to some component of the nuclei, and this was examined further.

The nuclear fraction was subjected to sonication, and after this incubated with proteinase and DNAase, finally being subjected to the TCA/methanol extraction. It is clear from Table 2.10 that of these treatments only DNAase releases the strongly bound ^{14}C from the nuclei. The DNA present was therefore isolated by the procedure described earlier, and the ^{14}C content of the DNA-enriched phase determined at each stage. Table 2.11 shows that the ^{14}C remained closely associated with the DNA up to the chloroform-octanol extraction, at which point 89% of the original ^{14}C was still present in the DNA-enriched phase. However, when the DNA was precipitated with ethanol, the ^{14}C was entirely lost from the DNA.

The ethanol phase was evaporated and after dissolving the residue in water subjected to t.l.c. in system A, and, the chromatogram scanned for ^{14}C. Two peaks were observed with R_F 0.10 and 0.92; that of R_F 0.10 gives a blue colour with naphthoresorcinol and disappeared upon treatment with β-glucuronidase with a corresponding increase in the peak of R_F 0.92, which corresponded to 2-naphthylacetic acid. 77% of the ^{14}C present was 2-naphthylacetic acid while its glucuronic acid conjugate accounted for the remaining 23%.

Table 2.10

Effect of enzyme treatment and sonication on the binding of 2-naphthylacetic acid to the nuclear fraction of rat liver.

	% ^{14}C remaining in pellet after TCA/methanol treatment.
Untreated	100
Sonication	94
Sonication + Proteinease	97
Sonication + DNAase	8

Table 2.11

Association of ^{14}C with liver nuclear DNA after administration of [^{14}C]-2-naphthylacetic acid to rats

	% original ^{14}C associated with DNA,
Original Nuclei	100
Sodium dodecyl sulphate	100
Cold phenol	94
Ribonuclease digestion of RNA	92
Chloroform - octanol treatment	89
Ethanol precipitation	2

Discussion

The metabolic fate and elimination of 2-naphthylacetic acid in the ferret and rat will be discussed separately, followed by comparison of the two species and a discussion of differences in the metabolic disposition of the isomeric 1-and 2-naphthylacetic acids.

Rat

After the i.p. injection of 100 mg/kg 2-naphthylacetic acid, rats excreted ^{14}C slowly and incompletely over a 6 day period, with 47% of dose in the urine and 13% in the faeces. The major urinary metabolites were the three amino acid conjugates, namely 2-naphthylacetyl-glycine, -glutamine and-taurine together with smaller amounts of the ester glucuronide and the free acid. The ratio of total amino acid conjugates to the glucuronic acid conjugate was about 5:1, indicating that the rat conjugates 2-naphthylacetic acid preferentially with amino acids.

Consideration of the rat data is however complicated by the fact that in this species 2-naphthylacetic acid undergoes extensive biliary excretion and enterohepatic circulation. This process can obscure the nature of the primary conjugation events since urinary conjugates may be products of secondary metabolic pathways occurring as part of the enterohepatic cycling process. 2-Naphthylacetic acid is extensively excreted in rat bile as its glucuronic acid conjugate and this is not surprising in view of its polarity and mol. wt. (362) (see Smith, 1973). Biliary excretion results in the setting up of a sizeable enteric

pool of the conjugate as shown by the fact that 24 h after the injection of the ^{14}C-labelled acid over 60% of the dose is found in the gut contents and intestinal wall. The major component of the pool is 2-naphthylacetylglucuronide together with small amounts of the glycine and glutamine conjugates which also appear in the bile. It is well known that glucuronic acid conjugates are hydrolysed in the gut by bacterial β-glucuronidase (see Smith & Millburn, 1975). Thus, the 2-naphthylacetylglucuronide will be broken down in the enteric pool liberating the free acid which is then absorbed. The acid may then undergo further amino acid or glucuronic acid conjugation, the conjugates partitioning themselves between urine and bile according to their relative affinities for these routes of elimination. This process will proceed until the acid has been removed from the enterohepatic circulation by urinary excretion. This sequence of events explains why 2-naphthylacetic acid is more rapidly eliminated by bile-duct cannulated rats, mainly as its glucuronide, than by intact animals, which excrete both glucuronic acid and amino acid conjugates more slowly in the urine.

Therefore extensive biliary excretion and enterohepatic circulation contribute to the slow elimination in the first 24 h after which the drug is not excreted appreciably in the bile (2.8% of the dose). The result of the faecal excretion should have reflected the effect of biliary excretion but as very small amount of the acid is detected in the faeces, it suggests that the drug is absorbed by the

gut wall.

The high affinity of 2-naphthylacetic acid for animal tissues may contribute to its slow elimination. Since it has been shown that 24 h post dose only a very small amount of the compound is excreted in the bile, it is reasonable to assume that most of the compound would be excreted within 48 h, but in fact only 60% was eliminated in 6 day period. It therefore appears that the remaining 40% binds to the tissues and this was confirmed by the tissue distribution experiment. Subcellular distribution studies suggest that 2-naphthylacetic acid has a marked affinity to bind to nuclear DNA.

Analysis of rat urine showed in addition to the unchanged acid, four metabolites namely, the glucuronic acid, taurine, glutamine and glycine conjugates, the major metabolite being 2-naphthylacetyltaurine.

The nature of the urinary metabolites present after the administration of 2-naphthylacetic acid to rats was surprising. It would be expected to be conjugated both with glycine and glucuronic acid, but the occurrence of glutamine and taurine conjugation were unexpected. Studies reported in the literature from this department and elsewhere of the metabolism of carboxylic acids have never previously detected the occurrence of glutamine conjugation in the rat. Table 2.12 summarizes these investigations.

This has led to previous authors asserting that glutamine conjugation does not occur in non-primates, being restricted to man and other primate species (Smith & Williams, 1972), but the high level of glutamine conjugation of 2-naphthyl-

Table 2.12

Amino acid conjugation of arylacetic acids in the rat

Compound	% Dose recovered	% Dose conjugated with				Reference
		Glycine	Glucuronic acid	Glutamine	Taurine	
Phenylacetic acid	95	94	-	-	1	2
1-Naphthylacetic acid	64	15	33	-	-	1
2-Naphthylacetic acid	35	9	6	6	10	
Hydratropic acid	81	trace	63	-	-	1
4-Nitrophenylacetic acid	89	69	-	-	-	2
4-Chlorophenylacetic acid	95	92	-	-	-	2
Indo-3-ylacetic acid	76	43	-	-	-	3
Diphenylacetic acid	48	trace	45	-	-	1

1. Dixon et al (1977)
2. James et al (1972)
3. Bridges et al (1974)

acetic acid in the rat shows that non- primates are able to perform this conjugation. The occurrence of taurine conjugation in the rat, while unexpected, is not without precedent, since there is a single previous report of taurine conjugation occurring in the rat (Table 2.12). However, the extensive use of taurine conjugation by the rat for 2-naphthylacetic acid is a finding worthy of note.

Ferret

The ferret also utilises mainly amino acids for the conjugation of 2-naphthylacetic acid rather than glucuronic acid. Thus, the ratio of total urinary amino acid conjugates (glycine, glutamine, taurine) to the glucuronic acid conjugate is about 5:1, suggesting that this acid is preferentially conjugated with amino acids by this species. This would be expected from other work on the fate of related acids in a range of carnivores (Caldwell et al., 1978). It was not possible to determine the biliary excretion of 2-naphthylacetic acid in this species, as bile duct-cannulated animals died soon after injection of the acid. Tissue distribution studies, however, suggested that biliary excretion is not extensive, since 24 h after the injection of the ^{14}C-labelled acid, only 7% of the dose was associated with the gut wall and contents. In contrast, nearly 50% of the dose was associated with the carcass in some, as yet undefined, form.

The major urinary metabolite of 2-naphthylacetic acid in the ferret is 2-naphthylacetyltaurine and smaller

amounts of the glycine and glutamine conjugates and the glucuronide are also formed. It is not surprising that extensive taurine conjugation should occur in the ferret. Studies of Idle et al.(1978) have showed that the ferret has a propensity to conjugate arylacetic acids with taurine, (Table 2.13). The utilisation of glutamine for the conjugation of 2-naphthylacetic acid is also of interest. Only one previous study has shown that the ferret is capable of conjugating arylacetic acids with glutamine (Hirom et al.,1977) (Table 2.13). The use of glutamine by the ferret and rat in the conjugation of this acid suggest that this mechanism maybe more widespread in terms of its species occurrence and not restricted to primates as previously thought.

Comparison of the metabolic fates of the isomeric 1-and 2-naphthylacetic acids

A comparison of the pattern of metabolism and elimination of 2-naphthylacetic acid with its 1-isomer reveals some remarkable differences. 1-Naphthylacetic acid is rapidly eliminated by most species including the rat and ferret as a glucuronic acid conjugate (Dixon et al.,1974, 1977*a*; Idle , 1976). In general amino acid conjugation is at a low level for this compound and when it occurs, glycine and taurine are the amino acids utilized. However, with the 2-isomer, the opposite holds for the rat and ferret where the acid is preferentially conjugated with amino acids rather than with glucuronic acid. This

Table 2.13

Conjugation of arylacetic acids in the ferret

Compound	% Dose recovered	% Dose conjugated with				Reference
		Glycine	Glucuronic acid	Glutamine	Taurine	
Phenylacetic acid	67	42	-	trace	14	1
1-Naphthylacetic acid	50	3	13	-	32	3
2-Naphthylacetic acid	33	5	5	6	15	
4-Nitrophenylacetic acid	22	2	-	-	14	3
4-Chlorophenylacetic acid	87	26	-	-	8	3
Indol-3-ylacetic acid	83	19	-	-	34	2
Diphenylacetic acid	38	-	24	-	-	3

1. James et al (1972)
2. Bridges et al (1974)
3. Idle et al (1978)

preference is not so clear in the rat where the process is complicated by enterohepatic circulation. Thus, for 2-naphthylacetic acid there is in general a shift in the pattern of metabolic conjugation away from one involving glucuronic acid conjugation as occurs for the 1-isomer to one dominantly of amino acid conjugation.

2-Naphthylacetic acid is the first carboxylic acid found to undergo in non-primate species simultaneous conjugation with three different amino acids, namely glycine, glutamine and taurine. This metabolic pattern is relatively uncommon since when amino acid conjugation of an arylacetic acid occurs this is usually with one particular amino acid. The ability of 2-naphthylacetic acid to expose the occurrence of these three amino acid conjugations concurrently suggests that it may usefully be employed as a probe for eliciting the species distribution of these mechanisms.

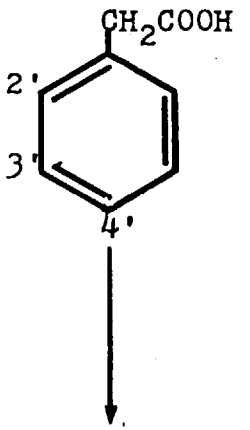
The marked differences in the metabolic conjugation of 1- and 2-naphthylacetic acids must be related in some way to differences in the structure of the two acids. The amino acid conjugation mechanisms appear to be more structure-dependent in terms of substrate requirements than the glucuronic acid conjugation mechanism (Caldwell, 1978). Thus, the glycine conjugation of arylacetic acids is inhibited by the presence of substituents on the α methylene group (Dixon et al., 1975; 1977a,b). Also the extent of glycine conjugation of nuclear substituted benzoic acids

is strongly influenced by the nature and position of the substituent in the ring. In general glycine conjugation is prevented, probably for steric reasons, by the presence of a relatively bulky group in the ortho position to the carboxyl group (Bray et al., 1955; Caldwell, 1978). It is suggested that the greater affinity of the 2-naphthylacetic acid for amino acid conjugation compared with the 1-isomer is related to steric hindrance factors influencing the conjugation. Thus, 1-naphthylacetic acid may be regarded as 2', 3'-benzophenylacetic acid i.e. an arylacetic acid derivative carrying a substituent in the ortho position which might be expected to interfere sterically with the amino acid conjugation. By contrast, 2-naphthylacetic acid may be regarded as 3', 4'-benzophenylacetic acid in which there is no ortho substituent and therefore no steric hindrance factor mitigating against amino acid conjugation. (Fig. 2.4).

Fig.2.4

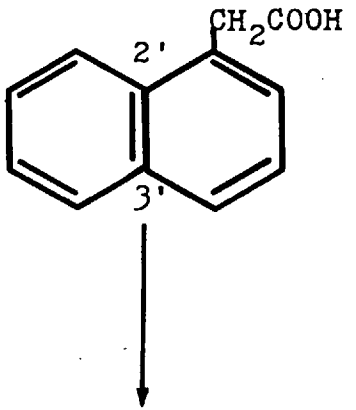
Chemical structure and principal pathways of conjugation of phenylacetic acid and isomeric naphthylacetic acids.

Phenylacetic acid



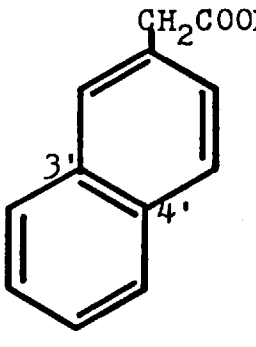
AMINO ACIDS

1-Naphthylacetic acid



GLUCURONIC ACID

2-Naphthylacetic acid



AMINO ACIDS

CHAPTER THREE

COMPARATIVE METABOLIC CONJUGATION OF THE ISOMERIC 1- AND 2-NAPHTHYLACETIC ACIDS IN VARIOUS SPECIES.

CHAPTER THREE

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Introduction

Previous studies on the metabolism of arylacetic acids showed that their metabolic conjugation is generally with glucuronic acid and/or with an amino acid (glycine, taurine and glutamine) depending on the species. Utilisation of glutamine is thought to be restricted to primates while conjugation with taurine, although occurring at random, is particularly well developed in carnivorous species (see James et al.,1972a; Dixon et al.,1977a,b,c, and Idle et al., 1978).

The results described in the previous chapter on the conjugation of 2-naphthylacetic acid have given a new dimension to the predictability of the metabolic conjugation of arylacetic acids. Although it appears that the generalisation concerning the utilisation of glucuronic acid and amino acids for conjugation is still valid, several unexpected features emerged from this study:

(a) the utilisation of glutamine by non-primate species for the conjugation of 2-naphthylacetic acid.

(b) the utilisation of multiple amino acids (glycine, glutamine and taurine) for the conjugation of 2-naphthylacetic acid and

(c) the extensive utilisation of taurine by non-carnivore species.

These new aspects of arylacetic acid conjugation were considered sufficiently unusual as to require further

investigation.

The metabolic behaviour of 2-naphthylacetic acid becomes even more intriguing when compared to the behaviour of its isomer,1-naphthylacetic acid.which shows a predictable pattern of conjugation with glucuronic acid and glycine in non-primate species. The objectives of the present investigations therefore were as follows:

(i) to determine the pattern of metabolic conjugation of 2-naphthylacetic acid in other species viz, guinea pig, mouse, hamster, gerbil and rabbit to see if the "irregular" pattern encountered in the rat and ferret extends to other species.

(ii) to determine the metabolic pattern of 1-naphthylacetic acid in the same species so that a comparison across the species of the metabolic fates of the isomeric naphthylacetic acids can be made.

Materials and Methods

For 2-naphthylacetic acid and [^{14}C]-2-naphthylacetic acid Materials and Methods are described in detail in Chapter Two and therefore only details of additional relevant materials and methods are given here.

[^{14}C]-1-Naphthylacetic acid, sp. act. 323 μCi/mg; radiochemical purity 99% was purchased from Radiochemical Centre Amersham U.K. 1-Naphthylacetic acid was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. and purified prior to use (m.p. 131°). 1-Naphthylacetyl-glycine, -glutamine and -taurine were samples previously synthesized in this laboratory (Dixon et al., 1977a)

Animals

Female Dunkin-Hartley guinea pigs (body wt. 200-500g), golden hamsters (body wt. 70-80g), gerbils (body wt. 60-90g), T.O. strain mice (body wt. 15-20g) and Dutch rabbits (body wt. 2.1-2.5 kg) were purchased from local suppliers.

Administration of compounds

[^{14}C]-1-Naphthylacetic acid (100 mg/kg; 16 μCi/kg) (dissolved in aqueous 0.1 M sodium bicarbonate) was administered intraperitoneally while [^{14}C]-2-naphthylacetic acid was administered to rabbitsorally dissolved in propane-1, 2-diol.

Collection of excreta

Animals were housed individually in metabolism cages appropriate to their size with the exception of mice which were housed in groups of five. Urine and faeces were

collected at room temperature daily over a period of 1-6 days post dosing. They were allowed free access to water food being withheld for the first 24 hours after dosing to avoid contamination of the urine.

Reverse isotope dilution for 1-and 2-naphthylacetic acids and their conjugates

Reverse isotope dilution for 1-naphthylacetic acid, 1-naphthylacetyl-glycine, -glutamine, and -taurine was carried out as described by Dixon et al. (1977a) while for 2-naphthylacetic acid and its conjugates, this was performed as previously described (Chapter Two).

Chromatography

Thin-layer chromatography (t.l.c.) of 1- and 2-naphthylacetic acids and their conjugates was performed using plastic backed Silica gel 60 HF_{254} (Cat. No. 5735, Merck A.G. Darmstadt, W. Germany). In all cases, following application of compounds, the plates were developed by the ascending technique to 12 cm from the origin using the solvent systems described in Chapter 2 and in Table 3.1. Table 3.1 shows the chromatographic properties of 1-naphthylacetic acid and its conjugates while those of 2-naphthylacetic acid and conjugates were presented in Chapter Two (Table 2.3).

Identification of urinary metabolites of 2-naphthylacetic acid

Guinea Pig

Samples of the 0-24 h urine (0.1 ml) were subjected to

Table 3.1

Chromatographic properties of 1-naphthylacetic acid and its conjugates.

Compound	Solvent	R_F value A	B	G	Appearance beneath u.v.light (254nm)
1-Naphthylacetic acid		0.83	0.64	0.86	dark purple
1-Naphthylacetyltaurine		0.30	0.0	0.18	dark purple
1-Naphthylacetyl-L-glutamine		0.43	0.13	0.40	dark purple
1-Naphthylacetylglycine		0.60	0.37	0.63	dark purple

For composition of solvents A and B see Chapter 2:

G, Benzene / Acetone / Glacial Acetic Acid 6:2:1 by vol.

Chromatograms were run on silica gel on aluminium backed plates.

t.l.c. in solvent A and scanned for ^{14}C. Three peaks were observed with R_F values of 0.10, 0.78, 0.92. The peak at R_F 0.10 gave a blue colour with naphthoresorcinol and disappeared on treatment of urine samples with either M-NaOH or β-glucuronidase with corresponding increase of peak at R_F 0.92. Identification of other peaks was achieved as previously described in Chapter two and the peaks at R_F 0.10, 0.78 and 0.92 were thus identified as 2-naphthylacetyl-glucuronide, -glycine and 2-naphthylacetic acid respectively.

Similarly, radiochromatogram scans following t.l.c. of urine in Solvent B and C showed the presence of 2-naphthylacetyl-glucuronide,-glycine and 2-naphthylacetic acid.

Mouse and Gerbil

Samples (0.1 ml) of the 0-24 h urine of mice, and gerbils which had received $[^{14}C]$-2-naphthylacetic acid were subjected to t.l.c. in solvent A and scanned for ^{14}C. In each case, four peaks were observed with R_F values of 0.10, 0.22, 0.78, 0.92. The peak at R_F 0.10 gave a blue colour with naphthoresorcinol and disappeared on urine treatment with either M NaOH or β-glucuronidase with corresponding increases at R_F 0.92. Further identification of this peak and other peaks was achieved as previously described in Chapter Two and peaks at R_F 0.10, 0.22, 0.78 and 0.92 were thereby identified as 2-naphthylacetyl-glucuronide, -taurine and -glycine and 2-naphthylacetic acid respectively.

Similarly, radiochromatogram scans of chromatograms of urine in solvent B and C showed the presence of 2-naphthylacetyl-glucuronide, -taurine, glycine and 2-naphthylacetic acid.

Hamster and Rabbit

Radiochromatogram scans of chromatograms of 0-24 h urine of hamster and rabbit in solvent A showed 5 similar-sized peaks with R_F values of 0.1, 0.22, 0.56, 0.78 and 0.92. The peak of R_F 0.1 gave a positive naphthoresorcinol reaction and disappeared when the urine samples were treated with β-glucuronidase or M NaOH and its disappearance was accompanied by an increase in the size of the peak at R_F 0.92 (2-naphthylacetic acid). The peaks with R_F values 0.22, 0.56 and 0.78 corresponded to 2-naphthylacetyl-taurine, -glutamine and -glycine respectively. Chromatograms of the urines developed in solvent B were also scanned for ^{14}C and this revealed 5 radioactive peaks, with R_F values of 0.07, 0.42, 0.69, 0.84 and 0.93. The peak at R_F 0.07 disappeared when the urine was treated with β-glucuronidase or M NaOH and this resulted in an enlargement of the peak at R_F 0.93 (2-naphthylacetic acid). These identifications were confirmed by reverse isotope dilution as previously described in Chapter Two.

Identification of urinary metabolites of 1-naphthylacetic acid in various animal species

Following administration of [^{14}C]-1-naphthylacetic acid (100 mg/kg; as sodium salt) to guinea pigs, hamsters,

gerbils and mice three ^{14}C peaks were detected in their urines following radiochromatogram scanning of t.l.c. plates developed in solvent A. The properties of these peaks and their colour reactions were compared with those given by reference compounds (Table 3.2). Reverse isotope dilution, enzymic, alkaline and acid hydrolyses were carried out on these urine samples. On the basis of the results of these tests, the peak at R_F 0.1 was assigned to 1-naphthylacetyl-glucuronide while the peaks at R_Fs 0.63 and 0.86 corresponded to 1-naphthylacetylglycine and 1-naphthylacetic acid respectively.

Results.

2-Naphthylacetic acid

Guinea Pig

The excretion of ^{14}C in the urine of guinea pig after the administration of [^{14}C]-2-naphthylacetic acid is shown in Table 3.3. Most of the administered ^{14}C recovered was excreted in the first 24 h.

Analysis of the 0-24 h urine of the guinea pig revealed that in addition to the unchanged drug, conjugates of 2-naphthylacetic acid with glucuronic acid and glycine were observed. (Table 3.4).

Mice and Gerbils

The excretion of ^{14}C in the urine of mouse and gerbil after administration of [^{14}C]-2-naphthylacetic acid was also rapid. Over a period of 3 days, the mouse excreted 92% of the dose while the gerbil excreted 68% of the dose

Table 3.2

Chromatographic identification of metabolites in urine of mouse, gerbil, hamster and guinea pig after administration of $[^{14}C]$ -1-naphthylacetic acid. For details see text.

Urine Treatment	^{14}C peaks observed following t.l.c. in Solvent A	Colour Reaction	Observation	Conclusion
None	R_F 0.1	Blue spot with naphthoresorcinol		Presence of Glucuronic acid conjugate
	0.63	-	Corresponds to R_F of naphthyl-acetylglycine	Glycine conjugate present.
	0.8	-	Corresponds to the free acid	Presence of the unchanged compound
Treatment with β-glucuronidase or M NaOH followed by t.l.c.	R_F 0.86		Disappearance of R_F 0.1 peak, increase in peak at R_F 0.86	Increase in amount of 1-naphthylacetic acid due to hydrolysis of the glucuronide to glucuronic acid and 1-naphthylacetic acid.
Treatment with Conc. HCl.			Absence of peaks at R_Fs 0.1 and 0.63. Increase in size of peak at R_F 0.86.	All conjugates hydrolysed to the free acid.

Table 3.3

Excretion of ^{14}C by various species after the administration $[^{14}C]$-2-naphthylacetic acid. Values are means of at least 3 animals with ranges in parentheses. The results for the mouse are for a group of 5.

Species	Guinea Pig	Mouse	Gerbil	Hamster	Rabbit
No., Sex	3F	5F	3F	3F	3F
Dose, mg/kg (^{14}C μCi/kg)	100(16)	100(16)	100(16)	100(16)	100(16)
Route	i.p.	i.p.	i.p.	i.p.	p.o.
	% dose ^{14}C in urine				
Urine					
Day 0-1	83.1(79.8-85.9)	81.8	59.6(49.9-69.2)	52.3(42.7-59.5)	51.0(37.0-59.2)
1-2	9.6(7.9-11.5)	10.1	4.9(3.5-7.5)	20.3(17.7-23.1)	17.2(15.1-19.7)
2-3	1.3(1.1-2.2)	0.9	3.4(2.3-4.4)	7.2(4.6-10.2)	5.2(3.4-7.4)
3-4	-	-	- -	- -	1.1(0.9-1.3)
4-6	-	-	- -	- -	0.7(0.4-1.1)
Total	94.0(88.8-99.6)	92.8	67.9(55.7-81.1)	79.8(65-92.8)	75.8(60.0-85.0)

Table 3.4

Urinary Metabolites of [^{14}C] -2-Naphthylacetic Acid in the Guinea Pig, Mouse, Gerbil and Hamster (Details of dosing with [^{14}C] -2-Naphthylacetic as described in Table 3).

Compound	% Dose ^{14}C excreted in that form by				
	Guinea Pig	Mouse	Gerbil	Hamster	Rabbit
2-Naphthylacetic acid	19.8(18.2-21.4)	2.4	2.2(1.7-2.7)	8.6(7.7-9.4)	5.6(4.5-6.7)
2-Naphthylacetylglycine	31.7(27.3-34.3)	3.1	51(42.8-62.3)	16.7(15.1-17.7)	15.3(13.4-17.2)
2-Naphthylacetyltaurine	-	43.7	3.6(3.0-4.7)	4.7(2.9-5.7)	4.8(2.3-5.4)
2-Naphthylacetylglutamine	-	-	-	18.2(14.0-21.6)	13.1(8.2-15.1)
2-Naphthylacetylglucuronide	31.6(30.3-33.4)	32.6	2.9(2.2-3.9)	4.0(3.0-5.6)	12.2(8.5-13.7)
Total ^{14}C excreted in 0-24 h urine	83.1(75.8-89.1)	81.8	59.7(49.7-73.6)	52.2(42.7-60)	51.0(37.0-59.1)

(Table 3.3). The bulk of the recovered ^{14}C was excreted in the first day while smaller amounts were excreted each succeeding day.

Analysis of the 0-24 h urine of mice and gerbils showed that in addition to the unchanged compound, 2-naphthylacetyl-glucuronide, -taurine and -glycine were present (Table 3.4).

Rabbits and Hamsters

The elimination of ^{14}C in the urine of the rabbit and hamster following administration of [^{14}C]-2-naphthylacetic acid is shown in Table 3.3. Over a 3 day period in the hamster and a 6 day period in the rabbit, a substantial amount of the ^{14}C was recovered (80-84% of the dose). The bulk of the recovery was in the first 24 h with smaller amounts on each succeeding day.

The urinary metabolites of [^{14}C]-2-naphthylacetic acid in hamster and rabbit urine samples were identified by comparison of their chromatographic properties with those of authentic samples, as described above, and by reverse isotope dilution. In addition to unchanged 2-naphthylacetic acid, four conjugates were detected, namely, the glucuronic acid, glycine, glutamine and taurine conjugates (Table 3.4).

1-Naphthylacetic acid

The elimination of ^{14}C in the urine of guinea pig, mouse, hamster and gerbil after the administration of [^{14}C]-1-naphthylacetic acid is shown in Table 3.5. Over

Table 3.5

Urinary excretion of ^{14}C by various species after the administration of [^{14}C]-1-naphthylacetic acid.

Values are means of 3 animals with ranges in parentheses except in mice where the result is for a group of five animals. [^{14}C]-1-Naphthylacetic acid (100 mg/kg; 16 μCi) was administered intraperitoneally.

	% dose ^{14}C in urine in			
	Guinea Pig	Mouse	Gerbil	Hamster
Day 0-1	62(54-66.4)	76.5	75.5(72.3-78.6)	92.8(80.4-101
1-2	7.6(4.6-11.4)	3.2	1.2(0.5-1.8)	-
2-3	6.7(4.9-10.6)	1.5	2.6(2.0-3.1)	-
Total	76.3(70.3-82.9)	81.2	79.3(74.8-83.5)	92.8(80.4-101

the three days of the experiment, the bulk of the administered ^{14}C (76-93% of dose) was recovered in all four species. In all species most of the excreted ^{14}C was found in the 0-24 h urine with much smaller amounts on succeeding days. Faecal excretion was not investigated.

The urinary metabolites of 1-naphthylacetic acid were identified by comparison of their chromatographic properties with those of authentic samples, as described above, by reverse isotope dilution, and by colour tests. In addition to unchanged 1-naphthylacetic acid, two conjugates were detected in all four species, namely, the ester glucuronide and 1-naphthylacetylglycine. Quantitative data for their excretion by the four species are given in Table 3.6.

Discussion

The metabolism of 2-naphthylacetic and 1-naphthylacetic acids has been studied in five laboratory animal species. The results are summarised in Tables 3.7 and 3.8 with Table 3.7 giving the summary of the results of 2-naphthylacetic acid metabolism. The results of studies of the rat and ferret (Chapter 2) have been included for comparison purposes. Table 3.8 summarises the results of the metabolism of 1-naphthylacetic acid and data from other studies have also being included.

2-Naphthylacetic acid

The results of this study indicate that all the animal species studied (rabbit, guinea pig, hamster, gerbil and mouse) have the ability to conjugate 2-naphthyl-

Table 3.6

The urinary metabolites of 1-naphthylacetic acid in the guinea pig, mouse, gerbils and hamster

Dose 100 mg/kg 16 μCi/kg i.p. The 0-24 h urine of animals which received [^{14}C]-1-naphthylacetic acid was analysed as described in the text and the results are expressed as averages of at least 3 animals with ranges in parentheses, except for mice where the result is from a group of five animals.

	% Dose ^{14}C in urine as that compound			
Compound	Guinea pig	Mouse	Gerbils	Hamster
1-Naphthylacetic acid	8.7(6.7-9.7)	12.8	9.1(8.6-9.5)	19.4(16.8-21.7)
1-Naphthylacetylglucuronide	33.4(27.7-39.4)	51.9	28.3(32.5-33)	63.7(54.5-71.1)
1-Naphthylacetylglycine	20.0(16.7-23.6)	11.8	38.1(36.1-40.2)	9.7(8.2-12)
Total ^{14}C recovered in 0-24 hrs	62.1(51.1-72.7)	76.5	75.5(68.2-82.7)	92.8(79.5-104.8)

Table 3.7

Patterns of metabolic conjugation of 2-naphthylacetic acid in various species

Details of dosing as in Table 3.5

Species	^{14}C excreted % dose	% dose excreted in that form in 0-24h urine				
		Unchanged	Glutamine	Glycine	Taurine	Glucuronide
Rat	35	5.2	5.7	9	10	5.7
Rabbit	51	5.6	13	15	4.8	12
Ferret	30	1.4	6.3	5.4	1	5.4
Guinea Pig	83	20	-	32	-	32
Hamster	62	10	22	20	5.5	4.8
Mouse	82	2.4	-	3.1	44	3.3
Gerbils	60	2.4	-	51	3.6	2.9

acetic acid with glucuronic acid and glycine. This is not surprising as previous studies of arylacetic acids have shown that such acids are conjugated mainly with glucuronic acid and/or glycine by non-primate species (Dixon et al.,1977a,b,c; James et al.,1972a,b; Bridges et al.,1974).

The utilisation of taurine as a conjugating agent in the metabolism of 2-naphthylacetic acid occurs in all the species studied with the exception of the guinea pig. The level of taurine conjugation is considerably higher in these species compared to that found for phenylacetic acid which is conjugated with taurine at a low level (less than 3% of the dose) in the rabbit and other rodents (James et al., 1972a). Studies of other arylacetic acids have indicated that utilisation of taurine is not in general a major feature of arylacetic acid metabolism (Dixon et al.,1977a,b,c; Bridges et al.,1974; Idle et al.,1978). One surprising feature is the extensive utilisation by the mouse of taurine for the conjugation of 2-naphthylacetic acid. This is surprising as studies hitherto of arylacetic acid metabolism have not revealed the occurrence of taurine conjugation in the mouse (Bridges et al.,1974; James et al.,1972a).

The hamster and the rabbit conjugate 2-naphthylacetic acid with glutamine in addition to glycine, taurine and glucuronic acid. Glutamine conjugation of 2-naphthylacetic acid is extensive in these two species which is also surprising. This result further confirms that glutamine

conjugation is not restricted as was hitherto thought to primate species but can be found in non-primate species. There is the possibility that given the right substrate those species which apparently do not normally utilise glutamine in the conjugation of arylacetic acids might be capable of so doing, i.e, the absence is more apparent than real. There is also the possibility that a certain substrate level is necessary for this mechanism to be unveiled or that the animal species lack the necessary free glutamine and taurine concentration to enable the mechanism to operate (see Chapter 4).

The elimination of 2-naphthylacetic acid in 5 species, namely, guinea pig, mouse, hamster, gerbil and rabbit, is very rapid compared to the rat and ferret and this may be due to the occurrence of an enterohepatic circulation in the rat and of tissue accumulation in the ferret. In fact, extensive biliary excretion (60% dose) of 2-naphthylacetic acid has been shown to occur in the rat but this is low (3% dose) in the rabbit.

1-Naphthylacetic Acid

The urinary excretion products of 1-naphthylacetic acid in the guinea pig, mouse, gerbil, hamster were, in addition to the unchanged acid, 1-naphthylacetyl-glucuronide and -glycine. Dixon et al. (1977a) found a similar metabolic pattern for the rabbit and rat.

A combination of these results with those of Dixon et al. (1977a) and Idle (1976) provides a comprehensive overview of the metabolism of 1-naphthylacetic acid in

various species and illustrates the important role played by species in the metabolism of this acid (Table 3.8). With the exception of the cat, which is known to be deficient in the utilisation of glucuronic acid for the conjugation of some arylacetic acids other species studied utilise glucuronic acid predominantly for the conjugation of 1-naphthylacetic acid. Utilisation of multiple amino acids for the conjugation of 1-naphthylacetic acid seems to be limited to the primates and the carnivores while rodents utilise only one amino acid. The role played by species in the metabolism of this acid is shown by the variation of the amino acid utilised and the extent of their utilisation. The primates utilise glutamine which is not surprising, while the carnivores utilise taurine; glycine is utilised by both primate and non-primate species.

Comparison of the metabolism of isomeric 1- and 2-naphthylacetic acids

These studies have shown that the two isomeric 1- and 2-naphthylacetic acids differ considerably in respect of their metabolic fate in various non-primate species (Table 3.9). Fig. 3.1 shows their metabolic patterns. The 1-isomer is conjugated with glucuronic acid and glycine with the exception of the ferret. In the case of 2-naphthylacetic acid, it is conjugated with several amino acids (glutamine, taurine and glycine) and glucuronic acid.

A summary of a comparison of the metabolic fates of the isomeric 1- and 2-naphthylacetic acids are given below. In general, the conjugation options for 1-naphthylacetic acid

Table 3.8

Conjugates of 1-naphthylacetic acid in various species

		% Dose of 1-naphthylacetic acid excreted in 24 h as				
		Unchanged	Glutamine	Glycine	Taurine	Glucuronic Acid
Primates						
Man*		-	-	-	6.0	91.0
Rhesus*	Old World Monkeys	9.0	-	-	2.0	89.0
Cynomolgus*	Old World Monkeys	35.0	3.0	-	4.0	29.0
Squirrel*	New World Monkeys	10.0	2.0	6.0	6.0	7.0
Capuchin*	New World Monkeys	10.0	2.0	6.0	15.0	8.0
Marmoset* (Prosimians)		5.0	2.0	2.0	9.0	49.0
Non-Primates						
Rabbit* (Lagomorph)		6.0	-	5.0	-	71.0
Rat*	Rodents	17.0	-	15.0	-	33.0
Hamster	Rodents	19.0	-	10.0	-	64.0
Guinea Pig	Rodents	9.0	-	20.0	-	33.0
Mouse	Rodents	13.0	-	12.0	-	52.0
Gerbil	Rodents	9.0	-	32.0	-	28.0
Cat*	Carnivores	2.0	-	37.0	25.0	-
Ferret**	Carnivores	9.0	-	9.0	37.0	9.0

* Data from Dixon et al (1977a) ** Data from Idle (1976)

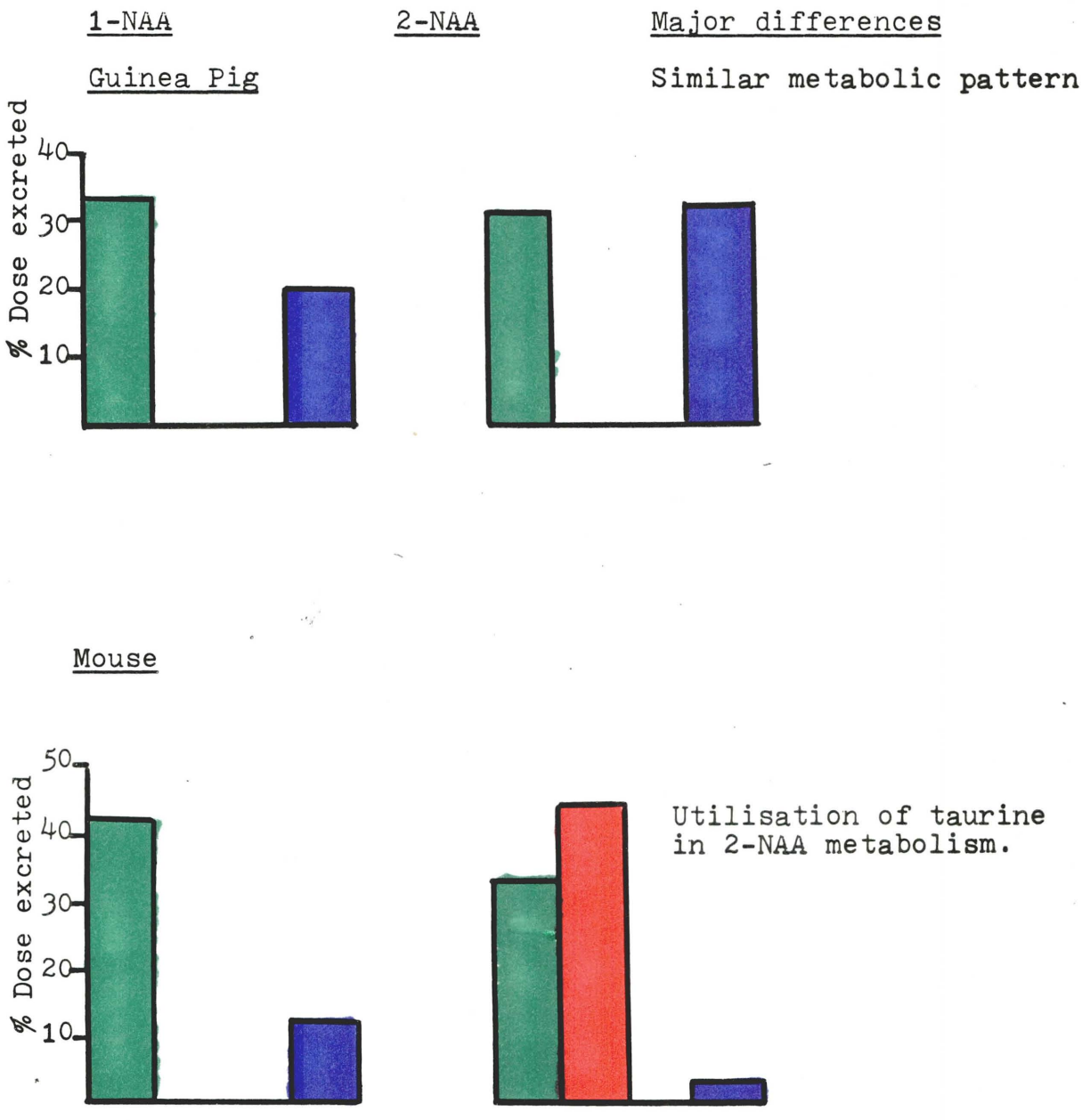
Table 3.9 Comparative metabolism of the isomeric 1- and 2- naphthylacetic acids in various species

Species	Compound	% ^{14}C Dose Recovered	% Dose Conjugates as			
			Glutamine	Taurine	Glycine	Glucuronide
Guinea Pig	1-Naa	62	-	-	20	33
	2-Naa	83	-	-	32	32
Mouse	1-Naa	77	-	-	12	52
	2-Naa	82	-	44	3.1	33
Hamster	1-Naa	93	-	-	38	28
	2-Naa	52	18	4.7	17	4
Gerbil	1-Naa	76	-	-	10	64
	2-Naa	60	-	3.6	51	2.9
Rabbit	1-Naa	81	-	-	4.5	71
	2-Naa	51	13	4.8	15	12
Rat	1-Naa	64	-	-	15	33
	2-Naa	35	5.7	10	8.6	5.7
Ferret	1-Naa	64	-	37	8.9	8.9
	2-Naa	30	6.3	15	5.4	5.4

Key = 1-Naa = 1-naphthylacetic acid, 2-Naa = 2-naphthylacetic acid.

Fig. 3.1

Comparison of the metabolic conjugation pattern of isomeric 1- and 2-naphthylacetic acids in various species

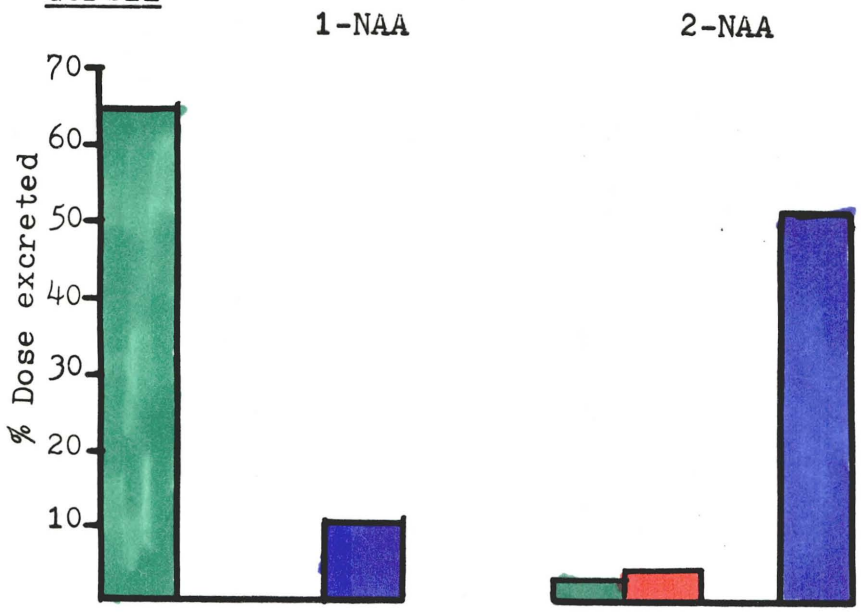


1-NAA = 1-naphthylacetic acid

2-NAA = 2-naphthylacetic acid

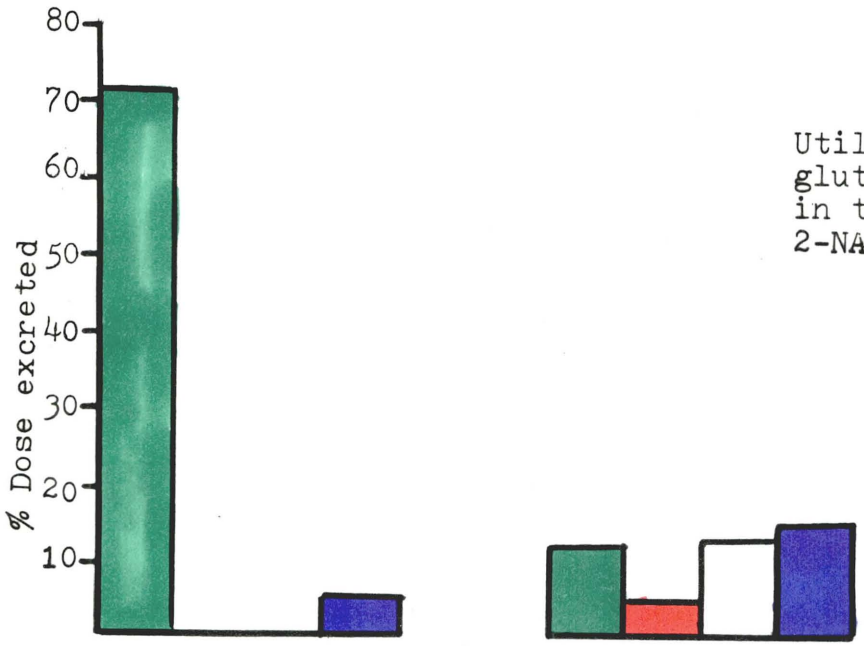
Fig. 3.1 contd.

Gerbil



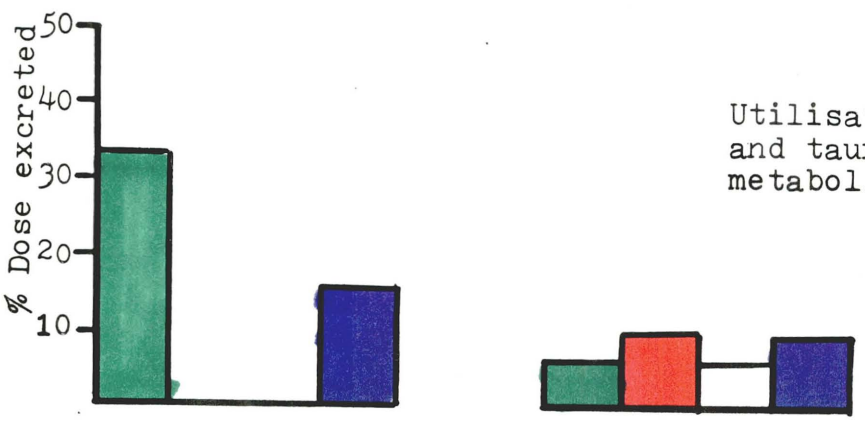
Taurine utilisation in 2-NAA metabolism

Rabbit



Utilisation of glutamine and taurine in the metabolism of 2-NAA.

Rat



Utilisation of glutamine and taurine in the metabolism of 2-NAA.

Fig. 3.1. contd.

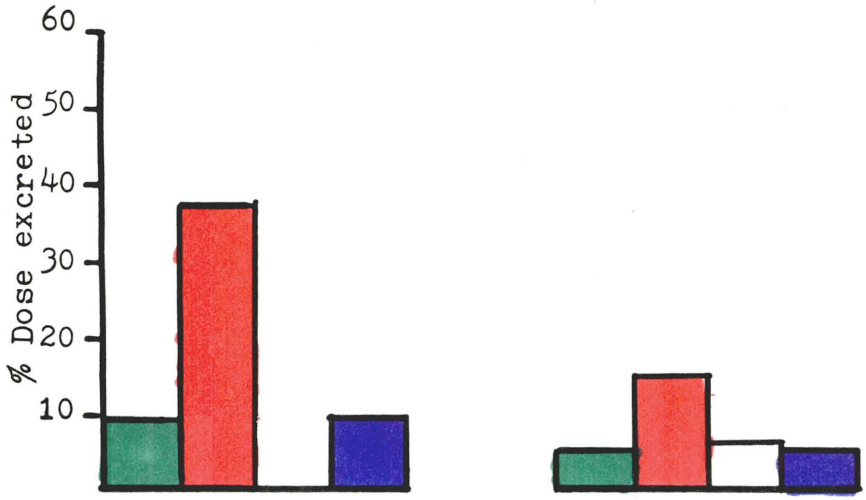
1-NAA

2-NAA

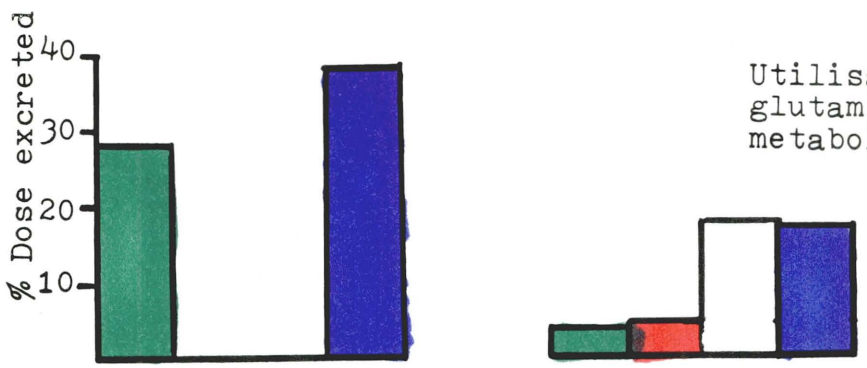
Major differences

Ferret

Utilisation of glutamine in 2-NAA metabolism



Hamster



Utilisation of taurine and glutamine in 2-NAA metabolism.

appear to be restricted to glucuronic acid and glycine in the species studied with the exception of the ferret while 2-naphthylacetic acid is capable of four conjugation options namely with glucuronic acid and glycine, glutamine and taurine. The influence of species on the metabolic conjugation of 2-naphthylacetic is more pronounced than for 1-naphthylacetic acid.

Guinea Pig

No significant difference in the metabolic conjugation of 1- and 2-naphthylacetic acid except for the relative amounts of the glycine and glucuronic acid conjugates formed.

Mouse

2-Naphthylacetic acid is conjugated with taurine, glucuronic acid and glycine with the taurine conjugate as the major metabolite. 1-Naphthylacetic acid is conjugated mainly with glucuronic acid.

Hamster

2-Naphthylacetic acid is conjugated mainly with glutamine and taurine; the 1-isomer however is conjugated mainly with glycine and glucuronic acid.

Gerbil

2-Naphthylacetic acid is conjugated mainly with glycine and to a smaller extent with taurine and glucuronic acid while the main conjugate of 1-naphthylacetic acid is the glucuronic acid conjugate.

Rabbit and Rat

2-Naphthylacetic acid is conjugated with taurine and glutamine in addition to glucuronic acid and glycine in both species. 1-Naphthylacetic acid is conjugated nearly exclusively with glucuronic acid by both species.

Ferret

In this species 2-naphthylacetic acid is conjugated with glutamine, taurine, glycine and glucuronic acids while the 1-isomer forms the same conjugateswith the exception of the glutamine conjugate.

The comparative metabolism of 1- and 2-naphthylacetic acids in a range of species presents a complex picture out of which nevertheless a number of generalisations may be perceived:

(i) both acids undergo conjugation with glucuronic acid and amino acids but the relative importance of the two conjugation systems and the nature of the amino acids utilised vary with compound and species.

(ii) In general, irrespective of species 1-naphthylacetic acid shows a greater affinity for glucuronic acid conjugation than does the 2-isomer. Thus the mean amino acid/glucuronic acid ratio for 8 species for 1-naphthylacetic was about 1 compared to a value of 6 for the 2-isomer.

(iii) the pattern of conjugation of 1-naphthylacetic acid was largely of the 'predicted type' i.e.

conjugation with glucuronic acid and glycine with taurine appearing in carnivores.

(iv) the pattern of amino acid conjugation of 2-naphthylacetic acid was most unusual, involving one or more of glutamine, taurine and glycine depending upon species.

(v) the strikingly different patterns of metabolic conjugation of 1- and 2-naphthylacetic acid emphasize the difficulties and dangers of attempting to predict metabolic patterns on the basis of chemical structure and species considerations.

CHAPTER FOUR

FACTORS AFFECTING THE METABOLIC CONJUGATION OF 2-NAPHTHYLACETIC ACID IN VARIOUS SPECIES.

CHAPTER FOUR

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CHAPTER FOUR

FACTORS AFFECTING THE METABOLIC CONJUGATION OF 2-NAPHTHYLACETIC ACID IN VARIOUS SPECIES.

I. INFLUENCE OF DOSE.

Introduction

The results presented in the previous two chapters showed that the metabolic conjugation of 2-naphthylacetic acid is unusual in a number of respects which may be summarized as follows:

i) it showed a complex pattern of conjugation involving in some species both glucuronic acid and several different amino acids (glycine, taurine and glutamine).

ii) some non-primate species utilize glutamine for the conjugation of 2-naphthylacetic acid, a metabolic reaction previously thought to be a feature of primate metabolism of arylacetic acids.

iii) the extensive utilisation of taurine by several non-carnivore species; once again this metabolic route was hitherto thought to be largely a characteristic of carnivore species.

iv) the marked disparity in the pattern of metabolism of the isomeric 1- and 2-naphthylacetic acids.

It seemed therefore that because of this unusual or 'aberrant' pattern of metabolism of 2-naphthylacetic acid that the subject was worthy of further study. In particular to focus attention on the possible reasons for the unusual and unpredictable pattern of conjugation of 2-naphthylacetic acid. For this purpose it was important to propose a number

of hypotheses capable of experimental investigation, to explain the unusual nature of the conjugation of 2-naphthylacetic acid.

Hypotheses to explain the unusual nature of the metabolic conjugation of 2-naphthylacetic acid

Four hypotheses are suggested to explain the unusual nature of the metabolic conjugation of 2-naphthylacetic acid as follows:

1. the manifestations of certain amino acid conjugations may be dose-dependent i.e. certain critical levels of a substrate may be necessary in order to saturate alternative conjugation pathways before their existence is made explicit. In other words there may be a concentration threshold for a substrate before a particular metabolic conjugation pathway is activated or unveiled. This hypothesis would therefore presuppose that common laboratory mammalian species such as the rat and guinea pig are mechanistically equipped for glutamine, taurine and glycine conjugation but that the exposure of the first two would require particular conditions of substrate concentration to expose them. The investigations described in this chapter are in fact an attempt to investigate this hypothesis.

2. The second hypothesis is that the variable

conjugation pattern encountered among the species is a reflection of species variations in the availability of the particular amino acid. This hypothesis is investigated in part B of this chapter.

3. A third possibility is that induction and inhibitory phenomena are at work to influence conjugation patterns. Thus, certain substrates (e.g. an arylacetic acid) may be able to induce the appearance of a particular amino acid conjugation pathway in the same way that the microsomal mono-oxygenase system may be induced. Alternatively, some pathways may be depressed by the presence of certain substances; the latter could include the arylacetic acid itself, an amino acid conjugate, or even the conjugating agent itself. Some of the possibilities are investigated and described in part 2.

4. The fourth hypothesis concerns the specificity of the enzymes, the amino acid transferases. It is possible that the nature of these and their activity varies with species and that this factor determines the particular pattern of amino acid conjugation of 2-naphthylacetic acid for a species.

Many drugs have been shown to be metabolised by a primary metabolic pathway at low dose levels but higher

doses unveil secondary metabolic routes. An example of such compound is benzoic acid which is metabolised at low doses (50 mg/kg) in the rat by conjugation with glycine while at higher dose (above 200 mg/kg) is metabolised by conjugation with glycine and glucuronic acid (Bridges et al. 1974).

Similarly 1-naphthylacetic acid exhibits this type of dose dependent metabolism. Thus at low dose, 1-naphthylacetic acid is conjugated mainly with glycine while at higher dose levels, the glycine conjugation pathway is saturated and a second metabolic pathway comes into prominence in this case glucuronic acid conjugation (Dixon et al.,1977a).

Materials and Method

Materials and Methods used are described in Chapters Two and Three and only new materials or methods will be described here.

Administration of Compounds

$[^{14}C]$-2-Naphthylacetic acid was administered by i.p. injection dissolved in propane-1, 2-diol to guinea pigs, ferrets, hamsters, and mice at doses of 5, 50, 100, and 200 mg/kg and their urine collected over 24h period. In the rat, the compound was administered to bile duct-cannulated animals over the same dose range as to those of other animals, and bile and urine collected over 3h period.

Results

Data for the urinary elimination of ^{14}C by mice, guinea pigs, hamsters and ferrets and for the rat urinary and biliary excretion following the administration (i.p.) of $[^{14}C]$-2-naphthylacetic acid at various dose levels is shown in Tables 4.1 and 4.2 and Fig. 4.1. The qualitative and quantitative data for each species are discussed below.

Guinea Pig

Over a 24h period 70-80% of the administered $[^{14}C]$-2-naphthylacetic acid was eliminated in the urine at the four dose levels used.

The urinary metabolites were the glucuronic acid and glycine conjugates as well as the unchanged acid (Table 4.2). At a low dose level (5 mg/kg) the major urinary metabolite was the glycine conjugate but as the dose level was increased, glucuronic acid conjugation increased with

Fig. 4.1

Influence of dose variation on the metabolic pattern of 2-naphthylacetic acid

Guinea Pig

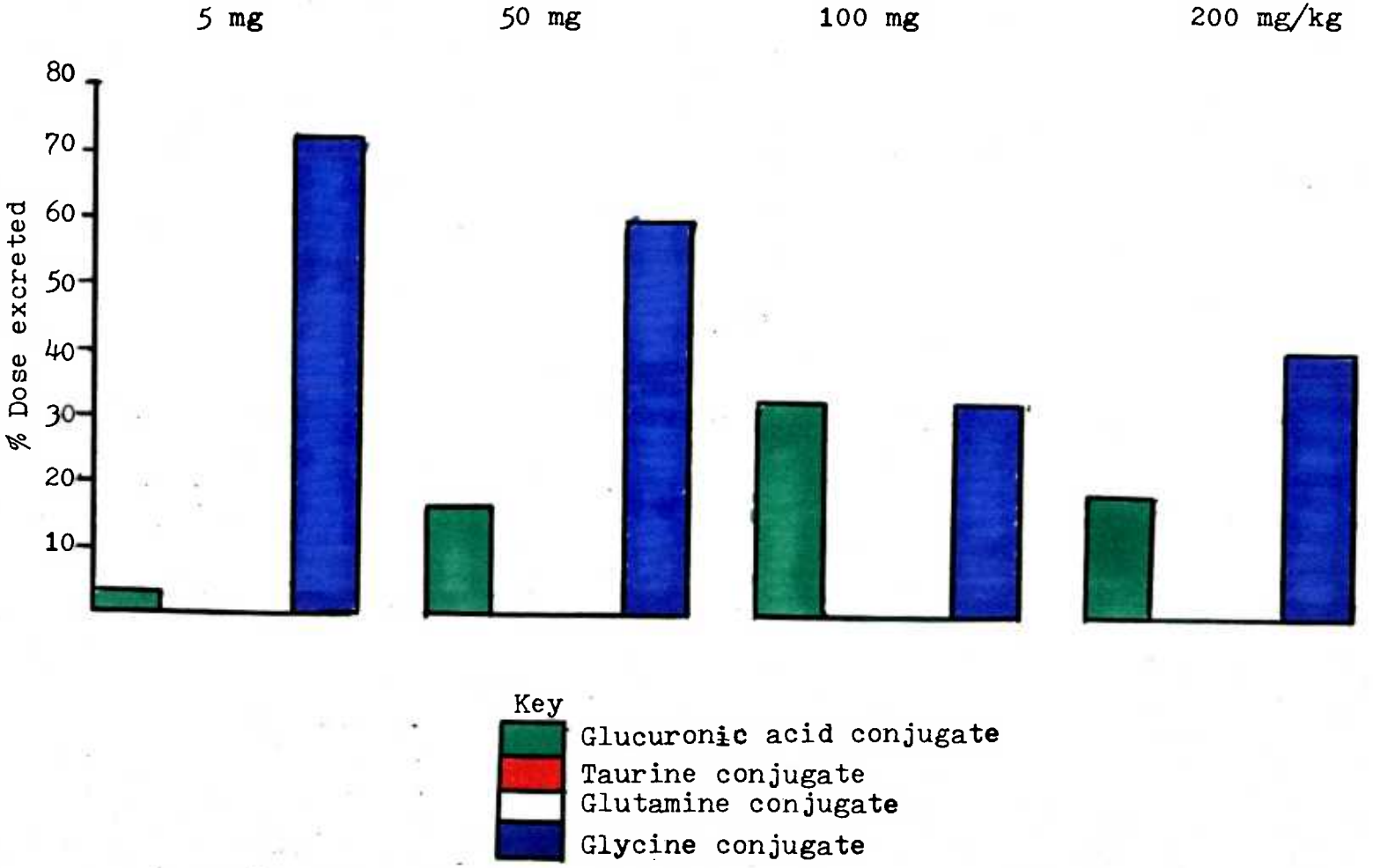


Fig. 4.1 contd.

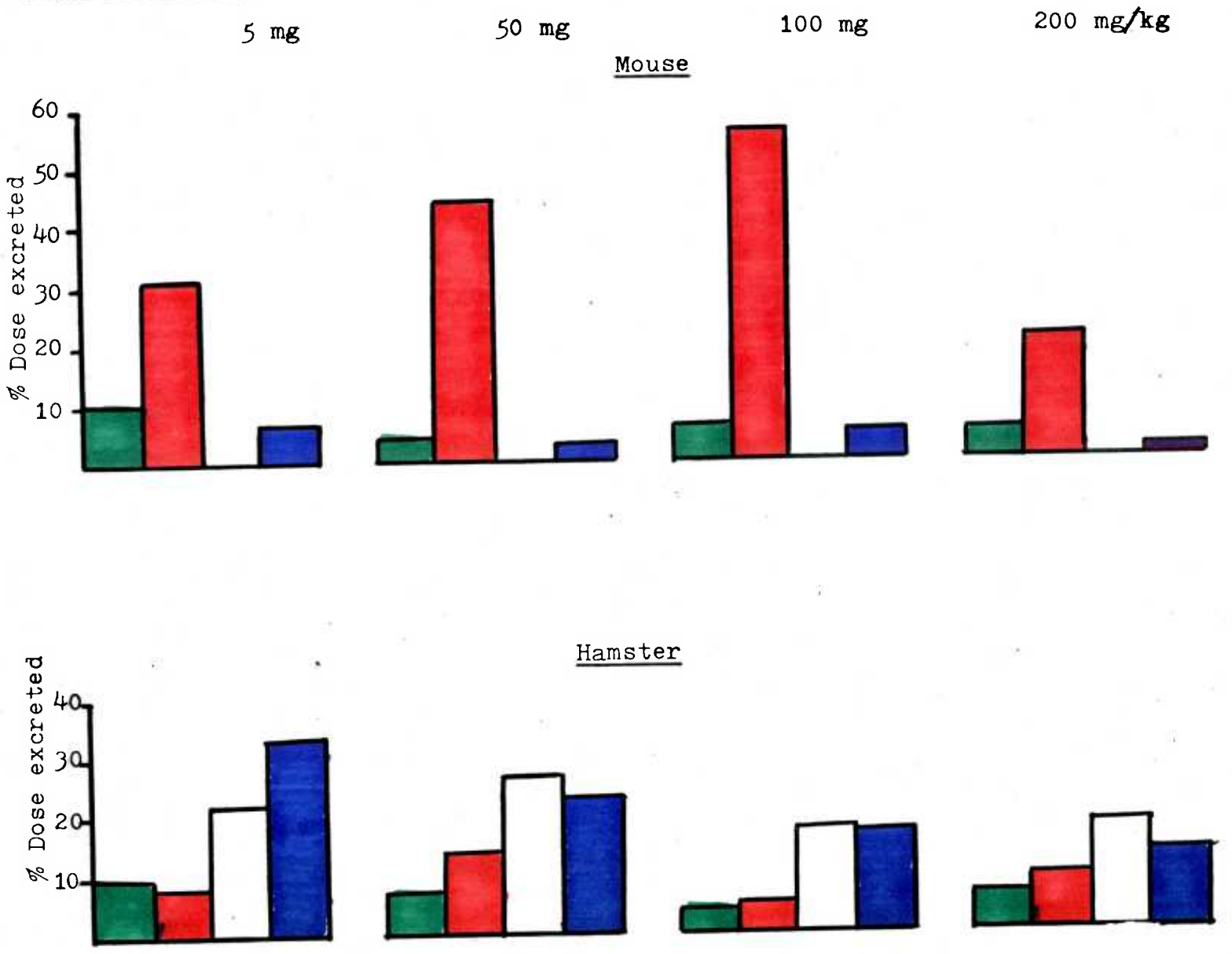

5 mg
50 mg
100 mg
200 mg/kg
Mouse
% Dose excreted
60
50
40
30
20
10
Hamster
% Dose excreted
40
30
20
10

Fig. 4.1 concluded

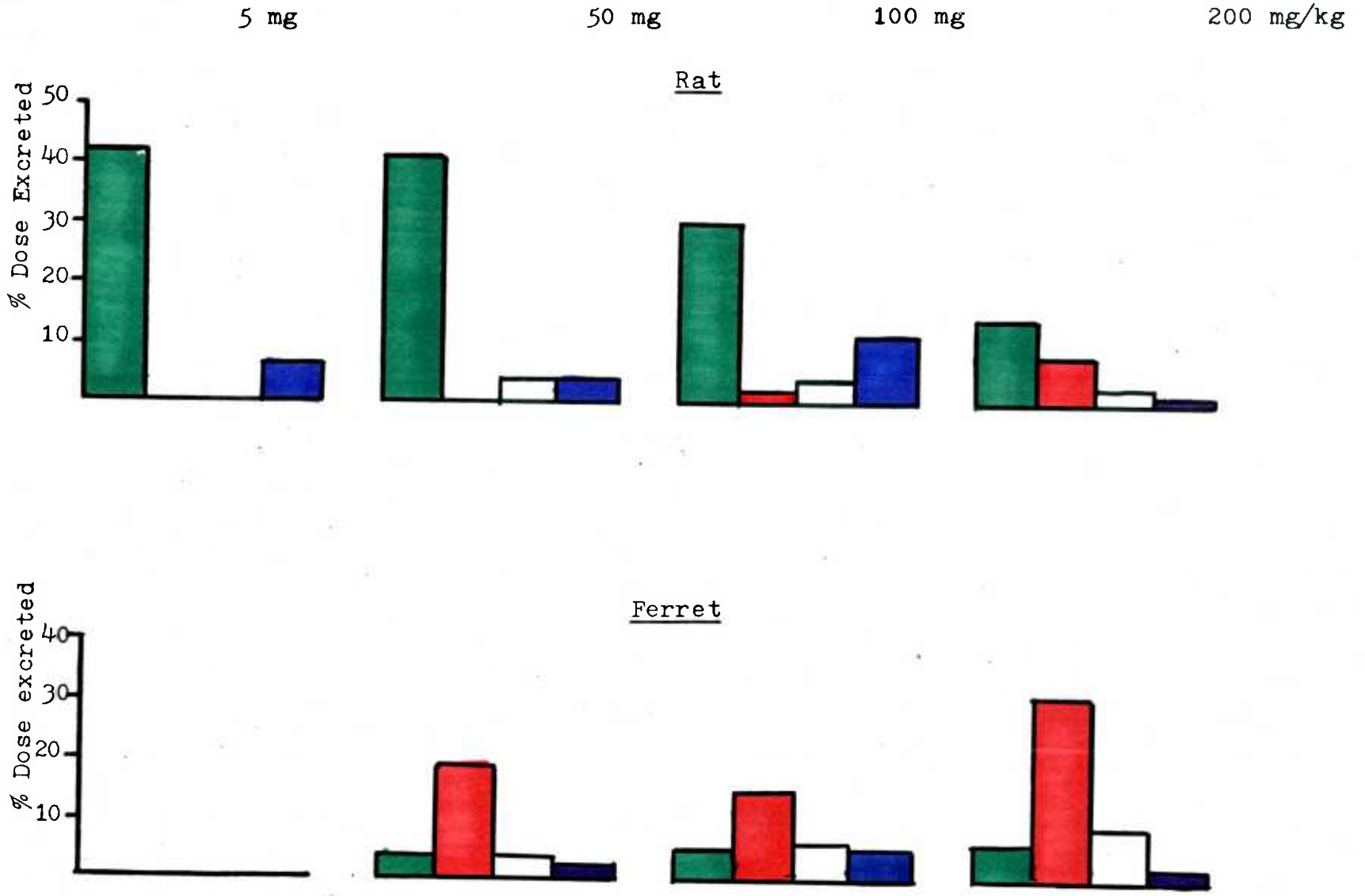


Table 4.1

Urinary ^{14}C recovery after the administration of [^{14}C]-2-naphthylacetic acid at various dose levels to variety of animal species. Values are mean of at least 3 animals (Data are for 0-24h period except where stated otherwise).

Dose mg/kg	Species	Guinea Pig	Mice	Hamster	Rat*	Ferret
		% Dose excreted				
5		76(71.9-80.3)	50.5	85.9(70.7-115)	40.3(37.2-41.6)	-
50		71.3(65.2-83.3)	52.9	73.0(68.5-77.1)	51.5(47.0-58.1)	33.0(27.0-35.3)
100		83.1(79.8-85.9)	70.3	52.3(42.7-59.5)	52.0(48.9-56.1)	33.0(31.1-35.6)
200		69.5(58.8-79.5)	28.1	50.5(48.1-54.9)	28.1(26.0-30.1)	49.2(47.3-53.1)

* 3h urinary and biliary excretion

\- No data.

Table 4.2

Dose-dependent conjugation of 2-naphthylacetic acid in various species.

Species	% Dose conjugated with	Dose Size (mg/kg) 5	50	100	200
Guinea Pig	Glucuronic acid	3.0	16	32	18
	Taurine	ND	ND	ND	ND
	Glutamine	ND	ND	ND	ND
	Glycine	72	59	32	40
	Unchanged	2.1	1.7	20	12
Mice	Glucuronic acid	9.6	4.2	6.3	4.8
	Taurine	31	44	56	21
	Glutamine	ND	ND	ND	ND
	Glycine	5.6	2.8	4.8	2.3
	Unchanged	4.7	2.1	3.9	0.4
Hamster	Glucuronic acid	9.7	7.1	4.2	6.6
	Taurine	8.2	14	4.6	9.9
	Glutamine	22	27	18	19
	Glycine	33	23	17	14
	Unchanged	13	2.0	8.7	1.3
Rat	Glucuronic acid	42	41	30	14
	Taurine	ND	ND	1.5	7.7
	Glutamine	ND	3.5	4.1	2.5
	Glycine	5.9	4.1	11	1.0
	Unchanged	ND	2.5	4.0	1.2
Ferret	Glucuronic acid	-	3.9	5.4	6.2
	Taurine	-	19	15	31
	Glutamine	-	3.6	6.4	9.1
	Glycine	-	2.2	5.4	2.1
	Unchanged	-	1.2	1.4	1.2

ND = Not detected.

- = Not investigated.

a concomitant decrease in the amount of the glycine conjugate (see Fig. 4.1).

Mouse

The urinary elimination of radioactivity in this species varied from about 50% of dose in 24h at a dose level of 5 and 50 mg/kg to only 28% at a dose level of 200 mg/kg.

The major metabolic products in the urine were the taurine conjugate together with smaller amounts of the glucuronic acid and glycine conjugates. It was observed that excretion of the taurine conjugate increased with increase of dose upto 100 mg/kg, above which however a fall occurred. This pattern parallels that seen for the overall elimination of [^{14}C]-2-naphthylacetic acid which also decreased with increase of dose to 200 mg/kg. This suggests that when saturation of the primary metabolic pathway for 2-naphthylacetic acid in the mouse (taurine conjugation) occurs it is not compensated for by an increase in the capacities of the alternative pathways of glucuronic acid and glycine conjugation so that overall elimination therefore falls.

Hamster

Over the dose levels investigated between 51-86% of the dose of [^{14}C]-2-naphthylacetic acid was recovered. Once again urinary recovery of ^{14}C declined with increase in dose level. This suggests once again that saturation of the metabolic mechanisms has occurred.

The excretion products were in addition to the

unchanged acid, the glucuronic acid, glycine, glutamine and taurine conjugates. (Table 4.2., Fig. 4.2). The relative amounts of the glutamine and glycine conjugates decreased with increase in dose from 5 to 200 mg/kg.

Rat

The data shown in Table 4.1 for this species is for the combined biliary and urinary values obtained 3h after the intraperitoneal injection of [^{14}C]-2-naphthylacetic acid. The overall recovery of ^{14}C at 5, 50 and 100 mg/kg was about 40-50% dose in 3h but this declined to only 28% at 200 mg/kg. The pattern of urinary elimination of the acid was notably dose-dependent. At the lowest dose level (5 mg/kg) the urinary excretion products were the glucuronic acid conjugate together with a small amount of the glycine conjugate. At 50 mg/kg, the glutamine conjugate was also excreted in addition to the glucuronic acid and glycine conjugate while at 100 and 200 mg/kg the taurine conjugate was also excreted in addition to the other three conjugates seen at 50 mg/kg.

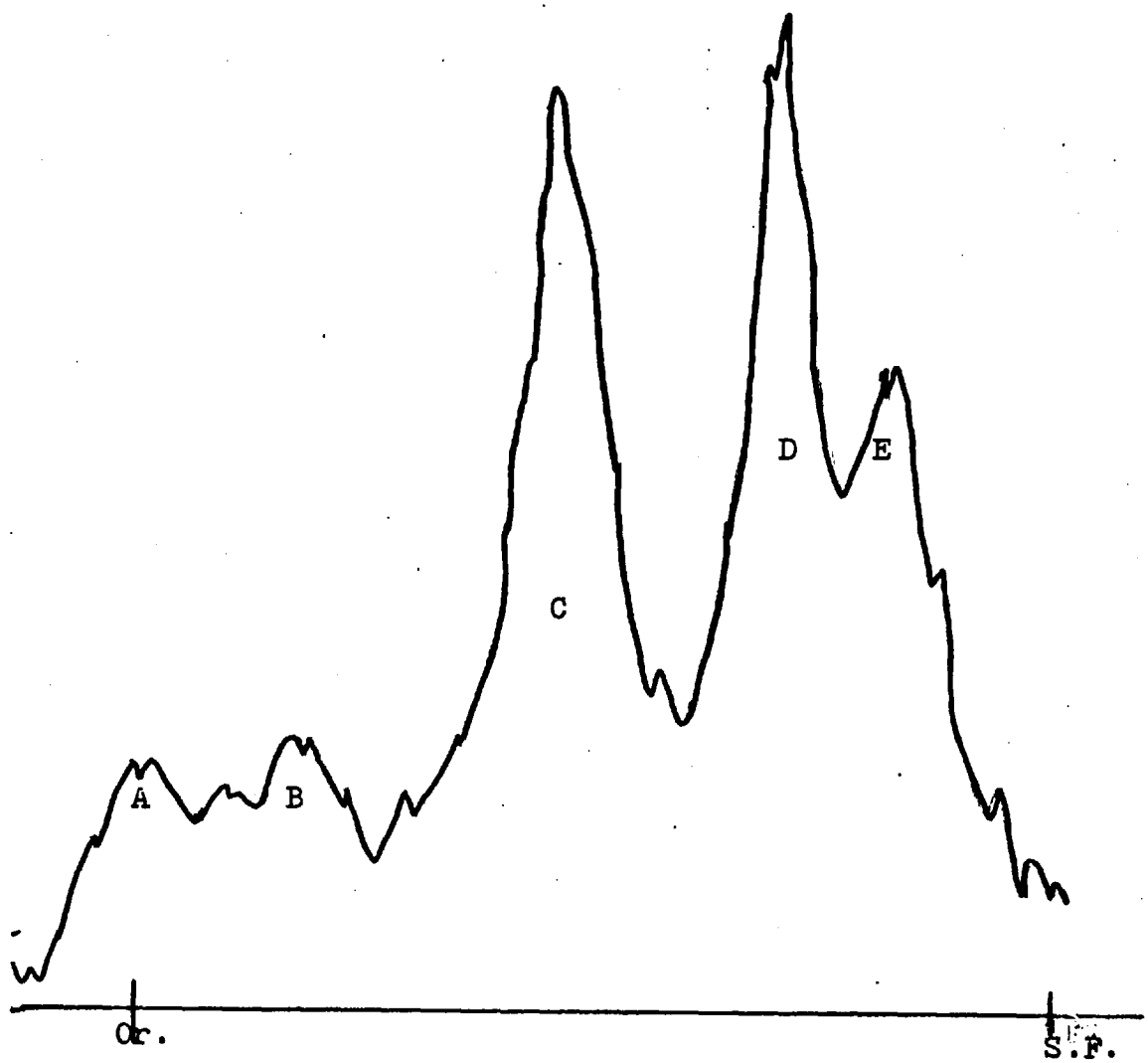
Ferret

The urinary recovery of radioactivity following the administration of [^{14}C]-2-naphthylacetic acid was 33-49% of dose for the three dose levels investigated (50, 100 and 200 mg/kg).

With increase in dose quantitative changes in the pattern of elimination were observed. Thus, with increase in dose the relative proportion of the dose excreted conjugated with glutamine increased while the proportion

Fig. 4.2

Radiochromatogram scan of urine of hamster dosed with [C^{14}]-2-naphthylacetic acid (5mg, 16 uCi/kg) developed in solvent A



A =2-Naphthylacetylglucuronide
B =2-Naphthylacetyltaurine
C =2-Naphthylacetylglutamine
D =2-Naphthylacetylglycine
E =2-Naphthylacetic acid

Or. = Origin S.F. =Solvent Front

eliminated as the taurine conjugate at 200 mg/kg (31%) was nearly double that seen at 50 mg/kg (19%).

Discussion

The influence of variation in dose level on the qualitative and quantitative aspects of the metabolic conjugation and elimination of [^{14}C]-2-naphthylacetic acid in five laboratory animal species has been studied and the results presented in this chapter. The primary objective of this particular study was to ascertain whether or not the manifestation of certain amino acid conjugation mechanisms in a species is dose-dependent and that a certain dose-threshold may be necessary to elicit evidence of their existence and potential. No attempt was made in these experiments to obtain a full recovery of the administered dose of 2-naphthylacetic acid. Comparison for the species at the different dose levels are based upon 24h urinary recoveries of ^{14}C except for the rat where the values are for combined values for urinary and biliary excretion. The total pattern for the species studies is a complex one as might be expected when attempting to relate several variables (species, dose and multiple conjugation pathways). No attempt will be made to discuss the details but rather attention will be focused on the major points particularly as they relate to the proposed hypotheses.

Two major points emerge from this study. Firstly, the qualitative metabolism of 2-naphthylacetic acid is dose-dependent in one of the five species investigated namely, the rat. In this species, a small dose (5 mg/kg) of

2-naphthylacetic acid is conjugated with glucuronic acid and glycine but as the dose-level is increased the conjugation pattern changes qualitatively with subsequent emergence of first the glutamine and then the taurine conjugates. This finding is noteworthy since it does support the view for the rat at least that certain amino acid conjugation mechanisms remain obscure or latent until exposed by challenge with a large dose of a drug which saturates the primary metabolic options available.

In the case of the other four species investigated however there was no evidence of changes in dose level eliciting a qualitative change in the pattern of conjugation. Nevertheless in some cases significant changes in the quantitative aspects of the conjugation patterns emerged pointing to the fact that some pathways in these species are dose-dependent. Thus, in the hamster the glycine pathway appears to be saturable as shown by the decrease in the excretion of this conjugate with increase of dose. In the ferret the relative importance of glutamine and taurine conjugation increased with increase of dose levels.

The overall findings would indicate therefore that the metabolic conjugation of 2-naphthylacetic acid is dose-dependent. The scope of the dose-dependency can vary from alterations in the relative importance of the various conjugation pathways (e.g. ferret and hamster) to eliciting qualitative changes in the pattern as with the rat.

The findings with the rat are of great interest since

they indicate for this species at least that 'unusual' conjugation pathways may be unveiled under appropriate challenge conditions as with size of dose. There is however the question as to why it did not prove possible to unveil these pathways in the other species examined by manipulation of dose. Two suggestions can be made to explain this. Firstly, the capacities and affinities of the major conjugation options are not readily open to saturation and it is difficult therefore to 'unveil' alternative pathway by dose manipulation. A second possibility of course is that they are not present and cannot be unveiled by any challenge.

CHAPTER FOUR

FACTORS AFFECTING THE METABOLIC CONJUGATION OF 2-NAPHTHYLACETIC ACID. 2. INFLUENCE OF VARIOUS PRETREATMENTS.

Introduction

In the first part of this chapter, some hypotheses were proposed to explain the unusual pattern found for the metabolic conjugation of 2-naphthylacetic acid. Two of these were that the variable conjugation pattern encountered among the species could be (i) a reflection of species variations in the availability for conjugation of the particular amino acid, or (ii) due to induction or inhibitory phenomena at work to influence conjugation patterns.

In the previous chapter it was shown that some species are capable of utilising both glutamine and taurine for conjugation of 2-naphthylacetic acid while some apparently are not. It is suggested that this could be due to the relative non-availability in these latter species of these particular amino acids for conjugation. Various studies have shown that glutamine and taurine are present in the free state in body fluids and the tissue of various species (Table 4.3). If these amino acids are present and are available for utilisation in conjugation reactions, this raises the problem of why some species are apparently unable to utilise them for conjugation purposes.

In this investigation, the guinea pig was used since it was the only animal studied which was unable to conjugate 2-naphthylacetic acid with glutamine and taurine.

Induction phenomena are important in drug metabolism; many studies have shown that the microsomal mono-oxygenase

Table 4.3

Levels of free glutamine and taurine in various animal tissues.

Species	Tissue	Free Amino Acid (mg/kg)		Reference
		Glutamine	Taurine	
Dog	Whole body	520-870	ND	1
Cat	"	770	ND	1
Rabbit	"	450	ND	1
Rat	"	480-780	ND	1,6.
Mouse	"	530-820	ND	1
Rabbit	Liver	ND	688	9,10
Dog	"	450	900	2,13
Rat	"	550	1625	5,11
Cat	"	540	1038	3,14
Mouse	"	350	ND	5,10
Guinea Pig	"	555	675	7,12
Dog	Kidney	113	ND	2
Rat	"	220	1050	5,11
Cat	"	350	388	3,14
Mouse	"	100	1400	5 8
Guinea Pig	"	ND	175	7,12
Rabbit	"	ND	363	9

ND = Not determined.

For References please turn over.

References for Table 4.3

1. Waelsch (1951)
2. Hamilton (1945)
3. Krebs *et al*. 1949
4. Waelsch and Schwerin (Unpublished data).
5. Schwerin *et al*. (1950)
6. Dawson (1949)
7. Christensen & Lynch, (1948)
8. Zachmann (1966)
9. Dubreuil & Timiras, (1953)
10. Kit and Awapara (1953)
11. Garvin (1960)
12. Awapara (1956)
13. Jacobsen & Smith (1963)
14. Tallan *et al*. (1954)

system is capable of being induced by many compounds (Conney 1967, Parke 1975). Various studies have shown that concomitant administration of exogenous glycine with benzoic acid or phenylacetic acid resulted in an increase in the amount of glycine conjugate excreted (Amsel and Levy 1970; Wan and Riegelman 1972). Attempt to increase the activity of glycine N-acyltransferase *in vitro*, in liver and kidney of both adult rats and immature rabbits by pretreatment with benzoic acid, salicylic acid or phenobarbital was abortive but oral administration of sodium salicylate and intraperitoneal administration of sodium benzoate and phenobarbital to both female and male rats increased the activities of glycine N-acyltransferases in both the liver and kidney (James and Bend 1978). Studies of Furst *et al.* (1977) showed that glycine conjugation is induced by salicylate in man thereby producing an increase in the formation of salicyluric acid. The guinea pig has also been used to evaluate the possibility that 2-naphthylacetic acid may be able to induce certain metabolic conjugation pathways i.e. exposure to this acid may induce or uncover the taurine and glutamine pathways.

This possibility seemed attractive since it has been shown that 2-naphthylacetic acid has a marked affinity to bind to nuclear DNA and is persistent. (see Chapter Two).

The conjugation of 2-naphthylacetic acid is very remarkable and so therefore it was of interest to investigate this possibility that it may be capable of inducing amino acid conjugation pathways.

It is noteworthy that the literature is apparently free of references to experimental attempts to induce the amino acid conjugation reactions.

This section is therefore concerned with the evaluation of two hypotheses (i) that the non-utilisation of glutamine and taurine by the guinea pig for the conjugation of 2-naphthylacetic acid is related to the relative non-availability of these compounds in the species for conjugation and (ii) that 2-naphthylacetic acid is able to induce glutamine and taurine conjugation in the guinea pig.

Materials and Method

Materials and methods have been described in Chapters 2,3, & 4 and only new materials and methods are presented here.

Administration of compounds

Taurine or glutamine (300 mg/kg) was administered dissolved in the appropriate amount of 0.1M sodium bicarbonate solution i.p. to guinea pigs daily for 5 days, and the last dose was given immediately after the administration of the dose of [^{14}C]-2-naphthylacetic acid (200 mg/kg). 2-Naphthylacetic acid (200 mg/kg) was administered i.p. to the guinea pigs daily for 4 days followed on the fifth day by a dose of [^{14}C]-2-naphthylacetic acid (200 mg/kg).

Identification of Metabolites

Chromatographic identification of urinary metabolites was as described in Chapter Two.

Results

Administration of glutamine or taurine prior to [^{14}C]-2-naphthylacetic acid to guinea pig

The excretion of ^{14}C and the nature and amounts of the urinary metabolites present after the administration of [^{14}C]-2-naphthylacetic acid i.p. to the guinea pig alone and after pretreatment with glutamine and taurine is given in Table 4.4.

When [^{14}C]-2-naphthylacetic acid was administered to the guinea pig, the urinary excretion of ^{14}C in 24 h

Table 4.4

Influence of Glutamine and Taurine on the fate of 2-naphthylacetic in guinea pigs.

Dose and other details in text. Values are mean of 3 animals with ranges in parentheses.

Pretreatment	% Dose Recovered	% of ^{14}C excreted as		
		Unchanged	Glucuronide	Glycine conjugate
None	62.0(60.0-66.4)	22.4(19.4-24.3)	37.3(37.3-39.0)	40.3(43.3-36.7)
Glutamine (300 mg/kg i.p. daily x 7)	33.4(33.4-33.5)	11.9(0.0-23.9)	34.6(31.7-37.3)	53.5(58.8-68.8)
Taurine - 1 -	69.4(65.8-73.0)	0(0-0)	8(6.2-9.3)	92(90.7-93.8)

(62% of dose) and the products were in addition to unchanged 2-naphthylacetic acid (22%), the glucuronic acid (37%) and glycine (40%) conjugates (Fig. 4.3).

The administration of taurine prior to administering 2-naphthylacetic acid did not influence the urinary elimination of [^{14}C]-2-naphthylacetic acid, with 69% being excreted in 24h. The urinary products were the glucuronic acid (8%) and glycine (92%) conjugates, and the unchanged acid was not present (Fig. 4.4).

Administration of glutamine prior to administration of [^{14}C]-2-naphthylacetic acid resulted in a much slower elimination of 2-naphthylacetic acid, only 33% of dose being found in the 24h urine (Fig 4.5).

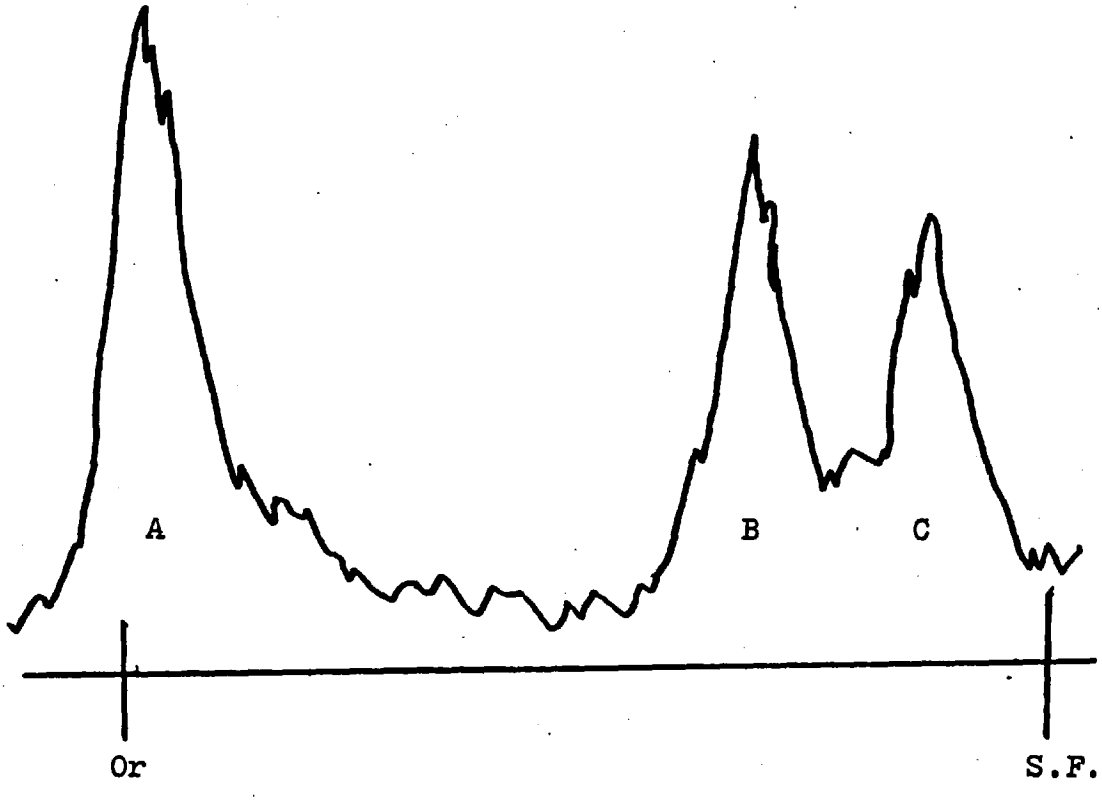
The urinary products were the glucuronic acid (35% of ^{14}C excreted) glycine (54% of ^{14}C excreted) conjugates and the unchanged 2-naphthylacetic acid (12%).

Attempt to activate glutamine and taurine conjugation in the guinea pig by administration of 2-naphthylacetic acid

The excretion of ^{14}C in the 0-24h urine after administration of 200 mg/kg 2-naphthylacetic acid daily for four days followed on the fifth day by one dose of 200 mg/kg [^{14}C]-2-naphthylacetic acid was 50% of the dose. The urinary metabolites, in addition to the unchanged compound (12%), were the glucuronic acid (10%) and glycine (28%) conjugates. These results are in agreement with results of dose-dependent studies (see Table 4.2).

Fig 4.3

Radiochromatogram scan of urine of guinea pig dosed with [^{14}C]-2-naphthylacetic acid (100 mg/kg i.p., 16 µCi) developed in Solvent A.



A = 2-Naphthylacetylglucuronide

B = 2-Naphthylacetylglycine

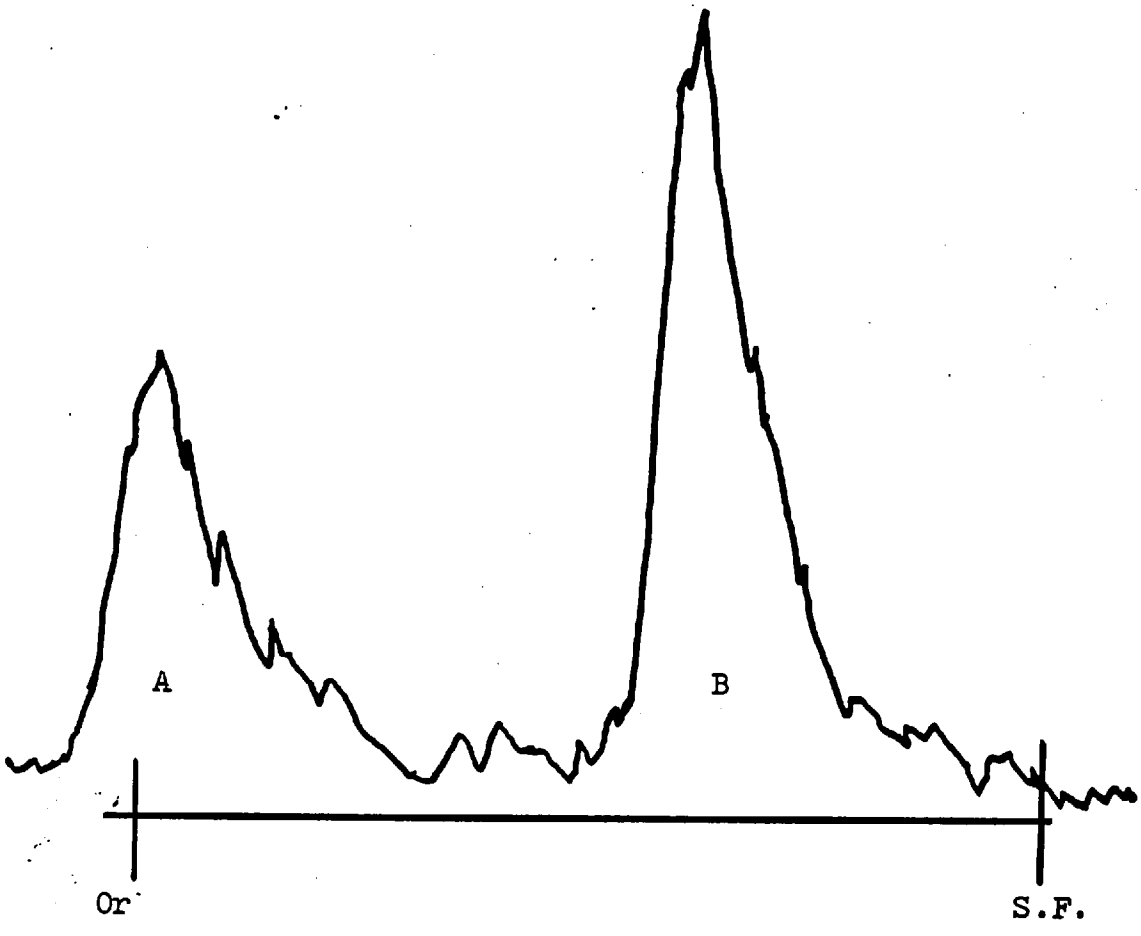
C = 2-Naphthylacetic Acid

Or = Origin

S.F. = Solvent Front.

Fig. 4.4

Radiochromatogram scan of urine of guinea pig dosed with [^{14}C]2-naphthylacetic acid and glutamine (see text) developed in Solvent A



A = 2-Naphthylacetylglucuronide

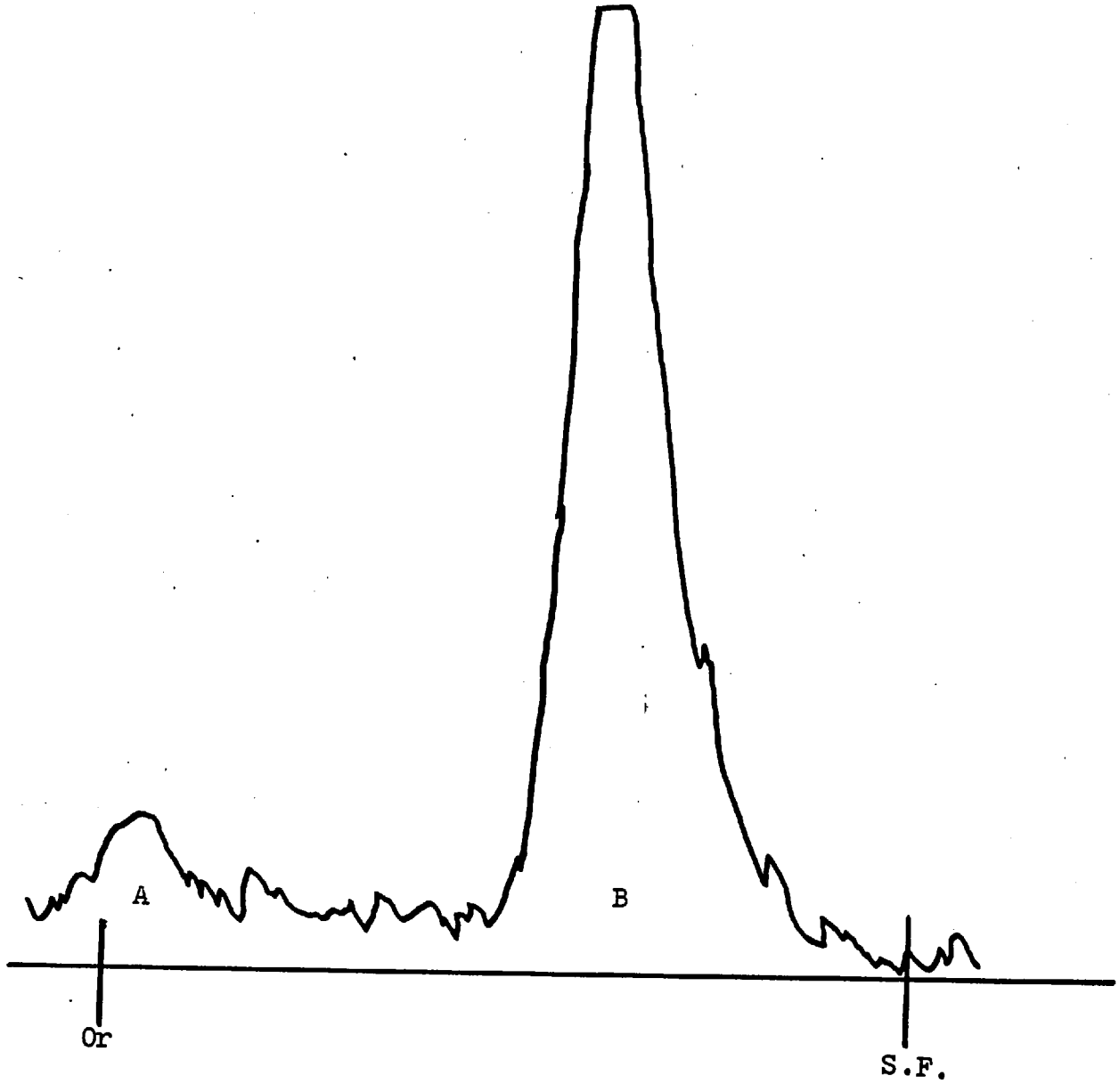
B = 2-Naphthylacetylglycine

Or = Origin

S.F. = Solvent Front.

Fig 4.5

Radiochromatogram scan of urine of guinea pig dosed with 2-naphthylacetic acid and taurine, (see text) developed in Solvent A



A = 2-Naphthylacetylglucuronide

B = 2-Naphthylacetylglycine

Or = Origin

S.F. = Solvent Front.

Discussion

The studies described in this chapter were carried out to determine the following:

i. whether or not the variable conjugation pattern encountered among the species studied is a reflection of variations in the availability of the particular amino acids (in this case glutamine and taurine for the conjugation of 2-naphthylacetic acid in the guinea pig).

ii. if induction and inhibition phenomena were influencing conjugation patterns.

The results obtained will be discussed on the basis of these two hypotheses. Various studies have established the level of various amino acids in the body fluids and tissues of several species (see Table 4.3). This table shows that the level of glutamine in the guinea pig and the rat liver are similar. However, the latter species is able to conjugate 2-naphthylacetic acid with glutamine. Therefore, it is difficult to attribute the non-conjugation of 2-naphthylacetic acid with glutamine by the guinea pig to the non-availability of glutamine but could be due to some other reasons which may be:

(a) Higher glutamine level requirement for conjugation or

(b) absence of the appropriate enzyme mechanism in this species.

The results of this study showed that supplementation of glutamine by administration of the amino acid over a five day period did not initiate glutamine conjugation in the guinea pig and therefore the apparent absence of this mechanism in this species is unlikely to be due simply to deficiency of the amino acid.

Conjugation of 2-naphthylacetic acid with taurine was not detected when a taurine supplement was administered to the guinea pig over a five day period. The result obtained here suggests that the taurine conjugation mechanism for 2-naphthylacetic acid is absent in the guinea pig. James et al. (1972a) showed the formation of phenylacetyl taurine by the guinea pig when dosed with phenylacetic acid, the quantity is very small (trace) also suggesting that this mechanism is latent or at a low level in the guinea pig. There is apparently no other published report of the taurine conjugation of arylacetic acids in the guinea pig.

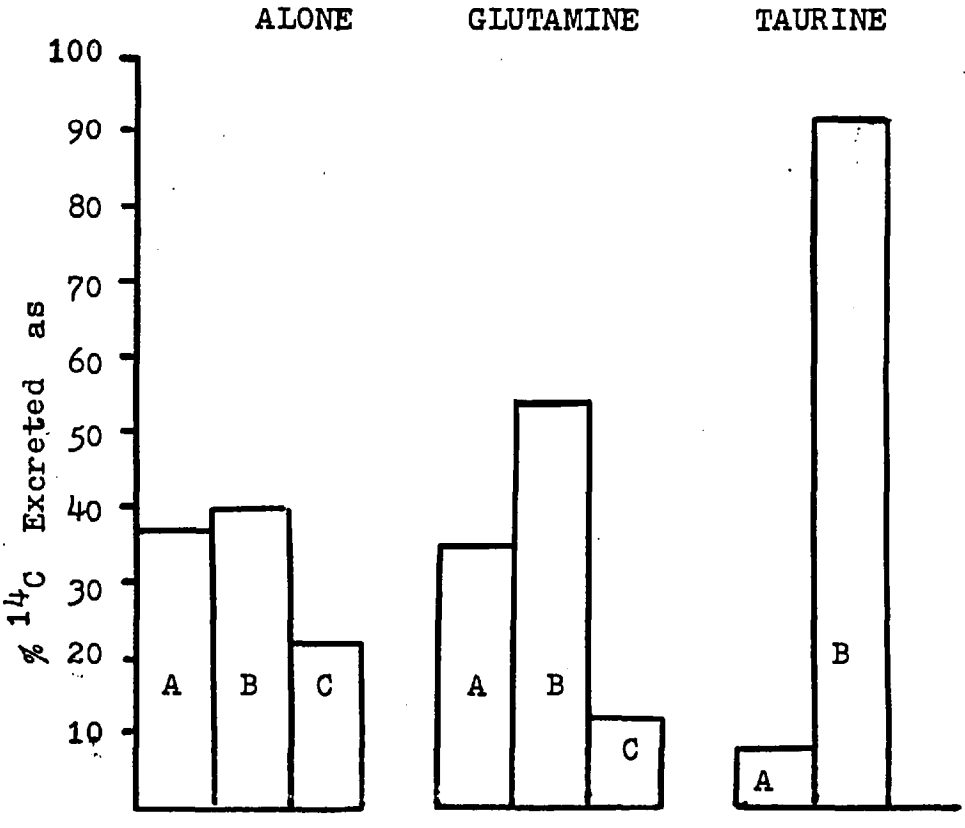
The attempts to induce the conjugation of 2-naphthylacetic acid with glutamine and taurine shows that these two conjugation mechanisms are not stimulated by administration of 2-naphthylacetic over several days. This suggests that these enzyme mechanisms are not induceable in the guinea pig by 2-naphthylacetic acid administration under the experimental conditions employed.

Influence of taurine administration on the conjugation of 2-naphthylacetic acid

The administration of glutamine and taurine

Fig. 4.6

Histogram of ^{14}C excreted after amino acid supplementation to guinea pig



A = 2-Napthylacetylglucuronide

B = 2-Naphthylacetylglycine

C = 2-Naphthylacetic acid

supplements to the guinea pig was not accompanied by the formation and excretion of 2-naphthylacetyl-glutamine and -taurine when 2-naphthylacetic acid was given. Nevertheless a most interesting effect of taurine but not glutamine administration on the pattern of conjugation was observed (Table 4.4 & Fig. 4.6). Thus following taurine administration the conjugation of 2-naphthylacetic acid was mainly with glycine due to almost complete disappearance of the glucuronic acid conjugate. This suggests that the glucuronic acid mechanism *in vivo* could be sensitive to available taurine levels. Furthermore, it suggests the possibility that the activities of the various conjugation pathways could be influenced by the availability of the conjugating agents for other reactions.

CHAPTER FOUR

FACTORS AFFECTING THE METABOLIC CONJUGATION OF 2-NAPHTHYLACETIC ACID. 3. ORIGIN OF THE TAURINE UTILISED IN THE METABOLIC CONVERSION OF 2-NAPHTHYLACETIC ACID TO 2-NAPHTHYLACETYLTAURINE.

Introduction

In recent years several studies have shown that taurine can be utilised for the metabolic conjugation of carboxylic acids in a wide range of species (see previous Chapters). Since the first observation of James et al. (1972a), who showed that the ferret was able to utilise taurine for the conjugation of phenylacetic acid, a number of arylacetic and aryloxyacetic acids and recently a benzoic acid derivative (3-phenoxybenzoic acid) have been shown to undergo taurine conjugation in various species. At present no pattern is clear concerning the species occurrence and substrate requirements of the taurine conjugation. There are suggestions that it may be more highly developed among carnivore species than other mammals investigated.

While little is known about the in vivo expression of the taurine conjugation even less is known about the mechanism and source of the taurine for conjugation. Indeed there is apparently no published work on the latter indicating whether or not taurine is used as such or in a precursor form for conjugation. A possibility that taurine itself may be used is indicated by the data of Morris and Price (1963) who observed that the taurine moiety of the glycyltaurine conjugate of quinaldic acid could be derived from exogeneously administered taurine. They showed that when [^{14}C]taurine and quinaldic acid were co-administered to cats labelled quinaldylglycyltaurine was excreted in the urine and they concluded that taurine is incorporated directly into quinaldylglycyltaurine.

These studies on the taurine conjugation of 2-naphthylacetic acid in the ferret are designed to establish the origin of taurine moiety in the formation of 2-naphthylacetyltaurine. If preformed taurine is incorporated directly into the conjugate, the [^{14}C] taurine, administered prior to administration of 2-naphthylacetic acid, should appear in the urine as radiolabelled 2-naphthylacetyltaurine. However if cysteine (cysteine is a starting compound for the biosynthesis of taurine in animal tissue) or another intermediate is first incorporated and then subsequently transformed to a taurine moiety then exogenously administered [^{14}C] taurine would not be expected to appear in the 2-naphthylacetyltaurine.

The present investigation on the source of taurine for conjugation has been carried out using the ferret since it is able to utilise taurine extensively in the conjugation of arylacetic acids (see Idle et al.,1978).

Materials and Methods

Materials and Methods are described in chapters 2,3 and 4 and only new materials and methods are presented here.

[^{14}C] taurine, sp. act. 0.91 mCi/mg, (radio-chemical purity $\geq$ 99%) was purchased from Radiochemical Centre, Amersham U.K.

Administration of compounds

2-Naphthylacetic acid (100 mg/kg) dissolved in propane-1,2-diol was administered i.p. to ferrets 2 hours prior to administration of 16 µCi of [^{14}C] taurine i.p. (100 mg/kg) dissolved in water (1 ml) to the animals.

Collection of Urine

Animals were kept singly in individual cages and urine collected for 24h after administration of the [^{14}C] taurine.

Identification of Metabolites

Chromatographic identification of urinary metabolites was as described in Chapter Two.

Reverse isotope dilution for [^{14}C] taurine

Urine samples (2 ml) of ferrets dosed with [^{14}C] taurine and 2-naphthylacetic acid was treated with 11.6M HCl (2 ml) and heated for 2h in hot water-bath at 100°C. The hydrolysate was cooled and taurine (1 g) added and left to stand at room temperature for 1h and then precipitated with a mixture (10 ml) of methanol/acetone

(2:1 by vol.). The precipitate was filtered, dried, counted for ^{14}C as already described. The crystals were redissolved in water and re-precipitated by the addition of the methanol/acetone mixture filtered and counted. This procedure was repeated several times until constant specific activity was achieved.

Results

The elimination of ^{14}C following i.p. administration of [^{14}C] taurine with/without 2-naphthylacetic acid in the ferret is shown in Table 4.5. Over a 24h period, 26% of the dose was recovered when [^{14}C] taurine alone was administered while 21% of the dose was recovered when the labelled taurine was administered with the 2-naphthylacetic acid.

Only one compound was detected when the urine of ferret dosed with [^{14}C] taurine was chromatographed and the plate scanned for ^{14}C. This had an R_F value 0.07 in solvent A (Fig. 4.7) which corresponded with the R_F of authentic taurine and its identity was further confirmed by reverse isotope dilution.

When [^{14}C] taurine and 2-naphthylacetic acid were administered to the ferret, radio t.l.c. of the urine in solvent A showed two peaks with R_F values of 0.06 and 0.18 (Fig. 4.8). The peak R_F with 0.06 corresponded to taurine while that with R_F 0.18 corresponded to 2-naphthylacetyl-[^{14}C] taurine. Treatment of the urine with 11.6M HCl caused disappearance of the peak at R_F 0.21 and a

Table 4.5

The elimination of ^{14}C by ferrets after the administration of [^{14}C] taurine alone and with 2-naphthylacetic acid

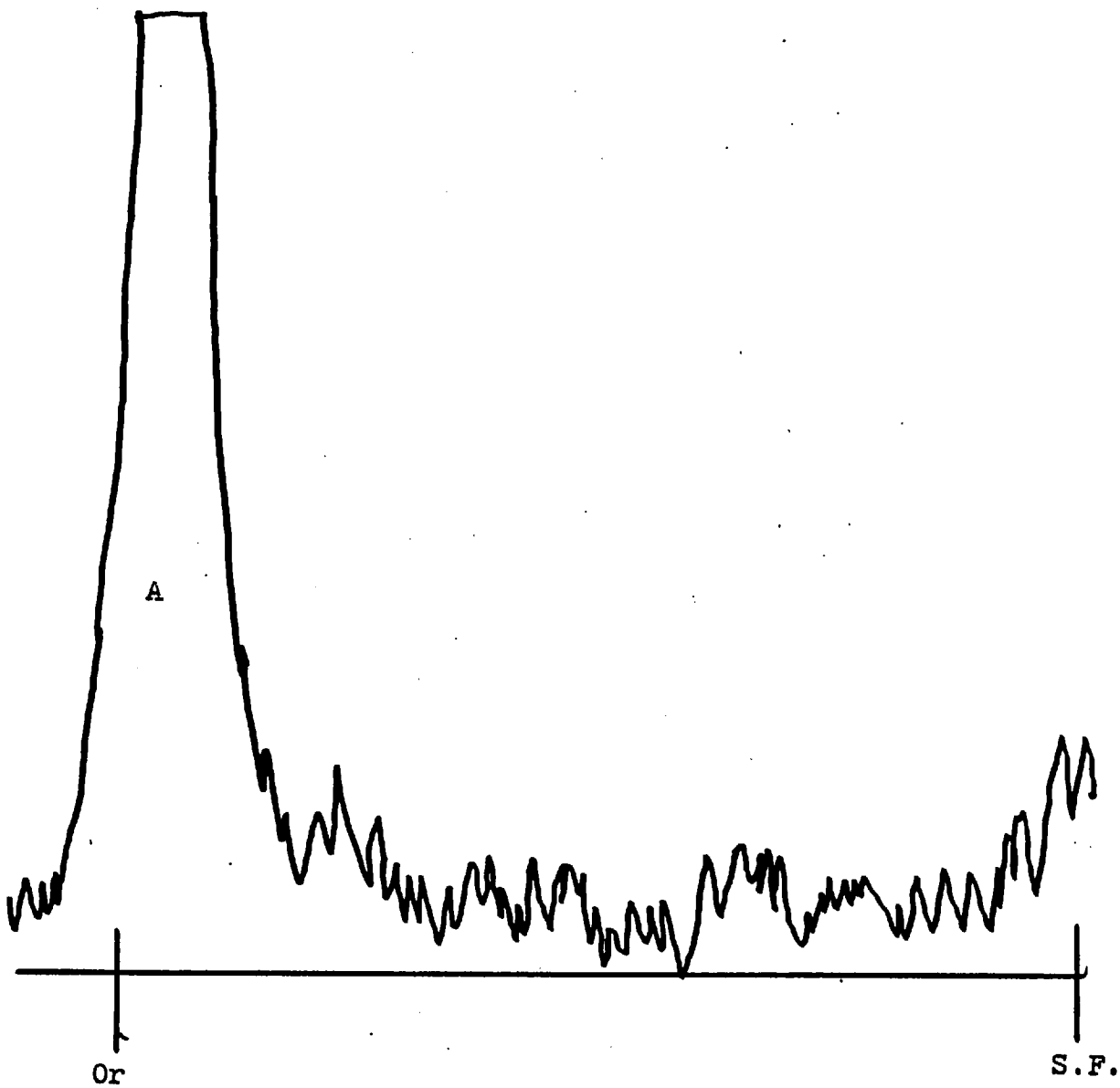
[^{14}C] Taurine (200 mg, 16 μCi/kg) dissolved in water was administered to ferrets i.p. as described followed 2 hours later by 2-naphthylacetic acid (100 mg/kg) in propane-1-2 diol was administered. Values are mean of at least 3 animals.

% ^{14}C dose excreted as	Compounds Administered	
	Taurine	Taurine + 2NAA
Taurine	26(20-31)	6(5-7)
2-Naphthylacetyl taurine	-	15(14-16)
Total ^{14}C excreted	26(20-31)	21(19-23)

2NAA = 2-Naphthylacetic Acid.

Fig. 4.7

Radiochromatogram scan of urine of ferret dosed with [^{14}C] taurine (see text) developed in Solvent A.



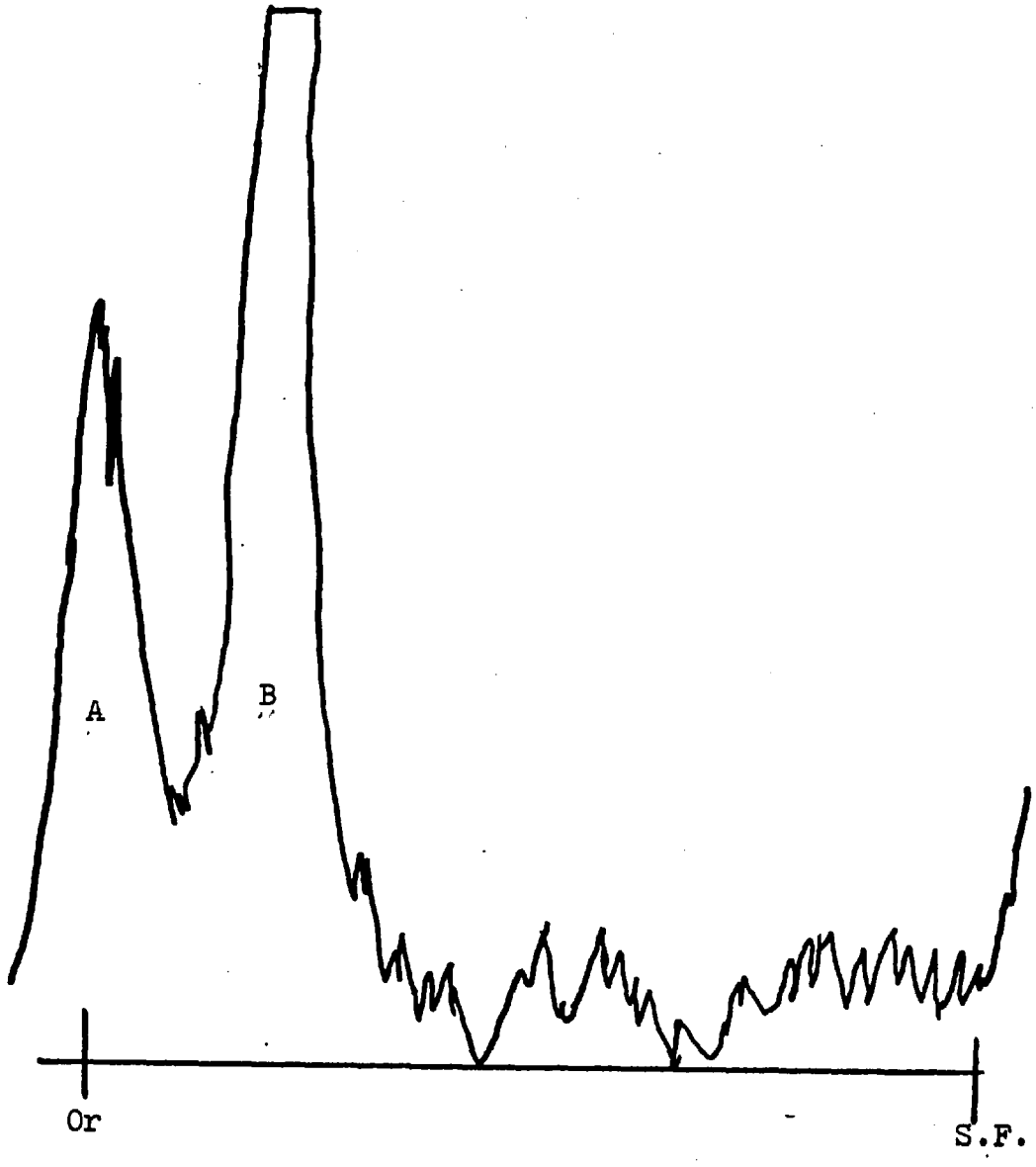
A = Taurine

Or = Origin

S.F. = Solvent Front.

Fig. 4.8

Radiochromatogram scan of urine of ferret dosed with [^{14}C] taurine and 2-naphthylacetic acid, (see text) developed in Solvent A.



A = Taurine

B = 2-Naphthylacetyltaurine

Or = Origin

S.F. = Solvent Front.

corresponding increase in the size of the peak at R_F 0.06 (Fig. 4.9). This finding and the positive result of a reverse isotope dilution for taurine confirmed that this peak was 2-naphthylacetyl-$[^{14}C]$-taurine.

The amount of 2-naphthyltaurine formed when $[^{14}C]$-2-naphthylacetic acid was administered to ferrets was 80 µmol/kg while when $[^{14}C]$ taurine was administered with cold 2-naphthylacetic acid the amount of labelled 2-naphthylacetyl-$[^{14}C]$-taurine formed was 120 µmol suggesting that all the 2-naphthylacetyltaurine formed was labelled.

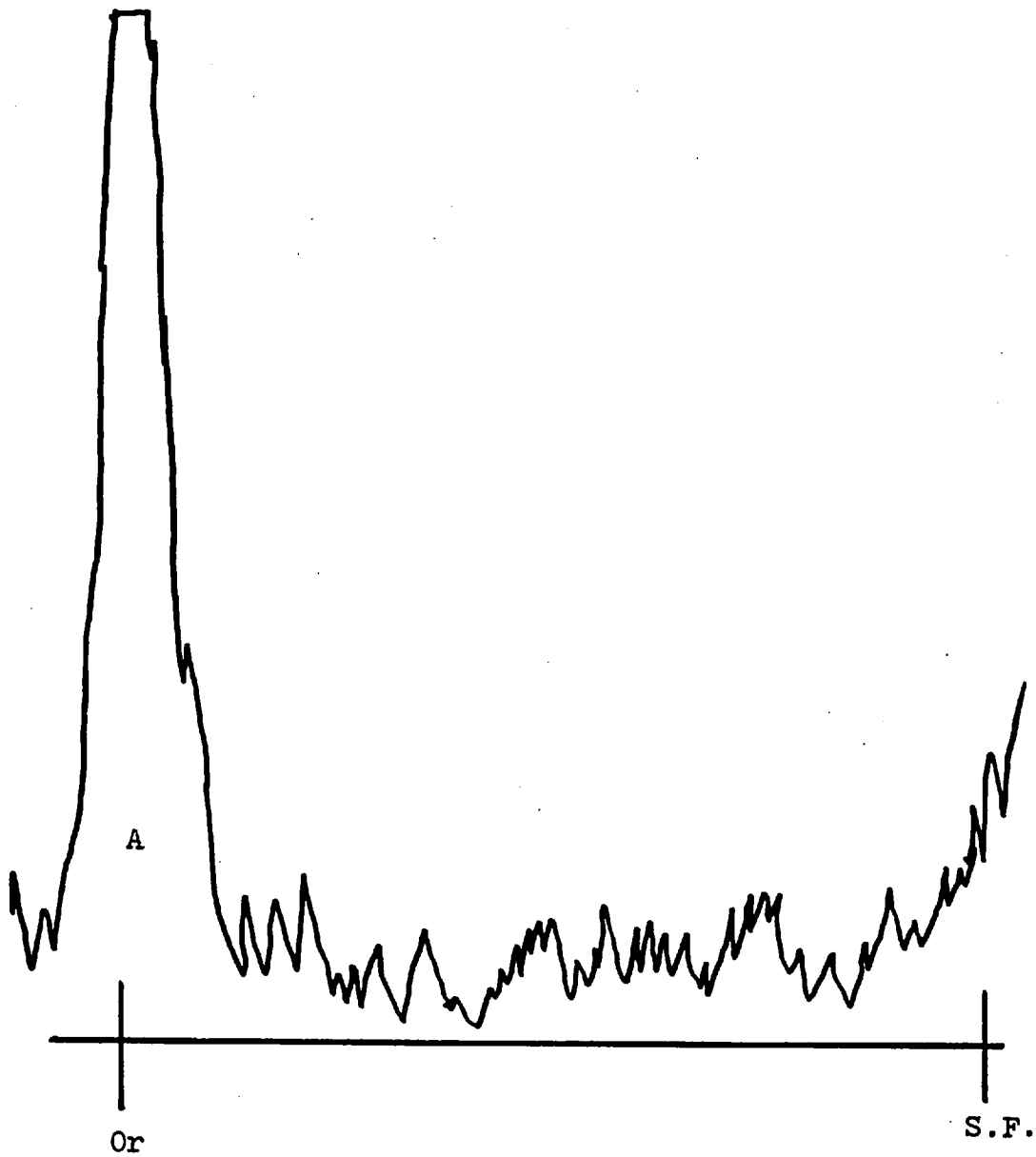
Discussion

The results of this study on the origin of the taurine moiety of 2-naphthylacetyltaurine suggest that taurine itself is used in the formation of this conjugate rather than a precursor. Thus, 2-naphthylacetic acid is not first conjugated with a precursor which is then converted to 2-naphthylacetyltaurine but is conjugated directly with taurine. This result agrees with the results of Morris and Price (1963) who showed that the cat utilises taurine directly in the conjugation of quinaldic acid to form the glycyltaurine conjugate.

At present appropriate *in vitro* methods have not been developed to study the mechanism of taurine conjugation as has been done for the glutamine and glycine conjugation mechanisms. Attempts in this laboratory to achieve this have so far achieved little success. Little is known therefore of the mechanism of the taurine conjugation of xenobiotic acids. The main salient fact to emerge from

Fig. 4.9

Radiochromatogram scan of hydrolysed urine of ferret dosed with [^{14}C]taurine and 2-naphthylacetic acid (see text), developed in Solvent A.



A = Taurine.

Or = Origin

S.F. = Solvent Front.

this study is that the ferret can utilise *in vivo* administered taurine in the biosynthesis of the taurine conjugate of 2-naphthylacetic acid. James & Bond (1978) have studied the synthesis of phenylacetyltaurine *in vitro* using fish liver as the enzyme source. They showed that the fish liver has the proper transferase which is capable of transferring taurine to phenylacetyl CoA to form phenylacetyltaurine and the release of the CoA from phenylacetyl CoA. Although this has been possible, this enzyme system has not been purified or isolated and this mechanism has not been established in mammalian species. For the time being it must be assumed that the mechanism is similar to that for glycine and glutamine conjugation.

CHAPTER FIVE

COMPARATIVE METABOLIC STUDIES OF CLOFIBRIC ACID AND CLOFIBRATE IN MAN AND FIVE NON-PRIMATE SPECIES.

CHAPTER FIVE

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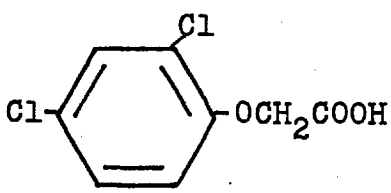
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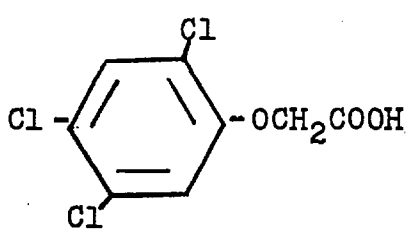
INTRODUCTION

The aryloxyacetic acids (general structure Ar. $O.CH_2COOH$) are an important group of compounds and find application in agriculture, as herbicides and growth hormones, and as drugs. Some examples are as follows:

Herbicides

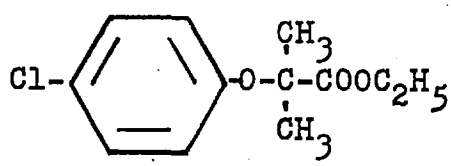


2,4-Dichlorophenoxyacetic acid

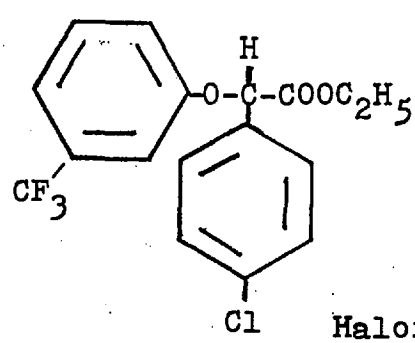


2,4,5-Trichlorophenoxy-acetic acid.

Drugs



Clofibrate



Halofenate

The widespread use of aryloxyacetic acids in medicine and agriculture naturally stimulated an interest in their metabolic fate both in animal and plant species. The earliest metabolic study was that of Levy and Lewis (1947) who reported that phenoxyacetic acid is metabolically inert in the rabbit and is excreted unchanged. Since this time the metabolic fate of a number of aryloxyacetic acids has been reported and the findings are summarised in Table 5.1. In general it appears that α-unsubstituted aryloxyacetic acids are excreted unchanged or conjugated to a small extent with glycine and/or taurine depending upon species. The

Table 5.1

Metabolism of some aryoxyacetic acids in various species.

Compound	Species	Metabolic Pattern	References
Phenoxyacetic acid	Rabbit	Unchanged	Levey and Lewis (1947)
o-Chlorophenoxyacetic acid	Rabbit	Unchanged	Levey and Lewis (1947)
p-Chlorophenoxyacetic acid	Rabbit	Unchanged	Levey and Lewis (1947)
2,4-Dichlorophenoxyacetic acid	Sheep	Unchanged	Clark (1964)
	Rat	Glycine (1.4%)	Khanna and Fang (1966)
		Taurine (1.4%)	Khanna and Fang (1966)
	Man	Unchanged	Kohli et al. (1974)
	Dogfish	Taurine	James and Bend (1976)
	Flounder	Taurine	James and Bend (1976)
2,4,5-Trichlorophenoxyacetic acid	Rat	Unchanged	Piper et al (1973)
	Dog	Unchanged	Piper et al (1975)
	Man	Unchanged	Gehring et al (1973)
	Dogfish	Taurine	Guarino et al (1977)
	Flounder	Taurine	Guarino et al (1977)

P.T.O.

Table 5.1 contd.

Clofibrate	Man	Glucuronic acid	Thorp and Waring (1962), Faed and McQueen (1978).
	Rat	Glucuronic acid	Thorp and Waring (1962).
	Dog	Glucuronic acid unknown conjugate	Cayen et al. (1977)

α-substituted acid clofibrate however undergoes extensive glucuronic acid conjugation in man and animals.

Clofibrate and Clofibric Acid

Clofibrate is the ethyl ester of clofibric acid (4-chlorophenoxyisobutyric acid) and is currently widely used as a hypocholesterolaemic agent in man. This application is based upon the studies of Thorp and Waring (1962) who showed that several aryloxyacetic acids including clofibric acid have the ability to reduce total lipid and cholesterol levels in blood and liver. It was introduced into the U.K. in 1962 by I.C.I. Ltd. under the trade name Atromid for promoting a reduction in circulating lipid and cholesterol levels.

Absorption, distribution and elimination

Clofibrate is well absorbed and distributed through the tissues of dogs, and rats following its oral administration; the highest level being found in the liver (Cayen et al. 1977). In man, it is also well absorbed when given orally and it rapidly appears in blood as the deesterified clofibric acid with a plasma concentration peak of the acid occurring 4h after oral administration.

The urinary elimination of clofibrate after oral administration seems to be species dependent; over a 24h period almost all the administered drug is recovered in the urine of rat (85%) and man (78%) while it is eliminated slowly in the dog (25%) with equal amount of the drug appearing in the faeces over the same time period.

In all the species investigated, clofibrate is rapidly converted to the free acid, clofibric acid, by esterases present in the blood and tissues. The acid is then conjugated with glucuronic acid both in the rat and man although the extent of conjugation differs with man apparently having a higher capacity compared to the rat where only half of the urinary material consists of the glucuronic acid conjugate. The excretion products in the dog differ in that in addition to conjugating it with glucuronic acid, it is capable of utilising another mechanism, since an unknown compound cleaved to clofibric acid by treatment with conc. KOH was also present.

Most studies reported on clofibrate in the literature have been directed towards its use as an antihyperlipidaemic drug in man. It is noteworthy that despite its widespread use, no full comparative metabolic study of clofibrate has been carried out and it is thus of interest to carry out such an investigation especially in view of dog data. There has also been a discussion in the literature concerning the structure of the glucuronic acid conjugate of clofibric acid present in human urine. Faed and McQueen (1978) have claimed that there are two types of glucuronic acid conjugate of clofibric acid while Thorp and Waring (1962) and others assumed that only one glucuronic acid conjugate is formed. The present study will therefore be concerned with the following:

i. To confirm the metabolic fate of clofibrate and clofibric acid in man and rat.

ii. To investigate the metabolic fate of clofibric acid and clofibrate in various other species; dog, ferret, cat, guinea pig and rabbit.

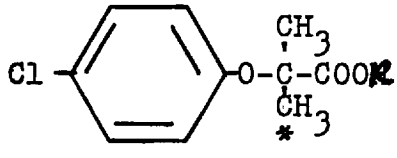
iii. To isolate and to determine the structure of clofibrylglucuronide isolated from the urine of man and rabbit following dosing with clofibric acid.

Materials and Methods

[^{14}C]-Clofibrate (ethyl 2-(4'-chlorophenoxy)-2-[^{14}C-methyl] propionate), sp. act. 7.3 μCi/mg, radiochemical purity 98%, an oil, and [^{14}C]-clofibric acid (2-(4'-chlorophenoxy)-2-[^{14}C-methyl] propionic acid, sp. act. 8.2 μCi/mg, m.p. 122° both were labelled on the methyl carbon (see below), were the gifts of ICI Pharmaceuticals Division, Alderley Park, Cheshire, U.K. All other compounds were purchased and purified as appropriate prior to use.

R = H Clofibric acid

R = C_2H_5 Clofibrate



* POSITION OF LABELLING.

Clofibrylglycine (2-(4'-chlorophenoxy)-2-methylpropionyl-glycine)

This compound was prepared by method described by Sheehan and Hess (1955) by directly coupling the carboxyl function of clofibric acid with the free amino group of glycine on treatment with N,N'-dicyclohexylcarbodiimide at

room temperature.

Clofibric acid (5 g) and glycine ethyl ester (2.4 g) dissolved in tetrahydrofuran (25 ml)were treated with a solution of dicyclohexylcarbodiimide (4.8 g) in tetrahydrofuran (10 ml), the reaction mixture left at room temperature with stirring for 24 h. Glacial acetic acid (5 ml) was added to decompose the excess carbodiimide, and the insoluble urea so formed was filtered and the filtrate reduced to dryness in vacuo. The residue was dissolved in ethyl acetate (50 ml), this washed with 2N-HCl (15 ml) and the organic solvent removed on the rotary evaporator. The residue was dissolved in M-NaOH (10 ml) and stirred at 40° for 4 h, filtered, the filtrate adjusted to pH2 with M-H_2SO_4, extracted with ethyl acetate (3 x 3 vol.) the combined extracts dried (anhyd. Na_2SO_4) and evaporated in vacuo. The residue was dissolved in the minimum volume of 2M-NH_4OH and 2M-HCl added slowly whereby white crystals separated. The solution was kept at 4° for 48 h and white crystals of the title compound harvested by filtration, m.p. 100-105°. Clofibrylglycine, $C_{12}H_{14}O_4$ NCl requires C, 53.0; H, 5.2; N, 5.2; Cl, 13.1. Found C, 52.7; H, 5.2; N, 5.2; Cl, 13.0.

Clofibryltaurine

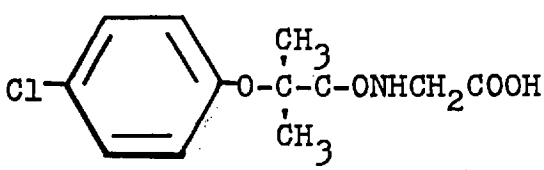
All efforts to synthesise this by the Schotten-Baumann reaction (see Chapter Two) and the above method proved abortive and clofibryltaurine was therefore obtained biosynthetically by isolation from urine of beagle dogs

dosed with clofibric acid.

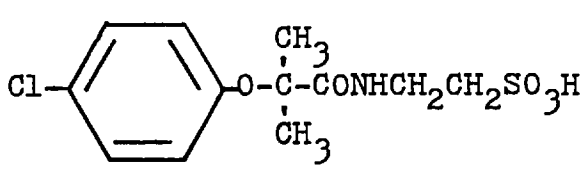
Urine (100 ml) of a dog dosed with [^{14}C]-clofibric acid (100 mg/kg 0.4 uCi/kg p.o) was adjusted to pH2 with glacial acetic acid and extracted with ethyl acetate (3 x 3 vol) to remove the free acid and its glucuronide. After separating the phases, dissolved ethyl acetate in the aqueous phase was removed at 40° on the rotary evaporator. The ethyl acetate free aqueous phase was then freeze-dried and the residue extracted with hot methanol (3 x 50 ml). The methanol was removed in vacuo and the residue dissolved in water (5 ml) and an aliquot chromatographed (t.l.c. Solvent B) and then scanned for ^{14}C. One peak was observed with R_F value of 0.10 in System H and 0.63 in System B.

Attempts to crystallise this material from the extract were abortive. Its identity and properties were therefore established as follows. Equal volumes (1 ml) of the extract and conc. HCl were taken, the mixture sealed in a thick-walled glass tube and heated in an oven at 120°C for 18 h. The tube was cooled, opened cautiously and the contents dried in a vacuum dessicator over NaOH pellets. The dry residue was dissolved in distilled water (2 ml). The solution was examined as follows. Aliquots were subjected to t.l.c. in systems A and H, and the chromatograms scanned for ^{14}C. In system A, one ^{14}C peak was observed with R_F 0.89 which corresponded to free clofibric acid, and when the plates were sprayed with ninhydrin a purple spot was seen

at R_F 0.10 corresponding to free taurine. In system H one ^{14}C peak was found with R_F 0.51, corresponding to clofibric acid and when the plates were sprayed with isatin a yellow spot at R_F 0.05 appeared corresponding to free taurine. Another aliquot was treated with dansyl chloride solution (see chapter 2) and chromatographed in solvent system D. On viewing under u.v. light, an intensely fluorescent spot with R_F 0.30 was observed, and this corresponded to dansyltaurine.



CLOFIBRYLGLYCINE



CLOFIBRYLTAURINE

Clofibrylglucuronide

This was isolated from the urine of a female rabbit which was given on each of two days a single dose of clofibric acid (1 g p.o). The urine was collected for three days and then freeze-dried. The residue was extracted with methanol (3 x 50 ml), the solvent removed *in vacuo* and the residue dissolved in water (20 ml). After adjusting to pH2 with 0.5M HCl, the solution was extracted with ether (3 x 50 ml), dried over anyhyd. Na_2SO_4 and the ether evaporated *in vacuo*. The residue was dissolved in dry methanol (20 ml) adjusted to pH7 with 0.1M KOH and the volume reduced to about 10 ml in a rotary evaporator. Cold dry ether was added slowly to the chilled solution until it became cloudy, and the solution was stored at -20° until a white precipitate of the potassium salt of clofibrylglucuronide had formed (about 48 h) (500 mg). This was filtered and recrystallised from ethanol/ether, m.p. 155-160°. This material gave an intense positive reaction with naphthoresorcinol and was shown chromatographically to be cleaved following incubation with β-glucuronidase to give clofibric acid. A full account of the properties of this conjugate is given later.

The glucuronide of clofibric acid was also isolated from the urine of a male volunteer who took orally 1.5 g of clofibric acid containing 1 μCi of ^{14}C. The 24 h urine (containing 49% of the dose) was adjusted to pH2, extracted with *n*- hexane to remove clofibric acid (4% of dose) and then with ethyl acetate, which removed all the remaining radioactivity. T.l.c. of this extract showed only one

compound to be present, which was isolated by preparative t.l.c. as described in chapter 2. The crystals obtained (200 mg) had a melting point of 157-161°.

Animals

Female Wistar albino rats (150-200g), female Dunkin-Hartley guinea pigs (200-250g), female Dutch rabbits (2-2.5kg) and female polecat ferrets (500-900g) were purchased from suppliers in the London area. Male mongrel cats (3.2-3.5kg), and male ICI beagle dogs (14.2-19.5 kg) were from stocks maintained by ICI Pharmaceuticals Division.

Human Volunteers

Six healthy male volunteers, who gave their informed consent, participated in the trials, ages 22-28 years, weights 70-80 kg.

Administration of compounds

Animals

$[^{14}C]$-Clofibric acid (100 mg/kg) and $[^{14}C]$-clofibrate (113 mg/kg) were administered in propane-1,2-diol to rats, guinea pigs, and ferrets by intraperitoneal injection and orally in propane 1-2-diol to rabbits.

Cats and dogs were dosed orally with $[^{14}C]$-clofibric acid in aqueous solution at ICI Pharmaceuticals Division and the urines transported frozen to London for analysis.

Collection of urine

Rats, guinea pigs, rabbits, ferrets, dogs and cats were kept in individual metabolism cages and food was withheld for the 24 h post-dosing. They had free access to water.

Urine was collected for periods of 1-3 days.

Human studies

[^{14}C]-Clofibric acid (500 mg or 1500 mg), [^{14}C]-clofibrate (565 mg) were taken orally in a hard gelatin capsule early in the morning after a light breakfast.

Human volunteers kept to their normal intake of fluids and solid food and collected their urine every 2 hours for the first 12h, and thence from 12-24 and 24-48 hours, into bottles containing 2 ml of acetic acid and chloroform mixture (1:1 by volume) as preservative.

Storage of Urine

All excreta were kept at -20^{o}without pH adjustment prior to analysis.

Chromatography

Thin-layer chromatography (t.l.c.) of clofibric acid and its conjugates, certain amino acids and their dansyl derivatives and sugars was performed using plastic backed silica gel 60 HF_{254} plates (Cat. No. 5735, Merck A.G. Darmstadt, W. Germany), except for the dansylamino acids for which non-fluorescent silica gel plates (Merck Cat. No. 5748) were used.

A	Benzene/acetone/glacial acetic acid	2: 2:1 by vol.
B	Chloroform/methanol/glacial acetic acid	24: 8:1 by vol.
D	Chloroform/methanol/glacial acetic acid	15: 4:1 by vol.
F	Butan-1-ol/acetic acid/water	4: 1:1 by vol.
H	Benzene/ethanol/glacial acetic acid	80:12:1 by vol.
I	Isopropanol/water	7:3 by vol.

The plates were developed for 12 cm from the origin in the above solvent systems. R_F values and colour reactions are presented in Table 5.2.

Nuclear Magnetic Resonance (NMR) Spectra

Proton NMR spectra were obtained using Varian EM 390 while 13C NMR spectra were obtained using Bruker HX 90E instruments at ICI. Concentration of clofibrylglucuronide isolated from rabbit urine used was 40 mg/0.5 ml D_2O.

Mass spectrometry

Mass spectra were obtained using a Varian-MAT CH5 instrument with Spectro-System 100 data system by direct insertion. The ionising voltage was 70 eV.

Infra red spectroscopy

A Perkin Elmer 157G infra-red spectrometer was used.

Radiochemical techniques.

These are described in detail in Chapter Two.

Treatment of urine samples

Mild alkaline hydrolysis and treatment with β-glucuronidase were as described in Chapter Two.

Ammonolysis

Isolated clofibrylglucuronide was spotted on t.l.c. plates and these placed in a tank containing 0.88 S.G. ammonia vapour for 24h. They were then developed in solvent system I and then viewed under u.v. light.

Reverse isotope dilution for clofibrylglucuronide

The clofibrylglucuronide present in urine (10 ml) of

Table 5.2 Chromatographic properties of Clofibric acid and related compounds

Compound	Solvent	R_F value A	B	H	I	Appearance beneath u.v.	Colour reactions with Naphthoresorcinol
Clofibric acid		0.89	0.91	0.51	-	dark purple	-
Clofibrylglycine		0.84	0.88	0.37	-	dark purple	-
Clofibryltaurine		-	0.63	0.10	-	dark purple	-
Clofibrylglucuronide		0.33	0.21	0.04	-	dark purple	purple
Xylose		-	-	-	0.46		purple blue
Ribose		-	-	-	0.42		purple blue
Glucose		-	-	-	0.26		purple blue
Glucuronic acid		-	-	-	0.07		purple

Chromatograms were run on silica gel plates.

rabbit dosed with [^{14}C]-clofibric acid was extracted into ethyl acetate and the extract evaporated to dryness *in vacuo*. The residue was dissolved in 1M NaOH (10 ml) and heated on water bath for 30 min. Clofibric acid (1g) was added to the solution and left at room temperature for 1h and the volume reduced by evaporation in a rotary evaporator and allowed to cool. The precipitate so formed was filtered and recrystalised from water to constant specific radioactivity.

Identification of metabolites of clofibric acid and clofibrate

Clofibrate, being an ester, is hydrolysed to clofibric acid in the animal body before conjugated and therefore the same identification procedures were used for the two compounds.

Rat, rabbit and guinea pig urine

Aliquots of the 24h urine (0.1 ml) of rat, rabbit and guinea pig were subjected to t.l.c. in solvent B and the chromatograms scanned for ^{14}C. In each case, two peaks were observed whose centres had R_F 0.21 and 0.90. The plates were sprayed with the naphthoresorcinol spray and the peak at R_F 0.21 gave a purple colour showing the presence of glucuronic acid. Treatment of the urine with M-NaOH or β-glucuronidase caused the disappearance of the peak at R_F 0.21 with an equivalent increase in the peak of R_F 0.90. Comparison of R_F values with those of authentic samples showed the peak at R_F 0.21 to be clofibrylglucuronide and that at R_F 0.90 was clofibric acid.

Dog and Ferret

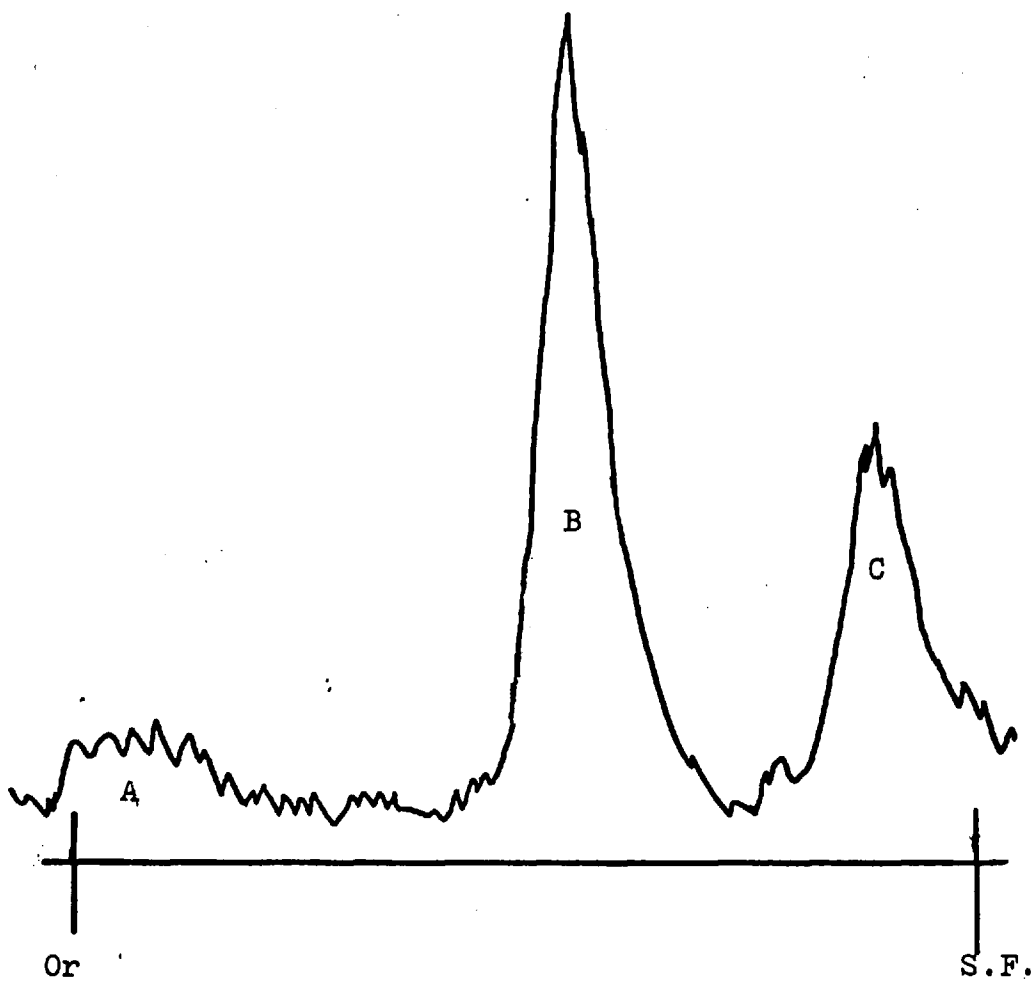
Aliquots of the 24h urine (0.1 ml) of dog and ferret were subjected to t.l.c. in solvent B and the chromatograms scanned for ^{14}C. This revealed three peaks whose centres had R_F 0.21, 0.65 and 0.91 (Fig. 5.1). The peak of R_F 0.21 gave a purple colour with naphthoresorcinol, and treatment of the urine with either M NaOH or β-glucuronidase resulted in its disappearance with a corresponding increase in that at R_F 0.91. The peak of R_F 0.21 was identified as the glucuronic acid conjugate of clofibric acid. The metabolite with R_F 0.63 was isolated following elution from chromatogram and hydrolysed with conc. HCl as previously described. An aliquot was chromatographed in system A and sprayed with isatin reagent. A yellow spot, R_F 0.07 revealed the presence of taurine in the hydrolysate. When the the hydrolysate was dansylated as previously described and chromatographed it revealed the presence of dansyl-taurine (R_F 0.32) in solvent C. The second metabolite in dog and ferret urine was therefore identified as clofibryltaurine.

Cat

Aliquots (0.1 ml) of the 0-24h urine of cats dosed with $[^{14}C]$-clofibric acid was subjected to t.l.c. in solvent B and the chromatogram scanned for ^{14}C. Two peaks were observed whose centres had R_F 0.65 and 0.91. The peak at R_F 0.91 corresponded to clofibric acid while that at R_F 0.65 was treated as described for dog and ferret and was identified as clofibryltaurine.

Fig 5.1

Radiochromatogram scan of urine of ferret dosed with [^{14}C]-Clofibric acid (100 mg/kg, i.p., 0.5 µCi) developed in Solvent A.



A = Clofibrylglucuronide B = Clofibryl taurine

C = Clofibric Acid.

Or = Origin S.F. = Solvent Front.

Man

Aliquots (0.1-0.5 ml) of urine samples obtained from volunteers dosed with [^{14}C] clofibric acid were examined by t.l.c. in solvent H, and the chromatograms scanned for ^{14}C. Two peaks were observed with R_F values 0.04 and 0.51 (Fig 5.2). The peak at R_F 0.04 gave a positive reaction to naphthoresorcinol and disappeared when urine was treated with either M NaOH or β-glucuronidase with a corresponding increase in the peak at R_F 0.51. This peak at R_F 0.04 was identified as clofibrylglucuronide while the peak at R_F 0.51 corresponded to clofibric acid.

Similarly, t.l.c. in solvent B and A showed the presence on scanning of two peaks at R_Fs 0.21 and 0.90, and 0.33 and 0.90 respectively. The peaks at R_F 0.21 in solvent B and R_F 0.33 in solvent A gave a positive reaction with naphthoresorcinol and disappeared on treatment of the urine with M NaOH or β-glucuronidase with concomitant increases in the peaks at R_F 0.90. The peaks were identified as clofibrylglucuronide and clofibric acid respectively.

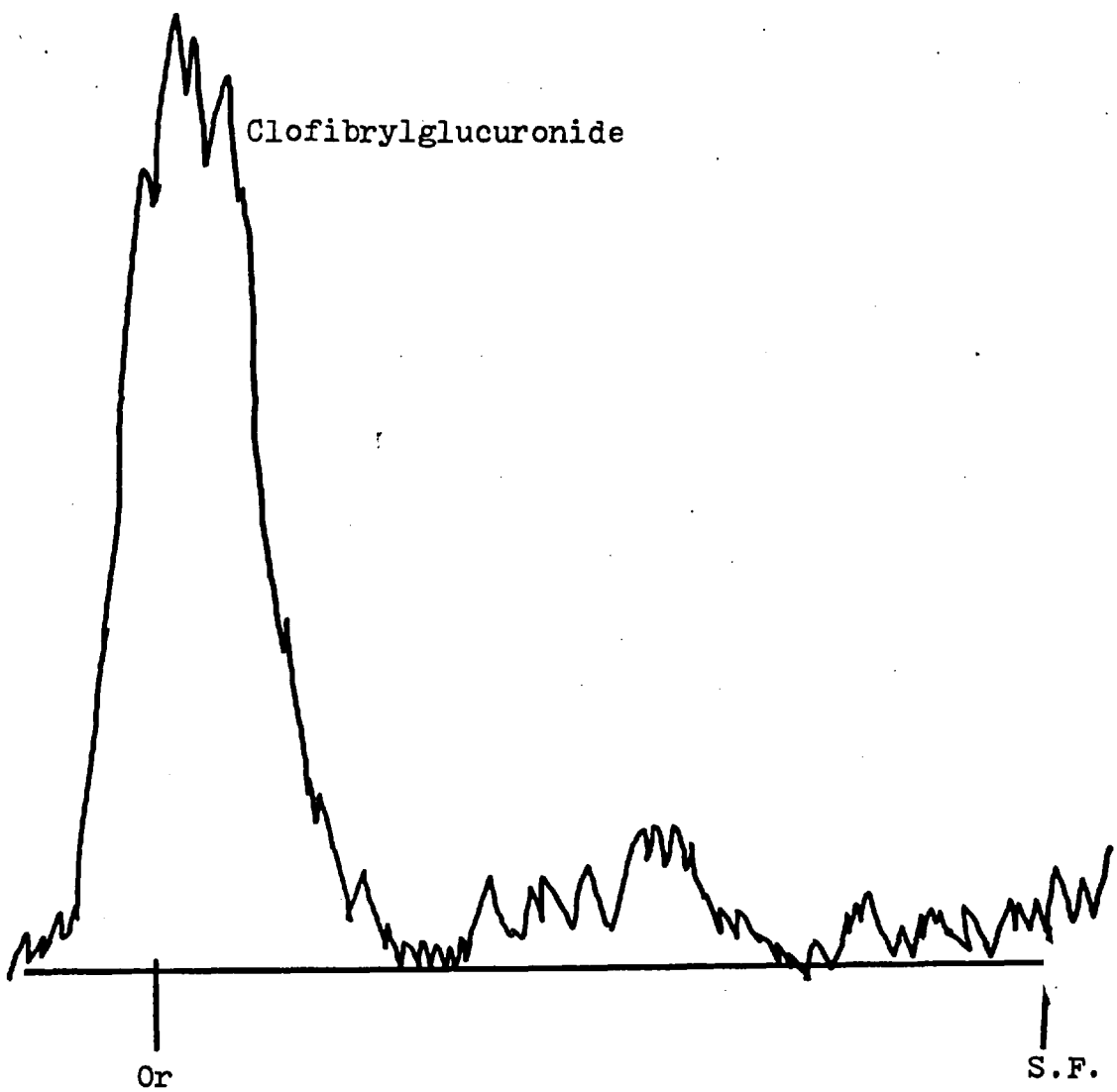
Results

Clofibrylglucuronide

Elemental analysis of the isolated potassium salt of clofibrylglucuronide gave C 43.1, 43.3; H 4.4, 4.4; Cl 7.53 which corresponds to C_{16} H_{18} O_9 Cl K. H_2O which requires C, 43.0; H, 4.4; Cl. 7.9;. The identity of the sugar moeity was shown by t.l.c. following ammonolysis(NaOH and β-glucuronidase hydrolysis)of the conjugate on t.l.c. plates

Fig 5.2

Radiochromatogram scan of urine of a human volunteer dosed with $[^{14}C]$-Clofibric acid (500 mg, p.o; 1 μCi) developed in System B.



Or = Origin S.F. = Solvent Front.

and these procedures resulted in the complete cleavage of the conjugate to glucuronic acid and clofibric acid. The R_F of the glucuronic acid corresponded in R_F to an authentic sample of glucuronic acid and reacted with naphthoresorcinol to give a purple colour.

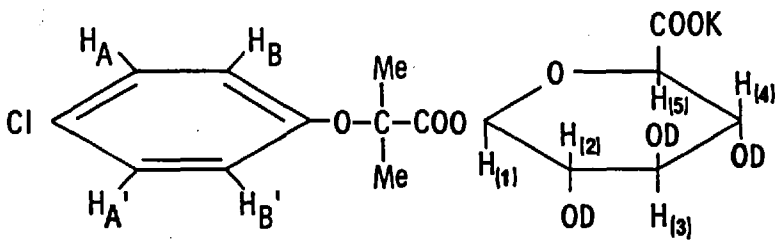
Final confirmation of the structure of the conjugate was given by proton and C-13 magnetic resonance spectroscopy (all chemical shifts are relative to TMS) (Fig. 5.3, 5.4). The proton spectrum (in HOD at 90mHz) showed characteristic AA'BB' signals (4 protons) at 7.3 δ and 6.95 δ due to the 4'-substituted benzene ring, and a singlet (6 protons) at 1.68 δ arising from the side chain methyl groups. The glucuronic acid moiety gives a multiplet (4 protons) at 3.4 δ, from the protons on C-2, 3, 4 and 5 of the sugar ring, and a doublet at 5.65 δ from the anomeric proton on C-1. A spectrum obtained at 220mHz confirmed that this signal was a doublet and the size of the coupling constant indicated that the protons on C-1 and C-2 were axial to each other, i.e. the sugar has the β-configuration. The hydroxyl protons are replaced by D from the solvent and are thus not seen.

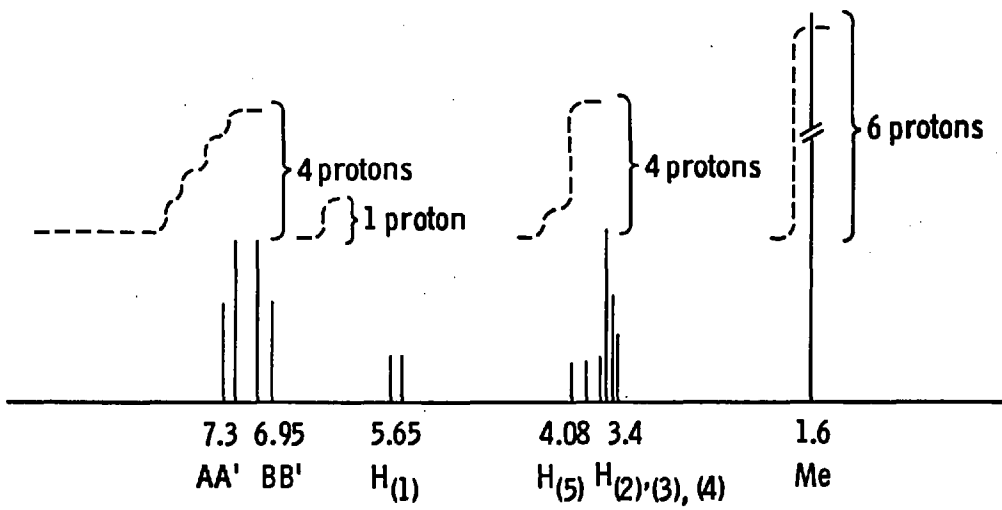
C-13 spectra were obtained with proton noise decoupling and with single frequency off resonance decoupling (S.F.O.R.D). The carbonyl carbons at C-6 of glucuronic acid and of CPIB appear at 176.0 δ and 175.5 δ. The carbons of the benzene ring appear at 153.8 δ (that bearing Cl), 129.6 δ (that bearing O) and those bearing protons at 130.6 δ and 123.6 δ. These latter become doublets

Fig. 5.3

Proton Magnetic Resonance Spectrum of Clofibrylglucuronide Isolated from Rabbit Urine (Ca 40mg/0.5ml D_2O)

A





B Integration

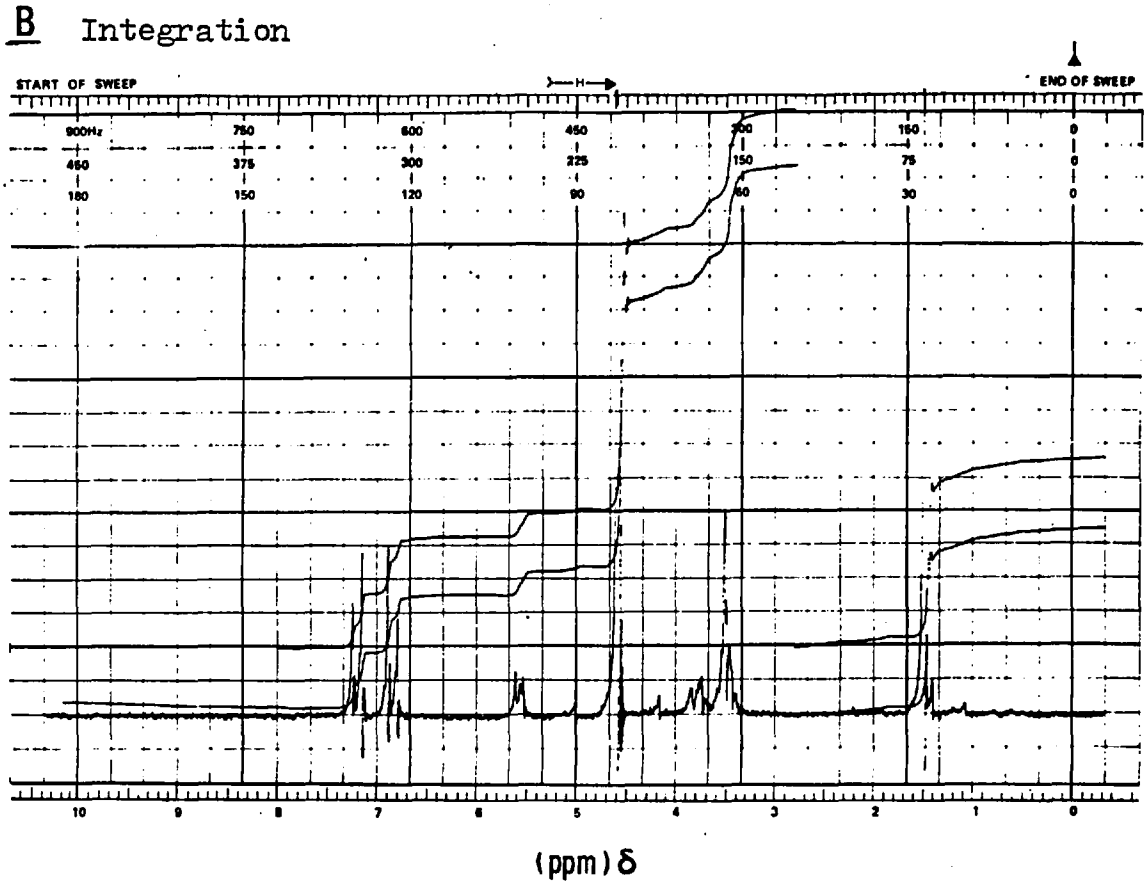
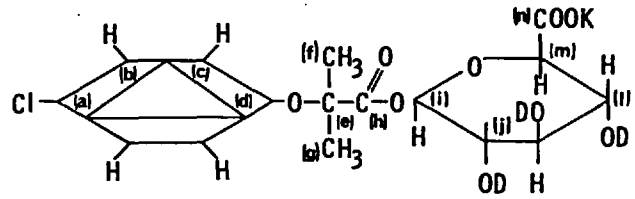
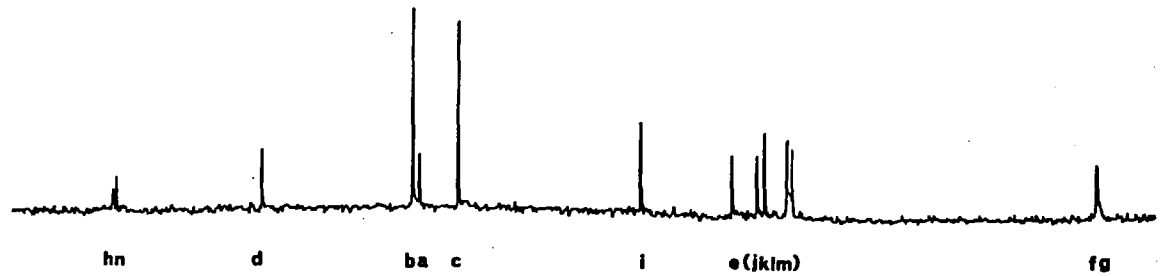


Fig. 5.4

Carbon 13 Pulse Spectrum (A) and Single Frequency off Resonance Decoupling (S.F.O.R.D.) Spectrum (B) of Clofibrylglucuronide Isolated from Rabbit Urine (40 mg/0.5 ml D_2O)

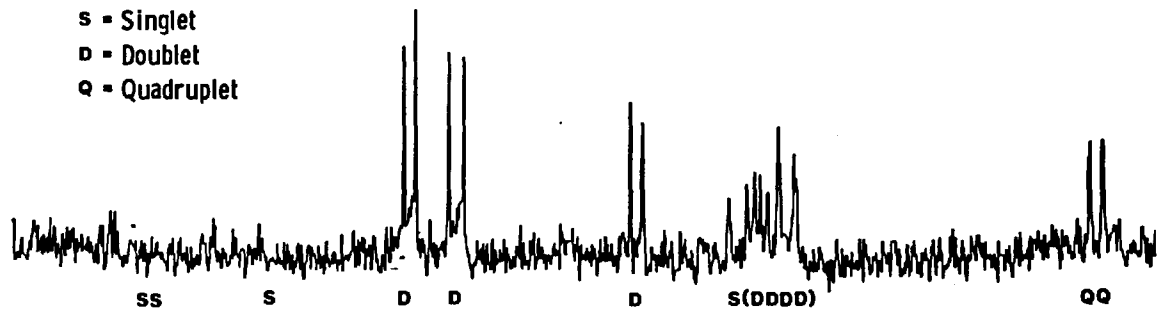


A



Assignment carbon

B



on S.F.O.R.D. The quaternary carbon of CPIB is seen at 81.8 δ (singlet on S.F.O.R.D) and the two methyl groups appear at 25.5 δ and 25.3 δ , the slight difference being due to the influence of the asymmetric centres in the molecule. The glucuronic acid ring carbons are at 95.9 δ (C-1) and 77.8 δ , 76.6 δ , 73.0 δ and 72.7 δ (C-2 to 5 respectively), all of which become doublets on S.F.O.R.D. Taken together, these data show that the conjugate of CPIB isolated from rabbit urine is potassium 1-O-(4'chlorophenoxyisobutyryl)-glucosiduronate, the structure shown in Fig. 5.5.

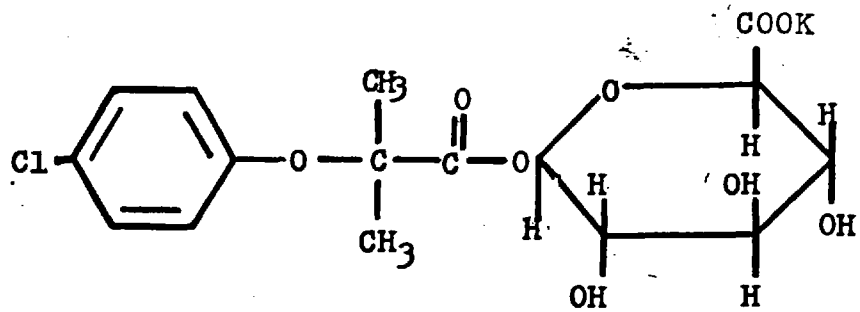
The compound isolated from human urine was identical in chemical reactions, chromatographic mobility and hydrolysis behaviour to that isolated from rabbit urine, and preliminary n.m.r. studies show the similarity of the two conjugates.

Rat, guinea pig, rabbit, cat, dog and ferret

The qualitative and quantitative aspects of the urinary elimination of [^{14}C] clofibric acid and [^{14}C] clofibrate by various species are shown in Tables 5.3 and 5.4. Table 5.3 gives results for the rat, guinea pig and rabbit while the findings for the three carnivore species (cat, dog and ferret) are shown in Table 5.4. In the case of the rat, guinea pig and rabbit there were no marked differences in the observed excretion patterns seen for clofibric acid and clofibrate. There is an indication that total urinary recovery is higher in the guinea pig and rabbit when clofibrate as opposed to clofibric acid, is given. For both compounds and all three species the urinary recovery

Fig. 5.5

Structure of isolated clofibrylglucuronide from rabbit urine



Potassium 1-O- (4'Chlorophenoxyisobutyryl) - glucosiduronate

Table 5.3 Urinary Metabolites of [^{14}C]-Clofibric acid and [^{14}C]Clofibrate in the Rat, Guinea Pig and Rabbit

Species	(No.,Sex)	Dose mg/kg and route (µCi/kg)	Clofibric acid % dose ^{14}C excreted in 0-24h urine	Unchanged	as glucuronide
Guinea Pig	(3F)	100 i.p.(16)	74(58-86)	27(18-32)	46(40-54)
Rat	(3F)	100 i.p.(16)	96(73-108)	46(23-67)	50(33-67)
Rabbit	(3F)	100 p.o.(16)	60(60-61)	5.4(4.9-6.0)	54(54-55)

Animals were dosed as indicated with [^{14}C]clofibric acid, [^{14}C]clofibrate dissolved in propane-1,2-diol as indicated. Urine was collected for 24h post-dosing and analysed for ^{14}C, unchanged acid and clofibrylglucuronide as described in text. Results are mean values for 3 animals with ranges in parentheses.

P.T.O.

Table 5.3 contd.

	Dose mg/kg and route (µCi/kg)	Clofibrate — % dose ^{14}C excreted in 0-24 urine	Unchanged	as glucuronide
Guinea Pig	113 i.p.(4)	98(98-102)	20(15-24)	78(78-79)
Rat	113 i.p.(4)	85(82-88)	15(10-20)	70(64-73)
Rabbit	113 p.o.(4)	91(89-93)	4.7(4.6-4.7)	(84-89)

Table 5.4 Urinary Metabolites of [^{14}C] Clofibric Acid in the Cat, Dog and Ferret

Species	(No.,Sex)	Dose mg/kg.,route 1 µCi/kg	% dose ^{14}C clofibric acid excreted in 0-24h urine*	unchanged	conjugated with: taurine	glucuronic acid
Cat	(3F)	100 i.p. (16)	24(20-26)	13(13-14)	10(7-13)	n.d.
Dog	(3F)	100 i.p.(16)	39(23-58)	12(7-21)	20(10-26)	7.5(5.7-11)
Ferret	(3F)	100 i.p.(16)	23(20-26)	5.6(3.2-8.0)	17(15-18)	1.1(1.0-1.2)

Animals were dosed with [^{14}C] clofibric acid dissolved in water as the sodium salt as indicated. Urine was collected daily for up to 3 days post dosing and analyzed for [^{14}C] clofibric acid and its metabolites as described in the text. Results are mean values for 3 animals with ranges in parentheses.

*The % dose recovered in the 24-48h period urine samples (mean values) were as follows: cat (26), dog (28) and ferret (35). The ferret excreted a further 14% during the 48-72h period. n.d. = not detected.

of ^{14}C was relatively high, amounting to 60-98% of the dose within a 24h period after dosing. In the rabbit which excreted 60% of the dose in the first 24h a further 14% of the dose was eliminated in the 24-48h period. It should be noted however that the compounds were given to the rabbit by the oral route and this route difference might account for the apparently slower rate of excretion of ^{14}C seen in this species following administration of radiolabelled clofibric acid.

The urinary metabolites of clofibric acid and its ester were identified by comparison of their chromatographic properties with those given by authentic samples, colour reaction and by reverse isotope dilution. The results showed that there were only two excretion products in the case of the rat, guinea pig and rabbit, these being the clofibric acid and its ester glucuronide. The three species varied in the relative proportions of the two compounds excreted.

The ester glucuronide was the major excretion product of both clofibric acid and clofibrate for the three species studied. These were however small species differences in the amounts of unconjugated clofibric acid excreted; this being low in the rabbit (5% dose) and substantially higher in the rat and guinea pig. No clofibryl-glycine or -taurine was detected in the urine of these three species.

There occur marked differences in the qualitative and quantitative aspects of the urinary excretion of $[^{14}C]$

clofibric acid in the cat, dog and ferret (Table 5.4) compared to the rat, guinea pig and rabbit. Table 5.4 shows that the urinary excretion of ^{14}C was only 20-40% of the dose in the first 24h with further substantive elimination (30-40%) occurring in the subsequent 24-48h period. For these species the main excretion products were the unchanged acid, the taurine conjugate (clofibryltaurine) and in the case of the dog and ferret small amounts of clofibryl-glucuronide. No glycine conjugate was detected in the urine of all three species.

Man

Clofibric Acid

The elimination of ^{14}C in the urine of 5 human volunteers receiving 500 mg [^{14}C]-clofibric acid orally is shown in Table 5.5. Most of the ^{14}C recovered was eliminated in the first 12h after administration, and over a two day period the majority of the administered ^{14}C was recovered (73-92% of dose).

Fig. 5.6 shows the cumulative excretion of clofibrylglucuronide on an individual basis for the five subjects investigated. The reason for doing this is to see whether or not there are marked interindividual differences in the excretion of the conjugate. It can be seen that the rate of excretion of the conjugate may be somewhat slower in one subject (subject 2) compared to the other four subjects which exhibited a relatively uniform pattern of excretion. Whether or not this slower excretion in subject 2 is a

Table 5.5

Excretion of ^{14}C and urinary metabolites following the administration of [^{14}C]-Clofibric acid (500 mg 1 Ci) to human volunteers.

	Subject 1 % Dose as			Subject 2 % Dose as			Subject 3 % Dose as		
Time (h)	Free	Glucuronide	Total	Free	Glucuronide	Total	Free	Glucuronide	Total
0-2	0.2	2.6	2.8	0.2	3.5	3.7	0.2	3.0	3.2
2-4	0.0	8.4	8.4	0.0	6.1	6.1	0.0	9.6	9.6
4-6	0.0	8.3	8.3	0.0	5.6	5.6	0.0	9.8	9.8
6-8	0.9	7.7	8.6	0.6	5.1	5.7	1.0	8.8	9.8
8-10	0.2	8.7	8.9	0.1	7.1	7.2	0.2	9.4	9.6
10-12	0.2	5.2	5.4	0.2	4.4	4.6	0.2	4.6	4.8
12-24	1.1	21.0	22.1	0.9	17.4	18.3	1.2	21.8	23.0
24-48	ND	ND	28.0	ND	ND	21.5	ND	ND	12.2
Total ^{14}C									
0-24	2.6	61.9	64.5	2.0	49.2	51.2	2.8	66.0	68.8
0-48	ND	ND	92.5	ND	ND	72.7	ND	ND	81.0

ND = Not Determined

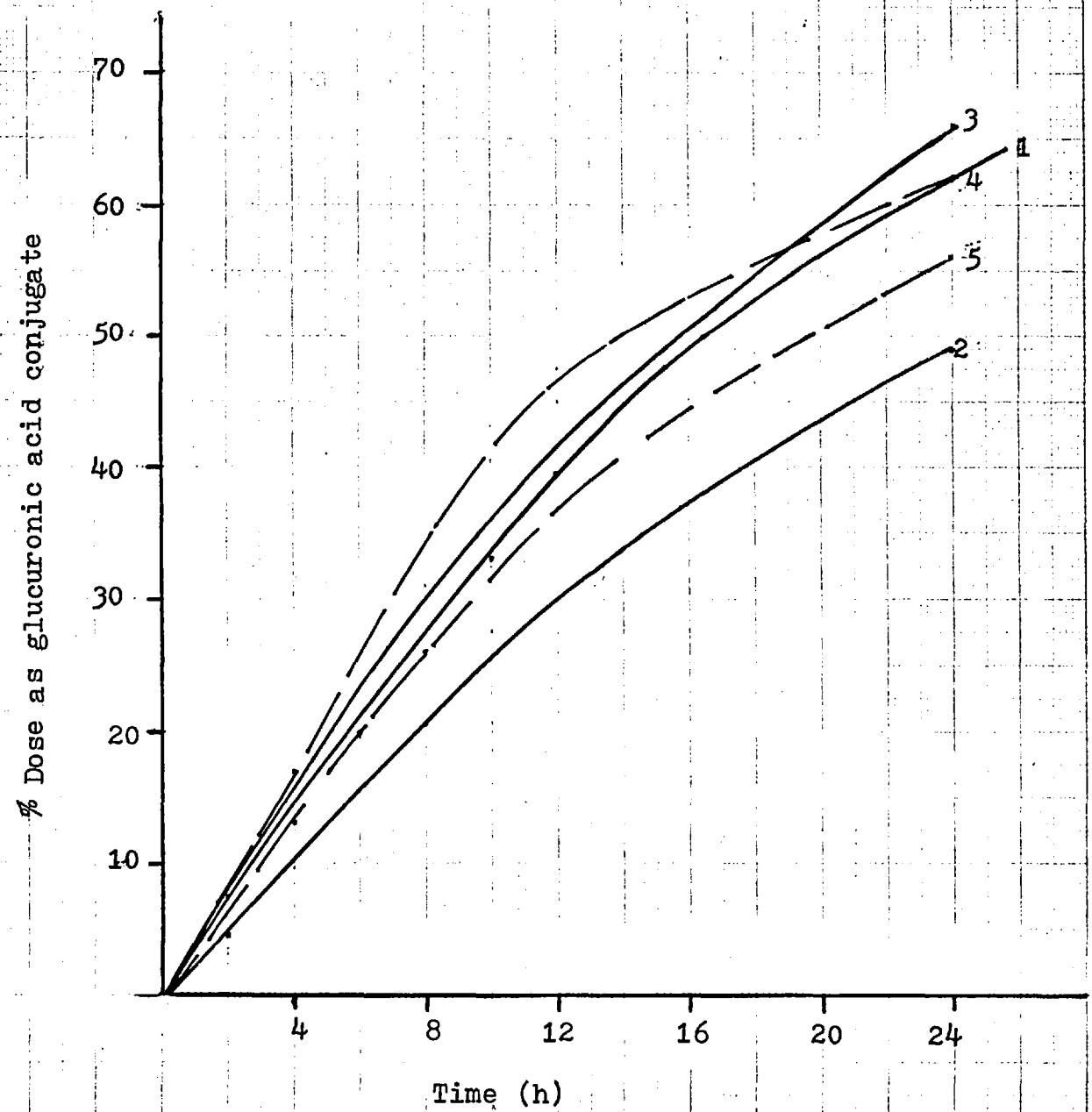
P.T.O.

Table 5.5 contd.

Time (h)	Subject 4 % Dose as			Subject 5 % Dose as			Mean % Dose as		
	Free	Glucuronide	Total	Free	Glucuronide	Total	Free	Glucuronide	Total
0-2	0.5	7.5	8.0	0.3	4.7	5.0	0.3	4.1	4.4
2-4	0.0	9.5	9.5	0.0	8.1	8.1	0.0	8.2	8.2
4-6	0.0	9.5	9.5	0.0	7.2	7.2	0.0	7.7	7.7
6-8	1.0	9.3	10.3	0.7	6.0	6.7	0.8	6.8	7.6
8-10	0.2	7.2	7.4	0.1	6.8	6.9	0.2	7.5	7.7
10-12	0.1	3.4	3.5	0.2	5.7	5.9	0.2	4.3	4.5
12-24	0.9	16.8	17.7	1.0	18.6	19.6	1.0	18.7	19.7
24-48	ND	ND	21.0	ND	ND	19.1	ND	ND	20.2
Total ^{14}C									
0-24	2.7	61.9	64.6	2.3	56.1	59.4	2.5	57.3	59.8
0-48	ND	ND	85.6	ND	ND	78.5	ND	ND	80.0

ND = Not determined

Fig 5.6 Plot of cumulative glucuronic acid conjugate excreted following oral administration of clofibric acid (500 mg) to human volunteers.



function of impaired conjugation cannot be decided on the basis of the present evidence but clearly the possibility exists.

In order to check the possibility that the formation and excretion of the glucuronic acid conjugate may be dose-dependent two volunteers were challenged with a larger dose (1500 mg) of clofibric acid and its elimination monitored. (Table 5.6). Most of the ^{14}C recovered was eliminated in the first 12h as was found for the lower dose of 500 mg. The only urinary metabolite detected was clofibrylglucuronide which was identified after isolation (see page 200) by comparison of its chromatographic properties with a sample of the glucuronide isolated from rabbit urine, by colour tests and by n.m.r. In addition to this, small amounts of free clofibric acid were found. The urinary metabolites after a 1500 mg dose are similar to those of 500 mg, with the ester glucuronide predominating while very small amounts of free clofibric acid are also eliminated.

Clofibrate

The excretion of ^{14}C in the urine of human volunteers after the oral administration of $[^{14}C]$-clofibrate (565 mg) is shown in Table 5.7. Over 3 day period, over half of the administered ^{14}C was eliminated (60-63% of dose). Nearly half of this was eliminated in the first 12h after administration (21-33% of dose). The cumulative excretion of clofibrylglucuronide on an individual basis for three subjects is shown in Fig. 5.7. The reason for doing this

Table 5.6

Excretion of ^{14}C following oral administration of 1500 mg of [^{14}C]-Clofibric acid in two human volunteers.

Subject	1	2
Time (h)	% Dose ^{14}C excreted	
0-2	5.1	2.3
2-4	9.2	9.2
4-6	8.0	7.4
6-8	7.5	6.3
8-10	5.5	6.2
10-12	5.0	2.2
Total 0-12	40.3	33.6
12-24	14.3	17.6
0-24	54.6	51.2
24-48	11.8	14.5
0-48	66.4	65.7

Table 5.7

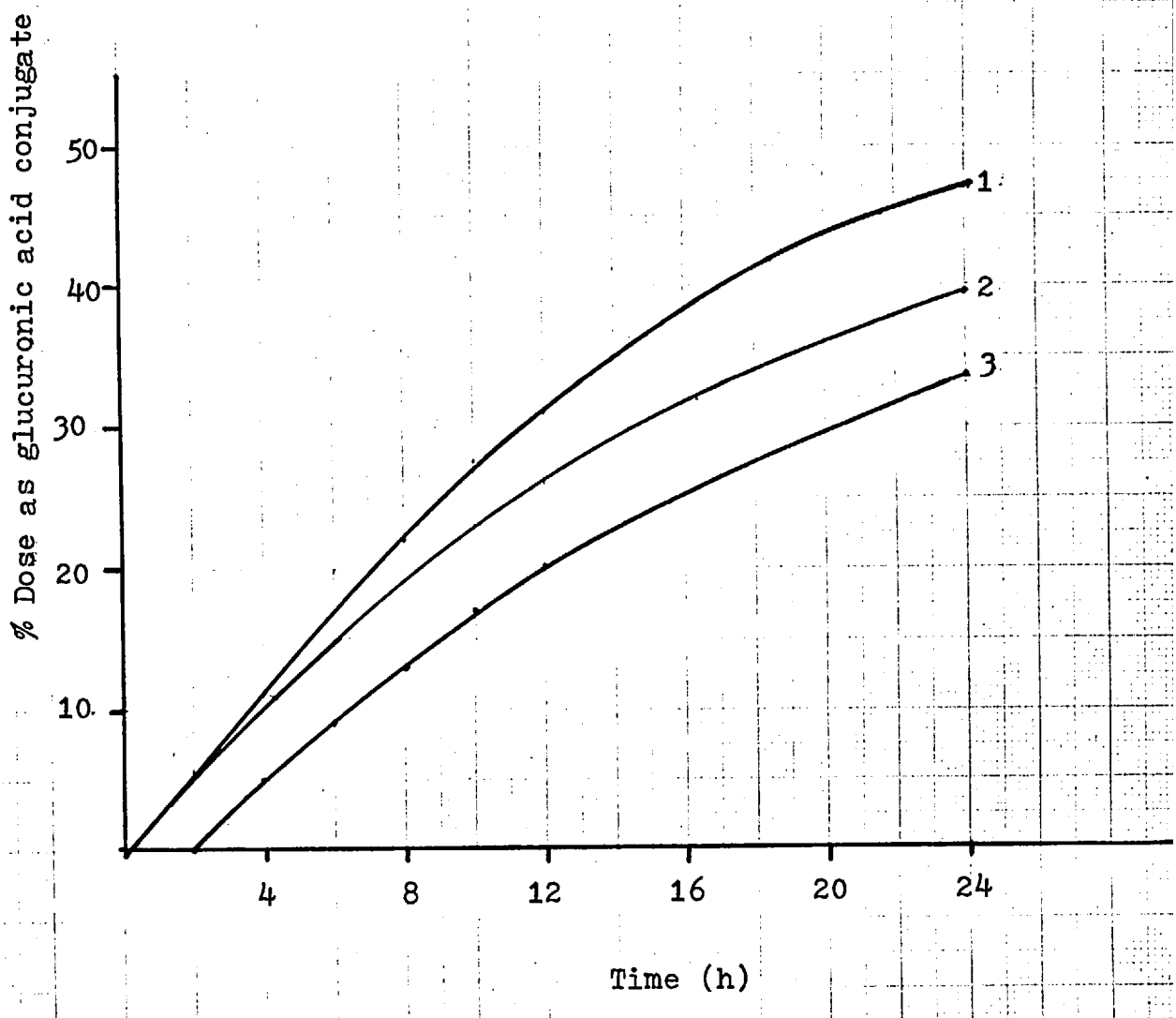
Urinary excretion of ^{14}C after oral administration of 565 mg of [^{14}C] clofibrate to human volunteers.

	Subject 1 % Dose as			Subject 2 % Dose as			Subject 3 % Dose as			Mean % Dose as		
Time (h)	Free	Glucuronide	Total	Free	Glucuronide	Total	Free	Glucuronide	Total	Free	Glucuronide	Total
0-2	0.2	4.2	4.4	0.2	5.7	5.9	0.0	0.1	0.1	0.1	3.4	3.5.
2-4	0.2	5.3	5.5	0.2	5.3	5.5	0.2	5.1	5.3	0.2	5.2	5.4
4-6	0.3	6.7	7.0	0.2	3.9	4.1	0.1	3.7	3.8	0.2	4.8	5.0
6-8	0.2	5.9	6.1	0.2	4.2	4.4	0.2	4.1	4.3	0.2	4.8	5.0
8-10	0.2	5.4	5.6	0.1	3.6	3.7	0.2	3.7	3.9	0.2	4.0	4.2
10-12	0.2	3.7	3.9	0.1	3.4	3.5	0.1	3.5	3.6	0.2	3.8	4.0
12-24	0.6	14.6	15.2	0.5	13.2	13.7	0.6	13.7	14.3	0.6	13.8	14.4
Total												
0-24	1.9	45.8	47.7	1.5	39.2	40.8	1.4	33.9	35.3	1.6	39.7	41.3
0-48	ND	ND	60.6	ND	ND	55.9	ND	ND	51.8	ND	ND	56.1
0-72	ND	ND	63.0	ND	ND	62.0	ND	ND	59.5	ND	ND	61.5

ND = Not Determined.

Fig. 5.7

Plot of cumulative glucuronic acid conjugate excreted following oral administration of Clofibrate (565 mg) to human volunteers.



was to compare the excretion pattern with those found for clofibric acid and also to confirm whether or not for subject 2 the rate of excretion of the conjugate is low as was found when this volunteer had been given clofibric acid in the previous investigation (p.219). This was not the case as the excretion was similar to that of subject 1. Subject 3 has an apparently slow excretion rate of this metabolite but this is suspected as being due to loss of sample in the first few hours by the subject which was not reported.

Two ^{14}C urinary excretion products were detected, namely clofibric acid and its ester glucuronide. The identity of the latter was established by comparison of its properties with those of an authentic sample and by colour reactions.

Discussion

The metabolism of clofibrate and clofibric acid has been studied in man and 6 laboratory animal species. The results of these studies are summarised in Tables 5.8 and 5.9 with Table 5.8 giving the summary of the results of clofibric acid metabolism while the findings for clofibrate metabolism are shown in Table 5.9. The metabolism of these compounds will be discussed separately and finally their results compared.

Clofibric acid

The urinary elimination of clofibric acid is species dependent. The elimination of ^{14}C in 24h varies from as

Table 5.8

24h Urinary excretion of ^{14}C and Urinary metabolites after the administration of clofibric acid.

Species	Dose (mg)	Route	% Dose Recovered (mean)	% Dose as glucuronide	% Dose as Taurine Conjugate	% Dose as Unchanged
Man	500mg/Man	Oral	60	57	-	2.8
Rat	100mg/kg	i.p.	96	50	-	46
Rabbit	100mg/kg	p.o.	60	54	-	5.4
Guinea Pig	100mg/kg	i.p.	74	46	-	27
Dog	100mg/kg	i.p.	39	7.5	20	12
Cat	100mg/kg	i.p.	24	-	10	13
Ferret	100mg/kg	i.p.	23	1.1	17	5.6

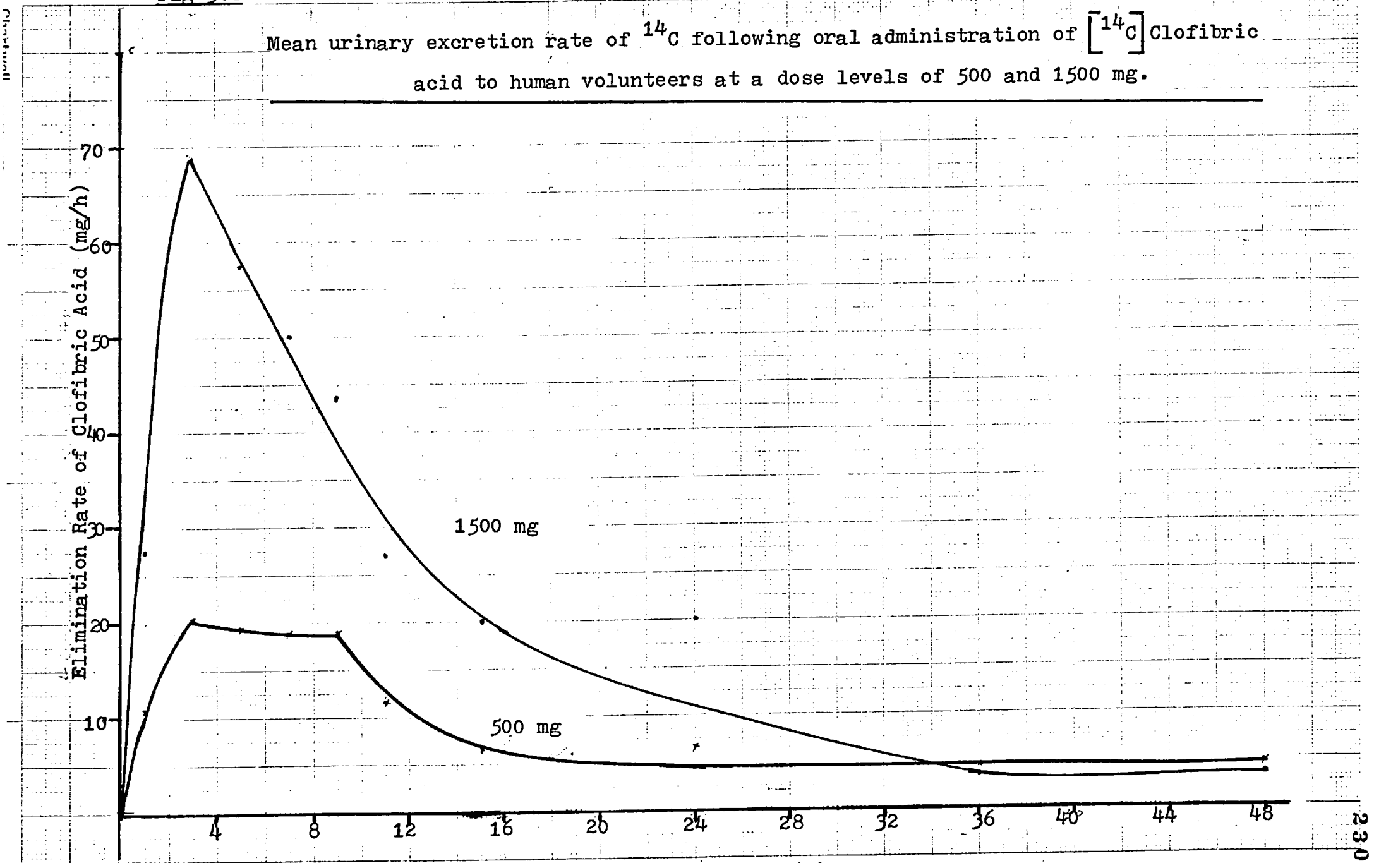
low as 23% in the cat to 96% of the dose in the rat (Table 5.8). The slow elimination is prominent with the carnivore species while by contrast rapid elimination occurs with man, the rodent species, and the rabbit. This slow elimination by carnivores also occurs with the metabolism of 2-naphthylacetic acid in carnivore species (Chapters Two and Three) and it may be one of the characteristics of these species with respect to the elimination of arylacetic and aryloxyacetic acids.

The results of this study indicate that man, rabbit and the rodent species have a high affinity for the metabolic conjugation of clofibric acid with glucuronic acid. The rodent species differ from man and the rabbit in the amount of the free drug excreted, which is higher for these species than for man and the rabbit. Thorp and Waring (1962) and others characterised a single glucuronic acid conjugate of clofibric acid in human urine while Faed and McQueen (1978) however, claimed to have detected two similar glucuronic acid conjugates of clofibric acid. The present study indicated the presence of only one glucuronic acid conjugate of clofibric acid both in human and rabbit urine and this was conclusively identified as the 1-O-acyl conjugate namely, 1-O-(4-chlorophenoxyisobutyryl)-glucuronic acid.

A close examination of the results of the human studies at a dose level of 500 mg showed that the excretion rate of clofibric acid apparently reached a maximum of about 20 mg/h between the third and ninth hour after dosing (Fig. 5.8). This suggests that the elimination pathway which involves

Fig 5.8

Mean urinary excretion rate of ^{14}C following oral administration of [^{14}C] Clofibric acid to human volunteers at a dose levels of 500 and 1500 mg.



conjugation with glucuronic acid may be saturated and therefore the metabolism of clofibric acid could be dose dependent but a study at a high dose level (1500 mg) showed this was not the case (Fig. 5.8). The metabolism of clofibric acid in man is therefore not dose dependent at least for the dose levels examined.

The metabolism of clofibric acid in man, rabbit and the rodent species differs from that of the carnivores in their ability to utilise glucuronic acid extremely well as the conjugating substrate when compared to the carnivores. Several studies have shown that the cat is deficient in glucuronic acid conjugation mechanism. The rabbit, man and the rodent species are apparently unable to conjugate clofibric acid with taurine while this is the major metabolic pathway of clofibric acid in the carnivore species. It should be noted that with man, rabbit and rat are capable of utilising taurine in the conjugation of certain arylacetic acids so that the mechanism is not absent from these species (Dixon et al.,1977; Khanna and Fang,1976). Utilisation of taurine appears to be well developed in carnivore species and seems to be a major conjugation pathway for many carboxylic acids in carnivores (Idle, 1977).

Clofibrate

The elimination of clofibrate in rat, rabbit, guinea pig and man is summarised in Table 5.9. In man it is eliminated slowly (42% in 24h) as compared to the other three species where it is eliminated more rapidly. In man and in all the three animal species studied (rat, rabbit,

Table 5.9

24 h Urinary excretion of ^{14}C and urinary metabolites after the administration of $[^{14}C]$-clofibrate.

	% Dose Recovered	% Dose as	
		glucuronide	unchanged
Man	42	40	2
Rat	85	70	15
Rabbit	92	87	5
Guinea Pig	98	78	20

and guinea pig), the only metabolite present was the glucuronic acid conjugate which accounted for nearly all the excreted material.

Clofibrate being an ester is first hydrolysed to the acid (Phase I metabolism) before being subsequently conjugated. Comparison of the elimination and metabolism of clofibric acid and its ester in the species studied showed that they are both eliminated rapidly with the exception of the man where it is lower in the case of clofibrate than that of clofibric acid. The rat results agree with the findings of Cayen et al. (1977) and suggest that the rate of clofibrate hydrolysis to the acid is very rapid that it does not limit the elimination of the drug.

The question arises as to what factors influence and favour the marked tendency for clofibrate and clofibric acid to undergo conjugation with glucuronic acid and taurine (depending upon species) when previous studies (Table 5.1) indicate that many aryloxyacetic acids are not conjugated and are excreted unchanged. The reason for this may be a structural one which favours a high affinity for glucuronic acid conjugation in non-carnivores and a high affinity for taurine conjugation in carnivore species.

Phenoxyacetic acid differs from phenylacetic acid by the presence of an oxygen atom between the aromatic and acetic acid side-chain. As stated in Chapter One the nature of the substituent on the methylene group of arylacetic acids

profoundly influences their subsequent pattern of metabolic conjugation. α-Substitution has a crucial effect in determining the relative extent and balance of conjugation with glucuronic acid and amino acids. This may also apply to aryoxyacetic acids such as clofibric acid, which undergoes extensive conjugation particularly with glucuronic acid and this is probably influenced by the presence of substituents on the α-methylene functions (see Table 5.10).

The above results and discussions indicate the following:

i. the structure of the glucuronic acid conjugate of clofibric acid isolated from the urine of humans and rabbits dosed with clofibric acid is that of 1-O-(4 chlorophenoxy isobutyryl)-glucuronic acid.

ii. the metabolic pattern for both clofibric acid and its ester clofibrate is similar in human volunteers and the animal species investigated although important species differences occur in respect of the pattern of metabolic conjugation of the two compounds.

iii. Clofibric acid is metabolised mainly by glucuronic acid conjugation in man, rat, guinea pig and rabbit but not in the cat. The latter species and also the ferret and dog form the taurine conjugate of clofibric acid as the major conjugate (fig 5.9).

Table 5.10

Influence of substitution on the methylene carbon of phenyl- and phenoxy-acetic acids.

Compound	R^1	R^2	% Glucuronidation in rat	% Glucuronidation in rabbit	Ref.
Phenylacetic acid	H	H	0	0	1
Hydratropic acid	H	CH_3	64	73	2
Phenoxyacetic acid	H	H	0	0	3
Clofibric acid	CH_3	CH_3	50	54	

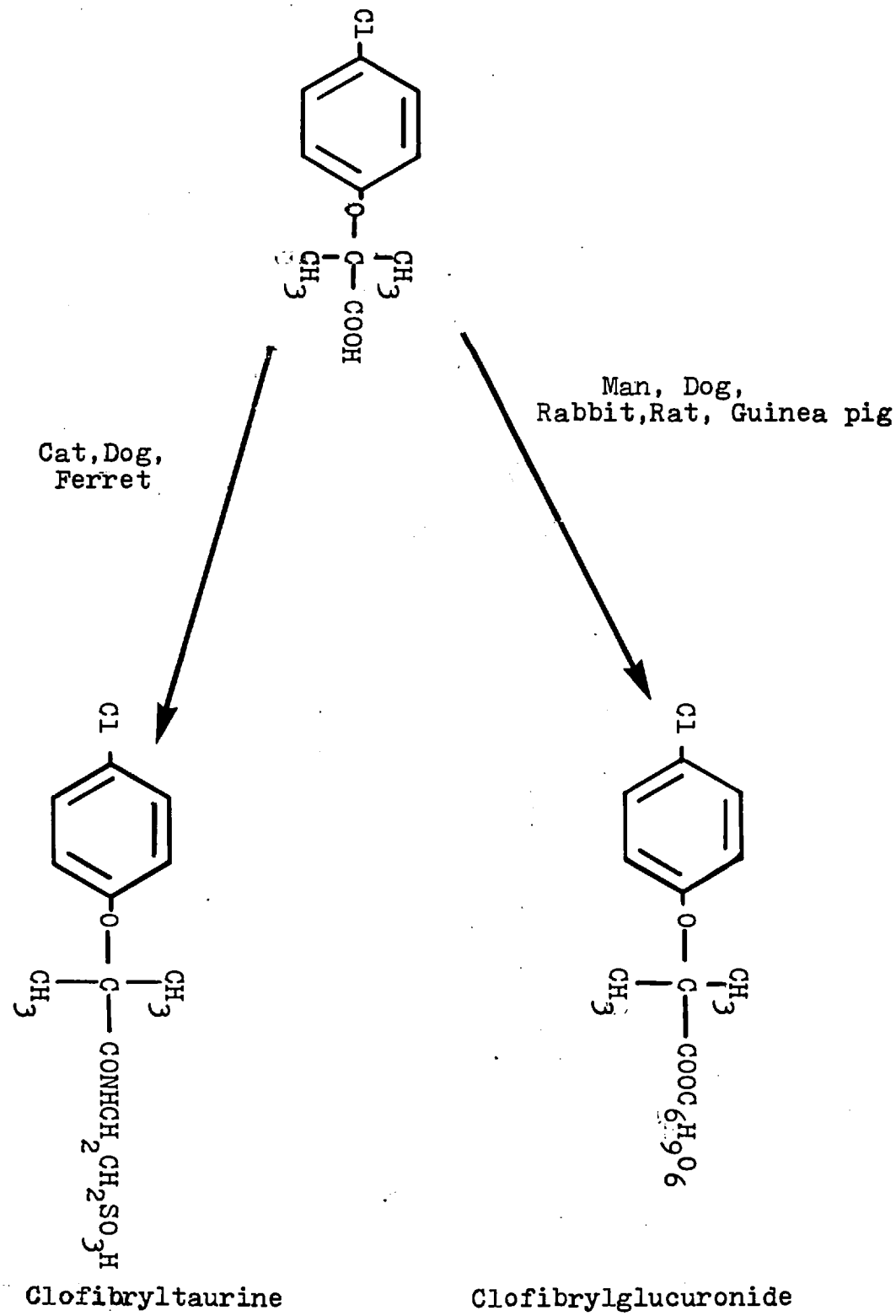
1 James et al. 1972

2 Dixon et al. 1977b

3 Levy et al. 1947

Fig. 5.9

Metabolic pattern of clofobric acid in various species



Clofibryltaurine

Clofibrylglucuronide

iv. In man, clofibric acid did not show dose-dependent metabolism when given orally at two dose levels (500 mg and 1500 mg).

v. Clofibric acid undergoes extensive metabolic conjugation (glucuronic acid and taurine) and this appears to be unusual for an aryloxyacetic acid many of which are metabolically resistant. The high affinity of clofibrate to undergo conjugation is suggested, by analogy with the arylacetic acids, to be associated with the presence of the substituents on the α-methylene function.

CHAPTER SIX

Discussion and Conclusion.

Discussion

In this discussion it is proposed to discuss two major issues which have emerged from the work on the comparative metabolic conjugation of 2-naphthylacetic acid. This work established that 2-naphthylacetic acid underwent conjugation *in vivo* with glucuronic acid and amino acids and in this respect it conformed to the normal and expected general pattern for an arylacetic acid of this type. However, the detailed pattern of conjugation was aberrant in a number of respects:

a). it showed a pattern of multiple amino acid conjugation involving two or three amino acids (glycine, glutamine and taurine);

b). non-primate species were found capable of utilising glutamine for the conjugation of 2-naphthylacetic acid;

c). non-carnivore species utilised taurine for the conjugation, a mechanism previously thought to be largely a feature of carnivore species;

d). the conjugation pattern of 2-naphthylacetic acid was profoundly different from that observed for the isomeric 1-naphthylacetic acid.

The two issues for discussion which arise from the comparative metabolic study are as follows:

(i) why is the metabolic conjugation pattern so different to that encountered for other aromatic and arylacetic acids?

(ii) what are the implications for this in the context of the predictability of carboxylic acid metabolism? Carboxylic acids of various types including arylacetic acids and aryloxyacetic acids find important technological applications and it is useful to know how far their metabolic fate in the animal body can be predicted. In the past such predictions could be conveniently made on the premise of simple generalisations but the question now is to how far this is affected by the unusual findings with 2-naphthylacetic acid.

The Unusual pattern of metabolic conjugation of 2-naphthylacetic acid

The unusual amino acid conjugation pattern encountered for 2-naphthylacetic acid is at variance with that encountered for many other acids even including the isomeric 1-naphthylacetic acid. The question arises as to why 2-naphthylacetic acid is able to elicit such a markedly different pattern. The finding of the formation of glutamine conjugates in non-primate species is curious especially since it was originally thought to be a conjugation mechanism largely confined in terms of its occurrence to man and anthropoid monkey species (rhesus, cynomolgus, marmoset, squirrel monkey and capuchin). A suggestion that this may not necessarily be the case is indicated by Hirom et al., (1977) who showed that phenylacetic acid forms to a small

extent a glutamine conjugate in the ferret. Clearly, in view of the experience with 2-naphthylacetic acid, glutamine conjugation can no longer be considered to be the prerogative of man and related simian species and it can be found in more lowly mammalian species. The reason why 2-naphthylacetic acid, (but not other arylacetic acids, including 1-naphthylacetic acid) elicits glutamine conjugation in the rat, ferret, hamster and rabbit is not clear. Various possibilities have been entertained:

(i) glutamine conjugation may not occur in some species because of a relative lack of glutamine for conjugation.

(ii) the existence of a glutamine conjugation mechanism in a species may only be 'uncovered' when sufficient substrate is given to saturate alternative pathways.

(iii) the glutamine conjugation mechanism may be 'induced' and therefore 'uncovered' by certain arylacetic acid substrate such as 2-naphthylacetic acid.

(iv) the glutamine conjugation mechanism may involve multiple forms of the various enzymes involved. If this is the case then clearly scope exists for the occurrence of multiple forms characterised by differences both in terms of their substrate requirement and species occurrence.

One might on this basis conceive of a family of enzymes

concerned with glutamine conjugation. It may be that some non-primate mammals possess the mechanism responsible for the glutamine conjugation of 2-naphthylacetic acid but not that for phenylacetic acid. By contrast it could be suggested that primate species possess multiple forms capable of the metabolic conjugation with glutamine of both 2-naphthylacetic acid and phenylacetic acid.

The balance of the data obtained and described in this thesis supports hypothesis (iv) as the most likely explanation. Attempts to elicit and manipulate the glutamine conjugation by attempted induction by substrate feeding, large dose administration to swamp alternative pathways and finally by glutamine supplementation all failed. The concept of multiple form or heterogeneity of the conjugation mechanism is by no means novel and has already been described to occur with other mechanism, such as glucuronidation. Wishart *et al*. (1978) and Del Villar *et al*. (1975) have isolated two forms of UDP-glucuronyltransferases which differ in their substrate specificities. Also various forms of glutathione *S*-transferases have been purified and at least seven distinct forms have been isolated in the rat liver while in man, five forms have been isolated (Jerina and Bend 1977).

Attempt to elucidate taurine conjugation mechanism *in vitro* has been abortive. Lately James and Bend (1978) have partially established this mechanism in fish species. In their experiments, the activation step was omitted, that is, already synthesised phenylacetyl-CoA was added to taurine in fish liver homogenate and the taurine conjugate was formed.

The results suggest that the various amino acid conjugation mechanisms may have different activation steps and possibly a particular chemical structure is needed to participate in the various steps. This may be the case with 2-naphthylacetic acid which is able to be appropriately activated in relation to taurine conjugation but this does not occur with the 1-isomer in non-carnivores. Thus differences in activation may explain why the 2-isomer but not the 1-naphthylacetic acid undergoes taurine conjugation in vivo. The fourth hypothesis, namely, existence of multiple forms of the various enzymes involved, raised for glutamine conjugation mechanism may also be applicable to taurine conjugation mechanism.

Implications for predictability of the metabolic conjugation of carboxylic acids

One of the aims of drug metabolism research has been to develop frameworks within which the fate of compounds in the organism can be anticipated correctly, and recently there have been indications that this aim may be achievable, for instance with the amphetamines (Caldwell, 1976) and a consideration of the available data on the fate of carboxylic acids similarly suggests that the level of prediction of the fate of such compounds is quite high.

The work reported in this thesis has very important implications for attempts to predict the fate of foreign compounds, in general and with respect to aromatic acids. Generally carboxylic acids are conjugated with glucuronic acid or amino acid depending on the chemical structure and species. However, the nature of the amino acids utilised

cannot be predicted, for example, phenoxybenzoic acid is conjugated with taurine in the mouse, a non-carnivore, extensively (Huston and Cassida, 1978). Although this acid is expected to be conjugated with amino acid, based on its structure, the particular amino acid utilised will be difficult to predict. Quite clearly, the work on 2-naphthylacetic acid and, to a lesser extent, clofibric acid, has revealed that reactions previously unreported may occur to very significant extents with these compounds.

Since at present all attempts to create predictive frameworks for the metabolism of groups of compounds is essentially retrospective, these findings upset quite dramatically our ideas about the way in which animal species and chemical structure determine the fate of aromatic acids *in vivo*.

Given the present paucity of knowledge on the enzymology of the various metabolic options open to such acids, it is impossible to rationalize the current findings with previous "dogma". An understanding of the basic principles linking the data reported here with work reported in the literature must need await advances on the molecular aspects of the enzymes involved in amino acid conjugations.

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The Reversibility of the *N*-Oxidation of Chlorphentermine *in vivo*

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Very little information is available on the metabolic fate of the *N*-oxidation products of aliphatic amines, which are now well established as biotransformation products of these compounds. The possible fates of these *N*-oxidation products are of interest when the reversibility of the *N*-oxidation process and the interrelationships between the products are being considered. The anorectic drug chlorphentermine (4-chloro-$\alpha\alpha$-dimethylphenethylamine) has been shown to be *N*-oxidized in a range of species to give its *N*-hydroxy and nitro analogues (Caldwell *et al.*, 1975), and we now report on the fate of these analogues in the rat.

Nitrochlor[^{14}C]phentermine (4-chloro-$\alpha\alpha$-di[^{14}C]methylphenyl-α-nitroethane) was prepared by the $KMnO_4$ oxidation of chlorphentermine, and *N*-hydroxychlor[^{14}C]-phentermine hydrochloride was synthesized from this nitro compound by controlled reduction (for methods, see Caldwell *et al.*, 1975). Nitrochlorphentermine was dissolved in propane-1,2-diol, and *N*-hydroxychlorphentermine hydrochloride was dissolved in water for intraperitoneal injection into female Wistar albino rats (body wt. 180–200g; dose 100mg/kg). The rats were housed in Metabowls (Jencons Ltd.) which enabled collection of urine, faeces and $^{14}CO_2$ in the expired air, the latter being trapped in ethanolamine/2-methoxyethanol (1:2, v/v). After the administration of *N*-hydroxychlor[^{14}C]phentermine, 74% of the administered dose was recovered in 6 days, with 66% in the urine, 3% in the faeces and 5% in the expired air as $^{14}CO_2$ (collected for 2 days). Dosage with nitrochlor[^{14}C]phentermine resulted in the elimination of 92% of the administered ^{14}C in 4 days, with 41% in the urine, 1% in the faeces and 50% as $^{14}CO_2$ in the expired air (collected for 2 days).

The analysis of metabolites in the urine was carried out after preliminary purification of the urine with Amberlite XAD-2 resin, the methanol eluate being used for paper and thin-layer chromatography. Chlorphentermine was assayed by reverse isotope dilution, and other metabolites examined by g.l.c. and g.c./m.s. These methods are described by Caldwell *et al.* (1975).

After the administration of both compounds, the only radioactive metabolites found in the urine were *N*-hydroxychlorphentermine and its glucuronic acid conjugate. For nitrochlorphentermine, these accounted for 41% of the dose (9% free, 34% conjugated), whereas for *N*-hydroxychlorphentermine, they totalled 66% (24% free, 42% conjugated). In both cases, no nitrochlorphentermine was found, and reverse isotope dilution showed the absence of chlorphentermine.

In separate experiments, bile-duct-cannulated rats excreted 22% of an injected dose of nitrochlor[^{14}C]phentermine (100mg/kg intraperitoneally) in the bile in 24h, with a further 10% in the urine. Again, the only metabolic product found was *N*-hydroxychlorphentermine, present only as its glucuronic acid conjugate in the bile and both free and conjugated in the urine.

These results show that *N*-hydroxychlorphentermine is metabolized in the rat principally by conjugation with glucuronic acid, which is to be expected, since the free hydroxylaminic hydroxyl group is readily available for conjugation. Nitrochlorphentermine is, by contrast, metabolized along two distinct pathways, one involving reduction of the nitro group to the hydroxylamine, excreted free and as its glucuronic acid conjugate, and another involving oxidation of the side-chain methyl groups to $^{14}CO_2$. It is clear that the *N*-oxidative metabolism of chlorphentermine is not fully reversible *in vivo*, since neither of the products of *N*-oxidation are reduced to chlorphentermine, but the nitro compound is reduced to *N*-hydroxychlorphentermine.

The oxidation of the side-chain methyl groups of nitrochlorphentermine is a reaction of some interest, but possible intermediates have not been identified. It is likely that the

nitrogen atom is lost before the oxidation, and this could involve the nitroso compound, which would be an expected intermediate in the reduction of the nitro compound to the hydroxylamine. Such nitroso compounds spontaneously eliminate the nitroso group, giving a dimethylstyrene (Lindeke *et al.*, 1975), which could then be oxidized, thereby giving rise to $^{14}CO_2$ in the expired air.

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Structure–Metabolism Relationships of Arylacetic Acids: The Metabolic Fate of Naphth-2-ylacetic Acid *in vivo*

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The pattern of metabolic conjugation of arylacetic acids is both species- (James *et al.*, 1972) and structure- (Dixon *et al.*, 1976) dependent. Whereas simple arylacetic acids, such as phenylacetic acid, are conjugated with amino acids, arylacetic acids carrying an α-substituent are metabolized mainly by conjugation with glucuronic acid. These structurally dependent differences in the pattern of metabolic conjugation have been related to differences in affinity for a specific submitochondrial binding site and the mitochondrial glycine-conjugating mechanism (Dixon *et al.*, 1976). We have now extended these observations on the relationship between structure and pattern of conjugation of arylacetic acids to an examination of the influence of nuclear substitution on the conjugation pattern. For this purpose we have studied the metabolic conjugation of naphth-2-yl[^{14}C]acetic acid in rat, rabbit and ferret and have compared the results with those previously obtained with the isomeric naphth-1-ylacetic acid (Dixon *et al.*, 1976; Idle *et al.*, 1976).

Naphth-2-yl[*carbonyl*-^{14}C]acetic acid was synthesized from 2-bromomethylnaphthalene and Na^{14}CN followed by acid hydrolysis of the intermediate naphth-2-ylacetonitrile. It was purified by recrystallization from aq. ethanol, m.p. 140°C. The labelled acid was administered to the animals dissolved in water containing the calculated amount of $NaHCO_3$; the dose and route of administration are given in Table 1. The urine and faeces were collected daily for up to 6 days. For rats, the expired air was drawn through a mixture of ethanolamine/2-methoxyethanol (1:2, v/v) to trap any $^{14}CO_2$ present. The ^{14}C content of the excreta was determined by liquid-scintillation counting and the urine analysed for unchanged naphth-2-ylacetic acid and its conjugates by quantitative radiochromatogram scanning and by reverse isotope dilution. The ester glucuronide was assayed by difference before and after hydrolysis with β-glucuronidase or 2M-NaOH (see Dixon *et al.*, 1974). The amino acid conjugates were identified both by comparison of chromatographic properties with those of authentic samples and by isolation. For the latter, the amino acid conjugates were isolated by preparative thick-layer chromatography and then hydrolysed with 6M-HCl. The amino acids liberated were identified by chromatography as such and as their dansyl derivatives.

Table 1 shows the qualitative and quantitative aspects of the excretion of naphth-2-ylacetic acid in the rat, rabbit and ferret and compares the data with that previously found

Table 1. *Conjugation patterns of naphthylacetic acids in vivo*

Naphth-2-yl[^{14}C]acetic acid was administered in dilute $NaHCO_3$ solution, dose 100mg/kg, by intraperitoneal injection. Values represent the means of at least three animals. Data for naphth-1-ylacetic acid in the rat and rabbit are taken from Dixon *et al.* (1974) and for the ferret from Idle *et al.* (1976). tr, Trace; 1-isomer and 2-isomer refer to naphth-1-yl- and naphth-2-yl-acetic acid respectively. The results are expressed as the percentage of the ^{14}C in the 0–24h urine sample excreted as that compound.

	Rat		Rabbit		Ferret	
Compound	1-isomer	2-isomer	1-isomer	2-isomer	1-isomer*	2-isomer
Unchanged acid	26	14	11	8	5	4
Ester glucuronide	50	17	86	24	26	16
Glycine conjugate	23	23	6	30	6	16
Taurine conjugate	tr	38	0	9	63	45
Glutamine conjugate	0	8	0	26	0	19
Percentage of ^{14}C dose in 0–24 h urine	64	35	81	51	78	33

* Results for naphth-1-ylacetic acid in the ferret are based on a 0–48 h urine collection.

for its 1-isomer. The three species excreted the ^{14}C slowly, 40–75% of the dose being recovered in the urine over 6 days, with a further 12–21% in the faeces. No $^{14}CO_2$ was detected in the expired air of the rat. In the three species examined, five radioactive compounds were found in the urine, these being unchanged naphth-2-ylacetic acid, naphth-2-ylacetylglucuronide and naphth-2-ylacetyl-glycine, -taurine and-glutamine.

There is a marked difference in the pattern of conjugation of naphth-2-ylacetic acid when compared with its 1-isomer and furthermore this same difference is evident in all three species examined. Thus in the rat, naphth-2-ylacetic acid is eliminated largely in the urine as amino acid conjugates with glycine, taurine and glutamine with some naphth-2-ylacetylglucuronide, By contrast, naphth-1-ylacetic acid is eliminated in the urine more rapidly by the rat, and the major urinary excretion product in this species is the glucuronic acid conjugate together with some naphth-1-ylacetylglycine. Similarly, in the rabbit the main urinary excretion products of naphth-2-ylacetic acid are the glycine, taurine and glutamine conjugates. By contrast, naphth-1-ylacetic acid is excreted by this species mainly as the glucuronic acid conjugate together with a small amount of the glycine conjugate. The ferret forms the glycine, taurine and glucuronic acid conjugates of naphth-1-yl- and naphth-2-yl-acetic acids, but, in addition, the latter also gives rise to a glutamine conjugate.

It is thus clear from these findings that in all three species there is a clear difference in the pattern of metabolic conjugation of naphth-1-yl- and naphth-2-yl-acetic acid, as judged by the relative proportions of the glucuronic acid and amino acid conjugates of the two arylacetic acids excreted in the urine. For these three species the extent of amino acid conjugation is both greater and more varied for naphth-2-ylacetic acid than for the 1-isomer, which in the main is conjugated with glucuronic acid.

Phenylacetic acid and its *para*-substituted derivatives are metabolized dominantly by amino acid conjugation. However, the pattern of conjugation is structure-dependent, since substitution of the α-methylene group of arylacetic acids leads to a marked change in conjugation pattern away from one involving amino acids to one involving glucuronic acid. The two isomeric naphthylacetic acids may be regarded as *o*-,*m*-(1-)benzo- and *m*,*p*-(2-)benzo-substituted phenylacetic acids and the difference in extent of their amino acid conjugation may be due to the fact that the amino acid conjugation of phenylacetic acids may be sensitive to the nature and position of nuclear substituents. The findings indicate that naphth-1-ylacetic acid has a lower affinity for amino acid conjugations than its 2-isomer and this may be related to the steric effects of the *o*-, *m*-benzo ring. It is noteworthy that certain *ortho*-substituted benzoic acids are less readily conjugated with glycine than their *m*- and *p*-isomers (Bray *et al.*, 1948) and this may be related to the steric hindrance by the *ortho*-substituent. These findings indicate that the amino acid conjugation of arylacetic acids may be influenced structurally by the position of nuclear substitution as well as by α-methylene substitution.

A point of interest to emerge from these studies is the utilization of glutamine for the conjugation of naphth-2-ylacetic acid by the rat, rabbit and ferret. It has been previously thought that glutamine conjugation of arylacetic acids was a metabolic reaction restricted in its occurrence to man and anthropoid species (James *et al.*, 1972); the formation of small amounts of phenylacetylglutamine from phenylacetic acid by the ferret has been found (Hirom *et al.*, 1977).

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The Structure of the Glucuronic Acid Conjugate of Clofibric Acid

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Clofibric acid [2-(4-chlorophenoxy)-2-methylpropionic acid] is the active metabolite of the hypolipidaemic drug, clofibrate. In man and animals, it is metabolized principally by glucuronic acid conjugation (Sedaghat & Ahrens, 1975; Cayen *et al.*, 1977; Houin & Tillement, 1978), but attention has been drawn to the apparent resistance of this conjugate to β-glucuronidase treatment (Houin & Tillement, 1978). Faed & McQueen (1978) reported the presence of two conjugates of clofibric acid in human urine, only one of which was cleaved by β-glucuronidase. Both were labile to dilute alkali, and they suggested that the β-glucuronidase-susceptible conjugate was the 1-*O*-acylglucuronide, whereas the resistant conjugate was a positional isomer (i.e. the 2-, 3-, or 4-*O*-acylglucuronide). This was by analogy with the known mobility under certain conditions of the acyl moiety of bilirubin-IXα glucuronide (Blankaert *et al.*, 1978). In view of this, it was thus of interest to characterize more fully the glucuronic acid conjugate of clofibric acid formed *in vivo*.

A female Dutch rabbit (3 kg body wt.) received two daily doses of 1 g of clofibric acid orally as its sodium salt. The urine was collected for 3 days, and the total volume was freeze-dried. A methanolic extract of the residue was evaporated, dissolved in water and this was extracted with diethyl ether at pH 2. After evaporation, the residue in methanol was adjusted to pH 7 (0.1 M-KOH), from which white crystals of the potassium salt of the glucuronic acid conjugate of clofibric acid as its monohydrate were precipitated by the addition of dry ether, and recrystallized from ethanol/diethyl ether, m.p. 155–160°C (Found C, 43.1, 43.3; H, 4.4, 4.4; Cl, 7.53; $C_{16}H_{18}O_9ClK,H_2O$ requires C, 43.0; H,4.4; Cl, 7.9%).

This material was strongly reducing to Fehling's and Benedict's reagents and gave an intense purple colour with naphthoresorcinol. It was hydrolysed by β-glucuronidase ('Ketodase'; General Diagnostics, Eastleigh, Hants., U.K.; 2500 units at 37°C for 18 h) and 2 M-NaOH (60°C for 5 min) to clofibric acid, as shown by t.l.c. The enzymic hydrolysis was inhibited by saccharo-1,4-lactone.

The identity of the sugar moiety was shown by t.l.c. after ammonolysis of the conjugate, on t.l.c. plates, and this procedure resulted in the complete cleavage of the conjugate to glucuronic acid and clofibric acid. Final confirmation of the structure of the conjugate was given by ^1H and ^{13}C n.m.r. spectroscopy. All chemical shifts are relative to tetramethylsilane. The ^1H n.m.r. spectrum (in $^1H^2HO$ at 90 mHz) showed characteristic AA'BB' signals (4 protons) at δ(p.p.m.) 7.3 and 6.95, due to the benzene ring substituted at C-4, and a singlet (6 protons) at δ1.68 arising from the side-chain methyl groups. The glucuronic acid moiety gives a multiplet (4 protons) at δ3.4, from the protons on C-3, -3, -4, and -5 of the sugar ring, and a doublet at δ5.65 from the anomeric proton on C-1. A spectrum obtained at 220 mHz confirmed that this signal was a doublet and the size of the coupling constant indicated that the protons on C-1 and C-2 were axial to each other, i.e. the sugar has the β-configuration. The hydroxy protons are replaced by ^2H from the solvent and are thus not observed.

^{13}C n.m.r. spectra were obtained with proton-noise decoupling and with single-frequency off-resonance decoupling. The carbonyl carbon atoms at C-6 of glucuronic acid and of clofibric acid appear at δ176.0 and 175.5. The carbon atoms of the benzene ring appear at δ153.8 (that bearing Cl), δ129.6 (that bearing an oxygen atom) and those bearing protons at δ130.6 and 123.6. These latter signals become doublets on single-

frequency off-resonance decoupling. The quaternary carbon atom of clofibric acid is seen at δ81.8 (singlet on single-frequency off-resonance decoupling) and the two methyl groups appear at δ25.5 and 25.3, the slight difference being due to the influence of the asymmetric centres in the molecule. The glucuronic acid-ring carbon atoms are at δ95.9 (C-1) and 77.8, 76.6, 73.0 and 72 (C-2–C-5 respectively), all of which become doublets on single-frequency off-resonance decoupling. Taken together, these results show that the conjugate of clofibric acid isolated from rabbit urine is potassium 1-*O*-[2-(4-chlorophenoxy)-2-methylpropionyl]glucosiduronate.

The glucuronide of clofibric acid was also isolated from the urine of a male volunteer who took orally 1.5g of clofibric acid containing 1 μCi of ^{14}C. The 24h urine sample (containing 49% of the dose) from the volunteer was adjusted to pH 2, extracted with hexane to remove clofibric acid (4% of the dose) and then with ethyl acetate, which removed all of the remaining radioactivity. T.l.c. of this extract showed only one compound to be present, which was isolated by preparative t.l.c. This compound was identical in chemical reactions, chromatographic mobility and hydrolysis behaviour with that isolated above from rabbit urine, and preliminary n.m.r. studies show the similarity of the two conjugates.

These results, taken together with other studies on the fate of clofibric acid in animals and man (Caldwell *et al.*, 1979), suggest that only one glucuronic acid conjugate of clofibric acid is formed *in vivo* and excreted in the urine, and that this is the 1-*O*-acyl-glucuronide.

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Amino Acid Conjugation Responses to 2-Naphthylacetic Acid in Rodents

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Arylacetic acids are commonly metabolized by conjugation with either glucuronic acid or an aliphatic amino acid. Among rodents, the latter compound is commonly glycine, but recent experience indicates that contrary to previous notions, alternative amino acid conjugation pathways can occur in the rat, particularly the extensive utilization of glutamine and taurine. We have shown (Emudianughe *et al.*, 1978) that 2-naphthylacetic acid elicits an unusual pattern of metabolic conjugation in non-primate species, undergoing simultaneous conjugation with glutamine and taurine, as well as the more usual glycine and glucuronic acid. This suggested that the compound might be used as a probe to reveal glutamine and taurine conjugation in rodent species hitherto regarded as deficient in these mechanisms. This communication reports on the fate of 2-naphthylacetic acid in the mouse, hamster, gerbil and guinea pig, and also presents results for 1-naphthylacetic acid to emphasize the remarkable conjugation pattern elicited by the 2-isomer.

Table 1. *Conjugation patterns of the isomeric naphthylacetic acids in five rodent species*

Both ^{14}C-labelled naphthylacetic acids were given by intraperitoneal injection in dilute $NaHCO_3$ (100mg/kg body wt. 15μCi/kg body wt.). Results are the means for at least three animals. The balance of the material was excreted unchanged. Abbreviations: tr, trace.

	Proportion of urinary ^{14}C conjugated (%)									
	Guinea pig		Gerbil		Mouse		Hamster		Rat*	
Isomer ...	1	2	1	2	1	2	1	2	1	2
Glycine	31	38	51	85	15	4	11	32	33	24
Glutamine	0	0	0	0	0	0	0	35	0	16
Taurine	0	0	0	6	0	53	0	9	tr	29
Glucuronic acid	51	38	37	5	68	40	69	8	54	16

* Data for 1-naphthylacetic acid from Dixon *et al.* (1977) and for 2-naphthylacetic from Emudianughe *et al.* (1978).

The metabolism of 1-naphthyl[^{14}C]acetic acid and 2-naphthyl[^{14}C]acetic acid was examined in female mice (T.O. strain, body wt. 15–20g), Dunkin Hartley guinea pigs body wt. 350g), golden hamsters (body wt. 70–90g) and gerbils (body wt. 50–60g). The experimental procedures were those described by Dixon *et al.* (1977) and Emudianughe *et al.* (1978), and other details are given in Table 1.

Table 1 shows quantitative and qualitative results from these studies, together with data previously obtained in the rat. In all species, some 60–90% of the ^{14}C dose was recovered in the urine from the time of administrations up to 24h. 2-Naphthylacetic acid underwent principally amino acid conjugation, with glycine in all five species, with taurine in the gerbil, hamster, rat and mouse, and with glutamine in the hamster and the rat. Ester glucuronidation was also found to occur, but in no case to a greater extent than amino acid conjugation. In contrast, the fate of 1-naphthylacetic acid was much simpler, with glucuronidation as the major reaction in all species, glycine conjugation as a minor pathway and a very small amount of taurine conjugation in the rat.

The use of 2-naphthylacetic acid as a probe for amino acid-conjugation mechanisms has revealed that both taurine and glutamine conjugation can occur extensively in certain rodent species. Taurine conjugation, although most extensive in carnivores, appears to be haphazard in its occurrence to particular combinations of acid and species, and is now seen to be particularly well developed in the mouse. Glutamine conjugation was previously considered to be a reaction peculiar to primate species (Smith & Caldwell, 1977), but the present work has shown that it can be an important pathway in rodent species towards an appropriate substrate.

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Xanthine Oxidase Activity in Extracts from Contact-Inhibited and Spontaneously Transformed Mouse Embryo Cells in Culture

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Many metabolic properties initially believed to be specific for cancer cells have proved to be markers for rapid growth rather than malignancy. Weber (1977*a*,*b*) has attempted to circumvent this problem by examining enzyme activities in a range of chemically induced rat hepatomas of different growth rates, and has proposed that a variety of metabolic alterations, which appear throughout this range of hepatomas, but not in foetal, adult or regenerating rat liver, are characteristic of the neoplastic state. Such characteristic changes could be of considerable value in therapy and diagnosis of cancer. It is therefore important to determine whether or not such changes are generally found in cancer cells.

One of the alterations reported by Weber's group was an elevated activity of glutamine phosphoribosyldiphosphate amidotransferase (EC 2.4.2.14) and a decreased activity of xanthine oxidase (EC 1.2.3.2), thus shifting the balance towards purine biosynthesis (Prajda *et al.*, 1976). Lowered xanthine oxidase activity in mouse mammary tumours, and antitumour activity of injected xanthine oxidase in mice, have been reported (Haddow *et al.*, 1953; Lewin *et al.*, 1957; Haddow *et al.*, 1958). Liver xanthine oxidase activity fell in rats fed on a low-protein diet, but this did not occur in tumour-bearing rats (Burton & Begg, 1957).

We have compared the activity of xanthine oxidase in contact-inhibited non-tumorigenic and spontaneously transformed tumorigenic mouse embryo cells. Balb/3T3 and 3T12 are respectively non-tumorigenic and tumorigenic lines derived from Balb/c mouse embryos; 3T3 and 3T6 are a corresponding pair derived from non-inbred Swiss mouse embryos (Aaronson & Todaro, 1968; Todaro & Green, 1963). Sources of cells and methods of enzyme extraction and assay have been described (Clynes *et al.*, 1979; Duke *et al.*, 1973). The results presented in Table 1 indicate (1) that the spontaneously transformed cell lines have higher xanthine oxidase activity than their normal counterparts grown under the same conditions and (2) that non-confluent Balb/3T3 and 3T12 cells contain lower xanthine oxidase activity than do confluent cells. The latter finding may be relevant to cell-cycle fluctuations in xanthine oxidase activity, or may be related to cell contact or to alterations in composition of the medium at high cell densities.

The results presented in Table 1 raise questions as to the generality of lowered xanthine oxidase activity in tumour cells, but several limitations in the experimental system used must be considered. (1) Although the assays were carried out under conditions of linearity with respect to time and enzyme concentration, the presence of competing enzymes in the extract has not been ruled out. (2) The contact-inhibited and transformed lines were developed by serial subculture of mouse embryo cells at low and high density

Table 1. *Xanthine oxidase activity in mouse embryo cell lines*

Xanthine oxidase activity is expressed as nmol of isoxanthopterin produced/min per mg of protein. Numbers given are arithmetic means±S.D., with numbers of experiments in parentheses. Abbreviations: NC, non-confluent; C, confluent.

Cell line		Xanthine oxidase activity	Range
Balb/3T3	NC	0.33±0.07 (5)	0.21–0.38
3T12	NC	1.00±0.23 (5)	0.71–1.32
Balb/3T3	C	0.81±0.28 (5)	0.42–1.28
3T12	C	1.96±0.28 (8)	1.60–2.43
3T3	C	0.50±0.03 (4)	0.45–0.56
3T6	C	1.16±0.26 (5)	0.68–1.37

Addition of ATP did not modify release of enzyme from cells. Whereas parathyrin hormone has been shown to inhibit alkaline phosphatase activity of cultured non-neoplastic bone cells (Luben *et al.*, 1976), parathyrin prostaglandin E_2 nor calcitonin influenced alkaline phosphatase output by cultured sarcoma cells.

Thus, although this tumour is hormone-responsive in terms of cyclic AMP production, this responsiveness has not yet been shown to extend to regulation of alkaline phosphatase activity. Other specialized functions of the cells may also be shown to be hormone-controlled.

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Affinity Labelling of the Ribonucleic Acid Polymerase of *Escherichia coli*

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RNA polymerase (EC 2.7.7.6) from *Escherichia coli* is known to possess two binding sites for nucleoside triphosphates (Wu & Goldthwait, 1969), one of which is presumed to bind the nucleotide that initiates RNA chains, whereas the other binds nucleotides involved in elongating the growing chains. The binding of various mucleotide analogues suggests that at least one of these sites is located on either the β or the β' subunit (Frischauf & Scheit, 1973; Wu & Wu, 1974; Armstrong *et al.*, 1976). Periodate oxidizes 1,2-diols to the corresponding dicarbonyl compounds, and this reaction has been used to make nucleotide affinity labels for enzymes such as pyruvate carboxylase (Easterbrook-Smith *et al.*, 1976) and galactosyltransferase (Powell & Brew, 1976). We have used this reaction to synthesize oxidized derivatives of all four ribonucleotides. The oxidized nucleotides all inhibited RNA polymerase under normal assay conditions with a K_I of about 1 mM.

When RNA polymerase was incubated with the nucleotide derivatives together with $NaBH_4$ the extent of inhibition increased with time. The reaction was then quenched with lysine and dialysed to remove unchanged label. A sample was electrophoresed on a 5% polyacrylamide gel containing sodium dodecyl sulphate. The gel was sliced and the slices were solubilized before being counted for radioactivity in toluene/diphenyloxazole. By comparison with a gel stained for protein with Coomassie Blue it was shown that the radioactivity had co-migrated with the α-subunit of the enzyme. Controls established that (*a*) failure to quench the reaction with lysine led to all the subunits being labelled, (*b*) omission of RNA polymerase from the reaction led to total loss of radioactivity during dialysis and (*c*) a sample of oxidized ATP alone run on the polyacrylamide gel did not give a peak of radioactivity in the same position as the α-band. We therefore conclude that these nucleotide derivatives are affinity labels which react with the α-subunit. Since all four oxidized nucleotides gave the same result, we tentatively suggest that the elongation site is located on or close to the α-subunit. This is not inconsistent with previous results if it is assumed that this site is located between two subunits. Since the primary sequence of the α-polypeptide is known (Ovchinnikov *et al.*, 1977), it is possible to characterize the exact lysine residue labelled.

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An Unusual Structural Feature of Non-Histone Chromosomal High-Mobility-Group Protein 1

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One of the most interesting features of the non-histone chromosomal high-mobility group protein ('protein HMG 1') is the fact that over 50% of the amino acids in the protein are charged. Some 25% of the amino acid residues are basic, which is similar to the situation found in the five histones. However, unlike the histones, protein HMG 1 also contains 29% aspartic and glutamic residues. We have isolated a CNBr peptide from this protein (peptide CB2) which contains 47% aspartic and glutamic residues. The peptide is approx. 120 residues in length and contains no homoserine or homoserine lactone. This peptide must therefore represent the *C*-terminal portion of the molecule. The sequence of the first 44 amino acid residues has been determined for this peptide. Since this sequence contains only ten aspartic and glutamic residues (23%), the majority of the aspartic and glutamic residues must therefore be concentrated in the *C*-terminal portion of this peptide. Evidence for this high concentration of aspartic and glutamic residues comes from a tryptic peptide from peptide CB2. This peptide is eluted just within the excluded volume on a Sephadex G-25 column, and has the amino acid composition Lys_3, Asx_{12}, Glx_{29}. The *N*-terminal sequence of this peptide has been determined on a protein sequenator and was shown to be Lys-Lys-Glu-Glu-Glu-Glu-Asp-Glu-Glu-Asp-Glu. This peptide was taken through 25 cycles on the sequenator, and, although unambiguous identification of the sequence beyond step 12 was not possible, no asparagine or glutamine residues were observed on any of the cycles. Additionally it is unlikely that any of the aspartic or glutamic residues in this peptide are amidated, since we have previously shown that only about 11 aspartic and glutamic residues are amidated in the total molecule, and we have found this number of amidated residues in other peptides during our sequence studies on this protein. The function of this highly unusual acidic region of the molecule has yet to be determined.

Binding of Naphth-2-ylacetic Acid to Rat Liver Deoxyribonucleic Acid

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A diversity of compounds, including polycyclic aromatic hydrocarbons, aromatic amines, numerous heterocyclic compounds and nitrosamines have been found to interact with nucleic acids (Farber & Epstein, 1970; Lawley, 1970; Weinstein *et al.*, 1971). During the course of an investigation of the metabolic fate of naphth-2-ylacetic acid, it was found that it accumulated in the tissues and that this, at least in part, arose from an affinity of the acid to bind to DNA (Emudianughe *et al.*, 1977). These findings

are reported below, since there appears to be little if any information reported hitherto about the binding of xenobiotic carboxylic acids to nucleic acids.

Female Wistar albino rats (180–220g body weight) were injected intraperitoneally with naphth-2-yl[^{14}C]acetic acid (100mg/kg; 5mCi/animal) and killed 3h later. Their livers were homogenized in 0.25M-sucrose, and determination of ^{14}C showed that 6.9% of the dose was present. The homogenate was separated into nuclear, mitochondrial, microsomal and cytoplasmic fractions as described by Dixon *et al.* (1978), which showed that the ^{14}C was distributed as follows: nuclei, 23%; mitochondria, 4%; microsomal fraction, 13%; cytoplasm, 60%. Each fraction was then precipitated with trichloroacetic acid and the pellet extracted exhaustively with methanol as described by Mitchell *et al.* (1973), to determine whether the ^{14}C present was merely loosely associated with the cellular macromolecules or covalently bound to them. When the mitochondrial, microsomal and cytoplasmic fractions were subjected to this trichloroacetic acid/methanol treatment, less than 5% of the original ^{14}C remained in the pellet after extraction. However, when the nuclear fraction was so extracted, some 40% of the ^{14}C was not removed by repeated trichloroacetic acid/mcthanol extraction, indicating that the radioactivity was closely associated with some constituent of the nuclear fraction. To study this binding further, samples of the nuclear fraction of livers of rats dosed with naphth-2-yl[^{14}C]acetic acid were sonicated, incubated with either proteinase (Sigma, type VI from *Streptococcus griseus*) or deoxyribonuclease (Sigma, grade I from ox pancreas) for 18h at 37°C and then subjected to the trichloroacetic acid/methanol extraction as before. Incubation with proteinase did not alter the ^{14}C content of the pellet compared with control samples carried through the procedure, but incubation with deoxyribonuclease caused the almost complete disappearance of ^{14}C from the pellet, with some 10% of control only remaining.

This therefore suggested that naphth-2-ylacetic acid or its metabolites was closely associated with DNA of rat liver and this was investigated by isolating DNA from livers of rats dosed with naphth-2-ylacetic acid as before. The nuclear fraction of homogenates of liver was treated successively with sodium dodecyl sulphate, ice-cold phenol, ribonuclease, and by chloroform/octanol extraction and ethanol precipitation to isolate DNA, and it was found that the fraction of ^{14}C present in the nuclear fraction, which was previously shown to be tightly bound, was carried through this procedure with DNA, right up to the chloroform/octanol extraction stage. However, when this DNA-rich aqueous phase was treated with 2vol. of ethanol to precipitate out the DNA, the ^{14}C was liberated into the liquid phase, and was not associated with the precipitated DNA. It thus appears that the ^{14}C, which is closely associated with rat liver DNA after the administration of naphth-2-yl[^{14}C]acetic acid, is not covalently bound to this macromolecule, but is physicochemically adsorbed on to the DNA. [Brookes & Lawley (1964) have reported similar findings; they showed that hydrocarbons bound to DNA *in vitro* could be removed when the DNA was precipitated with ethanol, but when the same hydrocarbons were administered to mice, their metabolites were covalently linked to DNA and could not be removed by ethanol precipitation.] The nature of the ^{14}C associated with the DNA was studied by t.l.c. of the final ethanolic phase, and comprised mainly unchanged naphth-2-ylacetic acid (77%) together with some naphth-2-ylacetyl glucuronide (23%). The possible significance of the binding of naphth-2-ylacetic acid to DNA requires investigation.

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Heterogeneity of Proteoglycans from Adult Human Costal Cartilage

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Changes in the glycosaminoglycan composition of human hyaline cartilages during maturation and aging (Kaplan & Meyer, 1959; Mathews & Glagov, 1966; Mason & Wusteman, 1970) suggest changes in the type of proteoglycan synthesized by chondrocytes at different times. Electrophoresis of adult human costal-cartilage proteoglycans on cellulose acetate membranes (Pedrini, 1969) separated two types of molecule; one, a predominently chondroitin sulphate proteoglycan; the other, a keratan sulphate proteoglycan. However, heterogeneity could have arisen from mechanical degradation of native proteoglycans during homogenization of the tissue (Sajdera & Hascall, 1969), or by the action of cartilage proteinases during extraction (Pearson & Mason, 1977). After precautions to prevent either physical or enzymic degradation, it has been shown that the major proteoglycan fraction of bovine nasal cartilage can be separated into two components by electrophoresis in large-pore composite agarose/polyacrylamide-gels (Pearson & Mason, 1977). The same methods have been applied to study the heterogeneity of adult human costal-cartilage proteoglycans.

Cartilage was obtained at necropsy within 5 h of death, frozen (−20°C, 2 h), stripped of perichondrium and shredded with a Surform blade. Grossly calcified tissue was discarded. Tissue slices were extracted with 0.15 M-KCl followed by 4.0 M-guanidine HCl at 1°C in the presence of proteinase inhibitors (see Pearson & Mason, 1977). The tissue residue was then powdered in liquid N_2 before extraction again with 4.0 M-guanidine HCl. This report deals with the proteoglycans solubilized by the first and second guanidine HCl extracts, which account for 12% and 6% respectively of the total tissue hexuronic acid.

A_1-proteoglycan fractions ($d > 1.70$) were isolated from the crude extracts by centrifugation in a caesium chloride density gradient under associative conditions, at 10°C, in the presence of proteinase inhibitors (Roughley & Mason, 1976; Pearson & Mason, 1977). Fractions from the first and second extracts are described as 'A1GuHCl,1' and 'A1GuHCl,2' respectively, in the nomenclature of Heinegård (1972). Each fraction contained about 70% of the total hexuronic acid of the extract. The methods for electrophoresis in agarose/polyacrylamide gels and spectrophotometric scanning of gels have been described (McDevitt & Muir, 1971; Roughley & Mason, 1976). Proteoglycan bands stained with Toluidine Blue were cut from the gel, placed in 2.0 M-$CaCl_2$, agitated by vortex-mixing, centrifuged and the supernatants retained. The residue was resuspended in 2.0 M-$CaCl_2$, sonicated (MSE sonicator; low power setting, amplitude 3, 20°C, 30 s) and the supernatant recovered after centrifugation. The pooled supernatants were dialysed against 2.0 M-$CaCl_2$ (1:50, v/v; 20°C, 18 h), then against water (1:50, v/v; 4°C, 18 h, repeated two times), freeze-dried and analysed for hexosamine content (Mayes *et al.*, 1973).

The A_1-proteoglycans from four cartilage samples (female, 47 years old; male, 59; male, 64; female, 76) all contained three electrophoretic components with mobilities (R_{BPB}) 0.8, 0.9 and 0.96 relative to bromophenol blue (BPB) (Fig. 1). Fractions from the two oldest subjects also contained a fourth component, R_{BPB} 1.04. Each electrophoretic component has a characteristic glucosamine:galactosamine ratio (Table 1). For a given component in fractions 'A1GuHCl,1' or 'A1GuHCl,2', from either cartilage, the ratio is very similar. The ratio indicates the relative proportions of keratan sulphate: chondroitin sulphate in a proteoglycan. Since the rate of migration of a multichain proteoglycan in large-pore gels is probably, predominantly, a function of its hydrodynamic size, the data show that the larger proteoglycan species (low R_{BPB}) contain relatively more chondroitin sulphate to keratan sulphate than the smaller species (high R_{BPB}).

The glucosamine:galactosamine ratio of fraction 'A1GuHCl,1' differs from that of fraction 'A1GuHCl,2' (Table 1). This difference appears to be due to a difference in the relative amounts of the electrophoretic components present in A_1-proteoglycan frac-

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Studies on the Metabolism of Arylacetic Acids

5. The Metabolic Fate of 2-Naphthylacetic Acid in the Rat, Rabbit and Ferret

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1. The fate of [^{14}C]-2-naphthylacetic acid has been studied in rat, ferret and rabbit and the findings compared with those for the 1-isomer.

2. 2-Naphthylacetic acid was conjugated in all three species with glycine, glutamine, taurine and glucuronic acid.

3. In rat, but not rabbit, 2-naphthylacetic acid is eliminated in the bile (50% dose) and undergoes enterohepatic circulation.

4. The pattern of metabolism and elimination of 2-naphthylacetic acid in rat is dose-dependent. Small doses (5 mg/kg) are excreted in urine and bile mainly as the glucuronic acid conjugate, while larger doses (250 mg/kg) are eliminated in the bile mainly as amino acid conjugates.

5. Metabolic conjugation of 1- and 2-naphthylacetic acids differs in the three species studied. 1-Naphthylacetic acid is conjugated largely with glucuronic acid and amino acid conjugation is minimal, whereas the converse is true of the 2-isomer. These differences in conjugation have been correlated with structural differences between the two acids.

6. 2-Naphthylacetic acid is the first carboxylic acid found to undergo simultaneous amino acid conjugation with glycine, glutamine and taurine in non-primate species. 2-Naphthylacetic acid may be a useful probe for exploring the species occurrence of the various conjugations.

Previous studies have shown that the pattern of metabolism of arylacetic acids depends upon their structure and in particular on the nature of the aryl substituent and substitution on the α-methylene group (Dixon *et al.*, 1974, 1976; Dixon, Caldwell & Smith 1977 a, b, c). We have now extended our observations concerning the relationship between the chemical structure of arylacetic acids and their pattern of metabolic conjugation to an investigation of the metabolic fate of 2-naphthylacetic acid in three species, namely, the rat, rabbit and ferret. This compound was chosen to compare its metabolic fate with that of the isomeric 1-naphthylacetic acid (Dixon *et al.*, 1977 a). Also, 2-naphthylacetic acid is more toxic, to both rats and ferrets, than 1-naphthylacetic acid (Idle, 1976) and this could arise from differences in metabolism. Part of these findings have been communicated (Emudianughe, Caldwell & Smith, 1977).

Materials and methods

Potassium [^{14}C]cyanide, sp. act. 40 Ci/mol, was purchased from the Radiochemical Centre, Amersham, U.K. 2-Naphthylacetic acid, m.p. 140–142° and 2-bromomethylnaphthalene, m.p. 51–54°, were purchased from Aldrich Chemical Co., Wembley, U.K.

[carbonyl-^{14}*C*]-2-*Naphthylacetic acid*

2-Bromomethylnaphthalene (1·19 g) and K^{14}CN (0·35 g; 3 mCi) in a mixture of ethanol (20 ml) and water (5 ml) were refluxed for 9 h. After removal of the ethanol on a rotary

evaporator, water (20 ml) was added and the reaction mixture extracted with ether (6 × 50 ml) until no further radioactivity was extracted. The bulked ether extracts, containing crude 2-naphthylaceto[^{14}C]nitrile, were dried (anhyd. Na_2SO_4), evaporated to dryness and the residue refluxed with 48% HBr (50 ml) for 3 h. The mixture was then extracted with ether (3 × 50 ml) and the bulked extracts re-extracted with 0·1 M NaOH (75 ml). The alkaline extract was acidified (pH 1) with conc. HCl, extracted with ether (3 × 50 ml), and these extracts combined and re-extracted with 0·1 M NaOH (75 ml). This procedure was repeated. The [*carbonyl*-^{14}C]-2-naphthylacetic acid was then precipitated by cautious addition of conc. HCl to the NaOH extract, filtered and recryst. from aq. ethanol, m.p. 143°, sp. act. 3·42 μCi/mg. This material gave a single radioactive spot with the same R_F as authentic 2-naphthylacetic acid when subjected to t.l.c. in systems A–D (Table 1) and was shown by reverse isotope dilution to have a radiochemical purity of > 99%.

Amino acid conjugates of 2-naphthylacetic acid

2-Naphthylacetyl-glycine, -glutamine and -taurine were synthesized from 2-naphthylacetyl chloride and the amino acid using the methods described by Dixon *et al.* (1977 a) for the corresponding 1-naphthylacetic acid derivatives. Analytical details only are quoted.

2-*Naphthylacetylglycine*, m.p. 145–146° from aq. ethanol. Equiv. wt. by titration (0·1 M NaOH) 245 (requires 243). $C_{14}H_{13}O_3N$ requires C, 69·1; H, 5·4 and N, 5·8. Found C, 68·8; H, 5·5 and N, 5·7. The mass spectrum of the methyl ester showed a molecular ion at *m/e* 257 (6%) with prominent peaks at *m/e* 225 (5%, loss of $-OCH_3$) 168 (35%), 141 (40%) and 115 (10%).

2-*Naphthylacetyl*-L-*glutamine*, m.p. 160–161° from water. Equiv. wt. by titration (0·1 M NaOH) 315 (requires 316). $C_{17}H_{18}O_4N_2$ requires C, 64·9; H, 5·7 and N, 8·9. Found C, 64·6; H, 5·8 and N, 9·0. The mass spectrum of the methyl ester showed a small molecular ion at *m/e* 328 (0·5%) with peaks at *m/e* 296 (3%, loss of $-OCH_3$), 168 (38%), 141 (47%) and 115 (33%).

2-*Naphthylacetyltaurine*, m.p. 293–294° for the Na salt as its hemihydrate, from methanol–acetone (2 : 1, v/v). The crystals gave a positive Na flame test and did not effervesce when treated with $NaHCO_3$. $C_{14}H_{14}NO_4SNa.\frac{1}{2}H_2O$ requires C, 51·9; H, 4·3; N, 4·3; S, 9·9; Na, 7·1. Found C, 52·2; H, 4·3; N, 4·3; S, 10·0; Na, 7·2. The mass spectrum of the methyl ester showed characteristic peaks at *m/e* 185 (5%), 167 (1%), 141 (13%) and 115 (4%).

Spectroscopic methods

Mass spectra were obtained using a Varian CH5 instrument with a Spectrosystem 100 data system, by direct insertion. The ionizing voltage was 70 eV.

Animals

Female Wistar albino rats (body wt. 150–200 g) and female Dutch rabbits (body wt. 2·2–2·7 kg) were used. Mongrel ferrets of both sexes (body wt. 400–800 g) were bred by mating mongrel albino ferrets with domestic polecat ferrets (both *Mustela furo*). [^{14}C]-2-Naphthylacetic acid (5–250 mg/kg) was administered in propane-1,2-diol, either orally or by intraperitoneal injection. Animals were housed in suitable cages and urine and faeces collected for up to 6 days. In one set of experiments, rats were injected intraperitoneally with [^{14}C]-2-naphthylacetic acid and housed in Metabowls (Jencons Ltd.) to permit collection of the expired air. The air from the cages was drawn through ethanolamine–2-methoxyethanol (1 : 2, v/v) to trap any $^{14}CO_2$ present. The excreta were stored at −20° until analysis. Bile-duct cannulation of rats and rabbits was as described by Abou-El-Makarem *et al.* (1967) and Hirom *et al.* (1972).

Chromatography

The chromatographic properties of 2-naphthylacetic acid, its conjugates and certain amino-acids are shown in Table 1. For radiochromatogram scanning, the urine (0·01–0·2 ml, containing about 1×10^4 d.p.m.) was chromatographed on 5 cm wide t.l.c. plates and developed in solvents A, B, C or D. After development and drying, the plates were scanned using a Packard radiochromatogram scanner, model 7200.

Radiochemical techniques

Urine and bile samples (0·01–1 ml) were counted in triplicate in a dioxan-based scintillation fluid, while faeces were homogenized and bleached before counting as above (Caldwell, Dring and Williams 1972). In some experiments, the chromatograms were cut into 0·5 cm pieces and each counted for ^{14}C as above, to allow the plotting of a histogram of ^{14}C content against chromatographic mobility. In all experiments a Packard Tri-Carb liquid scintillation spectrometer, model 3320, was used.

Table 1. Chromatographic properties of 2-naphthylacetic acid, its conjugates and certain amino acids

Compound	Solvent	R_F value A	B	C	D	E	Appearance beneath u.v.	Colour reactions with Ninhydrin	Isatin
2-Naphthylacetic acid		0·90	0·92	0·95	—	—	dark purple	—	—
2-Naphthylacetyl-taurine		0·21	0·44	0·32	—	—	dark purple	—	—
2-Naphthylacetyl-L-glutamine		0·53	0·69	0·44	—	—	dark purple	—	—
2-Naphthylacetyl-glycine		0·76	0·87	0·77	—	—	dark purple	—	—
Dansylglutamic acid		—	—	0·45	—	—	intense blue*	—	—
Dansylglycine		—	—	0·54	—	—	intense blue*	—	—
Dansyltaurine		—	—	0·32	—	—	intense blue*	—	—
Glycine		—	—	—	0·43	—	purple	purple	pink
Glutamic acid		—	—	—	0·63	0·24	purple	purple	pink
Taurine		—	—	—	—	0·18	purple	purple	yellow

T.l.c. plates were of plastic-backed silica gel 60 F 254 (Cat. No. 5735, Merck A.G., Darmstadt, W. Germany), except for the dansylamino acids for which non-fluorescent silica gel plates (Merck Cat. No. 5748) were used. The solvent systems were A, benzene–acetone–acetic acid (2 : 2 : 1, by vol.); B, ethanol–benzene–acetic acid (5 : 5 : 3, by vol.); C, chloroform–methanol–acetic acid (15 : 4 : 1, by vol.); D, ethanol–water (7 : 3, v/v); E, butan-1-ol–acetic acid–water (4 : 1 : 1, by vol). They were inspected under a Hanovia Chromatolite (254 nm). The ninhydrin and isatin sprays were both 5% solutions in methanol, and after spraying the plates were gently heated with a hot-air blower to reveal the coloured spots.

*These colours were highly fluorescent spots seen on the non-fluorescent plates.

Reverse isotope dilution for 2-naphthylacetic acid and its amino acid conjugates

For 2-naphthylacetic acid and its glycine and glutamine conjugates, 1 g of the appropriate acid, in aq. $NaHCO_3$, was added to urine samples containing 0·5–1 μCi of ^{14}C. The urine was adjusted to pH1 with 2 M HCl and the ppt. formed was filtered and recryst. from the appropriate solvent (see syntheses above) to constant sp. radioactivity. 2-Naphthylacetylglucuronide was estimated by difference following its conversion to 2-naphthylacetic acid by mild alkaline hydrolysis (see below). The difference between 'total' and 'free' naphthylacetic acid is a measure of the glucuronic acid conjugate. For 2-naphthylacetyltaurine, 1 g of the Na salt of the conjugate was added to urine and the whole reduced to dryness on a rotary evaporator. The residue was treated with hot methanol (5 ml), filtered and the methanol extract treated with acetone (3 ml). The white crystals that separated were filtered and recryst. from methanol–acetone (2 : 1, v/v) to constant sp. radioactivity.

Treatment of urine samples

Urine samples were analysed by t.l.c. as such and after mild alkaline hydrolysis or treatment with β-glucuronidase.

(*a*) *Alkaline hydrolysis*: urine (1 ml) was heated on a boiling water bath with M NaOH (1 ml) for 15 min and neutralized with 2 M HCl before chromatography.

(*b*) *β-Glucuronidase treatment*: urine (2 ml) was adjusted to pH 5 with glacial acetic acid and incubated with Ketodase (Warner-Chilcott Labs.) (1 ml) at 37° for 18 h. As a control to establish the activity of the enzyme, a tube was included containing phenolphthalein glucuronide (1 mg) dissolved in a second sample (1 ml) of urine. At the end of the experiment this tube was made alkaline with M NaOH, and the appearance of a deep purple colour showed that the enzyme was active.

Dansylation of amino acids

Small samples (ca. 500 mg) of glycine, glutamic acid and taurine in water (0·5 ml) were treated with a soln. of dansyl (5-dimethylamino-1-naphthalenesulphonyl) chloride (3 mM in acetone; 0·1 ml), left at room temp. for 1 h and chromatographed on non-fluorescent t.l.c. plates in solvent C.

Tissue distribution

[^{14}C]-2-Naphthylacetic acid was administered to rats and ferrets and after 24 h the animals were killed by a blow on the head. The vital organs were removed, and homogenized in 3 vol. water in an Ultra-Turrax homogenizer, bleached as for faeces and counted for ^{14}C. The carcass of each animal was then dissolved in 80% ethanolic KOH and aliquots counted for ^{14}C.

Chromatographic identification of urinary metabolites

Radiochromatogram scans of chromatograms of 0–24 h urine of rat, rabbit and ferret in solvent A showed 5 similar-sized peaks with R_F values of 0·1, 0·22, 0·56, 0·78 and 0·92. The peak with R_F 0·1 gave a positive naphtharesorcinol reaction and disappeared when the urine samples were treated with β-glucuronidase or M NaOH. Its disappearance was accompanied by an increase in the size of the peak at R_F 0·92 (2-naphthylacetic acid). The peaks with R_F values 0·22, 0·56 and 0·78 corresponded to 2-naphthylacetyl-taurine, -glutamine and -glycine respectively. Chromatograms of the urines developed in solvent B were also scanned for ^{14}C and this revealed 5 radioactive peaks, with R_F values of 0·07, 0·42, 0·69, 0·84 and 0·93. The peak at R_F 0·07 disappeared when the urine was treated with β-glucuronidase or NaOH and this resulted in enlargement of the peak at R_F 0·93 (2-naphthylacetic acid). The peaks with R_F values 0·42, 0·69 and 0·84 corresponded to 2-naphthylacetyl-taurine, -glutamine and -glycine respectively.

Larger volumes of the urine (2–3 ml) were treated with M NaOH and chromatographed on preparative thick-layer plates (20 × 20 cm, 1 mm thick) spread with silica gel GF_{254} (Merck A. G., Darmstadt, W. Germany) and developed in solvent C. A strip 1 cm wide down the middle of the plate was scraped off in 1 cm sections, the ^{14}C content of each determined and a histogram plotted to locate the ^{14}C peaks on the plate, which had R_F values of 0·32, 0·44, 0·77 and 0·95. These correspond to 2-naphthylacetyl-taurine, -glutamine and -glycine and 2-naphthylacetic acid respectively, and the areas of silica gel containing the amino acid conjugates were scraped off and eluted with methanol. The methanol was evaporated, the residue refluxed with conc. HCl for 2 h and neutralized before examination by t.l.c. The hydrolysates were divided into three parts and treated as follows (1) t.l.c. in systems A and B followed by radiochromatogram scanning, (2) t.l.c. in systems D and E followed by spraying with ninhydrin and isatin, (3) dansylation as described and examination by t.l.c. in solvent C.

The area with R_F 0·32 in solvent C on hydrolysis gave (1) single ^{14}C peaks in solvents A and B with R_F values 0·9 and 0·92 respectively, corresponding to 2-naphthylacetic acid,

(2) a spot of R_F 0·18 in solvent E, which gave a purple colour with ninhydrin and a yellow colour with isatin, and corresponded with taurine, (3) t.l.c. in solvent C of the hydrolysate after dansylation showed an intensely fluorescent spot of R_F 0·33 under u.v. light, which corresponded to dansyltaurine. This peak was therefore assigned to 2-naphthylacetyltaurine.

Similar treatment of the hydrolysates from the area of R_F 0·44 in solvent C showed (1) the presence of ^{14}C peaks corresponding to 2-naphthylacetic acid (2) spots of R_F 0·63 and 0·24 in solvents E and F, which gave purple colours with ninhydrin and pink with isatin, and corresponded with glutamic acid, (3) t.l.c. in solvent C of the hydrolysate after dansylation showed under u.v. light an intensely fluorescent spot with R_F 0·45, which corresponded to dansylglutamic acid. This peak was therefore assigned to 2-naphthylacetyl-glutamine or -glutamic acid, both of which would give rise to 2-naphthylacetic acid and glutamic acid on hydrolysis.

The hydrolysate of the area with R_F 0·77 in solvent C was examined as described and showed (1) the presence of ^{14}C peaks corresponding to 2-naphthylacetic acid, (2) a spot with R_F 0·4 in solvent E, which turned purple and pink on spraying with ninhydrin and isatin respectively, (3) after dansylation, t.l.c. in solvent C showed under u.v. light a highly fluorescent spot with R_F 0·55, which corresponded to dansylglycine. This peak was therefore assigned to 2-naphthylacetylglycine.

Results

Table 2 gives details of the elimination of ^{14}C in the urine and faeces by rat, rabbit and ferret after administration of [^{14}C]-2-naphthylacetic acid. Over the six days of the experiment only partial recovery was achieved, with 60% dose excreted by rat, 63% by ferret and 84% by rabbit. In separate experiments, $<0·1\%$ dose was found as $^{14}CO_2$ in the expired air of rats in five days after injection of [^{14}C]-2-naphthylacetic acid. In all three species, most of the excreted ^{14}C was in the urine with smaller amounts in faeces. The majority of the urinary ^{14}C was recovered in the 0–24 h urine, with progressively smaller amounts on succeeding days.

Table 2. Excretion of ^{14}C by various species after administration of [^{14}C]-2-naphthylacetic acid

[^{14}C]-2-Naphthylacetic acid was dissolved in propane-1,2-diol and administered to animals as described. Values are means of at least 3 animals with ranges in parentheses.

	Rat	Rabbit	Ferret
No., Sex	3F	3F	2M, 2F
Dose, mg/kg (^{14}C μCi/kg)	100(16)	100(16)	50(16)
Route	i.p.	p.o.	i.p.
	% ^{14}C dose in		
Urine			
Day 0–1	35·4(28·4–41·9)	51·0(37·0–59·2)	29·8(27·0–32·4)
1–2	8·2(2·8–19·1)	17·2(15·1–19·7)	6·0(5·7– 6·4)
2–3	1·6(0·7– 2·3)	5·8(3·4– 7·4)	3·2(1·7– 4·4)
3–4	0·8(0·5– 1·2)	1·1(0·9– 1·3)	2·6(2·5– 2·8)
4–6	0·9(0·5– 1·6)	0·7(0·4– 1·1)	
Total	46·9(40·5–52·6)	75·8(60·0–85·0)	41·6(39·6–47·0)
Faeces			
Day 0–3	11·7(6·2–18·1)	8·2(5·4–10·1)	13·3(11·7–15·9)
3–6	1·2(0·5– 2·6)		8·0(6·8– 9·4)
Total	12·9(6·8–18·6)	8·2(5·4–10·1)	21·3(20·3–22·7)
Total ^{14}C in excreta	59·8(54·5–59·1)	84·0(69·2–95·1)	62·9(61·2–67·4)

The urinary metabolites of [^{14}C]-2-naphthylacetic acid were identified by comparison of their chromatographic properties with those of authentic samples, as described above, and by reverse isotope dilution. In addition to unchanged 2-naphthylacetic acid, four conjugates were detected, namely the glucuronic acid, glycine, glutamine and taurine conjugates, and quantitative data for their excretion by the three species are given in Table 3.

Table 3. Urinary metabolites of [^{14}C]-2-naphthylacetic acid in various species

[^{14}C]-2-Naphthylacetic acid (100 mg/kg; 16 μCi/kg) was administered to the animals and their excreta collected. The 0–24 h urine was analysed for metabolites as described in the text. Results quoted are the means of at least 3 animals with ranges in parentheses.

Compound	% dose		
	Rat	Rabbit	Ferret
2-Naphthylacetic acid	5·2(2·1– 7·8)	5·6(4·5– 6·7)	1·4(1·1– 1·5)
2-Naphthylacetylglycine	8·6(7·9– 9·6)	15·3(13·4–17·2)	5·4(4·9– 5·7)
2-Naphthylacetyltaurine	10·2(8·8–12·8)	4·8(2·3– 5·4)	14·7(14·2–15·0)
2-Naphthylacetylglutamine	5·7(5·1– 6·2)	13·1(8·2–15·1)	6·3(5·5– 7·1)
2-Naphthylacetylglucuronide	5·6(4·9– 6·2)	12·2(8·5–13·7)	5·4(5·2– 5·5)
Total of above metabolites	35·3(28·2–41·9)	51·0(36·9–59·1)	33·2(31·0–35·5)
Total ^{14}C excreted in 0–24 h urine	35·4(28·4–41·9)	51·0(37·0–59·1)	33·4(31·1–35·6)

All three species formed mainly amino acid conjugates of 2-naphthylacetic acid, with smaller amounts of the glucuronic acid conjugate; the unchanged acid was also excreted. In the rat, the major urinary metabolite was 2-naphthylacetyltaurine (10% dose) with the glycine, glutamine and glucuronic acid conjugates and the free acid present in smaller (5–8%) amounts. The major urinary metabolites in rabbit were 2-naphthylacetyl-glycine (15%), -glutamine (13%) and -glucuronide (12%) with smaller amounts of the free acid and its taurine conjugate. The ferret formed 2-naphthylacetyltaurine as the major excretion product (15%) with the glycine, glutamine and glucuronic acid conjugates and the free acid also being present in the urine.

Table 4 shows quantitative and qualitative details of the excretion of [^{14}C]-2-naphthylacetic acid by bile-duct cannulated rats and rabbits. In 24 h, rats

Table 4. Biliary excretion of [^{14}C]-2-naphthylacetic acid in the rat and rabbit

Bile-duct cannulated rats and rabbits were injected intraperitoneally with [^{14}C]-2-naphthylacetic acid (100 mg/kg, 26 μCi/kg), excreta collected and analysed for ^{14}C and metabolites as described in the text. Figures represent the mean of 3 animals with ranges in parentheses. Collection period was 8 h for rabbits and 24 h for rats. n.d.=not detected.

Compound	% dose		
	Rat		Rabbit
	Urine	Bile	Bile
2-Naphthylacetic acid	2·7 (1·3– 4·4)	3·7 (2·4– 5·9)	0·6 (0·1– 1·1)
2-Naphthylacetylglycine	4·6 (2·3– 7·5)	10·2 (5·9–13·5)	n.d.
2-Naphthylacetyltaurine	5·6 (2·8– 9·1)	n.d.	n.d.
2-Naphthylacetylacetylglutamine	3·0 (1·6– 4·9)	4·3 (2·8– 5·3)	0·8 (0·1– 1·5)
2-Naphthylacetylglucuronide	n.d.	32·2(27·7–42·5)	4·3 (0·7– 8·1)
Total of above metabolites	15·7 (7·9–25·6)	50·4(43·1–65·0)	5·7 (0·9–10·7)
Total ^{14}C excreted	15·8 (7·9–25·7)	51·6(43·0–66·2)	5·8 (0·9–10·8)

Table 5. Influence of dose size on the disposition of 2-naphthylacetic acid in the rat and the ferret

Bile-duct cannulated rats were injected intraperitoneally with [^{14}C]-2-naphthylacetic acid, bile collected for 3 h and the urine collected by bladder puncture. Ferrets were injected intraperitoneally with [^{14}C]-2-naphthylacetic acid and their 0–24 h urines collected. Urine and bile were analysed for ^{14}C and metabolites as described in the text. Results given are the means of 3 animals with ranges in parentheses, expressed as percentage of dose.

Dose: (mg/kg)	50		100		200	
Excreta:	Urine	Bile	Urine	Bile	Urine	Bile
Rat						
2-Naphthylacetic acid	n.d.	2·4(1·9– 2·9)	0·6 (0·5– 0·6)	3·4(1·9– 4·3)	—	1·1 (1·1– 1·1)
2-Naphthylacetylglycine	0·5 (0·5– 0·6)	3·4(2·4– 4·8)	0·9 (0·9– 0·9)	9·6(7·2–13·4)	—	0·8 (0·8– 1·1)
2-Naphthylacetyltaurine	n.d.	n.d.	1·5 (1·4– 1·6)	n.d.	—	7·6 (6·4– 8·7)
2-Naphthylacetylglutamine	n.d.	3·4(2·4– 4·3)	0·3 (0·3– 0·3)	3·8(3·4– 4·8)	—	3·9 (3·6– 4·2)
2-Naphthylacetylglucuronide	2·4 (2·4– 3·2)	38·4(36·0–41·3)	0·7 (0·7– 0·8)	29·8(27·8–33·1)	—	14·0(12·9–15·1)
Total ^{14}C	3 (3 – 4)	48 (47 –50)	4 (2 – 6)	48 (43 –53)	0·1(0·1– 0·2)	28 (26 –30)
Ferret						
2-Naphthylacetic acid	1·2 (1·0– 1·4)		1·4 (1·1– 1·5)		1·2 (1·1– 1·2)	
2-Naphthylacetylglycine	2·2 (2·1– 2·4)		5·4 (5·2– 5·5)		2·1 (2·0– 2·1)	
2-Naphthylacetyltaurine	19·2(18·0–21·8)		14·8(14·7–14·8)		30·7(28·2–33·1)	
2-Naphthylacetylglutamine	3·6 (3·5– 4·5)		6·4 (6·0– 6·7)		9·1 (4·5–13·6)	
2-Naphthylacetylglucuronide	3·9 (3·7– 4·2)		5·4 (5·0– 5·7)		6·2 (6·0– 6·4)	
Total ^{14}C	30·0(27·0–32·4)		33·4(31·1–35·6)		49·2(48·6–49·9)	

n.d. = not detected, — = not determined.

excreted 52% of the dose in the bile with a further 16% in urine. Analysis of the bile by the techniques described revealed the presence of four radioactive excretion products, identified as 2-naphthylacetyl-glycine, -glutamine and -glucuronide and 2-naphthylacetic acid. In contrast with the urinary data given in Table 3, no 2-naphthylacetyltaurine could be detected in the bile, and the major biliary metabolite was 2-naphthylacetylglucuronide. In other experiments, rats were given [^{14}C]-2-naphthylacetic acid by intraperitoneal injection (100 mg/kg) 24 h before cannulation of the common bile duct, and their bile was collected for 24 h, i.e. 24–48 h after dosing. These animals excreted 2·0% (1·5–2·9%) dose in the bile during this period. In 8 h, rabbits excreted some 5% dose of [^{14}C]-2-naphthylacetic acid in the bile, principally as its glucuronic acid conjugate, with smaller amounts of the glycine conjugate and the free acid also present.

The influence of dose size on the metabolic fate of 2-naphthylacetic acid has been studied in rat and ferret (Table 5). In the rat, the dose increased, there was a decrease in the amount excreted in the urine, this being 3% at 50 mg/kg and only 0·1% at 200 mg/kg and above, with a lesser decline in the biliary excretion of ^{14}C. The excretion products present in both urine and bile changed with increasing dose. The percentage excreted as glucuronide decreased, and that excreted as the three amino acid conjugates increased, as the dose was increased. The glutamine conjugate was not present at doses of less than 50 mg/kg and accounted for increasing percentage of excreted ^{14}C with increasing dose. 2-Naphthylacetyltaurine was only formed at doses of 10 mg/kg and above.

In the ferret, the pattern of urine metabolites did not vary appreciably with dose. The major urinary metabolite was the taurine conjugate, with smaller amounts of the glycine and glutamine conjugates. The proportions of the dose excreted unchanged and conjugated with glucuronic acid did not change with dose. At the highest dose (200 mg/kg), the total recovery of ^{14}C in the 0–24 h

Table 6. Tissue distribution of ^{14}C in rat and ferret 24 h after intraperitoneal injection of [^{14}C]-2-naphthylacetic acid

Rats and ferrets were injected intraperitoneally with [^{14}C]-2-naphthylacetic acid (100 mg/kg) kept in metabolism cages for 24 h and then killed. After dissection, the ^{14}C content of each organ was determined as described in the text. Values given are means of 3 rats with ranges in parentheses and of 2 ferrets with individual values in parentheses.

	Rat		Ferret	
Organ	% dose in organ	% dose/g tissue	% dose in organ	% dose/g tissue
Liver	0·8 (0·7 – 1·1)	0·12	2·1 (2·1 – 2·2)	0·13
Heart	0·1 (0·05– 0·11)	0·13	0·1 (0·1 – 0·1)	0·04
Lung	0·1 (0·08– 0·1)	0·09	0·2 (0·2 – 0·2)	0·05
Brain	0·16 (0·13– 0·18)	0·12	0·1 (0·1 – 0·1)	0·02
Kidney	0·23 (0·17– 0·3)	0·15	0·34 (0·34– 0·35)	0·14
Gut wall	7·6 (6·1 – 9·1)	0·63	3·0 (3·0 – 3·0)	0·10
Gut contents	53·9 (50·9 –55·4)	—	3·7 (3·6 – 3·7)	—
Carcass	15·0 (14·2 –16·0)	0·10	47·2 (45·1 –49·3)	0·12
Blood*	n.d.	—	4·5 (4·3 – 4·7)	—
Urine (0–24 h)	16·1 (15·1 –16·9)	—	32·8 (32·0 –33·5)	—
Total	94·0 (90·1 –98·2)	—	94·0 (90·9 –97·1)	—

* Blood volume taken as 8% of body weight. n.d. = not determined.

urine was 49%dose, appreciably greater than at the two other doses examined, 30 and 33% at 100 and 50 mg/kg respectively.

In both rats and ferrets some 94% of the dose of [^{14}C]-2-naphthylacetic acid was accounted for when the ^{14}C remaining in the tissues (Table 6) was added to that excreted. The major differences in the tissue distribution of ^{14}C between the two species were found in the quantities present in the gut contents and carcass. Some 54% of the dose was present in the rat gut contents, compared with 4% in the ferret, while for the carcass, the figures were 15% in the rat and 47% in the ferret.

Discussion

Comparison of the pattern of metabolism and elimination of 2-naphthylacetic acid with its 1-isomer reveals some remarkable differences. 1-Naphthylacetic acid is rapidly eliminated by most species including the rat, rabbit and ferret as a glucuronic acid conjugate (Dixon *et al.*, 1974, 1977; Idle *et al.*, 1976). In general, amino acid conjugation is at a low level for this compound and when it occurs, glycine and taurine are the amino acids utilized. However, with the 2-isomer, the opposite holds for the rabbit and ferret, where the acid is preferentially conjugated with amino acids rather than with glucuronic acid. This preference is not so clear, in the rat where the process is complicated by enterohepatic circulation. Thus, for 2-naphthylacetic acid there is in general a shift in the pattern of metabolic conjugation away from one involving glucuronic acid conjugation, as occurs for the 1-isomer, to one dominantly of amino acid conjugation.

A second remarkable feature of the metabolism of 2-naphthylacetic acid is the formation of a glutamine conjugate of the acid by the rat, rabbit and ferret in addition to the glycine and taurine conjugates. Previously, it was considered that the glutamine conjugation was a feature of the metabolic conjugation of arylacetic acids by anthropoid species (James *et al.*, 1972). However, these findings indicate that the reaction may occur, apparently at a low level, in non-primate animals for particular arylacetic acids. Hirom, Idle & Williams (1977) have found that the ferret also utilizes glutamine to a small extent for the metabolic conjugation of phenylacetic acid.

2-Naphthylacetic acid is the first carboxylic acid found to undergo in non-primate species simultaneous conjugation with three different amino acids, namely glycine, glutamine and taurine. This metabolic pattern is relatively uncommon, since when amino acid conjugation of an arylacetic acid occurs this is usually with one particular amino acid. The ability of 2-naphthylacetic acid to expose the occurrence of these three amino acid conjugations concurrently suggests that it may be usefully be employed as a probe for eliciting the species distribution of these mechanisms.

The marked differences in the metabolic conjugation of 1- and 2-naphthylacetic acids must be related in some way to differences in the structure of the two acids (see Figure 1). The amino acid conjugation mechanisms appear to be more structure-dependent in terms of substrate requirements than the glucuronic acid conjugation mechanism. Thus, the glycine conjugation of arylacetic acids is inhibited by the presence of substituents on the α-methylene group (Dixon *et al.*, 1976, 1977 b, c). Also the extent of glycine conjugation of nuclear substituted benzoic acids is strongly influenced by the nature and position of the substituent in the ring. In general glycine conjugation is prevented, probably for steric reasons, by the presence of a relatively bulky group in the ortho position to the carboxyl group (Bray *et al.*, 1955; Hansch, Lien & Helmer, 1968). It is suggested that the greater affinity of the 2-naphthylacetic

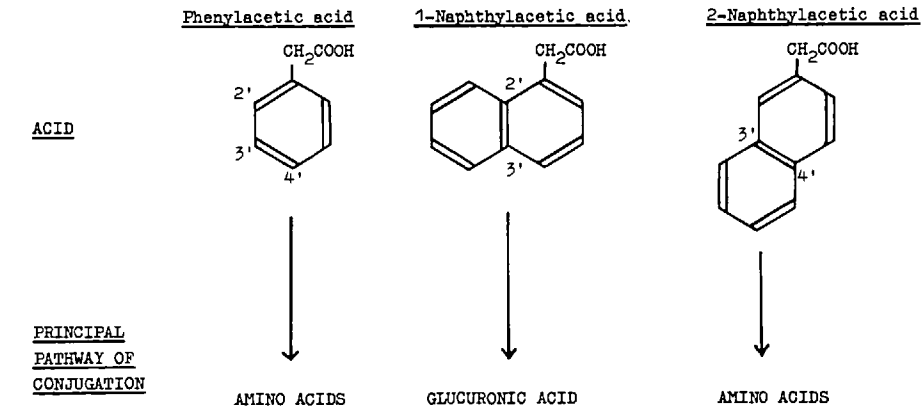


Figure 1. *Chemical structures and principal pathways of conjugation of phenylacetic acid and the isomeric naphthylacetic acids.*

acid for amino acid conjugation compared to the 1-isomer is related to steric hindrance factors influencing the conjugation. Thus, 1-naphthylacetic acid may be regarded as 2′, 3′-benzophenylacetic acid, i.e. an arylacetic acid derivative carrying a substituent in the *ortho* position which might be expected to interfere sterically with the amino acid conjugation. By contrast, 2-naphthylacetic acid may be regarded as a 3′, 4′-benzophenylacetic acid in which there is no *ortho* substituent and therefore no steric hindrance to amino acid conjugation.

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